I. RNA gel

Objective: to verify mRNA's integrity and confirm that there is no degradation

1. Prepare samples (400ng mRNA in H_2O) as described in spreadsheet:
2. Add 5ul loading dye (NOVEX TBE urea loading dye 5X, Invitrogen).
3. Heat samples 3mins at 70C.
4. Use ssRNA ladder (NEB N0364S) with loading dye as marker.
5. Run 6% TBE-Urea gel (Invitrogen), in 1X TBE (Invitrogen), 140V for 2hrs, DO NOT CUT GEL, stain with SYBR Safe (5ul for every 50ml of 1X TBE) for ~2mins.

Gel results:

Gel #1: Gel #2:

II. Preparation of HEK293T cells

1. Grow HEK293T cells up to ~70-80% confluency in a T75 flask.

2. Plate 100ul of cells in each well of a 96-well plate the night before transfection.
   For plating:
   i. Collect the cells by trypsinization -> *Trypsinize 2 T75 flasks
   ii. Centrifuge cells 1000rpm, 5mins.
   iii. Resuspend pellets into 10ml DMEM.
   iv. Make 1:3 dilution in 15ml tube= 3.33ml cells + 6.67 DMEM.
   v. Make 1:5 dilution in 15ml tube= 2ml cells + 8ml DMEM.
   vi. Prepare 1 opaque plate with 1:3 cells for all transfection conditions.
   vii. Prepare 1 opaque plate with 1:5 cells for all transfection conditions.
   viii. Prepare 1 clear bottom plate with 3 wells for 1:3 cells and 3 wells for 1:5 cells to check cells growth.

Plate layout

*use spreadsheet to edit layout

III. mRNA transfection

1. Check cells and choose the plate that is more confluent (either 1:3 plate or 1:5 plate).
   Chose _____ plate, _____ % confluent.
   Plate layout:
   *see template on spreadsheet
2. If not done beforehand, prepare RNAs in 7ul RNA+H_2O. If done, take them out to thaw on blue rack.

*use spreadsheet for calculations

*Note: If doing normalization, also add a second reporter construct (for example Firefly or Renilla).

3. Warm up Opti-MEM in bead bath, spin down Mirus TransIT®-mRNA Transfection Kit transfection reagents and warm up to RT on TC cart.
   a. Product #: MIR 2225
4. Prepare reaction tubes: Label the tubes and prepare a balance if needed (36uL, you can use the Opti-MEM. Remember to prepare a tube with 1mL of Opti-MEM.
5. Add 25ul Opti-MEM to reaction tubes.
6. Add 7ul RNAs + H_2O to reaction tubes (use spreadsheet to calculate).
7. Add 2ul Boost to all reaction tubes.
8. Mix well by pipetting up and down.
9. Add 2ul Transit to one tube, wait 30secs, add transit to next tube, and so on.
10. Mix well by pipetting up and down.
11. Incubate for 3 mins and spin down during incubation time. Start timer after adding transit to last well.
12. Load 10.2ul of reaction to each well of cells in each set of triplicates for 1 of the biological replicates. Wait 30secs. Load the next set of triplicates.
13. When finished with set 1 of biological replicates, put lid on plate and **tap the corners** of the plate to shake a bit. Take the lid back off and load the next set of biological replicates following steps 11 & 12 again. Remember to write the time you finished the transfections.
14. Incubate for 8 hours at 37C (in TC room incubator).

IV. Dual-glo Nano luciferase/ Firefly luciferase assay

Kit: Nano-Glo® Dual-Luciferase® Reporter Assay System, Cat. No.: N1620

1. Prepare the NanoDLR™ Stop & Glo® Reagent fresh for each use.
    a. Calculate the amount of NanoDLR™ Stop & Glo® Reagent needed to perform the desired experiments.
    b. In a new container, dilute the NanoDLR™ Stop & Glo® Substrate 1:100 into an appropriate volume of room-temperature NanoDLR™ Stop & Glo® Buffer.
    c. Mix by inversion. (If the NanoDLR™ Stop & Glo® Substrate has collected in the cap or on the sides of the tube, briefly spin the tubes in a microcentrifuge before dispensing).
    d. Example: If you need 5ml of NanoDLR™ Stop & Glo® Reagent, transfer 5ml of NanoDLR™ Stop & Glo® Buffer to a disposable container, such as a 15ml centrifuge tube, and add 50µl of NanoDLR™ Stop & Glo® Substrate.
2. Remove plates of cells from the incubator.
3. Remove 50ul of media from each well.
4. Add appropriate volume of Add ONE-Glo™ EX Luciferase Assay Reagent to each well. Ratio 1:1, so add 50ul. Pipet up and down to mix.
5. Cover plate with aluminum foil
6. Incubate at room temperature for 3 minutes (preferably with shaking).
7. Measure firefly luminescence immediately.
8. Add NanoDLR™ Stop & Glo® reagent to the plate and mix.
9. Cover plate with aluminum foil.
10. Incubate at room temperature for a minimum of 10 minutes, maximum of 20mins (with shaking).
11. Measure NanoLuc luminescence immediately.

***NOTE**: Luciferase activity is temperature dependent. The temperature of the samples and reagents should be kept constant while measuring luminescence. This is most easily accomplished by using reagents that are equilibrated to room temperature.

Short: Dual-glo Nano luciferase/ Firefly luciferase assay

1. Atleast an hour before incubation time ends, take ONE-Glo™ EX Luciferase Assay Reagent and NanoDLR™ Stop & Glo® buffer out to thaw. They are in 15mL tubes on the freezer door. Place in a beaker with water and cover with foil. Take out NanoDLR™ Stop & Glo® reagent from the luciferase box.
2. Remove plates of cells from the incubator.
3. With the multichannel pipette. Remove 50ul of media from each well.
4. Add ONE-Glo™ EX Luciferase Assay Reagent to the white trays. (Multiply 50x#of wells to get the amount).
5. Add appropriate volume of Add ONE-Glo™ EX Luciferase Assay Reagent to each well. Ratio 1:1, so add 50ul. Pipet up and down to mix.
6. Cover plate with aluminum foil.
7. Incubate at room temperature for 3 minutes (preferably with shaking).
8. Measure firefly luminescence immediately.
9. Add NanoDLR™ Stop & Glo® buffer (same amount of ONE-Glo™ EX Luciferase Assay Reagent) and mix with 1/100 of the reagent. ex. for 2mL add 20uL of the reagent.
10. Add NanoDLR™ Stop & Glo® reagent to the plate and mix.
11. Cover plate with aluminum foil.
12. Incubate at room temperature for a minimum of 10 minutes, maximum of 20mins (with shaking). Measure NanoLuc luminescence immediately.