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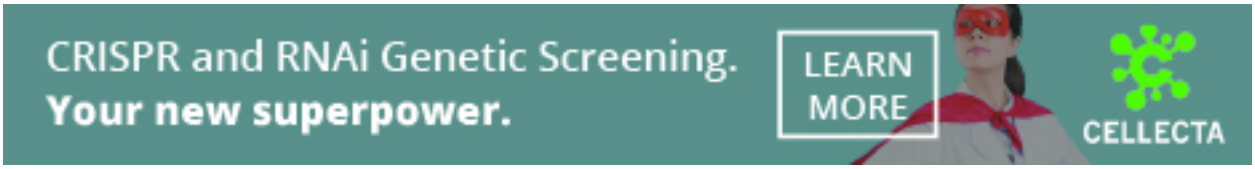

Use of Stock Solutions to Simplify mRNA Quantitation by Reverse Transcription–PCR Assays

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Measurement of small amounts of mRNA can be performed by combining the sensitivity of PCR with the specific generation of cDNA by reverse transcription (RT). RT-PCR is currently used to measure both relative and absolute amounts of mRNA (1–3).

Because the RT-PCR assay is carried out in a small volume that contains many reagents, experimental error can occur during the addition of microliter quantities of reagents to individual assays. Such experimental error can be greatly reduced by the use of premixed stock solutions that are frozen in unit-of-use aliquots. We describe six stock solutions that are sufficient to carry out the entire RT-PCR reaction; we also demonstrate that both *Taq* polymerase and AMV reverse transcriptase can be frozen in solutions appropriate for their reaction conditions. Note that the RT-PCR stock solutions described here are not simply commercial PCR buffer solutions; some of them are prepared with commercial PCR buffers.

By using frozen, unit-of-use aliquots of premixed reagents, we have found that the reproducibility of this sensitive assay is quite satisfactory. In addition, the RT-PCR experimental protocol itself is greatly streamlined, which again improves reproducibility.

MATERIALS AND METHODS

Cell Culture and RNA Isolation

Cells were maintained in 50% Dulbecco's MEM/50% Ham's F-12 containing 10% newborn calf serum plus 100 U/ml penicillin and 100 μg/ml of streptomycin. MG-63 human osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). Total RNA was isolated from subconfluent cultures of MG-63 cells by the single-step procedure of Chomczynski and Sacchi.[4]

RT-PCR

We have found that the RT-PCR assay can be performed with only six solutions (Table 1) and that both AMV reverse transcriptase and *Taq* DNA polymerase can be frozen in the appropriate reagents required for RT-PCR. Briefly, a frozen solution containing all of the reagents for RT (Table 1; solution 4, AMV mix) was thawed rapidly in a 37°C water bath, placed on ice, and divided into the desired number of assay tubes containing preannealed RNA and antisense primer in the appropriate volume of water. Whenever feasible, we added RNA and primers in as large a volume as possible to again reduce the possibility of pipetting errors. After a 30-min incubation at 42°C to generate cDNA, an aliquot of *Taq* DNA polymerase solution (Table 1; solution 6, *Taq* mix) and sense primers were added and PCR was carried out with a thermocycling program. A more detailed description of the protocol employed is presented in Table 1.

Measurement of Integrin mRNA Levels by RT-PCR

The sequences of integrin α_2 and α_5 PCR primers have been reported previously.[5] Primers were 5′-end-labeled with [γ-^{32}P]ATP (>3000 Ci/mmole) by T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH)[6] to a specific activity of at least 2×10^7 dpm/μg, and each reaction employed 2×10^6 to 4×10^6 dpm.

Integrin mRNA levels were determined using the protocol described in detail in Table 1. Following RT-PCR using ^{32}P-end-labeled primers, bands of PCR products were visualized by ethidium bromide staining and then excised from 5% polyacrylamide gels. The radioactivity in each band was determined by scintillation counting. Samples were obtained at PCR cycle numbers within the exponential range of amplification (cycles 22–24 in the case of both the α_2- and α_5-mRNA obtrained from MG-63 cells).

RESULTS AND DISCUSSION

The RT-PCR assay can be performed with only six solutions (Table 1); both AMV reverse transcriptase and *Taq* DNA polymerase can be frozen in the appropriate reagents required for RT-PCR. We compared freshly mixed AMV and *Taq* solutions with the same solutions after several cycles of freezing and thawing and observed little change in PCR product generated even after five cycles of freez-

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TABLE 1 Stock Solutions for RT-PCR

Solution 1. RNA sample
Solution 2. Antisense primer (200 ng/assay; stock solution = 200 ng/μl)
Solution 3. Diethylpyrocarbonate (DEPC)-treated water

Solution 4. **AMV mix**

Solution	Conc./assay	100 assays (μl)
a. 10× RT buffer (Cetus PCR buffer)[a]	0.8×	160
b. 1 M Tris (pH 8.3) (checked with pH paper)	50 mM[b]	84
c. 5 mg/ml of BSA (BRL)	5 μg	100
d. 0.5 M dithiothreitol (DTT)	12.5 mM	50
e. 0.1 M $MgCl_2$ (or as required by primer set)	8 mM[c]	136
f. 40 U/μl RNasin	20 units	50
g. dNTPs (25 mM each) (Pharmacia)	2 mM	40
h. 20 U/μl AMV (Life Sciences)	1 unit[d]	5
	Final volume	625

Store AMV stock solution frozen in tubes containing 6.5 assays per tube (40.6 μl/tube) and use 6.25 μl/assay. The RT reaction is carried out in a final volume of 20 μl.

Solution 5. Sense primer (either 200 ng of cold primer/assay or 100 ng of cold primer/assay + 100 ng ^{32}P-labeled primer/assay; working solution = 100 ng/μl).

Solution 6. ***Taq* mix**

Solution	Conc./assay	100 assays (μl)
a. Water	—	6870
b. 10× *Taq* DNA polymerase buffer (Promega)[e]	0.8×	800
c. 25 mM dNTPs (Pharmacia)	0.8 mM[f]	80
d. 5 U/μl *Taq* DNA polymerase (Promega)	2.5 units	50
	Final volume	7800

Store *Taq* stock solution frozen in tubes containing 6.5 assays per tube (507 μl/tube) and use 78 μl/assay + 2 μl/assay sense primer = 80 μl/assay final volume. The PCR final volume is 20 μl RT + 80 μl PCR (above) = 100 μl.

Presented are the solutions used for RT-PCR, their source, the final concentration of each reagent in the RT-PCR assay, the volume of each stock solution used to prepare 100 assays, and the volume of the assay mix sufficient for 6.5 assays. Concentrations of $MgCl_2$, dNTPs, primers, *Taq*, and AMV should be optimized for the primer set employed. The RT-PCR assay is carried out as described previously.[9] Note that Promega 10× *Taq* DNA polymerase buffer should not be used for the RT step owing to inhibition of reverse transcriptase by detergents. Briefly, 6.25 μl of AMV solution is added to 200 ng of antisense primer preannealed to RNA in 13.75 μl of DEPC-treated water to give a final volume of 20 μl. RNA should be added in as large a volume as practical. After 30 min of RT at 42°C, 78 μl of *Taq* solution and 2 μl containing 200 ng of sense primer (100 ng cold + 100 ng ^{32}P-labeled) are added and thermocycling is carried out for a predetermined number of cycles.

[a]Cetus 10× PCR buffer consists of 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, and 0.1% gelatin (wt/vol).

[b]The Tris-HCl in the 10× RT buffer plus the 1 M Tris-HCl supplement yields a final concentration of 50 mM Tris-HCL.

[c]The $MgCl_2$ in the 10× RT buffer plus the 0.1 M $MgCl_2$ supplement yields a final concentration of 8 mM $MgCl_2$ (6 mM free $MgCl_2$ + 2 mM dNTP bound $MgCl_2$).

[d]Recommend using 1 unit of AMV reverse transcriptase per assay for low to moderate abundance mRNAs and 10 units per assay for high-abundance mRNAs (one must optimize AMV reverse transcriptase for a particular target RNA).

[e]Promega 10× PCR buffer consists of 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 0.1% gelatin (wt/vol), and 1% Triton X-100.

[f]0.8 mM dNTPs in 100 μl final volume (note that the final solution will also contain unreacted dNTPs from the RT step).

ing and thawing (Table 2A) or storage at −70°C for a period of at least 11 months (Table 2B). Before a frozen stock is depleted, a new stock should be prepared and compared with an old stock to assure reliability and consistency of results. It is good practice to retain a reference stock as an aid in troubleshooting. Even though AMV and *Taq* solutions can be exposed to several freeze–thaw cycles (Table 2A), we recommend that the AMV and *Taq* solutions be frozen at −70°C in unit-of-use sizes and that the aliquoted stocks be thawed only once and the remainder discarded. This practice eliminates experimental error that could result from mishandling or contamination of a single stock solution during repeated use.

Because individual primer sets may have different optimal conditions for RT-PCR,[7,8] it may be necessary for more than one AMV and *Taq* solution to be prepared. For example, we determined that 1 unit of AMV reverse transcriptase per assay was adequate for moderately abundant messages (α_5-integrin subunit), whereas 10 AMV units per assay was required for more abundant messages (fibronectin and actin). The PCR step is quite sensitive to small differences in Mg^{2+} and *Taq* DNA polymerase concentrations.[7,8] Optimization of reagent concentrations improves results and economizes on reagents.

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TABLE 2 Stability of RT-PCR Solutions

A. Stability to repeated freeze–thaw cycles

	dpm in PCR product (α_5-subunit)		
		frozen and thawed	
Frozen solution	control	once	five times
Both not frozen	515 ± 87	—	—
AMV only	—	522 ± 35	437 ± 59
Taq only	—	502 ± 60	441 ± 27
AMV + *Taq*	—	496 ± 38	441 ± 35

B. Stability during long-term storage at −70°C

Time in freezer	cpm in PCR product (α_2-subunit)
Control (not frozen)	3274 ± 193
4 months	4138 ± 249
11 months	7805 ± 101

RT-PCR was carried out as described in Table 1 for 24 PCR cycles using a primer set for the integrin-α_5 subunit and 22 cycles using primers for the α_2-subunit.[5] The sense primer was end-labeled with ^{32}P as described previously, and radiolabeled PCR products were excised from polyacrylamide gels and counted.[5,9] Three individual trials were carried out, and the results are expressed as the mean ± S.E.

(*A*) The effects of repeated freeze–thaw cycles on the stability of AMV and *Taq* solutions were studied. Little, if any, loss in AMV or *Taq* activity was noted after five freeze–thaw cycles.

(*B*) The effect of time of storage on enzyme activity was studied. AMV mix (solution 4) and *Taq* mix (solution 6) were stored at −70°C for the times indicated and used to amplify the integrin-α_2 subunit in the same total RNA preparation. Both AMV and *Taq* mixes were found to be stable for at least 11 months. Because different commercial enzyme preparations were used to make the AMV and *Taq* mixes for each time point, it is not surprising that some difference in total PCR product generated was noted for each set of solutions studied. Nevertheless, as demonstrated by the standard errors for each time point, a given set of stock solutions produced quite reproducible results.

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