Rapid Isothermal Amplification and Multiplexed Surface-Based Detection of Short DNA Sequences

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ABSTRACT

We have coupled a novel isothermal amplification method for short oligonucleotides (EXPAR) with solution and surface-based detection involving DNA-functionalized gold nanospheres. The assay allows for different target sequences to be detected using the same set of DNA:Au nanospheres through appropriately designed reporter sequences. The solution based assay relies on simple visual detection through aggregation of DNA-Au nanospheres in solution, and is rapid, sensitive and sequence-specific, with minimal requirement for instrumentation. We have also implemented a multiplexed surface based variation of this assay using either fluorescence based detection on standard DNA microarrays, or atomic force microscopy (AFM) of DNA nanosphere immobilization on silicon substrates. Our ultimate goal is to utilize this assay for impedance based electronic DNA detection.

Keywords: isothermal DNA amplification, nanosphere colorimetric detection, impedance DNA detection, microarray DNA detection

1 INTRODUCTION

Amplification of specific DNA and RNA sequences is an essential step in nucleic acid based clinical diagnostics. The polymerase chain reaction (PCR) is the most common method used for amplifying DNA in both molecular biology and medical diagnostics [1,2]. Real time PCR enables quantitative detection of target DNA down to single digit copy numbers with a large dynamic range and excellent linearity [3,4]. However, most conventional real-time PCR requires several hours of amplification and specialized thermocycling equipment, which renders PCR-based molecular diagnostics costly and non-portable. Rapid PCR methods have been developed to decrease the total assay time. Rapid PCR can be performed on commercial instruments such as the Applied Biosystems Fast 7900HT real time PCR system, and on small-scale, portable microfluidic PCR devices [5]. Nevertheless, rapid PCR still requires sophisticated and expensive instrumentation. Isothermal nucleic acid amplification methods do not require thermocycling equipment and some techniques have much faster amplification times than conventional PCR.

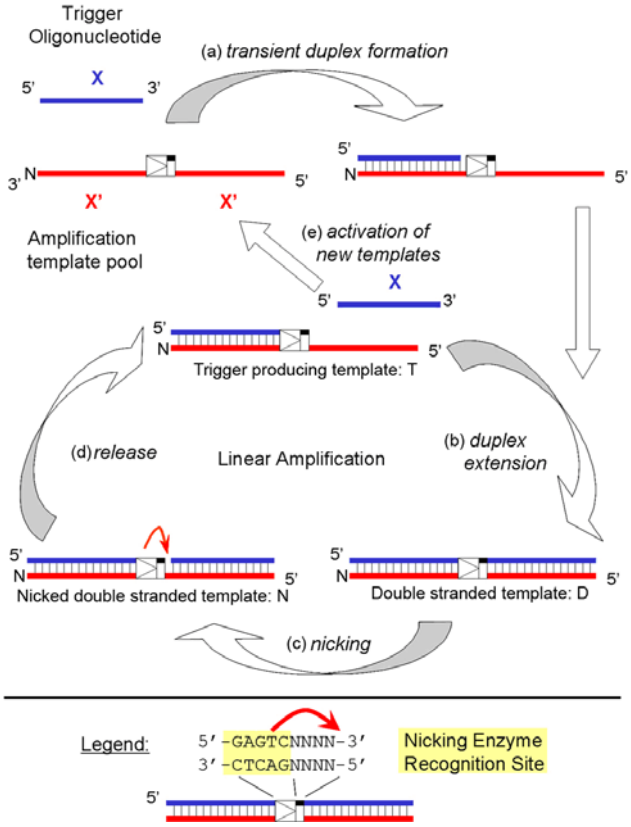

Figure 1: Overview of the exponential amplification reaction EXPAR

We have utilized a novel isothermal exponential nucleic acid amplification reaction (EXPAR) [6] to enable rapid, visual endpoint detection of short DNA sequences, facilitated by a color change due to DNA nanosphere aggregation [7]. Unlike other isothermal amplification reactions, EXPAR rapidly amplifies short oligonucleotides 10^6 to 10^9 fold in a manner of minutes. The EXPAR reaction occurs at 55°C, a temperature which permits activity and stability of the two thermophilic enzymes involved in the reaction: a polymerase (large fragment Bst polymerase) and a nicking endonuclease (Nt.BstNBI), which recognizes a double stranded GAGTC sequences and nicks the top strand four bases downstream. The EXPAR reaction is initiated by a short trigger oligonucleotide, which ultimately is to be generated from a genomic DNA target sequence of interest. The trigger X transiently binds to the complementary recognition sequence at the 3'-end of an amplification template (Figure 1a), and is extended through the polymerase (Figure 1b), forming the double

stranded nicking enzyme recognition site on the top strand, which is then cleaved through the nicking endonuclease (Figure 1c). At the temperature of the reaction (55°C), the newly formed trigger is released from the amplification template (Figure 1d), and the trigger-producing form of the template re-enters the linear amplification cycle. Newly formed trigger oligonucleotides activate additional template sequences (Figure 1e), giving rise to exponential amplification of the trigger X.

EXPAR shows great promise for the development of rapid point of care based nucleic acid diagnostic assays and devices. We herein report an increase in sensitivity for our previously described colorimetric DNA detection assay based on coupling EXPAR amplification with aggregation of DNA-functionalized gold nanospheres. We further introduce a multiplexed variation of the reaction suitable for surface-based detection. We have obtained preliminary proof of principle for this method through a standard fluorescence-based microarray format using a Cy3-labeled detection probe, or through atomic force microscopy (AFM), using DNA-functionalized nanospheres.

2 RESULTS

2.1 Real-Time Fluorescence

To assess the limit of detection and dynamic range of the EXPAR reaction, we have monitored the progression of isothermal DNA amplification in real time via SYBR Green fluorescence detection through the intercalating dye SYBR Green II. During an EXPAR reaction, the fluorescence intensity increases in a sigmoidal fashion as the amplification template is converted from single stranded to partially or completely double stranded DNA, and reaches a plateau once all single stranded template is converted into the active, trigger producing forms (Figure 2).

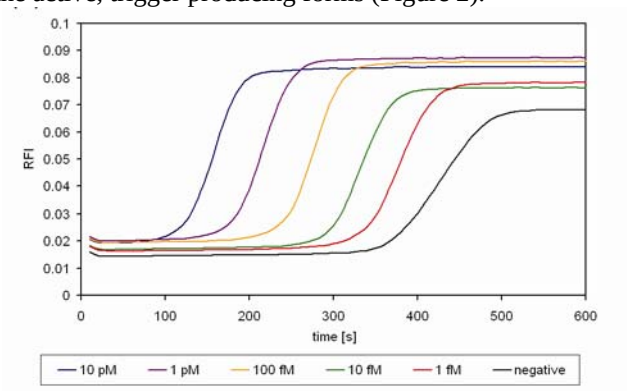

Figure 2: EXPAR amplification monitored through real time fluorescence detection mediated by SYBR Green II. Amplification curves for different starting trigger concentrations or in the absence of trigger (negative).

For EXPAR, we define the onset of efficient amplification as the point of inflection (POI) of these sigmoidal curves, which is analogous to the cycle threshold in PCR. As in real time PCR, we have observed a linear correlation between the POI and the logarithm of the starting trigger concentration for a 10-fold trigger dilution series from 10 pM to 1 fM. However, a blank containing all components of the EXPAR master mix except for trigger also displays amplification after approximately 7 minutes. This non-specific background amplification limits at the present time the sensitivity of EXPAR to 1 fM, equal to 30,000 copies of trigger in a 50 µl reaction volume. The cause of non-specific background amplification is currently under investigation.

2.2 Colorimetric Detection

Mirkin and co-workers have used gold nanospheres functionalized with a high density of oligonucleotides for simple colorimetric DNA detection based on nanosphere aggregation [8,9,10,11,12]. This assay involves hybridization of bridging oligonucleotides to two sets of DNA-functionalized gold nanospheres, causing aggregation and a red-to-blue color change of the solution due to plasmon resonance coupling. The color change can be enhanced through spotting onto a C18-modified silica thin layer chromatography (TLC) plate to permit direct visual readout. Visual read-out through nanosphere aggregation requires a higher than clinically relevant concentration of bridging oligonucleotides. The sensitivity can be enhanced through catalytic silver reduction [13,14,15,16], which however increases the complexity and prolongs the overall time required for the assay.

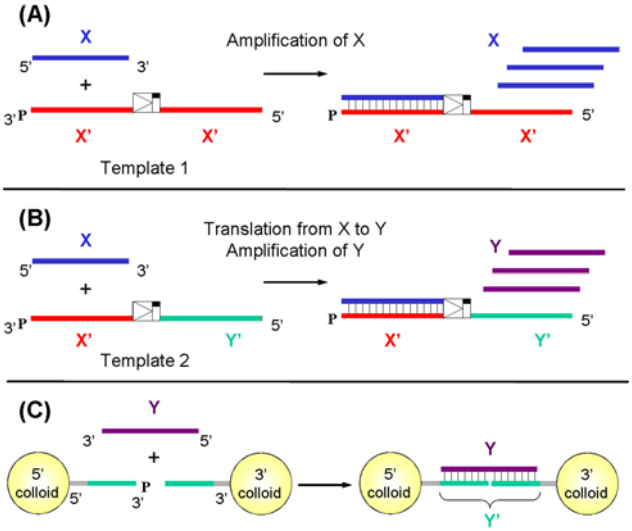

Figure 3: Overview of the two stage EXPAR reaction with detection through DNA:Au nanosphere hybridization: (A) exponential amplification of trigger X; (B) conversion of trigger X to reporter Y, and (C) DNA:Au nanosphere aggregation induced by the bridging reporter Y.

We have developed a two-stage EXPAR amplification reaction coupled with visual colorimetric detection based on DNA-Au nanosphere aggregation [7]. As shown in Figure 3, the reaction exponentially amplifies trigger X in the first stage, then linearly translates trigger X via a second

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amplification template into a universal reporter oligonucleotide Y in the second stage. Reporter Y then hybridizes to and aggregates two sets of DNA-Au nanospheres.

As previously described [7], the overall assay can be performed in ten minutes or less, with minimal reagent consumption and simple instrumentation. First, the EXPAR master mix containing trigger and both templates is heated for several minutes, depending on the desired sensitivity limit. Following EXPAR amplification, the master mix is combined with a DNA nanosphere detection reagent, incubated at room temperature for two minutes, and the solution is then spotted onto a C18-TLC plate. This reaction allows detection of different trigger sequences using the same set of DNA nanospheres, through appropriate design of the two amplification templates.

Previously, we have reported a limit of detection for this reaction of 100 fM starting trigger concentration. We have determined that the sensitivity of single and two-stage EXPAR can be improved through optimization of template sequences and reaction conditions. Using these refinements, we have been able to decrease the limit of detection for this reaction of 1 fM starting trigger in a 10 µl reaction volume (6000 copies) in a reproducible manner (Figure 4). We have observed even higher sensitivity for some reactions, however we are still improving the reproducibility at such low trigger concentrations.

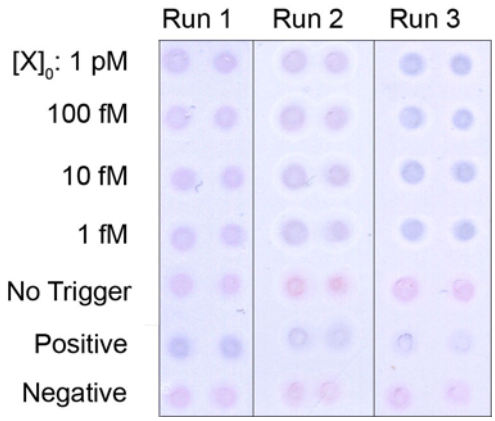

Figure 4: Visual detection of trigger oligonucleotide (positive: blue/purple, negative: red) by the two-stage EXPAR reaction combined with DNA-nanosphere based colorimetric detection through the spot test. The controls labeled "Positive" and "Negative" solely contain the nanosphere detection reagent and either 1 µM externally added reporter or no reporter oligonucleotide, respectively. These controls verify the stability of the DNA nanospheres and the specificity of the reaction. The second negative "No Trigger" control contains the EXPAR template, but no trigger, and verifies absence of non-specific background amplification. Runs 1-3 represent three different experiments conducted on separate days with different reagent batches.

2.3 Surface-Based Detection

We have expanded the two-stage EXPAR amplification reaction to enable multiplexed surface-based detection using a sandwich-type hybridization assay with a universal detection probe, coupled either to a fluorophore or to a gold nanosphere. Through this multiplexed version of the two-stage EXPAR reaction (Figure 5), different trigger sequences (e.g. X1 and X2) are amplified, and then converted to a generic set of bridging reporter oligonucleotides (e.g. Y1 and Y2). These reporters contain a modular 5' portion (labeled a or b) that hybridizes to a generic set of surface-bound probe oligonucleotides (a' or b'), and a universal 3' portion (u) which can hybridize to the detection probe u', which is conjugated either to Cy3 or immobilized on a gold nanosphere. To establish proof of principle for this multiplexed assay format, we have developed a set of four bridging reporter oligonucleotides Y1-Y4 containing the generic sequence u and specific sequences a, b, c and d, respectively.

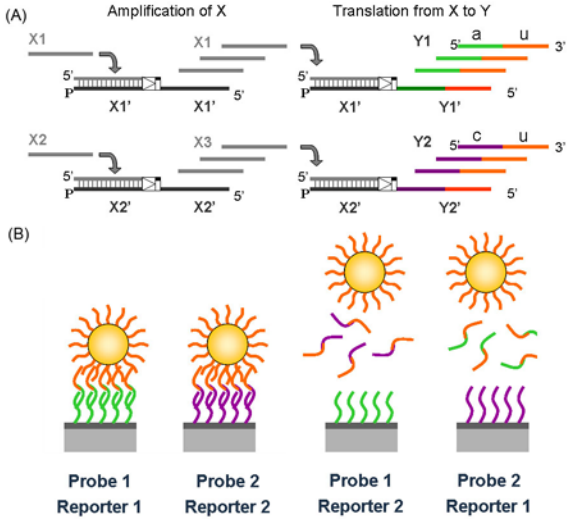

Figure 5: Surface-based detection scheme for the two-stage EXPAR amplification reaction. (A) Two-stage EXPAR amplification produces a reporter oligo, half of which is specific to the trigger sequences X1 or X2, the other half universal. (B) Co-immobilization of nanospheres (pictured) or fluorescently-labeled oligos occurs only when the reporter oligo produced in (A) is complementary to probes immobilized on a surface.

These reporters plus Cy3-u' hybridize in a specific manner to a printed microarray containing the corresponding probe sequences a', b', c' and d' (Figure 6A). Using AFM, we have confirmed that DNA nanospheres functionalized with u' are immobilized on silicon substrates functionalized with sequences a', b', c' and d' in a sequence-specific manner through bridging hybridization using these reporter sequences,. We have further obtained preliminary proof of principle for two-stage EXPAR amplification coupled to surface based

detection, using a simplified version of the scheme shown in Figure 5. In this case, four second stage templates were designed to generated the reporters Y1-Y4, but all starting from the same trigger sequence X (i.e. X'-Y1', X'-Y2', X'-Y3' and X'-Y4'). We have shown that using two stage EXPAR amplification with the template X'-X' and one of the templates X'-Yn', trigger X can be amplified and converted to the appropriate reporter Yn, which is subsequently detected on the microarray (Figure 6B and C). In these experiments, the negative no trigger control remained negative, showing no interference by non-specific background amplification.

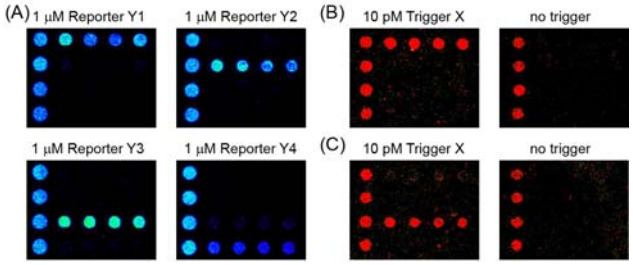

Figure 6. Spotted microarrays on glass slides containing a positive control oligonucleotide (Cy3-labeled) in column 1, and probe oligonucleotides a', b', c' and d' in rows a-d respectively of columns 2-5. (A) Verification of specific sandwich hybridization to Cy3-labeled oligonucleotide u', in the presence of 1 μM reporter Y1 (a-u), Y2 (b-u), Y3 (c-u) and Y4 (d-u). (B) two-stage EXPAR amplification using templates X'-X' and X'-Y1', starting with either 10 trigger X or no trigger. Reporter Y1 generated in the presence of trigger is detected on the array. (C) two-stage EXPAR amplification using templates X'-X' and X'-Y3', starting with either 10 trigger X or no trigger. Reporter Y3 generated in the presence of trigger is detected on the array.

Using AFM we have verified that this two-stage EXPAR reaction results in effective sequence-specific immobilization of DNA nanospheres on silicon substrates functionalized with the appropriate probe sequences, according to the reaction shown in Figure 5. Very little or no DNA nanosphere immobilization is observed on surface with non-complementary probe sequence or in the absence of EXPAR amplification.

3 CONCLUSIONS

The herein reported improvement in sensitivity of two-stage EXPAR amplification coupled with visual colorimetric readout through DNA nanosphere aggregation is an important step in rendering this assay clinically relevant. We are currently coupling this the two-stage EXPAR reaction with trigger generation from genomic DNA, and are continuing the refinement of reaction conditions in order to achieve optimal sensitivity and robustness.

We are developing a surface based multiplexed version of the two-stage EXPAR reaction to enable parallel detection of different trigger sequences and incorporation of internal controls. This multiplexed reaction scheme will enable DNA detection in a variety of formats. We are integrating the multiplexed two stage EXPAR reaction with impedance-based electronic DNA detection on semiconductor biosensor surfaces. Our ultimate goal is to develop a closed system microfluidic device incorporating trigger generation, amplification and multiplexed surface based detection to enable simple, rapid, and sensitive DNA detection with minimal instrumentation, suitable for point-of-care clinical diagnostic applications.

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