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*Original Article*

# A chemiluminescence based approach to nucleic acid testing to detect HBV, HCV, and HIV-1 in blood screening

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## ABSTRACT

Hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) are life-threatening blood-borne infections that can be unintentionally transmitted in transfused blood products. Here, we optimized a method for detecting HBV, HCV, and HIV-1 in blood screening using magnetic nanoparticles (MNPs) and polymerase chain reaction (PCR)-chemiluminescence. Viral genomes were extracted from donated blood samples using MNPs. The isolated viral DNA and RNA were amplified in a one-step parallel reverse transcription-PCR (RT-PCR). HBV, HCV, and HIV-1 were detected using complementary nucleic acid probes and quantified using a chemiluminescent substrate. 10 422 donated blood samples were tested both with the above method and Roche Cobas TaqScreen MPX Test. The lengths of the amplified PCR products for HBV, HCV, and HIV-1 were 119 bp, 220 bp, and 174 bp, respectively, indicating that the extraction methods successfully separated high-quality viral genomes from the serum samples. The probes and reaction conditions used for the chemiluminescent detection of HBV, HCV, and HIV-1 genomes in unknown samples were empirically optimized. Of the 10 422 blood samples screened, 12 were HBV positive and 2 were HCV positive. These results were consistent with those of a parallel-controlled study using Roche Cobas TaqScreen MPX Test. The assay described here is fast, accurate, and sensitive for detecting HBV, HCV, and HIV, which runs in parallel and has broad implications for blood screening and epidemiological studies.

**Keywords:** magnetic nanoparticle, chemiluminescence, HBV, HCV, HIV-1, genetic diagnosis

## INTRODUCTION

Advances in screening technology have improved the safety profiles of blood products used in transfusion medicine. However, there is still a risk of transmitting blood-borne pathogens. Studies have demonstrated that serologically negative donors who were infected with any of the three major transfusion-transmitted viruses (hepatitis B virus [HBV], hepatitis C virus [HCV], and human immunodeficiency virus

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[HIV-1]) could not be detected by serological screening[1]. Transfusion-associated virus transmission is primarily due to blood donation during the asymptomatic window early in infection. Blood containing viral contaminants can also pass screening tests due to low viral titer, viral variation, the formation of quasispecies, or operator error[2].

Blood is routinely screened using an enzyme immunoassay (EIA). However, there are inherent limitations in the EIA approach, resulting in some virus-positive samples being missed. The EIA approach relies on the immune responses elicited by the antigen and requires detectable high-titer antibodies that persist for a relatively long time. Therefore, EIA may miss infections in donors who produce low-quality or very few antibodies against a particular pathogen as a result of individual donor variation. The emergence of antigenic variants and rare viral subgroups can also be missed due to antigenic variation in the donor that may not be detected by the standard test (*e.g.*, low viral titer, virus variation, and quasispecies formation). Finally, there is a window period after infection and before seroconversion that limits detection[3]. For example, the average window period between infection and immune responses detectable by EIA for HBV, HCV, and HIV-1 is 56 days, 58 days, and 22 days, respectively[4–5]. Undetected infections during this window period can result in severe effects for transfusion recipients. Therefore, there is a clinical need for techniques to better detect virus infection in newly infected donors, thereby better preventing and controlling transfusion-transmitted viral infections.

Over the past twenty years, nucleic acid testing (NAT) has been widely applied to screen blood for transfusion. NAT detects the pathogen directly, and unlike EIA, is not dependent on the donor's immune response. Thus, the sensitivity and specificity of NAT are higher than those of EIA, and the window between infection and detection is shorter than the window required for EIA[6]. For example, the window period for detecting HBV, HCV, and HIV-1 is shortened to 14.9 days, 2.2 days, and 4.7 days, respectively[7]. This suggests that NAT effectively reduces the risk of transfusion-transmitted infections from these viruses. Reverse transcription-polymerase chain reaction (RT-PCR) is a common laboratory technique that uses reverse transcriptase (RT) to create cDNA from target RNA sequences, which are amplified *via* PCR. RT-PCR is either performed as a two-step or one-step assay. In a two-step assay, reverse transcription is performed first, followed by a separate PCR step. In a one-step assay, reverse transcription and PCR are performed in the same buffer using either a one or two enzyme system. One-step assay is faster, minimize the risk of sample contamination, and is simpler to be performed. Due to its simplicity and sensitivity, one-step RT-PCR is an important tool in nucleic acid research and the diagnosis of RNA-based infectious agents[8].

The primary method described here was previously established[9]. Therefore, this study aimed to optimize this method's ability to detect HBV, HCV, and HIV-1 in parallel utilizing a NAT technique that incorporates PCR-chemiluminescence[10–13]. This technique can be used to detect markers of infectious diseases in blood donors and patients, thereby reducing the risk of transfusion-associated infection.

## MATERIALS AND METHODS

### Blood samples

Blood samples were obtained from blood donors who provided informed consents at the Jiangsu Province Blood Center, Yangzhou Blood Center, and Suzhou Blood Center. 10 422 samples, which were negative for hepatitis B surface antigen (HBsAg), anti-HCV antibody, anti-HIV-1 antibody, and anti-Treponema pallidum (TP) antibody by EIA and had normal alanine aminotransferase (ALT) level, were included in this study to evaluate a new blood screening method.

The blood samples were screened for the presence of HBV, HCV, and HIV-1 in pools of eight donors. Donors were pooled for nucleic acid extraction, amplification, electrophoresis, and chemiluminescence detection. If a positive result was obtained, the pool was split, and the individual samples were tested.

The detection scheme was approved by the Medical Ethics Committee of Jiangsu Province Blood Center and registered in the Chinese Clinical Trial Registry (ChiCTR-DDT-14005151).

### Reagents and materials

Magnetic nanoparticles ($Fe_3O_4$@$SiO_2$, MNP) were obtained from the State Key Laboratory of Bioelectronics at Southeast University. Lysis and wash buffers were prepared in the laboratory. The PrimeScript One Step RT-PCR Kit was purchased from TAKARA Co., Ltd. (Beijing, China). The oligonucleotides were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). PCR reactions were performed using the 9902 system (Applied Biosystems, Waltham, USA). The chemiluminescent intensity was detected using a multi-function microwell plate reading machine (Enspire 2300 Multilabel Reader, Perkin Elmer, Waltham, USA).

Commercial blood screening kits were used as control (Cobas TaqScreen MPX Test, Roche, Basel, Switzerland).

### Nucleic acid extraction, PCR, hybridization, and chemiluminescence

The nucleic acid was extracted and purified, followed by PCR and electrophoresis. Then, hybridization and chemiluminescence were performed. These methods were conducted according to our previous study[9]. The primers were designed by Oligo 6.0. Mega 6 alignment tools were used to align major HBV, HCV, and HIV genotypes, and conserved region on HBV X gene, HCV 5' untranslated region, and HIV *gag* gene was selected to design the primers respectively. The PCR primers used are provided in ***Table 1***. ***Fig. 1*** illustrates the procedure and mechanism of nucleic acid isolation. ***Fig. 2*** showes the graphical presentation of the hybridization and chemiluminescence.

***Table 1*** **PCR primers used in this study**

| Target gene | Amplified fragments (bp) | Sequences |
|---|---|---|
| HBV X gene | 119 | F: 5'-TGC ACT TCG CTT CAC CTC T-3' |
| | | R: 5'-CAA GGT CGG TCG TTG ACA TT-3' |
| | | P1: 5'-AGC AAT GTC AAC GAC CGA CC-(15 t)-$NH_2$-3' |
| | | P2: 5'-AGG ACT CTT GGA CTC TCA-(15 t)-$NH_2$-3' |
| | | P3: 5'-GG ACT CTT GGA CT-(15 t)-$NH_2$-3' |
| | | P4: 5'-GCA GAG GTG AAG CGA AGT G-(15 t)-$NH_2$-3' |
| | | P5: 5'-GGT GGG CGT TCA CGG TGG-(15 t)-$NH_2$-3' |
| HCV 5' UTR | 220 | F: 5'-GTA TGA GTG TCG TGC AGC CT-3' |
| | | R: 5'-CAC TCG CAA GCA CCC TAT CA-3' |
| | | P6: 5'-ACT ATG GCT CTC-(15 t)-$NH_2$-3' |
| | | P7: 5'-CTG ATA GGG TGC TTG CGA T-(15 t)-$NH_2$-3' |
| | | P8: 5'-CTT GTG GTA CTG CCT GAT A-(15 t)-$NH_2$-3' |
| | | P9: 5'-TTC CGC AGA CCA CTA TGG CTC T-(15 t)-$NH_2$-3' |
| HIV-1 *gag* gene | 174 | F: 5'-AGG GGA AGT GAC ATA GCA GG-3' |
| | | R: 5'-TTC GCC CTT GTC TTA TGT CCA-3' |
| | | P10: 5'-ATA GTA AGA ATG TAT AGC CC-(15 t)-$NH_2$-3' |

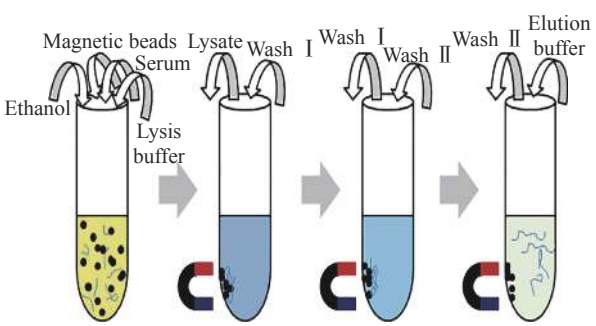


***Fig. 1*** **Schematic presentation of magnetic beads based nucleic acid extraction.**

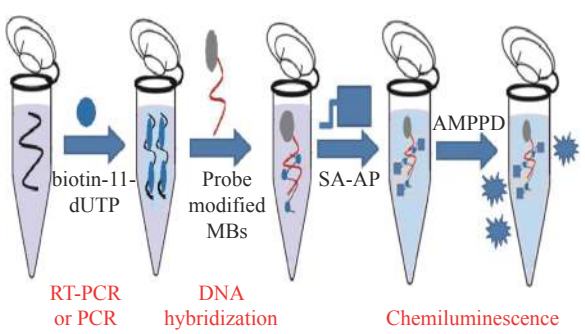


***Fig. 2*** **Graphical presentation of chemiluminescent detection procedure.**

### Probe optimization

Five HBV probes, four HCV probes, and one HIV-1 probe based on the conserved regions to capture the complementary biotin-labeled amplified product were designed. Only one probe was selected for the study based on empirical results. The reaction conditions were empirically determined using different concentrations of the probe and different hybridization times to optimize chemiluminescence. Each group was set up with triple duplicate wells ($n$=3).

### Sensitivity test

High concentrations of HCV and HIV-1 were added to different concentrations of HBV to detect the sensitivity of the assay. A sensitivity test was performed for HCV and HIV-1 according to the above method.

### Sample screening

The 10 422 blood samples were screened *via* the optimized method. In contrast with the commercial kits, these samples were also screened with Roche Cobas TaqScreen MPX Test according to the manufacturer's instructions.

### Statistical analysis

The data were expressed as mean ± SD. A Student's *t*-test was used to determine whether differences were significant in multiple comparisons. $P<0.01$ was considered statistically significant. The data were analyzed using SPSS 13.0 software.

## RESULTS

### Nucleic acid extraction, PCR, and gel electrophoresis

The average concentration of nucleic acid isolated was (282.72±188.94) ng/μL with an average A260/280 ratio of (1.77±0.11). PCR was performed to confirm that the nucleic acids were with high-quality for use in downstream applications. A representative image of the PCR products obtained for the HBV DNA is shown in ***Fig. 3A***. Lanes 1 and 2 show the positive and negative controls, while lanes 3 and 4 show blood samples collected for this study. The length of the PCR product was 119 bp. Two similar representative images are shown for HCV RNA and HIV RNA in ***Fig. 3B–C***, respectively. The lengths of PCR products were 220 bp and 174 bp, respectively, indicating successful HCV and HIV amplification. These results indicated that high-quality nucleic acids were isolated from the blood samples.

### Probe optimization

The intensity of the chemiluminescent signal was strongly dependent on the efficiency with which the probe captured the biotinylated target segment. The efficiency of the HBV probe was tested over a range of temperatures (***Fig. 4A***). Probe binding to the PCR amplicon was optimal at 45 ℃, and probe 1 (P1) and probe 5 (P5) were the most efficient binders. Therefore, these probes were used in the specificity test. The most intense chemiluminescence in the HBV sample was observed with P1; however, P1 was also strongly luminescent in the blank sample (***Fig. 4B***). In contrast, P5 was strongly luminescent in the HBV sample but not the blank. Therefore, P5 was further optimized. Chemiluminescence was tested over a range of probe concentrations (***Fig. 4C***) and hybridization times (***Fig. 4D***). Groups 5, 10, 15, and 20 had significant difference over groups 0.5 and 1 (***Fig. 4C***). Group 30 had significant difference over group 10 (***Fig. 4D***). The optimal probe concentration was 5 μmol/L, and 30 minutes was optimal for the hybridization reaction.

Similar optimization experiments were performed for the HCV probes testing the optimal temperature (***Fig. 5A***), binding specificity (***Fig. 5B***), probe concentration (***Fig. 5C***), and hybridization time (***Fig. 5D***). Groups 1, 5, 10, 15, and 20 had significant difference over group 0.5 (***Fig. 5C***). Groups 30, 40, and 50 had significant difference over group 10 (***Fig. 5D***). Probe 9 (P9) was found to be the best probe; the optimal concentration was 5 μmol/L; and the optimal hybridization time was 30 minutes.

There was only one HIV-1 probe, so the reaction conditions were optimized for the available probe. Group 35 had significant difference over groups 30, 40, 45, 50, and 55 (***Fig. 6A***). Groups 5, 10, 15, and 20 had significant difference over groups 0.5 and 1 (***Fig. 6C***). Groups 30 and 40 had significant difference over group 10 (***Fig. 6D***). The optimum temperature of hybridization was 35 ℃ (***Fig. 6A***); the probe was highly specific (***Fig. 6B***); the optimal concentration was 5 μmol/L (***Fig. 6C***); and the optimal hybridization time was 30 minutes (***Fig. 6D***).

### Sensitivity testing

The performance efficiencies of the HBV, HCV, and HIV-1 primers and the limit of chemilumi-

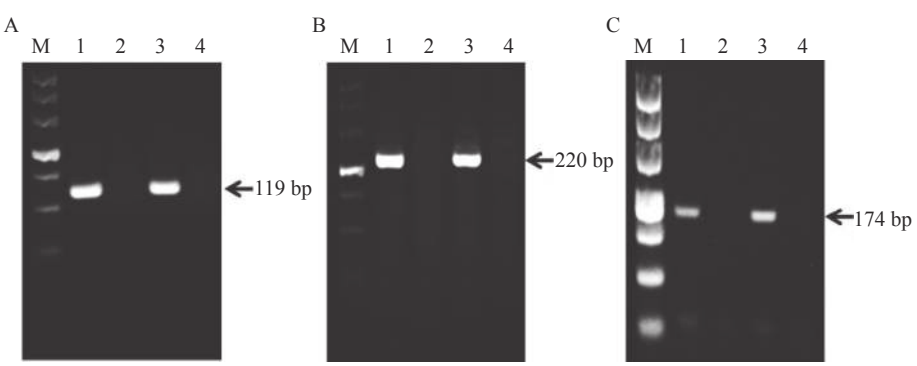


***Fig. 3*** **Representative electrophoresis results from the PCR amplification of HBV and HCV genomes.** A (HBV DNA), B (HCV RNA), and C (HIV-1 RNA) were amplified using specific primers from serum samples. M: Marker; Lane 1: positive control; Lane 2: negative control; Lane 3: representative positive serum sample; Lane 4: representative negative serum sample.

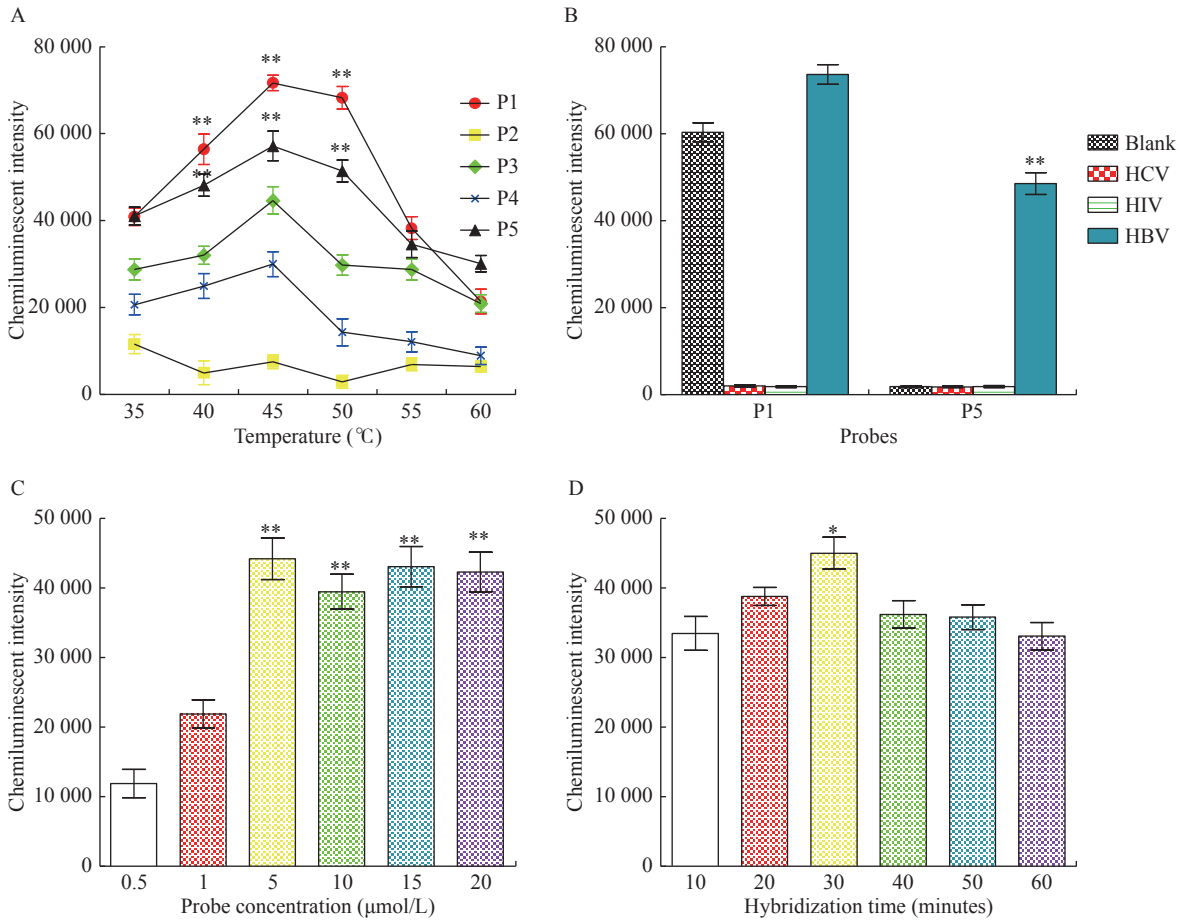


***Fig. 4* Optimization of probes used for chemiluminescent detection of HBV DNA in unknown blood samples.** A: Five HBV specific probes were tested for their ability to bind target HBV sequences. ***P*<0.01, compared with the P2, P3, and P4 groups. B: P1 and P5 were tested for HBV specificity in mixed reactions with known quantities of HCV and HIV-1 nucleic acids. ***P*<0.01, compared with the blank, HCV, and HIV groups. C: Relationship between probe concentration and chemiluminescence intensity. ***P*<0.01, compared with groups 0.5 and 1. D: Relationship between hybridization time and chemiluminescence intensity. **P*<0.05, compared with group 10.

nescence detection (LoCD) were assessed in serum in the presence of the other two viruses at a much higher viral load (***Table 2***). The results of the sensitivity tests are shown in ***Fig. 7***. The assay detected as few as 10 copies/mL of HBV and HCV, and 100 copies/mL of HIV-1.

### Sample screening

Of the 10 422 blood samples, 12 were HBV positive and 2 were HCV positive. The chemiluminescent signals from the HBV-positive and HCV-positive sample pools were similar to the positive control, but significantly stronger than the negative control and blank control. These results coincided with Roche Cobas TaqScreen MPX Test (***Table 3***).

## DISCUSSION

This study described an effective chemiluminescence-based PCR assay for the parallel detection of HBV, HCV, and HIV-1 in donated blood. The MNP based method for extraction of DNA had unique advantages. Compared with traditional nucleic acid extraction protocols which use organic solvents or several ultracentrifuge steps, the MNP based method had received great attention and significant interest due to its convenient manipulation, low cost, and potential for automation[9, 14–15]. Chemiluminescence was a heterogeneous immunoassay that can combine the antigen and antibody effectively. It had several advantages compared to EIA. Chemiluminescence was not susceptible to the hook effect seen in some immunoassays, nonspecific reactions, or false positive reactions caused by cross-contamination. Chemiluminescence was also stable, highly accurate, and controllable[16], and the assay sensitivity was increased due to the fact that the chemiluminescent intensity wasn't reduced by the combination of chemilumi-

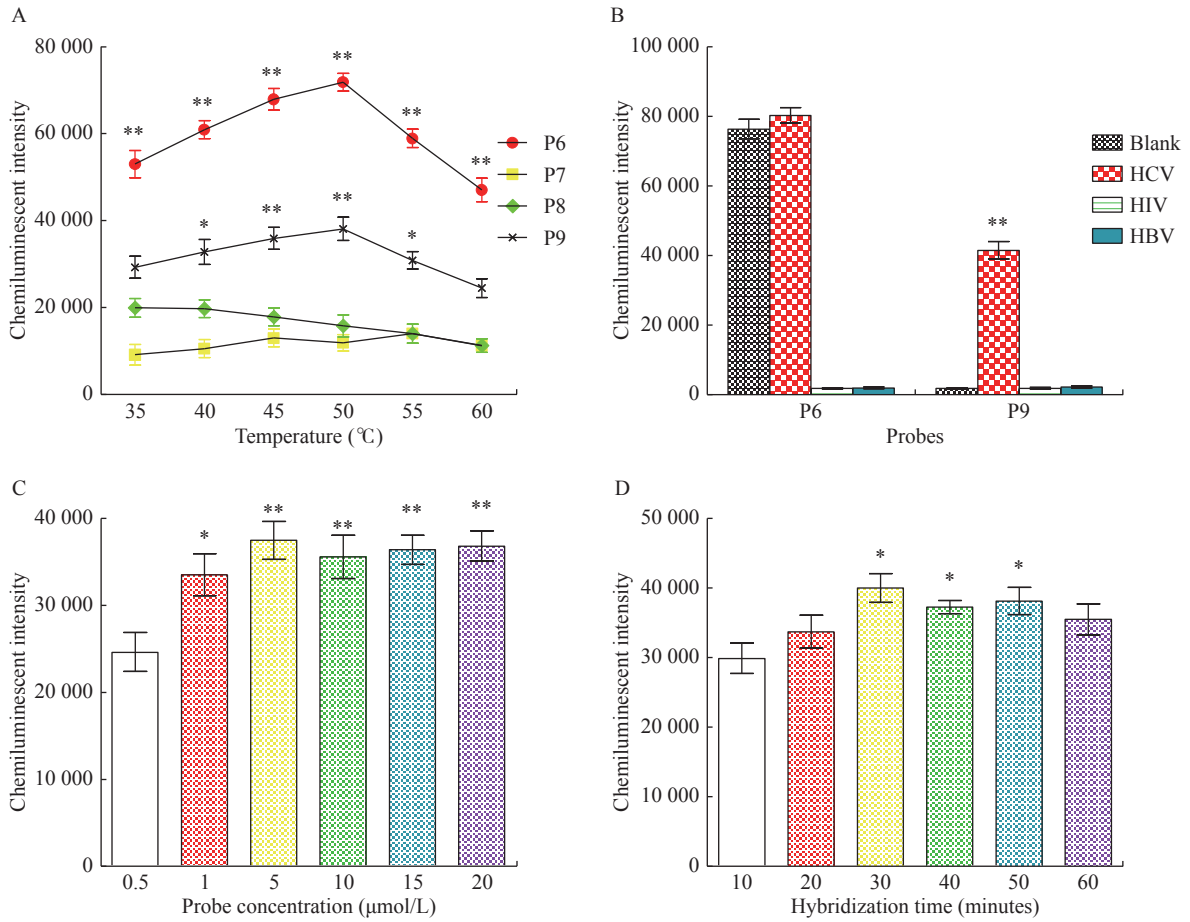


***Fig. 5* Optimization of probes used for chemiluminescent detection of HCV RNA in unknown blood samples.** A: Four HCV specific probes were tested for their ability to bind target HCV sequences. **P*<0.05, compared with the P7 and P8 groups; ***P*<0.01, compared with the P7 and P8 groups. B: P6 and P9 were tested for HCV specificity in mixed reactions with known quantities of HBV and HIV-1 nucleic acids. ***P*<0.01, compared with the blank, HBV, and HIV groups. C: Relationship between probe concentration and chemiluminescence intensity. **P*<0.05, compared with group 0.5; ***P*<0.01, compared with group 0.5. D: Relationship between hybridization time and chemiluminescence intensity. **P*<0.05, compared with group 10.

nescent substance and macromolecule[17]. Available NAT kits worldwide for blood screening were primarily based on fluorescent PCR and TMA. This assay combined high sensitivity and specificity to optimize the method for parallel detection of these viruses and has potentially broad implications for blood screening.

Many parameters could impact the final chemiluminescence output. One of the most important was the probe. HBV and HCV probes performed differently in terms of chemiluminescent detection. This might be attributable to the position of the probe on the target segment, the length of the probe, or the GC content. Therefore, these parameters should be taken into consideration when designing probes for chemiluminescence-based assays. P1 and P6 produced considerable chemiluminescent signals in the blank sample. These probes were positioned on the primer binding region of the PCR product. They wouldn't offer any specificity. If there was any amplification by primers during PCR (either mispriming or primer dimer formation), it would be detected by the probe. Therefore, it could be inferred that there was primer dimer formation to some level, which was detected by both P1 and P6 probes when blank samples were tested. For HIV, a probe which matched the well-conserved sequences among different HIV-1 genotypes was designed. When MNPs were modified with less than 5 μmol/L of the probe, the chemiluminescent signal was very low, likely because there was insufficient probe available to capture the PCR-amplified target sequences. However, minimal changes in chemiluminescence were observed with more than 5 μmol/L of the probe. The chemiluminescent signal decreased when the reaction was incubated for longer than 30 minutes. The loss of signal might be attributable to the loss of some MNPs as the reaction was monitored every 10 minutes to determine target-

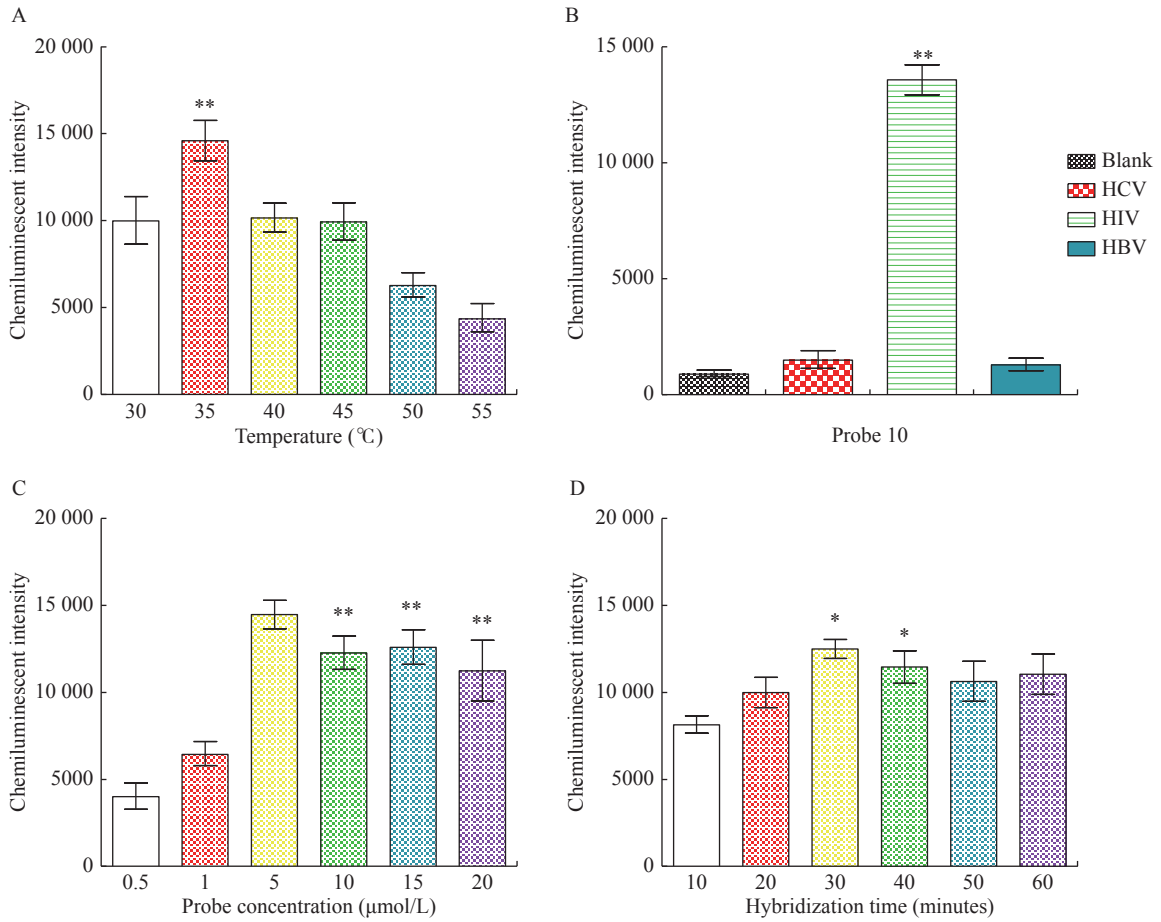


***Fig. 6* Optimization of probes used for chemiluminescent detection of HIV-1 RNA in unknown blood samples.** A: One HIV-1 specific probe was tested for the ability to bind target HIV-1 sequences. ***P*<0.01, compared with the rest of the other 5 groups. B: Probe specificity was tested in mixed reactions with known quantities of HCV and HBV nucleic acids. ***P*<0.01, compared with the blank, HBV, and HCV groups. C: Relationship between probe concentration and chemiluminescent intensity. ***P*<0.01, compared with groups 0.5 and 1. D: Relationship between hybridization time and chemiluminescent intensity. **P*<0.05, compared with group 10.

***Table 2* Viral genome copy numbers used to determine sensitivity in mixed reactions**

| Sample ID | HBV (copies/mL) | HCV (copies/mL) | HIV-1 (copies/mL) |
|---|---|---|---|
| A1 | 10 | 100 000 | 100 000 |
| A2 | 100 | 100 000 | 100 000 |
| A3 | 1000 | 100 000 | 100 000 |
| A4 | 10 000 | 100 000 | 100 000 |
| B1 | 100 000 | 10 | 100 000 |
| B2 | 100 000 | 100 | 100 000 |
| B3 | 100 000 | 1000 | 100 000 |
| B4 | 100 000 | 10 000 | 100 000 |
| C1 | 100 000 | 100 000 | 10 |
| C2 | 100 000 | 100 000 | 100 |
| C3 | 100 000 | 100 000 | 1000 |
| C4 | 100 000 | 100 000 | 10 000 |

probe hybridization.

The development of NAT that can detect viral infection early after infection is important for diagnostic research. In the present study, the screening results were consistent with those obtained using Roche Cobas TaqScreen MPX Test. This suggests that the assay is efficient and can be further optimized to reduce detection cost.

In summary, the method for parallel detection of HBV, HCV, and HIV-1 based on magnetic

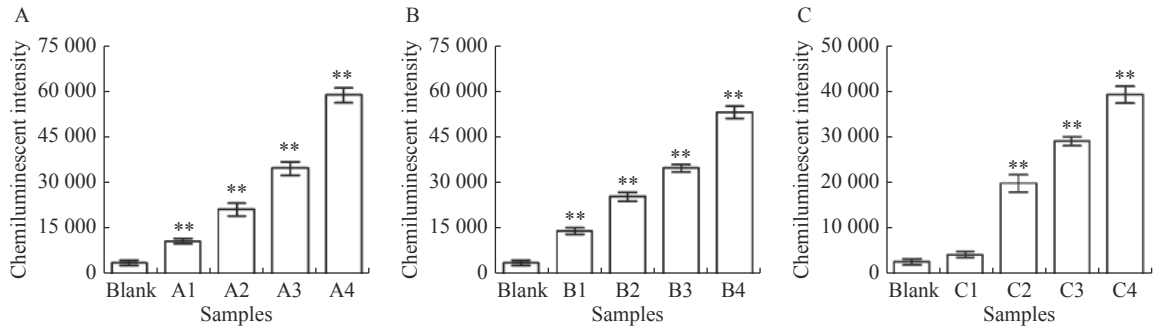


***Fig. 7* Sensitivity tests for HBV, HCV, and HIV-1 detection.** A: Sensitivity test for HBV detection in parallel PCR. B: Sensitivity test for HCV detection in parallel PCR. C: Sensitivity test for HIV-1 detection in parallel PCR. The sensitivity for HBV (A), HCV (B), and HIV (C) was determined in different ratios of standard serum dilutions as shown in *Table 2*. ***P*<0.01, compared with blank groups.

***Table 3*** **Comparison of sample screening in our protocol and Roche Cobas TaqScreen MPX Test**

| Method | HBV positive samples | HCV positive samples | HIV positive samples |
|---|---|---|---|
| Our protocol | 12 | 2 | / |
| Roche Cobas TaqScreen MPX Test | 12 | 2 | / |

The samples were the same for both methods.

nanoparticles and PCR-chemiluminescence was optimized. This method is highly sensitive, specific, and fast, making it suitable for large-scale blood screening. It provides an alternative for analyzing clinical samples in blood screening. However, since this is a preliminary study, further research with larger sample sizes is needed[18].

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