***JAX SATC Blastocyst assay for CRISPR/Cas9 Guide Testing***

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***Guide RNA annealing and ribonucleoprotein complex formation****.*

TLR2 crRNA was annealed with trRNA following IDT AltR System protocols. Briefly, both components were resuspended at 100 μM in IDT Duplex Buffer, combined in equal amounts, heated to 95°C for 5 mins, and allowed to cool passively to room temperature. Following this annealing step, concentration of annealed guide RNA was assayed by NanoDrop. Crispr:tracr guide RNA hybrid was complexed with AltR Cas9 at 37°C for 15 mins in a thermocycler.

***Guide RNA testing via blastocyst culture****.*

TLR2 gRNAs were individually tested *ex vivo* to assess reporter efficacy. Super-ovulated C57BL6NJ females were mated to reporter male studs (JR32672), and zygotes were harvested and electroporated with AltR-Cas9 (500 ng/μl), guide RNA (600 ng/μl), and ssDNA donor (2000 ng/μl). Following CRISPR/Cas9 electroporation, zygotes were cultured in Sydney Cleavage Medium (COOK Medical) at 37°C under CO2 in a benchtop incubator (COOK Medical). After 96 hours, blastocysts were collected for imaging and DNA extraction. Blastocysts were imaged on a Leica Dmi8 inverted fluorescent microscope at 10X magnification. For DNA extraction, blastocysts were collected in individual PCR tubes with 1.5 μl 25 mM NaOH / 0.2 mM EDTA at 95°C for 15 minutes and neutralized with an equal volume of 40 mM Tris HCl. The genomic region of interest was PCR amplified from 3 μl of DNA template, and product was Sanger Sequenced to assess CRISPR editing outcomes. Sanger sequences were analyzed using the ICE (Inference of CRISPR Editing) deconvolution tool from Synthego. (<https://ice.synthego.com>; Synthego Performance Analysis, ICE Analysis. 2019. v2.0.)

***CRISPR/Cas9 zygote electroporation For Tier2 Validation****.* All mouse procedures were conducted according to relevant national and international guidelines (AALAC and IACUC) and have been approved by the Jackson Laboratory Animal Care and Use Committee. For electroporation,1-cell zygotes from C57BL/6NJ female mice mated to reporter males were harvested and placed in a droplet of 10 μl TE with AltR-Cas9 (500 ng/μl) and guide RNA (600 ng/μl) combined with 10 μl low serum media (Opti-Mem, Sigma-Millipore), and transferred to an electroporation cuvette (Harvard Apparatus) with a 1mm gap electrode. Using a BTX ECM830 Electro Square Porator (Harvard Apparatus), embryos were electroporated with 6 3 ms pulses of 30V separated by 100 ms each. Oviduct transfers into pseudopregnant dams were performed immediately following electroporation.

***Guide Oligo Sequences***

**TLR2-R26-1 gRNA (fwd strand):** ACTCCAGTCTTTCTAGAAGA

**TLR2-R26-2 gRNA (rev strand):** CGCCCATCTTCTAGAAAGAC

**TLR2-R26-3 gRNA (rev strand):** TTGCAGCTCGAACTTCACCT

**TLR2-R26-52 gRNA (rev strand):** GGTAGCGGGCGAAGCACTGC

**TLR2-R26-59 gRNA (rev strand):** GACGTAGCCTTCGGGCATGG

**TLR2-R26-69 gRNA (fwd strand):** CAACTACAAGACCCGCGCCG

**Genotyping Primers:**

TLR-pCAG-GT-F2: gcctctgctaaccatgttca

TLR-pCAG-GT-R2: aactccagcaggaccatgtg

Expected product size: 732 bp (unedited), Ta~60-62C

Methods were adapted from Modzelewski et al. 2018, *Nature Protocols* PMID: [29748649](https://pubmed.ncbi.nlm.nih.gov/29748649)