mTRAP™
Maxi & Midi Kits

For mRNA isolation from cells and tissue

(version A2)

Catalog Nos. 23006 & 23506 (Maxi)
Catalog Nos. 23024 & 23524 (Midi)

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Overview

One of the most important aspects in the isolation of messenger RNA (mRNA) is to prevent degradation of the RNA during the isolation procedure. Because RNA breakdown is a universal feature of cells during RNA processing and metabolism, all cells possess a wide range of nucleases that have both specific and non-specific activities. Given the wide distribution of nucleases and the potential damage that they can inflict on mRNA during isolation, it is important to ensure that nuclease activity is totally inhibited, a problem made all the more difficult by the robust nature of many nucleases. Active Motif's Lysis Solution yields high-quality mRNA through the use of the powerful chaotropic agent guanidine thiocyanate (GTC) and a mix of detergents. Guanidine thiocyanate disrupts cells and separates proteins away from nucleic acids while the mixture of detergents and proteases efficiently inactivates ribonucleases.

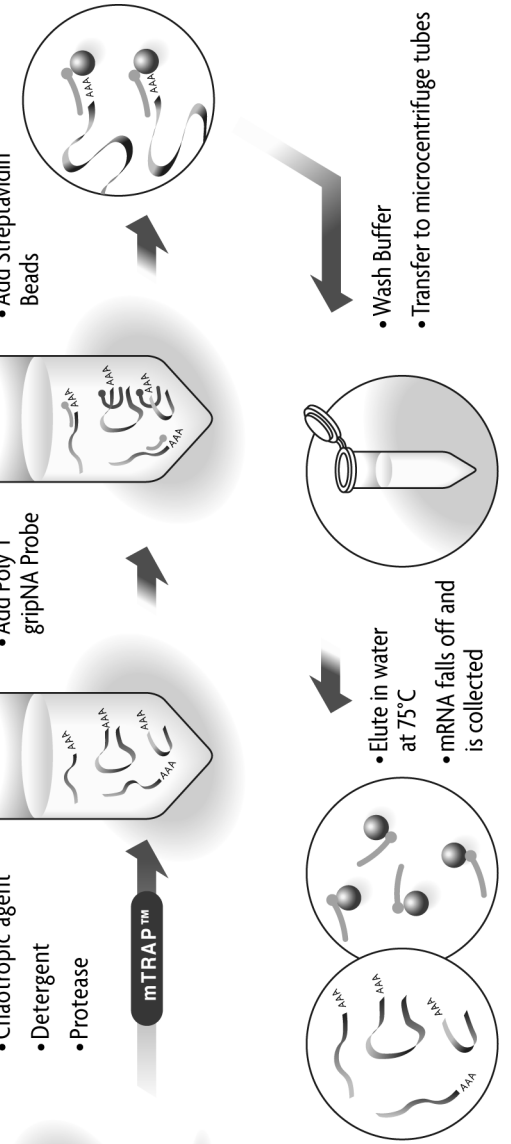
mTRAP™ Kits also use a novel Poly T gripNA Probe to capture the poly A mRNA. The Poly T gripNA Probe supplied with Active Motif's mTRAP Kits is optimized for capture of mRNA with high secondary structures and shorter poly A tails, resulting in a more representative mRNA population. The resulting pure, high-quality mRNA can be used for first-strand cDNA synthesis and PCR amplification, for generating cDNA libraries and for array technology.

mTRAP Kits are available in several different formats. mTRAP Maxi, mTRAP Midi and mTRAP 96 are designed for optimal isolation from tissue and tissue culture cell samples of different sizes, while mTRAP Total isolates mRNA from total RNA samples. For your convenience, many of the mTRAP components are sold separately (See Appendix, Section G).

product	capacity per rxn	yield mRNA*	format	cat. no.
mTRAP Maxi	0.5-2.0 x 10^8 cells / 0.4-1 g tissue	100-200 µg	6 rxns 5 x 6 rxns	23006 23506
mTRAP Midi	0.5-1.0 x 10^7 cells / 50-200 mg tissue	10-20 µg	24 rxns 5 x 24 rxns	23024 23524
mTRAP 96	0.5-1.0 x 10^6 cells / 10-20 mg tissue	100-300 ng	3 x 96 rxns	23096
mTRAP 96 with MAG-96 magnetic stand			3 x 96 rxns	23097
mTRAP Total	up to 500 µg total RNA	10-15 µg	12 rxns 5 x 12 rxns	23012 23512

* Yields may vary due to the type of sample, its developmental stage, growth conditions used, etc.

Flow Chart of Process



PNAs (Peptide Nucleic Acids)

The most common techniques for isolating mRNA require the hybridization of a DNA oligo dT to the poly A tail present on the 3´ end of mRNA molecules. Given the high amount of stable secondary structure in RNA resulting from RNA/RNA interactions, both within a single RNA molecule and between different RNA molecules, it is extremely difficult for oligonucleotides to penetrate these structures and hybridize to the target RNA sequence. Moreover, the high ionic strength conditions that are required for DNA to hybridize to RNA help preserve the mRNA in its natural, tightly folded structure. Thus, many mRNA molecules are inaccessible for oligo dT binding. Also, it is believed that a small proportion of poly A mRNA (~0.5-1%) have short (< 20 bases) poly A sequences. These mRNA form unstable complexes with natural oligonucleotides and thus may not be purified with oligo dT.

Peptide nucleic acids (PNAs) are a new class of molecule that overcome some of the problems associated with oligo dT. PNAs are DNA analogs where the four nucleosides, adenine (A), thymine (T), guanine (G) and cytosine (C), are attached to an *N*-(2-aminoethyl)glycine backbone instead of to a deoxyribose phosphate backbone, as in DNA[1-4]. Despite the dramatic change in chemical makeup, PNAs can hybridize to complementary DNA and RNA by Watson-Crick base pairing[1, 2], and they do so with affinities that are significantly higher than those of corresponding DNA oligonucleotides[5, 6]. Furthermore, PNAs have several additional advantages over DNA and RNA oligomers. For example, PNAs lack 3´ and 5´ polarity, enabling them to bind in parallel or antiparallel orientation to DNA or RNA[3]. It has been demonstrated that PNAs can bind double-stranded DNA by invading the DNA duplex and displacing one strand to form a stable D-loop structure[2, 7]. A further advantage of PNAs is that they are not susceptible to enzymatic degradation[8], and they are also stable in biological fluids[9]. Much of the current interest in PNAs has centered on their ability to act as antisense and antigene agents. However, poor water solubility and a tendency to self-aggregate have limited the biological applications of PNAs.

Efimov *et al* have designed novel classes of negatively charged PNA analogs that overcome some of the limitations of "classical" PNAs. Phosphono-PNA analogs (pPNAs) contain either *N*-(2-hydroxy-ethyl)phosphonoglycine or *N*-(2-aminoethyl)phosphonoglycine as the backbone while hydroxy-L-proline PNA analogs (HypNAs) contain *trans*-4-hydoxy-L-proline as the backbone molecule[10] (Figure 1). The introduction of negative charges into the PNA backbone leads to the significantly improved solubility characteristics of pPNAs and HypNAs while preserving the high affinity these mimics have for complementary RNA and DNA strands[11, 12].
Such high affinity to their target sequences has enabled the successful application of pPNAs as hybridization probes for DNA array technology[12]. In addition, it has been shown that two pPNA molecules bind to each molecule of RNA (or DNA) forming a pPNA2/RNA (or pPNA2/DNA) triplex (Figure 2).

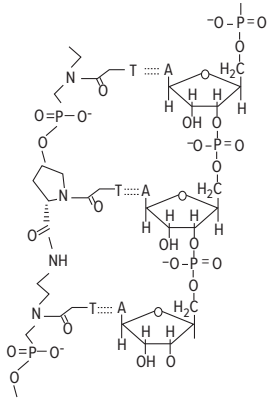


Figure 1: Chemical structure of Poly T gripNA Probe hybridizing to poly A mRNA.

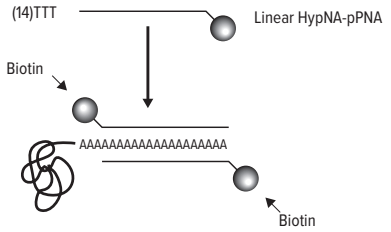


Figure 2: Two PNA molecules can form a triplex with the mRNA.

Advantages of Negatively Charged PNAs

Active Motif has developed a line of mRNA isolation kits that incorporate the pPNAs and HypNAs designed by Efimov *et al*. The Poly T gripNA Probe included in mTRAP mRNA Isolation Kits is a hetero-oligomer constructed of alternating thymine (T) pPNA and thymine (T) HypNA monomers in a 1:1 ratio with a biotin molecule at the 5´ (or N-terminal) end*.

A primary advantage of the Poly T gripNA Probe is that it binds with a much higher affinity to the poly A tails of mRNA as compared to oligo dT. This enables the mRNA binding and subsequent wash steps to be performed in buffers that contain significantly lower salt concentrations than conventional methods. The high-ionic strength conditions required by oligo dT-based methods can stabilize secondary structure in the mRNA, which prevents the poly A tails from being bound by the oligo dT. Moreover, washing under high-salt conditions can stabilize weak, non-specific interactions, leading to co-isolation of unwanted molecules.

Poly T gripNA Probe binds the mRNA in a buffer that contains less than half the NaCl concentration that is required by other methods. mTRAP Kits also utilize a low-salt Wash Buffer that destabilizes any weak, non-specific bonds that may have formed between nucleic acids and proteins with either the mRNA or the Poly T gripNA Probe. The elimination of mRNA secondary structure increases the yield of mRNA while the low-ionic strength wash greatly reduces ribosomal RNA and protein contamination. Consequently, there is no need to perform more than one enrichment with the Poly T gripNA Probe to eliminate unwanted ribosomal bands.

Another useful characteristic of the Poly T gripNA Probe is that it binds to complementary DNA with a higher affinity than it does to complementary RNA. The melting temperature for pPNA2/DNA triplexes is significantly higher than that for pPNA2/RNA triplexes. Melting curves generated in Active Motif's laboratories show that oligo dT releases synthetic poly A RNA and synthetic poly A DNA molecules at nearly identical temperatures. In contrast, Poly T gripNA Probe binds to RNA and DNA much more tightly, and the RNA triplexes melt at a lower temperature than the DNA (Table 1).

Table 1: Melting Data for RNA and DNA from Oligo dT and Poly T gripNA Probe in a Hybridization Buffer of 20 mM Tris pH 7.5, 100 mM NaCl and 10 mM MgCl2.

Probe	T_m for RNA	T_m for DNA
Oligo dT (16-mer)	47.4°C	46.0°C
Poly T gripNA Probe	72.5°C	81.5°C

Active Motif has taken advantage of this characteristic to enable selective elution of mRNA off the Poly T gripNA Probe. As genomic DNA can contain stretches of poly As, co-isolation of genomic DNA is frequently a problem when using oligo dT. Because the genomic DNA remains bound to Poly T gripNA Probe under the mRNA elution conditions, mTRAP Kits isolate high yields of mRNA with significantly reduced genomic DNA contamination. Further, because Poly T gripNA Probe is resistant to enzymatic degradation, an optional DNase step can be employed should the user wish to ensure the complete absence of genomic DNA in the isolated mRNA (See Appendix, Section A).

* Patent pending

Benefits of Poly T gripNA Probe

The mTRAP mRNA Isolation Kits have been designed to take advantage of the many features of Poly T gripNA Probe. These include:

1. The ability to hybridize under low-salt conditions that remove mRNA secondary structure. This effectively eliminates stem/loop structures, enabling hybridization to the poly A tails on all mRNA molecules. The result is both higher yields and the isolation of a more representative mRNA population.
2. The ability to perform wash steps under very low-salt conditions that destabilize weak, non-specific interactions and eliminate ribosomal RNA and protein contamination.
3. A higher affinity for complementary DNA than for complementary RNA strands, which enables differential elution of mRNA, significantly reducing genomic DNA contamination.
4. Because Poly T gripNA Probe is resistant to nucleases, an optional DNase step can be performed during the procedure, completely eliminating genomic DNA contamination.
5. Because Poly T gripNA Probe can form triplexes with the mRNA, it can stably hybridize to shorter poly A sequences, thus ensuring the efficient capture of mRNA molecules with short poly A tails.

Additional Benefits of mTRAP mRNA Isolation Kits

- Complete Kit with RNase-free solutions and plasticware
- Novel Lysing Solution that inhibits activity of RNases
- No total RNA isolation necessary
- Ability to precipitate very small quantities (1 µg) of mRNA without the need for carriers or freezing the sample through use of the included TouchDown™ Precipitation Reagent

Why has mTRAP™ replaced mVADER™?

Beginning in January 2002, mTRAP Kits have replaced Active Motif's original PNA-based mRNA Isolation Kits, mVADER. On-going PNA research has led us to change the composition of the PNA probes used to hybridize to the poly A tails on the mRNA. The Poly T gripNA Probe included in mTRAP mRNA Isolation Kits is a hetero-oligomer constructed of alternating thymine (T) pPNA and thymine (T) HypNA monomers in a 1:1 ratio. The old Maxi and Midi mVADER Kits utilized probe that was entirely thymine (T) pPNA. The old 96 and Total mVADER Kits utilized probe constructed of alternating thymine (T) pPNA and thymine (T) HypNA monomers in a 1:3 ratio.

Poly T gripNA Probe binds to the mRNA with higher affinity than the PNA probes used in the mVADER Kits. This has enabled us to change the composition of the Lysis and Wash Buffers and made possible elution by water at 75°C. Collectively, these changes have increased the yield of mTRAP Kits and reduced ribosomal RNA and genomic DNA contamination. Because of these many changes, we have replaced the mVADER product line with mTRAP.

Kit Contents

Reagents	Quantity (Maxi / Midi)	Composition	Storage
Lysis Buffer	100 ml / 50 ml	200 mM Tris, pH 7.5 200 mM NaCl 500 mM GTC Proprietary mix of detergents	Room Temperature
Wash Buffer	250 ml / 60 ml	20 mM Tris, pH 7.5 10 mM NaCl	Room Temperature
Protease (lyophilized)	20 mg / 8 mg		4°C short term -20°C long term
DEPC water	10 ml / 10 ml		Room Temperature
HPLC water	1 ml / 1 ml		Room Temperature
Poly T gripNA Probe (lyophilized)	6 rxns / 24 rxns (1 tube / 1 tube)		4°C short term -20°C long term
Streptavidin Beads	3 ml / 1.6 ml		4°C
1.7 ml microcentrifuge tubes	12 tubes / 48 tubes		Room Temperature
TouchDown™ Precipitation Reagent	400 mg / 400 mg		Room Temperature

Poly T gripNA Probe

Poly T gripNA Probe has been optimized to ensure efficient recovery of mRNA from cells, tissue and Total RNA. As PNAs in general are resistant to degradation by nucleases, Poly T gripNA Probe may be stored at 4°C for short-term use (weeks). We recommend that the Poly T gripNA Probe be kept at –20°C for long-term storage (months).

Streptavidin Beads

Streptavidin Beads are stable, nominally 1 µm microparticles with highly active streptavidin bound to the surface. They are supplied at approximately 1% solids in a storage buffer of 50 mM Tris, pH 8, 150 mM sodium chloride and 0.05% sodium azide. They also contain a stabilizing detergent at 0.1% concentration. The particles are free of RNase and DNase activity and are supplied in a form that can be directly added to the cell lysate. Store the Streptavidin Beads at 4°C. **Do not freeze the Streptavidin Beads.**

Binding Capacity: The binding capacity of the Streptavidin Beads is approximately 1 nmol of Poly T gripNA Probe per mg of particles, *i.e.* 10 pmol of probe/µl of particles.

Additional Materials Required

Solutions

Phosphate buffered saline (PBS) to wash tissue culture cells (See Appendix, Section E)
100% ethanol for TouchDown™ Precipitation Reagent

Equipment

Sterile 50 ml polypropylene centrifuge tubes (for mTRAP Maxi only)
45°C water bath
75°C water bath
95°C water bath (for optional DNase Treatment only)
Rocking platform
10-20 ml plastic pipettes (RNase free)
2 or 15 ml syringes with 18-21 gauge needles
Motor driven rotor-stator homogenizer for tissues (with a small shaft, *e.g.* 6 mm, to fit 1.7 ml tubes, for use with mTRAP Midi and tissue samples only)
Table-top centrifuge with speeds up to 12,000 x *g* (Maxi only)
Microcentrifuges at room temperature and 4°C
Mortar and pestle with liquid nitrogen (for use with tissue only)

Additional materials required for sodium acetate precipitation

Isopropanol or 100% ethanol
70% ethanol
2 M sodium acetate, pH 5.2 in DEPC water (See Appendix, Section E)
Mussel glycogen (2 µg/µl)

Protocols

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Important Precautions

Ribonucleases (RNases) are very stable enzymes responsible for RNA hydrolysis. RNases can be temporarily denatured by extreme conditions, but RNases readily renature. Therefore, RNases can easily survive autoclaving and other standard methods of protein inactivation. Because RNases are present in the oils of skin, fresh gloves should be worn at all times. Gloves will also protect the researcher from contact with the solutions. Protective eyewear should be worn at all times.

The reagents provided with mTRAP mRNA Isolation Kits are tested for RNase contamination prior to shipment. In addition:

- Keep lids on tubes until ready to use to avoid RNase contamination
- Use disposable, individually wrapped, sterile plasticware
- Use only sterile, new pipette tips (handled with gloves only) and microcentrifuge tubes
- Avoid equipment and areas of the lab that have contact with RNases, (*e.g.* centrifuge tubes used for DNA preparation, which may have contained concentrated RNase mixtures, or gel boxes that have been used for RNase-treated DNA samples)

Preparation of Protease Solution

Maxi Kit: Add 1 ml sterile water to the 20 mg of Protease. Use immediately to prepare Lysis Solution. Store Protease Solution at 4°C for short-term and at –20°C for long-term storage.

Midi Kit: Add 400 µl sterile water to the 8 mg of Protease. Use immediately to prepare Lysis Solution. Store Protease Solution at 4°C for short-term and at –20°C for long-term storage.

Preparation of Lysis Solution

The Lysis Buffer supplied with mTRAP has been optimized for use with the Poly T gripNA Probe. Prepare the Lysis Solution immediately prior to use by combining the appropriate amounts of Protease Solution and Lysis Buffer. Heat the Lysis Buffer at 45°C for 5-10 minutes prior to addition of the Protease Solution.

Warning: The Lysis Buffer contains the corrosive GTC, a strong denaturant.

Maxi Kit: For each isolation, add 150 µl Protease Solution to 15 ml Lysis Buffer.

Midi Kit: For each isolation, add 10 µl Protease Solution to 1.0 ml Lysis Buffer.

Complete disruption and homogenization of the starting material is essential for all intracellular RNA isolation procedures. Disruption involves breaking open the plasma membrane of cells and organelles to release all the RNA contained in the sample. Incomplete disruption reduces the RNA yield. Homogenization is necessary to reduce the viscosity of the cell lysate.

Homogeni-zation shears high molecular weight genomic DNA and other high molecular weight components, creating a homogeneous lysate. Some disruption methods simultaneously homogenize the sample while others require an additional homogenization step.

In general, when working with cultured cells the Lysis Solution disrupts the membranes of cells and organelles. A syringe and needle (18-21 gauge) are then used to homogenize the cell lysate. When working with tissue, a homogenizer is required to disrupt and homogenize the cell lysate.

Preparation of Poly T gripNA Probe

Store the lyophilized Poly T gripNA Probe at –20°C. Resuspend it in the supplied HPLC water, using 500 µl for the Maxi Kit and 400 µl for the Midi Kit. Store the resuspended Poly T gripNA Probe at 4°C for short-term use (weeks) and at –20°C for long-term storage (months).

Preparation of TouchDown™ Precipitation Reagent

mTRAP Kits contain a novel precipitation reagent, TouchDown. It enables the precipitation of nucleic acids without the addition of salt or mussel glycogen (or any carrier). TouchDown is ideal for precipitating small quantities of mRNA. Furthermore, with TouchDown, precipitation can be carried out at 4°C, eliminating the need to freeze the samples at –70°C.

TouchDown Precipitation Reagent is supplied in a high-density polyethylene (HDPE) bottle. Simply add 20 ml 100% ethanol to this bottle. You will also need an additional 10 ml 100% ethanol in a separate bottle (not supplied) for washing the RNA pellet.

At this stage, the manual is divided into two sections:

mTRAP Maxi mRNA Isolation, pages 10-12.
mTRAP Midi mRNA Isolation, pages 12-14.

mTRAP Maxi mRNA Isolation

Preparation of Sample from Animal Tissue

For higher yields, it is best to work with fresh tissue samples. When working with frozen tissue, it is important that it be thawed and homogenized in the Lysis Solution. This ensures immediate inactivation of any RNases that are released during cell lysis. Whether the tissue is fresh or frozen, homogenize it in Lysis Solution as quickly as is feasible. Lysis Solution cannot inactivate nucleases that are inside the tissue until the sample is homogenized. Complete homogenization is critical for complete cell lysis and inactivation of RNases. Tissue samples can be broken up with a mortar and pestle (using liquid nitrogen to keep the tissue frozen) prior to addition to the Lysis Solution and homogenization.

1. Prepare the Lysis Solution as described on page 8.
2. Place frozen tissue sample (0.4-1 g) into 15 ml Lysis Solution in a sterile 50 ml centrifuge tube.
3. Homogenize until the solution is uniformly suspended. Proceed to **Preparation of Messenger RNA.**

   Note: When using a mechanical homogenizer (such as a HandiShear Hand-Held Homogenizer, VirTis) begin homogenization at slow speeds until the tissue is broken into smaller pieces and then increase the speed to the maximum for 45-60 seconds. Avoid the generation of excess heat or foam.

Preparation of Sample from Tissue Culture Cells

We recommend that 0.5-2.0 x 10^8 cells be used for each mTRAP Maxi mRNA Isolation.

1. Wash the cells in 4°C phosphate buffered saline (PBS) solution (see Appendix, Section E).
2. Transfer the cells to a sterile 50 ml centrifuge tube. Pellet the cells by centrifuging at 2,500 x *g* for 5 minutes at 4°C. Continue with Step 3, or flash freeze the cells in liquid nitrogen and store the cells at –70°C.
3. Resuspend and lyse the cells by adding 15 ml Lysis Solution prepared as described on page 8. If pellet is frozen and does not thaw immediately, place the tube in a 45°C water bath for 1-2 minutes. Vortex to completely resuspend the cells. Proceed to **Preparation of Messenger RNA.**

Preparation of Messenger RNA

1. Incubate the cell lysate produced in Preparation of Sample at 45°C for 15-60 minutes. If insoluble material persists (usually in the case of tissue preparations), centrifuge at 12,000 x *g* (10,000 rpm for Sorval SS-34 rotors) for 10 minutes at room temperature and transfer the supernatant to a new tube. Incubation is important for complete digestion of ribonucleases and proteins. A 60-minute incubation is recommended for large samples of tissue (0.5-1 g), while smaller samples of tissue (50-200 mg) and tissue culture cells are effectively digested in 15-20 minutes. You may wish to optimize the time of incubation for your particular sample.

2. Following incubation at 45°C, homogenize the sample by passing the lysate through a sterile plastic syringe attached to an 18-21 gauge needle 4-5 times. This will yield a cleaner mRNA preparation.
3. Add 75 µl Poly T gripNA Probe (prepared as described on page 9) to the cell lysate.
4. Incubate at 75°C for 5 minutes.
5. Incubate at room temperature, with gentle shaking, for 15 minutes.
6. Add 470 µl Streptavidin Beads and incubate at room temperature on a rocking platform for 45 minutes. Proceed to **Washing the Streptavidin Beads.**

Washing the Streptavidin Beads

1. Centrifuge the sample at 2,500 x *g* (3,500 rpm for JS-4.2 and JS-3.0 rotors) for 10-15 minutes at room temperature in a table-top or similar centrifuge. When the Streptavidin Beads are completely pelleted, the lysate will be clear. Carefully remove the lysate supernatant with a 10-25 ml pipette.
2. Add 10 ml Wash Buffer. Resuspend the Streptavidin Beads by pipetting up and down with a pipette. If the beads are difficult to resuspend, gently vortex the sample at low speed.
3. Spin the sample at 2,500 x *g* for 5 minutes at room temperature and carefully remove the Wash Buffer.

   At this point you may proceed with the **Optional DNase Treatment** (Appendix, Section A), otherwise proceed with Step 4.
4. Resuspend the Streptavidin Beads in 10 ml Wash Buffer as described above.
5. Spin at 2,500 x *g* for 5 minutes at room temperature and carefully remove the Wash Buffer.
6. Repeat Steps 4 and 5 one more time if you performed the optional DNase Treatment. Increasing the number of washes reduces the amount of ribosomal RNA in the final mRNA preparation.
7. Resuspend the Streptavidin Beads in an additional 500 µl Wash Buffer. Transfer the beads into a sterile 1.7 ml microcentrifuge tube provided with the kit.
8. Centrifuge at 14,000 x *g* (full speed in a microcentrifuge) for 3 minutes at room temperature. Carefully remove the Wash Buffer. Proceed to **Elution of Messenger RNA.**

Elution of Messenger RNA

1. Resuspend the Streptavidin Beads in 150 µl DEPC water supplied in the kit. Ensure the beads are evenly resuspended using a sterile pipette tip.
2. Place the resuspended beads in a 75°C water bath for 2 minutes. This elutes the mRNA off the Poly T gripNA Probe.
3. Centrifuge at 14,000 x *g* for 2-3 minutes at room temperature. The mRNA is now in the supernatant.
4. Carefully transfer the supernatant to a sterile 1.7 ml microcentrifuge tube provided. This is your mRNA sample.
5. While most of the mRNA is eluted in one step, an additional ~10% may still be bound to the

Poly T gripNA Probe. If desired, repeat steps 1-3, then combine the two supernatants.

Note: If DNase Treatment was performed, the eluted sample should be placed in a 95°C waterbath for 3 minutes to ensure inactivation of any DNase that may have carried over through the washes.

The mRNA may be precipitated at this stage, see Appendix, Section B. We recommend using the supplied TouchDown Precipitation Reagent as it is fast, reliable and recovers 100% of the mRNA.

Following precipitation, proceed to Spectrophotometric Analysis and Agarose Gel Analysis of mRNA. See Appendix, Sections C and D.

mTRAP Midi mRNA Isolation

Preparation of Sample from Animal Tissue

For higher yields, it is best to work with fresh tissue samples. When working with frozen tissue, it is important that it be thawed and homogenized in the Lysis Solution. This ensures immediate inactivation of any RNases that are released during cell lysis. Whether the tissue is fresh or frozen, homogenize it in Lysis Solution as quickly as is feasible. Lysis Solution cannot inactivate nucleases that are inside the tissue until the sample is homogenized. Complete homogenization is critical for complete cell lysis and inactivation of RNases. Tissue samples can be broken up with a mortar and pestle (using liquid nitrogen to keep the tissue frozen) prior to addition to the Lysis Solution and homogenization.

1. Prepare the Lysis Solution as described on page 8.
2. Place frozen tissue sample (50-200mg) into 1 ml Lysis Solution in a sterile 1.7 ml microcentrifuge centrifuge tube provided with the kit.
3. Homogenize (using a homogenizer with a small shaft) until the solution is uniformly suspended. Proceed to **Preparation of Messenger RNA.**

Preparation of Sample from Tissue Culture Cells

We recommend that 0.5-1.0 x 10^7 cells be used for each mTRAP Midi mRNA Isolation.

1. Wash the cells in 4°C phosphate buffered saline (PBS) solution (see Appendix, Section E).
2. Transfer the cells into a sterile 1.7 ml microcentrifuge tube provided with the kit and pellet the cells at 14,000 x *g* (full speed in a microcentrifuge) for 3 minutes at 4°C. Continue with Step 3, or flash freeze the cells in liquid nitrogen and store the cells at –70°C.
3. Resuspend and lyse the cells by adding 1 ml Lysis Solution prepared as described on page 8. If pellet is frozen and does not thaw immediately, place the tube in the 45°C water bath for 1-2 minutes. Vortex to completely resuspend the cells. Proceed to **Preparation of Messenger RNA.**

Preparation of Messenger RNA

1. Incubate the cell lysate produced in Preparation of Sample at 45°C for 20-30 minutes. Incubation is important for complete digestion of ribonucleases and proteins. A 20-minute incubation is usually sufficient for digestion of small amounts of tissue (200 mg) and cultured cells. You may wish to optimize the time of incubation for your particular sample.
2. Homogenize the sample by passing the lysate through a sterile plastic syringe attached to an 18-21 gauge needle 4-5 times. This will yield a cleaner mRNA preparation.
3. Add 15 µl Poly T gripNA Probe (prepared as described on page 9) to the cell lysate.
4. Incubate at 75°C for 1 minute.
5. Incubate at room temperature, with gentle shaking, for 15 minutes.
6. Add 60 µl Streptavidin Beads and incubate at room temperature on a rocking platform for 45 minutes. Proceed to **Washing the Streptavidin Beads.**

Washing the Streptavidin Beads

1. Centrifuge the sample at 14,000 x *g* (full speed in a microcentrifuge) for 3-5 minutes at room temperature. When the Streptavidin Beads are completely pelleted, the lysate will be clear. Carefully remove the lysate supernatant with a sterile 1 ml pipette tip.
2. Add 750 µl Wash Buffer. Resuspend the Streptavidin Beads by pipetting up and down with a sterile pipette tip. If the beads are difficult to resuspend, gently vortex the sample at low speed.
3. Centrifuge the sample at 14,000 x *g* for 2-3 minutes at room temperature and carefully remove the Wash Buffer.

   At this point you may proceed with the **Optional DNase Treatment** (Appendix, Section A), otherwise proceed with Step 4.
4. Resuspend the Streptavidin Beads in 750 µl Wash Buffer as described above.
5. Spin at 14,000 x *g* for 2-3 minutes at room temperature and carefully remove the Wash Buffer.
6. Repeat Steps 4 and 5 one time if you performed the optional DNase Treatment. Increasing the number of washes reduces the amount of ribosomal RNA in the final mRNA preparation. Proceed to **Elution of Messenger RNA.**

Elution of Messenger RNA

1. Resuspend the Streptavidin Beads in 75 µl DEPC water provided in the kit. Ensure the beads are evenly resuspended using a sterile pipette tip.
2. Place the resuspended beads in a 75°C water bath for 2 minutes. This elutes the mRNA off the Poly T gripNA Probe.
3. Centrifuge at 14,000 x *g* for 2-3 minutes at room temperature. The mRNA is now in the supernatant.
4. Carefully transfer the supernatant to a sterile 1.7 ml microcentrifuge tube provided. This is your mRNA sample.
5. While most of the mRNA is eluted in one step, an additional ~10% may still be bound to the Poly T gripNA Probe. If desired repeat steps 1-3, then combine the two supernatants.

Note: If DNase Treatment was performed, the eluted sample should be placed in a 95°C waterbath for 3 minutes to ensure inactivation of any DNase that may have carried over through the washes.

The mRNA may be precipitated at this stage, see Appendix, Section B. We recommend using the supplied TouchDown Precipitation Reagent as it is fast, reliable and recovers 100% of the mRNA.

Following precipitation, proceed to Spectrophotometric Analysis and Agarose Gel Analysis of mRNA. See Appendix, Sections C and D.

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Appendix
Section A. Optional DNase Treatment

While the mTRAP Kits isolate mRNA that is relatively free of genomic DNA, certain sample types are susceptible to genomic DNA contamination. Moreover, some downstream applications require the absolute absence of genomic DNA contamination. As described in the introduction, one advantage of PNAs is that they are resistant to nucleases. Therefore, if desired, it is possible to perform a DNase digest of the material captured by the Poly T gripNA Probe (mainly mRNA and some DNA) while it is attached to the Streptavidin Beads.

Note: Use only RNase-free DNase (not provided). We recommend:
DNase I, Amplification Grade, Invitrogen Cat. No. 18068-015, or
DNase I (RNase-free), Ambion Cat. No. 2222

Following Step 3 in **Washing the Streptavidin Beads**, proceed with the following procedure. Values in **bold type** are to be used with the **mTRAP Midi** protocol.

1. Resuspend the Streptavidin Beads in 1 ml **(100 µl)** Wash Buffer using a pipette tip.
2. Add 3-5 Units **(1-2 Units)** RNase-free DNase.
3. Incubate at room temperature for 10 minutes.
4. Spin at 2,500 x *g* for 5 minutes **(14,000 x *g* for 2-3 minutes)** at room temperature to pellet the Streptavidin Beads. Carefully remove the supernatant.
5. Continue with **Washing the Streptavidin Beads** from Step 4 onward (page 11 or **13**).

Section B. Precipitation Protocols

Precipitation with TouchDown™ Precipitation Reagent

1. Add 5 volumes TouchDown Precipitation Reagent (prepared as described on page 9) to the eluted mRNA. Mix well by vortexing at high speed for 10 seconds in an inverted position.
2. Place the tube at 4°C for 20 minutes.
3. Centrifuge the sample at 14,000 x *g* (full speed in a microcentrifuge) at 4°C for 20 minutes. Carefully remove the supernatant. To prevent disturbing the mRNA pellet, leave ~5-10 µl of solution at the bottom of the tube.

   Caution: The mRNA pellet that forms with TouchDown is very loose and can easily be aspirated into the pipette tip. Be very careful when removing the supernatant and during the subsequent ethanol wash. It is a good idea to leave 5-10 µl of supernatant/ethanol at the bottom of the tube to minimize the disturbance of the pellet. The ethanol will evaporate in 5-10 minutes. When working with TouchDown for the first time, measure the mRNA concentration prior to precipitation. 100% of the mRNA should be recovered.

4. Add 200 µl 100% ethanol. Vortex briefly.
5. Centrifuge for 20 minutes at 14,000 x *g* at 4°C. Carefully remove the ethanol. Remove as much ethanol as possible without disturbing the pellet.
6. Air dry the pellet for 5-10 minutes, until the remaining ethanol has evaporated.

7. Resuspend the pellet in 20-50 µl DEPC water.
8. Heat the sample for 10 minutes at 70°C to aid in resuspension of the mRNA. Vortex to ensure complete resuspension.
9. Determine the concentration of the mRNA (See Appendix, Section C).

Following analysis, store the mRNA in 10 µl aliquots at –70°C.

Precipitation with Sodium Acetate, Ethanol/Isopropanol

1. Add 0.15 volumes of 2 M NaOAc, pH 5.2 to the eluted mRNA. Mix by vortexing.
2. Add either 2.5 volumes 100% ethanol or an equal volume of isopropanol. Mix by vortexing.
3. Incubate at -70°C for 20-30 minutes.
4. Centrifuge for 20 minutes at 14,000 x *g* at 4°C. Carefully remove the supernatant.
5. Wash pellet with 500 µl 70% ethanol.
6. Centrifuge for 5 minutes at 14,000 x *g* at 4°C. Carefully remove the supernatant.
7. Air dry the pellet for 5-10 minutes, until the remaining ethanol has evaporated.
8. Resuspend the pellet in 20-50 µl DEPC water.
9. Heat the sample for 5 minutes at 70°C to aid in resuspension of the mRNA. Vortex to ensure complete resuspension.
10. Determine the concentration of the mRNA (See Appendix, Section C).

Following analysis, store the mRNA in 10 µl aliquots at –70°C.

Note: In cases where the quantity of mRNA is very low (less than 2-3 µg), 8-10 µg of mussel glycogen (not provided) can be added as a carrier to aid in the recovery of the mRNA.

Section C. Spectrophotometric Analysis of Messenger RNA

To determine the concentration of the eluted mRNA, dilute 2 µl of the sample in 98 µl DEPC water. Use DEPC water to blank the spectrophotometer at 260 nm. Place the diluted sample in a 100 µl quartz microcuvette and read the absorbance at 260 nm. Use the formula below to determine the RNA concentration:

$$[\mathbf{RNA}] = (A_{260})\ (0.04\ \mu g/\mu l)\ D$$

D is the dilution factor (D = 50 in the above example). Determine the yield by multiplying the concentration by the volume of the mRNA sample. Note that the A_{260} must be > 0.05 to give an accurate RNA concentration.

A ratio of the readings at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the RNA with respect to contaminants that absorb in UV, such as protein.

Pure RNA has an A_{260}/A_{280} of 1.9-2.1 in 10 mM Tris, pH 7.5.

Section D. Agarose Gel Analysis of Messenger RNA

As well as checking the concentration and yield of the mRNA by spectrophotometric analysis, it is also necessary to check an aliquot of the mRNA on an agarose gel.

Designate one gel box in the lab for RNA analysis. Rinse this gel box with a dilute (1%) bleach solution followed by a number of rinses with distilled deionized water. Pour a 1% TAE agarose gel and allow it to solidify. Ensure that the 6X sample dye for RNA analysis has been prepared with DEPC water and is stored at 4°C. It is usually a good idea to keep aliquots of sample dye for RNA analysis separate from those for DNA analysis.

Load approximately 1 µg mRNA on the gel and run at 100 mAmps until the bromophenol blue dye from the sample buffer is approximately 2-2.5 inches into the gel.

Recipes for TAE and 6X sample dye are available in reference 13.

Section E. Composition of Solutions

1. **Phosphate Buffer Saline (PBS) Solution**

   137 mM NaCl
   2.7 mM KCl
   10 mM Na_2HPO_4
   1.8 mM KH_2HPO_4

   To prepare 1 liter, add: 8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2HPO_4

   to 800 ml of distilled water and mix until dissolved. Adjust the pH to 7.4 with 1 N HCl, and then bring the volume up to 1000 ml and autoclave. Store PBS at room temperature or at 4°C.

2. **DEPC Water**
   In a fume hood, add diethylpyrocarbonate (DEPC) to deionized water to a final concentration of 0.1% (v/v). Incubate overnight at room temperature in the fume hood. Autoclave for 20 minutes at least two times. Extra autoclaving will ensure complete removal of any DEPC.

   Caution: DEPC is a suspected carcinogen. Always use DEPC in a fume hood. Read and follow the manufacturer's safety instructions.

3. **2 M Sodium Acetate**
   Dissolve 136 g of sodium acetate•$3H_20$ in 400 ml water. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 500 ml with water. In a fume hood, add 500 µl diethylpyrocarbonate (DEPC). Incubate overnight at room temperature in a fume hood. Autoclave for 20 minutes at least two times. Extra autoclaving will ensure complete removal of any DEPC.

   Caution: DEPC is a suspected carcinogen. Always use DEPC in a fume hood. Read and follow the manufacturer's safety instructions.

4. **Mussel Glycogen**
   We recommend mussel glycogen from Roche Molecular Biochemicals (Boehringer Mannheim), Catalog No. 901-393 (20 µg/µl). Dilute to 2 µg/µl in DEPC water.

Section F. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
Low mRNA yield	Incomplete hybridization of mRNA to probe	Increase incubation time of the Poly T gripNA Probe with the cell lysate. Temperatures up to 40°C may be used to "speed up" the hybridization process
	Lysate too viscous for probe hybridization	Do not exceed 2 x 10^8 (10^7) cells or 1 g (200 mg) of tissue per 15 ml (1 ml) of Lysis Solution (Midi). In future preparations, reduce the amount of starting material and/or increase the volume of Lysis Solution
		Complete homogenization is required for direct mRNA isolation. Follow the recommendations regarding the use of a mortar and pestle (for tissue) and a syringe and needle to completely shear the sample
	Centrifugal force too low	Ensure that all the Streptavidin Beads are pelleted before removing the supernatant. This may require a longer or higher speed spin
	Loss of Streptavidin Beads during wash steps	Be sure not to aspirate any of the Streptavidin Beads from the pellet while removing the Wash Buffer
	Loss of mRNA during precipitation	If you anticipate that the mRNA to be precipitated is less than 1 µg, increase the amount of Touchdown Precipitation Reagent used to 10 volumes and incubate at -70°C for 20 minutes
Ribosomal RNA in final mRNA preparation	Insufficient washing	Ensure that the Streptavidin Beads are completely resuspended during the wash steps. For some samples, it may be necessary to use a pipette tip to resuspend the samples. If the samples do not resuspend easily, allow them to sit on the bench for a few minutes and then try again. Alternatively, gently vortex for 1-2 seconds 4-5 times. Some rRNA will always be present in the final mRNA sample
Genomic DNA contamination in the final mRNA preparation	The mRNA was eluted off the Poly T gripNA Probe at too high a temperature	Do not use a water bath that is > 80°C as bound genomic DNA will elute from the Poly T gripNA Probe
	Incomplete homogenization	Complete homogenization and shearing of lysate of the DNA helps reduce genomic DNA contamination. Follow the recommendations regarding the use of a mortar and pestle (for tissue) and a syringe and needle to completely shear the sample
	Problematic sample type	Some sample types are susceptible to genomic DNA contamination. Perform the Optional DNase Treatment (See Appendix, Section A)

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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