Notes for immunoprecipitation with dynabeads and transfection:

Day before:

* Seed well plates as stated. Eg. 1x 10^4 cells in 96 well or 2x10^5 cells in 6well. (Note. Scale up proportionate to the DharmaFect reagent)
* Eg. Split cells at 70% confluency, dilute 1:5, then 100uL of cell solution in 900uL media for 12well.
* Incubate overnight (24hrs) at normal conditions.

Day of transfection:

* Suspend the RNAs, XuL per nMole = 100uM stock.
* Make 5uM working, 5uL of 100uM in 95uL of RNAse-free water. Freeze.
* Make two master mixes (table is for PC3: need optimisation)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Tube 1. Diluted RNA per well | | Tube 2. Diluted Dharma per well | | Total transfection volume (uL) |
| No. Well | 5uM rna | SF media | DharmaFect | SF media |
| 96 | 0.5 | 9.5 | 0.2 | 9.8 | 100 |
| 12 | 5 | 95 | 2 | 98 | 1000 |
| 6 | 10 | 190 | 4 | 196 | 2000 |

* Mix the tubes separately and gently. Incubate 5minutes at RT.
* Add tube 1 contents to tube 2. Mix gentle and incubate for 20minutes at RT.
* Add 5%FBS/RPMI to Tube1/2 contents to make up to volume (eg. 1600uL for 6well)
* Remove old media from cells and add RNA/DharmaFect media.
* Incubate at normal conditions for 24-72hrs (max.).

\*\*\*transfection uses 25nM which is approximately 405pmol/100uL so only about 20pmol is used in transfection for 5uL of 5uM for 8.1nm sample.

Pull down>

* Collect supernatant as well in case candidate doesn’t bind.
* Make Washing buffer: 10mM tris-HCL (7.5pH), 1mM EDTA, 2M NaCl for 10mL.

1. Add biotin-mir to lysate, incubate for 20minutes with rotation. Meanwhile:
2. Resuspend beads.
3. Wash beads normally in new tube. Use 25uL per 10cm plate of lysate.
4. Add beads to tube and then add lysate.
5. Incubate for 15-30minutes while lightly rotating.
6. Place on magnet for 3minutes and collect supernatant in other tube.
7. Wash beads thrice.
8. Boil for 5minutes in 0.1% SDS to dissociate biotin-mirs. This will denature the protein. Try make about 100ul of solution.
9. Collect solution for analysis (need to assess protein concentration, then add sample buffer, then gel)

Pull down to do on Monday:

* Make lysate. NO BOIL.
* Do pull down with Biotin-148a, biotin-scrambled and no miR.
* Make sure you collect the supernatant and run on gel to assess whether anything was in the lysate at all.

Gel: Top is well, middle is content and last row is expectation. ‘Sup’ is supernatant.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Ladder | - | 148 | 148 sup | scr | Scr sup | neg | Neg sup | - | Ladder | - | 148 | scr | neg | - |
| Ladder | - | Band at 65 but maybe others | Multi band | No band at 65 but other bands | Multi band | No band | Multi band | - | ladder | - | For western: 148 should have band at 65 or 80. Others shouldn’t have this band. | | | - |

For optimisation: Change RNA concentration instead of dharma concentration.

* Perform with Cy5-Mir with the highest yield.
* So a 1:5 dilution x3 with different miRNAs (2.5 (0.5uL), 25 (5uL) and 50nM (10uL)).
* Negative control, with reagents but no RNA.
* Monday(18/7):
  + Set up 4 slides in 12 well plate. Later, seed with desired split (by 12).
  + Microscopy. 9-10: for CD9 and TSG101 and hnRNPK localisation.
  + Suspend and make working stock of miR mimics.
  + Do faux pull down (below) and measure with Bradford. If enough, go SDS-page, followed by transfer and coomasie and western.