Comparison of Protocols for Extracting Circulating DNA and RNA from Maternal Plasma

To the Editor:
We compared 2 column-based and an automated magnetic bead separation protocol for extracting circulating DNA and RNA from maternal plasma. We obtained peripheral blood from pregnant women at Prince of Wales Hospital, Hong Kong, and King’s College Hospital, London, with informed consent and institutional ethics approval.

Thirty second-trimester samples (median gestational age, 17.6 weeks) were divided into 2 aliquots for maternal plasma DNA extraction by either a QIAamp Mini Kit (Qiagen), with 800 µL of plasma applied per column and DNA elution into 50 µL of deionized water, or a MagNA Pure Total Nucleic Acid Large Volume Isolation Kit on the MagNA Pure LC instrument (Roche Diagnostics) (1) with DNA elution into 50 µL of elution buffer. β-Globin and SRY concentrations were quantified by real-time PCR assays (2).

With 5 µL of plasma DNA per PCR (2) for samples from women with male fetuses, SRY amplification was observed in 15 (100%) column-method samples and in 10 (67%) automated-protocol samples. The β-globin DNA concentration was significantly higher (P < 0.001, Wilcoxon signed-rank test; SigmaStat Ver. 3.0; SPSS) in the column extractions [median, 456 copies/µL; interquartile range (IQR), 276–744 copies/µL] than the automated protocol [median (IQR), 223 (169–350) copies/µL].

With 10 µL of plasma DNA extracted by the automated protocol per PCR (3), we detected all but 1 SRY-positive cases. Median (IQR) SRY concentrations for plasma DNA extracted by the column and automated methods were 40 (28–65) and 4 (1–9) copies/µL, respectively; median β-globin concentrations were 5257 (793–14,213) and 909 (239–5728) copies/µL, respectively. Maternal plasma SRY concentrations were significantly lower with the automated protocol (P = 0.002, Wilcoxon), but β-globin concentrations showed no significant difference (P = 0.087, Wilcoxon).

Maternal plasma was treated with Trizol LS (Invitrogen) to prevent RNA degradation (4), and 2 protocols for RNA extraction were compared. Trizol LS (4 mL; Invitrogen) was mixed with plasma (3.2 mL) before storage at −80 °C. Trizol-pre- served plasma was thawed and mixed with 0.8 mL of chloroform and centrifuged at 12,000g for 15 min at 4 °C. The upper aqueous layer was transferred into new tubes as 2 aliquots. RNA was extracted from 1 aliquot with 1 mL of the aqueous layer (equivalent to an original plasma volume of 272 µL) by the MagNA Pure Kit and instrument and eluted into 50 µL of elution buffer. We added 1 volume of 700 µL ethanol to 2.2 mL of the aqueous layer (equivalent to an original plasma volume of 1.6 mL) from the other aliquot and applied it to an RNAeasy Mini Kit minicolumn (Qiagen) as described previously (5). On-column DNase digestion was performed with 40 µL of the enzyme-buffer mixture from the RNA-Free DNase Set (Qiagen). β-Globin DNA, to assess DNA contamination, was detected with real-time PCR (2) in 3 of 10 samples in both protocols; concentrations were 98, 135, and 3419 copies/µL for the automated protocol and 30, 54, and 31 copies/µL for the column protocol. For another 10 samples, β-globin DNA was not detected in any samples after we used on-column DNase digestion with 80 µL of enzyme-buffer mixture from the RNA-Free DNase Set (Qiagen) for the column-based protocol and postextraction DNase treatment with a DNase I reagent set (Invitrogen) for the automated protocol. We used these methods to assess human chorionic gonadotropin β-subunit (βhCG) mRNA concentrations in 40 first-trimester maternal plasma samples (median gestational age, 13 weeks) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 20 of these samples, using intron-spanning reverse transcription-PCR assays as described previously (5, 6). The absence of DNA contamination was further confirmed with the AmpliTaq Gold enzyme (Applied Biosystems) for the βhCG assay without the reverse transcription step. Median (IQR) βhCG mRNA concentrations for the column-based and automated protocols were 447 (94–1478) and 893 (111–1606) copies/µL, respectively. Median GAPDH mRNA concentrations were 17,260 (9376–35,396) and 47,003 (37,704–102,520) copies/µL for the column-based and automated protocols, respectively. mRNA concentrations for both βhCG (P = 0.014, Wilcoxon) and GAPDH (P < 0.001, Wilcoxon) were significantly higher with the automated protocol.

Plasma DNA from the automated extraction was less concentrated than that from column extraction, whereas the contrary was true for RNA. Circulating DNA is nonparticulate, whereas RNA circulates in association with particulate matter (5–7), and different extraction methods may favor the isolation of certain physical forms of plasma nucleic acids. We demonstrated that the observed concentrations of circulating nucleic acids differ depending on the processing and analysis methods (7).

This study also reveals that careful evaluation of RNA extraction protocols and assay designs is necessary to avoid DNA contamination.

This work is supported by Ear-marked Research Grants from the Hong Kong Research Grants Council (CUHK 4395/03M for the DNA part and 4474/03M for the RNA part of the study).

References
Fetal Rhesus D mRNA Is Not Detectable in Maternal Plasma

To the Editor:

Detection of fetal-derived DNA and RNA molecules in maternal plasma is a promising approach for noninvasive prenatal diagnosis (1). Analysis of circulating fetal RNA, unlike analysis of fetal Y-chromosomal DNA, can be used in pregnancies with fetuses of either gender (2). Placenta-derived RNA species are readily detectable in maternal plasma (2).

The fetal hematopoietic compartment may be another source of nucleic acids in maternal plasma (3). Wataganara et al. (4) reported detection of γ-globin mRNA in maternal plasma. However, expression of γ-globin is not a fetus-specific phenomenon and is shared by maternal erythroid cells (5), and γ-globin mRNA is readily detectable in nonpregnant individuals (4). In this study, we assessed whether rhesus D (RHD gene) mRNA derived from fetal erythroid cells is detectable in the plasma of rhesus D-negative pregnant women.

Fifteen rhesus D-negative women at 11–40 weeks of gestation (Table 1), attending the University Women’s Hospital, Basel, were recruited with informed consent and institutional ethics approval. Trisomy 21 was subsequently confirmed in 1 pregnancy, which was excluded from the analysis. We collected 15 mL of maternal peripheral blood into EDTA tubes. Cord blood was collected from 3 of the term pregnancies after delivery. The blood samples were stored at 4 °C until processing within 3 h by centrifugation (2). Trizol LS reagent (4 mL) was mixed with 3.2 mL of plasma and stored frozen at −80 °C. The plasma samples were sent to Hong Kong in dry ice.

The maternal plasma and cord blood samples were analyzed for RHD, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and human placental lactogen (hPL) mRNA by 1-step real-time quantitative reverse transcription-PCR assays. The latter 2 assays had been described previously (2). The RHD mRNA assay was designed by use of Primer Express software, Ver. 2.0 (Applied Biosystems), with the fluorescent probe crossing the junction between exons 7 and 8 (GenBank accession no. BN000065). Specificity of the assay for RHD was conferred by the forward primer. The primer and probe sequences were as follows: forward primer, 5’-CTG TAT ATA CCG TCG GAG C-3’; reverse primer, 5’-TGA GTT CCC CAA TGC TGA GG-3’; fluorescent probe, 5’-(FAM) AAT GGC ATG ATT GCG TTC CAG GTC C (TAMRA)-3’, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. The reactions were set up according to the manufacturer’s instructions (EZ rTth RNA PCR reagent set; Applied Biosystems) in a reaction volume of 25 μL with 200 nM of each primer and 50 nM of the fluorescent probe. We used 5 μL of extracted plasma RNA for each reaction, which was performed in a thermal cycler with fluorescent detector (ABI Prism 7900; Applied Biosystems) at 50 °C for 2 min for the activation of uracil-N-glycosylase, 60 °C for 30 min for reverse transcription, and 95 °C for 5 min, followed by 45 cycles of 94 °C for 20 s and 1 min at 60 °C. The calibration curve was constructed with serial dilutions of an HPLC-purified single-stranded synthetic DNA oligonucleotide (5’-CTG TGT CTT GAT ACC GTC GGA GCC GGC AAT GGC ATG ATT GCG TTC CAG GTC CTC CTC AGC ATT GGG GAA CTC AGC TT-3’) at 2.5 × 10^7 to 2.5 copies. Specificity of the assay was confirmed by the lack of amplification for bulky coat RNA of rhesus D-negative individuals. Sensitivity of the assay was assessed by use of bulky coat and plasma RNA from 10 rhesus D-positive nonpregnant individuals. Positive amplification was noted from all samples, and the assay could detect 5 copies/mL of plasma.

Sample analysis was performed without knowledge of the fetal rhesus D status. GAPDH mRNA was detectable in all samples, whereas hPL mRNA, a placenta-specific transcript (2), was detectable in all maternal plasma samples (data not shown). RHD mRNA was detected in the cord plasma sample collected from a rhesus D-positive neonate, but in no maternal plasma samples (Table 1).

Although RHD mRNA has been detected in fetal erythroid cells isolated from maternal circulation (6, 7), we found no RHD mRNA in maternal plasma. We suggest that fetal erythroid cells are unlikely to contribute a large portion of the fetal mRNA in maternal plasma. We believe that detection of plasma RHD mRNA in rhesus D-negative preg-

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