**Method for Tier 2 Reporter Validation**

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***Guide RNA annealing and ribonucleoprotein (RNP) complex formation****.*

Reporter specific crRNA is annealed with trRNA following IDT AltR System protocols. Briefly, both components are 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 is assayed by NanoDrop. Crispr:tracr guide RNA hybrid is complexed with AltR Cas9 at 37°C for 15 mins in a thermocycler.

***CRISPR/Cas9 zygote electroporation for Tier2 Reporter 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 or C57BL/6J female mice mated to homozygous 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 six 3 ms pulses of 30V separated by 100 ms each. Oviduct transfers into pseudopregnant dams were performed immediately following electroporation. Pregnancies were allowed to progress to term and offspring were genotyped and sequenced (Sanger). Animals with edits predicted to activate the reporter were mated to inbred females and at least 3 edited offspring per sex per reporter subline are assessed. Tissues from each animal are collected, fixed in 4% PFA or NBF for 2-4 hours, cryoprotected in 30% sucrose and embedded in OCT medium and frozen on dry ice. Tissues are cryosectioned and either directly imaged or incubated with primary antibodies specific to the fluorophore (e.g. RFP) and corresponding secondary antibodies before staining with DAPI/Hoechst and imaged at 10x magnification. Images are assessed for reporter activation potential across tissues.

Tag RFP and Katushka2S proteins are detected using rabbit anti-RFP polyclonal antibody (Invitrogen Cat # R10367) and corresponding secondary antibodies such as Cy3-Donkey anti-Rabbit (Jackson ImmunoResearch # 711-165-152).

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