

RNA-STAT 60 REAGENT

For Isolation of Total RNA, DNA & Protein

I. ISOLATION OF RNA BY STAT 60.

1. INTRODUCTION

Recent progress in RNA isolation technology has made it possible to replace lengthy and laborious methods of total RNA isolation¹ by a single-step method^{2,3}. RNA STAT-60™ is a new and substantially improved version of the single-step method. It is a complete and ready to use reagent for isolation of total RNA from tissues and cells of human, animal, plant, yeast, bacterial, and viral origin. Extensive laboratory tests have shown that the RNA STAT-60™ is highly reliable and produces very consistent results.

The composition of RNA STAT-60™ (patent pending) includes phenol and guanidinium thiocyanate in a mono phase solution. A biological sample is homogenised in the RNA STAT-60™ using a glass-Teflon or Polytron homogenizer. Upon addition of chloroform, the homogenate separates into two phases: aqueous phase and organic phase. The total RNA remains exclusively in the aqueous phase while DNA and proteins are extracted into an organic phase and interphase. The total RNA is precipitated from the aqueous phase by addition of isopropanol, washed with ethanol and solubilized in water.

The entire procedure for RNA isolation using the RNA STAT-60™ can be completed in 1 hour. This is the most effective method of RNA isolation. The recovery of **undegraded mRNAs** using the RNA STAT-60™ is **30-150% greater** than with any other method of RNA isolation. RNA STAT-60™ offers:

- * **Total RNA/mRNA in under 60 minutes**
- * **Northern blot/PCR* - ready mRNA in under 60 minutes**
- * **No further purification required for use in subsequent procedures including Northern blotting and PCR***
- * **Extracts 30-150% more total RNA/mRNA than any other method**
- * **Cost effective method requiring less reagent/sample**

2. APPLICATION

The total RNA isolated by the RNA STAT-60™ is undegraded and free of protein and DNA contamination. It can be used for Northern analysis, dot blot hybridisation, poly A+ selection, in vitro translation, RNase protection assay, molecular cloning, and for polymerase chain reaction (PCR*) without additional treatment with DNase. The simplicity of the isolation using the RNA STAT-60™ makes it possible to process simultaneously a large number of samples, and the excellent recovery of RNA from very small biological samples (biopsies, etc.).

3. REAGENTS SUPPLIED

RNA STAT-60™: 100 ml or 200 ml bottle containing a red solution of RNA STAT-60™

PREPARATION Ready to use.

STORAGE Refrigerate at 2-8°C. Protect from exposure to light.

STABILITY 9 months. Refer to expiration date stamped on label.

4. REAGENTS REQUIRED, BUT NOT SUPPLIED

Chloroform (ACS grade) Isopropanol (ACS grade) Ethanol (ACS grade)

5. PROTOCOL

RNA/mRNA isolation by the RNA STAT-60™ method includes the following steps:

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1. **Homogenisation** RNA STAT-60™ (1 ml per 50-100 mg tissue, or 5-10 x 10⁶ cells)
2. **RNA Extraction** 1 vol. of homogenate +0.2 vol. of chloroform
3. **RNA Precipitation** 0.5 vol. of isopropanol
4. **RNA Wash** 75% ethanol

Unless stated otherwise the procedure is carried out at room temperature.

5.1 HOMOGENIZATION

A. TISSUES

Homogenise tissues samples in the RNA STAT-60™ (**1 ml/50-100mg tissue**) in a glass-Teflon or Polytron homogenizer. Sample volume should not exceed 10% of the volume of the RNA STAT-60™ used for homogenisation.

B. CELLS

Cells grown in mono layer are lysed directly in a culture dish by adding the RNA STAT-60™ (**1 ml/3.5cm petri dish**) and passing the cell lysate several times through a pipette. Cells grown in suspension are sedimented then lysed in the RNA STAT-60™ (**1 ml per 5-10 x 10⁶ cells**) by repetitive pipetting. **Washing calls before addition of the RNA STAT-60™ should be avoided as this increases the possibility of mRNA degradation.**

5.2 RNA EXTRACTION

Following homogenisation, store the homogenate for 5 min at room temp to permit the complete dissociation of nucleoprotein complexes. Next, add **0.2 ml of chloroform per 1 ml** of the RNA STAT-60™, cover the sample tightly, shake vigorously for 15 seconds and let it stay at **room temperature for 2-3 minutes**. Centrifuge the homogenate at **12,000g (max) for 15 minutes** at 4°C. Following centrifugation, the homogenate separates into two phases: **a lower red phenol chloroform phase** and the **colorless upper aqueous phase**. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interferes and organic phase. **The volume of the aqueous phase is about 60% of the volume of RNA STAT-60™ used for homogenization.**

5.3 RNA PRECIPITATION

Transfer the aqueous phase to a fresh tube and mix with isopropanol. Add 0.5 ml of isopropanol per **1 ml** of the RNA STAT-60™ used for homogenization. Store samples at **room temp for 5-10 minutes** and, centrifuge at **12,000g (max.) for 10 min at 4°C**. RNA precipitate (often visible before centrifugation) forms a white pellet at the bottom of the tube.

5.4 RNA WASH

Remove supernatant and wash the RNA pellet once with 75% ethanol by vortexing and subsequent centrifugation at **7,500g (max.) for 5 min** at 4°C. Add at least **1 ml of 75% ethanol per 1 ml of the RNA STAT-60™** used for the initial homogenization.

At the end of the procedure, dry the RNA pellet briefly by air-drying or in a **vacuum (5-10 min.)**. It is important not to let the RNA pellet dry completely as it will greatly decrease its solubility. Do not use the Speed-Vac for drying. Dissolve the RNA pellet in water or in 1 mM EDTA, pH 7, or 0.5% SDS solution. Vortex or pass the pellet a few times through a pipette tip. An incubation for **10-15 minutes at 55-60°C** may be required to dissolve RNA samples. Diethylpyrocarbonate (DEPC) treated RNase-free solutions¹ should be used for solubilization of RNA.

6. EXPECTED YIELD AND PURITY

Expected yield of total RNA:

- a.) Tissues (ug/mg tissue): liver, spleen, 7-10 ug; kidney, 3-4 ug; skeletal muscles, brain, 1-1.5 ug; placenta, 1-4 ug.
- b.) Cultured cells (ug/10⁶ cells): epithelial cells, 10-15 ug, fibroblasts, 5-7 ug. The final preparation of total RNA is free of DNA and proteins and has a 260/280 ratio > 1.8.

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7. NOTES AND COMMENTS

1. For isolation of RNA from a small amount of cells or tissue (1-10mg): homogenize samples in **0.8 ml** of the RNA STAT-60™, transfer the homogenate to the eppendorf tube and follow the isolation protocol with the exception of the RNA precipitation which should be carried out for **30 min at 4 °C**.
2. Following homogenization (before addition of chloroform) samples can be stored at **-70°C** for at least **2**

weeks.

3. An additional precipitation may be necessary to use RNA isolated by the RNA STAT-60™ in enzymatic assays. Following solubilization, precipitate RNA in the presence of **0.2 M** NaCl with **two volumes** of ethanol for **15 minutes at 4°C**. The PCR and RNase protection assays do not require this traditional precipitation step.
4. Hands and dust may be the major source of the RNase contamination. **Use gloves and keep tubes closed**. The use of sterile, disposable polypropylene tubes is recommended throughout the procedure.

8. SPECIAL HANDLING AND PRECAUTIONS

The RNA STAT-60™ contains poison (phenol) and irritant (guanidinium thiocyanate). CAN BE FATAL. When working with the RNA STAT-60™ **use gloves and eye protection** (shield, safety goggles). Do not get on skin or clothing. Avoid breathing vapor. Read also the warning note on the bottle. In case of contact immediately flush eyes or skin with a large amount of water for at least 15 minutes and seek immediate medical attention.

9. REFERENCES

1. Sambrook J., Fritsch E. F. and Maniatis T. (1989) *Molecular Cloning*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. Chomczynski P. and Sacchi N. (1987) *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction*. Anal. Biochem. 162, 156-189.
3. Kedzierski, W. and John Porter. 1991. *A Novel Non-enzymatic Procedure for Removing DNA Template from RNA Transcription Mixtures*. BioTechniques 10:210-214.

II. ISOLATION OF DNA BY STAT 60

The DNA is isolated from the interphase and phenol phase separated from the initial homogenate as described in the RNA isolation protocol. Following precipitation and a series of washes, the DNA is solubilized in 8mM NaOH neutralised and used for analysis. The DNA isolated by STAT 60 can be used for PCR, restriction and southern blotting. In addition the full recovery of DNA from tissue suspensions and cultured cells permits the use of STAT 60 for determination of the DNA content in analysed samples (P. Chomczynski, in preparation)

PROTOCOL

Reagents required, but not supplied: ethanol, sodium citrate and sodium hydroxide.

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- | | |
|------------------------------|---|
| 1. DNA PRECIPITATION | Phenol phase and interphase +0.3ml of Ethanol. (0.75ml of STAT 60) |
| 2. DNA WASH | 1 ml of 0.1M of 0.1M sodium citrate in 10% ethanol. 2ml, of 75% ethanol |
| 3. DNA SOLUBILIZATION | 8 mM NaOH |
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Note: Unless otherwise stated this procedure is carried out at room temperature.

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The protocol describes isolation of DNA from the phenol phase and interphase of samples homogenized (or lysed) in 1 ml of STAT 60

1. DNA PRECIPITATION

Remove the remaining aqueous phase overlying the interphase, and precipitate DNA from the interphase and organic phase with ethanol. Add 0.3 ml of 100% ethanol per 0.75ml of STAT 60 used for the initial homogenisation, and mix samples by inversion. Next, store the samples at room temperature for 2-3 minutes and sediment DNA by centrifugation at 2000g max for 5 minutes at 4°C.

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

2. DNA WASH

Remove the phenol- ethanol supernatant and save it at (4°C) for the protein isolation. Wash the DNA pellet twice in a solution containing 0.1M sodium citrate in 10% ethanol. Use 1 ml of the solution per 0.75 ml of STAT 60 used in the initial homogenisation. At each wash, store the DNA pellet in the washing solution for 30 minutes at room temperature (with periodic mixing) and centrifuge at 2,000g for 5 minutes at 4°C. Following these two washes, suspend the DNA pellet in 75% (1.5 – 2.0 ml of 75% ethanol per 1 ml of STAT 60), store for 10 - 20 minutes at room temperature (with periodic mixing) and centrifuge at 2,000g for 5 minutes at 4°C. *An additional wash in 100 mM sodium citrate – 10% ethanol is required for large pellets, containing >200ug DNA or large amounts of non-DNA material.*

3. DNA SOLUBILIZATION

Dry briefly the DNA pellet for 5 - 10 minutes and dissolve in 8 mM NaOH by slowly passing the pellet through the pipette. Add adequate amount of 8 mM NaOH to approach a DNA concentration of 0.2-0.3ug/ml. Typically, add 0.3 – 0.6 ml of 8 mM NaOH to the DNA isolated from 10^7 cells. The use of a mild alkaline solution assures full SOLUBILIZATION of the DNA pellet. At this stage, however the DNA preparations still contains insoluble gel-like material (fragments of membrane etc.). Remove insoluble material by centrifugation at 12,000g for 10 minutes and transfer the result supernatant containing DNA to a new tube. High viscosity of supernatant indicates the presence of high molecular weight of DNA.

4. QUANTITATION OF DNA.

Take an aliquot of the DNA preparation solubilised in 8mM NaOH, mix it with water and measure one A_{260} of the resulting solution

Calculate the DNA content using the A_{260} value for the double stranded DNA one A_{260} unit equals 50ug of double stranded DNA/ml

For calculation of a cell number in analysed samples assumes that the amount of DNA per 10^6 of diploid cells of human, at and mouse origin equals: 7.1ug, 6.5ug, and 5.8ug, respectively (1)

A typical preparation of DNA isolated from tissue suspension is composed of 60-100kb DNA (70%) and about 20kb DNA (30%). The DNA isolated from cells containing >80% of 60 - 100kb DNA and <10% of 20kb DNA.

The preparation of DNA is free of RNA and proteins and has a 260/280 ration >1.7.

5. AMPLIFICATION OF DNA BY PCR

Following SOLUBILIZATION in 8mM NaOH, adjust the pH of the DNA sample to 8.4 using HEPES. Add an aliquot of the sample (typically 0.1- 1.0 ug DNA) to PCR reaction mix, and perform PCR according to your standard protocol.

6. DIGESTION OF DNA BY RESTRICTASES

Adjust the pH of the DNA solution to a required value using HEPES. Alternatively, dialyse samples against 1mM EDTA, pH7 – pH8. Carry DNA restriction for 3 – 24 hours under optimal conditions for a specific

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restriction enzyme using 3-5 units of the enzyme per ug DNA. In a typical assay, 80- 90 % of the DNA is digested by restrictase.

(Reference 1: Ausubel F.M *et al.*, eds, 1990, *Current Protocols in Molecular Biology*, Greene Publishing Assoc. & Wiley-Interscience, New York, vol 2, p. A.1.5)

III: ISOLATION OF PROTEINS BY STAT 60

Proteins are isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol (**see step 1, in DNA precipitation**). The resulting precipitation can be analyzed for the presence of specified proteins by Western blotting.

PROTOCOL

Reagents required bur not supplied: guanidine hydrochloride, SDS, ethanol and isopropanol

1. PROTEIN PRECIPITATION	Phenol-ethanol supernatant +1.5ml isopropanol (0.75ml stat 60)
2. PROTEIN WASH	2 ml 0.3 M guanidine hydrochloride in 95%ethanol, 1 x 20min
3. PROTEIN SOLUBILIZATION	1% SDS

Unless otherwise stated the procedure is carried out at room temp

The protocol describes isolation of proteins from the phenol-ethanol supernatant obtained form a sample homogenized in 1 ml of STAT 60

1. PROTEIN PRECIPITATION

Precipitate proteins form the phenol-ethanol supernatant (approximate volume 0.8ml) with isopropanol. Add 1.5 ml of isopropanol per 0.75ml of Stat 60 used for the initial homogenization. Store samples for at least 10 minutes at room temperature and sediment the protein precipitate at12,000g for 10 minutes at 4°C

2. PROTEIN WASH

Remove supernatant and wash the protein pellet 3 times in a solution containing 0.3M guanidine hydrochloride in 95% ethanol. Add 2ml of the solution per 0.75 of STAT 60 used for the intial homogenization. At each wash, store samples in the washing solution for 20 minutes at room temperature and centrifuge at 7,500g for 5 minutes at 4°C . Next, vortex the protein pellet with 2 ml of ethanol (100%), store for 20 minutes at room temperature and centrifuge at 7,500g for 5 minutes at 4°C.

3. PROTEIN SOLUBILIZATION

Dry briefly the pellet for 5 – 10 minutes and dissolve it in 1 % SDS by pipetting. Incubation of the samples at 50°C may be required to complete solubilization of the protein pellet. Sediment any insoluble material by centrifugation at 10,000g for 10 minutes at 4°C and transfer the supernatant to a fresh tube. Use the protein solution immediately for Western blotting or store at -20°C

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PCR is subject of patents granted to Cetus Corporation.

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