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Real-time reverse transcription polymerase chain reaction detection and quantification of t(1;19) (*E2A–PBX1*) fusion genes associated with leukaemia

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Summary. A real-time reverse transcription polymerase chain reaction (RT-PCR) method is described that enabled the detection and quantification of *E2A–PBX1* fusion gene transcripts associated with t(1;19). The method was highly reproducible and offered exceptional sensitivity at 5 fg of fusion transcript per reaction, without the need for a nested PCR primer design. To illustrate the usefulness of this new technology the *E2A–PBX1* fusion gene transcript expression level for several human leukaemia cell lines that are positive and negative for cytogenetically detectable t(1;19) was determined. The RCH–ACV had a threefold higher expression of *E2A–PBX1* transcripts (600 transcripts per cell) than the other t(1;19) positive 697 (150 transcripts per cell). The only other cell line with detectable E2A–PBX1 was CEM, but the level of expression was < 1 transcript per cell.

Keywords: *E2A–PBX1*, real-time PCR, leukaemia, t(1;19), translocation.

Translocation t(1;19) is detected in 3–5% of childhood and 3% of adult acute lymphocytic leukaemia (ALL) cases (Crist *et al*, 1990; Rambaldi *et al*, 1996). This translocation fuses exons I to XIII of the *E2A* gene (19p13) with exons II to VIII of the *PBX1* (1q23) (Carroll *et al*, 1984). *E2A* codes for a helix-loop-helix transcription factor, whereas *PBX* encodes a homeobox gene, and the fusion protein produced has malignant potential (Kamps *et al*, 1991). A reverse transcription polymerase chain reaction (RT-PCR) method using a nested primer design has been described previously (van Dongen *et al*, 1999), and has demonstrated considerable detection capabilities. However, the assay does not permit quantification of the fusion gene transcripts for the evaluation of minimal residual disease and other uses. This report describes a single primer set design that uses real-time PCR for the detection and quantification of *E2A–PBX1* fusion gene transcripts. Using this method the level of fusion gene expression of two cell lines bearing t(1;19) was determined.

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METHODS

Construction of E2A–PBX1 fusion gene runoff transcripts. RNA was extracted from the 697 (CCL-119, American Type Culture Collection (ATCC, Manassas, VA, USA) cell line (1×10^7 cells) using a Qiagen RNAeasy™ mini column as directed (Qiagen, Santa Clara, CA, USA). First-strand cDNA was produced using the reverse transcription (RT) methods described by van Dongen *et al* (1999) with modifications as described. Whole RNA (1 μg) was primed with the 100 ng PBX1-E3 primer (Table I) (MWG Biotech, High Point, NC, USA), then mixed with 0·5 mmol/l dNTPs (Roche, Pleasanto, CA, USA), five units Stratascript™ reverse transcriptase (Stratagene, La Jolla, CA, USA), and 1 unit RNase inhibitor (Eppendorf, Hamburg, Germany) in 20-μl of 1× buffer (supplied with RT enzyme). This RT cocktail was reacted for 50 min at 42°C followed by 5 min at 95°C. From the initial cDNA strand, a smaller cDNA product was generated using PCR primers E2A–A and PBX1-B (Table I). PCR conditions were as described by van Dongen *et al* (1999), and produced a single 373 basepair band. This PCR product was cloned into a pPCR-Script™ Amp plasmid using a PCR cloning kit (Stratagene) as directed.

Bacterial colonies ($n = 20$) bearing an insertion vector were grown overnight in liquid broth containing ampicilin. Correct insert orientation was required, as the plasmid was

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Table I. Sequences of PCR primers and the Taqman™ probe used for real-time PCR.

Primer name	5′ Position (Size in bps)	Sequence (5′-3′)	Reference
PBX1-E3	748 (19)	TGAACTTGCGGTGGATGAT	van Dongen *et al* (1999)
PBX1-B	675 (19)	TCGCAGGAGATTCATCACG	van Dongen *et al* (1999)
E2A–A	1434 (19)	CACCAGCCTCATGCACAAC	van Dongen *et al* (1999)
E2APBX_F1	1436 (19)	CCAGCCTCATGCACAACCA	Current
E2APBX_D	411 (26)	CCTCCTCCTGGGCTCCTCGGATACTC	Current
E2APBX_R	467 (22)	CCTTCCGCTAACAGCATGTTGT	Current

used to generate runoff RNA transcripts of the *E2A–PBX1* fusion gene. To determine which plasmids contained a correctly orientated and sized insert, the colonies were screened using a PCR methodology. Using the M13 (−20) primer, which is proximal to the T7 promoter sequence of the plasmid, and an *E2A* specific primer (*E2A*–A), 1 μl of the overnight culture was directly assayed using PCR. The 20 μl PCR consisted of 0·25 mmol/l dNTPs, 30 ng of forward and reverse primer, and 2 units of AmpliTaq™ polymerase (Applied BioSystems, Redwood City, CA, USA) with the supplied buffer A. Products were run on a 1% agarose ethidium bromide-stained gel, and those reactions that generated a correctly sized product, 373 bp plus 223 bp of flanking plasmid sequences, were selected. Plasmid DNA was isolated from the selected overnight cultures using Qiagen Mini Plasmid extraction columns, as directed. Selected plasmid DNAs were sequenced by automated fluorescent sequencing and the sequences compared with the published *E2A* (Accession M31222, exons I–XIII) *PBX1* (Accession M86546, exons I-VIII) gene sequences. A single error-free plasmid was subsequently used to generate runoff RNA transcripts.

Table II. *E2A–PBX1* RT-PCR protocol.

1. *RT-reaction with random hexamers*
500 ng of RNA in 5·0 μl of H_2O
Add 120 ng Random Hexamers (5 μl)
Incubate at 65°C for 5 min
Cool to room temperature
RT buffer: 50 mmol/l Tris-HCl (pH = 8·3), 75 mmol/l KCl, 3 mmol/l $MgCl_2$
RNAasin 1·0 units
RT enzyme 5·0 units (Stratascript™)
dNTP 0·5 mmol/l
Incubate 5 min 25°C, 50 min 42°C, 5 min 95°C

2. *Real-time PCR*

5 μl volume of reacted RT reaction
10 μl of Taqman Universal Master Mix
100 ng E2APBX1-F
100 ng E2APBX-R
10 ng E2APBX-D Taqman probe to a final volume of 20 μl
5 min 50°C, 10 min 95°C, 45 cycles (95°C−15 s, 60°C−30 s)

Circular plasmid DNA was linearized by restriction of the single *Sac*I site located distal to the plasmid's T7 promoter site. Large amounts of runoff RNA transcripts were produced using the MegaShortScript™ T7 kit as directed (Ambion, Austin, TX, USA). To eliminate the contaminating plasmid DNA the runoffs were processed though a Qiagen RNA mini column and the product was then restricted with *Hae*III (several sites between the real-time primers), then treated with DNase I, and passed through an RNA mini column. Real-time PCR (below) was used to directly (no RT step) screen the runoff RNA transcripts to ensure that no residual plasmid DNA was present as a contaminant. The quantity of the runoff transcript was initially determined by spectrophotometer and then confirmed using a RiboGreen™ RNA quantification kit (Molecular Probes, Eugene, OR, USA).

Design of real-time primers. Real-time PCR primers were manually designed and then tested using PRIMER EXPRESS Software (Applied Biosystems) (Table I). Guidelines for primer design, as suggested by Applied Biosystems, were adhered to tightly. Real-time PCR primers E2A–PBX1-F1 and E2A–PBX1-R are able to detect the two different splice variants as originally described by van Dongen *et al* (1999). PCR primers were proofed using the selected *E2A–PBX1* fusion gene plasmid. A TaqMan™ probe (E2A–PBX1-D) was designed such that it anneals to the 5′ end of PBX1 exon II and was labelled 5′ with the reporter dye tetrachloro-6-carboxyfluorescein (TET) as well as 3′ with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) (MWG Biotech).

RT reactions. First-strand cDNA, generated from runoff RNA transcripts mixed in with background RNA (0·5 μg of human colon RNA per 20 μl of RT reaction) (Stratagene), was used to construct a standard curve for *E2A*–PBX1 fusion gene expression. The RT reaction cocktail consisted of 1× buffer (Stratagene), 0·5 mmol/l dNTPs, one unit RNAse inhibitor, 5 units Stratascript™ RT, 120 ng random hexamers (Gibco BRL, Carlsbad, CA, USA) in a 20-μl volume, incubated at 25°C for 5 min followed by 42°C for 50 min followed by 95°C for 5 min (Table II). Specific RT primers can also be used instead of random primers, although we noted no difference in sensitivity when using TAL-B-R (data not shown).

Real-time PCR reactions (Applied Biosystems) using 5 μl of the completed RT reaction were performed as directed by

Table III. Description and source of the human leukaemia cell lines used in this study (Kasumi-1 and RCH–ACV were used with permission).

Cell Line	t(1;19)	Type	Source #	Source
CEM	–	Acute lymphoblastic leukaemia	CCL-119	ATCC
RCH–ACV	+	Acute lymphoblastic leukaemia		Jack *et al* (1986)
Kasumi-1	–	Acute myeloid leukaemia		Asou *et al* (1991)
HL-60	–	Acute myelogenous leukaemia	CCL-240	ATCC
K-562	–	Chronic myeloid leukaemia in blast crisis	CCL-243	ATCC
REH	–	Acute lymphocytic leukaemia (non-T; non-B)	CRL-8286	ATCC
697	+	B-cell precursor leukaemia	ACC-42	DSMZ

the manufacturer except that reaction volumes were decreased from 50 μl to 20 μl so as to use less reagents. PCR primers E2APBX-F1 and E2APBX-R were added at 100 ng each, whereas the E2APBX-D Taqman™ probe was just 10 ng (again this used less of the expensive probe). Reactions were then subjected to 5 min at 50°C, 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. Data was collected and analysed with Applied Biosystem's SEQUENCE DETECTION software (Version 1·7).

Sample preparation. Whole cell RNA was extracted from several cell lines obtained from ATCC (Manassas, VA, USA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) (Table III). Kasumi-1 was kindly provided by Dr Nanao Kamada (Research Institute of Nuclear Medicine and Biology, Hiroshima, Japan) and used with permission. The RCH–ACV cell line was kindly provided by Dr Mignon Loh (Department of Paediatrics, University of California San Francisco, CA, USA). Colon RNA obtained from Stratagene served as a negative control in all experiments as well as background RNA in which the E2A–PBX1 runoff transcripts could be concealed to mimic the real life situation.

β2-Microglobulin (β2MG) mRNA expression was used to normalize results, and permitted RNA quantification and efficiency of the RT reaction to be determined. Primers for β2MG PCR were as previously described (Pallisgaard *et al*, 1999). After the RT reaction, 2 μl of the cDNA cocktail were added to a separate real-time PCR assay for β2MG expression. Briefly, the assay contained 10 μl of Taqman Universal Master Mix (Applied Biosystems), 60 ng of the forward and reverse primers and 6 ng of a TET-labelled TaqMan™ Probe. Known amounts of RNA (colon RNA used as a negative control) generated the standard Ct values for that amount of RNA. These sample β2MG Ct values were then divided by the average colon Ct values generating a factor to correct for variations in the amounts of sample RNA.

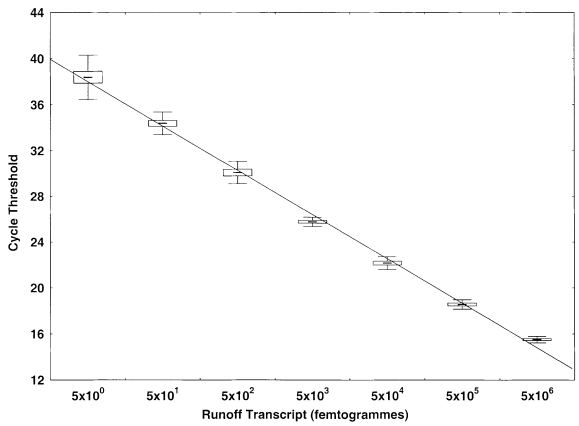

Fig 1. Standard curve of *E2A–PBX1* fusion gene expression. Standard curves from five separate experiments are combined to produce this curve ($R^2 = 0{\cdot}983$, d.f. = 88, $P < 10^{-6}$). Mean Ct values are indicated by bar with standard errors (boxes) and deviations (whiskers). The likelihood ratio test was unable to demonstrate any difference between any of the individual experiments. Although 500 ag of the runoff transcript could be detected with the assay, its reliability was inconsistent and this standard point has been excluded from the standard curve. The resolution of the assay is 5 fg of transcript in a background of 500 ng of RNA.

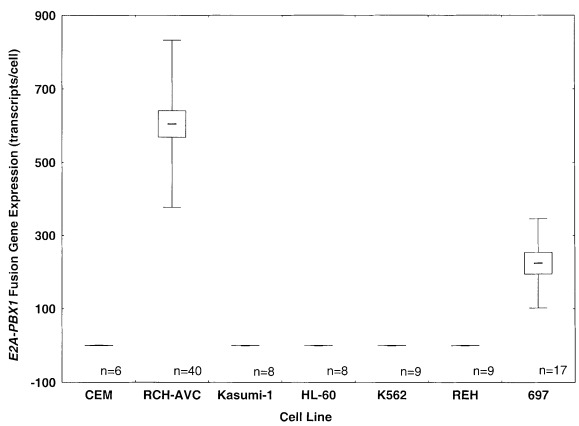

Fig 2. *E2A–PBX1* fusion gene expression determined for human leukaemia cell lines. Mean Ct values are indicated by bar with standard errors (boxes) and deviations (whiskers). The molecular weight of the 507 bases of the fusion gene runoff transcript (includes flanking plasmid sequences) was 162734·2 g/mol, thus 1 fg contains 3830 molecules. Approximately 4 pg per cell of whole cell RNA could be obtained from the cell lines. Kasumi-1, REH, K-562 and HL-60 cell lines did not exhibit any *E2A*–PBX1 expression. However, expression was consistently detected in the CEM cell line at 1 fg per 500 ng of whole RNA (0·3 ± SD 0·4 transcripts per cell).

RESULTS

Standard curves were prepared such that each standard point was replicated at the RT step four times. Linear regression analysis of the standard curve experiment data demonstrated considerable precision ($R^2 = 0{\cdot}99$, d.f. $= 26$, $P < 10^{-6}$). There was little or no difference between standard curves prepared for different experiments (likelihood ratio test, $\chi^2 = 0{\cdot}31$, d.f. $= 2$, $P = 0{\cdot}86$). Figure 1 shows the data from five separate standard curve experiments combined. Resolution of the real-time RT-PCR assay permitted 5 fg of *E2A–PBX1* fusion gene runoff transcript to be consistently detected. Attempts to detect attogramme (ag) amounts of the runoff transcript were unsuccessful although, occasionally, 500 ag of the fusion gene could be detected. The use of a nested PCR primer design would probably permit those levels to be routinely reached. However, such methods are increasingly susceptible to cross contamination and should, if possible, be avoided. Resolution demonstrated by the present method was sufficient to assay for transcript expression levels in several cell lines, and is probably sufficient for measuring minimal residual disease in patients whose leukaemic cells bear this translocation.

Minor variances in the RNA amounts assayed, owing either to measurement (by spectrophotometer) or pipette error, were accounted for by normalization against the β2MG gene. Known amounts of colon RNA were assayed in each experiment and consistently yielded the same Ct values (16·94 ± SE 0·07, SD 0·32). β2MG Ct values for samples were divided by that figure, thus providing a correction factor to apply against the sample RNA amounts (data not shown). These corrections were relatively minor in every case.

Cell lines 697 and RCH–ACV, both of which bear cytogenetically detectable t(1;19), had considerable *E2A–PBX1* expression levels, with hundreds of transcripts per cell (Fig 2). These calculations are based on the assumption that an average growing cell has 4 pg of whole cell RNA. For reasons that are unclear, RCH–ACV had a threefold higher level of fusion gene transcripts than 697 (*t*-test, $P < 10^{-6}$). In addition, RCH–ACV had considerable variation in *E2A–PBX1* expression levels, particularly between different RNA extractions. For this reason, this cell line was assayed numerous times (n = 40) to attempt to characterize the level of variation, the cause of which remained elusive. Several other cytogenetically negative cell lines were also assayed for the presence of *E2A–PBX1* transcripts. Only one, the CEM line, demonstrated some expression. The CEM cell line yielded barely detectable amounts of the fusion gene, at expression levels of less than one copy per cell. It is likely this expression level is the result of a mixed population of cells in the cell culture, with only a small fraction of the cells bearing the fusion gene, or that in this cell line the fusion gene message is relatively short lived compared with the other cell lines. Colon RNA, which was routinely assayed as a negative control, never demonstrated any expression of the fusion gene, nor did the HL-60, K-562, REH, or Kasumi-1 cell lines.

DISCUSSION

The *E2A–PBX1* assay described here (protocol provided in Table II) is relatively simple to undertake and is exceedingly reliable and reproducible. With the increased detection resolution provided by this single round real-time PCR design, the method described here should be useful for the detection of minimal residual disease. We also plan to use it for the detection of *E2A–PBX1* fusion gene transcripts generated *in vitro* after cell culture and chemical exposure and for determining the background frequency of t(1;19), if any, in normal human populations.

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REFERENCES

Asou, H., Tashiro, S., Hamamoto, K., Otsuji, A., Kita, K. & Kamada, N. (1991) Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8; 21 chromosome translocation. *Blood*, **77**, 2031–2036.

Carroll, A.J., Crist, W.M., Parmley, R.T., Roper, M., Cooper, M.D. & Finley, W.H. (1984) Pre-B cell leukemia associated with chromosome translocation 1; 19. *Blood*, **63**, 721–724.

Crist, W.M., Carroll, A.J., Shuster, J.J., Behm, F.G., Whitehead, M., Vietti, T.J., Look, A.T., Mahoney, D., Ragab, A. & Pullen, D.J. (1990) Poor prognosis of children with pre-B acute lymphoblastic leukemia is associated with the t(1;19)(q23; p13): a Pediatric Oncology Group study. *Blood*, **76**, 117–122.

van Dongen, J.J., Macintyre, E.A., Gabert, J.A., Delabesse, E., Rossi, V., Saglio, G., Gottardi, E., Rambaldi, A., Dotti, G., Griesinger, F., Parreira, A., Gameiro, P., Diaz, M.G., Malec, M., Langerak, A.W., San Miguel, J.F. & Biondi, A. (1999) 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. *Leukemia*, **13**, 1901–1928.

Jack, I., Seshadri, R., Garson, M., Michael, P., Callen, D., Zola, H. & Morley, A. (1986) RCH-ACV: a lymphoblastic leukemia cell line with chromosome translocation 1;19 and trisomy 8. *Cancer Genetics and Cytogenetics*, **19**, 261–269.

Kamps, M.P., Look, A.T. & Baltimore, D. (1991) The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. *Genes and Development*, **5**, 358–368.

Pallisgaard, N., Clausen, N., Schroder, H. & Hokland, P. (1999) Rapid and sensitive minimal residual disease detection in acute

leukemia by quantitative real-time RT-PCR exemplified by t(12;21) TEL–AML1 fusion transcript. *Genes Chromosomes and Cancer*, **26**, 355–365.

Rambaldi, A., Attuati, V., Bassan, R., Neonato, M.G., Viero, P., Battista, R., Di Bona, E., Rossi, G., Pogliani, E., Ruggeri, M., Amaru, R., Rivolta, A., Giudici, G., Biondi, A. & Barbui, T. (1996) Molecular diagnosis and clinical relevance of t(9;22), t(4;11) and t(1;19) chromosome abnormalities in a consecutive group of 141 adult patients with acute lymphoblastic leukemia. *Leukemia and Lymphoma*, **21**, 457–466.