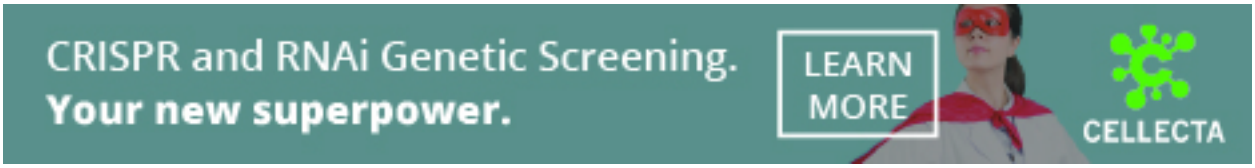
Quantitative analysis of CD34+ stem cells using RT-PCR on whole cells.

D A Molesh and J M Hall

Genome Res. 1994 3: 278-284



Quantitative Analysis of CD34$^+$ Stem Cells Using RT–PCR on Whole Cells

David A. Molesh and Jeff M. Hall

CellPro, Inc., 22322 20th Avenue SE, Suite 100, Bothell, Washington 98021

We have employed RT–PCR of whole cells to develop a quantitative method for estimating the number of rare cells expressing a unique mRNA in a large, mixed population of cells. We have demonstrated that RT–PCR can be done on whole cells without the need for extraction of the RNA. This allows for a great saving of time and effort, as well as allowing quantitative analysis to be based on the total number of cells analyzed in a given aliquot and the presence or absence of the specific RT–PCR product. We have employed a limiting dilution series on whole cells, with multiple aliquots at each cell concentration to achieve more statistical power in the analysis of a rare cell type. We have used a nested amplification of the CD34 mRNA to be able to detect a single cell expressing the CD34 mRNA in a larger population of non-CD34-expressing cells. We demonstrate that by using this technique, cells from blood and bone marrow containing the CD34 mRNA can be followed quantitatively during a multistep purification involving immunoadsorption followed by fluorescence-activated cell sorting. We also demonstrate that many cells that express the CD34 protein on their surface no longer contain detectable levels of CD34 mRNA, a phenomenon that appears to be developmentally regulated.

There are a variety of circumstances in which it is of great value to be able to determine the presence and amount of a rare cell type among other more numerous cells. Examples include the detection of tumor cells in blood or bone marrow,[1] the identification of donor and/or host cells in chimeras that have resulted from tissue transplantation,[2] the detection of fetal cells in maternal blood,[3] and the detection of virally infected cells present in a tissue in which the majority of cells are not infected.[4] In many of these cases, the rare target cell may be present at levels of 1 per 1×10^6 nontarget cells or less.

PCR has been shown to be capable of detecting a single target molecule or cell.[5,6] Providing that the target cell has a unique nucleic acid sequence relative to a nontarget cell type, PCR is capable of the amplification of the target molecule over a great excess of nontarget nucleic acid. Analysis of rare cell types by PCR amplification, such as detection of chromosomal translocations in examination of minimal residual disease, is increasingly being applied in the clinical setting.[7] Studies have shown that using purified DNA, PCR can detect one lymphoma cell present in 1,000,000 normal cells.[8] Incorporation of a reverse transcriptase (RT) step prior to PCR has extended such analysis to cellular RNA; however, a limitation in these studies is that they generally have used DNA or RNA extracted from a single, large aliquot of cells and then extrapolated back to achieve an estimate of the starting levels of the target cell. Therefore, a small number of cells containing a large amount of target nucleic acid will be indistinguishable from a large amount of cells containing low levels.

Two general methods have been proposed for quantitative PCR. One method consists of competitive coamplification of a standard sequence, a known amount of which has been introduced into the sample.[9,10] The second method relies on limiting dilution analysis of the sample, with the rate of disappearance of the target signal compared relative to that of another known product.[11] More recently, the use of PCR on whole cells in dilution series has allowed quantitation of a particular DNA sequence relative to the number of starting cells.[12–14]

The method described in this paper combines the concept of limiting dilution analysis of whole cells with nested RT–PCR, capable of detecting a single target cell over a background of large numbers of nontarget cells, to evaluate the presence and number of a rare cell type found in human blood, the hematopoietic stem cell. In this method, one first obtains a cell count of the starting amount of blood in which there exists a rare cell type with a unique mRNA that is only expressed in the target cells and that can be detected at single cell levels using nested RT–PCR amplification. In the example presented in this paper, we have detected the presence of the human hematopoietic stem cell by using RT–PCR of the mRNA for the CD34 cell-surface antigen.[15] A series of replicate aliquots containing decreasing numbers of cells from the total population is then set up, down to levels at which the rare cell is no longer present and thus no longer gives a PCR signal. By counting the number of wells with the PCR product at each concentration, it is possible to obtain an accurate estimate of the concentration and number of those rare cells in the initial population.

This paper demonstrates that the CD34$^+$ hematopoietic stem cell, present

3:278–284©1994 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/94 $5.00

at levels below 1/1000 in peripheral blood, can be followed quantitatively using RT–PCR of whole cells during a multistep purification protocol involving avidin–biotin immunoadsorption followed by fluorescent-activated cell sorting (FACS). We demonstrate that many cells with CD34 molecules on their surface contain no detectable CD34 mRNA and that this phenomenon may be related to pathways associated with hematopoietic stem cell maturation. This work demonstrates that RT–PCR can be used in a very simple manner on whole cells, with no extraction of nucleic acid required, and can be used to quantitate rare cells present at levels of 1 in 10^6.

MATERIALS AND METHODS

Peripheral blood monocytes and mononuclear cells from human marrow were taken from healthy volunteer donors and were isolated on a Ficoll gradient (density 1.077). Residual erythrocytes were then hemolysed for 2 min at room temperature in 0.84% ammonium chloride, 0.1% potassium bicarbonate, and 0.002% EDTA, and the remaining cells were washed in phosphate buffered saline (PBS) containing 1% bovine serum albumin. Cells were resuspended in PBS, counted on a hemocytometer, and put into a 96-well PCR tray at various numbers of cells per well. The total volume of cells put in each well was kept constant at 5 μl. Cell lines used as controls were KG1A ($CD34^+$) and HL-60 ($CD34^-$), obtained from the American Type Culture Collection.

Cells were lysed by heating at 95°C for 5 min. Eight microliters of DEPC-treated water was added to each tube, along with 1 μl of the 3′ outer primer (from a stock solution at 0.1 nmoles/μl). This mixture was incubated for 10 min at 65°C and then placed on ice. Six microliters of Master Mix 1 was then added to each reaction tube. Master Mix 1 consisted of 2 μl of 10× RT buffer (200 mM Tris-HCl at pH 8.4, 500 mM KCl, 25 mM $MgCl_2$, and 1 mg/ml of BSA), 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTPs, and 1 μl of RT (200 U/μl). This mixture was incubated at 42°C for 50 min, followed by 5 min at 95°C. The 20-μl volume was then brought to 100 μl with the addition of 80 μl of Master Mix 2. Master Mix 2 consisted of 1 μl of *Taq* polymerase (5 U/μl), 1 μl of each of the two outer primers (from stocks of 1 nmole/μl), 69 μl of water, and 8 μl of 10× PCR buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 25 mM $MgCl_2$). Thirty-five cycles of PCR ensued, with a temperature profile of 30 sec at 94°C, 55°C, and 72°C. One microliter of product from this first PCR round was added to 50 μl of Master Mix 3 (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM concentration of the two internal primers, and 25 U/ml of *Taq* polymerase) for a second round of PCR for 35 cycles, with a temperature profile of 30 sec at 94°C, 55°C, and 72°C. The PCR products were analyzed on a 3:1% NuSeive/GTG agarose gel (FMC). RT–PCR was performed using reagents supplied with the BRL superscript amplification system, whereas regular PCR was performed using reagents supplied by Boehringer Mannheim. All PCR reactions were performed by using a Perkin-Elmer 9600 thermocycler. Total cellular RNA was extracted as described previously.[16]

The sequences of the nested CD34 mRNA primers were CACCCTGTGTCTCAACATGG and GGGAGATGTTGCAAGGCTAG (outer pair, 5′ and 3′, respectively) and CAGTGTCTACTGCTGGTCTTGG and GAATAGCTCTGGTGGCTTGC (inner pair, 5′ and 3′, respectively). The product sizes for the two amplicons were 257 and 140 bp, respectively. Primers were obtained from Operon Technologies, Alameda, CA. Primers for detecting CD34 were designed based on the genomic DNA sequence;[17] the two 5′ primers were within exon 2, and the two 3′ primers were within exon 3.

Avidin–biotin immunoaffinity selection of $CD34^+$ cells from human blood and marrow was accomplished using the CellPro CEPRATE CD34 LC laboratory cell separation system (CellPro, Bothell, WA). Cell sorting was accomplished with a Colter EPICS Elite cell sorter, using the fluorescent-labeled antibodies HPCA (PE-labeled anti-CD34) and FITC-labeled anti-HLA–DR, both obtained from Becton-Dickinson.

Statistical Analysis

When multiple replicate aliquots containing a limiting number of target cells are being amplified, a statistical treatment of the resulting number of positive wells should be used to calculate the estimated target cell concentration. The variables needed to derive the target cell concentration are c_i = number of cells per well at dilution i, n = number of wells at each dilution, and k_i = the number of positive wells at the ith dilution. This assumes that the method will be used in situations where the target cells are relatively rare, for example, less than a few percent of the parent population. Under this model the Poisson distribution can be used. This method is based on calculating the maximum likelihood estimate of f, the fraction of target cells in the parent population.

Let X equal the number of target cells in a single well at dilution i. X has a Poisson distribution with a mean equal to $f \times xc_i$. The probability that a given well will be positive in this assay is given by equation 1.

$$P(X > 0) = 1 - \exp(-fc_i) \qquad (1)$$

The probability of observing the experimental outcome of k_i out of n wells positive at dilution i is given by equation 2, expressed in logarithmic form for convenience.

$$\log_e[P(k_i)] = \log[C(n,k_i)] + k_i \log[1 - \exp(-fc_i)] - fc_i(n - k_i) \qquad (2)$$

where $C(n,k_i)$ is the binomial coefficient for n and k_i. Taking the log of this likelihood function, given by equation 3.

$$\log L = \Sigma\log[C(n,k_i)] + \Sigma k_i \log[1 - \exp(-fc_i)] - \Sigma fc_i(n - k_i) \qquad (3)$$

Taking the first derivative of *logL* with respect to f and setting it equal to 0 will determine the value of f, the concentration of target cells that maximizes the probability of the observed outcome. Equation 4 is given, where N = the total number of cells in all the wells at all dilutions.

$$\Sigma k_i c_i/(1 - \exp - fc_i) = N \qquad (4)$$

An approximate solution for f can be found by using a quadratic approximation to the function on the left hand side of equation 4. The approximate solution is given by equation 5.

$$f = \Sigma k_i / [N - \Sigma(k_i \times c_i/2)] \quad (5)$$

To use this first approximation, only the dilutions that produce mixed positive and negative wells should be used along with the first neighboring higher and lower dilutions (if there are any) that produce all positive or all negative wells. A large series of dilutions that produce either all positive or all negative wells will seriously bias the estimation using equation 5.

RT–PCR on Whole Cells to the Single Cell Level

PCR of DNA from whole cells has been accomplished in the past from a variety of tissues.[18] We first investigated whether it was possible to perform RT–PCR on mRNA in whole cells that were lysed directly without extraction of the RNA. In experiments to determine the effect and sensitivity of RT–PCR on RNA from whole cells versus extracted RNA, KG1A cells, which contain the mRNA for CD34, were diluted from 10,000 cells to single cell levels. Using nested amplification of the CD34 message, the PCR signal generated from whole lysed cells was compared with that generated from purified cellular RNA. Figure 1 shows the RT–PCR product obtained from the amplification of the CD34 message in whole cells compared with the RT–PCR product from RNA extracted from KG1A cells. The amount of cells either extracted or used directly was 10,000, 1,000, 100, 10 (1 well each), and four replicates of 1 cell per well. Product was only observed in extracted mRNA when a minimum of 10,000 cells were used in the extraction. In contrast, in the eight lanes of Figure 1 in which RT–PCR of CD34 message was performed directly on whole cells, a signal was detected in all lanes except one of the four single-cell replicates. On the basis of the Poisson distribution presented in the statistics section of this paper, approximately one in three wells would be expected to be blank at the single cell level, in good agreement with the one in four observed.

Having shown that the direct amplification of RNA from whole cells was possible down to the single-cell level, we then demonstrated that this sensitivity was possible in the presence of large numbers of nontarget cells. Figure 2 shows the results when a single target KG1A cell was mixed with HL60 cells, which do not express the CD34 mRNA, at ratios of 1:10,000, 1:25,000, 1:50,000 and 1:100,000. The CD34 RT–PCR product was detectable at all the ratios, even at 1:100,000 KG1A/HL60 cells. Again, it will be noted that there are several blank wells among the duplicates at each of the four ratios, which again is most likely to be the chance outcome of attempting to put one target cell in a well.

Having shown that the mRNA from a rare target cell can be amplified in the presence of large numbers of nontarget cells, we demonstrated that it is possible to employ RT–PCR on a mixed population of cells to quantitate the level of a rare cell containing a unique mRNA within the general population. By performing multiple replicate wells of nested RT–PCR at a variety of cell numbers over a range of dilutions that go from saturation (one or more target cells in every well) to well below saturation level, it should be possible to calculate a value for the amount of the target cell type in the starting population.

To prove that this assay was quantitative and linear within the range described, the following experiment was performed. A mixture of 100 KG1A and 1×10^6 HL60 cells was made, giving a ratio of 1 KG1A cell per 10,000 HL60 cells. We then aliquoted 1,000, 5,000, 10,000, 20,000, and 50,000 total cells into 10 replicate wells at each concentration. A positive well must contain at least one target KG1A cell. Figure 3 shows the PCR results from this replicate dilution series. On the basis of the Poisson distribution, the expected number of positive wells at each of the concentrations should be 1/10 at 1,000 cells per well, 4/10 at 5,000 cells per well, 6/10 at 10,000, 9/10 at 20,000, and 10/10 at 50,000. As can be seen in Figure 4, the observed number of positive wells at each concentration of cells is in very close agreement to the expected values. Using the values generated in this example, equation 5 yields the following:

$$f = \frac{3 + 7 + 9 + 10}{860{,}000 - (765{,}000/2)} = 6.1 \times 10^{-5}$$

The value determined for f corresponds to a 1:16,400 dilution of target to nontarget cells, which is close to the 1:10,000 dilution that was set up for this mixing experiment.

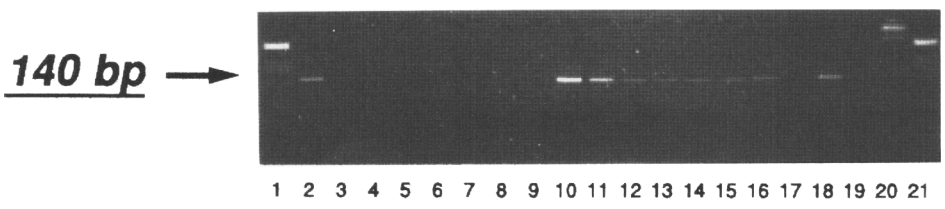


FIGURE 1 Comparison of RT–PCR of CD34 from extracted RNA and whole cells. (Lane *1*) Molecular weight size markers (*Hae*III digest of pBR322); (lanes *2–9*) RT–PCR on RNA extracted from 10^4, 10^3, 10^2, 10, and four lanes of 1 KG1A cells; (lanes *10–17*). RT–PCR performed directly on whole cells at levels of 10^4, 10^3, 10^2, 10, and four lanes of 1 KG1A cell; (lane *18*) RT–PCR on purified mRNA extracted from 10^7 KG1A cells; (lane *19*) H_2O negative control; (lane *20*) BRL positive control; (lane *21*) molecular weight markers.

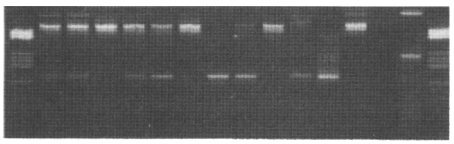


FIGURE 2 RT–PCR of a single $CD34^+$ KG1A cell over a background of $CD34^-$ HL60 cells. (Lane *1*) Molecular weight markers (*Hae*III digest of pBR322); (lanes *2,3*) RT–PCR of 1 KG1A cell in the presence of 100,000 HL60 cells; (lanes *4,5*) RT–PCR of 1 KG1A cell and 75,000 HL60 cells; (lanes *6,7*) RT–PCR of 1 KG1A cell and 50,000 HL60 cells; (lanes *8,9*) RT–PCR of 1 KG1A cell and 25,000 HL60 cells; (lanes *10,11*) RT–PCR of 1 KG1A cell and 10,000 HL60 cells; (lane *12*) RT–PCR of purified KG1A RNA; (lane *13*) RT–PCR of 100,000 HL60 cells; (lane *14*) H_2O; (lane *15*) BRL control; (lane *16*) molecular weight size markers.

mRNA in Stem Cells

Having validated the assay in a model system, we set out to quantitate the levels of $CD34^+$ cells in peripheral blood before and after purification by immu-

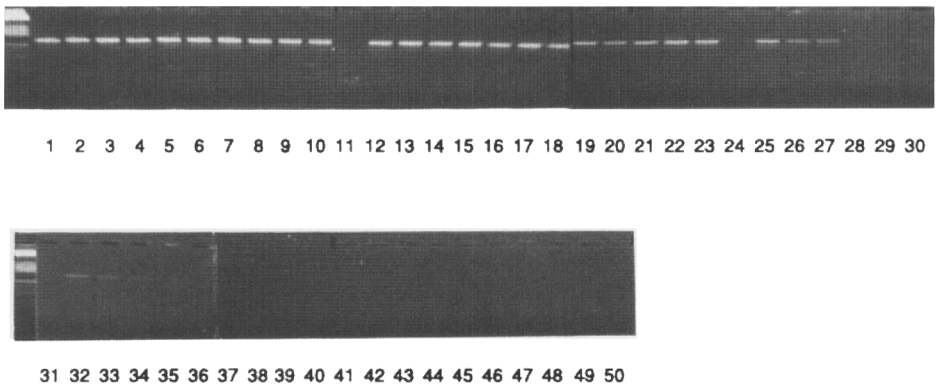


FIGURE 3 RT–PCR analysis of CD34 mRNA in a mixed cell population containing a ratio of 1 $CD34^+$ KG1A cell per 10,000 HL60 cells ($CD34^-$). Ten-well replicates at each of the five concentrations of cells indicated were amplified by RT–PCR for the CD34 mRNA. Molecular weight size markers shown in the *left*-most lane of each row were *Hae*III digested pBR322. Cells per well: (Lanes *1–10*) 50,000; (lanes *11–20*) 20,000; (lanes *21–30*) 10,000; (lanes *31–40*) 5,000; (lanes *41–50*) 1,000.

noaffinity chromatography of the CD34 fraction. The experimental design was as follows: the mononuclear fraction from 20 ml of adult human peripheral blood was isolated from a Ficoll gradient, hemolysed, and counted on a hemocytometer. Prior to CD34 selection, a small amount of the starting material was aliquoted into a total of 30 wells: 10 wells at 100,000, 10,000, and 1000 cells per well. The remaining 23×10^6 cells were then stained with a biotinylated CD34 antibody and passed over an avidin immunoaffinity column. The adsorbed, $CD34^+$ fraction was eluted from the column and counted. The adsorbed fraction contained 63,000 cells, or 0.27% of the starting material, and a portion of it was placed in 10 wells each of 100, 10, and 1 cell(s) per well. RT–PCR was performed on all 60 wells, and the results are shown in Figure 5. In the starting material, four wells are positive in the 10 lanes at 10^5 cells/well, with the remaining dilutions being completely negative. In the CD34-enriched, adsorbed fraction there were 10 of 10 positive wells at the 100 cells per well level, 6 of 10 positive wells at the 10 cells per well level, and 2 of 10 positive wells at the 1 cell per well level. This demonstrates a very clear enrichment of cells containing the CD34 mRNA in the adsorbed fraction. To quantitate the degree of enrichment achieved, the values for the number of positive wells were entered into equation 5. The calculated value for the frequency of cells containing CD34 mRNA in the starting material was 4.4×10^{-6}, or 1/227,000 while the frequency of cells containing CD34 mRNA in the adsorbed fraction was 0.031, or 1/32 cells, demonstrating a 7000-fold enrichment of cells containing the CD34 mRNA.

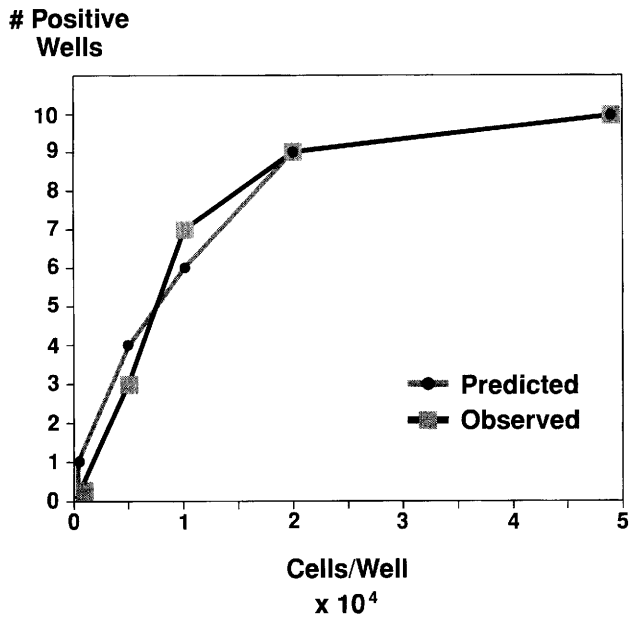


FIGURE 4 Comparison of the theoretical number of positive wells expected vs. the actual values obtained in Fig. 3 for RT–PCR amplification of various numbers of cells containing a ratio of 1 $CD34^+$ cell per 10,000 $CD34^-$ cells.

A portion of the adsorbed material was restained with a fluorescent CD34 antibody and analyzed by FACS for surface CD34 expression. The percent of cells in the adsorbed fraction expressing CD34 on their surface as judged by fluorescent antibody staining was 9.6%, a much higher level than the 3.1% determined above by the RT–PCR assay. This indicated that a sizable percent of the selected cells that had the CD34 protein on their surface lacked detectable levels of the CD34 mRNA. To confirm this finding, we examined $CD34^+$ cells from both blood and bone marrow after a two-step positive selection for $CD34^+$ cells consisting of avidin–biotin immunoaffinity chromotagraphy followed by flow cytometric sorting. This two-step purification yields a final $CD34^+$ population of >90% purity. The experimental design was as follows: adult human bone marrow and peripheral blood were processed and run over a CD34 immunoaffinity column as described previously. The $CD34^+$ adsorbed fractions were restained with a fluorescent-labeled CD34 antibody and flow sorted for $CD34^+$ cells. After the double selection, the $CD34^+$ cells from marrow and blood were plated in PCR wells at concentrations of 10, 1, and 0.1 cell(s) per well, with 10 wells at each concentration, for a total of 60 wells, and RT–PCR was performed. The results of this PCR, shown in Figure 6, were that at the 10 cells per well level, 9 of 10 wells were positive in both marrow and blood; at the 1 cell per well level, 5 of 10 wells were positive for marrow and 4 of 10 were positive for blood; and at the 0.1 cell per well level, one well was positive in both sources. Using equation 5, 23% of $CD34^+$ cells

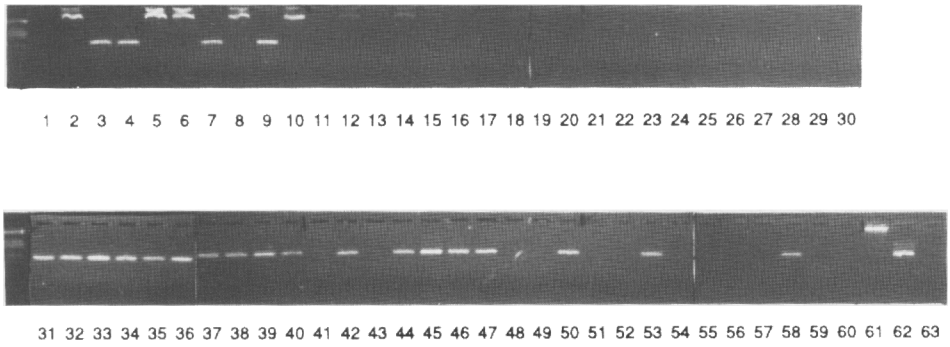


FIGURE 5 RT–PCR analysis of CD34 mRNA in human peripheral blood mononuclear cells before and after immunoaffinity enrichment of $CD34^+$ cells. Ten-well replicates at each of the three concentrations of cells indicated were amplified for the CD34 mRNA by RT–PCR. (Lanes *1–30*) Unfractionated blood (Start); (lanes *31–60*) the CD34-enriched, adsorbed fraction of the immunoaffinity column (Ads). The molecular weight markers shown in the *left*-most lane of each row were *Hae*III-digested pBR322. (Lanes *1–10*) 10^5 Start; (lanes *11–20*) 10^4 Start; (lanes *21–30*) 10^3 Start; (lanes *31–40*) 10^2 Ads; (lanes *41–50*) 10 Ads; (lanes *51–60*) 1 Ads; (lane *61*) BRL control; (lane *62*) KG1A mRNA control; (lane *63*) H_2O control.

containing the CD34 mRNA were from marrow and 22% from blood.

This demonstrates that in both bone marrow and blood a sizable percentage of $CD34^+$ cells have the CD34 protein on their surface but lack detectable CD34 mRNA. This phenomenon might be related to the temporal pathway from stem cells to the many lineages of the hematopoietic system. To determine whether this was the case, we preenriched the $CD34^+$ fraction from bone marrow on the CellPro avidin–biotin column and followed that by a double sort for $CD34^+$ cells and HLA–DR^+ and HLA–DR^- cells. The most primitive stem cells, making up <5% of the total $CD34^+$ population, do not express HLA–DR.[19] The sort windows for this experiment are shown in Figure 7. The number of cells sorted for each fraction were 10,129 $CD34^+/DR^-$ cells and 326,593 $CD34^+/DR^+$ cells. These fractions were diluted in PBS and plated in PCR wells at the following concentrations, with eight wells per concentration: 2 cells per well, 1 cell per well, and 0.5 cell per well. RT–PCR was performed on all 48 wells. As seen in Figure 8, the $CD34^+/DR^-$ fraction signal was observed in seven of eight wells in the 2-cell replicates, in seven of eight wells in the 1-cell replicates, and in four of eight wells in the 0.5-cell replicates. In the $CD34^+/DR^-$ fraction, signal was observed in seven of eight wells in the 2-cell replicates, in four of eight wells in the 1-cell replicates, and in none of the 0.5-cell replicates. Using equation 5, the values obtained were 1.09 for the $CD34^+/DR^-$ cells and 0.58 for the $CD34^+/DR^+$ cells.

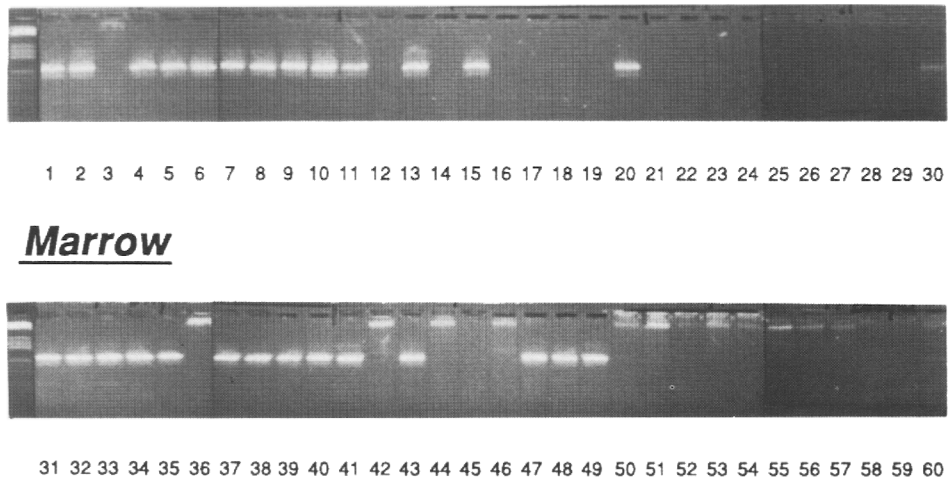


FIGURE 6 RT–PCR analysis of CD34 mRNA in human peripheral blood [(lanes *1–10*) 10 cells/well; (lanes *11–20*) 1 cell/well; (lanes *21–30*) 0.1 cell/well] and in bone marrow [(lanes *31–40*) 10 cells/well; (lanes *41–50*) 1 cell/well; (lanes *51–60*) 0.1 cell/well] after two enrichment steps. CD34 cells from blood and marrow were purified by immunaffinity column enrichment followed by fluoresence cell sorting. Ten-well replicates at each of the three concentrations of cells indicated were analyzed from each selection. The molecular weight markers shown in the *left*-most lane of each row were *Hae*III-digested pBR322.

DISCUSSION

The identification of subpopulations of cells by the presence of specific mRNAs is important for detecting and quantitating rare cells present in mixed cell populations. The method described in this paper has the ability to estimate frequencies of 1 target cell per 1,000,000 nontarget cells. This method can be easily applied to quantitate rare cell types that exhibit a difference in their RNA or DNA sequence from the majority cell type. This technique allows one to follow the levels of a rare cell type during manipulations either to purge or purify that cell type when the starting or ending levels of the target population fall below levels that other techniques, such as flow cytometry, Southern blot analysis, or visual inspection, can be used. This method also requires the sacrifice of very few cells to obtain estimation of the target cell type. In the first example given above of the isolation of $CD34^+$ cells from peripheral blood, 1,110 cells were used from the 63,000 obtained in the adsorbed $CD34^+$ fraction, or 1.7%, leaving the remaining material available for other analysis or uses.

This work demonstrates that many of the cells found in adult blood or bone marrow with CD34 on their surface no longer contain detectable levels of CD34 mRNA. By comparing DR^+ and DR^- subpopulations of $CD34^+$ stem cells, we have shown that the more primitive DR^- fraction contains a higher ratio of CD34 mRNA to surface protein expres-

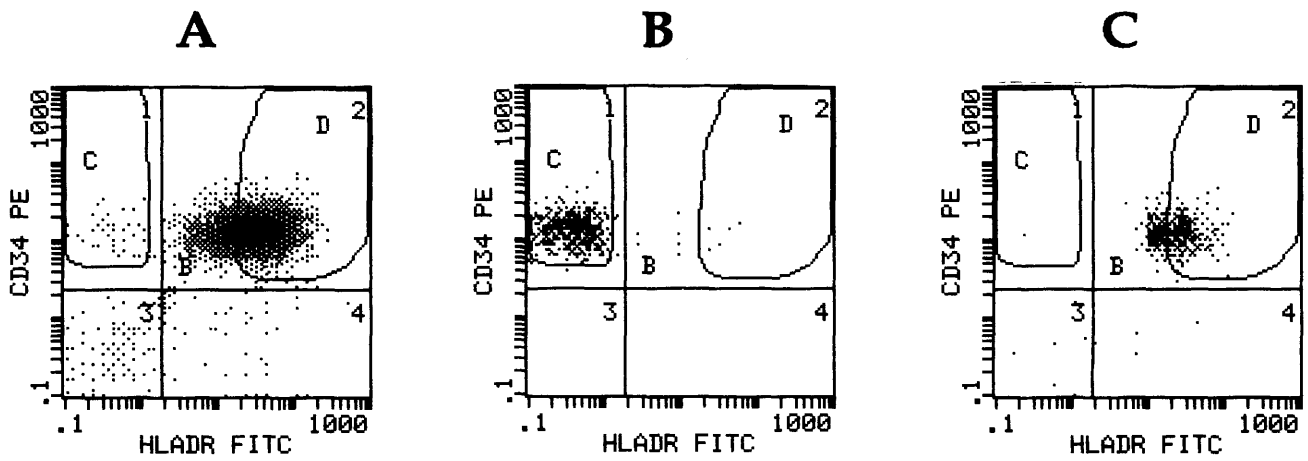


FIGURE 7 Sort windows and reanalysis after a CD34 column enrichment followed by a CD34/DR double sort. $CD34^+$ cells from bone marrow were enriched by immunoaffinity column purification and then restained with PE-labeled anti-CD34 and FITC-labeled anti-HLADR. (*A*) Sort windows for the CD34 cells after immunoaffinity enrichment. Window C contains $CD34^+/DR^-$ cells; window D contains $CD34^+/DR^+$ cells. (*B*) Reanalysis of the $CD34^+/DR^-$ sorted cells from *A*. (*C*) Reanalysis of the $CD34^+/DR^+$ sorted cells from *A*.

sion. This finding is consistent with the hypothesis that as the stem cell becomes committed to various lineages, the mRNA for CD34 is eliminated or turns over before the CD34 protein. It is likely that the more primitive $CD34^+/DR^-$ cells are resting stem cells, which contain the CD34 mRNA and continually replenish the CD34 protein on their outer membrane, whereas the $CD34^+/DR^+$ cells represent the current wave of progenitors that have recently become committed to various lineages, are in the process of losing their CD34 protein, and have already begun to lose the mRNA for CD34. By comparison, in cells selected in an identical two-step procedure for CD20, which is a mature B-cell marker, there was a direct one-to-one correlation with CD20 surface expression and detectable levels of the CD20 mRNA in every $CD20^+$ cell (data not shown).

The messages that we have amplified successfully from whole cells include those for CD20, CD34, CD44, the estrogen receptor, mucin, and Neu/Her-2. We were surprised at first that RT–PCR was possible on whole cells, owing to the presumed presence of RNases in any cell lysate. It is possible that the initial 5-min heat denaturation at 95° denatures cellular RNases sufficiently to prevent the exposed cellular RNA from being degraded during the subsequent RT step. It is also possible that the requirement for nested amplification may overcome moderate levels of RNA degradation that may occur. It may be that some messages exist in cells at such low levels that they would be difficult to detect reliably at the single cell level. In this regard, one problem we have experienced is that if multiple splice variants of a target mRNA exist, which can all be amplified by a set of primers, one may have such heterogeneity of product sizes that no final single product is made in quantities sufficient to be detectable on an ethdium bromide-stained gel. Also, owing to the inhibition of PCR by hemoglobin, it is very important to remove all heme from the preparation of cells from blood before attempting to amplify them by whole cell RT–PCR. To overcome this inhibition, we routinely use Ficoll followed by hemolysis with amonium chloride to remove the red blood cells.

The variability inherent in the manual dilution of cells can be a limit to the accuracy of this method, although the use of manual dilutions simplifies the performance of this assay. Use of a cell sorter to distribute exact numbers of cells obviously improves the accuracy of the values derived from this assay. Although error rates when depositing very small numbers of cells per well may be large, it is important to keep in mind that in the case of a rare cell type present in an abundance of nontarget cells, the number of cells per well should be relatively high, which should minimize this type of sampling error.

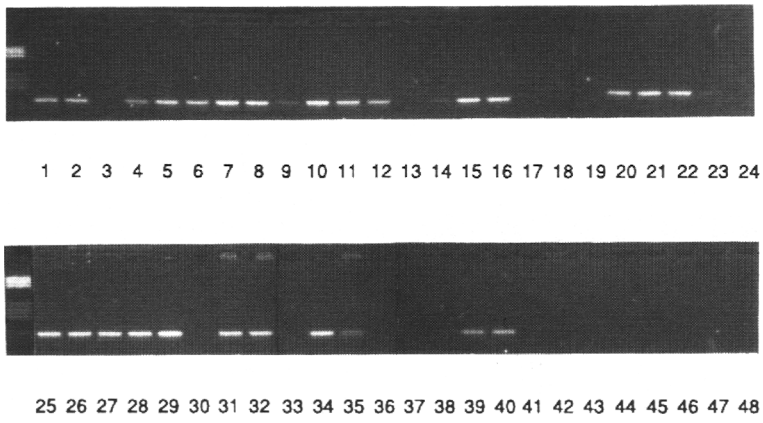


FIGURE 8 RT–PCR analysis of CD34 mRNA from the cells sorted in Fig. 7. Eight-well replicates at each of the three concentrations of cells indicated were amplified for the CD34 mRNA by RT–PCR. (Lanes *1–24*) $CD34^+/DR^+$ cells from window C in Fig. 7*B*; (lanes *25–48*) $CD34^+/DR^+$ cells from window D in Fig. 7*C*. The molecular weight markers shown in the *left*-most lane of each row were *Hae*III-digested pBR322. [Cells per well: (Lanes *1–8*) DR^-, 2; (lanes *9–16*) DR^-, 1; (lanes *17–24*) DR^-, 0.5; (lanes *25–32*) DR^+, 2; (lanes *33–40*) DR^+, 1; (lanes *41–48*) DR^+, 0.5.]

The use of statistical analysis in the generation of the target cell value is an important part of this method. As the variables in equation 5 indicate, this assay can be performed with wide ranges of total number of wells, number of cells in each well, number of dilutions used, and the range over which those dilutions extend. The most accurate statistical estimate for the fraction of target cells contained in the starting population can be obtained by optimizing the number of dilutions, the number of replicates done at each dilution, and the range of concentrations over which the dilutions are performed. When the target cell frequency is unknown in the starting cell population, it is best to sample a few wells at a wide range of dilutions and then narrow in on a circumscribed range of dilutions where there will be a mixture of positive and negative wells.

The ability to perform RT–PCR on cells directly, with no RNA extraction required, saves a great deal of time as well as minimizes possible loss of material during extraction procedures. In previous methods, when PCR has been performed on nucleic acid extracted from a large starting population, a small number of cells with a high level of target nucleic acid will be indistinguishable from a larger number of cells containing low levels of the target nucleic acid. By applying the methods described in this paper, the actual number of target cells and not the average amount of target nucleic acid relative to the entire population can be obtained. Finally, this method allows quantitation of rare cell types, such as tumor cells, when they are below levels at which currently available methods are quantitative.

ACKNOWLEDGMENTS

We thank Dr. Nayak Polissar of the Statistics and Epidemiology Research Corporation (Seattle, WA) for his invaluable assistance with the statistical analysis portion of this work. We also thank Mark Rehse for his help with cell sorting and analysis.

REFERENCES

1. Roth, M.S., J.H. Antin, E.L. Bingham, and D. Ginsburg. 1989. Detection of Philadelphia chromosome-positive cells by the polymerase chain reaction following bone marrow transplant for chronic myelogenous leukemia. *Blood* **74:** 882–885.
2. Brenner, M.K., D.R. Rill, R.C. Moen, R.A. Krance, J. Mirro, W.F. Anderson, and J.N. Ihle. 1993. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* **341:** 85–86.
3. Lo, Y.M.D., P. Patel, J.S. Wainscoat, P. Sampetre, M.D.G. Gillmer, and J. Flemming. 1989. Prenatal diagnosis using fetal cells in the maternal circulation. *Lancet* **339:** 1363–1365.
4. Ou, C.Y., S. Kwok, S.W. Mitchell, D.H. Mack, J. Sininsky, J.W. krebs, P. Feorino, D. Warfield, and G. Schochetman. 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. *Science* **238:** 295–297.
5. Saiki, R., S. Scharf, F. Faloona, K. Mullis, G. Horn, H. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:** 1350–1354.
6. Li, H., U.B. Gyllensten, X. Cui, R.K. Saiki, H.A. Erlich, and N. Arnheim. 1988. Amplification and analysis of DNA sequences in single human sperm and diploid cell. *Nature* **335:** 414–417.
7. Gribben, J.G., D. Neuberg, A.S. Freedman, C.D. Gimmi, K.W. Pesek, M. Barber, L. Saporito, S.D. Woo, F. Coral, N. Spector, S.N. Rabinowe, M.L. Grossbard, J. Ritz, and L.M. Nadler. 1993. Detection by PCR of residual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood* **81:** 3449–3457.
8. Gribben, J.G., A.S. Freedman, S.D. Woo, K. Blake, R.S. Shu, G. Freeman, J.A. Longtire, G.S. Pinkus, and L.M. Nadler. 1991. All advanced stage non-Hodgkin's lymphomas with a PCR amplifiable bcl-2 translocation have residual cells containing the bcl-2 rearrangment at evaluation and following treatment. *Blood* **78:** 3275–3280.
9. Volkenandt, M., A.P. Dicker, D. Banerjee, R. Fanin, B. Schweitzer, T. Horikoshi, K. Danenberg, and J.R. Bertino. 1992. Quantitation of gene copy number and mRNA using the polymerase chain reaction. *Proc. Soc. Exp. Biol. Med.* **200:** 1–6.
10. Jurriaana, S., J.T. Dekker, and A. Ronde. 1992. HIV-1 viral DNA load in peripheral blood mononuclear cells from seroconverters and long-term infected individuals. *AIDS* **6:** 635–641.
11. Ferre, F. 1992. Quantitative or semi-quantitative PCR: Reality versus myth. *PCR Methods Applic.* **2:** 1–9.
12. Mirsky, M.L., Y. Da, and H.A. Lewin. 1993. Detection of bovine leukemia proviral DNA in individual cells. *PCR Methods Applic.* **2:** 333–340.
13. Pozansky, M., B. Walker, W.A. Haseltine, J. Sodorski, and E. Langhoff. 1991. A rapid method for quantitating the frequency of peripheral blood cells containing HIV-1 DNA. *J. Acquired Immune Deficiency Syndromes* **4:** 368–373.
14. Brisco, M.J., J. Condon, P.J. Sykes, S.H. Neoh, and A.A. Morley. 1991. Detection and quantitation of neoplastic cells in acute lymphoblastic leukemia, by use of the polymerase chain reaction. *Brit. J. Haematol.* **79:** 211–217.
15. Berenson, R.J., R.G. Andrews, W.I. Bensinger, D.F. Kalamsz, G. Knitter, C.D. Buckner, and I.D. Bernstein. 1988. Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* **81:** 951–955.
16. Chomzynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
17. Satterthwaite, A.B., T.C. Burn, M.M. LeBeau, and D.G. Tenen. 1992. Structure of the gene encoding CD34, a human hematopoietic stem cell antigen. *Genomics* **12:** 788–794.
18. Kawasaki, E.S. 1990. Sample preparation from blood, cells, and other fluids. In *PCR protocols: A guide to methods and applications*, pp. 146–152. Academic Press, San Diego, CA.
19. Civin, C. and S. Gore. 1993. Antigenic analysis of hematopoiesis: A review. *J. Hematotherapy* **2:** 137–144.

Received November 22, 1993; accepted in revised form February 15, 1994.