

08. (August) 2019

Project: iGEM_Munich2019 Shared Project

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Johanna:

HiBit for hArc:

- samples:
 - 1 = Mock (V30)
 - 2 = Gag (V8)
 - 3 = hArc (V25)
- buffer preparation
 - Add Protease Inhibitor Cocktail (SigmaAldrich, P8340) 1:50 to VLB (VLP/exosomes lysis buffer: 1X PBS, 1 % Triton X-100), prepare 40 µl per well + 500 µl.
 - Add Protease Inhibitor Cocktail 1:50 to PBS4MIX (PBS for mixing supernatant unlysed samples 1:1), prepare 40 µl per well + 500 µl.
 - Add Protease Inhibitor Cocktail 1:100 to CLB (cell lysis buffer: 1X PBS, 0.5 % Triton X-100), prepare 400 µl per well + 500 µl.
 - Add Protease Inhibitor Cocktail 1:100 to PBS4DIL (PBS for diluting all samples 1:10), prepare 135 µl per well + 1.5 ml.
- **Supernatant harvesting**
 - Harvest 600 µl supernatant containing the VLPs 72 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 unlysed 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 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:

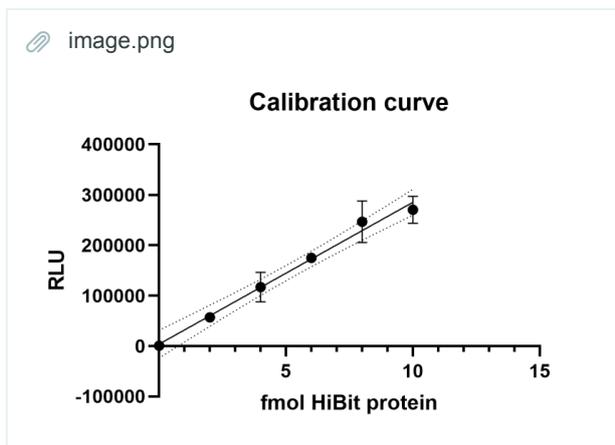
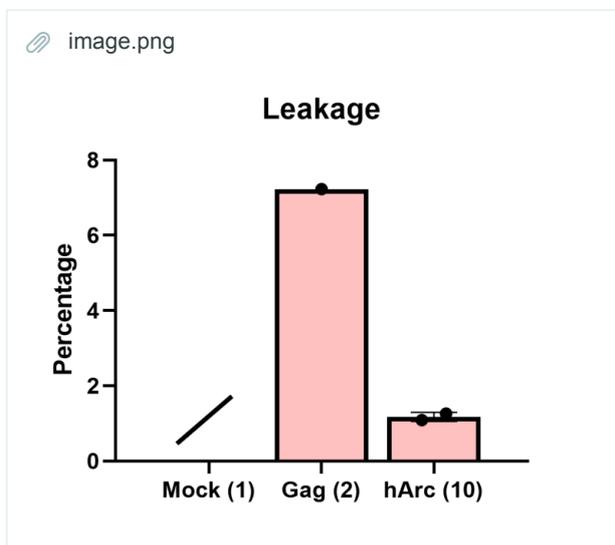
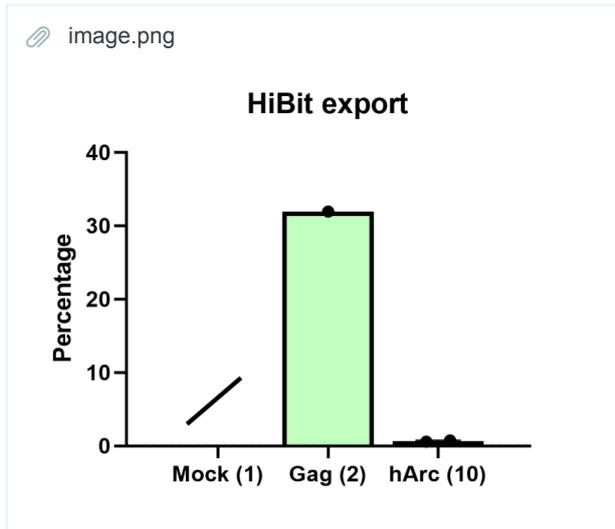
HiBit calibration curve dilution series 09.08.2019							
	A	B	C	D	E	F	G
1		1	2	3	4	5	
2	20 nM solution	0 µl	1 µl	2 µl	3 µl	4 µl	5 µl
3	PBS4DIL	300 µl	399 µl	398 µl	397 µl	396 µl	395 µl
4	HiBit Control Protein in 40 µl (1 well)	0 fmol	2 fmol	4 fmol	6 fmol	8 fmol	10 fmol

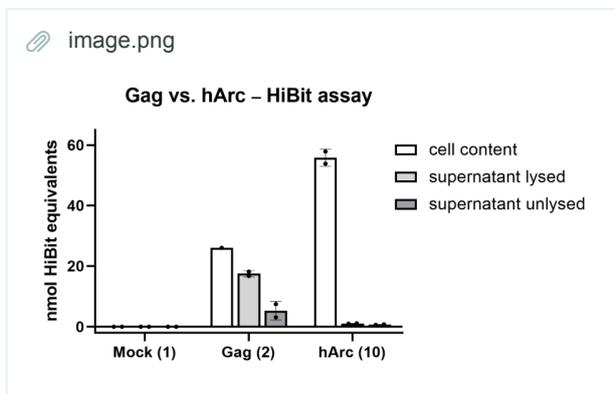
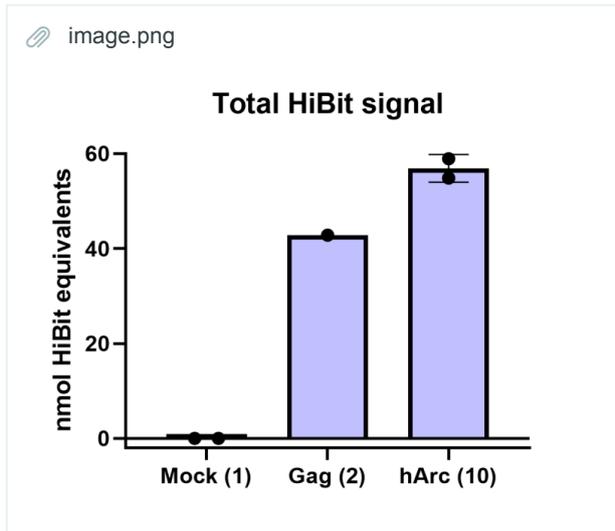
96-well plate:

HiBit for hArc 09.08.2019												
	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS		2 fmol		4 fmol		6 fmol		8 fmol		10 fmol	
B	1 (SN lysed)		2 (SN lysed)		3 (SN lysed)							
C	1 (SN unlysed)		2 (SN unlysed)		3 (SN unlysed)							
D	1 (cells lysed)		2 (cells lysed)		3 (cells lysed)							
E												
F												
G												
H												

- result: hArc does not work, the previous data could be confirmed

results hArc HiBit 09.08.2019														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1			1	2	3	4	5	6	7	8	9	10	11	
2	standardcurve	A	310	590	56950	2436670	137810	96200	175110	174130	275560	217080	251300	2890
3	SN lysed	B	810	2080	5903730	6407830	389050	357460						
4	SN unlysed	C	1400	2550	1092300	2635480	265880	214290						
5	Z lysed	D	2550	5570	18320360	144003320	40701780	37876860						
6														
7	always duplicates;		Mock	Mock	Gag	Gag	hArc	hArc						
8														
9			Calibration curve parameters											
10			Slope	28137										
11			Y-intercept	3830										
12														
13			Data standardized to 1 fmol HiBit protein											
14			-1,1E-01	-6,2E-02	2,1E+02	2,3E+02	1,4E+01	1,3E+01						
15			-8,6E-02	-4,5E-02	3,9E+01	9,4E+01	9,3E+00	7,5E+00						
16			-4,5E-02	6,2E-02	6,5E+02	5,1E+03	1,4E+03	1,3E+03						
17														
18			Data standardized to the whole well (19-well; 160 ul medium)											
19			-8,6E+00	-5,0E+00	1,7E+04	1,8E+04	1,1E+03	1,0E+03						
20			-6,9E+00	-3,6E+00	3,1E+03	7,5E+03	7,5E+02	6,0E+02						
21			-1,8E+00	2,5E+00	2,6E+04	2,0E+05	5,8E+04	5,4E+04						
22														
23			Unit change to nmol											
24			-8,6E-03	-5,0E-03	1,7E+01	1,8E+01	1,1E+00	1,0E+00						
25			-6,9E-03	-3,6E-03	3,1E+00	7,5E+00	7,5E-01	6,0E-01						
26			-1,8E-03	2,5E-03	2,6E+01	2,0E+02	5,8E+01	5,4E+01						
27														
28			Ordering											
29														
30			cell content		supernatant lysed		supernatant unlysed							
31		1	-0,0018197	0,0024736	-0,0085866	-0,0049757	-0,0069091	-0,0036393						
32		2	26,039066	204,71193	16,774781	18,208053	3,094772	7,4823897						
33		10	57,856843	53,840893	1,0952696	1,0054519	0,7450688	0,5983865						
34														
35			Total HiBit signal		Total HiBit signal									
36		1	-0,0104062	-0,002502		1	2	10						
37		2	42,813847	222,91998		-0,0104062	42,813847	58,952113						
38		10	58,952113	54,846345		-0,002502	222,91998	54,846345						
39														
40			Export		Export									
41		1	16,120219	53,409091		1	2	10						
42		2	31,9523	4,8114411		16,120219	31,9523	0,5940428						
43		10	0,5940428	0,7421925		53,409091	4,8114411	0,7421925						
44														
45			Leakage		Leakage									
46		1	66,393443	145,45455		1	2	10						
47		2	7,2284372	3,3565361		66,393443	7,2284372	1,2638542						
48		10	1,2638542	1,0910234		145,45455	3,3565361	1,0910234						





Johanna and Sarah

Biotin- and His-Purification

- preparation
 - Biotin (magnetic beads)
 - add 20 μ L Biotinbeads to 6 Eppis
 - wash 3x with 80 μ L PBS
 - His
 - 125 μ L new Ni-NTA-Beads
 - mix with 125 μ L Wash Buffer
 - spin down for 1 min at 1000g, remove supernatant -> repeat 3 times
 - last time: add wash Buffer and distribute 40 μ L to 6 Eppis
- Harvesting
 - harvest 700 μ L of the 6 wells, centrifuge 10 min at 2000 g
 - take a 40 μ L aliquot from each --> Supernatant
 - Biotin
 - add 200 μ L of the SN to 2 eppis prepared with 20 μ L beads --> technical duplicates (W1, W2, W3)
 - His
 - remove SN from beads after spinning 1 min at 1000 g
 - add 200 μ L of the SN to 2 eppis prepared with 20 μ L NiNTA-Beads --> technical duplicates (W4, W5, W6)
- Incubation
 - incubate 2 h at 4 $^{\circ}$ C, shaking

- Buffer
 - VLB
 - PBS4DIL
 - CLB
- wash and elution
 - Biotin
 - wash 2x with 200 μ L PBS
 - elute with 200 μ L CLB (10 min 60 °C, shaking)
 - His
 - wash 2x with 200 μ L Wash Buffer
 - elute with 200 μ L Elution Buffer
- HiBit
 - mix 40 μ L of elution with 40 μ L VLB -> 10 min at 60 °C
 - 5 μ L sample + 45 μ L PBS4DIL
 - 40 μ L of diluted sample + 40 μ L HiBit (Protein: 1:100, substrat: 1:50)

- results: The different ratios between gag and PGSL-1 (The purification-protein) don't influence the purification of the VLPs