WARNING: Toxic in contact with skin and if swallowed. Causes burns. After contact with skin, wash immediately with plenty of detergent and water. If you feel unwell, seek medical advice Immediately. (label where possible). Phenol (108-95-2) and Other Components (NJRTRN 80104437-30PF99).

TRIZOL® Reagent
Total RNA Isolation Protocol

Description
TRIZOL® Reagent (U.S. Patent No. 5,346,994) is a ready-to-use reagent for the isolation of total RNA from tissues and cells. The process is based on the ability of phenol and guanidine isothiocyanate, an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi. During sample homogenization or lysis, TRIZOL® Reagent maintains the activity of RNases by trapping RNase-containing cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase, the RNA is recovered by precipitation with isopropanol alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the precipitate, and an additional precipitation with isopropanol alcohol yields proteins from the organic phase. Copurification of the DNA may be observed for normalizing RNA yields from samples. This technique performs well with small quantities of tissue (50-100 mg) and cells (5 x 10^6), and large quantities of tissue (2 g) and cells (>10^7) of human, animal, plant, or bacterial origin. The simplicity of the TRIZOL® Reagent method allows simultaneous processing of a large number of samples. The entire procedure can be completed in one day and results from all samples can be obtained in 24 hours. TRIZOL® Reagent is highly effective for the isolation of protocollable RNA. It can be used for Northern blot analysis, dot blot hybridization, poly(A)* selection, in vitro translation, RNA protection assay, and molecular cloning. For use in the polymerase chain reaction (PCR®), treatment of the isolated RNA with amplification grade DNase (GIBCO BRL Cat. No. 10018) is recommended when the two primers lie within a single exon.

TRIZOL® Reagent facilitates isolation of a variety of RNA species of large or small molecular size. For example, RNA isolated from rat liver, ethophenolized on an agarose gel, and stained with ethidium bromide, shows discrete bands of high molecular weight RNA. Human RNA isolated from 15 kg in size, two predominant ribosomal RNA bands at -5 kb (28S) and at -2 kb (18S), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, SS). The isolated RNA has an A260/A280 ratio of 1.6-1.8. The expected yield of RNA isolated from the samples is 5-10 mg/ml liver and spleen, 1-2 mg/ml kidney, 3-4 mg/ml skeletal muscle and -1.5 mg/ml placenta, 1-4 mg/ml. The expected yield of RNA from 1 x 10^7 cultured cells -8-15 mg; fibroblasts, 3-7 mg.

cents required, but not supplied:
- chloroform (without any additives, such as isooamyl alcohol)
- diethylpyrocarbonate (DEPC®) treated water
- 75% Ethanol (in DEPC®-treated water)
- RNase-free water or 0.5% SDS solution (To prepare RNase-free water, draw water into the RNase-free syringe. Add diethylpyrocarbonate (DEPC®) to 0.01% (v/v). Let stand overnight and autoclave. The SDS solution must be prepared using DEPC-treated, autoclaved water)

Precautions for Preventing RNase Contamination:
RNase contamination can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction. The following guidelines should be observed when working with RNA:
- Always wear disposable gloves. Skin contains by RNases and RNAs that can concentrate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
- Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to prevent RNase contamination with RNases from shared equipment. For example, a laboratory that is using RNA probes will likely be using RNase A or T1 to reduce background of filters, and any nondisposable items (such as automatic pipettes) can be autoclaved on 10 minutes in 0.5 M NaOH, rinsed thoroughly with water, and autoclaved.

Other Precautions:
- Use of disposable tubes made of clear polypropylene is recommended when working with less than 2 ml volumes of TRIZOL® Reagent.
- Never allow RNase-free glassware or plasticware to come in contact with RNase-free glassware or plasticware. Discard RNase-free glassware or plasticware tubes, and test to be sure that the tubes can withstand 12,000 x g with TRIZOL® Reagent, chloroform, and chloroform and phenol. Do not use these tubes that leak or crack.
- Carefully evaluate the weights of the tubes prior to centrifugation.
- Glass tubes must be sealed with paraffin topped with a layer of foil, and polystyrene tubes must be capped before centrifugation.

NOTES:
1. To facilitate isolation of RNA from small quantities of sample (5 x 10^6 cells or 50 microgram) perform homogenization and chloroform and phenol phase separation, as described in step 2. Prior to precipitating the RNA with ethanol, add 5 mg of triethylamine (to neutralize the residual sodium bicarbonate (as current) to the aqueous phase. (THE GLASSMAX® RNA Microisolation Spin Cartridge System (GIBCO BRL Cat. No. 18325), it is also suggested for use in these cases. This system is particularly useful for use with small quantities of 5 x 10^6 cells (10) and tissues (250 mg), where the total RNA isolated is to be used in RT-PCR.
2. A higher ethanol precipitation step may be required for samples with high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tibial and skin parts. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 x g for 10 minutes at 2°C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. In samples from fat cells, it is necessary to use lipase to remove fat cells, as well as isopropyl alcohol which should be removed after precipitation. In this case, the supernatant is centrifuged at 10,000 x g for 5 minutes at 4°C, carefree removal of the aqueous phase is critical for the quality of the isolated RNA.

1. HOMOGENIZATION (see notes 1-3)
a. Tissues
Homogenize tissue samples in 1 ml of TRIZOL® Reagent per 50-100 mg of tissue using a glass-Teflon® or power homogenizer (Polytron, or Tekmar’s T-80). Mix tissue with TRIZOL® Reagent, and homogenate on medium speed. The homogenate can be kept in cold at -20°C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored at -70°C for at least one week, or at least one year at -10°C or below.

1. Table-top centrifuges that can maintain a maximum of 2,600 x g are suitable for use in these protocols if the centrifugation time is increased to 30-60 minutes in steps 2 and 3.

INSTRUCTIONS FOR RNA ISOLATION:
1. Homogenization

2. PHASE SEPARATION
Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml TRIZOL® Reagent. Cap sample tubes securely. Shake tubes vigorously by hand 15 to 20 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge samples at no more than 12,000 x g for 15 minutes at 2°C to 8°C. Follow centrifugation, the mixture separates into a lower red, phenol-chloroform phase, intermediate phase consisting of the upper aqueous phase. RIA DNA remains exclusively in aqueous phase. The volume of the aqueous phase is about 60% of the volume TRIZOL® Reagent used for homogenization.

3. RNA PRECIPITATION
Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase with mixing with isopropanol alcohol. Use 0.5 ml of isopropanol alcohol per 1 ml of TRIZOL® Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 x g for 10 minutes at 2°C to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA WASH
Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL® Reagent used for the initial homogenization. Wash the sample by vortexing and centrifugation at no more than 7,500 x g for 5 minutes at 2°C to 8°C. Partially dissolved RNA samples have an A260/A280 ratio < 1.6. Dissolve RNA with 0.5 SDS solution by placing the solution a few times through a glass pipet, and incubating for 10 minutes at 55 to 60°C.

INSTRUCTIONS FOR DNA ISOLATION:
Add 0.5 ml of chloroform to the aqueous phase, as described in the RNA isolation protocol, to dissolve the DNA. The DNA from the interphase and phenol phase from the initial homogenization may be isolated. Following precipitation and a series of washes, the DNA is solubilized in 8 M NaOH. Precipitation of the RNA from DNA is obtained by using DEPC treated TRIZOL® Reagent for the determination of the DNA content in analyzed samples. Simultaneous extraction of genomic DNA allows for normalization of the results of Northern analysis per genomic DNA. Depending on the sample, the DNA pellet obtained may require additional purification (e.g., phenol extraction) prior to other applications.

Ratios required, but not supplied:
- Ethanol
- 0.1 M Sodium citrate in 10% ethanol
- 75% Ethanol
- 8 M NaOH

Unless otherwise stated, the procedure is carried out at 15 to 30°C.
2. DNA WASH
Remove the phenol-ethanol supernatant, and if desired, save it for protein isolation. Wash the DNA pellet twice in a solution containing 0.1 M sodium citrate in 10% ethanol. Use 1 mL of the solution per 1 mL of TRIZOL Reagent used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 x g for 5 minutes at 2 to 8°C. Following these two washes, suspend the DNA pellet in 75% ethanol (1.5-2 mL of 75% ethanol per 1 mL of TRIZOL Reagent), store for 10-20 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 x g for 5 minutes at 2 to 8°C.

An additional wash in 0.1 M sodium citrate/10% ethanol solution is required for large pellets containing > 200 μg DNA or large amounts of a non-DNA material.

3. REDESOLVING THE DNA
Briefly dry the DNA pellet for 5-10 minutes under vacuum and dissolve in 8 mM NaOH by slowly passing the pellet through a pipette. Add an adequate amount of 8 mM NaOH to approach a DNA concentration of 0.2-0.3 μg/μL. Typically, add 0.3-0.6 mL of 8 mM NaOH to the DNA solution isolated from 50-70 mg of tissue or 1 x 10^8 cells. The use of a mild alkali solution assures full solubilization of the DNA pellet. At this stage, however, the DNA preparations (especially from tissues) still contain insoluble gel-like material (fragments of membranes, etc.). Remove the insoluble material by centrifugation at 12,000 x g for 10 minutes. Transfer the supernatant containing DNA to a new tube.

QUANTITATION AND EXPECTED YIELDS OF DNA
Take an aliquot of the DNA preparation solubilized in 8 mM NaOH, mix it with water and measure the A260 of the resulting solution. Calculate the DNA content using the A260 value for double-stranded DNA. One A260 unit equals 50 μg of double-stranded DNA/mL. For calculation of cell number in analyzed samples, assume that the amount of DNA per 1 x 10^6 diploid cells of human, rat, and mouse origins equals 7.1 μg, 6.5 μg, and 5.8 μg, respectively. The expected yield of DNA per mg of tissue is: 3-4 μg from liver and kidney; and 2-3 μg from skeletal muscles, brain and placenta. The expected yield of DNA per 1 x 10^6 cultured human, rat and mouse cells is 5-7 μg.

APPLICATIONS
Amplification of DNA by PCR
Add an optimized DNA in 8 mM NaOH, adjust the pH to 8.4 with 0.1 M HEPES (see table). Add 0.1 to 1.0 μg of the DNA sample to your PCR reaction mixture and perform the standard PCR protocol.
Restriction endonuclease reactions
Adjust the pH of the DNA solution to a required value using HEPES (see Table). Alternatively, samples may be dialyzed against 1 mM EDTA, pH 7 to pH 8.0. Use 3-5 units of enzyme per microgram of DNA. Use the conditions recommended by the manufacturer for the particular enzyme, and allow the reaction to proceed for 3 to 24 h. In a typical assay, 80-90% of the DNA is digested.

pH Adjustment of DNA Samples Dissolved in 8 mM NaOH
(For 1 mL of 8 mM NaOH use the following amounts of 0.1 M or 1 M HEPES, free acid.

<table>
<thead>
<tr>
<th>pH</th>
<th>Final pH</th>
<th>0.1 M HEPES</th>
<th>1 M HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.4</td>
<td>7.2</td>
<td>66</td>
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<tr>
<td>8.2</td>
<td>7.0</td>
<td>90</td>
<td>42</td>
</tr>
<tr>
<td>8.0</td>
<td>6.9</td>
<td>115</td>
<td>50</td>
</tr>
<tr>
<td>7.8</td>
<td>6.8</td>
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<td>65</td>
</tr>
<tr>
<td>7.6</td>
<td>6.7</td>
<td>180</td>
<td>80</td>
</tr>
</tbody>
</table>

Notes:
1. The phenol phase and interphase can be stored at 2 to 8°C overnight.
2. Samples suspended in 75% ethanol can be stored at 2 to 8°C for months.
3. Samples dissolved in 8 mM NaOH can be stored overnight at 2 to 8°C. For long-term storage, adjust the pH to 7-8, and adjust the EDTA concentration to 1 mM.

INSTRUCTIONS FOR PROTEIN ISOLATION
Proteins are isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol (see step 1, DNA PRECIPITATION). The precipitating agent can be analyzed for the presence of specific proteins by Western blotting. Reagents required, but not supplied:
- Aprotinin, 10 KIU/mL
- 0.3% Guanidine hydrochloride in 95% ethanol
- Carnitin, 10% SDS

1. PROTEIN PRECIPITATION
Precipitate proteins from the phenol-ethanol supernatant (approximate volume 0.8 mL, per 1 mL of TRIZOL Reagent) with isopropanol alcohol. Add 1.5 mL of isopropanol per 1 mL of TRIZOL Reagent used for the initial homogenization. Store samples for 10 minutes at 15 to 30°C, and sediment the protein precipitate at 12,000 x g for 10 minutes at 4°C.

2. PROTEIN WASH
Remove the supernatant and wash the protein pellet 3 times in a solution containing 0.3 M guanidinium hydrochloride in 95% ethanol. Add 2 mL of wash solution per 1 mL of TRIZOL Reagent used for the initial homogenization. During each wash cycle, store the protein pellet in the washing solution for 30 minutes at 15 to 30°C and centrifuge at 8,000 x g for 5 minutes at 2 to 8°C. After the final wash, vortex the protein pellet in 2 mL of wash solution. Store the protein pellet in ethanol for 20 minutes at 15 to 30°C and centrifuge at 15,000 x g for 5 minutes at 2 to 8°C.

3. RESOLVING THE PROTEIN PELLET
Vacuum dry the protein pellet for 5-10 minutes. Dissolve it in 1% SDS by pipetting. Complete dissolution of the protein pellet may require incubating the sample at 50°C. Sediment any insoluble material by centrifugation at 10,000 x g for 10 minutes at 2 to 8°C. Transfer the supernatant to a fresh tube. The sample is ready for use in Western blotting or may be stored at -5 to -20°C for future use.

For questions, please call the Life Technologies TECHLINE 800-555-6321.

For laboratory use only.

CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

July 1996

Form #370

TROUBLESHOOTING GUIDE
DNA ISOLATION
- Expected yield of RNA per mg of tissue or 1 x 10^8 cultured cells
- Liver and spleen, 6-10 μg
- Kidney, 3-4 μg
- Skeletal muscles, 1-1.5 μg
- Placenta, 1-4 μg
- Epithelial cells, 8-15 μg
- Fibroblasts, 5-7 μg

Low yield
- Incomplete homogenization or lysis of samples.
- Final RNA pellet incompletely resuspended.

Assays including 1 μg
- Sample homogenized in too small a reaction volume.
- Following homogenization, samples were not stored at room temperature for 5 minutes.
- The aqueous phase was contaminated with the phenol phase.
- Incomplete dissolution of the final RNA pellet.

RNA degradation
- Tissues were not immediately processed or frozen after removal from the animal.
- Samples used for isolation, or the isolated RNA preparations were stored at -5 to -20°C, instead of -60 to -70°C.
- Cells were dispersed by trypsin digestion.

Aqueous solutions or tubes were not RNase-free.

Formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.

DNA contamination
- Sample homogenized in too small a reaction volume.
- Sample isolated contained organic solvents (e.g., ethanol, DMSO), strong buffers, or alkaline solution.

Proteoglycan and polysaccharide contamination
- The following modification of the RNA precipitation (step 3) removes these contaminating compounds from the isolated RNA. Add to the aqueous phase 0.25 mL of isopropanol followed by 0.25 mL of a high salt precipitation solution (1.2 M sodium acetate and 0.8 mM NaCl) per 1 mL of TRIZOL Reagent used for the homogenization. Mix the resulting solution, centrifuge and proceed with the isolation as described in the protocol. The modified precipitation effectively precipitates RNA while maintaining polysaccharides and proteoglycans in a soluble form.

A combination of the modified precipitation with an additional centrifugation of the initial homogenate (note #5, RNA isolation protocol) is required to isolate pure RNA from plant material containing a very high level of polysaccharides.

DNA ISOLATION
- Expected yields of DNA per mg of tissue or 1 x 10^8 cultured cells
- Liver and kidney, 3-4 μg
- Skeletal muscles, brain and placenta 2-3 μg
- Cultured human, rat, and mouse cells, 5-7 μg
- Fibroblasts, 5-7 μg

Low yield
- Incomplete homogenization or lysis of samples.
- Final DNA pellet incompletely resuspended.

Assays including 1 μg
- RNA was not sufficiently removed from the DNA preparation. Wash the DNA pellet an additional time with 0.1 M sodium citrate in 10% ethanol.

DNA precipitation
- Tissues were not immediately processed or frozen after removal from the animal.
- Samples used for isolation, or the isolated RNA preparations were stored at -5 to -20°C, instead of -60 to -70°C.
- Samples were homogenized with a Polytron or other high speed homogenizer.

RNA contamination
- Incomplete removal of aqueous phase.
- DNA pellet insufficiently washed with 0.1 M sodium citrate in 10% ethanol.

Other applications
- Prior to use in PCR amplification, adjust the pH to 8.4.
- For digestion of the DNA with restriction endonucleases, adjust the pH to the desired value, use 3-5 units of enzyme per μg of DNA, and allow the reaction to go for 3-24 hours under optimal conditions for the particular enzyme.

Typically 80-90% of the DNA is digested.

PROTEIN ISOLATION
- Low yield
- Incomplete homogenization or lysis of samples.
- Final DNA pellet incompletely resuspended.

Protein degradation
- Tissues were not immediately processed or frozen after removing from the animal.
- Band deformation in PAGE
- Protein pellet insufficiently washed.

PCR is covered by a patent held by Hoffman LaRoche Corporation.