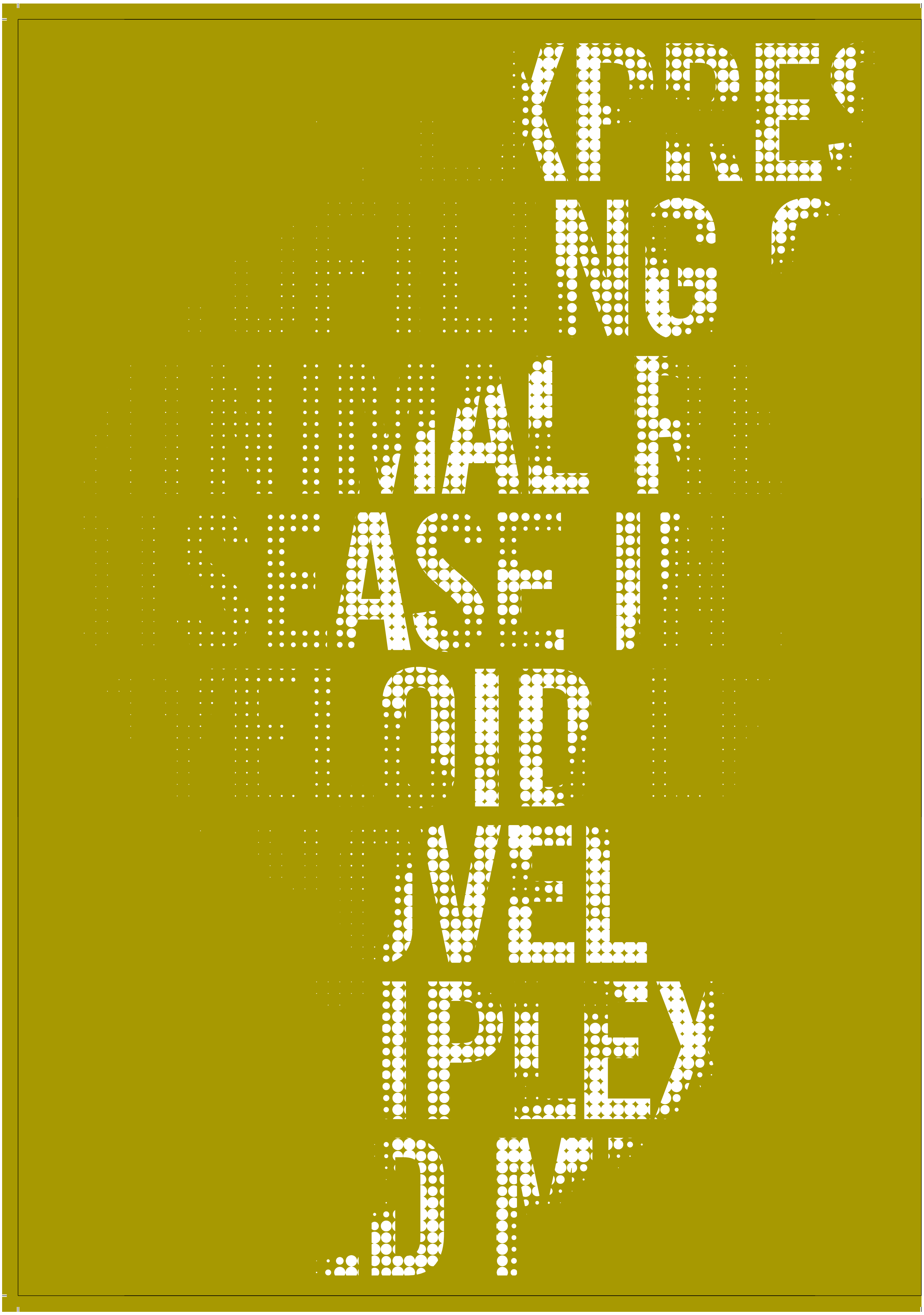

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GENE EXPRESSION PROFILING OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA BY NOVEL MULTIPLEX-PCR BASED METHOD

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Leukemia. 2004 Dec;18(12):1981-8

ABSTRACT

In acute myeloid leukaemia (AML) alterations in apoptotic pathways are crucial for treatment outcome, resulting either in refractoriness or in Minimal Residual Disease (MRD).

The apoptosis characteristics of MRD cells may differ from those at diagnosis and thereby determine the adequacy of further treatment. Such characteristics are largely unknown, since studies hereto are hampered by minimal cell availability.

This study explores the applicability of the recently described RT-multiplex Ligation-dependent Probe Amplification (RT-MLPA) for gene expression analysis of small amounts of RNA obtained from MRD cells.

Reproducibility and dilution experiments showed that the relative expression of 37 apoptosis related genes starting with only 1000 cells, could be measured with 12% variation; for 100 cells, 31/37 genes could still be quantified, though expression variation increased.

In practice 100-1000 leukemic cells can be obtained from bone marrow samples with clinically relevant MRD percentages of 0.01-0.1. Procedures often necessary to obtain AML blasts, that is, FACS-sorting, freeze-thawing or combinations are possible, provided that selected viable non-apoptotic cells are used.

Concluding, RT-MLPA allows accurate gene expression profiling of MRD cells. This method will help to gain insight in the processes of MRD emergence and persistence in AML, which may ultimately guide new therapeutic strategies in AML.

INTRODUCTION

High complete remission rates are currently achieved in patients with acute myeloid leukemia (AML)[1]. However, many patients eventually relapse due to the emergence, persistence and finally outgrowth of the so-called minimal residual disease (MRD).
In 80% of AML patients, MRD can be detected by multi-parameter flow cytometry on the basis of aberrant combinations of cell-surface markers, established at diagnosis and absent or present at very low frequencies on normal cells[2-6]. Detection and quantification of these low numbers of malignant cells enable to predict imminent relapse before clinical and hematological signs of recurrence[7,8].
Refinement of MRD characteristics is expected to lead to the defining of patient risk categories, which should ultimately permit the design of patient-tailored treatment strategies. Secondly, the study of MRD frequencies in time, would allow establishment of important time points for clinical intervention.
However, it is only the characterisation of the MRD cells that will enable to design the nature of such intervention: chemotherapy, immune therapy or therapy using signal inhibitors.
We have shown previously that it is possible to combine Immunophenotypical detection of (MRD) cells with functional characterisation[5,9]. Among others, this has shown transient changes in apoptosis-related protein expression in the course of disease[10]. A major limitation of these studies was the relatively low number of parameters that could be studied: since clinically relevant MRD percentages in bone marrow are in the range of 0.1-0.01% of MRD, the actual availability of MRD cells is in the order of a few thousand cells or less.
Recently part of our group (JPS, CJE) described the use of a single tube PCR-based MLPA method for the detection and quantification of >35 DNA sequences[11]. The technique has subsequently been adapted for mRNA expression[12].
We now report the adaptation of this method for expression profiling of samples with cell numbers as low as a hundred cells, without prior amplification steps or nested PCR.

MATERIALS AND METHODS

Human leukaemia bone marrow cells
Mononuclear cells were isolated by a Ficoll gradient 1.077 g/ml (Amersham Biosciences, Uppsala, Sweden). Erythrocytes were lysed for 10 min on ice, by adding 10 ml lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) to the cell pellet.
The mononuclear cells were frozen in RPMI1640 (Invitrogen, Breda, The Netherlands) medium containing 20% heat inactivated Fetal Calf Serum (FCS) (Gibco, Breda, The Netherlands) and 10% DMSO (Riedel-de Haën, Seelze, Germany) and stored in liquid nitrogen until analysis.
For analysis, samples were rapidly thawed by dilution with preheated (37°C) RPMI containing 40% FCS. To avoid aggregation of dead cells 20 ml of RPMI1640/40% FCS containing 200 µM $MgCl_2$ (Fluca, Buchs, Germany) and 0.1 mg/ml DNase 1 grade 1 (Roche, Mannheim, Germany) was added for 45 min at 37°C.

Cell lines
HL-60 (AML FAB M2), MV4;11 (AML FAB M5, t(4;11)) and RS4;11 (ALL L2, t(4;11)(q21;q23)) were obtained from DSMZ-GmbH (German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany).

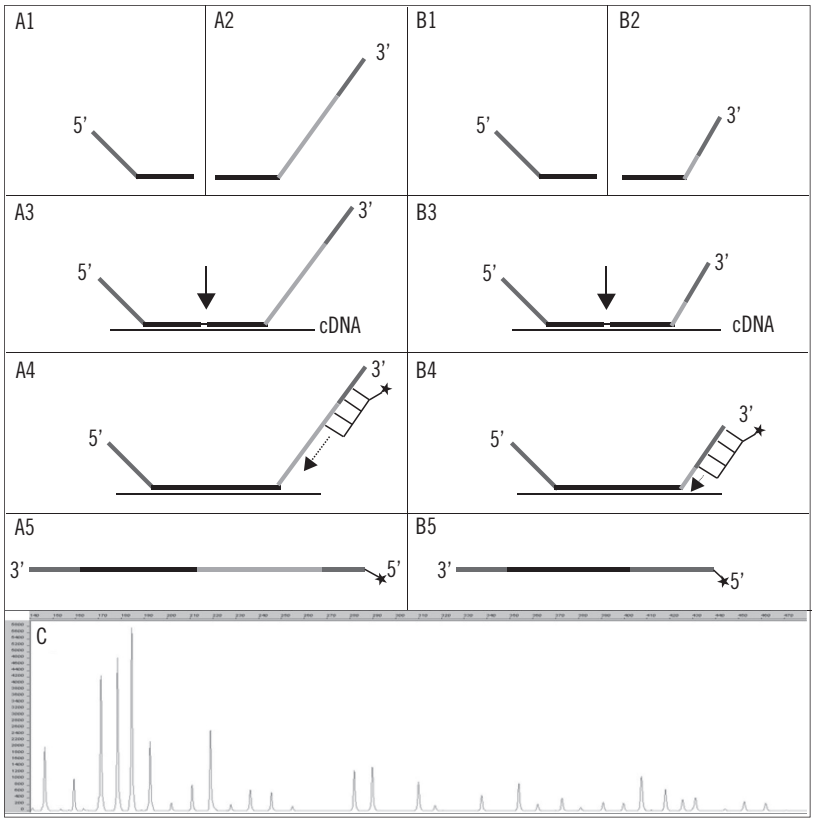

Figure 1. Outline of RT-MLPA.
Total RNA was converted to cDNA using specific RT primers designed directly downstream of the target probe hybridisation site[12,14]. For the detection and quantification of mRNA expression of target gene A, two cDNA binding probes are used together (A1+A2).
The first probe (A1) consisted of two synthetic sequences, the hybridising sequence depicted in black and the PRC primer-binding site depicted in dark grey. The second probe (A2) was constituted of two synthetic probes, depicted in black (hybridising sequence) and dark grey (PCR primer binding sites), joined to a non-hybridising M13 derived oligonucleotide depicted in light grey.
Each target gene specific probe combination contains a M13 derived oligonucleotide of unique length, e.g. for detection of target gene B, the M13 oligonucleotide B2 depicted in light grey is shorter than the M13 oligonucleotide used in probe A2 for detection of target gene A. In the end this resulted in products in the range of 130-472 nucleotides[11].
After hybridisation of the target specific probe combination (A3 and B3) to adjacent sites on the target sequence, the probes can be ligated by a thermo stable ligase (A4 and B4). All ligated probes can than be exponentially amplified by PCR, using only one primer pair of which the forward primer is fluorescently labelled.
The fluorescently labelled amplification products of unique length (A5 and B5) can than be quantified by means of capillary electrophoresis. Figure 1C shows a typical example of an RT-MLPA apoptosis probe panel. Expression levels of individual genes are characterised by different peaks, where the peak area's represent the amounts of fluorescently labelled amplification product of unique length. For relative quantification of gene expression peak area of target genes are divided by the peak area of house keeping genes.

HL-60 and MV4;11 were cultured in RPMI1640 medium supplemented with 10% FCS and maintained at 0.5-1.0 x 10^6 cells/ml. RS4;11 was cultured in alpha-MEM (Gibco, Breda, The Netherlands) with 10% FCS and maintained at 0.2-1.0 x 10^6 cells/ml.
All cell lines were incubated in 5% CO_2 humidified atmosphere at 37°C.

Sorting of viable CD45 dim cells
When using frozen-thawed AML samples at diagnosis, viable 7-AAD negative (7-AAD^-) blasts or viable non-apoptotic (7-AAD^-/$AnnexinV^-$) or apoptotic (7-AAD^-/$AnnexinV^+$) cell fractions were obtained as follows: cells were stained in PBS/0.1% BSA (Braun) for 15 min at room temperature with CD45-APC (Dako Corporation, Carpenteria, CA) 1:20 and 7-AAD (Via Probe™, San Diego, CA) 1:20.
Cells were washed and resuspended in binding buffer of the AnnexinV-FITC apoptosis-kit (Nexins Research, Kattendijke, The Netherlands) at a concentration of 10^5-10^6 cells/ml and incubated with AnnexinV-(FITC) 1:1000 for 15 min on ice.
A Becton Dickinson Vantage flow cytometer was used to select cell populations. Viable AML blasts were cold-sorted into 15 ml Falcon-tubes using PBS (Braun) as a sheet solution and kept on ice.
In specific cases (see Results), leukemic cells had to be identified in a background of non-AML cells. To establish such, leukaemia associated phenotypes (LAPs) were determined at diagnosis as previously described[5,8,13] and used to sort the populations of interest.

RNA preparation
After centrifuging the purified or non-purified cells in order to remove the sheet solution, total RNA was prepared using RNBee solution (Tel-test Inc., Friendswood, TX) according to the manufacturer's recommendations. Total RNA was stored at -80°C.

MLPA
MLPA method described by Schouten et al.[11] was adapted for use on RNA derived cDNA[12]. The method is outlined in short in Figure 1. The probe set used in this assay is targeted against apoptosis-related genes and described in Table 1.
In probe design, allowance has been made for possible DNA contamination as well as for the presence of splice variants[12].
In the case of highly abundant mRNA targets, which on all or most occasions causes off-scale signals, competitor oligonucleotides[11,12,14] were added to the probe mixture.

RT-MLPA procedure and analysis
Human total RNA samples were diluted in RNAse-free water (2 µl) and kept on ice. After addition of 1.0 µl SALSA-RT buffer (MRC-Holland, Amsterdam, The Netherlands) 0.5 µl RNAse free water and 1.0 µl RT-primers (0.5pM of each RT-primer) and dNTPs (4 fmol of each dNTP) at 0-4°C, RNA-samples were mixed, heated for 1 min at 80°C in 200 µl tubes in a thermocycler with a heated lid (MJ, Biozym) to melt the secondary structure of the mRNA and incubated for 5 min at 45°C.
Samples were maintained at 37°C while 1.5 µl 20 U/µl MMLV Reverse Transcriptase (Promega M1701, Madison, USA) was added. cDNA was synthesized for 15 min at 37°C. Inactivation of MMLV-RT enzyme was for 2 min at 98°C. Samples were cooled to 25°C and 1.5 µl SALSA-MLPA buffer (1.5 M KCl, 300 mM Tris-HCl pH 8.5 and 1mM EDTA) mixed with 2 µl probe mix (1-4 fmol of each synthetic probe oligonucleotide and each M13 derived oligonucleotide) in TE was added (RT-MLPA outline is shown in Figure 1). Samples were heated for 1 min at 95°C and incubated for 16h at

60°C to gain optimal hybridisation. Ligation of annealed oligonucleotides was performed by diluting the samples to 40 µl with dilution buffer (2.6 mM $MgCl_2$, 5 mM Tris-HCl pH 8.5, 0.0013% non-ionic detergents, 0.2 mM NAD) containing 1U Ligase-65 enzyme, and incubated for 15 min at 54°C. The ligase enzyme was inactivated by heating at 98°C for 5 min.
Ligation products were amplified by PCR and therefore 10 µl of the ligation reaction was added to 30 µl PCR buffer at room temperature.
While at 60°C, 10 µl of a buffered solution containing PCR primers (10 pmol), dNTPs and 2.5 U SALSA polymerase (MRC-Holland) was added. PCR Cycles 20s at 95°C, 30s at 60°C, 1 min at 72°C, one cycle 20 min at 72°C and cooling down to 4°C.
The samples were amplified with one reverse primer (GTGCCAGCAAGATCCA-ATCTAGA) and one forward FAM-labelled primer (GGGTTCCCTAAGGGTTGGA) and were analysed on the ABI 3100 Avant capillary electrophoresis system (Applied Biosystems, Warrington, UK) after addition of the GeneScan-500 ROX size standard. Data were analysed using Genotype and GeneScan software (Applied Biosystems).

Reference genes
As internal references we have selected five House Keeping Genes (HKGs), that is, $ß_2$-microglobulin (B_2M), ß-glucuronidase (GUS-B), thymosin ß-10 (TMSB-10), polyadenylate-binding protein cytoplasmic-1 (PABPC1) and ferritin light chain (FTL)[15].

Real-time PCR quantification
LightCycler RT-PCR (Roche, Penzberg, Germany) has been used to confirm the value of relative quantitative RT-MLPA measurements.
PCR amplification was performed on human reverse transcribed RNA using pd(N)6 random hexamer primers (Amersham Biosciences) after removal of DNA by means of treatment with RNAse free DNAse (Promega, M6101). LightCycler RT-PCR primers have been designed on the same sequence as the RT-MLPA probes in order to span the same exon boundaries. The following oligonucleotides were used as primers for LightCycler RT-PCR: Mcl-1 (AF118124); Mcl-1F 5'- CAAAACGGGACTGGCTAGTT-3'; Mcl-1R 5'- CAGCAACACCTGCAAAAGC-3'; Bax (L22473); Bax F 5'- TGGAGCT-GCAGAGGATGATT-3'; Bax R 5'- CAGTTGAAGTTGCCGTCAGA-3'; GUS-B (NM_000181); GUS-B F 5'- GAAAATATGTGGTTGGAGAGCTCATT-3'; GUS-B R 5'-. CCGAGTGAAGATCCCCTTTTTA-3'; B_2M (AB021288); B_2M F 5'-GAGTAT-GCCTGCCGTGTG-3'; B_2M R 5'- AATCCAAATGCGGCATCT-3'.
Total RNA derived cDNA samples from various leukemic cell lines (HL-60, MV4;11 and RS4;11) together with three patient derived BM samples obtained at diagnosis (FAB: M5b, M2, M2) were used as template.
Each reaction contained 2 µl of cDNA, 0.5 pmol/µl of forward and reverse primers, 2 µl of 10x LightCycler Fast Start DNA Master Mix SYBR Green (Roche) and H_2O added to a final volume of 20 µl.
The PCR conditions consisted of 10 min at 95°C, 45 cycles 15 s at 95°C, 10 s at 59°C and 10 s at 72°C.
For quantification, standard curves were prepared by serial 10-fold dilutions from 10^6 down to 10^2 molecules of either Mcl-1 or Bax PCR-products cloned into Promega T-easy vectors (Promega). LightCycler RT-PCR expression data were calculated relative to the expression of B_2M[16-20] and GUS-B[19], as advised in the Europe Against Cancer Program[21].

Statistical analysis
Differences in normalized target gene expression between different populations or conditions were calculated using the Student's *t*-test. Correlations between different conditions were determined by applying the Spearman rank correlation coefficient. Smallest detectable differences in reproducibility and dilution experiments were determined by applying Bland & Altman statistics. $P<0.05$ was considered significant in all analyses.

RESULTS

Reproducibility and sensitivity of RT-MLPA
For the assessment of reproducibility of RT-MLPA method and in order to determine whether for future experiments the assay had to be performed in duplicate or more, a large amount of RNA was isolated from viable blasts of an AML bone marrow (BM) sample.
Experiments were performed on 4 consecutive days with quadruplicate measurements per day, resulting in a total number of 16 data sets (each containing data on 37 apoptosis related genes). The mean of the 16 the values was considered to be the ideal value for each separate gene.
When using 100 ng RNA, the inter measurement correlation was excellent ($R=0.937$, $p<0.0001$). We then compared each individual value as well as all possible combinations of duplicates, triplicates and quadruplicates obtained on the same day with the ideal value. Results were obtained with Bland & Altman statistics and showed that performing single RT-MLPA measurements resulted in 72% expression variation, which declined to 53%, 37% and 31% when using duplicate, triplicate or quadruplicate measurements respectively.
The whole experiment was repeated using RNA from viable blasts of a different AML BM sample, with similar results ($R=0.924$, $p<0.0001$). Using duplicates offers a compromise between reproducibility on the one hand and effort in time and costs on the other. The reproducibility of results when using duplicate measurements was further confirmed in four subsequent independent experiments using BM samples of four other AML patients. In each of these four experiments the ideal value was the mean of the duplicate measurements of 100 ng RNA.
Here, the expression variation of the whole gene panel when using duplicate measurements and comparing 10000 cells (which is close to the 100 ng RNA previously tested) to 100 ng RNA was 66%, which approximates the 53% previously found.
To judge for future experiments, whether or not individual genes are differentially expressed between samples, smallest detectable differences for individual apoptosis-related genes were determined using 100 ng RNA and duplicate measurements and applying Bland & Altman statistics (Table 1).

Subsequently, we determined the minimum cell number that is still appropriate for measuring reliable and reproducible normalised gene expression. In dilution experiments containing 10 intermediate steps, RNA was isolated from a range of 50 to 10000 FACS-sorted cells (Table 2).
Comparison of the normalised gene expression profiles, showed 300 cells to be the minimal cell number required to determine the expression of all 37 apoptosis-related genes in the panel. Although excellent correlations existed between data obtained from 100 ng and as few as 100 cells ($p<0.0001$, Table 2), gene expression of less than 300 cells was significantly different compared to 100 ng RNA (last column Table 2).

The smallest detectable difference for the whole panel also increases from 53% when using 100 ng RNA, to 78% when using 800 cells and 136% when using 300 cells. This increase in variation is predominantly caused by a relative large increase of a few genes with relatively low expression.
It should be kept in mind that the increased variation can be reduced by performing experiments in triplicate or quadruplicate.
Thereby, RT-MLPA appears to be more distinctive than microarray analysis for which differential expression has to be at least 100%[22]. RT-MLPA thus offers the opportunity to reliably study small cell numbers.

Table 1. Smallest detectable difference in apoptosis related gene expression using 100 ng RNA and duplicate measurements

BCI-2 family	Bax-like	BH3-only	IAP-family	Assorted	Assorted	Remaining
Anti-apoptotic	**Pro-apoptotic**	**Pro-apoptotic**	**Anti-apoptotic**	**Pro-apoptotic**	**Anti-apoptotic**	
BCI-W (52%)	MCI-1-short (25%)	Noxa (31%)	NIAP/BIRC1 (63%)	AIF/PDCD8 (41%)	CFLAR (36%)	BLK (60%)
BCI-XI (27%)	Bax-long (46%)	Bad (843%)#	cIAP/BIRC2*	Apaf-1 (41%)	PI9 (48%)	PARN (24%)
BCI-2 (111%)#	Bax-short (48%)	Bak-1 (48%)	cIAP2/BIRC3 (84%)	Apaf-1L (41%)	Apaf-1XL (47%)	
BCI-2A1 (36%)	BCI-Rambo (55%)	Bim (54%)	XIAP/BIRC4 (39%)	Smac (45%)		
MCI-1-long (36%)	BCI-G*	Bid (46%)	Survivin/BIRC5 (397%)#			
		Harakiri*	Appolon/BIRC6 (26%)			
		Puma (42%)	Livin/BIRC7*			
			MAP-1 (45%)			
			BNIP3L/NIX (24%)			
			Bmf (61%)			

*Expression signals of BCI-G, Harakiri, BIRC2 and BIRC7 were extremely low or not detectable. #Expression signals of BCI-2, Bad and BIRC5 show large differences most probably due to relative low expression levels

Specificity of RT-MLPA

To determine how specifically the AML blast can be identified based on gene expression in an environment of abundant normal BM cells, such an MRD situation was mimicked.
We spiked 2000 FACS-purified AML blasts cells bearing a LAP, that is, a combination of aberrant cell surface makers, in this case $CD7^+/CD13^-/CD34^+$ (Figure 2a), at a final concentration of 0.1% into a BM sample of a cardiac surgery patient not bearing this LAP (Figure 2b). The spiking result is shown in Figure 2c.
Gene expression patterns of these spiked LAP^+ cells after purification by cell sorting were compared with sorted LAP^+ blasts before spiking (from Figure 2a) and revealed

excellent correlations, R=0,999, p<0,001, without significant changes in gene expression (p=0.728, comparison shown in Figure 2d).
The gene expression profiles of LAP$^+$ AML BM cells (obtained from Figure 2a) show completely different gene expression profiles when compared to the unsorted normal BM cells (obtained from Figure 2b; see Figure 2e). This indicates that gene expression in AML blasts identified by LAP and present at very low frequency in a background of normal cells can be determined very accurately.

Table 2. Sensitivity of RT-MLPA method

Cell number	N exp*	N values#	R§	P value‖	P valaue ‡
10,000	2	80	0.840	<0.0001	0.460
2,000	2	80	0.846	<0.0001	0.307
1.000	4	160	0.847	<0.0001	0.159
800	4	160	0.863	<0.0001	0.203
500	1	40	0.840	<0.0001	0.198
400	4	160	0.850	<0.0001	0.192
300	4	160	0.834	<0.0001	0.060
200	2	77	0.921	<0.0001	**0.014**
100	2	77	0.844	<0.0001	**0.001**
50	1	38	0.334	0.041	**<0.001**

Correlations between apoptosis related gene expression (n=37) and the HKGs (n=3) in a range of 50 to 10,000 FACS-sorted cells and the apoptosis profile of 100 ng of RNA, all obtained from the same AML sample at diagnosis

*Number of independent experiments each performed in duplicate. #Total number of obtained expression values for all genes tested in each of the four of conditions. Note that when using less than 300 cells, the number of values no longer is 40 per experiment, since genes with the lowest expression are now undetectable. §Correllation between the two tested conditions (Spearman rank correlation coefficient). ‖P-value of this correlation. ‡P-value of the difference between the tested conditions (Student's t-test)

House Keeping Genes
Five house keeping genes (HKGs) were tested for their expression stability under experimental conditions. These reference genes were tested for consistency of expression during experimental procedures necessary to obtain as well as to store primary AML blasts.
In order to distinguish between true target gene variability (37 genes) and HKG (5 genes) inconsistency, target gene expression as a ratio of the different HKGs was calculated (Figure 3). Gene expression of fresh AML patient BM samples, not used in previous validation experiments, was compared with those of frozen pellets.
We assume that in case of true variability of target genes, but not of the HKGs, the relationship between gene expressions in the two different experimental conditions should reveal similar characteristics for all 5 HKGs.
After elaborate testing of the HKGs, it appeared that two of the tested HKGs, TMSB-10 and PABPC had to be excluded from the apoptosis probe panel since their expression levels were too abundant and not in the range of target gene expression. Furthermore, FTL had to be excluded from further analysis because a DNA signal was measured at the FTL designed probe length.

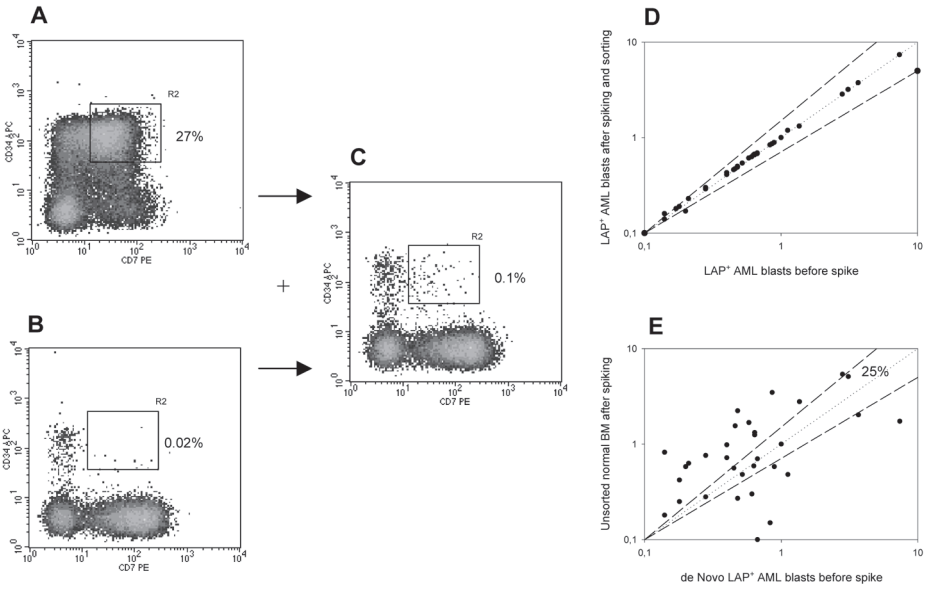

Figure 2. Simulation of MRD using AML blasts at diagnosis and normal bone marrow.
A. AML BM at diagnosis bearing the aberrant antigen combination $CD34^+/CD7^+/CD13^-$ on 27% of the total gated blast cells (gate R2).
B. BM of a cardiac patient with a LAP^+ background of 0.02% of total gated cells are depicted in gate R2.
C. A total of 2,000 FACS purified LAP^+ AML cells (purity 99%) were spiked into the cardiac marrow with a final frequency of 0.1%.
D. After retrieving the spiked AML blasts by means of FACS sorting on the LAP^+ cells, gene expression profiles were compared to the expression profiles of sorted LAP^+ AML blasts before spiking and showed excellent correlations ($R=0.999$, $p<0.001$). When applying 53% cut-off levels as defined for duplicate measurements, as much as 98% of the measurements fall within the interval
E. Comparison of the expression profile of the unsorted normal BM with that of sorted LAP^+ AML blasts reveals differences in gene expression of these populations. Only 25% of all genes showed similar (<53% differences) expression.

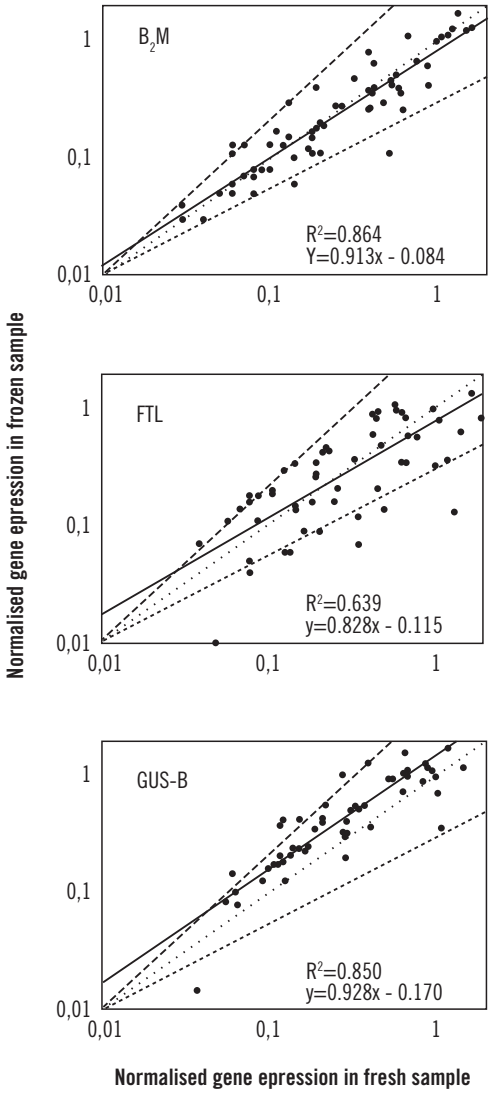

Figure 3. Role of HKGs in the normalisation of gene expression profiles in fresh and fresh-frozen cell pellets.

Apoptosis related gene expression of one AML sample normalised to three different HKG (B_2M, FTL, GUS-B). On the X-axis the normalised expression of the fresh sample is shown, on the Y-axis the normalised expression of the sample after storage as a pellet. Note that both conditions reveal almost identical expression for all 3 HKGs (accepting 53% inter measurement variation: indicated by the broken lines- - -).

Although falling within the inter measurement variation, in the case of GUS-B most of the genes are expressed at higher levels in the frozen pellet, indicating that the absolute expression of GUS-B itself was lower in the frozen pellet compared to the fresh sample.

In the case of inconsistency of a specific HKG, only the relationship using this particular HKG would show a significant difference in gene expression pattern between the two experimental conditions tested. Figure 3 is an example of such an approach and shows that gene expression between fresh and liquid nitrogen stored samples is close to y=x for B_2M and FTL normalised gene expression, while GUS-B shows higher expression for most of the normalised genes in the liquid nitrogen sample (y= x+0.17).
This indicates that the GUS-B itself is expressed at lower levels in frozen samples as compared to fresh samples.
When considering the method's inherent variability when using duplicate measurements (53%) still more than 90% of GUS-B normalised gene expression would fall within that safety interval, indicating that in this case GUS-B is still a reliable HKG.
The final choice for a specific HKG was made using LightCycler RT-PCR by comparing B_2M and GUS-B for absolute copy number (next paragraph).

External validation by Lightcycler RT-PCR
In a previous report RT-MLPA expression of several genes has been measured by microarray and compared with LightCycler RT-PCR[12].
As an extension, RT-MLPA expression levels of Bax and Mcl-1 were compared with LightCycler RT-PCR in leukemic cell lines as well as in three AML patient BM samples using B_2M and GUS-B as reference genes (Figure 4).
Both methods revealed a good correlation (R=0.88; p=0.02). Based on more stable Ct value in a larger series of myeloid leukemia samples (data not shown), GUS-B was selected to be the HKG of first choice for further comparison.

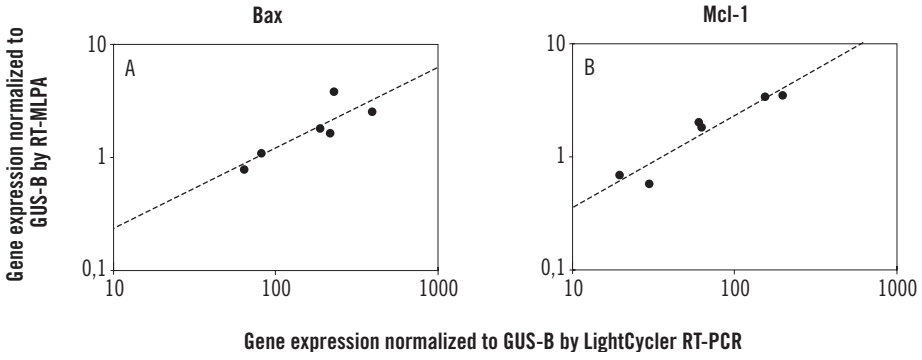

Figure 4. Comparison of gene expression by RT-MLPA versus Lightcycler RT-PCR.
Bax (panel A) and MCL-1 (panel B) expression measured by RT-MLPA (Y-axes) and LightCycler RT-PCR (X-axes) were normalised to GUS-B, in three patient BM samples as well as in three leukemic cell lines (RS4;11, HL-60, MV4;11). As template, RNA derived hexamer cDNA was used.
Good correlations between RT-MLPA and LightCycler RT-PCR were observed for both Bax and MCL-1 (R=0.88; p=0.02). This indicates that, although absolute copy numbers do not match, RT-MLPA technique provides adequate information on relative proportions.

Procedure induced changes in normalised gene expression

Procedures necessary to obtain AML blasts may induce changes in gene expression, while using the appropriate HKG for normalisation. FACS sorting is one of the most important procedures necessary to study AML blasts present in low frequencies in whole bone marrow samples. Therefore, we tested whether target gene expression would be influenced by this procedure.

To obtain analogous total cell populations for comparison of the sorted and nonsorted fraction, FACS sorting of AML patient BM not used in former experiments was performed without labelling and gate setting using comparable sorting times when testing different procedures.

An excellent correlation was found between normalised values (procedure 1, columns 4 and 5 of Table 3), but most important, no significant differences in gene expression existed between both procedures (Table 3, column 6).

To study whether AML patient BM material stored in liquid nitrogen was also appropriate, the same experiment was performed on frozen-thawed cells (procedure 2 of Table 3) with similar results.

To establish in a more direct way whether RT-MLPA results obtained from fresh and frozen-thawed samples are comparable, the following experiment was performed: Cells were sorted from fresh BM samples as well as from frozen-thawed BM samples. Since it is likely that the freeze-thawing process, which is accompanied by considerable viability changes, induces expression changes due to the presence of dead cells, sorting was performed on viable ($7AAD^-$) blasts ($CD45^{dim}$) cells.

To discriminate leukemic blasts from contaminating normal cells and thereby facilitating the analysis of blasts even when present in low frequencies, CD45 APC (Dako) or CD45 PE (Becton Dickinson) was used for gating on the CD45 dim population[23].

The results show good correlations between both conditions (Table 3, procedure 3, columns 4 and 5) and no significant differences (Table 3, last column).

Lastly, expression profiles of fresh AML BM samples were compared directly with fresh cells frozen as a pellet in liquid nitrogen. As expected no significant differences were found (Table 3, procedure 4).

Table 3. Procedure induced changes of gene expression

Tested procedures	N exp*	N values#	R§	P value‖	P value‡
1. Fresh no sort - fresh sort (without gate setting)	1	40	0.992	(<0.0001)	0.959
2. DMSO no sort - DMSO sort (without gate setting)	2	80	0.856	(<0.0001)	0.290
3. Fresh sort ($7AAD^-$/CD45dim) - DMSO sort ($7AAD^-$/CD45dim)	3	240	0.895	(<0.0001)	0.660
4. Fresh - frozen pellet	2	80	0.784	(<0.0001)	0.440

*Number of independent experiments each performed in duplicate. The total number of different samples was 3.
#Total number of obtained expression values for all genes tested in each of the four of conditions.
§Correlation between the two tested conditions (Spearman rank correlation coefficient).
‖P-value of this correlation.
‡P-value of the difference between the tested conditions (Student's t-test)

Gene expression in viable and apoptotic cells

Although similar gene expression levels were found in fresh and liquid nitrogen stored samples (Table 3, procedure 3) when gating only on $7\text{-}AAD^-$ populations, it has to be noted that the $7\text{-}AAD^-$ population may contain early apoptotic cells, that can be tracked using AnnexinV[24].

With considerable percentages of such early apoptotic cells (AnnexinV$^+$, 7-AAD$^-$) present, this population may have significant impact on the observed apoptosis-related gene expression.
To study this, we analysed six AML patient BM samples that, upon thawing, showed both viable, early apoptotic and dead cells. The expression profiles in FACS sorted 7-AAD$^-$/AnnexinV$^-$ and 7-AAD$^-$/AnnexinV$^+$ populations showed significant differences ($p=0.0001$; $N=6$), indicating that additional gating on the AnnexinV$^-$ population in the 7-AAD$^-$ population is obligatory in at least part of the samples. For all future experiments, sorting of the 7-AAD$^-$/AnnexinV$^-$/CD45^{dim} population should be applied to obtain relevant information on gene expression profiles in viable nonapoptotic AML blasts.

DISCUSSION

We present the use of Reverse Transcriptase-Multiplex Ligation-dependent Probe Amplification (RT-MLPA) technique for gene expression profiling using low numbers of cells.
The technique enables simultaneous detection and relative quantification of expression of 40 genes in a single PCR reaction[12]. Reliable and reproducible detection of amplification products of all 40 probes is already possible using input of as low as 300 cells. Taken into account that gene specific smallest detectable differences determine the presence of differential expression and can be considerable for low expressed genes. This method thus enables the studies of expression profiles of a predefined set of genes in small cell (sub) populations such as present in MRD conditions. The technique may be applied as well for other applications such as characterization of low frequency stem cell fractions or small lymphocyte subpopulations.

In our attempt to validate the application of multi parameter RT-MLPA for these small cell subpopulations, we have encountered several potential pitfalls for gene expression profiling, which include mainly the methodology needed for the use of the biological materials.
Given the highly dynamic nature of mRNA gene transcripts and the sensitivity of mRNA to enzymatic degradation adequate preservation of RNA in clinical samples is obligatory. The effects of tissue handling on mRNA levels have been extensively studied and appeared to have a variable impact on gene expression measurements[25-27]. In our hands the tested procedures, FACS sorting and freeze-thawing plus storage, did not induce significant changes in gene expression. Therefore sample profiles obtained through different (combined) strategies were merged in one data set.
In situations of complete remission in AML, frequencies of malignant cells, of about 0.01-5% are common[13,28-30]. To enable to study these particular MRD cells it is necessary to identify these by the presence of a LAP.
Low percentages of LAP$^+$ MRD cells can be a limiting factor that determines whether or not an MRD sample can be used for RT-MLPA.
With very low percentages of LAP bearing cells, FACS sorting will be extremely time consuming. In the case of a CD34$^+$ LAP, pre-purification of CD34$^+$ cells by MACS technology will be used, to prevent artificial changes of target gene expression due to time delay between sample aspiration and RNA extraction[27].
The amount of aspirated marrow can be a limiting factor as well. With MRD frequencies of 0.1%, 10^6 cells are needed to obtain 1000 LAP bearing MRD cells for analysis by the RT-MLPA method. Aspiration of the amounts of marrow needed to obtain such total cell quantities appears to be feasible in practice.

Given the ability to study multiple parameters on sequential patient samples, including low cell number MRD samples, the RT-MLPA offers a novel pioneering technique. The low frequencies of malignant cells in MRD hamper the use of microarray for gene expression profiling. Microarrays enable the explorative and functional study of thousands of transcripts, but require considerable amounts of mRNA, which, in the case of MRD, would require extensive pre-amplification of mRNA. In contrast to genome wide, laborious screening by micro-array, the more restricted but easy-to-perform-RT-MLPA-approach yields fast and selective information. RT-PCR, on the other hand, is an extremely sensitive technique for detection and quantification of samples down to a single cell. The drawback of RT-PCR is its inability to simultaneously quantify different templates in a single reaction.
A second crucial issue was the choice of suitable control gene(s). The latter is important in order to assess the quality and quantity of RNA, correct for variations and exclude samples of poor quality. In our study we ultimately selected GUS-B, although B_2M gave satisfactory results as well.
A multitude of factors determine the treatment response in patients with AML. Effectiveness of chemotherapy in AML is in part determined by blast susceptibility to undergo apoptosis after adequate treatment. For that reason an RT-MLPA probe panel consisting of genes important in the mitochondrial pathway of apoptosis, which in part determines the response to treatment with chemotherapy, was used. Probe sets detecting other distinct genes can be designed as well; hereby the RT-MLPA technique can contribute to the development and improvement of new as well as already existing prognostic parameters.

In conclusion, the new RT-MLPA technique permits relative assessment of 37 apoptosis-related genes in very low numbers of cells, such as obtained with MRD blasts in follow-up bone marrow.
Applying RT-MLPA method on sequential samples and large patient groups will clarify mechanisms that contribute to chemotherapy resistance and MRD emergence in AML. Ultimately this will lead to a better understanding of the nature of MRD and guide future MRD directed therapies.

ACKNOWLEDGEMENT
We are indebted to Dr. J. Berkhof for statistical assistance.

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