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GUIDELINES

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BSH guideline for the estimation of fetomaternal haemorrhage

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1 | METHODOLOGY

This guideline was compiled according to the British Society for Haematology (BSH) process at [https://b-s-h.org.uk/media/lvdbh3p5/2023-11-14-bsh-guidelines-development-process-updated-nov-2023-1.pdf?cf=638356449664970000]. Grading of Recommendations Assessment, Development and Evaluation (GRADE) nomenclature was used to evaluate levels of evidence and to assess the strength of recommendations. The GRADE criteria can be found at http://www.gradeworkinggroup.org and are also summarised in an appendix of the full BSH process guidance above.

2 | LITERATURE REVIEW DETAILS

A search of published literature between January 2005 and August 2023 was undertaken using Ovid MEDLINE, Ovid EMBASE and Web of Science Core Collection databases, using the terms: Fetomaternal haemorrhage; FMH; transplacental haemorrhage; allogeneic D; D incompatible transfusion; D incompatible transplant; Kleihauer; acid elution; flow cytometry; FITC; anti-HbF; anti-D; HDFN; HDN; haemolytic disease of newborn; rhesus immune globulin; circulating cell-free fetal DNA; CCF-DNA; non-invasive prenatal; NIPT

3 | REVIEW OF THE MANUSCRIPT

Review of the manuscript was performed by the British Society for Haematology (BSH) Transfusion Task Force and the BSH Guidelines Committee. It was also on the members section of the BSH website for comment.

Wherever possible, other BSH transfusion guidelines are cross referenced in this document to avoid duplication of information and potential inconsistency between guidelines. Ensure that the current versions of the BSH guidelines are referred to at all times.

4 | PURPOSE AND OBJECTIVES

The purpose of this guideline is to provide healthcare professionals with clear practical and technical guidance for estimating the volume of target red blood cells (RBCs) in an individual's circulation when using acid elution (AE) and flow cytometry (FC) methods. It is laboratory focused and recognises the difficulties involved in interpreting AE slides and the limitations of both the AE and FC techniques.

The emphasis is on accurate Feto-Maternal Haemorrhage (FMH) estimation in D negative females to ensure that a sufficient dose of anti-D immunoglobulin (Ig), in addition to any routine antenatal anti-D Ig prophylaxis (RAADP) is administered and maternal alloimmunisation prevented.[1] There is continued emphasis on the confirmation of FMH greater than 2 mL, the follow-up of FMH greater than or equal to 4 mL

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and the timely and effective communication of results to ensure appropriate administration of anti-D Ig prophylaxis. It also makes recommendations for FMH estimation in D positive females and the quantification of D positive RBCs in D negative individuals following transfusion or solid organ transplantation.

The key objectives are to provide guidance on:

- When testing is required
- Sample requirements
- Practical and technical aspects for performing screening and quantification by AE and FC methodologies
- The calculations required to determine the volume of fetal RBCs
- When to apply the uncertainty of measurement (UoM) to results
- The management of cases recognising the need for prompt investigation, reporting of results and follow-up sampling
- The limitations of each technique
- Resolving discrepancies between AE and FC results
- Reporting FMH results

This guideline does not include information on sample acceptance criteria, sample storage or the use of anti-D Ig to prevent sensitisation of D negative females of child bearing potential to the D antigen.

Readers must cross reference the current versions of other related guidelines and in particular the BSH guideline for use of anti-D immunoglobulin for the prevention of haemolytic disease of the fetus and newborn,[2] the BSH guideline for the investigation and management of red cell antibodies in pregnancy,[3] the BSH guideline for compatibility procedures in blood transfusion laboratories[4] and the BSH guideline for the administration of blood components.[5] All are available at https://b-s-h.org.uk/guidelines.

The full guideline with appendices can also be found on the BSH guidelines website.

5 | USE OF THE TERM GENDER

The writing group would like to clarify the use of terms relating to gender as applied to clinical transfusion practice. When the term gender is used in this guideline, we are referring to the genetic gender of the patient that is the sex at birth.

Transfusion laboratories should ensure that they are aware of, and align with, their local guidelines regarding the recording of gender at the point of patient registration. But equally, those responsible for patient registration guidance should be made aware of patient safety issues as they relate to blood transfusion samples. It is important to note that it is not legally permissible to record that a patient is transgender on an IT system without their explicit permission as it is a protected characteristic under the Equality Act (2010). Software providers can be approached to provide system upgrades to enable rules-based selection of components and application of anti-D prophylaxis to be applied on a case-by-case basis. It may be necessary to update the guideline when national guidance on the use of the term gender becomes available.

6 | INTRODUCTION

FMH may occur during pregnancy or at delivery. If the fetus / baby is D positive, D negative females can be sensitised to the D antigen. In subsequent pregnancies, this can result in haemolytic disease of the fetus and newborn (HDFN). In D negative females, it is important to assess the volume of FMH to determine the dose of anti-D Ig required to prevent sensitisation.

There is a risk of allo-immunisation to D antigen if screening does not correctly identify the presence of D positive fetal RBCs. This will result in samples not being referred for confirmation and quantification testing and potential failure to provide adequate anti-D Ig.

In the UK, the most common techniques for the screening and quantification of FMH are the AE method, a modification of the Kleihauer-Betke test[6] and FC.[7–10] The AE method is based on the different properties of haemoglobin F (HbF) in fetal RBCs and haemoglobin A (HbA) in adult RBCs. FC techniques are based on the detection of a minor population of fetal RBCs using anti-D and anti-HbF conjugated fluorochromes.

The AE method is most suited as a screening method for FMH and initial quantification of the volume of fetal RBCs, if a FMH is detected. The FC method is the recommended reference method to confirm and accurately quantify the volume of FMH. Other methods of estimating FMH have been described, but they are not in common use in the UK and do not provide accurate quantification.[11]

There is interest in using FC as an initial screening test for FMH when resources allow and appropriate advice has been included in these guidelines. Performance in UK NEQAS exercises has shown FC to be sufficiently sensitive to accurately detect FMH at a volume of ≤2 mL of packed fetal RBCs (0.1% fetal red cells) (K. Veale, UK NEQAS, personal communication 20/07/2023).

7 | KEY RECOMMENDATIONS

Recommendation 1: A FMH test must be performed following birth or a potentially sensitising event (PSE) after 20 weeks gestation to ensure that all necessary measures are taken to minimise maternal sensitisation to the D antigen and hence reduce the incidence of HDFN. (Section 8.2) Grade 1A.

Recommendation 2: The maternal EDTA blood sample should be taken at least 30 min after birth to ensure any fetal RBCs are distributed within the maternal circulation. (Section 9) Grade 2C.

Recommendation 3: A separate maternal EDTA sample should be obtained for FMH estimation that has not been used for other tests. When only one sample is available, the FMH test should be performed first. (Section 9) Grade 1B.

Recommendation 4: Tests should be performed to distinguish between the cord sample and the maternal sample where the ABO and D group on both is the same. (Section 9) Grade 2A.

Recommendation 5: Controls must be prepared using ABO compatible blood samples which have no history of irregular red cell antibodies and have a low risk of a raised HbF. (Section 11.2) Grade 1A.

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Recommendation 6: Controls should be performed with each batch of AE slides stained and should be treated in exactly the same way as the maternal sample. (Section 11.2) Grade 1A.

Recommendation 7: AE slides should be thoroughly washed using de-ionised or distilled water. Tap water should not be used. (Section 11.4) Grade 1A.

Recommendation 8: For manual AE quantification, it is essential to survey a minimum of 10 000 adult RBCs to achieve a reasonable coefficient of variation. (Section 11.8.2) Grade 1A.

Recommendation 9: When performing the AE technique, a historical assumption of the number of maternal 'ghost' cells per field must not be relied upon for FMH estimation. (Section 11.8.2) Grade 1A.

Recommendation 10: The sample should be referred for quantification of D positive fetal RBCs by FC if the FMH quantification is ≥2 mL by the AE method. (Section 11.9) Grade 1A.

Recommendation 11: An anti-D marker should be used for FC quantification when the FMH result is used to calculate the prophylactic anti-D Ig dose to be recommended. (Section 13.2) Grade 1A.

Recommendation 12: For FC quantification, a second marker should be used to exclude auto-fluorescent neutrophils from the calculation for FMH estimation. (Section 13.2) Grade 1A.

Recommendation 13: For FC quantification, at least two separate aliquots of whole blood should be taken from the sample and tested in parallel. (Section 13.3) Grade 1A.

Recommendation 14: The original maternal EDTA sample, screened by AE or FC, should be used for confirmatory FMH testing by FC. (Section 13.3) Grade 1A.

Recommendation 15: Controls for FC should include mixtures of D positive (R1r) and D negative RBCs. (Section 13.4) Grade 1A.

Recommendation 16: Controls for FC should discriminate between clinically significant bleed volumes, e.g., 1% D positive red cells; 0.2% D positive red cells as well as a larger FMH volume, e.g., 10% D positive red cells (220 mL). (Section 13.4) Grade 2C.

Recommendation 17: For FMH ≥2 mL, if FC quantification testing delays the administration of prophylactic anti-D Ig to >72 h post PSE, the AE quantification result should be used to calculate the appropriate level of anti-D prophylaxis. (Section 13.8) Grade 1A.

Recommendation 18: If the FC quantification test result is not expected to be available to determine the level of anti-D Ig administration, it is recommended that the AE quantification is repeated by a second operator before the sample is referred for FC quantification. (Section 13.8) Grade 1B.

Recommendation 19: Where a discrepancy between the AE and FC result remains following repeated testing, if possible, the D type of the fetus/neonate should be determined either by cell-free fetal DNA *RHD* genotyping or RBC D typing by serology. (Section 15.4) Grade 1A.

Recommendation 20: Staff should be trained to identify possible causes of discrepant results, e.g., D variant RBCs, hereditary persistence of fetal haemoglobin, errors in sample storage, sample preparation, assay technique or the limitations of each test. (Section 15.4) Grade 1A.

Recommendation 21: There must be a documented process in place for the management of anti-D Ig prophylaxis when discrepant results between AE and FC techniques occur. (Section 15.4). Grade 1A.

Recommendation 22: For an unresolved discrepant result between AE and FC, there should be a process in place to report an incident through a quality management system, investigate the discrepancy, and manage the case through discussion with a specialist clinical/laboratory staff member. (Section 15.4) Grade 1A.

Recommendation 23: When a repeat maternal sample is required, it should be taken and tested by a FMH screening test 72 h after the total dose of anti-D immunoglobulin by intramuscular injection (48 h if the anti-D Ig was given intravenously). This allows sufficient time for anti-D Ig to take effect and to check the volume of remaining fetal RBCs. (Section 17.2) Grade 1A.

Recommendation 24: For continuous uterine bleeding after 20 weeks' gestation, a FMH screening test should be performed every 2 weeks,[12] irrespective of previous repeat sampling following clinically significant bleed volumes. (Section 17.3) Grade 1A.

Recommendation 25: Where an estimated FMH exceeding 2 mL is detected by AE, a preliminary result should be communicated to the clinical team as soon as possible, prior to receiving the results of the confirmatory test, to alert clinicians to the possible need for additional anti-D Ig prior to discharge. (Section 19) Grade 1A.

Recommendation 26: When considering possible intravenous administration of anti-D Ig, refer to the BSH guideline for the use of anti-D Ig for the prevention of haemolytic disease of the fetus and newborn.[2] (Section 19) Grade 1A.

8 | PRINCIPLES OF FMH TESTING

8.1 | A test for FMH estimation should be undertaken on D negative females[12]

- Following the birth of a D positive baby.
- Following all potentially sensitising events after 20 weeks gestation where the fetal D group is unknown or has been predicted to be D positive by cffDNA *RHD* screening.

FMH tests should be completed and anti-D Ig administered within 72 h of a potentially sensitising event.[2]

8.2 | Tests for FMH estimation are not required[12]

- When the sensitising event is before 20 weeks gestation.
  The fetal blood volume is approximately 30 mL with a fetal packed red cell volume of 10–12 mL. Only a very small proportion crosses into the maternal system, even after a sensitising event – typically ≤0.1 mL of packed red cells.[13,14]
- When it has been conclusively established that immune anti-D is present.

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- When the fetus is predicted to be D negative according to the cffDNA *RHD* screen. (Refer to Section 18).
- When the baby is known to be D negative.
- When D positive status is confirmed (exceptions detailed in Section 8.3).

If there is any doubt of the maternal D typing result, arrange further testing at a reference laboratory and treat as D negative until the results of these tests are available.[3,4]

Refer to Appendix C.

RECOMMENDATION 1. A FMH test must be performed following birth or a potentially sensitising event (PSE) after 20 weeks gestation to ensure that all necessary measures are taken to minimise maternal sensitisation to the D antigen and hence reduce the incidence of HDFN. Grade 1A.

8.3 | FMH estimation in D positive females may be clinically useful in the following circumstances where identifying FMH may change clinical management

- Intrauterine fetal death/stillbirth[15,16]
- Trauma[16,17]
- Unexplained neonatal anaemia[16]
- Post intrauterine transfusion (IUT)/fetal blood sampling[2,18]

Refer to Section 21.

9 | SAMPLE REQUIREMENTS

Maternal sampling should be undertaken when sufficient time has elapsed to allow fetal RBCs to be distributed within the maternal circulation following delivery, manual removal of placenta, or other sensitising event, including the use of intraoperative cell salvage at delivery. A minimum of 30 min is considered adequate.[2] Contemporary studies obtained postpartum samples ~30–60 min after delivery and fetal cell kinetics demonstrated the highest detectable levels of FMH at ~30 min with subsequent decline. Sampling should ideally occur promptly (preferably within 2 h) and before discharge.[19–21]

RECOMMENDATION 2. The maternal EDTA blood sample should be taken at least 30 min after birth to ensure any fetal RBCs are distributed within the maternal circulation. Grade 2C.

Two maternal EDTA samples should be obtained; one for FMH estimation and a separate sample for ABO and D grouping. The sample must be suitable for testing with no evidence of aggregates or RBC lysis following visual inspection.

If only a single sample is available, the FMH test must be performed first, particularly where manual grouping techniques are used. Centrifugation will separate RBCs according to density with larger, less dense fetal RBCs remaining closer to the plasma: RBC interface.[22] If these are sampled when testing the maternal blood group, there is a theoretical risk that a FMH test performed on the same sample could underestimate the true FMH. There is also a remote possibility that inconsistencies in maternal blood grouping could occur if there is a large FMH. In addition, centrifugation makes thorough mixing for subsequent FMH testing more difficult.

RECOMMENDATION 3. A separate maternal EDTA sample should be obtained for FMH estimation that has not been used for other tests. When only one sample is available the FMH test should be performed first. Grade 1B.

At birth, a cord blood sample should be obtained from the placenta/umbilical vessels of the baby born to all D negative females to establish ABO and D group of the baby.

In addition to the required patient identifiers,[3] the samples should be clearly marked 'Cord' and 'Maternal' as appropriate. The request forms / orders should contain relevant clinical details and date and time of delivery or sensitising event.

Correct identification of the cord blood sample is critical. SHOT reports show that there are frequent near miss events where cord and maternal blood samples are mislabelled or transposed.[23] Maternal and cord samples are often taken at the same time by the same member of staff and there is considerable potential for sample transposition. If the maternal and cord ABO and D group are the same, an alternative method is required to ensure that a sample transposition has not occurred. Methods include a full blood count, the mean cell volume (MCV) being higher in the fetal sample[24] or an alkali denaturation test (Apt-Downey) to distinguish between fetal and maternal haemoglobin.[25,26]

If cord blood is unavailable, consideration should be given to obtaining a neonatal sample for blood grouping or utilisation of the cffDNA *RHD* genotyping result, if available. If this is not possible, then it should be assumed that the baby is D positive for the purposes of FMH determination and the administration of anti-D Ig prophylaxis.

RECOMMENDATION 4. Tests should be performed to distinguish between the cord sample and the maternal sample where the ABO and D group on both is the same. Grade 2A.

10 | QUALITY ASSURANCE

Laboratories performing FMH estimation should be accredited and operate under an effective quality management system with compliance demonstrated through participation in an accredited external

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quality assessment scheme, for example, UK NEQAS. Points for consideration:

- Equipment used as part of the testing procedure must be maintained and calibrated.
- All commercially available test kit manufacturer's instructions should be followed and reagents should be IVD-CE, CE UKNI or UKCA marked. Where unavailable, a risk impact assessment, as well as validation of the test should be completed before use. Techniques should meet the Medicines and Healthcare products Regulatory Agency (MHRA) requirements.
- All test kits and reagents should be validated before introduction into routine use.
- Laboratories must have a documented procedure for the acceptance of test kits and reagents.
- Any changes to the manufacturer's instructions resulting in an alteration to the testing method and any in-house methods should be validated before use.
- All laboratory testing procedures must be validated in compliance with published standards of accreditation.[27]
- Staff must be trained and have competency assessments relevant to the appropriate standard operating procedures.
- The validity of FMH test results must be verified by the acceptance of quality controls before results are reported.
- Sample tracking should be employed to allow reconciliation of FMH samples arriving into the laboratory, the completion of a FMH screening test and timely referral for quantification/confirmation testing.

11 | ACID ELUTION TECHNIQUE FOR ESTIMATION AND QUANTIFICATION OF FMH

11.1 | Principle

HbF is more resistant than HbA to both alkali denaturation and acid elution. When a dry maternal blood film is fixed and then immersed in an acid buffer solution, HbA is denatured and eluted, leaving RBC 'ghosts'. HbF is resistant to denaturation and elution and therefore fetal RBCs stain pink and stand out in the sea of maternal 'ghost' cells. There are many factors that influence the quality of the results; timings of elution and counterstaining, spreading of films and mixing of samples. Therefore, a meticulous technique and strict timing are essential. Additionally, white blood cells (WBC), especially lymphocytes and also adult RBCs containing HbF may stain darkly and be misinterpreted as fetal RBCs, compromising test interpretation.[28]

11.2 | Controls

Controls should be prepared to simulate FMH samples by adding aliquots of cord blood to adult whole blood samples. The adult whole blood and cord blood samples should be ABO compatible with no history of irregular red cell antibodies present. Selecting normal adult male blood samples will negate the risk of selecting a sample from a pregnant individual. The HbF content of adult RBCs is raised during pregnancy.[29–31] Refer to Section 11.10.

RECOMMENDATION 5. Controls must be prepared using ABO compatible blood samples which have no history of irregular red cell antibodies and have a low risk of a raised HbF. Grade 1A.

Control samples should be stored at 2–8°C for a maximum of 4 days, but fresh phosphate buffered saline (PBS) dilutions and slides should be made each time a batch of tests is performed. Deviations from this may be used provided they have been validated according to ISO 15189 standard.[27] Pre-made control samples should be mixed well prior to film preparation as per Section 11.3.

Negative control: Fresh EDTA adult whole blood.

Positive control: Fresh EDTA cord blood added to fresh adult whole blood (as above) to give a dilution of 1:100.

RECOMMENDATION 6. Controls should be performed with each batch of slides stained and should be treated in exactly the same way as the maternal sample. Grade 1A.

11.3 | Slide preparation

Samples should meet the requirements as described in Section 9.

All samples must be mixed thoroughly prior to dilution for 10 min on a rotary mixer or for an equivalent time when using alternative methods.

Thin films are best achieved by diluting an aliquot of the maternal whole blood sample. This is typically a whole blood: PBS dilution (1:2 or 1:3) that is gently mixed. An anaemic sample may require a modified dilution or spreading technique to achieve a film of appropriate thickness.

Thin blood films should be freshly made on clean dry slides; slides may require degreasing prior to use.

The thickness of the blood film is important and should result in RBCs touching but not overlapping when examined under a microscope. If films are too thick, overlapping cells may prevent proper elution, resulting in adult RBCs taking up some of the counterstain.

A modified technique where half of the slide is acid eluted can be used to demonstrate effective HbA elution technique, which is visualised after staining[32] (Figure 1).

11.4 | Staining

Staining should be performed according to the manufacturer's instructions for use, or an alternative validated technique according to ISO15189 standard.[27]

The effectiveness of staining is dependent on several factors including: temperature of the reagents, age of the sample, age of the

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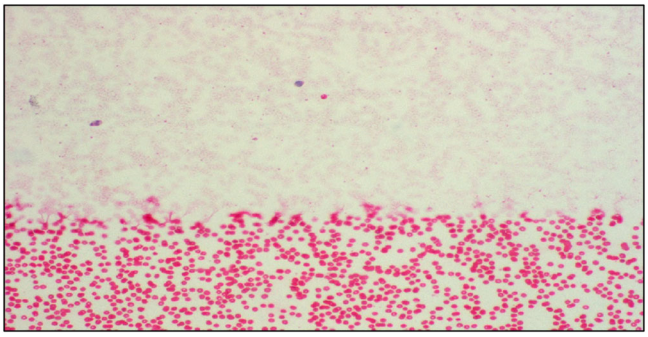

FIGURE 1 Acid elution slide using a modified technique where half the slide is eluted.[32]

stain, pH of the stain, pH of the water used for washing, timing of fixing, elution, and staining.

The washing stages should be thorough; clearer films may be obtained using de-ionised water or distilled water. The back of each slide should be wiped after staining to remove any excess stain deposit and it should be dried in an upright position.

RECOMMENDATION 7. The AE slides should be thoroughly washed using de-ionised or distilled water. Tap water should not be used. Grade 1A.

11.5 | Examination of the stained films

It is recommended that this is divided into **screening** (Section 11.6) and **quantification** (Section 11.8).

Controls should be examined first to ensure that the preparation and staining are satisfactory. If the controls do not show the expected result, the whole process must be repeated.

The ideal microscope settings for the examination of FMH slides may be different to the ideal settings for the examination of haematology morphology slides. It may be helpful to lower the condenser and to increase the light intensity on the microscope. A blue filter may be useful for differentiating white blood cells.

Select an area of the film where cells are evenly distributed and in a monolayer, usually near the thin end (feather edge).

Due to the elution of HbA from maternal RBCs, counting accurate numbers of 'ghost' cells can be difficult. A modified technique can improve visibility of maternal RBCs for counting, where half of the slide is eluted and then the entire slide stained[32] (Figure 1). Any alternative techniques should be validated according to ISO15189 standard.[27]

11.6 | Basic screening

1. Screen the control slides and the test slides using a ×10 eyepiece and a ×***10 objective*** to check for adequate staining and even distribution of fetal RBCs, if present.
2. Examine 25 low power fields. If any fetal RBCs are seen, then quantification must be performed.

11.7 | Semi-quantitative screening

A fully validated and controlled method should be used. Initial validation should be performed to ensure that the area viewed in a low power field (×10 eyepiece and ×***10 objective)*** is at least 16 times greater than that in a high power field (×10 eyepiece and ×***40 objective)*** (Figure 2).[33,34]

To use this semi-quantitative method, the power of the lenses used MUST be as described in the method.

1. Scan the control slides and the test slides using a ×10 eyepiece and a ×***10 objective*** to check for clear staining and even distribution of fetal RBCs, if present
2. Select an area of the film where the adult cells are touching but not overlapping and count the ***adult*** cells using a ×***40 objective***.

- ***If there are more than 100 adult cells in one high power field*** then proceed to count the fetal RBCs as per step 3.
- ***If there are fewer than 100 adult RBCs in one high power field***, either remake the film or proceed to full quantification if any fetal RBCs are seen in 25 low power fields as per Section 11.8.

3. Under low power field × **(10 objective)**, examine 25 fields and count the ***fetal*** RBCs.

- ***If a total of 10 or more fetal RBCs are seen***, then quantification must be performed (see Section 11.8).
- ***If fewer than 10 cells are seen*** in total then it can be assumed that the volume of fetal cells is less than 2 mL. This will be covered by the standard anti-D Ig dose and no further action is required (see Section 19 on how to report results).

11.8 | FMH quantification by AE

If using the semi-quantitative screen (Section 11.7) and 10 or more fetal RBCs are present in 25 low power fields, quantification should be performed.

If the semi-quantitative screen is not used, then quantification should be performed when ***any*** fetal RBCs are seen during examination of the slides.

For accurate quantification, it is recommended that a minimum of 10 000 maternal RBC 'ghosts' are examined using a ×***40 objective***. The use of a Miller square graticule (Figure 3) or an indexed grid is necessary to obtain an accurate ratio of fetal to maternal RBCs in each blood film.

11.8.1 | Using a miller square graticule

1. Using a ×10 eyepiece and ×40 ***objective***, select an area of the film where cells are touching but not overlapping.
2. Count **adult** RBCs (*A*) in the small square (Figure 3), including all cells that overlap the left hand or upper edges but not those overlapping the right hand or lower edges.

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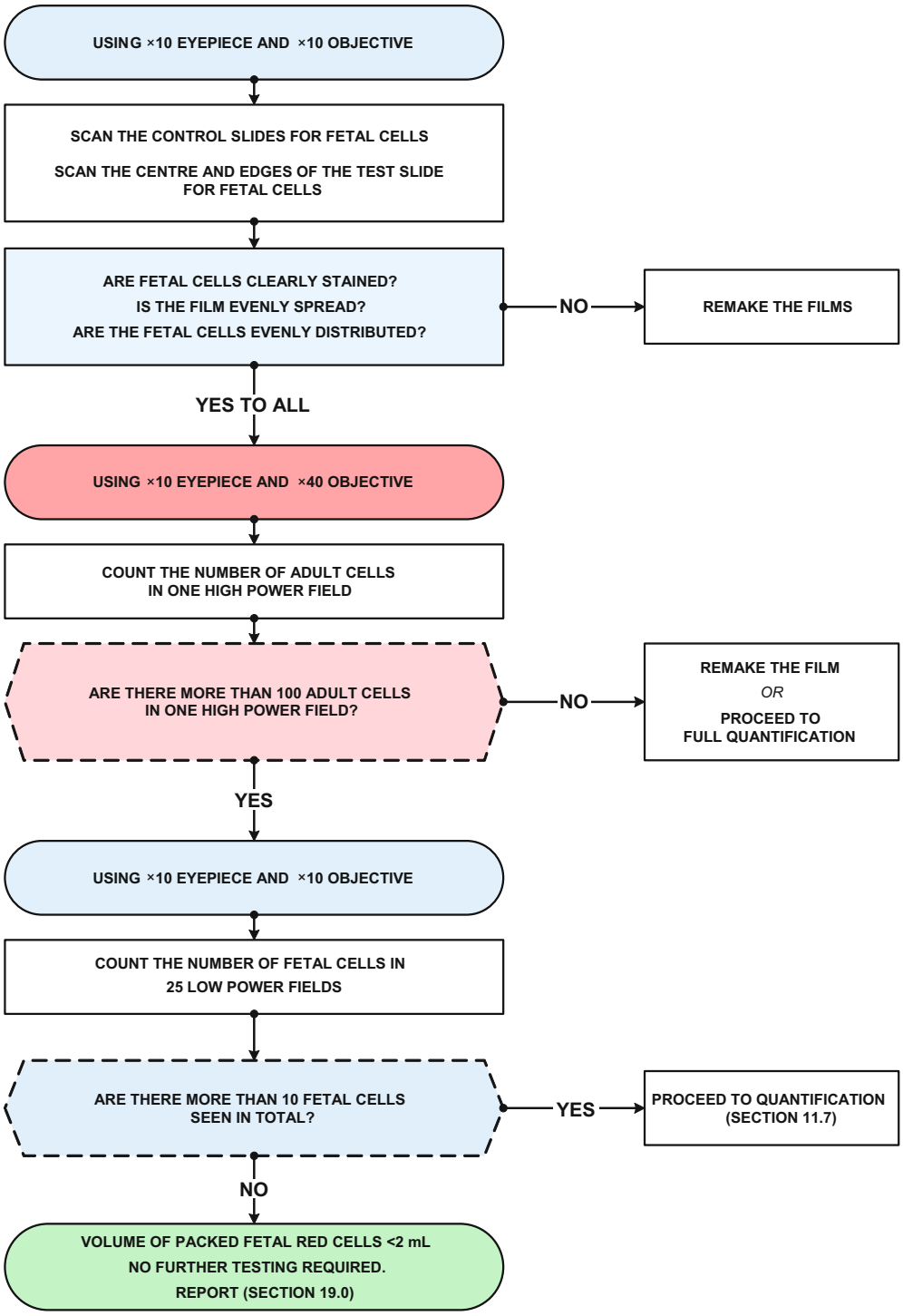

FIGURE 2 Flow diagram for semi-quantitative FMH screening by acid elution. **Important: Microscope objectives other than those stated below must not be used for this semi-quantitative screening method.**

3. Count **fetal** RBCs (*F*) in the large square (Figure 3), treating cells overlapping the edges as above.
4. Move across the slide so that the next area to fall within the counting grid is contiguous with the preceding one and repeat the procedure. For example, move in a staircase like motion, up and right and do not include cells already counted.
5. Count until 1111 adult cells have been counted in the small square (representative of 10 000 in the large square).
6. Assume the total adult cells scanned = $A \times 9$ and total fetal cells counted = F.
7. Calculate the FMH using the formula described by Mollison[35] (see Section 11.8.3).

11.8.2 | Using an indexed square

The procedure is as above except that adult cells are counted in 10 of the 100 squares visible in a 10 × 10 grid.

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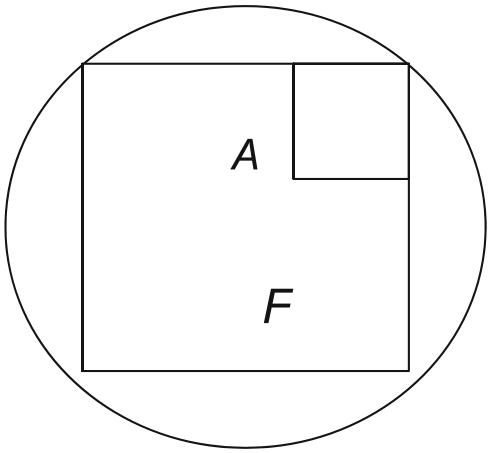

FIGURE 3 Miller square.

Count until 10 000 adult cells have been counted (1000 in the 10 squares being counted).

Assume the total adult cells scanned =

$A \times 10$ and total fetal cells counted = F.

RECOMMENDATION 8. For manual AE quantification, it is essential to survey a minimum of 10 000 adult RBCs to achieve a reasonable coefficient of variation (CV). Grade 1A.

RECOMMENDATION 9. A historical assumption of the number of maternal 'ghost' cells per field must not be relied upon for FMH estimation. Grade 1A.

11.8.3 | Calculation of FMH for the AE method

The packed fetal RBC volume should be calculated using Mollison's formula,[35] which assumes that:

The maternal RBC volume is 1800 mL.

Fetal RBCs are 22% larger than maternal RBCs.

Only 92% of fetal RBCs stain darkly.

$$\text{FMH(mL packed fetal RBCs)} = \frac{\text{Number of Fetal Cells Per High Power Field}}{\text{Number of Maternal Cells Per High Power Field}} \times 1800 \times \frac{1.22}{100} \times \frac{100}{92}$$

Or can be simplified to:

$$\text{FMH(mL packed fetal RBCs)} = \frac{\text{Number of Fetal Cells Per High Power Field}}{\text{Number of Maternal Cells Per High Power Field}} \times 2400$$

For example, if the number of fetal RBCs is 45 and maternal RBCs 10 000, then the fetal bleed will be calculated as follows:

$$\frac{45}{10000} \times 2400 = 10.8\,mL\ packed\ fetal\ RBCs$$

11.9 | Referral for confirmation by FC

UK NEQAS FMH exercise reports demonstrate the accuracy of counting low frequency events is associated with a higher variability by AE compared with FC techniques (refer to uncertainty of method median data, UK NEQAS reports 2023). The semi-quantitative AE screening method uses set objective lenses to identify and count a defined number of fetal RBCs in 25 low power fields (LPF), and full quantification must be performed if more than 10 fetal RBCs per LPF are observed. This is equivalent to an FMH of 2 mL of packed fetal RBCs. Therefore, the sample should be referred for quantification by FC if the FMH is ≥2 mL by the AE method[36] (Section 13).

RECOMMENDATION 10. The sample should be referred for quantification of D positive fetal RBCs by FC if the FMH is ≥2 mL by the AE method. Grade 1A.

11.10 | Testing in females with elevated HbF

The HbF content of maternal RBCs increases during pregnancy, mainly in the second trimester. This may cause problems with FMH estimation by the AE method.[21,29] Increased levels of HbF are also seen in various genetic disorders including thalassaemia, sickle cell disorder and hereditary persistence of fetal haemoglobin (HPFH). These false positive results are often characterised by a large variation in the density of staining in the RBCs containing HbF. There may be no clear distinction between fetal RBCs and these 'intermediate' stained cells. A standard dose of anti-D Ig should be given and the sample referred for FMH estimation by FC. An anti-D fluorochrome should be used to determine the level of D positive fetal RBCs present (see Section 13).

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12 | USE OF AUTOMATED SYSTEMS FOR AE CELL COUNTING

Manual analysis of AE slides is dependent on operator experience and can suffer from inter-observer and inter-laboratory variability.[30] Use of automated microscopes, computer vision algorithms to extract cell features, and machine learning models to distinguish fetal RBCs from maternal RBCs have been shown to improve efficiency, accuracy, and precision for FMH quantification when compared to the manual reading of AE tests.[37–40] Additionally, images of the AE slide can be stored and shared for recounting and can also be used for visual verification.

New automated methodology must be implemented through an established Quality Management System under Good Automated Manufacturing Practice (GAMP) principles.[41] A risk assessment must be performed and the system validated before being introduced into routine clinical use.

For routine testing, appropriate test controls must be used. Data migration and data back-up including verification must be performed where applicable to ensure data integrity of clinical results.

13 | METHODS OF FMH ESTIMATION—FLOW CYTOMETRY

This section describes the requirements for FMH estimation by FC and includes recommendations for using FC for FMH screening as well as for FMH quantification. A summary of the requirements for FC screening and FC quantification (confirmation) is given in Table 1. Specific guidance relevant to FC screening is provided in Figure 4.

TABLE 1 Summary of the requirements for FC Screening and FC quantification (confirmation).

	FC screening	FC quantification (Confirmation)
Fluorochromes	Anti-D Anti-HbF Anti-CD66b/Anti-CD45 (neutrophil/white cell marker)	Anti-D Anti-CD66b/Anti-CD45 (neutrophil/white cell marker)
Number of aliquots per patient sample	2	≥ 2
Controls	0.2% and 1% fetal RBC or D positive control. End of batch test control	10%, 1% and 0.2% D positive control.
Mollison's calculation	Yes	Yes
Result used to calculate level of anti-D Ig prophylaxis	No	Yes

13.1 | General principles

If the FMH result is to be used to calculate the level of anti-D prophylaxis required, an anti-D fluorochrome reagent should be used to label the D positive fetal RBCs (Table 1).

An HbF marker may detect and quantify all fetal RBCs and potentially any maternal F cells (maternal RBCs containing HbF) present, regardless of their D type.[42,43] The number of F cells and level of HbF present in maternal RBCs may increase during pregnancy.[21] If an HbF marker is used for these individuals or for an individual with hereditary persistence of HbF, the FMH result may be falsely elevated. This has an associated risk of inappropriate administration of anti-D Ig to D negative females following a sensitising event.

An HbF fluorochrome should only be used for supplementary FC testing to provide a resolution to discrepancies between AE results and FC results using an anti-D fluorochrome (Refer to Section 15).

An HbF fluorochrome may be used for FC screening, provided follow-on FC quantification for the purposes of anti-D administration is performed using an anti-D fluorochrome (Table 1).

FC results obtained using an anti-D fluorochrome reagent should not be used to assign a D phenotype to fetal RBCs and are not a replacement for D phenotyping of the cord sample at birth. Refer to Section 15.1.

WBC markers, or auto-fluorescence when fixation is employed, may be used to exclude white blood cells from the calculation for FMH (Refer to Section 13.2).

During validation, the robustness and limitations of any new FC screening assay must be fully established before use, determining an appropriate test batch size and appropriate use of controls.

All reagents must be fully validated according to ISO15189 before use. Appropriate flow cytometer instrument settings must be determined to ensure good separation between: D positive and D negative RBC populations; fetal RBC and adult RBC populations; RBC and white blood cell populations as appropriate. If there is overlap in the emission spectrum of the selected fluorochromes and they are detected on the same emission wavelength, compensation should be performed as per manufacturer's instructions.

Samples should be mixed thoroughly prior to testing. This should be for the equivalent of 10 min on an orbital mixer. Aliquots of whole blood should be removed immediately after mixing and receive a minimum of one wash before investigation. Do not use an automated cell washer. The loss of RBCs must be prevented and aliquots should be centrifuged adequately to ensure that all RBCs are sedimented (e.g.1000×*g* for 2 min). Centrifugation should be with a swing out, not fixed or semi-fixed rotor. Care should be taken when aspirating the wash solution to not disturb the pellet; do not decant.

13.2 | Selection of fluorochromes

A fluorochrome conjugated IgG monoclonal anti-D, known to have high avidity for the D antigen, is recommended. This information can be found in the manufacturer's reagent data sheet. When selecting reagents, consideration should be given to the specificity of the anti-

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Controls for FC Screening

If an HbF fluorochrome is used for FC screening the controls must consist of mixtures of fetal and adult RBCs. It is recommended that as a minimum, each batch of screening tests includes a 0.2% and 1% fetal RBC control. A 1% fetal RBC control sample can aid in protocol creation.

The number of test samples in a FC screening test batch has the potential to be greater than the number of test samples required for a FC confirmation test. This has an increased risk that artefacts relating to test procedures will detrimentally impact test results. To mitigate this risk, laboratories should consider the use of an 'end of batch test' 0.2% control to identify any such impact. Where used, this control must be the last tube set up and analysed during the test process. If the 0.2% 'end of batch test' control fails, testing must be repeated from sampling.

An isotype and fluorochrome matched inert control is not required when performing FMH screening by FC if a reagent that allows the exclusion of WBCs (particularly neutrophils) is used.

FIGURE 4 Guidance for flow cytometry screening.

D; ideally it should react with all D phenotypes capable of stimulating the production of immune anti-D. It is recognised that reagents currently available may not meet this criterion, but it is important that the performance characteristics and specificity of the anti-D reagent selected are known. The possibility that the anti-D reagent may not bind to or only bind weakly to D variant fetal RBCs should be taken into account when interpreting results.[44,45] This may cause discrepancies between screening (AE/FC) and FC quantification results (Refer to Section 15).

Changes in the maternal immune system during pregnancy result in a higher than normal WBC count, predominantly driven by a rise in neutrophils.[46,47] This has been identified as a cause of interference during FC investigation of FMH. A second marker should be used to exclude WBCs from the flow cytometry analysis, particularly auto-fluorescent neutrophils, from the calculation for FMH quantification, e.g., anti-CD45 or anti-CD66b[10].

RECOMMENDATION 11. An anti-D marker should be used for FC quantification when the FMH result is used to calculate the prophylactic anti-D Ig dose to be recommended. Grade 1A.

RECOMMENDATION 12. For FC quantification, a second marker should be used to exclude auto-fluorescent neutrophils from the calculation for FMH estimation. Grade 1A.

13.3 | Patient sample handling

At least two separate aliquots of whole blood should be taken from the sample and tested in parallel (Table 1). For FC quantification (confirmation testing), the aliquots must be taken from the original sample used for AE or FC screening. The aliquots should give similar results within intra-assay limits defined by the laboratory. Laboratories should determine the CV between patient replicates and define acceptable limits for their technique.[48] For results ≥2 mL a CV of ≤10% is expected. At lower thresholds of FMH, ≤2 mL (≤0.1% fetal cells), it should be accepted that the CV is likely to be greater. Discordant results should alert the operator to possible errors in sample preparation, testing and analysis.

RECOMMENDATION 13. For FC quantification, at least two separate aliquots of whole blood should be taken from the sample and tested in parallel. Grade 1A.

RECOMMENDATION 14. The original maternal EDTA sample, screened by AE or FC, should be used for confirmatory FMH testing by FC. Grade 1A.

13.4 | Control samples

Mixtures of D positive and D negative RBCs should be included in each test to adequately control the technique. It is recommended that as a minimum, each test includes a 0.2% and 1% D positive control. It is recommended that the test also includes a control that represents a larger FMH volume, e.g., 10% D positive RBCs. R1r (D + C + c + E−e+) RBCs should be used to create controls as they carry a heterozygous expression of the D antigen (Table 1). These RBCs will reflect the level of D expression on the fetal RBCs present in the maternal circulation and will generate a similar fluorescence intensity. This in turn will facilitate identification and gating

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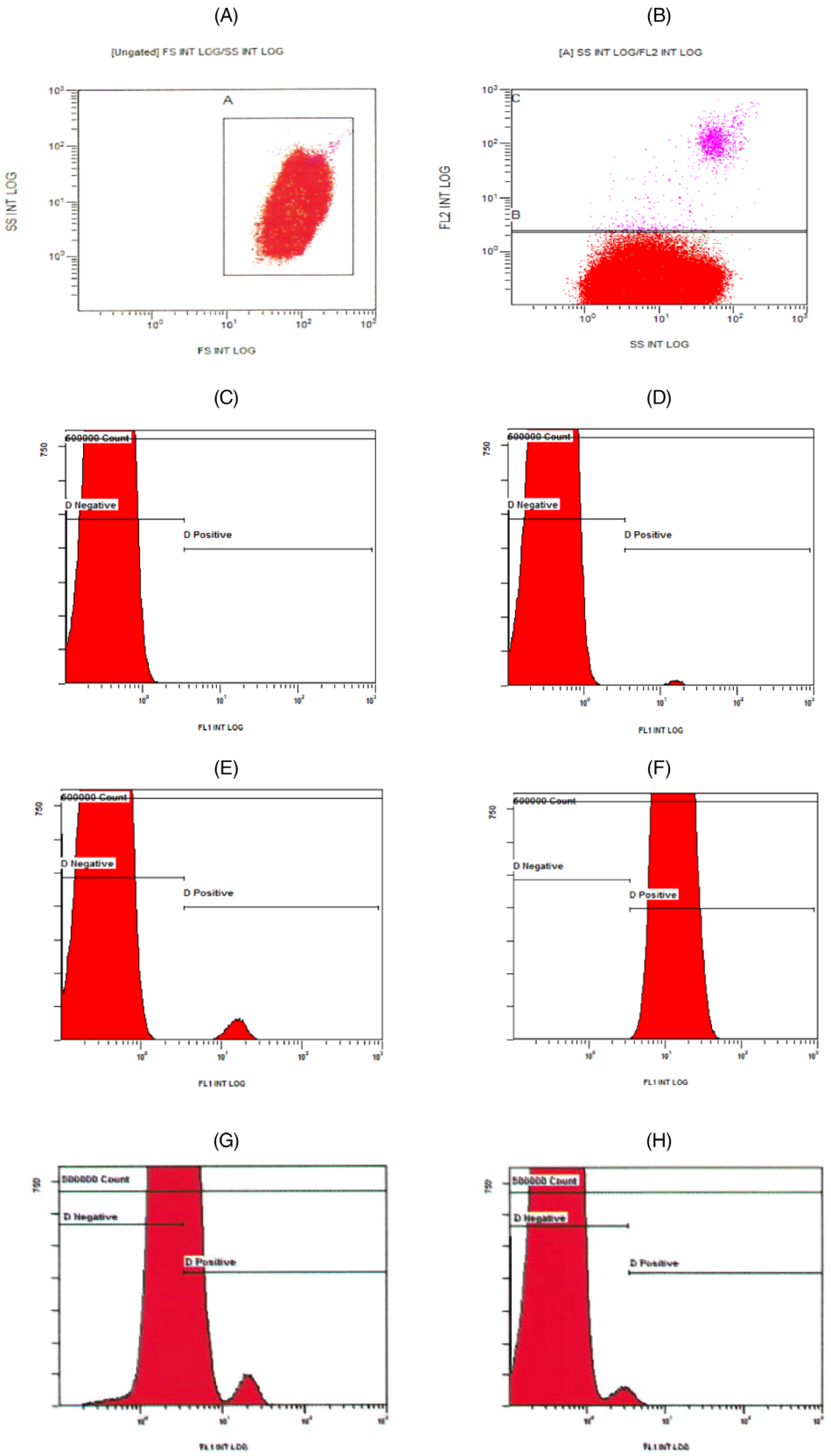

FIGURE 5 Histograms detailing gating of D positive and D negative RBCs. (A) Forward scatter/side scatter (FSC/SSC) RBC Gate. Threshold set to remove platelets and debris. (B) White Blood Cell Exclusion (when using anti-white blood cell reagent or autofluorescence). (C) No D positive fetal RBCs present. (D) Population of fetal RBCs present—0.2% positive control. (E) Population of fetal RBCs present—1% positive control. (F) D positive maternal sample. (G) D variant female with R1r baby. (H) D negative female with D variant baby.

of the fetal RBC population. The 0.2% control represents a level of 4 mL, above which an anti-D Ig dose of >500 IU would be required.[2] A 1% D positive RBC control sample can aid in protocol creation. Alternative controls may be used for flow cytometry confirmation if they better reflect the level of the FMH in the test samples.

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RECOMMENDATION 15. Controls for FC should include mixtures of D positive (R1r) and D negative RBCs. Grade 1A.

RECOMMENDATION 16. Controls for FC should discriminate between clinically significant bleed volumes, e.g., 1% D positive red cells; 0.2% D positive red cells as well as a larger FMH volume, e.g., 10% D positive red cells (220 mL). Grade 2C.

Non-specific uptake of antibody by cells included in the positive region can lead to falsely high results. Inclusion of an inert isotype and fluorochrome matched control is recommended to determine the proportion of background fluorescence present, e.g., fluorescein isothiocyanate (FITC) anti-D. The isotype control result must be subtracted from the test antibody result.

13.5 | Gating and analysis of control and patient samples

Along with appropriate instrument settings and compensation settings, gating strategies should be created during reagent validation. Populations relating to RBC and overlapping WBC events should be gated on a log scale for forward scatter and side scatter (Figure 5A). A loose primary gate should be set up around the RBC population that largely excludes platelets from the data.

Fixed gating strategies should be used where possible; ensuring across all results the events generated are contained within the appropriate gates (Figure 5A–H). Post analysis histograms and dot plots should be examined closely for each investigation. Fluorescence intensity/fluorochrome conjugated antibody binding can be adversely affected when D variant fetal RBCs are present, as well as by high levels of prophylactic anti-D Ig in the patient's circulation[44,49] (Figure 5G,H). Unusual cell populations can be encountered when testing samples from patients with a haemoglobinopathy or leukaemia. Where populations do not fall into fixed gating strategies, it is recommended that laboratories have processes in place to optimise the gating strategy for these patients (Refer to Section 15).

When a reagent to exclude WBCs from the analysis is used, the exclusion gating strategy should demonstrate clear separation between WBC and RBC populations (Figure 5D,E).

For creation of the histogram plot, photomultiplier voltages should be set so that no more than 1% of events are in the first fluorescent channel, allowing negative populations to be clearly seen. The 1% control should be used to set the gates (or regions) on the histogram to differentiate positive and negative events. These gates should be checked with samples containing a greater percentage of positive cell populations to ensure all populations will fall inside the gates as expected (Figure 5A–D). The number and percentage of cells in these two populations should be displayed and recorded. During patient sample investigation, no fewer than a total of 500 000 events (positive and negative) should be collected in the histogram plot from each individual control and patient test tube.

13.6 | Acceptance of control results

Acceptable ranges for positive controls should be determined by individual labs and should be nominally set at ± 2 standard deviations (SD).

13.7 | Calculation of FMH using FC methodologies

The packed fetal RBC volume should be calculated using Mollison's formula,[35] which assumes that:

- The maternal RBC volume is 1800 mL
- Fetal RBCs are 22% larger than maternal RBCs.

The FMH volume should be calculated using one of the following methods:

Using the percentage of fetal RBCs present:

$$\text{FMH (mL packed fetal RBCs)} = \text{PERCENTAGE FETAL CELLS} \times \frac{1800}{100} \times \frac{122}{100}$$

Which simplifies to:

$$\text{PERCENTAGE FETAL CELLS} \times 18 \times 1.22$$

For example, where 0.2% of the RBCs are fetal, then the FMH will be calculated as:

$$0.2 \times 18 \times 1.22 = 4.39 \text{ mL packed fetal RBCs.}$$

This result would be rounded up to 5 mL packed fetal RBCs.

Using the number of events present in each gate:

$$FMH(mL\ packed\ fetal\ RBCs) = \frac{Number\ Postive\ Events\ in\ Patient\ Test - Number\ Positive\ Events\ in\ Patient\ Isotype\ Control}{Total\ Gated'RedCell'events} \times 1800 \times 1.22$$

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Mollison's formula does not take into consideration that the patient may not have a packed RBC volume of 1800 mL.

Always round the FMH result up to the nearest mL when reporting results to ensure sufficient prophylactic anti-D Ig is administered. Anything greater than a whole number rounded up to the next whole number, e.g., 7.01 mL; 7.1 mL; 7.6 mL all round up to 8 mL.

The FMH result should be reported in mL of packed RBCs, without the inclusion of the UoM. Refer to Section 16 for further information on the application of UoM.

All samples above the defined cut off for FMH screening by FC must be referred for full quantification by FC (Section 13.8).

13.8 | Confirmatory testing following FMH screening by flow cytometry

During the initial validation of the FC FMH screening test, laboratories must establish a suitable cut off value for referral for FC confirmation. The cut off referral volume from FC screening to FC quantification may be greater than that for AE testing (2 mL). This is because UK NEQAS FMH exercises continue to show that FC testing is more accurate than AE, particularly at low bleed volumes (K Veale, personal communication 20/07/2023). The FC screening cut off value for referral for confirmation testing must be <4 mL. It is recommended that the UoM is added to the FMH FC screening result before rounding up to a whole number when considering referral for full quantification.

For FMH ≥2 mL, if the FC quantification test result is not expected to be available within a clinically relevant time frame, that is, to allow administration of prophylactic anti-D Ig within 72 h of the sensitising event, the AE quantification result should be used to calculate the appropriate level of anti-D prophylaxis. It is recommended that AE quantification is repeated before sample referral by a second operator. Anti-D Ig prophylaxis will be based on the AE quantification result and should be released under a documented process with clinical consultation. Further anti-D Ig top up should be provided where required following the results of FC quantification.

As FC screening tests do not include a patient negative control, the FC screening test should not be used to calculate the anti-D Ig dose to treat a significant fetal bleed (≥4 mL). In exceptional situations, results may be released under a consultant led concession process. In such circumstances, a repeat FC screening test is not required.

RECOMMENDATION 17. For FMH ≥2 mL, if FC quantification testing delays the administration of prophylactic anti-D Ig to > 72 h post PSE, the AE quantification result should be used to calculate the appropriate level of anti-D prophylaxis. Grade 1A.

RECOMMENDATION 18. If the FC quantification test result is not expected to be available to determine the level of anti-D Ig administration, it is recommended that the AE quantification is repeated by a second operator before the sample is referred for FC quantification. Grade 1B.

13.9 | Discrepancies between FC screening and FC confirmation results

The UoM of the confirmation and screening test should be taken into consideration when determining if the results are discrepant. When discrepancies between FC screening and FC confirmation results are outside of the UoM of the tests, repeat testing of the FC screen should be performed initially. All samples run within the same batch should be repeated to ensure transposition of samples has not occurred.

It is recommended that when running FC screening tests, results are not reported until confirmation testing is complete, and the result accepted. Repeat testing should also be considered where differences between confirmation and screening test results would lead to a different outcome for patient management or administration of anti-D Ig prophylaxis. If repeat testing would result in reporting a FMH result at >72 h post-sensitising event, discussion with specialist clinical/laboratory staff should take place to provide guidance for patient management.

If discrepancies in testing are identified, this should generate an incident report and a risk impact assessment should be performed

14 | SYSTEMS SUPPORTING FMH QUANTIFICATION AND PROPHYLACTIC ANTI-D IMMUNOGLOBULIN DOSE CALCULATION

Systems used to support good practice (GxP) and GxP critical processes, e.g., calculation of FMH results or the dose of anti-D Ig, should be regarded as an application and deemed to be GxP critical. They should be secure, risk assessed and appropriately validated. Formulae should be reviewed and the accuracy verified appropriately. Consideration should be given to purchasing future flow cytometry analysis software and blood transfusion laboratory information systems (LIMS) that are capable of calculating FMH results and the level of anti-D Ig dose advocated.

15 | DISCREPANCIES BETWEEN AE AND FC RESULTS FOR FMH ESTIMATION

Despite improvement in the accuracy of measurement of FMH, disparity between AE and FC results continues and is demonstrated by performance in the UK National External Quality Assessment Scheme (UK NEQAS) FMH exercises.[50] Fetal RBCs are often detected by the AE technique when none are present; the CV is higher for results

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obtained by AE than for those obtained by FC, and there is better intra-laboratory reproducibility for FC.[10,51,52]

It has been established that AE can both over and underestimate FMH, but most of the studies report the tendency of the AE tests to overestimate.[21,28,53]

Discrepancies between the AE result and the FC result occur due to errors in sample identification, storage and preparation, testing of inappropriate samples, poor technique, reagent/experimentation failure and also due to the limitations of each test.[52]

If unresolved discrepancies in testing are identified following repeat testing, a formal incident investigation and root cause analysis should be performed.

15.1 | Detection of HbF in D negative fetal cells

The AE test detects fetal RBCs due to the presence of HbF, whereas the FC test uses an anti-D fluorochrome to detect D positive RBCs. Discrepancies between AE and FC results, where the fetal D type is unknown, may result from the detection of HbF within the D negative fetal RBCs by the AE test. The D type of the fetus or neonate should be taken into consideration when interpreting results from different techniques. A cffDNA *RHD* genotyping result may be available to help resolve these discrepancies. Error in reagent use, where the anti-D fluorochrome is not added to test tubes, should be ruled out when discrepancy between AE and FC occurs. The use of a WBC exclusion reagent in FC testing, made up as a master mix with the anti-D reagent, can aid in mitigation of such errors.[10] FC results obtained using an anti-D fluorochrome must not be used to assign a D group to the fetus. Cases where the D phenotype of the fetus is unknown and the AE result and FC result remain discrepant should be referred to the clinical team for discussion.

15.2 | Adult red blood cells with HbF

The number of maternal adult RBCs with HbF increases during pregnancy to reach a peak between the first trimester and 18–22 weeks gestation.[30,31] The presence of maternal RBCs containing HbF can cause problems when performing FMH estimation by AE. False positive results or falsely high FMH estimations can be caused by intermediate staining of the maternal RBCs, and subsequently, these adult RBCs can be counted as fetal RBCs[42] (refer to Section 11.10). This could lead to the inappropriate administration of prophylactic anti-D Ig.

Elevated levels of adult RBCs with HbF occur in maternal haemoglobinopathies such as sickle cell disorder, β-thalassaemia and HPFH. However, HbF within maternal RBCs that are within the normal range and not consistent with HPFH can cause falsely positive or falsely high AE results.[54]

Staff should be aware that maternal RBCs with HbF could cause difficulties in interpreting AE slides and affect the accuracy of counting fetal RBCs. If elevated levels of adult RBCs with HbF are suspected, the sample should be referred for FMH estimation by FC. An anti-D fluorochrome should be used to determine the level of D positive fetal RBCs present. FC techniques using anti-HbF and anti-carbonic anhydrase may be useful in the investigation of intermediate staining in the AE technique and explain discrepancies between the FC and the AE FMH result.[55,56] However, the sole use of an anti-HbF fluorochrome should be avoided due to the risk of underestimation of the FMH volume.[57,58]

15.3 | D variant fetal red blood cells

Fetal RBCs that exhibit a serological altered expression of the D antigen, i.e., D variant, may not interact with the anti-D fluorochrome adequately to produce a fluorescence intensity high enough to effect a shift away from the D negative maternal RBCs. This will impact gating strategies upon result generation and will manifest as a positive AE test result with no apparent population of fetal RBCs observed in the FC test histograms. If the investigation of this discrepancy would result in the reporting of the FMH result at >72 h post-sensitising event, discussion with clinical/laboratory staff with specialist knowledge should take place to provide guidance for patient management. cffDNA *RHD* genotyping or serological determination of the paternal or infant D status may be helpful. A FC technique using a HbF fluorochrome will detect any fetal RBCs present irrespective of D antigen expression and could allow for more accurate anti-D Ig dosing until the fetal D type is resolved.[58,59]

The binding characteristics of the selected anti-D fluorochrome must be fully understood and it must be able to discriminate weak D in feto-maternal bleeds where the antigen site number is >1000.[59] Refer to the manufacturer's reagent data sheet for further information.

15.4 | Raised levels of white blood cells

During pregnancy, changes in the immune system result in a higher than normal WBC count, mainly driven by a rise in neutrophil count.[46] Raised levels of WBCs can cause problems in AE slide interpretation and miscounting darkly stained lymphocytes as fetal RBCs can cause an overestimation in FMH[28].

For all scenarios, if discrepancies in testing are identified, this should generate an incident report and root cause analysis should be performed. Discussion with specialist clinical/laboratory staff should be undertaken to provide guidance for patient management.

RECOMMENDATION 19. Where a discrepancy between the AE and FC result remains following repeated testing, if possible, the D type of the fetus/neonate should be determined either by cffDNA RHD genotyping screening test or RBC D typing by serology. Grade 1A.

RECOMMENDATION 20. Staff should be trained to identify possible causes of discrepant results, e.g., D

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variant RBCs, hereditary persistence of HbF, errors in sample storage, sample preparation, assay technique or the limitations of each test. Grade 1A.

RECOMMENDATION 21. There must be a documented process in place for the management of anti-D Ig prophylaxis when discrepant results between AE and FC techniques occur. Grade 1A.

RECOMMENDATION 22. For an unresolved discrepant result between AE and FC, there should be a process in place to report an incident through a quality management system, investigate the discrepancy, and manage the case through discussion with a specialist clinical/laboratory staff member. Grade 1A.

16 | UNCERTAINTY OF MEASUREMENT

UoM provides quantitative evidence that test results are reliable and 'fit for purpose' and can be used to drive standardisation across laboratories and improve test performance. UoM also has the potential to contribute to clinical care if it is reported alongside the test result and interpreted correctly.[60,61]

UoM for FMH results should be calculated and available in accordance with ISO 15189 standard.[27]

The UoM should be applied to either the FMH volume or the anti-D Ig prophylactic dose.[62,63] (refer to Appendix A). It is recommended that the FMH result is reported in 'mL of packed RBCs, as found', without the inclusion of the UoM of the testing methodology. Consideration should be given to including the UoM of the test in the user guide / on the website for the testing laboratory. A decision made not to apply the UoM should be fully risk assessed.

If the UoM is applied, the clinical interpretation of the final FMH result may differ, e.g., adding the UoM to the calculated FMH value may trigger referral for FC testing or trigger a clinical decision point for additional anti-D Ig when the original result did not. The application of the UoM can aid clinical decision making and optimise anti-D Ig administration to prevent sensitisation. Reporting UoM calls for more careful consideration of the FMH result and clinicians should be provided with the appropriate support needed to interpret the result correctly (refer to Section 19, Reporting FMH Results).

The accuracy of FMH estimation is impacted by factors such as sample processing, operator precision, change in reagent batch, test incubation conditions, routine maintenance, and room temperature. Therefore, UoM should be calculated at regular intervals, on a data set that encompasses all the possible routine changes that occur.

The UoM should be calculated using results from samples representing clinically significant FMH volume cut offs for the technique being employed, e.g., FMH quantification by AE = 2 mL, FMH screening by FC = greater than 2 mL, but less than 4 mL, FMH confirmation by FC = 4 mL.

Where practical, UoM samples should be tested by all laboratory scientists trained to the testing process, at various time points throughout the day and across a range of days.[62,63] Alternatively, the UoM may be calculated from quality control sample results and patient replicate results.

Where possible, validated software should be used to automatically apply the UoM of the testing methodology to the result.

Where the UoM is calculated using data from multiple laboratories, consideration should be given to applying an overarching level for the UoM that will remain appropriate for a reasonable period of time, e.g., Laboratory 1 = 9%; Laboratory 2 = 12%, Laboratory 3 = 15%; UoM set at 15% for all laboratories.[62,63]

17 | FURTHER FMH TESTING FOLLOWING ANTI-D PROPHYLAXIS

A flow diagram for FMH testing and subsequent actions is shown in Figure 6.

17.1 | FMH result confirmed by FC

- If the FMH is confirmed by FC as less than 4 mL, no further testing is required. Ensure a standard dose of anti-D Ig, sufficient to cover 4 mL has been administered.[2]
- If the FMH is confirmed by FC as ≥4 mL, this is considered to be a significant FMH and the patient requires further testing following the administration of the appropriate amount of anti-D Ig.
- Calculate the total dose of anti-D Ig required to clear the level of FMH present.[2]
- Ensure any additional anti-D Ig is administered, taking into account any postnatal anti-D Ig already given following birth. Do not take the routine antenatal anti-D prophylaxis (RAADP) dose into consideration or any anti-D Ig prophylaxis given during pregnancy because of a sensitising event.
- Always round up to the nearest vial size and where possible, use the same batch as the anti-D Ig given at birth to limit exposure to different batch products.[2]
- Refer to the BSH guideline for the use of anti-D immunoglobulin for the prevention of haemolytic disease of the fetus and newborn for further information.[2]

17.2 | Timing of follow-up samples for FMH ≥4 mL

- A follow-up maternal sample, 72 h after the most recent anti-D Ig IM administration must be requested to check for clearance of fetal RBCs by a FMH screening test.
- If anti-D Ig is administered intravenously, request the repeat sample 48 h post anti-D Ig administration.
- Where a FMH ≥4 mL is detected, it is recommended that the baby's haemoglobin level is measured following birth.

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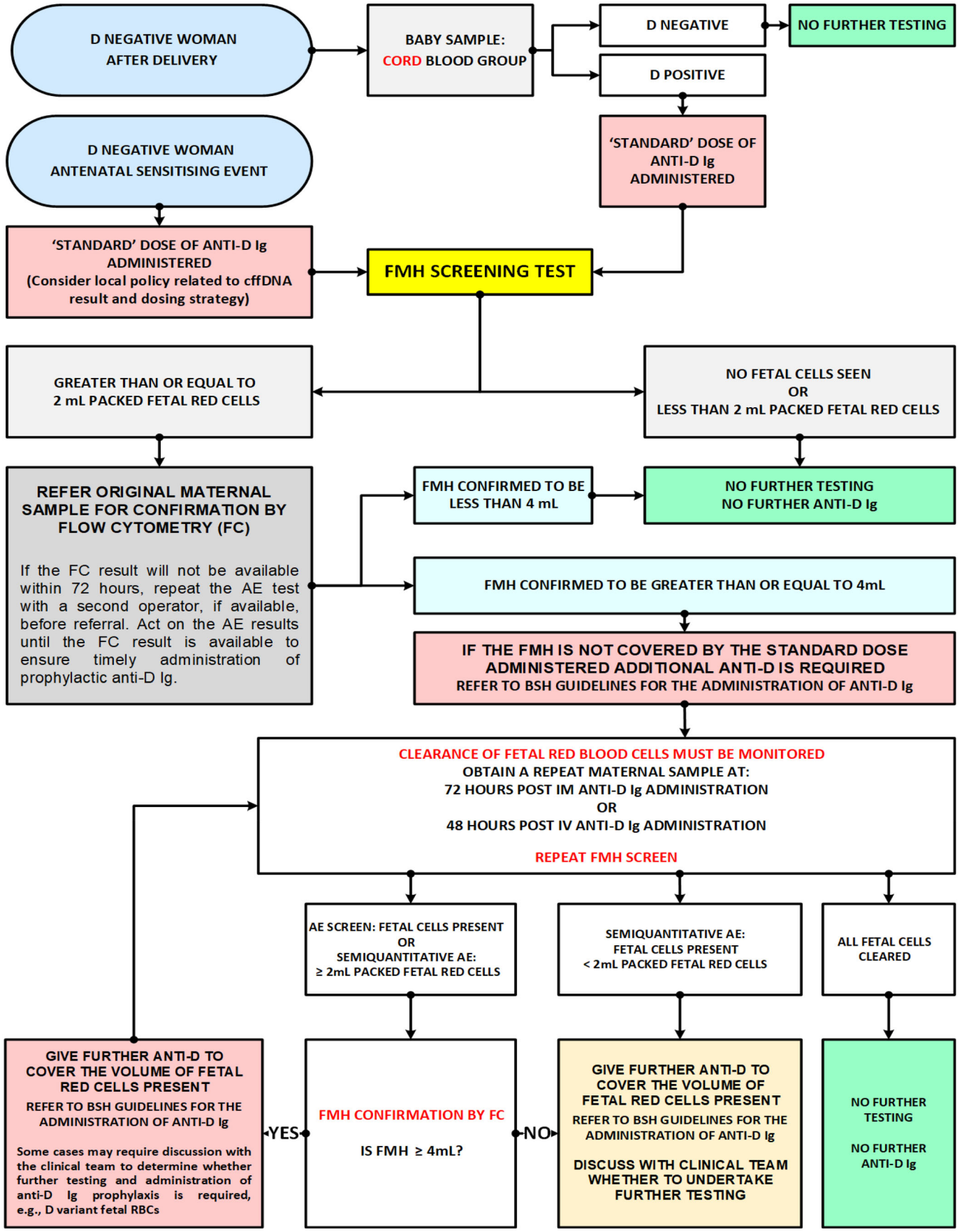

FIGURE 6 Flow diagram for FMH testing and subsequent actions.

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RECOMMENDATION 23. A repeat maternal sample should be taken and tested by a FMH screening test 72 h after the total dose of anti-D immunoglobulin IM injection (48 h if the anti-D Ig was given IV). This allows sufficient time for anti-D Ig to take effect and to check the volume of remaining fetal RBCs. Grade 1A.

17.3 | Timing of follow-up samples for continuous uterine bleeding

For continuous uterine bleeding after 20 weeks' gestation, a FMH screening test should be performed every 2 weeks,[12] irrespective of previous repeat sampling following clinically significant bleed volumes.

RECOMMENDATION 24. For continuous uterine bleeding after 20 weeks' gestation, a FMH screening test should be performed every 2 weeks,[12] irrespective of previous repeat sampling following clinically significant bleed volumes. Grade 1A.

17.4 | Action if fetal red blood cells have NOT cleared on follow-up sample

The presence of fetal RBCs in the follow-up sample by AE testing indicates that additional dosing of anti-D Ig is required.

Confirm that any previous recommended post-sensitisation event anti-D Ig has been administered.

- Send the follow-up maternal EDTA sample for testing by FC.
- Administer further anti-D Ig as dictated by the volume of fetal RBCs remaining.
- Discuss with specialist clinical/laboratory staff to determine requirements for further testing.
- If required, request a further maternal EDTA sample for screening, 72 h after the repeat anti-D Ig IM injection (48 h post-IV administration).

Situations may arise, where several doses of anti-D Ig have been administered but a positive test result persists. The circumstances of the original sample referral, e.g., delivery sample, and the total dose of anti-D Ig administered (confirmed by the patients notes) should be reviewed by clinical staff to determine the need for further follow up.

17.5 | Fetal RBCs have cleared on follow-up sample

If the fetal RBCs have cleared on the follow-up sample, **no further testing is required.**

There is no requirement at any stage of testing to check for free anti-D Ig in the patient's sample.

18 | cffDNA RHD GENOTYPING

Refer to local policy when considering the cffDNA *RHD* genotyping in relation to FMH testing protocols. There is a reported false-negative rate of 0.21% for high-throughput non-invasive prenatal screening testing for fetal *RHD* genotype.[64] Therefore, a full risk assessment recognising the clinical impact of cffDNA *RHD* genotype screening should be in place.

Also refer to the BSH guideline for blood grouping and antibody testing in pregnancy,[3] the BSH guideline for use of anti-D Ig for the prevention of HDFN[2] and the NICE guideline for high throughput non-invasive testing (NIPT) for fetal *RHD* genotype.[64]

The fetus should be assumed to be D positive for the purpose of FMH investigation when the cffDNA *RHD* genotyping result is indeterminate or there is no cffDNA *RHD* genotype available.

19 | REPORTING FMH RESULTS

Laboratories must have a policy to ensure the timely and effective communication of clinically significant FMH results to facilitate appropriate patient care.

The report must include:

- The test method (e.g., AE, FC using anti-D fluorochrome, FC using anti-HbF fluorochrome)
- The final result of the FMH test in 'mL packed fetal RBCs' rounded up to the nearest mL, e.g., 7.1 mL = 8 mL.
- The recommended dose of anti-D Ig.
- Request for follow-up samples required to check for clearance of fetal RBCs, if appropriate. Request for repeat samples 72 h post-IM anti-D Ig administration or 48 h post-IV administration.

Where the software of the LIMS allows, the report must also include:

- The reason for sample referral (e.g., post-delivery, sensitising event, follow-up of significant FMH, neonatal anaemia).
- Whether the sample has been referred for flow cytometry post AE investigation, if applicable.
- For D negative females, statements to guide the effective administration of anti-D Ig. These may include:
  - Advice about the total anti-D Ig dose required to cover the reported bleed.
  - A statement to administer additional anti-D Ig prophylaxis, if required.
  - Advice for dealing with larger bleeds, e.g., the use of IV anti-D Ig when multiple injections of IM anti-D Ig would be required.
  - Details of the person providing any clinical advice.

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- Results of confirmatory tests from the reference laboratory (or from repeat in-house testing by AE where FC results are not available within 72 h and the AE results are acted on in the interim).

The laboratory should consider including the following information in the report:

- Maternal D type
- Additional information that may explain a discrepancy between the AE result and the FC result, e.g., raised level of adult RBCs with HbF, D variant maternal or fetal RBCs.
- If no further samples are required for FMH testing
- If the test is performed outside its accredited scope, e.g., details of any concessionary investigation, such as solid organ transplant.

The laboratory should consider including the following in a user guide or on a website:

- A statement of how the UoM is applied to the FMH result, if applicable
- The method used to calculate the FMH result, e.g., state in the report that the FMH result has been calculated using Mollison's formula.

Examples of appropriate reports are detailed in Appendix B.

If a FMH result is also given/received by telephone, details must be fully documented. This must include details of the date, time, patient identification checks, FMH result, total dose of anti-D Ig prophylaxis required, person giving or receiving the information and also any difficulties encountered in providing/receiving the FMH result.

Ensure that the recipient of the report is aware of the total dose of anti-D Ig required to cover the FMH.

RECOMMENDATION 25. Where an estimated FMH exceeding 2 mL is detected by AE, a preliminary result should be communicated to the clinical team as soon as possible, prior to receiving the results of the confirmatory test, to alert clinicians to the possible need for additional anti-D Ig prior to discharge. Grade 1A.

RECOMMENDATION 26. When considering possible IV administration of anti-D Ig, refer to the BSH guideline for the use of anti-D Ig for the prevention of haemolytic disease of the fetus and newborn.[2] Grade 1A.

20 | QUANTIFICATION OF D POSITIVE RED BLOOD CELLS IN NON-PREGNANT INDIVIDUALS OF CHILD BEARING POTENTIAL

20.1 | Solid organ and tissue transplantation

The potential for sensitisation of D negative individuals following D positive solid organ transplantation (SOT) and some living donor bone grafts is recognised.[65–67] This results from donor D positive RBCs remaining in the organ's vasculature, even after flushing and in femoral head grafts.[66–68] If estimation of the D positive RBC volume is required, the formula in Section 20.2 should be used.[66,67] As the volume of red cells in a femoral head is anticipated to be below 4 mL then a dose of at least 500 IU anti-D per femoral head graft should be administered and an estimation of the D positive RBC volume is not required.

20.2 | Transfusion

Quantification of D positive RBCs should be performed by FC following intentional or inadvertent transfusion of D positive blood to D negative females of child bearing potential. Follow-up tests for D positive RBCs should be undertaken every 48 h if an IV dose of anti-D has been given or every 72 h if an IM dose has been given. If the D positive RBCs persist, further anti-D should be given and testing repeated until there are no detectable D positive RBCs in the circulation.

Where quantification takes place, it is important to consider that the correction factors used for FMH calculations in pregnant females are not relevant for the presence of adult RBCs. The recipient's packed RBC volume should be estimated based upon their height and weight using standard adult or paediatric calculations. Also, the kinetics of the donor RBCs are unknown; therefore, the effective timing of testing and anti-D Ig prophylaxis has not been established.[65,66,69]

The packed D positive adult RBC volume should be calculated using an adapted version of the Mollison formula[66] to guide the administration of anti-D Ig prophylaxis.

$$D\,positive\,adult\,RBCs\,(SOT\,and\,Transfusion) = \frac{Number\,Positive\,Events\,in\,Patient\,Test - Number\,Positive\,Events\,in\,Patient\,Isotype\,Control}{Total\,Gated\,'Red\,Cell'\,events} \times Packed\,RBC\,Volume\,of\,Patient$$

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21 | FMH ESTIMATION IN D POSITIVE FEMALES

Testing should be performed as described in Section 11 for AE screening and quantification. FMH estimation in D positive females by FC can be performed using an anti-HbF fluorochrome. Refer to Section 13 for guidance.

Staff should be trained and competency assessed in any techniques used for AE. Laboratories performing AE on D positive females should be accredited and operate under an effective quality management system. Compliance must be demonstrated through participation in an accredited external quality assessment scheme, e.g., UK NEQAS.

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CONFLICT OF INTEREST STATEMENT

The BSH paid the expenses incurred during the writing of this guidance. No additional funding was required for the update of these guidelines. All authors have made a declaration of interests to the B.S.H. which may be viewed on request. The following members of the writing group: L.P., M.H., J.E., K.V., D.H., L.J., S.A.F., C.G. and S.R. have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

DISCLOSURE

Review process: Members of the writing group will inform the writing group Chair if any new evidence becomes available that would alter the strength of the recommendations made in this document or render it obsolete. The document will be reviewed regularly by the relevant Task Force and the literature search will be re-run every 3 years to search systematically for any new evidence that may have been missed. The document will be archived and removed from the BSH current guidelines website if it becomes obsolete. If new recommendations are made, an addendum will be published on the BSH guidelines website (http://www.b-s-h.org.uk/guidelines). **Disclaimer:** While the advice and information in this guidance is believed to be true and accurate at the time of going to press, neither the authors, the BSH nor the publishers accept any legal responsibility for the content of this guidance.

AUDIT

Audits of clinical and laboratory practice should be undertaken on a continuing basis to ensure compliance with these guidelines and, where identified, variance or concerns in relation to compliance should be addressed. Examples of audits relevant to these guidelines.

- D negative females have had a FMH test at the appropriate time.
- Appropriate action has taken place when the FMH test result is positive.
- Comparison of confirmatory test results with initial screening result.
- Appropriate report comments in line with FMH result.

PATIENT CONSENT STATEMENT

Patient consent was not required for the update of this guideline.

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APPENDIX A: APPLICATION OF UOM

For the following worked examples, the UoM of the test = 15%.

Including UoM in the bleed volume:

Actual bleed volume = 12 mL.

Calculated UoM of bleed volume = 15% of 12 mL = 1.8 mL.

Reported bleed volume = Actual bleed volume + Calculated UoM of bleed volume = 13.8 mL.

Including UoM in the prophylactic anti-D dose:

Actual bleed volume = 12 mL.

Actual prophylactic anti-D dose = 1500 IU.

Calculated UoM of prophylactic anti-D dose = 15% of 1500 IU = 225 IU.

Minimum dose that can be administered is 500 IU.

Reported prophylactic anti-D dose =
Actual prophylactic anti-D dose + Calculated UoM of prophylactic anti-D dose (rounded up to nearest 500 IU dose) = 2000 IU.

APPENDIX B: EXAMPLES OF APPROPRIATE REPORT COMMENTS

Reports from the hospital laboratory to the clinical area.

No fetal cells present or FMH <2 mL on semi-quantitative screen by AE or FC.

- FMH test by {*insert method*}.
- Reason for sample referral, e.g., delivery, sensitising event,
- Administer standard prophylactic anti-D dose.
- Less than 2 mL fetal cells seen; this FMH will be covered by the standard dose of *{insert dose}* IU of prophylactic anti-D.
- No further testing is required.

FMH ≥2 mL of fetal cells and quantification has been performed. Confirmed FMH within the volume covered by the standard anti-D Ig dosage in use.

- FMH test by acid elution and flow cytometry.
- Reason for sample referral, e.g., delivery, sensitising event,
- Fetal cells seen.
- Estimated {*insert volume rounded up to nearest whole mL*} mL fetal red cells by acid elution.
- This has been sent for confirmation *{results of confirmatory test including methodology}*.
- This will be covered by the standard dose of {*insert dose}* IU of prophylactic anti-D.
- Please send a further maternal EDTA sample 72 h after the most recent administration of prophylactic anti-D (48 h post-IV administration) to check for clearance of fetal red cells.

Confirmed FMH exceeds the volume covered by the standard anti-D Ig dosage in use.

- FMH test by acid elution and flow cytometry.
- Reason for sample referral, e.g., delivery, sensitising event,
- Fetal cells seen.
- Estimated {*insert volume rounded up to nearest whole mL*} mL fetal red cells by acid elution,
- This has been sent for confirmation *{results of confirmatory test including methodology}*.
- The recommended total dose of {insert dose} IU of prophylactic anti-D is required immediately,
- Please administer any additional anti-D Ig prophylaxis required,
- Please send a further maternal EDTA sample 72 h after the most recent administration of prophylactic anti-D (48 h post-IV administration) to check for clearance of fetal red cells.

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Reference laboratory results to referring laboratory

FMH ≥2 mL Flow Cytometry Screening Report

- FMH test by flow cytometry.
- Reason for sample referral, e.g., delivery, sensitising event.
- This has been sent for confirmation *{results of confirmatory test including methodology}*
- FMH result: {*insert volume rounded up to nearest whole mL*} mL of packed fetal red cells.
- D type of maternal sample, if relevant.
- Level of adult red cells containing HbF present (if appropriate).

Flow Cytometry Confirmation Report.

- FMH test by flow cytometry.
- Reasons for sample referral, e.g., delivery, sensitising event, Acid Elution/Flow Cytometry screening test indicates FMH ≥2 mL or unable to interpret AE slide.
- FMH result: {*insert volume rounded up to nearest whole mL*} mL of packed fetal red cells.
- D type of maternal sample, if relevant.
- The recommended total dose of {insert dose} IU of prophylactic anti-D is required immediately.
- Please administer any additional anti-D Ig prophylaxis required.
- Consider the use of IV anti-D (if appropriate).
- Details of individual providing clinical advice (if appropriate).
- Please send a further maternal EDTA sample 72 h after the most recent administration of prophylactic anti-D to check for clearance of fetal red cells (48 h post-IV administration).
- Level of adult red cells containing HbF present (if appropriate).

The FMH result is calculated according to the Mollison formula which assumes that the patient is 75 Kg, the patient has a red cell volume of 1800 mL and the fetal red cells are 22% larger than adult red cells.[27]

Where a FMH estimation assay is used to perform quantification of D positive adult RBCs in:

- D negative SOT patients who have received D positive organs or tissue.
- Transfusion of D positive RBCs to D negative patients.

The final report should state – This is a concessionary test performed outside of the scope of the routine assay.

APPENDIX C: ADMINISTRATION OF PROPHYLACTIC ANTI-D AIDE MEMOIRE[12]

The Serious Hazards of Transfusion Aide Memoire is available at:
https://www.shotuk.org/resources/anti-d-immunoglobulin-ig-administration-in-pregnancy-an-aide-memoire/
or contact SHOT directly.

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