TECHNICAL MANUAL

PolyATtract® System 1000

Instructions for Use of Products
Z5400 and Z5420

Revised 8/16
TM228

PolyATtract® System 1000

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The PolyATtract® System 1000 isolates messenger RNA directly from crude cell or tissue lysates, eliminating the need for total RNA isolation. This system uses the Promega MagneSphere® technology to purify poly(A)+ RNA, eliminating the need for oligo(dT) cellulose columns.

The PolyATtract® System 1000 forgoes lengthy ethanol precipitation steps, phenol:chloroform extractions, overnight ultracentrifugation through cesium chloride gradients and lithium chloride (LiCl) precipitations (Figure 1). Increased mRNA yields are obtained because losses that occur during the organic extractions and precipitations are eliminated. The isolated mRNA is suitable for all molecular biology applications, including in vitro translation, cDNA synthesis, PCR analysis, ribonuclease (RNase) protection assays, primer extension and Northern blots. The increased yield of mRNA using this system allows detection of low copy number RNAs in relatively small amounts of material using Northern blot analysis.

The benefits obtained by eliminating the intermediate purification of total RNA include:

- **Time Savings:** Isolate mRNA in 45 minutes versus 4.5 hours with previous systems.
- **Increased Yields of Poly(A)+ RNA:** Up to twofold greater yields than with other methods.

The PolyATtract® System 1000 protocol can be adjusted to the amount of starting material, thus maximizing flexibility in the number of purifications that can be performed. Sufficient reagents are supplied to isolate mRNA from up to 2 grams of starting material in any combination of reaction sizes, with up to 1 gram per isolation. As little as 5mg of tissue or 1×10^6 cultured cells can be processed. All system components are guaranteed to be free of contaminating ribonucleases when used as directed and are thoroughly tested to assure optimal performance.

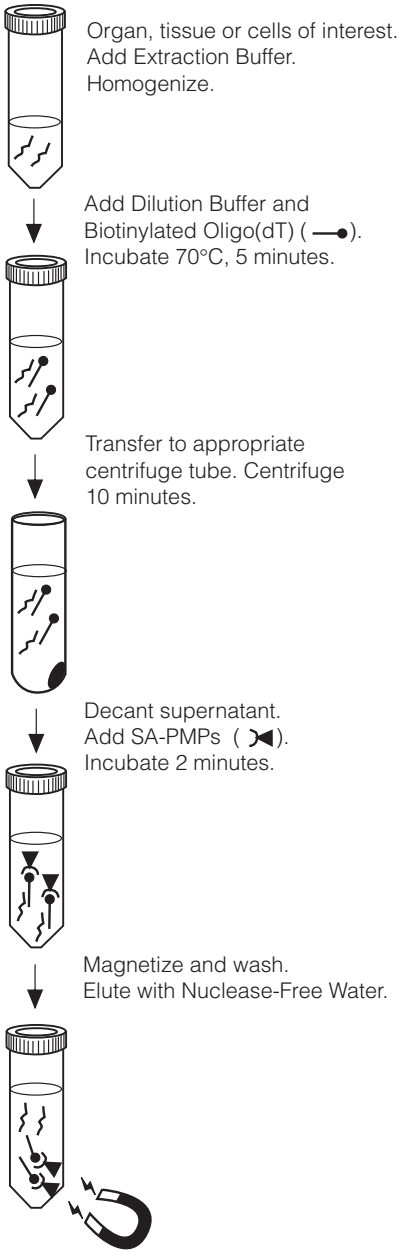

Figure 1. Schematic diagram of the PolyATtract® System 1000 Protocol.

2. Product Components and Storage Conditions

PRODUCT	CAT.#
PolyATtract® System 1000 with Magnetic Separation Stand	**Z5420**

Each system contains a Magnetic Separation Stand and sufficient reagents for the isolation of RNA from up to 2 grams of tissue or 4×10^8 cultured cells. Includes:

- 25ml Streptavidin MagneSphere® Paramagnetic Particles
- 20ml GTC Extraction Buffer
- 50µl Biotinylated Oligo(dT) Probe (50pmol/µl)
- 40ml Dilution Buffer
- 900µl β-Mercaptoethanol (97.4%)
- 25ml Nuclease-Free Water
- 375ml SSC 0.5X Solution (3 × 125ml)
- 1 PolyATtract® System 1000 Magnetic Separation Stand
- 1 PolyATtract® System 1000 Magnetic Stand Adapter (for 15ml tubes)
- 40 mRNA User Tubes

Note: The mRNA User Tubes are RNase-free.

PRODUCT	CAT.#
PolyATtract® System 1000 without Magnetic Separation Stand	**Z5400**

Each system contains sufficient reagents for the isolation of RNA from up to 2 grams of tissue or 4×10^8 cultured cells.

PRODUCT	CAT.#
PolyATtract® System 1000 Magnetic Separation Stand	**Z5410**

The magnetic stand has two positions, one for tubes of up to 2ml in size and the other one for 50ml tubes. A 15ml tube can be accommodated by using the supplied adapter in the position for the 50ml tube.

Storage Conditions: Store at 4°C. Do not freeze the Streptavidin MagneSphere® Particles; this will result in failure of the system.

3. General Considerations

3.A. Direct Purification of mRNA from Tissue

Successful isolation of intact RNA by any procedure requires that four important steps be performed: 1) effective disruption of cells or tissue; 2) denaturation of nucleoprotein complexes; 3) inactivation of endogenous RNase activity; and 4) purification of RNA from contaminating DNA and protein. The most important of these is the immediate inactivation of endogenous RNase activity, which is released from membrane-bound organelles upon cell disruption.

The PolyATtract® System 1000 combines the disruptive and protective properties of guanidine thiocyanate and β-mercaptoethanol to inactivate ribonucleases present in cell extracts (1). Guanidine thiocyanate (GTC), in association with SDS, acts to disrupt nucleoprotein complexes, allowing RNA to be released into solution and isolated free of protein. The final GTC concentration allows hybridization of the poly(A) sequence of most mature eukaryotic mRNA species to the synthetic Biotinylated Oligo(dT) Probe, yet maintains complete inhibition of cellular RNases. Hybridization occurs during the brief centrifugation step that removes cellular debris and precipitated proteins. After centrifugation, the Biotinylated Oligo(dT):mRNA hybrids are captured with Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs). The particles are washed at high stringency, and purified mRNA is eluted by the simple addition of Nuclease-Free Water (Cat.# P1193). This procedure yields an essentially pure fraction of mature mRNA after only a single round of magnetic separation, without organic extractions or precipitations. A summary of the procedure is provided in Figure 1.

The PolyATtract® System 1000 protocol was developed and optimized for mRNA isolation from tissues with a broad spectrum of mRNA expression levels. Because rigorous performance specifications require quantitative capture from tissues high in mRNA, the protocol is designed with an excess mRNA-binding capacity to assure recovery of all available mRNA. To maintain sufficient molar concentrations of the Biotinylated Oligo(dT) Probe and mRNA for hybridization, it is necessary to keep the volumes of extraction and dilution buffers to a minimum. Depending on the amount of starting material, one of two protocols should be followed. For sample sizes less than 125mg of tissue, perform the small-scale protocol. For sample sizes between 125mg and 1,000mg, perform the large-scale protocol. Do not use more than 1g of tissue because the increased lysate viscosity will lead to irreversible clumping of the particles during magnetic capture, resulting in lowered RNA yields. In addition, mRNA isolations can be performed with 10^6–10^8 cultured cells.

If the desired yield of mRNA is known in advance and starting material is not limiting, the PolyATtract® System 1000 can be tailored to give the desired yield. In this case, consult the top of the Probe and Particle Usage Charts (see Figure 3, Section 4.A, and Figure 4, Section 4.B), and purify mRNA from the corresponding amount of starting material indicated on the chart. Note that these charts were generated using mouse liver tissue (ICR strain), which has relatively high levels of mRNA per gram of tissue; the corresponding RNA yields are higher than those expected from other sources. Additional tissue can be processed (by performing multiple isolations with a maximum of 1g of tissue each) as necessary to supply the desired amount of mRNA.

3.B. Magnetic Particle Separation of Macromolecules

Paramagnetic particles incorporate iron oxide into submicron-sized particles that have no magnetic field but form a magnetic dipole when exposed to a magnetic field. The use of paramagnetic particles eliminates the need for traditional column chromatography, centrifugation or any other special equipment. These particles have been successfully used in the development of immunoassays (2) and probe diagnostic assays (3), and to measure RNA in cell lysates using oligo(dT)-tailed capture probes (4).

Unlike procedures that use direct coupling of probes to paramagnetic particles (3,4), the PolyATtract® System 1000 uses a biotinylated oligonucleotide probe to hybridize in solution to the targeted nucleic acid. The hybrids then are captured using coupled Streptavidin MagneSphere® Paramagnetic Particles. This approach combines the speed and efficiency of solution hybridization with the convenience and speed (<1 minute) of magnetic separation.

Promega uses its own highly purified streptavidin to produce the particles. These SA-PMPs exhibit a high binding capacity for biotinylated oligonucleotides and low nonspecific binding of nucleic acids. The binding capacity of the particles varies with the specific oligonucleotide probe used. For Biotinylated Oligo(dT) Probe, the calculated binding capacity is roughly 1nmol of free probe captured per milligram of SA-PMPs.

3.C. Downstream Applications

Messenger RNA purified with the PolyATtract® Systems is suitable for many molecular biology applications, including RT-PCR and PCR analysis, in vitro translation, cDNA synthesis, RNase protection assays, primer extension and Northern blot hybridizations. One such application is demonstrated in Figure 2. In this example, mRNA was isolated from murine tissues using the PolyATtract® System 1000. To evaluate the ability of the PolyATtract® System 1000 to prepare mRNA from small amounts of tissue homogenates, RT-PCR was used to examine expression of IL-1β and GAPDH message isolated from serial dilutions of mouse tissue. Figure 2 demonstrates the expression of both genes; amplified DNA for IL-1β and GAPDH was easily detected when mRNA was purified from as little as 5mg of mouse liver.

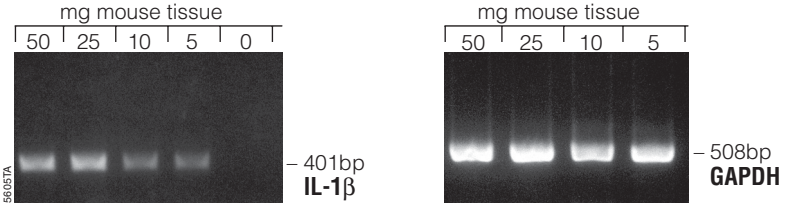

Figure 2. Detection of IL-1 and GAPDH transcripts in serial dilutions of mouse liver homogenates. Liver tissue from untreated BALB/c mice was homogenized in GTC Extraction Buffer, the homogenates serially diluted and mRNA isolated from the indicated amounts of tissue, using the PolyATtract® System 1000. cDNA was prepared from 20µl of each mRNA sample using 10–12 units of AMV Reverse Transcriptase (Cat.# M9004), Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511) and Oligo(dT)$_{15}$ Primer (Cat.# C1101). PCR was used to amplify a 401bp fragment from IL-1β and a 508bp fragment from GAPDH. *Taq* DNA polymerase was used for amplification. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. The primers for IL-1β span intron 6 and result in a 1,123bp product when genomic DNA is present in the sample. No 1,123bp bands were seen in cDNA amplified with IL-1β primers.
(Primer sequences and amplification protocols were provided by Dr. A.L. Oaklander, Johns Hopkins University.)

3.D. Creating a Ribonuclease-Free Environment

Ribonuclease is difficult to inactivate. Extreme care should be taken to avoid inadvertently introducing RNase activity into your RNA during or after the isolation procedure. This is especially important if the starting material was difficult to obtain or is irreplaceable. The following notes may help you to prevent accidental contamination of your sample.

1. Two of the most common sources of RNase contamination are the user's hands and bacteria or molds that may be present on airborne dust particles. To prevent contamination from these sources, sterile technique should be observed when handling reagents supplied with the kit. Gloves should be worn at all times.
2. Whenever possible, sterile disposable plasticware should be used to handle RNA. These materials are generally RNase-free and thus do not require pretreatment to inactivate RNase.
3. Nondisposable glassware and plasticware should be treated before use to ensure that it is RNase-free. Glassware should be baked at 200°C overnight, and plasticware should be thoroughly rinsed before use with 0.1N NaOH and 1mM EDTA followed by RNase-free water.

   Note: COREX® tubes (and similar centrifuge tubes) should be rendered RNase-free by treatment with diethyl pyrocarbonate (DEPC) and not by baking. Baking will reduce the failure rate of this type of tube during centrifugation. Alternatively, commercially available products such as RNase AWAY® may be used to remove contaminating RNases.
4. Solutions supplied by the user should be treated with 0.1% DEPC overnight at room temperature, then autoclaved for 30 minutes to remove any trace of DEPC.

   Note: Tris buffers cannot be treated with DEPC.
5. We have found that many good sources of distilled water are free of contaminating RNase activity. You may want to test your water source for the presence of RNase activity.

4. mRNA Isolation and Purification Protocols

Section 4 provides a step-by-step guide to purify mRNA from variously sized tissue and cell samples. Use **Table 1** to select the appropriate MagneSphere® Magnetic Separation Stand and tube sizes. Use **Table 2** (Section 4.A) to determine the quantity of reagents to use for small-scale tissue samples (5–100mg). Use **Table 3** (Section 4.B) to determine the quantity of reagents to use for large-scale tissue samples (125–1,000mg). See Section 4.C to isolate mRNA from small- and large-scale tissue samples, Section 4.D to isolate mRNA from 10^6–10^8 cultured cells, Section 4.E to precipitate and concentrate mRNA, and Section 4.F to determine mRNA concentration.

Notes:

1. Samples to be extracted should be as fresh as possible for optimal performance of this system. Alternatively, freeze samples in liquid nitrogen immediately after collection, and store at –70°C for future use. For valuable samples, we recommend saving a portion of the sample at –70°C in the event that sample loss occurs during processing.
2. Do not exceed the 1,000mg tissue capacity because the increased lysate viscosity leads to irreversible clumping of the SA-PMPs during magnetic capture and, thus, decreased system performance.
3. The SA-PMPs cannot be reused because Biotinylated Oligo(dT) remains attached, decreasing the capacity of the particles. In addition, trace amounts of nucleic acid contaminants may be present.
4. Centrifuges and rotors should be at room temperature.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.A.)

- Tissuemizer or equivalent homogenizer
- 50ml sterile screw-cap conical tubes
- 15ml sterile COREX® or other glass centrifuge tubes
- 70°C water bath
- Beckman Model J2-21 centrifuge or equivalent
- 1X PBS (for isolation from cell cultures)
- scale or balance (to weigh tissue samples)

Table 1. MagneSphere® Magnetic Separation Stands Compatible with the PolyATtract® System 1000.

Sample Size	Tube Size	MagneSphere® Magnetic Separation Stand (2-position) Cat.#	MagneSphere® Magnetic Separation Stand (12-position) Cat.#
5–10mg	0.5ml	Z5331	Z5341
5–35mg	1.5ml	Z5332	Z5342
35–100mg	12 × 75mm	Z5333	Z5343
100–1,000mg	50ml or 15ml[1]	Z5410	NA
1×10^6 cells	1.5ml	Z5332	Z5342
1×10^7–1×10^8 cells	50ml or 15ml[1]	Z5410	NA

NA: not applicable

[1]Use sterile, polypropylene tubes. We recommend 15ml or 50ml centrifuge tubes with caps (e.g., Corning® tubes) for sample sizes >100mg or 10^7–10_8 cells. The Adapter is provided for use with 15ml tubes. The Adapter snaps into the large hole of the Magnetic Separation Stand with the smaller hole of the Adapter closest to the magnet.

4.A. Table and Figure for Small-Scale mRNA Purification (5–100mg of Tissue)

Use Table 2 to determine the amount of PolyATtract® System 1000 reagents to use for small-scale isolation of mRNA, starting with 5–100mg of tissue. Alternatively, determine the amount of mRNA you wish to isolate, and use Figure 3 to determine the recommended amount of starting tissue.

Table 2. Volumes of PolyATtract® System 1000 Reagents Required for Various Starting Amounts of Tissue (Small-Scale).

	Tissue Amount (mg)[1]				
Component	**5**	**10**	**25**	**50**	**100**
GTC Extraction Buffer	40µl	80µl	200µl	400µl	800µl
Dilution Buffer	80µl	160µl	400µl	800µl	1.6ml
Oligo(dT) Probe[2]	5pmol	10pmol	25pmol	50pmol	100pmol
SA-PMPs	60µl	120µl	300µl	600µl	1.2ml
SSC 0.5X Solution[3]	500µl	500µl	1ml	1ml	1ml
Nuclease-Free Water	40µl	80µl	200µl	400µl	800µl

[1]For sample sizes that are not listed, use the reagent volumes for the next larger tissue size listed in this table. For example, for a 17–21mg tissue sample, use the reagent volumes listed for a 25mg tissue sample.

[2]When using <50pmol Biotinylated Oligo(dT) Probe per sample, dilute the probe 1:10 in Nuclease-Free Water.

[3]Volume shown is per wash (not the total wash volume) for the mRNA/SA-PMP complexes.

4.A. Table and Figure for Small-Scale mRNA Purification (5–100mg of Tissue; continued)

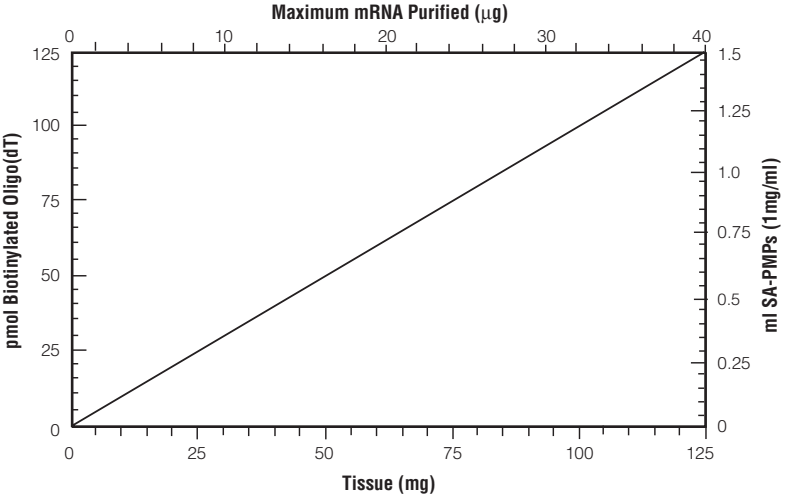

Figure 3. Probe/SA-PMP usage chart for small-scale protocol. Use the tissue weight determined in Section 4.C, Step 4, and locate this weight on the bottom axis of the chart. Read up to the diagonal line from this point. To determine the amount of Biotinylated Oligo(dT) Probe to add, read across to the left axis; to determine the amount of SA-PMPs to use, read across to the right axis. Alternatively, if a known amount of mRNA is desired, locate this number on the top axis. Read down from this point to the diagonal line. To determine the minimum amount of starting tissue required, read down to the bottom axis. To determine the amount of Biotinylated Oligo(dT) Probe and SA-PMPs to obtain the desired amount of mRNA, read to the left and right axes, respectively. **Note:** The yields of mRNA are based on isolation from mouse liver. Yields from other tissues may be significantly lower.

4.B. Table and Figure for Large-Scale mRNA Purification (125–1,000mg of Tissue)

Table 3 provides information on the amount of PolyATtract® System 1000 reagents to use to isolate mRNA when starting with 125–1,000mg of tissue. Alternatively, Figure 4 allows you to calculate the amount of starting tissue to isolate a particular quantity of mRNA.

Table 3. Volumes of PolyATtract® System 1000 Reagents Required for Various Starting Amounts of Tissue (Large-Scale).

	Tissue Amount (mg)[1]				
Component	**125**	**250**	**500**	**750**	**1,000**
GTC Extraction Buffer	1ml	1ml	2ml	3ml	4ml
Dilution Buffer	2ml	2ml	4ml	6ml	8ml
Oligo(dT) Probe	125pmol	250pmol	500pmol	750pmol	1,000pmol
SA-PMPs	1.5ml	3ml	6ml	9ml	12ml
SSC 0.5X Solution[2]	2ml	2ml	2ml	2ml	2ml
Nuclease-Free Water	1ml	2ml	3ml	5ml	6ml

[1]For sample sizes that are not listed, use the reagent volumes for the next larger tissue size listed in this table. For example, for a 525–575mg tissue sample, use the reagent volumes listed for a 750mg tissue sample.

[2]Volume shown is per wash (not the total wash volume) for the mRNA/SA-PMP complexes.

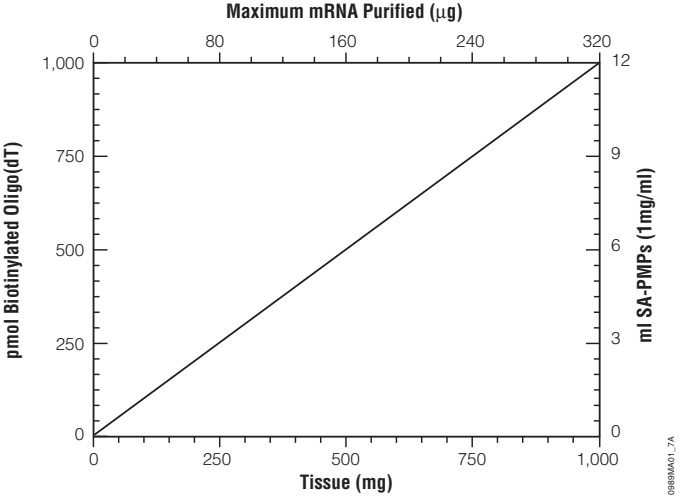

Figure 4. Probe/SA-PMP usage chart for large-scale protocol. Use the tissue weight determined in Section 4.C, Step 4, and locate this weight on the bottom axis of the chart. Read up to the diagonal line from this point. To determine the amount of Biotinylated Oligo(dT) Probe, read across to the left axis; to determine the amount of SA-PMPs, read across to the right axis. Alternatively, if a known amount of mRNA is desired, locate this number on the top axis. Read down from this point to the diagonal line. To determine the minimum amount of starting tissue required, read down to the bottom axis. To determine the amount of Biotinylated Oligo(dT) Probe and SA-PMPs to obtain the desired amount of mRNA, read to the left and right axes, respectively.

4.C. Protocol for mRNA Isolation from Tissue Samples

When isolating mRNA from tissue samples, refer to Table 1 for assistance in choosing the proper Magnetic Separation Stand and tube sizes. Use the tables and figures in Section 4.A, small-scale isolation, or Section 4.B, large-scale isolation, to determine the amount of reagents to use.

Sample Preparation

1. Remove the GTC Extraction Buffer, Biotinylated Oligo(dT) Probe, Nuclease-Free Water and SSC 0.5X Solution from the refrigerator, and warm to room temperature. Preheat the Dilution Buffer to 70°C. (Use caution when heating the Dilution Buffer; do not heat above 70°C and do not heat longer than necessary to reach 70°C. Excessive heating will cause the bottle to deform.)
2. In an appropriately sized tube (see Table 1), add 20.5µl of β-Mercaptoethanol (97.4%) per milliliter of Extraction Buffer (to make Extraction/BME Buffer). The final concentration of β-Mercaptoethanol is 2%. Use RNase-free pipettes, and wear gloves to reduce the chance of RNase contamination.

   Weigh the tube containing the buffer, and record the weight.

   ! β-Mercaptoethanol is toxic. Dispense in a fume hood, and wear appropriate personal protective equipment.
3. Working as quickly as possible, excise the tissue of interest, and place into the tube containing the Extraction/BME Buffer. Homogenize the tissue at high speed using a small homogenizer (such as a Tekmar Tissuemizer homogenizer) until no visible tissue fragments remain.

   If a small homogenizer is unavailable, quickly cut the tissue into small pieces with a sterile razor blade, freeze in liquid nitrogen and grind in a mortar and pestle under liquid nitrogen. Transfer the liquid nitrogen and ground tissue to a sterile 50ml screw-cap conical tube, allow the liquid nitrogen to evaporate, then immediately add the Extraction/BME Buffer. Mix thoroughly by inversion.
4. Weigh the tube containing the tissue in Extraction/BME Buffer. Calculate the tissue mass by subtracting the weight obtained in Step 2 from this new weight.

Probe Annealing

5. To determine the amount of Biotinylated Oligo(dT) Probe and SA-PMPs necessary for the tissue mass calculated in Step 4, refer to Section 4.A or 4.B. When adding <50pmol Biotinylated Oligo(dT) Probe per sample, dilute the Probe 1:10 in Nuclease-Free Water. The diluted probe is stable when stored at 4°C.
6. Dispense the preheated Dilution Buffer to a sterile tube, and add 10.25µl of β-Mercaptoethanol (97.4%) per milliliter of Dilution Buffer. The final concentration of β-Mercaptoethanol is 1%. Add this to the homogenate, and mix thoroughly by inversion. Add the amount of Biotinylated Oligo(dT) Probe determined in Step 5, and mix well by shaking. Incubate this mixture at 70°C for 5 minutes.

7. Transfer the lysate to a clean, sterile 15ml COREX® or other appropriately sized tube. Centrifuge at 12,000 × *g* for 10 minutes at room temperature to clear the homogenate of cell debris and precipitated proteins. During the centrifugation, completely resuspend the SA-PMPs by gently rocking the bottle. The particles should appear as a homogeneous mixture and be fully suspended in the liquid.

Rotors and centrifuges should be at room temperature to avoid precipitation of salts and detergents from solution.

Transfer the amount of SA-PMPs determined in Step 5 to a sterile 50ml or other appropriately sized, screw-cap conical tube, away from the Magnetic Stand. Place the tube with the SA-PMPs on the Magnetic Stand. (To use the Magnetic Separation Stand with 15ml tubes, snap the Adapter into the large hole of the Magnetic Stand with the smallest hole of the Adapter closest to the magnet.) Slowly move the stand toward the horizontal position until the particles are collected at the tube side. Carefully pour off the storage buffer by tilting the tube so that the solution runs over the captured particles. Pouring the storage buffer off in this manner decreases the chance of mixing the SA-PMPs into solution again, which would decrease yields.

8. Resuspend the SA-PMPs in SSC 0.5X Solution to the original SA-PMP volume used in Step 5. Capture the particles using the Magnetic Stand. Pour off the SSC Solution as described in Step 7. Repeat this wash step twice for a total of three times. Resuspend to the original volume with SSC 0.5X Solution.

Do not centrifuge the particles.

9. When centrifugation of the homogenate is complete, carefully remove the supernatant with a sterile pipette, avoiding the pellet. The homogenate will be translucent and brown in color. Add this cleared homogenate to the tube containing the washed particles in SSC 0.5X Solution away from the magnetic stand to ensure proper mixing of the homogenate and particles. Mix by inversion.

Do not disturb the cellular debris pellet while transferring the homogenate, as this results in decreased performance of the system. If the pellet is disturbed, recentrifuge as before.

10. Incubate the homogenate/SA-PMP mixture at room temperature for 2 minutes. Capture the SA-PMPs using the Magnetic Stand. Move the Magnetic Stand toward the horizontal position until the homogenate clears, then carefully pour off the supernatant as in Step 7. Save the supernatant in a sterile RNase-free tube on ice until you are certain that satisfactory binding and elution of mRNA has occurred.

Washes

11. Resuspend the particles in SSC 0.5X Solution to the volume indicated in Table 2 (Section 4.A) or Table 3 (Section 4.B) away from the Magnetic Stand. Mix by gently flicking the tube. Transfer the particle mixture to one of the 2ml mRNA User Tubes provided. Capture the particles by placing the tube on the Magnetic Stand. Carefully remove the SSC solution by pipetting, and discard. Repeat this wash step twice. After the final wash, remove as much of the SSC solution as possible without disturbing the SA-PMPs.

Wash steps should be performed **away** from the Magnetic Separation Stand.

4.C. Protocol for mRNA Isolation from Tissue Samples (continued)

mRNA Elution

12. To elute mRNA, add the amount of Nuclease-Free Water indicated in Table 2 (Section 4.A) or Table 3 (Section 4.B) to the SA-PMPs. Gently resuspend the particles by flicking the tube.

    Note: For larger volumes, multiple elutions will be necessary. Combine all eluted mRNA in one tube.

13. Magnetically capture the SA-PMPs by moving the Magnetic Stand toward the horizontal position as before. Transfer the liquid containing eluted mRNA to the appropriately sized sterile, RNase-free tube. Resuspend the particles in Nuclease-Free Water, and save on ice until mRNA yield and purity are determined. Proceed to Section 4.E for ethanol precipitation.

    Note: Particle carryover sometimes occurs with the supernatant transfer. To remove particles, centrifuge the supernatant at 12,000 × *g* for 1 minute in a microcentrifuge. Transfer the supernatant to a fresh tube, and immediately place on ice. Residual particles do not inhibit many common enzymatic reactions used to process RNA, and they actually help locate pellets after ethanol precipitations.

4.D. Protocol for mRNA Isolation from Cell Cultures

The following protocol should be used to isolate mRNA from 10^6–10^8 cultured cells. Refer to Table 4 for appropriate reagent quantities based on the starting number of cells. The starting number of cells and number of preps you wish to perform will affect which Magnetic Separation Stand you use. Refer to Table 1 for assistance in choosing the proper Magnetic Separation Stand and for proper tube sizes.

Table 4. Reagent Volumes Required for Various Starting Numbers of Cells.

	Cell Number	
Component	**1×10^6**	**1×10^7–1×10^8**
GTC Extraction Buffer	200µl	4ml
Dilution Buffer	400µl	8ml
Oligo(dT) Probe	30pmol	500pmol
SA-PMPs	500µl	6ml
SSC 0.5X Solution[1]	1ml	2ml
Nuclease-Free Water	100µl	1ml

[1]Volume indicated is per wash (not total wash volume) for the mRNA/SA-PMP complexes.

Sample Preparation

1. Remove the GTC Extraction Buffer, Biotinylated Oligo(dT) Probe, Nuclease-Free Water and SSC 0.5X Solution from the refrigerator, and warm to room temperature. Preheat the Dilution Buffer to 70°C. (Use caution when heating the Dilution Buffer; do not heat above 70°C, and do not heat longer than necessary to reach 70°C. Excessive heating will cause the bottle to deform.)
2. In a 50ml sterile screw-cap conical tube, add 20.5µl of β-Mercaptoethanol (97.4%) per milliliter of Extraction Buffer (to make Extraction/BME Buffer). The final concentration of β-Mercaptoethanol is 2%. Use RNase-free pipettes, and wear gloves to reduce the chance of contamination.

! β-Mercaptoethanol is toxic. Dispense in a fume hood, and wear appropriate personal protective equipment.

3. Collect 1×10^6–1×10^8 cells in a sterile 50ml conical centrifuge tube by centrifugation at $300 \times g$ for 5 minutes. Wash the cell pellet with 25ml of ice-cold, sterile 1X PBS, and centrifuge as above to collect the cells. Pour off the supernatant.
4. Add Extraction/BME Buffer to the cells. Homogenize the cells at high speed for 15–30 seconds using a small homogenizer (such as the Tekmar Tissuemizer homogenizer). Alternatively, vortex the cell pellet until cell lysis is complete.

Probe Annealing

5. Dispense the preheated Dilution Buffer to a sterile tube, and add 10.25µl of β-Mercaptoethanol (97.4%) per milliliter of Dilution Buffer. The final concentration of β-Mercaptoethanol is 1%. Add this to the homogenate, and mix thoroughly by inversion. Add the Biotinylated Oligo(dT) Probe (Table 4), and mix well. Incubate this mixture at 70°C for 5 minutes.
6. Transfer the lysate to a clean, sterile 15ml COREX® or other appropriately sized tube. Centrifuge at $12{,}000 \times g$ for 10 minutes at room temperature to clear the homogenate of cell debris and precipitated proteins. During the centrifugation, completely resuspend the SA-PMPs by gently rocking the bottle. The particles should appear as a homogeneous mixture and should be fully suspended in the liquid.

! Rotors and centrifuges should be at room temperature to avoid precipitation of salts and detergents from solution.

Transfer the amount of SA-PMPs, determined from Table 4, to a sterile 50ml screw-cap conical tube away from the Magnetic Stand. Place the tube and SA-PMPs on the Magnetic Stand. (To use the Magnetic Separation Stand with 15ml tubes, snap the Adapter into the large hole of the Magnetic Stand with the smallest hole of the Adapter closest to the magnet.) Slowly move the Magnetic Stand toward the horizontal position until the particles are collected at the side of the tube. Carefully pour off the storage buffer by tilting the tube so that the solution runs over the captured particles. Pouring the storage buffer off in this manner decreases the chance of mixing the SA-PMPs into solution again, which would decrease yields.

7. Resuspend the SA-PMPs in SSC 0.5X Solution to the original SA-PMPs volume (see Table 4). Capture using the Magnetic Stand. Pour off the SSC Solution as described in Step 6. Repeat the washings a total of three times. Resuspend in the original volume with SSC 0.5X Solution.

! **Do not** centrifuge the particles.

4.D. Protocol for mRNA Isolation from Cell Cultures (continued)

Probe Annealing (continued)

8. When centrifugation of the homogenate is complete, carefully remove the supernatant with a sterile pipette, avoiding the pellet. The homogenate will be translucent. Add this cleared homogenate to the tube containing the washed SA-PMPs in SSC 0.5X Solution away from the Magnetic Stand to ensure proper mixing. Mix by inversion.

Do not disturb the cellular debris pellet while transferring the homogenate, as this results in decreased performance of the system. If the pellet is disturbed, recentrifuge as before.

9. Incubate the homogenate/SA-PMP mixture at room temperature for 2 minutes. Capture the SA-PMPs using the Magnetic Stand. Move the Magnetic Stand toward the horizontal position until the homogenate clears, then carefully pour off the supernatant as in Step 6. Save this supernatant in a sterile tube on ice until you are certain that satisfactory binding and elution of the mRNA has occurred.

Washes

10. Resuspend the particles in SSC 0.5X Solution (volume indicated in Table 4) away from the Magnetic Stand. Mix by gently flicking the tube. Transfer the particle mixture to one of the 2ml mRNA User Tubes provided. Capture the SA-PMPs by placing the tube in the Magnetic Stand. Carefully remove the SSC solution by pipetting. Repeat this wash step twice. After the final wash, remove as much of the SSC solution as possible without disturbing the SA-PMPs.

Wash steps should be performed **away** from the Magnetic Separation Stand.

mRNA Elution

11. To elute mRNA, add the amount of Nuclease-Free Water indicated in Table 4 to the SA-PMPs. Gently resuspend the particles by flicking the tube.

12. Magnetically capture the SA-PMPs by moving the Magnetic Stand toward the horizontal position as before. Transfer the liquid containing the eluted mRNA to a sterile, RNase-free microcentrifuge tube. Resuspend the particles in Nuclease-Free Water, and save on ice until mRNA yield and purity are determined. Proceed to Section 4.E for ethanol precipitations.

    Note: Particle carryover sometimes occurs with the supernatant transfer. To remove particles, centrifuge the supernatant at 12,000 × *g* for 1 minute in a microcentrifuge. Transfer the supernatant to a fresh tube, and immediately place on ice. Residual particles do not inhibit many of the common enzymatic reactions used to process RNA, and they actually help in locating the pellets after ethanol precipitations.

4.E. Precipitation and Concentration of mRNA

1. **For cDNA cloning:** Add 0.1 volume of 3M sodium acetate (pH 5.2) and 1.0 volume of isopropanol to the eluate, and incubate at –20°C overnight.

   For translation in vitro: Add 0.1 volume of 3M potassium acetate and 1.0 volume of isopropanol to the eluate, and incubate at –20°C overnight.

2. Centrifuge at >12,000 × *g* for 10 minutes. Resuspend the RNA pellet in 1ml of 70% ethanol, and centrifuge again. Be careful when decanting the supernatant to avoid losing the RNA pellet.

3. **For short-term storage:** For storage of <30 days, dry the pellet in a vacuum desiccator for about 15 minutes, resuspend in RNase-free, deionized water at 0.5–1.0mg/ml and store at –70°C.

   For long-term storage: For storage of ≥30 days, store the RNA pellet in 70% ethanol at –70°C.

4.F. Determination of mRNA Concentration and Purity

The concentration and purity of eluted mRNA can be determined by spectrophotometry. Determine the absorbance readings at 230, 260 and 280nm (A_{230}, A_{260} and A_{280}). Absorbance readings should be between 0.1 and 1.0 A_{260} to ensure significance.

When mRNA is isolated from small amounts of tissue, the expected yields are such that absorbance must be read directly, without dilution of the sample. Be certain that cuvettes are RNase-free so that samples can be recovered after spectrophotometry. This can be done by washing the cuvettes briefly with 0.1N NaOH, 1mM EDTA followed by a brief rinsing with RNase-free water. For additional information on using cuvettes with small sample sizes, see Section 4.G.

Pure mRNA will have an A_{260}/A_{280} absorbance ratio of 2.0. To estimate mRNA concentration, assume that a 40µg/ml mRNA solution has an absorbance of 1 at 260nm. Also, determine the A_{260}/A_{230} ratio, which provides information about sample purity. An A_{260}/A_{230} ratio less than 2 indicates that GTC or β-Mercaptoethanol from the Extraction Buffer is still present. If this is the case, precipitate the RNA again.

The quality of isolated mRNA also may be checked by denaturing agarose gel electrophoresis (5). The mRNA should appear as a smear extending from approximately 8.0kb to approximately 0.5kb, depending on the tissue. The bulk of mRNAs should be clustered around 2.0kb. Expect to see very little ribosomal RNA using the PolyATtract® System 1000. However, the appearance of some ribosomal bands does not indicate poor performance of the system. Figure 5 shows a Northern blot of mRNA that contains visible amounts of both 28S and 18S ribosomal RNAs (see Panel A). The blot was probed with an alkaline phosphatase-oligonucleotide conjugate with a chemiluminescent substrate. Little or no hybridization is seen in the total RNA lane, whereas mRNA prepared using the PolyATtract® System 1000 shows a significant enrichment, despite the presence of minor amounts of ribosomal RNAs (see Panel B). A small amount of ribosomal contamination should not affect the functionality of the mRNA and is suitable for most applications.

4.F. Determination of mRNA Concentration and Purity (continued)

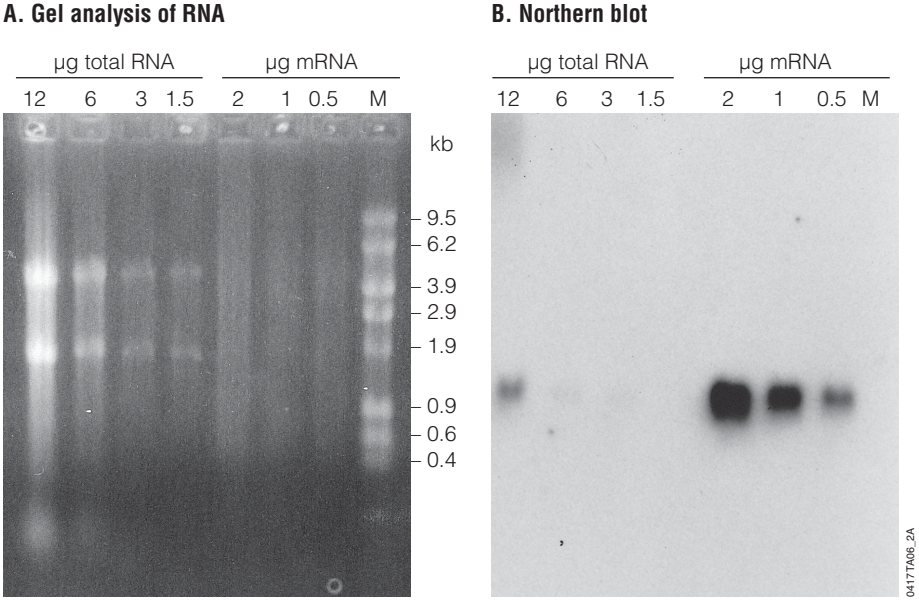

Figure 5. Northern blot of mRNA prepared using the PolyATtract® System 1000. Panel A. Photograph of ethidium bromide-stained gel containing samples of total RNA isolated from mouse liver using the RNAgents® System and poly(A)+ RNA (mRNA) isolated using the PolyATtract® System 1000, respectively. Amounts of RNA loaded are given at the top of the lanes. Lane M contains RNA Markers (Cat.# G3191), the sizes of which are given along the side of the gel. **Panel B.** Northern blot of the gel in Panel A. The probe used was an alkaline phosphatase-oligonucleotide conjugate specific to the α-1-proteinase inhibitor, which is an abundant message in liver. The probe was prepared and analyzed by a chemiluminescent detection method. The film was exposed to X-ray film for 2 hours at 37°C.

4.G. Spectrophotometry of Small Sample Volumes

Semi-Micro or Micro Cell cuvettes should be used for sample volumes of 1ml or less. Micro Short Cells (25mm tall) can be used to measure absorbance of 300–400µl samples, and 50–300µl sample absorbances can be measured in 96-well UV spectrophotometers using UV translucent plates and pathlength correction values. The minimum volume that can be measured in the cuvette depends upon the position of the light beam in the instrument. Refer to the owner's manual, or contact the manufacturer. Shorter pathlength cuvettes can be used to measure smaller volumes, but according to Beer's Law (ε × pathlength × concentration), a shorter pathlength requires a higher concentration of mRNA to give meaningful absorbance values. The standard pathlength of a cuvette is 1cm. Sub-Micro cells are available to measure sample volumes as small as 10µl. The compatibility of these cells with your spectrophotometer needs to be determined. Black masked cuvettes are preferable to clear-wall microcuvettes for a lower signal:noise ratio. The window material of the cuvette should be polished quartz with a usable wavelength of <200nm (e.g., Starna Spectrosil Far UV Quartz). Polystyrene and acrylic cuvettes are not suitable for measurements in the 230–280nm wavelength range, unless specified.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
No mRNA eluted	Salt was not eliminated during elution. Wash the final SA-PMP pellet again with Nuclease-Free Water, and check the A_{260} of this eluate.
	RNase contamination occurred during mRNA isolation. Repeat entire procedure. See Section 3.D, "Creating a Ribonuclease-Free Environment".
Low A_{260}/A_{280} ratios	Protein contamination. Several methods may be used to remove contaminating protein from RNA. The most expedient method is to perform a phenol:chloroform extraction of the purified RNA. Add an equal volume of phenol:chloroform to the final resuspended RNA pellet. This procedure should yield higher A_{260}/A_{280} ratios. However, some loss of RNA (up to 40%) can be expected.
Low A_{260}/A_{230} ratios	Contamination with guanidine thiocyanate or β-Mercaptoethanol. Precipitate the mRNA again as described in Section 4.E.
Low RNA yield	Sample size was too small. Re-isolate mRNA from a larger sample if microgram amounts are needed. **Note:** Very small amounts of mRNA can be detected by RT-PCR.
	Poor sample integrity. Make certain that tissues and cells are handled properly to minimize in vivo degradation. Freeze freshly isolated tissues immediately in liquid nitrogen, and store at –70°C until ready for use.
	Small amount of mRNA in cells. Some tissues yield lower amounts of mRNA. Increase the sample size, and re-isolate the mRNA.
	Partial mRNA degradation. Refer to Section 3.D. Also see "Poor sample integrity" above.

6. References

1. Chirgwin, J.M. *et al.* (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–9.
2. Birkmeyer, R.C. *et al.* (1987) Application of novel chromium dioxide magnetic particles to immunoassay development. *Clin. Chem.* **33**, 1543–7.
3. Morrissey, D.V. *et al.* (1989) Nucleic acid hybridization assays employing dA-tailed capture probes. I. Multiple capture methods. *Anal. Biochem.* **181**, 345–59.
4. Thompson, J. *et al.* (1989) A noise-free molecular hybridization procedure for measuring RNA in cell lysates. *Anal. Biochem.* **181**, 371–8.
5. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

7. Appendix

7.A. Composition of Buffers and Solutions

Note: The performance of this system is guaranteed when used with buffers provided with the system. For users who wish to make their own buffers, it is important that all reagents and equipment used be RNase-free (see Section 3.D).

Dilution Buffer

6X	SSC
10mM	Tris-HCl (pH 7.4)
1mM	EDTA
0.25%	SDS

20X SSC

87.7g	NaCl
44.1g	sodium citrate[1]

Dissolve in 400ml of Nuclease-Free Water. Adjust pH to 7.2 with HCl, and bring the volume to 500ml. Dispense into aliquots. Sterilize by autoclaving.

[1]Trisodium salt dihydrate

GTC Extraction Buffer

4M	guanidine thiocyanate
25mM	sodium citrate (pH 7.1)

10X PBS

11.5g	Na_2HPO_4
2g	KH_2PO_4
80g	NaCl
2g	KCl

Dissolve in 1 liter of distilled water. The pH of 1X PBS will be 7.4.

3M sodium acetate (pH 5.2)

408.1g	sodium acetate • $3H_2O$

Dissolve sodium acetate in 800ml of water. Adjust pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with water. Sterilize by autoclaving.

7.B. Related Products

Total RNA Isolation System

Product	Size	Cat.#
SV 96 Total RNA Isolation System	1 × 96 preps	Z3500
	5 × 96 preps	Z3505

Product	Size	Cat.#
SV Total RNA Isolation System	10 preps	Z3101
	50 preps	Z3100
PureYield™ RNA Midiprep System	10 preps	Z3740
	50 preps	Z3741

7.B. Related Products (continued)

Product	Size	Cat.#
Vacuum Adapters	20 each	A1331
Red Blood Cell Lysis Solution (CLB)	200ml	Z3141
RNA Lysis Buffer (RLA)	50ml	Z3051
Vac-Man® Jr. Laboratory Manifold, 2-sample capacity	1 each	A7660
Vac-Man® Laboratory Manifold, 20-sample capacity	1 each	A7231

mRNA Isolation from Total RNA

Product	Cat.#
PolyATtract® mRNA Isolation System II with Magnetic Stand	Z5200

Each system contains sufficient reagents for 3 separate mRNA isolations, each from 1–5mg of total RNA.

Product	Cat.#
PolyATtract® mRNA Isolation System I (Refill for Z5200)	Z5210

Each system contains sufficient reagents (excluding the Magnetic Separation Stand) for 3 separate mRNA isolations, each from 1–5mg total RNA.

Product	Cat.#
PolyATtract® mRNA Isolation System III with Magnetic Stand	Z5300

Each system contains sufficient reagents for 15 separate mRNA isolations, each from approximately 100–1,000µg of total RNA.

Product	Cat.#
PolyATtract® mRNA Isolation System IV (Refill for Z5300)	Z5310

Each system contains sufficient reagents (excluding the Magnetic Separation Stand) for 15 separate mRNA isolations, each from approximately 100–1,000µg of total RNA.

Product	Size	Cat.#
Biotinylated Oligo(dT) Probe (50pmol/µl)	35µl	Z5261
Streptavidin MagneSphere® Paramagnetic Particles	9ml (15 × 0.6ml)	Z5481
	25ml	Z5482
RNA Markers, 0.28–6.58kb	50µl	G3191
Access RT-PCR System	100 reactions	A1250
	500 reactions	A1280
Access RT-PCR Introductory System	20 reactions	A1260
GoTaq® PCR Core System I	200 reactions	M7660
GoTaq® PCR Core System II	200 reactions	M7665
Tth DNA Polymerase	100u	M2101
AMV Reverse Transcriptase	300u	M5101
AMV Reverse Transcriptase, High Concentration	600u	M9004
Recombinant RNasin® Ribonuclease Inhibitor	2,500u	N2511

7.C. Summary of Changes

The following changes were made to the 8/16 revision of this document:

1. The amount and concentration of the β-Mercaptoethanol component was changed, Sections 2, 4.C and 4.D.
2. The document design was updated.