

## **Guidelines for the Estimation of Fetomaternal Haemorrhage**

Working Party of the British Committee for Standards in Haematology, Transfusion Taskforce.

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### **Guideline Update**

This guideline replaces the previous BCSH guideline published in *Transfusion Medicine* (1999) 9, 87-92

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## Summary

The BCSH guidelines for the estimation of fetomaternal haemorrhage (FMH) were first published in 1999. This update has reviewed and extended the initial guidance.

The BCSH guidelines for the use of prophylactic anti-D immunoglobulin were published in 2006 and continue to recommend that a test for FMH is performed. These guidelines are currently under review (2009).

EQA exercises continue to show improvement in the accuracy of testing for FMH by acid elution and flow cytometry but errors are still made and laboratories undertaking these tests should have robust quality assurance systems in place.

Acid elution tests for fetal cells in the maternal circulation may be used outside the context of prevention of haemolytic disease of the newborn (HDN) and guidance is given where these tests might, and might not, be helpful. Likewise, flow cytometry tests for minor populations of D positive cells might be required outside the context of pregnancy and these are included.

Quantification by acid elution at low FMH bleed volumes is time-consuming and not always necessary. It is recognised that the accuracy of counting low frequency events is associated with a high coefficient of variation (CV) and therefore the cut-off for a 'significant' FMH is now set at 2mL. A semi-quantitative acid elution screening method has been described and validated. It is recommended that quantification is performed if the number of fetal cells seen by acid elution exceeds a certain level and that flow cytometry can be used to confirm these 'potentially significant' bleeds.

The flow cytometry method is no longer only provided by reference centres and a detailed description is given of this method. Recommendations are made about reagents.

The clear and timely communication of FMH results is essential to ensure that adequate anti-D immunoglobulin is given within the recommended 72 hours from delivery or other potentially sensitising event. Guidance is given as to the content of these verbal and written communications.

Follow up of confirmed significant fetomaternal haemorrhage is important. This is to check that adequate anti-D immunoglobulin has been given to clear a fetal bleed from the maternal circulation, by looking for fetal cells. Follow-up tests for maternal anti-D at 6 months may be of benefit but it is more important to record if a significant FMH has occurred in the previous pregnancy and to test for anti-D at booking in subsequent pregnancies.

Audit of FMH estimation is required as part of the process of preventing HDN. It is also important to audit discrepancies when a significant FMH is measured by two methods.

## Methods

The guideline group was selected to be representative of UK based medical experts. A search of published literature was undertaken using Pubmed, Cochrane Library and Ingenta databases. The following key words were used: Feto maternal haemorrhage, transplacental haemorrhage, Kleihauer, Kleihauer-Betke, haemolytic disease of the fetus and newborn, anti-D immunoglobulin, flow cytometry, acid elution. This covered the period 1999-2007.

The writing group produced the draft guideline which was subsequently revised by consensus by members of the Transfusion Task Force of the British Committee for Standards in Haematology, the Standing Advisory Committee for Immunohaematology and a sounding board of UK haematologists the BCSH (British Committee for Standards in Haematology) and the BSH Committee (British Society for Haematology) and comments incorporated where appropriate.

## 1. BACKGROUND

Transplacental or fetomaternal haemorrhage (FMH) may occur during pregnancy or at delivery and lead to immunisation to the D antigen if the mother is D negative and the baby D positive. This can result in haemolytic disease of the (fetus and) newborn (HDN) in subsequent pregnancies. It is important to assess the volume of FMH to determine the dose of anti-D immunoglobulin required by a D negative woman to prevent sensitisation.

The guidelines for the use of anti-D immunoglobulin for Rh prophylaxis (BCSH 2006a) state that at least 500 iu of anti-D immunoglobulin must be given to every D negative woman with no preformed anti-D within 72 hours of delivery of a D positive baby. This dose will be sufficient to prevent sensitisation from a bleed of up to 4mL fetal red cells. FMH greater than 4mL is rare but unpredictable; 1% of women have a FMH of greater than 4mL and up to 0.3% greater than 15mL and may not be protected by a 500 iu and 1500 iu dose of anti-D immunoglobulin, respectively. It is therefore important that the volume of FMH is accurately assessed so that, if necessary, a supplementary dose(s) of anti-D immunoglobulin can be administered and maternal alloimmunisation prevented.

Since the publication of the 1999 BCSH FMH guidelines (BCSH 1999), there has been considerable improvement in the accuracy of measurement of FMH. This has been demonstrated by improved performance in the UK National External Quality Assessment Scheme (UK NEQAS) exercises for acid elution (AE) and flow cytometry (FC). However, it has also been shown that the CV is higher for results obtained by AE, than for those obtained by FC. Accuracy in FMH testing remains important but there is increased focus in these guidelines on the confirmation of FMH greater than 2mL, the follow-up of FMH greater than 4mL and the timely and effective communication of results to clinicians so that appropriate action is taken. The National Institute for Clinical Excellence has issued guidance that all D negative women should be offered routine antenatal anti-D prophylaxis (RAADP) (NICE 2002). This has affected antenatal and postnatal serological testing strategies because of the presence of passive anti-D in D negative women after 28 weeks of pregnancy (BCSH 2006b). This guideline takes this into account and highlights the importance of continuing to test for FMH after 20 weeks of gestation and to give additional anti-D immunoglobulin for sensitising events, even if already given as RAADP.

The need for FMH measurement still exists because of the unpredictable nature of large bleeds as shown in this review of 134 published cases between 1966 and 1997 where 82% had no demonstrable cause (Giacoaia, 1997).

## 2. OBJECTIVES

These guidelines are a revision of those published in 1999 by the BCSH (BCSH 1999a).

The aim is to provide practical and technical advice for the estimation of a volume of fetal cells in the maternal circulation using the Kleihauer-Betke (hereafter referred to as 'acid elution') and flow cytometry methods.

These guidelines set out recommendations for best practice and include: quality assurance, recommendations for when and whom to test for FMH and methods for estimating FMH by acid elution (AE) and flow cytometry (FC). Guidelines are given for examination of the blood film, calculation of the fetal bleed volume, the format for communicating results to clinicians and checks to ensure that the fetal cells have been cleared from the circulation.

The aim is also to ensure that the FMH estimation is used to recommend an adequate dose of anti-D immunoglobulin to prevent sensitisation to the D antigen where the volume of FMH exceeds that covered by the 'standard' anti-D immunoglobulin dose in use locally. These guidelines should be used in conjunction with the current BCSH guidelines for the use of anti-D immunoglobulin for Rh prophylaxis (BCSH 2006a).

It is recognised that FC is used to detect minor D positive red cell populations in situations unrelated to pregnancy. Transfer of D positive donor red cells into a D negative recipient can occur as a result of solid organ transplants such as renal and heart-lung transplants. This has been reported to result in anti-D formation so anti-D immunoglobulin can be administered prophylactically with a flow cytometry test at 24 hours post transplant for the presence for D positive cells in the recipient's circulation (Mahes de Silva, personal communication). Testing is also useful after a D incompatible red cell transfusion to ascertain that adequate anti-D immunoglobulin has been administered (BCSH 2006a).

It may be helpful to estimate FMH by AE in D positive women who have had an intrauterine death or stillbirth particularly if the cause of death is unknown or fetal haemorrhage is suspected (Samadi 1999). Severe anaemia at birth may also warrant looking for occult FMH if the anaemia is otherwise unexplained.

### **3. QUALITY ASSURANCE**

Commercial kits used should be CE marked and the manufacturer's instructions followed. Any changes to the manufacturer's instructions and any in-house method used should be fully validated and standardised.

Staff should be trained to standard operating procedures. There should be a mechanism in place for ongoing assessment of staff competency.

All laboratories carrying out FMH estimation should participate in an accredited external quality assessment scheme, e.g. UK NEQAS.

#### **RECOMMENDATION 1**

The above should be included in the laboratory's quality manual

#### **Good practice point**

### **4. TESTS REQUIRED**

#### **4.1 A test for FMH estimation should be undertaken on:**

- D negative women, following delivery of a D positive baby.

Babies should be typed with anti-D reagents used for routine patient testing (saline reacting, IgM reagents which do not detect D<sup>V1</sup>) with no additional tests required (BCSH 2004). However, if found to be weak D or D variant using these reagents, a baby should be treated as D positive for the purposes of anti-D administration to the mother.

- Following all potentially sensitising events in D negative women after 20 weeks gestation (see Figure 1)

**Figure 1 Potentially Sensitising Events in Pregnancy after 20 weeks of gestation (BCSH 2006a)**

Amniocentesis, cordocentesis  
Antepartum haemorrhage/ PV bleeding in pregnancy  
External cephalic version  
Fall, abdominal trauma  
Intrauterine death and still birth  
In-utero therapeutic interventions (transfusion, surgery)  
Miscarriage  
Therapeutic termination of pregnancy

#### **RECOMMENDATION 2**

An FMH test in these situations will ensure that all measures are taken to minimise sensitisation to the D antigen and hence reduce the incidence of haemolytic disease of the newborn

**Evidence grade IIb, recommendation level B**

#### **4.2 Flow cytometry tests for a minor D positive population may be required:**

- Following a D positive RBC transfusion to a D negative woman of childbearing potential. To estimate or confirm the dose of anti-D immunoglobulin required, as part of the protocol given in the anti-D guidelines (BCSH 2006a), to prevent sensitisation to the D antigen.
- In solid organ transplantation when the donor is D positive and recipient D negative with childbearing potential.

#### **4.3 Tests for FMH estimation are not required:**

- When the sensitising event is before 20 weeks because the fetal blood volume is insufficient to exceed that covered by the minimum anti-D immunoglobulin dose in standard use.
- When the woman is known to have immune anti-D.

It is recognised, on occasions, that it is difficult to distinguish between passive and immune anti-D. In such cases, FMH estimation should be performed.

- When the fetus/baby is known to be D negative.
- When the woman is D positive

Maternal samples should be tested with saline reacting IgM anti-D reagents that do not detect D<sup>VI</sup> (BCSH 2006b, BCSH 2004). Women testing D positive using these reagents are unlikely to make anti-D that will adversely affect the baby. If there is any doubt, arrange further testing at a reference laboratory and treat as D negative until results of these tests are available

- In D positive women with unexplained abdominal pain in late pregnancy, FMH tests (by AE) are of limited diagnostic use. More sensitive and specific tests exist to investigate suspected placental abruption.

## 5.0 SAMPLE REQUIREMENTS

### 5.1 Samples required

#### At Delivery

**Maternal sample:** An EDTA sample for FMH estimation and, where possible, a separate ABO and D grouping sample.

However, it is advised that where the same sample is used for FMH estimation and maternal blood group, the FMH test is performed first, particularly where manual techniques are used. Centrifugation of red cells results in the larger fetal RBCs being located closer to the plasma:RBC interface. If these are sampled when testing the maternal blood group, there is a theoretical risk that an 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.

**Baby sample:** A test to estimate the volume of FMH should be performed only when the baby is D positive (a small number of exceptions exist, see above - section 4.3). Therefore, following delivery, a cord blood sample should be taken from the baby of a D negative woman to establish the ABO and D group. The sample should be taken with a syringe and needle from an umbilical cord blood vessel wherever possible.

If cord blood is unavailable, then consideration should be given to obtaining another sample for blood grouping. If this is not possible, then it should be assumed that the baby is D positive for the purposes of FMH determination, and administration of anti-D immunoglobulin prophylaxis.

#### During Pregnancy

Following a sensitising event after 20 weeks of gestation, a maternal EDTA sample is required for FMH estimation (see Figure 1).

Up to 28 weeks of gestation, the maternal blood group should be confirmed and antibody screen performed before any anti-D immunoglobulin is given.

After 28 weeks of gestation, anti-D immunoglobulin is still required even if RAADP has been given but antibody screening is not necessary (BCSH 2006b).

If recurrent uterine bleeding occurs in a D negative woman after 20 weeks gestation anti-D will be required at a minimum of 6 weekly intervals. An FMH test should be performed every 2 weeks and, if FMH is detected, additional anti-D will be required (BCSH 2006a) regardless of the presence or absence of passive anti-D. If FMH is detected, follow-up samples should be taken after 72 hours to check that the fetal cells have cleared (see section 9).

It is important to note that the blood group of the fetus is usually unknown during pregnancy and anti-D immunoglobulin will only clear D positive fetal cells. If the acid elution test remains positive, flow cytometry using anti-D can be performed to determine whether the fetal cells remaining in the maternal circulation are D positive or not (see section 8). It is envisaged that this situation will occur relatively infrequently. A test for FMH is not routinely recommended for D positive women with PV bleeding or ante-partum haemorrhage (see section 4.3).

#### **RECOMMENDATION 3**

An anti-D immunoglobulin injection is given in specific clinical situations in a standard dose according to local protocols. The FMH test is to determine whether an additional dose is required.

#### **Good practice point**

## 5.2 Sample labelling

The samples should be accurately labelled beside the patient in accordance with the BCSH guidelines for compatibility procedures in blood transfusion laboratories (BCSH 2004). In addition to the usual patient identifiers, the samples should be clearly marked 'Cord' and 'Maternal' as appropriate. The request forms should contain full demographic details in addition to the location, relevant clinical details and date and time of delivery or sensitising event.

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 D group is the same, an alternative method is required to ensure that a sample transposition has not occurred. Methods in use include an ABO group, a full blood count (the MCV being higher in the fetal sample) or an alkali denaturation test, known as the 'APT' test, to distinguish between fetal and maternal haemoglobin (Thomas 2006).

### RECOMMENDATION 4

It is essential to ensure that cord and maternal samples are correctly labelled

#### Good practice point

## 5.3 Timing of samples

The maternal sample for FMH estimation should be taken when sufficient time has elapsed to allow fetal cells to be distributed within the maternal circulation following delivery, manual removal of placenta or sensitising event. A period of 30-45 minutes is considered adequate (BCSH 2006a).

## 5.4 Laboratory testing of samples

The sample should be processed and results reported in sufficient time to ensure that a supplementary dose of anti-D immunoglobulin could be given within 72 hours of delivery or sensitising event if the FMH estimation exceeds the 'standard' anti-D immunoglobulin dose (BCSH 2006a).

## 6. METHODS OF ESTIMATION OF FMH

The techniques in most common use in the UK for the screening and quantification of FMH are the acid elution (AE) method, a modification of the Kleihauer-Betke test (Betke, 1958) and flow cytometry (FC) (Nance 1989, Johnson 1995, Lloyd-Evans 1996). These are described in detail below.

The acid elution method is based on the different properties of haemoglobin F (HbF) in fetal RBCs and haemoglobin A (HbA) in maternal RBCs. Flow cytometry methods in common use are based on the detection of a minor population of D positive cells with a fluorochrome conjugated IgG monoclonal anti-D reagent.

The AE method is most suited to screening for FMH and initial quantification of the volume of fetal cells, if FMH is detected. The FC method is the recommended reference method to confirm the volume of FMH after an initial positive AE screen.

Laboratories participating in UK NEQAS FMH exercises responded to a questionnaire in 2005 stating that 87% of laboratories use a commercial kit for AE, others using an in-house method (personal communication, UK NEQAS exercise 0503F 2005). These kits have been the subject of an MHRA evaluation (Parker-Williams and Carpenen 2005). Some recently described modifications of the AE technique are reported to make the counting of the maternal RBCs easier by eluting half the slide (Howarth 2002) (See figure 3).

Other methods of estimating FMH have been described, but they are not in common use in the UK. These include FC using anti-HbF instead of anti-D to identify the minor fetal RBC population (Davis 1998, Nelson 1998), detection of D positive cells by the rosette test

(Sebring 1982, Lafferty 2003), gel agglutination technology (Salama 1998, Fernandes 2000) and the use of fluorochrome conjugated anti-D with haematology analysers (Little et al 2005). These are not considered any further in this guideline although general comments regarding FMH estimation still apply.

## **7. ACID ELUTION TECHNIQUE**

### **7.1 Principle**

Fetal haemoglobin (HbF) is more resistant than adult haemoglobin (HbA) to both alkali denaturation and acid elution. When dry blood films are fixed and then immersed in an acid buffer solution, HbA is denatured and eluted, leaving red cell ghosts. Red cells containing HbF are resistant and the haemoglobin can be stained; these fetal cells stand out in a sea of ghost maternal cells (see figure 2).

There are many factors that influence the quality of the results. It is very important that particular attention is paid to detail and strict timing is essential.

### **7.2 Slide preparation**

Prior to any sampling, the maternal whole blood sample must be thoroughly mixed. Preferably use a separate sample to the one for ABO and D grouping (see section 5.1)

Thin blood films should be freshly made on clean dry slides previously degreased, if necessary. The thickness of the blood film is important and should result in red cells touching but not overlapping when examined under a microscope. Thin films are easier to read. If films are too thick, overlapping cells may prevent proper elution resulting in adult cells taking up some of the counterstain.

Thin films are best achieved by diluting an aliquot of the maternal whole blood sample before making the film. This is typically a 1:2 or 1:3 dilution with phosphate buffered saline, but might depend on the maternal PCV. The diluted sample should be well mixed before the film is made.

### **7.3 Controls**

**Negative control:** fresh EDTA blood (from suitable subject, not likely to have a raised HbF)

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

The control samples should be ABO compatible and should be mixed well before film preparation. The samples may be stored at 4°C for a maximum of 4 days but fresh slides should be made each time a batch of tests is performed.

#### **RECOMMENDATION 5**

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

#### **Good practice point**



## 7.4 Staining

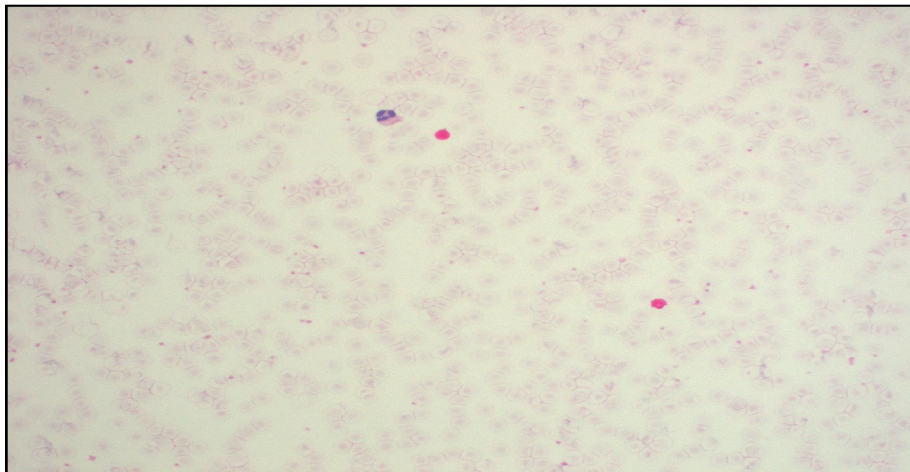
The effectiveness of staining is dependent on a number of factors including temperature of the reagents, age, quality and pH of the stain. The washing stages should be thorough. Clearer films maybe obtained using de-ionised water or distilled water rather than tap water. The back of each slide should be wiped after staining to remove any excess stain deposit, and dried in an upright position. The staining process should be progressed without any lengthy time delays to avoid fixation of haemoglobin.

## 7.5 Examination of the stained films

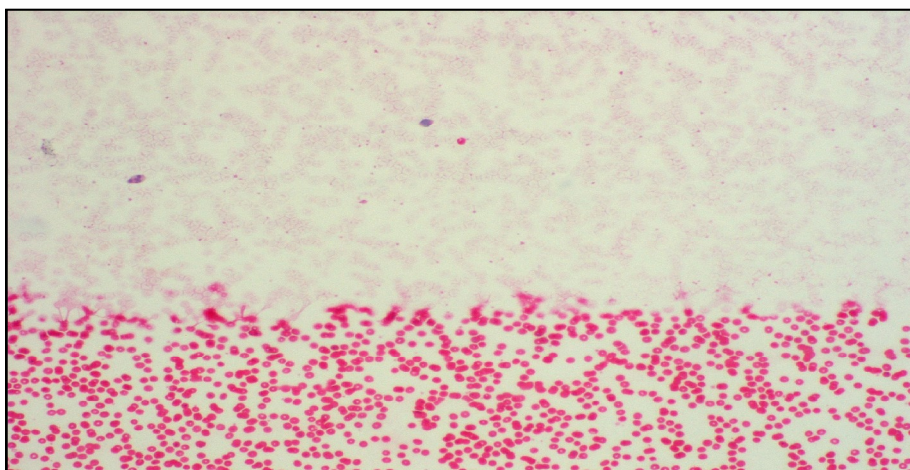
It is recommended that this is divided into **screening** and **quantification**. Controls should be examined first to ensure that the staining and preparation are satisfactory. If the controls are not to the required standard the whole process should be repeated.

It may be helpful to lower the condenser and to increase the light intensity on the microscope. Select an area of the film where cells are evenly distributed, usually near the thin end (feather edge).

**Figure 2 Acid elution slide**



**Figure 3 Acid elution slide using a modified technique where half the slide is eluted (after Howarth 2002).**



The 1999 version of these guidelines stated that if *any* fetal cells were seen whilst screening 25 low power fields (x10 eyepiece and x10 objective) then quantification should be performed. This recommendation should continue to be followed, unless the criteria below are met, in which case the semi-quantitative screen described below may be performed.

## 7.6 Screening

**This screening method has changed.** It reflects the need for a controlled, validated method to replace the practice by some laboratories of accepting the presence of 'occasional' fetal cells without proceeding to quantification, or reliance on screening methods where assumptions are made regarding the number of adult cells in a low power field (contrary to the 1999 guidelines). Using data from a supplementary UK NEQAS FMH exercise, this semi-quantitative screening method has been developed, with a 'worst case scenario' cut off point of 2mL.

Data from the UK NEQAS FMH scheme (UK NEQAS biennial report 2005-2006) shows that the minimum number of adult cells counted in a **high power field** is 100 (White 2006). Since the number of cells in a **low power field** (using x10 eyepiece and x10 objective) is 16 times that in a **high power field** (x10 eyepiece and x40 objective), the minimum number of adult cells in a **low power field**, from the same area of the film, can be extrapolated to be 1600. This allows the number of cells per **low power field** to be validated each time the test is performed.

### The following criteria must be met:

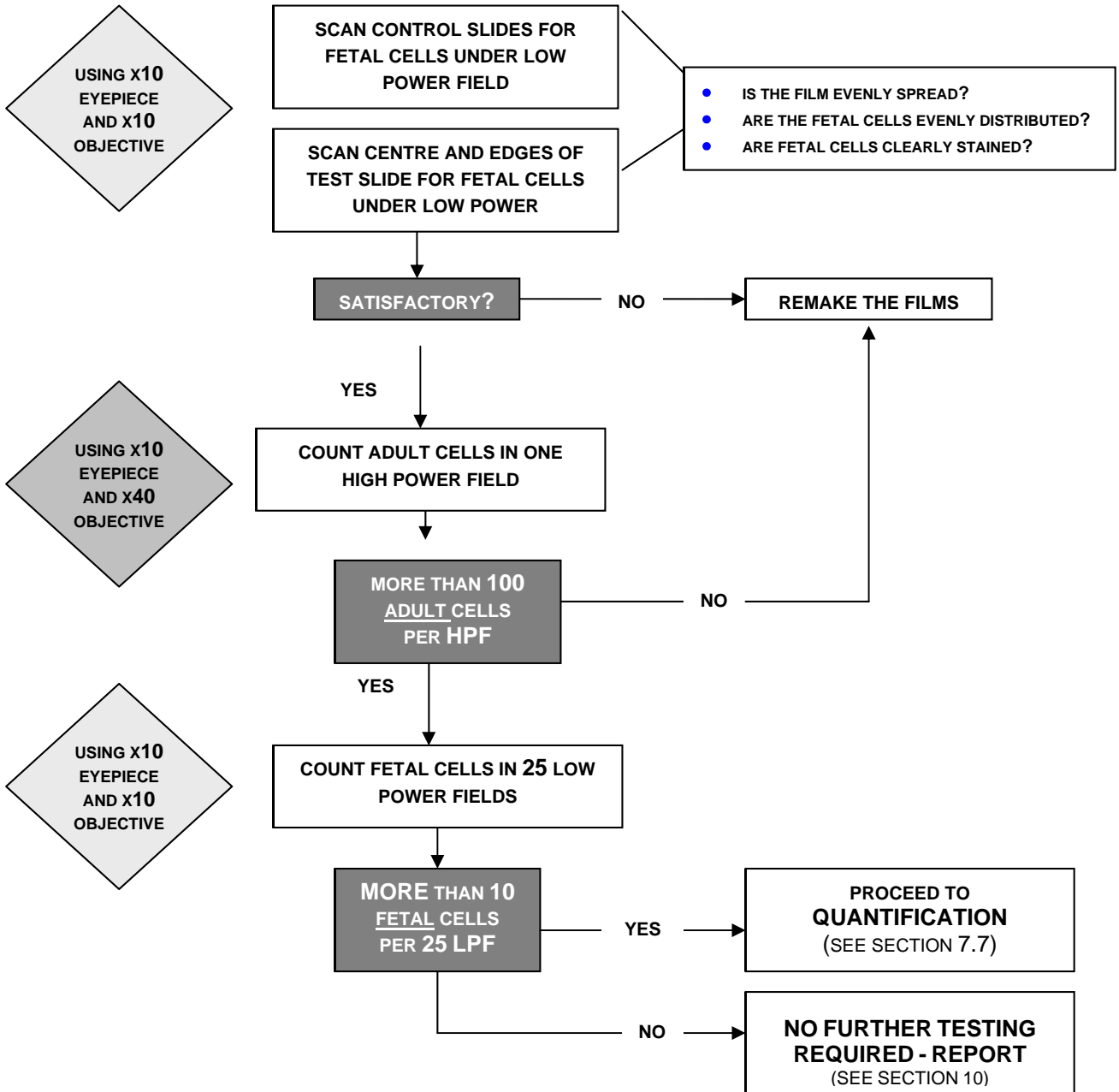
- A x10 eyepiece and x10 objective (**low power field**) should be used for screening.
- Initial validation should be performed to ensure that the area viewed in a low power field is at least 16 times greater than in a high power field.
- Each time this method is performed, adult cells in one **high power field** (x10 eyepiece and x40 objective) should be counted in the same area of the film as is to be screened, and there must be at least 100 adult cells present in this **high power field**.
- At least 25 **low power fields** should always be screened.

### Screening Method

1. Screen the test (and positive control) slides under **low power** using a x10 eyepiece and a x10 objective, to check for adequate staining and even distribution of fetal cells, if present.
2. Select an area of the film where the adult cells are touching but not overlapping and count **adult** cells under **high power**.
  - If there are more than 100 adult cells in one high power field then proceed to count fetal cells.
  - If there are fewer than 100 adult cells in one high power field, either remake the film or proceed to full quantification if any fetal cells are seen in 25 low power fields.
3. **Semi-quantitative screen** - go back to **low power** and examine 25 fields, counting **fetal** cells.
  - If a total of 10 or more fetal cells are seen, then quantification must be performed.
  - If fewer than 10 cells are seen in total then it can be assumed that the volume of fetal cells is less than 2mL. This will be covered by the standard anti-D immunoglobulin dose and no further action is required (see section 10 on how to report results).

**Figure 4** Flow diagram for semi-quantitative FMH screening by Acid Elution

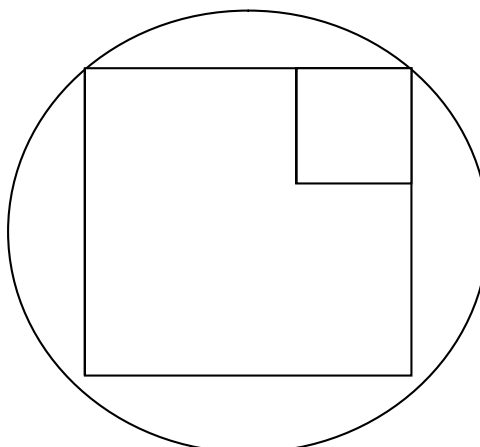
**IMPORTANT** – IF THE MICROSCOPE OBJECTIVES ARE DIFFERENT TO THOSE STATED HERE YOU **CANNOT** USE THIS SEMI-QUANTITATIVE SCREENING METHOD



## 7.7 FMH Quantification

Where using the semi-quantitative screen (described above) and 10 or more fetal cells are present in 25 low power fields, quantification should be performed. If the semi-quantitative screen is not used, then quantification should be performed where **any** fetal cells are seen during examination of the slides. For accurate quantification it is recommended that a minimum of 10,000 maternal red cell ghosts are examined using an x40 objective. The use of a Miller square or an indexed grid is necessary to obtain an accurate ratio of fetal to maternal cells in each blood film.

**Figure 5 Miller Square (see references for suppliers)**



### Using a Miller square

1. Using a x10 eyepiece and x40 objective, select an area of the film where cells are touching but not overlapping.
2. Count adult cells ( $A$ ) in the small square, including all cells which overlap the left hand or upper edges but not those overlapping the right hand or lower edges.
3. Count fetal cells ( $F$ ) in the large square, 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.
5. Assume the total adult cells scanned =  $A \times 9$  and total fetal cells counted =  $F$
6. Calculate the FMH using the formula described by Mollison (1972) (see section 7.8)

### Using an indexed square

The procedure is as above except that adult cells are counted in 10 of 100 squares in a 10x10 grid and the number of adult cells is then  $A \times 10$

#### RECOMMENDATION 6

For quantification, it is essential to survey a minimum of 10,000 cells to achieve a reasonable CV.

**Evidence level III, Recommendation grade B**

#### RECOMMENDATION 7

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

**Evidence level III, Recommendation grade B**

## 7.8 Calculation of FMH (using AE method)

This is calculated using the following formula (Mollison 1972), which assumes that:

- The maternal red cell volume is 1800mL
- Fetal cells are 22% larger than maternal cells
- Only 92% of fetal cells stain darkly

**The fetal bleed should be calculated as follows:**

$$\frac{\text{NUMBER OF FETAL CELLS PER HIGH POWER FIELD}}{\text{NUMBER OF MATERNAL CELLS PER HIGH POWER FIELD}} \times 1800 \times \frac{122}{100} \times \frac{100}{92}$$

**OR CAN BE SIMPLIFIED TO:**

$$\frac{\text{NUMBER OF FETAL CELLS PER HIGH POWER FIELD}}{\text{NUMBER OF MATERNAL CELLS PER HIGH POWER FIELD}} \times 2400$$

**For example, if the number of fetal cells is 9 and maternal cells 2000, then the fetal bleed will be calculated to be:**

$$\frac{9}{2000} \times 2400 = 10.8 \text{ ML PACKED FETAL RED CELLS}$$

## 7.9 False positives

Care should be taken to exclude false positive results. Although HbF increases during pregnancy, mainly in the second trimester, this rarely causes problems with FMH estimation by the AE method. Increased levels of HbF are seen in various genetic disorders including  $\beta$  thalassaemia, sickle cell anaemia and hereditary persistence of fetal haemoglobin (HPFH). These false positive results are often characterized by a large variation in the density of staining in the red cells containing HbF. There may be no clear definition between fetal cells and these 'intermediate' cells. Anti-D immunoglobulin should be given and the sample analysed using FC based on a minority population of D positive cells (see section 8), not on HbF. FC using HbF may be useful in rare cases for analysis of discrepant results (MacDonald, 2005).

### 7.10 Confirmation by FC

The sample should be referred for quantification of D positive cells by FC if the FMH is greater than 2 mL by the AE method.

## 8. FLOW CYTOMETRY

### 8.1 Principle

For the purpose of quantifying a minor population of D positive cells, a fluorochrome conjugated IgG monoclonal anti-D reagent is used to label the D positive fetal cells. Flow cytometry is used to detect and quantify the minor population in the D negative maternal sample.

Other tests using antibodies to alternative markers may be available but are not currently in routine use and are not considered further in this guideline.

## **RECOMMENDATION 8**

The original maternal EDTA sample (see Section 5.1) should be used for confirmatory FMH testing by FC.

### **Good practice point**

## **8.2 Staining**

Direct staining is recommended using a fluorochrome conjugated IgG monoclonal anti-D known to have high avidity for the D antigen. These reagents are commercially available.

When selecting reagents, consideration should be given to the specificity of the anti-D; ideally it should react with all D phenotypes capable of stimulating the production of 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 are known. The possibility that the anti-D reagent may not have detected D variant cord cells, although rare, should be taken into account when discrepancies arise between the results of AE and FC on a single sample.

## **8.3 Method**

Samples should be mixed thoroughly prior to testing. This should be for the equivalent of 10 minutes on an orbital mixer.

It is recommended that two aliquots of whole blood are taken from the original sample and are tested in parallel. The aliquots should give similar results within limits defined by the laboratory. Discrepant results should alert the operator to possible errors in sample preparation or counting.

These aliquots should be washed prior to staining to reduce the number of leucocytes and platelets present. However, to prevent the loss of fetal cells, a minimum number of washes should be applied and samples should be centrifuged adequately to ensure that all red cells are sedimented (e.g. 1000g for 2 minutes). Care should be taken when aspirating the wash solution; do not decant. Centrifugation should be with a swing out, not fixed or semi-fixed rotor. Do not use an automated cell washer.

## **8.4 Controls**

Non-specific uptake of antibody by cells included in the positive region can lead to falsely high results. Inclusion of an isotype and fluorochrome matched inert control is recommended to determine the proportion of background fluorescence. Once determined, this background should be subtracted from the count obtained in the 'positive' region.

The following semi-quantitative controls are included with each test batch and are tested against fluorochrome labelled anti-D.

### **Mixtures of D positive and D negative cells in these proportions:**

- **1% D positive**
- **0.2% D positive**

Acceptable ranges for positive controls should be determined by individual labs and should be nominally set at  $\pm 2SD$ .

The major cell population should be gated on log/log side and forward scatter. A loose gate should be drawn around the red cell population. Gating should aim to include all red cells and exclude platelets from the data.

Photomultiplier voltages should be set so that no more than 1% of negative events are in the first fluorescent channel so that the negative population can be clearly seen.

The 1% control should be used to set the regions on the histogram to differentiate positive and negative events. The 0.2% control is included to control the technique at the level at which anti-D above the minimum standard dose would be required. Markers (or regions) should be placed over the D positive and D negative populations. The number and percentage of cells in these two populations should be displayed and recorded.

No fewer than 500,000 events should be collected from each test against patient cells and a matched isotype control to ensure a CV of less than 10% for FMH >4mL. For the semi-quantitative control samples (1% and 0.2%), no fewer than 50,000 events should be collected.

### 8.5 Calculation of FMH using FC Method

This is calculated using the formula (Mollison 1972), which assumes that:

- The maternal red cell volume is 1800mL
- Fetal cells are 22% larger than maternal cells

**The fetal bleed should be calculated as follows:**

$$\frac{\text{PERCENTAGE FETAL CELLS}}{100} \times 1800 \times \frac{122}{100}$$

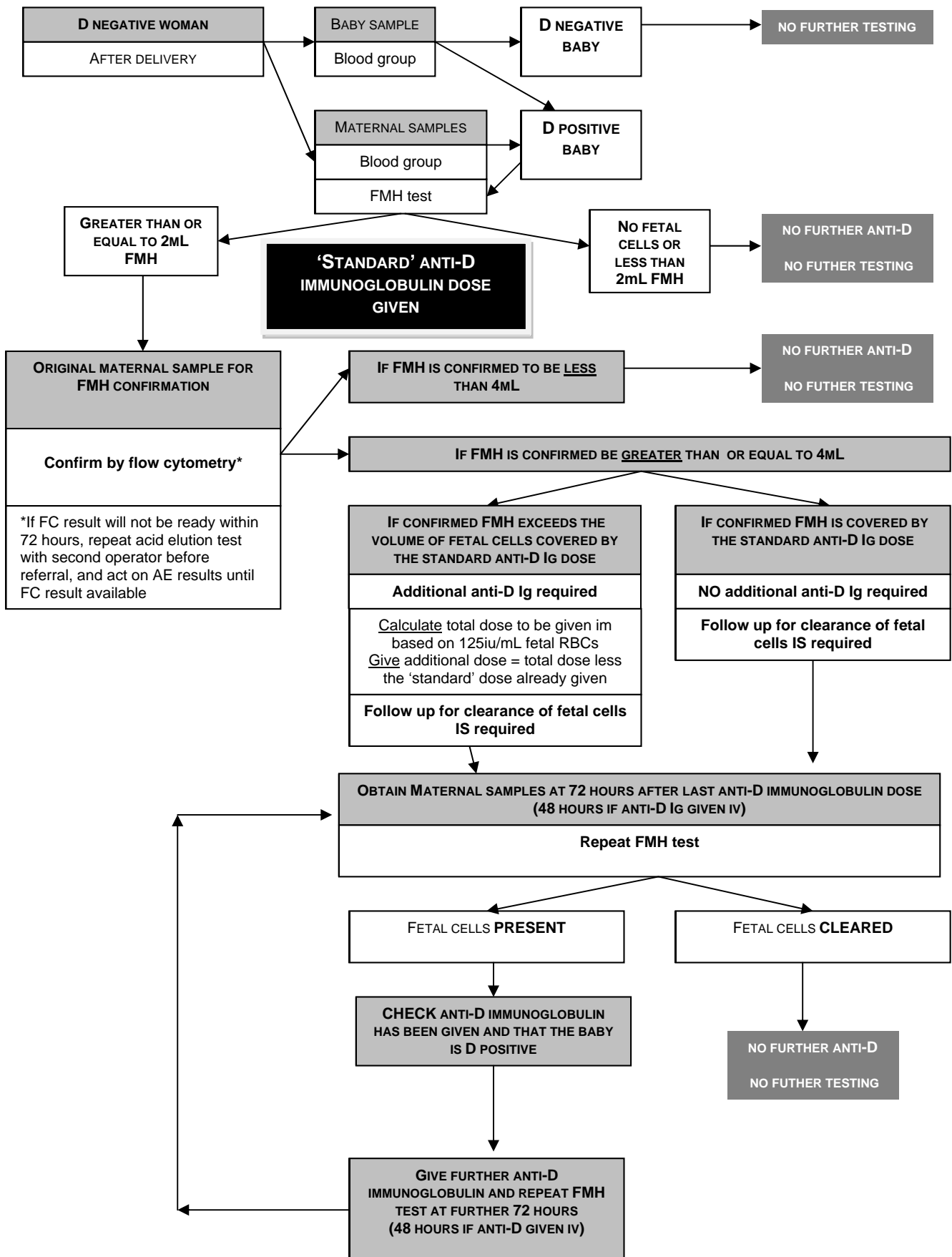
**OR CAN BE SIMPLIFIED TO:**

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

**e.g. where 0.5% of the red cells are fetal, then the FMH will be calculated as:**

$$0.5 \times 18 \times 1.22 = 10.98 \text{ mL PACKED FETAL RED CELLS}$$

**Figure 6** Flow diagram for FMH testing and subsequent actions





## 9. CONFIRMATION AND FOLLOW-UP

Also see flow diagram (Figure 6) for 'FMH testing and subsequent action' which is a summary of the following section

### 9.1 Confirmation

#### RECOMMENDATION 9

FMH of greater than or equal to 2mL by AE should be confirmed by the flow cytometry method (FC), using the original sample. If the FC result will not be available within 72 hours, the AE test should be repeated by a second operator before referral. In this case the AE results should be acted upon until the FC result is available.

#### Evidence level IIb, Recommendation grade B

If FC is not available within a clinically relevant time frame, either locally or at an accessible reference centre, then a different operator must repeat the AE test on the original sample before it is referred for flow cytometry.

### 9.2 Follow-up

#### 9.2.1 Follow-up of confirmed FMH

- a) If FMH confirmed by FC is less than 4mL and standard anti-D Ig has been given, **no further testing is required.**
- b) If FMH, confirmed by FC, is greater than or equal to 4mL but covered by the standard anti-D Ig dose in use, **additional anti-D Ig is not required.** However any confirmed FMH greater than or equal to 4mL is considered to be 'significant' and there should be a **follow-up maternal sample to check for clearance of fetal cells**, to confirm that the anti-D Ig has been given.
- c) If the confirmed FMH volume exceeds that covered by the 'standard' post-natal anti-D immunoglobulin dose already given, **an appropriate supplementary dose of anti-D immunoglobulin will be required and, follow-up maternal samples are required to check for clearance of fetal cells.**

**A 'standard' anti-D immunoglobulin dose of 500iu covers an FMH of up to 4mL fetal red cells, 1250iu up to 10mL and 1500iu up to 12mL when administered intramuscularly**

**Calculate the additional anti-D immunoglobulin dose according to the formula 125iu for every 1mL fetal cells, rounded up to the nearest vial size, taking into account the post-natal anti-D immunoglobulin dose already given (but not RAADP dose). It is advisable to use the same batch as the standard anti-D immunoglobulin dose where possible to limit exposure to different batch products (BCSH 2006a).**

For large FMH the use of iv anti-D immunoglobulin should be considered, and if used, specialist advice should be sought from a haematologist or reference centre before administration. Note that the dose calculation is different for intravenous anti-D immunoglobulin. It is good practice to advise checking the haemoglobin level on the baby in these cases.

#### 9.2.2 Timing of follow-up samples

The previous guidelines (BCSH 1999) recommended that a repeat sample was required 48 hours after the anti-D immunoglobulin. Where anti-D immunoglobulin is administered iv anti-D coated cells are likely to be cleared from the circulation within 48 hours, but where anti-D is administered im this might not always be the case, and a test at 72 hours can be more informative (Lubenko 1999). It is now recommended that a repeat sample should be obtained within 72 hours of administration of the previous dose of anti-D Ig if given im and 48 hours if given iv, and that circumstances such as the time required to obtain an FMH result,

availability of the patient, and the route of administration of previous dose(s) of anti-D should be taken into account when deciding on the timing of the sample.

#### **RECOMMENDATION 10**

A repeat maternal sample should be taken and screened 72 hours after the total dose of anti-D immunoglobulin injection (48 hours if the anti-D Ig was given iv). This is to check for clearance of fetal cells.

**Evidence level lib, Recommendation grade B**

#### **9.2.3 Action if fetal cells have NOT cleared on follow-up sample**

The presence of fetal cells in the follow-up sample may be indicative that insufficient anti-D immunoglobulin has been administered. If fetal cells are present in the follow-up sample;

- Confirm that the anti-D immunoglobulin has been administered.
- Confirm that the blood group of the baby is D positive.
- Send the follow-up maternal EDTA sample for testing by FC.

Give further anti-D immunoglobulin as dictated by the volume of fetal cells remaining and repeat the FMH test on a further maternal EDTA sample 72 hours after the repeat anti-D immunoglobulin injection. Repeat this sequence of immunisation and testing until no fetal cells are seen in the FMH test.

#### **9.2.4 Fetal cells have cleared on follow-up sample**

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

The 1999 guidance on the need for checking free anti-D if fetal cells have cleared has been removed. This due to concerns that the presence of free anti-D might be misinterpreted as a contraindication to further follow-up in cases where fetal cells are not cleared.

### **9.3 Longer term follow-up**

A maternal RBC antibody screen at six months is desirable but not always practicable. It provides the opportunity to counsel the woman about the possibility of sensitisation to the D antigen and the nature of HDN. It also provides an opportunity to perform an Rh phenotype on the partner, if not already undertaken. It is important to note that the absence of immune anti-D six months post delivery does not mean that the woman has not been sensitised. All women who have had FMH greater than 4mL detected should have this highlighted at booking in subsequent pregnancies.

## **10. REPORTING FMH RESULTS TO CLINICIANS**

It is important that FMH results are timely and effectively communicated. This will allow clinicians to manage the woman appropriately.

The report format needs to communicate the following;

- The reason for the sample (e.g. post-delivery, sensitising event, follow-up of significant FMH etc)
- The result of the FMH test in 'mL fetal red cells' rounded to the nearest mL
- Whether any supplementary anti-D immunoglobulin is required if a standard dose of anti-D immunoglobulin has already been administered

- Advice about the anti-D immunoglobulin dose required to cover the reported bleed.
  - Advice regarding follow-up samples to check for clearance of fetal cells
- Results of confirmatory tests from the reference lab (or from repeat in-house testing by AE where FC results are not available within 72 hours and the AE results are acted on in the interim.)

#### **RECOMMENDATION 11**

Where an estimated FMH exceeding 2mL is detected, a preliminary result should be communicated to the ward, prior to the results of the confirmatory test, to alert clinicians to the possible need for additional anti-D immunoglobulin prior to discharge.

#### **Good practice point**

**Figure 7a Suggested report format either if no fetal cells or less than 2mL fetal cells present on semi-quantitative screen**

#### **FMH test by acid elution**

**Give standard post-natal anti-D immunoglobulin dose**

**Less than 2mL fetal cells seen; this FMH will be covered by the standard dose of {insert dose} iu anti-D immunoglobulin**

**No further testing is required**

*The use of the term 'negative' is not used in this context because of the potential of communicating the wrong message to clinicians who may assume that a standard dose of anti-D immunoglobulin is not required.*

**Figure 7b Suggested report format if FMH volume greater than or equal to 2mL 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**

**Fetal cells seen**

**Estimated {insert volume rounded 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 anti-D immunoglobulin\***

**Send a further maternal EDTA sample 72 hours after the last anti-D injection to check for clearance of fetal red cells**

**Figure 7c** Suggested report format if confirmed FMH exceeds the volume covered by the standard anti-D Ig dosage in use.

**FMH test by acid elution**

**Fetal cells seen**

**Estimated {insert volume rounded to nearest whole mL} mL fetal red cells by acid elution**  
**This has been sent for confirmation {results of confirmatory test including methodology}**

**Give a further dose of {insert dose} iu anti-D immunoglobulin immediately**

**Send a further maternal EDTA sample 72 hours after the last anti-D injection (48 hours if iv anti-D has been given\*\*) to check for clearance of fetal red cells**

*\*\*For large FMH, additional comment should be made about the special formulations of anti-D immunoglobulin available.*

**11. AUDIT**

Clinical audit should be undertaken to ensure that all D negative women have had a FMH test at the appropriate time and, if the test is positive, that the appropriate action has been taken.

A laboratory audit of the results of confirmatory tests for FMH compared to the initial quantification should be undertaken.

In the small number of women requiring additional anti-D immunoglobulin, an audit of correct confirmatory and follow-up procedures should be undertaken.

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## **STATEMENTS OF EVIDENCE**

Ia Evidence obtained from meta-analysis of randomised controlled trials.

Ib Evidence obtained from at least one randomised controlled trial.

IIa Evidence obtained from at least one well-designed controlled study without randomisation.

IIb Evidence obtained from at least one other type of well-designed quasi-experimental study.

III Evidence obtained from well-designed non-experimental descriptive studies, such as comparative studies, correlation studies and case studies.

IV Evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities.

## **GRADES OF RECOMMENDATIONS**

**A Requires at least one randomised controlled trial as part of a body of literature of overall good quality and consistency addressing the specific recommendation.**

(Evidence levels Ia, Ib)

**B Requires the availability of well conducted clinical studies but no randomised clinical trials on the topic of recommendation.**

(Evidence levels IIa, IIb, III)

**C Requires evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities. Indicates an absence of directly applicable clinical studies of good quality.**

(Evidence level IV)