

# Isolation of fetal DNA from nucleated erythrocytes in maternal blood

(prenatal diagnosis/flow cytometry/Y chromosome/fetal blood)

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**ABSTRACT** Fetal nucleated cells within maternal blood represent a potential source of fetal genes obtainable by venipuncture. We used monoclonal antibody against the transferrin receptor (TfR) to identify nucleated erythrocytes in the peripheral blood of pregnant women. Candidate fetal cells from 19 pregnancies were isolated by flow sorting at 12½–17 weeks gestation. The DNA in these cells was amplified for a 222-base-pair (bp) sequence present on the short arm of the Y chromosome as proof that the cells were derived from the fetus. The amplified DNA was compared with standardized DNA concentrations; 0.1–1 ng of fetal DNA was obtained in the 20-ml maternal samples. In 7/19 cases, a 222-bp band of amplified DNA was detected, consistent with the presence of male DNA in the isolated cells; 6/7 of these were confirmed as male pregnancies by karyotyping amniocytes. In the case of the female fetus, DNA prepared from samples at 32 weeks of gestation and cord blood at delivery also showed the presence of the Y chromosomal sequence, suggesting Y sequence mosaicism or translocation. In 10/12 cases where the 222-bp band was absent, the fetuses were female. Thus, we were successful in detecting the Y chromosomal sequence in 75% of the male-bearing pregnancies, demonstrating that it is possible to isolate fetal gene sequences from cells in maternal blood. Further refinement in methodology should increase sensitivity and facilitate noninvasive screening for fetal gene mutations.

Various fetal cell types—trophoblasts, erythrocytes, and leukocytes—cross the placenta and circulate within maternal blood (1). Although these cells are rare, they have generated interest as a potential source of fetal genes for prenatal diagnosis.

Fetal trophoblasts in maternal blood were initially detected by microscopic studies (2). Flow cytometric analyses of cells binding a monoclonal antibody against trophoblast antigen, H315, were subsequently used to identify circulating trophoblast cells (3). Lymphocytes can adsorb this antigen; thus, this particular antibody is not specific for cells of fetal origin (4, 5). Lymphocytes with an XY karyotype have been demonstrated in peripheral blood from pregnant women carrying males (6). The methods used were effort-intensive, requiring visual screening of hundreds of maternal cells to detect a rare fetal cell.

In our previous study, we utilized the presence of fetal cell surface antigens and cytogenetic markers as independent means of identifying and enriching fetal lymphocytes flow-sorted from maternal blood (7, 8). The antenatal detection of HLA-A2-positive male fetuses was highly significant ( $P = 5.8 \times 10^{-6}$  by Fisher exact test) but clinical applications were limited by the scarcity of the fetal lymphocytes in maternal

blood and the resistance of these cells to undergo mitosis once isolated.

Fetal nucleated erythrocytes (NRBCs) were selected for sorting in the experiments described here because they are a cell type unlikely to be present in the peripheral blood of a normal adult. They are, however, abundant in fetal blood early in gestation (9, 10). In a given fetomaternal hemorrhage, the ratio of red cells to white cells should remain the same as in whole fetal blood, about 1000:1. As they are nucleated, the NRBCs contain a full complement of fetal genes.

Erythroblasts have been shown to express the transferrin receptor (TfR) (11) antigen on their cell surfaces from the burst-forming units, erythroid, stage up until and including nuclear extrusion (reticulocytes) (12). TfR is also present on activated lymphocytes (13), certain tumor cells (14), and trophoblast cells (15). Commercially available fluorescein-conjugated monoclonal antibodies against TfR were used in this study to enrich the proportion of NRBCs present in flow sorted samples.

The polymerase chain reaction (PCR) technique (16), with its capacity for DNA diagnosis from a single cell (17, 18), facilitates sensitive and specific detection of target gene sequences. We amplified Y chromosome specific sequences in sorted cells to prove that these cells originated in the fetus. The results of our experiments indicate that it is now possible to isolate candidate fetal cells from maternal blood, extract genomic DNA, and amplify uniquely fetal genes using PCR.

## MATERIALS AND METHODS

**TfR Analysis.** In the initial group of experiments, blood was collected in EDTA from six pregnant hospital employees at 10–33 weeks of gestation, six nonpregnant adults, and three newborn umbilical cords for TfR antigen analysis and microscopy of sorted cells. Umbilical cord blood was selected as a control because it contains large numbers of NRBCs. The blood was diluted 1:1 with Hanks' balanced salt solution (HBSS), layered over a Ficoll/Hypaque column (Pharmacia), and spun at 1400 rpm for 40 min at room temperature. The mononuclear cell layer was then isolated, washed twice with HBSS, and stained with either a 1:10 dilution of fluorescein-conjugated anti-TfR (Becton Dickinson catalog no. 7513) or a 1:20 dilution of phycoerythrin-conjugated anti-TfR (gift of Michael Loken, Becton Dickinson) in phosphate-buffered saline (PBS: 0.137 M NaCl/0.002 M KCl/0.008 M Na<sub>2</sub>HPO<sub>4</sub>/0.0015 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), 2% fetal calf serum, and 0.1% sodium azide on ice for 30 min. The cells were washed once in PBS prior to flow cytometry.

Abbreviations: PCR, polymerase chain reaction; NRBC, nucleated erythrocyte; TfR, transferrin receptor.

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Analysis and sorting were performed on a Becton Dickinson FACS-IV (19) with a Consort 40 program as described (20). The gain was standardized manually for each experiment by using identical laser power and fluorescent beads. TfR fluorescent (positive) and nonfluorescent (negative) cells were determined by physical separation on a logarithmic scale, sorted into tubes, and cytocentrifuged onto microscopic slides. Each sample was stained with Wright/Giemsa for morphology and the Kleihauer/Betke acid elution technique (21) to detect the presence of fetal hemoglobin. Microscopy was performed on a Leitz Orthoplan photomicroscope. Color prints were taken using Kodacolor VRG film. Between experiments, the sorter was sterilized by running a 10% household bleach solution in sterile distilled water through the tubing overnight.

**PCR Standardization.** To determine the minimum amount of male DNA detectable on autoradiographs, a series of standardization experiments were performed. DNA was prepared from five female and four male laboratory volunteers using conventional methods. For each individual, a series of six tubes containing 10-fold dilutions (from 1  $\mu$ g to 1 pg) of DNA and one tube containing no DNA were prepared for PCR.

PCR was performed using the standard reagents in the Gene Amp kit (Perkin-Elmer/Cetus catalog no. N801-0055) on a Perkin-Elmer DNA thermal cycler. Primers 411-01 (5'-TTT-AAT-GCC-AGC-ACT-AAT-TTA-CA) and 411-03 (5'-CCA-GAG-ACA-CAC-TAA-AAT-TCC-TC) define a 222-base-pair (bp) target sequence on the short arm of the Y chromosome. Y411 (U. Müller, personal communication) is identical to Y156 (22), a probe that detects a 10-fold repeat sequence that appears to be male specific on Southern blots. PCR was performed using the following modification of published methods (23): the night before amplification, reagents were incubated at 37°C with *Eco*RI, an endonuclease that has a restriction site within the target sequence. The enzyme was inactivated by heating to 95°C for 30 min. This has been shown to decrease problems with false positive amplification of contaminating DNA (18, 24, 25). All reactions were performed under sterile conditions wearing gloves and using positive displacement pipettes. We continuously monitored for contamination with male DNA by running nonpregnant female controls and reagent controls (containing no added DNA) with each reaction. During the course of these experiments one group of patient samples showed evidence of contamination in control lanes; those data are not included in this paper.

Each amplification cycle consisted of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C, for 18–24 cycles, with a 10-min extension in the last cycle. After amplification, the samples were ethanol precipitated, resuspended in 10  $\mu$ l of Tris-EDTA, and loaded into a 20-ml 2% agarose mini gel containing ethidium bromide (model Horizon 58, Bethesda Research Laboratories). Electrophoresis was at 50 mV for 90 min. The gels were inspected under ultraviolet light, photographed, denatured in 0.5 M NaOH/0.5 M NaCl for 5 min, and neutralized in three 5-min changes of 0.4 M Tris-HCl/0.08 M Tris base/0.6 M NaCl. The DNA was transferred to Hybond-N in 20 $\times$  SSC (0.10 M NaCl/0.01 M sodium citrate, pH 7) over 4 hr. Filters were baked in a vacuum oven at 80°C for 20 min. Each filter was placed in a buffer containing 5% dextran sulfate, 6 $\times$  SSC, 5 $\times$  Denhardt's solution (26), 5% SDS, 50% formamide, 20  $\mu$ g of sheared salmon sperm DNA per ml for a minimum of 4 hr at 42°C. The Y411 probe was oligonucleotide labeled with <sup>32</sup>P (27). Filters were incubated with 5  $\times$  10<sup>5</sup> counts per ml of labeled probe overnight, washed in seven changes of 0.2 $\times$  SSC/0.4% SDS at room temperature, and washed in three changes of the same solution at 55°C. After drying, filters were exposed to Kodak X-Omat AR film at

–70°C with Cronex (DuPont) intensifying screens. Radiographs were developed 24–72 hr after exposure.

**Detection of Male Fetal DNA in Pregnant Women.** Pregnant women between 12 and 17 weeks of gestation presenting to the Antenatal Diagnostic Center of Brigham and Women's Hospital were recruited for this study. All studies were conducted with human subjects' approval from committees at the Brigham and Women's Hospital and at the Children's Hospital. All of the women had a subsequent amniocentesis for either advanced maternal age or anxiety. No woman with a known elevation of maternal serum  $\alpha$ -fetoprotein was recruited because of the association with fetomaternal hemorrhage. One woman in the study was diagnosed as having twins (one male, one female) after her blood was drawn. Importantly, all blood samples were drawn prior to amniocentesis. One woman had an additional sample drawn at 32 weeks; cord blood from her infant was obtained at delivery. Twenty milliliters of venous blood was collected in EDTA and processed as described above in the TfR analysis.

Initially, 100,000 cells from each individual were analyzed for the percentage of TfR positive and negative cells. The remainder of the blood sample was sorted; TfR positive cells were counted and collected in a sterile 1.5-ml micro test tube and frozen at –20°C.

Prior to PCR, the cells were thawed at 37°C and spun for 10 min in an ultracentrifuge. The supernatant was removed, and the cell pellet was resuspended in 70  $\mu$ l of sterile double-distilled water. The mixture was boiled for 10 min and briefly cooled. The remainder of the reaction components were added as described above under PCR standardization. The presence of the 222-bp band was considered significant only if the female and reagent control lanes were blank on autoradiographs. Autoradiographs were analyzed blindly for the presence of the 222-bp target DNA sequence before results of amniocentesis became available.

## RESULTS

**TfR Analysis.** Analysis of histograms depicting fluorescence intensity versus light scatter revealed differences between the samples obtained from nonpregnant adults, pregnant women, and umbilical cord blood (Fig. 1). Though the six samples from the nonpregnant adults contained a small percentage of fluorescent (TfR<sup>+</sup>) cells (0.2–0.4; mean = 0.32), these cells were heterogeneous in size. On contour plot analysis, they did not coalesce into a discrete population (Fig. 1a). In contrast, the three cord samples contained large percentages of TfR<sup>+</sup> cells (23.3–65.2; mean = 39). Contour plot analysis revealed a large population of cells with intermediate light scattering properties (Fig. 1c). The six samples from the pregnant women contained more TfR<sup>+</sup> cells than the samples from the nonpregnant adults (0.3–1.8; mean = 0.83); contour plots revealed the consistent presence of a small but defined cell population (Fig. 1b).

**Microscopy.** The TfR<sup>+</sup> cells in the samples analyzed above were sorted onto microscope slides for morphologic identification and detection of fetal hemoglobin. Again, differences were seen between the three groups of samples. In the cord blood, large numbers of reticulocytes and NRBCs containing fetal hemoglobin were identified visually. In contrast, the samples from the nonpregnant adults consisted of lymphocytes, monocytes, and platelets (Fig. 2c). None of the isolated cells in this group contained fetal hemoglobin. In the samples from pregnant women, however, the sorted cells were overwhelmingly recently enucleated erythrocytes (reticulocytes). The origin of these cells cannot be determined. NRBCs containing fetal hemoglobin were occasionally identified (Fig. 2b). Monocytes, lymphocytes, erythrocytes containing adult hemoglobin, and platelets were only infrequently observed. Because trophoblast cells express the TfR

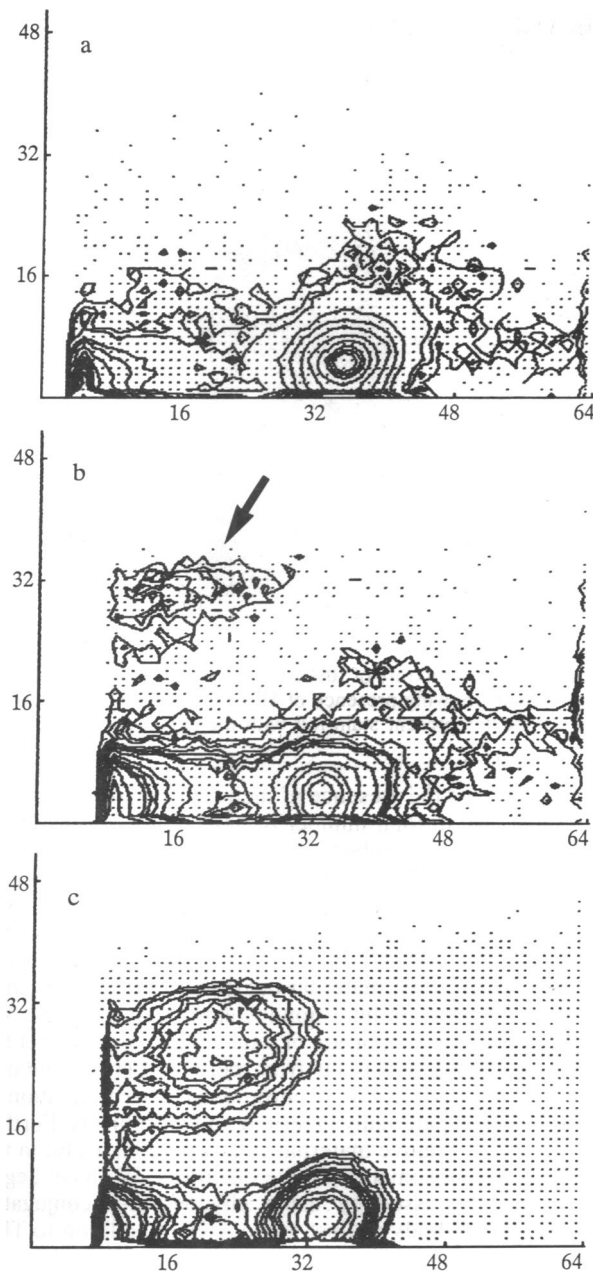


FIG. 1. Two-dimensional contour plot depicting forward angle light scatter (as an indication of cell volume) on the x axis versus fluorescence intensity on the y axis. The mononuclear cells have been stained with fluorescein-conjugated anti-TfR. (a) A nonpregnant adult. (b) A pregnant female at 16 weeks of gestation. (c) An umbilical cord sample. The low-fluorescence, low-scatter signals (from platelets) have been "gated" out of the analysis. The brightly fluorescent medium-sized population of cells including NRBCs can be clearly seen in c. The arrow in b refers to the TfR<sup>+</sup> population sorted in the pregnant women. The specific sorting gates used were vertical 21–35 and horizontal 7–60.

antigen, it was postulated that they might be present in the sorted population; none was detected.

**PCR Standardization.** Each of the four control males reproducibly amplified the 222-bp Y chromosomal sequence. Inspection of the ethidium bromide-stained gels under ultraviolet light revealed a visible band at 222 bp in the 100-ng and 10-ng lanes only. On autoradiographs, the sensitivity increased so that bands could be detected in the 1-ng and 100-pg lanes (Fig. 3). Hybridization to the Y probe was not observed in amplified samples from the five control females.

**Detection of Fetal DNA in Maternal Blood.** In the samples from the 19 pregnant women studied prospectively for detection of male fetal DNA, between 46,000 and 673,000 TfR<sup>+</sup> cells were sorted into micro test tubes. Upon visualization of the amplified DNA in agarose gels under ultraviolet light, bands at 222 bp were always present in the control male lane. They were never present at the gel level in samples from the control female, reagent control, or sorted cells from the pregnant women. After Southern transfer and hybridization, however, the 222-bp band became detectable in 7/19 pregnant women (Fig. 4). We interpreted this as presumptive evidence of male DNA and predictive of a male fetus; 6/7 women were diagnosed by karyotyping of cells obtained at amniocentesis as carrying males except for the woman carrying a male and a female. In one case, the band was present but the fetus was female. A repeat maternal sample obtained at 32 weeks of gestation and the infant's cord blood at delivery both revealed the presence of the 222-bp band. Further studies of this infant included karyotype of amniocytes and leukocytes from cord blood (46,XX), Southern blot analysis of unamplified *Hind*III-digested cord blood DNA probed with Y411, *in situ* hybridization using biotin-labeled Y411 (28), and PCR amplification of the 32-week maternal sample and cord blood using primers derived from probe p49a (29), which maps to the Y chromosome long arm. All were negative for the presence of the Y411 sequence or material from the Y chromosome. In 12/19 pregnant women, the 222-bp band was absent on autoradiographs; 10 of these women had female fetuses and the other 2 had males. The probability of obtaining these results by chance ( $P$ ) = 0.0063 by Fisher exact test.

To determine the quantity of fetal DNA being detected in the 20-ml maternal blood sample, the patient autoradiographs (Fig. 4) were compared with standardized reactions containing known concentrations of male DNA (Fig. 3). At 15–16 weeks of gestation, the amount of fetal DNA present was consistently 0.1–1 ng. By chance, the pregnancies studied at 12½–14 weeks of gestation were all female; thus, we were not able to observe male fetal DNA earlier than 15 weeks.

## DISCUSSION

We have shown that nucleated erythrocytes can be isolated from the peripheral blood of pregnant women after flow sorting. Such cells are obtained in a noninvasive manner and can be used to study fetal DNA.

We utilized the presence of fetal hemoglobin and the detection of amplified Y chromosomal sequences in male pregnancies as proof that the sorted nucleated erythrocytes are fetal in origin. We were able to prospectively identify male DNA in 75% (6/8) of male pregnancies. In the two pregnancies where we did not detect male DNA, there are several possibilities. There may have been fetomaternal blood group incompatibility contributing to the removal of fetal cells from the maternal circulation. Alternatively, the number of NRBCs present may have been below our limit of sensitivity to detect fetal DNA.

With the set of conditions described in these experiments, we could detect as little as 100 pg of fetal DNA or the equivalent of 15 fetal cells. By extending the number of cycles in the PCR, we can detect fewer cells (unpublished results). In the one case where the amplified DNA from three different experiments did not agree with the infant's karyotype, there may have been a low level of sex chromosome mosaicism, XX/XY chimerism (30), or the presence of the Y411 sequence on another chromosome. The possibility of cross-contamination with male DNA was small, as the three samples were amplified on three different occasions several weeks apart (see *Materials and Methods*).



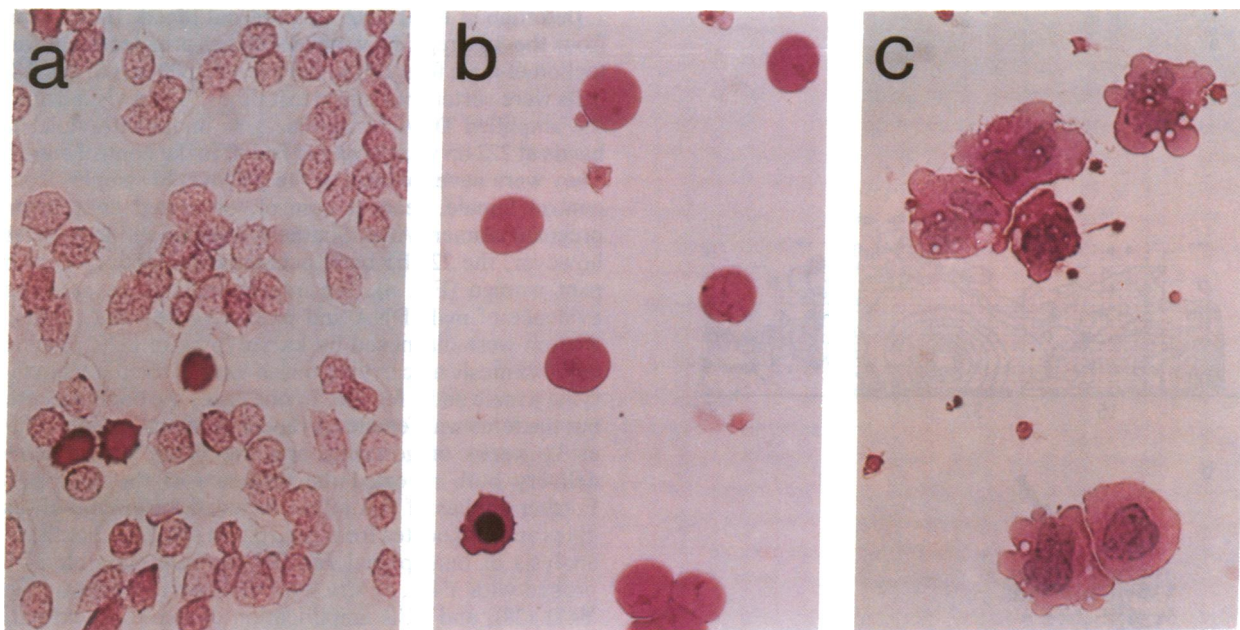


FIG. 2. Photomicrographs of cells stained by the Kleihauer/Betke technique. (a) Artificial mixture of 10% cord and 90% adult blood showing dark stain of cord cells containing HbF. (b) Tfr<sup>+</sup> cells sorted from a pregnant woman showing presence of HbF in nucleated and recently enucleated erythrocytes. (c) Tfr<sup>+</sup> cells sorted from a nonpregnant adult. The monocytes present stain red with the erythrosin used in this technique. ( $\times 640$ ).

The fetal cell sorting experiments described here provide additional information on the extent of fetomaternal hemorrhage in otherwise normal pregnancies. Medearis and colleagues (31) have shown between 28 and 564  $\mu$ l of fetal blood can be detected in the maternal circulation immediately postpartum, but we have seen no studies quantifying the fetal blood in maternal blood antenatally. We detected 1 ng of fetal DNA, which corresponds to 150 nucleated fetal cells. Using published values on the number of NRBCs in fetal blood at 16 weeks ( $3.6 \times 10^9$  per liter) (10, 32), we can calculate, by simple ratio, that there are 0.04  $\mu$ l of fetal blood in the 20-ml maternal sample. Thus, there are 10  $\mu$ l of fetal blood in the entire maternal circulation. This is a trivial amount when compared with the fetoplacental blood volume at 16 weeks of gestational age, about 20 ml.

Morphologic differences exist between Tfr<sup>+</sup> cells in mononuclear cell suspensions isolated from pregnant women, umbilical cord blood, and nonpregnant adults. We do not know if the increased percentage of Tfr<sup>+</sup> cells observed in the pregnant (as compared with nonpregnant) samples is due to the presence of fetal cells in maternal blood or simply an increased number of maternal cells expressing Tfr. Maternal synthesis of HbF in pregnancy has been previously docu-

mented (33), and the presence of such cells could explain part of the increase in total number of Tfr<sup>+</sup> cells. Large differences between the numbers of Tfr<sup>+</sup> cells sorted from pregnant women (46,000–673,000) and the average number of fetal nucleated cells identified by PCR and Southern blot (150) were seen. There are several possible reasons for this difference. The sorted Tfr<sup>+</sup> cells could be maternal nonnucleated erythrocytes, maternal nucleated erythrocytes, maternal leukocytes, or fetal nonnucleated erythrocytes. We have enriched the proportion of fetal NRBCs present but the sorted cell population is far from pure. Maternal contaminants do not interfere with Y chromosomal determination or the detection of paternally inherited polymorphisms. Further purification to remove maternal cells would be useful in the diagnosis of autosomal recessive disorders. We have begun to address this issue by using phycoerythrin-conjugated monoclonal antibody against leukocytes in addition to Tfr.

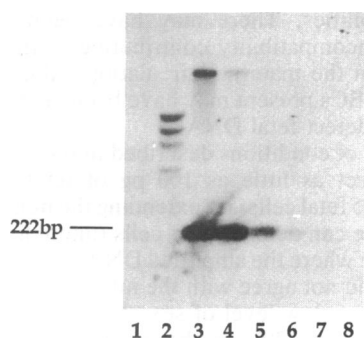


FIG. 3. Autoradiograph of diluted male DNA amplified for 222-bp sequence. Lane 1, reagent control; lane 2,  $\phi$ X174 molecular weight standard; lane 3, 100 ng; lane 4, 10 ng; lane 5, 1 ng; lane 6, 100 pg; lane 7, 10 pg; lane 8, 1 pg.

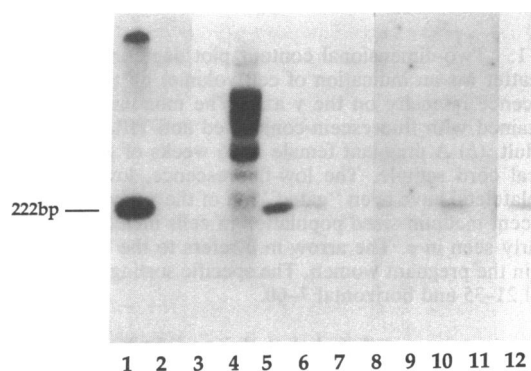


FIG. 4. Composite autoradiograph of amplified patient DNA. Lane 1, 10 ng, normal male; lane 2, 10 ng, normal female; lane 3, reagent control; lane 4,  $\phi$ X174; lane 5, sorted cells from patient 1 (male fetus); lane 6, sorted cells from patient 2 (male fetus); lane 7, sorted cells from patient 3 (female fetus); lane 8, sorted cells from patient 6 (female fetus); lane 9, sorted cells from patient 7 (male fetus); lane 10, sorted cells from patient 8 (male fetus); lane 11, sorted cells from patient 9 (female fetus); lane 12, cord blood from female infant whose cells were prenatally sorted in lane 8.

We have had success in preliminary experiments identifying and eliminating leukocytes from the Tfr<sup>+</sup> population by dual-color fluorescence-activated cell sorting. The degree of fetal cell enrichment already demonstrated would facilitate rapid screening for trisomy 21 in interphase nuclei by *in situ* hybridization with chromosome 21 probes (34).

The results of this paper confirm our prior findings that fetal cells can be sorted from maternal blood. While our original study used interphase Y chromatin to identify male cells, we can now show by PCR that specific Y chromosomal target gene sequences can be isolated and amplified from fetal cells.

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