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Product Information

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K5161 - TRIGent (Total RNA Isolation Reagent)

This Protocol Includes:

- Product Description
- Protocol for RNA Isolation
- Protocol for DNA Isolation
- Troubleshooting Guide

WARNING: Highly Toxic! This product contains Phenol.
Storage: Store at 4 °C for optimal performance. Protect from light.

Description

1. ***TRIGent*** is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the RNA isolation method developed by Chomczynski and Sacchi. During sample homogenization or lysis, *TRIGent* maintains the integrity of the RNA, while disrupting 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. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl 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 interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase. Co-purification of the DNA may be useful for normalizing RNA yields from sample to sample.
2. This technique performs well with small quantities of tissue (50~100 mg) and cells (5×10^6), and large quantities of tissue (≥1 g) and cells ($>10^7$), of human, animal, plant, or bacterial origin. The simplicity of *TRIGent* method allows simultaneous processing of a large number of samples. The entire procedure can be completed in one hour. Total RNA isolated by *TRIGent* is free of protein and DNA contamination. It can be used for Northern blot analysis, dot blot hybridization, poly $(A)^+$ selection, in vitro translation, RNase protection assay, and molecular cloning. For use in the polymerase chain reaction (PCR), treatment of the isolated RNA with amplification grade DNase I is recommended when the two primers lie within a single exon.
3. *TRIGent* facilitates isolation of a variety of RNA species of large or small molecular size. Starting material can be either fresh or frozen tissue, or cell samples of almost any size. For example, RNA isolated from rat liver, electrophoresed on an agarose gel, and stained with ethidium bromide, shows discrete bands of high molecular weight RNA between 7 kb and 15 kb in size, (composed of mRNA's and hnRNA's) 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, 5S). The isolated RNA has an $A_{260/280}$ ratio of 1.6~1.8 when diluted into distilled water. The expected yield of RNA per mg of tissue is: liver and spleen, 6~10 μg; kidney, 3~4 μg; skeletal muscles and brain, 1~5 μg; placenta, 1~4 μg. The expected yield of RNA from 1×10^6 Cultured cells is: epithelial cells, 8~15 μg; fibroblasts, 5~7 μg.

Reagents required, but not supplied:

1. Chloroform
2. Isopropyl alcohol
3. 75% Ethanol (in DEPC-treated water)
4. RNase-free water or 0.5% SDS solution. To prepare RNase-free water: Add 0.1% (v/v) DEPC to the water and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37 °C. Autoclave for 15 minutes to remove any trace of DEPC. SDS solution must be prepared by using DEPC-treated water.

Precautions for Preventing RNase Contamination:

RNases 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.

1. Always wear disposable gloves. Skin often contains bacteria and molds that can contaminate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
2. Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to prevent cross-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 on filters, and any nondisposable items (such as automatic pipettes) can be rich sources of RNases.
3. In the presence of *TRIGent*. RNA is protected from RNase. Downstream sample handling requires RNase-Free nondisposable glassware or plasticware. Glass items can be baked at 150°C for 4 hours, and plastic items can be soaked for 10 minutes in 0.5 M NaOH, rinsed thoroughly with water, and autoclaved.

Other Precautions

1. Use of disposable tubes made of clear polypropylene is recommended when working with less than 2 ml volumes of *TRIGent.*
2. For larger volumes, use glass (Corex) or polypropylene tubes, and test to be sure that the tubes can withstand 12,000×g with *TRIGent* and chloroform. Do not use tubes that leak or crack.
3. Carefully equilibrate the weights of the tubes prior to centrifugation.
4. Glass tubes must be sealed with parafilm topped with a layer of foil, and polypropylene tubes must be capped before centrifugation.

Notes for RNA Isolation:

1. Isolation of RNA from small quantities of tissue (1~10 mg) or Cell (10^2~10^4):
   Samples: Add 800 µl of *TRIGent* to the tissue or cells. Add 200µg glycogen directly to *TRIGent* (final glycogen concentration in *TRIGent* is 250 µg/ml). To reduce viscosity, shear the genomic DNA with 2 passes through a 26 gauge needle prior to chloroform addition. The glycogen remains in the aqueous phase and is co-precipitated with the RNA. It does not inhibit first-strand synthesis at concentrations up to 4 mg/ml and does not inhibit PCR.
2. After homogenization and before addition of chloroform, samples can be stored at -60~-70°C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 2~8°C for at least one week, or at least one year at -5 ~ -20°C.
3. Bench-top centrifuges that can attain a maximum of 2,600×g are suitable for use in these protocols if the centrifugation time is increased to 30~60 minutes in steps 2 and 3.

Protocol for RNA Isolation

CAUTION: When working with ***TRIGent*** use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood. Avoid breathing vapor.

Note: Unless otherwise stated, the procedure is carried out at 15~30°C, and reagents are at 15~30°C. See page 2 for reagents required but not supplied, and for precautions.

1. HOMOGENIZATION (see notes 1-3)

a. *Tissues Homogenize* tissue samples in 1ml of ***TRIGent*** per 50~100 mg of tissue using a glass-Teflon or power homogenizer (Polytron, or Tekmar's TISSUMIZER or equivalent). The sample volume should not exceed 10% of the volume of ***TRIGent*** used for homogenization.

b. *Cells Grown in Monolayer* Lyse cells directly in a culture dish by adding 1ml of *TRIGent* to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of ***TRIGent*** added is based on the area of the culture dish (1ml per 10 cm^2) and not on the number of cells present. An insufficient amount of ***TRIGent*** may result in contamination of the isolated RNA with DNA.

c. *Cells Grown in Suspension Pellet* cells by centrifugation. Lyse cells in *TRIGent* by repetitive pipetting. Use 1ml of the reagent per 5~10×10^6 of animal, plant or yeast cells, or per 1×10^7 bacterial cells. Washing cells before addition of

TRIGent should be avoided as this increases the possibility of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

2. OPTIONAL: An additional isolation step may be required for samples with high content of proteins, fat, polysaccharides or extra cellular material such as muscles, fat tissue, and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000×g for 10 minutes at 2~8°C. The resulting pellet contains extra cellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. In each case, transfer the cleared homogenate solution to a fresh tube and proceed with chloroform addition and phase separation as described.

3. PHASE SEPARATION

Incubate the homogenized samples for 5 minutes at 15~30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2ml of chloroform per 1ml of ***TRIGent***. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15~30°C for 2~3 minutes. Centrifuge the samples at no more than 12,000×g for 15 minutes at 2~8°C. Following centrifugation, the mixture separates into a lower, phenol-chloroform phase, a cloudy white interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of *TRIGent* used for homogenization.

4. 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 by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1ml of ***TRIGent*** used for the initial homogenization. Incubate samples at 15~30°C for 10 minutes and centrifuge at no more than 12,000×g for 10 minutes at 2~8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

5. RNA WASH

Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1ml ***TRIGent*** used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500×g for 5 minutes at 2~8°C.

6. REDISSOLVING THE RNA

At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5~10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an $A_{260/280}$ ratio<1.6. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55~60°C (Avoid SDS when RNA will be used in subsequent enzymatic reactions.) RNA can also be re-dissolved in 100% formamide (deionized) and stored at -70°C [See Bracete, et.al. (1998) Focus 20:3 p 82)].

Protocol for DNA Isolation

The procedure is carried out at 15~ 30 °C.

After complete removal of the aqueous phase, as described in the RNA isolation protocol, the DNA in the interphase and phenol phase from the initial homogenate may be isolated. Following precipitation and a series of washes, the DNA is solubilized in 8mM NaOH. Full recovery of DNA from tissues and culture cells permits the use of ***TRIGent*** for the determination of the DNA content in analyzed samples 2. Simultaneous extraction of genomic DNA allows for normalization of the results of Northern analysis per genomic DNA instead of the more variable total RNA or tissue weight. (Depending on the source, the DNA pellet obtained may require additional purification (e.g., phenol extraction) prior to other applications.

Reagents required, but not supplied:

- Ethanol
- 0.1 M Sodium citrate in 10% ethanol
- 75% Ethanol
- 8 mM NaOH

1. DNA PRECIPITATION

Remove the remaining aqueous phase overlying the interphase, and precipitate the DNA from the interphase and organic phase with ethanol. Add 0.3 ml of 100% ethanol per 1 ml of *TRIGent* used for the initial homogenization, and

mix samples by inversion. Next, store the samples at 15~30°C for 2~3 minutes and sediment DNA by centrifugation at no more than 2,000×g for 5 minutes at 2~8°C.

Careful removal of the aqueous phase is critical for the quality of isolated DNA.

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 1ml of *TRIGent* used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 minutes at 15~30°C (with periodic mixing) and centrifuge at 2,000×g for 5 minutes at 2~8°C. Following these two washes, suspend the DNA pellet in 75% ethanol (1.5~2 ml of 75% ethanol per 1 ml *TRIGent*), store for 10~20 minutes at 15~30°C (with periodic mixing) and centrifuge at 2,000×g for 5 minutes at 2~8°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 materia*l.

3. REDISSOLVING THE DNA

Air dry the DNA 5~15 minutes in an open tube (Do not dry using speed-Vac). Dissolve DNA in 8 mM NaOH such that the concentration of DNA is 0.2~0.3 μg/μl. Typically add 300~ 600 μl of 8mM NaOH to DNA isolated from 10^7 cells or 50~70 mg of tissue. Resuspending in weak base is HIGHLY recommended since isolated DNA does not resuspend well in water or in Tris buffers. The pH of the 8 mM NaOH is only ~9 and should be easily adjusted with TE or HEPES once the DNA is in solution. At this stage, the DNA preparations (especially from tissues) may contain insoluble gel-like material (fragments of membranes, etc.) Remove the insoluble material by centrifugation at >12,000 g for 10 minutes. Transfer the supernatant containing the DNA to a new tube. Stability of DNA in 8mM NaOH is several months at 4°C; greater than one year at -20°C; indefinitely at -70°C.

4. 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 A_{260} of the resulting solution. Calculate the DNA content using the A_{260} value for double-stranded DNA. One A_{260} unit equals 50 μg of double-stranded DNA/ml. For calculation of cell number in analyzed samples, assume that the amount of DNA per 1×10^6 diploid cells of human, rat, and mouse origin equals: 7.1 μg, 6.5 μg, and 5.8 μg, respectively[3]. The expected yield of DNA per mg of tissue is: 3~4 μg from liver and kidney; and 2~3μg from skeletal muscles, brain and placenta. The expected yield of DNA per 1×10^6 cultured human, rat and mouse cells is 5~7 μg.

Applications

1. **Amplification of DNA by PCR.**

After re-dissolving the DNA in 8 mM NaOH, adjust the pH to 8.4 with 0.1 M HEPES (see table). Add 0.1~1.0 μg of the DNA sample to your PCR reaction mixture and perform the standard PCR protocol.

2. **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~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 3~24 h. In a typical assay, 80~90% of the DNA is digestible. pH Adjustment of DNA Samples Dissolved in 8mM NaOH (For 1 ml of 8 mM NaOH use the following amounts of 0.1 M or 1 M HEPES, free acid.)

Final pH	0.1M HEPES (μl)	Final pH	1M HEPES (μl)
8.4	86	7.2	23
8.2	93	7.0	32
8.0	101		
7.8	117		
7.5	159		

Notes:

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

Troubleshooting Guide

RNA Isolation

• **Expected yields of RNA per mg of tissue or 1×10^6 cultured cells**

Liver and spleen	6~10 μg	Kidney	3~4 μg
Skeletal muscles and brain	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 re-dissolved.

• **$A260_{/280}$ ratio <1.65**

RNA sample was diluted in water instead of TE prior to spectrophotometric analysis. Low ionic strength and low pH solutions increase absorbance at 280nm. See Wilfinger, W. et.al, Biotechniques 22: 474-481 and Fox, D.K. (1998) Focus 20:2 p.37). Sample homogenized in too small a reagent 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 ~ -20 °C, instead of -60 ~ -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 reagent volume.
Samples used for the isolation 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 0.25ml of isopropanol followed by 0.25 ml of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 ml of *TRIGent* 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 #2, 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 1×10^6 cultured cells**

Liver and kidney	3~4 μg	Fibroblasts	5~7 μg
Skeletal muscles	2~3 μg	brain, and placenta	2~3 μg
Cultured human, rat and mouse cells		5~7 μg	

• **Low yield**

Incomplete homogenization or lysis of samples. DNA pellet is incompletely re-dissolved.

• **$A_{260/280}$ ratio <1.70**

DNA sample was diluted in water instead of TE prior to spectrophotometric analysis. Phenol 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 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 ~-20°C, instead of -60 ~ -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.