Review Article

Overview of non-invasive fetal blood group genotyping

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Abstract: Non-invasive prenatal testing (NIPT) is commonly used to identify feto-maternal blood group allele discrepancy. Fetal cell-free DNA (cfDNA) in maternal blood sample has two important uses: firstly, in genotyping for the diagnosis of hemolytic disease of the fetus and newborn (HDFN) and secondly, in targeting routine antenatal anti-D prophylaxis (RAADP) at women carrying an *RHD*-positive fetus. The aim of the current paper is to review technical aspects of cell-free fetal blood group genotyping including methods, controls, and accuracy of different assays. As a highly accurate technique, real-time PCR is overwhelmingly the most commonly used method for *RHD* screening tests. The antenatal *RHD* screening assays are exceedingly sensitive allowing reliable to target anti-D prophylaxis solely at women bearing an *RHD*-positive fetus, also after childbirth. Droplet digital PCR (ddPCR), capillary electrophoresis and massively parallel sequencing are powerful methods for fetal genotyping, and assays for blood groups within the blood group systems ABO, Rh, Kell, Fy, Jk and MNS have been implemented with reliable results. The massive abundance of maternal DNA in a sample, the small size of circulating cfDNA fragments and blood group genetics pose challenges for designing robust assays. Marker allele panels, epigenetic markers, total DNA and process controls are used to confirm the presence of fetal DNA in these assays.

Keywords: Non-invasive prenatal testing (NIPT); cell-free fetal DNA; molecular blood group typing; RHD screening

Received: 31 May 2021; Accepted: 27 September 2021; Published online: 05 November 2021.
doi: 10.21037/aob-21-41
View this article at: https://dx.doi.org/10.21037/aob-21-41

Introduction

Hemolytic disease of the fetus and newborn (HDFN) is caused by maternal red blood cell (RBC) antibodies of IgG class that cross the placenta and bind to RBCs of the fetus. These antibodies facilitate destruction of fetal RBCs, potentially leading to anemia and hyperbilirubinemia, sometimes even to fatal hydrops fetalis. Anti-D (Rh), anti-c (Rh), anti-K (Kell) create the most significant risk for a severe HDFN. Also, antibodies against other Rh antigens or Duffy and Kidd antigens may cause HDFN albeit less often and typically with milder symptoms. Anti-A and anti-B (ABO) antibodies may be a risk for HDFN after birth (1).

Even though anti-D is the most common cause of HDFN and one that results in the most severe form of the disease, anti-D immunization can be effectively reduced by anti-D prophylaxis given to RhD-negative women both ante- and postnatally. The risk for immunization due to fetal RBCs released to maternal circulation is highest in the third trimester of pregnancy and in delivery (2). To avoid maternal alloimmunization against fetal RhD antigen, postnatal anti-D prophylaxis has been a routine practice for decades. In addition, nowadays anti-D prophylaxis is often administered also antenatally to RhD-negative nonimmunized women, which has further reduced sensitization rates (2).

The goal of this review is to discuss blood group screening and genotyping assays that analyze fetal cell-free DNA (cfDNA). To this end, a literature search for publications in English highlighting recent studies (last 10 years) was performed. All genotyping platforms utilized in fetal blood group typing are included, and accuracy of

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the results is reviewed as well as the controls used in assays.

Blood group genetics

In Caucasians, the main cause of the RhD-negative phenotype is the *RHD* gene deletion. Screening methods are based on the absence of maternal *RHD* gene; they catch the *RHD* gene in a sample of the RhD-negative mother and assume it to be of fetal origin. In non-Caucasian populations, RhD-negativity is often caused by polymorphisms affecting gene expression, rather than a gene deletion. In the presence of a maternal *RHD* gene, detection of fetal allele requires several target sequences taking into account variant alleles. Also, the *RHD* and *RHCE* genes of the Rh system are highly homologous and pose a challenge for Rh genotyping. Blood group antigens A and B (ABO) are produced by a glycosyltransferase (coded by the ABO gene); the blood group O phenotype results from the unfunctional glycosyltransferase. The difference between *ABO*A1.01* (*A1*) and *ABO*B1.01* (*B1*) alleles is based on a few nucleotide exchanges in exon 7 of the *ABO* gene and the most common inactive *ABO*O.01* (*O1*) allele is caused by a frameshift deletion in exon 6. In addition, many other inactivating mutations occur in the *ABO* gene, therefore, designing robust genotyping methods for *ABO* blood group alleles is challenging (3).

The majority of blood group antigens, such as K, result from single nucleotide polymorphisms (SNPs), the smallest possible variation between genetic alleles. Therefore, a genotyping test must be able to detect the presence or absence of a SNP. A negative result is based on not detecting a positive signal, so it is essential to design and validate tests to ensure they are robust enough to avoid false negatives and positives. The main challenge rises from maternal DNA being much more abundant, thereby interfering with the genotyping test. Thus, particularly in the case of a negative fetus, the sample should be shown to really contain fetal DNA or otherwise ensure the result.

cfDNA

cfDNA of fetal origin is primarily derived from trophoblast cells of the placenta (4). As it is cleared promptly after delivery (5), previous pregnancies do not interfere with fetal genotyping. The discovery of circulating fetal cfDNA in 1997 (6) has significantly changed fetal genotyping procedures. As invasive methods, traditional amniocentesis and chorionic villus sampling carry a slight risk of miscarriage (7). The possibility to detect fetal cfDNA in a maternal sample has expanded the applications of non-invasive prenatal testing (NIPT).

While a sample taken from a mother contains both maternal and fetal cfDNA, maternal DNA is much more abundant. Fetal fraction refers to the proportion of fetal cfDNA of the total cfDNA in a maternal plasma sample. The amount of fetal cfDNA in the bloodstream of a pregnant woman increases with advancing gestational age (8). In addition, many technical and biological factors influence the fetal fraction, including the shipping time of the sample and the weight of the mother (9,10). The fetal cfDNA fraction ranges substantially (11), but the median is 10% at 10–13 gestation weeks (6,8), which is enough for reliable detection in genotyping assays.

Circulating cfDNA fragments are genomic and double-stranded but small, with the size of the main fragment population of fetal DNA being 143 base pairs and 166 base pairs for maternal DNA (12). As a result, amplicons are short, which must be taken into account in assay design.

Fetal blood group genotyping has two uses

Screening fetal RHD for targeting anti-D prophylaxis

Routine antenatal anti-D prophylaxis (RAADP) is recommended for all RhD-negative women at gestation weeks 28–34 to prevent immunization during the last trimester of pregnancy (13). Prophylaxis can be targeted solely at those RhD-negative women who benefit from it i.e., women who carry an RhD-positive fetus. The aim of targeting is to avoid unnecessarily giving a human-derived product to pregnant women, in addition to reducing the use of a limited commodity. There are also ethical considerations involved in the production of anti-D prophylaxis where donors are sensitized with a blood product.

The screening assays must be based on a high-throughput process to be cost-effective. The workflow should be automated also to prevent human errors. Mass testing allows savings in the purchase of consumables and staff resources. To reduce costs of a screening test, other issues to consider include sample logistics, DNA extraction method, test platform and electronic data transfer (14,15). Examples of how to implement a national screening and targeted RAADP program have been published from Denmark (16), Netherlands (17), Finland (15), and Norway (18). Based on them, an assay is not too difficult to set up in terms of technology, but challenges lie in the smooth co-operation and effective communication required between laboratories,

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maternity clinics, and delivery hospitals. Cost-effectiveness of targeted RAADP programs has been widely discussed in a review by Saramago *et al.* (19). The targeted RAADP may be cost-effective compared to the untargeted prophylaxis, depending mainly on the unit cost of the screening test and on whether the result of the test also guide post-partum administration of anti-D prophylaxis (19).

In their excellent review, Runkel *et al.* looked at fetal *RHD* screening studies (20). In a meta-analysis of 12 large prospective studies, they demonstrated a sensitivity of 99.9% [95% CI: (99.5%, 100%)] and a specificity of 99.2% [95% CI: (98.5%, 99.5%)]. Screening assays show very high accuracy and thus, they offer a reliable tool for targeting prophylaxis not only antenatally but also postnatally without the need for confirmatory typing of newborns.

Genotyping blood groups of fetuses of immunized women

If a mother has a clinically relevant antibody potentially causing HDFN and the father is heterozygous for the related blood group allele, diagnostic fetal blood group genotyping is needed. Careful fetal surveillance may be targeted solely at those pregnancies where a fetus is detected to be positive for the blood group allele. In case of a negative fetus, the mother can avoid unnecessary anxiety and health services do not incur unnecessary costs.

The main concern is false-negative results, which may cause serious consequences for the fetus. False-negative results are often due to the fetal DNA fraction falling below the detection limit in maternal plasma. To avoid risks, several practices (9,21,22) have been implemented, starting from pre-analytics: collecting blood samples in special cfDNA stabilizing tubes and processing them within a specific time limit (23,24). These measures help prevent lysis of maternal cells, which would otherwise induce an increase in total cfDNA and thereby reducing the fetal fraction (9). The fetal DNA fraction is very small in the early gestation but already at weeks 9–12, results have been shown to be reliable (8,10,21). In a case of negative result, confirmatory testing is often carried out after a couple of weeks. Assay design including controls, test validation, and algorithm for result interpretation, is critical.

Methods

DNA extraction

Extraction of cfDNA is a critical step in the fetal genotyping process. As cfDNA is fragmented, an extraction method must be suitable for small DNA fragments. Adamczyk *et al.* compared the BCSI SNAP card (Blood Cell Storage Inc.) and the MagnaPure large volume DNA isolation kit (Roche), both performed with an automated system (25). The BCSI SNAP card extraction is based on the binding of cfDNA on glass slides in the presence of chaotropic salts. The study demonstrated that this technique is a very efficient method for extracting fetal cfDNA.

Manfroi *et al.* tested three extraction kits QIAamp DNA DSP Blood Mini Kit, QIAamp DSP DNA Virus Kit and QIAamp Circulating Nucleic Acid Kit (Qiagen), all of which are used for manual extraction (26). They achieved the best extraction results with QIAamp Circulating Nucleic Acid Kit, which is specific for cfDNA. Londero *et al.* compared manual cfDNA extraction performed with the QIAamp Circulating Nucleic Acid Kit and automatic extraction accomplished with the QIAsymphony DSP Circulating DNA Kit (27). Both extraction methods were effective, but as they concluded, a manual method is more time-consuming and also more reliant on the competence of the operator than an automated process. Also, Ordonez *et al.* chose a steady automated system for routine use when they compared the manual QIAamp DSP Virus Kit and the automated COBAS AmpliPrep (Roche) method (28). Both methods were efficient and suitable for fetal cfDNA extraction.

In a recent study by Pedini *et al.*, four cfDNA extraction instruments with appropriate kits were compared (29). The kits tested were MagNA Pure 24 (Roche), IDEAL (IDSolution), LABTurbo 24 (Taigen) and Chemagic 360 (Perkin Elmer) with LABTurbo and IDEAL found to be slightly more efficient in fetal cfDNA extraction than the other two methods. However, they only studied five samples per each method, and the samples were not aliquots. Many instruments are only semi-automated and manual steps are therefore needed. If an instrument is not able to pipette plasma directly from a blood tube and plasma must be separated manually to another tube before DNA extraction process, a risk of errors such as sample mix-up is significant.

Genotyping methods

Several technological platforms are used for noninvasive fetal blood group genotyping. They are listed in *Table 1*.

Real-time PCR

The real-time PCR (qPCR) technique is based on collecting

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Table 1 Fetal genotyping assays by different technological platforms

Method	Target blood groups	Sensitivity (%)	Specificity (%)	Accuracy (%)	Total DNA control	Control for fetal DNA	Gestation weeks	Sample size	Reference
Sequencing	Kell			100			17–31	3	(30)
	ABO			100			12–35	26	(31)
	Rh, Kell, Fy, Jk and MNS			100			12–38	13	(32)
	RHD			100			9–30	8	(33)
ddPCR	RHD	100	100	100	GAPDH		12–36	35	(34)
	RHD	100	95.5–100	95.6–100		SRY+ TSPY1	28–30	46	(35)
	RHCE, Kell, Fy			100		SRY + RASSF1A	10–37	4–46	(22)
	Kell			94			12–35	32	(36)
Mass spectrometry	RHD			100		Y-STR	12–39	13	(37)
Capillary electrophoresis	RHD			100		52 SNPs	10–36	223	(38)
	RHD	95.2–98.6	100	97.9–99.4		AMELX/Y	7–23	337	(39)
	RHCE, Kell			100	CCR5		10–35	13–70	(40)
Real time PCR	RHCE	100	100	100	β-globin		12–28	46–100	(41)
	Rh, Kell	100	100	100	Albumin	SRY + set of 24 markers	7–38	49–168	(21)
	Rh, Kell	100	95.5–100	97.7–100	CCR5	SRY + set of paternal alleles	5–39	24–407	(42)
	ABO	96.9–100	88–97.7	93.2		RASSF1A + β-actin	12–25	73	(43)

No screening assays included; sensitivity, specificity and accuracy are given as by the authors in the references. ddPCR, droplet digital polymerase chain reaction.

data on fluorescence intensity in real time i.e., throughout PCR amplification. Fluorescence-labelled sequence-specific hydrolysis probes are at the core of the method. Amplification and detection are performed simultaneously with the same instrument, making this a fast, high-throughput method. Only basic PCR-laboratory equipment is needed. In addition, qPCR offers excellent sensitivity and specificity and thus, it is the preeminent technique to date.

A vast majority of *RHD* screening assays are based on qPCR. Recently, a number of large reviews of qPCR assays in fetal *RHD* screening have been published, for example Runkel *et al.* (20), Yang *et al.* (44) and Ontario Health Technology Assessment (45). These reviews demonstrate the high accuracy of laboratory developed qPCR tests which are widely used for *RHD* screening. Also, commercial kits are available including the newly published FetoGnost RhD real-time PCR assay (Ingenetix GmbH), which is not yet CE-approved (46). The other commercial qPCR kits are introduced below (CE-certified kits).

Besides *RHD*, genotyping of the *RHCE* gene alleles, and also *KEL*, is often requested in the care of immunized pregnancies. Finning *et al.* tested 70 samples for K, 13 for C, 44 for c and 46 for E (40). The accuracy was good, without any false-positive results among these 173 samples. However, one false-negative for K (sample of 17 gestation weeks) and altogether six inconclusive results were seen (samples of 12–18 gestation weeks). Gutensohn *et al.* genotyped samples taken at 12–28 gestation weeks using two different protocols for *RHCE* alleles (41). The sensitivity and specificity of the better-performing assay were 100% for C (46 samples), c (87 samples) and E (100 samples).

Scheffer *et al.* reported fully accurate diagnostic fetal genotyping results for D (168 samples), c (49 samples), E (85 samples) and K (60 samples) (21). The samples were

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drawn at 7–38 gestation weeks from immunized women and the results of only 11 samples were inconclusive. Orzińska *et al.* published diagnostic genotyping of D, C, c, E, and K blood groups for fetuses of immunized women (42). Only one false-positive result (K) was detected among 658 tested samples taken at 5–39 gestation weeks.

Song *et al.* genotyped prenatal samples (12–25 gestation weeks) from 73 women for ABO (43). They found the accuracy to be 83.9%, 96.8% and 100% when the fetus carried a paternally inherited A, B or O allele, respectively. In this ABO assay, allele-specific primers were utilized instead of more commonly used allele-specific probes. The aim was to avoid disturbance from maternal DNA by A and B allele-specific amplification. Cro' *et al.* designed a genotyping assay for Kell systems' alleles K1 and K2 (corresponding to K and k) (47). At the time of publication, they had just launched the assay but did not have any data from clinical samples.

CE certified RHD typing kits

At the moment, two commercial kits have CE certification (the European Directive 98/79/CE) in Europe and they both utilize qPCR technique. The Free DNA Fetal Kit RhD (Institute de Biotechnologies Jacques Boy) includes specific oligos for *RHD* exons 5, 7, 10 and for maize exon IVR2 (control) as well as positive and negative controls. Exogenous maize DNA is spiked to all samples before DNA extraction to function as an extraction and amplification control. The kit may be used from 11th gestation week. For example, Rouillac-Le Sciellour *et al.* (48), Manfroi *et al.* (26) and Londero *et al.* (27) have validated the kit with accurate results for targeting the anti-D prophylaxis. The other CE-approved option is the Devyser *RHD* kit (Devyser) which was developed in collaboration with Wikman *et al.* (49). This kit is designed for use from gestation week 10 and it is based on detecting just a single exon of the *RHD* gene (exon 4) with the *GAPDH* gene used as a control.

The situation regarding requirement of CE certification will change very soon, because a 5-year transition period of the Regulation (EU) 2017/746 on *in vitro* diagnostic medical devices (IVDR) will end 27th of May 2022. This new regulation restrains use of laboratory developed tests for the determination of the blood groups belonging to the ABO, Rh- (C, c, D, E, e), Kell, Kidd and Duffy -systems as they are classified to the highest risk category D. All the laboratories in the EU countries should utilize CE certified kits for fetal blood group allele genotyping in the above-mentioned blood group systems. New kits may be CE IVD certified as demand will grow. Interpretation of the Regulation 2017/746 is still unclear with regard to whether the requirement of CE certification also concerns fetal *RHD* screening tests. Wide group of experts agree, that fetal *RHD* screening for the purpose of targeting prophylaxis should be under the lower risk category C and the use of laboratory developed tests should be allowed in screening (50).

Mass spectrometry

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is based on differences in the mass of amplified sequences (51). Single allele-based extension reaction approach is an appropriate and sensitive method for the amplification of the fetal DNA sequence in the presence of abundant background maternal DNA. Li *et al.* set up an assay to detect KEL1 allele (corresponding K) with an accuracy of 94% (36). Bombard *et al.* (52), Tynan *et al.* (53) and Moise *et al.* (54) did pilot studies with a laboratory developed test, which later developed into a commercial test under the name of SensiGENE Fetal RHD Genotyping test (Sequenom). This multiplexed test detects three exons of the *RHD* gene. Moise *et al.* tested samples from over 400 pregnant women at three trimesters with >99% accuracy in a validation study of the test (55). In recent years, no studies on the mass spectrometry technique for fetal cfDNA genotyping have been published.

Droplet digital PCR (ddPCR)

ddPCR is based on the partitioning of a PCR reaction into thousands or even millions of uniform droplets. Each droplet is subjected to a separate quantitative PCR reaction. The aim is to dilute samples so that each droplet contains zero, a single or maximum a few copies of the template molecule. The starting concentration of the target is counted from the number of positive and negative droplets based on fluorescence using the Poisson statistics. ddPCR may not be the most convenient option for fetal *RHD* screening but the technique shows its power in fetal genotyping of blood groups which differ by only a single nucleotide. A cfDNA sample, containing a high abundance of maternal DNA, is diluted allowing the detection of just a few copies of paternal allele in fetal DNA (56). The advantages of the technique are that it is sensitive and accurate, enables the direct quantification of

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target molecules, and detects copy number differences, in addition to being tolerant to PCR inhibitors (57). ddPCR is technically more demanding and time consuming than qPCR, and therefore it is not commonly used for *RHD* screening purposes. Svobodová *et al.* have developed a ddPCR assay which is based on amplification of three exons of the fetal *RHD* gene (34). Sillence *et al.* proved that ddPCR is more sensitive than quantitative PCR, especially for suboptimal samples that contain a low fetal fraction of DNA (35). When the *RHD* gene of pregnant woman is not deleted but she carries an allele coding a variant RhD, detecting fetal DNA is even more difficult. Tsui *et al.* demonstrated (but with only two clinical samples) that digital PCR is able to amplify specifically *RHD* variant alleles differing by just one SNP (58). Recently, O'Brien *et al.* published a ddPCR genotyping assay for blood groups C, c, E (Rh), Fy and Kell and showed fully accurate results for 87 samples taken at gestation weeks 10–37 (22).

Sequencing

SNPs are technically challenging to detect accurately with PCR assays because, allele-specific amplification is no longer sufficiently specific after the several amplification cycles that are required to detect very low concentrations of fetal DNA among the abundant maternal DNA. Due to a low number of fetal DNA molecules in a sample, direct sequencing is not possible, and the amplification of the template is required. Unbiased PCR amplification is achieved by using a primer pair without allele specificity amplifying both antithetical alleles in same time (30). The strength of next-generation sequencing/massively parallel sequencing lies in possibility to multiplex the large number of samples and targets in a single test. While it is a sensitive method, its sensitivity depends on the sequencing depth. The remarkable homology between the blood group genes *RHCE* and *RHD* poses a significant challenge for detecting the difference between the alleles. Scrutinizing short haplotypes by sequencing enables fetal allele identification reliably (59). Because fetal fraction is crucial for the accuracy of the sequencing assay, it should be evaluated (60). As it is an inherent feature of sequencing (59) estimation of fetal fraction is relatively easy to implement. The recommended minimum fetal fraction is 4% (59). Sequencing as a technique is still rather laborious and, expensive and requires experience, and consequently the numbers of sequenced cases still remain small. However, the method is promising and under continuous development. Rieneck *et al.* have developed sequencing assays for Kell (30,61) and for ABO (31) blood groups from fetal cfDNA. Orzińska *et al.* succeeded in fetal blood group determination of the clinically most important blood group systems, Rh, Kell, Fy, Jk and MNS (32). Wienzek-Lischka sequenced indicative SNPs of these same systems but the assay was primarily intended for sequencing human platelet antigen 1, and the blood group alleles functioned as internal markers for fetal DNA (62). Takahashi *et al.* designed an assay based on sequencing to genotype *RHD*-positive D antigen-negative alleles in a population like Japanese where assays developed for deleted *RHD* are of no use (33).

Capillary electrophoresis

In this technique, fetal DNA is first amplified by specific primers and the resulting amplicons are then separated based on their size by capillary electrophoresis. The technique is more time-consuming than qPCR due to the separate electrophoresis step after amplification. In contrast to qPCR, no probes are needed. Kimura *et al.* were the first to test capillary electrophoresis in fetal *RHD* typing achieving accurate results, albeit with a small sample size (37). Doescher *et al.* exploited the possibility to multiplex PCR and used 52 SNPs as internal controls for indicating fetal DNA in *RHD* genotyping (38). A recent study, which also included samples from early gestation weeks compared endpoint quantitative fluorescent PCR with qPCR finding both methods sensitive and suitable for fetal *RHD* typing (39).

Controls used in assays

As the very low concentration of fetal DNA in a maternal sample is a significant risk for false-negative results, controls for the success of DNA extraction increase the assays' reliability. Especially in genotyping assays performed during the early gestation weeks, confirmation of the presence of fetal DNA is essential.

Markers specific to the father can indicate presence of fetal DNA (paternally inherited haplotype). One of the first controls utilized was the *SRY* gene in Y chromosome and it is still in use in many assays (*Table 1*) although its informativeness is solely limited to male fetuses. Other sex related markers include the genes *TSPY1* (35) and *AMELY* (39) on the Y chromosome.

Marker allele panels consist of a set of genetic markers that potentially differ between two individuals, in this context the mother and the father and consequently

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the fetus. The fetus may have inherited a few differing markers from the father and thus detection of these paternal markers indicates the presence of fetal DNA. Ideally, markers have an allele frequency of close to 50% but multiple markers are still needed. If several markers are tested, a sample from the father is not necessary but often both the mother and the father are genotyped. In Netherlands, if *SRY* and specific blood group allele are negative, a set of 24 markers is used to demonstrate fetal DNA in qPCR assay (21). This marker set consists of biallelic insertion/deletion polymorphisms (63). A set of 21 markers, including partly the same markers, is also used in a similar Rh and Kell assay in Poland (42). A capillary electrophoresis-based assay by Doescher *et al.* utilized a set of up to 52 SNPs to demonstrate the presence of fetal DNA (38).

One option is an epigenetic marker such as a tumor suppressor gene promoter *RASSF1A* which is said to be a universal fetal marker. This gene is hypermethylated in placental tissue but hypomethylated in maternal blood cells, thus there is epigenetic variation between the mother and the fetus. Maternal hypomethylated cfDNA may be removed by digestion with a methylation-sensitive restriction enzyme (64) and after that, only digestion-resistant fetal hypermethylated DNA is amplified. Because *RASSF1A* is not a direct fetal marker as such, a control for success of maternal DNA digestion is required. Chan *et al.* showed that the digestion efficiencies for hypomethylated *RASSF1A* and *beta-actin* sequences were equivalent (64). Thus, a *beta-actin* signal not detectable in a PCR assay indicates the completeness of the enzyme digestion. *RASSF1A* functions as a control, for example, in a ddPCR assay for Rh and Kell by O'Brien *et al.* (22).

For high-throughput *RHD* screening assays, the above-mentioned controls are either too laborious or, too expensive or have limited use. Additional targets increase the required sample volume if they are tested parallel. On the other hand, excessive multiplexing in the same reaction may interfere with amplification in qPCR assays. In a meta-analysis of 12 large screening assays, sensitivity was 99.9% (20), thus the frequency of false negatives is so low that it is acceptable to perform screening assays without a fetal DNA control. However, negative and positive run controls are needed in all PCR assays.

A total DNA control indicates the total amount of extracted cfDNA in a sample, including both maternal and fetal cfDNA. It is often used as a marker of the quality and quantity of the sample. Because the amount of total DNA is highly dependent on maternal DNA derived from lysed blood cells and maternal DNA may interfere with the detection of the fetal allele, a total DNA control may help to prevent false results. Basically, any genomic DNA sequence can function as a total DNA control. Examples of possible controls, including *GADPH*, *CCR5* and *albumin*, are listed in *Table 1*.

A process control is recommended, as it may serve as a control for DNA extraction and the overall amplification procedure. An exogenous DNA fragment is an option, for example maize DNA is used in The Free DNA Fetal Kit RhD (48). Besides, a synthetic DNA sequence, not belonging to any know organism is a feasible alternative. Exogenous DNA is added to samples before DNA extraction and detected by specific probes at the same time as the assay specific targets, so it is indicative of an appropriate process and reliable results. Homogeneous spiking of samples can be achieved with a pipetting robot or extraction instrument. A process control does not control fetal DNA directly, but its use is feasible and adequate in screening assays.

Quality assurance

An external quality assurance program is essential for quality management of all genotyping and screening assays. For example, Danish Institute for External Quality Assurance for Laboratories in the health sector (DEKS, https://deks.dk/en/laboratories/) provides an international external quality assurance scheme for fetal *RHD* genotyping from plasma samples (4,268 DK non-invasive fetal RhD genotyping). Also, private sample exchanging programs between two or a few laboratories are recommended to supplement external quality assurance.

The International Society of Blood Transfusion, which offers guidance and advice to users, established the cfDNA Subgroup on the Working Party of Red Cell Immunogenetics and Blood Group Terminology. In collaboration with international colleagues, the cfDNA Subgroup published recommendations for assay validation in order to secure clinical applicability of fetal genotyping (50). Recommendations are fully detailed and cover comprehensively issues from pre-analytics through performance evaluation, analytical detection limit, precision and robustness to quality assurance.

Conclusions

Fetal *RHD* screening is an established and widely used

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routine in many countries. The screening assays are extremely sensitive allowing the targeting of anti-D prophylaxis solely at women bearing an *RHD*-positive fetus, also after childbirth, eliminating the need for blood group typing of newborns. Fetal blood group genotyping in immunized pregnancies has replaced invasive methods, enhancing both fetal and maternal health. Blood group genetics and the overwhelming abundance of maternal DNA in a sample pose challenges for assays but recent technical advances have surpassed them, and the results are highly accurate.

For *RHD* screening assays, qPCR is distinctly the most commonly used method due to its accessibility and reliability. A wider selection of technical platforms is in use for fetal genotyping in immunized pregnancies. They all give accurate results, but exact comparisons are difficult due to preanalytical differences, small sample sizes, and differing gestation weeks of tested samples. Especially ddPCR and massively parallel sequencing seem promising genotyping methods for several blood group alleles already at early pregnancy.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editor (Frederik Banch Clausen) for the series "Blood Group Genotyping" published in *Annals of Blood*. The article has undergone external peer review.

Peer Review File: Available at https://aob.amegroups.com/article/view/10.21037/aob-21-41/prf

Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at https://aob.amegroups.com/article/view/10.21037/aob-21-41/coif). The series "Blood Group Genotyping" was commissioned by the editorial office without any funding or sponsorship. The author has no other conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/aob-21-41
Cite this article as: Haimila K. Overview of non-invasive fetal blood group genotyping. Ann Blood 2023;8:5.

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