AUTHOR:
Nicholas Marko

PRIMARY REVIEWERS:
Renee Rubio, Bryan Frank

1. PURPOSE

This protocol describes the procedure for amplifying RNA from a starting sample of total RNA to produce increased amounts of RNA having the sense orientation. This protocol is specifically designed for amplifying RNA that will be used with spotted oligonucleotide arrays or mixed cDNA/oligonucleotide spotted arrays, because it provides RNA in the appropriate (sense) orientation for interaction with both cDNA and oligonucleotide elements on the array.

2. SCOPE

This protocol is designed for use with the Human Genomics projects under the direction of John Quackenbush. This protocol is also applicable to other eukaryotic or prokaryotic genomics projects as described below.

3. MATERIALS

3.1	SuperScript II Kit (Invitrogen #18064014)
3.2	Superase-In RNAse Inhibitor (Ambion #2696)
3.3	DNTP Set, 100 mM (Invitrogen #10297018)
3.4	Second Strand Buffer (Invitrogen #10812014)
3.5	E. coli DNA Polymerase I (Invitrogen #18010025)
3.6	E. coli DNA Ligase (Invitrogen # 18052019)
3.7	T4 DNA Polymerase (Invitrogen #18005025)
3.8	Megascript T3 or T7 Kit (Ambion #1338 / #1334)
3.9	Minelute Reaction Cleanup Kit (Qiagen #28204)
3.10	RNEasy Mini Kit (Qiagen #74106)
3.11	EDTA, 0.5 uM
3.12	NaOH, 1N
3.13	HCl, 1N
3.14	PCR Tubes, 0.2mL [Thin-Walled, Nuclease-Free] (BioRad)
3.15	Caps for PCR Tubes [Nuclease-Free] (BioRad)
3.16	Peltier Thermal Cycler (with RAMP capability)
3.17	Nuclease-Free Water
3.18	Benchtop Centrifuge

THERMAL CYCLER PROGRAM(S)

70ºC	10:00
4ºC	5:00
4ºC	HOLD
42ºC	2:30:00
4ºC	2:00
4ºC	HOLD
65ºC	15:00
65ºC	HOLD
95ºC	3:00
50ºC	0:01
RAMP	0.4ºC /s
	to 4ºC
4ºC	2:00
4ºC	HOLD
16ºC	2:00:00
16ºC	HOLD
16ºC	5:00
16ºC	HOLD
37ºC	HOLD

4 SELECTION OF APPROPRIATE PRIMERS

4.1 Overview:

4.1.1 This protocol has been designed to afford the maximum amount of versatility in terms of the nature of the starting material and the type and orientation of amplified RNA that is produced.

4.1.2 The key to customizing the protocol to meet the user's individual needs is appropriate selection of the primer for the first strand and for the second strand cDNA synthesis reactions.

4.1.3 The same protocol can be used regardless of primer selection, so the primers are referred to as "Primer A" (the primer for first-strand cDNA synthesis) and "Primer B" (the primer for second-strand cDNA synthesis) in the text of the protocol.

4.1.4 The appropriate way to use this protocol is for the users to select which primers will be "Primer A" and "Primer B" (guided by the suggestions in Section 4.3) and to order and prepare primers with the appropriate sequence at the appropriate concentration (as described in Section 4.2).

4.2 Description of Primers

NOTE: When the following 6 primers are prepared as described, the volumes listed in the protocol will work with any combination of primers selected as "Primer A" and "Primer B".

4.2.1 Oligo$(dT)_{24}$ (Operon Custom) [100 ng/µL]:
5'-TTTTTTTTTTTTTTTTTTTTTTTT-3'

4.2.2 T3-Oligo$(dT)_{24}$ (Operon Custom) [100 ng/µL]:
5'-
GCGCGAAATTAACCCTCACTAAAGGGAGATTTTTTTTTTTTTTTTTTTTTTTT-3'

4.2.3 T7-Oligo(dT)$_{24}$ (Operon Custom or Ambion #5712) [100 ng/µL]:
5'GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTT
TTTTTTTTTT-3'

4.2.4 Random Hexamer Primers (Invitrogen #48190-011) [3 µg/µL]:
5'-NNNNNN-3'

4.2.5 T3 Random Nonamer (T3N9) (Operon Custom) [100 ng/µL]:
5'-GCGCGAAATTAACCCTCACTAAAGGGAGANNNNNNNNN-3'

4.2.6 T7 Random Nonamer (T7N9) (Operon Custom) [100 ng/µL]:
5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGNNNNNNNNN-3

4.3 Principles of Primer Selection

4.3.1 Type of Starting RNA

4.3.1.1 When starting with Eukaryotic RNA and attempting to amplify mRNA, the first strand primer is specific for the poly(A)$^+$ sequence. This will yield amplified mRNA only.

4.3.1.2 When starting from Prokaryotic RNA (therefore no poly(A)$^+$ sequence) or when amplified total RNA is the desired product, random primers are used for priming first strand synthesis. This will produce amplified total RNA.

4.3.1.3 When starting with partially degraded RNA from either Prokaryotic or Eukaryotic RNA, random primers are used for priming first strand synthesis. This will produce amplified total RNA, but may help to preserve the information coded on fragments of mRNA that have become separated from their poly(A)$^+$ sequence.

4.3.2 Orientation of the amplified RNA

4.3.2.1 Transcription from the first-strand cDNA will produce amplified RNA in the antisense orientation.

4.3.2.2 Transcription from the second-strand cDNA will produce amplified RNA in the sense orientation.

4.3.3 RNA polymerase selection

4.3.3.1 In some instances it is possible to select primers such that either a T3 or a T7 RNA Polymerase can be used to produce amplified RNA.

4.3.3.2 In theory, both of these polymerases have similar activity and specificity for their promoter, so this decision may be guided by which IVT enzyme system is readily available. In practice, it may help to test each system in order for the user to optimize yield.

4.3.3.3 The user should be sure to select the polymerase that matches the promoter used (T3 or T7).

4.3.4 Note on Second Strand Synthesis when antisense orientation is desired

4.3.4.1 When antisense orientation is desired for the amplified RNA, it is not necessary to prime second-strand cDNA synthesis with a promoter-modified primer.

4.3.4.2 In this instance, the user may proceed with the protocol as described and use random hexamers as “Primer B”, or

4.3.4.3 The user may omit the first-strand column cleanup and allow second-strand synthesis to prime with residual fragments of digested RNA (as described by Eberwine). This reaction is not as “clean,” but may save time and cost if specific, second-strand priming is not necessary.

4.3.5 Yield Considerations

4.3.5.1 In general, synthesis off of the first strand (antisense synthesis) proceeds ~5 times more efficiently than synthesis off of the second strand (sense synthesis).

4.3.5.2 For this reason, if orientation is not important but yield is a major concern, it is advisable to conduct synthesis following the antisense methods.

4.4 Primer Selection Strategies

4.4.1 When amplified mRNA is the desired product:

4.4.1.1 Antisense Orientation

4.4.1.1.1 Primer A: T3 or T7 Oligo$(dT)_{24}$

4.4.1.1.2 Primer B: Random Hexamers (or self-priming, as described above)

4.4.1.2 Sense Orientation

4.4.1.2.1 Primer A: Oligo$(dT)_{24}$ or T3 or T7 Oligo $(dT)_{24}$

NOTE: Although these promoters will not be used for synthesis, they will not interfere with antisense synthesis and may be used.

4.4.1.2.2 Primer B: T3N9 or T7N9

4.4.2 When amplified Total RNA is the desired product (or when starting with Prokaryotic RNA)

4.4.2.1 Antisense Orientation

4.4.2.1.1 Primer A: T3N9 or T7N9

4.4.2.1.2 Primer B: Random Hexamers (or self-priming, as described above)

4.4.2.2 Sense Orientation

4.4.2.2.1 Primer A: Random Hexamers

4.4.2.2.2 Primer B: T3N9 or T7N9

4.4.3 When starting from partially degraded Eukaryotic RNA, consider following the procedures for total RNA amplification in order to preserve sequence that has become separated from the Poly$(A)^+$ tail.

5 SETUP

5.1 Rigorous efforts to reduce RNAse contamination, including surface decontamination and use of RNAse-Free reaction tubes are required at all times throughout this protocol.

5.2 Reactions that require temperature control should be conducted in a thermal cycler with the heated lid turned "on." If the thermal cycler has not been programmed, enter a program that corresponds to this protocol before beginning the amplification process.

5.3 Note that when spinning down PCR tubes, do not exceed 4,000g as the thin-walled PCR tubes may crack or leak.

5.4 Configure the thermal cycler before beginning the procedure:

5.4.1 The heated lid should be turned on.

5.4.2 The "calculated" method should be used (when available) for optimal temperature control.

5.4.3 For optimal calculated temperature control, the thermal cycler program should be divided into six (6) independent programs so that the different reaction volumes can be entered prior to the incubations. The total program is listed above, and components of each sub-program are indicated by alternating shading. These sub-sections and their respective volumes correspond to the following parts of the amplification process:

5.4.3.1 Subprogram 1:	1st Strand Primer Annealing	18 µL
5.4.3.2 Subprogram 2:	1st Strand cDNA Synthesis	30 µL
5.4.3.3 Subprogram 3:	Template RNA Hydrolysis	50 µL
5.4.3.4 Subprogram 4:	2nd Strand Primer Annealing	21 µL
5.4.3.5 Subprogram 5:	2nd Strand cDNA Synthesis	35 µL
5.4.3.6 Subprogram 6:	In Vitro Transcription	40 µL

6 PROCEDURE

6.1 Step 1: First Strand cDNA Synthesis

6.1.1 In a 0.2µL PCR tube, combine the following:

> 0.5 µg of total RNA (in water)	(< 15.8 µL)
Superase-In (RNAse Inhibitor)	1 µL
Primer A	1 µL

6.1.2 Add Nuclease–Free water to bring the final volume to 17.8µL.

6.1.3 Heat at 70°C for 10 minutes in the thermal cycler.

6.1.4 Cool to 4°C for 5 minutes in the thermal cycler and then proceed immediately with the next step.

6.1.5 To the reaction, add the following (a master mix can be prepared and maintained on ice):

5X First Strand Buffer	6 µL
0.1 M DTT	3 µL
50mM DNTP Mix	1.2 µL
SuperScript II RT (200u/µL)	2 µL

6.1.6 Mix by pipetting or flicking, spin down, and place in the thermal cycler at 42°C for 2.5 hours.

6.1.7 Cool to 4°C for 2 minutes.

6.1.8 Mix by flicking the tubes and spin down briefly.

6.1.9 To terminate the reaction, add the following:

1N NaOH	10 µL
0.5 M EDTA	10 µL

6.1.10 Mix by flicking, spin down, and place in the thermal cycler at 65°C for 15 minutes.

6.1.11 Add 10µL of 1N HCl to normalize the pH.

6.1.12 Proceed immediately with the first strand cleanup.

6.2 Step 2: Cleanup of 1st Strand cDNA / Removal of RNA fragments

6.2.1 Cleanup the 1st strand cDNA using the Qiagen Minelute reaction cleanup kit. The following is an optimized modification of the original manufacturer's protocol.

6.2.2 To the reaction, add 300µL of Buffer ERC.

6.2.3 Verify that the color of Qiagen Buffer ERC remains yellow. If it turns pink/violet, the pH is incorrect. In this case, add 10µL of 3M NaOAC to adjust the pH (the buffer will turn yellow).

6.2.4 When the correct pH has been verified, transfer the sample to a Minelute column.

6.2.5 Centrifuge at >10,000g for 1 minute and discard the flow-through.

6.2.6 To wash, add 500µL of Qiagen Buffer PE and centrifuge at >10,000g for 1 minute.

6.2.7 Discard the collection tube and the flow-through and place the column in a fresh 2mL collection tube (user supplied).

6.2.8 Centrifuge at >10,000g for 1 minute to remove residual wash buffer.

6.2.9 Discard the collection tube and place the column in a fresh 1.5mL collection tube.

6.2.10 To elute, add 10µL of Qiagen Buffer EB (pH ~8.0). Incubate at room temperature for 1 minute and then centrifuge at >10,000g for 1 minute.

6.2.11 Elute a second time with 10µL of Qiagen Buffer EB, (pH ~8.0). Incubate at room temperature for 1 minute and centrifuge for 1 minute at >10,000g.

6.2.12 Proceed immediately with second strand synthesis.

6.3 Step 3: Second Strand cDNA Synthesis

6.3.1 In a fresh 0.2 mL PCR tube, combine the first strand cDNA from above with 2µL of Primer B.

6.3.2 Incubate at 95°C for 3 minutes in the thermal cycler.

6.3.3 Rapidly drop the temperature of the samples to 50°C, then slowly cool the samples from 50°C to 4°C over 2 minutes.

(**NOTE:** This can be accomplished using the thermal cycler RAMP function. First set the thermal cycler to cool from 95°C to 50°C and hold at 50°C for one second, then RAMP from 50°C to 4°C at 0.4°C/second [~120s]).

6.3.4 Maintain at 4°C for at least 2 minutes.

6.3.5 Add the following reagents to the cDNA/primer mix (a master mix can be made for use with multiple samples):

5X Second Strand Buffer	7 µL
25X DNTP Mix	2 µL
E. coli DNA Polymerase I	4 µL
E. coli DNA Ligase	1 µL

6.3.6 Mix by flicking, spin down, and incubate in a thermal cycler at 16°C for 2 hours.

6.3.7 After incubation, add 2µL of T4 DNA Polymerase to the reaction.

6.3.8 Incubate in a thermal cycler at 16°C for 5 minutes.

6.3.9 Terminate the reaction by adding 3.5µL of 0.5 M EDTA.

6.3.10 Proceed to cleanup of ds-cDNA.

6.4 Step 4: Cleanup of ds-cDNA

6.4.1 Cleanup the ds-cDNA using the Qiagen Minelute reaction cleanup kit. The following is an optimized modification of the original manufacturer's protocol.

6.4.2 To the reaction, add 300µL of Buffer ERC.

6.4.3 Verify that the color of Qiagen Buffer ERC remains yellow. If it turns pink/violet, the pH is incorrect. In this case, add 10µL of 3M NaOAC to adjust the pH (the buffer will turn yellow).

6.4.4 When the correct pH has been verified, transfer the sample to a Minelute column.

6.4.5 Centrifuge at >10,000g for 1 minute and discard the flow-through.

6.4.6 To wash, add 500µL of Qiagen Buffer PE and centrifuge at >10,000g for 1 minute.

6.4.7 Discard the collection tube and the flow-through and place the column in a fresh 2mL collection tube (user supplied).

6.4.8 Centrifuge at >10,000g for 1 minute to remove residual wash buffer.

6.4.9 Discard the collection tube and place the column in a fresh 1.5mL collection tube.

6.4.10 To elute, add 10µL of Qiagen Buffer EB (pH ~ 8.0). Incubate at room temperature for 1 minute and then centrifuge at >10,000g for 1 minute.

6.4.11 Elute a second time with Qiagen Buffer EB (pH ~ 8.0). This time, use only 7µL of buffer EB (add 1-2µL extra if you plan to quantitate or assay the ds-cDNA). Incubate at room temperature for 1 minute and centrifuge for 1 minute at >10,000g.

6.4.12 Proceed immediately with In Vitro Transcription.

6.5 Step 5: In Vitro Transcription

6.5.1 The Ambion MegaScript T3 or MegaScript T7 Kit (based on the promoter used) is used to synthesize and amplify sense RNA. The MegaScript reaction is run according to the manufacturer's protocol, but the reaction is doubled for each sample. Carry out the IVT reaction as follows:

6.5.2 Allow components of the master mix (below) to thaw. Maintain at room temperature while assembling the master mix (assembling on ice will coprecipitate the DNA in the presence of the spermidine in the reaction buffer).

6.5.3 Make a master mix for the reaction. Mix the components IN THE FOLLOWING ORDER:

ATP Solution	4 µL
CTP Solution	4 µL
GTP Solution	4 µL
UTP Solution	4 µL
10X Reaction Buffer	4 µL
T3 or T7Meagscript Enzyme Mix	4 µL

6.5.4 Add 24µL of the Master Mix to the 16µL sample of ds-cDNA template. Mix by flicking and spin down.

6.5.5 Incubate the reaction in a thermal cycler at 37°C for 8 hours to overnight (up to 16 hours).

NOTE: DNAse digestion should not be necessary because (1) the amount of template DNA is small relative to the amount of amplified RNA, and (2) purification with RNEasy Columns will remove the trace amounts of DNA. However, if reactions that are sensitive to DNA are planned, consider treatment of the sample with DNAse I according to manufacturer's instructions *[to the reaction mix, add 1 µL of DNAse I to destroy the template cDNA. Mix well, because the reaction mixture may be viscous. Incubate the reaction in a thermal cycler at 37°C for 15 minutes.]*

6.5.6 Proceed immediately with RNA purification.

6.6 Step 6: Purification of Amplified RNA

6.6.1 Amplified RNA is purified using either the Qiagen RNEasy MinElute Kit or the Qiagen RNEasy Mini Kit.

6.6.1.1 For amplifications that produce amplified RNA in the SENSE orientation, we suggest using the RNEasy MinElute Kits because yields are typically < 25µg.

6.6.1.2 For amplifications in the ANTISENSE orientation, either kit can be used because yields are typically 40 – 60µg. If there are concerns about potentially overloading the RNEasy MinElute column, the RNEasy Mini columns should be used.

NOTE: Estimating yield before cleanup (in an attempt to guide column selection) by measuring the OD 260 is inaccurate because the IVT reaction takes place in an excess of NTPs. These will falsely elevate the OD 260, making this method of estimation unreliable. We suggest that users determine empirically the anticipated yield and use the appropriate column. As a guide, please refer to the previous comments (6.6.1)

6.6.2 After selecting the appropriate Qiagen column, proceed with the RNA cleanup according to the manufacturer's specifications for that column.

NOTE: We recommend washing the RNA with no more than 500µL of wash buffers. While the manufacturer's protocol indicates that 750µL of buffer RPE (and 80% EtOH, for MinElute columns) of wash buffer should be used, we have found that 500µL volumes wash the RNA sufficiently and minimize potential carryover of wash buffer into the final RNA.

6.7 Step 7: Assess RNA Quantity and Quality

6.7.1 Measure the quantity of RNA by measuring the OD 260 on the Nanodrop.

6.7.2 RNA integrity may be assessed using the Bioanalyzer. Note, however, the amplified RNA will have a size range from ~300-1800 BP, creating a "smear" on the gel or a "bump" on the bioanalyzer graph. This is a function of the amplification and does not indicate RNA degradation.