SOP #: SOP017

TITLE: SOP of QPCR mRNA Preparation-Internal SOP

Version: 2.1

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Approval:	**Approved By**	**Date**
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	Dirk Dittmer, Director	**2018-07-14**

Revision History:	**Version**	**Effective Date**	**Description**
	1.0	8/31/2012	First Draft of SOP
	1.1	**5/17/13**	Updated several inconsistences
	2.0	5/5/2016	Added instructions for bead beating.
	2.1	2018-07-14	Expanded materials list

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Staff Signatures: (signing below indicates that you have read this SOP and understand the material contained in it)

Date	**Name (please print)**	**Signature**	**Date SOP 1st Performed**

1. Purpose

This document describes how to prepare samples from RNA isolation, enrich for mRNA, cDNA preparation, and submission to the Vironomics Core for real-time PCR (QPCR) arrays.

2. Introduction

This experiment is designed to isolate total RNA from cell pellets and prepare the samples for cDNA submission to the Vironomics Core facility for QPCR array. This procedure specifically utilizes enrichment of the mRNA poly-A tail. The Oligotex mRNA purification system (Qiagen, CA) exploits the observation that cellular mRNAs contain a polyadenylated (poly-A) tail of 20-250 adenosine residues. Since mRNAs are the only cellular RNAs that contain a poly-A tail, this feature can be taken advantage of to exclusively purify and enrich mRNA from a total RNA pool. The Qiagen Oligotex system uses a dT oligomer coupled to a solid phase matrix to bind the poly-A tail of mRNA while the remaining RNA, which does not contain a poly-A tail, is washed away. Hybridization of the poly-A tail to the dT oligomer is dependent upon high-salt conditions so the complex can be easily disrupted by lowering the ionic strength.
After mRNA enrichment, the reverse transcription creates cDNA, which will serve as the DNA template in the QPCR reaction. Before sample submission to the Vironomics Core, please pay special attention to the number of Reverse Transcription reactions per sample, and the total volume of cDNA required for submission. This volume is required for the QPCR reaction volume, in case you request duplicate QPCR of a sample, and for any repeat tests that are required.

3. Allowable Exceptions

The process for extracting total RNA from cultured cells is identical to that of extracting total RNA from tissues with the exception of using a tissue homogenizer. When isolating total RNA from culture cells a tissue homogenizer is not needed, simply re-suspending the cell pellet in TRI-reagent is sufficient to lyse the cells.

Labs may substitute their own procedure for extracting total RNA from cells if they are equivalent quality. Labs may also substitute another mRNA kit if it still has poly-A tail selective enrichment, as this is required for our QPCR arrays. Please contact Dr. Dirk Dittmer with questions regarding changes in protocols and kits.

4. Responsibilities

Submitting labs should contact the Vironomics Core lab manager before submission with estimate of number of samples, array type, and for an estimation of turn-around time. Submitting labs are responsible for submitting appropriate cDNA samples that meet quantity and quality requirements as laid out in this SOP.

5. Materials

5.1. **Section 6.1 Day1**

1. Valumax Extra-Safe lab coat, knit collar (Fisher, Catalog #239005***)
2. Nitrile Gloves, Kimberly-Clark Spring Green Powder Free (Fisher, Catalog #43438 or equivalent)
3. Wypall (Fisher, Catalog #1979001 or equivalent)
4. Eliminase, small (Fisher, Catalog #4-355-31)
5. Safety faceshield (Fisher, Catalog #19-181-60A or equivalent)
6. Clorox Bleach (Fisher Catalog #CLO 02490)
7. Ethanol 190 proof (Fisher, Catalog#04-355-266EA)
8. Blue absorbent pad/diaper/underpads with waterproof moisture barrier (VWR, Catalog # 56616-018 or equivalent)
9. Scientific Industries Vortex-Genie 2, variable speed 120V (Scientific Industries, Catalog # SI-0236)
10. Refrigerated bench-top microcentrifuge tube centrifuge, with microcentrifuge tube rotor, capacity of 4ºC, max speed 30,000 x g (Eppendorf or equivalent)

11. 2.0 ml flat bottom microcentrifuge tubes, autoclaved, max speed 18,000 x g (Fisher, Catalog #02-681-343 or equivalent)
12. 1.5 mL "conical" microcentrifuge tubes, autoclaved, max speed 30,000 x g (Fisher, Catalog #02-681-339 or equivalent)
13. Microcentrifuge Tube with o-ring screw cap, autoclaved (Fisher, Catalog #2-681-360 or equivalent)
14. 2.5 µL, 20 µL, 200 µL, and 1000 µL pipettes (Eppendorf, Catalog # 3120000909 or equivalent)
15. Aerosol resistant tips for 2.5 µL, 20 µL, 200 µL, and 1000 µL pipettes (Genesee, Catalog #24-401, 24-404, 24-412, 24-430, or equivalent)
16. Microcentrifuge tube racks (Fisher, Catalog #05-541-1 or equivalent)
17. BioSpec Mini-BeadBeater-1 (BioSpec Products #3110BX, or equivalent)
18. BioSpec 2.3MM Zirconia Beads (BioSpec Products # 11079125, or equivalent)
19. 100 ml TRI REAGENT™ (SIGMA; Saint Louis, MO-cat# T-9424 or equivalent)
20. 200 Phase Lock Gel Heavy 2mL tubes (5PRIME; Gaithersburg, MD-cat# 2302830)
21. GlycoBlue tracer (Applied Biosystems:Ambion-cat# 9515)
22. 500 ml Molecular Isopropanol (Sigma, MO-cat# I-9516 or equivalent)
23. Molecular Chloroform (Fisher Inc.; Pittsburgh, PA-cat# BP1145-1 or equivalent)
24. Phenol/Chloroform/Isoamyl alcohol **pH4.3** (Fisher Inc.; PA-cat# BP1754I-100 or equivalent) NOTE THE ACIDIC PH
25. Ice
26. Ice bucket
27. Timer
28. -80ºC Freezer

Additional items for other days

29. Hermle Labnet Z306 Centrifuge (Phenix, Catalog #C-0306 or equivalent)
30. Microcentrifuge rotor, 24x 1.5/2.0mL high speed, angle, Hermetically Sealed, for Hermle centrifuge Z306 (Genesee, Catalog # 33-116AR2H or equivalent)
31. ABI High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (200 rxn-cat#4368814 or 1000 rxn-cat#4368813) **You will need 3 reactions per sample**
32. Qiagen Oligotex mRNA Mini/Midikit (for 12 samples) (Qiagen cat#7 0042, kit) *Selection of Mini (≤ 0.25 mg) or Midi (0.25-0.50 mg) kit depends on total RNA concentration of sample before beginning Oligotex protocol. Both kits have the same reagent components.*
33. Bench top minicentrifuge (Bioexpress, Catalog #C-1301-PC or equivalent)
34. PCR certified water: Water, Sterile (for RNA work) DEPC-treated (Fisher Inc.; PA-cat #BP561 or equivalent)
35. PCR 8-strip with attached flat cap tubes (Bioexpress, Catalog #T-3196-1 or equivalent)
36. RNase H (New England Biolabs Inc.; Ipswich, MA-cat# M0297S)
37. 1X Tris-EDTA solution (TE), pH 7.4-8.0 (Fisher Inc.; PA-cat #BP2473, BP2476 or equivalent)
38. Agilent TapeStation 4200 System (Agilent, Catalog #G2991AA)
39. Agilent TapeStation foil or optical tube strip caps (Agilent, Catalog #5067-5154, 401425)
40. Agilent TapeStation sample plates or optical tube strips (Agilent, Catalog #5042-8502, 401428)
41. Agilent Loading Tips (Agilent, Catalog #5067-5598)
42. Agilent RNA ScreenTape (Agilent, Catalog #5067-5576)
43. Agilent RNA Sample Buffer (Agilent, Catalog #5067-5577)
44. Agilent RNA Ladder (Agilent, Catalog #5067-5578)
45. Thermal Cycler (Thermo Fisher Scientific, Veriti, Catalog #4375786 or equivalent)
46. Heat Block, capacity 35-75ºC
47. Thermometer, -1 to +101, dry block/incubator (Fisher, Catalog #13-201-558 or equivalent)
48. 4ºC Refrigerator
49. -20ºC Freezer

6. Procedure

6.1. RNA Isolation from Cells

The following protocol describes the isolation of total RNA from a cell pellet. Cells should be washed once in **ice-cold** PBS and pelleted for this procedure by centrifugation at 200xg for 10 minutes at 4°C. The expected yield is 5-10 µg total RNA/10^6 cells with an A_{260}/A_{280} ratio of 1.8-2.0.

RTminus recommendation: In order to check samples for gDNA contamination, we recommend you do your samples in duplicate if possible. If this is not possible, clinical samples, please see guideline in Section 6.3.

DAY 1:

1. *Tissues (see below for beat beating step). Cell pellets (optional or begin at step #2).*
   a. Keep samples on ice whenever possible.
   b. Samples must be in a flat bottom 2mL tube for bead beating.
   c. Centrifuge tumor samples that are in RNAlater at max speed for 30 seconds, at 4°C.
   d. Remove as much RNAlater or PBS as possible without disturbing the material.
   e. Add ~1/2-3/4 tube volume of zirconia/silica beads.
   f. Transfer 1000 µl of TRI-reagent into the tube of sample and beads.
   g. Bead beat for 1 minute (time =6 on our bead beater) at speed of 46. Ice. Repeat 2 more times for each sample if tissue (3 total), may not be necessary for cell pellets.
      i. Generally, perform in groups of ~4-6 tubes to allow for enough time to cool on ice.
   h. Transfer as much homogenate as possible to a new 2.0ml tube.

2. *Samples that skip step #1*: Transfer 1000 µl of TRI-reagent into the tube containing the cell pellet (~10^6 cells) and place the samples on ice. (If the samples are not already in a 2.0ml centrifuge tube they should be transferred once re-suspended in TRI-reagent.)

3. Vortex the samples for 30 seconds and place them on ice for 5 minutes.

4. Add 200 µl of Chloroform to each sample and mix well. This can be done by shaking the tubes, or by briefly vortexing the tubes.

5. After mixing, the sample should be centrifuged at full speed in a bench-top centrifuge for 15 minutes at 4°C.

6. When the tube is removed from the centrifuge three phases should be visible within the tube.

7. Spin down new phase lock tube at max speed for 30 sec.

8. **Remove the upper clear phase of Step 5 (350µl), that contains the RNA,**
   a. Note: at times it has been noted that the 3 layers are not immediately identifiable. Also, at times there may be a small floating pink TRI REAGENT layer on the very top. Avoid the pink layers and only remove the clear upper phase.
   b. *The middle phase contains the DNA from the sample, and the lower phase contains protein. The remaining two phases in the tube should be disposed of properly once the upper phase of interest is removed, as they are organic waste and must be handled as so.*

9. **and place into a new phase-lock tube** (Eppendorf, Brinkman Instruments, Westbury, NY).

10. Add 500 µl of Phenol/Chloroform/Isoamyl alcohol **pH4.3 (acidic)** to the sample in the phase lock tube and

11. Vortex for 1 minute.

12. Add 500 µl Chloroform and invert 2-5 times.

13. Incubate the samples on ice for 5 minutes.

14. Centrifuge the sample at full speed for 10 min at 4°C.

15. After centrifugation, transfer 200 µl of the clear upper phase into 1.5 mL conical microcentrifuge tube.

16. Add 1 µl GlycoBlue tracer (Ambion Inc.)
17. Add an equal volume (200 µl) of Isopropanol.
18. Mix the sample thoroughly, and store at -80°C at least overnight.

DAY 2:

Everything up to starting the cDNA reactions needs to be done in one day. Reverse transcription can be done overnight.

1. Remove RNA from freezer and allow the sample to thaw on ice (COMPLETELY).
2. Once the sample is thawed, centrifuge at full speed for 20 minutes at 4°C.
3. Carefully aspirate the supernatant without disturbing the pellet.
4. Air-dry the tube for 10 minutes at room temperature (RT).
5. Resuspend the RNA pellet in 50 µl of 0.1X TE, make using DEPC-treated sterile water.
6. Incubate at 65°C for 10 min.
7. Freeze 2 µl of RNA, which will be used for Agilent RNA integrity analysis (RIN).
8. Proceed to mRNA enrichment (polyA-selection) using Oligotex protocol.

6.2. **mRNA enrichment (polyA-selection), Qiagen Oligotex**

DO NOT USE RNA FROM MORE THAN 10^6 cells per one column.

Excerpt from pg 22-24 of Oligotex Handbook.

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).

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

Vironomics Core note, all our samples have been in this lowest range, use the highlighted volumes above.

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~~ *30* 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, *take the 30 µl of the first eluate and* pipet ~~another 20–100 µl~~ hot (70°C) ~~Buffer OEB~~ *onto the same* column *and microcentrifuge tube for second elution*. 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.

Note: Vironomics Core has validated steps #6 and 7 changes that are inserted and highlighted.

6.3. **Reverse Transcription**

Use the cDNA Kit according the manufacturers protocol. However, do **NOT** use the manufacturers RT conditions, but the ones below. You will need **3 x rxn** for a total volume of ***30µl 2xRTmix + 30 µl RNA (from 6.2 #2) to yield a total of 60 µl***.

An RTminus reaction should also be set-up for each sample to check for gDNA contamination. If there was not enough sample to do duplicate RNA extractions, then it will be best to pool like-with-like samples. For clinical trials we have generally pooled 3uL from 10 samples for each RTminus reaction.

Reverse Transcription Mix

Reagent	**RTminus sample/pool, per reaction (µl)**	**This protocol condition, per reaction (µl)**	*Manufacturer condition, per reaction (µl)*
10X RT Buffer	**6.0**	**6.0**	*2.0*
25X dNTP Mix (100uM)	**2.4**	**2.4**	*0.8*
10X RT Random Primers	**6.0**	**6.0**	*2.0*
MultiScribe Reverse Transcriptase	**NONE**	**3.0**	*1.0*
RNase Inhibitor	**3.0**	**3.0**	*1.0*
Nuclease-free water	**12.6**	**9.6**	*3.2*
Total	**30.0**	**30.0**	*10.0*
Sample	**30.0**	**30.0**	*10.0*

Cycling Conditions for Reverse Transcription-1 Cycle

Temperature	Time
30°C	15 minutes
42°C	15 minutes
52°C	15 minutes
70°C	10 minutes

6.4. **RNAseH digestion**

Following the RT reaction, the sample is prepared for PCR amplification by digestion of the remaining RNA. The RNA digestion is necessary to remove remaining RNA that might interfere with the subsequent PCR reaction. To perform the RNA digestion:

1. Add 39 µl of DEPC/0.1x Tris/EDTA (pH7.5) to the sample.
2. Add **1U** of RNase H (Invitrogen Inc. or Ambion Inc.) to the sample.
3. Vortex and spin down.
4. Incubate on thermal cycler
    a. 37°C for 30 min
    b. Heat the reaction at 95°C for 5 minutes
5. Vortex and spin down.
6. Store in –80.

6.5. **SHIP TO DITTMER LAB ON DRY ICE BY FEDEX.**

UNC VIRONOMICS CORE
UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
450 WEST DRIVE, LCCC RM 12-048
CHAPEL HILL, NC 27599-7290
PHONE: (919) 843-5292

For each shipment please prepare the following information but type into the current submission form that can be found on the website https://www.med.unc.edu/vironomics/

Name	Date	ID	Type	Array	Description / Cell	
Jo Pi	03/01/11	1	cDNA	KSHV	time 0,	BCBL-1
Jo Pi	03/01/11	2	RNA	KSHV	time 1hr	BCBL-1

Note: Once this protocol is complete, there should be 100µL of cDNA to send to the Vironomics Core. Less than 100µL may cause rejection of samples.

We currently run the following arrays (96 genes incl. controls):

- KSHV-DAv2018 *unpubl.*
- KSHVv2.0 *J Infect Dis. 2011, BMC Bioinform. 2010, Cancer Res. 2003*
- KSHV400bp *unpubl.*
- EBVv2.0 *J Virol. 2009, Blood. 2005, Nucleic Acids Res. 2005*
- HSV1v1.0 *unpubl.*
- HSV2v1.0 *unpubl.*
- NFkappaBv1.0 *Int J Cancer. 2011*
- RRVv1.0 *J Virol. 2009, J Virol. 2005*
- HCMVv1.0 *unpubl.*
- Pre-miRNAv1.0 *PLoS Pathog. 2009, Blood. 2008*
- *P53response12* *J Virol. 2007*

IMPORTANT: 3 MONTHS AFTER COMPLETION YOUR SAMPLES AND YOUR DE-IDENTIFIED DATA CAN BE INCORPORATED IN METHOD DEVELOPMENT AND OTHER STUDIES BY US WITHOUT AUTHORSHIP.

7. References

7.1. Qiagen, *Oligotex Handbook*. Oligotex mRNA Spin-Column Protocol. June 2012.
7.2. Applied Biosystems, *High Capacity cDNA Reverse Transcription Kit Protocol.* (Foster City, California, 2010).

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.