Appl Biochem Biotechnol (2009) 158:253–261
DOI 10.1007/s12010-008-8391-0

A Modified Method using TRIzol® Reagent and Liquid Nitrogen Produces High-Quality RNA from Rat Pancreas

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Received: 11 May 2008 / Accepted: 30 September 2008 /
Published online: 18 October 2008
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Abstract To establish an economical and reproducible method for the high-quality RNA extraction from pancreas, we isolated total RNA from rat pancreas with TRIzol® reagent and liquid nitrogen. In the initial stage, we optimized three influential factors, the way to homogenize pancreas, the time to collect the pancreatic tissue from animals, and the weight of the pancreatic tissue in 1 ml of TRIzol® reagent. The RNA quality was determined by detecting total RNA content and its absorbance at 260/280 nm wavelength, visualizing RNA in non-denatured agarose gel and performing RT-PCR of pancreas-specific genes. The A_{260}/A_{280} ratio of the total RNA extracted by grinding 20–30 mg of rat pancreatic tissue removed from the rats in liquid nitrogen within 1 min and then immersed in 1 ml of the TRIzol® Reagent was 1.75–1.89, and the ratio of 28S/18S ribosomal RNA bands was more than 1.8. Furthermore, full length of Pdx1 open-reading frame was amplified with RNA extracted from the grinding group rather than from the conventional group. The RT-PCR products of pancreas-specific genes from both exocrine and endocrine parts of pancreas were successfully derived from the extracted RNA. The results suggested that we successfully provided an economical, fast, and reproducible method to obtain the high-quality and intact RNA from rat pancreas with TRIzol® Reagent and liquid nitrogen.

Dongmin Li and Wuchao Ren equally contributed to this work. Shemin Lu and Tianbao Song are co-corresponding author.

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Humana Press

Keywords Rat pancreas · RNA · Total RNA extraction · TRIzol® Reagent · Liquid nitrogen

Introduction

The essential step to perform RNA-based researches is to obtain intact, high-quality RNA from target cells or tissues. In 1968, Cox first reported a method to isolate RNA with guanidinium chloride, a strong de-proteinization agent to have replaced commonly used phenol in nucleic acid purification [1]. Since then, guanidinium extraction has become the first-choice method for RNA purification. In 1980s, the most used method for RNA isolation from cells and tissues was described by Chirgwin et al. based on guanidinium thiocyanate, one of the most effective protein denaturant, which is able to efficiently denature endogenous ribonucleases. The method is very effective for separating undegraded RNA from different sources, even ribonuclease-rich tissues such as the pancreas, but requires over 20 h ultracentrifugation through a cesium chloride cushion [2]. In 1987, Chomczynski and Sacchi established a single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. The method provides a pure preparation of undegraded RNA in high yield and can be completed in less than 4 h by eliminating the ultracentrifugation step of the guanidinium thiocyanate–CsCl method. It is particularly useful for processing large numbers of samples and for isolating RNA from minute quantities of cell and tissue samples [3]. Since the advent of this method introduction, the "single-step" method has become widely used for isolating total RNA from biological samples of different sources. The single-step method has been developed into kits that are marketed in many countries under different trademarks [4]. TRIzol® reagent is one of the kits.

At present, although we can easily and successfully isolate RNA from most cells and tissues for many applications, the isolation of intact RNA from the pancreas usually is difficult because of autolysis and richness of endogenous ribonucleases [5]. A few researches on the method of RNA extraction from the pancreas have been reported so far [6, 7], but these techniques cannot be generally used due to their complexity and indefinite reproducibility [8]. Therefore, isolation of high-quality RNA from the pancreas is still a great challenge for biomedical researchers to study gene expression in this tissue. Since we tried in vain to use TRIzol® reagent and conventional protocol to extract RNA from the rat pancreas, we then optimized different conditions. After practicing more than 50 times, we successfully extracted the high-quality and intact RNA from the rat pancreas with TRIzol® reagent and our modified method. The isolated RNA can be used for RNA-based studies, such as RT-PCR, differential display genes expression and Northern blotting.

Materials and Methods

Animals

Eight female inbred E3 rats, weighing 220–250 g, were kept in our specific pathogen-free animal house with 12-h light/dark cycle. The rats were put in polystyrene cages and fed with the standard rodent chow and water ad libitum. The animal experiment was handled according to the Guide for the Care and Use of Laboratory Animals.

Reagents

TRIzol® reagent, Revertaid™ first strand complementary (cDNA) synthesis kit and Taq DNA polymerase were purchased from Invitrogen (Carlsbad, CA, USA), Co. MBI (Hanover, MA, USA), and Co. Takara (Dalian, Liaoning, China), respectively. PCR primers were designed and used Primerpremier 5 according to the gene sequences in the GenBank and synthesized by Shanghai SUNNY Company (Beijing, China). Their sequences are described in Table 1.

Methods

RNA Extracted from the Rat Pancreas with TRIzol® Reagent

Isolating Total RNA by Two Different Homogenizing Methods After anesthesia, 20–30 mg of the pancreatic tissue of each rat was cut as quickly as possible, placed in the DEPC-treated cryogenic vials, and then stored in liquid nitrogen. The samples were divided into two groups. In the grinding group, the pancreatic tissue was homogenized by grinding in liquid nitrogen, and the tissue powder was transferred into DEPC-treated 1.5 ml EP tubes filled with 1 ml of TRIzol® reagent. The tubes were shaken vigorously by hand for 30 s and then incubated at 25 °C for 5 min. In the conventional group, the tissue was homogenized directly in 1 ml of TRIzol® reagent with power homogenizer (IKA®—WERKE GMBH) and incubated as the same as that in the grinding group. Afterward, the procedure of the two groups followed the introduction described by the TRIzol® reagent manufacturer as usual. The RNA was dissolved in either RNase-free water for short-term usage or deionized formamide for long-term usage and stored at −80 °C.

Isolating Total RNA from Pancreatic Tissues Collected in Different Time Periods Five experimental groups (1 min group, 2 min group, 3 min group, 5 min group, and 7 min group) were designed according to the time period from cutting the pancreatic tissues to

Table 1 The primer sequence and size of the PCR products of the genes expressed in the rat pancreas.

Rat genes	Primer sequence	Size of PCR products (bp)
Insulin1	Sense primer: 5′-AGCAAGCAGGTCATTGTTCC-3′ Anti-sense primer: 5′-TTGCGGGTCCTCCACTTC-3′	209
Glucagons	Sense primer: 5′-ACCGTTTACATCGTGGCT-3′ Anti-sense primer: 5′-GTCTCTGGTGGCAAGGTTAT-3′	492
pancreas α-amylase	Sense primer: 5′-ACATTGGTGTAGCAGGGTT-3′ Anti-sense primer: 5′-CAAGGGCTCTGTCAGTAGG-3′	318
Pdx1 (ORF)	Sense primer: 5′-ATGAATAGTGAGGAGC AGTACTACG-3′ Anti-sense primer: 5′-CCGGGGTTCCTGCGGTC-3′	849
Pdx1	Sense primer: 5′-TAGTAGCGGGACAACGAGC-3′ Anti-sense primer: 5′-AGACCTGGCGGTTCACAT-3′	476
GlUT2	Sense primer: 5′- ACAACAACTCCGCACGC-3′ Anti-sense primer: 5′-GAGGGCTCCAGTCAACGA-3′	434
β-actin	Sense primer: 5′-CACCC GCGAG TACAA CCTTC-3′ Anti-sense primer: 5′-CCCAT ACCCA CCATC ACACC-3′	207

immersing them into the liquid nitrogen less than 1, 2, 3, 5, and 7 min, respectively. The RNA extraction procedure was the same as that in the grinding group mentioned above.

Isolating RNA from Different Weight of the Pancreatic Tissue Different weight of the pancreatic tissue was obtained from the similar part of the rat pancreas after anesthesia. We divided these samples into four groups. In the four groups, the weight of pancreatic tissue in 1 ml of TRIzol® reagent was 20–30, 50–60, 70–80, and 90–100 mg, respectively. The RNA extraction procedure was the same as that in the grinding group mentioned above.

Total RNA Quantification Evaluated by Spectrometer

The isolated RNA was diluted with DEPC-treated TE buffer at pH 8.0. The absorbance at 260 and 280 nm was then determined by microspectrometer (CE23-1, GENEQUEST), and the ratio of A_{260}/A_{280} was calculated.

Quality of the Total RNA Evaluated by 1% Non-denatured Agarose Gel Electrophoresis

The RNA electrophoresis tank and related equipments were treated with 3.3% H_2O_2 and washed completely with DEPC-treated water. Two microgram of total isolated RNA of each sample with different methods was electrophoresed on 1% non-denatured agarose/ethidium bromide (EB) gel for 30 min at 8 V/cm and visualized and photographed with gel imaging system (GeneSnap, Co. SYNGENE). The density of 28S and 18S ribosomal RNA (rRNA) bands was analyzed by using software (GeneTools from SYNGENE).

RNA Integrity Detected by RT-PCR with Special Primers Amplifying the Full Length of Pdx1 ORF

The full length of pdx1 open-reading frame (ORF) was amplified in the grinding group and in the conventional group by RT-PCR with special pdx1 (ORF) primers. Detail about the PCR condition and process was referred as the following part.

RT-PCR Analysis of the Total RNA Isolated from the Rat Pancreas

Several genes specially expressed in the rat pancreas, including insulin1, glucagon, pancreas α-amylase, pdx1, and GlUT2, and a housekeeping gene β-actin were analyzed. The cDNA was synthesized from 5 μg of the total RNA isolated from the rat pancreas with the method of the grinding group by utilizing MMLV reverse transcriptase and an oligo $(dT)_{18}$ primer according to the instruction of Revertaid™ First Strand cDNA Synthesis kit. The reverse transcription was performed in a 20 μl reaction volume system. PCR reaction system of 25 μl contained 0.5 μl of cDNA, 1 μl of 10 μM sense primer, 1 μl of 10 μM anti-sense primer, 2.5 μl of 10× PCR buffer, 2 μl of dNTP mixture (each 2.5 mM), and 0.125 μl of Takara Taq (5 U/μl). PCR was conducted at 94 °C for 5 min followed by 31 cycles of 94 °C for 1 min, 60.3 °C for 1 min (55 °C for 1 min for pdx1) and 72 °C for 1 min, and then 72 °C for 10 min. The amplification was performed by using PCR thermal cycler (PTC-200, Co. BIO-RAD). PCR products were run on 1% agarose/EB gel and visualized by gel imaging system.

Results

RNA Quality and Integrity Isolated with TRIzol® Reagent and Liquid Nitrogen

The A_{260}/A_{280} ratio of the total RNA, extracted by grinding the rat pancreatic tissue in liquid nitrogen and then immersing the tissue powder in TRIzol® reagent, was 1.75–1.89. The mean total RNA yield was 3–6 μg from 1 mg of the pancreatic tissue.

On the 1% non-denatured agarose/EB gel, there were clear 28S and 18S rRNA bands in the grinding group (see lane 1–4 of Fig. 1a). The ratio of 28S/18S rRNA bands was more than 1.8. In contrast, there was no clear band in the conventional group (see lane 5–8 of Fig. 1a), although the A_{260}/A_{280} ratio of the total RNA remained 1.92.

RT-PCR product of pdx1 ORF manifested a clear band between 750 and 1,000 bp (expected size, 849 bp) on the 1% non-denatured agarose/EB gel in the grinding group (see lane1–4 of Fig. 1b), but no band in the conventional group (see lane 5–8 of Fig. 1b).

Using this standard protocol, we have obtained highly pure and intact RNA, and we next investigated effects of collected time and weight of starting material on extracted RNA quality.

Effects of Time Period for Collecting the Pancreatic Tissue on RNA Extraction

The 28S and 18S rRNA bands of total RNA isolated from pancreatic tissue collected within 1 min were clearly visualized on the 1% non-denatured agarose/EB gel (see lane 1–2 of Fig. 2). With delay of time period for collecting the pancreatic tissue, 28S and 18S rRNA bands became lighter (see lane 3–10 of Fig. 2), and they were the lightest in the 7 min group (see lane 9–10 of Fig. 2). But 5S rRNA bands showed the opposite way.

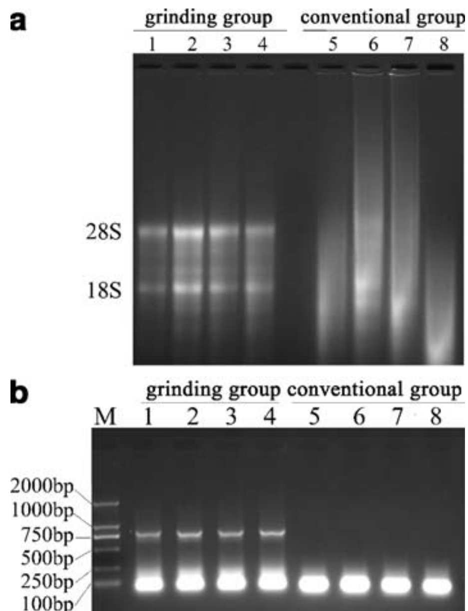

Fig. 1 Electrophoresis and RNA integrity analysis of total RNA isolated with TRIzol® reagent and liquid nitrogen by using two different homogenizing methods. **a** Pancreatic RNA isolated from four rats in the grinding group (*lanes 1–4*) and four rats in the conventional group (*lanes 5–8*), respectively. Two microgram of total RNA was run on 1% non-denatured agarose/EB gel. **b** The full length of pdx1 ORF (849 bp) amplified by RT-PCR

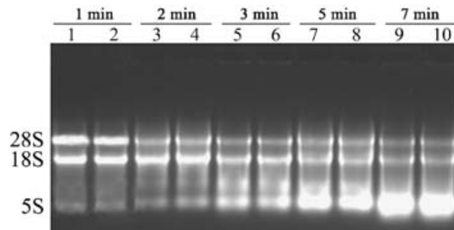

Fig. 2 Electrophoresis of total RNA isolated with TRIzol® reagent and liquid nitrogen from rat pancreatic tissue collected in different time periods (*lanes 1–2*, 1 min; *lanes 3–4*, 2 min; *lanes 5–6*, 3 min; *lanes 7–8*, 5 min; *lanes 9–10*, 7 min). Five microgram of total RNA was run on 1% non-denatured agarose/EB gel

Effects of Different Weight of the Pancreatic Tissue on RNA Extraction

The clear 28S and 18S rRNA bands of total RNA isolated from 20–30 mg of the pancreatic tissues in 1 ml of TRIzol® reagent was visualized on 1% non-denatured agarose/EB gel (see lane 1–2 of Fig. 3). With increase of weight of pancreatic tissue, 28S and 18S rRNA bands became lighter than those in the 20–30 mg group gradually (see lane 3–8 of Fig. 3). When 70–80 or 90–100 mg of the pancreatic tissue in 1 ml of TRIzol® reagent was used, the 28S and 18S rRNA bands were visible, but weak, and ratio of 28S/18S was less than 0.8. On the contrary, 5S rRNA bands were much clear and strong (see lane 5–8 of Fig. 3), indicating that RNA has been degraded.

RT-PCR Analysis of Pancreas-Specific Genes Utilized Total RNA from Grinding Group and Conventional Group

Finally, we chose six genes specifically expressed in endocrine and exocrine parts of rat pancreas to test RNA isolated from grinding group and conventional group.

In the grinding group, all genes had clear bands on 1% agarose/EB gel and were located in the expected positions, i.e., insulin 1 at ~250 bp and glucagon at ~500 bp (see Fig. 4a), α-amylase at ~250 bp, and GLUT2 at ~500 bp (see Fig. 4b), Pdx1 at 476 bp, and β-actin at ~250 bp (see Fig. 4c). But there was no any band at all except β-actin in the conventional group (see Fig. 4a,b,c).

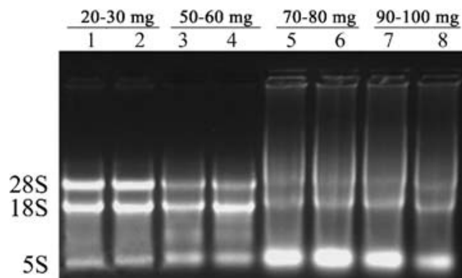

Fig. 3 Electrophoresis of total RNA isolated with TRIzol® reagent and liquid nitrogen from different weight of the pancreatic tissue of rats (*lanes 1–2*, 20–30 mg; *lanes 3–4*, 50–60 mg; *lanes 5–6*, 70–80 mg; *lanes 7–8*, 90–100 mg). Five microgram of total RNA was run on 1% non-denatured agarose/EB gel

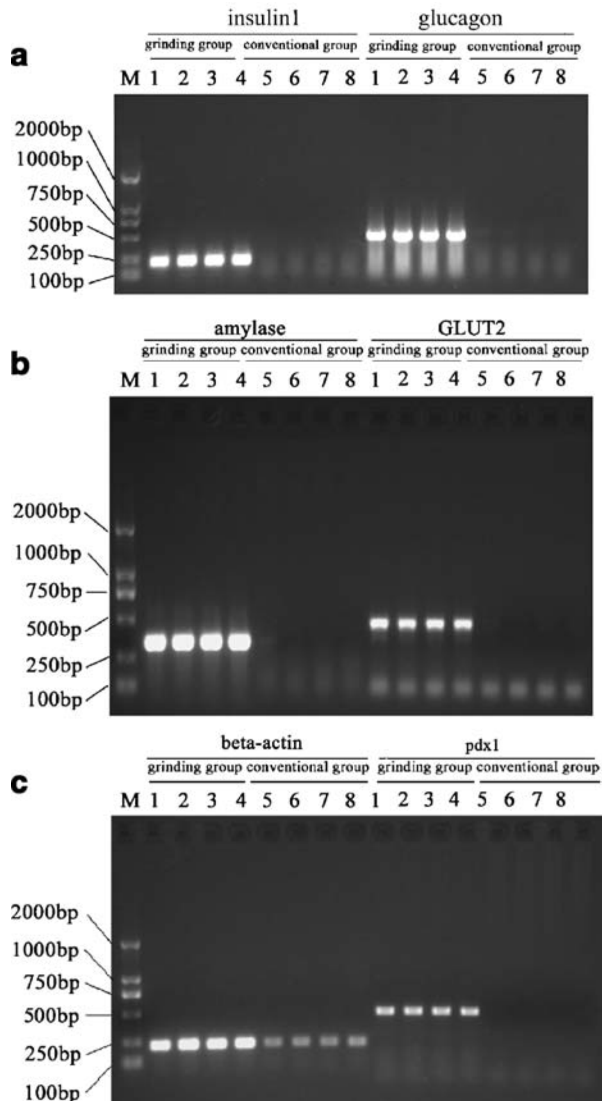

Fig. 4 RT-PCR analysis of mRNA isolated from the pancreatic tissue of four rats of the grinding group and the conventional group. **a** cDNAs from insulin1 and glucagon; **b** cDNAs from α-amylase and GLUT2; **c** cDNAs from β-actin and Pdx1

Discussion

RNA extraction is one of the most common techniques for gene function study. The mRNA is easily degraded, because it is susceptible to RNase, and RNase is stable and ubiquitously distributed in various tissues [9, 10]. The RNA extraction from common tissues and cells mainly requires reduction of the activity of RNase by dry-roasting experimental tools and treating related reagents with RNase inhibitors, such as DEPC. It is easy to isolate total RNA from most biological samples of different sources [10–12]. However, extraction of RNA from pancreas is even more difficult than that from other tissues, since the autolysis and incomplete inhibition of endogenous RNase exist [8]. Therefore, the key to RNA extraction from pancreas is to inhibit RNase activity efficiently. The method described by

Chomczynski and Saachi, based on lysis of cells with guanidine isothiocyanate and phenol, has proven to be a highly efficient method of preparing intact RNA from most tissues and is still the widely used method [3, 4]. However, it sometimes gives indefinite variable results from the pancreas, even when combined with the use of RNase inhibitors and cold-temperature homogenization or immediate snap-freezing of the pancreas before grinding the frozen tissue in a denaturing solution [6, 8]. In addition, Mullin et al. isolated high-quality intact RNA from the murine pancreas by in situ ductal perfusion of the pancreas with an RNase inhibitor before removal of the organ for RNA extraction [7]. However, the operation of in situ ductal perfusion of the pancreas is complicated and difficult.

In the present study, we modified the initial procedures of the TRIzol® reagent protocol and isolated high-quality RNA from the rat pancreas with TRIzol® reagent [13] and liquid nitrogen [14–16]. Firstly, we examined the effect of the different ways to homogenize the rat pancreas. We found that the RNA extraction by grinding rat pancreatic tissue in liquid nitrogen and then transferring the tissue powder into TRIzol® reagent had a good $A_{260/280}$ ratio (1.75–1.89) and clear 28S and 18S rRNA bands with their ratio more than 1.8. In the grinding process, it is essential to immerse frozen pancreatic tissue in liquid nitrogen to avoid RNA degradation that resulted from the tissue melting without TRIzol® reagent. When the very thin pancreatic tissue powder is transferred into TRIzol® reagent, pancreatic cells easily contact with TRIzol® reagent and are lysed, and RNase is quickly and completely inhibited. To evaluate RNA integrity, we amplified the full length of pdx1 ORF by RT-PCR. The pdx1 ORF was successfully amplified from its respective cDNA templates in the grinding group rather than the conventional group.

The pancreas has an extremely high level of RNase A [17–19], and RNA degradation appears to commence during dissection of the animal and removal of the pancreas. To increase great efficiency, performing the dissection is required as quickly as possible. In our study, the clear 28S and 18S bands of total RNA were observed and not degraded when the pancreatic tissues was obtained within 1 min. In contrast, there were very weak 28S and 18S rRNA bands and very strong 5S rRNA band when the time was prolonged to more than 3 min (5 and 7 min), suggesting that the RNA was completely degraded.

We also examined the effect of different weight of pancreatic samples on RNA extraction. Although 28S and 18S rRNA bands were obtained from total RNA isolated with 20–30, 50–60, 70–80, and 90–100 mg of the pancreatic tissue in 1 ml of TRIzol® reagent, the bands from the latter three groups were gradually weaker with a stronger 5S rRNA band with the weight increasing. The result suggested that the RNA should be partially degraded when mass of the pancreatic tissue is increased, probably because RNase in the pancreatic tissue more than 20–30 mg cannot be completely destroyed by denaturant in 1 ml of TRIzol® reagent. Therefore, time- and weight-collected pancreatic tissues are the most crucial for obtaining intact RNA.

To evaluate the profile of extracted RNA, we checked the expression of β-actin and some pancreas-specific genes, including insulin1, glucagon, pdx1, GLUT2, and amylase by RT-PCR. These genes were all successfully amplified from their respective cDNA templates in the grinding group. However, in the conventional group, there are no clear RT-PCR products except β-actin. Because we designed the primers spanning one or more introns to avoid genomic DNA template contamination and successfully amplified the full length of pdx1 ORF, the PCR products are causally derived from intact cDNA templates. These results suggested that pancreatic messenger RNA (mRNA) isolated with the method described above was undegraded, intact, and heterogenous RNA.

Taken together, we successfully extracted intact RNA by completely grinding a small quantity of the rat pancreatic tissue collected within 1 min in liquid nitrogen and then

transferring the tissue powder into 1 ml of TRIzol® reagent for further treatment. This modified method is economical, fast, and reproducible to obtain high-quality and intact RNA from the pancreas and will promote further studies on pancreatic gene functions.

Acknowledgments We are very grateful to Dr. Hongmin Li (School of Life Science, Northwest University, Xi'an, China) for her excellent technical assistance and to Mr. Muhanmude Shahzad for critical reading and linguistic correction of the manuscript.

Funding The project was supported by the National Natural Science Foundation of China (No. 30400249, 30571725 and 30630058), the Shaanxi Province International Cooperational Foundation of China (No.2007-kw-06), and the Shaanxi Province Natural Science Foundation of China (No. 2004C256).

Conflict of interest The authors declare no competing interests.

References

1. Cox, R. A. (1968). *Methods in Enzymology*, *12*, 120–129. doi:10.1016/0076-6879(67)12123-X.
2. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979). *Biochemistry*, *18*, 5294–5299. doi:10.1021/bi00591a005.
3. Chomczynski, P., & Sacchi, N. (1987). *Analytical Biochemistry*, *162*, 156–159. doi:10.1016/0003-2697(87)90021-2.
4. Chomczynski, P., & Sacchi, N. (2006). *Nature Protocols*, *1*, 581–585. doi:10.1038/nprot.2006.83.
5. Beintema, J. J., Campagne, R. N., & Gruber, M. (1973). *Biochimica et Biophysica Acta*, *310*, 148–160.
6. Mutter, G. L., Zahrieh, D., Liu, C., Neuberg, D., Finkelstein, D., Baker, H. E., et al. (2004). *BMC Genomics*, *5*, 88. doi:10.1186/1471-2164-5-88.
7. Mullin, A. E., Soukatcheva, G., Verchere, C. B., & Chantler, J. K. (2006). *BioTechniques*, *40*, 617–621.
8. Kiba, T., Kintaka, Y., Nakada, E., Suzuki, Y., Inoue, S., & Ishigaki, Y. (2007). *Pancreas*, *35*, 98–100. doi:10.1097/01.mpa.0000278690.93930.b6.
9. Evans, R., & Kamdar, S. J. (1990). *BioTechniques*, *8*, 357–360.
10. Peirson, S. N., & Butler, J. N. (2007). *Methods in Molecular Biology (Clifton, N.J.)*, *362*, 315–327. doi:10.1007/978-1-59745-257-1_22.
11. Salter, M. G., & Conlon, H. E. (2007). *Methods in Molecular Biology (Clifton, N.J.)*, *362*, 309–314. doi:10.1007/978-1-59745-257-1_21.
12. Chen, J., Byrne, G. E. Jr., & Lossos, I. S. (2007). *Diagnostic Molecular Pathology*, *16*, 61–72. doi:10.1097/PDM.0b013e31802f0804.
13. Xiang, X., Qiu, D., Hegele, R. D., & Tan, W. C. (2001). *Journal of Virological Methods*, *94*, 129–135. doi:10.1016/S0166-0934(01)00284-1.
14. Gill, S. S., Aubin, R. A., Bura, C. A., Curran, I. H., & Matula, T. I. (1996). *Molecular Biotechnology*, *6*, 359–362. doi:10.1007/BF02761714.
15. Wang, S. S., Sherman, M. E., Rader, J. S., Carreon, J., Schiffman, M., & Baker, C. C. (2006). *Diagnostic Molecular Pathology*, *15*, 144–148. doi:10.1097/01.pdm.0000213460.53021.cd.
16. Dekairelle, A. F., Van der Vorst, S., Tombal, B., & Gala, J. L. (2007). *Clinical Chemistry and Laboratory Medicine*, *45*, 1283–1287. doi:10.1515/CCLM.2007.281.
17. Bendayan, M., & Ito, S. (1979). *The Journal of Histochemistry and Cytochemistry*, *27*, 1029–1034.
18. Urban, E., Zingery, A. A., Bundrant, T., Weser, E., & Ziegler, D. M. (1982). *Journal of Pediatric Gastroenterology and Nutrition*, *1*, 267–272.
19. Potenza, N., Salvatore, V., Migliozzi, A., Martone, V., Nobile, V., & Russo, A. (2006). *Nucleic Acids Research*, *34*, 2906–2913. doi:10.1093/nar/gkl368.