Nastya in a big world of science

Presentation plan

1. LINE-1 expression vectors
2. Exosome IPs. WB, MS, MP
3. HEK-293 Flp-In T-REx EXOSC10-3xFLAG cells dox test
4. Jess experiments
5. Flp-In EXOSC10 HeLa cells

Preparing plasmids for future transfections with LINE-1 expression vectors

1.1 Midi/giga preps of MT302, MT289, MT/LD491, MT207, MT646

spreadsheet link in wiki:

https://macromolecule-child.rockefeller.edu/#Spreadsheet%20of%20extracted%20plasmid%20DNA%20by%20Midi%2Fgiga%20preps%20(Nastya%20and%20Lars)

Plasmid name	Amount ~ (need to be updated with Leila)
MT302	4.4 mg
MT289	4.4 mg
LD491	0.9 mg
MT207	0.3 mg
MT646 by giga prep	5,4 mg

plasmid	description
MT302	L1RP Orf2-PPX-3xFlag
MT289	L1RP untagged in pCEP-Puro-Tet
MT/LD491	ORFeus-HS ORF2-3xFlag Clean Tags

Preparing plasmids for future transfections with LINE-1 expression vectors

1.2 Cloning of MT646 (ORFeus-HS clean tag), MT647 (ORF2p-3xFlag alone clean tag)

The goal was to move from tet-on to CMV promoter

Protocols are in wiki

Seq results, plasmid maps are in BOX in .clc format)

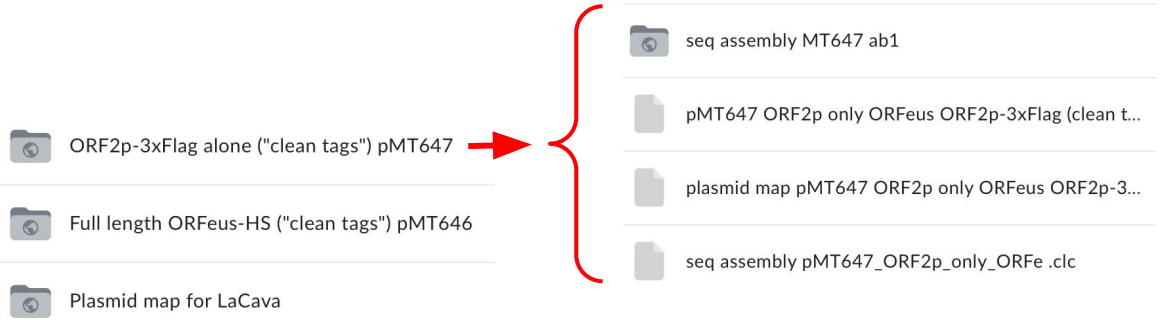
BOX > CLC_Files > L1 > **L1_nucleic_acids**

Anastasia Petrenko's Journal

admin 24th January 2021 at 9:21pm

Cloning project

> 12/08/2019_Cloning experiment MT646
>
> 12/08/2019_Cloning experiment MT647



Preparing plasmids for future transfections with LINE-1 expression vectors

1.3 Future plans

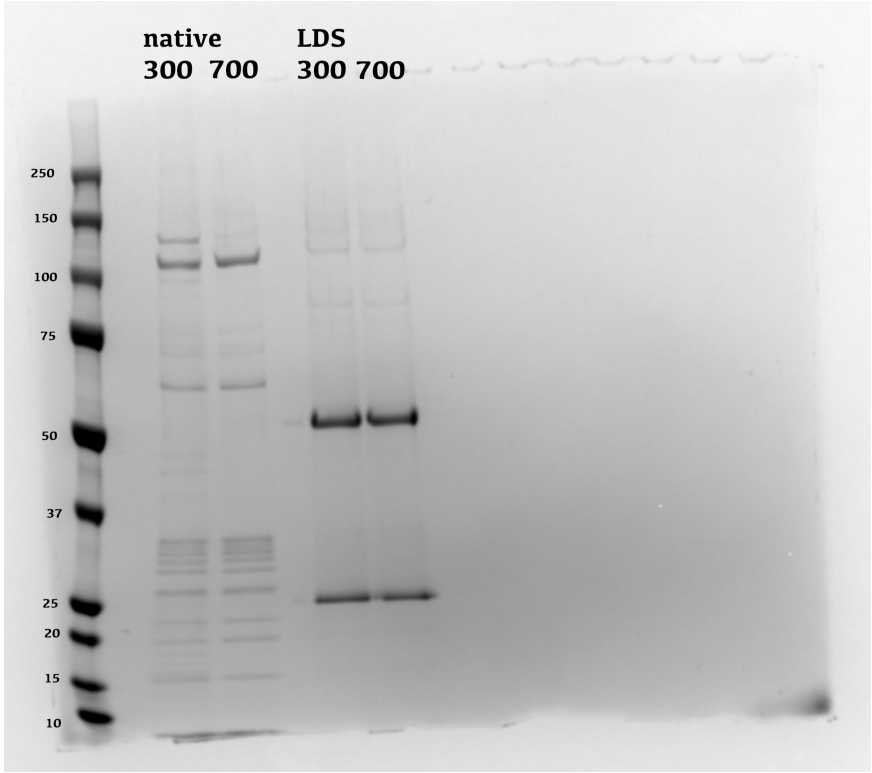
- MT646 version of L1RP
- pMT737 ORFeusHS ORF2-3C-3xF (H230A EN-) in pCEP-Puro CMV (EN- version of pMT646)
- RT-, both RT- and EN- of MT646 and MT647, also L1P1 version with mutations

2. Exosome IPs. WB, MS, MP

June

IP with NaCl 300/700 mM

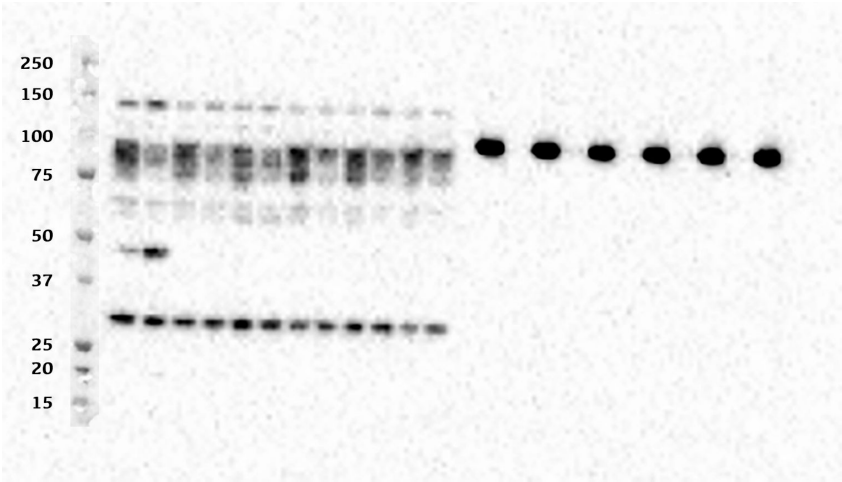
https://macromolecule-child.rockefeller.edu/#04%20June%202020_EXOSC10%20purification%20in%20NaCl%20300mM%20and%20700mM



June

anti-RRP6 antibody

1) Marker 5ul
2) 300mM NaCl SUP
3) 300mM NaCl FT
4) 300mM NaCl SUP
5) 300mM NaCl FT
6) 300mM NaCl SUP
7) 300mM NaCl FT
8) 700mM NaCl SUP
9) 700mM NaCl FT
10) 700mM NaCl SUP
11) 700mM NaCl FT
12) 700mM NaCl SUP
13) 700mM NaCl FT
14) Space
15) 300mM NaCl Native Elution
16) 300mM NaCl LDS Elution
17) 300mM NaCl Native Elution
18) 300mM NaCl LDS Elution
19) 300mM NaCl Native Elution
20) 300mM NaCl LDS Elution
21) 700mM NaCl Native Elution
22) 700mM NaCl LDS Elution
23) 700mM NaCl Native Elution
24) 700mM NaCl LDS Elution
25) 700mM NaCl Native Elution
26) 700mM NaCl LDS Elution



No signal from Sup/Ft, lots of background.

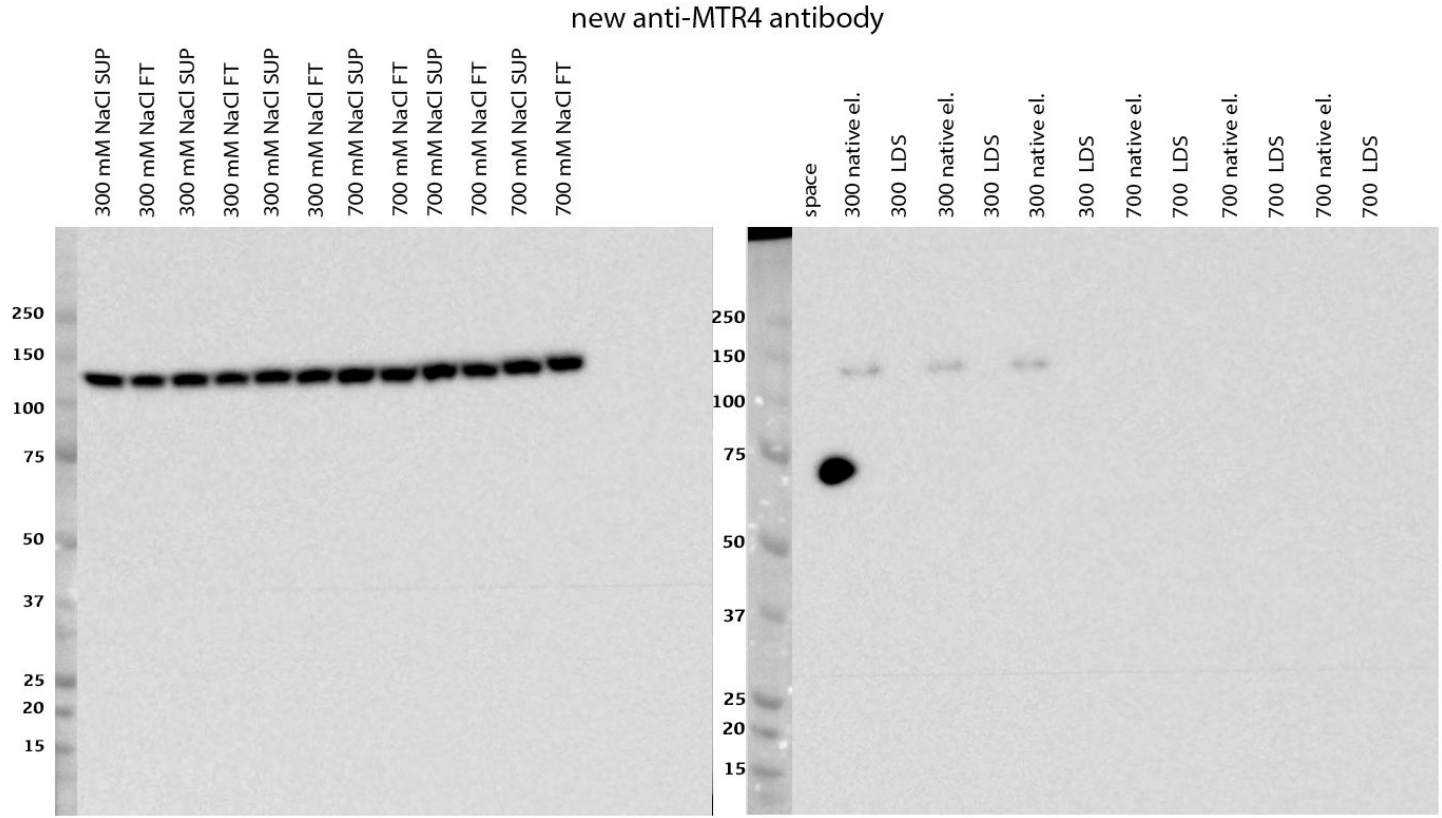
Everything 1 ul

LDS fraction 5 ul

https://macromolecule-child.rockefeller.edu/#04%20June%202020_EXOSC10%20purification%20in%20NaCl%20300mM%20and%20700mM

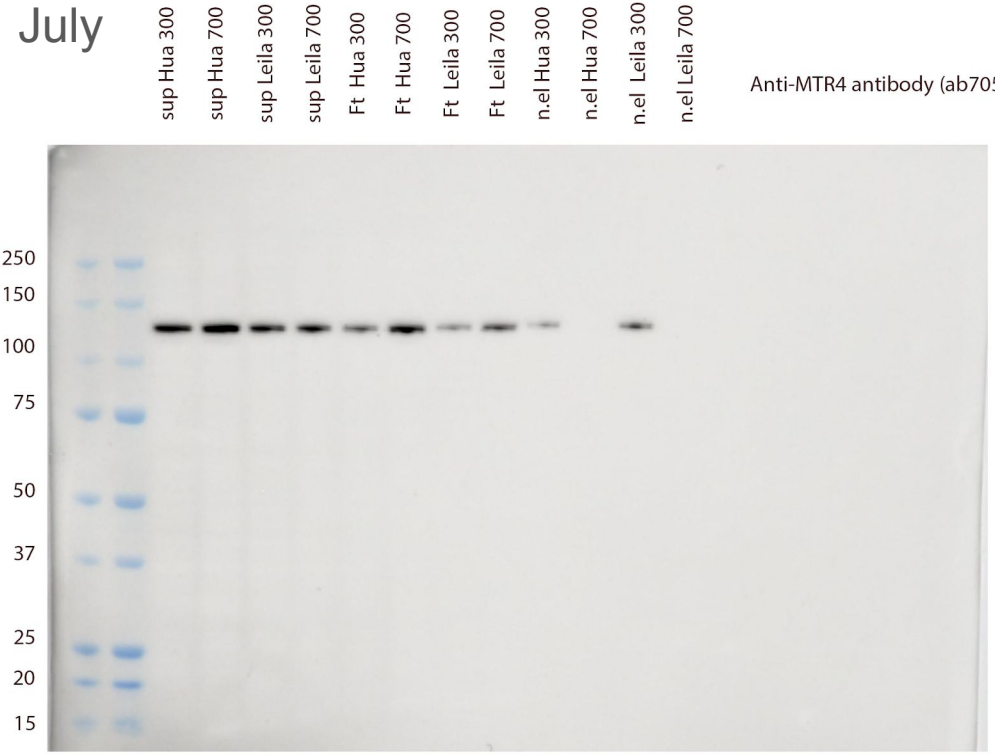
June

Ab70552



July

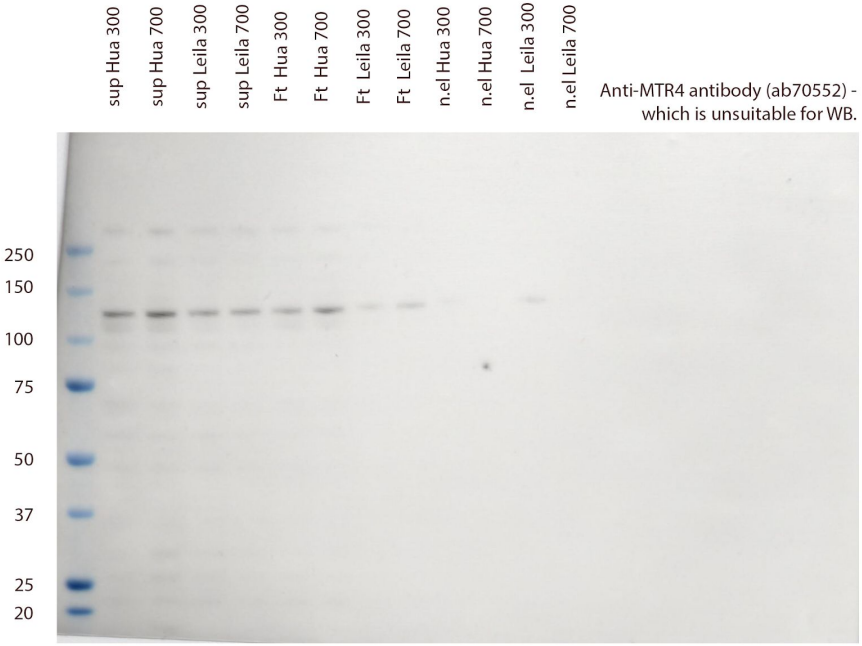
anti-MTR4 antibody comparison (not really fair one)

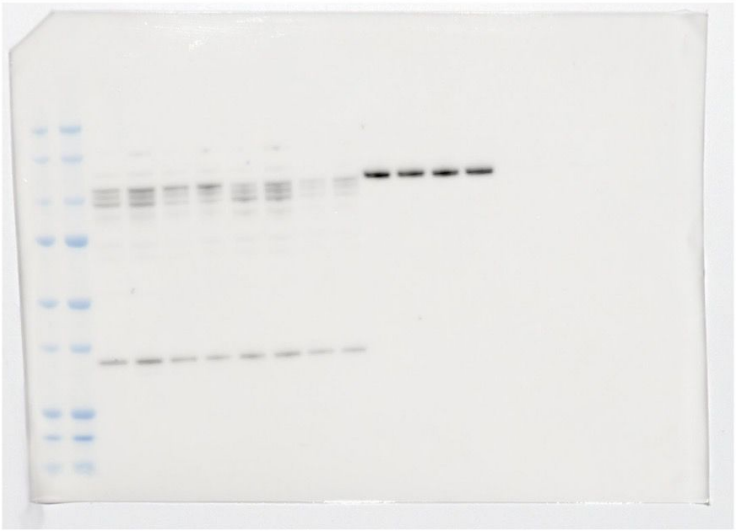


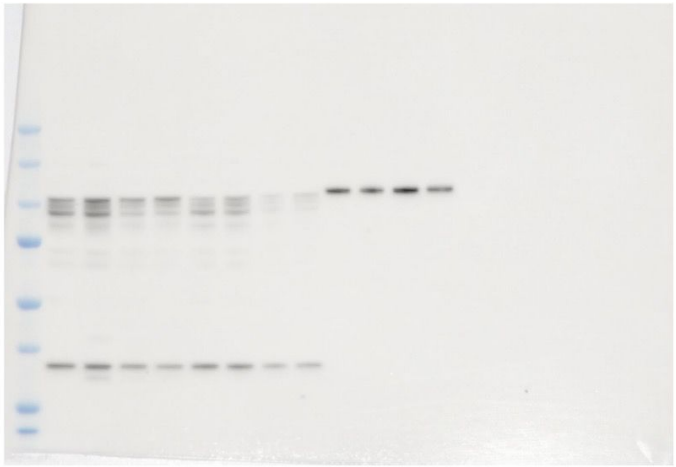
2 ul of sample in each fraction mix

For Ab70551 product concentration is 0,2 mg/mL, for Ab70552 - 1 mg/mL

1:4000 for Ab70551 and 1:20 000 (i didn't know that I should use same dilution factor)







Just the same blots with anti-rrp6 after stripping and reblocking

4 sup

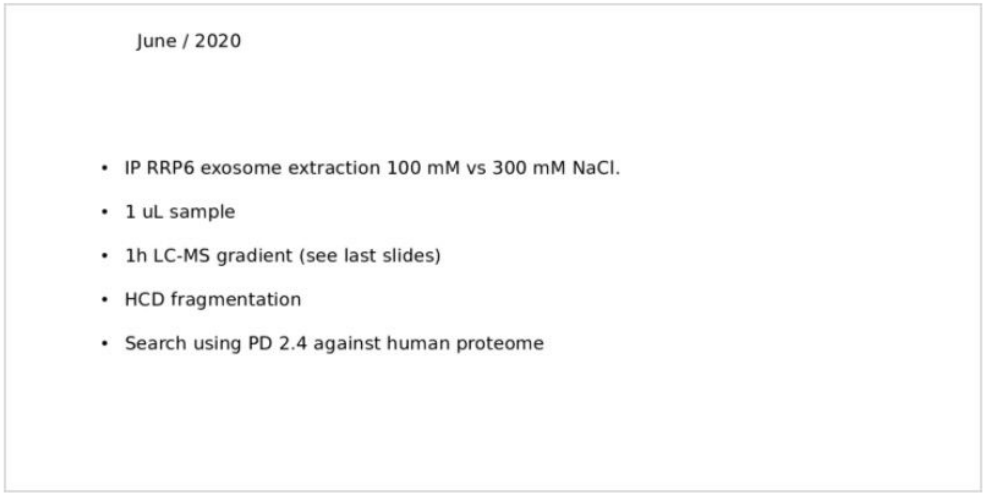
4 ft

4 n.el

4 LDS el

Then these IPs were analysed by MS

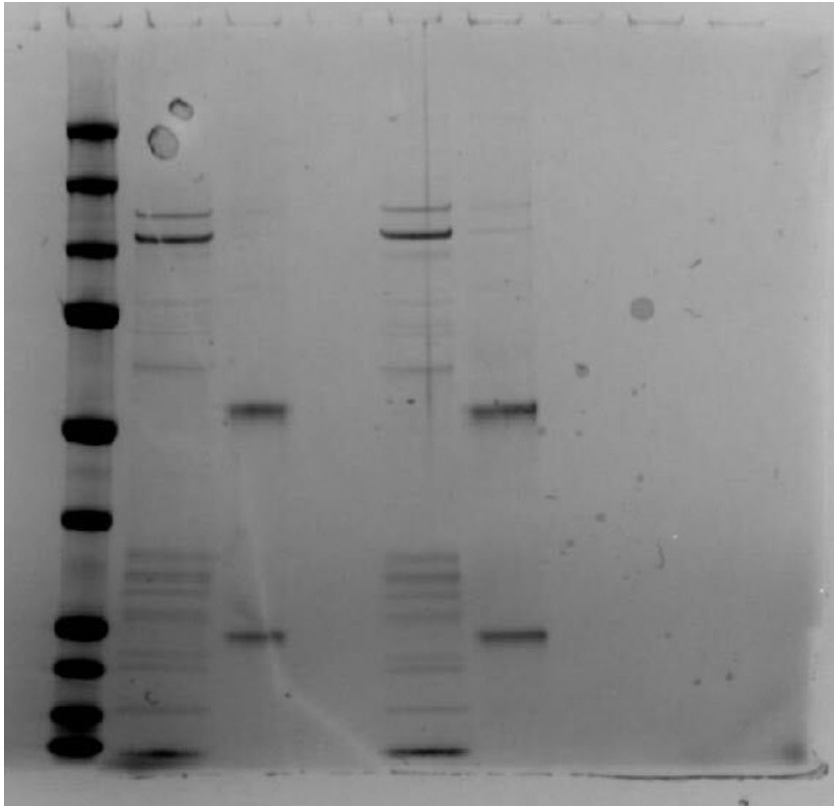
IPs June 2020.pptx
510 kB PowerPoint presentation



S-Trap Micro Ulra -High Recovery Protocol

https://macromolecule-child.rockefeller.edu/#16%2F06%2F2020_S-Trap%20Micro%20Ulra%20-High%20Recovery%20Protocol

Then we moved to MgCl2 samples (23.6.20)



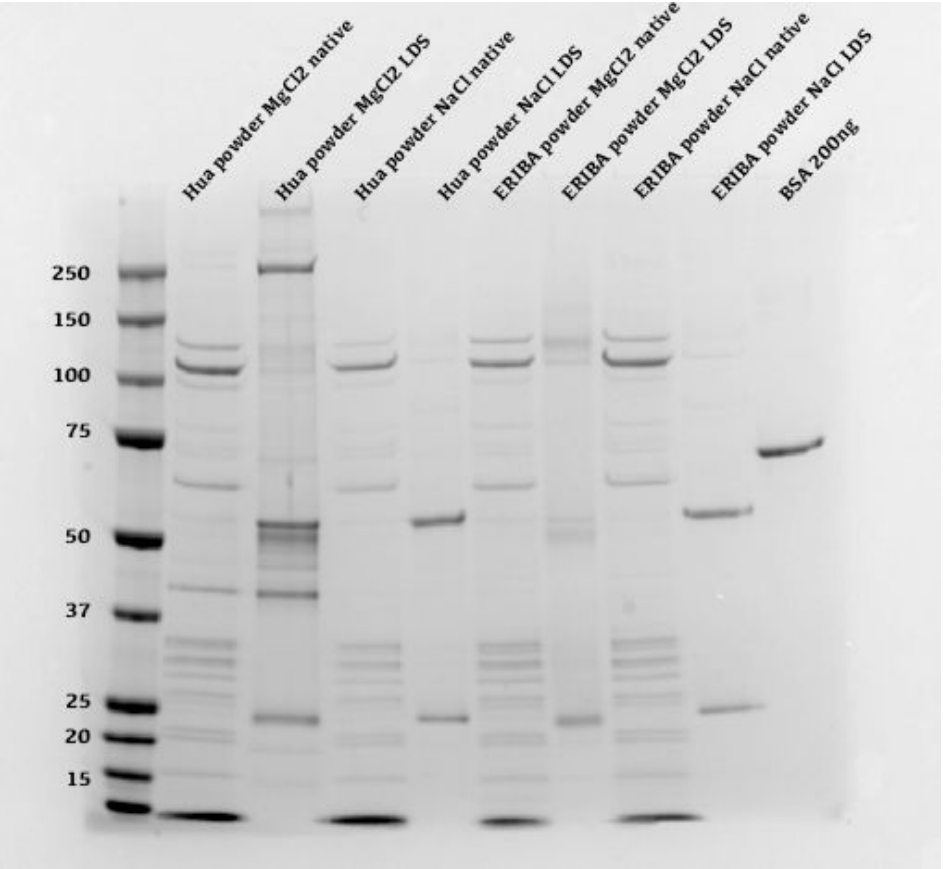
https://macromolecule-child.rockefeller.edu/#23%20June%202020_Preliminary%20Test%20for%20ZCCHC8

Hua's powder

First test

1. Marker_5ul prestained
2. Native RRP6_300mM NaCl (15ul, 50% of 100mg IP)
3. LDS RRP6_300mM NaCl (15ul, 50% of 100mg IP)
4. Native RRP6_100mM *MgCl2* (15ul, 50% of 125mg IP)
5. LDS RRP6_100mM *MgCl2* (15ul, 50% of 125mg IP)

MgCl2 samples (comparison Hua/Leila powder) 24.6.20



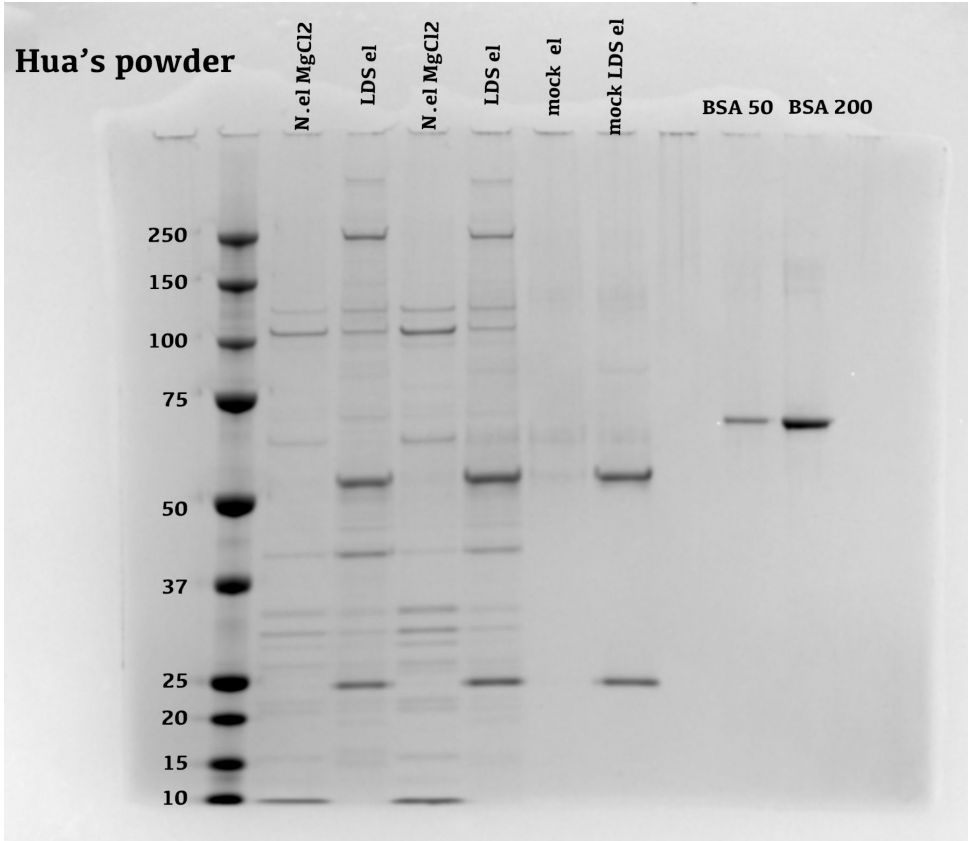
Got a contamination in 2nd line

https://macromolecule-child.rockefeller.edu/#24%20June%202020_Test%20for%20ZCCHC8%20Hua's%20and%20Leila's%20powder

Ladder

1. Hua powder MgCl2 native
2. Hua powder MgCl2 LDS
3. Hua powder NaCl native
4. Hua powder NaCl LDS
5. ERIBA powder MgCl2 native
6. ERIBA powder MgCl2 LDS
7. ERIBA powder NaCl native
8. ERIBA powder NaCl LDS
9. BSA 200ng

MgCl2 samples 30.6.20



https://macromolecule-child.rockefeller.edu/#30%20June%202020_Triplicate%20test%20for%20ZCCHC8

Luciano and I did triplicate MgCl2 IPs.

Rest of each sample was used for MS

Combined Native elution *MgCl2* = 5 ul of native elution from each replicate pooled. Total 15 ul

LDS combined elution = 6,5 ul of LDS elution from each replicate pooled. Total 19,5 ul

https://macromolecule-child.rockefeller.edu/#22%2F07%2F2020_S-Trap%20Micro%20Ulra%20-High%20Recovery%20Protocol%20(MgCl2) S-Trap Micro Ulra -High Recovery Protocol (MgCl2)

Then we ordered new chemicals, remade all buffers

https://macromolecule-child.rockefeller.edu/#15%2F7%2F20%20MgCl2%20100%20mM%204%20samples and other reports from 15.7.20, 22.7.20, 23.7.20

NaCl, MgCl2 IPs with new buffers (4 replicates, will be used for MS and for anti-ZCCHC8 Ab test)

I still have these NaCl 300/700 mM samples in case someone want to do WB.

https://macromolecule-child.rockefeller.edu/#22%2F07%2F2020_S-Trap%20Micro%20Ulra%20-High%20Recovery%20Protocol%20(MgCl2) S-Trap Micro Ulra -High Recovery Protocol (MgCl2 100 mM)

https://macromolecule-child.rockefeller.edu/#24%2F07%2F2020_S-Trap%20Micro%20Ulra%20-High%20Recovery%20Protocol%20(NaCl%20300%2F700%20mM) S-Trap Micro Ulra -High Recovery Protocol (NaCl 300/700 mM)

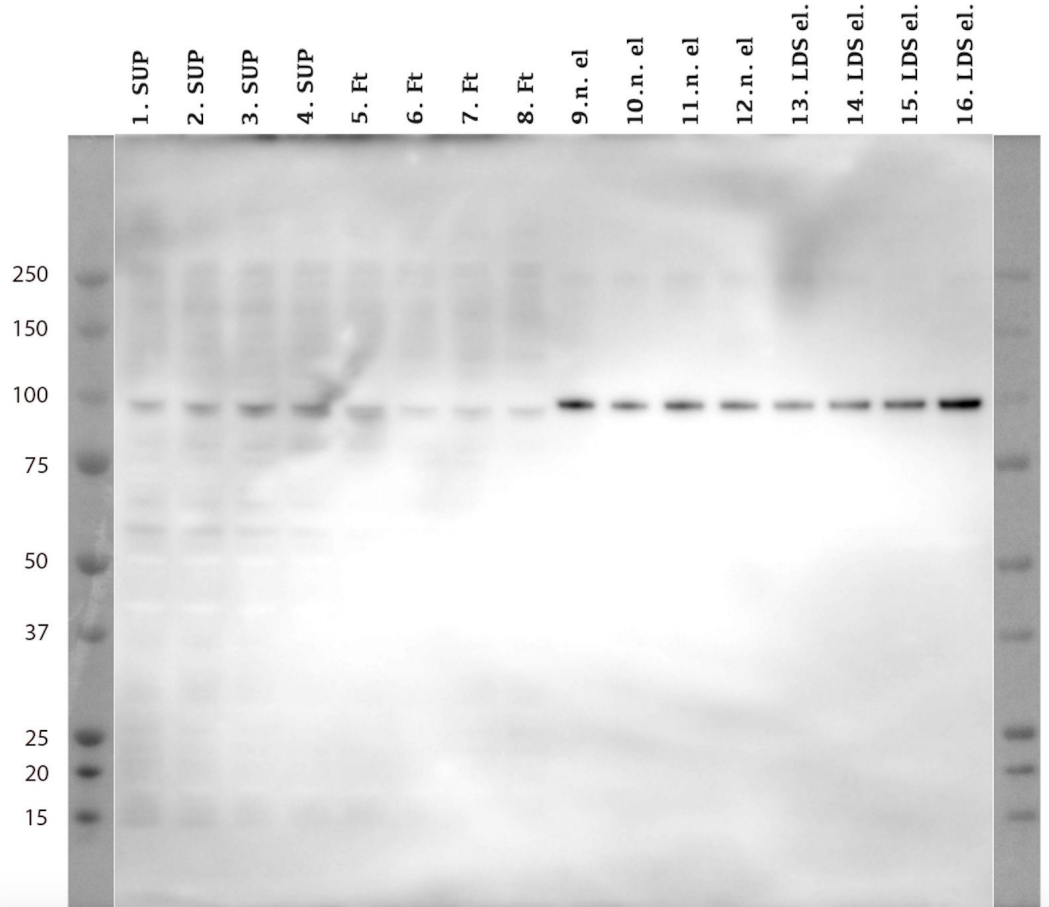
Here is a MS report

https://app.box.com/file/766357470481?s=9dilr83bz8qvr5aj44vmmuue5qj77qku&utm_campaign=collab%20auto%20accept%20user&utm_medium=email&utm_source=trans

WB with MgCl2 to test new ZCCHC8 Ab 1:1000

1 ul of SUP / Ft / n.el

9,5 ul of LDS el.



17/8/20 Triplicate experiment NaCl 300/700, MgCl2 100 mM

Wiki protocol

https://macromolecule-child.rockefeller.edu/#17%2F8%2F20%20NaCl%20300%2C%20700%20mM%20and%20MgCl2%20100mM%20protocol

Report

https://app.box.com/file/766357720572?s=gplnujc2oye33co0dpkwc7i9xta9r3av&utm_campaign=collab%20auto%20accept%20user&utm_medium=email&utm_source=trans

And that is it with IPs. We didn’t continue with that experiment.

N2102EP 24-well Hand Screen 11-2020

admin 25th January 2021 at 11:31pm

Image and conditions need to be uploaded on http://copurification.org/

Comments from Nastya

buffer preparation step:
19th buffer was really white and it was hard to filter it.

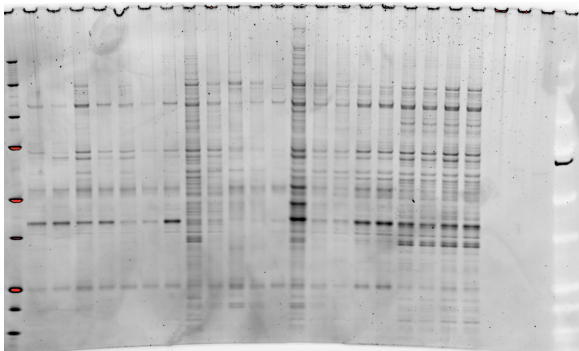
extraction step:
18th, 19th, 20th - the lysate started to separate. 18th, 19th, 21st, 22nd and 23rd buffers left a big pellet after centrifugation.

washing step:
18th buffer made a crystals while washing beads, so beads were washed with DPBS. 19th buffer was frozen touched with a tip, so beads for lysate with 19th buffer were also washed just with DPBS. 20th made crystals while washing beads - then beads were washed with DPBS.

glutaraldehyde:
22nd, 23rd and 24th buffers were with glutaraldehyde only for extraction and the protease inhibitor wasn't used here. All washing steps for these conditions were made without glutaraldehyde. glutaraldehyde tips: use cold water to disolve it.

This report was added to WIKI by Dennis Nanninga due to Nastya's leave.

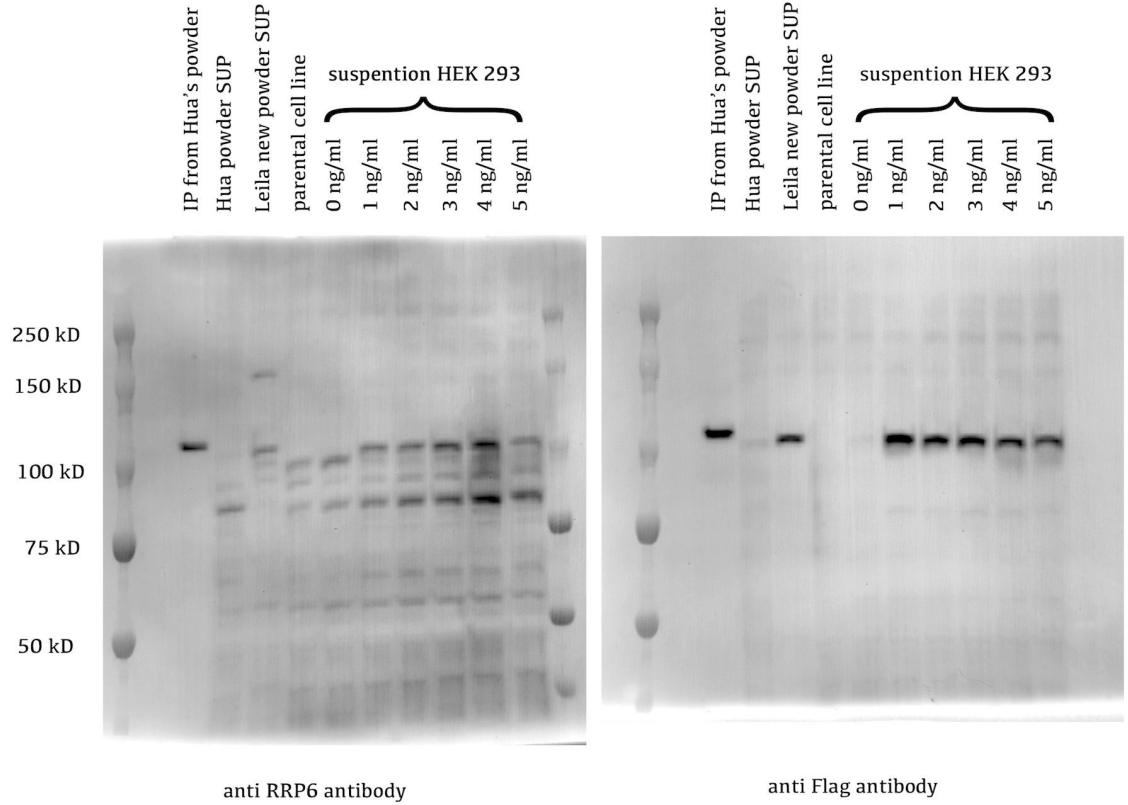
N2102EP 24-well Hand Screen



3. HEK 293 RRP6-3xFlag in suspension

*Note: We observed that 1 ng/ml of tetracycline in suspension culture was sufficient to give expression comparable to 5 ng/ml in adherent growth. However, we encourage researchers to test expression in their hands by carrying out an anti-RRP6 Western blot after induction (*Domanski et al., 2012*). This protocol can be miniaturized using smaller culture vessels for pre-tests of that nature.*

From “Affinity Purification of the RNA Degradation Complex, the Exosome, from HEK-293 Cells” Domanski, LaCava 2017



3x-Flag signal starts from 1 ng/ml and there no pattern when concentration of dox increases.

https://macromolecule-child.rockefeller.edu/#Oct%202020%20HEK%20293%20RRP6-3xFlag%20Flp-In%20TREX%20cell%20line%20DOX%20test%20induction

It’s impossible to tell where is a band of RRP6 without 3xFLAG on this blot

Suggestion - change Ab or use commercial pure RRP6 protein as a control

4. Jess experiments

Jess

> Jess experiment to test anti-rrp6 Ab
>
> Jess experiment with HEK 293 RRP6-3xFlag Flp-In TREX

Main info from these experiments:

- **Best sample concentration 0.4 ug/ul**
- **Best 1:250 of anti-rrp6 Ab**
- **35 min (maximum setting) it is not enough to separate 100 kDa and 103 kDa clearly in 12-230 kDa cassette. Might need to adjust other settings too.**

5. Hela S3 Flp-In TREX RRP6-3xFlag workflow

Goals - Growing cell in suspension. Why?

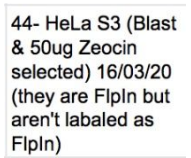
- It is cheaper - with the same volume of media you will get more cells, use less plastic (possible to reuse)
- It is easier to harvest suspension cells

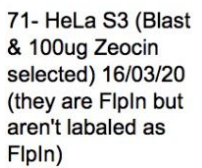
Disadvantages:

- Counting
- Not every cell line can grow well in suspension

5. Hela S3 Flp-In TREX RRP6-3xFlag workflow

1. John got this Hela S3 Flp-In TREX cell line from his collaborators in 2013
2. (before I came in March) Leila and Arianna selected cells with Zeocin (50 ug/ml and 100 ug/ml) and Blast (5 ug/ml)
3. In the middle of March we made stocks from these cells (day before lockdown)





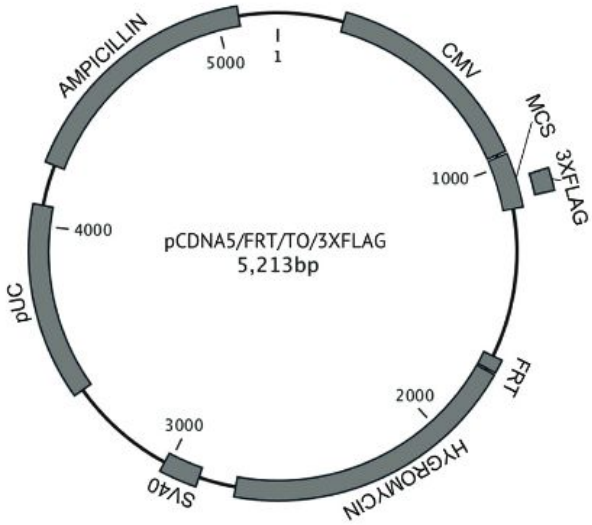
4. May-June I started to condition cells to suspension growing. Harvest them, made powder, keep few bottles of cells till August because Leila and Lars did some suspension transfection with them

5. Hela S3 Flp-In TREX RRP6-3xFlag workflow

5. 10th August we got plasmid

6. I made a transformation, inoculation and extracted plasmid DNA (2,3 mg)

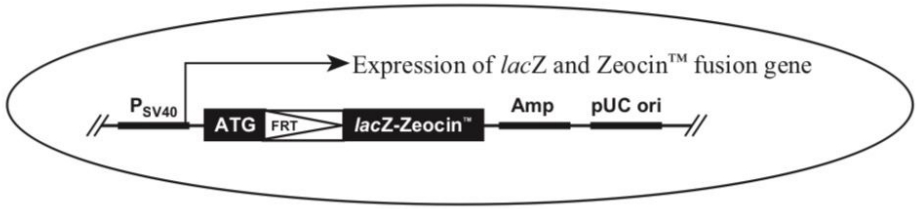
Name of Plasmid	date of isolation	colony number	volume (ul)	concentration (ng/ul)	amount (ug)	260/280
RRP6-3XFLAG	01/09/2020	2	200	1058	211,6	1.92
RRP6-3XFLAG	01/09/2020	2	156,6	2306	361,1196	1.90
RRP6-3XFLAG	01/09/2020	2	200	1864	372,8	1.91
RRP6-3XFLAG	01/09/2020	2	200	2753	550,6	1.93
RRP6-3XFLAG	18/08/2020	2	200	2079	415,8	1.92
RRP6-3XFLAG	18/08/2020	2	200	1400	280	1.93
RRP6-3XFLAG	18/08/2020	2	200	723	144,6	1.94



From John “the PNG I attached says CMV but actually it’s CMV-TetR.”

7. Since I had cells in suspension I asked Leila if it’s necessary to use stock for transection and we decided to seed cells from suspension on flasks and then use them.

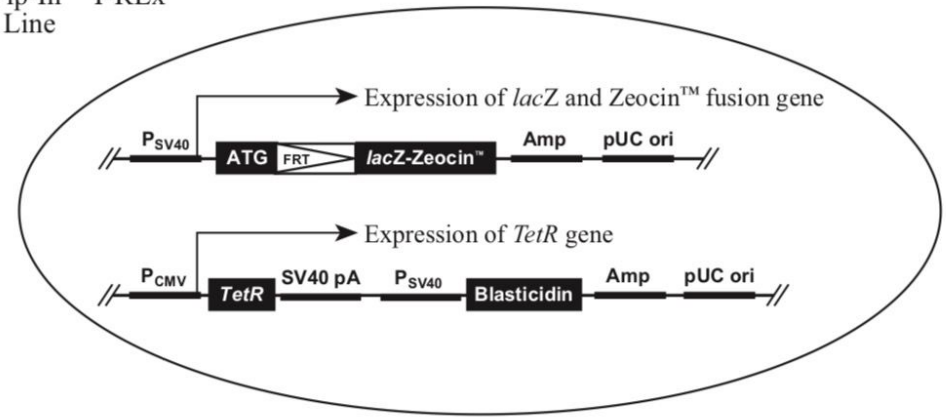
1. pFRT/*lac*Zeo is stably transfected into the mammalian cells of interest to generate the Zeocin™-resistant Flp-In™ Host Cell Line



Flp-In™ Host Cell Line

+ pcDNA6/TR©

2. The pcDNA6/TR© vector is stably transfected into the Flp-In™ Host Cell Line to generate the Zeocin-resistant, blasticidin-resistant Flp-In™ T-REx™ Host Cell Line



Flp-In™ T-REx™ Host Cell Line

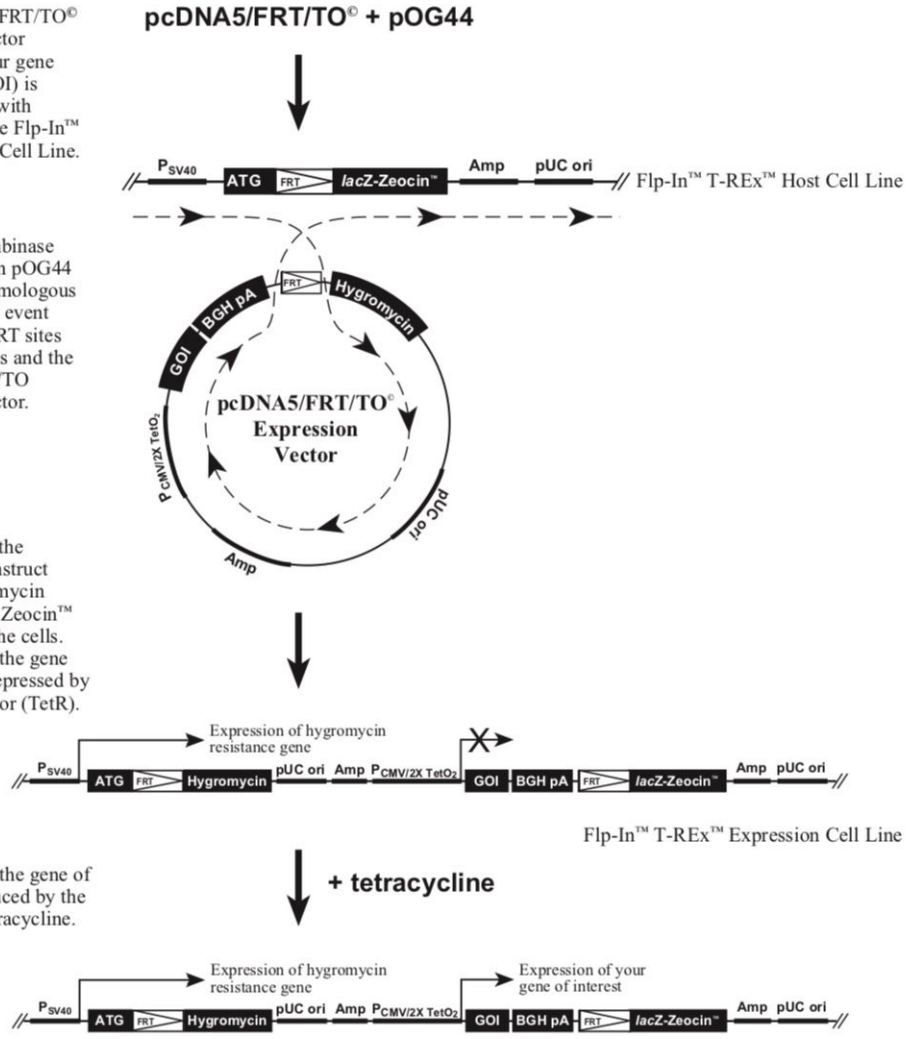
John's collaborators part of work

1. The pcDNA5/FRT/TO© expression vector containing your gene of interest (GOI) is cotransfected with pOG44 into the Flp-In™ T-REx™ Host Cell Line.

2. The Flp recombinase expressed from pOG44 catalyzes a homologous recombination event between the FRT sites in the host cells and the pcDNA5/FRT/TO expression vector.

3. Integration of the expression construct confers hygromycin resistance and Zeocin™ sensitivity to the cells. Expression of the gene of interest is repressed by the Tet repressor (TetR).

4. Expression of the gene of interest is induced by the addition of tetracycline.



Our part of work

5. Hela S3 Flp-In TREX RRP6-3xFlag workflow

15.9.20 Transfection (Lipofectamine 3,75 and 7,5 ul) - 2 T25 flasks. 80% confluency.

https://docs.google.com/document/d/1m4JZ3JHzneVUvlGO902WN-XFDr5BtZ4vKYKEMS6CvPw/edit (will be in wiki, Apostolis already have it, needs to be corrected)

16.9.20 Moved cells to T175 flask, after 3 hours change media to media with Hygromycin B selection (100 ug/ul). Lots of cells died, media was changed if necessary.

21.10.20 Cells were dence, I splitted each to 2 T175 (it was super bad decision because I used only ⅛ of T175 for new flask). After that cells were growing slowly under Hygromycin B (media was changed if necessary) before 24.10.20.

5. Hela S3 Flp-In TREX RRP6-3xFlag workflow

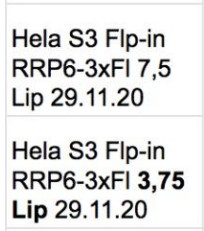
24.10.20 I checked with John if it is necessary to keep Hygro B+Blast in media and we decided to stop antibiotic selection.

In 3,75 Lip flask there were more colonies (but it maybe the splitting had cased this differences)

3 weeks after cells were more dense and I decided to make suspension and stocks (LaCava #1F (Flp-In))

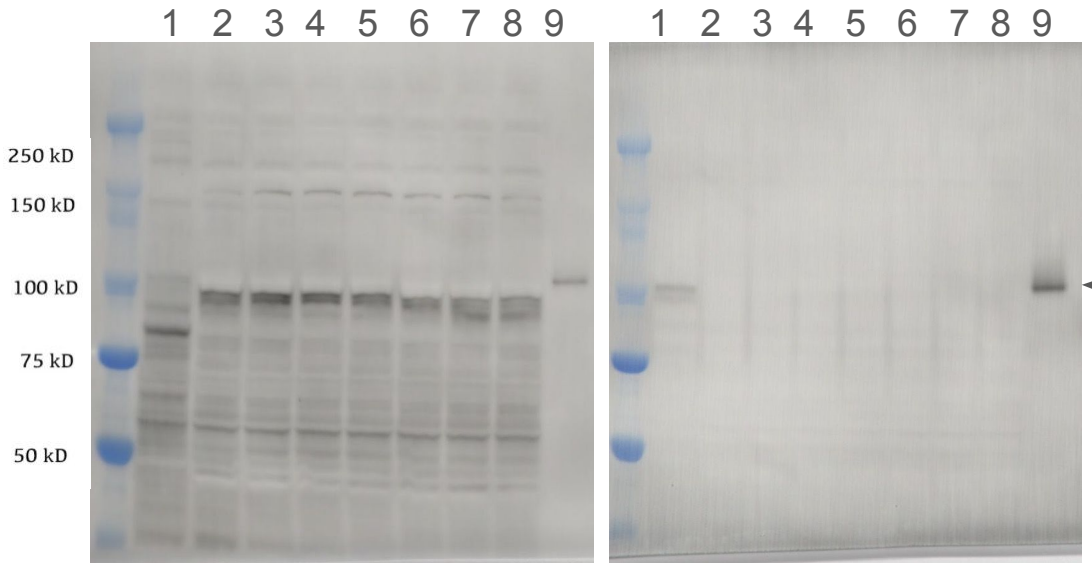
28.11.20 Cells from suspension were induced with different concentration of DOX

3.12.20 There were no RRP6-3xFlag in them



Hela S3 Flp-In TREX RRP6-3xFlag

0-12 ng/ml of DOX



1. Hua Sup 300mM NaCl
2. Parental cell line
3. 0 ng/ml Dox
4. 1 ng/ml Dox
5. 2 ng/ml Dox
6. 4 ng/ml Dox
7. 6 ng/ml Dox
8. 12 ng/ml Dox
9. N.el control

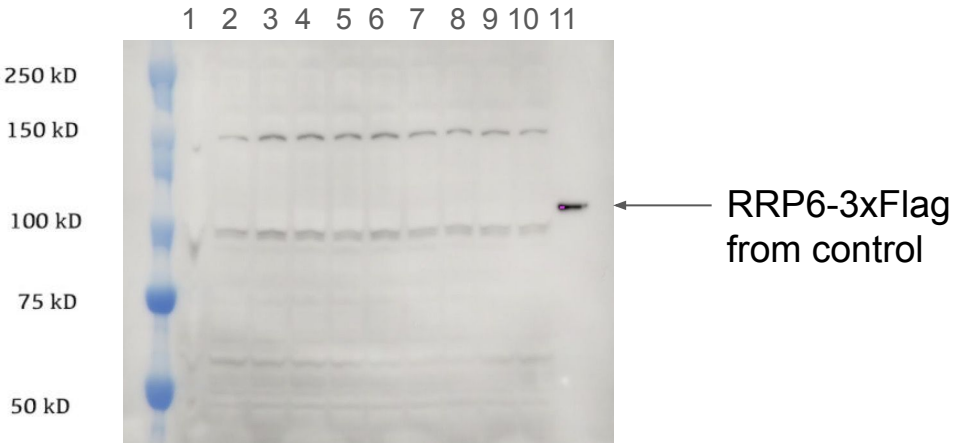
anti-RRP6 1:1000

anti-Flag 1:1000

Same blot

Hela S3 Flp-In TREX RRP6-3xFlag

0-640 ng/ml of DOX



1. Parental cell line
2. 0 ng/ml Dox
3. 5 ng/ml Dox
4. 10 ng/ml Dox
5. 20 ng/ml Dox
6. 40 ng/ml Dox
7. 80 ng/ml Dox
8. 160 ng/ml Dox
9. 320 ng/ml Dox
10. 640 ng/ml Dox
11. N.el control

anti-RRP6 1:1000

First WB by Apostolis

Hela S3 Flp-In TREX RRP6-3xFlag troubleshooting

I took cells for transfection NOT from stock but from suspension cell line that I was growing before. They were without antibiotics (Zeo/Blast) for a long time in suspension. After transfection I used Blast 5mg/ml with Hygro B 100 mg/ml. But maybe Hygro B 100 mg/ml it's not enough.

We didn't sequence plasmid (we have a seq file from Michael)

It was unnecessary to move from adherent to suspension while testing.

Ratio between DNA:Lipofectamine 3000 in my protocol was 10:3,75 and 10:7,5

It's a mistake. But I just used to much of DNA. In Lipofectamine protocol they used less DNA, same volume of Lipofectamine for same amount of cells 1*10^6.

DNA: transfection reagent ratio sub-optimal for cell line	Prepare complexes using a DNA (µg) to Lipofectamine® 2000 (µl) ratio of 1:2 to 1:3 for most cell lines. Optimization may be necessary. If so, vary DNA (µg): Lipofectamine® 2000 (µl) ratios from 1:0.5 to 1:5. If using a different transfection reagent, please consult the product manual.

Hela S3 Flp-In TREX RRP6-3xFlag troubleshooting

Hela S3 Flp In TREX #11+ blast 7/7/20
Hela S3 Flp In TREX #11+ blast 7/7/20
Hela S3 Flp In TREX #11+ blast 7/7/20
Hela S3 Flp In TREX #11+ blast 7/7/20

Stocks before conditioning

Timeline		Steps	Procedure Details
Day 0	1	Seed cells to be 70–90% confluent at transfection	
	2 (Vortex 2–3 sec)	Dilute Lipofectamine® 3000 Reagent in Opti-MEM® Medium – Mix well	
	3	Prepare master mix of DNA by diluting DNA in Opti-MEM® Medium, then add P3000™ Reagent – Mix well	
Day 1	4	Add diluted DNA to each tube of diluted Lipofectamine® 3000 Reagent (1:1 ratio)	
	5	Incubate	
	6	Add DNA-lipid complex to cells	
Day 2–4	7	Visualize/analyze transfected cells	

Component	96-well	24-well	6-well
Adherent cells	1–4 × 10^4	0.5–2 × 10^5	0.25–1 × 10^6
Opti-MEM® Medium	5 µL × 2	25 µL × 2	125 µL × 2
Lipofectamine® 3000 Reagent	0.15 and 0.3 µL	0.75 and 1.5 µL	3.75 and 7.5 µL
Opti-MEM® Medium	10 µL	50 µL	250 µL
DNA (0.5–5 µg/µL)	0.2 µg	1 µg	5 µg
P3000™ Reagent (2 µL/µg DNA)	0.4 µL	2 µL	10 µL
Diluted DNA (with P3000™ Reagent)	5 µL	25 µL	125 µL
Diluted Lipofectamine® 3000 Reagent	5 µL	25 µL	125 µL

Incubate for 5 minutes at room temperature.

Component (per well)	96-well	24-well	6-well
DNA-lipid complex	10 µL	50 µL	250 µL
DNA amount	100 ng	500 ng	2500 ng
Enhancer amount	0.2 µL	1 µL	5 µL
Lipofectamine®3000 Reagent used	0.15 and 0.3 µL	0.75 and 1.5 µL	3.75 and 7.5 µL

Incubate cells for 2–4 days at 37°C. Then, analyze transfected cells.

Seed 2 T25 flasks of cells to be **70-90%** confluent at transfection.

For each flask:

- 9 ug of pOG44 (433 ng/ul, so it will be **21 ul** of pOG44 in each transfection)
- 1 ug of RRP6-3XFLAG plasmid (1058 ng/ul, so it will be ~ **1 ul**)
- P3000™ Reagent (2 µL/µg DNA)
- 3.75 or 7.5 µL of Lipofectamine 3000

1. Seed cells to be 70–90% confluent at transfection
2. Dilute Lipofectamine™ 3000 Reagent in Opti-MEM™ Medium (2 tubes)– Mix well

Number of flask	1	2
Lipofectamine 3000	3.75	7.5 ul
Opti-MEM™ Medium	125 µL	125 µL

3. Prepare master mix of DNA by diluting DNA in OptiMEM™ Medium, then add P3000™ Reagent– Mix well

250 ul of Opti-MEM™ Medium + 42 ul of pOG44 + 2 ul of RRP6-3XFLAGplasmid + 40 ul of P3000TM Reagent (2 µL/µg DNA)

4. Add Diluted DNA to each tube of Diluted LipofectamineTM 3000 Reagent (1:1 ratio = 125 ul:125 ul)
5. Incubate for 10–15 minutes at room temperature.
6. Add DNA-lipid complex to cells
7. **Approx. 24 h later** split the cells and place them in T175 flask without antibiotic.
8. **Approx. 3 h later** aspirate media. Add media containing 100 ul/ml Hygromycin B and 10 ug/ul Blasticidin and 1% pen/strep.
9. Change media every 4-5 days
10. After 2 weeks colonies that are visible to the naked eye should have appeared.
11. Split cells and grow continuously in DMEM/10%FBS/1%pen/strep containing selection antibiotic.
12. Make a frozen stocks of obtained cell line