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Sensitive and selective viral DNA detection assay via microbead-based rolling circle amplification

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ABSTRACT

We report a sensitive and efficient magnetic bead-based assay for viral DNA identification using isothermal amplification of a reporting probe.

Rapid, specific, and high-throughput oligonucleotide diagnostic approaches to identify pathogenic and genetic diseases are of great importance to both medical and biosecurity fields.[1,2] Direct and sensitive DNA detection from samples collected directly from patients would avoid expensive and lengthy traditional assay approaches, which utilize antibodies and cell cultures. In recent years, there have been numerous reports about the development of ultra-sensitive DNA detection platforms.[3–9] Although sensitive, most of these assays require complex and time-consuming laboratory-based manipulation steps and expensive equipment. Here, we report a sensitive and efficient diagnostic magnetic bead-based assay for viral identification based on their DNA signatures. The readout process of our assay relies on isothermal amplification of the reporter DNA probe, requiring only a well-plate reader for sensitive detection. Collectively, this simple and rapid DNA sandwich capture assay enables both desired target binding and amplification of the readout process to render the required sensitivity for direct viral DNA detection in clinical samples with low-cost.

An illustration of our assay is shown in Scheme 1. For real-time monitoring of our target viral DNA, we first immobilize capture probe DNA onto ~1 μm diameter magnetic beads (DNA sequences are shown in Supplementary Information, Fig. S1). Blocking buffer is then added, which has been proven to be crucial for reducing non-specific DNA binding to both the beads and the side walls of the reaction microfuge tube (see Supplementary Information, Fig. S2A). After the addition of both target and reporter DNA, followed by extensive washing, the magnetic beads are then heated to release the reporter DNA into the supernatant. Once the released reporter DNA has been transferred to a 384-well plate, RCA is subsequently performed to elongate the reporter DNA. Amplification is monitored in real-time by SybrGreenII using a well-plate's fluorescent reader. The reporter DNA release step was necessary because we had previously observed that the presence of magnetic beads in the well-plate reader leads to inconclusive results when using SybrGreenII (see Supplementary Information, Fig. S2B).

The RCA amplification process has recently been used in a variety of assays to enable enhanced target detection.[10–13] Briefly, RCA is a polymerase-based technique that generates long ssDNA oligomers (>3–5 kbp in size) comprised of tandem repeats complementary in sequence to a circular DNA primer. Unlike PCR, the final ssDNA product remains attached to the initial RCA primer binding domain. One benefit of RCA is that it can be used for real-time detection as the amount of ssDNA present in the reaction increases with time. Thus, DNA elongation can then be monitored with SybrGreenII dyes, as the amount of fluorescence that the dyes emit will increase proportionally to the quantity of ssDNA present. An advantage of RCA, as opposed to PCR, is that it does not need to undergo thermal cycling to amplify the starting oligonucleotide, hence making it more applicable for low-cost high-speed assays.

After optimizing the RCA amplification, we first performed a detailed viral DNA sandwich capture assay with one of our DNA targets, Variola virus (VV).[7] After immobilizing the biotinylated VV capture DNA on streptavidin-coated magnetic beads, we performed extensive washes to remove unbound capture probes. To

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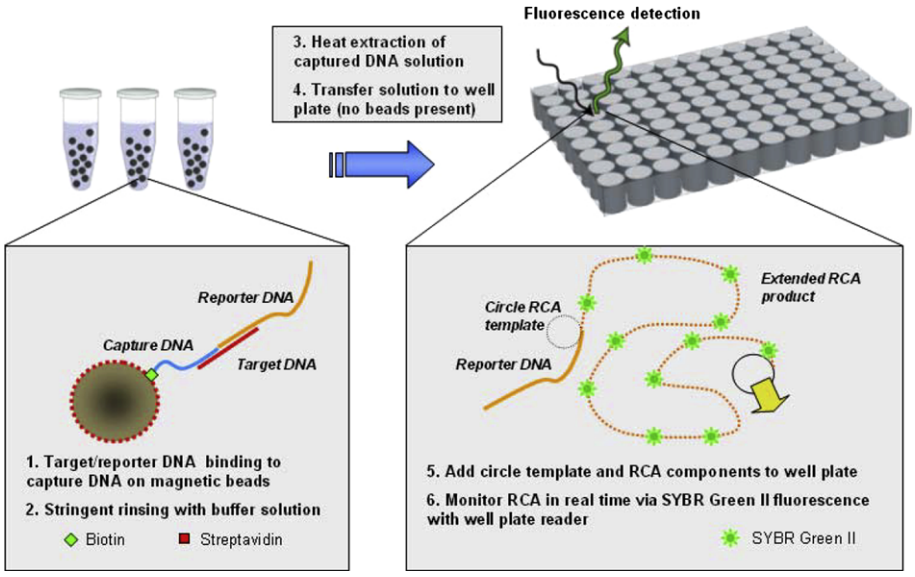

Scheme 1. An illustration of the magnetic bead-based DNA detection assay.

initiate the sandwich capture, we exposed the beads to six different concentrations of the VV DNA targets and reporter DNA for 30 min at room temperature (again with extensive washes in between steps). The magnetic beads were then suspended in RCA buffer and both the target and reporter strands previously bound to the magnetic beads were released at an elevated temperature (85 °C for 2 min). The extracted DNA strands were quickly transferred to a single well within a 384-well plate. After adding the necessary RCA components (in a final volume of 16 μL, see Experimental section in Supplementary Information for details), the available reporter DNA was subsequently extended and simultaneously monitored in real-time via a fluorescent well-plate reader.

As shown in Figure 1A, we observed that increasing the input target VV DNA concentration resulted in a corresponding increase in the measured fluorescence intensity after 60 min of RCA elongation. Furthermore, when the target VV DNA is absent (negative control), there is no observed RCA growth. This indicates that our blocking conditions utilized in the binding assay were optimal as there is minimal undesired non-specific binding during the assay. Conversely, the RCA growth can be monitored in real-time. Figure 1B illustrates the plots of three representative input VV target concentrations. We observed that after 1 h of RCA, there was no visible DNA elongation process stemming from the negative control, that is, no input VV target DNA. The trace at 100 pM VV DNA target input is clearly distinguishable from the negative control. Within 10 min after RCA initiation, the 10 nM VV target plot revealed a ~32-fold higher observed fluorescence intensity compared to the negative control (Fig. 1B). The RCA signal growth began to saturate after 30 min, at a fluorescence intensity is ~40-fold higher than the negative control. From Figure 1A and B, we observed that the lowest concentration of the input VV target that can successfully be amplified was ~100 pM. Consequently, we estimated the limit of detection (LoD) of this assay to be between 10 and 100 pM. We also observed that the fluorescence intensity saturated at concentrations above 10 nM (Fig. 2A). We reasoned that this is due to the limiting concentration of circular DNA molecules added to each reaction, thus the RCA plots from both input DNA concentrations, that is, both 10 and 100 nM, appeared to be similar.

To probe the specificity of our assay, we performed the interrogation in the presence of three DNA targets: Hepatitis-B virus

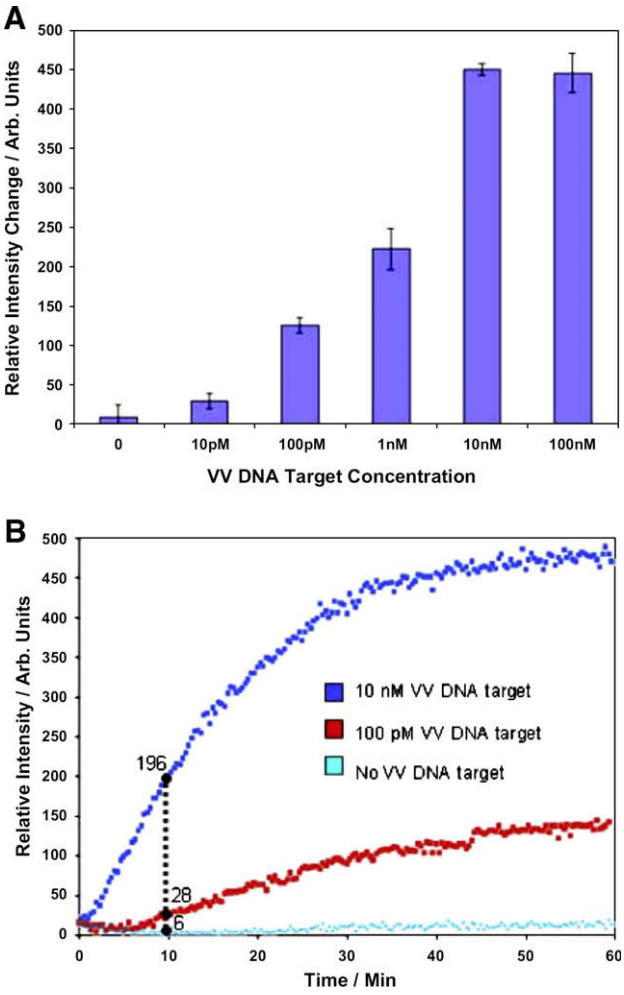

Figure 1. (A) Change in intensity, as measured after 60 min of RCA at various input concentrations of VV DNA target. (B) Representative real-time fluorescent intensity traces of the input VV target concentrations at 0, 100 pM, and 10 nM. The dotted black line illustrates the fluorescence intensity difference between the three traces at 10 min after RCA initiation.

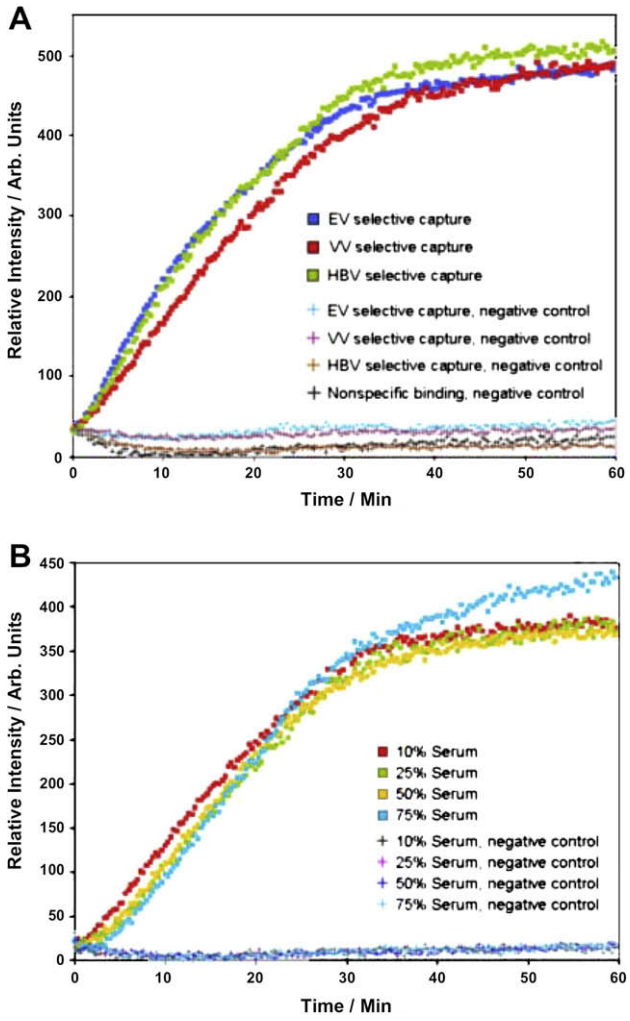

Figure 2. (A) Real-time traces of RCA fluorescent intensity from of a simultaneous input of three DNA targets, each at a concentration of 10 nM, for each viral set. The negative controls presented include incorrect target inputs and non-functionalized beads. (B) Real-time fluorescent intensity traces of EV DNA targets (10 nM) and negative controls in increasing serum concentrations.

(HBV), Ebola virus (EV), and Variola (smallpox) virus (VV).[5] Each of the three capture DNA-conjugated magnetic beads was exposed to all three intended DNA targets concurrently (target DNA concentrations fixed at 10 nM). We adopted the same experimental approach as was previously described, and RCA was performed with each of the extracted reporter DNA molecules. As shown in Figure 2A, each of the three capture beads was able to specifically recognize their intended target, as evident by the corresponding RCA amplification plots. The RCA growth for each input DNA target is observed to closely resemble the RCA amplification for VV detection as shown in Figure 1B, in which only one correct target was presented to the beads. These results indicate the assay's reliability for specific DNA target detection, even in a mixture of DNA targets.

To confirm the assay's specificity, we performed the following two control experiments (details are shown in Supplementary Information, Fig. S3): (1) In the presence of non-functionalized magnetic beads, that is, containing no capture DNA, no subsequent RCA growth was observed (Fig. 2A). Thus, there was no non-specific binding between the target or reporter probes to the magnetic bead. Again, the non-specific binding was suppressed by the addition of blocking buffer. (2) In situations where the intended target (from the pool of DNA targets) was removed, no subsequent RCA growth was observed (Fig. 2A). For example, when beads functionalized with EV capture probes were exposed to only VV and HBV targets and EV reporter probes, no subsequent RCA amplification was observed. This result indicates that the beads featuring EV-capturing DNA are specific to only the EV DNA target.

Finally, we examined the feasibility of performing this assay in a complex matrix. The same assay against EV DNA target (10 nM) was repeated, except that the input EV DNA target was diluted in solutions of fetal calf serum with concentrations ranging from 10% to 75% (v/v in binding buffer) during the sandwich capture steps of the assay. The samples were then washed, and the DNA was collected into appropriate buffer. RCA amplification was clearly demonstrated (Fig. 2B) at all tested concentrations of serum, indicating that the sensing portion of the assay is extremely robust in tolerating the input DNA target being suspended in serum. Both sensitivity and specificity of the DNA detection were unaffected by serum concentrations up to 75% (see Fig. S5 in Supplementary section for gel analysis image of RCA amplified DNA products and explanation for not attempting higher serum concentrations). To demonstrate the lack of increased background potentially induced by confounding components in fetal calf serum, negative control experiments were performed in the absence of input EV DNA targets. As expected, the negative control traces in serum did not exhibit any RCA amplification (as there is no target DNA present). These observations indicate that we can perform direct DNA detection in serum, which is an important criterion for directly monitoring potential viral infection in clinical blood samples.

In conclusion, we have described an assay that allows rapid, efficient, selective viral DNA target detection, in the presence of serum. Depending on the input target DNA concentration, we have observed that the amplification of the reporter DNA via RCA has enabled ~40- to 50-fold increase in the fluorescence intensity, and most of this amplification occurs within 30 min. The entire assay takes place in under 4 h (un-optimized), and requires little more than a portable well-plate reader. Furthermore, the reaction volume for RCA is minimal as each run is performed in a single well (within a 384-well plate), minimizing the use of costly reagents. Last, by performing the RCA reaction in a 384-well plate, we should be able to extend the assay to encompass up to 384 interrogations within a single run if desired.

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Supplementary data

Experimental details, results of both blocking buffer and presence of beads in the RCA reaction, circularizing of the RCA template, and agarose gel analysis of RCA products. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.064.

References and notes

1. Beaudet, A. L.; Belmont, J. W. *Annu. Rev. Med.* **2008**, *59*, 113.
2. Zhang, X. E.; Bi, L. J. *Methods Mol. Biol.* **2007**, *382*, 163.
3. Zhao, W.; Wang, L.; Tan, W. *Adv. Exp. Med. Biol.* **2007**, *620*, 129.
4. Zhang, H.; Zhao, Q.; Li, X. F.; Le, X. C. *Analyst* **2007**, *132*, 724.
5. Nam, J. M.; Stoeva, S. I.; Mirkin, C. A. *J. Am. Chem. Soc.* **2004**, *126*, 5932.

6. Stoeva, S. I.; Lee, J. S.; Smith, J. E.; Rosen, S. T.; Mirkin, C. A. *J. Am. Chem. Soc.* **2006**, *128*, 8378.
7. Stoeva, S. I.; Lee, J. S.; Thaxton, C. S.; Mirkin, C. A. *Angew. Chem., Int. Ed. Engl.* **2006**, *45*, 3303.
8. Stromberg, M.; Goransson, J.; Gunnarsson, K.; Nilsson, M.; Svedlindh, P.; Stromme, M. *Nano Lett.* **2008**, *3*, 816.
9. Armani, A. M.; Kulkarni, R. P.; Fraser, S. E.; Flagan, R. C.; Vahala, K. J. *Science* **2007**, *317*, 783.
10. Schweitzer, B.; Roberts, S.; Grimwade, B.; Shao, W.; Wang, M.; Fu, Q.; Shu, Q.; Laroche, I.; Zhou, Z.; Tchernev, V. T.; Christiansen, J.; Velleca, M.; Kingsmore, S. F. *Nat. Biotechnol.* **2002**, *20*, 359.
11. Fischer, N. O.; Tarasow, T. M.; Tok, J. B. *Anal. Biochem.* **2008**, *373*, 121.
12. Di Giusto, D. A.; Wlassoff, W. A.; Gooding, J. J.; Messerle, B. A.; King, G. C. *Nucleic Acids Res.* **2005**, *33*, e64.
13. Li, N.; Li, J.; Zhong, W. *Electrophoresis* **2007**, *29*, 424.