Utility of Linearly Amplified RNA for RT-PCR Detection of Chromosomal Translocations

Validation Using the t(2;5)(p23;q35) NPM-ALK Chromosomal Translocation

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The requirement for sufficient quantities of starting RNA has limited the ability to evaluate multiple transcripts using reverse transcriptase-polymerase chain reaction (RT-PCR). In this study, we demonstrate the utility of linear RNA amplification for RT-PCR analysis of multiple gene transcripts including a chromosomal translocation, using the t(2;5)(p23;q35) as a model. RNA from the t(2;5)-positive cell line, SU-DHL-1, and the t(2;5)-negative cell line, HUT-78, was extracted and exposed to two rounds of linear amplification. RT-PCR using cDNA from the resultant amplified (a) RNA and total RNA resulted in the 177 bp *NPM-ALK* fusion gene product from the SU-DHL-1 cell line, but not from aRNA or total RNA from the HUT-78 cell line. DNA sequencing of the RT-PCR products from total and aRNA of SU-DHL-1 cells demonstrated identical sequences corresponding to the *NPM-ALK* fusion gene. Evaluation of 25 snap-frozen tissue samples, including eight *NPM-ALK*-positive ALCLs demonstrated 100% concordance of t(2;5) detection between cDNA from total RNA and that from aRNA. Our results show that linear amplification of RNA can enhance starting RNA greater than 200-fold and can be used for rapid and specific detection of multiplex gene expression from a variety of sources. This method can generate a renewable archive of representative cDNA, which can be used for retrospective screening of stored samples as well as positive controls for the clinical molecular diagnostic laboratory. (*J Mol Diagn* 2004, 6:16–21)

Clinical samples generating nanogram quantities of RNA have been precluded from routine reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. The ability

to detect multiple chromosomal translocations, their resultant fusion transcripts and biomarkers from small tissue samples will be important for obtaining data useful for prognostic and diagnostic information. One potential approach is to enhance the levels of starting RNA. The amplification of RNA by *in vitro* transcription initially described by Eberwine et al^{1,2} has been shown to increase the amount of starting mRNA up to 200-fold and consistently preserve comparative mRNA levels when starting with 1 μ g of poly(A)RNA or 10 μ g of total RNA.^{3,4}

In vitro transcription-mediated linear amplification has emerged as a reliable method for generation of abundant quantities of RNA in which the pre-amplification relative proportions of individual transcripts are maintained in the amplified (a) RNA. aRNA has found utility in a variety of applications where enhancement of starting material is critical. aRNA has been used for gene expression analysis of single neurons,⁵ but it has been more commonly used to enhance starting material in complementary DNA (cDNA) microarray analyses,^{6–11} including our own studies.¹² Our RNA amplification method combines reverse transcription with *in vitro* transcription (IVT) to produce amplified RNA (aRNA). The RNA amplification method uses two primers for cDNA synthesis. The first primer is the dT/T7 primer. It is constructed (5' to 3') with 15 thymidine residues and the T7 promoter sequence. This primer binds to the poly adenosine tail of mRNA (mRNA) as a starting point for reverse transcription, preferential for mRNA. This also incorporates the T7 promoter sequence into the cDNA for the IVT. The template switch primer binds to the extra random nucleotides attached to the 3' end of the newly synthesized cDNA strand by the SuperScript II enzyme (Invitrogen, Carlsbad, CA). This allows for full-length reverse transcription of the mRNA population.

Supported by a grant from the National Leukemia Research Association to M.S.L., the ARUP Institute for Clinical and Experimental Pathology, and a grant CA 83984–01 from the National Institutes of Health to K.S.J. E.-J.

Accepted for publication August 28, 2003.

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To apply RT-PCR to minute samples for a variety of clinical testing, there is a need for robust methods which can amplify minute amounts of RNA without notably altering the information substance of the original RNA.^{13,14} In this study, we show the utility of a modified *in vitro* RNA amplification procedure as starting material for the detection of fusion transcripts that are associated with chromosomal translocations. We have used the *NPM-ALK* fusion gene characteristic of the t(2;5) chromosomal translocation as a model to characterize the sensitivity and specificity of the assay, however, this methodology should be applicable to all RT-PCR assays for chromosomal translocations.

The chromosomal translocation t(2;5)(p23;q31) results in the fusion of the catalytic domain of the anaplastic lymphoma kinase (*ALK*), to the 5'-end of the nucleophosmin gene (*NPM*) generating a constitutively activated oncogenic tyrosine kinase (*NPM-ALK*)^{15,16} and is detectable in up to 80% of anaplastic large cell lymphomas (ALCLs).¹⁷ Our results indicate that linear-amplified RNA can be used to enhance starting material for RT-PCR analysis from nanogram quantities of RNA. We demonstrate the utility of aRNA in the specific detection of the t(2;5) chromosomal translocation from a cell line and snap-frozen archived tissues in a rapid and straightforward manner. Furthermore, the global amplification generates an almost unlimited archive of representative cDNAs, which can be used to analyze multiple genes from archived tissue samples.

Materials and Methods

RNA Isolation

RNA was isolated from SU-DHL-1 and HUT-78 cell lines using the TRIzol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's protocol. Fifty to 100 μ g of snap-frozen tissue samples obtained from Primary Children's Medical Center, University of Utah (Institutional Review Board (IRB) number 10707) were homogenized and suspended in TRIzol. Briefly, 1 ml TRIzol was added for each million cells and cells were lysed on a rocker for 30 minutes at room temperature, until the solution was homogenized. Lysate was extracted with chloroform and precipitated with isopropanol overnight at -70°C . RNA was pelleted by centrifugation, washed twice in ethanol, and resuspended in DEPC-treated water. DNA was removed by DNase treatment and the resulting RNA was phenol/chloroform extracted and precipitated with sodium acetate and ethanol overnight at -70°C . RNA was pelleted, washed in ethanol, and resuspended in DEPC-treated water. The concentration and purity of RNA was determined based on O.D._{260/280} measurements. Total RNA quality was assessed by 2% agarose gel electrophoresis.

RNA Amplification

One μ g of total RNA obtained from cell lines and tissues was combined with 0.1 μ g oligo dT/T7 primer (5' AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT

AGG CGC T¹⁵ 3') (QIAGEN Operon, Valencia, CA), brought to a volume of 10 μ l, incubated at 70°C for three minutes, and cooled to room temperature. For first-strand cDNA synthesis, each sample was combined with 4 μ l 5X First-Strand Buffer, 2 μ l 0.1 mol/L DTT, 400U Superscript II (Invitrogen, Carlsbad, CA), 40U Rnasin (Promega, Madison, WI), 2 μ l 10 mmol/L dNTPs (Amersham Pharmacia, Piscataway, NJ), and 0.1 μ g of template switch primer (5' AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG 3'). Samples were incubated at 42°C for 90 minutes. Second-strand cDNA synthesis required each sample to be combined with 15 μ l Advantage PCR Buffer, Advantage Polymerase (Clontech, Palo Alto, CA), 2U RNase H (Invitrogen, Carlsbad, CA), and 10 mmol/L dNTPs. Samples were incubated at 37°C for 5 minutes, 94°C for 2 minutes, 65°C for 1 minute, and 75°C for 30 minutes. The reaction was terminated by adding 7.5 μ l 1 mol/L NaOH/2 mmol/L EDTA and incubating at 65°C for 10 minutes.

cDNA was extracted with linear acrylamide as a coprecipitant by phenol:chloroform:isoamyl alcohol extraction. cDNA was precipitated in 7.5 mol/L ammonium acetate and ethanol at -70°C overnight after which it was pelleted by centrifugation, washed twice with ethanol, dried, and resuspended in DEPC-treated water. cDNA was purified using Bio-6 chromatography columns (Bio-Rad, Hercules, CA) and flow-through was vacuum concentrated to 8 μ l.

cDNA was subjected to *in vitro* transcription with the T7 Megascript Kit (Ambion, Austin, TX) or the RiboAmp RNA Amplification Kit (Arcturus, Mountain View, CA) using manufacturer's protocols. RNA obtained from first round of amplification was extracted using TRIzol, and precipitated in isopropanol at -70°C overnight. aRNA was washed twice in ethanol and resuspended in 9 μ l DEPC-treated water.

Samples were subjected to a second round of amplification identical to the first round of amplification, except two aspects. In the second round of first-strand synthesis, the RNA was incubated at 70°C with random hexamers and 0.5 mg oligo dT/T7 primer was used to provide a priming site. Concentration and purity of total RNA and aRNA (after the second round of amplification) from cell lines and tissue samples was determined by O.D._{260/280} measurements and quality was assessed by electrophoresis on 2% agarose gels.

Reverse Transcription

Total RNA and aRNA from SU-DHL-1 and HUT-78 cell lines and tissue samples was reverse-transcribed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's protocol.

RT-PCR

cDNA from total and aRNA of cell lines and tissue samples were diluted to 50 to 1000 ng/ μ L. The primer set selected to amplify the t(2;5) translocation was: Forward: 5'-TCC CTT GGG GGC TTT GAA ATA ACA CC and Reverse: 5'-CGA GGT GCG GAG CTT GCT CAG C-3'

(Operon, Alameda, CA) (GenBank accession number S82740). The total *NPM-ALK* gene is 2043 bp and the RT amplicon is 177 bp, which spans the t(2;5) translocation breakpoint. The ubiquitously expressed gene, *GAPDH*, was amplified for each sample to ensure amplifiability of cDNA from respective RNA samples (Forward: 5'-CGA CCA CTT TGT CAA GCT CA- 3' Reverse 5'-AGG GGA GAT TCA GTG TGG TG-3') (GenBank accession number AF261085). The following are primer sequences for the housekeeping genes analyzed; *ABL* (Forward: 5'-CCC AAC CTT TTC GTT GCA CTG T- 3' Reverse: 5'-CGG CTC TCG GAG GAG ACG TAG A- 3') (GenBank accession number M14755); glucose-6-phosphate dehydrogenase (*G6PDH*) (Forward: 5'-CCG GAT CGA CCA CTA CCT GGG CAA G-3' Reverse: 5'-GTT CCC CAC GTA CTG GCC CAG GAC CA-3') (GenBank accession number AY158142); *ALAS* (Roche catalog number 3 302 504) and *HPRT* (Roche catalog number 3 261 891). RT-PCR was performed on a Perkin-Elmer 2600 Thermalcycler at the following conditions: denaturation at 94°C for 1 minute, annealing at 55°C for 40 seconds, and extension at 72°C for 40 seconds. Thirty cycles of amplification were performed with a 4°C-cooling step as conclusion.

DNA Sequencing

RT-PCR products from SU-DHL-1 total RNA and aRNA, amplifying *NPM-ALK*, were purified using QIAgen Nucleic Acid Purification Kit-Microcentrifuge (QIAgen, Valencia, CA), according to manufacturer's protocol. aRNA and total RNA cDNA templates were then diluted to 200 ng/ μ L in DEPC water. Six μ L of both samples was mixed with 8 μ L of 0.8 pmol/ μ L NPM primer and ALK primer (sequences and locations are listed in the section entitled "RT-PCR"), for a total of four samples (total RNA cDNA+NPM primer, total RNA cDNA+ALK primer, aRNA cDNA+NPM primer, and aRNA cDNA+ALK primer) and sequenced on an ABI Prism DNA Sequencer (Applied Biosystems, Foster City, CA).

Results

Linear RNA Amplification

In this study, we sought to establish the utility of aRNA as starting material for the RT-PCR analysis of gene expression using the *NPM-ALK* fusion transcript and housekeeping genes as targets. Various amounts of total RNA (50 to 1000 ng) isolated from the ALCL-derived t(2;5)-positive cell line, SU-DHL-1 and the t(2;5)-negative T-cell line HUT-78 were subjected to two rounds of linear amplification using a procedure adapted from Wang et al.⁶ Using 1000 ng of starting RNA template, we consistently observed approximately 15-fold amplification after the first round of amplification and an average of approximately 200-fold amplification after the second round (based on O.D._{260/280} measurements). Figure 1 illustrates total and aRNA from SUDHL-1, HUT-78, and a frozen tissue biopsy, size fractionated by 2% agarose gel electrophoresis and stained with ethidium bromide. The lanes

Figure 1. Agarose gel demonstrating total (lanes T) and amplified RNA (lanes A) from the t(2;5)-positive cell line, SUDHL-1, the t(2;5)-negative cell line, HUT-78, and a frozen tissue biopsy (case 3). Total RNA was extracted from cell lines and tissue sample and size fractionated on a 2% agarose gel. Total RNA was subjected to two rounds of linear amplification to yield aRNA and size fractionated on 2% agarose gels. Total RNA shows abundant 28s and 18s ribosomal RNA. Lanes of aRNA from cell lines and the tissue sample show a smear pattern ranging in size from 50-through 1000 bp, reflecting a spectrum of mRNA transcript sizes. Lane M is a 1000–50 bp size ladder.

containing total RNA (lanes entitled T) illustrates abundance of 28S and 18S ribosomal RNA while aRNA from all samples (lanes entitled A) show a smear ranging in size from 50 to 1000 bp, reflecting a spectrum of mRNA transcripts. RNA amplification of a frozen-tissue biopsy using the RiboAmp Kit from Arcturus yielded significantly higher amount of aRNA after even one round of amplification.

RT-PCR Analysis

To assess the fidelity of our RNA linear amplification procedure for RT-PCR analysis, we amplified the *NPM-ALK* fusion gene using cDNA obtained from total RNA and aRNA of the two cell lines, SU-DHL-1 and HUT-78. As shown in Figure 2, gel electrophoresis of RT-PCR products demonstrate a single band of 177 bp, corresponding to the *NPM-ALK* fusion transcript in cDNA derived from total RNA, (lane 1) and aRNA (lane 3,) obtained from the t(2;5)-positive cell line, SU-DHL-1 but not in total RNA and aRNA from the t(2;5)-negative HUT-78 cell line (lanes 5 and 7, respectively). The ubiquitous housekeeping gene, *GAPDH* was expressed in both cell lines, for each sample (lanes 2, 4, 6, and 8). DNA sequencing of RT-PCR products using total RNA and aRNA from the SU-DHL-1 cell line showed identical sequences matching that of the target *NPM-ALK* fusion gene transcript (data not shown).

In our next set of experiments, we determined the feasibility of this methodology on snap-frozen archived

Figure 2. Agarose gel electrophoresis of RT-PCR products using total and aRNA from the t(2;5)-positive cell line, SU-DHL-1 and the t(2;5)-negative cell line, HUT-78. After amplification of RNA, samples were subjected to reverse-transcription and the resultant cDNA for all samples was amplified for detection of the 177bp *NPM-ALK* fusion transcript. To verify RNA amplifiability and integrity, the 202-bp *GAPDH* gene was amplified for all samples. **Lanes 1 and 2** depict *NPM-ALK* and *GAPDH* gene transcripts obtained by RT-PCR using SU-DHL-1 cDNA from total RNA of SU-DHL-1 cells, respectively. **Lanes 3 and 4** depict *NPM-ALK* and *GAPDH* gene transcripts obtained from cDNA from aRNA of SU-DHL-1 cells, respectively. Amplification of *NPM-ALK* from cDNA obtained from total RNA of HUT-78 cells fails to show a RT-PCR product (**lane 5**) but show amplification of *GAPDH* (**lane 6**). Analysis of cDNA obtained from aRNA of HUT-78 cells also shows absence of *NPM-ALK* gene transcript (**lane 7**), but shows a band corresponding to the *GAPDH* transcript (**lane 8**). **Lanes labeled B** contain template-free (H₂O) controls. **Lane M** is a DNA size marker.

tissue samples. We analyzed snap-frozen tissue samples of 25 biopsies obtained from T-cell lymphoproliferative disorders including: three *NPM-ALK*-positive ALCLs (as determined by immunohistochemistry), five peripheral T-cell lymphomas not otherwise specified, two reactive lymph nodes with T-cell hyperplasias, and 15 large-cell lymphomas not otherwise specified. Of the 25 tissue samples analyzed, RT-PCR using cDNA obtained from aRNA, demonstrated a distinct 177-bp product corresponding to *NPM-ALK* fusion transcript from eight tissue samples, while the rest were negative (Table 1). A 100% concordance was observed between the RT-PCR detection of total RNA and aRNA for the *NPM-ALK* transcript as well as *GAPDH*. All samples were positive for the house-keeping gene, *GAPDH*, which ensured the adequate nature of the respective RNA sample.

To determine the level of sensitivity, we performed RT-PCR analysis using a 50 ng/ μ L concentration of cDNA obtained from total RNA and aRNA of the SU-DHL-1 cell line after serial dilutions as follows: 1:1, 1:10, 1:100, 1:500, and 1:1000. Subsequently, equal volumes (5 μ L) of the respective RT-PCR products were evaluated by agarose gel electrophoresis. Interestingly, we found that for *NPM-ALK* fusion transcript, the sensitivity was lower when using aRNA compared to total RNA. For example, use of a 50 ng/ μ L concentration of SU-DHL-1 cDNA from aRNA yielded little RT-PCR product, which was approximately 20 times less than when using approximately the same amount of total RNA cDNA (based on O.D._{260/280} measurements). Figure 3A illustrates the resulting RT-PCR products from serial dilutions of cDNA from total RNA of SU-DHL-1 cells. A clearly distinct band corresponding to *NPM-ALK* transcript (177 bp) can be identified in lanes labeled 1:1, 1:10, 1:100, and 1:500. The *GADPH* gene transcript was detectable at similar dilutions, however, as shown in Figure 3A, the levels were lower than that of *NPM-ALK*. cDNA from aRNA was also serially diluted in placental DNA. As shown in Figure 3B, *NPM-ALK* gene transcript was detectable at up to 1:1000 dilutions. Unlike cDNA from total RNA, the *GADPH* transcript levels from aRNA were consistently detectable at higher dilutions (1:1000) (see Figure 3B).

Table 1. Detection of NPM-ALK Fusion Transcript Using Total and aRNA from Archived Snap-Frozen Tissue Samples

Case no.	Diagnosis	RNA source	GAPDH	NPM-ALK
1	ALCL (<i>NPMALK</i> +)	Total RNA	+	+
	ALCL (<i>NPMALK</i> +)	aRNA	+	+
2	ALCL (<i>NPMALK</i> +)	Total RNA	+	+
	ALCL (<i>NPMALK</i> +)	aRNA	+	+
3	ALCL (<i>NPMALK</i> +)	Total RNA	+	+
	ALCL (<i>NPMALK</i> +)	aRNA	+	+
4	RLN	Total RNA	+	—
	RLN	aRNA	+	—
5	RLN	Total RNA	+	—
	RLN	aRNA	+	—
6	PTCL	Total RNA	+	—
	PTCL	aRNA	+	—
7	PTCL	Total RNA	+	—
	PTCL	aRNA	+	—
8	PTCL	Total RNA	+	—
	PTCL	aRNA	+	—
9	PTCL	Total RNA	+	—
	PTCL	aRNA	+	—
10	PTCL	Total RNA	+	—
	PTCL	aRNA	+	—
11	LCL	Total RNA	+	—
	LCL	aRNA	+	—
12	LCL	Total RNA	+	—
	LCL	aRNA	+	—
13	LCL	Total RNA	+	—
	LCL	aRNA	+	—
14	LCL	Total RNA	+	—
	LCL	aRNA	+	—
15	LCL	Total RNA	+	—
	LCL	aRNA	+	—
16	LCL	Total RNA	+	—
	LCL	aRNA	+	—
17	LCL	Total RNA	+	—
	LCL	aRNA	+	—
18	LCL	Total RNA	+	—
	LCL	aRNA	+	—
19	LCL	Total RNA	+	—
	LCL	aRNA	+	—
20	LCL	Total RNA	+	—
	LCL	aRNA	+	—
21	LCL	Total RNA	+	—
	LCL	aRNA	+	—
22	LCL	Total RNA	+	—
	LCL	aRNA	+	—
23	LCL	Total RNA	+	—
	LCL	aRNA	+	—
24	LCL	Total RNA	+	—
	LCL	aRNA	+	—
25	LCL	Total RNA	+	—
	LCL	aRNA	+	—

ALCL, anaplastic large cell lymphoma (CD30+, ALK+ T-cell lymphoma); RLN, reactive lymph node; aRNA, amplified RNA; PTCL, peripheral T cell lymphoma, not otherwise specified; LCL, large-cell lymphoma, not otherwise specified.

To address the lower sensitivity of aRNA compared to total RNA in RT-PCR detection of *NPM-ALK*, we performed additional experiments. We found that aRNA quality and quantity was strongly method-dependent. The use of the RiboAmp Kit from Arcturus not only enhanced the quantity of RNA after one round of amplification, but also enhanced the mRNA transcript length (data not shown). RT-PCR using aRNA obtained by the Arcturus Kit generally resulted in higher *NPM-ALK* transcript

Figure 3. Agarose gels show detection of *NPM-ALK* and *GAPDH* gene transcripts at increasing dilutions of cDNA from total and amplified RNA of SU-DHL-1 cells. *NPM-ALK* gene transcript is detected at 1:1; 1:10, 1:100, and 1:500 dilutions of cDNA obtained from total RNA (**A, upper panel**). Detection of *GAPDH* was also detectable up to 1:500 dilution (**A, lower panel**). Use of aRNA obtained from SU-DHL-1 cells demonstrated detection of *NPM-ALK* gene transcript at 1:1, 1:10, 1:100, 1:500, and 1:1000 dilutions (**B, upper panel**). Detection of *GAPDH* was also detectable up to 1:1000 dilution (**B, lower panel**).

levels compared to that obtained by the modified Eberwine method. In addition, extension of *in vitro* transcription from 4 hours to 6 hours resulted in higher quantities and longer mRNA transcripts. Finally, extension of reverse-transcription from 50 minutes to 60 minutes also resulted in higher *NPM-ALK* transcript levels.

To illustrate the potential utility of aRNA in the detection of gene transcripts other than *NPM-ALK*, we used total and aRNA obtained from sample number 3 for the analysis of five different genes, *ABL*, *GAPDH*, *G6PDH*, *ALAS*, and *HPRT*. As shown in Figure 4, distinct bands representing the respective transcripts were detected for all genes analyzed except for *G6PDH*. Comparison of gene transcript detection between total RNA and aRNA showed a high level of concordance although differences in relative levels of gene transcript were observed for example *ABL* (lanes 1 and 2). This set of data suggests that this approach is applicable for the detection of a wide variety of transcripts.

Discussion

The ability to analyze gene expression of small biopsies or even single cell populations is providing new insights into disease pathogenesis and may have potential applications in routine molecular diagnostics and disease prognostication. Expression profiling studies of lymphoma¹⁸ and breast cancer patients¹⁹ have revealed the clinical utility of a reduced set of genes, the expression of which may provide critical information regarding prognosis and response to therapy. The development of meth-

Figure 4. Agarose gel shows amplification of five control gene transcripts in addition to *NPM-ALK* from total and amplified RNA from frozen-tissue sample (ALCL number 3). **Lane M** represents DNA size markers. **Lanes 1 and 2** demonstrate *ABL* gene transcript from total RNA and aRNA, respectively; **lanes 3 and 4** demonstrate *GAPDH* gene transcript from total RNA and aRNA, respectively; **lanes 5 and 6** demonstrate absence of RT-PCR product for detection of *G6PDH* from total and aRNA; **lanes 7 and 8** demonstrate *ALAS* gene transcript from total and aRNA, respectively; **lanes 9 and 10** demonstrate *HPRT* gene transcript from total and aRNA, respectively; **lanes 11 and 12** show *NPM-ALK* gene transcript from total and aRNA, respectively. **Lanes** labeled **B** represent negative water controls.

ods that facilitate the rapid analysis of several gene transcripts with diagnostic and prognostic implications will be highly relevant in this regard. RNA amplification is an approach that has been used to increase starting RNA for global gene expression analysis. Most protocols involve the preparation of total cDNA using a specialized oligo-dT primer incorporating the sequence of an RNA polymerase promoter. In a subsequent *in vitro* RNA polymerase reaction, many copies of RNA are generated from each copy of cDNA. Amplified RNA has been used in several gene expression studies using microarray technology.⁶⁻⁸ It has also been reported in gene expression studies examining small population of cells with extremely low yields of total RNA. In this study, we report the feasibility of such an amplification technique that generates high quality aRNA for use in the RT-PCR detection of chromosomal translocation-associated fusion gene transcripts using the t(2;5) chromosomal translocation as a model.

By using two rounds of amplification, we consistently observed greater than 200-fold enhancement of RNA, when 0.5 to 1 μ g of starting RNA is used. Similar yields were obtained for RNA obtained from cell lines and archived snap-frozen tissue samples highlighting the wide clinical utility of the methodology. Importantly, we observed that different protocols resulted in different efficiencies of RNA amplification. In our experience, the RNA Amplification Kit from Arcturus was rapid, cost-effective, and yielded higher levels of aRNA than our home-brew modification of the Eberwine method (data not shown).

To determine the specificity of *NPM-ALK* fusion gene expression, we tested its expression in well-characterized cell lines that were either positive or negative for the t(2;5) translocation. Repeated experiments demonstrated our ability to detect the expression of the *NPM-ALK* fusion gene in the ALCL-derived SU-DHL-1 cell line but not in the HUT-78 cell line, highlighting the specificity of the reaction. DNA sequencing of the RT-PCR products from the SU-DHL-1 cell line confirmed the identity of the transcript as *NPM-ALK*. Furthermore, identical sequences were found in the RT-PCR products obtained from total RNA and aRNA, for *NPM-ALK*. In addition to cell lines, we were also able to detect the *NPM-ALK* transcript in aRNA samples obtained from snap-frozen tissue samples of three CD30-positive, *ALK*-positive anaplastic large cell lymphomas and five large-cell lymphomas, but not in 17 peripheral T-cell lymphomas, reactive lymph nodes, or large cell lymphomas of unspecified immunophenotype. More importantly, the 100% concordance with 0% false-positive and false-negative rate between the assay using total RNA and aRNA ensures high specificity in the detection of the fusion transcript even within archived snap-frozen tissue samples.

RT-PCR of the t(2;5) gene transcript was performed using serial dilutions of cDNA obtained from total and aRNA to determine the sensitivity of the reaction. We found that the sensitivity of RT-PCR detection of *NPM-ALK* was lower for reactions using cDNA obtained from aRNA (see Figure 3). We observed the requirement for 10 times cDNA from aRNA for similar results. This was however, dependent on the quality of starting RNA, the effi-

ciency of the amplification, and the target gene analyzed. As shown in Figure 4, relative expression levels of the housekeeping genes analyzed varied between aRNA and total RNA. For example, aRNA expression levels appeared to be higher than total RNA when the *GAPDH* and *HPRT* genes were amplified. However, the *ABL* and *ALAS* genes demonstrated the opposite effect, where expression levels were lower in aRNA than total RNA. Subsequent experimentation using primer sets with varying annealing locations on the *ABL* gene demonstrated higher expression levels with primer sets located further 3' of the target gene. These data highlight the importance of analysis of primer locations as well as the potential for greater sensitivity by using aRNA for RT-PCR. One potential explanation for the low sensitivity may be due to the processivity of the dT/T7 first-strand primer during RNA amplification.²⁰ In reactions where processivity is low, there may be overrepresentation of 3' ends of mRNA. While, if the transcript being detected is located far upstream (5'), it may be under-represented during RNA amplification. One possible solution for this would be to increase the time of first-strand cDNA synthesis, allowing the dNTP's additional time to anneal further 5' of mRNA. Alternatively, closer attention to design of the primers used for RT-PCR such that they are located more 3' would also improve the sensitivity. Future studies need to address improving the bias in the relative amplification of low-, medium-, and highly expressed transcripts.

aRNA derived from two rounds of amplification is adequate to perform between 20 and 50 reverse transcription reactions for use in RT-PCR. This would make it highly useful as a cDNA control bank for positive and negative controls for a clinical molecular diagnostics laboratory performing analyses for the detection of chromosomal translocations. Furthermore, aRNA can be used not only in the detection of chromosomal translocations, but can also be used for limited mRNA profiling studies. The application of this technology to clinical samples yielding small quantities of RNA, such as small excisional biopsies, fine-needle aspirates, and microdissected tissue samples may facilitate the standard use of these samples for RT-PCR analyses. This is the first report demonstrating the combined application of RNA amplification in the sensitive and potentially quantitative RT-PCR detection of a chromosomal translocation and holds promise in the routine molecular diagnostics laboratory setting.

Acknowledgments

We thank G. Chris Fillmore and Robert T. Abbott for providing cell cultures and Sam Page for DNA sequencing data.

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