

CAT

Critically Appraised Topic

Titel: Fetal Cell Count by Flow Cytometry.

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CLINICAL BOTTOM LINE

CLINICAL RELEVANCE: For an adequate prevention of alloimmunization of the mother during pregnancy, extra doses RhoGAM® are often needed in case of a massive fetomaternal transfusion (FMT). *'It is essential to **quantify the volume of FMT** to calculate the appropriate anti-D dosage for administration.'*

*Attachment 1: Evidence level II b – Recommendation grade B (BCSH 2006);
 Evidence level III – Recommendation grade B (SOGC 2003);
 Recommendation grade B (RCOG 2002).*

DIAGNOSTIC-THERAPEUTIC IMPACT: Guidelines recommend to report the quantitative result of FMT as soon as possible, **within 72 hours**. Having flow cytometry (FCM) readily available 24h/24h 7d/7d is not necessary: *'At no time should anti-D be withheld based upon, or pending, the results of a test to quantify FMT' (ANZSBT 2002). 'Following sensitising events anti-D should be injected as soon as possible and certainly within 72 hours of the event' (III-B BCSH 2006). 'Some protection may be offered up to 10 days after the sensitising event' (BSCH 2006; RCOG 2002; Lee et al 1999).*

Underestimation of FMT could have important health and medico-legal consequences. To confirm a massive FMT, a repeat estimation of the FMT should be carried out 48 h following the initial anti D injection (BCSH 1999). A duplicate test sample is a minimal requisite (BCSH 1999). Follow-up samples check that fetal cells are being removed from the circulation.

TECHNICAL PERFORMANCE: Flow cytometry is a reliable method for the detection of fetal cells in routine laboratory to quantify a massive FMT:

- FCM is **more objective & more precise (CV < 15%** in Davis 1998) to quantify FMT than the Kleihauer-Braun-Betke (KBB) method (CV 39,5% - 71,8% in Lafferty 2003). *'To minimize the coefficient of variation obtainable with FCM, no less than 500 000 events should be collected' (BCSH 1999).*
- Anti-HbF fluorescent labeled reagent is preferred above anti-D, because direct staining of D-positive cells can give FN results when the mother received RhoGAM®. Two parameter FCM can better discriminate between the fetal and the maternal red cell population (Pelikan 2006).
- A sample must be EDTA-anticoagulated blood from the mother.
- The analytical sensitivity must be at least 0,6% fetal red blood cells (fRBC), the clinical relevant level to detect a FMT of 15 ml fRBC or 30 ml of fetal blood. This can be neutralised by one dose of RhoGAM® 300 µg or 1500 I.U., as marketed in Belgium (CLSI H52-A 2001). The limit of detection reaches the clinical relevance: **The analytical sensitivity of FCM is 0,1% fRBC** or 2,4 ml fRBC or ~5 ml fetal blood (Pelikan 2006;

Davis 1998; Mollison 1993) when 50 000 events are collected (collection of 500 000 events would improve the sensitivity). The analytical sensitivity of KBB is 5 ml fetal blood (*Ochsenbein 2002*).

- Until now there is no laboratory method available that is able to detect minor FMT because none can reach the limit for sensitisation, corresponding to 0,1 ml fRBC or 0,004% fRBC (*SOGC 2003*).

DECISION MAKING: A clinical-diagnostic-therapeutic algorithm is proposed based upon the organisational structure of LAG, discussions with the gynaecologists of UZ Leuven and evidence-based literature. Although literature suggests the more accurate FCM test for massive FMT, the following arguments restrained us from implementing FCM in our routine laboratory:

- Only 0,4% of requests in UZ Leuven are FMT > 15 ml fetal blood and are taken into consideration for FCM analysis (~ 4 samples a year);
- Such a low turnover can not build up expertise;
- Expertise is essential because of the difficulties we experienced to interpret cytograms.

CLINICAL/DIAGNOSTIC SCENARIO

During pregnancy or at delivery FMT can cause alloimmunization of the mother, in response to contact with Rhesus (Rh) system antigens of the foetus. *SOGC Guidelines (2003)* mention 'even 0,1 ml of D-positive red blood cells can sensitize 3% of D-negative women'. Alloimmunization can cause haemolytic disease of the newborn (HDN) in subsequent pregnancies, with a high risk for mortality.

Guidelines recommend the administration of anti-Rh D immunoglobulin to Rhesus-negative woman to prevent sensitisation (*I-A SOGC 2003; IV-C BCSH*). In Belgium 300 µg or 1500 I.U. of RhoGAM® is used for routine antenatal anti-D prophylaxis (RAADP). 'The RAADP scheme should be regarded as supplementary to any anti-D administered for sensitising episodes' (*IIB-B BCSH 2006; NICE 2002; RCOG 2002*). The dose of anti-D should neutralise the volume of FMT. 300 µg RhoGAM® neutralises 15 ml of fetal red blood cells (fRBC), 30 ml of fetal blood, or 0,6% fRBC/adult RBC (*CLSI 2001*). In case of a massive FMT, quantification of FMT is recommended to adapt the dose of anti-D (*III-B SOGC 2003; IIB-B BCSH 2006; B RCOG 2002*). 'Transfusion of 30 ml or more of fetal blood occurs in 0,3 – 1,7% of all deliveries' (*Ochsenbein 2002; Chen 2002*).

There are different techniques for estimating the FMT, with different accuracy and precision. 'The Kleihauer-Braun-Betke (KBB) method is currently designated the Class B reference method in the *CLSI Guideline H52-A (2001)*, due to the anticipation that flow cytometric methods based upon HbF detection by monoclonal antibodies will gain acceptance as the more appropriate reference method, due to greater accuracy.' The KBB method is a subjective test with multiple sources of variability, resulting in an inter-CV of 39,5% - 71,8% (*2003 Lafferty Coag Trans Med*). An objective method like flow cytometry (FCM) can minimise inter-individual variability. In our current laboratory organisation, KBB is performed 24h/24h 7d/7d but flow cytometry is not readily available. This critically appraised topic evaluates whether flow cytometry is a reliable method for the detection of fetal cells in the routine laboratory.

QUESTION(S)

- 1) **CLINICAL RELEVANCE:** Which clinical indication(s) do Guidelines propose for fetal cell count in maternal blood?
- 2) **DIAGNOSTIC-THERAPEUTIC IMPACT:** Is it necessary to have flow cytometry 24h/24h 7d/7d available? If not, what is clinically acceptable?
What is the benefit of follow-up samples?
- 3) **TECHNICAL PERFORMANCE:** Is flow cytometry a reliable method for the detection of fetal cells in routine laboratory? Is it more accurate than the Kleihauer-Betke method? Can the limit of detection (analytical sensitivity) reach the clinical relevance?

SEARCH TERMS

- 1) *MeSH Database (PubMed Medline from 1966): MeSH term: 'Antigens, Rhesus D [immunology]; Foetus [immunology]; Immunity, Maternally-Acquired; Infant, Newborn; Maternal-Fetal Exchange; Prenatal Diagnosis; Prospective Studies; Retrospective Studies; Fetomaternal hemorrhage [prevention & control]; Fetomaternal hemorrhage AND Flow; Carbonic anhydrase AND Haemoglobin; Xmn-I polymorphism; Hereditary persistence of fetal haemoglobin; Transfus Med [ta] AND anti-D'*
- 2) *SUMSearch (<http://sumsearch.uthscsa.edu/>): Kleihauer RhoGAM®,*
- 3) *The National Institute for Clinical Excellence (<http://www.nice.org.uk/>),*
- 4) *Cochrane (<http://www.update-software.com/cochrane/>): No reviews by topic: 'Neonatal; Pregnancy and childbirth'; Clinical trials: search for 'fetomaternal hemorrhage; Kleihauer; trauma AND pregnancy'*
- 5) *National Committee for Clinical Laboratory Standards (NCCLS; <http://www.nccls.org/>),*
- 6) *UpToDate Online version 16.1 (2008) (www.uptodate.com) Search term: 'Rhesus alloimmunization': The initial prenatal assessment and routine prenatal care. Lockwood CJ. Last updated: February 14, 2008. / Management of Rhesus (Rh) alloimmunization in pregnancy. KJ Moise. Last updated: February 14, 2008. / Pathogenesis and prenatal diagnosis of Rhesus (Rh) alloimmunization. KJ Moise. Last updated: January 15, 2008. / Prevention of Rh(D) alloimmunization. KJ Moise. Last updated: November 21, 2006.*
- 7) *The Society of Obstetricians and Gynaecologists of Canada (SOGC; http://www.sogc.org/index_e.asp) Search terms: 'Rho(D) immune globulin; Rh iso- or allo-immunization; anti-D; anti-Rh; WinRho; RhoGAM®; pregnancy'.*
- 8) *<http://www.fda.gov> Search term: 'Fetal'.*
- 9) *KCE Federaal Kenniscentrum voor de Gezondheidszorg (<http://www.kce.fgov.be>)*
- 10) *National Guideline Clearinghouse (<http://www.guideline.gov>) Search term: 'alloimmunization'. Management of alloimmunization during pregnancy. American College of Obstetricians and Gynecologists (ACOG) 2006.*

RELEVANT EVIDENCE/REFERENCES

*Citations are marked 'in italic' in the full text.**Guidelines and Recommendations*

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- 3) *Guidelines on the prophylactic use of Rh D immunoglobulin. National Blood Authority (NBA) 2003: Announcement d.d. 2004*
- 4) *Nationale richtlijn prenatale zorg: een basis voor een klinisch pad voor de opvolging van zwangerschappen; KCE reports vol. 6A. Federaal Kenniscentrum voor de Gezondheidszorg (KCE) 2004.*
- 5) *Erythrocytenimmunisatie en zwangerschap: RICHTLIJN No 50. Nederlandse Vereniging voor Obstetrie en Gynaecologie (NVOG) 2003.*
- 6) *Prevention of Rh alloimmunization. Maternal-Fetal Medicine Committee, Genetics Committee: Fung Kee et al. J Obstet Gynaecol Can 2003; 25:765-73.*
- 7) *Prevention of Rh Alloimmunization. Society of Obstetricians and Gynaecologists of Canada (SOGC) 2003.*
- 8) *Use of Anti-D Immunoglobulin for Rh Prophylaxis. Royal College of Obstetricians and Gynaecologists (RCOG) 2002.*

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International Forum, Reviews, Thesis

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APPRAISAL

I. CLINICAL RELEVANCE

During pregnancy or at delivery fetomaternal transfusion (FMT) can cause alloimmunization of the mother, in response to contact with antigens on the surface of fetal red blood cells acquired from the father. The most immunogenic antigen systems are Rhesus D (RhD or D), followed by Rhesus c, Kell, Rhesus E, Kidd and Duffy¹. Alloimmunization can cause haemolytic disease of the newborn (HDN) when maternal antibodies of IgG-type cross the placenta. Sensitised fRBC's are destroyed by macrophages in the fetal spleen and by cellular cytotoxicity (NVOG 2003). HDN happens occasionally in the first pregnancy, but potentially more often in subsequent pregnancies, with a high risk for mortality.

Rhesus D alloimmunization of the mother means the Rhesus D-negative mother produces anti-D antibodies (Ab) against Rhesus D-positive fetal RBC's. The Rhesus D antigen is expressed on the surface of fRBC after 30 days of gestation. Prophylactic anti-D administration prevents significantly Rhesus D alloimmunization and reduces the risk of HDN. Since the availability of RhoGAM® in the 1960s, postpartum anti-D prophylaxis for RhD-negative mothers decreased alloimmunization from 14% to 2% in the USA (*Vox Sanguinis* 2003). Thanks to the introduction of routine antenatal anti-D prophylaxis (RAADP) in 1998 in The Netherlands and 2002 in UK alloimmunization decreased from 1,5% to 0,2%² (2006 *Proefschrift Pelikan*; 2003 *NVOG Richtlijnen*; 1999 *Lee Guidelines Transfus Med*) and to 0,1% in Finland, France, Italy and USA (*Vox Sanguinis* 2003). RAADP is cost effective in a first pregnancy, not in subsequent pregnancies (*Uptodate*; *REVIEW Crowther 1999*; *NICE 2002*; *BCSH 1999*; *NVOG 2003*; *VVOG 2007*). Anti-D prophylaxis is of no use to women who have already been sensitised to the D antigen (*NICE 2002*; *RCOG 2002*). D^{weak}-positive woman, having a D^u allelic variant, express small amounts of D antigen on the surface of their RBC's and do not need anti-D prophylaxis (*Hillyer eds 2001*).

Although the Rhesus D antigen is thought to elicit a strong immune response, the response varies considerably among individuals (*Uptodate 2008*). Factors influencing the primary immunological response are the immunogenicity of the fetal RBC's (homozygous have higher dose of D antigen compared to heterozygous), the frequency of the FMT and ABO-compatibility.

Transplacental FMT accounts for virtually all cases of maternal RhD-alloimmunization (*Uptodate 2008*). The clinical situations in which fetal cell detection is demanded, come down to the **quantification of the FMT volume**. There are two main clinical applications for **fetal cell count**. One application is to determine if there has been a traumatic event to the placenta. The other application, developed in the late 1960s, calculates the amount of anti-Rhesus D immune globulin such as RhoGAM® that should be given to prevent RhD-alloimmunization (*Mollison 1993*).

¹ Het College voor de Bloedtransfusie adviseert om c-, E- en K-negatief bloed te selecteren voor alle vrouwen <45 jaar (*NVOG 2003*).

² Despite a significant decrease of alloimmunization thanks to RAADP, there are still 170 complicated pregnancies a year in The Netherlands due to anti-D immunization (*NVOG 2003*). This is due to underestimation of FMT, underdosing of RhoGAM®, unrecognised spontaneous FMT, mothers refusing RAADP and incompatible transfusion or transplantation.

There is no strong evidence for the clinical application of fetal cell count for:

- Evaluation of haematopoiesis in anemia and myelodysplasia
- Simplified measurement of adult HbF-containing cells counting for hemoglobinopathies. Some authors report the use of FCM for the differential diagnosis of Hereditary Persistence of fetal haemoglobin (HPFH).
- Therapeutic monitoring of treatments intended to raise HbF levels in Sickle Cell Disease (eg. butyrate, hydroxyurea)
- Evaluation of pre-eclampsia risk

I.1. Placental trauma

*SOGC Guidelines mention 'Even 0,1 ml of D-positive red blood cells or fRBC can sensitize 3% of D-negative women' (SOGC Guidelines 2003). 0,1 ml fRBC is the same as 0,004% fRBC or 1/25 000 fRBC. All pregnancies release fetal cells in the maternal circulation, estimated at 1/50 000 fRBC (0,002% or 0,05 ml) à 1/20 000 fRBC (0,005% or 0,12 ml) (Warzynski 1997; Medearis 1984). **There is an overlap between the natural process of fetal cell release in any pregnancy (0,05 – 0,12 ml fRBC) and the volume that may cause sensitisation after a minor FMT (0,1 ml).** And adult HbF-containing RBC's are increased during pregnancy in normal conditions (Wiley 2007). In these circumstances, the detection of small volumina of FMT will have no impact on the clinical decision making regarding the administration of RhoGAM®. Thus, there is **no clear indication for fetal cell count in minor traumata.** All Rhesus D-negative woman are at risk for alloimmunization if they bear a Rhesus-positive child and should be offered RAADP without bothering if a minor trauma took place. **Guidelines recommend the administration of RAADP to all non-sensitised pregnant women who are Rhesus D-negative (2002 NHS/NICE; I-A SOGC 2003; IV-C BCSH 2006; IA Uptodate 2008).** Previous administration of RhoGAM® for a potentially sensitising event early in pregnancy is no contra-indication for RAADP (NICE 2002; RCOG 2002). But RhoGAM® should not be given to sensitised women with preformed anti-D antibodies (2003 NBA; 2002 RCOG). **In Belgium 300 µg or 1500 I.U. of RhoGAM® is used for RAADP at 28 weeks of gestation (KCE 2004).** As commented in section II.1.2. nine weeks later, i.e. at 37 weeks of gestation, approximately 5ng/ml of anti-D is left; which can still neutralize approximately 2,5 ml fetal blood. Attachment 2 gives an overview of international RAADP Guidelines.*

The size of the trauma during pregnancy elevates this risk for alloimmunization. **In macro-traumata, there is a clear evidence to quantify the volume of FMT to calculate the appropriate anti-D dosage for administration (Iib-B BCSH 2006; III-B SOGC 2003; B RCOG 2002).** Anti-D administered for sensitising episodes is independent of and supplementary to the RAADP scheme (Iib-B BCSH 2006; NICE 2002; RCOG 2002).

I.2. Therapy-dosage determination

The dose of anti-D should neutralise the volume of FMT (CLSI 2001):

300 µg RhoGAM® neutralises 30 ml of fetal blood, 15 ml fRBC or 0,6% fRBC.

⇒ Clinical-therapeutic action (i.e. augmentation of the RhoGAM® dose) has to be taken when FMT reaches 30 ml fetal blood, 15 ml fRBC or 0,6% fRBC. The analytical sensitivity should be at least that of the clinical usefulness, thus 0,6% (CLSI 2001).

We can presume the range of clinical usefulness goes from 0,6% to 5% fRBC (i.e. up to 250 ml fetal blood or 120 ml fRBC) (*CLSI 2001*). The upper range is deduced from an assumed fetal blood volume of roughly 100 ml/kg body mass and an assumed fetal weight of 2,5 kg approaching the end of the third trimester.

Martens F (presentation MaNaMa 2007):

	Blood volume (ml/kg body mass)
Premature	115
Neonatus	80 – 110
Baby, child	75 – 100
Adult	70

Doubilet PM (J Ultrasound Med 1997):

Foetus	Weight (kg)
20 weeks	0,3
28 weeks	1
36 weeks	2,6
41 weeks	3,6

Mollison's Blood Transfusion in Clinical Medicine 1993 arbitrarily defines a massive FMT > 50 ml fetal blood or > 25 ml fRBC. But in clinical practice, a FMT > 30 ml fetal blood or > 15 ml fRBC is thought to be of significant relevance and may be defined as massive FMT.

Risk factors for massive FMT are 'potentially sensitising events' that could cause the mother to produce antibodies against the D antigen. Guidelines recommend fetal cell count for the following **potentially sensitising events** (*2006 BCSH; 2003 SOGC; 2003 NBA; 1999 Lee e.a.; Uptodate*):

- Fall / abdominal trauma considered sufficient to cause FMT
- Invasive prenatal diagnosis:
 - amnio- and cordocentesis
 - chorionic villus sampling
- In-utero therapeutic intervention:
 - intrauterine transfusion
 - shunting
- Antepartum haemorrhage (APH)
- External cephalic version
- Ectopic pregnancy
- Intrauterine death, miscarriage, termination of pregnancy
- Spontaneous (increases with advancing gestational age and is highest at delivery)

Controversy exists about vaginal blood loss as a risk factor (*NVOG 2003 versus RCOG 1999*).

In case of a massive FMT, quantification of FMT is recommended to adapt the dose of anti-D (*III-B SOGC 2003; IIb-B BCSH 2006; B RCOG 2002*). Guidelines for therapy-dosage determination in case of possible sensitising events are summarized in the following tables:

AUSTRALIA / NEW ZEALAND (2003 NBA Guidelines; 2002 ANZSBT Guidelines):

1st trim	250 IU anti-D	no FMT test
2nd, 3d trim	625 IU anti-D neutralises 6 ml fRBC + extra 625 IU above 6ml fRBC	test FMT (KBB or preferable FCM) → Report ml FMT & number of vials (of 625IU) required
Follow-up large FMT	calculate supplemental dose	test FMT with FCM Sample 48h after i.m. anti-D

UNITED KINGDOM (1999 *Lee ea Recommendations of anti-D administration; 2006 BCSH Guidelines for anti-D administration; 2002 RCOG Guidelines*):

< 12 we	(250 IU anti-D)	no FMT test
12 – 20 we	250 IU anti-D	no FMT test
> 20 we	500 IU anti-D + extra 125 IU/ml FMT above 4ml fRBC	test FMT → KBB < 4ml fRBC → FCM also > 4ml fRBC
Follow-up large FMT	calculate supplemental dose	test FMT with FC Sample 72h after i.m. anti-D

CANADA (2003 *SOGC Guidelines RhD alloimmunization*):

< 12 we	600 IU anti-D	Quantitative FMT test when risk of > 15ml fRBC* => extra dose 50 IU / 0,5ml fRBC * Placental abruption, blunt trauma to abdomen, cordocentesis, placenta previa with bleeding
> 12 we	1500 IU anti-D	

THE NETHERLANDS (2003 *NVOG Richtlijnen*):

< 20 we	375 IU anti-D	no FMT test
20 – 26 we	375 IU anti-D	no FMT test
> 26 we	1000 IU anti-D + extra dosis > 20ml foetaal bloed	“Vóór anti-D toediening wordt aanbevolen de mate van FMT te objectiveren met KBB.”
- Neonatale anemie - tansfusie D+ donor	Bereken anti-D dosis	“Vóór anti-D toediening dient men KBB te verrichten EN bij FMT > 20 ml anti-D dosis te berekenen”

BELGIUM (2007 *VVOG. 2006 CEBAM Aanbevelingen Zwangerschaps-begeleiding; 2004 KCE Nationale richtlijn prenatale zorg; 2001 CLSI/NCCLS Guideline H52-A; RhoGAM® manufacturer's instruction*):

< 12 we	?	
12 – 20 we	?	
> 20 we	1500 IU anti-D + extra dosis > 30ml foetaal bloed	“Vóór anti-D toediening wordt aanbevolen de mate van FMT te objectiveren met KBB.”

Normal delivery is considered a sensitising episode. After delivery, a RhD-negative mother will receive RhoGAM® if her child is tested RhD-positive. ‘Administered RhoGAM® during pregnancy should not be a contra-indication to give the postnatal dose if the infant is RhD-positive’ (Lee 1999 *Transfus Med*). It is recommended to calculate the volume of FMT approximately 30 minutes after delivery (BCSH 2006). In UZ Leuven the sample is collected

1h à 1h30 after delivery (and a little later for a caesarean section). The calculated fetal cell count will determine the postpartum dose of RhoGAM®. *'Approximately 1 in 1000 deliveries will be associated with an excessive FMT; risk factors will identify only 50% of these'* (Ness 1987). *'Transfusion of 30 ml or more of fetal blood occurs in 0,3 – 1,7% of all deliveries'* (Ochsenbein 2002; Chen 2002).

GUIDELINES POST-PARTUM

AUSTR / NZ (2003 NBA Guidelines; 2002 ANZSBT Guidelines)

post partum	600 IU i.m.	
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UK (2006 BCSH Guidelines; 1999 Lee e.a. Guidelines Transfus Med)

post partum	500 IU i.m.	Calculate extra dose if FMH > 4 ml: 125 IU i.m. per extra ml FMH tested with FC Sampling 30 - 45 min after FMH
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NL (2003 NVOG Richtlijnen)

post partum	1000 IU i.m.	"Vóór anti-D toediening wordt aanbevolen de mate van FMT te objectiveren met KB."
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CAN / B / USA (2003 SOGC Guidelines; 2006 CEBAM Aanbevelingen Zwangerschapsbegeleiding; 2004 KCE Nationale richtlijn prenatale zorg; RhoGAM © manufacturer's instruction)

post partum	1500 IU i.m.	Calculate extra dose > 15ml FMH
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II. DIAGNOSTIC-THERAPEUTIC IMPACT

There are two causes of failure to prevent alloimmunization: either the dose of anti-D is insufficient or it is given too late (*Mollison 1993*). Therapy-dosage determination is essential in massive FMT. How fast RhoGAM® has to be administered and how fast the fetal cell count has to be available will be answered in this section. In the current laboratory organisation in UZ Leuven, KBB is performed 24h/24h 7d/7d but FCM is not readily available.

II.1. Clinical impact of fetal cell detection

II.1.1. Diagnostic aspect

The diagnosis of Rhesus D alloimmunization is based on an indirect Coombs test (screening for irregular antibodies) for the detection of anti-D Ab in maternal serum and the anti-D titer. A positive indirect Coombs test means that the foetus is at risk for HDN, not that it has occurred or will develop. A supplemental diagnostic test is necessary to detect circulating fetal red blood cells in maternal circulation. The quantification of those circulating fetal cells offers an idea about the volume of the FMT that has occurred. A large FMT can induce an increase in anti-D titer and a higher risk for HDN.

To adequately prevent Rhesus alloimmunization, the dose of RhoGAM® administered to the RhD-negative mother should neutralise the size of the FMT. For any FMT greater than the volume neutralised by one dosis of RhoGAM® an appropriate supplementary dose of RhoGAM® must be given immediately (*BCSH 1999*). **Clinical-therapeutic action (i.e. augmentation of the RhoGAM® dose) has to be taken when FMT reaches 30 ml fetal blood, 15 ml fRBC or 0,6% fRBC.** *‘Only 21/1248 patients (1,7%) had more than 30 ml of fetal blood detected in maternal blood’ (Chen 2002). ‘Transfusion of 30 ml or more of fetal blood occurs in 0,3% of all deliveries’ (Ochsenbein 2002).*

To confirm a massive FMT, ***‘a repeat estimation of the FMT should be carried out 48 h following the initial anti D injection and the serum plasma should be screened for anti-D’*** (*BCSH 1999*). When large doses of RhoGAM® are needed, fetal cell count is determined on **follow-up** samples to check that fetal cells are being removed from the circulation. *‘In the case of bleeds exceeding 5 ml fRBC, the errors in recommended RhD Ig doses could be quite large, perhaps leading to an under-dosing’ (Lubenko 1999).* Therefore, Lubenko et al. recommend that women receiving large doses of RhD Ig are regularly **monitored during the first week after delivery, preferably at 3, 5, 7 and 10 days** (*Lubenko 1999*). For the follow-up of a large FMT calculation of supplemental doses of RhoGAM® is required with FCM 48 hours after i.m. administration of RhoGAM® (*2003 NBA Guidelines or 2002 ANZSBT Guidelines*).

II.1.2. Treatment

RhoGAM® must be administered to the Rhesus D-negative mother prior to the development of maternal Rhesus D antibodies (*Hillyer eds 2001*). The aim of this passive immunization is to prevent maternal B-lymphocyte activation and memory cell formation. RhoGAM® is a human plasma-derived anti-D immune globulin type G with traces of IgA (< 15 µg). IgG

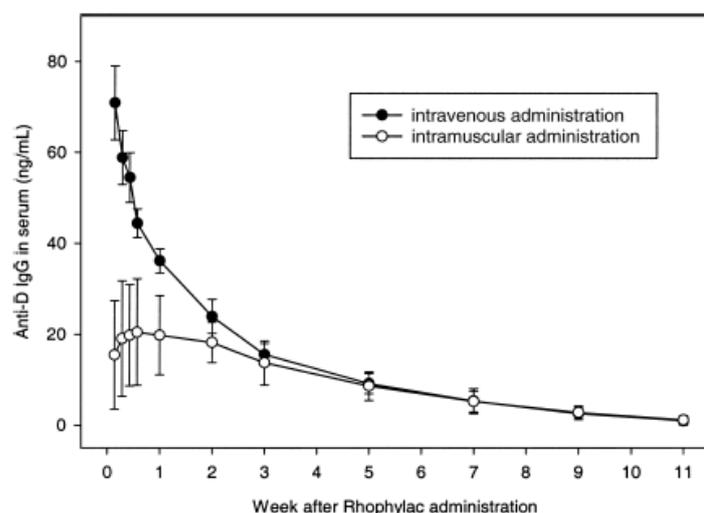
crosses the placenta; this may show a weak positive direct Coombs reaction on neonatal blood (and may mistakenly be interpreted as a sign of an immune mediated haemolytic anemia).

Adverse events may be an allergic reaction or anaphylaxis triggered by RhD immune globulin (*package insert RhoGAM*). IgA-deficient mothers may produce anti-IgA antibodies. There is no known incremental risk of adverse drug effects associated with delayed administration of additional anti-Rhesus D immune globulin, but the actual benefit has not been tested or proven in prospective clinical studies (*FDA website*).

On the Belgian market there is one dose of 300µg or 1500 I.U. available. The dose of 50µg or 250 I.U. is registered in Belgium but contains a package and insert conform the USA-norms. Other countries have access to different RhD immune globulin doses, which explains the different administration schedules in the international Guidelines.

RhoGAM® is obtained from previously-sensitized post-menopausal volunteers, so the neutralising power (# ml FMT) of 1500 I.U. has to be checked in the package insert. *'Since all plasma derived products are made from human blood, they may carry a risk of transmitting infectious agents, e.g., viruses, and theoretically the Creutzfeldt-Jakob disease (CJD) agent'* (*package insert RhoGAM*). The risk for transfer of serious diseases with RhoGAM® is extremely small (*NVOG 2003*). Depending on the manufacturing technique, aggregates of immunoglobulins may not be completely cleared. For those reasons RhoGAM® should be administered intramuscularly (i.m.) and not intravenously (*Mollison*). Via i.m. route RhoGAM® protects against alloimmunization over a period of many weeks. Two to seven days after i.m. injection, the plasmaconcentration of RhoGAM® peaks.

The mean elimination half-life is $17,6 \pm 5$ days (*Bichler 2003*). *'Na antenatale anti-D-toediening van 1000 I.U. mag gedurende ongeveer 8 weken een beschermend effect worden verwacht voor FMT < 20 ml foetaal bloed'* (*NVOG 2003*). After 9 weeks, approximately 5ng/ml of anti-D is left (25µg if the total blood volume is 5L), as shown in the picture on the right side (*Bichler ea. BJOG 2003*). 25µg can still neutralize 2,5 ml fetal blood. Remark: In case of a large FMT the clearing of RhoGAM® will be faster (*2007 VVOG*).



Adequate dosing will rapidly neutralize fetal cells in maternal circulation. Two examples in the thesis of Griet Luyckx showed the KBB detected no fetal cells any more 2 days after injection of adequate doses of RhoGAM® (*eindwerk Griet Luyckx*).

For any FMT greater than the volume neutralised by one dosis of RhoGAM® an appropriate supplementary dose of RhoGAM® must be given **as soon as possible, but surely within 72 hours** (cfr. Guidelines below; Attachment 4 Klinisch Zorgpad UZ Leuven - Gynaecologie):

- **within 48h**
 - 2003 NVOG Richtlijn
- **within 72h**
 - 1999 Lee e.a. Guidelines Transfus Med: *'ASAP after sensitising event, but always within 72h'*
 - 2002 RCOG Guidelines (Grade B recommendation)
 - 2003 NBA Guidelines (Level I evidence)
 - 2003 SOGC Guidelines (postpartum Level I-A recommendation) *'may be administered **within 28d**'*
 - 2006 BCSH Guidelines (Level III evidence, Grade B recommendation)
 - 2007 BCFI online
 - 2007 RhoGAM® manufacturer's instruction

'Some protection may be offered up to 10 days after the sensitising event' (BSCH 2006; RCOG 2002; Lee et al 1999). Recommendations have been made to administer it as late as 28 days after delivery, although with decreasing efficacy (Bowman 1985; Hillyer eds 2001).

Important is that *'at no time anti-D should be withheld based upon, or pending, the results of a test to quantify FMT' (2002 ANZSBT Guidelines)*. From this point of view and from the fact FCM nor KBB detect minor traumatata, the administration of the first dose of RhoGAM® will not depend on the KBB or FCM result. But supplemental doses of RhoGAM® will depend on the KBB or FCM result. In contrast to the above citation, NVOG says: *'Vóór anti-D toediening wordt aanbevolen de mate van FMT te objectiveren met KBB' (NVOG 2003)*. This opinion may rely on ideal sample collection, before RhoGAM® may interfere with circulating fetal cells. But the pharmacokinetics of i.m. administration show it lasts 3 days before RhoGAM® attains high serum concentrations.

II.1.3. Health outcome

Adequate doses of RhD immunoglobulin are reflected by the percentage of fetal RhD-positive red cells cleared (i.e. >50%) during the following 2–6 days (Lubenko 1999). Lubenko et al. believe that the **survival on days 2–3 of 40% or less of the fetal red cells initially present at delivery indicates that an adequate dose** of anti-D Ig for fetal red cell clearance has been given. If more than 40% of cells initially present still remain, then a further dose of anti-D calculated to cover the remaining volume of fetal RhD-positive cells should be given. Additionally, tests on maternal plasma for the presence of anti-D may also be useful. The low levels of anti-D (i.e. 0,2–0,5 I.U. ml) detected in follow-up samples taken after RhD Ig administration reflect adequate dosing.

Underestimation of FMT can cause ineffective anti-D prophylaxis and maternal alloimmunization. Underestimation of FMT has important implications for the health of the mother (risk of HDN in successive pregnancies) and for possible medico-legal consequences. Therefore, it is necessary to reliably assess FMT!

Overestimation of FMT results in unnecessarily higher dosing of RhoGAM® and the related undesirable costs as well as the increased risks of adverse reactions and disease transmission. Overdosing RhoGAM® occasionally causes allergic responses in the mother, but these are rare (NICE 2002). No other serious adverse events have been reported (Lubenko 1999).

II.2. Organizational impact

II.2.1. Impact in the hospital

15% of Caucasian mothers are Rhesus D-negative; the incidence is lower in other genders. KCE (Federaal Kenniscentrum voor de Gezondheidszorg) mentions n = 121 382 births in 2006 in Belgium (*KCE 2003*). From all those pregnancies, approximately n = 18 207 mothers were Rhesus D-negative. If we extrapolate those data, Belgian hospitals have to deal with roughly eighteen thousand pregnancies at risk for Rhesus D-alloimmunization per year.

II.2.2. Incorporated in Clinical Practice Recommendations/Guidelines?

Based upon the Guidelines about RAADP and actions to take in case of possible sensitising events, the table below summarizes antenatal and postnatal tests and the prevention of sensitisation, based upon BCSH Recommendations (Attachment 3). The clinical prenatal care path of Gynaecology in UZ Leuven follows NVOG Recommendations, which are also mentioned in the table (Attachment 4: Klinisch zorgpad UZ Leuven – Dienst Gynaecologie).

weeks of gestation	transfusion tests	fetal cell count
< 16 NVOG	No recommendations are specified	
< 20 BCSH	determine ABOD/anti-D of mother when potentially sensitising episode	/
> 20 BCSH or > 16 NVOG	1) determine ABOD/anti-D of mother when potentially sensitising episode	2) administer 1 dose of RhoGAM® 3) asses FMT when potentially sensitising episode 4) administer supplemental RhoGAM® if needed
28 BCSH or 30 NVOG	determine ABOD/anti-D of mother prior to RAADP administration to mother	/
birth	1) determine ABOD neonatus + mother	2) administer 1 dose of RhoGAM® 3) asses FMT if neonatus is RhD-positive 4) administer supplemental RhoGAM® if needed

This table is introduced into the algorithm in section V. Decision Making. A proposal for **introduction into the diagnostic prenatal care path** of the UZ Leuven would be to add ‘fetal cell count’ with a grey box (*‘test enkel zinvol bij specifieke risicogroep’*) at consultation weeks 20-24, 28 and 36 (Attachment 5: Diagnostisch zorgpad UZ Leuven – Dienst Laboratoriumgeneeskunde). Another adaptation: the blue box indicating *‘Indirecte coombs - zo bloedgroep rhesusnegatief’* at 28 weeks is evidence-based.

The management of D-negative woman consists of blood type (ABOD) and antibody screen at the first prenatal visit of each new pregnancy (*Grade IB Uptodate; Hillyer eds 2001*). It aims to confirm the mother is RhD-negative and she has no preformed anti-D. ABOD and antibody screen are repeated for any possible sensitizing event; an increasing anti-D Ab titer has to be monitored every 2 weeks (*Rode Kruis BTC*). A twofold increase in titer is significant (*NVOG 2003*). Serial titers are not useful for monitoring fetal status of hemolysis when the mother has had a previously affected foetus or neonate (*Level A Recommendation – National Guideline Clearinghouse*).

The assessment of FMT by fetal cell count is recommended for **possible sensitizing events** occurring after 20 weeks of gestation (*BCSH 2006*) / **after 16 weeks of gestation** (*NVOG 2003*). **The size of the FMT will determine the dose of RhoGAM® to administer** (*BCSH*

2006). Remark: RhoGAM® should be given to all non-sensitised RhD negative women who have a spontaneous complete or incomplete abortion after 12 weeks of pregnancy, who have therapeutic termination of pregnancy or ectopic pregnancy (*Grade B recommendation RCOG 2002*); in these cases assessment of FMT is not necessary.

In Belgium **RAADP of 300µg RhoGAM® (1500 I.U.) is offered at week 28 of gestation (KCE)**. Information leaflets should be made available to pregnant women to help with the informed consent process (*Grade C RCOG 2002*). KCE mentions in his prenatal care report: ‘*Gedetailleerde informatiebrochures moeten ter beschikking gesteld worden van elke Rh-negatieve vrouw*’. It is important that the 28-week antibody screening sample is taken prior to the RAADP injection being given because it is not possible to differentiate between administered prophylactic anti-D and immune anti-D in laboratory tests (*Good Practice Point BCSH 2006*). We can only speculate the administration of RhoGAM® provokes an anti-D titer up to 1:8 and alloimmunization a titer higher than 1:8 (*Rode Kruis BTC*). A titer of greater than 1:8 is considered a risk for fetal hemolysis (*Hillyer 2001*). *Blood Transfusion Centre Vlaams-Brabant-Limburg* considers a titer of 1:16 relevant for HDN.

Immediately following delivery another dose of 300µg RhoGAM® is administered to the mother if the antibody screen performed before delivery does not suggest an experienced alloimmunization and if the determination of the blood group of the neonatus turns out Rhesus-negative. After this, fetal cells are counted to evaluate the FMT for adequate protection against sensitization with an **extra dose RhoGAM®** if needed. The combination of the fetal cell count in maternal blood and the determination of the anti-D titre in maternal plasma is proposed in a diagnostic–therapeutic algorithm (*BCSH 1999*):

Acid elution/flow cytometry	Serum/plasma	Action
No fetal cells present	No free anti-D	Give further dose of anti-D Re-test the serum/plasma for presence of free anti-D in 48 h
No fetal cells present	Free anti-D	No further action
Fetal cells present	No free anti-D	Quantify and give appropriate further dose of anti-D Repeat FMH assessment in 48 h
Fetal cells present	Free anti-D	Repeat FMH assessment in 48 h

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When concern for Rh alloimmunization exists the initial management is determination of the paternal erythrocyte antigen status (*Level A Recommendation – National Guideline Clearinghouse*). Where a large FMT has been identified ‘*a sample should be taken from the mother 6 months after the sensitizing event and the serum or plasma tested for the presence of anti-D*’ (*BCSH 1999*). The anti-D antibody response is serologically detectable 5 to 15 weeks after immunization (*Uptodate 2008*). Screening for the formation of anti-D antibodies should be performed no earlier than 6 months after receiving a dose of RhoGAM®, as the drug may cause the antibody screening to be positive and not accurately indicate the patient's immune status (*FDA website*).

If an anti-D antibody is identified, measures consistent with normal clinical management of patients at risk of HDN should be instituted (*FDA website*). Icterus neonatorum has to be controlled by direct Coombs test and haemoglobin concentration in cord blood and bilirubinemia monitoring during the first days of life (*NVOG 2003; 2007 VVOG*). Mothers with a negative antibody test should be made aware of the fact that they could be sensitized and that antibodies and related complications might nonetheless develop in a subsequent pregnancy involving an RhD-positive foetus (*FDA website*).

III. TECHNICAL PERFORMANCE

There are different techniques for estimating the FMT, with different accuracy and precision. Only quantitative methods are relevant, not qualitative methods. *'The Kleihauer-Braun-Betke (KBB) method is currently designated the Class B reference method in the CLSI Guideline H52-A (2001), due to the anticipation that flow cytometric methods based upon HbF detection by monoclonal antibodies will gain acceptance as the more appropriate reference method, due to greater accuracy.'* The KBB method is a subjective test with multiple sources of variability, resulting in an inter-CV of 39,5% - 71,8% (2003 Lafferty Coag Trans Med). An objective method like flow cytometry can minimise inter-individual variability.

III.1. Calculation of FMT

The quantification of the FMT volume employs the ratio of counted fetal cells over the number of screened maternal RBC's. The formula of estimated FMT is based on assumptions, like an arbitrary volume of maternal blood (5000 ml – *Kleihauer 1957*) or an arbitrary maternal erythrocyte volume (1800 ml – *Mollison 1993*). Some formulas incorporate a correction for the larger size of fetal RBC (22%) compared to adult RBC and a correction for staining efficiency of the KBB test (92%) (*1993 Mollison; 2002 ANZSBT; 2004 BCSH*). Not all formulas use those corrections, resulting in a worldwide inter-laboratory variation of FMT calculation. FMT results from different labs can not be compared to each other.

1993 Mollison; 2002 ANZSBT; 2004 BCSH propose the formula underneath, using the arbitrary maternal erythrocyte concentration of 1800 ml, a correction for 22% larger fetal cells and a correction of 92% staining efficiency of the KBB test:

$$\# \text{ ml FMT (fetal RBC)} = \text{fRBC} / \text{maternal RBC} \times 1800 \text{ ml} \times 1,22 \times 1,09$$

Or the shortcut method:

$$\# \text{ ml FMT (fetal RBC)} = \text{fRBC} / \text{maternal RBC} \times 2400$$

For FCM analysis, the correction for staining efficiency is deleted (*BCSH 1999*):

$$\# \text{ ml FMT (fetal RBC)} = \text{fRBC} / \text{maternal RBC} \times 1800 \text{ ml} \times 1,22$$

In UZ Leuven the formula does not incorporate corrections and uses an arbitrary maternal blood volume instead of the maternal erythrocyte concentration:

$$\# \text{ ml FMT (fetal blood)} = \text{fRBC} / \text{maternal RBC} \times 4500 \text{ ml}^3$$

Or the shortcut method (when 150 000 maternal RBC's are screened):

$$\# \text{ ml FMT (fetal blood)} = \text{fRBC} \times 0,03$$

No consideration is made regarding the variation in maternal blood volumes arising from differences in body mass even though this can be quite large. *'For example, a 152-cm, 44-kg woman with a blood volume of 3077ml has 60% of the red cell mass of a 183-cm 86-kg mother with a volume of 5217 ml'* (*Lubenko 1999*). Neither the maternal haematocrit at the time of delivery, neither the maternal haemodilution occurring late in pregnancy are taken into account (*Lubenko 1999*). The question arises whether the FMT is a reliable parameter to report.

³ American Association of Blood Banks formula is # ml FMT (fetal blood) = fRBC/maternal RBC × 5000 ml

III.2. Flow Cytometry

Flow cytometry is the most commonly proposed alternative to the KBB and has a number of clear advantages including the ability to rapidly and reproducibly analyze large numbers of events, and good sensitivity in detecting small numbers of fetal red cells (*Little 2005*). *'It is generally accepted that flow cytometry is the method of choice to quantify FMT. Despite the lower number of users, unpublished figures from the RCPA QAP surveys and educational exercises demonstrate that flow cytometry produces results within a narrower range, smaller standard deviation and a reduced coefficient of variation [CV] compared to the Kleihauer test' (ANZSBT 2002).*

The FCM method uses hydrodynamic focussing to flush cell per cell trough a flow chamber. In the flow chamber each cell is detected by a combination of light scatter (forward scatter FSC and side scatter SSC) and fluorescence detection of monoclonal antibodies. The FITC or PE fluorescent-labeled antibodies used for fetal cell detection target Rhesus D or HbF. Both single parameter and two parameter FCM assays are commercially available:

- ANTI-D SINGLE TARGET: 'Quant-Rho ® FITC anti-D' assay (Alba Bioscience, Durham, North Carolina)
- ANTI-HbF SINGLE TARGET:
 - Caltag's Fetal Hemoglobin (Invitrogen, Carlsbad, CA);
 - Trillium Diagnostic's FMH QuikQuant (Trillium Diagnostics, Brewer, Maine) for flow cytometry or for the Abbott Sapphire.
- ANTI-HbF/CA DUAL TARGET:
 - 'Fetal Cell Count™ kit' (IQ Products) contains anti-HbF Ab plus anti-CA Ab. Adult RBC contain carbonic anhydrase (CA) isoenzymes I and II. HbF detects fetal RBC's and CA the maternal RBC's, which helps to discriminate between both populations.
 - € 376,31 / kit => € 15,05 / test (in total € 45,15 for the patient's sample + positive control + negative control)
 - ~~Currently no kit is available with anti-HbF Ab plus anti-i Ab.~~ The combination of these 2 fetal parameters has no benefit compared to a single parameter test because no discrimination is possible with maternal RBC's. The i antigen is present on fetal cells and disappears during the first year of life, to be replaced by the I antigen. Anti-i is an unspecific marker, as it is found transitorily in many patients of different ages suffering from infectious mononucleosis.

III.2.1. FCM targeting Rhesus D

This procedure targets membrane Rhesus D antigens, so it does not need a permeabilization step. Direct staining with FITC-labeled monoclonal anti-D antibodies should always be done on a **maternal sample prior to the administration of RhoGAM®. Otherwise false negative test results (FN)** will be created due to lysed fRBC's in response to the in vivo action of RhoGAM® and due to inhibition of the FITC-labeled anti-D binding (*REVIEW Crowther 1999*). It is recommended that where it is known that the mother has received RhoGAM®, an indirect flow cytometric test should be undertaken. The indirect FCM test is time-consuming. It uses a non-fluorescent polyclonal antiserum or monoclonal anti-D antibody and a second reagent consisting of FITC-labeled Fab antihuman IgG, to prevent agglutination of fetal RhD-positive red cells.

⇒ We can conclude that Rhesus D target is not the best option. When clinical information is lacking, the laboratory does not know whether the mother received RhoGAM® or not. This problem would be often encountered in a routine setting.

III.2.2. FCM targeting HbF

The HbF target is located intracellular in contrast to Rhesus D on the RBC membrane, so this procedure needs a permeabilization step. Fetal RBC's contain $\geq 90\%$ HbF ($\alpha_2 \gamma_2$), and at delivery the newborn has 70 – 80% HbF (*CLSI Guideline H52-A*). fRBC's are found in foetus, newborn and maternal circulation of pregnant female, but not in non-pregnant individuals. From the age of 1,5 year adult haemoglobin types are found in the following concentrations: 96 – 98% HbA ($\alpha_2 \beta_2$), $\leq 1\%$ HbF ($\alpha_2 \gamma_2$) and 1,5 – 3,2% HbA₂ ($\alpha_2 \delta_2$) (*CLSI Guideline H52-A*).

An important aspect of the FCM targeting HbF is its ability to distinguish fetal HbF-containing RBC's (in case of a FMT) from adult HbF-containing RBC's with a weaker fluorescence intensity. The presence of adult HbF-containing RBC's represent a more prevalent phenomenon than previously thought (*1997 Warzynski*). Hereditary persistence of fetal haemoglobin (HPFH), beta-thalassemia, other haemoglobinopathies (HbEE, heterozygote $\delta\beta^0$, homozygote $\delta\beta$), (re)activation of the erythropoiesis AML and rare syndromes have a prevalence of +- 25%, which is an important source of FP KBB test. FCM will detect maternal HbF excess as a distinct population, thanks to CA-detection. In beta-thalassemia major there is an increase of adult HbF-containing RBC's from 30% to 90% HbF (*Hoffbrand book*). In beta-thalassemia minor/intermediate the HbF is not always increased. In HPFH adults have 10 – 30% HbF. This is a condition of a persistent HbF production in adult life in the absence of major haematological abnormalities (normal red blood cell indices and morphology) (*Hoffbrand book*). The expression of the gamma globin gene of HbF persists at high levels in adult erythroid cells (*Uptodate 2008*). It may result from a major deletion of the beta globin gene cluster or a point mutation. The former is characterized by clearly increased levels of HbF (15 – 35% in heterozygotes; 100% in homozygotes), the latter by 10 – 15% HbF in heterozygotes. KBB shows pancellular distribution of HbF. There is also a third type of HPFH unlinked to the beta globin gene cluster, characterized by modest elevations in HbF (1 – 4%) (*Hoffbrand book*). The KBB shows a heterocellular distribution of HbF in an uneven fashion among the adult RBC's (*Uptodate 2008*).

III.2.2.1. Analytical performance characteristics of FCM (analytical validation report)

a. Preanalytical considerations (patient variables, sample stability)

Patient variables

In the formula for the calculation of FMT (cfr. III.1.), no consideration is made regarding the variation in maternal blood volumes, neither the maternal haematocrit at the time of delivery, neither the maternal haemodilution occurring late in pregnancy.

Sample stability

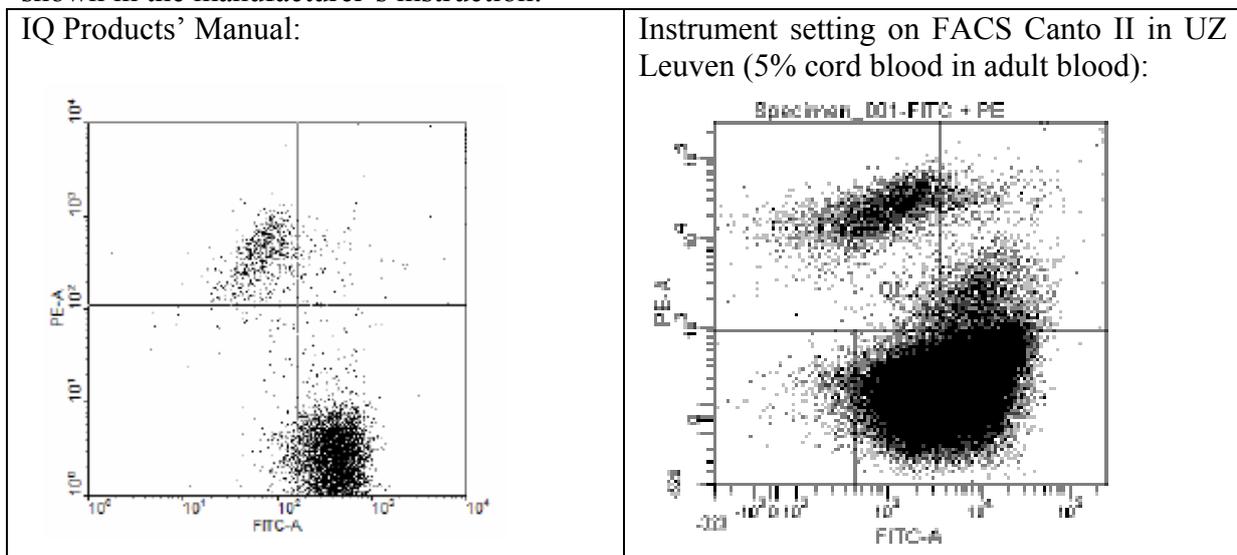
A sample must be EDTA-anticoagulated blood from the mother. Samples should be processed within 3 days of sample collection.

It is recommended that, as a minimum, duplicate test samples be analysed (*BCSH 1999*).

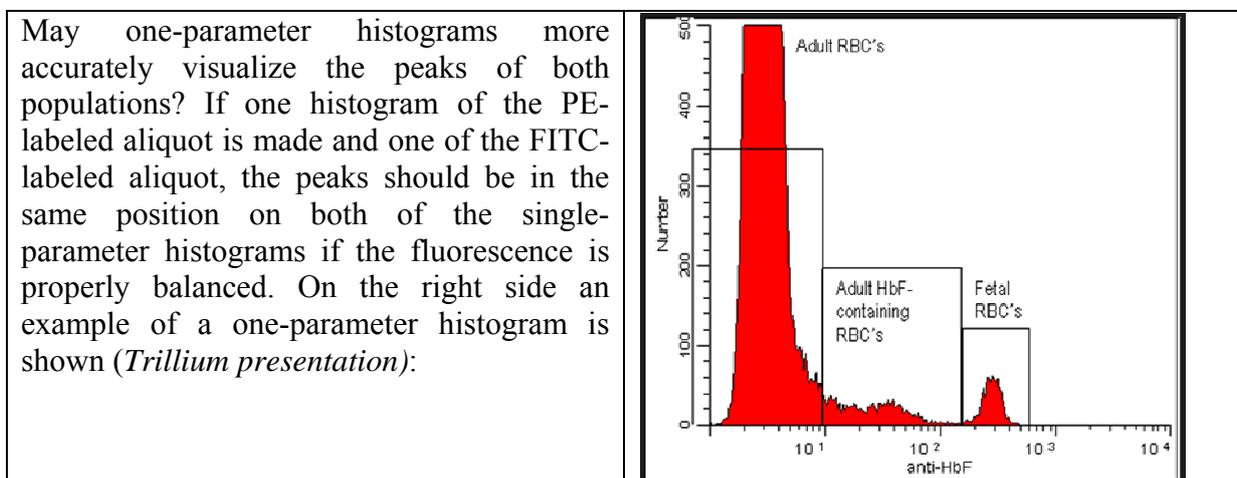
b. Analytical considerations (procedure, reproducibility, accuracy, correlation)

Instrument setup

Proper instrument setup prior the analysis of samples is pivotal for obtaining accurate results (*manufacturer's instruction IQ Products*). The PE/FITC compensation will be more than usual, because the fixation and permeabilization process makes the RBC's very autofluorescent (*Trillium presentation*). On the FACS Canto II the best PE-FITC compensation is 15,51 and the best FITC-PE compensation is 2,04. The setting of the quadrants during instrument setting of the FACS Canto II in UZ Leuven was not as evident as shown in the manufacturer's instruction:



The population that strongly expresses HbF was rather diffuse, showing interference with the FITC canal. The reason is probably not a technical problem but rather a physiological manifestation of carbonic anhydrase (CA). Although CA is expressed on adult RBC, the fRBC are not totally negative for CA. A small percentage of fRBC express CA in a level 100x lower than adult RBC. At about 40 weeks of gestation, CA production is switched on in fRBC (*Brady ea 1990*).



Test procedure

The procedure we tested is defined in manufacture's instruction of IQ Products' Fetal Cell Count™ kit. It contains fixation, permeabilization and labelling steps with PE-labeled anti-HbF and FITC-labeled anti-CA monoclonal antibodies. FCM analysis of 100 000 has to be performed within 30 minute.

Reproducibility

The variation coefficients mentioned in literature vary from CV 10% to 15% (*Chen 2002; Davis 1998*). 'To minimize the coefficient of variation obtainable, no less than 500 000 events should be collected' (*BCSH 1999; 1999 Lubenko*). Then an acceptable coefficient of variation can be obtained for a FMT of 0,02% fRBC (0,48 ml fRBC or 1 ml fetal blood): 'A minimum of 100 fRBC's has to be counted over 500 000 events to ensure a CV of 10% or less' (*1999 Lubenko*).

Accuracy (bias)

The fetal cell test on its own can not be calibrated, but **calibration** of the flow cytometer FACS Canto II occurs with beads with fluorochromes

The antibodies must be validated against mixtures of D-positive and D-negative cells to ensure that a clear discrimination between negative and positive populations can be made (*BCSH 1999*).

'Good laboratory practice requires that controls be run with every batch of patient samples or at least once per day of analysis'. **Controls** are ideally stabilized whole blood materials that can be used to provide precision monitoring of the analytical method, but mixtures of adult and umbilical cord blood are also allowed. There should be a negative control, a low-positive control of 0,1% - 0,3% fRBC to ensure assay sensitivity and a high-positive control of at least 1% fRBC to ensure adequate Ab saturation (*CLSI H52-A*).

FETALtrol (Trillium Diagnostics) is a CE marked IVD according to ISO13485:2003 that has been cleared by the FDA as a hematologic control for fetal red cell detection. This stabilized blood product is an assayed 3-level control set designed to validate and monitor the quality of procedures for the detection of FMT. Its stability is 3 months. It is compatible as a control for both manual KBB and FCM methods. The low-positive control contains 0 – 0,02% fRBC, the intermediate control 0,12 – 0,24% fRBC and the high control 0,79 – 1,58% fRBC. The high control can be used as a reference material for proper gating during instrument setup. FETALtrol is distributed by Prosan NV(prosan@skynet.be) and costs € 368 per set (IQP-370FT).

The correlation between the original KBB which is based on HbF detection and FCM by indirect anti-D staining is found to be poor ($r^2 = 0,714$) (*1995 Johnson J Clin Pathol*). In contrast, when both KBB and FCM used the same **HbF target, an excellent correlation** of $r^2 = 0,99$ ($y = 1,008 x - 1,612$) was found when 170 woman were tested in a randomised

controlled trial (2007 *Fernandes Pren Diagn*). In another study on 113 woman, FCM with the Caltag HbF method also correlated well with the KBB test ($r^2 = 0,926$) (1997 *Warzynski*).

Citations from different authors and Guidelines illustrate FCM is a more accurate and more reproducible method than KBB:

- '*KBB lacks adequate accuracy and precision for reliable quantification of FMT and could lead to 19,4% of inadequate anti-D prophylaxis when FMT > 10ml*' (2003 *Lafferty e.a. Am J Clin Pathol*)
- '*Our flow cytometric estimations on 25 of the 54 mothers indicated a low level (<4 mL) of fetal RhD-positive red cell bleeds that had been either incorrectly or inconclusively quantified using the Kleihauer test*' (Lubenko 1999).
- '*Fetal red blood cells were identified in 10 women by the KBB method, and in 26 women by FCM. FCM was both more sensitive and more timely for the quantitation of FMT than was KBB*' (2007 *Fernandes Pren Diagn*).
- '*Despite the lower number of users, unpublished figures from the RCPA QAP surveys and educational exercises demonstrate that FCM produces results within a narrower range, smaller standard deviation and a reduced CV compared to the KBB test*' (2002 *ANZSBT GUIDELINES*)
- '*FCM is accepted as the most accurate quantitative test for assessing FMT*' (2003 *NBA GUIDELINES*; 2002 *ANZSBT GUIDELINES*; 2001 *NCCLS/CLSI GUIDELINES H52-A*; 1999 *Lee e.a. GUIDELINES TRANSFUS MED*)
- '*The SSC believes that FCM is the method of choice for quantification if readily available*' (2003 *NBA GUIDELINES*; 2002 *ANZSBT GUIDELINES*)
- '*FCM is useful for follow-up of large FMT*' (1999 *Lee e.a. GUIDELINES TRANSFUS MED*)

c. Analytical range

The analytical sensitivity must be at least 0,6% fRBC, the clinical relevant level to detect a FMT of 15 ml fetal red blood cells or 30 ml of fetal blood. This can be neutralised by one dose of RhoGAM® 300 µg or 1500 I.U., as marketed in Belgium (*CLSI H52-A 2001*).

The **analytical sensitivity** of FCM when collecting 50 000 events is 0,1% fetal RBC or 2,4 ml fRBC or ~5 ml fetal blood (*Pelikan 2006*; *Davis 1998*; *Mollison 1993*). '*FCM is capable of detecting >0.1% fetal cells in maternal blood, below this level it is considered insensitive*' (*Proefschrift Pelikan 2006*). The collection of 500 000 events with FCM may potentially lower the sensitivity; this has to be tested in each laboratory.

Less evidence based literature report a lower analytical sensitivities of 0,05% fRBC or 1,2 ml fRBC or 2,5 ml fetal blood (*poster Davis 2008*) and a higher analytical sensitivity of 0,3% fRBC or 7,2 ml fRBC or 15 ml fetal blood (*Bayliss 1991*).

The difficult discrimination between the fetal and maternal red cell population may partially be solved by the use of two discriminating parameters. But a lower specificity of the second parameter antibodies may result in technical problems.

d. Turn around time (TAT)

ANZSBT Guidelines (2002) prescribe the result of fetal cell count should be available **within 72 hours**. *BCSH Guidelines (2004)* mention the KBB result needs to be available as soon as possible, and the test should be confirmed with FCM if the FMT exceeds the volume neutralised by one dose of RhoGAM®.

Important is that *'at no time anti-D should be withheld based upon, or pending, the results of a test to quantify FMT'* (2002 ANZSBT Guidelines). In this setting, our laboratory would be able to offer FCM tests during the week, not in the weekends or at night.

e. KAL (clinical tolerance limits)

Alert the clinician when a massive FMT > 15 ml fetal blood is detected.

III.2.2.2. Diagnostic performance of FCM

Few data are available regarding the consequences of administering doses of anti-RhD Ig based on flow cytometric estimations of FMT.

Sources of errors with FCM are:

- Not optimized instrument setup and compensation. The fixation/permeabilization step makes the RBC very autofluorescent. Therefore adjusting of the FITC-PE compensation is needed.
- Carry-over after a positive sample or positive control.
- RBC aggregates post-transfusion.

Until now there is no laboratory method available that is able to detect minor FMT because none can reach the limit for sensitisation, corresponding to 0,1 ml fRBC or 0,004% fRBC (*SOGC 2003*).

III.3. Kleihauer Betke: acid elution technique

In 1957 Kleihauer, Braun and Betke described an acid elution technique to differentiate RBC's with HbF (fetal cells) from RBC's with HbA (adult cells) (*Kleihauer ea. Klin Wochenschr 1957; 35:637-638*). It is based on the principle of elution of HbA out of the RBC's in acid medium, while HbF-containing RBC's resist cellolysis.

'The KBB method is currently designated the Class B reference method, due to the anticipation that flow cytometric methods based upon HbF detection by monoclonal antibodies will gain acceptance as the more appropriate reference method, due to greater accuracy. Class B reference method is believed to be of the caliber of a Class A reference method, except that the process of evaluation with a definitive method and a certified reference material has not yet been completed' (CLSI Guideline H52-A).

Commercially available kits for KBB are listed below (prices are incl. BTW; excl. transport costs and analyst labour):

- Immucor Gamma: 'Kleihauer Kit' 3 x 100 ml contains erythrosine B and haematoxylin
 - € 129,19 / kit => € 1,29 / test
- DiaMed: 'DiaMed HbF Screening Test' 3 x 100 ml contains celistine blue
 - € 135,76 / kit => € 1,36 / test
- DiaSorin: 'Kleihauer Test' 6 x 100 ml
 - € 309,76 / kit => € 1,55 / test
- ~~In-house preparations~~ (not recommended: prone to even larger variability)

The following kits have been recalled from the market:

- ~~Sure-Tech (distributed by Ortho Clinical Diagnostics): 'Fetal Hemoglobin'~~
 - € 419,30 / kit => € 2,80 / test => € 2800 / jaar
- ~~The KBB kit FETALSCREEN™ from Ortho Clinical Diagnostics~~ has been recalled because of possible false negative results: it is possible that some patients who experienced FMT > 30 ml fetal blood were not identified. *'Women who were screened with the recalled kits should undergo antibody testing at 6 months postpartum to identify those who have become sensitized'* (FDA website).

III.3.1. Analytical performance characteristics of KBB (analytical validation report)

a. Preanalytical considerations (sample stability)

A blood smear of fresh K₂EDTA anticoagulated blood of the mother is prepared within 48 hours (*Denise Pelikan*). The blood may be stored at 2 – 8°C if not immediately (*Product insert Immucor kit*).

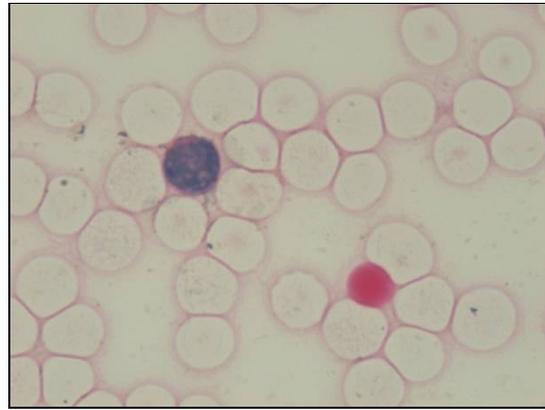
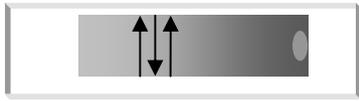
b. Analytical considerations (reproducibility, accuracy, report limit)

Procedure of Immucor Gamma's Kleihauer Kit

Adherence to the procedure is essential. The fixation (3 min in EtOH) – elution (30 sec in citrate-phosphate buffer) – colouring (2 min in erythrosine B) steps need to be chronometered exactly. Overfixation may inhibit elution. The elution step of 30 seconds is critical. Wash with

tap water, no reversed-osmosis water. The method has been validated for staining in Stretton Young jars (*validatiedossier d.d. 05-05-2008*).

The fetal cells are counted in 150 microscopic fields (ocular 10x and objective 50x). Fetal RBC's are coloured fuchsia, adult RBC's look like ghost cells. The Immucor Gamma kit has the advantage to make a differentiation with lymphocytes possible thanks to the blue-grey colour of the white blood cell nuclei.



In UZ Leuven the formula for calculating FMT is based on the assumption of an arbitrary maternal blood volume of 4500 ml. Three rows on the blood smear are screened, corresponding to 3 x 50 fields of 1000 RBC or a total of 150 000 adult RBC's. No correction factors (for the larger size of fRBC and for the staining efficiency) are taken into account.

$$\# \text{ ml FMT} = 4500 \text{ ml} \times \text{fRBC} / 150\,000 \text{ adult RBC}$$

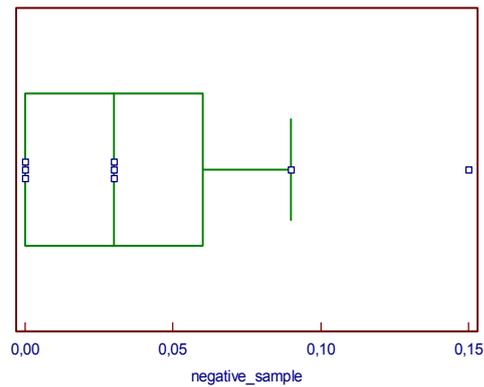
$$\# \text{ ml FMT} = 0,03 \times \text{fRBC}$$

Reproducibility

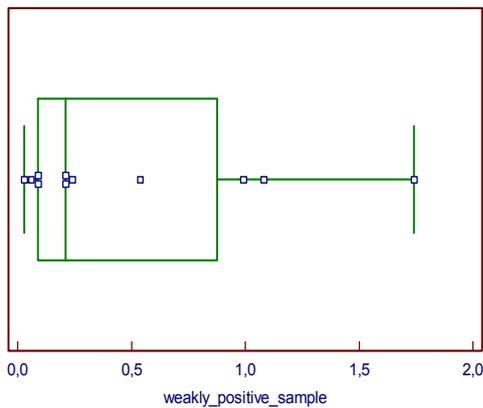
Literature emphasizes the imprecision of the KBB method and the large variation coefficient of varying between CV = 39,5% and 71,8% (2003 Lafferty *Coag Trans Med*; Duckett JR *Br J Obstet Gynaecol* 1997;104:845-846; Bromilow IM *Clin Lab Haem* 1997;19:137-142). This situation pleads for the introduction of a more precise confirmation test.

An in-house study compared the fetal cell count results of 11 analysts in the UZ Leuven laboratory. Five samples with a different FMT were microscopically evaluated after staining with KBB kit from Immucor Gamma. In-house inter-individual analysis showed the variation for a negative sample, for a weakly positive sample (FMT < 5 ml fetal blood) and for a strongly positive sample (FMT > 15 ml fetal blood) are unacceptable. Interpreting a negative sample is very subjective; some false positive results are released. No false negative results were observed with the Immucor staining, which is very important regarding the clinical impact. For a sample of approximately 12 ml fetal blood, we found an acceptable CV of 17% (normally distributed population). From a statistical point of view, mean \pm SD and %CV cannot be given for the other samples because our results were not normally distributed. The figures below show Box-and-Whisker plots with median [IQR]. IQR is the interquartile range from 25th percentile to 75th percentile.

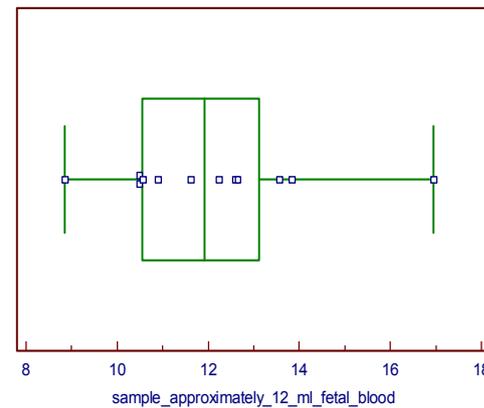
An in-house study compared the fetal cell count results of 11 analysts in the UZ Leuven laboratory. Five samples with a different FMT were microscopically evaluated after staining with KBB kit from Immucor Gamma. The results show a huge variation (imprecision) for negative, weakly positive and strongly positive samples. A sample of about 12 ml fetal blood shows an acceptable variation with the KBB technique: median [IQR] = 12 [10,5 - 13] ml fetal blood (*MedCalc®v9.4.1.0 Mariakerke, Belgium*).



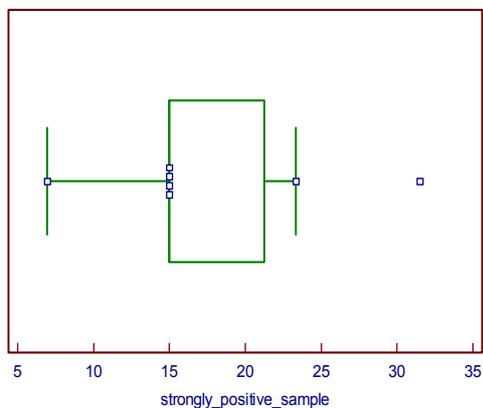
median [IQR] = 0,03 [0 – 0,06]



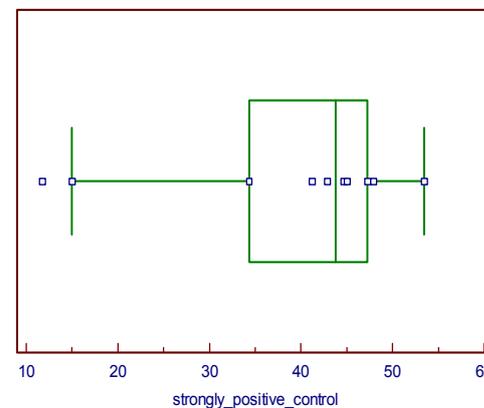
median [IQR] = 0,2 [0,1 – 0,9]



median [IQR] = 12 [10,5 - 13]



median [IQR] = 15 [15 - 21]



median [IQR] = 44 [34 - 47]

It is important to notice that a KBB result around 12 ml fetal blood contains a fault of approximately 1,5 ml fetal blood with the Immucor kit. When larger FMT > 15 ml is detected with KBB, the fault may reach 10 ml fetal blood! The limit of 30 ml fetal blood determines if a supplemental dose of RhoGAM® has to be given. However, with the KBB technique a result of 20 ml fetal blood could equal 30 ml fetal blood, taking the possible analytic fault into account. For this reason, 20 ml fetal blood has to be interpreted as an alarm value and the clinician has to be phoned.

The in-house study also compared the results of KBB staining with Immucor Gamma versus KBB staining with the Sure-Tech kit (Orhto Diagnostics), previously used for the routine analysis in UZ Leuven. Results show negative samples are more doubtful with Sure-Tech kit. A weakly positive sample was often released falsely negative with the Sure-Tech kit, which is unacceptable regarding the clinical impact. For a sample of 12 ml fetal

blood stained with Sure-Tech a fault of 3,5 ml has to be taken into account, which is larger than with the Immucor kit.

Accuracy (bias)

'Both underestimation and overestimation of the KBB are reported' (*Proefschrift Pelikan 2006*). In the case of bleeds exceeding 5 ml fRBC, the errors in recommended RhD Ig doses could be quite large, perhaps leading to an under-dosing of mothers with a larger BMI by a factor of two or more. Underestimation is of clinical significant concern, as it causes inadequate prevention of sensitization and risk for developing maternal anti-D and subsequent HDN. Overestimation does not result in clinically significant complications.

Controls should be stabilized whole blood materials or mixtures of adult and umbilical cord blood. There should be a low-positive control of 0,1% - 0,3% fRBC and a high-positive control of at least 1% fRBC (*CLSI H52-A*). CLSI Guideline gives no recommendation about a negative control for KBB. FETALtrol (Trillium Diagnostics) is a commercial control compatible for both manual KBB and FCM methods.

Introduction of a positive control in routine setting has been achieved with the release of the corrected SOP-042 d.d. 080508 (cfr. *validatiedossier d.d. 05-05-2008*). A validation report is set up for the introduction of the Immucor Gamma kit. Attention is drawn to a more uniform staining procedure and logging when reagents have been refreshed. The positive control in our routine laboratory is made of anticoagulated blood from a 1-month-old child for the reason of practicable work conditions (but we are not always satisfied). **Acquisition of the commercial FETALtrol control would certainly be an improvement.** It eliminates the need to acquire cord blood and prepare home brew controls, saving time precious to laboratory professionals and reducing exposure to untested potentially infectious material.

The positive control is used to check:

- the critical elution step
- the contrast between the fuchsia fetal cells (erythrosine B) and the eluted ghost cells
- the blue-grey counterstaining of the white blood cell nucleus (haematoxylin)

Report limit

The report limit was changed to **< 5 ml of fetal blood** at gynaecologists discretion. The reason was the huge inter-individual variability and the discrepancies found during a comparative study with routine samples in our laboratory. A 'negative' result or '0 ml' is not reported to prevent wrong interpretation. If the analyst counts zero fetal cells when screening 3 rows, it does not mean a minor FMT can be excluded. The cut-off of 5 ml was chosen in accordance with the analytical sensitivity of 5 ml fetal blood detected by *Ochsenbein et al (Ochsenbein 2002)*.

c. Analytical range

The **analytical sensitivity** of KBB is 5 ml fetal blood (*Ochsenbein 2002*). This corresponds to 2,4 ml fRBC or 0,1% fRBC. The upper limit of detection depends on the admissible CV. Actual quality norms do not accept the large CV's known with KBB technique. Our in-house

study showed unacceptable inter-individual variation for samples < 5 ml and > 15 ml fetal blood. So the analytical range is restricted to **5 – 15 ml fetal blood**.

d. Turn around time (TAT)

The joint working group of the British Blood Transfusion Society and the Royal College of Obstetricians and Gynaecologists mentions the KBB test should be available as soon as possible (1999 Lee *e.a. Guidelines Transfus Med*).

The current status in UZ clinical laboratory is TAT < 4 h and availability 24h/24h 7d/7d.

e. KAL (clinical tolerance limits)

Alarm value to phone the clinician is FMT \geq 15 ml fetal blood.

A query in UZ Leuven over 2 years from december 2005 until december 2007 showed n = 1913 requests for fetal cell count by KBB, mostly demanded by the department of maternity and sometimes from the department of gynaecology. 98,9% (N = 1892/1913) of the samples had a FMT < 5 ml fetal blood. N = 13/1913 (0,7%) had a FMT between 5 and 15 ml fetal blood. N = 1/1913 (0,05%) had a FMT between 15 and 20 ml fetal blood. And N = 7/1913 (0,4%) had a result > 20 ml fetal blood. The results revealed 7 massive FMT and 1 case of persistence of adult HbF in July 2007. Although in the total population sometimes follow-up samples were taken (26%), none of the massive FMT received follow-ups.

Query results:	ml fetal blood
May 2006	24,3
June 2006	22,5
July 2006	31,8
September 2006	29,3
November 2006	16,4
February 2007	28,8
June 2007	90,0
July 2007	51,3

The patients with massive FMT were retrospectively controlled.

- Two mothers seemed to be Rhesus D-positive, and two mothers were Rhesus D-negative with a Rhesus D-negative neonatus. Those 4 mothers did not need RhoGAM®. But other irregular antibodies may have represent a problem. One of these neonati was born preterm because of fetal anemia.
- The mother who had 22,5 ml fetal blood received 2 doses of RhoGAM®, which is theoretically enough to cover the FMT.
- The mother who had 29,3 ml fetal blood received one dose of RhoGAM®, although she may have needed a supplemental dose. She had a caesarean section.
- The mother with the highest FMT of 90 ml fetal blood did not receive RhoGAM® in UZ Leuven, but maybe in another hospital. We could not deduce a reason. Her child was anemic (Hb = 7,2 g/dl).

The query dec05-dec07 revealed only 7 FMT > 15 ml fetal blood out of 1913 requests (0,4%).

III.3.2. Diagnostic performance of KBB

The KBB technique is a source for multiple errors:

- Assumptions in the formula for calculating FMT
- Variability in thickness of bloodfilm
- Staining efficiency only 92%
- Inter-individual variability:
 - A different microscopic field for counting contains a different number of RBC's
 - Subjectivity for the interpretation of a fetal cell: the difficulty of classifying cells of intermediate staining.

IV. COST IMPACT

Actual cost

	KBB	FCM
Reagent cost per test (direct cost)	€ 1,29	€ 45,15 (incl. controls)
Primary activity of MLT	€ 30,21 (50 min)	€ 66,46 (1h50)
THEORETICAL COST PER TEST	€ 31,50	€ 111,61

KBB with Immucor Gamma's Kleihauer Kit:

- ◆ € 129,19 / kit (100 tests) => € 1,29 / test
- ◆ Technical activity is 50 min = staining (30 min) + microscopy (20 min)
Excl. slide drying (20 min)

FCM with IQ Products' Fetal Cell Count kit:

- ◆ € 376,31 / kit (25 tests) => € 15,05 / test
- ◆ Technical activity is 1h50 = wash (20 min*) + labelling (1h) + flow (30 min)
*these 20 minutes may be deducted because the wash step may be omitted.

Costs are excl. overhead and secondary activities like maintenance of flow cytometer.

Reimbursement (*Focus Diagnostica Nomenclature 2007 Art. 33bis included*)

Nomenclatuur regel 'erythrocyten – foetale, telling in het bloed van de moeder' B200.
Beperking: M-1 (maximum 1 bepaling uit te voeren per afname).

Honorarium	€ 5,82
Tarief bij onderaanneming	€ 5,82
Tarief voor ambulante & gehospitaliseerde patiënt	€ 1,45
RIZIV ambulante code	553136
RIZIV gehospitaliseerde code	553140

Cost impact on the society

The administration of RhoGAM® for RAADP represent a significant cost for the society and a risks of disease transmission and adverse reactions. RhD-negative mothers bearing a RhD-negative child have no benefit from RAADP. In the current RAADP schemes, all RhD-negative mothers should receive RhoGAM®. The Rhesus blood group typing of the foetus is reserved for relevant medical circumstances, where an invasive method for collecting fetal blood is ethically acceptable, e.g. amniocentesis when there is speculation about alloimmunization and the father is heterozygeous for the concerning antigen. The discovery of **cell-free fetal DNA (cffDNA)** in the maternal plasma in 1997 has led to a new non-invasive method for the determination of the fetal Rhesus status (*NVOG 2003; Lo 1997*). Rijnders from Utrecht showed invasive amniocentesis was avoided in 41,7% of patients (*Rijnders 2004*).

The molecular biological techniques used for the analysis of free fetal DNA are not accessible to all laboratories. An International forum of 10 countries (USA, Finland, France, Italy, Japan, The Netherlands, Poland, Spain, UK, Austria) revealed that most countries do not perform

cffDNA analysis; Poland and Spain very rarely (*Vox Sanguinis 2003*). UK tested more than 200 cases of cffDNA and showed good results (no FN nor FP). The Netherlands are enrolled in a NWO study. France compares cffDNA with PCR on amnion cells. It is important to use an internal control in the PCR reaction; for boys the *SRY* gene can be used; for girls a reliable gene still has to be found.

In Belgium ULB Erasmus restrained from implementing the cffDNA PCR because they did not receive enough requests. Because of the interest from the gynaecologists, the samples of ULB are sent to Dr. Costa in Paris since a couple of years.

CONTACTS:

In France, the laboratory of Dr. Costa, American Hospital of Paris (jean-marc.costa@ahparis.org) performs analysis from 6 weeks of gestation. They have experience with hundreds of samples.

In Belgium one laboratory performs cffDNA since 2002 (*Minon ea. Transfusion 2008*):

Dr. Minon, CHR De la Citadelle, Liège (tel 04 223 87 81). Samples can be analyzed from 8 weeks of gestation. The administrative procedure is easier than in Paris.

Remark: A commercial kit 'Fetal Cell/DNA Prenatal Gender Test' without mentioning the technique and without any guarantee of quality seems to be accessible to anybody via e-shopping.

Remark: The future is likely to bring less invasive testing using cell-free fetal DNA from maternal plasma for applications like (total) genome analysis.

V. DECISION MAKING

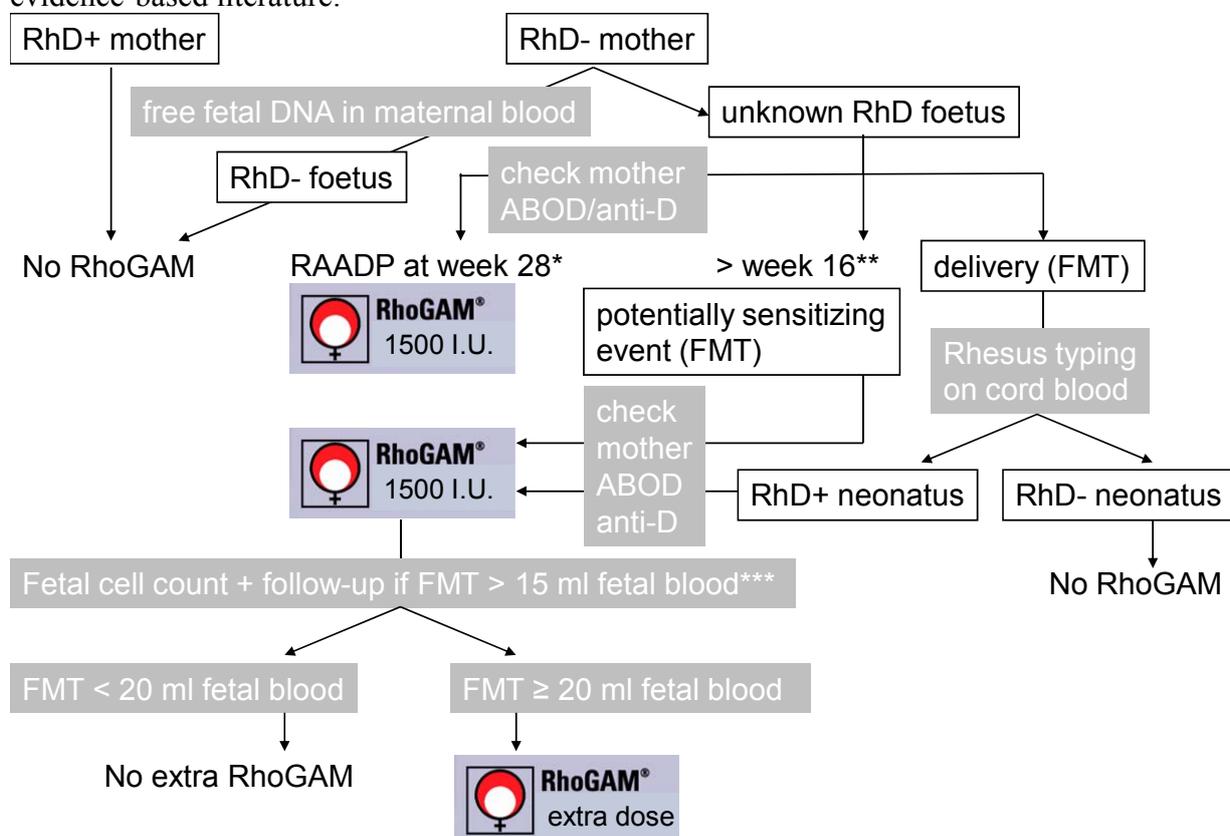
When is the administration of one dose of RhoGAM® necessary?

- RhD-negative mother, not previously sensitised:
 - RAADP in the first pregnancy
 - For any potentially sensitizing event (minor and massive FMT)
- When is fetal cell detection necessary?
- massive FMT
 - to calculate the size of FMT for supplemental RhoGAM® doses

When is the administration of RhoGAM® NOT APPROPRIATE?

- RhD-negative mother with RhD-negative foetus
 - RhD genotyping of foetus: cell-free fetal DNA in maternal plasma

A clinical-diagnostic-therapeutic algorithm is proposed for application in UZ Leuven, based upon the organisational structure of LAG, discussions with the gynaecologists of UZL and evidence-based literature:



* covers until week 37; NVOG recommends termination of pregnancy at week 37 – 38 in case of low alloimmunization activity (NVOG 2003; 2007 VVOG).

** 'Alhoewel placentapassage van irreguliere antistoffen reeds vóór een zwangerschapsduur van 16 weken plaatsvindt, is deze pas vanaf 16 weken van klinische betekenis. Foetale sterfte door bloedgroepenimmunisatie is vanaf 16 weken beschreven' (NVOG 2003) / The assessment of FMT by fetal cell count is recommended for possible sensitizing events occurring after 20 weeks of gestation (BCSH 2006)

*** alert limit, phone the clinician

Literature suggests the more accurate FCM test for massive FMT. A possible scenario would have been to screen for fetal cells with KBB 24h/24h 7d/7d and to quantify more accurately the FMT with flow cytometry within 72 hours in case of massive FMT > 15 ml fetal blood. But the following arguments restrained us from implementing FCM in our routine laboratory:

- Only 0,4% of requests in UZ Leuven are FMT > 15 ml fetal blood and are taken into consideration for FCM analysis (~ 4 samples a year);
- Such a low turnover can not build up expertise;
- Expertise is essential because of the difficulties we experienced to interpret cytograms.

These reasons may indicate why no Belgian lab and only one lab in The Netherlands performs FCM for fetal cell count in a routine setting. An International Forum with 10 countries revealed that only 3 countries actually perform FCM for massive FMT (UK, Finland, Poland) (*Vox Sanguinis* 2003), in contrast to the International Recommendations for accurate FCM testing.

Remark: It still stays interesting to perform FCM with the available test kit in our laboratory when we encounter rare massive FMT routine samples, in parallel with KBB. Then 500 000 events have to be analyzed with FCM.

TO DO/ACTIONS

- 1) In our current laboratory setting fetal cell count by flow cytometry will not immediately be implemented. A training to minimise the inter-individual variability of the Kleihauer-Betke test is offered to laboratory technicians.
- 2) Adjust **report limit to FMT < 5 ml fetal blood**. The protocol should mention 'ml of fetal blood', to be clear we do not report 'ml fRBC'.
- 3) **Alarm value of FMT > 15 ml fetal blood** has to be introduced into the SOP-042. The clinician has to be phoned and follow-up samples are recommended.
- 4) A suggestion for introduction of fetal cell count into the **diagnostic prenatal care path** is proposed at week 20-24, 28 and 36 with a grey box ('*test enkel zinvol bij specifieke risicogroep*'). Do the gynaecologists consider using the clinical-diagnostic-therapeutic algorithm?
- 5) Acquisition of the commercial FETALtrol control would be an improvement.