BIO 101 Protocol

The RNaid® KIT

1007-000	**10 preps**
1007-200	**200 preps**

- **Isolate and Purify RNA in Twenty Minutes from Agarose, solutions, and from Unincorporated Radioactive Label.**

- **Isolates and Purify from Polyacrylamide gels in less than an hour**

- **RNA is Suitable as a Substrate for Multiple Enzymatic Manipulations Including Reverse Transcription, RNase Protection Assays, and *in vitro* Translation.**

Shipping & Storage:

The RNaid® Kit is shipped at ambient temperature. Recommended storage is room temperature.

"Check with BIO 101... We have you time in mind!"

BIO 101

Revision #: *1007-999-7H03P*

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RNaid® Kit Protocol

Kit Components

1007-000 (10 preps)

Name	Volume	Catalog #
DEPC Treated Water	1.5 ml	1007-001
RNA Binding Salt	6 ml	1007-002
RNAWash Concentrate	12 ml	1007-003
RNAMATRIX®	200µl	1007-004
10% Acetic Acid	0.5 ml	1007-005

1007-200 (200 preps)

Name	Volume	Catalog #
DEPC Treated Water	15 ml	1007-201
RNA Binding Salt	60 ml	1007-202
RNAWash Concentrate	120 ml	1007-203
RNAMATRIX®	1.5 ml	1007-204
10% Acetic Acid	0.5 ml	1007-205

All references to user supplied reagents are italicized and bolded in the protocol for your convenience.

The* RNaid® Kit *and all of its reagents are for research use only. All kit components have been lot qualified for the isolation of undegraded RNA. To avoid contamination of samples and reagents with RNase, follow appropriate laboratory procedures, wear surgical or similar gloves, use sterile vials, pipets, and pipet tips etc. To prepare glass, ceramic, or metal homogenizers, clean and bake at 200°C for 2 hours to remove any contaminating RNase.

Introduction

The **RNaid**® Kit contains all of the solutions and reagents, except for ethanol, necessary for the isolation and purification of RNA in twenty minutes from agarose and polyacrylamide gels, from solutions, and from unincorporated radioactive label (see Protocol). The resulting RNA is suitable as a substrate for multiple enzymatic manipulations including reverse transcription, RNase protection assays, and *in vitro* translation. For isolation of total RNA from tissues/cells use **RNaid**® Plus Kit (Cat # 1109-200) or Total RNA SAFEKIT™ (Cat # 1008-200). For difficult to lyse samples use the **FASTRNA® Kits** (call Bio 101 for information). The **RNaid**® Kit provides sufficient reagents for two hundred or more RNA purifications.

Protocol

A. Isolation of RNA from Solution, Agarose or Polyacrylamide gels
B. Isolation of RNA from Agarose/Formaldehyde Gels
C. Purification of RNA from Transcription Reactions
D. Trouble Shooting Guide

A. Isolation of RNA from Solution, from Agarose or Polyacrylamide Gels Containing 0-6 M Urea

Note: Reagents for agarose/urea gels are not included with the kit.

For an agarose gel in 0.5x TAE buffer containing 6M urea, prepare two solutions.

Solution A

Dissolve 8M urea in water by heating to 60°C.
Cool to room temperature and adjust pH to 3.8 with solid citric acid.
Use approximately 0.8g citric acid/100 ml 8M urea; use free acid, not sodium salt.

Solution B

Prepare a 4x agarose solution in 2x TAE buffer, pH 6.0.
Melt agarose completely by boiling.

Mix Solution A with 1/4 volume melted Solution B and cast gel. The final concentration is 6M urea and 0.5x TAE at the desired agarose concentration. The gel will solidify within 30 to 60 minutes at 4°C. An agarose concentration of less than 1% may take overnight at 4°C to solidify. The gel will remain clear upon solidification. Load sample and run at 4°C. Heat denature RNA sample before loading by incubation at 60°C for 10 minutes in the presence of 50% formamide, or at 80°C for 10 minutes without formamide.

Isolation of RNA from Solution

1. Add 3 volumes of **RNA Binding Salt** and mix well.
2. Continue with step 3., below.

Isolation of RNA from Agarose

1. Excise desired RNA band from ethidium bromide stained gel and determine approximate volume by its weight (1 mg = 1 µl volume). Place gel slice in microcentrifuge tube.

2. Add 3 volumes of **RNA Binding Salt** (i.e. to 0.1 g gel slice add 0.3 ml of RNA Binding Salt). Mix and incubate at room temperature for 10 minutes to dissolve agarose. Alternatively, place tube in 45-55°C water bath to dissolve agarose more rapidly. Continue with step 3., below.

Isolation of RNA from Polyacrylamide

1. Excise band from ethidium bromide stained gel and determine approximate volume by weight. Place into microcentrifuge vial. If gel concentration is 10% or higher, crush or cut into small pieces. Add 3 volumes of **RNA Binding Salt**. Soak for 20 minutes at 60°C. Remove liquid with small bore pipet tip avoiding gel pieces, and transfer to new vial.

2. Add **2 µl of 10% Acetic Acid** per every 0.5 ml of liquid to change pH to 5.0-5.5 (check with pH paper). This will increase recovery efficiency.

3. Estimate the amount of RNA expected and add **1 µl of RNAMATRIX®** for every µg of RNA. Add a minimum of **5 µl of RNAMATRIX®**. Mix well and allow binding of RNA to the matrix for at least five minutes at room temperature. Mix occasionally to keep **RNAMATRIX®** in suspension during adsorption.

4. Spin for 1 minute in microcentifuge at maximum speed to pellet RNA/**RNAMATRIX®** complex. Remove supernatant and save aside; if supernatant contains residual RNA, more **RNAMATRIX®** can be added for complete recovery. Spin pellet again briefly and remove residual liquid with small bore pipet tip.

5. Add **500 µl RNA**Wash solution (remember to add ethanol before first use) and resuspend pellet by mixing with pipet tip. Spin for 1 minute in microcentrifuge at maximum speed and remove supernatant.

6. Repeat washing step 5. 1 or 2 times. After last wash, spin tube again briefly and remove residual liquid with small bore pipet tip.

**** Add 120 ml (1007-203) or 12 ml (1007-003) of 100% Ethanol and mix well before use.***

7. Resuspend pellet in RNase-free water (use kit-supplied **DEPC Treated Water**). Use 10-20 µl per 5 µl **RNAMATRIX®**. Mix thoroughly with pipet tip and elute RNA by incubating at 45-55°C for 5 minutes.

8. Spin for 2 minutes in microcentrifuge at maximum speed. The RNA will be in the supernatant. Remove supernatant containing RNA to sterile vial.

Note: Before spectrophometric analysis of the sample, spin tube for 1 minute to pellet any remaining RNAMATRIX® material, and remove RNA sample to new vial. Alternatively, pass RNA sample through a sterile 0.2 µm membrane attached to a syringe or in a spin column.

9. ***Optional:*** Repeat elution steps 7. and 8. to recover an additional 5-15% RNA.

B. Isolation of RNA from Agarose Gels Containing Formaldehyde

Note: Reagents for agarose/formaldehyde gels are not included with the kit.

10x Gel Buffer:
200 mM MOPS, pH 7.0 (adjust with NaOH)
10 mM EDTA
10 mM NaOAc.

Prepare 1.2% agarose gel containing 6.6% formaldehyde and 1x gel buffer. Do not add ethidium bromide to the gel, only to the RNA sample as described below. Run gel at 3-5 V/cm in 1x gel buffer with 6.6% formaldehyde at pH 7.0.

Preparation of RNA sample for gel:
10 µl formamide (deionized)
4 µl formaldehyde (37%/12.3 M)
2 µl 10x gel buffer
3 µl RNA (up to 20 µg)
1 µl ethidium bromide (400 µg/ml)

Heat at 65°C for 10 minutes before loading in well of agarose gel. Formamide is dense enough to allow the sample to be loaded without adding an additional dense liquid, however, a loading dye mixture can be used if preferred.

Isolation of RNA from Agarose/Formaldehyde Gel

1. Excise RNA band(s) from gel after electrophoresis. Visualize RNA with long wave UV for minimal length of time while cutting gel. Determine approximate volume of gel slice(s) by weight and place slice(s) into microcentrifuge tubes.

2. Adjust the pH of the **RNA Binding Salt** to pH 5.0 by adding **2 µl of 10% Acetic Acid** (included with kit) per **1 ml of RNA Binding Salt** and add 3 volumes to the gel slice (i.e. to 0.1 g gel slice add **0.3 ml Binding Salt/Acetic Acid** mixture). The lower pH will optimize the binding efficiency of RNA to the **RNAMATRIX**〉. Incubate at 37°C for approximately 10 minutes with occasional mixing to melt agarose.

3. When gel is completely melted, place vial at room temperature and add **1-2 µl of RNAMATRIX®** per µg of RNA (add a minimum of **5 µl of RNAMATRIX®**). Mix well and allow RNA to adsorb to the matrix for 10 minutes at room temperature with periodic mixing.

4. Centrifuge for 1 minute in microcentrifuge at maximum speed to pellet the RNA/**RNAMATRIX®** complex. Remove supernatant to new tube and save for possible re-adsorption. Briefly spin again to collect remaining liquid in bottom of the tube. Remove all traces of liquid with a small bore pipet tip.

5. Resuspend pellet in same amount of **RNA Binding Salt** as in step 2 to wash pellet and help remove remaining traces of agarose and formaldehyde. Mix thoroughly with pipet tip. Spin for 1 minute and remove supernatant. Pulse spin and remove traces of liquid with small bore pipet tip.

6. Resuspend pellet in **500 µl RNA**Wash solution (remember to add ethanol before first use*) by mixing with pipet tip. Spin for 1 minute and remove supernatant.

   **** Add 120 ml (1007-203) or 12 ml (1007-003) of 100% Ethanol and mix well before use.***

7. Repeat washing step 6 one or two times. Re-spin and remove traces of liquid as described in step 5.

8. Resuspend pellet completely in **DEPC Treated Water** by mixing with pipet tip. Use 10-20 µl of water per 5 µl **RNAMATRIX®**. Elute RNA by incubating at 80°C for 10 minutes. Spin tube for 2 minutes and remove supernatant with RNA. If using the **SPIN™** option, transfer suspension to a **SPIN™ Filter** and spin for 1 minute in microcentrifuge. The supernatant containing RNA will be in the catch tube.

Optional: A second elution will yield 5-15% more RNA.

9. Heat eluted RNA to 80°C for 10 minutes to further dissociate residual formaldehyde from RNA. This second heating step will reverse chemical modification of the RNA caused by formaldehyde (Boedtker, H., 1967, Biochemistry 6, 2718-2727) and will render RNA biologically active as substrate for modifying enzymes. Let cool to room temperature to allow RNA to renature, or place on ice immediately to avoid renaturation. The RNA is now ready for use in enzymatic manipulations.

C. Purification of RNA from Transcription Reactions

Note: Reagents for transcription, template removal, and hydrolysis are not included with the kit.

Transcription Reaction
(commercially available as a kit from several manufacturers)

1. Combine: 5 µl 5x transcription buffer
(200 mM Tris-HCl, pH 7.5 at 37°C, 30 mM $MgCl_2$, 50 mM NaCl, 10 mM spermidine)
1 µl RNase Inhibitor (1 unit/µl)
1 µl 50 mM DTT
1 µl each rATP, rGTP, rUTP (10 mM each)
4 µl radioactively labeled rCTP (200 µCi)
8 µl distilled water
1 µl DNA template (1µg/µl)
1 µl RNA Polymerase (i.e. T3, T7, or Sp6 Polymerase)
25 µl total reaction volume

2. Mix and incubate at 37°C for 1 hour. When completed, remove 1 µl of the reaction and determine TCA precipitable counts to calculate % incorporation.

Removal of DNA Template and Hydrolysis of RNA

Note: Depending on the size of the transcribed RNA and the purpose of use, it may be necessary to shorten the transcripts by hydrolysis with sodium hydroxide. After hydrolysis, it is crucial to neutralize the pH before purification of the RNA with RNAMATRIX® . If the pH of the sample is alkaline the RNA will not adsorb to the matrix.

3. Add 1 µl of RNase-free DNase (10 units/µl). Incubate at 37°C for 10 minutes and place on ice. If probe is to be hydrolyzed, continue with step 4. Otherwise, continue with **Purification of RNA**, step 7., page 10.

4. Add 50 µl ETS buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS), 1.7 µl 5M NaCl, and 1 µl 1M DTT. Mix.

5. Add 10 µl 2N NaOH for hydrolysis of RNA transcripts. Incubate on ice for 30 minutes for smaller transcripts (< 1kb) or for 60 minutes for larger transcripts (> 1 kb). As an example, a 1 kb transcript can be shortened to 150-220 bases by a 30-40 minute incubation at 4°C.

6. Warm to room temperature, then add 20 µl (2x volume) of 1 M MES buffer to neutralize pH and stop hydrolysis. Add MES buffer after warming tube to room temperature to prevent precipitation. Volume is approximately 110 µl at this point.

Purification of RNA with RNAMATRIX®

7. Add 3 volumes of **RNA Binding Salt** and mix. Do not precipitate or gel purify RNA prior to adding **RNA Binding Salt** and **RNAMATRIX®**. Pre-purifying the "hot" RNA can lead to very tight binding to the **RNAMATRIX®** and will be difficult to elute.

8. Estimate the amount of transcripts and add **1-2 µl of RNAMATRIX®** per µg of RNA; add a minimum of **5 µl of RNAMATRIX®**. Mix well and incubate at room temperature for 5 minutes with occasional mixing to allow adsorption of RNA to the matrix.

9. Spin for 1 minute in microcentrifuge at maximum speed and remove and discard supernatant which contains most of the unincorporated label. Follow precautions and regulations for handling and disposing of radioactive materials as specified in Radioactive Materials License.

10. Wash pellet two times with **500 µl RNA Wash Solution** (remember to add the correct amount of ethanol before first use, see page 8 for instructions) and resuspend pellet completely by mixing with pipet tip. Spin for 1 minute in microcentrifuge at maximum speed and remove supernatant.

11. Remove residual traces of liquid and elute RNA with appropriate volume of **DEPC-Treated Water** by carefully resuspending pellet and incubating at 50°C for 3 minutes. Spin for 1 minute and remove supernatant containing labeled RNA to new tube. If using the **SPIN™** option, transfer suspension to a **SPIN™ Filter** and spin for 1 minute in microcentrifuge. The supernatant containing RNA will be in the catch tube.

Optional: A second elution will yield 5-15% more RNA.

Note: If the RNAMATRIX® retains radioactive label, elute a second time by resuspending pellet in RNase-free water and incubating at 80°C for 3 minutes. Spin and remove supernatant to new tube. If the second elution does not contain any radioactive label and the RNAMATRIX® still retains radioactivity, all RNA has been eluted. Radioactivity retained by the RNAMATRIX® represents reagent decay products which bind to the RNAMATRIX®. If available, use fresh radioactive reagents no older than two weeks from the date of manufacture since the amount of decay products increases with age of radioactive materials (especially ^{32}P-labelled reagents). It is important to purify radioactively labelled RNA using the* **RNaid® Kit** *with* **SPIN™** *immediately after transcription reaction is complete to minimize binding of RNA radiolysis products to the RNAMATRIX®.

D.Trouble Shooting Guide

1. **Gel Purification**
2. **Adsorption to RNAMATRIX®**
3. **Spectrophotometer Readings**

1. Gel Purification

Technical Background: The right choice of gel separation system is extremely important for best results. Criteria are native versus denaturing conditions and agarose versus polyacrylamide gel matrix. Generally, recovery of RNA from agarose gels is more efficient and convenient than from polyacrylamide. However, separation properties of polyacrylamide are usually superior. To improve recovery efficiency from polyacrylamide, extract RNA at elevated temperature (55-65°C) and extended time (20 min). Both formaldehyde/agarose gels as well as TBE/polyacrylamide gels create unfavorable binding conditions for RNA to **RNAMATRIX®** as the pH increases upon dissociation of the gel. Adjustment to more acidic conditions is necessary. In both cases, optimal binding efficiency can be restored by adjusting the pH to an appropriate range using 10% acetic acid (kit-supplied). When using formaldehyde, do not exceed a final concentration of 6.6% in the gel and buffer, and prepare all solutions fresh just before use. Avoid adding ethidium bromide to the formaldehyde gel since it will fluoresce and render the detection of RNA bands extremely difficult. Instead, add ethidium bromide to the RNA sample before loading as described in Section **C**. Always remember to process RNA as quickly as possible, never leave RNA inside gel for long periods of time (> 1hr), remove immediately and store under proper conditions to avoid degradation.

Important Note: Agarose or acrylamide used for separation of RNA should be of highest purity available. The quality of chemicals used for gel buffers is equally critical for good separation results. When preparing urea solutions, always filter-purify through 0.45 μm membrane to remove insoluble particles. Pre-treat hardware, such as glassware, gel devices, combs, spacers, etc., with DEPC to remove contaminating RNases. Use sterile, individually wrapped and unopened disposable supplies if possible. Prepare all buffers and gels with* DEPC-Treated Water *and autoclave buffers.

Problem: Agarose gel slice does not dissolve completely.

Solution: Follow instructions and incubate agarose at 45-55°C for 10 minutes. If gel has not dissociated, add more RNA Binding Salt and continue incubation at same temperature for 10 minutes.

Problem: Liquid cannot be completely removed from polyacrylamide gel elution, or transferred liquid contain small pieces of polyacrylamide.

Solution: Remove gel pieces by forcing liquid trough a 0.2 µm membrane attached to a syringe (Acrodisc PF with 0.2 µm Supor and 0.8 µm Pre-filter works well, supplied sterile by Gelman Sciences). Alternatively, use a large pipet tip with a sterile cotton plug. ***Do not use glass wool***.

Problem: The RNA solution eluted from the gel has been checked with pH paper and shows a pH>7.5.

Solution: Use the kit-supplied **10% Acetic Acid** to adjust pH. Add 2 µl **10% Acetic Acid** per every 0.5 ml of eluted liquid to change pH to 5.0-6.0 and check with pH paper. This procedure should be used for both TBE/polyacrylamide and formaldehyde/agarose eluates. Once pH has been adjusted to optimal range, continue with RNA purification and add **RNAMATRIX**〉 to the eluted RNA.

2. Adsorption to RNAMATRIX®

Technical Background: The conditions for binding of RNA to the **RNAMATRIX®** can be varied according to lysis and/or purification method (see Gel Purification section) and desired RNA population or size of RNA molecules. The binding efficiency is influenced by ionic strength and pH. Adsorption in the presence of guanidine thiocyanate only (lysis solution) selects for larger RNA's. Addition of **RNA Binding Salt** during adsorption causes binding of smaller molecules. Optimal pH range for adsorption of RNA to **RNAMATRIX®** is Ph 5.5-7.5; higher pH conditions result in a loss of binding capacity. In general, lower pH (5.5-6.0) tends to increase binding of smaller RNA molecules, slightly higher pH (6.5-7-5) favors larger sizes.

Problem: After addition of RNAMATRIX® and mixing, the matrix forms aggregates and does not disperse evenly throughout the sample.

Solution: Aggregation of the **RNAMATRIX®** tends to occur at high concentrations of nucleic acid. Try breaking up aggregates with pipet tip or by rocking tube back and forth quickly. Small aggregates will not effect the procedure.

Problem: During RNA wash steps of the pellet, the RNAMATRIX® does not disperse evenly.

Solution: The pellet is as easily dispersed in the **RNA Wash** solution (containing ethanol) as in water. When adding the **RNA Wash** solution to the pellet, use same pipet tip and break-up pellet by stirring. Be careful to expel any trapped matrix from the tip back into the sample. Small aggregates will not affect the procedure.

Problem: During adsorption to RNAMATRIX®, not all RNA is bound and removed from solution.

Solution: The amount of **RNAMATRIX®** was not sufficient to adsorb all of the RNA. Transfer supernatant with unbound RNA to new vial, add 5-10 µl of new **RNAMATRIX®**. You may also re-use the matrix previously applied to this sample. Save supernatant until after the initially bound RNA is eluted from the matrix, then add supernatant back to the same matrix. Incubate, and follow wash procedures as described. Elute RNA with 5-10 µl **DEPC Treated Water** and combine with RNA eluted from the first adsorption process.

3 Spectrophotometer Readings

Technical Background: The amount of nucleic acid in the eluate from **RNAMATRIX®** can be determined by optical density at 260 nm. The **RNAMATRIX®** material absorbs over a wide range of the spectrum, including UV. This absorption is consistent and very low over a range from 210 nm to approximately 310 nm, absorbing 0.01 OD units. Residual matrix material can easily be removed by centrifugation and this will not contribute to any absorption readings.

Problem: OD_{260} measurement of the RNA Wash Solution supernatant shows UV absorption.

Solution: Spin vial with pellet of RNA/**RNAMATRIX**〉 complex for 10 seconds and remove traces of liquid with a small bore pipet tip. Resuspend pellet in 500 µl **RNA Wash** Solution and mix with pipet tip to resuspend the matrix evenly. Spin for 1 minute in microcentrifuge at maximum speed. Remove supernatant and measure OD_{260}. If absorption is still above background, repeat washing procedure. If no absorption is detectable, proceed with elution of the RNA as described.

Problem: Eluted RNA is contaminated with residual RNAMATRIX®.

Solution: Small amounts of **RNAMATRIX®** in the RNA sample are inert and will not effect enzymatic reactions. However, **RNAMATRIX®** absorbs UV light and might effect $OD_{260/280}$ measurements. To remove residual **RNAMATRIX®** from the eluted RNA, re-spin vial in microcentrifuge for 30 seconds at maximum speed and remove RNA solution with small bore pipet tip. Be careful not to touch bottom of tube near pellet area. For consistent and reliable measurement of multiple samples, complete removal of residual **RNAMATRIX®** particles can be accomplished by centrifugal filtration through a **SPIN™ Filter** unit, or by passing RNA sample through a sterile 0.2 µm membrane attached to a syringe.

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General Information

BIO 101 is a pioneer in developing kits for molecular biology research. We introduced the **GENECLEAN®** Kits in 1986 and have since been manufacturing products to bring convenience into your research. Our goal is to make your life easier by simplifying the complexities of lab work.

Technical Support and Ordering Information

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