Bridging Two Worlds: The Application of High-Throughput Screening to Structural Chemistry

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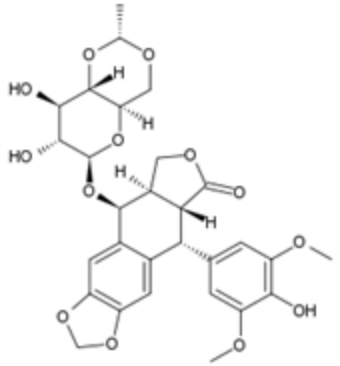
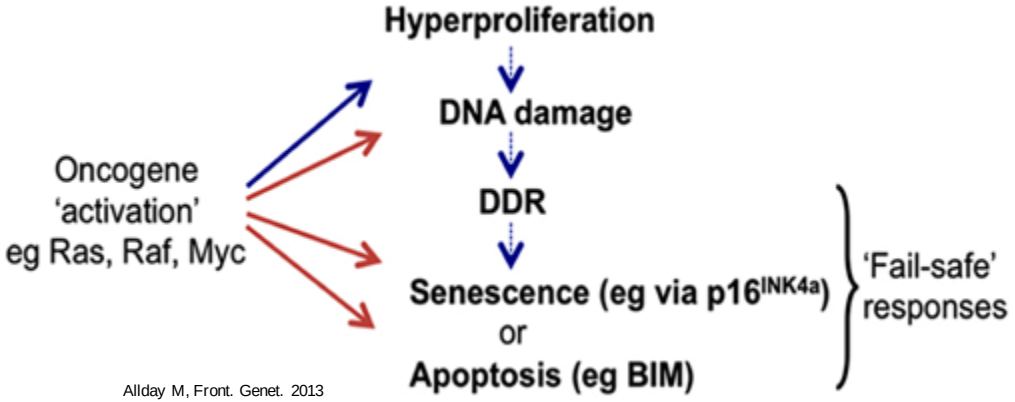
Investigating The Role Of Secreted Factors During The Induction Of Therapy And Oncogene Induced Senescence

- Senescence is defined as a stable proliferation arrest, that can happen in many cell types
- Commonly, Senescence is thought of as a cellular response to uninhibited growth, as a type of tumor suppressive activity
- However, there are many aspects of Senescence that can negatively affect cells
- Finding ways to curb the detrimental aspects of Senescence while reinforcing the benefits is an ongoing area of research

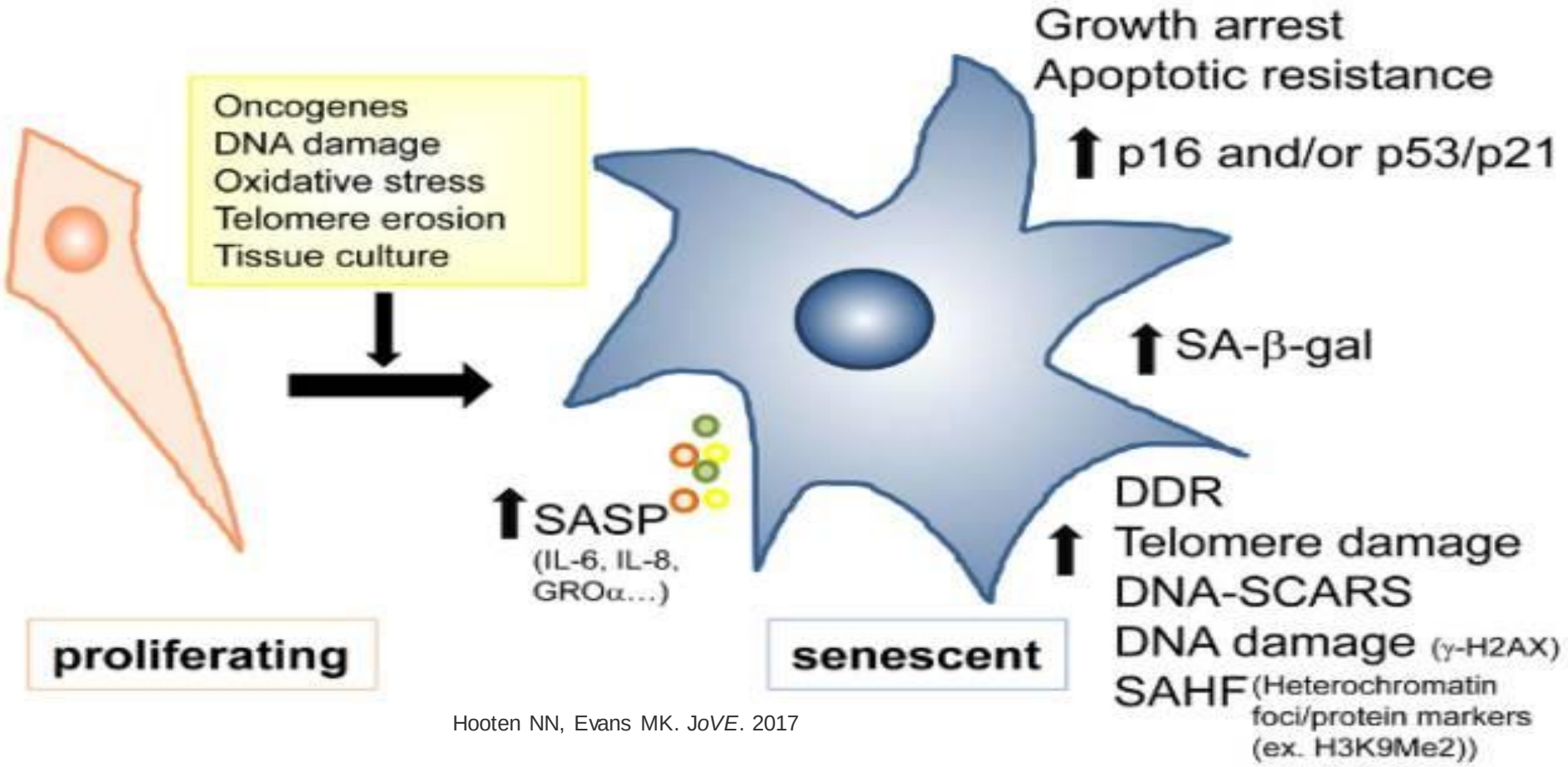


Skeletal Model of Etoposide (VP-16)



Allday M, Front. Genet. 2013

What is Senescence?

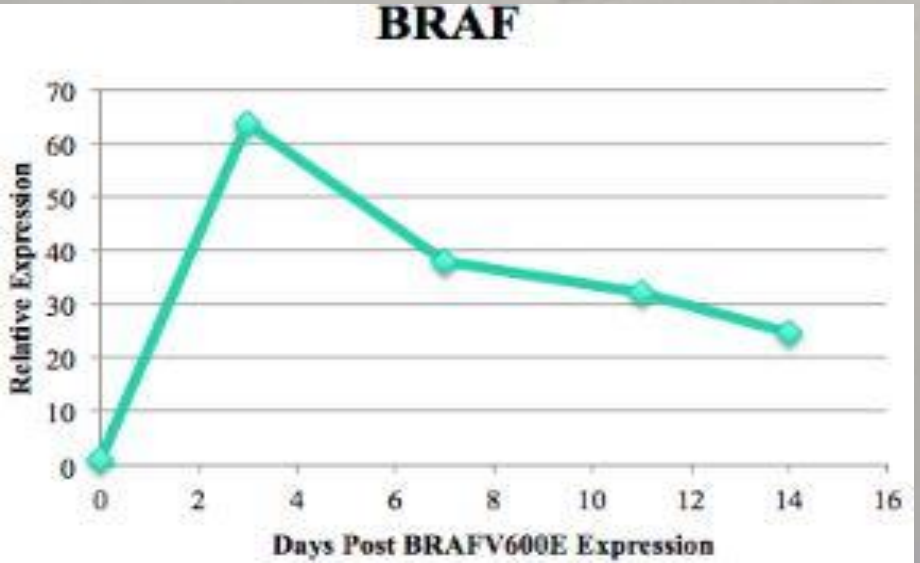


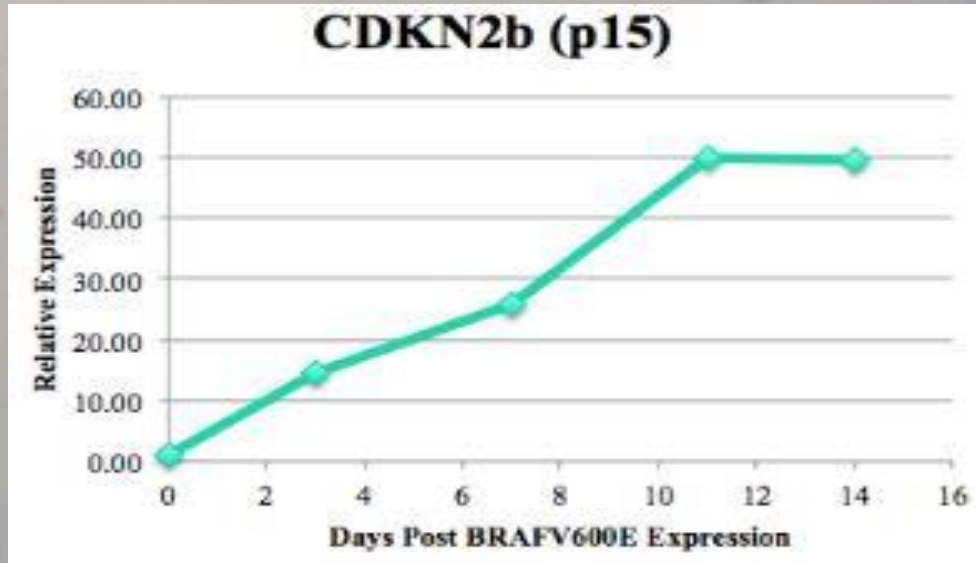
Hooten NN, Evans MK. J*oVE*. 2017

Classifying Senescence:
Gene Expression & Phenotypic Changes

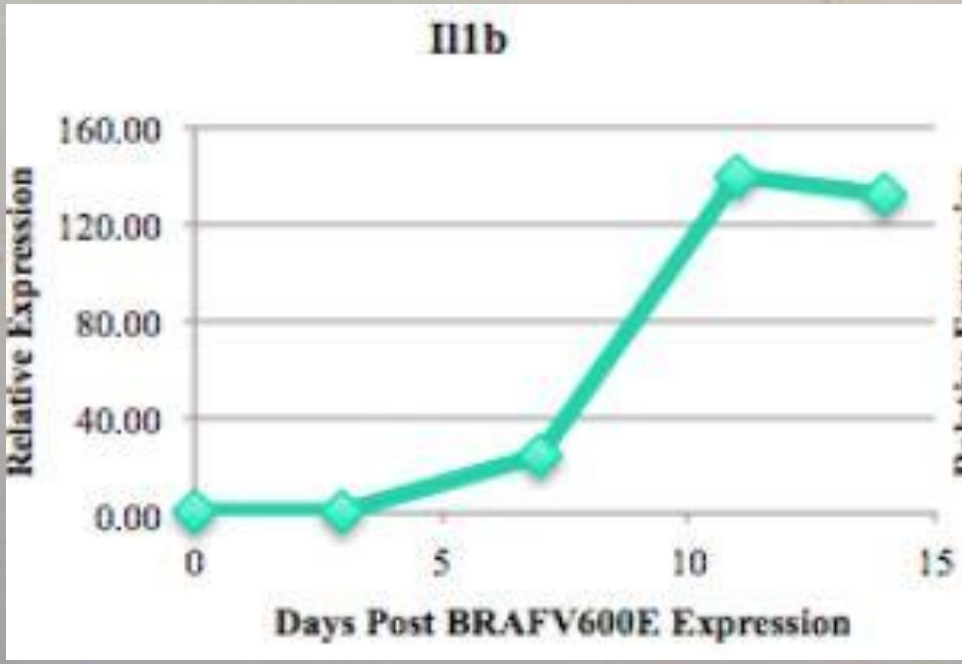
Figure 1 qPCR results for gene changes from day 0 senescence to day 14

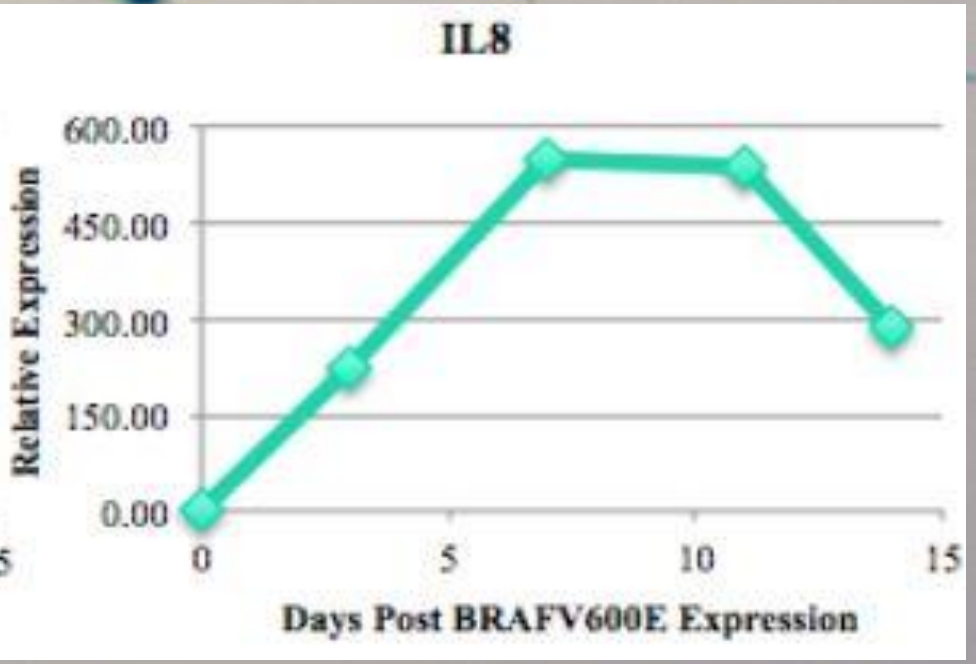
qPCR was performed on 5 different samples: uninfected melanocytes (day 0), and 4 $BRAF^{V600E}$ melanocytes from 4 different time points (days 3, 7, 11, and 14). CT values were normalized between the GAPDH (a housekeeping gene) values of day 0 and the other samples. Once this ratio was obtained, the other genes were compared. The relative expression is the fold change in expression of that gene in the cell.



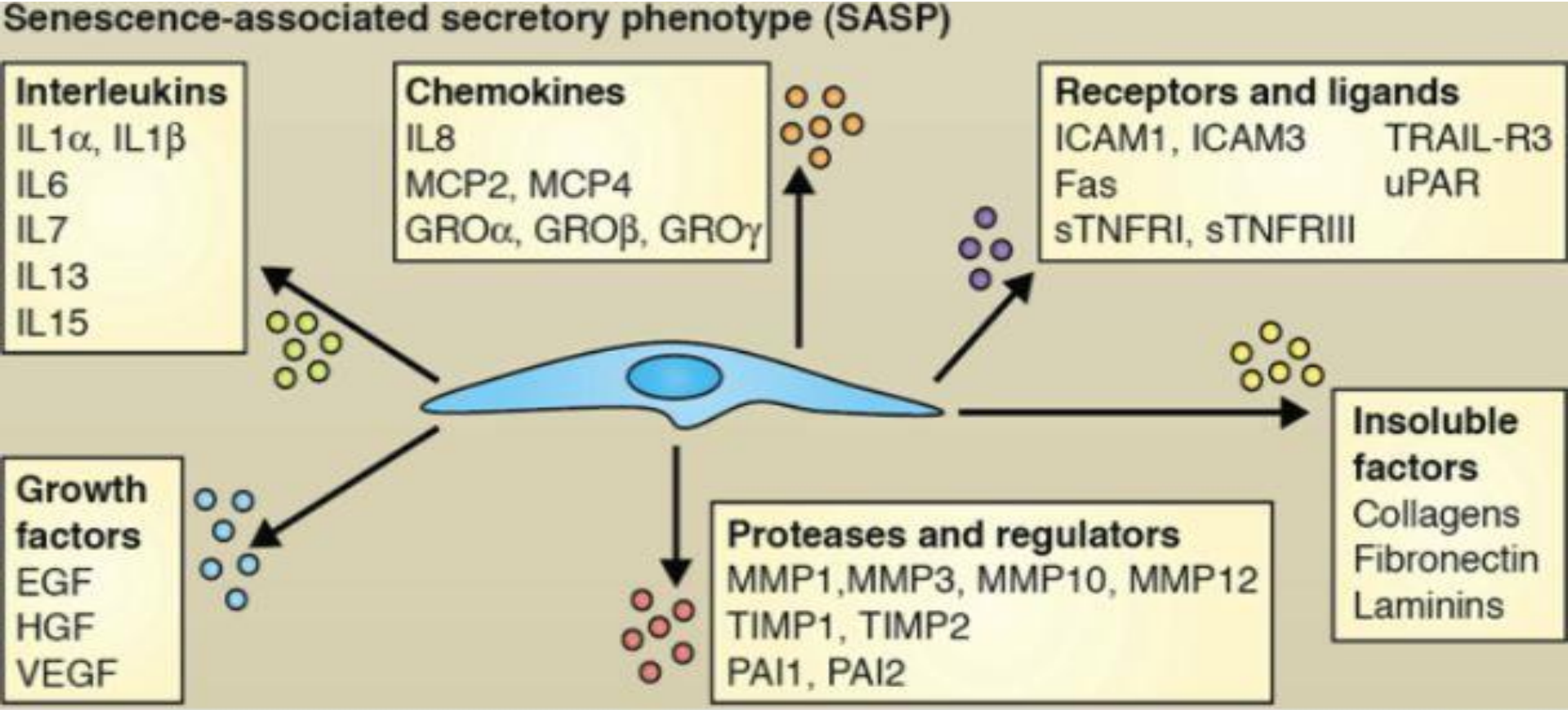


A Paradigm of Senescence: Overexpression of Cytokines





What Happens to Senescent Cells?



Pawlikowski et al., JCS, 2013.

Finding Inhibitors for the Inflammation Response in Senescent Cells: Pilot Screen Compounds

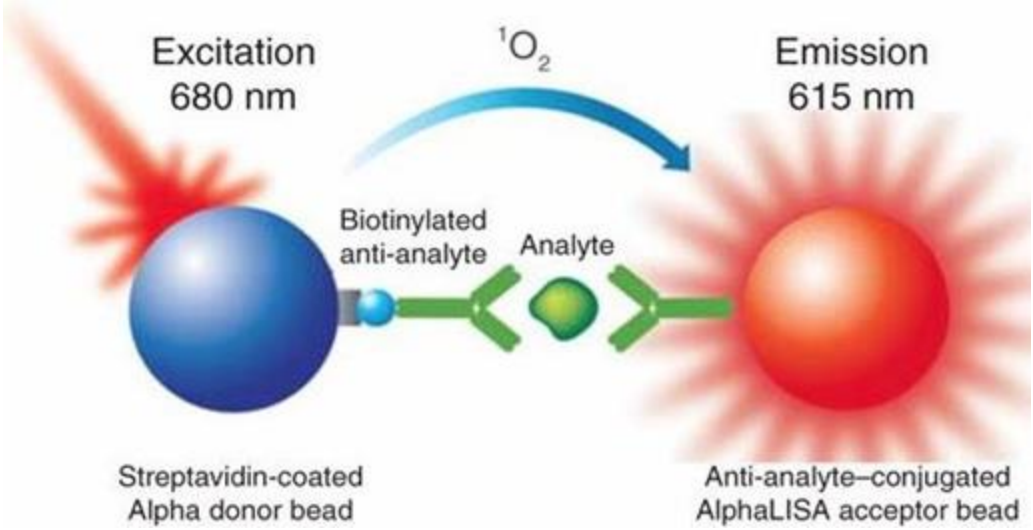
- MLL is a histone methyltransferase involved in the global regulation of gene transcription. The MLL-Menin interaction allows aberrant, uncontrolled cell division. MI-503 and MI-2-2 inhibit this interaction
- QNZ (EVP4593) is a potent NF-kB inhibitor. NF-kB is linked to DNA transcription and cell survival. Changes in NF-kB can lead to cancer and autoimmune disease
- Deoxycorticosterone and dexamethasone are both steroids that abate the inflammation response
- MGCD0103 and TSA are HDAC inhibitors, predicted to be tumor suppressors, due to their epigenetic effect on p21 and pRb
- Decitabine is a hypomethylating agent, inhibiting DNA synthesis

Drug Name	Target	FDA Approved	Indication	Concentration	Units	Box (AML)
MI-503	Menin		MLL leukemia	10	mM	8
MI-2-2	Menin		MLL leukemia	10	mM	8
QNZ (EVP4593)	NF-kB			10	mM	8
Deoxycorticosterone	GluR	Y		10	mM	9
Dexamethasone Acetate	Glucocorticoid	Y		10	mM	4
MGCD0103	HDAC	Y	DLBCL	10	mM	4
Trichostatin A	HDAC			1	mM	own box
Decitabine	DNA synthesis	Y	AML	10	mM	4

Finding Inhibitors for the Inflammation Response in Senescent Cells: Experimental Design

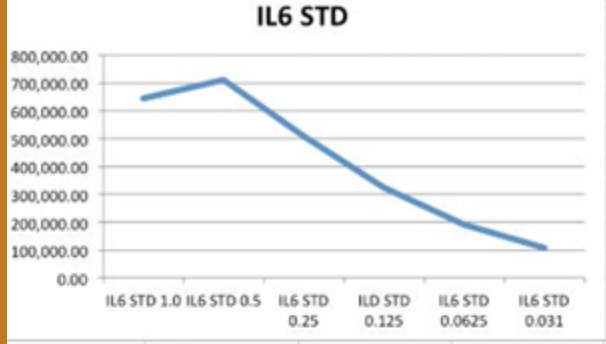
- IMR-90 cells (fibroblasts) were used for cytokine expression
- 96 & 384 well plates allowed for high-throughput processing
- Cells were cultured up to PD-40, and split often to prevent high confluency
- DMSO concentration was kept at 0.5%. Negative effects seen in culture were negligible
- Media additions / aspirations, and compound addition were performed using a multidrop combi, Biotek x405, and Janus workstation (respectively)

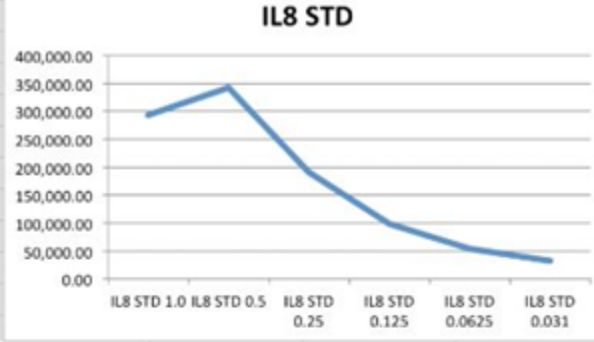
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	Monday	Tuesday
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	
Plate Cells	Etoposide /DMSO Treatment	Refeed Media + Compound				Serum-Free media change	Harvest supernatant/Assay

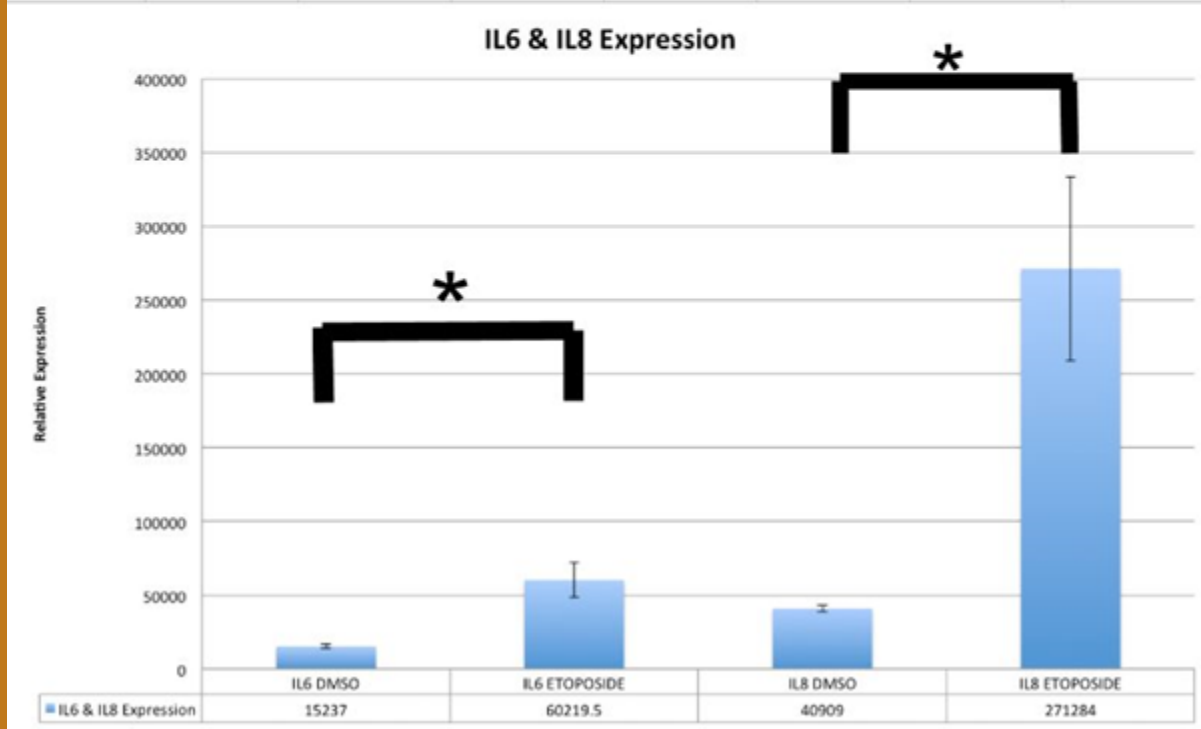


AlphaLISA Quantification of Cytokines:

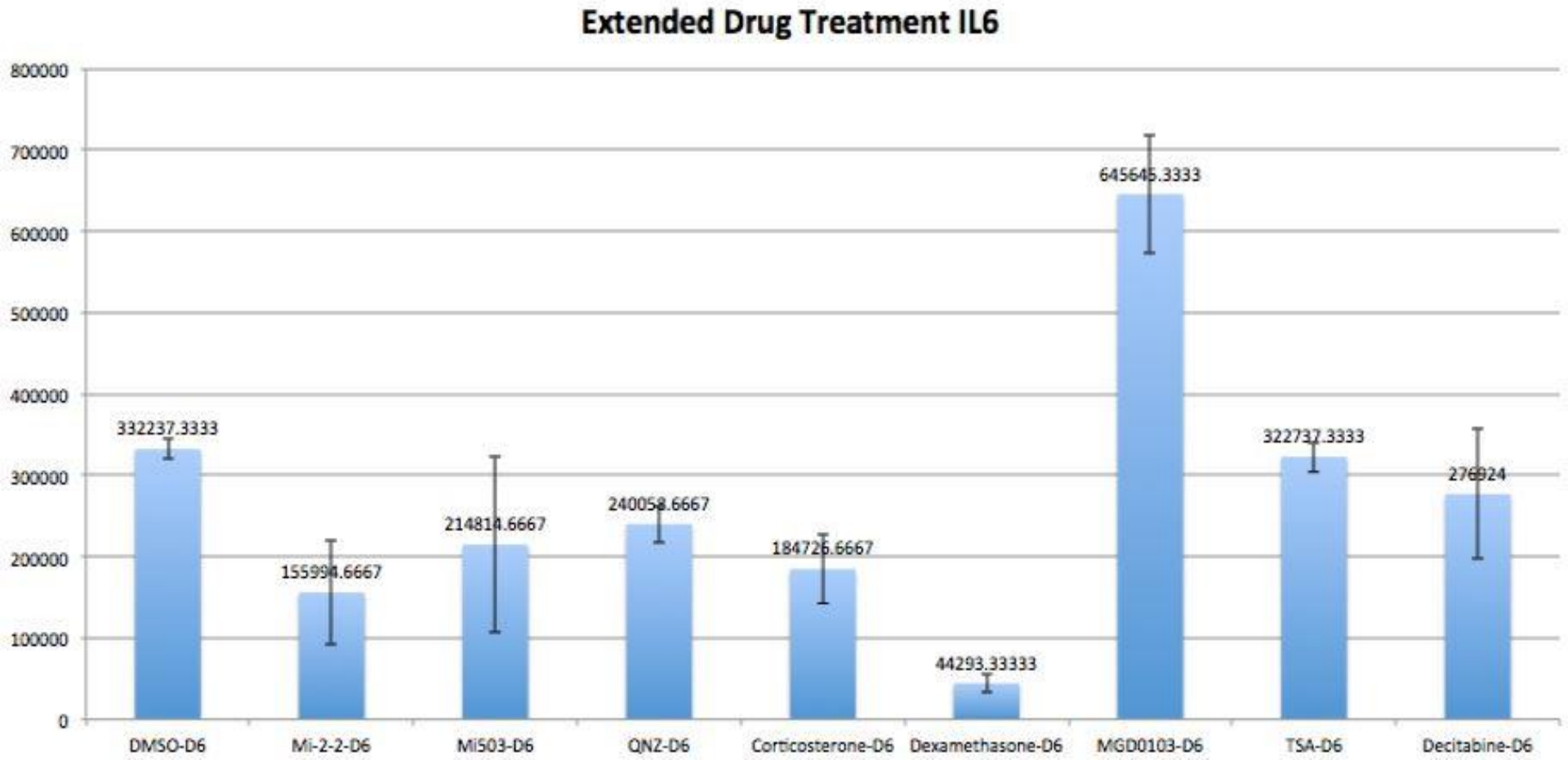
Assay Confirmation + Standardization



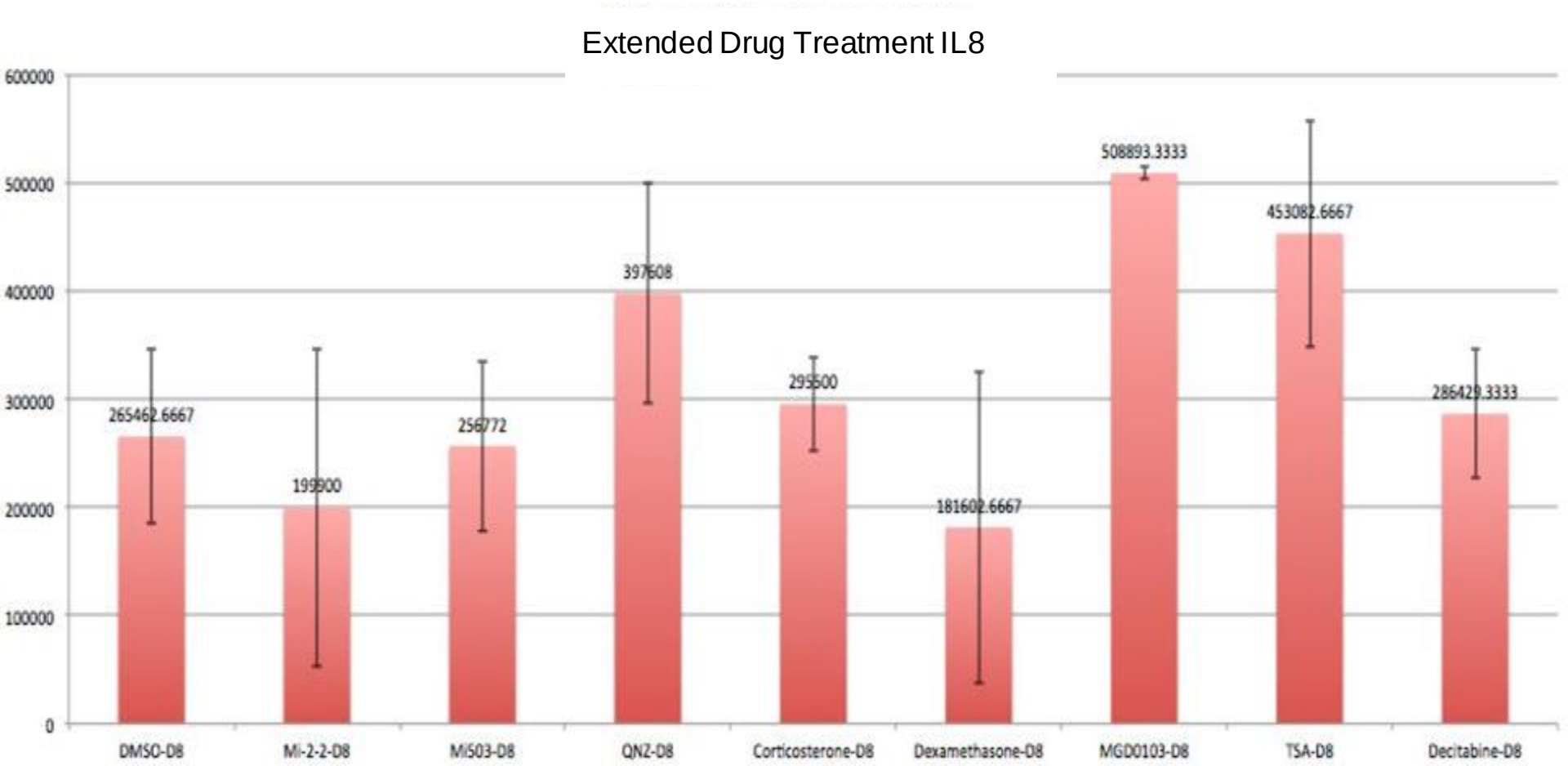




AlphaLISA Results – IL6: Compound VS Signal



AlphaLISA Results – IL8: Compound VS Signal



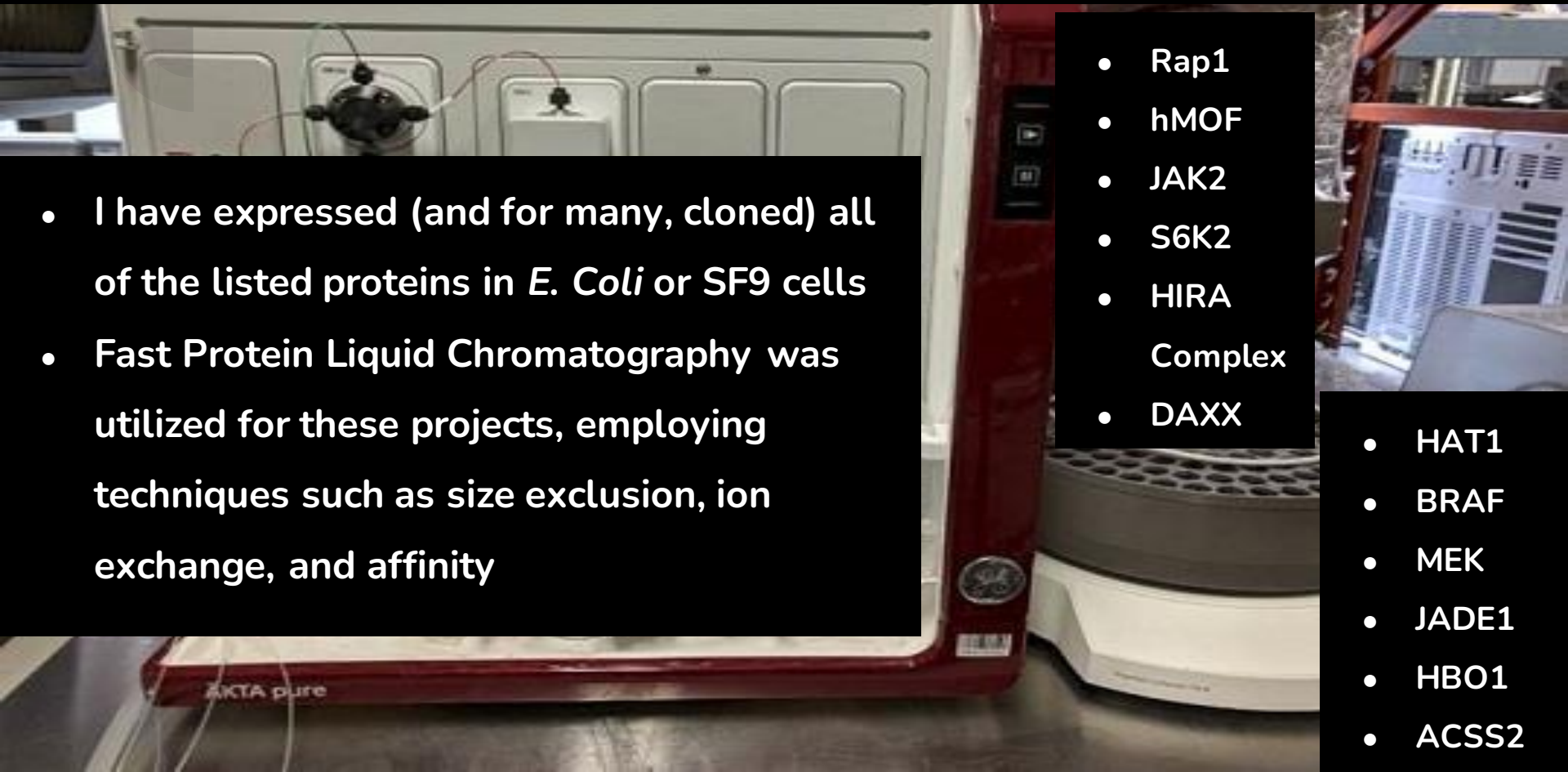
What is the bridge from HTS to Structural Chemistry at Penn?

Penn

EPIGENETICS INSTITUTE

- The UPenn Epigenetics Institute is a large group of researchers dedicated to working on epigenetics and age related diseases
- The two labs that I was a part of (HTS Core in Microbiology, Marmorstein Lab in Biochemistry) are integral to this collaboration

Purifying Proteins (a very long list)

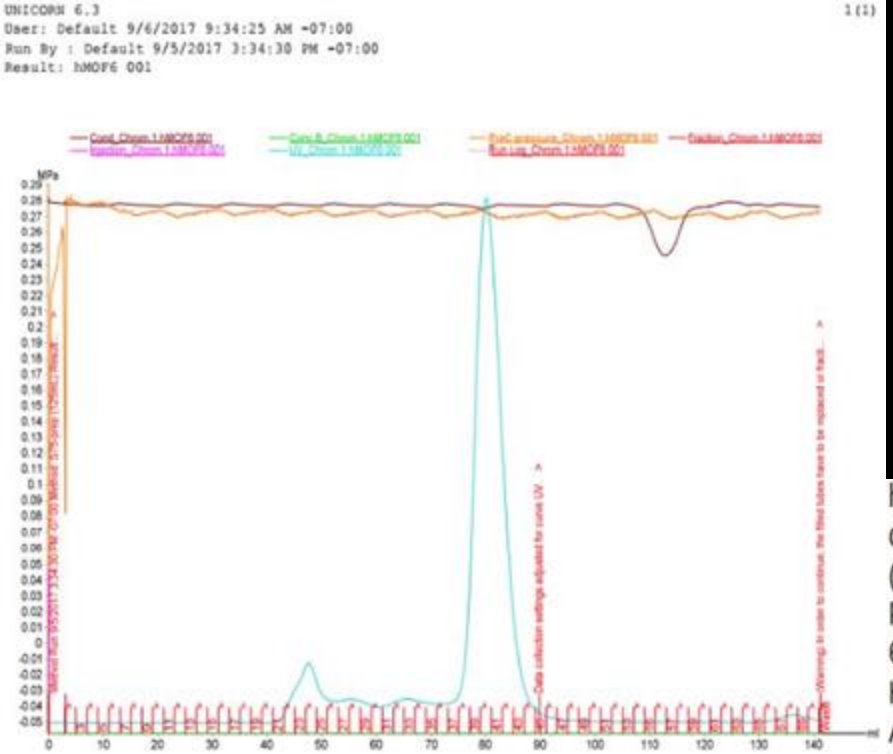


- I have expressed (and for many, cloned) all of the listed proteins in *E. Coli* or SF9 cells
- Fast Protein Liquid Chromatography was utilized for these projects, employing techniques such as size exclusion, ion exchange, and affinity

- Rap1
- hMOF
- JAK2
- S6K2
- HIRA Complex
- DAXX

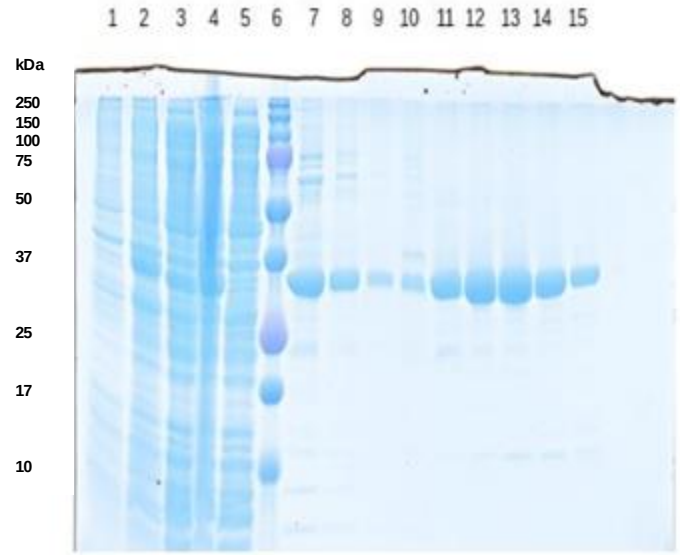
- HAT1
- BRAF
- MEK
- JADE1
- HBO1
- ACSS2

Purifying hMOF: A Relatively Straightforward Process



hMOF HAT domain (174-449)
Purification 6
His Nickel Affinity + Gel filtration FPLC
Coomassie Gel

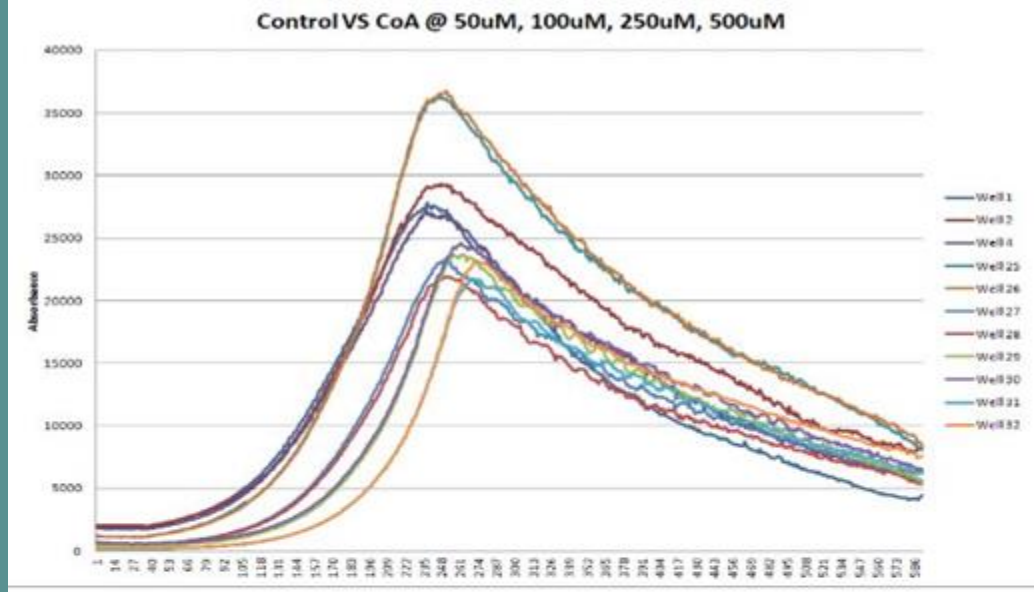
9/6/17

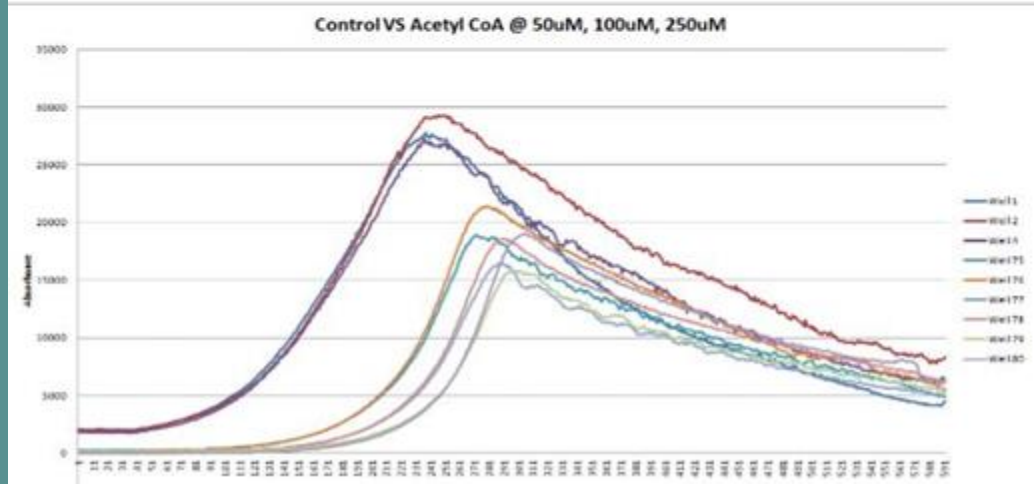


1-Uninduced
2-Induced
3-Soluble
4-Total
5-Flow through
6-MW marker
7-Elution 1
8-Elution 2
9-Elution 3
10-Beads
11-Fraction 39
12-Fraction 40
13-Fraction 41
14-Fraction 42
15-Fraction 43

Application: Assays

- Thermal shift assays determine the melting temperature of a protein due to various factors
- These may include: pH, Salinity, Substrate / Protein Concentration
- The higher melting temperature observed relative to baseline indicates a stabilization of the protein (or protein / compound complex)
- The assay requires minimal resources – widely available reagents, cost effective, and accommodates high-throughput applications
- A qPCR machine measures fluorescence (a dye, SYPRO orange, is the origin) as the assay plate is heated
- Over time, the dye (which binds to hydrophobic surfaces) is exposed to the reaction buffer. Signal increases until max temperature is reached, and gradually diminishes





Application:

Crystallize Protein

⇩

X-Ray Diffraction

⇩

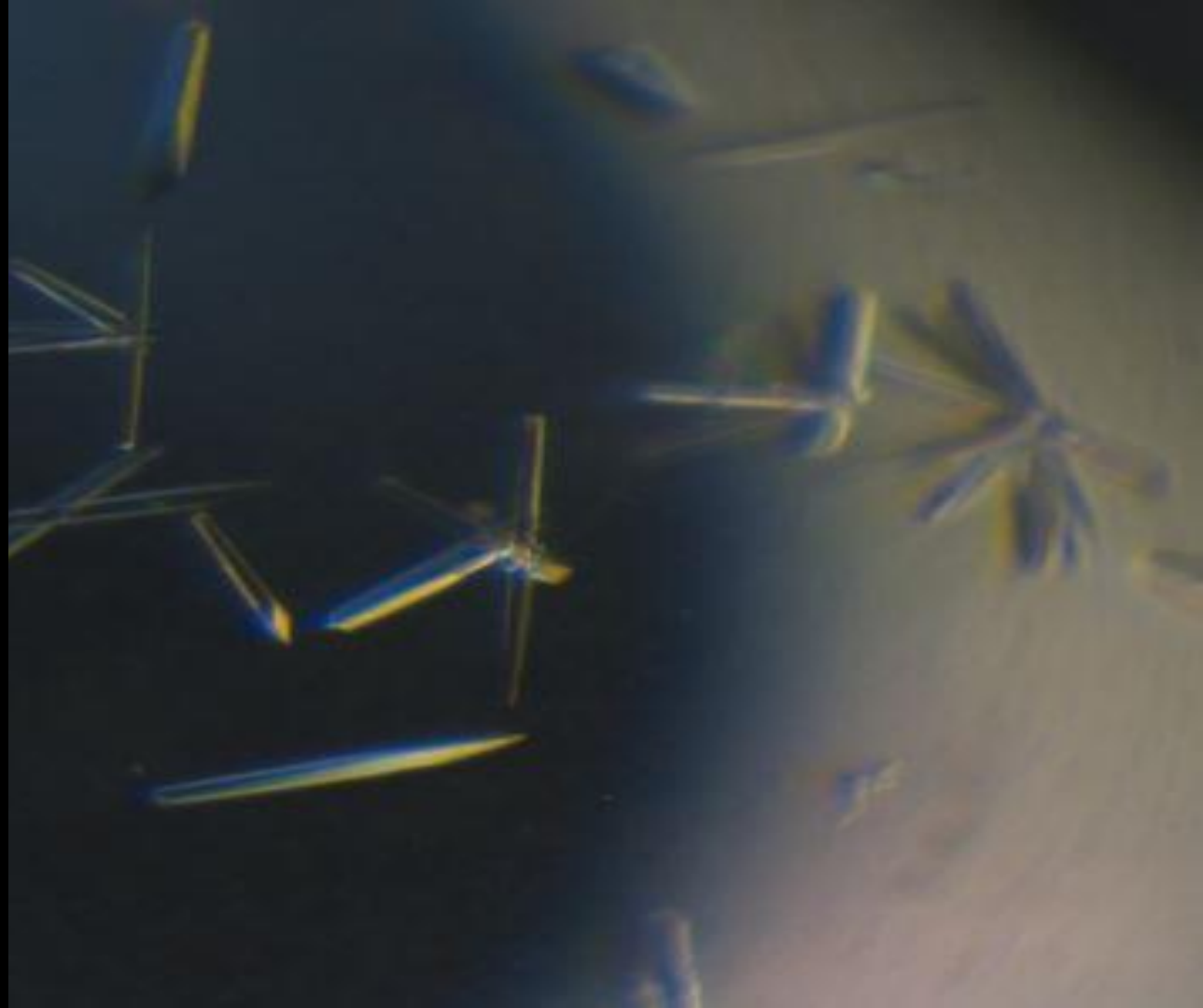
Data Analysis

⇩

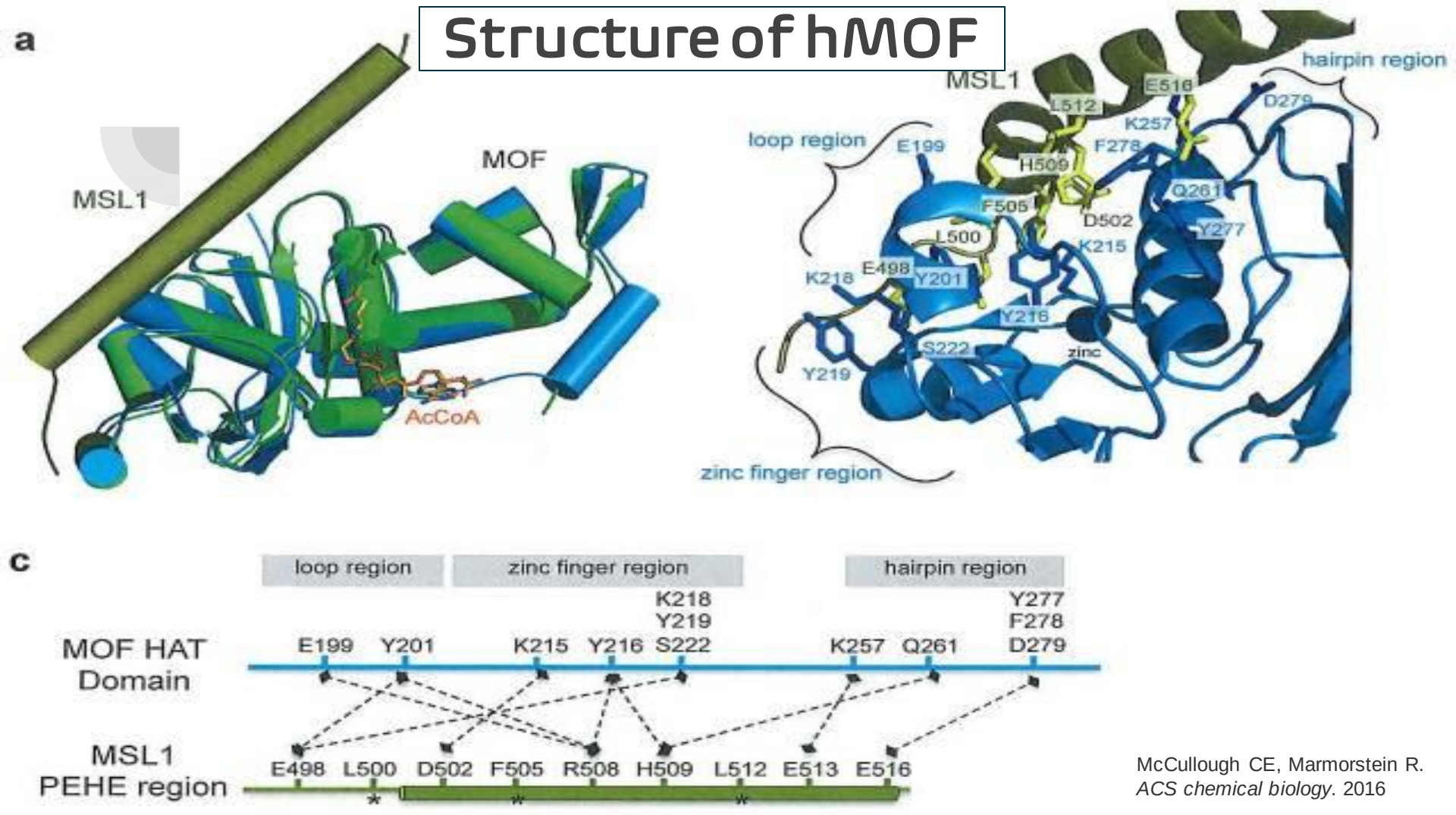
Solve Protein Structure

⇩

Advance Human Knowledge :D



Structure of hMOF

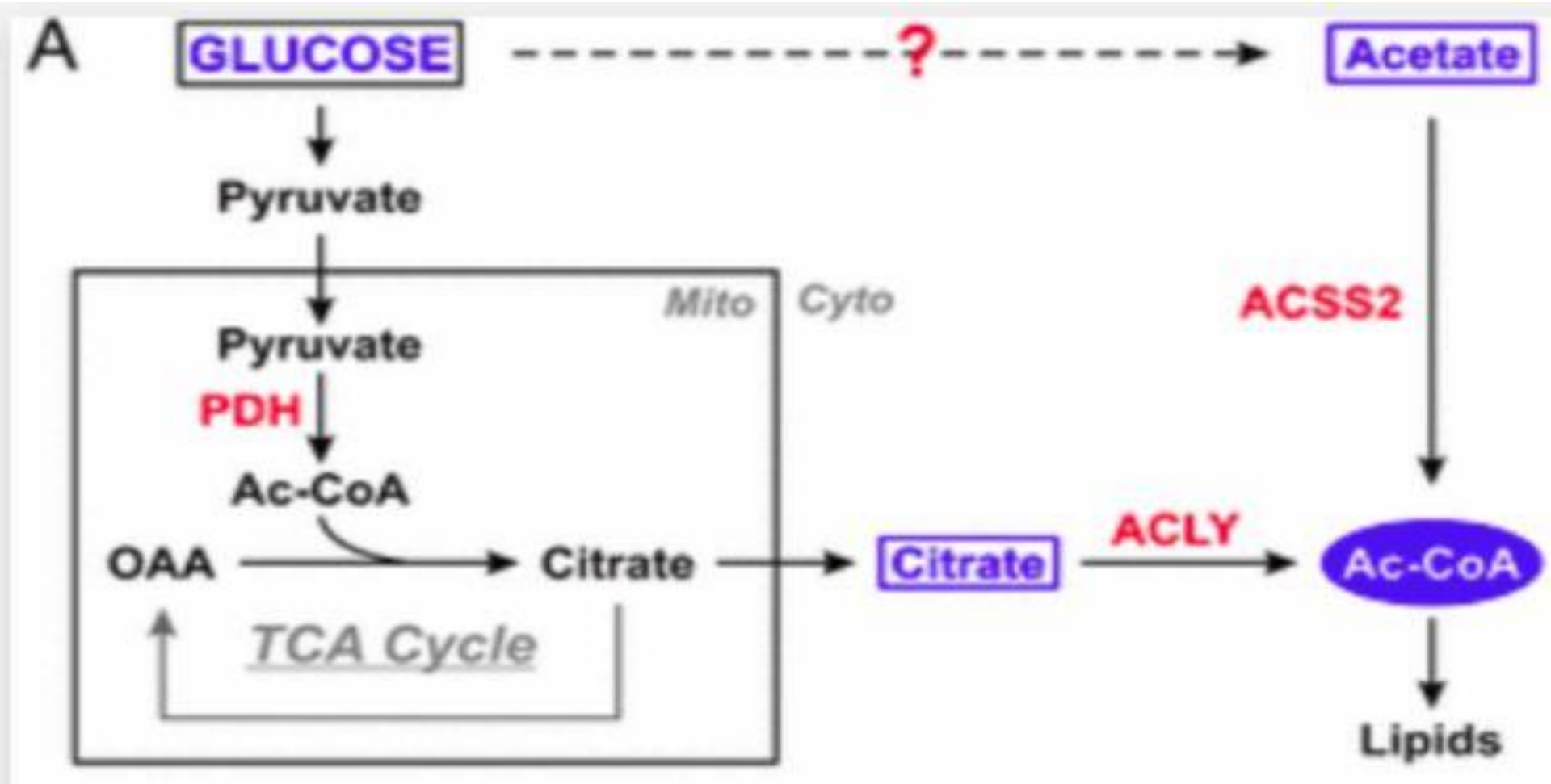


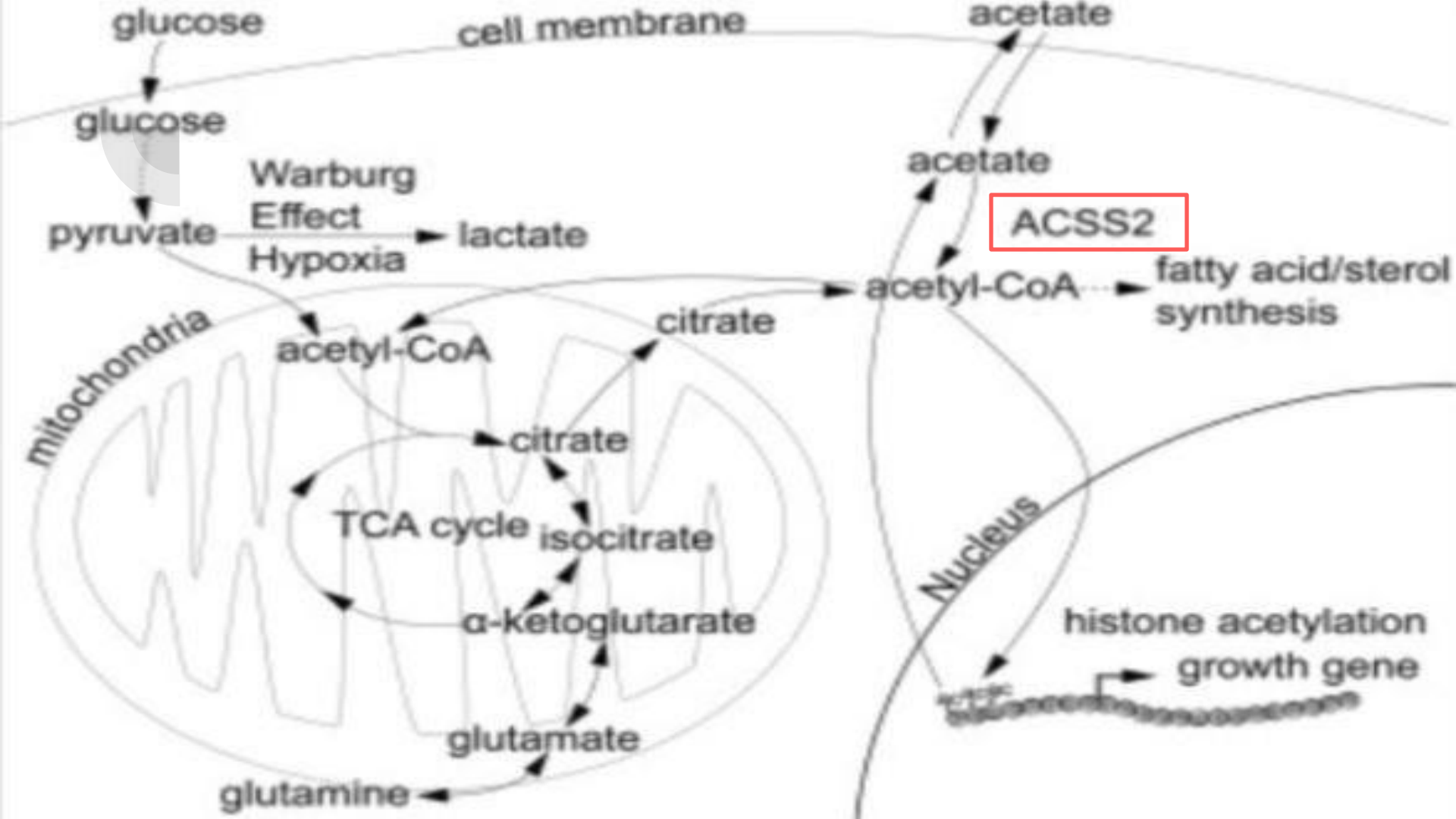
McCullough CE, Marmorstein R. *ACS chemical biology*. 2016

Plate	Well	MAYBRIDGE CODE	MolFormula	MolWeight	Parent MolWt	Salt_Info	CATALOGUE_Ref	TARGET WEIGHT (mgs)	ACTUAL WEIGHT (mgs)	Smiles	Moles	Concentration	Units	MeOH_50mM	Units
1	A2	CC08110	C2 H Br N2 S	165.0139	165.01		CC08110FL	5	4.94	S1C(=NN=C1)Br	0.0000299	50	mM	599.	uL
1	B2	CC38510	C10 H12 Br N	226.1158	226.12		CC38510FL	5	4.96	N1(c2cccc(c2)Br)CCCC1	0.0000219	50	mM	439.	uL
1	C2	MO07325	C6 H7 Br N2 O2	219.0373	219.04		MO07325FL	5	5.05	N1(C(=CN=C1Br)C(=O)OC)C	0.0000230	50	mM	461.	uL
1	D2	AW00238	C13 H9 Br F3 N3 O S	392.198	392.19		AW00238SC	5	5.07	n1c(nccc1C(F)(F)F)SCC(=O)Nc1ccc(cc1)Br	0.0000129	50	mM	259.	uL
1	E2	AW00727	C12 H16 Br F N2	287.1744	287.17		AW00727FL	5	4.97	N1(Cc2c(cc(cc2)Br)F)CCCNCC1	0.0000173	50	mM	346.	uL
1	F2	AW00960	C14 H14 Br N O2	308.174	308.17		AW00960SC	5	5.08	N1(c2ccc(cc2)Br)C(=O)CC2(C1=O)CCCC2	0.0000164	50	mM	330.	uL
1	G2	BTB00081	C20 H14 Br N O3	396.239	396.24		BTB00081SC	5	5	N1C(=O)C(c2c1ccc(c2)Br)(CC(=O)c1ccc2ccccc2c1)O	0.0000126	50	mM	252.	uL
1	H2	BTB00089	C18 H16 Br N O3	374.232	374.23		BTB00089SC	5	5.08	N1C(=O)C(c2c1ccc(c2)Br)(CC(=O)c1c(cc(cc1)C)C)O	0.0000135	50	mM	271.	uL
1	A3	BTB00099	C13 H12 Br N O2	294.1468	294.15		BTB00099SC	5	5.05	n1c2c(c(cc1C)C(=O)OCC)cc(cc2)Br	0.0000171	50	mM	343.	uL
1	B3	BTB00115	C11 H10 Br N3 O	280.124	280.12		BTB00115SC	5	4.92	n1c2c(c(cc1C)C(=O)NN)cc(cc2)Br	0.0000175	50	mM	351.	uL
1	C3	BTB00244											mM	243.	uL
1	D3	BTB00245											mM	252.	uL
1	E3	BTB00246											mM	247.	uL
1	F3	BTB00500											mM	265.	uL
1	G3	BTB00501											mM	257.	uL
1	H3	BTB00503											mM	464.	uL
1	A4	BTB00581											mM	406.	uL
1	B4	BTB00681											mM	315.	uL
1	C4	BTB00694	C14 H12 Br N	274.16	274.16		BTB00694SC	5	4.91	N[=CC1CCC(C1)Br)CC1CCCC1	0.0000179	50	mM	358.	uL
1	D4	BTB01061	C11 H7 Br O3	267.0773	267.08		BTB01061SC	5	5.07	O1C(=O)C(=Cc2cc(ccc21)Br)C(=O)C	0.0000189	50	mM	380.	uL
1	E4	BTB01179	C15 H15 Br N2 O3 S	383.264	383.26		BTB01179SC	5	5	S(=O)(=O)(Nc1ccc(c(c1)N=Cc1cc(ccc1O)Br)C)C	0.0000130	50	mM	261.	uL
1	F4	BTB01253	C10 H5 Br O4	269.0495	269.05		BTB01253SC	5	4.94	O1C(=O)C(=Cc2c1ccc(c2)Br)C(=O)O	0.0000183	50	mM	367.	uL
1	G4	BTB01338	C17 H17 Br N2 O4	393.235	393.24		BTB01338SC	5	4.93	N(=Cc1cc(ccc1O)Br)N=Cc1cc(c(c(c1)OC)OC)OC	0.0000125	50	mM	251.	uL
1	H4	BTB01340	C13 H9 Br N4 O	317.145	317.14		BTB01340SC	5	4.91	N1=Nc2cc(ccc2N1)N=Cc1cc(ccc1O)Br	0.0000154	50	mM	310.	uL
1	A5	BTB01804	C18 H14 Br N3 O2	384.232	384.23		BTB01804SC	5	5	n1c2c(c(cc1C)C(=O)NN=Cc1c(cccc1)O)cc(cc2)Br	0.0000130	50	mM	260.	uL
1	B5	BTB01924	C7 H5 Br2 N O2	294.9295	294.93		BTB01924SC	5	4.93	O=C(c1c(c(cc(c1)Br)Br)N)O	0.0000167	50	mM	334.	uL
1	C5	BTB01928	C12 H9 Br2 N O2	359.016	359.02		BTB01928SC	5	4.96	N1=C(OC(=O)c2cc(cc(c21)Br)Br)C(=CC)C	0.0000138	50	mM	276.	uL
1	D5	BTB01996	C12 H9 Br Cl N O3	330.564	330.56		BTB01996SC	5	4.91	n1c2c(c(c(c1)C(=O)OCC)O)cc(cc2Cl)Br	0.0000148	50	mM	297.	uL
1	E5	BTB02039	C14 H8 Br Cl F3 N O	378.574	378.58		BTB02039SC	5	4.98	N(=Cc1c(ccc(c1)Br)O)c1cc(ccc1Cl)C(F)(F)F	0.0000131	50	mM	263.	uL
1	F5	BTB02093	C9 H6 Br N O2	240.055	240.06		BTB02093SC	5	4.92	N1=C(C)OC(=O)c2cc(ccc12)Br	0.0000204	50	mM	410.	uL
1	G5	BTB02228	C19 H18 Br N3 O2	400.274	400.27		BTB02228SC	5	5.06	N1(c2c(cccc2)N2CCOCC2)C(=O)c2c(ccc(c2)Br)N=C1C	0.0000126	50	mM	253.	uL

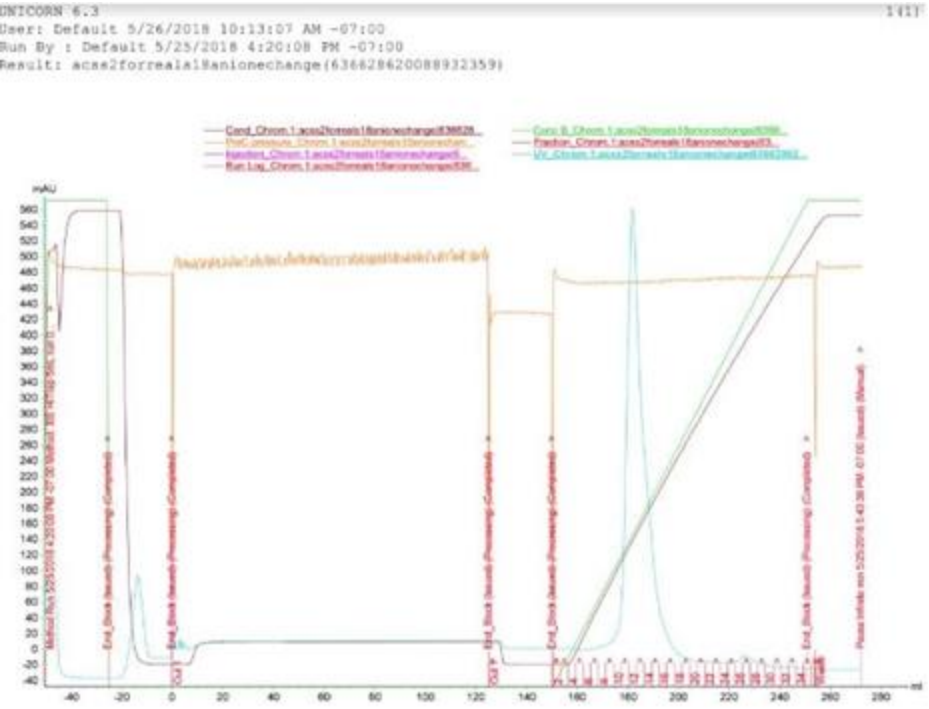
Compound Library Management, Organization, and Usage

ACSS2: From acetyl-CoA Production to Histone Acetylation

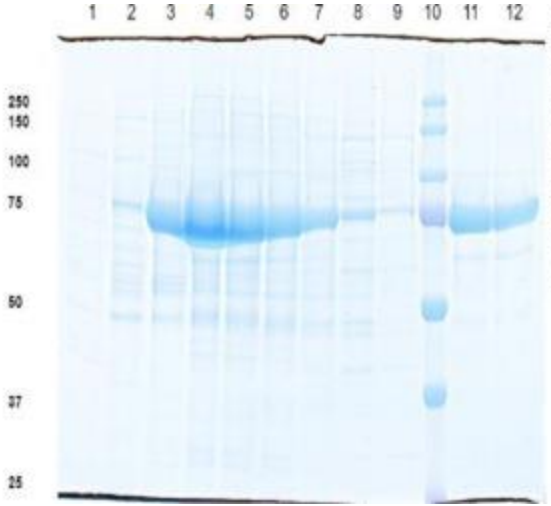



glucose
cell membrane
acetate
glucose
acetate
Warburg
Effect
Hypoxia
pyruvate
lactate
ACSS2
acetyl-CoA
fatty acid/sterol
synthesis
mitochondria
acetyl-CoA
citrate
citrate
TCA cycle
isocitrate
Nucleus
α-ketoglutarate
histone acetylation
growth gene
glutamate
glutamine

ACSS2 Purification (Part I): Anion Exchange Chromatography

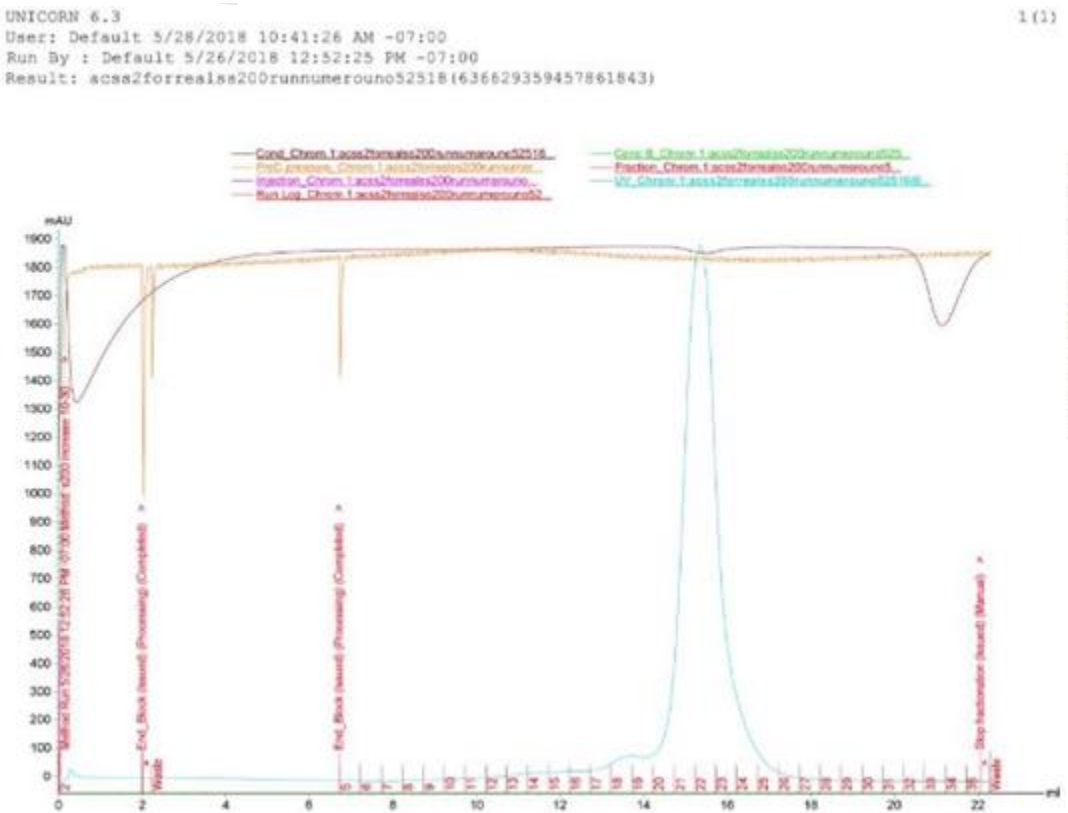


Hs ACSS2-30-701 codon optimized Purification 1 Talon nickel affinity--> Anion Exchange Coomassie gel

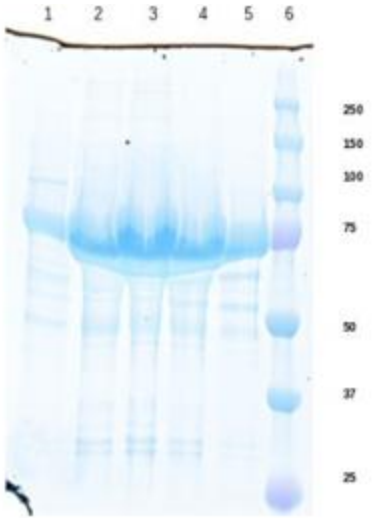


1-Fraction 9
2-Fraction 10
3-Fraction 11
4-Fraction 12
5-Fraction 13
6-Fraction 14
7-Fraction 15
8-Fraction 16
9-Fraction 17
10-MW marker
11-Fraction 12 (second run)
12-Fraction 13 (second run)

ACSS2 Purification (Part II): Size Exclusion Chromatography (S-200)



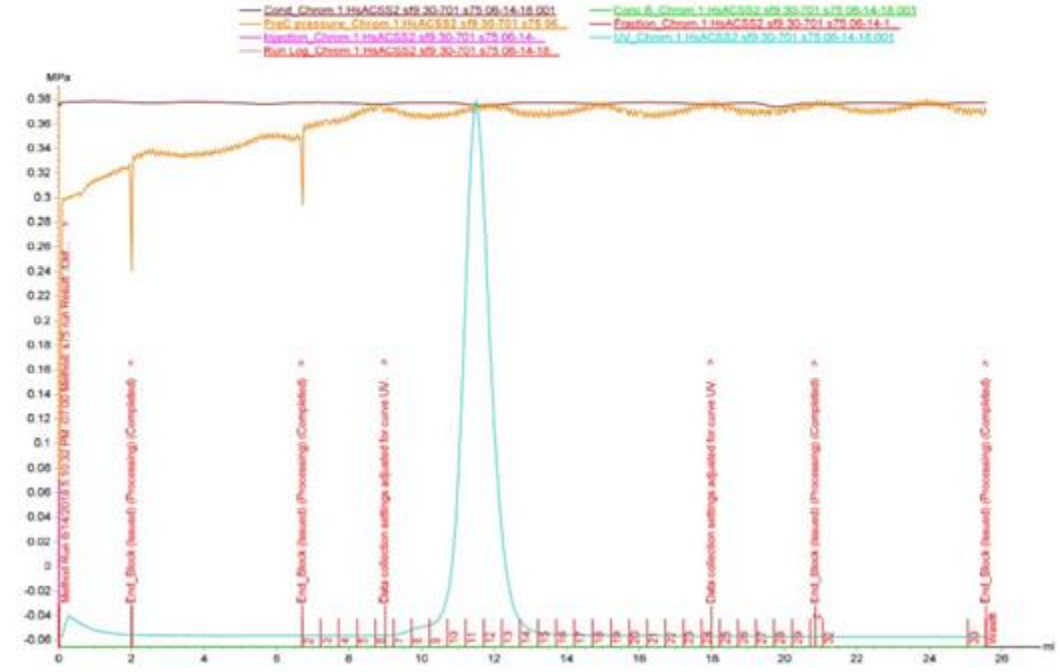
Hs ACSS2-30-701 codon optimized Purification 1 Anion Exchange--> Superdex 200 Coomassie gel 5/26/18



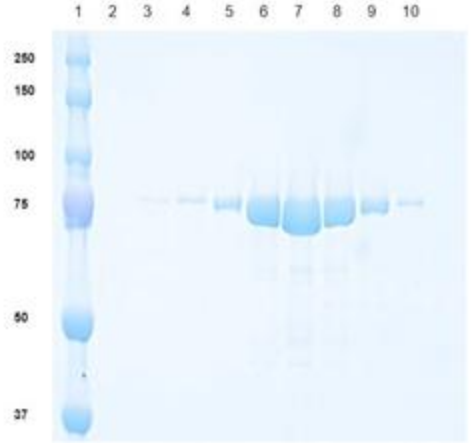
1-Fraction 20
2-Fraction 21
3-Fraction 22
4-Fraction 23
5-Fraction 24
6-MW Marker

ACSS2 Purification (Part III): Size Exclusion (Superdex 75)

User: Default 6/15/2018 10:33:30 AM -07:00
Run By : Default 6/14/2018 5:10:32 PM -07:00
Result: HsACSS2 sf9 30-701 s75 06-14-18 001



Hs
ACSS2-30-701
codon optimized
Purification 2
Superdex 75
Coomassie gel
6/14/18



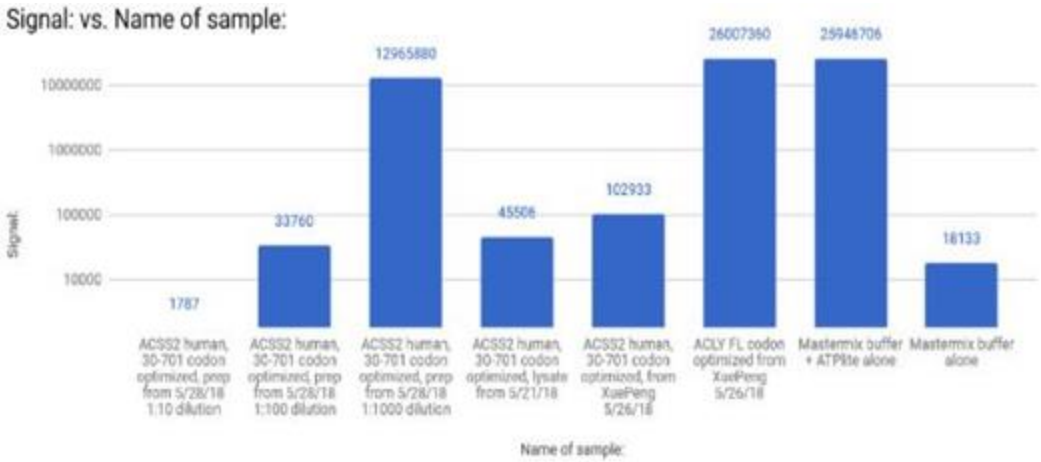
1-MW Marker
2-Fraction 6
3-Fraction 7
4-Fraction 8
5-Fraction 9
6-Fraction 10
7-Fraction 11
8-Fraction 12
9-Fraction 13
10-Fraction 14

Assaying for enzymatic activity: Quantifying production of acetyl-CoA from Acetate

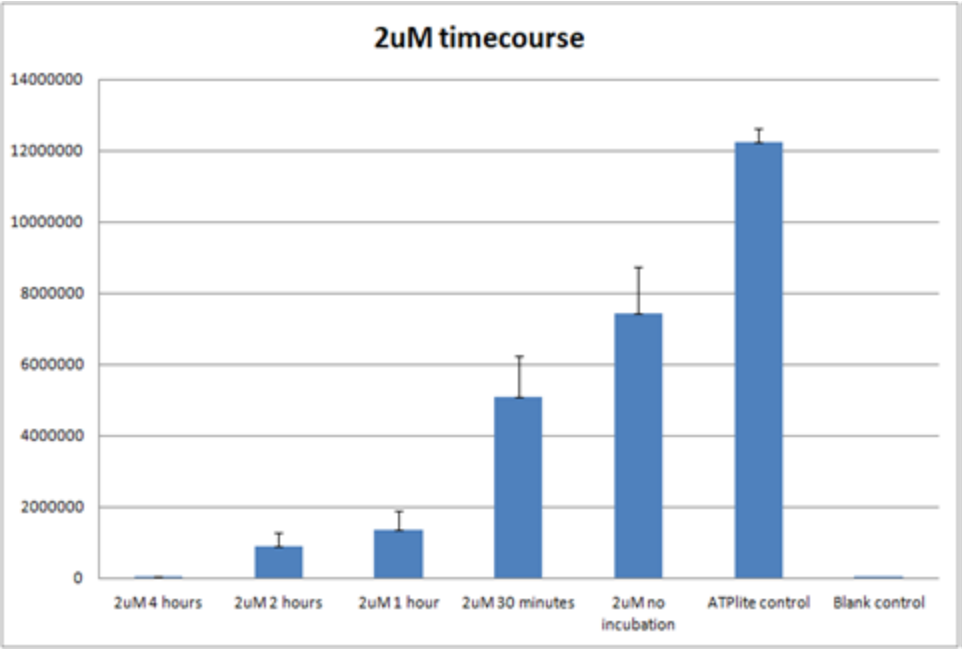
Experiment:

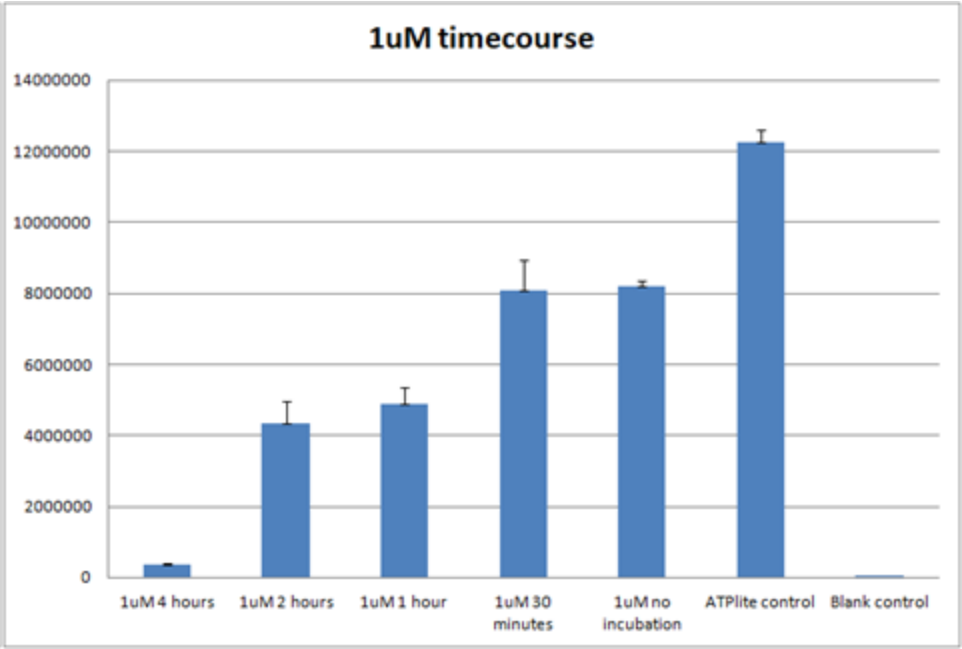
Component	Volume/Well	Final [ ]	For 20 reactions
10X Buffer	2.5 uL	1X	50 uL
5 mg/mL BSA	0.5 uL	0.1 mg/mL	10 uL
1 mM CoA	2.5 uL	100 uM	50 uL
1 mM ATP	0.5 uL	20 uM	10 uL
5X Enzyme (ACSS2)	5 uL	1X (calculate)	N/A
25 mM Acetate	5 uL	5 mM	100 uL
ddH2O	9 uL	N/A	180 uL
Total	25 uL	N/A	400 uL

1. 3-5 enzyme concentrations in triplicate
2. Prepare 500 (more or less) nM diluted enzyme in assay buffer, prepare serial dilutions
3. Prepare a master reaction mix minus enzyme
4. Add 20 ul of reaction mix to white opaque bottom plate (corning 3570) using 16-ch pipette
5. Add 5 ul of enzyme, manually
6. Seal and Tap spin plate for 1 min at 300xg
7. Incubate at room temp for 1-2 hrs
8. Add 25ul of 1X ATPlite
9. Tap spin for 1 minute at 300xg
10. Incubate for 5 minutes
11. Read luminescence

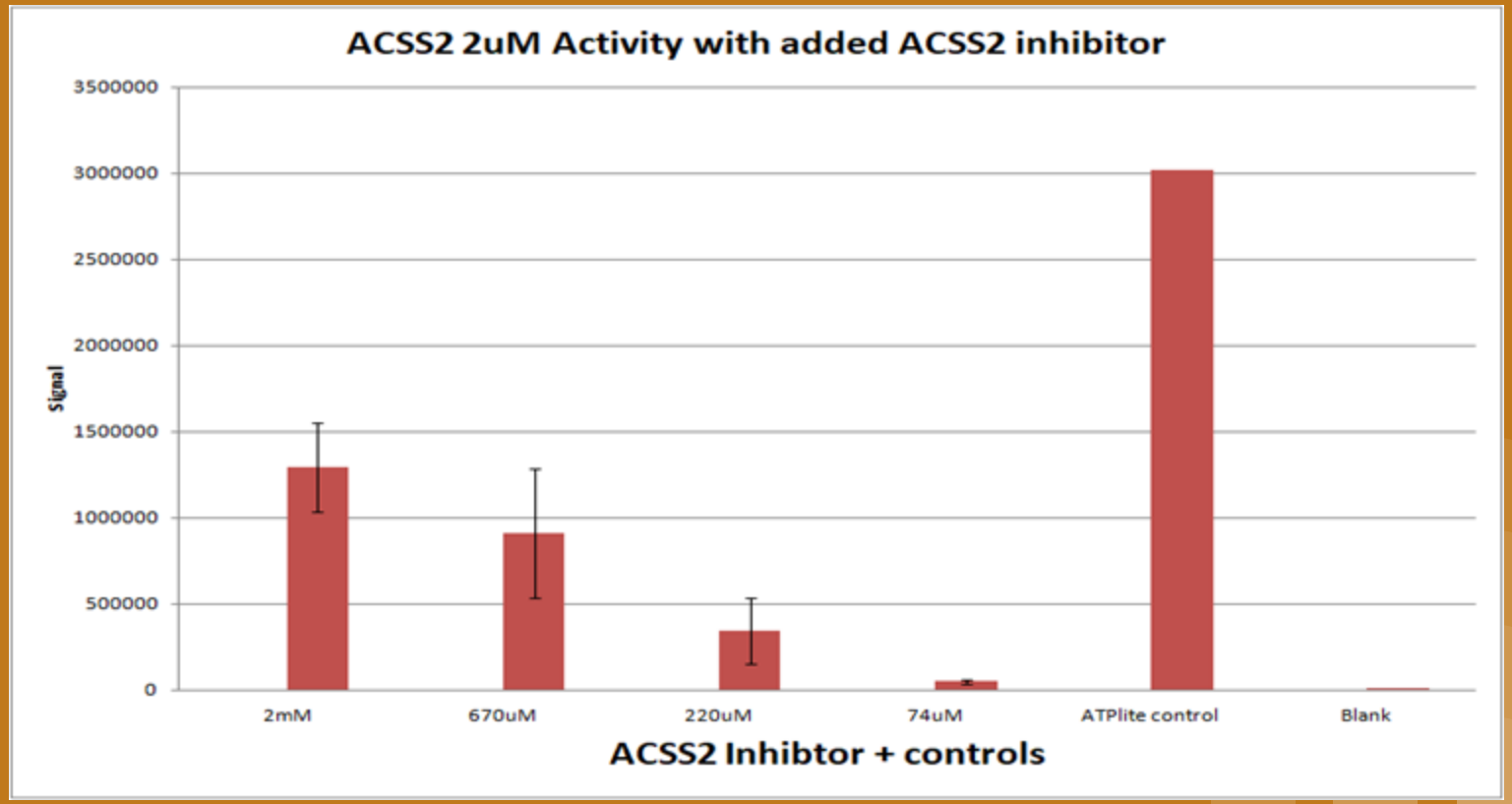


Refining the Assay: Concentration + Incubation Time





Screening for ACSS2 Inhibitors: It all comes down to this



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