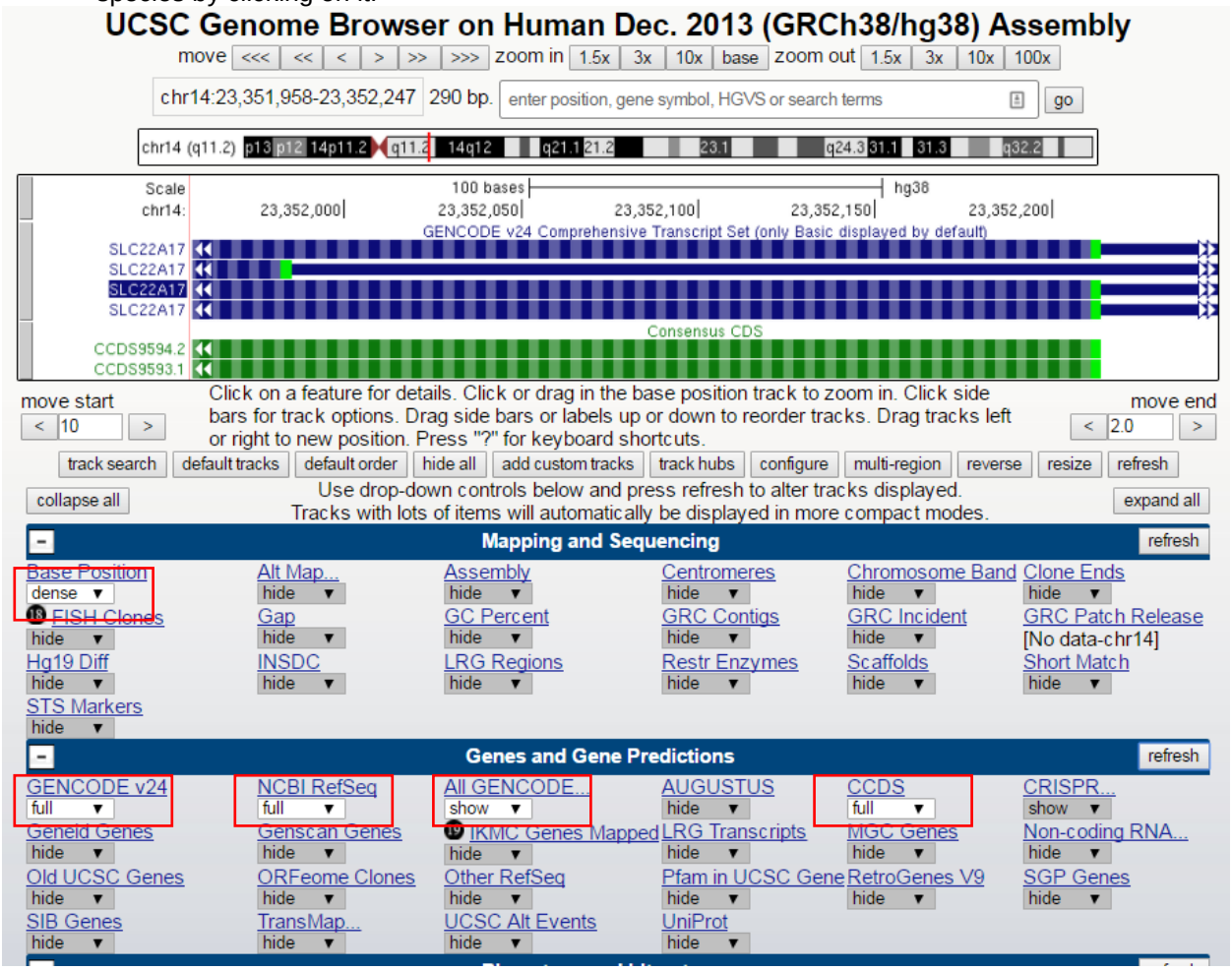
CRISPR Knock Out (KO) Protocol

The following protocol is tailored for generation of a KO for a gene for which you have an antibody.

1. Find genomic sequence of gene for Cas9 targeting
   This step is a prerequisite to designing gRNA that will direct the Cas9 to this first exon contained in all isoforms you want to target to create a double stranded break (DSB). The break is subsequently repaired by Non-homologous End Joining (NHEJ), creating indels resulting in a frameshift that will
2. Open Genome browser gateway: https://genome.ucsc.edu/cgi-bin/hgGateway
   a. Genome Browser Gateway: Enter gene name in 'Position/Search Term' and select desired species by clicking on it.

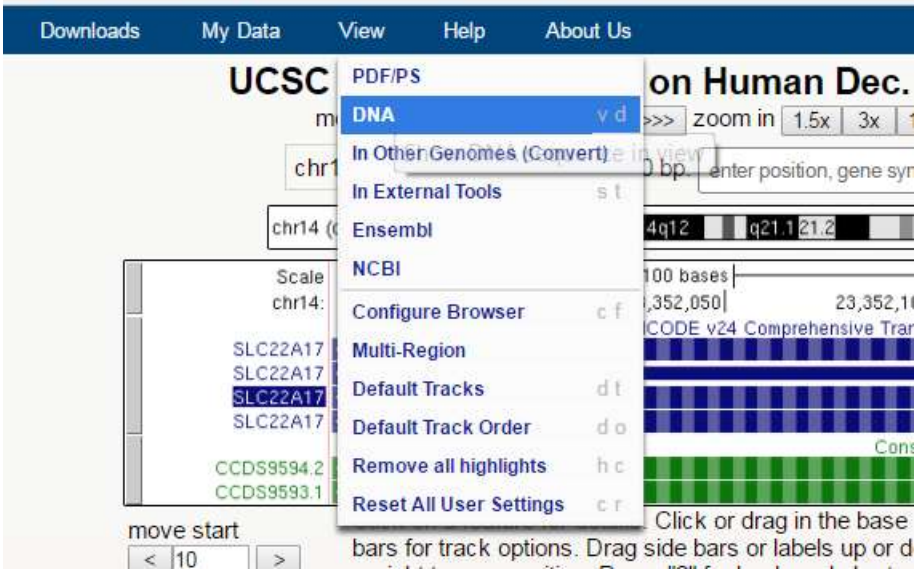


   b. Hide elements that you don't want to view, the ones we've used are highlighted in red.
   c. Find the first exon that is common in all isoforms that you want to target. Websites that can be helpful in finding splice isoforms are:
   http://projects.insilico.us/SpliceMiner/Gene.jsp
   http://www.ensembl.org/index.html
   https://www.ncbi.nlm.nih.gov/refseq/

   Note exons can run left to right or vice versa. Mouse-over exon to get #.

   d. On browser window, highlight (Command +Shift, drag→Mac; Shift + Click, drag→PC) the sequence making up the targeted exon. The view can be moved left or right by clicking and dragging.

e. To get DNA click “view” and the “DNA”, then select “Get DNA” on the next window
f. Create planning document and paste and label sequence



3. Create gRNAs from MIT CRISPR gRNA generation website: http://crispr.mit.edu/
   a. Select “unique genomic region- Up to 250bp”
   b. Upload up to 250bp from the desired exon
      a. Select 3-4 gRNAs that are not overlapping and have the highest score (which is inversely proportional to off target effects). Potential off targets can be pasted into the planning document if desired. The more mismatches present the less likely it will occur and the lower score it will receive. *-NGG at the end of the primer is important. But it should not appear in primer*

2. Add the compliment to then the appropriate 5’ overhangs

   Single locus (20 bp):

   pX459 (pSpCas9(BB)-2A-Puro):

   If your target sequence start with no G put one G at beginning to since it helps transcription:

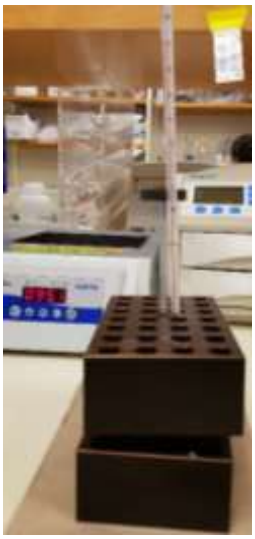
   5’-CACCGNNNNNNNNNNNNNNNNNNN-3’
   3’-CNNNNNNNNNNNNNNNNNNNCAAA-5’

   If your target sequence start with G:

   5’-CACCNNNNNNNNNNNNNNNNNNN-3’
   3’-NNNNNNNNNNNNNNNNNNNCAAA-5’

3. Order 3-4 sets of gRNA primers (std synthesis) in **5’ to 3’** orientation (again NO PAM included)

Cloning Guide Sequence



1. Annealing top and bottom strands:

1 ul	Top oligo (100 uM)
1 ul	Top oligo (100 uM)
1 ul	10X T4 Ligation Buffer (NEB)
7 ul	ddH2O
10 ul	Total

Take out heat blocks from 95C bath

Take blocks out and cool at R.T. till ~50C (one block on top of the other)

2. Ligate double strand oligo to px459 backbone vector:

1 ul	px459V2.0 (200ng/ul)
0.67 ul	Annealing product (1:20 dilution of above with 50mM NaCl)
1 ul	NEB Buffer 2.1
0.5ul	ATP (10mM)
6 ul	ddH2O
0.25ul	T4 DNA ligase (or rapid ligase but not Taq ligase)
0.5ul	BbsI (-80C)
10 ul	Subtotal

- T4 DNA ligase will ligase both sticky (no overhang length restriction) and blunt ends.
- Taq DNA ligase will only ligase sticky ends with ~15bp overhang but heat stable

Put tube in thermocycler: 12X (37C for 5mins, 21C for 5mins), 21C on hold

3. Transformation into DH5-alpha competent cells
   a. Thaw 50 ul competent cells
   b. Add maximum 5 ul (1/10 of total volume to not dilute [salt] too much) DNA and flick to mix,
   c. incubate on ice for 30 min
   d. Heat shock at 42 C for 90 sec
   e. Incubate on ice for 5 min
   f. Add 1ml LB
   g. Incubate at 37 C for 0.5 hr
   h. Centrifuge @ 3k g for 2mins, re-suspend in ~50ul LB
   i. Plate all on the plate with correct antibiotics
   j. Incubate overnight at 37 C

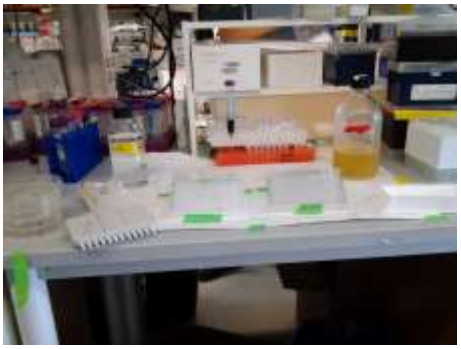
PCR screening

-this is a high-throughput version that is used for SNP KI screening, but can be scaled down for gRNA insertion screening

Purpose: We pick colonies from a bacterial plate, and run a PCR screening to amplify the DNA so we can see which samples (which wells from the 96well plates) are positive for what we are looking for (eg. Has the deletion or specific mutation)

Materials

1-96 well PCR plate (Can use PCR strips)
1-96 well plate (can use TC-treated plates, or PCR strips)
LB with Antibiotic
Bacterial plates
Cell trough
Multichannel pipette
2X Phire Green HSII PCR master mix (Fisher#F126LPM)
Forward primer (For px vectors use: taa aat gga cta tca tat gc) 10uM
Bottom guide oligo (unique for each guide) 10uM

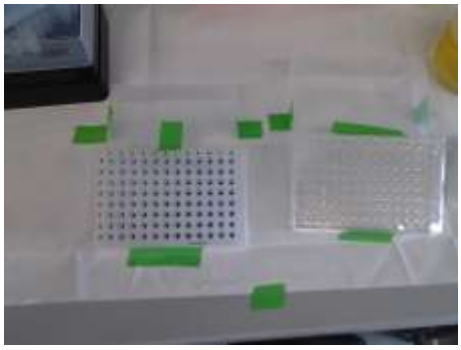


1. Get white paper and pipette tray covers. Tape onto bench.

2. Tape 96 well plates onto bench.

a. Put white plate on left

3. Grab white paper and tip covers. Tape onto bench.
4. Add 9.5ml of dH20 to trough. Then add 500ul DNA loading dye. Mix.
5. Set multichannel pipette to 10ul. Roll pipette onto tips.
6. Load 10ul (of DNA loading dye + water) into white plates (left plate)



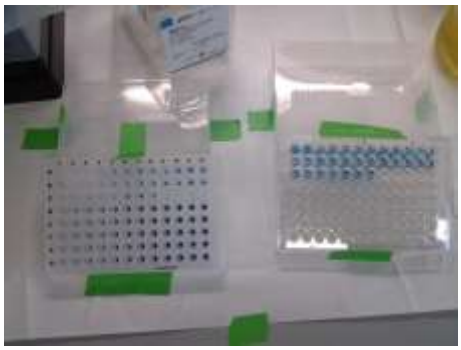
7. Rinse trough with DI water.
8. Pour LB onto trough.
9. Set pipette to 100ul
10. Load 100ul of LB into clear tray.
11. Set P10 pipette to 8ul.
12. Pick a colony (by lightly touching it), swirl, and mix into a well (from the white plates)

a. **try not to pick colonies close to each other

13. Take out 8ul and put it into the clear plates.

a. **write down which wells correspond to which gRNA constructs in your lab notebook

14. Repeat taking 3-6 colonies per guide
15. Take 8ul out of the well after the samples, this will serve as your no-template control



Running the PCR for colony screening

1. Grab ice bucket

2. Defrost PCR master mix *Don't do this on the 95C heat block it will ruin the Hot-start feature

3. Calculate how much PCR master mix you will need (25uL/reaction) and include 15% extra. Calculate how much primer you will need (1uL of 10uM/rxn) to add.

4. Add complete PCR mix to wells containing remaining bacterial sample (~2ul) and dH_2O control.

5. Grab 96 well plate (the white one, left) and aspirate out wells you didn't use

6. Seal plate clear sticker, rolling or scraping to ensure seal. **Pay close attention to the side wells!

Gently vortex (setting 4) to mix, if sample gets on the sticker lid, spin down plate.

7. Set the PCR machine using the following program:

94C for 5mins

98C for 10s
53C for 15s 27 cycles* too many cycles can yield false positives!
72C for 30S

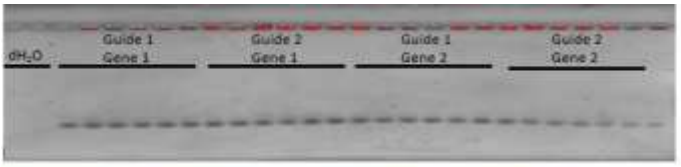
72C for 5mins
4C forever

8. Cast a 1.5%-2%agarose gel while PCR is running

9. Load 10-20ul of finished PCR product on to a gel with a 100BP ladder

10. Image gel (amplicon should be ~100bp) Forward primer (For px vectors use: taa aat gga cta tca tat gc)

11. Colonies that are positive should be recorded and the corresponding wells (containing the rest of the colony in LB) should be grown up in LB + antibiotics for miniprep



12. Send 1-2 colonies for each guide for sequencing using the same FWD primer used in colony screening (For px vectors use: taa aat gga cta tca tat gc)

Preparing cells for Flow Cytometry/ Sorting

You should freeze down the portion of the polyclonal plate that is not going to be sorted in case anything goes wrong

(Start preparing 30 minutes before booked appointment (booking is online))

Preparing 96 well plate (we usually sort 2 plates per guide):

1. Pour DMEM with 20% FBS + P/S in a reservoir for the multi-channel pipette.
2. Using a multichannel pipette, put 200µl of the media into each well of the 96-well plate.
3. Label plates with cell line, guide number and plate number

Preparing cells:

1. Rinse cells with PBS.
2. Trypsinize cells (0.25 ml for 1 well of 6 well plate, 1 ml for 10cm plate) then add FBS containing culture media.
3. Spin down all cells in 15mL falcon, 1.2K-2min.
4. Aspirate media, and resuspend the cells in 1 ml of PBS with 0.2% BSA (filter sterilized with 0.2uM syringe filter) or in Fluorobrite DMEM (in 4C).
5. Put the resuspended cells into a 15 ml Falcon tube or FACS tube. Keep it on ice. (It is best not to leave cells in tube on ice for more than 20 minutes before sorting)

Sorting fluorescent endogenous tagging knock-in: 1 plate per sample

Using mCherry positive knock-in for KI co-selection: 2-3 plates per sample

Sorting KO: 2 plates per guide

Sorting Knock-in: 3-4 plates per guide

~ 2mins per 96well plate ~5mins per polyclonal sorting