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TRIzol® Reagent and TRIzol® LS Reagent

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PRODUCT DESCRIPTION
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TRIzol Reagent:
TRIzol Reagent is a ready to use mixture of phenol, guanidine isothiocyanate, red dye and other proprietary components that can be used to isolate total RNA in 1 hour in a single step. DNA and proteins can be recovered with sequential precipitation from the organic phase. TRIzol was developed by Piotr Chomczynski. The red dye allows easy detection of the organic phase and is non-interactive with nucleic acids.

The TRI in TRIzol stands for Total RNA Isolation. It also signifies the fact that this reagent can be used in the purification of RNA, DNA and proteins from a single source.

Analytical Specifications:

pH of a 10X Dilution	4.8-4.9
Specific Conductivity	15.0-17.0 mS/cm
Refractive Index	1.4884-1.4502
Boiling Point	181.8°C
Melting Point	40.85°C
Specific Gravity at 41°C	1.058

The specific gravity or density of TRIzol is 1,075.1 g/L. This calculation can be made by weighing 1 ml of the reagent.

TRIzol LS Reagent:
TRIzol LS is a complete, ready-to use reagent for easy and simultaneous isolation of total RNA, DNA and proteins from liquid samples. The reagent, a mono-phasic solution of phenol and guanidinium thiocyanate, is an improvement to the single step RNA isolation method developed by Chomzcynski and Sacchi. TRIzol LS is similar to the original TRIzol in composition and results of use. However, TRIzol LS is designed for use with liquid samples such as serum and virus preparations in which large volumes of sample need to be processed while TRIzol is designed for cell suspensions or tissues. It is formulated to accommodate processing of more liquid sample per unit of reagent compared to the original formula.

Difference between TRIzol and TRIzol LS:
The only difference between TRIzol and TRIzol LS is in the concentration of components. TRIzol LS is slightly more concentrated formula to allow lower quantities of reagent to be used relative to sample. (TRIzol = 10:1 required, TRIzol LS = 3:1 required). The two reagents can be distinguished by color (TRIzol LS is darker – maroonish red) and by refractive index (TRIzol LS = ~1.45; Regular TRIzol = ~1.47).

.TRIzol LS can be used like TRIzol (on solid samples), however there will probably be a decrease in yield vs. using regular TRIzol. TRIZOL LS SHOULD NOT BE USED UNDILUTED WITH SOLID SAMPLES. To dilute: take 750 ul TRIzol LS + (50-100 mg tissue + water to make 250 ul).

Analytical Specifications:

pH of a 10X dilution	~ 4.84
Specific Conductance	~ 16.3 mS/cm (measured on 10 fold diluted sample)
Refractive Index	~ 1.47

SHIPPING CONDITIONS
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- Primary Shipping method is at room temperature.
- Secondary Shipping method is on wet ice.
- The UN# for TRIzol is UN2821.
- The tariff code for TRIzol is 3822.00.0000 (Laboratory Reagents).

STORAGE CONDITIONS
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Store TRIzol Reagent and TRIzol LS Reagent at 4°C.

STABILITY
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TRIzol Reagent and TRIzol LS Reagent are stable at 4°C for 2 years.

QC SPECIFICATIONS
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For QC Specifications for TRIzol Reagent and TRIzol LS Reagent, please refer to the Certificate of Analysis for each product.

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Applications and Yields
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- Isolation of RNA from tissues and cells from human, animal, plant leaves , plant roots, yeast, and bacterial origin. This RNA can be used in Northern Blots, Dot Blots, Poly A+ Selection, *in vitro* Translation, RNase Protection Assay, S1 Mapping and cDNA Synthesis. If the RNA is to be used for RT PCR, it should be treated with Amplification Grade DNase I (18068015). [See *Focus* 15.4, 99 and *BioTechniques* 15.3, 532].
- Isolation of DNA from tissues and cells from human, animal and bacterial origin. The DNA that is isolated is of sufficient quality for Restriction Enzyme digestion and Southern blotting. It should be > 20 kb.
- Purification of Protein from tissues and cells from human, animal plant and bacterial origin for Western blotting, and depending on the protein, recovery of enzymatic activity [See Gadotti and Seeds (1995) *Biotechniques* 18(1),46-50; and *Focus* 17:3, 98]. Proteins isolated with TRIzol can also be used for immunoprecipitation if the antibody used recognizes the denatured epitopes on the protein of interest.

Other Applications:

- Plasmid purification from *E. coli*. Marina Kniazeva reports in Elseveir's Trends Journal on the WWW (posting from 3/19/97) in an article entitled "TRIzol for Plasmid DNA Isolation" the purification of ~300 μg of plasmid from a 50 ml culture of DH5alpha. Briefly, the cells were pelleted and then lysed in 2 ml of TRIzol by passing them through a 21 gauge needle. After adding chloroform, the plasmid is isolated from the aqueous phase after treatment with RNase.
- Isolating RNA from enterovirus as reported by Knepp JH, Geahr MA, Forman MS, Valsamakis A. J Clin Microbiol. 2003 Aug;41(8):3532-6. Comparison of automated and manual nucleic Acid extraction methods for detection of enterovirus RNA.
- Isolation of RNA from virus. *Focus* 17:1 p 18-19 describes a method for isolation of RNA from virus.

Isolation of RNA from nuclear run-on assays. [Srivastava (1993) *BioTechniques* 15.2, 226].

Expected yields of RNA/DNA per mg of tissue or 1 x 10^6 cultured cells:

Tissue	**RNA**	**DNA**
Liver, Spleen	6-10 μg	3-4 μg
Kidney	3-4 μg	3-4 μg
Skeletal Muscles, Brain	1-1.5 μg	2-3 μg
Placenta	1-4 μg	2-3 μg
Epithelial Cells	8-15 μg	5-7 μg
Fibroblasts	5-7 μg	5-7 μg
Human Macrophages	5-25 μg	
Mouse Macrophages	2-5 μg	
Rat Lung	1.5-2 μg	

Note: RNA Yields from small (micro) amount of rat or mouse tissue, using 250 μg/ml glycogen in TRIzol. See *Focus* 21.2, 38 for more details.

10^2	12 +/- 1 ng
10^3	24 +/- 3 ng
10^4	140 +/- 17 ng

10^5	640 +/- 75 ng

Note: RNA Yields from 10^2-10^5 (small or micro amount) of Hela cells using 250 µg/ml final concentration glycogen in TRIzol as a carrier. See *Focus* 21.2, 38 for more details.

Tissue	Amount tissue	Yield in micrograms
Rat Liver	1 mg	16 +/- 4
	3 mg	23 +/- 5
	5 mg	38 +/- 2
	10 mg	77 +/- 3
Rat Brain	10 mg	21 +/- 3
	20 mg	33 +/- 2
	50 mg	75 +/- 13
Mouse Adrenal	6 mg	10 +/- 2
Glands	12 mg	20 +/- 3

RNA Isolation with TRIzol

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Quick overview of Protocol:

- Add TRIzol to the sample and homogenize.
- Add chloroform to the homogenate. Use 200ul of chloroform for every 1ml of TRIzol used. Invert and mix well. Centrifuge to get phase separation.
- RNA is in the upper aqueous phase. Remove upper aqueous phase and precipitate to get the total RNA. Use 0.5 ml isopropyl alcohol for every 1ml of TRIzol used. Incubate and centrifuge.
- Wash RNA pellet with 75% ethanol. Centrifuge.
- Redissolve the RNA pellet.

Detailed Protocol Steps RNA isolation with TRIzol

Homogenization
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Homogenize sample using a glass Teflon or power homogenizer (Polytron or Tekmar's Tissumizer) in a 1.5 microcentrifuge tube. Tissue culture cells do not have to be homogenized, but just mixed extensively with TRIzol. Sonication will work to lyse cells in TRIzol, but should only be used if the researcher does not plan to isolate DNA from the sample. Cells grown in monolayers can be lysed directly in the culture dish.

Recommended Sample to TRIzol volume

	Amount of Sample	**Amount of TRIzol**
Tissue	<10 mg	0.8 ml + 200 μg glycogen
	50 – 100 mg	1 ml
	1 g	10-20 ml
Monolayer Cells from	3.5 Dish (area = 10 cm^2)	1 ml
Suspension Cells	10^2-10^5 Animal, Yeast, or Plant Cells	0.8 ml + 200 μg glycogen
	5-10x10^6 Animal, Yeast, Plant Cells	1 ml
	1 x 10^7 Bacterial Cells	1 ml
	1 Volume pelleted bacterial cells	20 Volumes TRIzol
Whole blood	1 ml	10 ml

(1X10^7 RecA- bacterial cells is about 200 μl of culture with A_{550} ~0.4)

Possible stopping points and storage:
- After homogenization (before addition of chloroform) samples can be stored at -70°C for at least 1 year.
- The homogenated samples can also sit at room temperature for several hours before chloroform is added.
- Stability of RNA in TRIzol for field samples: If the biological sample is efficiently lysed in TRIzol and the reagent can inactivate the nucleases, RNA can be safely stored for 3 or 4 days at room temperature (20-25ºC). When TRIzol lysates are stored for 7 days or more, at room temperature, some evidence of degradation begins to appear based on northern analysis; however, these samples may still work fine for RT-PCR.
- For additional information on stopping points, see Focus 20:2 p. 36.

Considerations for sample:
- **Sample volume should NOT exceed 10% of the volume of the reagent.**
- **Use of NP40 or Triton in the sample:** The sample may contain up to 1.0% NP40 or Triton X 100 without interfering with isolation.
- **Scaling of reactions:** Reactions can be scaled up linearly with regards to amount of sample for tissues and suspension cells. Reactions for monolayer cells should be scaled up linearly based on the surface area of the plate NOT the amount of cells. Use at least 1 ml of TRIzol for every 10 cm^2 dish (i.e., 1 ml per 35 mm dish). The reason for this is that even after good aspiration, a thin layer of media remains on the plate. As the surface area increases, it is important to scale up as recommended to avoid dilution of TRIzol. Dilution of TRIzol may lead to incomplete lysis.
- **Insoluble material after homogenization:** There are two methods to remove insoluble material:
  1. RNA Isolation only: If a lot of insoluble material exists after homogenization and a 5 minute room temperature incubation, remove by centrifugation at 12,000 x g for 10' at 4°C before adding chloroform (a clear supernatant and jelly-like pellet should be seen). Remove the supernatant and proceed to the next step. Note: This should not be done if subsequent DNA isolation is to be done.
  2. RNA and DNA isolation: If a lot of insoluble material exists after homogenization and 5 min. room temperature incubation, the homogenate can be passed through a polypropylene mesh to remove insoluble material which may interfere with precipitation of DNA.

- **Addition of glycogen to the sample:** Glycogen can be included with sample, it often improves yield and remains with the RNA (glycogen is water soluble). Glycogen is available as catalog # 10814010.

Phase Separation
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Incubate sample for 5 minutes in TRIzol at room temperature. Add 0.2 ml of chloroform for every 1ml of TRIzol used. Shake vigorously for 15 seconds and incubate at room temperature for 2-3 min. Centrifuge samples 15 min. at 12,000 x g at 4°C.

- **Chloroform to use:** Use straight chloroform; no isoamyl alcohol is needed. Chloroform with 50 ppm amylene can be used. Chloroform solublizes lipids in the sample. .
- **BCP (1-bromo-2 chloropropane) can be used in the place of chloroform**. BCP has a higher density. Because of its higher density, the interface is more compact and therefore the aqueous layer is more easily recovered. (see Chomczynski, P. and Mackey, K. (1995) *Anal. Biochem*. 225, 163-164). The quantity and quality of RNA is the same with both reagents. The amount of BCP used for phase separation equals 10% of the TRIzol volume.
  Using lower centrifugation speeds: Centrifugation speeds as low as 5000 -6000 x g have been used at this step, however, the centrifugation time should be doubled to 30 min. to get similar yields.
- **Multiple phases observed upon addition of chloroform:** If you observe multiple phases after adding chloroform and mixing, these are most likely due to inadequate mixing of the chloroform with the reagent. Chloroform is much more dense than TRIzol and therefore will immediately sediment to the bottom of the tube. Simply vortexing the solution will not properly mix chloroform with the TRIzol solution and may give a partial phase separation. Whenever clear water-like solution on the bottom of the tube is observed, this may be chloroform. Simply remix the samples by shaking the contents vigorously as stated in the protocol and centrifuge the samples at 12000 g for 15 min. at 4ºC. .
- **Temperature for spin:** The 4°C spins are essential for phase separation. Room temperature spins may result in variable phase separation thus resulting in variable RNA yields.
- **Isopropanol used instead of chloroform:** If isopropanol is inadvertently added at this step instead of chloroform, add more isopropanol to precipitate everything, then resuspend the pellet in TRIzol and use the protocol as specified. RNA yields will be compromised, but it may be possible to obtain a product in RT-PCR (see next paragraph for protocol). Protocol for adding more isopropanol if isopropanol was used instead of chloroform:
  1. Add more isopropanol so that the total volume of isopropanol equals the volume of TRIzol used. Spin at 7500 x g for 10 min. at 4°C.
  2. Pour off supernatant; allow relatively compacted pellet to air dry Estimate the size of the pellet in µl; add at least 15-20 volumes of TRIzol (e.g., for a 100 µl pellet, add at least 1.5 ml TRIzol).
  3. Break the pellet up well (may have to use a hand-held homogenizer). Store the solution for 10-15 min. at room temperature; every 5 min. or so, shake it by hand to make certain it is well dispersed.
  4. Proceed with the TRIzol protocol as written (i.e., add chloroform). Results will not be optimal, but it may be possible to get a product in RT-PCR.
- **Addition of too much chloroform:** If a larger amount of chloroform than needed was inadvertently added, add more TRIzol so that the ratio of 0.2 ml chloroform: 1 ml TRIzol is maintained. If too much chloroform is added, this will drive the DNA, and eventually the protein, into the aqueous phase.
- **Back extraction during RNA isolation step:** It is not possible to back extract the RNA at this step to clean it up or remove DNA. Addition of more TRIzol to the aqueous phase will just cause it to become pinkish and one phase. It is best to proceed on and then clean up the RNA with DNase I or multiple ethanol washes.

RNA Precipitation
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Transfer the aqueous phase to a fresh tube. The aqueous phase is the colorless upper phase that corresponds to ~60% of the volume of TRIzol used. The interphase should be fairly well-defined.

Precipitate the RNA by mixing with 0.5 ml of isopropanol for every 1 ml of TRIzol used initially. (Ethanol may be substituted for isopropanol. Use 0.75 ml of ethanol per ml of TRIzol).

Incubate at room temperature for 10 min. and centrifuge for 10 min. at 12000 x g at 4°C. The RNA should be visible on the side of the tube.

- **Centrifugation at lower speeds:** Centrifugation speeds as low as 5000-6000 x g have been used at this step, but the centrifugation time should be doubled to 30 min. to get similar yields.
- **Cloudiness observed after addition of isopropanol:** A "cloudiness" may be observed upon the addition of isopropanol (regardless of its temperature--i.e. room temperature or -20°C) to some samples (for example, liver extraction samples). Continue to precipitate the RNA at room temperature for 10-15 min.
- **Sample storage after adding isopropanol:** , The RNA may be stored in isopropanol overnight at 4°C. Prolonged storage and this reduced temperature will not influence the yield of RNA appreciably. Do not store at -20°C, as salts will precipitate, or do not store prolonged at room temperature because the guanidine isothiocyanate can harm the RNA.
- **Using Salmon Sperm DNA as a carrier:** Salmon Sperm DNA can be used as a carrier and should be added during the precipitation of the aqueous phase. (

RNA Wash

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Remove the supernatant. Wash pellet with 1 ml 75% ethanol for every 1 ml of TRIzol used. Mix sample by flicking and inverting the tube or vortexing and centrifuge at 7500 x g for 5 min. at 4°C.

- **Storage of RNA in 75% ethanol:** RNA may be stored in 75% ethanol for 1 week at 4°C or 1 year at -20°C.
- **Isolating large RNAs:** If there is difficulty isolating large RNA from some cell lines, the pellet can be dissolved completely in 3 ml of a GTC Solution (4 M Guanidine IsoThiocyanate, 25 mM NaCitrate pH 7.0, 0.5% N Laurylsarcosine, and 0.1 M Beta-Mercaptoethanol) and precipitated with 3 ml of isopropanol. (This should be done twice). See *Focus* 20.3, 80; "Modified TRIzol Reagent Protocol for Large RNA" for more detail.

Redissolving the RNA

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It is preferred to air dry RNA as vacuum-drying nucleic acid precipitates may causing over-drying of pellets. These vacuum-dried pellets are then very difficult to disolve and the samples may be lost. Clean, sufficiently dried RNA pellets are usually white or may have a clear jelly-like appearance. RNA dried under vacuum is often degraded. If the pellet is overdried, you may resuspend RNA in RNase Free H_2O. Sample may have to be incubated at 55°C-60°C for 10-15 min. with repeated pipetting to completely redissolve. (As a general reference, 50 µl DEPC Water is generally used to resuspend the RNA from $5x10^6$ HeLa Cells).

- **Resuspension of RNA from RNase-rich tissues:** RNA from RNase-rich tissues (e.g., rat pancreas) or large RNA should be resuspended in 100% formamide to increase stability. Pancreas RNA samples directly resuspended in water or RNA samples that were in guanidine HCl and treated with proteinase K (to remove residual RNases) and then resuspended in water were degraded within 2 weeks; samples in 100% formamide were stable for as long as 3 years. See also *Focus* 20.3, 80, "Modified TRIzol Reagent Protocol for Large mRNA" or *Focus* 20.3, 82, "Isolation and Long Term Storage of RNA from Ribonuclease Rich Pancreas Tissue".
- **Long-term storage of RNA:** For long-term storage, it is recommended to resuspend RNA in stabilized formamide and storing at -70°C. To remove the formamide, add 4 volumes ethanol, and if less than 20 µg RNA also add NaCl to 0.2 M. Precipitate the RNA.

  For spectrophotometric readings or gel electrophoresis, the RNA may remain in formamide. For RT-PCR, the RNA should be diluted to a final concentration of 5%.

Additional Notes for RNA isolation with TRIzol

Isolation of RNA from Small Quantities of Tissue (1 to 10 mg) or Cells (100 to 10,000) Samples
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Protocol:

1. Add 800 µl TRIzol to the sample. Homogenize cells by pipetting repeatedly. Add 200 µg glycogen (Cat. No. 10814-010) directly to the TRIzol reagent. If processing tissue, pulverize in liquid nitrogen first and then add 800 µl TRIzol containing 200 µg glycogen (final concentration 250 µg/ml) followed by vigorous vortexing or power homogenization.
2. Place at room temperature, cap vial, and vortex at high speed for 10 sec. Make sure the TRIzol reagent wets the side of the vial in order to solubilize any sample that may be remaining on the walls.
3. Shear the genomic DNA in the sample by passing twice through a 26-gauge needle connected to a 1 ml syringe. Using the syringe, transfer the sample to a sterile 1.5 ml microcentrifuge tube.
4. Add 160 µl of chloroform (or 49:1 chlorform:isoamyl alcohol) to each sample and vortex up to 30 s. Spin at maximum speed in the microcentrifuge 5 minutes to separate the phases.
5. Transfer the upper aqueous phase to a fresh tube and add 400 µl ice-cold isopropanol. Allow the samples to precipitate at -20°C 1 hour - overnight. Pellet the RNA by centrifugation at maximum speed in the microfuge 15 min at room temperature.
6. Decant the supernatant. Wash the pellet in 200 µl of 70% ethanol and spin again 10 min at maximum speed. Decant the supernatant, removing as much as possible without disturbing the pellet. Dry RNA pellet.
7. Resolubilize the pellet in 30 - 50 µl RNAse-free deionized water. NOTE: If tissue is high in RNAses (e.g., adrenal gland, pancreas,) resuspend in 100% deionized formamide. Be sure to vortex or pipette up and down the sample to ascertain that pellet is resolubilized fully. Store at -70°C.

Note: 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. Nanogram amounts of total RNA can be obtained from as little as 100 cells. Microgram amounts of total RNA were isolated from 1 to 10 mg of tissue. Decreasing the concentration of glycogen below 250 µg/ml TRIzol resulted in lower and more variable yields of RNA.

Reference for Protocol: (Alma M. Bracete, Donna K. Fox, and Domenica Simms, 1998, Isolation and Long Term Storage of RNA from Ribonuclease-rich Pancreas Tissue, *FOCUS*® 20:3, p. 82).

Procedure for use when DNA is not needed, only RNA
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When isolating RNA with TRIzol, if you do not need to isolate the genomic DNA, centrifuge the sample following homogenization before adding chloroform at 12000 X g for 10 minutes at 4ºC to pellet the DNA. Add chloroform to the supernatant and proceed with the RNA isolation protocol. Also, after RNA isolation, you can treat with Amplification Grade DNase I. Using non-amplification grade DNase I has been shown to degrade RNA samples in some cases.

Expected A_{260}/A_{280} of total RNA isolated by TRIzol
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The value of the A_{260}/A_{280} ratio is a commonly used criteria for nucleic acid purification and the values usually given for pure DNA and RNA are 1.8 and 2.0, respectively (1-2). However, the absorbance of nucleic acids at these wavelengths is dependent upon the ionic strength and pH of the medium. For example, the A_{260}/A_{280} ratio of the same RNA preparation differs greatly depending on whether the RNA is diluted into distilled, DEPC-treated water or TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). When the RNA is diluted into and blanked against water the A_{260}/A_{280} ratio is 1.7-1.8; when TE is used the ratio becomes 2.2-2.3. (Table from Fox, D.K. (1998) *Focus* 20.2, 37; Measuring Absorbance of RNA Samples)

Diluent	**A_{260}**	**A_{280}**	**A_{260}/A_{280}**	**[RNA]µg/ml**
Cytoplasmic RNA	0.381	0.223	1.711	15.24

dissolved in distilled. H_2O				
Cytoplasmic RNA dissolved in TE.	0.335	0.145	2.310	13.4
RNA isolated by TRIzol and dissolved in distilled H_20.	0.585	0.328	1.785	23.4
RNA isolated by TRIzol and dissolved in TE.	0.544	0.247	2.206	21.76

This effect is independent of the method of purification. An extensive study of the effects of pH and ionic strength has been done by Wilfinger, *et al* (4) who show that the change in the A_{260}/A_{280} ratio is primarily due to a decrease in the absorbance at 280 nm when the ionic strength and/or pH is increased. Wilfinger, *et al* also note that these same effects are seen when the A_{260}/A_{280} ratio of DNA is measured. We recommend that RNA or DNA be diluted with TE or 1-3 mM Na_2PO_4 buffer at pH 8.0-8.5 (4) for spectrophotometric studies.

Although a high A_{260}/A_{280} ratio may not indicate an extremely pure preparation of nucleic acid, a low A_{260}/A_{280} ratio (1.7 for RNA) does indicate that the preparation is contaminated and may not be suitable for some applications.

References:

1. Gallagher, S.R. (1992) Current Protocols in Molecular Biology Vol 3. Ausubel, F.M. Brent,R. ., Kingston, R.E, Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K (Eds)., John Wiley and Sons, New York.
2. Sambrook, J., Fritch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Section E5, Coldspring Harbor Laboratory Press, Plainview, New York.
3. Wilfinger, W.W., Mackey, Karol and Chomczynski, P. (1997) *Biotechniques* 22(3): 474-481.
4. Manchester, K. (1995) *Biotechniques* 19(2):208-210

Isolation of RNA from cells stored in a guanidine solution

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When cells are stored in a guanidine solution at -70°C, TRIzol can still be used to isolate the RNA in a 1:10 dilution (i.e., 10 ml TRIzol per ml of cells); however, there may be chemical degradation of the RNA by guanidine after prolonged storage.

Samples with proteoglycans and/or polysaccharides

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If a sample is known to have a high content of proteoglycans and/or polysaccharides (such as rat liver, rat aorta, plants), the following modification of the RNA precipitation step should remove these contaminating compounds from the isolated RNA:

- Add 0.25 ml of isopropanol to the aqueous phase followed by 0.25 ml of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl (no pH adjustment necessary)) per 1 ml of TRIzol used for homogenization. Mix the resulting solution, centrifuge, and proceed with isolation as described in the protocol.

This modified precipitation effectively precipitates RNA and maintains proteoglycans and polysaccharides in a soluble form. To isolate pure RNA from plant material containing a very high level of polysaccharides, the modified precipitation should be combined with an additional centrifugation of the initial homogenate.

In general, it is not recommended to use the high-salt precipitation if polysaccharide or proteoglycan contamination is not a concern.

General RNA information

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The components of total RNA include: ribosomal RNA (rRNA) ~90% of total RNA; transfer RNA (tRNA) ~5% of total RNA; small nuclear RNAs ~1% of total RNA; messenger RNA ~1-3% of total RNA.

Predominant ribosomal bands from mammalian species: 28S (4,712 bp), 18S (1,950 bp), 5.8S (~160 bp), 5S (~120 bp).

Predominant ribosomal bands from *E. coli*: 23S (2,904 bp), 16S (1,541 bp), 5S (~120 bp).

Quantitation of Total RNA
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Remove a 10 µl aliquot of total RNA and dilute with 990 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0).

Read the A_{260} and A_{280} blanked against TE buffer and calculate the amount of RNA recovered.
The RNA recovered may be determined by the formula:

- Total RNA (ug) = (A_{260}) (40 ug/ml/A_{260}) (100) (0.6 ml)
- A_{260} is the absorbance of the solution at 260 nm
- 40 ug/ml/A_{260} is a fixed conversion factor relating absorbance to concentration for RNA
- 100 is the dilution factor, and 0.6 ml is the total volume. Concentration = Total/600 ul = ug/µl.

Tubes to use with TRIzol
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Polypropylene microcentrifuge tubes are the recommended vessels for the TRIzol or TRIzol LS protocol. Polypropylene tubes display very good chemical resistance to strong acids and a broad array of both aliphatic and aromatic hydrocarbon solvents. Polypropylene tubes are unaffected by TRIzol or TRIzol LS exposure and they also provide resistance to cracks during centrifugation at speeds of 10000 x g. In contrast, polycarbonate tubes are not recommended for applications that involve either aliphatic and aromatic hydrocarbon solvents. If TRIzol or TRIzol LS remains in contact with polycarbonate for a period of time, it most likely will dissolve the tubes. This could result in the fracture of the tubes during centrifugation and the loss of the samples.

Anti-foaming agents
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TRIzol contains components which function as anti-foaming agents. The addition of other anti-foaming agents has not been tested.

Troubleshooting RNA isolation with TRIzol

Low Yield of RNA/Degraded RNA
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- RNA may have been speed vac or lyophilized after the last ethanol precipitation. RNA that has been dried completely has decreased solubility. Additionally, if excess centrifugation speeds (speeds greater than 12000 x g) were used, it is harder to solubilize RNA/DNA.
- RNA pellet may not be completely solubilized. To increase rate of solubilization, pipet repeatedly in SDS or DEPC-treated water, then heat to 50-60°C. Sample may also have been rich in polysaccharides or proteoglycans. If so, isopropanol precipitation step should be done with 0.25 vol isopropanol and 0.25 volume high salt solution (see section on Samples with proteoglycans and/or polysaccharides).
- Cells were washed prior to the addition of TRIzol. Washing cells before the addition of TRIzol increases the possibility of mRNA degradation.

- Sample was not fully homogenized.
- Tissues not IMMEDIATELY processed or frozen after removal from animal.
- Tissues were not completely disrupted; if a centrifugation is done prior to adding chloroform, there should be a white mucus-like pellet. If there is a tan-colored precipitate, this is indicative that not all of the cells have been lysed.
- If a mortar and pestle was used to powder the tissue, RNA and DNA may stick nonspecifically to the mortar and pestle. It may be better to use a glass homogenizer and teflon pestle; add TRIzol to the homogenizer, then add frozen tissue and homogenize.
- RNA stored after isolation at -20°C instead of -70°C.
- Tissue culture cells were disrupted by trypsin.
- Homogenizing for too long and too continuously in a small volume (e.g., 1 ml) may cause heating of the sample; this may result in degradation of the RNA in the tissue. Samples should be cooled during homogenization, and homogenization should be done in on-off cycles (as opposed to continuously).
- OD reading may vary due to solution the sample is stored in AND what it was diluted in prior to quantitation. Wilfinger, et. al., in (1997) B*ioTechniques* 22.3, 478 reports that increases in ionic strength may significantly reduce nucleic acid absorbance, thereby affecting quantitative determinations based on A_{260}.

DNA Contamination in RNA Prep
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- A portion of the interphase was removed with the aqueous phase after the initial separation.
- An insufficient amount of TRIzol was added to the sample. In general, 1 ml of TRIzol should be used for every 0.05 g of tissue or every 10 cm^2 dish.
- Original sample had traces of other organic material to begin with (ethanol, DMSO, etc).
- RNA should be treated with Amplification Grade DNase I prior to RT-PCR.
- There were insolubles after the first homogenization that were not removed by centrifugation before chloroform extraction.

Poor A_{260}/A_{280} Ratios For RNA (< 1.65)
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- Sample homogenized in too small a volume of TRIzol.
- Samples not stored at room temperature for 5 minutes after homogenization
- Final RNA pellet was not fully dissolved. This may be the case if the RNA pellet was overdried (if the pellet is clear and not white, this indicates overdrying). To get the pellet to dissolve completely, heat to 55-60°C for 10-15 minutes and repeatedly pipet.
- There may be phenol contamination. This may occur if samples were centrifuged at room temperature instead of 4°C; phenol is more soluble in the aqueous phase at room temperature. If absorbance is seen at 270 nm (phenol), sample can be ethanol precipitated to remove residual phenol.
- Guanidine absorbs around 240 nm. Phenol has two peaks: one around 275 nm, the other is broad and covers from below 220 to around 240 nm. If a very large peak is observed in that range, it is recommended to precipitate and wash again. It is also recommended to prevent this to do the phase separation after addition of chloroform at 4°C.
- Residual chloroform; reprecipitate.
- In some samples dissolved in water, the ratio may be low due to the acidity of the water or the low ion content in the water. The ratios may go up if the sample is dissolved in TE and the spec is zeroed with TE. (or 1-3 mM Na_2PO_4, pH ~8.0). [See Wilfinger (1997) *BioTechniques* 22.3, 474]. The molar extinction coefficient of the nucleotides is given at neutral pH (see Maniatis), suggesting that the absorbance at 260 nm would be highest at neutral pH.
- If A_{280} is >0.5, the reading is out of the linear range; try making a dilution such that the A_{280} is below 0.5.

Yellowish-Brown or Pinkish Colored Aqueous Phase
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- This is common with skin samples. It is assumed that there is fat in these samples, and the fat micelles "try to spin to the top of the tube" during the centrifugation. In skin samples, the micelles pick up melanin pigment and cause the aqueous phase to appear colored. Fat micelles may also pick up pigment from the TRIzol itself and cause a pinkish color. If a sample is thought to contain fat, the sample homogenate in TRIzol may be centrifuged prior to addition of chloroform. The fat will appear as a clear layer at the top of the supernatant; this should be pipetted off and discarded.
- If a sample contains a lot of blood, the aqueous phase may appear cloudy and/or yellowish (this may be due to iron in the hemoglobin). If the centrifuge used is not cold, the organic phase will be a deeper maroon color; some of this color may come into the aqueous phase and cause it to appear orange or yellow.
- A pinkish aqueous phase may also be caused by over-dilution of the sample (i.e., the sample:TRIzol ratio > 1:10), as well as too much salt or protein in the sample. This can cause premature phase separation, which can be remedied by adding a bit more TRIzol to the sample. If the RNA is isolated from a pinkish aqueous phase, chances are that it will be contaminated with DNA.

Precipitate At the Bottom of the tube following centrifugation after adding Chloroform (before isopropanol is added)
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- This is most likely polysaccharides or cell membranes; DNA should be in the interphase. In samples containing blood (e.g., liver), a red viscous layer may be visible on top of the pellet. This is most likely due to blood products and should not be carried over with the supernatant.

Intensity of the Ribosomal RNA Bands from Prep to Prep is Inconsistent
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- Check the composition and freshness of the loading buffer. The composition should be such that the final concentration of formamide is 50% and the formamide must be fresh.

No Bands around 200 bp on a Northern Blot
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- Proteoglycans co-purify with RNA in TRIzol and can be transferred onto a Northern Blot. [Schick (1995)18.4, 575]. See steps above for removing proteoglycans at the isopropanol precipitation step.

A_{260}/A_{280} ratio >2.0 for RNA
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(back to RNA Isolation)

- Degraded RNA can cause an increased absorbance at 260 nm.

DNA Isolation with TRIzol
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Quick overview of Protocol:

1. Add 0.3 ml of ethanol to the interphase and organic phase for every 1ml of TRIzol used. Mix and spin.
2. Remove the supernatant and store for protein isolation. Wash the DNA pellet. Incubate and store. Spin pellet.
3. Dry the pellet and re-dissolve.

Detailed Protocol Steps for DNA Isolation with TRIzol

DNA Precipitation
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Add 0.3 ml of 100% ethanol to the red organic phase for every 1 ml of TRIzol Reagent used. Mix by inversion. Store at room temperature for 2-3 min. and sediment DNA at 2000 x g for 5' at 4°C.

- **Using isopropanol instead of ethanol:** Isopropanol may be used instead of ethanol; use 0.2 ml for every ml TRIzol. The use of isopropanol may lead to protein precipitating with the DNA therefore it is best to use ethanol.
- **Centrifugation at higher speed:** DNA can be precipitated at 5000 X G for 5 min. at 4°C to increase yield, but may make pellet more difficult to resuspend.
- **Possible stopping point:** The phenol phase and interphase can be stored at 4°C overnight.

DNA Wash
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Remove the phenol/ethanol supernatant. Wash DNA pellet 2X in 1 ml of a solution containing 0.1 M Sodium Citrate in 10% ethanol with pH 8.5.

Store the DNA pellet in the washing solution for 30 min. at room temperature before centrifuging at 2000 x g for 5 min. at 4°C. After these washes, wash DNA in 2 ml 75% ethanol for every 1 ml TRIzol used and store for 10-20 min at room temperature. Centrifuge 2000 x g for 5 min. at 4°C.

- **Note on sodium citrate:** Sodium citrate dihydrate can be used, but the pH will have to be adjusted.
- **Possible stopping points:** Samples can be stored in the washing solution (0.1 M Sodium Citrate in 10% ethanol) for at least a couple of hours.
- **Visible extracellular debris:** Extracellular debris may be visible at this step; this is only a wash step and will not solubilize the DNA and ECM, collagen, etc. Normally, the small pellet will be dislodged/ disrupted when the sodium citrate is added, but if the pellet is large, it should be disrupted using a pipet tip or spatula if necessary; this will improve the diffusion of phenol and guanidine out of the pellet.

Redissolving the DNA
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- Air Dry or dry the DNA pellet for 5-10 min. under vacuum and dissolve in 8 mM NaOH by slowly passing the pellet through a pipet. Add 0.3-0.6 ml 8 mM NaOH for every 50-70 mg tissue or 1 x10^7 cells such that the DNA concentration will be 0.2-0.3 µg/µl. The mild base allows full solubilization of the DNA pellet. Remove insolubles by centrifugation at 12000 x g for 10 min. Transfer the supernatant to a new tube and ethanol precipitate (or simply just adjust the pH to the desired pH).
- **Possible stopping points:** Samples suspended in 75% ethanol can be stored at 4°C for several months. Samples solubilized in 8mM NaOH can be stored overnight at 4°C. For prolonged storage, adjust samples with HEPES to pH 7-8 (see table below) and supplement with 1 mM EDTA. Once pH is adjusted, DNA can be stored at 4°C or -20°C.

- **Adjustment of pH:**
  After the DNA is solubilized in 8 mM NaOH, adjust the DNA solution to a desired pH by the addition of HEPES. Use the following amounts of 0.1 M or 1 M HEPES (free acid) per 1 ml of 8 mM NaOH:

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

- **DNA solubilization problems may be due to several different issues:**
  1. The most common problem relating to DNA solubilization occurs when the DNA pellets are over-dried. The use of vacuum suction devices to remove the wash solutions may cause overdrying of DNA pellet. Vacuum suction draws air through the pellet and almost always will over dry the DNA pellets. Avoid removing the wash solutions with any type of vacuum suction device and limit the drying time to < 5 min. If these simple instructions are followed many nucleic acid solubility problems can be avoided.
  2. Remove droplets of ethanol from the wall of the test tube with a sterile cotton swab. Additional ethanol can be removed by touching the pellet with a sterile capillary pipette tip. Excess ethanol will be drawn into the lumen of the pipette by capilarity. DNA solubility issues can usually be eliminated if the pellets are placed into either TE buffer or 8 mM NaOH before all of the ethanol has evaporated. The DNA pellets will become clear after 5-10 min. incubation as they begin to rehydrate. In order to solubilize the DNA completely, the solution must be pipetted before removing an aliquot for quantitation.
  3. DNA pellets that are over-dried can be solubilized but it may be necessary to put them into the refrigerator and pipet them periodically until they become clear and go into solution.

Additional notes for DNA Isolation with TRIzol

Alternative procedure for DNA isolation
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This alternative procedure replaces steps 1-2 of the DNA isolation procedure. Prepare a back extraction buffer containing: 4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris pH 8.0 (free base).

1. Following phase separation (RNA isolation procedure step 2), remove any remaining aqueous phase overlaying the interphase and add back extraction buffer to the interphase-organic phase mixture. Use 0.5 ml of back extraction buffer per 1.0 ml of TRIzol used for the initial homogenization. Vigorously mix by inversion for 15 sec and store for 10 min at room temperature.
2. Perform phase separation by centrifugation at 12000 x g for 15 min at 4°C.
3. Transfer the upper aqueous phase containing DNA to a clean tube and save the interphase and organic phase at 4°C for subsequent protein isolation.
4. Precipitate DNA from the aqueous phase by adding 0.4 ml of isopropanol per 1 ml of TRIzol used for the initial homogenization. The isopropanol volume should be 0.8 volumes of the aqueous phase.

5. Mix the tube contents by inversion and store for 5 min at room temperature. If the expected DNA yield is less than 20 μg, add a microcarrier such as glycogen to the aqueous phase prior to isopropanol addition and mix.
6. Sediment DNA by centrifugation at 12000 x g for 5 min at 4-25°C and remove the supernatant. Wash the DNA pellet with 1.0 ml of 75% ethanol and proceed with DNA solubilization as described in Step 3.

Note about pH
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The pH of the DNA should be adjusted to desired pH prior to further manipulation (i.e., PCR, restriction enzyme digestion, etc.). Once pH is adjusted, DNA can be used directly in PCR.

Single-stranded DNA and TRIzol
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Single-stranded DNA will separate with the DNA phase.

Use of DNA for restriction enzyme digests
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Restriction Enzyme digestions with DNA isolated by the TRIzol method may require excess enzyme (3-5 U/μg) and extra time (3-24 hrs).

Troubleshooting DNA isolation with TRIzol

Low Yield of DNA/DNA Degradation
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- Incomplete homogenization/lysis of samples. If any solid material remains after chloroform is added, this indicates that DNA yield may be poor, as DNA will remain trapped in the "unhomogenized" material.
- Final DNA pellet not fully redissolved. It can take several hours to resuspend the DNA. Some incubation at 37°C between pipettings will help. Also make sure that it is not too concentrated or it won't go back into solution. If the DNA is not fully redissolved, it will be lost during the final spin when removing the gel-like material.
- Tissues not IMMEDIATELY processed or frozen after removal from animal.
- Samples were homogenized with a high-speed homogenizer. DNA shearing happens.
- If expected yield is <10 μg, there are limitations to the physical action of precipitation which would lead to low yields. Microcarrier (glycogen, tRNA) may be included in the homogenization and/or wash steps, or samples may be pooled to increase expected yield.

A_{260}/A_{280} Ratio < 1.70 for DNA
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Phenol was not sufficiently removed from DNA preparation. Additional washes with 0.1 M Sodium Citrate in 10% ethanol should be done. It's not unusual for residual phenol from the extraction to remain. An OD 260:280 on the extracted material would show a higher than expected 280. It is recommended to do a second ethanol precipitation to remove remaining phenol. This will also remove any excess salt.

Large pellet after initial ethanol precipitation
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This pellet may contain connective tissue and protein, in addition to the DNA. If the pellet is reddish (it should be white), this indicates that there is unhomogenized tissue present. Proteins can leach out and cause a lower OD ratio (NaOH will solubilize protein). Centrifuging the sample at high speed once it is resuspended in the NaOH will pellet this tissue and protein.

Aqueous layer appeared after addition of 100% ethanol
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There are several possible reasons for this:

- If the aqueous phase was removed completely and the ethanol was added to the samples, it will remain on top of the TRIzol due to ethanol's lower density. If the samples were centrifuged without mixing the two liquids, the ethanol will remain on top of the TRIzol after centrifugation, the DNA will remain at the interface and the TRIzol will be localized to the red bottom organic fraction. If the ethanol was not mixed properly, proceed with mixing the samples, then centrifuge and proceed with step 1 of the DNA isolation protocol.
- If 70% ethanol was added accidentally, it may be possible to get a small volume of water on top of the organic fraction.
- The phase separation was not complete during the RNA isolation step. This can occur because the chloroform or BCP was not adequately mixed, the samples were not centrifuged at the proper g-force, for the required period of time or at the correct temperature. The net result is that significantly less than 600 µl of the RNA aqueous phase will be recovered from the sample. Phase separation problems usually occur when the chloroform or BCP are mixed by vortexing. Due to the large difference in density between TRIzol and the organic, the solutions are never mixed completely and only a portion of the aqueous phase will be recovered. When the ethanol is added and the samples are remixed sufficiently, the phase separation will go to completion and water could appear on top of the sample.

Protein Isolation with TRIzol
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(back to Protocol and Application Notes)

Quick overview of Protocol

1. Add isopropanol to the phenol/ethanol supernatant to precipitate the proteins. Incubate at room temperature and spin.
2. Wash pellet with guanidine/ethnaol solution. Spin.
3. Solubilize the protein pellet.

Detailed steps for Protein Isolation with TRIzol

Protein Precipitation
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Precipitate proteins from the phenol/ethanol supernatant (from DNA isolation procedure) by adding 1.5 ml isopropanol per 1 ml of TRIzol reagent used for initial homogenization. Store sample at room temperature for 10 min. and sediment by centrifugation at 12000 x g for 10 min. at 4°C.

- **Using acetone instead of isopropanol:** Proteins can be precipitated by the addition of isopropanol or acetone. Optimal protein yield can be achieved with acetone to phenol-ethanol ratios between 3:1 and 6:1 worked best (see *Focus* 17:3, 98).
- **Possible stopping point:** After precipitation of DNA, the protein-containing phenol/ethanol phase may be stored at -70°C for several months before proteins are isolated.

Protein Wash

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Remove supernatant and wash pellet 3x in 0.3 M guanidine hydrochloride (GuHCl) in 95% ethanol. Add 2 ml wash solution per 1 ml TRIzol used. In general, the pellet will not be completely dispersed, as it is a very hard and rubbery pellet. Try to break up the pellet by using a pipet tip or by vortexing. During each wash cycle, store the protein pellet in wash solution for 20 min at room temperature. Centrifuge 7500 x g for 5 min. at 4°C to re-acquire pellet. After the final wash, vortex protein pellet in 2 ml 100% ethanol. Store protein pellet in ethanol for 20 min. at room temperature (this is a continuing wash to remove phenol and dye) and then centrifuge at 7500 x g for 5 min. at 4°C.

- **Preparing 0.3M GuHCl in 95% ethanol:** 6 M GuHCl (in water) can be diluted to 0.3M with absolute ethanol . .
- **Possible stopping point:** The protein pellet suspended in 0.3 M GuHCl-95% ethanol or in ethanol may be stored for at least one month at 4°C or at least one year at -20°C. There was no significant difference between proteins that were freshly prepared or stored in the phenol ethanol solution at room temperature for 1 week, at 4°C for 1 month, or at -20°C for 3 months (see *Focus* 17:3, 98).
- **Alternative for guanidine hydrochloride:** Guanidine thiocyanate at 0.2 M in 95% ethanol may be substituted for 0.3 M GuHCl in 95% ethanol for the protein wash steps.

Redissolving the Protein Pellet

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The critical step in protein isolation is extraction of proteins from the pellet at the end of the protocol. The following 3 methods are alternative approaches allowing efficient recovery of proteins. Different classes of proteins, especially membrane proteins, may display different solubilities and may require different solutions or approaches.

- Remove the ethanol wash and air dry pellet for 5-10 min. at room temperature. Dissolve in 1% SDS by pipetting. Start by resuspending pellet in 5x volume of the pellet itself. Incubation of samples at 50°C may be required to efficiently extract soluble proteins from the pellet. The pellet is composed of soluble proteins and insoluble material such as tissue membranes, ECM, etc. Sediment insoluble material by centrifugation at 10000 x g for 10 min. at 4°C and transfer the supernatant to a clean tube. Use the protein solution immediately for Western blotting or store at -20°C.
- Remove the ethanol wash and air dry the protein pellet for 5-10 min. at room temperature. Dissolve the protein in 10 M urea/50 mM DTT. (Prepare fresh urea/DTT each time). Add around 50 µl of solution at a time and break up the pellet using a needle. The total volume of solution added should be varied to obtain the desired concentration of solubilized proteins. Store the sample for 1 hr at room temperature. Boil the sample for 3 min. and sonicate on ice using 10 short bursts. All proteins should be in solution at this time. If not, the boiling and sonication steps may be repeated up to two more times. After complete solubilization, use the protein solution for Western blotting or store at -20°C. This procedure was developed by Drs. Linda Varela and Margot Ip and reported in the following reference: (1996) *Endocrinology*, vol. 137, p. 4915.

- Dialyze the phenol ethanol supernatant (from the end of the DNA isolation protocol) against three changes of 0.1% SDS at 4°C. Centrifuge the dialysate at 10000 g for 10 min. at 4°C and use the clear supernatant for Western blotting. The phenol ethanol solution can dissolve some types of dialysis membranes (cellulose ester) so the dialysis tubing being used should be tested with the membrane to assess compatibility.

Note on using SDS for dissolving the pellet: 1% SDS is required to resolubilize the proteins from the pellet; a lower concentration will not be sufficient. After solubilization, the SDS may be diluted.

Additional Notes for Protein Isolation with TRIzol

Recovering phosphorylated proteins
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To recover Phosphorylated proteins, add a protease/phosphatase inhibitor to the 1% SDS before resuspending.

Membrane proteins
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Membrane bound proteins can also be extracted with TRIzol. Resolubilizing the protein is the difficult part depending on the protein's amino acid charge. A positively charged protein will go into solution easier than a lipophilic (hydrophobic) one. An SDS or urea solution might not allow solubilization of the protein of interest.

Analysis of proteins before precipitation
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It is recommended to take aliquots of the ethanol/TRIzol phase prior to protein precipitation and run directly on SDS PAGE to determine solubility/immuno reactivity of the proteins of interest. This eliminates the possibility of proteases being released when the pellet is homogenized. Electrophoresis will separate out the phenol and ethanol from the protein. It is recommended that the aliquot of the phenol/ethanol phase that is loaded must contain at least 10X the minimal amount of protein that is necessary for detection of immunoreactivity: a suggestion is 2.5, 5, 10 µl of supernatant diluted in sample buffer. See *Focus* 17:3, 98; Wu, Lai-Chu, Isolation and Long-Term Storage of Proteins From Tissues and Cells Using TRIZOL Reagent.

Interference of SDS with protein quantitation
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The SDS in the solubilization buffer can interfere with Bradford and Lowry Assays.Methods that do not have detergent-interface problems, and that do not rely on A260/A280 measurements may be used (traces of phenol may cause overestimation of protein concentrations).

Precipitation of SDS during dialysis
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If the SDS precipitates out during dialysis, remove the dialysis from the 4°C and place it at room temperature. This will allow the SDS to go back into solution.

TRIzol LS Reagent

Applications and Yields

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TRIzol LS is a ready-to-use reagent for the isolation of total RNA from liquid samples of human, animal plant, yeast, bacterial, and viral origin. The total RNA isolated using TRIzol LS can be used in RT-PCR, for Northern blot analysis, dot blot hybridization, poly(A)+ selection, *in vitro* translation, RNase protection assay, and molecular cloning. The reagent was also used for the simultaneous isolation of DNA and proteins from human leukocytes. The recovered DNA was used in PCR for the detection of three different genes.

TYPICAL RNA YIELDS: (The isolated RNA has an A260/280 ratio of 1.6-1.8)

Starting Material	**Amount of Sample**	**RNA Yield**
Human Blood*	250 ul	2.6-4.0 ug
	1 ml	15 – 20 ug
Human Leukocytes**	$7x10^7$ cells	60-70 ug
	$7x10^8$ cells	1109 ug
Rat Liver	1 g	5300 ug
New Tobacco Leaf		73 ug

Kidney	1 mg	3-4 ug
Skeletal muscles and brain	1 mg	1–1.5ug
Placenta	1 mg	1–4 ug
Epithelial cells	1 x 10^6 cells	8–15 ug
Fibroblasts	1 x 10^6 cells	5-7 ug

*Human Blood was from a single individual. RNA was isolated within 30 min. of being drawn from individual.
**Human Leukocytes were commercially prepared and pooled from 50 individuals.

TYPICAL DNA YIELDS:

Starting Material	**Amount of Sample**	**DNA Yield**
Human Leukocytes	7.0x10^8 cells	1.3 mg
Cultured human, rat, and mouse cells	1 x 10^6	5-7 ug
Fibroblasts	1 x 10^6	5-7 ug
Liver and Kidney	1 mg	3-4 ug
Skeletal muscles, brain, and placenta	1 mg	2-3 ug

*Human Leukocytes were commercially prepared and pooled from 50 individuals.

RNA Isolation with TRIzol LS
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(back to TRIzol LS Reagent)

Quick overview:

1. Add TRIzol LS to the sample and homogenize.
2. Add chloroform to the homogenate. Use 200ul of chloroform for every 0.75ml of TRIzol LS used. Invert and mix well. Centrifuge to get phase separation.
3. RNA is in the upper aqueous phase. Remove upper aqueous phase and precipitate to get the total RNA. Use 0.5 ml isopropyl alcohol for every 0.75ml of TRIzol LS used. Incubate and centrifuge.
4. Wash RNA pellet with 75% ethanol. Centrifuge.
5. Redissolve the RNA pellet.

Detailed Protocol Steps for RNA isolation with TRIzol LS

Homogenization
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Add 0.75 ml of TRIzol LS Reagent for each 0.25 ml of sample and homogenize. If using tissues or cells in suspension where the sample is <0.25 ml, adjust the volume to 0.25 ml with water (this is not necessary for cells grown in monolayer since the medium remaining will be sufficient). Use at least 0.75 ml of TRIzol LS Reagent per 5-10 x 10^6 cells. The volume ratio of TRIzol LS Reagent to sample should always be 3:1. (See product sheet for additional details).

- **Possible stopping point:** After homogenization and before addition of chloroform, samples can be stored at - 70ºC for at least one month. The phenol phase and interphase can be stored at 4ºC overnight.
- **Note for using whole blood with TRIzol LS:** Biological fluids such as whole blood which contain a high level of contaminating material may be diluted 1:1 with water. The innate buffering capacity of whole blood leads to the possibility of coprecipitating DNA with the RNA in blood samples. Blood collected with EDTA contains the highest level of DNA contamination, whereas blood collected with a trisodium citrate solution shows the least DNA contamination. Blood collected with heparin falls in between the two.
- **Isolation of RNA from small quantities of sample:** To facilitate isolation of RNA from small quantities of sample (<10^6 cells or < 10 mg tissue) perform homogenization (or lysis) of samples in 0.75 ml of TRIzol LS Reagent, and add 5-10 ug of glycogen (catalog # 10814010) as a carrier, and adjust the sample volume to 0.25 ml. Following homogenization, add chloroform and proceed with the phase separation, as described in step 2.
- **Samples with high content of polysaccharides, fat, and proteins:** An additional isolation step may be required for samples with high content of proteins, fat, polysaccharides, or extracellular material. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12000 X g for 10 min. at 4ºC. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernate 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 clean tube and proceed with chloroform addition and phase separation as described.
- **Samples with NP40 and Triton:** Samples may contain up to 1.0% NP40 or Triton X 100 without interfering with isolation.

Phase Separation
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Incubate the homogenized samples for 5 min. at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 0.75 ml of TRIzol LS Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at room temperature for 2 to 15 min. Centrifuge the samples at no more than 12000 X g for 15 min. at 4ºC.

- **Volume of the aqueous phase:** The volume of the aqueous phase is about 70% of the volume of the initial TRIzol LS Reagent used for homogenization.
- **Slower speed for centrifugation:** Table-top centrifuges that can attain a maximum of 2600 x g can be used if the centrifugation time is increased to 30-60 min.
- **Two phases are seen before adding chloroform:** The sample contained too much liquid. Add more TRIzol until only one phase appears.

RNA Precipitation
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Transfer the aqueous phase to a clean tube. Precipitate the RNA from the aqueous phase by adding 0.5 ml of isopropyl alcohol per 0.75 ml of TRIzol LS Reagent used for the initial homogenization. Incubate at room temperature for 10 min. and centrifuge at 12000 X g for 10 min. at 4ºC.

- **Addition of excess isopropyl alcohol:** Excess isopropanol will precipitate salt but it will also precipitate other contaminants that may have partitioned into the aqueous phase. Although the alcohol washes will remove salt contamination they may not remove other aqueous phase contaminants that are precipitated by the excess alcohol.

- **Slower speed for centrifugation:** Table-top centrifuges that can attain a maximum of 2600 x g can be used if the centrifugation time is increased to 30-60 min.

RNA Wash
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Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 0.75 ml of TRIzol LS Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7500 X g for 5 min. at 4ºC.

- **Possible stopping point:** The RNA precipitate can be stored in 75% ethanol at 4ºC for at least one week, or at least one year at -20ºC.

Redissolving the RNA Pellet
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(back to RNA Isolation with TRIzol LS)

Briefly dry the RNA pellet (air-dry preferred or vacuum-dry for 5min.). 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 min. at 55-60ºC.

- **Overdrying the RNA pellet:** Air drying the RNA pellet is preferred over drying by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility.

Additional Notes for RNA Isolation with TRIzol LS

RNAses
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In the presence of TRIzol LS Reagent, RNA is protected from RNase contamination.

Tubes to use with TRIzol LS
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See section about "Tubes to use with TRIzol".

Troubleshooting RNA Isolation with TRIzol LS

For additional information see Troubleshooting RNA Isolation with TRIzol Notes Section above.

Low Yield of RNA
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- Incomplete homogenization or lysis of samples.
- Final RNA pellet incompletely redissolved.
- OD reading may vary due to solution the sample is stored in AND what it was diluted in prior to quantitation. Wilfinger, et. al., in (1997) B*ioTechniques* 22.3, 478 report that increases in ionic strength may significantly reduce nucleic acid absorbance, thereby affecting quantitative determinations based on A_{260}.

- Incorrect storage of the samples. (For example, whole blood should NOT be stored. The blood should be collected and added to TRIzol immediately for RNA isolation.)

A260/280 ratio is <1.65
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- Sample homogenized in too small a volume of TRIzol.
- Samples not stored @ RT for 5 min. after homogenization. This may result in nuclear proteins not being dissociated.
- Final RNA pellet was not fully dissolved. This may be the case if the RNA pellet was overdried (if the pellet is clear and not white, this indicates overdrying). To get the pellet to dissolve completely, heat to 55-60ºC for 10-15 min. and repeatedly pipet.
- Phenol contamination (this may occur if samples were centrifuged at room temperature instead of 4ºC; phenol is more soluble in the aqueous phase at room temperature). If absorbance is seen at 270 nm (phenol), sample can be ethanol precipitated to remove residual phenol.
- Residual chloroform is present; reprecipitate.
- OD reading may vary due to solution the sample is stored in AND what it was diluted in prior to quantitation. Wilfinger, et. al., in (1997) B*ioTechniques* 22.3, 478 report that increases in ionic strength may significantly reduce nucleic acid absorbance, thereby affecting quantitative determinations based on A_{260}. (See additional notes in the Low Yield of RNA/Degraded RNA in TRIzol Notes section.)

RNA degradation
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- Tissues were not immediately processed or frozen after removing from the animal.
- Inefficient homogenization.
- 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.
- If TRIzol LS is not diluted by either the liquid sample or water to the appropriate concentration, contaminants may be precipitated that will affect the stability of the RNA.

DNA Contamination
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- 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.

DNA Isolation with TRIzol LS
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Detailed Protocol Steps for DNA Isolation TRIzol LS
    DNA Precipitation
    DNA Wash
    Redissolving the DNA
Additional Notes for DNA Isolation with TRIzol LS
    Alternative protocol for DNA isolation
Troubleshooting for DNA Isolation with TRIzol LS
    Low Yield of DNA

A260/280 Ratio is <1.70
DNA Degradation
RNA Contamination

Quick overview:

1. Add 0.3 ml of ethanol to the interphase and organic phase for every 0.75 ml of TRIzol LS used. Mix and spin.
2. Remove the supernatant and store for protein isolation. Wash the DNA pellet.
3. Dry the pellet and re-dissolve.

Detailed Protocol Steps for DNA Isolation TRIzol LS

DNA Precipitation
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(back to DNA Isolation with TRIzol LS)

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 0.75 ml of TRIzol LS Reagent used for the initial homogenization, and mix samples by inversion. Store the samples at room temperature for 2-3 min. and sediment DNA by centrifugation at no more than 2000 X g for 5 min. at 4ºC.

- **Increasing the centrifugation speed:** Precipitation step can be spun at 5000 X g for 5 min. at 4ºC to increase yield, but may make pellet more difficult to resuspend.
- **Addition of excess ethanol:** Adding more ethanol will precipitate proteins.

DNA Wash
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(back to DNA Isolation with TRIzol LS)

Remove the phenol-ethanol supernate. Wash the DNA pellet twice in 0.1 M sodium citrate in 10% ethanol. Use 1 ml of the solution per 0.75 ml of TRIzol LS used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 min. at room temperature (with periodic mixing) and centrifuge at 2000 X g for 5 min. at 4ºC. Resuspend the DNA pellet in 75% ethanol (1.5-2 ml of 75% ethanol per 0.75 ml TRIzol LS Reagent), store for 10-20 min. at room temperature and centrifuge at 2000 X g for 5 min. at 4ºC.

- **Wash for large pellets:** An additional wash in 0.1 M sodium citrate 10% ethanol solution is required for large pellets containing > 200 mg DNA .

Redissolving the DNA
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(back to DNA Isolation with TRIzol LS)

Briefly dry the DNA pellet for 5-10 min. under vacuum and dissolve in 8 mM NaOH by slowly passing the pellet through a pipet. Add an adequate amount of 8 mM NaOH to approach a DNA concentration of 0.2-0.3 ug/ml. Typically, add 0.3-0.6 ml of 8 M NaOH to the DNA isolated from 50-70 mg of tissue or 1 X 10^7 cells. Remove the insoluble material by centrifugation at 12000 x g for 10 min. Transfer the supernate containing DNA to a new tube.

- **Possible stopping point:** Samples dissolved in 8 mM NaOH can be stored overnight at 4ºC. For long-term storage, adjust the pH to 7-8, and adjust the EDTA concentration to 1 mM. Do not store at -20ºC.

- **Adjustment of pH:**
  After the DNA is solubilized in 8 mM NaOH, adjust the DNA solution to a desired pH by the addition of HEPES. Use the following amounts of 0.1 M or 1 M HEPES (free acid) per 1 ml of 8 mM NaOH.

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

Additional Notes for DNA Isolation with TRIzol LS

Alternative protocol for DNA isolation
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For an alternative protocol for DNA isolation, see the TRIzol Additional Notes for DNA Isolation section above under Alternative procedure for DNA isolation with TRIzol Reagent.

Troubleshooting DNA Isolation with TRIzol LS

For additional information see Troubleshooting DNA Isolation with TRIzol Notes Section Above.

Low Yield of DNA
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(back to Protocol and Application Notes)
(back to DNA Isolation with TRIzol LS)

- Incomplete homogenization or lysis of samples.
- Final DNA pellet incompletely redissolved.
- Increase precipitation centrifugation to 5000 x g. May increase yield but may also make pellet more difficult to solubilize. Confirm centrifugation at 2000-5000 x g, not rpm.
- OD reading may vary due to solution the sample is stored in and what it was diluted in prior to quantitation. Wilfinger, et. al., in (1997) B*ioTechniques* 22.3, 478 report that increases in ionic strength may significantly reduce nucleic acid absorbance, thereby affecting quantitative determinations based on A_{260}.

A260/280 ratio is <1.70
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(back to DNA Isolation with TRIzol LS)

- Phenol was not sufficiently removed from the DNA preparation. Wash the DNA pellet.
- In some samples dissolved in water, the ratio may be low due to the acidity of the water or the low ion content in the water. The ratios may go up if the sample is dissolved in TE and the spec is zeroed with TE (or 1-3 mM Na2PO4, pH ~8.0). [See Wilfinger(1997)BioTechniques 22.3, 474]. The molar extinction coefficient of the nucleotides is given at neutral pH (see Maniatis), suggesting that the absorbance at 260 nm would be highest at neutral pH.

DNA degradation
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- DNA is usually very stable and it is not markedly influenced by the time requirement following removal of the tissue sample from the animal. DNA degradation is more influenced by shearing during homogenization and vortexing the samples during isolation. Also, DNA can be sheared if the samples are exposed to multiple freeze-thaw cycles.

RNA contamination
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(back to DNA Isolation with TRIzol LS)

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

Protein Isolation with TRIzol LS
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(back to TRIzol LS Reagent)

Quick overview:

1. Add isopropanol to the phenol/ethanol supernatant to precipitate proteins. Incubate at room temperature and spin.
2. Wash pellet with guanidine/ethanol solution. Spin.
3. Solubilize the protein pellet.

Detailed steps for Protein Isolation with TRIzol LS

Protein Precipitation
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Precipitate proteins from the phenol-ethanol supernate (approximate volume 0.8 ml per 0.75 ml of TRIzol LS Reagent) with isopropyl alcohol. Add 1.5 ml of isopropyl alcohol per 0.75 ml of TRIzol LS Reagent used. Store samples for 10 min. at room temperature, and sediment the protein precipitate at 12000 X g for 10 min. at 4ºC.

Protein Wash
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(back to Protein Isolation with TRIzol LS)

Remove the supernate and wash the protein pellet three times in a solution containing 0.3M guanidine hydrochloride in 95% ethanol. Add 2 ml of wash solution per 0.75 ml of TRIzol LS Reagent used. During each wash cycle, store the protein pellet in the wash solution for 20 min. at room temperature and centrifuge at 7500 X g for 5 min. at 4ºC.

After the final wash, vortex the protein pellet in 2 ml of 100% ethanol. Store the protein pellet in ethanol for 20 min. at room temperature and centrifuge at 7500 X g for 5 min. at 4ºC.

- **Possible stopping point:** The protein pellet suspended in 0.3 M guanidine hydrochloride-95% ethanol or in ethanol can be stored for at least one month at 4ºC, or for at least one year at - 20ºC.

Redissolving the Protein Pellet
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Vacuum dry the protein pellet for 5-10 min. Dissolve in 1% SDS by pipetting (may need to heat at 50oC). Sediment insoluble material by centrifugation at 10000 x g for 10 min. at 4ºC. Transfer the supernatant to a clean tube.

- For other recommendations for solubilizing the protein pellet, please see Step 3 of Protein Isolation with TRIzol above.

Additional Notes for Protein Isolation with TRIzol LS

Interference of SDS with protein quantitation
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The SDS in the solubilization buffer can interfere with Bradford and Lowry Assay.Methods that do not have detergent-interface problems, and that do not rely on A260/A280 measurements may be used (traces of phenol may cause overestimation of protein concentrations).

PRODUCT DOCUMENTATION
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Brochures	**Citations**	**Cell lines**
COA	**FAQ**	**Licensing**
Manuals	**MSDS**	**Newsletters and Journals**
Vector Data		

REFERENCES
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TRIzol Reagent Key References:

Focus 15.4, 99; TRIzol: A New Reagent for Optimal Single Step Isolation of RNA.
Focus 17:3, 98; Isolation and Long-Term Storage of Proteins From Tissues and Cells Using TRIZOL Reagent.
Focus 20:2, 36 RNA Isolation with TRIzol Reagent (Helpful Tips and Stopping Points)
Focus 20:2, 37; Measuring Absorbance of RNA Samples
Focus 20:3, 80; Modified TRIzol Reagent Protocol for Large mRNA
Focus 20:3, 82; Isolation and Long Term Storage of RNA from Ribonuclease rich Pancreas Tissue.
Focus 21:2, 38; Isolation of Total RNA from Small Samples
Focus 21:3, 66; One-Step RT-PCR to Detect Cytokine/Chemokine Induction in Macrophages
Focus 21:1, 3; One-Step Analysis and Quantification of RNA by RT-PCR: Use of High Temp RT
Focus 21:1, 8; Improved SUPERSCRIPT™ One-Step RT-PCR Systems
Focus 22:1, 13 Answers to Frequently Asked Questions about RT-PCR

Chomczynski, et. al. (1987) Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Anal. Bioch.* 162, 156.

Subhasis Banerjee, Alan Smallwood, Anne E. Chambers and Kypros Nicolaides, 2003. Quantitative recovery of immunoreactive proteins from clinical samples following isolation of RNA and DNA. BioTechniques 35(3) 450-456).

TRIzol LS Reagent Key References:

Chomczynski, P., et al. (1994)A reagent for the single-step isolation of viral RNA from human serum and biopsy samples. J. NIH Res. 6,83.

PRODUCT NAME AND CATALOG NUMBERS
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Name	Size	Catalog Number
TRIzol Reagent	100 ml	15596026
	200 ml	15596018
TRIzol LS Reagent	100 ml	10296010
	200 ml	10296028

COMPONENTS
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Invitrogen supplies TRIzol Reagent and TRIzol LS Reagent in 100 ml and 200 ml quantities in brown, opaque bottles.

ASSOCIATED PRODUCTS
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TRIzol Max Bacterial RNA Isolation Kit Cat# 16096020
Max Bacterial Enhancement Reagent Cat# 16122012
