RNA Confirmation Procedure

Step One: Design Primers

- Using sequence from CMHD database and NeoL primer, design R primer from web resource such as Primer3
- Sequence from database should be blasted in NCBI/ensemble before primer is designed to make sure the sequence is good
- Using sequence of gene and new primer, determine expected length of PCR product

Step Two: RNA isolation/purification

- RNA is purified from expanded ES cells using the Machery-Nagel RNA kit

Step Three: cDNA synthesis

- Protocol follows

Step Four: PCR

- Protocol follows

Step Five: Check PCR products

- Run pcr products on 1.5% agarose gel

Step Six: Send for Sequencing

- If the PCR band is of expected size, send PCR product for sequencing using the primer listed in section below.

Step Seven: Blast sequence results against CMHD and NCBI/ensembl databases.

- When sequencing is returned, blast sequence against CMHD database to determine if the new sequence matches the old sequence
- Also, blast the new sequence through NCBI/ensembl to see if the correct gene is sequenced

RNA Confirmation Protocol

Part One: cDNA Synthesis

(Invitrogen M-MLV RT protocol)

1. Mix together:
   - 1 ul 3'CDS primer
   - 1 ul dNTP mix
   - 2 ul purified RNA
   - 8 ul H_2O

The 3CDS primer sequence is AAG CAG TGG TAA CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT TVN

2. Heat mixture to 65^{o}C for 5 minutes *(PCR program 655min)*. Chill on ice immediately afterwards.

3. Add:
   - 1 ul RNase OUT
   - 2 ul DTT
   - 4 ul 5x First-strand Buffer

4. Mix and incubate at 37^{o}C for 2 minutes *(PCR program 3702min).*

5. Add in 1 ul of M-MLV RT buffer.

6. Incubate 37^{o}C for 50 minutes *(PCR program RTLIA).*

7. Inactivate reaction by incubating at 70^{o}C for 15 minutes.

Part Two: PCR

(Invitrogen M-MLV RT protocol)

Master Mix:

ddH_2O	38.0ul
10x PCR Buffer	5.0ul
MgCl	1.5ul
dNTP mix	1.0ul
Left Primer	1.0ul
Right Primer	1.0ul
Taq	0.5ul
cDNA	2.0ul
Total	**50.0ul**

Cycling Conditons:

94^{o}C 2 minutes
94^{o}C 30 seconds
62^{o}C 30 seconds
72^{o}C 2 minutes
repeat steps 2-5 for 39 cycles
72^{o}C 2 minutes
hold at 4^{o}C

Program: RHOATR

Left primer (F) is listed below. Right primer (R) is your gene specific primer that you design.

Primers for Clone Confirmation

pGTlox4/4pA

- Left: (bsdL) GTTATGTGTGGGAGGGCTAAGCAAT
- 69bp to splice site
- Sequencing: bsdL GTTATGTGTGGGAGGGCTAAGCAAT

SD5

- Left: (NeoL) GCTATCAGGACATAGCGTTGGCTAC
- 193bp to splice site
- Sequencing: NeoL :

pMS

- Left: (NeoL) GCTATCAGGACATAGCGTTGGCTAC
- 385bp to splice site
- Sequencing: NeoL

NMDi

- Left: (NeoL) GCTATCAGGACATAGCGTTGGCTAC
- 192bp to splice site
- Sequencing: NeoL

pUPA

- Left: (NeoL) GCTATCAGGACATAGCGTTGGCTAC
- 918bp to splice site
- Sequencing: (UPAtrap 7) ATTCCAGACAAGTTTGTTGTTGGATATGCC
- 64bp to splice site