

## Magnetic bead technology in viral RNA and DNA extraction from plasma minipools

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**BACKGROUND:** Nucleic acid testing (NAT) of pooled plasma samples from individual blood donations for viral nucleic acids has become widely established. Full automation of such sample processing can overcome many of the problems associated with methods used so far.

**STUDY DESIGN AND METHODS:** In this study an automated extraction method for viral nucleic acids (parvovirus [PAV] B19 DNA, hepatitis B virus [HBV] DNA, and hepatitis A virus [HAV] RNA), starting directly from the minipool sample ( $n = 96$ , 9.6 mL), was evaluated. A magnetic separation module I (chemagic, Polymer Laboratories) in combination with the chemagic viral DNA and RNA kit special based on the use of magnetic beads was used for this purpose. More than 144 pools spiked with defined concentrations of reference material and an additional 102 pools negative for the analyte were extracted and amplified. The isolated viral nucleic acids were detected by polymerase chain reaction (PCR).

**RESULTS:** The assays were highly specific and obtained a 95 percent detection limit of 875 IU per mL of pooled single donation for PAV B19, 260 IU per mL for HAV, and 1274 IU per mL for HBV, respectively. The crossing points showed variation coefficients from 1.49 to 2.76 percent. The turnaround time for the whole process was 3 hours. Testing of subpools to determine an infected single donation would be possible with the same general extraction method. A total of 102 unspiked minipools ( $96 \times 100 \mu\text{L}$  per donation) were analyzed and none tested positive.

**CONCLUSION:** The automated magnetic bead-based extraction in combination with real-time PCR detection can be used to routinely screen blood donations for viremic donors to further increase the safety of blood products. Minipools as well as subpools can be directly processed.

The use of nucleic acid amplification tests (NAT) for the detection of viruses starting from pooled plasma samples from individual blood donations has gained in importance.<sup>1,2</sup> At present, single-donation testing is expensive<sup>3</sup> and in larger blood banks not feasible owing to the intensive workload. Dilution due to pool size and the need of highest sensitivity led to various methodologic protocols for improvement of the extraction efficiency, such as centrifugation<sup>4</sup> or the addition of agents that support precipitation of viral particles.<sup>5</sup> These modifications, however, are time-consuming and tricky, for example, resuspension of the invisible pelleted viral particles after centrifugation, which could result in the loss of nucleic acid, and are considerable disadvantages for routine blood donor screenings. Centrifugation steps are difficult to incorporate into automation.<sup>6</sup> We sought an automated extraction method for parvovirus (PAV) B19 DNA, hepatitis B virus (HBV) DNA, and hepatitis A virus (HAV) RNA that could start directly from the minipool sample ( $n = 96$ , 9.6 mL). Besides NAT for HCV and HIV-1, which are mandatory in Germany, corresponding tests for HBV, PAV B19, and HAV have often become release criteria in German Red Cross Blood banks to further increase safety of blood products and fulfill demands from plasma fractionation industry.

**ABBREVIATION:** PAV = parvovirus.

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Here we describe the evaluation of a magnetic particle-based extraction method for viral nucleic acids in combination with detection by real-time polymerase chain reaction (PCR) for B19, HAV, and HBV.

## MATERIALS AND METHODS

### Samples and standards

Minipool samples consisted of ethylenediaminetetraacetate-plasma derived from individual blood donations. WHO First International Standards for PAV B19 DNA NAT, HAV RNA NAT, and HBV DNA NAT were obtained from NIBSC (Hertfordshire, UK): First WHO International Standard for Parvovirus B19 NAT assays (NIBSC Code 99/800), First WHO International Standard for HAV RNA NAT assays (NIBSC Code 00/560), and First WHO International Standard for HBV DNA NAT assays (NIBSC Code 97/746).

### Pooling and spiking

Replicate pools from previously serologically and NAT-screened donations were used as a matrix for spiking experiments. Plasma samples of 100  $\mu$ L were processed in pools of 96 with a liquid handler (Genesis RSP150, Tecan Group Ltd, Maennedorf, Switzerland). Pools were spiked with 100  $\mu$ L of diluted reference material. From each dilution, eight replicate pools were spiked, individually extracted, and amplified. This spike experiment was performed 3 times on 3 days by different operators, and the limit of detection was determined by statistical analysis. Subpooling was performed via a x-y intersection approach from 96-well microtiter plates, pooling 100- $\mu$ L individual samples by row ( $n = 12$ ) and by column ( $n = 8$ ).

### Automated nucleic acid extraction

Simultaneous extraction of viral DNA and RNA was performed according to the manufacturers instructions with a viral DNA and RNA kit (chemagic, chemagen AG, Baesweiler, Germany) special in conjunction with a magnetic separation module I (chemagic, chemagen AG), whereby up to 12 samples could be processed in parallel. Briefly, 9.6 mL of pooled plasma was mixed with 9.6 mL of lysis buffer, containing 50  $\mu$ L of protease and 7  $\mu$ g of carrier RNA. After being vortexed for 10 seconds, the lysate was incubated for 5 minutes at 55°C. The lysate was then mixed with 30 mL of binding buffer containing 50  $\mu$ L of magnetic beads. The complete isolation process including binding, wash, and elution steps was then performed automatically on a chemagic magnetic separation module I. A binding time of 30 minutes and continual mixing ensured that the viral nucleic acids and the synthetic internal control were captured by the magnetic beads. Finally, viral nucleic acids were eluted in 100  $\mu$ L of elution buffer.

### Amplification and detection

Five microliters of eluate was used as a template for B19 DNA PCR and for HAV RNA reverse transcription-polymerase chain reaction (RT-PCR), respectively, with a real-time thermocycler (LightCycler, Roche, Indianapolis, IN). HBV-specific sequence was amplified from 30  $\mu$ L of template in a sequence detection system (ABI PRISM SDS 7700, Applied Biosystems, Foster City, CA). The PCR amplification and detection was performed according to the manufacturer's instructions (RealArt™ Parvo B19 LC PCR kit, RealArt HAV LC RT PCR kit, RealArt HBV™ PCR kit, artus GmbH, Hamburg, Germany). Internal controls monitor the validity of the PCR in each reaction.

### Statistical analysis

Probit analysis of the sensitivity hit rates was performed with computer software (SPSS, Chicago, IL); standard deviation (SD) and coefficient of variation (CV) were calculated with another computer program (Excel, Microsoft Corp., Redmond, WA).

## RESULTS

### Analytical sensitivity and 95 percent detection limit

The limit of detection was determined by preparing serial half-log dilutions of international standards for NAT. From each dilution, 8 replicate pools were spiked, individually extracted, and amplified. The results are shown in Table 1. The 95 percent detection limit from these data was calculated by probit analysis and yielded 875 IU per mL pooled single donation for PAV B19, 260 IU per mL pooled single donation for HAV, and 1274 IU per mL pooled single donation for HBV.

### Precision

Intra- and interassay precision define the variability of measured values within an assay and between different assays for the same analyte. SDs of the target crossing point-threshold  $C_p$  ( $C_p$  characterizes the PCR cycle where the fluorescence signal specifically increases over the baseline for the first time) of the different assays and the percentage of variation CV are shown in Table 2.

### Specificity

A total of 102 minipools, representing approximately 9950 blood donations that were replicates from routine NAT pools, were analyzed for PAV B19 DNA, HBV DNA, and HAV RNA. All pools tested negative for the presence of these viral sequences in concordance with results obtained from routine screening. None of these was invalid because of a negative result for the internal control indicating that the tests are highly specific. No cross-reac-

**TABLE 1. Analytical sensitivity for minipool samples (n = 96 samples/pool)**

Spiked concentration (IU/mL pooled single donation)	Series 1 (Day 1) PCR-positive	Series 2 (Day 2) PCR-positive	Series 3 (Day 3) PCR-positive	PCR-positive/pools tested	Percentage positive
<b>PAV B19*</b>					
50.0 × 10 <sup>3</sup>	8	8	8	24/24	100
15.8 × 10 <sup>3</sup>	8	8	8	24/24	100
5.0 × 10 <sup>3</sup>	8	8	8	24/24	100
1.58 × 10 <sup>3</sup>	8	8	8	24/24	100
500	7	5	8	20/24	83.33
158	6	4	1	11/24	45.83
<b>HAV†</b>					
8.0 × 10 <sup>3</sup>	8	8	8	24/24	100
2.531 × 10 <sup>3</sup>	8	8	8	24/24	100
800	8	8	8	24/24	100
253	8	8	8	24/24	100
80	4	4	6	14/24	58.33
25	2	2	7	11/24	45.83
<b>HBV‡</b>					
3.16 × 10 <sup>3</sup>	8	8	8	24/24	100
1.0 × 10 <sup>3</sup>	8	8	8	24/24	100
316	6	7	3	16/24	66.66
100	7	3	5	15/24	62.50
31	3	4	2	9/24	37.50
10	1	0	4	5/24	20.83

\* 95 percent detection limit was 875 IU per mL pooled single donation.

† 95 percent detection limit was 260 IU/mL pooled single donation.

‡ 95 percent detection limit was 1274 IU/mL pooled single donation.

**TABLE 2. Intraassay and interassay variability of the different tests\***

Virus	Mean crossing point (C <sub>P</sub> )	SD	%CV	Spiked concentration (IU/mL pooled single donation)
<b>Parvo B19</b>				
Intraassay variable	29.43	0.27	0.92	50.0 × 10 <sup>3</sup>
	33.26	0.58	1.76	1.58 × 10 <sup>3</sup>
Interassay variable	29.71	0.44	1.49	50.0 × 10 <sup>3</sup>
	33.32	1.16	3.49	1.58 × 10 <sup>3</sup>
<b>HAV</b>				
Intraassay variable	31.95	0.14	0.45	8.0 × 10 <sup>3</sup>
	36.66	0.49	1.34	253
Interassay variable	32.40	0.52	1.60	8.0 × 10 <sup>3</sup>
	37.12	0.68	1.82	253
<b>HBV</b>				
Intraassay variable	35.21	0.91	2.58	3.16 × 10 <sup>3</sup>
	36.96	1.00	2.71	1.0 × 10 <sup>3</sup>
Interassay variable	35.48	0.76	2.15	3.16 × 10 <sup>3</sup>
	37.13	1.03	2.76	1.0 × 10 <sup>3</sup>

\* Eight replicate pools were spiked with defined concentrations of reference material and tested on Day 1 and spiking was repeated on Day 2. Data were calculated from the crossing points of each run.

tivity could be observed when HAV spiked pools were analyzed in the HBV and B19 PCR and vice versa (see Table 3).

**Testing of subpools and performance qualification**

Mimicking subpool strategy in case of a reactive minipool, we spiked a minipool with 100 µL of diluted reference material for each virus and three different wells of a micro-titer plate with 240 µL for HBV, HAV, and PAV B19, respec-

tively. The 20 subpools were created and subsequently tested. Concentrations of the spiking material were set to 500 IU per mL single donation for HAV and HBV. For PAV B19 the concentration was set to 1.6 × 10<sup>5</sup> IU per mL single donation taking into account that rather high-titer samples may cause cross-contamination problems than analytes close to the detection limit would be an issue. The minipool and the two corresponding subpools tested positive for the spiked virus.

**Robustness and cross-contamination**

We investigated the risk of false-positive results caused by high-titer samples cross-contaminating negative samples

during the whole analysis. Therefore, we created two 3 × 4 matrices of alternating spiked and unspiked minipools as 12 pools can be processed simultaneously on the magnetic separation module I. PAV B19 titer of the spiked pools was set to 1.6 × 10<sup>8</sup> IU per mL single donation. Non-spiked pools were analyzed.

Eleven nonspiked pools tested negative in the B19 PCR. The internal control of one pool tested negative because of residuing magnetic particles in the eluate.

TABLE 3. Specificity\*

Minipool number	Spiked concentration (IU/mL pooled single donation)	HAV $1.00 \times 10^6$	PAV B19 600	HBV $3.61 \times 10^5$	HCV $12.1 \times 10^3$	HIV-1 $4.06 \times 10^3$
1	Positive control	X†	X	X	X	X
2	HAV negative		X	X	X	X
3	HAV negative		X	X	X	X
4	HAV negative		X	X	X	X
5	B19 negative	X		X	X	X
6	B19 negative	X		X	X	X
7	B19 negative	X		X	X	X
8	HBV negative	X	X		X	X
9	HBV negative	X	X		X	X
10	HBV negative	X	X		X	X
11	Negative control					
12	Negative control					

\* Twelve minipools were spiked with different combinations of virus and analyzed.

† X = virus spike and positive result in PCR.

Removing the residue from the eluate revealed a valid negative result in the B19 PCR.

## DISCUSSION

NAT of blood donation minipools requires handling large blood volumes and an inherent problem of decreasing sensitivity due to dilution by pooling. In the past, time-consuming and laborious steps such as centrifugation or precipitation have been included but incur the risk of pellet loss or at least incomplete resuspension of pelleted viral particles. We examined the technical and analytical feasibility of a highly sensitive automated extraction method that starts with the large volume of minipool samples without any concentration steps. The lysis of the viruses occurs directly within the plasma as a first step. The viral nucleic acids are then bound to the surface of the magnetic beads and by successive wash steps purified. The elution volume is 100  $\mu$ L, which is just 1/100 of the starting volume and enables the possibility for different PCR assays. Apart from the lysis step all following steps are performed automatically on the chemagic magnetic separation module I with an electromagnet and rotating rods.

The purified viral nucleic acids were analyzed with rapid cycling real-time PCRs. The sensitivity of detection for B19 and HAV is within the same range as reported from other minipool NAT procedures.<sup>7,8</sup> The expected sensitivity limit below 1000 IU for HBV was not achieved owing to bad performance in one of the test series resulting in an increase in the statistical 95 percent detection limit (see Table 1). From the subpool experiment and performance qualification study, we saw that 500 IU per mL single donation could be clearly detected in the minipools and in the subpools.

The turnaround time for the complete process of extraction as well as detection of, for example, B19 is approximately 3 hours. Subsequent identification of a

virus-positive single donation by testing of subpools is possible within the same working shift. As a result products with short life-time manufactured from nonreactive donations could be released without critical time delay. Deferral of labile products could be reduced.

The degree of automation could be further increased by implementing an automatic liquid handling system that dispenses the buffers and beads and a robotic manipulator that positions the filled buffer tubes on the chemagic magnetic separation module I. Automation of magnetic-based extraction is a promising attempt.<sup>9</sup>

From the data obtained it can be concluded that viral nucleic acid extraction from large volume minipool samples is realizable as a semiautomated process, showing high sensitivities, and offers the possibility to perform different PCR assays from one sample eluate.

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