In vitro translation protocol **AMGS 2020**

*use *In vitro translation calculations spreadsheet* as reference for calculations and notes

* protocol adapted from (Rakotondrafara and Hentze 2011)

I. Reagents

a. Translation mix (T-mix)

Reagent	Stock	Final concentration	Amount added
HEPES pH 7.5	1M	8.6mM	8.6ul
Creatine phosphate	1M	20mM	20ul
Creatine Kinase	1mg/ml	0.1ug/ul	100ul
Spermidine	0.1M	0.1mM	1ul
Amino acids	1mM	100uM	100ul
Water	-	-	770.4

b. 10X salt (no KOAc, no MgOAc)

Reagent	Stock	Final concentration	Amount added (ul)
HEPES pH 7.5	1M	273mM	273
KOAc	2M	900mM	0
MgOAc	2M	-	0
Spermidine	1M	2mM	2
Putrescine	1M	6mM	6
TCEP	1M	20mM	20
Water	-	-	699

c. 10X salt (600mM KOAc, 20mM MgOAc)

STOCK concentration K (M1)[mM]	*Stock volume (V2) [ul]*	*Individual reaction volume (ul)*	*Reaction stock concentration (X)*	*Total reaction volume (V2) [ul]*	*Final concentration (M2)per reaction [mM]*	*Final concentration per stock [mM]*	*Volume of 10X salt per reaction (V1) [ul]*	*Volume of 2M K stock to add to 10X salt mix (V1) [ul]*	*STOCK Mg concentration (mM)*	*Stock Mg concentration (mM)*	*Reaction Mg concentration (mM)*	*Volume of 100mM MgOAc stock to add to 10X salt mix (V1) [ul]*	*Volume of 10X salt mix (no K, no Mg) (ul)*
2000	1000	10	4	40	60	600	4	300	100	20	2	200	500

II. Preparation

1. Clean the following with RNAse free spray:
    a. Bench
    b. Pipettes on bench
    c. 20ul multi-channel pipette
    d. 200ul multi-channel pipette
    e. Ice buckets (2 or 3)
    f. Metal racks (2)
    g. Small centrifuges (both)
    h. Table top cold centrifuge (by PCR machines)
    i. PCR block

2. Locate and have ready low-binding RNAse free tips, low-binding RNAse free 2.0ml eppendorfs and low-binding RNAse free 250ul tubes.
   *use spreadsheet to take notes on reagents

3. Fast temp table top cold centrifuge to 4°C
4. Set up a PCR block to 30°C.
5. Make an inventory of all the necessary reagents before starting
6. Prepare & label the necessary low-binding RNAse free 250ul tubes and place on clean cold metal rack on ice.
   *4x250ul tubes per reaction (1 for master mix & 3 for aliquots)

7. Prepare & label the necessary low-binding RNAse free 2ml eppendorf tubes and place on ice.
   *1x2ml tube per cell lysate
   *1x2ml tube per mRNA

8. Take out all reagents and thaw on ice.
   *do not let the cell lysate thaw for more than 10mins

9. Vortex and spin down all reagents *EXCEPT CELL LYSATE AND MRNAS* and place back on ice.

10. Spin down quickly mRNA stocks and prepare mRNA dilutions.
    *use spreadsheet for calculations

III. Set up IVT reaction

1. Refer to reaction set-up on spreadsheet
   *use “IVT reaction general set-up” & “IVT reaction descriptions” tables on spreadsheet
   *It is always recommended to make a 4X concentrated master mix (40ul) and aliquot into 3x10ul reactions.

2. After cell lysate is thawed, centrifuge for 10secs at 11500rpm at 4°C. A small pellet will form. Transfer supernatant to a cold 2ml low binding Eppendorf.

3. Add *corresponding volume of* water to master mix reaction tubes.

4. Add *corresponding volume of* RNAse inhibitor to master mix reaction tubes.

5. Add *corresponding volume of* T-mix to master mix reaction tubes.

6. Add *corresponding volume of* 10X salt to master mix reaction tubes.

7. Add *corresponding volume of* 20mM ATP stock to master mix reaction tubes.

8. Add *corresponding volume of* 20mM GTP stock to master mix reaction tubes.

9. Add *corresponding volume of* 0.5M TCEP stock to master mix reaction tubes.

10. Add *corresponding volume of* mRNA (1000ng/ul dilution) to master mix reaction tubes.

11. Add *corresponding volume of* cell lysate to master mix reaction tubes.

12. Spin down master mixes very quickly and place back on the cold metal rack on ice immediately.

13. Mix master mixes by pipetting up and down. For this, set up the 200ul multichannel pipette to 30ul. Using the multichannel pipette will allow you to mix up to 8 reactions at a time. With the master mixes kept on the cold metal rack on ice, pipette up and down 30 times to mix them.

14. Aliquot the master mixes into 3 new 250ul low binding tubes (which should also be on a cold metal rack on ice) by pipetting 10ul of the corresponding master mix to each new tube.

15. Mix each aliquot by pipetting up and down. For this, set up the 20ul multichannel pipette to 5ul. Using the multichannel pipette will allow you to mix up to 8 reactions at a time. With the aliquots kept on the cold metal rack on ice, pipette up and down 30 times to mix them.

16. Spin down aliquots very quickly and place back on the cold metal rack on ice immediately.

17. Set timer to 30mins.

18. Put the IVT aliquots in PCR machine at 30°C for 30mins.

IV. NanoLuciferase assay

1. Find reagents and thaw at room temperature. Put reagents on rack at room temperature right after starting IVT reaction incubation.
   a. Kit: Nano-Glo® Luciferase Assay System, Cat. No.: N1120
   b. Reagents can be found in “Luciferase assays reagents” box in my -20°C freezer.

2. Locate white bottom 96 well plate
   a. Reusable
   b. In Angélica’s bench drawer

3. Locate media through.
   a. Common ones located on bottom shelf of common TC plates rack.

4. Prepare enough NanoGlo Buffer + Substrate (50:1) for all the samples and dispense on media through.
   a. Do this 5 minutes before IVT incubation time is up
   b. For 96-well plate: 50ul per well
   c. Example: For a plate with 10 wells = Will make for 12 wells = 600ul total -> 600ul NGlo buffer + 12ul substrate
   d. Amounts for your plate:

5. Once IVT incubation time is up, remove IVT aliquots from PCR machine, spin down quickly and place on room temperature rack (or pipette box).

6. Take 9.5ul of each IVT aliquot and put in a well in the white bottom 96 well plate. Repeat the same for all IVT aliquots.

7. Set up the 200ul multichannel pipette to 50ul.

8. Using the multichannel pipette, add 50ul of previously prepared NanoGlo Buffer + Substrate solution to each well. Pipet up and down 30 times to mix.

9. Read luminescence immediately using Spark plate reader.

10. Save data on USB drive and analyze using spreadsheet.