08. (August) 2019

Project: iGEM_Munich2019 Shared Project
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FRIDAY, 2/8/2019

Alejandro

VLP harvesting and HiBit

- all carried out in a 96-well plate
- materials:
  - Add Protease Inhibitor Cocktail (SigmaAldrich, P8340) 1:50 to VLB (VLP/exosomes lysis buffer: 1X PBS, 1 % Triton X-100), prepare 80 µl per well + 500 µl.
  - Add Protease Inhibitor Cocktail 1:50 to PBS4MIX (PBS for mixing supernatant unlysed samples 1:1), prepare 10 µl per well + 500 µl.
  - Add Protease Inhibitor Cocktail 1:100 to CLB (cell lysis buffer: 1X PBS, 0.5 % Triton X-100), prepare 160 µl per well + 500 µl.
  - Add Protease Inhibitor Cocktail 1:100 to PBS4DIL (PBS for diluting all samples 1:10), prepare 114 µl per well + 1.5 ml.
- protocoll:
  - **Supernatant harvesting**
    Harvest 120 µl supernatant containing the VLPs 72 h after transfection and 24 h after medium exchange into a black flatbottom 96-well plate (SN centrifugation plate).
    Centrifuge the plate at 2000 rcf 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 23 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:

standard curve HiBit 19/07/2019

	A	B	C	D	E	F	
1		1	2	3	4	5	
2	20 nM solution	0 µl	1 µL	2 µL	3 µL	4 µL	5 µL
3	PBS4DIL	400 µL	399 µL	398 µL	397 µL	396 µL	395 µL
4	HiBit Control Protein in 40 µl (1 well)	0 fmol	2 fmol	4 fmol	6 fmol	8 fmol	10 fmo

- luminescence signal integration time: 1 sec
- note: 10 min at 60 °C were not enough to lyse the VLPs, probably because the wells did not heat up in that short time. The incubation of the supernatant lysed plate was repeated for 30 min at 60 °C

Johanna:

Biotin-Purification

- used the other remaining sample from 19.07.2019
- aim: determine the best beads to samplevolume-ratio
- centrifuged the samples (5 min, 2000 g)
- VLP purification with biotin beads. ≙

  Protocol for harvesting from 6-well plates (2.2 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 the corresponding amout of biotin magnetic bead suspension per Eppi (see table).

biotin beads volume

	1	2	3
1	2.67 µL (≙ 10 µL for 1.5 mL sample)	8.01 µL (≙ 50 µL for 1.5 mL sample)	13.3 µL (≙ 50 µL for 1.5 mL sample)

II. Add PBS and mix well by inverting the tube.

PBS for equilibration

	1	2	3
1	27 µL	80 µL	130 µL

III. Put the tube on the magnet and remove the supernatant.
IV. Add PBS and resuspend the beads well by pipetting up and down.

c. Harvesting.
   I. Put the tube with the magnetic beads on the magnet and remove the PBS.
   II. Transfer 400 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 2 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 130 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) and add 130 µl PBS for a third time on the beads. Resuspend them well by pipetting up and down.
f. Elution/Lysis.
   I. Discard the rest of the supernatant.
   II. Add 400 µl CLB and resuspend the beads well by pipetting up and down.
   III. Incubate at 60 °C for 10 min.
   IV. Put the tube with the magnetic beads on the magnet.
   V. 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 black flatbottom 96-well plate and shake it at 300 min^{-1} for 10 min before measuring the luminescence.

- The aliquots for Flowthrough, W1 and W2 were only taken for condition 4, aliquots from Prepurification and Elution were taken from all three conditions (2,4,5)
- standard curve: 0, 2 fmol, 4 fmol, 6 fmol, 8 fmol, 10 fmol HiBit Protein
- results: no clear correlation between the ration of magnetic biotin beads and sample volume

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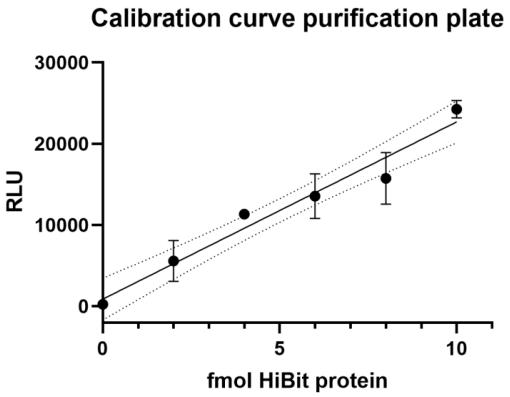

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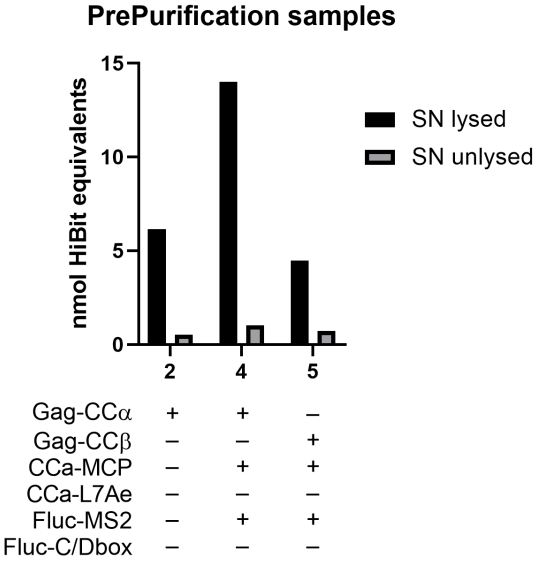

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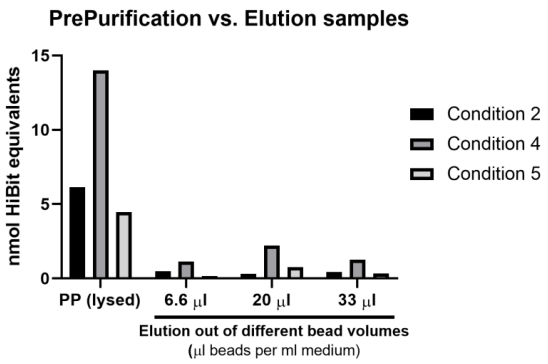

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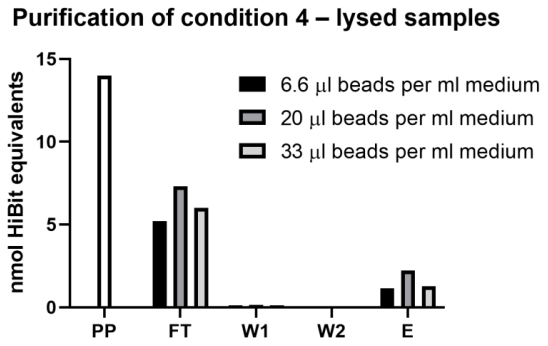

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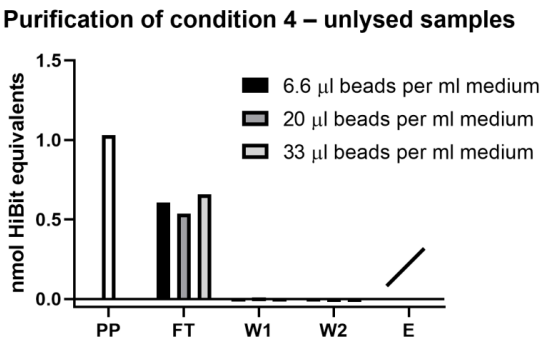

Moritz

Harvesting for qPCR

- At 12:00
- pooling 80 µL of upper supernatant from 10 wells into 1 eppi
- pooling 80 µL with cells from 10 wells into second eppi

--> 3x (for each condition)

cell culture

- Splitting

Sarah

His-Purification:

- samples:
  - mock
  - His-Tag
  - scAvidin
- 200 µL SN loaded onto His Beads
- 200 µL wash (20 mM Imidazole)
- 200 µL elution (250 mM Imidazole)
- HiBit