STRATEGIES FOR RAPID AND REAGENT-LESS ELECTROCHEMICAL DETECTION OF RPA PRODUCTS

Sallam Mohammed Ahmed Al-Madhagi

Strategies for rapid and reagent-less electrochemical detection of RPA products

Sallam Al-Madhagi

DOCTORAL THESIS

Tarragona

2018

Strategies for rapid and reagent-less electrochemical detection of RPA products

Sallam Al-Madhagi

DOCTORAL THESIS

Supervised by:

Prof. Ioanis Katakis

Prof. Ciara K. O´Sullivan

Department of Chemical Engineering

Tarragona, 2018

UNIVERSITAT ROVIRA i VIRGILI

Department of Chemical Engineering
Universitat Rovira i Virgili
Av. Països Catalans 26
43007 Tarragona, Spain
Tel: 977 55 96 58
Fax: 977 55 96 67

I STATE that the present study, entitled "Strategies for rapid and reagent-less electrochemical detection of RPA products", presented by Sallam Mohammed Ahmed Al-Madhagi for the award of the degree of Doctor, has been carried out under my supervision at the Department Chemical Engineering of this university.

Doctoral Thesis Supervisor

Prof. Ioanis Katakis

Prof. Ciara O'Sullivan

Tarragona, July 5th, 2018

Summary

Nowadays, there is a need to develop a rapid, simple, inexpensive and reliable DNA testing system for diagnosis in different fields such as genetic diseases, pathogens detection, forensics, and personalised medicine.

In this work, the isothermal amplification and modified tailed primers to simplify the steps required for the electrochemical DNA detection are combined.

Modified tailed primers are based on a single stranded oligonucleotide sequence linked to a carbon spacer, which effectively blocks elongation, prior to the primer sequence. Thus, resulting in an amplicon with a duplex flanked by two single stranded DNA tails. One of the tails was used to hybridise to a surface immobilised probe and the other to an enzyme or gold nanoparticles labelled reporter probe. Using these modified primers allowed us to detect DNA electrochemically without any need for post-amplification sample treatment decreasing the assay time and presenting an approach that can facilely find the application at the point of need.

In this work, three different methods have been proposed and investigated in parallel based on using modified primers and isothermal amplification.

In the first method (Chapters 2), an extremely rapid and sensitive DNA amplification and detection assay has been developed based on combining tailed primers and isothermal amplification. Using the HLA-DQB1*02 allele as a model system, the sensor demonstrated the ability to amplify and detect DNA in a few minutes without the need to perform any post-amplification treatments.

In the second method (Chapters 3), a geno-sensor has been developed for the direct detection of the RPA products without the need to purify, create ssDNA or perform labelling process. The sensor developed based on using a forward primer modified with ssDNA tail and reverse primer-linked with HRP. Using this combination of primers allow us to produce an amplicon of duplex target specific DNA, flanked by single-stranded DNA tail on one end and HRP on the other using karlodinium armiger DNA as a model system.

In the third method (Chapters 4), a reagent-less geno-sensor has been developed based on using tailed primers and AuNPs modified with thiolated ssDNA and 6-(Mercaptohexyl)ferrocene as an active redox label for electrochemical detection. Using these modified primers along with modified AnNPs allowed us to detect karlodinium armiger DNA electrochemically without any need for post-amplification sample treatment.

Chapter 1

Introduction

1.1. Biosensors

According to the IUPAC, the biosensor is a self-contained integrated device that uses specific biological recognition element-mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals to provide specific quantitative or semi-quantitative analytical information [1][2].

The biosensors consist of three main parts, a biological recognition element (biochemical receptor) which is retained in direct spatial contact with the transduction element, a transducer, and a signal processing system[1].

Biosensors can be classified based on transducer used four types to electrochemical, optical, piezoelectric and calorimetric [3].

Biosensors can be also classified based on the biological recognition element (biochemical receptor) to enzyme, immuno, DNA, cells, tissues, etc)

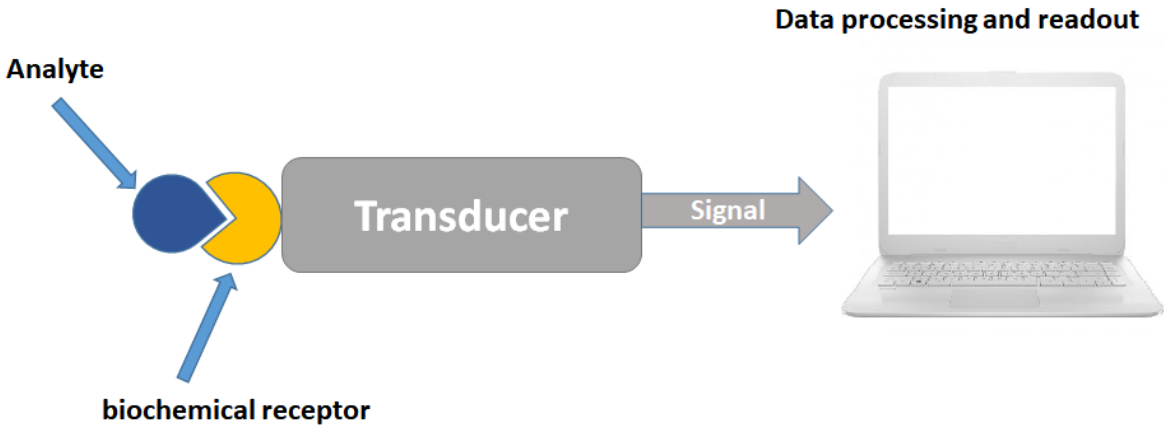


Figure 1.1. The main components of the biosensor.

1.2. DNA sensors

DNA biosensor (Geno-sensors) is the type of biosensors witch based on oligonucleotide probes immobilised over the signal transducer as a biological recognition element, this immobilised recognition single strand DNA (ssDNA) is normally used for the hybridisation to specific target sequences, converting it to a quantitive or qualitative signal[4].

1.2.1. Probe immobilisation

Probe immobilisation over the transduction element is an essential factor in the development and overall performance of the geno-sensors. A good DNA probe immobilization technique would promote a high sensitivity and selectivity via maximisation of the hybridisation efficiency and minimisation of non-specific adsorption. Several DNA probe immobilization techniques have been employed in electrochemical DNA sensing such as adsorption methods, covalent bonding, and avidin-biotin interaction[3][5][6][7].

1.2.1.1. Adsorption

Adsorption is the simplest techniques for DNA probes immobilization to build up the recognition part of the biosensor. Adsorbing DNA to the sensor surface does not require any chemical reagents or DNA probes modification[8][9][10][11][12][13][14][15].

This immobilisation occurs via electrostatic adsorption between the negatively charged DNA and positively charge transduction part. Positive potential could be applied to enhance the electrostatic interaction between the negatively DNA probe and the positively charged transducer surface[16].

1.2.1.2. Avidine/ Straptividine – Biotine interaction

The affinity between biotin and streptavidin (Ka= 1015 M^{-1}) is the strongest non-covalent biological interaction known. Each streptavidin monomer can bind to one biotin molecule, allowing a streptavidin protein to maximally bind four biotins[17].

The specificity, rapidity, Resistant to changes in temperature or pH leads it to be used in biosensors to build up the recognition element[18].

1.2.1.3. Covalent attachment

Chemisorption and covalent attachment are the two common covalent attachment methods for the immobilization of DNAs on the surface reported in the literature[19][20][21][22][23][24][12][25][26][27][28][29][30][31][32][33]. DNA probes immobilization via covalent attachment to the transducer surface demonstrated a good stability, flexible, highly binding strength and prevent desorption of DNA probe monolayer from the electrode surface[34][35]. On the other hand, covalent bonding technique provides a good vertical orientation where the end of DNA probe was grafted on the electrode surface which can result in high efficiency of DNA hybridization. In covalent bonding method, the DNA capture probe is typically linked with the group of thiols (SeH) or amines (NH2) at the end of 3′ or 5′ to bind covalently to the metal surface or specific functional group introduced to the electrode surface[21]. Gold and metal surfaces have been extensively used in biosensing applications, especially as thiols (R−SH), disulfides (R−S−S−R), and sulfides (R−S−R) strongly adsorb onto metal surfaces forming self-assembled monolayers (SAMs)[36]. Thiolated oligonucleotide probes have been commonly used for direct immobilisation on gold electrodes to form the recognition layer of the geno-sensor by making Self-assembled monolayers SAM through chemisorption by S linkages. The absence of impurities and contaminations from the substrate surface is preferable but not absolutely essential because thiols are able to detach contaminating compounds from the gold because of the high affinity of the sulfur groups to gold [36]. Self-assembled monolayers obtained utilizing thiol-tethered oligonucleotides mixed with alkanethiols such as mercaptohexanol represent a simple yet

effective means to control the density and availability of the capture probe. In addition, using alkanethiols displaces the non-specifically adsorbed probe molecules, while leaving the remaining ones in an upright position. Thus, the orderly arrangement of probes results in an increased hybridization efficiency. An additional feature of this immobilisation chemistry is the stability of the surface-attached monolayer of biomolecules.

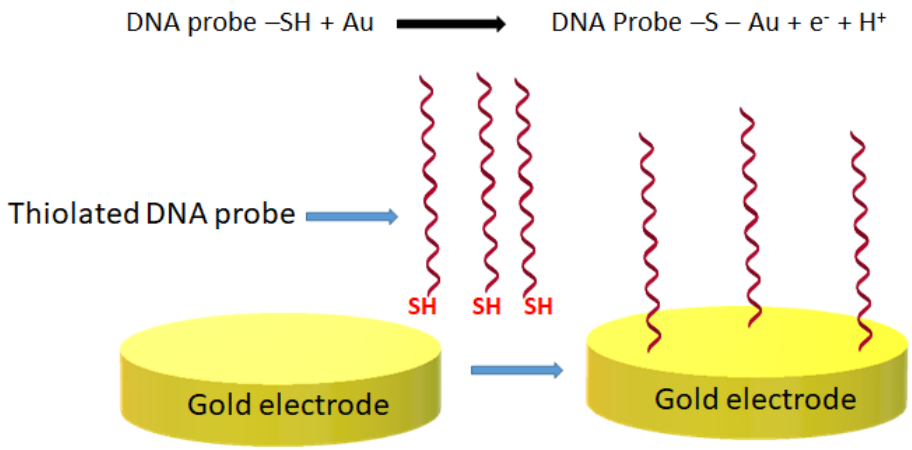


Figure 1.2. Immobilisation of the thaiolated DNA probe on gold electrodes.

Table 1.1. Comparative table of DNA probe immobilisation methods.

Immobilisation method	Sensor surface	DNA modification	Interactions	Advantages	Drawbacks	Ref.
Adsorption	Positively charge surface	Un modified DNA	Charge-charge interaction	• Simple • Fast • No need for linker molecules	• Random orientation • Desorption by detergent	[8][9][16]
Streptavidin-Biotin	Streptavidin Biotin	Biotin Streptavidin	Specific Streptavindin-Biotin interaction	• Improved orientation • High specificity and functionality • Well-controlled • Reversible	• Expensive, Slow • Use of biocompatible linker • Poor reproducibility	[8][9][16]
Covalent attachment	Au COOH CHO CHCH2O N=C=S $HC(CO)_2NH$ Si-R-SH	Thiols (-SH) Amines (-NH2) Amines (-NH2) Amines (-NH2) Amines (-NH2) Thiols (-SH) Thiols (-SH)	Chemical bonding	• Improved orientation • High specificity and functionality • Reversible	• Use of linker molecules • Slow • Irreversible • Island formation	[8][9][16]

1.3. Electrochemical biosensors

Electrochemical biosensors are the most commonly used class of biosensors due to its high sensitivity, minimal power requirements, simple design, suitability, portability and relatively low cost compared to other techniques[16][37] [38].

In general, electrochemical biosensor requires three electrodes: a working electrode, a reference electrode, and a counter or auxiliary electrode. The working electrode acts as the transduction element of the biochemical event, while the counter electrode establishes a connection to the electrolytic solution so that a current can be applied to the working electrode. The reference electrode is responsible for maintaining a known and stable potential at the working electrode.

Different electrochemical techniques used for developing biosensors including amperometric, voltammetric, conductimetric, potentiometric and electrochemical impedance spectroscopy, and these can be further classified into, direct and indirect electrochemical detection.

Amperometric

Amperometric biosensors are based on monitoring the current associated with oxidation or reduction of an electroactive species involved in the recognition process. The measurable current produced is linearly proportional to the concentration of the electroactive product, which in turn is proportional to the non-electroactive enzyme substrate[1] [2][5][39][40][41][42].

Potentiometric

Potentiometric measurement is based on the determination of the potential difference between an indicator and a reference electrode when zero or negligible current flows through them [43][35][38][44][41][45].

This potential difference provides information about the ion activity in an electrochemical process. The working electrode may be an ion selective electrode (ISE) based on a thin film or permselective membranes. ISEs can detect ions such as F^-, I^-, CN^-, Na^+, K^+, Ca^{2+},H^+, NH^{4-}, or gas (CO_2, NH_3) in complex biological matrices. ISEs are mainly used in clinical chemistry for the measurement of relevant electrolytes in physiological fluids and also in analytical chemistry and biochemical/biophysical research, where measurements of ion concentration in an aqueous solution are required[46].

Conductimetric

Conductimetric biosensor are based on the measurement of the electrical conductivity in a solution at a constant voltage, produced by biochemical reactions on the transducer part of the biosensor which specifically generate or consume ions[47][48][49].

Impedimetric

Impedimetric biosensors are based on the measurement of the complex electrical resistance of the system and are sensitive to surface phenomena and changes in bulk properties[50].

Based on the nature of the measuring signal, impedance biosensors can be classified into two main groups[51]:

A) *Capacitive*, where the surface of the electrode is completely covered by a dielectric layer and the whole electrode assembly behaves as an insulator. In this type of sensors, the presence of the redox probe is not required.

B) *Faradaic*, where the surface of the electrode, which is partially or wholly covered by an insulating layer which is able to catalyse a redox probe. In this case, the measured parameter is the charge transfer resistance.

Electrochemical Impedance Spectroscopy (EIS) is a promising method for developing a label-free DNA detection due to its ability to measure the electrical resistance that associated with the presence of the DNA on the working electrode surface[52][24][26].

1.4. DNA amplification

1.4.1. PCR

Polymerase chain reaction (PCR) has been developed by Kary Mullis in 1984. Amplifying DNA has revolutionised many life science applications and related areas such as clinical diagnoses, and medical, biological and forensic analyses[53].

PCR mechanism can be described briefly in three steps. Frist, Original double-stranded DNA (dsDNA) denaturation by heating at 90 to 96 °C. Second, annealing in which the primers bind to their complementary sequences in the single-stranded DNA (ssDNA) molecules.

The third step is the synthesis of a complementary nucleic acid strand-mediated by the polymerase starting from the 3'-end of the primer, at 72 °C, in a process referred to as elongation. The product is two new double helices, each composed of one of the original strands plus its newly assembled complement.

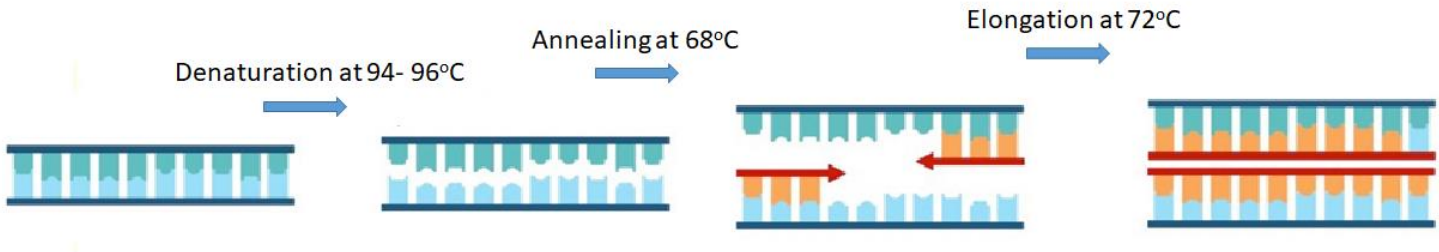


Figure 1.3. Polymerase chain reaction (PCR) mechanism

1.4.2. Isothermal Amplification

The thermal nature of PCRs that require a long time to produce copies of DNA ranges from one to two hours, this limits the possibility of transferring this technique to be used in the field or in limited sources eras[54]. Therefore, in recent years, several alternative techniques for isothermal DNA amplification have been developed[55][56][55][57][58][59][60][61].

1.4.3. loop-mediated amplification (LAMP)

loop-mediated isothermal amplification (LAMP) was originally reported by Notomi et al in 2000. LAMP. It is a one-step amplification reaction that amplifies a target DNA sequence under isothermal conditions (60°C to 65°C)[62][63][64][65][66][67][63].

LAMP mechanism starts with using a set of four target-specific primers, including a forward inner primer, backward inner primer, and two outer primers, to recognize six to eight distinct sites sequences on the target DNA. At (60°C to 65°C) one of the LAMP primers can anneal to the DNAs without a denaturing step because of the dynamic equilibrium of the double-stranded DNAs around this temperature [60]. An additional pair of "loop primers" can further accelerate the reaction by enabling the generation of a stem-loop DNA for subsequent complex LAMP cycling including self-priming reactions.

1.4.4. Exponential amplification reaction (EXPAR)

Exponential amplification reaction (EXPAR) has been devised by Galas et al. in 2003 [68] for amplification of short oligonucleotides by a combination of polymerase strand extension and single-strand nicking [69]. Exponential amplification reaction (EXPAR) normally occurs at a constant temperature around 60°C [68].

The isothermal exponential amplification reaction (EXPAR) as an alternative amplification technique has been used for the detection of DNA and miRNA[70].

1.4.5. Strand displacement amplification (SDA)

Displacement amplification (SDA) is an isothermal method developed in 1992[56][57][71][72]. The SDA reaction can be performed over a broad temperature range (37°C to 70°C). After the initial heating for the separation of the dsDNA, the primers bind sequence-specifically introducing a restriction site into the product.

Bumper primers, which bind adjacent to the first primer are elongated by a polymerase with strand displacement activity, releasing the first single stranded amplicon. In a second step, the reverse primer, including a nickable restriction site, is elongated. An endonuclease cleaves the restriction sites only at one strand, because thiol-modified nucleotides are incorporated to prevent cutting of the whole dsDNA strand. The free 3’-end is subsequently extended, displacing the new single stranded copy molecule. This process of nicking and displacing leads to exponential amplification of DNA.

1.4.6. Rolling circle amplification (RCA)

Rolling circle amplification (RCA) has been developed by Murakami et al. in 2009[56][73]. Rolling circle amplification (RCA) is an isothermal enzymatic process mediated by certain

DNA polymerases using a short DNA or RNA primer is amplified to form a long single-stranded DNA or RNA using a circular DNA template[74][75] exploiting the excellent strand displacement activity of a Phi29 bacteriophage polymerase[56].

Padlock probes are linear oligonucleotides containing two target specific sequences designed to circularise after hybridisation and subsequent ligation. The dual recognition in combination with a ligation reaction ensures specificity of detection. After that, the circular padlock probe serves as a template for the polymerase, which continuously elongates the product and displaces the generated strand [56].

1.4.7. Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence-based amplification, also known as self-sustained sequence replication (3SR) and transcription-mediated amplification TMA [60] was first described in 1991.

Nucleic acid sequence-based amplification (NASBA) is an isothermal amplification technique specifically designed for the detection of RNA targets [76]. Nucleic acid sequence-based amplification occurs at constant temperature 41ºC after initial strand separation step at 95ºC in the case of dsDNA and 56ºC in the case of the RNA amplification[77].

In the first phase, reverse DNA primers containing a T7 promoter region, bind to any available target sequence in the sample. The primers are extended by the reverse transcriptase. The resulting RNA-cDNA hybrids are degraded by the activity of RNase H, leading to cDNA single strands. A forward DNA primer hybridises to these targets forming a new template, which can be elongated by the reverse transcriptase. This step integrates the T7 promoter region into the produced DNA, allowing a T7 RNA polymerase to bind, generating complementary copies of RNA. During the cyclic process, each synthesised RNA will initiate a new round of duplication, leading to exponential amplification in around 1.5 h.

1.4.8. Helicase-dependent amplification (HDA)

HDA, firstly described in 2004. The HDA reaction is a three step cycle process (template separation, primer hybridization, and primer extension)[58][71][78]. The process initiates with helicase unwinding of dsDNA to which forward and reverse primers can bind, followed by polymerase-mediated elongation. Following elongation, helicase can again act on the freshly synthesized dsDNA and the cycle asynchronously repeats, with similar amplification kinetics to existing PCR, at 60–65ºC without further temperature steps[79].

1.4.9. Single primer isothermal amplification (SPIA)

Single primer isothermal amplification (SPIA) is an isothermal DNA amplification method developed in 2005[56][60]. This process occurs at a stable temperature (50°C) without the need of adding intermediate reagents and results in the linear amplification of DNA products within 90 min. In this isothermal amplification, chimeric RNA/DNA primers are used to bind with target regions and initiate polymerisation. The primer is engineered in such a way that RNase H degradation of the RNA portion of the chimeric primer will re-expose the binding site to allow a subsequent primer to anneal. Strand displacement activity of the polymerase removes the previously generated strand. This repeated cycle continuously generates new amplicons [79][80].

1.4.10. Signal-mediated amplification of RNA technology (SMART)

Signal-mediated amplification of RNA technology (SMART) is an isothermal nucleic acid amplification develped in 2001[81].

The SMART method consists of two single-stranded oligonucleotide probes (extension and template) that are annealed to a specific target sequence and then it forms a three-way junction (3WJ) structure. After the three-way junction formation, a DNA polymerase elongates the shorter extension probe, meanwhile synthesising the complementary strand of the template probe.

1.4.11. Recombinase polymerase amplification (RPA).

RPA has been developed in 2006 by Piepenburg et al[82]. The RPA method amplifies DNA sequences by using a recombinase, DNA polymerase and single-stranded DNA-binding proteins (SSBs) at constant temperature (37 ºC – 41ºC)[56].

The RPA cycle is initiated by the binding of a recombinase to the primers in the presence of ATP. The complex then interrogates double stranded DNA seeking a homologous sequence and promotes strand invasion by the primer at the cognate site. Its displacement activity creates a single strand, which is stabilized by SSBs. Finally, the recombinase disassembles and a strand displacing polymerase binds to the 3' end of the primer to elongate it in the presence of dNTPs[83][79].

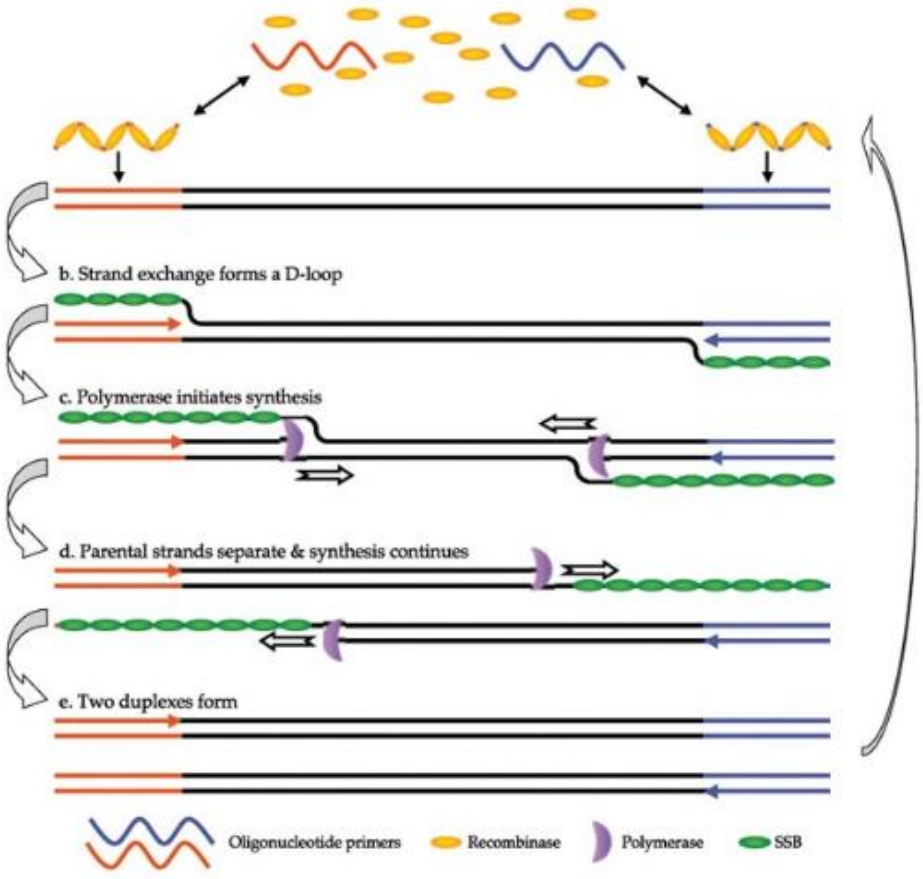


Figure 1.4. Recombinase polymerase amplification (RPA) mechanism.

Amplification method	Test temperature	LOD (Copies)	Primers	Average amplification time	Pre-heating	Number of enzymes	Target	Key publication	Year
NBSA	41°C	1	2	~ 60min	Yes (65 °C for RNA and 95 °C for DNA)	3	RNA, DNA	(Compton, 1991)	1991
SDA	37°C	10	4	~ 2h	Yes	2	DNA	(Walker et al., 1992b)	1992
RCA	23°C	1	1	~ 1.5h	No	1	DNA	Fire and Xu, 1995)	1995
LAMP	65°C	1	4, 6 or 8	~ 60min	No	1	DNA	(Notomi et al., 2000)	2000
SMART	41°C	1	2	~ 3h	Yes (95 °C)	2	DNA	Wharam et al., 2001)	2001
HDA	60°C -65°C	1	2	~ 1.5h	No	2	DNA	(Vincent et al., 2004)	2004

SPIA	45°C -50°C	-	1	~ 1.5 h	No	2-3	RNA	(Kurn et al., 2005 2005)
RPA	37°C -41°C	1	2	~ 20min	No	2	DNA	(Piepenburg et al., 2006 2006)

Table 1.2. Comparative table of DNA isothermal amplification methods.

1.5. Geno-sensors for amplification product analysis

Amplification of the specific sequence of interest is increasing the concentration of the sample. In the majority of DNA biosensors and microarrays, post-amplification single-stranded DNA (ssDNA) generation is necessary to hybridise it to the recognition element on the transducer surface. Several techniques have been used to generate ssDNA from double-stranded amplification product, including thermal denaturation, heat/alkaline treatment of avidin/biotin-dsDNA, triplex formation, asymmetric PCR and combined with magnetic beads for separation[84][85][16][20][26][48][37][86].

1.5.1. Tailed primers

In 2015 Joda et al. has developed a new strategy for detecting a DNA amplification products without the need to create ssDNA by modifying primers with carbon spacer, which effectively blocks elongation. Thus resulting produce a PCR product flanked by two single-stranded DNA tails for hybridization with probes immobilised on the surface of the electrode and with HRP labelled reporter probe[87]. This system shows the ability to detect PCR samples in few minutes without the need to de any post-amplification sample treatment.

This novel technology has been used in developing some other geno-sensors. Jauset-Rubio et al. has developed a lateral flow detection systems for DNA[88] and aptamers[89] via combining modified primers with RPA.

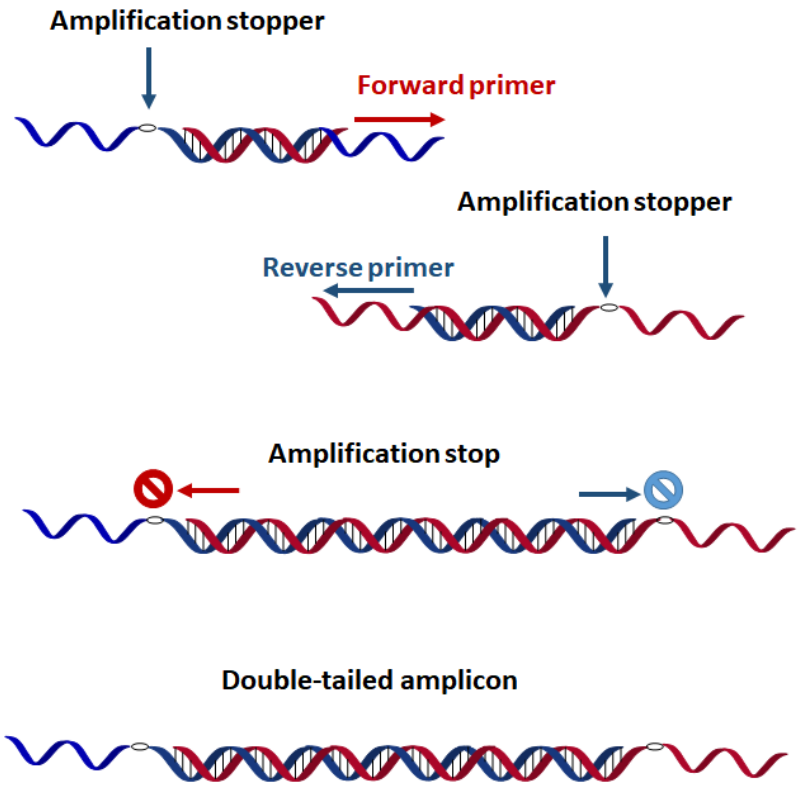


Figure 1.5. Modified tailed primers.

1.6. Recombinase polymerase amplification in combination with electrochemical detection

Since development of the RPA, many electrochemical detection systems have been developed to detect RPA products. Escosura et al. have developed an electrochemical biosensor based on using forward primers labelled with magnetic beads and reverse primers labelled with gold nanoparticles (AuNPs). The labelled amplification product immobilized on the electrode surface by a magnet then AuNPs detected directly through electrocatalytic hydrogen evolution[90].

Benjamin et al. have developed an electrochemical biosensor for the detection of tuberculosis. In their work, they have used biotin-dUTPs to produce tagged amplicons. Subsequently, amplicons tagged with biotin bonded with magnetic beads coated with streptavidin and HRP-streptavidin, followed by chronoamperometric electrochemical detection using TMB as substrate[91].

Benjamin et al. have developed another electrochemical biosensor for the detection of tuberculosis based on using biotin-dUTPs to produce tagged amplicons, at the same time streptavidin–AuNPs were bonded to the amplicons on an electrode surface, the gold nanoparticles oxidized to $AuCl^{4-}$, which can be detected by differential pulse voltammetry[92].

Another electrochemical biosensor has been developed by Lau et al. for plant pathogen detection. Modified primers have been used to generate double tagged amplicons with biotin at one end and an oligonucleotide overhang at the other. Streptavidin magnetic beads were used to purify the amplicon. Subsequently, the capture probe was used to bind to AuNP labelled with a complementary capture probe. After purification, the amplicons were drop-casted on screen-printed carbon electrodes and the gold of the AuNPs were measured using differential pulse voltammetry (DPV)[93].

Tsaloglou et al. have developed a new method for electrochemical detection of specific sequences of DNA present in trace amounts in serum or blood. In their work, they immobilized $Ru(NH_3)^6]^{3+}$ on the electrode surface. Subsequently, they monitor the decrease of the peak current with increasing the initial amount of DNA[94].

Another amplification and detection strategy has been developed based on a solid phase RPA.

Del Rio et al. have developed a solid phase RPA assay based on immobilisation of the forward primer on a gold electrode surface and reverse primer contained a biotin in the 5', with post-amplification detection achieved using streptavidin-HRP in the presence of a precipitating TMB substrate[95].

Del Rio et al. have been reported another solid phase RPA assay based on immobilisation of the forward primer on a gold electrode surface. DNA denaturation to produce ssDNA has been done after amplification on the electrode surface. Subsequently, reverse primer contained a biotin has been used to hybridize with the amplicon, detection achieved using streptavidin-HRP in the presence of a precipitating TMB substrate[96].

Table 1.3. Comparative table of electrochemical biosensors for the detection of the RPA products.

Organism	**Target**	**Amplification/ detection strategy**	**Label**	**Detection**	**Test temp.**	**LOD**	**Assay time**	**Ref.**
Leishmania	dsDNA	Liquid phase amplification/ solid phase detection	AuNPs	Chronoamperometry	37°C	0.8 parasite/ mL	10 min	[90]
Tuberculosis	dsDNA	Liquid phase amplification/ solid phase detection	HRP	Chronoamperometry	38°C	1 CFU	20 min	[91]
Tuberculosis	dsDNA	Liquid phase amplification/ solid phase detection	AuNPs	DPV	38°C	1CFU	20 min	[92]
P. syringae	dsDNA	Liquid phase amplification/ solid phase detection	AuNPs	DPV	37°C	200 pM	20 min	[93]
Mycobacterium smegmatis	dsDNA	Liquid phase amplification/ solid phase detection	$Ru(NH_3)^6]^{3+}$	cyclic voltammetry	37°C	0.040 ng/µL	20 min	[94]
Piscirickettsia salmonis	dsDNA	Solid phase amplification/ solid phase detection	HRP	Chronoamperometry	37°C	$5 \cdot 10^{-8}$ µg ml^{-1}	40 min	[95]
Francisella tularensis	dsDNA	Solid phase amplification/ solid phase detection	HRP	Chronoamperometry	37°C	$1.3x10^{-13}$ M	60 min	[96]

1.7. Thesis objectives

In this thesis, the overall objective of this work was to develop a rapid, easy to use, cost-effective diagnostic geno-sensor for the direct detection of the RPA products without the need to create a single strand DNA (ssDNA).

The first part of the thesis is focused on the development of electrochemical DNA sensors for the detection of the coeliac disease-associated HLA DQB1*02 allele by the combination of modified primers with isothermal RPA and electrochemical detection.

The second part of the thesis is focused on the development of reagent-less electrochemical DNA sensors for the detection of karlodinum armeger based on tailed primers and Au nanoparticles modified with ferrocene.

The third part of the thesis is focused on the development of one step detection system based on producing dsDNA labelled with HRP to be detected electrochemically without the need to create (ssDNA) or do post amplification labelling step.

1.8. References:

[1] D.R. Thévenot, K. Toth, R.A. Durst, G.S. Wilson, Electrochemical biosensors: Recommended definitions and classification, Biosens. Bioelectron. 16 (2001) 121–131. doi:10.1016/S0956-5663(01)00115-4.

[2] R. Monošík, M. Streďanský, E. Šturdík, Biosensors - classification, characterization and new trends, Acta Chim. Slovaca. 5 (2012) 109–120. doi:10.2478/v10188-012-0017-z.

[3] E.- Korotkaya-, Biosensors: Design, Classification, and Applications in the Food Industry, Foods Raw Mater. 2 (2014) 161–171. doi:10.12737/5476.

[4] C.L. Manzanares-Palenzuela, B. Martín-Fernández, M. Sánchez-Paniagua López, B. López-Ruiz, Electrochemical genosensors as innovative tools for detection of genetically modified organisms, TrAC - Trends Anal. Chem. 66 (2015) 19–31. doi:10.1016/j.trac.2014.10.006.

[5] J.I.A. Rashid, N.A. Yusof, The strategies of DNA immobilization and hybridization detection mechanism in the construction of electrochemical DNA sensor: A review, Sens. Bio-Sensing Res. 16 (2017) 19–31. doi:10.1016/j.sbsr.2017.09.001.

[6] T.M. Herne, M.J. Tarlov, Characterization of DNA Probes Immobilized on Gold Surfaces, J. Am. Chem. Soc. 119 (1997) 8916–8920. doi:10.1021/ja9719586.

[7] J.J. Gooding, V.R. Gonçales, Recent advances in the molecular level modification of electrodes for bioelectrochemistry, Curr. Opin. Electrochem. 5 (2017) 203–210. doi:10.1016/j.coelec.2017.09.018.

[8] S.B. Nimse, K. Song, M.D. Sonawane, D.R. Sayyed, T. Kim, Immobilization techniques for microarray: Challenges and applications, Sensors (Switzerland). 14 (2014) 22208–22229. doi:10.3390/s141222208.

[9] J.I.A. Rashid, N.A. Yusof, The strategies of DNA immobilization and hybridization detection mechanism in the construction of electrochemical DNA sensor: A review, Sens. Bio-Sensing Res. 16 (2017) 19–31. doi:10.1016/j.sbsr.2017.09.001.

[10] J. Chen, B. Fu, T. Liu, Z. Yan, K. Li, A Graphene Oxide-DNA Electrochemical Sensor Based on Glassy Carbon Electrode for Sensitive Determination of Methotrexate, Electroanalysis. 30 (2018) 288–295. doi:10.1002/elan.201700615.

[11] E.R. Ribeiro Teles, D.M. França dos Prazeres, J.L. de Lima-Filho, Electrochemical Detection of a Dengue-related Oligonucleotide Sequence Using Ferrocenium as a Hybridization Indicator, Sensors. 7 (2007) 2510–2518. doi:10.3390/s7112510.

[12] S. Liu, Y. Lin, L. Wang, T. Liu, C. Cheng, W. Wei, B. Tang, Exonuclease III-aided autocatalytic DNA biosensing platform for immobilization-free and ultrasensitive electrochemical detection of nucleic acid and protein, Anal. Chem. 86 (2014) 4008–4015. doi:10.1021/ac500426b.

[13] M.I. Pividori, A. Merkoçi, S. Alegret, Electrochemical genosensor design: Immobilisation of oligonucleotides onto transducer surfaces and detection methods, Biosens. Bioelectron. 15 (2000) 291–303. doi:10.1016/S0956-5663(00)00071-3.

[14] Z. Sun, T. Liao, Y. Zhang, J. Shu, H. Zhang, G.J. Zhang, Biomimetic nanochannels based biosensor for ultrasensitive and label-free detection of nucleic acids, Biosens. Bioelectron. 86 (2016) 194–201. doi:10.1016/j.bios.2016.06.059.

[15] F. Ahour, A. Shamsi, Electrochemical label-free and sensitive nanobiosensing of DNA hybridization by graphene oxide modified pencil graphite electrode, Anal. Biochem. 532 (2017) 64–71. doi:10.1016/j.ab.2017.06.004.

[16] M. Rahman, X.-B. Li, N. Lopa, S. Ahn, J.-J. Lee, Electrochemical DNA Hybridization Sensors Based on Conducting Polymers, Sensors. 15 (2015) 3801–3829. doi:10.3390/s150203801.

[17] A.M. Debela, M. Ortiz, V. Beni, S. Thorimbert, D. Lesage, R.B. Cole, C.K.O. Sullivan, B. Hasenknopf, Biofunctionalization of Polyoxometalates with DNA Primers , Their Use in the Polymerase Chain Reaction (PCR) and Electrochemical Detection of PCR Products, (2015) 17721–17727. doi:10.1002/chem.201502247.

[18] V.R.V. Montiel, R.M. Torrente-Rodrï¿½guez, G.G. de Rivera, A.J. Reviejo, C. Cuadrado, R. Linacero, F.J. Gallego, S. Campuzano, J.M. Pingarrï¿½n, Amperometric determination of hazelnut traces by means of Express PCR coupled to magnetic beads assembled on disposable DNA sensing scaffolds, Sensors Actuators, B Chem. 245 (2017) 895–902. doi:10.1016/j.snb.2017.02.041.

[19] B. Xu, D. Zheng, W. Qiu, F. Gao, S. Jiang, Q. Wang, An ultrasensitive DNA biosensor based on covalent immobilization of probe DNA on fern leaf-like α-Fe<inf>2</inf>O<inf>3</inf> and chitosan Hybrid film using terephthalaldehyde as arm-linker, Biosens. Bioelectron. 72 (2015) 175–181. doi:10.1016/j.bios.2015.05.015.

[20] X. Qin, S. Xu, L. Deng, R. Huang, X. Zhang, Photocatalytic electrosensor for label-free and ultrasensitive detection of BRCA1 gene, Biosens. Bioelectron. 85 (2016) 957–963. doi:10.1016/j.bios.2016.05.076.

[21] M. Amouzadeh Tabrizi, M. Shamsipur, A label-free electrochemical DNA biosensor based on covalent immobilization of salmonella DNA sequences on the nanoporous glassy carbon electrode, Biosens. Bioelectron. 69 (2015) 100–105. doi:10.1016/j.bios.2015.02.024.

[22] I. Grabowska, A. Stachyra, A. Góra-Sochacka, A. Sirko, A.B. Olejniczak, Z.J. Leśnikowski, J. Radecki, H. Radecka, DNA probe modified with 3-iron bis(dicarbollide) for electrochemical determination of DNA sequence of Avian Influenza Virus H5N1, Biosens. Bioelectron. 51 (2014) 170–176. doi:10.1016/j.bios.2013.07.026.

[23] Z. Shakoori, S. Salimian, S. Kharrazi, M. Adabi, R. Saber, Electrochemical DNA biosensor based on gold nanorods for detecting hepatitis B virus, Anal. Bioanal. Chem. 407 (2015) 455–461. doi:10.1007/s00216-014-8303-9.

[24] Y. Ye, J. Xie, Y. Ye, X. Cao, H. Zheng, X. Xu, Q. Zhang, A label-free electrochemical DNA biosensor based on thionine functionalized reduced graphene oxide, Carbon N. Y. 129 (2018) 730–737. doi:10.1016/j.carbon.2017.12.060.

[25] K.H. Lee, J.O. Lee, S. Choi, J.B. Yoon, G.H. Cho, A CMOS label-free DNA sensor using electrostatic induction of molecular charges, Biosens. Bioelectron. 31 (2012) 343–348. doi:10.1016/j.bios.2011.10.042.

[26] L. Ribovski, V. Zucolotto, B.C. Janegitz, A label-free electrochemical DNA sensor to identify breast cancer susceptibility, Microchem. J. 133 (2017) 37–42. doi:10.1016/j.microc.2017.03.011.

[27] Y. Lu, J. Zhong, G. Yao, Q. Huang, A label-free SERS approach to quantitative and selective detection of mercury (II) based on DNA aptamer-modified SiO2@Au core/shell nanoparticles, Sensors Actuators, B Chem. 258 (2018) 365–372. doi:10.1016/j.snb.2017.11.110.

[28] D.M. Mills, C.P. Martin, S.M. Armas, P. Calvo-Marzal, D.M. Kolpashchikov, K.Y. Chumbimuni-Torres, A universal and label-free impedimetric biosensing platform for discrimination of single nucleotide substitutions in long nucleic acid strands, Biosens. Bioelectron. 109 (2018) 35–42. doi:10.1016/j.bios.2018.02.059.

[29] W. Cai, S. Xie, J. Zhang, D. Tang, Y. Tang, An electrochemical impedance biosensor for

Hg2+detection based on DNA hydrogel by coupling with DNAzyme-assisted target recycling and hybridization chain reaction, Biosens. Bioelectron. 98 (2017) 466–472. doi:10.1016/j.bios.2017.07.025.

[30] H.M.R. Gonçalves, L. Moreira, L. Pereira, P. Jorge, C. Gouveia, P. Martins-Lopes, J.R.A. Fernandes, Biosensor for label-free DNA quantification based on functionalized LPGs, Biosens. Bioelectron. 84 (2016) 30–36. doi:10.1016/j.bios.2015.10.001.

[31] F. Heinrich, M. Riedel, F. Lisdat, Detection of abasic DNA by means of impedance spectroscopy, Electrochem. Commun. 90 (2018) 65–68. doi:10.1016/j.elecom.2018.04.005.

[32] J.E. Weber, S. Pillai, M.K. Ram, A. Kumar, S.R. Singh, Electrochemical impedance-based DNA sensor using a modified single walled carbon nanotube electrode, Mater. Sci. Eng. C. 31 (2011) 821–825. doi:10.1016/j.msec.2010.12.009.

[33] H. Subak, D. Ozkan-Ariksoysal, Label-free electrochemical biosensor for the detection of Influenza genes and the solution of guanine-based displaying problem of DNA hybridization, Sensors Actuators, B Chem. 263 (2018) 196–207. doi:10.1016/j.snb.2018.02.089.

[34] H.J. Lee, S.S. Lee, Label-free quantitative detection of nucleic acids based on surface-immobilized DNA intercalators, Sensors Actuators, B Chem. 241 (2017) 1310–1315. doi:10.1016/j.snb.2016.09.188.

[35] T. Bronder, C.S. Wu, A. Poghossian, C.F. Werner, M. Keusgend, M.J. Schöning, Label-free detection of DNA hybridization with light-addressable potentiometric sensors: Comparison of various DNAimmobilization strategies, Procedia Eng. 87 (2014) 755–758. doi:10.1016/j.proeng.2014.11.647.

[36] C. Wittmann, C. Marquette, DNA Immobilization, 2012. doi:10.1002/9780470027318.a9210.

[37] C. Yang, M.E. Denno, P. Pyakurel, B.J. Venton, Recent trends in carbon nanomaterial-based electrochemical sensors for biomolecules: A review, Anal. Chim. Acta. 887 (2015) 17–37. doi:10.1016/j.aca.2015.05.049.

[38] R. Eivazzadeh-Keihan, P. Pashazadeh-Panahi, B. Baradaran, A. Maleki, M. Hejazi, A. Mokhtarzadeh, M. de la Guardia, Recent advances on nanomaterial based electrochemical and optical aptasensors for detection of cancer biomarkers, TrAC - Trends Anal. Chem. 100 (2018) 103–115. doi:10.1016/j.trac.2017.12.019.

[39] J. Chen, Z. Liu, H. Peng, Y. Zheng, Z. Lin, A. Liu, W. Chen, X. Lin, Electrochemical DNA biosensor based on grafting-to mode of terminal deoxynucleoside transferase-mediated extension, Biosens. Bioelectron. 98 (2017) 345–349. doi:10.1016/j.bios.2017.07.012.

[40] M.U. Ahmed, I. Saaem, P.C. Wu, A.S. Brown, Personalized diagnostics and biosensors: A review of the biology and technology needed for personalized medicine, Crit. Rev. Biotechnol. 34 (2014) 180–196. doi:10.3109/07388551.2013.778228.

[41] G. Evtugyn, T. Hianik, Electrochemical DNA sensors and aptasensors based on electropolymerized materials and polyelectrolyte complexes, TrAC - Trends Anal. Chem. 79 (2016) 168–178. doi:10.1016/j.trac.2015.11.025.

[42] J. Lee, W. Yoshida, K. Abe, K. Nakabayashi, H. Wakeda, K. Hata, C.A. Marquette, L.J. Blum, K. Sode, K. Ikebukuro, Development of an electrochemical detection system for measuring DNA methylation levels using methyl CpG-binding protein and glucose dehydrogenase-fused zinc finger protein, (2016). doi:10.1016/j.bios.2016.09.060.

[43] J. Wang, X. Cai, G. Rivas, H. Shiraishi, Stripping potentiometric transduction of DNA hybridization processes, Anal. Chim. Acta. 326 (1996) 141–147. doi:10.1016/0003-2670(96)00042-6.

[44] J. Wang, Amperometric biosensors for clinical and therapeutic drug monitoring : a review, 19 (1999)

47–53.

[45] K. Cammann, Bio-sensors based on ion-selective electrodes, Fresenius' Zeitschrift Für Anal. Chemie. 287 (1977) 1–9. doi:10.1007/BF00539519.

[46] R. Paper, Electrochemical Biosensors - Sensor Principles and Architectures, (2008) 1400–1458.

[47] J. Ali, J. Najeeb, M. Asim Ali, M. Farhan Aslam, A. Raza, Biosensors: Their Fundamentals, Designs, Types and Most Recent Impactful Applications: A Review, J. Biosens. Bioelectron. 08 (2017) 1–9. doi:10.4172/2155-6210.1000235.

[48] N. Ahmed, Detecting food borne pathogens using electrochemical biosensors : An overview, (2018).

[49] S. Gs, A. Cv, B.B. Mathew, Biosensors : A Modern Day Achievement, 2 (2014) 26–39. doi:10.12691/jit-2-1-5.

[50] F. Lisdat, D. Schäfer, The use of electrochemical impedance spectroscopy for biosensing, (2008) 1555–1567. doi:10.1007/s00216-008-1970-7.

[51] M.I. Prodromidis, Impedimetric Biosensors and Immunosensors, Environ. Chem. Int. Semin. Anal. Sci. Pakistan J. Anal. Environ. Chem. 8 (2007) 69–71. http://www.ceacsu.edu.pk/PDF file/Journal Vol 8 No 1 and 2/69-71-PJAEC-P.pdf.

[52] G. Wang, R. Han, X. Su, Y. Li, G. Xu, X. Luo, Zwitterionic peptide anchored to conducting polymer PEDOT for the development of antifouling and ultrasensitive electrochemical DNA sensor, Biosens. Bioelectron. 92 (2017) 396–401. doi:10.1016/j.bios.2016.10.088.

[53] Y. Zhang, P. Ozdemir, Analytica Chimica Acta Microfluidic DNA amplification — A review, 638 (2009) 115–125. doi:10.1016/j.aca.2009.02.038.

[54] P. Gill, A. Ghaemi, Nucleic Acid Isothermal Amplification Technologies — A Review, 7770 (2017). doi:10.1080/15257770701845204.

[55] P. Gill, A. Ghaemi, Nucleic acid isothermal amplification technologies - A review, Nucleosides, Nucleotides and Nucleic Acids. 27 (2008) 224–243. doi:10.1080/15257770701845204.

[56] V.T. Katja Niemann, Isothermal Amplification and Quantification of Nucleic Acids and its Use in Microsystems, J. Nanomed. Nanotechnol. 06 (2015). doi:10.4172/2157-7439.1000282.

[57] Kumar, Isothermal Nucleic Acid Amplification System: An Update on Methods and Applications, J Genet Genom. 2 (2018) 1–5. https://www.omicsonline.org/open-access/isothermal-nucleic-acid-amplification-system-an-update-on-methods-andapplications.pdf.

[58] Y. Zhao, F. Chen, Q. Li, L. Wang, C. Fan, Isothermal Amplification of Nucleic Acids, Chem. Rev. 115 (2015) 12491–12545. doi:10.1021/acs.chemrev.5b00428.

[59] D. Morisset, D. Stebih, K. Cankar, J. Zel, K. Gruden, Alternative DNA amplification methods to PCR and their application in GMO detection: A review, Eur. Food Res. Technol. 227 (2008) 1287–1297. doi:10.1007/s00217-008-0850-x.

[60] J. Li, J. Macdonald, Advances in isothermal amplification: Novel strategies inspired by biological processes, Biosens. Bioelectron. 64 (2014) 196–211. doi:10.1016/j.bios.2014.08.069.

[61] X. Zhang, S.B. Lowe, J.J. Gooding, Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP), Biosens. Bioelectron. 61 (2014) 491–499. doi:10.1016/j.bios.2014.05.039.

[62] Y. Mori, H. Kanda, T. Notomi, Loop-mediated isothermal amplification (LAMP): Recent progress

in research and development, J. Infect. Chemother. 19 (2013) 404–411. doi:10.1007/s10156-013-0590-0.

[63] Y. Mori, T. Notomi, Loop-mediated isothermal amplification (LAMP): A rapid, accurate, and cost-effective diagnostic method for infectious diseases, J. Infect. Chemother. 15 (2009) 62–69. doi:10.1007/s10156-009-0669-9.

[64] Z.K. Njiru, Loop-mediated isothermal amplification technology: Towards point of care diagnostics, PLoS Negl. Trop. Dis. 6 (2012) 1–4. doi:10.1371/journal.pntd.0001572.

[65] P.J. Asiello, A.J. Baeumner, Miniaturized isothermal nucleic acid amplification, a review, Lab Chip. 11 (2011) 1420–1430. doi:10.1039/c0lc00666a.

[66] S. Fu, G. Qu, S. Guo, L. Ma, N. Zhang, S. Zhang, S. Gao, Z. Shen, Applications of loop-mediated isothermal DNA amplification, Appl. Biochem. Biotechnol. 163 (2011) 845–850. doi:10.1007/s12010-010-9088-8.

[67] K. Nagamine, K. Watanabe, K. Ohtsuka, T. Hase, T. Notomi, Loop-mediated isothermal amplification reaction using a nondenatured template [1], Clin. Chem. 47 (2001) 1742–1743. doi:10.1093/nar/28.12.e63.

[68] J. Van Ness, L.K. Van Ness, D.J. Galas, Isothermal reactions for the amplification of oligonucleotides, 2003 (2003) 0–5.

[69] H. Jia, Z. Li, C. Liu, Y. Cheng, Ultrasensitive detection of microRNAs by exponential isothermal amplification, Angew. Chemie - Int. Ed. 49 (2010) 5498–5501. doi:10.1002/anie.201001375.

[70] Y. Zhang, J. Hu, C. Zhang, Sensitive Detection of Transcription Factors by Isothermal Exponential Ampli fi cation-Based Colorimetric Assay, (2012).

[71] Y.J. Jeong, K. Park, D.E. Kim, Isothermal DNA amplification in vitro: the helicase-dependent amplification system., Cell. Mol. Life Sci. 66 (2009) 3325–3336. doi:10.1007/s00018-009-0094-3.

[72] C.A. Spargo, M.S. Fraiser, M. Van Cleve, D.J. Wright, C.M. Nycz, P.A. Spears, G.T. Walker, Detection of M. tuberculosis DNA using thermophilic Strand Displacement Amplification, Mol. Cell. Probes. 10 (1996) 247–256. doi:10.1006/mcpr.1996.0034.

[73] J. Kim, C.J. Easley, Isothermal DNA amplification in bioanalysis: Strategies and applications, Bioanalysis. 3 (2011) 227–239. doi:10.4155/bio.10.172.

[74] W. Zhao, M.M. Ali, M.A. Brook, Y. Li, Rolling circle amplification: Applications in nanotechnology and biodetection with functional nucleic acids, Angew. Chemie - Int. Ed. 47 (2008) 6330–6337. doi:10.1002/anie.200705982.

[75] M.M. Ali, F. Li, Z. Zhang, K. Zhang, D.K. Kang, J.A. Ankrum, X.C. Le, W. Zhao, Rolling circle amplification: A versatile tool for chemical biology, materials science and medicine, Chem. Soc. Rev. 43 (2014) 3324–3341. doi:10.1039/c3cs60439j.

[76] B. Deiman, P. Van Aarle, P. Sillekens, Characteristics and applications of Nucleic Acid Sequence-Based Amplification (NASBA), Appl. Biochem. Biotechnol. - Part B Mol. Biotechnol. 20 (2002) 163–179. doi:10.1385/MB:20:2:163.

[77] O. Mayboroda, I. Katakis, C.K. O'Sullivan, Multiplexed isothermal nucleic acid amplification, Anal. Biochem. 545 (2018) 20–30. doi:10.1016/j.ab.2018.01.005.

[78] M. Vincent, Y. Xu, H. Kong, Helicase-dependent isothermal DNA amplification, EMBO Rep. 5 (2004) 795–800. doi:10.1038/sj.embor.7400200.

[79] P. Craw, W. Balachandran, Isothermal nucleic acid amplification technologies for point-of-care

diagnostics: A critical review, Lab Chip. 12 (2012) 2469–2486. doi:10.1039/c2lc40100b.

[80] G. Xinzhong, G. Ying, Y. Shuai, S. Ting, Z. Linfu, A new molecular diagnosis method combined single primer isothermal amplification with rapid isothermal detection assay in detection of group B Streptococcus, 7 (2013) 4317–4322. doi:10.5897/AJMR2013.2534.

[81] M.J. Hall, S.D. Wharam, A. Weston, D.L.N. Cardy, W.H. Wilson, Use of signal-mediated amplification of RNA technology (SMART) to detect marine cyanophage DNA, Biotechniques. 32 (2002) 604–611.

[82] O. Piepenburg, C.H. Williams, D.L. Stemple, N.A. Armes, DNA detection using recombination proteins, PLoS Biol. 4 (2006) 1115–1121. doi:10.1371/journal.pbio.0040204.

[83] I.M. Lobato, C.K. O'Sullivan, Recombinase polymerase amplification: Basics, applications and recent advances, TrAC - Trends Anal. Chem. 98 (2018) 19–35. doi:10.1016/j.trac.2017.10.015.

[84] L. Tan, J. Ge, M. Jiao, G. Jie, S. Niu, Amplified electrochemiluminescence detection of DNA based on novel quantum dots signal probe by multiple cycling amplification strategy, Talanta. 183 (2018) 108–113. doi:10.1016/j.talanta.2018.02.063.

[85] S.Z. Mousavisani, J.B. Raoof, A.P.F. Turner, R. Ojani, W.C. Mak, Label-free DNA sensor based on diazonium immobilisation for detection of DNA damage in breast cancer 1 gene, Sensors Actuators, B Chem. 264 (2018) 59–66. doi:10.1016/j.snb.2018.02.152.

[86] N. Shoaie, M. Forouzandeh, K. Omidfar, Highly sensitive electrochemical biosensor based on polyaniline and gold nanoparticles for DNA detection, IEEE Sens. J. 18 (2017) 1835–1843. doi:10.1109/JSEN.2017.2787024.

[87] H. Joda, V. Beni, A. Willems, R. Frank, J. Höth, K. Lind, L. Strömbom, I. Katakis, C.K. ÓSullivan, Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles, Biosens. Bioelectron. 73 (2015) 64–70. doi:10.1016/j.bios.2015.05.048.

[88] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, A. Saeed, M.N. Abbas, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay, Sci. Rep. 6 (2016) 37732. doi:10.1038/srep37732.

[89] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Aptamer Lateral Flow Assays for Ultrasensitive Detection of β-Conglutin Combining Recombinase Polymerase Amplification and Tailed Primers, Anal. Chem. 88 (2016) 10701–10709. doi:10.1021/acs.analchem.6b03256.

[90] A. De La Escosura-Muñiz, L. Baptista-Pires, L. Serrano, L. Altet, O. Francino, A. Sánchez, A. Merkoçi, Magnetic Bead/Gold Nanoparticle Double-Labeled Primers for Electrochemical Detection of Isothermal Amplified Leishmania DNA, Small. 12 (2016) 205–213. doi:10.1002/smll.201502350.

[91] B.Y.C. Ng, W. Xiao, N.P. West, E.J.H. Wee, Y. Wang, M. Trau, Rapid, Single-Cell Electrochemical Detection of *Mycobacterium tuberculosis* Using Colloidal Gold Nanoparticles, Anal. Chem. 87 (2015) 10613–10618. doi:10.1021/acs.analchem.5b03121.

[92] B.Y.C. Ng, E.J.H. Wee, N.P. West, M. Trau, Naked-Eye Colorimetric and Electrochemical Detection of Mycobacterium tuberculosis - Toward Rapid Screening for Active Case Finding, ACS Sensors. 1 (2016) 173–178. doi:10.1021/acssensors.5b00171.

[93] H.Y. Lau, H. Wu, E.J.H. Wee, M. Trau, Y. Wang, J.R. Botella, Specific and sensitive isothermal electrochemical biosensor for plant pathogen DNA detection with colloidal gold nanoparticles as probes, Sci. Rep. 7 (2017) 1–7. doi:10.1038/srep38896.

[94] M.N. Tsaloglou, A. Nemiroski, G. Camci-Unal, D.C. Christodouleas, L.P. Murray, J.T. Connelly, G.M. Whitesides, Handheld isothermal amplification and electrochemical detection of DNA in resource-limited settings, Anal. Biochem. 543 (2018) 116–121. doi:10.1016/j.ab.2017.11.025.

[95] J.S. del Río, I.M. Lobato, O. Mayboroda, I. Katakis, C.K. O'Sullivan, Enhanced solid-phase recombinase polymerase amplification and electrochemical detection, Anal. Bioanal. Chem. (2017) 1–9. doi:10.1007/s00216-017-0269-y.

[96] J.S. del Río, M. Svobodova, P. Bustos, P. Conejeros, C.K. O'Sullivan, Electrochemical detection of Piscirickettsia salmonis genomic DNA from salmon samples using solid-phase recombinase polymerase amplification, Anal. Bioanal. Chem. 408 (2016) 8611–8620. doi:10.1007/s00216-016-9639-0.

Chapter 2

Isothermal amplification using modified primers for rapid electrochemical analysis of coeliac disease associated DQB1*02 HLA allele

Isothermal amplification using modified primers for rapid electrochemical analysis of coeliac disease associated DQB1*02 HLA allele

Sallam Al-Madhagi[a], Hamdi Joda[a¥], Miriam Jauset-Rubio[a], Mayreli Ortiz[a,] Ioanis Katakis[a*] and Ciara K. O'Sullivan[a,b*]

[a] Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona

[b] ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain

[¥]Current address: Miller School of Medicine, University of Miami, Miami, FL 33136, United States

2.1. Abstract

DNA biosensors are attractive tools for genetic analysis as there is an increasing need for rapid and low-cost DNA analysis, primarily driven by applications in personalized pharmacogenomics, clinical diagnostics, rapid pathogen detection, food traceability and forensics. A rapid electrochemical genosensor detection methodology exploiting a combination of modified primers for solution-phase isothermal amplification, followed by rapid detection via hybridization on gold electrodes is reported. Modified reverse primers, exploiting a C18 spacer between the primer-binding site and an engineered single stranded tail, are used in a recombinase polymerase amplification reaction to produce an amplicon with a central duplex flanked by two single stranded tails. These tails are designed to be complementary to a gold electrode tethered capture oligo probe as well as a horseradish peroxidase labelled reporter oligo probe. The time required for hybridization of the isothermally generated amplicons with each of the immobilized and reporter probes was optimised to be 2 minutes, in both cases. The effect of amplification time and the limit of detection were evaluated using these hybridization times for both single stranded and double stranded DNA templates. The best detection limit of 70 fM for a ssDNA template was achieved using 45 minutes amplification, whilst for a dsDNA template, just 30 minutes amplification resulted in a slightly lower detection limit of 14 fM, whilst both 20 and 45 minute amplification times were observed to provide detection limits of 71 and 72 fM, respectively, but 30 and 45 minute amplification resulted in a much higher signal and sensitivity. The genosensor was applied to genomic DNA and real patient and control blood samples for detection of the DQB1*02 HLA allele, as a model system, demonstrating the possibility to carry out molecular diagnostics, combining amplification and detection in a rapid and facile manner.

Keywords: DNA detection; modified primers; electrochemical detection; recombinant polymerase amplification**.**

2.2. Introduction

There is a mature need for a rapid, simple, inexpensive and reliable nucleic acid testing system for a plethora of applications in molecular diagnostics[1]. In the vast majority of techniques reported to date, DNA amplification is required prior to nucleic acid analysis. Conventional amplification systems such as the polymerase chain reaction require the use of a thermocycler instrument with inherent power requirements and a need for trained personnel. Moving towards achieving assays that meet the ASSURED criteria of affordability, sensitivity, specificity, user-friendliness, robustness, rapidity, equipment-free and deliverability to end users[2], several isothermal amplification techniques have been developed, including loop-mediated amplification (LAMP)[3] ,exponential amplification reaction (EXPAR)[4], strand displacement amplification (SDA)[5], rolling circle amplification (RCA)[6], nucleic acid sequence-based amplification (NASBA)[7], helicase-dependent amplification (HDA)[8] and recombinase polymerase amplification (RPA)[9].

RPA has several advantages over other isothermal techniques due to its simplicity, sensitivity and rapid amplification at a constant temperature between 25–42 ºC[10], consequently having immense potential capability in microsystems and lateral flow assays for point of need applications[11]. RPA takes advantage of the natural homologous recombination process, where a recombinase enzyme and single stranded binding proteins enable the scanning and invasion of a DNA template with primers, thus avoiding the need for thermal denaturation, with repeated primer extension via polymerase strand displacement activity, resulting in exponential amplification.

Despite the high efficiency, simplicity, and rapidity of RPA, the detection of RPA amplicons via hybridization normally requires the generation of single stranded DNA, adding cost and complexity to the assay. To address this, we previously developed an approach to produce PCR amplicons that could be directly detected post-amplification via hybridization[12]. This approach exploited the use of primers modified with a carbon spacer, which effectively blocks elongation, linked to a single stranded oligonucleotide sequence, thus resulting in a duplex amplification product flanked by two single stranded DNA tails. One of the tails were used to hybridize to a surface immobilized probe and the other to an enzyme labelled reporter probe, and the detection assay was completed in less than 5 min without any need for post-amplification sample treatment. Recently, we combined this approach with RPA for the lateral flow based detection of DNA[13] and aptamers[14].

In the work reported here, we describe the combination of these modified primers with isothermal RPA and electrochemical detection, with the main objective of the work being the achievement of extremely rapid amplification and detection of the coeliac disease associated HLA DQB1*02 allele. Amplification time and hybridization times with a surface tethered probe and an enzyme labelled probe, respectively, were evaluated and the final system applied to the detection of a real patient sample and control sample, highlighting the potential application of this approach to point-of-need diagnostics.

2.3. Materials and methods

2.3.1. Materials

Maleimide activated 96-Well plates and Proteinase K were obtained from Fisher Scientific (Madrid, Spain). RPA TwistAmp® basic kit was purchased from TwistDx Ltd. (Babraham, United Kingdom). Low range ultra-agarose gel powder was supplied from Bio Rad Laboratories S.A. (Barcelona, Spain). Phosphate buffered saline (50mM) pH 7.4 (PBS), containing 0.05% (v/v) Tween 20 (PBS-Tween), 6 mercapto-1-hexanol (MCH) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma–Aldrich (Barcelona, Spain). TMB enhanced one component HRP membrane was supplied by Diarect AG (Germany). Potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) were obtained from Scharlau (Barcelona, Spain). Dithiol 16-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15-pentaoxahexa-decane) (DT1) was obtained from SensoPath Technologies (Bozeman, MT, USA). DNA probes, DNA primers, Horseradish peroxidise (HRP) modified reporting sequences and synthetic analogues of DQB1*02 target were supplied by biomers.net GmbH (Ulm, Germany) as lyophilized powder and reconstituted in RNase and DNase-free water and used without further purification.

Table 2.1. The DNA target, probe and primers sequences used in this work were:

Name	Nucleotide sequence (from 5′ to 3′)
DQB1*02target	CGTGCGTCTTGTGAGCAGAAGCATCTATAACCGAGAAGAGATCGTGCGCTT CGACAGCGACGTGGGGGAGTTCCGGGCGGTGACGCTGCTGGGGCTGCCTGC CGCCGAGTACTGGAACAGCC
DQB1*02 capture probe	GTC GTG ACT GGG AAA AC TTT TTT TTT TTT TTT-SH
DQB1*02 tailed forward primer	GTT TTC CCA GTC ACG AC-Spacer-CGT GCG TCT CGT GAG CAG AAG
DQB1*02 tailed reverse primer	TGT AAA ACG ACG GCC AGT-Spacer-GGC TGT TCC AGT ACT CGG CGG
HRP-reporter probe	ACTGGCCGTCGTTTTACA-HRP

Both synthetic single stranded target DNA (ssDNA) and PCR amplified double stranded DNA (dsDNA) was used in this work. PCR amplification was carried out using non-tailed primers. PCR amplification was performed using DreamTaq DNA polymerase kit (Fisher Scientific- Spain) with following volumes: 10 µL 5x buffer, 5 µL dNTPs (2 mM), 5 µL $MgCl_2$ (50 mM), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5 µL BSA (50 mg/mL), 27 µL H_2O, 0.5 µL polymerase and 1 µL of 5 nM ssDNA template. PCR amplification was performed using an initial denaturation step at 96°C for 1min, followed by 25 cycles performing 95ºC for 30 sec, annealing for 30 sec at 61 °C and elongation at 72ºC for 30 sec, with a final extra step of 72ºC for 5 min. Gel electrophoresis analysis was carried out to confirm successful PCR amplification.

2.3.2. Liquid phase RPA / solid phase detection

The target (synthetic/genomic) was first amplified using isothermal recombinase polymerase amplification and modified tailed forward and reverse primers. Subsequently, detection was carried out by hybridisation to a capture probe surface tethered either on a microtiter plate or on gold electrodes, followed by hybridisation with a HRP labelled reporter probe.

2.3.3. RPA

RPA was performed using a TwistAmp Basic kit using the protocol recommended by the supplier with slight modifications. Briefly, 240 nM of modified forward and reverse primers, DNA template (DQB1*02), DNAse free water and 1× rehydration buffer was made up to a total volume of 100 µL and then divided into 25 µL /reaction before addition of 14 mM magnesium acetate to initiate the RPA reaction. To optimize the amplification temperature, amplification was carried out at 36, 37, 38, 39, 40 and 41°C.

2.3.4. Enzyme Linked Oligonucleotide Assay (ELONA)

Thiolated probe complementary to the single stranded DNA tail of the forward primer (500 nM) was prepared in 10 mM PBS, added to each well of a maleimide microtiter plate and left to incubate for 2 hrs at 37 °C. Any remaining maleimide groups were subsequently blocked with 100µM mercaptohexanol.

The RPA amplified DNA was diluted (1:3) in 50 mM Tris buffered saline containing 150 mM NaCl, added to each well of the microtiter plate to hybridise with the immobilised capture probe complementary to the ssDNA tail of the extended forward primer of the RPA product, and incubated for the reported time at 37 °C. Reporter HRP-DNA probe (10 nM) was then added to each well to hybridise to the complementary ssDNA tail of the extended reverse primer at the other end of RPA product, for the reported time at 37 °C. Between each step, the wells were washed with 200 µL of PBS-Tween 3 times. Finally, TMB substrate for ELISA was added and the reaction was stopped after 5 min using 1M H_2SO_4. The absorbance was then measured at 450nm using a microplate reader (SpectraMax, bioNovacientífica S.L.,Spain).

2.3.5. Electrochemical detection on gold electrode array

All electrochemical experiments were performed using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software program (Eco Chemie, The Netherlands), equipped with a MUX module (Eco Chemie B.V., The Netherlands) for sequential measurement of working electrodes that share the same reference and counter electrode.

2.3.6. Electrode chip design

The electrochemical cell consists of a customized electrode array chip designed in-house using AutoCAD software (Autodesk Inc, USA) and fabricated at Fineline GmbH (Hilden, Germany) using printed circuit board (PCB) technology with a soft gold surface finish. It consists of 32 working electrodes (diameter =1mm), each arranged with a gold counter electrode and gold reference electrode. This array was not specifically designed for the work reported here, but could be used for multiplexed detection of RPA amplicons. The electrode array was interfaced with a multiplexed potentiostat using a custom-designed connector.

2.3.7. Electrode surface functionalization and hybridization

Prior to functionalization, the electrode array was cleaned via exposure to UV/ozone for 10 min, followed by immersion in 25% v/v H_2O_2 containing KOH (50mM) for a further 10 min. Finally, the array was rinsed with MilliQ water and then with ethanol and finally dried in N_2 gas. Each electrode of the array was functionalized using a co-immobilization approach by spotting 1 µL of 100µM DT1 and 5 µM of thiolated DNA probe in 1 M KH_2PO_4 onto each electrode. Self-assembling was left to take place for 3 h at room temperature within a humidified chamber, and then the electrode array was rinsed with MilliQ water and dried with N_2 gas.

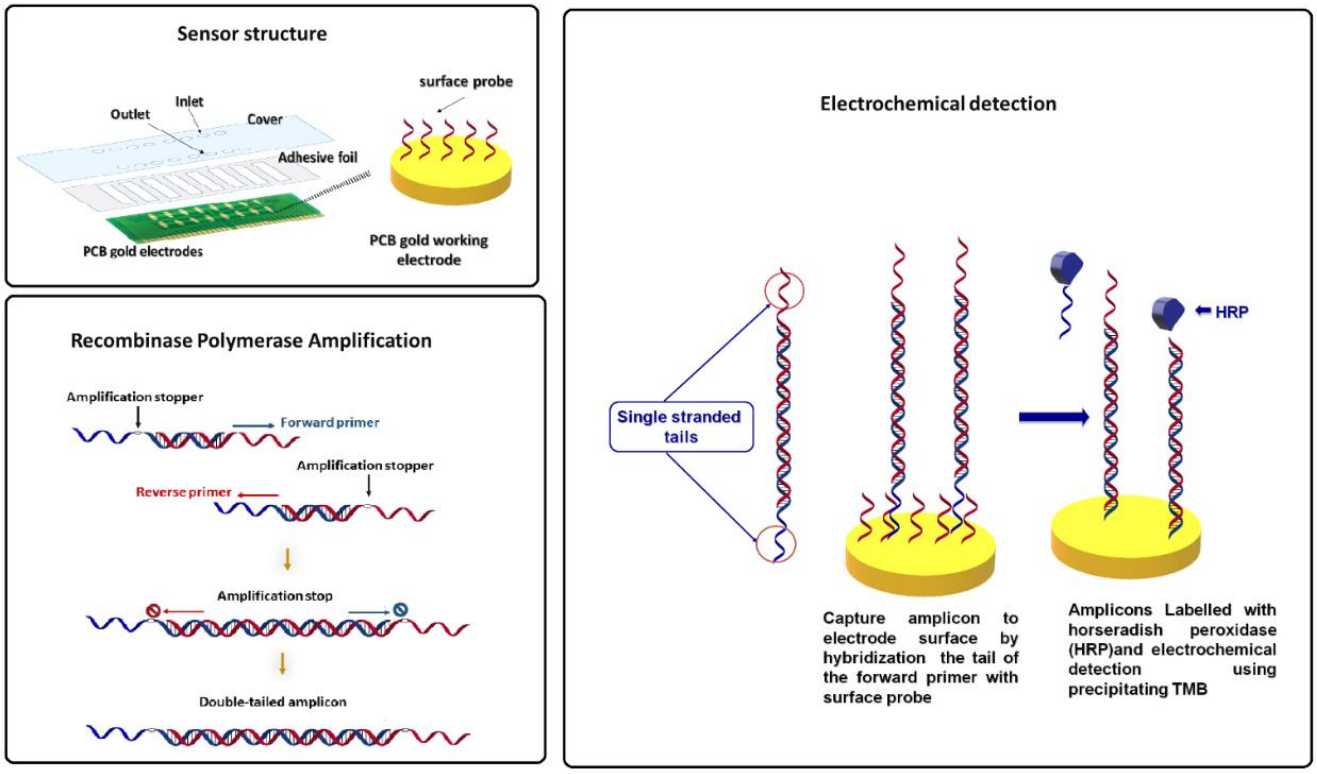


Figure 2.1. Schematic overview of of electrode array, functionalization and detection process.

The functionalized electrode array was then mounted within the fluidic device fabricated on 2 mm thick poly(methylmethacrylate) (PMMA) sheet using a Fenix CO_2 laser (Synrad Inc., USA). A laser patterned double-sided adhesive gasket was used to form 8-microchannel structures 1 mm in width.

mplified RPA product (10µL) was injected into the corresponding microfluidic channel and incubated for the reported time at 37 °C to hybridize with capture probe and subsequently reporter HRP-DNA probe (10 nM) was injected and again incubated for the reported time at 37 °C. Between each step, the channels were thoroughly washed with 2 x 200 µL of 50 mM Tris-buffered saline (TBS, pH 8.0). Finally, TMB enhanced one component HRP membrane substrate was added and electrochemical reduction of the HRP mediated product was measured using fast pulse amperometry (0 V for 10 ms followed by −0.2 V for 500 ms vs. Au built-in reference electrode).

2.4. Results and discussion

2.4.1. Liquid phase RPA / solid phase detection

RPA with tailed primers was performed using liquid phase amplification / solid-phase detection at a range of temperatures between 36 and 41 oC, and the highest efficiency of amplification was observed at 38 oC, but as the amplification levels were not significantly higher than those obtained at 37 oC, in order to maintain one constant temperature for the entire assay as 37 oC was the temperature used for hybridization, 37 oC was also thus used for amplification for all further experiments. Following RPA, the 25µL reaction mix was combined with 2 µL of 2 mg/mL Proteinase K, and the obtained amplicons then characterised using agarose gel electrophoresis (Figure 2.2). RPA products could also be purified using a purification kit, but either the use of Proteinase K or a purification kit is required prior to carrying out gel electrophoresis to avoid smearing on the gel. Lanes 2, 4, 6, 8, 10, 12 correspond to RPA amplification of a 2 nM template for 40, 30, 20, 10, 5 and 2 min, respectively, with Lanes 3, 5, 7, 9, 11 and 13 corresponding to non-template controls for 40, 30, 20, 10, 5 and 2 min, respectively. Intense amplification bands appear for RPA amplified for 40, 30, 20 and 10 min and a faint band could be observed for 5 min amplification, whilst no visible bands were observed for the NTC controls (NTC = water).

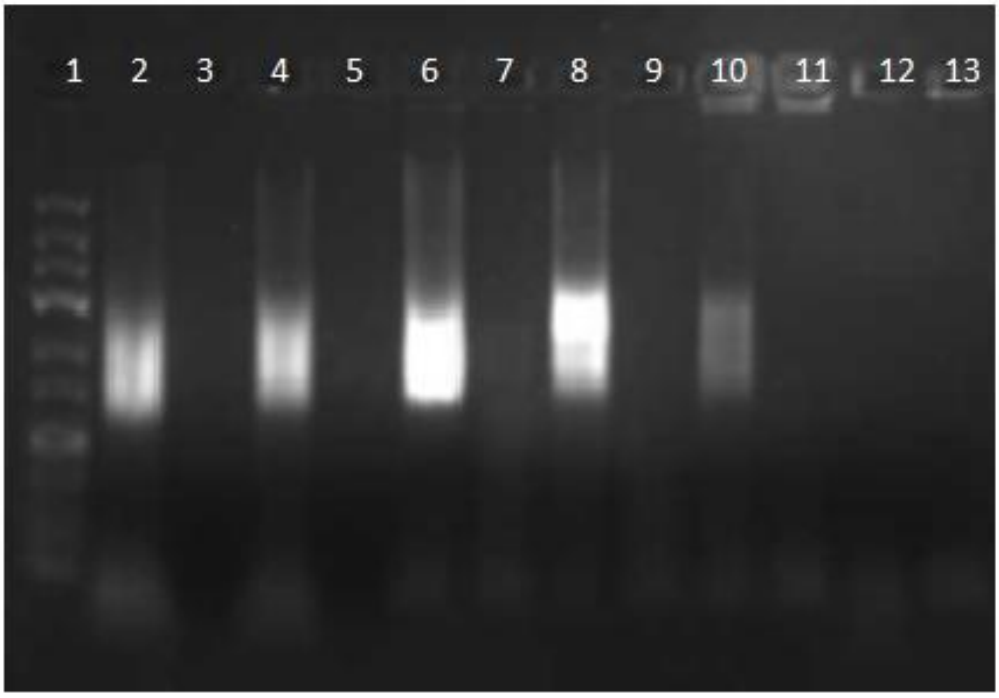


Figure 2.2. Gel electrophoresis analysis for RPA products amplification times. Lane 1, 10 bp ladder; lane 2, RPA for 40 min; lane 3, NTC 40 min; lane 4, RPA for 30 min; lane 5, NTC 30 min; lane 6, RPA for 20 min; lane 7, NTC 20 min; lane 8, RPA for 10 min; lane 9, NTC 10 min; lane 10, RPA for 5 min; lane 11, NTC 5 min; lane 12, RPA for 2 min; lane 13, NTC 2 min.

Unpurified RPA products (i.e. no Proteinase K step as used prior to electrophoresis) were detected using an enzyme linked oligonucleotide assay via solid-phase hybridization with a short thiolated DNA probe complementary to one ssDNA tail, which was surface immobilised on the well of a maleimide coated microtiter plate, whilst the second ssDNA tail was used to hybridize to a HRP-linked reporter probe. Samples were diluted 1:3 in 50 mM Tris buffered saline containing 150 mM NaCl and allowed to hybridise for 30 min at 37 oC. Following thorough washing, reporter DNA-HRP probe (10nM) was subsequently added and allowed to hybridize for a further 30 min at 37 oC. TMB substrate for ELISA was added and reaction was stopped after 5 min using 1M H2SO4. As can be seen in Figure 2.3, increasing amplification times resulted in a higher amount of amplified product, but even just 5 minutes of amplification resulted in a detectable amount of RPA amplicon.

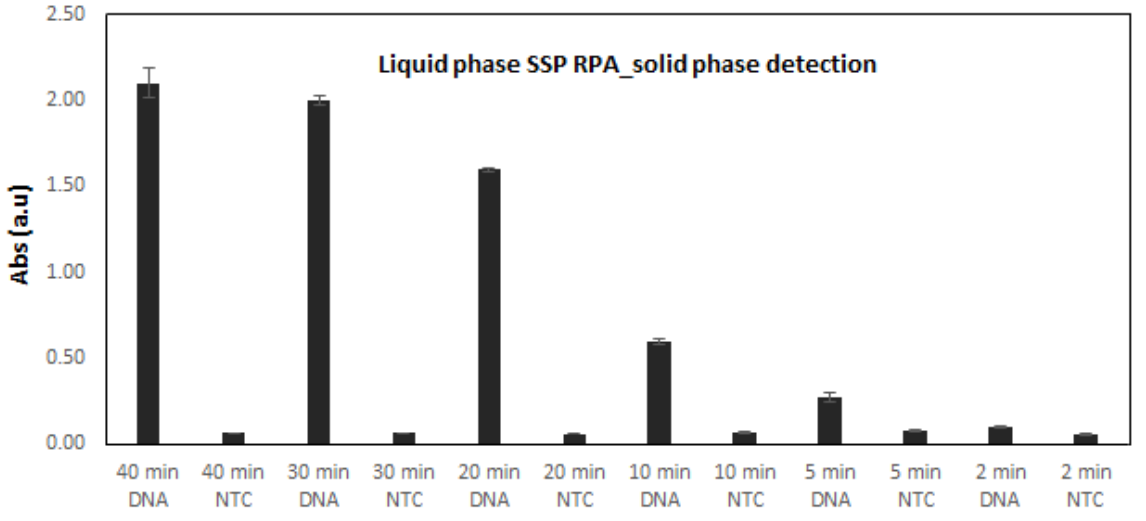


Figure 2.3. Colorimetric absorbance sign for liquid phase RPA products and NTC amplified for 40, 30, 20, 10, 5 and 2 min. Absorbance was measured at 450nm following addition of sulfuric acid. In all cases, hybridization with capture and reporter probes was 30 min.

2.4.2. Liquid phase RPA / electrochemical solid phase detection

Following demonstration of the ELONA based direct detection of the RPA tailed amplicons, electrochemical detection of solid-phase hybridisation was explored. Based on the ELONA results, synthetic target DNA (10 nM) was amplified for 10 minutes, and the unpurified RPA amplicon obtained was directly detected via hybridization with gold electrode immobilized capture probe, varying the hybridization time from 2 to 60 minutes at 37 ºC. Subsequently, DNA-HRP reporter probe was added and allowed to hybridize for an excessive amount of time (30 min), to ensure maximum signal. Based on our previous results [12], we expected a rapid hybridisation time, which can be attributed to the short length of the single stranded DNA tail. DNA hybridizes via a combination of collisional kinetics to find its complementary DNA, which is the rate limiting step, and is followed by rapid DNA zipping. Thus, the shorter the DNA that needs to be scanned for complementarity, the more rapid hybridization can occur, particularly as the only available single stranded DNA for binding is the complementary tail. As expected, the same levels of hybridization were obtained for the range of times interrogated, indicating that a 2 minute hybridization time with surface immobilized probe was adequate, although 10 minutes was where plateauing was observed (Figure 2.4a).

The effect of duration of hybridization between captured DNA duplex and the second ssDNA tail with DNA-labelled reporter probe was then evaluated, using an initial hybridization time with the capture probe of 10 minutes. As can be seen in Figure 2.4b, effective hybridization was already observed at just 2 minutes, rapidly reaching a plateau at longer hybridization times. As the objective of this work was to find the conditions for the most rapid amplification and detection of the HLA DQB1*02 allele, hybridization times of 2 minutes for capture probe and RPA amplicon, and 2 minutes subsequent hybridization time with the HRP labelled reporter probe were selected and used to detect amplicons produced following 2 to 45 minutes of isothermal amplification.

Either the use of Proteinase K or rapid freezing at -80 ^{o}C for 10 minutes was observed to efficiently stop the amplification reaction via denaturation / inactivation of the recombinase A, strand displacing polymerase and the single stranded binding proteins. Heating to 80 ^{o}C and was also tested as a means to stop the RPA and could also be used, but the use of proteinase K or freezing was simpler to implement, and here we exploited the use of rapid freezing to stop the RPA reaction.

As soon as amplification was stopped, the amplicons were purified. RPA products were purified using DNA Clean & Concentration^{TM} -5 kit (Zymo Research) by mixing 5 volumes of DNA binding buffer to each volume of the RPA products, the mixture transferred to the Zymo-Spin column and centrifuged for 30 seconds at 10000g, after which the column was washed three times with 200 µl DNA wash buffer and centrifuged for 30 seconds at 10000g. Finally, 15 µL of water added directly to the column matrix and incubated for one minute and then centrifuged for 30 seconds at 10000g to elute the dsDNA. . It should be noted that these steps of Proteinase K / freezing / purification would not be used in a final assay/device and is only implemented in this exploratory work to stop the RPA reaction and study the effect of amplification time on detection limit. In a final assay, as detailed in the study to optimize hybridization times detailed above, the RPA amplicon is directly detected via hybridization, without any need for purification / post-RPA processing.

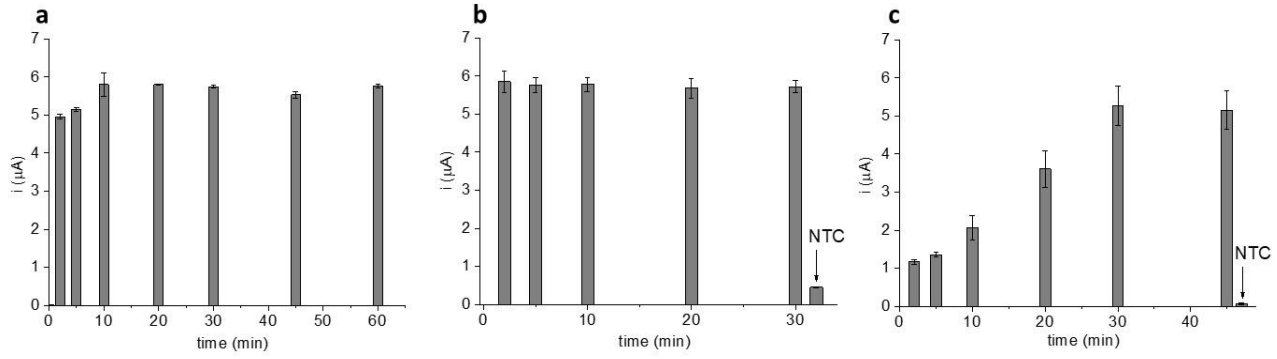


Figure 2.4. (a) Effect of duration of hybridization time of tailed RPA amplicon with capture probe surface tethered on gold electrode. 10 nM template DNA was used and 30 minutes hybridization with HRP labelled reporter probe. (b) Effect of duration of hybridization time of tailed RPA amplicon with HRP labelled reporter probe, following hybridization with surface immobilized capture probe for 10 minutes. 10 nM template DNA was used, amplified for 10 min. (c) Detection of RPA generated tailed amplicons following 2, 5, 10, 20, 30 and 45 minutes amplification. NTC indicates the signal obtained following amplification of a non-specific template for 40 minutes. 10 nM target was used as template and 2 min capture and reporter probe hybridization.

As was expected, and can be seen in Figure 2.4c, increasing amplification times resulted in increasing levels of amplicons produced, with tailed RPA products being detectable even after just 2 minutes amplification, reaching a plateau at ca. 30 minutes, after which further amplification did not improve the amplification yield. However, it must be noted that the starting concentration of DNA was 10nM, and it possible to directly detect this concentration without any amplification using the hybridization assay developed.

A combination of 2 minutes hybridization with each of capture probe and reporter probe was thus further explored amplifying a range of concentrations (2 fM – 2 nM) of template starting concentrations of both synthetic ssDNA and PCR amplified dsDNA using a range of amplification times between 2- 45 minutes (Figure 2.5).

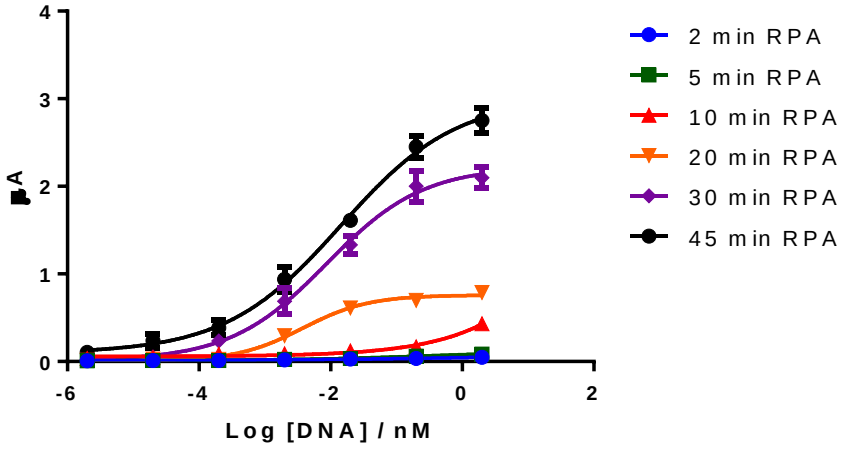


Figure 2.5. Calibration curves obtained amplifying a range of concentrations (2 fM – 2 nM) of template starting concentrations of synthetic ssDNA using a range of amplification times (2- 45 minutes). In all cases 2 min hybridization with capture and reporter probes was used.

As can be seen in Table 2.1, when using ssDNA as a target the detection limit decreased and the sensitivity increased with increasing amplification times. A four parameter logistic regression model was used to interpret the data from a sigmoidal curve, with the exception of the 10 minute amplification calibration curve, which did not fit well and led to ambiguous results.

Table 2.2. Effect of amplification time on IC50 and LOD using synthetic ssDNA template

Amplification time	R square	IC50	LOD
2 minutes	0.9977	1.137 +14.9 nM	1.3 nM
5 minutes	0.9976	0.3082 + 2.69 nM	718 pM
10 minutes	0.9859	Ambiguous	25 pM
20 minutes	0.9942	0.0038 ± 1.2986 nM	0.32 pM
30 minutes	0.9977	0,0087 ± 1.259 nM	0.11pM
45 minutes	0.9982	0.01430 ± 1.31 nM	70fM

The same experiment was then repeated using dsDNA that had been generated using PCR. The concentration of post-PCR DNA was determined using Nanodrop and using this known starting concentration, dilutions made to results in starting concentrations of 2 fM – 2 nM for use as templates for RPA (Figure 2.6).

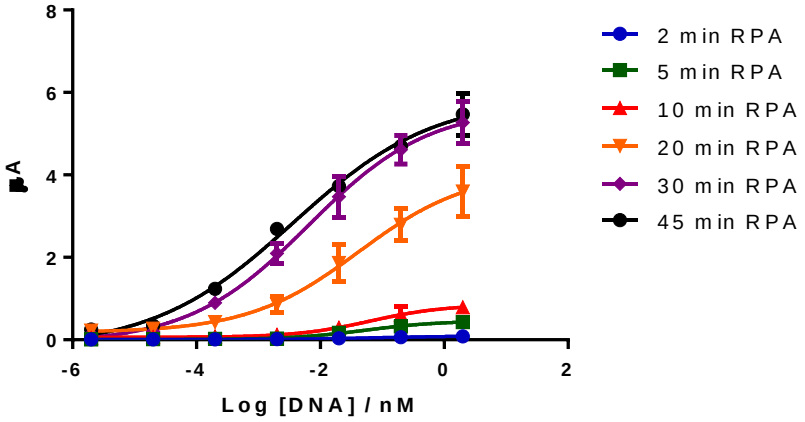


Figure 2.6. Calibration curves obtained amplifying a range of concentrations (2fM – 2nM) of template starting concentrations of PCR generated dsDNA using a range of amplification times (2- 45 minutes). In all cases 2 min hybridization with capture and reporter probes was used.

Table 2.**3**. Effect of amplification time on IC50 and LOD using PCR generated dsDNA template

Amplification time	R square	IC50	LOD
2 minutes	0.9955	0.1040 + 1.4135 nM	1pM
5 minutes	0.9966	0.05497 + 1.311 nM	1.22 pM
10 minutes	0.9991	0.06413 +1.157 nM	0.54 pM
20 minutes	0.9989	0.0416 + 1.2966 nM	72fM
30 minutes	0.9977	0.0006 + 1.213 nM	14fM
45 minutes	0.9955	0.0034 + 1.61 nM	71fM

As expected better detection limits were obtained using double stranded DNA target as this is the preferred target for RPA. With ssDNA as target, the first elongations are relatively slow, converting ssDNA to dsDNA, and after the ssDNA is converted to dsDNA, amplification increases in speed. This is reflected by the fact that approximately the same detection limit is achieved after 45 minutes using both ssDNA and dsDNA. Additionally, it can be seen that the kinetics of RPA appear to be slower with the tailed primers than with normal, non-tailed primers. However, this needs to be verified with a range of different targets, as in our experience, the time required for amplification is highly target dependent, with no direct correlation between amplicon length, or GC content of target/primers. In the case of the HLA DQB1*02 target, the optimum amplification time is deemed to be 30 minutes.

In order to attempt to further decrease the assay time, a study was carried out where the RPA amplicon and HRP-labelled reporter probe were pre-incubated and then added to the electrode array, or, alternatively, where the HRP labelled reporter probe and the tailed RPA amplicon were simultaneously added to the functionalized electrode array. However, as can be clearly seen in Figure 2.7, the highest signals are observed when a step-by-step approach is used.

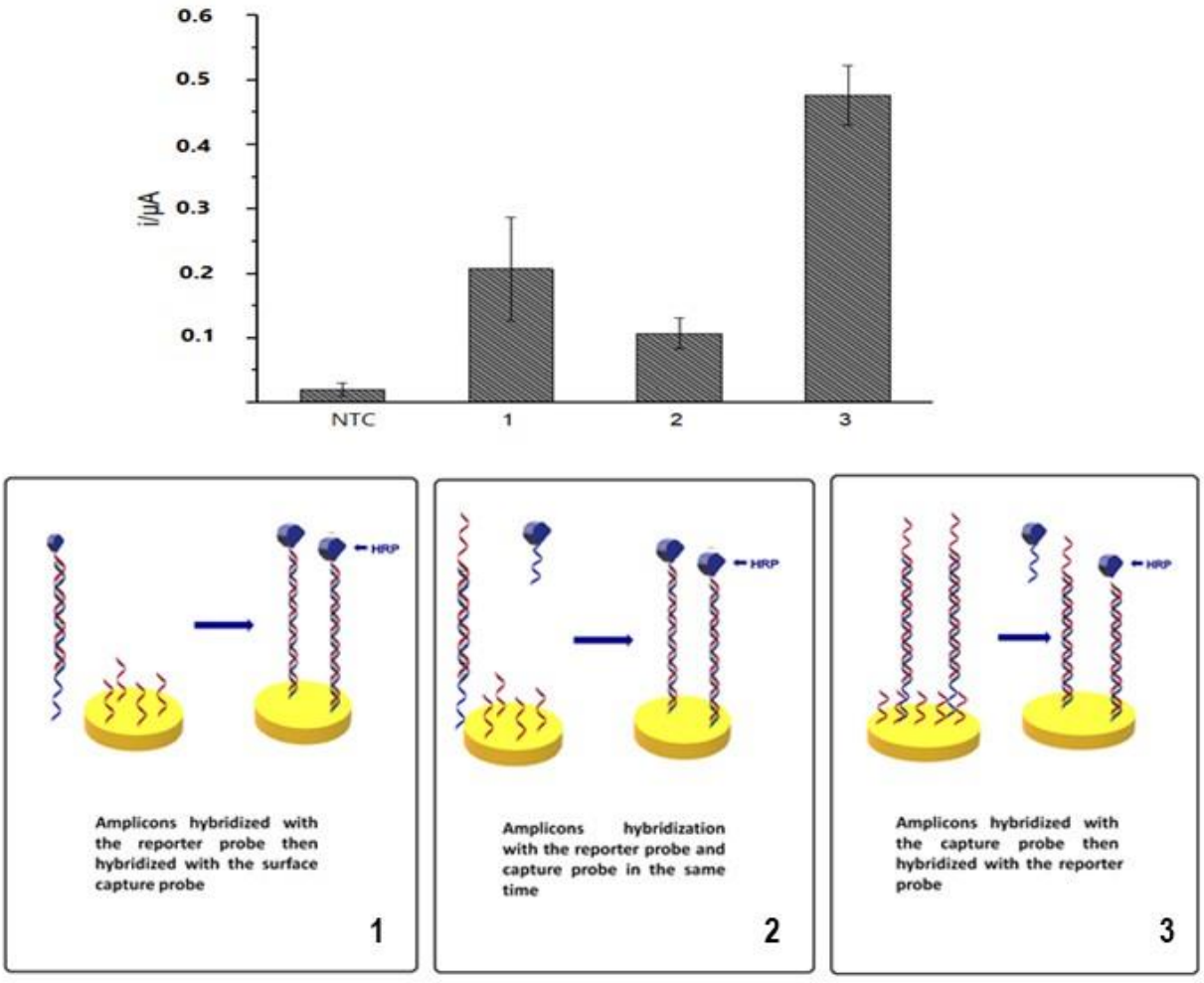


Figure 2.7. (1) Amplicons hybridized with the reporter probe then hybridized with the capture probe; (2) Amplicons hybridized with the reporter probe and the capture probe in the same time; (3) Amplicons hybridized with

Using the optimised assay conditions of 30 minutes amplification, 2 minutes hybridization with the surface immobilized probe and subsequently, 2 minutes hybridization with the HRP labelled reporter probe, the genosensor was used to detect the DQB1*02 in real patient samples. Genomic DNA was extracted from a human blood sample and provided by the Finnish Red Cross Blood Service, which had previously been genotyped and electrochemically analyzed [12]. DNA from a human sample containing DQB1*02 sequence (GE +ve) and a control sample not containing the DQB1*02 sequence (GE -ve) were analyzed, and as can be seen in Figure 2.8 amplification and detection of amplification products was only observed in the case of the positive sample. Furthermore, specificity of the amplification was evaluated using a non-specific target, ERBB2 (simply chosen as was available in the laboratory) and no amplification was observed (Figure 2.8 inset).

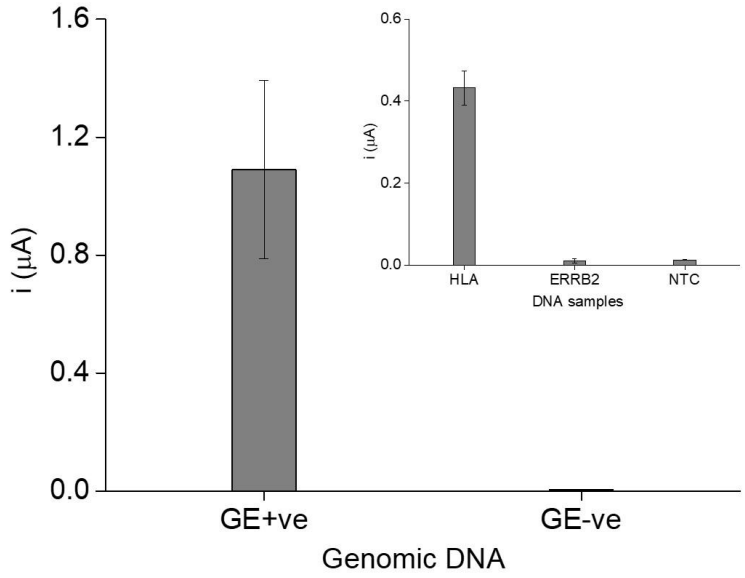


Figure 2.8. Application of genosensor to to real patient samples; Inset: Specificity of developed genosensor. Measurement conditions as described in text.

2.5. Conclusions

In the work reported here, we further explored the potential of the combination of tailed primers and isothermal amplification for the extremely rapid and sensitive combined amplification and detection of the HLA DQB1*02 coeliac-disease associated allele, which was used as a model target. Both forward and reverse primers were specifically designed to contain a C18 and a single stranded DNA tail. The C18 effectively stops polymerase mediated amplification, resulting in an amplicon of duplex target specific DNA, flanked by two single stranded DNA tails. We have previously reported this approach using the polymerase chain reaction, and recently reported the same approach using isothermal recombinase polymerase amplification, and here we further explored the possibility of minimizing hybridization and amplification times. Hybridization of just two minutes was observed to be adequate for hybridizing to both the surface immobilized capture probe and to the HRP labelled reporter probe. It was also attempted to co-hybridize to both the surface labelled probe and the reporter probe in one-step but there appeared to be a competitive phenomena between the probes for binding the RPA tailed amplicon, akin to the Hooke effect that is sometimes observed with antibodies in enzyme linked immunosorbent assays, and thus a step-by-step assay format was preferred. Whilst it is possible to use shorter amplification times and detect the RPA product, in the case of the HLA DQB1*02, a 30 minute amplification time was deemed optimum for testing a real patient sample. Ongoing work is focused on evaluating the effect of amplification time on detection limit using a range of different genomic targets.

2.6. Acknowledgements

This work has been carried out with partial financial support from the Spanish Ministerio de Economía y Competitividad (SEASENSING BIO2014-56024-C2-1, and CIGUASENSING BIO2017-87946-C2-1). SA acknowledges the Secretariat d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitatde Catalunya for his scholarship support (2015 FI_B 01072).

2.7. References

[1] J. Wang, From DNA biosensors to gene chips, 28 (2000) 3011–3016.

[2] R.W. Peeling, K.K. Holmes, D. Mabey, A. Ronald, Rapid tests for sexually transmitted infections (STIs): the way forward, Sex. Transm. Infect. 82 (2006) v1–v6. doi:10.1136/sti.2006.024265.

[3] T. Notomi, H. Okayama, H. Masubuchi,

T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA, 28 (2000).

[4] J. Van Ness, L.K. Van Ness, D.J. Galas, Isothermal reactions for the amplification of oligonucleotides, 2003 (2003) 0–5.

[5] G.T. Walker, M.S. Fraiser, J.L. Schram, M.C. Little, J.G. Nadeau, D.P. Malinowski, Strand displacement amplification-an isothermal , in vitro DNA amplification technique, 20 (1992) 1691–1696.

[6] T. Murakami, J. Sumaoka, M. Komiyama, Sensitive isothermal detection of nucleic-acid sequence by primer generation – rolling circle amplification, 37 (2009). doi:10.1093/nar/gkn1014.

[7] P. Gill, A. Ghaemi, Nucleic acid isothermal amplification technologies - A review, Nucleosides, Nucleotides and Nucleic Acids. 27 (2008) 224–243. doi:10.1080/15257770701845204.

[8] M. Vincent, Y. Xu, H. Kong, Helicase-dependent isothermal DNA amplification, EMBO Rep. 5 (2004) 795–800. doi:10.1038/sj.embor.7400200.

[9] O. Piepenburg, C.H. Williams, D.L. Stemple, N.A. Armes, DNA detection using recombination proteins, PLoS Biol. 4 (2006) 1115–1121. doi:10.1371/journal.pbio.0040204.

[10] R.K. Daher, G. Stewart, M. Boissinot, M.G. Bergeron, Review Recombinase Polymerase Amplification for Diagnostic Applications, 958 (2016). doi:10.1373/clinchem.2015.245829.

[11] V.A. Online, L.A. Tortajada-genaro, S. Santiago-felipe, M. Amasia, A. Russom, RSC Advances, (2015) 29987–29995. doi:10.1039/C5RA02778K.

[12] H. Joda, V. Beni, A. Willems, R. Frank, J. Höth, K. Lind, L. Strömbom, I. Katakis, C.K. ÓSullivan, Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles, Biosens. Bioelectron. 73 (2015) 64–70. doi:10.1016/j.bios.2015.05.048.

[13] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, A. Saeed, M.N. Abbas, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay, Sci. Rep. 6 (2016) 37732. doi:10.1038/srep37732.

[14] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Aptamer Lateral Flow Assays for Ultrasensitive Detection of β-Conglutin Combining Recombinase Polymerase Amplification and Tailed Primers, Anal. Chem. 88 (2016) 10701–10709. doi:10.1021/acs.analchem.6b03256.

Chapter 3

One step Nucleic acid electrochemical detection based on Recombinase Polymerase Amplification and modified primers

One step Nucleic acid electrochemical detection based on Recombinase Polymerase Amplification and modified primers

Sallam AL-Madhagi[a], Ioanis Katakis[a], Ciara O'Sullivan[a,b]

[a] Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona

[b] ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain

3.1. Abstract

An electrochemical geno-sensor based on direct detection of amplification products without the need to purify, create ssDNA or perform labelling step is reported. Modified primers were used to generate recombinase polymerase amplification (RPA) products with a central duplex flanked by single-stranded tail at one end of the duplex and horse-radish peroxidase (HRP) on the other. These single-stranded tail were used for direct hybridisation with surface immobilised capture probes.
The time required for hybridisation of the isothermally generated labelled amplicons with the immobilized capture probes was optimized to be 10 minutes. The effect of amplification time was optimized to be 60 min at 37°C. The limit of detection achieved was 48 fM. Presented geno-sensor was applied to the analysis of both synthetic and genomic DNA.

Keywords: DNA detection; modified primers; electrochemical detection; labelled amplicons

3.2. Introduction

The detection of specific DNA sequences becoming increasingly important in several areas such as diagnosis of genetic diseases[1], pathogens detection[2], forensics, food safety[3] and personalised medicine[4].

Conventional methods for the detection of specific DNA sequences are based on either direct sequencing or DNA hybridisation methods. Because of its simplicity, the hybridisation methods are more commonly used in DNA analysis[5]. The DNA hybridisation geno-sensores are the most widely used approach in the analysis of nucleic acids, which consisting of single-stranded DNA (ssDNA) probes layer immobilized on a transducer surface in order to recognize its complementary nucleic acid (target sequence) with high efficiency and extremely high specificity. In the vast majority of those DNA sensors, pre-detection treatments such as creating single strand DNA and labelling is essential.

The analysis of DNA requires amplification, because the DNA in many biological samples is present only at such low concentrations that it cannot be detected directly[6]. Polymerase chain reaction (PCR) is the most commonly used for amplification of DNA, the thermal nature of PCRs that require a long time to produce copies of DNA ranges from one to two hours, this limits the possibility of transferring this technique to be used in the field or in limited sources eras[54]. Therefore, in recent years, several alternative techniques for isothermal DNA amplification have been developed[7][8][7][9][10][11][12][13]. Among those isothermal amplification methods is Recombinase polymerase amplification (RPA), which has several advantages over other isothermal techniques due to its simplicity, sensitivity and rapid amplification at a constant temperature between 25–42 ºC[14].

In spite of the high efficiency, simplicity, and rapidity of RPA, the detection of RPA amplicons via hybridization normally requires the generation of single stranded DNA, followed by labelling step which increases the time of the detection and complicates the detection process. Thus, we previously developed an approach to produce PCR amplicons that could be directly detected post-amplification via hybridization[15] by using modified primers thus resulting in a duplex amplification product flanked by two single-stranded DNA tails. Those two tails have been used to hybridize to a surface immobilised probe and an enzyme labelled reporter probe. This novel technology has been combined with RPA for DNA electrochemical detection[16] and for the lateral flow based detection of DNA[17] and aptamers[18]. The use of modified primers has allowed us to avoid creating ssDNA, but labelling process remains essential.

Herein, we describe a highly sensitive diagnostic method by coupling one of these modified primers (tailed forward primer) and reverse primer liked to HRP with the RPA, with the main objective of simplifying the electrochemical detection of the RPA products by avoiding purification, creating ssDNA and pre-detection labelling.

3.3. Materials and methods

3.3.1. Materials

RPA TwistAmp® basic kit was purchased from TwistDx Ltd. (Babraham, United Kingdom). DreamTaq DNA polymerase kit was purchased (Fisher Scientific- Spain). DNA Clean & Concentration^{TM} -5 kit was purchased from (Zymo Research). TMB enhanced one component HRP membrane was supplied by Diarect AG (Germany). Potassium dihydrogen phosphate (KH2PO4) from Scharlau (Barcelona, Spain). Dithiol 16-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15-pentaoxahexa-decane) (DT1) was obtained from SensoPath Technologies (Bozeman, MT, USA). DNA probes, DNA forward primer, Horseradish peroxidise (HRP) modified revers primer and synthetic analogues of Karlodinum armiger (Ka) and HLA targets were supplied by biomers.net GmbH (Ulm, Germany) as lyophilized powder and reconstituted in RNase and DNase-free water and used without further purification. Genomic DNA were supplied by IRTA (Cataluña, Spain).

Table 3.1. DNA target, probe and primers sequences used in this work:

Name	Nucleotide sequence (from 5′ to 3′)
Karlodinum armiger (Ka) target	ata gct tca cag cag agg tta caa cac caa tgc tgc tcc gct acc cgc gat ctc atg cac cag gga agc ttc aag aca ccc cta ccc ccg tgc agg agc tca caa aga aag ttc aca gtg aga tgg ttg gat gtg tgt
Karlodinum armiger (Ka) capture probe	ttc att gag ttc gtc gta at ttt ttt ttt ttt ttt-SH
Karlodinum armiger (Ka) tailed forward primer	att acg acg aac tca atg aa – C3 Spacer - ata gct tca cag cag agg tta caa c
Karlodinum armiger (Ka) HRP-reverse primer	HRP- aca cac atc caa cca tYt cac tg
Karlodinum armiger (Ka) unmodified forward primer	aca cac atc caa cca tYt cac tg
Karlodinum armiger (Ka) unmodified reverse primer	ata gct tcg cag aca aag gtg aat c
Karlodinum vinificum (Kv) target	ata gct tcg cag aca aag gtg aat ccc aat gct gct cca cta ccc gcg aac tgc taa cgc cag ggt gcg gaa gag aac tac ccc aac ccc cgc gca aga gct cac aaa gaa gtt cac agt gaa atg gtt gga tgt gtg t
Wrong capture probe	gtc gtg act ggg aaa ac ttt ttt ttt ttt ttt-SH

3.3.2. PCR amplification of Ka ssDNA target to produce dsDNA template

For this assay, target was first amplified in conventional PCR to produce dsDNA.

PCR amplification was carried out using unmodified primers. PCR amplification was performed using DreamTaq DNA polymerase kit (Fisher Scientific- Spain) with following volumes: 10 µL 5x buffer, 5 µL dNTPs (2mM), 5 µL MgCl (50 mM), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5µL BSA (50 mg/mL), 27 µL H2O, 0.5 µL polymerase and 1 µL of 5nM ssDNA template. All PCR reactions were heated at 95 °C for 2 min, followed by 30 rounds of PCR, with a denaturing step at 95 °C for 30 s, annealing step at 58 °C for 30 s, and an elongation step at 72 °C for 30 s. A final extension step was performed at 72 °C for 5 min.

PCR amplification was carried out in a iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain). Double-stranded PCR products were analyzed using agarose gel electrophoresis. For each sample, 4 µl was run on the gel with 4 µl of loading buffer on a 3 % (w/v) agarose gel stained with GelRed™ nucleic acid stain and visualized with a UV lamp (λ = 254 nm).

PCR products were purified using DNA Clean & Concentration$^{TM\ -5}$ kit (Zymo Research) by mixing 5 volumes of DNA binding buffer to each volume of the RPA products, the mixture transferred to the Zymo-Spin column and centrifuged for 30 seconds at 10000g, after which the column was washed three times with 200 µl DNA wash buffer and centrifuged for 30 seconds at 10000g. Finally, 20 µL of water added directly to the column matrix and incubated for one minute and then centrifuged for 30 seconds at 10000g to elute the dsDNA. Purified dsDNA quantified by UV spectrophotometry with a NanoDrop 2000 at λ = 260 nm.

3.3.3. Liquid phase RPA / solid phase detection

The target (synthetic/genomic) was first amplified using isothermal recombinase polymerase amplification (RPA) utilizing tailed forward primer and HRP reverse primer. Subsequently, detection was carried out by hybridisation to a surface capture probe on gold electrodes.

3.3.4. RPA assay

The RPA amplification mixture was prepared according to the manufacturer's instructions, by mixing 2.4 µl of 10 µM HRP reverse primer and 2.4 µl of 10 µM tailed forward primer, 2 µl DNA template (Ka), 11 µl DNAse free water and 2.95 µl 1× rehydration buffer. Lyophilized pellet was then added to the mixture. The reaction was finally initiated by addition of 2.5 µl of 280 mM magnesium acetate to a final volume of 50 µl, and the solution was immediately mixed by vortex. The amplification was performed using iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain) for a given time at 37 °C.

3.3.5. Electrochemical RPA detection on gold electrode array

All electrochemical measurements were performed using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software program (GPES) (Eco Chemie, The Netherlands), equipped with a MUX module (Eco Chemie B.V., The Netherlands) for sequential measurement of working electrodes that share the same counter electrode, silver / silver chloride electrode was used as reference.

3.3.6. Electrode chip design and fabrication

The electrochemical cell consists of a customized electrode array chip designed “in- The electrochemical cell consists of a customized electrode array chip designed in-house using AutoCAD software (Autodesk Inc, USA) and fabricated at Fineline GmbH (Hilden, Germany) using printed circuit board (PCB) technology with a soft gold surface finish. It consists of 32 working electrodes (diameter =1mm), each arranged with a gold counter electrode and gold reference electrode. This array was not specifically designed for the work reported here, but could be used for multiplexed detection of RPA amplicons. The electrode array was interfaced with a multiplexed potentiostat using a custom-designed connector.

3.3.7. Electrode surface functionalization and electrochemical detection

Prior to functionalization, the electrode array was cleaned via exposure to UV/ozone for 10 min, followed by immersion in 25% v/v H_2O_2 containing KOH (50mM) for a further 10 min. Finally, the array was rinsed with MilliQ water and then with ethanol and finally dried in N_2 gas. Each electrode of the array was functionalized using a co-immobilization approach by drop-casting 1 µL of 100µM DT1 and 5 µM of thiolated DNA probe in 1 M KH_2PO_4 onto each electrode. Self-assembling was left to take place overnight at room temperature within a water-saturated atmosphere, and then the electrode array was rinsed with MilliQ water and dried with N2 gas.

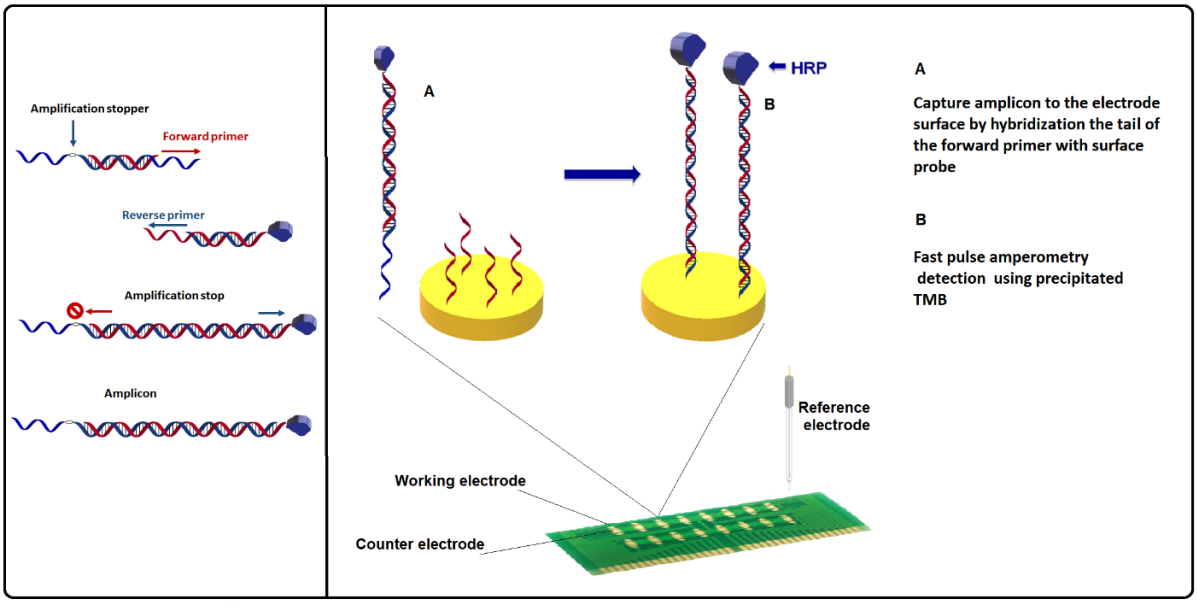


Figure 3.1. Electrode setup, functionalization and detection process.

Amplified RPA product (1 µL) was drop-casted into the electrode surface and incubated for the reported time at 37 °C within a water-saturated atmosphere to hybridize with the capture probe. Following incubation, the electrodes were sequentially washed with TRIS buffer saline (pH 8) under magnetic steering. For the electrochemical measurements, TMB enhanced one component HRP membrane substrate was added and allowed to react for 5 min. Finally, the oxidized/precipitated TMB was detected by fast pulse amperometry (0 V for 10 ms followed by −0.2 V for 500 ms vs. silver/silver chloride reference electrode).

3.4. Results and discussion

Optimizing the RPA time in this work was done by the amplification of a 2 pM dsDNA Ka template for 10, 20, 30, 40, 50 and 60 min using the protocol described above. Tailed forward primer and HRP reverse primer were used to perform the liquid phase amplification at 37ºC. A master mix of 100 µL was amplified in Ependorff using iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain), Subsequently, 15 µL aliquots were taken from the master RPA reaction after 10, 20, 30, 40, 50 and 60 min, then the RPA reactions stopped by freezing. Whilst, no template control (NTC) has amplified separately for 60 min with the same amplification conditions. Following RPA, 1 µL of the amplification products drop-casted on the electrode surface and kept to hybridise with the surface capture probe for 30 min at 37ºC within a water-saturated atmosphere. Subsequently, TMB enhanced one component HRP membrane substrate was added and allowed to react for 5 min.

As it can be seen in (Figure 2), increasing amplification times resulted in increasing levels of amplicons produced, while no positive electrochemical signal obtained after 10 min amplification, this indicates that incorporating HRP reverse primer in the RPA reaction needs minimum 20 min to produce amplicons labelled with HRP.

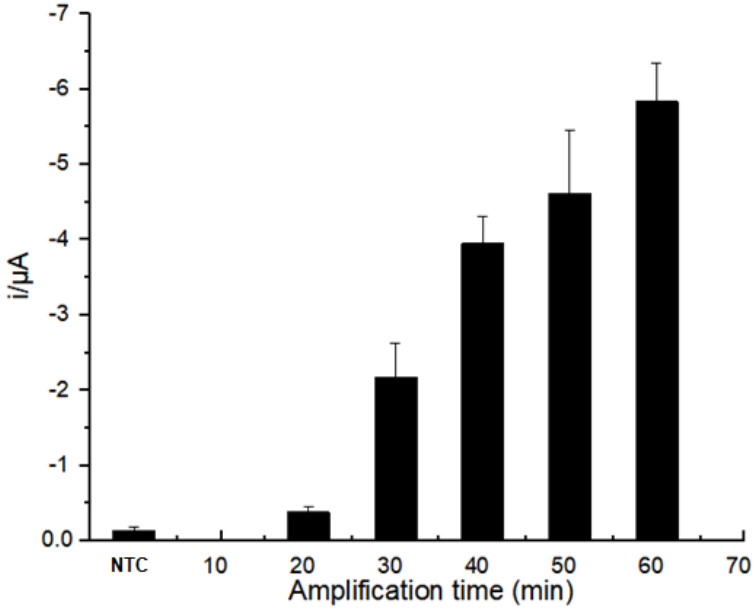


Fig 3.2. Electrochemical detection of RPA amplicons following 10, 20, 30, 40, 50 and 60 minute's amplification. NTC indicates the signal obtained following amplification of a non-specific template for 60 minutes. 2 pM target was used as template and 30 min capture hybridization.

Based on the results obtained from the amplification time optimization, 60 min RPA products of the 2 pM dsDNA Ka has been used to optimize the hybridisation time with the surface capture probe to ensure maximum signal. Unpurified RPA amplicon were directly detected via hybridisation with gold electrode immobilised capture probe, varying the hybridisation time from 2 to 30 minutes at 37 ºC within a water-saturated atmosphere. The hybridisation of the RPA labelled amplicons with 2 minute was adequate, although 10 minutes was where plateauing was observed (Figure 3.3).

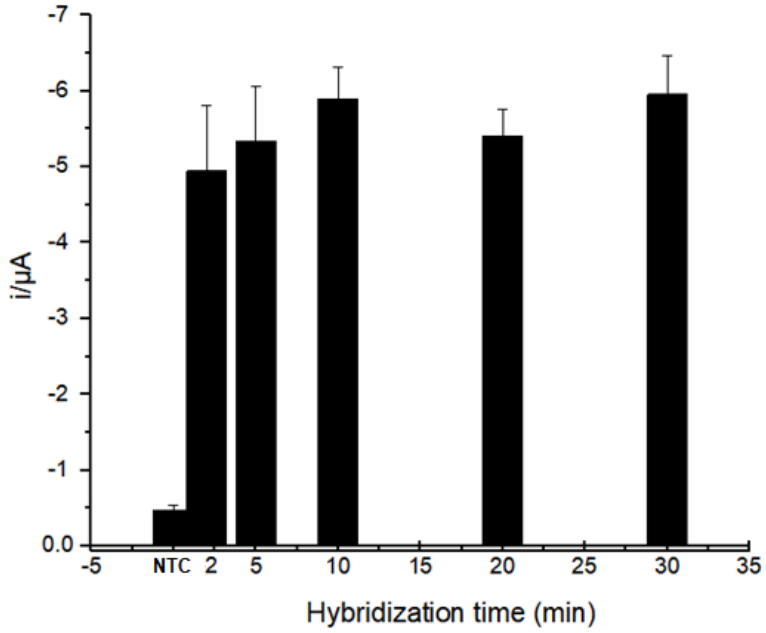


Fig 3.3. The effect of duration of hybridisation time of 60 min RPA amplicons with surface capture probe tethered on the gold electrode

DNA starting concentration is an important factor in the evaluation of any DNA detection system. Here we used 5 fold serial dilutions of the starting DNA template from 2 pM to 200 aM. The optimum conditions of the amplification and hybridisation from the experiments described above were used (60 min amplification and 10 min hybridization). The limit of detection (LOD) obtained using dsDNA was 48 fM determined as the concentration of the analyte at the mean blank signal plus three times the standard deviation of the blank.

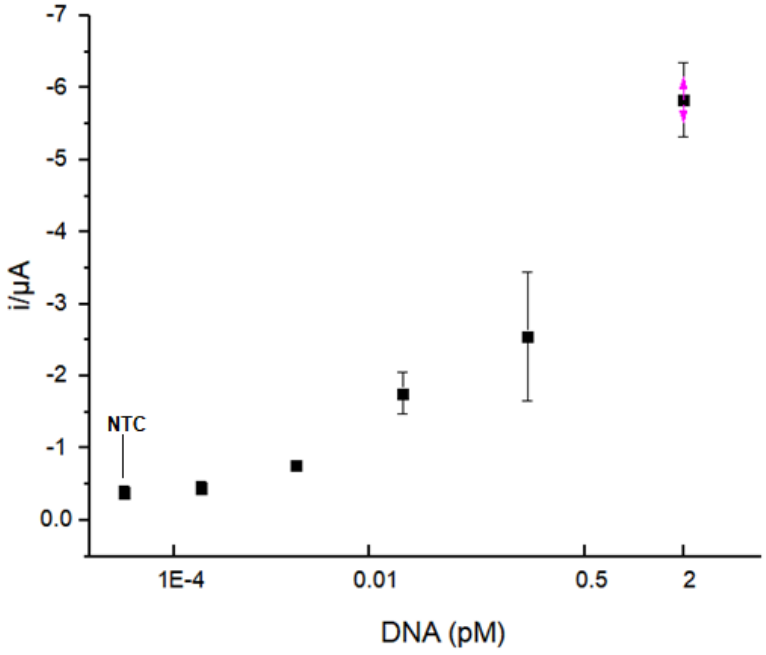


Fig 3.4. Calibration plot of 60 min RPA and 10 min hybridisation with capture probe.

In the interest of demonstrating assay specificity towards karlodinium armiger (Ka) target, two strategies have been used. Starting with using wrong capture probe on the electrode surface to demonstrate the selective capturing of the target to the electrode surface, subsequently, 60 min RPA products of Ka have hybridised with the wrong capture for 30 min, as it can be seen in (Figure. 3.5. A) only background signal was observed in this experiment. Furthermore, we challenged the assay with non-karlodinium armiger (Ka) targets by using HLA target (simply chosen as was available in the laboratory). As expected, only Ka positive sample gave a significant positive electrochemical signal (Fig 3.5. A).

Using the optimised assay conditions of 60 minutes of amplification, 10 minutes hybridisation with the surface immobilized probe, the developed geno-sensor was used to detect the Ka in genomic DNA extracted from seawater. Genomic DNA was extracted from seawater and provided by IRTA. DNA containing Ka sequence (GE +ve) and a control sample not containing the Ka sequence (GE -ve) were analysed, and as can be seen in (Figure 3.5.B) a significant positive electrochemical signal was observed in the case of the positive sample.

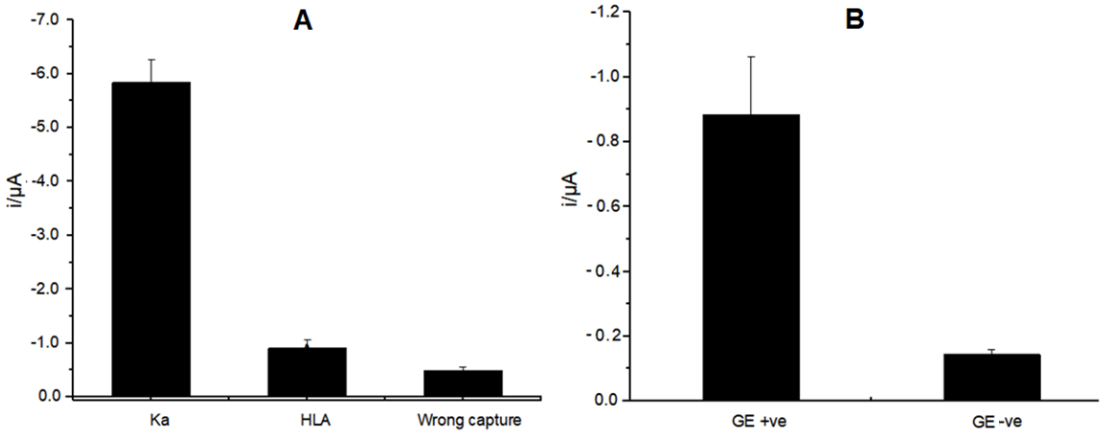


Fig 3.5. (A) Specificity of developed geno-sensor. (B)Application of geno-sensor to real samples. Measurement conditions as described in text.

3.5. Conclusions

Existing strategies of DNA electrochemical detection require multi-step procedures such as DNA purification, creating single strand DNA and labelling.

In this contribution, we have developed an electrochemical geno-sensor to detect RPA products without the need to purify, create ssDNA or perform labelling process. The geno-sensor in this work has been developed based on using a specifically designed forward primer with single stranded DNA tail mediated by a C3 chain. On the other hand, reverse primer linked with HRP has been used. Using this combination of primers allow us to produce an amplicon of duplex target specific DNA, flanked by single-stranded DNA tail on one end and HRP on the other. The sensor was used for the detection of both synthetic and genomic karlodinium armiger DNA samples. The LOD obtained following 60 min RPA and 10 min hybridisation with the surface capture probe and electrochemical detection was 48 fM of original synthetic DNA. At the same time, the selectivity of the sensor has been evidenced by using karlodinium veneficum genomic DNA and HLA DQB1*02 synthetic DNA controls.

3.6. Acknowledgment

This work has been carried out with partial financial support from Spanish Ministerio de Economía y Competitividad (SEASENSING BIO2014-56024-C2-1): “Seasensing: Microsystems for rapid, reliable and cost effective detection of toxic microalgae on-site and in real-time” SAM acknowledges the Secretariat d’Universitats i Recerca del Departament d’Economia i Coneixement de la Generalitatde Catalunya for his scholarship support (2015 FI_B 01072).

3.7. References

[1] S. Campuzano, M. Pedrero, J.M. Pingarrón, Electrochemical genosensors for the detection of cancer-related miRNAs, Anal. Bioanal. Chem. 406 (2014) 27–33. doi:10.1007/s00216-013-7459-z.

[2] Y. El Goumi, Electrochemical Genosensors : Definition and Fields of Application, 3 (2017) 14–16. doi:10.15406/ijbsbe.2017.03.00080.

[3] B. Martín-Fernández, C.L. Manzanares-Palenzuela, M. Sánchez-Paniagua López, N. de-los-Santos-Álvarez, B. López-Ruiz, Electrochemical genosensors in food safety assessment, Crit. Rev. Food Sci. Nutr. 57 (2017) 2758–2774. doi:10.1080/10408398.2015.1067597.

[4] R. Paper, Electrochemical Biosensors - Sensor Principles and Architectures, (2008) 1400–1458.

[5] M.I. Pividori, A. Merkoçi, S. Alegret, Electrochemical genosensor design: Immobilisation of oligonucleotides onto transducer surfaces and detection methods, Biosens. Bioelectron. 15 (2000) 291–303. doi:10.1016/S0956-5663(00)00071-3.

[6] M.N. Tsaloglou, A. Nemiroski, G. Camci-Unal, D.C. Christodouleas, L.P. Murray, J.T. Connelly, G.M. Whitesides, Handheld isothermal amplification and electrochemical detection of DNA in resource-limited settings, Anal. Biochem. 543 (2018) 116–121. doi:10.1016/j.ab.2017.11.025.

[7] P. Gill, A. Ghaemi, Nucleic acid isothermal amplification technologies - A review, Nucleosides, Nucleotides and Nucleic Acids. 27 (2008) 224–243. doi:10.1080/15257770701845204.

[8] V.T. Katja Niemann, Isothermal Amplification and Quantification of Nucleic Acids and its Use in Microsystems, J. Nanomed. Nanotechnol. 06 (2015). doi:10.4172/2157-7439.1000282.

[9] Kumar, Isothermal Nucleic Acid Amplification System: An Update on Methods and Applications, J Genet Genom. 2 (2018) 1–5. https://www.omicsonline.org/open-access/isothermal-nucleic-acid-amplification-system-an-update-on-methods-andapplications.pdf.

[10] Y. Zhao, F. Chen, Q. Li, L. Wang, C. Fan, Isothermal Amplification of Nucleic Acids, Chem. Rev. 115 (2015) 12491–12545. doi:10.1021/acs.chemrev.5b00428.

[11] D. Morisset, D. Stebih, K. Cankar, J. Zel, K. Gruden, Alternative DNA amplification methods to PCR and their application in GMO detection: A review, Eur. Food Res. Technol. 227 (2008) 1287–1297. doi:10.1007/s00217-008-0850-x.

[12] J. Li, J. Macdonald, Advances in isothermal amplification: Novel strategies inspired by biological processes, Biosens. Bioelectron. 64 (2014) 196–211. doi:10.1016/j.bios.2014.08.069.

[13] X. Zhang, S.B. Lowe, J.J. Gooding, Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP), Biosens. Bioelectron. 61 (2014) 491–499. doi:10.1016/j.bios.2014.05.039.

[14] I.M. Lobato, C.K. O'Sullivan, Recombinase polymerase amplification: Basics, applications and recent advances, TrAC - Trends Anal. Chem. 98 (2018) 19–35. doi:10.1016/j.trac.2017.10.015.

[15] H. Joda, V. Beni, A. Willems, R. Frank, J. Höth, K. Lind, L. Strömbom, I. Katakis, C.K. ÓSullivan, Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles, Biosens. Bioelectron. 73 (2015) 64–70. doi:10.1016/j.bios.2015.05.048.

[16] S. Al-Madhagi, H. Joda, M. Jauset-Rubio, M. Ortiz, I. Katakis, C.K. O'Sullivan, Isothermal amplification using modified primers for rapid electrochemical analysis of coeliac disease associated DQB1*02 HLA allele, Anal. Biochem. 556 (2018) 16–22. doi:10.1016/j.ab.2018.06.013.

[17] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, A. Saeed, M.N. Abbas, M.S. El-

Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay, Sci. Rep. 6 (2016) 37732. doi:10.1038/srep37732.

[18] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Aptamer Lateral Flow Assays for Ultrasensitive Detection of β-Conglutin Combining Recombinase Polymerase Amplification and Tailed Primers, Anal. Chem. 88 (2016) 10701–10709. doi:10.1021/acs.analchem.6b03256.

Chapter 4

Reagent-less electrochemical DNA sensor based on Recombinase Polymerase Amplification and modified primers

Reagent-less electrochemical DNA sensor based on Recombinase Polymerase Amplification and modified primers

Sallam AL-Madhagi[a],Mamantos Prodromidis[c], Ioanis Katakis[a], Ciara O'Sullivan[a,b]

[a] Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona

[b] ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain

[c] Department of Chemistry, University of Ioannina, 45110, Ioannina, Greece

4.1. Abstract

In this work, we designed a reagent-less DNA sensor based on modified primers, modified gold nanoparticles (AuNPs) and isothermal amplification. Colloidal gold nanoparticles were prepared by citrate reduction of $HAuCl_4$ with an average diameter of 13 nm. Then, these gold nanoparticles modified with thiolated ssDNA and 6-(Mercaptohexyl)ferrocene using self-assembly co-immobilisation method. Modified primers, exploiting a C3 spacer between the primer-binding site and an engineered single stranded tail, were used in a recombinase polymerase amplification (RPA) reaction to produce an amplicon with a central duplex flanked by two single stranded tails. These tails were designed to be complementary to a gold electrode tethered capture oligo probe as well as an oligo probe immobilised on the AuNPs. The time required for hybridisation of the target tailed DNA with the surface immobilised probe and reporter probe immobilised on the AuNPs was optimised to be 10 minutes, in both cases. Amplification time was further optimised to be 40 minutes, to ensure the maximum signal. Under optimal conditions, the biosensor could detect target dsDNA down to 7.2 fM. Furthermore, the developed DNA was used in the analysis of real samples, and it demonstrates the ability to amplify and detect target DNA in the genomic DNA samples.

Keywords: DNA detection; modified primers; electrochemical detection; labelled amplicons

4.2. Introduction

Electrochemical detection of DNA sequences offers a sensitive, low-cost, and portable method in biochemical and biomedical researches and gene diagnostics[1].The low detection limit of DNA sequences is a very important factor in developing DNA sensors[2]. Thus, DNA could be amplified to increase the target DNA concentration.

Polymerase chain reaction (PCR) is the most commonly used for amplification of DNA, the thermal nature of PCRs that require a long time to produce copies of DNA ranges from one to two hours, this limits the possibility of transferring this technique to be used in the field or in limited sources eras. Therefore, in recent years, several alternative techniques for isothermal DNA amplification have been developed[3][4][3][5][6][7][8][9]. Among those isothermal amplification methods is Recombinase polymerase amplification (RPA), which has several advantages over other isothermal techniques due to its simplicity, sensitivity and rapid amplification at a constant temperature between 25–42 ºC[10].

On the other hand, increasing the sensitivity of the detection system is highly desirable. One of the methods used to increase the sensitivity of the detection systems is labelling the target DNA with more than one electroactive label. Yusuke Hasegawa and co-workers have developed a methodology of conjugating the oligonucleotides with ferrocene for the electrochemical detection of DNA base mismatch[1]. Benjamin and co-workers have developed DNA detection assay based on using biotin-dUTP to produce RPA amplicons modified with biotin to bind it with streptavidin HRP to improve the sensitivity of the electrochemical detection[11][12].

We previously developed an approach to produce PCR amplicons that could be directly detected post-amplification via hybridisation[13] by using modified primers thus resulting in a duplex amplification product flanked by two single-stranded DNA tails. Those two tails have been used to hybridise to a surface immobilised probe and an enzyme labelled reporter probe. This novel technology has been combined with RPA for DNA electrochemical detection[14] and for the lateral flow based detection of DNA[15] and aptamers[16].
In this work, we describe a highly sensitive molecular diagnostic tool by coupling tailed primers with the RPA and modified AuNPs, with the main objective of developing highly sensitive and simple electrochemical platform for the detection of the RPA products without the need to purify or create ssDNA.

4.3. Materials and methods

4.3.1. Materials

RPA TwistAmp® basic kit was purchased from TwistDx Ltd. (Babraham, United Kingdom). DreamTaq DNA polymerase kit was purchased (Fisher Scientific- Spain). DNA Clean & Concentration™ -5 kit was purchased from (Zymo Research). TMB enhanced one component HRP membrane was supplied by Diarect AG (Germany). Potassium dihydrogen phosphate (KH2PO4) from Scharlau (Barcelona, Spain). DNA probes, DNA forward primer, Horseradish peroxidise (HRP) modified revers primer and synthetic analogues of Karlodinum armiger (Ka) and HLA targets were supplied by biomers.net GmbH (Ulm, Germany) as lyophilized powder and reconstituted in RNase and DNase-free water and used without further purification. Genomic DNA were supplied by IRTA (Cataluña, Spain).

Table 4.1. DNA target, probe and primers primers sequences used in this work:

Name	Nucleotide sequence (from 5′ to 3′)
Karlodinum armiger (Ka) target	ata gct tca cag cag agg tta caa cac caa tgc tgc tcc gct acc cgc gat ctc atg cac cag gga agc ttc aag aca ccc cta ccc ccg tgc agg agc tca caa aga aag ttc aca gtg aga tgg ttg gat gtg tgt
Karlodinum armiger (Ka) capture probe	ttc att gag ttc gtc gta at ttt ttt ttt ttt ttt-SH
Karlodinum armiger (Ka) tailed forward primer	att acg acg aac tca atg aa – C3 Spacer - ata gct tca cag cag agg tta caa c
Karlodinum armiger (Ka) HRP-reverse primer	tgt aaa acg acg gcc agt – C3 Spacer - aca cac atc caa cca tYt cac tg
Karlodinum armiger (Ka) unmodified forward primer	aca cac atc caa cca tYt cac tg
Karlodinum armiger (Ka) unmodified reverse primer	ata gct tcg cag aca aag gtg aat c
Karlodinum vinificum (Kv) target	ata gct tcg cag aca aag gtg aat ccc aat gct gct cca cta ccc gcg aac tgc taa cgc cag ggt gcg gaa gag aac tac ccc aac ccc cgc gca aga gct cac aaa gaa gtt cac agt gaa atg gtt gga tgt gtg t
Wrong capture probe	gtc gtg act ggg aaa ac ttt ttt ttt ttt ttt-SH

4.3.2. PCR amplification of Ka ssDNA target to produce dsDNA template

In this work, target was first amplified in conventional PCR to produce dsDNA to use it as template in the RPA assay or in the optimization experiments. PCR amplification was carried out using unmodified primers. PCR amplification was performed using DreamTaq DNA polymerase kit (Fisher Scientific- Spain) with following volumes: 10 µL 5x buffer, 5 µL dNTPs (2mM), 5 µL MgCl (50 mM), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5 µL BSA (50 mg/mL), 27 µL H2O, 0.5 µL polymerase and 1 µL of 5 nM ssDNA template. All PCR reactions were heated at 95 °C for 2 min, followed by 30 rounds of PCR, with a denaturing step at 95 °C for 30 s, annealing step at 58 °C for 30 s, and an elongation step at 72 °C for 30 s. A final extension step was performed at 72 °C for 5 min. PCR amplification was carried out in a iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain). Double-stranded PCR products were analyzed using agarose gel electrophoresis. 4 µl of PCR products was run in the gel with 4 µl of loading buffer in a 3 % (w/v) agarose gel stained with GelRed™ nucleic acid stain and visualized with a UV lamp (λ = 254 nm).

PCR products were purified using DNA Clean & ConcentrationTM-5 kit (Zymo Research) by mixing 5 volumes of DNA binding buffer to each volume of the RPA products, the mixture transferred to the Zymo-Spin column and centrifuged for 30 seconds at 10000g, after which the column was washed three times with 200 µl DNA wash buffer and centrifuged for 30 seconds at 10000g. Finally, 20 µL of water added directly to the column matrix and incubated for one minute and then centrifuged for 30 seconds at 10000g to elute the dsDNA. Purified dsDNA quantified by UV spectrophotometry with a NanoDrop 2000 at λ = 260 nm.

4.3.3. Preparation of colloidal gold nanoparticles

GNPs with an approximate average diameter of 13 nm were prepared by citrate reduction of $HAuCl_4$ as described in the literature[17]. All glassware was cleaned with aqua regia ($HCl:HNO_3$ = 3:1), rinsed with deionized water and dried before using. An aqueous solution of sodium citrate (2.0 mL, 1 wt.%) was rapidly added to a boiling $HAuCl_4$ solution (50 mL, 0.01 wt.%) under vigorous stirring. The colour of the solution changed slowly from purple to blue and finally to red, indicating formation of GNPs. The solution was refluxed for an additional 15 min under vigorous stir-ring. After the heat source was removed, the solution was stirred continuously until it reached room temperature. Finally, the cooled solution was stored in the refrigerator at 4 ˚C.

4.3.4. Preparation of ferrocene/reporter probe-AuNPs conjugation

Conjugation of reporter DNA to AuNPs was achieved via mixing thiolated reporter DNA probe and 6-(Mercaptohexyl)ferrocene with 1 ml of AuNPs. The solution was left to incubate for 24 hours at room temperature. Subsequently, the conjugate was centrifuged at 8000 rpm for 30 minutes at 22ºC and the pellet was re-suspended three times in 500 µL deionized water in order to clean the conjugate and to remove any free DNA or ferrocene. The conjugate was then re-suspended in deionized 50 µL water.

4.3.5. Liquid phase RPA / solid phase detection

The target (synthetic/genomic) was first amplified using isothermal recombinase polymerase amplification (RPA) utilizing tailed primers. Subsequently, detection was carried out by hybridisation to a surface capture probe on gold electrodes. Then with reporter probe.

4.3.5. RPA assay

The RPA amplification mixture was prepared according to the manufacturer's instructions, by mixing 2.4 µl of 10 µM tailed reverse primer and 2.4 µl of 10 µM tailed forward primer, 2 µl DNA template (Ka), 11 µl DNAse free water and 2.95 µl 1× rehydration buffer. Lyophilized pellet was then added to the mixture. The reaction was finally initiated by addition of 2.5 µl of 280 mM magnesium acetate to a final volume of 50 µl, and the solution was immediately mixed by vortex. The amplification was performed using iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain) for a given time at 37 °C.

4.3.6. Electrode surface functionalization and electrochemical detection

The gold electrode was prepared for modification by mechanical polishing with suspensions of 0.3 µm and then 0.05 µm alumina particles followed by rinsing with MilliQ water and then with ethanol and finally dried with N2 gas. Each gold working electrode was functionalized using a co-immobilization approach by drop-casting 4 µL of 100µM mercaptohexanol (MCH) and 5 µM of thiolated DNA probe in 1 M KH_2PO_4

onto each electrode. Self-assembling was left to take place overnight at room temperature within a water-saturated atmosphere, and then electrodes were rinsed with MilliQ water and dried with N2 gas.

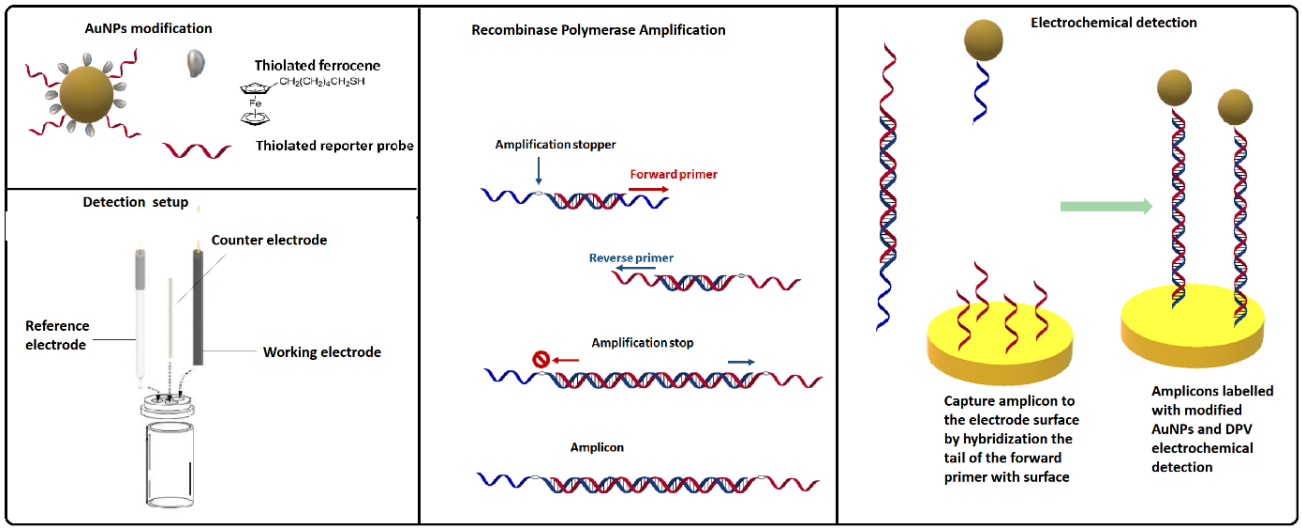


Fig 4.1. Electrode setup, functionalization and detection process.

Amplified RPA product (4 µL) was drop-casted into the electrode surface and incubated for the reported time at 37 °C within a water-saturated atmosphere to hybridize with the capture probe. Following incubation, the electrodes were sequentially washed TRIS buffer saline (pH 8) under magnetic steering. All electrochemical measurements were performed using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software program (GPES) (Eco Chemie, The Netherlands). The three-electrode system includes 2 mm gold working electrodes CHI 101 (CH Instruments) (Shanghai, China), Ag/AgCl electrode as the reference and platinum wire as the counter electrode. DPV was recorded in PBS (pH 7.4) solution at a potential range from 0 to 0.7 V.

4.4. Results and discussion

4.4.1. Gold nanoparticles characterization

The size, size distribution of the AuNPs was determined using TEM image of Au colloids. Later on, UV–vis scan from 800 nm to 200 nm were recorded with a spectrophotometer (Cary 100 Bio UV-visible spectrophotometer, Agilent) to determine the concentration (Figure 3.2).

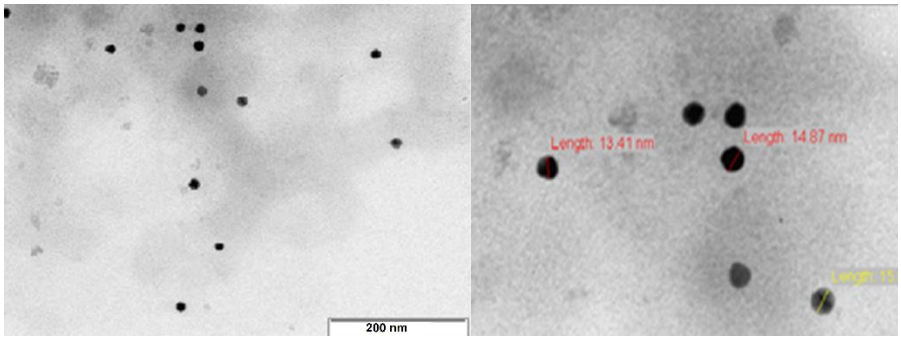


Figure 4.2.TEM image of Au colloids of Au colloids

4.4.2. Ferrocene/reporter probe-AuNPs conjugation optimization

AuNPs were modified with different ratios of ferrocene and thiolated reporter probe (Table 3.1), these ratios have been evaluated using DPV electrochemical detection.

Table 4.2. Ferrocene/reporter probe-AuNPs conjugations

	Ferrocene(3mM)	Reporter probe(100µM)	Gold nanoparticles (3.4 nM)
1	10 µL	20 µL	1mL
2	10 µL	10 µL	1mL
3	10 µL	5 µL	1mL
4	10 µL	2.5 µL	1mL
5	10 µL	1.25 µL	1mL

First, conventional PCR was performed (using the protocol described above) to produce dsDNA using modified primers to produce duplex amplification product flanked by two single-stranded DNA tails. Those two tails have been used to hybridise to a surface immobilised probe and reporter probe immobilised on the modified AuNPs. Then, PCR products purified using DNA Clean & Concentration^{TM-5} kit (Zymo Research) and quantified by UV spectrophotometry with a NanoDrop 2000 at $\lambda = 260$ nm. After that, 1 nM of the tailed amplicons has been prepared in 50 mM Tris buffered saline containing 1M NaCl. Subsequently, 4 µL of the prepared tailed dsDNA drop-casted on the electrode surface and kept for hybridisation with the surface capture probe then labelled with modified AuNPs, for 20 min at 37^{o}C within a water-saturated atmosphere in both cases. Finally, DPV was recorded in PBS (pH 7.4) at a potential range from 0.2 to 0.7 V

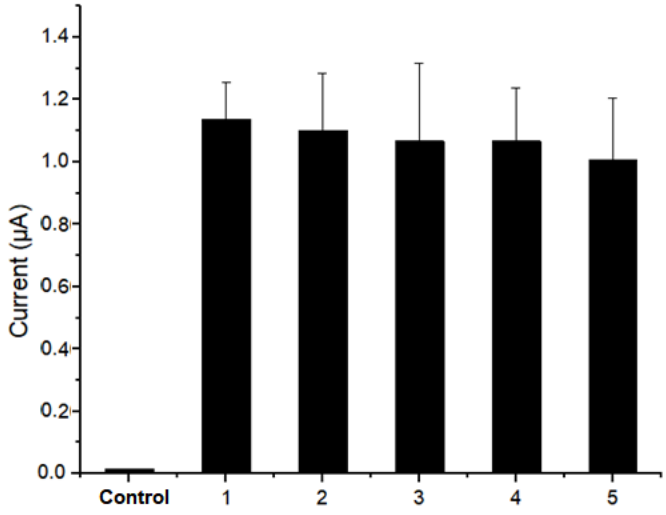


Figure 3.3. Electrochemical evaluation of the ferrocene/reporter probe-AuNPs conjugations

Electrochemical signals 1, 2, 3, 4, 5, correspond to the number of conjugation in (Table 3.2). while, the control is the electrochemical signal in absence of the tailed target. As it can be seen in figure 3.3. the electrochemical signal remains stable with the decrease of the thiolated reporter probe. For that reason, the conjugation with the lowest concentration of reporter probe has been used in the experiments of the optimisation and detection.

4.4.3. Optimization of experimental conditions

Because experimental conditions play a key role in the development of the DNA sensor, their optimisation the time needed for the hybridisation of the target with capture surface probe and reporter probe is very important. At first, 1 nM of the tailed Ka DNA prepared in 50mM Tris buffered saline containing 1M NaCl was used to optimise the time needed for the hybridisation of the tailed amplicons with capture surface. 4 uL was directly dropped on electrode surface and kept for hybridisation with the gold electrode immobilized capture probe, varying the hybridization time from 2 to 20 min at 37 °C and keeping the time of the hybridisation with the reporter probe constant (20 min) Figure 3.4.A. Subsequently, the time needed for the hybridisation of the target with reporter probe has been optimised using the same strategy by varying the hybridization time of the target with the reporter probe from 2 to 20 min, keeping the time of the hybridisation with the surface probe constant (20 min) Figure 3.4.B.

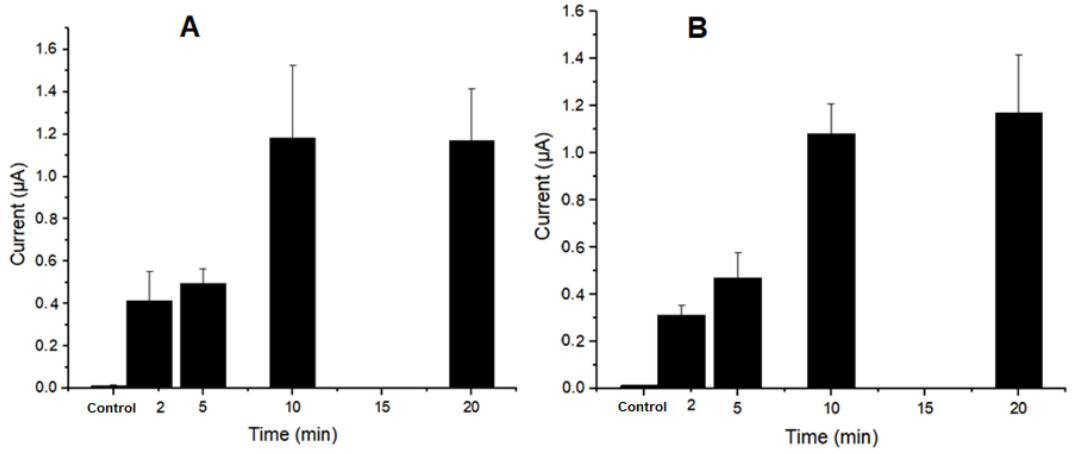


Figure 4.4. (a) Effect of duration of hybridization time of tailed 1nM Ka target with capture probe surface tethered on gold electrode, followed by 20 minutes hybridization with AuNPs. (b) Effect of duration of hybridization time of tailed 1nM Ka target AuNPs, following hybridization with surface immobilized capture probe for 10 minutes. 10 nM template DNA was used, amplified for 10 min.

4.4.4. Optimization of RPA time

Optimizing the RPA time in this work was done by the amplification of a 2 pM dsDNA Ka template for 5, 10, 20, 30 and 40 min using the protocol described above. Tailed primers were used to perform the liquid phase amplification at 37ºC. A master mix of 100 µL was amplified in Ependorff using iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain), subsequently, 15 µL aliquots were taken from the master RPA reaction after 5, 10, 20, 30, and 40 min and the RPA reaction stopped by freezing. Whilst, no template control (NTC) has amplified separately for 40 min with the same amplification conditions. Following RPA, 4 µL of the amplification products drop-casted on the electrode surface and kept to hybridise with the surface capture probe for 10 min at 37ºC within a water-saturated atmosphere. Subsequently, amplicons labelled with the modified AuNPs for 10 min. DPV measurements done using the conditions described above. As it can be seen in (Figure 3.5), increasing amplification times resulted in increasing levels of amplicons produced, reaching a plateau at ca. 30 minutes, after which further amplification did not improve the amplification yield.

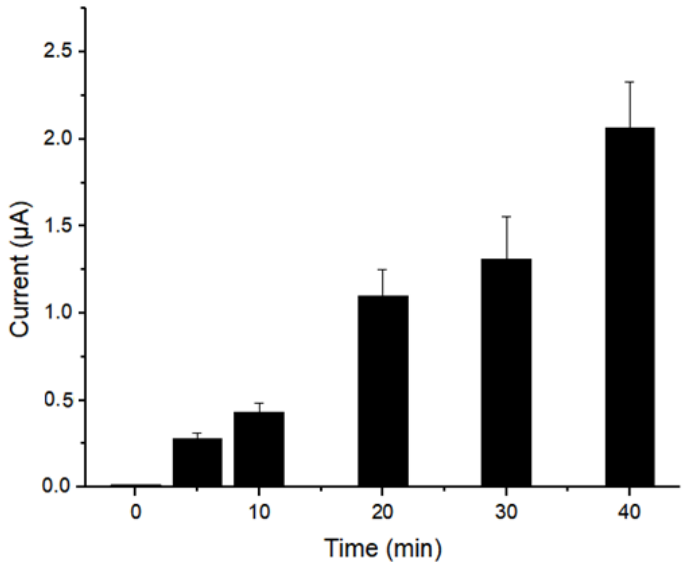


Fig. 4.2. Electrochemical detection of RPA amplicons following 5, 10, 20, 30 and 40 minute's amplification. NTC indicates the signal obtained following amplification of a non-specific template for 40 minutes. 2 pM target was used as template and 2 min capture and modified AuNPs hybridization

Based on the results obtained from the amplification time optimization, and using the optimum conditions of hybridisation the sensitivity has been evaluated using 5 fold serial dilutions of the starting DNA template from 2 pM to 200 aM. The limit of detection (LOD) obtained was 7.2 fM determined as the concentration of the analyte at the mean blank signal plus three times the standard deviation of the blank.

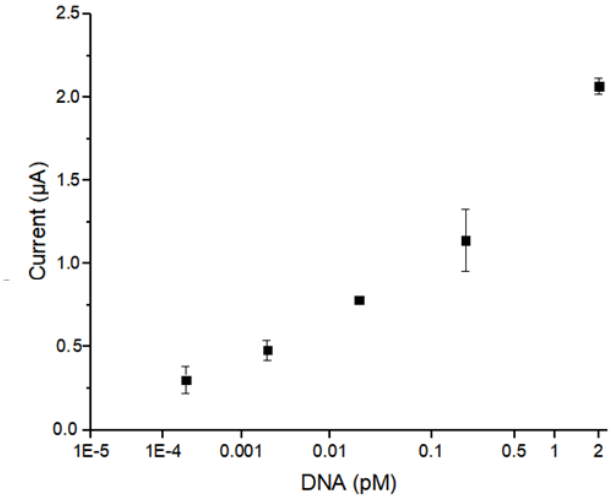


Fig. 4.3. Calibration curves obtained amplifying a range of concentrations (2 pM to 200 aM) of template starting concentrations after 40 min RPA and 10 min hybridization with capture probe and 10 min hybridization with reporter probe.

The specificity of this sensor towards karlodinium armiger (Ka) target has been evaluated using HLA target (simply chosen as was available in the laboratory). As expected, only Ka positive sample gave a significant positive electrochemical signal (Fig.4.4. A). Then optimised assay has been used to detect the Ka in genomic DNA extracted from seawater. Genomic DNA was extracted from seawater and provided by IRTA. DNA containing Ka sequence (GE +ve) and a control sample not containing the Ka sequence (GE -ve) were analysed, and as can be seen in (Figure) a significant positive electrochemical signal was observed in the case of the positive sample.

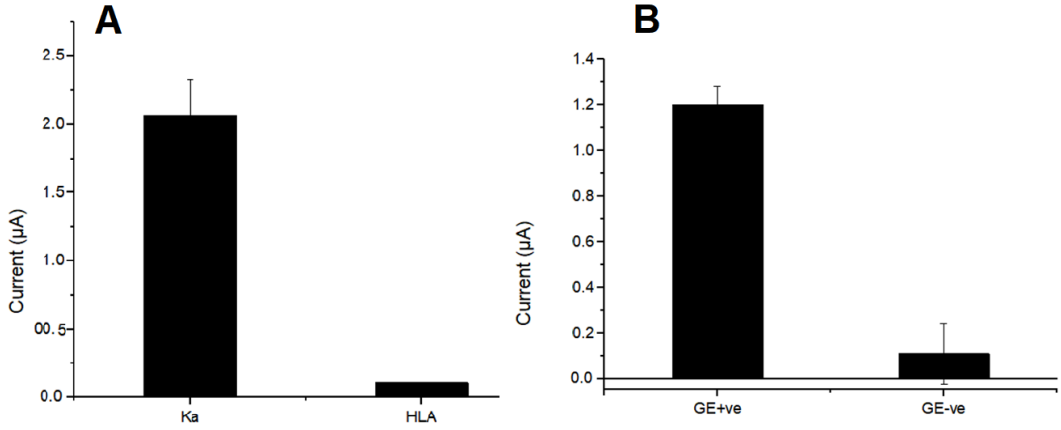


Fig. 4.4. (A) Specificity of developed geno-sensor. (B)Application of geno-sensor to real samples. Measurement conditions as described in text.

4.5. Conclusions

In this work, the use of the AuNPs modified with thiolated ssDNA and 6-(Mercaptohexyl)ferrocene and using this conjugation for electrochemical measurements was demonstrated, as an effective method to label each DNA target with more than one Ferrocene molecule, with the aim of developing highly sensitive DNA sensor. Modified primers were used to produce an amplicon with a central duplex flanked by two single stranded tails. These tails were designed to be complementary to a gold electrode tethered capture oligo probe as well as an oligo probe immobilised on the AuNPs. The time required for hybridisation of the target DNA with the surface immobilised probe and reporter probe was optimised to be 10 minutes, in both cases. Using the optimum conditions of amplification and hybridisation, the LOD obtained was 7.2 fM of original synthetic DNA. At the same time, the selectivity of the sensor has been evidenced by using karlodinium veneficum genomic DNA and HLA DQB1*02 synthetic DNA controls.

4.6. Acknowledgment

This work has been carried out with partial financial support from Spanish Ministerio de Economía y Competitividad (SEASENSING BIO2014-56024-C2-1): “Seasensing: Microsystems for rapid, reliable and cost effective detection of toxic microalgae on-site and in real-time” SAM acknowledges the Secretariat d’Universitats i Recerca del Departament d’Economia i Coneixement de la Generalitatde Catalunya for his scholarship support (2015 FI_B 01072).

4.7. References

[1] Y. Hasegawa, T. Takada, M. Nakamura, K. Yamana, Ferrocene conjugated oligonucleotide for electrochemical detection of DNA base mismatch, Bioorganic Med. Chem. Lett. 27 (2017) 3555–3557. doi:10.1016/j.bmcl.2017.05.049.

[2] H.B. Wang, H.D. Zhang, Y. Chen, K.J. Huang, Y.M. Liu, A label-free and ultrasensitive fluorescent sensor for dopamine detection based on double-stranded DNA templated copper nanoparticles, Sensors Actuators, B Chem. 220 (2015) 146–153. doi:10.1016/j.snb.2015.05.055.

[3] P. Gill, A. Ghaemi, Nucleic acid isothermal amplification technologies - A review, Nucleosides, Nucleotides and Nucleic Acids. 27 (2008) 224–243. doi:10.1080/15257770701845204.

[4] V.T. Katja Niemann, Isothermal Amplification and Quantification of Nucleic Acids and its Use in Microsystems, J. Nanomed. Nanotechnol. 06 (2015). doi:10.4172/2157-7439.1000282.

[5] Kumar, Isothermal Nucleic Acid Amplification System: An Update on Methods and Applications, J Genet Genom. 2 (2018) 1–5. https://www.omicsonline.org/open-access/isothermal-nucleic-acid-amplification-system-an-update-on-methods-andapplications.pdf.

[6] Y. Zhao, F. Chen, Q. Li, L. Wang, C. Fan, Isothermal Amplification of Nucleic Acids, Chem. Rev. 115 (2015) 12491–12545. doi:10.1021/acs.chemrev.5b00428.

[7] D. Morisset, D. Stebih, K. Cankar, J. Zel, K. Gruden, Alternative DNA amplification methods to PCR and their application in GMO detection: A review, Eur. Food Res. Technol. 227 (2008) 1287–1297. doi:10.1007/s00217-008-0850-x.

[8] J. Li, J. Macdonald, Advances in isothermal amplification: Novel strategies inspired by biological processes, Biosens. Bioelectron. 64 (2014) 196–211. doi:10.1016/j.bios.2014.08.069.

[9] X. Zhang, S.B. Lowe, J.J. Gooding, Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP), Biosens. Bioelectron. 61 (2014) 491–499. doi:10.1016/j.bios.2014.05.039.

[10] I.M. Lobato, C.K. O'Sullivan, Recombinase polymerase amplification: Basics, applications and recent advances, TrAC - Trends Anal. Chem. 98 (2018) 19–35. doi:10.1016/j.trac.2017.10.015.

[11] B.Y.C. Ng, W. Xiao, N.P. West, E.J.H. Wee, Y. Wang, M. Trau, Rapid, Single-Cell Electrochemical Detection of *Mycobacterium tuberculosis* Using Colloidal Gold Nanoparticles, Anal. Chem. 87 (2015) 10613–10618. doi:10.1021/acs.analchem.5b03121.

[12] B.Y.C. Ng, E.J.H. Wee, N.P. West, M. Trau, Naked-Eye Colorimetric and Electrochemical Detection of Mycobacterium tuberculosis - Toward Rapid Screening for Active Case Finding, ACS Sensors. 1 (2016) 173–178. doi:10.1021/acssensors.5b00171.

[13] H. Joda, V. Beni, A. Willems, R. Frank, J. Höth, K. Lind, L. Strömbom, I. Katakis, C.K. ÓSullivan, Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles, Biosens. Bioelectron. 73 (2015) 64–70. doi:10.1016/j.bios.2015.05.048.

[14] S. Al-Madhagi, H. Joda, M. Jauset-Rubio, M. Ortiz, I. Katakis, C.K. O'Sullivan, Isothermal amplification using modified primers for rapid electrochemical analysis of coeliac disease associated DQB1*02 HLA allele, Anal. Biochem. 556 (2018) 16–22. doi:10.1016/j.ab.2018.06.013.

[15] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, A. Saeed, M.N. Abbas, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay, Sci. Rep. 6 (2016) 37732. doi:10.1038/srep37732.

[16] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Aptamer Lateral Flow Assays for Ultrasensitive Detection of β-Conglutin Combining Recombinase Polymerase Amplification and Tailed Primers, Anal. Chem. 88 (2016) 10701–10709. doi:10.1021/acs.analchem.6b03256.

[17] L. Liu, H. Pan, M. Du, W. Xie, J. Wang, Glassy carbon electrode modified with Nafion-Au colloids for clenbuterol electroanalysis, Electrochim. Acta. 55 (2010) 7240–7245. doi:10.1016/j.electacta.2010.06.078.

Chapter 5

Conclusions

5.1. General Conclusions

Development of rapid, simple, inexpensive and reliable DNA testing system is desired for diagnosis of genetic diseases, pathogens detection, forensics, and personalised medicine.

Conventional methods for the detection of specific DNA sequences are based on either direct sequencing or DNA hybridisation methods. The DNA hybridisation geno-sensores are the most widely used approach in the analysis of nucleic acids, which consisting of single-stranded DNA (ssDNA) probes layer immobilized on a transducer surface in order to recognize its complementary nucleic acid (target sequence) with high efficiency and extremely high specificity. In the vast majority of those DNA sensors, pre-detection treatments such as creating ssDNA and Labelling is essential.

The multi-step procedures needed for the DNA analysis in the DNA hybridisation geno-sensores is one of the limitations in the development of portable molecular diagnostics devices. In this work, we combined isothermal amplification and modified primers to simplify steps required for electrochemical detection of the DNA.

Modified primers with a carbon spacer, which effectively blocks elongation, linked to a single stranded oligonucleotide sequence, thus resulting in a duplex amplification product flanked by two single stranded DNA tails. One of the tails were used to hybridize to a surface immobilized probe and the other to an enzyme or gold nanoparticles labelled reporter probe. Using these modified primers allowed us to detect DNA electrochemically without any need for post-amplification sample treatment.

Furthermore, post-amplification labelling of the target DNA has been avoided by coupling one of these modified primers (tailed forward primer) and reverse primer liked to HRP with the RPA. Using these modified primers allowed us to produce RPA labelled amplicons. Using this streamline of DNA amplification and detection can simplify the most challenging steps required in the electrochemical detection of the DNA such as purification, creating ssDNA and pre-detection labelling.

5.2. Future work

This work can be extended in the future by using modified primers with isothermal amplification to develop an integrated device for the amplification and detection of the DNA in limited-resource areas.

Furthermore, the integration of modified primers, isothermal amplification, and nanoparticles could be used to develop instrument-free colorimetric qualitative detection system which would be used as a point-of-care diagnostic tool.

Appendix

6.1. Appendix 1. List of figures

6.2. Appendix 2. List of tables

6.3. Appendix 3. List of abbreviations

AuNPs	Gold nanoparticles
CD	Coeliac disease
cDNA	Complementary DNA strand
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribose nucleoside triphosphate, also known as nucleotide
DPV	Differential pulse voltammetry
dsDNA	Double stranded DNA
DT1	(10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol
ELONA	Enzyme-linked oligonucleotide assay
GE+ve	Genomic DNA containing target sequence.
GE-ve	Genomic DNA not containing target sequence.
GPES	General purpose electrochemical system
HLA	Human leukocyte antigens.
HRP	Horseradish peroxidase
IUPAC	International Union of Applied Chemistry

KA	Karlodinium armiger
Kv	Karlodinium veneficum
LAMP	Loop mediated isothermal amplification
LOD	Limit of detection
MCH	6-mercapto-1-hexanol
NASBA	Nucleic acid sequence based amplification
NTC	Non-template control PBS Phosphate-buffered saline
NTC	No Template Control
PBS	Phosphate buffer saline.
PCB	Printed circuit board
PCR	Polymerase chain reaction
PMMA	Polymethylmethacrylate
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RPA	Recombinase Polymerase Amplification

SAM	Self assembled monolayer
SDA	Strand displacement amplification
ssDNA	Single stranded DNA
SSP	Sequence-specific primers
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TMB	3,3’,5,5’-tetramethylbenzidine
TRIS	Tris(hydroxymethyl)amino methane
UV	Ultraviolet

6.4. Appendix 4. Annex

Analytical Biochemistry 556 (2018) 16–22

Contents lists ScienceDir

Analytical Biochemistry

journal www.elsevier.com/locate/

Isothermal amplification using modified primers for rapid electrochemical analysis of coeliac disease associated DQB1*02 HLA allele

Sallam Al-Madhagi[a], Hamdi Joda[a,1], Miriam Jauset-Rubio[a], Mayreli Ortiz[a], Ioanis Katakis[a,**], Ciara K. O'Sullivan[a,b,*]

[a] Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007, Tarragona, Spain [b] ICREA, Passeig Lluís Companys 23, 08010, Barcelona, Spain

ARTICLEINFO

Keywords:
DNA detection
Modified primers
Electrochemical detection
Recombinant polymerase amplification

ABSTRACT

DNA biosensors are attractive tools for genetic analysis as there is an increasing need for rapid and low-cost DNA analysis, primarily driven by applications in personalized pharmacogenomics, clinical diagnostics, rapid pathogen detection, food traceability and forensics. A rapid electrochemical genosensor detection methodology exploiting a combination of modified primers for solution-phase isothermal amplification, followed by rapid detection via hybridization on gold electrodes is reported. Modified reverse primers, exploiting a C18 spacer between the primer-binding site and an engineered single stranded tail, are used in a recombinase polymerase amplification reaction to produce an amplicon with a central duplex flanked by two single stranded tails. These tails are designed to be complementary to a gold electrode tethered capture oligo probe as well as a horseradish peroxidase labelled reporter oligo probe. The time required for hybridization of the isothermally generated amplicons with each of the immobilized and reporter probes was optimised to be 2 min, in both cases. The effect of amplification time and the limit of detection were evaluated using these hybridization times for both single stranded and double stranded DNA templates. The best detection limit of 70 fM for a ssDNA template was achieved using 45 min amplification, whilst for a dsDNA template, just 30 min amplification resulted in a slightly lower detection limit of 14 fM, whilst both 20 and 45 min amplification times were observed to provide detection limits of 71 and 72 fM, respectively, but 30 and 45 min amplification resulted in a much higher signal and sensitivity. The genosensor was applied to genomic DNA and real patient and control blood samples for detection of the coeliac disease associated DQB1*02 HLA allele, as a model system, demonstrating the possibility to carry out molecular diagnostics, combining amplification and detection in a rapid and facile manner.

Introduction

There is a mature need for a rapid, simple, inexpensive and reliable nucleic acid testing system for a plethora of applications in molecular diagnostics [1]. In the vast majority of techniques reported to date, DNA amplification is required prior to nucleic acid analysis. Conventional amplification systems such as the polymerase chain reaction require the use of a thermocycler instrument with inherent power requirements and a need for trained personnel. Moving towards achieving assays that meet the ASSURED criteria of affordability, sensitivity, specificity, user-friendliness, robustness, rapidity, equipment-free and deliverability to end users [2], several isothermal amplification techniques have been developed, including loop-mediated amplification (LAMP) [3], exponential amplification reaction (EXPAR) [4], strand displacement amplification (SDA) [5], rolling circle amplification (RCA) [6], nucleic acid sequence-based amplification (NASBA) [7], helicase-dependent amplification (HDA) [8] and recombinase polymerase amplification (RPA) [9].

RPA has several advantages over other isothermal techniques due to its simplicity, sensitivity and rapid amplification at a constant temperature between 25 and 42 °C [10], consequently having immense potential capability in microsystems and lateral flow assays for point of need applications [11]. RPA takes advantage of the natural homologous recombination process, where a recombinase enzyme and single stranded binding proteins enable the scanning and invasion of a DNA template with primers, thus avoiding the need for thermal denaturation, with repeated primer extension via polymerase strand displacement activity, resulting in exponential amplification.

Despite the high efficiency, simplicity, and rapidity of RPA, the detection of RPA amplicons via hybridization normally requires the generation of single stranded DNA, adding cost and complexity to the assay. To address this, we previously developed an approach to produce PCR amplicons that could be directly detected post-amplification via hybridization [12]. This approach exploited the use of primers modified with a carbon spacer, which effectively blocks elongation, linked to a single stranded oligonucleotide sequence, thus resulting in a duplex amplification product flanked by two single stranded DNA tails. One of the tails was used to hybridize to a surface immobilized probe and the other to an enzyme labelled reporter probe, and the detection assay was completed in less than 5 min without any need for post-amplification sample treatment. Recently, we combined this approach with RPA for the lateral flow based detection of DNA [13] and aptamers [14].

In the work reported here, we describe the combination of these modified primers with isothermal RPA and electrochemical detection, with the main objective of the work being the achievement of extremely rapid amplification and detection of the coeliac disease associated HLA DQB1*02 allele. Amplification time and hybridization times with a surface tethered probe and an enzyme labelled probe, respectively, were evaluated and the final system applied to the detection of a real patient sample and control sample, highlighting the potential application of this approach to point-of-need diagnostics.

Materials and methods

Materials

Maleimide activated 96-Well plates and Proteinase K were obtained from Fisher Scientific (Madrid, Spain). RPA TwistAmp® basic kit was purchased from TwistDx Ltd. (Babraham, United Kingdom). Low range ultra-agarose gel powder was supplied from Bio Rad Laboratories S.A. (Barcelona, Spain).

Abbreviations: DNA, Deoxyribonucleic acid; dsDNA, Double stranded DNA; DT1, (10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol; ELONA, Enzyme linked oligonucleotide assay; ELISA, Enzyme linked immunosorbent assay; GE+ve, Genomic DNA containing DQB1*02 sequence; GE-ve, Genomic DNA not containing DQB1*02 sequence; HLA, Human leukocyte antigens; HRP, Horseradish peroxidise; MCH, 6-mercapto-1-hexanol; NTC, No Template Control; PCB, Printed circuit board; PCR, Polymerase chain reaction; PBS, Phosphate buffer saline; RPA, Recombinase Polymerase Amplification; SAM, Self assembled monolayer; ssDNA, Single stranded DNA; SSP, Sequence-specific primers; TBS, Tris-buffered saline; TMB, 3, 3′, 5,5′-tetramethylbenzidine

* Corresponding author. Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007, Tarragona, Spain. ** Corresponding author.
E-mail addresses: ioanis.katakis@urv.cat (I. Katakis), ciara.osullivan@urv.cat (C.K. O'Sullivan).
1 Current address: Miller School of Medicine, University of Miami, Miami, FL, 33136, United States.

https://doi.org/10.1016/j.ab.2018.06.013
Received 9 March 2018; Received in revised form 12 June 2018; Accepted 13 June 2018 Available online 18 June 2018
0003-2697/

Phosphate buffered saline (50 mM) pH 7.4 (PBS), containing 0.05% (v/v) Tween 20 (PBS-Tween), 6 mercapto-1-hexanol (MCH) and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma–Aldrich (Barcelona, Spain). TMB enhanced one component HRP membrane was supplied by Diarect AG (Germany). Potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) were obtained from Scharlau (Barcelona, Spain). Dithiol (16-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15pentaoxahexa-decane) (DT1) was obtained from SensoPath Technologies (Bozeman, MT, USA). DNA probes, DNA primers, Horseradish peroxidise (HRP) modified reporting sequences and synthetic analogues of DQB1*02 target were supplied by biomers.net GmbH (Ulm, Germany) as lyophilized powder and reconstituted in RNase and DNase-free water and used without further purification.

The DNA target, probe and primers sequences used in this work were:

DQB1*02 target:
CGTGCGTCTTGTGAGCAGAAGCATCTATA
ACCGAGAAGAGATCGT
GCGCTTCGACAGCGACGTGGGGGAGTTC
CGGGCGGTGACGCTGCT
GGGGCTGCCTGCCGCCGAGTACTGGAAC
AGCC DQB1*02 capture probe:
5′-GTC GTG ACT GGG AAA AC TTT TTT TTT TTT TTT-SH-3′ DQB1*02 tailed forward primer:

5′-GTT TTC CCA GTC ACG AC-Spacer-CGT GCG TCT CGT GAG CAG AAG-3′

DQB1*02 tailed reverse primer:
5′-TGT AAA ACG ACG GCC AGT-Spacer-GGC TGT TCC AGT ACT CGG CGG-3′

HRP-reporter probe:
5′ACTGGCCGTCGTTTTACA-HRP-3′

Both synthetic single stranded target DNA (ssDNA) and PCR amplified double stranded DNA (dsDNA) was used in this work. PCR amplification was carried out using non-tailed primers. PCR amplification was performed using DreamTaq DNA polymerase kit (Fisher Scientific- Spain) with the following volumes: 10 µL 5x buffer, 5 µL dNTPs (2 mM), 5 µL $MgCl_2$ (50 mM), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5 µL BSA (50 mg/mL), 27 µL H_2O, 0.5 µL polymerase and 1 µL of 5 nM ssDNA template. PCR amplification was performed using an initial denaturation step at 96 °C for 1min, followed by 25 cycles performed at 95 °C for 30 s, annealing for 30 sat 61 °C and elongation at 72 °C for 30 s, with a final extra step of 72 °C for 5 min. Gel electrophoresis analysis was carried out to confirm successful PCR amplification.

Liquid phase RPA/solid phase detection

The target (synthetic/genomic) was first amplified using isothermal recombinase polymerase amplification and modified tailed forward and reverse primers. Subsequently, detection was carried out by hybridization to a capture probe surface tethered either on a microtiter plate or on gold electrodes, followed by hybridization with a HRP labelled reporter probe.

RPA

RPA was performed using a TwistAmp Basic kit using the protocol recommended by the supplier with slight modifications. Briefly, 240 nM of modified forward and reverse primers, DNA template (DQB1*02), DNAse free water and 1 × rehydration buffer was made up to a total volume of 100 µL and then divided into 25 µL/reaction before addition of 14 mM magnesium acetate to initiate the RPA reaction. To optimize the amplification temperature, amplification was carried out at 36, 37, 38, 39, 40 and 41 °C.

Enzyme linked oligonucleotide assay (ELONA)

A thiolated probe complementary to the single stranded DNA tail of the forward primer (500 nM)

was prepared in 10 mM PBS, added to each well of a maleimide microtiter plate and left to incubate for 2 hat 37 °C. Any remaining maleimide groups were subsequently blocked with 100 μM mercaptohexanol.

The RPA amplified DNA was diluted (1:3) in 50 mM Tris buffered saline containing 150 mM NaCl, added to each well of the microtiter plate to hybridize with the immobilized capture probe complementary to the ssDNA tail of the extended forward primer of the RPA product, and incubated for the reported time at 37 °C. Reporter HRP-DNA probe (10 nM) was then added to each well to hybridize to the complementary ssDNA tail of the extended reverse primer at the other end of RPA product, for the reported time at 37 °C. Between each step, the wells were washed with 200 μL of PBS-Tween 3 times. Finally, TMB substrate for ELISA was added and the reaction was stopped after 5 min using 1 M H_2SO_4. The absorbance was then measured at 450 nm using a microplate reader (SpectraMax, bioNovacientífica S.L., Spain).

Electrochemical detection on gold electrode array

All electrochemical experiments were performed using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software program (Eco Chemie, The Netherlands), equipped with a MUX module (Eco Chemie B.V., The Netherlands) for sequential measurement of working electrodes that share the same reference and counter electrode.

Electrode chip design

The electrochemical cell consists of a customized electrode array chip designed in-house using AutoCAD software (Autodesk Inc, USA) and fabricated at Fineline GmbH (Hilden, Germany) using printed circuit board (PCB) technology with a soft gold surface finish. It consists of 32 working electrodes (diameter =1 mm), each arranged with a gold counter electrode and gold reference electrode. This array was not specifically designed for the work reported here, but could be used for multiplexed detection of RPA amplicons. The electrode array was interfaced with a multiplexed potentiostat using a custom-designed connector.

Electrode surface functionalization and hybridization

Prior to functionalization, the electrode array was cleaned via exposure to UV/ozone for 10 min, followed by immersion in 25% v/v H_2O_2 containing KOH (50 mM) for a further 10 min. Finally, the array was rinsed with MilliQ water and then with ethanol and finally dried in N_2 gas. Each electrode of the array was functionalized using a co-immobilization approach by spotting 1 μL of 100 μM DT1 and 5 μM of thiolated DNA probe in 1 M KH_2PO_4 onto each electrode. Self-assembling was left to take place for 3 hat room temperature within a humidified chamber, and then the electrode array was rinsed with MilliQ water and dried with N_2 gas.

The functionalized electrode array was then mounted within the fluidic device fabricated on 2 mm thick poly (methylmethacrylate) (PMMA) sheet using a Fenix CO_2 laser (Synrad Inc., USA). A laser patterned double-sided adhesive gasket was used to form 8-microchannel structures 1 mm in width. Amplified RPA product (10 μL) was injected into the corresponding microfluidic channel and incubated for the reported time at 37 °C to hybridize with capture probe and subsequently reporter HRP-DNA probe (10 nM) was injected and again incubated for the reported time at 37 °C. Between each step, the channels were thoroughly washed with 2 × 200 μL of 50 mM Tris-buffered saline (TBS, pH 8.0). Finally, a TMB enhanced one-component HRP membrane substrate was added and electrochemical reduction of the HRP mediated product was measured using fast pulse amperometry (0 V for 10 m s followed by −0.2 V for

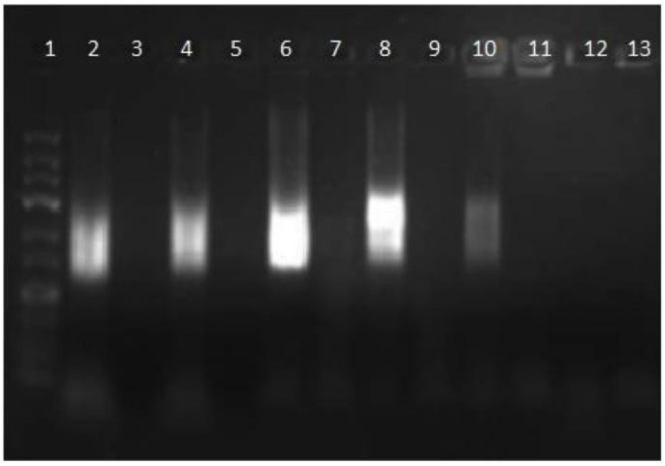


Fig. 2. Gel electrophoresis analysis for RPA products amplification times. Lane 1, 10 bp ladder; lane 2, RPA for 40 min; lane 3, NTC 40 min; lane 4, RPA for 30 min; lane 5, NTC 30 min; lane 6, RPA for 20 min; lane 7, NTC 20 min; lane 8, RPA for 10 min; lane 9, NTC 10 min; lane 10, RPA for 5 min; lane 11, NTC 5 min; lane 12, RPA for 2 min; lane 13, NTC 2 min.

500 m s vs. Au built-in reference electrode) (see Fig. 1).

Results and discussion

Liquid phase RPA/solid phase detection

RPA with tailed primers was performed using liquid phase amplification/solid-phase detection at a range of temperatures between 36 and 41 °C, and the highest efficiency of amplification was observed at 38 °C, but as the amplification levels were not significantly higher than those obtained at 37 °C, in order to maintain one constant temperature for the entire assay as 37 °C was the temperature used for hybridization, 37 °C was also thus used for amplification for all further experiments. Following RPA, the 25 μL reaction mix was combined with 2 μL of 2 mg/mL Proteinase K, and the obtained amplicons then characterised using agarose gel electrophoresis (Fig. 2). RPA products could also be purified using a purification kit, but either the use of Proteinase K or a purification kit is required prior to carrying out gel electrophoresis to avoid smearing on the gel. Lanes 2, 4, 6, 8, 10, 12 correspond to RPA amplification of a 2 nM template for 40, 30, 20, 10, 5 and 2 min, respectively, with Lanes 3, 5, 7, 9, 11 and 13 corresponding to nontemplate controls for 40, 30, 20, 10, 5 and 2 min, respectively. Intense amplification bands appear for RPA amplified for 40, 30, 20 and 10 min and a faint band could be observed for 5 min amplification, whilst no visible bands were observed for the NTC controls (NTC = water).

Unpurified RPA products (i.e. no Proteinase K step was used prior to electrophoresis) were detected using an enzyme linked oligonucleotide assay via solid-phase hybridization with a short thiolated DNA probe complementary to one ssDNA tail, which was surface immobilized on the well of a maleimide coated microtiter plate, whilst the second ssDNA tail was used to hybridize to a HRP-linked reporter probe. Samples were diluted 1:3 in 50 mM

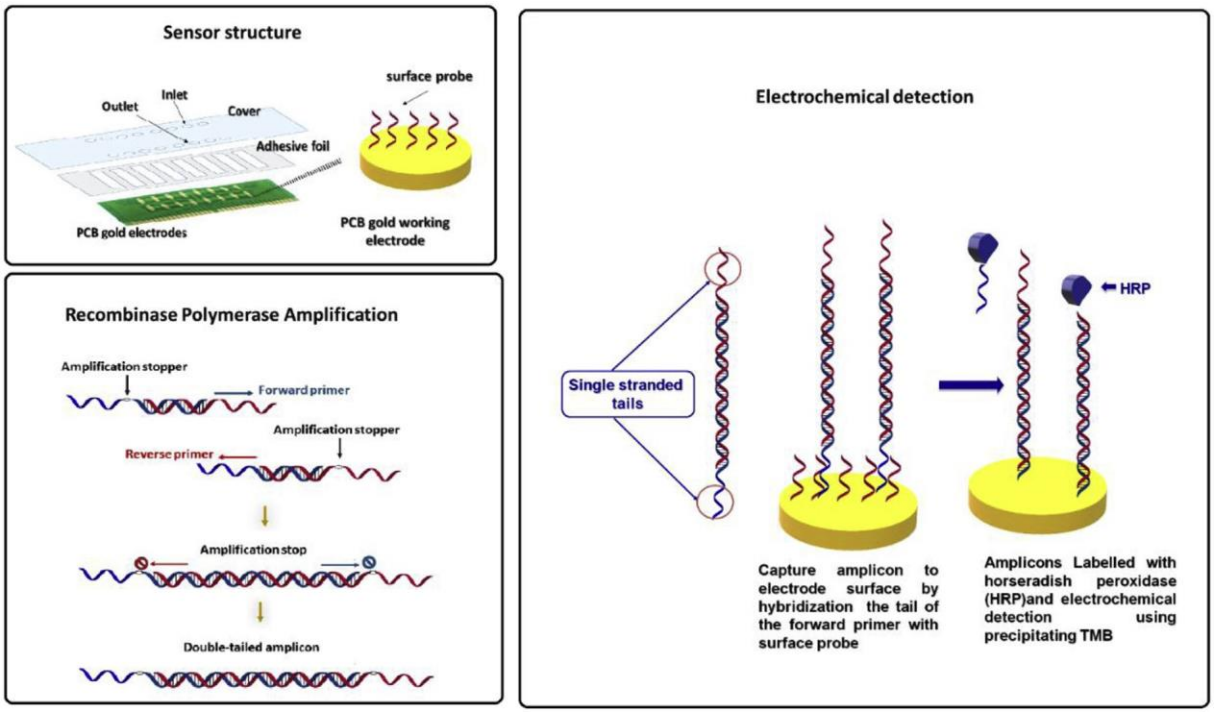


Fig. 1. Schematic overview of electrode array, functionalization and detection process.

Tris buffered saline containing 150 mM NaCl and allowed to hybridize for 30 min at 37 °C. Following thorough washing, the reporter DNA-HRP probe (10 nM) was subsequently added and allowed to hybridize for a further 30 min at 37 °C. TMB substrate for ELISA was added and reaction was stopped after 5 min using 1 M H_2SO_4. As can be seen in Fig. 3, increasing amplification times resulted in a higher amount of amplified product, but even just 5 min of amplification resulted in a detectable amount of RPA amplicon.

Liquid phase RPA/electrochemical solid phase detection

Following demonstration of the ELONA based direct detection of the RPA tailed amplicons, electrochemical detection of solid-phase hybridization was explored. Based on the ELONA results, synthetic target DNA (10 nM) was amplified for 10 min, and the unpurified RPA amplicon obtained was directly detected via hybridization with the gold electrode immobilized capture probe, varying the hybridization time from 2 to 60 minat 37 °C. Subsequently, the DNA-HRP reporter probe was added and allowed to hybridize for a lengthy amount of time (30 min), to ensure maximum signal. Based on our previous results [12], we expected a rapid hybridization time, which can be attributed to the short length of the single stranded DNA tail. DNA hybridizes via a combination of collisional kinetics to find its complementary DNA, which is the rate limiting step, and is followed by rapid DNA zipping. Thus, the shorter the DNA that needs to be scanned for complementarity, the more rapid hybridization can occur, particularly as the only available single stranded DNA for binding is the complementary tail. As expected, the same levels of hybridization were obtained for the range of times interrogated, indicating that a 2 min hybridization time with surface immobilized probe was adequate, although 10 min was where plateauing was observed (Fig. 4a).

The effect of duration of hybridization between captured DNA duplex and the second ssDNA tail with DNA-labelled reporter probe was then evaluated, using an initial hybridization time with the capture probe of 10 min. As can be seen in Fig. 4b, effective hybridization was already observed at just 2 min, rapidly reaching a plateau at longer hybridization times. As the objective of this work was to find the conditions for the most rapid amplification and detection of the HLA DQB1*02 allele, hybridization times of 2 min for capture probe and RPA amplicon, and 2 min subsequent hybridization time with the HRP labelled reporter probe were selected and used to detect amplicons produced following 2–45 min of isothermal amplification.

Either the use of Proteinase K or rapid freezing at −80 °C for 10 min was observed to efficiently stop the amplification reaction via denaturation/inactivation of the recombinase A, strand displacing polymerase and the single stranded binding proteins. Heating to 80 °C was also tested as a means to stop the RPA and could also be used, but the use of proteinase K or freezing was simpler to implement, and here we exploited the use of rapid freezing to stop the RPA reaction.

As soon as amplification was stopped, the amplicons were purified. RPA products were purified using DNA Clean & Concentration™−5 kit (Zymo Research) by mixing 5 vol of DNA binding buffer to each volume of the RPA products, the mixture transferred to the Zymo-Spin column and centrifuged for 30 sat 10000 g, after which the column was washed three times with 200 μL DNA wash buffer and centrifuged for 30 s at 10000 g. Finally, 15 μL of water was added directly to the column matrix and incubated for 1 min and then centrifuged for 30 s at 10000 g to elute the dsDNA. It should be noted that these steps of Proteinase K/ freezing/purification would not be used in a final assay/device and is only implemented in this exploratory work to stop the RPA reaction and study the effect of amplification time on detection limit. In a final assay, as detailed in the study to

optimize hybridization times detailed above, the RPA amplicon is directly detected via hybridization, without any need for purification/post-RPA processing.

As was expected, and can be seen in Fig. 4c, increasing amplification times resulted in increasing levels of amplicons produced, with tailed RPA products being detectable even after just 2 min amplification, reaching a plateau at ca. 30 min, after which further amplification did not improve the yield. However, it must be noted that the starting concentration of DNA was 10 nM, and it is possible to directly detect this concentration without any amplification using the hybridization assay developed.

A combination of 2 min hybridization with each of the capture probe and reporter probe was thus further explored amplifying a range of concentrations (2 fM – 2 nM) of template starting concentrations of both synthetic ssDNA and PCR amplified dsDNA using a range of

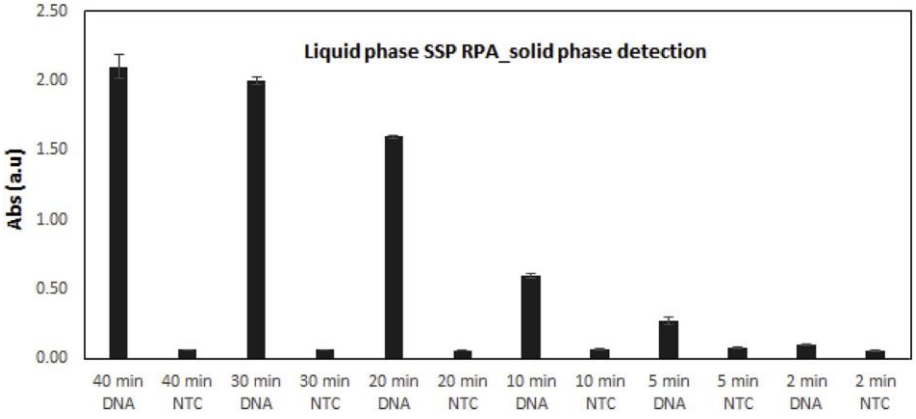


Fig. 3. Colorimetric absorbance signals for liquid phase RPA products and NTC amplified for 40, 30, 20, 10, 5 and 2 min. Absorbance was measured at 450 nm following addition of sulfuric acid. In all cases, hybridization with capture and reporter probes was 30 min.

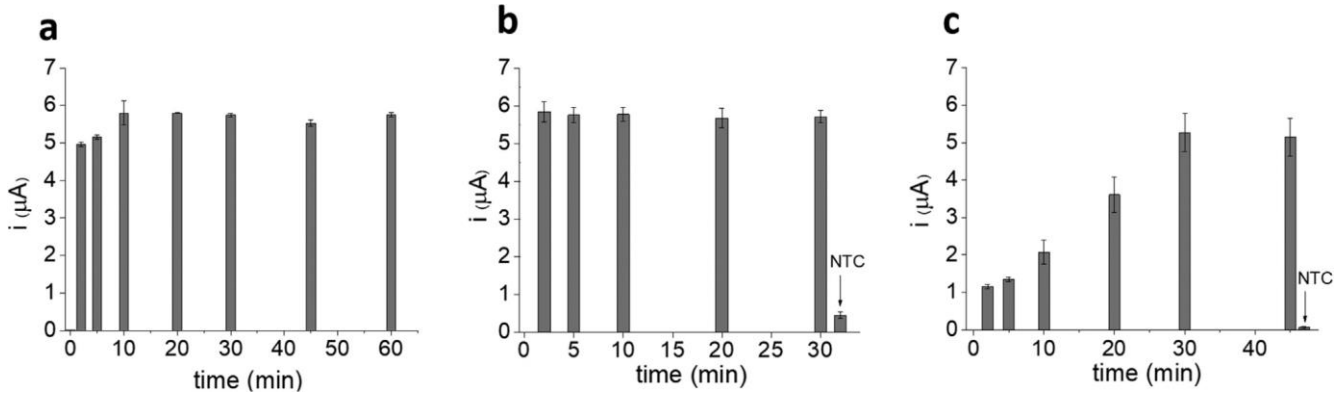


Fig. 4. (a) Effect of duration of hybridization time of tailed RPA amplicon with capture probe surface tethered on gold electrode. 10 nM template DNA was used together with 30 min hybridization with HRP labelled reporter probe. (b) Effect of duration of hybridization time of tailed RPA amplicon with HRP labelled reporter probe, following hybridization with surface immobilized capture probe for 10 min 10 nM template DNA was used, amplified for 10 min. (c) Detection of RPA generated tailed amplicons following 2, 5, 10, 20, 30 and 45 min amplification. NTC indicates the signal obtained following amplification of a non-specific template for 40 min 10 nM target was used as template and 2 min capture and reporter probe hybridization.

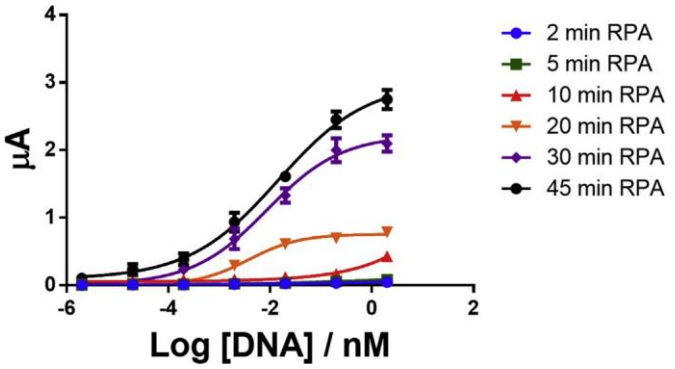


Fig. 5. Calibration curves obtained amplifying a range of concentrations (2fM – 2 nM) of template starting concentrations of synthetic ssDNA using a range of amplification times (2–45 min). In all cases 2 min hybridization with capture and reporter probes was used.

amplification times between 2 and 45 min (Fig. 5).

As can be seen in Table 1, when using ssDNA as a target the detection limit decreases and the sensitivity increases with increasing amplification times. A four parameter logistic regression model was used to interpret the data from a sigmoidal curve, with the exception of the 10 min amplification calibration curve, which did not fit well and led to ambiguous results.

The same experiment was then repeated using dsDNA that had been generated using PCR (Table 2). The concentration of post-PCR DNA was determined using Nanodrop and using this known starting concentration, dilutions were made to result in starting concentrations of 2 fM – 2 nM for use as templates for RPA (Fig. 6).

As expected better detection limits were obtained using a double stranded DNA target as this is the preferred target for RPA. With ssDNA as a target, the first elongations are relatively slow, converting ssDNA to dsDNA, and after the ssDNA is converted to dsDNA, amplification

Table 1
Effect of amplification time on IC50 and LOD using synthetic ssDNA template.

Amplification time	R square	IC50	LOD
2 min	0.9977	1.137 + 14.9 nM	1.3 nM
5 min	0.9976	0.308 + 2.69 nM	718 pM
10 min	0.9859	Ambiguous	25 pM
20 min	0.9942	0.004 ± 1.299 nM	0.32 pM
30 min	0.9977	0,009 ± 1.259 nM	0.11pM
45 min	0.9982	0.0143 ± 1.31 nM	70 fM

Table 2
Effect of amplification time on IC50 and LOD using PCR generated dsDNA template.

Amplification time	R square	IC50	LOD
2 min	0.9955	0.1040 + 1.414 nM	1pM
5 min	0.9966	0.055 + 1.311 nM	1.22 pM
10 min	0.9991	0.0641 + 1.157 nM	0.54 pM
20 min	0.9989	0.0416 + 1.297 nM	72 fM
30 min	0.9977	0.001 + 1.213 nM	14 fM
45 min	0.9955	0.003 + 1.61 nM	71 fM

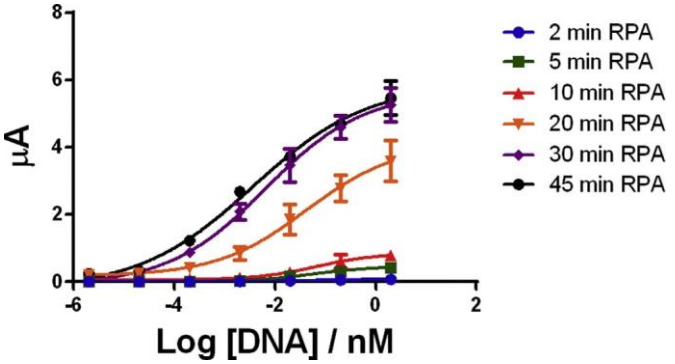
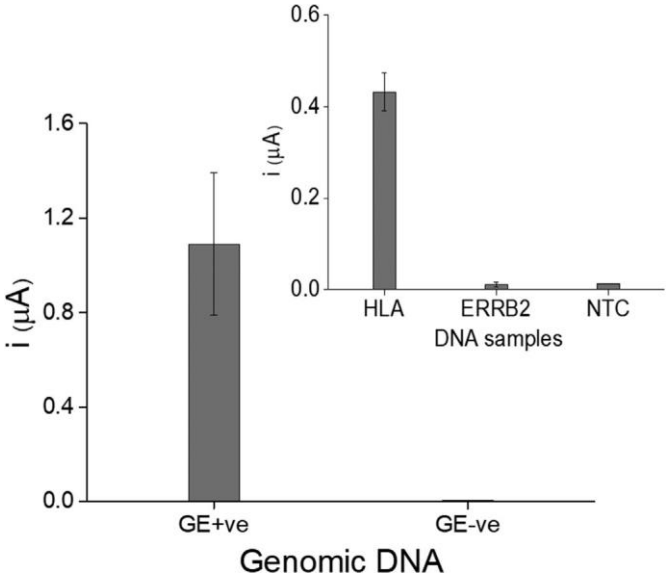


Fig. 6. Calibration curves obtained amplifying a range of concentrations (2 fM – 2 nM) of template starting concentrations of PCR generated dsDNA using a range of amplification times (2–45 min). In all cases 2 min hybridization with capture and reporter probes was used.

increases in speed. This is reflected by the fact that approximately the same detection limit is achieved after 45 min using both ssDNA and dsDNA. Additionally, it can be seen that the kinetics of RPA appear to be slower with the tailed primers than with normal, non-tailed primers. However, this needs to be verified with a range of different targets, as in our experience, the time required for amplification is highly target dependent, with no direct correlation between amplicon length, or GC content of target/primers. In the case of the HLA DQB1*02 target, the optimum amplification time is deemed to be 30 min.

In order to attempt to further decrease the assay time, a study was carried out where the RPA amplicon and HRP-labelled reporter probe were pre-incubated and then added to the electrode array, or, alternatively, where the HRP labelled reporter probe and the tailed RPA amplicon were simultaneously added to the functionalized electrode array. However, as can be clearly seen in Fig. 7, the highest signals are



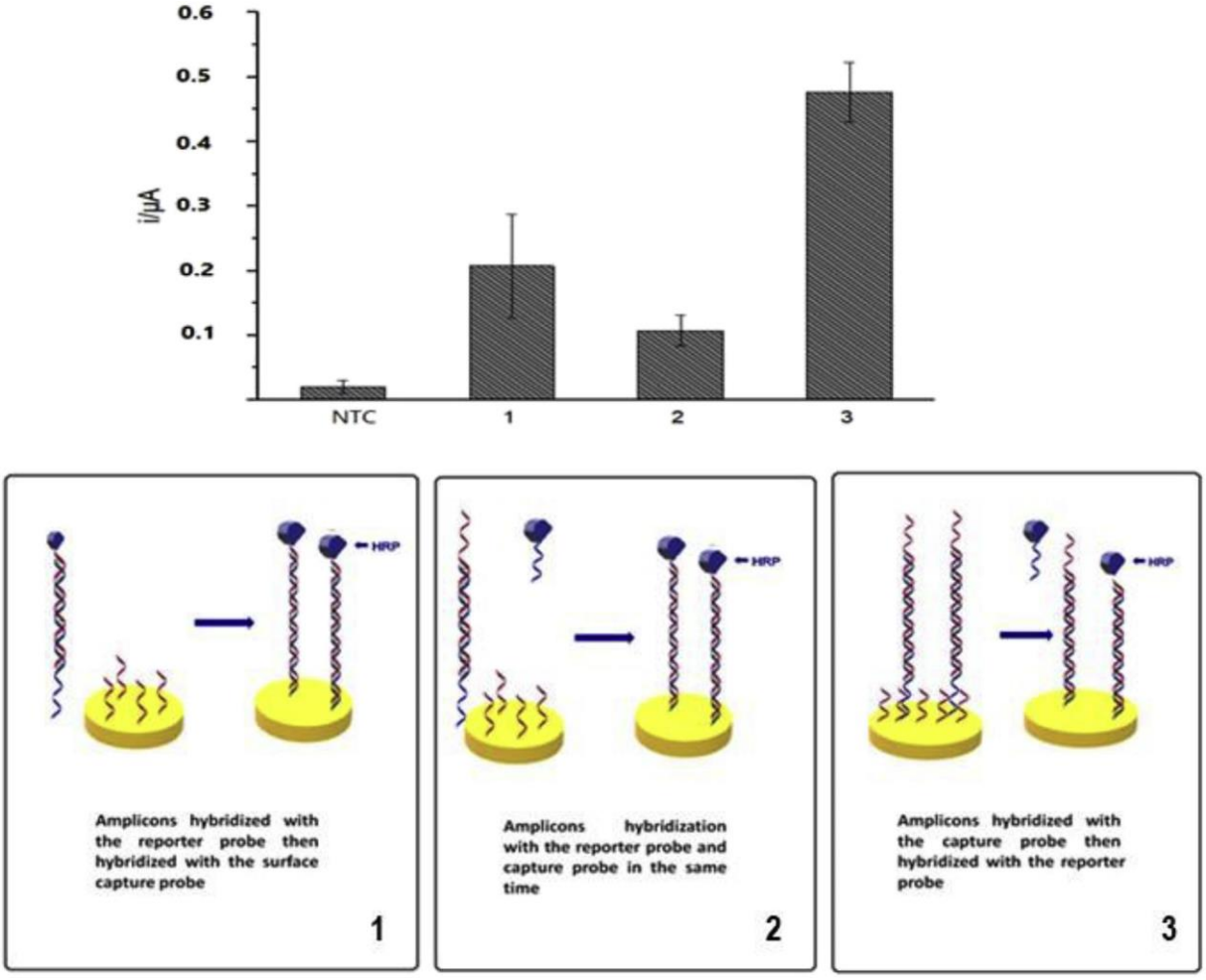


Fig. 7. (1) Amplicons hybridized with the reporter probe then hybridized with the capture probe; (2) Amplicons hybridized with the reporter probe and the capture probe in the same time; (3) Amplicons hybridized with the capture probe then hybridized with the reporter probe.
Fig. 8. Application of genosensor to real patient samples; Inset: Specificity of developed genosensor. Measurement conditions as described in text.

observed when a step-by-step approach is used.

Using the optimised assay conditions of 30 min amplification, 2 min hybridization with the surface immobilized probe and subsequently, 2 min hybridization with the HRP labelled reporter probe, the genosensor was used to detect the DQB1*02 in real patient samples. Genomic DNA was extracted from a human blood sample and provided by the Finnish Red Cross Blood Service, which had previously been genotyped and electrochemically analyzed [12]. DNA from a human sample containing DQB1*02 sequence (GE + ve) and a control sample not containing the DQB1*02 sequence (GE -ve) were analyzed, and as can be seen in Fig. 8 amplification and detection of amplification products was only observed in the case of the positive sample. Furthermore, specificity of the amplification was evaluated using a non-specific target, ERBB2 (simply chosen because it was available in the laboratory) and no amplification was observed (Fig. 8 inset).

Conclusions

In the work reported here, we further explored the potential of the combination of tailed primers and isothermal amplification for the extremely rapid and sensitive combined amplification and detection of the HLA DQB1*02 coeliac-disease associated allele, which was used as a model target. Both forward and

reverse primers were specifically designed to contain a C18 and a single stranded DNA tail. The C18 effectively stops polymerase mediated amplification, resulting in an amplicon of duplex target specific DNA, flanked by two single stranded DNA tails. We have previously reported this approach using the polymerase chain reaction, and recently reported the same approach using isothermal recombinase polymerase amplification, and here we further explored the possibility of minimizing hybridization and amplification times. Hybridization of just 2 min was observed to be adequate for hybridizing to both the surface immobilized capture probe and to the HRP labelled reporter probe. It was also attempted to cohybridize to both the surface labelled probe and the reporter probe in one-step but there appeared to be a competitive phenomena between the probes for binding the RPA tailed amplicon, akin to the Hooke effect that is sometimes observed with antibodies in enzyme linked immunosorbent assays, and thus a step-by-step assay format was preferred. Whilst it is possible to use shorter amplification times and detect the RPA product, in the case of the HLA DQB1*02, a 30 min amplification time was deemed optimum for testing a real patient sample. Ongoing work is focused on evaluating the effect of amplification time on detection limit using a range of different genomic targets.

Acknowledgements

This work has been carried out with partial financial support from the Spanish Ministerio de Economía y Competitividad (SEASENSING BIO2014-56024-C2-1, and CIGUASENSING BIO2017-87946-C2-1). SAM acknowledges the Secretariat d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitatde Catalunya for his scholarship support (2015 FI_B 01072).

References

[1] J. Wang, From DNA biosensors to gene chips, 28 (2000) 3011–3016.
[2] R.W. Peeling, K.K. Holmes, D. Mabey, A. Ronald, Rapid tests for sexually transmitted infections (STIs): the way forward, Sex. Transm. Infect. 82 (2006) v1–v6, http://dx.doi.org/10.1136/sti.2006.024265.
[3] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA, 28 (2000).
[4] J. Van Ness, L.K. Van Ness, D.J. Galas, Isothermal reactions for the amplification of oligonucleotides, 2003 (2003) 0–5.
[5] G.T. Walker, M.S. Fraiser, J.L. Schram, M.C. Little, J.G. Nadeau, D.P. Malinowski, Strand displacement amplification-an isothermal, in vitro DNA amplification technique, 20 (1992) 1691–1696.
[6] T. Murakami, J. Sumaoka, M. Komiyama, Sensitive isothermal detection of nucleicacid sequence by primer generation – rolling circle amplification, 37 (2009), http://dx.doi.org/10.1093/nar/gkn1014.
[7] P. Gill, A. Ghaemi, Nucleic acid isothermal amplification technologies - a review, Nucleos. Nucleot. Nucleic Acids 27 (2008) 224–243, http://dx.doi.org/10.1080/ 15257770701845204.
[8] M. Vincent, Y. Xu, H. Kong, Helicase-dependent isothermal DNA amplification, EMBO Rep. 5 (2004) 795–800, http://dx.doi.org/10.1038/sj.embor.7400200.
[9] O. Piepenburg, C.H. Williams, D.L. Stemple, N.A. Armes, DNA detection using recombination proteins, PLoS Biol. 4 (2006) 1115–1121, http://dx.doi.org/10.1371/ journal.pbio.0040204.
[10] R.K. Daher, G. Stewart, M. Boissinot, M.G. Bergeron, Review recombinase polymerase amplification for diagnostic applications, 958 (2016), http://dx.doi.org/10. 1373/clinchem.2015.245829.
[11] V.A. Online, L.A. Tortajada-genaro, S. Santiago-felipe, M. Amasia, A. Russom, RSC Adv. (2015) 29987–29995, http://dx.doi.org/10.1039/C5RA02778K.
[12] H. Joda, V. Beni, A. Willems, R. Frank, J. Höth, K. Lind, L. Strömbom, I. Katakis, C.K. ÓSullivan, Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles, Biosens. Bioelectron. 73 (2015) 64–70, http://dx.doi.org/10.1016/j.bios.2015.05.048.
[13] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, A. Saeed, M.N. Abbas, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay, Sci. Rep. 6 (2016) 37732, http://dx.doi.org/10.1038/srep37732.
[14] M. Jauset-Rubio, M. Svobodová, T. Mairal, C. McNeil, N. Keegan, M.S. El-Shahawi, A.S. Bashammakh, A.O. Alyoubi, C.K. O'Sullivan, Aptamer lateral flow assays for ultrasensitive detection of β-conglutin combining recombinase polymerase amplification and tailed primers, Anal. Chem. 88 (2016) 10701–10709, http://dx.doi. org/10.1021/acs.analchem.6b03256.