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

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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

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′-tetrame-thylbenzidine

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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). 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)-3,6,9,12,15-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.

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

DQB1*02 target:
CGTGCGTCTTGTGAGCAGAAGCATCTATAACCGAGAAGAGATCGT GCGCTTCGACAGCGACGTGGGGGAGTTCCGGGCGGTGACGCTGCT GGGGCTGCCTGCCGCCGAGTACTGGAACAGCC
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 s at 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 h at 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.