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Project: iGEM_Munich2019 Shared Project
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Johanna and Sarah

Heparin-Purification (min6 + HEK293T)

- Stock Solutions
  - 1 M HEPES, PH 7.2 (0.5 L: 119.15 g HEPES (free acid), MW: 238.3)
  - 5 M NaCl (0.5 L: 29.22 g NaCl, MW: 58.44)
  - 1 M NaOH (0.5 L: 20 g NaOH, MW: 40)
  - 30 % 2-Propanol
- Buffer A:
  - 50 mM HEPES, pH 7.2
- Buffer B
  - 50 mM HEPES, 2 M NaCl, pH 7.2
- Equilibration Buffer
  - 50 mM HEPES, 120 mM NaCl, pH 7.2
  - 5 CV = 5 mL
- Wash Buffer
  - 50 mM HEPES, 120 mM NaCl, pH 7.2
  - 10 CV = 10 mL
- Linear Salt Gradient: 6 - 100 % over 20 CV = 20 mL, 100 % B
- Regeneration
  - 1 M NaOH, 10 CV = 10 mL
  - 30 % 2-Propanol, 10 CV = 10 mL

Preparation

Prepare Stock Solutions, filter them!
Prepare Buffer A, Buffer B, Equilibration Buffer, Wash Buffer
Prepare Elution Buffer (20 x 10 mL -> label 20 x 15 mL Falcons)
Prepare XY x FT Eppis, 10 x Wash Eppis, 20 x Elution Eppis
Equilibrate the heparin resin with 5 CV **Equilibration Buffer**
Prepare the sample: harvest the supernatant form the 6-well plate. Centrifuge 10 minutes at 2000 g.
Filtration: Filter the harvested supernatant through a 0.8 µm syringe filter
Load the sample on the resin -> collect Flow-through = XY mL = XY fractions

Wash

Wash with 10 CV Was Buffer = 10 fractions

Elution

Elute with a salt gradient (120 - 20000 mM NaCl) with 20 CV = 20 fractions

Elution Pattern

	NaCl [mM]	Buffer A [µL]	Buffer B [µL]	Buffer A [mL]	Buffer B [mL]
1	150	925	75	**9.25**	**0.75**
2	200	900	100	**9**	**1**
3	300	850	150	**8.5**	**1.5**
4	400	800	200	**8**	**2**
5	500	750	250	**7.5**	**2.5**
6	600	700	300	**7**	**3**
7	700	650	350	**6.5**	**3.5**
8	800	600	400	**6**	**4**
9	900	550	450	**5.5**	**4.5**
10	1000	500	500	**5**	**5**
11	1100	450	550	**4.5**	**5.5**
12	1200	400	600	**4**	**6**
13	1300	350	650	**3.5**	**6.5**
14	1400	300	700	**3**	**7**
15	1500	250	750	**2.5**	**7.5**
16	1600	200	800	**2**	**8**
17	1700	150	850	**1.5**	**8.5**
18	1800	100	900	**1**	**9**
19	1900	50	950	**0.5**	**9.5**
20	2000	0	1000	**0**	**10**

10 CV at the end of the gradient with **100 % Buffer B**

Regeneration

10 CV 1 M NaOH

10 CV 30 % 2-Propanol

Data Analysis

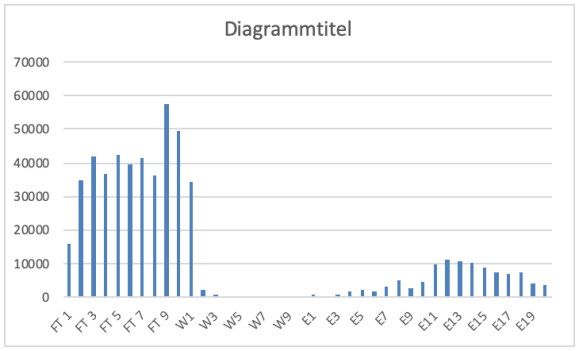
Measure A280 of each fraction (Flow-Through, Wash, Elution) .

SDS Page: L, FT, W, E1, E2, E3,... (total: XY combs?)

results:

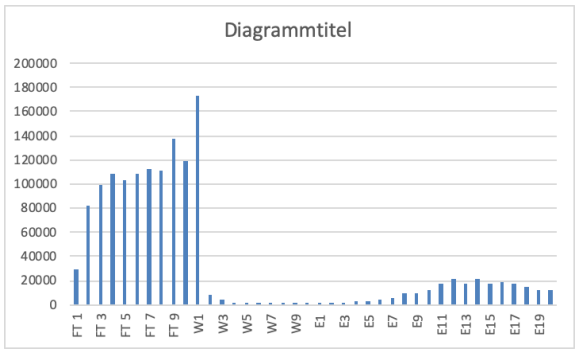
- min6 VLP empty:

image.png



- min6 VLP loaded:

image.png



- concentrate all elution-fractions with 30 kDa centricons

Alejandro

HiBit assay

- on 96-well plates with both HEK and min6 cells
- **VLP/exosomes Lysis Buffer:** Add Protease Inhibitor Cocktail (SigmaAldrich, P8340) 1:50 to VLB (1X PBS, 1 % Triton X-100), prepare 40 µl per well + 500 µl.
  - **PBS for mixing supernatant unlysed samples (1:1)**: Add Protease Inhibitor Cocktail 1:50 to PBS4MIX, prepare 40 µl per well + 500 µl.
  - **Cell Lysis Buffer**: Add Protease Inhibitor Cocktail 1:100 to CLB 1X PBS, 0.5 % Triton X-100), prepare 400 µl per well + 500 µl.
  - **PBS for diluting all samples (1:10)**:Add Protease Inhibitor Cocktail 1:100 to PBS4DIL, prepare 135 µl per well + 1.5 ml.
- **Supernatant harvesting**
  Harvest 120 µl supernatant containing the VLPs 72 h after transfection / exosomes 48 h after transfection and 24 h after medium exchange (VLPs) into a black flatbottom 96-well plate (= SN centrifugation plate).
  Centrifuge the plate at 2000 g for 10min to remove dead cells and bigger cell debris.
  Transfer 90 µl supernatant to a PCR 96-well plate (= SN lysed plate) and work on ice from this step on. Discard the plate with the pelleted cells.
  Add 10 µl PBS4MIX to a new PCR 96-well plate (= SN unlysed plate).
  Transfer 10 µl from the centrifuged supernatant to the SN unlysed plate and mix well (6 times up and down).
  Add 80 µl VLB to the SN lysed plate and mix well (6 times up and down). Put a plastic foil over the plate and make sure all the wells are well closed. Incubate then the plate at 60 °C for 15 min in a thermocycler and put it afterwards back on ice.

Dilute samples from both the SN lysed and SN lysed plates 1:10 by transferring 4 µl to two new white flatbottom 96-well plates containing 36 µl PBS4DIL (= SN lysed 4HiBit and SN unlysed 4HiBit plates). Aliquot 38 µl PBS4DIL with a multichannel pipette and push only until the pressure point to avoid adding bubbles. About 2 µl will remain inside the plastic tips. Push all the way through when adding the 4 µl sample. Mixing is not necessary.

You can freeze the remaining 155 µl sample in the SN lysed plate by putting it in the –80 °C freezer to do a qPCR with the same samples.

Analyze the sample content with the Nano-Glo® HiBiT Extracellular Detection System (Promega). Add 42 µl freshly prepared HiBiT Reaction Mix (push only until the pressure point) to the 40 µl diluted samples (equivalent to 1.25 % or 1/80 total supernatant) and shake the plates at 300 min^{-1} for 10 min at room temperature before measuring the luminescence.

Cell content analysis

Carefully remove the remaining 40 µl medium in the wells.

Add 160 µl CLB to each well and pipette up and down 16 times washing well the whole well area.

Centrifuge the cell culture plate at 3.000 rcf for 10 min.

Transfer 120 µl and into a new PCR 96-well plate (= CC plate) and work on ice from this step on. Discard the plate with the pelleted cell debris.

Put a plastic foil over the plate and make sure all the wells are well closed. Incubate the plate at 60 °C for 15 min in a thermocycler and then put the plate back on ice.

Dilute the samples 1:10 by transferring 4 µl to a new white flatbottom 96-well plate containing 38 µl PBS4DIL (= CC 4HiBit plate). Aliquot 38 µl PBS4DIL with a multichannel pipette and push only until the pressure point to avoid adding bubbles. About 2 µl will remain inside the plastic tips. Push all the way through when adding the 4 µl sample. Mixing is not necessary.

You can freeze the remaining 115 µl sample in the SN lysed supernatant plate by putting it in the –80 °C freezer to do a qPCR with the same samples.

Analyze the sample content with the Nano-Glo® HiBiT Extracellular Detection System. Add 42 µl freshly prepared HiBit Reaction Mix (push only until the pressure point) to the 40 µl diluted samples (equivalent to 2.5 % or 1/40 total supernatant) and shake the plate at 300 min^{-1} for 10 min at room temperature before measuring the luminescence.

HiBiT Calibration Curve

Dilute the 20 µM HiBiT Control Protein (Promega) 1:1000 in PBS4DIL (1 µl protein + 999 µl PBS4DIL) and mix well. You can use this 20 nM solution for several HiBiT assays. After step 19 freeze it at –20 °C.

Make a dilution series in six 1.5 Eppis according to the following table and measure each solution in replicates on each white flatbottom 96-well plate:

calibration curve

	A	B	C	D	E	F	G
1	20 nM solution	0 µl	3.75 µl	7.5 µl	11.25 µl	15 µl	18.75
2	PBS4DIL	300 µl	296 µl	293 µl	289 µl	285 µl	281
3	**Total Volume**	300 µl	300 µl	300 µl	300 µl	300 µl	300