Fetal Cell Count™ kit

Diagnostic of Feto-maternal Transfusion by flow cytometry

[REF]1 CL-IQP-379 25 tests I package insert

For In Vitro Diagnostic Use

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 1.5 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.

Kit content

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>Fixative Solution (A) - Containing &lt; 0.1% sodium azide</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Reagent B</td>
<td>Fixative Solution (B) - buffered Formaldehyde X, R20/21/22, 36/37/38, 40, 43</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Reagent C</td>
<td>Permeabilization Solution (C) - containing sodium dodecyl sulfate (SDS) X R20, 22, 36/37/38, 41, 42</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Reagent D (10x)</td>
<td>Washing Solution (10x) - concentrated - PBS containing heparin</td>
<td>1x50mL</td>
</tr>
<tr>
<td>Reagent E</td>
<td>Polyclonal antibody to human carbonic anhydrase conjugated with FITC, containing &lt; 0.1% sodium azide</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Reagent F</td>
<td>Monoclonal antibody to human fetal hemoglobin conjugated with R-PE, containing &lt; 0.1% sodium azide</td>
<td>1.3 mL</td>
</tr>
</tbody>
</table>

Laboratory material required but not included

Laboratory centrifuge; 5 mL sterile, conically bottomed test tube; sterile, conically bottomed microcentrifuge tubes; phosphate buffered saline (PBS), pH 7.4; demineralised water; blood collection tubes with anticoagulant; adjustable micropipettes and tips; vortex; hemocytometer 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.

I D K

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. Reagent C contains sodium dodecyl sulfate (SDS). SDS is an irritating 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 Cedarlane if the original test kit is damaged.

Specimen Collection and Preparation

Processing of the blood sample

Note 1 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 450 mL of filtered demineralised water to 50 mL of 10xD washing Solution. The total volume is 500 mL of 1xD Washing Solution. After dilution, Reagent D (1x) is stored at 2-8°C

- Collect 0.5 - 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.

Test Procedure Fetal Cell Count™ kit

Note 2 Stored blood (up to 3 days) or cord blood and maternal blood to be used for spiking experiments should be washed three times (3 x 2 mL reagent D) before starting the tests. When possible use the soft start and stop of the centrifuge Although not necessary, it is advised to wash fresh samples too.

A- Fixation and Permeabilization

1. Reagent C should be at room temperature (this will dissolve any precipitates).
2. Add 100 µL Reagent A to a 5 mL conically bottomed tube.
3. Add 10 µL EDTA-anticoagulated whole blood (or 5 µL of packed cells), mix and vortex.
4. Add 100 µL Reagent B and vortex.
5. Incubate the mixed cell suspension at room temperature for 30 minutes. Mix the suspension gently every 10 minutes.
6. Add 2 mL 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 Reagent D.
10. Resuspend the cell pellet and vortex gently.
11. Add 100 µL Reagent C and resuspend the cells by vortexing.
12. Incubate the cell suspension 3 to 4 minutes.
13. Add 2 mL Reagent D and mix the cells by inverting the tubes a few times.
14. Centrifuge the cell suspension at 300 g for 3 minutes.
15. Discard the supernatant.
16. Add 2 mL Reagent D and resuspend cell pellet by inverting the tubes a few times.
17. Centrifuge the cell suspension at 300 g for 3 minutes.
18. Discard the supernatant.
19. Resuspend the cell pellet in 1 mL Reagent D and resuspend the cells by gentle vortexing.
-B- Immunofluorescent staining

20. 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 19)

21. Incubate at room temperature for 15 minutes (Avoid direct light).
22. Add 2 mL Reagent D.
23. Centrifuge the cell suspension at 300 g for 3 minutes.
24. Discard the supernatant.
25. Resuspend the cell pellet in 500 µL Reagent D to perform the flow cytometry analysis.
26. The cells are now ready for data acquisition by flow cytometry. The cells should be assessed within 30 minutes.

-C- 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.

**Instrument Settings**

**Note 3** This procedure describes setting up the flow cytometer prior to acquisition and analysis of Fetal Cell Count™ kit data. Proper instrument setup is pivotal for obtaining accurate results with the Fetal Cell Count™ kit.

**Target cells**

- Approximately 5 % mix of cord blood in normal adult blood (v/v)

**Note 4** Prior to mixing, the cord blood and adult blood should be washed 3 times (3 x 2 mL) using Reagent D (300g for 3 minutes).

**Procedure**

- Follow the procedure as described in the section Test Procedure Fetal Cell Count™ kit
- **Carbonic anhydrase and Hemoglobin F staining**; Stain one sample of mixed blood with anti-human CA-FITC, one sample with anti-human HbF-R-PE and one sample with both antibodies.
- **Unstained Control**. One sample, 100 µL cell suspension (fixed and permeabilized) should be set aside as a negative cell control.

**Analysis**

**Note 5** Acquisition and analysis can be performed on scatter gating (gating on forward scatter (FSC) and side scatter (SSC)). Select logarithmic amplification for FSC and SSC gains.

1. Analysis of the **unstained control**. Select all erythrocytes by using a region and exclude debris and background noise by setting the appropriate FSC threshold (see Cytogram 1). Activate the region for all further steps in the evaluation.

2. **Unstained control** cells should also be used to adjust FL1 and FL2 photomultiplier tube (PMT) voltages. FL1/FL2 baseline signals should be depicted squarely in lower left corner in an FL1 vs. FL2 dot plot (see Cytogram 2).

3. 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 to adjust compensation of R-PE from FL1. FL2 positive signals (fetal red blood cells) should be depicted in the upper left quadrant in the FL1 vs FL2 dot plot (see Cytogram 3).

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

5. Finally the prepared mixed blood sample 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 Cytograms 5a ) and put the vertical axes directly left of the CA positive population (see Cytograms 5b). Fetal red blood cells are located in upper left quadrant of the dot plot (experiment shown; 5.04%), whereas interfering F-cells are located in the upper and lower right corners (see Cytograms 5b).

**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 complete absence of 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.

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.

**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.
- A decrease in Hbf and CA contents cannot be excluded when cells are stored at room temperature for more than 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 (polyclonal) is specific for the CA (Carbonic Anhydrase) antigen.

**Correlation to THE INDIRECT DETECTION version of the Fetal Cell Count™ kit (IQP-370)**

This version is the improved version of the Fetal Cell Count kit that was based on the indirect staining of the two used markers (IQP-370). Studies demonstrate identical performance of the versions whereas the direct detection version (CL-IQP-379) has a more sensitive detection limit of 0.014% (100,000 cells, at 3 SD) The correlation coefficient ($r^2$) between the two versions is > 0.99

**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 a 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 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 research@cedarlanelabs.com

- During the clinical evaluation this improved Fetal Cell Count™ kit has been compared to an earlier version of the Fetal Cell Count™ kit 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 performance by comparison with the generally used Kleihauer-Betke test. In this study 130 patient samples were screened.

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<thead>
<tr>
<th>Fetal Cell Count™</th>
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<th>-</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>Kleihauer-Betke</td>
<td>10</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>113</td>
<td>130</td>
</tr>
</tbody>
</table>

- In 7.7% (10/130) 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 10 patients (10/28 - 35.00%) contained true fetal cells using the Fetal Cell Count™ kit (range 0.17 to 11.2%).
- Using the Kleihauer-Betke test, 11 patients (11/28 - 39%) were positive whereas the Fetal Cell Count™ kit detected no fetal cells, indicating a non-occurred Feto-maternal transfusion.
- The last 7 positive blood patients (7/28 - 25%) had an non-typical Kleihauer-Betke test pattern with very faint staining of a number of cells. Fetal Cell Count™ kit was positive in the UL area (mean 0.09%) but showed the typical pattern for Thalassemia. These corresponding patients were diagnosed as being Thalassemic.

**Bibliography**

1. DIN EN ISO 980 Graphic symbols for use in the labelling of medical devices.


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. Cedarlane Laboratories Limited is not liable for property damage, personal injury, or economic loss caused by the product.