

Rapid ultra-sensitive isothermal DNA detection using RPA technology and a BMG LABTECH microplate reader

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- Isothermal nucleic acid amplification technology RPA is introduced
- Simple sample preparation procedure, constant temperature operation and homogenous format
- A BMG LABTECH microplate reader used for assay development

Introduction

Nucleic acid amplification techniques (NAATs) are central to molecular DNA tests, including clinical diagnostics, environmental testing, food testing, and many other applications. While PCR-based testing in laboratories is well established, thermo-cycling equipment that can monitor reaction kinetics is expensive, as well as power-demanding and generally unsuitable in providing cost-effective access to a large number of end-users.

Expansion of the user market requires reduction in the cost complexity and power requirement of devices and a simplification of operating procedures. The isothermal nucleic acid amplification technology Recombinase-Polymerase-Amplification (RPA), recently developed by TwistDX, is ideally positioned to fulfil these requirements. The constant low reaction temperature of RPA, its resistance to temperature fluctuation, and the integration of the DNA amplification step with proprietary detection probes means that it can be employed easily using standard laboratory equipment such as a BMG LABTECH microplate reader as well as small portable devices anticipated in the near future.

The technology achieves amplification from single template molecules to detectable levels in very short time-frames (down to less than 10 min) at a constant and low temperature.

Crucially for its application in non-laboratory settings, RPA can be combined with very simple sample preparation procedures and can utilise stabilised reaction components. Moreover, by employing a novel and proprietary fluorescent probe system, RPA can operate in a homogenous detection format that facilitates both, ease of application and great read-out speed.

Probes for the RPA system use a fluorophore and quencher separated by a nuclease target site which operates only on duplex DNA – thus fluorescence is seen to increase when specific amplification has occurred.

In this application note we describe the use of a BMG LABTECH microplate reader for fluorescence intensity measurements as a means of real-time monitoring of DNA detection reactions using RPA.

Materials & Methods

Reagents

The proteins used in this experiment were purified as described previously, except Creatine Kinase, which was obtained from Roche. PEG35000 was obtained from Calbiochem. Oligonucleotide amplification primers and probe (containing an internal FAM fluorophore and a corresponding BHQ1 quencher) were supplied by Eurogentec.

Lyophilisation

Complete RPA reaction components including probes and primers were lyophilised in microfuge tubes in a Virtis Genesis freeze dryer.

Sample preparation

Template material was prepared from buccal swabs by incubation in 0.3 M NaOH for 5 min at room temperature. The obtained lysis solution was used directly in amplification reactions. Control template (female and male human genomic DNA) was obtained from Promega.

Reaction set-up

For each reaction a rehydration solution containing buffer components and the template was added to lyophilised reagents and mixed, bringing the total volume of each reaction to 50 μ L. The template portion of the solution consisted of water or 300 copies human genomic DNA for the controls (female or male DNA; note that this corresponds to 150 copies of the Y-chromosome) or 1.5 μ L sample material. The completed reactions were transferred to a black 384-well plate with clear bottom from Greiner and read at 37°C.

Assay Principle

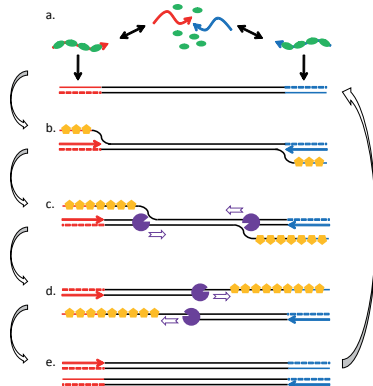


Fig. 1: RPA employs enzymes, known as recombinases [green], that are capable of interacting with amplification primers (red and blue arrows) and pairing them with homologous sequence in duplex DNA (indicated sections of black double line; a). Strand exchange and 'D-loop' formation is assisted by binding of single-strand DNA-binding protein (yellow) to the displaced strand (b). The 3' ends of the oligonucleotides are extended by strand displacing polymerases (purple), thereby copying the displaced strand (c, d). Both, the original template and the copy are then targets for subsequent recombination/extension events and an exponential amplification reaction is initiated (e). Using two gene-specific primers, the recombinase-driven RPA process ensures that DNA synthesis is directed only to defined target sites in a given template molecule.



Results & Discussion

The target for this validation experiment is located within the sex-determining region on the human Y-chromosome (Sry), and the amplification primers and detection probe were designed accordingly.

A total of 9 samples were tested for the presence of the Sry target sequence on the human Y chromosome. These included a 'no template' control (water), a positive control (male human genomic DNA), a negative control (female human genomic DNA), and material from buccal swaps obtained from 6 individuals (5 male, 1 female).

Reactions were set up as described under Material and Methods and monitored at 37°C for a total reaction time of 20 min. Readings were taken every 30 sec, following a 2 sec shaking cycle (double-orbital, 1 mm), and using excitation and emission filters of 485-12 nm and 520-10 nm wavelength, respectively. As shown in Figure 2 both negative controls and the sample prepared from a female buccal swap did not result in the generation of a fluorescent signal.

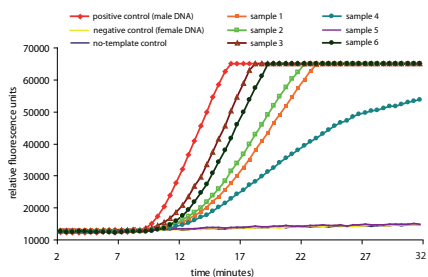


Fig. 2: A total of 3 controls and 6 samples were tested for the presence of the Y-chromosome target. The two negative controls and the sample derived from the buccal swap of a female individual (sample 5) did not produce a fluorescent signal above the baseline. The positive control and all swaps from 5 male probands generated a positive signal within 12 minutes of the start of the reaction. Note that the onset of amplification in positive control (300 copies of template DNA in the reaction, corresponding to 150 copies of the Y-chromosome) precedes the 5 test samples, indicating that the total amount of template in samples 1 to 5 is probably lower than 300 genomic copies.

By contrast, the positive control and all 5 male buccal swaps scored positive for the presence of the Sry target within less than 12 min of initiation of the reaction. The exact number of template molecules in the different sample preparations is likely to vary as buccal samples were not quantified, probably accounting for the differences in the time of onset of amplification and in the total signal strength between the six sample reactions.

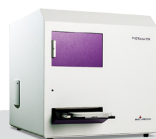
Conclusion

The experiment described in this application note shows the detection of a target sequence on the Y-chromosome in sample material prepared from buccal swaps, thus validating the feasibility of combining all important aspects of a working NAAT in a single integrated format: a very simple sample preparation procedure, a DNA amplification system requiring isothermal temperature conditions and a homogenous read-out system.

Furthermore, the established assay delivers the test reagents in a stabilised and easy-to-use formulation, demanding only very limited user input for operation (two pipetting/mixing steps), lending itself for deployment in non-laboratory settings. Eventually, the implementation of the technology in point-of-care and field-testing environments will require the use of compatible and cost-effective handheld fluorescent reading devices. However, the BMG LABTECH microplate reader acts as a valuable tool for both assay development programs and for the use of the TwistDX technology platform in laboratory and high-throughput applications.

Background

TwistDX is a biotechnology company located on the Babraham research campus near Cambridge, UK. The focus of the company lies in the integration of its proprietary nucleic acid amplification and detection technologies with user-friendly sample preparation procedures and test delivery formats, enabling portable DNA testing assays for the point-of-use market. TwistDX will also provide DNA amplification and detection alternatives for laboratory based nucleic acid testing in both, the research & development and applied markets in the near future.



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