Formulation development of a recombinant VAR2-antiCD3 protein for cancer immunotherapy

- Appendix -

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Appendix 1. In silico analysis

Appendix 1.1 Hotspots aCD3 heavy chain

Region	FRH1																						
KABAT	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23
Original	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Residues	Q	V	Q	L	Q	Q	S	G	A	E	L	A	R	P	G	A	S	V	K	M	S	C	K

							CDRH1					FRH2											
H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43	H44	H45	H46	H47
24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
A	S	G	Y	T	F	T	R	Y	T	M	H	W	V	K	Q	R	P	G	Q	G	L	E	W

		CDRH2																	FRH3				
H48	H49	H50	H51	H52	H52a	H53	H54	H55	H56	H57	H58	H59	H60	H61	H62	H63	H64	H65	H66	H67	H68	H69	H70
48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
I	G	Y	I	N	P	S	R	G	Y	T	N	Y	N	Q	K	F	K	D	K	A	T	L	T

H71	H72	H73	H74	H75	H76	H77	H78	H79	H80	H81	H82	H82a	H82b	H82c	H83	H84	H85	H86	H87	H88	H89	H90	H91
72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
T	D	K	S	S	S	T	A	Y	M	Q	L	S	S	L	T	S	E	D	S	A	V	Y	Y

			CDR-H3										FRH4										
H92	H93	H94	H95	H96	H97	H98	H99	H100	H100a	H100b	H101	H102	H103	H104	H105	H106	H107	H108	H109	H110	H111	H112	H113
96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
C	A	R	Y	Y	D	D	H	Y	C	L	D	Y	W	G	Q	G	T	T	L	T	V	S	S

Potential site for deamidation: Asparagine (N)	
Potential site for deamidation: Glutamine (Q)	
Amino acid promoting deamidation in position N+1	Serine (S), Threonine (T), Histidine (H)
Potential site for oxidation: Methionine (M)	
Potential site for light-induced oxidation -Tryptophan (W)	

Appendix 1.2 Hotspots aCD3 light chain

Region	FRL1																						
KABAT	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23
Original	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Residues	Q	I	V	L	T	Q	S	P	A	I	M	S	A	S	P	G	E	K	V	T	M	T	C

	CDR-L1										FRL2													
L24	L25	L26	L27	L29	L30	L31	L32	L33	L34	L35	L36	L37	L38	L39	L40	L41	L42	L43	L44	L45	L46	L47	L48
24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
S	A	S	S	S	V	S	Y	M	N	W	Y	Q	Q	K	S	G	T	S	P	K	R	W	I

	CDR-L2								FRL3															
L49	L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	L60	L61	L62	L63	L64	L65	L66	L67	L68	L69	L70	L71	L72
48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
Y	D	T	S	K	L	A	S	G	V	P	A	H	F	R	G	S	G	S	G	T	S	Y	S

																	CDR-L3							
L73	L74	L75	L76	L77	L78	L79	L80	L81	L82	L83	L84	L85	L86	L87	L88	L89	L90	L91	L92	L93	L94	L95	L96
72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
L	T	I	S	G	M	E	A	E	D	A	A	T	Y	Y	C	Q	Q	W	S	S	N	P	F

	FRL4									
L97	L98	L99	L100	L101	L102	L103	L104	L105	L106	L107
96	97	98	99	100	101	102	103	104	105	106
T	F	G	S	G	T	K	L	E	I	N

Potential site for deamidation: Asparagine (N)	
Potential site for deamidation: Glutamine (Q)	
Amino acid promoting deamidation in position N+1	Serine (S), Threonine (T), Histidine (H)
Potential site for oxidation: Methionine (M)	
Potential site for light-induced oxidation: Tryptophan (W)	

Appendix 1.3 Hotspots prone to deamidation in aCD3

If the +1 residue after Asn and Gln is a Gly, Ser, Thr or His, Asn and Gln are more prone to deamidation. Solvent exposure and protein flexibility
also determines the degree of deamidation.
In aCD3, HC Gln 6, HC Gln 43, HC Gln 105 and LC Gln 6 have an increased probability for deamidation. None of these residues are positioned
in the CDR regions.

Heavy chain segment[a]	Residue[b]	Comment	SASA (Å²)/relative SASA (%)
FRH1	Gln 6	Prone to deamidation, N+1 (Ser)	7.3/3.2
FRH2	Gln 43	Prone to deamidation, N+1 (Gly)	100/44.5
CDRH2	Asn 52	Potentially involved in epitope recognition	47.7/24.5
CDRH2	Asn 58	Potentially involved in epitope recognition	41.3/21.2
CDRH2	Asn 60	Potentially involved in epitope recognition	29.6/15.2
CDRH2	Gln 61	Potentially involved in epitope recognition	131.1/58.3
FRH4	Gln 105	Prone to deamidation, N+1 (Gly)	90.2/40.1
Light chain segment[A]	**Residue[b]**	**Comment**	**SASA (Å²)/relative SASA (%)**
FRL1	Gln 6	Prone to deamidation, N+1 (Ser)	19.9/8.9
CDRL1	Asn 34	Potentially involved in epitope recognition	0.8/0.4
CDRL3	Gln 89	Potentially involved in epitope recognition	0/0
CDRL3	Gln 90	Potentially involved in epitope recognition	0/0
CDRL3	Asn 94	Potentially involved in epitope recognition	93.1/47.7

[a] CDR regions are highlighted in grey.
[b] Amino acid numbers refer to the KABAT numbering.

Appendix 1.4 Hotspots prone to oxidation in aCD3

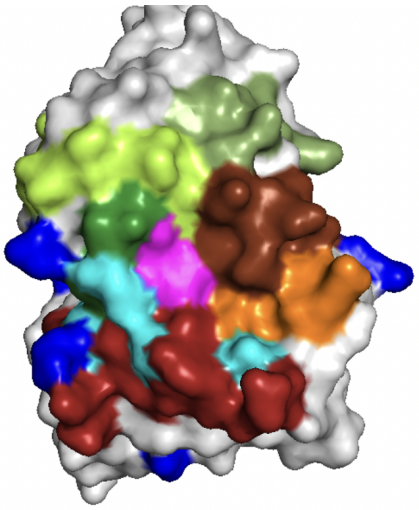
Surface exposed Met and Trp are more prone to oxidation, but also partially buried and buried residues can be prone to oxidation if they
are positioned in a flexible region of the protein. This table only covers the surface exposure.
In aCD3, HC Trp 103, LC Met 11 and LC Trp 91 may be prone to oxidation.

Heavy chain segment[a]	Residue[b]	Comment	SASA (Å²)/relative SASA (%)
FRH1	Met 20	Potentially prone to chemically induced oxidation.	5.3/2.4
CDRH1	Met 34	Potentially prone to chemically induced oxidation.	0.8/0.3
FRH2	Trp 36	Potentially prone to light-induced oxidation.	0/0
FRH2	Trp 47	Potentially prone to light-induced oxidation.	6.8/2.4
FRH3	Met 80	Potentially prone to chemically induced oxidation.	0.7/0.3
FRH4	Trp 103	Potentially prone to light-induced oxidation.	33.6/11.8
Light chain segment[A]	**Residue[b]**	**Comment**	**SASA (Å²)/relative SASA (%)**
FRL1	Met 11	Potentially prone to chemically induced oxidation.	40.5/18.1
FRL1	Met 21	Potentially prone to chemically induced oxidation.	0/0
CDRL1	Met 33	Potentially prone to chemically induced oxidation.	0/0
FRL2	Trp 35	Potentially prone to light-induced oxidation.	1.2/0.4
FRL2	Trp 47	Potentially prone to light-induced oxidation.	21.0/10.9
FRL3	Met 78	Potentially prone to chemically induced oxidation.	4.5/2.0
CDRL3	Trp 91	Potentially prone to light-induced oxidation.	49.6/17.41

[a] CDR regions are highlighted in grey.
[b] Amino acid numbers refer to the KABAT numbering.

Appendix 1.5 Surface-exposed residues

Blue = Gln
Teal = Met (Not shown)
Cyan = Asn
Magenta = Trp



LC-CDR1
LC-CDR2
LC-CDR3
HC-CDR1
HC-CDR2
HC-CDR3

Appendix 2. Lab protocols

Appendix 2.1 Buffer preparation

Buffer preparation protocol

The chosen buffers in the range pH 3.5-8 are shown in table 1 below.

Table 1: Chosen buffers

pH ideal	pH measured	Buffer	pH range	Additive salt
3.5	3.27	50 mM Citrate	3.0-6.2	150mM NaCl
4.5	4.6	50 mM Acetate	3.6-5.6	150mM NaCl
6.0	6.0	50 mM MES	5.5-6.7	150mM NaCl
6.0	6.0	50 mM Histidine	5.5-7.4	150mM NaCl
7.4	7.4	50 mM PBS	5.8-8.0	150mM NaCl
8.0	8.0	50 mM TRIS-HCl	7.0-9.2	150mM NaCl
6.0	6.0	50 mM Histidine	5.5-7.4	150mM Sucrose
8.0	8.0	50 mM TRIS-HCl	7.0-9.2	150mM Sucrose

Preparation protocols for the citrate and acetate buffers are seen in tables 2 and 3 below.

Table 2: Citrate buffer

Component	Amount	Concentration
tri-Sodium citrate (mw: 294.1 g/mol)	314 mg	10.7 mM
Citric acid (mw: 192.1 g/mol)	755 mg	39.3 mM

Total: 50 mM in 100mL

Table 3: Acetate buffer

Component	Amount	Concentration
Sodium acetate (mw: 82.03 g/mol)	185 mg	22.5 mM
Acetic acid (mw: 60.05 g/mol)	0.157 mL	22.5 mM

density: 1,05 g/mL	(= 165 mg)	

Total: 50 mM in 100mL

The step by step preparation for the buffers with 2 components follows as described below:

1. Add the salts/solutions in the amounts described in tables 2 and 3 above
2. Add MilliQ water up to a total volume of 80mL
3. Measure pH and adjust with more salt/solution.
4. Add 3 mL of 5M NaCl solution
5. Add MilliQ water to a total volume of 100mL, check with the measuring cylinder.
6. Sterile filter using a 0.22μm filter, pour into a new bottle
7. Label

Table 4: Histidine buffer

Component	Amount
Histidine	1.05 g

Total: 50 mM in 100mL

Table 5: MES buffer

Component	Amount
MES	0.98 g

Total: 50 mM in 100mL

Table 6: TRIS-HCl buffer

Component	Amount
TRIS-HCl	0.788 g

Total: 50 mM in 100mL

The step by step preparation for the buffers His, MES, TRIS-HCl follows as described below:

1. Add the salts in the amounts described in tables 4, 5, 6 seen above
2. Add MilliQ water up to a total volume of 60mL
3. Measure pH and adjust with HCL or NAOH
4. Add 3 mL of 5M NaCl solution OR Sucrose solution (see below)
5. Add MilliQ water to a total volume of 100mL, check with a measuring cylinder.
6. Sterile filter using a 0.22μm filter, pour into a new bottle
7. Label

Table 7: 5 M (mol/L) NaCl (58,44g/mol)

Component	Amount
NaCl	29,22 g

Total: 5M in 100mL

Table 8: 5 M (mol/L) Sucrose (342,3g/mol)

Component	Amount
Sucrose	171,15 g

Total: 5M in 100mL

Table 9: PBS

Component	Amount
PBS	Use already prepared solution

Protein purification

Database information & protein handling

1. Finding the protein in the database
    a. Q-drive
    b. CMP - VAR2CSA
    c. Databaser
    d. Protein database
    e. Note: name, concentration, buffer, extin. coeff., box in freezer, molecular weight
2. Protein handling (see protocol)
    a. Put the protein on ice
    b. Thaw by hand
    c. Centrifuge for 5 mins, 16 000 rpm at 4°C. If you see a pellet after centrifugation? → bad buffer.
    d. Put on ice again - always keep the protein on ice! (when the protein is thawed once, mark the lid with a cross).

Desalting & concentration

Materials

- Concentration columns - CC (can be found at the end of the corridor to the right). molecular sizes >10kDa will go through the membrane
- Saltning columns (can be find in the cold room, VAR2 shelf) - SC

Method - Concentration

a. Pretreat the concentration column with the buffer that the protein has been stored in (for example PBS). Just fill up a little bit.
b. Centrifuge at 3500g (rcf) for 5 min at 4°C.
c. Empty/discard the liquid that has gone through the column.
d. Observe the protein amount and pool two or more tubes if necessary. Keep at least 3,0 µL as a reference.
e. Centrifuge at 3500g for 5 min at 4°C. Repeat until you have approximately 100µL of the protein in each of the buffers. Fill up with more PBS if necessary. *OBS* Concentrate to a higher concentration than desired because it will be diluted in the saltning column (later step).
f. Take out the content from the CC and transfer to an Eppendorf tube.
g. Measure the protein concentration in Nanodrop. (see below)

Method - Desalting

a. Prepare x2 falcon tubes (15mL) for all the different buffers, mark them both on the LID *AND* the TUBE.
b. Check the filter size (kDa) on the concentration columns and the volume of the tube (depending on the volume of protein sample added to each tube) to follow the right row in the protocol (ex 0,5mL or 2ml 40kDA).
c. Break the end of the SC tubes and open & close the lid to release the pressure. Put the SC tubes into the falcon tubes.
d. Start the procedure as described on the paper, ex (0,5mL):
    - Remove storing buffer
    - Wash 1 min (with the new buffer)
    - Wash 1 min
    - Wash 2 min
    - Transfer to a *new* falcon tube!
    - Add 100µL of protein
    - Centrifuge for 2 min
    - Put the sample on ice
    - Transfer the liquid to an Eppendorf tube
    - Nanodrop (blank with the right buffer for every sample).

Method - Sterile filtration (No pre-treatment!)

a. Stand in the sterile bench hood in the cell lab in the “toxic room”
b. Pour the protein sample into the sterile filter
c. Centrifuge at maximum speed for 1 min
d. Aliquot - OBS print labels

Method - Nanodrop

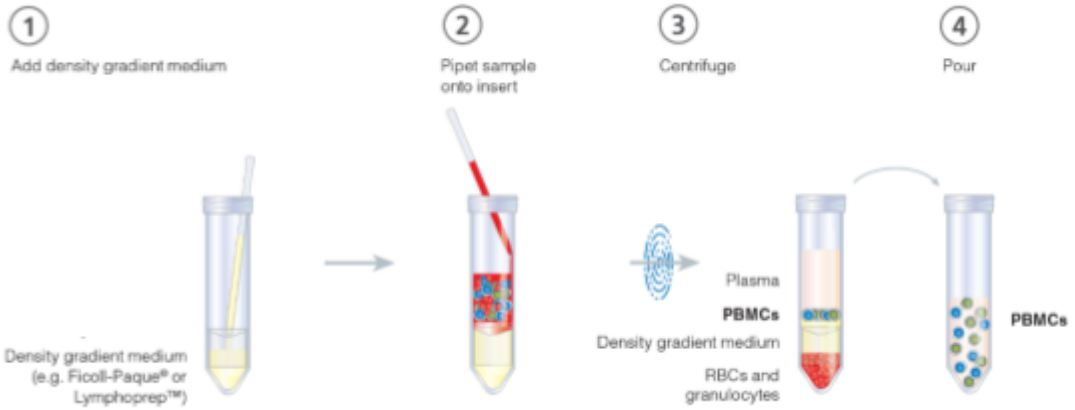
a. Open the Nanodrop program on the computer
b. Choose: Protein A280
c. Change “Ext Coff”, found in the sidebar to the right.
   OBS If 1,55 in the database: x10 here → 15,5.
d. Reverse pipette 2µL of the blank (same buffer as protein is stored in), close the arm
e. Click “blank”
f. Click “measure” and make sure the graph shows no Abs.
g. Open the arm, wipe off the instrument and reverse pipette 2µL of protein sample. Close the arm and click measure - note down the concentration and ensure that the peak is around 280nm → otherwise not protein.

Cell work

Cell work: PBMC purification and freezing & thawing cells

Purification of PBMCs: Order Blood: Drive → CTC team → Databaser → Weekly used blood

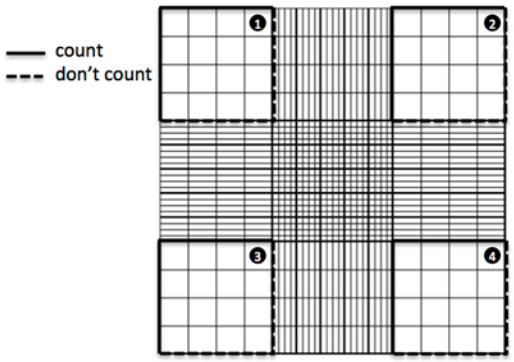
1. Warm lymphoprep in 37°C equal to 20 mL pr. EDTA tube of Blood
2. Warm PBS2 in 37°C equal to 1:1 volume of collected blood.
3. Collect the appropriate volume of blood where 1 mL ~ 1 million PBMC's.
    a. Use normal tubes with EDTA to avoid clumps
4. For each tube of blood, take one leucosep tube and add 20~ mL of lymphoprep.
    a. Place the pipette tip in the middle of the filter, gently push it through. Add little bit above the filter limit
5. Spin for 30 seconds at 800xg – this forces the lymphoprep beneath the leucosep filter.
6. Mix the blood and heated PBS2 in a 1:1 dilution (e.g. 15 mL blood & 15 mL PBS2).
7. Slowly add a maximum of 30 mL of blood-PBS solution into the prepared leucosep tube, by gently pressing the pipette tip on the side of the leucosep wall (figure 1 (2))
    a. This is to ensure that the blood gets on top of the filter and that it'll be separated correctly with as much purity as possible.
8. Spin for 20 minutes at 800xg at RT.
    a. To get a nice separation set to **deacceleration 3**.
    (This program will run for around 30min → good time to take a break)
9. Carefully collect the "cloudy layer". First, use a 1000µL pipette twice and transfer into a new 50mL tube. Then take a 10mL pasteur pipette and suck up the thin layer (approx. two times), *OBS* it doesn't matter if some of the serum above comes along with the cells, but it is important to get the **whole layer** of cells.
    a. Same donor? Fill up from different blood-tubes to 30mL and then add 20 mL PBS2.
10. Spin for 10 min at 200xg



11. FREEZE (See below) OR Remove the supernatant, by pouring and resuspend in 20 mL glutamax RPMI (10% FBS) (can be found in the fridge in the second cell lab from SDS-PAGE at VAR2 Shelf).
12. Spin for 6 min at 300xg
13. Remove the supernatant, by pouring and resuspend in 20 mL of the media, that the cancer cells need.
14. Put the tube on ice and count the cells;
    a. Use the glass slide found on the bench in the second cell lab (in a glass box filled with alcohol). Mix 20uL DPBS2 (dye, found in the fridge in the second cell lab) (RT) + 20uL cell suspension. Suck up a small volume and place one drop underneath the glass slide by gently pushing the liquid out with the tip tilted to the edge of the slide. Place the slide under the microscope and count cells in opposite corners of the hemocytometer; (ex: in right figure 1 & 4) 1 count = 1 million cells/mL
    (Alternatively count all 4 corners and divide by two if it looks less homogenous)

Example:

225	
	210



- 4,35 mio/mL (millon cells)

15. Use the cells for the FACS setup. (100 µl (=200.000 cells)/well → 2 million cells/plate)
16. Cells will fall down, pipette up and down before transfer 100 µl (=200.000 cells) to each well in a 96-well plate (round bottom) for flow cytometry (Nunc, cat.no. 163320).

Freezing down PBMC's:

1. Fill up the 50mL falcon tube (containing 45mL suspended PBMC's) with PBS2 to a total volume of 50mL
2. Centrifuge at 400xg for 5 minutes

In the meantime:

3. Calculate how many cells you have:
    a. Example: 4.35x10^6 cells/mL (from cell count) and 45mL suspended PBMC volume
    → 4.35x10^6 cells/mL x 45mL = 195.75x10^6 cells ≈ 200x10^cells
    b. We want 40x10^6 cells/ frozen cryo tube
    → 200x10^cells/40x10^6 cells = 5 cryo tubes
4. When the centrifugation is finished, discard the supernatant
5. Resuspend the pellet in a smaller volume of PBS2 (around 3mL)
6. Mix around by pipetting up and down a couple of times

7. Transfer fresh PBS2 up to a total volume of 50mL
8. Centrifuge at 400xg for 5 minutes

In the meantime:

9. Go to a stationary computer inside the lab and open up:
   Q drive → CMP → VAR2CSA → CANCER TEAM → cancer cell database
10. Scroll down and create a new TC number for the frozen cells (next number in the order). Information to enter the excel sheet under tab "In -180 freezer"
    a. TC number: *Example: TC826*
    b. Date
    c. Cell type *Example: PBMCs*
    d. Media *Example: RPMI + Glutamax + 10% FBS + 1% pen/strep*
    e. No. of tubes *Example: 5*
    f. Backup in master box *Example: No*
    g. Initials *Example: Frida & Maja /Nanna*
    h. Comment: *Example: Use for flow*
    i. Origin *Example: Blood (Nanna)*
11. Mark each cryo tube with;
    a. on side: TC-number, date and cell type
    b. on top: TC-number
12. When the centrifuge is finished, repeat steps 4-8 (centrifuge again)

In the meantime:

13. Calculate the amount of FBS and DMSO PBS to use:
    a. Example: 200x10^6 cells in total; 2.5mL FBS and 2.5mL 20% DMSO PBS (If 400x10^cells, use 5mL of each instead)
14. After last centrifugation: Discard supernatant and resuspend pellet in 2.5mL FBS. *Note: It is important that there are no clumps!*
15. From the resuspended pellet, transfer 2.5mL into a new 10mL falcon tube (the volume might increase due to leftovers in the pellet and it's important to be exact)
16. Add 2.5mL 20% DMSO PBS dropwise by leaning the pipette tip against the tube wall
17. Mix by pipetting up and down a couple of times
18. Distribute the mixture into the 5 cryo tubes with 1mL in each
19. Put tape over the label to avoid washing off the ink during freezing (Tape is found on Chumpol's shelf)
20. Put all cryo tubes in a cryo box (round, lime green color) and freeze in -80 freezer (OBS do NOT fast-freeze using liquid nitrogen)

The day after:

21. Open up the excel spreadsheet again in a stationary computer inside the lab (Q drive → CMP → VAR2CSA → CANCER TEAM → cancer cell database) and go to the second tab "boxes in -180"
22. Find a box with free spots for all cryo tubes that you are freezing and write in the sheet where you place them.

23. Also find the Immunotherapy sheet and fill in the information of the frozen samples (TCnr etc.)
24. When Mr Frosty has been in the -80 freezer for around 24h, go to floor 01 and put it into the -180 freezer.
25. Take left, go under the big stairs into the basement.
26. Take right and enter the second door.
27. Find the right nitrogen container (Look for a paper that says ”Morten” etc) in the middle; Rack 12 (at the top to the right), Rack 11 (at bottom to the left). Take the whole rack outside the container and close the freezer.
28. Find the right box and place the vials and finally put everything back.

Thawing cells:

1. Take out 2 50mL tubes of FBS, 5mL L-glutamine in a 10mL falcon tube & 5mL penicillin streptomycin in a 10mL falcon tube from the CTC team -20 freezer (in the cell lab close to the ImageQuant) and thaw in the 37degrees heating bath in the cell lab
2. Prepare 20 % FBS in RPMI medium (RPMI 20) for PBMC washing:
    a. Take a new RPMI medium from the cold room, first shelf to the right (looks like strawberry juice)
    b. Add all liquids from the thawed; 2 50mL tubes of FBS, 5mL L-glutamine & 5mL penicillin streptomycin and mix
3. Add 10 ml of the mixture to a 15 ml falcon tube and put into the heating bath, warm up to 37C.
4. Take a fresh aliquot of PBMC’s from the liquid nitrogen-freezer in the basement and put it in a box with dry ice.
5. Thaw the aliquot partly in the heating bath until some ice is still visible.
6. Thaw it the last part in your hand and pipette up and down a little bit until all ice is gone. Add the contents of the vial to the 10ml warm medium in the falcon tube using a Pasteur pipette. *OBS Add slowly.*
7. Centrifuge 500g, 10min
8. Pour out the supernatant (all in one motion so the cells don’t resuspend in the supernatant). Resuspend in fresh 1mL warm RPMI20 using a Pasteur pipette and add 8ml more of warm RPMI20, Centrifuge 500g, 10min
9. Pour out the supernatant (all in one motion so the cells don’t resuspend in the supernatant). Resuspend in fresh 1mL warm RPMI20 using a Pasteur pipette and add 8ml more of warm RPMI20
10. Count the cells (see above)

SDS-PAGE

SDS-PAGE

1. Use the Nanodrop results to calculate the right volumes,
   ex: if protein concentration from the Nanodrop was 2,5mg/mL and we want 1ug → 1 / 2,5 = 0,4uL and than multiplied by 2 since there will be duplicates of the sample (with and without DDT), which finally leads to a concentration 0,8 uL.
2. The total volume should be 20uL, meaning that in this case 19,2uL of the buffer is added to one the Eppendorf tubes. 0,8uL of the protein is then added.
3. Take 10uL out into the other duplicate Eppendorf tube.
4. In the hood, add 2uL of DTT+ or DTT- respectively. DTT can be found in the fridge close to SDS-page. Put DTT at 10s on a heating bath before use.
5. The total volume should now be 12 uL in every sample.
6. Important to label the sample well, if you are running two different gels! 1,1 (gel 1, sample 1) with black pencil and 2,1 (gel 2, sample 1) with red pencil and sample 1 could be for ex TRIS DTT+(black) and DTT- (red).
7. Put the sample on the heating block for 5 min. Centrifuge quickly (3-5sek) to spin down the samples.
8. SDS-PAGE information:
   a. There are 15 wells or 12 wells.
   b. Don't forget the ladder
   c. Always load in a asymmetric order so you can identify your samples afterwards
   d. You can run 2 gel each time.
   e. Don't run on Thursdays (crowded) and Mondays & Tuesdays are the best.
9. Remove the tape from the gel.
10. Add electrolyte (found in the large water container next to the heating block. In the middle: pour all the way up until it pour over into sides and there up to approximately 1/3.
11. Place the gel in the container.
12. Remove slowly and carefully the plastic coverage keeping the wells in place by pulling it upwards.
13. 10 s on ice, easier to load.
14. Load the gel with 11uL, keep the tip straight and slowly load the well. Don't push the pipette button further than to the stop - avoiding bubbles.
    a. If a small part of the sample floats over into the next well you can, with a new pipette tip, suck it up and discard it before adding the next sample
15. Put the cover on and connect the cables in the machine + to + and - to -.
16. Start, 55min, 0,07 A280
17. Check the gel as you start, if you see bubbles coming from the bottom - everything is fine! No bubbles - the tape wasn't removed / cables are connected incorrectly. Check the gel

after a while (approx 30 min) - CANNOT go to the end cause then the whole gel will be stained.

18. When the gel is done: disconnect the cables, take off the cover, loosen the white buckle on the back to open. Take out the gel, rinse with deionized water and put down the gel on a piece of paper, backwards. Use the spatula and carefully open the gel by cracking each side open.
19. Pour staining liquid Coomassie blue (found in fridge) in a plastic quadric box with a lid
20. Turn the gel upside down in the liquid by dragging the spatula in the slit. Put the lid on the box and place in the plate shaker in the hood for 15 min. *OBS* Avoid putting the box directly on the green sticky carpet.
21. Check the gel on the light plate (and take a photo with your phone) or go to the machine in the other part of the lab.
22. (See the protocol there) Use the spatula to take out the gel carefully and put on the WHITE plate. Try to place the gel in the middle, check by click on the “focusing” bottom.
23. Save & Print.

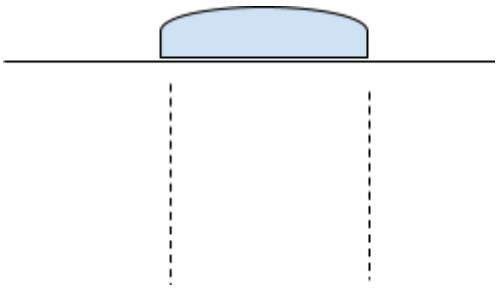
ImageQuant

ImageQuant (image analysis SDS PAGE)

1. Take picture of the SDS PAGE gel, to think about;
    a. Make sure the loading wells can be seen at the top (easier to identify the lanes in the program later)
    b. Edit the image with a;
        i. High contrast
        ii. As white background as possible
    c. Save the picture in .gel format
2. Open the ImageQuant program
    a. Choose 1D gel analysis
3. Upload the gel-picture (The program will ask you to save the analysis, press no)
4. Press Analysis (in the top bar) → Lane creation
    a. Choose Create lanes;
        i. Choose “Manual”
        ii. Settings:
            1. Number of Tiers: 3
            2. Number of Lanes: 15
            3. Lane % width: 100
        iii. Drag the purple lines to the top and bottom of the gel
        iv. Press and hold the computer mouse and drag out the 15 wells where they fit to the gel
    b. Choose Edit multiple lanes (where you before chose “Create lanes”)
        i. Settings:
            1. Bend/Resize Lane Box
        ii. Drag each of the 4 corners of the 15 wells so they fit the picture
    c. Press Accept
5. Analysis → Background subtraction
    a. Choose Background method to create a baseline:
        i. Settings:
            1. Radius: 200
            2. Background method: Rolling ball
    b. Press Subtract; *Check that a baseline appeared in all lanes by pressing the number of the wells in the Image Window (left figure) -> shows baseline in the lane window (right figure)*
6. Analysis → Band detection
    a. Choose Peak Detection options
        i. Settings:
            1. Minimum slope: 32

2. Median Filter: 4
3. % Max. Peak: 0
4. mark the box “Snap to Peak Editing”
5. mark the box Automatic detection
6. unmark the box “fixed width”
7. fixed width: 6

b. **Press Detect** (the computer tries to find the bands on its own)
c. To detect the other bands, click on each one of them
d. Click on each lane (on the well number) and adjust where each band stops and starts, either directly in the Image window by dragging the red lines or in the lane window (right figure) by dragging the dashed lines - make sure you have the whole band within the brackets:



7. Analysis → Molecular size calibration
    a. Choose “New marker 2018” or “M&F large proteins”. Make sure the size standards matches the ladder you used for the SDS-PAGE
    b. Choose Curve type: Linear log
    c. Mark the “Use Rf to propagate” box
    d. Click on a grey part of the gel in the first lane (within the 15 well-area) and yellow lines will appear that connect the protein ladder with its correct sizes.
    e. **Press Compute;** *Check that calculated MW have appeared for each lane in the Measurement window (bottom figure)*
8. Analysis → Quantity calibration
    a. Choose Curve: Linear log
    b. Unmark the “Force through origin” box
    c. Choose: Microgram
    d. Press one band that you know the quantity of and enter the amount in the box (ex; band in lane 8, 1ug)
    e. **Press Calibrate** → yellow diamonds should appear for all bands
    f. *Check that calculated volumes have appeared for each lane in the Measurement window (bottom figure)*
9. Analysis → Experiment Overview
10. Reports → Analysis report
    a. Wish to save? → yes!

CSA ELISA

Aim: analyze binding of recombinant VAR2CSA proteins to CSA attached to decorin & HSPG

1. Coat Falcon 96 well plates (351172, BD Biosciences) with 50 µl of decorin (3 µg/ml in PBS D8428, Sigma-Aldrich, stock concentration X (ex. 2,9mg/mL=2900ug/mL) mg/ml) and 50 µl of HSPG (2 µg/ml GAG content in PBS H4777, Sigma-Aldrich, check stock concentration on the product sheet, (16,5 uL in 5200uL PBS) or 1,06mg/mL (14,7uL in 5200uL PBS!) store overnight 4°C (no need shaking, wrapped in tin foil) at SDS-Page fridge, Benjamin's shelf.
    a. HSPG and Decorin can be found in the VAR2 fridge in the corridor at Benjamin's shelf.
    b. The plates are in the storage room, mark how many you take.
    c. Shake the plate a little bit after coating – remove bubbles and the bottom will be covered.
    d. Calculations ex Decorin:
        i. 50uL in 96 well = 4800uL
        ii. Do some extra 5200uL
        iii. (3ug/mL*5200uL)/(2900ug/mL) =5,4uL of decorin
        iv. (2ug/mL*5200uL)/631ug/mL) =16,5uL of HSPG
        v. Mix 5,4uL decorin + 5200uL PBS in a 15mL tube
        vi. Pour over into reservoir, 50uL in each well

2. Prepare TSM washing buffer (500ml) and TSM blocking & binding buffer (100ml) according to the mixing recipes at the end of the protocol.
3. Empty the wells after coating
4. Block the plate with a TSM Blocking buffer (150 µl/well) (gentle shaking, 130) for 1-2 hours at 37°C. (Cold room, VAR2 shelf, 20x)
5. While the plates are blocking, make the dilutions of proteins according to the ELISA sheet. Prepare a two-fold dilution series (1.56 nM to 100 nM) of each protein in the TSM binding buffer. This is made on one solution plate which will be enough for 2 ELISA plates (120uL divided to 50uL/ELISA plate).
    a. Start with adding 120uL of TSM binding buffer in each 96 well of both plates.
    b. After that add 80uL of TSM binding buffer into the top row (100nM concentration line), considering 200uL in the top row now.
    c. Add the (calculated) specific rest volume of the TSM binding buffer into each well respectively. Ex. Add 39,5uL → 239,5uL.
    d. Add the protein respectively to each well. Ex. 0,5uL protein → total 240 uL
    e. Use 6 pipet tips (mix 8 times in each row). Forward 120uL into every column below, mix 8 times.. etc. (LEAVE last row ! NO protein here, this is the blank).
    f. Put on ice, while waiting.
6. Empty the wells and add 50 µl/well of the protein preparation above. Easiest to use 8 pipet tips, take out 50uL of Ex. citrate 4°C from (100nM-blank) add to HSPG plate. Take out 50uL of the same row on the dilution/protein plate and add to the Decorin plate. Change pipettes and repeat for all 12 rows.
7. Incubate for 1 hour 37°C with gentle shaking (130rpm).

8. Prepare anti-V5-HRP antibody (AbCam, Ab1325, Rabbit pAb to V5) (1: 9000) (R96125, Life Technologies), found in the fridge in the freezer room, Chumpol's shelf in a yellow box ("Chumpol/Sabrine") in TSM binding buffer.
    a. 1:9000 anti-V5-HRP antibody in 10mL (enough for 2 plates).
    b. 10mL = 10 000uL → 10 000uL/9000= 1,11uL anti-V5-HRP antibody.
    c. Prepare a 15mL falcon tube with 10mL TSM binding buffer and add 1,11uL and mix.
    d. Wrap in aluminum foil
9. Empty the wells and wash 3X with a TSM washing buffer.
    a. Use the big, round reservoir and the electrical, 8 tip -pipette called "Susanne".
    b. Use the tips in the lilac box. Use the button to suck up the liquid, first click = air out and then just push the button and 1200uL of liquid will be added to each well.
    c. Fill the plate (needs 2 rounds with the pipettes for a 96 plate).
    d. Empty the wells and start over (repeat 2 more times).
    e. Tips! Use the same pipette tips for everything and if you are not prepared with the next step let the last washing liquid stay so the wells are not running dry.
10. Add 50 µl/well of the anti-V5-HRP antibody mixture and incubate for 1 hour 37°C with gentle shaking.
11. The TMB+ needs to be at room T. TMB+ can be found in the cold room, second bookcase to the right. Take out 10mL to 2 plates in a 15mL falcon tube. Wrap the tube in foil, sensitive for light.
12. Wash 3X with TSM washing buffer (same step as step 8).
13. Empty the wells.
14. Add 50 µl of the developing mixture (TMB+) to the wells. Allow to develop for 6-9 minutes without light. Start the time when you start adding in the first wells.
15. (DO NOT empty the wells) Stop the reaction with 50 µl/well of 0.2 M H_2SO_4, buffer already prepared in the hood. Add the stopping buffer in the same order as adding TMB. (can be standing like this in 30min if that is desirable but otherwise measure the plate directly.)

Read the ELISA plate

1. Login and open the ELISA program.
2. Click on "Available device".
3. Read at the ELISA reader at 450 nm, put the plate in the machine, click start.
4. Click on "XLS Export Raw Data (plate)", save it somewhere on the drive.
5. Click ok so you can see the excel file.
6. Don't throw away your plates before checking that the data is received on your personal computer.
7. Update the ELISA sheet with absorbance and any modifications to the protocols

CD3 ELISA

Aim: analyze binding of recombinant VAR2CSA proteins to CD3

1. Coat wells of a MaxiSorp Elisa plate with 50 µl of CD3 (Recombinant human CD3 protein (Active) abcam ab220577, lot: GR3309893-1, 0,1 mg/ml) in **2** µg/ml in PBS. Leave overnight at 4°C (no need for shaking, wrapped in tin foil). It can also be incubated 2-3 hrs at RT.
    a. See calculation at the end of the protocol.
2. Discard in sink and wash plate 3 times with 200 µl PBS+ 0.05% Tween (see manual below). Dry the plate very well after each wash by hitting it vigorously on a stack of paper.
3. Block wells with 150 µl blocking buffer and incubate for 1-2 hrs at room temp.
4. While the plates are blocking conjugate, make the protein dilution plate (aCD3-VAR2) and make the dilutions of proteins in the *binding buffer*.
    a. For a **five**-fold dilution series (200 nM to 0,0128 nM) of each protein prepare 120 uL all rows, EXCEPT in the first row.
    b. First row, do the calculations of the protein from Nanodrop (ex. 1,1uL protein needed, fill up with 118,9ul binding buffer to a total volume of 120uL)
    c. Mix 8 times in each row and start to take out 30 uL and dilute in 120 uL, EXCEPT blank!
5. Wash plates 3x with 200 µl PBS-T (washing buffer).
6. Add the prepared protein (from the dilution plate) to the plate, 50 µL/ well and incubate 1 hr at RT with gentle shaking.
7. Prepare the ab's, diluted in the binding buffer, see calculation below!
8. Take out the TMB-X buffer to let it become RT and wrap in foil/keep away from light.
9. Wash plates 3x with 200 µl PBS-T (washing buffer)
10. Incubate with anti-V5-HRP antibody (used for our protein and -control (VAR2), and rabbit anti mouse IgG (Used for the +control aCD3), use 50 µL/well and incubate for 1 hour at RT with gentle shaking.
11. Wash plates 3x with 200 µl PBS-T (Washing buffer).
12. Develop – add 50 uL TBM-X to each well and let it sit until the most positive wells are a deep blue (~8 mins).
13. Stop the reaction with 50 µl/well of 0.2 M H_2SO_4.
14. Read at the ELISA reader at 450 nm.

Wash buffer: PBS + 0,05% Tween20 (1 plate: 500mL PBS + 250uL)

Binding/blocking Buffer: Wash buffer + 5% skim milk powder (1 plate: 100mL Wash buffer + 5g skim milk (can be found in the fridge in the chemical room))

CD3 preparation:
Located: -20 freezer in the hall (VAR2) the shelf at very bottom.
Aliquots: 8 vials with 30uL each (200ug/mL)

Calculations:

i. 50ug CD3 mixed with 250uL alliquoted MQ water → 200ug/mL
ii. 30uL in each vial → 8 vials
iii. Each plate should be coated with 1,1ug/mL and 5mL is needed for 1 plate (96wells*50uL =4800ul=4,8mL). The concentration in the vial is 200ug/mL=0,2ug/uL.
5mL*1,1ug/mL=0,2ug/uL*X → 27,5uL of the CD3 vial. PBS needed: 5000-27,5= 4972,5uL.

ab preparation:

1. anti-V5-HRP antibody (AbCam, Ab1325, R96125, Life Technologies, 1:9000, same as CSA ELISA, used for our protein and -control (VAR2), can be found in the fridge in the freezer room, Chumpol's shelf).
    a. prepare 10mL in total
    b. 1,11uL ab + 10mL binding buffer
2. rabbit anti mouse IgG (Used for the +control aCD3, P260, 1:1000, found close to QuantImage, -20 freezer, top shelf, Rack to the left, Box "Var2 ab")
    a. prepare 1mL in total
    b. 1 uL ab + 1mL binding buffer

FACS

FACS with PBMC's

Protein preparation: (dilution plate)

1. Measure the concentrations of the proteins on the nanodrop (remember to change the extension coefficient for each different construct).
2. Insert the concentrations and the name of the proteins of the FACS sheet on the folder VAR2CSA/databases/Nanna/Labbog. Remember to include one control protein per plate.
3. Mix proteins of the following concentrations in a deep 96-well plate:
   200 nM (to obtain saturated binding)-0,05 nM in a 4-fold dilution in PBS2.
4. Add the protein and the buffer to row A (240uL in total) and add 120 µl of PBS2 to row B-G.
5. Make a 4-fold dilution by transferring 40 µl from row A to row B. Mix at least 8 times with the multipipette for each row. Continue doing the same until row G. Do not discard the extra. LEAVE row H as BLANK!

Row H

- On the last row there will be the controls:

Blank	CD8 (APC-Cy7)	CD4(PE-Cy7)	V5 (FITC) 66,66 nM VAR2-aCD3	Blank	Blank	VAR2 200nM	66,67nM	22,22nM	7,41nM	2,47nM	0,82nM

  - 3 blanks = only PBS2 (120uL)
  - CD8 (APC-Cy7)= only PBS2 (120uL)
  - CD4 (PE-Cy7)= only PBS2 (120uL)
  - V5 (FITC) 66nM VAR2-aCD3 (see excel sheet, use one protein buffer)
  - VAR2 dilution serie Use mp4112 and do similar serie as above (200nM-0,05nM)

6. Leave the proteins diluted on ice covered with a ELISA sticker.
7. Set the plate centrifuge in the downstairs lab at 5°C and close the lid.

Cells (FACS plate)

8. After the plate has been shaking at 130rpm in 37 degrees for 1h. KEEP ON ICE!
9. Centrifuge cells at 600xg/4 min at 5°C. (Have the top row out)
10. Carefully remove supernatant by turning it gently upside down in the sink. Without turning it back put it on a piece of paper and turn it on the right way again. NOT throw it like ELISA!
11. Add 200 µl PBS2 with automatic multipipette to the side of the wells. After adding the PBS2 gently to each well. Pipette each row eight times then shift pipette.
12. Centrifuge cells at 600xg/4 min at 5°C. (Have the top row out)
13. Carefully remove supernatant by turning it gently upside down in the sink. Without turning it back put it on a piece of paper and turn it on the right way again. NOT throw it like ELISA!
14. Keep cells on ice while on bench

Protein and antibodies: (FACS plate)

15. Add protein solution/buffer 100 µl/well from the dilution plate, prepared before. Mix 8 times.
16. Incubate plate on whipping board in cold room for 30 min.
17. Centrifuge cells at 600xg/4 min at 5°C. (Have the top row out)
18. Carefully remove supernatant by turning it gently upside down in the sink. Without turning it back put it on a piece of paper and turn it on the right way again. NOT throw it like ELISA!
19. Add 200 µl PBS2 with automatic multipipette to the side of the wells. After adding the PBS2 gently to each well. Pipette each row eight times then shift pipette.
20. Centrifuge cells at 600xg/4 min at 5°C. (Have the top row out)
21. Repeat the washing step!
22. Resuspend cells in 100 ul/well **antibodies diluted in** PBS2
    a. Ab can be found in the freezer room, in the fridge, Chumpols box. Choose the ones for FACS.
        i. APC-Cy7 (CD8+) 557760 (fridge in the freezer room, top shelf, pink box, Anti-human)
        ii. PE-Cy7 (CD4+) 580644 (fridge in the freezer room, top shelf, pink box, Anti-human)
        iii. V5 (aCD3-VAR2+) 460308 (fridge in the freezer room, Chumpols box)
        iv. 16.6uL V5 + 8283,4uL PBS2 (vortex) → add on row A, C, D, E, F, G
        v. All above - mixture → add on the second row, B
            3uL of each ab (=9uL) + 1491uL PBS2 (vortex)

Row H:

Blank	CD8 (APC-Cy7)	CD4(PE-Cy7)	V5 (FITC) 66,66 nM VAR2-aCD3	Blank	Blank	VAR2 200nM	66,67nM	22,22nM	7,41nM	2,47nM	0,82nM

    b. Amounts for Ab'r preparations:
        i. 3 blanks = V5 (aCD3-VAR2+) from 8300uL prepared above
        ii. CD8 = 0,4uL APC-Cy7 (CD8+) (directly from the vial) + 100uL PBS2
        iii. CD4 = 0,4uL PE-Cy7(CD4+) (directly from the vial) + 100uL PBS2
        iv. V5 (FITC) 66nM VAR2-aCD3 = V5 (aCD3-VAR2+) from 8300uL prepared above
        v. VAR2 dilution serie = V5 (aCD3-VAR2+) from 8300uL prepared above
23. Incubate plate on whipping board in cold room for 30 min.
24. Centrifuge cells at 600xg/4 min at 5°C. (Have the top row out)
25. Carefully remove supernatant by turning it gently upside down in the sink. Without turning it back put it on a piece of paper and turn it on the right way again. NOT throw it like ELISA!
26. Add 200 µl PBS2 with automatic multipipette to the side of the wells. After adding the PBS2 gently to each well. Pipette each row eight times then shift pipette.
27. Centrifuge cells at 600xg/4 min at 5°C. (Have the top row out)
28. Repeat the washing step!
29. Resuspend cells in 200 µl PBS2

FC500 FACS machine:

30. Before turning on the FACS machine check if there is enough Cleanse fluid and Sheath fluid and that there is space in the waste container.
31. Use setups in Mette A's folder (password: var2csa10)
32. Make a cleanse cycle. Go to plate wizard and chose 40 well plate and drag the cleanse setup from the folder panels to the first four tubes. Make sure that the first tube is filled with cleanse solution (has to be changed every day) and the next three tubes with water.
33. When shutting down: when cleanse cycle has finished click on the cleanse button  . Wait until finished and the press idle mode  .
34. Go to the desktop and choose "FACS off"
35. Turn off the computer.

Analyze the data - simple program

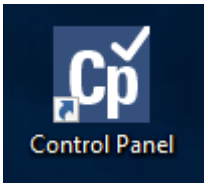
1. CD8 not very good positive signal (APC-CY7)
2. Plot with area and width (FSC-A and FSC-W or FSC-H) --> dot plot tells you if it is a monomer or duplette (our is collected to one long thin area--> monomer --> good
3. Gate lymphocytes, gate for single cells (from dot plot above)
4. Add static, geom mean, Alexa flour)
5. Add for the whole sheet

Floor 14 data program

1. Drag and drop
2. Heart → layout annotation → choose sample name
3. Start with positive control: gate for lymphocytes (FSC-A against SSC-A)
4. Gate for single cells (FSC-A against FSC-H)
5. Gate for Alexa Flour 488-A against Histogram
6. Statistic → sign lika statistic mean → geometric mean (choose the fluorophore)
7. Right click → copy analysis to all group
8. Layout editor → drop and drag → save as PDF
9. You can also draw the "single cell" line into the program on top of each other to see how it shifts. Right click the graf, choose "properties", "geometric mean", mark cells. Click on "T" and choose customize to get the peaks in a similar broad area.
10. Take one of the samples from the B row, Comp -PE -Cy 7 against Comp -APC-Cy7
    a. Than choose alexa on the X axis (against the gating for only APC + cells) where we can see the CD8 +
    b. Than choose the alexa on the y axis (against the gating for only PE+ cells) where we can see the CD4 +
    c. OR skip a & b and just gate all together since we just want to see all T cells anyway
11. Copy these setting for all in row B which have all ab detection
12. Click on the picture and than legend if it not appear

SE - HPLC

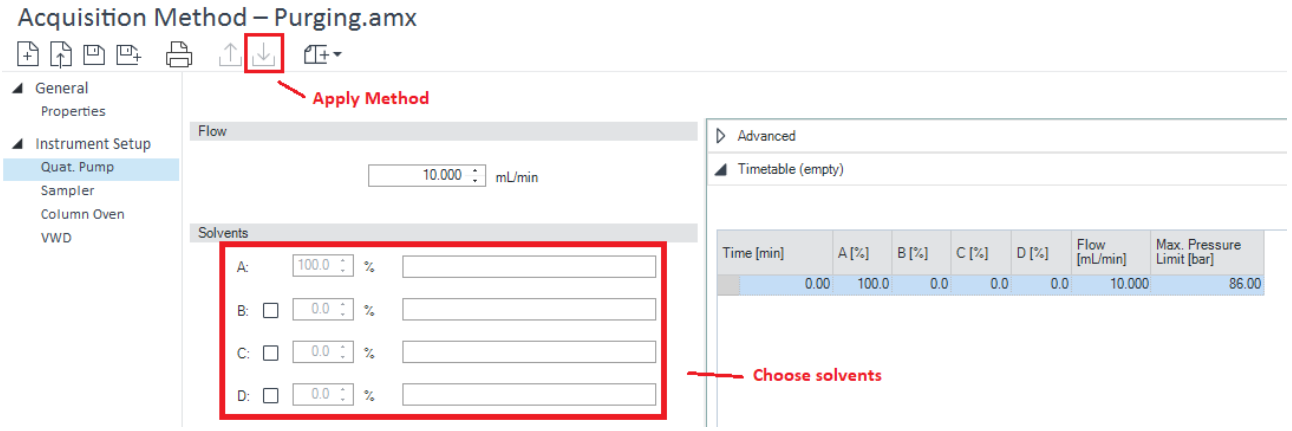
Initiating



1. Turn on the HPLC modules, using the "On" button shown. There are three modules that needs to be turned on: The pump, the column oven/auto sampler and the detector.
2. Turn on the computer (password: 3000hanover) and open the Control Panel (instrument).
3. Choose the appropriate project and press Launch (VAR2 QC). This opens the Acquisitions panel.
4. In the Acquisition panel turn on all devices.
5. NOTE: if the column device has not been used, start early, UV light takes time to heat up.

Purge

6. Open the valve by turning counterclockwise. This redirects the flow to bypass the column.
7. Go to Methods in the Acquisition panel and open the Acquisition method "Purging.amx"
8. Put the inlet tubes (A-D) in the applicable solvents and purge one by one.
    a. Set solvent A at 100%.
    b. Press "Send the current method to the instrument" to apply method
    c. Go to Status and turn on the Quat. Pump.
    d. Let it run for approximately 1 min at 1mg/mL.

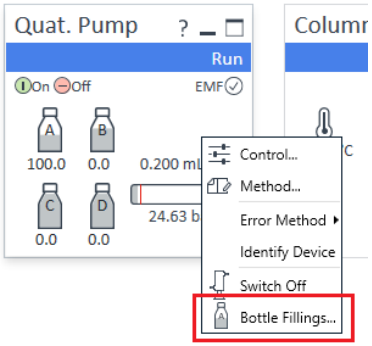


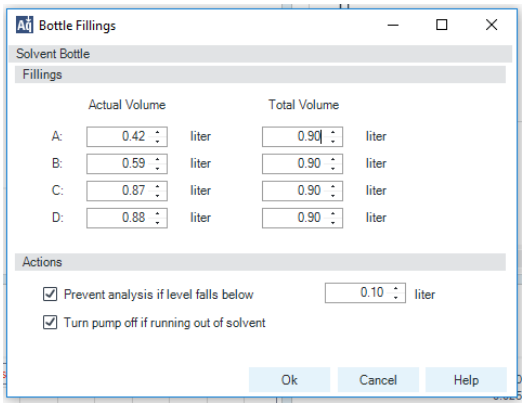
9. Repeat step a-d for each solvent inlet, before turning off the pump.
10. When it is closed set the flow to 0.04mg/mL

Set Bottle Fillings

11. Go to Status, right-click the Quat. Pump panel and choose "Bottle Fillings..."

12. Insert the current volumes of solvents and make sure that the “Actions are filled out as below. (Normally works digitally and nothing needs to be changed).





Programming an Acquisition Method

The parameters of given analysis are defined in the Acquisition Method that is being used. The following is a guide to set up a new Acquisition Method.

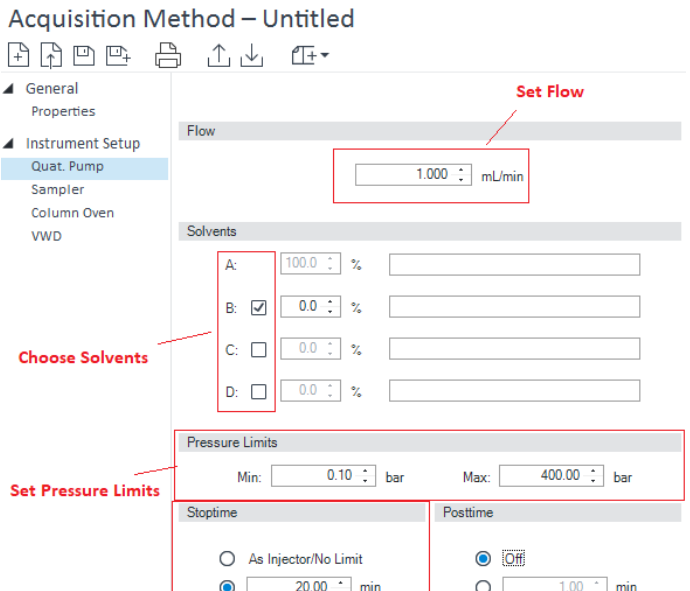
1. Open the Acquisition Panel, go to Method, and open a new Acquisition Method.

Here you can customize the setting for the 4 different modules: Quat. Pump, Sampler, Column Oven, and detector (VWD).

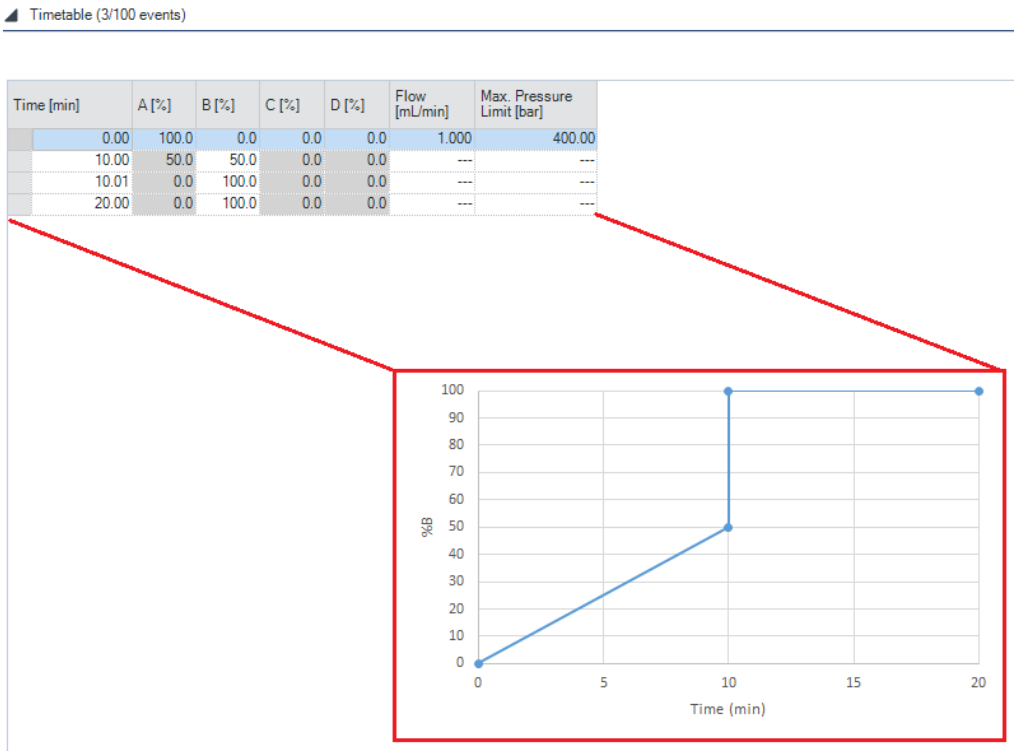
The Pump

open
set flow - 0.05 mg/mL SEC flow?
max 50 bar
stop time - 50 min
Posttime 10 min
sample loading in vials around 10-20uL (always take 10 uL extra than it says in protocol)in the bottoms of the vials.

2. Go to Quat. Pump, and start by defining the pressure limits.
    a. The maximum limit should be adjusted to fit the recommended limits of the column used.
    b. The Minimum limit should always be set to a minimum of 0.1 bar, as a lower pressure than this means that air has entered the system.
3. Insert the flow that you want to use for the method. Set to 0.05.
4. Set the Stoptime of your method. This should be the total time you want your method to run. **IMPOTANT:** If you do not set a stoptime the method will keep running until the system runs out of buffer. If the box “As Injector/No Limit” is ticked, untick it. 50 minutes.

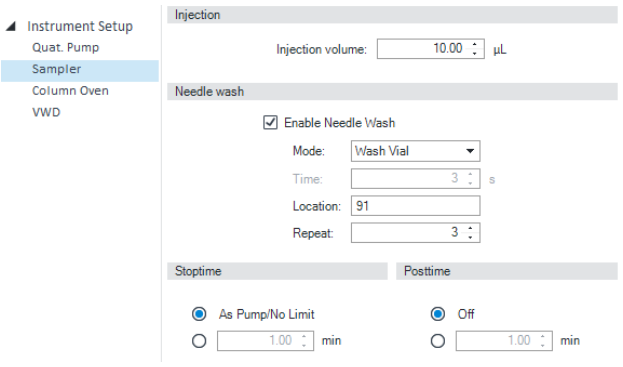


5. Tick of the solvents you want to use in your method.
6. Setup the timetable for your program.
    a. Add the needed steps. The software automatically makes a gradient between different buffer compositions. To make a step elution insert a step with the new buffer composition 0.01 min after the previous step.
    b. Below is shown an example of a random timetable and the corresponding solvent curve.



The Sampler

7. Insert your injection volume. The sampler is able to inject volumes in the 0-100µL range. In protein samples with a concentration of *>1mg/mL 5-10µL usually give a good signal.* **Use 10µL.**
8. **Take everything times 2 ! (20uL) in total**
9. **OBS ! Don't forget protein standards, can be found in the freezer VAR2 in the corridor, top shelf, to the right. 5uL needed**



HPLC	
Protein	Buffer

4,90	5,10
5,05	4,95
4,74	5,26
4,66	5,34
5,10	4,90
5,28	4,72

10. For the needle wash experience has shown that the *Wash Vial mode* works the best. To use the mode make a vial containing your sample buffer, and place it in position 91. Then type 91 in the location field. (Position 91 is the one usually used for the wash-vial.
11. Make sure the stoptime is marked "As Pump/No Limit" this will tie the stoptime of the sampler to the stoptime of the pump.
12. If needed it is possible to change the injection parameters further but the preset works fine.
13. Injection volume (Should as the protocol and not with the 10uL extra that we have added).
14. PBS always at place 91.

The Column Oven

15. (If you want to control the temperature of the column, you can set the column oven to temperature between 10°C and 80°C.) Use "not controlled"
16. Make sure the stoptime is marked "As Pump/No Limit" this will tie the stoptime of the column oven to the stoptime of the pump.

The Detector (VWD)

17. The detector has the ability to measure absorbance in the 190nm to 600nm range. It can detect a signal either at a single wavelength or at two wavelengths.
18. To get a signal at dual wavelengths, tick "Enable Dual-Wavelength" and insert the wavelength of signal A and B.
19. Högerklicka på devicen, välj method, välj 280nm to 260nm, save

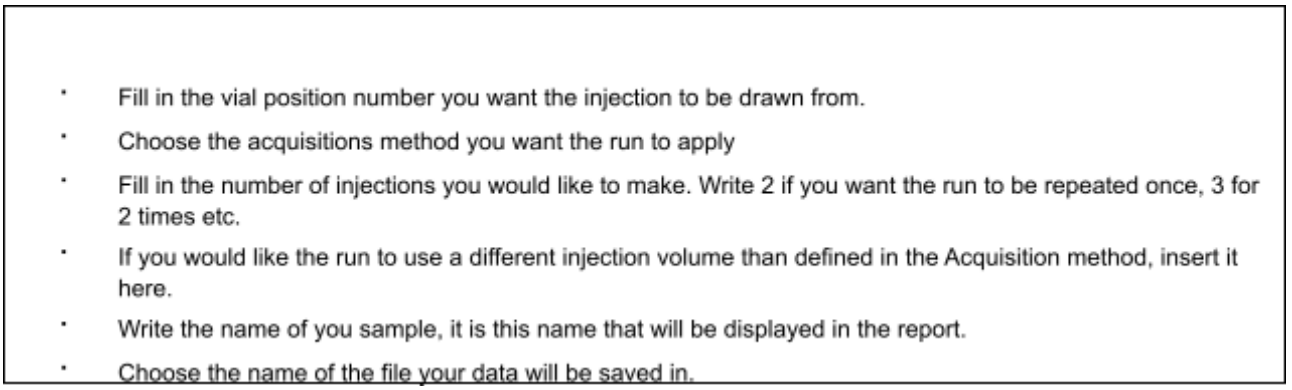
Running a Method

There are two ways of running a method: setup a sequence or setup a single run. Running a sequence is the preferable option, as you will always need to run a blank run before you run a sample. A single sample run can be used if you want to add a single sample to a sequence you have already started.

Setting up a sequence

1. Create a new sequence and save it as a sequence file (.sqx)

2. Insert a Blank Run as the first sample, and then insert you other samples. All samples should be filled out as shown on the picture below.



3. IF you want to auto fill an entire column right click the value you want to use and choose "Fill Down".
4. When everything has been filled out press "Run" to start the sequence.

IMPORTANT: When you have started a sequence, you need to apply a shutdown method. If you do not the system will keep running until it runs out of buffer. "Status" -shutdown method (look how the buffers are located (A, B, C, D). Need to run first water and than ethanol at 0.04mg/mL (100% C & 100%D eg).

Cycle ended

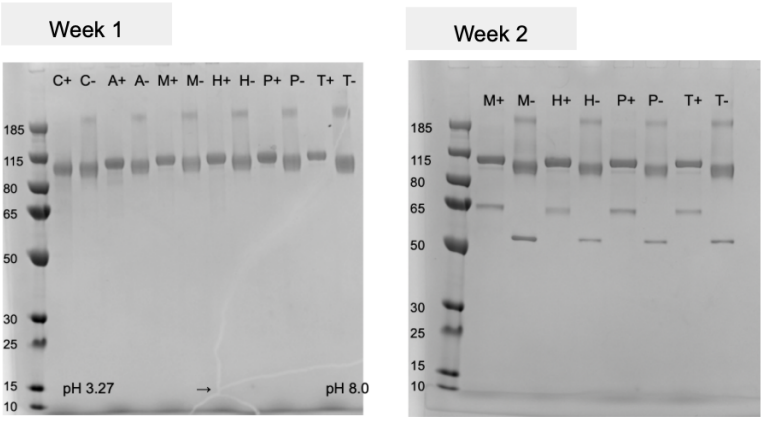
1. Right click the test that you have run in Acquisition Panel (The program where you can see all the pumps etc), click "launch..." (a new program will open "Data Panel")
2. (We are now in Data Selection) Mark all samples (Ctrl + shift), right click, click "load"
3. At the bottom to the left, click "Data processing"
4. Mark all samples
5. Choose method called "GL_LC Percent Default Method"
6. Mark all samples
7. At the top to the left, choose "Processing", than click "link method"
8. At the bottom to the left, click "Reporting"
9. Mark all samples, double click on "separate dual signal chromatography"
10. Ctrl + S (save as PDF)
11. Use an USB to transfer the data

VDW- wash, Ethanol - storage of the column, PBS - run the samples in (91), Protein standard -should appear 4 peaks (contains 4 proteins)

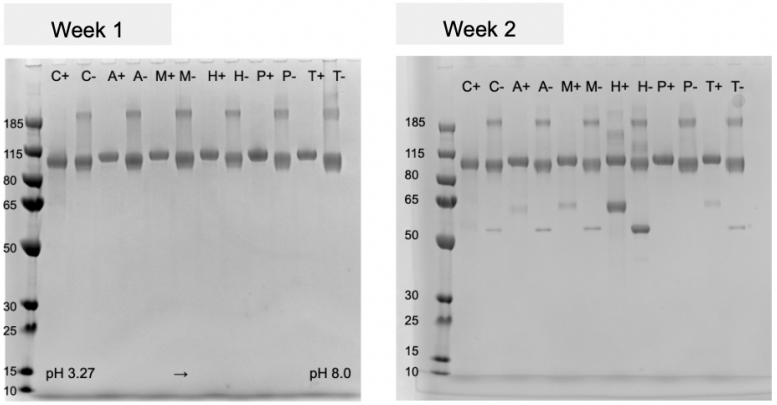
Appendix 3. Initial study

Appendix 3.1 SDS PAGE

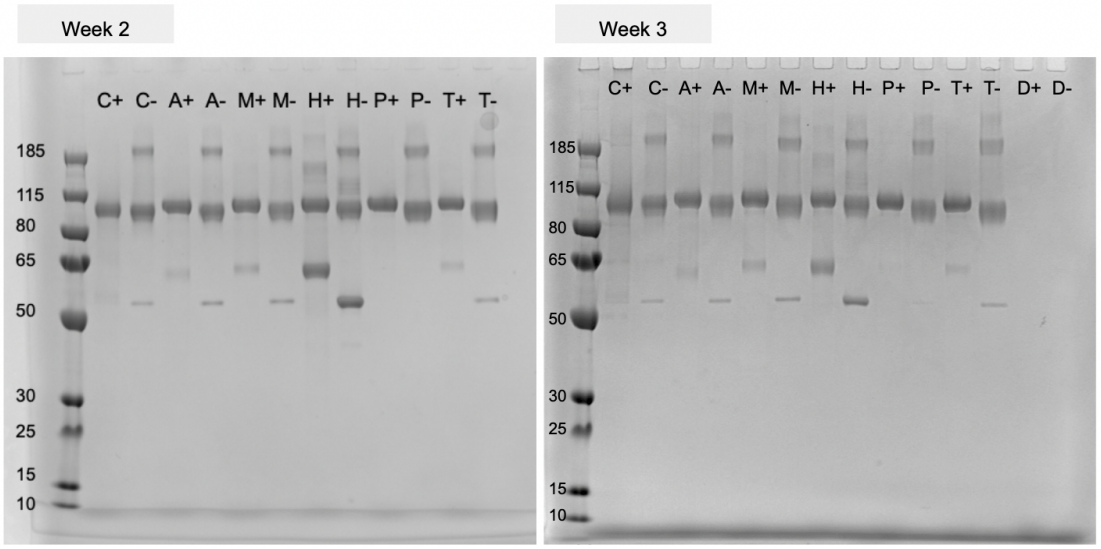
Appendix 3.1.1 Week 1 & 2, 4°C



Appendix 3.1.2 Week 1 & 2, -80°C

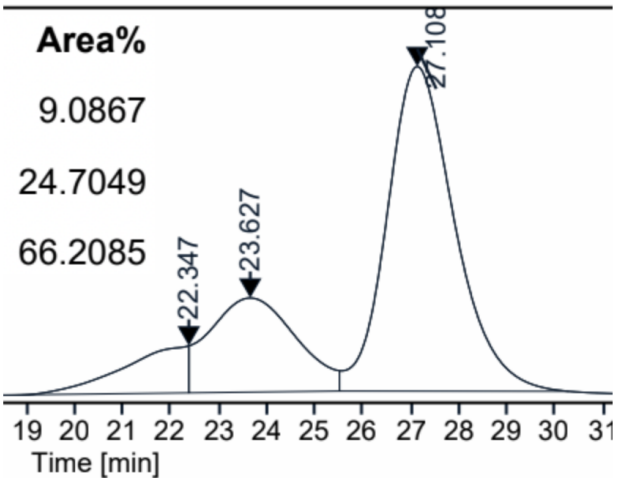


Appendix 3.1.3 Confirmation test, week 2 & 3



Appendix 3.2 HPLC

Appendix 3.2.1 Original protein, mp4111



Appendix 3.3 CSA ELISA

Appendix 3.3.1 Raw data, week 1

PLATE 1 -Decorin	Citrate (4°C)	Acetate (4°C)	MES (4°C)	HIS (4°C)	PBS (4°C)	TRIS-HCl (4°C)	Citrate (-80°C)	Acetate (-80°C)	MES (-80°C)	HIS (-80°C)	PBS (-80°C)	TRIS-HCl (-80°C)
	1	2	3	4	5	6	7	8	9	10	11	12
100	2,6751	1,9757	1,949	1,8863	1,936	2,083	2,304	1,955	2,1299	1,997	2,039	0,073

					5	5				8		
50	2,6515	1,7929	1,7313	1,7095	1,8557	1,8234	2,291	1,7361	1,7531	1,8654	1,8354	0,0671
25	2,0535	1,331	1,1336	1,4282	1,2856	1,3121	1,6644	1,4868	1,4664	1,3882	1,3121	0,0492
12,5	1,7386	0,9064	0,7425	0,9952	0,8241	0,8692	1,0738	1,0301	1,0392	0,8822	1,0471	0,0488
6,25	1,1141	0,5737	0,4075	0,6628	0,4512	0,4934	0,7904	0,7724	0,6567	0,6053	0,6111	0,0528
3,125	0,6714	0,2933	0,223	0,4571	0,2745	0,2797	0,4688	0,4869	0,3752	0,3542	0,3542	0,0476
1,5625	0,4013	0,1724	0,1311	0,3163	0,1053	0,1494	0,2437	0,3274	0,2145	0,2054	0,1966	0,046
0	0,0452	0,0492	0,0493	0,0508	0,0465	0,0458	0,0547	0,0477	0,0466	0,0466	0,0473	0,0508

PLATE 2 -HSPG	1	2	3	4	5	6	7	8	9	10	11	12
	Citrate (4°C)	Acetate (4°C)	MES (4°C)	HIS (4°C)	PBS (4°C)	TRIS-HCl (4°C)	Citrate (-80°C)	Acetate (-80°C)	MES (-80°C)	HIS (-80°C)	PBS (-80°C)	TRIS-HCl (-80°C)
100	1,9394	0,619	0,5637	0,5389	0,086	0,7665	0,9245	0,1657	0,6282	0,627	0,7008	0,9927
50	1,1781	0,3639	0,3359	0,3344	0,3877	0,4557	0,6772	0,381	0,4436	0,3977	0,5158	0,6212
25	0,5357	0,2177	0,1807	0,2362	0,206	0,2332	0,3808	0,2443	0,2426	0,2306	0,2759	0,3727
12,5	0,369	0,1441	0,1254	0,1462	0,1424	0,141	0,2277	0,1576	0,1722	0,1697	0,1715	0,2312
6,25	0,2566	0,1003	0,095	0,1162	0,102	0,1046	0,1606	0,1319	0,1189	0,12	0,1336	0,1577
3,125	0,1614	0,0905	0,0832	0,0985	0,0815	0,0777	0,1112	0,0934	0,1401	0,0826	0,0883	0,1232
1,5625	0,1085	0,0751	0,0744	0,0796	0,0785	0,0711	0,0925	0,0788	0,0927	0,0803	0,0937	0,1002
0	0,069	0,0664	0,0782	0,0927	0,0628	0,0608	0,0605	0,0632	0,0765	0,089	0,128	0,1057

Appendix 3.3.2 Raw data, week 2

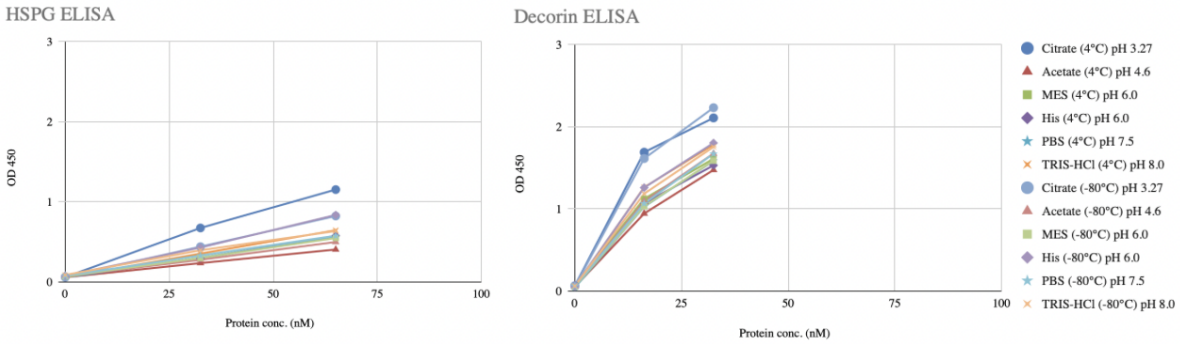
PLATE 1 -HSPG	1	2	3	4	5	6	7	8	9	10	11	12
	Citrate (4°C)	Acetate (4°C)	MES (4°C)	HIS (4°C)	PBS (4°C)	TRIS-HCl (4°C)	Citrate (-80°C)	Acetate (-80°C	MES (-80°C)	HIS (-80°C)	PBS (-80°C)	TRIS-HCl (-80°C

								)				)
65	1,1538	0,4056	0,5594	0,5775	0,5565	0,6467	0,8238	0,4992	0,5486	0,837	0,5777	0,6361
32,5	0,675	0,2365	0,3025	0,2941	0,301	0,3544	0,4415	0,2784	0,2993	0,4304	0,3309	0,3963
0	0,0604	0,0575	0,0593	0,0584	0,0569	0,0586	0,0632	0,0701	0,0702	0,0798	0,0766	0,088

PLATE 1 - Decorin												
32,5	2,1093	1,4757	1,6113	1,5321	1,5862	1,7841	2,2334	1,6757	1,5882	1,8035	1,6725	1,7562
16,25	1,6915	0,9451	1,1241	1,0682	1,0283	1,264	1,6149	1,0935	1,0335	1,2603	1,0676	1,1861
0	0,0649	0,0528	0,0629	0,0712	0,044	0,0536	0,058	0,0555	0,0535	0,0592	0,0597	0,0628

Appendix 3.3.3 Results, week 2

Week 2

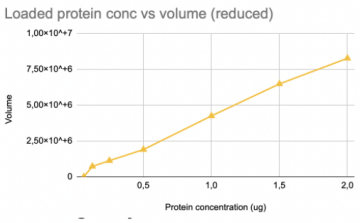


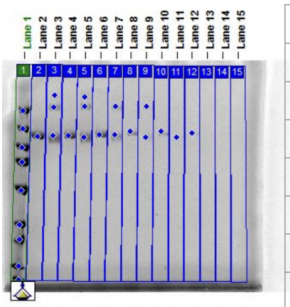
Appendix 4. Tonicity study

Appendix 4.1 SDS PAGE

Appendix 4.1.1 Linear range, original protein mp4206

Reduced



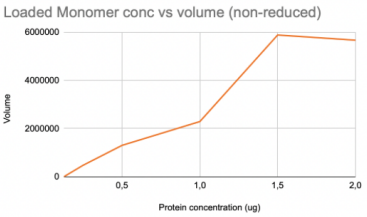


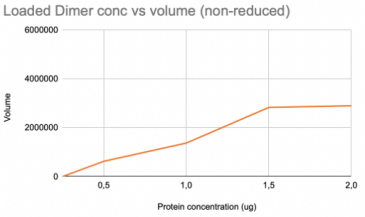
	ImageQuant (ug)	Loaded (ug)
1		Protein ladder
2	2	1,95
4	1,5	1,53
6	1,0	1,0 (set to this)
8	0,5	0,45
10	0,25	0,26
12	0,125	*~0,17*
14	0,0625	-

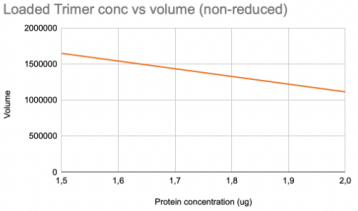
Comments:

- Linearity can be seen
- Trimers only seen in 2,0ug & 1,5ug

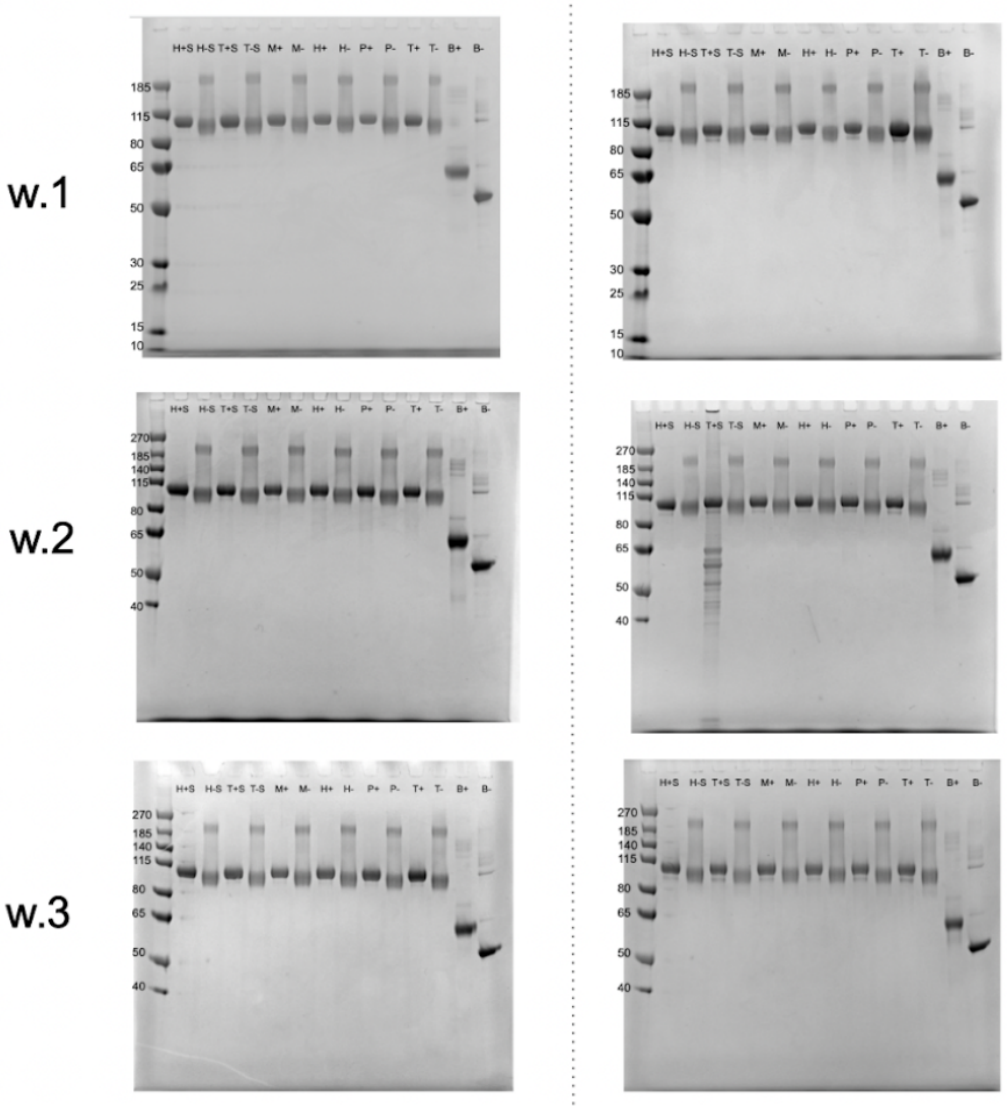
Non-reduced





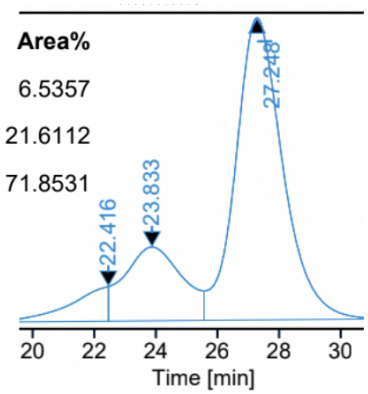


Appendix 4.1.2 Week 1, 2 & 3 gels

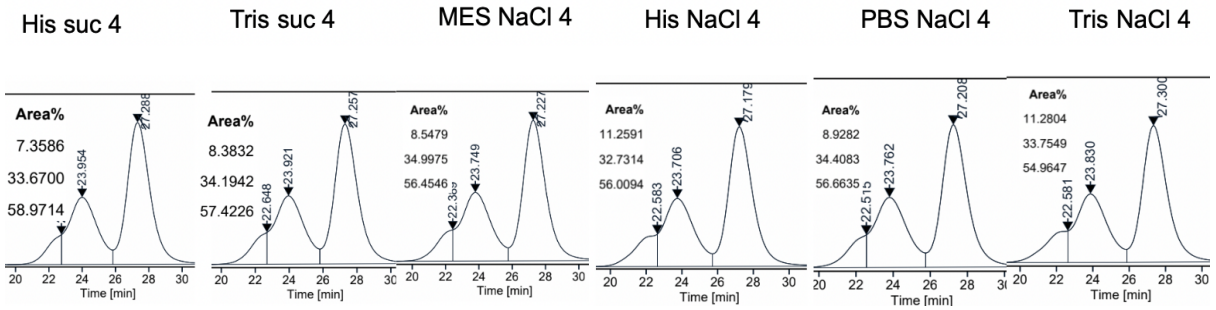


Appendix 4.2 HPLC

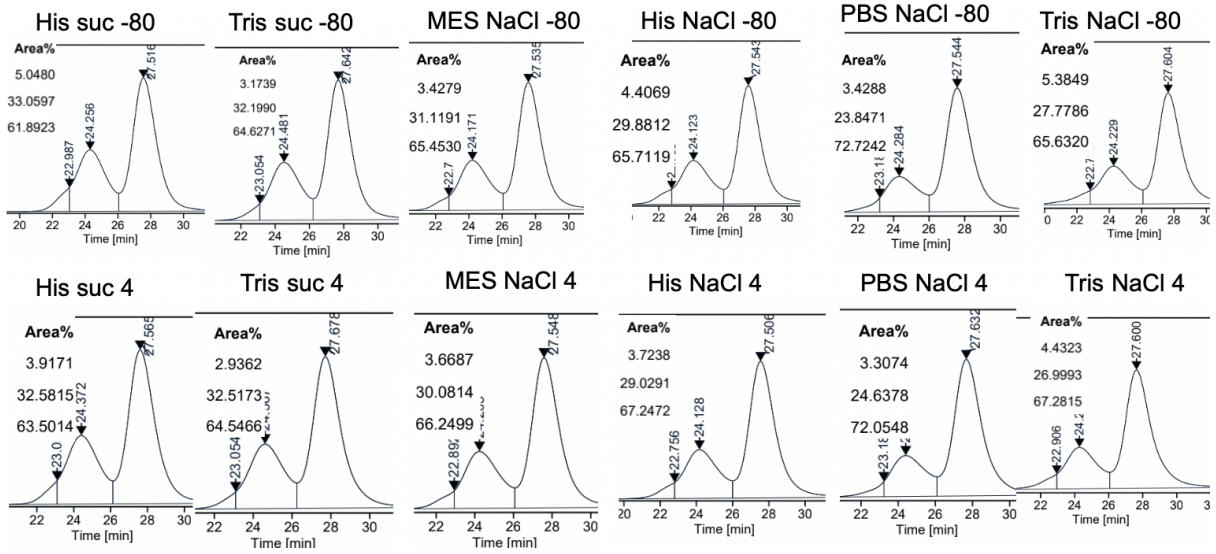
Appendix 4.2.1 Original protein, mp4206



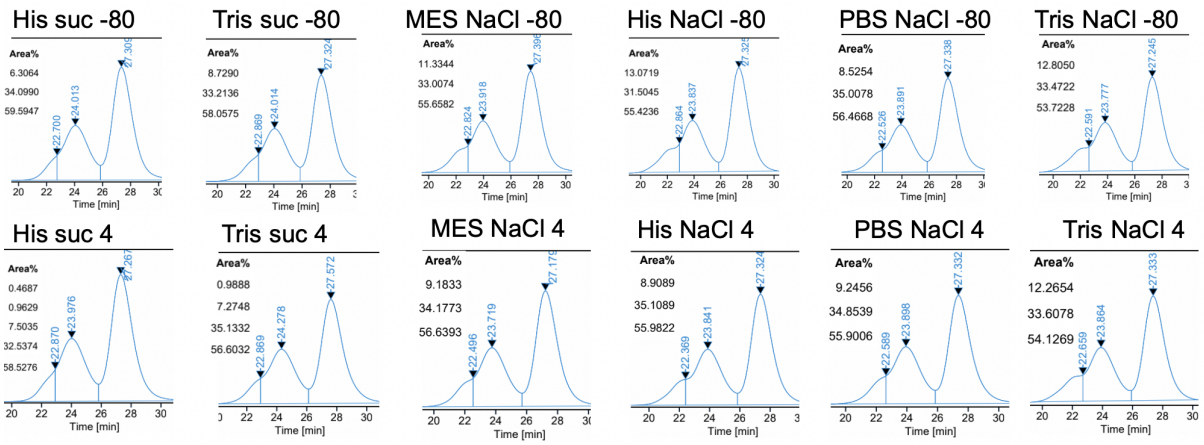
Appendix 4.2.2 Week 0, 4°C



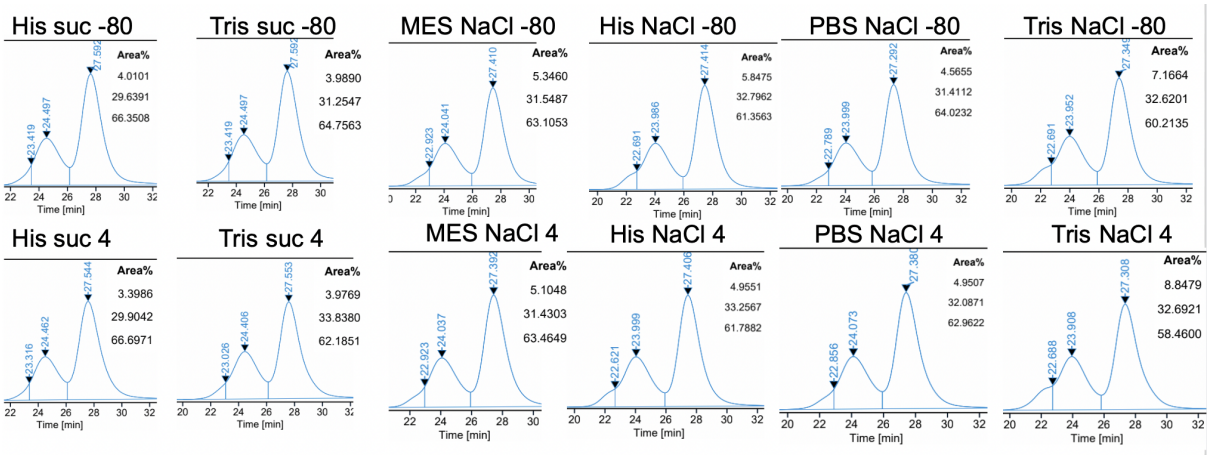
Appendix 4.2.3 Week 1, 4°C & -80°C



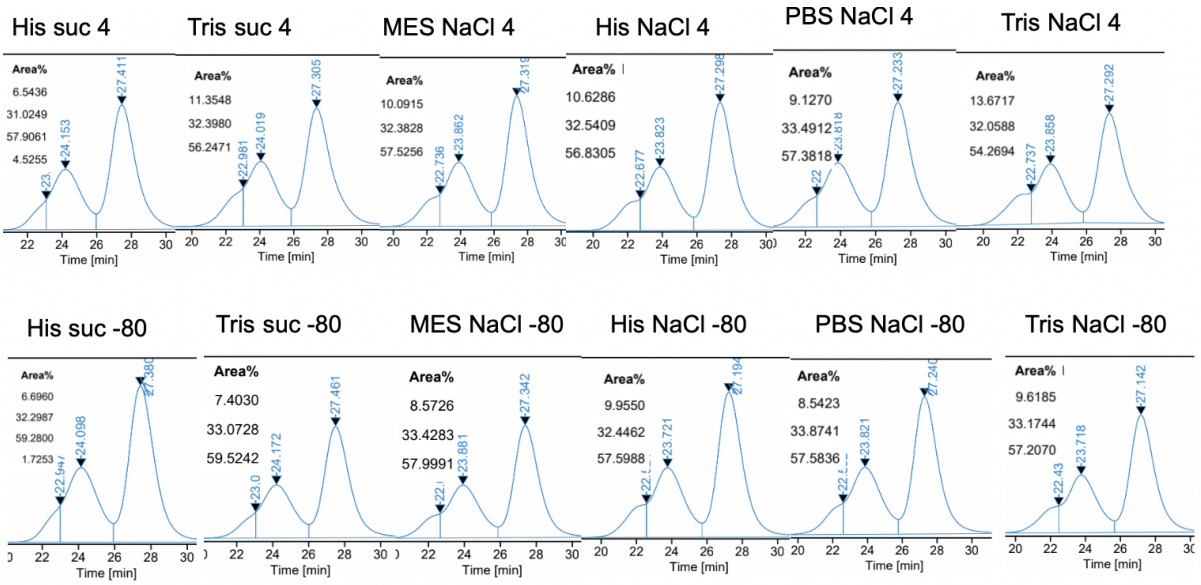
Appendix 4.2.4 Week 2, 4°C & -80°C



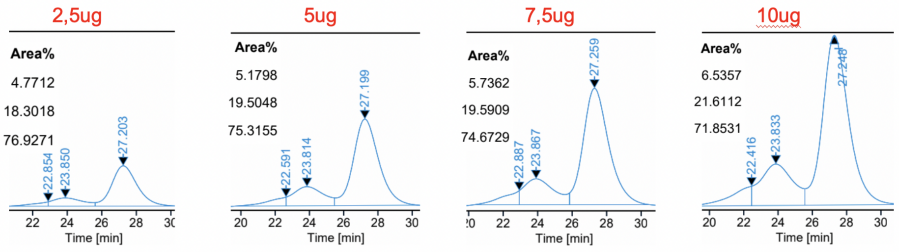
Appendix 4.2.5 Week 3, 4°C & -80°C

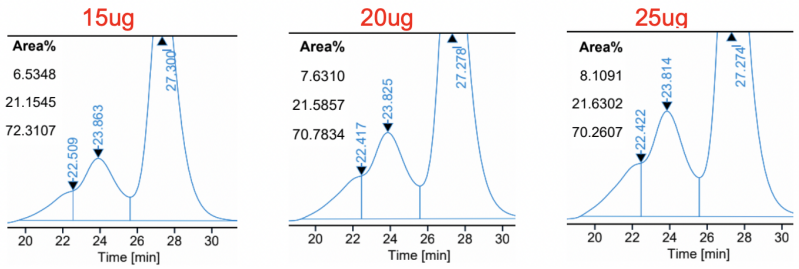


Appendix 4.2.6 Week 4, 4°C & -80°C



Appendix 4.2.7 Linear range, original protein mp4206





Appendix 4.3 CSA ELISA

Appendix 4.3.1 Week 0, Raw data (repeated)

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-80°C)	HIS+ NaCl (-80°C)	PBS + NaCl(-80°C)	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	3,4233	3,3231	3,3258	3,3323	3,3223	3,1925	3,3278	3,2971	3,3063	3,311	3,2401	3,5646
50	3,4226	3,3697	3,3455	3,3826	3,2679	3,3033	3,2772	3,298	3,2536	3,2744	3,3681	3,388
25	3,3926	3,3745	3,364	3,3524	3,3008	3,2475	3,2747	3,2193	3,2856	3,2629	3,2806	3,4096
12,5	3,2971	3,2097	3,1	3,2505	3,1999	3,1248	3,1182	3,1567	3,0355	3,1392	3,0697	3,0966
6,25	3,0522	3,0529	2,9793	3,0456	3,0855	2,9494	2,9123	2,9549	2,9592	3,0927	2,7905	2,8669
3,125	2,7768	2,7835	2,7835	2,7156	2,6509	2,693	2,665	2,587	2,6223	2,7076	2,3882	2,676
1,5625	2,317	2,2138	2,2906	2,3006	2,2126	2,114	2,3066	2,1389	2,204	2,3976	2,4634	2,1643
0	0,1345	0,1205	0,1205	0,1573	0,1373	0,1041	0,1047	0,1093	0,087	0,0946	0,1043	0,0981

PLATE 2 -HSPG	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-80°C)	HIS+ NaCl (-80°C)	PBS + NaCl(-80°C)	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	3,2993	3,2292	3,1066	3,1442	3,1827	3,1639	2,8032	2,9471	3,1264	3,1377	2,9403	3,1758
50	2,9859	2,8467	2,9547	2,9399	2,7333	2,8174	2,6324	2,6374	2,7953	2,7561	2,7878	3,1019
25	2,6464	2,4364	2,5403	2,5851	2,5576	2,5293	2,3247	2,2054	2,4713	2,5846	2,4751	2,4667
12,5	2,2037	2,1326	1,9309	1,9955	2,0411	1,9778	1,9229	1,896	2,0183	2,0822	2,1799	2,1349
6,25	1,3935	1,3571	1,3569	1,4002	1,3834	1,3962	1,4013	1,3223	1,4504	1,6952	1,444	1,4256
3,125	0,8104	0,7455	0,7118	0,7564	0,7728	0,7782	0,9104	0,7496	0,7485	0,8716	0,818	0,7598
1,5625	0,5341	0,4586	0,4281	0,4259	0,4736	0,4695	0,4276	0,4525	0,4573	0,5357	0,5213	0,4647
0	0,275	0,211	0,2427	0,2064	0,2121	0,2226	0,1787	0,1937	0,1981	0,203	0,2634	0,202

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-80°C)	HIS+ NaCl (-80°C)	PBS + NaCl(-80°C)	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	2,1249	2,1016	2,1678	2,1017	2,1934	2,1433	1,9761	2,0789	2,1847	2,116	2,1319	2,3539
50	1,886	1,8767	1,8465	1,8097	1,8258	1,9536	1,774	1,9143	1,951	2,0311	2,01	2,002
25	1,4607	1,3874	1,4448	1,3862	1,4418	1,4458	1,2702	1,4775	1,5245	1,565	1,4266	1,4593
12,5	0,9933	0,9306	0,9683	0,9745	0,9934	1,0434	0,795	1,0476	1,0119	1,1446	0,9175	0,9149
6,25	0,6075	0,5379	0,5633	0,6258	0,6042	0,622	0,4752	0,6425	0,6339	0,8548	0,556	0,6929
3,125	0,339	0,2984	0,3111	0,3649	0,3277	0,3191	0,2429	0,3436	0,3691	0,5785	0,3024	0,3993
1,5625	0,1834	0,1563	0,1706	0,2093	0,1653	0,1711	0,1404	0,1808	0,1965	0,3235	0,1792	0,1925
0	0,0443	0,05	0,0494	0,0456	0,0472	0,0454	0,0505	0,0501	0,0515	0,0534	0,0516	0,0521

PLATE 2 -HSPG	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-80°C)	HIS+ NaCl (-80°C)	PBS + NaCl(-80°C)	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	0,7592	0,0804	0,8155	0,7344	0,8242	0,9459	0,5261	0,9301	0,9626	0,9464	0,8212	0,896
50	0,4006	0,0586	0,4023	0,3596	0,4199	0,4662	0,3	0,5044	0,4621	0,5019	0,4617	0,48
25	0,2367	0,0478	0,2265	0,2115	0,2448	0,2503	0,1605	0,2671	0,254	0,2839	0,2623	0,2865
12,5	0,1501	0,0512	0,1494	0,1396	0,1662	0,1638	0,1105	0,1687	0,1622	0,188	0,1504	0,176
6,25	0,1095	0,0453	0,1047	0,1034	0,1089	0,1137	0,094	0,1225	0,1188	0,1337	0,114	0,1282
3,125	0,0919	0,0452	0,084	0,0852	0,0869	0,09	0,1143	0,0911	0,0882	0,0994	0,0875	0,1169
1,5625	0,0879	0,0551	0,0741	0,0767	0,0767	0,0785	0,0741	0,0775	0,079	0,0921	0,0776	0,0897
0	0,0777	0,043	0,0683	0,0596	0,059	0,0734	0,0608	0,0662	0,0692	0,0661	0,0639	0,0668

Appendix 4.3.2 Week 1, Raw data

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	2,1267	2,1218	2,1393	2,1587	2,2464	2,132	1,9993	2,16	2,0364	2,1616	2,1465	2,3242
50	1,7285	1,7238	1,967	1,7586	1,7555	1,9503	1,7708	1,8643	1,9597	2,0584	1,8819	2,001
25	1,1678	1,1657	1,2272	1,3554	1,138	1,3801	1,1825	1,3181	1,3033	1,5842	1,3129	1,2737
12,5	0,7328	0,7449	0,7917	0,9478	0,6933	0,9851	0,7977	0,8295	0,9549	1,1057	0,9206	0,8319
6,25	0,4151	0,4189	0,5762	0,5157	0,4593	0,5617	0,4883	0,5234	0,6038	0,9392	0,5765	0,5116
3,125	0,2331	0,2168	0,3451	0,2798	0,2598	0,2905	0,2661	0,2647	0,3627	0,5781	0,3313	0,2946
1,5625	0,1199	0,1373	0,1863	0,1571	0,146	0,1693	0,1484	0,1575	0,2038	0,0622	0,1778	0,1685
0	0,0453	0,0486	0,045	0,0453	0,0438	0,0561	0,0435	0,0442	0,0438	0,0477	0,0416	0,0416

PLATE 2 -HSPG	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	0,4629	0,605	0,7261	0,708	0,8194	0,743	0,5582	0,7301	0,9083	0,8566	0,8608	0,8262
50	0,2597	0,3341	0,3693	0,3411	0,3492	0,3745	0,2654	0,3486	0,4157	0,4164	0,4209	0,3868
25	0,1543	0,1673	0,1946	0,1724	0,1709	0,1791	0,1532	0,1937	0,2147	0,233	0,2272	0,2212
12,5	0,1089	0,1013	0,1298	0,1051	0,113	0,1207	0,0989	0,1281	0,1372	0,1642	0,152	0,1487
6,25	0,0761	0,08	0,0913	0,0791	0,0847	0,0939	0,0841	0,0977	0,102	0,1178	0,1103	0,1226
3,125	0,068	0,0703	0,0734	0,0677	0,0715	0,0732	0,067	0,0733	0,0803	0,0983	0,0775	0,0923
1,5625	0,0659	0,0679	0,0737	0,0612	0,0645	0,0672	0,0653	0,0653	0,0681	0,0556	0,0711	0,0761
0	0,0641	0,07	0,0675	0,0584	0,0628	0,056	0,057	0,0562	0,0552	0,0634	0,0565	0,061

Appendix 4.3.3 Week 2, Raw data

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	2,0464	2,1281	2,0608	2,1797	1,8955	1,9117	1,6736	1,9329	1,923	2,0126	1,9858	1,9528
50	1,741	1,7971	1,9201	1,8716	1,8885	1,7699	1,5991	1,7475	1,6561	1,7354	1,7339	1,7572
25	1,2799	1,3329	1,4072	1,5295	1,2805	1,2856	1,1565	1,2924	1,2356	1,3581	1,327	1,2648
12,5	0,8867	0,8517	0,9876	1,1118	0,9357	0,9456	0,7133	0,806	0,8394	0,8704	0,862	0,8883
6,25	0,5121	0,5107	0,5687	0,6362	0,5361	0,5315	0,4566	0,4866	0,5025	0,5211	0,5494	0,5142
3,125	0,2979	0,2833	0,3092	0,37	0,297	0,3069	0,2454	0,2767	0,2777	0,3069	0,2949	0,2969
1,5625	0,1628	0,1521	0,1643	0,2152	0,1648	0,1743	0,1437	0,1523	0,1538	0,1776	0,1747	0,1661
0	0,0463	0,0497	0,0501	0,048	0,0472	0,0539	0,0547	0,0572	0,0488	0,0531	0,0536	0,0526

PLATE 2 -HSPG	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	0,6276	0,7199	0,8303	0,8536	0,8516	0,7886	0,5209	0,8487	0,658	0,8591	0,7788	0,8407
50	0,3191	0,3516	0,4148	0,4203	0,4249	0,4054	0,3134	0,4433	0,3578	0,4341	0,4338	0,4445
25	0,1868	0,1937	0,2379	0,2429	0,232	0,2349	0,1594	0,2497	0,2001	0,2387	0,2431	0,2485
12,5	0,1267	0,1284	0,1447	0,1628	0,1458	0,1461	0,1158	0,1619	0,1351	0,1481	0,1573	0,1582
6,25	0,0946	0,0942	0,099	0,1101	0,1041	0,1076	0,096	0,1163	0,107	0,1179	0,1137	0,1323
3,125	0,0831	0,0788	0,0862	0,0862	0,0848	0,0889	0,077	0,0906	0,0944	0,091	0,0989	0,1097
1,5625	0,0814	0,0767	0,0797	0,0751	0,0814	0,0814	0,0757	0,0801	0,0741	0,0946	0,0839	0,1
0	0,067	0,0767	0,0654	0,3917	0,0642	0,0659	0,0694	0,074	0,0711	0,075	0,0753	0,0854

Appendix 4.3.4 Week 3, Raw data

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	2,11	2,1731	2,0463	2,0571	2,0445	2,0836	1,9319	2,0806	1,9856	2,0484	2,015	2,1931
50	1,777	1,8176	1,9463	1,8328	1,6844	1,7779	1,6647	1,7806	1,7575	1,7581	1,9144	1,905
25	1,3754	1,3402	1,5208	1,387	1,2648	1,3634	1,2233	1,4104	1,3903	1,4488	1,3918	1,4627
12,5	0,8449	0,8837	1,0716	0,9303	0,7813	0,8702	0,8755	0,9576	0,9374	1,0151	0,9618	1,0001
6,25	0,5235	0,543	0,5891	0,539	0,4421	0,5278	0,4728	0,5496	0,5682	0,6058	0,5662	0,5989
3,125	0,2996	0,295	0,3417	0,2958	0,2465	0,2795	0,2719	0,3101	0,3249	0,3425	0,313	0,3504
1,5625	0,1644	0,1682	0,188	0,1594	0,1382	0,1686	0,1443	0,1727	0,187	0,2045	0,1755	0,1949
0	0,0589	0,0532	0,0608	0,0517	0,0513	0,0512	0,053	0,0529	0,0538	0,0563	0,0538	0,0543

PLATE 2 -HSPG	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	0,6311	0,7466	0,8937	0,813	0,6908	0,8107	0,5952	0,9877	0,876	1,0143	1,1515	1,1911
50	0,3133	0,3675	0,4457	0,4006	0,3433	0,4013	0,2852	0,4878	0,4514	0,5114	0,5773	0,6121
25	0,1728	0,199	0,2268	0,2135	0,1876	0,214	0,1578	0,2586	0,2288	0,2635	0,2821	0,3489
12,5	0,1145	0,1246	0,1329	0,1288	0,1178	0,1336	0,1011	0,1445	0,134	0,1521	0,1741	0,2084
6,25	0,0932	0,0891	0,0982	0,0931	0,0854	0,0947	0,0794	0,1038	0,1035	0,1103	0,1246	0,1523
3,125	0,0784	0,0851	0,0789	0,0808	0,0747	0,0778	0,0747	0,0812	0,0878	0,0903	0,095	0,1135
1,5625	0,0914	0,0793	0,0868	0,0777	0,0763	0,0736	0,0733	0,0782	0,0868	0,0837	0,0823	0,0886
0	0,0728	0,081	0,0782	0,0786	0,0729	0,0841	0,0668	0,0775	0,0768	0,0821	0,0843	0,0765

Appendix 4.3.5 Week 4, Raw data

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	1,892	1,8623	1,87	1,8401	1,8213	1,7265	1,6498	1,6863	1,7981	1,7074	1,808	1,9667
50	1,4879	1,422	1,6312	1,5608	1,5278	1,3901	1,3276	1,2733	1,4591	1,3306	1,5341	1,5803
25	1,0329	1,0259	1,2392	1,0962	1,0899	1,0458	0,8879	0,9376	1,0725	0,9941	1,1678	1,1469
12,5	0,6685	0,6351	0,7962	0,7193	0,6903	0,6793	0,5754	0,6396	0,6848	0,628	0,7329	0,7759
6,25	0,3734	0,3639	0,4682	0,4182	0,3904	0,4037	0,2929	0,3524	0,3982	0,3623	0,4268	0,4538
3,125	0,2002	0,1943	0,2562	0,23	0,2293	0,2151	0,179	0,1959	0,225	0,2138	0,2533	0,2527
1,5625	0,1339	0,1232	0,1446	0,1425	0,1375	0,1379	0,1114	0,1149	0,1399	0,1219	0,1512	0,1428
0	0,0502	0,0849	0,0534	0,0537	0,0557	0,064	0,0584	0,0586	0,0539	0,0529	0,0533	0,0624

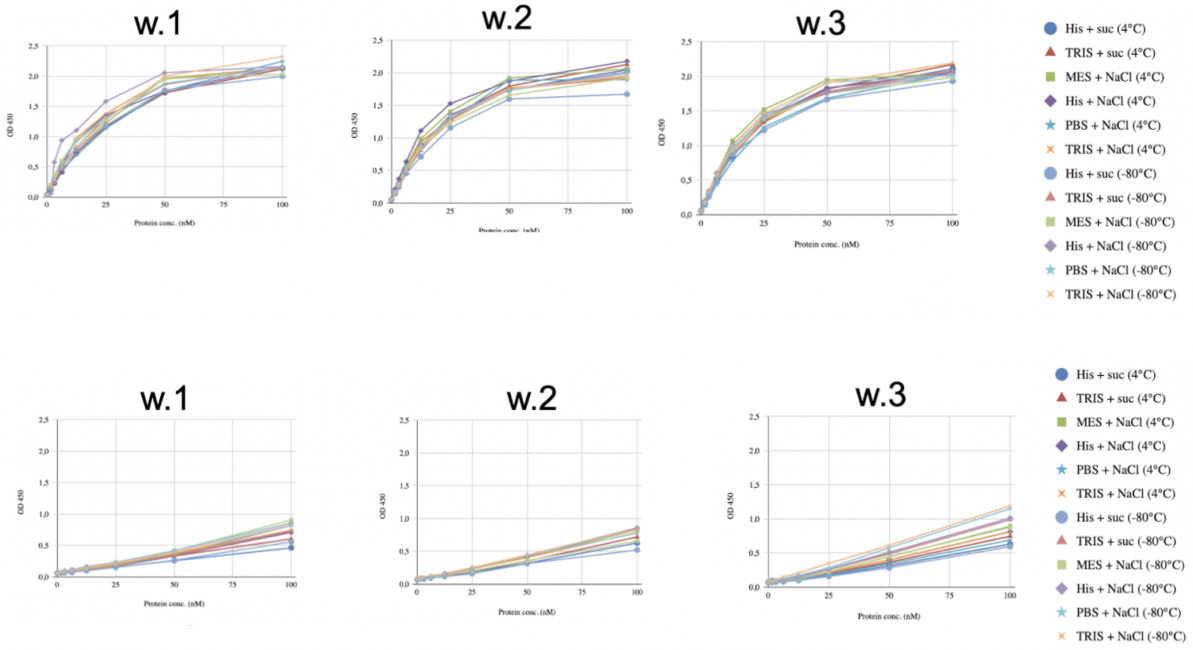
PLATE 2 -HSPG	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4'	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	0,4584	0,5791	0,8332	0,7629	0,7638	0,7563	0,4612	0,7056	0,7468	0,7175	0,9254	0,8396
50	0,2225	0,26	0,376	0,3469	0,3281	0,3611	0,2209	0,3536	0,3518	0,3553	0,4656	0,4278
25	0,1301	0,151	0,207	0,202	0,1865	0,1896	0,1282	0,1825	0,1945	0,196	0,2519	0,2268
12,5	0,0962	0,1081	0,1288	0,1238	0,1182	0,1338	0,0914	0,1216	0,1252	0,141	0,1569	0,1446
6,25	0,0736	0,0861	0,0898	0,0892	0,0883	0,0889	0,0833	0,088	0,0973	0,0976	0,1119	0,1175
3,125	0,0738	0,071	0,0827	0,0716	0,0729	0,0754	0,0657	0,0798	0,0899	0,0821	0,0951	0,0957
1,5625	0,0717	0,0732	0,0761	0,0826	0,0737	0,082	0,0772	0,0769	0,0764	0,0814	0,0869	0,1266
0	0,1203	0,0625	0,066	0,0777	0,0664	0,0703	0,058		0,0763	0,0966	0,08	0,1427

Appendix 4.3.6 Week 12, Raw data

PLATE 1 -Decorin	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	1,9775	2,1379	1,9761	1,9966	2,075	2,0969	1,9495	1,9874	1,9979	2,0589	2,0371	2,0626
50	1,7991	1,8409	1,8046	1,79	1,8149	1,754	1,7462	1,7541	1,7861	1,8965	1,8391	1,8613
25	1,4804	1,4654	1,4791	1,5433	1,4492	1,3924	1,4104	1,4777	1,5202	1,5551	1,5541	1,5855
12,5	1,0678	1,0594	1,051	1,0888	1,1128	0,9655	1,0122	1,0256	1,1354	1,1781	1,0663	1,2181
6,25	0,6532	0,6523	0,6824	0,725	0,6931	0,6211	0,6854	0,6861	0,8249	0,8154	0,745	0,8863
3,125	0,3731	0,3625	0,3762	0,4144	0,3976	0,3303	0,3766	0,3987	0,472	0,4812	0,4391	0,4845
1,5625	0,2012	0,1682	0,1463	0,1691	0,1669	0,1601	0,1684	0,1728				
0	0,0508	0,0538	0,0625	0,0546	0,0511	0,0488	0,0478	0,0521	0,0485	0,0574	0,066	0,0518

PLATE 2 -HSPG												
	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4	HIS + NaCl (4°C)	PBS + NaCl (4	TRIS-HCl + Na	HIS + suc(-80°C)	TRIS-HCl + su	MES + NaCl (-80°	HIS+ NaCl (-80°	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	0,6854	0,7483	0,8377	0,9033	0,8873	0,6778	0,7229	0,9793	1,027	1,0638	1,071	1,2181
50	0,3591	0,3606	0,4035	0,4451	0,4565	0,3687	0,3563	0,5034	0,5449	0,5309	0,5304	0,7085
25	0,1869	0,1889	0,208	0,2482	0,2349	0,1932	0,1846	0,2575	0,2815	0,2846	0,2952	0,3518
12,5	0,1257	0,1251	0,1349	0,1434	0,1417	0,1286	0,1206	0,1567	0,1647	0,1661	0,1694	0,2038
6,25	0,0868	0,0904	0,1005	0,0997	0,0981	0,0889	0,0907	0,1051	0,1087	0,1111	0,1127	0,1364
3,125	0,0712	0,0692	0,0773	0,074	0,0726	0,0652	0,066	0,0743	0,0788	0,0826	0,0856	0,0905
1,5625	0,0657	0,0655	0,0909	0,1533	0,0641	0,0658	0,0622	0,0625	0,1362	0,0669	0,0684	0,074
0	0,0637	0,0646	0,0693	0,0727	0,0639	0,0599	0,0684	0,0634	0,0595	0,0751	0,0603	0,0607

Appendix 4.3.6 Week 1, 2 and 3 Results



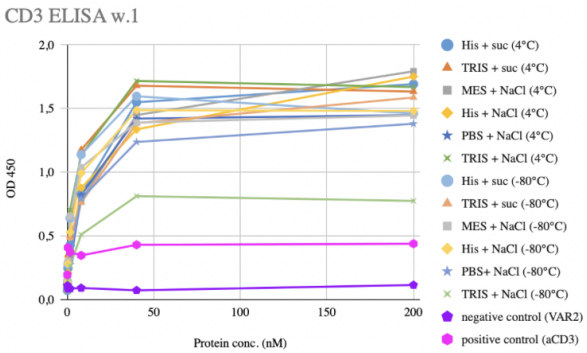
Appendix 4.4 CD3 ELISA

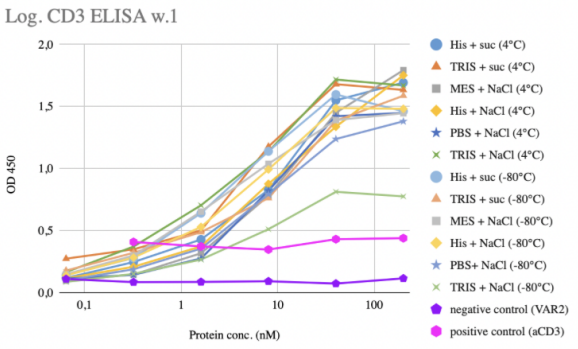
Appendix 4.4.1 Week 1, Set up & Raw Data

	1	2	3	4	5	6	7	8	9	10	11	12	
Plate 1	HIS + suc (4°C)	TRIS-HCl + suc (4°C	MES + NaCl (	HIS + NaCl (4°C)	PBS + NaCl (	TRIS-HCl + NaC	HIS + suc(-80°C)	TRIS-HCl + suc (-	MES + NaCl	HIS + NaC	PBS + NaCl(-8	TRIS-HCl + NaCl (-80°C)	
200													
40													
8													
1,6													
0,32													
0,064													
	Blank						Blank						
VAR2(-)	200	40	8	1,6	0,32	0,064	200	40	8	1,6	0,32	Blank	aCD3 (+)
												aCD3 (+)	

	HIS + suc (4°C)	TRIS-HCl + suc (4°C	MES + NaCl (	HIS + NaCl (4°C)	PBS + NaCl (	TRIS-HCl + NaC	HIS + suc(-80°C)	TRIS-HCl + suc (-	MES + NaCl	HIS + NaC	PBS + NaCl(-8	TRIS-HCl + Na	VAR2	aCD3
200	1,6925	1,6339	1,7931	1,7516	1,449	1,6694	1,4618	1,5871	1,4462	1,4817	1,381	0,7755	0,1148	0,4388
40	1,55	1,68	1,4494	1,3372	1,4217	1,7172	1,5967	1,3855	1,3906	1,4877	1,2382	0,8127	0,0731	0,4306
8	0,8271	1,1767	0,7804	0,8765	0,8213	1,1362	1,1391	0,7618	1,0383	0,9928	0,7929	0,5111	0,0916	0,3472
1,6	0,4283	0,5014	0,3171	0,3725	0,2751	0,7038	0,64	0,4873	0,6531	0,5292	0,3547	0,2661	0,0863	0,3724
0,32	0,2464	0,3488	0,1459	0,2084	0,1436	0,3711	0,2788	0,3211	0,2988	0,282	0,1882	0,1511	0,0848	0,4083
0,064	0,1175	0,2737	0,0959	0,1147	0,1132	0,1651	0,1408	0,1775	0,1474	0,1388	0,0986	0,0863	0,1096	
0	0,0734	0,074	0,0925	0,0906	0,0933	0,1265	0,0957	0,0892	0,0826	0,1015	0,0644	0,0662		0,1948

Appendix 4.4.2 Week 1, logarithmic scale



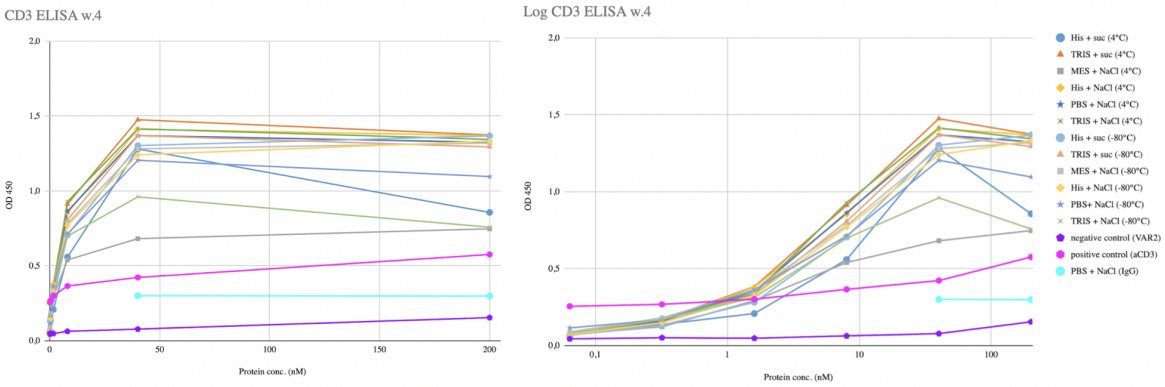


Appendix 4.4.3 Week 4, Set up & Raw Data

1	2	3	4	5	6	7	8	9	10	11	12	
HIS + suc (4°C)	TRIS-HCl + suc (4°C	MES + NaCl (	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4°	HIS + suc(-80°C)	TRIS-HCl + suc (-80°C)	MES + NaCl	HIS + NaCl (	PBS + NaCl(-8	TRIS-HCl + NaCl (-80°C)	
			Ab for VAR2 and VAR2-aCD3 detection: anti-V5-HRP antibody									
Blank				200 (PBS 4, IgG	40 (PBS 4, IgG	Blank						
200	40	8	1,6	0,32	0,064	200	40	8	1,6	0,32	Blank	aCD3 (+)
											aCD3 (+)	

	HIS + suc (4°C)	TRIS-HCl + suc (4°C	MES + NaCl (	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4°	HIS + suc(-80°C)	TRIS-HCl + suc (-80°C)	MES + NaCl	HIS + NaCl (	PBS + NaCl(-8	TRIS-HCl + Na	VAR2	aCD3	
200	0,8556	1,3724	0,7464	1,3677	1,3233	1,3426	1,3666	1,2927	1,3182	1,3315	1,095	0,757	0,1537	0,5751	0,2976
40	1,279	1,4741	0,6807	1,41	1,3686	1,4134	1,3019	1,3679	1,28	1,2401	1,2036	0,9595	0,0771	0,4217	0,3
8	0,5579	0,9107	0,5388	0,8538	0,8623	0,9279	0,705	0,806	0,7803	0,7685	0,712	0,6971	0,0624	0,3647	
1,6	0,2076	0,382	0,291	0,3781	0,3519	0,3371	0,2807	0,3299	0,3559	0,3247	0,3618	0,3123	0,0472	0,3003	
0,32	0,1372	0,1674	0,1216	0,1554	0,1596	0,1698	0,1317	0,1498	0,1799	0,147	0,1666	0,1698	0,0504	0,2673	
0,064	0,0667	0,0713	0,0724	0,0721	0,0698	0,073	0,0667	0,0745	0,0885	0,0704	0,1143	0,0813	0,0432	0,2546	
0	0,0518	0,0484	0,0477	0,0473			0,0617	0,0473	0,0594	0,0492	0,0507	0,0507			

Appendix 4.4.4 Week 4, logarithmic curve

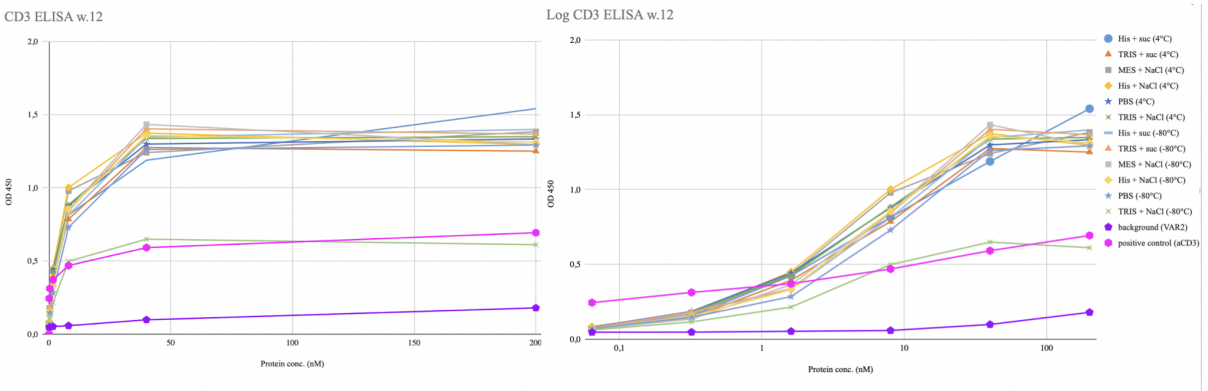


Appendix 4.4.5 Week 12, Set up & Raw Data

	1	2	3	4	5	6	7	8	9	10	11	12		
Plate 1	HIS + suc(-80°C)	TRIS-HCl + suc (	MES + NaCl (	HIS + NaCl (-8	PBS + NaCl(-80°C	TRIS-HCl + NaCl (-8	HIS + suc (4	TRIS-HCl + suc (4°C	MES + NaCl	HIS + NaCl (4°	PBS + NaCl (4°C)			
200														
40														
8				Ab for VAR2 and VAR2-aCD3 detection: anti-V5-HRP antibody										
1,6														
0,32														
0,064														
	Blank						Blank							
VAR2(-)	200	40	8	1,6	0,32	0,064	200	40	8	1,6	0,32	Blank	aCD3 (+)	
												aCD3 (+)		

	HIS + suc(-80°C)	TRIS-HCl + suc (	MES + NaCl (	HIS + NaCl (-8	PBS + NaCl(-80°C	TRIS-HCl + NaCl (-8	HIS + suc (4	TRIS-HCl + suc (4°C	MES + NaCl	HIS + NaCl (4°	PBS + NaCl (4°	TRIS-HCl + Na	VAR2	aCD3
200	1,5407	1,2507	1,3836	1,2956	1,334	1,3493	1,3994	1,3672	1,2964	1,313	1,2927	0,6119	0,1801	0,6937
40	1,1885	1,2752	1,2408	1,3742	1,2991	1,3372	1,3471	1,4032	1,4335	1,3582	1,2613	0,6497	0,099	0,592
8	0,8151	0,7866	0,9791	1,0021	0,8793	0,884	0,8138	0,8434	0,8525	0,8545	0,7301	0,4988	0,0588	0,4696
1,6	0,424	0,3944	0,4332	0,4503	0,4427	0,4283	0,3346	0,3379	0,3651	0,3318	0,2852	0,215	0,0532	0,3714
0,32	0,1554	0,1465	0,1674	0,179	0,1863	0,1807	0,1807	0,1827	0,1353	0,1537	0,1472	0,116	0,0478	0,3131
0,064	0,0683	0,066	0,0758	0,0843	0,0803	0,076	0,073	0,0756	0,0676	0,0726	0,0717	0,0622	0,0473	0,2448
0	0,0449	0,0454	0,0457	0,053	0,0498	0,0489	0,0661	0,0514	0,0506	0,0586	0,0553	0,0491	0	0

Appendix 4.4.6 Week 4, logarithmic curve



Appendix 4.5 FACS

Appendix 4.5.1 Week 2, Set up & Raw data

PLATE 1	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-80°C)	HIS+ NaCl (-80°C)	PBS + NaCl(-80°C)	TRIS-HCl + NaCl (-80°
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
400												
200	CD8 (APC-Cy7)	CD4(PE-Cy7)	V5 (FITC) 200nM VAR2-aCD3									
100												
50												
25												
12,5												
200nM Protein + CSA												
Compensations	Blank	CD8 (APC-Cy7)	CD4(PE-Cy7)	V5 (FITC) 200nM VAR2	Blank	Blank	VAR2 400nM	200nM	100nM	50nM	25nM	12,5nM

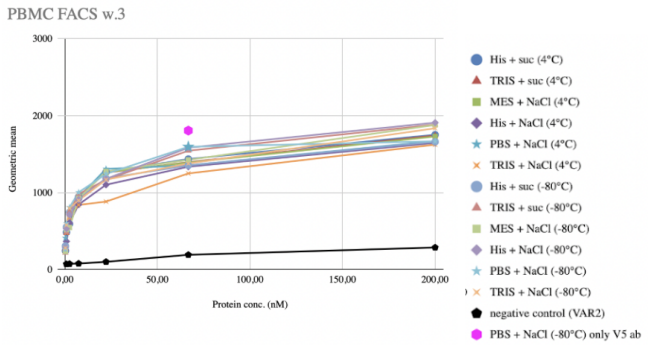
protein conc (nM)	HIS + suc (4°C)	TRIS-HCl + suc (	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-80°C)	HIS+ NaCl (-80°C)	PBS + NaCl(-80°C)	TRIS-HCl + Na(	VAR2	PBS + NaCl (-80°C) only V5
400	1612	1592	1518	1381	1421	1381	1342	1347	1317	1340	1340	1202	109	
200													96,9	2514
100	1116	1111	1137	1104	1117	1111	1060	1130	1130	1160	1193	1152	79,8	
50	1030	1002	990	977	990	993	959	960	942	972	988	912	71,9	
25	915	852	889	829	873	819	812	832	778	808	852	790	67,3	
12,5	767	726	757	745	781	718	719	754	727	733	762	712	63,1	
200nM Protein + CSA	1083	1021	1040	1065	1085	998	1007	1023	1064	1039	1039	1054		
VAR2/ Controls	64,2	60,7	68,5	1332										
CD+ CD8 +														
200nM	2438	2702	2456	2585	2440	2720	2559	2716	2545	2628	2514	2710		

Appendix 4.5.2 Week 3, Set up & Raw data

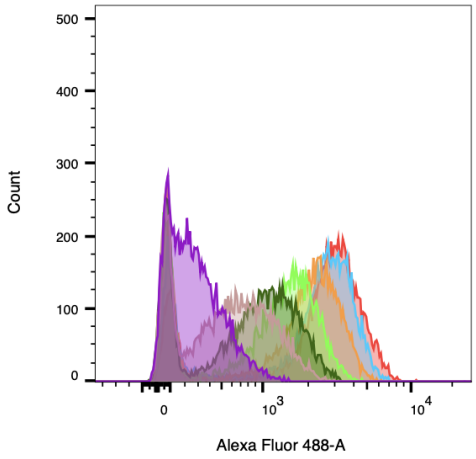
PLATE 1	HIS + suc (4°C)	TRIS-HCl + suc (4'	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-8	HIS+ NaCl (-	PBS + NaCl(-:	TRIS-HCl + NaCl (-80°C)
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
200,00												
66,67	CD8 (APC-Cy7) +	CD4(PE-Cy7) +	V5 (FITC) with	VAR2-aCD3								
22,22												
7,41												
2,47												
0,82												
0,27												
Compensations	Blank	CD8 (APC-Cy7)	CD4(PE-Cy7)	V5 (FITC) 66,66 nM VAR2-aCD3	Blank	CD8 (APC-Cy7) &	VAR2 200nM	66,67nM	22,22nM	7,41nM	2,47nM	0,82nM

	His suc 4	Tris suc 4	MES NaCl 4	His NaCl 4	PBS NaCl 4	Tris NaCl 4	His suc -80	Tris suc -80	MES NaCl -80	His NaCl -80	PBS NaCl -80	Tris NaCl -80
200,00	1752	1742	1729	1646,0	1669,0	1623,0	1660	1884	1879	1909,00	1667	1836
66,67	1436	1395	1390	1336	1354	1249	1354	1544	1425	1583	1595	1378
22,22	1269	1259	1264	1102,0	1308,0	883,0	1184	1167	1274	1178,00	1243	1168
7,41	944	956	949	844,0	937,0	839,0	924	980	922	909,00	1003	897
2,47	592	718	738	604,0	740,0	676,0	744	752	546	712,00	808	797
0,82	484	475	518	366,0	542,0	475,0	552	487	519	531,00	599	577
0,27	243	239	260	255,0	410,0	292,0	306	218	236	280,00	262	279

Appendix 4.5.3 Week 3, Results

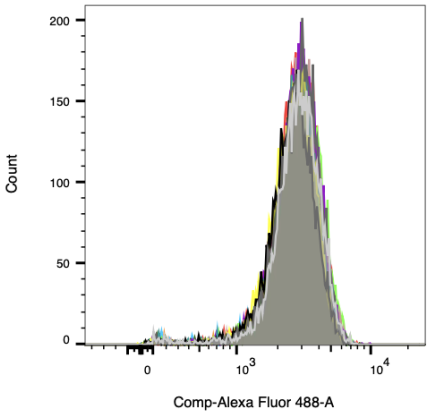


Appendix 4.5.4 Week 3, Concentration pattern, His + NaCl 80°C, 200nM-0.27nM



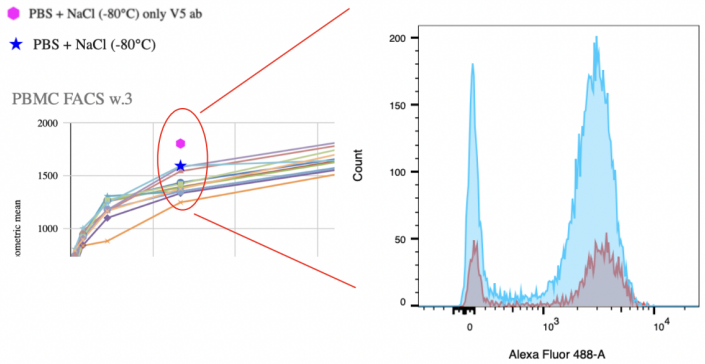
	Sample Name	Subset Name	Count	Geometric Mean : Comp-Alexa Fluor 488-A
	His NaCl -80_G10_G10_075.fcs	Single Cells	9600	280
	His NaCl -80_F10_F10_074.fcs	Single Cells	9554	531
	His NaCl -80_E10_E10_073.fcs	Single Cells	9521	712
	His NaCl -80_D10_D10_072.fcs	Single Cells	9518	909
	His NaCl -80_C10_C10_071.fcs	Single Cells	9568	1178
	His NaCl -80_B10_B10_070.fcs	Single Cells	9587	1581
	His NaCl -80_A10_A10_069.fcs	Single Cells	9671	1909

Appendix 4.5.5 Week 3, All buffer at 66,67nM, overlap



	Sample Name	Subset Name	Count	Geometric Mean : Comp-Alexa Fluor 488-A
	Tris suc 4_B2_B02_014.fcs	T cells	6016	2726
	Tris suc -80_B8_B08_056.fcs	T cells	6625	2740
	Tris NaCl 4_B6_B06_042.fcs	T cells	5877	2487
	Tris NaCl -80_B12_B12_084.fcs	T cells	6235	2543
	PBS NacL 4_B5_B05_035.fcs	T cells	6045	2643
	PBS NaCl -80_B11_B11_077.fcs	T cells	6758	2715
	MES NaCl 4_B3_B03_021.fcs	T cells	6046	2752
	MES NaCl -80_B9_B09_063.fcs	T cells	6203	2626
	His suc 4_B1_B01_007.fcs	T cells	6120	2835
	His suc -80_B7_B07_049.fcs	T cells	5954	2657
	His NaCl 4_B4_B04_028.fcs	T cells	6043	2613
	His NaCl -80_B10_B10_070.fcs	T cells	6760	2694

Appendix 4.5.6 Week 3, Ab's competing



	Sample Name	Subset Name	Count
	PBS NaCl -80_B11_B11_077.fcs	Single Cells	9624
	Compensation Controls_Alexa Fluor 488 Stained Control_H04_004.fcs	Single Cells	2266

Appendix 4.5.7 Week 4, Set up & Raw data

PLATE 1	HIS + suc (4°C)	TRIS-HCl + suc (4°	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-8	HIS+ NaCl (-	PBS + NaCl(-	TRIS-HCl + NaCl (-80°C)
protein conc (nM	1	2	3	4	5	6	7	8	9	10	11	12
A 200,00												
B 50,00												
C 12,50												
D 3,13												
E 0,78												
F 0,20												
G 0,05												
H	Blk	Blk	Blk	Blk	Blk	200	50,00	12,50	3,13	0,78	0,20	0,05

	His suc 4	Tris suc 4	MES NaCl 4	His NaCl 4	PBS NaCl 4	Tris NaCl 4	His suc -80	Tris suc -80	MES NaCl -80	His NaCl -80	PBS NaCl -80	Tris NaCl -	VAR2
200,00	2120	2161	2103	2095	1992	2061	1845	1918	2008	1901	2058	1950	539
50,00	1722	1765	1714	1662	1642	1602	1545	1515	1603	1486	1623	1495	167
12,50	1375	1324	1325	1289	1289	1203	1213	1170	1256	1171	1197	1156	105
3,13	1084	1067	1039	1034	963	1052	931	946	941	920	1006	932	92,7
0,78	783	764	807	810	737	838	745	729	750	728	846	692	83,5
0,20	456	346	415	403	384	540	380	355	389	363	500	356	83,8
0,05	219	150	198	189	184	295	179	168	181	181	224	170	85,1

Appendix 4.5.8 Rested VS non-rested cells, Set up

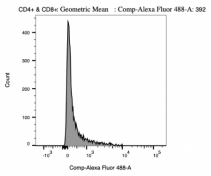
PLATE 2	1	2	3	4	5	6	7	8	9
non-rested	Mix 1	Mix 1	Mix 1 no viability	Mix 2	Mix 2	Mix 2 no viability	VAR2 + V5	Blk + V5	Blk
rested	Mix 1	Mix 1	Mix 1 no vibility	Mix 2	Mix 2	Mix 2 no viability	VAR2 + V5	Blk + V5	Blk
compensations	Blk + V5	aCD3	aCD4	aCD8	BV510/viability	VAR2-aCD3 + V5			

Plate 1: rested & non-rested cells

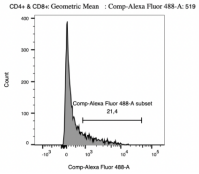
- Mix 1: 100nM VAR2 (mp4112) detected by:
  - APC (CD3+)
  - APC-Cy7(CD8+)
  - PE-Cy7(CD4+)
  - BV510 (viability) *(mix with and without)*
  - V5 (VAR2-aCD3)
- Mix 2: 100nM VAR2-aCD3 (mp4207, PBS, -80) detected by:
  - APC-Cy7(CD8+)
  - PE-Cy7(CD4+)
  - BV510 (viability) *(mix with and without)*
  - V5 (VAR2-aCD3)

Appendix 4.5.9 Rested VS non-rested cells, Background binding

Non-rested cells



Rested cells



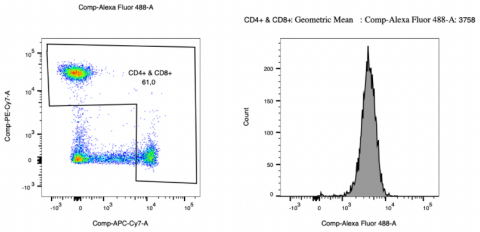
Well nr.	Rested or Non-rested	Geometric mean (background)
A1	Non-rested	392
A2	Non-rested	408
B1	Rested	519
B2	Rested	523

Comment:

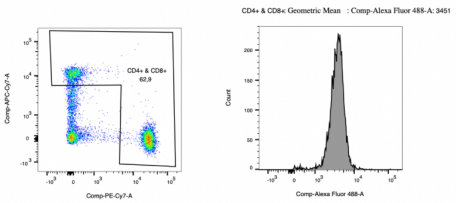
- Non-rested cells have a lower geometric mean → a lower background binding

Appendix 4.5.10 Rested VS non-rested cells, VAR2-aCD3 binding

Non-rested cells



Rested cells



Well nr.	Rested or Non-rested	Geometric mean (background)
A4	Non-rested	3758
A5	Non-rested	3777
B4	Rested	3451
B5	Rested	3411

Comment:

- Non-rested cells have a higher geometric mean → more binding

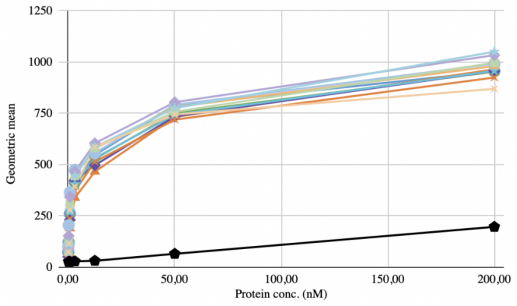
Appendix 4.5.11 Week 12, Set up & Raw data

PLATE 1	HIS + suc (4°C)	TRIS-HCl + suc (4	MES + NaCl (4°C)	HIS + NaCl (4°C)	PBS + NaCl (4°C)	TRIS-HCl + NaCl (4	HIS + suc(-80°C)	TRIS-HCl + suc (-8	MES + NaCl (-8	HIS+ NaCl (-8	PBS + NaCl(-8	TRIS-HCl + NaCl (-80°C)
protein conc (nM	1	2	3	4	5	6	7	8	9	10	11	12
200,00												
50,00												
12,50												
3,13												
0,78												
0,20												
0,05												
	Blk + V5	aCD4	aCD8	viability	BV510/viability	Blk + V5	200,00	12,50	3,13	0,78	0,20	0,05

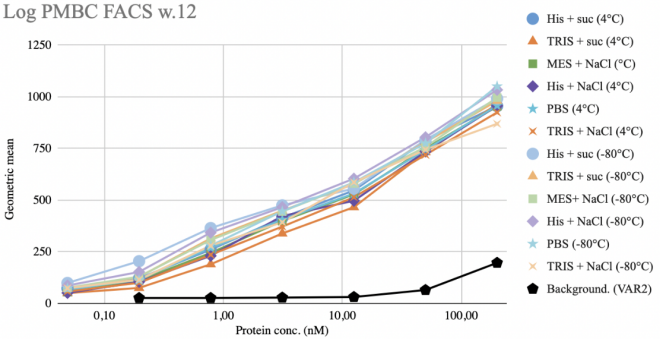
	His suc 4	Tris suc 4	MES NaCl 4	His NaCl 4	PBS NaCl 4	Tris NaCl 4	His suc -80	Tris suc -80	MES NaCl -	His NaCl -8	PBS NaCl -8	Tris NaCl -8	VAR2
200,00	954	963	952	955	956	923	990	979	995	1032	1049	868	196
50,00	787	731	749	734	742	718	790	779	756	803	774	743	64,7
12,50	546	465	527	496	531	513	555	577	580	603	585	585	30,8
3,13	414	338	398	422	405	371	474	448	444	465	445	392	28,1
0,78	260	189	244	230	268	235	364	314	306	342	279	279	26,1
0,20	121	74,8	111	104	117	99,6	204	128	129	152	113	115	26,1
0,05	72,3	49,6	54,4	50,5	64,4	57,3	98,7	71,6	75,5	86,2	73	73,5	

Appendix 4.5.12 Week 12, Results

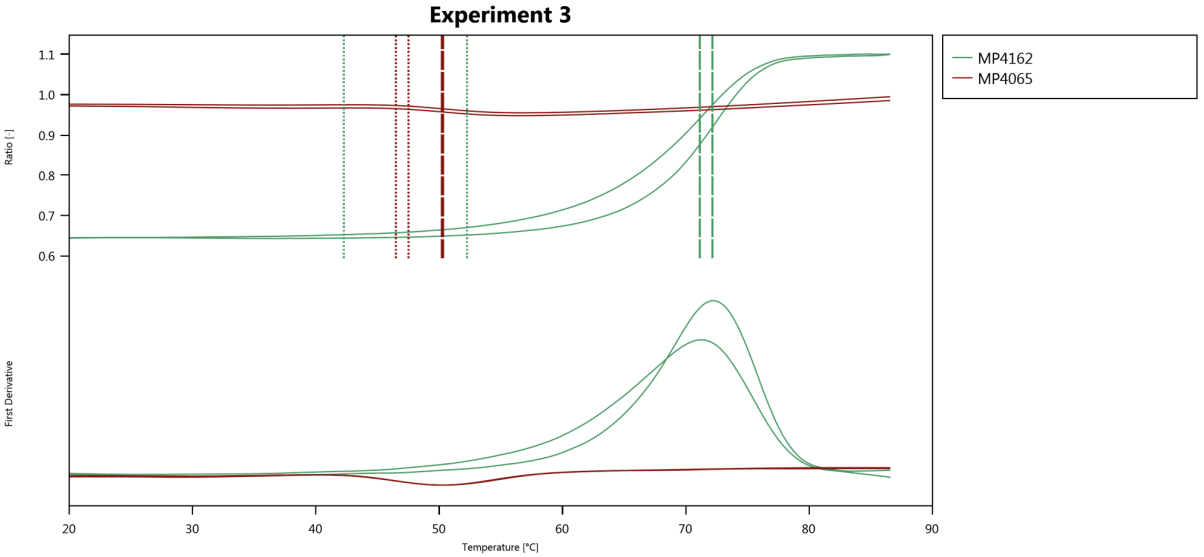
PMBC FACS w.12

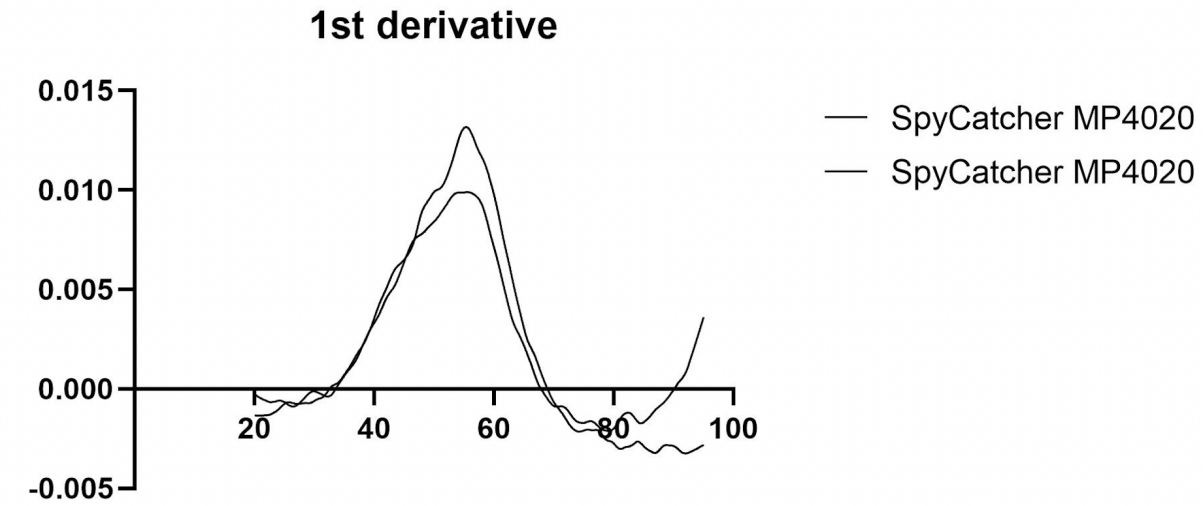


Log PMBC FACS w.12



Appendix 4.6 NanoDSF of VAR2 & aCD3-spycatcher & Spycatcher

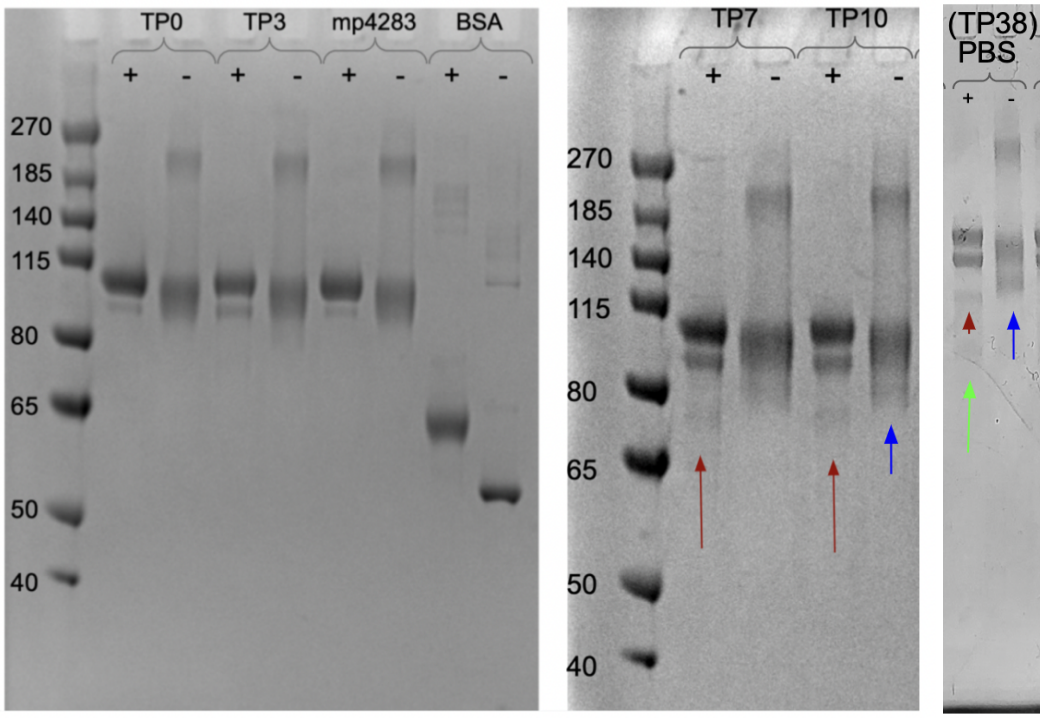


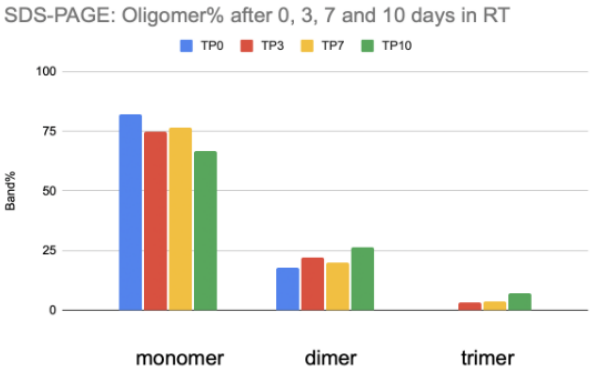


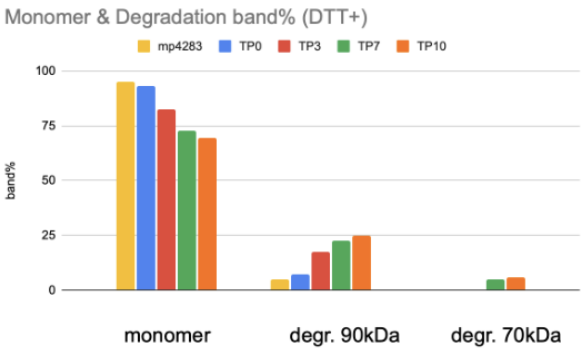
Appendix 5. Stress study

Appendix 5.1 SDS PAGE

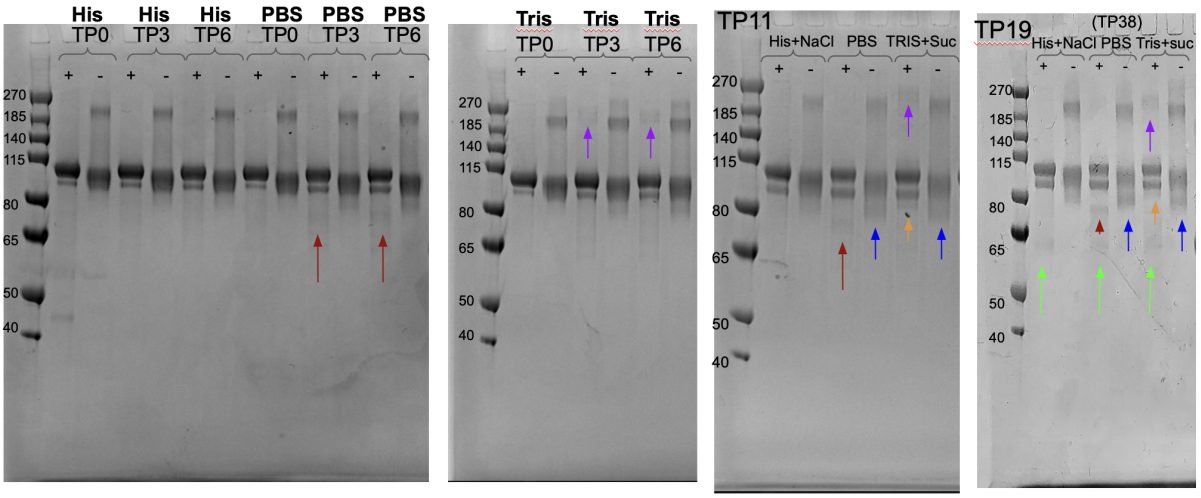
Appendix 5.1.1 Pre-study - Room Temperature



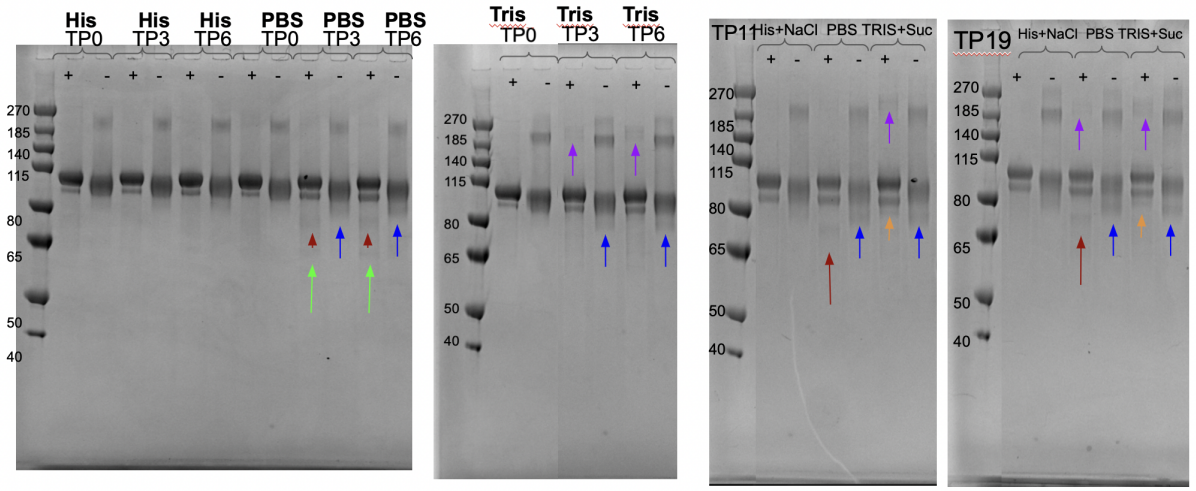




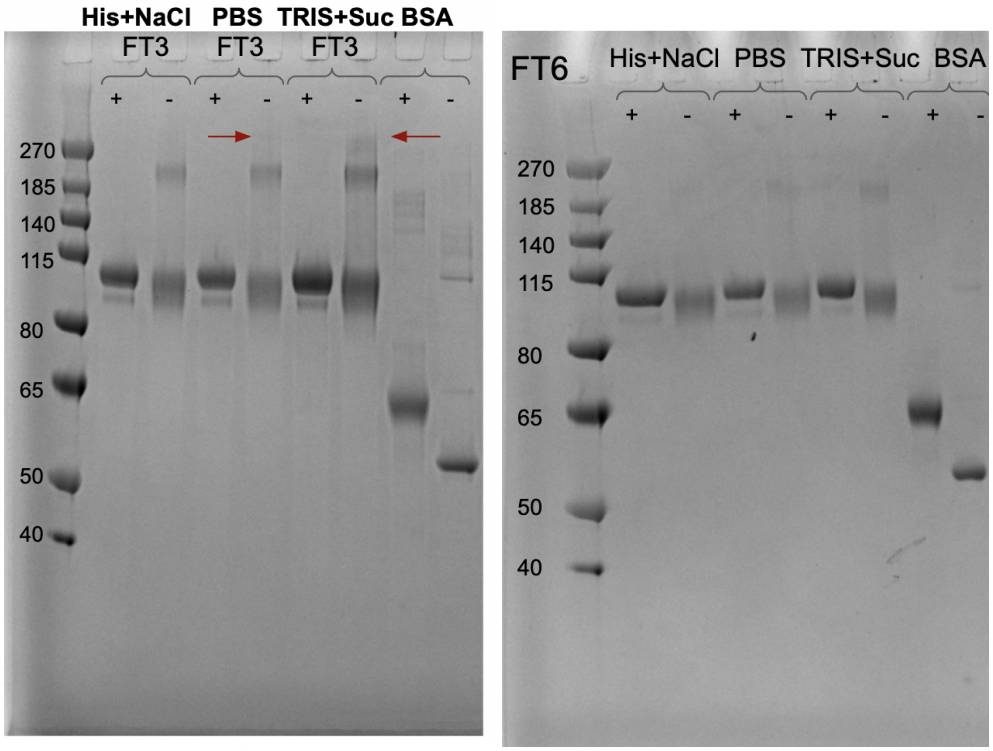
Appendix 5.1.2 Stress test 1 - Room Temperature



Appendix 5.1.3 Stress test 2 - Room Temperature + 30min rotation/ day (shear stress)

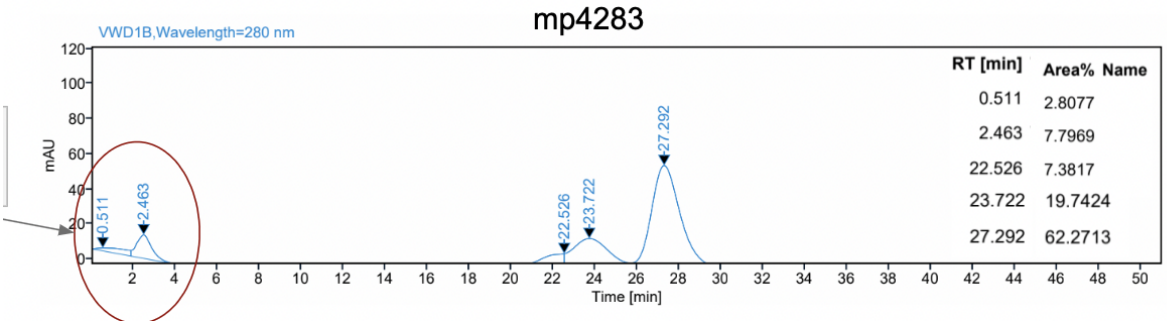


Appendix 5.1.4 Stress test 3 - Freeze & Thaw

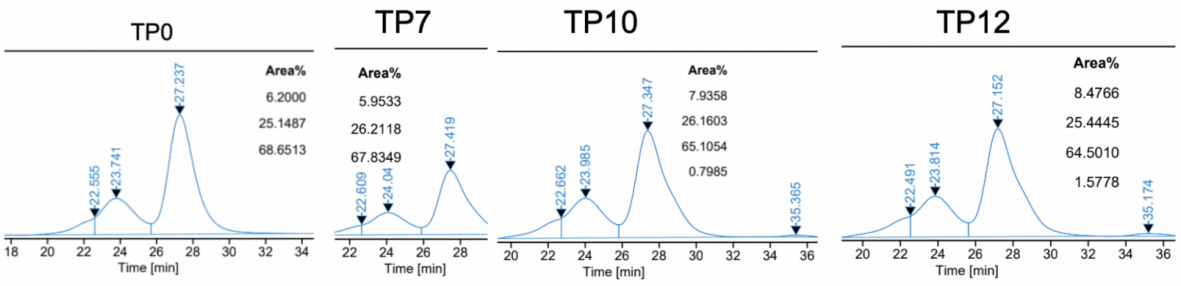


Appendix 5.2 HPLC

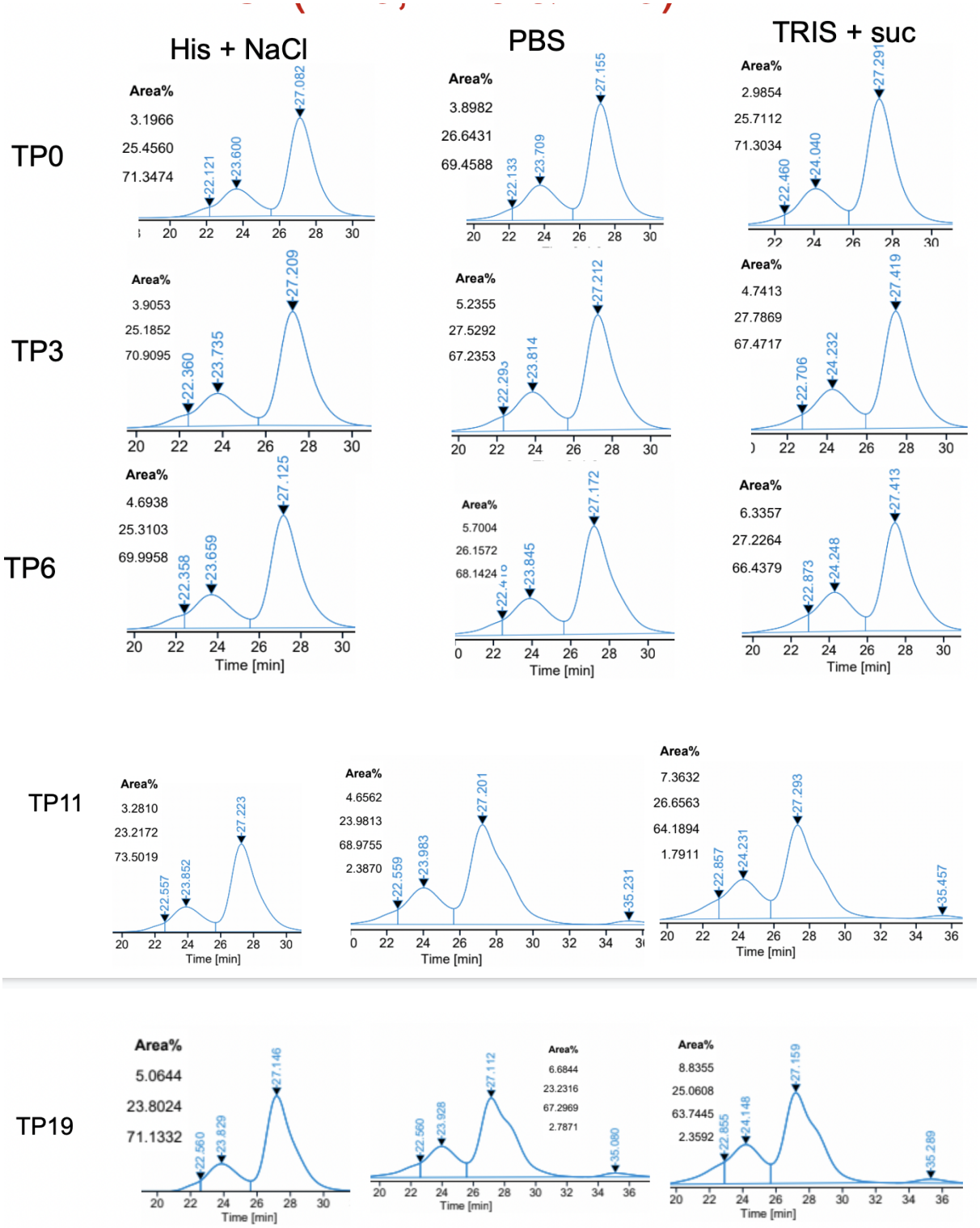
Appendix 5.2.1 Original protein, mp4283



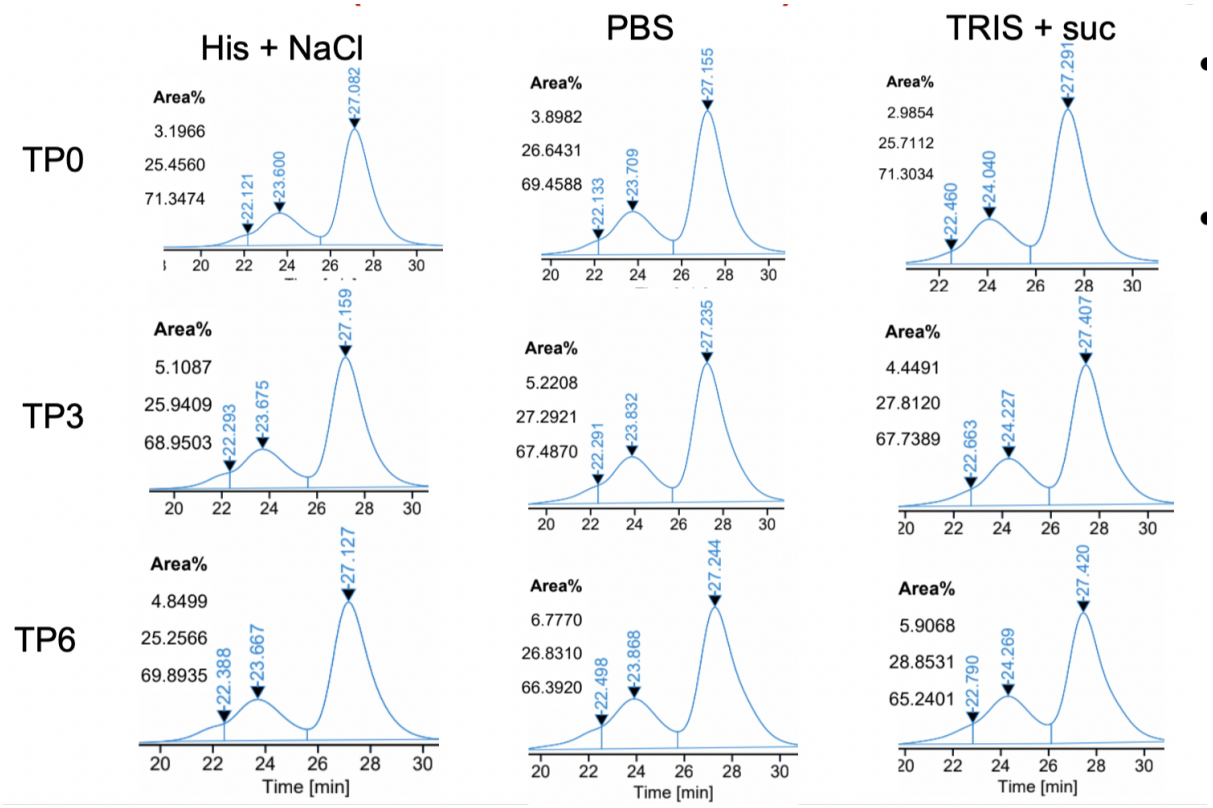
Appendix 5.2.2 Pre study - Room Temperature

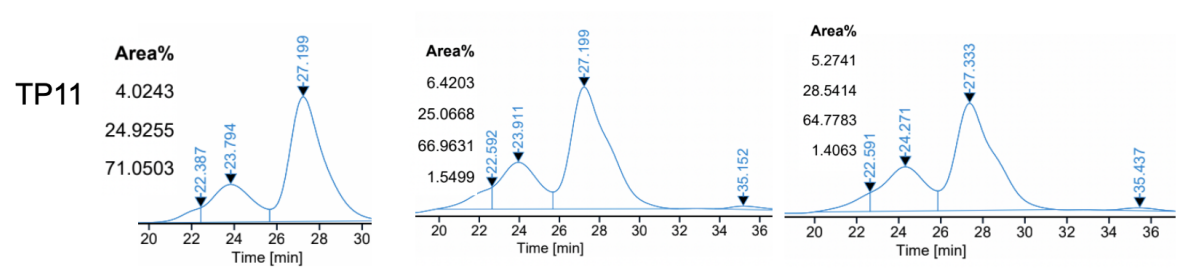


Appendix 5.2.3 Stress test 1 - Room Temperature

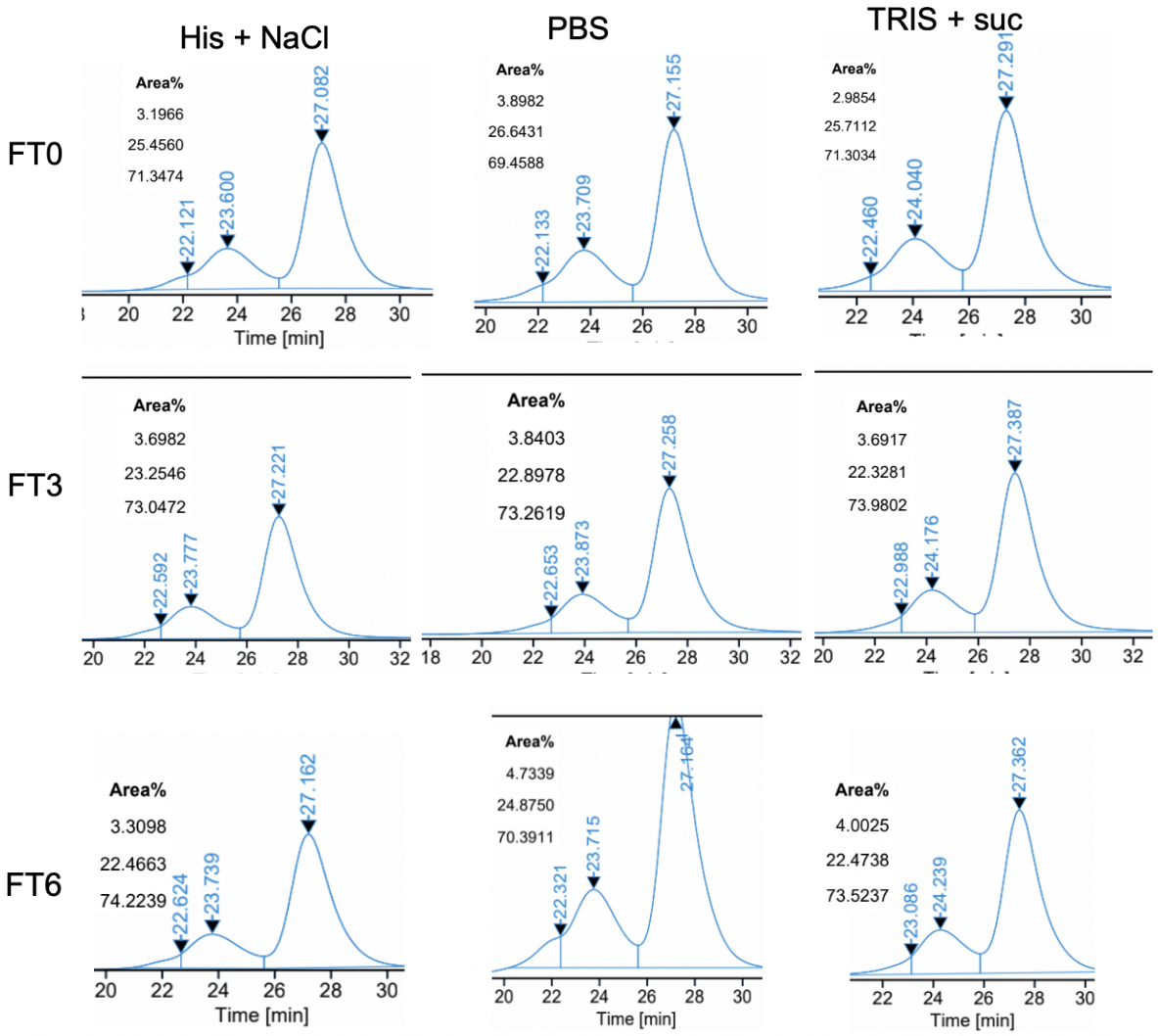


Appendix 5.2.4 Stress test 2 - Room Temperature + 30min rotation/ day (shear stress)



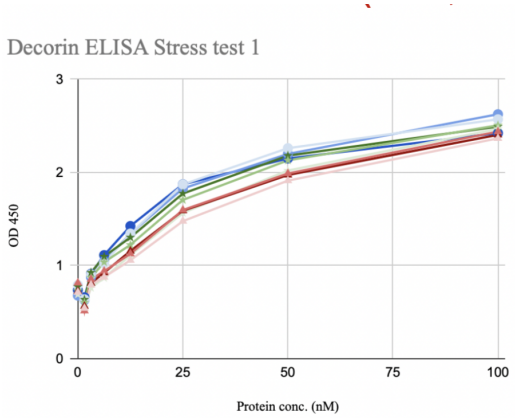


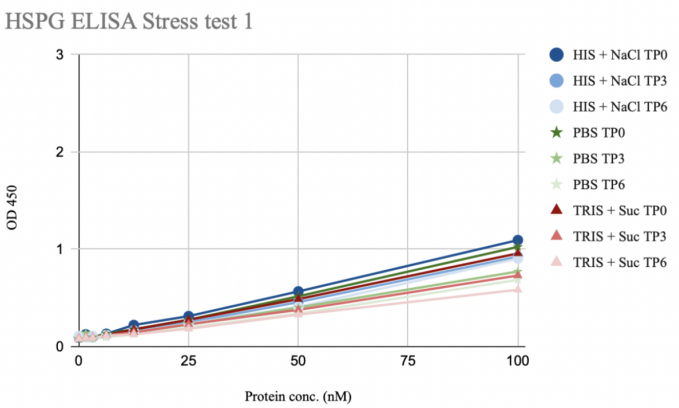
Appendix 5.2.5 Stress test 3 - Freeze & Thaw

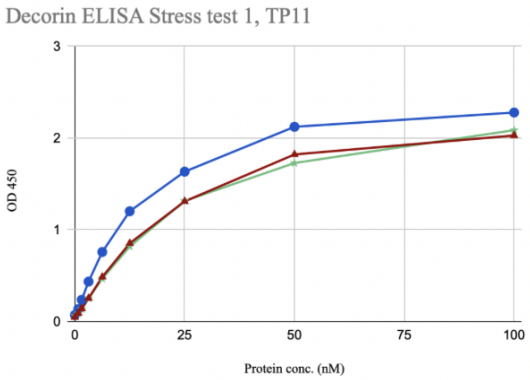


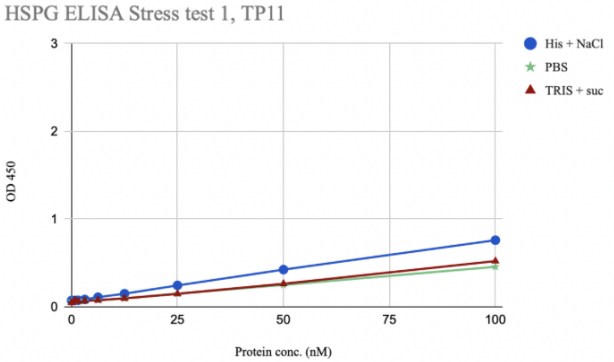
Appendix 5.3 CSA ELISA

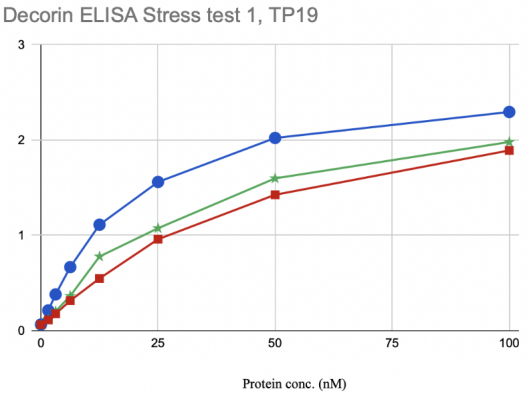
Appendix 5.3.1 Stress test 1 - Room Temperature

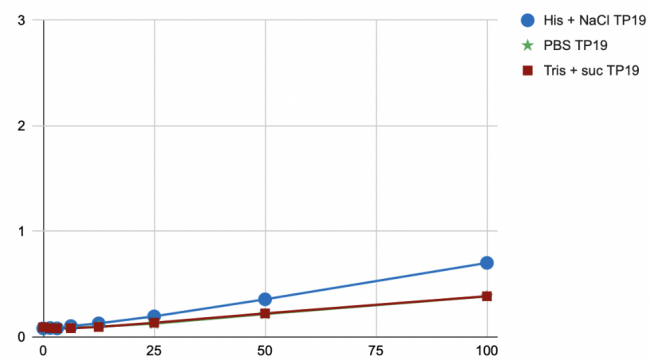












	HIS + NaCl TP0	HIS + NaCl TP3	HIS + NaCl TP6	PBS + NaCl TP0	PBS + NaCl TP3	PBS + NaCl TP6	TRIS+ suc TP0	TRIS+ suc TP3	TRIS+ suc TP6
protein conc (nM)	1	2	3	4	5	6	7	8	9
100	2,4222	2,6246	2,5703	2,4919	2,5059	2,4675	2,4035	2,4384	2,3656
50	2,1492	2,1987	2,2603	2,18	2,1294	2,0204	1,9725	1,9885	1,9119
25	1,8728	1,8277	1,8714	1,7731	1,7049	1,565	1,5885	1,5961	1,477
12,5	1,4245	1,3432	1,3425	1,3022	1,2222	1,1117	1,1564	1,1265	1,0571
6,25	1,1118	1,0462	1,027	1,0977	1,0369	0,8678	0,9234	0,9402	0,8761
3,125	0,9113	0,8748	0,9044	0,9248	0,8694	0,7613	0,8113	0,8527	0,792
1,5625	0,6598	0,6284	0,6374	0,6352	0,6031	0,553	0,5638	0,5142	0,5417
0	0,7369	0,6785	0,79	0,7727	0,7288	0,6903	0,7243	0,8136	0,7133

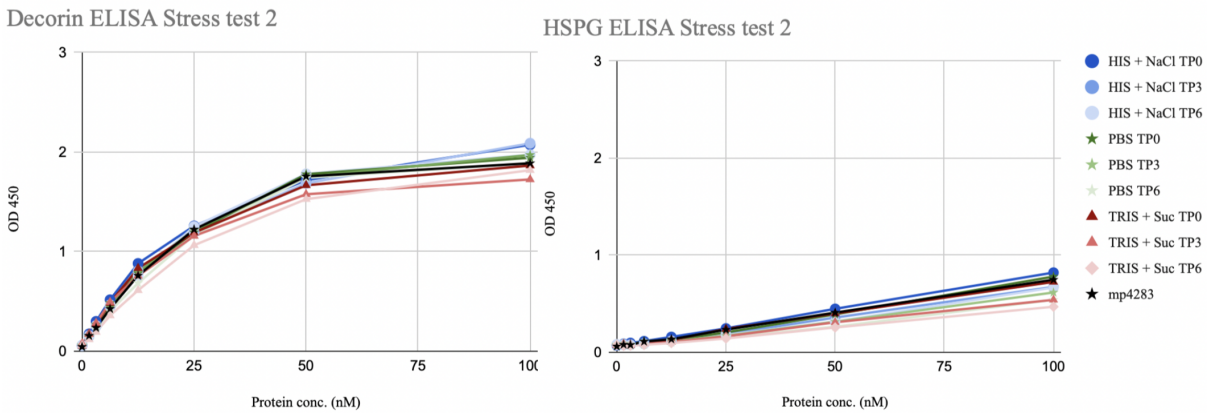
	HIS + NaCl TP0	HIS + NaCl TP1	HIS + NaCl TP4	PBS + NaCl TP0	PBS + NaCl TP1	PBS + NaCl TP0	TRIS+ suc TP0	TRIS+ suc TP3	TRIS+ suc TP6
protein conc (nM)	1	2	3	4	5	6	7	8	9
100	1,094	0,9247	0,9045	1,0245	0,7714	0,6856	0,9576	0,7304	0,5839
50	0,5667	0,457	0,408	0,5167	0,4021	0,3387	0,4881	0,3775	0,328
25	0,3127	0,2492	0,2358	0,2757	0,2263	0,1958	0,2748	0,2294	0,183
12,5	0,2215	0,1667	0,1499	0,177	0,1453	0,1234	0,1768	0,1467	0,1261
6,25	0,1315	0,121	0,1154	0,1316	0,1057	0,097	0,1189	0,1172	0,1121
3,125	0,1013	0,0981	0,1098	0,0951	0,089	0,0786	0,0941	0,0907	0,0916
1,5625	0,1256	0,101	0,0937	0,0895	0,1388	0,086	0,0905	0,0926	0,0895
0	0,0966	0,1011	0,1072	0,0799	0,0843	0,0928	0,0782	0,0847	0,0777

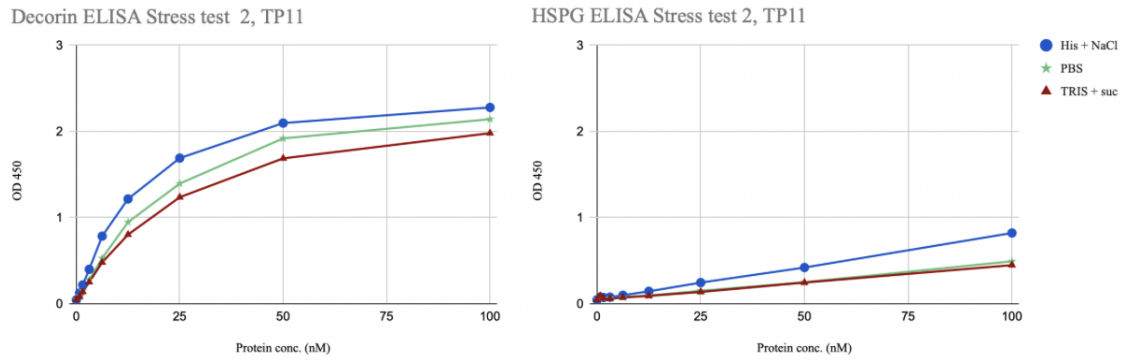
	ST 1. HIS + NaCl TP	ST1. PBS + NaCl TP	ST 1. TRIS + suc TP	ST 2. HIS + NaCl TP	ST2. PBS + NaCl TP	ST 2. TRIS + suc TP	ST 3. HIS + NaCl FT	ST3. PBS + NaCl FT	ST3. TRIS + suc FT6
protein conc (nM)	1	2	3	4	5	6	7	8	9
100	0,7596	0,4563	0,5212	0,8214	0,4926	0,4482	0,8186	0,866	0,9126
50	0,4241	0,2493	0,2629	0,4207	0,2507	0,2462	0,4475	0,499	0,5055
25	0,244	0,1504	0,1486	0,2455	0,1513	0,1373	0,2493	0,2817	0,274
12,5	0,1497	0,0972	0,0961	0,1446	0,0949	0,0914	0,1464	0,167	0,1589
6,25	0,1103	0,0764	0,076	0,0988	0,0764	0,0734	0,1029	0,1122	0,1099
3,125	0,0843	0,0694	0,0672	0,0767	0,0676	0,0588	0,0784	0,0842	0,0828
1,5625	0,0754	0,0714	0,0635	0,072	0,0642	0,0646	0,073	0,0771	0,0776
0,78125	0,0746	0,0712	0,0725	0,0781	0,0745	0,0913	0,0764	0,0839	0,0868

	ST 1. HIS + NaCl TP	ST1. PBS + NaCl TP	ST 1. TRIS + suc TP	ST 2. HIS + NaCl TP	ST2. PBS + NaCl TP	ST 2. TRIS + suc TP	ST 3. HIS + NaCl FT	ST3. PBS + NaCl FT	ST3. TRIS + suc FT6	
protein conc (nM)	1	2	3	4	5	6	7	8	9	
100	2,2785	2,0861	2,0281	2,2785	2,1419	1,9797	2,1047	2,26	2,1766	0
50	2,123	1,7282	1,8215	2,0972	1,9194	1,6874	1,893	2,1038	1,9771	0
25	1,6339	1,3153	1,3101	1,6906	1,395	1,2366	1,4965	1,6333	1,5597	0
12,5	1,2028	0,8182	0,8534	1,2166	0,947	0,8028	1,0534	1,1155	1,0792	0
6,25	0,7588	0,4689	0,4864	0,7848	0,5287	0,48	0,6623	0,6987	0,6934	0
3,125	0,436	0,2526	0,2546	0,3999	0,2765	0,2518	0,37	0,355	0,3241	0
1,5625	0,2376	0,1377	0,1418	0,2193	0,1499	0,1391	0,195	0,2022	0,2065	0
0,78125	0,1369	0,0872	0,0922	0,1251	0,0923	0,0826	0,1125	0,1271	0,1269	0
0	0,072	0,0655	0,0479	0,0456	0,0512	0,0459	0,044	0,0639	0,0706	

	ST 1. HIS + NaCl TP	ST1. PBS + NaCl TP	ST 1. TRIS + suc TP	ST 2. HIS + NaCl TP	ST2. PBS + NaCl TP	ST 2. TRIS + suc TP	ST 1. HIS + NaCl TP	ST1. PBS + NaCl TP	ST 1. TRIS + suc TP	ST 2. HIS + NaCl	ST2. PBS + N	ST 2. TRIS + su
protein conc (nM)	1	2	3	4	5	6	7	8	9	10	11	12
100	2,2946	1,9794	1,891	2,1549	1,94		0,7018	0,385	0,3862	0,6377	0,4041	0,4241
50	2,0217	1,5979	1,4258	1,8255	1,6049	1,2238	0,3568	0,2171	0,2235	0,3101	0,2264	0,2364
25	1,5601	1,0748	0,9594	1,3122	1,0952	0,866	0,1955	0,1259	0,1348	0,1669	0,1404	0,1439
12,5	1,1115	0,7782	0,5479	0,8318	0,6768	0,4808	0,129	0,098	0,0949	0,1114	0,1018	0,0987
6,25	0,6667	0,364	0,3151	0,5031	0,3855	0,2598	0,1038	0,087	0,0829	0,1022	0,0905	0,0921
3,125	0,3805	0,2035	0,1773	0,2644	0,2046	0,1564	0,0819	0,0813	0,0823	0,0859	0,0814	0,0813
1,5625	0,2129	0,1145	0,1133	0,1572	0,1165	0,0917	0,0854	0,0896	0,0848	0,0867	0,0887	0,0848
0	0,0649	0,057	0,0638	0,0613	0,0611	0,0584	0,0818	0,0871	0,0929	0,0964	0,0906	0,088

Appendix 5.3.2 Stress test 2 - Room Temperature + 30min rotation/ day (shear stress)





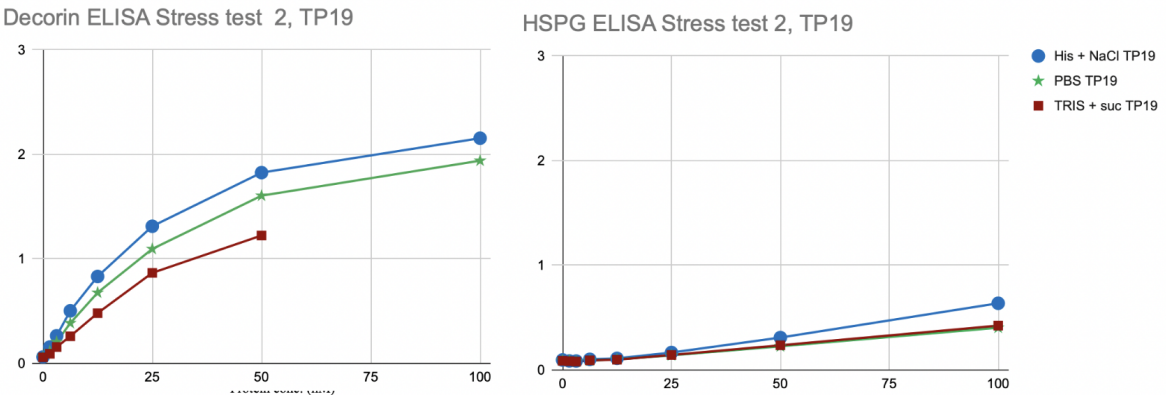
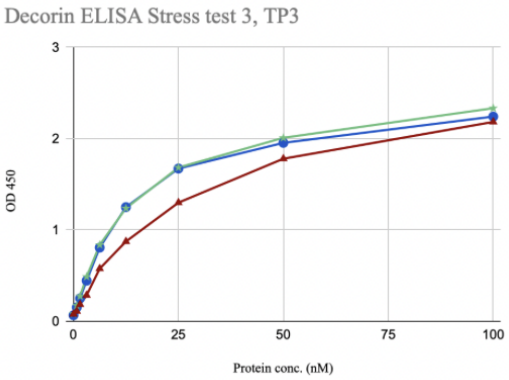
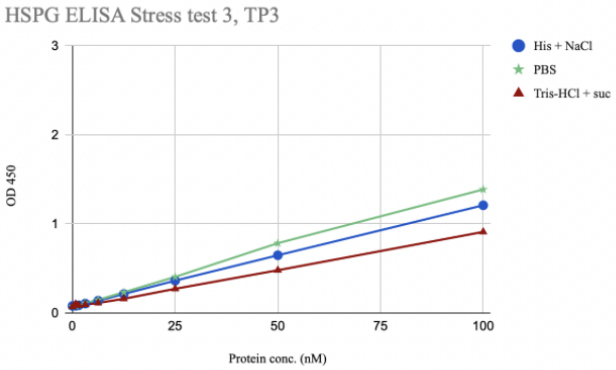


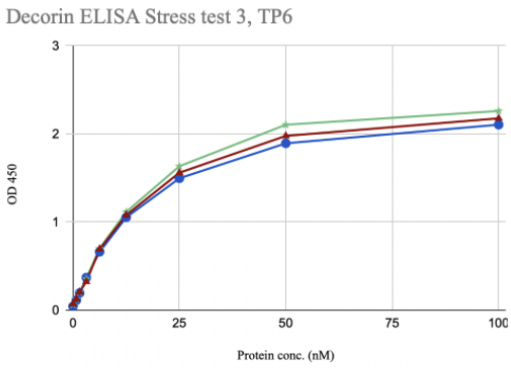
PLATE 1 -Decor	HIS + NaCl TP0	HIS + NaCl TP3	HIS + NaCl TP6	PBS + NaCl TP0	PBS + NaCl TP3	PBS + NaCl TP6	TRIS+ suc TP0	TRIS+ suc TP3	TRIS+ suc TP6	mp4283
protein conc (nM)	1	2	3	4	5	6	7	8	9	10
100	2,0739	2,0882	1,9732	1,9438	1,9709	1,8895	1,8648	1,7252	1,818	1,8875
50	1,7179	1,6866	1,7808	1,7775	1,7519	1,7408	1,6663	1,5761	1,5272	1,7561
25	1,2577	1,1958	1,2517	1,1929	1,2158	1,1657	1,1902	1,1574	1,0643	1,223
12,5	0,8811	0,8054	0,7598	0,7818	0,7928	0,6837	0,8335	0,7569	0,6117	0,7596
6,25	0,5164	0,448	0,4652	0,4844	0,4403	0,4028	0,4843	0,493	0,3582	0,426
3,125	0,2993	0,2516	0,2575	0,2688	0,2505	0,2232	0,2736	0,2839	0,1922	0,235
1,5625	0,1735	0,1487	0,1567	0,1549	0,1439	0,1315	0,1746	0,1699	0,1185	0,1554
0	0,057	0,0549	0,0569	0,0575	0,0591	0,0639	0,0544	0,0771	0,059	0,0437

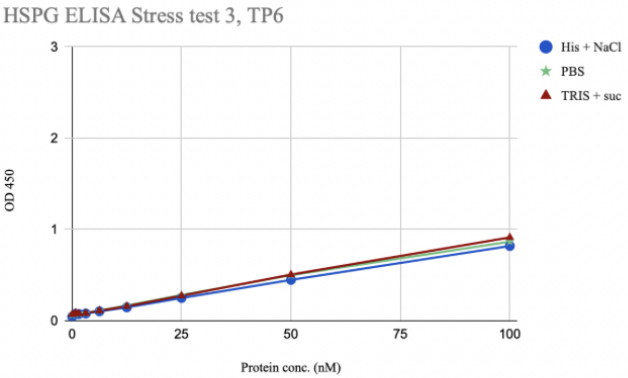
	HIS + NaCl TP0	HIS + NaCl TP3	HIS + NaCl TP6	PBS + NaCl TP0	PBS + NaCl TP3	PBS + NaCl TP6	TRIS+ suc TP0	TRIS+ suc TP3	TRIS+ suc TP6	mp4283
protein conc (nM)	1	2	3	4	5	6	7	8	9	10
100	0,8213	0,674	0,6675	0,7804	0,6163	0,5406	0,7253	0,5404	0,4694	0,748
50	0,448	0,3577	0,3161	0,391	0,3148	0,2637	0,4	0,3092	0,2556	0,4089
25	0,2462	0,1887	0,1792	0,2043	0,1703	0,1525	0,2406	0,1645	0,1422	0,233
12,5	0,1586	0,1206	0,1149	0,1247	0,1109	0,0978	0,1265	0,1058	0,0955	0,1348
6,25	0,1145	0,0931	0,0853	0,1134	0,0814	0,0788	0,0947	0,0873	0,0764	0,1102
3,125	0,0965	0,0783	0,0769	0,0784	0,0739	0,0665	0,0777	0,0713	0,0681	0,0768
1,5625	0,0872	0,0782	0,0813	0,0811	0,0899	0,0835	0,0778	0,0866	0,0667	0,0793
0	0,0717	0,0753	0,0826	0,0746	0,0797	0,0832	0,0737	0,0756	0,0746	0,0612

Appendix 5.3.3 Stress test 3 - Freeze & Thaw



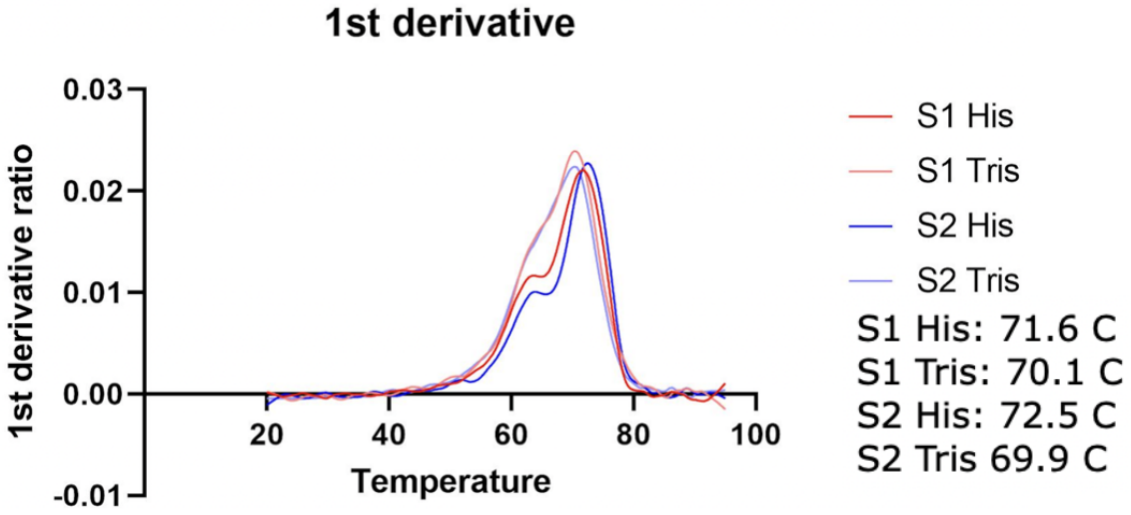






Decorin & HSPG	HIS + NaCl FT4	PBS FT4	TRIS + suc FT4				HIS + NaCl FT4	PBS FT4	TRIS + suc FT4	
protein conc (nM)	1	2	3	4	5	6	7	8	9	
100	1,2088	1,3863	0,9103	0,0789	0,0862	0,0872	2,2414	2,333	2,1828	0
50	0,648	0,7836	0,4798	0,0747	0,0839	0,0834	1,9537	2,0088	1,78	0
25	0,3629	0,4049	0,2712	0,0685	0,0678	0,0804	1,6722	1,6836	1,299	0
12,5	0,2127	0,2328	0,1589	0,0662	0,07	0,0707	1,2501	1,2375	0,8737	0
6,25	0,1376	0,1477	0,1127	0,0821	0,0698	0,0727	0,8066	0,8368	0,5766	0
3,125	0,1055	0,1132	0,0924	0,0776	0,0759	0,0694	0,4449	0,4881	0,2847	0
1,5625	0,0866	0,0889	0,0856	0,0754	0,0769	0,0738	0,2503	0,275	0,1844	0
0,78125	0,0802	0,0791	0,1009	0,092	0,0914	0,0835	0,1521	0,173	0,1101	0

Appendix 5.4 NanoDSF



Appendix 6. Protein

Appendix 6.1 Protein data

Experiment	Construct	Size (kDa)	Conc. (mg/mL)	Storage buffer	Expression system	Date produced
Initial analysis	human anti-CD3 (ID1ID2a)	97	0,43	PBS	Baculo	2020-09-30
Tonicity test	Human anti-CD3 ID1ID2a	97	3,39	25mM NaP ~150mM NaCl	Baculo	2020-12-15
	Human antiCD3-Fc-SpyCatcher (AB815)	68,3	4,17	1xPBS	ExpiCHO	2020-11-04
	ID1-ID2a	68	1,21	25mM NaP ~150mM NaCl	Shuffle	2020-09-30
Physical stress tests	Human anti-CD3 ID1ID2a	97	0,60	25mM NaP ~150mM NaCl	Baculo	2021-02-10

Appendix 6.2 Stress test: protein concentration in PBS buffer

	Prestudy (mg/mL)	ST1 (mg/mL)	ST2 (mg/mL)	ST3 (mg/mL)
Start (TP0)	0,809	0,809	0,809	0,809
TP19 (FT6)	1,058	1,040	1,588	1,34

Appendix 7. Aliquots

Appendix 7.1 Tonicity study

	−80	4
HIS + SUC	50uL, 0w	50uL, 0w
	50uL, 1w	50uL, 1w
	50uL, 2w	50uL, 2w
	70uL, (take out for 3w, 4w)	100uL, (take out for 3w, 4w)
TRIS + SUC	50uL, 0w	50uL, 0w
	50uL, 1w	50uL, 1w
	50uL, 2w	50uL, 2w
	50uL, 3w	50uL, 3w
	-	50uL, 4w
	50uL, LT, 3 months	50uL, LT, 3 months
	50uL, LT, 6 months	50uL, LT, 6 months
	100uL precipitation (take out for 4w)	100uL precipitation
MES + NaCl	50uL, 0w	50uL, 0w
	50uL, 1w	50uL, 1w
	50uL, 2w	50uL, 2w
	-	50uL, 3w
	-	50uL, 4w
	50uL, LT, 3 months	50uL, LT, 3 months
	50uL, LT, 6 months	50uL, LT, 6 months

	100uL precipitation (take out for 3w+ 4w)	100uL precipitation
HIS + NaCl	50uL, 0w	50uL, 0w
	50uL, 1w	50uL, 1w
	50uL, 2w	50uL, 2w
	-	50uL, 3w
	-	50uL, 4w
	50uL, LT, 3 months	50uL, LT, 3 months
	50uL, LT, 6 months	50uL, LT, 6 months
	100uL precipitation (take out for 3w+ 4w)	100uL precipitation
PBS + NaCl	50uL, 0w	50uL, 0w
	50uL, 1w	50uL, 1w
	50uL, 2w	50uL, 2w
	50uL, 3w	50uL, 3w
	50uL, 4w	50uL, 4w
	50uL, LT, 3 months	50uL, LT, 3 months
	50uL, LT, 6 months	50uL, LT, 6 months
	100uL precipitation (take out for 3w+ 4w)	100uL precipitation
TRIS + NaCl	50uL, 0w	50uL, 0w
	50uL, 1w	50uL, 1w
	50uL, 2w	50uL, 2w
	50uL, 3w	50uL, 3w
	50uL, 4w	50uL, 4w
	50uL, LT, 3 months	50uL, LT, 3 months
	50uL, LT, 6 months	50uL, LT, 6 months
	100uL precipitation (take out for 3w+ 4w)	100uL precipitation

Appendix 7.2 Stress test

	Intial study (PS)	Stress 1 (S1)	Stress 2 (S2)	Stress 3 (S3)
HIS + NaCl		**40uL, TP 0**		200uL, TP 0
pH 6.0		100uL, TP 1	100uL, TP 1	
(H)		160uL, TP 4	160uL, TP 4	
PBS	100uL, TP 3	**40uL, TP 0**		200uL, TP 0
pH 7.4	Mon: SDS, HPLC 40uL, TP0	40uL, TP 1	40uL, TP 1	
(P)	mp4283, 100uL	100uL, TP 4	100uL, TP 4	
TRIS + suc		**40uL, TP 0**		200uL, TP 0
pH 8.0		100uL, TP 1	100uL, TP 1	
(T)		160uL, TP 4	160uL, TP 4	

Appendix 8. MS

Sequence data:

:Amino Acids

Intensity Coverage: 1.4 % (4109 cnts)
Sequence Coverage MS/MS: 21.6%
Sequence Coverage MS: 21.6%
pI (isoelectric point): 9.3

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        10         20         30         40         50         60         70         80         90        100
GQVQLVQSGA EVKKPGESLK VSCKASGYTF TSYYMHWVRQ APGQGLEWMG IINPSGGSTS YAQKFQGRVT MTRDTSTSTV YMELSSLRSE DTAVYYCARG

       110        120        130        140        150        160        170        180        190        200
SAYYYDFADY WGQGTLVTVS SGGGGSGGGG SGGGGSGGGS YVLTQPSSVS VAPGQTATIS CGGHNIGSKN VHWYQQRPGQ SPVLVIYQDN KRPSGIPERF

       210        220        230        240        250        260        270        280        290        300
SGSNSGNTAT LTISGTQAMD EADYYCQVWD NYSVLFGGGT KLTVLGSAGG SGGDSITHSS IKTNKKKECK DVKLGVREND KDLKICVIED TSLSGVDNCC

       310        320        330        340        350        360        370        380        390        400
CQDLLGILQE NCSDNKRGSS SNDSCDNKNQ DECQKKLEKV FASLTNGYKC DKCKSGTSRS KKKWIWKKSS GNEEGLQEEY ANTIGLPPRT QSLYLGNLPK

       410        420        430        440        450        460        470        480        490        500
LENVCEDVKD INFDTKEKFL AGCLIVSFHE GKNLKKRYPQ NKNSGNKENL CKALEYSFAD YGDLIKGTSI WDNEYTKDLE LNLQNNFGKL FGKYIKNNNT

       510        520        530        540        550        560        570        580        590        600
AEQDTSYSSL DELRESWWNT NKKYIWTAMK HGAEMNITTC NADGSVTGSG SSCDDIPTID LIPQYLRFLQ EWVENFCEQR QAKVKDVITN CKSCKESGNK

       610        620        630        640        650        660        670        680        690        700
CKTECKTKCK DECEKYKKFI EACGTAGGGI GTAGSPWSKR WDQIYKRYSK HIEDAKRNRK AGTKNCGTSS TTNAAASTDE NKCVQSDIDS FFKHLIDIGL

       710        720        730        740        750        760        770        780        790        800
TTPSSYLSNV LDDNICGADK APWTTYTTYT TTEKCNKERD KSKSQSSDTL VVVNVPSPLG NTPYRYKYAC QCKIPTNEET CDDRKEYMNQ WSCGSARTMK

       810        820        830        840        850        860        870        880        890
RGYKNDNYEL CKYNGVDVKP TTVRSNSSKL DSGRGELEGK PIPNPLLGLD STRTGWSHPQ FEKGGGSGGG SGGSSAWSHP QFEK
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Appendix 9. NGC