- sen, J. Nienhuis and E.W. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* 62:1943-1945.
3. Borriello, F. and K.S. Krauter. 1990. Reactive site polymorphism in the murine protease inhibitor gene family is delineated using modification of the PCR reaction (PCR + 1). *Nucleic Acids Res.* 18:5481-5487.
4. Gussow, D. and T. Clackson. 1989. Direct clone characterization from plaque and colonies by the polymerase chain reaction. *Nucleic Acids Res.* 17:4000.
5. Jensen, M.A. and N. Straus. 1993. Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. *PCR Methods Appl.* 3:186-194.
6. Moré, M.I., J.B. Herrick, M.C. Silva, W.C. Ghiorse and E.L. Madsen. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl. Environ. Microbiol.* 60:1572-1580.
7. Parkinson, J.M., J.R.G. Pöhler and D.M.J. Lilley. 1998. Catalytic and binding mutants of the junction-resolving enzyme endonuclease I of bacteriophage T7: role of acidic residues. *Nucleic Acids Res.* 27:682-689.
8. Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., p. 1.86 and E.12. CSH Laboratory Press, Cold Spring Harbor, NY.
9. Smit, E., P. Leeflang, B. Glandorf, J. Dirk van Elsas and K. Wernars. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* 65: 2614-2621.
10. Torsvik, V.L., J. Goksøyr and F.L. Daae. 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* 56:782-787.
11. Vitousek, P.M., J.D. Aber, R.W. Howarth, G.E. Likens, P.A. Matson, D.W. Schindler, W.H. Schlesinger and D. Tilman. 1997. Human alteration of the global nitrogen cycle: causes and consequences. *Issues in Ecology*, Spring. Ecological Society of America, Washington, D.C. 1:1-15.
12. Wenger, R.H. and P.J. Nielsen. 1991. Reannealing of artificial heteroduplexes generated during PCR-mediated genetic isotyping. *Trends Genet.* 7:178.
13. White, T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 314-322. In M.A. Innis, D.H. Gelfand, and J.J. Sninsky (Eds.), *PCR protocols: A Guide to Methods and Applications*. Academic Press, San Diego, CA.
14. Wintzingerode, F.V., U.B. Göbel and E. Stackebrandt. 1997. Determinations of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* 21:213-229.

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Relative Quantitative RT-PCR Protocol for *TrkB* Expression in Neuroblastoma Using *GAPD* as an Internal Control

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ABSTRACT

An RT-PCR protocol for the relative quantitative measurement of *TrkB* transcripts using glyceraldehyde-3-phosphate-dehydrogenase (*GAPD*) transcripts as an internal control is described. Both *TrkB* and *GAPD* sequences were co-amplified in the exponential phase of amplification using 5' biotinylated primers. The PCR products were subjected to PAGE, electro-transferred to nylon membrane and detected by a chemiluminescent procedure using alkaline phosphatase conjugated with avidin. Signals detected on X-ray film were analyzed by densitometry. The ratio between *TrkB* and *GAPD* expression levels was determined to normalize the expression levels of *TrkB* transcripts. Initially, strong signals of *GAPD* transcripts led to overexposure of X-ray film compared to those of *TrkB*, which

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causes difficulties in the accurate determination of the TrkB/GAPD ratio. To circumvent this problem, uniformly biotinylated GAPD primers were replaced with a mixture of biotinylated and non-biotinylated GAPD primers of the same sequence and concentration. GAPD signals detected on X-ray film were proportionally decreased as the amount of biotin-labeled primers was reduced in the total GAPD primers. This modification enabled both GAPD and TrkB signals to be analyzed within the linear range of X-ray film detection without affecting the amplification efficiency of TrkB sequence. Use of composite primers may have a wide range of applicability in quantitative analysis of nucleic acids.

INTRODUCTION

PCR is a well-established technique for the rapid amplification of specific DNA sequences in vitro. Amplification of RNA can also be achieved by a combination of reverse transcription (RT) and subsequent PCR. RT-PCR (1,8) is more sensitive than traditional RNA analysis techniques such as Northern blot analysis (11) and is able to detect rare RNA species and transcripts from small numbers of cells or small amounts of tissues. However, quantitative analysis by RT-PCR can be difficult because of the exponential nature of PCR. A small variation during the assay might yield a marked change in the amount of the final product (2). The use of internal standards is therefore desirable in quantitative RT-PCR analysis to correct variations in RT, PCR as well as product detection steps (3). An ideal endogenous standard would be a transcript in which the expression is constant during the cell cycle, between cell types or in response to external stimuli. A housekeeping gene *GAPD* that is transcribed constitutively in most cell types and tissues has been commonly used as an invariant control. Previous studies on *Trk* expression in neuroblastoma cell lines and tumors showed that *GAPD* was a suitable control (9,13).

To analyze PCR products, agarose or polyacrylamide gel electrophoresis is commonly used. PCR products separated on gels are visualized by ethidium bromide staining. More sensitive detection of PCR products is achieved by use

of blotting-based techniques in which the PCR products are first transferred to a membrane and are detected by hybridization with radioactively labeled probes. Alternatively, 5'-biotinylated primers are used to label PCR products, and the biotinylated PCR products are detected by chemiluminescence using horseradish peroxidase or alkaline phosphatase conjugated with avidin (4). In addition, 5'-fluorophore-labeled primers can also be used for the detection of small amounts of PCR products. A sensitive detection of PCR products is essential for quantitative RT-PCR analysis because PCR has to be performed at a reduced number of cycles to ensure that the amplification is in the exponential phase. Under these conditions, the amount of PCR products generated is too small to be detectable by dye staining.

We describe here a method for relative quantification of RNA expression by RT-PCR. This protocol addresses one of the common problems in the relative quantitative RT-PCR: the difference between expression levels of targets and internal controls. Using composite primers (a mixture of 5'-labeled and nonlabeled primers of the same sequence and concentration), we were able to modulate the apparent signal intensity of internal control detected on X-ray film. As a result, both signals of the gene of interest and internal control can be analyzed within the linear range of X-ray film detection. We also showed that the use of composite primers in relative quantitative PCR did not compromise the principle of the assay. Furthermore, the use of composite primers may have a wide range of applicability in the quantitative analysis of nucleic acids.

MATERIALS AND METHODS

Cell lines

All cell lines used in the study were from the collection of the cell bank at the Children's Hospital of Philadelphia.

Isolation of RNA

Total cellular RNA was extracted from neuroblastoma cell lines using the Totally RNA™ kit (Ambion, Austin,

TX, USA) according to the manufacturer's instructions. The concentration of RNA in these samples was determined by optical density (OD) of the samples at 260 nm and converting it to the RNA concentration using the cofactor of 1 OD = 40 µg/mL.

First-Strand cDNA Synthesis

The RT reaction was carried out using 2.5 µg of total RNA in a total volume of 20 µL containing 150 ng random hexamers (Life Technologies, Gaithersburg, MD, USA), 0.5 mM dNTPs, 10 mM dithiothreitol, 200 U SUPERSCRIPT™ II reverse transcriptase (Life Technologies) in the reaction buffer consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂. Initially, the total RNA was denatured at 70°C for 10 min and immediately chilled on ice. First-strand cDNA was obtained after 10 min at 23°C and 50 min at 42°C, followed by 70°C for 15 min. Two units of RNase H (Life Technologies) were then added to each reaction, and the resultant mixture was further incubated at 37°C for 20 min.

PCR

PCR was carried out in a final volume of 10 µL, containing 0.5 U of *Taq* Gold® DNA polymerase (PE Biosystems, Foster City, CA, USA), 200 µM dNTPs, 0.4 µM of each primer, in a buffer consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl₂ and 1 µL of the RT product (reverse-transcribed total RNA). The 5' biotin-labeled primers used for the amplification of a *TrkB* were 5'-GCAATG-ATGAT-GACTCTGCC-3' (sense) and 5'-GGA-ACACTTTTCCAAAGGCT-3' (antisense). The 5' biotin-labeled and nonlabeled primers used for the amplification of *GAPD* were 5'-CATC-AAGAAGGTGGTGAAGC-3' (sense) and 5'-GAGCTTGACAAAGTGGTC-GT-3' (antisense). *GAPD* primers were added together with *TrkB* primers in the same PCR. All samples were overlaid with mineral oil. Amplification was performed on a PTC-100™ programmable thermal controller (MJ Research, Woburn, MA, USA). The samples were first denatured at 95°C for 12 min, followed by 5, 10, 15 or 20 cycles of 95°C

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for 30 s, 55°C for 30 s and 72°C for 90 s. The final cycle was followed by a 5 min extension step at 72°C to ensure that the amplified DNA was double-stranded. The absence of cellular DNA contaminants was routinely checked by RT-PCR assays of negative control samples (no RT had been added). The following compositions of *GAPD* primers were used: (i) *GAPD* 1: 100% biotiny-

lated, (ii) *GAPD* 1:24: 4% biotinylated primers + 96% nonbiotinylated primers, (iii) *GAPD* 1:49: 2% biotinylated primers + 98% nonbiotinylated primers and (iv) *GAPD* 1:99: 1% biotinylated primers + 99% nonbiotinylated primers.

Detection of PCR Products

PCR products (10 µL) were subject-

ed to non-denaturing 6% PAGE (75 min, 90 V) in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3). DNA bands were electro-transferred onto nylon membrane (Hybond®-N⁺; Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 4°C overnight at 20 V in 0.25× TBE buffer. DNA was immobilized on the membranes by baking the blots at 80°C in a vacuum oven for 1 h and then exposing them to UV irradiation for 1 min using a UV-transilluminator (UVP, San Gabriel, CA, USA).

Detection of biotin-labeled DNA was performed according to the Southern-Light™ protocol (Tropix, Bedford, MA, USA) at room temperature. The blots were washed 2 × 5 min in the blocking buffer (0.5 mL/cm²) consisting of 0.2% I-Block™ reagent (Tropix), 0.5% SDS and 1× MBS (0.01 M MOPS, 0.15 M NaCl, 0.005 M NaOH) and incubated further in the blocking buffer (1 mL/cm²) for 10 min. Alkaline phosphatase-conjugated streptavidin in buffered 50% glycerol (AvidX-AP™; Tropix) was diluted 1:5000 in the blocking buffer. The blots were incubated for 20 min with constant agitation in the diluted avidin-alkaline phosphatase conjugate solution (0.2 mL/cm²), followed by 1 × 5 min wash in the blocking buffer and 3 × 5 min wash in the wash buffer (1 mL/cm²) consisting of 1× MBS and 0.5% SDS. After 2 × 2 min wash in 20 mM Tris-HCl (pH 9.8) containing 1 mM MgCl₂, the blots were briefly drained and placed on Saran® wrap. A thin layer of CSPD-ready-to-use-substrate solution (Tropix) was pipetted onto the blots (3 mL/100 cm²) and incubated for 5 min. After removal of the excess CSPD solution, the blots were placed into development folders (14 × 19 cm² clear polypropylene sheets; Tropix) and exposed to X-Omat™-AR film (Eastman Kodak, Rochester, NY, USA) for 5–10 min. Signals on X-ray film were captured by a Model 4910 high-performance charge-coupled device (CCD) camera (COHU, San Diego, CA, USA). The captured images were analyzed by NIH Image version 1.55 software.

Northern Blot Analysis

Expression of *TrkB* was examined by Northern hybridization using the 3.9

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kbp *TrkB* cDNA clone as a probe (7). Twenty micrograms of each RNA preparation were subjected to Northern blot analysis as previously described (10). The blot was washed in 0.1× standard saline citrate containing 1% SDS at 63°C and was exposed to X-ray film at -70°C overnight.

RESULTS AND DISCUSSION

The quantitative measurement of *Trk* family gene transcripts expressed in neuroblastoma cell lines and tumor specimens has traditionally been performed by Northern blot analysis using *ACTIN* or *GAPD* transcripts as invariant controls (5,12). Northern blot analysis requires at least 10 µg of total cellular RNA per sample, but there are times when available amounts of tumor RNA are limited. We therefore developed a duplex quantitative RT-PCR that requires only 1–2.5 µg RNA per sample. In our protocol, *TrkB* and *GAPD* (i.e., an internal control) sequences were amplified together in the same PCR. To accurately determine the normalized expression of *TrkB* (i.e., the ratio between *TrkB* and *GAPD* expression), PCR was performed in the exponential phase of amplification for both *TrkB* and *GAPD* sequences. To detect the small amounts of PCR products generated, PCR primers were biotin-labeled at their 5' ends, and the biotinylated PCR products were detected by an alkaline phosphatase-based chemiluminescence assay.

We found that the use of uniformly biotinylated *GAPD* primers in the duplex relative quantitative PCR produced much stronger *GAPD* signals on X-ray film as compared to those generated by *TrkB*. This effect was due to relatively high levels of *GAPD* transcripts expressed in the cell, which caused inaccurate determination of the *TrkB*/*GAPD* ratio. To circumvent this problem, we made a simple modification to this protocol to reduce *GAPD* signals detected on X-ray film without compromising the principle of relative quantitative PCR.

In the modified protocol, only a fraction of the *GAPD* primers used was biotinylated, while the rest were nonlabeled. The overall concentration of

GAPD primers in the PCR remained the same. We defined a mixture of 5'-labeled and nonlabeled primers of the same sequence and concentration as the composite primers. The use of composite *GAPD* primers made it possible for PCR amplification of both *TrkB* and *GAPD* sequences to be performed in primer excess conditions. Lowering *GAPD* primer concentrations to obtain less intensive signals was not desirable because the amplification kinetics of *GAPD* became completely different from that of the *TrkB* sequence (data not shown).

We first investigated the effect of partly biotinylated *GAPD* primers in relative quantitative RT-PCR. The expression of *TrkB* transcripts in the neuroblastoma cell line SMS-KCNR was examined using four different compositions of *GAPD* primers (Figure 1). *TrkB*

and *GAPD* sequences were amplified individually or together to compare the influence of different *GAPD* primer compositions on the amplification of a *TrkB* sequence (Figure 1). PCR was also carried out for 5, 10, 15 and 20 cycles to determine the optimal number of PCR cycles (Figure 1). Figure 1 shows that RT-PCR products were detected at the expected size both for the *GAPD* (160 bp) and the *TrkB* (247 bp). When 100% biotinylated primers were used, *GAPD* generated strong signals that overexposed X-ray film (*GAPD* 1, Figure 1A). *GAPD* signals were decreased proportionally as the amount of biotin-labeled primers was reduced in the total *GAPD* primers. We found that the composition of *GAPD* 1:49 (2% biotinylated + 98% nonbiotinylated primers) was optimal because the signal intensities of both *TrkB* and *GAPD* were ap-

Figure 1. Co-amplification of *GAPD* and *TrkB* sequences using composite *GAPD* primers. Expression levels of co-amplified *GAPD* and *TrkB* sequences were compared with those of individually amplified *TrkB* and *GAPD* sequences. RT-PCR products of *TrkB* (247 bp) and *GAPD* (160 bp) from the SMS-KCNR cell line after 5, 10, 15 and 20 cycles of PCR were analyzed on 6% polyacrylamide gels, followed by chemiluminescent detection. Four different compositions of the *GAPD* primers were used: (A) *GAPD* primers 1 (100% biotinylated); (B) *GAPD* primers 1:24 (4% biotinylated); (C) *GAPD* primers 1:49 (2% biotinylated) and (D) *GAPD* primers 1:99 (1% biotinylated). All four blots (A–D) were analyzed under the same conditions to minimize variations in transfer and detection. The PCR products were electrotransferred onto nylon membrane at the same time. DNA bands were detected simultaneously using the chemiluminescence procedure (see Materials and Methods), and the four blots were exposed simultaneously to the same X-ray film. Lane 1: molecular weight marker (biotinylated, 50–1000 bp); lanes 2–5: *GAPD* amplified individually; lanes 6–9: *GAPD* and *TrkB* amplified together; lanes 10–13: *TrkB* amplified individually. *GAPD* signals decrease proportionally to the composition of the biotin-labeled primers included in the total *GAPD* primers. *TrkB* signals show a similar intensity in all four *GAPD* primer compositions used. Minor bands appeared at the position slightly larger than the *TrkB* signal in panel A at 20 cycles are likely due to the signal of unprocessed *GAPD* transcripts.

proximately the same (Figure 1C). In addition, 20 PCR cycles were found to be optimal (lanes 9 and 13 of Figure 1, A–D). Furthermore, it was found that the intensity of *TrkB* signals was not affected by the co-amplification of *GAPD*. Similar levels of *TrkB* signal were observed when the *TrkB* sequence was individually amplified (Figure 1, A–D, lanes 12 and 13) or when the *TrkB* sequence was co-amplified with different compositions of *GAPD* primers (Figure 1, A–D, lanes 8 and 9).

Figure 2 shows the *TrkB* signals after 10, 15 and 20 PCR cycles of amplification using the four different compositions of biotinylated *GAPD* primers (columns in gray). These data were compared to those amplified in the absence of the internal control *GAPD* (columns in black). As shown in Figure 2, there was no significant difference between signals from both groups. The maximal variation of *TrkB* amplified individually was 14%, while the maximal variation of *TrkB* co-amplified with four different compositions of biotin-labeled *GAPD* primers was 10%. These data collectively indicate that the levels of *TrkB* expression determined were not affected by the composition of the *GAPD* primers used.

To further examine the validity of this RT-PCR protocol for quantitative analysis, we analyzed the ratio between *TrkB* and *GAPD* signals when four different compositions of *GAPD* primers were used. Figure 3 shows that when the composite *GAPD* primers (1%, 2% and 4% biotinylated primers in the total *GAPD* primers) were used, there was a linear relationship between the *TrkB*/*GAPD* signal ratio and the proportion of biotinylated *GAPD* primers. In contrast, when 100% biotinylated *GAPD* primers were used, the *TrkB*/*GAPD* ratio was out of the linear plot, which indicates that the *GAPD* signal that was detected exceeded the linear range of X-ray film detection. The data also indicated that there was no preferential usage of either biotinylated or nonlabeled *GAPD* primers in the PCR. Taken together, the ratio between the *TrkB* and the *GAPD* signal can represent the relative level of *TrkB* expression.

We also confirmed that PCR was in the exponential phase of amplification both for *GAPD* and *TrkB* sequences at PCR cycles used for quantitative analysis (20 cycles). The *TrkB* sequence was amplified together with the *GAPD* sequence using the *GAPD* primer composition of 1:49 (2% biotinylated) at dif-

Figure 2. *TrkB* signals under various PCR conditions using *TrkB* primers alone or with different compositions of *GAPD* primers. Gray columns show *TrkB* signals after 10, 15 and 20 PCR cycles of PCR amplification using four different compositions of biotinylated *GAPD* primers. These data were compared to those amplified in the absence of the internal control *GAPD* (black columns).

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ferent PCR cycles. The OD units of *GAPD* and *TrkB* were then plotted against PCR cycles (Figure 4). Figure 4 shows that PCR was in the exponential phase of amplification for both *GAPD*

Figure 3. Relationship between the *TrkB*/*GAPD* ratio and the proportion of biotinylated primers in the total *GAPD* primers. Relative quantitative RT-PCR analysis was performed using 20 PCR cycles. The ratio between *TrkB* and *GAPD* signals was plotted against the proportion of biotin-labeled primers in the total *GAPD* primers. The linear regression analysis shows that the two parameters have a linear relationship ($r = 0.95$) when the composite *GAPD* primers were used (1%, 2% and 4%).

Figure 4. PCR amplification kinetics of *TrkB* and *GAPD*. *TrkB* and *GAPD* sequences were co-amplified at the PCR cycles indicated. The composition of *GAPD* used was 1:49 (biotin-labeled:nonlabeled). Signal intensities of *GAPD* and *TrkB* were plotted against PCR cycles used, and the regression analysis was then performed. The results showed that both *GAPD* and *TrkB* sequences were amplified exponentially between 15 and 22 cycles of PCR.

and *TrkB* between 15 and 22 cycles.

Finally, we examined whether the results of our PCR-based assay for quantitative transcript analysis were consistent with those of Northern blot analysis (Figure 5). RNA samples from three neuroblastoma cell lines (SMS-KCNR, NBL-S and NLF) were first analyzed by Northern blot analysis. As shown in Figure 5, both full-length and truncated forms of *TrkB* (9 kb and 8.5 kb, respectively) were detected in SMS-KCNR, but not in NBL-S or NLF by Northern blot analysis. Similarly, the results of the RT-PCR showed high-level expression of *TrkB* in SMS-KCNR, very low-level expression in NBL-S and no expression in NLF. Un-

der the experimental conditions used, the RT-PCR method was more sensitive than the Northern blot technique and thus was able to detect very low levels of *TrkB* expression in NBL-S. It should be mentioned that the *TrkB* primers used in the RT-PCR experiments were specific for the full-length form, and the truncated form of *TrkB* was not detected. Taken together, the data by these two methods were consistent. We confirmed this by examining the expression of *TrkA* and *MYCN* in a cohort of neuroblastoma tumor specimens by our method and comparing our data with the results previously generated by Northern blot (6) (Tang and Ikegaki, unpublished data).

In summary, we describe here a method for relative quantitative analysis of RNA transcript expression using the RT-PCR technique. The relative quantitative RT-PCR assay described met two important criteria. First, PCR was in the exponential phase of amplification for both the internal control and sequence of interest. Second, signal intensities of the internal control and sequence of interest were within the linear range of X-ray film detection. This was achieved by the use of composite primers for the internal control. These features ensured accurate determination of the normalized expression of transcripts of interest. The protocol is simple to set and requires no major equipment to perform and so would be useful for relative quantification of transcript levels expressed in cells and tissues. Furthermore, the use of composite primers may have a wide range of applicability to quantitative nucleic acid analyses.

REFERENCES

1. Chelly, J., J.C. Kaplan, P. Maire, S. Gautron and A. Kahn. 1988. Transcription of the dystrophin gene in human muscle and non-muscle tissue. *Nature* 333:858-860.
2. Gilliland, G., S. Perrin, K. Blanchard and H.F. Bunn. 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 87:2725-2729.
3. Horikoshi, T., K.D. Danenberg, T.H. Stadlbauer, M. Volkenandt, L.C. Shea, K. Aigner, B. Gustavsson, L. Leichman et al. 1992. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the poly-

Figure 5. Comparison of the RT-PCR procedure with Northern blot analysis for *TrkB* expression in neuroblastoma cell lines. (A) Northern blot analysis was performed on total RNA samples of SMS-KCNR, NLF and NBL-S neuroblastoma cell lines. A full-length cDNA clone of human *TrkB* was used as a probe, which detected transcripts encoding full-length (9 kb) and truncated (8.5 kb) forms of *TrkB*. (B) RT-PCR analysis was performed to examine the expression of *TrkB* transcripts in the same set of cell lines, using *TrkB* primers alone (lanes 1–3) or *TrkB* and *GAPD* primers together (lanes 4–6). The composition of *GAPD* used was 1:49 (biotin-labeled:nonlabeled).

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- merase chain reaction. *Cancer Res.* 52:108-116.
4. **Martin, C.S., L. Butler and I. Bronstein.** 1995. Quantitation of PCR products with chemiluminescence. *BioTechniques* 18:908-913.
5. **Nakagawara, A., M. Arima, C.G. Azar, N.J. Scavarda and G.M. Brodeur.** 1992. Inverse relationship between trk expression and N-myc amplification in human neuroblastomas. *Cancer Res.* 52:1364-1368.
6. **Nakagawara, A., M. Arima-Nakagawara, N.J. Scavarda, C.G. Azar, A.B. Cantor and G.M. Brodeur.** 1993. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N. Engl. J. Med.* 328:847-854.
7. **Nakagawara, A., X.G. Liu, N. Ikegaki, P.S. White, D.J. Yamashiro, L.M. Nycum, J.A. Biegel and G.M. Brodeur.** 1995. Cloning and chromosomal localization of the human *TRK-B* tyrosine kinase receptor gene (NTRK2). *Genomics* 25:538-546.
8. **Rappolee, D.A., D. Mark, M.J. Banda and Z. Werb.** 1988. Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. *Science* 241:708-712.
9. **Svensson, T., M. Ryden, F.H. Schilling, C. Dominici, R. Sehgal, C.F. Ibanez and P. Kogner.** 1997. Coexpression of mRNA for the full-length neurotrophin receptor trk-C and trk-A in favourable neuroblastoma. *Eur. J. Cancer* 33:2058-2063.
10. **Tang, X.X., J.A. Biegel, L.M. Nycum, A. Yoshioka, G.M. Brodeur, D.E. Pleasure and N. Ikegaki.** 1995. cDNA cloning, molecular characterization, and chromosomal localization of NET(EPHT2), a human EPH-related receptor protein-tyrosine kinase gene preferentially expressed in brain. *Genomics* 29:426-437.
11. **Wang, A.M., M.V. Doyle and D.F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 86:9717-9721.
12. **Yamashiro, D.J., X.G. Liu, C.P. Lee, A. Nakagawara, N. Ikegaki, L.M. McGregor, S.B. Baylin and G.M. Brodeur.** 1997. Expression and function of Trk-C in favourable human neuroblastomas. *Eur. J. Cancer* 33:2054-2057.
13. **Yamashiro, D.J., A. Nakagawara, N. Ikegaki, X.G. Liu and G.M. Brodeur.** 1996. Expression of TrkC in favorable human neuroblastomas. *Oncogene* 12:37-41.
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