Rapid visual identification of PCR amplified nucleic acids by centrifugal gel separation: Potential use for molecular point-of-care tests

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**Article info**

**Abstract**

Recently, nucleic acid amplification and detection techniques have progressed based on advances in microfluidics, microelectronics, and optical systems. Nucleic acids amplification based point-of-care test (POCT) in resource-limited settings requires simple visual detection methods. Several biosensing methods including lateral flow immunoassays (LFIA) were previously used to visually detect nucleic acids. However, prolonged assay time, several washing steps, and a need for specific antibodies limited their use. Here we developed a novel, rapid method to visualize amplified nucleic acids with naked eyes in clinical samples. First, we optimized conditions based on separation using very low centrifugal force and a density medium to detect human papillomavirus (HPV)-16 DNA in cervical specimens. After DNA extraction, HPV16 PCR was performed with biotin-labeled forward primer and Cy3-labeled reverse primer. PCR amplicon was mixed with streptavidin-magnetic beads, introduced into the density medium. After two-minute centrifugation, the result was visually identified. This system showed identical results with commercial HPV real-time PCR for 30 clinical samples and could detect up to $10^5$ copies/mL of HPV DNA without any optical instruments. This robust and sensitive visual detection system is suitable for non-specialist personnel and point-of-care diagnosis in low-resource settings.

**Keywords:** DNA separation, Molecular POC, Biosensing, Gel-separation, Centrifugation, Visual detection

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**1. Introduction**

Rapid and accurate diagnostic point-of-care testing (POCT) on the field is important for diagnosis of infectious diseases in developing countries where referral to a centralized laboratory is limited. Recently, POCT has progressed based on advances in microfluidics, microelectronics, optical systems, and chip-based nucleic acid amplification and detection techniques (Jani and Peter, 2013).

The World Health Organization (WHO) developed the ASSURED criteria (Affordable by those at risk of infection, Sensitive, Specific, User-friendly, Rapid and robust, No equipment, Delivered to those who need it) for an ideal POCT in resource-limited settings (Drain et al., 2014). As the simplicity in use and cost of a molecular POCT is critical (Tomazelli Coltro et al., 2014), molecular POCT in resource-limited settings requires simple visual detection methods without the need for instruments, such as an expensive light source, filters, and imaging capture devices. Appropriate requirements of visualization with the naked eye for molecular POCTs are short incubation time (<30 min), no washing steps, minimal procedural steps for color development, precision, and high reproducibility. Although several visual detection techniques have been reported (Jung et al., 2010; Zhang et al., 2015), they involve several washing steps, relatively long incubation time, or additional steps for color development.

In this study, we used a gel column (Ortho BioVue system poly cassettes, Ortho-Clinical Diagnostics, Raritan, NJ) and magnetic beads for visual detection of amplicons after polymerase chain reaction (PCR). The gel column based on column agglutination technology (Lapierre et al., 1990) has been widely used in blood banking in Europe and America since 1991 for direct and indirect
antiglobulin tests, ABO/Rh typing, red blood cell phenotyping, and detection of unexpected antibodies (South, 1993). The gel column separates the antigen–antibody complex (agglutination) according to size, shape, and charge of agglutination. However, this column agglutination technology as itself is ineffective for visual detection of amplified DNA. Magnetic beads have been widely used for the separation of biomolecules in biomedicine (Borlido et al., 2013). Since magnetic beads have a brown color and appropriate density in the mobile phase, higher than that of the gel column, a magnetic bead suspension was used as the mobile phase to carry the amplicon in this study.

We achieved visualization with the naked eye, of amplified DNA using magnetic beads and a ready-to-use gel column without probe addition, incubation, or dilution steps. This simple, separation-free visual detection of target DNA (SPIN-DNA) was applied to detect human papillomavirus (HPV) DNA and assess the feasibility of our method.

2. Materials and methods

2.1. Sample collection and DNA extraction

For evaluation of potential diagnostic feasibility of this visual detection method for HPV detection, we used 30 cervical swab specimens that had been stored after a routine HPV DNA test using Roche 4800 HPV test (Roche Molecular Diagnostics, Pleasanton, CA). Out of the 30 specimens, 24 were HPV16-positive and the rest contained other HPV types and were used as negative samples. Nucleic acids from the samples were prepared using cobas x 480 (Roche Molecular Diagnostics, Pleasanton, CA), an automated instrument for nucleic acid extraction. Using 1 mL cervical specimen in Roche collection media per subject, the instrument yielded 150 μL nucleic acid eluted sample, and this elution was used for HPV DNA PCR.

2.2. PCR

For concept of rapid visual identification of target DNA amplicons, PCR was performed for HPV. The primers were designed based on HPV-type 16 L1 DNA (Supplementary Table S1). Reverse primers were labeled with Cy3 or TAMRA at the 5′ end of the sequence. To attach Cy3 to the 5′ end of the reverse primer, the primer was synthesized by N-hydroxysuccinimide (NHS) ester modification (Mujumdar et al., 1993). Biotin was labeled at the 5′ end of forward primers to be captured by streptavidin-labeled magnetic beads (Dynabead MyOne Streptavidin C1, Life Technologies, Grand Island, NY, USA). Modified primers were synthesized by Bioneer (Daejeon, Korea).

For HPV PCR, amplification was performed using the Hot-StarTaq Plus Master Mix Kit (QIAGEN, Valencia, USA), as previously described previously (Lee et al., 2015). Reaction mixtures contained 10 μL master mix, 250 nm of each dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, and 5 μL sample DNA. A GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, USA) was used for DNA amplification. The thermal cycling conditions used were as follows: 10 min at 95 °C; 40 cycles at 95 °C for 30 s, 50 °C for 30 s, and 60 °C for 45 s; followed by 60 °C for 10 min. The effect of HPV DNA amplicon length on detection of DNA using magnetic bead-based gel column detection was also studied, to determine the effect of DNA amplicon size on gel separation (Supplementary Fig. S1). HPV DNA was amplified using biotin-labeled forward primer and Cy3-labeled reverse primer for 95-, 141-, or 302-bp sized amplicons (Supplementary Table S1).

2.3. Visual detection of amplified DNA by gel column (SPIN-DNA)

The principle of the rapid visual detection system (SPIN-DNA) is illustrated in Fig. 1. Final optimized protocol included the following steps. After PCR amplification, a mixture of 4 μL biotinylated and Cy3-labeled amplified DNA, 4 μL magnetic bead suspension, and 1 μL GelRed (20 x) were pipetted into each gel column and incubated for 5 min at 37 °C. After incubation, gel card was centrifuged at 55 x g for 2 min. No migration showed negative results (non-sensitized magnetic beads). A positive result indicated that the amplicon-carrying magnetic beads (sensitized magnetic beads) had moved through the gel matrix to the bottom of the gel column.

To determine whether the specificity and/or resolution of visual detection of amplicon by gel column could be improved, a series of steps were taken. After PCR amplification, the mixture of 4 μL biotinylated and Cy3-labeled amplified DNA, 4 μL magnetic bead suspension, and 1 μL GelRed (20 x) were pipetted into each gel column and incubated for 5 min at 37 °C. After incubation, gel card was centrifuged at 55 x g for 2 min. No migration showed negative results (non-sensitized magnetic beads). A positive result indicated that the amplicon-carrying magnetic beads (sensitized magnetic beads) had moved through the gel matrix to the bottom of the gel column.
experiments regarding amplicon separation in the gel column were performed using different conditions such as incubation time (5 min, 10 min, 15 min), concentrations of Sepharose 4B (Sepharose, Sigma-Aldrich, St. Louis, MO, USA), with or without intercalating dyes, and levels of centrifugal force. Confocal microscopy was performed using Carl Zeiss LSM510 META (Carl Zeiss, Germany) to assess the mechanism of binding of non-sensitized magnetic beads to the Sepharose beads by intercalating dye, GelRed.

2.4. Evaluation of detection sensitivity and performance of SPIN-DNA

To determine the sensitivity of SPIN-DNA method, a 10-fold dilution series (10^1–10^7 copies/mL) of 1st WHO International Standard for HPV16 DNA (NIBSC code: 06/202, HPV16 DNA WHO standard) was prepared. Under optimal conditions, we evaluated the performance of the SPIN-DNA system to detect HPV16 DNA in 30 clinical samples.

3. Results

3.1. Difference in mobility of sensitized versus non-sensitized magnetic beads in gel column

First, we observed the differences in mobility between target amplicon-carrying magnetic beads (sensitized) and those not carrying the target amplicon (non-sensitized) in the gel column (Fig. 2). Sensitized magnetic beads with the target amplicon captured via streptavidin-biotin conjugation were transported to the bottom of the gel card column by low-speed centrifugation (55 × g) (Supplementary Fig. S2). The migration velocity of sensitized magnetic beads was altered and faster than that of non-sensitized magnetic beads under the same low-speed centrifugation (55 × g).

3.2. Effect of Cy3-labeled DNA duplex on magnetic bead mobility

To evaluate the effect of Cy3-labeling on the mobility of streptavidin-magnetic beads, target DNA was amplified with/without Cy3-labeled reverse primer. Gel separation was then performed as described above. We demonstrated that the difference in mobility of streptavidin-magnetic beads was observed only when the Cy3-labeled amplicon was captured on streptavidin-magnetic beads. When amplicon without Cy3-labeled primer was mixed with streptavidin-magnetic beads (Fig. 3), Mobility was the same as control (negative PCR mixture) when the amplicon was not labeled with Cy3 to reverse primer.

3.3. Effects of intercalating dye on the mobility of non-sensitized magnetic beads in gel column

When GelRed was added into the column prior to loading the amplicon-magnetic beads complex into gel column, migration of the non-sensitized magnetic beads complex was not observed and formed a flat line at the loading area of the gel matrix. However, sensitized magnetic beads moved through the affinity matrix to the bottom of the gel column (Fig. 4). At high concentrations of GelRed, sensitized magnetic beads also bound to the gel matrix and did not move to the bottom of the gel column (Supplementary Fig. S3).

3.4. Mechanism of intercalating dye on mobility of non-sensitized magnetic beads by confocal imaging

Without GelRed, primers could not enter the porous Sepharose beads, exclusively (Supplementary Fig. S4). When GelRed was added, however, the biotinylated forward primers were trapped in the Sepharose beads. In the non-sensitized state, streptavidin-magnetic beads conjugated with biotinylated forward primers. These primer-conjugated magnetic beads were trapped in the Sepharose beads by addition of GelRed. As a result, the non-sensitized magnetic beads did not migrate to the bottom of the gel column and formed a flat line in the gel matrix of loading area.

3.5. Evaluation of detection sensitivity and performance of SPIN-DNA

It was determined that the concentration of HPV16 DNA must be at least 10^2 copies/mL to be detectable in the gel column (Fig. 5). The results were in good agreement with those obtained using a conventional RQ-PCR kit (kappa statistic = 1.000, Supplementary Fig. S5). The SPIN-DNA system detected HPV DNA in all 24 RQ-PCR-positive samples (100%) while no HPV DNA was detected in all six RQ-PCR-negative samples (100%).

![Fig. 2. Gel separation of non-sensitized (1) and sensitized (2) magnetic beads. After PCR amplification of target DNA (HPV16), 4 μL amplicon, and 4 μL magnetic bead suspension were dispensed into a gel column and incubated for 5 min at 37 °C. After incubation, the gel column was centrifuged at 55 × g. Visual identification was performed every 1 min for 5 min, followed by every 5 min for 10 min. Images captured at 0, 1, 2 minutes are shown in Fig. 2, while those at other time points are shown in Supplementary Fig. S2. 1; non-sensitized magnetic beads (negative control) and 2; sensitized magnetic beads (positive control, 1 x 10^5 copies/mL HPV16 DNA standard).](image-url)
4. Discussion

Our simple visual detection is accomplished by gel-based centrifugation for the first time. We used streptavidin-magnetic beads and gel column centrifugation for DNA detection after PCR. This new, rapid method for visualization of target DNA (captured by streptavidin-magnetic beads) is based on gel-separation principle by low-speed centrifugal force, performed within 2 min. This method is simple to understand, easy to perform, and the results are highly reproducible.

The detection principle of this method is creating a difference in centrifugal mobility between target amplicon-carrying magnetic beads (sensitized) and non-target magnetic beads (non-sensitized) in the gel-based column. The sedimentation of sensitized magnetic beads was faster than non-sensitized magnetic beads under the same conditions.

4.1. Advantages of the visual detection system

This visualization method has several advantages. There were no prolonged incubation time (< 10 min) and no washing steps, rather a one-step procedure (one mixing step and then, centrifugation). Most visual detection techniques require capture antibodies, several washing steps, relatively long incubation time or additional steps of color development. Moreover, results of other detection techniques based on color change are often not discernible by the naked eye near the lower limit of detection of DNA concentration. The results of our method were more objective than those with color-change principle.

Lateral flow immunoassay (LFIA) is currently, the most simple and successful rapid diagnostic platform for antigen/antibody detection. However, LFIA requires 2–3 types of antibodies and it is difficult to be manufactured as other types than strip type. Our detection technology does not require capture antibodies and it can be integrated into centrifugal microfluidics with various structural modifications.
We observed that the mobility difference of streptavidin-magnetic beads was altered only when the biotin-labeled amplicon was captured on streptavidin-magnetic beads (Supplementary Fig. S6) and this is one of the main key features to visualize the amplified DNA in this study. The density of streptavidin-magnetic beads used in this study was 1.4 g/mL, which is higher than that of the medium (glass beads) of the gel column and very low centrifugal force at 55 × g caused sedimentation of streptavidin-magnetic beads. In addition, there was no need of additional color development because the magnetic beads are inherently visible.

4.2. Factors affecting migration of streptavidin-magnetic beads

The gel column is generally used to detect red blood cell agglutination (Reis et al., 1993). This poly-cassette gel column contains minute glass beads, density reagent, anti-human globulin, and anti-IgG-, anti-C3-polyspecific antibodies (Byrne et al., 1996). In our detection system, Sepharose was added to the top of gel column to trap the non-sensitized streptavidin-magnetic beads. The average diameter of Sepharose was 90 μm (45–165 μm) with an average pore diameter of approximately 30 nm (Miller, 2001). The concentration of Sepharose affects the migration of streptavidin-magnetic beads through the matrix of the gel (Supplementary Fig. S7). Additionally, the speed of migration could be affected by the size, conformation, and charge of the sensitized and non-sensitized magnetic beads.

Of the several factors affecting migration of streptavidin-magnetic beads, the Cy3-labeled primer was thought to be important for mobility of sensitized magnetic beads, as the difference in mobility of sensitized and non-sensitized magnetic beads disappeared when the amplicon was not labeled with Cy3 at the reverse primer (Fig. 3).

To investigate whether other fluorescent dyes could also produce the same effect on the mobility of sensitized magnetic beads in the gel column, we tested labeling the reverse primer with TAMRA instead of Cy3, for target amplification. The mobility of TAMRA-labeled amplicon was found to be different from that of the Cy3-labeled amplicon, but the same as the reference control (amplicon without dye-labeled reverse primer) (Supplementary Fig. S8). Thus, the phenomenon of mobility of sensitized magnetic beads with Cy3-labeled amplicon is thought to be unique in the matrix of the gel column. Although fluorescent dyes have certain effects on the stability of the DNA duplex (Moreira et al., 2005; Moreira et al., 2015), the mechanism of the effect of Cy3-labeled amplicon on the mobility of streptavidin-magnetic beads is unclear.

Another main key feature of our method to visualize the amplified DNA was trapping non-sensitized magnet beads in Sepharose. We trapped non-sensitized magnetic beads (on which the biotinylated forward primer was attached) in the porous gel (Sepharose) using intercalating dye, GelRed (Fig. 4). When optimal concentration of GelRed was added into the column prior to loading the amplicon-magnetic bead complex into gel column, the non-sensitized magnetic bead complexes did not migrate through the matrix to the bottom and the non-sensitized magnetic beads formed a flat line at the gel matrix loading area.

It is well known that intercalating dyes alter the structure and mechanical properties of DNA (Biebricher et al., 2015; Smith et al., 1992). The unsymmetrical cyanine dyes, thiazole orange homodimer (TOTO) binds to single-stranded (ss) DNA (ssDNA, M13mp18 ssDNA) to form a fluorescent complex that is stable (Rye and Glazer, 1995). Although the size of primers is small enough to be trapped within the porous Sepharose matrix (average pore diameter of approximately 30 nm) (Miller, 2001), the primers and the Sepharose were repulsive each other in the absence of GelRed (Supplementary Fig. S4). When GelRed was added in this study, the biotinylated forward primers were adsorbed into the Sepharose beads. Intercalated primers such as GelRed could be trapped in the porous Sepharose due to neutralization of the net charge of primer-dye (Nyberg et al., 2013; Zhang et al., 2008).

Several intercalating dyes (SYBR green (Lonz, Allendale, NJ, USA), SYTOX (Invitrogen, Carlsbad, CA, USA), SYTO9 (Invitrogen, Carlsbad, CA, USA), BOBO3 (Invitrogen, Carlsbad, CA, USA), TOTO1 (Invitrogen, Carlsbad, CA, USA)) were evaluated for their influence on the mobility of streptavidin-magnetic beads. Compared to the mobility of non-sensitized magnetic beads in the gel column without intercalating dye, both mono-intercalating (SYBR green, SYTOX and SYTO9) and bis-intercalating dye (BOBO3, TOTO1, and GelRed) groups enhanced the non-sensitized magnetic beads to be inhibited to move down (Supplementary Fig. S9). However, the migration patterns of sensitized and non-sensitized magnetic beads in gel separation are slightly different with different intercalating dyes and their concentrations. Especially, the effect of bis-intercalating dye groups on the mobility of non-sensitized magnetic beads was more prominent than that of mono-intercalating groups.

As the amplicon size increased, the mobility of the sensitized magnetic beads decreased. However, the mobility of the sensitized magnetic beads was not proportional to the size of amplicon. Evaluations using several sizes of PCR products at different concentrations further confirmed that SPIN-DNA could be used to accurately determine the presence of target DNA amplicon just within 2 min of centrifugation.

4.3. Performance evaluation with clinical samples and summary of the visual detection system

The concentration of HPV16 DNA must be at least 10^2 copies/mL to be detectable in the SPIN-DNA system. We evaluated the performance of the SPIN-DNA system for detecting HPV16 DNA in clinical samples. The results were in good agreement with those obtained using a conventional RQ-PCR kit (kappa statistic = 1.000). We observed the same results with different target DNA (IS6110, MTB standard DNA, 10^5 copies/μL, Supplementary Fig. S10).

In this study, we explored the feasibility of using SPIN-DNA as a DNA biosensor for detecting the target DNA sequence of HPV or MTB for potential applications in molecular diagnostics. We evaluated the performance of the SPIN-DNA system in comparison to the commercial HPV RQ-PCR kit. To the best of our knowledge, no previous studies have performed to detect amplicon visually by gel-based centrifugation. The visual detection method developed in this study does not require separation or color development steps, making it a suitable molecular POCT for the analysis of clinical samples. Moreover, nanoparticles other than magnetic beads can be used to transport the amplicon as long as the sedimentation conditions including density, viscosity, and centrifugal force are optimized.

Further studies that are beyond the scope of this work need to be performed, to clarify the mechanisms underlying the observed phenomenon. Since our visual detection system is now in a proof-of-concept state and lacking clinical evaluation for validation, further studies are needed for use in clinical diagnostic fields.

5. Conclusion

We developed a novel and simple, gel-based visual detection system and demonstrated its application to detect HPV DNA. In this study, we showed that the performance of our detection system using a simple gel column was comparable to that of conventional RQ-PCR. Improvements and optimization of this
system are challenges that merit further attention. It is a challenge to integrate sample preparation and processing and amplification on our detection system. Our visual detection system with naked eye is suitable and readily integrated on centrifugal microfluidic platforms and accelerate centrifugal microfluidic platform for use in resource-limited settings.

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.bios.2016.01.006](http://dx.doi.org/10.1016/j.bios.2016.01.006).

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