Fetal Cell Count™ Kit II

The Fetal Cell Count™ Kit II is intended for the discrimination and quantitative detection of human fetal red blood cells in maternal blood. The Fetal Cell Count™ Kit II is based on a sensitive and accurate flow cytometric method, which offers a dual fluorescent detection of two intracellular located antigens, Hemoglobin F (HbF) and Carbonic Anhydrase (CA).

PACKAGE INSERT
25 Samples
(IQP-370)

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# Fetal Cell Count™ Kit II

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Intended Use

The Fetal Cell Count™ Kit II is intended for the discrimination and quantitative detection of human fetal red blood cells in maternal blood. The Fetal Cell Count™ Kit II 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.

Summary and Explanation

Diagnosis of FMH
The detection of circulating fetal cells in maternal blood represents an important area of laboratory support to the obstetrical management of women. The quantification of fetal red blood cells (RBC’s) is most commonly used to estimate the extent of fetomaternal hemorrhage (FMH), either in case of trauma with suspected placental injury or in the situation of an RhD incompatibility between the fetus and the mother for prevention of hemolytic disease of the new-born (HDN).

To date, routine FMH assessment and quantification is performed by the Kleihauer-Betke (KB) test. Although this test is inexpensive and easy to perform, it is a tedious procedure with subjective read out. In contrast to the Fetal Cell Count™ Kit II, the KB test does not distinguish between maternal RBC’s containing HbF (F-cells) and fetal RBC’s, which may result in an overestimation of the fetal RBC’s. The latter may have consequences for prophylactic or therapeutic treatment of the pregnant women.

In recent years, several reports have demonstrated the use of flow cytometry methods as an accurate, sensitive and reliable substitute for the Kleihauer-Betke test. The Fetal Cell Count™ Kit II quantitates human fetal red blood cells in maternal blood. The test is based on the sensitive and accurate flow cytometric detection of HbF and CA present in different red blood cells populations.

Principles of the Procedure

The Fetal Cell Count™ test is based on a combination of two antibodies. One is directed against fetal hemoglobin (HbF), which is present in fetal RBC’s and in a small percentage of adult RBC’s (called F-cells). The second antibody is directed against Carbonic Anhydrase (CA), an enzyme only present in adult RBC’s 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 RBC’s results in low background staining, negligible HbF leakage, and minimal cell clumping.
The Fetal Cell Count™ II method consists of the following steps:

1. Fixation and Permeabilization of the RBC’s
2. Immunofluorescent staining
3. Data acquisition by flow cytometry

Materials and Reagents

Reagents supplied in the Fetal Cell Count™ Kit II

Ready-to-use reagents for 25 samples. Upon receipt, store the Kit at 2 - 8 °C. Reagents stored according to stated storage instructions are stable until the expiration date indicated on the labels.

<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</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Reagent C</td>
<td>Permeabilization Solution – containing Sodium Dodecyl Sulfate (SDS)</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Reagent D</td>
<td>Washing Solution – PBS containing heparin</td>
<td>2 x 200 mL</td>
</tr>
<tr>
<td>Reagent E</td>
<td>Polyclonal antibody to human Carbonic Anhydrase 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 containing &lt;0.1% sodium azide</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Reagent G</td>
<td>Goat anti-Rabbit IgG (H+L) FITC conjugated containing &lt;0.1% sodium azide</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Reagent H</td>
<td>Goat Fab’ anti-Mouse IgG (H+L) R-PE-conjugated containing &lt;0.1% sodium azide</td>
<td>1.3 mL</td>
</tr>
</tbody>
</table>

Laboratory materials and equipment required

(Not included in the Fetal Cell Count™ Kit II)

- Laboratory centrifuge
- 5 mL sterile, conically bottomed test tube
- Sterile, conically bottomed microcentrifuge tubes
- Blood collection tubes with anticoagulant
- Adjustable micropipettes and tips
- Vortex
Warnings and Precautions

The Fetal Cell Count™ Kit II was developed FOR RESEARCH USE ONLY.

Caution:
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.

Specimen Collection and Preparation

Processing of the blood sample

Collect 0.5 – 1.0 mL venous blood into an EDTA or Heparin-treated tube, using aseptic venipuncture. The blood sample should be kept at room temperature (20 - 25 °C) until processing. Before use the blood samples can be stored at room temperature up to 3 days on a roller-mixer. Testing of the blood sample a second or third day, requires the cells to be washed with reagent D (3 x 2 mL) from the Fetal Cell Count™ kit II before starting the test.

Test Procedure Fetal Cell Count™ Kit II

Note: Stored blood 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. If possible use the soft start and stop of the centrifuge.
I. 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 20 µL 2.5 x 10³ cells/µL), mix and vortex.
4. Add 100 µL Reagent B and vortex.
5. Incubate the mixed cell suspension at room temperature for 30 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 aggregated cells and vortex.
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 mix the cells 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 suspension in 1 mL Reagent D and resuspend the cells by vortexing.

II. Immunofluorescent staining

20. Add together in a new conical bottomed tube and mix well:
   - 50 µL Reagent E - Rabbit anti-human CA.
   - 50 µL Reagent F - Mouse anti-human HbF.
   - 25-50 µL Erythrocytes suspension. (Cell suspension 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 suspension in 100 µl Reagent D.
26. Add 50 µL Reagent G (Goat anti-Rabbit IgG (H+L) FITC) to the cell suspension.
27. Add 50 µL Reagent H (Goat Fab’ anti-Mouse IgG (H+L) R-PE) to the cell suspension.
28. Incubate at room temperature for 15 minutes. (Avoid direct light).
29. Add 2 mL Reagent D to the cell suspension.
30. Centrifuge the cell suspension at 300 g for 3 minutes.
31. Discard the supernatant.
32. Resuspend the cell suspension in sufficient Reagent D to perform the flow cytometry analysis.
33. The cells are now ready for data acquisition by flow cytometry. The cells should be assessed immediately or within 10 minutes.
III. Data Acquisition

List mode files of 25,000 – 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.

For Patient Samples: at least 100,000 erythrocytes
Cord blood or maternal blood: 25,000 erythrocytes or more
Spiking experiments of cord blood in maternal blood: at least 100,000 erythrocytes.

Time table:

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>30 min.</td>
</tr>
<tr>
<td>Wash</td>
<td>3 min.</td>
</tr>
<tr>
<td>Permeabilization</td>
<td>3 min.</td>
</tr>
<tr>
<td>2x Wash</td>
<td>6 min.</td>
</tr>
<tr>
<td>Incubation Primary antibody</td>
<td>15 min.</td>
</tr>
<tr>
<td>Wash</td>
<td>3 min.</td>
</tr>
<tr>
<td>Incubation secondary antibody</td>
<td>15 min.</td>
</tr>
<tr>
<td>Wash</td>
<td>3 min.</td>
</tr>
<tr>
<td>Total Hands on Time</td>
<td>12 min.</td>
</tr>
<tr>
<td>Total Time FCC II kit</td>
<td>90 Minutes</td>
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Instrument Settings

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

Cells:
- Cord blood
- Normal adult blood
- Approximately 1 - 2 % mix of cord blood in normal adult blood (v/v).
(Prior to mixing, the cord - and normal blood should be washed with PBS three times.)

Procedure:
Follow the procedure as described on page 4-5.

Carbonic anhydrase and Hemoglobin F staining:
Stain one sample of cord blood, one sample of normal blood and one sample of mixed blood with CA and HbF.

Unstained Control:
From one sample, 100 µL cell suspension (fixed and permeabilized) should be set aside as a negative cell control.
**Analysis:**
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. First analyze the **unstained control**. Select all erythrocytes and exclude debris and background noise by setting the appropriate FSC threshold (Cytogram 1.1).

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 the third decade in an FL1 vs. FL2 dot plot (Cytogram 1.2).

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 **stained cord blood** to adjust compensation of R-PE from FL1. FL2 positive signals (fetal red blood cells) should be depicted in the first decade in the FL1 vs FL2 dot plot (Cytogram 1.3); a small double positive signal in the second decade may occur.
4. To adjust compensation of FITC from FL2, the stained adult blood should be analyzed. FL1 positive signals (adult red blood cells) should be depicted in the fourth decade in the FL1 vs. FL2 dot plot (Cytogram 1.4a); double positive F-cells are located in the second decade in the same dot plot (Cytogram 1.4b).

5. Finally the prepared mixed blood sample should be analyzed to check the cytometer settings being made. Fetal red blood cells are located in the first decade whereas interfering F-cells are located in decade two. (Cytogram 1.5a& 1.5b)
Results

The results of the evaluation of patient blood samples are a quantitative and reliable source to determine the concentration of fetal RBC's in the maternal blood circulation. Fetal RBC's are recognized by their bright HbF expression in combination with a complete absence of CA expression. This in contrast to maternal RBC's with no HbF 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 II are presented on page 9 - 12. The accuracy of the fetal RBC count was evaluated on mixed-field populations of adult and cord blood RBC's. The cytograms clearly demonstrate the usefulness of a second red blood cell marker, CA, for an accurate discrimination between the different RBC populations in maternal blood. Without the use of the CA marker, discrimination between the fetal RBC’s and the variable concentrations of maternal F-cells becomes problematic.

In addition, the obtained results and percent fetal RBC’s may be used to calculate the total volume of fetal RBC’s in the maternal blood circulation.

Examples of the Fetal Cell Count™ Kit II

The results are representative examples obtained with the Fetal Cell Count™ Kit II. The following five cytograms represent the data obtained from a spiking experiment with cord blood mixed with normal adult blood containing a HIGH percentage of F-cells. Cytogram 2.1 shows the double labeling with HBF and CA. Cytogram 2.2 and 2.3 illustrate the difficulty to obtain a clear separation between fetal RBC’s and F-cells, and therefore to accurately quantify the former.

Cytogram 2.1:
Quadrant A1 shows the clear separation of the following 3 cell populations:
A1; No CA and high HbF content (fetal RBC’s), A2; CA and low HbF content (F-cells), A4; CA and no HbF content (adult RBC’s). The plot shows that, although the population of F-cells is considerable, the population of real fetal RBC’s can be distinguished accurately.
Cytogram 2.2:
Region I in the dot plot shows the total percentage of HbF positive cells (fetal cells plus F-cells) and the problematic discrimination between the two populations based on HbF content alone.

Cytogram 2.3:
The histogram shows that identification of fetal RBC's based on HbF content alone can be difficult. Marker D shows all HbF expressing cells, whereas marker J shows the arbitrarily identified highly HbF containing cells. The latter leads to a significant overestimation (~160%) of the fetal RBC population.
Cytogram 2.4 and 2.5: The dot plot and histogram show the percentage of CA positive adult RBC’s and the percentage of fetal RBC’s not staining for CA.

The following three cytograms represent data obtained from a spiking experiment with cord blood mixed with normal adult blood containing a LOW percentage of F-cells. All three cytograms demonstrate the clear separation of HbF positive fetal cells (A1) from the CA positive F-cells (A2) and adult cells (A4).

Cytogram 3.1: This cytogram shows a blood sample with low F-cell contamination. Again, 3 cell populations are clearly distinguished. A1; No CA and high HbF content (fetal RBC’s), A2; CA and low HbF content (F-cells), A4; CA and no HbF content (adult RBC’s).
Cytogram 3.2:
Region I in the dot plot shows the total percentage of HbF positive cells (fetal cells plus F-cells) and the discrimination between the two populations based on HbF content alone.

Cytogram 3.3:
The histogram shows the percentage of all HbF containing RBC’s (D), and the percentage of RBC’s with high HbF content (J). As the percentage of contaminating F-cells is only marginal in this example (see cytogram 2.1), the overestimation introduced by identification through HbF content alone is of course proportionally low.
The percentage of contaminating F-cells to be expected in a patient sample will be ranging between and around the two examples shown in this booklet (see cytogram 2.1 and 3.1).
Quality Control

All reagents in the Fetal Cell Count™ Kit and as well as the linearity and accuracy of the fetal red blood cell count have been tested on different mixed-field populations of adult and cord blood RBC’s.

Limitations of the Procedure

- Personnel experienced in aseptic techniques should perform the collection of the blood sample.
- The Fetal Cell Count™ Kit II is intended for detection using flow cytometry and not for use with immunofluorescent microscopy.
- The efficacy of the Fetal Cell Count™ Kit II with samples other than human RBC’s has not been established.
- The Fetal Cell Count™ Kit II is intended for Research Use Only.
- 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

Studies have been performed to analyze the binding specificity of the different antibodies for HbF and CA. The obtained results indicated that the antibody directed against HbF (Fetal hemoglobin) recognizes only the γ chain of hemoglobin F, while the second polyclonal antibody is very specific for the CA antigen.

Bibliography


**WARRANTY**

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Cat. No. IQ P-370

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Results with Fetal Cell Count™ kit II

The following cytograms represent cord blood spiked in maternal blood with a low percentage of maternal F-cells (cytogram 1) and a high percentage of maternal F-cells (cytogram 2), obtained with the Fetal Cell Count™ kit II. The use of the anti-CA allows the clear discrimination between Fetal RBC’s (HbF ++), maternal F-cells (HbF + / CA ++) and, maternal cells (CA ++).

The Fetal Cell Count™ kit II is a complete kit based on the detection and monitoring of HbF-containing fetal red blood cells (RBC’s) in maternal peripheral blood using dual fluorescent flow cytometry.

It enables:
- The quantitative detection of fetal RBC’s containing intracellular fetal hemoglobin (HbF) and maternal RBC’s containing intracellular Carbonic-Anhydrase (CA) including maternal F-cells.
- Accurate discrimination of fetal RBC’s from maternal F-cells
- Detection and monitoring of patients with suspected feto-maternal hemorrhage (FMH)

The complete Fetal Cell Count™ kit II contains the following reagents:
- Reagents for fixation and permeabilization of RBC’s
- Wash solution
- Immunofluorescent reagents for simultaneous detection of intracellular HbF and CA.
  - Anti-CA
  - Anti-HbF
- FITC and R-PE conjugated secondary antibodies.

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