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Additional Information

Allele-specific ligation and recombinase polymerase amplification for the detection of single nucleotide polymorphisms

Ana Lázaro[a], Eric Seiti Yamanaka[a], Ángel Maquieira[a,b,c]*, Luis A. Tortajada-Genaro[a,b,c]*

(a) Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022, Valencia, Spain

(b) Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València-Universitat de València, Valencia, Spain

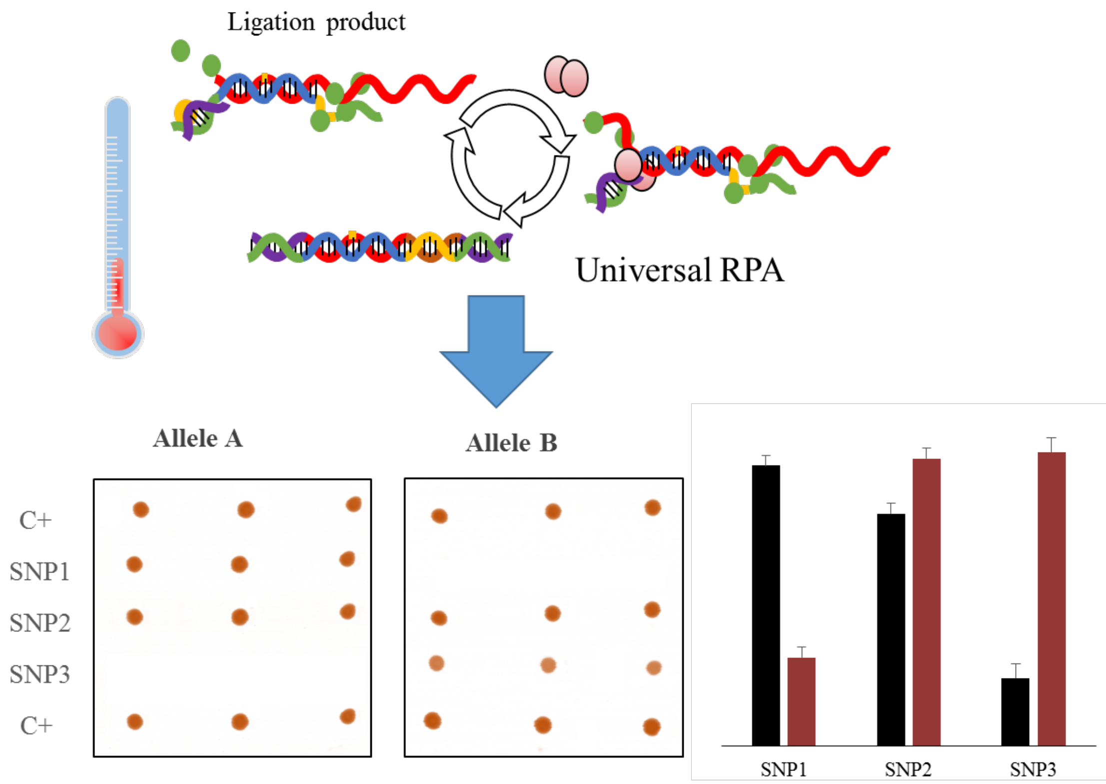
(c) Unidad Mixta UPV-La Fe, Nanomedicine and Sensors, IIS La Fe, Valencia, Spain

ABSTRACT

A novel multiplex detection of single nucleotide polymorphisms (SNPs), with point-of-care testing as its aim, is reported for supporting pharmacogenetic-based decisions. The strategy relies on allele-specific ligation to discriminate base sequence variations at the SNP site and the extension of generated products by isothermal amplification and recombinase polymerase amplification (RPA). Having demonstrated the assay principle, the variables for the adequate integration of the ligation-amplification process were studied and compared to a conventional PCR approach. One key result was the development of RPA in a universal format using short-length primers, which enabled detection based on selective hybridisation on a barcode-DNA chip and a low-cost optical sensor. As proof of concept, we successfully discriminated genetic variants related to cardiovascular diseases and the adequate prescription of oral anticoagulant antagonists of vitamin K (genes *CYP2C9* and *VKROC1*).

Keywords: universal RPA; ligation; SNP; optical sensor; hybridization chip; personalised healthcare.

31 **GRAPHICAL ABSTRACT**



32

33

34 **INTRODUCTION**

35 The implementation of personalised healthcare needs better accessibility to genetic
36 information given current technological and economical barriers [1]. Although the use of
37 sequencing techniques increases every year, this approach is expensive or unaffordable
38 in many clinical scenarios [2]. Thus simple, accurate and cost-effective solutions are also
39 required as alternatives. The excellent performance of ligase-mediated detection makes
40 these techniques one of the preferred alternatives for routine genotyping applications
41 [3,4]. In order to achieve the required sensitivity, ligation has been coupled to an
42 amplification step prior to a quantification technique, such as capillary electrophoresis
43 [5], chemiluminescence [6], bead-based colorimetric detection [7], chip-based
44 fluorescence [8], real-time fluorescence [9] and chip-based reflection [10]. However, a
45 pending challenge is a faster response time and an easier operation system to be integrated
46 into and miniaturised on a compact platform.

47 Isothermal amplification approaches, which replicate nucleic acids at a constant
48 temperature, are a powerful alternative to conventional PCR, and they open up new ways
49 to achieve the required sensitivity in point-of-care devices [11]. In the last few decades,
50 particular rolling circle amplification (RCA) features have been extensively exploited for
51 ligase-based genotyping [12,13]. Recently, loop-mediated isothermal amplification
52 (LAMP) has been integrated into a ligation method for the end-point detection of
53 microRNAs [14].

54 This study addresses the recombinase polymerase amplification (RPA) of allele-
55 specific ligation products. This isothermal technique combines enzymes to facilitate the
56 binding of primers to the DNA target and the stabilisation of reaction intermediates to
57 avoid heating double-stranded nucleic acid for template separation [15]. With the
58 adequate integration, the potential advantages are a simplification of the assay platform
59 (materials, dimensions and bonding technique), and the demanded equipment [16,17]. In
60 fact, this technique is also compatible with fast-response diagnostic and equipment-free
61 approaches [18].

62 Our research aims to face the complex challenge of the simultaneous amplification
63 of several products generated in an enzyme-mediated ligation, despite the limited
64 multiplexing capability of RPA [19]. The research hypothesis states that generic primers
65 might enable an increment in copy numbers for all the formed ligation products (universal
66 RPA). Regarding detection, RPA has been combined with conventional techniques [19]
67 until advanced hybridisation methods on several platforms, such as microtiter plates [20],

68 microdevices [21] and arrays [22]. In the present study, a novel approach based on a
69 barcode chip and a portable optical sensing device is developed. The expected advantages
70 are high-throughput capability and easy selection of hybridisation conditions, a flexible
71 working range, and better assay selectivity and sensitivity [23,24]. In fact a similar
72 approach has recently been successfully applied for ligation-PCR products [10].
73 As the RPA-based method should require a shorter incubation time (30-90 min), a
74 lower constant temperature (35-40ºC) and a simpler protocol (i.e. single incubation step),
75 an integrated assay would be more amenable for point-of-care applications. As
76 demonstrated, the proposed methodology was applied to the genotyping of single
77 nucleotide polymorphisms (SNPs) in the pharmacogenetics field. These methods are
78 required for the discrimination of adverse drug reactions given their significant impact on
79 public health in terms of patient status, death rates and healthcare costs [25].
80

81 **MATERIALS AND METHODS**

82 **Principle of SNP genotyping**

83 The method combines a ligation and an isothermal amplification in a universal
84 format (Fig. 1a and 1b). The first stage is based on using ligase and two specific
85 oligonucleotides per genomic variant. In the presence of the target nucleotide, the left
86 probe with a 5′-tail (forward universal primer) ligates to the right probe with a 3'-tail
87 (reverse universal primer). In a second stage, these specific tails enable isothermal
88 amplification. Indeed the mechanism is based on the action of binding proteins and
89 recombinase to yield a cyclic process of primer annealing and enzymatic extension. The
90 universal design of the primers leads to the amplification of all the products
91 simultaneously with a single primer pair.
92 One main advantage of this method is its multiplexing capability. The incorporation
93 of a barcode into the ligation tail enables detection based on the selective hybridisation
94 of each ligation amplification. In a third stage, each allele-specific product hybridises to
95 the complementary probe attached to the chip surface (Fig. 1c). The resulting image
96 pattern can be related to a precise genetic profile.
97

98 **Ligation**

99 Two reaction solutions contained the correspondent ligation probes at 50 nM (allele
100 wild-type or mutant mixture) and 30 ng genomic DNA in Tris-EDTA buffer (Tris-base
101 10 mM, EDTA 1 mM, pH 8) was prepared. After probe annealing (5 min, 98°C and 30

102 min, 65ºC), the ligase (Salsa Ligase-65, MRC-Holland, The Netherlands) was added. The
103 solutions were incubated for the ligation process (54°C, 15 min) and enzyme deactivation
104 (98°C, 5 min). In each assay batch, the ligation control analysed the genomic DNA
105 samples together. For optimisation purposes, another ligase (ampligase, Lucigen-
106 Epicentre, USA) was also studied.
107
108 **Isothermal amplification**
109 The reagents used for universal RPA were TwistAmp Basic RPA kit (TwistDx,
110 UK). Two reaction mixtures (12.5 μL) were prepared with rehydrated buffer, 14 mM of
111 magnesium acetate, 400 nM of the associated upstream primer
112 (ACTTCGTCAGTAACGGAC or GAGTCGAGGTCATATCGT), 400 nM of the
113 downstream primer (GACTCACTATAGGCAGAC), 10 μM of digoxigenin dUTP and
114 1.25 μL of the ligation product. The solutions were heated at 37ºC for 40 min in an oven.
115 In each assay batch, the amplification controls were analysed together with the ligation
116 products.
117
118 **Detection for single assays**
119 The RPA products from the discrimination assay of a single polymorphism were
120 visualised by agarose gel electrophoresis. After clean-up by silica-gel membrane
121 adsorption (PCR purification kit, Jena Bioscience, Germany), electrophoretic separation
122 was done in 3% agarose gel, at 110 V, fluorescent dye (Realsafe Nucleic acid Staining
123 Solution 2×, Real Lab., Spain). Products were detected after both the addition of the
124 fluorescent dye and the measurement of the response in a microplate reader (Wallac,
125 model Victor 1420 multilabel counter, Finland).
126
127 **Detection for multiplexed assays**
128 The amplification products of several polymorphisms were simultaneously detected
129 by a hybridisation assay based on Blu-Ray technology (chip and reader). The layout of
130 the barcode probes enabled the simultaneous analysis of 36 samples per disc
131 (Supplementary Information). The ligation-RPA product (6 μL) was mixed with
132 hybridisation solution (21 μL), composed of SSC buffer 3× (sodium citrate 45 mM, NaCl
133 450 mM, pH 7), 20% formamide, and 2.5× Denhardt's reagent. A positive hybridisation
134 control (labelled amplification product of *ACTB* gene) was added (3 μL). The solution

135 was denatured at 92°C for 10 min and transferred to the array surface. Discs were
136 incubated at 37°C for 45 min in a conventional oven, and gently washed for 1 min first
137 with SSC 0.1× and then with SSC 0.01×.

138 Chip colorimetric staining was based on incubation with conjugated antibodies
139 (anti-digoxigenin) and the addition of colorimetric substrate (Supplementary
140 Information). Finally, the disc was placed into the Blu-Ray drive and scanned by the
141 pickup laser (405 nm). The reflected light was collected and digitised to generate
142 monocromatic images (tagged image file format with resolution of 16 bit). The optical
143 intensity signals of each spot were quantified using in-home software.

144

145 **Sample analysis**

146 The assay performances were evaluated by applying the method for the genotyping
147 of single-nucleotide changes from human samples. The biosensing method was applied
148 to the SNPs associated with widely prescribed drugs in primary care, such as heart
149 diseases [26]. The target variants were rs1057910, rs1799853 and rs9923231 located in
150 genes *CYP2C9* and *VKORC1*. Primers and probes were designed according to the
151 thermodynamic parameters associated with the perfect-match and mismatched duplexes
152 (Supplementary Information). Several quality controls were carried out to ensure that
153 each step and the entire assay were correctly performed.

154 Subjects (n=30) were recruited for the present study according to ethical guidelines.
155 The DNA extracts from buccal smear samples were diluted to 4 ng/µL (1,300 copies) and
156 analysed as described in the previous sections (multiplexed format).

157 For assignation purposes, a genotype decision rule was constructed based on a spot
158 signal-to-noise ratio for both allele-specific products. The automated algorithm enabled
159 signal transformation and sample classification. Firstly, responses were converted into
160 polar coordinates ($\theta_{normalized}$, r). Then patients were grouped into the correspondent
161 population based on their relative position.

162

163 ## RESULTS AND DISCUSSION

164 ### 1. Set-up of the ligation-universal amplification method

165 The main research challenge to discriminate single-base changes in several targeted
166 genes is the combination of selective ligation and RPA in a universal format. The *in-silico*
167 method was employed to select probes by considering that the ligation oligonucleotides
168 must incorporate primer sequences as a tail for their latter isothermal amplification. The
169 thermodynamic-based design provided sequences without homology to other sequences
170 in the human genome or stable secondary structures. Initial experiments were performed
171 using artificial ligation products to confirm the correct selection of oligonucleotides
172 (Supplementary Information). One relevant result was about RPA primer length
173 compared to conventional approaches that recommend values between 30 and 35 [15].
174 Shorter sequences (18 nucleotides) provided sensitive and precise amplification yields (>
175 10^7) with no loss of selectivity. These performances agreed with those previously
176 reported, where short nucleotides can be used for certain biosensing applications [27]. In
177 short, the isothermal amplification of ligation products, which included the selected tails,
178 was feasible.

179 The following experiments focused on ligation by studying two approaches that
180 rely on the selective activity of ligase-65 and ampligase, respectively. The first enzyme
181 is used in the technique called multiplex ligation-dependent probe amplification (MLPA)
182 [5]. The second enzyme has been employed in different ligation-based techniques [9].
183 The objective was to establish the conditions for which two probes were bound adjacently
184 on a target sequence, ligated and amplified. Negative responses (signal-to-noise ratios
185 below 3) were obtained in all the phials with incomplete reaction mixtures or non-
186 complementary templates. Positive assays were reported for the reaction, which included
187 perfect-match probes to the DNA template, and demonstrated that both ligation
188 approaches were compatible to the RPA mechanism-based amplification (Fig. 2a).
189 Nevertheless, the ligase-65 reactions (enzyme 1U, 60ºC, 15 min) provided higher and
190 more reproducible signals than the ampligase reactions in the presence of the
191 complementary target DNA.

192 Regarding the RPA conditions, the saturated signals for the perfect-match duplexes
193 were achieved after a 40-minute incubation with universal primers and ligation products.
194 This amplification kinetics agreed with previous studies based on conventional RPA [15].
195 Figure 2b shows that the primer concentration for the amplification based on a universal
196 format was comparable to previous studies (400 nM). It is worth emphasising the low

197 responses of the negative controls because one important drawback of ligation-based
198 methods is false-positives (i.e. absence of template DNA) [4]. Another relevant result was
199 the amount of ligation solution required for correct isothermal amplification (Fig. 2c).
200 The best results were achieved for a dilution factor of 1:5 because excessive dilution
201 reduces the final number of copies, and higher ligation solution values drastically
202 modified the RPA conditions, probably due to buffer capacity and the inhibiting
203 environments of the ligation components. A parallel study was performed to compare the
204 amplification yields in a thermal cycling regime (PCR) and the isothermal mode (RPA).
205 Statistics analyses confirmed that both methods provided comparable mean signals (t-
206 test, $p>0.05$) and comparable standard deviations (F-test, $p>0.05$) (Fig. 2d). Compared to
207 ligation-PCR methods, ligation-RPA requires less thermal variations and less precise
208 heating/cooling technologies. These features could favour an easier miniaturisation and
209 automation (i.e. centrifugal-based microfluidic system) [11,17,28].
210

211 **2. Set-up of optical detection**

212 The detection of a single polymorphism or mutations was easily achieved by
213 conventional approaches, such as gel electrophoresis and fluorescent reading. The
214 detection of a band or a fluorescent response indicated the presence or the absence of
215 nucleotide change. However, the simultaneous detection of several targets required a
216 sensing assay with multiplexing capability. Among the different techniques currently
217 available for DNA diagnostics, the detection of ligated-amplified products was
218 approached using a solid-phase hybridisation assay for its excellent performance [10,20].
219 The proposed method was based on inserting a barcode into the ligation
220 oligonucleotide. Firstly, the experiments proved that its presence did not modify the
221 ligation or amplification yields (t-test, $p>0.05$). Under the selected conditions, the
222 combination of both the isothermal enzymatic processes (ligation-amplification) was
223 feasible, and opened up an innovative path to increase assay sensitivity based on universal
224 hybridisation probes. Secondly, detection in an array format based on Blu-ray technology
225 (BD) was studied for its potential for point-of-care applications [29]. The main challenge
226 was to select suitable conditions, as described in Supplementary Information. By
227 considering the assay restrictions, different labelled-reagents and immunostaining
228 methods were compared (Fig. 3). In all the approaches, the platform preserved the general
229 optical/mechanical properties to enable the correct reading of the generated spots, with
230 sensitivity in the order of fmol. However, the staining based on labelled-

231 nucleotides/alkaline phosphatase-antibody/fast red substrate displayed better analytical
232 performance than other combinations.
233 The BD-based method was successfully compared with the DNA assay performed
234 on planar plastic chips and the later imaging using a conventional scanner. Nevertheless,
235 BD technology displayed appealing features compared to other analytical platforms
236 [4,30]. Firstly, the high-hydrophobic nature of the disc surface is interesting due to the
237 RPA mixture composition (low background signals). Secondly, a high-resolution reader
238 is suitable for high-density arrays of several barcode probes. Thirdly, chip/reader features
239 (optical, mechanical, etc.) match an accurate, versatile and affordable detection system
240 for point-of-care scenarios [11]. In summary, the developed method is an interesting
241 approach as portability, simplicity and low cost in both acquisition and maintenance are
242 required in diagnostic systems that use nucleic acids as biomarkers [25].
243
244 **3. Multiplex evaluation**
245 The simultaneous discrimination of several polymorphisms was studied. The first
246 task was to select compatible ligation probes based on a similar estimated stability of
247 hybrids (template-ligation probe and RPA product-barcode) and comparable alteration
248 due to the presence of a single-nucleotide mismatch (Supplementary Information). The
249 determined features estimated a feasible discriminatory analysis of three targets
250 simultaneously given the low cross-reactivity probability.
251 The following activities focused on the experimental confirmation. The optimised
252 method was tested using different formats from an assay run for the genotyping of a single
253 SNP (1-plex) to an assay for three polymorphisms (triplex). Table 1 shows the recorded
254 spot signals in each case, indicating that no molecular recognition was produced for the
255 mismatched templates (null target-to-target cross-reactivity). The recognition profiles
256 corresponded precisely with the expected perfect-match complexes (probe-template),
257 independently of assay multiplexing. The results demonstrated that all the sets of ligation
258 oligonucleotides were correctly ligated and amplified in the same solution to yield
259 unequivocal hybridisation. Therefore, our method involves a higher multiplexing
260 capability approach compared to other approaches, such as performing an oligonucleotide
261 ligation assay after the isothermal amplification of a specific region [30].
262

263 **4. Demonstration of accurate SNP scoring**

264 Method performances were assessed for the classification of patients suffering
265 cardiovascular diseases. In a pilot study, 30 different individuals were genotyped by
266 identifying the specific variant for each targeted polymorphism according to the
267 hybridisation patterns (Supplementary Information). A heterozygote patient led to
268 positive amplifications for both RPA reactions and consequent hybridisation in both
269 probes. A homozygous patient produced single positive amplification and consequent
270 hybridisation in the specific probe. Nevertheless, an automated algorithm was applied to
271 reduce patient classification uncertainty due to residual signals. The assignation was
272 based on its position on the discrimination map (Fig. 4). The output was that a nucleotide
273 change was detected in 12 (40%), 12 (40%) and 19 (63%) cases for rs1799853 (*CYP2C9*
274 gene), rs1057910 (*CYP2C9* gene) and rs9923231 (*VKORC1* gene), respectively.
275 Therefore, the uncertainty of a drug treatment's individual efficacy could be minimised
276 based on the specific genetic profile. A revision of anticoagulant drug doses is
277 recommendable for mutant individuals [26].

278 Assay reproducibility was determined from the replicated samples and was
279 expressed as a relative standard deviation. The values were 6% for the intra-assay and
280 19% for the inter-assay. Assay accuracy was validated by the agreement of the genotypes
281 assigned by the independent sequencing of patient samples (Supplementary Information).
282 Although further research is needed, the investigated assay showed certain advantages
283 compared to sequencing methods. It provided key genetic information for personalised
284 diagnostic, prognostic and therapy selection tools more simply and cheaply. The
285 developed approach can be potentially extrapolated to other screening genomic
286 applications because the assay principles are based on universal formats. The biosensing
287 assay is compatible to the technologies used for decentralised scenarios, such as a doctor's
288 office, remote locations, emergency needs and ubiquitous low-resource health systems
289 [2,3,11,19,29].

290

291 **CONCLUSIONS**

292 The developed method exploits the advantages of ligation-mediated discrimination
293 as an effective tool to detect single base-pair variations. Nevertheless, its novelty lies in
294 the way that the required detection sensitivity is achieved: an isothermal and universal
295 method. Multiplexing capabilities and integration potentiality (i.e. combination to
296 portable instruments) are higher than other PCR-mediated methods.

Regarding the health impact, the assay allows a screening DNA diagnostic and opens up new avenues of personalised medicine from tailored therapies to direct-to-patient approaches. Given its excellent versatility, the method can be useful for the simultaneous detection of a large number of single base-pair polymorphisms or mutations. The recommendation for effective development is a conscientious design of oligonucleotides to guarantee the specific ligation, amplification and hybridisation of targeted variations.

Finally, the integrated platform fulfils the demanded requirements for next-generation genetic testing devices. The resulting system is robust, simple, sensitive and cost-effective, and can be easily converted into a biosensing device for onsite detection with limited laboratory infrastructure.

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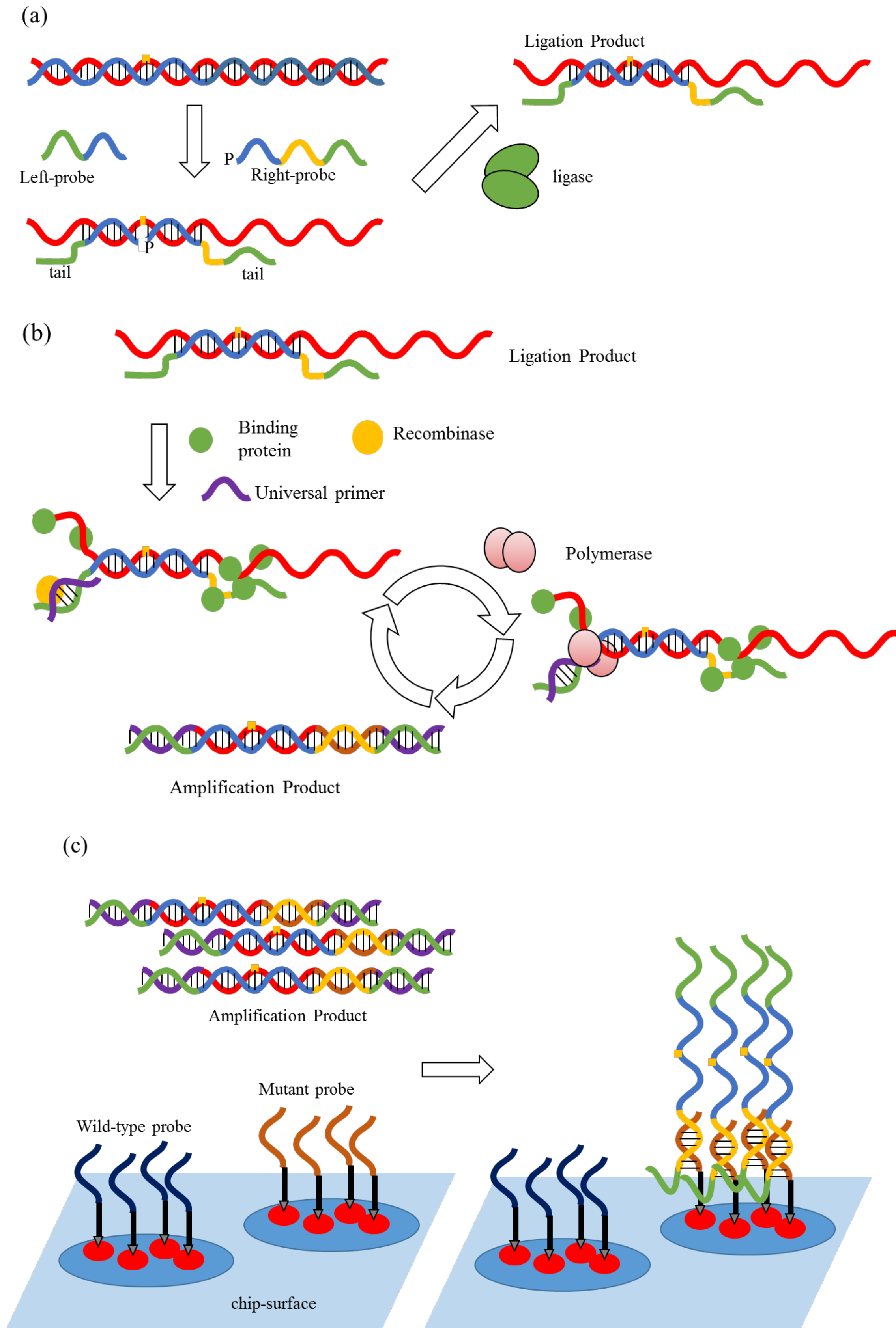

Fig. 1. Scheme of the multiplexed discrimination assay for single-base changes (polymorphisms or mutations): (a) allele-specific ligation. (b) Universal RPA. (c) Hybridisation in an array format.

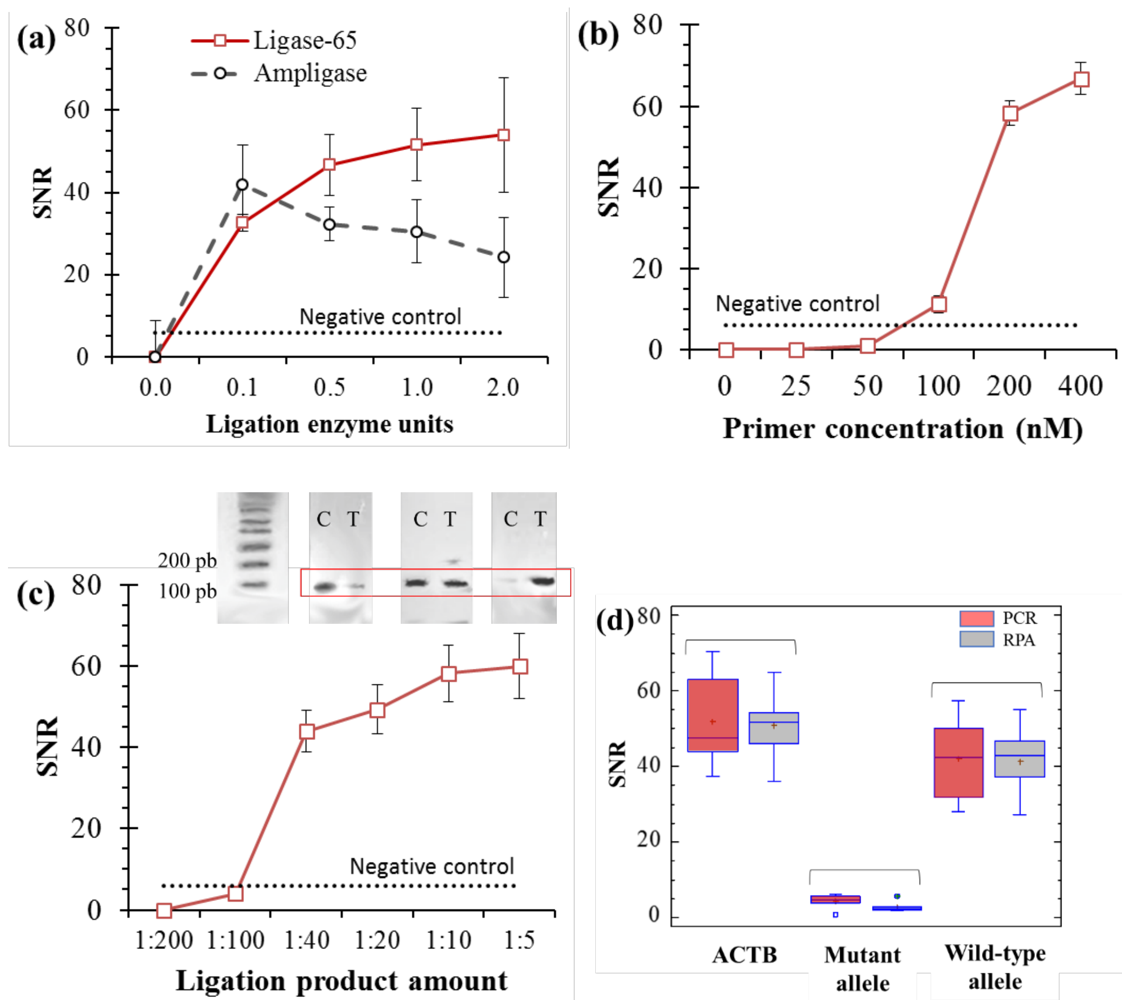

Fig. 2. Integration of ligation and universal RPA. (a) Ligation enzyme concentration. (b) Amplification primer concentration. (c) Ligation product amount. (d) Comparison of PCR (red) and RPA (blue) for different reaction mixtures. SNR: signal-to-noise ratio. DNA template: 1000 copies (wild-type). Reference genes: *ZFX* and *ACTB*. Tested gene: *VKORC1*. Expected product length: 103 pb.

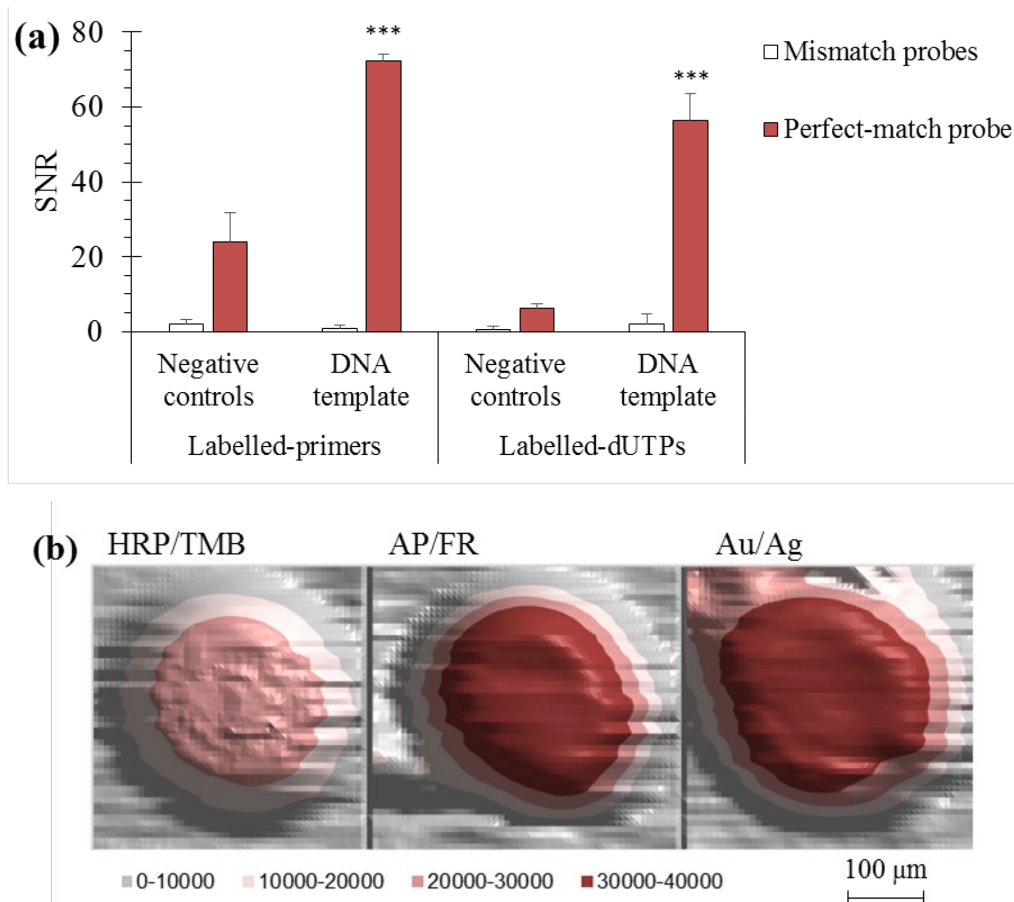

Fig. 3. Optical detection of RPA products (array format). (a) Effect of digoxigenin-labelling method during amplification by comparing 5'-functionalised universal forward primers and labelled 2'-deoxyuridine 5'-triphosphate (dUTPs). (b) Response curve of an array spot depending on the staining method. DNA template: 1,000 copies. Sample: genomic DNA mutant in the *VKORC1* gene. ***: p-value <0.001. SNR: signal-to-noise ratio. HRP/TMB: Horseradish peroxidase system. AP/FR: Alkaline phosphatase system. Au/Ag: Nanoparticle system.

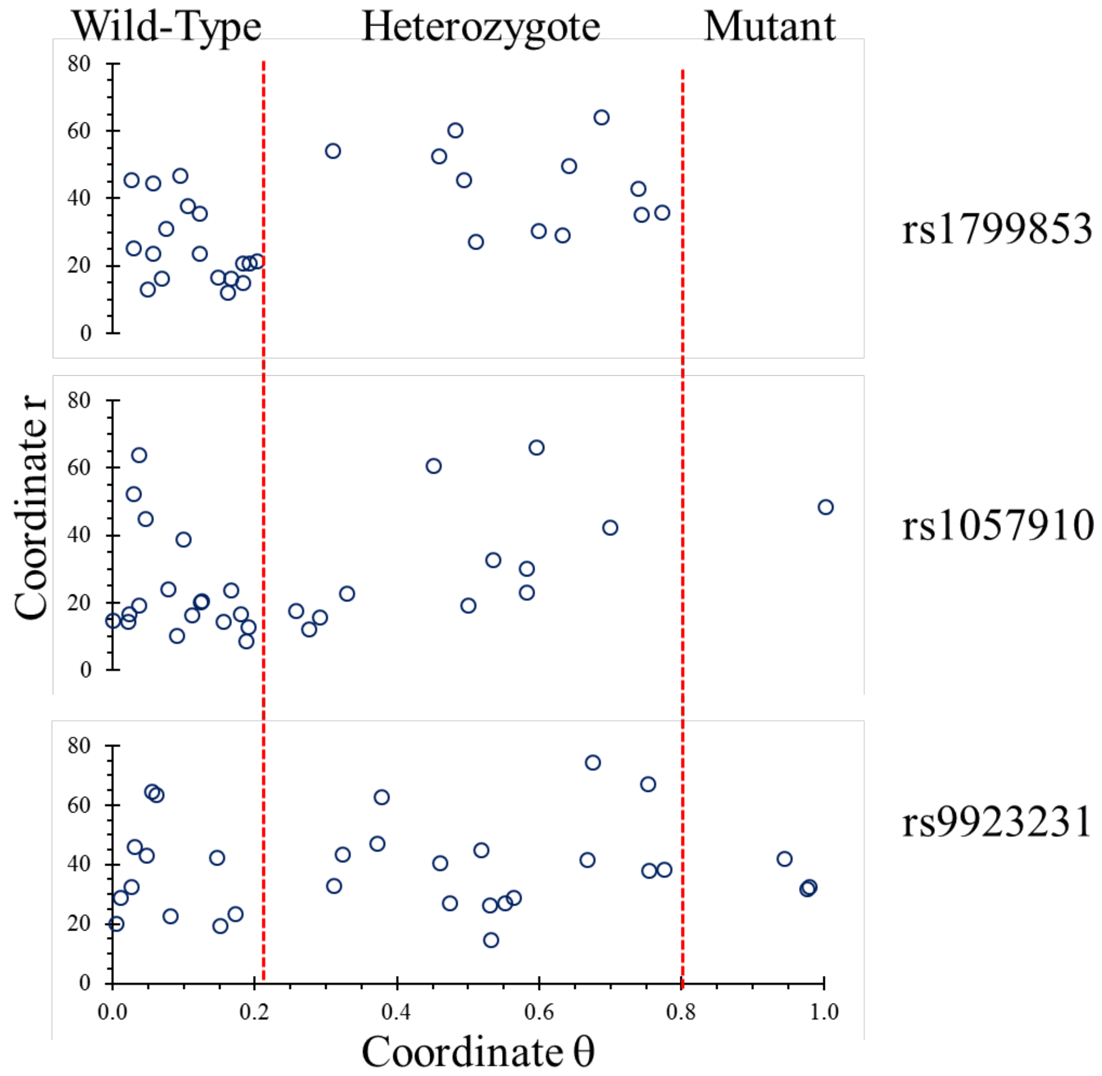

Fig. 4. Discrimination map plotted from the response signals of patients (wild-type and mutant reactions) for each polymorphism.

Table 1. Spot mean SNRs depending on the ligation mixture (single, duplex or triplex) and the forward primer used in the amplification reaction (wild-type or mutant). Patient 1: heterozygous in rs1799853, wild-type in rs1057910, and wild-type in rs9923231. Patient 2: wild-type in rs1799853, wild-type in rs1057910, and heterozygous in rs9923231.

			Array probe[b]					
			Wild-type RPA			**Mutant RPA**		
	Mix[a]		**rs1057910**	**rs1799853**	**rs9923231**	**rs1057910**	**rs1799853**	**rs9923231**
Patient 1	**single**	**1**	46±10	6±3	2±3	46±3	2±3	4±2
		2	4±2	34±7	3±2	5±2	6±5	2±1
		3	4±2	6±6	61±4	5±9	6±3	7±1
	duplex	**1-2**	67±8	64±5	7±3	45±2	5±3	6±5
		1-3	74±9	6±6	73±10	65±7	8±2	7±3
		2-3	7±3	58±7	80±5	6±3	6±4	6±3
	triplex	**1-2-3**	57±6	30±4	52±4	55±5	10±3	5±2
Patient 2	**single**	**1**	26±5	4±2	3±1	9±5	6±3	2±1
		2	3±1	29±1	2±1	3±2	3±2	3±2
		3	1±2	4±2	57±3	2±2	2±3	27±8
	duplex	**1-2**	42±12	25±5	3±2	5±5	4±2	3±2
		1-3	60±13	3±2	44±10	5±5	3±2	66±6
		2-3	4±1	39±5	67±7	1±2	4±1	48±3
	triplex	**1-2-3**	49±8	35±3	56±1	7±4	3±3	37±2

[a] Ligation mixtures, 1: rs1799853, 2: rs1057910, 3: rs9923231

[b] Underlined values indicate a significant difference from the negative control response (t-test, p-value > 0.05).