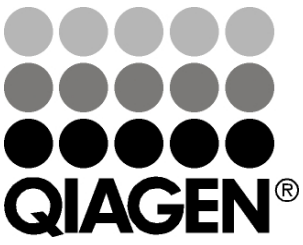
June 2012

Oligotex® Handbook

For purification of poly A^+ RNA from total RNA and directly from cultured cells or tissues as well as purification of polyadenylated in vitro transcripts



Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Contents

Kit Contents

Oligotex mRNA Kits	**Mini**	**Midi**
Catalog no.	**70022**	**70042**
Kit capacity (total RNA)	**3 mg**	**12 mg**
Oligotex Suspension*	200 µl	700 µl
Buffer OBB (binding buffer)† ‡	7 ml	7 ml
Buffer OW2 (wash buffer)†	2 x 19 ml	2 x 19 ml
RNase-free water	10 ml	10 ml
Buffer OEB (elution buffer)†	2 x 1.5 ml	2 x 1.5 ml
Small Spin Columns	12	12
Large Spin Columns	–	–
Microcentrifuge Tubes (1.5 ml)	36	36
Quick-Start Protocol	2	2

* Oligotex Suspension contains 0.1% sodium azide (NaN_3) as a preservative. Sodium azide is highly toxic and may react explosively with lead or copper drain pipes. Take appropriate safety measures and wear gloves when handling. Dispose of azide-containing solutions according to your institution's waste-disposal guidelines.

† Oligotex buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

‡ Not compatible with disinfecting reagents containing bleach. Contains a guanidine salt, which is an irritant. Take appropriate safety measures and wear gloves when handling.

Oligotex Direct mRNA Kits	Mini	Midi/Maxi
Catalog no.	**72022**	**72041**
Kit capacity	**See page 8**	
Oligotex Suspension*	420 µl	1 ml
Buffer OL1 (lysis buffer)† ‡	16 ml	16 ml
Buffer ODB (dilution buffer)†	35 ml	35 ml
Buffer OW1 (wash buffer 1)†	5 ml	5 ml
Buffer OW2 (wash buffer 2)†	19 ml	19 ml
Buffer OEB (elution buffer)†	3 ml	4.5 ml
Small Spin Columns	12	–
Large Spin Columns	–	6
Microcentrifuge Tubes (1.5 ml)	36	18
Quick-Start Protocol	2	2

* Oligotex Suspension contains 0.1% sodium azide (NaN_3) as a preservative. Sodium azide is highly toxic and may react explosively with lead or copper drain pipes. Take appropriate safety measures and wear gloves when handling. Dispose of azide-containing solutions according to your institution's waste-disposal guidelines.

† Oligotex buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

‡ Not compatible with disinfecting reagents containing bleach. Contains a guanidine salt, which is an irritant. Take appropriate safety measures and wear gloves when handling.

Storage

Store kits and Oligotex Suspension at room temperature (15–25°C). Some settling may occur during storage. The Oligotex particles may be easily resuspended by heating to 37°C and shaking before use. Oligotex Suspension and kits are stable for 12 months at room temperature. Oligotex should be stored only in the storage buffer provided. Do not freeze.

Intended Use

The Oligotex mRNA Mini Kit and the Oligotex Direct mRNA Mini Kit are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste when using Oligotex Direct mRNA Kits.

Buffer OL1, included in Oligotex Direct mRNA Kits, contains guanidine thiocyanate, which can form highly reactive compounds when combined with bleach.

If liquid containing this buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the Oligotex mRNA Kit and Oligotex Direct mRNA Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Kit descriptions

Oligotex mRNA Kits

Oligotex mRNA Kits contain spin columns and all necessary reagents and buffers for isolation of pure poly A^+ mRNA from total RNA preparations in less than 30 minutes with >90% recovery. Isolation of poly A^+ mRNA from total RNA usually provides slightly greater enrichment than direct isolation from cells or tissues, since there is no risk of interference by other cellular components. For sensitive applications, the Oligotex mRNA procedure is generally preferred over the Oligotex Direct mRNA procedure. Oligotex Suspension is also available separately. See ordering information, page 95.

Oligotex Direct mRNA Kits

Oligotex Direct mRNA Kits allow isolation of pure poly A^+ mRNA directly from cells, tissues, or small amounts of blood in less than 1 hour (standard protocols) or less than 30 min (optional shortened protocols). The Oligotex Direct mRNA purification procedure utilizes rigorous denaturing lysis conditions to generate an immediate RNase-free environment for the isolation of intact mRNA. The optimal design of Oligotex particles, in combination with RNA-protecting lysis and hybridization conditions, results in efficient purification of poly A^+ mRNA in a short time with greater than 90% recovery. Oligotex Direct mRNA Kits contain reagents, buffers, and spin columns for the purification of poly A^+ mRNA directly from cells, tissues, or small amounts of blood. See ordering information, page 95.

Kit capacities

Oligotex mRNA Kits and Oligotex Direct mRNA Kits are designed for specific ranges of starting material, and also offer flexibility for use with widely varying amounts. For example, the Oligotex Direct mRNA Micro Kit contains spin columns and reagents for 12 micro preps ($\leq 1 \times 10^6$ cells each). Alternatively, as many as 2×10^7 cells can be processed at one time with this kit. Preps of this size require larger volumes of reagents, and so only 2 preps of this size can be performed with the Oligotex Direct mRNA Micro Kit. Table 1 (below), Table 2 (page 10), and Table 3 (page 11) provide guidelines on how to customize Oligotex Kits to the desired prep size.

Table 1 (below) and Table 2 (page 10) give the maximum number of preps per kit for the Oligotex mRNA Kits with different starting amounts of total RNA. Up to 1 mg total RNA can be processed per prep with the small spin columns included in the Oligotex mRNA Mini and Midi Kits. Using the Oligotex mRNA

batch protocol (see page 25), this amount can be scaled up to 3 mg per prep. With the Oligotex mRNA Maxi kit, up to 3 mg total RNA can be processed using either a batch procedure or the large spin columns included in the kit.

Table 1. Kit capacities for Oligotex mRNA Kits (spin column protocol)

Amount of total RNA in starting material per prep, mg	Maximum number of preps per kit		
	Mini	Midi	Maxi
≤0.25	12 (13)*	12 (28)*	6 (17)*
0.25–0.50	6	12 (14)*	6 (14)*
0.50–0.75	4	12 (14)*	6 (14)*
0.75–1.00	3	12	6 (12)*
1.0–1.5	–	–	6 (8)*
1.5–2.0	–	–	6
2.0–2.5	–	–	5
2.5–3.0	–	–	4

* Maximum number of preps per kit with purchase of additional spin columns. Small spin columns (recommended for all preps with <1.0 mg total RNA) are included in Oligotex mRNA Mini and Midi Kits and can be ordered separately for use with the Oligotex mRNA Maxi Kit. See page 98 for ordering information. Additional 1.5 ml microcentrifuge tubes may be required.

Table 2. Kit capacities for Oligotex mRNA Kits (batch protocol)

Amount of total RNA in starting material per prep, mg	Maximum number of preps per kit		
	Mini	Midi	Maxi
≤0.25	13	14	7
0.25–0.50	6	14	7
0.50–0.75	4	14	7
0.75–1.00	3	12	7
1.0–1.5	2	8	7
1.5–2.0	1	6	6
2.0–2.5	1	5	5
2.5–3.0	1	4	4

Table 3 (below) gives the maximum number of preps for the Oligotex Direct mRNA Kits using different amounts of starting material. Up to 2 x 10^7 cells or 100 mg tissue per prep can be processed with the Oligotex Direct mRNA Micro or Mini Kits. Up to 1 x 10^8 cells or 1 g tissue can be processed per prep with the Oligotex Direct mRNA Midi/Maxi Kit. Use of larger amounts may result in reduced yields and impure mRNA preparations.

Table 3. Kit capacities for Oligotex Direct mRNA Kits

Amount of starting material per prep	Maximum number of preps per kit		
	Micro	Mini	Midi/Maxi
Number of cells			
100*– 2 x 10^5	12	12 (14)†	6 (14)†
2 x 10^5 – 5 x 10^5	12	12 (14)†	6 (14)†
5 x 10^5 – 1 x 10^6	12	12 (14)†	6 (14)†
1 x 10^6 – 5 x 10^6	7	12	6 (14)†
5 x 10^6 – 1 x 10^7	3	6	6 (14)†
1 x 10^7 – 2 x 10^7	2	3	6 (9)†
2 x 10^7 – 3 x 10^7	–	–	6 (7)†
3 x 10^7 – 5 x 10^7	–	–	6
5 x 10^7 – 7 x 10^7	–	–	4
7 x 10^7 – 1 x 10^8	–	–	2
Tissue, mg			
≤10	12	12 (14)†	6 (14)†
10–25	7	12 (14)†	6 (14)†
25–50	3	6	6 (14)†
50–100	2	3	6 (9)†
100–150	–	–	6 (7)†
150–250	–	–	6
250–500	–	–	4
500–750	–	–	2
750–1000	–	–	2

* Lowest amount tested.

† Maximum number of preps per kit with purchase of additional spin columns. Small spin columns (recommended for all preps with <2 x 10^7 cells or <100 mg tissue) are included in Oligotex Direct mRNA Micro and Mini Kits and can be ordered separately for use with the Oligotex Direct mRNA Midi/Maxi Kit. See page 98 for ordering information. Additional 1.5 ml microcentrifuge tubes may be required.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

For Oligotex mRNA Kits or Oligotex Direct mRNA Kits:

- Water bath or heating block
- Sterile, RNase-free pipet tips
- Microcentrifuge
- Disposable gloves

For Oligotex Direct mRNA Kits only:

- Equipment for disruption and homogenization (see "Disruption and homogenization of starting material", page 39)
- Sterile, RNase-free, 2 ml microcentrifuge tubes
- 14.3 M β-mercaptoethanol (β-ME)* (**Note**: Stock solutions are usually 14.3 M.)
- Sterile polypropylene or glass (Corex®) centrifuge tubes (Direct midi or maxi preps only)
- QIAGEN Centrifuge 4–16K or equivalent (Direct midi/maxi preps only)

* β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

Important Notes

RNA abundance

A typical mammalian cell contains 10–30 pg total RNA. The majority of RNA molecules, however, are tRNAs and rRNAs. mRNA accounts for only 1–5% of the total cellular RNA although the actual amount depends on the cell type and physiological state. Approximately 360,000 mRNA molecules are present in a single mammalian cell, with approximately 12,000 different mRNA species per cell. Some mRNAs comprise as much as 3% of the mRNA pool whereas others account for less than 0.01%. These "rare" or "low abundance" messages may have a copy number of only 5–15 molecules per cell. However, these rare species may account for as much as 11,000 different mRNA species, comprising 45% of the mRNA population. For more information, see Alberts, B. et al. (1994) *Molecular Biology of the Cell*, 3rd ed., New York: Garland Publishing, Inc.

Due to the low proportion of mRNA in the total cellular RNA pool, reducing the amount of rRNA and tRNA in a total RNA preparation increases the relative amount of mRNA. mRNA enrichment is essential for construction of cDNA libraries and other applications where intact mRNA is highly desirable. The probability of selecting the right clone is greatly increased by reducing the amount of unwanted rRNA and tRNA. With pure, intact mRNA preparations, even low level messages can easily be detected by in vitro translation, northern hybridization, S1 analysis, expression-array and expression-chip analysis, or SAGE technology. Isolation of pure, intact mRNA is of great importance when characterizing mRNA species with these techniques.

RNA stabilization in tissue

RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological materials is necessary because, directly after harvesting the biological sample, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes in gene expression pattern need to be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses, quantitative RT-PCR, such as TaqMan® and LightCycler® technology, or other nucleic acid-based technologies, such as NASBA® and bDNA analysis or any other related technologies.

RNA*later*® RNA Stabilization Reagent (see page 97 for ordering information) represents a new technology enabling rapid and reliable preservation of gene-expression patterns in biological material so as to provide reliable gene-expression analysis. Alternatively, use Allprotect Tissue Reagent, which provides

immediate stabilization of DNA, RNA, and protein in tissues samples at room temperature.

RNA*later* technology is designed for stabilization and protection of cellular RNA in animal tissues. The samples are harvested and immediately submerged in RNA*later* RNA Stabilization Reagent for storage for:

- 1 day at 37°C
- 7 days at 18 to 25°C
- 4 weeks at 2 to 8°C
- Archival storage at –20°C or –80°C

During storage in RNA*later* RNA Stabilization Reagent, even at elevated temperatures (e.g., at room temperature, 15–25°C, or 37°C), the cellular RNA remains intact and undegraded. RNA*later* technology replaces current inconvenient, dangerous and equipment-intensive methods like snap-freezing of samples in liquid nitrogen, storage at –80°C, cutting and weighing on dry ice, or immediate processing of the harvested samples.

RNA*later* RNA Stabilization Reagent cannot be used for stabilization of RNA in whole blood, plasma, or serum.

Oligotex description

Oligotex Suspension is a affinity reagent for the detection, isolation, purification, and enzymatic modification of nucleic acids containing polyadenylic acid sequences. Oligotex Suspension consists of polystyrene–latex particles of uniform size (1.1 µm diameter) and a perfect spherical shape (Figure 1). dC10T30 oligonucleotides are covalently linked to the surface of the polystyrene–latex particles via a condensation reaction. This linkage is very stable to heat, alkali, and formamide, allowing easy dissociation of isolated poly A^+ nucleic acids.

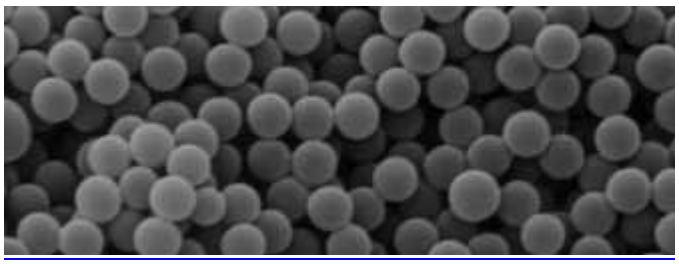


Figure 1. Scanning electron micrograph of Oligotex particles at 7500x magnification.

The size, composition, and surface structure of Oligotex particles have been optimized for uniform dispersion and minimal centrifugation time. The particles form a stable suspension that provides a large surface area for rapid and efficient binding of polyadenylic acids. Larger particles would tend to sediment during incubation, decreasing their availability for hybridization, and smaller

particles would require longer centrifugation times. With Oligotex particles, the higher capacity and accuracy of hybridization provides superior purification of poly A^+ mRNA compared to other oligo-dT matrices.

Binding capacity

For a typical 1900-nucleotide mature mRNA molecule, 1 pmol of mRNA corresponds to approximately 0.6 µg. The maximal binding capacity of Oligotex particles is 10 pmol per mg of resin. A 10 µl volume of the 10% Oligotex Suspension contains 1 mg of resin. So the maximal theoretical binding capacity is 6 µg mRNA per 10 µl Oligotex Suspension.

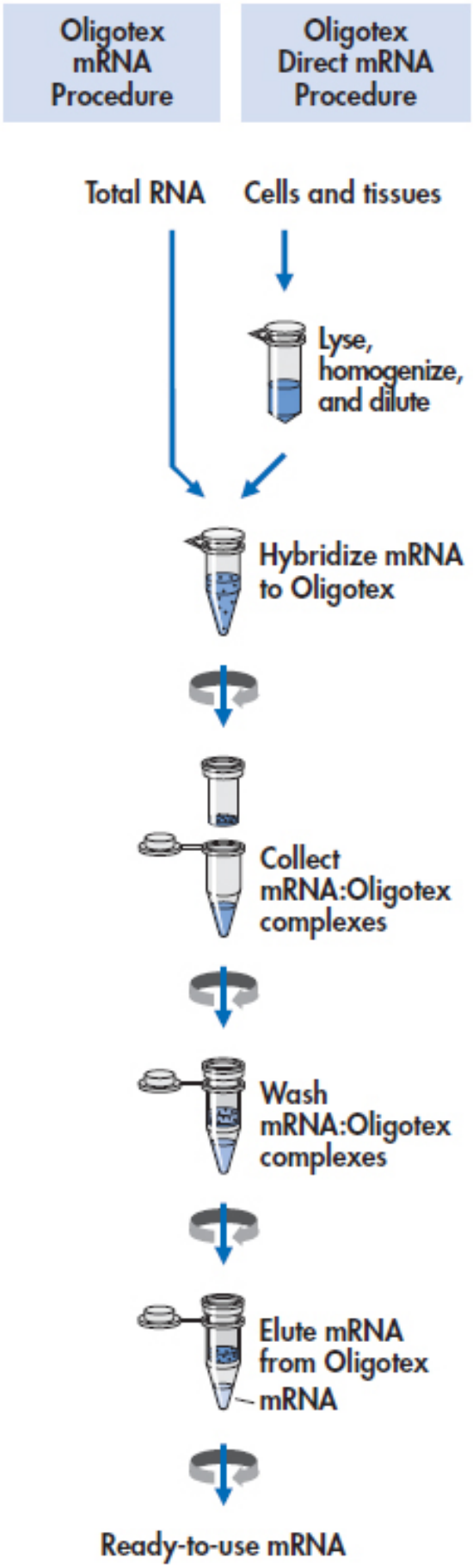
The Oligotex principle

The Oligotex procedure for isolation, purification, and manipulation of poly A^+ RNA takes advantage of the fact that most eukaryotic mRNAs (and some viral RNAs) end in a poly-A tail of 20–250 adenosine nucleotides. This poly-A tail is added to the RNA transcript in the nucleus following transcription. In contrast, rRNAs and tRNAs, which account for over 95% of cellular RNAs, are not polyadenylated. The poly-A tail provides a useful tool for separation, selective isolation, or specific enzymatic modification of eukaryotic mRNAs (1–5% of total cellular RNA). Poly A^+ mRNA can be purified by hybridizing the poly-A tail to a dT oligomer coupled to a solid-phase matrix. rRNA and tRNA species, without a poly-A tail, do not bind to the oligo-dT and are easily washed away. Since hybridization requires high-salt conditions, the poly A^+ mRNA can then easily be released by lowering the ionic strength and destabilizing the dT:A hybrids.

The optimized design of Oligotex particles provides superior poly A^+ mRNA-binding capacity through fast and efficient hybridization. mRNA recoveries are consistently greater than 90%. The Oligotex purification procedure minimizes loss of poly A^+ RNA, eliminates the risk of degradation by RNases, and requires minimal hands-on time. This makes it highly suited for simultaneous handling of multiple samples.

Oligotex technology overcomes the many difficulties associated with oligo-dT cellulose. Oligotex Suspension is a ready-to-use suspension that eliminates the need to equilibrate powder or dissolve tablets. It consists of small polystyrene–latex particles that do not require the extensive washing steps associated with oligo-dT cellulose. With Oligotex technology, mRNA can be eluted in a small volume, making alcohol precipitation unnecessary. Poly A^+ mRNA purified with Oligotex technology is ready for immediate use in any downstream applications, such as:

- RT-PCR
- cDNA synthesis
- cDNA library construction
- In vitro translation
- SAGE technology
- Expression-array and expression-chip analysis
- RNase and S1 nuclease protection
- Primer extension
- RNA northern, dot, and slot blotting
- Microinjection
- RNA-Seq
- Next-generation sequencing (NGS)


Oligotex mRNA Procedure
Oligotex Direct mRNA Procedure
Total RNA
Cells and tissues
Lyse, homogenize, and dilute
Hybridize mRNA to Oligotex
Collect mRNA:Oligotex complexes
Wash mRNA:Oligotex complexes
Elute mRNA from Oligotex
mRNA
Ready-to-use mRNA

Additional applications

Besides the standard protocols described in this handbook, Oligotex technology works well for a variety of additional applications involving poly A^+ RNA (see Figure 2). Oligotex particles provide an excellent support for use in cDNA synthesis, subtractive hybridization, and cDNA cloning. Poly A^+ RNA hybridized to Oligotex particles can be directly transcribed into cDNA by reverse transcriptase. cDNAs immobilized on Oligotex particles can then be directly amplified for analytical RT-PCR, used for subtractive hybridization, or used for subtractive hybridization following PCR. Oligotex technology makes subtractive hybridization simple and convenient, even with small amounts of RNA. Contact QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com) for basic protocols based on the published literature and customer data.

Oligotex technology has also been used successfully for cleanup of poly-A tailed oligonucleotides.

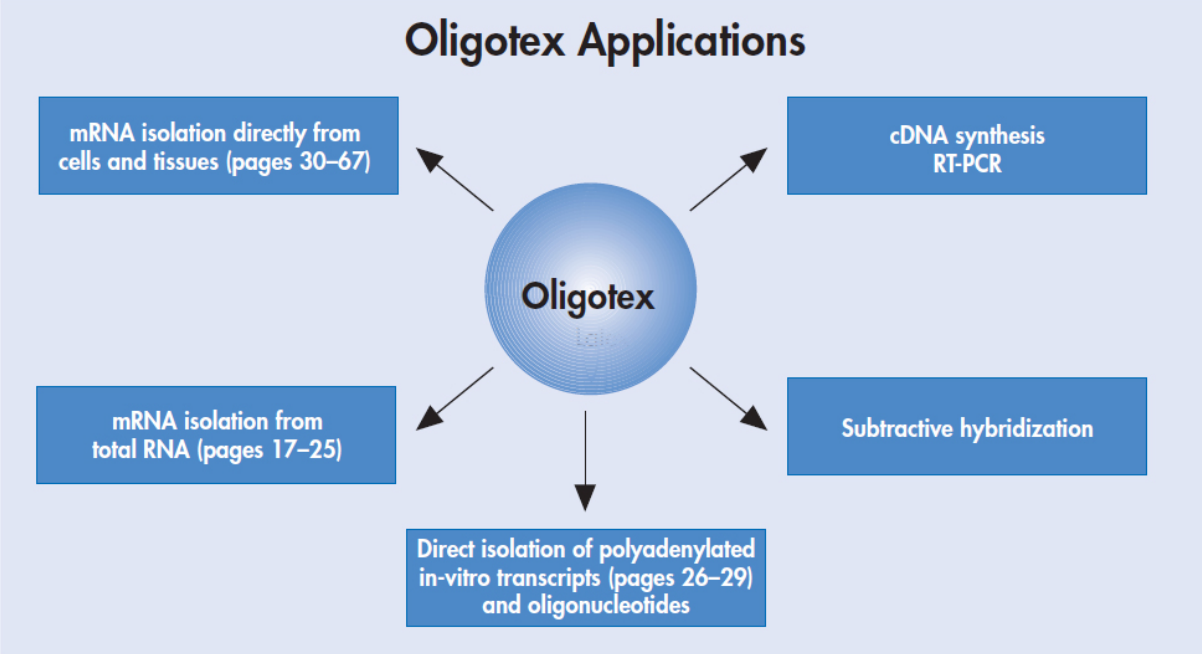


Figure 2. Oligotex applications.

Oligotex mRNA Purification Protocols

Description of protocols

Purification of mRNA with the Oligotex mRNA spin column protocol (page 22) is an enrichment of poly A^+ RNA from total RNA. Oligotex resin can be used with total RNA purified by various methods, but better results are generally obtained with purer starting material. Experiments at QIAGEN suggest that RNA purified

in the absence of phenol is typically purer for most downstream applications, including subsequent mRNA purification. For best results, we recommend starting with total RNA purified using silica-membrane technology. For example, RNeasy® Kits can be used to isolate total RNA from a variety of starting materials and provide high-quality RNA ideal for use with Oligotex technology. See page 98–99 for ordering information.

Although the spin column protocol is generally recommended, the Oligotex mRNA batch protocol (page 25) may be necessary if you are using impure total RNA or if you are unsure about the purity of your total RNA. Many isolation procedures do not remove contaminants such as protein that can clog Oligotex spin columns. Better results are generally obtained with purer starting material.

The Oligotex mRNA spin column protocol for purification of in vitro transcripts (page 28) can be used for purification of poly A^+ in vitro transcripts, such as those generated by nuclear-extract transcription systems. In vitro transcripts from most bacterial systems (e.g., T3 or T7) are not polyadenylated and will not be selected in the Oligotex procedure.* For purification of in vitro transcripts that are not polyadenylated, RNeasy Kits can be used (see pages 98–99 for ordering information).

Although the spin column protocol is generally recommended, the Oligotex mRNA batch protocol for purification of in vitro transcripts (page 31) may be necessary for in vitro transcription reactions that are especially viscous or contain large amounts of protein or other contaminants that could clog Oligotex spin columns.

The Oligotex mRNA protocol using Oligotex Direct mRNA buffers (Appendix D, page 87) can be used for purification of poly A^+ RNA from total RNA using buffers from the Oligotex Direct mRNA Kits. Although these buffers are designed for direct isolation of mRNA from animal cells and tissues (see "Oligotex mRNA Purification Protocols", page 18), equal yields and RNA quality are generally obtained as with the buffers provided in the Oligotex mRNA Kits.

Poly A^+ mRNA purified with Oligotex technology does not require further purification and is ready to use in any standard application such as northern, dot and slot blotting, expression-array and expression-chip analysis, in vitro translation, S1 nuclease analysis, RNase protection, primer extension, cDNA synthesis, RT-PCR, microinjection, cDNA library construction, subtractive cDNA cloning, and SAGE technology.

* Unless the transcript itself contains a poly-A tract. This could be included either in the vector or in the insert. For example, a eukaryotic cDNA insert may include part of the poly-A tail, and some bacterial transcription vectors include a poly-A tract at the end of the transcript.

Quantification of starting RNA

The concentration of RNA should be determined prior to Oligotex mRNA purification by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml ($A_{260}=1 \Rightarrow 40$ µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. As discussed below (see "Purity of starting RNA"), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1M NaOH, 1 mM EDTA followed by washing with RNase-free water (see "Solutions", page 82). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris, pH 7.0 (1/50 dilution).

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free).

A_{260} = 0.55

Concentration of RNA sample	= 40 µg/ml x A_{260} x dilution factor
	= 40 µg/ml x 0.55 x 50
	= 1100 µg/ml
Total amount	= concentration x volume of sample in ml
	= 1100 µg/ml x 0.1 ml
	= 110 µg of RNA

Purity of starting RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

1.9–2.1* in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution in which the RNA is diluted.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 $\Rightarrow$ 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of starting RNA", above).

In the Oligotex mRNA protocols, better results are generally obtained with purer starting material. Experiments at QIAGEN suggest that RNA purified in the absence of phenol is typically purer for most downstream applications, including subsequent mRNA purification. For best results we recommend starting with total RNA purified using silica-membrane technology.

Oligotex terminology for mRNA preparations from total RNA

For convenience, we have classified the size of mRNA preparation according to the amount of total RNA processed. Oligotex mRNA Kits are not restricted to their nominal prep size. The kits offer flexibility for use with widely varying amounts of starting material. See Table 1 (page 9) and Table 2 (page 10) for guidelines to customize Oligotex mRNA Kits to the desired prep size.

- Mini prep <250 µg total RNA
- Midi prep 250 µg – 1 mg total RNA
- Maxi prep 1–3 mg total RNA

* Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

Protocol: Purification of Poly A$^+$ mRNA from Total RNA using Spin Columns

Important notes before starting

- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- Read "Introduction" (page 8) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Determine the amount of total RNA in the RNA sample (see "Quantification of starting RNA", page 20).
- Buffer OBB may form a precipitate upon storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- ▲ denotes mini/midi prep volumes (with ≤1.0 mg total RNA); ●denotes maxi prep volumes (with 1.0–3.0 mg total RNA).

Procedure

1. **Determine the amount of starting RNA. Do not use more than ▲ 1 mg or ● 3 mg total RNA. Pipet total RNA into an RNase-free 1.5 ml microcentrifuge tube, and adjust the volume with RNase-free water (if necessary) to the volume indicated in Table 4, below.**

   Note: The initial volume of the RNA solution is not important so long as the volume can be brought up to the indicated amount with RNase-free water. If starting with precipitated RNA, dissolve the RNA pellet in the appropriate amount of RNase-free water by heating the tube for 3 min at 60°C followed by vortexing for 5 s and sharply flicking the tube. Repeat at least twice.

2. **Add the appropriate volume of Buffer OBB and Oligotex Suspension (see Table 4, below).**

   Mix the contents thoroughly by pipetting or flicking the tube.

Table 4. Buffer amounts for Oligotex mRNA spin column protocol

Total RNA	Add RNase-free water to final volume, µl	Buffer OBB, µl	Oligotex Suspension, µl	Prep size
▲ ≤0.25 mg	250	250	15	Mini
▲ 0.25–0.50 mg	500	500	30	Midi
▲ 0.50–0.75 mg	500	500	45	Midi
▲ 0.75–1.00 mg	500	500	55	Midi
● 1.0–1.5 mg	650	650	85	Maxi
● 1.5–2.0 mg	650	650	115	Maxi
● 2.0–2.5 mg	650	650	135	Maxi
● 2.5–3.0 mg	650	650	175	Maxi

1. **Incubate the sample for 3 min at 70°C in a water bath or heating block.**

   This step disrupts secondary structure of the RNA.

2. **Remove sample from the water bath/heating block, and place at 20–30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

3. **Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed (14,000–18,000 x *g*), and carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

4. **Resuspend the Oligotex:mRNA pellet in ▲ 400 µl or ● 600 µl Buffer OW2 by vortexing or pipetting, and pipet onto a ▲ small spin column or a ● large spin column placed in a 1.5 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed.**

   Small spin columns are supplied with the Oligotex mRNA Mini and Midi Kits and can be purchased separately (see page 95 for ordering information). Large spin columns are supplied in the Oligotex Maxi Kits.

5. **Transfer the spin column to a new RNase-free 1.5 ml microcentrifuge tube, and apply ▲ 400 µl or ● 600 µl Buffer OW2 to the column. Centrifuge for 1 min at maximum speed and discard the flow-through.**
6. **Transfer spin column to a new RNase-free 1.5 ml microcentrifuge tube. Pipet 20–100 µl hot (70°C) Buffer OEB onto the column, pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.**

   Note: The volume of Buffer OEB used depends on the expected or desired concentration of poly A^+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.
7. **To ensure maximal yield, pipet another 20–100 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.**

   To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Protocol: Purification of Poly A$^+$ mRNA from Total RNA using a Batch Procedure

Important notes before starting

- This protocol may be necessary if you are using impure total RNA or if you are unsure about the purity of your total RNA. Many isolation procedures do not remove contaminants such as protein that can clog Oligotex spin columns. Better results are generally obtained with purer starting material.
- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- Read "Introduction" (page 8) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Determine the amount of total RNA in the RNA sample (see "Quantification of starting RNA", page 20).
- Buffer OBB may form a precipitate upon storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).

Procedure

1. **Determine the amount of starting RNA. Do not use more than 3 mg total RNA. Pipet total RNA into an RNase-free 1.5 ml microcentrifuge tube, and adjust the volume with RNase-free water (if necessary) to the volume indicated in Table 5, below.**

   Note: The initial volume of the RNA solution is not important so long as the volume can be brought up to the indicated amount with RNase-free water. If starting with precipitated RNA, dissolve the RNA pellet in the appropriate amount of RNase-free water by heating the tube for 3 min at 60°C followed by vortexing for 5 s and sharply flicking the tube. Repeat at least twice.

2. **Add the appropriate volume of Buffer OBB and Oligotex Suspension (see Table 5, below).**

   Mix the contents thoroughly by pipetting or flicking the tube.

Table 5. Buffer amounts for Oligotex mRNA batch protocol

Total RNA, mg	Add RNase-free water to final volume, µl	Buffer OBB, µl	Oligotex Suspension, µl	Prep size
≤0.25	250	250	15	Mini
0.25–0.50	500	500	30	Midi
0.50–0.75	500	500	45	Midi
0.75–1.00	500	500	55	Midi
1.0–1.5	650	650	85	Maxi
1.5–2.0	650	650	115	Maxi
2.0–2.5	650	650	135	Maxi
2.5–3.0	650	650	175	Maxi

3. **Incubate the sample for 3 min at 70°C in a water bath or heating block.**

   This step disrupts secondary structure of the RNA.

4. **Remove sample from the water bath/heating block, and place at 20 to 30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

5. **Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed (14,000–18,000 x *g*), and carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

6. **Resuspend the Oligotex:mRNA pellet in 1 ml Buffer OW2 by vortexing or pipetting. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed, and carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure.

7. **Repeat step 6 once.**

8. **Add 20–100 µl hot (70°C) Buffer OEB. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A^+ mRNA, to another RNase-free tube.**

   Note: The volume of Buffer OEB used depends on the expected or desired concentration of poly A^+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with Oligotex and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

9. **To ensure maximal yield, add another 20–100 µl hot (70°C) Buffer OEB to the Oligotex pellet. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A^+ mRNA, and combine the eluates.**

Protocol: Purification of In Vitro Transcripts using Spin Columns

Important notes before starting

- This protocol can be used for purification of poly A^+ in vitro transcripts, such as those generated by nuclear-extract transcription systems. In vitro transcripts from most bacterial systems (e.g., T3 or T7) are not polyadenylated and will not be selected by the Oligotex procedure.* For purification of in vitro transcripts that are not polyadenylated, RNeasy Kits can be used (see pages 98–98for ordering information).
- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- Read "Introduction" (page 8) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Buffer OBB may form a precipitate upon storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).

Procedure

1. **Adjust volume of the in vitro transcription sample with RNase-free water to 200 µl.**

   Note: Any volume up to 200 µl can be used. For samples <200 µl, add RNase-free water to 200 µl final volume.

* Unless the transcript itself contains a poly-A tract. This could be included either in the vector or in the insert. For example, a eukaryotic cDNA insert may include part of the poly-A tail, and some bacterial transcription vectors include a poly-A tract at the end of the transcript.

2. **Add 200 µl Buffer OBB and the appropriate amount of Oligotex Suspension (see equation below).**

   Determine the volume of Oligotex Suspension required (in microliters) according to the following equation:

   3 x transcript yield (µg)/transcript length (kb) = volume of Oligotex Suspension (µl)

   Example: For an expected yield of 5 µg of a 1.5 kb transcript, use 10 µl Oligotex

   Suspension (3 x 5 µg/1.5 kb = 10 µl).

3. **Mix the contents by pipetting or flicking the tube. Incubate for 3 min at 70°C in a water bath or heating block.**

   This step disrupts RNA secondary structure.

4. **Remove sample from the water bath/heating block, and place at 20 to 30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

5. **Transfer contents of the tube to a small spin column or a large spin column placed in a microcentrifuge tube. Centrifuge for 1 min at maximum speed.**

   Small spin columns are supplied with the Oligotex mRNA Mini and Midi Kits and can be purchased separately (see page 98 for ordering information). Large spin columns are supplied in the Oligotex Maxi Kits.

   Note: Save the flow-through until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

6. **Transfer the spin column to a new RNase-free 1.5 ml microcentrifuge tube, and apply 400 µl Buffer OW2 onto the column. Centrifuge for 1 min at maximum speed and discard the flow-through.**
7. **Repeat step 6 once using the same microcentrifuge tube.**
8. **Transfer the spin column to a new RNase-free 1.5 ml microcentrifuge tube, and pipet at least 20 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.**

   Note: The volume of Buffer OEB used depends on the expected or desired concentration of the in vitro transcript. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

9. **To ensure maximal yield, pipet another 20 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.**

   To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute into the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Protocol: Purification of In Vitro Transcripts using a Batch Procedure

Important notes before starting

- This protocol can be used for purification of poly A^+ in vitro transcripts, such as those generated by nuclear-extract transcription systems. In vitro transcripts from most bacterial systems (e.g., T3 or T7) are not polyadenylated and will not be selected by the Oligotex procedure.* For purification of in vitro transcripts that are not polyadenylated, RNeasy Kits can be used (see pages 98–98 for ordering information).
- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- Read "Introduction" (page 8) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Buffer OBB may form a precipitate upon storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).

Procedure

1. **Adjust volume of the in vitro transcription sample with RNase-free water to 200 µl.**

   Note: Any volume up to 200 µl can be used. For samples <200 µl, add RNase-free water to 200 µl final volume.

* Unless the transcript itself contains a poly-A tract. This could be included either in the vector or in the insert. For example, a eukaryotic cDNA insert may include part of the poly-A tail, and some bacterial transcription vectors include a poly-A tract at the end of the transcript.

2. **Add 200 µl Buffer OBB and the appropriate amount of Oligotex Suspension (see equation below).**

   Determine the volume of Oligotex Suspension required (in microliters) according to the following equation:

   3 x transcript yield (µg)/transcript length (kb) = volume of Oligotex Suspension (µl)

   Example: For an expected yield of 5 µg of a 1.5 kb transcript, use 10 µl Oligotex

   Suspension (3 x 5 µg/1.5 kb = 10 µl).

3. **Mix the contents by pipetting or flicking the tube. Incubate for 3 min at 70°C in a water bath or heating block.**

   This step disrupts RNA secondary structure.

4. **Remove sample from the water bath/heating block, and place at 20 to 30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

5. **Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed (14,000–18,000 x g), and carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

6. **Resuspend the Oligotex:mRNA pellet in 1 ml Buffer OW2 by vortexing or pipetting.**

   Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed, and carefully remove the supernatant by pipetting.

7. **Repeat step 6 once.**

8. **Add at least 20 µl hot (70°C) Buffer OEB. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A^+ mRNA, to another RNase-free tube.**

   Note: The volume of Buffer OEB used depends on the expected or desired concentration of poly A^+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with Oligotex resin and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

9. **To ensure maximal yield, add another 20 µl hot (70°C) Buffer OEB to the Oligotex pellet. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A^+ mRNA, and combine the eluates.**

Oligotex Direct mRNA Purification Protocols

Description of protocols

The Oligotex Direct mRNA protocols are designed for isolation of poly A^+ RNA from animal cells, tissue, or small amounts of blood. Poly A^+ mRNA purified with Oligotex does not require further purification and is ready to use in any standard application such as northern, dot and slot blotting, in vitro translation, S1 nuclease analysis, RNase protection, primer extension, cDNA synthesis, RT-PCR, microinjection, cDNA library construction, and subtractive cDNA cloning.

Compared to mRNA isolation from total RNA fractions, direct mRNA isolation from cell or tissue lysates may copurify small amounts of rRNA. This slight contamination with rRNA, however, does not indicate poor quality of mRNA or poor performance of the kits, and the presence of small quantities of rRNA does not affect the function of the mRNA.

For animal cells, three different direct protocols are provided. The Oligotex Direct mRNA protocol for isolation of Poly A^+ mRNA from animal cells (page 43) is the standard protocol for isolation of poly A^+ RNA directly from cells or small amounts of blood. Samples are first lysed and homogenized in the presence of a highly denaturing guanidine-isothiocyanate (GITC) buffer, which immediately inactivates RNases to ensure isolation of intact mRNA. Oligotex Suspension is added, and hybridization takes place between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA. Contaminants are then washed away, and high-quality poly A^+ RNA is eluted.

For applications that require mRNA in its mature form, where unspliced or partially spliced RNA is not desirable, the Oligotex Direct mRNA protocol for mRNA purification from cytoplasm of cultured cells (page 51) is particularly advantageous. This protocol also avoids potential problems of excessive lysate viscosity due to high-molecular-weight genomic DNA. In the protocol, cells are gently lysed using a non-ionic detergent (Nonidet P-40). The nuclei remain intact and can be pelleted to remove the genomic DNA. The cytoplasmic supernatant is then used for direct isolation of the mature mRNAs. Cytoplasmic RNA accounts for approximately 85% of total cellular RNA so yields are not significantly reduced. The cytoplasmic lysis Buffer OCL and dilution Buffer OCD must be prepared by the user (see Appendix C, page 85).

For many cell lines, the Oligotex Direct mRNA protocol for mRNA purification from animal cells using urea–SDS buffer (page 69) is useful for reducing rRNA background. It should be used if rRNA is a problem in the standard protocol. In addition, this protocol is designed for Proteinase K digestion following lysis and may be useful for biological material, such as blood, or cell lines with a high protein content. The conditions for lysis and RNase inactivation are less rigorous

though, and cells cannot be directly lysed in the cell culture vessel. The urea–SDS lysis Buffer OL2 must be prepared by the user (see Appendix C, page 85).

For animal tissue, two different direct protocols are provided. The Oligotex Direct mRNA protocol for isolation of Poly A^+ mRNA from animal tissues (page 63) is the standard protocol for isolation of poly A^+ RNA directly from tissues. Samples are first lysed and homogenized in the presence of a highly denaturing guanidine-isothiocyanate (GITC) buffer, which immediately inactivates RNases to ensure isolation of intact mRNA. Oligotex Suspension is added and hybridization takes place between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA. Contaminants are then washed away, and high-quality poly A^+ RNA is eluted.

The Oligotex Direct mRNA protocol for isolation of Poly A^+ mRNA from animal tissues using urea–SDS buffer (page 69) is designed for Proteinase K digestion following lysis and may be useful for tissues, such as heart or muscle, that have a high protein content. The conditions for lysis and RNase inactivation are less rigorous though than in the standard protocol. In general, equal yields and RNA quality are obtained using the urea–SDS buffer as with the standard lysis buffers. The urea–SDS lysis Buffer OL2 must be prepared by the user (see Appendix C, page 85).

The buffers included in Oligotex Direct mRNA Kits can also be used to isolate poly A^+ mRNA from total RNA with the Oligotex mRNA protocol using Oligotex Direct mRNA buffers (Appendix D, page 87). Oligotex mRNA Kits are, however, specifically designed for this application (see page 95 for ordering information). For protocols using the Oligotex mRNA Kits, see page 95.

Spin column format

Spin columns that are supplied in the Oligotex Direct mRNA Kits are recommended in all protocols for the most convenient handling when working with Oligotex. The spin column protocols are described in detail on pages 43–69. Small spin columns can also be ordered separately (see page 95 for ordering information).

Batch format

With the following guidelines, any of the Oligotex Direct mRNA protocols can be adapted to batch format in which spin columns are omitted. In general, spin column protocols are recommended over batch protocols, but occasionally, better results are obtained with a batch protocol. For example, if the initial lysate is extremely viscous, a batch protocol is recommended in order to avoid the risk of clogging the spin column.

Instead of collecting the Oligotex resin in a spin column, the polystyrene–latex particles can be pelleted by centrifugation for 2 min at maximum speed (14,000–18,000 x g) in a microcentrifuge if a microcentrifuge tube is used or

10 min at 10,000 x *g* in a QIAGEN Centrifuge 4–16K or equivalent centrifuge, if larger tubes are used. The supernatant or eluate should be removed by carefully aspirating, pipetting, or decanting, in order to avoid carryover of Oligotex particles.

In the wash steps, the Oligotex:mRNA pellet should be resuspended in the appropriate wash buffer, then pelleted by centrifugation, and the supernatant discarded as described above.

In the elution steps, the pellet should be resuspended in heated elution buffer and pelleted by centrifugation as described above. The supernatants containing the eluted mRNA should then be carefully transferred to a new RNase-free microcentrifuge tube.

Oligotex terminology for direct mRNA preparations

For convenience, we have classified the size of mRNA preparation according to the amount of cells or tissue processed. Oligotex Direct mRNA Kits are not restricted to their nominal prep size. The kits offer flexibility for use with widely varying amounts of starting material. See Table 3 (page 11) for guidelines to customize Oligotex Direct mRNA Kits to the desired prep size.

- Micro prep $\leq 1 \times 10^6$ cells or ≤ 10 mg tissue
- Mini prep $1 \times 10^6 – 2 \times 10^7$ cells or 10–100 mg tissue
- Midi prep $2 \times 10^7 – 5 \times 10^7$ cells or 100–250 mg tissue
- Maxi prep $5 \times 10^7 – 1 \times 10^8$ cells or 250–1000 mg tissue

Determining the amount of starting material

Counting cells or weighing tissue is the most accurate way to determine the amount. However, the following may be used as a guide.

Animal cells: The expected numbers of HeLa cells in various cell culture vessels is indicated in Table 6, below.

Animal tissue: A 3 mm cube of rat kidney (27 mm^3) weighs 30–35 mg. A 5 mm cube (125 mm^3) weighs 150–175 mg. Entire mouse organs can be used with the Oligotex Direct mRNA Midi/Maxi Kit provided they do not exceed 1 g. The following are average weights of various adult mouse organs:

- Kidney: 180–250 mg
- Spleen: 100–160 mg
- Lung: 190–210 mg
- Heart: 100–170 mg
- Liver: 1–1.8 g (up to 1 g can be processed with the Oligotex Direct mRNA maxi procedure)

Table 6. Growth areas and number of HeLa cells in various cell culture vessels

Cell culture vessel	Growth area (cm^2)*	Number of cells
Multiwell plates†		
96-well	0.32–0.6	$4–5 \times 10^4$
48-well	1.0	1.3×10^5
24-well	2.0	2.5×10^5
12-well	4.0	5×10^5
6-well	9.5	1.2×10^6
Dishes		
35 mm	8	1×10^6
60 mm	21	2.5×10^6
100 mm	56	7×10^6
145–150 mm	145	2×10^7
Flasks		
40–50 ml	25	3×10^7
250–300 ml	75	1×10^7
650–750 ml	162–175	2×10^7
900 ml	225	3×10^7

* Growth area varies slightly depending on the supplier. Confluent growth is assumed for multiwell plates and dishes.

† Values per well are reported.

Expected amounts of RNA in cells and tissues

The expression pattern and amount of poly A^+ RNA in cells or tissues varies with the cell or tissue type, developmental stage, and differentiation status as well as the influence of environmental factors, such as growth conditions or disease. As a guide, the expected amount of total RNA and mRNA in various cells or tissues is shown in Table 7, below.

Table 7. Amounts of total RNA and mRNA in a variety of cells and tissues

Source	Total RNA,* µg	mRNA,* µg
Cell cultures		
Standard (10^7 cells)	30–500	0.3–25
HeLa	150	3
NIH/3T3	120	3
COS-7	350	5
Mouse (100 mg tissue)		
13-day old embryo	450	13
Mouse tissue (adult)	50–400	1–15
Brain	120	5
Heart	120	6
Intestine	150	2
Kidney	350	9
Liver	400	14
Lung	130	6
Spleen	350	7

* Amounts can vary due to developmental stage, growth conditions used, etc.

Handling and storage of starting material

RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents.

For stabilization in RNA*later* RNA Stabilization Reagent, samples should be submerged in the appropriate volume of the reagent immediately after harvesting the material. This prevents unwanted changes in the gene-expression pattern due to RNA degradation or new induction of genes. The relevant procedures for harvesting and stabilization should be carried out as quickly as possible.

Alternatively, samples can be immediately flash frozen in liquid nitrogen and stored at –70°C as soon as they are harvested or excised. Frozen tissue should

not be allowed to thaw during handling or weighing, but cell pellets can partially thaw enough to allow them to be dislodged by flicking. The relevant procedures should be carried out as quickly as possible.

Samples can also be stored at –70°C in lysis buffer (Buffer RLT) after disruption and homogenization. Frozen samples are stable for months.

Note: For isolation of cytoplasmic mRNA from eukaryotic cells (page 51), only freshly harvested cells can be used.

Disruption and homogenization of starting material

Efficient disruption and homogenization of the starting material is an absolute requirement for all nucleic acid purification procedures. Disruption and homogenization are 2 distinct steps.

- **Disruption**: Complete disruption of cell walls and plasma membranes of cells and organelles is absolutely required to release all the nucleic acids contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced DNA and RNA yields.
- **Homogenization**: Homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. Homogenization shears carbohydrates to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of nucleic acids to QIAGEN silica membranes and magnetic particles and therefore significantly reduced DNA and RNA yields.

Cellular disruption is one of the most critical steps in nucleic acid purification. Disruption in lysis buffer alone, without physical shearing, may result in nucleic acid degradation by endogenous DNases and RNases. Incomplete disruption prevents the lysis buffer, which inactivates nucleases, from contacting nucleic acids within the intact cells. Furthermore, cellular debris that is not disrupted can result in decreased yield and increases the risk of clogging the purification column. After sample disruption, there should be no visible particulates (except when disrupting materials containing hard, noncellular components, such as connective tissue, bone, or woody plant tissue). QIAGEN kits and protocols contain recommendations for the most appropriate method of sample disruption and homogenization to maximize the yield and quality of your DNA, RNA, and protein preparation.

Table 8. Disruption and homogenization methods

Starting material	Disruption method	Homogenization method	Comment
Cultured animal cells	Addition of lysis buffer	a) TissueRuptor b) QIAshredder homogenizer* c) Syringe and needle	If ≤5 x 10^5 cells are processed, the lysate can be homogenized by pipetting or vortexing for 30 seconds. No homogenization is needed for cytoplasmic RNA protocol.
Animal tissue	TissueRuptor	TissueRuptor	Simultaneously disrupts and homogenizes
	Stainless steel in a TissueLyser II or TissueLyser LT with lysis buffer	Stainless steel in a TissueLyser II or TissueLyser LT	Bead-milling simultaneously disrupts and lysis buffer homogenizes; bead-milling cannot be replaced by vortexing
	Mortar and pestle	a) Syringe and needle b) QIAshredder homogenizer*	TissueRuptor homogenization usually gives higher yields than other homogenization methods

* QIAshredder spin columns can be purchased separately for preps with ≤2 x 10^7 cells or ≤100 mg ground tissue. See page 96 for ordering information.

Disruption and homogenization using the TissueRuptor or other rotor–stator homogenizer

The TissueRuptor thoroughly disrupts and simultaneously homogenizes, in the presence of lysis buffer, animal cells or tissues in 20–90 seconds depending on the toughness and amount of the sample. The TissueRuptor can also be used to homogenize cell lysates. The rotor turns at a very high speed causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels and by keeping the tip of the TissueRuptor submerged.

Disruption and homogenization using bead mills

In bead-milling, cells and tissues can be disrupted by rapid agitation in the presence of beads. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the sample. Disruption efficiency is influenced by:

- Size and composition of beads
- Ratio of buffer to samples (if buffer is used)
- Amount of starting material
- Speed and configuration of agitator
- Consistency of sample
- Type of disruption vessel
- Temperature of the samples

The optimal beads to use are 0.1–0.6 mm glass beads for bacteria, yeast, etc., and 3–7 mm stainless steel beads for animal tissues. It is essential that glass beads are pretreated by washing in concentrated nitric acid. All other disruption parameters must be determined empirically for each application. Please refer to suppliers' guidelines for further details.

Disruption using a mortar and pestle

To disrupt tissue using a mortar and pestle, freeze the sample immediately in liquid nitrogen, and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into an RNase-free, liquid-nitrogen–cooled polypropylene or Corex tube. Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw. Add lysis buffer and continue as quickly as possible with homogenization using QIAshredder or a syringe and needle as described below.

Note: Grinding the sample using a mortar and pestle will disrupt the sample but not homogenize it. Homogenization must be performed separately.

Homogenization using QIAshredder homogenizers

Use of QIAshredder modules is a fast and efficient way to homogenize cell or ground-tissue lysates without cross contamination of the samples. QIAshredder homogenizers can be used in Oligotex Direct protocols for lysates of up to 2×10^7 cells or 100 mg ground tissue in ≤ 700 µl volume. The cell or ground-tissue lysate is loaded onto the QIAshredder spin column placed in a 2 ml collection tube, spun for 2 min at maximum speed in a microcentrifuge, and the homogenized lysate collected. QIAshredder spin columns can be purchased separately for use with Oligotex Kits (see page 95 for ordering information). Contact QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com) for further details.

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. High-molecular-weight DNA can be sheared by passing the lysate through a 20-gauge (0.9 mm diameter) needle, attached to a sterile plastic syringe, at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize loss.

Protocol: Isolation of Poly A$^+$ mRNA from Animal Cells

Important notes before starting

- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- β-Mercaptoethanol (β-ME) must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl β-ME per 1 ml Buffer OL1. Buffer OL1 is stable for 1 month after addition of β-ME.
- Read "Introduction" (page 8) and "Oligotex mRNA Purification Protocols" (page 18) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Buffer OW1 and Buffer OL1 may form a precipitate during storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly to allow the pellet to be dislodged by flicking in step 2. Homogenized cell lysates (in Buffer OL1, step 3) can be stored at –70°C for several months. To process frozen lysates, thaw samples for ■ 10 min (for ≤2 x 10^7 cells), ▲ 15 min (for 2 x 10^7 to 5 x 10^7 cells), or ● 20 min (for 5 x 10^7 to 1 x 10^8 cells) at 37°C in a water bath to dissolve salts. Continue the protocol with step 4.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- Unless otherwise indicated, all centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- To perform this protocol in batch format, see page 35.
- ■ denotes micro/mini prep volumes (for ≤2 x 10^7 cells); ▲ denotes midi prep volumes (for 2 x 10^7 to 5 x 10^7 cells); ● denotes maxi prep volumes (for 5 x 10^7 to 1 x 10^8 cells).

Procedure

1. Prepare samples according to step 1a or 1b below.

1a. Sample preparation for cells grown in suspension. Do not use more than ■ 2 x 10^7, ▲ 5 x 10^7, or ● 1 x 10^8 cells.

Determine the number of cells. Spin down the desired number of cells for 5 min at 300 x *g* in an RNase-free glass or polypropylene centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and continue with step 2 of the protocol.

Note: Incomplete removal of the supernatant will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT_{30} of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

1b. Sample preparation for cells grown in a monolayer. Do not use more than ■ 2 x 10^7, ▲ 5 x 10^7, or ● 1 x 10^8 cells.

Cells grown in a monolayer in cell culture dishes can either be lysed directly in the culture dish or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer in cell culture flasks should always be trypsinized.

To lyse directly in cell-culture dishes: Determine the number of cells. (Table 6, page 37, gives approximate cell numbers for various cell culture vessels.) Completely aspirate the cell culture medium, and continue immediately with step 2 of the protocol.

Note: Incomplete removal of the cell culture medium will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT_{30} of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

To trypsinize cells: Determine the number of cells. (Table 6, page 37, gives approximate cell numbers for various cell culture vessels.) Aspirate medium, and wash cells with PBS. Aspirate PBS and add 0.10–0.25% trypsin in PBS to trypsinize the cells. After cells detach from the dish or flask, add medium (containing serum, to inactivate the trypsin), transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and pellet by centrifugation at 300 x *g* for 5 min. Completely aspirate supernatant, and continue with step 2 of the protocol.

Note: Incomplete removal of the supernatant will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT30 of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

2. **Disrupt cells by addition of room-temperature Buffer OL1.**

   For pelleted cells, loosen the cell pellet by flicking the tube. Add the appropriate volume of Buffer OL1 (see Table 9, page 46). Vortex for 5–10 s or pipet up and down to mix, and proceed at once with step 3.

   Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields. β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

   For direct lysis, add the appropriate amount of Buffer OL1 (see Table 9, page 46) to the cell culture dish. Collect cell lysate with a rubber policeman. Transfer to a 2 ml (or larger) centrifuge tube (not supplied). Vortex for 5–10 s or pipet up and down to mix. Proceed at once with step 3.

   Do not exceed the recommended number of cells. If too much sample or too little lysis buffer is used, a viscous sample will result, potentially clogging the spin column and causing reduced yields and impure mRNA preparations. If lysing cells directly in 145 mm cell culture dishes, the amounts of Buffer OL1 and Buffer ODB must be increased. See Table 9, below.

   Note: β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

Table 9. Buffer volumes for Oligotex Direct mRNA purification from cells

Number of cells	Buffer OL1*, ml	Buffer ODB, ml	Oligotex Suspension, µl	Prep size
■ 100† – 2 x 10^5	0.4	0.8	20	Micro
■ 2 x 10^5 – 5 x 10^5	0.5	1.0	20	Micro
■ 5 x 10^5 – 1 x 10^6	0.6	1.2	20	Micro
■ 1 x 10^6 – 5 x 10^6	0.6	1.2	35	Mini
■ 5 x 10^6 – 1 x 10^7	0.6/1.0‡§	1.2/2.0‡	70	Mini
■ 1 x 10^7 – 2 x 10^7	0.6/1.0‡§	1.2/2.0‡	110	Mini
▲ 2 x 10^7 – 3 x 10^7	0.6/1.0‡	1.2/2.0‡	130	Midi
▲ 3 x 10^7 – 5 x 10^7	2.0	4.0	165	Midi
● 5 x 10^7 – 7 x 10^7	3.0	6.0	250	Maxi
● 7 x 10^7 – 1 x 10^8	5.0	10.0	500	Maxi
Whole blood (≤50 µl)	0.6	1.2	35	Mini

* Be sure that β-ME has been added to Buffer OL1 before use (30 µl β-ME per 1 ml Buffer OL1).

† Lowest amount tested.

‡ If using 145 mm cell culture dishes, 0.6 ml Buffer OL1 is not sufficient to cover the entire surface. Cells can be processed with 0.6 ml Buffer OL1 and 1.2 ml Buffer ODB only if they are trypsinized and pelleted first. These volumes are the maximum amount that can be used with a single microcentrifuge tube. If lysing cells directly in the culture vessel, increase the amounts of Buffer OL1 and Buffer ODB to 1.0 ml and 2.0 ml, respectively.

§ QIAshredder homogenizers can be used for homogenization of up to 0.7 ml cell lysate. If using 1.0 ml Buffer OL1 for lysis, add 0.7 ml of the lysate at first, and homogenize with QIAshredder homogenizers. Then transfer the homogenized lysate to a larger tube, add the remaining 0.3 ml lysate to the QIAshredder column, and homogenize. Combine the two homogenized lysates, and add 2.0 ml Buffer ODB. Do not use QIAshredder homogenizers for >2 x 10^7 cells.

3. Homogenize the sample.

Three alternative methods (a, b, or c) may be used to homogenize the sample. After homogenization, proceed with step 4. See "Disruption and homogenization of starting material", page 39, for a more detailed description of homogenization methods.

Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of Oligotex spin columns. Homogenization with the TissueRuptor or QIAshredder homogenizers generally results in higher RNA yields than with a syringe and needle.

a. Homogenize cells using the TissueRuptor homogenizer until the sample is homogeneous (typically ■ 20–30 s, ▲ 30–45 s, or ● 60–90 s at maximum speed).

or

b. Pipet the lysate directly onto a QIAshredder spin column placed in ■ 2 ml collection tube, and centrifuge for 2 min at maximum speed. Do not use QIAshredder homogenizers for >2 x 10^7 cells or >700 µl lysate. (Exception: see Table 9, page 46, note §)

or

c. Pass the lysate at least 5–10 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.

4. Add the appropriate volume of Buffer ODB (see Table 9, page 46) to the lysate, and mix thoroughly by pipetting. Centrifuge in a microcentrifuge for 3 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Transfer the supernatant to a new RNase-free tube.

This step removes remaining cell debris and protein and creates optimal conditions for hybridization of poly A^+ mRNA to Oligotex. Be sure not to transfer any of the precipitate since this leads to clumping of the Oligotex resin in the following step, decreased performance of the procedure, and low yields of poly A^+ mRNA. If some of the cell debris or protein has been transferred, collect the precipitate again by centrifugation. Then continue with step 5.

Note: Always centrifuge, even if no precipitate is visible after addition of Buffer ODB.

5. Add the appropriate amount of Oligotex Suspension to the sample (see Table 9, page 46). Mix thoroughly by pipetting or vortexing, and place at 20 to 30°C for 10 min.

This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

6. **Pellet the Oligotex:mRNA complex by centrifuging in a microcentrifuge for 5 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

   Optional: If time is a restricting factor and slight rRNA contamination is not a concern, the protocol can be shortened at this point by proceeding directly with step 10. The shortened procedure still provides significant enrichment of poly A^+ RNA. However, for best results, and particularly for applications where further enrichment of mRNA is necessary (e.g., cDNA library construction), the full protocol is recommended.

7. **Resuspend the Oligotex:mRNA pellet thoroughly in ■ 100 µl, ▲ 200 µl, or ● 300 µl Buffer OL1 by vortexing or pipetting.**
8. **Add ■ 400 µl, ▲ 800 µl, or ● 1200 µl Buffer ODB, incubate at 70°C for 3 min and then place at room temperature (15–25°C) for 10 min.**

   Note: This step significantly enriches for poly A^+ RNA by decreasing the amount of rRNA.

9. **Pellet the Oligotex:mRNA complex by centrifugation in a microcentrifuge for 5 min at maximum speed, and carefully remove the supernatant by pipetting.**

   ● Maxi protocol: Centrifuge for 10 min at 10,000 x *g*, and carefully remove the supernatant by pipetting.

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

10. **Resuspend the pellet in ■ 350 µl, ▲ 600 µl, or ● 1 ml Buffer OW1 by vortexing or pipetting.**

11. Pipet the sample onto a ■ small spin column or ▲ large spin column placed in a ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed. Discard the flow-through.

● Maxi protocol: Do not use a spin column. Transfer the resuspended pellet directly into a ● 2 ml microcentrifuge tube. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

● **Maxi protocol**: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

12. Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 350 µl or ▲ 600 µl Buffer OW2 onto the column. Centrifuge for 1 min at maximum speed, and discard the flow-through.

● **Maxi protocol**: Resuspend the pellet in ● 1 ml Buffer OW2. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

● **Maxi protocol**: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

13. Repeat step 12 once, using the same microcentrifuge tube.

14. Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 20–100 µl or ▲ 50–300 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.

● **Maxi protocol**: Resuspend the pellet in ● 100–500 µl hot (70°C) Buffer OEB. Load onto a ● large spin column placed in a new RNase-free ● 2 ml microcentrifuge tube, and centrifuge for 1 min at maximum speed.

Note: The volume of Buffer OEB used depends on the desired or expected concentration of poly A^+ mRNA (see "Expected amounts of RNA in cells and tissues", page 37). Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

15. To ensure maximal yield, pipet another ■ 20–100 µl, ▲ 50–300 µl, or ● 100–500 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin. Centrifuge for 1 min at maximum speed.

To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Protocol: Isolation of Poly A$^+$ mRNA from Cytoplasm of Cultured Cells

Important notes before starting

- This protocol is particularly advantageous for applications that require RNA in its mature form, where unspliced or partially spliced RNA is not desirable. See page 34 for more information.
- Prepare Buffers OCL and OCD (see Appendix C, page 85).
- Chill Buffer OCL to 4°C.
- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- β-Mercaptoethanol (β-ME) must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl β-ME per 1 ml Buffer OL1. Buffer OL1 is stable for 1 month after addition of β-ME.
  (**Note**: Buffer OL1 is not required in the optional shortened protocol.)
- Read "Introduction" (page 8) and "Important Notes" (page 13) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Buffer OW1 and Buffer OL1 may form a precipitate during storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Only use freshly harvested cells. Ice crystals form during freezing and thawing and destroy the nuclear membranes, releasing DNA and other nuclear molecules.
- It is important to perform the cell lysis in step 2 on ice and the following centrifugation step at 4°C. If centrifugation at 4°C is not possible, centrifugation may be carried out at 20 to 30°C. However, the risk of RNA degradation will increase slightly, especially for cell lines with a high RNase content. With the exception of Buffer OCL, buffers should not be prechilled.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- Unless otherwise indicated, all centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- To perform this protocol in batch format, see page 35.

■ denotes micro/mini prep volumes (for ≤2 x 10^7 cells); ▲ denotes midi prep volumes (for 2 x 10^7 to 5 x 10^7 cells); ● denotes maxi prep volumes (for 5 x 10^7 to 1 x 10^8 cells).

Procedure

1. Prepare samples according to step 1a or 1b below.

1a. Sample preparation for cells grown in suspension. Do not use more than ■ 2 x 10^7, ▲ 5 x 10^7, or ● 1 x 10^8 cells.

Determine the number of cells. Spin down the desired number of cells for 5 min at 300 x *g* in an RNase-free glass or polypropylene centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and continue with step 2 of the protocol.

Note: Incomplete removal of the supernatant will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT_{30} of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

1b. Sample preparation for cells grown in a monolayer. Do not use more than ■ 2 x 10^7, ▲ 5 x 10^7, or ● 1 x 10^8 cells.

Cells grown in a monolayer in cell culture flasks or dishes should always be trypsinized.

To trypsinize cells: Determine the number of cells. (Table 6, page 37, gives approximate cell numbers for various cell culture vessels.) Aspirate medium, and wash cells with PBS. Aspirate PBS and add 0.10–0.25% trypsin in PBS to trypsinize the cells. After cells detach from the dish or flask, add medium (containing serum, to inactivate the trypsin), transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and pellet by centrifugation at 300 x *g* for 5 min. Completely aspirate supernatant, and continue with step 2 of the protocol.

Note: Incomplete removal of the supernatant will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT30 of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

2. **Loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer OCL (see Table 10, below), and carefully resuspend by flicking the tube. Incubate on ice for 5 min. Proceed with step 3.**

   The suspension should clear rapidly, indicating lysis of the plasma membrane. It is important to perform this incubation on ice using prechilled Buffer OCL.

   Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields. For isolation of cytoplasmic RNA, freshly harvested cells must be used.

Table 10. Buffer volumes for Oligotex Direct mRNA purification from cytoplasm of cells

Number of cells	Buffer OCL, ml	Buffer OCD, ml	Oligotex Suspension, µl	Prep size
■ 100* – 3 x 10^5	0.2	0.2	20	Micro
■ 3 x 10^5 – 1 x 10^6	0.4	0.4	20	Micro
■ 1 x 10^6 – 5 x 10^6	0.6	0.6	35	Mini
■ 5 x 10^6 – 1 x 10^7	0.6	0.6	70	Mini
■ 1 x 10^7 – 2 x 10^7	0.6	0.6	110	Mini
▲ 2 x 10^7 – 3 x 10^7	0.6	0.6	130	Midi
▲ 3 x 10^7 – 5 x 10^7	1.0	1.0	165	Midi
● 5 x 10^7 – 7 x 10^7	1.3	1.3	250	Maxi
● 7 x 10^7 – 1 x 10^8	1.5	1.5	500	Maxi

* Lowest amount tested.

3. **Transfer lysate to a microcentrifuge tube and spin for 2 min at 300–500 x *g* at 4°C. Pipet the supernatant into an appropriately sized RNase-free centrifuge tube.**

   The supernatant contains the cytoplasmic extract. It is generally slightly cloudy and yellow-white, depending on the cell type. The pellet contains the nuclei and cell debris. The pellet is white and considerably smaller than the whole cell pellet obtained in step 1.

   Note: Do not centrifuge at higher speeds. This could destroy the nuclei, releasing DNA and other nuclear molecules. If centrifugation at 4°C is not possible, centrifugation may be carried out at 20 to 30°C. However, the risk of RNA degradation will increase slightly, especially for cell lines with a high RNase content.

4. **Add the appropriate volume of Buffer OCD and Oligotex Suspension (see Table 10). Mix thoroughly by vortexing or pipetting.**

5. **Incubate for 3 min at 70°C in a water bath or heating block, and then place at 20 to 30°C for 10 min.**

   The incubation at 70°C disrupts the secondary structure of the RNA while the incubation at room temperature (15–25°C) allows hybridization between the oligo dT_{30} linked to the Oligotex particles and the poly-A tail of the mRNA.

6. **Pellet the Oligotex:mRNA complex by centrifuging in a microcentrifuge for 5 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

   Optional: If time is a restricting factor and slight rRNA contamination is not a concern, the protocol can be shortened at this point by proceeding directly with step 10. The shortened procedure still provides significant enrichment of poly A^+ RNA. However, for best results, and particularly for applications where highly purified mRNA is necessary (e.g., cDNA library construction), the full protocol is recommended.

7. **Resuspend the Oligotex:mRNA pellet thoroughly in ■ 100 µl, ▲ 200 µl, or ● 300 µl Buffer OL1 by vortexing or pipetting.**

   Note: β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

8. **Add ■ 400 µl, ▲ 800 µl, or ● 1200 µl Buffer ODB, incubate at 70°C for 3 min and then place at room temperature for 10 min.**

   Note: This step significantly enriches for poly A^+ RNA by decreasing the amount of rRNA.

9. **Pellet the Oligotex:mRNA complex by centrifugation in a microcentrifuge for 5 min at maximum speed, and carefully remove the supernatant by pipetting.**

   ● Maxi protocol: Centrifuge for 10 min at 10,000 x g, and carefully remove the supernatant by pipetting.

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

10. **Resuspend the pellet in ■ 350 µl, ▲ 600 µl, or ● 1 ml Buffer OW1 by vortexing or pipetting.**

11. **Pipet the sample onto a ■ small spin column or ▲ large spin column placed in a ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed. Discard the flow-through.**

    ● Maxi protocol: Do not use a spin column. Transfer the resuspended pellet directly into a ● 2 ml microcentrifuge tube. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

    ● Maxi protocol: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

12. **Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 350 µl or ▲ 600 µl Buffer OW2 onto the column. Centrifuge for 1 min at maximum speed, and discard the flow-through.**

    ● Maxi protocol: Resuspend the pellet in ● 1 ml Buffer OW2. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

    ● Maxi protocol: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

13. **Repeat step 12 once, using the same microcentrifuge tube.**

14. **Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 20–100 µl or ▲ 50–300 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.**

    ● **Maxi protocol**: Resuspend the pellet in ● 100–500 µl hot (70°C) Buffer OEB. Load onto a ● large spin column placed in a new RNase-free ● 2 ml microcentrifuge tube, and centrifuge for 1 min at maximum speed.

    Note: The volume of Buffer OEB used depends on the desired or expected concentration of poly A^+ mRNA (see "Expected amounts of RNA in cells and tissues", page 37). Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

15. **To ensure maximal yield, pipet another ■ 20–100 µl, ▲ 50–300 µl, or ● 100–500 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin. Centrifuge for 1 min at maximum speed.**

    To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Protocol: Isolation of Poly A$^+$ mRNA from Animal Cells Using Urea–SDS Buffer

Important notes before starting

- For many cell lines, this protocol is useful for reducing rRNA background. It should be used if rRNA is a problem in the standard protocol. In addition, this protocol is designed for proteinase K digestion following lysis and may be useful for biological material, such as blood, or cell lines with a high protein content. See page 34 for more information.
- Prepare Buffer OL2 (see Appendix C, page 85). β-ME (or DTT) must be added before use.
- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat Buffer OL2 to 50°C to dissolve salt precipitates.
- If performing the optional proteinase K digestion in step 4, heat a water bath or heating block to 56°C.
- Heat Buffer OEB to 70°C in a water bath or heating block.
- Read "Introduction" (page 8) and "Important Notes" (page 13) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly to allow the pellet to be dislodged by flicking in step 2.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- Unless otherwise indicated, all centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- To perform this protocol in batch format, see page 35.
- ■ denotes micro/mini prep volumes (for ≤2 x 10^7 cells); ▲ denotes midi prep volumes (for 2 x 10^7 to 5 x 10^7 cells); ● denotes maxi prep volumes (for 5 x 10^7 to 1 x 10^8 cells).

Procedure

1. Prepare samples according to step 1a or 1b below.

1a. Sample preparation for cells grown in suspension. Do not use more than ■ 2 x 10^7, ▲ 5 x 10^7, or ● 1 x 10^8 cells.

Determine the number of cells. Spin down the desired number of cells for 5 min at 300 x *g* in an RNase-free glass or polypropylene centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and continue with step 2 of the protocol.

Note: Incomplete removal of the supernatant will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT_{30} of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

1b. Sample preparation for cells grown in a monolayer. Do not use more than ■ 2 x 10^7, ▲ 5 x 10^7, or ● 1 x 10^8 cells.

Cells grown in a monolayer in cell culture flasks or dishes should always be trypsinized.

To trypsinize cells: Determine the number of cells. (Table 6, page 37, gives approximate cell numbers for various cell culture vessels.) Aspirate medium, and wash cells with PBS. Aspirate PBS and add 0.10–0.25% trypsin in PBS to trypsinize the cells. After cells detach from the dish or flask, add medium (containing serum, to inactivate the trypsin), transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and pellet by centrifugation at 300 x *g* for 5 min. Completely aspirate supernatant, and continue with step 2 of the protocol.

Note: Incomplete removal of the supernatant will dilute the lysate, inhibiting lysis and affecting hybridization between mRNA and the oligo-dT_{30} of the Oligotex particle in subsequent steps. Both effects may reduce mRNA yield.

2. Loosen the cell pellet by flicking the tube. Add the appropriate volume of Buffer OL2 (see Table 11, below). Vortex for 5–10 s or pipet up and down to mix, and proceed at once with step 3.

Do not exceed the recommended number of cells. If too much sample or too little lysis buffer is used, a viscous sample will result, potentially clogging the spin column and causing reduced yields and impure mRNA preparations.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields. β-ME (or DTT) must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of 14.3 M β-ME per 1 ml Buffer OL2.

Table 11. Urea–SDS buffer volumes for Oligotex Direct mRNA purification from cells

Number of cells	Buffer OL2, ml	Oligotex Suspension, µl	Prep size
■ 100† – 3 x 10^5	0.3	20	Micro
■ 3 x 10^5 – 1 x 10^6	0.5	20	Micro
■ 1 x 10^6 – 5 x 10^6	0.6	35	Mini
■ 5 x 10^6 – 1 x 10^7	0.6	70	Mini
■ 1 x 10^7 – 2 x 10^7	1.0‡	110	Mini
▲ 2 x 10^7 – 3 x 10^7	1.0	130	Midi
▲ 3 x 10^7 – 5 x 10^7	1.8	165	Midi
● 5 x 10^7 – 7 x 10^7	3.0	250	Maxi
● 7 x 10^7 – 1 x 10^8	5.0	500	Maxi
Whole blood (≤50 µl)	1.5	40	Mini

* Be sure that β-ME (or DTT) has been added to Buffer OL2 before use (10 µl β-ME per 1 ml Buffer OL2). See Appendix C (page 85).

† Lowest amount tested.

‡ QIAshredder homogenizers can be used for homogenization of up to 0.7 ml cell lysate. If using 1.0 ml Buffer OL2 for lysis, add 0.7 ml of the lysate at first, and homogenize with QIAshredder homogenizers. Then transfer the homogenized lysate to a larger tube, add the remaining 0.3 ml lysate to the QIAshredder column, and homogenize. Combine the two homogenized lysates. Do not use QIAshredder homogenizers for >2 x 10^7 cells.

3. Homogenize the sample.

Three alternative methods (a, b, or c) may be used to homogenize the sample. See "Disruption and homogenization of starting material", page 39, for a more detailed description of homogenization methods. After homogenization, proceed with the optional proteinase K digestion (step 4) or directly with step 5.

Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of Oligotex spin columns. Homogenization with the TissueRuptor or QIAshredder homogenizers generally results in higher RNA yields than with a syringe and needle.

a. Homogenize cells using the TissueRuptor until the sample is uniformly homogeneous (typically ■ 20–30 s, ▲ 30–45 s, or ● 60–90 s at maximum speed).

or

b. Pipet the lysate directly onto a QIAshredder spin column placed in ■ 2 ml collection tube, and centrifuge for 2 min at maximum speed. Do not use QIAshredder homogenizers for >2 x 10^7 cells or >700 µl lysate. (Exception: see Table 11, page 59, note ‡)

or

c. Pass the lysate at least 5–10 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.

4. **Proteinase K digestion (optional): Add 5 µl QIAGEN Proteinase K solution* per milliliter of Buffer OL2. Mix and incubate at 56°C for 15 min. Then continue with step 5.**

   Buffer OL2 is optimal for Proteinase K digestion. Although for most samples, protein digestion is unnecessary, it may increase the RNA yield with some cell types. In addition, it may be helpful for biological material, such as blood, with a high protein content.

5. **Centrifuge for 3 min at maximum speed in a microcentrifuge (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Transfer supernatant to a new RNase-free tube.**

   This step removes remaining cell debris and protein. Be sure not to transfer any of the precipitate since this leads to clumping of the Oligotex resin in the following step, decreased performance of the procedure, and low yields of poly A^+ mRNA. If some of the cell debris or protein has been transferred, collect the precipitate again by centrifugation. Then continue with step 6.

   Note: Always centrifuge, even if no precipitate is visible.

6. **Add the appropriate amount of Oligotex Suspension to the sample (see Table 11, page 59). Mix thoroughly by pipetting or vortexing, and place at 20 to 30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

* See page 90 for ordering information. If using proteinase K from another supplier, use 100 µg proteinase K per milliliter of Buffer OL2.

7. **Pellet the Oligotex:mRNA complex by centrifuging in a microcentrifuge for 5 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

8. **Resuspend the pellet in ■ 350 µl, ▲ 600 µl, or ● 1 ml Buffer OW1 by vortexing or pipetting.**
9. **Pipet the sample onto a ■ small spin column or ▲ large spin column placed in a ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed. Discard the flow-through.**

   ● ***Maxi protocol***: Do not use a spin column. Transfer the resuspended pellet directly into a ● 2 ml microcentrifuge tube. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

   ● ***Maxi protocol***: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

10. **Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 350 µl or ▲ 600 µl Buffer OW2 onto the column. Centrifuge for 1 min at maximum speed, and discard the flow-through.**

    ● ***Maxi protocol***: Resuspend the pellet in ● 1 ml Buffer OW2. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

    ● ***Maxi protocol***: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

11. **Repeat step 10 once, using the same microcentrifuge tube.**

12. Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 20–100 µl or ▲ 50–300 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.

● **Maxi protocol**: Resuspend the pellet in ● 100–500 µl hot (70°C) Buffer OEB. Load onto a ● large spin column placed in a new RNase-free ● 2 ml microcentrifuge tube, and centrifuge for 1 min at maximum speed.

Note: The volume of Buffer OEB used depends on the desired or expected concentration of poly A^+ mRNA (see "Expected amounts of RNA in cells and tissues", page 37). Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

13. To ensure maximal yield, pipet another ■ 20–100 µl, ▲ 50–300 µl, or ● 100–500 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin. Centrifuge for 1 min at maximum speed.

To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Protocol: Isolation of Poly A$^+$ mRNA from Animal Tissues

Important notes before starting

- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- β-Mercaptoethanol (β-ME) must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl β-ME per 1 ml Buffer OL1. Buffer OL1 is stable for 1 month after addition of β-ME.
- Read "Introduction" (page 8) and "Important Notes" (page 13) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Buffer OW1 and Buffer OL1 may form a precipitate during storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- For best results, stabilize animal tissues immediately in RNA*later* RNA Stabilization Reagent. Tissues can be stored in RNA*later* RNA Stabilization Reagent for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C.
- Fresh, frozen, or RNA*later* stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer OL1. Homogenized tissue lysates (in Buffer OL1, step 2) can also be stored at –70°C for several months. To process frozen lysates, thaw samples and incubate for ■ 10 min (for ≤100 mg tissue), ▲ 15 min (for 100–250 mg tissue), or ● 20 min (for 250–1000 mg tissue) at 37°C in a water bath to dissolve salts. Continue the protocol with step 3.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- Unless otherwise indicated, all centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- To perform this protocol in batch format, see page 35.
- ■ denotes micro/mini prep volumes (for ≤100 mg tissue); ▲ denotes midi prep volumes (for 100–250 mg tissue); ● denotes maxi prep volumes (for 250–1000 mg tissue).

Procedure

1. **Determine the amount of tissue. Do not use more than ■ 100 mg, ▲ 250 mg, or ● 1 g tissue.**

   Weighing tissue is the most accurate way to determine the amount. See also page 36 for some general guidelines on tissue weights.

   Note: RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 2. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. For more information about stabilization of RNA in tissues using RNA*later* RNA Stabilization Reagent, see the *RNA*later *Handbook*, the *RNeasy Mini Handbook*, or the *RNeasy Midi/Maxi Handbook*.

   Note: Do not exceed the recommended amount of tissue. If too much sample or too little lysis buffer is used, a viscous sample will result, potentially clogging the spin column and causing reduced yields and impure mRNA preparations.

2. **Disrupt tissue and homogenize lysate.**

   Four alternative methods (a, b, c, or d) may be used for disruption and homogenization of animal tissue. See page 39 for a more detailed description of disruption and homogenization methods.

   Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the Oligotex spin column. Homogenization with the TissueRuptor generally results in higher RNA yields than with other homogenization methods.

   a. To simultaneously disrupt and homogenize the sample using lysis buffer and the TissueRuptor: Place tissue in a suitably sized vessel for the homogenizer. Add the appropriate volume of room-temperature Buffer OL1 (see Table 12, page 66), and homogenize using the TissueRuptor until the sample is homogeneous (typically ■ 20–45 s, ▲ 45–90 s, or ● 60–90 s at maximum speed). Continue the protocol with step 3.

   Note: β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

or

b. To disrupt the sample with a mortar and pestle and homogenize using QIAshredder homogenizers (■ micro/mini preps only): Immediately place the weighed, fresh or frozen tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of room-temperature Buffer OL1 (see Table 12, page 66). Pipet the lysate directly onto a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Continue the protocol with step 3. Do not use QIAshredder homogenizers for >100 mg tissue or >700 µl lysate.

Occasionally a salt precipitate may form when lysis Buffer OL1 is added to the frozen, ground tissue. If this happens, heat the lysate to 37°C for 5 min to redissolve salts before pipetting into the QIAshredder spin column.

Note: β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

or

c. To disrupt the sample with a mortar and pestle and homogenize using a needle and syringe: Immediately place the weighed, fresh or frozen tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of room-temperature Buffer OL1 (see Table 12, below), and homogenize by passing the lysate at least 5–10 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Continue the protocol with step 5.

Occasionally a salt precipitate may form when lysis Buffer OL1 is added to the frozen, ground tissue. If this happens, heat the lysate to 37°C for 5 min to redissolve salts before homogenizing with a needle and syringe.

Note: β-ME must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl of 14.3 M β-ME per 1 ml Buffer OL1.

Table 12. Buffer volumes for Oligotex Direct mRNA purification from tissue

Amount of tissue, mg		Buffer OL1,* ml	Buffer ODB, ml	Oligotex Suspension, µl	Prep size
■	≤10	0.6	1.2	20	Micro
■	10–25	0.6	1.2	35	Mini
■	25–50	0.6	1.2	70	Mini
■	50–100	0.6	1.2	110	Mini
▲	100–150	1.0	2.0	130	Midi
▲	150–250	2.0	4.0	165	Midi
●	250–500	3.0	6.0	250	Maxi
●	500–750	4.0	8.0	360	Maxi
●	750–1000	5.0	10.0	500	Maxi

* Be sure that β-ME has been added to Buffer OL1 before use (30 µl β-ME per 1 ml Buffer OL1).

3. **Add the appropriate volume of Buffer ODB (see Table 12, page 66) to the lysate, and mix thoroughly by pipetting. Centrifuge in a microcentrifuge for 3 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Transfer the supernatant to a new RNase-free tube.**

   This step removes remaining tissue debris and protein and creates optimal conditions for hybridization of poly A^+ mRNA to Oligotex. Be sure not to transfer any of the precipitate since this leads to clumping of the Oligotex resin in the following step, decreased performance of the procedure, and low yields of poly A^+ mRNA. If some of the tissue debris or protein has been transferred, collect the precipitate again by centrifugation. Then continue with step 4.

   Note: Always centrifuge, even if no precipitate is visible after addition of Buffer ODB.

4. **Add the appropriate amount of Oligotex Suspension to the sample (see Table 12, page 66). Mix thoroughly by pipetting or vortexing, and place at 20 to 30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

5. **Pellet the Oligotex:mRNA complex by centrifuging in a microcentrifuge for 5 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

   Optional: If time is a restricting factor and slight rRNA contamination is not a concern, the protocol can be shortened at this point by proceeding directly with step 9. The shortened procedure still provides significant enrichment of poly A^+ RNA. However, for best results, and particularly for applications where highly purified mRNA is necessary (e.g., cDNA library construction), the full protocol is recommended.

6. **Resuspend the Oligotex:mRNA pellet thoroughly in ■ 100 µl, ▲ 200 µl, or ● 300 µl Buffer OL1 by vortexing or pipetting.**

   Note: Ensure that β-ME is added to Buffer OL1 before use (see "Important notes before starting").

7. **Add ■ 400 µl, ▲ 800 µl, or ● 1200 µl Buffer ODB, incubate at 70°C for 3 min and then place at room temperature (15–25°C) for 10 min.**

   Note: This step significantly enriches for poly A^+ RNA by decreasing the amount of rRNA.

8. **Pellet the Oligotex:mRNA complex by centrifugation in a microcentrifuge for 5 min at maximum speed, and carefully remove the supernatant by pipetting.**

   ● Maxi protocol: Centrifuge for 10 min at 10,000 x *g*, and carefully remove the supernatant by pipetting.

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

9. **Resuspend the pellet in ■ 350 µl, ▲ 600 µl, or ● 1 ml Buffer OW1 by vortexing or pipetting.**

10. **Pipet the sample onto a ■ small spin column or ▲ large spin column placed in a ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed. Discard the flow-through.**

    ● **Maxi protocol**: Do not use a spin column. Transfer the resuspended pellet directly into a ● 2 ml microcentrifuge tube. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

    ● **Maxi protocol**: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

11. **Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 350 µl or ▲ 600 µl Buffer OW2 onto the column. Centrifuge for 1 min at maximum speed, and discard the flow-through.**

    ● **Maxi protocol**: Resuspend the pellet in ● 1 ml Buffer OW2. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

    ● **Maxi protocol**: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

12. **Repeat step 11 once, using the same microcentrifuge tube.**

13. **Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 20–100 µl or ▲ 50–300 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.**

    ● **Maxi protocol**: Resuspend the pellet in ● 100–500 µl hot (70°C) Buffer OEB. Load onto a ● large spin column placed in a new RNase-free ● 2 ml microcentrifuge tube, and centrifuge for 1 min at maximum speed.

    Note: The volume of Buffer OEB used depends on the desired or expected concentration of poly A^+ mRNA (see "Expected amounts of RNA in cells and tissues", page 37). Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

14. **To ensure maximal yield, pipet another ■ 20–100 µl, ▲ 50–300 µl, or ● 100–500 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin. Centrifuge for 1 min at maximum speed.**

    To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Protocol: Isolation of Poly A$^+$ mRNA from Animal Tissues using Urea–SDS Buffer

Important notes before starting

- This protocol is designed for proteinase K digestion following lysis and may be useful for tissues, such as heart or muscle, that have a high protein content. See page 35 for more information.
- Prepare Buffer OL2 (see Appendix C, page 85). β-ME must be added before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of 14.3 M β-ME per 1 ml Buffer OL2.
- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat Buffer OL2 to 50°C to dissolve salt precipitates.
- If performing the optional proteinase K digestion in step 3, heat a water bath or heating block to 56°C.
- Heat Buffer OEB to 70°C in a water bath or heating block.
- Read "Introduction" (page 8) and "Important Notes" (page 13) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- For best results, stabilize animal tissues immediately in RNA*later* RNA Stabilization Reagent. Tissues can be stored in RNA*later* RNA Stabilization Reagent for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C.
- Fresh, frozen, or RNA*later* stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer OL2.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- Unless otherwise indicated, all centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- To perform this protocol in batch format, see page 35.
- ■ denotes micro/mini prep volumes (for ≤100 mg tissue); ▲ denotes midi prep volumes (for 100–250 mg tissue); ● denotes maxi prep volumes (for 250–1000 mg tissue).

Procedure

1. **Determine the amount of tissue. Do not use more than ■ 100 mg, ▲ 250 mg, or ● 1 g tissue.**

   Weighing tissue is the most accurate way to determine the amount. However, as a general guide, a 3 mm cube of rat kidney (27 mm^3) weighs 30–35 mg, and a 5 mm cube (125 mm^3) weighs 150–175 mg. Weights for whole mouse organs are given on page 38.

   Note: RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 2. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. For more information about stabilization of RNA in tissues using RNA*later* RNA Stabilization Reagent, see the *RNA*later *Handbook*, the *RNeasy Mini Handbook*, or the *RNeasy Midi/Maxi Handbook*.

   Note: Do not exceed the recommended amount of tissue. If too much sample or too little lysis buffer is used, a viscous sample will result, potentially clogging the spin column and causing reduced yields and impure mRNA preparations.

2. **Disrupt tissue and homogenize lysate.**

   Four alternative methods (a, b, c, or d) may be used for disruption and homogenization of animal tissue. See page 39 for a more detailed description of disruption and homogenization methods.

   Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the Oligotex spin column. Homogenization with the TissueRuptor generally results in higher RNA yields than with other homogenization methods.

   a. To simultaneously disrupt and homogenize the sample using lysis buffer and the TissueRuptor: Place tissue in a suitably sized vessel for the homogenizer. Add the appropriate volume of Buffer OL2 (see Table 13, page 72), and homogenize using the TissueRuptor until the sample is uniformly homogeneous (typically ■ 20–45 s, ▲ 45–90 s, or ● 60–90 s at maximum speed). Continue the protocol with the optional proteinase K digestion (step 3), or proceed directly with step 4.

   Note: β-ME must be added to Buffer OL2 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of 14.3 M β-ME per 1 ml Buffer OL2.

   or

b. To disrupt the sample with a mortar and pestle and homogenize using QIAshredder homogenizers (■ micro/mini preps only): Immediately place the weighed, fresh or frozen tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen– cooled tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer OL2 (see Table 13, page 72). Pipet the lysate directly onto a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Continue the protocol with the optional proteinase K digestion (step 3), or proceed directly with step 4. Do not use QIAshredder homogenizers for >100 mg tissue or >700 µl lysate.

Occasionally a salt precipitate may form when lysis Buffer OL2 is added to the frozen, ground tissue. If this happens, heat the lysate to 37°C for 5 min to redissolve salts before pipetting into the QIAshredder spin column.

Note: β-ME must be added to Buffer OL2 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of 14.3 M β-ME per 1 ml Buffer OL2.

or

c. To disrupt the sample with a mortar and pestle and homogenize using a needle and syringe: Immediately place the weighed, fresh or frozen tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer OL2 (see Table 13, below), and homogenize by passing the lysate at least 5–10 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Continue the protocol with the optional proteinase K digestion (step 3), or proceed directly with step 4.

Occasionally a salt precipitate may form when lysis Buffer OL2 is added to the frozen, ground tissue. If this happens, heat the lysate to 37°C for 5 min to redissolve salts before homogenizing with a needle and syringe.

Note: β-ME must be added to Buffer OL2 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of 14.3 M β-ME per 1 ml Buffer OL2.

Table 13. Urea–SDS buffer volumes for Oligotex Direct mRNA purification from tissue

Amount of tissue, mg	Buffer OL2,* ml	Oligotex Suspension, µl	Prep size
■ ≤10	0.6	1.2	20
■ 10–25	0.6	1.2	35
■ 25–50	0.6	1.2	70
■ 50–100	0.6	1.2	110
▲ 100–150	1.0	2.0	130
▲ 150–250	2.0	4.0	165
● 250–500	3.0	6.0	250
● 500–750	4.0	8.0	360
● 750–1000	5.0	10.0	500

* Be sure that β-ME has been added to Buffer OL2 before use (10 µl β-ME per 1 ml Buffer OL2).

3. **Proteinase K digestion (optional): Add 5 µl QIAGEN Proteinase K solution* per milliliter of Buffer OL2. Mix and incubate at 56°C for 15–60 min. Then continue with step 4.**

   Buffer OL2 is optimal for Proteinase K digestion. Although for most samples, protein digestion is unnecessary, it should always be used for tissues, such as heart or muscle, that have a high protein content. In addition, it may increase the RNA yield with other tissues.

4. **Centrifuge for 3 min at maximum speed in a microcentrifuge (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Transfer supernatant to a new RNase-free tube.**

   This step removes remaining tissue debris and protein. Be sure not to transfer any of the precipitate since this leads to clumping of the Oligotex resin in the following step, decreased performance of the procedure, and low yields of poly A^+ mRNA. If some of the cell debris or protein has been transferred, collect the precipitate again by centrifugation. Then continue with step 5.

   Note: Always centrifuge, even if no precipitate is visible.

* See page 90 for ordering information. If using proteinase K from another supplier, use 100 µg Proteinase K per milliliter of Buffer OL2.

5. **Add the appropriate amount of Oligotex Suspension to the sample (see Table 13, page 72). Mix thoroughly by pipetting or vortexing, and place at 20 to 30°C for 10 min.**

   This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

6. **Pellet the Oligotex:mRNA complex by centrifuging in a microcentrifuge for 5 min at maximum speed (14,000–18,000 x *g*) if microcentrifuge tubes are used, or 10 min at 10,000 x *g* if larger tubes are used. Carefully remove the supernatant by pipetting.**

   Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube (up to 100 µl if larger tubes are used). The remaining solution will not affect subsequent steps in the procedure.

   Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

7. **Resuspend the pellet in ■ 350 µl, ▲ 600 µl, or ● 1 ml Buffer OW1 by vortexing or pipetting.**

8. **Pipet the sample onto a ■ small spin column or ▲ large spin column placed in a ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed. Discard the flow-through.**

   ● **Maxi protocol**: Do not use a spin column. Transfer the resuspended pellet directly into a ● 2 ml microcentrifuge tube. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

   ● **Maxi protocol**: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

9. **Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 350 µl or ▲ 600 µl Buffer OW2 onto the column. Centrifuge for 1 min at maximum speed, and discard the flow-through.**

   ● **Maxi protocol**: Resuspend the pellet in ● 1 ml Buffer OW2. Centrifuge for 5 min at maximum speed, and carefully discard the supernatant.

   ● **Maxi protocol**: Loss of the Oligotex resin can be avoided if approximately 100 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect subsequent steps in the procedure.

10. **Repeat step 9 once, using the same microcentrifuge tube.**

11. Transfer the spin column to a new RNase-free ■ 1.5 ml or ▲ 2 ml microcentrifuge tube. Pipet ■ 20–100 µl or ▲ 50–300 µl hot (70°C) Buffer OEB onto the column. Pipet up and down three or four times to resuspend the resin, and centrifuge for 1 min at maximum speed.

● **Maxi protocol**: Resuspend the pellet in ● 100–500 µl hot (70°C) Buffer OEB. Load onto a ● large spin column placed in a new RNase-free ● 2 ml microcentrifuge tube, and centrifuge for 1 min at maximum speed.

Note: The volume of Buffer OEB used depends on the desired or expected concentration of poly A^+ mRNA (see "Expected amounts of RNA in cells and tissues", page 37). Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

12. To ensure maximal yield, pipet another ■ 20–100 µl, ▲ 50–300 µl, or ● 100–500 µl hot (70°C) Buffer OEB onto the column. Pipet up and down three or four times to resuspend the resin. Centrifuge for 1 min at maximum speed.

To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Clogged spin column	
Oligotex mRNA protocols	
a) Spin column overloaded	Do not use more than 1 mg total RNA per preparation with the small spin columns in the Oligotex mRNA Mini or Midi Kit. Do not use more than 3 mg total RNA per preparation with the large spin columns in the Oligotex mRNA Maxi Kit.
b) Partially undissolved total RNA	If starting with precipitated RNA, dissolve the RNA pellet in the appropriate amount of RNase-free water by heating the tube for 3 min at 60°C followed by vortexing for 5 second and sharply flicking the tube. Repeat at least twice.
c) Impure starting RNA	Better results are generally obtained with purer starting material. We recommend using total RNA isolated or cleaned up using RNeasy Kits (see pages 98–99 for ordering information). The Oligotex mRNA batch protocol is recommended if you are unsure about the purity of your total RNA.
d) Centrifugal force too low	Ensure that points (a), (b), and (c) above are not a factor. Increase centrifugal force or centrifugation time.
Oligotex Direct mRNA protocols	
a) Insufficient homogenization	Homogenization is an absolute requirement for direct mRNA isolation (see "Disruption and homogenization of starting material", page 39). To continue, see "Rescue of sample" below.

	Comments and suggestions
b) Too many cells or too much tissue per volume of lysis Buffer OL1	The viscosity of the lysate is critical for direct mRNA isolation. Do not exceed the recommended amount of tissue or number of cells (see Table 3, page 9). To continue, see "Rescue of sample" below. In future preparations, use batch format (see page 35), reduce the amount of starting material, and/or increase the volume of lysis buffer.
c) Diluted lysate preparation contaminated with cell debris or protein precipitate	Be sure not to transfer any of the cell-debris pellet with the supernatant, as this leads to clumping of the Oligotex resin in the following step, decreased performance of the procedure, and low yields of poly A^+ mRNA. If some of the cell debris or protein has been transferred, collect the precipitate again by centrifugation. To continue, see "Rescue of sample" below.
d) Centrifugal force too low	Ensure that points (a)–(c) above are not a factor. Increase centrifugal force or centrifugation time. Rescue of sample for points (a)–(c): To rescue the sample, transfer the flow-through and the clogged column, upside-down, to a 15 ml polypropylene centrifuge tube. Add enough buffer to cover the spin column: either 1 part lysis Buffer OL1 plus 2 parts dilution Buffer ODB (standard protocols), or equal parts lysis Buffer OCL plus dilution Buffer OCD (cytoplasm protocol), or lysis Buffer OL2 alone (urea–SDS protocols). Vortex and spin down Oligotex:mRNA complexes. Discard column. Resuspend Oligotex pellet, hybridize mRNA for 10 min at room temperature (15–25°C), and spin down the Oligotex:mRNA complexes. Aspirate supernatant, and resuspend Oligotex resin in 1 ml Buffer OW1. Transfer to a microcentrifuge tube, and continue the procedure in batch format (see page 35).

Low or no yield of RNA

Oligotex mRNA and Oligotex Direct mRNA protocols

a) Elution volume too low	Repeat elution steps with another aliquot of hot (70°C) Buffer OEB.

	Comments and suggestions
b) Temperature of elution Buffer OEB too low	Be certain that Buffer OEB has been heated to 70°C. Remember that small volumes cool down quickly. Buffer OEB should therefore be added immediately to the sample. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex resin, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.
c) Insufficient elution	Be sure to follow the full elution procedure: add hot (70°C) Buffer OEB or eluate to the spin column, pipet up and down to resuspend Oligotex resin, and spin to elute.
d) RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the procedure or later handling. See Appendix A, page 81.
e) Insufficient hybridization time	Repeat the procedure with the supernatant saved during the procedure. Add fresh Oligotex Suspension, and repeat the protocol beginning with the 70°C incubation (Oligotex mRNA protocols and Oligotex Direct mRNA cytoplasmic protocol) or room-temperature hybridization step (Oligotex Direct mRNA protocols). Extend hybridization time if necessary.
f) Lysate or starting RNA solution too viscous or concentrated during hybridization	Be sure not to exceed the recommended amount of total RNA (Oligotex mRNA protocols) or cells and tissue (Oligotex Direct mRNA protocols). Dilute the supernatant saved during the procedure. Add fresh Oligotex, and repeat the protocol beginning with the 70°C incubation (Oligotex mRNA protocols and Oligotex Direct mRNA cytoplasmic protocol) or room-temperature hybridization step (Oligotex Direct mRNA protocols).

	Comments and suggestions
g) Temperature too high during hybridization	Hybridization should be carried out at 20 to 30°C as indicated in the respective protocols. If the ambient temperature is higher, a cooled water bath may be necessary. Repeat the procedure with the supernatant saved during the procedure. Add fresh Oligotex Suspension, and repeat the protocol beginning with the 70°C incubation (Oligotex mRNA protocols and Oligotex Direct mRNA cytoplasmic protocol) or room-temperature hybridization step (Oligotex Direct mRNA protocols).
Oligotex Direct mRNA protocols	
a) Insufficient homogenization	If any cell clumps or chunks of tissue were visible, the sample was not homogenized properly, and the mRNA inside the clumps could not be isolated. Homogenize until the tissue or cells are uniformly suspended.
b) Co-precipitation of nucleic acid with proteins when Buffer ODB is added to the lysate (standard protocols)	Make sure that the initial centrifugation to remove cell debris and protein is performed at 20–30°C. Repeat the initial centrifugation step if necessary.
c) Incomplete mixing after addition of Buffer ODB	Mix thoroughly after addition of Buffer ODB.
mRNA degradation	
a) RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the procedure or later handling. See Appendix A, page 81.
b) Sample inappropriately handled	Ensure that samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent. For frozen cell pellets or tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the protocol quickly, especially the first few steps. See Appendix A (page 81), and "Handling and storage of samples" (page 38).

Poor downstream performance of mRNA

Carryover of salts or cellular components	Be sure to thoroughly resuspend the Oligotex pellet as described in the protocols following centrifugation steps. Incomplete resuspension can leave salts or cellular components trapped in the Oligotex pellet. If necessary, also carefully resuspend the Oligotex resin on the spin column during the Buffer OW2 wash steps.

mRNA eluate volume greater than expected

Carryover of Buffer OW2	Some Buffer OW2 may carry over from the final wash step. Although this will not affect downstream applications, it will slightly increase the volume of the eluate. For maximum removal of Buffer OW2, carry out the Buffer OW2 washes and elution in a batch mode. Alternatively, after the final Buffer OW2 wash step, place the spin column in a new microcentrifuge tube, and centrifuge for 1 min at maximum speed. Discard the microcentrifuge tube, and proceed with the elution steps.

rRNA contamination

Oligotex mRNA protocols

Standard protocols	The standard procedures provide significant enrichment of poly A^+ RNA. However, for a somewhat higher enrichment of mRNA, the following enrichment steps can be added. For all Oligotex mRNA protocols: following step 5, add equal amounts of RNase-free water and Buffer OBB. (If following the Oligotex mRNA protocol using Oligotex Direct mRNA buffers, use 1 part Buffer OL1 plus 4 parts Buffer ODB.) Mix the contents thoroughly by pipetting or flicking the tube. Repeat steps 3–5, and then continue the relevant protocol with step 6.

	Comments and suggestions
Oligotex Direct mRNA protocols	
a) Shortened protocol used	The shortened procedure still provides significant enrichment of poly A^+ RNA. However, for best results, and particularly for applications where highly purified mRNA is necessary (e.g., cDNA library construction), the full protocol is recommended. To clean up a sample with too much rRNA, do another round of purification following the optional Oligotex mRNA protocol using Oligotex Direct mRNA buffers (Appendix D, page 87) or using one of the Oligotex mRNA Kits.
b) Standard protocol used	For many cell lines, the urea–SDS protocol is useful for reducing rRNA background. It should be used if rRNA contamination is a problem in the standard protocol. To clean up a sample with too much rRNA, do another round of purification following the optional Oligotex mRNA protocol using Oligotex Direct mRNA buffers (Appendix D, page 87) or using one of the Oligotex mRNA Kits.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Appendix A: General Remarks for Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipets and electrophoresis tanks), use of RNaseKiller (cat. no 2500080) from 5 PRIME (www.5prime.com) is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 82), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (SDSs), available from the product supplier.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix B: Storage, Quantification, and Determination of Quality of Poly A$^+$ mRNA

Storage

mRNA may be stored at –20°C or –70 °C in elution buffer. Under these conditions, no degradation of RNA has been detected, even after 2 to 3 years.

Quantification and purity of poly A$^+$ RNA

See "Quantification of starting RNA" and "Purity of starting RNA" (page 20). Since poly A$^+$ mRNA accounts for only 1–5% of the total RNA, amounts may be difficult to determine photometrically. Fluorimetric determination* or quantitative RT-PCR are more sensitive and more accurate methods, especially for low amounts of RNA. Small amounts of RNA can be accurately quantified using an Agilent® 2100 Bioanalyzer®, quantitative RT-PCR, or fluorometric quantification.

Integrity of RNA

Because Oligotex captures mRNA at the 3' end, integrity of the RNA in the starting material is absolutely crucial for representation of complete transcript sequences in the isolated poly-A RNA.

The integrity of total RNA as starting material for isolation of mRNA with Oligotex technology can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining† or by using the QIAxcel® system or Agilent 2100 Bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

The Agilent 2100 Bioanalyzer also provides an RNA Integrity Number (RIN) as a useful measure of RNA integrity. Ideally, the RIN should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

The integrity and size distribution of the mRNA purified with Oligotex technology can be checked by denaturing agarose gel electrophoresis or by using the

* Ausubel, F.M. et al., eds. (1991) *Current protocols in molecular biology*. New York: Wiley Interscience, p. A.3D.1.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

QIAxcel system or Agilent 2100 Bioanalyzer. Isolated mRNA should appear as a smear ranging from 200 nt to 8 kb. The bulk of the mRNA should lie between 1.5 and 4 kb. If only small amounts of mRNA are purified, visualization may be difficult.

The integrity and size distribution of the mRNA purified with Oligotex technology can be checked by denaturing agarose gel electrophoresis. Ethidium bromide-stained mRNA should appear as a smear ranging from 200 nt to 8 kb. The bulk of the mRNA should lie between 1.5 and 4 kb. If only small amounts of mRNA are purified, visualization by ethidium bromide staining may be difficult.

Poly A^+ mRNA can be visualized on a northern blot of the gel hybridized with a labeled oligo-dT probe. The mRNA integrity can also be analyzed by hybridization of the northern blot with a probe for a specific mRNA present in the RNA pool. A discrete band indicates intact mRNA.

Appendix C: Composition of Buffers and Solutions

Oligotex Suspension

10% (w/v) suspension (= 1 mg/10 µl) Oligotex particles in:

10 mM	Tris·Cl, pH 7.5
500 mM	NaCl
1 mM	EDTA
0.1%	SDS
0.1%	NaN_3 (sodium azide)*

Wash Buffer OW2

10 mM	Tris·Cl, pH 7.5
150 mM	NaCl
1 mM	EDTA

Elution Buffer OEB

5 mM	Tris·Cl, pH 7.5

Buffer for Oligotex mRNA protocols

Buffer OBB

20 mM	Tris·Cl, pH 7.5
1 M	NaCl
2 mM	EDTA
0.2%	SDS

* Sodium azide is highly toxic and may react explosively with lead and copper drain pipes. Take appropriate safety measures and wear gloves when handling. Dispose of azide-containing solutions according to your institution's waste-disposal guidelines.

Buffers for direct mRNA purification from cytoplasm of cultured cells

Buffer OCL

10 mM	Tris·Cl pH 7.5
140 mM	NaCl
5 mM	KCl
1 %	Nonidet P-40*

Add before use:

1000 U/ml	RNase inhibitor (optional)
1 mM	DTT (optional)

Buffer OCD

1 M	LiCl
20 mM	Tris·Cl, pH 7.5
2 mM	EDTA
1%	SDS

Buffer for direct mRNA purification using urea–SDS buffer

Buffer OL2

700 mM	urea
500 mM	NaCl
50 mM	HEPES, pH 7.8
1 %	SDS

Add before use:

1% (v/v)	β-mercaptoethanol (or 10 mM DTT)†
5 µl/ml	QIAGEN Proteinase K solution‡ (optional)

Note: Buffer OL2 is stable for 2 weeks at room temperature (15–25°C). If stored for longer periods of time, the pH should be adjusted to 7.8 immediately prior to use. Do not autoclave.

* Nonidet P-40 is no longer manufactured. It can be replaced with Nonidet P-40 Substitute (Fluka, cat. no. 74385) or Igepal® CA-630 (SIGMA, cat. no. I 3021).

† β-Mercaptoethanol (β-ME) is recommended for the animal-cell protocol although, for most cell lines, DTT (dithiothreitol) may be used instead. For cell lines containing high RNase activity and for the animal-tissue protocol, only β-ME can be used. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing.

‡ See page 90 for ordering information. If using proteinase K from another supplier, use 100 µg proteinase K per 1 ml of Buffer OL2.

Appendix D: Oligotex mRNA Protocol Using Oligotex Direct mRNA Buffers

For isolation of poly A^+ mRNA from total RNA using buffers from the Oligotex Direct mRNA Kits

Important notes before starting

- Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature (15–25°C).
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- β-Mercaptoethanol (β-ME) must be added to Buffer OL1 before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 30 µl β-ME per 1 ml Buffer OL1. Buffer OL1 is stable for 1 month after addition of β-ME.
- Read "Introduction" (page 8) before starting.
- If working with RNA for the first time, please read Appendix A (page 81).
- Determine the amount of total RNA in the RNA sample (see "Quantification of starting RNA", page 20).
- Buffer OW1 and Buffer OL1 may form a precipitate upon storage. If necessary, redissolve by warming at 37°C, and then place at room temperature.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C.
- All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).
- To perform this protocol in batch format, see page 35.
- ▲ denotes mini/midi prep volumes (with ≤1.0 mg total RNA); ● denotes maxi prep volumes (with 1.0–3.0 mg total RNA).

Procedure

D1. Determine the amount of starting RNA. Do not use more than ▲ 1 mg or ● 3 mg total RNA. Pipet total RNA into an RNase-free 1.5 ml microcentrifuge tube, and adjust the volume with Buffer ODB (if necessary) to the volume indicated in Table 14, below.

Note: The initial volume of the RNA solution is not important so long as the volume can be brought up to the indicated amount with Buffer ODB. If starting with precipitated RNA, dissolve the RNA pellet in the appropriate amount of Buffer ODB by heating the tube for 3 min at 60°C followed by vortexing for 5 s and sharply flicking the tube. Repeat at least twice.

D2. Add the appropriate volume of Buffer OL1 and Oligotex Suspension (see Table 14). Mix the contents thoroughly by pipetting or flicking the tube.

Table 14. Buffer amounts for Oligotex mRNA purification using Oligotex Direct mRNA buffers

Total RNA, mg	Add Buffer ODB to final volume, µl	Buffer OL1, µl	Oligotex Suspension, µl	Prep size
▲ ≤0.25	400	100	15	Mini
▲ 0.25–0.50	800	200	30	Midi
▲ 0.50–0.75	800	200	45	Midi
▲ 0.75–1.00	800	200	55	Midi
● 1.0–1.5	1040	260	85	Maxi
● 1.5–2.0	1040	260	115	Maxi
● 2.0–2.5	1040	260	135	Maxi
● 2.5–3.0	1040	260	175	Maxi

D3. Incubate the sample for 3 min at 70°C in a water bath or heating block.

This step disrupts secondary structure of the RNA.

D4. Remove sample from the water bath/heating block, and place at 20 to 30°C for 10 min.

This step allows hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA.

D5. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed, and carefully remove the supernatant by pipetting.

Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure.

Note: Save the supernatant until certain that satisfactory binding and elution of poly A^+ mRNA has occurred.

D6. Resuspend the Oligotex:mRNA pellet in ▲ 400 µl or ● 600 µl Buffer OW1 by vortexing or pipetting, and pipet onto a ▲ small spin column or a ● large spin column placed in a 1.5 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed.

Small spin columns are supplied with the Oligotex Direct mRNA Micro and Mini Kits and can be purchased separately (see page 95 for ordering information). Large spin columns are supplied in the Oligotex Direct mRNA Midi/Maxi Kits.

D7. Transfer the spin column to a new RNase-free 1.5 ml microcentrifuge tube, and apply ▲ 400 µl or ● 600 µl Buffer OW2 to the column. Centrifuge for 1 min at maximum speed and discard the flow-through.

D8. Repeat step 7 once.

D9. Transfer spin column to a new RNase-free 1.5 ml microcentrifuge tube. Pipet 20–100 µl hot (70°C) Buffer OEB onto the column, pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.

Note: The volume of Buffer OEB used depends on the expected or desired concentration of poly A^+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

D10. To ensure maximal yield, pipet another 20–100 µl hot (70°C) Buffer OEB onto the column. Pipet up and down 3 or 4 times to resuspend the resin, and centrifuge for 1 min at maximum speed.

To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C, and elute in the same microcentrifuge tube. However, for maximal yield, the additional volume of Buffer OEB is recommended.

Appendix E: Disruption and Homogenization of RNA*later* Stabilized Tissues using the TissueLyser LT

Important points before starting

- Ensure that you are familiar with operating the TissueLyser LT by referring to the *TissueLyser LT User Manual*.
- After storage in RNA*later* RNA Stabilization Reagent or Allprotect Tissue Reagent, tissues become slightly hard. If disrupting in Buffer RLT, we recommend increasing the volume of this buffer according to the protocols in the *RNeasy Mini Handbook*. Precooling of tubes and samples (steps E1–E3) is not necessary for stabilized tissue.
- When disrupting tough or very tough samples, we recommend using one or two 7 mm stainless steel beads, respectively, instead of one 5 mm stainless steel bead to guarantee optimal disruption.

Procedure

E1. Place 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm mean diameter) on dry ice for at least 15 min. Keep the insert of the TissueLyser LT Adapter at room temperature (15–25°C).

E2. Transfer up to 30 mg fresh or frozen tissue to the precooled tubes and incubate for another 15 min on dry ice.

If handling tissue samples stabilized with RNA*later* RNA Stabilization Reagent or Allprotect Tissue Reagent, cooling on dry ice is not necessary.

E3. Place the tubes into the insert of the TissueLyser LT Adapter, and incubate at room temperature for 2 min to avoid freezing of lysis buffer in step 4.

Do not incubate for longer than 2 min, otherwise the tissue will thaw, resulting in potential RNA degradation.

E4. Immediately add the appropriate volume of lysis buffer (e.g., Buffer RLT, Buffer RLT Plus, or QIAzol Lysis Reagent) to each tube.

Note: If using Buffer RLT Plus, we recommend adding Reagent DX to prevent excessive foaming.

E5. Place the insert with sample tubes into the base of the TissueLyser LT Adapter, which is attached to the TissueLyser LT. Place the lid of the TissueLyser LT Adapter over the insert, and screw the knob until the lid is securely fastened.

E6. Operate the TissueLyser LT for 2–5 min at 50 Hz.

The duration of disruption and homogenization depends on the tissue being processed and can be extended until no tissue debris is visible.

If processing fiber-rich tissues, complete disruption and homogenization may sometimes not be possible. However, small amounts of debris have no effect on subsequent RNA purification with QIAGEN kits and are usually digested in the proteinase K step.

E7. Proceed with RNA purification.

Do not reuse the stainless steel beads.

Appendix E: Guidelines for RT-PCR and Real-Time RT-PCR

RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. RT and PCR can be carried out either sequentially in the same tube (1-step RT-PCR) or separately (2-step RT-PCR).

One-step RT-PCR requires gene-specific primers. For this application, QIAGEN offers the QIAGEN OneStep RT-PCR Kit, which enables one-step RT-PCR of any RNA template without optimization (see page 99 for ordering information).

Two-step RT-PCR is generally carried out using oligo-dT and ramdom primers in the RT step and gene-specific primers in the PCR step. For the RT step, QIAGEN offers the QuantiTect® Reverse Transcription Kit for efficient and sensitive reverse transcription.

For the PCR step, QIAGEN offers enzymes that minimize PCR optimization:

- *Taq* DNA Polymerase — for PCR without a hot start
- HotStarTaq® DNA Polymerase — for PCR with a hot start
- HotStarTaq Plus DNA Polymerase — for PCR with a hot start and a fast 5 min enzyme activation time

For more information on QIAGEN products for one-step RT-PCR and two-step RT-PCR, visit www.qiagen.com/products/pcr.

Real-time RT-PCR

The range of QuantiTect Kits and Assays guarantee highly specific and sensitive results in real-time RT-PCR on any real-time cycler and require no optimization of reaction and cycling conditions. QuantiTect Kits are available for two-step and one-step RT-PCR and are compatible with detection by SYBR® Green I dye or by sequence-specific probes (e.g., TaqMan and FRET probes). Multiplex RT-PCR of up to 4 targets is also possible. Predesigned, validated QuantiTect Assays are supplied as primer sets or primer–probe sets and are easily ordered online at www.qiagen.com/GeneGlobe. For more information on QuantiTect Kits and Assays, visit www.qiagen.com/geneXpression.

Appendix F: Protocol for Formaldehyde Agarose (FA) Gel Electrophoresis

The following protocol for formaldehyde agarose (FA) gel electrophoresis is routinely used at QIAGEN and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols (e.g., Sambrook, J. et al., eds. (1989) *Molecular cloning — a laboratory manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

FA gel preparation

To prepare FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, mix:

1.2 g agarose

10 ml 10x FA gel buffer (see composition below)

Add RNase-free water to 100 ml

If smaller or larger gels are needed, adjust the quantities of components proportionately.

Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde* and 1 µl of a 10 mg/ml ethidium bromide* stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer (see composition below) for at least 30 min.

RNA sample preparation for FA gel electrophoresis

Add 1 volume of 5x loading buffer (see composition below) to 4 volumes of RNA sample (for example 10 µl of loading buffer and 40 µl of RNA) and mix.

Incubate for 3–5 min at 65°C, chill on ice, and load onto the equilibrated FA gel.

Gel running conditions

Run gel at 5–7 V/cm in 1x FA gel running buffer.

* Toxic and/or mutagenic. Take appropriate safety measures.

Composition of FA gel buffers

10x FA gel buffer

200 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

Adjust to pH 7.0 with NaOH.

1x FA gel running buffer

100 ml 10x FA gel buffer

20 ml 37% (12.3 M) formaldehyde*

880 ml RNase-free water

5x RNA loading buffer

16 µl saturated aqueous bromophenol blue solution†

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde*

2 ml 100% glycerol

3.084 ml formamide

4 ml 10 x FA gel buffer

Add RNase-free water to 10 ml.

Stability: Approximately 3 months at 4°C

* Toxic and/or mutagenic. Take appropriate safety measures.

† To make a saturated solution, add solid bromophenol blue to distilled water. Mix and continue to add more bromophenol blue until no more will dissolve. Centrifuge to pellet the undissolved powder, and carefully pipet the saturated supernatant.

Ordering Information

Product	Contents	Cat. no.
Oligotex mRNA Mini Kit (12)	For 12 mRNA minipreps: 200 µl Oligotex Suspension, Small Spin Columns, Collection Tubes (1.5 ml), RNase-Free Reagents and Buffers	70022
Oligotex mRNA Midi Kit (12)	For 12 mRNA midipreps: 700 µl Oligotex Suspension, Small Spin Columns, Collection Tubes (1.5 ml), RNase-Free Reagents and Buffers	70042
Oligotex Direct mRNA Mini Kit (12)	For 12 mRNA minipreps: 420 µl Oligotex Suspension, Small Spin Columns, Collection Tubes (1.5 ml), RNase-Free Reagents and Buffers	72022
Oligotex Direct mRNA Midi/Maxi Kit (6/2)	For 6 mRNA midi or 2 maxipreps: 1 ml Oligotex Suspension, Large Spin Columns, Collection Tubes (1.5 ml), RNase-Free Reagents and Buffers	72041
Oligotex Suspension (0.5 ml)	0.5 ml for mRNA isolation from up to 8 mg of total RNA	79000
Accessories		
Small Spin Columns (24)	24 RNase-free spin columns for Oligotex spin procedures	79523
QIAGEN Proteinase K (2 ml)	2 ml (>600 mAU/ml, solution)	19131
QIAGEN Proteinase K (10 ml)	10 ml (>600 mAU/ml, solution)	19133

Product	Contents	Cat. no.
Centrifuges		
Centrifuge 4–16	Universal laboratory centrifuge with brushless motor	81300* 81310† 81325‡ 81320§
Centrifuge 4–16K	Refrigerated universal laboratory centrifuge with brushless motor	81400* 81410† 81425‡ 81420§
Related products		
QIAshredder — for simple and rapid homogenization of cell and tissue lysates		
QIAshredder (50)¶	50 disposable cell-lysate homogenizers for use in nucleic acid minipreps, caps	79654
TissueLyser II — for medium- to high-throughput sample disruption for molecular analysis		
TissueLyser II	Bead mill, 100-120/220-240 V, 50/60 Hz; requires the TissueLyser Adapter Set 2 x 24 or TissueLyser Adapter Set 2 x 96 (available separately)**	85300
TissueLyser Adapter Set 2 x 24	2 sets of adapter plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser II	69982
TissueLyser Adapter Set 2 x 96	2 sets of adapter plates for use with Collection Microtubes (racked) on the TissueLyser II	69984

* Japan. † North America. ‡ UK. § Rest of the world.

¶ Other kit sizes are available; see www.qiagen.com.

** The TissueLyser II must be used in combination with the TissueLyser Adapter Set 2 x 24 or TissueLyser Adapter Set 2 x 96.

Product	Contents	Cat. no.
TissueLyser LT — for low- to medium-throughput sample disruption for molecular analysis		
TissueLyser LT	Compact bead mill, 100-240 V AC, 50-60 Hz; requires the TissueLyser LT Adapter, 12-Tube (available separately)*	85600
TissueLyser LT	Adapter, 12-Tube Adapter for disruption of up to 12 samples in 2 ml microcentrifuge tubes on the TissueLyser LT*	69980
TissueRuptor — for low-throughput sample disruption for molecular analysis		
TissueRuptor (120 V, 60 Hz, US/JP)	Handheld rotor–stator homogenizer, 120 V, 60 Hz (for North America and Japan), 5 TissueRuptor Disposable Probes	9001271
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor	990890
RNA*later* RNA Stabilization Reagent — for immediate stabilization of RNA in tissues		
RNA*later* RNA Stabilization Reagent (50 ml)†	50 ml RNA*later* RNA Stabilization Reagent for stabilization of RNA in 25 x 200 mg tissue samples	76104
Allprotect Tissue Reagent — for immediate stabilization of DNA, RNA, and protein in tissues		
Allprotect Tissue Reagent (100 ml)	100 ml Allprotect Tissue Reagent, Allprotect Reagent Pump	76405

* The TissueLyser LT must be used in combination with the TissueLyser LT Adapter, 12-Tube.

† Other kit sizes are available; see www.qiagen.com.

Product	Contents	Cat. no.
RNeasy Protect Kits — for stabilization and purification of up to 100 µg total RNA from tissues		
RNeasy Protect Mini Kit (50)*	RNA*later* RNA Stabilization Reagent (50 ml), 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74124
RNeasy Protect Maxi Kit (12)	RNA*later* RNA Stabilization Reagent (100 ml), 12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-free Reagents and Buffers	75182
RNeasy Kits — for purification of total RNA from cells, tissues, and yeast		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Midi Kit (10)*	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75142
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-free Reagents and Buffers	75162
QIAamp® RNA Blood Mini Kit — for purification of cellular RNA from fresh whole blood		
QIAamp RNA Blood Mini Kit (50)*	For 50 RNA preps: 50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	52304
RNeasy Plant Mini Kit — for purification of total RNA from plants and fungi		
RNeasy Plant Mini Kit (20)*	20 RNeasy Mini Spin Columns, 20 QIAshredder Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74903

* Other kit sizes are available; see www.qiagen.com.

Product	Contents	Cat. no.
RNeasy 96 Kit — for 96-well purification of total RNA from cells		
RNeasy 96 Kit (4)*	For 4 x 96 total and cytoplasmic RNA preps: 4 RNeasy 96 Plates, Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, RNase-Free Reagents and Buffers	74181
Omniscript RT Kit — for reverse transcription of 50 ng to 2 µg RNA per reaction		
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-free water	205110
Sensiscript RT Kit — for reverse transcription of less than 50 ng RNA per reaction		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-free water	205211
QIAGEN OneStep RT-PCR Kit — for highly sensitive and successful one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 x 50 µl reactions: QIAGEN OneStep RT-PCR Enzyme Mix (1 x 50 µl), 5x QIAGEN OneStep RT-PCR Buffer (1 x 250 µl), dNTP Mix (1 x 50 µl, 10 mM each), 5x Q-Solution® (1 x 400 µl), RNase-free water (1 x 1.9 ml)	210210
HotStarTaq DNA Polymerase — for highly specific amplification with minimal optimization		
HotStarTaq DNA Polymerase (250 U)*	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$	203203
***Taq* DNA Polymerase — for standard and specialized PCR applications**		

Product	Contents	Cat. no.
Taq DNA Polymerase (250 U)	250 units *Taq* DNA Polymerase, 10x PCR Buffer, 10x CoralLoad® PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$	201203
QuantiTect Rev. Transcription Kit — for fast cDNA synthesis enabling sensitive real-time two-step RT-PCR for gene expression analysis		
QuantiTect Rev. Transcription Kit (50)*	For 50 x 20 µl reactions: 100 µl 7x gDNA Wipeout Buffer, 50 µl Quantiscript Reverse Transcriptase, 200 µl 5x Quantiscript RT Buffer, 50 µl RT Primer Mix, 1.9 ml RNase-Free Water	205311
QuantiTect Probe RT-PCR Kit — for one-step qRT-PCR using sequence-specific probes for gene expression analysis		
QuantiTect Probe RT-PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Probe RT-PCR Master Mix, 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204443
QuantiTect SYBR Green RT-PCR Kit — for one-step qRT-PCR using SYBR Green I for gene expression analysis		
QuantiTect SYBR Green RT-PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green RT-PCR Master Mix, 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204243

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

* Other kit sizes are available; see www.qiagen.com.

Notes

Notes

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