Screening for the Rhag KO

Screening the KO insertion system is less practical than screening for replacement homologous recombination as the Bradley lab 3'hprt insertion vector system depended on gap repair targeting as part of a strategy to identify correct insertions during ES selection, and the deletion is repaired when the insertion is in the correct gene.
We used two types of PCR for each KO, one detecting the KO insertion and one detecting the presence of a WT allele. The two are necessary for differentiating KO from heterozygotes At the time, attempts at setting up multiplex assays to test for both alleles simultaneously were unfortunately not successful (a drawback to correct identification if the DNA doesn't amplify). In any case we always verified protein expression.

1. Screening for the **Rhag KO vector insertion**:

We use a primer at the extremity of the rhag sequence in exon 6:
5'-TGC-TGC-TAT-GAC-CAT-TGG-AA-3'
and a primer in 3'hprt, 115bp from the PvuII site:
5'-ATT-GCA-TCG-CAT-TGT-CTG-AG-3'

the PCR cycle is
94°C-2' /
94°C-1';56°C-2'; 72°C-1' for 35cycles/
72°C-7'

the amplified fragment is **196bp**.

2. Screening for the **Rhag wt allele**

We developed a test for a polymorphism between 129 and C57Bl/6 in the Rhag sequence : The 'polymorphism' PCR was designed to detect **a difference in intron 3 of the Rhag sequence between the sequence of 129 origin (which includes a 14nt insertion) and C57Bl/6** WTsequence using the following primers which amplify a **570bp WT** fragment and do not amplify the 129 sequence containing allele:

5'-CCA-CTA-TAT-TTT-AAG-CTT-TGT-AAT-GTA-AT-3' and
5'-GAA-AAC-AAG-CCC-CAT-TCA-AA-3'

the PCR cycle is
94°C-2' /
94°C-1';58°C-1'; 72°C-1' for 35cycles/
72°C-7'

3. Alternative screening for the **Rhag wt allele** :

To distinguish homo- and heterozygotes we, initially, used a PCR which amplifies the region homologous to the Rhag insert in the vector on the wt chromosome, yielding **a #7.2kb amplicon**. This long PCR is less handy, and necessitates well purified DNA – phenol/chloroform extraction was suggested by the manufacturer, but we used DNA prepared with Nucleospin (Macherey-Nagel). Best results were seen with the Takara LA enzyme (apparently now marketed by Sigma Aldrich).

We used
- a primer in intron1 which is 2014bp 5' to exon2 and 65 bp 5' to the extremity of the homologous region contained in the knockout vector:
5'-CCA-TCT-CTC-CAG-CCT-AGC-AAT-TTT-C-3'
and
-a primer in intron 6 at 176 bp from the 3' end of exon 6 (the rhag sequence contained in the vector stops at 109bp in exon 6)
5'-AAG-TCA-GGA-AAG-AGT-CAT-TGC-ATG-G-3'

the PCR cycle is
94°C-1' /
94°C-20s'; 68°C-15' for 14 cycles/
94°C-20s'; 68°C-15'+15s/cycle for 16cycles/
72°C-10'