Handbook

Dynabeads® mRNA DIRECT™ Micro Kit

Cat. no. 610.21

mRNA isolation for
RT-PCR amplification

Rev. no 003

www.invitrogen.com/dynal

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invitrogen bead separations

Introduction

The Dynabeads® mRNA DIRECT™ Micro Kit provides a fast and simple way to combine poly(A)$^+$ RNA (mRNA) isolation and PCR. The kit isolates highly purified and intact mRNA directly from small cells and tissue samples. Direct mRNA isolation is completed in only 15 minutes in a single tube, without the need to prepare total RNA or any other purifications steps. Use the isolated mRNA directly for reverse transcription into cDNA and then amplify the transcripts by PCR.

The kit contains Dynabeads® Oligo $(dT)_{25}$, uniform and superparamagnetic beads with oligo (dT) sequences covalently bound to their surface. The isolation relies on base pairing between poly (A)$^+$ residues of most mRNA and the oligo (dT) sequences on the bead surface. The bead-bound oligo (dT) is used both to capture the mRNA and as a primer for reverse transcriptase synthesis into first strand cDNA. The isolated mRNA will not need to be eluted from the beads, but rather used directly for reverse transcription and PCR amplification. The combination of direct mRNA isolation and one-tube reverse transcription allows for fast and reliable PCR detection.

The Dynabeads mRNA DIRECT™ Micro Kit provides enough reagents for 100 mRNA isolations from up to 2.5 x 10^4 mononuclear cells, up to 1 x 10^4 cultured cells or up to 5 mg tissue (depending on the tissue).

Contents

1. Product Description

Use the Dynabeads mRNA DIRECT™ Micro Kit for simple and rapid isolation of mRNA from small cell and tissue samples, for direct use in reverse transcription PCR.
The direct mRNA isolation is completed in only 15 minutes, with no need for preliminary total RNA purification. The oligo (dT) bound to the bead surface is used both to capture the mRNA and as a primer for the reverse transcriptase to synthesise the first strand cDNA.

1.1 Kit components

- **Dynabeads Oligo $(dT)_{25}$ (2 x 1 ml):**
  Supplied as approx. 5 mg/ml bead-suspension in phosphate-buffered saline (PBS) pH 7.4, containing 0.02% NaN_3 as a preservative:
  137mM NaCl
  2.7 mM KCl
  4.3 mM Na_2HPO_4 x $7H_2O$
  1.4 mM KH_2PO_4
  0.02% NaN_3

- **Lysis/Binding Buffer (15 ml):**
  100 mM Tris-HCl, pH 7.5
  500 mM LiCl
  10 mM EDTA, pH 8.0
  1% LiDS
  5 mM dithiothreitol (DTT)

- **Washing Buffer A (30 ml):**
  10 mM Tris-HCl, pH 7.5
  0.15 M LiCl
  1 mM EDTA
  0.1% LiDS
- **Washing Buffer B (30 ml):**
  10 mM Tris-HCl, pH 7.5
  0.15 M LiCl
  1 mM EDTA
- **10mM Tris-HCl (15 ml):**
  10 mM Tris-HCl, pH 7.5

Note: All reagents used are of analytical grade and RNase-free.

Note: If the buffers precipitate, warm to room temperature and mix until the precipitate dissolves.

1.2 Dynabeads Oligo $(dT)_{25}$ characteristics

Diameter: 2.8 µm (C.V max 5%)
Density: ~1.3 g/cm^3
Magnetic mass susceptibility: 16 ± 3 x 10^{-5} m^3/kg
Surface area: 4-8 m^2/g

1.3 Storage and stability

Store Dynabeads mRNA DIRECT™ Micro Kit at 2-8°C. The components in the kit are guaranteed stable until the expiry date stated on the label when stored unopened at 2-8°C.

Dynabeads Oligo $(dT)_{25}$ are stable in a pH range of 4-13.

Note: Dynabeads Oligo $(dT)_{25}$ may be frozen in the buffer they are supplied in. Avoid repeated freezing and thawing. Do not store or freeze the Dynabeads in distilled water.

The Dynabeads suspension and the buffers provided in the kit are ribonuclease-free and tested for optimal performance. Before use, resuspend Dynabeads Oligo $(dT)_{25}$ thoroughly by shaking the vial gently to disperse the beads in solution.

Note: Store the vials of Dynabeads upright to avoid drying of the beads. If the Dynabeads by accident have dried in the vial, resuspend the beads in the buffer they are supplied in by placing the vial on a mixer overnight (4°C).

1.4 Additional materials needed

- Dynal magnet: see www.invitrogen.com/magnets
- Sterile, RNase-free micro centrifuge tubes
- Sterile, RNase-free pipette tips
- Sample mixer

When working with tissue samples, the following additional materials are needed:

- Liquid nitrogen
- Manual tissue grinder
- Syringe and needle (see section 4)

All reagents used should be analytical grade and RNase-free.

1.5 Product Performance

The Dynabeads mRNA DIRECT™ Micro Kit provides enough reagents for 100 microscale mRNA isolations directly from up to 2.5 x 10^4 mononuclear cells, up to 1 x 10^4 cultured cells or up to 5 mg tissue (depending on the tissue type). The isolation is completed in only 15 minutes, without the need for intermediate total RNA isolation or other purification steps. A strong RNase inhibiting agent and stringent hybridization and washing buffers ensure isolation of intact, high purity mRNA even from crude samples rich in RNases. Enzymatic downstream applications are not inhibited by the presence of the beads.

1 ml of Dynabeads Oligo $(dT)_{25}$ can isolate 10 µg of mRNA. The specific yield will depend on the tissue or cell type. 20 µl of Dynabeads Oligo $(dT)_{25}$ are used per micro-scale isolation. 1 x 10^4 cultured cells contain 0.3 - 25 ng mRNA depending on the cell type and state.

2. Isolation Protocol

Add 100 µl Lysis/Binding Buffer to your starting sample and homogenize (e.g. by pipetting) until complete lysis is obtained.

Transfer the lysate to a tube containing 20 µl pre-washed Dynabeads® Oligo $(dT)_{25}$

Mix 5 minutes on a roller at room-temperature for mRNA to hybridize to the oligo-dT on the beads.

Place on magnet and remove supernatant. Add 100 µl Washing Buffer A and resuspend by careful pipetting.

Wash once in 100 µl Washing Buffer A and twice in 100 µl Washing Buffer B. The magnet allows for easy washing and change of buffers.

Resuspend the Dynabeads®-mRNA complex in 100 µl ice-cold Tris-HCl and store on ice for reverse transcription and downstream applications. (Elution is optional at this step.)

Immediately prior to adding the reverse transcription PCR-mix to the Dynabeads® -mRNA complex, apply the magnet and remove the supernatant.

The Dynabeads®-mRNA complex is now ready for reverse transcription and PCR.

2.1 Isolation of mRNA from cells for PCR

Please read Initial Check-list and Technical Tips (section 3) before starting the mRNA isolation protocol.

Note: Bring all buffers, except the 10 mM Tris-HCl, to room temperature prior to use.

To prevent RNA degradation by RNase contamination: USE GLOVES AND CHANGE THEM FREQUENTLY.

A. Preparation of Dynabeads Oligo $(dT)_{25}$

1. Resuspend the Dynabeads thoroughly before use.
2. Transfer Dynabeads needed for all samples (using 20 µl Dynabeads per mRNA isolation) from the stock tube suspension, to an RNase-free microcentrifuge tube.
3. Place the tube on a Dynal magnet.
4. After 30 seconds (or when the suspension is clear) remove the supernatant.
5. Remove the tube from the magnet and pre-wash Dynabeads by resuspending in Lysis/Binding Buffer to the original volume by pipetting.
6. Place the tube on the magnet and remove supernatant.

Note: Do not allow the Dynabeads to dry, as this may lower their capacity.

7. Remove the tube from the magnet and resuspend the beads in Lysis/binding buffer to the original volume. Aliquot 20 µl suspension to each sample tube.

B. Preparation of lysate from cultured cells and cell suspensions

This protocol is recommended for samples containing up to 1 x 10^4 cultured cells or up to 2.5 x 10^4 mononuclear cells.

1. Wash the cell suspension in phosphate-buffered saline (PBS) prior to preparing a cell pellet by centrifugation. The cell pellet can be used immediately, or frozen in liquid nitrogen and stored at -80°C for later use.
2. Add 100 µl Lysis/Binding Buffer to the fresh/frozen cell pellet. Perform a repeated passage of the solution through a pipette tip to obtain complete lysis. The lysate may be frozen (-80°C) and stored for later use.
3. Combine the lysate with Dynabeads Oligo $(dT)_{25}$ in the mRNA isolation protocol below, step 2.

C. Protocol for mRNA isolation for PCR amplification

1. Prepare the Dynabeads and the lysate as described in section 2.1. A and B.
2. Transfer the clear lysate to the tube containing 20 µl pre-washed Dynabeads.
3. Mix by pipetting up and down a few times.
4. Place the tube on a sample mixer or roller for 5 min. at room temperature to allow the mRNA to anneal to the Dynabeads with continuous rotation.
5. Place the sample tube on the magnet and discard the supernatant.

6. Remove the sample tube from the magnet and resuspend the Dynabeads-mRNA complex in 100 µl Washing Buffer A by careful pipetting.
7. Place the sample tube on the magnet and discard the supernatant.
8. Repeat steps 6 - 7 once.
9. Resuspend the Dynabeads-mRNA complex in 100 µl Washing Buffer B.
10. Transfer the suspension to a new tube.
11. Place the new sample tube on the magnet and discard the supernatant.
12. Resuspend the Dynabeads-mRNA complex in 100 µl Washing Buffer B.
13. Place the sample tube on the magnet and discard the supernatant.
14. Remove the sample tube from the magnet and resuspend in 100 µl of ice-cold 10mM Tris-HCl.
15. Place the sample tube on ice prior to PCR amplification. We recommend preparing the reverse transcription PCR mix before starting the mRNA isolation protocol.
16. Immediately before adding the reverse transcription PCR mix, place the tube on the magnet and discard the supernatant.
17. For one-tube PCR, resuspend the Dynabeads-mRNA complex in 50 µl reverse transcritption PCR mix and transfer to PCR-tube. For two-step PCR, resuspend the Dynabeads-mRNA complex in the reverse tran-

scription PCR reaction mix according to the manufacturers recommendation.

18. Perform cDNA synthesis as recommended by the manufacturer of the reverse transcriptase. When using a thermostable reverse transcriptase and the bead-bound oligo (dT) as primer for first strand cDNA synthesis, an initial incubation at 50°C for 5 minutes is necessary before proceeding at the recommended temperature. (See section 5.)

2.2 Isolation of mRNA from tissues for PCR

A. Preparation of lysate from plant and animal tissues

1. Grind frozen tissue sample (up to 5 mg depending on the tissue type) in a microcentrifuge tube, using a manual tissue grinder (see section 1.4). Work quickly. Aliquot (weigh) the animal or plant tissue while frozen, to avoid mRNA degradation. Use the specified amount of tissue, since an excess of tissue will reduce the mRNA yield and purity.
2. Keep the sample frozen by dipping the sample tube in liquid nitrogen.
3. Add 100 µl Lysis/Binding Buffer and thaw sample while continuing to grind until complete lysis is obtained (approx. 1-2 min). A rapid lysis in the Lysis/Binding Buffer is critical to obtain undegraded mRNA. If the raw extract is noticeably viscous a shearing step might be beneficial (see section 4).

4. Spin the lysate for 30-60 seconds in a microcentrifuge to remove debris. The lysate can be frozen and stored at -80°C for later use.

B. mRNA isolation for PCR amplification

The 100 µl lysate is combined with the 20 µl prewashed Dynabeads Oligo $(dT)_{25}$ (from section 2.1 A). Continue with the protocol for mRNA isolation for PCR amplification (section 2.1 C, step 3).

2.3 Tumour cell enrichment followed by mRNA isolation and PCR

Dynabeads® Epithelial Enrich (Cat. no. 161.01/02) will enrich epithelial tumour cells from blood or MNC. 5 ml anticoagulated whole blood samples or MNC at a concentration of 1-2 x 10^7 cells/ml are mixed with the Dynabeads coated with the monoclonal antibody BerEP4 against the human epithelial antigen (HEA). Epithelial cells bind to the beads in a 30 minute incubation. The isolated mRNA can also be used in PCR for other genes.

A. Preparation of lysate from tumour cells isolated from whole blood

1. Follow the protocol for positive isolation of tumour cells from whole blood using Dynabeads Epithelial Enrich
2. After adding the final washing solution, the bead suspension is transferred to a microcentrifuge tube. Keep the Dynabeads-cell complex on ice for immediate mRNA isolation and PCR.
3. Immediately before use, place the sample tube on the magnet for 2-3 minutes and remove supernatant.
4. Lyse the cells by resuspending the Dynabeads-cell complex in 100 µl Lysis/Binding Buffer.
5. Mix by pipetting up and down 2-3 times.

B. Preparation of lysate from tumour cells isolated from MNC

1. Follow the protocol of Dynabeads Epithelial Enrich for positive isolation of tumour cells from MNC.
2. After adding the final Washing solution, the bead suspension is transferred to a microcentrifuge tube. Keep the Dynabeads-cell complex on ice for immediate mRNA isolation and PCR.
3. Immediately before use, place the sample tube on the magnet for 2-3 minutes and remove supernatant.
4. Lyse the cells by resuspending the Dynabeads-cell complex in 100 µl Lysis/Binding Buffer.
5. Mix by pipetting up and down 2-3 times.

C. mRNA isolation for PCR amplification

1. Place the sample tube with the lysed cells on the magnet for 3 minutes.
2. Transfer the supernatant (clear lysate containing released mRNA) to a microcentrifuge tube containing 20 µl pre-washed Dynabeads Oligo $(dT)_{25}$ (from section 2.1 A).
3. Continue with the protocol for mRNA isolation for PCR amplification (section 2.1 C, step 2).

3. Initial Check-list and Technical Tips for Dynabeads mRNA DIRECT™ Micro kit

You are strongly advised to read this section before starting your mRNA isolation protocol

3.1 Check-list

1. Bring all buffers except the 10 mM Tris-HCl to room temperature before use. Store the 10 mM Tris-HCl on ice or 2-8°C prior to use.
2. Ensure that the Dynabeads Oligo $(dT)_{25}$ have been fully resuspended before use. Resuspend by brief vortexing or pipetting.
3. Check that your lysis buffer has not precipitated. If any precipitation is observed, warm to room temperature and shake to dissolve.
4. Prepare your reverse transcription PCR mix before the mRNA isolation and keep on ice.

5. When working with cells isolated by immunomagnetic separation (IMS), make sure that all IMS-Dynabeads are removed from the lysate before adding Dynabeads Oligo $(dT)_{25}$ (section 2.3 C, steps 1 and 2).
6. A rapid lysis in Lysis/binding buffer is critical for obtaining undegraded mRNA. Avoid thawing of frozen material prior to lysis.

3.2 Technical Tips

- Keep the vials of Dynabeads Oligo $(dT)_{25}$ in an upright position to ensure that the beads are covered with buffer, as drying will reduce their performance. Should the Dynabeads Oligo $(dT)_{25}$ by accident have dried, the beads can be resuspended in the buffer they are supplied in by placing the vial on a roller or equivalent overnight (4°C). This treatment will restore their complete functionality.
- Wear disposable gloves and change them frequently.
- Use sterile, RNase-free microtubes and pipette tips.
- RNase inhibitors may be added to the protocol at any step.

Note: Adding an RNase inhibitor is normally redundant.

- If elution of mRNA is necessary, add 10-20 µl 10 mM Tris-HCl and incubate at 65-80°C for 2 minutes. Place the tube on the magnet and immediately transfer the supernatant to a new microcentrifuge tube. The eluate may be used directly for reverse transcription or frozen for later use (-80°C).
- We recommend to immediately use the Dynabeads-mRNA complex for reverse transcription. If storage is needed, elute the mRNA off the beads and freeze the mRNA-containing supernatant. When storing mRNA, it is critical that no RNase is present in your sample.
- If needed, the concentration of mRNA can be measured by reading the absorbance of eluted mRNA at 260 nm. The solution must be free of Dynabeads Oligo $(dT)_{25}$ as the beads will interfere with the spectrophotometrical readings.
- For mRNA isolation from larger sample volumes, use the Dynabeads mRNA DIRECT™ kit.

4. Troubleshooting

4.1 Viscosity reduction by DNA-shearing

If the raw extract is noticeably viscous due to released DNA, a DNA-shear step should be included in your protocol. Use force to shear the DNA properly by passage through a syringe. In addition, be aware that repeated shearing causes foaming of the lysate due to a detergent in the buffer. However, this should not affect the mRNA yield. The foam can be reduced by a 30 second centrifugation.

4.2 DNA contamination

Direct mRNA isolation methods have a potential risk of DNA contamination. The Dynabeads mRNA DIRECT™ Micro kit protocol applies only small amounts of cells and tissue are used and consequently this potential problem is minimised. If high viscosity is observed, it is important to reduce this either by diluting the sample or by DNA-shearing as described above. There are several possibilities for controls to ensure the detection of cDNA and not genomic DNA:

- Choose the primers in neighbouring exons to obtain different amplicon sizes for cDNA and genomic DNA.
- Include a negative control with no reverse transcriptase added.
- Use genomic DNA specific primers to detect any contamination.
- Optional: Use RNase-free DNase to treat the mRNA sample before cDNA synthesis to get rid of any DNA contamination (ref.1).

5. Downstream Applications

5.1 Solid-phase cDNA synthesis and one-tube reverse transcription PCR

Enzymatic downstream applications are not inhibited by the presence of Dynabeads Oligo $(dT)_{25}$, hence the bead-bound mRNA can be used directly for solid-phase cDNA synthesis and reverse transcription PCR (Ref. 2,3,4,5,6,7).

The oligo (dT) sequence on the Dynabeads is used not only to capture the mRNA, but also as a primer for the subsequent reverse transcriptase synthesis into cDNA. The resulting first strand cDNA is covalently linked to the surface of the Dynabeads, and may be used for cDNA amplification. The Dynabeads solid-phase technology is compatible with most cDNA-synthesis kits commercially available. Perform cDNA synthesis as recommended by the manufacturer of the reverse transcriptase. When using thermostable reverse transcriptase and the bead-bound oligo (dT) as primer for first strand cDNA synthesis, an initial step of incubation at 50°C for 5 minutes is necessary before proceeding at recommended temperature. The PCR is not inhibited by the presence of the Dynabeads.

cDNA synthesis and PCR can be performed sequentially in one tube, i.e. in the same reaction buffer using an enzyme capable of both RNA and DNA dependant polymerization. The combination of direct mRNA isolation using Dynabeads Oligo $(dT)_{25}$ and one-tube reverse transcription PCR offers a convenient system for fast and reliable PCR detection.

5.2 Construction of immobilised cDNA libraries for multiple PCR amplifications

A reusable solid-phase cDNA library can be made directly on the bead-surface. The first strand cDNA (using the bead-bound oligo (dT) as primer) is covalently-linked to the Dynabeads (Ref. 2,5,6,7,8,9), and can be reused for multiple PCR amplifications of specific transcripts. The different transcripts are amplified by adding different specific primer sets in successive PCR reactions using the solid-phase cDNA library as template.

Some of the advantages are:

- Allows multiple analysis of precious materials and small samples.
- One extraction allows the amplification of several gene transcripts.
- Enables simple and rapid buffer changes required to optimise the conditions for specific enzymes.

5.3 PCR amplification from a reusable solid-phase cDNA library

1. Add PCR-mix with primers and Taq polymerase, and resuspend the cDNA-Dynabeads properly.
2. Cycle twice to generate enough template for further amplification with a 5 min. extension at 72°C.
3. Melt the strands at 94°C for 2 min, place on magnet and transfer the supernatant with the amplification product to a new PCR tube. Continue the cycling reaction.

4. Wash the cDNA-Dynabeads twice in 10 mM Tris-HCl or 1 x PCR buffer and reuse them by adding a new PCR-mix for amplification of a different transcript. For storage of the cDNA library use TE-buffer or equivalent
5. Amplified PCR-products are analysed by standard molecular methods.

Note: If it is not necessary to reuse the cDNA-Dynabeads, just run the PCR with the beads present through the cycling reactions as described.

6. Other Dynabeads® Oligo $(dT)_{25}$ Products

Cat. no.	Product description
610.11/12	Dynabeads® mRNA DIRECT™ Kit For direct isolation of mRNA from cells, animal and plant tissue.
610.06	Dynabeads® mRNA Purification Kit For mRNA purification from total RNA.
610.02	Dynabeads® Oligo $(dT)_{25}$, (2 x 1 ml)
610.05	Dynabeads® Oligo $(dT)_{25}$, (5 x 1 ml)

7. References

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   Reusable cDNA libraries coupled to magnetic beads.
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5. Raineri I, *et al.*
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   HIV-1 promotor insertion revealed by selective detection of chimeric provirus-host gene transcripts.
   Nucleic Acids Res. 1992;20:6261-6266

7. Sharma *et al.*
PCR-based construction of subtractive cDNA library using magnetic beads.
BioTechniques 1993;15:610-611
8. Fellmann F, *et al.*
Simplified Protocol of Solid-Phase cDNA Libraries for Multiple PCR Amplication
BioTechniques 1996;21:766-770
9. Karrer EE, *et al*.
In Situ isolation of mRNA from individual plant cells: Creation of cell-specific cDNA libraries.
Proc. Natl. Acad. Sci. USA 1995;92:3814-3818

8. Warnings and Limitations

Invitrogen Dynal AS complies with the Quality System Standards ISO 9001:2000 and ISO 13485:2003.

The Dynabeads® mRNA DIRECT™ Micro Kit is guaranteed stable until the expiry date stated on the label when stored unopened at 2-8°C.

The Dynabeads mRNA DIRECT™ Micro Kit is for research use only.

The phosphate-buffered saline (PBS) contains 0.02% sodium azide (NaN_3) as a preservative. Sodium azide may react with lead and copper drain-pipes to form highly explosive metal azides. When disposing through drains, flush with large volumes of water to prevent azide build-up. Sodium azide is toxic if ingested. Avoid pipetting by mouth.

RNase contamination should be prevented by standard

procedures during the preparation of starting material and during the experiment.
Prior to use, the Dynabeads Oligo $(dT)_{25}$ should be washed once in Lysis/binding buffer as described in the protocol. Resuspend the Dynabeads Oligo $(dT)_{25}$ well to obtain a homogenous dispersion of beads in solution.
Material Safety Data Sheet is available from www.invitrogen.com.
Certificate of Analysis/Compliance is available upon request.

9. Warranty

The products are warranted to the original purchaser only to conform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Invitrogen Dynal's obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Invitrogen Dynal expense, of any products which shall be defective in manufacture, and which shall be returned to Invitrogen Dynal, transportation prepaid, or at Invitrogen Dynal's opinion, refund of the purchase price. It shall be the responsibility of the purchaser to pack returned item(s) in a manner to avoid shipping damage to the unit. Claims for merchandise damaged in transit must be submitted to the carrier.
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SPEC-06241