CRISPR/RNP based knock in and magnetofection

Introduction

Cas9/sgRNA RNP comlex assembly and its deliverywith ssDNA based donor to cells by magnetofection

Materials

- Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT cat. n. 1081061)
- guideRNA (gene specific Alt-R CRISPR crRNA + Alt-R CRISPR tracrRNA)
- ssDNA donor
- Opti-MEM Media
- (Nocodazol); (RS1) to increase HDR efficiency
- Nuclease-Free Water, Nuclease-Free IDTE Buffer or Nuclease-Free Duplex Buffer.
- CRISPRMAX lipofectamine or other (cell line dependent) Improved delivery of Cas9 protein/gRNA complexes using lipofectamine CRISPRMAX
- magnetic nanoparticles Combimag (OZ Biosciences)
- magnetic plate (home-made from neodymium magnets or commertial OZ Biosciences)

Procedure

Prepare RNA oligos

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) in Nuclease-Free IDTE Buffer or Nuclease-Free Duplex Buffer. We suggest resuspending the RNA oligos to 100 µM stock concentrations

   100 uM stock: Normalized amount delivered 2 (nmol) + 20 ul of resuspension buffer (5 nmol + 50 ul atd.) and prepare 1 µM working concentrations from those stock resuspensions.
   Note: Store resuspended RNA oligos at –20°C.
   Resuspension Calculator
   ssDNA HDR template purchased as ultramer dilute to 0.3 uM stock

2. Mix the crRNA and tracrRNA in equimolar concentrations in a sterile microcentrifuge tube. For example, create a final duplex concentration of 1 µM:

Table1

	A	B	C
1	**Component**	**µl**	**Final**
2	100 µM Alt-R CRISPR-Cas9 crRNA	1	1 uM
3	100 µM Alt-R CRISPR-Cas9 tracrRNA	1	1 uM
4	Nuclease-Free Duplex Buffer:	98	
5	Total	100	

Alternatively: keep working stocks of crRNAs and tracrRNA at 10 µM concentration in TE (10 mM Tris, pH 7.5, 0.1 mM EDTA), in which case mix 1 µL of crRNA and 1 µL of tracrRNA with 8 µL of Duplex Buffer.

3. Heat at 95º C for 5 min.

4. Remove from heat and allow to cool to room temperature (20–25ºC) on your bench top.

5. If needed, dilute the complexed RNA or sgRNA to a working concentration (for example, 1 µM) in Nuclease-Free Duplex Buffer or IDTE Buffer.

   Note: crRNA:tracrRNA duplexes are stable for at least 6 months with no loss in activity when stored at –20°C at a concentration of ≥1 µM.

Prepare cells day before

6. 1 day prior to magnetofection, seed adherent cells in 96-well plates at 10,000–30,000 cells per well.

   CRITICAL Note: Ideally, cells should be confluent (but not overgrown) on the day of magnetofection

   If needed after cell attachment, cells can be treated with 200 ng/ml of nocodazol for 15 h before transfection to increase HDR efficiency, but it can harm magnetofection efficiency. We also did not observe any increase in HDR efficiency of A2780 if standard lipofection was used.

Form the RNP complex

7. Dilute Alt-R S.p. Cas9 enzyme to a working concentration (for example, 1 µM) in OptiMEM media

   Note: The molecular weight of Alt-R S.p. Cas9 enzymes is 162,200 g/mol. All Alt-R S.p. Cas9 enzymes are provided at a stock concentration of 62 µM (10 mg/ml).

8. To produce the RNP for each well in the 96-well plate, combine the following:

Table2

	A	B
1	**Component**	**CRISPRMAX reagent Volume per well (µL)**
2	Guide RNA oligos [1 µM]	1.5
3	Diluted Cas9 enzyme [1 µM]	1.5
4	Cas9 PLUS™ Reagent (from CRISPRMAX kit)	0.6
5	Opti-MEM Media (or Cas9 buffer)	19.9
6	TOTAL	23.5

CRITICAL This guide describes optimized conditions for lipofection based magnetofection of Alt-R CRISPR-Cas9 RNP into A2780, but seems to work with other cell lines (10 nM RNP). The protocol can be used for other adherent, immortalized, eukaryotic cell lines, but may require further optimization. Here are presented conditions that demonstrate maximal editing efficiency and minimal cell toxicity. Thanks to magnetofection, the level of transfection reagent can be dropped dramatically. For difficult-to-transfect cells or non-dividing cells, another delivery method, such as electroporation, may be required.

For optimization 96-well plate transfections, vary the ranges of the following components:
CRISPR-Cas9 RNP complex: 3, 10, 30 nM
CRISPRMAX or other reagent: 0.2-2.0 (0.5) uL

9. Incubate at room temperature for 5 min to assemble the RNP complexes.

   Note: The RNP complex can be stored for up to 4 weeks at 4°C or for up to 6 months at –80°C.

10. For co-delivery of donor DNA, add 1.5 ul ssoDNA ultramer oligonucleotide (0.3 uM konc) or 70 ng of single strand DNA oligonucleotide prepared by IVT method or 10-15 ng prepared by PCR with biotinylated primer

    CRITICAL Note: Dilute your ssDNA in TE. It may need further optimization of the amount of ssDNA. It is critical step as ssDNA excess can cause unwanted non-specific integrations usually accompanied with increase of cell death

11. Proceed with magnetofection

Magnetofection of the RNP complex with ssDNA

This guide describes optimized conditions for magnetofecion/lipofection of Alt-R CRISPR-Cas9 RNP into A2780 cells using forward transfection protocol. We found that although a reverse transfection protocols work usually better than forward, combination with magnets made forward transfection surperior. Furthermore is simplify the process as cells does not have to be washed from trypsin which can contain RNAse residuals which can degrade the guide RNAs

12. Aspirate media from cells and add 150 µl of fresh media (RPMI) with 10 % serum as needed / well of 96-well

    This media can be supplemented with RS1 drug (final 20 µM) as it can increase HDR efficiency when short (~100 b) homologous arms are used withind ssDNA donor

13. For each well of a 96-well plate, combine the following, and incubate at room temperature (20–25°C) for 20 (10-15) min to form transfection complexes:

Table3

	A	B
1	**Component**	**Amount µL**
2	RNP+ssDNA	25
3	CRISPRMAX transfection reagent	0.5
4	Opti-MEM Media	24.3
5	Combimag	0.2
6	TOTAL	50

Ideally, prepare 3 tubes (RNP+ssDNA, CRISPRMAX in Opti-MEM, Combimag) and mix them quickly together (Combimag at last). For highest efficiency, all should be mixed and ready withing 3 minutes upon CRISPRMAX addition

14. Incubate the mix for 20 min

    CRITICAL Note: Proceed to step 15 within 30 minutes

15. When incubation is complete, add 50 µL of transfection complexes (from step 13) to the wells of a 96-well tissue culture plate (have 150 µl media) = total 200 µl

16. Immediately, transfer 96-well plate on magnetic plate and incubate for 20 min (Incubator, 37 °C,5% CO2)

17. Remove magnetic plate and leave them for 24 h (Incubator, 37 °C,5% CO2)

    Note: Dox can be supplemented even at this stage

18. 24h after exchange media to remove RNP complexes, let them 4h to recover and proceed as needed

    Note: Knock in outcome can be visible 24 h after (or later…72h) magnetofection depending on the kinetics of modified gene

19. Within 24-94 h, check the localization by fluorescent microscopy and select wells with best results

    Note: Ideally, it is the best to transfect independently many wells with different conditions

20. Cultivate cells as long as needed for FACS sorting