RNA Isolation mit Trizol (Sigma TRI Reagent T9424)

Introduction:
Yields to expect: 1-3 µg of total RNA/mg of tissue. Keep in mind that mRNA represents 1-3 % of total RNA. If large amounts of total RNA (more than 500 µg) are required, use protocol P 5 (one mouse liver for example gives 4-5 mg of total RNA).
Whatever method you use, RNA is always stored as EtOH precipitate. This means that the final RNA, resuspended in ultrapure Sigma water, is reprecipitated with 0.1 vol. of 3 M NaAc, 2 vol. EtOH and stored at -20°C. If RNA is needed, vortex this suspension and remove appropriate amount in fresh tube. Then spin as usual, wash with 70 % EtOH, air-dry and resuspend in Sigma water for further use.

Nachfolgende Mengen sind für 1 konfluente 6cm-Schale. Bei abweichenden Mengen bitte genau die Anweisungen des Herstellers bezüglich Zellzahl/Gewebemenge beachten!

- *bevor die ganze Prozedur beginnt: Gelkammern in NaOH (10 M, 10 min) einweichen, 2 min Fließwasser, 2 x deion. Wasser; Gel gießen mit frischem Kolben und Zentrifuge vorkühlen: 4°C*

- Zellen: konfluente 6 cm Schale (Zugabe von TRI-Reagent ist abhängig von der Zellzahl - siehe Produkt Information im Anhang)
- Gewebe: Überschuß Flüssigkeit an Einmalhandtuch abtropfen lassen; Gewebe schnell in N_2 einfrieren und bei -20 oder 80° aufbewahren. Alternativ: sofort in 2 ml TRI Reagent + Vanadyl-Ribonucleoside (NEB S1402 , Endkonzentration 5 mM) mit Ultra Turrax in 15- oder 50 ml-Falcon-Tube zerkleinern und weiterverarbeiten. (Stab dekontaminieren mit 10N NaOH, gründlich mit sterilem Wasser nachspülen, zwischen den einzelnen Proben mit H_2O spülen)
- Zellen 3 x mit sauberem Zellkultur-PBS waschen > sehr gut absaugen
- je Schale **1 ml** TRI Reagent (Sigma T9424)
- □ mit Zellschaber abkratzen und schnell in 15 ml-Röhrchen überführen; 1 min auf Eis mit Ultraturrax homogenisieren; hohe Geschwindigkeit
- > in **2 ml Tube** überführen
- Inkubation 5 min bei RT
- Zugabe von **0,2 ml Chloroform**, ca. 15 s invertieren > 3 min RT
- Zentrifugieren: 12.000 g (=RCF), 10 min, 4 °C □ Phasentrennung
    - untere rote Phase = Phenol-Chloroform (organisch)
    - Interphase
    - obere Phase (farblos) = RNA
- obere Phase vorsichtig in neues 1,5 ml Tube überführen
- *Präzipitation:*
    - Zugabe von 0,5 ml Isopropanol
    - Invertieren 3-5x
    - 10 min bei RT Inkubieren
    - Zentrifugieren: 12.000 g, 10 min 4°C
    - RNA-Pellet sollte sichtbar sein
- *waschen:*
    - Überstand entfernen
    - 2x 75% EtOH waschen (1 ml per 1 ml TRI Reagent)
    - kurz vortexen, so das Pellet sich ablöst, aber nicht zerfällt
    - zentrifugieren: 14.000 rpm, 5 min, 4°C
    - vorsichtig und vollständig EtOH abnehmen
    - Pellet 5 - 10 min lufttrocknen oder kurz in Heizblock bei 55 °C trocknen
    - ***weiter mit zusätzlicher Phenol-Chloroform-Extraktion oder DNAse-Verdau***

- *für DNAse-Verdau in 30-50 µl S-H20 lösen - je nach Pellet*

- *Kontrollgel:*
  - Agarosegel:
    - 1 µl RNA
    - 4 µl 6x Ladepuffer
    - 19 µl Sigma-H2O

A Phenol-Chloroform-Extraktion

(Diese Extraktion muss nicht durchgeführt werden, wenn noch ein DNAse Verdau erfolgt!!)

- **Pellet in 400 µl Sigma-H2O** aufnehmen, auf- und abpipettieren
- 10 min 55°C im Heizblock um RNA vollständig zu lösen
- *Phenol-Chloroform-Extraktion:*
  - Add 320 µl Phenol/Chloroform (0,8 Volumina; Verhältnis 1:1; Phenol pH 4.0 Applichem A1624.0250)
  - vortexen und 5 min bei 14.000 rpm, 4°C zentrifugieren
  - Überstand in neues 1,5 ml Tube
  - Add 320 µl Chloroform
  - vortexen und 5 min bei 14.000 rpm, 4°C zentrifugieren
  - Überstand in neues 1,5 ml Tube
- *Fällung:*
  - Add 1 µl Glycogen (RNA grade, Fermentas R0551)
  - Add 40 µl 3M NaAc
  - Add 800 µl 100% EtOH
  - 30 min auf Trockeneis inkubieren
  - 10 min bei 14.000 rpm, 4°C zentrifugieren
  - 2 x 70% EtOH waschen, danach jeweils 10 min, 14.000 rpm, 4°C zentrifugieren
  - letzten Überstand vollständig abnehmen und Pellet bei RT trocknen lassen
  - Pellet in 30 µl Sigma-H_2O aufnehmen
  - 3 min bei RT stehen lassen
  - 10 min 55°C Heizblock

Konzentrationsmessung:

- Nanodrop 1 µl
- Agarosegel:
  - 1 µl RNA
  - 4 µl 6x Ladepuffer
  - 19 µl Sigma-H2O

B DNase-Verdau:

- Kit: EN0521 von Fermentas
- **12 µg RNA** + 12 µl 10x reaction buffer with MgCl2
  + 12 µl DNaseI 1U/µl
  + auffüllen auf 120 µl Sigma-H_2O
- 30 min 37°C Heizblock
- ***Phenol/Chloroform Extraktion*** **:**
  - Ad 80 µl S-H_2O
  - Add 160 µl Phenol/Chloroform (1:1; 0,8 Volumina)
  - vortexen und 10 min 14.000 rpm, 4°C zentrifugieren
  - Überstand in neues 1,5 ml Tube

- + 200 µl Chloroform
- vortexen und 10 min 14.000 rpm, 4°C zentrifugieren
- Überstand in neues 1,5 ml Tube
- <u>Fällung:</u>
  - + 1 µl Glycogen (RNA grade)
  - + 20 µl 3M NaAc pH 5,5
  - + 500 µl 100% EtOH, invertieren
  - 10 min bei 14.000 rpm, 4°C zentrifugieren
  - 2 x 70% EtOH waschen, danach jeweils 5 min, 14.000 rpm, 4°C zentrifugieren
  - letzten Überstand vollständig abnehmen und Pellet bei RT trocknen lassen
  - in 12 µl S-H_2O lösen.
  - 1.5 µl 3M NaAc pH 5.5, 30 µl 100% EtOH zugeben, invertieren und bei -20 oder -80°C°C aufbewahren.

<u>Konzentrationsmessung</u>:

- Nanodrop 1 µl
- Agarosegel:
  - 1 µl RNA
  - 4 µl 6x Ladepuffer
  - 19 µl Sigma-H2O

RT-PCR

<u>First strand cDNA Synthesis; Fermentas K1632</u>

- Primer: 1:1-Mix aus oligo $(dT)_{18}$ - und Random hexamer primern

2 µg RNA (Dnase verdaut)
+ 0,5 µl Random hexamer primer
+ 0,5 µl oligo $(dT)_{18}$ (100 µM)
auffüllen auf 13 µl Sigma-H_2O (100 µM)

→ 65 °C, 5 min, kurz zentrifugieren und auf Eis stellen

+ 4 µl 5x Reaktion Buffer
+ 2 µl dNTP´s (10mM)
+ 1 µl Reverse Transcriptase

→ 5 min 25 °C
→ 60 min 45 °C
→ 5 min 70 °C

- cDNA 1:10 verdünnen : 20µl cDNA + 180 µl S-H_20 > da 2 µg RNA umgeschrieben↘ c= 10 ng/µl
- Lagerung bei -80 °C

qPCR

<u>Maxima SYBR Green qPCR Master Mix (2x) Fermentas K0251(200rxns); K0252(1000rxns); K0253(4000rxns)</u>

1 Reaktion	finale Konzentration
12,5 µl SYBR Green	
1,0 µl ROX (von 1:200 Verdünnung, 250nM); Stock 50µM; Verdünnung in S-H_2O; Einsatz von ROX vom Cycler abhängig.	10,00 nM
4,5 µl S-H_2O	
1,5 µl Forward primer (10 µM)	0,75 µM
1,5 µl Reverse primer (10 µM)	0,75 µM
4,0 µl Template (c: cDNA 10 ng/µl)	40,00 ng
∑25,0 µl Ansatz	

[Template Konzentration ist von der Expressionsstärke des Gens anhängig > bei GAPDH etl. weniger einsetzen!]
AB 7500, TRM; Serial-Nr. 275003780

Set up:

→ Gerät immer vor PC anschalten!
→ Guest auswählen, kein Passwort

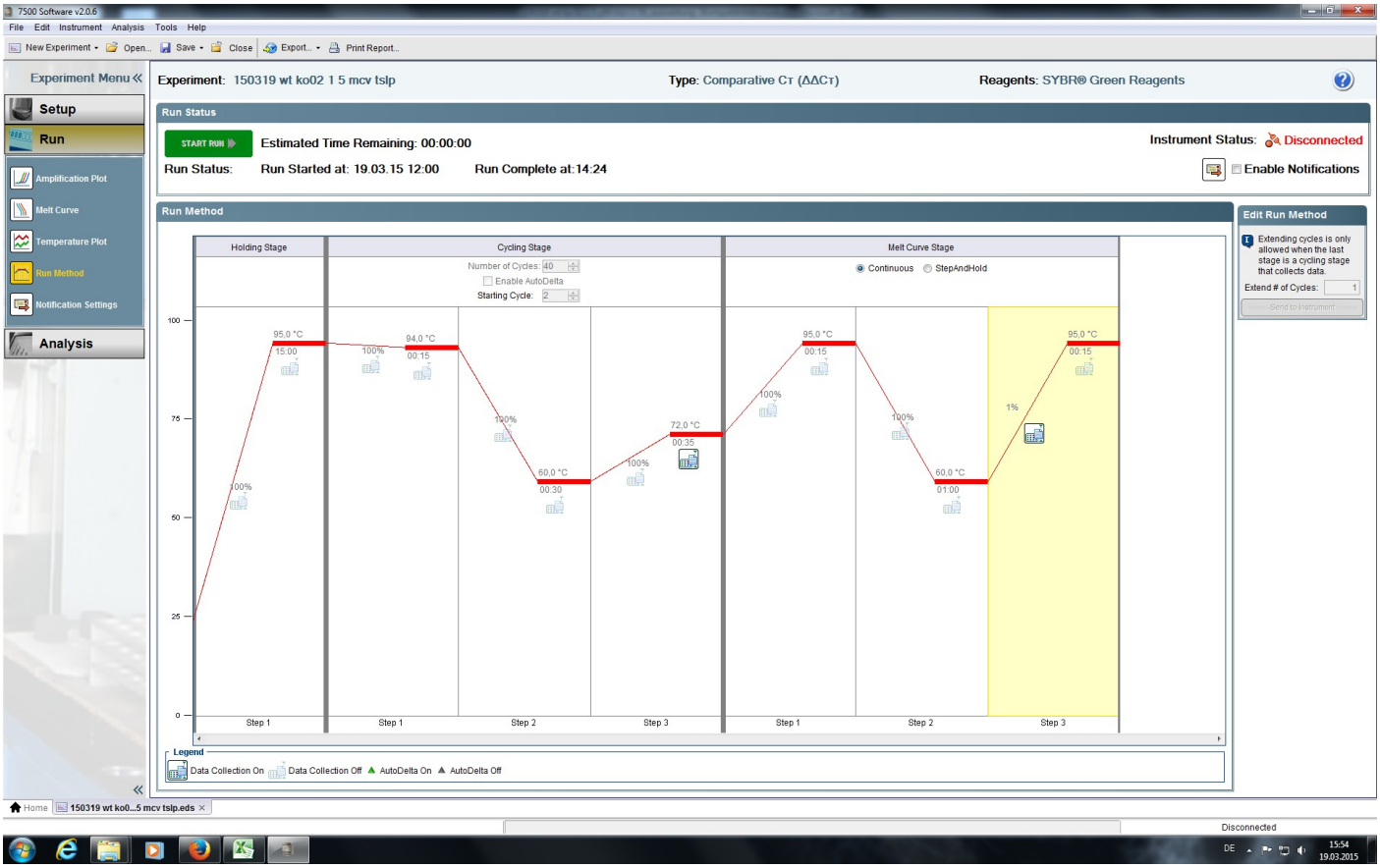
- advanced setup – 7500 (96-well) [nicht fast wählen!]
- quantification > comparative ct > SYBR green reagent
- plate setup:
  - targets wählen(primer)
  - sample wählen (wt, ko…)
  - biological group > nichts wählen!

[Step 1: 50°C 2min uracyl glycosylane > nicht notwendig, da nicht in unserem Kit enthalten]

Program [open run method - AG Magin Janina]

- 40 Zyclen
- 25 µl Volumen eingeben
- melt curve nicht vergessen

Thermoprofil:


7500 Software v2.0.6
File Edit Instrument Analysis Tools Help
New Experiment Open... Save Close Export... Print Report...
Experiment Menu
Setup
Run
Amplification Plot
Melt Curve
Temperature Plot
Run Method
Notification Settings
Analysis
Experiment: 150319 wt ko02 1 5 mcv tslp
Type: Comparative CT (ΔΔCT)
Reagents: SYBR® Green Reagents
Run Status
START RUN
Estimated Time Remaining: 00:00:00
Instrument Status: Disconnected
Run Status: Run Started at: 19.03.15 12:00 Run Complete at: 14:24
Enable Notifications
Run Method
Holding Stage
Cycling Stage
Melt Curve Stage
Number of Cycles: 40
Enable AutoDelta
Starting Cycle: 2
Continuous StepAndHold
95.0 °C 15:00
94.0 °C 00:15
60.0 °C 00:30
72.0 °C 00:35
95.0 °C 00:15
60.0 °C 01:00
95.0 °C 00:15
100
75
50
25
0
Step 1 Step 1 Step 2 Step 3 Step 1 Step 2 Step 3
Edit Run Method
Extending cycles is only allowed when the last stage is a cycling stage that collects data.
Extend # of Cycles:
Send to Instrument
Legend
Data Collection On Data Collection Off AutoDelta On AutoDelta Off
Home
150319 wt ko0...5 mcv tslp.eds
Disconnected
DE
15:54
19.03.2015

Anhang

SIGMA-ALDRICH®
sigma-aldrich.com
3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

TRI Reagent®
For processing tissues, cells cultured in monolayer or cell pellets

Catalog Number **T9424**
Store at room temperature.

TECHNICAL BULLETIN

Product Description
TRI Reagent is a quick and convenient reagent for use in the simultaneous isolation of RNA, DNA, and protein. Successful isolations from human, animal, plant, yeast, bacterial, and viral samples can be obtained. A convenient single-step liquid phase separation results in the simultaneous isolation of RNA, DNA, and protein.[1] This procedure is an improvement of the single-step method reported by Chomczynski and Sacchi[2] for total RNA isolation. TRI Reagent performs well with large or small amounts of tissue or cells and many samples can be simultaneously extracted.

This product, a mixture of guanidine thiocyanate and phenol in a monophase solution, effectively dissolves DNA, RNA, and protein on homogenization or lysis of tissue sample. After adding chloroform or 1-bromo-3-chloropropane and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases. One ml of TRI Reagent is sufficient to isolate RNA, DNA, and protein from 50–100 mg of tissue, 5–10 $\times$ 10^6 cells, or 10 cm^2 of culture dish surface for cells grown in monolayer.

This is one of the most effective methods for isolating total RNA and can be completed in only 1 hour starting with fresh tissue or cells. The procedure is very effective for isolating RNA molecules of all types from 0.1–15 kb in length. The resulting RNA is intact with little or no contaminating DNA and protein. This RNA can be used for Northern blots, mRNA isolation, *in vitro* translation, RNase protection assay, cloning and polymerase chain reaction (PCR).

The DNA is in the interphase and phenol phase, which forms after the addition of chloroform or 1-bromo-3-chloropropane to the TRI Reagent in Sample Preparation, step 2. After precipitation and multiple washes, the DNA is dissolved in 8 mM NaOH. The solution is neutralized and the DNA is ready for analysis. The resulting DNA is suitable for PCR, restriction enzyme digestion, and Southern blotting.

After precipitating the DNA with ethanol (DNA Isolation, step 1), the proteins can be removed from the phenol-ethanol supernatant. The isolated material can be probed for specific proteins by Western blotting.[1]

Reagents Required but Not Provided

RNA Isolation:
- Chloroform, Catalog Number C2432, or 1-Bromo-3-chloropropane, Catalog Number B9673
- 2-Propanol, Catalog Number I9516
- 75% Ethanol
- 1 mM sodium phosphate, Catalog Number S3264, pH 8.2, 0.5% SDS solution, Catalog Number L4522, diluted 20-fold, formamide, **or** diethylpyrocarbonate-treated water

DNA Isolation:
- 8 mM NaOH
- 0.1 M trisodium citrate, 10% ethanol solution
- Absolute ethanol
- 75% ethanol
- EDTA

Protein Isolation:
- 2-Propanol, Catalog Number I9516
- Absolute ethanol, Catalog Number E7023
- 95% Ethanol, Catalog Number E7148
- 1% SDS, Catalog Number L4522, diluted 10-fold
- 0.3 M Guanidine hydrochloride, Catalog Number G3272, in 95% ethanol

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
Store the product at room temperature.

Procedures
Sample Preparation

1A. Tissue:
Homogenize tissue samples in TRI Reagent (1 ml per 50–100 mg of tissue) in a Polytron® or other appropriate homogenizer.
Note: If minimal shearing of the DNA is desired, use a loosely fitting homogenizer, not a Polytron (see DNA Isolation, step 3, note b). The volume of the tissue should not exceed 10% of the volume of the TRI Reagent.

1B. Monolayer cells:
Lyse cells directly on the culture dish. Use 1 ml of the TRI Reagent per 10 cm^2 of glass culture plate surface area. After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogenous lysate.
Note: TRI Reagent is **not** compatible with plastic culture plates.

1C. Suspension cells:
Isolate cells by centrifugation and then lyse in TRI Reagent by repeated pipetting. One ml of the reagent is sufficient to lyse 5–10 × 10^6 animal, plant, or yeast cells, or 10^7 bacterial cells.
Notes:
a. If samples have a high content of fat, protein, polysaccharides, or extracellular material such as muscle, fat tissue, and tuberous parts of plants an additional step may be needed. After homogenization, centrifuge the homogenate at 12,000 × *g* for 10 minutes at 2-8 °C to remove the insoluble material (extracellular membranes, polysaccharides, and high molecular mass DNA). The supernatant contains RNA and protein. If the sample had a high fat content, there will be a layer of fatty material on the surface of the aqueous phase that should be removed. Transfer the clear supernatant to a fresh tube and proceed with step 2. Recover the high molecular mass DNA from the pellet by following DNA Isolation, steps 2 and 3.
b. Some yeast and bacterial cells may require a homogenizer.
c. After the cells have been homogenized or lysed in TRI Reagent, samples can be stored at –70 °C for up to 1 month.

2. Phase Separation: To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.1 ml of 1-bromo-3-chloropropane or 0.2 ml of chloroform (see Phase Separation, notes a and b) per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 2–15 minutes at room temperature. Centrifuge the resulting mixture at 12,000 × *g* for 15 minutes at 2-8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).
Notes:
a. 1-Bromo-3-chloropropane is less toxic than chloroform and its use for phase separation decreases the possibility of contaminating RNA with DNA.[4]
b. The chloroform used for phase separation should not contain isoamyl alcohol or other additives.
c. For isolation of poly A^+ fraction from the aqueous phase see Appendix I.

RNA Isolation

1. Transfer the aqueous phase to a fresh tube and add 0.5 ml of 2-propanol per ml of TRI Reagent used in Sample Preparation, step 1 and mix. Allow the sample to stand for 5–10 minutes at room temperature. Centrifuge at 12,000 × *g* for 10 minutes at 2-8 °C. The RNA precipitate will form a pellet on the side and bottom of the tube.
Note: Store the interphase and organic phase at 2-8 °C for subsequent isolation of the DNA and proteins.

2. Remove the supernatant and wash the RNA pellet by adding a minimun of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in Sample Preparation, step 1. Vortex the sample and then centrifuge at 7,500 × *g* for 5 minutes at 2-8 °C.
Notes:
a. If the RNA pellets float, perform the wash in 75% ethanol at 12,000 × *g*.
b. Samples can be stored in ethanol at 2-8 °C for at least 1 week and up to 1 year at –20 °C.

3. Briefly dry the RNA pellet for 5–10 minutes by air-drying or under a vacuum. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Do not dry the RNA pellet by centrifugation under vacuum (Speed-Vac®). Add an appropriate volume of formamide, water, or a 0.5% SDS solution to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60 °C for 10–15 minutes.
   Notes:
   a. Final preparation of RNA is free of DNA and proteins. It should have a A_{260}/A_{280} ratio of ≥1.7.
   b. Typical yields from tissues (μg RNA/mg tissue): liver, spleen, 6-10 μg; kidney, 3-4 μg; skeletal muscle, brain, 1-1.5 μg; placenta, 1-4 μg.
   c. Typical yields from cultured cells (μg RNA/10^6 cells): epithelial cells, 8-15 μg; fibroblasts, 5-7 μg.
   d. Ethidium bromide staining of RNA in agarose gels visualizes two predominant bands of small (2 kb) and large (5 kb) ribosomal RNA, low molecular mass (0.1–0.3 kb) RNA, and discrete bands of high molecular mass (7–15 kb) RNA.

DNA Isolation

1. Carefully remove the remaining aqueous phase overlaying the interphase and discard. To precipitate the DNA from the interphase and organic phase, add 0.3 ml of 100% ethanol per 1 ml of TRI Reagent used in Sample Preparation, step 1. Mix by inversion and allow to stand for 2–3 minutes at room temperature. Centrifuge at 2,000 × *g* for 5 minutes at 2-8 °C.
   Note: Removal of the remaining aqueous phase before DNA precipitation is a critical step for the quality of the isolated DNA.

2. Remove the supernatant and save at 2-8 °C for protein isolation. Wash the DNA pellet twice in 0.1 M trisodium citrate, 10% ethanol solution. Use 1 ml of wash solution for every 1 ml of TRI Reagent used in Sample Preparation, step 1. During each wash, allow the DNA pellet to stand (with occasional mixing) for at least 30 minutes. Centrifuge at 2,000 × *g* for 5 minutes at 2-8 °C. Resuspend the DNA pellet in 75% ethanol (1.5–2 ml for each ml TRI Reagent) and allow to stand for 10–20 minutes at room temperature.
   Notes:
   a. **Important:** Do not to reduce the time samples remain in the washing solution. Thirty minutes is the absolute minimum time for efficient removal of phenol from the DNA.
   b. If pellet contains >200 μg of DNA or large amounts of non-DNA material, an additional wash in 0.1 M trisodium citrate, 10% ethanol solution is required.
   c. Samples suspended in 75% ethanol can be stored at 2-8 °C for several months.

3. Dry the DNA pellet for 5–10 minutes under a vacuum and dissolve in 8 mM NaOH with repeated slow pipetting with a micropipette. Add sufficient 8 mM NaOH for a final DNA concentration of 0.2–0.3 μg/μL (typically 0.3–0.6 ml to the DNA isolated from 50–70 mg of tissue or 10^7 cells). This mild alkaline solution assures complete dissolution of the DNA pellet. Centrifuge at 12,000 × *g* for 10 minutes to remove any insoluble material and transfer the supernatant to a new tube.
   Notes:
   a. A viscous supernatant indicates the presence of high molecular mass DNA.
   b. The size of the DNA will depend on the force exerted during homogenization. Avoid using a Polytron homogenizer.
   c. Samples dissolved in 8 mM NaOH can be stored at 2-8 °C overnight. For long term storage, adjust the pH value to between 7 and 8 and supplement with EDTA (final concentration 1 mM).
   d. To determine DNA concentration, remove an aliquot, dilute with water, and measure the A_{260}. For double stranded DNA, 1 A_{260} unit/ml = 50 μg/ml.
   e. To calculate cell number, assume the amount of DNA for 10^6 diploid cells of human, rat, and mouse equals 7.1 μg, 6.5 μg, and 5.8 μg, respectively.
   f. Typical yields from tissues (μg DNA/mg tissue): liver, kidney, 3–4 μg; skeletal muscle, brain, and placenta, 2–3 μg.
   g. Typical yields from cultured human, rat, and mouse cells: 5–7 μg DNA/10^6 cells.

To Amplify DNA by PCR

After dissolving in 8 mM NaOH, adjust to pH 8.4 using HEPES (add 86 μL of 0.1 M HEPES, free acid/ml of DNA solution). Add sample (generally 0.1–1 μg) to PCR mix and follow PCR protocol.

To Digest DNA with Restriction Enzymes

Adjust the pH of the DNA solution to that needed for the restriction enzyme digestion using HEPES, or dialyze samples against 1 mM EDTA, pH 7–8. Allow the restriction enzyme digestion to continue for 3–24 hours under optimal conditions. It is recommended that 3–5 units of enzyme be used per 1 μg of DNA. Typically, 80–90% of the DNA is digested.

Protein Isolation

1. Precipitate proteins (see note) from the phenol-ethanol supernatant (DNA Isolation, step 2) with 1.5 ml of 2-propanol per 1 ml of TRI Reagent used in Sample Preparation, step 1. Allow samples to stand for at least 10 minutes at room temperature. Centrifuge at 12,000 × *g* for 10 minutes at 2-8 °C.
   Note: For some samples, the protein pellet may be difficult to dissolve in 1% SDS (step 3). Use this alternate procedure to correct the problem:
   a. Dialyze the phenol-ethanol supernatant against 3 changes of 0.1% SDS at 2-8 °C.
   b. Centrifuge the dialysate at 10,000 × *g* for 10 minutes at 2-8 °C.
   c. The clear supernatant contains protein that is suitable for use in Western blotting procedures.

2. Discard supernatant and wash pellet 3 times in 0.3 M guanidine hydrochloride/95% ethanol solution, using 2 ml per 1 ml of TRI Reagent used in Sample Preparation, step 1. During each wash, store samples in wash solution for 20 minutes at room temperature. Centrifuge at 7,500 × *g* for 5 minutes at 2-8 °C. After the 3 washes, add 2 ml of 100% ethanol and vortex the protein pellet. Allow to stand for 20 minutes at room temperature. Centrifuge at 7,500 × *g* for 5 minutes at 2-8 °C.
   Note: Protein samples suspended in 0.3 M guanidine hydrochloride/95% ethanol solution or 100% ethanol can be stored for 1 month at 2-8 °C or 1 year at –20 °C.

3. Dry protein pellet under a vacuum for 5–10 minutes. Dissolve pellet in 1% SDS aided by working the plunger of micropipette with tip in the solution. Remove any insoluble material by centrifugation at 10,000 × *g* for 10 minutes at 2-8 °C. Transfer supernatant to a new tube. The protein solution should be used immediately for Western blotting or stored at –20 °C.

Troubleshooting Guide

1. RNA Isolation:
   A. Low yield may be due to:
      - incomplete homogenization or lysis of samples.
      - the final RNA pellet may not have been completely dissolved.
   B. If the A_{260}/A_{280} ratio is <1.65:
      - the amount of sample used for homogenization may have been too small.
      - samples may not have been allowed to stand at room temperature for 5 minutes after homogenization.
      - there may have been contamination of the aqueous phase with the phenol phase.
      - the final RNA pellet may not have been completely dissolved.
   C. If there is degradation of the RNA:
      - the tissues may not have been immediately processed or frozen after removing from the animal.
      - the samples used for isolation or the isolated RNA preparations may have been stored at –20 °C instead of –70 °C as specified in the procedure.
      - cells may have been dispersed by trypsin digestion.
      - aqueous solutions or tubes used for procedure may not have been RNAse-free.
      - formaldehyde used for the agarose gel electrophoresis may have had a pH value <3.5.
   D. If there is DNA contamination:
      - the volume of reagent used for the sample homogenization may have been too small.
      - samples used for the isolation may have contained organic solvents (ethanol, DMSO), strong buffers or alkaline solution.

2. DNA Isolation:
   A. Low yield may be due to:
      - incomplete homogenization or lysis of samples.
      - the final DNA pellet may not have been completely dissolved.
   B. If the A_{260}/A_{280} ratio is <1.70:
      - phenol may not have been sufficiently removed from the DNA preparation. Try one more wash of the DNA pellet with the 0.1 M trisodium citrate, 10% ethanol solution.

C. If there is degradation of the DNA:
- the tissues may not have been immediately processed or frozen after removing from the animal.
- the samples used for isolation may have been stored at –20 °C instead of –70 °C as specified in the procedure.
- samples may have been homogenized with a Polytron or other high speed homogenizer.

D. If there is RNA contamination:
- there may have been too much aqueous phase remaining with the organic phase and interphase.
- the DNA pellet may not have been washed sufficiently with 0.1 M trisodium citrate, 10% ethanol solution.

3. Protein Isolation:

A. Low yield may be due to:
- incomplete homogenization or lysis of samples.
- the final protein pellet may not have been completely dissolved.

B. If there is degradation of the protein:
- the tissues may not have been immediately processed or frozen after removing from the animal.

C. If PAGE shows band deformation:
- protein pellet may not have been washed sufficiently.

Appendix

I. Isolation of Poly A^+ RNA

After the RNA has been precipitated with 2-propanol (RNA Isolation, step 1), dissolve the pellet in poly A^+ binding buffer and pass through an oligo-dT cellulose (Catalog Number O3131) column to selectively remove mRNA according to the procedure of Aviv and Leder.[3]

II. Isolated RNA is to be used in RT-PCR

1. Modifying the procedure by performing the additional centrifugation step in the initial Sample Preparation, step 1B, note c further minimizes the possibility of DNA contamination in the RNA extracted by TRI Reagent LS.
2. A more complete evaporation of ethanol is required when RNA samples are to be used in RT-PCR. This is especially critical for small volume samples (5–20 µl), which may contain a relatively high level of ethanol if not adequately dried.

References

1. Chomczynski, P., *BioTechniques*, **15**, 532-537 (1993).
2. Chomczynski, P., and Sacchi, N., *Anal. Biochem.*, **162**, 156-159 (1987).
3. Aviv, H., and Leder, P., *Proc. Natl. Acad. Sci. USA*, **69**, 1408-1412 (1972).
4. Chomczynski. P., and Mackey, K., *Anal. Biochem.*, **225**, 163-164 (1995).

RC,JC,PHC 07/11-1