

PCR protocol for KOMP-CSD derived Mice & ESC

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I DNA extraction from mouse tissues (following manufacturers suggestions)

Materials

DNeasy® Tissue Kit 250 (Qiagen 69506)
100% Ethanol (Gold Shield Chemical Company DSP-CA-151)

Procedure

1. Make a cocktail of ATL with 180uL buffer ATL and 20uL Proteinase K. Add 200uL to each tube. Be sure tail snip is submerged.
2. Incubate at 55°C for 6-12 hours in heat block or water bath.
3. Remove tubes from heat source and vortex.
4. Add 400 ul of AL/Ethanol mixture to each tube. Vortex.
5. Pour liquid into an appropriately labeled Qiagen spin column. Centrifuge at 8,000 rpm for 1 minute. Note-AL/Ethanol mixture is 1 part AL buffer to an equal part of Ethanol. Usually made 50 ml at a time, with 25 ml of AL and 25 ml of 200 proof (100%) Ethanol.
6. Transfer spin column to a new collection tube and add 500 ul of AW1. Be sure ethanol (200 proof) has been added to AW1 buffer; if you add ethanol to AW buffer, be sure to mark the bottle. Spin at 8,000 rpm for 1 minute.
7. Transfer spin column to a new collection tube. Add 500 ul of AW2. Again, be sure 200 proof ethanol has been added. Spin at max speed (14,000 rpm) for 3 minutes.
8. *Carefully* remove tubes from centrifuge. Transfer to a 1.5 ml microtube. Check each spin column for Ethanol before placing in the microtube! If ethanol is still on the spin column, pour off the fluid from the collection tube and spin again for 1 minute at 14,000 rpm. Residual ethanol may inhibit PCR!
9. Add 200 ul of Buffer AE (elution buffer) to spin columns in microcentrifuge tubes. Incubate at room temp for 2 minutes. Spin at 8,000 rpm for 1 minute.
10. Once you have eluted twice (for a total of 200 ul in each tube), label each tube with the sample info that was put on the spin column. Be sure to date the first, last, and approximately every fifth tube for each batch of tails. Store in a cardboard box in the 4°C refrigerator for short term and -20°C for long term.

II PCR strategy and reaction

Oligos (5' to 3')

Common-loxP-F: GAGATGGCGCAACGCAATTAAT

Design a reverse compliment oligo in the arm of homology ~200bp downstream or more from the Common-loxP-F oligo.

Common-en2-R: CCAACTGACCTTGGGCAAGAACAT

Design a forward direct oligo in the arm of homology ~300bp upstream or more of Common-en2-R oligo.

Common-3'F: CACACCTCCCCCTGAACCTGAAA

Access to vector 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. Access the Genbank File by clicking on "view" under ES Cell Clones bar. This will provide sequence to use for amplicon size expectation.

Materials and equipment

AmpliTaq® DNA Polymerase w/ 10X Buffer II (ABI N808-0156)
dNTP set (Fisher BP2564-4)
Peltier Tetrad2 thermal cycler (BioRad).
Oligos (Sigma Genosis)

Procedure

1. Prepare the following master mix on ice:

Components	Volume per rxn
Molecular grade H ₂ O	18.6 ul
10 X PCR buffer II	2.5 ul
25mM MgCl	1.7 ul
dNTP (10mM each)	0.5 ul
Oligo's (20uM each)	0.5 ul
Amplitaq® Polymerase (5U/uL)	0.2 ul
Total Master Mix	24 ul

2. Briefly vortex master mix and transfer 24uL of to each 200ul thin walled reaction tube on ice.
3. Briefly vortex DNA and input 1uL of ~100 ng DNA into reaction tube.
4. PCR with the following thermal conditions

Temp	Duration	Repetitions
94 °C	5 min	1x
94 °C	15 sec	10X (decrease 1C/cycle)
65 °C	30 sec	
72 °C	40 sec	
94 °C	15 sec	30X
55 °C	30 sec	
72 °C	40 sec	
72 °C	5 min	1x
15 °C	forever	

III Data Analysis

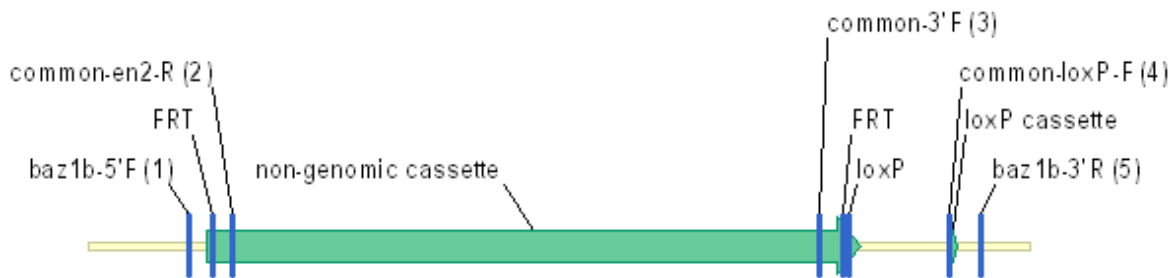
Materials and Equipment

Loading Dye (15ml glycerol; 35ml H ₂ O, 125mM each Bromophenol Blue/Xylene Cyanol).
1 Kb plus DNA Ladder (Invitrogen 10787-026)
GenePure LE Agatose (ISCBioExpress E-3120-500)
Gel Logic Imaging System 100 (Kodak)

Procedure

1. Prepare a 1.5% TBE 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. Inject 50% reaction volume into well (12.5 ul).
4. Run gel at 120 volts for 1.5 hours in TBE.
5. Image under UV and adjust and store image for record.
6. Controls: non-template control, isogenic wild type, 1kb plus ladder. (positive control if available).

PCR Strategy example



Example: primer combinations for KOMP-CSD targeted Baz1b gene

test	bax1b-5' (primer 1)	common-en2-R (primer 2)	common-3'F (primer 3)	common-loxP-F (primer 4)	baz1b-3'R (primer 5)	amplicon size
target allele 3'				x	x	302bp
target allele 5'	x	x				401bp
allele after cre			x		x	536bp
allele after flp-e	x				x	1.4kb
after cre & flp-e	x				x	495bp
Wild type	x				x	1.2kb

Alternatively: For standard loxP genotyping that determines zygosity— design forward and reverse oligos flanking the loxP cassette that will react with both wild type and mutant allele. First, verify that the loxP cassette amplicon and wild type amplicon will give a greater than ~30 bp mobility shift of a relatively small amplicon (<300bp). If this is not possible then primers 1-5 will usually give zygosity of any combination of treatment rather it is untreated mutant, flipped only, cre excised only, or flipped and cre excised.