UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INSTITUTO DE RECONOCIMIENTO MOLECULAR
Y DESARROLLO TECNOLÓGICO

Tesis Doctoral

Integración de técnicas basadas en ADN para el desarrollo de biosensores aplicados en seguridad alimentaria

Presentada por **Sara Santiago Felipe** para optar al grado de Doctor por la Universitat Politècnica de Valencia

Directores
Dr. Ángel Maquieira Catalá
Dra. Rosa Puchades Pla
Dr. Luis Antonio Tortajada Genaro

Valencia, Octubre de 2015

D. Ángel Maquieira Catalá, Catedrático de Universidad del Departamento de Química de la Universitat Politècnica de València.

Dª. Rosa Puchades Pla, Catedrática de Universidad del Departamento de Química de la Universitat Politècnica de València.

D. Luis Antonio Tortajada Genaro, Profesor Contratado Doctor del Departamento de Química de la Universitat Politècnica de València.

CERTIFICAN:

Que el trabajo que presenta Sara Santiago Felipe, en esta memoria, con el título "Integración de técnicas basadas en ADN para el desarrollo de biosensores aplicados en seguridad alimentaria" ha sido realizado bajo nuestra dirección en el Instituto de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) de la Universitat Politècnica de València" para optar al grado de Doctor por la Universitat Politècnica de Valencia.

Para que así conste, firman el presente certificado en Valencia, Octubre de 2015.

Dr. Ángel Maquieira Catalá Dra. Rosa Puchades Pla Dr. Luis Antonio Tortajada Genaro

A ti papá

"Vive como si fueras a morir mañana.
Aprende como si fueras a vivir siempre."
Mahatma Ghandi

Agradecimientos

Dicen que las personas entran en tu vida por alguna razón. Bien por una temporada, o bien para siempre, llegan para enseñarnos y acompañarnos en el aprendizaje de la vida. Tras más de 5 años en el IDM, ha llegado el momento de finalizar mi tesis doctoral, y echando la vista atrás, han sido muchas las personas que me han acompañado y enseñado en algún momento del camino.

En primer lugar, quiero darles las gracias a Ángel y a Rosa, por haberme dado la oportunidad de formar parte de este grupo de investigación, por haberme dedicado su tiempo y esfuerzo, y por todo lo que me han enseñado.

Gracias Luis, por haberte dedicado a esta tesis en cuerpo y alma. Gracias por tu guía y apoyo, por tantas horas invertidas tanto dentro como fuera del laboratorio, y gracias también a Esther, Jaime y Ana por compartir tu tiempo libre con mi tesis.

Durante estos años, he tenido la suerte de coincidir y trabajar con personas estupendas. Sergio, Jesús, Tania, Ernest, Fran y Vicky, gracias por vuestra acogida y ayuda en mis inicios en el laboratorio. Eva y María José, gracias por animarme siempre, tanto en lo profesional como en lo personal. Sergi, gracias por introducirme en el mundo de los CDs, por tus consejos y ayuda a lo largo de este tiempo y por hacerlo siempre con una sonrisa. Gracias también a todo la gente (de ambos lados del pasillo) que sin trabajar conmigo directamente, siempre tienen un minuto para ayudar, saludar y dedicarte unas palabras amables. Raquel, Noelle, Eric, Paulina, Miquel, Jorge, Alba, Gabi, Ahmed, Julia, Marichu, Miguel Ángel, Patricia, y otras tantas personas maravillosas.

Quiero dar las gracias especialmente a mis compis de "campana y termociclador", por vuestra amistad dentro y fuera del IDM. Eli, nadie sabe sacar el mal genio con tanta gracia y cariño, y nadie convierte tan bien los ng de ADN en CFUs como tú. Salva, tu ayuda y sabiduría no tienen

precio, y aunque te hayamos tenido que compartir entre 3, siempre serás un miembro destacado de mi tesis. Regina, son tantas las cosas compartidas, que solo puedo darte un millón de gracias y decirte que eres increíble. Anita, mi Cliff, tu marcha del labo marcó un antes y un después. Fue un regalo poder ir a trabajar juntas a diario y lo es poder contar contigo desde entonces.

También quiero agradecer a la gente del lab de arriba su amabilidad y ayuda a lo largo de estos años. Félix, gracias por preocuparte por mí como si fuera una de tus doctorandas. Andrea y Yoli, gracias por vuestras sonrisas y por vuestro buen rollo contagioso. Elena, Patri y María, en vosotras he encontrado 3 amigas increíbles, sois estupendas y tengo mucha suerte de haberos conocido. Estos años de gym, viajes a alumnado y comidas en el italiano no habrían sido iguales sin vosotras.

En el plano personal, me siento la persona más afortunada del mundo, ya que tengo la suerte de caminar muy bien "acompañada".

Mis amigas del alma, Patri, Sandra, Esther y Marta, a parte de que siempre habéis mostrado interés por la tesis y por entender mi trabajo, me habéis enseñado que el nivel de amistad puede sobrepasarse y os habéis convertido en parte de mi familia. Siempre estáis ahí, en primera fila, para celebrar conmigo las cosas buenas y no dejándome nunca en las malas. Gracias por todo nenis.

Creo que si hubiera podido elegir a mi "familia política" no lo hubiera acertado más. Quiero daros las gracias por vuestro apoyo, vuestro amor y por intentar entenderme cuando os explico "lo que hago" en el laboratorio. Yaya Lola, Carmen, Mañón, cuñaos, locas y mis 4 perlas, gracias por quererme tanto.

Gracias a mi familia. Cada uno de vosotros sois un pilar en mi vida que me ayuda a mantenerme en pie. Gracias tía Ana, Nora y Adrián, porque siempre que miro hacia atrás, ahí estáis vosotros compartiendo mi felicidad y mis lágrimas.

Gracias Nacho, el mejor hermano que podría tener. Por creer que soy más lista de lo que soy y por estar orgulloso de "lucecita", ahora ya casi casi si que soy "la doctora de la familia…" jaja! Gracias por haberte casado con mi Pico y habernos dado a Valentina, un regalo que ha venido a alegrarnos la vida justo cuando más falta nos hacía.

Mamá y papá, gracias por todo. Sin vosotros no sería lo que soy. Gracias por la educación que me habéis dado, los valores que me habéis transmitido y por ser mi modelo en la vida. Os quiero mucho. Mamá, tu amor y generosidad incondicional no tiene nombre, eres la bondad en persona. Papá, siempre has sido un ejemplo de trabajo y constancia, y en los últimos años nos has demostrado aún más tu fortaleza y capacidad de lucha y superación. Sólo tú sabes lo que te echo de menos. Sé que desde ahí arriba, con los yayos y el padre de Pepe, vas a ver mi tesis en primera fila, espero que estés tan orgulloso de mí como yo lo estoy de ti.

Pepe, el pilar principal de mi vida, nada tendría sentido si no pudiera compartirlo contigo. Me encanta cuando te hablo de mis "bichos" y mis "ADNs" y haces como que me entiendes. Gracias por estar ahí siempre de manera incondicional. Contigo las alegrías se multiplican y las penas se reducen. La vida a tu lado es más fácil y feliz, y a partir del próximo noviembre, seguro que más emocionante. Te quiero.

Pepito, aún no has llegado y ya nos alegras la vida. Gracias pequeñín por venir a hacernos felices.

Gracias a todos por formar parte de mi vida.

Resumen

La seguridad alimentaria está garantizada cuando se dispone de alimentos suficientes, nutritivos e inócuos. Esta garantía, además, ha de cumplirse a lo largo de todo el proceso productivo, lo que se conoce como "seguridad de la granja a la mesa". Nace así una nueva forma de abordar el problema, con un enfoque global y un tratamiento integral.

Para afrontar este reto, las técnicas moleculares basadas en el empleo de ácidos nucleicos son, actualmente, utilizadas en la detección de amenazas alimentarias, como por ejemplo, alérgenos, microorganismos, organismos genéticamente modificados (OGM), o la autentificación de especies.

Sin embargo, muchos de los métodos en uso presentan limitaciones, ya que son costosos, complicados, y requieren personal y equipamiento especializado. La tecnología de biosensores es una aproximación adecuada, ya que proporciona resultados fiables de manera sencilla y rápida, y con capacidades añadidas como portabilidad y automatización para llevar a cabo los ensayos directamente en puntos de control (POC).

Esta tesis se ha centrado en el desarrollo de un sistema biosensor, basado en la tecnología de disco compacto, para la detección de ácidos nucleicos en aplicaciones de seguridad alimentaria adaptable a POC. Las investigaciones llevadas a cabo han permitido obtener nuevos conocimientos en tecnologías génicas, pudiendo efectuar aportaciones metodológicas de interés caracterizadas por su miniaturización, integración y automatización.

En este sentido, una parte de la investigación aborda la simplificación de la etapa de amplificación del ADN diana, eludiendo el termociclado mediante el empleo de técnicas alternativas a la reacción en cadena de la polimerasa (PCR). Para ello, se han estudiado dos técnicas de amplificación isoterma, la amplificación por recombinasa polimerasa (RPA) y la amplificación por desplazamiento múltiple (MDA). La detección se lleva a cabo mediante ensayos de hibridación con sondas de ADN inmovilizadas en formato micromatriz sobre la superficie de policarbonato de un DVD. Además, la amplificación por RPA se ha

combinado con la detección mediante inmunoensayo ezimático (ELISA) para la determinación simultánea de múltiples analitos.

En otra aproximación, se han integrado las fases de amplificación e hibridación en una única etapa, simplificando aún más el proceso analítico. Para ello, la amplificación isoterma por RPA se realiza en fase sólida sobre la superficie del disco en diferentes formatos: gota, cámaras microfluídicas o microreactores. En los dos primeros formatos, las reacciones tienen lugar en la superficie del DVD y la medición se realiza registrando la intensidad del haz láser del lector reflejado, mientras que en el tercer caso la reacción se lleva a cabo en micropocillos integrados en la estructura del DVD y la lectura se realiza midiendo la intensidad de la señal transmitida.

Finalmente, se ha desarrollado una metodología para monitorizar la síntesis de ADN en tiempo real, integrando así las etapas de amplificación y cuantificación. Para ello, se emplea la reacción de amplificación isoterma mediada por bucle (LAMP), registrando secuencialmente el avance de la reacción mediante cambios turbidimétricos o colorimétricos en el medio de reacción. De este modo, se relaciona el perfil de cada ensayo con el número de copias de cada gen diana, permitiendo así su cuantificación.

Para cada metodología se han establecido las propiedades analíticas, y los resultados obtenidos han sido validados por comparación con técnicas de referencia y mediante el empleo de muestras certificadas. Como prueba de concepto, los diferentes desarrollos se han aplicado a la detección y determinación de la presencia de alérgenos (avellana, cacahuete, soja, tomate y maíz), organismos modificados genéticamente (p35S, tNOS y Bt-11), bacterias patógenas (*Salmonella* spp., *Cronobacter* spp. y *Campylobacter* spp.) y hongos (*Fusarium* spp.), así como la identificación de especies cárnicas.

Resum

La seguretat alimentària està garantida quan es disposa d'aliments suficients, nutritius i innocus. Esta garantia, a més, ha de complir-se al llarg de tot el procés productiu, el que es coneix com a "seguretat de la granja a la taula". Naix així una nova forma d'abordar el problema, amb un enfocament global i un tractament integral.

Per a afrontar este repte, les tècniques moleculars basades en la utilització d'àcids nucleics són, actualment, usades en l'anàlisi de certes amenaces alimentàries, com per exemple, la detecció d'al·lèrgens, microorganismes, organismes genèticament modificats (OGM), o l'autentificació de determinades espècies.

No obstant això, molts dels mètodes en ús presenten limitacions, ja que són costosos, complicats, i requerixen personal i equipament especialitzat. La tecnologia de biosensors és una aproximació adequada, ja que proporciona resultats fiables de manera senzilla i ràpida, i amb capacitats afegides com portabilitat i automatització per a dur a terme els assajos directament en punts de control (POC).

Esta tesi s'ha centrat en el desenvolupament d'un sistema biosensor, basat en la tecnologia de disc compacte, per a la detecció d'àcids nucleics en aplicacions de seguretat alimentària adaptable a POC. Les investigacions dutes a terme han permés obtindre nous coneixements en tecnologies gèniques, podent efectuar aportacions metodològiques d'interés caracteritzades per la seua miniaturització, integració i automatització.

En este sentit, una part de la investigació aborda la simplificació de l'etapa d'amplificació de l'ADN diana, eludint el termociclat per mitjà de l'utilització de tècniques alternatives a la reacció en cadena de la polimerasa (PCR). Per a això, s'han estudiat dos tècniques d'amplificació isoterma, l'amplificació per recombinasa polimerasa (RPA) i l'amplificació per desplaçament múltiple (MDA). La detecció es du a terme per mitjà d'assajos d'hibridació amb sondes d'ADN immobilitzades en format micromatriu sobre la superfície de policarbonat d'un DVD. A més, l'amplificació per RPA s'ha combinat amb la detecció per mitjà

d'inmunoassaig ezimátic (ELISA) per a la determinació simultània de múltiples anàlits.

En una altra aproximació, s'han integrat les fases d'amplificació i hibridació en una única etapa, simplificant encara més el procés analític. Per a això, l'amplificació isoterma per RPA es realitza en fase sòlida sobre la superfície del disc en diferents formats: gota, cambres microfluídicas o microreactors. En els dos primers formats, les reaccions tenen lloc en la superfície del DVD i el mesurament es realitza registrant la intensitat del feix làser del lector reflectit, mentres que en el tercer cas la reacció es du a terme en micropouets integrats en l'estructura del DVD i la lectura es realitza mesurant la intensitat del senyal transmesa.

Finalment, s'ha desenvolupat una metodologia per a monitoritzar la síntesi d'ADN en temps real, integrant així les etapes d'amplificació i quantificació. Per a això, s'utilitza la reacció d'amplificació isoterma mitjançada per bucle (LAMP), registrant sequencialment l'avanç de la reacció per mitjà de canvis turbidimétrics o colorimétrics en la reacció. D'esta manera, es relaciona el perfil de cada assaig amb el nombre de còpies de cada gen diana, permetent així la seua quantificació.

Per a cada metodologia s'han establit les propietats analítiques i els resultats obtinguts han sigut validats per comparació amb tècniques de referència i per mitjà de l'utilització de mostres certificades. Com a prova de concepte, els diferents desenvolupaments s'han aplicat a la detección i determinació de la presència de al·lèrgens (avellana, cacauet, soja, tomata i dacsa), organismes modificats genèticament (p35S, tNOS i Bt-11), bacteris patògens (*Salmonella* spp., *Cronobacter* spp. i *Campylobacter* spp.), fongs (*Fusarium* spp.), així com en la identificació d'espècies càrniques.

Abstract

Food security is guaranteed when there is sufficient, safe and nutritious food. This assurance must be satisfied throughout the entire production process, which is known as "safety from farm to fork". This results in a new way of addressing the problem with a global and comprehensive approach.

To address this challenge, molecular techniques based on the use of nucleic acids are used in the analysis of certain food threatens, such as allergens, microorganisms, genetically modified organisms (GMOs), or food authentication.

However, some of the described methods still have limitations, since they are expensive, complicated, and require specialized staff and equipment. Alternatively, biosensor technology provides reliable results in a simple fast way, with added capabilities such as portability and automation, allowing to perform the analysis directly at points-of-control (POC).

This thesis has focused on developing a biosensor system, based on compact disc technology, for the detection of nucleic acids in food safety applications and adaptable to POC needs. The carried out investigations have yielded new insights into gene technology, making interesting methodological contributions characterized by miniaturization, integration and automation.

The first part of the research deals with the simplification of the amplification step, eluding the thermocycling by using alternatives to the polymerase chain reaction (PCR). To this end, two isothermal amplification techniques have been studied: the recombinase polymerase amplification (RPA) and the multiple displacement amplification (MDA). The detection was performed by hybridization assays with DNA probes immobilized in microarray format on the polycarbonate surface of a DVD. Furthermore, RPA amplification has been combined with detection by an immunoenzymatic assay (ELISA) for the simultaneous detection of multiple analytes.

In another approach, amplification and hybridization have been integrated in a single step, further simplifying the analytical process. For that, the isothermal RPA amplification is performed in solid phase on the surface of the disc in different formats: drop, microfluidic chambers or micro-reactors. In the first two formats,

the reactions take place at the surface of the DVD and the measurement is performed by recording the intensity of the reflected laser. In the third case the reaction is carried out in micro-wells embedded in the DVD substrate and the measurement is performed by measuring the intensity of the transmitted signal.

Finally, a method to real time monitoring DNA synthesis has been developed, integrating the amplification and quantification steps. To this end, the isothermal loop mediated isothermal amplification reaction (LAMP) has been used. The monitoring of the reaction progress is performed by sequentially measuring colorimetric or turbidimetric changes in the reaction mixture. Thus each profile is related to the number of copies of each target gene, allowing their quantitation.

The analytical properties (have been established for each methodology and the obtained results have been validated by comparison with reference techniques and by using certified samples. As proof of concept, the different developments have been applied to the detection and determination of the presence of allergens (hazelnut, peanut, soybean, tomato and maize), genetically modified organisms (p35S, tNOS and Bt-11), pathogenic bacteria (*Salmonella* spp., *Cronobacter* spp. and *Campylobacter* spp.), fungi (*Fusarium* spp.), as well as meat authentication.

Publicaciones

Los resultados de la presente tesis doctoral han dado lugar a las siguientes publicaciones científicas:

Luis A. Tortajada-Genaro, **Sara Santiago-Felipe**, Sergi Morais, José Antonio Gabaldón, Rosa Puchades, Ángel Maquieira, *"Multiplex DNA Detection of Food Allergens on a Digital Versatile Disk"*, J. Agric. Food Chem., **2012**, 60, 36-43.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Recombinase polymerase and enzyme linked immunosorbent assay as a DNA amplificationdetection strategy for food análisis*", Anal. Chim. Acta, **2014**, 811, 81- 87.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Sergi Morais, Rosa Puchades, Ángel Maquieira, "*One-pot isothermal DNA amplification - Hybridisation and detection by a disc-based method*", Sens. Actuators B, **2014**, 197, 385-404.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Sergi Morais, Rosa Puchades, Ángel Maquieira, "*Isothermal DNA amplification strategies for duplex microorganism detection*", Food Chem., **2015**, 174, 509-515.

Luis A. Tortajada-Genaro, **Sara Santiago-Felipe**, Mary Amasia, Aman Russom, Ángel Maquieira, "*Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs)*", RSC Adv., **2015**, 5, 29987-29995.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Parallel solid-phase isothermal amplification and detection by optical disc technology*", Anal. Chem, **2015**, Submitted.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Javier Carrascosa, Rosa Puchades, Ángel Maquieira, "*Micro-reactors on compact discs for real-time loop-mediated isothermal amplification*", Analyst, **2015**, Submitted.

Solicitud de patente:

Ángel Maquieira, Luis A. Tortajada-Genaro, **Sara Santiago-Felipe,** Sergi Morais, "*Dispositivo integrado para el seguimiento de reacciones basado en microreactores en disco óptico*". Presentado el 20-03-2015. En fase de evaluación por la OEPM (P201530371).

Comunicaciones en congresos nacionales e internacionales:

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Extracción de ADN genómico de muestras alimentarias para la detección de alérgenos*", IV Workshop on Sensors and Molecular Recognition, ISBN 978-84-694-0303-7, Valencia, **2010**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Sergi Morais, Tania Arnandis-Chover, Rosa Puchades, Ángel Maquieira, Garbiñe Olabaria, Josu Berganza, "*Multiplex pathogen detection by compact disk technology*", IECB Young Scientist Simposium, Burdeos, **2011**.

Luis A. Tortajada-Genaro, **Sara Santiago-Felipe**, Sergi Morais, Rosa Puchades, Ángel Maquieira, "*Detection of food allergens by micro-arraying on digital versatile disk*", European Lab Automation 2011, Hamburgo, **2011**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Detección del alérgeno alimentario Corylus avellana mediante tecnologías de biosensado de bajo coste*", V Workshop on Sensors and Molecular Recognition, ISBN 978-84-8363-771-5, Valencia, **2011**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Strategies of DNA amplification for food pathogen detection*", 13as jornadas de análisis instrumental, Barcelona, **2011**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Comparación de métodos de aplificación de ADN aplicados a la detección de Salmonella spp. y Cronobacter sakazakii en leche*", VI Workshop on Sensors and Molecular Recognition, ISBN 978-84-695-5262-9, Valencia, **2012**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Combinación de la amplificación isoterma de ácidos nucleicos con revelado enzimático. Prueba de concepto para detección de amenazas en seguridad alimentaria.*", XVIII Reunión SEQA. Sociedad Española de Química Analítica, ISBN 978-84-616-4849-8, Jaén, **2013**.

Luis A. Tortajada-Genaro, **Sara Santiago-Felipe**, Regina Niñoles, Salvador Mena, Sergi Morais, Rosa Puchades, Ángel Maquieira, "*Alternativas en la obtención de información genómica utilizando biosensores basados en discos compactos.*", XVIII Reunión SEQA. Sociedad Española de Química Analítica, ISBN 978-84-616-4849-8, Jaén, **2013**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Amplificación isoterma mediada por bucle como estrategia de cuantificación de ADN*", VII Workshop on Sensors and Molecular Recognition, ISBN 978-84-695-8811-6, Valencia, **2013**.

Luis A. Tortajada-Genaro, **Sara Santiago-Felipe**, Mary Amasia, Aman Russom, Rosa Puchades, Ángel Maquieira, "*LAB-ON-A-DVD: plataforma integrada para el análisis de organismos genéticamente modificados*", VIII Workshop on Sensors and Molecular Recognition, ISBN 978-84-697-1302-0, Valencia, **2014**.

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira, "*Amplificación isoterma en fase sólida para el biosensado de ácidos nucleicos*", VIII Workshop on Sensors and Molecular Recognition, ISBN 978-84-697-1302-0, Valencia, **2014**.

Sara Santiago-Felipe, Mary Amasia, Aman Russom, Ángel Maquieira, Luis A. Tortajada-Genaro, "*DVD discs as Low-cost diagnostics for GMO screening*", 18th International Conference on Miniaturized Systems for Chemistry and Life Sciences (μTAS 2014), Texas, **2014**.

Luis A. Tortajada-Genaro, **Sara Santiago-Felipe**, Rosa Puchades, Ángel Maquieira, "*Sistemas integrados para el desarrollo y seguimiento de reacciones bioanalíticas aplicadas en el análisis genético.*", XX Reunión SEQA. Sociedad Española de Química Analítica, Santiago de Compostela, **2015**.

Abreviaturas y acrónimos

AAPPCC	Análisis de peligros y puntos de control crítico
ADN	Ácido desoxirribonucleico (DNA, deoxyribonucleic acid)
AFM	Microscopio de fuerza atómica
ANOVA	Análisis de la varianza
antiDig	Anticuerpo anti digoxigenina
AP	Fosfatasa alcalina
APN	Ácido peptidonucleico (PNA, peptidonucleic acid)
ARN	Ácido ribonucleico (RNA, ribonucleic acid)
ATCC	Colección americana de cultivos tipo
BCIP	5-Bromo-4-cloro-3-indolil fosfato
BD	Disco Blu-Ray
BLAST	Basic Local Alignment Search Tool
Btn	Biotina
CCD	Dispositivo de carga acoplada
CCGU	Colección de cultivos de la Universidad de Goteborg
CD	Disco compacto
CD-R	Disco compacto grabable
CD-RW	Disco compacto regrabable
CFU	Unidades formadoras de colonia
CE	Comisión Europea
CECT	Colección española de cultivos tipo
CRM	Material certificado de referencia
Ct	Ciclo umbral
CTAB	Bromuro de hexadeciltrimetilamonio
Dig	Digoxigenina
DMSO	Dimetilsulfóxido
dsADN	ADN de cadena doble
dNTPs	Desoxinucleótidos
Dig-dUTPs	Desoxinucleótidos marcados con digoxigenina
DVD	Disco versátil digital

ECDC	Centro Europeo para la Prevención y el Control de las Enfermedades
EDTA	Ácido etilendiaminotetraacético
EFSA	Agencia Europea de Seguridad Alimentaria
ELISA	Ensayo por inmunoabsorción ligado a enzimas
FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura
HDA	Amplificación dependiente de helicasa
HRP	Peroxidasa de rábano picante
IDM	Instituto Interuniversitario de Reconocimiento Molecular y Desarrollo Tecnológico
IR	Infrarrojo
LAMP	Amplificación isoterma mediada por bucle
LED	Diodo emisor de luz
LMG	Colección belga de cultivos
LOD	Límite de detección
LPA	Ligación dependiente de sonda
MDA	Amplificación por desplazamiento múltiple
M-Disc	Disco Millennial
NASBA	Amplificación basada en la secuencia de ácidos nucleicos
NBT	Nitroazul de tetrazolio
OGM	Organismo genéticamente modificado (GMO, genetically modified organism)
OPD	Ortofenildiamina
pb	Pares de bases (bp, base pairs)
PBS	Tampón fosfato salino
PC	Policarbonato
PCR	Reacción en cadena de la polimerasa
PE	Poliestireno
PEG	Polietilenglicol
PIF	Leche maternizada

PMMA	Polimetilmetacrilato de metilo
POC	Punto de control
QCM	Microbalanza de cristal de cuarzo
RCA	Amplificación en círculo rodante
RPA	Amplificación por recombinasa polimerasa
RSD	Desviación estándar relativa
rt-PCR	PCR en tiempo real
SDA	Amplificación por desplazamiento de hebra
spp.	Varias especies del mismo género
SPR	Resonacia de plasmón superficial
sp-RPA	RPA en fáse sólida
ssADN	ADN de cadena simple
ssARN	ARN de cadena simple
SSB	Proteínas de unión a cadena simple
SSC	Tampón salino de citrato de sodio
SNR	Relación señal ruido (S/N)
TBE	Tampón tris-borato-EDTA
TMB	3,3',5,5'-tetrametilbencidina
UE	Unión Europea
UV-Vis	Ultravioleta visible
µTAS	Microsistemas de análisis total
WGA	Amplificación del genoma completo

Índice

Índice de Figuras

Índice de Tablas

1. Introducción

1.1 Seguridad alimentaria

Una alimentación suficiente y sana posibilita el crecimiento, la regeneración y reparación de tejidos, aportando la energía necesaria para los procesos vitales. La Declaración Universal de Derechos Humanos y la Constitución Española recogen el derecho a la protección de la salud, y obligan a los poderes públicos a garantizar la seguridad y salud de los consumidores. Por tanto, la seguridad alimentaria constituye un derecho de todos los seres humanos que ha de ser reconocido.

La Cumbre Mundial de Alimentos organizada en 1996 por la FAO, definió que "*Existe seguridad alimentaria cuando todas las personas tienen en todo momento acceso físico y económico a suficientes alimentos inocuos y nutritivos para satisfacer sus necesidades alimentarias y sus preferencias en cuanto a los alimentos a fin de llevar una vida activa y sana*".[1] El concepto de seguridad en esta definición es doble, implicando por una parte, seguridad en el acceso, y por otra, seguridad respecto a la inocuidad de los alimentos, la cual se ve amenazada en no pocas ocasiones por elementos de riesgo.

Las graves crisis alimentarias sufridas a lo largo de la historia han provocado una mayor sensibilización en materia de seguridad alimentaria. Por un lado, los métodos de control se han ampliado a toda la cadena del proceso productivo, incluyendo elaboración, empaquetado, distribución, venta y consumo del producto final. Surge así una nueva forma de abordar el problema con un enfoque global y un tratamiento integral del consumo de alimentos que va "de la granja a la mesa".[2] Por otra parte, la mayor sensibilización del consumidor respecto de la seguridad alimentaria ha provocado un mayor nivel de exigencia, de modo que, cada vez más, los consumidores tratan de influir en qué se produce, y cómo se produce y comercializa.

A consecuencia de ello, aparece la necesidad de establecer la trazabilidad de los alimentos, entendida ésta como la capacidad de poder identificar el origen de

[1] Cumbre Mundial sobre la Alimentación, 1996. Plan de Acción de la Cumbre Mundial sobre la Alimentación, 13-17 de noviembre de 1996, FAO, Roma.
[2] Libro Blanco sobre Seguridad Alimentaria. 2000, Comisión de las comunidades europeas, Bruselas.

un alimento y su recorrido a lo largo de toda su vida útil. Esto facilita localizar e identificar aquellos puntos de la cadena donde se produce una ruptura de la seguridad alimentaria y evitar, además, actividades fraudulentas. Por otro lado, la obligatoriedad de la implantación de sistemas de Análisis de Peligros y Puntos de Control Crítico (APPCC) en las industrias alimentarias, contribuyen considerablemente a disminuir el riesgo de exposición de los consumidores. Todo ello ha dado lugar a que se ponga en práctica una normativa muy amplia y una metodología de control muy importante.

Las tecnologías disponibles actualmente se presentan como potentes herramientas de control de los sistemas de seguridad alimentaria y trazabilidad. Las nuevas tecnologías de la información y la comunicación han revolucionado tanto el etiquetado como la gestión de la información, mejorando extraordinariamente los sistemas de trazabilidad actuales. Por otra parte, los avances científicos ofrecen enormes posibilidades para mejorar los sistemas de seguridad alimentaria. Así, mientras que los avances dirigidos a la optimización de procesos productivos, conservación o envasado de alimentos, inciden de forma indirecta en una mejora de la seguridad alimentaria, las nuevas herramientas analíticas presentan una elevada sensibilidad y una mayor versatilidad en sus posibilidades de aplicación, contribuyendo a mejorar directamente los sistemas de control.[3]

1.2 Agentes que amenazan la inocuidad de los alimentos

La inocuidad de los alimentos se ve amenazada por la presencia de diferentes agentes que pueden ser propios o ajenos al mismo, añadidos con o sin intención, o aparecer tras ciertos tratamientos durante el procesado industrial, y que originan graves consecuencias cuando se ingieren.[3,4] Estos agentes pueden clasificarse en diferentes grupos según su naturaleza (Figura 1). La presente tesis

[3] AESA (Agencia Española de Seguridad Alimentaria) y Genoma España. 2005, ISBN: 84-609-5044-1.
[4] RASFF (Rapid Alert System for Food and Feed). 2013, Directorate general for health and consumer protection, European Commission, Brussels.

profundiza en aquellos agentes que pueden ser detectados, principalmente, mediante metodologías génicas.

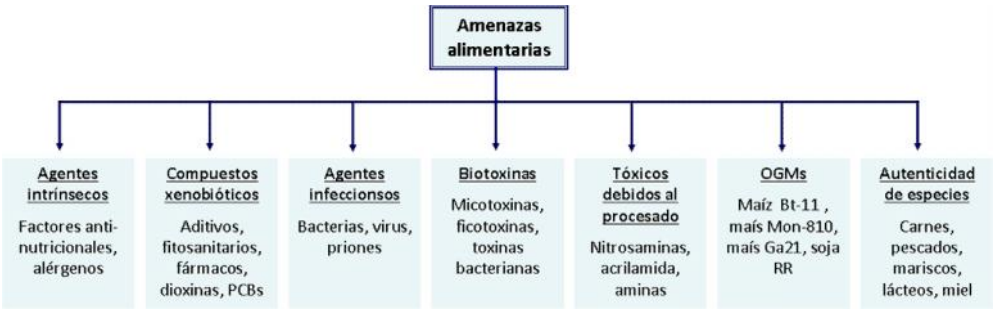


Figura 1. Clasificación de las posibles amenazas en seguridad alimentaria.

1.2.1 Agentes intrínsecos

Se encuentran en los alimentos de manera natural, es decir, no son añadidos ni debidos a procesos industriales o contaminaciones. Se pueden distinguir dos tipos: factores antinutricionales y alérgenos.

Los alérgenos alimentarios son aquellos componentes que pueden inducir una reacción de hipersensibilidad (alergia) en personas susceptibles, debido a su ingestión, contacto o inhalación. Esta reacción no se relaciona con ningún efecto fisiológico del alimento, sino que es de patogenia inmunitaria, es decir, sólo se produce en algunos individuos tras la toma de incluso muy pequeñas cantidades del alérgeno. Todos los alimentos pueden ser potencialmente alergénicos, y su prevalencia está en relación con los hábitos de alimentación, pudiendo variar con la edad y, fundamentalmente, con los procesos de maduración inmunológica y fisiológica.[5,6]

Considerando que los alérgenos alimentarios pueden causar serios problemas a dosis muy bajas y que no existe una terapia curativa para los procesos de hipersensibilidad, es evidente que los individuos afectados deben evitar completamente los alimentos que desencadenan en su organismo la reacción alérgica. Por ello, en el ámbito europeo es obligatorio indicar, en el etiquetado,

[5] AESAN (Agencia Española de Seguridad Alimentaria y Nutrición). Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición sobre Alergias Alimentarias, 2007.
[6] Burks, W., Ballmer-Weber, B. K. *Mol. Nutr. Food Res.*, 2006, 50, 595-603.

los ingredientes que pertenezcan a alguno de los catorce grupos alimentarios incluidos en el anexo de la Directiva 2007/68/CE,[7] con independencia del porcentaje en peso que representen en el producto final. Estos grupos de alérgenos potenciales son: cereales que contengan gluten (trigo, centeno, cebada, avena, espelta, kamut o sus variedades híbridas); crustáceos y productos a base de crustáceos; moluscos y derivados; huevos y productos a base de huevos; leche y derivados; pescado y productos a base de pescado; cacahuetes y productos a base de cacahuetes; soja y productos a base de soja; altramuces y derivados; frutos de cáscara (almendras, avellanas, nueces, anacardos, pecanas, castañas de Pará, pistachos, nueces de macadamia) y derivados; apio y derivados; mostaza y derivados; granos de sésamo y derivados; y anhídrido sulfuroso y sulfitos (en concentraciones superiores a 10 mg/kg o 10 mg/L).

Además, hay que considerar que los consumidores pueden verse expuestos a los llamados alérgenos "ocultos", denominados así por la dificultad de poder identificar su existencia en el alimento antes de su ingestión (larvas de anisakis, látex, etc.). Estos, entre otras causas, pueden ser el resultado de contaminaciones cruzadas o de fraudes.[8] En este contexto, el desarrollo de sistemas de control efectivos, puede disminuir significativamente los casos de exposición de los consumidores que padecen este tipo de alergias.

1.2.2 Agentes infecciosos

Se conocen como agentes infecciosos aquellos microorganismos (bacterias, virus, hongos o priones) capaces de producir una infección o enfermedad transmisible. En los últimos años, se ha documentado un incremento en la incidencia de afecciones alimentarias producidas por la ingestión de microorganismos. Según el Centro Europeo para la Prevención y el Control de las Enfermedades (ECDC) y la Agencia Europea de Seguridad Alimentaria (EFSA), en el

[7] Directiva 2007/68/CE de la comisión de 27 de noviembre de 2007 que modifica el anexo III bis de la Directiva 2000/13/CE del Parlamento Europeo y del Consejo por lo que se refiere a determinados ingredientes alimentarios.
[8] Anibarro, B., Seoane, F. J., Mugica, M. V. *J. Invest. Allerg. Clin.*, 2007, 17, 168-172.

año 2012 se registraron 5363 brotes de enfermedades transmitidas por alimentos en la Unión Europea, de los cuales 477 correspondieron a España.[9]

Entre las bacterias más frecuentes que producen este tipo de enfermedades se encuentran *Salmonella* y *Campylobacter* (Figura 2a y Figura 2b), que suelen causar casi el 40 % de los casos de gastroenteritis benignas. Existen otros agentes, como *Cronobacter sakazakii* (Figura 2c), que aunque tienen menor importancia numérica causan una elevada mortalidad en recién nacidos.

Los principales virus transmitidos a través de los alimentos y el agua son los virus Norwalk o norovirus, causantes de gastroenteritis, con una incidencia de aproximadamente el 14% de los casos registrados en 2012.[9]

Los hongos pueden estar presentes en una gran variedad de alimentos y su principal peligro radica en las toxinas que producen. Las micotoxinas más relevantes desde el punto de vista de la salud son las aflatoxinas, ocratoxina A, fumonisina B1, tricotecenos y zearalenona,[4] y su aparición suele estar ligada a un tipo concreto de producto, como por ejemplo, las fumonisinas en maíz.

Los países de la Unión Europea participan de un sistema común de medidas de protección contra enfermedades alimentarias que se recogen en la Directiva 2003/99/CE.[10]

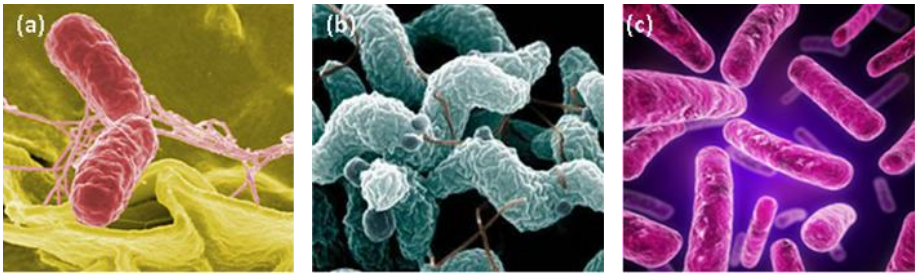


Figura 2. Ejemplo de bacterias patógenas:[11] (a) *Salmonella typhimurium*; (b) *Campylobacter jejuni*; (c) *Cronobacter sakazakii.*

[9] EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA Journal*, 2014, 12, 3547-3859

[10] Directiva 2003/99/CE del Parlamento Europeo y del Consejo de 17 de noviembre de 2003 sobre la vigilancia de las zoonosis y los agentes zoonóticos y por la que se modifica la Decisión 90/ 424/CEE del Consejo y se deroga la Directiva 92/117/CEE del Consejo.

[11] https:// www.medicalxpress.com (Fecha de consulta Enero/2015)

1.2.3 Organismos genéticamente modificados

Un organismo genéticamente modificado (OGM) es un organismo al cual se le ha alterado su genoma mediante técnicas de ingeniería genética. Son variedades de especies conocidas a las que se les ha conferido alguna capacidad funcional (detectable y heredable) a partir de la incorporación de factores hereditarios (genes) de otras especies. El material genético transferido se denomina transgén, y consiste en una secuencia promotora, el gen de interés, y una secuencia terminadora.

La transformación genética puede contribuir en sí misma al aumento de la seguridad alimentaria mediante la obtención de variedades más resistentes o productivas. Sin embargo, esta tecnología comporta una serie de riesgos potenciales que han de tenerse en cuenta desde el punto de vista de la seguridad alimentaria, como por ejemplo, flujo incontrolado de genes, pérdida de biodiversidad, o aparición de resistencias y alergias. Por ello, la legislación europea es muy estricta en cuanto a la aprobación de nuevas variedades de OGMs y el etiquetado de los alimentos que los contienen, siendo obligatorio en alimentos con más de un 0,9% de OGM. [12,13]

La detección de los OGMs se basa en la determinación de diversos elementos genéticos (Figura 3).[13,14] Los elementos de *screening* permiten identificar la secuencia del promotor o del terminador del transgén, y su detección permite identificar la mayoría de los transgénicos. Los elementos específicos de taxón permiten identificar los genes de la planta huésped, mejorando la caracterización de las especies involucradas y aumentando así la selectividad. Entre los elementos que permiten la detección del propio transgén se encuentran los específicos de gen -que identifican el propio gen introducido-, los específicos de construcción -

[12] Reglamento (CE) 1830/2003 del Parlamento Europeo y del Consejo de 22 de septiembre de 2003 relativo a la trazabilidad y al etiquetado de organismos modificados genéticamente y a la trazabilidad de los alimentos y piensos producidos a partir de éstos, y por el que se modifica la Directiva 2001/18/CE.

[13] Marmiroli, N., Maestri, E., Gullì, M., Malcevschi, A., Peano, C., Bordoni, R., De Bellis, G. *Anal. Bioanal. Chem.*, 2008, 392, 369-384.

[14] Elenis, D. S., Kalogianni, D. P., Glynou, K., Ioannou, P. C., Christopoulos T. K. *Anal. Bioanal. Chem.*, 2008, 392, 347-354.

que permiten identificar el promotor usado y el propio transgén-, y los específicos de evento -que permiten identificar una parte del transgén y del sitio de integración-.

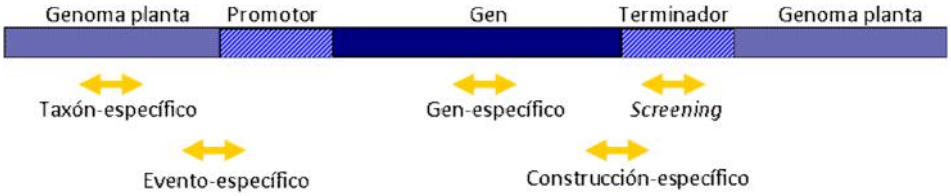


Figura 3. Esquema de las partes de un transgén y los principales elementos de detección.

1.2.4 Autenticidad de especies

La identificación y autentificación de especies (pescados, aves, ganado, vegetales, frutos, especias, etc.), y la detección de su presencia en productos alimentarios, es de gran importancia en el ámbito de la seguridad y calidad alimentaria. Esto permite la detección de fraudes, en los que se ha sustituido algún ingrediente por otro de menor precio. Además, cuando se modifica la composición de un alimento se pueden originar problemas de salud, como reacciones alérgicas, intoxicaciones o la transmisión incontrolada de enfermedades, como la encefalopatía bovina espongiforme.[15] También hay que considerar los posibles conflictos religiosos y/o culturales que pueden surgir.[16]

Los productos relacionados con mayor frecuencia con fraudes alimentarios son, principalmente, los derivados cárnicos, pescados, mariscos y derivados, productos lácteos y aceite.[17]

[15] Calvo, J. H., Rodellar, C., Zaragoza, P., Osta, R. *J. Agric. Food Chem.*, 2002, 50, 5262-5264.
[16] Asensio, L., González, I., García, T., Martín, R. Food Control, 2008, 19, 1-8.
[17] Mafra, I., Ferreira, I. M., Oliveira, M. B. P. *Eur. Food Res. Technol.*, 2008, 227, 649-665.

1.3 Métodos moleculares empleados en seguridad alimentaria

Las técnicas de detección empleadas en seguridad alimentaria han de cumplir dos requisitos fundamentales. En primer lugar, han de ser técnicas analíticas exactas y sensibles, que permitan la detección de los compuestos en los límites deseados. En segundo lugar, debido a la complejidad y diversidad de las matrices en que se encuentran, han de presentar una selectividad adecuada, que permita efectuar el análisis sin que interfieran los numerosos contaminantes presentes en la muestra.

Las técnicas de referencia -químicas y microbiológicas- son susceptibles de importantes mejoras en cuanto a rapidez, prestaciones, sencillez y robustez. Por ello, existe una demanda en cuanto al desarrollo de métodos alternativos que superen estas limitaciones.

Las técnicas basadas en bioreconocimiento molecular se están empleando con éxito en el campo de la seguridad alimentaria, ya que son rápidas, sencillas, sensibles, selectivas, abaratan el coste de análisis y permiten su portabilidad, lo que aumenta las oportunidades de aplicación.[18,19]

Los dos grupos de técnicas moleculares más establecidas en seguridad alimentaria son las inmunológicas y las génicas.[16]

1.3.1 Métodos inmunológicos

En este apartado se encuentran los ensayos basados en la unión específica de un anticuerpo a un antígeno. Un anticuerpo es una proteína producida por el organismo como respuesta inmunológica a un “invasor” extraño, mientras que el antígeno es la sustancia que el cuerpo está tratando de eliminar o bloquear mediante la respuesta inmunológica. El factor determinante de estos métodos es la elección de los inmunoreactivos apropiados, de los que dependerá tanto la sensibilidad como la selectividad del ensayo.

[18] Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., Uyttendaele, M. *Food Microbiol.*, 2010, 27, 710-730.
[19] Arvanitoyannis, I. S., Kotsanopoulos, K. V., Papadopoulou, A. *Crit. Rev. Food Sci.*, 2014, 54, 1473-52.

En general, los inmunoensayos son técnicas selectivas y sensibles, y tienen la ventaja de poder detectar prácticamente cualquier analito. Así pues, son numerosos los inmunoensayos disponibles para la detección y cuantificación de diferentes analitos alimentarios. Diversos autores han revisado la aplicación de este tipo de técnicas para la autentificación de especies, y la detección de alérgenos, GMOs y bacterias patógenas.[16,20-23]

Entre los formatos más comunes se encuentran los Western-blot, los tests de aglutinación o las tiras reactivas, siendo el ensayo por inmunoabsorción ligado a enzimas (ELISA) el más ampliamente utilizado. Este ensayo se basa en la detección de la proteína inmovilizada sobre una fase sólida mediante su unión específica a anticuerpos marcados.[3,16] El formato ELISA más empleado es el denominado "sándwich", en el que el antígeno es reconocido por dos anticuerpos, demostrando una elevada sensibilidad y reproducibilidad.[22] Los sucesivos avances en investigación han permitido el desarrollo de numerosos kits comerciales basados en este formato. [16,21,22]

Sin embargo, los inmunoensayos poseen ciertas limitaciones. Se ha demostrado que tras algunos tratamientos de procesado industrial, el contenido proteico de las muestras es más inestable que el contenido en ácidos nucleicos, lo que puede influir negativamente en la sensibilidad y robustez del análisis.[24] Esto se ve confirmado por diversos estudios en los que con métodos basados en ácidos nucleicos se obtienen sensibilidades mejores o comparables a las obtenidas mediante técnicas inmunológicas.[16] En la Tabla 1 se comparan los límites de detección alcanzados por ambas técnicas para la detección de diferentes analitos. Además, se ha visto que mediante las técnicas de ácidos nucleicos se alcanza una mejor selectividad que con las inmunoquímicas. Esto se debe a que las técnicas basadas en ácidos nucleicos amplifican la concentración de analito en lugar de la señal, lo que conlleva una menor aparición de falsos positivos.[22,25]

[20] Besler, M. *Trends Anal. Chem.*, 2001, 20, 662-672.
[21] van Hengel, A. J. *Anal. Bioanal. Chem.*, 2007, 389, 111-118.
[22] Zhao, X., Lin, C. W., Wang, J., Oh, D.H. *J. Microbiol. Biotechnol.*, 2014, 24, 297-312.
[23] Deisingh, A. K., Badrie, N. *Food Res. Int.*, 2005, 38, 639-649.
[24] Arlorio, M., Cereti, E., Coïsson, J. D., Travaglia, F., Martelli, A. *Food Control*, 2007, 18, 140-148.
[25] Scharf, A., Kasel, U., Wichmann, G., Besler, M. *J. Agric. Food Chem.*, 2013, 61, 10261-10272.

Tabla 1. Comparación de resultados obtenidos mediante métodos de detección basados en ácidos nucleicos e inmunoensayo.

Analito	**Límite de detección**	
	Basados en ADN*	**Basados en inmunoensayo***
Aeromonas spp.	1 CFU/mL[26]	10^3 CFU/mL[26]
E. coli O157 (toxina shiga)	30 CFU/mL[27]	$1{,}2\cdot10^3$ CFU/mL[28]
Salmonella spp.	$2\cdot10^2$ CFU/mL[29]	$9{,}2\cdot10^3$ CFU/mL[30]
C. jejuni	$4\cdot10^1$ CFU/mL[29]	10^4 CFU/mL[31]
Altramuz	10 ppm[32]	10 ppm[33]
Cacahuete	< 10 ppm[34]	< 10 ppm[34]
Avellana	< 10 ppm[35]	< 10 ppm[35]

*Referencia bibliográfica.

1.3.2 Métodos génicos

Se basan en la detección de una secuencia de ADN concreta y específica de la especie a detectar. Normalmente incluyen una reacción de amplificación que aumenta exponencialmente la concentración de ADN de interés, lo que permite alcanzar una elevada sensibilidad. El método más empleado para la amplificación del ADN es la reacción en cadena de la polimerasa (PCR).[36] Se basa en el empleo de una enzima polimerasa termoestable que sintetiza una cadena de ADN complementaria a una cadena de ADN sencilla. Para ello, necesita la presencia de secuencias cortas de ADN, denominadas cebadores, que deben ser

[26] Arora, S., Agarwal, R. K., Bist, B. *Int. J. Food Microbiol.*, 2006, 106, 177-183.
[27] Fach, P., Perelle, S., Grout, J., Dilasser, F. *J. Microbiol. Meth.*, 2003, 55, 383-392.
[28] Zhang, Y., Tan, C., Fei, R., Liu, X., Zhou, Y., Chen, J., Chen, H., Zhou, R., Hu, Y. *Anal. Chem*., 2014, 86, 1115-1122.
[29] Hong, Y., Berrang, M. E., Liu, T., Hofacre, C. L., Sanchez, S., Wang, L., Maurer, J. J. *Appl. Environ. Microb*., 2003, 69,3492-3499.
[30] Park, S., Kim, Y. T., Kim, Y. K. *BioChip J.*, 2010, 4, 110-116.
[31] Xu, F., Xu, D., Ming, X., Xu, H., Li, B., Li, P., Aguilar, Z. P., Cheng, T., Wu, X., Wei, H. *J. Nanosci. Nanotechnol.*, 2013, 13, 4552-45529.
[32] Ecker, C., Ertl, A., Pulverer, W., Nemes, A., Szekely, P., Petrasch, A., Linsberger-Martin, G., Cichna-Markl, M. *Food Chem.*, 2013, 141, 407-418.
[33] Galan, A. M. G., Brohée, M., Scaravelli, E., van Hengel, A. J., Chassaigne, H. *Eur. Food Res. Technol.*, 2010, 230, 597-608.
[34] Stephan, O., Vieths, S. *J. Agric. Food Chem.*, 2004, 52, 3754-3760.
[35] Holzhauser, T., Stephan, O., Vieths, S. *J. Agric. Food Chem.*, 2002, 50, 5808-5815.
[36] Mullis, K. B., Faloona, F. A. *Method. Enzymol.*, 1987, 155, 335-350.

complementarias de los extremos de la secuencia que se desea copiar. La reacción consta de tres fases que se repiten un número determinado de ciclos:

- Desnaturalización: la doble hélice de ADN se separa en dos hebras de cadena simple.
- Alineamiento: los cebadores se unen a sus zonas complementarias del ADN de cadena sencilla.
- Elongación: se sintetiza la cadena complementaria de ADN a partir de los cebadores.

En la Figura 4 se muestran las fases de cada ciclo y el aumento exponencial del número de copias de la región de interés.

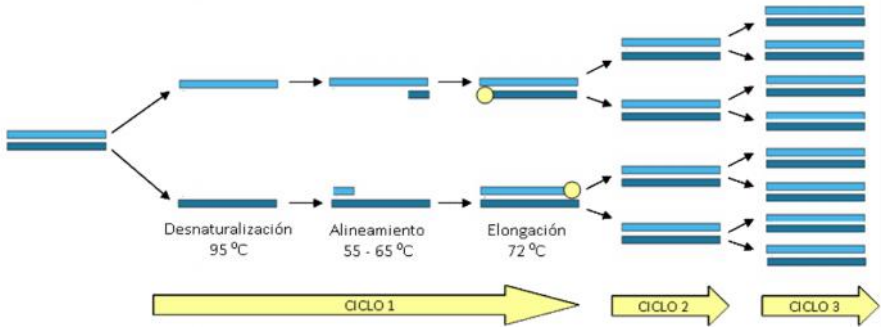


Figura 4. Fases de la PCR.

La sencillez y versatilidad de la técnica han permitido su aplicación a múltiples áreas (clínica, industrial, etc.), siendo numerosos los trabajos aplicados en el campo de la seguridad alimentaria.[17,21,22,37] En aplicaciones microbiológicas ha supuesto una revolución, ya que problemas tales como la dificultad de detección de determinadas dianas o la imposibilidad de aislamiento *in vitro* de algunos microorganismos, pueden obviarse mediante PCR. Además, esta técnica permite realizar ensayos múltiples, es decir, detectar varias especies o varios genes de una especie en una misma muestra y reacción, con el consiguiente ahorro de tiempo y reactivos. Sin embargo, la combinación de más de un analito en la reacción puede

[37] Shrestha, H. K., Hwu, K. K., Chang, M. C. *Trends Food Sci. Tech.*, 2010, 21, 442-454.

reducir la sensibilidad y selectividad del ensayo, debido a la competencia de los cebadores e interacciones cruzadas.[38]

Existen numerosas técnicas de identificación de los fragmentos amplificados, como la secuenciación, o la electroforesis en gel de agarosa seguida de la tinción con un fluoróforo intercalante. No obstante, dos de las técnicas más extendidas son la hibridación en formato de micromatriz y la PCR en tiempo real (rt-PCR).[3,39]

a) Micromatrices de ADN

Los ensayos en formato de micromatriz utilizan un soporte sobre el que se sitúa el material biológico formando una matriz ordenada, de manera que las coordenadas de cada punto de la matriz están totalmente identificadas, permitiendo su análisis y posterior interpretación (Figura 5). Según sea el material inmovilizado, se tienen los ADN *arrays* o *genechips*, los *proteinchips* y los *tissuechips*.

El formato de *microarray* de ácidos nucleicos se basa en la unión de una cadena de secuencia conocida con su cadena complementaria. Permiten trabajar con una elevada densidad de sondas o moléculas de reconocimiento, y la posibilidad de gestionar grandes cantidades de información en un tiempo reducido y al menor coste posible, proponiéndose como una de las plataformas de discriminación más eficaces. Estos dispositivos han sido ampliamente empleados en el campo de la genómica, contando con numerosas aplicaciones clínicas, farmacéuticas y agroalimentarias.[39]

El primer *microarray* comercialmente disponible fue producido por la empresa Affymetrix en el año 1994, con una capacidad de 16.000 sondas de ADN, que permitía el análisis simultáneo de miles de genes.[40,41] Desde entonces, ha habido un importante crecimiento de la oferta comercial (Agilent, Applied Microarrays,

[38] Edwards, M. C., Gibbs, R. A. *Genome Res.*, 1994, 3, S65-S75.
[39] Pirrung, M. C. *Angew. Chem. Int. Edit.*, 2002, 41, 1276-1289.
[40] Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X. C., Stern, D., Winkler, J., Lockhart, D., J., Morris, M. S., Fodor,Fodor, S. P. *Science*, 1996, 274, 610-614.
[41] Goffeau, A. *Nature*, 1997, 385, 202.

Roche NimbleGen, CombiMatrix's CMOS,…), existiendo en el mercado una gran diversidad de biochips. [42,43]

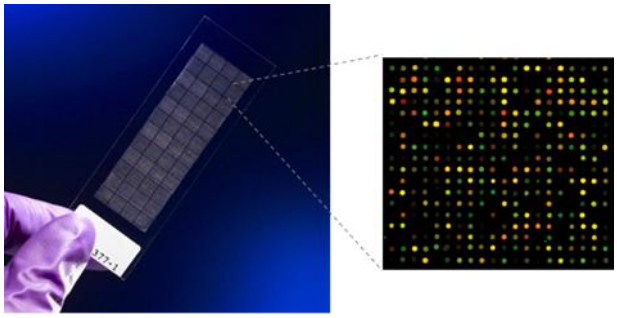


Figura 5. Ejemplo de biochip comercial.[44]

Los ácidos nucleicos de las muestras a analizar se marcan normalmente en el proceso de amplificación, y se incuban sobre la micromatriz de sondas permitiendo la hibridación (reconocimiento y unión entre cadenas complementarias) y, por tanto, la identificación y cuantificación de los ácidos nucleicos presentes en la muestra (Figura 6).

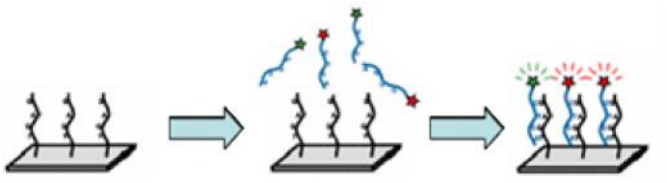


Figura 6. Esquema del proceso de hibridación en micromatrices de ácidos nucleicos.

b) <u>PCR en tiempo real</u>

La PCR en tiempo real (rt-PCR), también llamada PCR cuantitativa (qPCR), se caracteriza por realizar la detección y cuantificación de los productos amplificados a medida que la reacción tiene lugar. Para ello, la amplificación es monitorizada a partir de una señal generada a medida que el proceso avanza y que, en la mayoría de los casos, es una señal de fluorescencia. De este modo, el aumento de la señal

[42] Hardiman, G. *Pharmacogenomics*, 2004, 5, 487-502.
[43] Dixit, C. K. *J. Biochips Tiss. Chips*, 2013, 3, e124.
[44] http://www.affymetrix.com (Fecha de consulta Enero/2015)

en cada ciclo se corresponde con un incremento de la concentración del producto amplificado. El número de ciclo en el cual la señal amplificada alcanza un valor por encima del ruido de fondo (ciclo umbral, Ct "threshold cycle") está relacionado directamente con la cantidad de ácido nucleico diana inicial (Figura 7).[45] Se trata de una técnica rápida y sencilla, que disminuye las posibilidades de contaminación cruzada al eliminar el análisis postamplificación de la PCR convencional. Sin embargo, la determinación simultánea de varios analitos presenta el inconveniente de requerir un fluoróforo diferente para cada diana, lo que reduce las posibilidades de multiplexado.[46] Además, su uso requiere equipos particulares, que en conjunto suponen una inversión de 30.000-60.000 €, y dificultan su automatización y portabilidad.

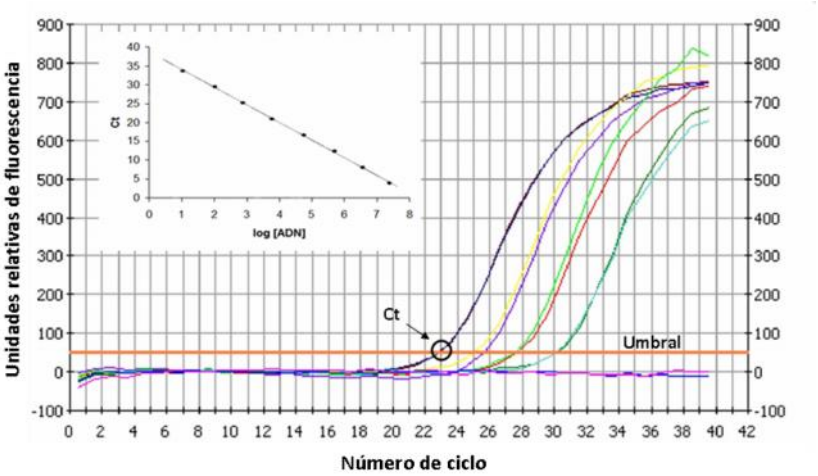


Figura 7. Curvas de amplificación de una PCR en tiempo real: señal de fluorescencia con respecto al número de ciclos. Inserto: relación lineal del ciclo umbral (Ct) frente al log de la concentración de ADN.[45]

[45] http://www.analyticalbiotech.es (Fecha de consulta Enero/2015)

[46] Pierik, A., Boamfa, M., van Zelst, M., Clout, D., Stapert, H., Dijksman, F., Broer, D., Wimberger-Friedl, R. *Lab Chip*, 2012, 12, 1897-1902.

1.3.3 Técnicas mixtas

Estas técnicas combinan los métodos inmunológicos y génicos en el mismo proceso, de manera que un método interviene en la etapa de reconocimiento del analito y el otro en la detección de dicho bioreconocimiento. De este modo, se combina la gran selectividad de los inmunoensayos con la elevada sensibilidad asociada a la amplificación por PCR, dando lugar a métodos con mejores prestaciones. Dos de las técnicas más extendidas son la inmuno-PCR y la PCR-ELISA.

En la inmuno-PCR, en primer lugar, se realiza el bioreconocimiento del analito mediante un anticuerpo conjugado a un oligonucleótido y, a continuación, se lleva a cabo la PCR como sistema de detección.[47,48] Se ha demostrado que esta técnica es especialmente útil para la detección de ciertos virus y toxinas alimentarias, obteniéndose límites de detección entre 100 y 10.000 veces superiores a los obtenidos mediante ELISA o rt-PCR. Sin embargo, se ha observado que esta elevada sensibilidad en ocasiones puede generar falsos positivos.[49]

La técnica PCR-ELISA permite la inmunodetección de los productos de la PCR tras ser inmovilizados en una microplaca. La principal ventaja de este método es su bajo coste en comparación con otras técnicas similares (micromatrices en soporte de vidrio, rt-PCR, electroforesis capilar…).[50] Su alta selectividad y sensibilidad le confieren un enorme potencial como herramienta analítica.

1.3.4 Aplicaciones en seguridad alimentaria

Tal como se ha indicado, los métodos inmunológicos, génicos y las técnicas mixtas, se han empleado con éxito en el control de la seguridad alimentaria. No obstante, la selección de un método u otro ha dependido principalmente del analito estudiado.

---

[47] Niemeyer, C. M., Adler, M., Wacker, R. *Trends Biotechnol.*, 2005, 23, 208-216.
[48] Lei, J., Li, P., Zhang, Q., Wang, Y., Zhang, Z., Ding, X., Zang, W. *Anal. Chem.*, 2014, 86, 10841-10846.
[49] Adler, M., Wacker, R., Niemeyer, C. M. *Analyst*, 2008, 133, 702-718.
[50] Sue, M. J., Yeap, S. K., Omar, A. R.,Tan, S. W. Application of PCR-ELISA in Molecular Diagnosis. *BioMed Res. Int.*, 2014, Article ID 653014, 6 pages, doi:10.1155/2014/653014.

Hasta la fecha, se han descrito numerosos inmunoensayos en diferentes formatos, especialmente para la detección de alérgenos y toxinas.[16,21] Los métodos basados en tiras reactivas se han propuesto para, por ejemplo, la detección de alérgenos,[51] toxinas bacterianas,[52] proteínas expresadas por transgenes[53] o proteínas de origen animal.[54] Estos ensayos destacan por su rapidez y sencillez, aunque generalmente sólo permiten realizar análisis cualitativos unianalito, lo que limita sus aplicaciones.[16]

El formato Western-blot se considera más una técnica propia de labores de investigación que de análisis rutinarios, siendo útil en la detección de proteínas insolubles.[55] Así, se ha empleado en algunas aplicaciones de detección de alérgenos[56] o proteínas transgénicas, [57] con límites de detección similares a los obtenidos mediante otros inmunoensayos.

El ensayo ELISA utilizando placas de poliestireno (PE) y detección colorímetrica es el formato más extendido. Existen numerosos trabajos aplicados a la detección de alérgenos,[32,35] bacterias patógenas,[26,58] proteínas de expresión de transgenes,[57] proteínas de leche[59] y otras proteínas de origen animal.[60] Destacan aplicaciones desarrolladas mediante detección luminiscente[28] o inmuno-PCR,[61-63] con sensibilidades superiores a las obtenidas mediante ELISA clásico.

A pesar de que los inmunoensayos son prácticos y efectivos, en general son menos sensibles que los métodos basados en ácidos nucleicos (Tabla 1 y Tabla

[51] Stephan, O., Moller, N., Lehmann, S., Holzhauser, T., Vieths, S. *Eur. Food Res. Technol.*, 2002, 215, 431-436.
[52] Aldus, C. F., van Amerongen, A., Ariens, R. M. C., Peck, M. W., Wichers, J. H., Wyatt, G. M., *J. Appl. Microbiol.*, 2003, 95, 380-389.
[53] Fagan, J., Schoel, B., Haegert, A., Moore, J., Beeby, J. *Int. J. Food Sci. Tech.*, 2001, 36, 357-367.
[54] Myers, M. J., Yancy, H. F., Farrell, D. E., Washington, J. D., Frobish, R. A. *J. Food Protect.*, 2005, 68, 2656-2664.
[55] Ahmed, F. E. *Trends Biotechnol.*, 2002, 20, 215-223.
[56] Blais, B. W., Phillippe, L. *J. Food. Prot.*, 2001, 64, 895-898.
[57] Kuiper, H. A. *Food control*, 1999, 10, 339-349.
[58] Kumar, S., Balakrishna, K., Batra, H. V. *Biomed. Environ. Sci.*, 2008, 21, 137-143.
[59] López-Calleja, I. M., González, I., Fajardo, V., Hernández, P. E., García, T., Martín, R. *Int. Dairy J.*, 2007, 17, 87-93.
[60] Liu, L., Chen, F. C., Dorsey, J. L., Hsieh, Y. H. P. J. *Food Sci.*, 2006, 71, M1-M6.
[61] Henterich, N., Osman, A. A., Mendez, E., Mothes, T. *Mol. Nutr. Food Res.*, 2003, 47, 345-348.
[62] Zhang, W., Bielaszewska, M., Pulz, M., Becker, K., Friedrich, A. W., Karch, H., Kuczius, T. *J. Clin. Microbiol.*, 2008, 46, 1292-1297.
[63] Roth, L., Zagon, J., Ehlers, A., Kroh, L. W., Broll, H. *Anal. Bioanal. Chem.*, 2009, 394, 529-537.

2).[17] Además, hay que tener en cuenta que el procesado de alimentos puede alterar la estructura de las proteínas, perjudicando el reconocimiento antígeno-anticuerpo.[17,24,26] En el caso particular de los OGMs, puede ocurrir que la proteína no este suficientemente expresada a pesar de contener el transgén, dando lugar a falsos negativos.[17,55] Por ello, existe una tendencia a desarrollar aplicaciones de seguridad alimentaria basadas en la detección del ADN.[16]

La amplificación por PCR, combinada con diferentes métodos para la detección de los productos, es la técnica más extendida. Se han descrito numerosos trabajos que emplean la electroforesis en gel de agarosa para la separación de los fragmentos amplificados, por ejemplo, para la detección de bacterias patógenas,[26] OGMs,[64] o la identificación del origen de leches.[65] Sin embargo, en estas aplicaciones, la capacidad de multiplexado puede verse limitada. Destaca el empleo de la electroforesis capilar, la cual permite una mayor separación y automatización de los análisis.[14,66] Se han publicado trabajos que combinan la separación por electroforesis capilar con la amplificación simultánea de múltiples dianas mediante un único par de cebadores,[67,68] aunque, la sensibilidad alcanzada es ligeramente inferior a la obtenida mediante otras metodologías génicas.

Se ha demostrado que la amplificación enzimática que tiene lugar en la PCR-ELISA mejora considerablemente la sensibilidad.[49] Además, esta técnica permite realizar un análisis múltiple a un coste relativamente bajo, habiéndose aplicado

[64] Hernandez, M., Rodriguez-Lazaro, D., Zhang, D., Esteve, T., Pla, M., Prat, S. *J. Agric. Food Chem.*, 2005, 53, 3333-3337.
[65] Maudet, C., Taberlet, P. *J. Dairy Res.*, 2001, 68, 229-235.
[66] García-Cañas, V., González, R., Cifuentes, A. *Electrophoresis*, 2004, 25, 2219-2226.
[67] Unterberger, C., Luber, F., Demmel, A., Grünwald, K., Huber, I., Engel, K. H., Busch, U. *Eur. Food Res. Technol.*, 2014, 239, 559-566.
[68] Moreano, F., Ehlert, A., Busch, U., Ángel, K. H. *Eur. Food Res. Technol.*, 2006, 222, 479-485.

para la detección de alérgenos,[35,69] bacterias patógenas,[70,71] GMOs[72] o autentificación de especies.[73]

Los ensayos de hibridación sobre micromatrices de ADN se han consolidado como una técnica excelente para la realización de ensayos masivos y protocolos de barrido (*screening*), debido a su sencillez, rapidez y elevada selectividad. Se han publicado un gran número de estudios para la detección múltiple de alérgenos,[74] bacterias patógenas,[75-77] OGMs,[78-80] y la autentificación de especies animales[81] y vegetales,[82] alcanzándose sensibilidades comparables a las obtenidas mediente otras metodologías génicas de referencia como la rt-PCR.

La PCR en tiempo real, basada en ensayos fluorométricos de hibridación, es la técnica más utilizada en aplicaciones de seguridad alimentaria,[14] sobre todo, en aquellos casos en que es necesario cuantificar los analitos. Así, la rt-PCR se ha empleado, con excelentes resultados, en numerosos trabajos de control de etiquetado,[17] detección de diferentes tipos de analitos como alérgenos,[32,34,83-85]

[69] Stephan, O., Vieths, S. *J. Agric. Food Chem.*, 2004, 52, 3754-3760.
[70] Perelle, S., Dilasser, F., Malorny, B., Grout, J., Hoorfar, J., Fach, P. *Mol. Cell. Probe.*, 2004, 18, 409-420.
[71] Yuanhong, L., Lin, C., Chong, Z., Qiming, C., Fengxia, L., Xiaomei, B., Zhaoxin, L. *Int. Dairy J.*, 2013, 33, 27-33.
[72] Glynou, K., Ioannou, P. C., Christopoulos, T. K. *Anal. Bioanal. Chem.*, 2004, 378, 1748-1753.
[73] Taboada, L., Sánchez, A., Velasco, A., Santaclara, F. J., Perez Martín, R. I., Sotelo, C. G. *J. Agric. Food Chem.*, 2014, 62, 5699-5706.
[74] Rossi, S., Scaravelli, E., Germini, A., Corradini, R., Fogher, C., Marchelli, R. *Eur. Food Res. Technol.*, 2006, 223, 1-6.
[75] Suo, B., He, Y., Paoli, G., Gehring, A., Tu, S. I., Shi, X. *Mol. Cell. Probe.*, 2010, 24, 77-86.
[76] Wang, M., Cao, B., Gao, Q., Sun, Y., Liu, P., Feng, L., Wang, L. *J. Clin. Microbiol.*, 2009, 47, 3178-3184.
[77] Hong, B. X., Jiang, L. F., Hu, Y. S., Fang, D. Y., Guo, H. Y. *J. Microbiol. Meth.*, 2004, 58, 403-411.
[78] Leimanis, S., Hernández, M., Fernández, S., Boyer, F., Burns, M., Bruderer, S., Glouden, T., Harris, N., Kaeppeli, O., Philipp, P., Pla, M., Puigdomènech, P., Vaitilingom, M., Bertheau, Y., Remacle, *J. Plant Mol. Biol.*, 2006, 61, 123-139.
[79] Xu, J., Zhu, S., Miao, H., Huang, W., Qiu, M., Huang, Y., Fu, X., Li, Y., *J. Agric. Food Chem.*, 2007, 55, 5575-5579.
[80] Germini, A., Rossi, S., Zanetti, A., Corradini, R., Fogher, C., Marchelli, R. *J. Agric. Food Chem.*, 2005, 53, 3958-3962.
[81] Peter, C., Brünen-Nieweler, C., Cammann, K., Börchers, T. *Eur. Food Res. Technol.*, 2004 219, 286-293.
[82] Rønning, S. B., Rudi, K., Berdal, K. G., Holst-Jensen, A. *J. Agric. Food Chem.*, 2005, 53, 8874-8880.
[83] Hupfer, C., Waiblinger, H. U., Busch, U. *Eur. Food Res. Technol.*, 2007, 225, 329-335.
[84] Pafundo, S.; Gullì, M.; Marmiroli, N. Food Chem., 2009, 116, 81-815.

microorganismos (bacterias,[86,87] hongos,[88,89] virus[90]) y OGMs,[91-94] y en la identificación de carne,[95] fruta,[96] pescado[97] o leche.[98]

En la Tabla 2 se recogen las características de los métodos moleculares aplicados en seguridad alimentaria aplicados en diferentes matrices.

1.3.5 Tendencias actuales

Como respuesta a las demandas de los consumidores y productores, cada vez más interesados en aspectos relacionados con calidad, autenticidad, salud y bienestar, los desarrollos y legislación en materia de seguridad alimentaria han experimentado cambios profundos en los últimos años. En la Unión Europea, el énfasis se ha centrado en garantizar la seguridad e inocuidad de los alimentos, incluyendo la evaluación científica como referencia para la toma de decisiones.[3] La industria alimentaria ha reaccionado, en primer lugar, proporcionando una mayor información sobre los componentes de los alimentos y, con más o menos rigor, publicitando la inocuidad y efectos beneficiosos de sus productos.

[85] Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., Waiblinger, H. U. *Eur. Food Res. Technol.*, 2010, 230, 367-374.
[86] Hyeon, J. Y., Park, C., Choi, I. S., Holt, P. S., Seo, K. H. *Int. J. Food Microbiol.*, 2010, 144, 177-181.
[87] Wang, X., Zhu, C., Xu, X., Zhou, G. *Food Control*, 2012, 25, 144-149.
[88] Suanthie, Y., Cousin, M. A., Woloshuk, C. P. *J. Stored Prod. Res.*, 2009, 45, 139-145.
[89] Scauflaire, J., Godet, M., Gourgue, M., Liénard, C., Munaut, F. *Fungal Biolog.*, 2012, 116, 1073-1080.
[90] Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N., Katayama, K. *J. Clin. Microbiol.*, 2003, 41, 1548-1557.
[91] Cottenet, G., Blancpain, C., Sonnard, V., Chuah, P. F. *Anal. Bioanal. Chem.*, 2013, 405, 6831-6844.
[92] Xu, W., Yuan, Y., Luo, B., Bai, W., Zhang, C., Huang, K. *J. Agric. Food Chem.*, 2009, 57, 395-402.
[93] Lee, S.H., Kim, J. K., Yi, B. Y. *J. Agric. Food Chem.*, 2007, 55, 3351-3357.
[94] Köppel, R., Sendic, A., Waiblinger, H. U. *Eur. Food Res. Technol.*, 2014, 239, 653-659.
[95] Tasara, T., Schumacher, S., Stephan, R. *J. Food Prot.*, 2005, 68, 2420-2426.
[96] Serradilla, M. J., Hernández, A., Ruiz-Moyano, S., Benito, M. J., López-Corrales, M., de Guía Córdoba, M. *Food Control*, 2013, 30, 679-685.
[97] Lopez, I., Pardo, M. A. *J. Agric. Food Chem.*, 2005, 53, 4554-4560.
[98] López-Calleja, I., González, I., Fajardo, V., Martín, I., Hernández, P. E, García, T., Martín, R. *Food Control*, 2007, 18, 1466-1473.

Tabla 2. Características de los métodos moleculares desarrollados para aplicaciones de seguridad alimentaria.

Agente	Analito	Método de detección	LOD*	Referencia
Alérgenos	Avellana y cacahuete	Tira reactiva	0,0001%	51
	Nuez de Brasil	Western-blot	0,0005%	56
	Avellana	ELISA	<0,001%	35
	Altramuz	ELISA	0,001%	33
	Gluten	Inmuno-PCR	0,000016%	61
	Marisco, pescado y crustáceos	MLPA y electroforesis capilar	0,01%	67
	Avellana	PCR-ELISA	<0,001%	35
	Cacahuete	PCR-ELISA	0,001%	69
	Avellana y cacahuete	*Microarray* de ADN	65 fmol	74
	Altramuz	rt-PCR	0,001%	32
	Cacahuete	rt-PCR	0,0001%	34
	Apio	rt-PCR	0,0005%	83
	Almendra, cacahuete, anacardo, nuez y sésamo	rt-PCR	5-0,5 pg	84
	Avellana, cacahuete, mostaza, soja, huevo, leche, almendra y sésamo	rt-PCR	0,01%	85
Agentes infecciosos	*E. coli*	Tiras reactivas	10^4 CFU/mL	52
	Aeromonas spp.	ELISA	10^3 CFU/mL	26
	Salmonella spp.	ELISA	10^4 CFU/mL	58
	E. coli	Inmunoensayo quimioluminiscente	10^3 CFU/mL	28
	E. coli	Inmuno-PCR	> 10 pg/mL	62
	Salmonella spp.	PCR-ELISA	10^3 CFU/mL	70
	Cronobacterspp.	PCR-ELISA	10^2 CFU/mL	71
	E. coli, *S. enterica*, *L. monocytogenes*, y *C. jejuni*	*Microarray* de ADN	10^3 CFU/mL	75
	E. sakazakii, S. enterica, K. pneumoniae, K.a oxytoca, S. marcescens, A.baumannii, B. cereus, L. monocytogenes, S. aureus, y E. coli	*Microarray* de ADN	10^4 CFU/mL	76
	E. coli, C. jejuni, S.dysenteriae, V. cholerae, V.parahaemolyticus, P. vulgaris, B.cereus, L. monocytogenes, C.botulinum, S. enterica, Y.enterocolitica, S.aureus, C. perfringens y S.pyogenes	*Microarray* de ADN	10 CFU/mL	77
	Salmonella spp. y *Cronobacter spp.*	rt-PCR	10^3 CFU/mL	86
	Cronobacter spp.	rt-PCR	10^3 CFU/mL	87

Tabla 2. Continuación.

Agente	Analito	Método de detección	LOD*	Referencia
	Aspergillus spp., *Penicillium spp.*, y *Fusarium spp.*	rt-PCR	1 pg	88
	Fusarium spp.	rt-PCR	5-50 pg	89
	Novovirus	rt-PCR	10 copias	90
OGMs	Genes cp4 epsps y Cry1Ab	Tiras reactivas	<10%	53
	Gen cp4 epsps	Western-blot	0,25-1%	57
	Gen cp4 epsps	ELISA	0,25-1,4%	57
	Maíz	Inmuno-PCR	6 amol	63
	Maíz	PCR y electroforesis en gel de agarosa	0,005%	64
	Maíz y soja	MLPA y electroforesis capilar	0,1%	68
	Genes p35S y tNOS	PCR-ELISA	0,05%	72
	Genes p35S, tNOS y npt11	*Microarray* de ADN	< 0,3%	78
	Soja y maíz	*Microarray* de ADN	0,5-1%	79
	Maíz y soja	*Microarray* de ADN	65 fmol	80
	Maíz, soja, patata, arroz, colza y algodón	rt-PCR	<0,04%	91
	Maíz	rt-PCR	0,1%	92
	Maíz	rt-PCR	0,05%	93
	Genes p35S, tNOS, PFMV, PAT, cp4 epsps y CPT2	rt-PCR	0,1%	94
Autenticidad	Proteína animal	Tiras reactivas	2%	54
de especies	Leche de vaca	ELISA	1%	59
	Cerdo	ELISA	0,5-0,05%	60
	Ternera	ELISA	0,05%	60
	Leche de vaca	PCR y electroforesis en gel de agarosa	0,1%	65
	Bacalao, abadejo y maruca	PCR-ELISA	123 pg	73
	Ternera, cerdo, pollo, pavo, oveja y cabra	*Microarray* de ADN	0,1%	81
	Trigo, cebada, avena, arroz, maíz y centeno	*Microarray* de ADN	20-40 ng	82
	Ternera	rt-PCR	0,001-0,1%	95
	Cerezas	rt-PCR	10 pg	96
	Atún	rt-PCR	0,25 ng	97
	Leche de cabra y oveja	rt-PCR	0,5%	98

*LOD: Límite de detección expresado en porcentaje (%), concentración (CFU/mL, pg/mL) o cantidad (fmol, pg, copias, ng), según los resultados de cada trabajo.

Esto ha propiciado el desarrollo de mejores procesos de producción y la demanda de métodos rigurosos de evaluación analítica que garanticen la seguridad e inocuidad de los alimentos y permitan la detección fiable y a tiempo de sus componentes. Además, esto ha de cumplirse a lo largo de los diferentes puntos de control de la cadena de producción. Así pues, existe la necesidad de contar con nuevas tecnologías que sean rápidas, selectivas y sensibles, y que permitan su portabilidad y automatización, sin suponer una pérdida en sus prestaciones y un incremento en el coste.

En los últimos años se ha observado un aumento en la actividad investigadora en el área de los biosensores. Esta tecnología puede proporcionar la misma fiabilidad que los métodos establecidos, pero de manera más sencilla y con menores tiempos de respuesta. Sin embargo, la mayoría de los biosensores disponibles comercialmente son de aplicación médica, por lo que en ámbitos como el agroalimentario, todavía hay mucho trabajo por hacer antes de que estos sistemas sensores se conviertan en una alternativa real competitiva.[99]

1.4 Biosensores

Un biosensor se define como un dispositivo integrado capaz de proporcionar información analítica cuantitativa o semicuantitativa. Está compuesto por un elemento de reconocimiento biológico (ácido nucleico, anticuerpo, enzima, tejido, etc.) que se encuentra en contacto con un elemento de transducción, el cual permite procesar la señal generada por la interacción entre el elemento de reconocimiento y el analito.[100] Como resultado de esta unión se produce la variación de una o varias propiedades físico-químicas (pH, transferencia electrónica, calor, potencial, masa, propiedades ópticas, etc.) que son detectadas por el transductor, el cual transforma la respuesta en una señal secundaria, generalmente eléctrica, que posteriormente se procesa para ser presentada en forma de información específica (Figura 8).

[99] Van Dorst, B., Mehta, J., Bekaert, K., Rouah-Martin, E., De Coen, W., Dubruel, P., Blust, R., Robbens, J. *Biosens. Bioelectron.*, 2010, 26, 1178-1194.
[100] Thévenot, D. R., Toth, K., Durst, R. A., Wilson, G. S. *Biosens. Bioelectron.*, 2001, 16, 121-131.

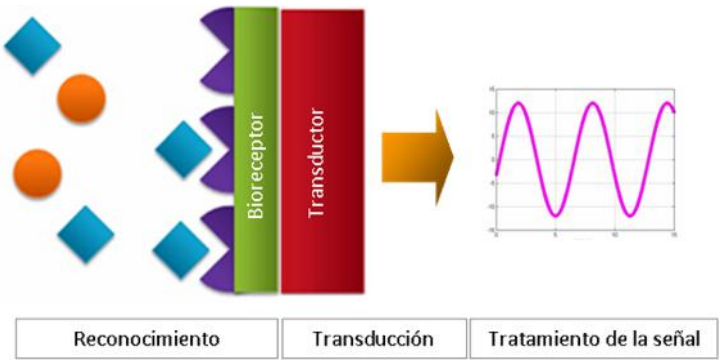


Figura 8. Esquema del funcionamiento básico de un biosensor.

El concepto básico de sensor biológico apareció en 1962, cuando Clark y Lyons desarrollaron una técnica electroquímica pionera para la determinación de la concentración de O_2, CO_2, iones, azúcar y urea, directamente en sangre.[101] Sin embargo, fue en 1977 cuando se desarrolló el primer dispositivo utilizando microorganismos vivos inmovilizados en la superficie de un electrodo sensible a amonio. Este dispositivo se utilizaba para detectar el aminoácido arginina y sus creadores lo denominaron "sensor bio-selectivo".[102] Posteriormente, se denominó "biosensor", término que ha permanecido desde entonces.

Los biosensores constituyen una herramienta analítica muy útil debido a propiedades tales como:[103]

- Elevada selectividad y sensibilidad.
- Robustez y reproducibilidad.
- Bajo coste de producción.
- Tiempos de análisis reducidos.
- Pretratamiento de muestra sencillo o innecesario.
- Manejo fácil que no requiere personal cualificado.
- Portabilidad, que permite realizar análisis *in situ*.
- Automatización, que facilita su integración dentro de sistemas de monitorización.

[101] Clark, L. C., Lyons, C. *Ann. NY Acad. Sci.*, 1962, 102, 29-45.
[102] Rechnitz, G. A., Kobos, R. K., Riechel, S. J., Gebauer, C. R. *Anal. Chim. Acta*, 1977, 94, 357-365.
[103] González-Rumayor, V., García-Iglesia, E., Ruiz-Galán., O. Aplicaciones de biosensores en la industria agroalimentaria. CEIM/Dirección General de Universidades e Investigación, 2005, pp. 94.

- Miniaturización.
- Bajos requerimientos operativos y de mantenimiento.
- Mayor capacidad multianálisis.

No obstante, existe una amplia variedad de biosensores y no todos poseen cada una de las características citadas anteriormente. La combinación de varias de ellas sitúa a los biosensores en una posición ventajosa frente a otras técnicas de análisis.

1.4.1 Clasificación de los biosensores

Los sistemas biosensores se clasifican según el tipo de interacción que se establece entre el elemento de reconocimiento y el analito, el método utilizado para detectar dicha interacción, la naturaleza del elemento de reconocimiento, el formato de ensayo o el sistema de transducción (Tabla 3).

1.4.1.1 Tipo y modo de detección de la interacción

Los biosensores se categorizan, de acuerdo al tipo de interacción sonda-diana, en sensores biocatalíticos o de bioafinidad. En los primeros, el elemento de reconocimiento cataliza una reacción en la que, directa o indirectamente, se encuentra involucrado el analito de interés. La detección se realiza midiendo la aparición de productos o la desaparición de reactivos en el medio de reacción. Los sensores de bioafinidad se basan en la interacción del analito con el elemento de reconocimiento, formando un complejo analito-receptor.

En ambos casos, la detección de la interacción puede realizarse de dos maneras, directa e indirecta. La detección directa o sin marcaje (label-free) se basa en los cambios producidos en una propiedad físico-química relacionada con la biointeracción, o en la aparición de un fenómeno relacionado con la misma, por ejemplo, variación del índice de refracción, generación de una especie luminiscente, o estimación de la impedancia eléctrica.

En los sistemas de detección indirecta, se recurre al marcaje. Los marcadores empleados son muy diversos, pudiendo utilizarse enzimas, elementos

radioactivos, fluoróforos, moléculas quimioluminiscentes, partículas metálicas o cofactores enzimáticos, entre otros.

Tabla 3. Elementos para la clasificación de los biosensores.

Tipo de interacción	**Detección de la interacción**
- Biocatalítica	- Directa
- Afinidad	- Indirecta
Elemento de reconocimiento	**Sistema de transducción**
- Proteínas	- Electroquímico
· Enzimas	· Conductimétricos
· Anticuerpos	· Potenciométricos
· Receptores celulares	· Amperométricos
- Ácidos nucleicos	· Impedimétricos
· ADN	- Óptico
· ARN	· Resonancia de plasmón superficial
- Elementos supramoleculares naturales	· Fibra óptica
· Células u orgánulos celulares	· Interferometría
· Tejidos	· Absorción UV-Vis
· Bacterias	· Fluorescencia
· Virus	- Piezoeléctrico
· Fagos	· Microbalanza de cristal de cuarzo
- Elementos biomiméticos	· Ondas acústicas
· Polímeros de impresión molecular (MIPs)	· Magnetoelástico
· Aptámeros	- Entalpimétrico
· Ácidos peptidonucleicos (APNs)	- Nanomecánico

1.4.1.2 Elementos de reconocimiento

Se clasifican en cuatro categorías principales (Tabla 3), siendo las proteínas y los ácidos nucleicos los más empleados en el desarrollo de biosensores.[104]

Los biosensores de ácidos nucleicos utilizan el fenómeno de hibridación de sondas. Esta unión, formada a través de puentes de hidrógeno entre las bases nitrogenadas, es altamente estable, selectiva y reversible.[105]

[104] Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C. *Biotechnol. Adv.*, 2010, 28, 232-254.
[105] Perumal, V., Hashim, U. *J. Appl. Biomed.*, 2014, 12, 1-15.

En la actualidad se pueden encontrar gran cantidad de aplicaciones basadas en biosensores de ADN, incluyendo la identificación de organismos modificados genéticamente, alérgenos alimentarios y agentes patógenos.[99,103,104]

Como alternativa al uso de sondas de ácidos nucleicos naturales, se han desarrollado otros elementos de reconocimiento, como los aptámeros y los ácidos peptidonucleicos (APNs). Un aptámero es un oligonucleótido de cadena sencilla sintetizado artificialmente, capaz de reconocer de forma específica y con alta afinidad diversas moléculas diana mediante un plegamiento tridimensional de su cadena (Figura 9a).[99] Los APNs son moléculas sintéticas similares a los ácidos nucleicos en las que el esqueleto de azúcar-fosfato se ha reemplazado por una cadena de 2-(N-aminoetil)-glicina, unidas por enlace peptídico (Figura 9b).[106] Estas moléculas presentan una mayor estabilidad y una mayor capacidad de hibridación que los ácidos nucleicos naturales, sin embargo, su aplicación práctica posee serios inconvenientes, ya que son caros y, dependiendo de su secuencia, tienden a formar agregados y son difíciles de purificar.[104] Estas moleculas se han utilizado en diversas aplicaciones agroalimentarias, muchas de las cuales se recogen en una revisión de Sforza *et al.*[106]

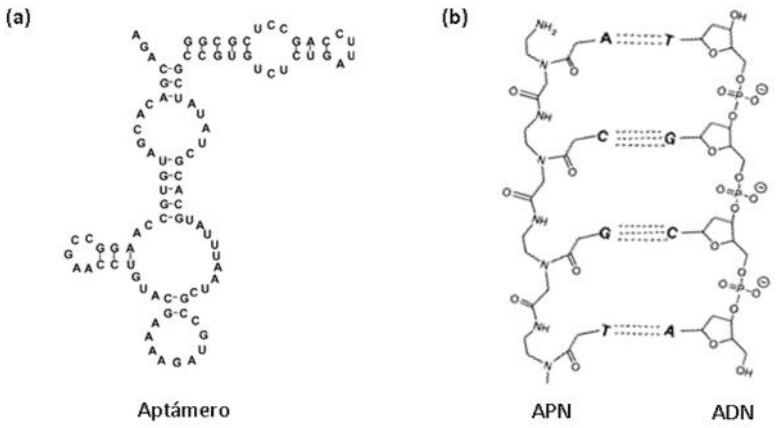


Figura 9. Esquema de (a) la estructura de un áptamero[107] y (b) de la unión entre una hebra de ADN y APN.[108]

[106] Sforza, S., Corradini, R., Tedeschi, T., Marchelli, R. *Chem. Soc. Rev.*, 2011, 40, 221-232.
[107] Li, N., Nguyen, H. H., Byrom, M., Ellington, A. D. *PLoS One*, 2011, 6, e20299.
[108] Pellestor, F., Paulasova, P., Macek, M., Hamamah, S. *Hum. Reprod*., 2004, 19, 1946-1951.

1.4.1.3 Sistema de transducción

Existen muchos tipos de transductores, los cuales convierten las variaciones de la propiedad físico-química utilizada para poner de manifiesto la interacción bioreceptor-analito en una señal analítica que puede ser amplificada, almacenada y registrada (Tabla 3). Los electroquímicos, piezoeléctricos y ópticos son los transductores más comunes en biosensores de ADN.[99] La elección del transductor generalmente viene determinada por las exigencias particulares de cada estudio o aplicación.

Los transductores electroquímicos se basan en la medida de cambios en la corriente, potencial o impedancia inducidos a causa del reconocimiento entre el bioreceptor y el analito. Estos sensores son económicos, rápidos y fácilmente miniaturizables, por lo que se han empleado en múltiples trabajos.[99,109]

Los sistemas de transducción piezoeléctricos o másicos miden cambios de masa inducidos por la formación del complejo bioreceptor-analito. Estos sistemas se han utilizado en diversas aplicaciones, muchas de las cuales se recogen en las revisiones de Van Dorst *et al.*[99] y Singh *et al.*[109] Su principal ventaja radica en que permiten la detección directa sin marcaje con una elevada sensibilidad. Sin embargo, son caros (detector y electrodos) y su capacidad de multiplexado es limitada.[103]

Los transductores ópticos se basan en la medición de las variaciones que se producen en las propiedades de la luz como consecuencia de la interacción bioreceptor-analito. Los registros pueden deberse a cambios en fluorescencia, absorción, interferometría, luminiscencia, dispersión, reflexión o refracción en la superficie de reconocimiento. Avances recientes en el campo de la nanotecnología han contribuido significativamente al desarrollo de nuevos biosensores colorimétricos. La conjugación de biomoléculas con nanopartículas ha dado lugar a biosensores más competitivos, sensibles y selectivos, siendo particularmente destacable el empleo de nanopartículas de oro.[110] También destacan los biosensores basados en resonancia de plasmón superficial (SPR), que

[109] Singh, R., Mukherjee, M. D., Sumana, G., Gupta, R. K., Sood, S., Malhotra, B. D. *Sensor. Actuat. B-Chem.*, 2014, 197, 385-404.
[110] Fu, Z., Zhou, X., Xing, D. *Sens. Actuat. B-Chem.*, 2013, 182, 633-641.

ofrecen una respuesta rápida, sencilla y directa.[22,26,104] Además, el desarrollo de la resonacia plasmón superficial por imagen (iSPR), ha permitido aumentar la capacidad de multiplexado de estos sistemas.[111,112] Sin embargo, su principal inconveniente radica en su elevado coste.

1.4.2 Ejemplos de biosensores de ADN en seguridad alimentaria

Hasta la fecha, se han descrito múltiples biosensores electroquímicos, siendo la detección amperométrica, voltamétrica e impedimétrica las más comunes.[104] Estos dispositivos se han empleado con éxito para la detección de toxinas,[113,114] xenobióticos,[115-117] bacterias patógenas,[118] alérgenos,[119] autentificación de carnes[120] y OGMs,[121] alcanzándose elevados niveles de sensibilidad (Tabla 4).

Entre los biosensores piezoeléctricos, el más empleado en detección de ADN es la balanza de microcristal de cuarzo (QCM), destacando aplicaciones para detección unianalito de microorganismos patógenos[122-124] y OGMs.[125]

Los biosensores ópticos son los más utilizados para determinación de ácidos nucleicos en aplicaciones de seguridad alimentaria. Uno de los más extendidos es la SPR, encontrándose múltiples trabajos aplicados a la detección de OGMs[126,127] y

[111] Homola, J., Vaisocherová, H., Dostálek, J., Piliarik, M. *Methods*, 2005,37, 26-36.
[112] Piliarik, M., Párová, L., Homola, J. *Biosens. Bioelectron.*, 2009, 24, 1399-1404.
[113] Siontorou, C. G., Nikolelis, D. P., Miernik, A., Krull, U. J. *Electrochim. Acta*, 1998, 43, 3611-3617.
[114] Wei, F., Ho, C. M. *Anal. Bioanal. Chem.*, 2009, 393, 1943-1948.
[115] Babkina, S. S., Ulakhovich, N. A. *Bioelectrochemistry*, 2004, 63, 261-265.
[116] Kim, Y. S., Jung, H. S., Matsuura, T., Lee, H. Y., Kawai, T., Gu, M. B. *Biosens. Bioelectron.*, 2007, 22, 2525-2531.
[117] Kim, Y. S., Niazi, J. H., Gu, M. B. *Anal. Chim. Acta*, 2009, 634, 250-254.
[118] Liu, N., Gao, Z. X. Chin. *J. Anal. Chem.*, 2007, 35, 638-642.
[119] Bettazzi, F., Lucarelli, F., Palchetti, I., Berti, F., Marrazza, G., Mascini, M. *Anal. Chim. Acta*, 2008, 614, 93-102.
[120] Ahmed, M. U., Hasan, Q., Hossain, M. M., Saito, M., Tamiya, E. *Food Control*, 2010, 21, 599-605.
[121] Yang, J., Yang, T., Feng, Y. Y., Jiao, K. *Anal. Biochem.*, 2007, 365, 24-30.
[122] Wu, V. C., Chen, S. H., Lin, C. S. *Biosens. Bioelectron.*, 2007, 22, 2967-2975.
[123] Tombelli, S., Mascini, M., Sacco, C., Turner, A. P. *Anal. Chim. Acta*, 2000, 418, 1-9.
[124] Chen, S. H., Wu, V. C. H., Chuang, Y. C., Lin, C. S. *J. Microbiol. Methods*, 2008, 73, 7-17.
[125] Mannelli, I., Minunni, M., Tombelli, S., Mascini, M. *Biosens. Bioelectron.*, 2003, 18, 129-140.
[126] D'Agata, R., Corradini, R., Ferretti, C., Zanoli, L., Gatti, M., Marchelli R., Spoto, G. *Biosens. Bioelectron.*, 2010, 25, 2095-2100.
[127] Mariotti, E., Minunni, M., Mascini, M. *Anal. Chim. Acta*, 2002, 453, 165-172.

microorganismos patógenos.[128,129] Algunos de ellos se encuentran disponibles comercialmente para la detección de *E. coli* 0157:H7 (Spreeta™) y para la identificación de *L. monocytogenes* y *Salmonella* spp. (Biacore 3000). [104]

A pesar de que la detección bioluminiscente es poco empleada por la dificultad de implantarla en biosensores compactos, se encuentran algunos estudios de identificación de toxinas[130] o bacterias patógenas,[131] en los que se alcanzan límites de detección comparables o mejores a los obtenidos mediante otros sistemas transductores (Tabla 4).

La detección colorimétrica destaca en aplicaciones que requieren una fase de revelado enzimático o metalográfico. Así, se ha empleado para la detección de alérgenos,[35,69] OGMs[72,132] y autentificación de cafés,[133] principalmente basadas en ensayos PCR-ELISA o tiras reactivas.

La detección fluorescente es la más extendida, ya que es el sistema de detección por excelencia en ensayos en tiempo real y en micromatrices. En el contexto de la seguridad alimentaria se han descrito una gran cantidad de estudios para la detección múltiple de alérgenos,[85] OGMs,[78,79] microorganismos patógenos,[77,134] toxinas,[135] y la autentificación de especies vegetales[82] y animales,[95] permitiendo alcanzar, en general, elevados niveles de sensibilidad (Tabla 4).

En cuanto al bioreconocimiento, en general, la gran mayoría de los biosensores descritos se basan en la afinidad que poseen las hebras simples de ADN (ssADN) por sus cadenas complementarias, siendo, por tanto, las ssADN los principales elementos empleados como sondas de reconocimiento. Sin ambargo, destacan

[128] Zezza, F., Pascale, M., Mulè, G., Visconti, A. *J. Microbiol. Meth.*, 2006, 66, 529-537.
[129] Zhang, D., Yan, Y., Li, Q., Yu, T., Cheng, W., Wang, L., Ju, H. Ding, S. *J. Biotechnol.*, 2012, 160, 123-128.
[130] Tang, J. J., Yu, T., Guo, L., Xie, J.W., Shao, N. S., He, Z. K. *Biosens. Bioelectron.*, 2007, 22, 2456-2463.
[131] Baeumner, A. J., Cohen, R. N., Miksic, V., Min, J. H. *Biosens. Bioelectron.*, 2003, 18, 405-413.
[132] Kalogianni, D. P., Koraki, T., Christopoulos, T. K., Ioannou, P. C. *Biosens. Bioelectron.*, 2006, 21, 1069-1076.
[133] Trantakis, I. A., Spaniolas, S., Kalaitzis, P., Ioannou, P. C., Tucker, G. A., Christopoulos, T. K. *J. Agr. Food Chem.*, 2012, 60, 713-717.
[134] Joshi, R., Janagama, H., Dwivedi, H.P., Kumar, T. M. A. S., Jaykus, L.A., Schefers, J., Sreevatsan, S. *Mol. Cell. Probes*, 2009, 23, 20-28.
[135] Cruz-Aguado, J. A., Penner, G. *J. Agric. Food Chem.*, 2008, 56, 10456-10461.

algunas aplicaciones en que se utilizan cadenas dobles de ADN (dsADN) o hebras de ARN para la detección de toxinas[113] o bacterias patógenas,[131,134] que han alcanzado límites de detección inferiores a los obtenidos mediante ssADN en aplicaciones similares (Tabla 4). Recientemente, se han desarrollado diversos biosensores de elevada sensibilidad basados en el uso de APNs y aptámeros, principalmente para la detección de toxinas[114,130,135] y compuestos xenobióticos,[116,117] los cuales no es posible detectar mediante sondas de ácidos nucleicos convencionales.

En la Tabla 4 se recogen ejemplos de biosesores de ADN desarrollados para aplicaciones en seguridad alimentaria, clasificados en función del transductor empleado.

1.4.3 Tendencias actuales

Con la integración de los avances alzancados en biotecnología, nanotecnología, física, química, electrónica y bioingeniería, se están desarrollando biosensores cada vez más competitivos. Sin embargo, hay pocos biosensores comercializados para uso en el campo agroalimentario.[103]

Según el tipo de biosensor y la aplicación a la que se destine, los requisitos que debe cumplir el dispositivo varían. Así, en algunos casos prima la selectividad y sensibilidad, mientras que en otros se trata del tiempo de respuesta o la capacidad de trabajo (multiplexado). En los últimos años, las principales líneas de investigación en tecnologías de biosensores de ADN abordan los siguientes aspectos:

- Miniaturización de los dispositivos hasta una escala micro y nanométrica.
- Desarrollo de dispositivos compactos integrables en sistemas complejos.
- Aumento de la portabilidad.
- Integración de las diferentes fases del proceso analítico para reducir costes y tiempos de ensayo.

Tabla 4. Biosensores de ADN para aplicaciones de seguridad alimentaria.

Transductor	Detección	Analito	Sonda	LOD	Ref.
Electroquímico	Amperometría	Aflatoxina	dsADN	0,5 nM	113
	Amperometría	Plomo, cobre y hierro	ssADN	10^{-10} - 10^{-7} M	115
	Amperometría	*Staphylococcus* spp.	ssADN	20 ng/mL	118
	Voltametría	Estradiol	Aptámero	0,1 nM	116
	Voltametría	Toxina botulínica	Aptámero	40 pg/mL	114
	Voltametría	Tetraciclina	Aptámero	1 nM	117
	Voltametría	Avellana	ssADN	0,1 - 0,3 nM	119
	Voltametría	Ternera, pollo y cerdo	ssADN	23 pg/µL	120
	Impedimetría	Gen pat	ssADN	10^{-11} M	121
Piezoeléctrico	QCM	*E. coli*	ssADN	10 ng	122
	QCM	*A. hydrophila*	ssADN	0,1 µM	123
	QCM	*E. coli*	ssADN	10^{2} CFU/mL	124
	QCM	Genes p35S y tNOS	ssADN	1%	125
Óptico	Luminiscencia	*E. coli*	ARN	40 CFU/mL	131
	Luminiscencia	Toxina abrina	Aptámero	1 nM	130
	iSPR	Soja	APN	18 fmol	126
	SPR	Genes p35S y tNOS	ssADN	1 nM	127
	SPR	*Fusarium* spp.	ssADN	0,06 pg/ 30 ng	128
	SPR	*Salmonella* spp.	ssADN	10^{2} CFU/mL	129
	Colorimetría	Avellana	ssADN	<0,001%	35
	Colorimetría	Cacahuete	ssADN	0,001%	69
	Colorimetría	Genes p35S y tNOS	ssADN	0,05%	72
	Colorimetría	Genes p35S y tNOS	ssADN	0,1%	132
	Colorimetría	Café	ssADN	5%	133
	Fluorescencia	Avellana, cacahuete, mostaza, soja, huevo, leche, almendra y sésamo	ssADN	0,01%	85
	Fluorescencia	Genes p35S, tNOS y npt11	ssADN	< 0,3%	78
	Fluorescencia	Soja y maíz	ssADN	0,5-1%	79
	Fluorescencia	*E. coli, C. jejuni, S.dysenteriae, V. cholerae, V.parahaemolyticus, P. vulgaris, B.cereus, L. monocytogenes, C.botulinum, S. enterica, Y.enterocolitica, S.aureus, C. perfringens y S.pyogenes*	ssADN	10 CFU/mL	77
	Fluorescencia	*S. enterica*	ARN	10^{1} CFU/g	134
	Fluorescencia	Ocratoxina A	Aptámero	1 nM	135
	Fluorescencia	Trigo, cebada, avena, arroz, maíz y centeno	ssADN	20-40 ng	82
	Fluorescencia	Ternera	ssADN	0,001-0,1%	95

- Monitorización en tiempo real.
- Aumento de la capacidad de multiplexado.
- Incorporación de nuevos elementos de reconocimiento que incrementen la sensibilidad y la estabilidad.
- Desarrollo de mejores procedimientos de inmovilización de sondas.
- Desarrollo de nuevos materiales, técnicas de fabricación y métodos de detección.

Los dispositivos de óptica integrada están adquiriendo gran importancia debido a su elevada sensibilidad, estabilidad mecánica, fabricación a gran escala y posibilidad de miniaturización e integración en microsistemas. Dentro de este tipo de dispositivos, la tecnología de disco compacto se ha convertido en una herramienta analítica de gran potencial.[136] El trabajo de investigación realizado en la presente tesis se ha centrado en esta tecnología y su desarrollo, teniendo en cuenta alguna de las tendencias arriba destacadas.

1.5 Tecnología de disco compacto

1.5.1 Fundamento de la tecnología

El disco compacto es un soporte digital óptico utilizado para almacenar cualquier tipo de información (audio, vídeo, aplicaciones, documentos, etc.). Bajo esta denominación se incluyen varios tipos de discos ópticos. El disco compacto (CD) fue desarrollado por Sony y Philips y, aunque puede variar ligeramente la composición de los materiales empleados en su fabricación, todos los dispositivos siguen unas especificaciones comunes, recogidas en los llamados "Rainbow books".[137] Los discos compactos están compuestos de un sustrato de plástico transparente, generalmente de policarbonato (PC), cubierto con una capa metálica reflectiva de aluminio, plata u oro, protegido por una película de una laca

[136] Maquieira, A. Biosensors and Molecular Technologies for Cancer Diagnostics, Taylor & Francis, New York, USA. 2012, 417-440.
[137] Standard IEC60908, International Electrotechnical Comission, 1999.

polimérica, generalmente polimetilmetacrilato de metilo (PMMA) y, opcionalmente, una etiqueta (Figura 10a). Tanto el PC como el PMMA son materiales transparentes a la radiación visible, presentan baja distorsión óptica, alta resistencia mecánica, buena estabilidad térmica y gran hidrofobicidad. Los CDs grabables (CD-R) o regrabables (CD-RW) presentan, además, una capa de colorante orgánico entre el substrato de carbonato y la capa metálica.

El disco estándar más utilizado tiene un diámetro de 12 cm, un grosor de 1,2 mm y un agujero central de 15 mm, aunque en el mercado existen discos de menor tamaño y presentación (Figura 10b).

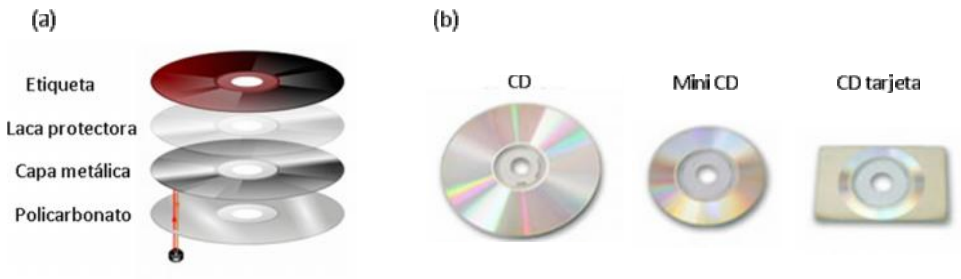


Figura 10. (a) Esquema de un disco compacto. (b) Ejemplo de diferentes tamaños y formatos.

En los discos compactos, la información se almacena en forma de *pits* que se imprimen durante el proceso de fabricación (discos grabados) o se generan durante la grabación (discos grabables). Cada *pit* tiene una profundidad de aproximadamente 125 nm, una anchura de 500 nm, y una longitud que oscila entre 830 y 3.500 nm. Los *pits* están ordenados en el disco a lo largo de una única espiral continua que constituye la pista de datos (*data track*), la cual es barrida por el haz láser desde el interior hasta el exterior del CD, en sentido levógiro. El espacio entre pistas (distancia radial entre una revolución de la espiral y la siguiente) es de 1600 nm. Los espacios entre *pits* a lo largo de la misma pista de datos se conocen como *lands* (Figura 11).

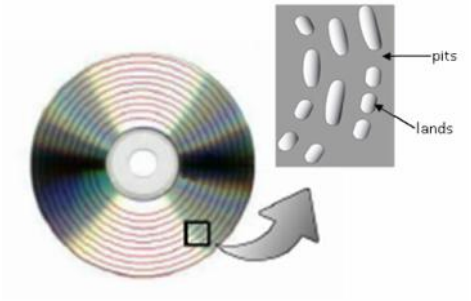


Figura 11. Espiral de pits y lands que conforman la pista de datos de un disco óptico.

La información codificada en los *pits* y *lands* se registra empleando un lector de discos compactos (Figura 12), compuesto por:

- Cabezal óptico de lectura o *pick-up*. Consta de un láser de emite a 780 nm, en el caso de los CDs, y una lente que lo focaliza para ajustar la distancia donde se encuentra la pista de datos. Además, lleva incorporado un sensor opto-electrónico (fotodiodo) que detecta la luz reflejada en la película metálica del disco.
- Motor de arrastre. Hace girar el disco controlando de forma precisa la velocidad de giro.
- Mecanismo rastreador. Mueve el dispositivo láser a lo largo de un eje radial fijo, de modo que el haz de luz puede seguir la pista de datos en espiral mientras el disco gira. Este sistema mueve el láser con una resolución de micrómetros.

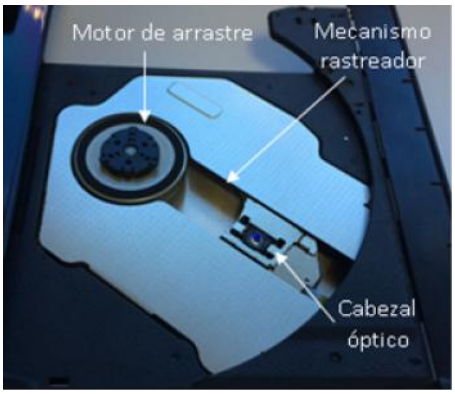


Figura 12. Esquema del sistema lector de discos compactos.

La lectura de la información grabada en el disco se realiza a partir de la modificación de la señal del láser reflejado. El lector utiliza el fenómeno de interferencia con la finalidad de proporcionar información binaria. La luz láser emitida atraviesa un divisor de haz que triplica el haz de entrada. Los tres haces se enfocan sobre la superficie del CD a través de un sistema óptico, de manera que el haz central se mantiene sobre la pista y lo otros son usados para el sistema de seguimiento automático de la pista (*autotracking*). La luz incidente atraviesa la capa de policarbonato y se refleja en la película metálica. La profundidad de los *pits* es la cuarta parte de la longitud de onda del láser, de manera que, mientras que a lo largo de un *pit* o de un *land* no hay cambios, cuando se produce una transición *pit-land* o *land-pit* hay un desfase de media longitud de onda entre la luz emitida y la reflejada (¼ + ¼ = ½) (Figura 13). Los fotodetectores miden este cambio en la intensidad luminosa como una señal digital (1-0).

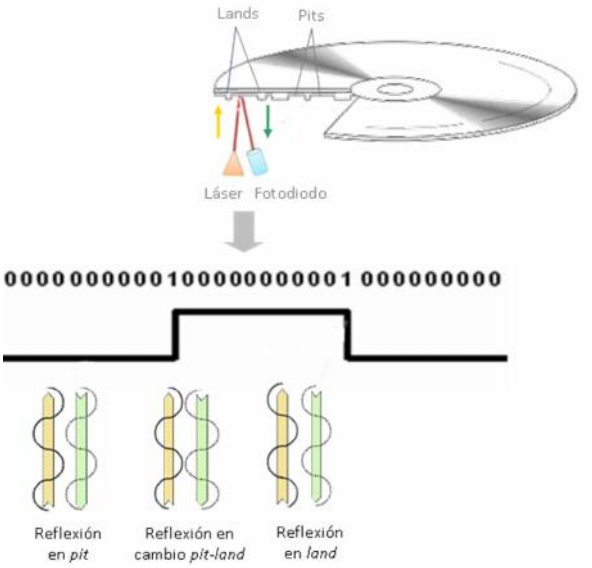


Figura 13. Esquema del fenómeno de interferencia en el que se basa el sistema de detección del lector de CDs.

Con posterioridad a los CDs se fueron desarrollando otro tipo de discos como soportes de grabación/reproducción de audio-vídeo. Así, en la década de los noventa, apareció el llamado Disco Versátil Digital o DVD. La principal ventaja del DVD frente al CD es su mayor velocidad de lectura y capacidad de

almacenamiento de datos. Esto se consigue gracias a un láser de mayor resolución, que emite a 650 nm, lo que permite disminuir las dimensiones de los *pits* y los *lands* y el espacio entre pistas (740 nm) (Figura 14). Otra ventaja de los DVDs es la posibilidad de almacenar información en ambas caras del disco; además, cada cara puede estar constituida por dos capas, lo que multiplica por cuatro la capacidad de almacenamiento de este tipo de discos ópticos. Posteriormente, se desarrollaron otros formatos de disco compacto, como el Blu-Ray (BD). Este formato trabaja con un láser de mayor resolución (405 nm), lo que aumenta todavía más su capacidad de almacenamiento de información con un menor ancho de pista (320 nm).

Existen ciertos discos que destacan por sus cualidades particulares, como el Light-Scribe o los discos Millennial (M-Disc). El Light-Scribe consiste en un CD o DVD convencional con un revestimiento termocrómico en la parte superior, que permite dibujar cualquier combinación de textos o imágenes empleando el láser del lector. Los M-disc se encuentran en formato DVD o BD y se caracterizan por poseer una resistencia extraordinaria antre cualquier tipo de agresión, debido a que incorporan una capa de un material de gran dureza y resistencia. En la Tabla 5 se comparan las particularidades más destacadas de los principales dispositivos ópticos.

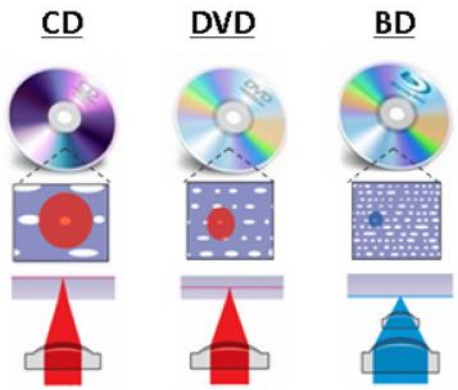


Figura 14. Esquema comparativo de la resolución óptica de los discos tipo CD, DVD, y BD.

Tabla 5. Características de los principales tipos de disco óptico.

	CD	**DVD**	**BD**
Material base	Policarbonato	Policarbonato	Policarbonato
Espesor del PC (mm)	1,2	2 × 0,6	1,2
Almacenamiento (GB/capa)	0,7	4,7	27
Transferencia de datos (Mbit/s)	0,15	11,1	36
$\lambda_{láser}$ (nm)	780	650	405
Apertura de la lente	0,45	0,6	0,85
Ancho de pista (nm)	1600	740	320
Resitencia al rayado	Baja	Baja	Alta

1.5.2 Empleo del disco compacto como plataforma analítica

Los materiales poliméricos, como los que constituyen los discos compactos (PC, PMMA, etc.) han demostrado ser una buena alternativa a otros como vidrio, silicio y óxido de silicio para ser empleados como plataforma biosensora. Hay un gran número de trabajos que utilizan plataformas poliméricas en forma circular como soporte analítico, los cuales pueden clasificarse siguiendo dos enfoques diferentes. El primero utiliza plataformas centrífugas microfluídicas (*lab-on-a-disc*) y los resultados se leen con detectores convencionales (fotómetros, fluorímetros, etc.) adaptados a una geometría circular.[138] El segundo enfoque emplea la tecnología de audio-vídeo tanto para la realización de los ensayos (discos) como para su lectura (lector/grabador).[136]

a) Plataformas centrífugas (*lab-on-a-disc*)

Básicamente están constituidas por discos poliméricos de varios milímetros de espesor, con una red de microcanales, depósitos y válvulas, que permiten llevar a cabo una o varias etapas del proceso analítico (tratamiento de muestra, amplificación, detección, etc.) (Figura 15). Además, utilizan la fuerza centrífuga como sistema impulsor para mover los fluidos y desarrollar los ensayos, logrando

[138] Gorkin, R., Park, J., Siegrist, J., Amasia, M., Lee, B. S., Park, J. M., Cho, Y. K. *Lab Chip*, 2010, 10, 1758-1773.

una mayor miniaturización y automatización de los métodos. Madou *et al.*,[139] Gorkin *et al.*,[138] Burger *et al.*[140] y Nolte[141] han revisado las principales aplicaciones de dichas plataformas, denominadas *lab-on-a-disc*. Utilizando esta tecnología, se han desarrollado numerosos trabajos en el campo del análisis celular, abarcando desde la etapa de cultivo de microorganismos hasta ensayos de lisis.[139,140,142] También se ha estudiado su aplicación en inmunoensayos y en ensayos génicos.[138,143,144]

Se han comercializado, con mayor o menor éxito, dispositivos centrífugos tipo *lab-on-a-disc* por compañías como Abaxis,[145] Gyros,[146] o Biosurfit;[147] centrados principalmente en aplicaciones de análisis de sangre y caracterización de proteínas para la industria farmacéutica.[138] En el campo del análisis de ácidos nucleicos, hay varios grupos prominentes trabajando en el desarrollo de dispositivos *lab-on-a-disc* para la detección de ADN, como por ejemplo, el grupo de Madou, o las empresas Samsung e IMTEK.[138]

Sin embargo, en la mayoría de estos trabajos, el resultado final del ensayo es medido utilizando detectores convencionales como densímetros, fotómetros, fluorímetros, etc., adaptados a una geometría circular. En ningún caso se hace uso del principio de lectura de los reproductores de discos compactos, ni discos compactos comerciales. Por ello, a pesar de todos los avances realizados en este campo, especialmente en la automatización del proceso analítico, aún no se ha explotado todo el potencial de los sistemas basados en la tecnología de disco compacto.

[139] Madou, M., Zoval, J., Jia, G., Kido, H., Kim, J., Kim, N. *Annu. Rev. Biomed. Eng*., 2006, 8, 601-28.
[140] Burger, R., Kirby, D., Glynn, M., Nwankire, C., O'Sullivan, M., Siegrist, J., Kinahan, D., Aguirre, G., Kijanka, G., Gorkin, R., Ducrée, J. *Curr. Opin. Chem. Biol.*, 2012, 16, 409-414.
[141] Nolte, D. D. *Rev. Sci. Instrum.*, 2009, 80, 101101-101124.
[142] Vázquez, M., Brabazon, D., Shang, F., Omamogho, J. O., Glennon, J. D., Paull, B. *TrAC-Trends Anal. Chem.*, 2011, 30, 1575-1586.
[143] Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle, R., von Stetten, F. *Lab Chip*, 2010, 10, 887-893.
[144] Kim, T. H., Park, J., Kim, C. J., Cho, Y. K. *Anal. Chem.*, 2014, 86, 3841-3848.
[145] http://www.gyros.com/ (Fecha de consulta Febrero/2015)
[146] http://www.abaxis.com/ (Fecha de consulta Febrero/2015)
[147] http://www.biosurfit.com/ (Fecha de consulta Febrero/2015)

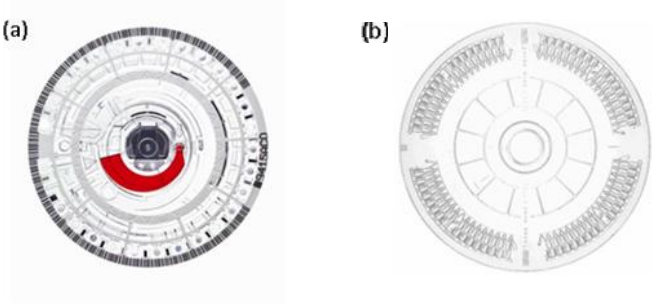


Figura 15. Lab-on-a-CD comercializado por (a) Abaxis y (b) Gyros.

b) Discos compactos de audio-vídeo

La utilización de discos compactos comerciales (CD, DVD o Blu-Ray) con fines analíticos presenta importantes ventajas. Se trata de soportes con extraordinarias propiedades físicas tales como gran resistencia y estabilidad a altas temperaturas. Además de sus buenas propiedades ópticas, son fácilmente derivatizables químicamente y pueden manipularse para crear dispositivos portátiles. A esto hay que añadir su gran superficie de análisis (94 cm^2) y su bajo coste (0,20 €/disco y 50-300 €/lector). Además, con motivo de la celebración del 25 aniversario del nacimiento del CD en 2007,[148] se estimó que había más de 200 billones de discos y más de 600 millones de lectores en circulación. Actualmente, se mantienen esas cifras debido al auge de la tecnología BD. Esto da una idea de la utilidad de la tecnología y de las ventajas que supone su fabricación en masa y su ubicuidad.

Kido *et al*.[149] fueron los primeros en emplear CDs comerciales para la detección de plaguicidas mediante inmunoesayos de inhibición. Sin embargo la detección se realizó mediante fluorescencia, no empleando un lector de discos.

Alexandre *et al*.[150] desarrollaron un disco compacto dividido en dos secciones, una para realizar ensayos de hibridación de ácidos nucleicos y otra para almacenar la información numérica. El ensayo se llevó a cabo en discos de baja reflectividad empleando las dos caras del mismo. La lectura de la información numérica se realizó en una de las caras mediante un lector convencional, y la

[148] Philips celebrates 25th anniversary of the compact disk. 2007. http://news.ecoustics.com/
[149] Kido, H., Maquieira, A., Hammock, B. D. *Anal. Chim. Acta*, 2000, 411, 1-11.
[150] Alexandre, I., Houbion, Y., Collet, J., Hamels, S., Demarteau, J., Gala, J. L., Remacle, J. *Biotechniques*, 2002, 33, 435-439.

información genómica se obtuvo en la cara opuesta mediante detección colorimétrica a partir de un fotodiodo situado perpendicularmente al emisor láser.

Otro de los trabajos utilizando un lector de CDs convencional fue el desarrollado por La Clair y Burkart,[151] en el que la unión del analito al disco se detectó mediante las propias rutinas de determinación de errores que incorporan los lectores. Se trata de una estrategia interesante porque la información analítica se obtiene con un lector de CDs estándar utilizando programas informáticos comerciales. Sin embargo, como cada fabricante aplica un modo distinto de corrección de errores, la sensibilidad de los ensayos se ve alterada haciendo difícil la comparación de resultados.

Otro ejemplo del empleo de discos y lectores convencionales es el desarrollado por Potyrailo *et al*.,[152] que utilizaron CDs y DVDs como plataformas sobre las que depositan películas reactivas para la detección de aniones y cationes. El resultado obtenido con lectores de CD y DVD es una imagen cuya intensidad se relaciona con la concentración de analito en la muestra. Sin embargo, esta aproximación no trabaja directamente sobre la superficie del disco y se aleja de los formatos de ensayo de alta densidad.

Desde la aparición de esta tecnología, el grupo de investigación en el que se ha realizado la presente tesis está desarrollando numerosos avances empleando discos compactos como plataforma analítica y los lectores como detectores para el desarrollo de ensayos basados en reconocimiento molecular tipo sonda-diana, de proteínas y ácidos nucleicos.

Tomando como base de ensayo la tecnología de disco compacto, en una primera aproximación, se demostró que la utilización del láser del lector de CDs como fuente de barrido puede generar señales químicas *in situ* sin interrumpir la lectura normal del disco, lo que tiene un elevado potencial analítico.[153,154] Para

[151] La Clair, J. J., Burkart, M. D. *J. Org. Biomol. Chem.*, 2003, 1, 3244-3249.
[152] Potyrailo, R. A., Morris, W. G., Leach, A. M., Sivavec, T. M., Wisnudel, M. B., Boyette, S. *Anal. Chem.*, 2006, 78, 5893-5899.
[153] Morais, S., Tamarit-López, J., Carrascosa, J., Puchades, R., Maquieira, A. *Anal. Bioanal. Chem.*, 2008, 391, 2837-2844.
[154] Morais, S., Carrascosa, J., Mira, D., Puchades, R., Maquieira, A. *Anal. Chem.*, 2007, 79, 7628-7635.

ello, se emplearon discos de baja reflectividad constituidos por policarbonato y una película de oro parcialmente transparente, de modo que parte del haz láser del lector de CDs era transmitido y detectado mediante un lector modificado con un fotodetector colocado en la cara opuesta (Figura 16a). La cantidad de luz que alcanza el fotodetector es proporcional al producto de reacción y, por tanto, la señal medida puede ser correlacionada con la concentración de analito. Siguiendo esta línea, también se estudió la modificación de los discos depositando polímeros o materiales reflectantes mediante técnicas de *spin-coating*. Esto permitió disponer de una película uniforme de polímero (poliestireno o PMMA) sobre la capa de oro, con el objeto de efectuar ensayos sobre un material diferente al policarbonato, ampliando así el campo de utilización analítica de la tecnología de CDs.[155-157]

En investigaciones posteriores, se apostó por simplificar el proceso utilizando el lector estandar de CDs, de modo que el propio fotodiodo del cabezal de lectura actuara como detector (Figura 16b). Así, la atenuación de la señal reflejada está correlacionada con la extensión de la reacción analítica. Esta aproximación proporcionó mejores resultados que la anterior, habiéndose aplicado a la determinación de residuos de plaguicidas y marcadores tumorales mediante inmunoensayos competitivos.[153]

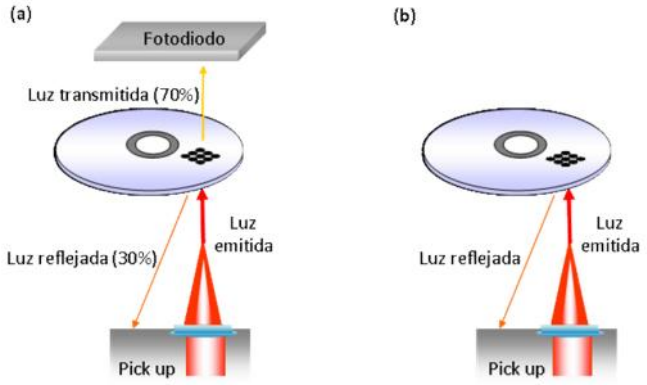


Figura 16. Tipos de detección empleando la tecnología de CD. (a) Detección por transmisión; (b) Detección por reflexión.

[155] Bañuls, M. J., González-Pedro, V., Puchades, R., Maquieira, *Bioconjugate Chem.*, 2007, 18, 1408-1414.
[156] Brun, E. M., Puchades, R., Maquieira, A. *Anal. Chem.*, 2013, 85, 4178-4186.
[157] Morais, S., Marco-Molés, R., Puchades, R., Maquieira, A. *Chem. Commun.*, 2006, 22, 2368-2370.

Más recientemente, se han desarrollado estudios empleando dispositivos ópticos con mejores prestaciones, como los DVDs o Blu-Rays, obteniéndose resultados relevantes en diversos campos. Así, se han llevado a cabo con éxito inmunoensayos en formato micromatriz para la detección múltiple de toxinas y residuos de plaguicidas en aguas y zumos.[158-160] También se han efectuado aportaciones para la detección de ácidos nucleicos mediante ensayos de hibridación con fragmentos de ADN amplificados por PCR y oligonucleótidos inmovilizados en formato micromatriz sobre la superficie del disco. Hasta la fecha y de forma paralela a esta tesis, se han obtenido resultados muy interesantes en la detección de *Salmonella* spp. y *Cronobacter sakazakii*, empleando DVDs y Blu-Rays como plataformas sensoras, alcanzándose límites de detección de alrededor de 10 CFU/mL.[161-164]

Los resultados obtenidos hasta el momento convierten la tecnología de discos compactos en una herramienta muy potente para ser empleada como biosensor en la realización de análisis génicos con un enfoque integrado.

1.6 Sistemas integrados para detección de ácidos nucleicos

Los métodos de determinación de ácidos nucleicos convencionales se desarrollan en varias etapas (extracción, amplificación, reconocimiento y detección) y requieren volúmenes de reactivos relativamente altos (50-1.000 µL), lo que, entre otros inconvenientes, conlleva un incremento significativo del coste y tiempo de análisis, y aumenta el riesgo de contaminación cruzada. Por ello, uno

[158] Morais, S., Tamarit-López, J., Puchades, R., Maquieira, A. *Environ. Sci. Technol.*, 2010, 44, 9024-9029.
[159] Morais, S., Tortajada-Genaro, L. A., Arnandis-Chover, T., Puchades, R., Maquieira, A. *Anal. Chem.*, 2009, 81, 5646-5654.
[160] Navarro, P., Morais, S., Gabaldón, J. A., Pérez, A. J., Puchades, R., Maquieira, A. *Anal. Chim. Acta*, 2013, 784, 59-64.
[161] Tamarit-López, J., Morais, S., Puchades, R., Maquieira, A. *Bioconjugate Chem.*, 2011, 22, 2573-2580.
[162] Arnandis-Chover, T., Morais, S., Tortajada-Genaro, L. A., Puchades, R., Maquieira, A., Berganza, J., Olabarria, G. *Talanta*, 2012, 101, 405-412.
[163] Peris, E., Bañuls, M. J., Puchades, R., Maquieira, A. *J. Mater. Chem. B*, 2013, 1, 6245-6253.
[164] Arnandis-Chover, T., Morais, S., González-Martínez, M. Á., Puchades, R., Maquieira, A. *Biosens. Bioelectron.*, 2014, 51, 109-114.

de los principales retos en investigación en el área de los biosensores es el desarrollo de sistemas integrados miniaturizados. Estos dispositivos, llamados también microsistemas de análisis total (μTAS, *micro total analysis system*), son ampliamente demandados como herramienta analítica ya que, entre otras ventajas, permiten:[165-167]

- Efectuar todas las etapas del análisis automáticamente.
- Disminuir la escala de trabajo y consumir menos muestra y reactivos, lo que, además de reducir el coste, minimiza los residuos medioambientales generados.
- Mejorar el transporte de energía, masa y mezcla de reactivos, lo que implica una reducción en los tiempos de análisis y un menor consumo energético.
- Albergar un mayor número de muestras en la misma plataforma.
- Desarrollar dispositivos más compactos, con mayor grado de automatización y portabilidad para la realización de ensayos *in situ* en puntos de control.
- Producir los microsistemas en masa, lo que reduce los costes por unidad.

De las diferentes etapas a implementar en estos microsistemas, la amplificación del ADN es una de las más críticas, ya que de ella depende que la detección sea sensible y selectiva. Se han desarrollado algunos sistemas capaces de detectar ADN sin una amplificación previa; sin embargo, a nivel práctico aún no se ha consolidado.[168] Así pues, los principales esfuerzos se han centrado en el desarrollo de dispositivos miniaturizados que permitan la amplificación de ácidos nucleicos de manera exitosa, proponiendo diferentes aproximaciones en los últimos años.

En la bibliografía se recogen numerosos estudios basados en el empleo de dispositivos microfluídicos para la amplificación de ácidos nucleicos. Zhang y

[165] Whitesides, G. M. *Nature*, 2006, 442, 368-373.
[166] Ahmad, F., Hashsham, S. A. *Anal. Chim. Acta*, 2012, 733, 1-15.
[167] Zanoli, L. M., Spoto, G. *Biosensors*, 2013, 3, 18-43.
[168] Craw, P., Balachandran, W. *Lab Chip*, 2012, 12, 2469-2486.

Ozdemir,[178] McCalla y Tripathi,[169] Ahmad y Hashsham,[166] Chang *et al.*[170] y Wu *et al.*[171] han publicado revisiones sobre los avances actuales en el desarrollo de microreactores para llevar a cabo la amplificación en aplicaciones *point-of-control*.

a) Amplificación por PCR

Los primeros dispositivos miniaturizados para la amplificación por PCR fueron propuestos por Northrup *et al*. en 1993[172] y Wilding *et al*. en 1994.[173] Ambos sistemas proponían una amplificación estática consistente en un chip de silicio con un micropocillo donde tenía lugar la reacción y un dispositivo termoeléctrico con efecto Peltier para regular la temperatura (Figura 17a). Siguiendo este modelo, diversos grupos de investigación desarrollaron dispositivos que realizan la reacción de PCR en menos de 7 min, e incluso la monitorizan en tiempo real (rt-PCR).[174,175]

Otros grupos han propuesto un diseño alternativo para llevar a cabo reacciones de amplificación de manera continua o en flujo (Figura 17b). Este planteamiento, introducido por Nakano *et al*. en 1994,[176] consistió en un canal serpenteante que atraviesa zonas a diferente temperatura, de modo que los reactivos de PCR son bombeados a través del circuito. La principal ventaja de este tipo de dispositivos frente a los anteriores es que presentan un mayor ratio superficie/volumen, permitiendo que la regulación de la temperatura sea más rápida y que el tiempo de cada ciclo de PCR pueda reducirse a segundos. Sin embargo, para su diseño hay que tener en cuenta factores como las dimensiones y forma del canal, y el ratio de flujo o el volumen, lo que los convierte en dispositivos más caros y complejos.

[169] McCalla, S. E., Tripathi, A. *Annu. Rev. Biomed. Eng.*, 2011, 13, 321-343.
[170] Chang, C. M., Chang, W. H., Wang, C. H., Wang, J. H., Mai, J. D., Lee, G. B. *Lab Chip*, 2013, 13, 1225-1242.
[171] Wu, J., Kodzius, R., Cao, W., Wen, W. *Microchim. Acta*, 2014, 181, 1611-1631.
[172] Northrup, M. A., Ching, M. T., White, R. M., Watson, R. T. *Transducers*, 1993, 93, 924-926.
[173] Wilding, P., Shoffner, M. A., Kricka, L. J. *Clin. Chem.*, 1994, 40, 1815-1818.
[174] Auroux, P. A., Koc, Y., Manz, A., Day, P. J. R. *Lab Chip*, 2004, 4, 534-546.
[175] Carlborg, C. F., Gylfason, K. B., Kaźmierczak, A., Dortu, F., Bañuls, M. J., Maquieira, A., Kresbach, G. M., Sohlstrom, H., Moh, T., Vivien, L., Popplewell, J., Ronan, G., Barrios, C. A., Stemme, G., van der Wijngaart, W. *Lab Chip*, 2010, 10, 281-290.
[176] Nakano, H., Matsuda, K., Yohda, M., Nagamune, T., Endo, I., Yamane, T. *Biosci. Biotechnol. Biochem.*, 1994, 58, 349-352.

En la bibliografía se recogen numerosos dispositivos en flujo que realizan la PCR en tiempos muy reducidos (<10 min), aunque los límites de detección, en general, son peores que los obtenidos mediante PCR estática (10^6 y 10^2 copias de ADN diana, respectivamente). Esto es debido al elevado ratio superficie/volumen, que produce una mayor adsorción inespecífica de los reactivos, especialmente de la enzima polimerasa, lo que afecta negativamente a la eficiencia de la reacción. Para reducir esta limitación se ha estudiado el efecto de diversos tratamientos de superficie (silanización o deposición de capas de óxido) o la adición de reactivos a la mezcla de reacción (polietilenglicol, polivinilpirrolidona o albúmina de suero bovino).[167]

Otra aproximación para la miniaturización de la PCR es el empleo de gotas como microreactores, también llamada digital-PCR.[177] Para ello, la mezcla acuosa de reacción se dispersa en una fase inmiscible en agua, formándose una emulsión de gotas de la disolución de PCR, de entre unos pocos y cientos de micrométros de diámetro. Actualmente existen en el mercado diversos dispositivos para la realización de la PCR digital, como los comercializados por Fluidigm Corporation, Bio-Rad, Life Technologies o RainDance. Se trata de instrumentos caros (>100.000€), y al ser los microreactores gotas acuosas, su integración en sistemas de análisis más complejos puede verse dificultada. [178]

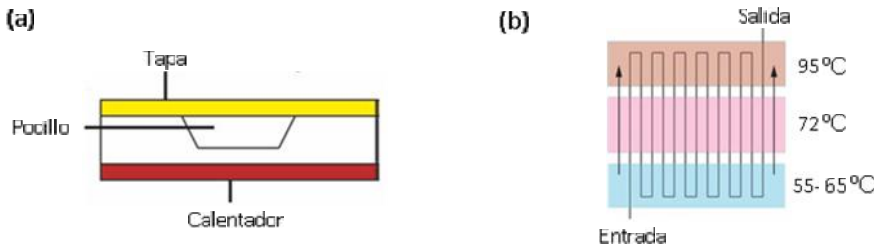


Figura 17. Esquema de dispositivos miniaturizados para la amplificación de ADN (a) estática y (b) en flujo.

[177] Baker, M. *Nat. Methods*, 2012, 9, 541-544..
[178] Zhang, Y., Ozdemir, P. *Anal. Chim. Acta*, 2009, 638, 115-125.

b) Alternativas a la PCR

A pesar de que la PCR tradicional se considera el método de referencia para la amplificación de ácidos nucleicos, presenta una serie de limitaciones que dificultan su integración en sistemas miniaturizados. Requiere un estricto control de la temperatura, lo que dificulta el diseño de los dispositivos y conlleva un gasto energético elevado. Además, debido a la alta temperatura necesaria para la etapa de desnaturalización (95 °C), los materiales a emplear están limitados y pueden producirse fenómenos de evaporación y formación de burbujas indeseables.[168,179]

Con el fin de evitar estos inconvenientes, se desarrollaron técnicas de amplificación isoterma, las cuales permiten sintetizar ácidos nucleicos a temperatura constante, mimetizando los sistemas celulares *in vivo*. Para ello, emplean enzimas que se encargan de llevar a cabo las fases de separación e hibridación de las cadenas de ADN. En las reacciones isotermas, el factor limitante es la actividad enzimática y no la velocidad de termociclado como ocurre en la PCR, por lo que los sistemas de amplificación isoterma miniaturizados emplean normalmente microcámaras estáticas, ya que aunque el diseño en flujo optimice el transporte de calor, no acelera la reacción.[179] Esto da a los sistemas de amplificación isoterma la ventaja de presentar un diseño más simple, robusto y con menor requerimiento energético.

1.7 Reacciones de amplificación isoterma

Actualmente, se han descrito numerosas técnicas de amplificación isoterma con características muy diversas. En la bibliografía se encuentran diferentes revisiones que recogen las principales aportaciones desarrolladas hasta la fecha.[180-182] En esta memoria se recogen las más empleadas en el desarrollo de sistemas integrados.

[179] Asiello, P. J., Baeumner, A. J. *Lab Chip*, 2011, 11, 1420-1430.
[180] Gill, P., Ghaemi, A. *Nucleos. Nucleot. Nucl.*, 2008, 27, 224-243.
[181] Karami, A., Gill, P., Motamedi, M. H. K., Saghafinia, M. *J. Global Infect. Dis.*, 2011, 3, 293-302.
[182] Yan, L., Zhou, J., Zheng, Y., Gamson, A. S., Roembke, B. T., Nakayama, S., Sintim, H. O. *Mol. BioSyst.*, 2014, 10, 970-1003.

a) Amplificación basada en la secuencia de ácidos nucleicos (NASBA)

La reacción NASBA fue descrita por primera vez por Compton en 1991,[183] y se basa en la amplificación de moléculas de ARN según el mecanismo de la replicación retroviral.

La elongación del cebador que contiene el promotor de la enzima ARN polimerasa T7 forma, durante el proceso de amplificación, un ADN complementario al ARN molde, a partir del cual la ARN polimerasa T7 sintetizará múltiples copias de ARN, que a su vez servirán de molde para nuevos ciclos de síntesis (Figura 18).

La NASBA se puede emplear para la amplificación de ADN, aunque para ello requiere una enzima adicional (ADN transcriptasa) que traduzca el ADN a ARN, y una fase previa de desnaturalización a 95 °C, lo que complica el proceso.

En la bibliografía hay diversos trabajos que desarrollan la NASBA en sistemas *lab-on-a-chip*.[168] Destaca el trabajo de Gulliksen *et al*.,[184] que integra en un sistema microfluídico las fases de preconcentración y extracción de la muestra, amplificación por NASBA y posterior detección en tiempo real. A nivel comercial, la empresa CorisBioconcept[185] ha incorporado la amplificación mediante NASBA a un sistema de detección basado en tiras reactivas para determinar *Trypanosomabruceil cruzi* y *Leishmania* spp.

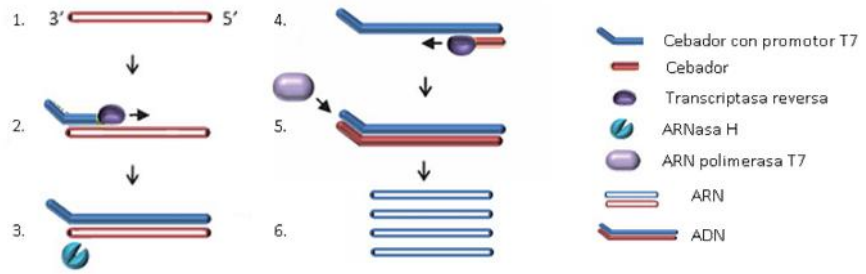


Figura 18. Esquema de amplificación mediante NASBA.

[183] Compton, J. *Nature*, 1991, 350, 91-92.
[184] Gulliksen, A., Keegan, H., Martin, C., O'Leary, J., Solli, L. A., Falang, I. M., Grønn, P., Karlgård, A., Mielnik, M. M., Johansen, I. R., Tofteberg, T. R., Baier, T., Gransee, R., Drese, K., Hansen-Hagge, T., Riegger, L., Koltay, P., Zengerle, R., Karlsen, F., Ausen, D., Furuberg, L. *J. Oncol.*, 2012, 2012, 1-12.
[185] http://www.corisbio.com/ (Fecha de consulta 02/02/2015).

b) Amplificación por desplazamiento de la hebra (SDA)

La SDA fue descrita por primera vez por Walker *et al.* en 1992,[186] y se basa en el empleo de un par de cebadores con un sitio de restricción en su secuencia, un par de cebadores para desplazar a los anteriores, y una enzima ADN polimerasa con actividad de desplazamiento de hebra.

Tras una desnaturalización inicial a 95 °C, las dos parejas de cebadores hibridan con el ADN molde, de manera que al elongarse se produce una hebra de ADN de cadena doble con los sitios de restricción por donde cortará la endonucleasa de restricción. La extensión de la polimerasa a partir de los sitios de corte liberará una cadena de ADN simple, que servirá como molde para el siguiente ciclo de síntesis (Figura 19).

La mayoría de los trabajos basados en SDA se han desarrollado para aplicaciones clínicas, concretamente para la detección de enfermedades infecciosas.[187]

A pesar de que la amplificación por SDA es una técnica sensible que trabaja a temperaturas próximas a la ambiental (37 °C), su uso en dispositivos integrados *point-of-control* no está muy extendido. Esto probablemente se debe a su elevado tiempo de análisis (2 h) y baja selectividad, requiriendo métodos de detección post-amplificación, como la hibridación, para diferenciar los productos obtenidos.[168,170]

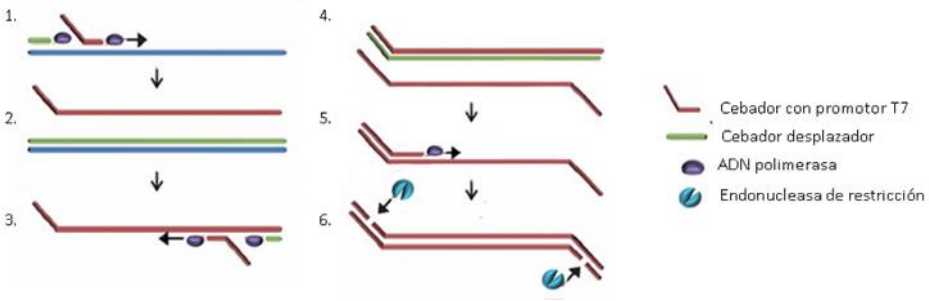


Figura 19. Esquema de amplificación mediante SDA.

[186] Walker, G. T., Fraiser, M. S., Schram, J. L., Little, M. C., Nadeau, J. G., Malinowski, D. P. *Nucleic Acids Res.*, 1992, 20, 1691-1696.
[187] Gill, P., Ghaemi, A. *Nucleos. Nucleot. Nucl.*, 2008, 27, 224-243.

c) Amplificación en círculo rodante (RCA)

LA RCA fue descrita en 1995 por Fire y Xu.[188] Se basa en el empleo de la enzima ADN polimerasa ϕ29, la cual presenta una elevada procesividad y actividad de desplazamiento de hebra, para amplificar de manera continua una molécula de ADN de cadena circular. De este modo, el producto final es una cadena larga de ADN simple con repeticiones sucesivas de la secuencia del ADN circular.

La baja temperatura de reacción (30 °C) la convierten en una buena alternativa a emplear en dispositivos µTAS; sin embargo, al amplificar preferentemente ADN molde monocatenario circular, sus aplicaciones se ven limitadas a plásmidos, fagos, virus o viroides. Para su uso en aplicaciones de ADN de doble cadena se requiere una etapa previa de circularización, que se lleva a cabo mediante el empleo de "sondas candado" (Figura 20).

En el mercado no hay kits comerciales basados en la RCA, sin embargo en la bibliografía se encuentran diversos estudios que la emplean en dispositivos microfluídicos.[167,168,179] Mahmoudian *et al*.[189] desarrollaron un chip de PMMA con pocillos de 10 µL para la detección integrada de *V. cholerae* mediante amplificación por RCA y posterior electroforesis. Sato *et al*.[190] emplearon la RCA en fase sólida para obtener productos de amplificación de *Salmonella* spp. anclados en microesferas de Sefarosa de 34 µm de diámetro.

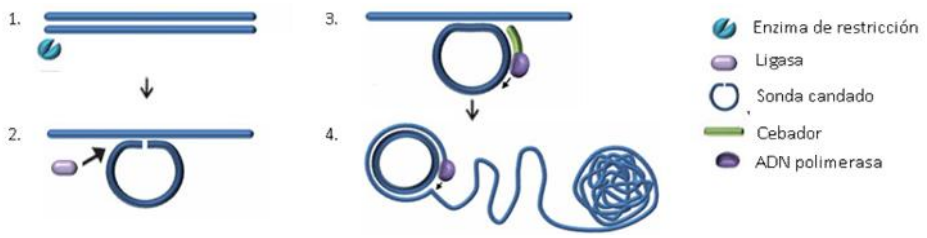


Figura 20. Esquema de amplificación mediante RCA.

[188] Fire, A., Xu, S. Q. *P. Natl. Acad. Sci. USA*, 1995, 92, 4641-4645.
[189] Mahmoudian, L., Kaji, N., Tokeshi, M., Nilsson, M., Baba, Y. *Anal. Chem.*, 2008, 80, 2483-2490.
[190] Sato, K., Tachihara, A., Renberg, B., Mawatari, K., Sato, K., Tanaka, Y., Jarvius, J., Nilsson, M., Kitamori. *Lab Chip*, 2010, 10, 1262-1266.

d) Amplificación por desplazamiento múltiple (MDA)

La reacción MDA, descrita por Dean et al.,[191] se basa en el empleo de polimerasa ϕ29 y oligonucleótidos de 6 pares de bases (hexámeros) que hibridan al azar en distintas regiones del genoma. Esto da lugar a una estructura ramificada de cadena sencilla a la que pueden unirse nuevos hexámeros (Figura 21).

Diversos estudios han demostrado que la MDA es un método muy sensible, capaz de amplificar material genético a partir de una única célula.[167] Sin embargo, de todo el producto amplificado, sólo aproximadamente el 30% es específico, ya que la MDA amplifica todo el ADN presente en la muestra. Se ha visto que una reducción del volumen de reacción disminuye la amplificación de los ADN interferentes, aumentando por tanto la selectividad. Así, Marcy *et al*.[192] lograron una mayor selectividad al amplificar ADN de células de *E. coli* en un microreactor de 60 nL, que al hacerlo en un vial de 50 µL.

La MDA se emplea fundamentalmente para realizar amplificaciones del genoma completo (WGA) en secuenciación y análisis de mutaciones.[193,194] También se ha empleado para el desarrollo de sistemas *lab-on-a-chip*, destacando el trabajo de Rhee *et al*.,[195] que propone un dispositivo de PDMS para la lisis y amplificación por MDA de ADN de *E. coli* en un volumen de reacción de 200 pL, y en otras aplicaciones.[194,196]

[191] Dean, F. B., Hosono, S., Fang, L., Wu, X., Faruqi, A. F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S. F., Egholm, M., Lasken, R. S. *P. Natl. Acad. Sci. USA*, 2002, 99, 5261-5266.

[192] Marcy, Y.; Ishoey, T.; Lasken, R. S.; Stockwell, T. B.; Walenz, B. P.; Halpern, A. L.; Beeson, K. Y.; Goldberg, S. M. D.; Quake, S. R. *PloS Genet*., 2007, 3, 1702-1708.

[193] Li, J., Macdonald, J. *Biosens. Bioelectron.*, 2015, 64, 196-211.

[194] Liang, J., Cai, W., Sun, Z. *J. Genet. Genomics*, 2014, 41, 513-528.

[195] Rhee, M., Light, Y. K., Yilmaz, S., Adams, P. D., Saxena, D., Meagher, R. J., Singh, A. K. *Lab Chip*, 2014, 14, 4533-4539.

[196] http://www.qiagen.com/es/

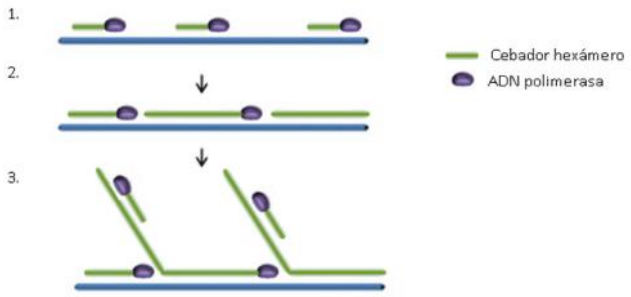


Figura 21. Esquema de amplificación mediante MDA.

e) Amplificación dependiente de helicasa (HDA)

La reacción HDA fue descrita por primera vez en 2004 por Vicent *et. al.* [197] Esta reacción emplea una proteína ADN helicasa para separar la doble hebra de ADN, y proteínas de unión a cadena simple (SSB) para estabilizar la horquilla de replicación (Figura 22).

La HDA posee una cinética de reacción muy similar a la de la PCR, sin embargo, cuando el número de copias iniciales de ADN es < 100, la velocidad de la reacción decae considerablemente.[168] Además, el rendimiento de amplificación depende de las condiciones del medio de reacción y de la longitud de los fragmentos amplificados, disminuyendo a partir de fragmentos > 100 pb, siendo necesaria una optimización exhaustiva de la reacción.[167]

Son numerosos los trabajos que emplean la HDA para realizar la amplificación en fase sólida en formato micromatriz.[198] También se han desarrollado sistemas microfluidicos basados en HDA. Así, Mahalanabis *et al.*[199] desarrollaron un dispositivo integrado para la preparación de muestra y detección en tiempo real de *E. coli* en menos de 50 min. Por otro lado, Ramalingam *et al.*[200] propusieron un chip de PDMS para la amplificación en paralelo de 4 reacciones de 5 µL, demostrando las múltiples prestaciones de esta reacción.

[197] Vincent, M., Xu, Y., Kong, H. *EMBO Rep.*, 2004, 5, 795-800.
[198] Andresen, D., Nickisch-Rosenegk, M. V., Bier, F. F. *Clin. Chim. Acta*, 2009, 403, 244-248.
[199] Mahalanabis, M., Do, J., ALMuayad, H., Zhang, J. Y., Klapperich, C. M. *Biomed. Microdevices*, 2010, 12, 353-359.
[200] Ramalingam, N., San, T. C., Kai, T. J., Mak M. Y. M., Gong, H. *Microfluid. Nanofluid.*, 2009, 7, 325-336.

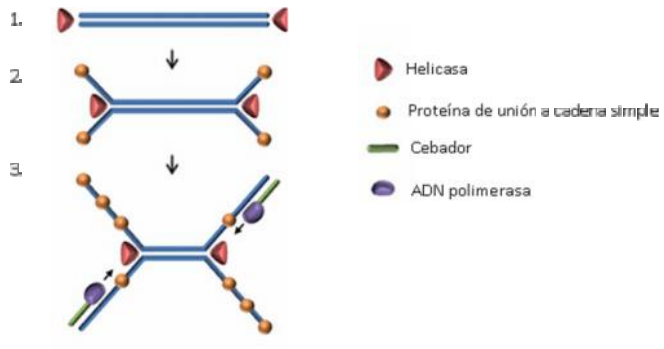


Figura 22. Esquema de amplificación mediante HDA.

f) Amplificación por recombinasa polimerasa (RPA)

LA RPA fue propuesta por Piepenburg *et al*. en 2006,[201] pero ha sido en los últimos años, en paralelo al desarrollo de la presente tesis doctoral, cuando su empleo como técnica de amplificación ha experimentado un importante crecimiento. Su mecanismo se basa en el empleo de recombinasas para facilitar la hibridación de los cebadores, y proteínas SSB (Single Strand Binding) para estabilizar la horquilla de replicación (Figura 23).

Su empleo en sistemas miniaturizados ha sido ampliamente demostrado,[168] destacando su uso en dispositivos *lab-on-a-disc*. Lutz *et al*.[143] propusieron un dispositivo para el análisis simultáneo de 30 muestras en reactores de 10 µL. Más recientemente, se ha descrito un microsistema de análisis total para la detección de *Salmonella enteritidis* que integra, en un único disco, las fases de extracción de ADN, amplificación por RPA y detección de los fragmentos amplificados mediante hibridación con sondas de ácidos nucleicos inmovilizadas sobre tiras reactivas.[144]

Esta reacción es una de las más compatibles para ser utilizadas en microsistemas miniaturizados, destacando su rapidez (40 min) y baja temperatura de trabajo (37-42 °C). El único inconveniente es que puede dar lugar a pre-amplificaciones inespecíficas a temperatura ambiente, que pueden evitarse controlando rigurosamente las condiciones de la reacción.[167]

[201] Piepenburg, O., Williams, C. H., Stemple, D. L., Armes, N. A. *PloS Biol.*, 2006, 4, 1115-1121.

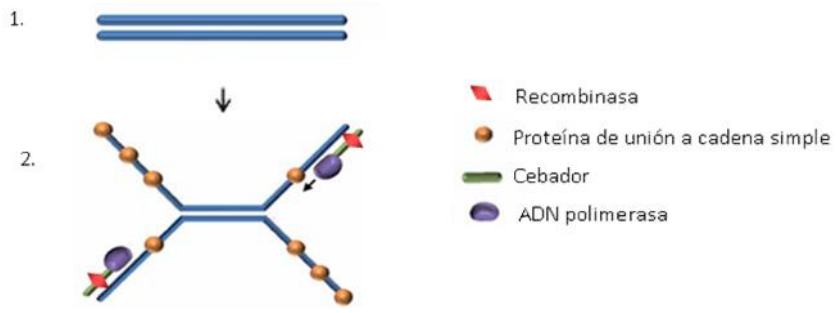


Figura 23. Esquema de amplificación mediante RPA.

g) Amplificación isoterma mediada por bucle (LAMP)

La LAMP, descrita por Notomi et al.,[202] se basa en el empleo de un sistema compuesto por dos cebadores internos, FIP (F1c+F2) y BIP (B2+B1c), y dos cebadores externos, F3 y B3, que reconocen un total de seis secuencias distintas en el ADN molde. Durante la amplificación, se forma una estructura en doble asa, a partir de la cual se genera una mezcla de productos de doble cadena con diferentes longitudes y múltiples bucles (Figura 24).

La LAMP es la técnica de amplificación isoterma más empleada en trabajos de investigación y patentes, ya que posee una elevada selectividad y capacidad de sintetizar grandes cantidades de ADN (10^9 copias/h). Como resultado de este alto rendimiento se producen elevadas concentraciones de iones pirofosfato, que al reaccionar con el magnesio del medio genera un precipitado blanco, facilitando la detección visual. El pirofosfato de magnesio producido está correlacionado con la concentración de ADN inicial, lo cual permite realizar el análisis cuantitativo de las muestras.[203]

En el mercado existen diversos kits comercializados por la empresa Eiken Chemical Company (Tokio) para amplificar ADN y ARN mediante LAMP.[204] Con respecto al desarrollo de sistemas miniaturizados, son numerosos los trabajos que emplean la LAMP como técnica de amplificación. Destacan diversos estudios,

[202] Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. *Nucleic Acids Res.*, 2000, 28, e63.
[203] Mori, Y., Nagamine, K., Tomita, N., Notomi, T. *Biochem. Biophys. Res. Commun.*, 2001, 289, 150-154.
[204] http://www.eiken.co.jp (Fecha de consulta Febrero/2015)

como el de Hataoka *et al.*,[205] que desarrollaron un microchip de PMMA para la amplificación por LAMP y posterior detección por electroforesis. Sin embargo, la mayoría de los trabajos proponen un sistema de detección óptico, basado en la medida de la fluorescencia o de la turbidez. Fang *et al.*[206] propusieron un dispositivo microfluidico para la detección turbidimétrica cuantitativa de 8 muestras en paralelo. Zhou *et al.*[207] desarrollaron un *lab-on-a-disc* para la amplificación en tiempo real de 10 muestras en paralelo mediante detección fluorescente. También destacan algunas aplicaciones, como la de Wu *et al.*,[208] que integran la fase de purificación, amplificación y detección, en un único dispositivo. En general, todas las aplicaciones mencionadas se caracterizan por obtener elevada sensibilidad en tiempos cortos.

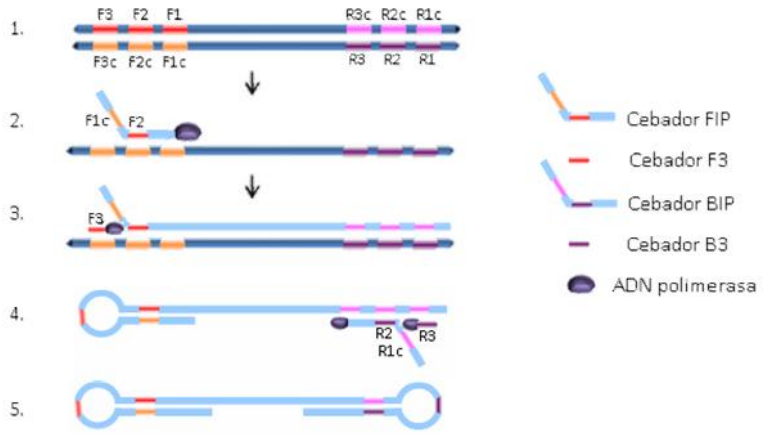


Figura 24. Esquema de amplificación mediante LAMP.

[205] Hataoka, Y., Zhang, L. H., Mori, Y., Tomita, N., Notomi, T., Baba, Y. *Anal. Chem.*, 2004, 76, 3689-3693.
[206] Fang, X., Liu, Y., Kong J., Jiang, X. *Anal. Chem.*, 2010, 82, 3002-3006.
[207] Zhou, Q. J., Wang, L., Chen, J., Wang, R. N., Shi, Y. H., Li, C. H., Zhang, D. M., Yan, X. J., Zhang, Y. J. *J. Microbiol. Meth.*, 2014, 104, 26-35.
[208] Wu, Q., Jin, W., Zhou, C., Han, S., Yang, W., Zhu, Q., Jin, Y., Mu, Y. *Anal. Chem.*, 2011, 83, 3336-3342.

h) Consideraciones generales para la amplificación en sistemas integrados

En la Tabla 6 se recogen las características más relevantes de las reacciones de amplificación isoterma descritas. A continuación se describen los principales aspectos a tener en cuenta para su integración en sistemas de análisis miniaturizados.[168]

- **Tipo de analito**. Si la molécula a detectar es una cadena de ADN, todas las reacciones permiten llevar a cabo la amplificación, pero en el caso de NASBA y SDA, se requiere una etapa previa para separar la doble cadena, dando lugar a protocolos de análisis más complejos.

- **Temperatura de reacción**. Las reacciones NASBA y SDA en aplicaciones de ADN requieren una etapa de desnaturalización inicial a 95 °C, previa a la reacción a la temperatura de trabajo. Otras reacciones como RCA, MDA y RPA presentan menores requerimientos energéticos al operar a una única temperatura próxima a la ambiental (30-42 °C), lo que simplifica y facilita su integración en sistemas analíticos.

- **Producto de amplificación**. La estructura de los fragmentos amplificados va a determinar la elección del método de detección posterior. Las amplificaciones mediante NASBA, SDA, HDA y RPA producen fragmentos amplificados de tamaño conocido y determinado, siendo compatibles con la mayoría de técnicas de detección aplicables a la PCR (electroforesis, hibridación, etc.). Por el contrario, la RCA, MDA y LAMP producen moléculas de tamaño desconocido que, en algunos casos, pueden formar estructuras secundarias, dificultando su posterior detección. En el caso de la LAMP, la formación de productos secundarios permite la detección cuantitativa sin ningún tratamiento adicional.

- **Manejo**. Las reacciones que requieran menor manipulación y menor número de etapas serán más sencillas y reproducibles. En este sentido, la RPA destaca por su simplicidad y rapidez.

Tabla 6. Comparación de las características de las principales técnicas de amplificación isoterma descritas.

Propiedad	NASBA	SDA	RCA	MDA	HDA	RPA	LAMP
Amplificación de ADN	Sí	Sí	Sí	Sí	Sí	Sí	Sí
Amplificación de ARN	Sí	Sí	Sí	Sí	Sí	Sí	Sí
Temperatura (°C)	37-42	37	30	30	60-65	37-42	60-65
Tiempo (min)	60-120	120	60-90	100-300	100-120	40-60	45-65
Tolerancia a inhibidores	Baja	Baja	Baja	Baja	Alta	Baja	Alta
Desnaturalización	Sí	Sí	No	No	No	No	No
Proteínas involucradas*	2-3	2	1-2	1	3	3	1
Diseño de cebadores	Simple	Complejo	Simple	Simple	Simple	Simple	Complejo
Amplificación múltiple	Sí	No	Sí	No	Sí	Sí	No
Producto de amplificación	ssARN	dsADN	ssADN	ssADN+dsADN ramificado	dsADN	dsADN	dsADN en bucles

* Polimerasas, RNAsas, helicasas, SSBs, transcriptasas, ligasas, recombinasas.

- **Capacidad de multiplexado**. La propiedad de amplificar múltiples analitos en una misma reacción es altamente demandada. Las reacciones NASBA, RCA, HDA y RPA son capaces de analizar simultáneamente más de un analito; sin embargo, hay que tener en cuenta que las reacciones múltiples pueden requerir tediosas etapas de optimización (diseño de cebadores, tiempos, temperaturas) para evitar amplificaciones preferentes o baja selectividad (reactividad cruzada).

- **Tolerancia a posibles inhibidores**. Las técnicas isotermas presentan una mayor tolerancia que la PCR a ciertos interferentes presentes en las muestras, lo que reduce la necesidad de introducir etapas de purificación en la preparación de muestra. Concretamente, la HDA y la LAMP han mostrado una mejor respuesta en muestras complejas.[182]

- **Sensibilidad**. Prácticamente todas las reacciones alcanzan un rendimiento de amplificación similar al de la PCR (10^8 copias/h), siendo la LAMP la que presenta una mayor capacidad de síntesis de ADN (>10^9 copias/h, reacciones de 25 µL). No obstante, la reducción del volumen de reacción que conlleva la miniaturización de los dispositivos integrados puede reducir la sensibilidad del método.

- **Rapidez y flexibilidad**. Es deseable que la reacción tenga lugar en el menor tiempo posible y que sea altamente adaptable, es decir, capaz de realizar nuevos ensayos con los mínimos cambios en el medio de reacción. En cuanto a rapidez de reacción, la RPA y la LAMP son las técnicas más destacadas, siendo su tiempo de reacción inferior a 60 min, frente a los 90-120 min requeridos en la PCR. Por otro lado, la HDA ha demostrado ser muy susceptible a pequeños cambios en el medio de reacción, lo que reduce su adaptabilidad a protocolos de ensayo integrados.

Las reacciones RPA, MDA y LAMP, pueden ser consideradas como las de mayor potencial para su aplicación en sistemas biosensores integrados basados en la tecnología de disco compacto, ya que ofrecen ventajas como rapidez, sencillez, bajo requerimiento energético, elevado rendimiento y posibilidad de realizar análisis en tiempo real.

2. Objetivos

El **objetivo principal** de la presente tesis doctoral es el desarrollo de nuevas técnicas de biosensado aplicadas a la determinación de analitos relacionados con la seguridad alimentaria, concretamente la detección e identificación de regiones específicas de ADN, de modo que se disponga de sistemas integrados, basado en la tecnología de disco compacto, con capacidad de multiplexado adaptables a aplicaciones *point-of-control*.

Para ello se plantean los siguientes objetivos **particulares**:

1. Puesta a punto de ensayos de hibridación de productos de amplificación sobre micromatrices de sondas de ácidos nucleicos.

2. Estudio de técnicas de amplificación isoterma con fines bioanalíticos. Optimización y análisis comparativo con respecto a la PCR, empleando como técnica de detección la hibridación en microplaca ELISA y disco compacto.

3. Integración de las fases de amplificación e hibridación de ácidos nucleicos en fase sólida.

4. Integración de las fases de amplificación y detección mediante un dispositivo basado en un sistema dinámico que permita llevar a cabo la detección cuantitativa en tiempo real.

3. Parte experimental y resultados

Capítulo 1. Amplificación por PCR y detección mediante ensayos de hibridación

En este capítulo, se investiga en el desarrollo de un sistema de análisis de micromatrices basado en la tecnología de disco compacto. Estos sistemas han demostrado ser una alternativa novedosa, que integran el reconocimiento biomolecular en la superficie de un disco compacto estándar, y la posterior detección/cuantificación mediante un lector/grabador comercial. Como se indicó en la Introducción, se han publicado diversos estudios que combinan esta tecnología con la hibridación de productos de la PCR, sin embargo, los ensayos descritos hasta ahora detectan un solo analito por reacción.

En el trabajo, titulado "*Multiplex DNA Detection of Food Allergens on Digital Versatile Disk*", se propone por primera vez la integración de la tecnología de disco compacto con un ensayo de amplificación e hibridación múltiple, es decir, de varios analitos en el mismo análisis. El estudio, aplicado a la detección de tres alérgenos alimentarios (avellana *Corylus avellana* L., cacahuete *Arachis hypogaea* y soja *Glycine max*), se ha basado en la amplificación simultánea por PCR y en la detección de los productos formados con sondas de oligonucleótidos inmovilizadas en la superficie de un DVD en formato micromatriz (Biosensor 1). El registro de las señales se ha realizado mediante un lector/grabador de DVDs estándar (Figura 25).

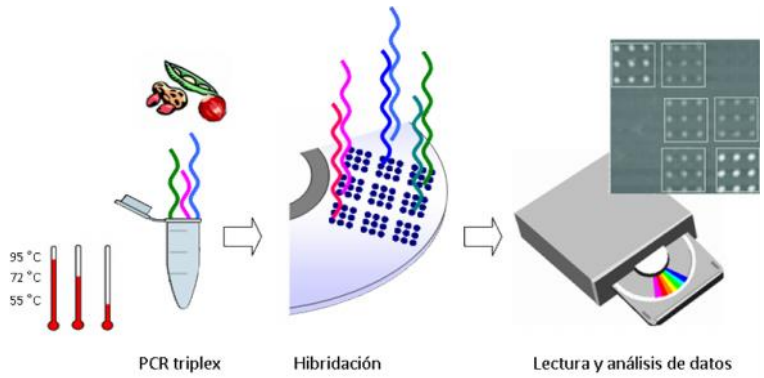


Figura 25. Esquema de detección simultánea de alérgenos (avellana, soja y cacahuete) y la hibridación sobre micromatrices de ADN en disco compacto.

Para ello, se han abordado las siguientes etapas:

- Selección del método de extracción de ADN genómico.
- Diseño de los cebadores y las sondas.
- Optimización de la reacción de PCR triplex en vial.
- Optimización del protocolo de hibridación.
- Determinación de los parámetros analíticos del método.
- Estudio de su viabilidad en análisis de muestras alimentarias.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Multiplex DNA detection of food allergens on digital versatile disk

Luis A. Tortajada-Genaro,[a] Sara Santiago-Felipe,[a] Sergi Morais,[a] José Antonio Gabaldón,[b] Rosa Puchades,[a] and Ángel Maquieira[a]

[a] *Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain*

[b] *Departamento de Tecnología de Alimentos. Universidad Católica San Antonio de Murcia, Murcia, Spain*

Reprinted with permission from ***J. Agric.Food Chem., 2012, 60, 36-43.***

Abstract

The development of a DNA microarray method on a digital versatile disk (DVD) is described for the simultaneous detection of traces of hazelnut (*Corylus avellana* L.), peanut (*Arachis hypogaea*), and soybean (*Glycine max*) in foods. After DNA extraction, multiplex PCR was set up using 5′-labeled specific primers for *Cor a 1*, *Ar h 2*, and *Le* genes, respectively. Digoxin-labeled PCR products were detected by hybridization with 5′-biotinylated probes immobilized on a streptavidin-modified DVD surface. The reaction product attenuates the signal intensity of the laser that reached the DVD drive used as detector, correlating well with the amount of amplified sequence. Analytical performances showed a detection limit of 1 μg/g and good assay reproducibility (RSD 8%), suitable for the simultaneous detection of the three targeted allergens. The developed methodology was tested with several commercially available foodstuffs, demonstrating its applicability. The results were in good agreement, in terms of sensitivity and reproducibility, with those obtained with ELISA, PCR-gel agarosa electrophoresis, and rt-PCR.

Keywords: Allergen, food, hazelnut, peanut, soybean, DNA microarraying, DVD.

1. Introduction

Individuals with food allergies, 2-4% of the total world population, can react adversely to the ingestion of very low amounts of allergens. Food labeling plays a crucial role in the prevention of the reactions by providing the consumer access to the information of health risks.[1] In this way, regulations of several countries oblige producers to indicate allergic food included in priority lists (e.g., EU Directive 2007/68/EC). The major allergenic ingredients come from milk, eggs, fish, peanuts, tree nuts, soybean, and cereals. However, most reported food-allergic events do not come from packaged foods because they have the major ingredient controlled. The problem is the inadvertent presence of food allergens, frequently present at low levels. Then, the challenge is the detection of traces of such allergens.

Detection methods of allergens are used for confirmatory or screening analysis. Mass spectrometry is a confirmatory tool for the unambiguous identification and/or characterization of food allergens.[1-4] Application of proteomics strategies allows the analysis of intact proteins or the use of accurate peptide mass for high-throughput analysis. Specialized personnel, efficiency of protein separation, and biomarkers requirements, together with the high cost of instrumentation, limit the routine application of most of these methods. Screening methods can be easily integrated as part of Hazard Analysis and Critical Control Point programs of industries or regulation agencies. They are classified according to the target molecule: specific protein or coding DNA.[5] Both have advantages and disadvantages. Immunoassays are still preferred methods because they directly detect the compound responsible for triggering the allergic events and they are simple and timeefficient.[3] Also, some industrial derivatives are extrudates with high content in potentially allergenic protein and extremely low DNA content, for example, soy derivatives. Therefore, enzyme-linked immunosorbent assays (ELISAs) or dipsticks are widely used in the food industry.[6] However, methods based on polymerase chain reaction (PCR) techniques provide interesting benefits. The concentration of DNA is nearly independent of the variety and growth conditions. The DNA molecule is relatively stable, allowing its extraction from all kinds of tissues, and the stability of reagents is also higher than

for proteins. Although the minimal eliciting doses, the lowest concentration to provoke allergic reaction, are still unknown, the detection limit of PCR-based methods is lower. Intensive processed and heat-treated foods are characterized by a higher degradation of proteins compared to DNA.[7] Also, DNA is efficiently extracted from food matrices and is less affected by extraction conditions as compared to proteins. Then, in recent years, the number of proposed DNA methods for food allergen detection has been growing.

End-point PCR or real-time-PCR methods have been reported for the detection of hazelnut,[8-10] peanut,[11-13] soybean,[14,15] sesame,[16] celery,[16] mustard,[16] lupine,[17] pistachio,[18] almond,[19] or other nuts.[20] However, food safety agencies and the food-processing industry are subjected to increasing scrutiny of their allergen control programs. Then, the multiplex methods offer the possibility to detect several allergens in one run with advantages such as saving time, reducing reagent costs, and lowering the probability of cross-contamination. Although an enzyme immunoassay has been described for detecting several allergen proteins, more advances have been achieved detecting targeted oligonucleotide sequences. Recently, simultaneous detection of relevant allergens has been described by multiplex real-time PCR methods.[21-23] The limitation of this technique is the reduced number of fluorophores for determining a few genes simultaneously. Ligation-dependent probe amplification (LPA) has been proposed for the simultaneous determination of 10 allergens.[24] The color channel limitations of real-time PCR are overcome, because the capillary electrophoretical separation after the common PCR amplification allows the use of the same fluorescent label for all allergens. However, the cost of instruments used in real-time PCR or LPA methods limits the application in several food industries or the close monitoring during manufacturing or distribution activities. In this way, microarrays allow an alternative analysis of multiple sequence targets in a single assay by PNA or DNA chips.[25,26]

An innovative approach is using a compact disk-based microarray system.[27,28] The advantages are multiple. First, compact disks (CDs) and digital versatile disks (DVDs) are the most cost-effective platforms with the ability both to conduct assays and to read, and even to record data, from the same disk using standard

drives. Second, commercial disks have shown good properties for probe immobilization (proteins and nucleic acids) and performing bioanalytical assays (e.g., chemically resistant, nonspecific interactions). Third, the surface of a single standard disk (94 cm^2) can hold thousands of spots (e.g., analytes, replicates, calibration standards, controls).

The aim of this study was to develop a simple, cost-effective, reliable, and highly sensitive methodology for allergen detection. DVD technology (disks and drives) was selected as the analytical platform, including appropriate controls to avoid false positives and guarantee high-quality results. Moreover, a new immobilization-detection system is proposed for DNA hybridization on disks. Previous studies used chemical or UV activation for the attachment of amino-modified probes in glass or polycarbonate disks; meanwhile, the detection of model sequences was usually performed by fluorescent scanners and CCD cameras or measuring errors by a CD driver.[27,29,30] In this study, several novelties have been incorporated. First, 5′- biotinylated probes and streptavidin were directly printed in a microarray format on the polycarbonate surface of a DVD. Second, targets were PCR products from food samples labeled during amplification. Third, multiplex detection was performed using a common developer for all targets based on peroxidaselabeled digoxigenin antibody. Fourth, the results are shown as an easily interpretable microarray image. In this paper, as a proof of concept, we present the PCR amplification combined with DVD microarraying that allows the simultaneous detection of three relevant allergens (hazelnut, peanut, and soybean) included in the priority list of the European Union (EU), the United States, and other countries. These seeds are considered to be the most difficult allergens to be analyzed, especially by protein-based techniques, due to extraction issues.[3]

2. Materials ans Methods

2.1 Chemicals and Food Products. Cetyltrimethylammonium bromide (CTAB), tris(hydroxymethyl)aminomethane (Tris), ethylendiaminetetraacetic acid (EDTA), β-mercaptoethanol, and 3,3′,5,5′- tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich (Madrid, Spain). Horseradish peroxidase-labeled digoxigenin

antibody (antiDIG-HRP) was from Abcam (Cambridge, U.K.). Tris-borate- EDTA buffer (TBE), RNase, proteinase K, agarose, electrophoresis loading buffer, and 50 bp and 1 kb ladders were purchased from Fisher Scientific (Madrid, Spain). Isopropanol, sodium chloride, chloroform, isoamyl alcohol, and ethanol were from Scharlau (Barcelona, Spain), and SYBR-Safe was from Invitrogen (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore). The oligonucleotides were from Roche Diagnostics (Barcelona, Spain) (Table 1). Several types of foods from different commercial brands were bought in local stores. Seeds, cookies, cereals, chocolates, pastas, soups, and snacks were stored at room temperature in the dark. Dairy products and jams were stored at -4 °C until use. Ice cream and frozen ready meals were stored at -20 °C.

Table 1. Primer, probe, and control sequences.

Target		Sequence 5'-3' [a]	Tm (°C)
Hazelnut	Primer F	Dig-ACTACATAAAGCAAAAGGTTGAAG	53.5
	Primer R	TCGTAATTGATTTTCTCCAGTTTG	55.2
	Probe	BtnTg-TTTTTCGGACAAAGCATCGCCTTCAATCA	67.1
Peanut	Primer F	Dig-CTAGTAGCCCTCGCCCTTTT	59.9
	Primer R	GGCATCTTCTGTCTCCTTGG	59.8
	Probe	BtnTg-TTTTTAGTTCCCACTGCTGCCTC	62.6
Soybean	Primer F	Dig-TCCACCCCCATCCACATTT	59.2
	Primer R	GGCATAGAAGGTGAAGTTGAAGGA	58.8
	Probe	BtnTg-TTTTTTTTTTCGAAGCTGGCAACGCTACCGGTT	74.1
C+ immob	Probe	Dig-TTTTTTTTTTTTTTTTGTCATGGGCCTCGTGTCGGAAAACC-BtnTg	81.0
C+ hybrid	Target	Dig-AGGGTCGTACACCGGCTGTAATCAAA	72.2
	Probe	BtnTg-TTTTTTTTTTTTTGATTACAGCCGGTGTACGACCCT	75.9
C- hybrid	Probe	BtnTg-ACCGTCGCGCACTATCTGATTTCAAA	73.3

[a]Dig: digoxigenin labeled, BtnTg: BiotinTEG labeled, C+ immob: immobilization positive control, C+ hybrid: hybridization positive control and C- hybrid: hybridization negative control.

2.2 DNA Extraction. Two extraction methods based on CTAB and column purification were tested. For the CTAB extraction, 100 g of sample was ground in a kitchen blender (Thermomix Vorwerk, Madrid, Spain) until homogenization. A 100 mg aliquot was mixed with 700 µL of extraction solution previously preheated at 65 °C (20 mg/mL CTAB, 1.4 M NaCl, 0.1 M Tris, 0.02 M EDTA, pH 8), 1.4 µL of β-mercaptoethanol, 10 µL of RNase (10 mg/mL), and 10 µL of proteinase K (20

mg/mL) and incubated at 65 °C for 10 min. After the addition of 100 µL of chloroform/isoamyl alcohol (24:1, v/v), a further incubation step was carried out at 65 °C for 30 min. After centrifugation for 10 min at 12,000 rpm (model 5415D, Eppendorf, Westbury, NY), the supernatant was transferred into a 1.5 mL tube, and 700 µL of chloroform/isoamyl alcohol (24:1, v/v) was added. Then, the mixture was centrifuged for 10 min at 12,000 rpm and the aqueous phase transferred into a new tube. After the addition of 700 µL of ice-cold isopropanol, the tubes were cooled for 10 min at -20 °C and centrifuged for 10 min at 12,000 rpm. The supernatant was removed, and the pellet was mixed with 500 µL of ice-cold ethanol. After a further centrifugation step (10 min at 1200 rpm), the supernatant was discarded and the pellet was dried for 60 min at room temperature. Finally, the dried pellet was dissolved in 100 µL of buffer (0.01 M Tris, 0.001 M EDTA, pH 8). The extracted DNA was stored at -20 °C until analysis. For column extraction, aliquots of 5-20 g of homogenized sample were extracted using a GMO extraction kit from Applied Biosystems (Madrid, Spain), according to the manufacturer's instructions. The extracted DNA was also stored at -20 °C until analysis.

For DNA extracts, the absorbances at 260 nm (A_{260}) and at 280 nm (A_{280}) were measured with a spectrophotometer (Agilent 8453 model, Madrid, Spain). An aliquot of the extract with loading buffer (Fisher Scientific, Madrid, Spain) was dispensed on a 0.7% (w/v) agarose gel. The molecular weight of the extracted DNA was determined by comparison with a 1 kb ladder, and the electrophoresis was carried out with TBE buffer at 120 V and room temperature. Gels were stained for 30 min with TBE containing SYBR-Safe at 0.01% (v/v), and bands were visualized on a UV transilluminator.

2.3 PCR Amplification. PCR reactions were carried out in a total volume of 50 µL using a thermocycler TC-400 from Bibby Scientific (Staffordshire, UK) and kit PCR Core from Roche. Single reactions were performed in ultrapure water, 1× Tris-KCl buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 mM $MgCl_2$, 200 µM dNTPs, 1.25 units of Taq DNA polymerase, 0.5 µM of both primers of only one allergen, and 150 ng of the genomic DNA. The thermal program was as follows:

initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final elongation at 72 °C for 5 min. Multiplex reactions were carried out similarly, but 0.5 µM of three primer pairs (hazelnut, peanut, and soybean) was added to the PCR mix. The absence of inhibitors in the DNA extracts was verified by parallel amplification of bacteriophage Lambda (Fisher Scientific, Madrid, Spain). An aliquot of PCR products was mixed with loading buffer, and the solution was loaded on a 3% (w/v) agarose gel. The size of the amplicons was determined by comparison with a 50 pb ladder, and electrophoresis was carried out with 1× TBE buffer at 120 V at room temperature. Gels were stained for 30 min with 1× TBE containing SYBR-Safe at 0.01% (v/v), and the bands were visualized on a UV transilluminator.

2.4 Microarraying. Bulk DVD-R disks were purchased from MPO Iberica (Madrid, Spain). The disks were first conditioned by gentle ethanol washing and water rinsing and then dried by centrifugation. Streptavidin (10 mg/L) and biotinylated probes (50 nM), diluted in printing buffer (50 mM carbonate buffer, pH 9.6, and 10% glycerol (v/v)), were transferred to the disk (50 nL) with a noncontact printing device (AD 1500 BioDot, Inc., Irvine, CA) in microarray format, controlling the working temperature and relative humidity at 25 °C and 90%, respectively.

2.5 DNA Array Hybridization and Data Analysis. The assays were carried out as follows: 10 µL of PCR product was mixed with 90 µL of 5× hybridization buffer (1× saline sodium citrate, NaCl 150 mM, sodium citrate 15 mM, pH 7) containing 10 nM positive hybridization control. Subsequently, the solution was denatured by heating at 95 °C during 5 min and transferred onto the DVD surface. After 120 min of incubation at 37 °C, 1 mL of antiDIG-HRP solution in PBS-T (1:250) was homogeneously dispensed on the washed disk. After 30 min at room temperature, the disk was washed and the product was developed by dispensing 1 mL of TMB solution. The reaction was stopped by washing the disk with water

after 2 min. The results were directly read by the DVD drive,[27] the reaction product optical density being related to the allergen amount.

2.6 Reference Methods. Identification of hazelnut and peanut was performed using ELISA kits from R-Biopharm AG (Darmstadt, Germany) according to the manufacturer's instructions. Absorbance was read with a Spectra MAX340pc microplate reader from Molecular Devices (Toronto, Canada) at 450 nm. The presence of soybean was detected by rt-PCR method with forward primer, 5′-TCCACCCCCATCCACATTT-3' reverse primer, 5′-GGCATAGAAGGTGAAGTTGAAGGA-3′; and probe, FAM-AACCGGTAGCGTTGCCAGCTTCG-TAMRA. The reaction conditions were as follows: 10 min at 95 °C for enzyme activation, 50 cycles of denaturation at 95 °C (15 s), and annealing at 60 °C (1 min). Readings were taken using a real-time sequence detection system 5700 from Applied Biosystems. The threshold was set at 10 times the standard deviation of the mean baseline emission calculated between the 3rd and 15th cycles. Gel electrophoresis was also used for the detection of three allergens. An aliquot of end-point-PCR products (8 μL) was mixed with 2 μL of 5× glycerol loading dye, and the solution was loaded on a 3% (w/v) agarose gel. DNA standard of known size (50 bp DNA) was used for the determination of the DNA fragment size. Electrophoresis was run in 1× TBE buffer solution at 120 V and room temperature. PCR product bands were stained with SYBR-Safe at 0.01% (v/v), and bands were visualized on a UV transilluminator.

3. Results

3.1 DNA Extraction. Analyte extraction is considered to be the most critical step in food allergen analysis, especially in processed foods. The extraction of genomic DNA from complex matrices, such as fat-rich and polyphenol-rich foods, is especially difficult because these compounds can potentially be coextracted and inhibit the PCR. Another problem is achieving a representative fraction from a food sample containing the trace allergen. A high-quality template DNA is an important requirement to facilitate the detection of the target sequences.[31] For that, two genomic DNA extraction methods based on different principles (CTAB-

based and column purification) were compared in food samples with different processing techniques and compositions. The DNA concentration was calculated according to the equation c (ng/μL) = A_{260} × 50 × dilution factor. The ratio A_{260}/A_{280} was used to evaluate the quality of the isolated DNA. The results of the extraction study are shown in Table 2. The amounts of DNA extracted were similar using the column method or the CTAB method. In terms of quality, the ratio of absorbance A_{260}/A_{280} was evaluated, being higher than 1.5 for all samples (raw or processed) using both methods. The integrity of the DNA extracts was assessed through agarose gel electrophoresis. Welldefined bands were observed with good accordance between the two extraction methods. PCR sensitivity is strongly dependent on the amount and quality of the DNA template, but inhibition was not observed in any extract, allowing the correct amplification and detection of allergen in spiked samples, as explained in the following sections. Because a good extraction yield, a high purity, and a good amplification of the target sequences were obtained, both extraction protocols can be used for DNA-based allergen detection.

Table 2. Yield and purity of DNA extracted using the CTAB and column methods.

	«g DNA/g of food		A_{260}/A_{280}	
	CTAB	**Column**	**CTAB**	**Column**
Raw hazelnut	32.2 ± 1.6	25.5 ± 1.0	1.50 ± 0.03	1.93 ± 0.19
Soy cookies	34.5 ± 0.5	18.0 ± 2.0	1.56 ± 0.04	1.71 ± 0.10
Dried soup	36.9 ± 1.6	19.7 ± 0.9	1.51 ± 0.09	1.67 ± 0.08
Soybean seeds	35.9 ± 1.1	61.4 ± 1.3	1.55 ± 0.08	1.74 ± 0.07
Soybean flour	38.6 ± 1.5	59.0 ± 1.9	1.50 ± 0.11	1.69 ± 0.12
Cocoa powder	40.4 ± 1.4	74.0 ± 1.9	1.63 ± 0.07	1.55 ± 0.06
Cow milk	21.1 ± 3.0	17.5 ± 1.6	1.66 ± 0.18	1.64 ± 0.15
Peanut butter	29.5 ± 1.3	15.4 ± 1.1	1.56 ± 0.05	1.56 ± 0.04
Chocolate spread	38.7 ± 1.7	47.8 ± 1.9	1.49 ± 0.07	1.59 ± 0.05
Soy yogurt	37.5 ± 1.0	21.9 ± 0.6	1.65 ± 0.10	1.70 ± 0.07

3.2 Optimization of PCR. According to PCR product specificity, minimal self-annealing, and similar melting temperatures, the target sequences were selected from the allergen genes (GenBank database). The selected genes were *Cor a 1* (accession no.Z72440) of hazelnut (*Corylus avellana* L.), *Ara h 2* (accession no. L77197.1) of peanut (*Arachis hypogaea*), and *Le* (accession no. K00821) of

soybean (*Glycine max*). The design of a novel peanut-specific primer pair was performed on the Ara h 2 coding region sequence using the software program Primer3Plus. The primer sequences of soybean and hazelnut were selected from the literature,[21,32] ensuring the absence of cross-dimers. All primers and probes were successfully checked for relevant homologies by BLASTNr search. Before the setup of triplex assay, the efficiency of each PCR system was tested. For that, an experimental design was carried out to optimize the PCR conditions, studying the response from dilution series and mixtures of allergens in blank food samples. A temperature gradient was performed from 56 to 65 °C to determine a common annealing temperature; it was optimal at 60 °C. The concentrations of primers tested were from 0.3 to 0.7 μM; it was optimal at 0.5 μM. Each PCR reaction generated the predicted product (109 bp for hazelnut, 82 bp for peanut, and 81 bp for soybean). The optimization of multiplex PCR was performed with mixtures of DNA extracted from the allergens and blank food samples. Under the working conditions (0.5 μM for each primer), any preferential amplification was detected, even for mixtures in which an allergen was present in a minor amount and other allergens were in excess.

3.3 DNA Array Hybridization and Detection. Multiplexed PCR products were thermally denatured and dispensed on the DVD modified surface. For that, the array layout on the DVD surface consisted of nine blocks of nine dots each: three blocks corresponding to single-target systems (specific allergen); two blocks corresponding to multitarget systems (three allergens); two positive controls (immobilization and hybridization); and two negative controls (immobilization and hybridization). A total of 10 arrays were printed on the polycarbonate surface of the DVD-R, allowing the simultaneous analysis of 10 samples (Figure 1). In this configuration, spots are 500 μm in diameter with a track pitch (center-to-center distance) of 1.5 mm, achieving an array density of 1.0 spot/mm^2. The storage stability of the modified DVD was tested at 4 °C until 7 weeks. The same analytical performances were observed.

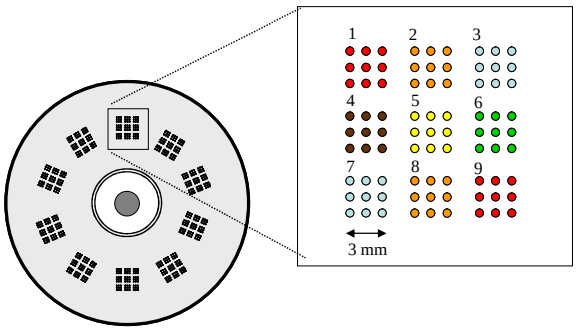


Figure 1. Scheme of DVD arrays. The blocks correspond to hybridization positive control (1), allergen probe mix (2), hybridization negative control (3), hazelnut probe (4), peanut probe (5), soybean probe (6), immobilization negative control (7), allergen probe mix (8), and immobilization positive control (9).

The hybridization assays were carried out at different incubation conditions (25-55 °C, 30-180 min) and concentrations of hybridization buffer (1-20×) to achieve the maximum yield. The best results on the basis on relative signal intensity were obtained using hybridization buffer 5× at 37 °C during 120 min. Next, the digoxigenin-labeled hybridization product was developed by the HRP-antidigoxigenin antibody-TMB system (Figure 2). The resulting solid changed the intensity of reflected light at 650 nm, allowing the quantification by the DVD drive. To verify that the assay steps are well-developed, several assay controls were implemented. The immobilization controls correspond to a double-labeled probe (positive) and a nonprobe block (negative). The hybridization controls correspond to a specific probe to digoxigenin-labeled oligonucleotide added together with PCR products (positive) and a noncomplementary oligonucleotide (negative). Non-cross-hybridization or nonspecific responses were observed.

Although specificity was successfully checked by BLAST search, DNA from several plant species and other food ingredients was isolated and tested in the selected multiplex PCR system. Almond, barley, bean, Brazil nut, cashew, chestnut, chickpea, corn, egg, kidney bean, lentil, lupine, oat, olive oil, pea, pecan, pistachio, powdered milk, rice, rye, sesame, sunflower seed, tomato, walnut, and wheat were tested. No cross-reactivity was observed, negative responses, confirming the high specificity of the developed assay.

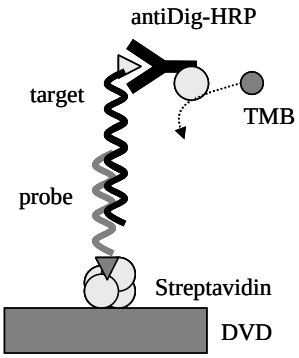


Figure 2. Scheme of the assay format developed on DVD surface.

The detection limit (LOD) was determined in two ways, by analyzing serially diluted DNA extracted from allergenic foods and the whole food product spiked with certain amounts of allergens. In both cases, allergen-free food samples were selected by obtaining blank responses with the DVD-based method or reference methods (ELISA or rt-PCR). They included cookies, jams, sponge cake, powdered milk, and frozen ready meals. First, mixtures of DNA extracted from three allergens were diluted with allergen-free food extracts, keeping a total DNA concentration constant (30 ng/µL). Figure 3 shows the images after scanning for a 10-fold serial dilution. Although the aim of this research was not the quantification of allergen, a correlation between the DNA amount of food allergens and the optical intensity measured by DVD detector was found. Nevertheless, the sensitivity of the screening assay was related to the signal-to-noise ratio (SNR) calculated from background signal. The 1 µg/g dilution was positive for three allergens, because SNR was >3, but lower concentrations were not detected. Second, commercial food products free from the three allergens were spiked with different combinations of hazelnut, peanut, and soybean until 10% of each one. The spiked samples were extracted, amplified, and analyzed. The measured LODs were 1 µg/g (0.0001%) for the three allergens, demonstrating the absence of inhibition effects during extraction. Also, method sensitivity was independent of food processing, the relative amount of allergen, or the nature of the food in which these allergens are usually contained as ingredients. These

values were similar to or better than those calculated by rt-PCR methods or the LPA method for allergen detection (>1 μg/g).[3]

Assay reproducibility was also determined from the optical density of spots. To determine intradisk and disk-to-disk relative standard deviations (RSDs) of positive controls, three DVDs were tested, each with 10 arrays and 9 replicates per array. The intradisk RSD varied from 3.6 to 5.3%, whereas the disk-to-disk RSD ranged from 5.8 to 7.3%, indicating their suitability. Assay reproducibility was also investigated by analyzing replicates of DNA extracts (n= 3), varying from 3.1 to 14.5%. No dependence of allergen nature was statistically confirmed by an ANOVA test. Therefore, the results were suitable for routine qualitative detection of food allergens.

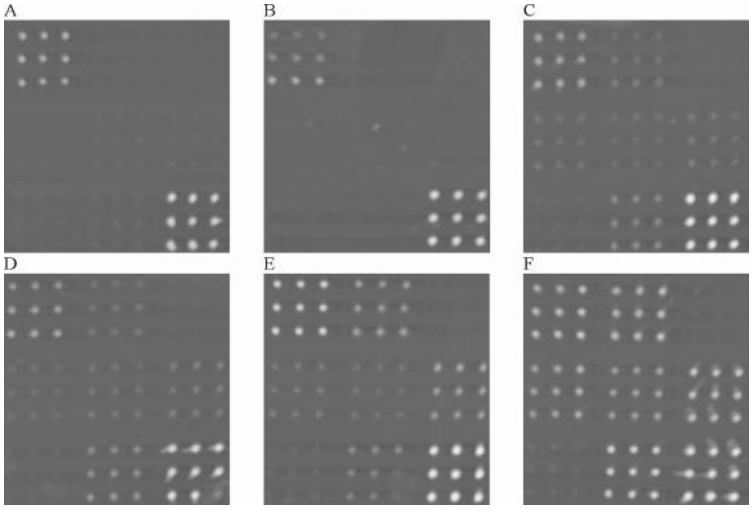


Figure 3. Optical density images from different assays. Panels A-F correspond to 0, 0.1, 1, 10, 100, and 1,000 μg/g of allergen mixture (hazelnut, peanut, and soybean), respectively.

3.4 Determination of Allergen Presence in Food Samples. A two-laboratory validation study was performed by analyzing 10 blind samples (unknown allergen content for participants). The methods were based on DVD technology (three allergens, simultaneously) and ELISA (hazelnut and peanut, individually) or rt-PCR (soybean). Detection limits of both ELISA methods were 0.00025%, and that of the rt-PCR method was 0.0005%. Table 3 shows the excellent agreement between the

proposed triplex assay and the three reference methods developed in an external laboratory. No allergen was detected in three food samples; meanwhile, one allergen was detected in the positive food samples. It is worth mentioning that the multiplexed analysis by DVD technology is able to analyze simultaneously several allergens, a goal that is very difficult to reach with screening protein-based methods.

Table 3. Comparison of screening results of allergens in commercial foodstuffs analyzed by DVD and reference methods (n=3).

Category	**Food**	**DVD-based method** [a]			**ELISA method**	**ELISA method**	**rt-PCR method**
		Hazelnut	Peanut	Soybean	Hazelnut	Peanut	Soybean
Jam	Quince paste 1	+	-	-	+	-	-
	Quince paste 2	-	-	-	-	-	-
	Quince paste 3	-	+	-	-	+	-
	Quince paste 4	-	-	-	-	-	-
Frozen ready meal	Meatballs	-	-	+	-	-	+
	Ham & Cheese escalope	-	-	+	-	-	+
	Chicken nuggets	-	-	+	-	-	+
	Vegetable Rice	-	-	+	-	-	+
	Cauliflower	-	-	-	-	-	-
	Scrambled eggs with shrimp	-	-	-	-	-	-

[a] Detection: + presence; - absence

Twenty-five food samples were analyzed for determining the presence of DNA from allergens using the triplex PCR method and DVD technology. Foodstuff was selected including several categories of them and a large variety of food-processing methodologies. Figure 4 shows examples of results from food samples. Besides positive controls, when only one allergen was present in food sample, the signal was observed in both multitarget block and specific block. In the case of the presence of some allergens, the associated blocks were detected. For all cases, specific hybridizations were obtained, because each target sequence hybridized to their corresponding specific capture probe. The presence of allergens was assessed according to the declaration on the label (Table 4). Allergen DNA was not found in sponge cake, corn flakes, cocoa powder, strawberry yogurt, cow's milk, and noodles. Hazelnut was detected in one cookie sample, cereal bars, and

several chocolate products. Peanut was detected in several cookies, one cereal product, flat breadsticks, and peanut butter. Twelve samples were positive to soybean, including cookies, chocolate products, ice cream, soups, and snacks. To check the performances of our technique, some protein derivatives of soybean were included. In the case of cookies containing traces of soybean lecithin, it was not detected by any DNA-based technique. Cookies containing protein extrude (<1%), an industrial derivative with extremely low DNA content, were positive by DVD technology. These results confirm the reliability and sensitivity of the proposed method in the qualitative detection of allergens even in some samples having a low DNA content (impurities of protein isolation process).

Several allergens were detected in seven food samples (37% of positive samples), demonstrating the importance of multiplexing to reduce the efforts of screening methods. The same food samples were also analyzed using an agarose gel electrophoresis technique to establish the presence or absence of specific bands. Good agreement was observed when allergen was found at high concentrations. However, in several cookies, ice cream, dried soups, and flat breadsticks, the presence of at least one allergen was detected only by the DVD technique.

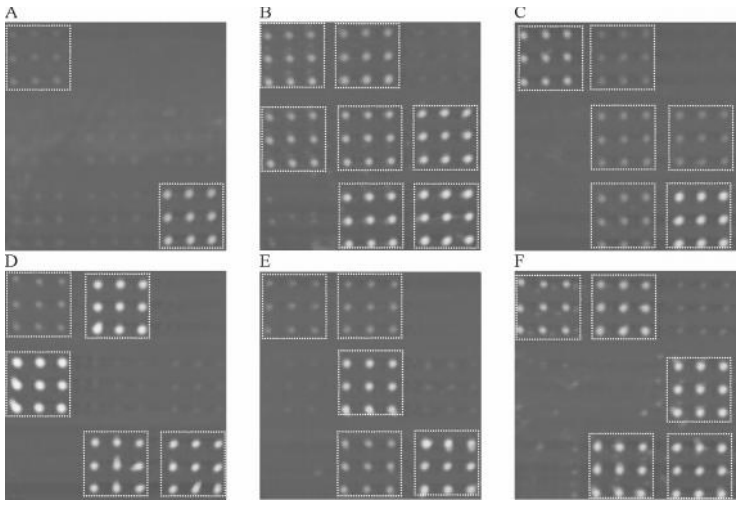


Figure 4. Optical density images from food samples: noodles (A); chocolate wafers (B); flat breadsticks (C); chocolate bar (D); peanut butter (E); ice cream (F).

Table 4. Comparison of screening results of allergens in commercial foodstuffs analyzed by DVD and electrophoresis techniques (n=3).

Category	Food	Declared Allergen [a]			Detected Allergen [b]					
					DVD-based method			Electrophoresis		
		Hazelnut	Peanut	Soybean	Hazelnut	Peanut	Soybean	Hazelnut	Peanut	Soybean
Seeds	Raw hazelnut	+	-	-	+	-	-	+	-	-
	Roasted peanut	-	+	-	-	+	-	-	+	-
	Soybean flour	-	-	+	-	-	+	-	-	+
Cookies	Chocolate cookies	±	±	+	-	+	+	-	+	+
	Wheat cookies	-	±	±	-	+	+	-	-	-
	Muesli cookies	±	±	+	+	+	+	-	-	+
	Butter cookies	-	±	±	-	+	+	-	-	-
	Sponge cake	-	-	-	-	-	-	-	-	-
	Soy cookies	-	-	+	-	-	+	-	-	+
	Soy lecithin cookies	-	-	+	-	-	-	-	-	-
	Soy protein cookies	-	-	+	-	-	+	-	-	-
Cereals	Cereals bar	+	±	-	+	+	-	+	+	-
	Corn flakes	-	±	-	-	-	-	-	-	-
Chocolates	Chocolate bar	+	-	-	+	-	-	+	-	-
	Chocolate spread	+	-	-	+	-	-	+	-	-
	Cocoa powder	-	-	-	-	-	-	-	-	-
	Chocolate wafer	±	+	+	+	+	+	+	+	+
Dairy products	Strawberry yogurt	-	-	-	-	-	-	-	-	-
	Soy yogurt	-	-	+	-	-	+	-	-	+
	Cow milk	-	-	-	-	-	-	-	-	-
	Ice cream	-	-	±	-	-	+	-	-	-
Soups	Dried soup	-	-	±	-	-	+	-	-	-
Pasta	Noodles	-	-	-	-	-	-	-	-	-
Snaks	Flat breadsticks	-	+	±	-	+	+	-	+	-
Butters	Peanut butter	-	+	-	-	+	-	-	+	-

[a] Declaration: + hazelnut, peanut, or soybean listed; - hazelnut, peanut, or soybean not listed; ± may contain trace levels.

[b] Detection: + presence; - absence

4. Discussion

A multiplexed DNA-based method using compact disk technology has been developed, demonstrating the usefulness for allergen determination. The proposed oligonucleotide microarrays are capable of detecting target genes of regulated allergens, including controls that guarantee the reliability of the analysis. As has been demonstrated, low-cost DVD technology can be used as a simple screening test of their presence/absence in foodstuffs of different nature. The assay specificity was good, showing no cross-reactivity in the experiments, and high reproducibility reinforces the use of this methodology for screening purposes. The multiplexed analysis on DVD is flexible to increase significantly the number of samples, replicates, or allergen targets to be simultaneously detected. A change in array layout is totally compatible with proposed immobilization-detection systems, and it would allow the determination of other priority or emergent allergens at one time. Besides, the universal nature of PCR and DVD technology suits the needs of the food industry, production or distribution areas, having significant concerns about food allergenic consumer complaints.

The analytical performances of DVD microarray technology were compared to other methods reported for screening determination of food allergens. A limitation of our approach is that allergenic protein is not directly analyzed, because it is based on the detection of a specific gene of the allergen. However, it was demonstrated that DNA from allergens was successfully extracted and detected in several processed foods, which is difficult for some protein-based methods. Also, some researchers have pointed out that DNA-based methods are more suitable than ELISA when closely related species have to be analyzed.[3] The detection limit, analysis time, reproducibility, and/or multiplexing capacity are the main advantages of the proposed method against gel electrophoresis analysis, ELISA, or single rt-PCR.

Multiplex rt-PCR is a potential competitive technique, because similar analytical performances have been reported achieving a tetraplex quantitative assay.[22] This technique without post-PCR steps provided shorter assay times, minimizing the risk of cross-contamination, although the detection limits are better for DVD technology. However, the main drawback of rt-PCR could be the

maximum number of allergens than can be determined, including internal control, to guarantee the correct analysis performance. Fluorescent detection is limited by the availability of different labeling dyes for probes. Multiplexed analysis on DVD is flexible to increase significantly the number of samples, replicates, or allergen targets to be simultaneously detected, due to the spatial separation of probes. Another potential competitive method is LDA, which allows the simultaneous determination of up to 10 allergens with similar selectivity, sensitivity, or reproducibility.[24] The main limitation is the analysis time (the duration of the ligation-hybridization step is 17-18 h), the sequential detection mode, and cost by reaction. Both alternatives (rt-PCR and LDA) are less portable and more expensive than DVD technology. Therefore, the proposed method is especially suitable for screening applications in the point-of-control facilities with a low availability of equipment or resources and maintaining the analytical performances. On the other hand, the compact disk based PCR microarray technology could be quite compatible with isothermal amplification or other simple developing approaches, to improve the competitiveness and simplify the working protocols.

Funding

This research was funded through projects FEDER MICINN CTQ2010-15943 and by Generalitat Valenciana (GV/2009/ 028 and PROMETEO/2010/008). The Spanish Ministerio de Educación y Ciencia provided S.S.-F. with a grant for her Ph.D. studies.

References

1. van Hengel, A. J. *Anal. Bioanal. Chem.*, 2007, 389, 111-118.
2. Monaci, L.; Visconti, A. *Trends Anal. Chem.*, 2009, 28, 581-591.
3. Monaci, L.; Visconti, A. *Trends Food Sci. Technol.*, 2010, 21, 272-283.
4. Heick, J.; Fischer, M.; Pöpping, B. *J. Chromatogr., A*, 2011, 1218, 938-943.
5. Besler, M. *Trends Anal. Chem.*, 2001, 20, 668-672.
6. Schubert-Ullrich, P.; Rudolf, J.; Ansari, P.; Galler, B.; Führer, M.; Molinelli, A.; Baumgartner, S. *Anal. Bioanal. Chem*., 2009, 395, 69-81.
7. Scaravelli, E.; Brohée, M.; Marchelli, R.; van Hengel, A. J. *Anal. Bioanal. Chem.*, 2009, 395, 127-137.
8. Holzhauser, T.; Wangorsch, A.; Vieths, S. *Eur. Food Res. Technol.*, 2000, 211, 360-365.

9. Arlorio, M.; Cereti, E.; Coïsson, J. D.; Travaglia, F.; Martelli, A. *Food Control*, 2007, 18, 140-148.
10. Piknová, L.; Pangallo, D.; Kuchta, T. *Eur. Food Res. Technol.*, 2008, 226, 1155-1158.
11. Hird, H.; Lloyd, J.; Goodier, R.; Brown, J.; Reece, P. *Eur. Food Res. Technol.*, 2003, 217, 265-268.
12. Stephan, O.; Vieths, S. *J. Agric. Food Chem.*, 2004, 52, 3754-3760.
13. Scaravelli, E.; Brohée, M.; Marchelli, R.; van Hengel, A. J. *Eur. Food Res. Technol.*, 2008, 227, 857-869.
14. Soares, S.; Mafra, I.; Amaral, J. S.; Oliveira, M. B. P. P. *Int. J. Food Sci. Technol.*, 2010, 45, 2581-2588.
15. Pedersen, M. H.; Holzhauser, T.; Bisson, C.; Conti, A.; Jensen, L. B.; Skov, P. S.; Bindslev-Jensen, C.; Brinch, D. T.; Poulsen, L. K. *Mol. Nutr. Food Res.*, 2008, 52, 1486-1496.
16. Mustorp, S.; Engdahl-Axelsson, C.; Svensson, U.; Holck, A. *Eur. Food Res. Technol.*, 2008, 226, 771-778.
17. Demmel, A.; Hupfer, C.; Hampe, E. I.; Busch, U.; Engel, K. H. *J. Agric. Food Chem.*, 2008, 56, 4328-4332.
18. Breqná, B.; Dudánová, H.; Kuchta, T. *Eur. Food Res. Technol.*, 2008, 228, 197-203.
19. Pafundo, S.; Gullì, M.; Marmiroli, N. *Food Chem.*, 2009, 116, 811-815.
20. Röder, M.; Filbert, H.; Holzhauser, T. *Anal. Bioanal. Chem.*, 2010, 398, 2279-2288.
21. Schöringhumer, K.; Redl, G.; Cichna-Markl, M. *J. Agric. Food Chem.*, 2009, 57, 2126-2134.
22. Köppel, R.; Dvorak, V.; Zimmerli, F.; Breitenmoser, A.; Eugster, A.; Waiblinger, H. U. *Eur. Food Res. Technol.*, 2010, 230, 367-374.
23. Pafundo, S.; Gullì, M.; Marmiroli, N. *Anal. Bioanal. Chem.*, 2010, 396, 1831-1839.
24. Mustorp, S.; Drømtorp, S. M.; Holck, A. L. *J. Agric. Food Chem.*, 2011, 59, 5231-5239.
25. Rossi, S.; Scaravelli, E.; Germini, A.; Corradini, R.; Fogher, C.; Marchelli, R. *Eur. Food Res. Technol.*, 2006, 223, 1-6.
26. Bettazzi, F.; Lucarelli, F.; Palchetti, I.; Berti, F.; Marrazza, G.; Mascini, M. *Anal. Chim. Acta*, 2008, 614, 93-102.
27. Morais, S.; Marco-Moles, R.; Puchades, R.; Maquieira, A. *Chem. Commun.*, 2006, 22, 2368-2370.
28. Morais, S.; Tortajada-Genaro, L. A.; Arnadis-Chover, T.; Puchades, R.; Maquieira, A. *Anal. Chem.*, 2009, 81, 5646-5654.
29. Chen, H.; Wang, L.; Li, P. C. H. *Lab Chip*, 2008, 8, 826-829.
30. Li, Y.; Wang, Z.; Ou, L. M. L.; Yu, H. Z. *Anal. Chem.*, 2008, 80, 8216-8223.
31. Demeke, T.; Jenkins, G. R. *Anal. Bioanal. Chem.*, 2010, 396, 1977-1990.
32. International Organization for Standardization. 21569:2005 Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products- qualitative nucleic acid based methods. International Organization for Standardization, Geneva, Switzerland, 2005.

Capítulo 2. Amplificación isoterma y detección mediante ensayos de hibridación

En este capítulo, se explora el potencial de las reacciones de amplificación isoterma como alternativa a la PCR previa a un ensayo de hibridación. Dado que salvan los inconvenientes que presenta la PCR, las reacciones isotermas presentan un gran potencial para el desarrollo de biosensores en aplicaciones génicas.

Se ha estudiado la compatibilidad de estas reacciones en ensayos de hibridación con sondas de ácidos nucleicos. Para ello se han seguido diferentes estrategias.

En un primer trabajo, "*Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food análisis*", se propone la amplificación por RPA combinada con la detección mediante ensayos de hibridación en una microplaca ELISA y revelado inmunoenzimático (Figura 26). Este método innovador se ha denominado RPA-ELISA (Biosensor 2).

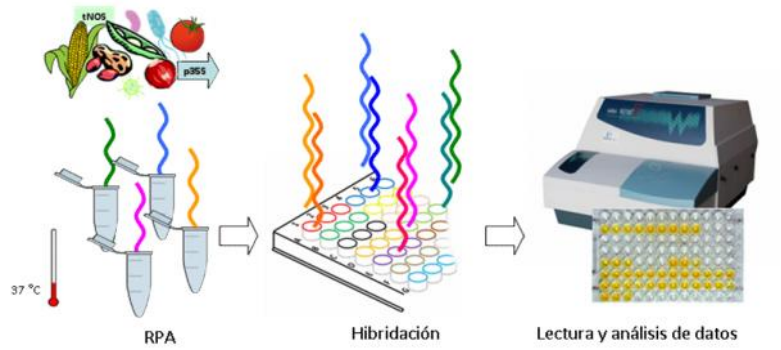


Figura 26. Esquema de la amplificación isoterma y detección mediante hibridación sobre micromatrices de ADN en placa ELISA.

El estudio, aplicado a la detección de diversas amenazas alimentarias (alérgenos, OGMs y bacterias y hongos patógenos), consta de las siguientes etapas:

- Puesta a punto de las reacciones de amplificación por PCR y RPA para cada analito en vial.
- Optimización del protocolo de hibridación de los fragmentos amplificados con las sondas de ADN inmovilizadas en la placa.
- Optimización de los ensayos de revelado y detección.

- Comparación de los resultados obtenidos para cada técnica de amplificación.
- Determinación de los parámetros analíticos del método.
- Estudio de viabilidad para el análisis de muestras alimentarias.

Los resultados obtenidos en este estudio llevan al desarrollo de un nuevo trabajo en el que se propone la combinación de las reacciones de amplificación isoterma con la detección mediante la tecnología de disco compacto. En la publicación, titulada "*Isothermal DNA amplification strategies for duplex microorganism detection*", se recogen los principales resultados del estudio de dos tecnologías isotermas de amplificación, RPA y MDA, combinadas con la posterior detección de sus productos utilizando la tecnología de DVD (Biosensores 3 y 4, respectivamente) (Figura 27).

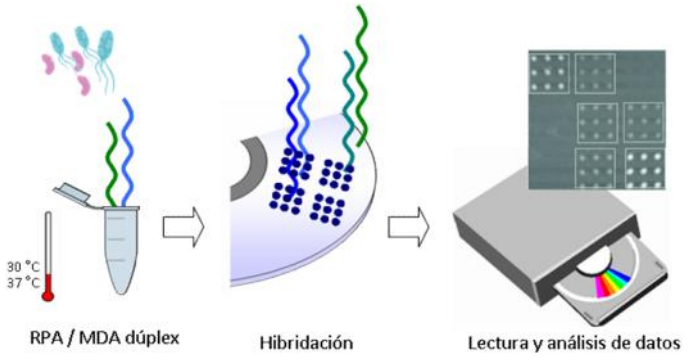


Figura 27. Esquema de la amplificación isoterma dúplex y detección mediante ensayos de hibridación en formato micromatriz sobre DVD.

El trabajo, aplicado a la detección dúplex de las bacterias patógenas *Salmonella* spp. y *Cronobacter* spp., consta de las siguientes etapas:

- Optimización de las reacciones de amplificación RPA, MDA y PCR en vial.
- Evaluación de protocolos post-amplificación para los fragmentos amplificados por MDA.
- Optimización de los ensayos de hibridación con las sondas de ADN inmovilizadas sobre la superficie del DVD.

- Estudio del efecto de posibles inhibidores presentes en la matriz alimentaria sobre las diferentes polimerasas.
- Comparación de los resultados obtenidos para cada técnica de amplificación.
- Determinación de los parámetros analíticos del método.
- Estudio de viabilidad en el análisis de muestras alimentarias.

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis

Sara Santiago-Felipe, Luis Antonio Tortajada-Genaro, Rosa Puchades, Ángel Maquieira

Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain

Reprinted from ***Anal. Chim. Acta, 2014, 811, 81-87****.*

Abstract

Polymerase chain reaction in conjunction with enzyme-linked immunosorbent assay (PCR-ELISA) is a well-established technique that provides a suitable rapid, sensitive, and selective method for a broad range of applications. However, the need for precise rapid temperature cycling of PCR is an important drawback that can be overcome by employing isothermal amplification reactions such as recombinase polymerase amplification (RPA). The RPA-ELISA combination is proposed for amplification at a low, constant temperature (40 °C) in a short time (40 min), for the hybridisation of labelled products to specific 5'-biotinylated probes/streptavidin in coated microtiter plates at room temperature, and for detection by colorimetric immunoassay. RPA-ELISA was applied to screen common safety threats in foodstuffs, such as allergens (hazelnut, peanut, soybean, tomato, and maize), genetically modified organisms (P35S and TNOS), pathogenic bacteria (*Salmonella* spp. and *Cronobacter* spp.), and fungi (*Fusarium*

spp.). Satisfactory sensitivity and reproducibility results were achieved for all the targets. The RPA-ELISA technique does away with thermocycling and provides a suitable sensitive, specific, and cost-effective method for routine applications, and proves particularly useful for resource limited settings.

Keywords: Isothermal amplification, ELISA, allergen, GMO, pathogen, food safety.

Highlights

• Recombinase polymerase amplification is a powerful DNA method operating at 40 °C.

• The combination RPA-ELISA gives excellent performances for highthroughput analysis.

• Screening of food safety threats has been done using standard laboratory equipment.

• Allergens, GMOs, bacteria, and fungi have been successfully determined.

1. Introduction

Analytical methods for fast, reliable, sensitive and cost-effective detection are highly demanded in many areas, including food safety. Methods based on the detection of nucleic acids offer interesting benefits because DNA molecules show constant concentrations, stability, and better extraction yields (even from processed and heattreated samples) than proteins.[1,2] Furthermore, the amplification by polymerase chain reaction (PCR) ensures the required sensitivity levels. Consequently in recent years, the PCR-based methods, e.g., rt-PCR, digital PCR or microarray, are the gold standard for the analysis of nucleic acids.[2-4]

The demand for sequence specific approaches that do not require laborious or expensive detection technologies has led to the hyphenation of PCR with enzyme-linked immunosorbent assay (PCR-ELISA).[5-7] This combines the high selectivity of DNA-based methods with ELISA sensitivity. This integration involves the hybridisation of labelled amplification products to specific captured probes in each microtiter well, as well as their immunodetection. Although sensitivity can be improved using fluorometric or chemiluminiscent substrates, PCR-ELISA methods normally employ colorimetric detection because better reproducibility, cost-by-assay, and stability are achieved.[6-8] Therefore, colorimetric PCR-ELISA is a method that is capable of processing up to 96 or 384 assays simultaneously, it is potentially automatable, and only requires the basic instruments present in any diagnostic laboratory.

The thermal PCR technique has its limitations, such as requiring precise temperature control and rapid thermocycling steps among the temperatures of dissociation (95 °C), annealing (55-65 °C) and elongation (70 °C).[6] Yet the use of other enzymes (or a combination of enzymes) to mimic DNA replication in vivo has emerged as a solution to conventional PCR polymerases.[9,10] At the moment, two isothermal amplification reactions in combination with ELISA have been described: loop mediated isothermal amplification (LAMP-ELISA)[11] and helicase dependent amplification (HDA-ELISA).[12] They do not require expensive amplification equipment and have been seen to be very flexible and capable of simultaneously processing up to several hundreds of samples in a few hours. However, these reactions are performed far from room temperature (60-65 °C),

require an initial denaturation step at 95 °C (HDA) or need complex primers design (LAMP).

An innovative isothermal amplification called recombinase polymerase amplification (RPA) offers interesting advantages.[13,14] This technique facilitates the binding of oligonucleotide primers to template DNA. Primers are elongated by a strand-displacing DNA polymerase, called *Bsu* polymerase, while single-stranded DNA-binding proteins stabilise amplification reaction intermediates. Compared to other amplification enzymes, *Bsu* polymerase maintains similar activity in inhibiting environments, requires a shorter incubation time, operates at lower temperatures, is easy to use and the amplified products do not need post-amplification treatment. The integration of DNA amplification and the detection step entails adjusting several critical variables. For instance, RPA buffer contains Carbowax 20M, a high-molecular-weight polyethylene glycol, as a crowding agent to influence the recombinase kinetics. However, it is known that these molecules may affect the solubility, melting temperature or viscosity of the reaction mix, and hence the amplified product.[15]

In this study, the hyphenation of the RPA and ELISA methodologies (RPA-ELISA) is developed for food safety applications. The technical implementation of integrated screening methods in food industry can vastly help to simplify the process and to reduce costs, thus making analytical procedures friendlier.[1] Here the simultaneous detection of different common food threats is proposed. Hazelnut, peanut and soybean have been selected as representative examples of the allergens included in priority lists.[16] Tomato and maize have also been included because there is growing concern about their allergenicity associated with their current widespread use.[17,18] Promoter 35S and terminator NOS are widely used for screening genetically modified organisms (GMOs) since 72% of GMOs contains at least one of these sequences.[19,20] *Salmonella* spp., *Cronobacter* spp. are frequently detected as being responsible for food contamination. According to the Food and Agriculture Organization of the United Nations and the World Health Organization, they are considered to be included among the most relevant pathogens and their absence is required in food safety analysis.[21,22] Finally, *Fusarium* spp. is one of most frequent fungi found in food and feed

derivates, and it produces mycotoxins that cause serious health problems in both humans and livestock.[23,24]

2. Experimental

2.1 Target genes. The selected genes for hazelnut, peanut, soybean, tomato, maize, GMO promoter-P35S, GMO terminator-TNOS, *Salmonella* spp., *Cronobacter* spp., and *Fusarium* spp. are shown in Table 1. All the primers and probes were successfully checked for relevant homologies by a BLASTNr search (http://blast.ncbi.nlm.nih.gov/).

2.2 Bacterial and fungal strains, foodstuff and DNA extraction. *Salmonella typhimurium* group B (CECT 443) and *Cronobacter sakazakii* (ATCC BBA894) were used as reference strains. They were isolated and provided by the GAIKER Technology Centre (Bizkaia, Spain). Viable samples were obtained by overnight culture on nutrient agar plates (0.5% Peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, pH 7) at 37 °C. Bacterial inoculation assays were prepared by adding 10-fold serial dilutions of an 18-h culture of each pathogen in sterile saline solution (0.8% NaCl), covering a range from 0 to $4 \cdot 10^4$ CFU/mL. *Fusarium moniliforme* (CECT 2982) was used as the reference fungal strain. It was isolated and provided by the Instituto Agroforestal Mediterráneo (IAM), Universitat Politècnica de València (UPV). Viable samples were obtained by culturing 4 days on nutrient agar plates at 25 °C. Fungi inoculation assays were prepared by adding fungal mycelium (10^3-10^5 mg of mycelium per g of food) from a 4-day culture. The certified reference materials (CRM) containing 0.05% of transgenic Bt11 maize (ERMBF412f) and 0.01% of transgenic RRS soybean (ERMBF410gk) were purchased from the Institute for Reference Material and Measurements (Geel, Belgium). Food products were bought in local stores. Genomic DNA was extracted from bacterial cultures, fungal mycelium, and food samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc., CA).

Inoculation assays were assessed after taking into account their common concentration in contaminated foods (e.g., *Salmonella* spp. $>10^2$ CFU/mL).[28]

Table 1. The primers, probes, and control sequences used for amplification procedures.

Target ***(gene)***		**Sequence 5'-3'**	**Tm (°C)**	**Amplicon size (bp)**	**Ref.**
Hazelnut	FP	Dig-ACTACATAAAGCAAAAGGTTGAAG	53.5	109	4
(Cor a1)	RP	TCGTAATTGATTTTCTCCAGTTTG	55.2		
	Probe	BtnTg-$(T)_5$-CGGACAAAGCATCGCCTTCAATCA	67.1		
Peanut	FP	Dig-CTAGTAGCCCTCGCCCTTTT	59.9	82	4
(Ara h2)	RP	GGCATCTTCTGTCTCCTTGG	59.8		
	Probe	BtnTg-$(T)_5$AGTTCCCACTGCTGCCTC	62.6		
Soybean	FP	Dig-TCCACCCCCATCCACATTT	59.2	81	4
(Le)	RP	GGCATAGAAGGTGAAGTTGAAGGA	58.8		
	Probe	BtnTg--$(T)_{10}$CGAAGCTGGCAACGCTACCGGTT	74.1		
Tomato	FP	Dig-AGACCACGAGAACGATATTTGC	66.8	92	20
(Lat 52)	RP	TTCTTGCCTTTTCATATCCAGACA	58.4		
	Probe	BtnTg-$(T)_5$ACTCTCTTTGCAGTCCTCCCTTGGG	57.6		
Maize	FP	Dig-CGTCGTTTCCCATCTCTTCCTCC	64.2	136	22
(adh 1)	RP	CCACTCCGAGACCCTCAGTC	63.5		
	Probe	BtnTg-$(T)_5$-CCTCACCAGTTACGAAACCAATCGATCCAA	67.1		
GMO promoter	FP	Dig-CCACGTCTTCAAAGCAAGTGG	59.8	132	23
(35S)	RP	TCCTCTCCAAATGAAATGAACTTCC	59.7		
	Probe	BtnTg-$(T)_5$-ATATAGAGGAAGGGTCTTGCGAAGGATA	64.8		
GMO terminator	FP	Dig-GCATGACGTTATTTATGAGATGGG	59.3	118	23
(NOS)	RP	GACACCGCGCGCGATAATTTATCC	64.4		
	Probe	BtnTg-$(T)_5$-TTTGCGCGCTATATTTTGTTTTCTATCGCG	64.8		
Salmonella spp.	FP	Dig-TACCAAAGCTAAACGCGCAGCT	62.1	152	8
(hns)	RP	TGATCAGGAAATCTTCCAGTTGC	61.1		
	Probe	BtnTg-$(T)_{10}$-TTTGATTACAGCCGGTGTACGACCCT	75.9		
Cronobacter spp.	FP	Dig-GTTGGATCACCTCCTTACCTGC	64.2	190	8
(16S-23S rDNA)	RP	AGTTAAACCTCTTCAACTCCTG	58.4		
	Probe	TGTGAGCACGCGAGGTTGTATCTTGCA-$(T)_{10}$-BtnTg	64.0		
Fusarium spp.	FP	Dig-CCGAGTTTACAACTCCCAAA	62.7	180	5
(ITS 1)	RP	ACAGAGTTTAGGGGTCCTCT	58.4		
	Probe	BtnTg-$(T)_{10}$-TTACCGGGAGCGGGCTGAT	67.4		
Positive control	Probe	Dig-$(T)_{15}$-GTCATGGGCCTCGTGTCGGAAAACC-BtnTg	81.0		
Negative control	Probe	BtnTg-ACCGTCGCGCACTATCTGATTTCAAA	73.3		

FP: forward primer, RP: reverse primer, Dig: digoxigenin labelled, BtnTg: biotin labelled.

2.3 RPA-ELISA. RPA assays were carried out in a total volume of 25 µL using the TwistAmp Basic kit (TwistDX, Cambridge, UK). Reactions contained 480 nM of each 5'-digoxigenin labelled primer (Table 1), 15 ng of genomic DNA, 14 mM of Mg acetate, and 1× rehydration buffer. First, all the reagents except for the DNA template and Mg acetate were prepared in a master mix, which was distributed into each 0.2 mL reaction tube containing the enzyme and the nucleotides in a dried pellet. Then, DNA was added into the tubes, and Mg acetate was dispensed lastly. Since the RPA reaction starts as soon as magnesium is added, the tubes were immediately placed into a heating oven (Memmert, model UF30, Germany) at 40 °C for 40 min.

Amplification products were analysed in 96-well microtiter ELISA plates (Corning, USA). For this purpose, 100 µL of streptavidin (0.2 mg/L) and biotinylated probes (20 nM), diluted in coating buffer (50 mM carbonate buffer, pH 9.6), were incubated overnight at 4 °C. Double labelled oligonucleotide (5'-biotin and 3'-digoxigenin) was used as the positive control and non-target biotinylated oligonucleotide was the negative control (not complementary to any target). Microtiter plates were washed 3 times with PBS-T (phosphate-buffered saline containing 0.05% (v/v) tween 20, pH 7.4) plus deionised water, and were stored at 4 °C to become stable at less 1 month. Amplified products (1 µL) were mixed with 99 µL of 5× hybridisation buffer (SSC, 1× saline sodium citrate: NaCl 150 mM, sodium citrate 15 mM, pH 7) and heated at 95 °C for 5 min to denature into single strains. Then denatured products (100 µL) were dispensed into each well and incubated at 37 °C for 45 min. After washing the plate 3 times with PBS-T and deionised water, 100 µL per well of anti-digoxigenin antibody labelled with horseradish peroxidase (anti-Dig-HRP) solution in PBS-T (1:2,000) were dispensed and incubated at room temperature for 25 min. After a washing step with PBS-T and deionised water, 100 µL of TMB solution (0.25 g/L of 3,3',5,5'-tetramethylbenzidine and 0.002 M of hydrogen peroxide in citrate buffer, pH 5.5) were dispensed and incubated at room temperature for 10 min. Finally, the reaction was stopped with 50 µL of 2.5 M sulphuric acid and absorbance was measured at 450 nm (reference wavelength: 650 nm) with a microtiter plate

reader (Wallac, model Victor 1420 multilabel counter, Finland). A sample was considered positive when the optical response was higher than the cut-off value.

2.4 PCR-ELISA. PCR mixtures (25 µL) contained 15 ng of extracted genomic DNA, 1× Tris-KCl buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 mM $MgCl_2$, 200 µM dNTPs, 1.25 units of Taq DNA polymerase (Roche, Germany) and 400 nM of each 5'-digoxigenin labeled primer (Table 1). Reactions were carried out in a TC400 thermocycler (Bibby Scientific, Staffordshire, UK) and applying the thermal programme: denaturation (95 °C, 5 min) followed by 40 cycles of denaturation (95 °C, 30 s), annealing (62 °C, 30 s) and elongation (72 °C, 30 s), with a final elongation step (72 °C, 5 min). The immunoassay was performed as is described above.

2.5 Comparison with other detection techniques. Amplification products were checked by electrophoresis on a 3% (w/v) agarose gel at 110 V and room temperature. Gels were stained for 30 min with 0.5× TBE buffer (Tris/Borate/EDTA) containing fluorophore RealSafe (Real Laboratories, Spain) at 0.01% (v/v), and bands were visualised with a UV transilluminator. Product size was determined by comparison with a 50 bp ladder (Fermentas, Lithuania). Amplification yields were calculated from the fluorescence measurements with SYBRSafe at 0.01% (v/v) in a microtiter plate reader.

2.6 Comparison of developing strategies. Other developing immunoassay steps were also tested independently. O-Phenylenediamine dihydrochloride (OPD) was used as an alternative colorimetric substrate to TMB. The addition of digoxigenin-dUTPs (35 µM) in the RPA mixtures was an alternative to obtain labelled amplified products. Three antigen-antibody recognition assays were also compared. The first approach was based on digoxigenin-labelled primers, an anti-digoxigenin antibody produced in sheep (anti-Dig) as a primary antibody at the 1/20,000 dilution, and anti-sheep conjugated with horseradish peroxidase (anti-sheep-HRP) as a secondary antibody at the 1/4,000 dilution. The second approach used Cy5-labelled primers, an anti-Cy5 antibody produced in mouse (anti-Cy5) at

the 1/2,000 dilution, and an anti-mouse conjugated with horseradish peroxidase (anti-mouse-HRP) at the 1/500 dilution. The third approach was based on Dig-labelled primers, sheep anti-Dig antibody as a primary antibody at the 1/5,000 dilution, and anti-sheep conjugated with alkaline phosphatase (anti-sheep-AP) as a secondary antibody diluted at 1/250, using the nitroblue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate solution (BCIP/NBT) as the colorimetric substrate. Data analysis was performed with the statistical package SPSS for Windows, v. 16.0.

3. Results and discussion

3.1 Adaptation of the RPA protocol. The reaction conditions (primer concentrations, temperature and time) were studied to achieve the same amplification conditions for 10 analytes: hazelnut, peanut, soybean, and maize seeds, tomato fruit, P35S and TNOS from the CRM, and pure cultures of *Salmonella* spp., *Cronobacter* spp., and *Fusarium* spp. The optimal RPA conditions were 480 nM for the forward and reverse primers, and incubation at 40 °C for 40 min. A single protocol was achieved for the parallel amplification of all tested analytes, which considerably cut the total analysis time. Amplified products were characterized by agarose gel electrophoresis for product size determination. RPA reactions generated the predicted product length, according to the proposed primers, these being: 109 bp for hazelnut, 82 bp for peanut, 81 bp for soybean, 92 bp for tomato, 136 bp for maize, 123 bp for P35S, 118 for TNOS, 152 bp for *Salmonella* spp., 190 bp for *Cronobacter* spp., and 180 bp for *Fusarium* spp.

3.2 Hybridisation assays: Integration of RPA and ELISA. Several factors influence hybridisation efficiency to the specific probe immobilised on the microplate, and later the detection response. For this purpose, multivariable experimental designs were made to optimise the main parameters during the assays.

The hybridisation process on solid supports, including polystyrene used in microplate wells, depends on probe coverage density. An indirect immobilisation reaction (streptavidin/biotinlabelled probe) was chosen. To that end, coating

conditions were optimised by varying the streptavidin concentration from 0.002 to 2 mg/L, and the probe concentration from 0.2 to 200 nM, and 0.2 mg/L of streptavidin and the 20 nM probe were selected (Figure 1A). A streptavidin concentration above 0.2 mg/L increased the signal, but drastically reduced assay reproducibility. Probe coverage was calculated using double labelled oligonucleotides (5'-biotin and 3'-Cy5) and a homemade surface fluorescence reader. Probe density was 0.08 fmol/mm^2. Additionally, the hybridization yield changed according to temperature. Hybridisation time was tested every 10 min for 1 h, and the temperature range was 25-50 °C. The maximum signal was achieved after 45 min at 37 °C (Figures 1B and C).

The effect of the reaction volume (12.5-200 mL/well) and product dilution (1/50 to 1/5,000) was also evaluated (Figure 1D). The best results were obtained at the 1/100 dilution of amplification product and using 100 mL as the reaction volume. Higher concentrations of amplification product reduced the signal. This effect can be explained by the presence of Carbowax 20 M (5%) in the RPA buffer. No other effects of amplification reagents or the washing protocol were observed, so it was unnecessary to perform further treatments with the RPA products in the proposed method. After testing some solutions (water, SSC, PBST, and PBS) and cycles (1-5), three cycles with PBST and one cycle with deionised water were chosen as the appropriate washing protocol.

3.3 Optimisation of the detection step. The ELISA assays based on the recognition digoxigenin-labelled RPA products by an anti-Dig-HRP antibody were optimised by analysing the DNA extracted from hazelnut, soybean seeds and transgenic maize. The highest signal was achieved at the 1/2,000 dilution, and no significant signal improvement was accomplished at higher concentrations (Figure 1E). The antibody incubation time was optimised, with the best results obtained at 25 min (Figure 1F). Three PBS-T washing steps and further deionised water rinsing were sufficed to eliminate any excess reagents.

For the immunoenzymatic detection of the hybridisation complex, a colorimetric reaction (λ=450 nm, $\lambda_{background}$= 650 nm) was employed by comparing two common HRP substrates, 3,3',5,5'-tetramethylbenzidine (TMB) and o-

phenylenediamine dihydrochloride (OPD), at different concentrations up to 2 g/L. As no remarkable differences were observed during the achieved analytical performances between both substrates, the use of TMB at 0.25 g/L was selected because it offers advantages, such as low cost, easy manipulation, greater stability and lower toxicity.[8]

Alternative strategies to the digoxigenin-labelled primers/anti-Dig-HRP/TMB system were adopted and compared to conventional methods: electrophoresis and in-well fluorescence by the SYBR-Safe DNA stain (Figure 2). Firstly, digoxigenin-dUTPs in the PCR mixtures were tested as an example of nucleotide labelling. This option either combined or not with digoxigenin-labelled primers, increases assay sensitivity, but at a slightly higher cost-by-reaction.[8] Secondly, the immunoassay can be performed using different combinations of primary and secondary antibodies. Two approaches were tested: digoxigenin-labelled primers/sheep anti-Dig/anti-sheep-HRP and Cy5-labelled primers/mouse anti-Cy5/antimouse-HRP. By keeping digoxigenin as the label group, the use of anti-Dig-HRP or anti-Dig/antisheep-HRP reagents yielded similar results. Yet the incorporation of Cy5 as the labelling group provided significantly poorer responses, probably due to the less effective antigen recognition of the anti-Cy5 antibody. Thirdly, the enzyme conjugated to the antibody is another open field that extends the number of available enzymatic substrates, such as antibodies conjugated with alkaline phosphatase (AP). To that end, the digoxigenin-labelled primers/sheep anti-Dig/anti-sheep-AP/BCIP/NBT approach was tested. The conjugation of antibodies to AP gave worse than HRP conjugation results. The statistics study of the results by an ANOVA test revealed that there were significant differences among the detection formats. All the RPA-ELISA approaches provided better sensitivity than the electrophoresis and fluorescence methods. The three systems based on digoxigenin-labelling and HRP/TMB detection gave better results than those obtained with the Cy5-labelled primers or with AP/BCIP/NBT detection ($F=39.49 > F_{6,14}=2.85$, p-value < 0.05).

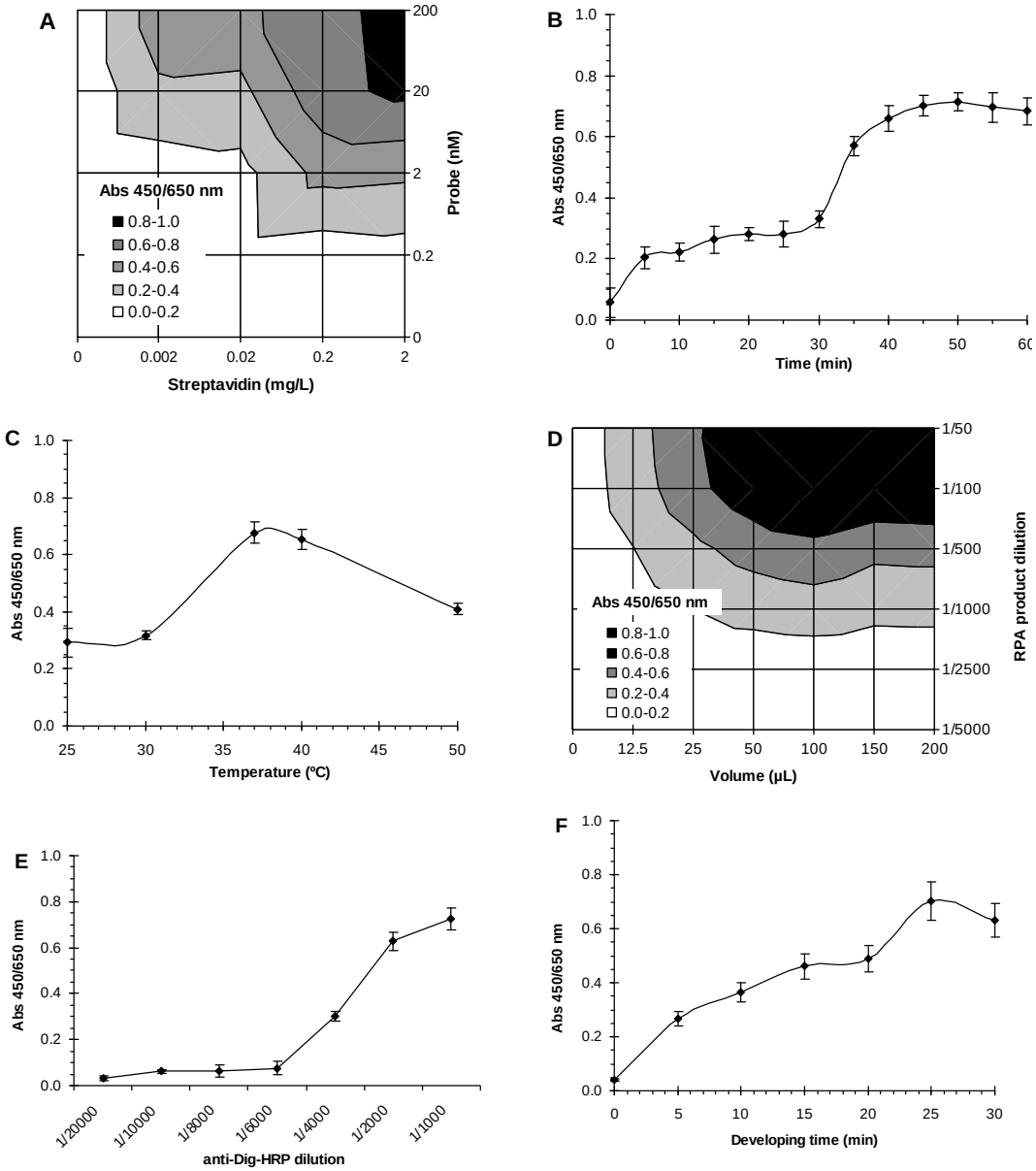


Figure 1. RPA-ELISA optimisation: effect of different experimental variables on optical intensity (A) Coating conditions (streptavidin and probe concentrations); (B) Hybridisation time; (C) Hybridisation temperature; (D) Hybridisation solution (dilution RPA solution and total volume); (E) Developing agent dilution (dilution of anti-Dig-HRP antibody); (F) Developing time (antibody incubation). Signal corresponds to 1.5 ng of target genomic DNA.

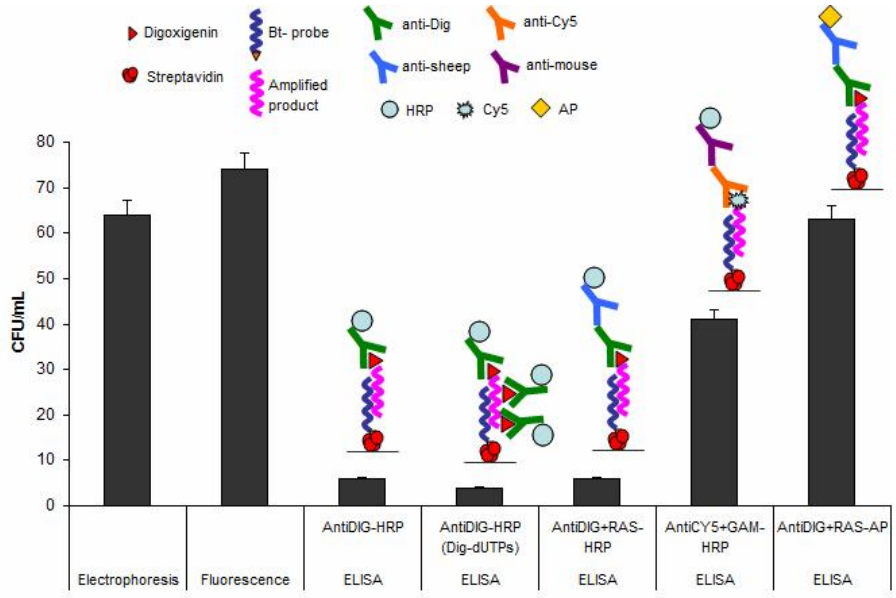


Figure 2. Comparison of detection strategies for RPA products. Bt-probe: biotinilated probe; anti-Dig: anti-digoxigenin antibody produced in sheep; anti-sheep: anti-sheep antibody; anti-Cy5: anti-Cy5 antibody produced in mouse; anti-mouse: anti-mouse antibody; HRP horseradish peroxidase; AP: alkaline phosphatase. Signal corresponds to the sensitivity of each format (calculated in CFU/mL), determined by analysing serially diluted DNA extracts from *Salmonella* spp.

3.4 Analytical performances. The analytical performances of RPA-ELISA (selectivity, limit of detection and reproducibility) were established and compared to those of PCR-ELISA. Firstly, assay selectivity was excellent, showing no cross-reactivity in any case, which reinforces its use for screening purposes.

Assay sensitivity was determined by analysing serially diluted DNA extracts. Genomic DNA, extracted from each ingredient (hazelnut, peanut, soybean and maize seeds, fruit tomato and both CRMs) or pure culture (*Salmonella* spp., *Cronobacter* spp., and *Fusarium* spp.), was 10-fold diluted with free-analyte extracts (wheat flour), and the total DNA concentration remained constant (30 ng/mL). Mixtures were amplified using both methods and were detected by ELISA. The limits of detection for each analyte were 1.3-5.3 µg/g for ingredients and 6-13 CFU/mL for pathogen cultures without an enrichment step (Table 2). The results were similar, or even better, than others obtained by the techniques available

only in full-equipped facilities, such as realtime PCR or DNA microarrays for foods and for microorganisms.[4,8,29,30]

Assay reproducibility was also determined from the optical density of replicates. The intra-day and inter-day RSDs for RPA-ELISA were lower than 6.6% and 12.0%, respectively. The results were similar to PCR-ELISA, demonstrating that the isothermal approach is a powerful alternative that does not compromise analysis quality. Therefore, the analytical performance of the RPA-ELISA method mean that it is suitable for routine DNA-based analyses in a broad range of applications.

Table 2. Comparison of limits of detection and reproducibility obtained by RPA-ELISA and PCR-ELISA.

		RPA-ELISA	PCR-ELISA
Limit of detection	Hazelnut (µg/g)*	1.29	5.80
	Peanut (µg/g)	11.21	13.27
	Soybean (µg/g)	2.01	1.47
	Tomato (µg/g)	9.45	6.63
	Maize (µg/g)	14.36	2.00
	P35S (µg/g)	8.36	1.24
	TNOS (µg/g)	19.74	6.69
	Salmonella spp. (CFU/mL)	6.00	5.00
	Cronobacter spp. (CFU/mL)	13.00	12.00
	Fusarium spp. (µg/g)	5.93	31.96
Mean Reproducibility ** **(%)**	Intra-day	1.4 - 6.6	3.4 - 7.2
	Inter-day	7.9 - 11.3	8.5 - 14.5

* µg/g refers to µg of analyte per g of food

** Reproducibility was calculated from the samples containing 0.1 % of analyte (n=3)

3.5 Analysis of food samples. Twelve commercial foodstuffs were studied in order to evaluate the reliability of the method for its application in large-scale screening. The evaluation set was selected by including several categories and a large variety of food processing methodologies (e.g., raw, baked, etc.) (Table 3).

All the samples were negative for pathogenic bacteria (*Salmonella* spp. and *Cronobacter* spp.) and fungi (*Fusarium* spp.), but the samples from the inoculation assays were positive. Although colorimetric responses increased with analyte concentrations, it was not possible to obtain an exact quantification of the samples, rather only an approximate result was obtained. This was probably due

to the variability associated with the DNA extraction process or with the endpoint amplification technique. Therefore as previously described, cutoff values were established from the negative control readings.[6,12] The absorbance values higher than or equal to 0.10 were considered positive for RPA-ELISA.

The occurrence of the different analytes was simultaneously determined in a single plate by common recognition and developing reagents (Table 3). Nevertheless, the naked-eye detection of food threatens is also possible, as shown in Figure 3 (an example of the microplate image). The yellow colour appeared for positive samples, whereas negative samples showed a noncolour.

Positive results were observed in all cases for the analytes declared, even at trace levels, or in spiked samples (40/40). Thus, traces of hazelnut in chocolate wafer, soya in chocolate wafer and soup, and maize in cookies gave a signal with low absorbance values (<0.2). Low absorbance signals corresponded to minor ingredients, such as peanut in chocolate wafer or soya in muesli cookies. Greater absorbance signals (>0.5) were recorded for major ingredients such as tomato or maize in ketchup. In the inoculated samples, low absorbance signals were related to concentrations of up to $4{\cdot}10^1$ CFU/mL in bacteria and of up to 10^3 μg/g (0.1%) in fungus. This was observed for *Salmonella* spp. in chocolate wafer and for *Fusarium* spp. in ketchup. The higher absorbance values corresponded to concentrations above $4{\cdot}10^3$ CFU/mL in bacteria, which occurs for *Salmonella* spp. in tomato or feed samples, and for *Cronobacter* spp. in skimmed powdered milk or powdered infant formula. In fungal inoculations, higher absorbance values corresponded to concentrations of up to 10^5 μg/g (10%); for example, in muesli cookies or baby food.

Negative results were found in most of the samples declared to be analyte-free (78/80). The one exception was muesli cookies in which, despite not having declared any GMO, positive results were obtained for both the P35S and TNOS analyses. Their detection can be explained because it is not required to declare GMO ingredients for European food labelling unless they are above 0.9%.[31]

The reliable and sensitive results achieved indicate that the proposed RPA-ELISA method is useful for the detection of the most important food threats in a broad set of samples.

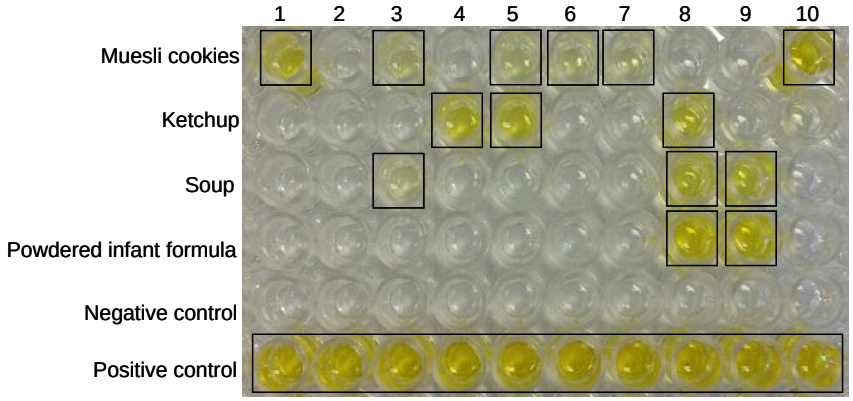


Figure 3. Naked-eye results of commercial food samples in microplates. Each sample (row) is tested for each analyte (columns): (1) hazelnut; (2) peanut; (3) soybean; (4) tomato; (5) maize; (6) P35S; (7) TNOS; (8) *Samonella* spp.; (9) *Cronobacter* spp.; (10) *Fusarium* spp. Highlighted rectangles indicate positive samples (absorbance > cut-off value).

4. Conclusions

RPA, as an isothermal amplification method, offers numerous advantages. This reaction does away with the need for thermocycling and allows the use of simple technology such as heaters or ovens, is inexpensive and allows minimal maintenance control. Specifically, RPA has proven to have interesting properties, such as tolerance to temperature fluctuations, working near room temperature, cost-effectiveness, short amplification time, reliability and simplicity. Besides, its combination with ELISA for the detection of nucleic acid amplified products offers other advantages, such as sensitivity enhancement. Two other approaches that combine isothermal DNA amplification with ELISA detection have been described. However, RPA has demonstrated to provide equal or better analytical performance with greater simplicity of operation (one single and a lower temperature, and easier primer design).

Table 3. Screening results of the analytes in commercial food samples analysed by RPA-ELISA.

	Declared analyte[a] / Detected analyte[c]							**Spiked analyte[b] / Detected analyte[c]**		
Food	Hazelnut	Peanut	Soybean	Tomato	Maize	P35S	TNOS	*S*. spp.	*C*. spp.	*F*. spp.
Muesli cookies	+ / ++	- / nd	+ / +	- / nd	± / +	<0.9% / +	<0.9% / +	- / nd	- / nd	10% / +++
Chocolate wafer	± / +	+ / +	± / +	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4{\cdot}10^{1}$ / +	- / nd	- / nd
Ketchup	- / nd	- / nd	- / nd	+ / ++	+ / ++	<0.9% / nd	<0.9% / nd	$4{\cdot}10^{3}$ / ++	- / nd	0.1% / +
Feed	- / nd	- / nd	- / nd	- / nd	+ / +	+ / ++	+ / +	$4{\cdot}10^{4}$ / +++	- / nd	1% / ++
Tomato	- / nd	- / nd	- / nd	+ / +++	- / nd	+ / ++	+ / ++	$4{\cdot}10^{3}$ / ++	$4{\cdot}10^{2}$ / ++	- / nd
Baby food	- / nd	- / nd	- / nd	- / nd	+ / +++	<0.9% / nd	<0.9% / nd	$4{\cdot}10^{4}$ / +++	- / nd	10% / +++
Soup	- / nd	- / nd	± / +	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4{\cdot}10^{2}$ / ++	$4{\cdot}10^{2}$ / ++	- / nd
Skimmed powdered milk	- / nd	- / nd	- / nd	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4{\cdot}10^{2}$ / ++	$4{\cdot}10^{4}$ / +++	- / nd
Powdered infant formula	- / nd	- / nd	- / nd	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4{\cdot}10^{4}$ / +++	$4{\cdot}10^{3}$ / +++	- / nd
CRM (RRS 5%)	- / nd	- / nd	+ / +++	- / nd	- / nd	+ / ++	+ / +	- / nd	- / nd	- / nd
CRM (Bt11 Maize 5%)	- / nd	- / nd	- / nd	- / nd	+ / +++	+ / ++	+ / +	- / nd	- / nd	- / nd
Sweet corn	- / nd	- / nd	- / nd	- / nd	+ / +++	<0.9% / nd	<0.9% / nd	- / nd	- / nd	1% / ++

[a] Declared: + analyte listed; - analyte not listed; ± may contain trace levels; <0.9% labelling not required (GMO-EU regulation).
[b] Spiked analyte correspond to *Salmonella* spp. *(S.* spp.*), Cronobacter* spp. *(C.* spp.*),* and *Fusarium* spp. *(F.* spp.*).*
[c] Used code: +, detected at low level; ++, detected at medium level; +++, detected at high level; nd, non detected.

The present method has demonstrated its usefulness in the food safety area as a screening assay capable of detecting target genes of potential food threats, such as allergens, GMOs, pathogens, or undeclared food intolerance ingredients. This fast, lowcost technology for semi-quantitative analyses has shown excellent analytical performances (selectivity, sensitivity, reproducibility, and high throughput). After the DNA extraction step, the assay can be performed in 2 h and all the samples can be processed simultaneously with only one amplification condition and the same detection technique. It is worth mentioning that our approach also proves flexible to help significantly increase the number of analysed samples and/or replicates, or to simultaneously detect agents of a different nature. The results are also obtained by naked-eye examination in some applications. Therefore, the proposed method is especially suitable for screening applications in point-of-control facilities and does not compromise analytical performance.

Acknowledgements

This research has been funded through Projects GV/2009/028 (Generalitat Valenciana) and CTQ/2010/15943 (MICINN). The Spanish Ministry of Education and Science provided S.S.F. with a grant for her PhD studies. The fungal cultures were supplied by B. Mora-Sala and J. García-Jiménez from the Instituto Agroforestal Mediterráneo, UPV.

References

1. Asensio, L., González, I., García, T., Martín, R. *Food Control*, 2008, 19, 1-8.
2. Scaravelli, E., Brohée, M., Marchelli, R., van Hengel, A. J. *Anal. Bioanal. Chem.*, 2009, 395, 127-137.
3. Shen, F., Davydova, E.K., Du, W., Kreutz, J. E., Piepenburg, O., Ismagilov, R. F. *Anal. Chem.*, 2011, 83, 3533-3540.
4. Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldón, J. A., Puchades, R., Maquieira, A. *J. Agric. Food Chem.*, 2012, 60, 36-43.
5. Luk, J. M., Kongmuang, U., Tsang, R. S. W., Lindberg, A. A. *J. Clin. Microbiol.*, 1997, 35, 714-718.
6. Musiani, M., Venturoli, S., Gallinella, G., Zerbini, N. *Nat. Protoc.*, 2007, 2, 2502-2510.
7. Holzhauser, T., Stephan, O., Vieths, S. *J. Agric. Food Chem.*, 2002, 50, 5808-5815.

8. Nascimento, L. D., Passos, S. R. L., Mouta-Confort, E., Santiago, M. A., Alves, A. S., Madeira, M. F., Schubach, A. O., Marzochi, M. C. A. *J. Clin. Lab. Anal.*, 2009, 23, 152-156.
9. Ahmad, F., Hashsham, S. A. *Anal. Chim. Acta*, 2012, 733, 1-15.
10. Asiello, P. J., Baeumner, A. J. *Lab Chip*, 2011, 11, 1420-1430.
11. Ravan, H., Yazdanparast, R. *Anal. Chim. Acta*, 2012, 733, 64-70.
12. Gill, P., Amini, M., Ghaemi, A., Shokouhizadeh, L., AbdulTehrani, H., Karami, A., Gilak, A., *Diagn. Micr. Infec. Dis.*, 2007, 59, 243-249.
13. Piepenburg, O., Williams, C. H., Stemple, D. L., Armes, N. A. *PLoS Biol.*, 2006, 4, 1115-1121.
14. Rohrman, B. A., RichardsKortum, R. R. *Lab Chip*, 2012, 12, 3082-3088.
15. Lavery, P. E., Kowalczykowski, S. C., *J. Biol. Chem.*, 1992, 267, 9307-9314.
16. Commission, Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients, Off. J. Eur. Communities: Legis. 310 (2007) 11-21.
17. Pafundo, S., Gullì, M., Marmiroli, N. *J. Agric. Food Chem.*, 2011, 59, 10414-10424.
18. Fonseca, C., Planchon, S., Renaut, J., Oliveira, M. M., Batista, R. *J. Proteomics*, 2012, 75, 2027-2037.
19. von Götz, F. *Anal. Bioanal. Chem.*, 2010, 396, 1961-1967.
20. Commission Regulation, (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired, Off. J. Eur. Communities: Legis. 268 (2011) 24-28.
21. Commission Regulation, (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, Off. J. Eur. Communities: Legis. 338 (2005) 1-26.
22. Arnandis-Chover, T., Morais, S., Tortajada-Genaro, L. A., Puchades, R., Maquieira, A. Berganza, J., Olabarria, G. *Talanta*, 2012, 101, 405-412.
23. Grimm, C., Geisen, R. *Lett. Appl. Microbiol.*, 1998, 26, 456-462.
24. Commission Regulation, (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, Off. J. Eur. Communities: Legis. 364 (2006) 5-24.
25. Yang, L., Pan, A., Jia, J., Ding, J., Chen, J., Chen, H., Zhang, C., Zhang, D. *J. Agric. Food Chem.*, 2005, 53, 183-190.
26. International Organization for Standardization, Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-qualitative nucleic acid based methods, in: ISO 21570:2005, International Organization for Standardization, Geneva, Switzerland, 2005.
27. International Organization for Standardization, Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-qualitative nucleic acid based methods, in: ISO 21569:2005, International Organization for Standardization, Geneva, Switzerland, 2005.
28. World Health Organization, International Course: Epidemiology of Foodborne Diseases, Autonomous University of Yucatan, Merida, Mexico, 2004, pp. 25-36.
29. Köppel, R., van Velsen-Zimmerli, F., Bucher, T. *Eur. Food Res. Technol.*, 2012, 235, 843-853.

30. Zhou, P. P., Zhang, J. Z., You, Y. H., Wu, Y. N. *Biomed. Environ. Sci.*, 2008, 21, 53-62.
31. Regulation, (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC, Off. J. Eur. Communities: Legis. 166 (2003) 9-15.

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Isothermal DNA amplification strategies for duplex microorganism detection

Sara Santiago-Felipe, Luis Antonio Tortajada-Genaro, Sergi Morais, Rosa Puchades, Ángel Maquieira

Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain

Reprinted from ***Food Chem., 2015, 174, 509-515****.*

Abstract

A valid solution for micro-analytical systems is the selection of a compatible amplification reaction with a simple, highly-integrated efficient design that allows the detection of multiple genomic targets. Two approaches under isothermal conditions are presented: recombinase polymerase amplification (RPA) and multiple displacement amplification (MDA). Both methods were applied to a duplex assay specific for *Salmonella* spp. and *Cronobacter* spp., with excellent amplification yields (0.2-8.6·10^8 fold). The proposed approaches were successfully compared to conventional PCR and tested for the milk sample analysis as a microarray format on a compact disc (support and driver). Satisfactory results were obtained in terms of resistance to inhibition, selectivity, sensitivity (10^1-10^2 CFU/mL) and reproducibility (below 12.5%). The methods studied are efficient and cost-effective, with a high potential to automate microorganisms detection by integrated analytical systems working at a constant low temperature.

Keywords: Isothermal DNA amplification, pathogens, milk, microarraying.

1. Introduction

The development of effective detection methods for pathogenic microorganisms covers several areas, such as food safety, environmental monitoring and clinical diagnostics. Microbiological methods are being replaced with those based on DNA, where amplification with polymerase chain reaction (PCR) is the most widespread approach. For instance, automation of DNA amplification enables the use of portable microdevices, multiplexing, reduced sample volumes and reagents, and reduces contamination risks.[1] Efforts are being made to overcome its drawbacks for extended point-of-need applications; for example, increasing multiplexing ability or reducing costs, time analysis and technical requirements. Yet the integration of nucleic acid amplification into microdevices, such as digital PCR or labon-a-chip, is complex, and several issues must be resolved. PCR demands accurate temperature control and rapid thermocycling at between 55 °C and 95 °C. When initiating a specific step in the PCR, temperature fluctuation results in over- and under-shooting.[2] High temperatures also lead to variations in the volume reaction and gas bubble formation, which are the main causes of PCR failure in lab-on-a-chip devices.[3] Consequently, the design of simple, cost-efficient systems is no trivial matter, particularly when integrating sample preparation and/or multiplex detection into the same platform.

The use of enzymes mimicking DNA replication in vivo conditions is an alternative to conventional DNA polymerases.[4] Thus amplification can be performed using a simple thermoblock, peltier or oven at a fixed temperature. The commonest isothermal methods are strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), helicase-dependent amplification (HDA), isothermal recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and multiple displacement amplification (MDA).[5,6] The performance of RPA[7] and MDA[8] offers an interesting high-throughput analytical system, as demonstrated in a bright approach (digital RPA) proposed for the detection of a single pathogen on a chip.[9] However, these methods have not been described for multiplex strategies.

Several factors should be considered when integrating an amplification reaction for a high-capacity analytical platform, such as microarrays, including the compatibility of the amplification mechanism with multiplexing detection because other isothermal reactions are intrinsically limited to one target analyte (e.g., LAMP). Very few data describing analytical performances are available given the novelty of these amplification methods for analytical purposes. For instance, information about properties, such as temperature tolerance or the effect of inhibitors from a sample matrix, is scarce or even null.

The present research work deals with the potential integration of RPA (sequence-specific method) and MDA (massive method) into a duplex system. This study, for which *Salmonella* spp. and *Cronobacter* spp. were the chosen targets, is based on needs for food safety and environmental monitoring or clinical diagnostics.[10-13] As there is clear evidence for a causal association between the presence of *Salmonella* spp. and *Cronobacter* spp. in food and illness in humans, infections with these microorganisms have been documented as both sporadic cases and outbreaks.[14] Several methods have been proposed to determine the presence/absence of these specific bacterial pathogens. Contamination of infant milk has been extensively reported, based on traditional microbiological examination and DNA-based techniques, including culture enrichment and PCR amplification. The method was applied in milk samples to evaluate duplex isothermal amplification. Furthermore, DVDs (Digital Versatile Discs) have been used as low-cost, integrated effective microarray platforms and detection technology to demonstrate the concept by simultaneously quantifying both pathogens with a view to future screening applications (allergens, GMOs, species identification, etc.).[15,16]

2. Material and methods

2.1 Amplification protocols. The target gene *hns*, which codes for a DNA-binding protein, was selected because it is conserved in all the *Salmonella* spp. The specific target for the *Cronobacter* spp. species was located in the 16S-23S rDNA internal transcribed spacer sequence.

PCR mixtures (25 µL) consisted of 5 ng of extracted genomic DNA, 1× Tris-KCl buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 mM $MgCl_2$, 200 µM dNTPs, 1.25 units of Taq DNA polymerase (Roche, Mannheim, Germany) and 400 nM of each primer (Table S-1, see supplementary information). The thermal program was: denaturation (95 °C, 7 min) followed by 40 cycles of denaturation (95 °C, 30 s), annealing (59 °C, 30 s) and elongation (72 °C, 30 s), and a final elongation (72 °C, 4 min).

RPA reactions (25 µL) were performed by adding 5 ng of genomic DNA from inoculated milk samples and 240 nM of the primer pairs (Table S-1, see supplementary information) to the reconstituted solution of enzymes, nucleotides and buffer (TwistDx, Cambridge, UK). The duplex reactions were carried out in an oven (40 °C, 40 min).

MDA reactions (25 µL) were performed with final concentrations of 2 ng of genomic DNA, 1× MagniPhi buffer reaction (X-Pol Biotech, Madrid, Spain) and 50 µM of random hexamer primer. After heating (95 °C, 3 min), 500 µM dNTPs, plus phi29 polymerase (1 U), were added and the reaction was run in an oven (35 °C, 4.5 h) until a final inactivation step at 65 °C (10 min).

2.2 Bacterial strains, milk samples and DNA extraction. *Salmonella* serovar Typhimurium group B (CECT 443) and *Cronobacter sakazakii* (ATCC BBA-894) were used as reference strains (positive controls). Milk products, bought in local food stores, were inoculated with both pathogens. Inoculation assays were prepared by adding 10-fold serial dilutions of an 18-h culture in sterile saline solution (0.8% NaCl) to cover a range from 0 to $4 \cdot 10^4$ CFU/g for each pathogen. Genomic DNA was extracted from bacterial cultures and samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA, USA).

2.3 Analysis of amplification products. Amplification products were separated by electrophoresis on 3% (w/v) agarose gel, 1× TBE buffer (89 mM Tris base, 89 mM borate, 2 mM EDTA, pH 8) at 120 V and room temperature. Gels were stained for 30 min with 1× TBE containing 0.01% (v/v) of SYBR-Safe (Life Technologies, Carlsband, CA), and bands were visualized on an UV transilluminator. Size was

determined by comparing with a 50-bp ladder. Single amplification yields were calculated from the fluorescence measurements with SYBR-Safe at 0.01% (v/v) in a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Turku, Finland).

2.4 Post-amplification protocol. Two post-amplification protocols were assayed: restriction enzyme digestion and sonication.

The EcoNI enzyme (Fermentas, Vilnius, Lithuania), also called XagI, cuts double-stranded DNA at the specific recognition sequence CCTNN-N-NNAGG. Both pathogens have this restriction site closed to their target regions, at a distance of 41 bp for *Salmonella* spp. and 92 bp for *Cronobacter* spp. The digestion conditions were optimised: temperature (30-45 °C), time (1-16 h) and restriction enzyme units (1-4 U). The optimal protocol (37 °C, 8 h and 2 U of restriction enzyme) was performed in a total volume of 32 µL by adding 3.2 µL of 10× digestion buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA, pH 8.5), 10 µL of amplification product and 3.2 µL of the EcoNI enzyme solution (20 U). After the reaction, the restriction enzyme was inactivated by incubation at 65 °C for 20 min.

For the second option, small DNA fragments were obtained by sonication (UP200S ultrasonic disruptor, Hielscher, Teltow, Germany) using a microtip (1 mm in diameter) by applying 10 cycles (30 s ON, 30 s OFF) at 24 the kHz operating frequency, pulse 0.5 s and 70% amplitude. Vials were cooled in an ice bath to maintain sample integrity.

2.5 Inhibition assays. The amplification yield was evaluated in the presence of potential inhibitors. To that end, skimmed cows' milk samples and powdered infant formulas were added to the amplification mixtures to obtain a final concentration ranging from 0.05% to 20% (v/v). The effect of the Ca^{2+} ion was determined by adding $CaCl_2$ (0.2-8 mM) to the amplification solutions.

2.6 Addressing a biosensor based on DVD technology. Amplification products were analyzed by DVD-technology.[17] Streptavidin (10 mg/L) and 50-biotinylated probes (50 nM), listed in Table S-1 (see supplementary information), were spotted on the polycarbonate surface of a digital versatile disk (DVD), and 10 arrays were printed (6 × 6). Amplified products (1 µL) were mixed with 49 µL of hybridisation buffer (NaCl 750 mM, sodium citrate 75 mM, formamide 25%, pH 7), heated (95 °C, 5 min) and dispensed onto sensing arrays to perform the simultaneous analysis of 10 samples. After hybridisation (37 °C, 60 min) and washing, 1 mL of anti-digoxigenin antibody-HRP solution in PBS-T (1:500) was dispensed onto the DVD (room temperature, 30 min). After washing, 1 mL of the 3,3',5,5'-tetramethylbenzidine solution was dispensed and incubated for 8 min. After the recognition process and the developer reaction, the disc was placed into the DVD-drive and scanned by laser, and reflected light was measured. In the absence of a solid deposit (reaction product), the reflection properties of the DVD surface remained unchanged and the maximum intensity of the reflected beam was collected by the DVD drive (background signal). However, when the laser hit a microarray spot, the reflected laser beam attenuated and, consequently, the laser beam intensity that reached the photodiode of the DVD pickup diminished (Figure S-1, see supplementary information). By means of data acquisition software, a microarray image was generated, and the signal of each spot correlated with pathogen concentration. Assay sensitivity was established by analysing the bacterial DNA extracts obtained by serial dilution (0.1 to 10^5 CFU/mL). Limits of detection (LODs) were calculated as the pathogen concentration that produced a signal-to-noise ratio of 3. Assay reproducibility, expressed as relative standard deviations (RSDs), was calculated from triplicates. The microarray layout (6 × 6) on a compact disc consisted of four blocks (specific for *Salmonella* spp., specific for *Cronobacter* spp., positive control and negative control) of nine dots each. With this arrangement, the 50-nL printing solution yielded spots of 500-µm diameter and a track pitch (centre to centre distance) of 1.5 mm. An array density of about 1.0 spot/mm^2 was achieved (Figure S-2, see supplementary information).

2.7 Quality control. Firstly, a parallel analysis of control samples was performed.[18] Blank cultures and non-inoculated milk samples were included as negative controls. A mixture with the genomic DNA from the cultures of both pathogens (*Salmonella* spp. and *Cronobacter* spp.) was used as the positive sample. The results show the right amplification, hybridisation and detection protocol (external control). Secondly an oligonucleotide, non-complementary to the target pathogens, (negative block) and a digoxigenin-labelled oligonucleotide (positive block) were added in the array layout. These internal controls indicated whether the detection protocol from an unknown sample was successful.

The duplex amplifications performed with the specific primers of the two target pathogens were compared to the single amplifications containing the specific primers for one pathogen (specific *Salmonella* spp. or specific *Cronobacter* spp.). No performance differences were observed between both study approaches.

3. Results and discussion

3.1 Amplification methods. In the RPA reaction, multi-variable initial experiments for the duplex assay (*Salmonella* spp. and *Cronobacter* spp.) revealed that primer concentration, incubation temperature and reaction time were the most critical parameters. Figure 1A shows that the individual concentration of the primers in a duplex assay can be less when compared to those used in the single assay, probably because the maximum reaction rate was achieved. Regarding kinetic behaviour, the amplification process reached a stationary phase after 40 min (Figure 1B). A similar amplification yield was obtained when the oven was working within the 37-42 °C range, showing high tolerance to temperature fluctuations. Therefore, the selected concentrations of primers were 240 nM and the largest number of copies was reached at 40±2 °C for 40 min, which corresponds to an amplification yield of $8.6 \cdot 10^8$. No false-positive results were obtained due to pre-initiation or non-specific amplification.

Furthermore, RPA is a sequence-specific amplification method that requires the design of primers, similarly to PCR, to control product properties or selectivity. The initial experiments showed that the distance between the forward and reverse primers had a major effect on amplification yield. Therefore, product length varied, and the forward primer specific to *Salmonella* changed, while the rest of the reaction conditions remained constant. Figure 1C shows that an increment of product length decreased the number of copies but increased the reaction rate. These results were interpreted according to the RPA mechanism[7] and polymerase processivity; i.e., measurement of the global number of nucleotides added per time unit.[19] A short product cuts the time proteins are bound to the DNA compared to the proteins that are free in the solution, and the total number of nucleotides incorporated are less. However, template replication finished early and, consequently, the exponential amplification was favoured, and the number of copies for the target sequences increased. It is worth mentioning that this effect is important for isothermal methods because PCR can be controlled by changing the elongation step time.

RPA specificity was checked at different levels. Firstly, an alignment against closely related species was performed by the Blastn software (NBCI databank). The forward primer of *Salmonella*, used to amplify the 100-bp product, was rejected because it aligned against other pathogens, such as *Escherichia*, *Shigella* and *Photorhabdus*. The set of specific oligonucleotides to the 152-bp product for *Salmonella* spp. and the 190-bp product for *Cronobacter* spp. were selected for the duplex amplification method. Secondly, the analysis of pure bacterial cultures, listed in Table S-2 (see supplementary information), was satisfactory and provided negative amplification results for the nontarget pathogens.

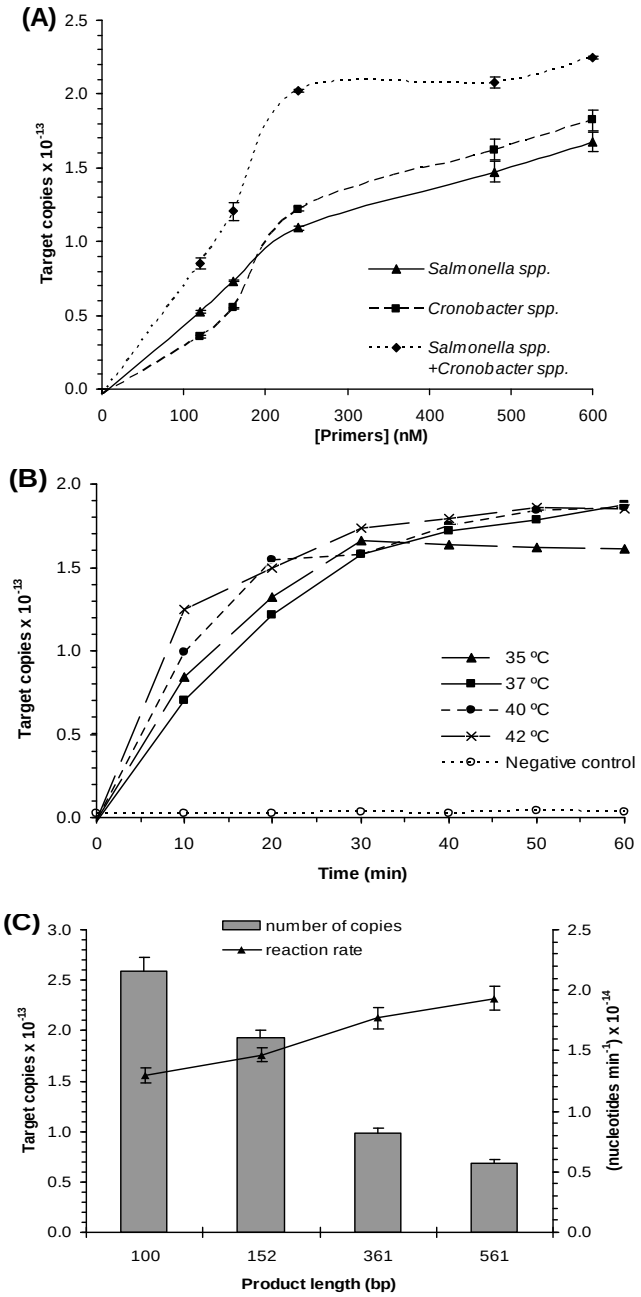


Figure 1. Effect on amplification efficiency for the duplex RPA method (*Salmonella* spp. $4{\cdot}10^1$ CFU/mL and *Cronobacter* spp. $4{\cdot}10^1$ CFU/mL): (A) individual primer concentration, (B) temperature and time, and (C) amplification product length varied, while the forward primer specific to *Salmonella* changed, and the rest of working conditions remained constant.

In the MDA reaction, a non-primer design was necessary because phi29 polymerase combined with a random hexamer randomly amplified the whole genome (massive amplification method). The mechanism involved strand displacement DNA synthesis on single- and double-strand DNA templates by primer annealing at multiple sites. Therefore, the amplification yield depended mainly on primer concentration, primer nature, temperature and reaction time. Reproducible results were obtained for hexamer concentrations under 50 μM, which was the optimum value (Figure 2A). The primers resistant to the exonuclease activity of phi29 polymerase (the thiophosphate linkage for two 3' terminal nucleotides) provided good efficiencies as compared to the exosensitive primers (non-internal modification). The results also indicated that phi29 polymerase was less tolerant to temperature fluctuations because the number of copies changed according to the working temperature (Figure 2B). Finally, the reaction time study showed that a stationary phase was reached after 270 min. Therefore, the best yield, $9.8 \cdot 10^4$ in genomic units, was reached at 35.5 ± 0.5 °C for 4.5 h.

The results of both duplex methods were comparable to single pathogen approaches, and amplification yields were highly reproducible (variation <5%). Low liquid evaporation and gas-bubble formation were observed at working temperatures. Thus these amplification methods are technically simpler for miniaturized systems, and are less sensitive to temperature fluctuations than PCR.

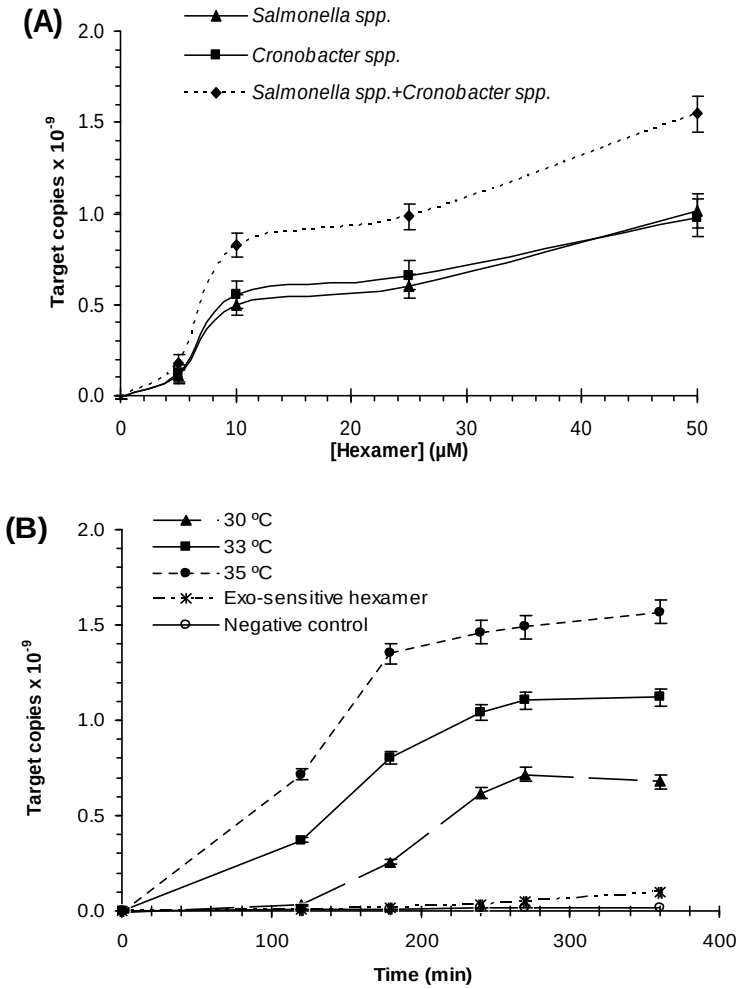


Figure 2. Effect on amplification efficiency for the duplex MDA method (*Salmonella* spp. $4{\cdot}10^1$ CFU/mL and *Cronobacter* spp. $4{\cdot}10^1$ CFU/mL): (A) primer concentration and (B) temperature and time.

3.2 The post-amplification protocol. Duplex RPA reactions yielded the two predicted products, as confirmed by agarose gel electrophoresis. The multibranched polymerization mechanism of MDA leads to a massive amplification method that provides several products. The bands in the electrophoretic separation appeared as smears, which were evenly distributed from 0.3 to 4 kbp for the mixtures of both pathogens. In this case, integration with

microarraying platforms was limited because the good stability of the large-sized products resulted in low hybridisation yields, as described for PCR-based methods.[20]

Further fragmentation of amplification products prior to the hybridisation assays was required. To that end, enzymatic and physical protocols were assayed. For the first option, restriction enzyme EcoNI was selected because a common sequence (CCTNN-N-NNAGG) was presented for both target regions (gene *hns* in *Salmonella* spp. and the 16S-23S rDNA internal transcribed spacer sequence in *Cronobacter* spp.). This enzyme provided fragments from 0.1 to 0.8 kbp after 8 h of digestion. Comparable amounts of small-sized oligonucleotides (Figure 3) were obtained by sonication (high frequency acoustic waves >20 kHz). Although sonication is a fast automatable option for MDA, the absence of a post-amplification treatment in the RPA approach simplifies its integration into a high-throughput platform.

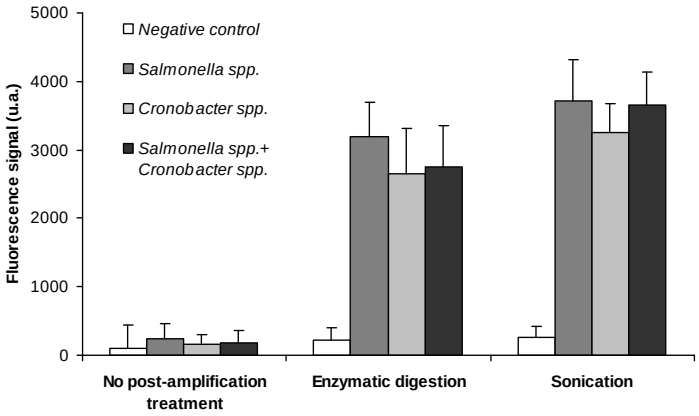


Figure 3. Fluorescence response of small-sized products (<800 bp) measured after post-amplification treatment and gel electrophoresis separation.

3.3 Analytical features. Addressing a biosensor based on DVD technology. To date, the RPA method has been reported only for single determinations using end-point fluorescent detection, lateral-flow strips or microfluidic chips.[21] The MDA method has been used for whole genome amplification in combination with functional gene arrays.[8,22] The LODs achieved were between 10 and 1,000 copies/mL. However, very few described approaches are quantitative methods and they have not been applied as real multiplex approaches, such as microarray platforms.

By way of example of a simple portable detection system, the combination of isothermal amplification with compact disc technology was firstly studied. The results indicated that this analytical platform and the detector were highly compatible with isothermal amplification approaches because no further DNA product treatment, for example purification, was required. A duplex assay to simultaneously detect *Salmonella* spp. and *Cronobacter* spp. was also easily implemented in a microarray format on a DVD surface following the protocols described in previous studies.[17] The positive and negative controls were included to guarantee the reliability of the pathogen analysis.

The results were compared to those obtained with the PCR-based method, and showed a good correlation between the amount of DNA and the optical signal. Thus, the LODs for *Salmonella* spp. and *Cronobacter* spp. were 17-32 copies/mL for PCR, 10-48 copies/mL for RPA and 7-31 copies/mL for MDA (Table 1). Although the amplification factor of MDA was lower than RPA, the LODs were similar in the microarraying format, probably because of the amplification mechanism. In RPA, denaturation was performed by a mixture of enzymes and polymerisation generated double-strand DNA.[7] Amplification by the MDA method involved strand displacement DNA synthesis on templates.[8] Then the formation of single-stranded regions during multibranched polymerisation should increase the hybridisation yield. Intra-day reproducibility was lower than 8.5% for PCR, and below 12.5% for RPA and MDA. Inter-day reproducibility ranged from 6.3% to 16.8%. The analytical performances obtained with both amplification methods, without an enrichment culture, were similar, or better, than those obtained by rt-PCR, glass microarrays or traditional microbiological methods.

Table 1. Comparison of the experimental protocols and analytical performances obtained by the PCR, RPA, and MDA techniques.

Amplification conditions	**PCR**	**RPA**	**MDA**
Polymerase	Taq DNA	Bsu DNA	Phi29
Number of primers	2 by target	2 by target	Random hexamer
Design of primers	Yes	Yes	No
Denaturing agent	Heat	SSB proteins	Polymerase
Tolerance to temperature fluctuation	Low	High	High
Resistance to inhibition	High	High	High
Multiplex amplification	Yes	Yes	Yes
Thermal equipment	Thermocycler	Heater (oven)	Heater (oven)
Thermal equipment prize	>5,000 € (96 samples)	1,500 € (>1,000 samples)	1,500 € (>1,000 samples)
Price per assay (€)	2.63	2.83	2.18

Experimental protocols	**PCR**	**RPA**	**MDA**
Initial template denaturation	Yes (95 °C)	No	Yes (95 °C)
Working temperature (°C)	Cycle (95, 60, 72)	42	35
Final enzymatic inactivation	No	No	Thermal (95 °C)
Post-amplification treatment	No	No	Recommended
Reaction time (min)	100	40	270

Analytical performances*	**PCR**	**RPA**	**MDA**
Signal *Salmonella* spp. (10^2 CFU/mL)	1832 (S/N=8)	1529 (S/N=6)	3366 (S/N=12)
Signal *Cronobacter* spp. (10^2 CFU/mL)	2146 (S/N=10)	2154 (S/N=9)	3120 (S/N=11)
LOD *Salmonella* spp.	32 CFU/mL	48 CFU/mL	31CFU/mL
LOD *Cronobacter* spp.	17 CFU/mL	10 CFU/mL	7 CFU/mL
Intra-day reproducibility	6.1 - 8.5 %	9.6 - 12.5 %	5.9 - 12.5 %
Inter-day reproducibility	10.9 - 12.1 %	10.4 - 14.3 %	10.1- 16.8 %

* Format: Microarraying on compact disk and CD driver detection
S/N: Signal-to-noise ratio, LOD: Limit of detection

3.4 Milk sample analysis. It is well-known that some enzymes used for amplification are not compatible with the specific substances present in the sample matrix, which diminishes their activity and, subsequently, assay sensitivity.[23] For this reason, resistance to inhibition for the three studied amplification approaches was assessed in milk samples (Table 2). The RPA method amplified even in presence of 15-25% of milk, while the MDA method proved less tolerant of the matrix (14-16%). Moreover, all the amplification methods studied showed similar inhibition to those caused by Ca^{2+}, where 6.3-7.2 mM was the maximum concentration tolerated. This inhibitory effect has been previously reported for PCR,[24] but has never been described in isothermal polymerases.

Table 2. Maximum calcium inhibition concentrations tolerated during the duplex DNA amplification reactions.

	PCR (Taq DNA polymerase)		**RPA (Bsu DNA polymerase I)**		**MDA (phi29 polymerase)**	
	Salmonella spp.	*Cronobacter* spp.	*Salmonella* spp.	*Cronobacter* spp.	*Salmonella* spp.	*Cronobacter* spp.
% Skimmed milk (v/v)	26.9 ± 0.7	24.2 ± 0.8	23.6 ± 1.5	25.5 ± 0.9	16.2 ± 1.7	16.7 ± 2.0
% PIF (v/v)	18.1 ± 1.0	19.2 ± 0.6	14.9 ± 0.9	18.6 ± 0.4	13.0 ± 2.1	13.4 ± 2.3
Ca^{2+} (mM)	6.8 ± 0.2	7.2 ± 0.1	6.3 ± 0.4	7.0 ± 0.2	6.9 ± 0.5	7.0 ± 0.5

PIF: powdered infant formula.
Values correspond to the 10^2 CFU/mL of pathogen concentration.

Powdered infant formulas and skimmed milk were spiked with *Salmonella* spp. and *Cronobacter* spp. (0 to $4 \cdot 10^4$ CFU/mL). Noninoculated milks were negative, whereas all the inoculated samples were positive, and a correlation between pathogen amount and optical intensities was found (Figure 4). The recovery levels achieved were in good agreement with the spiked concentration in all cases (Table 3). It is worth mentioning that the methods did not require an overnight enrichment step, which cut considerably the analysis time, and allowed duplex detection of samples with pathogens less than 40 CFU/mL. The proposed approaches open up an advantageous form of pathogen determination using automated devices. The development of a competitive, portable, low-energy analytical system that integrates all the steps is underway.

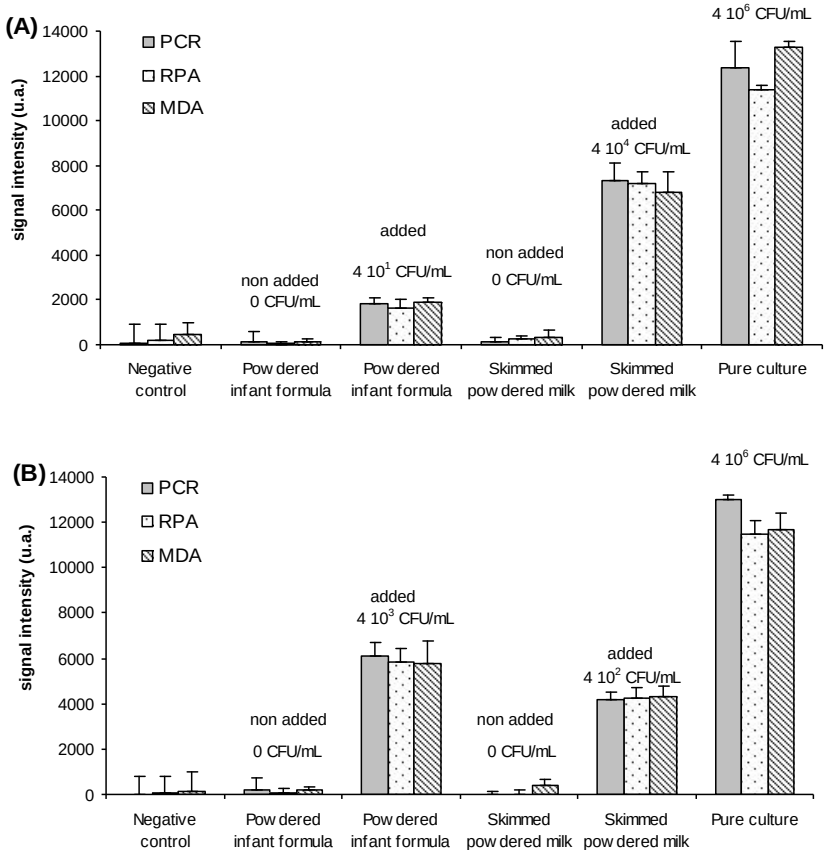


Figure 4. The microarray signals yielded by amplification techniques for inoculated milk samples with both pathogens: (A) average spot density for *Salmonella* spp. and (B) average spot density for *Cronobacter* spp.

Table 3. Recovery results for the milk samples.

Sample	Spiked level (log_{10} CFU/mL)		Detected level (log_{10} CFU/mL)					
			PCR		RPA		MDA	
	***S.* spp.**	***C.* spp.**	***S.* spp.**	***C.* spp.**	***S.* spp.**	***C.* spp.**	***S.* spp.**	***C.* spp.**
Skimmed powdered milk	0	0	ND	ND	ND	ND	ND	ND
	2.6	0	2.8	ND	2.8	ND	2.7	ND
	4.6	0	4.2	ND	4.2	ND	3.4	ND
	0	2.6	ND	2.9	ND	2.5	ND	2.0
	0	4.6	ND	4.1	ND	4.9	ND	4.0
PIF 1	0	0	ND	ND	ND	ND	ND	ND
	3.6	2.6	3.1	2.8	3.4	2.8	3.9	2.0
PIF 2	0	0	ND	ND	ND	ND	ND	ND
	1.6	0.6	1.7	0.6	1.3	1.2	1.8	1.5
	2.6	4.6	2.7	4.9	2.7	4.9	2.4	4.2
PIF 3	0	0	ND	ND	ND	ND	ND	ND
	1.6	3.6	1.9	3.6	1.1	3.9	1.4	3.3

PIF: Powdered Infant Formula, *S.* spp.*: Salmonella* spp.*, C.* spp.*: Cronobacter* spp.
ND: No Detected (signal-to-noise ratio<3)

4. Conclusions

RPA and MDA are two innovative methods that offer several advantages for the automation of DNA assays in wide range of point-of-need applications. Isothermal amplifications do not require sophisticated hardware for accurate temperature control against thermocycling PCR-based methods. Moreover, both enzymes operate near room temperature if compared to other isothermal reactions (e.g., LAMP at 60 °C). The results reveal that both methods offer portability without compromising analytical performance, tolerance to inhibitors or price per assay. Nevertheless, some properties, such as short times or lack of a post-amplification protocol, indicate RPA has a higher potential than the MDA method for point-of-need applications. MDA is also an interesting method for high-multiplexing determination because RPA, such as PCR, is limited by a restrictive primer design and to a small number of targets (<10 genes).

A duplex system using these isothermal amplifications is proposed for the first time. This study also demonstrates that the integration of nucleic acid amplification and detection into analytical devices, such as compact discs (bio-

recognition and reading), is technically possible and allows high-throughput analyses. Therefore, the low-cost detection of different targets in parallel with minimal manipulation is achievable with these approaches.

Acknowledgements

Funding projects MINECO CTQ2013-45875-R and GV PrometeoII/2014/040. MECD provided S.S.F with a PhD grant.

References

1. Asiello, P. J., Baeumner, A. J. *Lab Chip*, 2011, 11, 1420-1430.
2. Kim, Y. H., Yang, I., Bae, Y. S., Park, S. R. *BioTechniques*, 2008, 44, 495-505.
3. Nakayama, T., Hiep, H. M., Furui, S., Yonezawa, Y., Saito, M., Takamura, Y., Tamiya, E. *Anal. Bioanal. Chem.*, 2010, 396, 457-464.
4. Gill, P., Ghaemi, A. *Nucleos. Nucleot. Nucl.*, 2008, 27, 224-243.
5. Yan, L., Zhou, J., Zheng, Y., Gamson, A. S., Roembke, B. T., Nakayama, S., Sintim, H. O. *Mol. BioSyst.*, 2014, 10, 970-1003.
6. Zanoli, L. M., Spoto, G. *Biosensors*, 2013, 3, 18-43.
7. Piepenburg, O., Williams, C. H., Stemple, D. L., Armes, N. A. *PLoS Biol.*, 2006, 4, 1115-1121.
8. Dean, F. B., Hosono, S., Fang, L. H., Wu, X. H., Faruqi, A. F., Bray-Ward, P., Sun, Z Y., Zong, Q. L., Du, Y. F., Du, J., Driscoll, M., Song, W. M., Kingsmore, S. F., Egholm, M., Lasken, R. S. *P. Natl. Acad. Sci. USA*, 2002, 99, 5261-5266.
9. Shen, F., Davydova, E. K., Du, W., Kreutz, J. E., Piepenburg, O., Ismagilov, R. F. *Anal. Chem.*, 2011, 83, 3533-3540.
10. Cahill, S. M., Wachsmuth, I. K., Costarrica, M. L., Embarek, P. K. B. *Clin. Infec. Dis.*, 2008, 46, 268-73.
11. Derzelle, S., Dilasser, F. *BMC Microbiol.*, 2006, 6, 100-112.
12. Hyeon, J., Park, C., Choi, I., Holt, P. S., Seo, K. *Int. J. Food Microbiol.*, 2010, 144, 177-181.
13. Wang, M., Cao, B., Gao, Q., Sun, Y., Liu, P., Feng, L., Wang, L. *J. Clin. Microbiol.*, 2009, 47, 3178-3184.
14. Codex Alimentarius Commission. Code of hygienic practice for powdered formulae for infants and young children CAC/RCP 66 - 2008.
15. Morais, S., Tortajada-Genaro, L. A., Arnandis-Chover, T., Puchades, R., Maquieira, A. *Anal. Chem.*, 2009, 81, 5646-5654.
16. Siegrist, J., Peytavi, R., Bergeron, M., Madou, M. *IVD Technol.*, 2010, 16, 22-26.
17. Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldón, J. A., Puchades, R., Maquieira, A. *J. Agric. Food Chem.*, 2012, 60, 36-43.

18. Arnandis-Chover, T., Morais, S., Tortajada-Genaro L. A., Puchades, R., Maquieira, Á, Berganza, J, Olabarria, G. *Talanta*, 2012, 101, 405-412.
19. Zhuang, Z., Ai Y. *Biochim. Biophys. Acta*, 2010, 1804, 1081-1093.
20. Halperin, A., Buhot, A., Zhulina, E. B. *J. Phys. Condens. Matter*, 2006, 18, S463-S490.
21. Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle, R., von Stetten, F. *Lab Chip*, 2010, 10, 887-893.
22. Erlandsson, L., Rosenstierne, M. W., McLoughlin, K., Jaing, C., Fomsgaard, A. *PloS One*, 2011, 6, e22631.
23. Wilson, I. G. *Appl. Environ. Microb.*, 1997, 63, 3741-3751.
24. Al-Soud, W. A., Rådström, P. *Appl. Environ. Microb.*, 1998, 64, 3748-3753.

SUPPLEMENTARY INFORMATION

Isothermal DNA amplification strategies for duplex microorganism detection

Sara Santiago-Felipe, Luis Antonio Tortajada-Genaro, Sergi Morais, Rosa Puchades, Ángel Maquieira

Table S-1. Probes and primers used for the studied amplification methods.

		Target	Sequence 5'-3' [a]	Bases (mer)	Tm (°C)
PCR and RPA	Primer-L	*Salmonella* spp.	Dig-TACCAAAGCTAAACGCGCAGCT	22	62.1
	Primer-R	*Salmonella* spp.	TGATCAGGAAATCTTCCAGTTGC	23	61.1
	Primer-L	*Cronobacter* spp.	Dig-GTTGGATCACCTCCTTACCTGC	22	64.2
	Primer-R	*Cronobacter* spp.	AGTTAAACCTCTTCAACTCCTG	22	58.4
MDA	Primer	Massive	Dig-NNNN*N*N / Dig-NNNNNN	6	-
Array	Probe	*Salmonella* spp.	BtnTg-$(T)_{10}$-TTTGATTACAGCCGGTGTACGACCCT	36	75.9
	Probe	*Cronobacter* spp.	TGTGAGCACGCGAGGTTGTATCTTGCA$(T)_{10}$-BtnTg	27	64.0
	Probe	Negative control	BtnTg-$(T)_{10}$-CGAAGCTGGCAACGCTACCGGTT	33	71.3
	Probe	Positive control	Dig-$(T)_{10}$-TTTTTGTCATGGGCCT CGTGTCGGAAAACC-BtnTg	40	81.0

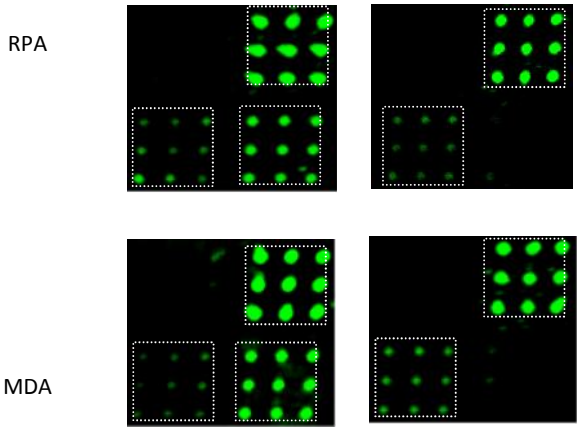
[a] Dig: digoxigenin labeled, BtnTg: BiotinTEG labeled, *: thiophosphate linkages
Oligonucleotide source: Arnandis-Chover et al. Talanta 2012, 101, 405-412.

Table S-2. Selectivity for different related microorganisms.

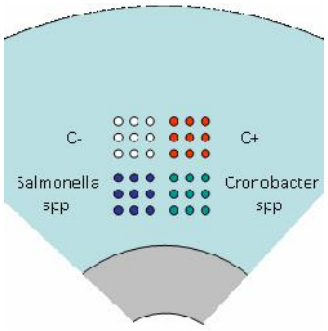
Species	**Strain**	*Salmonella* spp.	*Cronobacter* spp.
Salmonella serotype Typhimurium	CECT 443	+	-
Salmonella serotype Typhimurium	CCUG 21390	+	-
Salmonella serotype Lille	CCGU 12647	+	-
Salmonella serotype Dublin	CECT 4152	+	-
Salmonella serotype Anatum	CCUG 36820	+	-
Salmonella serotype Minnesota	CCUG 21390	+	-
Cronobacter sakazakii BBA	CECT 894	-	+
Cronobacter sakazakii	CECT 29544	-	+
Cronobacter dublinensis subsp. dublinensis	LMG 23823T	-	+
Cronobacter malonaticus	LMG 23826T	-	+
Cronobacter turicensis	LMG 23827T	-	+
Campylobacter jejuni	CCUG 17696	-	-
Citrobacter freundii	CECT401	-	-
Enterococcus faecalis	CECT 4081	-	-
Enterobacter aerogenes	CECT 684T	-	-
Escherichia coli	CECT 10536	-	-
Hafnia alvei	CEC 157	-	-
Klebsiella pneumoniae	CECT 142	-	-
Proteus vulgaris	CECT 484	-	-
Pseudomonas aeruginosa	CECT 116	-	-
Serratia marcescens	ATCC 25419	-	-
Staphylococcus aureus	CECT 239	-	-

+: Positive amplification, -: Negative amplification

(a)	**Powered infant formula**	**Skimmed powdered milk**
Amplification method	*Salmonella* spp. $4\ 10^1$ CFU/mL *Cronobacter* spp. $4\ 10^3$ CFU/mL	*Salmonella* spp. $4\ 10^2$ CFU/mL *Cronobacter* spp. 0 CFU/mL



(b)



C-: negative control
C+: positive control

Figure S-1. (a) Images displayed on DVD by RPA and MDA assays for inoculated milk samples. (b) Microarray layout on DVD.

Capítulo 3. Amplificación isoterma en fase sólida

En el capítulo anterior se ha demostrado que la RPA es una estrategia de amplificación adecuada para llevar a cabo reacciones en vial de modo sencillo, rápido y robusto, con prestaciones analíticas comparables a las obtenidas mediante PCR.

En este capítulo, se ha analizado si la RPA es idónea para el desarrollo de biosensores con un mayor grado de integración. Con este fin, se ha abordado el estudio de la reacción RPA en fase sólida, en la que la amplificación e hibridación tienen lugar simultáneamente. Esta innovadora técnica, denominada sp-RPA, se ha combinado con la tecnología de disco compacto (plataforma y detector), estableciendo una estrategia progresiva respecto a los formatos de ensayo.

En el primer trabajo, "*One-pot isothermal DNA amplification-hybridisation and detection by a disc-based method*", se propone la amplificación sp-RPA en formato micromatriz sobre la superficie del disco (Biosensor 5). Para ello, se adiciona la mezcla de reacción sobre la micromatriz con los cebadores anclados en el disco, logrando un sistema *lab-on-a-CD* de gran sencillez (Figura 28).

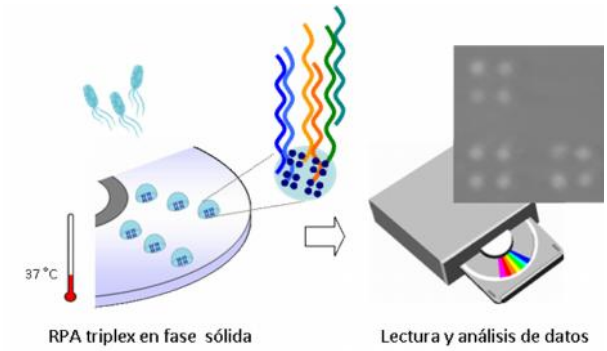


Figura 28. Esquema de la amplificación isoterma triplex en fase sólida sobre la superficie del disco y lectura de las micromatrices.

Como prueba de concepto, el ensayo se ha aplicado a la detección simultánea de tres genes específicos de *Salmonella* que engloban los principales serotipos virulentos, y consta de las siguientes etapas:

- Estudio del comportamiento de las gotas en la superficie del DVD.
- Puesta a punto de la reacción RPA en fase sólida.
- Estudio comparativo de diferentes sistemas de calentamiento.
- Optimización de la reacción simultánea para 3 genes.

- Comparación de los resultados con los obtenidos mediante el formato convencional en 2 etapas (amplificación+hibridación).
- Determinación de los parámetros analíticos del método.
- Estudio de viabilidad para el análisis de muestras alimentarias.

Las buenas prestaciones obtenidas con la sp-RPA, han llevado al desarrollo de un sistema *lab-on-a-disc* con un mayor grado de integración y automatización. En el segundo trabajo, titulado "*Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs)*", se propone un sistema multicapa compuesto por cámaras microfluídicas adheridas a la superficie del disco (Figura 29). De este modo la reacción sp-RPA tiene lugar en formato micromatriz en el interior de las cámaras, logrando mayor reproducibilidad y una reducción del volumen de reacción y del riesgo de contaminación cruzada (Biosensor 6).

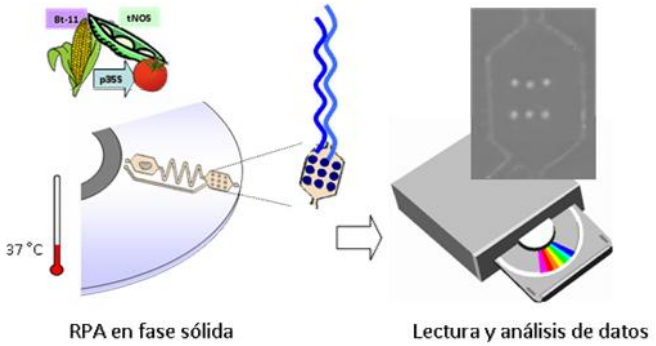


Figura 29. Esquema de la amplificación isoterma en fase sólida en cámaras microfluídicas y lectura de las micromatrices.

El método se ha aplicado para la discriminación de 5 genes específicos de OMGs (2 específicos de taxón, 2 de screening y 1 específico de construcción), y consta de las siguientes etapas:

- Diseño y fabricación de las cámaras microfluídicas.
- Caracterización de los parámetros microfluídicos.
- Puesta a punto de la reacción RPA en fase sólida.

- Optimización de los protocolos de lavado y revelado.
- Determinación de los parámetros analíticos del método.
- Estudio de viabilidad para el análisis de muestras alimentarias.

A pesar de las múltiples ventajas obtenidas en los anteriores sistemas *lab-on-a-disc*, el aumento del número de genes analizados simultáneamente continuaba siendo un desafío. En el tercer trabajo, titulado "*Parallel solid-phase isothermal amplification and detection by DVD technology*", se desarrolla una plataforma microfluídica integrada, compuesta por canales y micropocillos perforados en el sustrato del disco, que ha permitido llevar a cabo 90 reacciones sp-RPA en paralelo (Biosensor 7). De esta manera las reacciones sp-RPA ocurren de manera aislada en el interior de microreactores y de modo paralelo para cada analito (Figura 30).

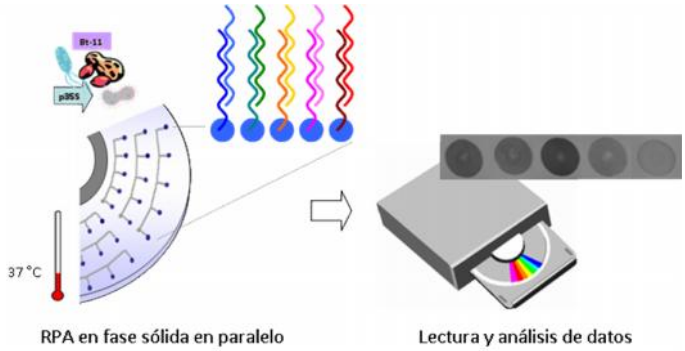


Figura 30. Esquema de la amplificación isoterma en fase sólida en paralelo en el interior de las estructuras microfluídicas y lectura de los resultados.

El método, aplicado a la detección simultánea de bacterias, alérgenos y OMGs, consta de las siguientes etapas:

- Diseño y fabricación de los dispositivos integrados.
- Caracterización de los parámetros microfluídicos.
- Puesta a punto de la reacción múltiple sp-RPA.
- Optimización de los protocolos de lavado y revelado.
- Determinación de los parámetros analíticos del método.
- Estudio de viabilidad para el análisis de muestras alimentarias.

One-pot isothermal DNA amplification- hybridisation and detection by a disc-based method

Sara Santiago-Felipe, Luis Antonio Tortajada-Genaro, Sergi Morais, Rosa Puchades, Ángel Maquieira

Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain

Reprinted from ***Sens. Actuators B, 2014, 197, 385-404****.*

Abstract

An integrated sensor comprising isothermal DNA amplification and in situ detection is presented. The method principle is based on recombinase polymerase amplification (RPA) and detection in the microarray format by compact disc technology as a highthroughput sensing platform. Primers were immobilised on the polycarbonate surface of digital versatile discs (DVD) and, after hemi-nested amplification, multiplexing identification of each tethered product was achieved by optical scanning with a 650 nm-laser ofthe DVD drive. The efficiency of one-pot hybridisation/elongation/detection depended strongly on probe density and other factors such as the concentration of the unbound primers present in solution. The optimised conditions provided equivalent amplification factors (7.3×10^8-8.9×10^8 fold) to those obtained by conventional reactions performed in vials. The proposed method was applied to *Salmonella* detection (generic by *hns* and *oriC* genes, and specific for subspecies I by *STM4507*

gene). A triplex assay was satisfactorily compared to the non-integrated protocols. Food and vaccine samples were analysed in a shorter time with less handling. The results indicate that the multiplex DVD assay is a simple, competitive, isothermal, portable system that is particularly useful for microbiological routine analysis.

Keywords: Isothermal, solid-phase amplification, compact disc, pathogen, microarray.

1. Introduction

The detection of specific DNA targets is interesting in genetic or infectious diseases diagnosis, bioterrorism, food safety and forensic areas. Traditionally, polymerase chain reactions (PCR) combined with techniques such as gel electrophoresis or solid hybridisation, have been used to reveal the presence of single-copy genes. However, these two-step protocols can also lead to sample contamination.[1]

Different methods have been proposed for the integration of amplification and hybridisation. The most relevant strategy is the use of real-time PCR, which allows the rapid, quantitative identification of DNA targets through the use of specific probes. Yet expensive non-portable instrument is required for sensitive fluorescent measurements, which restricts this technique for point-of-need applications. This limitation has been overcome with the application of microfabrication technologies integrating PCR reactors in a microfluidic device.[2,3] However, these solutions are useful for the simultaneous determination of a small number ofgenes.

Sensors based on integrated microarrays seem to be an ideal approach since their miniaturised size allows the arrangement of numerous probes in a relatively small space and reaction volume. An interesting category is lab-on-a-chip systems that incorporate different connected chambers for each analytical step, including one for hybridisation in a microarray format.[4,5] In these systems, the control of changes in evaporation or temperature is crucial because performance might be affected.[6]

A technique called solid-phase PCR was developed for the integration of both amplification and hybridisation processes in a single step.[7-10] To this end, one or both primers is/are attached on a solid support (beads, microplate, or a microarray format on a flat surface), while the other PCR components remain in the liquid phase. Enzymatic extension of the primer directly produces a tethered amplification product. The advantages are high throughput, ease of operation, and specific fast detection. However, amplification yields are lower than those of conventional solution-phase reactions.[8] It has been demonstrated that the addition of forward and/or reverse primers to the reaction mixture can enhance

amplification efficiency because the reaction proceeds in both the liquid and solid phases.[9] When the attached primers differ from those in solution, the mechanism is called heminested solid-phase amplification. Here the amplification of an extended nucleotide sequence is followed by the amplification of a region located within the first amplification product, which provides improved selectivity and sensitivity.[11]

The throughput of the above-discussed PCR-based methods is limited by thermal constraints because an imprecise temperature leads to lower amplification efficiency. In the last few years, two approaches have been considered to overcome this drawback. Firstly, the development of devices with accurate temperature control and fast transitions between stages, using low thermal conductivity materials.[5,12] Secondly, the use of other polymerases (or combination of enzymes) allows for isothermal amplification, thus simplifying the heating device.[13] In the latter option, there are some relevant examples based on strand displacement amplification (SDA), helicase-dependent amplification (HDA) and recombinase polymerase amplification (RPA), which have been combined with detection by silicon microring resonators, on-chip fluorescence, electrochemical devices or lateral flow strips.[8,14-17] However, these methods are performed far from room temperature (60-65 °C), require a time-consuming step for surface treatment and functionalisation, or need a complex fabrication process of devices and expensive detectors.

The aim of this study was to develop an integrated system that provides both the flexibility of isothermal amplification and the multiplexed capacity of microarrays in a homogenous assay. According to our experience, in-tube RPA is readily compatible with subsequent solid-hybridisation. The studied one-pot approach involves the development of the solid-phase RPA reaction in a microarray format onto the sensing surface. As proof-of-concept, the assay is done on an optical low-cost portable analytical device and does not use microfluidics. Hybridisation/elongation is performed on the surface of a digital versatile disc (DVD) and the products are detected with a standard DVD drive.[18-19] The principles and benefits of this innovative simple sensor are showcase through

detecting *Salmonella* bacteria in different clinical and food products. Nevertheless, this strategy can be easily extended to other targets, layouts and detection systems.

2. Methods

2.1 DNA targets. The assay was developed for the simultaneous detection of three genes: *hns* and *oriC* for the generic identification of *Salmonella* spp., and *STM4057* for the specific detection of *Salmonella* subspecies I. The primers used in *hns* gene detection were 5'-digoxigenin-TACCAAAGCTAAACGCGCAGCT-3' (forward), 5'-TGATCAGGA-AATCTTCCAGTTGC-3' (solution reverse), and 5'-biotin-TEG-T10-TTTGATTA-CAGCCGGTGTACGACCCT-3' (surface reverse). The primers used in *oriC* gene detection were 5'-digoxigenin-TTATTAGGATCGCGCCAGGC-3' (forward), 5'-AAA-GAATAACCGTTGTTCAC-3' (solution reverse), and 5-biotin-TEG-T10-GCTAGTG-ATCCTTTCCAACGCATTG-3' (surface reverse). The primers used in *STM4057* gene detection were 5'-digoxigenin-GGTGGCCTCGATGATTCCCG-3' (forward), 5'-CCCACTTG-TAGCGAGCGCCG-3' (solution reverse), and 5'-biotin-TEG-T10-GCCCGGCCTCC-GGTGAAGGTAATTT-3' (surface reverse). Food and clinical samples were analysed. Inoculation assays were also prepared by adding 10-fold serial dilutions of an 18-h culture of each pathogen in sterile saline solution (0.8% NaCl) covering a range from 0 to $4{\cdot}10^4$ colony forming units per millilitre (CFU/mL). The protocols for DNA extraction, the description of the cells and the other oligonucleotides used in this study are available in the supplementary information.

2.2 Microarraying. Recordable DVDs were purchased from MPO Iberica (Spain). Discs were firstly conditioned by gentle ethanol washing, water rinsing, and dried by centrifugation. Primers were immobilized by passive adsorption by means of streptavidin-biotin interaction. For that, each mixture of streptavidin (10 mg/L) and biotinylatedsurface primer (50 nM) in printing buffer (50 mM carbonate buffer, pH 9.6 and 1% glycerol (v/v)) was transferred to the polycarbonate disc surface (50 nL) with a non-contact printer (AD 1500 BioDot Inc., CA, USA), and the working temperature and relative humidity were

controlled at 25 °C and 90%, respectively. Different microarray layouts, or distributions of primers on the surface, were examined (see supplementary information). The final layout consisted of 36 arrays of 25 dots each (5 × 5) with a 1-mm track pitch: four spots corresponding to *hns* gene, four dots corresponding to *oriC* gene, four dots corresponding to *STM4057* gene, four positive controls, and nine negative controls (immobilisation and hybridisation).

Immobilisation densities of primers were determined by using a dual labelled oligonucleotide (biotin at the 5'-end and Cy5 at the 3'-end), and fluorescence was measured by a homemade reader device. Excitation of the Cy5-marker was achieved by light emitting diodes (Toshiba TLOH157P) at an angle of 55°, illumination of 150 mW and wavelengths of 595-615 nm. Emission intensity was collected by a high sensitivity charge-coupled device (Qimaging Inc., Canada). Then, the amount immobilised on the disc was estimated from a calibration curve obtained from serial dilutions of the Cy5-labelled oligonucleotide (0.1-100 nM).

2.3 Integrated procedure. The amplification enzymes, nucleotides and buffer (TwistAmp Basic from TwistDx, UK) were mixed with 160 nM of both primer pairs (forward and reverse solution primers) for the three target genes and 10 ng of genomic DNA, extracted sample in triplicate. Denhardt's reagent (2.5×, Life Technologies) and inert oil (8% v/v, Sigma) were also added to reduce the non-specific background and to generate an inert overlay for minimising the evaporation phenomenon, respectively. Later, mixtures (25 µL) were dispensed with a multi-channel micropipette to form a spherical sessile droplet on the corresponding microarray layouts. Positive and negative amplification controls were also included. The droplet dimensions were measured using a Dino-Lite Digital Microscope (BigC.com, CA) with a resolution of 1.3 megapixels (1280 × 1024 pixels). Accordingly, the volume of droplets was established [Eq. (1)] by three parameters: contact radius (*r*), droplet height (*h*), and contact angle (¯).

$$V = \frac{®}{6} h\left(3r^2 + h^2\right) = \frac{®r^3}{3} \frac{f(¯)}{\sin^3 ¯} \qquad \text{[eq. 1]}$$

where $f(¯) = 2 - cos\ ¯\ (3 - cos^2\ ¯) = (2 + cos\ ¯)(1 - cos\ ¯)^2$.

The disc was introduced into a container (standard DVD plastic box) in a water-saturated atmosphere, and the solid-phase amplification reactions were carried out at 37 °C for 40 min in a heating oven. The immobilised product was developed according to the protocol described in Reference 18.

2.4 Sensing principle. The amount of pathogen was related to the optical density of the reaction product, which was read directly by an adapted DVD drive from LG Electronics Inc. (Englewood Cliffs, NJ, USA) and controlled by custom software, with a reading time lower than 10 min.[18] The measurement principle is based on the optics of the DVD drive and the variation of the reflection properties of the DVD surface given the presence of the biorecognition product. Briefly during DVD scanning following the disc track (rotation speed 4 × ≡ 13.46 m/s, 26 dB gain, 1.7 megasamples/s), the laser strikes the product which attenuates laser beam intensity that reaches the photodiode of the pickup. If there is no solid product, the reflection properties of the DVD surface remain unchanged and the maximum intensity of reflected beam is collected by theDVD drive (background signal). The analogue signals are acquired directly from the photodiode of the DVD drive. Then grey-scale images (the tagged image file format, colour depth 16 bit, scale0-65,535) were generated and the optical intensity signals of each spot were quantified using in-home software. Image processing (feature gridding, addressing, segmentation and quality assurance) was automatically performed in less than 5 min per disc.

2.5 Conventional procedure. In the first step, triplex RPA reactions were performed in vials (25 µL) by adding 160 nM of each primer and 10 ng of the genomic DNA from extracted sample to the reconstituted RPA solution. Furthermore, deoxynucleotides (200 µM) were added to obtain the correct amplification yield for the multiplex assay. Tubes were placed inside a heating oven (Memmert UF30, Germany) at 37 °C for 40 min. In the second step, the hybridisation assays on the disc were carried out with 10 µL of the amplified product mixed with 90 µL of 5 × hybridisation buffer (1× saline sodium citrate, NaCl 150 mM, sodium citrate 15 mM, pH 7) containing 10 nM of the positive hybridisation control. Subsequently, the solution was denatured by heating at 95 °C for 5 min and transferred to the microarray on the DVD polycarbonate surface. After 40 min of incubation at 37 °C, the array was washed with pure water. Products were developed and imaging was performed as described above.

2.6 Data processing. The amplification efficiency (*E*) in the PCR, or the increase of product per cycle, might be described by the equation $E= (N/N_0)^{1/n}$, where N_0 and N are the initial and final number of targeted molecules, and n is the total number of cycles. *E*-values vary between 1 and 2 per cycle. For the RPA (isothermal amplification reaction), efficiency was associated with the average number of primer extensions by *Bsu* DNA polymerase I. Therefore the *E*-value, or the average increase in product by the time unit, was calculated from the global amplification yield described by the equation $E= (N/N_0)^{1/t}$, where t is the reaction time (in min).

The statistical package SPSS for Windows v. 16.0 was used for the data analysis of the optical signals.

3. Results and discussion

3.1 Droplet format. A first study to establish the behaviour of the reaction droplets on the sensing surface was developed by dispensing the RPA mixtures on DVDs. The high hydrophobic nature of polycarbonate generates spherical sessile droplets (Figure 1). The statistical analysis confirmed that the right and left contact angles were the same ($F=0.04 < F_{1,6}=5.99$, p-value > 0.05).

Several factors were studied to reduce droplets evaporation due to the gas/liquid equilibrium. The variation of the buffer composition ($MgCl_2$ range 5-20 mM, formamide range 0-25%, Tween range 0-0.5%) did not yield satisfactory results. However, the water saturated ambient (DVD box) and the presence of inert oil in the reaction mixture (8% v/v) forming an external lipid layer, reduced water evaporation. Regarding volume, small droplets increased the assays per disc and cut the analysis cost, but a poor response was produced because the amplification reaction was partially inhibited. Hence, a droplet volume of 25 µL was selected, with 5.16 ± 0.02 mm being the diameter on the polycarbonate surface. Under these conditions, the evaporation regime in accordance with temperature (25-55 °C) was established (Figure 1). The drop volume diminished linearly with time, just as Pittoni et al. reported for the polycarbonate surface.[20] The initial droplet had a contact angle of 59.5 ± 1.4°, a height of 1.72 ± 0.04 mm, and a contact radius of 2.87 ± 0.12 mm. After 40 min heating in an oven at 37 °C (the working temperature of the RPA), the contact angle and height were reduced to 43.1 ± 0.5° (-27%) and 1.14 ± 0.02 mm (-38%), respectively, but increased the contact radius to 2.95 ± 0.04 mm (+3%). These changes in the droplet dimensions (<20% in volume) did not modify the polymerase activity.

An assay with 36 droplets was chosen (Figure 1). The positions of the arrays were chosen to directly dispense the reagents with a multi-channel micropipette (distance between flanking droplets: 1 cm). Moreover, the hydrophobic nature of the sensing surface (polycarbonate, contact angle of ~90°) allowed correct platform manipulation without any cross-contamination effect between the adjacent droplets. The available area for each microarray was 20.9 ± 0.2 mm^2.

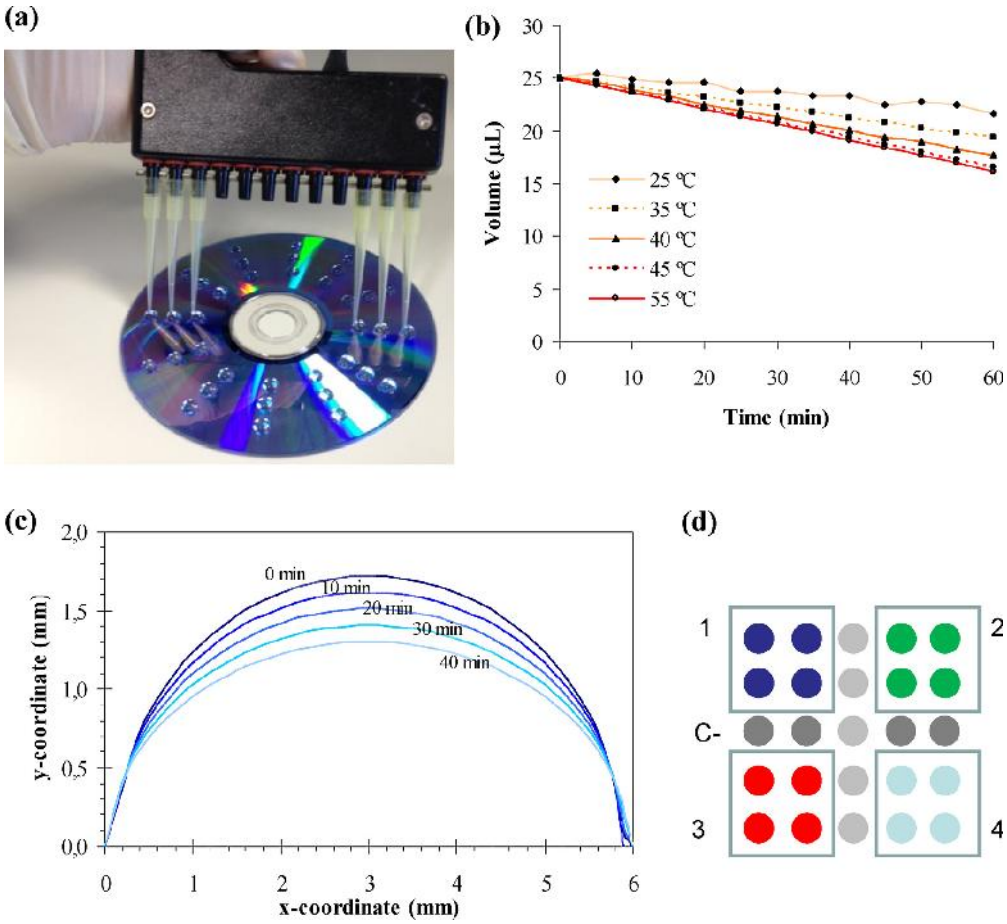


Figure 1. (a) Photography of the RPA-mixture dispensation by a multi-channel micropipette on DVD (36 reactions). (b) Time and temperature effect on droplet volume. Error < 5% (four replicates). (c) Evolution of droplet profiles at 37 °C. (d) Microarray layout: the *oriC* gene (block 1), the *STM4057* gene (block 2), the positive control (block 3), the *hns* gene (block 4) and negative control (C-).

3.2 Optimisation of the solid-phase RPA format. Different solid-phase amplification approaches were studied to check the absence of false-positive or false-negative responses and to compare spot intensities.

The first approach was based on immobilising primers on the solid support and on dispensing other reaction components in the liquid phase onto the disc. The results obtained were very poor interms of sensitivity (signal-to-noise lower than 5 for 2 ng DNA/mL). In a second approach, the addition of unbound primers to the reaction mixture led to amplification in both phases (in liquid and on the surface), and gave satisfactory results (signal-to-noise higher than 10 for 2 ng DNA/mL). The liquid fraction analysis confirmed the presence of the amplification product.

Spot intensities depended on the immobilisation density of the primer. To this end, different amounts of reverse primer were anchored on the surface. The effect of the attached primer concentration (immobilisation densities from 0 to 0.13 fmol/mm^2) to the optical signal is plotted in Figure 2a. The highest signal was obtained for 50 nM (0.03 fmol/mm^2). Higher concentrations led to a decreased signal, probably due to the steric effects; i.e., hindrance, repulsion, etc., between attached primers and/or amplified products, as demonstrated for solid-phase hybridisation on glass surfaces.[10]

The effect of immobilising a single primer (asymmetric amplification) or both primers (bridge amplification) onto the solid surface was also assessed (Figure 2b). The analysis of variance (ANOVA) test showed that there was no significant difference attaching the forward primer, the reverse primer, or both ($F=2.1 < F_{2,12}=3.89$, p-value > 0.05). For further experiments, the reverse primer was immobilised. As a negative control, a primer with a dideoxynucleotide at the 3'end was used to avoid primer elongation. Since the signal was comparable to the background, non-specific hybridisation processes were done. Thus the optical signal recorded with conventional primers was completely due to the solid-phase amplification.

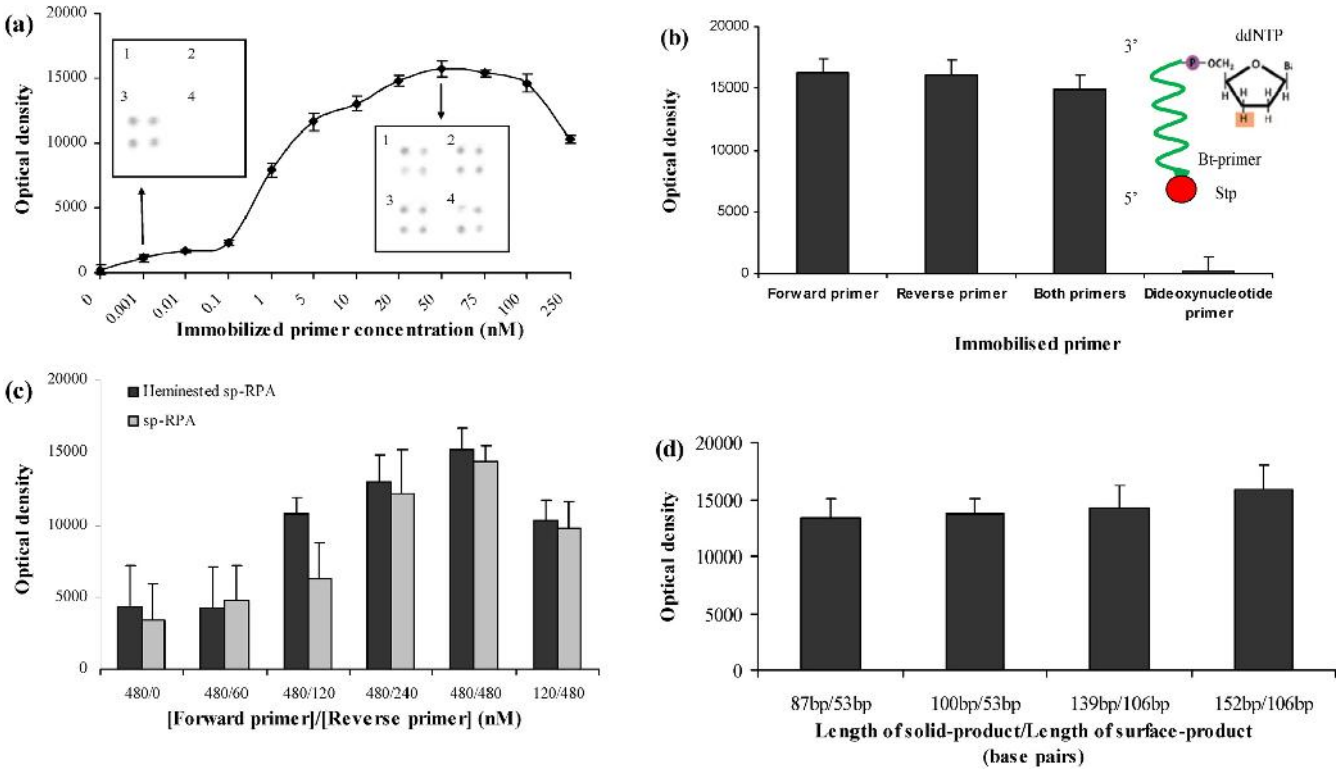


Figure 2. Effect of experimental factors on optical intensity: (a) Concentration of the attached primer. Inserts: array images corresponding to the *oriC* gene (block 1), the *STM4057* gene (block 2), the positive control (block 3) and the *hns* gen (block 4); (b) type of immobilised primer; (c) primer concentration in the liquid phase for the heminested sp-RPA and sp-RPA format; (d) length of the heminested-amplified products (solution/surface). Pathogen concentration, 10^2CFU/mL.

Figure 2c also shows the results for the two solid-phase approaches according to the unbound reverse primer sequence if compared to the attached one. Conventional amplification (sp-RPA) involved the formation of the same product in the liquid phase as in solid phase, because the same sequence is used for both phases. Meanwhile, the heminested approach (heminested sp-RPA) led to a shorter immobilised product than that formed in the liquid phase, because the attached primer was designed to be specific for a region located within the first product. Then the initial amplification (in solution), was followed by the nested solid-phase reaction. No signal difference was observed when the reverse unbound primer was equal to (sp-RPA) or differed from (heminested sp-RPA) the attached primer on the surface and, consequently, similar *E*-values were reached. The heminested approach was chosen because this format guarantees higher selectivity since three specific regions of the target gene are involved in the assay.

Our previous vial-RPA studies have demonstrated that as product length increases, the number of copies lowers and the reaction rate improves due to enzyme processivity; i.e., measurement of the global number of nucleotides added per time unit. In the heminested sp-RPA, a similar effect was expected because DNA polymerase elongated both primers (solution and surface). Then, the size of both reaction products was studied by changing the forward and/or the reverse primer used, which led to product lengths of between 87 bp and 152 bp for the solution-products, and of between 53 bp and 106 bp for the surface-products (Figure 2d). The ANOVA test showed that the signals for the one-pot format were comparable ($F=0.877 < F_{3,12}=3.49$, p-value > 0.05). These results indicated that enzyme processivity affected the global amplification yield, but these variations were not observed on the surface reaction.

3.3 AFM imaging. The AFM imaging of DVD surface revealed changes in the topography of the surface, as consequence of the biomolecules immobilisation (Figure S-5, see supplementary Information). Raw polycarbonate exhibited a smooth and featureless surface, with a maximum peak depth at 1.34 nm. After primer immobilisation, by means of streptavidin-biotin interaction, a homogenous spread of globular particles appeared. The analysis of the dimensions of these

particles reported a minimum diameter of around 11 nm, corresponding to the streptavidin as it has been previously described.[22] After the solid-phase amplification, an increase of the height was observed from 6.3 to 6.8 nm. This variation may be due to two effects related to the amplification reaction, an increase of the density of the attached molecules and the presence of double-strand DNA.[23] Previous studies have reported similar height increments for the hybridisation of a 150 pb amplified product.[22]

3.4 Effect of temperature and reaction time. Figure 3 shows the optical signal with the temperature and reaction time for the integrated reaction when compared to the optical signal recorded by the two-step protocols. The maximal response for the heminested sp-RPA format was accomplished in 40 min at 37 °C, but the signal was approximately 20% lower than that obtained when amplification and hybridisation took place separately in avial and on a disc, respectively. This decrease is in agreement with those reported in previous studies based on solid-phase PCR, which shows that the amplification rate is lower than for the two-step protocols.[11] This fact can be explained if we consider that the high negative charge density of the immobilised primers on the solid support may disturb polymerase functioning on the surface, and/or may repel the target DNA in solution, to reduce hybridization and extension efficiency. Additionally in the solid-phase formats, the reagents (enzyme, nucleotides, and especially the DNA template) have to be transported to the surface before elongation from the attached primer starts. Subsequently, a concentration gradient is formed between the bulk solution close to the surface, which diminishes amplification efficiency (limiting reactant).

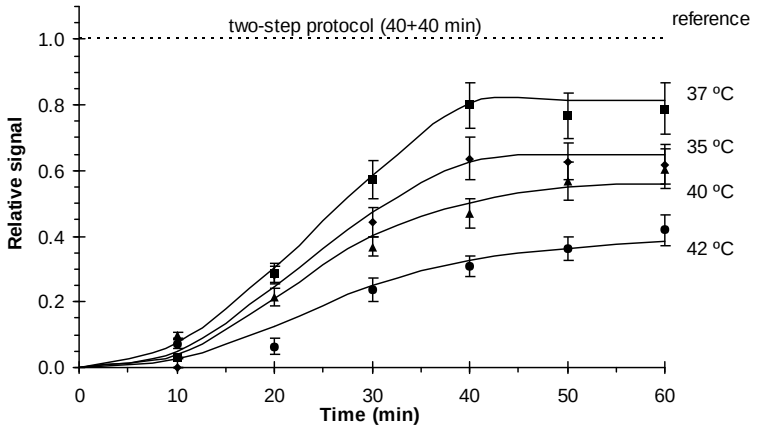


Figure 3. Amplification kinetics for the integrated method at different reaction temperature and compared with the two-step protocol. Pathogen concentration: 10^2CFU/mL; probe density: 0.03 fmol/mm^2.

The heminested sp-RPA format proved more sensitive to temperature than the amplification in the vial. This worse temperature tolerance (5-fold lower) might be due to the reagent concentration variations caused by the droplet evaporation process, rather than by modified enzymatic activity. Then different heating systems were examined to control the isothermal process. Assay performance was evaluated using several low-cost heating devices: oven, infrared lamp, hot plate, water bath. The obtained droplet volumes and optical signals are shown in Table 1. The ANOVA test was performed and showed two groups of devices if compared to signal intensity. Higher responses were achieved for the non-contact heating systems (oven and IR lamp), if compared to systems based on heating by contact ($F=17.58 > F_{4,5}=5.19$, p-value < 0.05). These differences are explained by thermal conductivity (λ=0.241 Wm^{-1}K^{-1} at 38 °C) and diffusivity ($\alpha=1.53\cdot10^{-7}$ m^{2}s^{-1} at 38 °C) of polycarbonate.[24] A temperature gradient appeared between the upper side of the DVD (surface with the reaction solution) and the bottom (hot surface), introducing a time delay before reaching the optimal amplification temperature on the analytical surface. The oven (non-contact heating device) provided a low evaporation percentage and high signals, and was selected for further experiments. Under these conditions, reproducible assays were also achieved (variation on the optical signal: 4-6%) and the equivalent amplification

factor of the targeted DNA was $7.3 \cdot 10^8$-$8.9 \cdot 10^8$-fold, the equivalent to an average amplification efficiency of 1.11 per minute. This low *E*-value is common in solid-phase amplifications, as described above.

Table 1. Droplet parameters and evaporation percentage (n=8) displayed by each heating device (37 °C, 40 min).

Parameter*	Initial	Oven	Infrared lamp	Hot plate	Water bath
θ_L (°)	59.6 ± 1.2	47.0 ± 0.6	44.8 ± 1.7	40.5 ± 1.4	50.6 ± 0.3
θ_R (°)	59.5 ± 1.5	47.1 ± 0.3	44.3 ± 1.7	40.6 ± 0.1	51.2 ± 0.9
r (mm)	2.87 ± 0.12	2.95 ± 0.04	3.07 ± 0.36	3.03 ± 0.07	2.91 ± 0.01
h (mm)	1.72 ± 0.04	1.30 ± 0.02	1.24 ± 0.15	1.11 ± 0.05	1.39 ± 0.05
V (μL)	25.0 ± 1.1	18.8 ± 0.8	19.4 ± 1.4	16.8 ± 1.4	19.9 ± 0.9
% evaporation	-	25 ± 5	22 ± 8	33 ± 8	20 ± 5
optical density	-	16279 ± 1045	14491 ± 566	10746 ± 694	10886 ± 580

* θ_L: left angle, θ_R: right angle, r: droplet radius, h: droplet height, V: volume calculated according to equation [1]

3.5 Proof of concept: detection of *Salmonella*. Although the one-pot reaction can be performed by different sensors, the use of compact disc technology provides major advantages. Firstly, the mass production of discs and readers creates a high quality, low-cost, and high-access sensor. Secondly, polycarbonate substrates have excellent bioanalytical properties, such as high immobilisation yield of primers, minimal droplets displacement, and absence of non-specific backgrounds. Thirdly, high-working capability has been achieved due to the huge sensing area. Finally, the optical reader properties (laser drive), such as light weight (<500 g) or reduced volume (a few centimetres), are compatible with its implementation in different settings.

The analytical performance of the one-pot format based DVD technology was established and compared to those of the two-step approach. A triplex assay was tested for the generic detection of *Salmonella* spp. (genes *hns* and *oriC*) and for specific detection of *Salmonella* subspecies I (gene *STM4057*), and 432 independent spots per DVD (3 genes × 4 replicates × 36 zones) were printed. No primer-primer dimer, false positive or cross-contamination problems were observed.

A correlation between the amount of DNA extracted from pure cultures and the optical signal was obtained for the three genes up to a concentration of 200 ng/mL. As seen in Table 2, the lowest detectable concentration was 0.12-0.15 ng/mL, the equivalent to 24-30 copies/mL. The slightly worse results obtained for the heminested sp-RPA as compared to the two-step format (6-25 copies/mL) is explained by the poorer amplification efficiency of the solid-phase approach. The reproducibility of the integrated format, expressed as the relative standard deviation of three replicates, was 7.8-20.7%. These results were slightly higher than the two-step approach (5.9-16.3%), but were adequate for pathogen determination.

Table 2. Analytical performance of the one-pot (A) and two-step systems (B) for the different target genes.

	hns gene		***oriC gene***		***STM4057 gene***	
	A	**B**	**A**	**B**	**A**	**B**
Working range (ng/mL)	$0.12\text{-}2\cdot10^4$	$0.03\text{-}2\cdot10^4$	$0.15\text{-}2\cdot10^4$	$0.07\text{-}2\cdot10^4$	$0.13\text{-}2\cdot10^4$	$0.13\text{-}2\cdot10^4$
LOD (CFU/mL)	24	6	30	15	25	25
RSD (%)*	9.3-14.7	6.3-8.1	7.8-15.2	8.8-9.5	10.4-12.8	6.6-7.9

*Relative standard deviation calculated from a pure culture 10^3 CFU/mL (n=3).

The reliability of the method for its application in large-scale screening was evaluated by the detection of *Salmonella* strains in food and clinical samples. The set was composed of raw samples and was spiked with several serotypes of *Salmonella* spp. and the non-target pathogen (*Cronobacter sakazakii*). Thirty-six samples/replicates were simultaneously analysed by a single assay to yield the results shown in Figure 4. The samples inoculated with *C. sakazakii* or non-inoculated food samples were negative for the three genes. Industrial sample (chicken carcass) and vaccines (human: *S. thyphi* and veterinary: *S. typhimorium* and *S. enteritidis*) were positive and showed a semi-quantitative correlation between the amount of pathogen and optical intensities, expressed as a signal/noise ratio for the microarray spots (Table 3). Hence an integrated amplification-detection of *Salmonella* spp. and subspecies I was achieved in a broad set of samples.

Numerous studies have been described for *Salmonella* detection. Conventional methods include microscopy, culture and serology, but they are time consuming

and have worse sensitivity that our proposed approach.[25] Techniques based on PCR amplification are the most commonly used and show similar (or better) analytical performances but reduced portability.[25,26] In recent years, there has been much research activity in the area of biosensors, suchas quartz crystal microbalance or latex agglutination assays, which have improved limits of detection and reduced time assay.[25-27] Recent biosensors have been published applying solid-phase RPA combined with silicon microring resonance, on-chip fluorescence, lateral flow strips or electrochemical detection.[15-17] These approaches have reported similar analytical performances, but have shown some drawbacks. For instance, the number of samples that can be detected simultaneously is smaller, time-consuming surface treatments are required and they need complex devices and expensive detectors. Conversely, no blocking/modification procedure was necessary for the polycarbonate surface of DVDs to the avoid inhibition of polymerases or the non-specific adsorption of amplification reagents. Also, in the DVD technology, the simultaneous analysis of 3 genes in 36 samples was allowed, and the DVD-drive is used as a low-cost, portable, sensitive and reproducible optical detector.

The analysis time of the solid-phase amplification was 40 min. The two-step strategy took approximately 88 min in all: in-tube amplification (40 min), hybridisation solution preparation (~2 min), thermal denaturing (5 min), and the hybridisation process (40 min). Therefore, the one-pot strategy dramatically reduces sample handling and the assay time without compromising the results.

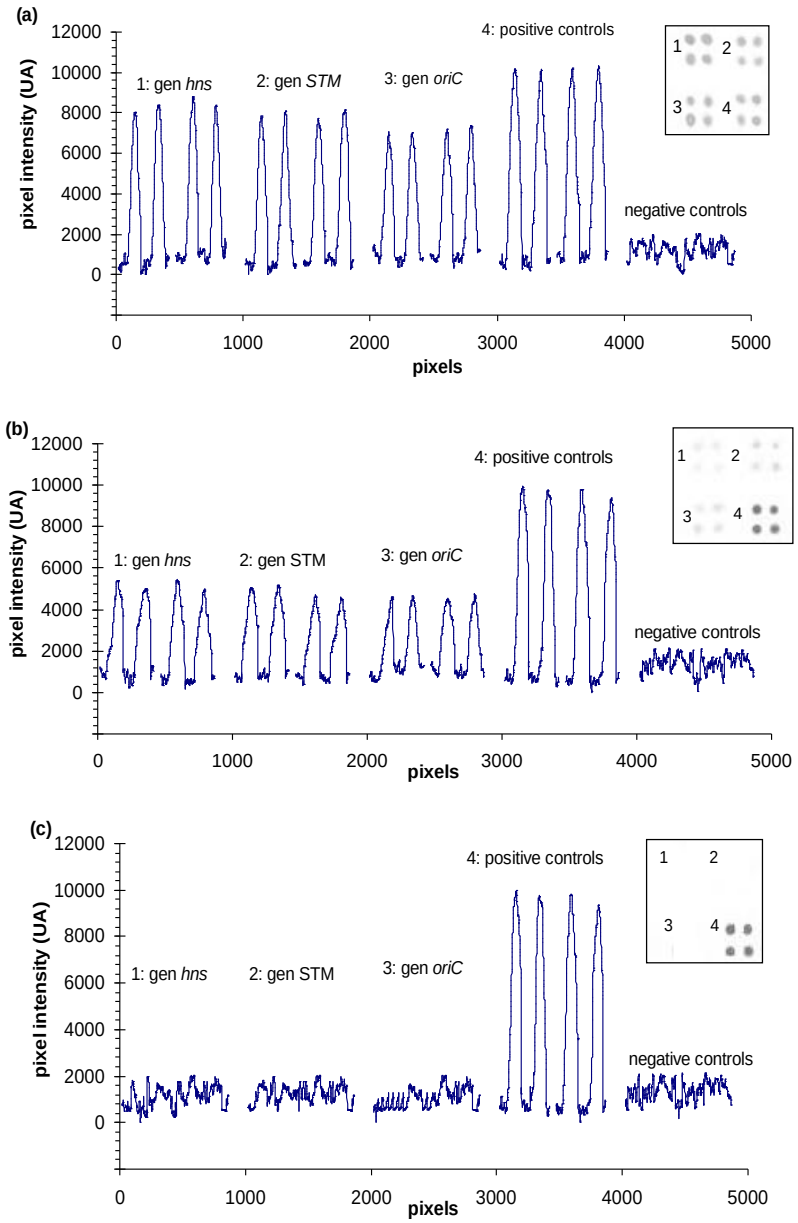


Figure 4. Smoothed profiles of spots and optical density of microarrays: (a) veterinary vaccine (dilution $1/10^5$), (b) skimmed milk (*S. llandoff* 10^2CFU/mL), and (c) skimmedmilk (*Cronobacter sakazakii* 10^2CFU/mL).

Table 3. Microarray signals and analysis results of the samples: genes *hns* and *oriC* for the generic identification of *Salmonella* spp. and *STM4057* gene for the specific detection of *Salmonella* subspecies I.

Sample	Signal			Result*		
	hns gene	*oriC* gene	*STM4057* gene	*hns* gene	*oriC* gene	*STM4057* gene
Skimmed powdered milk	305	256	1337	-	-	-
Skimmed milk	217	1511	846	-	-	-
Powered infant milk 1	584	321	298	-	-	-
Powered infant milk 2	1257	278	678	-	-	-
Powered infant milk 3	816	363	1438	-	-	-
Chicken	758	1276	314	-	-	-
Powered infant milk 3 (*C. sakazakii* 10^6 CFU/mL)	1248	800	697	-	-	-
Chicken neck skin (*S. enteritidis* 10^6 CFU/mL)	9474	8738	8172	++++	++++	++++
Human vaccine (dilution $1/10^6$)	7176	6323	6733	+++	+++	+++
Human vaccine (dilution $1/10^7$)	5948	4160	5793	++	++	++
Veterinary vaccine (dilution $1/10^5$)	8177	7698	7521	++++	++++	+++
Veterinary vaccine (dilution $1/10^7$)	3474	2131	2597	+	+	+
Skimmed milk (*S. llandoff* 10^2 CFU/mL)	3825	5335	4468	++	++	++
Powered infant milk 1 (*S. malmoe* 10^3 CFU/mL)	6375	5540	3696	+++	+++	++
Powered infant milk 2 (*S. london* 10^4 CFU/mL)	8401	5695	7612	++++	+++	++++
Powered infant milk 3 (*S. typhimorium* 10^3 CFU/mL)	6876	5612	6702	+++	+++	+++

* -: <1500; +: 1500-3500; ++: 3500-5500; +++: 5500-7500; ++++: >7500.

4. Conclusions

A simple method, an alternative to conventional tools (quantitative PCR or glass-slides microarray formats), has been developed to increase access to genomic information in non-specialised laboratories. The proposed system integrates DNA amplification and hybridisation in one process and on one platform. Moreover, the isothermal nature of the solid-phase RPA protocol simplifies the required heating system.

This is the first time that a solid-phase RPA approach in a microarray format is presented. The obtained data have shown that the system is a competitive, portable and robust sensor that integrates the amplification and hybridisation steps in a one-pot reaction to allow a multiplex analysis (e.g., number of genes and samples). It is worth mentioning that the proposed platform does not require microfluidic assemblies, which immensely simplifies the analytical process or the design/construction of the sensing platform. Furthermore, this method can be extended to other isothermal reactions (e.g., HDA), other detection approaches (e.g., membrane microarrays), or can be used to integrate microfluidic elements (e.g., reaction chambers).

The implementation of the one-pot method has been achieved for food safety control where multi-step approaches are time-consuming and prone to contamination. The reliable identification (inclusivity and exclusivity) of regulated infectious microorganisms is an important issue because disinfection techniques are target designed. It should be noted that the detection of *Salmonella* strains has been done by processing 36 samples of genomic DNA per disc in under 90 min. Despite the simplicity of the approach, the results demonstrate that this assay can be applied without compromising analytical performance and that it well suits routine genomic analysis (diseases diagnosis, bioterrorism, food safety and forensic areas).

Acknowledgements

This research has been funded through Projects GVA-PROMETEO/2010/008 (Generalitat Valenciana) and CTQ/2013/45875-R (MINECO). The Spanish Ministry of Education and Science provided S.S.F. with a grant for her PhD studies.

References

1. Zhang, C., Xing, D. *Nucleic Acids Res.*, 2007, 35, 4223-4237.
2. Zhang, Y., Ozdemir, P. *Anal. Chim. Acta*, 2009, 638, 115-125.
3. Henry, O. Y. F., O'Sullivan, C. K. *Trends Anal. Chem.*, 2012, 33, 9-22.
4. Wang, L., Li, P.C. *Anal. Chim. Acta*, 2011, 687, 12-27.
5. Carlborg, C. F., Gylfason, K. B., Kazmierczak, A., Dortu, F., Banuls, M. J., Maquieira A., Kresbach, G. M., Sohlstrom, H., Moh, T., Vivien, L., Popplewell, J., Ronan, G., Barrios, C. A., Stemme, G., van der Wijngaart W. *Lab Chip*, 2010, 10, 281-290.
6. Hoang, V. N., Kaigala, G. V., Atrazhev, A., Pilarski, L. M., Backhouse, C. J. *Electrophoresis*, 2008, 29, 4684-4694.
7. Adessi, C., Matton, G., Ayala, G., Turcatti, G., Mermod, J. J., Mayer, P., Kawashima, E. *Nucleic Acids Res.*, 2000, 28, e87.
8. Westin, L., Xu, X., Miller, C., Wang, L., Edman, C. F., Nerenberg, M. *Nat. Biotechnol.*, 2000, 18, 199-204.
9. Tillib, S. V., Strizhkov, B. N., Mirzabekov, A. D. *Anal. Biochem.*, 2001, 292, 155-160.
10. Pemov, A., Modi, H., Chandler, D. P., Bavykin, S. *Nucleic Acids Res.*, 2005, 33, e11.
11. Araujo, D. B., Langoni, H., Almeida, M. F., Megid, J. *BMC Res. Notes*, 2008, 1, 17-22.
12. Ahmad, F., Hashsham, S. A. *Anal. Chim. Acta*, 2012, 733, 1-15.
13. Andresen, D., von Nickisch-Rosenegk, M., Bier, F. F. *Clin. Chim. Acta*, 2009, 403, 244-248.
14. Zanoli, L. M., Spoto, G. *Biosensors*, 2013, 3, 18-43
15. Shin, Y., Perera, A. P., Kim, K. W., Park, M. K. *Lab Chip*, 2013, 13, 2106-2114.
16. Sabaté del Río, J., Adly, N. Y., Acero-Sánchez, J. L., Henry, O. Y. F., O'Sullivan, C. K. *Biosens. Bioelectron*., 2014, 54, 674-678.
17. Kersting, S., Rausch, V., Bier, F. F., von Nickisch-Rosenegk, M. *Malar. J.*, 2014, 13, 99-107.
18. Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldon, J. A., Puchades, R., Maquieira, A. *J. Agric. Food Chem.*, 2012, 60, 36-43.
19. Arnandis-Chover, T., Morais, S., Tortajada-Genaro, L. A., Puchades, R., Maquieira, A., Berganza, J., Olabarria, G. *Talanta*, 2012, 101, 405-412.
20. Pittoni, P. G., Chang, C., Yu, T., Lin, S. *Colloids Surf. A*, 2013, 432, 89-98.
21. Campuzano, S., Pedrero, M., Garcia, J. L., Garcia, E., Garcia, P., Pingarron, J. M. *Anal. Bioanal. Chem.*, 2011, 399, 2413-2420.
22. Neish, C. S., Martin, I. L., Henderson, R. M., Edwardson, J. M. *Brit. J. Pharmacol*., 2002, 135, 1943-1950.
23. Mirmomtaz, E., Castronovo, M., Grunwald, C., Bano, F., Scaini, D., Ensafi, A. A., Scoles, G., Casalis, L. *Nano Lett.*, 2008, 12, 4134-4139.
24. Zhang, X., Hendro, W., Fujii, M., Tomimura, T., Imaishi, N. *Int. J. Thermophys.*, 2002, 23, 1077-1090.

25. Singha, R., Das Mukherjeed, M., Sumana, G., Guptab, R. K., Soode, S., Malhotra, B. D. *Sens. Actuators B*, 2014, 197, 385-404.
26. Donhauser, S. C., Niessner, R., Seidel, M. *Anal. Chem.*, 2011, 83, 3153-3160.
27. Van Dorst, B., Mehta, J., Bekaert, K., Rouah-Martin, E., De Coen, W., Dubruel, P., Blust, R., Robbens, J. *Biosens. Bioelectron.*, 2010, 26, 1178-1194.

SUPPLEMENTARY INFORMATION

One-pot isothermal DNA amplification-hybridisation and detection by a disc-based method

Sara Santiago-Felipe, Luis Antonio Tortajada-Genaro, Sergi Morais, Rosa Puchades, Ángel Maquieira

1. Assay principle. The scheme of the assay principle is shown in Figure S-1. In the two-step protocol, the extracted DNA from the lysed cells is mixed with RPA reagents and the isothermal amplification is performed in tube. Then, recombinase enzymes facilitate the strand exchange of non-template strand and primer. Single-stranded DNA molecules are stabilized by single-stranded DNA binding proteins (gp32). Primer elongation using *Bsu* DNA polymerase I from the *Bacillus subtilis* is performed and the process is repeated yielding the amplified product. After denaturing by incubation in hybridisation buffer (95 °C), the labeled product hybridizes with the immobilized probes on streptavidin modified surface following a microarray arrangement. In the integrated protocol, the amplification mixture is dispensed over the microarray and incubated at constant temperature. The amplification process is carried out both in solution (amplification with the floating primers) and on the surface (amplification with the immobilized primer) as described above (Figure S-1). The liquid-phase amplification and the specific solid-phase elongation are combined into a one-step reaction. In this method, classified as 'active arrays', immobilized oligonucleotides participate as primers, annealing to template and being elongated by polymerase. Furthermore, since the immobilized primers are attached in microarray format, the multiplex identification of the amplified targets is possible due to their positions in the array. Therefore, the special feature of integrated assay is that the elongation process, and further detection, is directly performed on the surface. The principle of detection is described in the manuscript and represented in Figure S-2.

Furthermore, a video showing the entire protocol has been included as supplementary material (http://dx.doi.org/10.1016/j.snb.2014.07.073.).

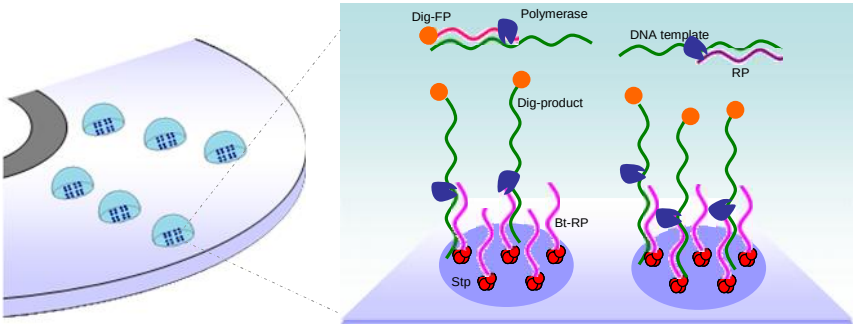


Figure S-1. Scheme of the one-pot assay: simultaneous isothermal amplification and hybridisation on DVD surface. Bt-RP: biotin-labeled reverse primer, FP: forward primer, Stp: streptavidin, Dig-PF: digoxigenin labeled forward primer, Dig-product: digoxigenin amplification product.

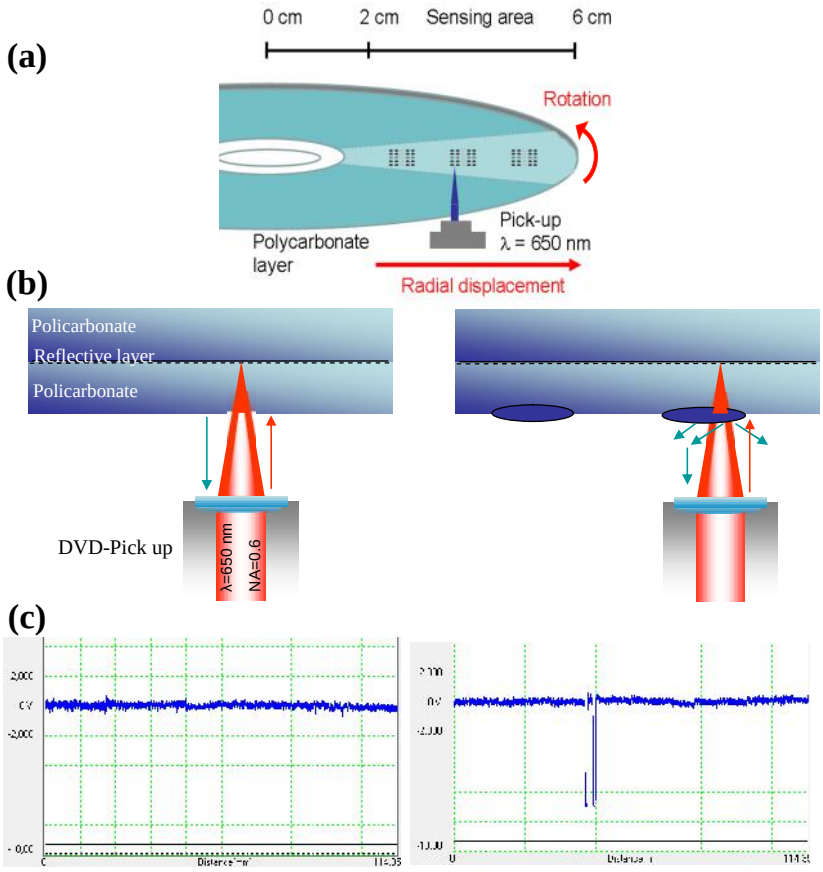


Figure S-2. (a) Scheme of DVD disc showing area available for biosensing. (b) Scheme of the scanning process of the DVD surface (transversal cross-cutting) by the DVD pick-up. Red arrows indicate the emitted laser light and green arrows the reflected/dispersed light from the scanned surface. (c) Screen shots of electronic signal collected by the data acquisition software. Baseline is registered for the spot absence and a signal variation (proportional to the density of solid deposit) is measured in the presence of the spots.

2. Samples and DNA extraction. *Salmonella* spp. strains were isolated and provided by GAIKER Technology Centre (Bizkaia, Spain) and AINIA Technology Centre (Valencia, Spain). Viable cultures were obtained overnight in nutrient agar plates (0.5% peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, pH 7) at 37 °C. Food samples bought in local supermarkets were direct analyzed or inoculated with different serotypes (*S. typhimorium*, *S. llandoff*, *S. enteritidis*, *S. malmoe*, *S. london*). Regulated microbiological standard method (ISO 6579:2003), and quantitative PCR method, indicated the presence of *S. typhimorium* in an industrial sample of chicken carcass, provided by a manufacturing company. The enrichment protocol was performed on buffered peptone water (Panreac, Barcelona, Spain). The proposed method was also used to analyze a human vaccine (Vivotif) containing *S. thyphi* and a veterinary vaccine (Avisan Secure) containing *S. typhimorium* and *S. enteritidis*. No pre-enrichment protocol was applied for vaccines. Genomic DNA was extracted from bacterial cultures, food samples, and vaccines using the DNeasy Blood & Tissue Kit (Qiagen, Inc., CA).

3. Oligonucleotides. The analytical platform was designed for simultaneous detection of specific regions in three genes: *hns* and *oriC* genes generic for *Salmonella* spp., and *STM4057* gen specific for *Salmonella* subspecies I. The oligonucleotides were provided by Eurofins (Ebersberg, Germany). Table S-1 shows the primers, probes, and controls used. Cy5-labeled oligonucleotide (reverse complementary to immobilized primer of *hns* gene) was employed as control to determine immobilisation density (control 1). Double labeled oligonucleotide (5'-biotin and 3'-digoxigenin) was spotted on discs as positive control of process (control 2). 5'-Biotin-labeled oligonucleotide with 3'-dideoxynucleotide (reverse primer of hns gene) was used as negative control of on-disc amplification (control 3). 5'-Biotin-labeled oligonucleotide was also immobilized as negative control (control 4).

Table S-1. List of oligonucleotides. FP: forward primer, R1: solution reverse primer, R2: solid reverse primer, C: controls.

Use	Target	Sequence (5'-3')	Tm (°C)	Ref
	Gene *hns*			
FP	*Salmonella* spp.	[DIG]-AACGGTGAAACTAAAACCTGGACTGG	66.2	own
FP	*Salmonella* spp.	[DIG]-TACCAAAGCTAAACGCGCAGCT	62.1	[1]
R1	*Salmonella* spp.	TTCCAGTTGCTTACCTTGTTCTTCCA	64.6	own
R1	*Salmonella* spp.	TGATCAGGAAATCTTCCAGTTGC	61.1	[1]
R1	*Salmonella* spp.	[DIG]-TGATCAGGAAATCTTCCAGTTGC	61.1	[1]
R2	*Salmonella* spp.	[BTN-TG]- TTTTTTTTTTAGGGTCGTACACCGGCTGTAATCAAA	75.9	own
R2	*Salmonella* spp.	[BTN-TG]-TTTTTTTTTTTTTGATTACAGCCGGTGTACGACCCT	75.9	own
	Gene *oriC*			
FP	*Salmonella* spp.	[DIG]-TTATTAGGATCGCGCCAGGC	59.4	[2]
R1	*Salmonella* spp.	AAAGAATAACCGTTGTTCAC	51.2	[2]
R2	*Salmonella* spp.	[BTN-TG]-TTTTTTTTTTGCTAGTGATCCTTTCCAACGCATTG	64.8	own
	Gene *STM4057*			
FP	S. subspecie I	[DIG]-GGTGGCCTCGATGATTCCCG	63.5	[3]
R1	S. subspecie I	CCCACTTGTAGCGAGCGCCG	65.5	[3]
R2	S. subspecie I	[BTN-TG]-TTTTTTTTTTGCCCGGCCTCCGGTGAAGGTAATTT	68.3	[3]
	Controls			
C1	-	[Cy5]-TTTTTTTTTTTTTGATTACAGCCGGTGTACGACCCT-[BTN-TG]	66.1	own
C2	+	[DIG]-TTTTTTTTTTTTTTTTGTCATGGGCCTCGTGTCGGAAAACC-[BTN-TG]	81.0	own
C3	*Salmonella* spp.	[BTN-TG]-TTTTTTTTTTTTTGATTACAGCCGGTGTACGACCCT-[DDC]	75.9	own
C4	-	[BTN-TG]-TTTTTTTTTTCGAAGCTGGCAACGCTACCGGTT	74.1	own

[1] Jones, D., Law, R., Bej, A. K. *J. Food Sci.*, 1993, 58, 1191-1197.
[2] Widjojoatmodjo, M. N., Fluit, A. C., Torensma, R., Keller, B. H., Verhoef, *J. Eur. J. Clin. Microbiol. Infect. Dis.*, 1994, 10, 935-8.
[3] Kim, H. K., Park, S. H., Kim, H. Y. *Appl. Environ. Microbiol.*, 2006, 72, 6142-6151.

4. Microarray layout. The simultaneous analysis of a huge number of genes, samples and replicates involved reducing printing volumes and distances between flanking spots. However, the spot density was limited by the intrinsic properties of microarray format such as the composition of printing solution related to the surface hydrophobicity or the detector resolution. In case of DVD system, the polycarbonate is a hydrophobic substrate and the optical resolution of the compact disk driver as analytical detector on polycarbonate layer is about 500 µm with a pixel resolution about 8.2 µm/pixel. Additionally, the spatial separation of primers was a compromise between minimizing the interferences in simultaneous amplification reactions and increasing the multiplexing capability.

The configuration of arrays on plastic surface was also studied. The spatial separation of droplets should prevent the cross-contamination between flanking RPA mixtures (samples). In case of DVD, the sensing surface available for immobilisation of primers is defined by the scanning area (region of disk with track structure). For a conventional compact disk, the track covers a radius range between 2 and 6 cm (Figure S-2a).

Given the dimensions of droplets (20.9 $\pm$ 0.2 mm^2) and the high analytical surface of DVD-platform ($\varnothing$ = 12 cm, approximately 5,000 mm^2), dozens of arrays can be printed allowing a high number of different targets and/or samples detected in a single assay. Nevertheless, a low-density format was chosen for this preliminary study, using of thirty-six droplets per DVD. The separation between droplets (samples or replicates) was 1 cm from centre to centre (Figure S-3a). The advantage of this configuration was that reagents were dispensed using a multi-channel micropipette (inter-tip distance of 1 cm), simplifying the working protocol.

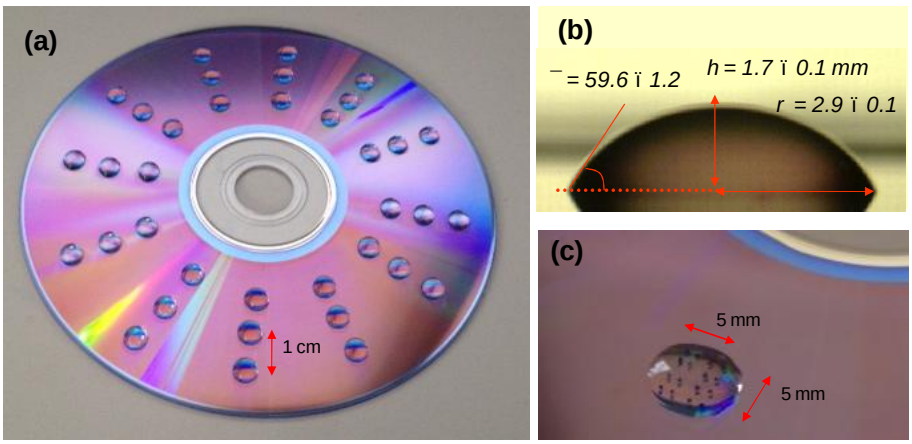


Figure S-3. (a) Image of DVD layout (36 reactions), (b) Digital microscopy image of 25 µL-droplet of RPA reaction mixture on plastic surface including droplet parameters data: θ contact angle, r droplet radius, and h droplet height (image magnification factor = 350). (c) Image of assay layout: twenty-five dots (5×5) with a track pitch (center to center distance) of 1 mm, corresponding to 9 negative spots and 16 positive spots.

5. Microarray images. Calibration curves were obtained from serial diluted pure cultures of *S. thyphimurium*. Figure S-4 shows the images corresponding to a concentration range of pathogen between 0 and 10^4 CFU/mL.

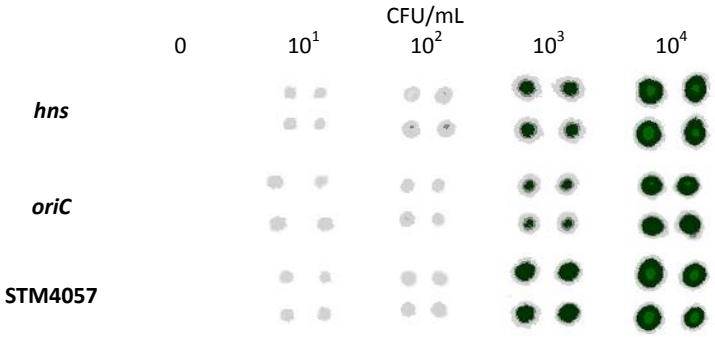


Figure S-4. Images for different target genes (0-10^4 CFU/mL).

6. AFM images. Figure S-5 shows the AFM images corresponding to a raw DVD surface, after primer immobilization, and after solid-phase RPA of a sample containing 10^2 CFU/mL. Atomic force microscopy (AFM) images were obtained with a Veeco model Dimension 3100 Nanoman from Veeco Metrology (Santa Barbara, CA) operating in tapping mode in air at room temperature.

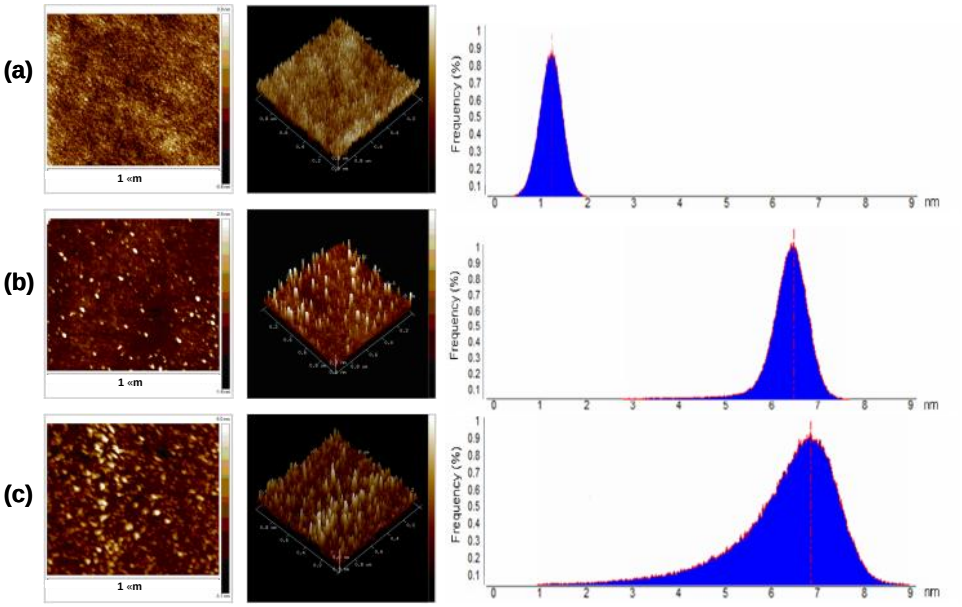


Figure S-5. AFM images of DVD surface (1 µm × 1 µm) recorded in air in 2-D representation (Left), 3-D representation (Middle), and depth histogram plots (Right): (a) raw DVD, (b) after primer immobilization, and (c) after solid-phase RPA. Pathogen concentration, 10^2 CFU/mL.

RSC Advances

PAPER

View Article Online
View Journal |View Issue

Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs)

Luis A. Tortajada-Genaro,[a] Sara Santiago-Felipe[a], Mary Amasia,[b] Aman Russom,[b] Ángel Maquieira[a]

[a] *Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain*

[b] *Div. of Nanobiotechnology, KTH Royal Institute of Technology, Stockholm, Sweden.*

*Reproduced from **RSC Advances, 2015, 5, 29987-29995**, with permission from The Royal Society of Chemistry.*

Abstract

A new advancement in massive DNA-based screening in limited-resource settings is demonstrated through the incorporation of easy-to-fabricate microfluidic chambers on digital versatile discs (DVDs) to perform isothermal recombinase polymerase amplification (RPA) in a microarray format. Standard un-modified DVD discs and commercial drives are used for the low-cost detection method. DNA primers were printed in a microarray format on the polycarbonate surfaces of DVDs with integrated control spots to guarantee the absence of false-negatives and false-positives. The solid-phase amplification assay, including the washing protocols and development reaction, was performed by the dispensation of solutions through the inlet and by controlling the flow-movement by DVD drive centrifugation. The final disc with reaction products was inserted into a DVD player and microarray images were captured and automatically processed. This

simple approach was applied for the screening of genetically modified organisms (GMOs) in food samples. The limit of detection was 7 μg/g, which is well below the EU regulation limit for GMOs in food products. Therefore, the only required materials for food safety monitoring were standard store-bought DVDs, plastic chambers, tips, pipettes, an oven, and a standard DVD drive. The proposed strategy allows an integrated microarray system with low manipulation, reduced sample volume, and portability, which are beneficial for low-resource settings.

1. Introduction

The development of DNA biosensors is related to the adequate selection and integration of support, probes, assay format, and transduction phenomena that are needed to perform and detect a biorecognition. Unlike silicon chips, plastic polymers, as analytical platforms, offer the advantages of being transformed easily and cheaply into devices that combine operations of sample treatment, fluid management, and detection.[1] However, in some cases, the proposed platforms are not useful for real applications because the systems for fluid management and signal detection are not easily adaptable to a wide range of scenarios.

Several research groups and companies have been working on the development of biosensors based on the use of compact discs or 'lab-on-a-CD' systems. There are two main approaches depending on the nature of the disc used.[2] The first one involves the use of plastic substrates that are several mm thick with circular shapes, which are integrated in a microfluidic system (such as, microchannels, valves, and chambers).[3,4] Some of them, known as micro-total analysis systems (μTAS), integrate all the analytical steps that are required for genomic assays.[5] Other lab-on-a-CD devices present a lower integration level, and the procedures involve some handling steps.[6] In both the cases, the measurement is generally performed with instruments, such as colorimeters or expensive static detectors such as fluorescence microscopes or other complex non-integrated systems.

The second approach directly uses audio-video compact discs as the support for carrying out bioassays, and the detection is based on the scanning of a focused laser that is present in conventional disc drives.[7] The main advantage of these technologies (namely, CD, DVD, and Blu-Ray) is that they are mass-produced for the consumer electronic market with highquality standards and cost-effective prices. Our group has demonstrated that it is possible to use a CD player/writer as a detector, using low-reflective discs (transmission/reflection mode) or conventional discs (reflection mode); moreover, the chemical modification of the surfaces can be incorporated. These systems show a higher sensitivity and

working capacity (e.g. multiplexing), allowing the implementation of extremely inexpensive optical devices for biological applications.[8-10]

The development of DNA hybridization assays has been addressed using centrifugal disc platforms. The procedures include flow hybridization in various types of reservoirs, such as double-spiral,[11] channel,[12,13] or chamber,[14] and all of them were combined with fluorescence detection. An interesting approach is the integration of isothermal amplification and fluorescent real-time detection with commercially available centrifugal disc and analyzer.[6] Moreover, hybridization assays on a microarray format have been performed based on DVD[9] and BD[10] technology (e.g., disc and detector). The experimental steps are similar to those when glass or other solid supports are used. A distinct advantage is that the use of expensive and bulky scanners, which typically image the spots, is avoided, paving the way for the widespread use of the microarray technology. Nevertheless, integrated approaches are required to reduce the number of steps and the manipulation of samples.

In a recent study, Santiago-Felipe *et al.*[15] demonstrated the advantages of isothermal recombinase polymerase amplification (RPA) combined with DVD hybridization and detection using a technique called as solid-phase amplification. In this approach, one primer is attached onto the polycarbonate surface of a DVD (bottom layer), while the other amplification components are retained in the liquid phase. The polymerase extension of the primer produces a tethered and detectable amplification product. The results have shown that this can be a new strategy to integrate amplification and hybridization into a same platform at a constant low temperature, avoiding the use of devices with technologically complex heating/cooling systems. On the other hand, Russom *et al.*[16] have shown how merging optical discs and microfluidics is a new step towards the low-cost point-of-care applications. Low reflectivity DVDs were fabricated from 0.6 mm DVD substrates, including a spiral groove of 0.74 mm track pitch, which was coated with a 10 nm thick layer of SiO_2. The microfluidic layers, containing microchannels and other fluidic reservoirs, were incorporated over the metallic layer of the discs (top layer). Then, the integration of assay development, controlled by spinning rate, and reading (transmission mode) in only one platform

was achieved. The system was applied to low-cost HIV diagnostics by counting CD4+ cells isolated from whole blood. However, this approach requires some disc modifications and incorporation of a planar photodiode into the DVD drive to detect the transmitted light.

In this study, a semi-automated DNA assay in microarray format is proposed based on the integration of a simple adhesive microfluidic layer onto the polycarbonate surface of conventional DVDs (bottom layer). Primers are pre-printed onto the DVD surface, and lyophilized RPA reagents are stored within a spinning platform. The rest of the reagents are hand-dispensed and the solid-phase amplification is performed, resulting in the attachment of the amplified products onto the DVD surface. After the development of the amplification products, the microfluidic layer is removed and the disc is read by reflection mode (i.e., conventional DVD drive). The presence of the amplification product modifies the light intensity of the scanning laser of the DVD drive (reflection mode), and using a data acquisition software, a microarray image is generated. As proof of concept, the method has been applied for the low-cost, reliable, rapid screening of genetically modified organisms (GMOs).

2. Methods

2.1 Target genes. GMO testing was based on the determination of several genetic elements. Screening elements were the two most common transgenic genes (namely, 35S-promoter from cauliflower mosaic virus, or p35S, and nopaline synthase terminator, or tNOS), allowing the detection of most of the authorized or unauthorized lines.[17,18] Taxon-specific elements detect genes that are specific for plants, such as lectin (*Le1*) for *Glycine max* (soybean), alcohol dehydrogenase 1 (*adh1*) for *Zea mays* (maize), and LAT52 protein (*LAT52*) for *Solanum lycopersicum* (tomato). These elements increase the characterization of involved transgenic ingredients, allowing increased selectivity for certain GMOs.[19] Construction-specific elements are included for the complete identification of GMO events such as Bt-11 construction, which involves a junction region between the intron 6 (IVS6) from a maize alcohol dehydrogenase 1 gene (*adh1-1S*) and a synthetic *cryIA(b)* gene.[20]

2.2 Integrated DVD system design. The DVD-based bioanalytical platform consists of two disc substrates (namely, optical layer and microfluidic layer) bonded together (see supplementary information). The optical layer substrate used is a standard store-bought DVD-ROM disc purchased from MPO Iberica (Spain). According to the DVD specifications, a standard blank disc is composed of two 0.6 mm thick polycarbonate substrates with a middle layer of highly reflective metallic material (thickness 1,000-1,500 Å). The polycarbonate layer at the bottom had an injection molded spiral microguide (track pitch of 0.74 mm) to guide the subsystems of the detector laser (λ=650 nm) along the data track.

The microfluidic substrate was fabricated using a 0.2 mm pressure-sensitive adhesive (PSA) (adhesive transfer tape 91022, 3M, USA) bonded to a disc-shaped piece of polycarbonate plastic (thickness=0.6 mm) with drilled access through-holes (diameter=1 mm). Ten identical fluidic structures, containing microfluidic components, including channels and reservoirs, were radially arrayed to enable multiplexed assays on a single disc using a CO_2 laser cutter (Hylax Hypertronics). A scheme of this device is shown in Figure 1 with details of the two chambers and channel locations. Each structure has a chamber for pre-amplification mixing (Figure 1A, Chamber 1) and a chamber for solid-phase amplification and detection (Figure 1A, Chamber 2). The dimensions of the chambers are 5.5 mm in length, 5.5 mm in width and 0.2 mm in height, and therefore can contain a sample volume of 6 μL. The two chambers are connected by a 0.6 mm wide mixing channel as the hydrophobic valve. The disc was designed to enable these simple fluidic steps at low spinning rates (<1,500 rpm) that are achievable in commercial DVD drives.

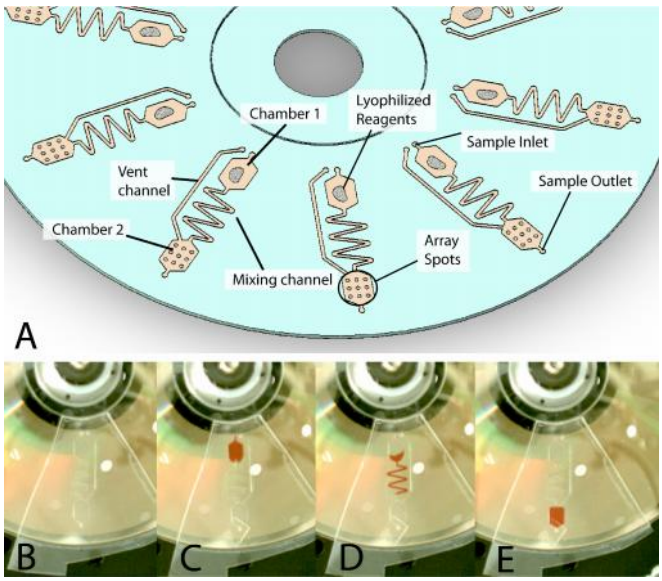


Figure 1. (A) Schematic of the two-chamber microarray DVD substrate (B) Single device affixed to DVD surface, with overhanging plastic tab for easy user removal (C) Sample is loaded into Chamber 1. Lyophilized reagents are reconstituted upon contact with liquid. (D) Spinning the DVD at 2,000 rpm opens capillary valve and transfers the sample through a mixing channel into Chamber 2. (E) After spinning for 20 seconds, the sample is fully transferred into Chamber 2 where solid-phase amplification and detection occurs.

Standard commercial DVD-ROMs were firstly conditioned by gentle ethanol washing, water rinsing, and drying by centrifugation. Biotinylated primers (Table S-1, see supplementary information) were immobilized on passively adsorbed streptavidin. For immobilization, each mixture of streptavidin (5 mg/L) and biotinylated-labelled primer (100 nM) in 50 mM carbonate buffer, pH 9.6, and 1% glycerol (v/v) was printed on a polycarbonate disc surface (50 nL) with a non-contact printer (AD 1500 BioDot Inc., CA). Working temperature and relative humidity were adjusted at 25 °C and 90%, respectively. Because this arrayer is traditionally used for printing on standard glass slides, a custom printing-layout was developed to print multiple arrays. The printing area for each chamber was 4 × 4 mm^2 with an allowance for minor misalignment with the printer. In a single run, 10 arrays of 9 spots (3 × 3) for primers, negative controls, and positive controls were spotted in the Chamber 2 region with a 1 mm track pitch. Pre-stored lyophilized reagents (0.8 mg) for amplification were dispensed into

Chamber 1 with a spatula. The reagent mixture composed of 2 mM DTT, 5% Carbowax 20 M, 200 mM dNTPs, 3 mM ATP, 50 mM phosphocreatine, 100 ng/µL creatine kinase, 30 ng/µL Bsu, and recombinase proteins (namely, 900 ng/µL gp32, 120 ng/µL uxsX, and 30 ng/µL uvsY) was supplied by TwistDx (UK). Finally, the microfluidic layer was aligned with the DVD-ROM surface and affixed to enable a bubble-free flat disc. The outer holes were sealed with PCR sealer tape (Corning, USA), and the disc was stored at -20 °C until use.

2.3 DVD drive. The assay performed on the disc was controlled and measured by an adapted DVD drive from LG Electronics Inc. (Englewood Cliffs, USA). The device incorporated a data acquisition board (model DT9832A-02-OEM, Data Translation, Germany) with a sample rate of up to 2 megasamples per second. A standard DVD drive has a motor to rotate the disc, an optical system with a laser (λ=650 nm), and a servo focus/tracking system to centre and focus the beam on the spiral track. For the acquisition of data stored on the track, the laser scans the entire disc surface and reads the reflected intensity. Our device takes advantage of these components in two ways. First, the DVD drive is used as a centrifuge, which controls the spinning rate of the disc. Second, the drive is used as a detector, which captures the signal variation during surface scanning due to the presence of biochemical interactions (solid products).

The performances of the optical disc drive were controlled by customized software, written in Visual C++, running on a laptop connected to it through a universal serial bus (USB 2.0) interface. During the disk scanning, only the signals coming from selected areas are processed for digitization and stored in the computer (5 MB size file). The signals for each track and microarray are deconvoluted into an image. The image analysis of the microarray was also performed by the software.

2.4 Samples and extraction of genomic DNA. The certified reference materials (CRMs) were purchased from the Institute for Reference Material and Measurements (Belgium). Food products were bought from local stores. For genomic DNA extraction, aliquots of 5-20 g of the homogenized sample were

extracted using a GMO-extraction kit based on column purification according to the manufacturers' instructions (Applied Biosystems, Spain). The extracted DNA was quantified by spectrophotometry (NanoDrop 2000/2000c, Thermo Scientific Inc., USA), and stored at -20 °C until analysis.

2.5 Amplification and development. Each sample was analyzed in quintuplicate, including positive and negative amplification controls. Solid phase RPA was performed on the disc (10 reactions per disc, see supplementary information). Reaction mixtures (6 µL) contained 480 nM of each 5'-digoxigenin labelled primer (Table S-1, see supplementary information), 5 ng of genomic DNA, 14 mM of Mg-acetate, and 1× rehydration buffer. Denhardt's reagent (2.5×, Life Technologies, Spain) was also added to reduce the non-specific background. Subsequently, the mixtures were dispensed with a micropipette onto the corresponding microfluidic structure through the inlet holes. The outer hole was sealed with PCR sealer tape (Corning, USA). Then, the disc was inserted into the DVD player and a slow spin was performed (<600 rpm for 10 s) to lead the reaction mixture to Chamber 1. After a proper reconstitution and mixing of the reagents, the spin was increased to 1,000 rpm to fully move the sample into Chamber 2, covering the pre-printed array. Subsequently, the disc was introduced into a container (standard DVD plastic box) in a water-saturated atmosphere, and the solid-phase amplification reactions were carried out at 37 °C for 40 min in an oven (model UF30, Memmert, Germany). After removing the sealer tape, the chamber was emptied by spinning at 1,000 rpm for 10 s. The dispensation of solutions and reagents were carried out through the inlet with the movement of solutions controlled by centrifugation as described above. The array was washed by dispensation of 0.1× washing solution (SSC, 1× saline sodium citrate, containing 150 mM of NaCl and 15 mM of sodium citrate, pH 7) and water through the inlet holes (6 µL). The chamber was emptied by spinning at 1,000 rpm for 10 s. The detection was carried out using a mixture of anti-digoxigenin antibody produced in sheep (1/4,000) and anti-sheep conjugated with horseradish peroxidase (1/500) in PBS-T (phosphate buffered saline and 0.05% (v/v) Tween 20, pH 7.4) (6 µL). The developing reagent was 3,3',5,5'-tetramethylbenzidine (TMB) (6 µL). The array

was washed with PBS-T plus deionised water as described above. Finally, the fluidic layer was removed and the disc was inserted into the DVD player.

2.6 DVD surface scanning. Because the inner structure of the optical disc remained unaltered, the microarrays on the surface were correctly read. The disc was scanned at a rotation speed of 4× ≡ 13.46 m/s and the signal was acquired at 26 dB gain and 1.7 megasamples/s with a reading time lower than 10 min. Then, 10 microarray images were created (tagged image file format, grey-scale with 16 bit-colour depth, scale 0-65,535). Optical intensity signals of each spot, corresponding to the amount of reaction product, were quantified. In the absence of a solid biorecognition product, the reflection properties of the disc surface were unchanged and the beam intensity collected by the DVD drive was at the maximum, corresponding to the background signal of the image. However, when the laser hit the deposit of TMB product, the intensity of the laser beam that reached the photodiode decreased, corresponding to a signal for the microarray spot. In this configuration, the mean intensity of each spot was 1963 pixels (spot diameter = 500 mM). After subtracting the local background, any spot displaying a signal-to-noise ratio higher than three was considered to be positive. The reading and image processing (i.e., feature gridding, addressing, segmentation, and quality assurance) was automatically performed in less than 15 min by the disc. Used discs were discarded following the same laboratory safety guidelines as those for ELISA plates.

2.7 Complementary measurements. Digital images of the fluidic process, shown in Figure 1B-E, were obtained using a custom visualization stand composed of a motor (EC-Max 40 Series DC) and controller (ESCON 5/50) supplied by Maxon (USA) and a CMOS camera (uEYE camera model UI-3360CP) supplied by IDS (Germany).

Amplification products recovered from the reaction chamber were checked by electrophoresis on a 3% (w/v) agarose gel at 110 V and room temperature. Gels were stained for 30 min with 0.5× TBE buffer (Tris/Borate/EDTA) containing the fluorophore RealSafe (Real Laboratories, Spain) at 0.01% (v/v), and bands were

visualized with a UV transilluminator. Product size was determined by comparison with a 50 bp ladder (Fermentas, Lithuania).

3. Results and discussion

3.1 Microfluidic DVD characterization. A simple system composed of a reaction/detection chamber (microarray) connected by a channel to a loading chamber for the dispensation of reagents was designed (Figure 1). Fluid propulsion on the DVD disc platform was achieved through controlling the centrifugally induced pressure on the fluid as the disc spins.[21] The flow rate was dependent mainly on the rotational speed of the disc, the location of the fluid, the geometry of the fluidic channels, and the specific fluidic properties (namely, density, viscosity, and surface energy). Through the utilization of combinations of different channel geometries and spin speeds, precise flow rates ranging from nanoliters to millilitres per second were modulated. The fluidic valves were designed for use in the 0-1,500 rpm range to allow for easy automation within a standard DVD drive.

Process monitoring was performed with a custom visualization stand for the optimization of the process. Regarding the reagent loading, the images showed that the fluidic samples were perfectly introduced into the loading chamber (Chamber 1 of Figure 1C) and held in place via a capillary valve. A critical challenge studied in the development of an integrated system was the storage of amplification reagents and their release. The microfluidic structure included a hydrophobic valve to allow the rehydration of reagents after sample loading, and the active life of the in-disc reagents was verified for at least two weeks.

After the initial set of tests, a serpentine mixing channel was designed to ensure the complete mixing of the lyophilized reagents with the inserted sample before they reached the reaction chamber. The selected dimensions were a width of 600 μm and a total linear length of 38 mm containing six elbows. With this configuration, the capillary valve was open when the disc was spun at 2,000 rpm for 20 seconds, and the sample was fully transferred into the reaction chamber (Chamber 2 of Figure 1D and E). The entire reaction chamber was completely

covered with solutions. No formation of bubbles or other artefacts were observed.

In addition, the microfluidic structure was designed for washing the reaction chamber by controlling the disc rotation. To characterize the optimum washing speeds, the flow rate through the structures was modelled using the governing equation [Kellogg] as follows:

$$Q = A\frac{D_h{}^2 \rho \omega^2 \bar{r} \Delta r}{32 \mu L}$$

where D_h is the hydraulic diameter of the channel, ° is the liquid density, μ is the angular velocity of the spinning CD, $\bar{r}$ is the average distance of the liquid element from the CD centre, a*r* is the radial extent of the fluid, μ is the fluid viscosity, and *L* is the length of the liquid in the channel. The hydraulic diameter of the channel, D_h is defined as 4*A/P*, where *A* is the cross-sectional area of a rectangular channel and *P* is the wetted perimeter of the channel. In repeated trials using a 600 μm serpentine channel, the washing liquid was fully transferred in 6 seconds at 500 rpm, corresponding to a flow rate of 1 μL per second. This result closely matches the calculated theoretical flow rates when the channel design specifications are known.

3.2 Optimization of the solid-phase RPA format. The isothermal solid-phase amplification was addressed according to the assay that was recently published based on a heminested mechanism.[15] It consisted of immobilising primers, following a microarray layout, on the support of the reaction chamber. Other amplification components in the liquid phase were dispensed from the loading chamber. After the reaction chamber was filled, the amplification was produced in the static mode, i.e. rotation was not carried out during the reaction. As unbound primers were added to the reaction mixture, the amplification was produced in both the phases (in liquid and on the surface). Then, centrifugal microfluidics was just used for the semi-automated reconstitution of lyophilized reagents, for mixing them with samples and their dispensation, minimizing the contamination and increasing the reproducibility of assays. Nevertheless, merging DNA assays on

microfluidic discs required the selection of robust chemistry that is able to resist the solution flow. Three processes -sensitive to flow action- were studied: (i) immobilization of primers, (ii) washing protocols, and (iii) developing reaction.

In regards to the anchoring of primer, an indirect adsorption mode was chosen based on streptavidin/biotin recognition. The main advantage was the simplicity of the process because the microarraying of biotinylated primers and streptavidin could be directly performed on bulk discs without previous surface treatment of DVD or blocking steps. Under these working conditions, immobilized primers were resistant to a flow up to 13,200 nL/s (at 2,000 rpm). Moreover, the amplification process on solid supports depended on the immobilization density of the primer. To that end, coating conditions were optimized by varying the streptavidin concentration from 5 to 20 ppm and the primer concentration from 50 to 200 nM. The highest signal was obtained for a primer concentration of 100 nM (0.06 fmol/mm^2) (Figure 2A).

The set-up of post-amplification steps was important for the integration of the assay in the microfluidic device. Compared to PCR mixtures, an RPA mixture is a highly viscous solution with a higher number of components, making the washing protocol more crucial. After the solid-phase amplification, the rest of the reaction components must be effectively removed without the release of the products that are immobilized on the disc surface of the reaction chamber. The composition of washing solutions was optimized in conventional DVDs -without microfluidic structures- by controlling parameters such as pH, ionic strength and astringency. SSC buffer and PBS-T buffer were selected for washing after the amplification process and after the incubation of antibodies, respectively. In the microfluidic discs, two protocols were assayed for both the post-amplification and the post-antibody incubation steps. In the static protocol, referred as 0 rpm, the washing method was based on completely filling the array chamber, incubation, and fast removal of the liquid. In the in-flow washing method, the washing buffers were passed continuously through the array (disc spin 0-1,500 rpm). Because the in-flow washing protocol significantly decreased the signal, the static protocol was chosen (Figure 2B). Then, the washing cycles (1-5) were studied for both the washings (namely, post-amplification and post-antibody incubation). Figure 2C

shows that a higher number of washing cycles produced a significant decrease in the signal due to the release of the product.

Two signal enhancement reactions were compared for developing the digoxigenin-labelled products that were immobilized in the reaction chamber. The first was a metallographic reaction based on the dispensation of anti-digoxigenin antibody produced in a rabbit (1/7,500 dilution) and anti-rabbit antibody conjugated with gold (1/100 dilution) using silver as the developing reagent. The second approach consisted of an enzymatic reaction that dispensed anti-digoxigenin antibody produced in sheep (1/4,000 dilution) and anti-sheep conjugated to horseradish peroxidase (1/500 dilution), followed by the addition of TMB as the developing reagent.

Both reaction sequences (namely, metallographic and enzymatic approaches) produced a detectable precipitate that modified the laser intensity of the DVD drive (λ=650 nm) during the disc scanning (Figure 2D).

However, the metallographic reaction provided high and variable background signals, which resulted in low and irreproducible signal-to-noise ratios and false-positives, which increased the number of washing cycles as well. The unusually high background signal observed during metallographic developing may be due to an incomplete washing of components present in the RPA mix (such as carbowax and proteins) or developing reagents, i.e. antibodies, which could enhance the reduction of silver, leading to nonspecific depositions. Because the enhancement reaction using TMB was less sensitive to the rest of the RPA components (absence of nonspecific signal), the enzymatic reaction was selected.

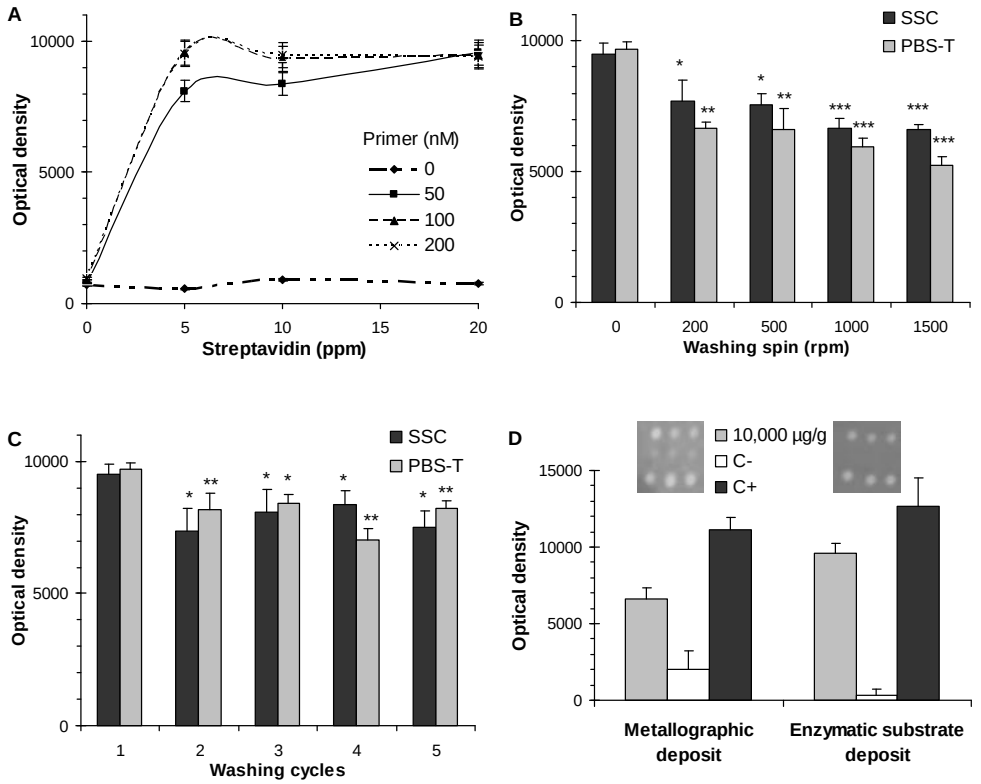


Figure 2. Results from the amplification of p35S gene from Bt-11 maize at 1%: (A) Effect of coating conditions (streptavidin and primer concentration) on the signal intensity. (B) Effect of washing RPMs with the SSC and PBS-T buffers on signal intensity; *P<0.05, **P<0.01, ***P<0.001; one-way ANOVA (C) Effect of washing cycles with the SSC and PBS-T buffers on signal intensity; *P<0.05, **P<0.01; one-way ANOVA (D) Comparison between developing reactions.

3.3 Analytical performances for GMO detection. The method was applied for the screening of GMOs including screening, plant-specific, and construction-specific elements. The increasing production of transgenic crops and the concern regarding the safety of derived foods has led to the extensive monitoring of foodstuff that could contain GMOs. Therefore, the development of low-cost, reliable, rapid analytical methods for their detection and quantification through the entire production chain is of great importance.

The sensitivities of the assays were determined in two ways: by analyzing serially diluted genomic DNA (10-fold dilutions) and by analyzing samples with

different concentrations of transgenic ingredient. For the first approach, the concentration of DNA dispensed into the inlet chamber was varied from 100,000 μg/g (10%) to 0 μg/g (0%). An excellent correlation between the concentration of the transgenic template and the optical intensity measured by the DVD detector was found (r^2=0.976) (Figure 3).

For the second approach, the sensitivity of the method was assessed by simultaneously determining samples with decreasing concentrations of transgenic foods in relation to non-GMOs foods from 0% (w/w) to 10% (w/w). A *t*-test revealed that there was no significant difference between the slopes of the calibration curves (*p*-value = 0.806 > 0.05).

The detection limits (LODs) were calculated as the lowest amount of DNA that is able to produce a signal that could be distinguished from the blanks (NTC: control solution without template or food without transgenic ingredient). All the target genetic elements were detected at a concentration of 110-460 μg/g (0.011-0.046%).

The estimation of the sensitivity of the method in terms of copy numbers can be made theoretically as described in ref. 20. Taking into account the genome size of the plants and considering them in their haploid form, 100 ng of DNA would contain 36630 copies of the genome for the maize and 88496 copies for the soybean. The limit of detection of 0.01% GMO in that amount of genomic DNA (100 ng) would then correspond to about 3.7-8.8 haploid copies of target sequences for the different plant species. These results are similar to or better than others obtained by rt-PCR (1-16 copies)[22] or by a PCR-microarray (37-88 copies).[20] Thus, the system can reliably comply with the legal requirement of 0.9% limit of detection set by the EU.

Assay reproducibility, expressed as relative standard deviation (RSD), was determined from the optical density of spots from samples analyzed in triplicate, each in three DVDs. The intra-day RSD varied from 3.0% to 6.7%, whereas the inter-day RSD varied from 5.4% to 11.4%.

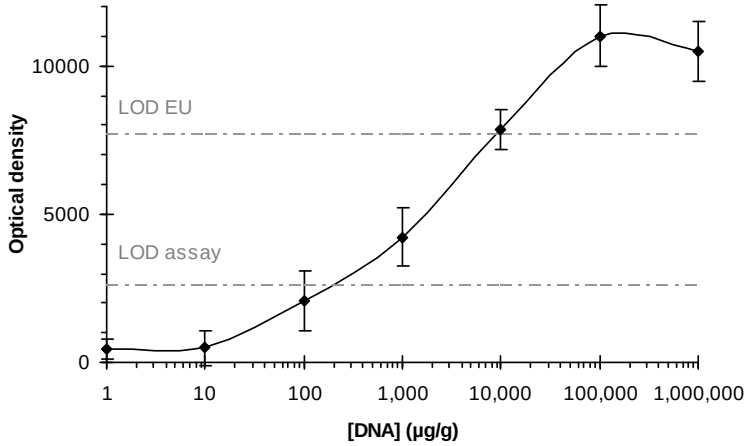


Figure 3. Calibration curve of GMO detection on the microfluidic disc (five replicates from Bt11 maize). Dashed line above indicates the labeling limit regulated in EU (the most restrictive worldwide GMO regulation) and the lower, the limit of detection of the assay. Positive samples between the two limits could be detected although the labeling is not mandatory in the EU.

3.4 Detection of GMOs in certified reference material and food samples. The capabilities of the method were evaluated by detecting GMOs in 7 certified reference materials: maize Bt176 (ERMBF411), maize Bt11 (ERM-BF412b and ERM-BF412f), maize GA21 (ERM-BF414b), maize MON810 (ERM-BF413ck), maize Bt176 (ERM-BF411) and Roundup ReadyTM Soya (ERMBF410dk). All the elements of the different GMOs were detected, and they corresponded to the expected patterns (Table 1). The four lines of transgenic maize provided positive responses for the maize-specific gene (namely, *adh1* gene) and negative for the rest of the taxon probes. The transgenic line of soybean (RRS) provided positive responses for the soybean-specific gene (*Le1* gene) and negative for the rest of the taxon probes. Screening elements and construct-specific elements were correctly detected in all the cases and no cross-contamination between adjacent chambers was observed (n=50). A sample was considered positive when the optical response was higher than the cut-off value (namely, an optical density of 2550).

Table 1. Detection patterns obtained (5 replicates): Certified reference materials and food samples.

Sample	Certified/Declared GMO content	DVD Detection					
		Taxon			Screening		Construction
		Le1	*adh1*	*LAT52*	p35S	tNOS	Bt-11
CRM Bt-176 maize	2.00 ± 0.11 %	-	+	-	+	-	-
CRM Bt-11 maize	0.98 ± 0.29 %	-	+	-	+	+	+
CRM Bt-11 maize	4.89 ± 0.21 %	-	+	-	+	+	+
CRM GA21 maize	0.10 ± 0.08 %	-	+	-	-	+	-
CRM MON810 maize	5.00 ± 0.11 %	-	+	-	+	-	-
CRM RRS soya	10.00 ± 0.10 %	+	-	-	+	+	-
Tomato NahG	Presence	-	-	+	++	++	-
Feed	Presence	-	+	-	++	++	++
Cookies	No declared	+	+	-	+	-	-
Ketchup	No declared	-	+	+	-	-	-
Soy sauce	No declared	+	-	-	-	-	-
Soy sauce+RRS	0.5%	+	-	-	+	+	-
Sweet corn	No declared	-	+	-	-	-	-
Sweet corn+MON810	0.1%	-	+	-	+	-	-
Cereals baby food	No declared	-	+	-	-	-	-
Noodles	No declared	-	-	-	-	-	-

The method was also applied for the detection of food samples. Figure 4 shows an example of the optical signals registered by the DVD drive. As can be seen, samples containing GMO ingredients were detected because positive responses were observed in the corresponding spots. Table 1 proves that positive results were observed in all the cases for the analytes declared, even at trace levels, or in spiked samples. Negative results were found in most of the samples declared to be analyte-free. The only exception was for cookie samples, in which despite not having any declared GMOs, positive results for p35S were obtained. This can be explained because its concentration might be lower than 0.9% (EU regulation).

The reliable and sensitive results achieved indicate that the proposed method is useful for the detection GMO in routine food-safety monitoring.

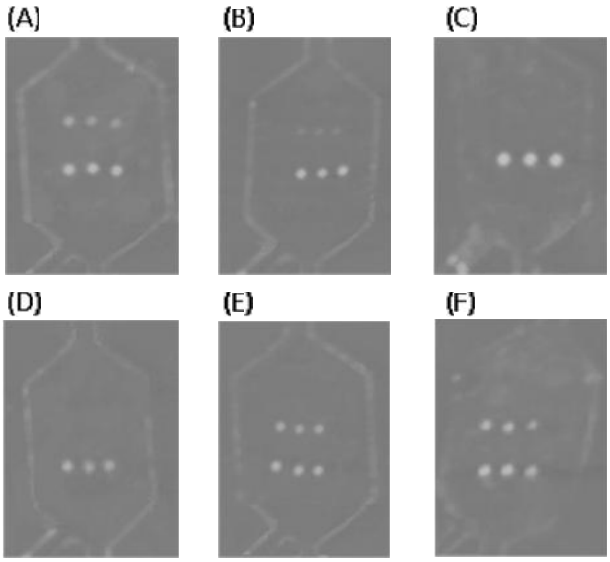


Figure 4. Examples of 3×3 microarray DVD image results obtained from six food samples: (A) tomato (target p35S), (B) feed (target BT11), (C) noodles (target *Le1*), (D) sweet corn (target tNOS), (E) cereals baby food (target *adh1*), and (F) ketchup (target *LAT52*). Upper row: specific probes, middle row: negative controls, bottom row: positive controls.

4. Conclusions

Screening protocols require analytical platforms with properties such as high working capacity, sensitivity, and reliability. This study is aligned with recent investigations that are focused on merging optical discs and centrifugal microfluidics to create a new step towards increased automatization, reduced sample consumption and low cost diagnostics. The proposed system integrates microfluidic chambers on digital versatile discs (DVDs) to perform an isothermal DNA solid-phase amplification in a microarray format. The novelty is that microfluidics is in the bottom layer of the DVD and the measurement is performed by reflection using a standard DVD player (which has small dimensions, light weight, and connectability to internet or telephone network device). Developed discs, due to their properties such as high mechanical resistance, good thermal stability and hydrophobicity, have demonstrated to be an excellent option as a bioanalytical platform. The prospective costs of the system (<2€ per disc and <500€ per reader) are below that of the state-of-the-art equipment, i.e. qPCR

plates and fluorescence-thermocyclers. In addition, the method is easy to operate by locally trained staff and requires inexpensive and unspecific equipment (namely, extraction columns, pipettes, oven, DVD drive, and laptop). The properties of the proposed system make it suitable to be applied in a wide-range of ambits such as low resource settings, satellite/decentralized laboratories and production plants.

As proof-of-concept, the device was applied in the detection of GMOs because their reliable identification is an important issue as their labelling is legally regulated. Screening methods are especially needed due to the high number of samples and genes to be controlled. This low-cost technology for semiquantitative analyses has shown excellent analytical performances (namely, selectivity, sensitivity, reproducibility, and high throughput). The integration of the amplification and hybridisation steps into a one-pot reaction allows the processing of the samples in less than 90 min, reducing manipulation, reagent consumption, and risks of cross-contamination. Despite the simplicity of the approach, the results demonstrate that this screening assay can be applied without compromising analytical performance and that it suits routine genomic analysis.

Acknowledgements

This research has been funded through the projects FP7 Digital sequencing (European Commission), PAID-00-12 (UPV, GVA), FEDER PrometeoII/2014/040 (GVA) and CTQ/2013/45875 R (MINECO). The Spanish Ministry of Education and Science provided S.S.F. with a grant for her PhD studies.

References

1. Haeberle, S., Mark, D., von Stetten F., Zengerle, R. *Microsystems and Nanotechnology*, Springer Berlin Heidelberg, 2012, pp. 853-895.
2. Nolte, D. *Rev. Sci. Instrum.*, 2009, 80, 101101.
3. Vázquez, M., Brabazon, D., Shang, F., Omamogho, J. O., Glennon, J. D., Paull, B. *Trends Anal. Chem.*, 2011, 30, 1575-1586.

4. Ducree, J., Haeberle, S., Lutz, S., Pausch, S., von Stetten, F., Zengerle, R. *J. Micromech. Microeng.*, 2007, 17, S103-S115.
5. Roy, E., Stewart, G., Mounier, M., Malic, L., Peytavi, R., Clime, L., Madou, M., Bossinot, M., Bergeron, M. G., Veres, T. *Lab Chip*, 2015, 15, 406-416.
6. Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle R., von Stetten, F. *Lab Chip*, 2010, 10, 887-893.
7. Hua-Zhong, Y., Yunchao, L., Ou, L. M. L. *Acc. Chem. Res.*, 2013, 46, 258-268.
8. Morais, S., Tortajada-Genaro, L. A., Arnandis-Chover, T., Puchades, R., Maquieira, A. *Anal. Chem.*, 2009, 81, 5646-5654.
9. Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldón, J. A., Puchades, R., Maquieira, A. *J. Agric. Food Chem.*, 2012, 60, 36-43.
10. Arnandis-Chover, T., Morais, S., González-Martínez, M. A., Puchades, R., Maquieira, A. *Biosens. Bioelectron.*, 2014, 51, 109-114.
11. Peng, X. Y., Li, P. C. H., Yu, H., Parameswaran, M., Chou, W. L. *Sens. Actuators, B*, 2007, 128, 64-69.
12. Li, C., Dong, X., Qin, J., Lin, B. *Anal. Chim. Acta*, 2009, 640, 93-99.
13. Jia, G., Ma, K., Kim, J., Zoval, J. V., Peytavi, R., Bergeron, M. G., Madou, M. *Sens. Actuators, B*, 2006, 114, 173-181.
14. Rupp, J., Schmidt, M., Münch, S., Cavalar, M., Steller, U., Steigert, J., Stumber, M., Dorrer, C., Rothacher, P., Zengerle, R., Daub, M. *Lab Chip*, 2012, 12, 1384-1388.
15. Santiago-Felipe, S., Tortajada-Genaro, L. A., Morais, S., Puchades, R., Maquieira, A. *Sens. Actuators, B*, 2014, 204, 273-281.
16. Ramachandraiah, H., Amasia, M., Cole, J., Sheard, P., Pickhaver, S., Walker, C., Wirta, V., Lexow, P., Lione, R., Russom, A. *Lab Chip*, 2013, 13, 1578-1585.
17. Elenis, D. S., Kalogianni, D. P., Glynou, K., Ioannou, P. C., Christopoulos, T. K. *Anal. Bioanal. Chem.*, 2008, 392, 347-354.
18. Querci, M., van den Bulcke, M., Žel, J., van den Eede, G., Broll, H. *Anal. Bioanal. Chem.*, 2010, 396, 1991-2002.
19. Marmiroli, N., Maestri, E., Gullì, M., Malcevschi, A., Peano, C., Bordoni, R., De Bellis, G. *Anal. Bioanal. Chem.*, 2008, 392, 369-384.
20. Hamels, S., Glouden, T., Gillard, K., Mazzara, M., Debode, F., Foti, N., Sneyers, M., Esteve-Nuez, T., Pla, M., Berben, G., Moens, W., Bertheau, Y., Audéon, C., Van den Eede, G., Remacle, J. *Eur. Food Res. Technol.*, 2009, 228, 531-541.
21. Gorkin, R., Park, J., Siegrist, J., Amasia, M., Lee, B. S., Park, J. M., Kim, J., Kim, H., Madou, M., Cho, Y. K. *Lab Chip*, 2010, 10, 1758-1773.
22. Cottenet, G., Blancpain, C., Sonnard, V., Chuah, P. F. *Anal. Bioanal. Chem.*, 2013, 405, 6831-6844.

SUPPLEMENTARY INFORMATION

Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs)

Luis Antonio Tortajada-Genaro, Sara Santiago-Felipe, Mary Amasia, Aman Russom, Ángel Maquieira

1. Disc structure. The DVD-based bioanalytical platform consists on a multi-layer disc, as it is described in Figure SI.1. The bottom layer is a standard DVD disc, composed of two thick-polycarbonate substrates and a middle layer of highly reflective metallic material. The upper layer is the microfluidic substrate composed by pressure sensitive adhesive foil and a disc-shape polycarbonate plastic. The holes, channels, and reservoirs have been fabricated on this substrate. Finally, the inlet holes (sample/reagent loading) and outlet holes (waste removing) are sealed with an inert tape.

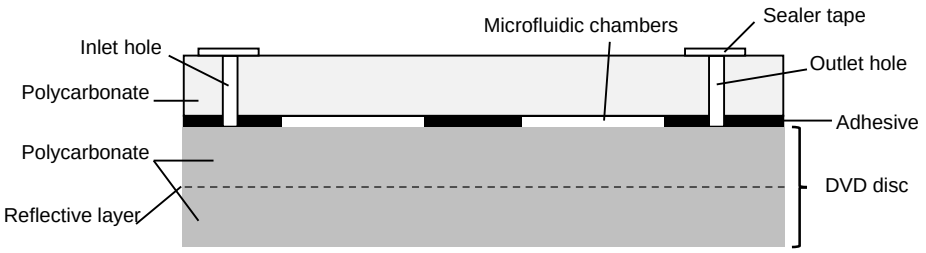


Figure S-1. Scheme of the optical disc containing microfluidic structures.

2. Solid-phase amplification on disc. The isothermal amplification was based on solid-phase recombinase polymerase amplification (RPA) in the reaction chamber. For that, the forward primer (FP-I) was immobilized on the DVD following a microarray format via streptavidin-biotin interaction. The sample and other reaction components were dispensed in the liquid phase to the reaction chamber by centrifugation. This reaction mixture included digoxigenin-labelled forward primer (Dig-FP-S) and the reverse primer (RP). Table S-1 shows the oligonucleotide sequences of primers used.

Table S-1. List of oligonucleotides. RP: reverse primer, FP-I: forward primer immobilized on DVD surface, FP-S: forward primer in solutions, C: controls.

Gen	Use	Sequence 5'-3'	Len	Tm	ref
Control	C-	[BtnTg]-T10-AGGGTCGTACACCGGCTGTAATCAAA	46	75.9	own
	C+	[DIG]-T10-TTTTGTCATGGGCCTCGTGTCGGAAAACC-[BtnTg]	40	81.0	own
Endogenous					
Le1	FP-I	[BtnTg]-T10-CGAAGCTGGCAACGCTACCGGTT	33	74.1	[a]
Le1	FP-S	[DIG]-TCCACCCCCATCCACATTT	19	59.2	[a]
Le1	RP	GGCATAGAAGGTGAAGTTGAAGGA	24	58.8	[a]
adh1	FP-I	[BtnTg]-T10-CCTCACCAGTTACGAAACCAATCGATCCAA	35	67.1	own
adh1	FP-S	[DIG]-CGTCGTTTCCCATCTCTTCCTCC	23	64.2	[a]
adh1	RP	CCACTCCGAGACCCTCAGTC	20	63.5	[a]
Lat52	FP-I	[BtnTg]-T10-ACTCTCTTTGCAGTCCTCCCTTGGG	25	66.8	own
Lat52	FP-S	[DIG]-AGACCACGAGAACGATATTTGC	22	58.4	[b]
Lat52	RP	TTCTTGCCTTTTCATATCCAGACA	24	57.6	[b]
Screening					
p35S	FP-I	[BtnTg]-T10-TATAGAGGAAGGGTCTTGCGAAGGATA	35	64.8	own
p35S	FP-S	[DIG]-CCACGTCTTCAAAGCAAGTGG	21	59.8	[c]
p35S	RP	TCCTCTCCAAATGAAATGAACTTCC	25	59.7	[c]
tNOS	FP-I	[BtnTg]-T10-GCGTATTAAATGTATAATTGCGGGACT	27	63.7	own
tNOS	FP-S	[DIG]-GCATGACGTTATTTATGAGATGGG	24	59.3	[c]
tNOS	RP	GACACCGCGCGCGATAATTTATCC	24	64.4	[c]
Construct					
Bt-11	FP-I	[BtnTg]-T10-TTCTGGGTTACTCAAGCAGTTGTATGG	30	66.6	own
Bt-11	FP-S	[DIG]-AAAAGACCACAACAAGCCGC	20	58.4	[a]
Bt-11	RP	CAATGCGTTCTCCACCAAGTACT	23	62.9	[a]

[BtnTg]: Biotin with triethylene glycol spacer; T10: Thymine tail (10 nucleotides); [Dig]: digoxigenin

Then, the initial amplification (in solution) was followed by the extension of immobilized primers (solid-phase reaction) and the formation of attached

digoxigenin-labelled product (Dig-product). As the FP-I was designed to be specific for a region located within the first product, the heminested approach led to a shorter immobilised product than that formed in the liquid phase. Selectivity and sensitivity of this amplification method is improved compared to conventional solid-phase approaches [d]. Figure S-2 shows the amplification principle on disc.

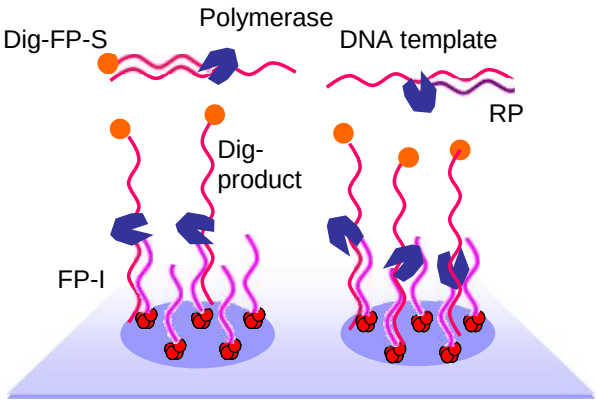


Figure S-2. Scheme of solid-phase isothermal amplification on disc based on RPA.

[a] International Organization for Standardization, Geneva, Switzerland (2005) ISO 21570:2005. Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-quantitative nucleic acid based methods

[b] Yang, L., Pan, A., Jia, J., Ding, J., Chen, J., Cheng, H., Zhang, C., Zhang, D. *J. Agr. Food Chem.*, 2005, 53, 183-190.

[c] International Organization for Standardization, Geneva, Switzerland (2005) ISO 21569:2005. Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-qualitative nucleic acid based methods.

[d] Santiago-Felipe, S., Tortajada-Genaro, L. A., Morais, S., Puchades, R., Maquieira, A. *Sensor. Actuat. B-Chem.*, 2014, 204, 273-281.

analytical chemistry Article
pubs.acs.org/ac

Parallel solid-phase isothermal amplification and detection of multiple DNA targets by DVD technology

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Rosa Puchades, Ángel Maquieira

Instituto interuniversitario de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain

Submitted to Anal. Chem., September 2015

Abstract

An integrated methodology based on a centrifugal microfluidic platform is proposed for parallel detection of multiple DNA target sequences using a standard digital versatile disc (DVD) technology. Microstructures embedded on a DVD disc allow samples and reagents management using both capillary effect and centrifugal force induced by disc rotation. Recombinase polymerase amplification (RPA), in bridge solid phase format, takes place into separated compartments, modifying their optical properties. Then, the DVD drive registers the modifications of the beam intensity emitted by disc drive and transmitted through the platform. The proposed strategy allows tens of genetic determinations simultaneously, in less than 2 h, with small sample volume (3 µL), low manipulation and cost. This easy-to-use method was applied to high-throughput screening of relevant safety threats (allergens, GMOs and pathogenic bacteria) in food samples, reaching satisfactory results in terms of sensitivity (48.7 fg of DNA) and reproducibility (below 18%). The developed platform enables cost-effective multiplex amplification/detection useful for a highly demanded field such the massive screening of specific nucleic acid targets.

1. Introduction

Multiplex PCR is one of the most used technologies for detecting DNA because of its capability of simultaneously amplifying different targets in a single reaction.[1] Furthermore, many of the most established high-throughput methods are based on its combination with oligonucleotide microarrays.[2] However, because of the intrinsic interference and competition between primer pairs, the targeted sequences are seldom amplified equally, even resulting in the absence of some products in the final mixture. Thus, multiple rounds of optimization are required resulting in time-consuming and labor-intensive protocols.[3]

To overcome this limitation, several strategies, such as algorithms for primer design or the use of universal primers, have been proposed.[1] However, the better results have been achieved by physically isolating the primer pairs, the compartmentalization of the amplification reactions being the most innovative approach.[1,4] In the literature, the generation of water-in-oil droplets, named digital-PCR, or the fabrication of reactors have been proposed to isolate the reagents, allowing to simultaneously increase the number of assays, improve the amplification efficiency, and reduce the reaction volumes.[1,5,6,7] However, the current developed systems may present several drawbacks. First, the high temperature requirements of PCR may form gas bubbles, leading to volume variations and amplification failures. Second, most employ fluorescent detection or perform it outside of device, requiring cost hardware equipment or labor consuming procedures to recover the amplified products before detection. In addition, some present complex fabrication manifolds or equipment, such as external valves and pumps.[1,6,8]

Solid-phase amplification approaches have been a promising alternative to avoid post-amplification treatments of the PCR products. Here, one or both primers are grafted on a solid support while keeping other reaction components in the liquid phase. Enzymatic extension of the primers directly produces a tethered amplification product onto the substrate surface. This format provides several advantages such as spatially resolved products, high throughput, ease of operation and specific detection.[9] On the other hand, the introduction of 3D chips has demonstrated to provide higher surface-density ratio, increasing the primers

immobilization density and enhancing target accessibility. However, an imprecise heat distribution can lead to lower amplification efficiency.[2] Examples of 3D systems include gel-based chips from polyacrilamide gel pads, conical dendrons, micropillars, single threads, bead microarrays or microreactors.[2,6,11]

Isothermal amplification methods, where nucleic acids are synthesized at constant temperature, can overcome the above discussed thermal constraints. Among the developed reactions, the recombinase polymerase amplification (RPA) has many interesting properties, such as short reaction time (20-60 min), robustness, and low energetic requirements (close to room temperature). Several researches demonstrate that RPA is readily compatible with integrated systems such as solid-phase amplification combined with silicon microring resonance,[12] lateral flow strips,[14] electrochemical detection modes,[13] or microfluidic devices based on fluorescence detection.[15]

Recently, our research group has developed two approaches that perform heminested solid-phase RPA (sp-RPA) in 2D-chip format using compact disc technology as support and detector. In the first study, spherical sessile droplets were dispensed onto the surface of the disc to simultaneously detect three genes of *Salmonella* spp.[16] In the second approach, single detection of genetically modified organisms (GMOs) was performed using microfluidic system, fabricated in pressure-sensitive adhesive, bound to a DVD.[17]

In this study, a support with 3D structures, composed by channels and wells embedded on a standard DVD disc, is presented for parallel DNA detection. The proposed device combines the use of centrifugal microfluidics to automatically meter, split and dispense reagents, with the detection by a DVD drive. The main challenges were the fabrication of these structures, keeping the performances of the optical disc, and the development of a high-multiplexing DNA assay.

As proof-of-concept, five food safety threats have been simultaneous detected. The selected targets are intensively controlled along the food production chain, according to the recommendations of Food and Agriculture Organization and the regulations of many countries. Peanut is representative example of the allergens included in worldwide priority lists.[20] Promoter 35S and construction Bt-11 are used for screening authorized or unauthorized GMOs and

for the identification of specific GMO events, respectively.[17] *Salmonella* spp. and *Campylobacter* spp. are among the most relevant pathogens due to their high prevalence and toxic effects in humans.[20]

2. Methods

2.1 Oligonucleotides and samples. The specific primers of selected genes are shown in Table 1. All the primers were successfully checked for reliable inclusivity (specific target analyte detection) and exclusivity (lack of interference from non-target analytes).

Salmonella Typhimurium group B (CECT 443) and *Campylobacter jejuni* (CCUG17696) were used as reference strains. Bacterial isolation and inoculation assays were prepared as described by Santiago-Felipe *et al*.[20] The certified reference materials (CRM), containing 0.05% of transgenic Bt11 maize (ERM-BF412f) and 0.01% of transgenic RRS soybean (ERM-BF410gk), were purchased from the Institute for Reference Material and Measurements (Geel, Belgium). Food products were bought in local stores. Genomic DNA was extracted from bacterial cultures and food samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc., CA).

Table 1. Primers sequences used for amplification procedures

Target (*gen*)	Use	Sequence 5'-3'	Tm (°C)	Ref.
Peanut	FP	BtnTg-T10-CTAGTAGCCCTCGCCCTTTT	53.8	21
Ara h2	RP	BtnTg-T5-AGTTCCCACTGCTGCCTC	52.6	
GMO promoter	FP	BtnTg-T10-CCACGTCTTCAAAGCAAGTGG	54.4	22
35S	RP	BtnTg-T5-TTATATAGAGGAAGGGTCTTGCGAAGGATA	58.9	
GMO construction	FP	BtnTg-T10-AAAAGACCACAACAAGCCGC	51.8	23
Bt-11	RP	BtnTg-T10-TTCTGGGTTACTCAAGCAGTTGTATGG	58.2	
Salmonella spp.	FP	BtnTg-T10-TGATCAGGAAATCTTCCAGTTGC	53.5	16
hns	RP	BtnTg-T10-AGGGTCGTACACCGGCTGTAATCAAA	59.5	
Campylobacter spp.	FP	BtnTg- T10-CACGTGCTACAATGGCATAT	49.7	This work
16S rDNA	RP	BtnTg- T10-GGCTTCATGCTCTCGAGTT	51.1	

FP: forward primer, RP: reverse primer, BtnTg: biotin labelled

2.2 Fabrication of microstructured DVDs. The analytical platform was composed by DVD disc with the microfluidic structures and a sealing layer. A computer numerical control drilling machine (Bungard CCD, Karo 5410, Germany) was used to hole the channels and microwells on a store-brought DVD-R discs thought the upper polycarbonate (PC) layer. The feed speed and rotational rate of the tungsten carbide drill were 2,000 mm/s and 48,000 rpm, respectively. The precision of the fabrication process was ± 0.1 µm. Fluidic structures were holed radially (three radius) to enable eighteen assays on a single disc (Figure 1). Each structure has a comb shape to aliquot the reagents solutions to five microwells. The aliquoting structure is formed by inlet chamber (1.5 mm in diameter and 0.4 mm in depth) and a primary channel (1 mm in width and 0.4 mm in height) connected with the microwells by other five distribution channels acting as hydrophobic valves (5 mm in length, 0.8 mm in width and 0.4 mm in height). The lengths of primary channel were 35, 45 and 50 mm, giving volumes of 17, 21, and 23 µL, for the internal, middle and external radius, respectively. The microwells were drilled at 35 mm, 45 mm and 55 mm respect to the disc center and their dimensions were 2 mm in diameter and 1.1 mm in depth.

The sealing layer was fabricated using pressure sensitive adhesive (PSA) (ARcare 90445, Adhesives Research, Ireland) affixed to an acetate sheet, including access and emptying through-holes. The structures in PSA foil (0.08 mm depth) were made using a cutter-plotter (Graphtec, Japan-Graphtec CE-2000), replicating shapes performed in the DVD disc. Instead of the microwells, square chambers (3.2 mm × 3.5 mm) were holed.

The fabrication uniformity and quality of the microstructured disc were checked using Dino-Lite Digital Microscope (BigC.com, California) with a resolution of 1.3 megapixel (1280x1024 pixels), and a SMZ-1B Stereoscopic Microscope (Nikon Corporation, Japan) with a total magnification between 16-70x and real field of 13.1-3 mm. Surface contact angles were calculated from dispensing microdrops at room conditions by quintuplicate.

Before layer bounding, biotinylated primers were immobilized on passively adsorbed streptavidin on the microwell surface. For that, a mixture of streptavidin (20 mg/L) and forward and reverse primers of a single targeted gene (200 nM) in

50 mM carbonate buffer at pH 9.6, was dispensed in each microwell (2.5 µL) and incubated overnight at 4 °C. Microwells were washed with PBS-T (phosphate-buffered saline containing 0.05% (v/v) tween 20, pH 7.4), plus deionised water. Then, the sealing layer was aligned to the coated DVD and affixed to enable a bubble-free flat disc. Assembled discs were stored at -20 °C.

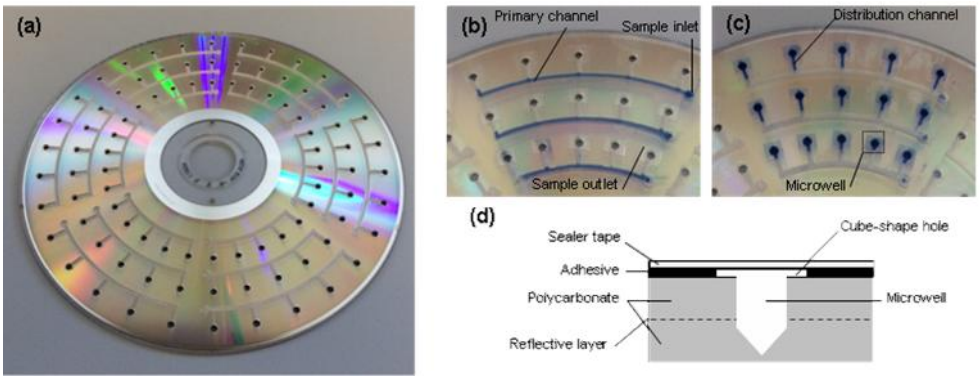


Figure 1. (a) Picture of the microfluidic DVD platform for 18 samples (5 genes) (b) Picture of three samples loaded into primary channel through the inlet hole. (c) Picture of three samples after spinning the DVD at 500 rpm that opens capillary valve and the samples are aliquoted into parallel microwells, where solid-phase amplification and detection occurs. (d) Scheme of the microwell embedded into the multi-layer system of a standard DVD. Note: the buffer was colored with blue ink to achieve a higher quality of the photographs.

2.3 Assay principle. The parallel isothermal amplification is based on an innovative assay called bridge solid-phase RPA (bridge sp-RPA), performed in a centrifugal microfluidic disc. The sample and other reaction components are dispensed thought the inlet hole. The disc is designed to enable simple steps at low spinning rates (< 1,500 rpm), easily achieved by the stepper motor integrated in commercial DVD drives. As the disc spins, each sample simultaneously splits into five aliquots and is dispensed into the microwells for parallel amplifications. Hence, each specific reaction takes place into a physically separated compartment.

In the proposed solid-phase amplification format, a nucleic acid bridge is formed between two extended strands.[24,25] For that, forward and reverse primers are 5'-end immobilized on the microwell, allowing the 3'-end free for DNA

synthesis (Figure 2). The genomic DNA hybridizes with the immobilized primers and the recombinase enzymes and single-stranded DNA binding proteins (gp32) facilitates the strand exchange of non-template strand and primer. Then, primers are elongated by *Bsu* DNA polymerase I, forming surface bound amplified products. These surface bound copies can also hybridize to other attached primers in the vicinity, allowing bridge-formation amplification. The generated products, labeled during the amplification and developed by an immunoenzimatic reaction, are detected by a photonic system based on DVD technology (disc and drive).

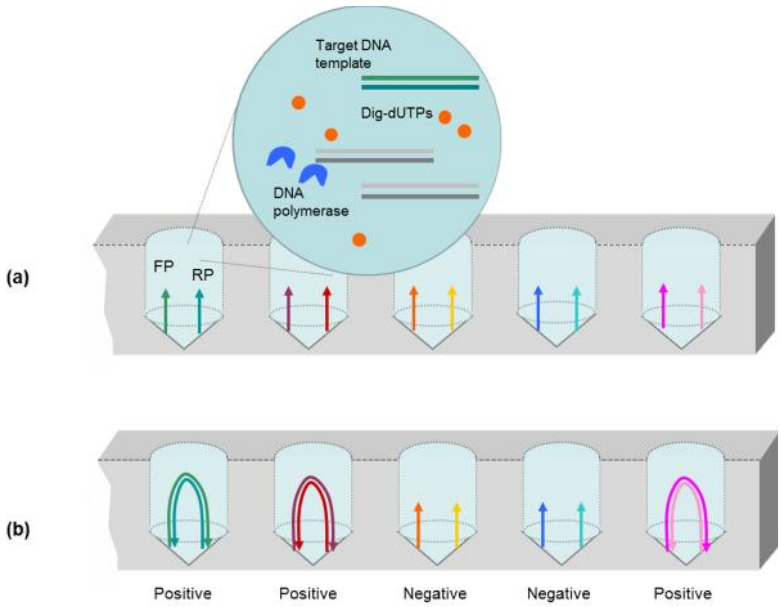


Figure 2. Schematic representation of the amplification assay (bridge sp-RPA). (a) Initial microwells embedded on DVD disc containing tethered primers for five targeted genes. Insert shows reaction mixture containing sample with different DNA molecules (targets or not) and RPA reagents. (b) Final microwells with the immobilized products (bridge structures). Dig-dUTPs: digoxigenin labeled deoxynucleotide, FP: forward primer, RP: reverse primer

According to the specifications, a standard DVD disc is composed of two 0.6 mm thick- PC substrates, with a middle layer of highly reflective metallic material (thickness 1,000 - 1,500 Å). The bottom PC layer has an injection molded spiral microguide (0.74 mm track pitch) across the whole disc for surface scanning and

data storage. The microwells are fabricated to cross the metallic layer from the top PC substrate; meanwhile none of other microfluidic structure went through it. Then, the laser beam intensity emitted by the DVD pick-up is only transmitted through the transparent microwells. The disc surface scanning is possible because the DVD drive contains an optical system with a laser (λ=650 nm) and a servo focus/tracking system to center and focus the beam on the spiral track. In addition, the DVD drive integrates an error correction system to avoid burst errors caused by imperfections on the disc surface like scratches or fingerprints. Thus, the presence of microwells does not disturb the disc scanning, and the signal variation, due to the development of biochemical interactions, is performed during a conventional disc reading.

2.4 Assay protocol: amplification and development. The amplification mixtures, developing reagents and washing solutions were dispensed with a micropipette on the primary channels through the inlet hole (17-23 µL). The stepper motor of DVD drive rotated the disc, controlling the spinning rate and the reagent flow to the microwells.

Amplification enzymes, nucleotides and buffer (TwistDx, UK) were reconstituted, mixed with 10 µM digoxigenin-dUTPs and 6 ng of genomic DNA from the extracted sample, and loaded into the disc. The outer holes were tape sealed (Corning, USA) and slowly spun (500 rpm for 3 s) by the DVD drive to lead the solutions to microwells. Next, the disc stored into a water-saturated atmosphere container (standard DVD plastic box) was introduced in an oven (model UF30 Memmert, Germany) to carry out solid-phase amplification reactions at 37 °C for 45 min. After removing the sealer tape, the emptying of the microwells was done by spinning at 1,000 rpm during 5 s by the DVD drive. The microwells were washed three times with 0.1× washing solution (SSC, 1× saline sodium citrate: NaCl 150 mM, sodium citrate 15 mM, pH 7) and water, by dispensation and emptying through centrifugation as described above.

The enzymatic developing reaction was a mixture of anti-digoxigenin antibody produced in sheep (1/4,000) and anti-sheep conjugated with horseradish peroxidase (1/500) in PBS-T. After a washing step with PBS-T and deionised water,

a mixture of 3,3',5,5'-tetramethylbenzidine (TMB) (0.25 g/L) and hydrogen peroxide (0.002 M) in citrate buffer at pH 5.5, was dispensed and incubated at room temperature for 10 min.

2.5 Assay protocol: DVD reading and data analysis. The equipment consisted in a conventional DVD drive (LG Electronics Inc., USA) provided with an upper planar photodiode (model SLCD-61N2, Silonex, USA) and a data acquisition board (DAQ, model USB-2527, Measurement Computing Corporation, USA). The planar photodiode (5.1 mm × 5.1 mm) had a spectral sensitivity of 0.55 A/W at 940 nm, a spectral range between 400 and 1100 nm and an acceptance half-angle of 60°. The photonic system detected the transmitted laser beam and converted it into an analog electrical signal. Then, the DAQ digitized the analog signals from the detection areas and transferred them to the computer for processing.

The disc was scanned at a rotation speed of 4× (equivalent to 13.46 m/s) with an acquisition signal of 26 dB gain and a sample rate up to 1.7 Msps. Then, microwells images were created (tagged image file format, grey-scale with 16 bit-colour depth, scale 0-3) and the optical intensity signals related with the amount of reaction product. In absence of reaction, the maximum intensity of transmitted beam is collected by the photodiode (background signal). When the laser hit the colored product, the intensity of laser beam that reached the photodiode decreased, corresponding to the signal of the microwell. Used discs were discarded following the same laboratory safety guidelines than ELISA plates.

The optical disc drive control (centrifugation and detection) and the image analysis were performed by custom software, written in Visual C++, running on a laptop connected to it through a USB2.0 universal serial bus interface. The reading and image processing (feature gridding, addressing, segmentation, and quality assurance) was automatically done in 14 min by disc. An assay was considered positive when the optical response was higher than the cut-off value, calculated as three times the standard deviation of the negative control.

Statistical package Statgraphics Centurion for Windows v. 16.1.15 was used for data analysis.

3. Results and discussion

3.1 Set-up of the microfluidic disc. The properties of the microfluidic structure (microwells and channels) were optimized to obtain proper aliquoting, low unspecific adsorption, and maximum response signal.

For the design of microwells inside DVD, several diameters (up to 4 mm), depths (600 to 1100 µm) and shapes were tested using a blue-dye solution. The optical signal registered by the photodiode detector was similar for different diameters, but values higher than 2 mm leaded a reading error caused by the loss of the disc track during DVD reading. Meanwhile, the measured signal increased in relation to the microwell depth due to the optical pathway variation (Figure 3a). The best results were achieved by a microwell (2 mm in diameter) with a conic shape on bottom (height 400 µm), cylinder on top (height 700 µm), and a cube-shape hole in the sealing layer (height 800 µm), so the total volume of each compartment was 3.2 µL (Figure 1d). This design probably provided the best thermal transfer between solution, support and environment. The proposed system showed relevant advantages compared to previous approaches based on assays performed on the disc surface.[26] The optical path length was longer and as the reflective metallic layer was crossed, the total laser intensity from the DVD pick up focused in the solution.

The distance between microwells was chosen considering that the error correction system of a DVD can read up to a burst of defective 2,900 bits. To ensure an appropriate tracking during DVD reading, a simple platform was fabricated with 30 microwells per radius (6 samples×5 genes), the distances between microwells being 7, 9 and 11 mm for the internal, middle and external radius, respectively.

The comb structure (inlet hole, primary channel and distribution channels) was designed to hold the loading reagents, distribute the solution, and fill all the microwells. Regarding the flow reagent movements, the condition for spontaneous filling or valving is highly sensitive to materials and the aspect ratio of the structures.[27] The proposed channels had a rectangular shape, composed of PC in three walls and acetate film in the top, the contact angles being $\theta_{PC} \sim 90°$ (hydrophobic) and $\theta_{acetate} \sim 60°$ (hydrophilic), respectively. Assuming a constant

cross-section, the dimension restrictions of the microchannel, height (H) and width (W), can be expressed as:[28]

$$\frac{W}{H} \geq \frac{-2 \cdot \cos^{-}_{PC}}{\cos^{-}_{acetate} + \cos^{-}_{PC}}$$

This equation reveals that wider and shallower channels are more favorable for liquid filling. The selected dimensions of the primary channel were 1.1 mm in width, 400 µm in height, and 35-50 mm in length, depending on radius. The distribution channels were 5 mm in length, 8 mm in width and 400 µm in height. Under these conditions, buffered solutions were loaded in the primary channel through the inlet, spontaneously moved forward along by capillary action, and stopped at the junctions of the narrower distribution channels (Figure 1b). In this point, the surface tension turned out to be a retarding force that kept the fluid from moving forward, acting as capillary valve.

The flow rate through the structures, controlled by disc rotation, was modeled as described by Tortajada-Genaro *et al*.[17] Taking into account the characteristics of the centrifugal platform and the fluid, a rate of 500 rpm was chosen, reaching a flow rate of 1.00 ± 0.13 µL per second. The spinning of the DVD allowed the liquid to overcome the capillary pressure barrier, pushing the fluid into the distribution channels. The samples were then completely transferred to microwells in less than 3 seconds, and the excess volume remained in the distribution channels (Figure 1c). Compared to other microfluidic devices fabricated using hydrophobic substrates, no bubbles or other artifacts were observed.[29] There was no preferential distribution among channels or ratios, since all samples were uniformly and simultaneously dispensed to the microwells.

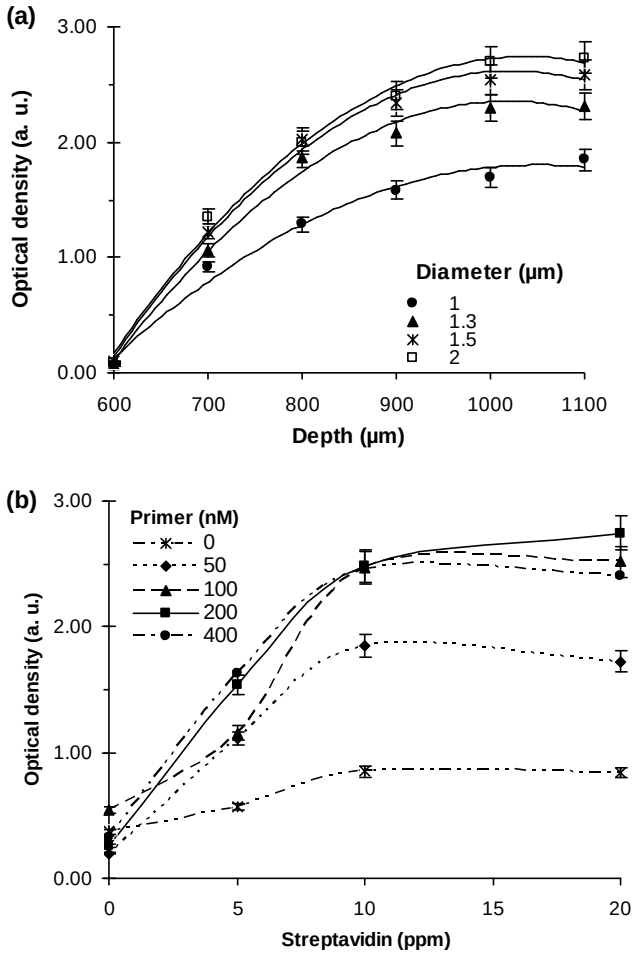


Figure 3. (a) Effect of the microwell dimensions on optical intensity. (b) Effect of coating conditions (streptavidin and primer concentration) on signal intensity. Results from the amplification of p35S gene from Bt-11 maize at 1%.

3.2 Optimization of assay format. Several reaction formats for solid-phase amplification are compatible with high-throughput screening, using the developed platform. A first option was to attach one primer in the microwell and add the other to the reaction mixture. However, the unspecific formation of primer dimers can take place, limiting the multiplexing capabilities. The attenuation of possible non-specific byproducts is a crucial factor for isothermal amplification reactions and in particular for those working at lower temperatures where

mismatches are more likely to occur.[32] For that, a bridge solid-phase approach was selected, where both primers are attached on the support.

The amplification process on solid supports highly depends on the immobilization density of the primer.[31] An indirect adsorption mode was chosen based on the streptavidin/biotin recognition without previous surface treatment or blocking steps. Coating conditions were optimized by varying the streptavidin concentration from 5 to 20 ppm, and the biotinilated-primer concentration from 50 to 400 nM. The highest signal was obtained for a primer concentration of 200 nM, yielding a density of 1.2 fmol/mm^2 (Figure 3b). Higher concentrations led a decrease of signal probably due to steric hindrance, which can reduce the hybridization of DNA in solution and/or the attachment of the DNA polymerase to the tethered oligonucleotides.[11,31,33] The immobilization density was higher than others reported for conventional solid-phase RPA performed on polycarbonate surfaces (0.3-0.6 fmol/mm^2).[16,17] This can be explained because in bridge sp-RPA greater densities are required to allow the formation of bridges between the immobilized primers.[34] Streptavidin/biotin immobilized primers were resistant to microfluidic flows achieved by the proposed centrifugal platform.

Non-specific adsorptions were studied related to wall effects of reaction vessels and the addition of blocking agents. Regarding the first factor, the developed microfluidic structure had a surface-to-volume ratio of 5 nm^{-1} and 7 nm^{-1} for channels and microwells, respectively. These values are the same order of magnitude as those obtained for a 10 µL reaction performed in a standard PCR tube (0.002 µm^{-1}), and significantly lower (~100 times) than those obtained in other microfluidic devices, in which considerable surface adsorption of reagents (DNA and proteins) has been reported.[6]

Also, the addition of BSA (0.5%), PEG (1%) or Denhardt's reagent (2.5×) was studied because this strategy has been widely proposed to prevent non-specific adsorptions in similar platforms.[6,8] The presence of those reagents in the reaction mixtures did not increase the measured signal in any case (ANOVA, p-value>0.05). These results were in agreement with those observed in other DNA assays on PC surfaces where the non-specific adsorption was negligible.[16,21,35] Therefore, both

microfluidic design and DVD composition minimizes the effect of surface adsorption.

Different strategies were evaluated for developing the amplification products formed in the microwells and latter detection by DVD drive. Compared to previous approaches based on reflection reading, the proposed platform enabled the reading of the transmitted laser beam through the microwells. Therefore, the developing reaction, based on immunoenzymatic system, was done in both homogeneous (liquid) and heterogeneous (solid) formats, yielding a detectable product. The signal variations of the laser intensity (λ=650) during the disc scanning were 2.36 ± 0.17 and 0.59 ± 0.05, respectively. The observed differences between formats (t-test: p-value<0.01) were probably caused because the precipitate of the heterogeneous format was not absorbed enough to withstand washing flows. Therefore, the enzymatic reaction in the homogeneous format was selected for the proposed approach.

The absence of cross contamination among microwells was analyzed to ensure success in multiplexing applications. To that end, primers were alternately coated in adjacent microwells, the sample was loaded and amplified to produce alternately positive and negative reactions. Results showed that all the negative reactions produced signals lower than the limit of detection, confirming that no liquid transfer or diffusion between microwells takes place (t-test: p-value<0.01).

3.3 Analytical performances for food safety applications. The determination of targeted genes (peanut, p35S, Bt-11, *Salmonella* spp. and *Campylobacter* spp.) was examined following a large-scale screening approach.

Assay sensitivity was determined by analyzing serially diluted genomic DNA (10-folds dilution) by triplicate. A correlation between the concentration of DNA template and the optical signal measured by DVD detector was found for each analyte (Figure 4). The detection limits (LODs) were calculated as the lowest amount of DNA able to produce a distinguishable signal from the cut-off value (optical density of 0.8). The lowest detected concentrations were between 49-918 fg of extracted genomic DNA, which corresponded to 335-810 µg of analyte/g of food, for allergen and GMOs, and 92-176 CFU/mL for pathogenic bacteria. These

results comply with the EU legal requirements for GMOs detection (0.9 %), and are useful for allergen detection and pathogen screening. Assay reproducibility, expressed as relative standard deviation (RSD), was determined from the optical density of microwells from samples analyzed in triplicate. The intra-day RSD varied from 4.6 to 16.2 %, and the inter-day RSD from 10.0 to 18.4 %.

In summary, the attachment of both primers on the surface provided slightly worse analytical performances than those obtained by other solid-phase approaches.[16,17] These results were probably due to the lower amplification yield of the bridge format.[10,11] However, the proposed assay offered advantages such as higher multiplexing capability, null cross-contamination, non primer interferences and lower handling, since a single reaction mixture was required to analyze all target genes.

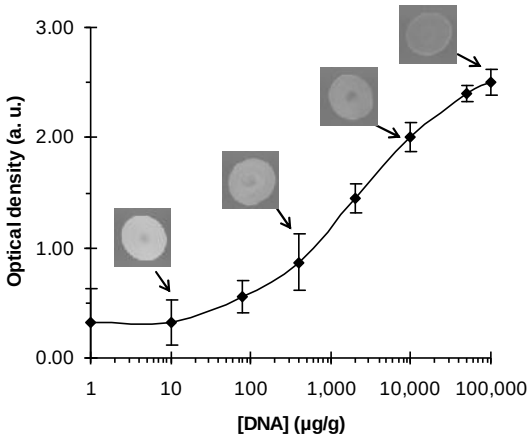


Figure 4. Calibration curve of peanut DNA (n=3). Inserts: Microwell images of the developed products corresponding to the indicated DNA concentration.

3.4 Proof of concept. The method was applied for the simultaneous screening of food common threats in representative samples to demonstrate its capability in a highly-throughput DNA analysis. With the proposed conditions (90 parallel reactions per disc), five genes were amplified-detected for 18 samples/replicates simultaneously, just with one pump of a pipette per sample. Samples containing analytes at high concentrations were easily identified by nacked eye detection, according to the microwell color (Figure 5). A more accurate measurement was achieved by registering optical intensities by DVD drive. The results were

satisfactorily compared to declared composition of foods and those obtained by the reference methods, individually analyzed (Table 2). The targeted genes were detected in all cases where the analytes were present, even at trace levels, and negative results were found in all the samples with no genes.

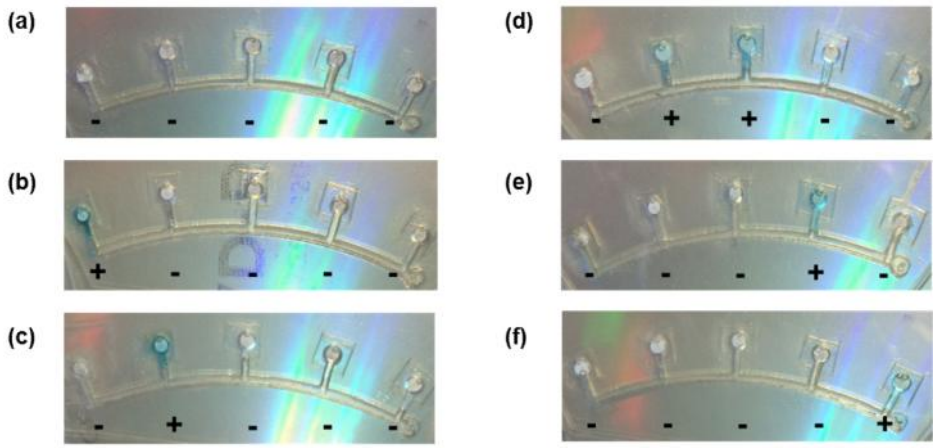


Figure 5. Images from the analysis of six food samples: (a) Muesli cookies, (b) Chocolate wafer, (c) MON 810 maize, (d) Bt-11 maize, (e) Powdered infant formula, and (f) Chicken carcass. Microwells contained primers for the specific detection of (from left to right): peanut, p35S, Bt-11, *Salmonella* spp. and *Campylobater* spp.

Selected application demonstrates that a large-scale DNA screening is possible integrating approaches such isothermal solid-phase, 3D-chips, and compact disc technology, being a competitive solution according to the state-of-the art.

Planar on-chip platforms, using bridge solid-phase amplification, report analytical performances similar to those of the present proposal.[10,13,25] But the reactions are not physically isolated, what may cause cross-contamination problems. An advanced solution is chips containing microreactors (3D-chips) for the simultaneous amplification of higher number of samples (e.g. more than 100).[4,6,7] However, the described approaches, compared to the proposed solution, are less automated, present higher surface-to-volume ratio and include tedious surface treatments to avoid non-specific adsorption of reagents. Also, time-consuming post-amplification procedures are required, such as gel electrophoresis or DNA hybridization, increasing the response time and the risk of

cross contamination.[1,6,7] On the other hand, several microfluidic systems have been developed, demonstrating automation advantages such as ease of operation, minimal cross-contamination, detection in less than 30 min and greater reproducibility.[15,18,19,28] Compared to these devices, the presented strategy is a less integrated platform, in terms of pre-storage of reagents or number of steps. However, some of them show low multiplexing capability or require advanced and/or expensive manufacturing techniques and specific equipments to control flows.

Most of the described approaches are based on PCR amplification, demanding an accurate temperature control.[1,4,6,10,19,25] The use of isothermal RPA simplifies the procedure, reduces the analysis time and avoids bulky thermalcyclers, being suitable for *in situ* applications. In addition, fluorescence detection in current methods requires specific and expensive equipment (fluorescence scanners, fluorimeters), what highly limits their costs and portability.[4,6,7,15,18,19,25] Hence, the main advantage of the proposed system comes from the detector, since DVD drives are cheap, portable, sensitive and robust devices, manufactured under high quality standards of massive-production. Furthermore, their light weight (<500 g) or reduced dimensions (a few centimetres), are compatible with their implementation in different settings. Therefore, the combination of the selected DNA methodology with a powerful detection technology leads a system with prospective costs (<3 € disc and <500 € reader) below the state-of-art, i.e. qPCR plates and fluorescence-thermocyclers.

Table 2. Results of the analysis of the samples using DVD technology (n=3).

Sample	Analyte presence	DVD detection [d]				
		Peanut	p35S	Bt-11	*Salmonella* spp.	*Campylobacter* spp.
Muesli cookies	Non declared [a,b]	-	-	-	-	-
Chicken carcass	Non declared [b]	-	-	-	-	-
Chocolate wafer	Peanut [a,b]	++	-	-	-	-
Cereals bar	Peanut at trace levels [a,b]	+	-	-	-	-
MON810 maize	GMO at 5.00 ± 0.11 % [c]	-	+++	-	-	-
Bt-11 maize	GMO at 0.98 ± 0.29 % [c]	-	+	++	-	-
Feed	GMO [a,b]	-	++	++	-	-
Powdered infant formula	*S. Typhimorium* at $4 \cdot 10^2$ CFU/mL [b]	-	-	-	++	-
Chicken neck skin	*S. Enteritidis* at 10^6 CFU/mL [b]	-	-	-	+++	-
Chicken carcass	*S. Edimburg* [b]	-	-	-	+++	-
Chicken carcass	*C. coli* at $2 \cdot 10^2$ CFU/mL [b]	-	-	-	-	+
Chicken carcass	*C. jejuni* at $5 \cdot 10^3$ CFU/mL [b]	-	-	-	-	++

[a] Listed in the labelling. [b] Determined by qPCR in a reference laboratory. [c] Certified reference material.
[d] Detection: Signal<0.8: -, 0.8<Signal≤1.5: + ; 1.5<Signal≤2.5: ++; Signal>2.5: +++

4. Conclusions

Parallelized systems provide advantages such reduced sample volumes, fast reaction times, and high-throughput detection. The last point is of particular importance for screening analysis, where multiple samples and targets must be analyzed as soon as possible. In this work, several elements have been integrated to achieve this goal. First, a simple microfluidic system based on the combination of capillarity forces and centrifugation is used to manipulate the motion of liquids by simply pipetting and spinning. Second, the isothermal nature of the RPA amplification reaction avoids the use of thermalcyclers, bubble formation and other related problems. Third, the integration of microstructures in the same optical discs and the detection based on disc technology is achieved, proving the way to work with commercially affordable analytical systems as those demanded by the end-users and market.

The novelty also comes from the detection approach using a DVD drive, because the results are monitored by measuring the laser transmitted through the transparent areas of the disc. Bridge solid-phase RPA is also an innovative format for highly parallel DNA amplification, without compromising the results quality. The combination leads to a versatile and economical platform, where tens of samples are simultaneously analyzed with minimal manipulation, drastically reducing the manual working steps, contamination risk, and errors.

The complete benefits of the method have not been reached yet, because an increase of the working capacity is truly possible, due to the scalability of DVD technology and associated methodology. Further developments can be designed for higher multiplexing, exploiting more the high area of the DVD and the tolerance of DVD drive. In the same way, the use of more accurate instruments, such as CO_2 laser cutter, can improve the fabrication process, achieving better reproducibility. It is worth mentioning that this approach can be extended to other disc formats and drivers such as compact disc (CDs), expanding the types of developing substrates that can be detected optimally (λ=780 nm). Considering the selected application (screening of food safety threats), excellent analytical performances (selectivity, sensitivity, reproducibility, ease of operation and high

throughput) have been achieved. This approach should be also suitable for many applications in health, forensic, food safety or environment fields.

Acknowledgements

This research has been funded through projects FEDER PrometeoII/2014/040 (GVA), and CTQ/2013/45875-R (MINECO). The Spanish Ministry of Education and Science provided S.S.F. with a grant for PhD studies.

References

1. Li, Y., Guo, S. J., Shao, N., Tu, S., Xu, M., Ren, Z. R., Ling, X. Wang, G. Q., Lin, Z. X., Tao, S. C. *Lab Chip*, 2011, 11, 3609-3618.
2. Ng, J. K., Chong, S. Multiplexing Capabilities of Biosensors for Clinical Diagnostics. *INTECH Open Access Publisher*, 2011.
3. Shrestha, H. K., Hwu, K. K., Chang, M. C. *Trends Food Sci. Tech.*, 2010, 21, 442-454.
4. Shao, N., Jiang, S. M., Zhang, M., Wang, J., Guo, S. J., Li, Y., Jiang, H. W., Liu, C. X., Zhang, D. B., Yang, L. T., Tao, S. C. *Anal. Chem.*, 2014, 86, 1269-1276.
5. Baker, M. *Nat. Methods*, 2012, 9, 541-544.
6. Hoffmann, J., Trotter, M., von Stetten, F., Zengerle, R., Roth, G. *Lab Chip*, 2012, 12, 3049-3054.
7. Gole, J., Gore, A., Richards, A., Chiu, Y. J., Fung, H. L., Bushman, D., Chiang, H. I., Chun, J., Lo, Y. H., Zhang, K. *Nat. Biotechnol.*, 2013, 31, 1126-1132.
8. McCalla, S. E., Tripathi, A. *Annu. Rev. Biomed. Eng.*, 2011, 13, 321-343.
9. Westin, L., Xu, X., Miller, C., Wang, L., Edman, C. F., Nerenberg, M. *Nat. Biotechnol.*, 2000, 18, 199-204.
10. Khan, Z., Poetter, K., Park, D. J. *Anal. Biochem.*, 2008, 375, 391-393.
11. Khodakov, D. A., Ellis, A. V. *Microchim. Acta*, 2014, 181, 1633-1646.
12. Shin, Y., Perera, A. P., Kim, K. W., Park, M. K. *Lab Chip*, 2013, 13, 2106-2114.
13. Kersting, S., Rausch, V., Bier, F. F., von Nickisch-Rosenegk, M. *Malaria J.*, 2014, 13, 99-107.
14. del Río, J. S., Yehia Adly, N., Acero-Sánchez, J. L., Henry, O. Y., O'Sullivan, C. K. *Biosens. Bioelectron.*, 2014, 54, 674-678.
15. Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle, R., von Stetten, F. *Lab Chip*, 2010, 10, 887-893.
16. Santiago-Felipe, S., Tortajada-Genaro, L. A., Morais, S., Puchades, R., Maquieira, A. *Sensor. Actuat. B-Chem.*, 2014, 204, 273-281.
17. Tortajada-Genaro, L. A., Santiago-Felipe, S., Amasia, M., Russom, A., Maquieira, A. *RSC Adv.*, 2015, 5, 29987-29995.
18. Zhou, Q. J., Wang, L., Chen, J., Wang, R. N., Shi, Y. H., Li, C. H., Zhang, D. M., Yan, X. J., Zhang, Y. J. *J. Microbiol. Meth.*, 2014, 104, 26-35.

19. Focke, M., Stumpf, F., Roth, G., Zengerle, R., von Stetten, F. *Lab Chip*, 2010, 10, 3210-3212.
20. Santiago-Felipe, S., Tortajada-Genaro, L. A., Puchades, R., Maquieira, A. *Anal. Chim. Acta*, 2014, 811, 81-87.
21. Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldón, J. A., Puchades, R., Maquieira, A. *J. Agr. Food Chem.*, 2011, 60, 36-43.
22. International Organization for Standardization, Geneva, Switzerland (2005) ISO 21569:2005. Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-qualitative nucleic acid based methods.
23. International Organization for Standardization, Geneva, Switzerland (2005) ISO 21570:2005. Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-quantitative nucleic acid based methods.
24. Adessi, C., Matton, G., Ayala, G., Turcatti, G., Mermod, J. J., Mayer, P., Kawashima, E. *Nucleic Acids Res.*, 2000, 28, e87.
25. Shin, Y., Kim, J., Lee, T. Y. *Sensor. Actuat. B-Chem.*, 2014, 199, 220-225.
26. Morais, S., Tamarit-López, J., Carrascosa, J., Puchades, R., Maquieira, A. *Anal. Bioanal. Chem.*, 2008, 391, 2837-2844.
27. Blanco-Gomez, G., Glidle, A., Flendrig, L. M., Cooper, J. M. Anal. Chem., 2009, 81, 1365-1370.
28. Li, G., Chen, Q., Li, J., Hu, X., Zhao, J. *Anal. Chem.*, 2010, 82, 4362-4369.
29. Liu, Y., Ganser, D., Schneider, A., Liu, R., Grodzinski, P., Kroutchinina, N. *Anal. Chem.*, 2001, 73, 4196-4201.
30. Tillib, S. V., Strizhkov, B. N., Mirzabekov, A. D. *Anal. Biochem.*, 2001, 292, 155-160.
31. Palanisamy, R., Connolly, A. R., Trau, M. *Bioconjugate Chem.*, 2010, 21, 690-695.
32. Kersting, S., Rausch, V., Bier, F. F., von Nickisch-Rosenegk, M. *Microchim. Acta*, 2014, 181, 1715-1723.
33. Carmon, A., Vision, Y. J., Mitchell, S. E., Thannhauser, T. W., Muller, U., Kresovich, S. *BioTechniques*, 2002, 32, 410-421.
34. Shapero, M. H., Leuther, K. K., Nguyen, A., Scott, M., Jones, K. W. *Genome Res.*, 2001, 11, 1926-1934.
35. Wang, Z., Li, R. X. *Nanoscale Res. Lett.*, 2007, 2, 69-74.

Capítulo 4. Amplificación isoterma en tiempo real

Los resultados descritos en los capítulos anteriores han demostrado que los métodos basados en la sp-RPA, a pesar de sus excelentes prestaciones, sólo permiten llevar a cabo una detección semicuantitativa, ya que la detección de los productos amplificados al final de la amplificación (fase estacionaria de la curva de reacción) no permite determinar con exactitud la concentración de ADN inicial.

En este capítulo, se propone la monitorización en tiempo real de la reacción de amplificación. De esta manera, se permite registrar la acumulación de los fragmentos amplificados a lo largo de la reacción y, por tanto, establecer una cuantificación directa al igual que en la rt-PCR.

Así, se presentan los resultados de las investigaciones para integrar la amplificación y la detección de ácidos nucleicos en tiempo real. En general, la monitorización de la RPA se lleva a cabo mediante detección fluorescente, la cual no puede llevarse a cabo mediante un lector de discos estandar. Por ello, se propone como alternativa la amplificación isoterma mediada por bucle (LAMP), que permite la monitorización turbidimétrica o colorimétrica de la amplificación, siendo esta aproximación compatible con el empleo del lector de DVDs como detector.

En el siguiente trabajo, titulado "*Micro-reactors on compact disks for real-time loop-mediated isothermal amplification*", se investiga la propuesta de crear una metodología y sistema analítico donde efectuar y detectar la rt-LAMP, utilizando como plataforma de trabajo los discos compactos (Biosensor 8). La presencia del ADN diana produce un cambio en las propiedades turbidimétricas o colorimétricas del medio de reacción, y su progreso se monitoriza mediante la medición de los cambios en la intensidad del láser del lector (Figura 31).

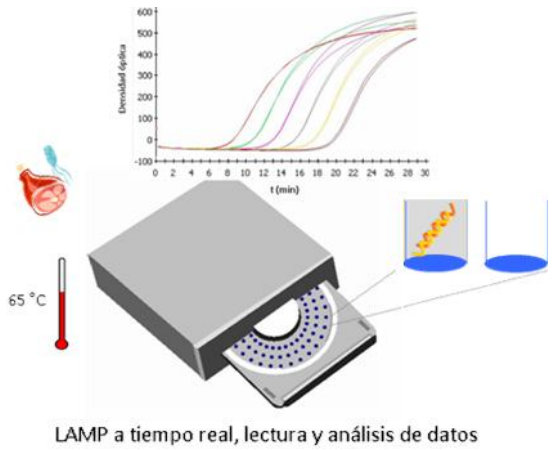


Figura 31. Esquema de la amplificación isoterma en tiempo real en el interior de los microreactores.

El método, aplicado a la detección de la bacteria patógena *Salmonella* spp. y la identificación de especies cárnicas (ternera), consta de las siguientes etapas:

- Diseño y fabricación del soporte de trabajo (disco con microreactores).
- Caracterización de los productos de amplificación.
- Comparación de diversas estrategias de detección.
- Determinación de los parámetros analíticos del método.
- Estudio de viabilidad para el análisis de muestras alimentarias.

Analyst

PAPER View Article Online View Journal

Micro-reactors on compact discs for real-time loop-mediated isothermal amplification

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Javier Carrascosa, Rosa Puchades, Ángel Maquieira

Instituto Interuniversitario de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) - Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain

Submitted to Analyst, September 2015

Abstract

An integrated device composed by micro-reactors embedded on compact discs is proposed for targeted DNA detection. The method principle is based on in-disc loop-mediated isothermal amplification (iD-LAMP) and simple quantitative optical read-out by a disc drive. In presence of target, the turbidimetric or colorimetric properties of reaction solution change, modifying the transmitted intensity of disc drive laser as function of reaction yield. The monitoring of real-time curves allowed the quantitative determination of DNA template amounts. The best amplification/detection results were obtained with micro-reactors (2 mm diameter and 1.1 mm in depth) drilled on digital video disc (DVD) and detection based on colorimetric mode. As proof of concept, the proposed assay was applied to the detection of the pathogenic bacteria *Salmonella* spp. and the identification of bovine meat in different food samples. Ninety-six samples were simultaneously analyzed in less than 15 min, with high specificity and sensitivity (5 CFU/mL and 10 µg/g for bacteria and meat, respectively). The in-disc results were comparable to those obtained using conventional LAMP or qPCR approaches. The proposed

device allows low sample and reagent consumption (3 µL of reaction), portability, ease-of-use, and rapid and high-throughput analysis at low cost.

1. Introduction

Nucleic acid-based methods are preferred for analytical detection in several areas, such as food safety, environmental monitoring, and clinical diagnostics. A key step in nucleic acid detection is the selective amplification because it generates a large number of target copies, greatly increasing assay sensitivity and controlling specificity.[1] The most widespread method for amplification is the polymerase chain reaction (PCR) performed in benchtop thermocyclers. However, efforts are being made to develop inexpensive, user-friendly and reduced-size devices for point-of-care applications.[2] Miniaturized and microfluidic reactors fit with these goals, as they allow reduced reaction time, less consumption of samples and reagents, increased portability and potential for automation.[2-4]

Although different PCR-based devices have been developed, some limitations hinder their integration into miniaturized systems.[5] PCR demands an accurate temperature control and rapid thermocycling between 55 °C and 95 °C. The fluctuation of temperature when initiating a specific step results in overshooting or undershooting.[1] Besides, high temperatures during denaturation steps lead to variations in the volume reaction and in gas-bubble formation, one of the main drawbacks in lab-on-a-chip. These constraints can be overcome through isothermal amplification methods, where nucleic acids are synthesized at constant temperature by using proteins that separate the DNA strands.[1,4] In isothermal amplification, the reaction time is controlled by the enzyme activity rather than the rate of thermal cycling and heat transport, as occurs in PCR-based methods. This gives the advantage of a simple design and less energy consumption, which makes miniaturized amplification compatible with portable detection systems.

Among the developed isothermal amplification reactions, the loop-mediated isothermal amplification (LAMP) has become a good option due to its rapidity and high sensitivity. LAMP uses *Bst* DNA polymerase to create loop structures that facilitate exponential sequence-specific amplification.[6-7] Under isothermal conditions (60-65 °C), this method generates large amounts of amplified product (>10^9 amplification factor) within 1 h. The use of multiple target sequence regions

(4-6 primers) confers high specificity. Therefore, LAMP is a very sensitive, easy, and time saving method being successfully applied for multiple screening tests.[6-11]

Some devices have been described based on end-point detection or real-time monitoring of LAMP reaction, carrying out on-chip electrophoresis, detection of turbidity change and charge-coupled device-based fluorescence imaging systems.[2,12-16] However, all these approaches require relatively sophisticated and expensive optical equipment.

In this study, a novel method, called in-disc LAMP (iD-LAMP), has been developed integrating LAMP amplification and compact disc technology. There are previous genomic assays performed in centrifugal devices, integrating all analytical steps by controlling rotation rate[17] or involving some handling steps.[18] This "lab-on-a-disc'' systems generally consist of microstructured circular platforms (channels, valves, chambers, etc.) where the reaction takes place, but the measurement is performed with benchtop instruments, such as colorimeters, or expensive static detectors, such as fluorescence microscopes, scanners or other complex non-integrated systems. The present method is based on the direct use of standard audio-video discs (CD, DVD, Blu-Ray) as support for carrying out amplification, and the results are read by optical scanning with the laser of the disc drive. The main advantage is that both support and detector are mass-produced with high-quality standards and cost-effective price. Compared to DNA biosensors based on detection by a disc drive,[19-22] the progress of the amplification reactions is real-time monitored in the current innovative method.

As proof-of-concept, the detection of a pathogenic bacterium (*Salmonella* spp.) and the identification of meat species (bovine) have been selected. Specific LAMP reactions take place into the microstructured disc and targeted gene quantification is achieved by continuously measuring of changes in the transmitted light intensity.

2. Methods

2.1 Discs. The amplification platform was composed by three layers (Figure 1). The first is a standard optical disc purchased from MPO Iberica (Madrid, Spain): compact disc (CD), digital versatile disc (DVD), or Blu-Ray discs (BD). A computer

numerical control drilling machine (Bungard CCD, Karo 5410, Germany) was used to hole 96 micro-reactors on bulk discs, drilled at a radius from 35 mm to 45 mm respect to the disc center. The feed speed and rotational rate of the tungsten carbide drill were 2,000 mm/s and 48,000 rpm, respectively. The dimensions of each micro-reactor were 2.00 mm in diameter and 1.10 mm in depth. After micro-reactors drilling, oxygen plasma exposure activated the discs enhancing hydrophilicity. Discs were introduced inside a microwave plasma reactor PVA Tepla 200 Plasma System (Feldkirchen, Germany) operating at 2.45 GHz and continuous 100 W power for 30 s. Oxygen pressure inside the reactor was 120 Pa.

The second layer of the device consisted of a double-sided pressure-sensitive adhesive of 0.08 mm in depth (ARcare 90445, Adhesives Research, Ireland). Chambers of 5×4.5 mm were cut using a cutter-plotter (Graphtec, Japan-Graphtec CE-2000). Once the disc and adhesive layer had been machined, they were aligned and laminated together. An acetate sheet composed the third layer used to seal the system.

2.2 Detector. Commercial CD (Premium, Plextor America, USA), DVD (LG Electronics Inc., USA) and BD drives (LG Electronics Inc., USA) were used. A standard recorder/reader drive integrates a motor to rotate the disc, an optical system with a laser, and a servo focus/tracking system to center and focus the beam on the spiral track across the whole disc surface. An upper planar photodiode (model SLCD-61N2, Silonex, USA) and a data acquisition board (DAQ, model USB-2527, Measurement Computing Corporation, USA) were home-implemented to register the transmitted laser light. The photodiode (5.1 mm × 5.1 mm) has a spectral sensitivity of 0.55 A/W at 940 nm, a spectral range between 400 and 1100 nm and an acceptance half-angle of 60°. The DAQ, with a sample rate up to 1 Msps, digitized the analog signals from the detection areas and transferred them to the computer for processing. The measurement principle is based on the optics of the disc drive and the variation of the optical properties of the disc due to the presence of the amplification reaction in the micro-reactor (Figure 1). The detector measured the changes in the light intensity of the drive

laser transmitted through the solutions during a cyclic scanning of surface (disc rotation), registering the reaction progress.

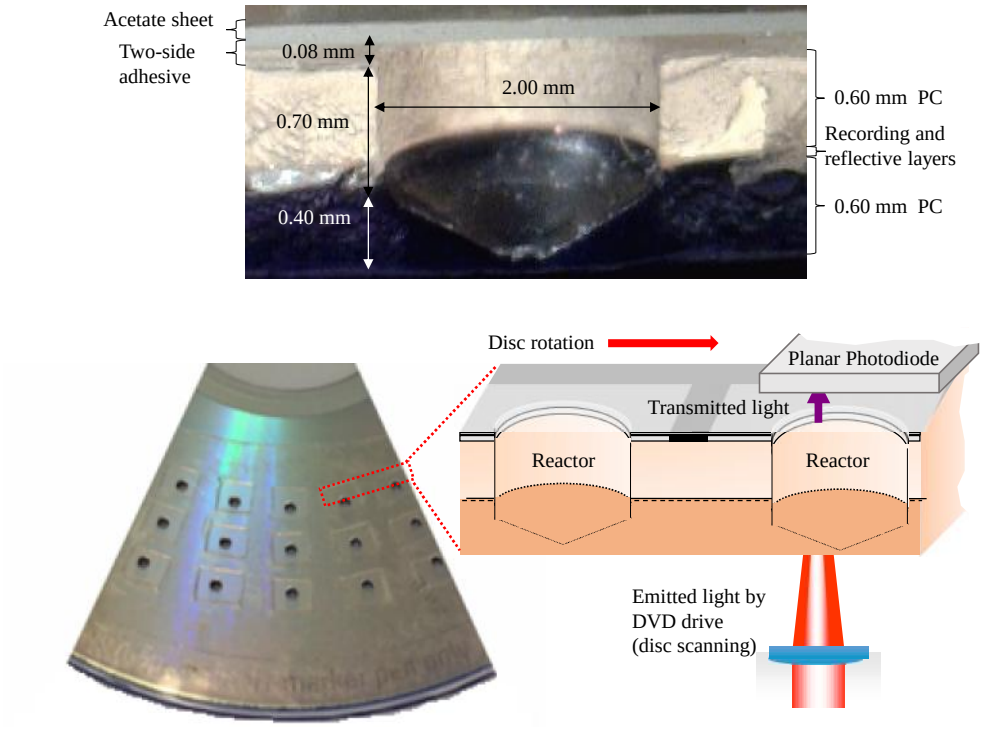


Figure 1. (a) Microphotography of the micro-reactor fabricated as a multi-layer system. (b) Photograph of analytical platform based on micro-reactors embedded on DVD disc and schematic diagram of detection system by DVD drive.

The device is enabled and configured to the specified disc rotational speed (1,000 rpm), the laser power (1 mW), and the focus of the laser beam to the disc surface. A continuous data acquisition is performed in the radius interval where the samples are deposited (35-45 mm respect to the disc center) at equal time intervals during the amplification (2 min). In each cycle, an automatic adjustment of the laser power is carried out, correcting the power drift due to the temperature conditions (65 °C) and intrinsic variations of the laser temperature. The transmitted optical density of the drive laser is recorded at an acquisition signal of 26 dB gain and 1 mega-samples per second. The data stream is divided into peaks that correlate with the microreactors of the disc, synchronized at the

same start point through a footprint at the outer edge of the disc. The averaged peaks (ten readings per cycle) are recorded in a character-separated values file (CSV).

2.3 Samples. The sequences of the LAMP primers for detecting Salmonella spp. and bovine specie are shown in Table S-1. Reference bacterial strains (targeted and non-targeted serovars and organisms) were supplied by CECC (Spain), NCTC (UK) and ATCC (USA). Bacterial isolation and inoculation assays were prepared as described by Santiago-Felipe *et al*.[23] Meat products were bought in local stores. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Inc., USA).

2.4 In-disc LAMP reaction and detection. Amplification reactions were carried out in 3 µL micro-reactors. The reaction mixture contained 1× ThermoPol reaction buffer (New England BioLabs, USA), 6 mM $MgSO_4$, 1.2 mM dNTPs, 0.2 µM F3 and B3, 1.6 µM FIP and BIP, 0.64 M betaine, 16 U of *Bst* DNA polymerase (New England BioLabs), 2.5 ng of DNA template, and inert oil (8%, v/v, Sigma) to prevent evaporation. For *Salmonella* spp. amplification, the mixture also included 0.8 µM LF and LB primers. Depending on detection strategy, additional reagents were added: no reagent for direct turbidimetry, 1 µM polyethylenimine (PEI) at the end of the reaction for end-point turbidimetry, and 120 µM hydroxynaphthol blue (HNB) in the reaction mixture for colorimetry.

The disc was sealed and inserted into disc drive, thermostating all the system at 65 °C into an oven (Memmert, model UF30, Germany). The disc was cyclically scanned during the isothermal incubation (duration: 30 min, cycle frequency: 2 min^{-1}). The data processing and the concentration calculation were automatically done. A sample was considered positive when the optical response was higher than the cut-off value (threshold), calculated as three times the standard deviation of the negative control. The concentration was calculated from the threshold time, the time needed for each sample to exceed the quantification

signal, measured for samples and standards. Used discs were discarded following the same laboratory safety guidelines than ELISA plates.

2.5 Complementary/Secondary measurements. The micro-reactor dimensions and contact angle values were measured using a Dino-Lite Digital Microscope (BigC.com, Torrance, USA) with a resolution of 1.3 megapixel (1280×1024 pixels). The fabrication uniformity and quality of the micro-reactors were checked using a SMZ-1B Stereoscopic Microscope (Nikon Corporation, Japan) with a total magnification between 16-70x (real field 13.1-3 mm). The particle size distribution of the produced magnesium pyrophosphate and DNA-PEI precipitate in the micro-reactors was determined by a Zetasizer Nano ZS (Malvern, UK) studying the dynamic light scattering of the sample.

The results obtained applying iD-LAMP method in 96-reactor disc were compared to conventional approaches based on end-point and real-time detection. Regarding to end-point detection, LAMP reactions in benchtop experiments, or in-tube amplification, were carried out using reaction mixtures described above and prepared in 200 µL-polypropylene tubes. Tubes (total volume 25 µL) were incubated at 65 °C for 30 min into a thermoblock (Digital Heatblock EU230V, VWR, Spain). Then, reaction solutions from in-tube and in-disc amplifications were analyzed by spectrophotometric analysis with a NanoDrop 2000/2000c (Thermo scientific, USA) and by electrophoresis on a 3% (w/v) agarose gel at 110 V and room temperature. Resulting gels were stained for 30 min with 0.5× TBE buffer (Tris/Borate/EDTA) containing the fluorophore Real-Safe (Real Laboratories, Spain) at 0.01% (v/v), and bands were visualized with a UV transilluminator.

For comparing with fluorescence real-time measurements, 1 µL of 1:10 diluted original SYBR green dye I (Invitrogen, Spain) was added to in tube LAMP reactions (96-wells plate and total volume 25 µL) before incubation at 65 °C using a 7500 Real-Time System (Applied Biosystems, Spain). Fluorescence readings were obtained every 2 min and values were considered positive when the fluorescence reading exceeded a threshold value (40,000 units).

Statistical package Statgraphics Centurion for Windows v. 16.1.15 was used for data analysis.

3. Results and discussion

3.1 Design of the micro-reactors. Three optical disc technologies (CD, DVD, and BD) were tested for developing the iD-LAMP method. First, the compatibility of laser reading with the fabrication of micro-reactor inside the disc was studied. Particularly, the dimensions of the micro-reactors could alter the physical properties of disc and the capability to follow the data track, present in the grove polycarbonate. Then, a two-factor experimental design was performed varying diameter (0.8 to 4 mm) and depth (0.6 to 1.1 mm) of embedded micro-reactors. In that configuration, the reflective metallic layer was locally removed, allowing the transmission of the light through specific areas of the structured disc. Although the disc integrity did not change, none of the BD discs with microreactors, regardless of the diameter or depth, was recognized by the BD drive. In case of CDs or DVDs, micro-reactors with a diameter larger than 2 mm also led to an error aborting the reading caused by a loss of spiral track. However, micro-reactors with lower diameters were compatible with the reading, and the scanning of full disc surface was possible.

The differences observed between the technologies were associated to the error tolerance. In optical recording roughly two types of errors can be distinguished: single and burst errors. Single (or random) errors affect one or two bytes and are caused by noise in combination with other sources of signal deterioration such as tilt of the disc or defocus of the laser spot on the disc. Burst errors are associated to defects on the disc surface like scratches, dust, fingerprints etc. The error correction system depends on the physical properties of the support on which the data is stored. Blu-Ray discs, due to its small spot, the thin cover layer and the high numerical aperture, are more sensitive to burst errors than CD and DVD ones. For the proposed analytical application, the micro-reactors in the disc are repetitive burst errors, modifying the reflexion of laser beam in the metallic layer to the pick-up. Therefore, the laser followed normally

the spiral track in surface regions without micro-reactors. When the laser focused on a given in-disc micro-reactor, the beam was not reflected, but the disc scanning followed (or not) if it was capable (or not) of correcting that error. In conclusion, the same defect (micro-reactor) on a BD affected more than on a CD or DVD, therefore being less robust to cope with microstructures in the disc.

The following experiments were focused on investigating the sensing capabilities based on detecting transmitted light (Figure 1). For that, the in-disc micro-reactors were filled with a blue dye solution (colorimetric detection mode). In surface regions without micro-reactors, non response was detected in the photodiode (absence of transmitted light). For negative control solutions, the focused laser crossed the micro-reactor reaching the photodiode and the maximum intensity of transmitted beam was collected (background signal). In presence of a colored compound in the micro-reactor, the intensity of the laser beam reaching the photodiode decreased. The results, expressed as average optical signal registered, showed that the response highly depended on the micro-reactor depth due to the optical pathway variation (Figure S-1). The selected dimensions were 2 mm in diameter and 1.1 mm in depth (Figure 1). Besides, kinetic measurements were also possible by the proposed device because the detector was able to capture the signal in the in-disc micro-reactors by cyclic surface scanning (rotation speed: 1,000 rpm).

Therefore, these experiments demonstrated that the micro-reactors embedded in CDs and DVDs did not disturb the track scanning, facilitating the measurement of reaction solutions (end-point or real-time) by means of conventional disc drives.

3.2 Disc treatment. The hydrophobic nature of the disc substrate (polycarbonate, contact angle ~ 90°) may hinder the reagents dispensation inside of in-disc micro-reactors and may lead to additional problems, such as non-specific adhesion to the walls of the micro-reactors. As it has been previously demonstrated for microfluidic channels,[24] micro-reactor surface was hydrophilized to overcome these drawbacks.

In a first approach, liquid treatments were assayed, whereby particle removal is achieved at the same time as surface wetting. Structures were treated with the surfactant Hellmanex II (120 min at room temperature with a solution of 2% in demineralized water), acid treatment (20 % HNO_3 + 5 % H_2SO_4 for 10 min at room temperature), and alkaline treatments (NaOH 1M during 30 min at 60 °C). These laborious methods only reduced hydrophobicity by 12 % (Table 1). Then, physical treatments (UV irradiation and plasma exposure) were studied.[24,25] The exposition to UV-light with mercury capillary lamp (6 mW/cm^2) at two different wavelengths (371 and 254 nm) during 240 min, decreased significantly the contact angle (Table 1). This reduction was greater for the shorter wavelength, reaching a reduction percentages of 55 % and 81 % for λ=371 nm and λ=254 nm, respectively. For oxygen plasma activation, the discs were introduced into a plasma system operating at 2.45 GHz, 100 W and 120 Pa during 30 s. In those conditions, a hydrophilic surface was achieved with 20° contact angle (Table 1). The surface topography was not modified after plasma treatment, keeping unchanged the optical properties of the disc. The aging effect over the treated discs was minimum, being the surface hydrophilic after a month of storage. After disc treatment, the reagent solutions were easily loaded in the in-disc micro-reactor and no surface interaction was observed. In conclusion, compared to the tested methods, oxygen plasma exposure was the fastest, cleanest, easy-going, and most effective method.

Table 1. Contact angle measurements and reduction percentage as a function of surface treatment. Replicates: 5 droplets.

	θ (°)	θ Reduction (%)	Time (min)
Raw PC	81.1 ± 0.5	-	-
Hellmanex	72.1 ± 0.5	11*	120
Acid solution	71.1 ± 0.3	12*	10
Alkaline solution	71.4 ± 0.6	12*	60
UV irradiation (371 nm)	36.3 ± 0.7	55*	240
UV irradiation (254 nm)	15.4 ± 0.6	81*	240
Oxigen plasma	20.6 ± 0.4	75*	0.5

* t-test: p-value <0.001

3.3 Characterization of iD-LAMP products. The amplification reactions for both studied targets (*Salmonella* spp. and bovine specie) were performed on in-disc micro-reactors (reaction volume of 3 μL) and compared to in-tube amplification (reaction volume of 25 μL). The ladder-like patterns of gel electrophoretic separation were comparable for amplification products obtained in-disc or in-tube reaction (Figure S-2). The expected lengths for the smallest products, according to the designed primers, were 191 and 173-mer for *Salmonella* spp. and bovine genes, respectively. No small fragments, related to the slow heat transfer described in some amplification devices,[28] were observed. Here, *Bst* DNA polymerase elongated the LAMP product in the in-disc micro-reactors under stable conditions. The key point of an effective in-disc elongation by *Bst* DNA polymerase is that the disc substrate (1.2 mm-polycarbonate) has a great thermal conductance (thermal conductivity of 0.241 $W \cdot m^{-1} \cdot K^{-1}$ and thermal diffusivity constant of $1.53 \cdot 10^{-7}$ $m^2 \cdot s^{-1}$ at 38 °C). Experimental investigations showed that estimated amplification yields for in-disc or in-tube reaction were comparable for both *Salmonella* spp. gene and bovine gene (t-test, p-value> 0.05). The results confirmed the consistency between iD-LAMP method and the traditional in-tube system. Both reactions took place with the same mechanism, specificity, and amplification efficiency.

3.4 Detection strategies of iD-LAMP products. Several strategies were compared to quantify products generated in-disc micro-reactors by disc drives.

The first approach involved turbidity measurements based on the sensing of magnesium pyrophosphate, a by-product of DNA amplification, or combined with the insoluble complex between synthetized DNA products and polyethylenimine (PEI). However, this reagent inhibited the LAMP reaction and only could be added after the amplification.[26] As other turbidimetric instruments, the effective measurement of beam dispersion required that particle sizes were higher than λ/4, λ being the disc driver laser wavelength. The size distribution of particles produced in LAMP reactions that contained specific DNA (0.03-30,000 fg/μL) were determined by a light scattering instrument (Figure S-3). Results indicated that a stable suspension was obtained containing submicro-particles of magnesium

pyrophosphate with a mean diameter of 336 ± 119 nm, demonstrating that the size of some particles was enough to disrupt the laser beam from disc drives (CD λ=780 nm and DVD λ=650 nm). The study of submicro-particles formed by DNA-PEI interaction indicated that the proportion of high-diameter particulates was higher (mean diameter 660 ± 261 nm). On other hand, visible spectra of amplified products were recorded by a spectrophotometer (Figure 2a). Both approaches increased the optical signal in the range from 500 to 800 nm. Nevertheless, a higher increment was achieved after the end-point addition of PEI in the range of 0.5-10 µM. Similar results were obtained studying the in-disc detection by the proposed devices in transmission mode (CD and DVD readers). For direct turbidity, only the highest amounts of DNA template (10,000-30,000 fg/µL) produced a detectable decrease of transmitted optical signal during the reaction progress performed in discs. Meanwhile, the addition of PEI 1 µM after the amplification of DNA template at concentrations higher than 30-500 fg/µL produced a detectable signal variation.

The second strategy tested was colorimetric detection mode based on the addition of hydroxynaphthol blue (HNB) to the initial LAMP reaction mixture. HNB, a metal ion indicator, has been proposed for end-point or real-time monitoring LAMP reaction.[27] During the amplification, the formation of magnesium pyrophosphate produces the destruction of red/purple magnesium-HNB complex, so the HNB returned to its uncomplexed blue form under the buffered reaction conditions (pH 8). The visible spectrum showed an overlap between reduction of transmitted light associated to the formation of MgP_2O_7 particles and an absorption peak, centered in 650 nm, associated to the formation of blue Mg-HNB complex (Figure 2a). This wavelength coincides with that of the DVD driver (650 nm). In fact, an important variation of optical intensity signals was registered by the disc drive for amplifications performed in the micro-reactors. From the assayed range (0-200 µM), the highest response was reached for 120 µM of HNB using DVD platform, allowing the detection of low DNA template concentrations (0.03-25 fg/µL).

Results indicated that both detection methods, turbidity (DNA-PEI complex) and colorimetric (magnesium-HNB complex), can be suitable for the detection of LAMP amplified products, in terms of sensitivity and reproducibility (Figure 2b). However, the post-amplification addition of PEI increased the contamination risk and difficulties for automation and/or integration processes. Then, the initial addition of HNB to reaction mixture was selected as the best detection strategy using DVD platform.

3.5 End-point detection. The analytical performances of iD-LAMP method via end-point analysis were determined using initial addition of HNB to reaction mixture and DVD platform. The sensitivity of the assays was examined by analyzing series of 10-fold diluted genomic DNA from targeted organisms (*Salmonella* spp. and bovine specie). Figure 2c shows the optical responses for end-point amplified solutions measured by the DVD drive after 30 min reaction. There was a correlation between the optical density registered and the logarithm of DNA template concentration, for both genes (Figure S-4). The lowest concentrations detected were 65 fg DNA/µL (equivalent to 13 CFU/mL) and 0.3 fg DNA/µL (equivalent to 9 µg/g sample) for *Salmonella* spp. and bovine genes, respectively. Reproducibility, expressed as relative standard deviation of five replicates (10^2 CFU/mL and 10^3 µg/g for *Salmonella* spp. and bovine, respectively), was 8.5-14.2 % for intra-day experiments and 16.3-20.4 % for inter-day experiments.

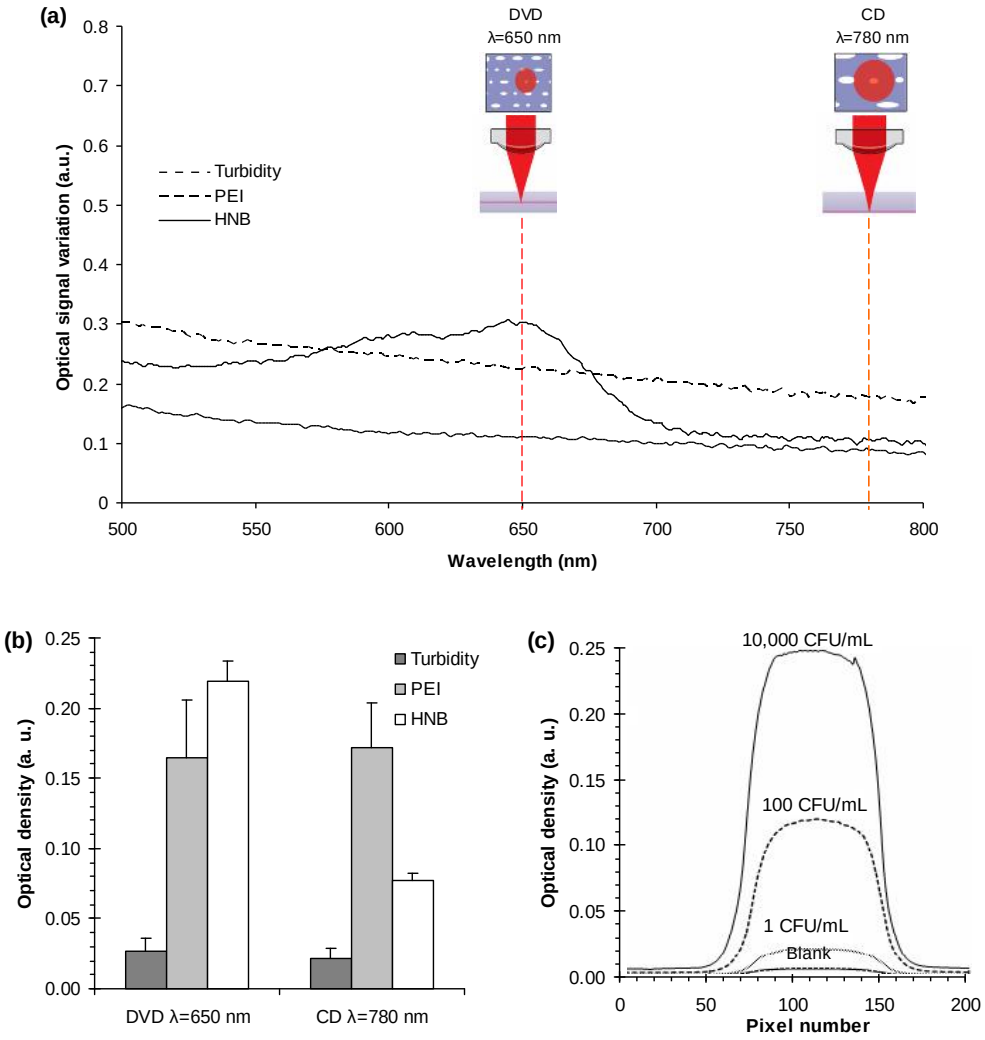


Figure 2. (a) Absorption spectra of the iD-LAMP products, obtained by a spectrophotometer, using different detection methods: turbidity based on magnesium pyrophosphate precipitate, turbidity based on DNA-PEI complex, and colorimetry based on the formation of Mg-HNB complex. Color dashed lines indicate the optimal wavelength of each compact disc drive: Digital Versatile Disc (DVD) and Compact Disc (CD). (b) Signals obtained by CD and DVD drives using turbidity based on magnesium pyrophosphate precipitate and DNA-PEI complex, and colorimetry based on the formation of Mg-HNB complex (c) Signals obtained by DVD drive based on the formation of Mg-HNB complex for different DNA concentrations.

3.6 Real-time detection. The end-point findings proved that the color changes detected by the DVD drive reflected the DNA yield during the progress of the amplification reaction. This meant that it was possible to use the proposed methodology to follow the DNA synthesis by continuous read out. To monitor iD-LAMP amplification kinetics, genomic DNA of both Salmonella and bovine standards (10-fold series) were amplified in-disc registering the color changes (cycle: 2 min). The same samples were amplified in-tube and monitored via fluorescence marker using a conventional thermal cycler. The signal-time registers obtained for both methods are shown in Figure 3a and 3b. Although the final amplification yields were comparable, the in-disc assay showed a higher amplification rate than in-vial. Probably, the enlarged surface-to-volume ratio increased thermal transfer and thus reduced cycling times, as demonstrated in other miniaturized devices.[29] Besides, an increase in the quantity of initial template DNA led to shorten the threshold time, or the reaction time necessary to reach positive signals above the baseline (optical density 0.07 for DVD drive and 40,000 units for conventional thermal cycler).

The calibration plot (threshold time versus logarithm of the initial DNA template concentration) showed a linear relationship for both *Salmonella* spp. and bovine systems (r^2=0.987 and r^2=0.985, respectively) (Figure 3c and 3d). The detection limits were 5 CFU/mL and 10 µg/g sample for *Salmonella* spp. and bovine, respectively. The relative standard deviation in five replicates was 6.5-17.1%. These results revealed that iD-LAMP method showed an adequate sensitivity, working range, and reproducibility for quantitative DNA analysis in less than 15 min.

The reliability of the iD-LAMP method for its application in large-scale screening was evaluated by the analysis of different pathogen cultures and foodstuffs and by comparing with reference method (qPCR). Specific genes for *Salmonella* spp. (*invA* gene) and bovine specie (mitochondrial 12S rRNA gene) were analyzed. Table 2 summarizes the results from iD-LAMP (96-micro-reactors-DVD) and qPCR methods. For the *invA* gene amplification, the non-target pathogens (*Cronobacter sakazakii* and *Campylobacter jejuni*) were negative, whereas all the *Salmonella* subspecies I were positive (*S. Typhimurium* and *S.*

Enteritidis). For 12S rRNA gene amplification, only foodstuffs containing beef were positive. In the positive amplifications, the iD-LAMP concentrations were in good agreement with qPCR concentrations for both analytes. Hence, quantitative detection of *Salmonella* spp. and bovine meat was achieved in a broad set of samples.

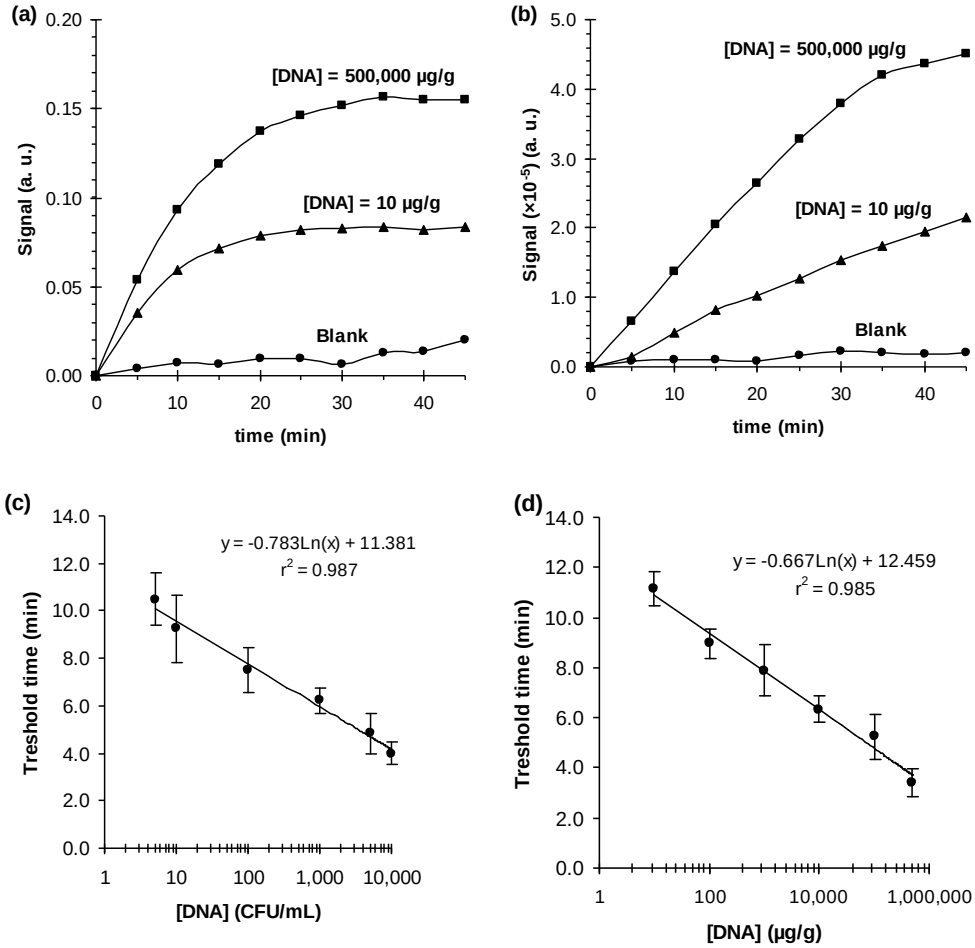


Figure 3. (a) In-disc LAMP response monitored via colorimetric detection by the DVD reader (b) In-tube LAMP response monitored via fluorescence using an Applied Biosystem 7500 thermal cycler. (c). Relation between the threshold times and the logarithm of the DNA template amount for *Salmonella* spp. (d) Relation between the threshold times and the logarithm of the DNA template amount for bovine. Five replicates

Table 2. Quantitative results for the analyzed samples by iD-LAMP and reference method: (A) *Salmonella* spp. and (B) bovine.

A	Concentration (log_{10} CFU/mL)		**B**	Concentration (log_{10} µg/g)	
Pathogen	iD-LAMP	qPCR[a]	Sample	iD-LAMP	qPCR[a]
C. sakazakii [c]	ND[b]	ND	Sautéed shrimp	ND[b]	ND
C. jejuni [d]	ND	ND	Vegetable soup	ND	ND
C. jejuni [d]	ND	ND	Ground pork	ND	ND
C. jejuni [d]	ND	ND	Ground turkey	ND	ND
S. Enteritidis	4.76	4.48	Ground beef	5.83	5.90
S. Enteritidis	4.12	3.57	Ground beef	5.60	5.70
S. Enteritidis	4.32	4.39	Ground beef	4.48	4.70
S. Enteritidis	4.76	4.25	Ground beef	4.21	4.00
S. Typhimurium	4.89	4.40	Feed 1	ND	ND
S. Typhimurium	4.62	4.00	Feed 2	ND	ND
S. Typhimurium	2.90	2.00	Meatloaf	5.61	5.54

[a] Determined by qPCR in a reference laboratory; [b] ND: No Detected
[c] Tested concentrations of *Cronobacter* sp.: 10^2-10^7 CFU/mL
[d] Tested concentrations of *Campylobacter* sp.: 10^2-10^7 CFU/mL

3.7 Comparison to other approaches. Numerous methods have been described for DNA detection using LAMP reactions in conventional plates or innovative devices. For real-time monitoring, techniques based on turbidity, fluorescence or electrochemical detection are the most commonly used.[10-15,28] Compared to iD-LAMP, these techniques have reported similar and, in few cases, better analytical performances (i.e. detection limits between 10-1,000 fg/µL), but have shown some drawbacks. In some cases, the number of samples that can be analyzed simultaneously is smaller. The methods need higher time assay and complex expensive equipment, what highly limits their costs and portability.

On the other hand, the proposed solution introduces important novel performances compared to previous "lab-on-a-chip" devices[2,9,10,12-14,29] or disc-shape approaches[15,18,30] for DNA assays (partially or totally automated). The device takes advantage of the intrinsic disc photonics (semiconductor diode, laser positioning/focusing system, etc.) combined with a complementary photodetector. The resulting analytical support and detector is compact, cheap, portable, and mass manufactured under high quality standards. Respect to other CD/DVD/BD approaches,[19-22,31] the versatility of in-disc assays performed in micro-reactors is higher than surface assays performed on top layer of opaque disc,

because transmission mode is incorporated. Compared to a possible system based on chambers fabricated on the top layer of semi-transparent DVDs,[32] better signal-to-noise ratios will be registered using our approach because their longer optical pathway and lower background (transparent support).

Therefore, the use of iD-LAMP device provides some advantages for real-time analysis. Firstly, the mass production of discs and readers assures a high quality, low-cost, and high-access sensor. The optical reader properties (laser drive), such as light weight (<500 g) or reduced volume (a few centimeters), are competitive with systems developed for its implementation in different settings. The prospective costs of the detection system (<3 €/disc and <300 €/reader) are below the state-of-art, i.e. qPCR plates and fluorescence-thermal cyclers. It is worth mentioning that the use of the proposed detection system based on a high intensity DVD laser beam, instead of other approaches such as a source of monochromatic light at 650 nm and a camera, leads to a more compact instrument, yielding high-quality images. Secondly, polycarbonate substrates, treated by oxygen plasma, have excellent bioanalytical properties compared to other plastics, such as minimal unspecific adsorption and low signal backgrounds. Thirdly, as it is based on an isothermal amplification technique, the method does not require fast accurate temperature changes, expensive reagents or time-consuming steps. iD-LAMP has demonstrated high specificity amplification in short reaction time. Furthermore, the technician “hands-on” time for the iD-LAMP assay is estimated to be one third that of a manual qPCR test. Fourthly, working capability for real-time monitoring is higher than conventional in-vial or alternative cartridge approaches. As proof-of-concept, the assayed device had 96 micro-reactors, but the number of samples simultaneously analyzed can increase due to the dimensions of disc (surface 94 cm^2) and micro-reactors (surface 3.1 mm^2).

4. Conclusions

Simple, effective, low-cost and fast response devices for DNA-based biosensing are needed. The proposed system iD-LAMP is a real-time non-fluorescent development that meets those requirements. Research efforts of this study have been focused on testing the features of the LAMP technique combined to optical disc technology owing to its simplicity of use. The integration of micro-reactors in the internal structure of optical discs and later sensing of the transmitted light by the disc drive has been demonstrated. So, the detection of targeted DNA based on an economically affordable analytical system, can be achieved both by end-point and real-time measurements. In addition, the isothermal nature of LAMP reaction avoids the use of thermalcyclers and prevents some thermal constraints associated to PCR.

The feasibility of the proposed approach has been assessed by determining two different kinds of analytes, such pathogen detection and identification of species. This technology for quantitative analyses has shown excellent analytical performances (selectivity, sensitivity, reproducibility, and high throughput), the results being comparable to in-vial LAMP and qPCR.

The iD-LAMP method, using a competitive, portable, and low-energy consumption analytical system, can be extended to other genomic assays in food safety, environmental, clinical or forensic areas. The proposed approach open up an advantageous form of DNA quantification, suitable to be applied in a wide range of ambits -in lab and out of lab- such as low resources settings, satellite/decentralized laboratories and production plants.

Acknowledgements

This research has been funded through projects FEDER PrometeoII/2014/040 (GVA) and CTQ/2013/45875-R (MINECO). The Spanish Ministry of Education and Science provided S.S.F. with a grant for her PhD studies.

References

1. Asiello, P. J., Baeumner, A. J. *Lab Chip*, 2011, 11, 1420-1430.
2. McCalla, S. E., Tripathi, A. *Annu. Rev. Biomed. Eng.*, 2011, 13, 321-343.
3. Auroux, P. A., Koc, Y., deMello, A., Manz, A., Day, P. J. R. *Lab Chip*, 2004, 4, 534-546.
4. Zanoli, L. M., Spoto, G. *Biosensors*, 2013, 3, 18-43.
5. Focke, M., Stumpf, F., Roth, G., Zengerle, R., von Stetten, F. *Lab Chip*, 2010, 10, 3210-3212.
6. Craw, P., Balachandran, W. *Lab Chip*, 2012, 12, 2469-2486.
7. Hara-Kudo, Y., Yoshino, M., Kojima, T., Ikedo, M. *FEMS Microbiol. Lett*., 2005, 253, 155-161.
8. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. *Nucleic Acids Res*., 2000, 28, e63.
9. Chen, L., Guo, J., Wang, Q., Kai, G., Yang, L. *J. Agric. Food Chem*., 2011, 59, 5914-5918.
10. Hsieh, K., Patterson, A. S., Ferguson, B. S., Plaxco, K. W., Soh, H. T. *Angew. Chem. Int. Ed*., 2012, 51, 4896-4900.
11. Tanner, N. A., Zhang, Y., Evans, T. C. *BioTechniques*, 2012, 53, 81-89.
12. Wastling, S. L., Picozzi, K., Kakembo, A. S. L., Welburn, S. C. *PLoS Negl. Trop. Dis*., 2010, 4, e865.
13. Safavieh, M., Ahmed, M. U., Ng, A., Zourob M. *Biosens. Bioelectron*., 2014, 58, 101-106.
14. Fang, X., Liu, Y., Kong, J., Jiang, X. *Anal. Chem*., 2010, 82, 3002-3006.
15. Ahmad, F., Seyrig, G., Tourlousse, D. M., Stedtfeld, R. D., Tiedje, J. M., Hashsham, S. A. *Biomed. Microdevices*, 2011, 13, 929-937.
16. Zhou, Q., Wang, L., Chen, J., Wang, R., Shi, Y., Li, C., Zhang, D., Yan, X., Zhang, Y. *J. Microbiol. Meth*., 2014, 104, 26-35.
17. Mori, Y., Kitao, M., Tomita, N., Notomi, T. *J. Biochem. Bioph. Meth*., 2004, 59, 145-157.
18. Roy, E., Stewart, G., Mounier, M., Malic, L., Peytavi, R., Clime, L., Madou, M., Bossinot, M., Bergeron, M. G., Veres, T. *Lab Chip*, 2015, 15, 406-416.
19. Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle, R., von Stetten, F. *Lab Chip*, 2010, 10, 887-893.
20. Morais, S., Tortajada-Genaro, L. A., Arnandis-Chover, T., Puchades, R., Maquieira, A. *Anal. Chem.*, 2009, 81, 5646-5654.
21. Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldón, J. A., Puchades, R., Maquieira, A. *J. Agr. Food Chem*., 2012, 60, 36-43.
22. Tortajada-Genaro, L. A., Santiago-Felipe, S., Amasia, M., Russom, A., Maquieira, A. *RSC Adv*., 2015, 5, 29987-29995.
23. Santiago-Felipe, S., Tortajada-Genaro, L. A., Puchades, R., Maquieira, A. *Anal. Chim. Acta*, 2014, 811, 81-87.
24. Liu, Y., Ganser, D., Schneider, A., Liu, R., Grodzinski, P., Kroutchinina, N. *Anal. Chem*., 2001, 73, 4196-4201.
25. Tamarit-López, J., Morais, S., Puchades, R., Maquieira, A. *Bioconj. Chem*., 2011, 22, 2573-2580.
26. Mori, Y., Hirano, T., Notomi, T. *BMC Biotechnol*., 2006, 6, 3-12.

27. Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K. *BioTechniques*, 2009, 46, 167-172.
28. Mori, Y., Nagamine, K., Tomita, N., Notomi, T. *Biochem. Bioph. Res. Co*., 2001, 289, 150-154.
29. Lee, S., Huang, J., Chuang, T., Sheu, J., Chuang, Y., Holl, M., Meldrum, D. R., Lee, C., Lin, C. *Sensor. Actuat. B-Chem*., 2008, 133, 493-501.
30. Gorkin, R., Park, J., Siegrist, J., Amasia, M., Lee, B. S., Park, J., Kim, J., Kim, H., Madou, M., Kyoung-Cho, Y. *Lab Chip*, 2010, 10, 1758-1773.
31. Santiago-Felipe, S., Tortajada-Genaro, L. A., Morais, S., Puchades, R., Maquieira, A. *Sensor. Actuat. B-Chem*., 2014, 204, 273-281.
32. Ramachandraiah, H., Amasia, M., Cole, J., Sheard, P., Pickhaver, S., Walker, C., Wirta, V., Lexow, P., Lione, R., Russom, A. *Lab Chip*, 2013, 13, 1578-1585.

SUPPLEMENTARY INFORMATION

Micro-reactors on compact discs for real-time loop-mediated isothermal amplification

Sara Santiago-Felipe, Luis A. Tortajada-Genaro, Javier Carrascosa, Rosa Puchades, Ángel Maquieira

1. Primers. Six primers, two inner, two outer and two loop primers (LF and LB), targeting *Salmonella enterica* invasion protein gene (*invA* gene) were used for *Salmonella* spp. detection. Four primers, forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3) and backward outer primer (B3) were used to amplify bovine mitochondrial DNA (12S rRNA gene). All the primers and probes were successfully checked for relevant homologies by a BLASTNr search (http://blast.ncbi.nlm.nih.gov/).

Table S-1. Primers used for amplification procedures.

Target		Sequence (5'-3')	Length (bp)	Tm (°C)
Salmonella spp.*	FIP	GACGACTGGTACTGATCGATAGTTTTTCAACGTTTCCTGCGG	42	73.3
	BIP	CCGGTGAAATTATCGCCACACAAAACCCACCGCCAGG	37	75.0
	F3	GGCGATATTGGTGTTTATGGGG	22	60.3
	B3	AACGATAAACTGGACCACGG	20	57.3
	LF	GACGAAAGAGCGTGGTAATTAAC	23	58.9
	LB	GGGCAATTCGTTATTGGCGATAG	23	60.6
Bovine**	FIP	ACACCTTGACCTAACGTTTTTATGT-CTATATACCGCCATCTTCAGC	46	77.9
	BIP	TGAAATGGGAAGAAATGGGCTAC-CCTCCTTTGGTTATTGGTTTC	44	77.9
	F3	CACCAATTCTTGCTAATACAGT	36	56.4
	B3	CACTCTATTCTTAGTTTACTGCTAA	32	59.2

* Hara-Kudo, Y., Yoshino, M., Kojima, T., Ikedo, M. *FEMS Microbiol. Lett*., 2005, 253, 155-161.

** Ahmed, M. U., Hasan, Q., Hossain, M. M., Saito, M., Tamiya, E. *Food Control*, 2010, 21, 599-605.

2. Micro-reactors. The micro-reactors were drilled in the top layer of commercial optical disc. Standard CDs, DVDs and BDs substrates are a 1.2 mm thick multilayer structure that contains all the operational information required to read the disc with a standard disc drive. Although all technologies use polycarbonate as substrate and have dielectric materials and reflective metal

layers, the structure varies between CDs, DVDs, or BDs. In case of DVD, according to the standard specifications, the bottom layer consists of a 0.6 mm thick-polycarbonate and has an injection molded spiral microguide (0.74 mm track pitch) in order to guide subsystems of the detector laser (λ=650 nm) to be kept on the data track. The middle layer is a highly reflective metallic material (thickness 1,000-1,500 Å), and the top layer is a 0.6 mm thick-polycarbonate substrate that guarantees the physical robustness (high-rate rotation, manipulation).

A two-factor experimental design was performed, varying diameters (0.8 to 4 mm) and depth (0.6 to 1.1 mm). The in-disc micro-reactors were tested using a blue-dye solution (colorimetric detection mode), and the results are shown in Figure S-1. The selected dimensions were 2 mm in diameter and 1.1 mm in depth. The microscope measures indicated a conic shape on bottom (height 0.40 mm) and cylinder on top (height 0.70 mm), so the micro-reactor volume was 2.3 µL (0.4 µL cone and 1.9 µL cylinder). The reaction chamber included a cube-shape on top delimited by PSA and acetate layers (5 × 4.5 × 0.08), so the final volume of each reaction chamber was 3.0 µL.

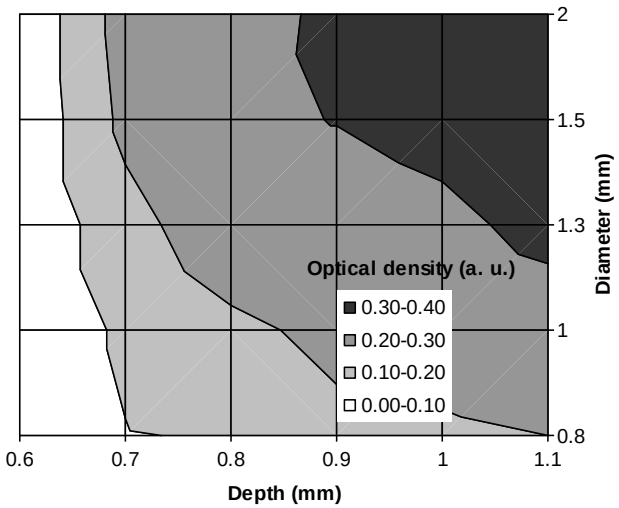


Figure S-1. Effect of the micro-reactor diameter and depth on optical intensity.

3. Agarose gel electrophoresis. Figure S-2 shows the gels after electrophoretic separation of the LAMP products obtained in the micro-reactors of optical discs (in-disc) and those obtained in typical reaction 200-µL tubes (in-tube). The characteristic ladder pattern is due to the formation of a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed after the annealing between alternately inverted repeats of the target sequence in the same strand.

Besides, amplified DNA was quantified from the fluorescence measurements with SYBR-Safe (Invitrogen, Spain) at 0.01% (v/v) in a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Finland). Amplification yields were calculated in reference to the initial DNA (ng of amplified DNA/ng of initial DNA×100). The estimated amplification yields were $(4.6 \pm 0.7)\cdot10^8$ and $(4.0 \pm 0.6)\cdot10^8$, for *Salmonella* spp. in tube and in disc amplification, respectively; and $(1.6 \pm 0.3)\cdot10^8$ and $(1.3 \pm 0.3)\cdot10^8$, for bovine in tube and in disc amplification. Hence, the results demonstrated that the isothermal conditions of LAMP reaction (65 °C) were reached and kept in the micro-reactors in the same way than in tube did.

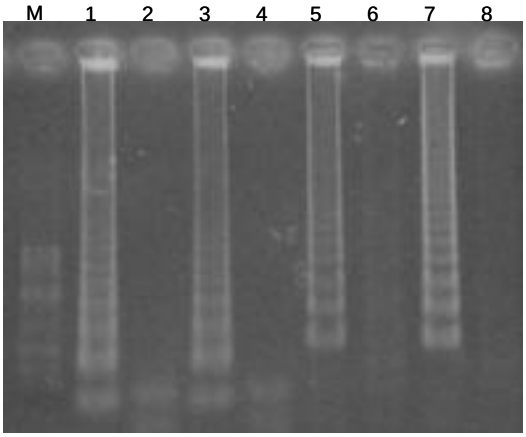


Figure S-2. Agarose gel electrophoresis of the LAMP products. Lane M: 100 base pair DNA ladder. Lanes 1-4: amplified products of *Salmonella Typhimurium*: in tube 10^2 CFU/mL (1), in tube 0 CFU/mL (2), in disc 10^2 CFU/mL (3), and in disc 0 CFU/mL (4). Lanes 5-8: amplified products of bovine meat: in tube 1% (5), in tube 0% (6), in disc 1% (7) and in tube 0% (8).

4. Particle diameter measurement. As other turbidimetric instruments, changes in detected light intensity were ascribed to changes in total scattering, considering that the intensity of the input light was constant, the samples had no absorbing constituents, and there are no changes in the light transmission caused by surface coatings. Then, particle sizes higher than λ/4 are required for the effective measurement of beam dispersion, being λ the sensing wavelength.

A Zetasizer Nano ZS instrument was used for determining the particle size distribution of the produced magnesium pyrophosphate and DNA-PEI precipitate in the micro-reactors. This technique studies the dynamic light scattering of the sample, measuring the diffusion of particles moving under Brownian motion, and converting this to size distribution using the Stokes-Einstein relationship.

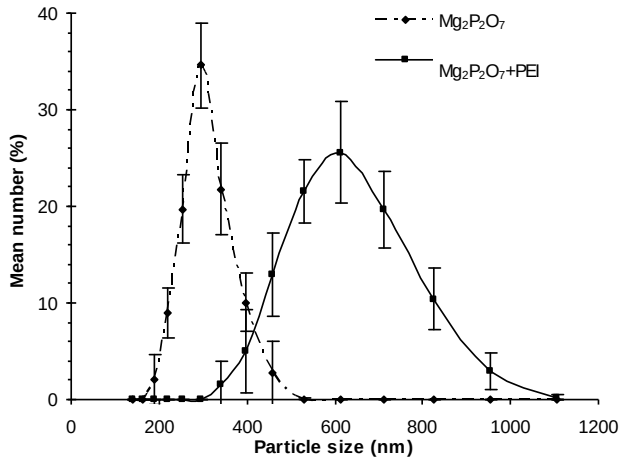


Figure S-3. Particles size of the magnesium pyrophosphate after LAMP reaction and LAMP reaction with precipitation of the amplified DNA. (DNA template=500 fg/µL).

5. End-point calibration curves. Series of 10-fold diluted DNA from *S. Typhimurium* culture and bovine meat were prepared and added to reaction mixtures. The solutions were dispensed on the micro-reactors and the disc was incubated at 65 °C during 30 min (five replicates). A correlation between the blue color of solutions in the micro-reactors and the DNA amount was visually observed. The lowest detectable concentration by naked eye was approximately 500 fg/µL. The results measured by the DVD drive showed that there was a linear relationship between the optical density registered and the logarithm of DNA

template concentration, for both *Salmonella* spp. and bovine genes (r^2=0.989 and r^2=0.971, respectively). The observed variations of sensitivity for the different targets could be due to little differences in amplification efficiency (sequence-dependent). The fluctuations between runs might be due to minor uncertainties in enzymatic activities or in the low-scale fabrication process of discs.

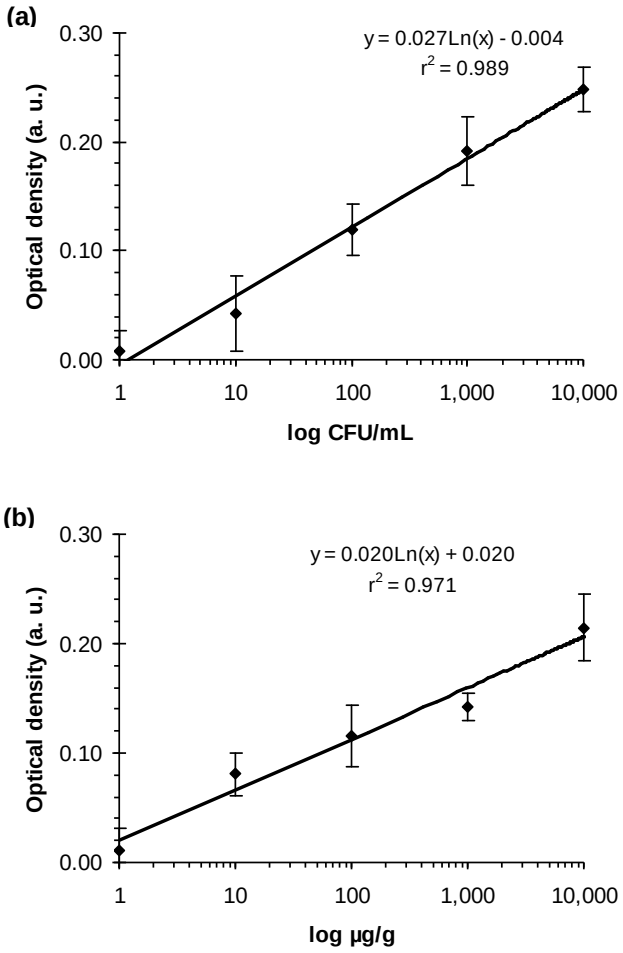


Figure S-4. iD-LAMP in end-point format. Relation between the optical density registered in DVD photodiode detector and the concentration of target DNA template: (a) *Salmonella* spp. and (b) bovine.

4. Resumen de resultados y discusión

Las investigaciones realizadas en esta tesis doctoral han tenido como principal objetivo el desarrollo de sistemas biosensores integrados y versátiles para la detección de amenazas alimentarias mediante ensayos génicos. Para ello, se han estudiado diversas técnicas de amplificación de ADN y diferentes soportes y formatos de ensayo. La mayoría de las investigaciones desarrolladas tienen en común la utilización de la tecnología de disco compacto tanto en lo que concierne al soporte (DVD) como al detector (lector de discos). Además, los sistemas desarrollados han permitido la determinación semicuantitativa o cuantitativa de los analitos estudiados en muestras de alimentos, incorporando un elevado grado de miniaturización, integración y automatización, y con prestaciones excelentes.

Biosensores desarrollados:

Biosensor 1: Amplificación por PCR y detección mediante ensayos de hibridación directa en la superficie del disco.

Biosensor 2: Amplificación por RPA y detección mediante ensayos de hibridación en placa de poliestireno.

Biosensor 3: Amplificación por RPA y detección mediante ensayos de hibridación directa en la superficie del disco.

Biosensor 4: Amplificación por MDA y detección mediante ensayos de hibridación directa en la superficie del disco.

Biosensor 5: Amplificación por RPA en fase sólida en gotas sobre la superficie del disco.

Biosensor 6: Amplificación por RPA en fase sólida en cámaras microfluídicas adheridas a la superficie del disco.

Biosensor 7: Amplificación por RPA en fase sólida en microreactores integrados en el sustrato del disco.

Biosensor 8: Amplificación por LAMP en tiempo real en microreactores integrados en el sustrato del disco.

En la Tabla 7 se recogen las principales características de los distintos sistemas desarrollados, y en la Tabla 8 se comparan las propiedades más destacadas de los mismos.

Tabla 7. Características de los sistemas biosensores desarrollados.

Sistema	Analito	Soporte	Reacción de amplificación	Amplificación múltiple	Nº etapas	V reacción (µL)	LOD	RSD (%)	Nº muestras	t (min)
1	Alérgenos	DVD	PCR	3	3	50-100	1 µg/g	5,8 - 7,3	10	250
2	Alérgenos, OGMs, baterias y hongos	Placa PE	RPA	1	3	25-150	1,3 - 5,3 µg/g 6 - 13 CFU/mL	7,9 - 11,3	96	130
3	Bacterias	DVD	RPA	2	3	25-50	10 - 48 CFU/mL	9,6 - 12,5	10	150
4	Bacterias	DVD	MDA	2	4	25-50	7 - 31 CFU/mL	10,4 - 14,3	10	400
5	Bacterias	DVD	sp-RPA	3	2	25	24 - 30 CFU/mL	7,8 - 15,2	36	90
6	OGMs	DVD	sp-RPA	1	2	6	7 µg/g	5,4 - 11,4	10	90
7	Alérgenos, OGMs y bacterias	DVD	Bridge sp-RPA	5	2	3	335 - 810 µg/g 92 - 176 CFU/mL	10,0 - 18,4	18	95
8	Bacterias y especies cárnicas	DVD	LAMP	1	1	3	10 µg/g 5 CFU/mL	6,5 - 17,1	96	15

Tabla 8. Resumen de las propiedades de los sistemas biosensores desarrollados.

Ventaja	Sistema Biosensor							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Análisis multianalito	++	-	+	+	++	-	+++	-
Posibilidad de automatización	-	+	-	-	+	++	+++	++
Capacidad de trabajo	+	+++	+	+	++	+	+++	+++
Rapidez de análisis	-	+	+	-	++	++	++	+++
Bajo coste	++	++	+++	+++	+++	+++	+++	+++
Reproducibilidad	+++	++	++	++	++	++	+	+
Sensibilidad	+++	+++	+++	+++	+++	+++	++	+++
Selectividad	+++	+++	+++	+++	+++	+++	+++	+++
Portabilidad	-	-	++	++	++	++	++	+++
Manejo sencillo	-	+	+	+	++	++	++	+++
Bajo requerimiento energético	-	++	++	++	+++	+++	+++	++
Nivel de integración	+	+	+	-	++	++	++	+++

Se observa que la reacción de amplificación condiciona drásticamente tanto las características del biosensor génico como las prestaciones. Todas las reacciones estudiadas en esta tesis, PCR, RPA, MDA y LAMP, han proporcionado sensibilidades adecuadas y comparables con las encontradas en la literatura. Sin embargo, la PCR, por su complejidad operativa (Biosensor 1), y la MDA, por necesitar una etapa de tratamiento post-amplificación (Biosensor 4), han demostrado ser poco idóneas para el desarrollo de sistemas integrados de altas prestaciones. Por el contrario, la RPA se ha mostrado como una opción excelente (Biosensores 2, 3, 5, 6 y 7). Ventajas como su gran versatilidad y operar a temperaturas próximas a la ambiente, han permitido su integración en sistemas de análisis multianalito, siendo aplicada con éxito en diferentes formatos de ensayo. Se ha demostrado que es posible llevar a cabo la amplificación en fase homogénea mediante RPA, siendo compatible con la etapa posterior de hibridación en disco (Biosensor 3). El desarrollo de la reacción en fase sólida (sp-RPA) ha permitido disminuir las etapas del proceso analítico y los tiempos de respuesta, logrando un mayor nivel de integración, sin perjudicar las prestaciones analíticas del ensayo (Biosensores 5 y 6). En este sentido, la reacción en formato "bridge" ha permitido aumentar el nivel de multiplexado, es decir, el número de analitos analizados simultáneamente (Biosensor 7). Sin embargo, los resultados obtenidos en términos de sensibilidad y reproducibilidad han sido ligeramente inferiores a los alcanzados mediante otros formatos, aunque suficientes.

A pesar de las excelentes prestaciones de los formatos basados en RPA, esta estrategia de ensayo sólo ha permitido la detección semicuantitativa de las dianas objeto de estudio. La reacción LAMP ha resultado ser una opción óptima para llevar a cabo análisis cuantitativos en tiempo real. Mediante este sistema, se ha logrado la integración total del proceso analítico, alcanzando unas excelentes prestaciones analíticas (Biosensor 8).

En cuanto al soporte y sistema de detección propuestos, el empleo de placas de poliestireno para la detección de los fragmentos amplificados presenta una alta capacidad de trabajo y bajo coste (Biosensor 2). Sin embargo, su baja versatilidad y requerir un lector de placas, no permite su integración en el desarrollo de sistemas miniaturizados, quedando limitado a aplicaciones en el

laboratorio. La tecnología de disco compacto ha demostrado ser una opción excelente. Tanto los discos como las unidades lectoras/grabadoras son dispositivos baratos, portátiles, sensibles y resistentes, fabricados bajo elevados estándares de calidad y producción masiva. Además, su peso y dimensiones reducidas los hacen compatibles con su aplicación en diferentes contextos. Esto confirma que el éxito en el desarrollo de un biosensor integrado depende, en gran medida, de las características del material de trabajo y de los sistemas de detección empleados.

En función de la categoría del disco se han obtenido diferentes aproximaciones. El uso de DVDs como soporte y detector para el desarrollo de ensayos de hibridación sobre micromatrices de ácidos nucleicos ha resultado ser una herramienta analítica de enorme potencial (Biosensores 1, 3 y 4). Ventajas como una gran superficie, baja absorción inespecífica, y buena sensibilidad y reproducibilidad, los convierten en una alternativa competitiva frente a los soportes tradicionalmente empleados en formato micromatriz.

Dotar a los discos compactos de estructuras microfluídicas ha permitido aumentar enormemente las capacidades de los sistemas biosensores. Estos dispositivos abren nuevos caminos ya que consiguen aprovechar más el potencial de la tecnología de CDs sin requerir modificaciones adicionales.

Con el empleo de estructuras microfluídicas *ad hoc* se ha logrado reducir el volumen de reacción, los riesgos de contaminación cruzada y la manipulación, e incluso pre-almacenar parte de los reactivos del ensayo (Biosensor 6).

El desarrollo de estructuras embebidas en el propio sustrato del disco ha permitido obtener una mayor compartimentación de los ensayos, aumentando considerablemente el multiplexado manteniendo las prestaciones analíticas (Biosensor 7).

Finalmente, la integración de micro reactores en el disco también ha permitido llevar a cabo la monitorización en tiempo real de las reacciones tipo LAMP (Biosensor 8). De esta manera, además de las ventajas anteriormente mencionadas, se ha logrado la integración total del proceso analítico, reduciendo aún más el tiempo total de ensayo. La mayor ventaja de este sistema es que permite llevar a cabo el seguimiento de las reacciones de amplificación en tiempo

real, monitorizando su cinética. Las prestaciones analíticas alcanzadas con la rt-LAMP han sido comparables a las de la PCR en tiempo real (rt-PCR).

Los resultados obtenidos para los sistemas desarrollados demuestran que la combinación de las reacciones de amplificación isoterma con la tecnología de disco compacto constituye una herramienta prometedora para la detección de ácidos nucleicos en aplicaciones de seguridad alimentaria, siendo una alternativa altamente competitiva frente a las técnicas de referencia.

5. Conclusiones

En la presente tesis se han estudiado nuevas técnicas de biosensado, concretamente de ADN, para el desarrollo de sistemas integrados adaptables a aplicaciones *point-of-control* en seguridad alimentaria. A lo largo de este trabajo, se han expuesto los resultados experimentales y se han evaluado sus propiedades y prestaciones según la problemática y aplicación concreta.

A continuación, se resumen las conclusiones más relevantes, clasificadas según los objetivos parciales planteados inicialmente.

- Se ha puesto a punto la integración de la tecnología de disco compacto con ensayos de amplificación e hibridación múltiple. Se ha demostrado que los ensayos de hibridación sobre micromatrices de ácidos nucleicos empleando la tecnología de disco compacto (plataforma y detector) constituyen una herramienta con alto potencial analítico, alcanzándose buenos límites de detección.

  En el caso de la amplificación por PCR, se ha detectado hasta 1 μg/g en el análisis múltiple de 3 alérgenos en 10 muestras simultáneamente.

- Se ha comprobado la compatibilidad de las reacciones de amplificación isoterma con los ensayos de hibridación con sondas de ácidos nucleicos, tanto en microplaca ELISA como en disco compacto. Se han obtenido resultados satisfactorios en términos de sensibilidad (<5 μg/g y <50 CFU/mL, para ingredientes y microorganismos patógenos, respectivamente) y reproducibilidad (<15%), siendo comparables a los obtenidos mediante amplificación por PCR.

  La integración de la amplificación por RPA con la tecnología de disco compacto ha resultado la mejor opción, ya que tanto el volúmen de muestra empleado como el tiempo del ensayo pueden reducirse a la mitad sin comprometer las prestaciones analíticas.

- Se ha descrito por primera vez la amplificación RPA en fase sólida sobre la superficie de un disco compacto tipo DVD. Mediante esta técnica, las fases de amplificación e hibridación tienen lugar en una única etapa, reduciendo a 90 min el tiempo total del ensayo y alcanzando límites de detección de 335 µg/g y 24 CFU/mL para ingredientes y bacterias patógenas, respectivamente.

  Siguiendo esta estrategia, se han optimizado diferentes formatos de amplificación en fase sólida. El formato "anidado" ha mostrado las mejores prestaciones analíticas (sensibilidad, selectividad y reproducibilidad). Mediante el formato "puente" se ha obtenido un mayor nivel de multiplexado, pudiendo analizar hasta 5 analitos simultáneamente.

  La aplicación de estas reacciones en el desarrollo de sistemas *lab-on-a-CD* ha permitido aumentar el grado de integración y automatización. Con el empleo de cámaras microfluídicas adheridas a la superficie del disco las reacciones tienen lugar en el interior de cámaras independientes, logrando mejorar la reproducibilidad y reducir el volumen de reacción y el riesgo de contaminación cruzada. El desarrollo de un sistema microfluídico (canales y microreactores) integrado en el propio sustrato del disco ha permitido, además, aumentar el número de muestras que pueden ser analizadas simultáneamente.

- Usando la amplificación isoterma LAMP, se han integrado por primera vez las fases de amplificación y detección en un dispositivo basado en la tecnología de disco compacto, permitiendo la monitorización en tiempo real de la reacción.

  Esta estrategia ha permitido llevar a cabo la detección cuantitativa de 96 muestras en 15 min, alcanzando límites de detección de 10 µg/g y 5 CFU/mL, para carne de ternera y *Salmonella* spp., respectivamente.

Estos resultados son comparables a los obtenidos mediante los métodos de referencia (rt-PCR), con la ventaja de ser económicamente mucho más competitivos.

Los sistemas biosensores propuestos demuestran que la combinación de las reacciones de amplificación isoterma con la tecnología de disco compacto constituyen una herramienta prometedora para llevar a cabo ensayos rutinarios en puntos de control. Además, las estrategias propuestas no requieren de personal especializado y equipos complejos, haciéndolos muy adecuados para su aplicación en ámbitos tales como laboratorios descentralizados, plantas de producción o en campo.