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Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: Quality assurance on behalf of SIOPEN-R-NET

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ABSTRACT

The clinical utility of detecting minimal residual disease (MRD) in children with neuroblastoma (NB) by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) is not clear. This in part reflects the lack of uniform methodology for analysis and reporting. Reference laboratories across Europe have therefore established standard operating procedures (SOPs) for the detection of NB cells by QRT-PCR. Haemopoietic samples are collected into PAXgene™ blood RNA tubes, which stabilise mRNA for 48 h at room temperature and more than 6 months at –80 °C. Tyrosine hydroxylase (TH) was selected as the target for NB cell detection, expression is normalised to β2-microglobulin and reported using the $\Delta\Delta C_t$ method. The sensitivity of QRT-PCR increased from 58% to 90% following the development of SOPs. A robust, transferable, objective method for the detection of NB cells by QRT-PCR has been defined to improve the power and consistency of studies on MRD in children with NB.

1. Introduction

The presence of metastatic disease at diagnosis in children with neuroblastoma (NB) is an important indicator of poor prognosis, consequently accurate and sensitive assessment of disease status is essential in these children. The methods currently used in clinical practice largely depend on radiological imaging to identify characteristic lesions and/or

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histological examination of bone marrow smears or trephines. Although these methods are sensitive for initial staging according to the International Neuroblastoma Staging System (INSS), they do not detect the small volume disease implicated in the relapse and metastatic process.

The use of reverse transcriptase polymerase chain reaction (RT-PCR) to detect tumour or tissue-specific RNA as a marker of circulating tumour cells has been described for a number of different cancer types, including NB.[1,2] This approach assumes that non-haemopoietic cells are not normally present in the compartment to be studied and requires the identification of a target mRNA expressed in tumour cells but not haemopoietic cells. Because catecholamines are produced by NB cells, the first enzyme in the catecholamine synthesis pathway tyrosine hydroxylase (TH), has been used as a target for the detection of disease by RT-PCR. Although other targets for the detection of NB cells by RT-PCR have been described, TH mRNA is currently the single most rigorously evaluated useful target for the detection of disease in clinical samples.[3–5] Using RT-PCR for TH mRNA clinically significant disease has been detected in peripheral blood[6–9] and bone marrow[7,10–13] samples from children at diagnosis, on therapy, on follow up and at relapse. Furthermore, the technique has been used to detect NB cells in autologous peripheral blood stem cell harvests from children with high-risk disease.[10,14]

Despite this literature, the clinical utility of this technique has not yet been defined. This may in part reflect the small number of children studied, but also the lack of uniform methodology and documentation for the assessment of this minimal disease.

The inconsistency of methods and reporting make it difficult to compare results from different centres and countries, limiting any meta-analyses (NHS Health and Technology Assessment (HTA) grant number 97/15/03; for further details of results see http://www.prw.le.ac.uk/epidemio/personal/rdr3/paed2.html). This emphasises the need to study the clinical impact of minimal disease detected by RT-PCR in children with NB in a large, multi-centre quality controlled clinical outcome study.

Children diagnosed with high-risk NB over the age of 1 year from 19 European countries are currently entered into HR-NBL1/ESIOP (for more information see http://www.siopen-r-net.org/). This provides a unique opportunity to evaluate the clinical significance of NB cells detected by RT-PCR in bone marrow (BM), peripheral blood (PB) and peripheral blood stem cell harvests (PBSC) of children with high-risk NB, and to assess the efficacy of minimal residual disease treatment strategies including 13-*cis* retinoic acid alone and in combination with anti-GD^2 monoclonal antibody therapy. Therefore an observational, blinded biological study has been incorporated into the current European high risk study (HR-NB1/ESIOP) for children with NB to investigate the clinical utility of RT-PCR detection of circulating NB cells.

Given the conflicting data in the published literature on the clinical value of RT-PCR detection of NB cells, we have established standard operating procedures (SOPs) to collect, transport, store, process and analyse BM, PB and PBSC samples for the detection of NB cells by quantitative (Q)RT-PCR across Europe (Fig. 1). SOPs have been defined following four main phases of work undertaken by six reference laboratories:

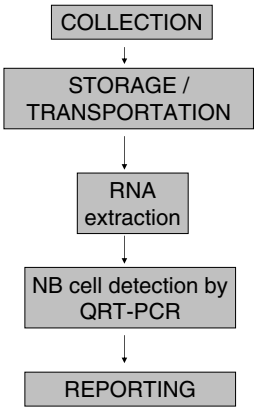


Fig. 1 – Workflow for the establishment of SOPs.

1. Assessment of the sensitivity and specificity of routine laboratory RT-PCR methods for the detection of NB cells using a series of blinded quality control samples.
2. Evaluation of methods that could be applied across all participating European centres for the collection, transportation and processing to RNA of whole BM, PB and PBSC.
3. Identification of the optimal primer/probe sets for detection of TH mRNA and the house-keeping gene β2-microglobulin (β2 M) by QRT-PCR, using the ABI 7700 platform (Applied Biosystems).
4. Define standard methods of reporting data from QRT-PCR analyses.

2. Subjects and methods

2.1. *Cell lines*

IMR-32 NB cells were purchased from the European Collection of Animal Cell Cultures (PHLS, UK), and maintained in 1 part Dulbecco's minimum essential medium (DMEM, Sigma): 1 part RPMI 1640 (Sigma) supplemented with 10% (v/v) FCS (Sera Lab). For cell spiking experiments cells were harvested using EDTA and trypsin, and viable cell number was counted by the trypan blue exclusion assay.

2.2. *Haemopoietic samples*

Known numbers of IMR-32 cells were added to a sample of PBSC harvest (12 ml) from a leukaemia patient and PB (48 ml) from a healthy volunteer, these were added into either 2 mM EDTA or PAXgene™ blood RNA tubes (BD, cat. No. 762165), to compare the efficiency of TH mRNA stabilisation.

To investigate the effect of time and storage conditions on RNA integrity, PB (14×2 ml) from healthy volunteers ($n = 5$) collected in PAXgene™ blood RNA tubes were analysed immediately, frozen at –80 °C or stored at room temperature for 2–120 h. The stability of samples stored frozen at –80 °C was evaluated up to 6 months.

RNA isolated from PB of adult volunteers with no evidence of malignant disease ($n = 30$) collected into PAXgene™ blood RNA tubes was used to evaluate the expression of TH and β2M mRNA in normal PB. Two of these 30 donors gave additional PB samples (54×2 ml) into which known numbers

(10–10,000) of IMR-32 cells were added by micro-manipulation. These were used to assess the sensitivity of NB cell detection by QRT-PCR for TH and for Series 8 and 9 quality control cell spike samples (see Section 2.6).

The RNA yield from 58 BM and 36 PB samples collected into PAXgene™ blood RNA tubes from 26 children from Italy and UK throughout the course of treatment on HR-NBL1/ESIOP was investigated. BM samples (n = 73) from children with NB in Belgium were collected into 2 mM EDTA and mononuclear cells isolated by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway) according to the manufacturer's instructions. Cell number was counted using an automated haematology analyser (Sysmex SE 9500, Toa Medical Electronics, Kobe, Japan) and RNA isolated using TRIzol (InVitrogen, Belgium) according to the manufacturer's instructions.

Informed consent was obtained for the collection and use of all clinical material for research. All clinical samples were obtained from children and adults in the United Kingdom unless otherwise stated. Ethical approval was obtained from the Leeds Teaching Hospital Trust Local Research Ethics Committee, Trent Multi Research Ethics Committee, and the Ethical Committee of the Ghent University Hospital, Belgium.

2.3. Sensitivity and specificity of RT-PCR for TH mRNA

The sensitivity and specificity of TH mRNA detection was evaluated by

(a) adding known numbers of IMR-32 cells (0–100) to PB (2 ml) from healthy volunteers or PBSC (0.5 ml) from a patient with leukaemia, or
(b) adding RNA from IMR-32 cells to RNA from PB from healthy volunteers or a non-NB cell line.

$$\text{Sensitivity} = \frac{\text{Number of samples positive for TH mRNA by RT-PCR}}{\text{Number of samples containing tumour cells or IMR-32 RNA}} \times 100$$

$$\text{Specificity} = \frac{\text{Number of samples negative for TH mRNA by RT-PCR}}{\text{Number of samples not containing tumour cells or IMR-32 RNA}} \times 100$$

2.4. RNA extraction

RNA was extracted according to the manufacturer's instructions from samples taken into EDTA using Ultraspec™ (Biogenesis, Bournemouth, UK),[6] and into PAXgene™ blood RNA tubes using the PAXgene™ blood RNA kit (Qiagen, cat. no.762134). RNA concentration and purity were evaluated by measuring the optical density at 280 and 260 nM using the Nanodrop ND-1000 (www.labtech.co.uk). The remaining samples were aliquoted into 10 μl and stored in a –80 °C freezer.

2.5. QRT-PCR method

As with all RNA assays caution was taken to avoid contamination of samples; samples were processed to RNA in an isolated room, separate to that where RT-PCR was performed. Single-use aliquots of dNTPs, primers and probes were used to avoid artefacts due to freeze–thaw, all consumables were RNAse-free and gloves were worn to prevent degradation of RNA by RNAses on the hands.

2.5.1. Reverse transcription (RT)

Reverse transcription was performed in thin-wall PCR eppendorf tubes (Scientific Laboratory Supplies Ltd, Cat No.GN-PCR-05-C). Prior to the reverse transcription reaction, RNA (in 10 μl) was heated at 95 °C for 5 min and cooled immediately on ice. Ten microlitre of RT mix was added to each sample: RT mix = 2 μl of 10× PCR reaction buffer (1×; Applied Biosystems), 2 μl of 10 mM dNTPs (1 mM; Amersham Biosciences), 1.6 μl of 100 mM $MgCl_2$ (8 mM; Sigma–Aldrich Ltd.), 1 μl of 0.6 μg/μl of random hexamer primers (0.3 μg/ml; Life Technologies Ltd.), 0.5 μl RNA Guard (16 U; Amersham), 1.34 μl MMLV reverse transcriptase (20 U; Amersham, cat. no. 27-0925-02) and 1.56 μl RNase-free H_20. Samples in a final reaction volume of 20 μl were heated in a block at 37 °C for 1 h. The efficiency of cDNA synthesis using random hexamer primers or poly dT primers (0.1–0.5 μg), and MMLV reverse transcriptase (5, 10, 15 and 20 U) from Amersham (Product no.27-0925-02) and Pharmacia (Product no. 27-0925-02) was evaluated by QRT-PCR for TH and β2M mRNA. Negative controls in which reverse transcriptase enzyme was absent were included in each assay. cDNA samples were analysed immediately by QPCR.

2.5.2. Analysis by QPCR

QPCR for the amplification of TH and β2M was performed using the TaqMan assay (Perkin–Elmer, Applied Biosystems); final concentration of reaction components is given in brackets where appropriate. Following cDNA synthesis the RT enzyme was inactivated by incubating samples at 95 °C for 5 min. The samples were incubated at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C × 15 s, 60 °C × 1 min, using the ABI PRISM™ 7700 Sequence detector. The threshold cycle, or C_t value, is defined as the number of cycles it takes to reach a point at which the fluorescence generated is first recorded as substantially above background (during the exponential amplification phase). When no amplification occurs, due to no detectable or absence of target mRNA, C_t values of 40 are recorded. Forward and reverse primers were designed in exons separated by an intron to minimise amplification of genomic DNA. The amplicon generated for TH mRNA was 72 bp and for β2M 110 bp. The optimal primer and probe sets were determined by comparing the efficiency and reproducibility of detecting TH or β2M in RNA isolated from: (i) IMR-32 cells; and (ii) PB into which known numbers of IMR-32 cells had been added.

TH forward primer 5′-ATTGCTGAGATCGCCTTCCA-3′ (exon 6, accession no. NM_199292.1); TH reverse primer 5′-AATCT-

CCTCGGCGGTGTACTC-3′ (exon 7); TH Probe 5′FAM-ACAGGCACGGCGACCCGATTC–3′TAMRA.[15]

β2M forward primer 5′-GAGTATGCCTGCCGTGTG-3′ (exon 2, accession no. NM_004048.2); β2M reverse primer 5′-AATCCAAATGCGGCATCT-3′ (exon 3); β2M Probe 5′FAM-CCTCCATGATGCTGCTTACATGTCTC–3′TAMRA.[16]

For the analysis of haemopoietic samples collected in PAXgene™ blood RNA tubes, 400 ng of total RNA extracted using the PAXgene™ blood RNA kit was reverse transcribed in a total volume of 20 μl. Sixteen microlitre of cDNA was added to 64 μl of TH PCR mix containing 40 μl TaqMan Universal PCR Master Mix (1×; Applied Biosystems), 0.8 μl of each 10 μM forward and reverse primer (100 nM), 0.4 μl of 20 μM 5′FAM-labelled probe (100 nM) and 22 μl RNase-free water. Three aliquots of 25 μl were transferred to a 96-well Optical Reaction Plate (Applied Biosystems); each replicate contained the equivalent of 5 μl cDNA generated from 100 ng of total RNA.

For relative quantification and to check the integrity of mRNA, the expression of β2M was measured by adding 4 μl of the remaining cDNA sample (80 ng total RNA) to 1 μl of RNase-free water and 20 μl of β2M PCR mix, containing 12.5 μl TaqMan Universal PCR Master Mix (1×; Applied Biosystems), 0.25 μl of each 10 μM forward and reverse primer (100 nM), 0.125 μl of 20 μM 5′FAM-labelled probe (100 nM) and 6.875 μl RNase-free water.

For the generation of RNA standard curves, total RNA (40 ng) from the IMR-32 cell line was reverse transcribed in a total volume of 20 μl. Tenfold serial dilutions of cDNA were used in the subsequent PCR step. To assess the variation between and within experiments, data from nine independent standard curves performed in triplicate were evaluated.

2.5.3. *Reporting of results*

When reporting on the analysis of clinical samples from children entered into the HR-NB1/ESIOP study, it was agreed that reference laboratories would use the comparative C_t method.[17] The expression of target (TH) normalised to the expression of a house-keeping gene (β2M) is reported relative (fold-difference) to a chosen positive control sample according to the formula:

$$2^{-\Delta\Delta C_t}, \text{ where } \Delta\Delta C_t = [(C_t\text{TH} - C_t\beta2\text{M})_{\text{Sample}} - (C_t\text{TH} - C)_t\beta2\text{M})_{\text{Positive control}}].$$

The positive control is cDNA reverse transcribed in 20 μl from 800 pg of IMR-32 RNA in 400 ng of RNA from PB from a healthy volunteer. To avoid variation generated by preparation of the positive control in individual laboratories, single use aliquots of control sample are provided by the central reference laboratory at the University of Leeds. This control is analysed in parallel with every clinical sample, allowing an assessment of inter-assay variability and standardisation of the threshold used for accurate $\Delta\Delta C_t$ calculation. ΔC_t is reported as the difference between the mean of the triplicate C_t values for TH and the β2M C_t value. When all three TH C_t values = 40, $\Delta C_{t\,\text{Sample}}$ is not calculated and the results are reported as negative for TH expression.

2.6. *Quality control*

The analysis of quality control samples was used to assess sensitivity and specificity of reference laboratory RT-PCR methods and to establish SOPs. The samples, mailed to participating reference laboratories on dry ice and analysed blind, consisted of

1. cDNA generated from RNA isolated from NB (IMR-32) cells [Series 1–5].
2. RNA from NB cells mixed with RNA isolated from a non-NB cell line or RNA from normal blood [Series 3–7].
3. NB cells (0–100) added to 2 ml of normal blood from healthy volunteers in PAXgene™ blood RNA tubes [Series 8 and 9].

2.7. *Statistical methods*

To assess the variation in the efficiency of amplification (slope of standard curves) by QRT-PCR for TH and β2M in IMR-32 RNA between experiments, random effects linear regression (fitting for an overall linear curve and random effects for each experiment) was performed using the SAS statistical package (SAS Institute, USA).

For comparison of RNA yield from BM, ANOVA was performed at each sampling point to examine the total between patient variation in comparison to the total within patient variation.

3. Results

3.1. *Collection and transportation of samples*

The efficiency of TH mRNA stabilisation in samples collected in 2 mM EDTA or PAXgene™ blood RNA tubes was compared. The presence of TH mRNA in PB or PBSC containing 10 or 100 IMR-32 cells was used as a measure of RNA integrity.

Detection was 100% when samples were taken into either 2 mM EDTA or PAXgene™ blood RNA tubes and stored at –80 °C prior to RNA extraction. However, following storage of samples at room temperature for 2 d, detection of 10 and 100 tumour cells was only possible in those samples stabilised in PAXgene™ blood RNA tubes. These results demonstrate that stabilisation of RNA in PB and PBSC samples is optimal when samples are taken into PAXgene™ blood RNA tubes for 2 d at room temperature, or frozen immediately at –80 °C.

In addition, β2M was stabilised in PB samples taken into PAXgene™ blood RNA tubes and incubated at room temperature for up to 2 d, or at –80 °C for long-term storage up to 6 months. The mean C_t value and SD for detection of β2M in 8 PB samples (from five individuals) maintained at –80 °C for 1–60 d was 16.70, SD 0.34. When incubated for more than 2 d at room temperature, the C_t values increased consistent with a decrease in the level of β2M mRNA (Table 2), demonstrating that the samples should not be stored at room temperature for longer than 48 h if the quality and yield of RNA is to be maximally stabilised. The quantity and quality of RNA extracted from PB samples collected into the anticoagulants EDTA or heparin prior to transfer into a PAXgene™ blood RNA tube within an hour of collection was constant (results not shown).

Table 1 – Original methods employed by reference laboratories

Laboratory	RNA isolation	RT priming	Reverse Transcriptase enzyme	TH PCR primers	Number of PCR cycles	Detection
1	RNeasy blood mini Kit (Qiagen)	Oligo(dT)	AML (Clontech)	Corrias *et al.*[5]	Nested PCR (2 × 30)	Ethidium Bromide agarose gel
2	TRIzol (InVitrogen)	PCR primers	MuLV (Applied Biosystems)	Swerts *et al.*[15]	50	Fluorescent (TaqMan probe)
3	RNeasy blood mini Kit (Qiagen)	Random hexamers	MuLV (InVitrogen)	Trager *et al.*[18]	40	Fluorescent (TaqMan probe)
4	RNeasy blood mini Kit (Qiagen)	Oligo(dT)/random hexamers	MuLV (Applied Biosystems)	Oltra *et al.*[19]	50	Fluorescent (TaqMan probe)
5	TRIzol (Gibco BRl)	Random hexamers	MuLV (Applied Biosystems)	Miyajima *et al.*[20]	35	Ethidium bromide agarose gel
6	Ultraspec™ (Biogenesis)	Random hexamers	MMLV (Amersham)	Burchill *et al.*[21]	50	Ethidium bromide agarose gel
SOPs	PAXgene™ blood RNA Kit (Qiagen)	Random hexamers	MMLV (Amersham)	Swerts *et al.*[15]	40	Fluorescent (TaqMan probe)

Table 2 – Stability of RNA in PB stored at room temperature in PAXgene™ blood RNA tubes

Number of days	Volunteer				
	1	2	3	4	5
0	16.69	16.80	16.66	16.84	16.74
1	16.50	16.82	16.87	16.71	16.84
2	16.30	16.24	16.51	16.92	16.90
3	*22.72*	16.11	*26.92*	*26.87*	*26.52*
4	*34.34*	*32.86*	*33.14*	*34.72*	*30.39*
5	*37.12*	*38.36*	*34.19*	*36.35*	*39.58*

RNA was extracted from PB samples from volunteers 1 to 5 after 2 h (number of days = 0) or at intervals between 1 and 5 d after incubation at room temperature. The integrity of RNA (100 ng) was analysed by QRT-PCR for β2M; mean C_t values from triplicates are shown. RNA was stable at least 60 d when stored at –80 °C (mean ± SD, 16.70 ± 0.34); however, although RNA was stabilised up to 2 d at room temperature, at 3–5 d the mean Ct values for β2M increased (shown in italics) consistent with deterioration of RNA.

3.2. *RNA extraction*

The yields and integrity of total RNA isolated from samples stabilised in PAXgene™ blood RNA tubes and extracted using the PAXgene™ blood RNA kit were tested on a panel of PB (2 ml; $n = 36$) and BM (0.5 ml; $n = 58$) samples from children with NB. The samples were taken at diagnosis (R0), first randomisation (R1), final randomisation (R2) and at the end of the treatment; more detail on the HR-NBL1/ESIOP study protocol are available on the study website (http://www.siopen-r-net.org/). The range of total RNA yield from PB samples was 0.02–14.8 μg (Mean ± SD; 4.9 ± 4.6 μg); OD 260:280 ratio 1.8–2.1. The yield of RNA from BM was highly variable, ranging from 0.02 to 18.1 μg (Mean ± SD 4.9 ± 4.8 μg; OD 260:280 ratio 1.9–2.2) (Fig. 2i). The variation in yield of RNA from BM was greater between patients than the yield from multiple samples within a single patient (ANOVA test). This variability in RNA yield was also observed in BM samples separated using Ficoll gradient (Fig. 2ii); there was no direct correlation between BM cell number and RNA yield ($r = 0.42$–0.53). Since the maximum isolation capacity of the PAXgene™ blood RNA column is 100 μg (manufacturer's information), these studies demonstrate that the stabilisation and extraction capacity of the PAXgene™ blood RNA tubes and columns is sufficient to isolate RNA from PB (2 ml), PBSC (0.5 ml) and BM (0.5 ml).

3.3. *NB detection by QRT-PCR and reporting*

Optimisation of the QRT-PCR assay using the primer/probe sets described was achieved by establishing standard curves for the detection of TH and β2M mRNA in serial dilutions of IMR-32 cDNA (Fig. 3i). Using data from nine independent experiments, within which triplicate analyses were carried out, the mean slope for the amplification of TH mRNA was –3.64 with no significant variation between experiments (slope ranged from –3.52 to –3.86), indicating high PCR efficiency. The assay was highly reproducible as indicated by low standard deviation of C_t values within (SD = 0.15–0.91) and between (SD = 0.34–0.49) experiments (Fig. 3i).

Using the standard procedures described for RNA extraction and QRT-PCR analysis, it was possible to reliably detect 10 IMR-32 cells in 2 ml of PB, this corresponds to the detection of a single IMR-32 cell in approximately 10^6 white blood cells (Fig. 3ii). TH mRNA was detected in 1/3 replicates from 5/30 PB samples from healthy volunteers, demonstrating the importance of performing three replicates for each QRT-PCR assay; the significance of TH mRNA detected in a single replicate out of three is not clear. With a difference of at least three C_t values, this assay discriminates between 2 ml PB samples containing 0 ($C_t = 39.8$), 10 ($C_t = 36.8$) or 100 ($C_t = 33.7$) IMR-32 cells (Fig. 3ii), consistent with the hypothesis that TH is a good target to detect NB cells in PB. The analysis of 100 ng of total RNA was sufficient to reliably detect >1 cell in 10^6 haemopoietic cells. Increasing the amount of RNA above 100 ng did not increase the sensitivity of the assay; mean TH C_t value when 100 ng of RNA from samples of 10 IMR-32 cells added to 2 ml of PB was 37.3 compared to a C_t value of 37.4 when 250 ng was analysed, and 23.9 compared to 24.9 for the detection of

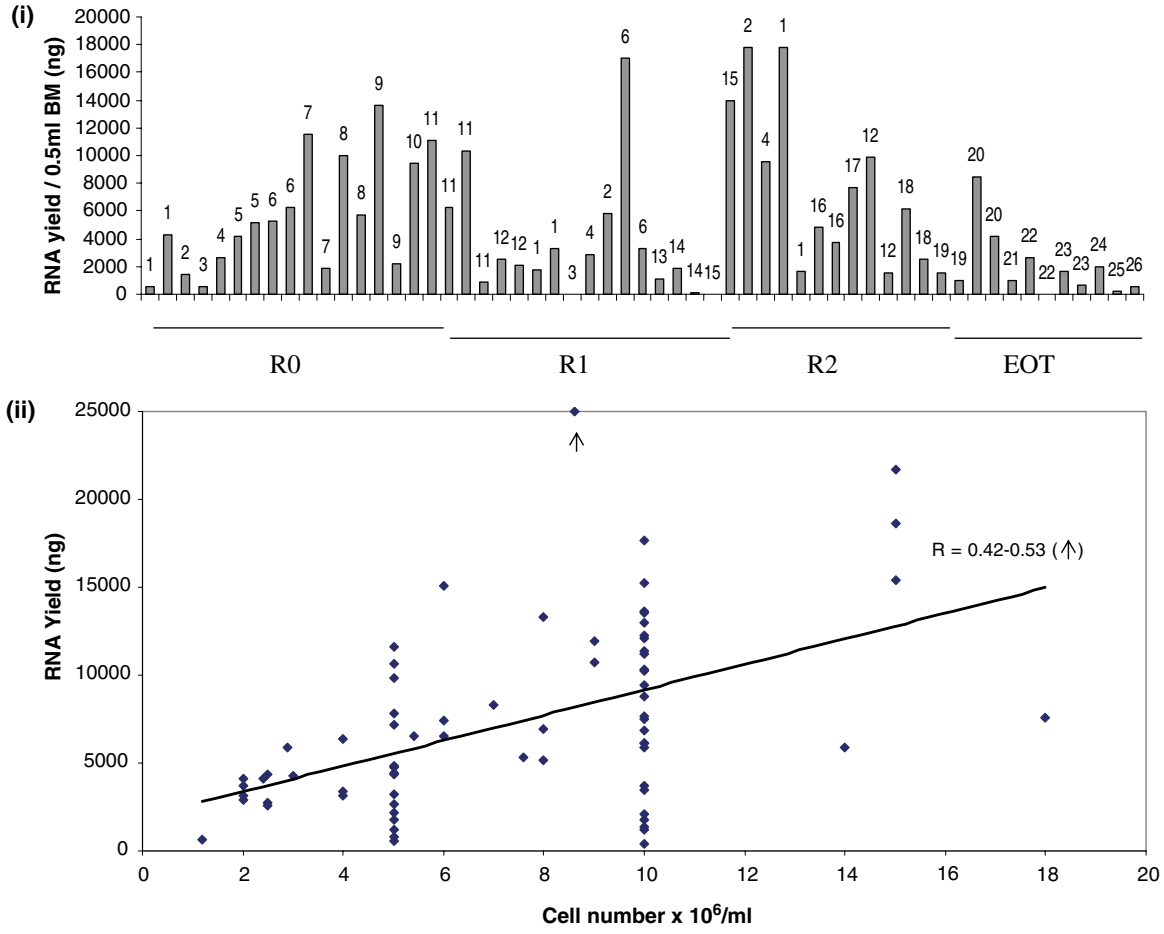


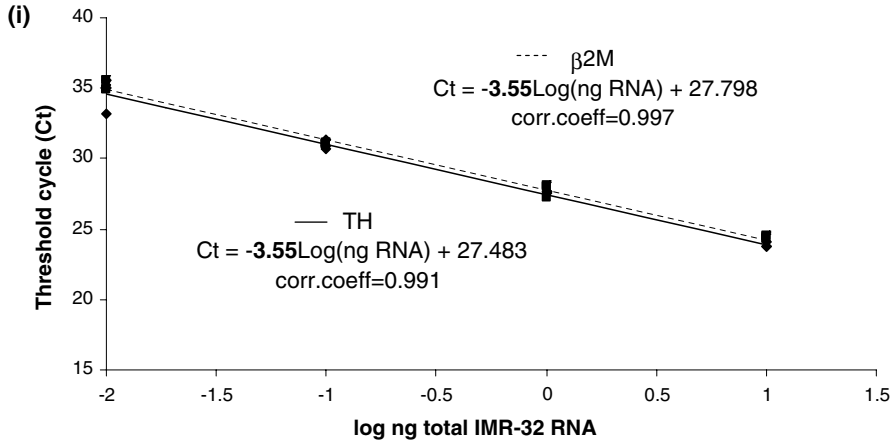
Fig. 2 – Variability in the yield of total RNA isolated from BM samples from children with NB using: (i) PAXgene™ blood RNA kit. Time points: RO, diagnosis; R1, first randomisation; R2, final randomisation; and EOT, end of treatment. Each number identifies samples from a single patient or (ii) TRIzol after isolation of cells by density gradient centrifugation. The relationship between cell number and RNA yield was limited, demonstrated by the moderate correlation coefficient (0.42); this increased to 0.53 if the maverick point indicated by arrow is omitted.

1×10^5 IMR-32 cells in 2 ml of PB. In duplicate samples from the same or from a different donor, the maximum variation in C_t values for β2M mRNA in PB samples from healthy volunteers was within one C_t value (data not shown), demonstrating the stability of β2M mRNA in haemopoietic samples. This is consistent with previous studies describing the use of β2M as a house-keeping gene in PB, BM and leukocyte samples.[22]

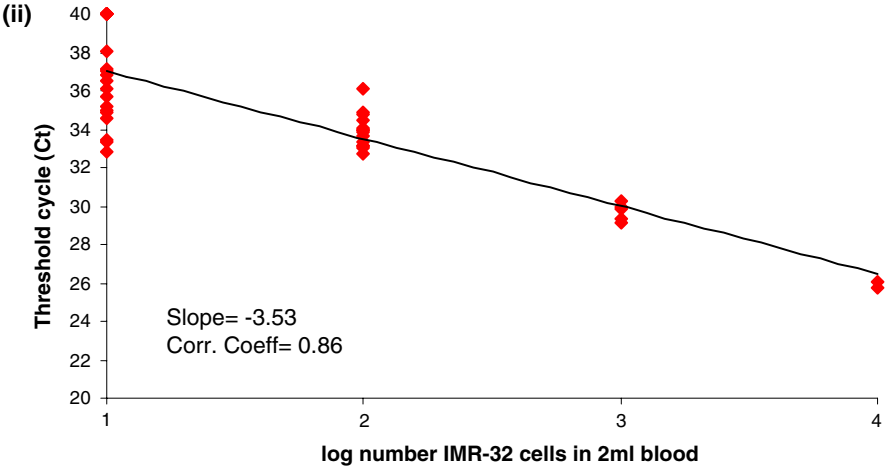
The importance of standardisation of RT-PCR protocols is illustrated in the results from the quality control series (Table 3). All participating laboratories were capable of performing PCR to detect TH mRNA in cDNA generated from 10 pg of RNA isolated from IMR-32 cells (Series 1–5), demonstrating the robust nature of the PCR amplification methods used in each laboratory. However, when analysing RNA from RNA mixing experiments the laboratories reported false negatives (sensitivity 40–60%), reinforcing the need for a standard method for the generation of cDNA from RNA. Standardisation of the reverse transcription protocol contributed to improved sensitivity (series 7, 79%) across all six laboratories (TH mRNA detection to 20 pg in 6/6 laboratories and to 10 pg in 3/6 laboratories). Standardisation of the amount of RNA and introduction of the quantitative assay (Series 8 and 9) improved overall sensitivity (90%); the maximum sensitivity of 10pg was achieved in 5/6 laboratories. Comparing the data from mixed RNA samples (Series 3–9), there was a trend to increased overall sensitivity ($p < 0.01$, logistic regression). Using the standardised procedures for processing and analysis of cell spikes, specificity was 100% across five laboratories (the sixth laboratory did not analyse the samples). With the exception of one laboratory, all other laboratories reported detection of 2 or 10 IMR-32 cells in 2 ml PB.

4. Discussion

All the reference laboratories participating in this study have specific experience and expertise on the detection of minimal disease in children with NB using RT-PCR for TH mRNA. However, the early quality control rounds clearly demonstrate that the sensitivity and specificity of NB cell detection by RT-PCR in these laboratories varied, emphasising the need for SOPs. Modifications of protocols for cDNA synthesis and PCR were made during the course of the quality control series



IMR-32 RNA (ng)	Mean	Within Exp SD	Between Exp SD	Overall SD
0.01	34.17	0.91	0.49	0.89
0.1	30.67	0.21	0.37	0.40
1	26.81	0.24	0.52	0.54
10	23.41	0.15	0.34	0.35



Number of NB cells	Mean Ct value (SD)
0	39.8 (0.9)
10	36.8 (2.5)
100	33.7 (0.9)
1,000	29.7 (0.5)
10,000	25.9 (0.3)

Fig. 3 – Sensitivity, specificity and reproducibility of QRT-PCR: (i) Standard curves obtained for TH and β2M by QRT-PCR. Triplicate C_t values from one experiment were plotted against the logarithm of 10-fold dilutions of IMR-32 cDNA. Reproducibility of the assay for the detection of TH mRNA is indicated by the within and between experiment standard deviation (SD) (n = 9 experiments, three replicates per experiment). (ii) QRT-PCR for TH to detect IMR-32 cells in PB. Known numbers of IMR-32 cells were added to 2 ml of PB and placed into PAXgene™ blood RNA tubes, and analysed using SOPs.

to arrive at the optimised SOPs described in this manuscript; original laboratory methods are summarised in Table 1. Consistent with the previous studies,[23,24] the greatest source of variation between laboratories was the method of sample collection, RNA isolation and cDNA synthesis. Such pre-analytical processes become increasingly important when the target is liable to degradation, as is the case of mRNA.

In this study, the PAXgene™ blood RNA collection tube was shown to stabilise mRNA from BM or PBSC (0.5 ml) and PB (2 ml) at room temperature for 48 h, and at –80 °C for up to 6 months. Furthermore, the isolation capacity of the PAXgene™ blood RNA kit was sufficient to isolate RNA from these sample types and volumes; the RNA yields are comparable to those described in previous studies.[25,26] The stabilisation efficiency of the PAXgene™ blood RNA collection tube will be limited by the cellular content of the sample; however, over the three different clinical sample types and volumes studied we identified no robust correlation between cell number and RNA yield.

Previous studies have suggested that mRNA integrity in PB samples taken into PAXgene™ blood RNA tubes is stabilised for up to 5 d at room temperature.[27,28] This is not consistent with the data presented in this manuscript and should be interpreted with caution.[26,29] However, stabilisation of TH and β2M mRNA was achieved when the samples were frozen at –80 °C for up to 6 months. Importantly, the stabilisation of mRNA in PAXgene™ blood RNA tubes for up to 48 h at room temperature allows the collection of PB (2 ml), BM or PBSC samples (0.5 ml) at any hospital to be shipped at room temperature to reference centres for analysis, without loss of sensitivity of tumour cell detection. If samples are not analysed within 48 hours of collection into PAXgene™ blood RNA tubes,

Table 3 – Results of quality control series

	IMR-32 cells added					IMR-32 RNA (pg) added						IMR-32 cDNA (pg)						Overall RNA summary	
	100	50	10	2	0	200	80	40	20	10	0	200	80	40	20	10	0	Sensitivity	Specificity
Lab no.																			
Series 3																			
1						–	–	–	–	–	–	+	+	–	–	+	–	58%	100%
2						+	–	–	–	–	–	+	+	+	+	+	–	(14/24)	(5/5)
3						+	+	+	+	+	–	+	+	+	+	+	–		
4						nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt		
5						nt	–	+	+	+	–	–	–	–	+	+	–		
6						+	+	+	+	+	–	+	+	+	+	+	–		
Series 4																			
1						+	+	+	+	+	+	+	–	–	–	–	–	60%	83%
2						–	–	–	–	–	–	+	+	+	+	+	–	(18/30)	(5/6)
3						+	+	+	+	+	–	+	+	+	+	+	–		
4						–	–	–	–	–	–	+	+	+	+	+	–		
5						+	+	–	+	+	–	+	–	–	–	–	–		
6						+	+	+	–	+	–	+	+	+	–	+	–		
Series 5																			
1						–	–	–	–	–	–	–	–	–	–	–	–	40%	50%
2						nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	(8/20)	(2/4)
3						+	+	+	+	+	+	+	+	+	+	+	–		
4						–	–	–	–	–	+	+	+	+	+	+	–		
5						nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt		
6						+	+	+	–	–	–	+	+	+	+	+	–		
Series 6																			
1						+	+	+	+	+	–							40%	83%
2						–	–	–	–	–	–							(12/30)	(5/6)
3						+	+	–	+	–	–								
4						–	–	–	–	–	–								
5						–	+	–	–	–	+								
6						+	+	+	–	–	–								
Series 7																			
1						+	+	+	+	–	+		SOPs					79%	83%
2						+	+	+	+	+	–		Reverse transcription					(23/29)	(5/6)
3						nt	+	+	+	–	–								
4						–	–	–	–	–	–								
5						+	+	+	+	+	–								
6						+	+	+	+	+	–								
Series 8																			
1	+	+	+	–	–	+		+	+	–	–							83%	67%
2	nt	nt	nt	nt	nt	+		+	+	+	–							(20/24)	(4/6)
3	+	+	–	+	–	+		+	+	+	+								
4	nt	nt	nt	nt	nt	+		+	+	+	+								
5	nt	nt	nt	nt	nt	+		–	–	–	–								
6	+	+	+	–	–	+		+	+	+	–								
Series 9																			
1	+	+	+		–	+				+	–		SOPs					90%	100%
2	+	+	+		–	+				+	–		QRT-PCR					(9/10)	(5/5)
3	nt	nt	nt		nt	nt				nt	nt								
4	+	–	–		–	+				+	–								
5	nt	nt	nt		nt	+				–	–								
6	+	+	+		–	+				+	–								

Series of quality control samples prepared as described in methods were mailed to participating laboratories 1–6; the samples were analysed blind. Series 1 and 2 consisted of cDNA samples only; results not shown. Detection of TH by RT-PCR is indicated as positive (+), negative (–) or not tested (nt).

they must be stored at –80 °C. The collection, storage and transportation of samples according to SOPs (Fig. 4) ensures preservation of RNA integrity. The collection of haemopoietic samples directly into PAXgene™ blood RNA tubes and transportation to the laboratory at room temperature within 48 hours are rapid and easy to perform, and therefore compli-

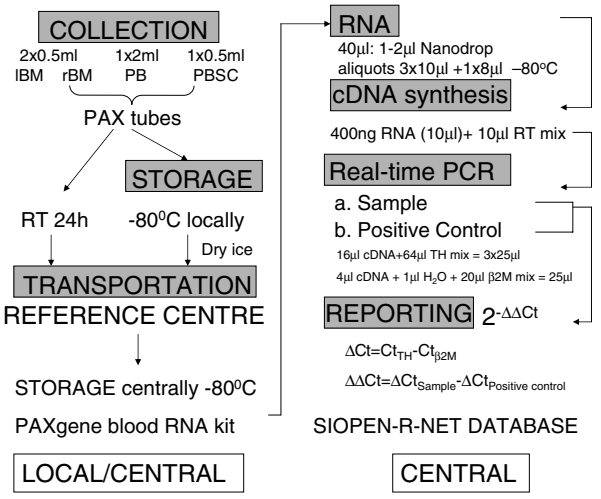


Fig. 4 – SOPS for sample collection, transportation, storage and sample analyses. BM: bone marrow; l: Left; r: right; PB: peripheral blood; PBSC: peripheral blood stem cell harvest. The SIOPEN-R-Net database contains results of RNA yield, quality, standard reporting of RT-PCR data, and blinded clinical information.

ance across multiple centres and countries is most likely to be achieved.

Many studies have used immunocytology, often for GD_2,[30] to detect minimal disease in children with NB. Although some studies suggest that this method is as sensitive as RT-PCR for the detection of rare tumour cells,[5,30] interpretation of such assays is subjective and can lead to inter-observer variation. This is a particular challenge when identifying a single or clumps of 2–10 NB cells when cells do not produce GD_2[31] or if antigen shed by NB cells is taken up by macrophages (which will stain positive with alkaline phosphatase potentially leading to false positives). The introduction of SOPs for sample processing, analysis and reporting can reduce this variability.[32] However interpretation of QRT-PCR is objective, lending itself more readily for multi-centre studies.

The use of QRT-PCR may allow a more precise risk assessment in monitoring minimal disease, although the high sensitivity and challenge of defining what Ct value constitutes a true positive can lead to the so-called detection of illegitimate target gene transcripts and an increased number of false positives. Consequently, defining how to report the results of the QRT-PCR has been critical, and must be applicable across all participating laboratories. The reporting of QRT-PCR by absolute Ct values is not possible across multiple laboratories, especially when different machines and/or different thresholds are used. However, the results can be standardised by using the $\Delta\Delta C_t$ method, in which levels of TH mRNA are normalised to a house-keeping gene and expressed relatively to a positive control sample. The selection of an optimal reference gene against which the expression of the target can be normalised is essential;[16] we have chosen β2M as its expression is stable in normal haemopoietic samples.[16,17] For the $\Delta\Delta C_t$ calculation to be valid, the efficiencies of TH and β2M amplification must be approximately equal as demonstrated using the SOPs method described in this paper; slope of the log cDNA dilution *versus* ΔC_t (CtTH-C_tβ2M) is close to zero (–0.045, data not shown) indicating equal amplification efficiencies.[15] Using the $\Delta\Delta C_t$ method also eliminates the need for standard curves, which would be more costly and require the use of more valuable clinical material. The accuracy of relative quantification is improved by the use of a positive control sample comparable to the clinical samples analysed, e.g. PB from healthy volunteers into which NB cells have been added. To remove the challenge of defining what constitutes a positive and negative result on an individual test basis, selection of the minimum clinically significant level is best defined by statistical analyses of the clinical study results. Together with the use of standard procedures for sample collection and processing to RNA, these characteristics meet the necessary criteria for the establishment of a robust normalisation strategy, critical for the generation of more accurate and clinically meaningful data.

Although RT-PCR detects the level of target mRNA within NB cells, it is not a measure of absolute cell number. Furthermore, it is clear that tumour cells are not detected in BM or PB of all children with high-risk disease, which has been interpreted by some as a 'false' negative result. However, this may reflect the biology of tumour cell shedding, dilution of tumour cells within the compartment and technical features such as the volume and frequency of samples analysed. However, in some cases this may be because the current targets used for the detection of NB cells by QRT-PCR are suboptimal; target expression in cells may not be abundant or homogeneous (reflecting the heterogeneity of the NB cell), may be expressed at low levels in BM or PB, or expression could be modified under different conditions, e.g. following exposure to chemotherapeutic agents. This emphasises the need to identify and evaluate targets for the sensitive and specific detection of NB cells by QRT-PCR in the compartment to be studied e.g. PB or BM. The detection of NB cells using multiple targets may improve both sensitivity and specificity of NB cell detection.

Analysis and interrogation of results from quality control series by the European RT-PCR reference laboratories has improved the sensitivity and specificity of detecting NB cells by QRT-PCR for TH mRNA, resulting in a robust, reliable, transferable, objective method. Centralised processing and analysis of clinical samples for multi-centre studies of minimal disease according to the established SOPs is essential to reduce inter-laboratory variability, and consequently improve the power and reliability of studies of MRD in children with NB.

Conflict of interest statement

None declared.

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REFERENCES

1. Burchill SA, Selby P. Early detection of circulating solid tumour cells using molecular technology. *Cancer Topics* 1999;**10**:1–4.
2. Burchill SA, Selby PJ. Molecular detection of low-level disease in patients with cancer. *J Pathol* 2000;**190**:6–14.
3. Gilbert J, Norris MD, Marshall GM, Harber M. Low specificity of PGP9.5 expression for detection of micrometastatic neuroblastoma. *Br J Cancer* 1997;**75**:1779–81.
4. Cheung IY, Cheung NK. Detection of microscopic disease: comparing histology, immunocytology, and RT-PCR of tyrosine hydroxylase, GAGE, and MAGE. *Med Pediatr Oncol* 2001;**36**:210–2.
5. Corrias MV, Faulkner LB, Pistorio A, et al. Detection of neuroblastoma cells in bone marrow and peripheral blood by different techniques: accuracy and relationship with clinical features of patients. *Clin Cancer Res* 2004;**10**:7978–85.
6. Burchill SA, Bradbury FM, Selby P, Lewis IJ. Early clinical evaluation of neuroblastoma cell detection by reverse transcriptase-polymerase chain reaction (RT-PCR) for tyrosine hydroxylase mRNA. *Eur J Cancer* 1995;**31**:553–6.
7. Kuroda Saeki M, Nakano M, Mizutani S. Clinical application of minimal residual neuroblastoma cell detection by reverse transcriptase-polymerase chain reaction. *J Pediatr Surg* 1997;**32**:69–72.
8. Shono K, Tajiri T, Fujii Y, Suita S. Clinical implications of minimal disease in the bone marrow and peripheral blood in neuroblastoma. *J Pediatr Surg* 2000;**35**:1415–20.
9. Burchill SA, Lewis IJ, Abrams KR, et al. Circulating neuroblastoma cells detected by reverse transcriptase polymerase chain reaction for tyrosine hydroxylase mRNA are an independent poor prognostic indicator in stage 4 neuroblastoma in children over 1 year. *J Clin Oncol* 2001;**19**:1795–801.
10. Miyajima Y, Horibe K, Fukuda M, et al. Sequential detection of tumor cells in the peripheral blood and bone marrow of patients with stage IV neuroblastoma by the reverse transcription-polymerase chain reaction for tyrosine hydroxylase mRNA. *Cancer* 1996;**77**:1214–9.
11. Seeger RC, Reynolds CP, Gallego R, Stram DO, Gerbing RB, Matthay KK. Quantitative tumor cell content of bone marrow and blood as a predictor of outcome in stage IV neuroblastoma: a Children's Cancer Group Study. *J Clin Oncol* 2000;**18**:4067–76.
12. Fukuda M, Miyajima Y, Miyashita Y, Horibe K. Disease outcome may be predicted by molecular detection of minimal residual disease in bone marrow in advanced neuroblastoma: a pilot study. *J Pediatr Hematol Oncol* 2001;**23**:10–3.
13. Horibe K, Fukuda M, Miyajima Y, et al. Outcome prediction by molecular detection of minimal residual disease in bone marrow for advanced neuroblastoma. *Med Pediatr Oncol* 2001;**36**:203–4.
14. Burchill SA, Kinsey SE, Picton S, et al. Minimal residual disease at the time of peripheral blood stem cell harvest in patients with advanced neuroblastoma. *Med Pediatr Oncol* 2001;**36**:213–9.
15. Swerts K, De Moerloose B, Dhooge C, et al. Potential application of ELAVL4 real-time quantitative reverse transcription -PCR for detection of disseminated neuroblastoma cells. *Clin Chem* 2006, Epub ahead of print.
16. Beillard E, Pallisgaard N, van der Velden VHJ, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) – a Europe against cancer program. *Leukemia* 2003;**17**:2474–86.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) method. *Methods* 2001;**4**:402–8.
18. Träger C, kogner P, Lindskog M, et al. Quantitative analysis of tyrosine hydroxylase mRNA for sensitive detection of neuroblastoma cells in blood and bone marrow. *Clin Chem* 2003;**49**:104–12.
19. Oltra S, Martinez F, Orellana C, et al. The doublecortin gene, a new molecular marker to detect minimal residual disease in neuroblastoma. *Diagn Mol Pathol* 2005;**14**:53–7.
20. Miyajima Y, Kato K, Numata S, et al. Detection of neuroblastoma cells in bone marrow and peripheral blood at diagnosis by the reverse transcriptase polymerase chain reaction for tyrosine hydroxylase mRNA. *Cancer* 1995;**75**:2757–61.
21. Burchill SA, Bradbury FM, Smith B, et al. Neuroblastoma cell detection by reverse transcriptase-polymerase chain reaction (RT-PCR) for tyrosine hydroxylase mRNA. *Int J Cancer* 1994;**57**:671–5.
22. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**:1–11.
23. Keilholz U, Willhauck M, Rimoldi D, et al. Reliability of reverse transcription-polymerase chain reaction (RT-PCR)-based assays for the detection of circulating tumour cells: a quality-assurance initiative of the EORTC Melanoma Cooperative Group. *Eur J Cancer* 1998;**34**:750–3.
24. Burchill SA, Lewis IJ, Selby P. Improved methods using the reverse transcriptase polymerase chain reaction to detect tumour cells. *Br J Cancer* 1999;**79**:971–7.
25. Thach DC, Lin B, Walther E, et al. Assessment of two methods for handling blood in collection tubes with RNA stabilizing agent for surveillance of gene expression profiles with high density microarrays. *JIM* 2003;**283**:269–79.
26. Breit S, Nees M, Schaefer U, et al. Impact of pre-analytical handling on bone marrow mRNA gene expression. *Br J Haematol* 2004;**126**:231–43.
27. Müller MC, Merx K, Weißer A, et al. Improvement of molecular monitoring of residual disease in leukemias by bedside RNA stabilisation. *Leukemia* 2002;**16**:2395–9.
28. Rainen L, Oelmueller U, Jurgensen S, et al. Stabilization of mRNA Expression in Whole Blood Samples. *Clin Chem* 2002;**48**:1883–90.
29. Kådegal B, Lindqvist M, Farnebäck M, Lenner L, Peterson C. Failure of the PAXgene™ Blood RNA System to maintain mRNA stability in whole blood. *Clin Chem Lab Med* 2005;**43**:1190–2.
30. Beiske K, Ambros PF, Burchill SA, et al. Detecting minimal residual disease in neuroblastoma patients-the present state of the art. *Cancer Lett* 2005;**228**:229–40.
31. Schumacher-Kuckelkorn R, Hero B, Ernestus K, Berthold F. Lacking immunocytological GD2 expression in neuroblastoma: report of 3 cases. *Pediatr Blood Cancer* 2005;**45**:195–201.
32. Swerts K, Ambros PF, Brouzes C, et al. Standardization of the immunocytochemical detection of neuroblastoma cells in bone marrow. *J Histochem Cytochem* 2005;**53**:1433–40.