August 9, 2007

RIBOPROBE GENERATION

MATERIALS

DIG RNA Labelling Kit (SP6/T7) (Roche Applied Science catalog # 11 175 025 910)
dNTPs
FastStart Taq DNA Polymerase DNTP Kit (Roche)
37°C Heat Block
PCR Thermal Cycler
Pipettors, Pipette tips, Microcentrifuge Tubes
Primers

NOTES/PRIMER DESIGN

1. Take your plasmid to the core lab for sequencing. I usually sequence using their stock T7 and T3 primers (or whatever are appropriate for the plasmid). The sequencing request form can be found at: http://www2.creighton.edu/medschool/medicine/departments/biomedicalsciences/research/technologyequipment/molecularbiologyresearchcorefacility/index.php
2. Find your gene's sequence at Pubmed:
   http://www.ncbi.nlm.nih.gov/sites/entrez
2. Note the location of the coding sequence (CDS).
3. Swtich to "FASTA" instead of "GENE" and copy the sequence to a word document. Now you can use "format" and "word count" to determine where the coding sequence is and highlight the sequence.
4. Blast your sequencing results from the core lab against the genome to ensure that you have the correct gene. Note what portion of the gene the plasmid contains. http://www.ncbi.nlm.nih.gov/projects/genome/seq/BlastGen/BlastGen.cgi?pid=9559
5. Also check to make sure there is no significant overlap with other genes.
6. Determine the sequence you wish to make a riboprobe with, which should be ~500+ bases, but should have no more than ~100 bases of overlap with other genes. This will ensure your probe's specificity under the current conditions used for in situ hybridizations. For genes that have multiple family members (ex. Lmx1a and Lmx1b) use the align tool to make sure that there is no significant alignment of your probe with an overlapping gene. http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi
7. Design your primers using the following rules: Tm between 56-58 degrees Celsius, G/C content of 50-66%, starting with a G or C if possible, not ending with more that 2 G's or C's, length of 18-30 bases (not counting the T7 promoter that is added to the reverse primer), and not containing more that three of any particular base in a row.
8. Analyze your primers using: http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx
9. Add the T7 promoter primer sequence (**TAA TAC GAC TCA CTA TAG GG)** to your reverse primer and order your primers.

10. In the following protocol, to make a larger batch, I increase the transcription size to 50µl, since some of the transcription efficiency will be reduced with the gel purification.

PROCECURE

1. Dilute your plasmids to 25ng/µl.
2. Prepare the following PCR reaction for each sample (100µl):
   1 µl Sample (plasmid 25ng/uL)
   10 µl Forward primer
   10 µl Reverse primer
   10 µl 10X Buffer with $MgCl_2$
   2 µl DNTPs
   1 µl FastStart Taq DNA Polymerase
   66 µl Sterile ultrapure water
3. Incubate for 4 minutes at 94°C.
4. Incubate 20 cycles of amplification consisting of incubation at:
   95°C for 30 seconds
   55°C for 30 seconds
   72°C for 60 seconds
5. Incubate for 10 minutes at 72°C.
6. To check purity, prepare 5% of the reaction to run out on an agarose gel (10µl total volume)
   5 µl PCR Sample
   2 µl 80% Glycerol BPB loading dye
   1 µl 10X SYBR Green
   2 µl DEPC-treated H_2O
7. Run the samples on a 1% agarose gel, with 5-10 µL of 100 bp ladder at each end.
8. Image the gel on a gel doc to check size and product fidelity.
9. If the reactions look pure, ethanol precipitate the remaining 95 µl of PCR product for each sample.
   95 µl PCR product
   9.5 µl 3 M Sodium Acetate (final concentration 0.3 M)
   250 µl 100% Ethanol
10. Incubate on ice for 15 minutes. Centrifuge at max speed for 15 minutes. Remove supernatant. Wash pellet with 250 µl of 70% ethanol. Centrifuge at max speed for 5 minutes. Remove supernatant and allow pellet to dry for 5 minutes. Redissolve in 30 µl water.
11. Add 2 µL purified template DNA to a reaction vial and 11 µL DEPC-treated H20.
12. Place the reaction vial on ice and then add the following:
    2 µl 10X NTP labeling mixture (Vial 7)
    2 µl 10X Transcription buffer (Vial 8)
    1 µl Protector RNase Inhibitor (Vial 10)
    2µl RNA Polymerase T7 (Vial 12)
13. Mix gently and centrifuge briefly. Incubate for 2 hours at 37°C.

14. Add 2 µl DNase I (Vial 9) to remove the template DNA. Incubate for 15 minutes at 37°C.
15. Stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0). Place the samples on ice (or at -20°C) until they are ready for purification.
16. Purify the samples using an acrylamide gel. Cleaniliness is key! Use RNase Zap on all working surfaces.
17. Mix 0.1 g Ammonium Persulfate in 1 mL of sterile water (10% APS).
18. Prepare a 6% acrylamide gel: Add 28 mL Sequel NE Part B + 12 mL Sequel NE Part A. Gently swirl the mixture to avoid air bubbles.
19. Add 200 µL (5 µL/mL) of 10% APS to the Sequel (A + B) solution. Gently swirl the solution.
20. Clamp the plates down properly to avoid leakage.
21. Apply the 6% acrylamide gel solution between the glass plates until full.
22. Place the comb into the gel. Be careful not to get air bubbles under the comb.
23. Let the gel polymerize 1 hour.
24. Add 24 µl gel loading buffer to each transcription reaction and purify on a 6% acrylamide gel.
25. Run the gel at 20-25 watts. Run xylene cyanol (top band) until it is ~2/3 down the gel.
26. Excise RNA by UV shadowing, cut into ~1mm cubes and elute RNA into 400 µl crush soak buffer overnight at 4°C. Depending on the amount of transcribed product, you may need to separate this into two tubes.
27. Remove supernatant from the gel cubes and precipitate RNA.
28. Precipitate RNA by addition of 1 mL 100% Ethanol.
29. Incubate on ice for 15 minutes. Centrifuge at max speed for 15 minutes. Remove supernatant. Wash pellet with 250 µl of 70% ethanol. Centrifuge at max speed for 5 minutes. Remove supernatant and allow pellet to dry for 5 minutes. Redissolve in 25 µl water.
30. Quantify on the nano-drop spectrophotometer.

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SOLUTIONS

1% Agarose (100 mL)

1 g	Agarose (Fisher catalog # BP-1356-100)
10 mL	10X TBE
90 mL	DEPC Water

- Place in flask and microwave until boiling. Mix well to ensure that all the agarose is melted evenly.

100 bp DNA Ladder (100 µL)

10 µL	1 µg/µL 100 bp Ladder (Invitrogen catalog # 15628-050)
20 µL	10X TBE
150 µL	DEPC Water
20 µL	80% Glycerol BPB dye

Crush Soak Buffer

5 mL	1 M HEPES
20 mL	5 M NaCl
1 mL	0.5 M EDTA

- Sterile ultrapure water to 500 mL
- Autoclave

DEPC Water (1 Liter)

1 mL	DEPC (Sigma catalog # D5758)
1 L	Sterile ultrapure water

- Autoclave.

2X Loading Buffer (2xLB)

4 g	Sucrose
10 mg	Bromophenol blue
10 mg	Xylene Cyanole FF
200 µL	10% SDS
2 mL	10X TBE
8.8 g	Urea

- Sterile ultrapure water to 20 mL
- Aliquot and store at $4^{o}C$

10X Sybr Green (1 mL)

1 µL	10,000X Sybr Green (Sigma catalog # S-9430)
999 µL	DEPC-treated Water

1X TBE Running Buffer (1 Liter)

100 mL	10X TBE
900 mL	Ultrapure Water

10X TBE (4 Liters)

432 g	Tris Base [0.9 M] (Fisher catalog #BP152-5)
220 g	Boric Acid [0.9 M] (Fisher catalog # BP168-1)
14.9 g	EDTA [10 mM] (Fisher catalog # BP120-500)

- Adjust volume with DEPC water to 4 Liters. Autoclave in 1 L aliquots.