IGI Protocol prepared by Chong Park

Application of Cas9/sgRNA RNP complex for Genome-Editing

A. sgRNA template synthesis by PCR

Goal is to make in vitro transcription (IVT) template by one PCR reaction. Mix one gene-specific 60mer and three common primers in one reaction. This is modified from the Doudna lab eLIFE paper (PMID 25497837). Gene-specific primer and BS6+10 have 20 nt overlap to extend.

sgRNA template sequence:
TAATACGACTCACTATAGGNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGGAAACAGCATAGC
AAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT (red-T7 promoter sequence & blue-N20 gRNA sequence) (in my experience, no difference up to 4G, GGGG)

Gene-specific primer (60mer): insert 20bp sgRNA seq for N20.
TAATACGACTCACTATAGGNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGGAAA
T25 (18mer): TAATACGACTCACTATAG
BS6+10 (90mer):
AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAAACTTGCTATGCTG
TTTCCAGCATAGCTCTTA
BS7 (22mer): AAAAAAAGCACCGACTCGGTGC

Set up 50ul PCR reaction. Make master mix without gene-specific primer and add 2ul of gene-specific primer into 48 ul of master mix.

	stock	final	volume in 50ul
gene-specific primer	500nM	20nM	2
B6+10	500nM	20nM	2
T25	10uM	1uM	5
BS7	10uM	1uM	5
dNTP mix	10mM	200uM	1
Phusion enz (NEB)	2U/ul	1U/50ul	0.5
HF buffer	5X	1X	10
water			24.5

Do 30 cycles of PCR and run 5ul on 2% agarose gel.

95˚C	0:30	
95˚C	0:10	⎫
57˚C	0:10	⎬ 30x
72˚C	0:10	⎭
72˚C	5min	
12˚C	∞	

If PCR looks good on gel, do PCR purification using any spin column kits (Spin columns from Zymo, MN, and Quiagen all work fine). Elute in ~30ul volume (the concentration will be about 40~50 ng/ul, total of ~1.2ug). From this spin column steps, you need all reagents designated for RNA work only. If you use a 96-well format, using SPRIselect (Beckman Coulter) might be easier. This protocol is shown in sgRNA purification step.

Some successfully used PCR product without any purification for in vitro sgRNA synthesis. You might be able to skip the purification step.

B. sgRNA synthesis and purification

We used a homemade T7 polymerase. The protocol to make T7 RNA polymerase can be easily found online. You can buy T7 polymerase from NEB or any vendors.

Set up 50ul reaction with a T7 polymerase.

	stock	final	volume in 50ul
Template	30~40ng/ul	500ng (~100nM)	In 15ul
NTP mix	25mM each	5mM	10
T7 10X buffer	10X	1X	5
T7 enzyme			0.5
water			19.5

T7 10X buffer: 400 mM Tris pH 7.9, 200 mM $MgCl_2$, 50 mM DTT, and 20 mM spermidine (Sigma 85558))

Incubate at 37°C for 2~4 hours.

Add 2.5ul of Turbo DNaseI (Life Technologies AM2238 or equivalent) and incubate at 37°C for 15 minutes.

Purify sgRNAs by either spin column (Megaclear or Zymo) or a simple ethanol ppt or SPRIselect. All methods worked well. Zymo column is preferred over Megaclear, since elution volume is smaller (15ul vs 50ul). From 50ul IVT, we get about 100~150 ug of sgRNA by column purification. Ethanol ppt gives better yield (~2 fold more). SPRIbead gives similar yield to Zymo column. You need highly concentrated sgRNAs to make a RNP complex so pay attention to final elution volume.

Zymo RNA Clean & Concentrator-5 columns (#R1016) purification

- Follow manual and then elute in pre-warmed 15ul 10mM Tris solution, twice. I usually get 3~5ug/ul concentration.
- Take 1ul and dilute it 1:10 to measure concentration. Then run 200ng on a 10% Urea-TBE gel to make sure the quality of sgRNAs. Mix sgRNAs with 2X RNA loading dye (95% fomamide, 0.025% xylene cyanol, 0.025% bromphenol blue, 18mM EDTA, and 0.025% SDS). Denature RNAs by boiling at 95~100 degree for 3min before loading to gel

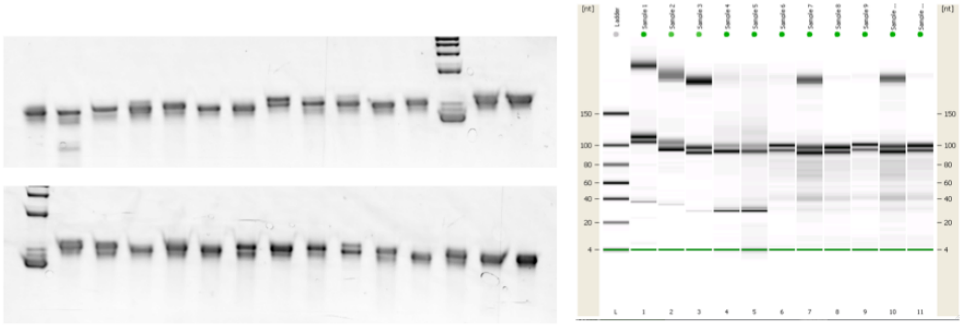
Ethanol ppt purification

- Bring up the volume to 100ul by adding 40ul water and 10ul 5M ammonium acetate solution and mix well. Then add 200ul of cold ethanol and mix. Store it on ice about one hour and spin down. Spin down at max speed and rinse with 70% ethanol once. Resuspend in 30ul 10mM Tris solution. The yield is about 8ug/ul in my experience.
- Take 1ul and dilute it 1:10 to measure concentration. Then run 200ng on a 10% Urea-TBE gel to make sure the quality of sgRNAs.

SPRI Bead (96 well plate format)
If you want to make your own SPRI beads, please request a protocol.
1) IVT sgRNA 10 uL or 20 uL (Input volume)
2) Bring volume to 200 uL with 100% EtOH (this helps binding of small fragments)
3) Add 5X SPRI
    5*10 (IVT sgRNA)= 50 uL of SPRI Beads
    5*20 (IVT sgRNA)= 100 uL SPRI Beads
4) Pipette mix 10X.
5) Incubate 5m at RT

6) Place on magnetic stand, 5 min
7) Remove supernatant, and discard
8) Add 200 uL, 80% EtOH. Wait 2 min. Remove EtOH. Repeat.
9) Air dry 5-10 min (pellet will change from a glossy/wet to matte/dry.
10) Elute 20 uL of water or Tris solution. Pipette mix 10X.
11) Incubate 2min at RT
12) Place on magnetic stand, 5 min
13) Keep Supernatant. Transfer to a new plate.

Shown below is the image sgRNAs on either 10% TBE/Urea gel (left) or bioanalyzer (right). Some of them on bioanalyzer have upper band, which could be due to non-denaturing condition of gel matrix).



C. Cas9 RNP complex electroporation

Cas9 protein: we used cas9 protein from UC Berkeley Macrolab and the Joe DeRisi lab here at UCSF. Both works great! If you want to make your own cas9 protein, please request a protocol.

We use Amaxa Nucleofactor 96-well shuttle system from Lonza. You need to figure out which kit to use depending on your cell line from its website (http://bio.lonza.com/resources/product-instructions/protocols/) and follow the instruction for electroporation step.

Cas9/sgRNA RNP complex should be prepared immediately before electroporation by incubating cas9 protein and sgRNAs at 1:1.3 molar ratio with cas9 buffer (20 mM Tris (pH 7.5), 150 mM KCl, 1 mM MgCl2, 10% glycerol and 1 mM DTT) at 37°C for 10 min. For homology-deriven repair (HDR), add HDR templates into the RNP complex. Keep this on ice until cells are ready for electroporation. 30pmol to 100pmol range of cas9 worked well for NHEJ and HDR in general.

For example, Nucleofection of HEK293T cells can be performed using SF cell- kits and program CM130 in an Amaxa 96-well Shuttle system. Each Nucleofection reaction consisted of approximately 2 X 10^5 cells in 20 μl of Nucleofection reagent and mixed with 10 μl of Ca9/sgRNA/donor complex. After electroporation, plate them onto a 24 well plate. It takes ~2 days to become confluent. Depending on your plan, you can harvest some cells to test editng efficiency and/or expand & freeze the rest for future functional analysis.

You can find more details regarding RNP electroporation in accompanying protocol by Manuel Leonetti.

D. Checking the efficiency of genome editing

Make genomic DNAs from harvested cells. Confluent 24 well yields in general 20~30ug of genomic DNA. If you are just checking efficiency of sgRNAs, you can make genomic DNAs from cells on a 96-well (you can easily get 10ug yield). You need only 200ng to set up 50ul PCR reaction to amplify target areas.

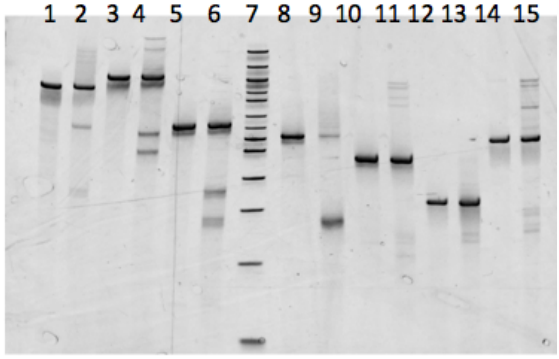
Optimize PCR for target area, ahead of time. Design 2 forward and 2 reverse primers by using Primer 3 program surrounding a target site. The product size ranging from 300-600bp is ideal for T7E1 assay. Eliminate primers that bind onto other genomic areas by using Blat search.

Do PCR optimization with temperatures gradient option for annealing temperature (set up a 50ul reaction and aliquot it to 15ul each to 3 PCR tubes. Pick three annealing temps, ranging for 58-61-64 degree). For genomic DNA PCR, I generally include DMSO and use GC buffer. Do 30-35 cycles of PCR and run a gel to identify the best primer pair and the condition.

	stock	final	volume in 50ul
Forward primer	10uM	0.5uM	2.5
Reverse primer	10uM	0.5uM	2.5
genomic DNA		200ng	X
dNTPs, 10mM	10mM	200uM	1
GC buffer	5X	1X	10
DMSO	100%	3%	1.5
phusion enz (NEB)	2U/ul	1U	0.5
water			50-(X+18)

T7E1 assay for NHEJ

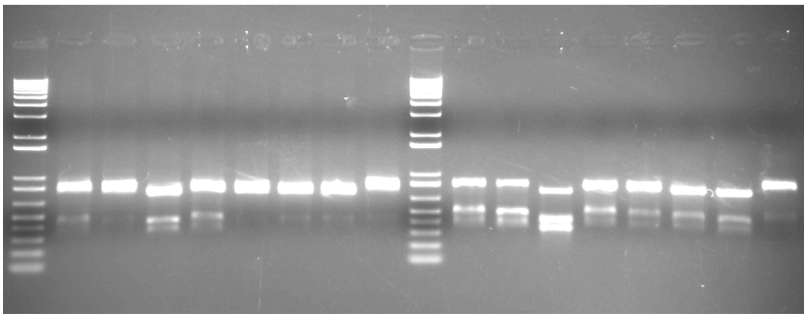
1. Run 50ul of PCR reaction with 200ng of genomic DNA from edited and control cells to amplify target site.
2. Run 5ul of PCR product to verify correct amplification.
3. Purify the rest with any spin column and nanodrop them.
4. Resuspend 200ng in 1X NEB buffer2 in 10 or 20ul final volume.
5. Make a duplex by denaturing and reannealing in a thermal cycler (95, 10min; 95-85 at -2/s. 85-25 at -0.1/s; hold at 4 °C).
6. Add 1ul of T7 Endonuclease (NEB) and incubate it at 37 degree for 1 hour.
7. Add 6X dye to stop the reaction and load it onto either 5% TBE acrylamide gel or 2% agarose gels depending on sizes you expect.
8. Stain gels and do imaging. Examples are shown below on 5% TBE-acrylamide gel.



1. CEL –
2. CEL +
3. C4BPB –
4. C4BPB +
5. EMX1 -
6. EMX1 +
7. Marker
8. SOX2 –
9. SOX2 +
10. BAG3 –
11. BAG3 +
12. KCNH2 –
13. KCNH2 +
14. MYBPC3 –
15. MYBPC3 +

Analysis for Homology-deriven Repair (HDR)

If insertion sequence is short and it contains unique enzyme sites, you can use same primers to amplify up knockin area. After purification, digest 200ng of PCR product to check the insertion (as shown below).



HindIII assay (measures HR) T7E1 assay (measures NHEJ)

If insertion sequence is long, it might be easier to design junction PCRs (one primer binding on outside of homology arm and the other on inserted sequence). In this case, you need to check both junctions to make sure correct insertion.