**RNA Immunoprecipitation (RIP) Protocol from Natalie McAdams**

**Turn on track changes for Laurie/Natalie Q and A**

**RNA Immunoprecipitation of MRB3010-PTP or TbRGG2-HTM**

**Cell growth and mitochondrial prep (all buffers contain RNase inhibitor)**

* Scale up PF cells with the tagged construct to 1L (PTP3010) or 2L (TbRGG2-HTM) per condition (1x107cells/ml) - you are growing up enough to do 2 technical replicates for each condition
* Grow cells to ~1x107 cells/ml and spin at 5,000rpm for 10min at 4°C
* Wash pellet with 25ml SBG (150mM NaCl, 20mM Glucose, 20mM NaHPO4 pH 7.9) and spin again at 6,000g for 10min at 4°C
* Resuspend pellet in 30ml DTE (1mM Tris pH8, 1mM EDTA, Complete protease inhibitor cocktail (Roche), RNase inhibitor)
* Dounce homogenize (Type A) on ice 10x back and forth and add 5ml 60% sucrose and mix
* Spin at 15,800g for 10min at 4°C
* Resuspend in 6.8ml STM (250mM sucrose, 20mM Tris pH8, 2mM MgCl2, 0.2mM CaCl2) add 1µl/1ml DnaseI and incubate on ice ~1hour
* Add 6.9ml STE (250mM sucrose, 20mM Tris pH8, 10mM EDTA), mix and spin 15,800g for 10min at 4°C
* Store pellet at -80°C until ready to do IP

**Lysis of mitochondria (all buffers contain RNase inhibitor)**

* Resuspend pellet in 18ml (per condition) N150 [50mM Tris pH 8, 150mM NaCl, 0.1%NP40, 5mM βME, and Complete protease inhibitor cocktail (Roche), RNase inhibitor]
* Add 2ml 10% Triton X-100 and place on ice for 20min
* DNase treat samples adding 10µl DNaseI and 20 µl 1M CaCl2
* Spin lysate at 14,000g for 15 min at 4°C, keep supernatant

**Preclear**

* Incubate supernatant with 100µl 50% slurry Superdex200 at 4°C for 45 min while rocking
* Use column and gravity to separate lysate from the beads leaving the beads in the column
* Divide sup (pre-cleared lysate from the step above) into 2 technical replicates (10ml per replicate) if doing -/+ tet you will have 4 total
	+ Take 2ml out of each and pool for a mock (negative control IP)
	+ You will then have a total of 5 samples with 8ml in each (1)mock, (2)tet- rep1, (3)tet-rep2 (4)tet+rep1, (5)tet+rep2

**Immunoprecipitation**

* Pre-equilibrate 150µl beads (50% slurry) in N150 [50mM Tris pH 8, 150mM NaCl, 0.1%NP40, 5mM βME, and Complete protease inhibitor cocktail (Roche), RNase inhibitor]
	+ PTP-use IgG beads, Superdex200 as a mock IP
	+ TbRGG2-HTM use ICL myc beads, HA-coupled beads as a mock IP
* Incubate sup and beads at 4°C 2+hours while rocking
* Use mini columns and gravity to elute the flow and wash 10ml N150+RNase inhibitor (no protease inhibitor!!)
* Transfer beads to an eppendorf tube and bring volume up to 500 µl (save 30 µl and run a Western to compare the amount of protein pulled down between the samples)
	+ Freeze the beads until after Western, remove beads if more protein pulled down in one sample so that the a comparable amount of protein is in each samples
* Once normalized and in a total volume of 500µl per reaction, add DnaseI 50µl 1mg/ml, 0.5µl 1M CaCl2 and incubate at 37°C for 30 min
* Add 50µl 10% SDS, and 38.7µl proteinase K and incubate at 55°C for 30min
* Add 5µl 0.5M EDTA and mix
* Let beads settle and remove liquid and place into a new tube
* Can add in spiked in RNA (pET41a) if want an additional normalization gene for precipitation
* Phenol/chloroform 2x and ethanol precipitate with salt and 1µl glycogen
* Resuspend RNA pellet in 30µl DEPC’d H20
	+ Take ½ the RNA and Dnase treat again using Ambion Dnase Kit at 37°C (1µl Dnase in 50µl total volume) for 30 min, add an additional 1µl Dnase and incubate at 37°C for 1 hour
	+ Phenol/chloroform and ethanol precipitate using salt and 1µl glycogen
	+ Resuspend final Dnased RNA pellet in 30µl DEPC’d H20

**cDNA and Preamplifcation**

* Nanodrop the DNased RNA to determine the concentration of RNA isolated
* Use BioRad iScript cDNA synthesis kit to convert 500ng of RNA (per sample) using gene specific primers (10µM-use a pool of primers in one reaction, only add the reverse primers!!!!) -20µl total volume (as per kit instructions for gene specific cDNA)
* Use the BioRad PreAmp Solution to preamplify before qPCR
	+ Add 5µl of primer mix (contains all qPCR primers,both forward and reverse primers 500nM)
	+ Add 25µl of PreAmp and run PCR (as per kit instructions)
* Use the pre-amp cDNA for qPCR reactions, depending on the gene add 2.5µl of cDNA dilution (ex 1:10dil) into 22.5µl syrb green/primer mix

**Calculations for RIP Analysis (adapted from RNA 2:540 (2018) Suppl. Methods)**

Fold enrichment compared to the mock RIP was calculated for each gene of interest (GOI) using the ΔΔCt method comparing the tet uninduced sample to that of the mock IP (that is, comparing the Ct value from the qPCR of the cDNA that was made from the RNA that was isolated in the IP of the tet uninduced experimental sample to the Ct value from the qPCR of the cDNA that was made from the RNA that was isolated the IP of the mock). Fold change to mock IP = 2(ΔΔCt), where ΔΔCt = (CtGOI-Mock - Ct18S-Mock) - (CtGOI-Uninduced - Ct18S-Uninduced). To determine the fold change in the RNA associated when MRB7260 was depleted, the RNA detected in the tet-induced sample was compared to the uninduced IP using the ΔΔCt method. Fold change with MRB7260 knockdown = 2(ΔΔCt), where ΔΔCt = (CtGOI-Uninduced - Ct18S-Uninduced) - (CtGOI-Induced - Ct18S-Induced).

**Notes on Calculations for RIP Analysis**

1) First sentence, second line, delta/delta CT :  what is the formula and where is it?

The delta delta CT method is a standard method for normalizating qPCR, subtraction of Ct values.  I have attached a spread sheet used for the calculation of delta/deltaCt.  You plug in your Ct values from qPCR and it normalizes the levels of your GOI to the normalization gene and then gives you a fold change between the samples.

It involves first comparing the level of your gene of interest to that of your normalization gene (this is the first delta). You subtract one value from the other value!

Examples:

CtGOI-Mock - Ct18S-Mock

CtGOI-Uninduced - Ct18S-Uninduced

CtGOI-Induced - Ct18S-Induced

Then you compare the normalized levels from above (delta1) between the samples giving you the second delta (get it-delta delta Ct)

For fold enrichment, you compare the normalized levels between the uninduced and the mock

Fold change to mock IP = 2(ΔΔCt), where ΔΔCt = (CtGOI-Mock - Ct18S-Mock) - (CtGOI-Uninduced - Ct18S-Uninduced)

For change with a protein knockdown you compare the normalized levels between the uninduced and induced samples

Fold change with MRB7260 knockdown = 2(ΔΔCt), where ΔΔCt = (CtGOI-Uninduced - Ct18S-Uninduced) - (CtGOI-Induced - Ct18S-Induced)

2)  In your formulas, we deduce that "-" actually means "in".  so its not "GOI minus Mock", rather it's "GOI in the Mock", correct?
Yes this means "in" the superscript

3)  Between isolation of RNA and qRT-PCR.....your example states use 2.5 ul of cDNA dilution (ex 1:10 dilution).  Is 1:10 where you would normally start?

That is usually were I started.  It will be different for each gene so I would use this to start and then adjust on future plates if needed.