

Array-based Nano-amplification Technique Was Applied in Detection of Hepatitis E Virus

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A rapid method for the detection of Hepatitis E Virus (HEV) was developed by utilizing nano-gold labeled oligonucleotide probes, silver stain enhancement and the microarray technique. The 5'-end -NH₂ modified oligonucleotide probes were immobilized on the surface of the chip base as the capture probe. The detection probe was made of the 3'-end -SH modified oligonucleotide probe and nano-gold colloid. The optimal concentrations of these two probes were determined. To test the detection sensitivity and specificity of this technique, a conservative fragment of the virus RNA was amplified by the RT-PCR/PCR one step amplification. The cDNA was hybridized with the capture probes and the detection probes on microarray. The detection signal was amplified by silver stain enhancement and could be identified by naked eyes. 100 fM of amplicon could be detected out on the microarray. As the results, preparation of nano-gold was improved and faster. Development time also was shortened to 2 min. Thus, considering high efficiency, low cost, good specificity and high sensitivity, this technique is alternative for the detection of HEV.

Keywords: HEV, Microarray, Nano-gold, Sensitivity, Visual detection

Introduction

Hepatitis E is a non-A and non-B hepatitis, which is caused by hepatitis E virus (HEV). The acute forms of hepatitis and large-scale epidemics are its basic characters. The first outbreak of hepatitis E happened in New Delhi during 1955~1956, when 97,000 persons were infected and 29,300 persons suffered from jaundice. Recently it is confirmed HEV

was responsible for decompensation of chronic liver disease (Kumar *et al.*, 2004). Thus, detection of HEV was attracting increasing attention. Until now, many related detection techniques have been established. They can be divided into two categories: Immuno-reaction and gene detection. The former method is to detect anti-HEV IgA, IgM and IgG by western blot, RIA (radioimmunoassay), RIBA (radioimmuno-binding assay), immunochromatographic test and so on (Li *et al.*, 1994; Ha *et al.*, 2004; Chen *et al.*, 2005; Hurtado *et al.*, 2005; Takahashi *et al.*, 2005). The latter is the rapidly developed molecular biological detection method, such as reverse transcription polymerase chain reaction (RT-PCR) (Huang *et al.*, 2002), nest-RT-PCR (Choi *et al.*, 2004), real-time quantitative PCR (Orri *et al.*, 2004) and FQ-RT-PCR (fluorescence labeled quantitative RT-PCR) (Jameel *et al.*, 1992). But there remains a need to develop an efficient detection method for HEV gene with high sensitivity, high speed and low cost.

Microarray as a novel biological technique has been used in gene detection widely. Good stability, high throughput and good sensitivity are its impressive advantages. But this method needs improvement, considering expensive probe labeling material and valuable signal scanner. So a visual detection technique was established by applying sandwich hybridization and silver stain (Taton *et al.*, 2000). This technique was 100 times as sensitive as LCS (laser confocal scanning) and made it possible to develop a rapid and cheap detection method for HEV.

Increasing studies revealed the application of gold nanoparticle as oligonucleotide label was wide (Patolsky *et al.*, 2000; Hong *et al.*, 2002; Urban *et al.*, 2003). And further development of gold-DNA based detection methods were performed by Mirkin and co-workers (Elghanian *et al.*, 1997; Taton *et al.*, 2000; Taton *et al.*, 2001; Park *et al.*, 2002). Cumulative evidences showed gold labeled probe was efficient and could be alternative marker in microarray assay. On the other hand, the silver stain enhancement, a technique companied with nano-gold labeling oligonucleotide, was used to amplify detection signal of nano-gold (Alexandre *et al.*, 2001; Csaki *et*

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al., 2003). This technique made detection signal visual (Taton *et al.*, 2000). And its sensitivity of gene detection was approximately 1000-fold high as that of Cy3-based fluorescence (James *et al.*, 2004).

Employing nano-gold label, silver enhancement and sandwich hybridization, we previously built a simultaneous detection method of Hepatitis B virus and Hepatitis C Virus (Wang *et al.*, 2003). Recently, Hepatitis A Virus (HAV) detection technique was established by us too (Wan *et al.*, 2005). Based on these work and with some improvements, the detection technique of Hepatitis E Virus (HEV) was built.

Materials and Methods

Materials of HEV detection microarray. Used materials were described as follows: Silanizing coupling reagent --- γ -aminopropyltriethoxysilane (APTES) came from Organosilicon Engineering Research Center of MOE (Wuhan). 1, 4-phenylene diisothiocyanate (PDITC, Aldrich), M-MLV reverse transcriptase (Biostar Com.), Taq DNA polymerase, uracil-DNA glycosylase (UDG, Gene Co. Ltd.) and dNTPs (dATP + dGTP + dCTP + dTTP + dUTP + Promega) were used. Other chemicals and biological reagents were analytical grade reagent (AR). Reagents and the glassware were prepared as previous work (Wang *et al.*, 2003). All solutions were added with 0.1% diethyl pyrocarbonate (DEPC, Sigma). The glasswares were pre-cleaned by immersing in aqua regia (HCl:HNO₃ = 3:1) followed by rinsing with deionized water and then dried at 180°C for 8 h. Finally, all Ep tubes and tips were sterilized under 121°C for 30 min.

The primers and probes for HEV gene were designed with software called DNAsis, which is based on HEV genome sequence in GenBank (Table 1). Used positive serum samples of hepatitis E and negative samples, which had been confirmed by ELISA, were collected from Wuhan No. 7 hospital, the Epidermal Disease hospital and the Zhongnan hospital (Wuhan).

The traditional development solution containing gelatine was prepared as pioneering work (Wan *et al.*, 2005). 60 ml of 1% gelatine solution, 10 ml of citric acid buffer (pH 3.5) and 1.7 g of hydroquinone dissolved in 30 ml water were filtrated respectively and mixed prior to use. Then, 50 mg of AgNO₃ dissolved in 2 ml of water was added to the mixed solution. The freshly prepared

solution was kept away from light. Citric acid buffer (pH 3.5) was obtained by dissolving 2.55 g citric acid (C₆H₈O₇ · H₂O) and 2.35 g sodium citrate (C₆H₅Na₃O₇ · H₂O) in 100 ml distilled water. The other silver development solution contained all of the components except for gelatine.

Amplification of HEV gene. The RT-PCR/PCR one-step amplification was created to amplify the gene fragments of HEV RNA. 100 μ l of Serum was mixed with protein denaturalization solution (1% SDS, 2% NP-40, 1% Tuwen-20, PBS pH 7.5 and DEPC). The mixed solution was boiled, following centrifugation. The supernatant as PCR template was added into PCR system (10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 200 μ M dNTPs, 20 pmol A1 and A2, 10 units of *Taq* DNA polymerase). PCR amplification was carried out in 100 μ l reaction mixture. The first step of PCR was incubation at 37°C for 15 min. Then Following denaturalization for 4 min at 94°C, 35 amplification circles of PCR (94°C 45 s, 45°C 45 s, 72°C 45 s) were performed. The last step of PCR was 5-min incubation at 72°C. The result was determined by electrophoresis assay.

Preparation for nano-Au, the detection probes and the capture probes. The method for preparation of gold nano-particles described by Grabar (Grabar *et al.*, 1995) was used in our experiment and some improvements were performed. Glasswares were soaked in aqua regia, washed with deionized water and then dried. 41.2 mg of HAuCl₄ · 4H₂O was dissolved in 100 ml of triple-distilled water and then the solution was boiled in microwave, to which 10 ml of 38.8 mM sodium citrate solution was added while stirring. Then, the mixture was boiled for 15 min in microwave. After cooling to room temperature, the solution was recovered to the original volume with distilled water and then filtrated with 0.45 μ m nylon film. The filtrate was desired gold nano-particles.

The immobilization of capture probe and the preparations of detection probe were carried out as description in previous work (Wang *et al.*, 2003). 50 μ l of Different concentrations (3.4 \times 10⁻⁵ M, 3.4 \times 10⁻⁶ M, 3.4 \times 10⁻⁷ M, 3.4 \times 10⁻⁸ M) of SH-modified probe were added to 1 ml of 0.01% nano-gold solution. After 16 h, 260 μ l of 5% BSA was added to the above system and then the solution was kept at 4°C for 10 min.

To determine the optimal concentration of the capture probe, The original solution of amino-modified probe (3.6 \times 10⁻⁵ M) (the capture probe) was diluted respectively at 10¹, 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷ times. Based on previous conclusion that the best concentration

Table 1. Sequence of primers and probes

	Sequence
RT-PCR primers E1	5'-CATGGTCGAGAAGGGCCAGG-3'
RT-PCR primers E2	5'-GCGGAAATCATAACAGTGGG-3'
Capture probe of HEV (P1)	5'-H ₂ N-(CH ₂) ₆ -O-(PO ₃)-AAGCTT-CATGGTCGAGAAGGGCCAGG-3'
Detection probe of HEV (S1)	5'-GGGTGACAATACTGAAGGCGGGATCCAAAA-(CH ₂) ₆ -SH-3'
The primers of positive control Y1	5'-GGAATTCATGAAGGATTATGTAATGGAA
The primers of positive control Y2	5'-ACGGTACAGCCGTTGATCTGGCTATG
Capture probe of positive control (P2)	5'-H ₂ N-(CH ₂) ₆ -O-(PO ₃)-CTTTAAACATCTCCCTGAACCGTTCCGCATTTCG-3'
Detection probe of positive control (S2)	5'-CGTTCTCTAACTATTTCTTTGATACCACGCAGG-SH-3'
Capture probe of negative control:	5'-H ₂ N-(CH ₂) ₆ -O-(PO ₃)-(T) ₁₀ -GAAGCTTTTAAACTTCTTTAAGTTTTCGCG-3'
Detection probe of negative control:	5'-GCTCAGTTTACTAGTCCATTTG-(T) ₁₀ -SH-3'

of 3'-sulfhydryl-oligonucleotide probe might be 3.4×10^{-7} M or 3.4×10^{-8} M (Wang *et al.*, 2003), the gold-labeled probes (the detection probe) made of 0.01% nano-gold particle colloid and 3'-sulfhydryl-oligonucleotide probe (3.4×10^{-7} M) was used as the detection probe in this experiment.

Preparation of detection microarray for HEV. The preparation was based on our previous work (Wang *et al.*, 2003). Glass slides for microarray were washed in hot concentrated HNO_3 for 10 min. After dried, the slides were soaked for 2 h in the APTES solution (distilled water : acetone : APTES = 15 : 280 : 6). Then they were rinsed with acetone and subsequently dried. The slides were treated by 0.2% of PDITC solution. At last, these slides were rinsed and dried before use. The synthesized amino-modified HEV probes were heated in boiling water and rapidly cooled. Then, 1 μl of the pretreated single-stranded probe samples were dropped onto the pretreated glass slides. The slides with the DNA probe array were kept in a moist environment at 37°C for 2 h and then rinsed with distilled water, blocked with salmon sperm DNA (1%) for 10 min. Finally, the prepared microarray was stored at $4\text{--}8^\circ\text{C}$.

Hybridization and staining. A rapid detection strategy for HEV gene diagnosis was designed by using nano-gold labeling, sandwich hybridization and silver staining enhancement. In this strategy, target cDNA was the RT-PCR/PCR product for HEV. The HEV capture probes were spotted on glass as design. Subsequently, 100 ml of HEV amplicon was heated at 98°C for 5 min and added to hybridization solution (30 mM NaCl, 1 mM PBS (pH7.0)) with the gold-labeled HEV-specific probes. The mixture was cooled quickly and poured on the microarray. The hybridization on the microarray was performed at 50°C for 10 min. After hybridization, the chip was rinsed and soaked in 0.2 M pH 3.5 citric acid buffer. Following development, the color on the chip was observed by the naked eye or a standard flatbed scanner.

Results and Discussions

Preparation of nano-gold particles. Nano-gold labeling was good label for microarray technique. Its cost was less than fluorescence label (Alexander *et al.*, 2001). So nano-gold label was used widely. In our previous works, preparation of nano-gold was key factor to affect the application of the detection microarray. Based on these improvements, the preparation approach of nano-gold was shortened from 56 h to 17 h. Due to microwave, these nano-gold particles still had good size distribution (14 ± 2 nm) and stable maximum absorbance at 520 nm. Furthermore, there was no deposition in prepared nano-gold solution at 4°C in 60 days. It was speculated this development was due to using BSA to stabilize nano-gold particles. The longer preservation than Mirkin's method (Taton *et al.* 2000) provided more changes for clinical application of HEV detection microarray.

Determining optimal concentrations of capture probe and detection probe of HEV. In original experiment, the result obtained showed the optimal concentration of the capture

probes was 3.6×10^{-9} M (Fig. 4). But there was less difference between 3.6×10^{-8} M and 3.6×10^{-9} M of probe. When the concentrations were lower than 3.6×10^{-9} M, higher concentrations of probes produced darker stains; 3.4×10^{-7} M or higher concentration had a negative correlativity with the stains. And the lowest of workable concentrations of the capture probe was 3.6×10^{-12} M.

To determine the optimal concentration of the detection probe of HEV, the gold-labeled probes (the detection probe) were prepared with 0.01% gold nano-particle colloid and the 3'-sulfhydryl-modified oligonucleotide probes, of which the concentrations were 3.4×10^{-5} M, 3.4×10^{-6} M, 3.4×10^{-7} M, 3.4×10^{-8} M respectively. These prepared probes were used to hybridize and stain. The result of developing speed showed the speed of the gold-labeled probe made with 3.4×10^{-7} M 3'-sulfhydryl-oligonucleotide probes was the fastest and that of the gold-labeled probe made with 3.4×10^{-8} M 3'-sulfhydryl-oligonucleotide probes was the slowest (Fig. 5). The result of 10 min-developing indicated that the intensity of the spots in the microarray, using the gold-labeled probe made with 3.4×10^{-8} M 3'-sulfhydryl-oligonucleotide probes, was lower. For 10 min-development, there was no difference among other detection probes. It was confirmed that the best concentration of 3'-sulfhydryl-oligonucleotide probes used to prepare for gold-tagged probes was 3.4×10^{-7} M.

In our laboratory, a detection platform for hepatitis virus had been built by using sandwich hybridization, nano-gold labeling and silver stain enhancement (Wang *et al.*, 2003; Wang *et al.*, 2004; Wan *et al.*, 2005). Considering its good specificity, high sensitivity and less cost, this technique was applied to detect HEV in serum. Optimal concentrations of labeling nano-gold, capture probe and detection probe for the detection of HEV were determined. These optimal concentrations of probes of HEV were different with those of HAV (hepatitis A virus), HBV (hepatitis B virus) and HCV (hepatitis C virus). These differences were speculated to attribute to the character of probe. The probe with higher T_m often had less optimal concentration in microarray assay. But there remained other unknown factors influencing the optimal concentration of the probe.

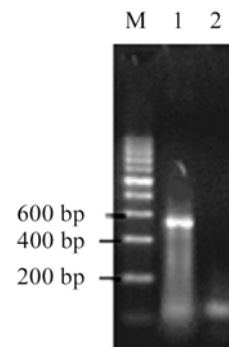


Fig. 1. Agarose gel electrophoresis analysis of HEV RT-PCR product. Lane 1 is DNA marker; Lane 2 shows the prospective 500 bp band; Lane 3 is negative control.

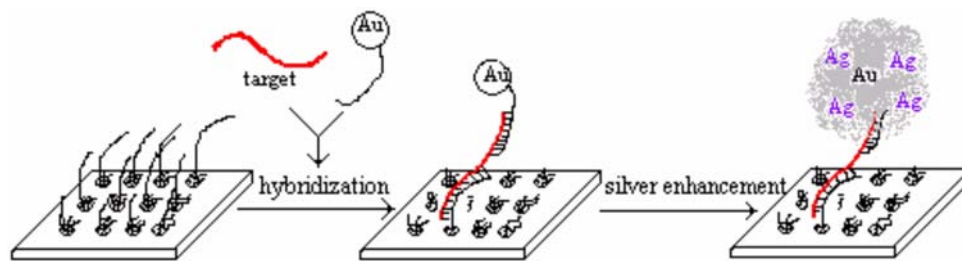


Fig. 2. Schematic presentation of nano-gold labeled DNA probes/silver stain enhancement method.

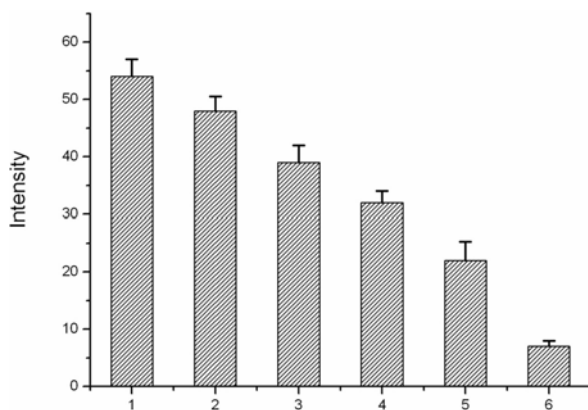


Fig. 3. Intensities of different concentrations of HEV amplicons on the detection microarray. The concentrations of used HEV amplicons as follows: 1) 10,000 fM; 2) 5,000 fM; 3) 1,000 fM; 4) 500 fM; 5) 100 fM; 6) 50 fM.

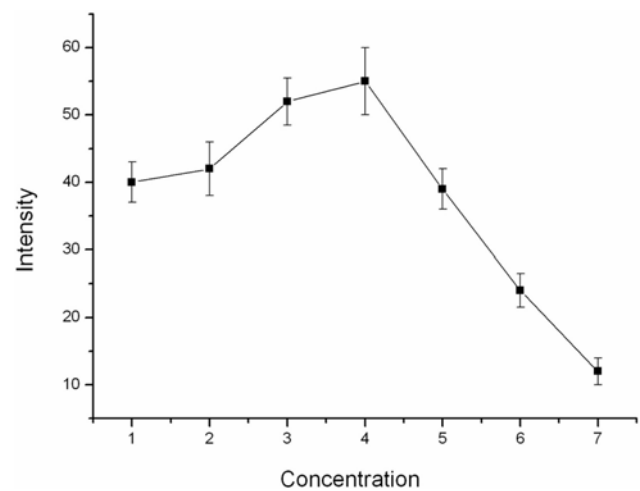


Fig. 4. The intensity of different concentrations of capture probes. The numbers 1,2,3,4,5,6,7 represent the concentration of 3.6×10^{-6} M, 3.6×10^{-7} M, 3.6×10^{-8} M, 3.6×10^{-9} M, 3.6×10^{-10} M, 3.6×10^{-11} M, 3.6×10^{-12} M respectively.

Amplification of HEV gene and determining sensitivity of the micorarray. As the result of the RT-PCR/PCR one step amplification, an expected 500 bp band appeared on a 1.5% agarose gel (Fig. 1). To test the sensitivity of this microarray, different concentrations of HEV amplicons ranging from 50 fM~10,000 fM were used on separate microarrays. A similar result to our previous work (Wan *et al.*, 2005) showed signal intensities were enhancing with the increasing of HEV amplicon concentrations (Fig. 3). 100 fM or higher concentrations of HEV amplicons had obvious detection signals.

In this HEV detection technique, this simpler approach of the RT-PCR/PCR one step amplification resulted in less contamination of PCR product and a faster operation for detection. This RT-PCR-based amplification had similar sensitivity and specificity to traditional RT-PCR. But due to the deposition of a great deal of silver particles on the surface of nano-gold particles of the detection probe (Nano-amplification technique) (Fig. 2), the detection signal was amplified for several hundred times when HEV was present. It suggested this technique had lower detection limitation than other RT-PCR-based detection technique. In quantity experiment, 100 fM of amplicon could be detected (Fig. 3). It suggested one copy of HEV in 1 l (liter) serum could be detected out. On the other hand, using nano-gold labeled probe and sliver stain enhancement linked to DNA

hybridization, the rapid detection technique for hepatitis virus exhibited high specificity as well as reproducibility, similar to those of quantitative fluorescence detection technique (Wang *et al.*, 2004). These made it possible the positive result would be obtained when HEV appeared in serum with no symptom of acute hepatitis E.

Improvement of development solution. The comparison of two kinds of development solutions showed presence of gelatin made developing approach longer. Thus, to increase detection speed of microarray, gelatin was gotten rid of the component list of the development solution. The developing speed of detection was accelerated from 10~15 min (with gelatin) to 2~3 min (without gelatin). The intensity discriminations between positive detection spots and negative detection spots were the most notable after 2-min development. Passing 9 min of development, the intensity of spots of samples, controls and the background would otherwise become hard to discriminate (Fig. 6). This quick development made the detection microarray of HEV easier to be applied. But the related mechanism remains unclear.

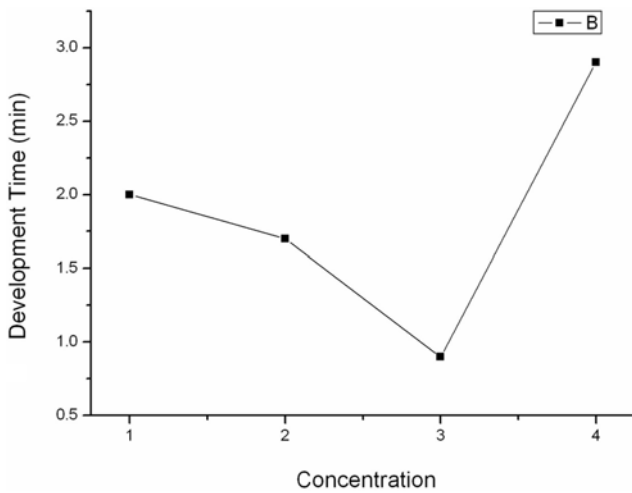


Fig. 5. Development time of the detection probes made of different concentrations of 3'-sulfhydry-oligonucleotide probes and 0.01% nano-gold particle colloid. The numbers 1,2,3,4, represent the detection probes made of the concentrations of 3.4×10^{-5} M, 3.4×10^{-6} M, 3.4×10^{-7} M, 3.4×10^{-8} M of 3'-sulfhydry-oligonucleotide probes respectively.

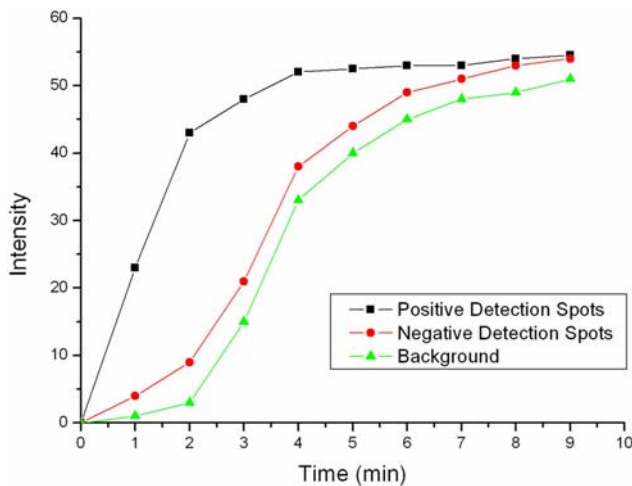


Fig. 6. The intensity and development time using the development solution without gelatin. The horizontal numbers stand for minutes of development time while the perpendicular numbers stand for relative intensities.

Rapid detection of HEV gene with nano-amplification technique. Based on the above results, the approach of detection method was confirmed as follows: The gene fragment of tryptophan was designed as a positive control. The two kinds of amplification products (target and positive control) were hybridized for 10min with detection probes of HEV (0.01% gold nano-particle labeling 3.4×10^{-7} M probe) and detection probe of positive control product on the microarray, on which the capture probes had been were immobilized. The chip was rinsed with PBN (1.2 M NaNO_3 /10 mmol/L $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.0) three times (2 min

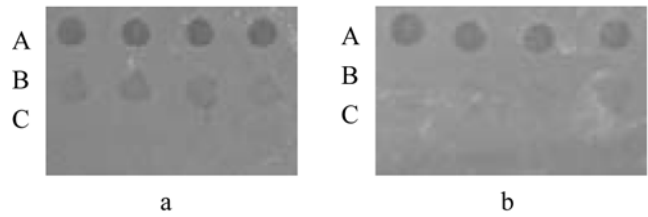


Fig. 7. The detection result of HEV detection microarray. A) Positive control; B) Target checking line; C) Negative control. a) Positive serum sample; b) Negative serum sample.

for each time) followed by Silver stain developing for 2 min. The result showed that rows A and B were stained and row C was not developed (Fig. 7a). When there was no HEV in serum samples, however, both row B and C were not developed while row A was still developed (Fig. 7b). It suggests that the gene detection technique, based on double-probe sandwich hybridization, nano-gold labeling and silver-staining enhancement, could be applied for the specific detection of HEV.

After all, a rapid detection technique for HEV based on Mirkin's approach and our previous works was built. And a few improvements had been performed. Due to the performance on the detection and adaptation to operation, this technique could be used to monitor HEV not only in serum but also in food or water.

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