**METHODS FOR DETECTING AND ANALYZING INDIVIDUAL RARE CELLS IN A POPULATION**

**Abstract**

Disclosed is a method for detecting a specific type of target cell contained in a mixture of cell types, comprising detecting the desired cell type based on its pattern of differential gene expression, said pattern being substantially absent from the other cell type(s) in the mixture. Also disclosed are methods for using that detection method for detecting and analyzing fetal cells present in a blood sample drawn from a pregnant woman.
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METHODS FOR DETECTING AND ANALYZING INDIVIDUAL RARE CELLS IN A POPULATION

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application Serial No. 08/127,170 filed September 27, 1993.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods for detecting a specific target cell in a mixture of cell types based on the pattern of differential gene expression of the target cell. Further, this invention relates to a method for prenatal diagnosis, wherein fetal cells are enriched from maternal blood, detected by their protein and/or nucleic acid components, and analyzed. The invention also relates to methods for fixing erythroid cells prior to in situ hybridization.

2. Description of Related Art

Prenatal diagnostic procedures presently utilized to identify chromosomal aberrations in the developing fetus require either amniocentesis or chorionic villus sampling (CVS). These methods involve obtaining fetal cells through invasive procedures which, although generally considered to be safe to both mother and fetus when performed by well trained personnel, are associated with an incidence of spontaneous abortion of 1 in 200-300 (Adinolfi, et al.,

-1-
Consequently, prenatal diagnostics are performed only on women who are considered at risk of having a baby with a specific defect. Women who are of advanced maternal age (35 years and older) are considered to be at high risk of having an affected child. Because there is a significantly greater number of low risk pregnancies, most genetic defects are undetected until birth. Thus, a present goal is to develop a non-invasive prenatal diagnostic technique which is applicable to all women, without the risks of existing techniques.

It has long been known that occasional fetal cells find their way into the maternal circulation (Adinolfi, et al., (1991) Prenatal Diagnosis, 11:799-804; Chueh & Golbus (1990) Seminars in Perinatology 14: 471-482). These cells may include platelets, trophoblasts, erythrocytes and leukocytes. In the past, distinguishing fetal cells from maternal cells has been hampered because of the small number of fetal cells in maternal blood, and because fetal and maternal cells differ only slightly morphologically, if at all.

Recent studies have provided evidence for the presence of fetal cells in the maternal circulation as early as six weeks of gestation (Chakravarty, et al. and Liou, et al., abstracts in Fetal Cells In Maternal Blood: Prospects for Noninvasive Prenatal Diagnosis, The New York Academy of

Cell Types:

To date, researchers have studied three fetal cell types as possible candidates for isolation. The advantages and disadvantages of each of these are summarized below:

Lymphocytes: The major advantage to lymphocytes is the presence of specific fetal HLA markers (Herzenberg, et al. (1979) Proc. Natl. Acad. Sci. USA 76:1453-1455). Fetal lymphocytes have been the focus of many antibody-based techniques for isolating fetal cells. In addition, once isolated, lymphocytes are relatively easy to grow in a cell culture system, thus providing an unlimited supply of cells. However, a disadvantage to using lymphocytes is the fact that fetal lymphocytes are present for up to five years after birth (Adinolfi, et al., (1991) Prenatal Diagnosis, 11:799-804; Chueh & Golbus (1990) Seminars in Perinatology 14: 471-482) and thus may potentially cause problems when analyzing cells from a women during
subsequent pregnancies. In addition, for this method to be useful, tests must first be conducted on the mother and father to determine their respective HLA type.

**Trophoblasts:** Trophoblasts are found in the tissue that attaches the ovum to the uterine wall (Adinolfi, et al., (1991) *Prenatal Diagnosis, 11:799-804*). These are found very early in pregnancy and therefore may be particularly useful for prenatal diagnosis. Unfortunately, they are found in very small numbers, and high affinity trophoblast-specific antibodies have proven difficult to obtain. In addition, these cells have a mucoprotein layer that coats the cell and tends to mask cell surface antigens, and trophoblast antigens may be absorbed on the surface of maternal cells (Adinolfi, et al., (1991) *Prenatal Diagnosis, 11:799-804*).

**Fetal Nucleated Erythrocytes:** The major advantage of fetal nucleated erythrocytes (RBCs; also known as normoblasts) is that they are present in large numbers. There are approximately 1000 times more erythrocytes than white blood cells in fetal circulation (Bianchi et al *Proc. Natl. Acad. Sci. USA* (1990) **87:3279-3283*). Assuming that fetal cells "leak" into maternal circulation in numbers proportional to their representation in the fetus, one would expect to find 1000 times more fetal RBCs than lymphocytes. The major problem with fetal erythrocytes is that they become enucleated as they mature (Bianchi et al *Prenatal Diagnosis* (1991) **11:523-528*; Salafia et al *Pediatric Pathology* (1988) **8:495-502*). However, the
proportion of nucleated to enucleated fetal RBCs is much higher earlier in gestation, which is ideal for prenatal diagnosis. There are significant differences between nucleated and enucleated RBCs (differential gene expression, surface transferrin receptor density, cell morphology, cell density, susceptibility to hypotonic lysis and susceptibility to lysis by ammonium chloride, for example) that could provide the basis of an enrichment protocol.

In fetal circulation, there are approximately $10^9$ erythrocytes per ml blood, and $10^6$ white blood cells per ml. Although there are no unequivocal data on the number of fetal cells in maternal circulation, the numbers in literature range from 1:50,000 to 1:10,000,000 (see Holzgreve et al The Journal of Reproductive Medicine (1992) 37(5):410-418; Price et al Am. J. Obstet. Gynecol. (1991) 165(6):1731-1737). Adult blood contains approximately $10^9$ RBCs per ml (M. Nikinmaa, Vertebrate Red Blood Cells: Adaptations of Function to Respiratory Requirements, New York:Springer-Verlag (1990). If 1:10,000,000 are fetal cells, there are approximately 100 fetal RBCs per ml of maternal blood. Therefore, if these cells can be isolated, 10-20 ml of maternal blood should provide enough fetal cells for prenatal diagnosis using technology currently available.

Current and proposed methods for performing prenatal analysis using maternal blood samples depend upon purification of the target fetal cell population to
homogeneity, using specific antibodies to cell surface antigens present on the cell of interest (the transferrin receptor, for example) in conjunction with positive selection using affinity columns, magnetic beads, or Fluorescence Activated Cell Sorting (FACS). The present method is unique in not requiring such a high level of physical purification, in that the target cells need only be slightly enriched, and are then identified and scored based on the individual cells specific protein or nucleic acid composition.

Thus, in view of the aforementioned deficiencies attendant with prior art methods of prenatal diagnosis, it should be apparent that there still exists a need in the art for a more accurate method of identifying DNA sequences of interest in fetal cells in maternal blood.

**SUMMARY OF THE INVENTION**

In its broadest aspect, the present invention is directed to a method for detecting a specific type of target cell contained in a mixture of cell types, comprising detecting the desired cell type based on its pattern of differential gene expression, said pattern being substantially absent from the other cell type(s) in the mixture.

The present invention also relates to using the foregoing detection method for detecting and analyzing fetal cells present in a blood sample drawn from a pregnant woman.
The present invention further relates to a method for enriching the concentration of fetal cells in a sample of maternal blood taken from a pregnant woman, which comprises subjecting the blood to a density gradient centrifugation, followed by treatment with a reagent which causes undesired cells to lyse. Another enrichment method, which may be termed "panning", comprises separating target cells from undesired cells by means of at least two reagents. The first reagent preferentially binds to at least some undesired cells in a mixture, and the mixture is then contacted with an immobilized second reagent, which preferentially binds to the first reagent, thereby effecting a separation of unwanted cells from target cells by negative selection. Alternatively, a positive selection may be accomplished when the first reagent preferentially binds to the desired cells.

The nature of the invention may be more clearly understood by reference to the following detailed description of the preferred embodiments of the invention and to the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a field with 3 definitive erythroid chicken cells, hybridized with PCR generated digoxigenin labeled probe to β̂-globin sequence of chicken. The signal indicates β̂-globin mRNA. Figure was taken with a 40X objective.

Figure 1B is a field with 2 definitive erythroid
chicken cells hybridized with PCR generated digoxygenin labeled probe to CAT sequence (Chloramphenicol Acetyl Transferase). Absence of signal indicates lack of hybridization due to absence of CAT mRNA in cells. Figure was taken with a 40X objective.

Figure 1C is a bright field image of cells in Figure 1A, showing preservation of cell morphology. Figure was taken with a 40X objective.

Figure 1D is a bright field image of cells in Figure 1B, showing preservation of cell morphology. Figure was taken with a 40X objective.

Figure 2A is an image of chicken definitive cells hybridized to PCR generated digoxygenin labeled probe to βA-globin chicken mRNA. Figure was taken with a 40X objective.

Figure 2B is an image of chicken definitive cells hybridized to PCR-generated digoxygenin labeled probe to CAT-mRNA. The low level of fluorescence is background. Figure was taken with a 40X objective.

Figure 2C is an image of chicken definitive cells which have been pre-treated with RNase and then hybridized to PCR generated digoxygenin labeled probe to βA-globin chicken RNA. The low level of fluorescence is background. Figure was taken with a 40X objective.

Figure 2D is an image of chicken definitive erythroid cells pre-treated with RNase and then hybridized with probes to CAT mRNA. Figure was taken with a 40X objective.

Figure 3 is a field of cord blood from the mononuclear
layer after separation on Histopaque 1077 medium. Cells are stained by May Grünwald-Giemsa methodology. All cell types may be distinguished on size, shape and color. Figure was taken with a 40X objective.

Figure 4 is a field of cord blood cells from the mononuclear layer after separation on Histopaque 1077 medium and subsequent lysis with 250mM NH₄Cl. Cells are stained by May Grünwald-Giemsa methodology. Membranes of mature fetal erythrocytes are visible as transparent “ghosts”. Figure was taken with a 40X objective.

Figure 5 is the same slide (different field) as in Figure 3, but with a 100X objective.

Figure 6 is the same slide (different field) as in Figure 4, but with a 100X objective.

Figure 7 shows adult whole blood, May Grünwald-Giemsa stained, prior to treatment with 250mM NH₄Cl. Figure was taken with a 40X objective.

Figure 8 shows adult whole blood, May Grünwald-Giemsa stained, after treatment with 250mM NH₄Cl. Figure was taken with a 40X objective.

Figure 9 shows May Grünwald stained fetal normoblasts, x,y coordinate scored, prior to hybridization with chromosome probes for FISH. Figure was taken with a 40X objective.

Figure 10 shows the a normoblast cell from the same slide as in Figure 9, with FISH using 3 alpha biotin/18 alpha digoxygenin chromosome probes. Figure was taken with
a 100X objective. The probes were visualized with avidin-fluorescein and anti-digoxigenin-rhodamine.

Figure 11 is a fluorescent image of human cord blood cells hybridized with human gamma globin cDNA probes. The figure was taken with a 400X objective using a triple band pass filter.

Figure 12 is the same image as Figure 11 except that it uses a DAPI filter.

Figure 13 is the same image as Figures 11 and 12 except that it is under bright field conditions.

Figure 14 shows a nucleated erythrocyte from maternal blood stained by benzidine/hematoxylin stain.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted above, one aspect of the present invention is the ability to detect a specific type of target cell in a mixture of cell types based on the pattern of differential gene expression of the target cell. For present purposes, pattern of differential gene expression includes analysis of RNA products, protein products, metabolic products or structural components. Differential gene expression may also be detected based on a different staining character from the other cell type(s). For present purposes, target cells may also be detected based on a feature of gene expression in common with the non-target cells, but present in a different amount. It will be appreciated that the target cell should have a pattern of gene expression different from the other cell types contained in the
mixture. Any known method for detecting a pattern of gene expression may be used, but it is preferred to use an in situ hybridization scheme with nucleic acid probes which hybridize to target cellular mRNA.

It will be apparent to one skilled in the art that the present detection method is applicable to detecting all sorts of target cells contained in a mixture of cell types, provided that the target cells have a pattern of gene expression different from the other cell types in the mixture. For example, this method could be used to detect leukemic cells in peripheral blood based on specific oncogenic mRNA's such as MYC or MYB produced by the leukemic cells; and hematopoietic progenitors in peripheral blood based on higher levels of transcription factor hGATA-1 produced by the progenitor cells. The porphobilinogen deaminase gene is another example of a gene whose expression level is highest in immature erythroid cells. In addition, the present method is useful to detect expression of transfection vectors into primary tissue cells present in small number in a population of cells, and also to detect infectious disease-causing organisms in human tissue.

The present method is particularly suitable for detecting the presence of fetal cells in a maternal blood sample taken from a pregnant woman. A maternal blood sample will generally have present maternal red cells and white cells, as well as some fetal white cells, fetal nucleated red cells, and fetal enucleated red cells. It is
useful to take advantage of the fact that maternal cells express $\beta$-globin, whereas fetal cells express $\gamma$-globin

(Evans et al Ann. Rev. Cell Biol. (1990) 6:95-124; Dalyot et al Ex. Hematol. (1992) 20:1143-1145). Thus, fetal cells may be identified by a hybridization pattern using a detectable probe which hybridizes to mRNA encoding $\gamma$-globin, but does not hybridize in any significant amount to mRNA encoding $\beta$-globin.

Probes suitable for use in the in situ hybridization methods of the present invention are preferably in the range of 150-500 nucleotides long. Probes may be DNA or RNA, preferably DNA.

The probes may be prepared by any method known in the art, including synthetically or grown in a biological host. Synthetic methods include oligonucleotide synthesis, riboprobes, and PCR. Probes may be generated and labeled in one step via a polymerase chain reaction [PCR] reaction as described in more detail below.

The probe may be labeled with a detectable marker by any method known in the art. Methods for labelling probes include random priming, end labeling, PCR and nick translation. Enzymatic labeling is conducted in the presence of nucleic acid polymerase, three unlabeled nucleotides, and a fourth nucleotide which is either directly labeled, contains a linker arm for attaching a label, or is attached to a hapten or other molecule to which a labeled binding molecule may bind. Suitable direct
labels include radioactive labels such as $^{32}\text{P}$, $^3\text{H}$, and $^{35}\text{S}$ and non-radioactive labels such as fluorescent markers, such as fluorescein, Texas Red, AMCA blue, lucifer yellow, rhodamine, and the like; cyanin dyes which are detectable with visible light; enzymes and the like. Labels may also be incorporated chemically into DNA probes by bisulfite-mediated transamination or directly during oligonucleotide synthesis.

Specifically, fluorescent markers may be attached to nucleotides with activated linker arms which have been incorporated into the probe. Probes may be indirectly labeled by the methods disclosed above, by incorporating a nucleotide covalently linked to a hapten or other molecule such as biotin or digoxygenin, and performing a sandwich hybridization with a labeled antibody directed to that hapten or other molecule, or in the case of biotin, with avidin conjugated to a detectable label. Antibodies and avidin may be conjugated with a fluorescent marker, or with an enzymatic marker such as alkaline phosphatase or horseradish peroxidase to render them detectable. Conjugated avidin and antibodies are commercially available from companies such as Vector Laboratories (Burlingame, California) and Boehringer Mannheim (Indianapolis, Indiana).

The enzyme can be detected through a colorimetric reaction by providing a substrate for the enzyme. In the presence of various substrates, different colors are produced by the reaction, and these colors can be
visualized to separately detect multiple probes. Any substrate known in the art may be used. Preferred substrates for alkaline phosphatase include 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT). The preferred substrate for horseradish peroxidase is diaminobenzoate (DAB).

In a further embodiment of the present invention, the target cells may be subjected to detection and analysis at the same time. For example, during the hybridization reaction of the probes to the target cell mRNA, probes useful in analyzing target cell DNA may be added to the cell mixture. While the probes for analyzing DNA may hybridize to DNA in non-target cells to yield a detectable signal, the relevant signal from the target cells will be accompanied by the signal indicative of differential gene expression, thus providing a basis for differentiating between target and non-target cells.

In another embodiment of the present invention, the concentration of nucleated fetal cells is enriched in a sample of maternal blood taken from a pregnant woman. In such method, the cells in the blood sample are contacted with an effective amount of a reagent which lyses at least a portion of the enucleated maternal and fetal cells in the sample, thereby enriching the concentration of the fetal nucleated cells. The reagent may be any compound which selectively lyses enucleated cells. Specific examples include detergents, and salts such as ammonium chloride. The optimal amount of the reagent to be used may be
determined by routine experimentation. However, when the reagent is an ammonium chloride solution, a solution in the range of about 100 to about 500 mM in water is preferred, with about 150 to about 300 mM being particularly preferred. Alternatively, the ammonium chloride may be dissolved in a buffer to stabilize the pH from about 7.0 to about 7.5. Suitable buffers include, but are not limited to, Tris and HEPES. The lysis step may optionally be preceded by a centrifugation step in which at least a portion of the enucleated red blood cells originally present in the sample is removed.

Following the lysis step, the cells may then be subjected to a detection step. One suitable detection step includes staining the different cell types remaining in the mixture different colors. For example, May-Grunwald Giemsa stain will stain lymphocytes violet while staining erythrocytes pink. Other suitable staining reagents may be determined without undue experimentation. For example, another suitable stain is Benzidine, which stains hemoglobin red. Benzidine may be used in conjunction with Hematoxylin, which stains nuclei blue.

In a further embodiment, fetal cells may be enriched by a “panning” technique which comprises separating target cells from undesired cells by means of at least two reagents. The first reagent preferentially binds to at least some undesired cells in a mixture, and the mixture is then contacted with with an immobilized second reagent, which preferentially binds to the first reagent, thereby
effecting a separation of unwanted cells from target cells by negative selection. Alternatively, a positive selection may be accomplished when the first reagent preferentially binds to the desired cells.

The panning technique may be used alone, or in conjunction with a density gradient centrifugation enrichment as described above, in which case the panning may either precede or follow the centrifugation. If it follows the centrifugation (which is preferred), the appropriate cell layer is isolated, which in the case of maternal blood from a pregnant female contains a mixture of fetal and maternal nucleated red and white cells and enucleated red cells. The mixture is then treated with a first reagent which preferentially binds to at least some cells other than nucleated red blood cells in the mixture, for example white cells. The mixture may then be exposed to an immobilized second reagent, which preferentially binds to the first reagent, thereby effecting a separation of unwanted white cells from target cells. The first and second reagents may be any reagents which exhibit preferential binding properties. In a preferred embodiment, the reagents are antibodies; most preferably the first reagent comprises one or more monoclonal antibodies, and the second reagent is a polyclonal antibody. In an alternative embodiment, the first reagent preferentially binds to at least some of the target nucleated red cells.

The second reagent may be immobilized on a solid
surface according to procedures well known in the art, including attachment to magnetic particles or adsorption onto a plastic surface, preferably polystyrene. Preferably, it is coated onto a plastic plate.

In a further embodiment, after enrichment but before staining, the cell mixture may be treated with an acid or alkali solution which will specifically elute adult hemoglobin (see Raper, J. of Med. Lab. Tech., 287-92 (1964)). The treating solution preferably contains about 200 mM acid or alkali. It is preferred that the acid solution be at a pH in the range of about 3.0 to about 4.0, and the alkali solution is preferably at a pH of at least 9.0. The acid solution preferably contains a buffer, preferably a citrate/phosphate buffer.

Another suitable detection step includes detection of a pattern of differential gene expression as discussed above.

After the cells are detected, they may be analyzed by any analysis method including in situ hybridization utilizing at least one nucleic acid probe which hybridizes to at least a portion of a chromosome in the detected cells. The cells may be prepared and the hybridization carried out using methods known in the art (for example, see "The ACT Cytogenetics Laboratory Manual", 2d Ed., Raven Press, 1991). The probes may be used to screen the cells for a variety of genetic abnormalities, including chromosomal aneuploidy, chromosomal deletions, and translocations. Particularly preferred is to analyze the
detected cells for trisomy 21, trisomy 13, trisomy 18 and numerical aberrations of the X and Y sex chromosomes. Any of the foregoing analyses may be done using probe kits presently commercially available (ONCOR, Inc., Gaithersburg, MD) following the manufacturer’s instructions.

Another aspect of the present invention resides in the discovery that erythroid cells may be fixed prior to in situ hybridization and that cell morphology and nucleic acid content are substantially maintained by contacting the cells with an effective amount of solution comprising formaldehyde and methanol. Preferably, the solution contains about 0.4% formaldehyde in methanol on a volume basis.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

**EXAMPLE 1**

**PREPARATION OF PROBES**

a) Preparation of Unlabeled Fragments

This example uses erythroid cells isolated from chick embryos. Those cells are a good human model because they are similar to the human fetal normoblast cells in that they retain their nuclei. They are also present in near homogeneity in chick embryo peripheral blood, and their globin gene expression patterns are well characterized.
Those features make them an ideal model system for the development of the hybridization techniques to be used in this method. The similarities between chick and human erythrocytes make it likely that methods developed with chicken cells will be applicable to human fetal erythrocytes.

Plasmid pHb1001 was constructed by insertion of chicken βA globin cDNA (full length) into the PstI site of the plasmid vector PBR322 (gift of Dr. G. Felsenfeld, National Institutes of Health), and was used as a template in a PCR reaction in which the following primers were used:

5′-GGCAAGGTCA ATGTGGCCGA A-3′ [SEQ ID NO:1]
5′-CACATGCAGC TTGTCACAAT G-3′ [SEQ ID NO:2]

The primers were synthesized by phosphoramidite chemistry on a Millipore Cyclone automated oligonucleotide synthesizer. The following reagents were added to a 100μl reaction vessel: plasmid template (10μl (10 ng/μl)); 10μl of 10 X PCR buffer (100 mM Tris pH 8.3, 500 mM KCl, 17mM MgCl₂); 10μl of dNTP mix (2mM ACGT); 10μl of each primer (20μM solution); 10μl of Thermus Aquaticus DNA polymerase (Perkin Elmer-Cetus; diluted 1:10 in PCR buffer) and sterile dH₂O to make 100μl. Initial denaturation was at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. This yielded a 294 base pair product. Fragment size was verified by agarose gel electrophoresis using a 10μl sample. Primers were removed using Centricon
30 spin columns with two 0.5 ml washes of sterile water.

Negative control probes were prepared in the same manner using the following PCR primers:

5′-CAATGGCACG TAAAGAACA TTTTGAGGC-3′ [SEQ ID NO:3]

5′-AAACTGCGG AATCGTCGT GGTATTCACT-3′ [SEQ ID NO:4]

The template was plasmid pUC18CAT, which was constructed by cloning the E. coli Chloramphenicol Acetyl Transferase (CAT) gene into the Pst 1 site of the pUC18 cloning vector multilinker (gift of Dr. J.E. Hesse, National Institutes of Health).

b) Preparation of Labeled Fragments

Labelled fragments were prepared using the unlabelled fragments produced in step a) above as a template. The preparation steps were the same as above except for the following changes in nucleotides:

- dNTP (ACG) 10μl (2 mM)
- dTTP 13.3 μl (1 mM)
- DIG-dUTP 6.6 μl (1 mM)

Incorporation of digoxygenin labelled nucleotide was verified by gel electrophoresis and detection of digoxygenin on a dot blot. Both the globin and CAT probes were equally incorporated. The dot blot was done after determination of DNA concentration by a fluorometric assay using Hoechst dye (Cesarone, et al., Analytical Biochemistry 100, pp. 188-197 (1979)).

-20-

SUBSTITUTE SHEET (RULE 26)
EXAMPLE 2

DETECTING CELLS BASED ON GENE EXPRESSION PATTERN

a) Cell Fixation

Fifty μL of a solution containing 7.5 X 10^7 cells per
ml of chicken erythrocytes was mixed with 50 μL of FCS
(Fetal Calf Serum). It is important to include the FCS or
other suitable protein in the smear, as leaving it out
could result in extensive loss of cells from the slide with
cell damage. Ten μL of this mix was placed in a line on
slide, and smeared with one pass using a glass Pasteur
pipette. The slide was allowed to air dry for
approximately 10 minutes.

The cell smear is next fixed in a solution of 0.4%
formaldehyde in methanol at 4°C for 10 minutes. Following
fixation, the slides may be transferred to -20°C methanol
for storage of up to 1 week.

b) Cell Pretreatment

Slides to be used were removed from storage and
allowed to air dry at room temperature for 10 minutes.

c) Hybridization

i) Denaturation of Slides and Probe

Slides were placed in 70% formamide/2XSSPE (Saline
Sodium Phosphate EDTA) at pH 7.0 for 2 minutes. They were
then dehydrated by passage through 70%, 80%, and 95%
ethanol at -20°C, 2 minutes each, then air dried. At this
point, the probes to be used (from Example 1) are denatured
for 5 minutes at 72°C.

ii) Quick Hybridization
The probes to be used are first added to a HYBRISOL® 1 brand hybridization solution (600 ng/30 µL, sold by Oncor, Inc., Gaithersburg, MD). The probe solution was then added to the slide containing the fixed cells (10 µL/half standard slide, 30 µL/whole slide). The probe mix on the slide was covered with a coverslip without sealing with cement. The mix was incubated for 30 minutes at 37°C in a humidified chamber. The coverslips were then floated off in 2XSSPE; it is important not to pull coverslips off, as this will damage cells. The slides were then washed for 5 minutes in 1XSSPE (pH 7.0) at 72°C, then soaked 2X1 minute in 1XPBD (from 10X stock of Phosphate Buffered Detergent sold by Oncor, Inc., Gaithersburg, MD).

**d) Development**

Fifty µL of anti-Digoxigenin antibody Fab fragment (Boehringer Mannheim, Indianapolis, IN, Catalog number 10207741) in ONCOR blocking reagent I protein blocking solution (Oncor, Inc., Gaithersburg, MD, Catalog number s1352-3) were added to the slide, and incubated for 5 minutes at 37°C in a humidified chamber. The slides were then rinsed three times for two minutes each in 1XPBD, followed by counterstaining with 12.5 µL of 0.025 µg/mL DAPI (1:4 dilution of stock solution sold by Oncor, Inc.). A coverslip was then placed on the slide, and the slide was viewed under a Zeiss Axioskop equipped with a cooled color CCD camera from Oncor Instrument Systems, Gaithersburg, MD.

The results are shown in Figures 1 and 2. Figure 1A
shows the hybridization pattern of the probe to chicken β adult globin mRNA in chicken definitive erythroid cells. Figure 1B shows the same cells in bright field. Figure 1C shows the hybridization pattern using the CAT mRNA probe as a negative control. Figure 1D shows the same cells in bright field.

The Figure 2 pictures establish that the signal is caused by hybridization to RNA. Figure 2A shows the hybridization pattern using the probe to β adult globin mRNA. In Figure 2B, similar cells were pretreated with RNase prior to hybridization. No signal is evident. Figures 2C and D are analogous to 2A and B except that the probe used was the negative control CAT probe.

Figures 1 and 2 thus demonstrate that it is possible to specifically detect β adult globin mRNA in cells treated as set forth in Example 2.

EXAMPLE 3
ENRICHMENT OF FETAL CELLS

a) Collection of Cord Blood

Blood from the umbilical cord is collected at birth into the anticoagulant ACD (acid-citrate-dextrose) and stored at room temperature. The blood is processed within 24 hours of collection.

b) Density Gradient Centrifugation

The anticoagulated cord blood is subjected to density gradient centrifugation using Histopaque 1077 density gradient medium (density of 1.077 g/ml; Sigma Chemical Co.,
St. Louis, MO) following the protocol provided by the manufacturer. All steps are performed at room temperature. A volume of cord blood (2 to 20 ml) is mixed with an equal volume of PBS (pH 7.2). The cell suspension is layered over an equal volume of Histopaque and centrifuged for 30 min at 700 X g in a swinging bucket rotor. During centrifugation, the enucleated red blood cells pellet to the bottom of the tube while the mononuclear cells, including the nucleated erythrocytes, migrate to the interface between the serum and the Histopaque. After centrifugation, the cells at the interface are collected and washed 2 times with PBS containing 0.1% fetal calf serum. After each wash, the cells are collected by centrifugation for 10 min at 300 X g.

c) Ammonium Chloride Lysis of Non-Nucleated Erythrocytes

One million cells from the Histopaque interface from step b) above are pelleted by centrifugation for 15 seconds at 2000 X g and resuspended in 1 ml of 250 mM ammonium chloride. The suspension is then incubated at room temperature for 15 minutes. During this time, the mature (enucleated) erythrocytes are lysed. The unlysed cells are collected by centrifugation (15 seconds at 2000 X g) and resuspended in PBS containing 0.1% fetal calf serum. The cells are then analyzed by May-Grunwald staining as described below.

d) May-Grunwald Giemsa Stain Protocol for Blood

The collected unlysed cells from step c) are diluted 1:2 with undiluted Fetal Calf Serum (FCS). Ten μl of that
dilution are placed in a line on a glass slide perpendicularly to the length of the slide, smeared with one pass using a glass Pasteur pipette, and air dried for approximately 10 minutes. The smear is then fixed in methanol for 5 minutes, rinsed four times in tap water, then dried by blowing heated air on the slide.

The slides are then placed in a Coplin jar containing May-Grunwald stain (Sigma Chemical Co.; prepared using non-polychromed (non-oxidized) methylene blue and eosin Y (prepared for Sigma)) for 5 minutes, and removed. Excess dye is drained off by tipping edge of the slide onto a paper towel. The slide is then placed directly into 50% May-Grunwald stain (all dye dilutions are into distilled H2O) in a Coplin jar for 2 minutes, with brief agitation at the start. The slide is then removed and excess dye drained off by tipping the edge of the slide onto a paper towel.

The slide is then placed directly in a 1:20 dilution of Giemsa stain (Sigma Chemical Co.) in a Coplin jar for 20 minutes. The slide is then removed and excess dye drained off by tipping the edge of the slide onto a paper towel. The slide is next placed directly into a Coplin jar containing methanol to wash off excess stain. The slide is then removed and excess dye drained off by tipping the edge of the slide onto a paper towel. The slide is then dried by blowing heated air over it, then mounted with a coverslip and examined under a Zeiss Axioskop equipped with a cooled color CCD camera from Oncor Instrument Systems,
Gaithersburg, MD. Normoblast cells were identified by cell staining and morphology, and the localization coordinates recorded. The slide was then removed from the microscope stage, subjected to fluorescent in situ hybridization (FISH) analysis, then replaced on the stage. The normoblasts were then selected using the previously recorded coordinates, and the fluorescence was detected.

Using the protocol described in example 3, a 5 ml sample of cord blood was processed and analyzed. Results are shown in Figures 3-8. All of the smears shown in Figures 3-8 have been stained with the May Grünwald-Giemsa stain described above.

Figure 3 shows the cord blood sample after the separation on Histopaque and before lysis with NH₄Cl.

Figure 4 shows the same sample after Histopaque and after lysis with NH₄Cl. Notice that the field shown in Figure 4 contains approximately equal numbers of enucleated erythrocytes and white blood cells. Also shown in this figure is a single fetal normoblast (enucleated erythrocyte). In whole blood prior to the density gradient centrifugation step, there are 1000 enucleated RBCs per white blood cell. After the centrifugation, the ratio of RBC to WBC is 1:1. All of the enucleated red cells remaining after the density gradient centrifugation have lysed and all that are left are the red cell “ghosts” (cell membrane devoid of most cytoplasm).

Figures 5 and 6 are the same cells shown in Figures 3 and 4 respectively, but Figures 5 and 6 were taken with a
100X objective. Figures 5 and 6 show that the morphology of the fetal normoblasts has been maintained throughout the purification.

Figures 7 and 8 are the results of adult blood that was subjected to the NH₄Cl lysis. The purpose of this experiment was to show that this lysis works not only on the cord blood, but also on the adult blood. As can be seen in Figure 7, almost all the cells in the smear of adult blood are enucleated red cells with a few lymphocytes. Figure 8 shows that after the lysis, the field shows the same number of lymphocytes, but all of the enucleated erythrocytes have been lysed.

Figure 9 is the result of a cord blood sample that was subjected first to the centrifugation through Histopaque 1077 followed by NH₄Cl lysis. As can be seen in Figure 9, the field shows one enucleated erythrocyte. Once again, these are nucleated fetal erythrocytes from cord blood. Figure 10 shows the same cell that was hybridized to alpha satellite probes to chromosomes 3 and chromosome 18 after the May Grünwald-Giemsa staining. As can be seen in Figures 9 and 10, these nucleated fetal erythrocytes can be hybridized with the probes to the chromosomes.

Cord blood contains approximately 10⁹ enucleated erythrocytes, and 10⁶ white blood cells per ml. The number of nucleated erythrocytes is variable and approximately 1:100,000. After density gradient centrifugation and lysis with ammonium chloride, there are no enucleated erythrocytes and the cell suspension is approximately 1%
nucleated erythrocytes. Therefore the number of nucleated erythrocytes increases from 1:100,000 to 1:100, which means the nucleated erythrocytes have been enriched 1000 fold. The yield of nucleated erythrocytes for the ammonium chloride lysis step is approximately 50% when compared to the yield of lymphocytes.

**EXAMPLE 4**

**RNA IN SITU HYBRIDIZATION WITH HUMAN GAMMA GLOBIN PROBE**

a) **Cell Preparation**

Histopaque 1083 (density of 1.083 g/ml; Sigma Chemical Co., St. Louis, MO) was warmed to room temperature. Human cord blood was added to an equal amount of Phosphate Buffered Saline (PBS, pH 7.2) and gently mixed. The mix was then placed on top of an equal amount of Histopaque solution and centrifuged for 30 minutes at 1400 rpm. The interface monolayer was removed. The cells were washed with 5 ml of PBS and the cell pellet was collected by centrifugation for 10 minutes at 1200 rpm. The wash step was repeated once.

b) **Cell Fixation**

The cell pellet was diluted with Hank's Balanced Salt Solution (HBSS) + 10% FCS to a concentration of 1.2 x 10⁶ cells/ml. Each Cytospin slide (Shandon Lipshaw Inc., Pittsburgh, PA) was pre-wetted by centrifuging at 500 rpm with 50 µl HBSS + 10% FCS for 5 minutes. Then 250µl of the cell suspension was applied and spun for 5 minutes at 500 rpm. The slides were air dried for 10 minutes. Then the
slides were fixed for 15 minutes in 100% methanol at room temperature and stored dry at 4°C.

c) Cell Pretreatment

Slides to be used were removed from storage and allowed to air dry at room temperature for 10 minutes.

d) Hybridization

PCR digoxigenin-labeled human gamma globin probes were generated essentially as in Example 1, except that the following primers were used:

5'-ATGGTCATT TCACAGAGG-3'  [SEQ ID NO:5]
5'-TTGTATTGC TGCCGAATAA AGCC-3'  [SEQ ID NO:6]

and the template is a plasmid containing the human gamma globin cDNA sequence. The probe (600 ng for each slide) was dried and resuspended in 20 µl of hybridization mix (50% formamide, 2X SSPE, 10% dextran sulfate, 0.5 µg/µl herring testes DNA) for 2 hours at 52°C. The probe was denatured 5 minutes at 72°C, and then cooled on ice for 10 minutes. The probe mix was then added to the slide containing fixed cells. The probe solution on the slide was then covered with a cover slip and sealed with rubber cement. The control slide was carried out in the same way except no gamma globin probe was added in the hybridization solution. The slides were then incubated overnight at 37°C in a humidified chamber. All slides were soaked in 1X SSPE to remove coverslips. Then the slides were washed in 50% formamide/2XSSPE (pH 7.0) at 37°C for 5 minutes, followed by 2X SSPE at 37°C for 5 minutes, and 1X SSPE at room temperature for 5 minutes.

-29-

SUBSTITUTE SHEET (RULE 26)
e) Development

The slides were detected with 50 μl of fluorescein conjugated anti-Digoxigenin antibody Fab fragment for 5 minutes at 37°C in a humidified chamber. Slides were rinsed three times for 2 minutes each in 1X PBD. Finally, the slides were counterstained with 18 μl of 1:2 dilution of DAPI stock. A coverslip was then placed on the slide. The slide was then viewed under a Zeiss Axioskop equipped with a cooled color CCD camera (from Oncor Instrument Systems, Gaithersburg, MD).

Figures 11-13 show the results of human cord blood hybridized with human gamma globin cDNA probes. In Figure 11, one fetal cell was hybridized specifically to the human gamma probe above the background level. Figure 12 shows the same image as Figure 11, except with the DAPI filter. All the nucleated cells were counterstained under that filter. The cell which hybridized specifically to the human gamma probe showed the presence of the nuclei. Under the Figure 13 bright field, the nucleus and cytoplasm of the cells are outlined. Only one of the many cells in the field was specifically hybridized to the human gamma globin probe. Those figures also show that the probe hybridized specifically to the cytoplasm, which is consistent with the location of the mRNA.
EXAMPLE 5

ENRICHMENT OF NUCLEATED RED BLOOD CELLS BY PANNING

1) Cells

Peripheral blood (PB) samples from three pregnant women at 15-16 weeks of gestation were pooled and subjected to density gradient centrifugation using Histopaque 1083. The maternal PB (diluted to 2-3 times with PBS) is layered over Histopaque and centrifuged for 30 minutes at 600 x g in a swinging bucket rotor. The mononuclear cells including nucleated red blood cells (nRBC's) together with some red blood cells (RBC's) are recovered at the interface while the majority of RBCs and neutrophils are pelleted at the bottom. The collected interface cells are washed twice with Hank's Balanced Salt Solution (HBSS) containing 0.1% FCS and are suspended in the Staining buffer at the cell concentration of 2 x 10^7 /ml.

2) Binding of the primary antibodies

a) Mouse monoclonal antibodies anti-CD45 and anti-CD32 (both antibodies are obtained from Caltag Laboratories, South San Francisco, CA) are added to the cell suspension at the concentration of 200 ng per 10^6 cells. After incubating at room temperature for 30 minutes the cells are washed once with staining buffer (HBSS, 2% BSA, 0.1% NaN_3), once with panning buffer (PBS, 0.5mM EDTA, 2% FCS, 0.1% NaN_3) and finally resuspended in the panning buffer at 5 x 10^6 /ml. Usually 1 ml of the cell suspension can be plated over 35 mm diameter plastic plate coated with goat anti-
mouse IgG antibody (Caltag).

3) Coating plastic plates

2 ml of Goat anti-mouse IgG antibody 20μg/ml in 100mM Tris-HCl, pH 9.0, is added to a plastic plate (35 mm diameter) and the plates are incubated at 37°C for 2 hours. The plates with antibody solution can be stored at 4°C for at least 2 weeks before usage. Following rinsing with staining buffer, 2 ml of staining buffer is added and incubated for 2 hours at 37°C. The plate is rinsed with panning buffer twice before addition of cell suspension.

4) Panning

The antibody treated cell suspensions are added to the secondary antibody-coated plate and are incubated at room temperature for 30 minutes, shaken gently, and incubated for another 30 minutes. The supernatant and the rinse of the plate are combined and the unattached cells population are recovered by centrifugation at 300 x g. Typically, 80-90% of mononucleated lymphocytes are attached to the plate while nRBC’s are recovered in the supernatant fraction, effecting a 5-10 fold enrichment of nRBC’s. This extent of enrichment allows one to apply samples obtained from 5 ml PB in the 6 mm diameter spot on a Cytospin slide.

EXAMPLE 6

IMMUNOAFFINITY CELL PARTITIONING

1) Isolation of nucleated red blood cells from maternal blood.

Peripheral blood from three pregnant women of 15-16
weeks of gestation were combined and subjected to a 
Histopaque 1083 density gradient as described in Example 
4a. The nucleated red blood cells in the interface were 
enriched by panning as described in Example 5. The cells 
unattached to the plates were isolated, and centrifuged to 
pellet the cells.

2) Modification of antibodies with TMPEG

Tresylated monomethoxy PEG (TMPEG) was made 
essentially as described by Delgado et al. (Biotechnol. 
Appl. Biochem. 12 119 (1990)). Briefly, 4.5 g of dried 
MPEG (Union Carbide) was mixed with 11.5 ml of 
dichloromethane, 285 μl of pyridine and 250 μl of tresyl 
chloride (Fluka) and reaction was continued for 1.5 hours 
on ice. After the dichloromethane was evaporated, the 
mixture was left overnight under vacuum. The dried TMPEG 
was redissolved in methanol/HCl (500:1) and TMPEG 
precipitate was formed by leaving the solution at 20°C for 
2 hours. The resulting white pellet was washed extensively 
with methanol/HCl (1000:1) at 4°C and finally the pyridine-
free TMPEG was recovered after drying overnight under 
vacuum.

Monoclonal antibodies anti-CD71 (Becton Dickinson) and 
anti-CD34 (Caltag) at a concentration of 50 μg/ml in PBS 
were mixed with 2 mg of TMPEG and incubated for 2 hours at 
room temperature. The excess of TMPEG was quenched by 
adding equal amount (w/w) of BSA.

-33-

SUBSTITUTE SHEET (RULE 26)
3) Partitioning of cells

a) Treatment of cells

The cell pellet isolated in step 1 were treated with MPEG-modified antibodies, anti-CD71 (transferin receptor) and anti-CD34.

b) PEG/Dextran sulfate 2 phase system

4.75% (w/w) PEG, 4.75% (w/w) 2-phase system in 0.15 M NaCl, and 10 mM sodium phosphate buffer pH 6.8 was prepared by mixing aqueous stocks solutions: 40% (w/w) PEG 8000 (Sigma), 20% (w/w) Dextran T-500 (Pharmacia), 1M NaCl and 0.4M sodium phosphate, pH 6.8. After mixing, the solution was left overnight at room temperature to allow two phases to be formed, and the top phase (PEG-rich phase) and bottom phase (dextran-rich) were stored separately.

c) Cell partitioning

The 2 phase system was reconstituted by mixing 0.5 ml of top phase and 0.5 ml of bottom phase after cell pellet from step 3a above (10^6-10^7 cells) was first suspended in the top phase. The suspension was mixed extensively by inversion and 2 phases were formed by leaving the mixture at room temperature for 20 minutes. Cells in top phase and bottom phase were recovered and cell partitioning to each phase were determined by Coulter Counter. The cells partitioned to upper PEG phase were collected and slides were prepared by Cytospin centrifugation.

d) Staining

The cells from the upper phase (3c above) were smeared by mixing with fetal calf serum (1:2) and smearing blood
down a microscope slide using a glass Pasteur pipet. The slides were air dried for 10 minutes and placed in a coplin jar of Benzidine stain (3,3'-dimethoxybenzidine; Fast Blue B (Sigma, D-9143) made to 1% solution in absolute methanol) for 2 minutes. The slides were removed, and excess liquid was allowed to run off. The slides were next placed in a coplin jar of 0.2% H₂O₂/70% ethanol for 1 minute, then removed, allowing excess liquid to run off. The slides were then placed in a coplin jar of distilled water for 1 minute, then removed, allowing excess liquid to run off. The slides were then placed in a coplin jar of Hematoxylin (Harris hematoxylin 7.5 g/L (Sigma, HHS-32) for 1-2 minutes, removed and placed under running tap water until excess dye was completely removed, then dried with a blower set at 400°C. The slide was then viewed under a Zeiss Axioskop equipped with a cooled color CCD camera (from Oncor Instrument Systems, Gaithersburg, MD). Figure 14 shows one nucleated red blood cell isolated by this technique.

While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.
SEQUENCE LISTING

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ANALYZING INDIVIDUAL RARE CELLS IN A POPULATION

(iii) NUMBER OF SEQUENCES: 6

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(A) MEDIUM TYPE: DISKETTE-3.50 INCH, 1.44 Mb
STORAGE
(B) COMPUTER: APPLE MACINTOSH POWERBOOK 165
(C) OPERATING SYSTEM: MACINTOSH
(D) SOFTWARE: WORD PERFECT 2.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US94/10971
(B) FILING DATE: 27-SEP-1994
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/127,170
(B) FILING DATE: 27-SEP-1993

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(2) INFORMATION FOR SEQ ID NO. 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

-36-
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
GGCAAGGTTCA ATGTGCCGA A

(2) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
CACATGCAGC TTGTCAACAT G

(2) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
CAATGCATC GTAAGAACA TTTTGAGGC

(2) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
AAAATGCCG AAATCTCGT GTATTCACCT

(2) INFORMATION FOR SEQ ID NO. 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
ATGGTCATTC TCACTAGAGG

(2) INFORMATION FOR SEQ ID NO. 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
TTGTATGGCT TGCAGAATAA AGCC
WHAT IS CLAIMED IS:

1. A method for detecting a specific type of target cell contained in a mixture of cell types, said method comprising detecting the desired cell based on its pattern of differential gene expression, said pattern being substantially absent from the other cell type(s) in the mixture.

2. The method of claim 1, wherein the target cell is detected based on an RNA product.

3. The method of claim 1, wherein the target cell is detected based on a protein product.

4. The method of claim 1, wherein the target cell is detected based on a metabolic product.

5. The method of claim 1, wherein the target cell is detected based on a structural component.

6. The method of claim 1, wherein the cell is detected based on a feature of gene expression present in a different amount than in the non-target cells.

7. The method of claim 1, wherein the target cell is detected based on a different staining character from the other cell type(s).

8. The method of claim 7, wherein the target cell is a fetal nucleated erythrocyte and the other cell type is a maternal nucleated erythrocyte.

9. The method of claim 8, wherein the fetal nucleated erythrocyte is detected by May-Grunwald stain.

10. The method of claim 2, wherein the pattern is

-39-

SUBSTITUTE SHEET (RULE 26)
detected by in situ hybridization with at least one nucleic acid probe.

11. The method of claim 10, wherein the probe hybridizes to RNA in the target cell.

12. A method for fixing erythroid cells prior to in situ hybridization such that cell morphology and nucleic acid content are substantially maintained which comprises contacting the cell with an effective amount of a solution comprising methanol and formaldehyde.

13. The method of claim 12 wherein the solution contains about 0.4% formaldehyde in methanol on a volume basis.

14. A method for enriching the concentration of nucleated fetal cells in a sample of maternal blood taken from a pregnant woman, said sample comprising maternal red blood cells, maternal white blood cells, fetal white blood cells and fetal nucleated and enucleated red blood cells, said method comprising contacting the cells within the sample with an effective amount of a reagent which lyses at least a portion of the enucleated cells, thereby enriching the concentration of the nucleated fetal cells.

15. The method of claim 14, wherein the reagent is selected from the group consisting of a detergent, a salt, and mixtures thereof.

16. The method of claim 15, wherein the reagent is a salt.

17. The method of claim 16, wherein the salt comprises a mixture of ammonium and chloride ions.
18. The method of claim 14, further comprising subjecting the blood sample, prior to contacting with the reagent, to centrifugation such that at least some of the enucleated cells are separated from the mixture.

19. A method for analyzing fetal cells which comprises:
   a) obtaining a sample of blood from a pregnant woman, said sample comprising maternal red blood cells, maternal white blood cells, fetal white blood cells and fetal nucleated and enucleated red blood cells;
   b) enriching the concentration of nucleated fetal cells in said sample by contacting the cells within the sample with an effective amount of a reagent which lyses at least a portion of the enucleated cells, thereby enriching the concentration of the nucleated fetal cells;
   c) detecting the desired cell based on its pattern of differential gene expression, said pattern being substantially absent from the other cell type(s) remaining in the mixture; and
   d) analyzing said detected cells.

20. The method of claim 19, further comprising subjecting the sample from step a) to a centrifugation step prior to step b) to thereby remove at least a portion of the enucleated cells originally present.

21. The method of claim 19, wherein the detection step detects an RNA product in the desired cell.

22. The method of claim 19, wherein the detection step detects a protein product.
23. The method of claim 19, wherein the detection step detects a metabolic product.

24. The method of claim 19, wherein the detection step detects a structural component.

25. The method of claim 19, wherein the detection step detects a different staining character from the other cell type(s).

26. The method of claim 25, wherein a stain selected from the group consisting of May-Grunwald-Giemsa, Benzidine and Hematoxylin is used in the detection step.

27. The method of claim 25, further comprising treating the enriched cells with an acid or alkali solution to elute adult hemoglobin prior to staining.

28. The method of claim 27, wherein an acid solution is used which has a pH of about 3.0 to about 4.0.

29. The method of claim 27, wherein an alkali solution is used which has a pH of at least about 9.0.

30. The method of claim 21, wherein the RNA product is mRNA encoding γ-globin and is detected with a detectable nucleic acid probe which hybridizes to the mRNA.

31. The method of claim 19, wherein the detected cells are analyzed by at least one nucleic acid probe which hybridizes to at least a portion of a chromosome in the detected cell.

32. The method of claim 19, wherein steps c) and d) are performed at the same time.

33. The method of claim 32, wherein the detection
step detects a RNA product with a nucleic acid probe which
hybridizes to said RNA product, and wherein the analysis
step is performed with at least one nucleic acid probe
which hybridizes to at least a portion of a chromosome in
the detected cell.

34. A method for enriching the concentration of
nucleated fetal cells in a sample of maternal blood taken
from a pregnant woman, said sample comprising maternal red
blood cells, maternal white blood cells, fetal white blood
cells and fetal nucleated and enucleated red blood cells,
said method comprising:

a) treating the mixture with a first reagent which
preferentially binds to the one of either the fetal
nucleated cells or any other cell type in the mixture; and

b) exposing the treated mixture to an immobilized
second reagent, which second reagent preferentially binds
to the first reagent, thereby effecting a separation of
fetal nucleated cells from other cell types.

35. The method of claim 34, wherein at least one of
the first and second reagents is an antibody.

36. The method of claim 35, wherein the first reagent
preferentially binds to at least some fetal nucleated red
blood cells.

37. The method of claim 35, wherein the first reagent
preferentially binds to at least some cells other than
fetal nucleated red blood cells.

38. The method of claim 34, further comprising
subjecting the maternal blood sample to a density
centrifugation step which enriches the concentration of fetal nucleated red blood cells in the mixture.

39. The method of claim 38, wherein the centrifugation is performed prior to treatment with the first and second reagents.

40. The method of claim 38, wherein the centrifugation is performed subsequent to step b).

41. The method of claim 34, further comprising after step b), detecting the desired cell based on its pattern of differential gene expression, said pattern being substantially absent from the other cell type(s) remaining in the mixture; and analyzing said detected cell.
Cord Blood 9/21/93-Male
1877 Mononuclear Layer
Pre-Lysis
40 X

Fetal Enucleated Red Blood Cells

Fetal Lymphocytes

Fetal Normoblast

Figure 3
Figure 4

Cord Blood 9/21/93-Male
1077 Mononuclear Layer
Post-Lysis
40 X
Cord Blood 9/21/93-Male
1077 Mononuclear Layer
Pre-Lysis
100 X

Fetal Lymphocytes
Fetal Normoblast
Fetal Enucleated Red Blood Cell
Fetal Lymphocyte

Figure 5
Figure 6

Cord Blood 9/21/93-Male
1077 Mononuclear Layer
Post-Lysis
100X

Enucleated Fetal Red Blood Cell Ghost

Fetal Normoblasts

Fetal Lymphocytes

Fetal Normoblast
Figure 7
Whole Adult Blood S/10/53-Male
Unseparated
Post-Lysis
40 X

Adult Lymphocytes

Adult Red Blood Cell Ghosts

Adult Neutrophil

Figure 8
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12Q 1/00, 1/68
US CL : 435/4, 6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/2, 4, 6, 91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS, BIOSIS, MEDLINE, APS
search terms: nucleated fetal cells, maternal blood, fetal globulin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US, A, 5,153,117 (SIMONS) 06 October 1992, see entire document.</td>
<td>1-41</td>
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<td>Y</td>
<td>Proceedings of the National Academy of Sciences USA, Volume 87, issued May 1990, D. W. Bianchi et al., &quot;Isolation of fetal DNA from nucleated erythrocytes in maternal blood&quot;, pages 3279-3283, see entire document.</td>
<td>1-41</td>
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<tr>
<td>Y</td>
<td>Blood, Volume 75, No. 11, issued 01 June 1990, C. Camaschella et al., &quot;Prenatal diagnosis of fetal hemoglobin Lepore-Boston disease on maternal peripheral blood&quot;, pages 2102-2106, see entire document.</td>
<td>1-41</td>
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</tbody>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  *A* document defining the general state of the art which is not considered to be of particular relevance
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  *P* document published prior to the international filing date but later than the priority date claimed

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<th>Date of the actual completion of the international search</th>
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<td>09 JAN 1995</td>
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<tr>
<td>Y</td>
<td>Experimental Hematology, Volume 20, issued 1992, N. Daylot et al., &quot;Adult and neonatal patterns of human globulin gene expression are recapitulated in liquid cultures&quot;, pages 1141-1145, see entire document.</td>
<td>1-41</td>
</tr>
<tr>
<td>Y,P</td>
<td>US, A, 5,275,933 (TENG ET AL.) 04 January 1994, see entire document.</td>
<td>1-41</td>
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