IDENTIFICATION OF AN EWS-PSEUDOGENE USING TRANSLOCATION DETECTION BY RT-PCR IN EWING'S SARCOMA

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Summary: The presence of a t(11;22)(q24;q12) translocation is one of the characteristic features of the Ewing family of tumors. The detection of the fusion gene product by RT-PCR using primers at both sides of the breakpoints has been advocated as a diagnostic tool. By applying this technique appropriate internal controls are required. We found that the use of normal non-rearranged EWS mRNA as an internal control for RNA quality may lead to conflicting data. We obtained PCR products of the expected size for the normal EWS mRNA in both RNA and DNA samples, suggesting the existence of one or more EWS pseudogenes. A 109 bp sequence at the 5' end of this PCR-product contained a correctly spliced exon junction and was 97% homologous to the EWS cDNA sequence. Similarly two such junctions were found in a 346 bp sequence of the 3' end, which was 89% homologous. Hence EWS should not be used as an internal control for the RNA quality in a RT-PCR based test for the presence of the translocation.   © 1995 Academic Press, Inc.

The Ewing tumor family, consisting of typical and atypical Ewing's sarcoma and peripheral primitive neuroectodermal tumor (PNET), shows little evidence of differentiation at the light microscopic level. Therefore, distinction from other small blue round cell tumors, like embryonal rhabdomyosarcoma, lymphoma or neuroblastoma is difficult. O13 immunohistochemistry is an additional diagnostic tools to identify selectively Ewing's sarcoma and PNET (1). However, O13 immunoreactivity is not restricted to these tumors and has been detected in some rhabdomyosarcomas and lymphomas as well (2-5). One highly specific feature of the Ewing tumor family is the presence of the reciprocal chromosomal translocation t(11;22)(q24;q12) in the tumor cells, present in 85-90% of the

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cases (6-9). The breakpoint has been cloned (10) and the two genes involved, FLI1 on chromosome 11q24 and EWS on chromosome 22q12, were identified (11,12). Several reports have described the RT-PCR detection of the resulting EWS-FLI1 fusion transcript as a valuable diagnostic tool to identify Ewing's sarcoma and PNET within the other small blue round cell tumors (13-17). Using this technique even fusion transcripts resulting from subtle structural abnormalities of 11q24 and 22q12 not found by cytogenetic analysis can be detected. The EWS-FLI1 fusion transcript can be identified in up to 95% of Ewing's sarcoma and PNET (18). In the remaining percentage of these tumors another translocation, t(21;22)(q22;q12), is found, resulting in an EWS-ERG fusion transcript which can also be detected by RT-PCR (18-20). Detection of the fusion transcript is highly specific since several other tumors, including rhabdomyosarcomas, small cell osteosarcomas and neuroblastomas are negative (13,15-17). We applied the RT-PCR translocation detection method as described (13-18) for implementation in routine diagnostic procedures. In this manuscript we demonstrate that the use of the wild type EWS gene, recommended as a control for RNA quality by Delattre *et al* (18), may lead to spurious signals when the RNA sample is contaminated with DNA. Pre-treatment of RNA with RNAse or DNAse before RT-PCR resulted in the identification of a novel EWS related product which turned out to be most likely an intronless EWS pseudogene. Since it is difficult to discriminate between PCR products of EWS-RNA and the EWS-pseudogene, false negative diagnoses may result from these misleading signals.

METHODS

Patient material: Snap frozen tissue, stored at -80°C, of 7 Ewing's sarcomas and several other tumors was studied. The diagnosis Ewing's sarcoma was based on histological, clinical-radiological and immunohistochemical data; all 7 cases studied showed immunoreactivity for the O13 antibody directed against the MIC2 protein. In addition, we used a Ewing's sarcoma cell-culture with cytogenetically proven t(11;22)(q24;q12) as a positive control. As a control for specificity, irrelevant normal, or tumoral tissue was used of 15 patients (including normal colon, ovarian carcinoma, melanoma of the eye, cervical carcinoma, papillary thyroid carcinoma, colon carcinoma, carcinoma of the breast, squamous cell carcinoma, rhabdomyosarcoma and small cell osteosarcoma).

DNA and RNA extraction: Total RNA extraction was performed with TRIzol (Gibco BRL Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's recommendation. This commercial guanidinium/phenol based reagent is claimed to be an improvement to the single-step RNA isolation

method developed by Chomczynski and Sacchi (21). 15 sections of 20 μm of snap frozen tissue were cut using a microtome. The microtome blade was thoroughly cleaned with 70% ethanol between cutting of individual specimens to minimize cross contamination. A cell suspension of 6×10^6 cultured Ewing sarcoma cells was centrifuged and the pellet was used for RNA isolation. DNA isolation from irrelevant freshly collected blood samples was performed according to the method of Miller *et al* (22). The DNA and RNA concentration and purity was determined by measuring the absorption at 260 and 280 nm at a spectrophotometer (Ultrospec plus, LKB Biochrom, Bromma, Sweden).

Reverse transcription: One or two μg of total RNA was reverse transcribed using 100 ng oligo dT (Boehringer Mannheim, Germany) in a total volume of 20 μl containing 1U RNAsin, 1 mM dNTP, 100 mM Tris-HCl, pH 8.3, 80 mM KCl, 12 mM $MgCl_2$, 2 mM DTT and 5U AMV-reverse transcriptase (Boehringer Mannheim, Germany). The tubes were incubated at 37°C for 1 hour. cDNAs were stored at -20°C.

Polymerase Chain Reaction: One μl of the resulted cDNA was used directly for amplification. PCR was performed in a total volume of 50 μl, containing 30 pmol of each primer, 0.2 mM dNTP (all purchased from Pharmacia, Woerden, the Netherlands), 50 mM KCl, 10 mM Tris (pH 8.3), 0.2 mg/ml Bovine Serum Albumin (BSA), 2 mM $MgCl_2$ and 1U Ampli-Taq (Perkin Elmer, Norwalk, CT, USA). All standard precautions were taken to prevent contamination during PCR-reaction. Thermal cycling was performed in a programmable heat block (Perkin Elmer Cetus, Norwalk, CT, USA). The amplification conditions for all primer sets used were as described (18); 30 cycles with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 68°C for 1 min and elongation at 72°C for 1 min. Ten μl of each PCR-product was subjected to electrophoresis in 1.2% agarose gels. A 100 bp length marker (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) was used to estimate the sizes of the obtained PCR-products. Amplification products were visualized by staining with ethidium bromide. To test for the presence of the EWS-FLI1 hybrid transcript, amplification was carried out with primers 22.8 and 11.11. These primers were prepared according to published sequences (18,20). EWS-FLI1 fusion PCR products vary in sizes ranging from 205 to 820 basepairs (12-14).

Controls: For confirmation that each RNA sample tested could potentially yield products after RT-PCR, transcripts of the wild type EWS or the housekeeping gene HPRT were amplified simultaneously under the same conditions as demanded for detection of fusion transcripts. For amplification of part of the wild type EWS cDNA, the same EWS forward primer (22.8) was used in combination with a reverse primer (22.4) (18). HPRT cDNA was amplified with primers hum1 (5'-ACCGGCTTCCTCCTCCTGAGCAGTC-3') and hum2 (5'AG-GACTCCAGATGTTTCCAAACTCAACTT-3'). To exclude PCR products resulting from DNA rather than RNA, two approaches were used. First, to test for the presence of contaminating genomic DNA in the RNA samples, each RNA sample was also incubated with a similar mixture without addition of AMV reverse transcriptase. Second, RNAse and DNAse treatments were performed; 2 μg of total RNA or DNA were treated with 1U RQ1 RNAse-free-DNAse (Promega, Madison, WI, USA) or RNAse (Stratagene, La Jolla, CA, USA) in a total volume of 12 μl containing 50 mM KCl, 10 mM Tris (pH 8.3), 0.2 mg/ml BSA, 2.5 mM $MgCl_2$ and 40 U RNAsin (Promega, Madison, WI, USA). Samples were incubated at 37°C for 1 hour and DNAse was inactivated by incubation at

80°C for 10 min. As a positive control for the EWS-FLI1 fusion transcript, RNA of cultured Ewing cells, with cytogenetically confirmed t(11;22) was included in each test. As a negative control H_2O was used as a template in each test to exclude contamination of primers, water or reagents.

Sequence analysis: The approximately 1200 bp PCR product that resulted from amplification of genomic DNA using M13-tailed primers 22.4 and 22.8 (CGAC-GTTGTAAAACGACGGCCAGT-22.8 and CAGGAAACAGCTATGAC-22.4) was sequenced. A second PCR was done with biotin labeled M13 primers using the product of the first PCR as template. The resulting PCR products were purified using the EasyPrep kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's recommendations. PCR products were alkali-denatured and single-strand fragments were captured using Dynabeads (ITK diagnostics, Uithoorn, the Netherlands). Sequencing reactions were performed on the Automated Laser Fluorescence DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's recommendations for the Autoread kit (Pharmacia biotech, Uppsala, Sweden). Computer assisted analysis of the obtained results was performed. Another approach was made using primers 22.4 and 22.8 (without M13 tails) which were radioactively labeled with $[^{32}P]$-dATP using T4 polynucleotide kinase. Sequencing was performed using the USB cycle sequencing kit (U.S.B., Cleveland, Ohio, USA) and thermal cycling took place in a programmable heatblock (Perkin Elmer, Norwalk, CT, USA) with 30 cycles consisting of annealing at 65°C and denaturation at 95°C. Samples were subjected to electrophoresis on standard denaturing 6% polyacrylamide gels containing 7M urea. Autoradiography was performed by exposition to X-ray films (Kodak) for 24-72 hours. Nucleotide sequences were determined by visual inspection. Obtained sequences were not verified by sequencing the opposite strand.

RESULTS

Reverse transcription and PCR reaction: In all seven Ewing's sarcomas tested and the Ewing's cells in culture with a proven t(11;22), EWS-FLI1 fusion transcripts were detected (Fig.1). No products were seen in the negative controls (H_2O) as well as in 15 carcinomas and sarcomas not belonging to the Ewing tumor family. The EWS internal control yielded a PCR product of the size as expected from previous studies (18) in all RNA samples tested. However, omission of reverse transcriptase, amplification of genomic DNA and pretreatment of DNA with RNAse revealed a product of the same size (Fig. 2A). After DNAse pretreatment of RNA samples, no PCR product was obtained anymore. These results indicate that the obtained products resulted from amplification of DNA, instead of reverse transcribed RNA. When we used the hypoxanthine phosphoribosyltransferase (HPRT) control as suggested by Foss *et al* (23), PCR products of the expected size (747 bp) were obtained only when RNA, after reverse transcription, was used as template (Fig. 2B). After
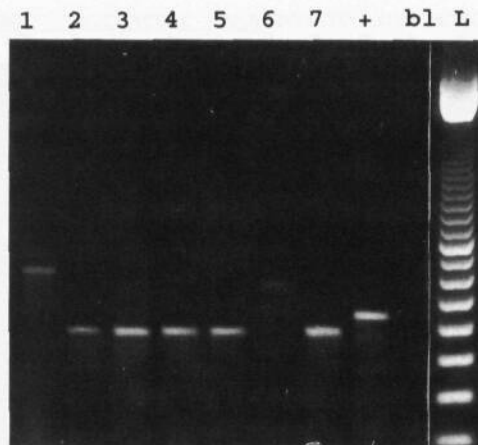
Figure 1. RT-PCR analysis of Ewing's sarcomas. Total RNA was extracted from archival fresh frozen tumor tissue, followed by RT-PCR analysis using primers on exon 7 of EWS and exon 9 on FLI1. Products were electrophoresed on a 1.2% agarose gel and visualized by staining with ethidium bromide. 1-7: Ewing's sarcomas, +: Ewing's sarcoma with cytogenetically proven t(11;22)(q24;q12), bl: H_2O as a negative control, L: 100 bp size marker.

pretreatment of DNA with RNAse and with omission of reverse transcriptase, no PCR product could be obtained.

Sequence analysis: From both the 5' and the 3' end of the EWS PCR product a partial nucleotide sequence could be obtained (Fig. 3). A sequence of 109 basepairs was obtained using the forward primer which showed 97% homology to the published sequence of the EWS-cDNA (12). In this region a correctly spliced junction between exons 7 and 8 in EWS (24) is present. From the 3' end, a nucleotide sequence of 346 basepairs could be obtained. Thirty-two mutations were found in this part relative to the EWS cDNA sequence (89% homology). Two splice junctions at the predicted positions in EWS, one between exons 14 and 15 and one between exons 15 and 16, are present. If the obtained sequences are translated in the same reading frame as the original EWS gene, various premature stops are observed.

DISCUSSION

The presence of either the EWS-FLI1 or the EWS-ERG fusion transcript is predicted to become the diagnostic criterium for the Ewing's tumor family in the future. The application of the RT-PCR translocation detection test for diagnostic purposes therefore requires appropriate controls for the validity of the test. To
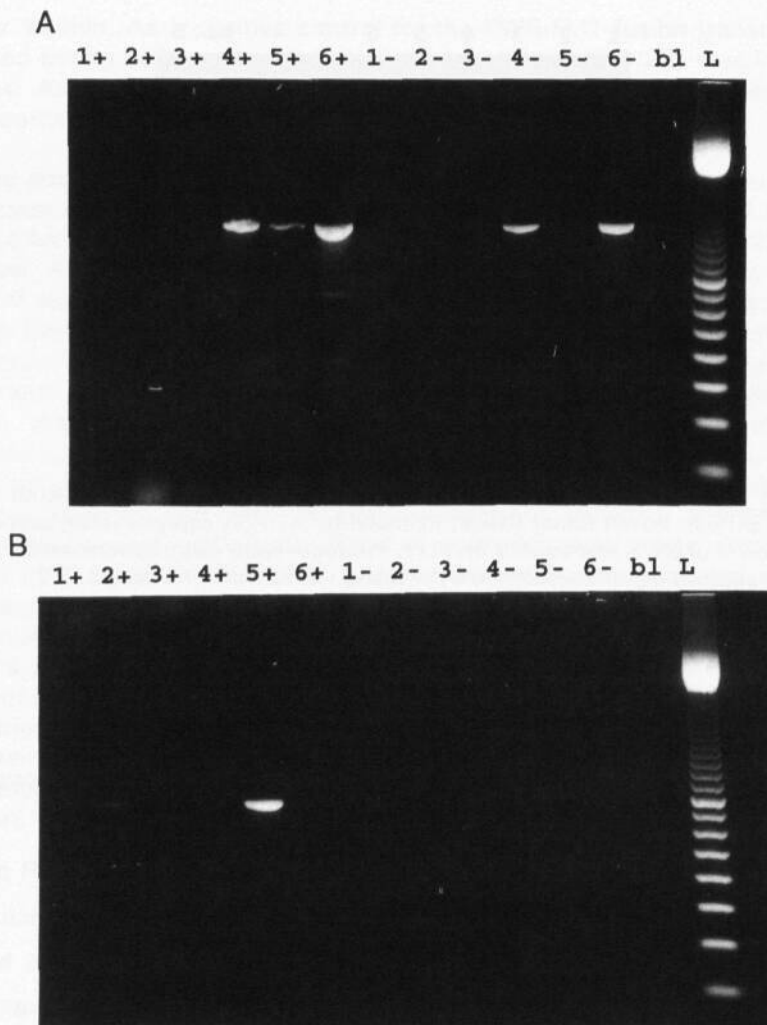
Figure 2. Testing and comparison of two RT-PCR internal controls for RNA quality and succeeding of the reverse transcription. Products were electrophoresed through 1.2% agarose gels and visualized using ethidium bromide staining. +: reverse transcriptase added in RT step, -: H_2O added instead of reverse transcriptase in RT step, 1: RNA treated with DNAse and RNAse, 2: RNA treated with DNAse, 3: DNA treated with DNAse, 4: DNA treated with RNAse, 5: RNA not treated, 6: DNA not treated, bl: H_2O as a negative control, L: 100 bp size marker. Both RNA and DNA were extracted from Ewing's sarcoma cells in culture using TRIzol.
A: Amplification of EWS internal control, as suggested by Delattre *et al.*(18). **B:** amplification of the HPRT housekeeping gene.

exclude contamination, a sample with H_2O should be included in each test. To exclude false negative diagnoses, an appropriate and reliable internal control to ensure RNA quality and reverse transcription is required since a positive internal

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751   CCCACTAGT  TACCCACCCC  AAACTGGATC  CTACAGCCAA  GCTCCAAGTC
      22.8 (forwardprimer)                             |||||
                                                       AAGTC

801   AATATAGCCA  ACAGAGCAGC  AGCTACGGGC  AGCAGA|GTTC  ATTCCGACAG
      |*||||||*|  ||||||||||  ||||||||||  ||||||||||  ||||*|||||
      AGTATAGCTA  ACAGAGCAGC  AGCTACGGGC  AGCAGA|GTTC  ATTCTGACAG

851   GACCACCCCA  GTAGCATGGG  TGTTTATGGG  CAGGAGTCTG  GAGGATTTTC
      ||||||||||  ||||||||||  ||||||||||  ||||||||||  ||||||||||
      GACCACCCCA  GTAGCATGGG  TGTTTATGGG  CAGGAGTCTG  GAGGATTTTC

901   CGGACCAGGA  GAGAACCGGA  GCATGAGTGG  CCCTGATAAC  CGGGGCAGGG
      ||||
      CGGA

951   GAAGAGGGGG  ATTTGATCGT  GGAGGCATGA  GCAGAGGTGG  GCGGGGAGGA
1001  GGACGCGGTG  GAATGGG|CAG  CGCTGGAGAG  CGAGGTGGCT  TCAATAAGCC
1051  TGGTG|GACCC  ATGGATGAAG  GACCAGATCT  TGATCTAG|GC  CCTCCTGTAG
1101  ATCCAGATGA  AGACTCTGAC  AACAGTGCAA  TTTATGTACA  AGGATTAAAT
1151  GACAGTGTGA  CTCTAGATGA  TCTGGCAGAC  TTCTTTAAGC  AGTGTGGGGT
1201  TGTTAAG|ATG  AACAAGAGAA  CTGGGCAACC  CATGATCCAC  ATCTACCTGG
1251  ACAAGGAAAC  AGGAAAGCCC  AAAGGCGATG  CCACAGTGTC  CTATGAAGAC
1301  CCACCCACTG  CCAAGGCTGC  CGTGGAATGG  TTTGATG|GGA  AAGATTTTCA
1351  AGGGAGCAAA  CTTAAAGTCT  CCCTTGCTCG  GAAGAAGCCT  CCAATGAACA
1401  GTATGCGGGG  TGGTCTGCCA  CCCCGTGAGG  GCAGAGGCAT  GCCACCACCA
1451  CTCCGTGGAG|  GTCCAGGAGG  CCCAGGAGGT  CCTGGGGGAC  CCATGGGTCG
1501  CATGGGAGGC  CGTGGAGGAG  ATAGAGGAGG  CTTCCCTCCA  AGAGGACCCC
1551  GGGGTTCCCG  AGGGAACCCC  TCTGGAGGAG  GAAACGTCCA  GCACCGAGCT
1601  GGAGACTGGC  AGTGTCCCAA  TCC|GGGTTGT  GGAAACCAGA  ACTTCGCCTG
                            |*          |||||||||||  |**|*|*|||  ||||||||||
                            AT          TCC|GGGTTGT  GAGACCGAGA  ACTTCGCCTG

1651  GAGAACAGAG  TGCAACCAGT  GTAAGGCCCC  AAAGCCTGAA  GGCTTCCTC C
      ||||||||||  |||*|*|||  |||||||*||  |||||||**|  |||||||||↓|
      GAGAACAGAG  ?GCACCGAGT  GTAAGGCTCC  AAAGCCTAGA  GGCTTCCTCCC

1701  CGCCACCCTT  TCCGCCCCCG  G|GTGGTGATC  GTGGCAGAGG  TGGCCCTGGT
      *|||||||||  *||*||||||  |||||  |*|||  ||||||||||  ||||||||||
      GGCCACCCTT  CCCACCCCCG  G|GTG.TAATC  GTGGCAGAGG  TGGCCCTGGT

1751  GGCATGCGGG  GAGGAAGAGG  TGGCCTCATG  GATCGTGGTG  GTCCCGGTGG
      ||||||*|||  ||||||||||  |||||*||||  ||||*|||||  ||||||||||
      GGCATGTGGG  GAGGAAGAGG  .AGCCTCATG  GATCATGGTG  GTCCCGGTGG

1801  AATGTTCAGA  GGTGGCCGTG  GTGGAGACAG  AGGTGGCTTC  CGTGGTGGCC
      ||||||||||  |||||*|||  ||*|||||||  |*||||||||  ||||||||| |
      AATGTTCAGA  GGTGGCTGTG  GTAGAGACAG  AAGTGGCTTC  CGTGGTGG.C

1851  GGGGCATGGA  CCGAGGTGGC  TTTGGTGGAG  GAAGACGAGG  TGGCCCTGGG
      *|||||||||  ||||||||||  ||||||||||  |||||*|||  ||||||||||
      TGGGCATGGA  CCGAGGTGGC  TTTGGTGGAG  GAAGACAAGG  TGGCCCTGGG

1901  GGGCCCCCTG  GACCTTTGAT  GGAACAGATG  GGAGGAAGAA  GAGGAGGACG
      |||||||*|  |||**|**||  |*|||**|||  ||||||||||  ||||||||||
      GGGCCCCCAG  GACTATGTAT  GTAACCAATG  GGAGGAAGAA  GAGGAGGACG

1951  TGGAGGACCT  GGAAAAATGG  ATAA|AGGCGA  GCACCGTCAG  GAGCGCAGAG
      ||*||||**|  |||||||              22.4 (reverse-
      TGAAGGAGGT  GGAAAAA

2001  ATCGGCCC
      primer)
```

Figure 3. Nucleotide sequences of parts of the EWS pseudogene (in italics) obtained by DNA sequencing are compared with the EWS cDNA sequence which was retrieved from the genome database (accession nr X66899). Exon junctions (24) are indicated with bars and primer sequences are underlined. (?: nucleotides that could not be determined .: deletion *: mutation ↓: insertion). A high percentage of homology (89% and 97%), three correctly spliced exon junctions and numerous stop codons were found.

control with a negative result for the fusion product determines that the diagnosis should be reconsidered.

In this manuscript we report the existence of an EWS pseudogene. Pseudogenes are homologs of active genes but they do not contain any of the introns and a polyA tail at the 3'-end can be found (25). Pseudogenes originate from retrotransposition (25) and the EWS mRNA may have been converted into DNA, which in turn was inserted into the genome. Pseudogenes do not encode functional proteins and the percentage of homology to the cDNA of the original gene decreases during evolution. A wide range of genes, especially the housekeeping genes, have been shown to have one or several retrotransposed homologs in the mammalian genome like, for instance, human ferrochelatase (26), dihydrolipoyl succinyltransferase (E2k) (27) and glyceraldehyde-3-phosphate dehydrogenase (23). To our knowledge no such genes have been reported to exist for EWS so far. We show that the length of the EWS PCR-products obtained by amplification of genomic DNA is identical to the size expected on basis of the cDNA sequence. By sequencing the PCR product obtained by amplification of DNA extracted from an irrelevant blood sample, we could determine a nucleotide sequence at both ends of the PCR product. These parts revealed a high percentage (97% and 89% respectively) of homology to the sequence of the EWS cDNA. Furthermore, three splice junctions were present at predicted positions and multiple stop codons were found. These results strongly indicate the presence of at least one EWS pseudogene in the human genome. Future studies should reveal whether this pseudogene is transcribed and where it is located in the genome.

Pseudogenes complicate the PCR analysis of (parts of) the original gene (23,26,28). Consequently, primersets which detect such pseudogenes are not recommended as internal control for RT-PCR based tests. An appropriate control for the RT-PCR translocation detection would be a gene, consistently expressed across a wide range of physiologic conditions in all cells and tumor cells. Therefore we tested PCR amplification of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) mRNA as suggested by Foss *et al* (23). Amplification of HPRT could be performed under exactly the same conditions as amplification of the EWS-FLI1 fusion transcript. PCR products of the expected size were only observed when RNA, after reverse transcription, was used as template. Therefore we recommend the HPRT primer set used in this study as

an appropriate and reliable control for application in the RT-PCR translocation detection for the Ewing tumor family.

With the molecular genetic elucidation of specific translocations like t(X;18)-(p11.2;q11.2) in synovial sarcoma (29), t(12;22)(q24;q12) in Clear Cell Sarcoma (30) and t(3;12)(q35;q14) in alveolar rhabdomyosarcoma (31), it might be expected that RT-PCR procedures will be increasingly implemented in routine diagnostic procedures. Therefore, in general we would suggest three different controls for translocation detection protocols; 1.) as a positive control RNA of a tumor (preferably of a tumor cell line for continuous supply) with a cytogenetically and/or molecular proven hybrid transcript; 2.) as a negative control H_2O as template to exclude contamination; 3.) an internal control to ensure RNA quality and reverse transcription for each test sample; preferably a housekeeping gene that can be amplified under exactly the same PCR conditions as the fusion product. In any way primer sets to be used for internal control should be thoroughly tested to ascertain that no pseudogenes can be detected, thus to exclude false positive internal control results which may lead to false negative diagnoses. This is the only way RT-PCR translocation detection tests can be reliably implemented in routine diagnostic procedures.

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