

LEADING ARTICLE

Standardization and quality control studies of ‘real-time’ quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – A Europe Against Cancer Program

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Detection of minimal residual disease (MRD) has proven to provide independent prognostic information for treatment stratification in several types of leukemias such as childhood acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and acute promyelocytic leukemia. This report focuses on the accurate quantitative measurement of fusion gene (FG) transcripts as can be applied in 35–45% of ALL and acute myeloid leukemia, and in more than 90% of CML. A total of 26 European university laboratories from 10 countries have collaborated to establish a standardized protocol for TaqMan-based real-time quantitative PCR (RQ-PCR) analysis of the main leukemia-associated FGs within the Europe Against Cancer

(EAC) program. Four phases were scheduled: (1) training, (2) optimization, (3) sensitivity testing and (4) patient sample testing. During our program, three quality control rounds on a large series of coded RNA samples were performed including a balanced randomized assay, which enabled final validation of the EAC primer and probe sets. The expression level of the nine major FG transcripts in a large series of stored diagnostic leukemia samples ($n=278$) was evaluated. After normalization, no statistically significant difference in expression level was observed between bone marrow and peripheral blood on paired samples at diagnosis. However, RQ-PCR revealed marked differences in FG expression between transcripts in leukemic

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The EAC network was composed of the following additional laboratories:

Laboratory	Institution	Town, country	Representative
17. Haematology Department	Hammersmith Hospital, Imperial College School of Medicine	London, UK	J Kaeda
18. Institute of Pathology	HMDS	Leeds, UK	P Evans
19. Department of Hematology	Hôpital Necker-Enfants malades	Paris, FR	E Macintyre
20. Department of Hematology	Hôpital Calmette – CHU	Lille, FR	C Preudhomme
21. Department of Exp. Immuno-Hematology	CLB	Amsterdam, NL	CE van der Schoot
22. Department of Medical Genetics	Erasmus Campus	Brussels, BE	P Heimann
23. Department of Hematology and Oncology	Hannover Medical School	Hannover, DE	J Krauter
24. Children’s Cancer Research Institute	St Anna Children’s Hospital	Vienna, AT	T Lion
25. Department of Onco-hematology and Molecular Diagnostics	Università la Sapienza	Rome, IT	F Lo Coco
26. Department of Hematology	Ospedali Riuniti	Bergamo, IT	A Rambaldi
27. Institute of Hematology and Medical Oncology ‘Seragnoli’	University of Bologna	Bologna, IT	G Martinelli
28. Department of Hemo Cytogenetics	Institut Paoli Calmettes	Marseille, FR	M Lafage-Pochitaloff

A total of 25 laboratories were involved in the first three phases of the study with the collaboration of Applied Biosystems (no. 3) and the Department of Medical Information (no. 13). During the last phase (phase IV) laboratory no. 28 joined the group. UK = United Kingdom, FR = France, NL = The Netherlands, BE = Belgium, AT = Austria, IT = Italy, DE = Germany.

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samples at diagnosis that could account for differential assay sensitivity. The development of standardized protocols for RQ-PCR analysis of FG transcripts provides a milestone for molecular determination of MRD levels. This is likely to prove invaluable to the management of patients entered into multicenter therapeutic trials.

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Introduction

Current treatment protocols for acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are based on prognostic factors, which contribute to therapy stratification.^{1–3} Key prognostic factors identified in leukemia over the years include pretreatment characteristics such as age, WBC count, immunophenotypic profiles, specific chromosomal abnormalities,^{4,5} aberrant fusion genes (FGs), and mutations, such as *FLT3* gene alterations in AML.^{6,7} Response to initial therapy provides a further well-known prognostic marker, in particular the presence or absence of blasts in the bone marrow after induction therapy in ALL and AML,^{8–12} or cytogenetic response in CML patients.^{13,14}

However, it is important to stress that patient outcome cannot be reliably predicted on the basis of such classical parameters, thereby underlining the potential importance of minimal residual disease (MRD) testing. Over the last 10 years, technological developments have enabled the detection of leukemic cells beyond the threshold of cytomorphology or karyotyping.^{15,16} Three different techniques are currently used with a sensitivity of at least one leukemic cell in a background of 10^3 normal cells: (1) immunophenotyping; (2) polymerase chain reaction (PCR) using genomic DNA for detection of clonal rearrangements of immunoglobulin (Ig) and T-cell receptor (TCR) genes in ALL; and (3) reverse-transcriptase-PCR (RT-PCR) for detection of gene rearrangements, mainly FG transcripts resulting from chromosomal translocations. While the first two directly measure the tumor load, the latter method measures gene expression.

MRD information in leukemia patients has been clearly established as an independent prognostic factor in three pathological situations: (1) childhood ALL, in particular after induction therapy;^{17–19} (2) *BCR-ABL* detection in CML patients after allogeneic stem cell transplantation,²⁰ enabling direction of donor leukocyte infusions; and (3) *PML-RARA* detection in acute promyelocytic leukemia (APL) patients after consolidation therapy,²¹ conferring benefit for pre-emptive therapy at the point of molecular relapse in comparison to frank relapse.²² These data have led to the introduction of molecular monitoring in the stratification strategy in some current multicenter therapeutic trials. However, the clinical impact of MRD detection in other types of leukemias remains to be demonstrated in large series of patients.

In order to tackle the problem of lack of standardized diagnostic methodology, 8 years ago European laboratories conducted a collaborative program through a BIOMED-1 Concerted Action.²³ This Concerted Action led to the development of standardized nested RT-PCR assays achieving sensitivities of at least 10^{-4} (RNA diluted into RNA) suitable for detection of MRD as well as diagnostic screening.²⁴ However, 'end point' PCR analyses do not permit precise quantification of MRD levels. This limitation was underlined by the finding of low levels of *AML1-ETO*,^{25,26} *CBFB-MYH11*¹ or *PML-RARA*^{1,27} transcripts in patients in long-term clinical remission and by the detection of *BCR-ABL* mRNA at very low levels in healthy

individuals.^{28,29} Quantitative PCR analysis has been achieved by competitive PCR.³⁰ Expert laboratories showed that this technique enables accurate prediction of relapse suggesting that such analysis could be used for adapting treatment in *BCR-ABL*-positive CML^{31,32} or in AML patients with *CBFB-MYH11* or *AML1-ETO* transcripts.^{33,34} However, this competitive PCR is labor intensive and time consuming which prohibits both standardization and large-scale multicenter analysis.

More recently, real-time quantitative PCR (RQ-PCR) has been introduced.^{35,36} There have been numerous manuscripts from individual laboratories demonstrating the reliability of this technology and its potential clinical value for MRD studies using FG transcripts as PCR targets such as *BCR-ABL* in CML^{37–39} or ALL,⁴⁰ *PML-RARA*,⁴¹ *AML1-ETO*^{42–44} and *CBFB-MYH11*^{45,46} in AML and *TEL-AML1*^{47–49} in ALL patients. However, standardized RQ-PCR procedures are warranted in order to apply this innovative technology for large-scale MRD studies within multicenter therapeutic trials.

In 1999, 25 university laboratories from 10 European countries designed a joint project of the health and consumer protection of the European Commission (SANCO) via the 'Europe Against Cancer' (EAC) program in order to develop standardization and quality control analysis for RQ-PCR, based on the ABI 7700 platform (Applied Biosystems, Foster City, USA) as it was the first such technology available. The major aim was to establish a standardized protocol allowing comparison of MRD data in order to assess the relative efficiency of each therapeutic strategy for leukemia bearing an appropriate molecular marker. The most frequently occurring FG transcripts in leukemia were selected, covering up to 30–40% of childhood and adult ALL and AML and more than 95% of CML patients.

Organization

A total of 25 laboratories were involved throughout the whole program. One laboratory (#28) joined the group for the latest phase. This EAC Concerted Action was supported by Applied Biosystems for the design and synthesis of most TaqMan probes and primers. For the extensive statistical analysis, the Concerted Action obtained support from the Department of Medical Information of the Université de la Méditerranée (Marseille, France).

The participating laboratories were divided into seven national networks (Austria–Germany, Belgium–The Netherlands, Denmark–Sweden, France, UK, Italy and Spain). For optimal efficiency, one control gene (CG) network (six laboratories) and nine FG networks (with 4–12 laboratories) were created, responsible for testing one particular target (Table 1). Each laboratory was involved in up to four gene networks.

Our program was divided into four main phases (I–IV) each completed by meetings to monitor progress made and to plan the experiments for the next phase. Common 96-well RQ-PCR reaction plate 'set-up' and presentation forms were designed in order to streamline the data analysis and the reporting of results. Work tasks were divided as follows (Table 2):

- The aim of phases I and II was the initial selection of primers and probes in addition to training of the members since the majority only started to use this RQ-PCR methodology through involvement in this consortium.
- During phase IIIa, different standard curves were compared (ie generated using RNA, cDNA or DNA plasmids) and the

Table 1 Organization of EAC Concerted Action via nine networks for the optimization of the detection of the main fusion gene transcripts in leukemias plus one network focusing on control genes

Chromosome aberration	RQ-PCR target	Positive controls		Networks for optimization of primers and probes	
		Cell lines ^a	Patients ^b	Coordinating laboratory	Number of collaborating laboratories ^d
t(1;19)(q23;p13)	<i>E2A-PBX1</i>	697	+	JM Cayuela <i>et al</i>	5
t(4;11)(q21;q23)	<i>MLL-AF4</i>	RS4;11, MV4-11, ALL-PO	+	E Beillard <i>et al</i>	6
t(12;21)(p13;q22)	<i>TEL-AML1</i>	REH	+	H Cavé <i>et al</i>	6
t(9;22)(q34;q11)	<i>BCR-ABL m-bcr</i>	TOM-1	+	F Pane <i>et al</i>	9
t(9;22)(q34;q11)	<i>BCR-ABL M-bcr</i>	K-562	+	G Barbany <i>et al</i>	12
del(1)(p32p32)	<i>SIL-TAL1</i>	CEM, RPMI8402 ^a	+	VHJ van der Velden <i>et al</i>	4
t(15;17)(q22;q21)	<i>PML-RARA</i>	NB-4	+ ^c	G Cazzaniga <i>et al</i>	8
inv(16)(p13q22)	<i>CBFB-MYH11</i>	ME-1	+ ^c	G Saglio <i>et al</i>	5
t(8;21)(q22;q22)	<i>AML1-ETO</i>	KASUMI-1	+	D Grimwade and J Aerts <i>et al</i>	6
—	Control genes	HL-60, RS4;11	+	E Beillard and Pallisgaard <i>et al</i>	6

^aThe cell lines used during phases I–IVa are listed; for *SIL-TAL* target, four other cell lines have been tested during phase IVb only (Table 22).

^bAnalyzed diagnostic samples during phase IVb.

^cFor *PML-RARA* bcr2, bcr3 and *CBFB-MYH11* type D and E, patient samples have also been analyzed in the previous phases.

^dDuring phases I, II, IIIa and IVa, including the coordinating laboratory; additional laboratories involved during phase IVb are cited in each of the nine sections.

Table 2 Phases of the EAC network with work tasks

Period	Work task (s)	Distributed material	Meetings
Phase I (March 99–June 99)	Training	RNA dilutions	Rotterdam (NL)
Phase II (July 99–February 00)	Optimization	Plasmid dilutions, RNA dilutions	Barcelona (ES), Capri (IT), Paris ^a (FR)
Phase IIIa (March 00–June 00)	Final validation, QC1	Plasmid dilutions, RNA and cDNA dilutions, 3 coded RNA samples (negative, 10 ⁻³ , 10 ⁻⁴)	Lisbon (PT)
Phase IIIb (July 00–October 00)	Balanced randomized assay, QC2	Plasmid dilutions, 5 coded RNA samples (2 negative, 10 ⁻¹ , 10 ⁻³ , 10 ⁻⁴)	Turin (IT)
Phase IVa (Nov 00–June 01)	<i>E. coli</i> experiments, QC3	Plasmid dilutions, 5 coded RNA samples (2 negative, pure, 10 ⁻⁴ , random)	Vienna (AT), Rotterdam ^a (NL), Marseille ^b (FR)
Phase IVb (July 01–Dec 01)	Patient testing	Plasmid dilutions	Turin ^a (IT), Stockholm (SE), Rotterdam ^a (NL)

FG networks were involved for testing during phases I, II, IIIa and IVa. Networks for each FG target were randomly designed during phase IIIb. During phase IVb, patient testing was conducted by individual institutions (FG networks and other individual laboratories) and pooled for statistical analysis. NL=The Netherlands, ES=Spain, IT=Italy, FR=France, PT=Portugal, AT=Austria, SE=Sweden.

^aNetwork leader meeting.

^bInternational Symposium.

final validation of selected primer/probe sets within specific networks was made. In parallel, we performed our first quality control round (QC1).

- During phase IIIb, testing of selected FG primer/probe sets was undertaken by all laboratories on centrally prepared coded quality control samples (QC2). This testing of the FG transcript targets was performed by randomly selected laboratories outside the original FG transcript networks.
- During phase IVa, we performed the third quality control round (QC3) including undiluted cell line RNA samples.
- During phase IVb, we determined the reference values of normalized FG transcript levels in leukemic cell lines and patient samples using the EAC primer/probe sets according to the EAC standardized protocol.
- In addition, reference ranges were established for the CGs in normal peripheral blood (PB), bone marrow (BM) and PB stem cells in fresh samples (see accompanying manuscript by Beillard *et al*⁵⁰).

Materials and methods

Principle

Currently available RQ-PCR technologies allow detection of fluorescence emission during the PCR reaction from one (TaqMan) or two (Light Cycler) internal oligonucleotide probes, or a fluorescent dye, the detected fluorescence being proportional to the amount of target present in the sample.^{51,52}

We decided to run our EAC protocol using the ABI 7700 platform with TaqMan probes since this was the first robust RQ-PCR technology available permitting analysis of a large number of samples in a single run (96-well plate format). The 5' nuclease assay (TaqMan technology) uses a single internal oligonucleotide probe bearing a 5' reporter fluorophore (eg 6-carboxy-fluorescein) and 3' quencher fluorophore (eg 6-carboxy-tetra-methyl-rhodamine). During the extension phase, the TaqMan probe is hydrolyzed by the nuclease activity of the *Taq* polymerase, resulting in separation of the reporter and quencher fluorochromes and consequently in an increase in fluorescence (Figure 1). In the TaqMan technology, the number of PCR cycles necessary to detect a signal above the threshold is called the cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the reaction (Figure 2a). The ΔRn corresponds to the increase in fluorescence intensity when the plateau phase is reached. Using standards or calibrators with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample (Figure 2b). The theoretical slope of the standard curve is -3.32 for a PCR reaction with maximum efficiency. Using a known amount of DNA molecules for the standard curve, the intercept point on the Y-axis defines the number of cycles theoretically needed to detect one molecule.

Molecular targets

Assays were designed to detect nine leukemia-associated FGs, including their more common breakpoint variants giving rise to 15 RNA targets: *E2A-PBX1*, *MLL-AF4* (variants exon 9-exon 5, exon 10-exon 4 and exon 11-exon 5), *TEL-AML1*, *BCR-ABL* (M-bcr and m-bcr), *SIL-TAL1*, *PML-RARA* (bcr1, bcr2 and bcr3), *CBFB-MYH11* (type A, D and E) and *AML1-ETO*.

In addition, 14 housekeeping genes were evaluated for their suitability to serve as CGs for sample to sample quality

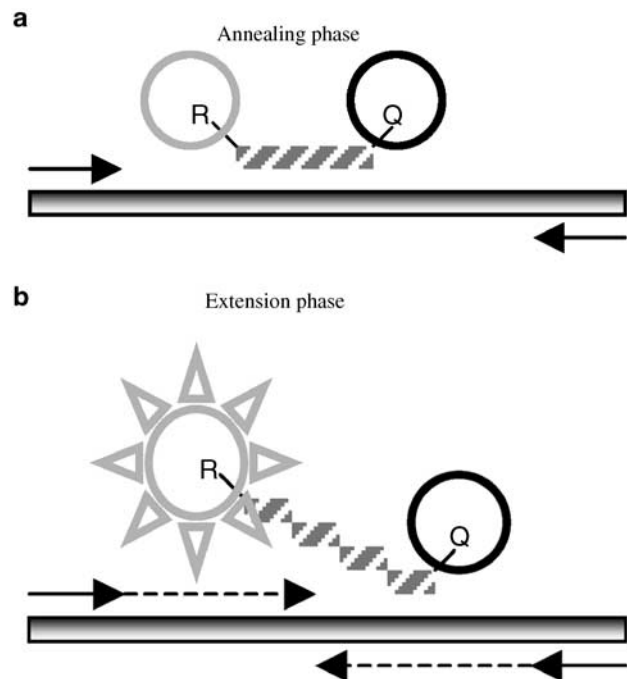


Figure 1 Principle of RQ-PCR detection using hydrolysis probe (TaqMan[™]). (a) The bi-fluorescent probe (R: reporter, Q: quencher) hybridizes on the complementary nucleotide sequence of the template during the PCR reaction. (b) The probe is degraded during the extension phase by the DNA polymerase enzyme resulting in an increasing fluorescence detected by the machine in the PCR tube.

variations and gene expression quantification (see accompanying manuscript by Beillard *et al*⁵⁰). Three CGs were ultimately selected (*ABL* (Abelson gene), *B2M* (beta-2-microglobulin) and *GUS* (beta-glucuronidase)); we analyzed the expression of the *ABL* gene during phases II–III and all three selected CGs (*ABL*, *B2M* and *GUS*) during phase IV.

Primer and probe design

Primers and probes were designed using Primer Express software (Applied Biosystems) based on their location on two separated exons and on the sequence of the amplicon generated by the primer sets described in the BIOMED-1 program.²⁴ They are depicted in the schematic diagram of the exon/intron structure of the corresponding FG (see Sections 1–9). During the first two meetings, an initial selection of primers and probes was made; newly designed sets for each molecular target as well as already available 'in house' sets from experienced laboratories were evaluated. The set selection was based on: (1) the absence of nonspecific amplification artifacts; (2) a good efficiency with a slope close to -3.32 (100% theoretical efficiency); (3) a good sensitivity (at least 10^{-4} RNA dilution or 100 copies for plasmid dilution); and (4) the robustness of the reaction with a ΔRn value >1.0 at the plateau phase for the highest dilutions (Table 3). Such results had to be reached in at least 80% of the participating laboratories for a particular primer/probe set to be selected. Potential primer/probe sets were tested in parallel on serial dilutions of cell lines and plasmids, and the set with the best performance profile, particularly in terms of sensitivity, was selected. If none of the primer/probe sets satisfied the selection criteria, new sets were designed and evaluated. Overall, starting

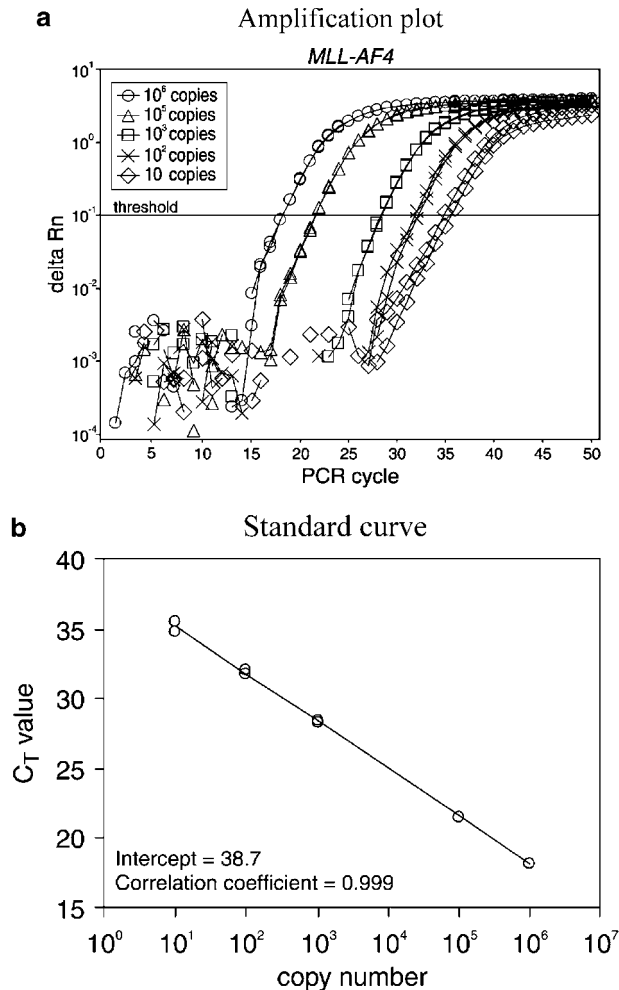


Figure 2 Amplification of *MLL* (ex 10)-*AF4* (ex 4) plasmid using EAC primer and probe sets. (a) Amplification plots (\log_{10} scale) of five DNA plasmid dilutions (10^6 – 10^1 molecules). (b) Standard curve obtained with the five plasmid dilutions.

from 47 primer sets and 44 probes that were tested during the first phases, 12 primer sets and nine probes were finally selected for the 15 targets (see figures in individual sections). In each case, the sensitivity of the TaqMan-based RQ-PCR analysis appeared to be comparable to previously standardized nested RT-PCR analysis.²⁴ On the *BCR* gene, Y (cytidine or thymidine) appears on the *BCR* primer at position 3188, according to the polymorphism recently described.⁵³

RNA and cDNA from cell lines and leukemia samples at diagnosis

RNA and/or cDNA samples were prepared centrally by the FG network leaders (Table 1) and distributed on dry ice to members of their respective networks during phases I–IVa. FG transcript-positive cell line RNA was commonly used (see Table 1 for specific cell lines), except for rare FG transcripts (*PML-RARA* bcr2 and bcr3 and *CBFB-MYH11* type D and E) for which patient RNA was provided by the network leader. These RNA samples were diluted in PB lymphocytes (PBL) RNA or FG transcript-negative cell line RNA. Cell lines were purchased from the DSMZ (Braunschweig, Germany), ATCC (Manassas,

Table 3 Results observed for selected EAC primer and probe sets

Fusion gene targets (n = 15)	RNA dilution (log)	Plasmid dilution (copy number)
Sensitivity	–4/–5	10 molecules
Reproducibility	–3/–4	10 molecules ^a
	CV(Ct) <6%	CV(Ct) <4% ^b
Robustness		
ΔRn	> 1	> 1
Slope	3.45±0.3	3.3±0.3

EAC criteria are defined in the Materials and methods section.

^aA total of 100 molecules only for FG variants (tested only during phase IIIa): *PML-RARA* bcr2 and bcr3, *CBFB-MYH11* type D and E.

^bCV of the 10 copies plasmid. CV was below 2.5% for the other dilutions.

VA, USA) or directly provided by academical laboratories (TOM-1, ME-1 and PF382) and cultured according to the supplier's instructions. During phase III, network leader laboratories prepared equivalent dilution series of cDNA. During phase IVb, patient sample RNAs, positive for the relevant FG transcript and that had been stored for less than 18 months, were analyzed locally undiluted and/or diluted in a solution of 1 $\mu\text{g}/\mu\text{l}$ *E. coli* 16S and 23S rRNA (Roche, Meylan, France) in duplicate. Overall, we analyzed 278 samples (BM and PB) mainly from different patients and 57 paired samples from a harvest of the same patient at the same time. Details are given in each FG section and corresponding tables. Although BM/PB data have some redundancy, analyses of BM and PB samples give an impression about the comparability of the results, particularly when paired BM/PB samples were used.

Plasmid DNA calibrators containing the target gene sequences

PCR products of the 15 different FG transcripts were generated from cell line or patient RNA by RT-PCR using BIOMED-1 A and B primers, as previously described.²⁴ PCR products were cloned into the PCR II TOPO vector (Invitrogen, Groningen, The Netherlands). The selected plasmid clones were sequenced for confirmation of their insert (Genome Express, Grenoble, France). After subsequent bulk production, the plasmids were extracted using the QIAFILTER Plasmid MIDI kit (Qiagen, Courtaboeuf, France) and quantified spectrophotometrically. The copy number for 1 μg was estimated according to the molecular weight of the vector and the insert. Then 20 μg of plasmid was linearized with *Bam*HI or *Hind*III restriction enzymes for 1 h at 37°C under agitation. The digested plasmid was serially diluted in a solution of Tris 10 mM, EDTA 1 mM pH 8, containing 20 ng/ μl of *E. coli* 16S and 23S rRNA (Roche). Five successive dilutions (200 000, 20 000, 200, 20 and 2 copies/ μl) were prepared. The corresponding standard curve generated a mean slope of –3.3 and an intercept of 39.8±1 Ct. A mean Ct value of 22.5±1 was obtained for the 20 000 copies/ μl dilution. The plasmid dilutions were centrally prepared in Marseille (J Gabert's laboratory) during phases I–III. Thereafter, Ipsogen (Marseille, France) kindly provided plasmid dilutions for phase IV.

Standardized RT-PCR protocol

A common EAC protocol was established for all molecular targets (FG and CG transcripts) during phases I and II and

then used by each laboratory throughout phases III and IV (Table 4).

RT step: This reaction was adapted from the BIOMED-1 protocol.²⁴ Starting from 1 μ g of total RNA, the main modifications involved alteration of the concentrations of random hexamers (25 μ M) and of reverse transcriptase (100 U) either murine Moloney leukaemia virus or Superscript (Invitrogen, Roche), which significantly enhanced the sensitivity of the assay (see accompanying manuscript by Beillard *et al*⁵⁰).

RQ-PCR step: All the RQ-PCR reactions were performed on a 7700 ABI platform (Applied Biosystems, Foster City, USA) using primers and TaqMan probes kindly provided by Applied Biosystems in conjunction with the TaqMan Universal Master Mix purchased from the same manufacturer. The number of amplification cycles was 50 (detailed protocol is described in Table 4).

Optimization of RQ-PCR assay (phase II): In order to optimize the results and save costs, phase II involved assessment of the influence of primer and probe concentrations (900 and 300 nM for primers and 200 and 100 nM for probes) and the reaction volume (50 vs 25 μ l) on the sensitivity of the assay.

After extensive testing within FG networks using serial dilutions of FG-positive cell line RNA in FG-negative RNA (10^{-1} – 10^{-5}) and plasmid (10^6 –10 copies), optimal results were obtained using a 25 μ l volume, with 300 nM of primers and a 200 nM concentration of probe, except for *AML1-ETO* for which a 100 nM probe was chosen (see Section 9).

Table 4 Standardized EAC RT and RQ-PCR protocols

1. RT reaction ^a
1 μ g of total RNA in 10 μ l of H ₂ O
Incubate at 70°C for 10 min
Cool on ice and add other reagents to a final volume of 20 μ l
Reverse transcriptase (either MMLV or Superscript I or II): 100 U
RT buffer (according to the RTase used)
dNTP: 1 mM
DTT: 10 mM
Random hexamers: 25 μ M
RNAse inhibitor: 20 U
Incubate subsequently at: room temperature for 10 min
42°C for 45 min
99°C for 3 min
Place the sample at 4°C after the RT step
Dilute the final cDNA with 30 μ l of H ₂ O
2. RQ-PCR reaction ^a
Final volume: 25 μ l
5 μ l of final cDNA (100 ng RNA equivalent)
Primers: 300 nM each
Probe: 200 nM except for the <i>AML1-ETO</i> probe (100 nM)
Master Mix: 12.5 μ l (1 \times)
Incubate the sample
at 50°C for 2 min
at 95°C for 10 min
Followed by 50 cycles
95°C for 15 s
60°C for 1 min
3. Standardized procedure for data analysis
Set threshold at 0.1 except for <i>PML-RARA</i> (0.05)
Set baseline between cycles 3 and 15 except for <i>B2M</i> (3–10)

^aAll mentioned concentrations are for the final volume of the reaction.

For comparative data analysis, a common threshold set at 0.1 was selected in order to be in the exponential phase. Such a threshold value typically lies above the so-called ‘creeping curves’ that were rarely observed in some negative controls; ‘creeping curves’ are defined as the amplification curve from a negative sample rising slowly during the PCR reaction. While the mechanism underlying the latter phenomenon is not entirely clear, examination of the ‘multicomponent’ view reveals that it is not indicative of specific amplification (see below). However, for *PML-RARA*, the threshold was fixed at 0.05 because of the relatively short exponential phase of the PCR amplification (low Δ Rn). The baseline was calculated from cycles 3–15 except for high-expression CGs where cycles 3–10 were used (see accompanying manuscript by Beillard *et al*⁵⁰). Additionally, we used dilutions of positive RNA (patient or cell line) into normal PBL or an FG-negative cell line RNA (10^{-1} – 10^{-6}) during phases I–IIIa and identical dilution series using cDNA during phase IIIa.

Standard curve comparison (phase IIIa)

A comparison between RNA, cDNA and plasmid standard curves was performed in order to determine the influence of the RT step on the results (standard curve slope, sensitivity and reproducibility) for each laboratory. The FG network leaders sent to the different laboratories within their target network (4–12 members, see Table 1) centrally prepared serial dilutions of the FG transcript-positive cell line RNA and the corresponding cDNAs, in addition to serial dilutions of the corresponding FG control plasmid (200 000, 20 000, 200, 20 and 2 copies/ μ l).

Quality control (QC) rounds on coded samples

General organization: QC1 (phase IIIa) and QC3 (phase IVa) were performed by laboratories within their original FG networks (Table 1), whereas QC2 (phase IIIb) involved randomly chosen laboratories (see Balanced randomized assay section).

Control samples and definition of (false-) positivity and (false-) negativity: The positive controls in all experiments and QC rounds concerned well-defined cell lines and patient samples (Tables 1 and 2). Two types of negative controls were used: (1) coded FG-negative RNA samples and (2) known negative controls for checking contamination of PCR products. These latter contamination controls concerned no-amplification controls (NAC), which contained *E. coli* RNA instead of human cDNA, and no-template controls (NTC), which contained water instead of human cDNA. Particularly, the NAC and NTC negative controls were regarded to be of utmost importance for identification of cross-contamination of PCR products, because this problem is frequently underestimated.

A positive well was defined as a sigmoidic amplification (log scale) with a Ct value below the Y-intercept Ct value of the plasmid standard curve + one Ct. Amplification on RNA samples of the FG was performed in triplicate and in duplicate for the CG expression. A false-negative sample was defined as a positive RNA sample with less than 50% of positive wells (0/2, 0/3 or 1/3). A false-positive result was defined as a negative sample, with at least 50% of positive wells (1/2, 2/3 or 3/3).

Sensitivity and reproducibility of the experiments: The criteria for sensitivity and reproducibility were defined during QC experiments via coded samples for each FG transcript

(Table 3). An experiment was assumed to be reproducible for a particular dilution if more than 80% of the laboratories detected at least two positive wells with a Ct difference less than 1.5 Ct. The sensitivity of the experiment was defined as the last dilution showing at least 50% of positive wells in more than 80% of the laboratories whatever the Ct values were.

Balanced randomized assay (phase IIIb): This assay was designed by the Department of Medical Information (Marseille, France). The aim was to evaluate to what extent RQ-PCR results were comparable between laboratories for MRD quantification of FG transcripts in a clinical setting according to the EAC protocol. The study focused on two main points: (1) comparison of the results between laboratories for a particular FG transcript which is crucial for multicenter studies and (2) the linearity of the RQ-PCR methodology in dilution experiments which is important to assess the potential tumor load during treatment. The balanced randomized assay appeared to be an appropriate statistical study to assess these two points without performing all the RQ-PCR analyses ($n=15$) in each participating laboratory ($n=25$).

The statisticians randomly assigned the 25 participating laboratories to nine networks. Each of the nine main FG transcripts were tested in 11 laboratories (except for *CBFB-MYH11* network, $n=12$), including the involved FG transcript network leader, making a total of 100 RQ-PCR experiments. Each laboratory tested five coded samples for four different targets, making a total of 500 coded samples analyzed in this QC2 study (Table 2). FG transcript-positive control RNAs were diluted (10^{-1} , 10^{-3} and 10^{-4} dilutions) in a negative RNA sample (HL60 or PBL).

A common plate design was used: the CG (*ABL*), the FG transcript and plasmid dilutions (*ABL* $n=3$, *FG* $n=5$) were amplified in triplicate, whereas four negative controls (NAC and NTC for *ABL* and *FG* targets) were run in duplicate. The raw data were collected and analyzed by each FG network leader. Common Excel worksheets were designed to collect the results within each FG network and were then forwarded to Marseille for subsequent statistical analysis (see below). The only exclusion criterion for coded RNA samples was an *ABL* Ct value outside the normal range [22–29.3] as defined by assays conducted within the CG network during phase IIIa (see accompanying manuscript by Beillard *et al*⁶).

Statistical analysis

General methodology: The CG and the FG RQ-PCR data were collected and analyzed by each FG network leader. The mean Ct or mean Δ Ct (mean Ct [FG]–mean Ct [CG]) values and the mean value of the \log_{10} of the copy number (CN) for each gene were used. The normalized copy number (NCN) was defined as the CN of the FG per one copy of the CG transcript (mean value of \log_{10} [FG CN]–mean value of \log_{10} [CG CN]) except for normalization to *B2M* gene transcript level for which the results were expressed per 100 copies. Since CN did not show a normal distribution, the logarithmic value of the CN was used for statistical analysis. For an easier comprehension, results obtained in a logarithmic way were subsequently converted into decimal values. The level of significance was set at $P<0.05$. In tables and figures, when the P -value was between 0.05 and 0.1, even if not statistically significant, the numbers are noted. When the P -value was >0.1 , the result appears as not significant (NS). The calculation of CV from the Ct's results in an underestimation of the real variation, since Ct's are based on log of the transcript

numbers. The correlations between the expression level of different genes were measured by the Pearson correlation coefficient (r). The box-plots used for presenting data show the median value (dark line) within a box containing 50% of the samples (25–75th percentile). The statistical analysis was performed in Marseille, France, using the SPSS 10.1 Software (SPSS Inc., Chicago, USA).

Balanced randomized assay (phase IIIb): Detection of significant differences in transcript quantification between laboratories: Four parameters per sample (Ct, Δ Ct, CN and NCN) were tested using a global linear model. The laboratory number was set as a random effect and data were analyzed. When the results were not comparable between laboratories ($P<0.05$) for the selected parameter and transcript, a *post hoc* analysis (Tukey method) was used to evaluate the number of laboratories reproducible with others ($P\geq 0.05$) defining a subgroup. For this, two criteria were chosen to estimate the reproducibility of the results: the number of subgroups (S_n) and the number of laboratories (L_n) reproducible with at least two other laboratories as defined by a nonsignificant difference between the mean ($P\geq 0.05$) according to the Tukey method. When a particular laboratory was present in two or more subgroups, it was counted only once. The best parameter to compare results between laboratories was the one with the highest L_n value and the lowest S_n value. Ideally, only one subgroup ($S_n=1$) containing all the laboratories ($L_n=11$ or 12 per network or $L_n=25$ for the whole assay) was expected.

Evaluation of the linearity: The Pearson correlation coefficient was chosen to measure the linearity of the quantification of the FG transcripts using the three coded positive samples. For each FG transcript, the best parameter ($\Delta\Delta$ Ct or NCN) to assess the results was the one with the highest correlation coefficient.

Reference values at diagnosis (phase IVb): Leukemia samples were excluded from the analysis if the CG amplification was not within the normal range for fresh samples defined as follows: *ABL* Ct [21.8–29.4], *B2M* Ct [15.6–24.9] and *GUS* Ct [20.8–28.0] (see accompanying manuscript by Beillard *et al*⁶). The comparison between PB and BM was performed with nonparametric tests (Wilcoxon paired test for at least five paired samples or Mann–Whitney U-test for unpaired samples). At least five paired samples were expected per FG transcripts. The 95% range of expression for NCN refers to the range between the 3rd and the 97th percentile for the selected gene. Correlation coefficients between the FG CN and each CG CN are given before normalization by the CG. For cell line(s), the median value obtained on the same sample in eight different laboratories is shown.

All the figures, tables and raw data are available on-line at the following address: <http://meidia.nord.univ-mrs.fr/EAC/publications.html>. This web site will stay available for a long-term period, at least 5 years.

1. t(1;19)(q23;p13) with the *E2A-PBX1* fusion gene transcript

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1.1. Background

The leukemogenic FG transcript *E2A-PBX1* results from fusion of the *E2A* and *PBX1* (formerly *prl*) genes, through the t(1;19)(q23;p13).^{54–56} The t(1;19)(q23;p13) is found in 3–5% of childhood and in 3% of adult precursor-B-ALL.⁵⁷ In 95% of the cases, *E2A-PBX1* transcripts are expressed.⁵⁸ This expression is tightly associated with detection of cytoplasmic Ig μ chains.^{59,60} The remaining t(1;19) precursor-B-ALL are *E2A-PBX1* negative and show no rearrangement of the *E2A* gene.⁶¹

The *E2A* gene, located on chromosome 19, encodes the helix–loop–helix Ig enhancer binding factors E12 and E47 and the *PBX1* gene on chromosome 1 encodes a DNA-binding homeobox protein.^{55,56} The genomic organization of *E2A* is well defined and breakpoints occur almost exclusively in the 3.5 kb intron between exons 13 and 14.^{54–56} The genomic organization of *PBX1* is not yet fully known and the breakpoints are dispersed over an intronic region of about 50 kb between exons 1 and 2. The majority of cases with *E2A-PBX1* FG transcripts show a constant junction of *E2A* exon 13 to *PBX1* exon 2 (Figure 3).⁶² A variant FG transcript has been described in about 5–10% of *E2A-PBX1*-positive patients. It is characterized by insertion of a stretch of 27 nucleotides at the *E2A-PBX1* junction.^{61,63} This inserted sequence probably arises from an alternatively spliced exon of *PBX1*.

The FG encodes a chimeric transcriptional activator containing the N-terminal transcriptional activator domain of *E2A* joined to the C-terminal DNA-binding homeobox domain of *PBX1*.⁶⁴ The transforming activity of *E2A-PBX1* proteins has been demonstrated both *in vitro* and *in vivo*.^{65–67}

Several studies using RT-PCR amplification of *E2A-PBX1* FG transcripts to assess MRD have been reported.^{68–72} All of them were performed with a qualitative assay showing a detection threshold of up to $10^{-4/-5}$. In none of these reports does the presence or absence of *E2A-PBX1* transcripts during follow-up predict treatment outcome. The largest series including 71 patients, published by Hunger *et al*,⁷⁰ found no difference in event-free survival between PCR-positive and PCR-negative patients analyzed at the end of consolidation treatment. All these studies pinpoint to the limitations of qualitative assessment of MRD in monitoring t(1;19)-positive ALL patients, and

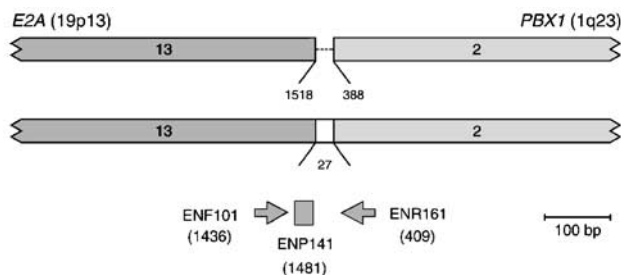


Figure 3 Schematic diagram of the *E2A-PBX1* FG transcript covered by the RQ-PCR primer and probe set (ENF101–ENP141–ENR161). The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 5).

underline the importance of quantitative approaches such as RQ-PCR methods.

1.2. EAC data

1.2.1. Primer design and optimization (phases I and II): Among two primer and probe sets tested, one was chosen: ENF101, ENR161 and ENP141. Positions and nucleotide sequences are shown in Figure 3 and in Table 5, respectively. This set was selected on the basis of higher ΔR_n values at the plateau phase with high dilutions of the *E2A-PBX1*-positive cell line 697 (also known as ACC42) RNA in HL60 RNA. During phase II and phase III experiments, this set of primers and probe allowed us to detect a 10^{-4} dilution of 697 RNA in HL60 RNA in seven out of seven laboratories; an example of typical amplification plots (10^{-1} , 10^{-3} and 10^{-4} cell line RNA dilutions) is shown in Figure 4. We conclude that ENF101, ENR161 and ENP141 are suitable for quantification of all *E2A-PBX1* fusion transcripts, including the variant form.

1.2.2. *E2A-PBX1* expression in 697 cell line and diagnostic patient samples (phase IV): One cell line, 697 and 27 diagnostic samples (14 BM and 13 PB samples) were analyzed. Blast percentages (defined morphologically) were

Table 5 Sequences and positions of the *E2A-PBX1* primers and probe

EAC code ^a	Primer/probe localization, 5'–3' position (size)	Sequence 5'–3'
ENF101	<i>E2A</i> , 1436–1454 (19)	CCAGCCTCATGCACAACCA
ENP141	<i>E2A</i> , 1481–1502 (22)	CCCTCCCTGACCTGTCTCGGCC
ENR161	<i>PBX1</i> , 409–389 (21)	GGGCTCCTCGGATACTCAAAA

^aENF=European network forward primer, ENP=European network TaqMan probe, ENR=European network reverse primer.

^bPositions according to accession numbers M31222 (*E2A*) and M86546 (*PBX1*).

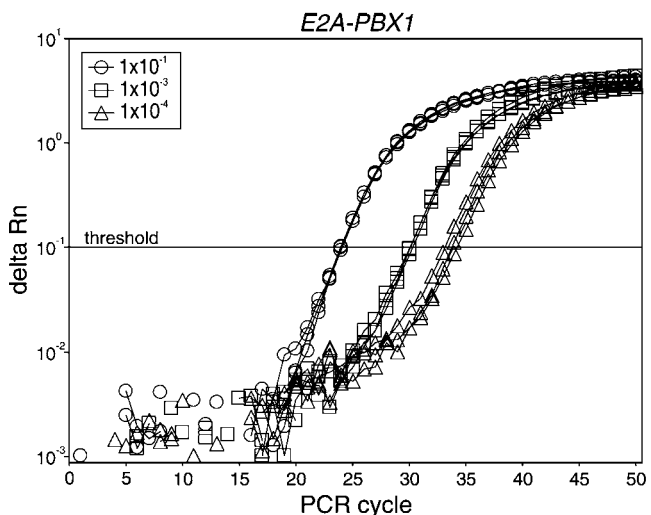


Figure 4 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of an *E2A-PBX1*-positive RNA sample in a negative RNA sample.

Table 6 Expression values of the *E2A-PBX1* FG transcript in cell line 697 and patients at diagnosis (phase IV)

Samples	Ct values				E2A-PBX1 NCN		
	E2A-PBX1	ABL	B2M	GUS	Per ABL	Per 100 B2M	Per GUS
Cell line 697	20.6	24.3	19.4	25.4	5.0	33.9	9.0
Patients							
BM (n = 14)	20.7 [18.4–22.6]	24.4 [22.6–28.2]	20.2 ^a [16.7–22.5]	24.9 [22.3–27.7]	7.6 [2.1–19.5]	44.6 ^a [10.5–101.0]	8.9 [1.6–17.8]
PB (n = 13)	21.0 [18.0–22.6]	25.2 [22.9–28.5]	19.5 [16.1–22.6]	25.8 [22.8–26.3]	9.4 [3.8–72.4]	42.7 [2.2–115.9]	13.6 [2.5–67.6]
Corr. coeff.					0.83	0.62	0.66

^aOne value was not available in one BM sample out of 14 for *B2M*. For patient samples and cell lines, median values [95% range] are indicated.

available in all but two samples (one PB and one BM) and ranged from 74 to 100% (median = 96%). Median values and 95% range for CGs and *E2A-PBX1* Ct values, as well as normalized *E2A-PBX1* copy number (corrected according to the blast percentage), are reported in Table 6. Ct values detected for *E2A-PBX1* and CG in the 697 cell line and patient samples were comparable indicating that they are expressed at similar levels. The highest correlation coefficient was observed between *E2A-PBX1* and *ABL* transcripts. Among PB and BM samples, 10 were paired samples, harvested in the same patient at presentation of the disease. No statistically significant difference could be observed between BM and PB, in terms of Ct or NCN in paired samples (Figure 5).

1.2.3. QC rounds (phases IIIa–IVa): During the various QC rounds, 11 negative samples (five negative RNA, three NAC and three NTC) and five positive samples (10^{-3} (two samples) and 10^{-4} (three samples) dilutions of 697 cell line RNA in HL60 RNA) were tested in 8–10 labs (Table 7). *E2A-PBX1* amplification of the negative samples accounts for 156 wells. Three wells (corresponding to three different samples) were found positive (2%), but none of these samples were considered positive according to the criteria defined in the Materials and methods section. *E2A-PBX1* amplification of the positive samples accounts for 78 wells. Only one well was found negative (1%). According to the criteria defined in the Materials and methods section, all five positive samples were found positive, as expected from the sensitivity threshold defined in the previous phases.

2. t(4;11)(q21;q23) with the *MLL-AF4* fusion gene transcript

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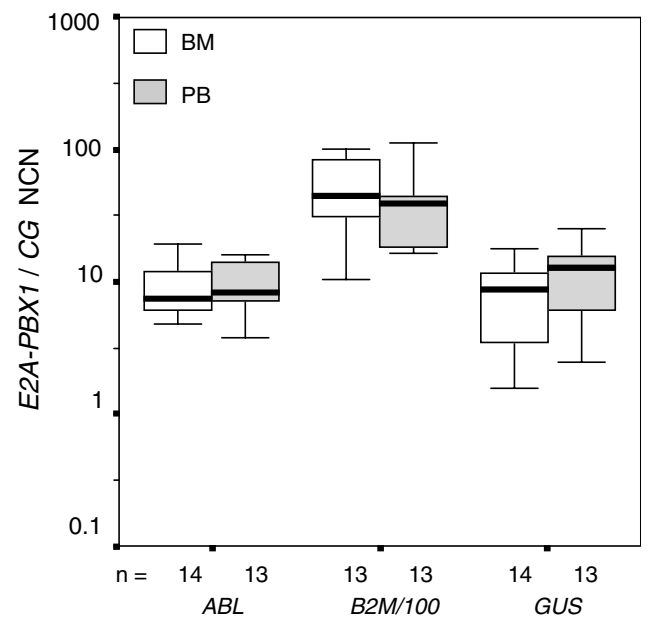


Figure 5 Comparison of *E2A-PBX1* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *E2A-PBX1* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. All differences between BM and PB per CG are not significant on paired samples ($n = 10$, Wilcoxon test). n : number of patient samples.

Table 7 Results of the QC rounds for *E2A-PBX1* FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10^{-3}	10^{-4}	FG neg. control	NAC/NTC
IIIa	0% (0/8)	0% (0/8)	0% (0/8)	0% (0/16)
IIIb	0% (0/10)	0% (0/10)	0% (0/20)	0% (0/20) ^b
IVa	ND	0% (0/8) ^a	0% (0/16)	0% (0/16)
All phases	0% (0/18)	0% (0/26)	0% (0/44)	0% (0/52)

Results are proportions of false-positive or -negative samples. A total of 10^{-3} and 10^{-4} are RNA dilutions of ACC42 cell line RNA in HL60 RNA. ND = not done.

^aOut of 24 wells, 23 were positive.

^bOut of 60 wells, 57 were negative.

2.1. Background

The t(4;11)(q21;q23) is the most frequent 11q23 translocation in precursor-B-ALL and involves *MLL* (*HRX*, *Htrx*, *ALL1*) and *AF4* (*FEL*) genes.⁷³ While *MLL-AF4* positivity is observed in 5% of pediatric and adult ALL cases, this subgroup accounts for 40–60% of infant and therapy-induced ALL. The function of mammalian *MLL* is still largely unknown, but it seems to play a central role in segmentation during development.⁷⁴ The recent mouse *AF4* knockout suggested that this gene encodes a putative transcription factor that is involved in the ontogeny of the lymphoid lineage.⁷⁵ Increased resistance of *MLL-AF4*-positive leukemia to stress-induced cell death shown *in vitro* has been suggested to contribute to their poor prognosis.⁷⁶

At the molecular level, breakpoints in *MLL* and *AF4* genes are spread within introns, between exons 8 and 12 (*MLL*) and exons 3 and 7 (*AF4*), some transcripts being more frequent in either adult or infant ALL.²⁴ Uckun *et al*,⁷⁷ using a nested RT-PCR technique with 10⁻⁴ sensitivity, reported low expression levels of *MLL-AF4* transcripts in up to 13% of pediatric ALL at diagnosis, some of them being negative for the *MLL-AF4* rearrangement by Southern analysis, and in around 25% of fresh normal BM or fetal liver samples. Based on these data, the authors suggested that RT-PCR assays for the *MLL-AF4* FG transcripts were not suitable for MRD monitoring. However, these remarkable findings have not been confirmed so far by other groups or by other techniques.^{78,79}

Nevertheless, in the same period, the first prospective MRD study, using nested RT-PCR, on 25 *MLL-AF4*-positive patients showed a significant correlation between PCR positivity, relapse and survival.⁸⁰ The heterogeneity of the *MLL-AF4* FG transcripts, their relatively low incidence in childhood and adult ALL and their poor prognosis with classical therapy explain the scarce number of reported MRD studies for this RT-PCR target.

2.2. EAC data

2.2.1. Primer design and optimization (phases I and II): From the three probes and six primer sets tested, a common probe ENP242 and a common reverse primer ENR262, both located on *AF4* exon 5, were adopted (Figure 6 and Table 8). Due to the large variety of *MLL-AF4* FG transcripts, a common primer/probe set for all *MLL-AF4* variants could not be designed. Two forward primers on the *MLL* gene were used in order to amplify at least 90% of non-infant and 60% of infant *MLL-AF4* FG transcripts: ENF207 (exon 9) and ENF208 (exon 10).²⁴ To our knowledge, no primer/probe sets for RQ-PCR have been published to date for *MLL-AF4* or any other *MLL* FG transcripts.

Three cell lines were available for testing: RS4;11 (*MLL* exon 10-*AF4* exon 4), MV4-11 (*MLL* exon 9-*AF4* exon 5) and ALL-PO expressing two alternative transcripts (*MLL* exons 10 and 11-*AF4*

exon 5). Three plasmids were constructed: *MLL-AF4* exon 9-exon 5, exon 10-exon 4 and exon 11-exon 5. An example of typical amplification plots (10⁻¹, 10⁻³ and 10⁻⁴ cell line RNA dilutions) is shown in Figure 7; our sensitivity data on cell line material were comparable to those previously observed for detection of *MLL-AF4* FG transcripts using nested PCR assay.²⁴ The 'creeping-curve' phenomenon was a rare event within the negative samples and mostly laboratory related (see the Materials and methods section and Section 9).

2.2.2. *MLL-AF4* expression in cell lines and diagnostic patient samples (phase IV): Among the 22 samples included (14 BM and eight PB, including two paired samples), most of them (*n*=19) were recruited from the French therapeutic protocols LALA-FRALLE for ALL and tested in Marseille. The blast cell percentage in the samples was not known for all the samples; so results were not corrected according to this proportion. The normalized *MLL-AF4* FG transcript expression (NCN) appeared to be similar between cell

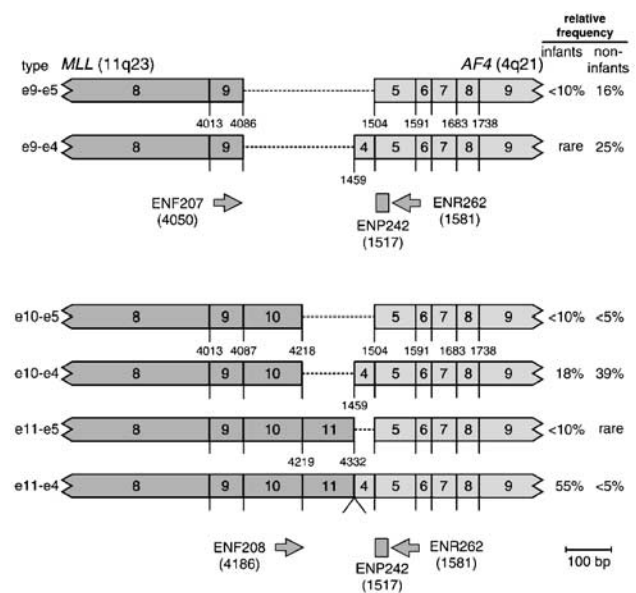


Figure 6 Schematic diagram of the *MLL-AF4* FG transcripts covered by the RQ-PCR primer and probe set. Two RQ-PCR sets are available with a common probe and a common reverse primer on *AF4* exon 5 (ENR262–ENP242). The first set uses a forward primer on *MLL* exon 9 (ENF207) and the second set uses a forward primer on *MLL* exon 10 (ENF208). *MLL-AF4* FG transcripts lacking *AF4* exon 5 cannot be amplified by the EAC sets. Taken together, the sets cover approximately 75% of the *MLL-AF4* variants either in infant or non-infant patients (see relative frequency on the right side of the diagram). The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 8).

Table 8 Sequences and positions of the *MLL-AF4* primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3'
ENF207	<i>MLL</i> , 4050–4074 (25)	CCCAAGTATCCCTGTAAAAACAAAA
ENF208	<i>MLL</i> , 4186–4208 (23)	GATGGAGTCCACAGGATCAGAGT
ENP242	<i>AF4</i> , 1517–1538 (22)	CATGGCCGCCTCCTTTGACAGC
ENR262	<i>AF4</i> , 1581–1560 (22)	GAAAGGAAACTTGGATGGCTCA

^aENF = European network forward primer, ENP = European network TaqMan probe, ENR = European network reverse primer.

^bPositions according to accession numbers L04284 (*MLL*) and L13773 (*AF4*).

lines ($n=3$) and patients (Table 9). No differences in *MLL-AF4* NCN were observed between PB and BM samples (Figure 8). The 95% range of expression was reduced when using either *ABL* or *GUS* as CG (Table 9) compared to the results obtained with *B2M* or without any CG (see web site). Furthermore, the correlation between *MLL-AF4* and *ABL* transcript levels was the highest observed among the three CGs tested in diagnostic samples (Table 9).

2.2.3. QC rounds (phases IIIa–IVa): Few false-negative samples (6%, 7/110) were observed for 10^{-3} and 10^{-4} dilutions. The amplification of *MLL-AF4* cDNA within negative samples (FG-negative samples, NAC and NTC) also called false positivity was limited to 3% (5/176) and restricted to individual laboratories (Table 10). The Ct value in the false-positive wells was always more than 30 and most of the times higher than 37.

3. t(12;21)(p13;q22) with the *TEL-AML1* fusion gene transcript

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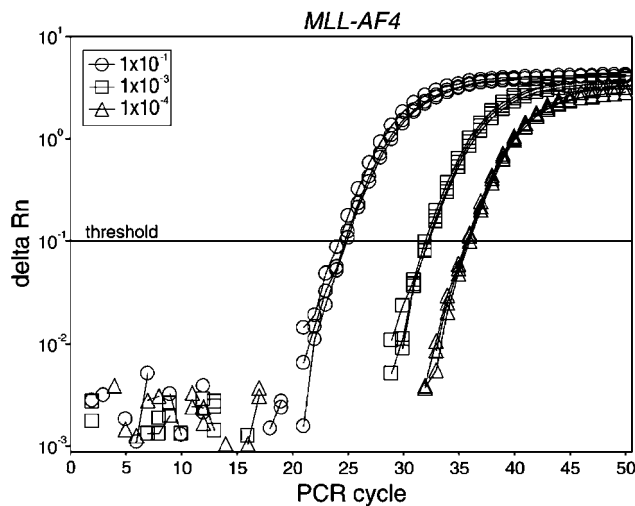


Figure 7 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of an RS4;11 cell line RNA in a negative PB MNC RNA sample.

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3.1. Background

The *TEL(ETV6)-AML1(CBFA2,RUNX1)* FG transcript results from the cryptic t(12;21)(p13;q22) and is found in about 25% of childhood precursor-B-ALL.^{81–83} Both *TEL* and *AML1* encode nuclear transcription factors, which are critical for normal hematopoiesis. Their fusion leads to leukemogenesis by disrupting the normal function of *TEL* and/or creating a transcriptional repressor that impairs *AML1* target gene expression.⁸⁴ Two recurrent translocation breakpoints have been described. The major one breaks within *TEL* intron 5 and *AML1* intron 1, generating *TEL* exon 5-*AML1* exon 2 FG transcripts. The minor one is found in about 10% of *TEL-AML1*-positive ALL and breaks within *AML1* intron 2, generating *TEL* exon 5-*AML1* exon 3 FG transcripts which are 39 bp shorter (Figure 9). This latter FG transcript, together with transcripts lacking *AML1* exon 3, is also detected in cases with *AML1* exon 2 fusion, as a result of alternative splicing.⁸⁵ Cases with breakpoints in *TEL* intron 4 have been described but remain exceptional.

The prognosis of *TEL-AML1*-positive ALL is still controversial. There is agreement that presence of the *TEL-AML1* FG transcripts is associated with a high probability of 4-year event-free survival. However, some authors,^{86,87} but not others,^{88–90} reported relapse rates similar to those of ALL in general, with a majority of relapses occurring off-therapy. Whether these variations from one study to another are due to methodological bias or differences in efficacy of chemotherapy regimens is still unclear.

Few studies have reported MRD results for patients with *TEL-AML1*-positive ALL so far. Most of these studies relied on a qualitative or semiquantitative evaluation of the transcript level.^{85,90,91} The high frequency of *TEL-AML1* transcript positivity in precursor-B-ALL prompted several groups to develop quantitative RT-PCR strategies targeted on this

Table 9 Expression values of the *MLL-AF4* FG transcript in cell lines and patients at diagnosis (phase IV)

Samples	Ct values				MLL-AF4 NCN values		
	MLL-AF4	ABL	B2M	GUS	Per ABL	Per 100 B2M	Per GUS
Cell lines							
RS4;11	24.4	23.1	19.5	22.8	0.35	4.4	0.29
MV4-11	25.4	23.9	20.6	24.2	0.56	9.3	0.63
ALL-PO	24.7	23.1	19.7	23.0	0.62	9.3	0.59
Patients							
BM ($n=14$)	26.1 [24.7–29.6]	24.6 [23.1–26.1]	19.1 [17.2–22.0]	23.5 [21.3–25.7]	0.46 [0.09–0.98]	1.2 [0.07–8.3]	0.32 [0.04–0.65]
PB ($n=8$)	27.1 [23.8–31.0]	25.0 [22.9–29.4]	18.6 [18.9–22.6]	23.4 [21.6–27.9]	0.60 [0.16–0.91]	1.4 [0.32–2.9]	0.27 [0.09–0.58]
Corr. coeff.					0.79	0.67	0.76

For patient samples and cell lines, median values [95% range] are indicated.

transcript to follow MRD.^{47–49,92} Preliminary data show that MRD is still detectable after induction therapy in 40–50% of patients, and that high MRD levels are found in some patients.

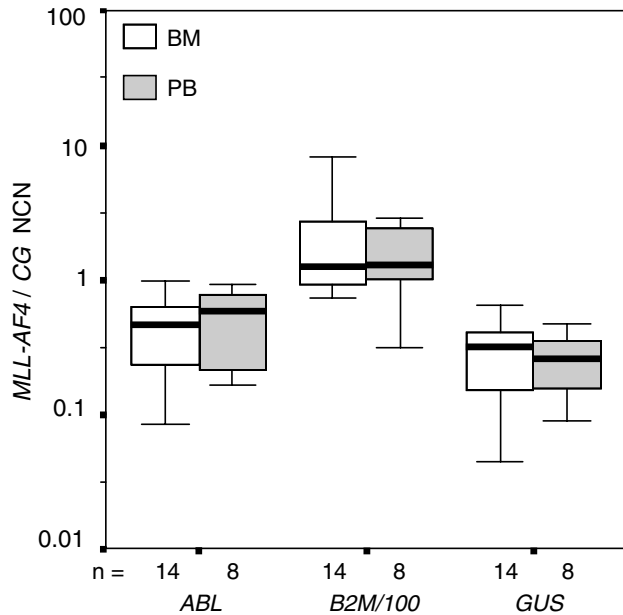


Figure 8 Comparison of *MLL-AF4* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *MLL-AF4* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. *n*: number of patient samples.

Table 10 Results of the QC rounds for *MLL-AF4* FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10 ⁻³	10 ⁻⁴	FG neg. control	NAC/NTC
IIIa	5% (1/21)	10% (2/21)	0% (0/21)	5% (2/42) ^a
IIIb ^b	9% (1/11)	9% (1/11)	0% (0/22)	0% (0/22)
IVa	0% (0/23)	9% (2/23)	2% (1/46)	9% (2/23) ^a
All phases	3.6% (2/55)	9% (5/55)	1.1% (1/89)	4.6% (4/87)

Results are proportions of false-positive or -negative samples. 10⁻³ and 10⁻⁴ are the RNA dilutions of a positive *MLL-AF4* cell line RNA in a negative RNA (PB MNC).

^aFor NAC and NTC samples, only one well out of two was positive in each case.

^bOnly RS4;11 was tested during phase IIIb.

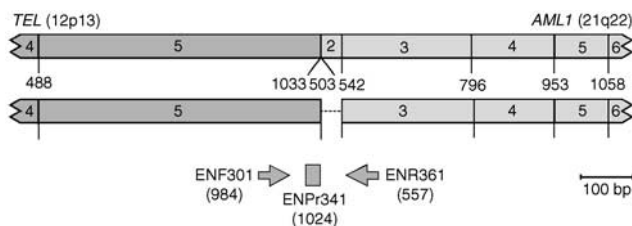


Figure 9 Schematic diagram of the *TEL-AML1* FG transcript covered by the RQ-PCR primer and probe set (ENF301–ENPr341–ENR361). The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 11).

However, despite the relatively high frequency of the *TEL-AML1* fusion, only small series of patients have been analyzed so far (always less than 30 patients), and the rarity of relapses in addition to their possible late occurrence made it difficult to make any clinical correlations so far.

3.2. EAC data

3.2.1. Primer design and optimization (phases I and II): The cell line used for testing was REH,⁹³ which displays a *TEL* exon 5–*AML1* exon 2 fusion. Two plasmid constructs were also used, one containing the 'long transcript' (*TEL* exon 5–*AML1* exon 2) and the other containing the 'short transcript' (*TEL* exon 5–*AML1* exon 3) (see the Materials and methods section).

At the end of the optimization phase, one primer/probe set (ENF301 on *TEL* exon 5, ENR361 on *AML1* exon 3 and ENPr341 on *TEL* exon 5) was selected from the five sets that were initially tested (Figure 9 and Table 11). An example of typical amplification plots (10⁻¹, 10⁻³ and 10⁻⁴ cell line RNA dilutions) is shown in Figure 10; the optimized RT and PCR conditions permitted us to detect a 10⁻⁴ dilution of the REH cell line in PBL RNA and 10 copies of plasmids in 100% of cases, which is consistent with the results obtained previously in the BIOMED-1 Concerted Action.²⁴ It has to be noticed that molecular variants such as transcripts with a *TEL*-exon 4 fusion remain undetected using this set of primers.

Table 11 Sequences and positions of the *TEL-AML1* primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3'
ENF301	<i>TEL</i> , 984–1002 (19)	CTCTGTCTCCCCGCCTGAA
ENPr341	<i>TEL</i> , 1024–1005 (20)	TCCCAATGGGCATGGCGTGC
ENR361	<i>AML1</i> , 557–542 (16)	CGGCTCGTGTGGCAT

^aENF=European network forward primer, ENPr=European network TaqMan reverse probe (in order to select the C-rich strand), ENR=reverse primer.

^bPositions according to accession numbers U11732 (*TEL*) and D43969 (*AML1*).

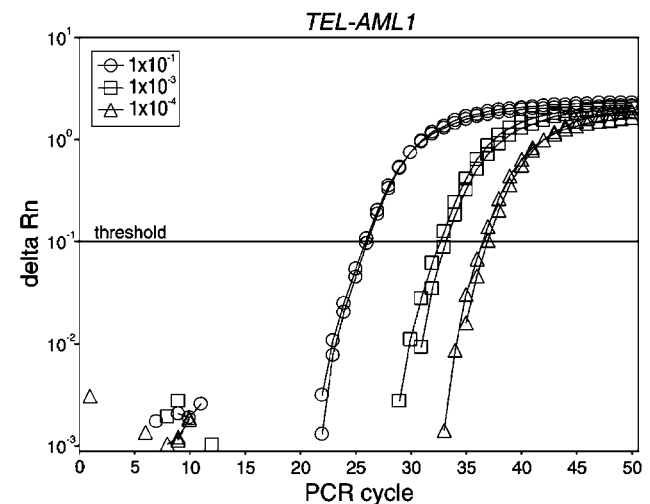


Figure 10 Amplification plots of 10⁻¹, 10⁻³ and 10⁻⁴ dilutions of a *TEL-AML1*-positive RNA sample in a negative RNA sample.

Table 12 Expression values of the *TEL-AML1* FG transcript in cell line and patients at diagnosis (phase IV)

Samples	Ct values				TEL-AML1 NCN ^a		
	TEL-AML1	ABL	B2M	GUS ^b	Per ABL	Per 100 B2M	Per GUS ^b
Cell line REH	21.7	23.4	18.7	23.5	3.2	27	2.5
Patients							
BM (n = 30)	22.6 [20.0–27.9]	25.5 [23.5–27.7]	19.2 [18.0–23.0]	25.2 [23.7–27.3]	4.45 [0.46–32.7]	23 [0.63–132]	3.19 [0.27–10.3]
PB (n = 27)	23.9 [21.6–27.9]	25.4 [24.4–28.3]	19.1 [17.9–22.7]	25.2 [24.5–26.3]	4.71 [0.20–15.2]	12 [0.39–99]	2.63 [0.50–9.0]
Corr. coeff.					0.66	0.42	0.66

^aCorrected according to the blast percentage in the sample.

^bOnly 13 PB and 17 BM samples were tested for *GUS*. For patient samples and cell lines, median values [95% range] are indicated.

3.2.2. TEL-AML1 expression in REH cell line and diagnostic patient samples (phase IV): *TEL-AML1* expression was studied in the REH cell line and in 57 *TEL-AML1*-positive precursor-B-ALL, 30 BM and 27 PB samples, including 23 paired samples (Table 12). Samples contained 18–100% leukemic blasts. A majority of these samples were obtained from patients of Hôpital Saint-Louis (Paris, France). NCN were calculated and adjusted for the percentage of blasts present in each sample.

TEL-AML1 expression in the REH cell line was within the range detected in primary leukemia samples.

No significant difference for *ABL* NCN was observed in *TEL-AML1* expression when comparing PB and BM samples collected at diagnosis in the same patients (Figure 11).

3.2.3. QC rounds (phases IIIa–IVa): As expected from the sensitivity threshold defined in previous phases, 10^{-3} and 10^{-4} RNA dilutions of the REH cell line were always found to be positive (Table 13) according to the criteria defined in the Materials and methods section. A 5×10^{-5} dilution was found to be positive in 7/7 laboratories. During the various QC rounds, 11 negative samples (six negative RNA and five NAC or NTC samples) were tested in 7–11 labs, corresponding to a total of 279 amplification wells (Table 13). Nine of these wells (3%) were found falsely positive, corresponding to three false-positive samples out of 93 (3%) according to the criteria mentioned above. These false-positive wells were observed in six different labs. No case of false positivity with three positive wells was observed, and Ct values were always higher than 39. All false-positive wells corresponded to negative RNA, while NAC and NTC were always negative. This observation suggests that these false-positive results could be due to contaminations achieved prior to amplification, such as the RT step.

4. t(9;22)(q34;q11) with the *BCR-ABL* m-bcr fusion gene transcript

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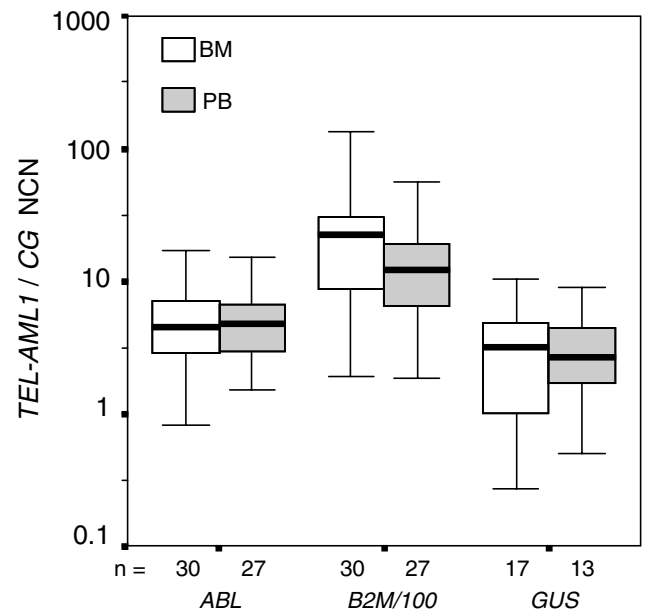


Figure 11 Comparison of *TEL-AML1* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *TEL-AML1* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. All differences between BM and PB per CG are not significant on paired samples ($n=23$, Wilcoxon test). n : number of patient samples.

Table 13 Results of the QC rounds for *TEL-AML1* FG transcript detection (phases III and IV)

Phase	False negativity			False positivity	
	10^{-3}	10^{-4}	5×10^{-5}	FG neg. control	NAC/NTC
IIIa	0% (0/7)	0% (0/7)	ND	0% (0/14)	0% (0/14)
IIIb	0% (0/11)	0% (0/11)	ND	9% (2/22)	0% (0/22)
IVa	ND	0% (0/7)	0% (0/7)	7% (1/14)	0% (0/7)
All phases	0% (0/18)	0% (0/25)	0% (0/7)	6% (3/50)	0% (0/43)

Results are proportions of false-positive or -negative samples. 10^{-3} , 10^{-4} and 5×10^{-5} are the RNA dilutions of REH cell line RNA in a negative RNA. ND = not done.

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4.1. Background

The *BCR-ABL* FG is associated with the formation of the Philadelphia translocation (Ph) and is one of the most common genetic abnormalities detected in leukemias.⁹⁴ In ALL, Ph is detected in 25–30% of adult and 2–5% of childhood cases.⁹⁵ Less frequently, it is associated with AML.⁹⁶ In the ALL subset, this genetic lesion is known to confer a very poor prognosis,^{95,97} and, consequently, its detection is important in planning aggressive therapies, including allogeneic bone marrow transplant. In addition, the Ph chromosome is found in more than 95% of CML cases and is the hallmark of this disease.⁹⁸ At the molecular level, the Ph chromosome or t(9;22) results in the juxtaposition of the 5’ part of the *BCR* gene (chromosome 22) to the 3’ part of the *ABL* gene (chromosome 9). In the vast majority of patients, the breakpoints in the *BCR* gene are clustered within three well-defined regions: (i) a 55 kb sequence of the first intron, called the minor breakpoint cluster region (m-bcr),⁹⁹ (ii) a 5.8 kb region spanning exons 12–16, called the major breakpoint cluster region (M-bcr),⁹⁹ and finally (iii) intron 19, called μ -bcr.^{100,101} Analysis of μ -bcr breakpoints will not be discussed further due to their extreme rarity. In the case of m-bcr breakpoints, the first exon of the *BCR* gene (e1) is juxtaposed to the second exon of the *ABL* gene (a2). The resultant fusion transcript (e1-a2) encodes a 190 kDa chimeric protein (p190).⁹⁹ This type of *BCR-ABL* FG is found in 65% of adults and 80% of children with Ph-positive ALL.¹⁰² Only in sporadic cases is the p190 encoding *BCR-ABL* gene found in CML.¹⁰³

Despite huge efforts, the molecular mechanisms by which the hybrid *BCR-ABL* protein gains transforming capability are still not fully understood. However, the *BCR-ABL* protein shows an increased and deregulated tyrosine kinase activity⁹⁸ and it seems to deregulate the normal cytokine-dependent signal transduction pathways leading to the inhibition of apoptosis and by growth factor independent growth.¹⁰⁴

All the studies about the clinical value of MRD in Ph+ ALL patients indicate that *BCR-ABL*-positive cells cannot be eradicated even by intensive chemotherapy.^{105–107} In a series of 36 Ph+ ALL patients treated by SCT, Radich *et al*¹⁰⁸ reported that RT-PCR assessment of MRD was, by multivariate analysis, the best prognostic indicator for continuous complete remission (CR). Recently, Yokota *et al*⁴⁰ reported the follow-up by RQ-PCR analysis of 13 m-bcr-positive ALL patients. All these data suggest that, in Ph+ ALL patients, quantitative monitoring of residual leukemic cells could prove more valuable than their qualitative detection to assist in clinical decision-making.

4.2. EAC data

4.2.1. Primer design and optimization (phases I and II): The efficiency of four different primer/probe sets, designed using the ‘Primer Express™’ software, was tested in 1:10 serial dilution experiments of RNA from the TOM-1 cell line (e1-a2 junction or m-bcr) into RNA from HL60 cells. All primer/probe sets were free from nonspecific amplification artifacts, but two sets were superior in terms of sensitivity and ΔR_n value at plateau phase. Both sets had comparable amplification efficiencies and reached 10^{-5} sensitivity and $\Delta R_n > 3.0$ at the 10^{-1} cell line dilution. After extensive testing, the set that included ENF402 (located in *BCR* exon 1), ENR561 and probe ENP541 (both located in *ABL* exon 2) was selected. Both the reverse primer and probe are common to the set used for the RQ-PCR detection of *BCR-ABL* (M-bcr) FG transcripts (Figure 12 and Table 14). An example of typical amplification plots (10^{-1} , 10^{-3} and 10^{-4} cell line RNA dilutions) is shown in Figure 13.

4.2.2. *BCR-ABL* m-bcr FG expression in TOM-1 cell line and diagnostic patient samples (phase IV): To establish reference intervals of m-bcr transcripts, we determined FG expression in 17 BM samples and seven PB samples from sequential ALL patients at diagnosis as well as centrally prepared and distributed RNA from the TOM-1 cell line (Table 15). For the FG and the three CG transcripts assayed, separate series of

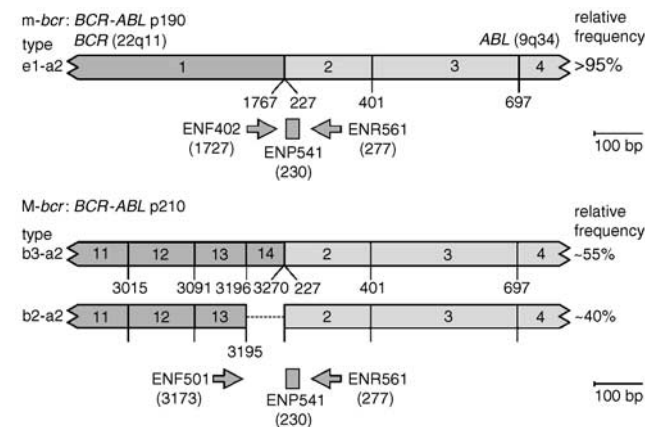


Figure 12 Schematic diagram of the *BCR-ABL* FG transcript covered by the RQ-PCR primer and probe set. For m-bcr, set: ENF402–ENP541ENR561; and for M-bcr, set: ENF501–ENP541–ENR561. The number under the primers and probe refers to their 5’ nucleotide position in the normal gene transcript (see Tables 14 and 17). Relative frequency refers to the proportion of e1-a2 transcript among m-bcr variants and the proportion of b3-a2 and b2-a2 transcripts among M-bcr variants.

Table 14 Sequences and positions of the *BCR-ABL* m-bcr primers and probe

EAC code ^a	Primer/probe localization, ^b 5’–3’ position (size)	Sequence 5’–3’
ENF402	<i>BCR</i> , 1727–1744 (18)	CTGGCCCCAACGATGGCGA
ENP541	<i>ABL</i> , 230–254 (25)	CCCTTCAGCGGCCAGTAGCATCTGA
ENR561	<i>ABL</i> , 277–257 (21)	CACTCAGACCCTGAGGCTCAA

^aENF = European network forward primer, ENP = European network TaqMan probe, ENR = European network reverse primer.

^bPositions according to accession numbers X02596 (*BCR*) and X16416 (*ABL*).

plasmid dilutions were amplified in each experiment to calculate transcript copy numbers. Although samples with *ABL* Cts >29.3 were excluded from the analysis because they were not suitable for amplification, the Ct values of all transcripts were generally lower in cell lines than in patient material (both BM and PB), most probably due to low quality of some of the patient RNAs. However, after normalization to CG expression, m-bcr transcript levels were comparable in patients and the TOM-1 cell line, and very similar in four paired BM and PB samples (Figure 14).

The primers and probe used to amplify *ABL* mRNA are also able to amplify *BCR-ABL* FG mRNA, and hence the assay of *ABL* gene expression used to normalize data may be affected by the levels of m-bcr transcript in the samples.

4.2.3. Quality control rounds (phases IIIa–IVa): Only 3/129 wells gave false-negative results (see the Materials and methods section for details) in the three QC rounds, and in all cases the false negativity was obtained for 10^{-4} TOM-1 dilutions. Furthermore, false-positive results were detected in 5.3% of PCR tests (13/246, Table 16).

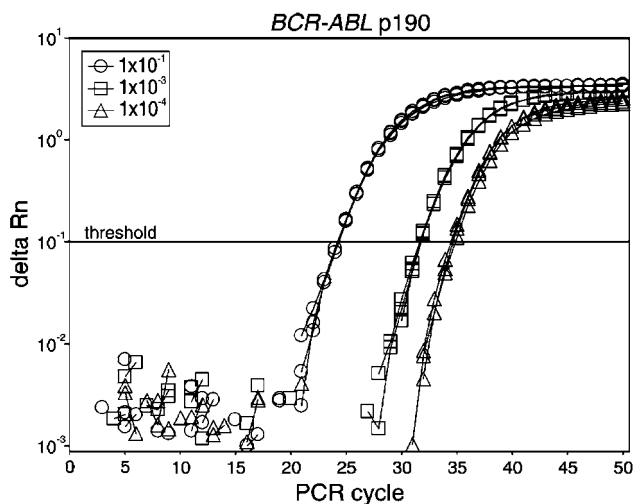


Figure 13 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of a *BCR-ABL* m-bcr-positive RNA sample in a negative RNA sample.

5. t(9;22)(q34;q11) with the *BCR-ABL* M-bcr fusion gene transcript

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5.1. Background

Most cases of CML are associated with the presence of t(9;22) resulting in a small derivative chromosome 22 known as the Ph.¹⁰⁹ As a consequence, the *ABL* proto-oncogene on chromosome 9 is fused to the *BCR* gene on chromosome 22.¹¹⁰ In CML patients and approximately 35% of Ph-positive adult ALL patients, the breakpoint on chromosome 22 is located between exons 12 and 16 of the *BCR* gene, in the so-called major breakpoint cluster region (M-bcr).⁹⁹ The breakpoint on chromosome 9 is located in most cases between exons 1 and 2 in the *ABL* gene. The transcription product of this *BCR-ABL* FG is an 8.5-kb aberrant fusion RNA with two junction variants b2a2 and/or b3a2 that gives rise to the *BCR-ABL* chimeric protein (p210), a tyrosine kinase with deregulated activity.¹¹¹ Rare cases with b2a3 and b3a3 *BCR-ABL* transcripts can be observed.

Because of its high sensitivity, qualitative RT-PCR has been extensively used to monitor residual disease in CML, yielding partially contradictory results. Sequential analysis of patients who received allogeneic BM transplantation (BMT) showed that repeated PCR positivity correlated with an increased risk of relapse.^{112,113} On the contrary, other studies did not find any correlation between PCR positivity and subsequent relapse, and showed that long-term survivors of allogeneic BMT could be

Table 15 Expression values of the *BCR-ABL* m-bcr FG transcript in cell line and ALL patients at diagnosis (phase IV)

Samples	Ct values				BCR-ABL NCN values		
	BCR-ABL m-bcr	ABL ^a	B2M	GUS	Per ABL ^a	Per 100 B2M	Per GUS
Cell line							
TOM-1	22.8	21.8	18.1	23.8	0.44	4.69	1.59
Patients							
BM (n = 17)	24.7 [21.3–27.1]	24.5 [21.7–27.1]	20.4 [16.6–22.1]	25.8 [22.1–28.9]	0.80 [0.35–1.38]	2.70 [0.58–26.3]	1.48 [0.24–16.6]
PB (n = 7)	23.6 [21.1–29.1]	22.7 [21.0–27.0]	18.2 [15.3–19.5]	24.5 [21.5–25.5]	0.68 [0.39–1.81]	3.36 [0.03–13.75]	1.63 [0.18–9.34]
Corr. coeff.					0.96	0.82	0.80

^aThe *ABL* EAC RQ-PCR set can amplify both *BCR-ABL* and *ABL* transcripts. Thus the values are impaired by the presence of the FG. For patient samples and cell lines, median values [95% range] are indicated.

PCR positive even years after transplant without ever relapsing.^{114–117}

A competitive RT-PCR method to quantify the level of *BCR-ABL* FG transcripts was developed by several groups^{118,119} in an effort to improve the predictive value of *BCR-ABL* mRNA detection. The sequential analysis of patients who had undergone BMT showed that monitoring of *BCR-ABL* FG transcripts

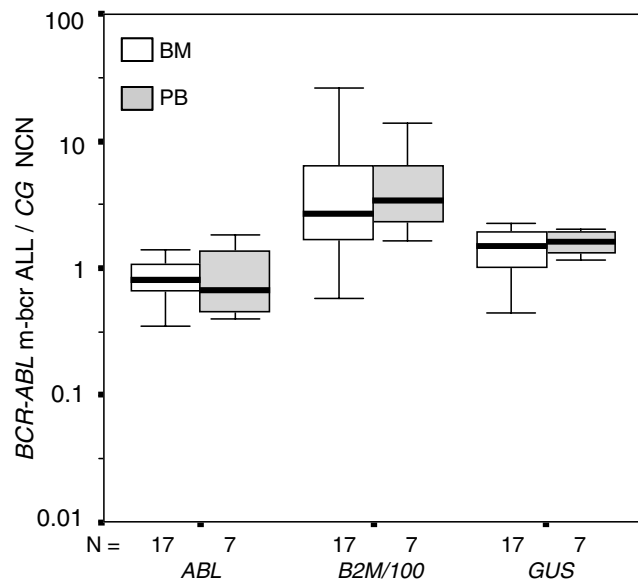


Figure 14 Comparison of *BCR-ABL* m-bcr FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *BCR-ABL* m-bcr per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. *n*: number of patient samples.

Table 16 Results of the QC rounds for *BCR-ABL* m-bcr FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10 ⁻³	10 ⁻⁴	FG neg. control	NAC/NTC
IIIa	0% (0/24)	0% (0/24)	13% (3/24)	8% (5/64)
IIIb	0% (0/30)	0% (0/30)	5% (3/60)	5% (2/42)
IVa	ND	14% (3/21)	0% (0/42)	0% (0/14)
All phases	0% (0/54)	4% (3/75)	4.8% (6/126)	5.8% (7/120)

Results are proportions of false-positive or -negative samples. 10⁻³ and 10⁻⁴ are the RNA dilutions of TOM-1 *BCR-ABL* m-bcr-positive cell line in HL-60 RNA. ND = not done.

Table 17 Sequences and positions of the *BCR-ABL* M-bcr primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3' ^c
ENF501	<i>BCR</i> , 3173–3193 (21)	TCCGCTGACCATCAAYAAGGA
ENP541	<i>ABL</i> , 230–254 (25)	CCCTTCAGCGGCCAGTAGCATCTGA
ENR561	<i>ABL</i> , 277–257 (21)	CACTCAGACCCTGAGGCTCAA

^aENF = European network forward primer, ENP = European network TaqMan probe, ENR = European network reverse primer.

^bPositions according to accession numbers X02596 (*BCR*) and X16416 (*ABL*).

^cY (cytidine or thymidine) appears on the *BCR* primer according to the polymorphism recently described on the *BCR* gene.⁵²

levels can be useful to predict an impending relapse while the patient is still in hematological and cytogenetic remission.^{31,120}

Based on quantitative RT-PCR studies, two groups have proposed to define a 'molecular relapse' parameter.^{31,121} In addition, quantification of *BCR-ABL* FG transcripts has also proven useful to monitor response to α -interferon¹²² and imatinib-treated patients.^{123,124}

Despite many encouraging reports, monitoring of *BCR-ABL* FG transcripts with competitive RT-PCR has had limited clinical impact, partly due to the fact that this approach is difficult to standardize. Since the advent of real-time PCR, several groups have published reports that describe the feasibility of monitoring CML patients with this technique.^{37–39,125–127} However, most RQ-PCR studies included too few patients or patients from different therapeutic protocols that together with methodological differences make it difficult to evaluate the clinical impact and to define general guidelines to monitor CML patients with RQ-PCR. The availability of a standardized protocol for RQ-PCR will facilitate data comparison among different centers, making it possible to define a threshold where a patient is likely to relapse and ultimately to assess the impact of an early therapeutic intervention based on the kinetics of *BCR-ABL* FG transcripts.

5.2. EAC data

5.2.1. Primer design and optimization (phases I and II):

Two alternative forward *BCR* primers, one located on *BCR* exon 13 (exon b2) and the second on *BCR* exon 14 (exon b3), and a reverse *ABL* primer and probe, both on the second exon of the *ABL* gene, were designed (Figure 12). This set, together with five comparable sets already available within the network, was evaluated for sensitivity as well as for robustness of the PCR reaction (see the Materials and methods section). Based on these parameters, the set designed by the EAC group was chosen. Since dilution series of the K-562 cell line was amplified with equal efficiency with either of the two forward primers, one common forward primer ENF501, located on *BCR* exon 13 (exon b2), was selected to amplify both fusion variants *BCR-ABL* b3-a2 and b2-a2 (Figure 12, Table 17). An example of typical amplification plots (10⁻¹, 10⁻³ and 10⁻⁴ cell line RNA dilutions) is shown in Figure 15.

5.2.2. *BCR-ABL* M-bcr expression in K-562 cell line and diagnostic patient samples (phase IV)

5.2.2.1. For CML, in K-562 and patients at diagnosis:

The expression of *BCR-ABL* M-bcr transcripts was quantified in 29 CML patients in order to establish the range of FG expression levels in diagnostic samples (Table 18 and Figure 16a). These samples included 15 BM and 14 PB samples. The expression of the *BCR-ABL* FG relative to the CG was very similar between BM and PB. No large variations in *BCR-ABL*

M-bcr expression levels were found between individual patients. Expression of *BCR-ABL* mRNA in the K-562 cell line was found to be higher than CML diagnostic samples by two logs relative to *B2M* and one log relative to *GUS* gene (see Table 18).

5.2.2.2. In *BCR-ABL* M-bcr-positive ALL: In addition to diagnostic samples from CML patients in chronic phase, *BCR-ABL* M-bcr expression was quantified in diagnostic *BCR-ABL* M-bcr-positive ALL samples (Table 19 and Figure 16b). Expression of *BCR-ABL* relative to *B2M* and *GUS* was found to be higher in ALL samples compared to CML samples (Tables 18 and 19). We observed a statistical difference in the FG level of expression between M-bcr ALL and CML patients either with *B2M* or *GUS* as CG ($P=0.001$), while no difference was observed between m-bcr and M-bcr in ALL (Figure 17).

The primer set designed to amplify the *ABL* CG is located on exon 2 and also amplifies the *BCR-ABL* FG. For this reason, the use of *ABL* as a CG could introduce a bias for quantifying *BCR-ABL* in CML and Ph+ ALL samples when a large proportion of the cells express *BCR-ABL*. Using the ratio (*BCR-ABL*/*ABL*) would theoretically lead to an underestimation of the tumor load

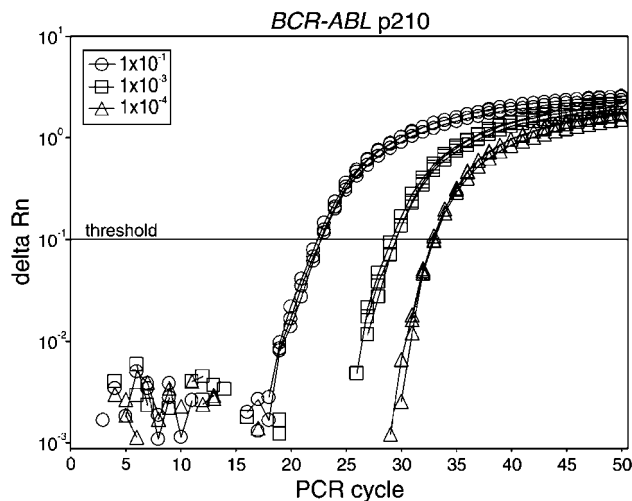


Figure 15 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of a *BCR-ABL* M-bcr-positive RNA sample in a negative RNA sample.

in these samples since the maximum ratio is one. However, this bias had a minor impact on relative quantification of FG transcripts at diagnosis (see Section 11.1.3). We found values up to 3.0 in BM and up to 4.4 in PB samples of CML patients at diagnosis (Table 18), although all median *BCR-ABL*/*ABL* ratios were below 1, except for PB CML patients at diagnosis (Tables 15 and 18). These unexpected results obtained with plasmid calibrators were confirmed without calibrators (ΔCt method, see web site) and clearly illustrate the limits of accuracy of gene transcript quantification by RQ-PCR. Similar results were observed in a oligocenter context.^{125,128}

5.2.3. Quality control rounds (phases IIIa–IVa): The percentage of false negatives was 4.9% (14/285) for the first and second quality control rounds (Table 20). The laboratories that showed the false negatives had a consistent reduction in sensitivity for all the targets in a particular phase, which indicated that the cause for the lower sensitivity was a lower RT efficiency rather than a PCR-related problem.

In the third quality control round (phase IVa), a rate of false positivity (5.5%, 6/110) similar to the previous phases was observed despite a particularly high frequency (18%, 4/22) of false-positive results within FG-negative samples (Table 20). This observation is possibly explained by the inclusion of the undiluted K-562 RNA among the coded samples instead of the 10^{-1} dilution and thereby increasing the risk of accidentally contaminating neighboring wells when pipeting the cDNA onto the PCR plate. It should be noted that the majority of the false-positive samples (NAC/NTC) were concentrated in individual laboratories while the rest of the laboratories (one laboratory per QC round) showed only occasionally single false-positive wells or no false positives.

6. Intrachromosomal microdeletion on 1p32 with the *SIL-TAL1* fusion gene transcript

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¹Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands; ²Hôpital Necker, Paris, France;

Table 18 Expression values of the *BCR-ABL* M-bcr FG transcript in cell line and CML patients at diagnosis (phase IV)

Samples	Ct values				<i>BCR-ABL</i> NCN		
	<i>BCR-ABL</i> M-bcr	<i>ABL</i> ^a	<i>B2M</i>	<i>GUS</i>	Per <i>ABL</i> ^a	Per 100 <i>B2M</i>	Per <i>GUS</i>
Cell line K-562	20.5	20.7 ^b	20.6	21.5	1.0	155 ^b	0.79
Patients							
BM (n = 15)	25.1 [21.5–27.0]	25.2 [20.7–26.8]	18.1 [15.9–21.5]	22.4 [21.6–27.9]	0.86 [0.44–3.0]	0.95 [0.43–5.3]	0.12 [0.04–0.87]
PB (n = 14)	23.1 [21.9–25.8]	23.7 [22.6–26.7]	17.6 [16.8–21.5]	21.7 [20.7–24.8]	1.17 [0.48–4.4]	2.8 [0.56–6.2]	0.22 [0.08–0.41]
Corr. coeff.					0.81	0.63	0.76

^aThe *ABL* EAC RQ-PCR set can amplify both *BCR-ABL* and *ABL* transcripts. Thus the values are impaired by the presence of the FG. We found values up to 3.0 in BM and up to 4.4 in PB samples of CML patients at diagnosis. These unexpected results were obtained with plasmid standard curve and without standard (ΔCt method, see web site).

^bK-562 cell line was tested in Uppsala and Marseille Nord on two independent cultures to confirm the results. For patient samples and cell lines, median values [95% range] are indicated.

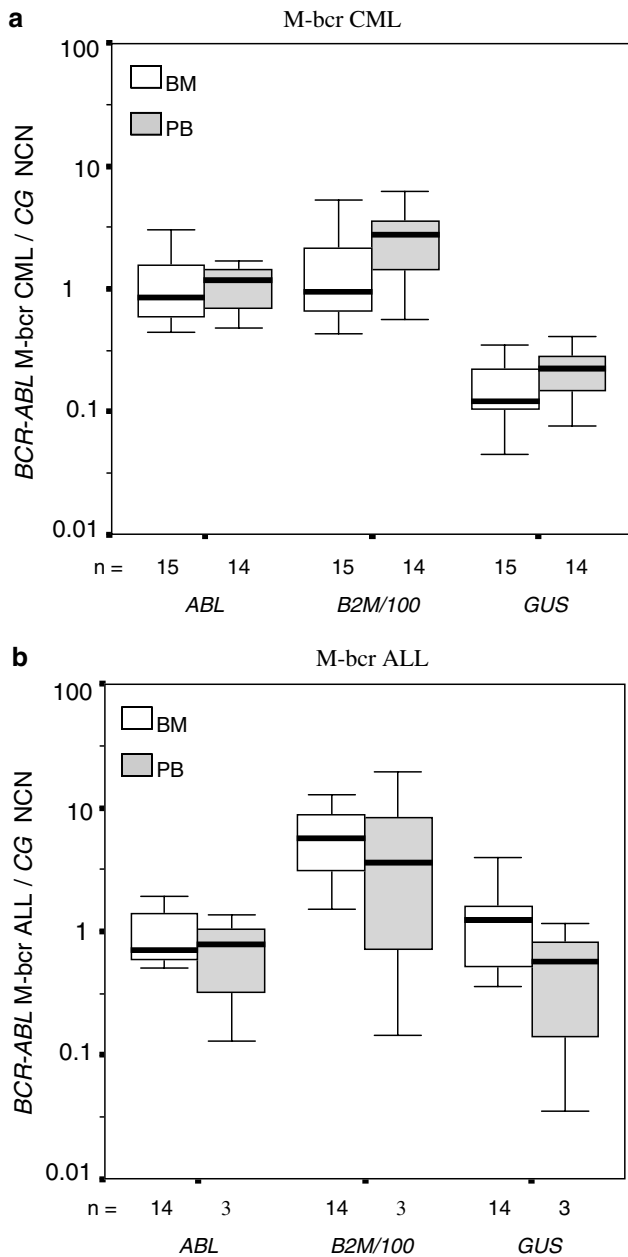


Figure 16 Comparison of *BCR-ABL* M-bcr FG transcript expression between BM and PB using either *ABL*, *B2M* or *GUS* as CG in (a) CML samples, (b) ALL samples. The ratio (NCN) is defined as the ratio between the copy number of *BCR-ABL* M-bcr per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. *n*: number of patient samples.

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6.1. Background

The microdeletion on 1p32 is the most frequent chromosome aberration found in childhood T-ALL.^{129–135} The microdeletion involves the *TAL1* gene (T-cell acute leukemia 1 gene, also

known as stem cell leukemia (*SCL*) or T-cell leukemia gene 5 (*TCL5*) and the *SIL* gene (*SCL* interrupting locus), which is located approximately 90 kb upstream.¹³³ As a result, the *TAL1* coding sequences are placed under the control of the *SIL* promoter, which is expressed in T cells, and consequently the *TAL1* gene becomes ectopically expressed in the involved T-ALL.

The *TAL1* gene, in particular exons 4–6, encode a 42 kDa protein, which is a basic helix–loop–helix (bHLH) transcription factor. The *TAL1* protein can heterodimerize with other bHLH transcription factors, including members of the E2A family, and is an essential factor for the development of all hematopoietic lineages.^{136–138} The *SIL* gene is a member of the immediate-early gene family, but its function in hematopoietic cells is not yet well defined.^{129,139}

Although both the *SIL* and *TAL1* genes contain several conserved deletion breakpoints, most cases ($\geq 95\%$) involve the sildb1 breakpoint in combination with the taldb1 or taldb2 breakpoint.^{131,132,140} By alternative splicing, three different *SIL-TAL1* transcripts can be formed, of which the type II transcript is the most predominant one.¹³⁴ There is no apparent relationship between the occurrence of *SIL-TAL1* transcripts and prognosis or outcome.¹³⁰

SIL-TAL1 transcripts are exclusively found in T-ALL, in which they are present in 5–25% of the patients.^{129,132,134,141} The frequency is related to the immunophenotype of the T-ALL (the presence of the *SIL-TAL1* FG is restricted to CD3[−] and TCR $\alpha\beta$ ⁺ T-ALL) and the occurrence of *TCRD* gene deletions.^{132,142} The *SIL-TAL1* FG transcripts seem to be more frequent in children compared to adults.¹³⁴

The detection of *TAL1* deletions at the DNA level has already been described.^{143–145} A recent report described a TaqMan-based RQ-PCR method for the detection of *TAL1* deletions at the DNA level in T-ALL patients.¹⁴⁵ In that report, the forward primer and probe were positioned in *SIL* exon 1b (and part of the following intron) and the reverse primer was located in *TAL1* exon 1b. Using the CEM cell line, a sensitivity of 10^{-5} could be obtained, which is equivalent to a single leukemic genome. To our knowledge, no RQ-PCR primers/probe sets for *SIL-TAL1* FG transcripts have been published so far.

6.2. EAC data

6.2.1. Primer design and optimization (phases I and II): Initially, three TaqMan probes (located in *SIL* exon 1a, *TAL1* exon 4 and *TAL1* exon 5), two forward primers (both in *SIL* exon 1a) and five reverse primers (two in *TAL1* exon 3, two in *TAL1* exon 4 and one in *TAL1* exon 6) were tested for specificity and efficiency.

A single forward primer (ENF601; located in *SIL* exon 1a), reverse primer (ENR664; located in *TAL1* exon 3) and probe (ENP641; located in *SIL* exon 1a) were selected (Figure 18 and Table 21). An example of typical amplification plots (10^{-1} , 10^{-3} and 10^{-4} cell line RNA dilutions) is shown in Figure 19.

The selected primer/probe set will detect virtually all *SIL-TAL1* transcripts (the most common type II as well as type III), but will not detect *TAL1* translocations or ‘aberrant’ expression of the *TAL1* gene without apparent rearrangements.

6.2.2. *SIL-TAL1* expression in cell lines and diagnostic patient samples (phase IV): Undiluted RNA of six different *SIL-TAL1*-positive cell lines was tested in duplicate for CG expression (*ABL*, *B2M* and *GUS*) and in triplicate for *SIL-TAL1* FG expression (Table 22). In three laboratories (Erasmus MC in

Table 19 Expression values of the *BCR-ABL* M-bcr FG transcript in ALL patients at diagnosis (phase IV)

Samples	Ct values				BCR-ABL NCN		
	<i>BCR-ABL</i> M-bcr	<i>ABL</i> ^a	<i>B2M</i>	<i>GUS</i>	Per <i>ABL</i> ^a	Per 100 <i>B2M</i>	Per <i>GUS</i>
Patients							
BM and PB ^{a,b} (n = 17)	24.1 [21.5–29.9]	24.0 [21.6–26.4]	19.0 [16.3–20.5]	24.8 [20.8–27.1]	0.71 [0.13–1.93]	4.90 [0.14–19.5]	1.15 [0.03–3.89]
Corr. coeff.					0.90	0.56	0.60

^aThe *ABL* EAC RQ-PCR set can amplify both *BCR-ABL* and *ABL* transcripts. Thus the values are impaired by the presence of the FG. For patient samples, median values [95% range] are indicated.

^bOne laboratory (Uppsala) analyzed all the samples.

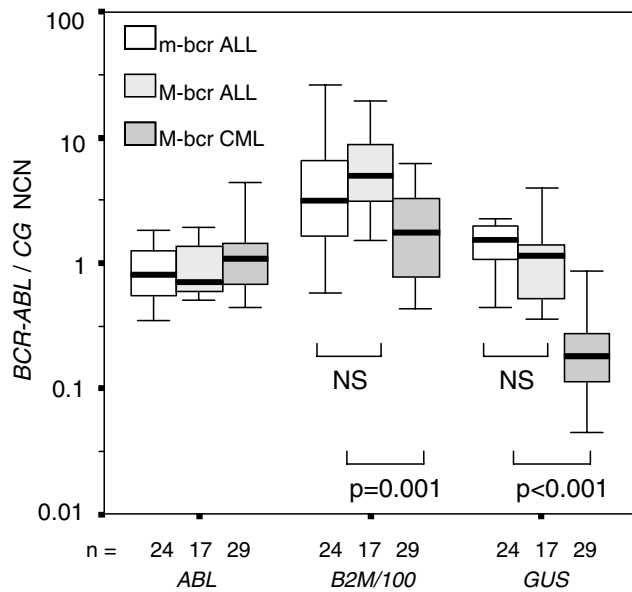


Figure 17 Comparison of *BCR-ABL* FG transcript expression between m-bcr ALL, M-bcr ALL and M-bcr CML at diagnosis using either *ABL*, *B2M* or *GUS* as CG. PB and BM were pooled according to each group to compare *BCR-ABL* FG expression between m-bcr ALL, M-bcr ALL and M-bcr CML. No significant difference was observed between m-bcr and M-bcr ALL either with *B2M* or *GUS* CG (Mann-Whitney test). A highly significant difference was found between *BCR-ABL*-positive ALL ($n=41$) and CML ($n=29$) samples either with *B2M* or *GUS* CG (Mann-Whitney test). n : number of patient samples.

Rotterdam, Hôpital Necker and Hôpital St Louis in Paris), a total of 16 *SIL-TAL1*-positive patients at diagnosis were also included (10 BM and 10 PB samples, including five pairs).

Ct values for the CG transcripts were significantly lower in the cell lines as compared to the patient samples (Table 22), which could be due to the fact that stored patient samples were used or alternatively that their expression is higher in cell lines than in primary patient samples. *SIL-TAL1* transcript expression (Ct, CN and NCN) was comparable between the six cell lines tested, but in patients a slightly larger variation in *SIL-TAL1* FG transcript expression was found (Table 22). Nevertheless, the normalized *SIL-TAL1* FG transcript expression did not differ between cell lines and patients. Furthermore, comparison between BM and PB samples (including five pairs) showed that *SIL-TAL1* transcript expression was similar in both compartments (Figure 20).

Table 20 Results of the QC rounds for *BCR-ABL* M-bcr FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10^{-3}	10^{-4}	FG neg. control	NAC/NTC
IIIa	0% (0/11)	9% (1/11)	9% (1/11)	4% (5/120)
IIIb	0% (0/11)	9% (1/11)	5.5% (1/22)	5% (7/132) ^a
IVa	0% (0/11)	0% (0/11)	18% (4/22)	2% (2/88)
All phases	0% (0/33)	6.1% (2/33)	10.9% (6/55)	4.1% (14/340)

Results are proportions of false-positive or -negative samples. 10^{-3} and 10^{-4} are the RNA dilutions of K-562 cell line RNA in a negative RNA.

^aOne lab had all their NAC+NTC contaminated, thus contributing to 50% of false-positive results.

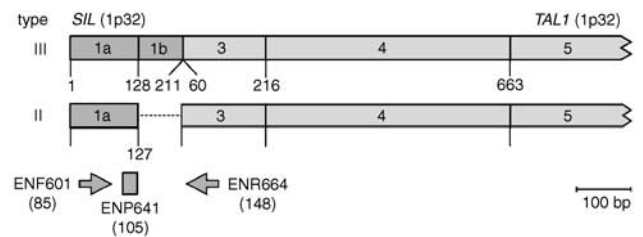


Figure 18 Schematic diagram of the *SIL-TAL1* FG transcript covered by the RQ-PCR primer and probe set (ENF601–ENP641–ENR664). The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 21).

6.2.3. Quality control rounds with blind samples (phases IIIa–IVa): False positivity was observed in two out of 46 FG-negative samples (4.3%) and in none out of 162 NAC/NTC wells (0%) resulting in a total false positivity of 1.0% (2/208). False negativity was only observed in the first QC round (phase IIIa), but was absent in the next two QC rounds (Table 23). Therefore, false negativity does not seem to be a problem, although this may be dependent on the level of *SIL-TAL1* FG transcripts in the leukemic cell sample.

7. t(15;17)(q22;q21) with the *PML-RARA* fusion gene transcript

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Table 21 Sequences and positions of the *SIL-TAL1* primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3'
ENF601	<i>SIL</i> , 85–103 (19)	CGCTCCTACCCTGCAAACA
ENP641	<i>SIL</i> , 105–126 (22)	ACCTCAGCTCCGCGGAAGTTGC
ENR664	<i>TAL</i> , 1 148–130 (19)	CCGAGGAAGAGGATGCACA

^aENF = forward primer, ENP = TaqMan probe, ENR = reverse primer.

^bPositions according to accession numbers M74558 (*SIL*) and S53245 (*TAL1*).

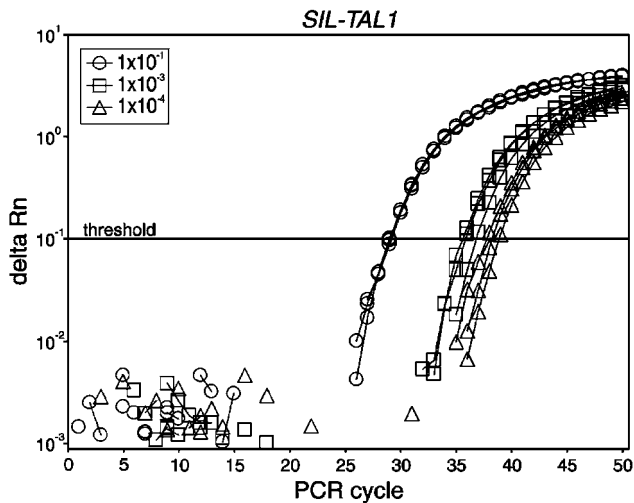


Figure 19 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of a *SIL-TAL1*-positive RNA sample in a negative RNA sample.

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¹Ospedale San Gerardo, Monza, Italy; ²Guy's, King's & St Thomas' School of Medicine, London, UK; ³Hospital Clínico Universitario, Salamanca, Spain; ⁴Institut Paoli Calmettes and present affiliation: Hôpital Universitaire Nord, Marseille, France; ⁵CLB, Amsterdam, The Netherlands; ⁶Karolinska Hospital, Stockholm, Sweden; ⁷Institut Paoli-Calmettes, Marseille, France; ⁸Medical School, Hannover, Germany; and ⁹Università la Sapienza, Roma, Italy

7.1. Background

The *PML-RARA* FG transcripts, which are the molecular result of the t(15;17)(q22;q21) translocation, are associated with the majority of APL cases, a distinct AML subset with M3 cytomorphology.¹⁴⁶

APL accounts for 10–15% of *de novo* AML in younger adults in Southern Europe (reviewed in Biondi et al¹⁴⁷). Among pediatric patients, the incidence of APL is usually considered to be lower and accounting for 3–9%, although published data from Italian cooperative studies indicate that APL occurs in Italian children with the same incidence as observed in adults. Moreover, several small series from different countries in Central and South America have noted a higher-than expected frequency of pediatric APL.¹⁴⁷

The two genes fused in the t(15;17) are *PML*, located on chromosome 15,^{148–151} and the retinoic acid receptor α (*RARA*) gene on chromosome 17.^{152,153} Other genes have been shown to be fused to *RARA* in rare instances of morphological APL cases negative for the t(15;17), such as *PLZF* on chromosome 11q23, *NPM* on 5q35, *NUMA* on 11q13 and *STAT5B* on 17q21.¹⁵⁴

The chimeric *PML-RARA* protein is a transcriptional repressor. In the absence of ligand (retinoic acid, RA), it binds DNA together with co-repressors such as SMRT (silencing mediator for RAR and TR) and N-CoR (nuclear receptor co-repressor) and renders chromatin inaccessible to transcriptional activators or basal transcription machinery.^{154,155}

RARA breakpoints always occur in intron 2 which is 17 kb in length (Figure 21).¹⁴⁹ By contrast, three regions of the *PML* locus are involved in the t(15;17) translocation breakpoints: intron 6 (bcr1; 55% of cases), exon 6 (bcr2; 5%) and intron 3 (bcr3; 40%) (Figure 21). As a consequence, there are three possible *PML-RARA* isoforms, referred to as long (L, or bcr1), variant (V, or bcr2) and short (S, or bcr3). It should be noted that the size of the PCR products varies in bcr2-positive cases, because of the variable breakpoint positions in exon 6 of the *PML* gene and inclusion of a variable number of *RARA* intron 2-derived nucleotides in the FG transcript.¹⁵⁶ Chimeric *PML-RARA* and *RARA-PML* transcripts are formed as a consequence of the reciprocal translocation between the *PML* and *RARA* loci. However, the observation that *RARA-PML* FG transcripts are present in most but not all APL cases, has favored the use of *PML-RARA* FG transcripts as PCR target for detection of APL cells at diagnosis and during monitoring. Standardized conditions for RT-PCR analysis of *PML-RARA* FG transcripts have been developed by the BIOMED-1 Concerted Action.²⁴ Primer sets have been designed that allow the detection of the various *PML-RARA* FG transcripts, generated by the existence of different *PML* breakpoint regions as well as the presence of alternative splicing between central exons of *PML*.

In the last decade, the availability of differentiation therapy with all-*trans* retinoic acid (ATRA) has produced a remarkable improvement in the outcome of patients with APL (reviewed in Grimwade¹⁵⁵). The challenge is how to identify the relatively small subgroup of patients at particular risk of relapse who cannot be reliably distinguished on the basis of pretreatment characteristics and who could potentially benefit from more intensive treatment in first remission. Overall, there is general agreement that a positive *PML-RARA* test after consolidation is a strong predictor of subsequent hematological relapse, whereas repeatedly negative results are associated with long-term survival in the majority of patients.^{21,157} The Italian GIMEMA group (Gruppo Italiano Malattie Ematologiche Maligne Adulto) reported that recurrence of PCR positivity, detected by 3-monthly BM surveillance performed after completion of therapy, was highly predictive of relapse.²¹ Using such a strategy, approximately 70% of relapses were successfully predicted.²¹ A

Table 22 Expression values of the *SIL-TAL1* FG transcript in cell lines and patients at diagnosis (phase IV)

Samples	Ct values				<i>SIL-TAL1</i> NCN ^a		
	<i>SIL-TAL1</i>	<i>ABL</i>	<i>B2M</i>	<i>GUS</i>	Per <i>ABL</i>	Per 100 <i>B2M</i>	Per <i>GUS</i>
Cell lines ^b							
CEM	26.1	23.3	20.6	22.9	0.12	1.38	0.07
ALL1	25.4	21.4	16.9	21.2	0.11	0.79	0.09
Molt-15	28.3	24.2	18.0	23.2	0.10	0.27	0.06
Molt-16	26.0	21.7	16.6	22.1	0.08	0.45	0.11
HSB-2	25.5	24.4	19.5	24.2	0.41	3.47	0.44
PF382	26.1	21.5	16.7	22.2	0.07	0.47	0.11
Patients							
BM (n = 10)	27.1 [25.2–29.4]	24.3 [22.2–25.0]	19.7 [17.4–21.1]	24.5 [22.9–25.3]	0.12 [0.02–0.23]	1.24 [0.03–4.86]	0.15 [0.02–0.44]
PB (n = 10)	28.4 [26.5–29.4]	24.3 [23.6–25.9]	19.3 [17.1–20.8]	24.7 [23.8–25.2]	0.09 [0.02–0.21]	0.86 [0.05–3.28]	0.12 [0.01–0.26]
Corr. coeff.					0.68	0.19 ^c	0.57

^aCorrected according to the blast percentage in the sample.

^bThe CEM cell line was analyzed in duplicate in eight laboratories, whereas the other cell lines were analyzed in duplicate in single laboratories.

^cNo significant correlation. For patient samples and cell lines, median values [95% range] are indicated.

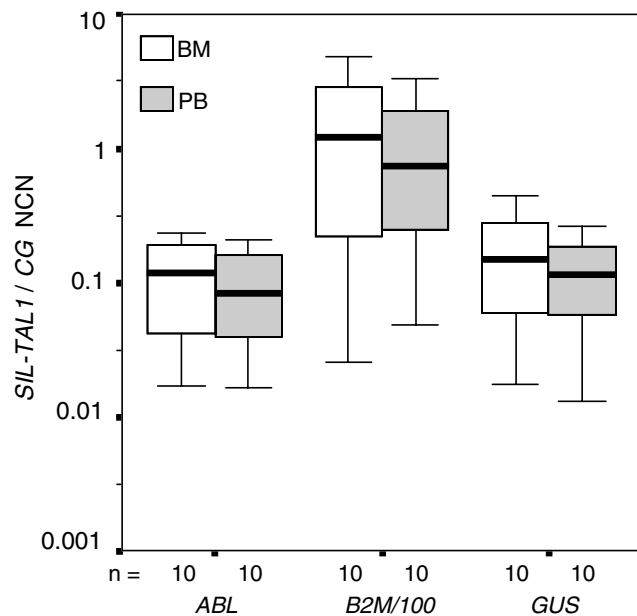


Figure 20 Comparison of *SIL-TAL1* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *SIL-TAL1* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. All differences between BM and PB per CG are not significant on paired samples ($n=5$, Wilcoxon test). n : number of patient samples.

different perspective in the application of MRD to identify APL patients at higher risk of relapse has been used by the MRC ATRA trial,¹⁵⁸ where the kinetics of achieving a molecular remission was evaluated. Finally, the benefit of early treatment at the time of molecular relapse has still to be proven, but preliminary evidence supports such a strategy.²²

Table 23 Results of the QC rounds for *SIL-TAL1* FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10^{-3}	10^{-4}	FG neg. control	NAC/NTC
IIIa	14% (1/7) ^a	43% (3/7) ^a	0% (0/8)	0% (0/64)
IIIb	0% (0/10) ^a	0% (0/10) ^a	5% (1/22)	0% (0/66)
IVa	0% (0/8)	0% (0/8)	6% (1/16)	0% (0/32)
All phases	4% (1/25)	12% (3/25)	4.3% (2/46)	0% (0/162)

Results are proportions of false-positive or -negative samples. 10^{-3} and 10^{-4} are the RNA dilutions of a positive *SIL-TAL1* cell line (CEM or Molt-15) in a negative RNA (HL-60 or PB MNC) sample. NEG: negative RNA (HL-60 mRNA, U937 mRNA or PB MNC RNA).

^aOne sample was excluded due to *ABL* Ct values > 29.

Among the different methods (conventional karyotyping, FISH and PML immunostaining with specific antibodies), RT-PCR detection of the *PML-RARA* FG transcripts appears to be the only approach suitable for MRD detection.²⁴ Moreover, quantitative PCR could provide information on the correlation between different levels of disease at early phases of therapy and clinical outcome. However, there have been relatively few studies reporting the use of RQ-PCR in APL patients.^{41,159–161}

Although the molecular diagnosis and monitoring of APL patients represents one of the most relevant examples of the impact of molecular genetics in clinical hematology, further investigations are still needed.

7.2. EAC data

7.2.1. Primer design and optimization (phases I and II): One probe and two reverse primers on *RARA* gene exon 3 in combination with seven forward primers on the *PML* gene were evaluated. Five forward primers were designed in *PML* exon 6, two and three specific primers for bcr1 and bcr2 breakpoints respectively, while two primers for bcr3 were designed in *PML* exon 3. Based on published data on the

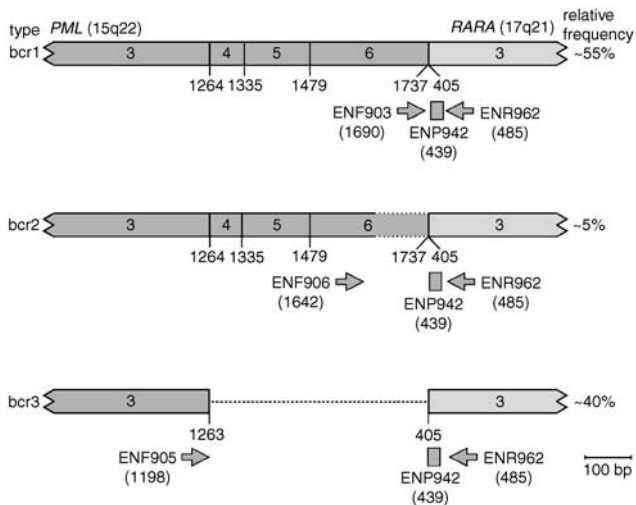


Figure 21 Schematic diagram of the *PML-RARA* FG transcript covered by the RQ-PCR primer and probe set. Three sets are available to cover all the *PML-RARA* transcript variants. A common probe (ENP942) and a common reverse primer (ENR962) on *RARA* gene exon 3 are used. BCR1, BCR2 and BCR3 variants are respectively amplified with ENF903, ENF906 and ENF905 forward primers. The relative frequency of each particular transcript appears on the right side of the diagram. The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 24).

localization of *bcr2 PML* breakpoints,^{162–164} the respective forward primers on *PML* exon 6 were designed in order to cover at least 80% of *bcr2* cases. The only cell line available for testing was NB-4,¹⁶⁵ which has a *bcr1 PML* breakpoint; for the evaluation of *bcr2* and *bcr3* primer/probe sets, diagnostic patient BM RNA was used. Plasmid constructs for all three *PML-RARA* breakpoint variants were made (see the Materials and methods section).

After extensive testing on cell line and patient RNA and plasmid dilutions, three specific primer/probe sets were selected, based on the maximum sensitivity: the probe *RARA* ENP942, the common reverse primer *RARA* ENR962 and three *PML* forward primers ENF903 (for *bcr1*), ENF906 (for *bcr2*) and ENF905 (for *bcr3*), respectively (Figure 21). Sequences are listed in Table 24. Although ENF906 can potentially be used to amplify *bcr1* as well as *bcr2* cases, direct comparison of ENF906 and ENF903 for amplification of *PML-RARA* in serial dilutions of the NB-4 cell line revealed the latter primer to provide a more sensitive assay for amplification of *bcr1* cases. An example of typical amplification plots (10^{-1} , 10^{-3} and 10^{-4} NB-4 RNA (*bcr1*) in PBL RNA) is shown in Figure 22.

7.2.2. *PML-RARA* expression in cell lines and diagnostic patient samples (phase IV): *PML-RARA* expression was studied in the NB-4 cell line and in 16 positive AML-M3 *bcr1* patients (Table 25 and Figure 23). Patient samples consisted of 14 BM and nine PB samples, including six paired BM/PB samples, obtained at diagnosis. Samples contained 10–100% of leukemia blasts. NCN were calculated and adjusted for the percentage of blasts present in each sample. *PML-RARA* expression in the NB-4 cell line was within the range of FG expression detected in primary leukemia samples.

PML-RARA expression was studied in six *bcr3*-positive patients at diagnosis, consisting of six BM and four PB samples

Table 24 Sequences and positions of the *PML-RARA* primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3'
ENF903	<i>PML</i> , 1690–1708 (19)	TCTTCCTGCCCAACAGCAA
ENF906	<i>PML</i> , 1642–1660 (19)	ACCTGGATGGACCGCCTAG
ENF905	<i>PML</i> , 1198–1216 (19)	CCGATGGCTTCGACGAGTT
ENP942	<i>RARA</i> , 439–458 (20)	AGTGCCCAGCCCTCCCTCGC
ENR962	<i>RARA</i> , 485–465 (21)	GCTGTAGATGCGGGGTAGAG

^aENF = forward primer, ENP = TaqMan probe, ENR = reverse primer.
^bPositions according to accession numbers M73778 (*PML*) and X06538 (*RARA*).

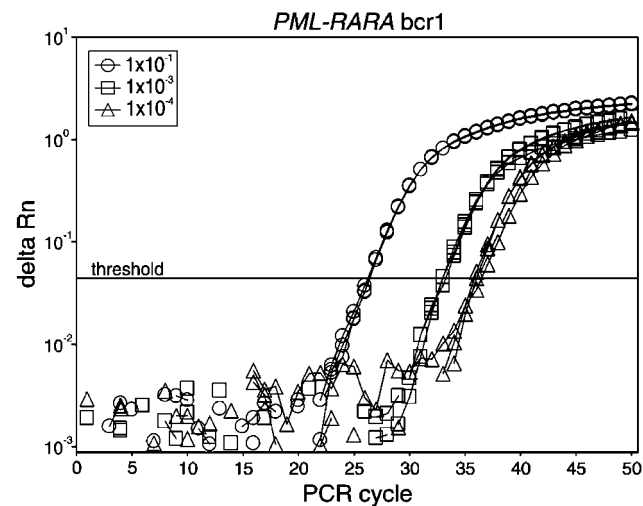


Figure 22 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of a *PML-RARA* BCR1-positive RNA sample in a negative RNA sample.

(including four paired BM/PB samples). Although the number was very limited, no significant difference was observed in *PML-RARA bcr3* expression when comparing PB and BM on paired samples except for *B2M* normalized results (see web site and Figure 23).

7.2.3. QC rounds (phases IIIa–IVa): During the various QC rounds, 145 negative samples were tested in 7–11 labs during the three phases (Table 26). Five out of 100 NAC/NTC samples (5%) and five out of 45 FG-negative samples (11%) were falsely positive for *bcr1* amplification (Table 26). Overall, the frequency of false positivity was 6.9% (10/145). The so-called false positivity was limited to individual laboratories and the Ct value in the false-positive well was always more than 30 and most of the time higher than 35.

By contrast, according to the criteria mentioned above, no false-negative samples ($n = 96$) for 10^{-3} and 10^{-4} dilutions were observed, either for *bcr1*, *bcr2* or *bcr3*. None of the 42 wells tested independently for *bcr2* and *bcr3* at 10^{-4} dilution falsely resulted as negative. Only eight out of 180 wells (4.4%) tested for *bcr1* at 10^{-4} dilution falsely resulted as negative.

Table 25 Expression values of the *PML-RARA* FG transcript in NB-4 cell line and *bcr1* patients at diagnosis (phase IV)

Samples	Ct values				PML-RARA NCN		
	PML-RARA	ABL	B2M	GUS	Per ABL	Per 100 B2M	Per GUS
Cell line NB-4	24.7	23.7	17.1	22.0	0.2	0.4	0.1
Patients							
BM (n = 14)	25.6 [23.1–27.5]	24.5 [21.7–28.5]	17.7 [16.0–24.9]	22.2 [20.3–26.7]	0.30 [0.09–1.82]	0.72 [0.19–3.18]	0.08 [0.02–0.56]
PB (n = 9)	25.7 [23.7–29.4]	24.6 [22.0–27.4]	17.9 [16.5–20.0]	23.0 [20.6–25.4]	0.36 [0.11–0.78]	0.26 [0.07–0.60]	0.05 [0.02–0.13]
Corr. coeff.					0.80	0.54	0.73

For patient samples and cell lines, median values [95% range] are indicated. NB-4 cell line was analyzed in triplicate (FG) or duplicate (CG) in two laboratories.

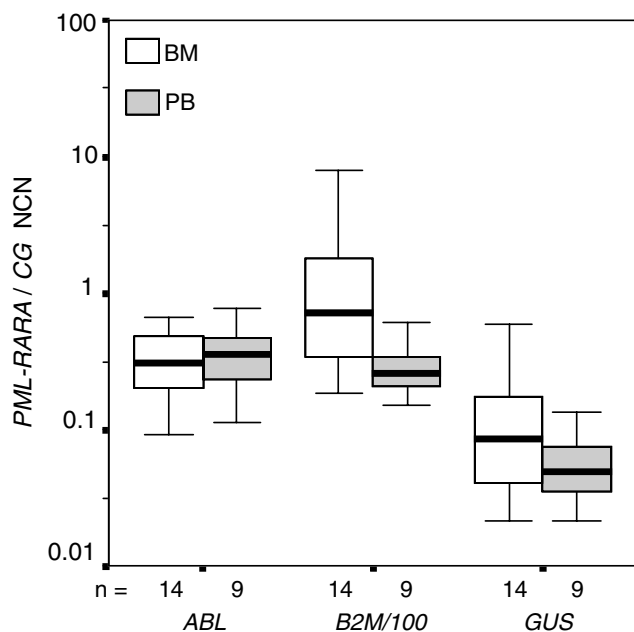


Figure 23 Comparison of *PML-RARA* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *PML-RARA* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. All differences between BM and PB per CG are not significant on paired samples ($n = 6$, Wilcoxon test) except for *B2M* NCN ($P = 0.03$). n : number of patient samples.

Table 26 Results of the QC rounds for *PML-RARA* *bcr1* FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10^{-3}	10^{-4}	FG neg. control	NAC/NTC
IIIa	0% (0/7)	0% (0/7)	29% (2/7)	7.1% (2/28)
IIIb	0% (0/11)	0% (0/11)	4.5% (1/22)	0% (0/44)
IVa	0% (0/11)	0% (0/10)	13% (2/16)	11% (3/28)
All phases	0% (0/29)	0% (0/28)	11% (5/45)	5% (5/100)

Results are proportions of false-positive or -negative samples. 10^{-3} and 10^{-4} are the dilutions of NB-4 cell line RNA in a negative RNA.

8. Inv(16) (p13q22) with the *CBFB-MYH11* fusion gene transcript

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8.1. Background

Pericentric inversion of chromosome 16, *inv(16)(p13q22)*, is found in about 8–9% of newly diagnosed AML cases.¹⁶⁶ The *inv(16)*-positive AMLs are included with those with *t(8;21)* translocation in a group generally referred to as 'core binding factor' (CBF) leukemias, as both are characterized by rearrangements of genes that code for components of the heterodimeric transcription factor CBF, which plays an essential role in hematopoiesis.¹⁶⁷ *Inv(16)* or the rarer *t(16;16)(p13;q22)* leads to fusion of the *CBFB* chain gene with the smooth muscle myosin heavy chain gene *MYH11*.¹⁶⁸ The resulting FG mRNA can be detected by RT-PCR and represents a suitable molecular marker for both diagnostic and monitoring studies.^{169–171} So far, 10 different *CBFB-MYH11* FG transcripts have been reported. The nomenclature used is derived and updated from the review by Liu *et al.*¹⁶⁷ More than 85% of positive patients have the type A transcript; type D and E transcripts each represent nearly 5%, whereas all other types occur in sporadic cases. *CBFB-MYH11*-positive AML are usually considered to have a favorable prognosis, with more than 50% of patients obtaining long-term CR.⁴ Such favorable results with conventional chemotherapy led some authors to consider that allo-BMT is not indicated to consolidate first CR in these patients, even when a suitable donor is available.^{46,172,173} Nevertheless, the relapse rate is still high indicating that reliable methods to detect MRD during hematologic CR are needed in order to better adapt the intensity of postremission therapy to specific cohorts of patients. So far, the use of qualitative RT-PCR-based methods employed to detect *CBFB-MYH11* FG transcripts did not allow consistent

discrimination of prognostic subgroups of patients in CR.^{169,171,174,175} In fact, the use of standard nested RT-PCR has produced conflicting MRD results: while in most reports the vast majority of patients in prolonged CR were found to be PCR-negative, a few long-term survivors never converted to RT-PCR negativity. Moreover, 10–20% of PCR-negative patients eventually relapsed, suggesting that the achievement of PCR negativity is not synonymous with cure. Some of the difficulties in interpreting the above results may derive from lack of standardization of methodologies involved. Quantitative RT-PCR studies using competitive PCR or RQ-PCR enabled monitoring of the decrease in *CBFB-MYH11* FG transcripts during early phases of induction and consolidation therapies.^{33,45,46,176,177} However, due to the low number of patients so far examined, it was not possible to define a kinetic or a cutoff level for predicting relapse.¹⁷⁸

8.2. EAC data

8.2.1. Primer design and optimization (phases I and II): During phase I, we tested six primer/probe sets: three for the A, two for D and one for the E form. As *CBFB-MYH11* transcripts type A, D and E represent approximately 95% of all cases, in order to amplify these transcripts we decided to use a common forward primer located on *CBFB* exon 5 (ENF803) and a common probe located on *CBFB* exon 5 (ENPr843). Three different reverse primers located respectively on *MYH11* exon 12 for type A (ENR862), *MYH11* exon 8 for type D (ENR863) and *MYH11* exon 7 for type E (ENR865) (Figure 24 and Table 27) were chosen. An example of typical amplification plots (10^{-1} , 10^{-3} and 10^{-4} RNA dilutions) is shown in Figure 25 for all three types of *CBFB-MYH11* transcripts.

8.2.2. *CBFB-MYH11* expression in the ME-1 cell line and diagnostic patient samples (phase IV): Pure RNA of the ME-1 cell line (type A) was tested in eight different laboratories (Table 28). In addition, diagnostic BM or PB samples of 24 type A patients, three type D and four type E were analyzed (Figure 26). The values of FG transcripts at diagnosis were similar in all three categories of patients, but show a range of variation from patient to patient of one log.

8.2.3. QC rounds (phases IIIa–IVa): No false-negative results for the 10^{-3} dilution were observed, whereas at the 10^{-4} dilution a maximum of 12% of false-negative results were observed (Table 29). False positivity was absent during all phases in NAC and NTC wells (0%, $n=104$), whereas a single case (two wells out of 16) of false positivity was observed during phase IVa in a coded FG-negative sample and was due to contamination (Table 29).

9. t(8;21)(q22;q22) with the *AML1-ETO* fusion gene transcript

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9.1. Background

The *AML1(CBFA2, RUNX1)-ETO (MTG8)* gene fusion results from the t(8;21)(q22;q22), which is the commonest chromoso-

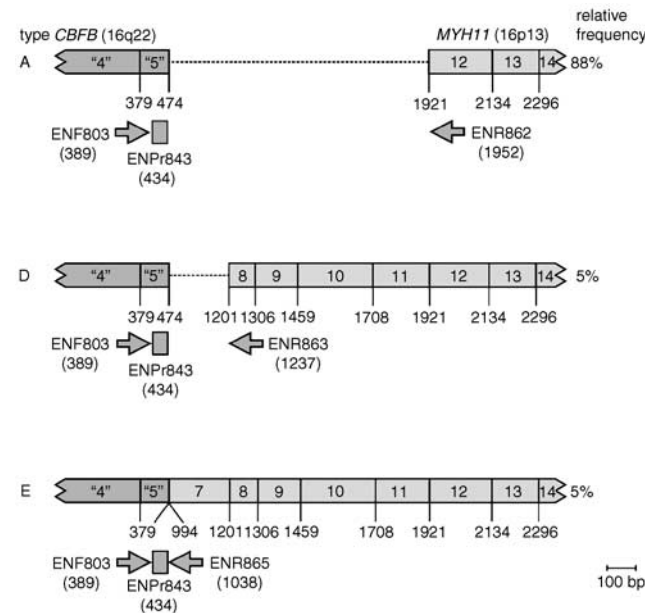


Figure 24 Schematic diagram of the *CBFB-MYH11* FG transcripts covered by the RQ-PCR primer and probe set. (a) Type A: set ENF803–ENPr843–ENR862, (b) type D: set ENF803–ENPr843–ENR863, (c) type E: set ENF803–ENPr843–ENR865. The relative frequency of each particular transcript appears on the right side of the diagram. The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 27).

Table 27 Sequences and positions of the *CBFB-MYH11* primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3'
ENF803	<i>CBFB</i> , 389–410 (22)	CATTAGCACAAACAGGCCTTGA
ENPr843	<i>CBFB</i> , 434–413 (22)	TCCGCTGTCTCTCCGAGCCT
ENR862	<i>MYH11</i> , 1952–1936 (17)	AGGCCCCGCTTGGACTT
ENR863	<i>MYH11</i> , 1237–1217 (21)	CCTCGTTAAGCATCCCTGTGA
ENR865	<i>MYH11</i> , 1038–1016 (23)	CTCTTCTCCAGCGTCTGCTAT

^aENF = forward primer, ENPr = TaqMan reverse probe (in order to select the C-rich strand), ENR = reverse primer.

^bPositions according to accession numbers L20298 (*CBFB*) and D10667 (*MYH11*).

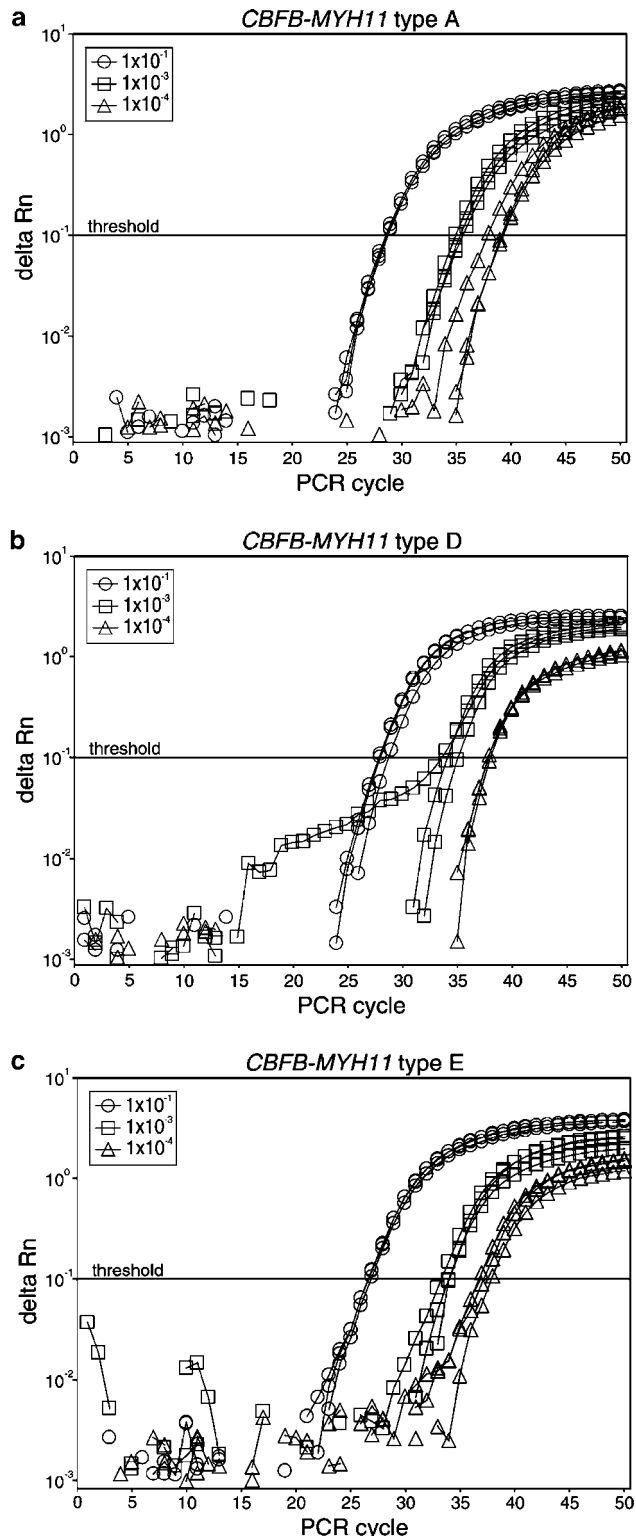


Figure 25 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of a *CBFB-MYH11*-positive RNA sample in a negative RNA sample: (a) Set type A on ME-1 cell line, (b) set type D on patient sample, (c) set type E on patient sample.

mal rearrangements associated with AML, being detected in approximately 8% of AML cases in children and young adults.⁴ The *AML1* gene encodes the $\alpha 2$ subunit of the heterodimeric transcription factor CBF, which is critical for hemopoietic

development and whose β subunit is disrupted by the *inv(16)/t(16;16)* which leads to the *CBFB-MYH11* FG (see Section 8).¹⁷⁹

As shown in Figure 27, *AML1* breakpoints are located within intron 5, while *ETO* breakpoints occur upstream of exon 2. This gives rise to a single type of *AML1-ETO* FG transcript in which *AML1* exon 5 is fused to *ETO* exon 2,²⁴ thereby simplifying molecular screening strategies and MRD monitoring as compared to FG with multiple breakpoint regions, such as *t(4;11)* with *MLL-AF4*, *t(15;17)* with *PML-RARA* and *inv(16)* with *CBFB-MYH11*.

AML1-ETO is an important PCR target for MRD detection in view of the generally favorable outcome of patients with the *t(8;21)*, such that routine use of BMT in first CR has been shown to confer no overall survival benefit.^{172,173} Therefore, it is of paramount importance to identify the relatively small subgroup of patients at high risk of relapse who could benefit from additional therapy. However, the role of MRD detection in *AML1-ETO*-positive AML has been somewhat controversial in view of the detection of FG transcripts in patients in long-term remission following chemotherapy, autologous BMT/PBSCT and even alloBMT.^{25,180,181} The detection of residual transcripts in patients who are cured of their disease has been seen as providing evidence that *AML1-ETO* alone is insufficient to mediate AML, and has recently been shown to relate to a fraction of stem cells, monocytes and B cells present in remission marrow.¹⁸² Hence, the relatively frequent reports of PCR positivity in patients considered to be cured of *t(8;21)*-positive AML is likely to reflect the higher levels of sensitivity commonly achieved for *AML1-ETO* RT-PCR assays (typically 1 in 10^5 to 1 in 10^6), as compared to those for other AML FG targets (typically 1 in 10^4 – 10^5). Despite the fact that a recent study has shown that conventional qualitative RT-PCR has the potential to provide an independent prognostic factor in *AML1-ETO*-positive AML,¹⁸³ there has been some concern regarding the suitability of sensitive 'end point' assays for MRD detection as a means of determining treatment approach in this subgroup of patients.

Over the last few years, quantitative RT-PCR methods have been investigated to determine whether they can more reliably identify the relatively small subgroup of patients destined to relapse.^{184–186} Competitive RT-PCR assays have revealed variation in *AML1-ETO* expression relative to *ABL* between cases at diagnosis (10-fold in BM, 32-fold in PB) and suggest that *AML1-ETO* and *ABL* mRNAs have comparable stability.¹⁸⁶ Furthermore, these studies revealed varying kinetics of FG transcript reduction following chemotherapy. Patients with low or undetectable levels of *AML1-ETO* transcripts were associated with maintenance of CCR, while high or rising transcript numbers predicted relapse.¹⁸⁶ These promising preliminary data suggest that RQ-PCR is likely to be valuable for MRD monitoring in this subset of AML, with the added advantages that the latter technique is less labor intensive, more reproducible and amenable to standardization lending itself to use in large-scale clinical trials. Preliminary studies of RQ-PCR^{42–44,187,188} have essentially confirmed the results obtained via competitive RT-PCR, revealing a 3.5- to 20-fold variation in *AML1-ETO* FG expression levels in diagnostic BM that was not related to blast percentage and which needs to be taken into account when assessing response to therapy. Furthermore, variability in kinetics of response to chemotherapy was noted, and interestingly *AML1-ETO* transcripts were also detected in patients in long-term remission from AML. However, the predictive value of RQ-PCR remains to be established in a large number of patients subject to a consistent treatment approach.

Table 28 Expression values of the *CBFB-MYH11* FG transcript in cell line and patients at diagnosis (phase IV)

Samples	Ct values				<i>CBFB-MYH11</i> NCN values		
	<i>CBFB-MYH11</i>	<i>ABL</i>	<i>B2M</i>	<i>GUS</i>	Per <i>ABL</i>	Per 100 <i>B2M</i>	Per <i>GUS</i>
Cell line ME-1	24.7	25.1	19.4	19.4	0.90	4.64	0.40
Patients							
Type A ^a (n = 30)	24.8 [22.2–27.8]	25.0 [22.5–27.7]	18.8 [16.3–24.0]	23.3 [21.6–26.8]	1.35 [0.13–14.8]	3.98 [1.45–447]	0.42 [0.07–4.57]
Type D ^b (n = 3)	24.4 [24.0–25.7]	24.2 [24.2–26.0]	19.2 [18.6–19.8]	24.2 [22.4–24.6]	1.0 [0.78–1.07]	4.0 [3.0–4.7]	0.32 [0.25–0.55]
Type E ^c (n = 4)	26.0 [25.2–28.4]	25.6 [23.7–27.0]	19.2 [18.2–19.5]	24.2 [23.5–25.7]	0.88 [0.62–1.55]	2.1 [0.72–2.57]	0.35 [0.21–0.37]
Corr. coeff.					0.76	0.65	0.76

^aSet ENF803–ENR862–ENP843.

^bENF803–ENR863–ENP843.

^cSet ENF803–ENR865–ENP843. Due to few PB samples (n = 4), data have been analyzed according to the type of transcript. For patient samples and cell lines, median values [95% range] are indicated.

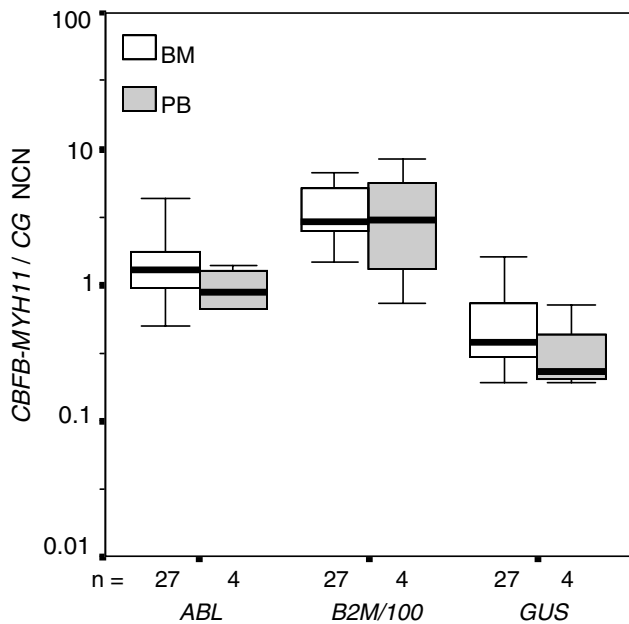


Figure 26 Comparison of *CBFB-MYH11* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. Samples have been pooled according to their origin (BM or PB) independently of the transcript type. The ratio (NCN) is defined as the ratio between the copy number of *CBFB-MYH11* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. n: number of patient samples.

9.2. EAC data

9.2.1. Primer design and optimization (phases I and II): Initially, the primer and probe sequences published by Marcucci et al⁴² were tested together with two ‘in house’ sets. While the former primer/probe set was superior in terms of sensitivity, we observed significant recurrent background signals in the negative control samples. We therefore decided to design two new probes compatible with this primer set leading to the

Table 29 Results of the QC rounds for *CBFB-MYH11* FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10 ⁻³	10 ⁻⁴	FG neg. control	NAC/NTC
IIIa	0% (0/14)	7% (1/14)	0% (0/7)	0% (0/28)
IIIb	0% (0/11)	18% (2/11)	0% (0/22)	0% (0/44)
Iva	ND	13% (1/8)	6.3% (1/16)	0% (0/32)
All phases	0% (0/25)	12% (4/33)	2.2% (2/45)	0% (0/104)

Results are proportions of false-positive or -negative samples. 10⁻³ and 10⁻⁴ are the dilutions of ME-1 cell line RNA in a negative RNA. ND = not done.

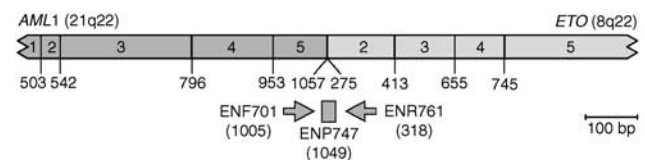


Figure 27 Schematic diagram of the *AML1-ETO* FG transcript covered by the RQ-PCR primer and probe set. EAC codes (ENF701, ENP747, ENR761). The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript (see Table 30). The probe is located on the breakpoint.

selection of ENP747 positioned on the breakpoint, in conjunction with the forward primer ENF701 positioned on *AML1* exon 5 and the reverse primer ENR761 on *ETO* exon 2 (Figure 27 and Table 30).²⁴ The cell line available for testing was KASUMI-1.¹⁸⁹ The *AML1-ETO* RQ-PCR assay was found to be particularly robust, associated with a relatively high ΔR_n (see Figure 28a). Reduction in the probe concentration from 200 to 100 nM was therefore evaluated. The decrease in probe concentration did not affect assay sensitivity, and the ‘baseline creeping’ artifact was only very rarely observed (Figure 28b).

9.2.2. *AML1-ETO* expression in KASUMI cell line and diagnostic patient samples (phase IV): Undiluted RNA of

Table 30 Sequences and positions of the *AML1-ETO* primers and probe

EAC code ^a	Primer/probe localization, ^b 5'–3' position (size)	Sequence 5'–3'
ENF701	<i>AML1</i> , 1005–1026 (22)	CACCTACCACAGAGCCATCAAA
ENP747	<i>AML1</i> , 1049–295 (30)	AACCTCGAAATCGTACTGAGAAGCACTCCA ^c
ENR761	<i>ETO</i> , 318–297 (22)	ATCCACAGGTGAGTCTGGCATT

^aENF = forward primer, ENP = TaqMan probe, ENR = reverse primer.

^bPositions according to accession numbers D43969 (*AML1*) and D14289 (*ETO*).

^cLocation of the breakpoint.

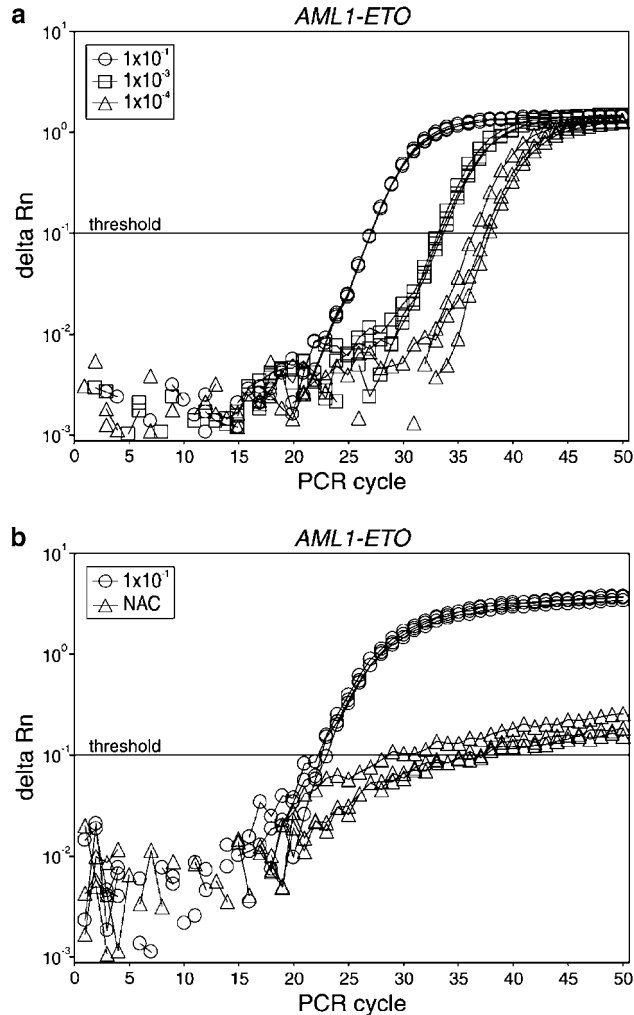


Figure 28 Amplification plots of 10^{-1} , 10^{-3} and 10^{-4} dilutions of an *AML1-ETO*-positive RNA sample in a negative RNA sample. (a) Classical amplification plot. (b) Creeping curve phenomenon observed on NAC samples.

the KASUMI-1 cell line was tested in five different laboratories (Table 31). Analysis for archived diagnostic RNA from 22 patient samples was undertaken by four different laboratories. All differences between BM and PB per CG were not significant on paired samples ($n=10$, Wilcoxon test). After applying the exclusion criteria, 12 PB and 10 BM samples were evaluated (Table 31).

AML1-ETO FG transcript expression (Ct and CN) was higher in the KASUMI-1 cell line than in the patient samples (median difference of approximately two Ct values). Among the patient samples, no difference was seen in the expression of the *AML1-ETO* FG transcript. With regard to the CGs, the expression of *ABL* was comparable between the cell line and patient samples. Significant variation was seen in the expression of *B2M*, both between patient samples and cell line and between BM and PB samples. For *GUS*, intermediate results were observed (Table 31 and Figure 29).

9.2.3. QC rounds (phases IIIa–IVa): No false-negative results (out of a total of 112 analyzed samples) for 10^{-3} and 10^{-4} dilutions were observed, which is in line with the good sensitivity of the RQ-PCR assay (Table 32). Overall, the frequency of false positivity was 9.7% (15/154). Six out of 108 NAC/NTC samples (5.6%) and nine out of 46 FG-negative samples (20%) were falsely positive for *AML1-ETO* amplification (Table 32). In coded FG-negative samples, false positivity was in most cases restricted to individual laboratories.

10. Overall results

10.1. Standard curve comparison (phase IIIa)

No significant differences were observed in Ct values for the lowest RNA, cDNA (10^{-3} and 10^{-4}) and plasmid (10^6 , 10^5 and 10^3 copies) dilutions, even between centrally and locally prepared cDNA samples. For the highest dilutions, the Ct values were more reproducible between laboratories for centrally distributed cDNA (10^{-3} and 10^{-4} dilutions) and plasmid (10 and 100 copies) than for RNA dilutions. The respective CV were below 5% for cDNA and plasmids and 11% for RNA at the highest dilution. In two target-networks (*AML1-ETO* and *CBFB-MYH11 type A*), this observation even resulted in significantly fewer positive results for the samples for which RT was performed locally compared to the centrally prepared cDNA samples. The slopes established with cDNA or plasmid dilutions were close to the theoretical slope -3.32 (100% efficiency) for the vast majority of the participating laboratories. In contrast, slopes from RNA dilutions were indicative of lower reaction efficiency probably due to RNA degradation during transportation. Finally, the sensitivity levels of RNA dilutions for all targets were comparable to standardized nested PCRs designed in the BIOMED-1 program²⁴ and were even better for *bcr2* and *bcr3* variants of *PML-RARA*. A total of 10 plasmid copies could generally be detected by all laboratories (see web site, phase IIIa and b).

Table 31 Expression values of the *AML1-ETO* FG transcript in cell line and patients at diagnosis (phase IV)

Samples	Ct values				AML1-ETO NCN		
	AML1-ETO	ABL	B2M	GUS	Per ABL	Per 100 B2M	Per GUS
Cell line KASUMI-1	22.2	26.2	23.3	24.1	5.46	80	1.42
Patients							
BM (n = 10)	24.9 [20.1–28.0]	26.4 [20.7–27.7]	18.8 [16.8–21.9]	23.8 [20.8–26.7]	2.1 [0.29–5.3]	10.0 [0.5–38.9]	1.32 [0.03–25.1]
PB (n = 12)	23.9 [18.6–27.6]	26.1 [22.5–28.6]	19.6 [16.5–23.6]	23.6 [21.4–27.4]	4.2 [1.1–20.0]	12.0 [2.5–33.1]	1.1 [0.01–159]
Corr. coeff.					0.81	0.80	0.44

For patient samples and cell lines, median values [95% range] are indicated.

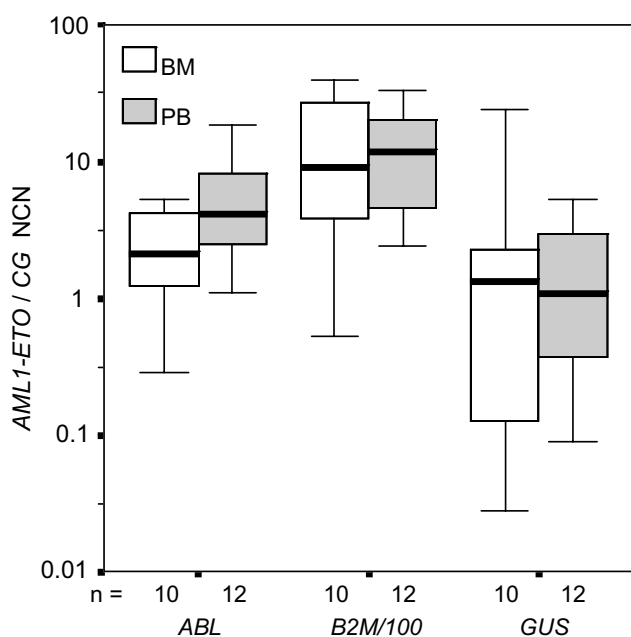


Figure 29 Comparison of *AML1-ETO* FG transcript expression between BM and PB at diagnosis using either *ABL*, *B2M* or *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of *AML1-ETO* per one copy of *ABL* or *GUS* gene and 100 copies of *B2M* gene. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. All differences between BM and PB per CG are not significant on paired samples ($n = 10$, Wilcoxon test). However, the use of *GUS* gene is not recommended due to the large range of values observed for the ratio *AML1-ETO*/*GUS*. n : number of patient samples.

10.2. Balanced randomized assay (phase IIIb)

This assay was set up in order to detect differences in transcript quantification between laboratories. The laboratories were randomly chosen to amplify four different FG transcripts, generally outside their original FG networks (see the Materials and methods section). This methodology is also of importance as a QC round to detect if false negativity and positivity are proportionally identical to other phases for which only laboratories focusing on a particular FG were performing the experiments.

Table 32 Results of the QC rounds for *AML1-ETO* FG transcript detection (phases III and IV)

QC phase	False negativity		False positivity	
	10^{-3}	10^{-4}	FG neg. control	NAC/NTC
IIIa	0% (0/21)	0% (0/21)	13% (1/8) ^a	8% (3/32)
IIIb	0% (0/11)	0% (0/11)	14% (3/22) ^b	0% (0/44)
IVa	0% (0/24)	0% (0/24)	25% (5/16) ^c	8% (3/32)
All phases	0% (0/56)	0% (0/56)	20% (9/46)	5.6% (6/108)

Results are proportions of false-positive or -negative samples. 10^{-3} and 10^{-4} are the RNA dilutions of KASUMI-1 cell line RNA in HL-60 RNA.

^aFalse positivity was due to three false-positive replicates in a single lab.

^bFalse positivity was due to three single positive wells in three different laboratories.

^cTwo labs out of 12 make up 4/5 false-positive samples, thus contributing to 80% of false positivity.

10.2.1. ABL amplification

10.2.1.1. On plasmid dilutions: We observed very similar results for the three *ABL* plasmid dilutions among the 25 laboratories. We found, for the *ABL* 10^5 copies plasmid dilution, 22.30 ± 0.38 (Ct \pm s.d., $n = 296$) and a corresponding CV of 1.7%. We found a significant laboratory effect (set as a random effect) on *ABL* Ct measurement for the three *ABL* plasmid dilution ($P < 0.001$, $n = 296$). But the difference between opposites laboratories was no more than 1.2 Ct. Such a difference might well not be relevant in a clinical point of view. According to the criteria defined in the Materials and methods section, all laboratories had reproducible results for Ct value of each *ABL* plasmid dilution (see web site).

10.2.1.2. On coded RNA samples: In the same way, we focused within each network on a laboratory effect for quantification of a given gene transcript. We used *ABL* gene amplification as a model to investigate this. No significant difference was found within networks between highly diluted samples (10^{-3} and 10^{-4}) and negative samples when *ABL* expression was compared using Ct or CN values (results of PML-RARA network given as an example in Figure 30a), except for the *CBFB-MYH11* network ($P = 0.03$). On the contrary, we found highly significant differences ($P < 0.001$) between laboratories within each network for the same samples (results of PML-RARA network given as an example in Figure 30b). Thus Ct and CN values were dependent on the testing laboratory and not on

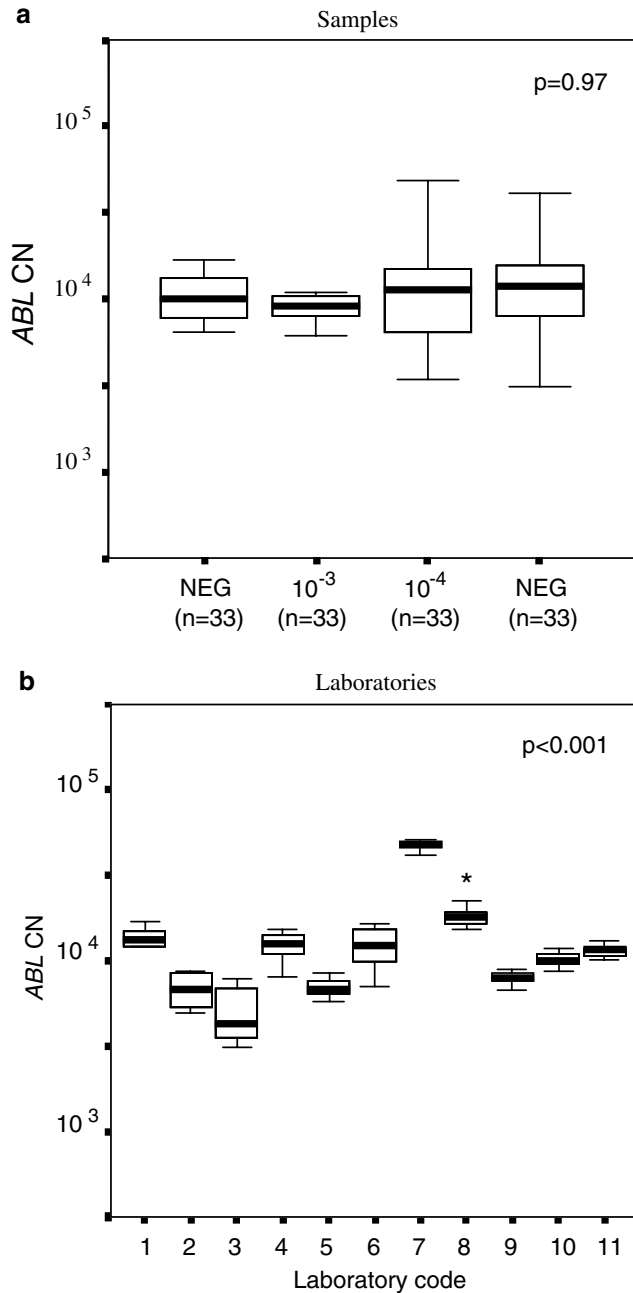


Figure 30 Influence of the pre-PCR steps on *ABL* gene transcript quantification of four coded samples tested in 11 randomly assigned laboratories during the balanced randomized assay (phase IIIb, *PML-RARA* network). (a) No significant difference between coded samples using a global linear model. (b) Highly significant difference between laboratories for the same samples. (*) Network leader who sent out the coded samples to other laboratories. The 10^{-1} dilution was excluded from the analysis because it could not be considered as identical to the four others. The 10^{-3} and 10^{-4} diluted samples also appear in Figures 31 and 32.

the sample. These data strongly suggest that variations occur during the pre-PCR steps (transportation and RT efficiency).

10.2.2. Fusion gene transcript amplification

We focused on the best parameter (Ct, CN, Δ Ct or NCN) to express RQ-PCR results in a serial dilution model. We

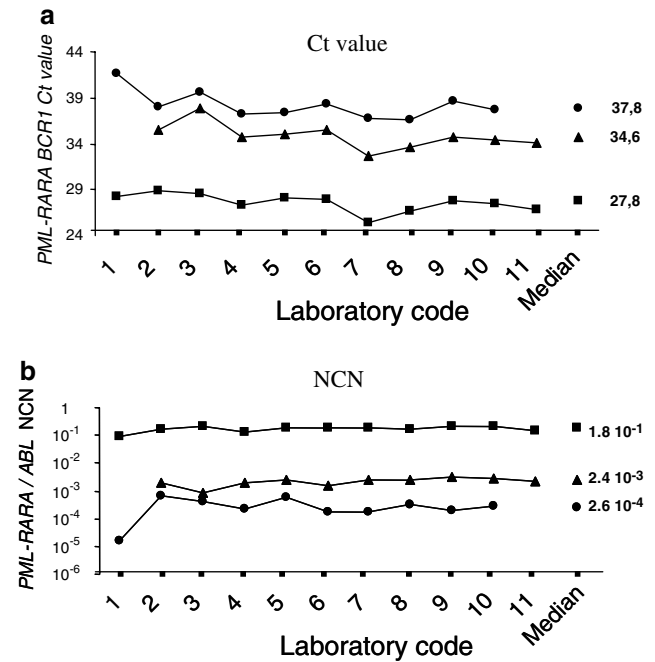


Figure 31 Results per laboratory of the amplification of *PML-RARA* FG transcript in three coded samples during the balanced randomized assay (phase IIIb). (a) Ct value of the FG transcript. (b) *ABL* NCN of the FG transcript of the three coded samples. Dilutions were 10^{-1} (■), 10^{-3} (▲) and 10^{-4} (●) of NB-4 RNA into a negative RNA sample. The CG improved greatly the linearity of the results (see also Figure 32). The 10^{-3} and 10^{-4} diluted samples also appear in Figure 30.

calculated the corresponding correlation coefficients on three diluted samples measured in 11 different laboratories (*PML-RARA* network as an example in Figure 31). The correlation coefficients were higher for the NCN method in five FGs, slightly higher with the Δ Ct method in two FGs, whereas both methods were equivalent for the remaining two FGs (Table 33). Only for quantification of *E2A-PBX1* transcripts, *ABL* appeared not to be helpful as CG to normalize the RQ-PCR results. Overall, use of *ABL* gene transcripts for normalization was found to greatly improve the linearity of the results (*PML-RARA* network as an example in Figure 32) and avoided overlap in FG Ct and CN values observed between different dilutions (see web site).

Finally, we checked the results of this model for MRD quantification. The correlation curve between Ct value (Y-axis) and CN value (\log_{10} , X-axis) for wells related to coded FG-positive samples ($n = 824$, covering all nine FG targets) showed a mean slope and an intercept of -3.35 and 39.7 , respectively. These good results indicated that even with nine different plasmid sets (one per transcript), the quantification of the FG transcripts in the coded RNA samples was similar whatever the plasmid set was.

10.3. Fusion gene expression levels in diagnostic leukemia samples

We compared (Wilcoxon test) the 55 paired BM and PB samples taken at the time of diagnosis, mainly from *E2A-PBX1*- and *TEL-AML1*-positive patients. Two additional pairs in *MLL-AF4* network were not analyzed due to insufficient number. The

Table 33 Correlation coefficients obtained during the balanced randomized assay on diluted samples (phase IIIb)

Fusion transcript Type	E2A-PBX1	MLL-AF4 Ex10-Ex 4	TEL-AML1	BCR-ABL m-bcr	BCR-ABL M-bcr	SIL-TAL1	PML-RARA bcr1	CBFB-MYH11 Type A	AML1-ETO
FG Ct	-0.99	-0.78	-0.97	-0.98	-0.85	-0.84	-0.91	-0.86	-0.91
FG CN	0.99	0.72	0.95	0.98	0.80	0.80	0.86	0.82	0.91
Delta Ct	-0.96	-0.74	-0.98	-0.99	-0.92	-0.86	-0.90	-0.88	-0.94
ABL NCN	0.96	0.91	0.98	0.98	0.98	0.99	0.98	0.97	0.93

Correlation coefficients were calculated on three diluted FG-positive RNA samples. Details appear in Figures 31 and 32. Bold values denote the highest absolute value(s) observed.

statistical analysis revealed that sample source had no significant impact on the expression level at diagnosis of the relevant FG expressed as NCN, except for *PML-RARA* using *B2M* or *GUS* as the CG (see web site). These analyses would suggest that either source of diagnostic material could act as a suitable reference for MRD studies. Overall, median NCN of each FG transcript was variable, ranging from 8.5 copies (*E2A-PBX1*) down to 0.1 copy (*SIL-TAL1*) per copy of *ABL* gene transcript (Figure 33a). Such result was also observed with *B2M* and *GUS* CG. Relative expression levels of the different FG were generally consistent irrespective of the CG used for normalization (Figure 33b,c), although some heterogeneity was observed in a few cases (see *PML-RARA*, *AML1-ETO* and *SIL-TAL1* sections). In most cases, NCN of the FG transcript in patient samples were comparable to those observed in the corresponding cell line controls (see each section). These results were corrected according to the blast cell proportion in the sample when the information was available.

10.4. RNA dilution series in *E. coli* RNA

FG-positive cell line or patient RNA was diluted in *E. coli* RNA, using 10-fold dilution steps (10^{-1} – 10^{-6} , see the Materials and methods section). We observed a concordance in the limiting dilution experiments between the last positive dilution of the cell line or patient RNA and the sensitivity predicted on the basis of FG and CG levels in the corresponding undiluted RNA (see Section 11.3). These data indicated that the quantification of the CG and the FG with CN in the pure sample was correct. A linear regression analysis on the same dilutions showed that the FG/CG ratio did not change significantly (less than a factor two) over 3–5 logarithmic dilutions depending on the FG expression level in the pure sample. These results indicated equal RQ-PCR amplification efficiencies for the FG and the CG on a large dilution range.

11. Discussion

As molecular analysis plays an increasing role in therapeutic stratification in clinical trials, standardization and external QC programs become mandatory. This large-scale collaborative study of leading laboratories in the field of RQ-PCR-based detection of FG transcripts is the first ever published on this scale in the molecular oncology field. It is an excellent example on how European collaboration can stimulate progress. Furthermore, this initiative has recently been complemented by a collaboration involving 47 expert European laboratories aiming to achieve standardization in the detection of Ig and TCR gene rearrangements as DNA targets for molecular

diagnosis in lymphoproliferative disorders (BIOMED-2 Concerted Action).

11.1. Overall achievements

The EAC program revealed that the PCR component of the RQ-PCR assay was very robust as demonstrated by use of the plasmid calibrators. The *ABL* plasmid amplification during phase IIIb suggested that the threshold of interlaboratory reproducibility for gene expression quantification is a factor two (one Ct). Such precision should be sufficient for clinical application. EAC standardization was obtained leading to the development of a common RQ-PCR protocol followed by the 26 member laboratories. Such common protocols (Table 4) and the use of robust calibrators by each participant enabled reliable comparison of results. We have selected EAC primer and probe sets (see tables in each particular section) allowing the detection by RQ-PCR of 15 different FG transcripts (nine FGS, with six breakpoint variants), corresponding to the main leukemia-associated translocations. The selected primer and probe sets using the common protocol showed a sensitivity of 10 plasmid molecules or 10^{-4} RNA cell line dilution for the majority of the targets. Three reference genes (*ABL*, *B2M* and *GUS*) have also been selected out of 17 tested based on their high expression stability in PB and BM of normal and patient samples (see accompanying manuscript by Beillard et al⁵⁰). This program was designed for MRD studies and the EAC primer and probe sets have not been designed for the detection of each FG variant at initial diagnosis. Consequently, the EAC sets could lead to false-negative results if used for molecular screening at diagnosis, failing to detect approximately 25% cases with underlying *MLL-AF4* FG transcripts as well as some rare variants of *BCR-ABL*, *CBFB-MYH11* and *PML-RARA*. Therefore for screening at initial diagnosis, the classical BIOMED-1 primers might be used.²⁴

11.1.1. QC data: false positivity and false negativity:

The QC analysis was performed on a large series of coded samples. False negativity was 1% for the 10^{-3} dilution (316 tested samples) and 3.8% for the 10^{-4} dilution (366 tested samples) (Table 34).

False positivity was observed in 6.0% (34/546) of the FG-negative RNA samples tested, always with low expression (Ct > 30) and mainly restricted to few laboratories and FG networks (Table 35), suggesting a cross-contamination during the experimental set-up (Table 34). The false positivity was lower in NAC/NTC samples (3.2%) than in FG-negative RNA samples (6.0%), and could be related to the additional RT step associated with an increasing risk of contamination. False positivity was clearly a potential problem for the reliable detection of low levels of FG transcripts. One likely explanation

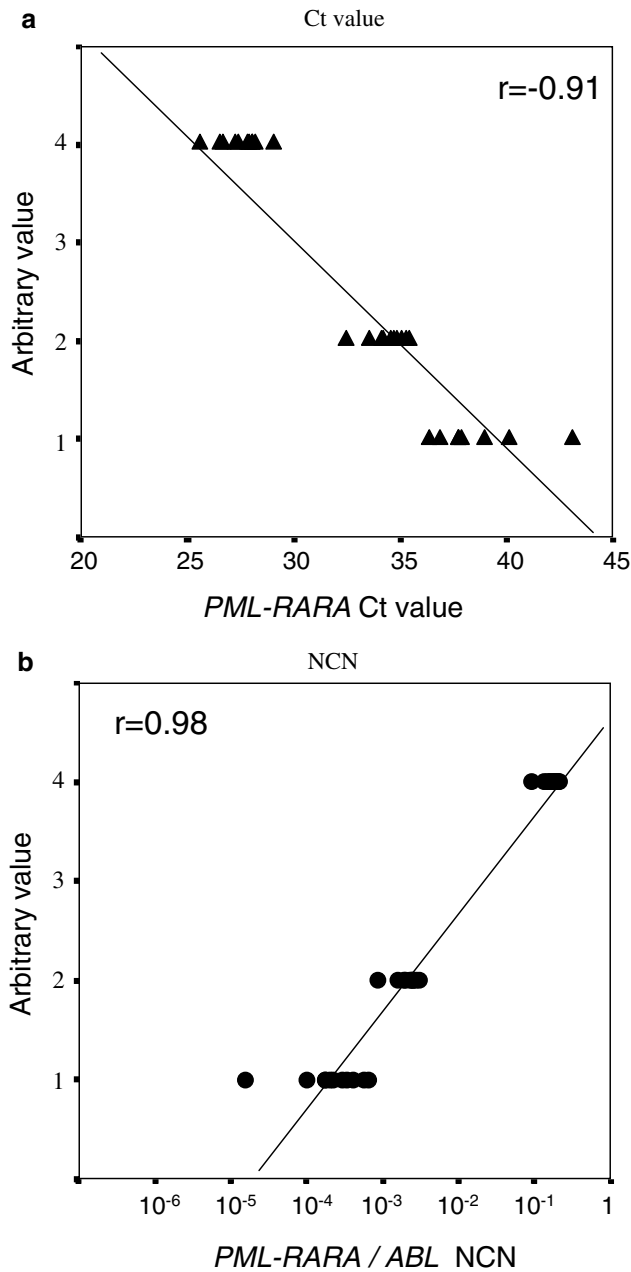


Figure 32 Schematic diagram of the results obtained during the balanced randomized assay (phase IIIb) within the *PML-RARA* network. Three coded *PML-RARA*-positive samples: 10^{-1} (Y-axis=4), 10^{-3} (Y-axis=2) and 10^{-4} (Y-axis=1) were analyzed in 11 different laboratories. Results on the X-axis: (a) Ct value or (b) the ratio *PML-RARA/ABL* of each coded sample according to dilution. These samples are identical to those appearing in Figures 30 and 31. Correlation coefficient r and regression curve calculated from these samples appear in the figure. In order to compare both figures, an identical scale was used for the X-axis. The same analysis has been performed for each FG network during phase IIIb for Ct and delta Ct values and CN and NCN. Results appear in Table 33.

is the large number of samples tested per laboratory in each experiment, with the inherent risk of cDNA carry-over between adjacent wells of the reaction plate. In any case, our data emphasize the need for very strict manipulation rules, even when using a closed system such as in RQ-PCR. Furthermore,

negative controls (NTC, NAC) are mandatory in each test but patient sample analysis should also ideally include 'RT negative' controls, whereby patient RNA is subjected to all stages of the RQ-PCR protocol with the omission of the RT enzyme in the cDNA synthesis step.

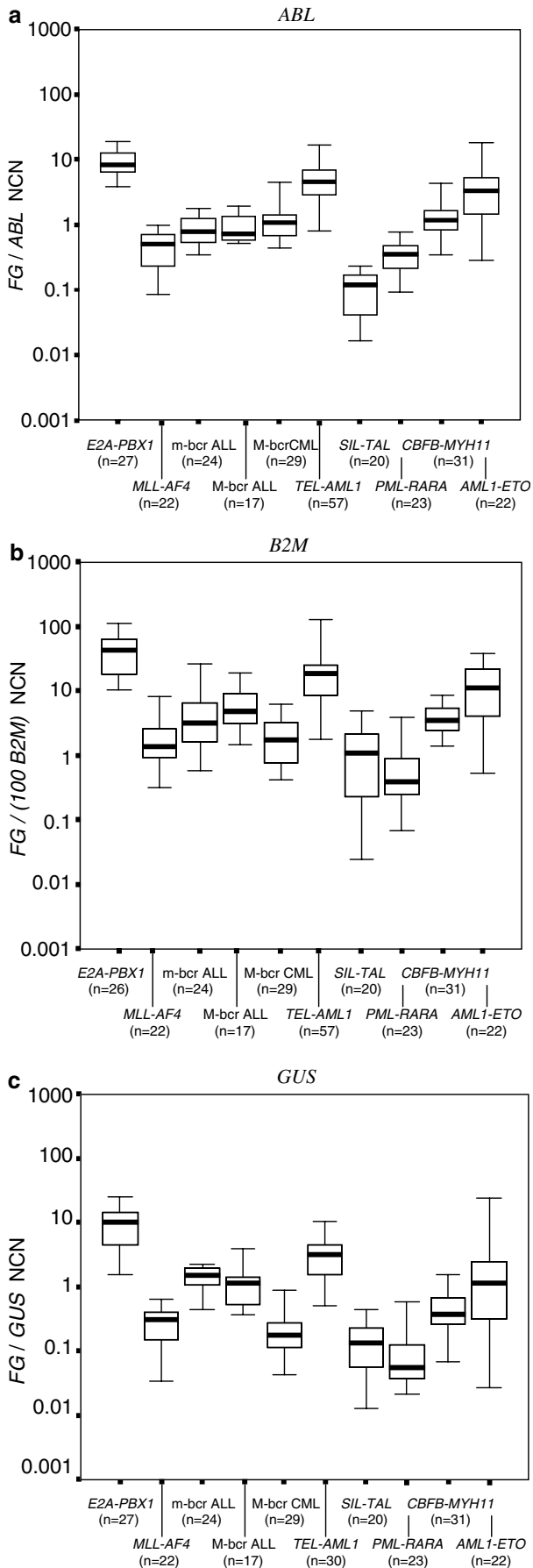
11.1.2. QC data: reproducibility of RQ-PCR results: The reproducibility of the RQ-PCR experiments was assessed by the balanced randomized assay (phase IIIb). Despite all our efforts to reduce variability, we still noticed significant differences between results for some laboratories. We observed that these discrepancies were related to the pre-PCR steps and are likely to have resulted from RNA degradation during storage and shipment, and/or intra- and interlaboratory variations in RT efficiency. The use of a CG to normalize results (Table 33) greatly improved the data reproducibility between laboratories suggesting that variations in RNA quality/quantity and RT efficiency can at least in part be compensated by normalization.

Even though normalization can reduce the effect of RNA degradation or inefficiency of the RT or PCR steps, it should be kept in mind that RNA degradation and RT efficiency can dramatically reduce the sensitivity of MRD detection.

During phase IIIb, the QC round was performed by a number of laboratories with no previous experience of PCR amplification of the involved FG transcripts. We did not notice significant changes in false positivity or false negativity proportions compared to other phases. These results suggest that the RQ-PCR protocol can be easily used for MRD detection by any other laboratory with the same sensitivity.

11.1.3. Expression level of fusion gene transcripts at diagnosis: We have for the first time compared the level of expression of the main FG transcripts in cell lines and a large series of leukemic samples at diagnosis ($n=278$) correlating FG expression with that of three reference genes (*ABL*, *B2M*, *GUS*). RQ-PCR revealed that marked differences in FG expression between transcripts in leukemic samples at diagnosis (Figure 33) could account for differential assay sensitivity. It would probably contribute to 'false-negative' results described in the literature for FG that are less expressed (eg *PML-RARA* or *SIL-TAL1*) and to the detection of FG transcripts in long-term remission in situations in which the FG is more highly expressed (eg *AML1-ETO*). Such information is likely to prove invaluable for clinical laboratories involved in MRD monitoring and also for basic research focusing on gene expression profiles (using DNA microarrays for example). However, we restricted the analyses to recently stored samples; hence it is currently unclear to what extent RNA degradation could influence the quantification. Over-time stability experiments are in progress to assess whether the degradation kinetics of FG and CG transcripts are similar (V van der Velden et al, in preparation). Furthermore, we compared FG expression in a large series of paired and unpaired diagnostic BM and PB samples. We observed that after correction for blast cell percentage and normalization by a CG, the relative expression of the FG transcript did not differ significantly between different sample sources. This suggests that either BM or PB at diagnosis can be used to estimate the NCN of the FG transcripts in individual cases of leukemia, serving as a reference for subsequent MRD assessments during follow-up.

11.1.4. Choice of the control gene: In the accompanying manuscript⁵⁰ we show that *ABL* gene expression did not differ significantly between normal and leukemic samples at diagnosis. Moreover, of the three extensively tested CGs, *ABL* gene



expression had the highest correlation with the FG transcripts in diagnostic samples. In our study, in a model of MRD detection (phase IIIb), normalization of FG expression to that of *ABL* as the CG improved the reproducibility of FG transcript results obtained in comparison to raw (not normalized) Ct or CN values. Therefore, we propose to use *ABL* as the CG of first choice for normalization in diagnostic and follow-up samples. As second choice, we recommend to use either *B2M* or *GUS* depending on their relative correlation with the respective FG transcript expression and the variability of the NCN at diagnosis. In the context of BCR-ABL quantification, the use of *ABL* should give only values up to 1.0 from a theoretical point. Our data, in accordance with the literature, show that practice is different. The values higher than 1.0 are most probably caused by the use of separate PCR methods and separate standards for BCR-ABL and *ABL*. In practice, identification of isolated samples with a low expression level of CG suggests RNA degradation or presence of inhibitors in such samples. On the other hand, observation of reduced or absent CG amplification for all samples tested is indicative of a reagent problem during the RT or PCR reactions. Finally, the CG transcript CN for each patient RNA sample allows normalization of efficiency of the pre-PCR steps.

11.2. Expression of RQ-PCR data

MRD monitoring by RQ-PCR analysis is becoming a tool for decision-making in multicenter therapeutic trials. For this reason, it is of capital importance for RQ-PCR results to be expressed in a uniform way. In the literature, no study addresses the issue of multicenter RQ-PCR analysis on a large scale (26 laboratories in our EAC network). Most publications show data from a single laboratory, but, to our knowledge, just one study involves the standardization between three laboratories for one target.¹⁵⁹ The majority of publications used a copy number ratio between the FG and the CG with a standard curve, this ratio being expressed as a decimal value or a percentage. To obtain the standard curve, laboratories used either cell line cDNA^{37,39,128,190} or plasmid DNA.^{38,42,125,191} So far, few authors used the $\Delta\Delta C_t$ method^{49,192} and, to our knowledge, a standard curve of diagnostic cDNA has not been used so far.

In our EAC network, we discussed four possibilities: (1) cell line RNA dilutions, (2) percentage of positive cell number relative to the diagnostic sample, (3) copy number ratios and (4) the $\Delta\Delta C_t$ method.

(1) The cell line RNA dilutions appeared to be very sensitive to degradation during transportation as shown in our study. The variability of expression for one cell line can be subject to large variations. Such potential variation depends on the source of the cell line, the timing of cell culture when RNA extraction has been done, and finally the RT efficiency. For multicenter studies, the option to overcome such difficulties could be to centrally prepare and distribute the cDNA.

Figure 33 Comparison of FG transcript expression in leukemic samples at diagnosis: (a) using *ABL* as CG, (b) using *B2M* as CG, (c) using *GUS* as CG. The ratio (NCN) is defined as the ratio between the copy number of the FG transcript per one copy of *ABL* or *GUS* gene transcript or 100 copies of *B2M* gene transcript. The median value corresponds to the black bold line. The box refers to the range defined by the 25th and the 75th percentile. BM and PB samples are pooled per transcript. *n*: number of patient samples for each particular transcript.

Table 34 Overall results of the QC rounds on coded RNA samples within the EAC network (phases III and IV)

QC phase ^a	False negativity		False positivity	
	10 ⁻³	10 ⁻⁴	FG neg. control	NAC/NTC
IIIa	1.7% (2/120)	4.6% (6/130)	6.5% (7/108)	4.2% (17/408)
IIIb	0.8% (1/119)	1.7% (2/116)	4.7% (11/234)	2.1% (9/436)
IVa	0% (0/77)	5% (6/120)	7.6% (15/198)	3.7% (10/272)
All phases	1.0% (3/316)	3.8% (14/366)	6.0% (33/546) ^b	3.2% (36/1116)

Results are proportions of false-positive or -negative samples.

^aDuring phase IIIb, coded samples were tested in randomly assigned laboratories.

^bFalse-positive results were observed in all the three QC rounds for *BCR-ABL* M-bcr, *PML-RARA* and *AML1-ETO* networks.

Table 35 False positivity per FG network during the QC rounds (phases III and IV)

Fusion transcript	FG neg. control	NAC/NTC
<i>E2A-PBX1</i>	0% (0/44)	0% (0/52)
<i>MLL-AF4</i>	1.1% (1/89)	4.6% (4/87)
<i>TEL-AML1</i>	6% (3/50)	0% (0/43)
<i>BCR-ABL</i> , m-bcr	4.8% (6/126)	5.8% (7/120)
<i>BCR-ABL</i> , M-bcr	10.9% (6/55)	4.1% (14/340)
<i>SIL-TAL1</i>	4.3% (2/46)	0% (0/162)
<i>PML-RARA</i>	11% (5/45)	5% (5/100)
<i>CBFB-MYH11</i>	2.2% (1/45)	0% (0/104)
<i>AML1-ETO</i>	20% (9/46)	5.6% (6/108)
All FG	6.0% (33/546) ^a	3.2% (36/1116)

Results are proportions of false-negative samples.

^aWhen excluding *AML1-ETO* network, the proportion decreases to 4.8% (24/500).

(2) Results expressed as frequency of positive cells would have the huge advantage to allow direct comparison with other MRD techniques. Furthermore, it would enable more reliable determination of kinetics of FG transcript reduction within individual patients, given the variability in FG transcript expression levels between patients as observed in this study. However, such an approach is dependent on availability of diagnostic material against which relative levels of MRD can be judged. Since the precise level of FG expression and its variations during treatment at the single cell level are not entirely clear, we ultimately decided to express results in terms of ratios between the target (FG transcript) and the reference (CG transcript) in our experiments.

(3) The ratio (NCN) was expressed as FG copies per copy of *ABL* or *GUS* gene transcript and per 100 copies of *B2M* gene transcript due to its high expression level. This ratio should be independent of the starting RNA quantity. For this purpose, we used plasmid standard curves, which offer the possibility to quantify directly the copy number of the transcripts. Our data show that plasmids are suitable calibrators for inter- or intralaboratory normalization of RQ-PCR analysis. Plasmid DNA is probably a good option providing stability and robustness, which is unlikely to be achieved when using large-scale production of cDNA as a potential QC material.

The potential drawbacks of this method are as follows. (i) The risk of contamination, although we used plasmid dilutions containing FG copies within the same range as patient samples. Usual rigorous precautions for PCR analysis are always required for limiting this risk. (ii) The use of plasmid calibrators reduces

the number of wells available for patient samples and slightly increases the cost. (iii) The calibrators introduce additional steps/calculations potentially increasing the variability. (iv) DNA plasmids do not directly assess the RT efficiency, but the CG expression level in patient samples clearly represents a control of the pre-PCR steps.

(4) The $\Delta\Delta C_t$ method (Applied Biosystems User's bulletin #2) does not have these disadvantages but has its own limitations. The method relies on the relative efficiencies of the FG and CG assays being comparable and consistent from plate to plate; therefore, it is critical that positive RNA or cDNA standards are routinely included, to enable deterioration in assay performance to be detected by a rise in Ct value as encountered once in our study for the analysis of 70 CML samples, including 17 paired samples. This method can be very efficient in expert laboratories and can be used to determine the relative level of MRD in comparison to the diagnostic sample. There are concerns that this approach may not lend itself to assessment of interexperimental variations in the intra- or interlaboratory setting. This may create difficulties in comparing RQ-PCR data between different groups, particularly when different machines are used (there are at least eight providers today).

11.3. Proposal for assessing the sensitivity level of RQ-PCR experiments based on EAC data

11.3.1. Background: When one encounters an absence of FG transcript amplification in a patient sample during follow-up, it is necessary to assess the detection limit for the particular assay to determine the reliability and clinical relevance of the result obtained. To address this issue, we propose two formulae to calculate the sensitivity level of a given experiment. While the $\Delta\Delta C_t$ method is explained in the accompanying manuscript,⁵⁰ we report here the use of copy number values.

11.3.2. Calculation: The formula is based on the results of *E. coli* dilution experiments and the correlation between FG CN and CG CN with a slope close to 1 in diagnostic samples (see each particular section). In this formula, the sensitivity is directly related to the NCN of the FG at diagnosis and the CG CN of the sample. Ideally, the calculation should be based on the patient's diagnostic NCN, after correction for blast percentage. If not available, EAC data can be used. In this model, 10 copies of the FG plasmid should be amplified for any particular fusion transcript. If only 100 copies can be amplified, the sensitivity should be reduced by one log₁₀:

$$\text{SENS} = -\log_{10}(\text{NCN}) - \log_{10}(\text{CGCN})$$

In this formula, SENS is the sensitivity (\log_{10}) of the experiment for the diagnostic sample and should be expressed as 10^{SENS} . NCN is either the ratio of the patient sample at diagnosis or if not available the corresponding median NCN from the EAC data.

The formula is valid for all CG at diagnosis, but one should be aware of the bias toward underestimation for *BCR-ABL/ABL* ratio for samples containing a high level of leukemic cells. However, only the *ABL* gene did not show any significant difference between BM and PB and between normal samples and leukemic samples at diagnosis (see accompanying manuscript).⁵⁰ Thus this formula can be used only with *ABL* as CG without any correction for assessing the sensitivity level of the experiment during the follow-up.

11.3.3. Three examples through real cases

11.3.3.1. At diagnosis: Patient A presents a pediatric T-ALL at diagnosis. The search of *SIL-TAL1* FG transcript in its PB sample remains negative. The quantification of *ABL* gene transcript using RQ-PCR with our EAC protocol is 46 000 copies. Thus the estimated sensitivity of the experiment based on EAC data for the median *SIL-TAL1* FG transcript expression in PB (0.09, Table 22) at diagnosis is

$$\text{SENS} = -\log_{10}(0.09) - \log_{10}(46\,000) = -3.6 \text{ (or } 10^{-3.6}\text{)}$$

11.3.3.2. At relapse: Patient B presents a late relapse of a *TEL-AML1*-positive precursor-B-ALL. The quantification of *TEL-AML1* FG transcript in its PB sample is 419 000 copies. The quantification of *ABL* gene transcript in the same sample is 17 000 copies. The *TEL-AML1/ABL* ratio for this patient is 25 (419 000/17 000). Thus the estimated sensitivity based on *TEL-AML1* FG expression in this patient is

$$\text{SENS} = -\log_{10}(25) - \log_{10}(17\,000) = -5.6 \text{ (or } 10^{-5.6}\text{)}$$

11.3.3.3. During follow-up: The same patient B is followed 3 months later by RQ-PCR for the detection of *TEL-AML1* FG transcript in PB and BM samples. *TEL-AML1* FG transcript is not detected by RQ-PCR in both samples. The results of *ABL* gene transcript quantification on the PB and BM samples are respectively 7700 and 13 700 copies. Based on the observation that *TEL-AML1* NCN does not differ significantly between PB and BM (Figure 11), the ratio in this patient at relapse (419 000/17 000) is used to calculate the sensitivity of this experiment:

$$\begin{aligned} \text{SENS(BM)} &= -\log_{10}(419/17) - \log_{10}(13\,700) \\ &= -5.5 \text{ (or } 10^{-5.5}\text{)} \end{aligned}$$

$$\begin{aligned} \text{SENS(PB)} &= -\log_{10}(419/17) - \log_{10}(7700) \\ &= -5.3 \text{ (or } 10^{-5.3}\text{)} \end{aligned}$$

Compared to classical RT-PCR FG transcript follow-up, the sensitivity in this case relies on patient and not on cell line samples. The sensitivity threshold calculated with this methodology is clearly more accurate than the one based on classical RT-PCR on cell line dilutions.²⁴

11.4. EAC protocol in clinical laboratories involved in current therapeutic trials for leukemias

Current therapeutic protocols for leukemias tend to be prognosis adapted, one consequence being that large number of institu-

tions are required to enroll patients to ensure that sufficient sample sizes are reached in order to address reliably which randomized treatment approaches afford a superior outcome. Analysis of MRD appears to be a key prognostic indicator allowing treatment optimization. Today, there is no standardized technique for fusion transcript detection during MRD follow-up. Our EAC protocol could provide the basis for an international reference of MRD studies using RQ-PCR analysis of FG transcripts. Indeed, up to 80 laboratories from 30 countries have already applied, upon a confidentiality agreement, for details of the methods employed in this study. Now, we are in a position to assess the clinical relevance of MRD analysis for the main FG transcripts in leukemia within therapeutic protocols. One direct application will be therapeutic trials with the innovative tyrosine kinase inhibitor (Imatinib) for *BCR-ABL*-positive ALL in Europe where the efficacy of each protocol will be followed at the molecular level using the EAC protocol. We would like to suggest that in the future, therapeutic strategies will be adapted according to such standardized MRD evaluations. It would be a great improvement if such biological data assist in therapeutic decision-making, such as in unrelated allogeneic transplant, infusion of donor lymphocytes or basically the choice of the most efficient therapeutic strategy.

In this large multicenter study, we aimed to standardize the only quantitative step of a sample RQ-PCR analysis: the PCR step. Assays were performed by the CG group on the RT step (see accompanying manuscript⁵⁰). This standardization effort is clearly a first step since notably all the previous steps from harvesting to RNA extraction were not studied. We believe that such standardization protocol on the PCR step should allow data comparison by measuring the quality of the sample.⁵⁰ Clearly in order to improve the quality of samples for such analysis in a routine laboratory, a large effort of optimization and standardization remains to be done.

Our EAC protocol has been set up using an ABI 7700 platform, but the TaqMan technology (5' nuclease assay) employed in this study can be in principle applied to various machines available today. The next step will be an inter-RQ-PCR machine standardization using robust calibrators. Furthermore, this standardization and QC program in leukemia could be a model for other biological markers in onco-hematology and more broadly in the oncology field. Standardization and quality control programs for novel technologies such as semiautomated MRD detection today, but also high-throughput chip DNA technologies tomorrow, are mandatory in ensuring that advances achieved through innovative genomic methodologies yield maximal benefit in improving the outcome of patients with leukemia.

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Supplementary Information

Supplementary Information accompanies the paper on Leukemia website (<http://www.nature.com/leu>).

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