

Loss of Native Allele Protocol for KOMP-Regeneron derived ESC lines
January 9th 2009

I. Alkaline DNA extraction in 96 well format

Solutions

50mM NaOH
1M Tris-HCl pH 8.0

Procedure

1. Grow ES cells in a 96-well TC plate to confluence. Store at -20 until use.
2. Bring to room temperature and add 50ul of 50mM NaOH to each well.
3. Agitate at room temperature for 10 minutes.
4. Transfer to a sterile 96 well reaction plate (gently scrape bottom and mix 3 times with pipette tips) and seal transfer plate with plate cover.
5. Heat at 95° C on thermal cycler with heated lid at 100° C for 10 minutes.
6. Bring to room temperature and carefully remove plate seal and add 20 ul of 1M Tris-HCl pH 8.0.
7. Reseal with new plate cover and briefly vortex (slow to fast to slow).
8. Centrifuge at 6K rpm for 2 minutes.
9. Store at -4° C until use or -20° C if storing more than a day.

II. Oligos and Taqman probes

Access to vector data

Access to clone specific vector and oligo data:

1. From <http://www.KOMP.org> type official gene symbol into search bar and click on correct gene symbol when list populates.
2. Click on the 7 digit project ID "VGXXXXX" relating to your order.



The screenshot shows the KOMP Repository website interface. At the top, there is a search bar and navigation tabs including Home, MyKOMP, Logout, Utilities, Storage, Projects, Reports, Catalog, DCC, and Blog. The main content area displays the gene **Nat8l** (N-acetyltransferase 8-like) with its MGI number 2447776. A diagram shows the gene structure with exons and introns, and a red arrow indicates the location of the KO junction. The status summary shows: ES cell colonies screened / QC positives, KOMP Target: Yes, and Up to date as of 01/09/09. A progress bar at the bottom shows the stages: Pre-pipeline, Design Strategy, Vector Construction, ESC Electroporation, Germline Transmission, and Cryo-preservation. The current stage is ESC QC Positives, which is highlighted in blue. A red arrow points to the project ID VG11213.

3. Use the "2,000 Bases Upstream" and "2,000 Bases downstream" to verify the KO junctions.
4. Blast this sequence and align against genomic contig to characterize wildtype sequence that would be replaced by vector or missing in the recombined allele
5. Design a Taqman target system with 6FAM on 5' of probe (TAMRA on 3')
6. Design a Taqman reference system with VIC on 5' of probe (TAMRA on 3') from a single copy conserved endogenous gene (Primer Express, ABI)
7. Validate the two systems together by multiplexing and running both against a 2 fold DNA dilution series in triplicate for ~6 dilutions. The slope of ΔC_t Vs. $-\log[\text{DNA}]$ needs to be <0.1 .

III Multiplexed Real-time PCR via Relative Cycle Threshold Method

Reference: ABI User Bulletin #2 (updated 10/2001) for multiplex in same tube and validation of each assay to match relative efficiencies of reference and target primer/probe combinations.

Materials and equipment

Taqman 2X Universal PCR Mix, ABI 4364340
96 well reaction plate with bar code, ABI 4306737
Optical adhesive cover, ABI 4311971
Snap-On Compression Pad, ABI 4333292
ABI 7900HT Realtime PCR system

Procedure

1. Bring DNA plates to room temperature.
2. Prepare the following realtime reaction mix in order (low lighting) and briefly vortex: (note the reference and target final concentrations may vary dependent on assay design)

Components	per rxn	per plate
Master Mix (2X)	12.5 ul	1,250 ul
biology grade H2O	10 ul	1,000 ul
Reference forward (20uM)	0.25 ul	25 ul
Reference reverse (20uM)	0.25 ul	25 ul
Reference VIC labeled probe (10uM)	0.25 ul	25 ul
Target forward (20uM)	0.25 ul	25 ul
Target forward (20uM)	0.25 ul	25 ul
Target FAM labeled probe (10uM)	0.25 ul	25 ul
Total Reaction Mix	24 ul	2,400 ul

3. Transfer 24ul of reaction mix to each well of labeled 96 well reaction plate with bar code.
4. Carefully remove plate cover from DNA plate and transfer 1ul of DNA to each corresponding reaction plate (in duplicate).
5. Seal top with optical adhesive cover and snap on compression pad for automated handling (if more than one plate).
6. Briefly vortex to mix (slow to fast to slow).
7. Transfer sample location data as Tab-delimited text for each plate onto a jump drive (ABI specific software).

8. Open SDS2.2.1 (ABI) on ABI 7900HT and select Relative Quantification ($\Delta\Delta Ct$)

9. Scan the bar code and import data related to the particular plate for each plate in the study.

10. Designate the proper parameters for each reference and target system and designate the reference as "endogenous reference" and set volume as 25ul.

11. Commence the following program on ABI7900HT for each plate and send to queue for automatic handling:

Temp	Duration	Repetitions
50° C	2 minutes	1x
95° C	10 minutes	1x
95° C	15 seconds	40 times
60° C	1 minute	

IV. Data Analysis

Procedure

1. Export all plates for analysis onto jump drive
2. Open SDS.2.2.1 (ABI) and select Relative Quantification ($\Delta\Delta Ct$) Study.
3. Add all plates in the study from jump drive and click Analyze.
4. Go to Analysis Settings and set Cycle Threshold to AutoCt. (if this provides poor data separation then manually set the Ct to optimal location and select automatic baseline).
5. Export the Results Table (tab-delimited text).
6. In Excel, open the Results Table
7. Graph the Average ΔCt values on Y axis and look for data separation of 1 Ct (indicating a 2 fold difference).
8. Take the average of the wild type population (lower ΔCt) and add 1 Ct value to attain expected heterozygous ΔCt .
9. Homologous recombination candidates will fall into the larger ΔCt population.
10. Expand candidates for Southern confirmation.

Example Data Separation

