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Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease

Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia

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Prospective studies on the detection of minimal residual disease (MRD) in acute leukemia patients have shown that large-scale MRD studies are feasible and that clinically relevant MRD-based risk group classification can be achieved and can now be used for designing new treatment protocols. However, multicenter international treatment protocols with MRD-based stratification of treatment need careful standardization and quality control of the MRD techniques. This was the aim of the European BIOMED-1 Concerted Action '*Investigation of minimal residual disease in acute leukemia: international standardization and clinical evaluation*' with participants of 14 laboratories in eight European countries (ES, NL, PT, IT, DE, FR, SE and AT). Standardization and quality control was performed for the three main types of MRD techniques, ie flow cytometric immunophenotyping, PCR analysis of antigen receptor genes, and RT-PCR analysis of well-defined chromosomal aberrations. This study focussed on the latter MRD technique. A total of nine well-defined chromosome aberrations with fusion gene transcripts were selected: t(1;19) with *E2A-PBX1*, t(4;11) with *MLL-AF4*, t(8;21) with *AML1-ETO*, t(9;22) with *BCR-ABL* p190 and *BCR-ABL* p210, t(12;21) with *TEL-AML1*, t(15;17) with *PML-RARA*, inv (16) with *CBFB-MYH11*, and microdeletion 1p32 with *SIL-TAL1*. PCR primers were designed according to predefined criteria for single PCR (external primers A ↔ B) and nested PCR (internal primers C ↔ D) as well as for 'shifted' PCR with a primer upstream (E5′ primer) or downstream (E3′ primer) of the external A ↔ B primers. The 'shifted' E primers were designed for performing an independent PCR together with one of the internal primers for confirmation (or exclusion) of positive results. Various local RT and PCR protocols were compared and subsequently a common protocol was designed, tested and adapted, resulting in a standardized RT-PCR protocol. After initial testing (with adaptations whenever necessary) and approval by two or three laboratories, the primers were tested by all participating laboratories, using 17 cell lines and patient samples as positive controls. This testing included comparison with local protocols and primers as well as sensitivity testing via dilution experiments. The collaborative efforts resulted in standardized primer sets with a minimal target sensitivity of 10^{-2} for virtually all single PCR analyses, whereas the nested PCR analyses generally reached the minimal target sensitivity of 10^{-4}. The standardized RT-PCR protocol and primer sets can now be used for molecular classification of acute leukemia at diagnosis and for MRD detection during follow-up to evaluate treatment effectiveness.

Keywords: acute myeloid leukemia (AML); acute lymphoblastic leukemia (ALL); PCR; standardization; fusion genes; chromosome aberrations; minimal residual disease (MRD); Concerted Action; BIOMED program

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Preface

Monitoring of acute leukemia patients during and after treatment for the presence of remaining leukemic cells ('minimal residual disease', MRD) has been shown to give major insight into the effectiveness of treatment.[1–6] In particular the (semi-) quantitative measurement of the decrease of the leukemic cell load during the first phases of treatment has high prognostic value. In childhood acute lymphoblastic leukemia (ALL) this application of MRD measurement has been proven to identify an unprecedented large group of low-risk patients (40–45%) with a 4-year relapse rate of only 2%, and a high-risk group (15%) with a 4-year relapse rate of 80%.[6] The remaining intermediate-risk group had an overall relapse rate of 23%, but at a later stage during maintenance treatment this group could be divided into an MRD-negative group with a 10% relapse rate and an MRD-positive group with a 67% relapse rate.[6]

It is clear that prospective MRD studies are feasible and that clinically relevant MRD-based risk group classification can be achieved and can now be used for designing new treatment protocols. However, multicenter international treatment protocols with MRD-based stratification of treatment need careful standardization and quality control of the techniques used for MRD detection.

Currently three different techniques with sufficient sensitivity of at least 10^{-3} (one leukemic cell between 10^3 normal cells) are used for MRD detection: (1) flow cytometric immunophenotyping, which is based on the detection of abnormal or unusual phenotypes; (2) PCR analysis of patient-specific junctional regions of rearranged immunoglobulin (Ig) or T cell receptor (TCR) genes; and (3) PCR analysis of breakpoint fusion regions of chromosome aberrations.[7,8] Each of these MRD techniques has its advantages and disadvantages with respect to technical complexity, applicability, and sensitivity, but all of them need standardization and quality control when used in multicenter studies. This was the aim of the European BIOMED-1 Concerted Action '*Investigation of minimal residual disease in acute leukemia: international standardization and clinical evaluation*'.[9] A total of 14 different laboratories from eight European countries (ES, NL, PT, IT, DE, FR, SE and AT) participated in this collaborative study.[9] Standardization and quality control was performed for flow cyto-

Table 1 RT-PCR analysis of fusion gene transcripts in acute leukemia

Chromosome aberration	*RT-PCR target*	*Positive controls*		*Design and initial testing of primers*	
		Cell lines[a]	*Patients*	*Responsible laboratory*	*Collaborating laboratory*
t(1;19)(q23;p13)	*E2A-PBX1*	697,SUP-B27,RCH-ACV	+	A Rambaldi *et al*	A Biondi *et al*
t(4;11)(q21;q23)	*MLL-AF4*	RS4;11,MV4-11	+	F Griesinger *et al*	A Biondi *et al* and EA Macintyre *et al*
t(8;21)(q22;q22)	*AML1-ETO*	KASUMI-1	+	EA Macintyre *et al*	F Griesinger *et al* and JA Gabert *et al*
t(9;22)(q34;q11)	*BCR-ABL* p190	TOM-1, ALL/MIK	+	G Saglio *et al*	A Rambaldi *et al*
t(9;22)(q34;q11)	*BCR-ABL* p210	BV173,KCL22,K562,LAMA-84	+	JA Gabert *et al*	M Gonzalez *et al*
t(12;21)(p13;q22)	*TEL-AML1*	REH	+	JA Gabert *et al*	G Saglio *et al* and A Biondi *et al*
t(15;17)(q22;q21)	*PML-RARA*	NB4	+	A Biondi *et al*	P Gameiro *et al*
inv(16)(p13;q22)	*CBFB-MYH11*	ME-1	+	EA Macintyre *et al*	JA Gabert *et al*
del(1)(p32;p32)	*SIL-TAL1*	CEM,RPMI8402	+	EA Macintyre *et al*	JJM van Dongen *et al*

[a]Kindly provided by Dr HG Drexler, DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (see Ref. 40).

metric immunophenotyping, PCR analysis of Ig and TCR genes, and PCR analysis of fusion gene transcripts in acute leukemia.[9–11] This report describes the successful standardization of PCR primers and PCR protocols for the sensitive detection of fusion gene transcripts of nine frequently occurring chromosome aberrations in acute leukemia.

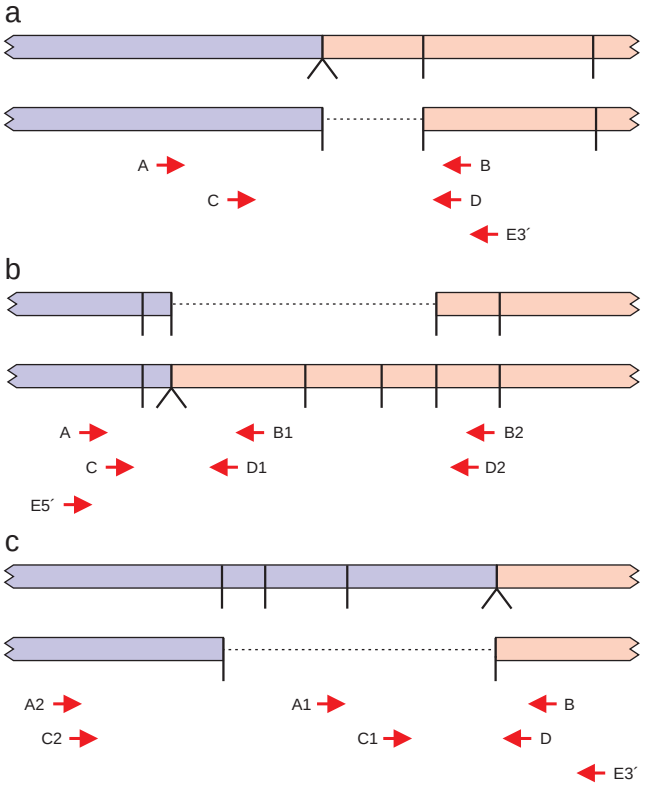

Figure 1 Three possibilities of primer design for RT-PCR detection of fusion gene transcripts, dependent on the presence or absence of more than one breakpoint cluster region in one of the two fusion gene partners. (a) In principle for each fusion gene transcript two external primers (A and B), two internal primers (C and D), and one 'shifted' primer (primer E) were designed (see text). (b) If more than one breakpoint cluster region occurs in the downstream fusion gene partner, extra B and D primers were designed. (c) If more than one breakpoint cluster region occurs in the upstream fusion gene partner, extra A and C primers were designed.

Introduction

Since the discovery of the first fusion gene, *BCR-ABL* in t(9;22),[12,13] many other chromosome aberrations with fusion genes have been and are being continually identified.[14–16] The genes involved in the genetic aberrations in acute leukemias play key roles in the development and function of lymphoid and myeloid cells. They frequently encode transcription factors, but also cell cycle regulators, signal transduction molecules, receptors, or Ig and TCR molecules.[14,17]

Several clinical studies have shown that chromosome aberrations in ALL and acute myeloid leukemia (AML) can be used for risk group classification.[18–22] For instance, t(9;22) and 11q23 aberrations such as t(4;11) in ALL are associated with poor prognosis,[18,19,21] whereas t(12;21) in ALL as well as t(8;21), t(15;17) and inv(16) in AML are associated with good prognosis.[19,20,23,24] In addition to this cytogenetic/molecular classification of acute leukemia at diagnosis, chromosome aberrations with leukemia-specific fusion gene regions can also be used as PCR targets for detection of MRD during follow-up, with sensitivities of 10^{-3} to 10^{-6}.[7,8]

PCR analysis of fusion genes is based on the design of oligonucleotide primers at opposite sides of the breakpoint fusion regions so that the PCR product contains the tumor-specific fusion sequences. In most types of chromosome aberrations with fusion genes the breakpoints in different patients are spread over 10 kb or more, distances which are difficult to cover by DNA PCR on a routine basis. This would imply that the precise breakpoint recombination sites at the DNA level have to be determined for each individual patient, in order to perform reliable sensitive PCR analyses. However, in many acute leukemias, the fusion gene is transcribed into a fusion mRNA, which can serve as the PCR target after reverse transcription (RT) into cDNA.

The standardization and quality controls in this study focussed on nine well-defined chromosome aberrations with fusion gene transcripts, which frequently occur in acute leukemia: t(1;19)(q23;p13) with the *E2A-PBX1* fusion gene,[25,26] t(4;11)(q21;q23) with the *MLL-AF4* fusion gene,[27–29] t(8;21)(q22;q22) with the *AML1-ETO* fusion gene,[30,31] the two main types of t(9;22)(q34;q11) with *BCR-ABL* fusion genes,[12,13,32] t(12;21)(p13;q22) with the *TEL-AML1* fusion gene,[33,34] t(15;17)(q22;q21) with the *PML-RARA* fusion gene,[35,36] inv(16)(p13;q22) with the *CBFB-MYH11* fusion

gene,[37] and the intrachromosomal microdeletion on 1p32 with the *SIL-TAL1* fusion gene.[38,39]

It was decided that the standardization and quality control should not only concern the primers, but also the RT reaction and the PCR protocol as well as the usage of common positive controls (cell lines and some patient samples) and a common negative control (HL60 cell line) (Table 1).[40]

Primer design for RT-PCR analysis

The collaborative study aimed at the positioning of the primers in such a way that they can be used in several different types of classical PCR approaches (Figure 1).

(1) PCR for molecular genetic classification of acute leukemia at diagnosis: This is generally performed by single PCR (primers A ↔ B) followed by agarose gel electrophoresis or polyacrylamide gel electrophoresis for detection of PCR products of a particular size. Alternatively, gel electrophoresis followed by blotting and hybridization with an oligonucleotide probe (eg internal primer) can also be performed for reliable identification of the PCR product.

(2) Sensitive PCR for MRD detection: This can be achieved by nested PCR with a set of external primers (A ↔ B) and internal primers (C ↔ D), followed by evaluation of the size of the obtained PCR products in agarose gel electrophoreses or polyacrylamide gel electrophoresis.

(3) Control PCR for confirmation or for exclusion of false positive results: Extra control or confirmation of results can be obtained by use of two independent single PCR analyses with two different primer sets (A ↔ B plus C ↔ E3′ or E5′ ↔ D), which result in partly overlapping ('shifted') PCR products (Figure 1). The 'shifted' primers should be positioned upstream (E5′ primer) or downstream (E3′ primer) of the external A ↔ B primers, eg dependent on the suitability of the upstream and downstream sequences for primer design. In order to reduce the risk of contamination, use of the 'shifted' E primers should be restricted to confirmation of positive results obtained with the A ↔ B primers.

In some chromosome aberrations the breakpoint region spans several introns, whereas in other aberrations multiple breakpoint regions occur. Consequently, the corresponding fusion gene transcripts will have different exon compositions, which might not easily be covered by the same set of PCR primers. RT-PCR detection of such fusion gene transcripts requires design of extra primers, which are positioned further downstream (Figure 1b) or upstream (Figure 1c), as in the case of t(9;22), inv(16) and t(15;17). For these translocations, seven primers had to be designed, whereas for the other translocations the design of five primers was sufficient. In t(9;22), the two main types of breakpoint cluster regions in the *BCR* gene are associated with different disease categories, ie ALL and chronic myeloid leukemia (CML).[12,13] Therefore they are addressed separately in this report as t(9;22) *BCR-ABL* p190 (almost exclusively occurring in ALL) and as t(9;22) *BCR-ABL* p210 (found in virtually all CML and some ALL).[12,13] As a consequence, two different sets of *BCR* primers but a single set of *ABL* primers were designed for PCR analysis of the two t(9;22) variants (see Sections 4 and 5).

Table 2 Database references per gene

Chromosome aberration	*Gene*	*Name*	*Accession number*	*Date*
t(1;19)	*E2A*	HUME12A	M31222	07 Nov 1994
	PBX1	HUMPBX1AB	M86546	07 Jan 1995
t(4;11)	*MLL*	HUMHRX	L04284	31 Dec 1994
	AF4	HUMAF4Y	L13773	31 Dec 1994
t(8;21)	*AML1*	HUMAML1C	D43969	11 Oct 1996
	ETO	HUMMTG8	D14289	27 Jan 1993
t(9;22)	*BCR*	HSBCRR	X02596	02 Jun 1994
	ABL	HSABL	X16416	12 Sep 1993
t(12;21)	*TEL*	HSU11732	U11732	17 Jul 1994
	AML1	HUMAML1C	D43969	11 Oct 1996
t(15;17)	*PML*	HUMPML	M73778	08 Jan 1995
	RARA	HSRAR	X06538	12 Sep 1993
inv(16)	*CBFB*	HUMCBFB	L20298	28 Sep 1993
	MYH11	HUMMHCAAA	D10667	18 Jan 1996
del(1p32)	*SIL*	HUMSIL	M74558	13 Jan 1995
	TAL1	S53245	S53245	07 May 1993

After initial design procedures and parallel testing of old and new primer sets, special guidelines were developed for primer design with the OLIGO5.0 software program (Dr Wojciech Rychlik, Cascade, CO, USA): (1) The melting temperature (Tm) should be 70–75°C (preferably 70–72°C). The Tm calculations should be according to the nearest neighbour method taking into account agreed PCR conditions such as salt concentration, etc: forward primer: 400 nM; reverse primer: 400 nM; monovalent cation: 50 mM; free Mg^{2+}: 1.7 mM. (2) The GC content of the PCR product should be <70%. (3) The ΔG values of the 3′ end of each primer should be relatively low, ie 3′ terminal pentamers should be less stable than –9 kcal/mol to avoid false priming. (4) The Δ Tm (Tm of PCR product minus Tm of primers) should be <24°C. (5) No hairpin formation should occur, particularly at the 3′ end. (6) The most stable 3′ terminal dimer should not exceed –4 kcal/mol; the most stable overall dimer is not so important. (7) The position of the primers should be as in Figure 1, resulting in PCR products of limited size: external primers A ↔ B should result in PCR products of 200–600 bp; the internal primers C ↔ D in PCR products of 120–350 bp; and the shifted primers E5′ ↔ D or C ↔ E3′ in PCR products of 200–600 bp. (8) In principle, the upstream primers A, C and E5′ are sense, whereas the downstream primers B, D and E3′ are anti-sense.

Based on the published germline sequences of the involved normal genes, we made virtual fusion transcript sequences, which were used for the primer design procedures. The data base references of the germline sequences used are given in Table 2.

RT-PCR protocol

During the first meetings of the BIOMED-1 Concerted Action, various local RT and PCR protocols were compared in order to understand the rationale of each difference and to take advantage of the experience of each laboratory. Subsequently, a common protocol was designed, tested and adapted again resulting in the standardized RT-PCR protocol (Table 3),

Table 3 Standardized RT-PCR protocol

1. RT-reaction with random hexamers
- 1 μg of RNA (or 0.1 μg of mRNA) in 9.5 μl of H_2O
- incubation at 70°C for 10 min
- cool on ice and add other reagents to final volume of 20 μl:
  RT buffer: 20 mM Tris HCl, 50 mM KCl, pH 8.3
  $MgCl_2$: 5 mM
  DTT: 10 mM
  random hexamers: 5 μM
  RNAasin: 20 units
  RT enzyme: 200 units Superscript (200 units per μl)
  dNTP: 1 mM
- temperatures and incubation times:
  room temperature for 10 min
  42°C for 45 min
  99°C for 3 min
  4°C at end of RT step

2. Single PCR or first round of nested PCR
- final volume of 50 μl
- 2–3 μl of cDNA (ie 10–15% of RT mixture)
- primers: 400 nM final concentration
- dNTP: 200 μM final concentration
- PCR buffer: 20 mM Tris HCl, 50 mM KCl, pH 8.3
- $MgCl_2$: 2.5 mM (to be optimized in each laboratory)
- *Taq* enzyme[a]: 1 unit per 50 μl volume[b]

3. PCR temperatures and cycle times
- initial melting: 95°C for 30 s
- PCR cycles[c]:
  94°C for 30 s (melting)[b]
  65°C for 60 s (annealing)
  72°C for 60 s (extension)
- number of cycles: 35
- no final extension needed
- stop of PCR: 16°C (or room temperature)[d]

4. Second round PCR for nested PCR
- 1 μl of first round PCR
- same volume, reagents and cycle conditions as for first round PCR, using the internal (nested) C ↔ D primers

[a]During the final phase of the study it appeared that usage of *AmpliTaq* Gold (PE Biosystems, Foster City, CA, USA) further improves the sensitivity.
[b]If the GC content of the PCR product is high (>70%), it is advised to use 2 units of *Taq* enzyme per 50 μl and to increase the melting temperature to 95°C.
[c]The melting, annealing and extension times might be shortened, if a new generation of rapid PCR machine is used.
[d]For evaluation of the PCR products, 15 μl of the PCR mixture is used for agarose gel electrophoresis.

which was used in all laboratories throughout the testing of the newly designed primer sets.

Testing and standardization procedures

For each chromosome aberration, two or three laboratories performed the design and initial testing of the new PCR primers (Table 1). The testing was performed in parallel to locally available PCR primers and protocols. In addition to locally available patient material, the same set of 17 cell lines was used as standard positive controls in all laboratories (Table 1). The 17 cell lines with well-defined chromosome aberrations were kindly provided for the collaborative study by Dr HG Drexler (DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).[40] Large-scale culturing and RNA extraction was performed in Rotterdam and subsequently ethanol-precipitated RNA samples were distributed to all participating laboratories. The primer testing was performed via dilution experiments (RNA in RNA), in which the patient RNA or cell line RNA was diluted into HL60 RNA as negative control. The dilution was performed in 10-fold steps, from undiluted to 10^{-6} or 10^{-7}.

The aims of the testing and standardization were to obtain clearly positive PCR results without aspecific background, which might hamper the interpretation of the results, and to reach sufficient sensitivity for each primer set in the dilution experiments with cell line RNA and patient RNA. We aimed at a target sensitivity of at least 10^{-2} for single PCR with the A ↔ B, C ↔ D, C ↔ E3′, and E5′ ↔ D primers and at a target sensitivity of at least 10^{-4} for the nested PCR with A ↔ B plus C ↔ D primers.

As soon as the responsible laboratories had approved the primer sets, the combined primer sets for all nine chromosome aberrations were sent to each participating laboratory. New dilution experiments were performed by all laboratories for each chromosome aberration. As soon as at least 70% of the laboratories reached the minimal target sensitivity, the primer set was approved. In some cases this was difficult and new primers were designed.

Exchange of personnel between the participating laboratories and a total of nine meetings (May 1994 in Lisbon, February 1995 in Münster, October 1995 in Monza, May 1996 in Stockholm, October 1996 in Paris, November 1996 in Rotterdam, April 1997 in Lisbon, November 1997 in Salamanca, and July 1998 in Rotterdam) were needed to complete the collaborative study. The results of this collaborative effort are described per chromosome aberration by the responsible scientists in nine sections.

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

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Background

The t(1;19)(q23;p13) was first reported in 1984 by different groups in some cases of pre-B-ALL[41–43] and the literature data so far reported indicate that this translocation can be found in a balanced (25%) or unbalanced (75%) form –19, +der(19)t(1;19) in which two normal chromosomes 1 are present. The t(1;19) is detected in about 5–6% of childhood ALL and in about 3% of adult ALL.[42,44–47] In both pediatric and adult patients this translocation occurs almost exclusively in pre-B-ALL expressing cytoplasmic Igμ, even though it has been reported sporadically in pro-B-ALL and common ALL (<1%) as well as in rare cases of T-ALL and AML.[44,45] Most cases carrying the t(1;19) express a typical immunophenotype with homogeneous expression of CD19, CD10, CD9, complete absence of CD34, and at least partial absence of CD20.[48] Moreover, the t(1;19) correlates with the presence of known clinical high-risk features, such as elevated cell count, high serum lactate dehydrogenase levels and central nervous system involvement.[44]

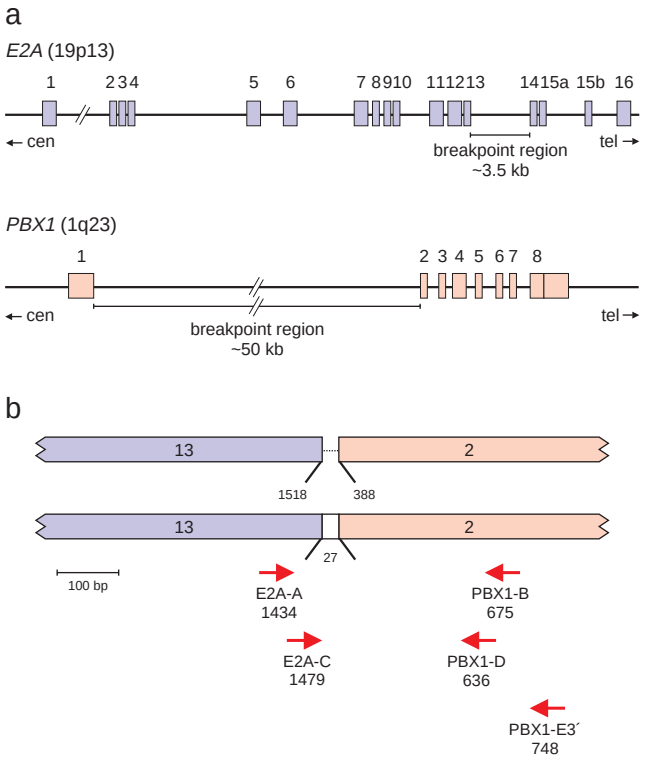

Figure 2 (a) Schematic diagram of the exon/intron structure of the *E2A* and *PBX1* genes, which are involved in t(1;19)(q23;p13). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. (b) Schematic diagram of the classical *E2A-PBX1* fusion transcript (upper) and the variant transcript (lower) with an insertion of 27 nucleoides (see Refs 50–51). The numbers under the fusion gene transcript refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The arrows indicate the relative position of the primers; the numbers refer to the 5′ nucleotide position of each primer (see Table 4).

Table 4 Primers for RT-PCR analysis of t(1;19)(q23;p13) with the *E2A-PBX1* fusion gene

Primer code	*5′ Position[a] (size)*	*Sequence 5′–3′*
E2A-A	1434 (19)	CACCAGCCTCATGCACAAC
PBX-B	675 (19)	TCGCAGGAGATTCATCACG
E2A-C	1479 (19)	CACCCTCCCTGACCTGTCT
PBX-D	636 (19)	GGCCTGCTCGTATTTCTCC
PBX-E3′	748 (19)	TGAACTTGCGGTGGATGAT

[a]See Table 2 for complete sequence information.

As shown in Figure 2a this translocation involves the *E2A* gene on chromosome 19 (band p13.2-p13.3) and the *PBX1* gene (also known as *PRL*) on chromosome 1(q23).[25,26,49] The genomic organization of *E2A* is well-defined and breakpoints occur almost exclusively in a 3.5-kb intron region between exon 13 and 14.[25,26,49] The genomic organization of the *PBX1* gene is not yet fully known and the breakpoints are dispersed over an intronic region of about 50 kb between exons 1 and 2.[25,26,49] In the majority of cases the *E2A-PBX1* fusion transcript shows a constant junction site of exon 13 (nucleotide 1518) of *E2A*[26] to the second exon (nucleotide 388) of *PBX1* (KA Monica, unpublished results, Accession number M86546)

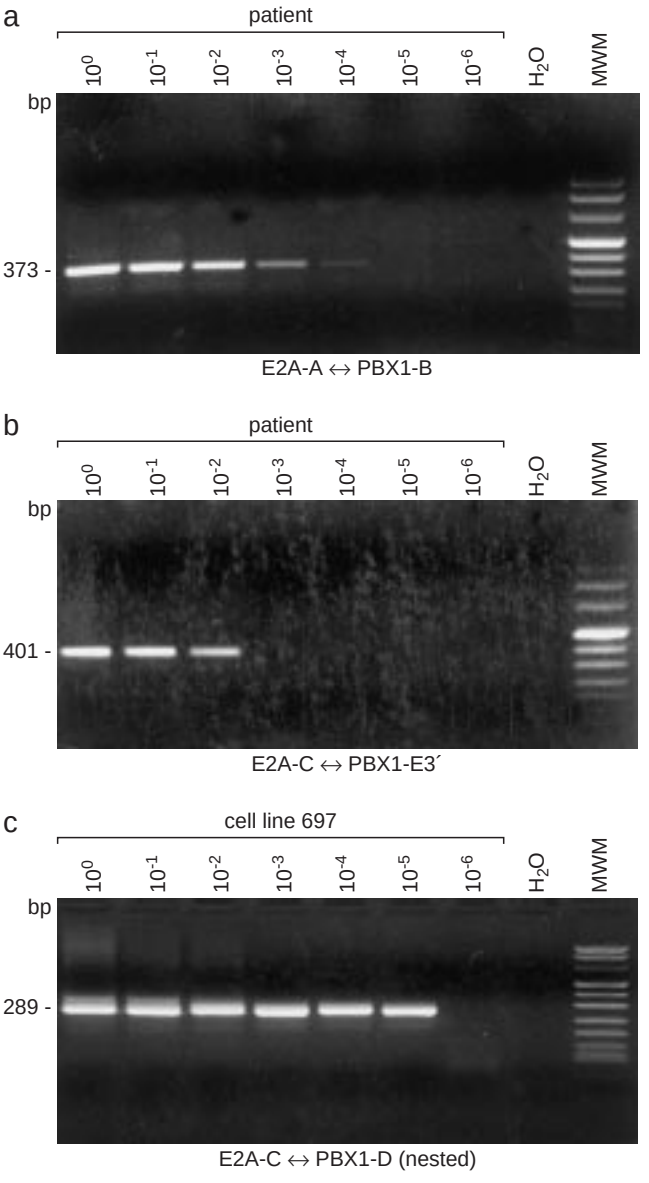

Figure 3 Ethidium bromide-stained agarose gels showing dilution experiments with single A ↔ B PCR (a), shifted C ↔ E3′ PCR (b), and nested PCR (c) using RNA from t(1;19)-positive patients or the 697 cell line. A tube without RNA was used as negative control (H_2O).

Table 5 Sizes of PCR products and log sensitivities of *E2A-PBX1* primer sets in RT-PCR testing

	A ↔ B	*C ↔ D*	*A ↔ B + C ↔ D*	*C ↔ E3′*
Sizes of PCR products in bp				
standard	373	289	289	401
variant (+27bp)[a]	400	316	316	428
697	–3	–4	–5	–3
SUP-B27	–3	–4	–5	–3
RCH-ACV	–3	–3	–4	–3
Patients	–4	–4	–5	–3

[a]Splice variant with insertion of 27 bp.

(Figure 2b). A variant fusion transcript is described in about 5–10% of t(1;19)-positive ALL.[50–52] It is characterized by a stretch of 27 nucleotides inserted at the usual junction point between nucleotides 1518 and 388 of the *E2A* and *PBX1* genes, respectively (Figure 2b). These additional nucleotides, which are identical in each case, appear to arise from a differentially spliced exon of either *E2A* or *PBX1* genes, but their exact derivation still remains unknown.[50–52]

Results of PCR primer design and testing

Table 4 shows the position and sequence of each primer relative to the *E2A* and *PBX1* genes. Primer E2A-A, derived from *E2A* gene exon 13 and primer PBX-B, derived from *PBX1* gene exon 2, were used in a first cycle PCR using the standardized PCR protocol of the BIOMED-1 project. This first round PCR reaches a sensitivity level of $10^{-3}/10^{-4}$, when the assay is performed using RNA obtained from control cell lines (697, SUP-B27, RCH-ACV) as well as ALL patients. When a second round of amplification is performed using the internal primers E2A-C and PBX-D, a reproducible sensitivity level of $10^{-4}/10^{-5}$ is obtained in both cell lines and ALL patients (Table 5). PCR products run in 1.5% agarose gels and visualized by staining with ethidium-bromide appear as clear bands of 373 bp and 289 bp for the first and the nested PCR, respectively. No extra bands are usually found with the possible exception of a small size band occasionally occurring at dilution of 10^{-5} (Figure 3). Finally, the shifted PCR employing the primer E2A-C and PBX-E3′, used to exclude false positive results, generates a clear band of 401 bp with a sensitivity of at least 10^{-2}, when RNA from cell lines and ALL patients is used (Figure 3b).

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

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Background

Molecular studies have shown that t(4;11)(q21;q23) involves the *MLL* (*HRX*, *Htrx*) and *AF4* (*FEL*) genes.[27–29] *MLL-AF4*-positive leukemias are observed in 50–70% of infant ALL cases and in approximately 5% of pediatric and adult ALL cases.[19,53,54] The presence of a t(4;11)(q21;q23) has been associated with the pro-B-ALL phenotype (CyCD79a$^+$, CD19$^+$, CD10$^-$, CD24$^-$)[54,55] and with coexpression of myeloid differentiation antigens (CD15 and CD65)[55–57] as well as the NG2 antigen.[58] The t(4;11)(q21;q23) fuses the 5′ portion of *MLL* to the 3′ portion of *AF4* (Figure 4).

The *MLL* gene is composed of 37 exons spanning a region of >1 MB. There are two numbering systems of exons; the most recent system will be used in this report (Figure 4).[59] The MLL protein contains a region of three AT-hooks, which have binding capacity to the minor groove of DNA. A domain with binding capacity to hemimethylated DNA is located in the 5′ portion of the protein encoded by exon 8. A zinc finger domain is encoded by exons 11 to 16 and a drosophila trithorax homology domain is located at the 3′ part of the gene.

The *MLL* gene has been found to be involved in approximately 30 different translocations, of which approximately 20 with identified partner genes.[15] Although some of the involved fusion partners may share sequence homology, fusion partners of completely different predicted functional properties have been characterized. *MLL* fusion genes are found in precursor-B-ALL, AML, myelodysplastic syndrome (MDS), some cases of T-ALL, and in secondary leukemia.[60–66]

In addition to *MLL* fusion genes generated by translocations, deletions of exon 11 have been associated with T-ALL[67] and tandem duplications of the gene have been observed in both *de novo* and secondary AML[68] as well as in normal peripheral blood and bone marrow mononuclear cells.[69]

The *AF4* gene is composed of 20 exons and encodes a serine–proline-rich protein.[28,70] The function of this protein has not been determined, and the functional domains are not well characterized.

MLL-AF4 fusion genes have been found in virtually all t(4;11) and in a significant number of cases where the t(4;11) was not detected by conventional cytogenetics.[57] Reciprocal *AF4-MLL* transcripts have been found in 70% of cases.[57] In contrast to the t(8;21) or the t(12;21), where only one or two variant fusion transcripts are observed, at least 10 different *MLL-AF4* fusion transcripts have been found, due to breakpoints in different introns (Figure 4a). Furthermore, differential splicing is a common phenomenon, leading to more than one fusion transcript in a leukemia. All fusion transcripts described in this paper are in frame (Figure 4b).[57]

The major breakpoint region of the *MLL* gene has been well characterized and is located on a *Bam*HI fragment of 8.3 kb. The breakpoints cluster in the 6.5 kb region between exons 8 and 12 (Figure 4a); breakage occurs most frequently in introns 9 and 10 in pediatric and adult ALL, and in intron 11 in infant ALL, leading to fusion genes involving exons 9 and 10 in adults and pediatric patients, and exon 11 in infants (Figure 4b).[71] The breakpoint cluster region of the *AF4* gene is larger, spanning a region of 40 kb (Figure 4a). The most frequent fusion point in *MLL-AF4* fusion genes is exon 4; in rare cases exons 5, 6 and 7 are fused to the *MLL* gene (Figure 4b).

MLL-AF4 has been identified as an adverse prognostic factor in infant leukemia by several study groups.[19,53,72,73] Also, it has been associated with a bad prognosis in adults, but prognosis seems to have improved with the introduction of high-dose Ara-C in the induction therapy of adult ALL.[54] In pediatric cases, there is some suggestion that different age groups have different prognoses.[19,53] Whether there is a correlation with different types of *MLL-AF4* fusion genes is still unclear.

Table 6 Primers for RT-PCR analysis of t(4;11)(q21;q23) with the *MLL-AF4* fusion gene

Primer code	*5′ Position[a] (size)*	*Sequence (5′–3′)*
MLL-A	3916 (17)	CCGCCTCAGCCACCTAC
AF4-B	1714 (20)	TGTCACTGAGCTGAAGGTCG
MLL-C	3936 (18)	AGGACCGCCAAGAAAAGA
AF4-D	1677 (20)	CGTTCCTTGCTGAGAATTTG
MLL-E5′	3793 (18)	AAGCCCGTCGAGGAAAAG

[a]See Table 2 for complete sequence information.

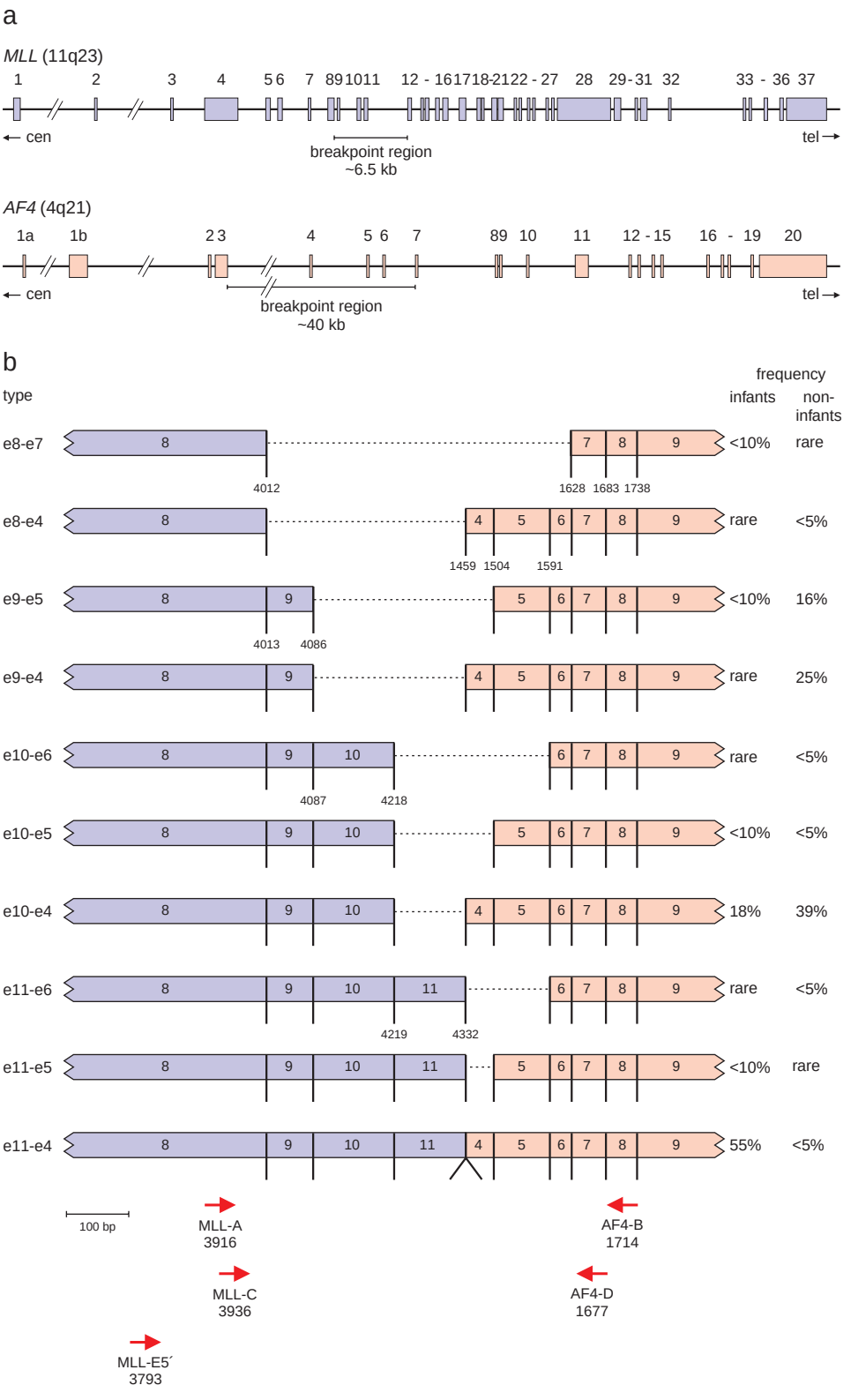

Figure 4 Schematic diagram of the exon/intron structure of the *MLL* and *AF4* genes, which are involved in t(4;11)(q21;q23). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. (b) Schematic diagram of the various types of *MLL-AF4* fusion transcripts. The numbers under the fusion gene transcripts refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The relative frequencies of the fusion transcripts differ between infants and non-infants (children and adults) and are caused by differences in the location of the breakpoints (see text). The arrows indicate the relative position of the primers, which can amplify all indicated fusion transcripts; the numbers refer to the 5′ nucleotide position of each primer (see Table 6).

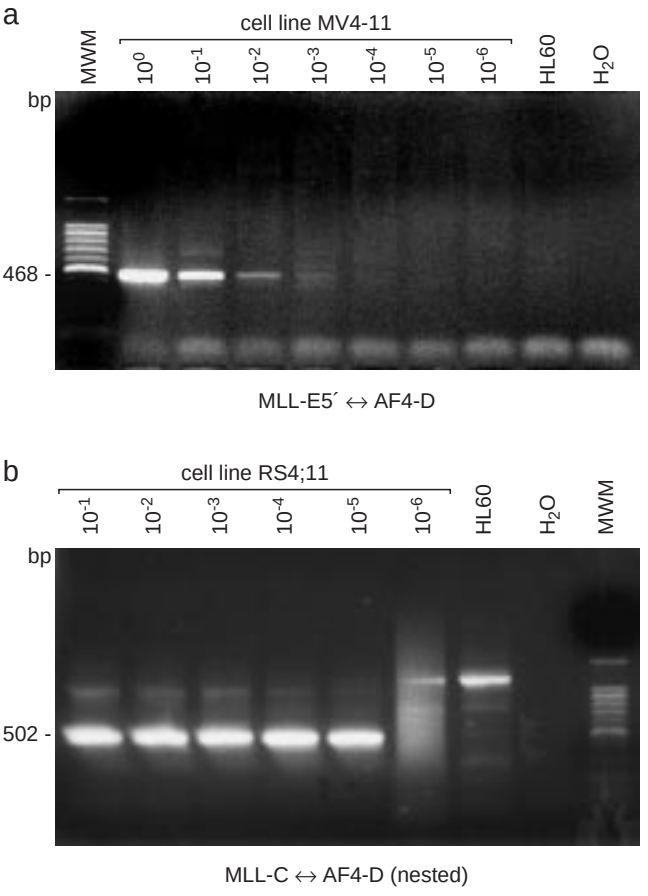

Figure 5 Ethidium bromide-stained agarose gels showing dilution experiments with the 'shifted' E ↔ D PCR (a) and the nested PCR (b), using RNA from the t(4;11)-positive cell lines MV4-11 and RS4;11, respectively. HL60 RNA and H_2O were used as negative controls. The C ↔ D primer set gave a large aspecific background band, which could easily be discriminated from the correct 502 bp PCR products.

Table 7 Sizes of PCR products and log sensitivities of *MLL-AF4* primer sets in RT-PCR testing

	A ↔ B	*C ↔ D*	*A ↔ B + C ↔ D*	*E5′ ↔ D*
Sizes of PCR products in bp				
e8-e7	184	127	127	270
e8-e4	353	296	296	439
e9-e5	382	325	325	468
e9-e4	427	370	370	513
e10-e6	427	370	370	513
e10-e5	514	457	457	600
e10-e4	559	502	502	645
e11-e6	541	484	484	627
e11-e5	628	571	571	714
e11-e4	673	616	616	759
MV4–11 (e9-e5)	–3	–3	–5	–3
RS4;11 (e10-e4)	–3	–3	–5	–3
Patients	–2	–3	–4	–2

Only limited data are available for MRD monitoring in patients with the t(4;11) by molecular analysis. The few cases which have been studied so far, suggest, that the *MLL-AF4* fusion gene disappears below the threshold of sensitivity in patients remaining in continuous complete remission.[74,75] However, more patients need to be studied in order to draw final conclusions.

Results of PCR primer design and testing

The primers for detection of *MLL-AF4* transcripts were designed in *MLL* exon 8 and *AF4* exon 7, which allows detection of all known *MLL-AF4* fusion gene transcripts (Figure 4b). The precise primer sequence information and position of the primers is summarized in Table 6.

Single PCR with A ↔ B, C ↔ D, and E5′ ↔ D primer sets reached sensitivities of 10^{-3} in case of the RS4;11 and MV4–11 cell lines and 10^{-2} in case of patients (Figure 5). The nested PCR resulted in sensitivities of 10^{-4} to 10^{-5} (Figure 5 and Table 7).

SECTION 3. t(8;21)(q22;q22) with the *AML1-ETO* fusion gene

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Background

The t(8;21)(q22;q22) was first described in 1973.[76] It is found primarily in *de novo* AML of FAB-M2 subtype and has been identified in the cell line Kasumi-1, derived from an AML-M2 patient at relapse.[77] The t(8;21) fuses the *AML1* gene (acute myeloid leukemia 1 gene), also identified as *PEBP2a* (polyoma enhancer binding protein 2 subunit a) or *CBFA2* (core binding factor subunit A2) to the *ETO* gene (eight twenty one gene), also identified as *CDR* (cyclin D-related gene) or *MTG8* (myeloid translocation gene on chromosome 8). The *AML1* gene is composed of nine exons spanning a region of 150 kb and the *ETO* gene is composed of 13 exons, spanning 87 kb (Figure 6a).

The *AML1* gene is not only involved in t(8;21) but also in several other translocations, including t(3;21)(q26.2;q22) with fusion to *EVI1*, *EAP* or *MDS1*, t(12;21)(p13;q22) with fusion to *TEL* (also called *ETV6*), and t(16;21) with fusion to *MTG16*.[78–80] Other chromosomal bands have been shown to be rearranged to the *AML1* locus, including 1p36, 5q13, 14q22, 15q22, 17q11 and 20p13.[81,82]

AML1-ETO fusion transcripts are found by RT-PCR in virtually all cases of t(8;21)-positive AML. They generate predominant PCR products of a constant size, corresponding to an in-frame fusion of *AML1* exon 5 to *ETO* exon 2. *AML1-ETO* fusion transcripts are not only found in virtually all cases of t(8;21), but also in cases with complex translocations and in a significant proportion of t(8;21)-negative AML.[83,84] *AML1* breakpoints are located between exon 5 and exon 6 (Figure 6a), primarily in a 20 kb *Bam*HI region. *ETO* breakpoints are located upstream of exon 2. To our knowledge, variant *AML1-*

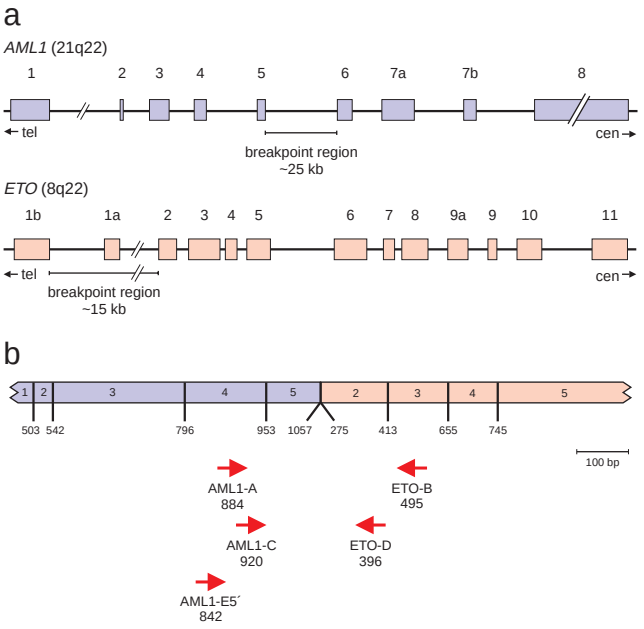

Figure 6 (a) Schematic diagram of the exon/intron structure of the *AML1* and *ETO* genes, which are involved in t(8;21)(q22;q22). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. (b) Schematic diagram of the *AML1-ETO* transcript with *AML1* exon 5 fused to *ETO* exon 2. The numbers under the fusion gene transcript refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The arrows indicate the relative position of the five primers; the numbers refer to the 5′ nucleotide position of each primer (See Table 8).

Table 8 Primers for RT-PCR analysis of t(8;21)(q22;q22) with the *AML1-ETO* fusion gene

Primer code	*5′ Position[a] (size)*	*Sequence (5′–3′)*
AML1-A	884 (21)	CTACCGCAGCCATGAAGAACC
ETO-B	495 (21)	AGAGGAAGGCCCATTGCTGAA
AML1-C	920 (22)	ATGACCTCAGGTTTGTCGGTCG
ETO-D	396 (22)	TGAACTGGTTCTTGGAGCTCCT
AML1-E5′	842 (24)	TGGCTGGCAATGATGAAAACTACT

[a]See Table 2 for complete sequence information.

ETO fusion transcripts derived from variant breakpoints have not been described. Reciprocal *ETO-AML1* fusion transcripts have not been identified.

The *AML1-ETO* RT-PCR product is usually present as a single band but additional minor PCR products of variable intensity can be seen. Two groups have identified three alternatively spliced, out-of-frame variants consisting of additional small *ETO* alternative exons (of 46, 68 or 82 nucleotides in length) between *AML1* exon 5 and *ETO* exon 2.[85,86]

The t(8;21) is associated with a relatively good prognosis, with a particularly good response to certain therapeutic agents, notably cytosine arabinoside.[87,88] These observations have paved the way towards the use of risk-adapted therapy based on cytogenetic and/or molecular genetic assessment.

Cytogenetically, the t(8;21) represents nearly 7% of *de novo* AML, being more common in younger patients. It is most

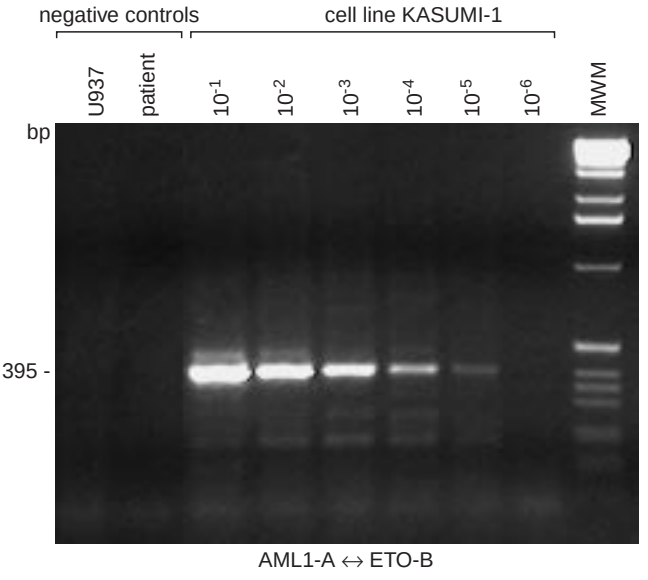

Figure 7 Ethidium bromide-stained agarose gel with a dilution experiment of RNA from the t(8;21)-positive cell line KASUMI-1 using the external A ↔ B primers. RNA from a t(8;21)-negative AML patient and from the cell line U937 were used as negative controls.

Table 9 Sizes of PCR products and log sensitivities of *AML1-ETO* primer sets in RT-PCR testing

	A ↔ B	*C ↔ D*	*A ↔ B + C ↔ D*	*E5′ ↔ D*
Sizes of PCR products in bp	395	260	260	338
KASUMI-1	–4	–4	–5	–4
Patients	–3	–4	–4	–3

frequent in the AML-M2 subtype, where it is found in 20–40% of cases.[83] It has also been described in rare cases of AML-M1 and AML-M4 and in therapy-related AML (t-AML). The latter may be related to the presence of a consensus topoisomerase II cleavage motif approximately 440 bp upstream of *AML1* exon 6, the same region which is involved in the fusion with the *ETO* gene.[89] The incidence of RT-PCR *AML1-ETO* positivity varies from 8 to 12% of AML,[83,84,90] thus representing a significant increase compared to, albeit historical, cytogenetic incidences.

The interest of *AML1-ETO* RT-PCR in the follow-up of AML patients is controversial. Certain groups have reported the persistence of *AML1-ETO* transcripts in long-term remission after allogeneic bone marrow transplantation (BMT) using nested PCR,[91] whereas others have suggested that residual positivity may have prognostic significance.[92] PCR negativity, including after nested PCR, has been reported.[93] The use of reproducible, quantitative RT-PCR will determine whether these differences are due to clinical or technical variables.

Results of PCR primer design and testing

Table 8 shows the position and sequence of each primer relative to the *AML1* and *ETO* genes. The three AML1-A, AML1-C and AML1-E5′ primers are located in *AML1* exon 4,

whereas the ETO-B primer is located in exon *ETO* 3 and the ETO-D primer in exon 2 (Figure 6b).

All three single PCR tests with A ↔ B, C ↔ D and E5′ ↔ D primers gave good sensitivities of 10^{-3} to 10^{-4}; the nested PCR reached a sensitivity of 10^{-4} to 10^{-5} (Figure 7 and Table 9).

SECTION 4. t(9;22) (q34;q11) with the *BCR-ABL* p190 fusion gene

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Background

The Philadelphia chromosome (Ph), besides being the hallmark of CML, also occurs in approximately 5% of childhood ALL and in 20–50% of adult ALL with an incidence progressively increasing with age.[94–96] Molecular analyses have established that the Ph translocation always results in the joining of 3′ sequences of the tyrosine kinase c-*ABL* proto-oncogene on chromosome 9 to the 5′ sequences of the *BCR* gene on chromosome 22.[13,97] Whereas the breakpoints on chromosome 9 are generally 5′ to *ABL* exon 2, the breakpoints on chromosome 22 differ in their position within the *BCR* gene, giving rise to fusion transcripts with different types of *BCR-ABL* junctions.[97]

In CML, the breakpoints on chromosome 22 are restricted to a central region of the *BCR* gene called 'major breakpoint cluster region' (M-*bcr*), leading to transcripts with different types of *BCR-ABL* junctions, dependent on the position of the breakpoint in *BCR* intron 13 or intron 14.[98,99] These fusion transcripts encode a BCR-ABL protein of 210 kDa, called $p210^{BCR/ABL}$ (see Section 5).

A second breakpoint cluster region in the *BCR* gene has been identified almost exclusively in Ph^+ ALL.[13] In fact, whereas approximately 40% of Ph^+ ALL show the same molecular rearrangements as in CML, in the remaining 60% of Ph^+ ALL the *BCR* breakpoints are located in the so-called 'minor breakpoint cluster region' (m-*bcr*) between the two alternative exons and exon 2 (Figure 8a). The breakpoints in the *ABL* gene are virtually all located in the large intron region (~200 kb) between *ABL* exon 1b and exon 2 (also called exon a2). As a consequence of the m-*bcr* breakpoints only the first exon of the *BCR* gene (also called exon e1) is joined to *ABL* exon 2 (e1–a2 junction) (Figure 8b).[13] This results in the production of a BCR-ABL protein of 190 kDa in molecular weight ($p190^{BCR/ABL}$).[100] Although the e1–a2 type of transcript has been mainly associated with ALL, sporadic cases of CML expressing only this type of transcript have also been reported.[101] In addition, it has been found that virtually all CML patients at diagnosis, besides the usual *BCR-ABL* p210 transcipts, through a mechanism of alternative splicing also express low amounts of e1–a2 transcripts, whose clinical and pathogenetic significance still waits to be elucidated.[102,103]

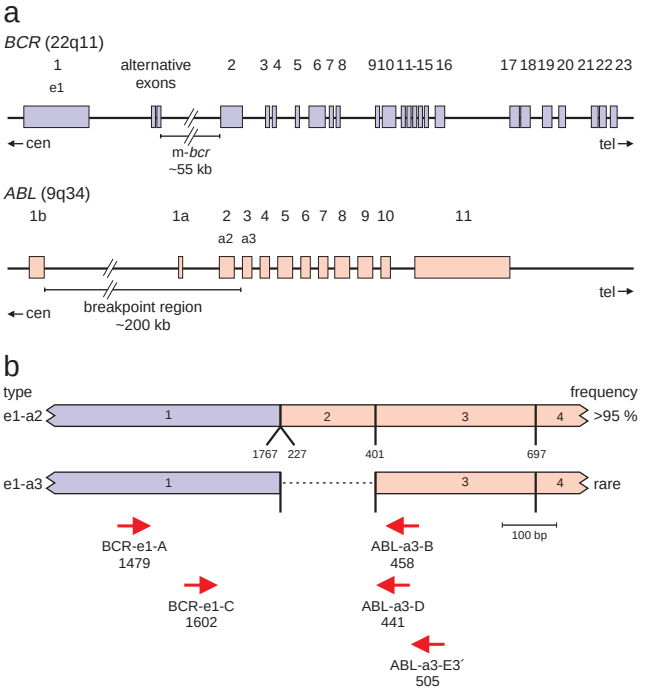

Figure 8 (a) Schematic diagram of the exon/intron structure of the *BCR* and *ABL* genes, involved in t(9;22)(q34;q11) with focus on the minor breakpoint cluster region (m-*bcr*). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. The old nomenclature for *BCR* exon 1 and *ABL* exons 2 and 3 is also indicated. (b) Schematic diagrams of the *BCR-ABL* p190 transcripts. The numbers under the fusion gene transcript refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The e1–a2 fusion transcript is found most frequently (>95%), but sporadic cases with e1–a3 transcripts have been reported (see Ref. 106). The arrows indicate the relative position of the primers; the three *ABL* primers are identical to those used for detection of *BCR-ABL* p210 transcripts (see Figure 10b). The numbers refer to the 5′ nucleotide position of each primer (See Table 10).

Table 10 Primers for RT-PCR analysis of t(9;22)(q34;q11) with the *BCR-ABL* p190 fusion gene

Primer code	*5′ Position*[a] *(size)*	*Sequence (5′–3′)*
BCR-e1-A	1479 (21)	GACTGCAGCTCCAATGAGAAC
ABL-a3-B	458 (21)	GTTTGGGCTTCACACCATTCC
BCR-e1-C	1602 (21)	CAGAACTCGCAACAGTCCTTC
ABL-a3-D	441 (23)	TTCCCCATTGTGATTATAGCCTA
ABL-a3-E3′	505 (23)	TGACTGGCGTGATGTAGTTGCTT

[a]See Table 2 for complete sequence information.

Detection of *BCR-ABL* transcripts in ALL patients

In ALL, the Ph-chromosome and consequently the *BCR-ABL* rearrangement is an independent unfavorable prognostic factor, which affects both hematological complete remission rate and probability of disease-free survival.[104–106] The poor results of the conventional treatment protocols have prompted the extensive use of more aggressive consolidation approaches, which include allogeneic or autologous transplantation with either bone marrow or peripheral blood stem cells.[107,108] In these treatment protocols, RT-PCR detection of *BCR-ABL* fusion transcripts certainly represents the method of choice for monitoring MRD during the follow-up.[109] The clinical validity

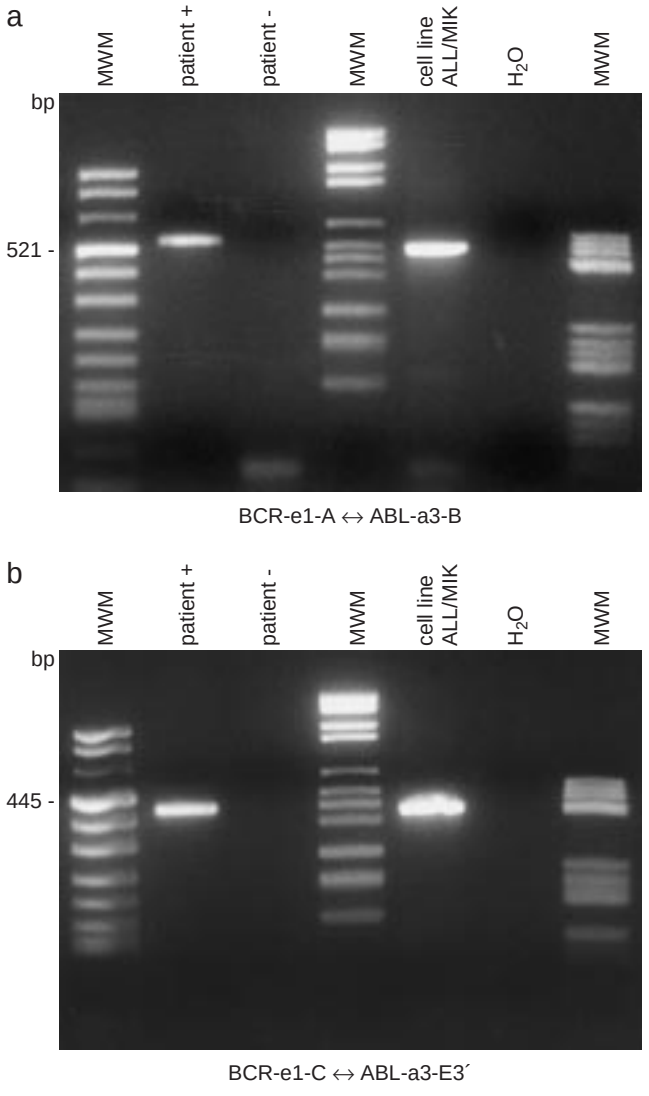

Figure 9 Ethidium bromide-stained agarose gels showing the positive PCR results with the external A ↔ B primers and 'shifted' C ↔ E3′ primers in a t(9;22)-positive patient and cell line ALL/MIK. A t(9;22)-negative ALL patient and H_2O were used as negative controls.

Table 11 Sizes of PCR products and log sensitivities of *BCR-ABL* p190 primer sets in RT-PCR testing

	A ↔ B	*C ↔ D*	*A ↔ B + C ↔ D*	*C ↔ E3′*
Sizes of PCR products in bp				
p190 e1-a2	521	381	381	445
p190 e1-a3[a]	347	207	207	271
TOM-1	–3	–3	–4	–3
ALL/MIK	–3	–3	–4	–2
Patients	–3	–4	–5	–3

[a]The rare p190 e1-a3 PCR product is generally caused by a breakpoint in *ABL* intron 2.

of this method in Ph+ ALL has been recently demonstrated for patients receiving BMT.[110] In these ALL patients the reappearance of RT-PCR positivity after BMT was significantly associated with the occurrence of a hematological relapse. Moreover, the possibility of identifying patients at high risk of relapse will eventually allow testing of the efficacy of new therapeutic strategies aiming at the decrease of post-transplant relapse rates, such as adoptive immunotherapy, antibody-based therapies, or interferon.

Results of PCR primer design and testing

Table 10 shows the position and sequence of each primer relative to the first exon of the *BCR* gene (e1) and the third exon of *ABL* gene (a3). The choice of reverse primers located on *ABL* exon 3 rather than on *ABL* exon 2, is based on the finding that in sporadic cases the *BCR-ABL* junction takes place between the *BCR* exon 1 and *ABL* exon 3 (also called exon a3) as a consequence of a breakpoint in *ABL* intron 2, resulting in the rare e1–a3 junction (Figure 8b).[106]

Primer BCR-e1-A and primer ABL-a3-B were used in a first round of PCR using the standardized RT-PCR protocol. The sensitivity reached at this stage was 10^{-3}. When a second round of amplification (nested PCR) was performed using the internal primers BCR-e1-C and ABL-a3-D, a reproducible sensitivity level of $10^{-4}/10^{-5}$ was obtained (Table 11). The C and D primers tested in a first round of RT-PCR showed a sensitivity of $10^{-3}/10^{-4}$. Finally, the shifted RT-PCR, employing ABL-a3-E3′ as reverse primer in conjunction with BCR-e1-C as forward primer, showed a sensitivity of $10^{-2}/10^{-3}$.

In all the reactions, after the first round of RT-PCR, the amplified products visualized on ethidium-bromide stained agarose gels showed only single bands of the expected molecular size (Figure 9 and Table 11). However, when testing patients expressing both p190 and p210 types of *BCR-ABL* transcripts, a very high molecular weight band (approximately 1800 bp) can be observed. This band corresponds to the amplification of the entire cDNA sequences included between the *BCR* exon 1 and *ABL* exon 3 in the p210 types of transcripts. Finally, a band of approximately 500 bp can be observed on the gel after the nested RT-PCR reaction, representing the amplified product derived from the first round of RT-PCR (521 bp).

SECTION 5. t(9;22)(q34;q11) with the *BCR-ABL* p210 fusion gene

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Background

The molecular consequence of t(9;22)(q34;q11) is the formation of two hybrid genes: *BCR-ABL* on the Ph chromosome and *ABL-BCR* on 9q+.[12,32] The *BCR-ABL* fusion gene encodes a protein with elevated tyrosine kinase activity which seems to exert its effects by interfering with cellular signal transduction

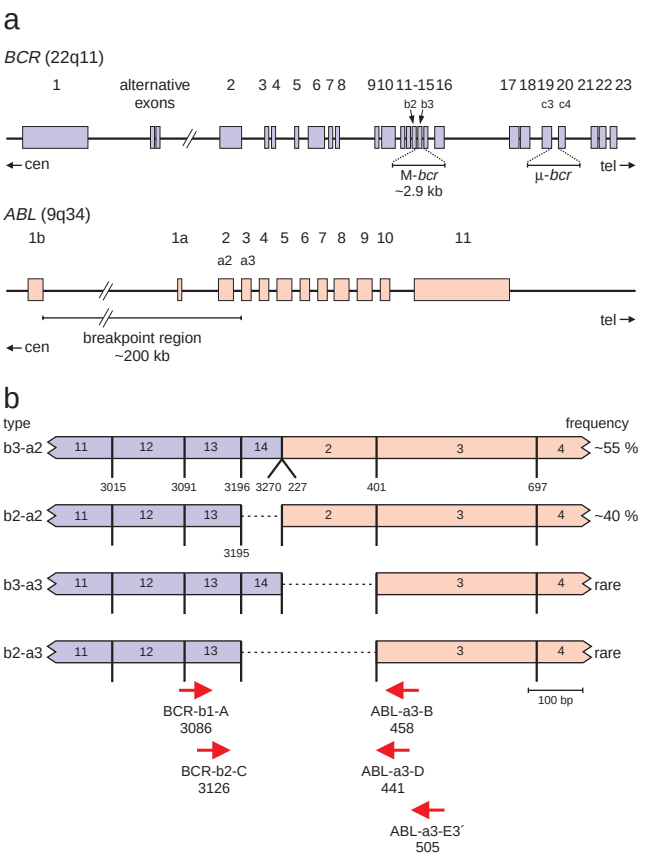

Figure 10 (a) Schematic diagram of the exon/intron structure of the *BCR* and *ABL* genes, involved in t(9;22)(q34;q11) with focus on the major breakpoint cluster region (M-*bcr*). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated, including the micro breakpoint cluster region (*μ-bcr*). The old nomenclature of the *BCR* and *ABL* exons is partly indicated. (b) Schematic diagram of the *BCR-ABL* p210 transcripts. The numbers under the fusion gene transcripts refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The b3–a2 and b2–a2 transcripts are found most frequently, but sporadic cases with b3–a3 and b2–a3 transcripts have been reported. The arrows indicate the relative position of the primers; the three *ABL* primers are identical to those used for detection of *BCR-ABL* p190 transcripts (see Figure 8b). The numbers refer to the 5′ nucleotide position of each primer (see Table 12).

Table 12 Primers for RT-PCR analysis of t(9;22)(q34;q11) with the *BCR-ABL* p210 fusion gene

Primer code	*5′ Position*[a] *(size)*	*Sequence (5′–3′)*
BCR-b1-A	3086 (22)	GAAGTGTTTCAGAAGCTTCTCC
ABL-a3-B	458 (21)	GTTTGGGCTTCACACCATTCC
BCR-b2-C	3126 (21)	CAGATGCTGACCAACTCGTGT
ABL-a3-D	441 (23)	TTCCCCATTGTGATTATAGCCTA
ABL-a3-E3′	505 (23)	TGACTGGCGTGATGTAGTTGCTT

[a]See Table 2 for complete sequence information.

pathways normally involved in the control of cell death and proliferation and cell–cell adhesion, involving domains from both BCR and ABL proteins.[111] The reciprocal *ABL-BCR* fusion gene has been studied less extensively, but some studies show that over two-thirds of CML patients produce *ABL-BCR* mRNA. The role of *ABL-BCR*, if any, remains unclear. More than 95% of CML patients have a *BCR-ABL* gene in their leukemic cells. However, it is not exclusive to CML, since it is found in about 30% (20–50%) of adult ALL and in 2–10% of childhood ALL, as well as in occasional cases of AML (<2%), lymphoma and myeloma.[19,95–97,112–116]

The BCR-ABL fusion protein can vary from 190 kDa to 230 kDa, depending on the site of the breakpoint in the *BCR* gene. This diversity seems to be correlated with the leukemia phenotype and aggressiveness.[106,112] In almost all CML as well as in 30–50% of adult Ph+ ALL and a small proportion (20–30%) of childhood Ph+ ALL, the breakpoints in the *BCR* gene are found within the M-*bcr* region.[113,117,118] Initially this was defined as a 5.8 kb region spanning exons 12 to 16 (originally called b1 to b5) of the *BCR* gene.[98] Vitually all CML patients have their breakpoint in the 2.9 kb region between *BCR* exons 13 and 15 (also called exons b2 and b4), that fuses to the large intron between *ABL* exons 1b and 2 (Figure 10a).[119]This results in a hybrid *BCR-ABL* transcript of 8.5 kb containing either *BCR* exon b2 or b3 and *ABL* exon 2 (also called exon a2). This mRNA encodes the 210 kDa BCR-ABL protein ($p210^{BCR/ABL}$). The majority of CML patients have transcripts with the b3–a2 (55%) or b2–a2 (40%) junctions (Figure 10b).[120,121] In 5% of cases, both b3–a2 and b2–a2 transcripts can be formed as a result of alternative splicing.[112,120,121] So far, no clear difference in clinical outcome has been reported between the two *BCR-ABL* p210 transcripts.[112,120]

Occasionally in CML patients other breakpoints both in *BCR* and *ABL* genes have been described. In very rare cases of Ph+ CML (<10 cases reported), the breakpoint in the *BCR* gene involves the ALL-associated m-*bcr* region, which results in the exclusive production of the smaller $p190^{BCR/ABL}$ fusion protein (see Section 4).[101] Clinically p190+ CML patients present with a prominent relative and absolute monocytosis and have clinical findings intermediate between CML and chronic myelomonocytic leukemia.[122]

A very small proportion of Ph+ CML patients displays a larger *BCR-ABL* fusion transcript that results from a fusion between *BCR* exon 19 (originally named c3) and *ABL* exon 2. This is caused by breakpoints in the micro breakpoint cluster region (*μ-bcr*) between *BCR* exon 19 and 20 (Figure 10a). This *BCR-ABL* fusion gene encodes a large 230 kDa BCR-ABL protein that seems to disrupt the normal process of granulocytic differentiation leading to a disorder characterized by an indolent clinical course in most cases.[123] In addition, some sporadic Ph+ and Ph− CML with *BCR* breakpoints outside the three previously commented cluster regions have been reported, involving several other introns (2, 5, 6, 8 and 10).[112] Finally, in rare Ph+ CML cases and also in some Ph+ ALL (<5%), the *BCR* sequences in the hybrid *BCR-ABL* transcript are fused to *ABL* exon a3 instead of a2 (Figure 8b and Figure 10b).[106]

Detection of *BCR-ABL* transcripts in CML patients

The use of RT-PCR for detection of *BCR-ABL* transcripts is possible in virtually all CML (>95%), even in the cases missed by standard cytogenetics (up to 10% of cases).[124] In most of the remaining CML cases the underlying molecular events still have to be discovered. RT-PCR is a useful tool for detection of MRD during follow-up.[125] Since 1989 several MRD-PCR studies have been performed, mainly in CML patients who underwent allogeneic BMT in order to evaluate the incidence and significance of PCR-positive cells.[126–129] The majority of these studies found that negative PCR results clearly predict a

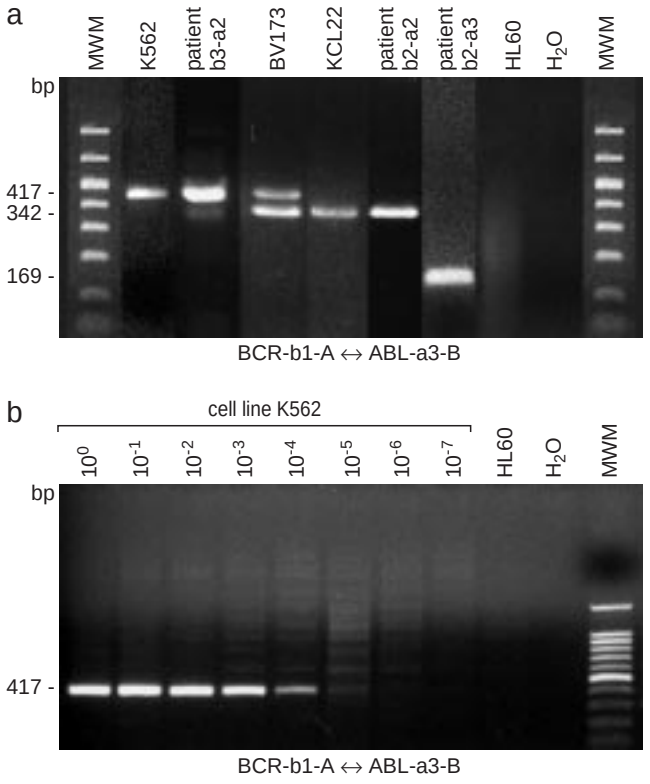

Figure 11 Ethidium bromide-stained agarose gel with several variants of *BCR-ABL* p210 transcripts as detected with the external A ↔ B primers (a). This concerns the b3–a2 transcripts in cell line K562 and a patient, the b2–a2 transcripts in cell line KCL22 and a patient, and cell line BV 173 with both transcripts. Finally, a patient with the rare b2–a3 transcript is presented. Results of a dilution experiment with RNA from the cell line K562 using the A ↔ B primer set (b).

Table 13 Sizes of PCR products and log sensitivities of *BCR-ABL* p210 primer sets in RT-PCR testing

	A ↔ B	*C ↔ D*	*A ↔ B + C ↔ D*	*C ↔ E3′*
Sizes of PCR products in bp				
p210 b3–a2	417	360	360	424
p210 b2–a2	342	285	285	349
p210 b3–a3	243	186	186	250
p210 b2–a3	168	111	111	175
BV173 (b2–a2 and b3-a2)	–4	–4	–6	–4
KCL22 (b2–a2)	–4	–4	–5	–4
Patients (b2–a2)	–3	–4	–4	–3
K562 (b3–a2)	–4	–4	–5	–4
LAMA-84 (b3–a2)	–3	–3	–5	–3
Patients (b3–a2)	–3	–3	–5	–3

favorable outcome, but a positive result is difficult to interpret. While most patients (>75%) are PCR positive with no clear clinical impact during the first 6 months post-BMT, PCR positivity seems to be a good marker of relapse risk 6 months to 1 year post-BMT (20–40% of patients).[128] In half of the cases, PCR negativity seems to correlate to graft-versus-host disease and withdrawal of immunosuppressive treatment.[127] In the late post-transplant period, the existence of PCR-positive cells again does not correlate with relapse, since PCR-positive cells have been observed even several years after BMT without any sign of disease progression, which may suggest the establishment of an equilibrium between these cells and the immune system.[129] The limitations of qualitative PCR for predicting relapse in individual patients has initiated the development of quantitative PCR (Q-PCR) assays that allow monitoring of *BCR-ABL* transcript levels. It has been suggested that Q-PCR can be regarded as an early predictor of outcome and that clinical decisions can be based on Q-PCR results after allogeneic transplant[130] or during interferon treatment.[131] This is particularly important in patient candidates for donor-lymphocyte infusion since, apparently, response correlates with residual tumor burden.[132,133]

Results of PCR primer design and testing

Five primers were designed for *BCR-ABL*, p210. Their position and location in the *BCR* and *ABL* genes as well as their sequences are given in Table 12 and Figure 10b. In order to detect the rare *BCR-ABL* variants b3–a3 and b2–a3, the initial primers in *ABL* exon a2 were substituted with primers in the *ABL* exon a3 (B, D and E3′ primers). In fact, the same three *ABL* primers are used for detection of both *BCR-ABL* p210 and *BCR-ABL* p190, ie the M-*bcr* and m-*bcr* variants, respectively (Figures 8b and 10b).

The sizes of PCR products obtained according to the different types of M-*bcr* breakpoints (b3–a2, b2–a2, b3–a3 and b2–a3) and the different primer pairs are given in Table 13 and partly illustrated in Figure 11a. The sensitivity of the different primer combinations and PCR strategies is also shown in Table 13. When a single PCR was used, the sensitivity ranged between 10^{-3} and 10^{-4}, either with the use of external (A ↔ B) or internal (C ↔ D) primers as well as the 'shifted' primer set (C ↔ E3′) (Figure 11b). This sensitivity increased to $10^{-5}/10^{-6}$ when nested PCR was performed (Table 13). In all cases, the sensitivity was independent of the type of transcript present in the translocation (b2–a2 or b3–a2).

SECTION 6. t(12;21)(p13;q22) with the *TEL-AML1* fusion gene

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Background

The t(12;21)(p13;q22) was first reported by two different groups in 1995.[33,34] Various later studies demonstrated that this translocation, not detectable by conventional cytogenetics, constitutes the most frequent rearrangement in childhood ALL. It occurs in approximately 25% of childhood ALL.[23,24,34,134,135] The majority of positive patients range in age between 1 and 12 years at diagnosis, with a peak between 2 and 5 years; all display a precursor-B cell immunophenotype,

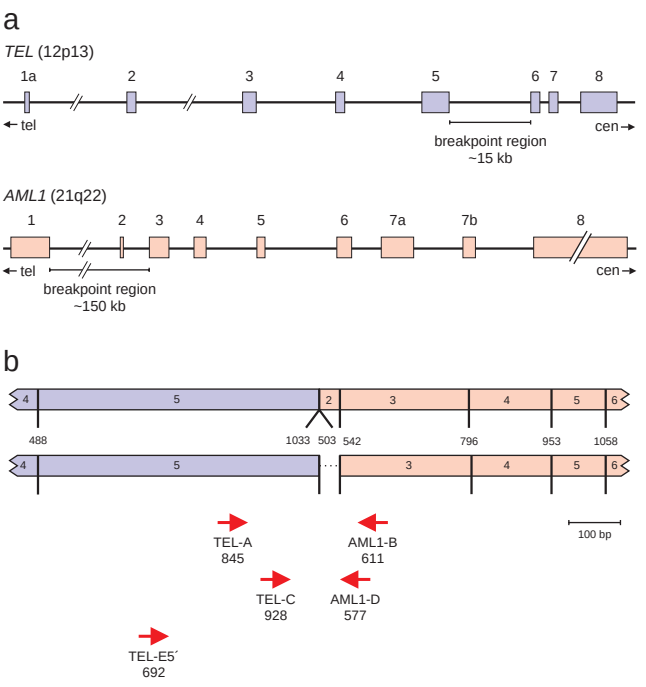

Figure 12 (a) Schematic diagram of the exon/intron structure of the *TEL* and *AML1* genes, involved in t(12;21)(p13;q22). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. (b) Schematic diagram of the *TEL-AML1* fusion transcripts. The numbers under the fusion gene transcripts refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. Most t(12;21)-positive patients have the larger transcript because of a breakpoint in *AML1* intron 1, but alternative splicing can cause skipping of *AML1* exon 2, leading to two PCR products in some patients (see text). In a minority of patients the *AML1* breakpoint is located in intron 2, resulting in a shorter transcript without *AML1* exon 2. The arrows indicate the relative position of the five primers; the numbers refer to the 5′ nucleotide position of each primer (see Table 14).

Table 14 Primers for RT-PCR analysis of t(12;21)(p13;q22) with the *TEL-AML1* fusion gene

Primer code	*5′ Position*[a] *(size)*	*Sequence (5′–3′)*
TEL-A	845 (20)	TGCACCCTCTGATCCTGAAC
AML1-B	611 (19)	AACGCCTCGCTCATCTTGC
TEL-C	928 (22)	AAGCCCATCAACCTCTCTCATC
AML1-D	577 (18)	TGGAAGGCGGCGTGAAGC
TEL-E5′	692 (20)	CGCACCAGGAGAACAACCAC

[a]See Table 2 for complete sequence information.

in particular common ALL and pre-B-ALL, more rarely pro-B-ALL. In addition, these patients are characterized by low WBC count at diagnosis (<50 000/l). Interestingly, the vast majority of patients have a non-hyperdiploidy DNA content (DNA index = 1), most have coexpression of myeloid markers[24] and the majority (70–80%) show deletion of the non-rearranged *TEL* allele.[136,137] So far, t(12;21) has not been found in T-ALL or AML. It has never been described in infant leukemias (age less then 1 year) and the frequency in adult leukemic patients is low (<2%).[138,139]

Several studies reported favorable outcomes for t(12;21)-positive patients,[23,24] in both retrospective studies and prospective studies with relatively short follow-up (reviewed in

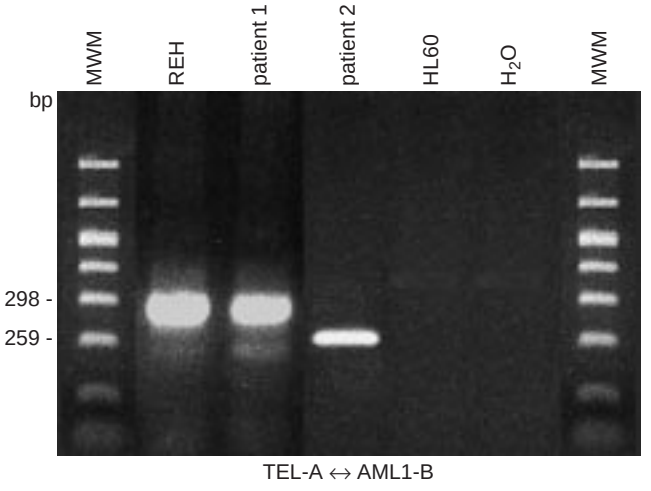

Figure 13 Ethidium bromide-stained agarose gel showing the two types of *TEL-AML1* transcripts in cell line REH and two patients using the external A ↔ B primers. Patient 1 contains both transcripts: the lower (faint) band is derived from the alternative splice variant (see Figure 12).

Table 15 Sizes of PCR products and log sensitivities of *TEL-AML1* primer sets in RT-PCR testing

	A ↔ B	*C ↔ D*	*A ↔ B + C ↔ D*	*E5′ ↔ D*
Sizes of PCR products in bp				
standard	298	181	181	417
variant (–39 bp)[a]	259	142	142	378
REH	–3	–3	–4	–3
Patients	–2	–3	–3	–4

[a]This variant can be caused by alternative splicing or by alternative breakpoints in *AML* intron 2.

Ref. 140). However, analyses of patients with relapsed ALL have shown that the frequency of this translocation is similar to that at diagnosis.[141,142] Nevertheless, the t(12;21)-positive patients retain a good prognosis and seem to have a more delayed time of relapse.[141,142] By contrast, other groups reported a very low incidence in relapsed ALL.[21,140,143] This apparent discrepancy must be clarified by larger prospective studies with a long follow-up.

As shown in Figure 12a, t(12;21) involves the *TEL/ETV6* gene on chromosome 12 and the *AML1/CBFA2* gene on chromosome 21.[33,34,144,145] The *AML1* gene is also involved in t(8;21), as described in Section 3 (Figure 6). The chromosome 12 region where the *TEL* gene is located is completely sequenced and available in the database (Table 2). The *TEL* gene is very large and consists of eight exons, but the breakpoints cluster in a 15 kb region between exons 5 and 6 (Figure 12a).[146] Only two cases have been described as having a breakpoint in intron 4,[24,134] which would be missed with the PCR analysis described here. The genomic organization of the *AML1* gene has not yet been completely unravelled,[147] and the breakpoints can occur either in the very large intron 1 (most frequently) or in intron 2. In most cases, the *TEL-AML1* fusion transcript shows a joining of exon 5 (nucleotide 1033) of *TEL* to the second exon (nucleotide 503) of *AML1* (Figure

12b). Alternative splicing causes the skipping of *AML1* exon 2 (39 bp) in a minority of transcripts, resulting in two PCR bands from the same patient. Less frequently, the breakpoint occurs in *AML1* intron 2, also resulting in the junction of *TEL* exon 5 to the third exon of *AML1* (Figure 12b). However, the two derivative fusion proteins retain the same frame and structure.

Results of PCR primer design and testing

Table 14 shows the position and sequence of each primer relative to the *TEL* and *AML1* mRNA. Primer TEL-A, located on *TEL* exon 5, and primer AML1-B, located on *AML1* exon 3, were used in a first cycle PCR using the standardized RT-PCR protocol. This first round PCR allowed a sensitivity level to be reached of 10^{-3}, when RNA of the REH control cell line was used and 10^{-2} using RNA from ALL patients. When a second round of amplification (nested PCR) was performed using the internal primers TEL-C and AML1-D, a reproducible sensitivity level of 10^{-4} or 10^{-3} was obtained in the REH cell line or patients, respectively (Table 15). The TEL-C and AML1-D primers (generally only used in nested PCR), tested in a first round PCR, allowed a sensitivity of 10^{-3} to be reached in both REH and patient RNA. Finally, the 'shifted' PCR with the TEL-E5′ and AML1-D primers showed a sensitivity of 10^{-3} in cell line REH and 10^{-4} in patient RNA. The PCR products obtained with A ↔ B primers can show the following patterns in agarose gels:

- one PCR band of 298 bp in case of a breakpoint in *AML1*-intron 1 (cell line REH in Figure 13);
- two PCR bands of 298 bp and 259 bp (less intense) in case of a breakpoint in *AML1* intron 1 and an alternative splicing variant lacking *AML1* exon 2, respectively (patient 1 in Figure 13);
- only one band of 259 bp in case of a breakpoint in *AML1* intron 2 (patient 2 in Figure 13).

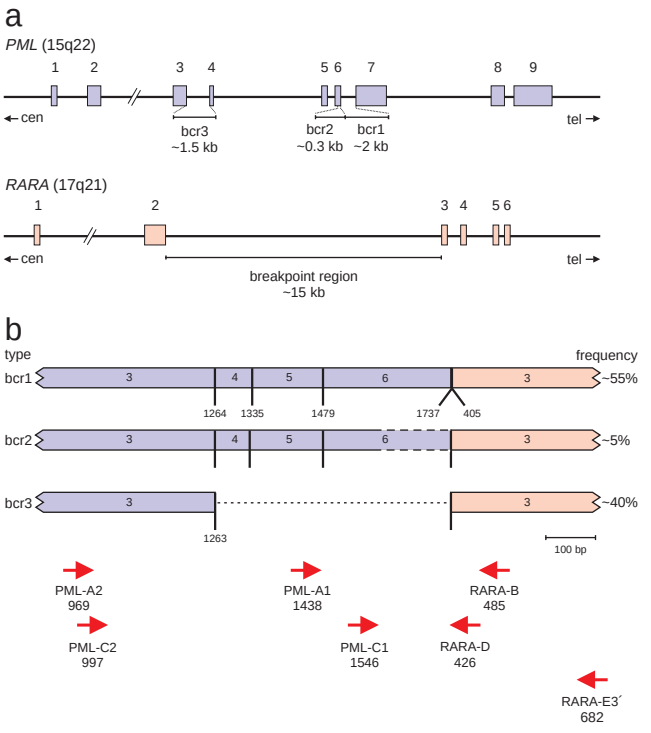

Figure 14 (a) Schematic diagram of the exon/intron structure of the *PML* and *RARA* genes, involved in t(15;17)(q22;q21)**.** The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. The bcr1 and bcr2 breakpoint regions are juxtaposed in intron 6 and exon 6, respectively. (b) Schematic diagram of the three types of *PML-RARA* transcripts, related to the different *PML* breakpoint regions. The size of the bcr2 transcript is dependent on the position of the breakpoint in *PML* exon 6. The numbers under the fusion gene transcripts refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The seven arrows indicate the relative position of the primers; the numbers refer to the 5′ nucleotide position of each primer (see Table 16).

Table 16 Primers for RT-PCR analysis of t(15;17)(q22;q21) with the *PML-RARA* fusion gene

Primer code	*5′ Position[a] (size)*	*Sequence (5′–3′)*
PML-A1	1438 (21)	CAGTGTACGCCTTCTCCATCA
PML-A2	969 (18)	CTGCTGGAGGCTGTGGAC
RARA-B	485 (20)	GCTTGTAGATGCGGGGTAGA
PML-C1	1546 (21)	TCAAGATGGAGTCTGAGGAGG
PML-C2	997 (19)	AGCGCGACTACGAGGAGAT
RARA-D	426 (20)	CTGCTGCTCTGGGTCTCAAT
RARA-E3′	682 (20)	GCCCACTTCAAAGCACTTCT

[a]See Table 2 for complete sequence information.

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

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Background

The t(15;17) is associated with acute promyelocytic leukemia (APL), a distinct AML subset with M3 cytomorphology.[148] The chromosomal breakpoints regions have been variously mapped to 15q22-q24 and 17q11-q21. The chromosomal break sites were isolated by four groups using distinct experimental approaches.[149–152] The two genes involved in t(15;17) are *PML*, coding for a putative novel transcription factor, on chromosome 15[149–152] and the retinoic acid receptor-α (*RARA*) gene on chromosome 17.[153,154] The chromosome 17 breakpoints are localized within a 15 kb DNA fragment of the *RARA* intron 2 (Figure 14a). By contrast, three regions of the *PML* locus are involved in the translocation breakpoints: intron 6 (bcr1; 55% of cases), exon 6 (bcr2; 5%), and intron 3 (bcr3; 40%). Chimeric *PML-RARA* and *RARA-PML* transcripts are formed as a consequence of the reciprocal translocation between the *PML* and *RARA* loci. The existence of different breakpoint regions in the *PML* locus and the presence of alternative splicing of *PML* transcripts are responsible for

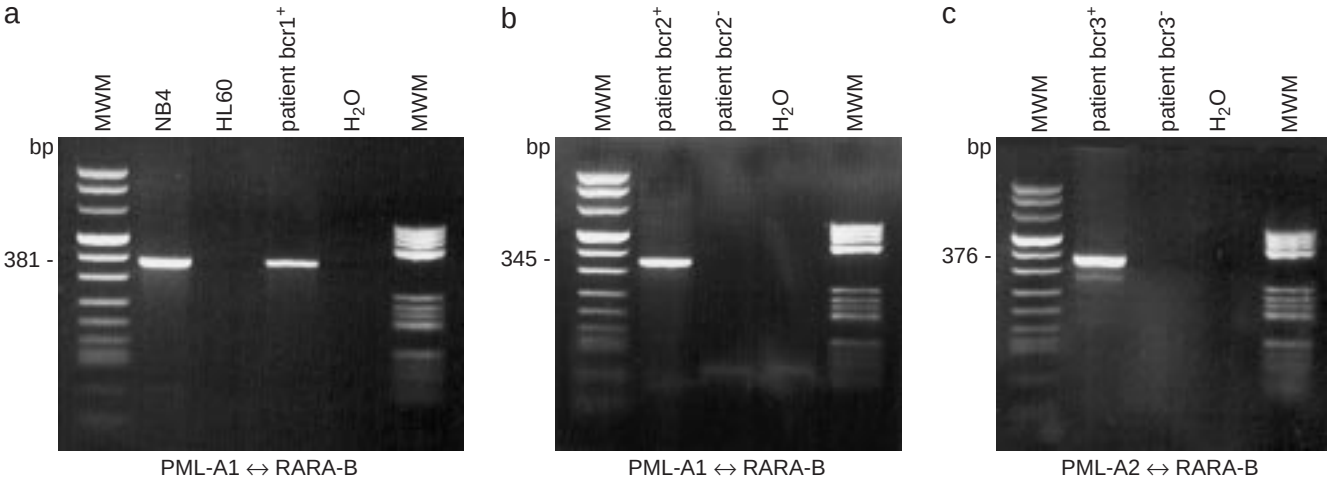

Figure 15 Ethidium bromide-stained agarose gels showing PCR products, derived from the three types of *PML-RARA* fusion genes using the external A ↔ B primers. RNA from t(15;17)-negative patients, the HL60 cell line, and H_2O were used as negative controls.

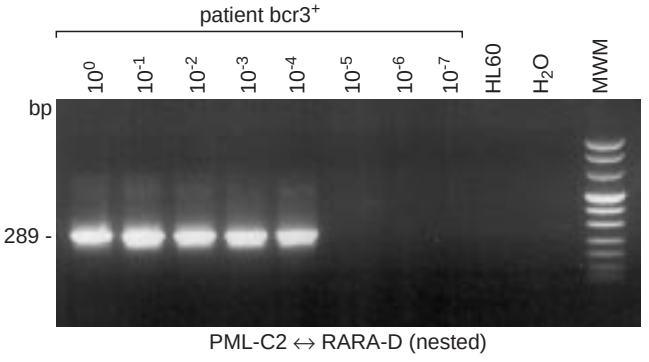

Figure 16 Ethidium bromide-stained agarose gel showing a dilution experiment with RNA from a t(15;17)-positive patient with bcr3 type *PML-RARA* transcripts. A sensitivity of 10^{-4} was reached with the nested PCR approach.

the great heterogeneity of *PML-RARA* junctions observed among APL patients (reviewed in Ref. 155). Moreover, the alternative usage of two *RARA* polyadenylation sites generates extra *PML-RARA* transcripts of different size.[155] A set of primers has been initially defined that allowed the amplification of *PML-RARA* junctions in all APL patients.[156] The observation that *RARA-PML* transcripts are present in most but not all APL cases, has favored the use of *PML-RARA* transcripts as PCR target for detection of APL cells at diagnosis and during monitoring.

Several studies attempted to correlate the type of *PML-RARA* transcript either with clinico–biologic features at diagnosis or with treatment response and outcome (reviewed in Ref. 157). The vast majority of analyzed series compared the two major *PML-RARA* isoforms, referred to as long (L) transcripts (including *PML* bcr1 and bcr2) and short (S) transcripts (*PML* bcr3). Because bcr2 (also referred to as 'variant' or V form) and bcr1 are located in *PML* exon 6 and intron 6, respectively, sequencing of all L transcript cases would be needed to clearly distinguish these two isoforms. Such distinction is usually not reported in clinical studies with a large number of patients.[157] At diagnosis no correlations were found with respect to sex, platelet count, presence of coagulopathy or retinoic syndrome, when comparing patients with L-type or S-type *PML-RARA* transcripts.[157] However, patients with S-type transcripts had significantly higher white blood cell counts and more frequently M3v morphology. Although S-type transcripts correlated with established adverse prognostic features (ie hyperleucocytosis, M3v), this association did not translate into poorer outcome as compared to patients with L-

Table 17 Sizes of PCR products and log sensitivities of *PML-RARA* primer sets in RT-PCR testing

	A1 ↔ B	*A2 ↔ B*	*C1 ↔ D*	*C2 ↔ D*	*A1 ↔ B + C1 ↔ D*	*A2 ↔ B + C2 ↔ D*	*C1 ↔ E3′*	*C2 ↔ E3′*
Sizes of PCR products in bp								
bcr1	381	(1329)	214	(688)	214	(688)	470	(944)
bcr2	345[a]	(819)[a]	178[a]	(652)[a]	178[a]	(652)[a]	434[a]	(908)[a]
bcr3	—	376	—	289	—	289	—	545
NB4	–3	NA[b]	–3	NA	–4	NA	–2	NA
Patients	–2	–2	–3	–2	–3	–4	–1	–2

[a]The sizes of the PCR products in bcr2+ APL patients are variable due to variable breakpoint positions in exon 6 of the *PML* gene.
[b]NA: not applicable.

type transcripts, in the context of combined ATRA and chemotherapy regimens.

Results of the PCR primer design and testing

In order to cover all three breakpoints in the *PML* gene, two extra forward primers were designed. Primer sets A1 ↔ B and C1 ↔ D cover the bcr1 and bcr2 breakpoints, whereas primer sets A2 ↔ B and C2 ↔ D can detect *PML-RARA* transcripts derived from *PML* breakpoints in bcr3 (Figures 14 and 15). The precise sequence information and relative position of the seven primers is given in Table 16. Table 17 summarizes the expected sizes of the PCR products per type of breakpoint cluster and per primer set. It should be noted that the size of the PCR products varies in case of bcr2$^+$ patients, because of the variable breakpoint positions in exon 6 of the *PML* gene.

The sensitivity tests were performed with RNA obtained from APL patients as well as with RNA from the NB4 cell line, which has the *PML* breakpoint in the bcr1 region. The positive RNAs were serially diluted in negative RNA obtained from the HL60 cell line.

A relatively high sensitivity of 10^{-4} was achieved with the nested amplification of the bcr1 transcripts obtained from the NB4 cell line and the bcr3 transcripts of APL patients (Figure 16). The bcr1 nested amplification was one log less sensitive (10^{-3}) when using RNA from positive patients (Table 17).

Single PCR was able to detect the different types of *PML-RARA* transcripts but with at least one log less sensitivity compared to the nested PCR: 10^{-2} when using RNA from positive patients and 10^{-3} with RNA from the NB4 cell line. A similar result was obtained with the nested primers alone, except for bcr1 transcripts, which could be detected at dilution levels of 10^{-3}, implying that the C1 ↔ D primer set is very efficient. The 'shifted' PCR was equally sensitive in detecting the bcr1 transcripts of the NB4 cell line and the bcr3 transcripts of patients, but gave a log sensitivity of 10^{-1} when amplifying the bcr1 transcripts of APL patients (Table 17).

No extra bands are usually found in PCR products run in 1.5% agarose gels and visualized with ethidium-bromide staining. However, multiple bands can appear in PCR products from bcr1$^+$ patients, if primers are used for bcr3 breakpoint identification. These extra bands are caused by alternative splicing of *PML* exons.[155]

SECTION 8. inv(16)(p13;q22) with the *CBFB-MYH11* fusion gene

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Background

A del(16)(q22) was initially reported in AML in 1982. This was rapidly shown to correspond to a pericentric inversion of chromosome 16, ie inv(16)(p13q22), which appeared to be associated with AML-M4 with abnormal eosinophils (M4Eo). The t(16;16)(p13;q22) was identified as a variant aberration. The inv(16)(p13q22) and t(16;16)(p13;q22) fuse the *CBFB* gene (core binding factor β subunit), also identified as *PEBP2b* (polyoma enhancer binding protein 2β), located on chromosome 16q22 to the *MYH11* gene (myosin heavy chain 11

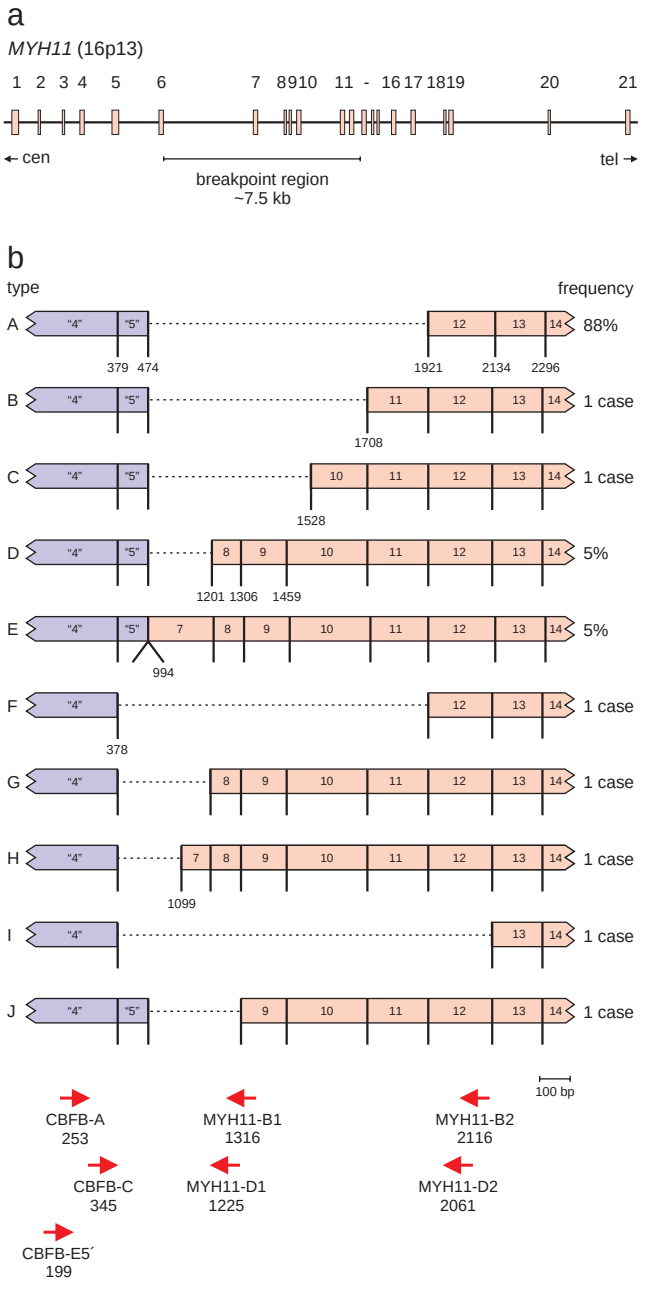

Figure 17 (a) Schematic diagram of the exon/intron structure of the *MYH11* gene, which is involved in inv(16)(p13;q22). The centromere (cen) and telomere (tel) orientation, exon numbering, and the breakpoint region are indicated. (b) Schematic diagram of the 10 different types of *CBFB-MYH11* fusion transcripts. The numbers under the fusion gene transcripts refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The different types of transcripts are mainly caused by breakpoints in different introns of the *MYH11* gene. Fusion transcripts type A, D and E together represent approximately 98% of all patients, while the other types concern single cases. The seven arrows indicate the relative position of the primers; the numbers refer to the 5′ nucleotide position of each primer (see Table 18).

Table 18 Primers for RT-PCR analysis of inv(16)(p13;q22) with the *CBFB-MYH11* fusion gene

Primer code	*5′ Position[a] (size)*	*Sequence (5′–3′)*
CBFB-A	253 (22)	GCAGGCAAGGTATATTTGAAGG
MYH11-B1	1316 (20)	TGAAGCAACTCCTGGGTGTC
MYH11-B2	2116 (22)	TCCTCTTCTCCTCATTCTGCTC
CBFB-C	345 (20)	GGGCTGTCTGGAGTTTGATG
MYH11-D1	1225 (20)	TCCCTGTGACGCTCTCAACT
MYH11-D2	2061 (18)	CTTGAGCGCCTGCATGTT
CBFB-E5′	199 (21)	CAGGGAGAACAGCGACAAACA

[a]See Table 2 for complete sequence information.

gene), whose protein product is also identified as SMMHC (smooth muscle myosin heavy chain) and is located on chromosome 16p13.[37] The inv(16)(p13q22) has been described in the ME-1 cell line (*CBFB-MYH11* type A transcript).[158]

The genomic structure of the human *CBFB* gene is not published. A putative map can be derived from the genomic map of the murine *CBFB* gene: the majority of breakpoints are located in a 15 kb intron between 'exon 5' and 'exon 6', here named intron 5. The 5′ to 3′ orientation is centromere to telomere. CBFB protein expression is ubiquitous and forms a heterodimeric transcription factor together with CBFA1, CBFA2 (called AML1; see Section 3), or CBFA3.

The *MYH11* gene is composed of 21 exons spanning 37 kb (Figure 17a). The 5′ region of the *MYH11* gene is frequently deleted during the inversion process. By consequence, only the *CBFB-MYH11* fusion gene is reproducibly expressed in cases of inv(16)(p13q22).

Ten different *CBFB-MYH11* fusion transcripts have been reported. The nomenclature used in this report is derived and updated from the review by Liu *et al*.[159] More than 85% of the positive patients have type A transcript; two other transcripts (D and E) represent nearly 5% each, whereas all others represent unique cases.[160–163] The relative incidences given in Figure 17b for types A, D and E are based on a literature compilation of approximately 200 reported *CBFB-MYH11* fusion transcripts. Two different classes of breakpoints can be seen in the *CBFB* gene. Due to the absence of a human *CBFB* genomic map, the two classes will be referred to as the HUMCBFB nucleotide 378 (also referred to as 399), whose breakpoints are probably located in intron 4, and HUMCBFB nucleotide 474 (also referred to as 495), whose breakpoints are probably located in intron 5. The latter breakpoints occur in nearly 99% of cases. Breakpoint heterogeneity is much more marked within the *MYH11* gene, since seven different exons (from exons 7 to 13) are variably included in fusion transcripts, as indicated in Figure 17b. All fusion breakpoints other than types C and H occur at exon boundaries.

The inv(16)(p13q22) is generally associated with a relatively good prognosis,[164,165] although this is not universal.[166] The subtle nature of the karyotypic changes in inv(16) render their cytogenetic detection difficult, particularly after R banding or when their presence is not suspected from morphological findings. For these reasons, *CBFB-MYH11* RT-PCR positivity but inv(16) negativity is not uncommon.[167–169] *CBFB-MYH11* transcripts are found in nearly 10% of *de novo* AML.[170] Although most (~50%) of these leukemias correspond to AML-M4Eo, *CBFB-MYH11* transcripts have also been found in many other AML types, including M4 without eosinophilic abnormalities, M2, M5 and, less frequently, M1, M6 and M7.[170] The inv(16)(p13q22) is also found in rare cases of CML in blast crisis, myelodysplastic syndromes, and therapy-related AML.[171,172]

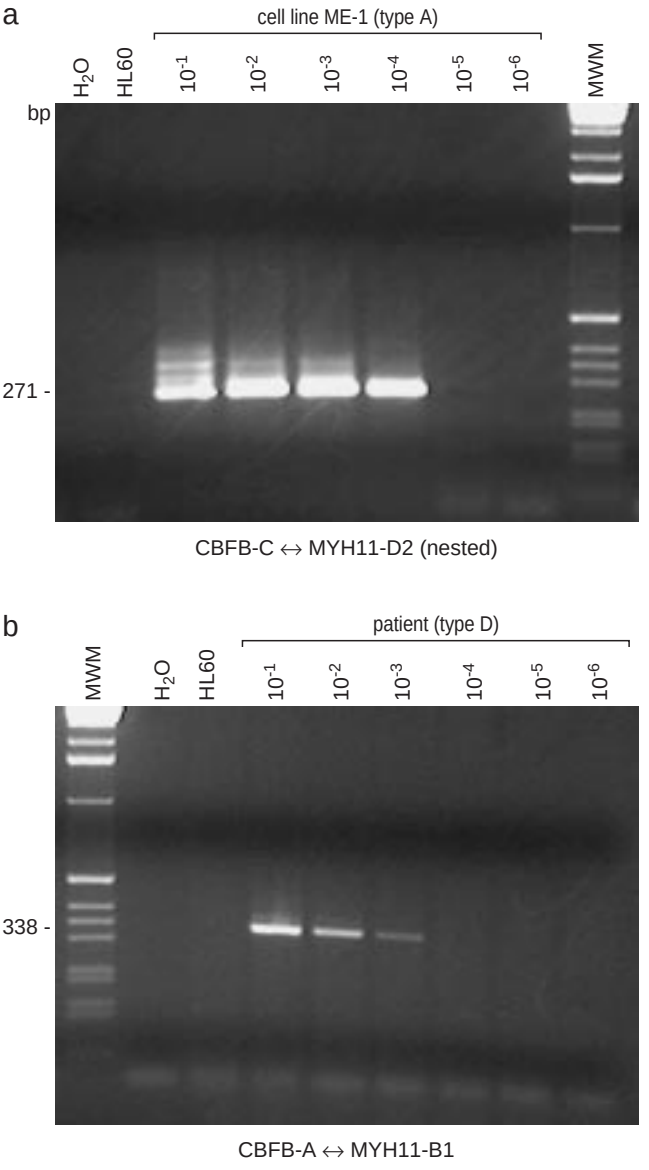

Figure 18 Ethidium bromide-stained agarose gels showing a dilution experiment of the ME-1 cell line (*CBFB-MYH11* type A transcripts) with the nested PCR approach (a) and a dilution experiment of an inv(16)-positive patient (*CBFB-MYH11* type D transcripts) with external A ↔ B primers (b).

The value of detection of *CBFB-MYH11* transcripts during follow-up is difficult to assess due to limited numbers of patients analyzed in each series. One group detected the persistence of *CBFB-MYH11* in long-term remission after allogeneic BMT using nested PCR.[173] In contrast, others have reported the disappearance of *CBFB-MYH11* transcripts in patients with long-term follow-up.[174–178] It will be important to determine the clinical value of *CBFB-MYH11* monitoring in larger series.

Table 19 Sizes of PCR products and log sensitivities of *CBFB-MYH11* primer sets in RT-PCR testing

	A ↔ B1	*A ↔ B2*	*C ↔ D1*	*C ↔ D2*	*A ↔ B1 + C ↔ D1*	*A ↔ B2 + C ↔ D2*	*E5 ↔ D1*	*E5 ↔ D2*
Sizes of PCR products in bp								
type A	—	418	—	271	—	271	—	417
type B	—	630	—	483	—	483	—	679
type C	—	811	—	664	—	664	—	810
type D	338	(1138)	155	(991)	155	(991)	301	(1137)
type E	545	(1345)	362	(1198)	362	(1198)	508	(1344)
type F	—	322	—	175	—	175	—	321
type G	242	(1042)	59	(895)	59	(895)	205	(1041)
type H	344	(1144)	161	(997)	161	(997)	307	(1143)
type I	—	—	—	—	—	—	—	—
type J	—	1033	—	886	—	886	—	1032
ME-1 (type A)	NA[a]	–2	NA	–3	NA	–4	NA	–3
Patients	–2	–3	–2	–3	–5	–4	–2	–3

[a]NA: not applicable.

Results of the PCR primer design and testing

The primers A, C and E5′ are located in 'exon 4' of the *CBFB* gene so that both types of *CBFB* breakpoints can be covered (Figure 17b). The B1 and D1 primers are positioned at the junction of *MYH11* exons 8 and 9 and in exon 8, respectively. This implies that they can be used for detection of *CBFB-MYH11* transcripts of type D, E, G and H, altogether representing 10–15% of cases. The B2 and D2 primers are positioned in *MYH11* exon 12 and can therefore be used for virtually all other *CBFB-MYH11* variants except for type I. The precise position of the seven primers and their sequences are given in Table 18.

Two weaker alternative splice variants of type A have been identified, with alternative addition of 31 nucleotides, due either to an alternative splice donor site from the last *CBFB* exon or to alternative splicing of *MYH11* exon 11.[163]

The sensitivity testing was performed via dilution experiments with the ME-1 cell line (inv(16) type A) and several patients carrying inv(16) type A, D or E. The single PCR dilution experiments consistently reached sensitivities of $10^{-2}/10^{-3}$, whereas the nested PCR experiments reached $10^{-4}/10^{-5}$ (Figure 18 and Table 19).

SECTION 9. Intra-chromosomal microdeletion on 1p32 with the *SIL-TAL1* fusion gene

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Background

The t(1;14)(p32;q11) translocation was first characterized in 1984.[179] It involves the *TCRD* locus at chromosome 14q11 and the *TAL1* gene (T cell acute leukemia gene 1), also identified as *SCL* (stem cell leukemia) or *TCL5* (T cell leukemia gene 5), at chromosome 1p32.[180] The translocation does not modify the *TAL1* reading frame but induces aberrant *TAL1* expression. A much more frequent mode of *TAL1* deregulation is a site-specific deletion between *TAL1* and a gene located approximately 90 kb upstream, *SIL* (*SCL* interrupting locus).[181] This deletion places the *TAL1* reading frame under the control of the *SIL* promoter, which is expressed in T cells. A further mode of *TAL1* deregulation is 'aberrant' expression of *TAL1* without apparent genetic rearrangement, estimated to occur in 5–10% of T-ALL.[182]

The *TAL1* gene is composed of eight exons spanning 16 kb (Figure 19). The first four are alternative exons (1a, 1b, 2a and 2b). The *TAL1* reading frame is located in exons 4, 5 and 6, giving a protein of 42 kDa, which is a transcription factor of the basic helix–loop–helix (bHLH) family. It heterodimerizes with ubiquitous bHLH members of the E2A family (E12, E47, HEB) and is essential for the development of all hematopoietic lineages, but its function in T-ALL is not clearly defined.[183]

The *SIL* gene is composed of 18 exons spanning 70 kb. The first two exons (1a and 1b) are non-coding. *SIL* function is not well-defined but it constitutes a member of the immediate–early gene family.[184]

SIL-TAL1 fusions are illegitimate V(D)J rearrangements, which are not visible by classical cytogenetics. *SIL* contains three donor deletion sites (sildb1 to sildb3), one of which (sildb1) is used in the vast majority (98%) of cases. *TAL1* contains seven acceptor deletion sites (taldb1 to taldb7), with two being involved in nearly all cases (taldb1 and taldb2).[38,39,181,185–188] In all but one case, these rearrangements create alternative fusion transcripts containing the entire *SIL* exon 1a and *TAL1* exon 4. These transcripts code for a normal TAL1 protein of 42 kDa. A single case with a deletion donor site located between *TAL1* exons 4 and 5 (taldb6) has been described.[185] *SIL* exon 1 is consequently fused directly to *TAL1* exon 5; this transcript codes for the shorter 22 kDa TAL1 protein. It should, however, be noted that the TAL1 proteins in T-ALL blasts with *TAL1* deletions have only been analyzed in a limited number of cases.[182] Numerous *SIL-TAL1*-positive T cell lines have been characterized, including RPMI8402, CEM, HSB2 and MOLT-16.[39]

The DNA breakpoints of the three donor-deletion sites in the *SIL* gene and the seven acceptor-deletion sites in the *TAL1* gene are shown in Figure 19. These are clustered at the site of heptamer-like recombinase recognition signal sequences. The vast majority (nearly 90%) of *SIL-TAL1* deletions are located between sildb1 and taldb1, tald1 (*TAL1*-deletion type 1).[38,39,186] Another frequently involved deletion is between sildb1 and taldb2, tald2.[38,39] All other deletion sites have been

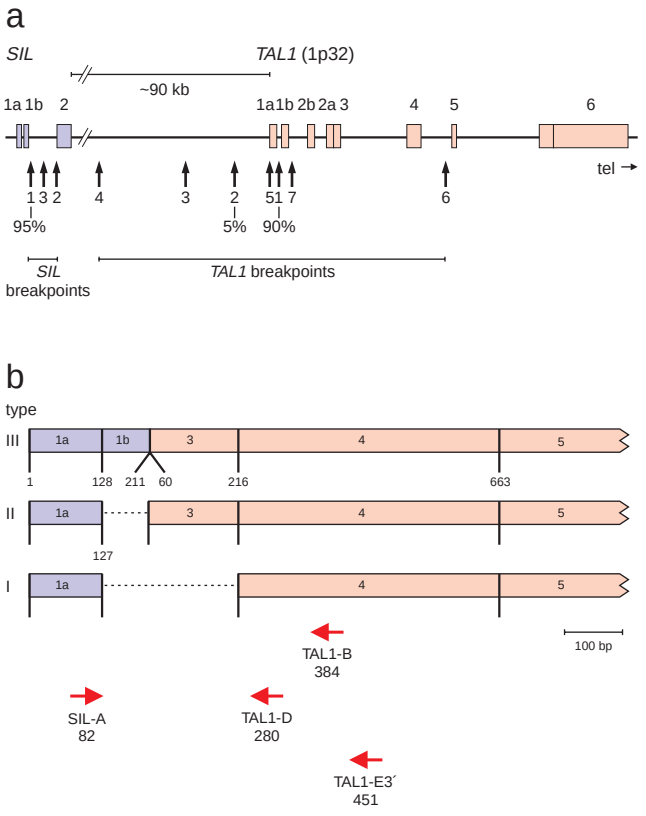

Figure 19 (a) Schematic diagram of the exon/intron structure of the *SIL* and *TAL1* genes, which are involved in the microdeletion on 1p32. The exon numbering and the position of the various breakpoints in the *SIL* and *TAL1* genes are indicated. In the vast majority of cases the *SIL* breakpoint 1 is used in combination with *TAL1* breakpoint 1 or 2. (b) Three main types of *SIL-TAL1* fusion transcripts are formed via alternative splicing of *SIL* exon 1b and *TAL1* exon 3. The numbers under the fusion gene transcripts refer to the first (5′) nucleotide of the involved exon, except when the last (3′) nucleotide of the upstream gene is indicated. The arrows indicate the relative position of the four primers. Because of the small GC-rich *SIL* exon 1a only one *SIL* primer could be designed, implying that a semi-nested PCR approach is used for sensitive detection of *SIL-TAL1* transcripts. The numbers under the primer codes refer to the 5′ nucleotide position of each primer (see Table 20).

Table 20 Primers for RT-PCR analysis of del(1)(p32;p32) with the *SIL-TAL1* fusion gene

Primer code	*5′ Position[a] (size)*	*Sequence (5′–3′)*
SIL-A	82 (19)	TCCCGCTCCTACCCTGCAA
TAL1-B	384 (18)	CGCGCCCAGTTCGATGAC
TAL1-D	280 (18)	CCGCGTCCCGTCCCTCTA
TAL1-E3′	451 (19)	CGTCGCGGCCCTTTAAGTC

[a]See Table 2 for complete sequence information.

found in unique cases only: tald3 (sildb1-taldb4),[39] tald4 (sildb1-taldb5),[39] tald5 (sildb2-taldb3),[187] tald6 (sildb1-taldb6)[185] and tald7 (sildb3-taldb7).[188]

Three different *SIL-TAL1* transcripts are formed by alternative splicing of the two *SIL* exons 1a and 1b and *TAL1* exons 3 and 4.[188] The predominant transcript (type II) contains *SIL* exon 1a spliced to *TAL1* exons 3 and 4. The two others forms contain *SIL* exon 1a and exon1b spliced to *TAL1* exons 3 and 4 (type III) or *SIL* exon 1a directly spliced to *TAL1* exon 4 (type I) (Figure 19b and Table 21).[189]

SIL-TAL1 transcripts are exclusively found in T-ALL.[188,189] The *SIL-TAL1* RT-PCR described here detects all *SIL-TAL1* deletions other than the single reported case with a *TAL1* deletion between exon 4 and exon 5, since the *TAL1* primers are located on exon 4 (Figure 19b). It also does not detect *TAL1* translocations or 'aberrant' expression of *TAL1* without apparent rearrangement. Data concerning the incidence of *SIL-TAL1* deletions are largely based on genomic detection of tald1 and tald2. The incidence in childhood T-ALL is estimated at approximately 5–25% of cases.[38,39,188] A single study suggested that these genomic deletions are absent in adult T-ALL.[190] *SIL-TAL1* transcripts have been found in 26% of childhood T-ALL and 16% of adult T-ALL, predominantly in younger adults.[188]

Detection of tald1 and tald2 at the DNA level did not have prognostic significance in a retrospective series of childhood T-ALL,[185] but these targets have been successfully used for follow-up.[6,191] The value of *SIL-TAL1* RT-PCR detection in prognostic evaluation and follow-up is unknown, but is currently being analyzed in a prospective manner (French LALA94 trial coordinated by JA Gabert, Marseille, France).

Results of PCR primer design and testing

It is important to note that *SIL-TAL1* RT-PCR is strictly dependent on the presence of DMSO 10%. It was not possible to identify acceptable pairs of primers allowing a true nested RT-PCR, mainly because the *SIL* primers had to be designed in the small GC-rich *SIL* exon 1a (~200 bp). For this reason, a semi-nested protocol with a single *SIL* primer was developed (Table 20 and Figure 19b). Table 21 summarizes the size of the RT-PCR products of the different *SIL-TAL1* transcript splice variants using the various primer combinations. Single PCR analysis with the A ↔ B, A ↔ D, or A ↔ E3′ primer combinations resulted in sensitivities of 10^{-2} to 10^{-3}, whereas the

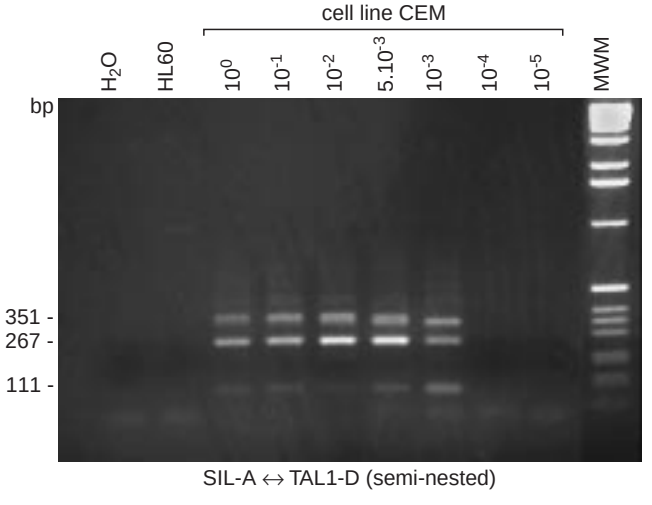

Figure 20 Ethidium bromide-stained agarose gel showing a dilution experiment of RNA from the *SIL-TAL1*-positive CEM cell line using the semi-nested PCR approach with the A ↔ B and A ↔ D primer combinations. The HL60 cell line and H_2O were used as negative controls. *SIL-TAL1* splice variants type II and type III were detected in the CEM cell line (see Figure 19 and Table 21).

Table 21 Sizes of PCR products and log sensitivities of *SIL-TAL1* primer sets in RT-PCR testing

	A ↔ B	*A ↔ D*	*A ↔ B + A ↔ D*	*A ↔ E3′*
Sizes of PCR products in bp				
type III splice variant (1a.1b–3.4)	455	351	351	522
type II splice variant (1a–3.4)	371	267	267	438
type I splice variant (1a–4)	215	111	111	282
RPMI8402	–2	–3	–4	–2
CEM	–3	–2	–4	–2
Patients	–3	–2	–4	–2

Table 22 Summary of the standardized PCR primer sets and their applicability

Chromosome aberration	*Fusion gene*	*No. of PCR primers*	*Estimated sensitivity*		*Recommended for molecular diagnosis*	
			single PCR	*nested PCR*	*ALL*	*AML*
t(1;19)(q23;p13)	*E2A-PBX1*	5	$10^{-3}/10^{-4}$	$10^{-4}/10^{-5}$	+	–
t(4;11)(q21;q23)	*MLL-AF4*	5	$10^{-2}/10^{-3}$	$10^{-4}/10^{-5}$	+	+/–[b]
t(8;21)(q22;q22)	*AML1-ETO*	5	$10^{-3}/10^{-4}$	$10^{-4}/10^{-5}$	–	+
t(9;22)(q34;q11)	*BCR-ABL*, p190	5 } 7[a]	$10^{-2}/10^{-3}$	$10^{-4}/10^{-5}$	+	+/–[b]
t(9;22)(q34;q11)	*BCR-ABL*, p210	5 }	$10^{-3}/10^{-4}$	$10^{-4}/10^{-6}$	+	+/–[b]
t(12;21)(p13;q22)	*TEL-AML1*	5	$10^{-2}/10^{-4}$	$10^{-4}/10^{-5}$	+[c]	–
t(15;17)(q22;q21)	*PML-RARA*	7	$10^{-2}/10^{-3}$	$10^{-3}/10^{-4}$	–	+
inv(16)(p13;q22)	*CBFB-MYH11*	7	$10^{-2}/10^{-3}$	$10^{-4}/10^{-5}$	–	+
microdeletion 1p32	*SIL-TAL1*	4	$10^{-2}/10^{-3}$	10^{-4}	+[d]	–

[a]The reverse primers (B, D and E3′) for *BCR-ABL* p190 and *BCR-ABL* p210 are identical.
[b]*MLL-AF4* fusion genes are rarely observed in AML (Refs 60, 61). *BCR-ABL* fusion genes are observed in 1–2% of AML (Refs 114–116).
[c]Only a few cases of adult ALL with t(12;21) have been reported; these patients were all younger than 30 years.
[d]So far *SIL-TAL1* fusion genes have only been detected in T-ALL.

semi-nested A ↔ B/A ↔ D PCR resulted in sensitivities of 10^{-4} (Figure 20 and Table 21).

Conclusion

The extensive collaborative studies by the 10 laboratories over a period of 4 years have resulted in a standardized RT-PCR protocol (Table 3) and PCR primer sets for the detection of nine well-defined chromosome aberrations. The protocol and primers can be used for molecular classification at diagnosis, ie for risk group classification, as well as for MRD detection, ie for evaluation of treatment effectiveness.

Table 22 summarizes information about the standardized PCR primer sets and their differential application for molecular classification of ALL and AML at diagnosis. For efficient PCR analysis at diagnosis a 96-well PCR machine allows collective analysis of approximately 14 ALL or 10 AML samples using the A ↔ B primer sets and including all relevant positive and negative controls for each chromosome aberration. All positive patient samples should be confirmed with the 'shifted' (control) C ↔ E3′ or E5′ ↔ D primer sets. While the primers and standardized protocols presented here provide a useful basis for good laboratory practice, it should be emphasized that each laboratory should test the primers and protocols with positive and negative controls in order to assess their performance under local conditions.

The minimal target sensitivity of 10^{-2} was generally reached for virtually all single PCR analyses, whereas the nested PCR analyses generally reached the minimal target sensitivity of 10^{-4}, except for occasional sensitivity levels of 10^{-3} in the case of *PML-RARA*, which is known to be a difficult target for sensitive detection (Table 22). During the final phase of the study it appeared that usage of *AmpliTaq* Gold (PE-Biosystems, Foster City, CA, USA) might further improve the sensitivity.

The participants of the BIOMED-1 Concerted Action decided to optimize and standardize the RT-PCR assays using nested PCR, as most of the members are currently using this technique. However, because of the risk of contamination by PCR product carry over, an alternative strategy can be used: one round PCR followed by blotting and hybridization with an internal probe,[124] which could be primer C or D. This strategy was tested by one laboratory (Marseille) and showed overall similar sensitivities as the nested PCR procedure.

The exchange of experience between the participating laboratories, the central production of the standardization reagents and control RNAs, and the regular meetings played an important role in the successful completion of this European Concerted Action.

The complexity of standardization and quality control in international clinical studies is frequently underestimated and therefore not addressed appropriately. The protocols and primers presented in this report can be efficiently used in multi-

center studies and therefore are highly valuable for clinical studies, particularly multicenter MRD studies. Nevertheless, regular meetings, each followed by a report with the agreements and action points, remain essential for preserving the quality and the continuous innovation of molecular diagnostics.

Over the last 2 years several large-scale PCR-based MRD studies have clearly demonstrated that quantitative information about MRD levels is important for MRD-based risk group classification.[2,3,5,6] The so-called real-time quantitative PCR (RQ-PCR) techniques offer the possibility for rapid and reproducible quantitation of MRD levels without post-PCR handling, thereby preventing contamination with PCR products.[133,192,193] The molecular laboratories of the BIOMED-1 Concerted Action have therefore now initiated a large study group of 25 laboratories from 10 European countries in order to standardize the RQ-PCR analyses of chromosome aberrations using the TaqMan technique.[194]

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