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**Joel A. Malek, Sofiya Y.
Shatsman, Bola A.
Akinretoye and John E. Gill**
*The Institute for
Genomic Research
Rockville, MD, USA*

Irreversible Heat Inactivation of DNase I without RNA Degradation

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In many applications of RT-PCR, residual genomic DNA in total RNA preparations is amplified together with

RNA, which results in false-positive data. One way to overcome this problem is to design primers that span a region containing one or more introns, thereby creating a diagnostic size difference between the amplification products originating from the RNA or the DNA. However, this method will not work in many organisms containing genes without introns (6). Additionally, in quantitative or semi-quantitative RT-PCR, the contaminating DNA will compete with cDNA for primers, which can lead to misinterpretation of the results. Preexisting DNA can also result in the appearance of additional differential bands during differential display (8) or cDNA-AFLP (1). In known sequences, DNA contamination can be eliminated by restriction endonuclease digestion before the amplification of cDNA (5). In short target regions, however, the choice of restriction enzymes is very limited, and because sequence knowledge is required, this method is not generally applicable. Moreover, these restriction fragments may still act as amplification primers and produce a nonspecific smear.

The most effective and universal way to eliminate genomic DNA is enzymatic treatment with DNase I. In the presence of magnesium ions, DNase I cleaves each DNA strand independently, while in the presence of manganese ions, both strands are cleaved at approximately the same site (10). Manganese ions can be used at a concentration ten times lower (0.66 mM) than magnesium ions, and they can prevent the production of artifacts in RT-PCR arising from the recombination of overlapping fragments that are created by DNase I treatment using magnesium as a cofactor (2). In addition, at commonly used concentrations, magnesium ions can significantly influence the thermostability of DNase I and contribute to the reactivation of the enzyme after heat treatment (3).

For reverse transcription of RNA, irreversible inactivation of DNase I can be achieved in two ways. First, the RNA can be extracted with phenol/chloroform, but this method is time consuming, results in RNA loss and can be problematic at low RNA concentrations. A second method consists of heat denaturation of DNase I at 75°C for 5 min. Using this method, no significant

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RNA loss was detected with quantitative RT-PCR (7). However, it has been described that RNA is hydrolyzed in the presence of divalent cations (4). We demonstrate here, by visualization of RNA on agarose gels and by RT-PCR, that RNA is rapidly degraded after DNase I inactivation at 75°C for 5 min in the presence of divalent cations and that this degradation can be overcome by adding the chelating agent EDTA (or EGTA, results not shown) before the heat denaturation step.

Total RNA was extracted from 300 mg liquid nitrogen-frozen banana leaf tissue (*Musa* sp.) using the RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) with two modifications to the manufacturer's instructions. To the RLT extraction buffer from the kit, 2% polyvinylpyrrolidone was added, and the sample was homogenized for 2 × 5 s in this buffer using an Ultra-Turrax® blender (Janke & Kunkel, Staufen, Germany). All glassware, plastic tubes and solutions used for manipulation of

RNA were rendered RNase-free by standard treatments (10). For DNase I treatment, 2 µg RNA was incubated at 37°C for 30 min with 1 µL 2 U/µL RNase-free DNase I (Ambion, Austin, TX, USA) in 1× manganese buffer containing 0.66 mM MnCl₂ and 10 mM Tris-HCl, pH 7.8, in a final volume of 10 µL. Then, for heat inactivation of DNase I, 2 µL 15 mM EDTA was added, followed by incubation at 75°C for 5 min. This 2.5-mM final concentration of EDTA was selected to have a slight excess over the final concentration of divalent cations (2.2 mM) in the reaction mixture including those in the DNase I storage buffer (20 mM HEPES, pH 7.5, 10 mM CaCl₂, 10 mM MgCl₂, 1 mM DTT and 50% glycerol).

Electrophoresis of RNA was performed after incubation at 55°C for 1 h on 2 µg RNA mixed with 1.5 volume of glyoxal reaction mixture [60% DMSO, 8% deionized glyoxal, pH >5.0 (Amresco, Solon, OH, USA), 1.2× BTPE (36 mM Bis-Tris, 12 mM PIPES and

1.2 mM EDTA), 4.8% glycerol and 200 µg/mL ethidium bromide] on a 1% agarose gel in 1× BTPE. DNase I-treated RNA with EDTA added before heat inactivation (Figure 1A, lane RNA 4) and untreated RNA (Figure 1A, lane RNA 2) showed intact ribosomal RNA bands, while DNase I-treated RNA without EDTA (Figure 1A, lane RNA 3) was visibly degraded. When a commercial DNase I buffer (Ambion) containing 2.5 mM MgCl₂, 0.1 mM CaCl₂ and 10 mM Tris-HCl, pH 7.5, was used instead of the manganese buffer, similar results were obtained (data not shown). These observations demonstrate (i) the verification of rapid hydrolysis of RNA in the presence of divalent cations at 75°C (9) and (ii) the prevention of this degradation by adding EDTA (or EGTA) before the heat inactivation step.

When DNase I-treated RNA is used in enzymatic reactions, residual EDTA might have an inhibitory effect. To verify total DNA degradation and test the influence of the chelating agent, PCR was performed. One-intron-spanning primers were designed based on the sequence derived from a partially cloned actin gene from banana and synthesized commercially (Eurogentec, Herstal, Belgium). These primers span a fragment of 260 bp or 170 bp, with or without the intron, respectively. The nucleotide sequences are 5'-ACCGAAGCCCCCTCTTAACCC-3' for the forward primer and 5'-GTATGGCTGAC-ACCATCACC-3' for the reverse primer. Reactions (20 µL) contained 200 µM dNTPs (HT Biotechnology, Cambridge, UK), 0.5 µM primers, 1× PCR buffer (Qiagen) containing 1.5 mM MgCl₂, 0.5 U *Taq* DNA polymerase (Qiagen) and 1 µL (160 ng) DNase I-treated RNA (yielding a final EDTA concentration of 0.125 mM). Cycling conditions were 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 56°C for 20 s and 72°C for 20 s, followed by 72°C for 2 min. No PCR product was observed for DNase I-treated RNA with EDTA added before heat inactivation (Figure 1B, lane RNA 4) or for DNase I-treated RNA with no EDTA added before heat inactivation (Figure 1B, lane RNA 3), indicating that DNA elimination is complete. In the case of DNase I-treated RNA with EDTA added before heat in-

Figure 1. Denaturing RNA gel electrophoresis, PCR and RT-PCR products of DNase I-treated RNA. (A) Denaturing gel electrophoresis was performed with equal amounts of RNA. Intact RNA is indicated by the presence of ribosomal RNA bands. PCR (B) and RT-PCR (C) were performed with equal amounts of RNA using actin-specific primers. Lane RNA 1, untreated RNA with EDTA added; lane RNA 2, untreated RNA; lane RNA 3, DNase I-treated RNA with no EDTA added before heat inactivation and lane RNA 4, DNase I-treated RNA with EDTA added before heat inactivation. MWM indicates the molecular weight marker (Smartladder Small Fragments, Eurogentec), DNA indicates the positive DNA control and H₂O indicates the negative water control.

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activation, to confirm that the absence of a visible product was not caused by inhibition of the PCR by residual EDTA (0.125 mM), PCR was performed under identical conditions on untreated RNA (Figure 1B, lane RNA 1). This resulted in a 260-bp band of similar intensity as the PCR product amplified from untreated RNA with no residual EDTA (Figure 1B, lane RNA 2).

To see the effect of the DNase I treatment on reverse transcription, RT-PCR was performed with the same primers in the linear range of amplification after optimizing the template amount and number of cycles. These reactions (20 µL) contained 300 µM dNTPs (PE Biosystems, Ueberlingen, Germany), 0.5 µM primers, 1× EZ buffer (PE Biosystems), 2.5 mM Mn(OAc)₂ (PE Biosystems), 20 U ANTI-RNase (Ambion), 2 U *rTth* polymerase (PE Biosystems) and 1 µL (160 ng) DNase I-treated RNA. Cycling conditions were as previously defined for PCR but with 24 cycles instead of 40 and preceded by re-

verse transcription at 60°C for 30 min. All PCR and RT-PCR amplifications were performed in a Mastercycler® Gradient (Eppendorf, Hamburg, Germany). For DNase I-treated RNA with no EDTA added before heat inactivation (Figure 1C, lane RNA 3), a 170-bp band of lower intensity was observed than for DNase I-treated RNA with EDTA added before heat inactivation (Figure 1C, lane RNA 4). RT-PCR was not inhibited at the residual EDTA concentration (0.125 mM) used because when performed under identical conditions on untreated RNA containing 2.5 mM EDTA (Figure 1C, lane RNA 1), it yielded a band with the same intensity as the RT-PCR product amplified from untreated RNA or from DNase I-treated RNA with EDTA added before heat inactivation (Figure 1C, lanes RNA 2 and 4). In addition, the presence of RT-PCR products also indicates that DNase I is not reactivated under the given buffer conditions.

Repeated RT-PCR experiments resulted in the same banding pattern, demonstrating the reproducibility of the method (data not shown). The data presented here confirm that RNA is hydrolyzed in the presence of divalent cations at 75°C, yet the hydrolysis is not complete since the 170-bp fragment could still be partially amplified. This contradicts the results of Huang et al. (7), who detected no significant RNA loss with quantitative RT-PCR when the DNase I buffer contained 5 mM MgCl₂. It should be stressed that our experiments have been performed at pH 7.8 (the optimal pH for the vendor's DNase I) while Huang et al. (7) performed their experiments at pH 8.3.

In summary, rapid hydrolysis of RNA resulting from heat inactivation following DNase I treatment can be avoided by adding a slight excess of EDTA over divalent cations before the heat inactivation step. The inactivation of the DNase I enzyme is irreversible, and residual EDTA does not inhibit subsequent reverse transcription or PCR. This method provides a single-step elimination of DNA contaminating RT-PCR or differential display.

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Ilse Wiame, Serge Remy, Rony Swennen and László Sági
Catholic University of Leuven, Heverlee, Belgium