

Long Range PCR protocol for KOMP-CSD derived ESC lines

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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 70°C for 10 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 $_6000 \times g$ (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 $_6000 \times g$ (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 $\times g$ (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

Oligos: (5' to 3')

KOMP-loxPcom-F: GAGATGGCGCAACGCAATTAAT

Design a reverse compliment oligo in genomic region outside the downstream arm of homology

KOMP-en2comR: CCAACTGACCTTGGGCAAGAACAT

Design a forward direct oligo in genomic region outside the upstream arm of homology

Access to vector data:

1. From Product Detail File "PDF" for a particular clone at www.komp.org, click on the "CSD Vector Design" hotlink
2. Under "final_vec_qc" click link in column with same design plate # (DP) as your clone.
3. After "Synthetic Vector" click link for genbank
4. Copy the sequence from beginning of 5' arm to end of 3' arm
5. Blast this sequence to align and characterize the vector arm of homology with genomic contig region and build an oligo outside the arm of homology

Materials and equipment

Expand Long Template PCR System, Roche 1681934.
Peltier Tetrad2 thermal cycler, BioRad.
Oligos, Sigma Genosis.

Procedure

1. Thaw and equilibrate all buffers at 37°C
2. Prepare the following master mix on ice:

Components	Volume per rxn
Sterile H2O	17.8 ul
10 X PCR buffer 1 Roche	2.5 ul
dNTP (10mM each) Roche	0.85 ul
downstream primer (20uM)	0.75 ul
upstream primer (20uM)	0.75 ul
Expand Long Template enzyme mix (5U/ul) Roche	0.35 ul
Total Master Mix	23 ul

3. Briefly vortex master mix and transfer 23 ul of to each 200ul thin walled reaction tube on ice.
4. Briefly vortex DNA and input 2 ul of 250 ng (input total 500 ng) into reaction tube.
5. PCR with the following thermal conditions:

Temp	Duration	Repetitions
94°C	2 minutes	1x
94°C	10 seconds	10X
55-65°C	30 seconds	
68°C	3 minutes	25X
94°C	10 seconds	
55-65°C	30 seconds	
68°C	3 min + 20 sec each successive cycle	1x
68°C	7 minutes	
4°C	forever	

III Data Analysis

Materials and Equipment

Loading Dye (15ml glycerol; 35ml H ₂ O, 125mM each Bromophenol Blue/Xylene Cyanol).

1 Kb plus DNA Ladder, Invitrogen 10787-026.

GenePure LE Agarose, ISCBioExpress E-3120-500.
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Gel Logic Imaging System 100, Kodak.

Procedure

- 1.** Prepare a 1% Agarose gel with 0.25ug Ethidium Bromide per ml agarose.
2. Line pipette tips with Loading Dye and mix with finished PCR reaction.
3. Inject 50% reaction volume into well (12.5 ul).
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).