Original Article

Microarray Analysis Using a Limited Amount of Cells

(RNA isolation / RNA amplification / cDNA microarray / $CD34^+$ cells)

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Abstract. cDNA microarray technology is widely used in various biological and medical disciplines to determine gene expression profiles. Unfortunately, this technology requires a large quantity of input RNA. Since there is an increasing need for more precise analyses of defined cell subpopulations with low cell counts, working protocols using a minimal number of input cells are required. Optimal RNA isolation and its accurate amplification are crucial to the success of these protocols. The HL-60 cell line was used in the search for a suitable protocol that can be used for clinical samples of $CD34^+$ haematopoietic cells obtained from bone marrow. The goal was to discover the best method for isolating and amplifying RNA from a small number of cells. Our evaluation of various methods and kits available in the market revealed that the combination of RNAqueous™ Kit for RNA isolation and the SenseAmp Plus Kit for one-round RNA amplification produced the best results. This article presents a verified protocol describing a reliable and reproducible method for obtaining enough input RNA for microarray experiments when the number of cells is limited.

Introduction

The haematopoietic stem cells, which are a subpopulation of $CD34^+$ bone marrow cells, are a topic of many studies (e.g. Ohmine et al., 2001; Steidl et al., 2002; Yong et al., 2006). Many of these studies are dealing with $CD34^+$ haematopoietic cells obtained from the bone marrow of chronic myeloid leukaemia patients. Unfortunately, $CD34^+$ cells make up only 1.5 % of all cells in bone marrow (Krause et al., 1996), and one is usually able to obtain only 5,000–50,000 $CD34^+$ cells from one patient without causing any harm. Therefore, there is a need to design a protocol that will allow performing microarray analysis using a limited number of input cells. Current literature of the leukaemia field focuses on studies using $CD34^+$ cells in the blastic phase of leukaemia (Zheng et al., 2006), which is characterized by rapid expansion (≥ 30 % blasts in bone marrow or ≥ 50 % in peripheral blood) of the myeloid or lymphoid differentiation-arrested progenitor $CD34^+$ cells (Ilaria, 2005; Zheng et al., 2006). However, this work focuses on pluripotent $CD34^+$ stem cells, which is a much smaller cell population. Unfortunately, there are only a few published studies using RNA from $CD34^+$ cells obtained from bone marrow, and the methods are not described in deep detail (Villuendas et al., 2006; Diaz-Blanco et al., 2007; Guglielmelli et al., 2007). One example is the study by Guglielmelli et al. (2007), which reported the pooling of RNA from $CD34^+$ cells of five different normal donors or patients in order to overcome the lack of input material. In this article, we describe a combination of RNA isolation and amplification methods that presents a reliable way for performing microarray analysis from a limited number of cells.

The quantity and quality of input RNA are crucial to the success of standard microarray protocols. A typical microarray experiment requires 5–50 μg of total RNA (Baugh et al., 2001; Feldman et al., 2002; Jenson et al., 2003; Livesey, 2003; King et al., 2005; Patel et al., 2005) or 0.1–5 μg of poly(A) mRNA (Duggan et al., 1999; Spiess et al., 2003) isolated from several millions of cells or a few hundred milligrams of tissue. However, the limited cell count in clinical samples often prevents researchers from obtaining sufficient amounts of RNA for a microarray experiment. In order to discover methods that produce the highest quantity and quality of RNA, we evaluated three commercially available methods for isolation of total RNA from a small number of cells: TriReagent, RNAqueous Kit, and RNeasy Micro Kit. The evaluation was focused mainly on the yield and

Received August 19, 2008. Accepted January 6, 2009.

This study was generously supported by the Ministry of Education of the Czech Republic grants MSM-0021622430, NPVII (2B06052) and LC535.

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Abbreviations: FACS – fluorescence-activated cell sorting, RIN – RNA integrity number.

purity of isolated RNA. We tested these methods using the HL-60 cell line. The yield and purity of RNAqueous Kit were the best and because of that this protocol was subsequently applied to the $CD34^+$ cells isolated from patients. However, the amount of total RNA isolated from a limited number of cells in clinical samples was still insufficient for a microarray analysis. Thus, it is necessary to amplify the RNA before subjecting it to microarray analysis.

We chose to use the Genisphere SenseAmp Plus Kit based on the study by Goff et al. (2004). This kit works by amplifying linear mRNA and produces the sense-strand mRNAs needed for microarray analysis. There are different approaches for evaluating the accuracy of RNA amplification (Goff et al., 2004; Stoyanova et al., 2004; Li et al., 2005; Patel et al., 2005; Kurimoto et al., 2006; Nygaard and Hovig, 2006). In this study, we compared microarray expression profiles of samples amplified from various amounts of RNA with expression profiles of unamplified samples.

In short, the objectives of this study were: (i) to assess the yield and quality of RNA isolated from specific numbers of HL-60 cells using different RNA isolation methods, (ii) to assess the yield of amplified RNA obtained from small amounts of input RNA, (iii) to check the integrity and quantity of amplified RNA using a spectrophotometer and an Agilent 2100 Bioanalyzer, (iv) to evaluate the fidelity of the amplification process by microarray gene expression analysis using amplified RNA versus unamplified RNA, and (v) to apply this protocol to clinical $CD34^+$ cell samples.

Material and Methods

Cell line and clinical samples

The human promyelocytic leukaemia HL-60 cell line was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Pan-Biotech, Aidenbach, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Pan-Biotech), 100 U/ml penicillin (Pan-Biotech) and 100 µg/ml streptomycin (Pan-Biotech) at 37 °C in 5% CO_2 and 100% humidity. Cells were counted in the Bürker counting chamber.

Bone marrow samples were taken from patients diagnosed with chronic myeloid leukaemia after written informed consent. Mononuclear cells were isolated from all clinical samples using fluorescence-activated cell sorting (FACS). The numbers of $CD34^+$ cells in the bone marrow samples and the enriched cell fractions were analysed by a FACSVantage SE flow cytometer (Becton Dickinson, San Jose, CA) using monoclonal anti-CD34 antibody (PE, 8G12, Becton-Dickinson, Franklin Lakes, NJ).

RNA isolation

Total RNA was isolated from 5×10^3 to 2.5×10^5 cells using the RNAqueous™ Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The total RNA samples were dissolved in 12 µl of nuclease-free water.

The quantity of total RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

RNA later (Ambion) was used for RNA preservation. This reagent can penetrate the cell membrane and inactivate cellular and other contaminating RNases.

RNA amplification

RNA amplification was performed as described in the Genisphere SenseAmp Plus manual (Genisphere, Hatfield, PA) with the following modification: for amplification, the amounts of all the reagents used were halved and the ratio of dT24 RT primer and oligo (dT)-T7 promotor primer, respectively, to the starting template was 1 : 10 because excess primer can lead to the appearance of template-independent products. cDNA was purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). *In vitro* transcription was carried out at 37 °C for 16 h.

Amplified RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen). RNA samples were dissolved in 14 µl of nuclease-free water. The quality of amplified RNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer.

The purified RNA (at least 100 ng) was used in the Poly(A) tailing reaction. The tailed sense RNA was purified using RNeasy MinElute Cleanup Kit and dissolved in 20 µl of nuclease-free water.

RNA labelling and microarray hybridization

Expression analysis was performed on SS-H19k8 microarray slides (UHN Microarray Centre, Toronto, Ontario, Canada). These slides contain 19,200 human cDNAs. The gene list for this microarray is available at http://www.microarrays.ca.

Twenty µl of tailed sense RNA (100 ng) or 20 µg of unamplified total RNA were transcribed into cDNA. The reverse transcription was performed in a 40-µl mixture containing 8 µl of 5× first-strand buffer, 2 µl of $(dT)_{23}$ primer (Sigma-Aldrich, St. Louis, MO), 3 µl of 20 mM dNTP (without dCTP), 1 µl of 2 mM dCTP, 4 µl of 0.1 M DTT, 1 µl of 2 mM Cy3-dCTP or Cy5-dCTP (Amersham, Pisacataway, NJ) and nuclease-free water. The reaction mixture was incubated for 5 min at 65 °C and 4 min at 42 °C. Subsequently, 2 µl of SuperScript II (200 U/ml, Invitrogen, Carlsbad, CA) and 1 µl of RNasin (Promega, Mannheim, Germany) were added to the reaction. The tube was incubated for 3 h at 42 °C and chilled on ice. RNA was hydrolysed by adding 4 µl of 50 mM EDTA (Sigma-Aldrich) and 2 µl of 10 N NaOH (Lach-Ner, Neratovice, Czech Republic). The mix was incubated for 20 min at 60 °C. The solution was neutralized using 4 µl of 5 M acetic acid (Penta, Prague, Czech Republic). The Cy3- and Cy5-labelled cDNAs were

pooled and then purified using Illustra CyScribe GFX Purification Kit (GE Healthcare, Munich, Germany) and dissolved in 40 µl of elution buffer.

The microarray slide was incubated in a prehybridization solution [5× SSC, 0.1% SDS (Sigma-Aldrich) and 10 mg/ml BSA (PAN-Biotech)] for 1 h at 37 °C. The slide was then rinsed thoroughly, twice in water for 7 min at 37 °C followed by a rinse in cold 96% ethanol, and carefully dried.

The purified labelled cDNAs (40 µl) were mixed with 40 µl of preheated (at 65 °C for 2 min) hybridization solution [100 µl of DIG Easy Hyb Granules (Roche Applied Science, Mannheim, Germany), 5 µl of 10 mg/ml Yeast tRNA (Invitrogen) and 5 µl of 10 mg/ml Calf Thymus DNA Solution (Invitrogen)] and heated for 2 min at 65 °C. The mixture was allowed to hybridize to the slide under a glass cover slip at 37 °C for 16–19 h in a hybridization chamber.

The hybridized microarray slides were washed three times in 1× SSC with 0.1% SDS for 10 min at 50 °C. The slides were scanned in a ProScanArray HT Microarray Scanner (PerkinElmer, Waltham, MA).

Image analysis

Image analysis software JABS-I (Masaryk University, Brno, Czech Republic, http://cbia.fi.muni.cz) (Koutna et al., 2007) was used. This software enables convenient slide analysis and data visualization. The intensities were extracted using a fixed-circle segmentation method performed on noise-filtered images. The average pixel intensity for each spot area on the slide was obtained and used for statistical comparison between the slides.

Normalization

The data were normalized in two ways for transparent data comparison. JABS-N software (Masaryk University) (Koutna et al., 2007) was used. This software performs both normalization procedures and also provides convenient data visualization. The first step represents data normalization within the same slide. This was performed because of intensity differences between channels on a given microarray slide caused e.g. by different dye (typically Cy3 and Cy5) incorporation. The well-established lowess method, which results in two normalized channels per slide (Quackenbush, 2002; Yang et al., 2002), was used for all slides. We approached each comparison of unamplified versus amplified samples individually. The samples were hybridized on the slide in the following way: on the first microarray, sample one was labelled with the Cy3 dye and sample two was labelled with the Cy5 dye, on the second microarray the dyes were reversed.

The second normalization step was performed to take into account the fact that each slide had been prepared under different conditions and scanned using different scanner settings. In order to control these variables, normalization between different slides was required. This normalization step was performed using the quantile method (Bolstad et al., 2003).

Slide comparison

The data obtained for different microarray slides were normalized in order to allow between-slide comparison (as described in the previous section). We compared normalized data using fold change cutoff. We used this technique to evaluate the matching factor (measured as a percentage) of two compared slides. Fold change cutoff measures the number of spots with significant intensity differences, both relative and absolute. The cutoff value for the intensity ratio and cutoff for absolute value of difference has to be set. We used a 1.5-fold change as the cutoff ratio and 1000 intensity units as the absolute value of the cutoff difference. Spots having the absolute difference (minimum intensity subtracted from maximum intensity) less than 1000 units and relative difference less than 1.5 (maximum intensity divided by minimum intensity) were marked as identical (i.e. no significant change in gene expression between slides). The percentage of these spots represented the matching factor.

Results

RNA isolation

The yield and quality of the RNA isolated from specific numbers of HL-60 cells using three different methods were compared. These three methods allow the RNA isolation from limited numbers of cells. Firstly, we used the TriReagent kit (Sigma-Aldrich), but the RNA obtained was generally contaminated with proteins and genomic DNA. Moreover, a large amount of RNA was lost during the purification step. Therefore, this method is unsuitable for RNA isolation from a limited number of cells. Next, we tested the RNeasy Micro Kit (Qiagen) and the RNAqueous™ Kit (Ambion). Both kits combine the selective binding properties of a silica-based membrane with the speed of microspin technology. We found that these kits were easier to use, and the procedures can be performed more rapidly and were more efficient in isolating RNA.

Total RNA was isolated from 5×10^3 to 2.5×10^5 HL-60 cells. The median RNA yields are shown in Table 1. Our calculations show that one cell contains 4.5–11.8 pg of total RNA. We chose the RNAqueous™ Kit because it produced a slightly higher yield, better 260/280 ratio, and is therefore more suitable for isolating RNA for microarray analysis. The 260/280 ratios were within the range of 1.70–1.98 for all samples (Table 1). The ratios declined as the number of cells was decreased. However, the 260/280 ratio is influenced considerably by pH. To obtain more accurate absorbance values, we measured absorbance in 10 mM Tris·Cl, pH 7.5. RNA isolated from samples was visualized using an Agilent 2100 Bioanalyzer. The electropherograms revealed two clear peaks representing the 28S rRNA and 18S rRNA, respectively (Fig. 1). The 28S/18S ribosomal RNA ratio was in the range of 1.7 to 2.5, and the RIN (RNA Integrity Number) was in the range of 8.5 to 9.9.

Table 1. Quantity and quality of RNA after isolation of HL-60 cells using RNAqueous™ Kit

Amounts of HL-60 cells	RNA quantity after isolation [ng]	260/280 nm
5 000	49	1.70
10 000	72	1.78
20 000	171	1.83
30 000	226	1.88
50 000	251	1.89
100 000	574	1.93
150 000	1050	1.96
200 000	1527	1.98
250 000	1938	1.98

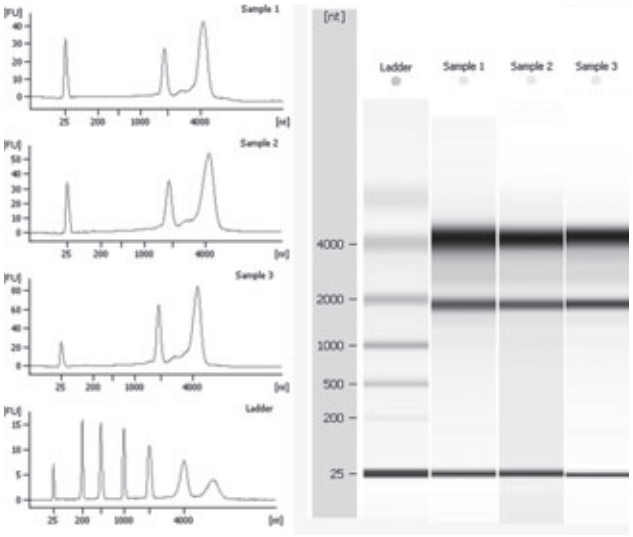

Fig. 1. Agilent 2100 Bioanalyzer electropherograms and gel image of isolated total RNA from HL-60 cells
The samples were diluted to the maximum concentration of 5 ng/µl and the RNA 6000 Pico LabChip® was used. The resulting electropherograms show two clear peaks (18S and 28S). For all samples, the 28S is higher than the 18S peak, indicating good-quality RNA. The RNA gel shows two bands, representing the 28S and 18S rRNA. Lane 1, total RNA isolated from 20,000 cells (5 ng/µl); lane 2, total RNA isolated from 10,000 cells (4 ng/µl); lane 3, total RNA isolated from 5,000 cells (3 ng/µl).

Amplification

We used 20 µg of total RNA for the microarray slides. Since it is not possible to obtain such an amount from a clinical sample, we amplified the RNA using the linear amplification method prior to labelling. A variety of RNA amplification methods are available. We chose the SenseAmp Plus Kit because it synthesizes polyadenylated sense-strand mRNA, which is nearly identical to the original mRNA. Therefore, RNA amplified using this method is of high quality and can be used as input material for downstream amplification or labelling procedures which require the dT-primed reverse transcription. Total RNA was amplified following the manufacturer's protocol with slight modifications (see Material and Methods). Various amounts of total RNA (25 ng, 50 ng, 100 ng, 200 ng, 300 ng, and 500 ng) isolated from the HL-60 cell line were subjected to one-round amplification (Table 2). The quality and quantity of amplified mRNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer. The 260/280 ratios of the products were within the range of 2.01–2.08 (Table 2), indicating successful amplification. It was found that the RNA smears of the amplified samples on gel lay in the 200–3000 nucleotide range, also indicating effective amplification (Fig. 2). We tested the effect of incubation time of *in vitro* transcription (4 h, 8 h, 12 h, 16 h, and 20 h) on the yield of amplified RNA. The highest amplification yields were obtained when *in vitro* transcription lasted 8 h or longer. We chose 16-h *in vitro* incubation time. However, using longer incubation time (more than 16 h) increases yield yet more, but it also increases the risk of generating shorter RNA transcripts.

Verification of RNA amplification using cDNA microarray

The amplification fidelity was verified by comparing expression profiles of amplified and unamplified RNA samples isolated from HL-60 cells (Fig. 3). Correlation studies of six pairs of amplified and unamplified samples were performed on four microarray slides for each sample in two independent experiments. In these experiments, an equal amount of total RNA from amplified and unamplified RNA originating from the same pool of isolated RNA was used to avoid the fluctuation of data. Spots with significantly different expression were analysed to identify genes that were differentially expressed in more than one slide. Our analysis revealed 17 differentially expressed genes among all slide pairs. This means that the error is random and not caused by the

Table 2. Yields and purity of HL-60 amplified RNA generated from known amounts of input total RNA after one-round amplification

Total RNA input	mRNA (2 % of total RNA)	Average yield of amplified mRNA	260/280 nm	Fold amplification
25 ng	0.5 ng	207 ng	2.05	414
50 ng	1.0 ng	485 ng	2.08	485
100 ng	2.0 ng	1 204 ng	2.07	602
200 ng	4.0 ng	2 011 ng	2.01	503
300 ng	6.0 ng	2 973 ng	2.02	496
500 ng	10.0 ng	5 626 ng	2.02	563

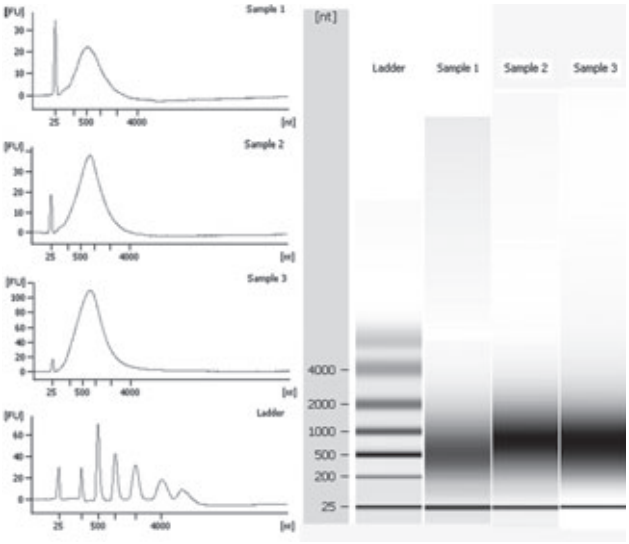

Fig. 2. Electropherograms and gel images of amplified RNA from HL-60 cells
The samples were diluted to the maximum concentration of 5 ng/µl and RNA 6000 Pico LabChip® was used. The RNA gel shows a smear, indicating the range of RNA transcript sizes. Sample 1, RNA amplified from 25 ng of total RNA; sample 2, RNA amplified from 50 ng of total RNA; sample 3, RNA amplified from 100 ng of total RNA. The electropherogram on the left shows the profile of a successful amplification. The major features of a successful amplification are one marker peak followed by a broad hump, which corresponds to the range of fragment sizes of the amplified RNA.

amplification itself. All matching factors were calculated from all spots on microarray slides according to the criteria described in previous section Slide comparison.

Table 3. Matching factor for inter-sample variability

Comparison	Matching factor ± SD [%]
unamplified vs. amplified from 25 ng	95.8 ± 1.2
unamplified vs. amplified from 50 ng	96.5 ± 0.9
unamplified vs. amplified from 100 ng	97.7 ± 0.9
unamplified vs. amplified from 200 ng	98.2 ± 0.7
unamplified vs. amplified from 300 ng	98.3 ± 0.8
unamplified vs. amplified from 500 ng	98.9 ± 0.7

The matching factors for the comparison of unamplified versus amplified samples are shown in Table 3. As expected, the matching factor drops with decreasing amount of total RNA added to the amplification reaction. However, the values of matching factors are still high and this suggests that the results are highly reproducible.

CD34⁺ cells

Based on these reliable and reproducible results using RNA samples from HL-60 cells, we proceeded to perform the same experiments on the clinical samples of CD34⁺ cells. Total RNA was extracted from various amounts of CD34⁺ cells (1.5×10^4, 2.5×10^4, 4×10^4, 10^5, 2×10^5) using the RNAqueous™ Kit (see Table 4 for results). The 260/280 ratio of isolated RNA was within the range of 1.7–1.86, and the RIN was around 9.

In the next step, various amounts of input total RNA (25 ng, 50 ng, 100 ng, 200 ng, 300 ng, and 500 ng) were subjected to the one round of amplification (Table 5). The quality and quantity of amplified RNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer. The ratio 260/280 was within

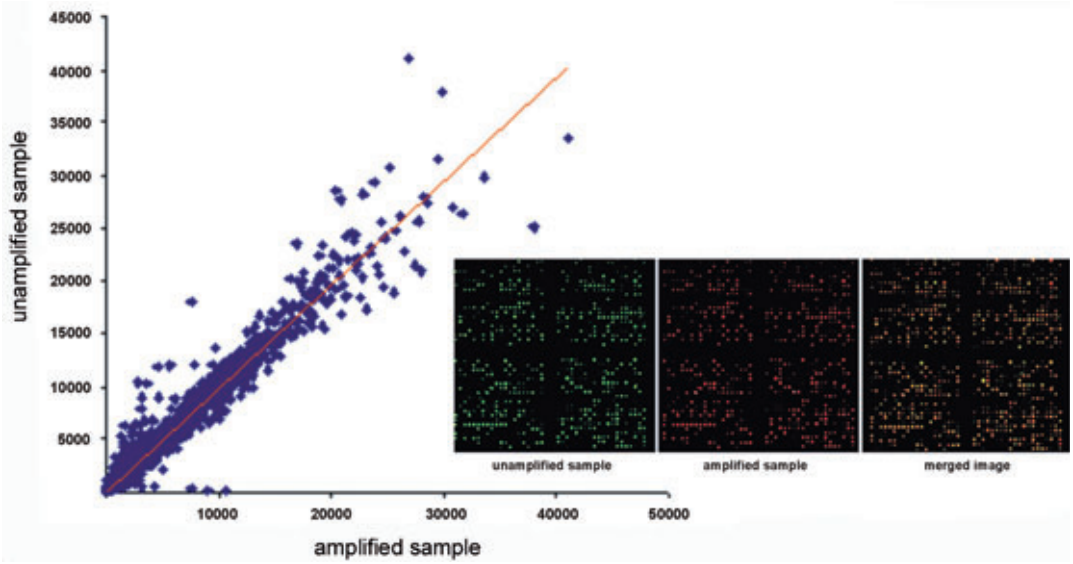

Fig. 3. 2D scatter plot showing expression profiles of both amplified (from 25 ng of total RNA) and unamplified RNA from HL-60 cells
Each dot in this plot represents the average expression level for the amplified (x-axis) and unamplified samples (y-axis). Genes with similar expression level are located close to the plot axis (x = y). The three boxes represent visual comparison of the same image area on different microarray slides. The left box represents amplified sample (Cy3 cDNA), the middle box represents unamplified sample (Cy5 cDNA), and the right box is a combination of these two.

Table 4. Quantity and quality of RNA after isolation of CD34⁺ cells using RNAqueous™ Kit

Amounts of CD34⁺ cells	RNA quantity after isolation [ng]	260/280 nm
15 000	58	1.72
25 000	95	1.70
40 000	289	1.73
100 000	458	1.86
200 000	821	1.82

the range of 1.98–2.2 (Table 4) for all samples and the RNA smear of amplified samples was between 200–3000 nucleotides. These parameters show that the amplification reactions were successful.

Amplified RNA was transcribed into labelled cDNA and hybridized to SS-H19k8 microarray slides. The obtained data were normalized by the lowess method (within the slide) and quantile method (between slides). The normalized expression profiles from different amplifications were compared among each other and the matching factor was calculated. The average matching factor between amplified CD34⁺ cells was 98.2 ± 0.6 %, which is a value that corresponds to the matching factor of two replications of the same cDNA microarray experiment. The comparison between amplified versus unamplified CD34⁺ samples could not be achieved because it is not possible to obtain enough starting material from patients for the direct hybridization to the slide. Therefore, the entire protocol was tested on the cell line HL-60 first.

Based on these results we can summarize that the amount of amplified RNA obtained from 25 ng of total RNA is sufficient for performing successful cDNA microarray analysis. Even such a small amount of starting total RNA does not influence the microarray results in principle. The expression profiles obtained from the CD34⁺ clinical samples were of high quality.

Discussion

Reliable methods for the precise monitoring of global gene expression in various types of cells are crucial in the research of many biological and medical disciplines. Yong et al. (2006) isolated RNA from 68 clinical samples, but a sufficient amount of total RNA for expression profiling was isolated only from 19 samples. This example illustrates that RNA isolation from clinical material in the quality and quantity required for experimental use is a complicated task. In the present study, we searched for a reliable and reproducible method of isolating sufficient amounts of total RNA for microarray analysis from a limited number of cells. Lack of RNA caused by the lack of starting clinical material is a common obstacle in biomedical research. In our case, CD34⁺ pluripotent stem cells represent only 1–4% of bone marrow mononuclear cells (Krause et al., 1996; Attia et al., 2003). In addition, the microarray technology we use requires 5–50 µg of input total RNA (Baugh et al., 2001; Feldman et al., 2002; Jenson et al., 2003; Livesey, 2003; King et al., 2005; Patel et al., 2005) or 0.1–5 µg poly(A) mRNA (Duggan et al., 1999; Spiess et al., 2003) in order to produce reliable gene expression profiles. This impediment motivates development of experimental protocols that allow application of microarray technologies in situations where the amount of starting biological material is limited.

In this study, we isolated total RNA from 5×10^3 to 2.5×10^5 of HL-60 cells using three different methods/kits – the TriReagent, RNAqueous Kit and RNeasy Micro Kit. RNA isolation using the TriReagent produced RNA of significantly lower purity than the other two methods. The quantity and purity of RNA isolated by the other two kits were satisfactory. We chose to use the RNAqueous™ Kit because in this method, the cells are ruptured and RNA is released directly into lysis solution without any loss. Endogenous ribonucleases are inactivated simultaneously. Furthermore, this method minimizes the chance of RNase contamination and yields products with better A260/280 ratios than the RNeasy Micro Kit.

Our results indicate that the variability in cellular RNA yields is present across different cell types as well as between different physiological stages of the same cell populations. A typical mammalian cell contains 10–30 pg of total RNA (Copois et al., 2003; Wang, 2005), while our calculations show that one cell contains 4.5–11.8 pg of total RNA. This is probably due to the fact that the loss of RNA during the isolation process is much higher when working with small numbers of cells compared to the large ones.

RNA amplification is a common method used to increase the number of mRNA molecules for hybridization on a microarray slide due to the fact that the yield of

Table 5. Yields and purity of CD34⁺ amplified RNA generated from known amounts of input total RNA after one-round amplification

Total RNA input	mRNA (2 % of total RNA)	Average yield of amplified mRNA	260/280 nm	Fold amplification
25 ng	0.5 ng	203 ng	2.20	406
50 ng	1.0 ng	381 ng	2.14	381
100 ng	2.0 ng	878 ng	1.98	439
200 ng	4.0 ng	1 503 ng	2.06	376
300 ng	6.0 ng	2 864 ng	2.10	477
500 ng	10.0 ng	4 483 ng	2.03	448

RNA after isolation is often insufficient for microarray analysis. To evaluate the amplification procedures available, some authors applied serial dilution of total RNA to obtain sequentially smaller RNA samples and subjected these samples to amplification (Jenson et al., 2003; Kurimoto et al., 2006). However, the results obtained from these studies are only informative at best because of the diverse experimental conditions. More importantly, diluting RNA samples isolated from a large number of cells is different from isolating RNA directly from a small number of cells. Therefore, in our evaluation, we limited the number of HL-60 cells used for RNA isolation to mimic the situation where the amount of the starting clinical material is limited.

We chose to use the SenseAmp Plus Kit for amplification. This kit synthesizes the sense-strand mRNAs, which are nearly identical to the original ones. We used oligo(dT)-T7 promotor primer at a ratio of 1 : 10 to the starting template, based on our observations and findings of Jenson et al. (2003). Higher primer concentrations lead to multimerization of primers and can also result in increased background hybridization (Kenzelmann et al., 2004).

Amplified and unamplified RNA were transcribed into labelled cDNAs and applied to microarray slides. After scanning, image analysis and normalization, the data were compared. As expected, the matching factor drops with the decreasing amount of total RNA added to the amplification reaction, as has also been shown by Baugh et al. (2001). However, the values of matching factors are still satisfactory even for the samples with little starting RNA and indicate that RNA amplification does not significantly alter the expression profile. Therefore, this procedure can be used in cases when microarray analysis is required and only a limited number of cells is available.

The results obtained from amplified and unamplified RNA samples have also been compared in many reports. Nygaard and Hovig (2006) showed that linear amplification provides reliable data for correct microarray analysis even if gene expression ratios are not completely preserved between amplified and unamplified material. Similarly, Jenson et al. (2003) and Patel et al. (2005) demonstrated that linear RNA amplification of small amounts of input RNA and use of this amplified RNA in microarray experiments yield results that are relatively comparable to results from experiments using unamplified RNA. Likewise, the studies of Baugh et al. (2001) and Stoyanova et al. (2004) indicated that the amplification does not reduce the overall sensitivity and has only a minor effect on fidelity. Taken together, these microarray experiments using amplified RNA generate reproducible results with only small differences compared to results obtained using unamplified RNA.

Many protocols allow amplification of less than 10 ng of total RNA, although they require two to three rounds of amplification. For instance, Li et al. (2005) used 10 ng of total RNA for a two-round amplification, and Baugh et al. (2001) reduced the starting material to 2 ng of total RNA for a two-round amplification procedure. Patel et al. (2005) attempted to amplify 500 pg of total RNA in three-round amplification. Nonetheless, these authors concluded that the level of agreement between amplified and unamplified material was high, although the correlation value was slightly reduced, indicating that variability increased as the quantity of RNA amplified was reduced. Furthermore, Feldman et al. (2002) and Jenson et al. (2003) observed that additional rounds of amplification resulted in truncation of the transcript. Spiess et al. (2003) strongly advised against extending the *in vitro* transcription step to increase the amounts of amplified RNA because the prolonged incubation time leads to decreased quality of amplified RNA. Also according to our results, the incubation longer than 16 hours increased the yield but also the amount of short RNA transcripts. Nonetheless, the amplification step is generally a multistep and laborious procedure, which requires a few days to complete.

The findings mentioned above indicate that the method described in this article is reliable and the results are reproducible, especially in cases where numbers of cells are limited. In our case we successfully applied this method to $CD34^+$ stem cells from clinical samples and we obtained enough RNA to produce high-quality microarray expression profiles. We conclude that the combination of RNAqueous Kit for RNA isolation and the SenseAmp Plus Kit for one-round RNA amplification leads to high-fidelity and reproducible microarray expression data even when a limited number of cells is used.

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