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Short note

FORMAzol® as an RNA storage medium: A cautionary note when performing RT-PCR

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Abstract

The labile nature of RNA demands careful procedures for its extraction, purification, and storage. Generally, RNA is solubilized in aqueous buffers or organic solvents, or precipitated with alcohol and then kept at −20 °C or colder. A commercially available product for RNA storage is FORMAzol® (Molecular Research Center). We began using FORMAzol because the Application Notes from the Product Description sheet claims that reverse-transcription (RT) is not inhibited so long as FORMAzol does not exceed 5% (v/v) in the reaction mix. This is ostensibly more convenient than having to precipitate RNA, resolubilizing it in water or buffer, and then proceeding with RT-PCR. However, amplicon yields for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were poor when using RNA directly from FORMAzol, even though its final concentration was typically much less than 5%. By contrast, satisfactory RT-PCR products were obtained with RNA stored frozen in water or that had been resolubilized from alcohol precipitates. When RT-PCR was then performed on ethanol-precipitated and resolubilized RNA from FORMAzol, yields of GAPDH amplicons were acceptable. Although a revision to the FORMAzol Product Description sheet is now available at the manufacturer's website (http://www.mrcgene.com/formazol.htm), if users of the product implicitly follow the directions found in the package insert sheet—not being aware of the inhibitory effects of formamide (the denaturant in FORMAzol)—unsatisfactory results may be

Abbreviations: DEPC, diethylpyrocarbonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOPS, morpholinopropanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction; TBE, Tris Na-acetate EDTA; TE, Tris EDTA.

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obtained from RT-PCR experiments. It is suggested that FORMAzol only be used for RNA storage and that RNA be precipitated with alcohol, washed, and resolubilized prior to use.

Keywords: FORMAzol; RT-PCR; GAPDH

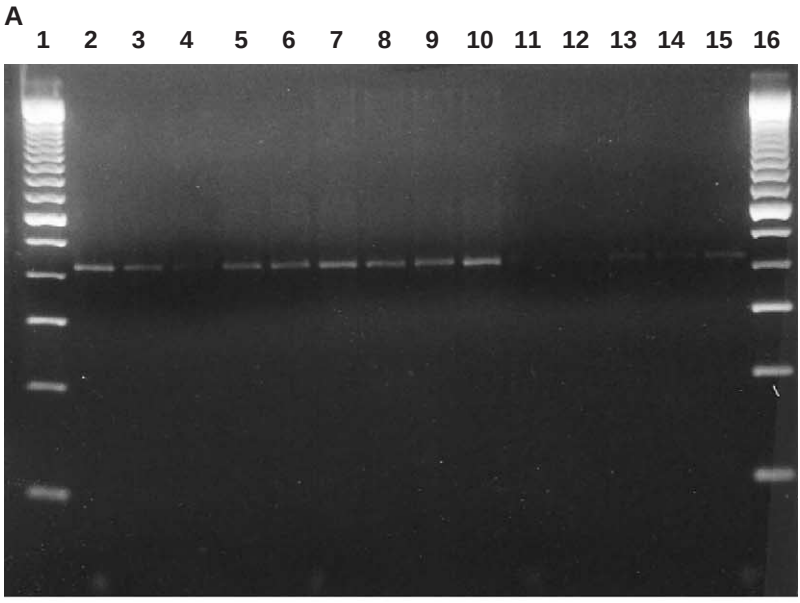
For studies involving RNA, the methods used in its extraction, purification, and storage are critically important because of the ubiquitous presence of ribonucleases. Once very commonly used RNA extraction and purification methods such as phenol–chloroform, high salt concentrations, SDS, proteinase K, and others [1] have given way to more efficient reagents that provide high yields of RNA free of contaminants (e.g., DNA, protein, glycogen) and with minimal RNA degradation. Perhaps the most popular RNA isolation method today is the acid guanidinium isothiocyanate phenol–chloroform extraction method [2], which has become a commercial product and is sold under several trade names such as TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH). Proper storage conditions of the purified RNA are also important [3], whether it is frozen in aqueous solution at −70 °C or kept as an alcohol precipitate at −20 °C. Not surprisingly, a number of commercial products are specifically formulated for RNA storage. These issues involving RNA quality are especially underscored when performing RT-PCR.

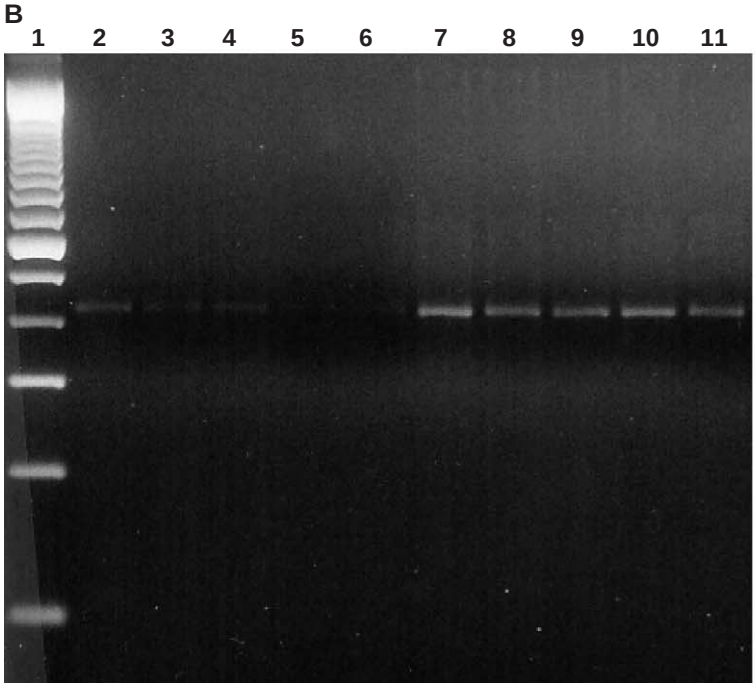
In general, investigators will use methods adapted from the research literature. The primary reason for doing so is usually one of cost: home-made reagents are far less expensive to make than purchasing commercial products. On the other hand, investigators may prefer to use commercial products because many have been developed to simplify their use (e.g., color-coded reagents) and because company quality control measures usually ensure lot-to-lot consistency in performance.

Unless one is knowledgeable about the function and characteristics of each reagent in a commercial product, most investigators (and especially less experienced ones) will carefully follow the instructions for use of the product, making the tacit assumption that the company had rigorously tested it and that performance claims are reliable. This may not always be the case.

We had been conducting RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a commonly used “housekeeping” or reference marker gene for normalization

Fig. 1. Panel (A) FORMAzol can affect RT-PCR. Total RNA was extracted and purified from brown adipose tissue of 5 rats and stored either as an ethanol-precipitate at −20 °C, solubilized in DEPC-water and frozen at −75 °C, or solubilized in FORMAzol® and frozen at −20 °C. RNA (2 μg) was reverse-transcribed, PCR-amplified with GAPDH primers, and amplicons were run on 2% agarose TBE-gels and visualized with ethidium bromide. Lanes 1 and 16, 100 bp ladder; lanes 2–5, RNA solubilized from an ethanol precipitate; lanes 6–10, RNA solubilized and frozen in DEPC-water; lanes 11–15, RNA stored in and taken directly from FORMAzol. Panel (B) RNA precipitated from FORMAzol improves RT-PCR yield. RT-PCR for GAPDH of RNA (2 μg) taken directly from FORMAzol or after RNA had been precipitated with ethanol, washed, and resolubilized in DEPC-water. Amplicons were run on 2% agarose TBE gels and visualized with ethidium bromide. Lane 1, 100 bp ladder; lanes 2–6, RNA from FORMAzol; lanes 7–11, RNA precipitated from FORMAzol with ethanol, washed, and solubilized in DEPC-water.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


B
1 2 3 4 5 6 7 8 9 10 11

purposes in gene expression studies because it is constitutively expressed. Earlier, we kept solubilized RNA in diethylpyrocarbonate (DEPC) treated-water at −75 °C, or as an ethanol precipitate at −20 °C; but recently, we began using FORMAzol® (Molecular Research Center Inc; http://www.mrcgene.com; catalog no. FO-121) for storing solubilized RNA at −20 °C. After the switchover, we noticed inconsistent results which prompted this investigation.

Five rats were sacrificed by CO_2 inhalation and interscapular brown adipose tissue pads were quickly excised and freeze-clamped between aluminum blocks kept on dry ice. Total RNA was extracted using TRI Reagent (Molecular Research Center; catalog no. TR118) according to the manufacturer's directions. RNA was quantified by spectrophotometry at 260 nm and its structural integrity was confirmed by visual inspection of ethidium bromide fluorescence of 28 and 18 S rRNA in formaldehyde-MOPS 1% agarose gels. Purified RNA was stored in three different ways: (1) solubilized in DEPC-water and frozen at −75 °C, (2) precipitated with ethanol and stored at −20 °C, and (3) solubilized in FORMAzol and then frozen at −20 °C as directed by the manufacturer.

Primers were designed for GAPDH from the NCBI rat genome database and synthesized by IDT (Integrated DNA Technologies; http://www.idtdna.com); the expected size of the amplified product was 438 bp. The primers were:

forward 5′-TGTCAGCAATGCATCCTGCACCACC-3′
reverse 5′-GAAGTCACAGGAGACAACCTGGTCGTCC-3′

Two micrograms of input RNA was reverse-transcribed (SuperScript First Strand Synthesis System, Invitrogen; http://www.invitrogen.com; catalog no. 11904-018). The RNA was taken from thawed aliquots solubilized in DEPC-water, or after centrifugation of ethanol-precipitates and re-solubilization in TE buffer, or directly from FORMAzol as suggested by the manufacturer. PCR amplification conditions were denaturation at 95 °C (5 min), 30 cycles of 95, 58, and 72 °C (1 min each), and a final elongation step of 72 °C (10 min) using a Biometra T-gradient thermocycler. PCR products were visualized on ethidium bromide-stained 2% agarose-TBE gels.

As shown in panel A of Fig. 1, RT-PCR of RNA reconstituted from ethanol precipitates (lanes 2–5) and of RNA solubilized and stored frozen in DEPC-water (lanes 6–10) yielded satisfactory quantities of amplicons. By contrast, when RNA stored in FORMAzol was used for RT-PCR, product yield was consistently poor (lanes 11–15). This observation suggested that either the RNA had degraded or FORMAzol was interfering with the reaction.

We first tested integrity of RNA stored in FORMAzol. RNA was precipitated with ethanol, centrifuged, and the supernatant removed; the pellet was washed with ethanol and solubilized with DEPC-water (panel B of Fig. 1, lanes 7–11). RT-PCR yielded the quantity of product we previously obtained with RNA solubilized only with DEPC-water and stored at −75 °C, or with RNA kept as an ethanol precipitate at −20 °C. Thus, the quality of RNA kept in FORMAzol was not an issue: it had not degraded. Next, the possibility of FORMAzol interference of PCR was considered. The Application Notes from the Product Description sheet for the product claims that reverse transcription is inhibited if FORMAzol exceeds 5% (v/v). However, the amount of FORMAzol in our reactions

ranged from 2.5–4% (v/v), and yet our results were unsatisfactory (panel B of Fig. 1, lanes 2–6). It therefore appears that FORMAzol, even in amounts less than that claimed to interfere with reverse transcription, is the cause of poor RT-PCR performance.

Interestingly, Molecular Research Center's website for product information for FORMAzol [4] has an updated Application Note which addresses the problem we have described. Availability of the on-line revision notwithstanding, it is likely that investigators who do not have extensive experience in molecular biology techniques will implicitly follow the user directions of the FORMAzol Product Description hard copy sheet, without realizing that formamide (the denaturant in FORMAzol) has adverse effects on reverse transcriptase reactions, and also being unaware of the newer protocol available at the company website. As a consequence, unsatisfactory results from RT-PCR studies may be obtained, even though the amount of FORMAzol in the reaction is less than the claimed limit of 5% (v/v).

In summary, we show that when RNA is stored in FORMAzol and then used for RT-PCR, the amount of amplification can be significantly affected. Investigators should therefore consult the updated protocol from the Molecular Research Center website [4] about its use to avoid problems. Further, it is recommended that investigators use FORMAzol for RNA storage purposes only and that RNA be ethanol-precipitated and then resolubilized in water or buffer prior to RT-PCR.

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