Fetal Cell Count™ kit

Diagnosis of feto-maternal transfusion by flow cytometry

REF[1] IQP-363 25 tests package insert
IVD CE ***In Vitro Diagnostic medical device***

Intended use

The Fetal Cell Count™ kit is intended for the discrimination and quantitative detection of human fetal red blood cells in maternal blood. The Fetal Cell Count™ kit is based on a sensitive and accurate flow cytometric method, which offers a dual fluorescent detection of two intracellular antigens, Hemoglobin F (HbF) and Carbonic Anhydrase (CA). Both HbF and CA are detected in red blood cells obtained from EDTA anti-coagulated or Heparin-treated human peripheral whole blood. The complete dual-color staining and analysis of up to 5 samples can be concluded within 2 hour from blood collection.

Principle of the test

The Fetal Cell Count™ methodology is based on a combination of two antibodies. One is directed against fetal hemoglobin (HbF), which is present in fetal RBCs and in a small percentage of adult RBCs (called F cells). The second antibody is directed against Carbonic Anhydrase (CA), an enzyme only present in adult RBCs and very late stage fetal cells. The dual-color flow cytometric method allows simultaneous detection of these two intracellular antigens, while the use of formaldehyde as fixative and sodium dodecyl sulfate (SDS) for permeabilization of fixed RBCs results in low background staining, negligible HbF leakage, and minimal cell clumping.

With every patient sample an adult (male) blood sample spiked with 5% cord blood as a positive control and without the cord blood spike as a negative control should be included with the test.

Kit content

Reagent A	Fixative Solution (A) - Containing < 0.1% sodium azide	2.5 mL
Reagent B	Fixative Solution (B) - buffered Formaldehyde DANGER	2.5 mL
Reagent C	Permeabilization Solution (C) – containing sodium dodecyl sulfate (SDS)	2.5 mL
Reagent D (10x)	Washing Solution (10xD), 10x concentrated - PBS containing heparin	1x50 mL
Reagent E	Monoclonal antibody to human Carbonic Anhydrase conjugated with FITC, containing < 0.1% sodium azide	1.3 mL
Reagent F	Monoclonal antibody to human fetal hemoglobin conjugated with R-PE, containing < 0.1% sodium azide	1.3 mL

Each kit contains sufficient reagents to perform 25 tests.

Laboratory material required but not included

Laboratory centrifuge; 5 mL sterile, test tubes; sterile, conically bottomed micro centrifuge tubes; phosphate buffered saline (PBS), pH 7.4; demineralized water; blood collection tubes with anticoagulant; adjustable micropipettes and tips; vortex; hemo cytometer or automated cell counter; stop watch/timer.

Storage

Upon receipt, store reagents at 2-8 °C. Avoid direct sunlight. Reagents stored according to stated storage instructions are stable until the expiration date indicated on the label. For repeatedly testing store the reagents immediately after usage at 2-8 °C.

Warning and precautions

Reagents containing sodium azide may react with lead or copper plumbing to form explosive metal azides. On disposal, flush with large amounts of water to prevent azide build-up. All reagents should be handled in accordance with good laboratory practices using appropriate precautions. In addition, handle all patient samples with appropriate precautions. Do not pipette by mouth and wear gloves during the procedure. Reagent B contains formaldehyde, a highly toxic allergenic and potentially carcinogenic reagent, which should be handled in accordance with good laboratory practices using appropriate precautions. Avoid skin or eye contact.
The test must be performed by well-trained and authorized laboratory technicians. Please contact the manufacturer if the original test kit is damaged.

Specimen collection and preparation

Reagent preparation

- Prior to testing the 10x concentrated washing solution (10x reagent D) should be diluted. Per sample about 16 mL of 1x reagent D is needed. 18 mL of 0.2 µm filtered demineralized water to 2 mL of 10x reagent D washing solution. The total volume is 20 mL of 1x D washing solution (maximum volume). For example, when testing a patient sample, a negative and a positive control a total of 60 mL of 1x reagent D is used.
- All reagents should be at room temperature before use. Especially reagent C should be at room temperature (any precipitates should be dissolved before use).

Collection and processing of a patient sample

- Collect (at least) 1.0 mL venous blood into an EDTA or Heparin-treated tube, using aseptic venapuncture. Blood samples should be stored at either 2-8 °C or at room temperature (20 – 25 °C) until processing. After 12 hours the sample should be stored at 2-8 °C and should be tested within 72 hours.
- A patient sample that was stored (12 – 72 hours), should be washed three times using 1x reagent D (3 x 2 mL at 300 g for 3 minutes, low brake) before starting the tests. When possible use the soft start and stop of the centrifuge.

Processing of cord blood and adult blood to be used for spiking experiments

- Cord and adult blood to be used for spiking experiments may also be stored up to 72 hours.
- Cord and adult blood should always be washed three times using 1x reagent D (3 x 2 mL at 300 g for 3 minutes, low brake) before spiking and the start f the staining procedure. When possible use the soft start and stop of the centrifuge.

Control samples

Always run a positive and negative control sample with every patient sample. A mix of cord blood and adult (male) blood is advised as positive control sample. When no cord blood is available FETALtrol (FH101) can be used. Adult (male) blood without spike is advised as negative control sample.

Positive control and to use for set up of cytometer

- Mix approximately 5% cord blood in normal adult blood (v/v). Only washed cord blood and adult blood should be mixed.
- When the mixture is not only to be used for set up and control, but also for an accurate quantification of the spiked cells the erythrocytes in both cord and adult blood samples should be counted on an hematology analyser. From these numbers the spike can be calculated accurately.

Negative control (no fetal cells)

- As a negative control it is advised to use blood from an adult man. Treat this material as patient sample in procedure.

Test procedure Fetal Cell Count™ kit

Fixation and Permeabilization
control (spiked) sample and patient sample

1. Label for each patient sample and the positive and negative external controls a separate 5 mL conical bottom centrifuge tube.
2. Add 100 µL Reagent A to each tube.
3. Add 10 µL EDTA-anticoagulated whole blood, mix and vortex. *When FetalTrol is used as a control sample 5µl should be used.*
4. Add 100 µL Reagent B and vortex.
5. Incubate the mixed cell suspension at room temperature for exactly 30 minutes. Mix the suspension gently every 10 minutes.
6. Add 2 mL 1x reagent D and mix the cells by inverting the tubes a few times.
7. Centrifuge the cell suspension at 300 g for 3 minutes.
8. Discard the supernatant.
9. Add 100 µL 1x reagent D.
10. Resuspend the cell pellet and vortex gently.
11. Add 100 µL reagent C and vortex (the incubation time of exactly 3 minutes is started with the first tube). Reagent C should be at room temperature (any precipitates should be dissolved before use).
12. After exactly 3 minutes: add 2 mL 1x reagent D and mix the cells by inverting the tubes a few times.
13. Centrifuge the cell suspension at 300 g for 3 minutes.
14. Discard the supernatant.
15. Add 2 mL 1x reagent D and resuspend cell pellet by inverting the tubes a few times.
16. Centrifuge the cell suspension at 300 g for 3 minutes.
17. Discard the supernatant.
18. Resuspend the cell pellet in 1 mL 1x reagent D and resuspend the cells by gentle vortexing.

Immunofluorescent staining control samples

19. Label four conical bottom tubes which can be used with the flow cytometer with S1, S2, S3, and S4.
20. Add the different components to the tubes following table 1. and mix.
21. Incubate at room temperature for 15 minutes in the dark (avoid direct light).

Tube	5% spiked sample	Reagent E	Reagent F
S1	50 µl	---	---
S2	50 µl	50 µl	---
S3	50 µl	---	50 µl
S4	50 µl	50 µl	50 µl

Table 1. Components to add together for adjustment of the settings of the flow cytometer.

22. Add 2 mL 1x reagent D and centrifuge the cell suspension at 300 g for 3 minutes.
23. Discard the supernatant.
24. Resuspend the cell pellet in 500 µL 1x reagent D.
25. The cells are now ready for data acquisition by flow cytometry. The cells should be assessed within 30 minutes.

Immunofluorescent staining patient sample

26. Add together in a new conical bottomed tube and mix well:
    a) 50 µL Reagent E - anti-human CA FITC
    b) 50 µL Reagent F - anti-human HbF-R PE
    c) 50 µL Erythrocyte suspension (the obtained cell suspension from step 18)
27. Incubate at room temperature for 15 minutes in the dark (avoid direct light).
28. Add 2 mL 1x reagent D and centrifuge the cell suspension at 300 g for 3 minutes.
29. Discard the supernatant.
30. Resuspend the cell pellet in 500 µL 1x reagent D.
31. The cells are now ready for data acquisition by flow cytometry. The cells should be assessed within 30 minutes.

Data Acquisition

- List mode files of at least 100,000 events should be collected for log FSC, log SSC, and log fluorescence signals for both fluorochrome conjugated antibodies with the region gated at the erythrocytes.
- Less than 100,000 events will influence the accuracy of the assay.
- To prevent coincidence of a fetal and a maternal cells passing the laser it is advised to run the samples at a low to medium speed.

Instrument Requirements

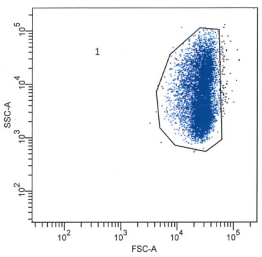
- Make sure that the flow cytometer is calibrated correctly according to manufacturer's instruction.
- It is advised to perform instrument calibration and maintenance on regular basis.
- The flow cytometer should be operated by a technician skilled in the art. Evaluation of the results should be done by someone skilled in the interpretation of flow cytometric data.

Instrument settings

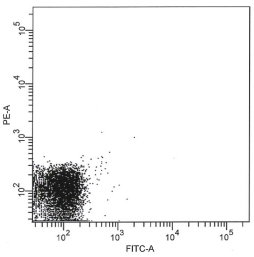
This procedure describes setting up the flow cytometer prior to acquisition and analysis of Fetal Cell Count™ kit data.
During analysis it is easier to interpret the data when the number of events in each dot plot is limited to 10,000 events.

Analysis

1. Select all erythrocytes in the **negative control cells (S1; *unstained control)*** by using a region and exclude debris and background noise by setting the appropriate FSC threshold (see cytogram 1). Select logarithmic amplification for FSC and SSC gains. Activate the region for all further steps in the evaluation.



Cytogram 1

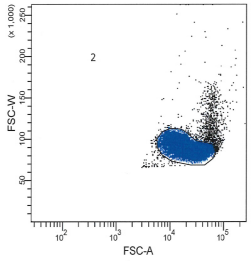


Cytogram 2

2. The same sample (S1; unstained control) should also be used to adjust FL1 and FL2 photomultiplier tube (PMT) voltages. FL1/FL2 baseline signals should be located in lower left corner in an FL1 vs. FL2 dot plot (see cytogram 2).

3. Doublets can be excluded by making a positive region on the single events, excluding the doublets in FSC-area vs FSC-width dot plot (see cytogram 3).

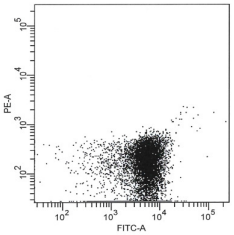
Use the combination of region 1 (events) and region 2 (single events) in all other steps and for all samples in the evaluation.



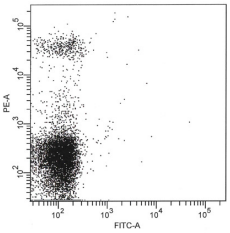
Cytogram 3

4. To adjust compensation of FITC from FL2, the ***sample stained with anti-Carbonic Anhydrase FITC* (S2)** should be analyzed. FL1 positive signals (*adult red blood cells*) should be in the lower right quadrant of the FL1 vs. FL2 dot plot (see cytogram 4).

5. Fluorescence compensation settings between the FITC and R-PE fluorescence signals should be optimized to separate the *fetal cells from maternal F cells*. Analyze the ***sample stained with only anti-HbF R-PE (S3)*** to adjust compensation of R-PE from FL1. FL2 positive signals (*fetal red blood cells*) should be in the upper left quadrant in the FL1 vs. FL2 dot plot (see cytogram 5).

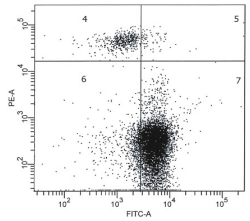


Cytogram 4



Cytogram 5

6. Finally the prepared **5% spiked blood sample (S4)** should be analyzed to check if the appropriate cytometer settings are obtained. Put the horizontal axes of the quadrant to evaluate the sample directly under the HbF positive population (see cytogram 6) and put the vertical axes directly left of the CA positive, but HbF negative, population. *Fetal red blood cells* are located in upper left quadrant of the dot plot, whereas *interfering (maternal) F cells* are located in the lower right corner together with the rest of the maternal erythrocytes.



Cytogram 6

7. The set up is completed and the patient sample(s) can be run and analyzed. When the positive control sample, as mentioned in the section Control samples, does not show staining of the fetal cells for HbF (PE-channel) the assay is invalid and should be run again.

Results

The results of evaluation of patient blood samples are a quantitative and reliable source to determine the concentration of fetal RBCs in the maternal blood circulation. Fetal RBCs are recognized by their bright HbF expression combined with a weaker CA expression. This in contrast to maternal RBCs having no HbF signal combined with bright CA expression, and maternal F cells with low HbF and bright CA expression. After approximately week 32 of gestation the CA expression in the fetal cells will get stronger. In week 38 of gestation and later the fetal cells might already express CA to the same extend as the maternal cells.

Typical results obtained with the Fetal Cell Count™ kit are presented in the sections **Instrument settings** and **Performance characteristics**. The accuracy of the fetal RBC count was evaluated on mixed-field populations of adult and cord blood RBCs. The cytograms clearly demonstrate the usefulness of a second red blood cell marker, CA, for accurate discrimination between the different RBC populations in maternal blood. Without CA as marker, discrimination between fetal RBCs and variable concentrations of maternal F cells becomes problematic.
In addition, obtained results and percent fetal RBCs may be used to calculate the total volume of fetal RBCs in the maternal blood circulation.

Quality control

All reagents in the Fetal Cell Count™ kit as well as linearity and accuracy of the fetal red blood cell count have been tested on different mixed-field populations of adult and cord blood RBCs [17,18].

Limitations of the procedure

- Personnel experienced in aseptic techniques should perform the collection of the blood sample.
- The Fetal Cell Count™ kit is intended for detection using flow cytometry and *not* for use with immunofluorescent microscopy.
- The efficacy of the Fetal Cell Count™ kit with samples other than human RBCs has not been established.
- The Fetal Cell Count™ kit is intended for *In vitro diagnostic use* in the countries that belong to the European Community. In all other countries this should be checked or considered to be labeled "*For research use only*".
- Accurate results with flow cytometric procedures depend on correct alignment and calibration of laser as well as proper gate setting.
- Lysis of erythrocytes and a decrease in HbF and CA contents cannot be excluded when cells are stored at room temperature for more than 72 hours (3 days). Therefore, preparation of the cells and incubation should always be performed within 3 days from blood collection.

Performance characteristics

***Antibody binding specificity* –** In-house study results concluded that the antibody directed against HbF (Fetal hemoglobin) recognizes only the γ chain of hemoglobin F, while the second antibody is specific for the CA (Carbonic Anhydrase) antigen.

Correlation to the improved version of the Fetal Cell Count™ kit (IQP-363) - This version is the improved version of the Fetal Cell Count™ kit that was based on the direct staining of the two used markers (IQP-379). Studies demonstrate identical performance of the versions. The correlation coefficient (r^2) between the two versions is > 0.999.

Linearity – Measurement of artificial mixtures for the (theoretical) concentration range 0.02 – 5.0 % (v/v) show a high correlation (r = 0.999), when 100,000 cells are measured. This correlation increases when larger number of cells are evaluated.

Specificity - Tested samples from control blood donors did not show staining in the upper left (UL) area. These data demonstrate that in there is no interference in the UL area leading to inaccurate counting of fetal cells.

Detection limit – The detection limit of the assay is based on the measurement of artificial mixtures and determined to be 0.014% when 100.000 cells are evaluated. Accuracy is improved when the number of events is increased.

Clinical evaluation – In total a series of 737 samples have been tested during two different clinical studies. Only part of the studies is represented here. The publications containing all data can be obtained via *marketing@iqproducts.nl*

- During the clinical evaluation the Fetal Cell Count™ kit (IQP-379) has been compared to an earlier version of the Fetal Cell Count™ kit (IQP-370) that was based on the indirect staining of the markers. The correlation between the two versions has shown to be $r^2 > 0.995$
- A clinical evaluation was performed to study the Fetal Cell Count™ kit (IQP-370) performance by comparison with the generally used Kleihauer-Betke test. In this study 130 patient samples were screened.

		Fetal Cell Count™		
		+	-	Total
Kleihauer-Betke	+	17	11	28
	-	0	102	102
	Total	17	113	130

- In 13,1% (17/130) of the cases feto-maternal transfusion was detected using both methods.
- On a total of 130 patients, 28 (28/130 – 21.59%) were shown to contain fetal cells by using the Kleihauer-Betke test. Of these, only 17 patients (17/28; 61%) contained true fetal cells using the Fetal Cell Count™ kit (range 0.17 to 11.2%) .
  The other 11 positive tested patients (11/28; 39%) had a non-typical Kleihauer-Betke test pattern with very faint staining of a number of cells.
- Out of the 11 Kleihauer-Betke positive and Fetal Cell Count™ kit negative patients 7 had a non-typical Kleihauer-Betke test pattern with very faint staining of the cells. These samples showed a typical pattern for thalassemia. These corresponding patients were diagnosed as being thalassemic.

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Explanation of used symbols

	Consult instructions for use
REF	Catalogue number
	Sufficient for
IVD	In Vitro Diagnostic medical device
	Caution, consult accompanying document
	Keep away from (sun)light
	Biological risks
	Temperature limitation (°C)
RUO	For Research Use Only
LOT	Batch code
	Use by yyyy-mm-dd
	Manufacturer
EC REP	Authorized Representative in the European Community

Contact information

IQ Products BV
www.iqproducts.nl
Rozenburglaan 13a
9727 DL Groningen
The Netherlands
T +31 (0)50 5757000
F +31 (0)50 5757002
marketing@iqproducts.nl

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