WM05 DUOX2 point mutation

General note;

- I design all projects using the DNA editing software Snapgene, which is commercially available
- I supply relevant files and maps as snapgene .dna files.
- These files can usually be opened in other DNA editing software, or you can download the free snapgene viewer software http://www.snapgene.com/products/snapgene_viewer/
- With the free version you cannot make sequence edits but you can view the maps and all annotated features and primers.

Guides for WM05 and OTEs

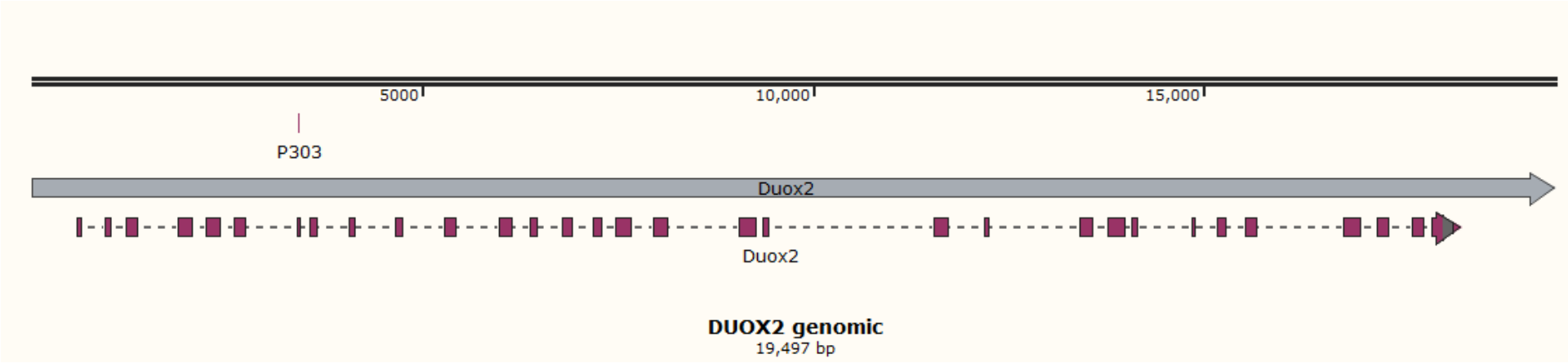
- Earlier publications in the CRISPR field (e.g. Fu et al, 2013, Nature Biotechnology and Hsu et al, 2013, Nature Biotechnology) raised concerns about off target effects (OTEs), and OTEs continue to be a controversial subject within the genome editing community (see O'Geen et al, 2015 for review). These earlier studies, whilst demonstrating the capacity of Cas9 to bind and cut unintended target sites, contain flaws in experimental design that we are now familiar with, including poor guide design, expression of Cas9 and sgRNA for extended periods of time, and the use of cancer-based cell lines, which often have mutated or imperfect DNA repair mechanisms.
- Since these earlier publications many have demonstrated that in more 'natural' cells such as primary cells, mES cells or iPSCs, that OTEs are very rare, and may not occur above background levels of cultured-acquired mutations (Tan et al, 2015 ; Kim et al, 2014). Of particular note is the study by Iyer et al, 2014, Nature Methods , which used whole genome sequencing to analyse CRISPR modified mice generated by pronuclear zygote injection, and demonstrated OTE mutation to be extremely rare in Cas9 modified mice. Additionally, because unlinked mutations segregate through breeding phenotyping of two independent founder animals (we routinely generate between 2 and 6 founders for knock in projects) would be sufficient to establish correct function and phenotype.
- The number of webtools available to assist in guide design has greatly increased, and we routinely use these to exclude guides that may have OTE risk. We select gRNAs with strict criteria using the http://www.sanger.ac.uk/htgt/wge/ site, and sequences predicted to have 0, 1 or 2 mismatches (MM) elsewhere in the genome are not be considered. Cutting at sequences that are 3 MM different to the guide sequence is very unlikely (Kim et al, 2015, Nature Methods) but we do not consider any 3 MM gRNAs found in the exons of other genes. We also microinject Cas9 recombinant protein, which, given its short window of expression (typically turned over in 12-18 hours), has been shown to be associated with minimal OTE generation (Kim et al, 2014).
- To summarise, updates in the literature, the use of informed design, use of Cas9 protein and the 'normal' cellular system of a zygote, we and others in the transgenic community do not consider OTEs to be a significant issue in the generation of CRISPR modified mice.

- http://www.sanger.ac.uk/htgt/wge/crispr/440427843

Strategy

- DUOX2 spans 20kb on chr2
- http://www.ncbi.nlm.nih.gov/gene/214593

- Replicate mutation from human in mouse; P303R



Strategy

g843 cuts in appropriate region and adheres to design criteria (see next slide)
ssDNA repair template (designed asymmetrically see Corn et al, Nature Biotechnology 2016 for details) introduces 2 single base mutations to generate point mutation AND inactivate PstI restriction site, shield mutation to inactivate PAM site and prevent re-cutting of repaired DNA

Guides were designed using Sanger website using stringent criteria for off target predictions (guides with mismatch (MM) of 0, 1 or 2 for elsewhere in the genome were discounted. MM3 were tolerated if predicted off targets were NOT exonic)
http://www.sanger.ac.uk/htgt/wge/crispr/313044620

An Alt-R crRNA (IDT) oligo for this guide (agcttcctgcagaaaactcc) was ordered and resuspended in sterile, Rnase free Injection buffer (TrisHCl 1mM, pH 7.5, EDTA 0.1mM) and annealed with tracrRNA (IDT) by combining 2.5ug crRNA with 5ug tracrRNA and heating to 95oC. The mix was allowed to slowly cool to room temperature. After annealing the complex was mixed with 1000ng Cas9 recombinant protein (labomics; final conc 20ng/ul) and incubated at RT for 15', before adding Cas9 mRNA (final conc; 20ng/ul) and the ssDNA PAGE purified repair template (IDT; final conc 100ng/ul) in a total injection buffer volume of 50ul. The injection mix was centrifuged for 10' and RT and the top 40ul removed to another tube for injection.

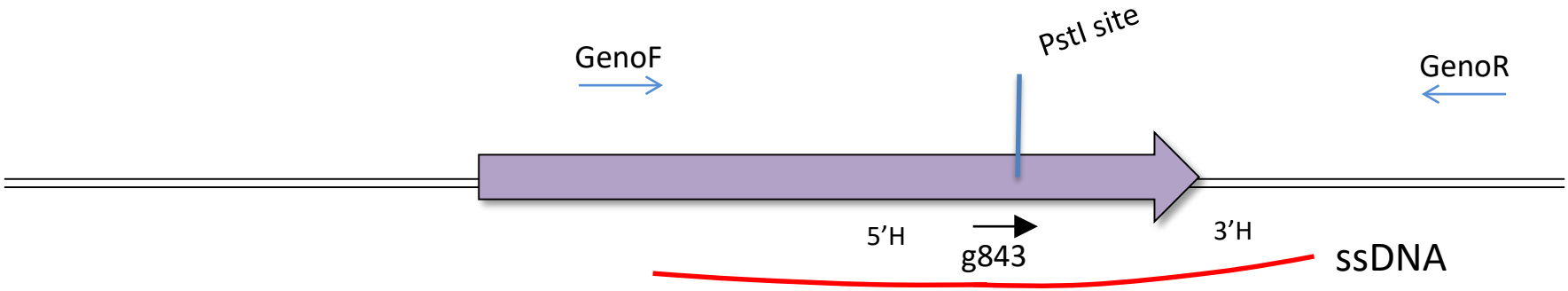
WM05 asymmetric ssDNA repair template (red base is specific base change to generate point mutation, blue base is silent, shield mutation to inactivate PAM site and prevent re-cutting of repaired DNA, and delete PstI restriction site –

tctgcacccaagtcttccttccccaaaggctcaacccaacccctcctccccttctaatcctcagaacattgctctatacca
atggctgcGcagcttcctgcaAaaaactcctccagagtattcaggtaatg

Pronuclear injection

- 2 Days of microinjections (22/23.06.2016) using AltR crRNA:tracrRNA:Cas9 complex (20ng/ul; 20ng/ul; 20ng/ul respectively), Cas9 mRNA (20ng/ul), and ssDNA HDR template (100ng/ul)
- This mix was pronuclear microinjected into one-day single cell mouse embryos. Zygotes were cultured overnight and the resulting 2 cell embryos surgically implanted into the oviduct of day 0.5 post-coitum pseudopregnant mice.
- Pups born c.July 2016
- 16 mice in total
- Genomic DNA extracted using Sigma sigma redextract-n-amp tissue pcr kit, using the RedEx PCR mix supplied for PCR

Genotyping 1



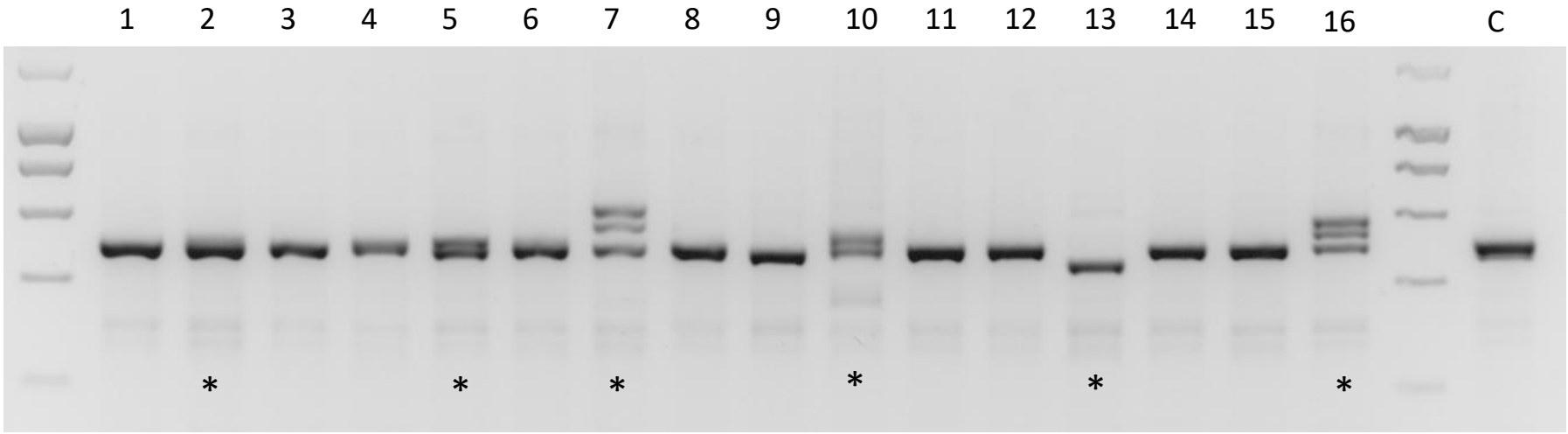
Ear punch DNA were extracted using Sigma sigma redextract-n-amp tissue pcr kit and subjected to PCR using the following primers

GenoF ctcccctccctagttgtggc

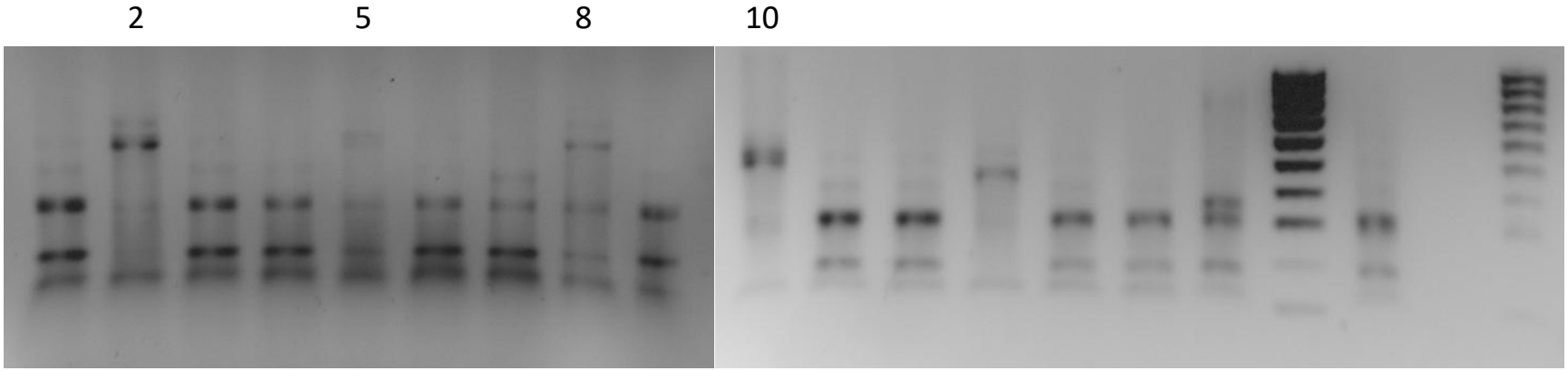
GenoR cagaggccaccacgaactcc

Amplicon = 500bp, if mutation successful the amplicon can no longer be digested by PstI (note; this is only a rough indication, PstI loss could also occur if NHEJ repair happened instead of HDR)

Undigested PCR – note the presence of multiple bands, or altered band sizes. These indicate CRIPSR cutting and NHEJ mediated repair rather than the HDR we are hoping for. I've marked with an asterisk lines with clear InDels but this is likely to be an underestimate due to lack of resolution in the agarose gel electrophoresis.



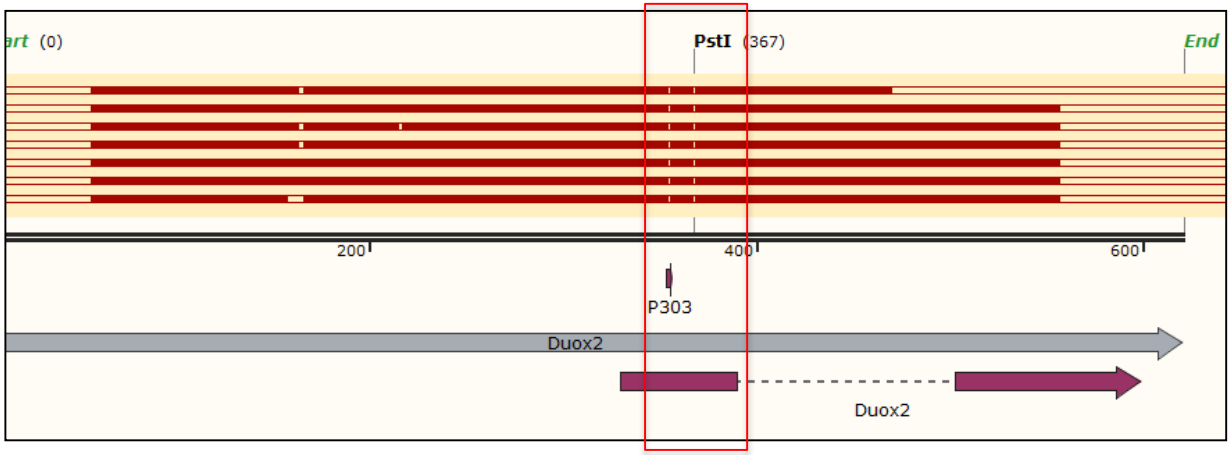
PstI digests – not the prettiest digest but L2, 5, 8 and 10 maintain the large 500bp band. L5 and L8 have probably one digested allele, L2 and L10 have no digested allele



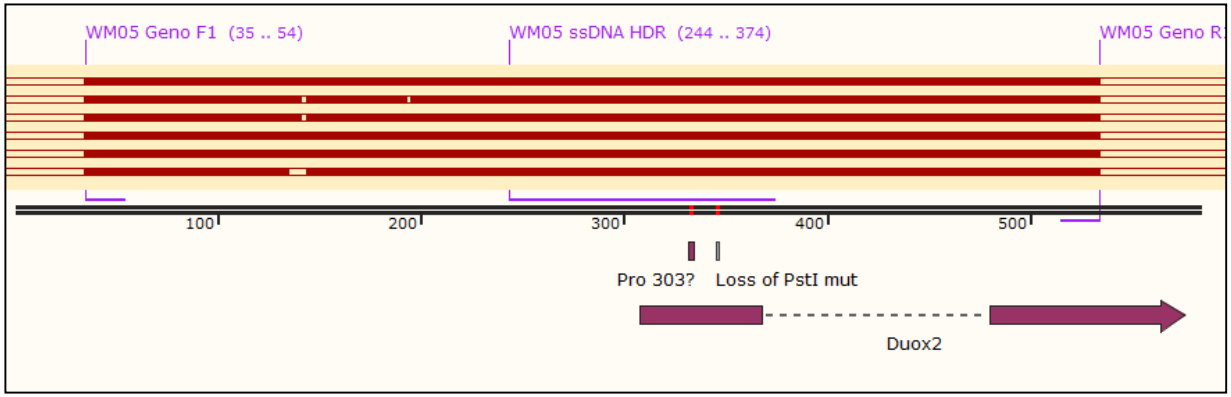
Genotyping 2

- Lines 2, 5, 8 and 10 was re-amplified with high fidelity Phusion polymerase
- Phusion amplified PCR reactions cloned into subsidiary vector (pCR-Blunt)
- 8 colonies per line were miniprepped, Sanger sequenced and aligned with WT genomic sequences

Alignment of sequenced colonies with WT sequence



Alignment of sequenced colonies with predicted mutated sequence



Note; 6 colonies were sequenced,
Filled red bars indicate alignment
Gaps indicate non-alignment
Note the gaps present in WT but predicted, indicating the desired changes have been made

Alignment of sequenced colonies with WT sequence

Alignment of sequenced colonies with predicted mutated sequence

Note the gaps present in WT but predicted, indicating the desired changes have been made

Line	Allele 1	Allele 2	Notes
2	P303R	P303R	Possible homozygote for the point mutation
5			Sequencing failed, amplified random region on chr12
8	P303R	Indel (-1bp/substitution)	Heterozygote for the point mutation, CRISPR targeting on second allele by NHEJ, PstI site maintained
10	P303R	Indel (-7bp)	Heterozygote for the point mutation, CRISPR targeting on second allele by NHEJ, PstI site lost (i.e. gives appearance of homozygote but false positive)

Further Breeding

- These mice were created on a C57BL/6 background. We recommend breeding with WT BL6 and confirming GLT using the genotyping primers previously described. Official nomenclature for the line would be:
- **C57BL/6J.DUOX2^{Em1Uman} (Tg2)**
- We routinely expect very high levels of germline transmission as we inject into the single cell stage, however if the mutation occurs after a few rounds of cell division some mosaicism is possible
- We will confirm GLT of these lines in-house (£500)
- Several independent lines that will essentially be identical, therefore it might be desirable to freeze some lines (service provided by TgU) and maintain a single colony (TBD)
- We recommend that in the initial characterisation of the mouse line to confirm the gene mutation by RT-PCR and cDNA sequencing.
- Perform your experiments!

GLT test

- Gel image

- Animals 22 and 26 from founder Tg? Both sequenced as one allele WT, one P303R (Het)