Cloning and Analyzing cDNA's from the Genome

By Jim Collins 9-10-15

Extract RNA: Trizol
(follow manufactures protocol)

DNase Treatment: Zymo DNA-free RNA kit

5 µg RNA
5 µl DNA Buffer
2.5 µl DNase I
to 25 µl w/RNase Free H_2O
10 min at 37 °C

Purify on column

cDNA Synthesis: BioRad iScript Kit

4 µl 5x iScript Mix (includes oligo dT and Random hexamers)
1 µl Rev. Transcriptase
1 µg DNA-Free RNA
To 20 µl with H_20
Incubate on thermocycler:
5 min @ 25°C
30 min @ 42°C
5 min @ 85°C

Dilute Rxn to 200ul with H_2O

PCR Amplification

Design primers to predicted coding regions using Primer 3 w/ 60°C annealing. 500-1000 bases is a good size for RNAi and *in situ*.

Reagents/RXN

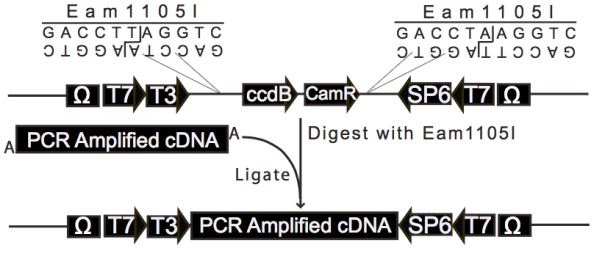
2.5 µl 10x Platnum Taq PCR buffer
0.5 µl 10mM dNTPs
1.5 µl 50mM $MgCl_2$
1.0 µl Forward Oligo (10 µM) or 0.1 (100 µM)
1.0 µl Reverse Oligo (10 µM) or 0.1 (100 µM)
1.0 µl cDNA
0.125 µl Platnum *taq*
To 25 µl with H_2O

PCR Conditions
Step 1: 4 min @ 95 °C
Step 2: 30 sec @95 °C
Step 3: 30 sec @ 55 °C
Step 4: 1 min @ 72 °C
Step 5: Go to 2 39 times
Hold at 4°C

Run on 1% Agarose Gel

Vector Preparation

Digest pJC53.2 (available from Addgene https://www.addgene.org/26536/) with Eam1105I, this leaves T-overhang



Digest
1 µg pJC52.3 (enough for multiple cloning reactions)
1 µl Eam1105I (Fermentas Fast Digest)
2.0 µl FastDigest Buffer
to 20 µl with H_20

Incubate 60 min at 37°C

Clean rxn with Zymo Clean and Concentrate Kit

Quantify and run ~50ng on Gel with PCR products. The digest should have two bands: 1600 (pJC53.2 insert ccdB and CamR) and 3200 (T-tailed vector). No further purification needed.

Ligation

4.0 µl unpurified PCR product
150 ng pJC53.2 (digested)
to 10 µl with H_2O

10 ul 2x Rapid Ligation Buffer
Mix
Add 1 µl NEB t4 DNA Ligase
Incubate for ~30-60 min on bench

Transformation

5 µl Ligation
50 µl Competent DH5α
Incubate on ice for 30 min, 90 sec heat shock at 42 °C, incubate on 2 min ice, add 1ml LB, shake for 1hr at 37 °C
Plating whole transformation on kanamycin (or ampicillin) plate will give several hundred colonies

Colony PCR

For in situ analysis you want the T3 promoter to transcribe the anti-sense strand, so use forward primer and T3 primer to determine which clones have correct insert and orientation.

2.5 µl 10x Platnum Taq PCR buffer
0.5 µl 10mM dNTPs
1.5 µl 50mM $MgCl_2$
1.0 µl Forward Oligo (10 µM) or 0.1 (100 µM)
0.1 µl 100µM EY (GAA TTA ACC CTC ACT AAA GGG AGA CC, modified T3 Primer)
0.125 µl Platnum *taq*
To 25 µl with H_2O

Pick colony with pipette tip and streak a small “line” on a labeled amp or kan plate. Drop pipette tip into the PCR mix. Pick 5 colonies for each transformation as a starting point.

Step 1: 10 min @ 95 °C For cell lysis and nuclease inactivation)
Step 2: 30 sec @95 °C
Step 3: 30 sec @ 55 °C
Step 4: 1 min @ 72 °C
Step 5: Go to 2 35 times
Hold at 4 °C

Run gel and identify clones of interest by the presence of bands ~25 bp larger than was observed in original PCR.
Pick clones for mini preps and submit for sequencing.
Sequence confirmed clones can be used for RNAi and/or Riboprobe synthesis with T3 RNAP.