Vezina Lab DIG-UTP Riboprobe Synthesis

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1 Works for me This protocol is published without a DOI.

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PROTOCOL CITATION

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Design of Polymerase Chain Reaction (PCR) Primers

1 Extract cDNA sequence from Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez).

2 Use Primer3 program (http://frodo.wi.mit.edu/primer3/) to design PCR primers against the 3'-region of the cDNA sequence.

The following parameters are used to assist in the design of high-quality, specific PCR primers:
A. Mispriming Library (repeat library): RODENT_AND_SIMPLE.
B. PCR Product Size Range: 500-800 bp.
C. Maximum allowable weighted similarity to ectopic sites in the cDNA sequence (Template Mispriming): 10.
D. Primer Size Minimum: 18 base pairs (bp), Optimum: 20bp , Maximum: 27 bp.
E. Primer melting temperature (Tm) Minimum: 57°C, Optimum: 60°C, Maximum: 63°C.
F. Maximum acceptable Tm difference between left and right primers: 100°C.
G. Primer GC content: Minimum:20%, Optimum :50%, Maximum: 80%.
H. Maximum allowable 3'-anchored global alignment score for self-complimentarity: 1.
I. Maximum allowable length of homonucleotide repeat (Poly-X): 3. J. Number of guanines or cytosines required at 3' end of left and right primer (GC Clamp): 1.
K. The following sequence is added to the 5'-end of the right primer: CGATG**TTAATACGACTCACTATAGGG** . This sequence includes a 5bp leader sequence followed by the T7 RNA polymerase recognition sequence (shown in bold-faced, underlined text).

3 Specificity of the PCR primers for the sequence is assessed by the Primer Blast program. Input the Primer3 derived sequences (without the leader and T7 sequence), use the default primer parameters, and blast against the Mus musculus organism RefSeq database. A specific Primer pair will elicit a perfect match to only the gene of interest.

4 Specificity of the PCR product for the cDNA sequence is assessed with the MegaBLAST Program (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090). A PCR sequence is considered specific for the cDNA when, using an EXPECT threshold of 0.01, it does not align with other members of the RefSeq

RNA database

5 Primers are synthesized by a commercial vendor and are resuspended in nuclease free water at a concentration of 100µM.

Synthesis of Labeled Riboprobes

6 **Prepare cDNA.**

a.To make cDNA, you need to first isolate RNA from: embryonic day (E)16.5 male urogenital sinus, E16.5 female urogenital sinus, and P50 male prostate (all lobes).Add Molecular Grinding Resin (G-Biosciences #786-138PR) to the frozen tissues, mechanically homogenize tissue with a pestle, and further disrupt tissue by passing homogenate through QIAshredder Columns (Qiagen # 79654).Isolate total mRNA with the RNeasy Mini Kit (Qiagen # 74104) and quantify mRNA by spectrophotometry.

b.Combine 2ug of RNA from E16.5 male UGS, 2ug RNA from E16.5 female UGS and 2ug from P50 male prostate. Use this combined RNA pool to make cDNA.

c.Synthesize cDNA with oligo d(T) primers, according to the instructions of the SuperScript® III First-Strand Synthesis System (Invitrogen # 18080-051). Combine the following items into PCR tube:
i.x µl 500ng pooled RNA
ii.1 µl of50 mM oligo $(dT)_{20}$
iii.1 µl of 10 mM dNTP and
iv.make up final volume to 10 µl with DEPC treated water.

d.Incubate at 65C for 5 min and then place on ice for at least 1 min

e.To each tube then add
i.2 µl 10X RT buffer,
ii.4 µl 25mM MgCl2,
iii.2 µl 0.1M DTT,
iv.1 µl RNaseOUT (40U/µl),
v.1 µl Superscript III RT(200 U/ µl).

f.Mixgently then incubate at 50C for 50 min

g.Terminate the reaction at 85C for 5 min. Chill on ice

h.Add 1 µl of RNase H to each reaction, mix and then incubate at 37C for 20 min

i.Once that is done, store at -20C and label as “pooled CDNA from E16.5M, E16.F and adult prostate) or proceed to PCR protocol immediately.

7 **Amplify PCR Product.**

a.PCR reaction components are optimized for each cDNA sequence.A typical 50 µl reaction contains:
1. 27.6 µl nuclease-free H_2O.
2. 5 µl of 10X Qiagen buffer,
3. 10 µl of 5X Buffer Q
4. 1.25 µl of 25 mM $MgCl_2$,
5. 0.4 µl of 25mM dNTPs (Roche Applied Science 11969064001),
6. 1.25 µl of 10 µM primers
7. 0.5 µl of Qiagen Taq DNA polymerase (Qiagen #201203)
8. 4.0 µlcDNA,

b.Thermocycling conditions on the Perkin Elmer GeneAmp 9600 are optimized for each cDNA sequence. A typical thermocycling protocol is: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C 30s, 57°C 30s, 72°C 1min and a final extension at 72°C 10 min.

8 Gel Purify PCR Product

a.Separate products in a 1% agarose gel

b.Visualize gel with an ultraviolet transilluminator and excise DNA bands with a clean razor blade.

c.Purify excised DNA bands from gel slice with a gel extraction kit (Qiagen # 28704).

d.Elute DNA in 40 µl nuclease free H_2O.

e.Quantify product by spectrophotometry.Expected yield is 1.2 - 3.6 µg.

9 Transcribe PCR product into labeled riboprobe.

a.*In vitro* transcription reaction (40 µl) contains:

- _ µlof 400 ng clean PCR product,
- And either
- a.4 µl 10X DIG labeling mix (Roche Applied Science # 11277073910) **or**
- b.4 µl 10X Fluorescein labeling mix (Roche Applied Science # 11685619910)
- 4 µl 10X transcription buffer ,
- 1µl of RNase inhibitor (Roche Applied Science #03335399001),
- 4 µl of 80U T7 RNA polymerase (Roche Cat # 10881767001),
- __ µl nuclease-free H_2O to make final volume to 40 µl
- Incubate 3-4 hr at 37°C (agitate samples every 30 min).

b. Purify riboprobes with the Qiagen RNEASY Mini kit (Qiagen #74104), according to the directions for RNA cleanup with DNase digestion with the following modifications. To do this:

- i.First make RLT containing BME: Add 10 µl BME/ 1ml RLT. You need 350 µl RLT/ PCR tube and Master mix for step
- ii. Adjust transcription volume to a final volume of 100 µl with RNase free water.
- iii. Add 350 µlBuffer RLT (containing BME) and mix well
- iv. Add 250 µl Ethanol. Mix well with pipetting and then transfer the 700 µl to an RNeasy Mini spin column placed in a 2ml collection tube. Centrifuge 15s at 10,000 rpm. Discard flow through.
- v. Now perform on column DNAse digestion: Add 350 µl Buffer RW1 to spin column, centrifuge for 15s at 10,000rpm. discard flow through.
- vi. Add to the center of column 70 µl of mix containing:
- a.7 µlReaction buffer+
- b.4 µlDNase 1[Promega #M610A]).
- c.59 µlwater
- vii. Incubate columns for 20 min at 37°C.
- viii .Apply 350 µl Buffer RW1, centrifuge for 15s at 10,000rpm and discard flow through.
- ix. Add 500 µl Buffer RPE centrifuge for 15s at 10,000rpm and discard flow through
- x. Repeat: Add 500 µl Buffer RPE centrifuge for 15s at 10,000rpm and discard flow through
- xi. Centrifuge at full speed for 1 min to wash column
- xii. Then transfer column to a clean 1.5ml tube, apply 40 µl nuclease-free H_2O to the center of each column. Incubate for 15 min at 60°C. Centrifuge for 1min at 10,000rpm
- xiii. Collect flow through, reapply flow-through to column. Incubate for 15 min at 60°C.
- xiv. Centrifuge for 1min at 10,000 rpm and collect flow through (purified RNA).

c. Quantitate by spectrophotometry. Expected yield is 4-20 µg.

d. Verify probe quality by separating probe by electrophoresis on a 1.5% non-denaturing agarose gel. To prepare probe for electrophoresis: Transfer 2 µl probe + 7 µl water and 1 µl 10X loading dye into microfuge tube. Heat in water bath at 65C for 5 minutes. Then immediately place in ice. Once chilled, load onto gel.

e. High quality probes migrate as distinct bands with minimal smearing.