Cell growth and cell extract preparation for cell-free translation system

(According to Rakotondrafara, A.M. and Hentze, M.W. (2011) An efficient factor-depleted mammalian *in vitro* translation system. *Nature Protocols*, 6: 563-571 with modifications)

1. Thaw one vail of frozen HEK293T pSB-HygB-GADD34-K3L cells (~1.5 × 10^6) in T25 plate (CellStar, 07000226) in a total volume of 4 ml of complete DMEM (Gibco, 11995-065) supplemented with 10% Tet-system approved Fetal bovine serum (Gibco, A47364-01), Penicillin (100 u/ml final) – Streptomycin (100 µg/ml final) (Gibco, 15140-122) and GlutaMax (1x, 2 mM L-alanyl-L-glutamine dipeptide) (Gibco, 35050061). Place the dish in the humidified 37 °C, 5% CO2 incubator.
2. The next day, check cells adherence and change the media. Supplement the media with 200 µg/ml Hygromycin B (final concentration) (Invitrogen, 10687010).
3. The next day, cells should be close to 100% confluency, transfer all cells in T75 plate (Corning, 430641U) in a total volume of 25 ml of complete media. Supplement the media with 200 µg/ml Hygromycin B. 4. In two days, cells should be close to 100% confluency, transfer half of cells from T75 in T175 plate (Falcon, 353112) in a total volume of 50 ml of complete media. Supplement the media with 200 µg/ml Hygromycin B. The rest of the cells might be passaged in T75 or T175 plate in case multiple sequential rounds of extract preparations are planned.
5. In two days, cells should be close to 100% confluency. Spit all cells in 25x 150 mm plates (Corning, 430599) in a total volume of 20 ml of complete media in each plate. VERY IMPORTANT! Don't put too many cells (~1.2 – 1.5 million of cells per plate is optimal!). One T175 at 100% confluency is a perfect number of cells to seed 25x 150 mm plates! Don't supplement the media with Hygromycin B!
6. The next day, add 20 µl of 1 mg/ml water solution of Doxycycline (Takara, 631311) to each plate. Mix Dox with media by tilting and rotations plates for ~20 sec to ensure the equal drug distribution in the media volume.
7. The next day, place plates on ice, remove growth media and detach cells by scraping. 8. For every 5 plates, wash with 5 ml of ice-cold DPBS (Gibco, 14190-144) twice, collect cells in two 50 ml centrifuge tubes.
9. Pellet cells by centrifugation at 1000 g for 5 min at 4 °C.
10. Remove supernatant and resuspend pellet in ice-cold DPBS. Divide cells into 3-4 1.5 ml Eppendorff tubes (~1 ml in each tube).
11. Pellet cells by centrifugation at 1,000g for 5 min at 4 °C.
12. The size of each pellet should be approximately 250–300 µl. Remove DPBS and resuspend the cell pellet in ice-cold lysis buffer in a 1:1 volume ratio. VERY IMPORTANT! To ensure translational activity of the cell extracts, carefully estimate the volume of the packed cells and add the same volume of lysis buffer. Lysis buffer (all concentrations are final): 10 mM HEPES-KOH pH 7.6, 10 mM KOAc, 0.5 mM $Mg(OAc)_2$, 5 mM DTT.
13. Incubate the cells for hypotonic swelling in lysis buffer for ~45 min on ice. VERY IMPORTANT! To ensure translational activity of the extract, perform cell lysis with ice-cold buffer.
14. Homogenize the cells by forcing the cell suspension through a 1-ml syringe with a 26G needle about 15 times (BD PrecisionGlide Needle, 26G x ½ (0.45 mm x 13 mm), 305111). VERY IMPORTANT! Avoid excessive handling of the extract, as it can have detrimental effects on the activity of the extract as a result of protein denaturation.
15. Mix all 3-4 cell lysates together and centrifuge the lysate at 15000 g for 1 min to remove the cell debris and nuclei, collect the supernatant. Check extract concentration – it should be A260 ~50-60. Make 55 µl aliquots, flash freeze in liquid nitrogen and store it at −80 °C until needed. VERY IMPORTANT! Don't adjust the volume of the cell extract with lysis buffer at this stage!