DNA Linear Amplification

Version 3.13, 4/21/2003, by Chih Long Liu

Note: *Before beginning this protocol, if your source DNA was sheared, you may need to CIP-treat the samples to remove 3' phosphate groups. Failure to do so will likely halve the yield of your amplification products.*

CIP Treatment of samples with terminal 3' phosphate groups (optional)

Reagent (for every 10 ul volume)	Volume	Final Conc.
2.5 U enzyme (NEB #M0290S)	0.25 ul	0.25 U/ul
10X NEB Buffer 3	1 ul	1X
Template DNA (max. 500 ng)	8.75 ul	max 50 ng/ul
Total	**10 ul**	

Each reaction can be scaled up to 100 ul per tube.

1. Incubate at 37°C for 1 hour
2. Clean up reaction with the Qiagen MinElute kit (# 28004; see end of protocol). Elute in 20 ul.

Tailing Reaction with TdT

Reagent	Volume	Final Conc.
20U TdT enzyme (NEB #M0252S)[1]	1 ul	2 U/ul
5X TdT buffer[2]	2 ul	1X
8% 100 uM ddCTP in 100uM dTTP mix‡	0.5 ul	5 uM dTTP (8% ddCTP)
5 mM $CoCl_2$	1.5 ul	0.75 mM
Template DNA (max 50ng)[3]	5 ul	max 5 ng/ul
Total	**10 ul**	

‡ *Ensure that the dNTP mixes do not go through more than 3 freeze-thaw cycles. Additional freeze-thaw cycles will further degrade the dNTPs and will reduce the efficiency of the reaction.*

1. Add 1-2 drops mineral oil to the top of the mixture, to prevent evaporation loss during incubation.
2. Incubate at 37°C for 20 minutes.
3. Stop reaction by adding 2 ul (per 10 ul reaction volume) 0.5M EDTA pH 8.0.
4. Clean up reaction with the Qiagen MinElute kit. If you started with 10ul, you may have to add 10 ul water to bring the volume up to 20 ul. Elute in 20 ul volume.

Second Strand Synthesis with Klenow Fragment Polymerase

---

[1] *Use a recombinant enzyme, and NEB if possible (NEB #M0252S or Roche #3 333 566). An enzyme derived from a natural source (e.g. Roche #220 582) may have lot-dependent variation and may result in unpredictable or lowered IVT yields. The Roche recombinant TdT produces a 50% drop in yield when used at the volumes specified in this protocol.*

[2] *If using the NEB enzyme, do **NOT** use the supplied NEB Buffer 4, since the DTT in the buffer will precipitate the $CoCl_2$ and inhibit the reaction. Use the cacodylate buffer (1M potassium cacodylate, 125mM Tris-HCl, and 1.25 mg/ml BSA, pH 6.6) supplied with the Roche enzyme. Take precautions in handling this arsenic-containing buffer, and employ waste disposal practices appropriate for your institution.*

[3] *Aim for ~1 pmol of template molecules. Lower limit is 2.5 ng DNA. Scale up the reaction volume accordingly for higher starting amounts.* ***For ChIP samples, if you are not sure how much DNA you have, you should scale up to a 20 ul volume.*** ***Note:*** *if insufficient enzyme is used, the efficiency of subsequent steps in the protocol will be significantly affected and result in significantly reduced yields (as little as 5-10% of normal expected yields).*

Reagent*	Volume	Final Conc.
25 uM T7-A_{18}B primer[4]	0.6 ul	300 nM
10X EcoPol buffer[||]	5 ul	1X
5.0 mM dNTP mix[‡]	2 ul	200 uM
water	20.4 ul	
T-Tailed DNA	20 ul	
Total	**48 ul**	

** If production of template-independent product is a significant problem, scale down the reaction volume, while keeping the reagent concentrations (except for the T-Tailed DNA) constant.*
‡ Ensure that the dNTP mixes do not go through more than 3 freeze-thaw cycles. Additional freeze-thaw cycles will degrade the dNTPs and will reduce the efficiency of the reaction.
|| Ensure any DTT in the buffer has completely dissolved before using. Pre-warm to 37°C if necessary.

Do NOT use mineral oil. Trace amounts of mineral oil appears to interfere with cleanup and IVT.

Use the following program in a thermal cycler:
94°C, 2 minutes to melt → ramp -1°C/sec to 35°C, then hold for 2 minutes to anneal → ramp –0.5°C/sec to 25°C → hold for 45 seconds (pause here if needed) and add 2 ul (50U) Klenow DNA polymerase (NEB# M0210S) during this time → 37°C, 90 minutes to extend.

Stop reaction by adding 5 ul 0.5M EDTA, pH 8.0 (end conc. 50 mM).

Second Strand DNA Cleanup

Clean up reaction with the Qiagen MinElute kit and elute in 20 ul.

In Vitro Transcription (IVT)

(uses the Ambion T7 Megascript Kit #1334)

dsDNA preparation

The IVT requires that the dsDNA be in 8 ul volume. Dry down the eluate from 20 to 8 ul in a speedvac at medium heat for 10-12 minutes.

Reagent	Volume
75 mM NTP Mix (A, G, C, & UTP)*	8 ul
Reaction buffer (warm to RT first!)**	2 ul
Enzyme mix (RNase inhibitor and T7 RNA Pol)	2 ul
Template dsDNA	8 ul
Total	**20 ul**

** If new kit, combine NTPs into one tube, then aliquot back out into the 4 tubes. In the first three freeze-thaw cycles, yields drop approximately 10-15% after each cycle. If the NTPs go through more than 3 freeze-thaw cycles, each subsequent freeze-thaw cycle may drop the yield by as much as 50%.*
*** If you add cold buffer and dsDNA, you may risk precipitation of your DNA.*

Incubate at 37°C O/N (acceptable range is 5-20 hours). Incubate in thermal cycler with heated lid, or in air incubator, and in 0.2 ml RNase-free PCR tubes to minimize vapor volume.

[4] T7-A_{18}B primer: (5'- GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAG$(A)_{18}$[B], where B refers to C, G or T). This should be obtained with HPLC, PAGE, or equivalent purification.

aRNA Purification Using Qiagen RNeasy Columns

Use the Qiagen RNeasy Mini kit (#74104) for this procedure. You may eventually have to order additional Buffer RPE if you use the entire kit for this purpose.

Make up RLT w/ β-ME and H_2O Master Mix:

Reagent	Volume
β-ME (14.2M stock solution)	3.5 ul
RNase-free water	80 ul
Buffer RLT (from Qiagen RNeasy Mini kit)	350 ul
Total	**433.5 ul**

1. Pre-aliquot the mix to 1.5 ml RNase-free tubes.
2. Transfer contents of the IVT mix to the RNase/DNase-free tube and vortex briefly.
3. Add 250ul ethanol (95-100%) and mix well by pipetting. (Do not spin here!)

If you have a Qiagen vacuum manifold, skip to step 8.

No Vacuum Manifold:

4. Apply sample (700ul) to RNeasy mini spin column sitting in a collection tube. Centrifuge 15 sec at ≥ 8000 × g. Discard flow through.
5. Transfer RNeasy column to a new 2-ml collection tube (supplied). Add 500ul Buffer RPE (which must contain ethanol) and centrifuge 15 sec at ≥ 8000 × g. Discard flow-through but re-use tube.
6. Pipet 500ul Buffer RPE onto RNeasy column and centrifuge for 2 min at maximum speed.
7. Remove flow through and pipet another 500ul Buffer RPE onto column. Centrifuge for 2 min at maximum speed. [This is an additional wash that is not in the Qiagen protocol which we have found necessary because of GITC contamination in the eluted RNA.] **Skip to Step 12.**

Vacuum Manifold:

8. Apply sample (700ul) to RNeasy mini spin column, attached to vacuum manifold. Apply vacuum.
9. Remove vacuum and pipet 500ul Buffer RPE onto RNeasy column. Apply vacuum.
10. Repeat Step 9, and transfer columns to 2-ml collection tubes (supplied). Spin 1 min at full speed.
11. Return columns to vacuum manifold and apply 500ul Buffer RPE. Apply vacuum.

12. (Transfer columns back to the 2-ml tubes;) Spin at full speed for 1 min to completely dry column.
13. Transfer RNeasy column into a new 1.5-ml collection tube (supplied) and add 30ul RNase-free water directly onto membrane. Centrifuge for 1 min at ≥ 8000 × g to elute. Repeat if expected yield is ≥ 30 ug.
14. Check RNA concentration and quality by measuring A_{260} and A_{260}/A_{280}.
15. Synthesize/label probe with random primer in RT reaction; hyb to arrays

Qiagen MinElute Kit protocol

The MinElute kit removes free nucleotides and anything below 40nt. Things between 40-70nt might also be removed but at a lower efficiency.

1. In a sample of volume 20-100 ul, add 300 ul Buffer ERC and mix thoroughly. If the sample is in less than 20 ul, bring up volume with ddH_2O. If the sample is in more than 100 ul, split sample and do in parallel.

2. Add sodium acetate (pH 5.0) if the buffer color is orange or purple (i.e. pH > 7.5). If the buffer is yellow, no additional sodium acetate is necessary.

3. Apply sample to column. Spin 1 min. at maximum speed in microcentrifuge.

4. Discard flow-through and add 750 ul Buffer PE (which must contain ethanol). Spin 1 min. at maximum speed in microcentrifuge.

5. Discard flow-through. Spin 1 min. at maximum speed in microcentrifuge to dry column.

6. Transfer columns to fresh 1.5 ml tubes. Pipette 10-20ul Buffer EB or water directly onto column membranes. Let stand for 1 minute, then spin 1 min. at maximum speed in microcentrifuge to elute.

7. Note on elution volumes: when working with amounts less than 100ng of DNA, the 10ul elution volume in Qiagen's MinElute protocol may recover less than the 80% claimed by Qiagen. Increase elution volume to 15-20 ul, and dry the volume down if necessary. At the post-SSS step, an elution volume of 20ul increased yields by 30-40% for a 50ng sample!

Time per protocol step

Step	Time
TdT Tailing and Cleanup:	30-40 minutes
Second Strand Synthesis and Cleanup:	2-2.5 hours
IVT:	5.5-20.5 hours
IVT Cleanup:	15-30 minutes
Gel (QC) and Spec (quantitation):	40-60 minutes
Total:	**9.5-25 hours**