09. (September) 2019

Project: iGEM_Munich2019 Shared Project
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FRIDAY, 20/9/2019

Johanna + Sarah

Heparin-Purification

- Materials
  - HiTrap Heparin HP
    - **(Flow Rate: 0.5 mL/min)**
    - **Fraction size: 1 ml**
  - 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

Purification 20/09/2019

	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**

Regeneration

10 CV 1 M NaOH

10 CV 30 % 2-Propanol

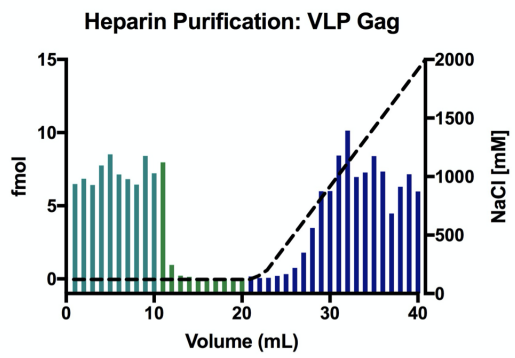
Data Analysis

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

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

- readout: HiBit
- results
  -

image.png



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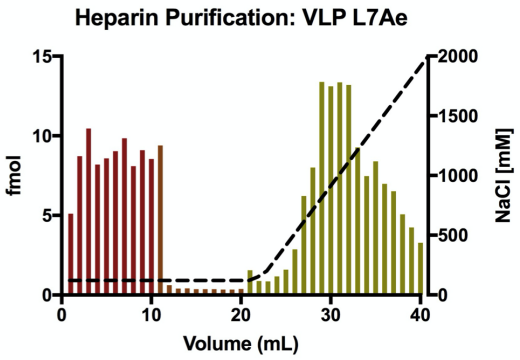

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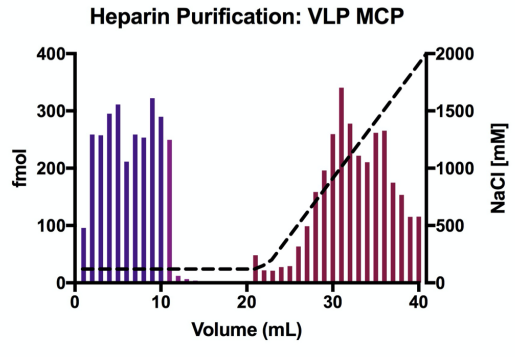

Alejandro

cell culture

- Splitting: passage 37
- medium exchange over MIN6-cells

Moritz

VLP-harvesting and HiBit on MIN6-cells

- Harvesting from 24-well plates
  - Buffers:
    - **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 600 µl supernatant containing the VLPs 72 h after transfection / exosomes 48 h after transfection and 24 h after medium exchange into 1.5 mL Eppis.
    Centrifuge at 5500 rpm (2029 g in *Eppendorf MiniSpin*) for 10min to remove dead cells and bigger cell debris.
    Transfer 400 µl supernatant to a new 1.5 mL Eppi and work on ice from this step on. Discard the plate with the pelleted cells.
    Transfer 2 X 40 µl from the centrifuged supernatant to two new 1.5 ml Eppis, one containing 40 µl PBS4Mix (unlysed supernatant) and the other one containing 40 µl VLB (lysed supernatant). Mix well, but do not vortex to avoid foam formation in the lysed supernatant samples.
    Incubate the lysed supernatant samples at 60 °C for 10 min. Then put the samples on ice.
    Shock-freeze the remaining 320 µl centrifuged supernatant and store it at –80 °C to do a qPCR with the same samples.
    Dilute both the non-lysed and the lysed samples 1:10 in cold PBS with Protease Inhibitor Cocktail 1:100 (5 µl sample + 45 µl PBS = PBS4DIL) and mix well by pipetting up and down.
    Analyze the sample content with the Nano-Glo® HiBiT Extracellular Detection System (Promega). Transfer 42 µL of the samples (push only until the pressure point) into a white 96 well plate. 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 200 µl medium in the wells.
    Add 400 µl CLB to each well and pipette up and down 16 times washing well the whole well area.
    Resuspend and transfer the cell lysate to 1.5 ml Eppis and centrifuge the tubes at 16.000 rcf in the cooled centrifuge for 10 min at 4°C.
    Collect first 80 µl and then 200 µl of the supernatants into two new 1.5 ml Eppis. Discard the cell debris pellet. Put the 80 µl aliquots on ice and shock-freeze the remaining 200 µl one to store it at –80 °C and do a qPCR with the same samples.
    Incubate the 80 µl cell lysates at 60 °C for 10 min. Then put the samples on ice.
    Dilute the lysed samples 1:10 in cold PBS with Protease Inhibitor Cocktail 1:100 (5 µl sample + 45 µl PBS = PBS4DIL) and mix well by pipetting up and down.
    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. Transfer 42 µL of the samples (push only until the pressure point) into a white 96 well plate. 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 HiBit

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

Alejandro

VLP purification over biotin beads and fluorescence microscopy

- **Protocol for harvesting from 6-well plates (2.5 ml medium)**
  a. Buffer preparation.
     I. Prepare 4 ml PBS per well in a 15/50 ml Falcon tube (put it on ice).
     II. Add Protease Inhibitor Cocktail (SigmaAldrich, P8340) 1:100 to CLB (cell lysis buffer: 1X PBS, 0.5 % Triton X-100), prepare 220 µl per well (put it on ice).
     III. Add Protease Inhibitor Cocktail 1:50 to VLB (VLP lysis buffer: 1X PBS, 1 % Triton X-100), prepare 280 µl per well (put it on ice).
     IV. Add Protease Inhibitor Cocktail 1:50 to PBS4MIX (PBS for mixing supernatant unlysed samples 1:1), prepare 280 µl per well (put it on ice).
     V. Add Protease Inhibitor Cocktail 1:100 to PBS4DIL (PBS for diluting samples 1:10), prepare 550 µl per well (put it on ice).
  b. Equilibration.
     I. Use 100 µl of biotin magnetic bead suspension per 2.0 ml Eppi.
     II. Add 1 ml PBS and mix well by inverting the tube.
     III. Put the tube on the magnet and remove the supernatant.
     IV. Add another ml PBS and resuspend the beads well by pipetting up and down.
  c. Harvesting.
     I. Harvest 2x 1 ml supernatant (containing the VLPs 65 h after transfection and 40 h after medium exchange) per well into 2.0 ml Eppis and centrifuge at 5500 rpm (2029 g in *Epp. MiniSpin*) for 10min to remove dead cells and bigger cell debris.
     II. Take 2X 50 µl aliquots (PrePurification) into new 1.5 ml Eppis.
     III. Put the tube with the magnetic beads on the magnet and remove the 1 ml PBS.
     IV. Transfer 800 µL centrifuged supernatant into the tubes with the magnetic beads, resuspend the beads well by pipetting up and down.
  d. Binding.
     I. Incubate the medium with the beads while spinning at 4 °C for 3 h min (take the rotating device to the cold room).
  e. Wash.
     I. Put the tube with the magnetic beads on the magnet.
     II. Take 2X 50 µl aliquots (Flowthrough) into new 1.5 ml Eppis and discard the rest of the supernatant.
     III. Add 600 µl PBS and resuspend the beads well by pipetting up and down.
     IV. Put the tube with the magnetic beads on the magnet.
     V. Take 2X 50 µl aliquots (Wash 1) into new 1.5 ml Eppis and discard the rest of the supernatant.
     VI. Repeat steps c. through e. (Wash 2)
  f. Elution/Lysis.
     I. Add 200 µl CLB and resuspend the beads well by pipetting up and down.
     II. Incubate at 60 °C for 10 min.
     III. Put the tube with the magnetic beads on the magnet.
     IV. Transfer as much as possible into a new 1.5 ml Eppi (Elution) without taking any magnetic beads along.
  g. HiBit assay.
     I. Analyze one part of the sample content with the HiBit Extracellular Detection Kit.

II. Add 50 µl VLB to one of the PrePurifiaction, Flowthrough, and Wash 1-3 samples, mix well, and incubate the samples at 60 °C for 10 min (--> lysed samples).
III. Add 50 µl PBS4MIX to one of the PrePurifiaction, Flowthrough, and Wash 1-3 samples, and mix well (--> unlysed samples).
IV. Dilute 5 µl Elution sample in 45 µl PBS4DIL in a new 1.5 Eppi.
V. Dilute 5 µl PrePurifiaction, Flowthrough, and Wash 1-3 samples (both lysed and unlysed) in 45 µl PBS4DIL in new 1.5 Eppis.
VI. Mix 40 µl freshly prepared HiBit Reaction Mix and 40 µl of the diluted samples (equivalent to 0.091 % of PrePurification and Flowthrough samples, 0.4 % of Wash samples, and 2 % of the Elution sample) on a white flatbottom 96-well plate and shake it at 300 min^{-1} (black shaker on Jeff's bench area) for 10 min before measuring the luminescence.

- Fluorescence microscopy: 11 mL Advanced DMEM were supplemented with 1.1 µL Hoechst 33342 (1:10000) and 11 µL ALexa-Fluor 546 biocytin (1:1000)
- The medium over the cells was removed and 2 mL/well of DMEM + fluorescent stuff was added
- the plate was incubated for 20 min at 37 °C and then the cells were washed 3 times with 2 mL warm PBS and then imaged on the EVOS