

Application of in situ ductal perfusion to facilitate isolation of high-quality RNA from mouse pancreas

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A technique to isolate high-quality intact RNA from murine pancreas is described. This technique involves in situ ductal perfusion of the pancreas with an RNase inhibitor prior to removal of the organ for RNA extraction. In this way, the pancreatic RNases are inhibited in situ allowing good yields of intact RNA, suitable for studies on pancreatic gene transcription by real-time PCR or microarray analysis, to be obtained in a reliable way.

INTRODUCTION

Isolation of intact RNA from the pancreas has always represented a particular challenge for molecular biologists interested in gene expression in this tissue. This is due to extremely high levels of RNase A, estimated for rat pancreas at 200 µg/g tissue (1), that are present in secretory granules disrupted during the isolation procedure. The technique of Chomczynski and Saachi (2), which involves lysis of cells in the strongly denaturing cocktail of guanidine isothiocyanate and phenol, has proven to be a highly efficient method of preparing intact RNA from the majority of tissues and is still the method of choice for most applications. However, it gives, at best, variable results with the pancreas, even when combined with the use of RNase inhibitors and cold-temperature homogenization (3,4) or immediate snap-freezing of the pancreas (5) prior to grinding the frozen tissue in a denaturing solution like TRIzol[®] reagent.

The major problem with all these techniques is that RNA degradation appears to commence during dissection of the animal and removal of the pancreas. Therefore the major variable is likely to be the efficiency with which the tissue is removed and exposed to RNase inhibitors. To address this problem, we have developed a novel method for RNA isolation that involves

pretreatment of the mouse pancreas by in situ ductal perfusion with RNAlater[®] prior to removal of the tissue. The RNA was subsequently isolated using TRIzol reagent and was obtained in consistently high yield. A comparison of pancreatic RNA isolated by this method to RNA isolated from NIT-1 pancreatic β cells using the TRIzol method confirmed that RNA of equally high quality was isolated by this novel approach. When coupled with the Chomczynski and Saachi method (2), this perfusion step significantly improves the reliability of isolation of high-quality pancreatic RNA.

MATERIALS AND METHODS

Animal Care and In Situ Ductal Perfusion of Mouse Pancreas with RNAlater

SJL/J mice (5- to 6-week-old), weighing 18–20 g (from Jackson Laboratories, Bar Harbor, ME, USA) were housed in a certified animal care facility and handled according to the *Guide for the Care and Use of Laboratory Animals* (6).

All equipment used in organ dissection was first treated with RNaseZap[®] (Ambion, Austin, TX, USA) according to the manufacturer's instructions. To circumvent RNA lysis

by RNases that may be released in the mouse pancreas when the animal is stressed, all procedures were conducted as swiftly as possible after each mouse was sacrificed. The mice were anesthetized with Avertin[®] consisting of 1.25% (w/v) 2,2,2-tribromoethanol, 2.5% (v/v) 2-methyl-2-butanol in distilled water at a dose of 0.02 mL/g body weight administered intraperitoneally (7). The mice were then euthanized by cervical dislocation. The abdominal and chest cavities were opened, and the lower lobes of the liver were moved aside to expose the pancreas and bile duct. Under a Model SMZ645 stereoscopic zoom dissecting microscope (4× magnification; Nikon Canada, Mississauga, ON, Canada), the bile duct was clamped with a bulldog clamp at the Sphincter of Oddi, where it enters the duodenum. A 3-mL syringe with a slightly bent 27-G needle was inserted into the bile duct near the liver and 1–2 mL RNAlater (Ambion) was slowly injected (Figure 1). As the pancreas was perfused, it visibly ballooned and became more translucent. Once completely perfused, there was resistance to injecting further liquid. At this time, it was excised with scissors and placed in 5 volumes of RNAlater on ice. The time to perfuse the pancreas was found to be <1 min in the hands of an experienced technician. The technique is similar to collagenase perfusion for islet isolation. In cases where snap-freezing of the pancreas was performed, the tissue was placed in a precooled 1.5-mL cryovial, and this was immediately put in liquid nitrogen.

Isolation of Total RNA

Extraction of total RNA from 5 × 10⁶ NIT-1 pancreatic β cells (ATCC, Manassas, VA, USA) was conducted in parallel to RNA isolation from the perfused pancreas. Previous experience had demonstrated that we could obtain high yields of RNA from NIT-1 β cells using TRIzol (Invitrogen, Carlsbad, CA, USA) (8), and these cells were therefore used as our positive control for RNA yield and integrity. NIT-1 cells were cultured in F12/K medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin, streptomycin, and neomycin. The cells were harvested in

1 mL TRIzol prior to RNA isolation. The perfused pancreas was placed in 5 mL TRIzol and homogenized for 20 s using an Ultra-Turrax® T18 basic homogenizer (IKA Works, Wilmington, NC, USA) at level 4. RNA was then isolated from an aliquot of the homogenized pancreas and the NIT-1 cell pellet, as described below.

Briefly, 1 mL homogenized pancreatic tissue or 1 mL NIT-1 β cells was placed in a 1.5-mL microcentrifuge tube, and the remainder of the homogenized pancreas sample was stored at -80°C . Chloroform (0.2 mL) was added, and the sample was shaken for 15 s prior to incubation at room temperature for 3 min. The sample was then centrifuged for 15 min at $16,800\times g$, and the supernatant was placed in a new microfuge tube containing 0.5 mL isopropanol to precipitate the RNA. The sample was mixed by inversion, incubated at room temperature for 10 min, and the RNA was then pelleted by centrifuging at $14,500\times g$ for 10 min at 4°C . The pellet was washed with 75% ethanol (1 mL) and reprecipitated at $9650\times g$ for 5 min at 4°C . The supernatant was removed, and once the pellet was dried, it was resuspended in 30 μL 0.1% diethylpyrocarbonate

(DEPC)-treated water. The integrity of the RNA was tested by electrophoresis on a 0.8% agarose gel and using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The concentration was determined by spectrophotometry at 260 and 280 nm.

cDNA Synthesis/Reverse Transcription

A reverse transcription reaction was performed on total pancreatic RNA or control mouse liver RNA (Ambion) using SuperScript™ II (Invitrogen) and random hexamers (Invitrogen) according to the manufacturer's instructions. Specifically, an aliquot containing 2 μg total RNA in approximately 2 μL sterile distilled water (treated with DEPC) was mixed with 2 μL random primer and DEPC-treated distilled water to give 10 μL . This was denatured by incubation at 70°C for 5 min. RNase Inhibitor (1 μL ; Invitrogen), 4 μL 5 \times First Strand Buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2 mM dNTPs, and 2 μL 0.1 M dithiothreitol (DTT) were added to each reaction and warmed to 42°C . SuperScript II (1 μL) was then added, and the mixture was incubated

first at 42°C for 20 min, then at 70°C for 15 min. A reaction in the absence of RNA was included as a control. Transcription products were analyzed following electrophoretic separation on a 0.8% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

This study was aimed at developing a robust technique for isolating RNA from mouse pancreatic tissue that was of sufficiently high quality to be suitable for microarray analysis and real-time PCR. Our results show that in situ ductal perfusion of the mouse pancreas with RNAlater, prior to its excision, increased the amount of high-quality RNA extracted by the Chomczynski and Saachi method (2) by over 10-fold. The Chomczynski and Saachi protocol for RNA isolation is widely and easily applied to the isolation of RNA from a broad range of organs, tissue, and cell culture. The presence of guanidium isothiocyanate in the homogenization buffer serves as a broadly effective protein denaturant and inhibitor of RNases (5,9). However, high levels of endog-

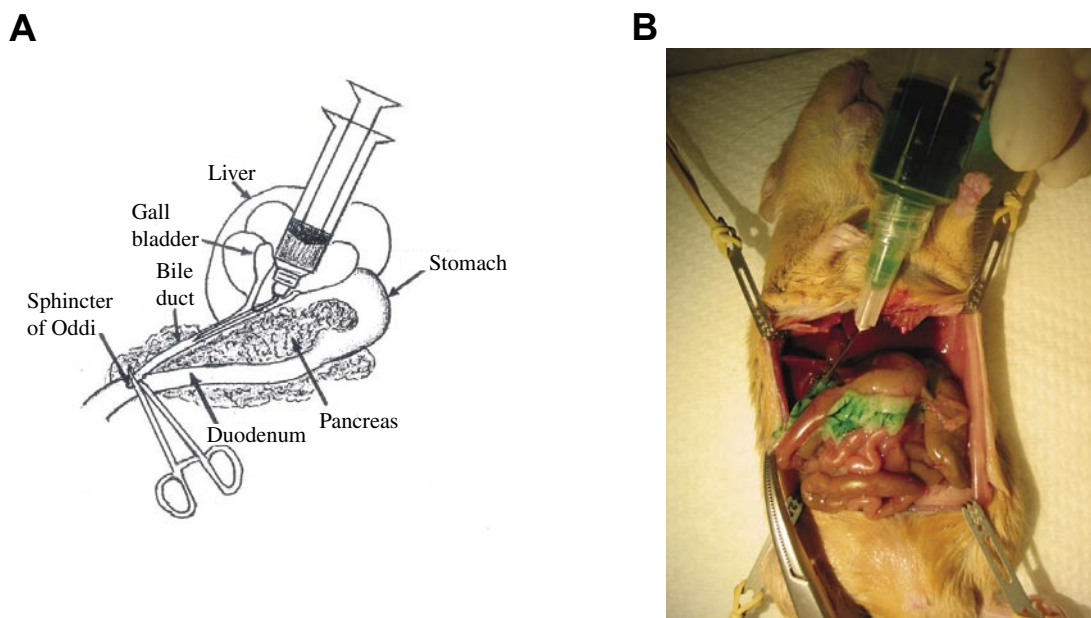


Figure 1. Perfusion of pancreas. (A) The diagram shows the position to clamp the bile duct where it enters the duodenum at the Sphincter of Oddi. Following cannulation of the bile duct, the RNase inhibitor, RNAlater is then injected into the duct near the liver, and the pancreas balloons as it fills with solution. Following perfusion, the pancreas is excised and placed in 5 volumes of RNAlater on ice. (B) Photograph showing ductal perfusion of pancreas with a green dye. Note the perfusion of all parts of the pancreas (as indicated by the dye) and inflation of the organ.

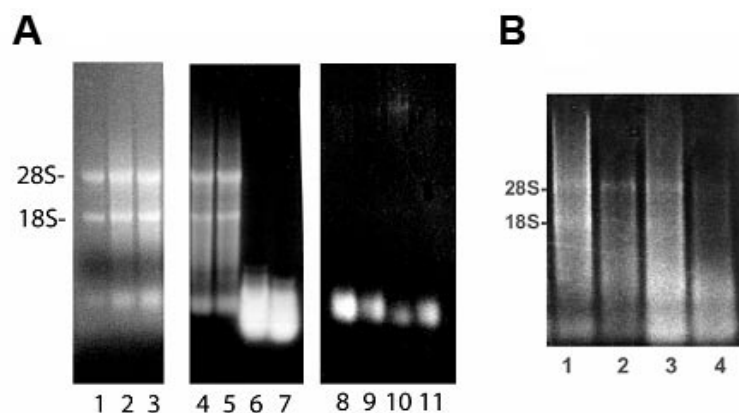


Figure 2. Agarose gel electrophoresis of total RNA. (A) RNA was isolated from NIT-1 β cells (lane 1) or mouse pancreata using TRIzol (lanes 2–11). Samples from mice that were pretreated by in situ ductal perfusion with RNAlater are shown in lanes 2–5 and following perfusion with phosphate-buffered saline (PBS) alone in lanes 8–11. RNA isolated from pancreata snap-frozen in liquid nitrogen prior to homogenization in TRIzol and RNA isolation are in lanes 6 and 7. The ribosomal 28S and 18S RNAs are indicated and show the high integrity of the RNA. (B) Agarose gel electrophoresis of total RNA isolated from mouse pancreas using RNAlater and TRIzol without perfusion. In lanes 1 and 2, the RNA from pancreas was placed in RNAlater immediately following dissection and then homogenized in TRIzol for RNA isolation. In lanes 3 and 4, RNA from pancreas was placed in RNAlater and then ground in liquid nitrogen.

enous nucleases in the pancreas have made consistent isolation of intact RNA from this tissue very difficult. It seems that the RNA is highly susceptible to degradation from the moment the animal is euthanized, and great efficiency in performing the dissection is required. Even then, without perfusion with an RNase inhibitor, we obtained variable yields. Some degree of inactivation of the endogenous RNases can be obtained by keeping the sample at 0°C or less (4,5). However in our hands, special attention to sample and equipment temperature did not consistently improve the quality of RNA, indicating that RNases were still present and active in sample degradation (Figure 2A, lanes 6 and 7, and 2B).

Other protocol modifications have previously been proposed to improve yield. For example, Chirgwin et al. (5) proposed the addition of 0.1 M 2-mercaptoethanol to guanidinium isothiocyanate to break protein disulfide bonds. Buffers containing aurintricarboxylic acid (ATA), an effective inhibitor of enzymes including RNases, have been successfully applied in the isolation of RNA from rat tissues (although pancreas was excluded) (10). However, ATA binds nucleic acid and must be removed by Sephadex® column chromatography. Notably, none of these protocol modifications attempt

to inhibit tissue RNases until the tissue is placed in the guanidinium isothiocyanate homogenization buffer.

We improved the isolation of RNA from the mouse pancreas with the TRIzol method by developing a technique for treating the pancreas with a powerful RNase inhibitor, RNAlater prior to homogenization, and RNA extraction. We reasoned that inhibiting pancreatic nucleases as early as possible would improve the likelihood of extracting intact RNA. Pancreatic RNA isolated by this in situ ductal perfusion method was compared with that isolated by various other methods and to RNA extracted from NIT-1 β cells (Figure 2 and Table 1). Under UV illumination, RNA isolated from NIT-1 β cells with TRIzol contained clear 28S and 18S ribosomal RNA (Figure 2A, lane 1) and also a low intensity smear of RNA reflecting the broad range of sizes of mRNA in the total RNA sample. In contrast, only small degraded RNA products were observed in two separate RNA samples extracted from mouse pancreata that were snap-frozen in liquid nitrogen and carefully kept frozen until homogenization (Figure 2A, lanes 6 and 7). In comparison, the RNA isolated from pancreata perfused with RNAlater is shown in Figure 2A, lanes 2–5, and following perfusion with phosphate-buffered saline (PBS)

in Figure 2, lanes 8–11. The RNA isolated from the four PBS-perfused organs consisted of only small degraded RNAs. However, perfusion of the mouse pancreas with RNAlater repeatedly facilitated isolation of high-quality intact RNA.

In comparison with these results, Figure 2B shows the yields we obtained in several experiments without perfusion. In these examples, the pancreas was placed in RNAlater immediately following dissection, and some samples (Figure 2B, lanes 3 and 4) were snap-frozen in liquid nitrogen prior to RNA isolation using TRIzol. The yields were substantially lower than following perfusion and extremely variable. We have also found that immediate snap-freezing of the pancreas in liquid nitrogen followed by transitioning in RNAlater-ICE (Ambion) gave little or no yield.

In order to determine the concentration of the intact RNA samples, the absorbance at 260 and 280 nm of a fixed dilution of each sample was measured by spectrophotometry, and the ratio 260/280 was calculated (Table 1). High concentrations of RNA were routinely isolated from mouse pancreas using this method, corresponding to approximately 32 μ g RNA/mg tissue from an estimated 30 mg pancreas.

RNA quality and integrity was further confirmed by cDNA synthesis from total RNA (Figure 3). Since reverse transcription is sensitive to impurities and contaminating DNA, cDNA serves as one indicator of high-quality RNA. Reverse transcription reactions were conducted with total RNA and were primed with random hexamers. The resulting cDNA products were separated by agarose gel electrophoresis alongside a ladder of DNA fragments of known lengths. Transcription products varying in size from 0.2 to >4 kb were produced, indicative of high-quality RNA.

In summary, we have described a technique that reliably improves the amount and quality of RNA extracted from the mouse pancreas, an RNase-rich organ. The RNA that we isolated using this technique was suitable for microarray analysis as well as for use in real-time PCR (Mullin and Chantler, manuscript in preparation) indicating

its high quality. Moreover, the lack of RNA degradation surmounts the problem of analyzing the differential expression of genes that may vary in their susceptibility to degradation, thereby skewing the results.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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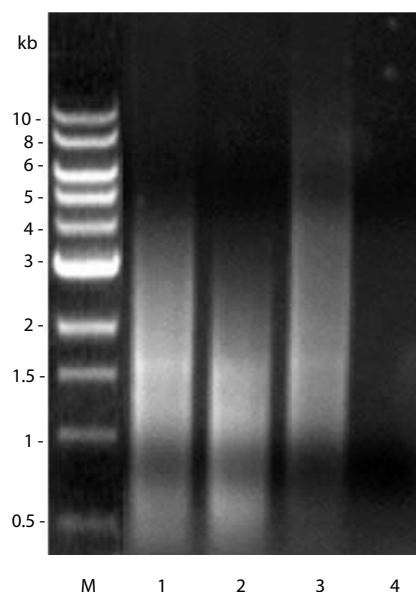


Figure 3. Agarose gel electrophoresis of cDNA from total mouse pancreas RNA. cDNA was synthesized using random hexamers and then separated on an ethidium-bromide agarose gel. Reverse transcription reactions were primed with RNA isolated from pancreata that were perfused with RNAlater as described in the text. Lanes 1 and 2 represent the cDNA reactions primed with two different pancreatic RNA samples. Equivalent amounts of the reverse transcription reactions are included in each lane. In lane 3, the reverse transcription reaction was primed with 2 μg mouse liver RNA, and in lane 4, RNA was replaced with an equivalent volume of nuclease-free distilled water. M is the 1-kb ladder (New England Biolabs, Ipswich, MA, USA).

Table 1. Analysis of RNA Isolated from NIT-1 β Cells and from Mouse Pancreata Perfused with RNAlater

RNA Source	Lane (Figure 2)	Ratio (260/280)	Concentration (μg/mL)
NIT-1 β cells	1	1.42	4.26
Pancreas SJL/J mouse	2	1.48	7.75
Pancreas SJL/J mouse	3	1.43	3.44
Pancreas C57/Bl6 mouse	4	1.37	4.03
Pancreas C57/Bl6 mouse	5	1.36	4.65

The yield of RNA from pancreas isolated from pancreas of both SJL/J and C57/Bl6 was found to be equivalent to and in one case higher than the yield from NIT-1 β cells.