

### ***Pulse Chase Assay (In vivo)***

#### Plasmids:

pCDNA (for balance)  
pA3F c-Myc  
pCDNA E3C 1-992

#### Cells:

293 cells are preferred; 293T cells have the large T antigen that modulates Sel10 activity.  
12.5 x10<sup>6</sup> cells/ transfection

#### RIPA Buffer:

10mM Tris (pH 8.0)  
1% NP40  
2mM EDTA  
150mM NaCl  
Supplemented with Aprotinin, Leupeptin, Pepstatin and MG132

#### DMEM without Methionine and Cystine

<sup>35</sup>S Labeled Methionine/Cystine mix

Sample	Vector (ug)	cMyc (ug)	E3C (ug)
1	12.5	12.5	-
2	-	12.5	12.5

Each sample is repeated 4 times for 4 different time points.

- Cells are mixed with DNA in 450uL complete medium (total volume including DNA) and transfected in 0.4cm gap electroporation cuvettes at 210V and 975uF
- Cells are incubated for 24 hrs, harvested with trypsin, similar transfections mixed and replated to account for slight variability in transfection efficiencies.

Note: Replating is time consuming but worth it for consistency

- 12hrs later, growth medium is replaced by DMEM without Methionine and Cystine.
- 45min later, depleted medium is replaced by growth medium supplemented with <sup>35</sup>S Labeled Methionine/Cystine mix (150uCi/sample) and MG132 (15ug/mL).

Note: Supposedly, MG132 is instantly reversible and addition definitely helps boost signal.

- 2hrs later all samples are washed with prewarmed PBS and regular growth medium is added to the cells.
- Zero time point cells are harvested in ice cold PBS using shear.
- Pelleted and the pellet dislodged by tapping.
- Cells are lysed in 600uL RIPA buffer by continuous vortexing for 1 min.

- Lysates are incubated on ice for 1hr, with intermittent vortexing every 10 min.
- Samples are harvested in Cold PBS every 30min and lysed.

Note: Each set of 2 IP's follows every ~30min, so the IP times are slightly different (overnight, overnight + 30min, overnight + 60min and overnight + 90min).

- Lysates are transferred to a 1.7mL Eppendorf tube and cleared by centrifuging at top speed for 10min.
- Lysates are transferred to fresh tube and precleared by adding 20uL Protein-A Sepharose Beads and rotating at 4°C for 45min.
- Protein-A sepharose beads are spun down and precleared lysates transferred to fresh tubes.
- 40uL lysate is saved from each sample (7.5%) and 2ug anti-Flag M2 antibody is added to each sample. Input lysates as well as the IP samples are rotated at 4°C overnight.

Note: 2ug of antibody is used in this experiment because we are normally operating in an antibody limited regime and IP's tend to equalize differences somewhat. It is better to have the antibody in excess to get true differences.

- The next morning, 30uL of 1:1 protein A/G beads are added to the IP samples and the samples are rotated for an additional 3hrs.
- Immunoprecipitated complexes are washed 5X with ice cold RIPA buffer (without MG132).
- Input and IP samples are boiled with SDS loading dye for 10min and resolved on a 7% acrylamide gel.
- Western blots are probed for flag tagged cMyc using 1ug/mL anti-flag M2 antibody, followed by IR800 conjugated anti-mouse secondary.
- After scanning, the blots are probed for E3C using anti-E3C A10 and IR800 conjugated anti-mouse secondary.
- IP gel is dried and exposed on a phosphoimager plate.
- Radiolabeled bands are quantified using Imagequant software