

SideStep mRNA Enrichment Kit

INSTRUCTION MANUAL

Catalog #400902

Revision B.0

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400902-12

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SIDESTEP MRNA ENRICHMENT KIT

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SideStep mRNA Enrichment Kit

MATERIALS PROVIDED

Materials provided	Quantity
SideStep Lysis and Stabilization Buffer	10 ml
Absolutely mRNA Purification Kit ^a (Catalog #400806)	1 kit
Absolutely mRNA oligo (dT) Magnetic Particles ^b	500 µl
Absolutely mRNA Hybridization Buffer	4 ml
Absolutely mRNA Wash Buffer ^b	4 ml
Absolutely mRNA Elution Buffer	4 ml

^a Sufficient reagents are provided to isolate mRNA from 10 cell lysate samples (100 µl each).

^b Buffer contains 0.05% sodium azide.

STORAGE CONDITIONS

SideStep Lysis and Stabilization Buffer: Store at –20°C upon receipt. After thawing, store at 4°C.

Absolutely mRNA Purification Kit: Store all components at room temperature. **Do not freeze the Absolutely mRNA oligo (dT) magnetic particles.**

ADDITIONAL MATERIALS REQUIRED

PBS, cold (see *Preparation of Media and Reagents*)

RNase- and DNase-free H₂O

Microcentrifuge tubes

Magnetic bead separation stand (e.g., Magnetight Separation Stand, available from VWR, Catalog #80031-368.)

Note *The separation stand should be configured to collect magnetic particles on the side, rather than the bottom, of a microcentrifuge tube.*

INTRODUCTION

The SideStep mRNA Enrichment Kit couples the SideStep Lysis and Stabilization Buffer* with the Absolutely mRNA Purification Kit for efficient mRNA enrichment directly from cells without first purifying total RNA.

SideStep technology achieves mammalian cell lysis and nucleic acid stabilization in the same buffer, eliminating the need for RNA purification prior to QRT-PCR experiments. This system is ideally suited for applications involving the analysis of a large number of samples. Frequently, it is desirable to assay mRNA levels in a large number of samples without isolating RNA, and then to isolate mRNA from the small subset of samples of interest for further analysis such as microarray target labeling.

The simple SideStep lysate preparation protocol takes approximately 10 minutes to perform and includes a single PBS wash followed by cell lysis in the SideStep lysis buffer. This buffer inactivates cellular nucleases and other enzymes, and the nucleic acids released into the buffer are stabilized and suitable for QRT-PCR and QPCR analysis or mRNA purification for up to 6 months.

The Absolutely mRNA purification kit allows rapid isolation of high-quality and exceptionally pure polyA⁺ RNA from cell lysates containing up to 10⁶ cell equivalents. The kit technology is based on specially engineered oligo (dT) magnetic beads which have a larger surface area compared to standard magnetic beads and deliver higher yields of pure RNA. The microparticles have fast magnetic response times, allowing for efficient and effective purification, delivering pure mRNA from cell lysates in less than 20 minutes.

The purification protocol is summarized in Figure 1. In step 1, the Absolutely mRNA oligo (dT) magnetic particles are combined with the SideStep cell lysate. PolyA⁺ RNA hybridizes to the microparticles under optimized buffer conditions. In step 2, a magnetic field is applied to pull the microparticle-mRNA complexes out of solution. Contaminants are removed by aspiration, and then the microparticles are thoroughly washed. In step 3, purified mRNA is eluted from the microparticles in an aqueous solution, providing maximum flexibility for downstream applications.

The protocol may be adapted for use without a magnetic separation stand by substituting centrifugation for magnetic force to achieve separation of mRNA from contaminants. See *Appendix 1* for a complete protocol.

* Patent pending.

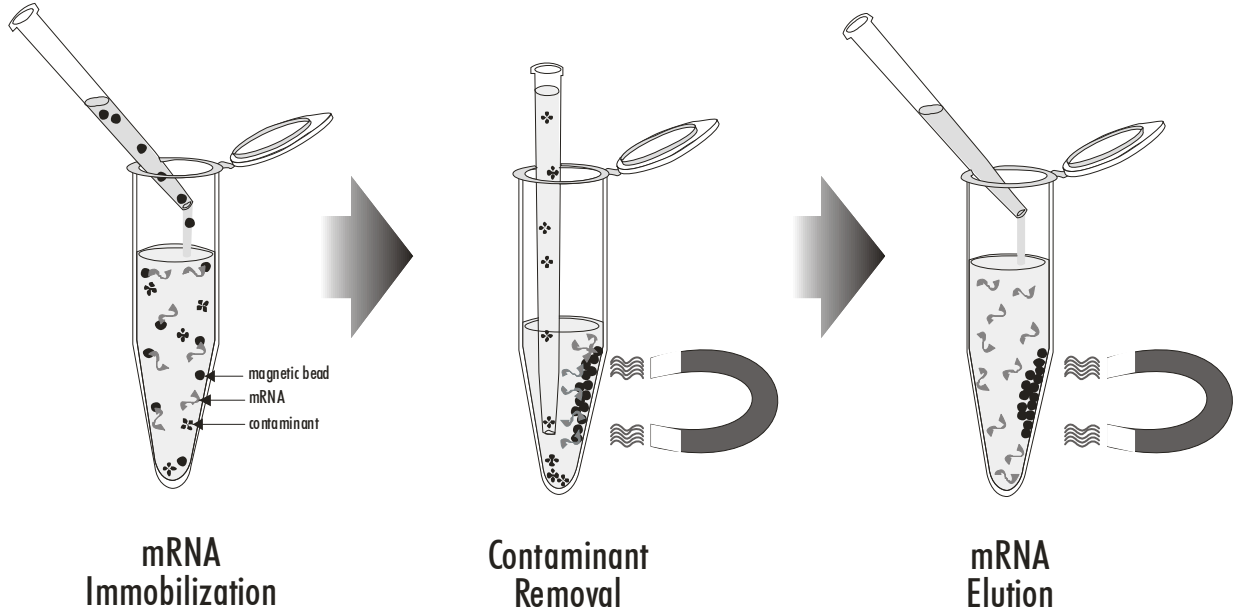


FIGURE 1 Absolutely mRNA purification method.

Preparation of SideStep Cell Lysates

Note *The SideStep lysis and stabilization buffer may be used to prepare lysates from a variety of mammalian cell lines. Commonly-used cell culture and harvesting methods are compatible with the SideStep buffer protocols, and those methods routinely used by your laboratory for the specific cell line should be employed.*

Cell Density Considerations

Lysates may be prepared with cell densities of up to 10^4 cell equivalents/ μl lysate. When sufficient cultured cells are available, we recommend preparing the lysate at the maximum cell density (10^4 cells/ μl), for maximum mRNA yield and flexibility in downstream applications. When analyzing a small number of cells in downstream applications such as QRT-PCR, the lysate may be diluted in RNase- and DNase-free H_2O prior to addition to the QRT-PCR reaction.

Cells are washed once in cold PBS in the protocol below. The density of the PBS suspension in step 4 will equal the final cell density of the lysate.

Preparation of Lysates from Cultured Cells

1. Harvest the cultured cells, using a method appropriate to the properties of the cell line and the growth vessel.

Note *If trypsin is used for cell harvesting, it must be inactivated before proceeding.*

2. Count the cells in an aliquot of the cell suspension.
3. Pellet the cells at $500\text{--}1,000 \times g$ at 4°C for 5 minutes. Aspirate the supernatant.
4. Resuspend the pelleted cells in at least 100 μl of cold PBS to a final concentration of $\leq 10^4$ cells/ μl .

Note *The density of the PBS cell suspension will equal the final cell density in the lysate. We recommend using the maximum cell density of 10^4 cells/ μl , for maximum mRNA yield and flexibility in downstream applications.*

5. Place 100 μl of the cell suspension ($\leq 10^6$ cells) in a 1.5-ml microfuge tube.
6. Pellet the cells at $500\text{--}1,000 \times g$ at 4°C for 5 minutes. Aspirate the supernatant. Remove as much of the PBS as possible without disturbing the pellet.

7. Add 100 μ l of SideStep lysis and stabilization buffer to the cell pellet.
8. Vortex for 1 minute to lyse the cells.
9. Process the lysate using the appropriate application or store the lysate according to the following considerations. Nucleic acids in the lysate are stable at room temperature for up to 4 hours, at 4°C for 1 month, and at -20°C for 6 months. Nucleic acids are no longer stabilized after dilution of the SideStep buffer. If you prepare dilutions of the lysate for QPCR or QRT-PCR analysis, do not store the dilutions or isolate mRNA from the diluted lysate.

Enrichment of mRNA

The mRNA enrichment protocol given below is for purification of mRNA from 100 μ l of SideStep cell lysate containing up to 10^6 cell equivalents. Reactions may be scaled down to 0.5 \times (for 50 μ l of lysate) or up to 10 \times (for up to 1 ml of lysate) in a single reaction tube. When scaling up or down, simply increase or decrease the volumes of all components, including the Absolutely mRNA oligo (dT) magnetic particles.

1. Swirl or roll the vial of Absolutely mRNA oligo (dT) magnetic particles to ensure that the particles are in a homogeneous suspension. Transfer 50 μ l of the particle suspension to an RNase-free microcentrifuge tube. Wash the particles twice with 100 μ l of Absolutely mRNA hybridization buffer. During each wash, mix by pipetting and then collect the beads using a magnetic separator stand, according to the manufacturer's instructions.

Notes *The hybridization buffer may precipitate when stored at cooler temperatures. Store the buffer at room temperature and do not place the buffer on ice. If a precipitate is present, warm the buffer to 50°C and swirl the solution to redissolve the components.*

Whenever the particles are collected using the magnetic separator, leave the tube in the magnetic stand while removing the supernatant.

2. Resuspend the washed particles in 100 μ l of Absolutely mRNA hybridization buffer by repeated pipetting.
3. Add 100 μ l of SideStep cell lysate to the resuspended magnetic particles. Mix by repeated pipetting.
4. Incubate the mixture at room temperature with gentle agitation for 5 minutes to allow hybridization of the polyA-RNA to the particles.
5. Collect the magnetic particles complexed with mRNA using a magnetic separator stand.

6. Remove the supernatant with a pipet tip.

Note *The supernatant, containing the unbound fraction of the total RNA may be retained, if desired, to facilitate troubleshooting or may be discarded at this step.*

7. Wash the mRNA-bound magnetic particles four times using 100 μ l of Absolutely mRNA wash buffer for each wash. Gently resuspend the particles during each wash, and then re-collect the particles using the magnetic separator stand. Be sure to remove all of the wash buffer when completing the final wash.
8. Remove the tube from the magnetic stand and then add 100 μ l of Absolutely mRNA elution buffer to the particles. Incubate the tube at room temperature with gentle agitation for 5 minutes to release the mRNA from the particles.
9. Collect the magnetic particles using a magnetic separator stand.
10. Draw off the 100- μ l eluate, containing the purified mRNA, and transfer the solution to a fresh, RNase-free tube. The RNA can be stored at -20°C for up to one month or at -80°C for long-term storage.

RNA Quantification

To quantify mRNA isolated from 10^6 cell equivalents, a highly sensitive fluorescence-based system (e.g., RiboGreen[®] RNA quantitation kit, Molecular Probes, Inc.) may be used.

TROUBLESHOOTING

Observation	Suggestion
RNA is degraded	Wear gloves throughout the procedure and when handling equipment and solutions used for RNA work. See <i>Appendix 2: Preventing Sample Contamination</i> for additional recommendations.
	Ensure that the SideStep lysate is not diluted prior to mRNA enrichment.
Final mRNA solution is too dilute for downstream applications	Purified mRNA may be concentrated by vacuum centrifugation (e.g. using a SpeedVac® concentrator).
mRNA is contaminated with excess rRNA	Ribosomal RNA may co-purify as the result of mRNA-rRNA interactions. Ensure that the hybridization and wash steps are carried out at room temperature. If rRNA levels are unacceptably high for downstream applications, the mRNA preparation can be subjected to a second round of purification using fresh beads. Note that performing an additional round of purification is expected to reduce the mRNA yield.
	Using the alternative protocol for particle separation by centrifugation (<i>Appendix 1</i>) instead of separation by magnetic force may increase the extent of rRNA contamination.
Magnetic particles are trapped in lid or in drops on the sides of microcentrifuge tubes or stock vial	Briefly spin the tube in a microcentrifuge. Resuspend the pelleted particles by swirling or rolling the tube, or by repeated pipetting, prior to continuing the protocol.
Purified mRNA concentration measurement by optical density (OD ₂₆₀) or the OD ₂₆₀ /OD ₂₈₀ ratio is unexpectedly low	The presence of residual magnetic particles in the purified mRNA solution interferes with measurement of optical density. Return the sample to the magnetic stand to collect the residual particles and then remeasure OD ₂₆₀ and OD ₂₈₀ .
The purified mRNA solution has a brownish coloration	Brown coloration is indicative of incomplete magnetic particle removal. Return the sample to the magnetic stand to collect the residual particles and then transfer the mRNA to a fresh tube, taking care to aspirate the supernatant with a pipet tip without disturbing the magnetic particle pellet.
	Use a magnetic stand that contains a stronger magnet. See <i>Additional Materials Required</i> for stand recommendations.
Hybridization buffer contains a precipitate	Components of the hybridization buffer may precipitate when stored at cooler temperatures. If a precipitate is present, warm the buffer to 50°C and swirl the solution to redissolve the components.

PREPARATION OF MEDIA AND REAGENTS

PBS (Phosphate Buffered Saline)

150 mM NaCl
 20 mM Na₂HPO₄
 adjust to pH 7.4 with HCl

APPENDIX 1: ALTERNATE PURIFICATION PROTOCOL USING PARTICLE CENTRIFUGATION

The mRNA enrichment protocol given below is for purification of mRNA from 100 μ l of SideStep cell lysate containing up to 10^6 cell equivalents. Reactions may be scaled down to 0.5 \times (for 50 μ l of lysate) or up to 10 \times (for up to 1 ml of lysate) in a single reaction tube. When scaling up or down, simply increase or decrease the volumes of all components, including the Absolutely mRNA oligo (dT) magnetic particles.

1. Swirl or roll the vial of Absolutely mRNA oligo (dT) magnetic particles to ensure that the particles are in a homogeneous suspension. Transfer 50 μ l of the particle suspension to an RNase-free microcentrifuge tube. Wash the particles twice with 100 μ l of Absolutely mRNA hybridization buffer. During each wash, mix by pipetting and then collect the beads by centrifugation at 10,000 $\times g$ for 2 minutes.

Notes *The hybridization buffer may precipitate when stored at cooler temperatures. Store the buffer at room temperature and do not place the buffer on ice. If a precipitate is present, warm the buffer to 50°C and swirl the solution to redissolve the components.*

Whenever the particles are collected by centrifugation, remove supernatant carefully to avoid disturbing the pelleted particles.

2. Resuspend the washed particles in 100 μ l of Absolutely mRNA hybridization buffer by repeated pipetting.
3. Add 100 μ l of SideStep cell lysate to the resuspended magnetic particles. Mix by repeated pipetting.
4. Incubate the mixture at room temperature with gentle agitation for 5 minutes to allow hybridization of the polyA-RNA to the particles.
5. Collect the magnetic particles complexed with mRNA by centrifugation at 10,000 $\times g$ for 2 minutes.
6. Remove the supernatant with a pipet tip.

Note *The supernatant, containing the unbound fraction of the total RNA may be retained, if desired, to facilitate troubleshooting or may be discarded at this step.*

7. Wash the mRNA-bound magnetic particles four times using 100 μ l of Absolutely mRNA wash buffer for each wash. Gently resuspend the particles during each wash, and then re-collect the particles by centrifugation at 10,000 $\times g$ for 2 minutes. Be sure to remove all of the wash buffer when completing the final wash.

8. Add 100 μ l of Absolutely mRNA elution buffer to the particles. Incubate the tube at room temperature with gentle agitation for 5 minutes to release the mRNA from the particles.
9. Collect the magnetic particles by centrifugation at $10,000 \times g$ for 2 minutes.
10. Draw off the 100- μ l eluate, containing the purified mRNA, and transfer the solution to a fresh, RNase-free tube. The RNA can be stored at -20°C for up to one month or at -80°C for long-term storage.

RNA Quantification

To quantify mRNA isolated from 10^6 cell equivalents, a highly sensitive fluorescence-based system (e.g., RiboGreen[®] RNA quantitation kit, Molecular Probes, Inc.) may be used.

APPENDIX 2: PREVENTING SAMPLE CONTAMINATION

Preventing RNase Contamination

Ribonucleases are very stable enzymes that hydrolyze RNA. RNase A can be temporarily denatured under extreme conditions, but it readily renatures. RNase A can therefore survive autoclaving and other standard methods of protein inactivation. The following precautions can help prevent RNase contamination:

- ♦ **Wear gloves at all times** during the procedures and while handling materials and equipment, as RNases are present in the oils of the skin.
- ♦ Exercise care to ensure that all equipment and supplies (e.g., centrifuge tubes, etc.) are free from contaminating RNases. Avoid using equipment or workspaces that have been exposed to RNases. Use only sterile tubes and micropipet tips.
- ♦ Micropipettor bores can be a source of RNase contamination, since material accidentally drawn into the pipet or produced by gasket abrasion can fall into RNA solutions during pipetting. Clean micropipettors according to the manufacturer's recommendations. Rinse both the interior and exterior of the micropipet shaft with 70% ethanol or 70% methanol.

Sterilizing Labware

Disposable Plasticware

Disposable sterile plasticware is generally free of RNases. If disposable sterile plasticware is unavailable, components such as microcentrifuge tubes can be sterilized and treated with diethylpyrocarbonate (DEPC), which chemically modifies and inactivates enzymes, according to the following protocol:

Caution *DEPC is toxic and extremely reactive. Always use DEPC in a fume hood. Read and follow the manufacturer's safety instructions.*

1. Add DEPC to deionized water to a final concentration of 0.1% (v/v) and mix thoroughly.
2. Place the plasticware to be treated into a separate autoclavable container. Carefully pour the DEPC-treated water into the container until the plasticware is submerged.
3. Leave the container and the beaker used to prepare DEPC-treated water in a fume hood overnight.

4. For disposal, pour the DEPC-treated water from the plasticware into another container with a lid. Autoclave the bottle of waste DEPC-treated water and the container with the plasticware for at least 30 minutes. Aluminum foil may be used to cover the container, but it should be handled with gloves and cut from an area untouched by ungloved hands.

Nondisposable Plasticware

Remove RNases from nondisposable plasticware with a chloroform rinse. Before using the plasticware, allow the chloroform to evaporate in a hood or rinse the plasticware with DEPC-treated water.

Electrophoresis Gel Boxes

To inactivate RNases on electrophoresis gel boxes, treat the gel boxes with 3% (v/v) hydrogen peroxide for 10–15 minutes and then rinse them with RNase-free water.

Glassware or Metal

To inactivate RNases on glassware or metal, bake the glassware or metal for a minimum of 8 hours at 180°C.

Treating Solutions with DEPC

Treat water and solutions (except those containing Tris base) with DEPC at 0.1% (v/v). During preparation, mix the 0.1% solution thoroughly, incubate the solution overnight at room temperature, and then autoclave it prior to use. If a solution contains Tris base, prepare the Tris solution with autoclaved DEPC-treated water.

Preventing Nucleic Acid Contamination

If the isolated RNA will be used for cDNA synthesis for cDNA library construction or PCR amplification, it is important to remove any residual nucleic acid from equipment that was used for previous nucleic acid isolations.

ENDNOTES

RiboGreen® is a registered trademark of Molecular Probes, Inc.
SpeedVac® is a registered trademark of Savant Instruments, Inc.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at <http://www.stratagene.com/MSDS/>. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.