^{35}S-LABELING OF MITOCHONDRIAL TRANSLATION PRODUCTS

Perform pulse labeling to assess the rate of mitochondrial protein synthesis or chase labeling to assess the stability of the mitochondrial-encoded proteins.

SOLUTIONS: LABELING MEDIUM (stored at 4^{o}C for several months):

- DMEM without methionine and without cysteine; such formulations also lack glutamine
- 1X glutamax
- 10% dialyzed serum
- 110 mg/L sodium pyruvate (certain formulations of DMEM lacking methionine and cysteine contain sodium pyruvate; check before adding)

INHIBITOR OF CYTOPLASMIC TRANSLATION (prepared fresh, prior to use):

- 2 mg/ml of either EMETINE (for pulse labeling) or ANISOMYCIN (reversible, for chase labeling) in PBS or in DMEM lacking met and cys. Filter sterilize.

^{35}S-METHIONINE OR ^{35}S-(MEHIONINE + CYSTEINE) MIXTURE (stored at -80^{o}C):

- Pure ^{35}S-methionine will give the strongest signal and the best signal/noise ratio. A comparable result (approximately 75% of the signal intensity resulting with pure ^{35}Smethionine) can be obtained with the PRO-MIX (less expensive) mixture from Amersham.

1X PBS

DMEM + 10% FBS

CHLORAMPHENICOL (CAP); for chase labeling only (stored at 4^{o}C for one week):

- 1 mg/ml in regular DMEM without serum. Add warm medium to the powder and incubate at 37^{o}C until the powder goes completely into solution. Filter sterilize.

2X GEL LOADING BUFFER (room temperature):

- 186 mM Tris-HCl, pH=6.7-6.8
- 15% glycerol
- 2% SDS
- 0.5 mg/ml bromophenol blue

• 6% ∂-mercaptoethanol NOTES BEFORE STARTING:

• Cells (fibroblasts, myoblasts, myotubes) are grown in 60 mm tissue culture (t.c.) plates and labeled directly in the plates. On the day of the experiment, dividing cells should be between 75 and 90% confluent. Starting with less cells might result in insufficient protein amounts. At the same time, cells must be less than 100% confluent, as they should still be dividing during the labeling.

• Preincubate the required volumes of labeling medium (2ml/plate) and of DMEM+10% FBS (5ml/plate) for at least 30 minutes in a t.c. plate, in the t.c. incubator. This step will allow the media to equilibrate to 5% CO_2 and 37^{O}C.

• During the labeling procedure it is important that individual plates be placed directly on the shelf of the incubator, rather than stacked on top of each other. This ensures that each plate takes the same time to equilibrate in terms of temperature and CO_2 concentration.

• For chase labeling, add 200 µl CAP solution/plate (total volume of ~5ml, final CAP concentration of 40 µg/ml) 22-24 hours prior to the start of the labeling procedure.

PROCEDURE:

1. Wash cells twice with 3 ml PBS.

2. Incubate for 30 minutes in labeling medium (2ml/plate). During this time, prepare and sterilize the appropriate cytoplasmic inhibitor solution.

3. Add 100 µl of emetine OR anisomycin (final concentration of 100 µg/ml) to each plate.

4. Incubate for 5 minutes prior to labeling.

5. Add 400 µCi of ^{35}S-methionine or PRO-MIX to each plate (final concentration of 200 µCi/ml) and incubate for 60 minutes.

6. For pulse labeling, chase cells in DMEM+10% FBS (5 ml/plate) for 10 minutes. For chase labeling, wash cells once with DMEM+10% FBS, then chase cells in DMEM+10% FBS (5 ml/plate) for 17 hours.

7. Wash cells three times with PBS (gently, mostly for myotubes).

8. Scrape cells in 1-1.5 ml ice-cold PBS and transfer to an Eppendorf tube. Collect cells by centrifugation at 4^{O}C, 1500g, for 10 minutes. Alternatively, in the case of myotubes, an enriched population of fused cells can be obtained by selective trypsinization: trypsinize cells for about 2 minutes, or until fused cells start lifting (unfused cells will take at least 5 minutes to trypsinize). Dilute trypsin by adding 5ml PBS to the plate and transfer trypsinized cells to a 15 ml Falcon tube. Rinse plate with another 5 ml PBS and add to the same 15 ml tube. Collect cells by centrifugation at 1500g for 5 minutes. Resuspend pellet in 1-1.5 ml cold PBS, and transfer to an Eppendorf tube. Collect cells by centrifugation at 4^{O}C, 1500g for 10

minutes.

9. Resuspend pellet in 200 µl ice-cold PBS. Samples can now be stored at -80^{O}C, or the procedure may be continued.

10. Use duplicates of 5 µl and 10 µl to determine the protein concentration of each sample, by using the Micro-BCA kit. For each sample, the values for the protein concentration as calculated from each of the duplicates must be within no more than 1015% of each other, otherwise the measurement should be repeated.

11. Spin down the desired amount of protein (usually 50 µg) by centrifugation at 4^{O}C, full speed, for 20 minutes.

12. Resuspend pellet in 10 µl of 2X gel loading buffer and 10 µl of deionized water (room temperature).

13. Sonicate samples for 3-8 seconds at 60%.

14. Spin samples at full speed, room temperature, for 10-15 minutes, or until bubbles disappear.

15. Load samples on a large 12-20% gradient polyacrylamide gel (use 37:1 acrylamide stock), and run at 10 mA for 15 hours.

16. Dry gel (60^{O}C, under vacuum, for one hour) and expose to Phospholmager cassette for 3 days or longer.

Note:

A more detailed protocol can be found in the following article:

Leary SC, Sasarman F. Oxidative phosphorylation: synthesis of mitochondrially encoded proteins and assembly of individual structural subunits into functional holoenzyme complexes. Methods Mol Biol. 2009;554:143-62. PDF