Optimization of RNA Purification and Analysis for Automated, Pre- Symptomatic Disease Diagnostics

Anjali Vaidya, Summer Student June – August 2004, Stanford University

Shanavaz Nasarabadi, Research Mentor

Fred Milanovich, Supervisor

JUNE 28, 2005

Abstract

When diagnosing disease, time is often a more formidable enemy than the pathogen itself. Current detection

methods rely primarily on post-symptomatic protein production (i.e. antibodies), which does not occur in noticeable levels until several weeks after infection. As such, a major goal among researchers today is to expedite pre-symptomatic disease recognition and treatment.

Since most pathogens are known to leave a unique signature on the genetic expression of the host, one potential diagnostic tool is host mRNA. In my experiments, I examined several methods of isolating RNA and reading its genetic sequence. I first used two types of reverse transcriptase polymerase chain reactions (using commercial RNA) and examined the resultant complementary DNA through gel electrophoresis. I then proceeded to isolate and purify whole RNA from actual human monocytes and THP-1 cells using several published methods, and examined gene expression on the RNA itself. I compared the two RT-PCR methods and concluded that a double step RT-PCR is superior to the single step method. I also compared the various techniques of RNA isolation by examining the yield and purity of the resultant RNA. Finally, I studied the level of cellular IL-8 and IL-1 gene expression, two genes involved in the human immune response, which can serve as a baseline for future genetic comparison with LPS-exposed cells. Based on the results, I have determined which conditions and procedures are optimal for RNA isolation, RT-PCR, and RNA yield assessment.

The overall goal of my research is to develop a flow-through system of RNA analysis, whereby blood samples can be collected and analyzed for disease prior to the onset of symptoms. The Pathomics group hopes to automate this process by removing the human labor factor, thereby decreasing the procedure's cost and increasing its availability to the general population. Eventually, our aim is to have an autonomous diagnostic system based on RNA analysis that would significantly mitigate the effects of disease and as a close parallel, of large-scale bioterrorism.

This document was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor the University of California nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or the University of California, and shall not be used for advertising or product endorsement purposes.

This work was performed under the auspices of the U.S. Department of Energy by University of California, Lawrence Livermore National Laboratory under Contract W-7405-Eng-48
UCRL: UCRL-TR-213336

Introduction

Recent evidence has underlined the importance of RNA, specifically small RNAs, as diagnostic targets of various infectious diseases. As a result, interest in isolating, purifying, and identifying RNA genes has increased tremendously. However, accurate RNA analysis is rendered difficult by the molecule's fragility and sensitivity: Gene transcripts can easily degrade, and improper sample handling can cause unintended gene induction. Standard RNA isolation techniques have typically been optimized for only the largest molecules and are often unsuitable for smaller species.

Promising methods for RNA collection include phenol and trysol based extractions, as well as glass fiber filter (GFF) based binding. Several reputable biotechnology companies such as Ambion and Qiagen have published methods of RNA isolation from cells. In all methods, a chaotropic agent is first used to lyse cells and inactivate endogenous RNases. A phenol or trysol reagent disrupts cells and tissues and separates RNA from contaminants using a combination of denaturants and Rnase inhibitors. An alcohol based solution can then be used to precipitate out the RNA and/or make it competent for binding to glass fiber filters. Gel electrophoresis, Northern blot analysis, and RT PCR can subsequently be used to identify specific strands of RNA.

Methods

RT-PCR

- 2-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
 - Reverse Transcription:
 - For each sample: Mix 2 µl random decamers, 2 µl 10x RT buffer, 4 µl dNTPs, 1 µl Rnase Inhibitor, 1 µl RNA sample, 1 µl Reverse Transcriptase, and 20 µl nuclease free water
 - Incubate at 44 °C for 1 hr., then at 92 °C for 10 min to inactivate Reverse Transcriptase
 - PCR
 - For each sample: Add 1 µl RT reaction, 5 µl 10x PCR buffer, 2.5 µl dNTP mix, 2.5 µl PCR primers, 2 µl Taq Polymerase, to 50 µl nuclease free water
 - Denature at 95 °C for 4 min, cycle 30 times at 94 °C for 20 sec and 72 °C for 40 sec., then complete at 72 °C for 5 min.
- 1-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
 - For each sample: Mix 1 µl template RNA, 6.5 µl dNTP mix, 2 µl random decamers, and 73 µl nuclease free water. Heat at 70 °C for 3 minutes. Replace tubes on ice.
 - Add: 10 µl 10x PCR Buffer, 1 µl Rnase Inhibitor, 5 µl PCR primers, 1 µl Reverse Transcriptase, 1 µl Taq polymerase. Incubate at 42 °C for 1 hr, 94 °C for 5 min, spin through 30 cycles of PCR at 94 °C for 20 sec and 72 °C for 40 sec., then at 72 °C for 5 min.

RNA Isolation and Stabilization

Ambion RiboPure™ - Blood

- For each sample: Add 500 µl cell solution to 1.3 ml RNAlater to preserve the RNA from degrading. Centrifuge sample for 1 min in microcentrifuge and remove supernatant by aspiration. Add 800 µl lysis solution and 50 µl sodium acetate solution to lyse cells. Vortex vigorously. Add 500 µl Acid-Phenol: Chloroform to extract RNA, then vortex. Store at room temperature for 5 min, centrifuge for 1 min, and transfer the aqueous phase containing RNA to a new tube.
- Pass the sample through a filter cartridge 700 µl at a time. Centrifuge for 10 sec in microcentrifuge to pass liquid through filter and discard flow-through. Wash twice with 700 µl 1x Tris-EDTA, then elute twice with 50 µl elution solution to recover RNA.

Ambion RNAqueous®

- For each sample: Add 200-700 µl lysis/binding solution for 100 – 10⁷ cells. Pipette lysate up and down. Reduce the viscosity of the lysate by homogenizing if necessary. Add an equal volume of 64% ethanol to the lysate and mix.
- Apply the lysate/ethanol mixture to the filter in 700 µl spurts. Centrifuge for 1 minute at room temperature at 10,000 x g to pass the mixture through the filter. Discard the flow through. Wash with 700 µl Wash Solution#1. Centrifuge at 10,000 x g for 1 minute and discard flow through. Then wash twice with 500 µl Wash Solution#2/3. Repeat centrifugation. Transfer filter to a fresh collection tube and elute with 40 µl Elution Solution. Centrifuge for 30 seconds at 10,000 x g. Elute with a second 10 µl aliquot of elution solution. Centrifuge for 30 seconds at 10,000 x g

PreAnalytix (QIAGEN) PAXgene™Blood RNA Kit

Figure 1 The PAXgene Blood RNA procedure

- For each sample: Pipet the sample into PAXgene column and incubate overnight. Then centrifuge the PAXgene Blood RNA tube for 10 min at 5000x g. Remove supernatant and add 5 ml RNase-free water to the pellet. Vortex, then centrifuge for 10 min at 5000x g. Remove and discard the entire supernatant. Resuspend pellet in 360 µl buffer BR1. Then add 300 µl Buffer BR2 and 40 µl Proteinase K, Vortex and incubate at 55 °C for 10 minutes. Centrifuge for 3 minutes at max speed.
- Add 350 µl 100% ethanol. Vortex. Then add 700 µl to PAXgene column sitting in processing tube. Centrifuge for 1 minute at 8000x g and discard flow-through. Add remaining sample to the column and repeat.
- Apply 700 µl Buffer BR3 to column and centrifuge for 1 minute at 8000x g. Discard flow-through. Apply 500 µl Buffer BR4 to the column. Centrifuge for 1 minute at 8000x g. Discard flow-through. Add another 500 µl Buffer BR4 to the column, centrifuge for 3 minutes at max speed, then transfer the column to an elution tube.
- Pipet 40 µl Buffer BR5 to the PAXgene column membrane. Centrifuge for 1 minute at 8000x g. Repeat the elution with 40 µl Buffer BR5. Incubate the eluate at 65 °C for 5 minutes. Chill immediately afterwards on ice.

TRIZOL RNA Extraction

- To a 250 µl sample, add 750 µl TRIZOL (GIBCO BRL). Homogenize sample using an 18-Gauge syringe and needle. Incubate at room temperature for 5 minutes. Add 200 µl chloroform. Shake vigorously for 15 seconds.
- Leave at room temperature for 15 minutes. Centrifuge at 12,000x g for 10 minutes at 4 °C. Transfer aqueous phase to a clean tube. Add 500 µl isopropanol and mix by inversion. Incubate at room temperature for 10 minutes.
- Centrifuge at 12,000x g for 10 minutes at 4 °C. Remove the supernatant and add 1 mL of 75% ethanol. Centrifuge at 7,500x g for 5 minutes at 4 °C. Remove supernatant and vacuum dry for 10 minutes.
- DNase treat sample by first resuspending the pellet in 8 µl water. Add 1 µl DNase buffer, and 1 µl DNase. Leave for 15 minutes at room temperature and then for 10 minutes at 65 °C. Store at -20 °C

Results

RT-PCR on commercial RNA control template

2-Step RT-PCR

1-Step RT-PCR

Lane 1: Reverse Transcription Negative Control

Lane 2: No RNA sample (Neg Control)

Lane 3: RNA Control Template

Lane 4: Reverse Transcription Negative Control

Lane 5: No RNA sample (Neg Control)

Lane 6: RNA Control Template

Lane 7: PCR Negative Control

Lane 8: PCR Negative Control

Lane 9: No RNA sample (Neg Control)

Lane 10: No RNA sample (Neg Control)

Lane 2: Reverse Transcription Negative Control

Lane 3: No RNA sample (Neg Control)

Lane 4: RNA Control Template

Lane 5: Reverse Transcription Negative Control

Lane 6: No RNA sample (Neg Control)

Lane 7: RNA Control Template

RNA Isolation and Purification

RNA Isolated using RiboPure™ Method

Lane 1: Isolated RNA
Lane 2: Isolated RNA
Lane 3: Ladder

No RNA is visible in the samples.

PAXGene Purified RNA from Leukocytes

Lane 1: PAXGene Purified RNA at 1:3 dilution
Lane 2: PAXGene RNA Sample 2 at 1:3 dilution
Lane 3: Positive Control Total RNA at 1:3 dilution
Lane 4: PAXGene Purified RNA concentrate
Lane 5: PAXGene RNA Sample 2 concentrate
Lane 6: Positive Control Total RNA concentrate

Only the positive controls exhibit distinct 18s and 28s RNA fragments, visible as two dark bands on the gel.

PAXGene Purified RNA from Monocytes

Lane 1: PAXGene Purification 1
 Lane 2: PAXGene Purification 1
 Lane 3: PAXGene Purification 1
 Lane 4: PAXGene Purification 1
 Lane 5: Positive Control Total RNA
 Lane 6: Positive Control Total RNA
 Lane 7: PAXGene Purification 2
 Lane 8: PAXGene Purification 2
 Lane 9: Positive Control ATCC RNA
 Lane 10: Positive Control Ambion RNA
 Lane 11: PAXGene Purification 2 with DNase treatment
 Lane 12: PAXGene Purification 2 with DNase treatment

We performed the PAXgene protocol twice, the second time with greater attention to sanitation and a subsequent DNase treatment.

Still, only the positive controls exhibit distinct 18s and 28s RNA fragments.

Trizol Extraction and RNAqueous® procedure

Lane 1: Trizol Extracted Monocyte RNA
 Lane 2: Trizol Extracted THP-1 RNA

Lane 3: RNAqueous® Extracted THP-1 cell RNA

Lane 4: RNAqueous® Extracted THP-1 cell RNA with DNase treatment
 Lane 5: THP-1 cell Total RNA Positive Control
 Lane 6: Ambion Control Template RNA Positive Control

The RNAqueous® procedure yields 18s and 28s bands as clear as those of the positive controls.

Quantifying the RNA Yield in the RNAqueous[®] procedure

RNAqueous[®] Extracted THP-1 cell RNA

THP-1 cell Total RNA Positive Control

Graph 1: RT-PCR on commercial RNA stock. This RT-PCR was performed on commercial RNA samples from Ambion and ATCC to determine the amount and rate of amplification when high purity RNA is used in a real time RT-PCR setting. The positive amplification in the Reverse Transcript negative control indicates some level of DNA contamination in the ATCC RNA sample.

Graph 2: RT-PCR on cellular extracted RNA. This RT-PCR was performed on RNA extracted from cells (human monocytes and THP-1 cells) using the TRIZOL based extraction and the RNAqueous® extraction.

Graphs 3 and 4: RT-PCR on IL-1 and IL-8 Genes. Using the RNA samples from the previous RT-PCR, these two experiments were conducted to amplify the IL-8 and IL-1 gene targets, both of which are involved in the human immune response.

Discussion

Based upon the gel results for the 1-step and 2-step RT-PCR, the yield and purity of DNA is superior in a 2-step process. The DNA bands on the 2-step gel are thicker and brighter. Dividing the process into an RT and a subsequent PCR step allows the complementary DNA to stabilize before amplification. In the 1-step procedure, there is greater enzyme competition since the Reverse Transcriptase and Taq Polymerase are operating in the same sample at the same time. This increased competition reduces the efficiency of the RT-PCR, consequently reducing the product.

Among the various methods of isolating and purifying RNA from cells, Ambion's RNAqueous[®] seems the best. Both the results of the RNA gels and the RT-PCR were considered when evaluating the different RNA isolation processes. In all methods except Ambion's RNAqueous[®] glass fiber filtration procedure, purified RNA was highly degraded, forming a smear towards the bottom of the gel. Only the Ambion procedure resulted in RNA that clearly separated into the 18s and 28s RNA fragments, similar to the RNA control templates which served as positive controls.

In terms of RNA yield, the TRIZOL-based extraction and Ambion's RNAqueous[®] procedure both produced RNA that could be successfully reverse transcribed and amplified. The RiboPure[™] and PAXgene procedures were run through similar RT-PCRs but demonstrated very little amplification and low DNA product (results not shown). When combining the results of the gels, which demonstrate RNA purity, and the RT-PCRs, which demonstrate RNA yield, RNAqueous[®] strong showing in both categories substantiates it being the best procedure for total RNA isolation.

With regard to IL-8 and IL-1 immune response genes, amplification rate and amount of gene expression varied depending upon which RNA extraction method was used. A controlled experiment in which one extraction method is used on both healthy and infected cells will provide an accurate comparison of genetic induction caused by LPS exposure. Nonetheless, these results demonstrate that expression of these two genes in healthy cells is relatively low, as amplification did not begin until the 20th cycle or later in all samples. Sample handling methods may also need to be revised to ensure that handling techniques do not cause IL-1 and IL-8 gene induction.

Future Work

With successful recovery and purification of cellular RNA, and a baseline established for IL-8 and IL-1 gene expression, future experiments should examine the genetic differences between cells exposed to various antigens and their healthy counterparts in order to expand the library of pathogenic genetic signatures. Subsequent experimentation should focus on automation, whereby the insertion of a blood sample triggers an RT-PCR or gel electrophoresis and an accurate genetic analysis of the subject. Such a machine, if available on a large scale, would revolutionize disease diagnostics as we know it.

Acknowledgements & References

I would like to thank Hitesh Kapur for the usage of his Agilent BioAnalyzer for my RNA gel analysis

I would also like to thank Shalini Mabery for her guidance and instruction with the TRIZOL RNA Extraction

Chacko, Sapna and Emmanuel Labourier. *Challenges of Small-RNA Purification and a Strategy to Meet Them*. March 2004.

Abbas, Abul K. Lichtman, Andrew H. Pober, Jordan S. *Cellular and Molecular Immunology 4th Edition*. 2000.

PreAnalytix. *PAXgeneTMBlood RNA Handbook*. April 2001.

Ambion. *RNAqueous[®] Small Scale Phenol-Free Total RNA Isolation Kit Instruction Manual*.