



Routine application of genotyping a step closer: direct PCR on plasma

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It is almost 70 years since Mandel and Metais (1) reported that nucleic acids are present in circulating plasma. This report of cell-free or cfDNA in plasma lay dormant for decades until the discovery that circulating tumour DNA is present as an admixture of normal DNA in the plasma (2,3). Today, genomic technologies, such as massively parallel sequencing (MPS), are being studied to realise predictions that these early discoveries would lead to clinical translation for cancer diagnosis, monitoring responses to treatment and prognosis (4).

It is some 20 years since Lo *et al.* showed that cell-free fetal DNA (cffDNA) is present in plasma (5) opening new pathways such as non-invasive prenatal testing (NIPT) for screening for fetal chromosomal anomalies such as Down's syndrome (6). Genomic MPS technologies are now being implemented in over 60 countries (6).

Within transfusion medicine and immunohematology, the earliest clinical application for NIPT cffDNA testing was fetal *RHD* blood group genotyping. This is now applied in a diagnostic setting for high risk alloimmunised RhD negative women and, in some jurisdictions, as a screen to target routine anti-D immunoglobulin prophylaxis for D-negative women carrying a D-positive baby. A limitation to high throughput has been that NIPT assays require a cfDNA extraction step before genotyping. In contrast to cancer genetics and anomaly screening, currently *RHD* blood group genotyping techniques are almost exclusively based on PCR technologies (6).

Surprisingly, there has been little application of plasma-derived cfDNA testing for blood group genotyping outside of fetal medicine. Blood group genotyping for blood donors

and patients is applied using microarray technologies that have been successfully integrated into tertiary red cell reference laboratories and, in some instances, applied for screening to prepare panels of genotyped donors (7). These accredited platforms, using genomic DNA as the target, are accurate and robust. For application in the clinical environments, however, the turn-around time of 6 to 8 hours is a limitation, with the need for DNA extraction a rate limiting step.

This issue of protracted turnaround time for blood group genotyping has been addressed in a recent paper by Dr. Wagner *et al.* (8) who report direct testing of cfDNA to detect 2 blood group single nucleotide variants (SNVs) for the most common Duffy and Kidd blood group variants. A PCR approach using plasma or serum without DNA extraction was applied to provide a group genotype within 40 minutes. This reduced processing time was achieved by review and optimisation of each step in this process.

The first step was by directly testing cfDNA in plasma in a PCR reaction mix without a cfDNA extraction step. Miniaturisation, with 1 μ L of plasma in a 10 μ L PCR reaction mix enabled the authors to adapt a rapid-cycle capillary PCR with an automated high-resolution melt (HRM) analysis as the end point using oligonucleotide probes. Oligonucleotide probes are included into the PCR-HRM which increases specificity and sensitivity of genotyping assays (9). A further step included redesign of primers, when necessary, to promote higher annealing temperatures in the PCR and thus reduce the time-lapse for cooling to the annealing temperature for each PCR cycle. The authors noted in their report that the thermocycling

conditions were pushed to the extreme.

This proof of principle was established by genotyping two sets of 100 donor samples, collected in EDTA or serum, for the antithetical Duffy blood group antigens (Fy^a and Fy^b), and Kidd antigens (Jk^a and Jk^b). Genotyping was tested in a blinded fashion and matched with serology only after genotyping had been completed. The turn-around time of 40 minutes from first pipetting step to interpretation of results is comparable to that for serology-based methods in routine use in hospital blood banks. With the optimisation steps described it becomes practical to employ genotyping to determine the choice of compatible blood units (particularly for patients who have already been transfused). There is further potential with multiplexing to target a range of clinically important blood group polymorphisms. It should be noted however that the throughput reported, three samples per run, to achieve a 40-minute turn-around, was low.

The genotyping scores reported were impressive, but were not 100%. All positive predictive values by genotyping were 100% however false negative assignments were observed for analysis of samples derived from both EDTA and serum tubes. Using plasma samples negative predictive values (NPVs) were 97% and 96% for Fy^a and Fy^b and 100% for Kidd antigens. Using serum samples NPVs were 94% and 100% for Fy^a and Fy^b and 84% and 96% for Jk^a and Jk^b which suggests further review of sample pre-analytics and of test optimisation would be of value.

A key problem with cfDNA is the small size of the fragments in circulation (average 165-bp) which needs to be considered in the PCR design. There is also potential for individual variation in cfDNA concentration, which may become significant without an extraction step. The study was performed on donors and the authors note that the reliability after massive plasma transfusion, in pregnant patients and in aplastic patients is yet to be established.

While the proof of principle was limited to two SNVs associated with clinically significant blood group antigens, there are over 350 registered blood group antigens, many with population specific associations. One other paper reports a direct blood PCR approach to type for the GP. Mur glycoprotein variant frequent in the East Asian population with the application intended for donor screening for blood group variants (10). There is clearly a clinical need for rapid genotyping for variants not easily typed serologically, for example Dombrock, also allowing rapid typing for patients already transfused.

This proof of principle paper by Wagner *et al.* opens a

doorway for blood group genotyping by directly testing cfDNA using a PCR-HRM approach, particularly in situations where timely results are required. The application of cfDNA testing for blood groups also suggests a future for blood group typing using technologies analogous to fetal anomaly screening and modern cancer genomics.

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