Ubiquitylation Assay (In vivo)

Plasmids:
pCDNA (for balance)
pCDNA HA-Ub
pA3F c-Myc (substrate)
pCDNA E3C 1-992

Cells:
293 cells are preferred; 293T cells have the large T antigen that modulates Sel10 activity.
12.5 x10^6 cells/ transfection

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

Stripping Buffer:
62.5mM Tris (pH 6.8)
98mM β–Mercaptoethanol
2% SDS

Sample	Vector (ug)	cMyc (ug)	Ub (ug)	E3C (ug)	MG132
1	20	-	-	-	+
2	15	5	-	-	+
3	5	5	10	-	+
4	0	5	10	5	+
5	10	5	-	5	+
6	5	5	10	-	-
7	0	5	10	5	-
8	10	5	-	5	-

- 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 36 hrs, before MG132 is added to the required samples (15ug/mL).
- MG132 addition is followed by further incubation for 2-3 hrs.
- 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.
- 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^{o}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 1ug anti-Flag M2 antibody is added to each sample. Input lysates as well as the IP samples are rotated at 4^{0}C overnight.
- 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.
- Plots 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.
- Blots are stripped by incubating in stripping buffer for 1hr at 58^{o}C
- Probed for HA tagged ubiquitin, using anti-HA 12CA5 and IR800 conjugated anti-mouse secondary.
- Bands are quantified using Odyssey 2.1 software