Long Range PCR protocol for KOMP-CSD derived ESC lines

7/2018

Warning/Recommendation: <u>All</u> mutant mice (lacZ+ or short range PCR+) derived from CSD & EUCOMM ES cell created chimeras should be confirmed for the targeted allele using 5′ long range PCR. Alternatively, targeting may be confirmed using quantitative (Taqman) LOA for CSD targets. All KO firsts should be confirmed for the presence of the distal loxP cassette.

I DNA extraction from ES cells (following manufacturers suggestions)

Materials

DNeasy® Tissue Kit 250 (Qiagen 69506)
PBS (50mM potassium phosphate; 150mM NaCl)

Procedure (12-well plates)

1. Wash the monolayer with cold PBS (4°C).

2. Scrape the cells with 0.5 ml of cold PBS using a rubber policeman, and transfer to 1.5ml tube on ice.

3. Add 20 µl proteinase K and 200 µl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 20 min. It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

4. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

5. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at _6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at _6000 x g (8000 rpm). Discard flow-through and collection tube.

7. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions.

8. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 8000 rpm to elute.

9. Store at -4° C until use or -20° C if storing more than a day.

II Long Range PCR

Universal Oligos: (5' to 3')

3' Universal (CSD-Neo-F): GGGATCTCATGCTGGAGTTCTTCG (fwd from genetrap cassette)

5' Universal (LR-5En2frt-R): GGTGGTGTGGGAAAGGGTTCGAAG (rev from genetrap cassette)

Access to clone specific vector and oligo data:

1. From KOMP.org type official gene symbol into search bar and click on correct gene symbol when list populates.

2. Under "Show All Projects" click on the 8 digit project ID "CSDXXXXX" relating to your order.

3. Use any or all 5' Gene Specific (GF) in conjunction with 5' Universal

4. Use any or all 3' Gene Specific (GR) in conjunction with 3' Universal

5. Access the Genbank File by searching KOMP.org by gene and click on the "Project ID" link from KOMP.org.

6. Redirects to IMPC and then click on the “MGI Allele Name” link.

7. Go to “Targeting Vectors” and click on “Design Oligos” link.

8. search for GF3 or GF4 (5’ primers) and/or GR3 or GR4 (3’ primers) as exampled below.

GF3	-1	-1	1	GACAGAGCCACTTGTGGAATGTAGCCTCAG
GF4	-1	-1	1	CTCATGTCATGAATTGAACATGATATGAAC
EX52	-1	-1	1	GAGGCCAAGTACCTGCCCAGCAGC
EX32	-1	-1	1	CAGGATGCCAACTGCAAAGCAGATTATAC
GR3	-1	-1	1	CAAGACAGAGCCGAGTGTGCCGTGCACTC
GR4	-1	-1	1	GCCGAGTGTGCCGTGCACTCTTAC

Materials and equipment

SequelPrep long PCR kit, Invitrogen A10498
Peltier Tetrad2 thermal cycler, BioRad.
Oligos, Invitrogen

Procedure

1. Thaw and equilibrate all buffers at room temperature

2. Prepare the following master mix on ice (1X shown):

Components	Volume per rxn
Sterile H20	13.24
10X Buffer (green tube)	2
Enhancer A (Red tube)	1
Enhancer B (Yellow tube)	1
DMSO (brown tube)	0.2
gene specific primer (10uM)	0.5
universal primer (10uM)	0.5
Enzyme (black tube)	0.36
total cocktail	19
template	1
reaction volume	20

3. Briefly vortex master mix and transfer 19 ul of to each 200ul thin walled reaction tube on ice.

4. Briefly flick DNA tube and input 1 ul of ~100 ng into reaction tube.

5. PCR with the following thermal conditions:
Note: may adjust elongation time to 1min/kb amplicon.

Temp	Duration	Repetitions
94°C	2 minutes	1x
94°C	10 seconds	10X
65°C	30 seconds -1°C/cycle	
68°C	10 minutes	
94°C	15 seconds	25X
55°C	30 seconds	
68°C	10 min +20 sec/cycle	
4°C	hold	

III Data Analysis

Materials and Equipment

Loading Dye (15ml glycerol; 35ml H20, 125mM each Bromophenol Blue/Xylene Cyanol).
1 Kb plus DNA Ladder, Invitrogen 10787-026.
GenePure LE Agatose, ISCBioExpress E-3120-500.
Gel Logic Imaging System 100, Kodak.

Procedure

1. Prepare a 1% Agarose gel with 0.25ug Ethidium Bromide per ml agarose. (non toxic comparable would be SYBRsafe, Invitrogen S33102).

2. Line pipette tips with Loading Dye and mix with finished PCR reaction.

3. Load 15ul of reaction into well.

4. Run gel at 120 volts for 2 hours.

5. Image under UV and adjust and store image for record.

6. Controls: non-template control, isogenic wild type, 1kb plus ladder. (no positive control available).