Fetal Cell Count™ kit

for flow cytometry

REF[1] IQP-363 25 tests package insert

[RUO] ***for Research Use Only***

UDI-DI 87179530223IQP-363NG

Barcode GS1 (GTIN): 8717953022332

PACKAGE INSERT

Fetal Cell Count™ kit

for flow cytometry

REF[1] IQP-363 25 tests package insert

RUO ***For Research Use Only. Not for use in diagnostic procedures.***

Intended use

The Fetal Cell Count™ kit is intended for the discrimination and quantitative detection of human fetal red blood cells (fRBC) within red blood cells. The Fetal Cell Count™. kit is based on a sensitive and accurate, non-automated flow cytometric method, which offers a dual fluorescent detection of two intracellular antigens, fetal hemoglobin (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, that must be executed and interpreted by well-trained and authorized laboratory technicians, and can be concluded within 90 minutes from blood collection. For Research Use Only. Not for use in diagnostic procedures.

Principle of the test

The Fetal Cell Count™ methodology is based on a combination of two antibodies. One is directed against HbF, which is present in fetal RBCs and in a small percentage of adult RBCs (called F cells). The second antibody is directed against 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.

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
- Demineralized water
- Blood collection tubes with anticoagulant
- Adjustable micropipettes and tips
- Vortex
- Hematology analyzer or automated cell counter
- Stopwatch or timer
- Flow cytometer

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 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. For detailed information please find the Safety Data Sheet on: www.iqproducts.nl.
The test must be performed by well-trained and authorized laboratory technicians. Please contact the manufacturer if the original test kit is damaged. Please be aware of the obligation of users of this kit to notify the manufacturer and designated authorities about incidents concerning this product.

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.

Configuration of the flow cytometer

The flow cytometer settings have to be optimized for analysis of RBCs. For these steps, proceed to page 4 to *'Configuration of the setting of the flow cytometer'* before measuring test samples.

Specimen collection and preparation

Reagent preparation

All reagents should be at room temperature before use. Especially reagent C should be at room temperature (any precipitates should be dissolved before use).

Reagent D

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. Add 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. For example, when testing a test sample, a negative and a positive control, a total of 60 mL of 1x reagent D is used.

Collection and processing of test sample

Collect (at least) 1.0 mL venous blood into an EDTA or heparin-treated tube, using aseptic venapuncture.

Storage

Blood samples should be stored at either 2-8 °C or at room temperature (20–25 °C) until processing. After 12 hours, store the sample at 2-8 °C. The sample must be tested within 72 hours.

A test 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 stop of the centrifuge.
Cord and adult blood to be used for spiking experiments must be stored separately.

Control sample preparation
Always run a positive and negative control sample with every test sample. A mix of (5%) cord blood and adult (male) blood is advised as positive control sample. The spike for the quality control of the kit is made with the appropriate volume of washed cells from 0.5 mL cord blood and adult blood.
When no cord blood is available FETALtrol (FH101 and FH102, IQ Products B.V.) can be used as a positive control. Adult (male) blood without spike is advised as negative control sample.

Positive control
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. When possible use the soft stop of the centrifuge. The positive control (spiked sample) should always be made (mixed together) on the day of use.

Mix approximately 5% cord blood in normal adult blood (v/v). When the mixture is not only to be used for setup and control, but also for an accurate quantification of the spiked cells, the erythrocytes in both cord and adult blood should be counted on a hematology analyzer. 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.

Test procedure Fetal Cell Count™ kit

Fixation and Permeabilization
1. Label a 5 mL conical bottom centrifuge tube for each test sample and the positive and negative external controls.
2. Add 100 µL reagent A to each tube.
3. Add 10 µL EDTA-anticoagulated whole blood or control sample, and vortex. *When FETALtrol is used as a control sample only 5 µl should be used.*
4. Add 100 µL reagent B and vortex.
5. Incubate the vortexed cell suspension at room temperature for exactly 30 minutes. Vortex the suspension every 10 minutes and make sure there are no cells on the bottom of the tube.
6. Add 2 mL 1x reagent D and vortex the tubes.
7. Centrifuge the cell suspension at 300 g for 3 minutes (low brake).
8. Discard the supernatant.
9. Vortex the tube a few seconds and add 100 µL 1x reagent D. Vortex 3 seconds at maximum speed. Make sure the cell pellet is completely resuspended.
10. Add 100 µL reagent C and vortex. Reagent C should be at room temperature (any precipitates should be dissolved before use). Incubate the mixed cell suspension at room temperature for exactly 3 minutes. *Note: the incubation time of exactly 3 minutes is started with the first tube.*
11. After exactly 3 minutes: add 2 mL 1x reagent D and vortex the tubes.
12. Centrifuge the cell suspension at 300 g for 3 minutes (low brake).
13. Discard the supernatant.
14. Add 2 mL 1x reagent D and resuspend cell pellet by vortexing.
15. Centrifuge the cell suspension at 300 g for 3 minutes (low brake).
16. Discard the supernatant.
17. Resuspend the cell pellet in 1 mL 1x reagent D and resuspend the cells by vortexing. Make sure the cell pellet is completely resuspended.

Immunofluorescent staining
18. Add the different components to the tubes following table 1 and vortex.
19. Incubate at room temperature for 15 minutes in the dark (avoid direct light).
20. Add 2 mL 1x reagent D and centrifuge the cell suspension at 300 g for 3 minutes (low brake).
21. Discard the supernatant.
22. Resuspend the cell pellet in 500 µL 1x reagent D.
23. The cells are now ready for data acquisition by flow cytometry. The cells should be assessed within 30 minutes. Measure at least 100,000 events.

Table 1. Components to add together for measuring test samples (P) and controls (C1 and C2).

Tube	Adult male blood	5% spiked sample	Test sample	Reagent E	Reagent F
P1	---	---	50 µl	50 µl	50 µl
C1	50 µl	---	---	50 µl	50 µl
C2	---	50 µl	---	50 µl	50 µl

Configuration of the settings of the flow cytometer

This part describes how the flow cytometer has to be configured for the use of the Fetal Cell Count™.
For the configuration of the flow cytometer a 5% cord blood spiked sample is needed (FETALtrol *cannot* be used). Follow the next steps:

1. Label a 5 mL conical bottom centrifuge tube for the 5% cord blood spiked sample.
2. Follow step 2 to 17 from the "Test procedure Fetal Cell Count™ kit".
3. Label four conical bottom tubes which can be used with the flow cytometer with S1, S2, S3, S4.
4. Add the different components to the tubes following table 2 and vortex.
5. Incubate at room temperature for 15 minutes in the dark (avoid direct light).
6. Add 2 mL 1x reagent D and centrifuge the cell suspension at 300 g for 3 minutes (low brake).
7. Discard the supernatant.
8. Resuspend the cell pellet in 500 µL 1x reagent D.
9. The cells are now ready for measurement by flow cytometry. The cells should be assessed within 30 minutes.

Table 2. Components to add together for the configuration of the settings of the flow cytometer.

Tube	5% spiked sample	Reagent E	Reagent F	Reagent D
S1	50 µl	---	---	100 µl
S2	50 µl	50 µl	---	50 µl
S3	50 µl	---	50 µl	50 µl
S4	50 µl	50 µl	50 µl	---

Flow cytometer configuration

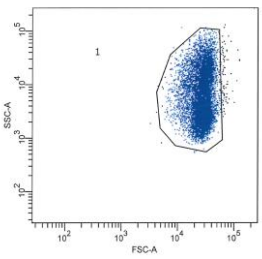
This procedure describes setting up the flow cytometer prior to analysis of test samples with the Fetal Cell Count™ kit.

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. Exclude debris and background noise by setting an appropriate FSC threshold and select the appropriate parameters to be able to exclude doublets in the data analysis phase.

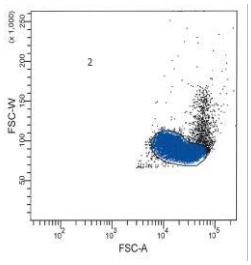
To prevent coincidence of a fetal and an adult cell passing the laser it is advised to run the samples at a low to medium speed.

Note: During analysis it is easier to interpret the data when the number of events in each dot plot is limited to 10,000 events.

1. Select all erythrocytes in the **negative control cells (*S1; unstained control)*** by using a region (see cytogram 1). Select logarithmic amplification for FSC and SSC gains.
2. Doublets can be excluded by making a positive region on the single events, excluding the doublets in FSC-A(rea) vs FSC-W(idth) dot plot (see cytogram 2).
   Use the combination of region 1 (events) and region 2 (single events) in all other steps and for all samples in the evaluation.

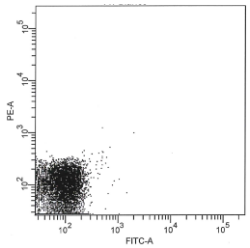


Cytogram 1



Cytogram 2

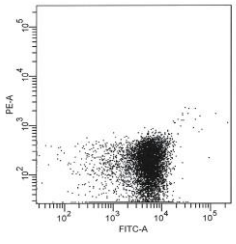
3. S1 (unstained control) should also be used to adjust FL1 and FL2 photomultiplier tube (PMT) voltages. FL1/FL2 baseline signals should be located in lower left corner in an FL1 vs. FL2 dot plot (see cytogram 3).



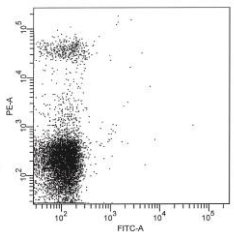
Cytogram 3

4. To adjust compensation of FITC from FL2, the **sample S2 stained with only reagent E *(anti-CA FITC)*** 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 adult F cells*. Analyze the **sample S3 stained with only reagent F *(anti-HbF R-PE)*** 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).

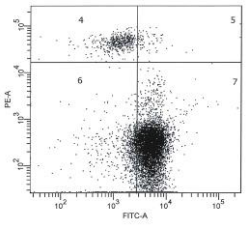


Cytogram 4



Cytogram 5

6. Finally, the prepared **5% spiked blood sample (*S4*)** should be analyzed to check if the appropriate cytometer settings are obtained. Place the horizontal axes of the quadrant 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 quadrant together with the rest of the maternal erythrocytes.



Cytogram 6

The setup is completed and the settings can be stored as a protocol and used with each new analysis of a test sample. Subsequently, a test sample(s) can be run and analyzed.

Results Analysis

The results of evaluation of test blood samples are a quantitative and reliable source to determine the concentration of fRBCs in the adult blood circulation. Fetal RBCs are recognized by their bright HbF expression combined with a weaker CA expression. This in contrast to adult RBCs having no HbF signal combined with bright CA expression, and adult F cells with low HbF and bright CA expression.

In addition, obtained results and percent fRBCs may be used to calculate the total volume of fRBCs in the adult blood circulation. Please be aware that guidelines for this calculation differ per lab.

When the positive control sample (spiked sample or FETALtrol) does not show staining of the fetal cells for HbF (PE-channel) the assay is invalid and should be run again.

**Important note*: For typical FETALtrol results please visit our website: https://www.iqproducts.nl/FETALtrol/

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. 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.

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.
- 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 recognizes only the γ chain of HbF, while the second antibody is specific for the CA antigen.

Accuracy – In-house study results have shown that both repeatability and reproducibility are optimal with coefficient of variance of 18.3% and 6.3% respectively for artificial mixtures with 1% fetal cells.

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.

Regulatory Status

At this time, the Fetal Cell Count™ kit is labeled "for research use only" in the US and Canada.

Bibliography

1. EN ISO 15223-1 Medical devices – Symbols to be used with information to be supplied by the manufacturer - Part 1: General requirements.
2. Sebring, E.S., Polesky, H.F. 1990. Fetomaternal hemorrhage: incidence, risk factors, time of occurence, and clinical effects. Transfusion 30: 344-357.
3. Garratty, G., and Arndt, P.A. 1999. Applications of flow cytofluorometry to red blood cell immunology. Cytometry (Communications in Clinical Cytometry) 38: 259-267.
4. Nance, S.J., Nelson, J.M., Arndt, P.A., et al. 1989. Quantitation of Feto-maternal hemorrhage by flow cytometry, a simple and accurate method. Am.J.Clin.Pathol. 91: 288-292.
5. Hadley, A.G. 1998. A comparison of in vitro tests for predicting the severity of haemolytic disease of the fetus and newborn. Vox Sang. 74: 375-383.
6. Lee, D., Contreras, M., Robson, S.C., Rodeck, C.H., Whittle, M.J. 1999. Recommendations for the use of anti-D immunoglobulin for Rh prophylaxis. Transf.Med. 9: 93-97.
7. Kleihauer, P., Braun, H., and Betke, K. 1957. Demonstration of fetal hemoglobin in erythrocytes of a blood smear. Klin.Wochenschr. 35: 637-638.
8. Davis, B.H., Olsen, S., Bigelow, N.C., Chen, J.C. 1998. Detection of fetal red cells in fetomaternal hemorrhage using a fetal hemoglobin monoclonal antibody by flow cytometry. Immunohematology 38: 749-756.
9. Johnson, P.R., Tait, R.C., Austin, E.B., et al. 1995. Flow cytometry in diagnosis and management of large fetomaternal haemorrhage. J.Clin.Pathol.48: 1005-1008.
10. Nelson, M., Popp, H., Horky, K., Forsyth, C., Gibson, J. 1994. Development of a flow cytometric test for the detection of D-positive fetal cells after fetolmaternal hemorrhage and a survey of the prevalence in D-negative women. Immunohematology 10: 55-59.
11. Corsetti, J.P., Cox, C., Leary, J.F., Cox, M.T., Blumberg, N., Doherty, R.A. 1987. Comparison of quantitative acid-elution technique and flow cytometry for detecting fetomaternal hemorrhage. Ann.Clin.Lab.Sci. 17: 197-206.
12. Navenot, J.M., Merghoub, T., Ducrocq, R., Krishnamoorthy, R., Blanchard, D. 1998. A new method for quantitative determination of fetal hemoglobin-containing red blood cells by flow cytometry: application to sickle cell disease. Cytometry 32: 186-190.
13. Nelson, M., Zarkos, K., Popp, H., Gilson, J. 1998. A flow-cytometric equivalent of the Kleihauer test. Vox Sang. 75: 234-241.
14. Navenot, J.M., Blandin, A.M., Willem, C., Bernard, D., Muller, J.Y., Blanchard, D. 1995. In situ detection of fetal hemoglobin by flow cytometry: evaluation of a simple procedure for quantitating fetal erythrocytes in maternal peripheral blood. In: International Society of Blood Transfusion – 5th Regional Congress; Venice 2-5 July, abstract POS 309, p239.
15. Blanchard, D., Bernard, D., Loirat, M.J., Frioux, Y., Guimbretière, J., Guimbretière, L. 1992. Caractérisation d'anticorps monoclonaux murins dirigés contre les érythrocytes foetaux. Rev.Fr.Transfus.Hémobiol. 35: 239-254.

16. Brady, HJ.M., Edwards, M., Linch, D.C., 1990 Expression of the human carbonic anhydrase I gene is activated late in fetal erythriod development and regulated by stage-specific trans-acting factors. British Journal of Haematology, 76, 135-142.
17. Bernaud, J., Rigal, D., Porra, V., Follea, G., Blanchard, D. Fetal Cell Count™ - a commercial kit for quantification of fetal cells in maternal blood by flow cytometry. 5th Euroconference on Clinical Cell Analysis. Athens, Greece, 20-25 September 2005.
18. Porra, V., Bernaud, J., Gueret, P., Bricca, P., Rigal, D., Follea, G., and Blanchard, D. 2007 Identification and uantification of fetal red blood cells in maternal blood by a dual-color flow cytometric method: evaluation of the Fetal Cell Count kit. Transfusion, 47:7 , 1281 – 1289.
19. Leers MP, Pelikan HM, Salemans TH, Giordano PC, Scharnhorst V. Discriminating fetomaternal hemorrhage from maternal HbF-containing erythrocytes by dual-parameter flow cytometry. Eur J Obstet Gynecol Reprod Biol. 2006 Jul 24.
20. de Wit H, Nabbe KC, Kooren JA, Adriaansen HJ, Roelandse-Koop EA, Schuitemaker JH, et al. 2011 Reference values of fetal erythrocytes in maternal blood during pregnancy established using flow cytometry. Am.J.Clin.Pathol.136(4):631-6.

Warranty

Products sold hereunder are warranted only to conform to the quantity and contents stated on the label at the time of delivery to the customer. There are no warranties, expressed or implied, which extend beyond the description on the label of the product. IQ Products BV is not liable for property damage, personal injury, or economic loss caused by the product.

Explanation of used symbols [1]

	Consult instructions for use
REF	Catalogue number
	Sufficient for
[RUO]	Research Use Only
	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
CE	Consult instructions for use
UK RP	Authorized Representative in the United Kingdom
UK CA	United Kingdom Conformity Assessed
CH REP	Authorized Representative for Switzerland

Contact information

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

UK RP **Advena Limited**
Plato Close, Tachbrook Park
Warwick. CV34 6WE. UK

CH REP **Casus Switzerland GmbH**
Hinterbergstrasse 49
Steinhausen, 6312, Switzerland