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Isolation of total RNA with the illustra RNAspin Mini Isolation Kit

Key words: *illustra • total RNA • microarray • quantitative reverse transcription polymerase chain reaction (QRT-PCR) • primer extension • Northern blot • sample preparation*

illustra™ RNAspin Mini Isolation Kit can be used to isolate total RNA from cultured cells, tissue, bacteria or yeast. The isolated RNA is of a high enough quality and quantity (up to 100 µg) for multiple downstream experiments and sensitive enzymatic applications such as RT-PCR, primer extension, and RNase protection assays. This application note describes the isolation of high-quality total RNA from several types of source material including hard-to-lyse bacterial strains, and the subsequent use in downstream applications, such as quantitative reverse transcription polymerase chain reaction (QRT-PCR), Northern blotting, and microarray experiments.

Materials

Products used

illustra RNAspin Mini Isolation Kit	28-0500-70
CodeLink™ Expression Assay Reagent Kit	320012
CodeLink Rat Whole Genome Bioarrays, six-pack	300031-6PK
CyScribe™ GFX™ Purification Kit	27-9606-01

Other materials used

RNAlater™ RNA Stabilization Solution (Ambion)
 RNAlater-ICE™ Frozen Tissue Transition Solution (Ambion)
 TaqMan™ Reverse Transcription Reagents (Applied Biosystems)
 TaqMan TAMRA primer/probe sets (Applied Biosystems)
 Agilent 2100 bioanalyzer (Agilent)
 ABI PRISM 7900HT Sequence Detection System (Applied Biosystems)

Protocol

The RNAspin Mini RNA Isolation Kit was used to purify total RNA for several different applications according to the protocol illustrated in Figure 1. To reduce the risk of clogging the RNA-binding column and the potential loss in sample yield and purity, the lysates in these experiments were prefiltered using column filters included in the kit. Incubation with DNase I, also included in the kit, was efficiently carried out on-column under conditions optimized by the Membrane Desalting Buffer.

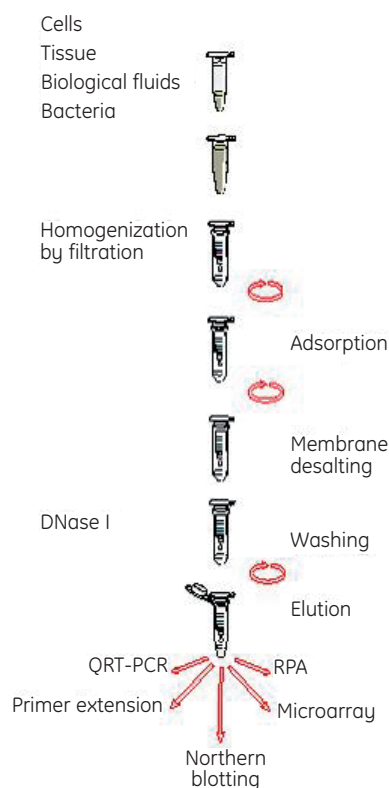


Fig 1. Schematic representation of the RNAspin Mini Isolation Kit protocol.



Assessment of RNA quality

Total RNA was isolated as described in Figure 1 from two cultures of 10^6 HeLa cells, grown according to standard procedures (1). The cell cultures were pooled and divided into equal aliquots for RNA isolation. In both cases, total RNA was eluted with 70 μ l of RNase-free water. Total RNA (20 μ l; 5–6 μ g) was run on a denaturing formaldehyde agarose gel. A PCR reaction using β -actin-specific primers was performed with 1 μ l (300 ng) of total RNA. The PCR products were analyzed on a TAE-buffered, 1% agarose gel electrophoresis (1).

Isolation of RNA from hard-to-lyse bacterial strains

A culture of *Pseudomonas putida* in LB broth (1.2 ml; OD₆₀₀ 0.9) was grown and harvested by centrifugation. The pellet was resuspended and divided into three different aliquots of 100- μ l TE containing 0.05, 0.2, and 0.8 mg/ml of lysozyme, respectively. The mixtures were incubated at 37 °C for 10 min, and further processing to isolate total RNA was carried out according to the standard protocol included in the kit. An aliquot of the eluted product (6 μ l) was analyzed on a 1.2% formaldehyde gel (1).

Application of the total RNA product in RT-PCR

Total RNA was isolated from different amounts of HeLa cells at varying cell number amounts: 10^5 , 10^4 , 10^3 , 330, 100, 33, 10, 3, and 0 (control), using the RNeasy Mini Isolation Kit (Fig 1). Prefiltration of the lysate, and subsequent treatment with DNase I were performed according to the protocol. The total RNA product was eluted with 40 μ l of buffer and 3 μ l of this was used in an RT-PCR reaction with β -actin primers.

Application of the total RNA product in QRT-PCR

A 100-mg aliquot of rat liver tissue was frozen and subsequently stored in RNA/later-ICE solution. Total RNA was extracted using the RNeasy Mini RNA Isolation Kit and the cDNA was generated using TaqMan Reverse Transcription Reagents and TaqMan TAMRA primer/probe sets with 1 μ g of the RNA templates (1). The probe/primer set targeted the rat thyroid hormone sulfotransferase (*ST1B1*) gene. *GAPDH* was used as a control transcript. The reactions were then purified using the CyScribe GFX Purification Kit. Various amounts (4.0 ng, 0.4 ng, and 0.04 ng) of the purified cDNA were used per QRT-PCR with HotStarTaq Kit (Qiagen) on the ABI 7900 instrument.

Total RNA was extracted from three different amounts (30 mg, 3 mg, and 0.3 mg) of mouse kidney cortex tissue with the RNeasy Mini RNA Isolation Kit. The tissue was either fresh frozen or preserved using RNA/later solution. The RNA eluate (10 μ l out of a total volume of 60 μ l) was used as a template for the reverse transcriptase reaction using primers specific for α -actinin-4. *GAPDH* was used as a control transcript.

Application of the total RNA product in a Northern blot experiment

The RNeasy Mini Isolation Kit was used to isolate total RNA from two treatment groups of macrophage cells. A signaling cascade within these cells ($\sim 2 \times 10^6$ each) was induced by treatment with lipopolysaccharide (LPS). After two hours, the LPS-treated cells were subjected to Actinomycin D (ActD) treatment and then harvested after 0, 30, 60, and 90 min. One group of cells was treated with an inhibitor of the phosphorylation signal cascade one hour prior to LPS treatment; whereas the other group received no inhibitor treatment. The expression profiles from each sample group were evaluated by Northern blot (1).

Application of the total RNA product in microarray gene expression analysis

The RNeasy Mini Isolation Kit was used to purify total RNA from five independent rat liver and five independent rat kidney tissue samples for comparative gene expression analysis. After isolation in parallel, 2 μ g of each of the total RNA products was used to generate separate preparations of biotin-labeled cRNA using the CodeLink Expression Assay Reagent Kit. The purity and integrity of the labeled cRNA was evaluated by A_{260}/A_{280} spectrophotometry and on the Agilent 2100 bioanalyzer. The cRNA was then hybridized onto Rat Whole Genome Bioarrays using the standard codeLink bioarray hybridization and detection protocol.

Results

The quality of the total RNA products in all the isolation experiments was determined via the A_{260}/A_{280} ratio and in all cases, this ratio was between 1.9–2.1 (data not shown). Where the RNA quality was assessed using the Agilent 2100 bioanalyzer, RNA Integrity Number (RIN) values were greater than 8.0. The rRNA bands were sharp with no visible, degradative bands; the 28S band was about twice as intense as the 18S band.

Effect of DNase I treatment on total RNA quality

An important feature of total RNA purity is the presence or absence of contaminating genomic DNA (gDNA) post-isolation. The membrane desalting step of the RNeasy Mini kit creates an optimal chemical environment for efficient on-column DNase I digest. Therefore, the isolated total RNA is free of gDNA. This is especially critical for enzymatically sensitive, downstream applications in which contaminating gDNA may interfere and cause spurious product formation and/or low product yield. The difference in RNA purity with or without DNase I treatment is shown in Figures 2A and 2B. Without DNase I treatment, residual gDNA is present (A). To determine whether trace amounts of gDNA was present even in the DNase I-treated samples, a more sensitive method was employed.

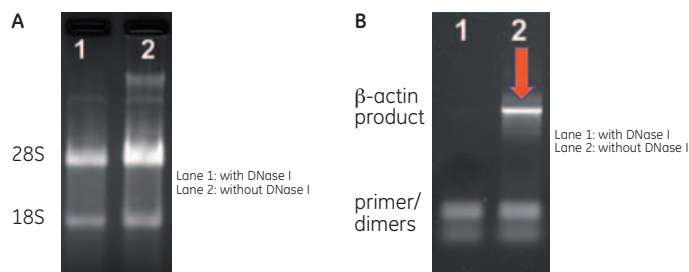


Fig 2. The DNase I digest of the RNAspin Mini kit efficiently eliminates genomic DNA contamination. (A) RNA purity with and without DNase I treatment ran on a denaturing gel. (B) PCR detection of DNA contamination in total RNA prepared with or without DNase I treatment visualized on an agarose gel.

PCR using β -actin specific primers was performed and analyzed on a 1% agarose gel. The results demonstrate that the DNase I treatment was very effective; the 750-bp PCR product of chromosomal β -actin was only seen in the sample that was not treated with DNase I (Fig 2B, lane 2, red arrow).

Examination of total RNA from hard-to-lyse bacterial strains

The RNAspin Mini Isolation Kit provides supplemental instructions for the enzyme/heat treatments required for effective RNA isolation from bacteria and yeast. The optimum lysozyme enzyme concentration may have to be empirically determined in order to maximize the yield from bacterial strains. Such a lysozyme concentration titration schema was used to successfully isolate high-quality total RNA from the hard-to-lyse bacterial strain, *Pseudomonas putida* (Fig 3).

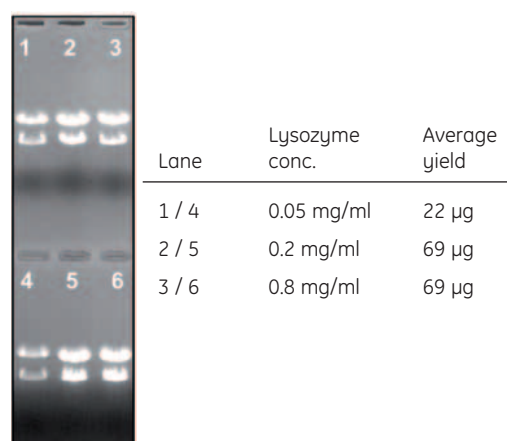


Fig 3. Yield and quality assessment of total RNA obtained from a hard-to-lyse bacterial strain and visualized on a 1.2% formaldehyde gel.

Application of the total RNA product in RT-PCR

RT-PCR results using total RNA isolated from different amounts of HeLa cells are shown in Figure 4. The 626-bp β -actin RT-PCR product could be detected from as few as 10 HeLa cells.

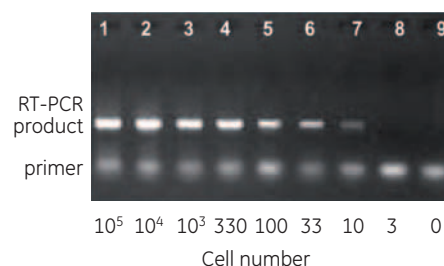


Fig 4. The RNAspin Mini Isolation kit can be used to detect transcripts from ≤ 10 HeLa cells.

Detection of RNA transcripts by QRT-PCR

The rat thyroid hormone sulfotransferase, encoded by the *ST1B1* gene, plays an important role in the regulation of thyroid hormone metabolism. As with other important transcripts, precise measurement of the relative expression level in the liver is crucial to the elucidation of the biological function of *ST1B1*. The data depicted in Figure 5 demonstrates that the RNA output of the RNAspin Mini is of a high enough caliber to perform well in sensitive downstream applications like QRT-PCR.

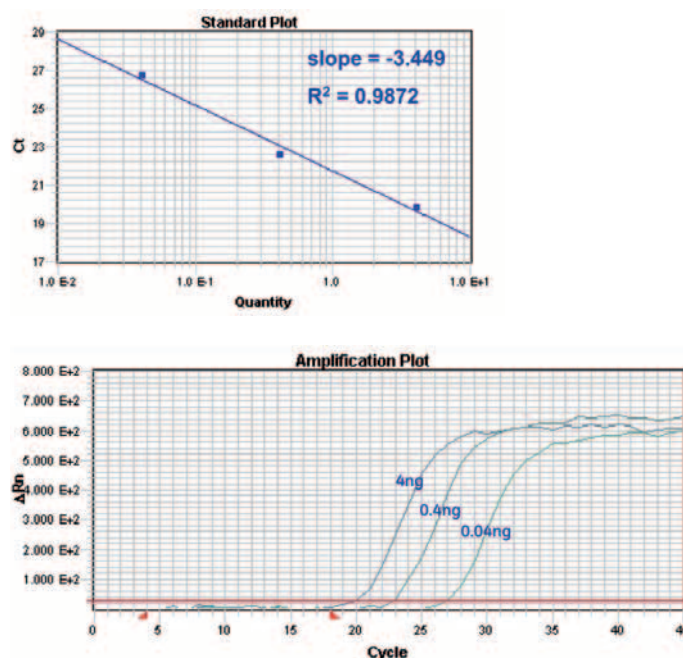


Fig 5. The RNAspin Mini Kit total RNA product performs well in sensitive downstream applications such as QRT-PCR. Titration of an RNAspin Mini total RNA sample (NAP274) with TAQ100 primer/probe set (rat thyroid hormone sulfotransferase, *ST1B1* gene). (A) QRT-PCR standard curve indicating the appropriate 10-fold differences in the reaction dilutions; (B) QRT-PCR C_T curves, showing typical response curves for clean RNA template.

Mutations in the actin-binding protein, actinin-4, have been associated with the nephrotic syndrome called focal segmental glomerulosclerosis (FSGS). In addition, mutations in actinin-4 have been associated with the promotion of the metastases of colorectal cancer. The differential expression level of an associated transcript in a given tissue—even with only minute amounts of tissue—provides critical information for understanding the metastatic state of colorectal cancer.

Here we see that the isolation of different amounts of sample material with the RNASpin Mini kit did not influence the measured expression profile (Fig 6). RNA can be isolated from samples as small as 0.3 mg and subsequently analyzed via QRT-PCR. Fresh-frozen tissue samples as well as samples stored in RNA*later* solution yielded similar results.

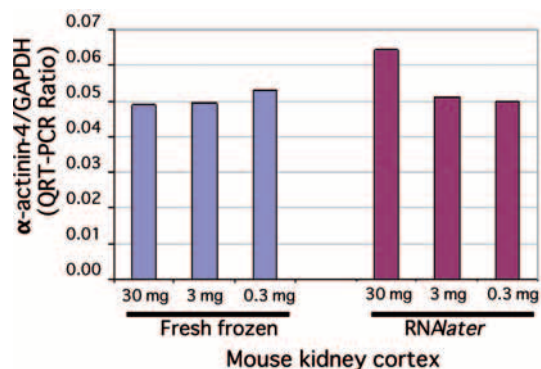


Fig 6. QRT-PCR detection of the α -actinin-4 transcript from even small tissue amounts. (Data kindly provided by A. Henger and S.M. Blattner, Div. of Nephrology, Dept. of Internal Medicine, University of Michigan, Ann Arbor, MI, USA).

Use of the total RNA product in Northern blots

Northern blot analysis remains a mainstay of gene expression analysis. To evaluate phosphorylation-mediated regulation, two macrophage cell cultures were stimulated by LPS and ActD treatments. Prior to stimulation, one culture was treated with an inhibitor of the phosphorylation signal cascade and the other was not. Total RNA from the different macrophage cultures was isolated using the RNASpin Mini kit, and analyzed by Northern blot (Fig 7, lanes 1–4 represent untreated cells and lanes 5–8 show inhibitor-treated cells). The RNASpin Mini kit was capable of yielding sufficient quality and quantity of RNA for Northern blot analysis. The data shows that with or without signaling inhibition, the amount of RNA binding protein mRNA (the transcript labeled in this experiment) declined steadily over the 90 min timeframe after ActD treatment.

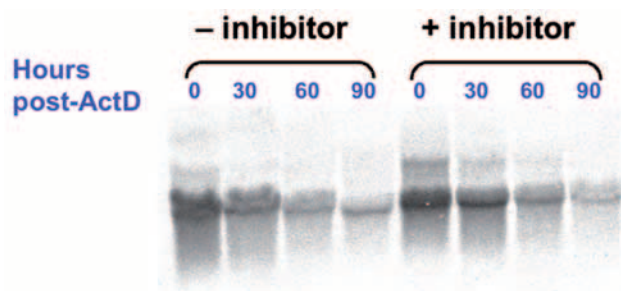


Fig 6. Purified RNA, from the RNASpin Mini Isolation kit, is of sufficient quality and yield for Northern blot analysis. (Data kindly provided by E. Hitti, Medizinische Hochschule Hannover, Institut für Physiologische Chemie, Hannover, Germany.)

Use of the total RNA product in microarray gene expression analysis

The average A_{260}/A_{280} ratios of the cRNA generated from rat liver and rat kidney RNASpin Mini total RNA were 2.17 and 2.14, respectively. Analysis of the cRNA on an Agilent 2100 bioanalyzer (Fig 7) showed that the labeled cRNA was consistently of high quality and good yield. Thus, the total RNA successfully generated labeled cRNA in a reproducible manner.

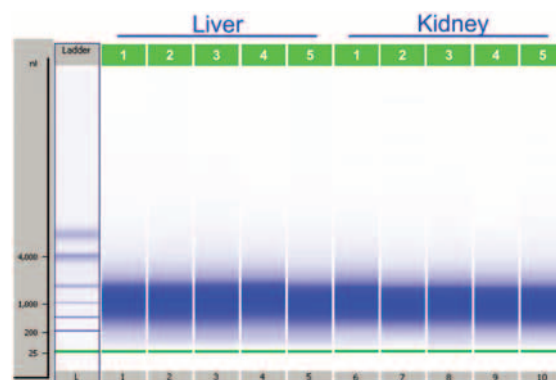


Fig 7. Agilent 2100 bioanalyzer analysis of labeled cRNA quality. The highly reproducible cRNA quality and yield is due in large part to the quality of the input total RNA.

Each of these ten independent labeled cRNA samples was then hybridized to a separate CodeLink Rat Whole Genome Bioarray. An examination of the replicate arrays (5 arrays per tissue—liver or kidney) showed excellent correlation of the normalized probe signals (Fig 8B) and low background for all (Fig 8A). This demonstrates that the individual total RNA samples were highly uniform in their quality and transcript population representation, which is crucial for microarray experiments. Additionally, the correlation of differential expression ratios between rat kidney and rat liver was > 0.97 for typical comparisons of two randomly selected replicate ratios (Fig 9).

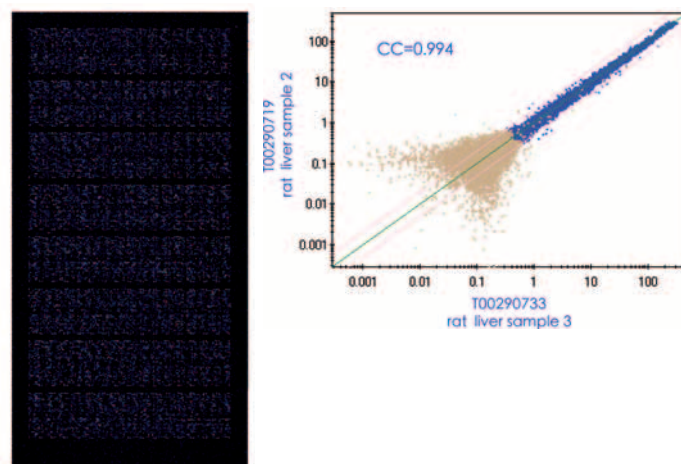


Fig 8. Microarray evaluation of total RNA prepared using the RNASpin Mini kit shows the high level of preparation reproducibility. Labeled cRNA (Fig 6) was run on CodeLink Rat Whole Genome Bioarrays. (A) The low level of background signal is indicated by the whole hybridized array image shown; (B) The normalized signal intensity correlation between replicate independent cRNA samples was high (Pearson correlation coefficient of 0.994).

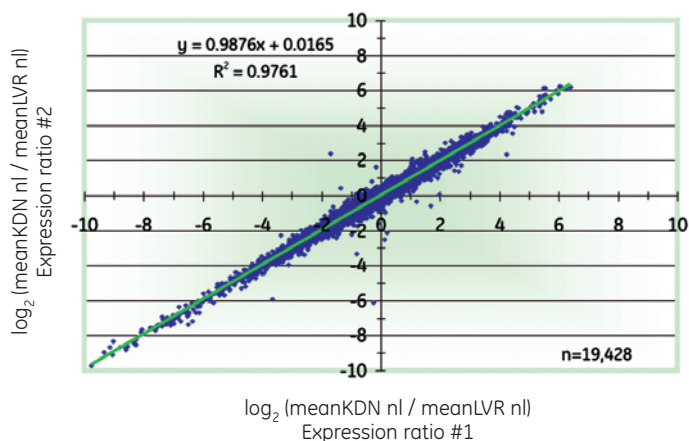


Fig 9. Differential expression analysis of the hybridized cRNA targets using CodeLink Rat Whole Genome Bioarrays. The figure shows the high concordance between two kidney:liver expression ratios, independently derived from 4 randomly chosen bioarrays. The $n = 19\,428$ number represents the number of concordantly "Good" probes used in the correlation calculation.

Conclusions

- The RNAspin Mini RNA Isolation kit yields RNA of high quality and integrity with little or no contaminating genomic DNA.
- The RNA product can be purified in varying amounts from a diverse range of sample types, including samples that are difficult to process. Typical input tissues include rat and mouse tissue (liver, kidney, brain), different tumor cell lines, *Streptococci*, *Actinobacillus pleuropneumoniae*, and *Pseudomonad* species.
- The RNAspin Mini RNA Isolation Kit is very effective at isolating high-quality RNA from small amounts of input tissue.
- The quality, integrity, and yield of total RNA is high enough for the most sensitive of downstream applications such as QRT-PCR, Northern blotting, and microarray analysis.

Reference

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