

Identify guideRNA (gRNA)

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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.

Reference Genome	Mechanism	Enzyme
<input type="radio"/> Human GRCh38 (NCBI RefSeq v.109.20210514)	<input checked="" type="radio"/> CRISPRko	<input checked="" type="radio"/> SpyoCas9 (NGG)
<input type="radio"/> Human GRCh38 (Ensembl v.104)	<input type="radio"/> CRISPRa	<input type="radio"/> SaurCas9 (NNGRR)
<input type="radio"/> Human GRCh37 (NCBI RefSeq v.105.20201022)	<input type="radio"/> CRISPRi	<input type="radio"/> AsCas12a (TTTV)
<input checked="" type="radio"/> Mouse GRCm38 (NCBI RefSeq v.108)		<input type="radio"/> enAsCas12a
<input type="radio"/> Mouse GRCm38 (Ensembl v.102)		
<input type="radio"/> Rat Rnor_6.0 (NCBI RefSeq v.106)		

Target(s)
 Quick lookup Bulk Upload file

Ankrd1 ✓

Ankrd1 107765 - ankyrin repeat domain 1 (cardiac muscle)

Ankrd13a 68420 - ankyrin repeat domain 13a

Ankrd16 320816 - ankyrin repeat domain 16

Ankrd17 81702 - ankyrin repeat domain 17

Ankrd11 77087 - ankyrin repeat domain 11

Ankrd13c 433667 - ankyrin repeat domain 13c

Ankrd10 102334 - ankyrin repeat domain 10

Ankrd13d 68423 - ankyrin repeat domain 13 family, member D

Ankrd12 106585 - ankyrin repeat domain 12

5 This tool will recommend the top N candidates according to:
• Raw ranking • Cut position • Mutual spacing

Report unpicked sequences?

Once the searching job is complete, download the “Picking Results” 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 could 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) (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).

CRISPR-Cas9 guide RNA design checker

Assess on- and off-targeting potential of protospacer designs of your own or from publications before ordering guide RNAs (gRNAs, such as crRNA and sgRNA) that are synthesized using our Alt-R gRNA modifications. For HDR experiment designs, please see the following HDR design tool.

Search for predesigned gRNA Design custom gRNA **CRISPR-Cas9 gRNA checker**

Species: Homo sapiens

Input format: FASTA Sequence

Paste/Type input Upload file

Enter up to 99 FASTA Sequences.
Please enter sequences in standard FASTA formatting.

```
>ExampleFASTA
ATGCGCTATGCCGACTGCTAGTAGCTAGCTA
```

This field is required.

CHECK

CLEAR AND RESET

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 should 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 cellular 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 culture 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 culture 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 could be checked in tumor nodules with the following method.

Validate the gRNA efficiency *in vivo*:

1. Isolate the tumor nodules, 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 should 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 results with TIDE. The gRNA total efficiency should be higher than 30%.

RAPID AND EASY QUANTITATIVE ASSESSMENT OF GENOME EDITING




TIDE

- For non-templated Cas9 editing
- Input: 2 Sanger sequence traces
- Output: Quantitative spectrum of indels around the cut site

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TIDER

- For template-directed Cas9 editing
- Input: 3 Sanger sequence traces
- Output: Quantification of templated mutations plus the spectrum of non-templated indels

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