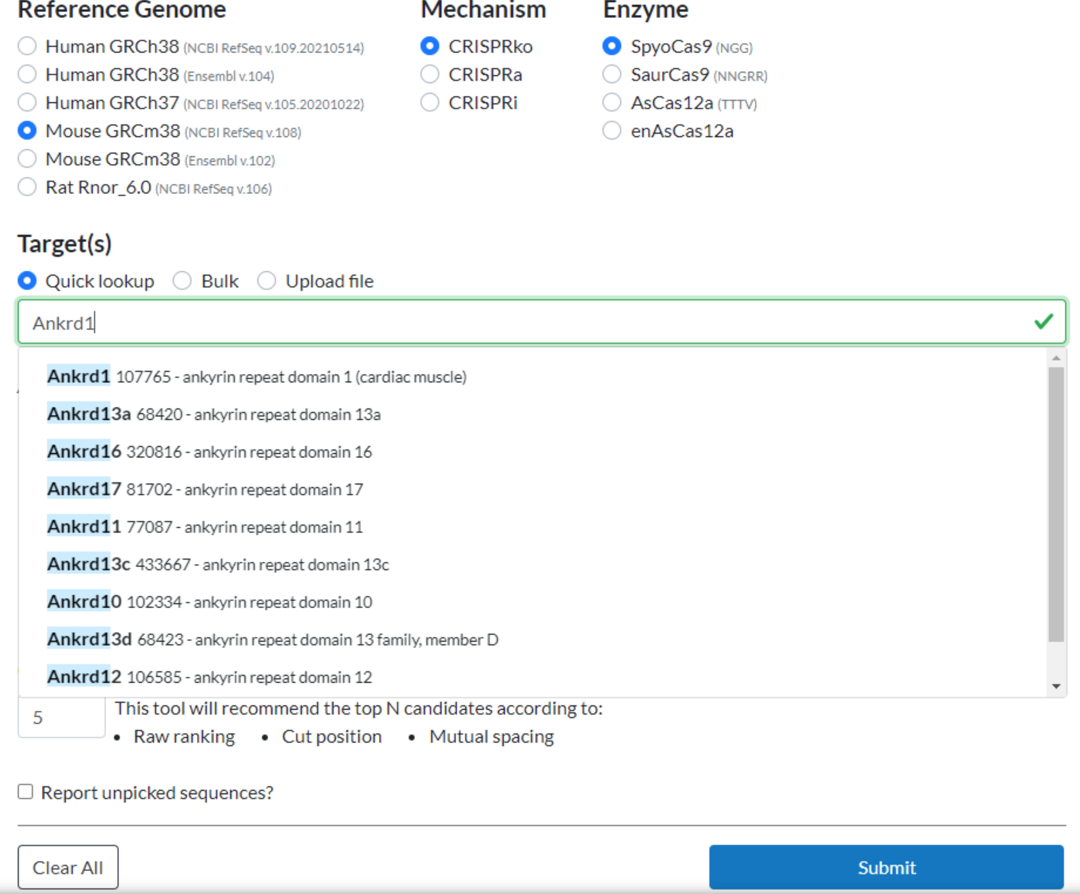
Identify guideRNA (gRNA)

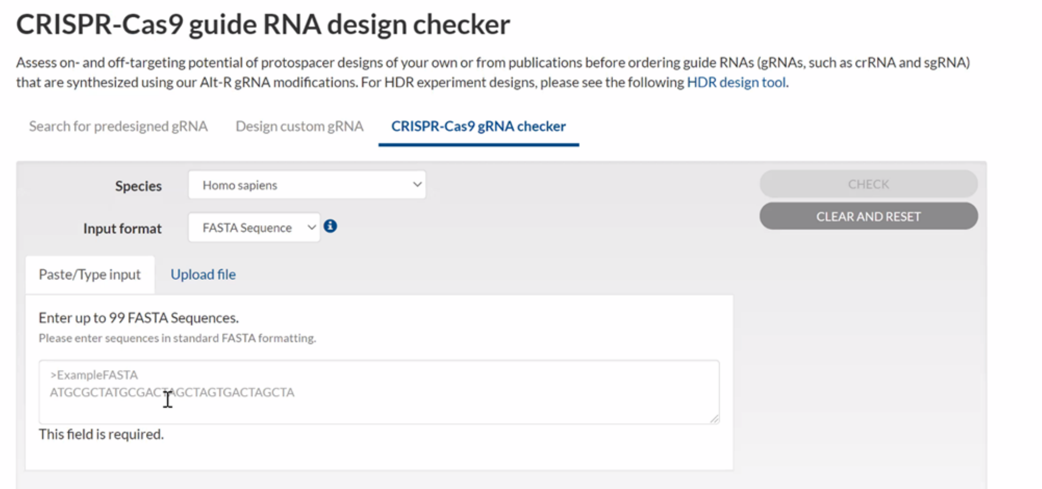
Xue Wang and Shanshan Deng, 2022

Using CRISPick (https://portals.broadinstitute.org/gppx/crispick/public) to select candidate gRNA sequences with great on-target and limited off-target activities. Select the following setting as shown below (e.g. mouse sgANKRD1) and hit submit. We can select top 10 gRNA candidates in stead of 5 shown in the picture.



Once the searching job is complete, download the “Picking Resμlts” file and open with the Excel. The gRNAs will be ranked based on the On-Target Efficacy Score. The gRNA in the sense strand or in the exons near the start position are preferred than those in the antisense strand or exons near the end position. Once we select the candidate gRNAs, we coμld go ahead with the prediction step.

Predict the gRNA efficiency online: Assess the on- and off-targeting scores on the IDT website (https://www.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE). Better to select the gRNA sequences with good scores regarding on-target and off-target effects (If the gRNA sequences have good prediction, the website will show green light to them).



Design the oligo sequence:

Forward oligo: CACCG+ the sequence we get from the above procedure;
(Note: If the first nucleotide is G in the sequence, just add CACC before the sequence)

Reverse oligo: AAAC + reverse complement of the sequence we get + C
(Note: If the first nucleotide is G in the sequence, do not add C after the sequence).

Plasmid construction.

1. Digest and dephosphorylate 3μg of the H138 lentiviral CRISPR plasmid with BsmBI for 1 h at 55 °C (can incubate longer):

3 μg H138
2 μl NEB BsmBI
2 μl NEB buffer
1 μl 20 mM DTT (final concentration: 1 mM)
X μl ddH2O
20 μl total

2. Gel purify digested plasmid using Zymo Gel recovery Kit and elute in elution buffer.

If BsmBI digested, a ~13kb filler piece shoμld be present on the gel. Only gel purify the larger band. Leave the 13kb band.

3. Phosphorylate and anneal each pair of oligos:

1 μl Oligo 1 (100 μM)
1 μl Oligo 2 (100 μM)
1 μl 10X T4 Ligation Buffer (NEB)
6.5 μl ddH2O
0.5 μl T4 PNK (NEB M0201S)
10 μl total
Please use the T4 Ligation Buffer since the buffer supplied with the T4 PNK enzyme does not include ATP (or supplement to 1mM ATP).

4. Put the phosphorylation/annealing reaction in a thermocycler using the following parameters:

37°C 30 min
95°C 5 min and then ramp down to 25°C at 5°C /min

5. Dilute annealed oligos from Step 3 at a 1:200 dilution into sterile water or elution buffer.

6. Set up ligation reaction and incubate at room temperature for more than 3 h or overnight if the enzymes expired:

X µl BsmBI digested plasmid from Step 2 (50ng)
1 µl diluted oligo duplex from Step 4
5 µl 2X Quick Ligase Buffer (NEB)
X µl ddH2O
10 µl subtotal
1 µl Quick Ligase
11 µl subtotal

Or

X µl BsmBI digested plasmid from Step 2 (50ng)
2 µl diluted oligo duplex from Step 4
2 µl 10X DNA ligase buffer
1 µl T4 ligase
X µl ddH2O
20 µl subtotal

7. Transformation and pick the colonies, then sequencing.

Validate the gRNA efficiency *in vitro*:

1. **Plasmid construction.** Insert the gRNA into the H138 lentivirus plasmid.
2. **Virus production.**
   Day 1: Transfection. Transfect the gRNA-lentivirus plasmid together with virus packaging plasmids into HEK293T cells.
   Day 2: Harvest of the first batch of virus. Replace the transfection medium with fresh medium in the morning, and collect the first batch of cell supernatant 8 hours later.
   Day 3: Harvest of the second and third batches of virus. Collect the cell supernatant in the early morning and late afternoon, respectively. Filter the lentiviral supernatant using a 45 µm pore filter to remove any remaining cellµlar debris. Use it immediately or aliquot virus to store at -80°C.
3. **Virus infection.**
   Day 1: Cell preparation. Seed the murine cells in 6-well plate, and cµlture in incubator overnight.
   Day 2: Infection. Combine 8 mg/mL polybrene reagent with virus medium at 1:1000. Infect the cells with virus medium containing polybrene in the early morning and late afternoon, respectively.
   Day 3: Repeat Infection. Infect the cells with virus medium containing polybrene in the early morning and then change the medium with cell cµlture medium in the late afternoon to let the cells recover overnight.
   Day 4: Puromycin selection. Replace with selection medium in the morning.

Change and replace the selection medium every 2 days for a week.

4. **Validation of editing efficiency.** After one week selection, validate the knockout efficiency by western blot.

If the knockout efficiency is high, then the gRNA will be inserted into px330 plasmid for *in vivo* experiments. The px330 plasmid together with oncogenes will be injected into mice to establish HCC model. The knockout efficiency coμld be checked in tumor nodμles with the following method.

Validate the gRNA efficiency *in vivo*:

1. Isolate the tumor nodμles, snap frozen in liquid nitrogen and then store the samples at –80°C.
2. Extract the genome DNA with DNeasy Blood & Tissue Kit (Qiagen).
3. Design a pair of PCR primers to amplify the target sequence. The amplicon size is between 300~500 bp, and the gRNA targeting site shoμld be at the center of the amplicon.
4. Gel purify the amplicon and sanger sequence the purified PCR product by using either forward or reverse primer.
5. Analyze the sequencing resμlts with TIDE. The gRNA total efficiency shoμld be higher than 30%.