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A Comparison of Methods for RNA Extraction from Lymphocytes for RT–PCR

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Various methods have been described to extract RNA from nonadherent mammalian cells or are provided in protocols accompanying various commercially available reagents.[(1–10)] Reverse transcriptase–PCR (RT–PCR) allows the amplification, even the quantitation, of previously undetectable amounts of mRNA.[(11,12)] In this burgeoning field, some major points of interest have not yet been investigated: (1) comparison of the cited methods (given the importance of RT–PCR, limiting dilutional assays of cDNA transcripts is a better end point than other RNA parameters like spectrophotometry, Northern blotting, etc.); (2) whether total RNA or poly(A) RNA extraction is more favorable for subsequent RT–PCR; and (3) a detailed comparison of cDNA synthesis with oligo(dT), random hexamer, or RT–PCR downstream primers.

We have addressed these questions by extracting RNA from human lymphocytes. The extracted RNA [either poly(A) or total] was reverse-transcribed, and the cDNA was subjected to PCR-amplifying β-actin, CD3, and γ/δTCR rearrangement Vδ2Jδ1.[(13–15)]

MATERIALS AND METHODS

Lymphocytes

Peripheral blood mononuclear cells (PBMC) from two healthy males (31 and 32 years old) were isolated by the Ficoll Hypaque method.[(16)] Cells were counted, and the cell suspension was either processed immediately or frozen in liquid nitrogen. We harvested 1.6×10^6 and 3×10^6 PBMC/ml. The cells were cryoconserved in RPMI 1640 containing 10% fetal calf serum and 30% dimethylsulfoxide (DMSO) (all reagents from Sigma, St. Louis, MO). Cells were either pelleted by centrifuging for 5 min at 12,000*g* or exposed to the lysis agent without centrifugation (volume 1:1). To estimate the effect of cell washing, one aliquot was washed three times with Hanks' balanced saline solution (HBSS) and one was left unwashed.

RNA Extraction

Nine different methods to extract total RNA and five to extract poly(A) RNA were applied to aliquots of the samples of PBMC (Table 1, Refs. 1–10). The following commercially available kits were

TABLE 1 Comparison of RNA Extraction Methods

Method	According to	Ref.	RNA	β-actin[a]	CD3[a]	Vδ2Jδ1[a]	Labor intensity[b]
1	Chomczynski	1	total	N.D.	10^{-3}	N.D.	2
2	Siebert and Chenchik	2	total	N.D.	10^{-3}	N.D.	4
3	Vauti + Siess	6	total	N.D.	10^{-2}	N.D.	2
4	Kamdar + Evans	5	total	N.D.	10^{-2}	N.D.	3
5	Zolfaghari	8	total	N.D.	10^{-2}	N.D.	3
6	Tavangar	3	total	N.D.	10^{-2}	N.D.	1
7	Iowa Biotech Corp.	10	total	10^{-6}	10^{-5}	10^{-2}	6
8	MRC, Inc.	9	total	10^{-6}	$10^{-4.75}$	10^{-2}	5
9	Biotecx, Inc.	—	total	10^{-6}	$10^{-4.75}$	10^{-2}	5
10	Hartmann; Celano	4, 7	poly(A)	N.D.	$10^{-3.5}$	N.D.	5
11	Amresco, Inc.	—	poly(A)	N.D.	10^{-4}	N.D.	4
12	Invitrogen Corp.	—	poly(A)	10^{-6}	10^{-5}	10^{-2}	4
13	Dynal, Inc.	—	poly(A)	N.D.	10^{-4}	N.D.	5
14	Quiagen, Inc.	—	poly(A)	N.D.	10^{-4}	N.D.	6

Fourteen different methods of RNA extraction [either total or poly(A)] were applied with limiting dilutional assays of three different transcripts (β-actin, CD3, Vδ2Jδ1).

[a]The maximum dilution of cDNA with positive amplification (as measured by ethidium bromide-stained agarose gels of PCR products) in 50% of replicate assays is given. The figures were obtained from 10^6 lymphocytes. RNA was dissolved in 50 μl of DEPC water. Ten microliters was reverse-transcribed with oligo(dT) in a total volume of 20 μl. One microliter of the latter was subjected to PCR or subsequently diluted. At least four replicate assays per dilutional step were run. (N.D.) Not determined.

[b]Subjective rating: (1) Most laborious; (6) most rapid and time saving. Criteria were total amount of time employed, number of centrifugation and washing steps, and convenience in terms of heating/cooling reagents and reactions.

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TABLE 2 Different PCR Assays Employed

β-actin (Ref. 13)
Primer XAHR20: 5′-ACCCACACTGTGCCCATCTA-3′
Primer XAHR17: 5′-CGGAACCGCTCATTGCC-3′
2.0 mmoles of $MgCl_2$, 15 pmoles of each primer, 30 cycles of 92°C(1′)/55°C(1′)/72°C(2′), 1 unit of *Taq* polymerase (Amplitaq, Perkin Elmer Corp., Norwalk, CT), 50 μl of master mix

CD3 (Ref. 14)
Primer CD3 us[a] 5′-CTGGACCTGGGAAAACGCATC-3′
Primer CD3 ds[a] 5′-GTACTGAGCATCATCTCGATC-3′
1.5 mmole of $MgCl_2$, 20 pmoles each primer, 38 cycles of 95°C(40″)/55°C(40″)/72°C(1′), 1 unit of *Taq* polymerase, 50 μl of master mix.

Vδ2Jδ1 (Ref. 15)
Vδ2 5′-ACCCTCAGGTGCTCCATGAA-3′
Jδ1 5′-TCCACAGTCACACGGGTTC-3′
3.0 mmoles of $MgCl_2$, 25 pmoles each primer, 38 cycles of 95°C(40″)/60°C(40″)/72°C(1′), 1 unit of *Taq* polymerase, 50 μl of master mix.

[a](US) Upstream; (ds) downstream.

applied: Catrimox 14 (Iowa Biotechnology Corp., Oakdale), Dynabeads mRNA purification kit (Dynal Inc., Lake Success, NY), Microfast Track mRNA isolation kit (Invitrogen Corp., San Diego, CA), Oligotex direct mRNA kit (Quiagen Inc., Chatsworth, CA), rapid mRNA purification kit (Amresco Inc., Solon, OH), Tri Reagent [Molecular Research Center (MRC) Inc., Cincinatti, OH], and Ultraspec RNA isolation system (Biotecx Laboratories Inc., Houston, TX). Total RNA or poly(A) RNA was dissolved in 50 μl of DEPC-treated water (the DEPC water was heat inactivated after 12 hr of incubation).

cDNA

Ten of the 50-μl samples were reverse-transcribed in a 20-μl reverse transcriptase (RT) master mix using Superscript II RNase H$^-$ reverse transcriptase kit reagents and protocol (GIBCO BRL, Gaithersburg, MD). Random hexamer primers (150 pmoles) (Perkin Elmer Corp., Norwalk, CT), oligo(dT18) primer (150 pmoles) (New England Biolabs, Beverly, MA), or the downstream primer of the subsequent PCR (75 pmoles) (syn-

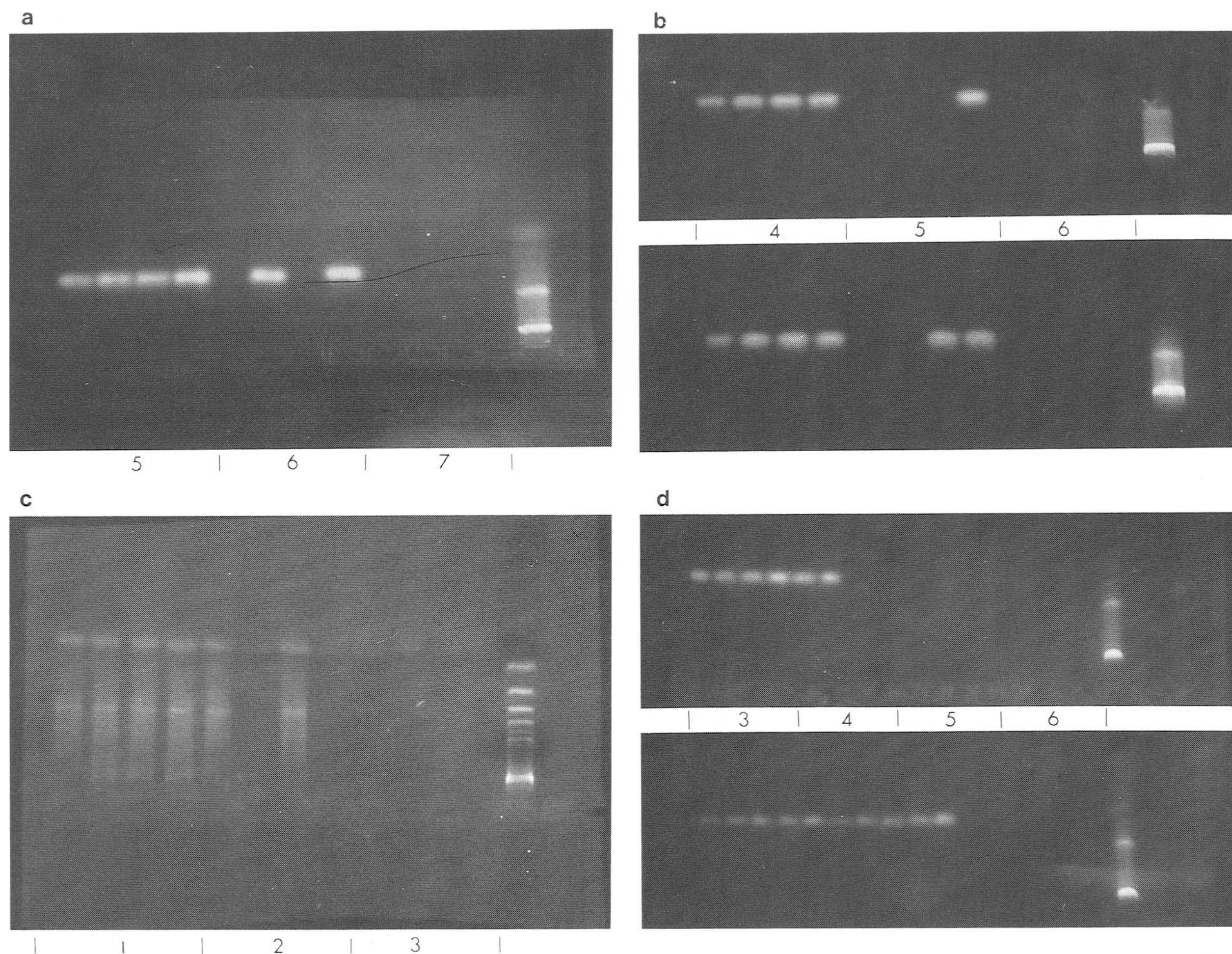

FIGURE 1 Ethidium bromide-stained 2% agarose gels of the PCR products. Quadruplicate assays per dilutional step were run. Dilution 10^{-x} indicated. Molecular size marker: 100-bp ladder. (*a*) β-Actin; (*b*) T-cell marker CD3 (*top* gel, method 8; *bottom* gel, method 7); (*c*) Vδ2Jδ1 T-cell receptor rearrangement; (*d*) reverse transcription with random hexamer primer (*top* gel) as compared with oligo(dT) (*bottom* gel), CD3 amplicon.

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thesized by Gene Link Inc., Thornwood, NY) were used to initiate cDNA synthesis.

PCR

Three different PCR assays were run; details of the assays are shown in Table 2. For each PCR, the master mix was prepared with either potassium chloride (500 mM in 10× buffer) or with potassium glutamate (1000 mM in 10× buffer).[17] Ethidium bromide-stained agarose gels showing PCR products are depicted in Figure 1.

RESULTS AND DISCUSSION

From this study, it became apparent that total RNA extraction with time-saving commercially available reagents and subsequent reverse transcription with either oligo(dT) or the RT–PCR downstream primer were the methods of choice for RT–PCR. In this setting the isolation of poly(A) RNA offered no particular advantage when compared with total RNA extraction.

Dilutional end points of β-actin, CD3, and Vδ2Jδ1 transcripts and subjective ratings of labor intensity are given in Table 1 (for examples, see Figure 1). Hence, methods 7–9 and 12 are the methods of choice. Method 12 was more laborious and expensive. We consider the small differences in dilutional end points between these methods as nonsignificant. Labor intensity rendered method 7 the most favored when processing larger sample numbers. In addition, no phenol waste was produced and samples could be processed conveniently at room temperature. Additional points of importance include the observation that after reverse transcription with oligo(dT) or RT–PCR downstream primer, the dilutional end point of the subsequent PCR was 10-fold lower than with random hexamer primer (Fig. 1d) and that washed pelleted cells offered no advantage over unwashed cells. In addition, pelleting of the cells led to 10–100 lower dilutions than did direct exposure to the lysis agent. We also found that the same dilutions could be reached with cryoconserved or immediately processed cells and that DNA contamination could not be detected in total RNA processed according to methods 1–14. Moreover, a temperature of 43°C for reverse transcription slightly increased the yield of cDNA in comparison with 37°C. Finally, a PCR master mix containing potassium glutamate instead of potassium chloride offered no particular advantage.

Thus, although all methods had their merits, the method of choice for the work described is total RNA extraction with commercially available reagents.

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