5' RACE Protocol For Generation of Seq. Tags

7/28/03

All reactions can be performed in a 96-well format using multichannel pipettors and thermocyclers.

First Strand cDNA Synthesis:

Number of Reactions: 210

Reaction conditions	Vol per rxn(ul)	Tot. Volume
RNA ~5-10ug	10	
First Strand cDNA oligo 300 (10ng)	1	210
DEPC-dH2O (Ambion cat#9922)	1	210
	2	420
total volume per rxn	12	

Incubate 70C, 5 minutes
Spin down. On ice add:

Reaction conditions	Vol per rxn(ul)	Tot. Volume
5x First strand buffer	4	840
0.1M DTT	2	420
10mM dNTP	1	210
	1	210
Superscript II (200 units/ul, Gibco/BRL cat#18064071)		
	8	1680
Total Volume	20	

Incubate 37C 1hr.
to hydrolyze: Add 2.2ul 1M NaOH

Incubate 65C 20min.
to neutralize: Add 2.4ul 1M HCL

First Size selection:

Load entire sample onto a 0.025mm (Fisher Scientific (Millipore) cat#VSWP01300) filter floating (shiny side up) in 6mls of TE(pH8.0) (Ambion cat#9858) in a small petri dish 35x10mm.

Dialyze for minimum of 2hrs to 3hrs maximum.

Take up reaction sample in 96-well PCR plates.

Make up lost volume by adding dH2O to filter for a total volume of 20ul.

Can store at -20C indefinitely.

Poly A addition:

Add on ice

Number of Reactions: 210

Reaction conditions	Vol per rxn(ul)	Tot. Volume
1st strand cDNA (sized selected)	20	
5x TdT Buffer	6	1260
2mM dATP	2	420
TdT (15units/ul, Gibco/BRL cat#10533032)	2	420
	10	2100
Total Volume per rxn	**30**	

Incubate 20 minutes at 37C
Incubate 5 minutes at 70C
Spin to collect samples.

2nd Strand cDNA Synthesis:

Number of Reactions: 210

Reaction conditions	Vol per rxn(ul)	Tot. Volume
Poly A tailed cDNA	30	
10X Buffer M (Roche cat#1417983)	4	840
10mM dNTP	2	420
T-tailed 2nd strand anchor oligo 301T (10ng)	2	420
Klenow (5units/ml, Roche cat#759724)	1	210
dH2O	1	210
	10	2100
Total Volume per rxn	40	

Incubate 25 minutes room temperature
Incubate 25 minutes 37C
Incubate 10 minutes 70C

2nd Size Selection: Using Glycogen Carrier

Add 10ul of 0.1ug/ul glycogen carrier to each sample (final concentration 0.06ug/ul glycogen), Total Volume approx. 50ul.

Pack Sephacryl S-500 High Resolution Resin (Amersham Pharmacia cat#17-0613-10) into Multiscreen-HV (Millipore cat#MHVBN4550) 96-well filtration plate

1. Mix 1:1 S500 resin to dH20.
2. Apply 300 ul 1:1 resin mixture to wells.
3. Centrifuge 600rcf x g, 2 minutes.
4. Apply 200 ul 1:1 resin mixture to wells.
5. Centrifuge 600rcf x g, 2 minutes.
6. Apply 200 ul 1:1 resin mixture to wells.
7. Centrifuge 600rcf x g, 2 minutes.
8. Wash 3 times with 100 ul dH20. Last centrifugation 800rcf x g, 2minutes.
9. Apply all 50ul of 2nd strand/glycogen mix to center of packed wells.
10. Centrifuge samples 800rcf x g, 2 minutes.
11. Elution vol. should be approx. 55 ul.

1st PCR:

Number of Reactions: 210

Reaction conditions	Vol per rxn(ul)	Tot. Volume
2nd Strand cDNA in dH20 (size selected)	55	
10X PE Buffer II	10	2100
25mM MgCl2	8	1680
25mM dNTP's	0.8	168
Oligo 301 (100ng)	2	420
Oligo 302 (100ng)	2	420
Amplitaq (5units/ml, Applied Biosystems cat#N808-0156)	1.2	252
	24	5040
Total Volume	79	

Cycling:

94C				1 minute
94C				30 seconds*
60C				30 seconds*
72C				1 minute*
72C				2 minutes
10C				Hold
*35 cycles				

Third Size Selection:S500 without Glycogen

Pack Sephacryl S-500 High Resolution Resin (Amersham Pharmacia cat#17-0613-10)
into Multiscreen-HV (Millipore cat#MHVBN4550) 96-well filtration plate

1. Mix 1:1 S500 resin to dH20.
2. Apply 300 ul 1:1 resin mixture to wells.
3. Centrifuge 600rcf x g, 2 minutes.
4. Apply 200 ul 1:1 resin mixture to wells.
5. Centrifuge 600rcf x g, 2 minutes.
6. Apply 200 ul 1:1 resin mixture to wells.
7. Centrifuge 600rcf x g, 2 minutes.
8. Wash 3 times with 100 ul dH20. Last centrifugation 800rcf x g, 2minutes.
9. Apply all 79 ul of1st PCR product to center of packed wells.
10. Centrifuge samples 800rcf x g, 2 minutes.
11. Elution vol. should be approx. 85 ul.

Semi Nested (2nd) PCR:

Number of Reactions: 210

Reaction conditions	Vol per rxn(ul)	Tot. Volume
1st PCR product (size selected)	15	
10X PE Buffer II	5	1050
25mM MgCl2	4	840
25mM dNTP's	0.4	84
Oligo 301 (100ng)	1	210
Oligo 303 (100ng)	1	210
dH20	23.1	4851
Amplitaq (5units/ml, Applied Biosystems cat#N808-0156)	0.5	105
	35	7350
Total Volume	50	

Cycling:

94C				1 minute
94C				30 seconds*
60C				30 seconds*
72C				1 minute*
72C				2 minutes
10C				Hold
*35 cycles				

Shrimp Alkaline Phospahatase/ Exonuclease I treatment

(removes excess oligos and dNTPs):

Number of Reactions: 210

Reaction conditions	Vol per rxn(ul)	Tot. Volume
Semi-nested PCR product	10	
Shrimp Alkaline Phosphatase (2units/ul, USB cat#70092X)	2	420
Exonuclease I (10units/ul, USB cat#70073X)	2	420
	4	840
total volume per rxn	14	

Incubate 37C, one hour
Incubate 95C, 15 minutes

Sequence template per 5'RACE sequencing protocol with sequencing oligo 305.

5'RACE Primers(For pGTpfs & pGTlxf only)

#300 5'-TAA TGG GAT AGG TTA CG-3'
#301T 5'-GGT TGT GAG CTC TTC TAG ATG GTT TTT TTT TTT TTT TTT-3'
#301 5'-GGT TGT GAG CTC TTC TAG ATG G-3'
#302 5'-AGT ATC GGC CTC AGG AAG ATC G-3'
#303 5'-ATT CAG GCT GCG CAA CTG TTG G-3'
#305 5'-CGA CGG GAT CCT CTA GAG TC-3'