Description (UNVALIDATED):

Standard protocol for nucleofecting.

Materials:

1. sgRNA (100 uM)
2. Cas9 protein (50 uM)
3. ssODN (100 uM) – if applicable
4. donor plasmid – if applicable
5. pMax GFP or SNP61 TdTomato plasmid (for sort selection)

Methods:

RNP Complexing Guide

RNP Complexing has been optimized at a 3:1 ratio of sgRNA to Cas9, respectively. uM corresponds to pmol/uL. Thus, **100 uM = 100 pmol/uL**.

Example: For this experiment we are going to use 150 uM of our sgRNA to make a KO cell line. We would use 1.5 uL of our sgRNA that has been re-suspended at 100 uM = 150 pmol. We then divide the total pmol of our sgRNA by 3 for our 3:1 ratio. 150 pmol/3 = 50 pmol. Our 3X NLS Cas9 protein concentration is 50 uM = 50 pmol/uL. We then divide what we need by what we have, $\frac{50\ \text{pmol}}{50\ \text{pmol/uL}} = 1\ \text{uL}$ Cas9 protein.

Complexing Ratio: $\frac{1.5\text{uL sgRNA (100 uM stock)}}{1\ \text{uL Cas9 (50 uM stock)}}$

Can also use 1 uL of sgRNA = 100 pmol. 100 pmol/3 = 33.3 pmol. Our Cas9 is at 50 uM, so dividing what we need by what we have $\frac{33.3\ \text{pmol}}{50\ \text{pmol/uL}} = 0.67\ \text{uL}$ Cas9 protein

Complexing Ratio: $\frac{1\ \text{uL sgRNA (100 uM stock)}}{0.67\ \text{uL Cas9 (50 uM stock)}}$

General Complexing Procedure

1. **Collect Reagents**
    a. Briefly vortex RNA and spin down to collect solution at bottom of the tube.
    b. Flick Cas9 aliquots and spin down to mix and collect protein at bottom of the tube.

2. **Complex RNP**
    a. Add gRNA with Cas9 protein at volumes listed below**.**

        ***Note 1: Use a master mix whenever possible to ensure all nucleofections with the same reagents are complexed the same. This also reduces pipetting error. For example: using 1 gRNA for several nucleofections for a cell line evaluation. Complex 1.5x total amount of sgRNA and Cas9 and aliquot for respective nucleofections.**

        ***Note 2: Multiple sgRNAs can be complexed in the same tube. Make sure to calculate Cas9 needed based on total amount of sgRNA being complexed.**

    b. Once all of reagents are added, mix RNA/protein solution by flicking tube and spinning down using a microfuge or microcentrifuge. Let the mixture incubate at room temperature for 10 min. After 10 min, RNP complexes should be formed.
    c. Transfer RNP complexes to ice or keep at 4°C until ready for nucleofection.

3. **Nucleofection**
    a. Nucleofect with appropriate Lonza 4D nucleofector program. Several cell line protocols can be found on their website.

Quick Transfection Examples by Project

*Transfection Protocol optimized for Lonza 4D Nucleofector with the Small 16 well cuvette.

1. 1 sgRNA Transfection (KO)
2. 2 sgRNA Transfection (Deletion/Translocation)
3. 1 sgRNA Transfection with Donors (KI)

4. 2 sgRNA Transfection with Donor (Deletion/Translocation)

1. 1 sgRNA Transfection (e.g. KO)

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
sgRNA (100 uM)	1.5
Cas9 protein (50 uM)	1
Total	2.7

Can be scaled down to 1 uL sgRNA if you're doing a cell line evaluation or don't have much RNA.

2. 2 sgRNA Transfection (e.g. Deletion/Translocation)

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
sgRNA1 (100 uM)	0.75
sgRNA2 (100 uM)	0.75
Cas9 protein (50 uM)	1
Total	2.7

3. 1 sgRNA Transfection with Donors (KI)

a. ssODN Donor (e.g. Point Mutation)

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
ssODN (100 uM)	1
sgRNA1 (100 uM)	1
Cas9 protein (50 uM)	0.67
Total	2.87

*Amounts can be adjusted to suit preference for the project.

ssODN can be increased while decreasing sgRNA:Cas9 or vice versa. Max volume for a small cuvette is 3.5uL.

b. Plasmid Donor (e.g. Large Insertion)

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
plasmid donor (1ug/mL)	1
sgRNA1 (100 uM)	1
Cas9 protein (50 uM)	0.67
Total	2.87

c. Plasmid Donor/ssODN Block or ssODN Mut/ssODN Block combo

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
ssODN mut (100 uM)	0.5
ssODN block (100 uM)	0.5
sgRNA1 (100 uM)	1
Cas9 protein (50 uM)	0.67
Total	2.87

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
plasmid (1-3 ug/mL)	0.5
ssODN block (100 uM)	0.5
sgRNA1 (100 uM)	1
Cas9 protein (50 uM)	0.67
Total	2.87

4. 2 sgRNA Transfection with Donor (e.g. Deletion/Translocation)

Reagent	Vol (uL)
pMax GFP (1 ug/mL)	0.2
ssODN (100 uM)	1
sgRNA1 (100 uM)	0.5
sgRNA2 (100 uM)	0.5
Cas9 protein (50 uM)	0.67
Total	2.87

General Nucleofection Protocol

Steps to be done before starting the process

1. Check for the best nucleofection program for the cell line

2. Add 2-3 mL of fresh media into wells of a 6 well plate – one well for each reaction **plus 1 well for the control**

Protocol

1. If using suspension cells, take 10 uL of sample for counting cells. If adherent cells, use trypsin or appropriate solution to lift off cells and then count the cells.
2. Count the cell density using Cell Countess and aliquot the required number of cells for nucleofection into a separate tube.
    a. Mix 10 uL of cells with 10uL of trypan blue
    b. Load 10 uL of cell/trypan blue mixture into Countess cuvette
3. Spin the cells at low speed (ex. 100-250 x g depending on the cell type) for 5 mins.
4. Aspirate the supernatant leaving the cell pellet as intact as possible.
5. Wash with 1 mL of PBS. At this step, the cells can be transferred to a 1.5 mL microcentrifuge tube.
6. Spin the cells as mentioned above.
7. Aspirate PBS and **repeat the wash step.**
8. Add 20 uL of nucleofection solution per nucleofection reaction (check the cell line evaluation for the best solution to be used for that cell line).
9. Mix gently and pipette 20 uL of the cell suspension into the tube containing DNA (pMax plasmid, donors, etc…). Leave the remaining cell suspension to be used as a control.
10. Add the cells + DNA mixture to the Cas9 + gRNA mix.
11. Mix gently 1-2x and transfer mix to the nucleofection cuvette/strip.
12. Use the appropriate program on the Lonza 4D nucleofector and nucleofect the samples.
13. Once the process is complete, transfer each reaction to an individual well containing media in a 6-well plate.
14. Add the remaining control cells from Step 9 to the control well. This will be used as a control for PCR, sorting, or analysis by FACS.

TROUBLESHOOTING

1. If you are getting excess death after nucleofecting, you can try
    a. Cutting down the amount of donor used (for a KI line)
    b. Changing the nucleofection solution
    c. Increasing the number of cells
    d. Contacting Lonza for more programs to try if none of these work

Note: If cell death continues to be a problem, this cell line may not be amenable to editing and should be placed on the Cell Line Blacklist