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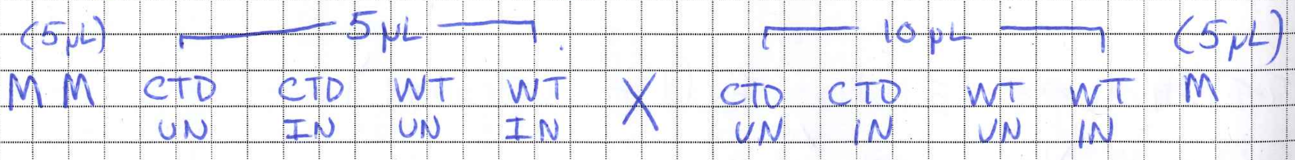
Inoculating Pin1 constructs in LB

	Pin1 - WT	Pin1-CTD	
O/N	4.685	5.865	(corrected w/ dilution)
Add to 1L	10.7 mL	8.5 mL	
8:54a	0.050	0.045	
9:55a	0.138	0.155	
10:31a	0.364	0.366	
10:46a	0.503	0.507	← Induced 1mM IPTG
5:22	4.86	2.74	← harvest

Spun down cells, I and U sample. Each had OD ≈ 0.5 and 1mL cells. Will resuspend in Urea (6M) / 20 mM Tris / 50 mM NaCl. Some volume left over in LB, I'll add 70 μL, then dye.

Making my own stock of loading dye. Lab stock is 4x 4x non reducing. I need to add 15 μL BME to 1 mL dye.

2.86 g of CTD cells, 3.43 g of WT cells.



Note: Samples were mixed 1:1 w/ 2x LDS dye + BME and heated to 90 °C for 5min before loading. Not viscous @ all.

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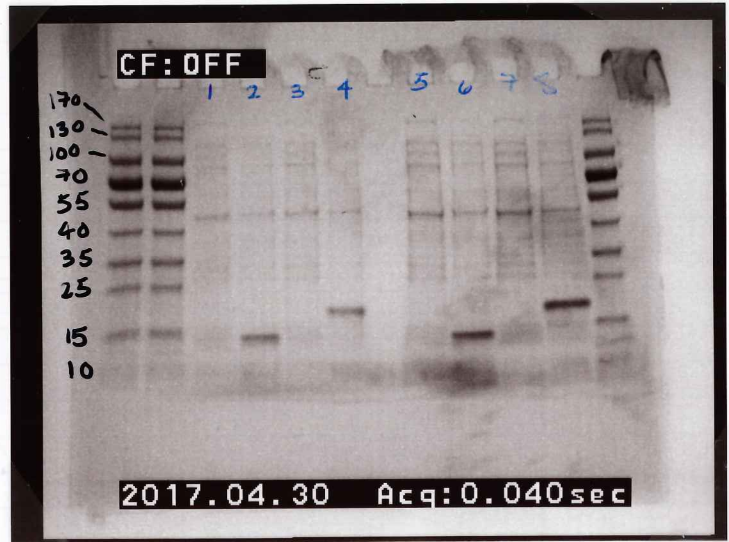

Gel lanes are on prev. page. Pattern is CTD U-I, WT U-I. CTD is measuring 14 kDa, FL WT pin1 is around ~~18~~ 15-20 kDa. This is pretty close to the expected values (see next page.)

5/1/2017

Lysis Buffer / Wash Buffer (1 L)

			Stock ~~5M~~
150 mM NaCl	→ 30 mL	30 mL	5M
20 mM ~~PIPES~~ HEPES (pH 7.5)	→	20 mL	1M
20 mM Imidazole (pH 7.5)	→	5.7 mL	3.5M
2 mM DTT		0.31 g (solid)	~~1M~~

~~Wash Buffers~~ Elution Buffer (500 mL)

150 mM NaCl → ~~30~~ 15 mL
20 mM HEPES → 10 mL
800 mM Imid → 115 mL
2 mM DTT → 0.16 g

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sPin1 Full Length Complete Sequence:

```
     H  M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R /G  S
1    catatgggcagcagccatcatcatcatcatcatagcagcggcctggtgccgcgtggcagc  60

-1   H  G  M  A  D  E  E  K  L  P  P  G  W  E  K  R  M  S  R  S   18
61   cacggcatggcggatgaagaaaaactgccgccgggctgggaaaaacgtatgagccgtagc  120

19   S  G  R  V  Y  Y  F  N  H  I  T  N  A  S  Q  W  E  R  P  S   38
121  agcggccgtgtgtattattttaaccatattaccaacgcgagccagtgggaacgtccgagc  180

39   G  N  S  S  S  G  G  K  N  G  Q  G  E  P  A  R  V  R  C  S   58
181  ggcaacagcagcagcggtggtaaaaacggtcagggcgaaccggcgcgtgtgcgttgcagc  240

59   H  L  L  V  K  H  S  Q  S  R  R  P  S  S  W  R  Q  E  K  I   78
241  catctgctggtgaaacatagccagagccgtcgtccgagcagctggcgtcaggaaaaaatt  300

79   T  R  T  K  E  E  A  L  E  L  I  N  G  Y  I  Q  K  I  K  S   98
301  acccgtaccaaagaagaagcgctggaactgattaacggctatattcagaaaattaaaagc  360

99   G  E  E  D  F  E  S  L  A  S  Q  F  S  D  C  S  S  A  K  A   118
361  ggcgaagaagatttgaaagcctggcgagccagtttagcgattgcagcagcgcgaaagcg  420

119  R  G  D  L  G  A  F  S  R  G  Q  M  Q  K  P  F  E  D  A  S   138
421  cgtggcgatctgggcgcgtttagccgtggccagatgcagaaaccgtttgaagatgcgagc  480

139  F  A  L  R  T  G  E  M  S  G  P  V  F  T  D  S  G  I  H  I   158
481  tttgcgctgcgtaccggcgaaatgagcggcccggtgtttaccgatagcggcattcatatt  540

159  I  L  R  T  E  *  L  E
541  attctgcgtaccgaataactcgag  564
```

Protein Sequence as Expressed:

```
MGSSHHHHHH SSGLVPRGSH GMADEEKLPP GWEKRMSRSS GRVYYFNHIT
NASQWERPSG NSSSGGKNGQ GEPARVRCSH LLVKHSQSRR PSSWRQEKIT
RTKEEALELI NGYIQKIKSG EEDFESLASQ FSDCSSAKAR GDLGAFSRGQ
MQKPFEDASF ALRTGEMSGP VFTDSGIHII LRTE
```

Quick Facts:

```
Number of amino acids: 184
Molecular weight:      20463.65    (20332.46 without first Met)
Theoretical pI:        9.20
Ext. coefficient:      20970 (reduced cysteine)

Atomic composition:
Carbon      C        878
Hydrogen    H       1371
Nitrogen    N        277
Oxygen      O        277
Sulfur      S          7
```

After Cleavage:

```
Number of amino acids: 167
Molecular weight:      18581.60
Theoretical pI:        8.96
Ext. coefficient:      20970 (reduced)

Atomic composition:
Carbon      C        799
Hydrogen    H       1255
Nitrogen    N        245
Oxygen      O        256
Sulfur      S          6
```

CT Domain (PPI) Complete Sequence:

```
     H  M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R /G  S
1    catatgggcagcagccatcatcatcatcatcatagcagcggcctggtgccgcgtggcagc  60
     H  G  M  E  P  A  R  V  R  C  S  H  L  L  V  K  H  S  Q  S
61   cacggcatggaaccggcgcgtgtgcgttgcagccatctgctggtgaaacatagccagagc  120
     R  R  P  S  S  W  R  Q  E  K  I  T  R  T  K  E  E  A  L  E
121  cgtcgtccgagcagctggcgtcaggaaaaaattacccgtaccaaagaagaagcgctggaa  180
     L  I  N  G  Y  I  Q  K  I  K  S  G  E  E  D  F  E  S  L  A
181  ctgattaacggctatattcagaaaattaaaagcggcgaagaagatttgaaagcctggcg  240
     S  Q  F  S  D  C  S  S  A  K  A  R  G  D  L  G  A  F  S  R
241  agccagtttagcgattgcagcagcgcgaaagcgcgtggcgatctgggcgcgtttagccgt  300
     G  Q  M  Q  K  P  F  E  D  A  S  F  A  L  R  T  G  E  M  S
301  ggccagatgcagaaaccgtttgaagatgcgagctttgcgctgcgtaccggcgaaatgagc  360
     G  P  V  F  T  D  S  G  I  H  I  I  L  R  T  E  *  L  E
361  ggcccggtgtttaccgatagcggcattcatattattctgcgtaccgaataactcgag  417
```

Protein Sequence as Expressed:

```
MGSSHHHHHH SSGLVPRGSH GMEPARVRCS HLLVKHSQSR RPSSWRQEKI
TRTKEEALEL INGYIQKIKS GEEDFESLAS QFSDCSSAKA RGDLGAFSRG
QMQKPFEDAS FALRTGEMSG PVFTDSGIHI ILRTE
```

Before Thrombin Cleavage:

```
Number of amino acids: 135
Molecular weight:      15039.81    (20332.46 without first Met)
Theoretical pI:        8.69
Ext. coefficient:      6990 (reduced cysteine)

Atomic composition:
Carbon      C        646
Hydrogen    H       1021
Nitrogen    N        203
Oxygen      O        201
Sulfur      S          6
```

After Cleavage:

```
Number of amino acids: 118
Molecular weight:      13157.76
Theoretical pI:        8.02
Ext. coefficient:      6990 (reduced)

Atomic composition:
Carbon      C        567
Hydrogen    H        905
Nitrogen    N        171
Oxygen      O        180
Sulfur      S          5
```

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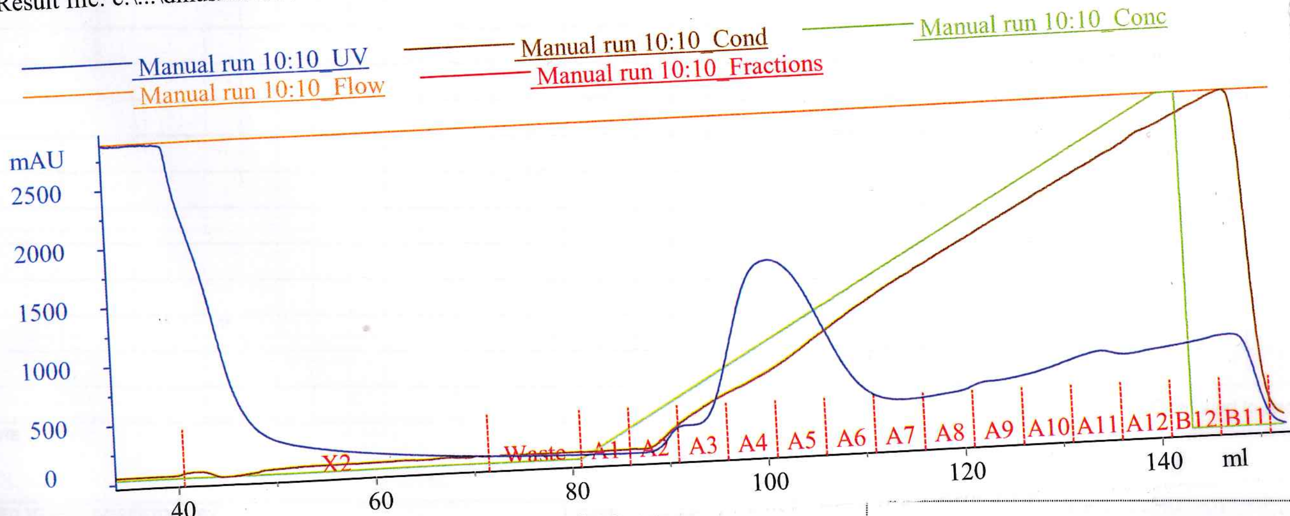
Add ~40 mL lysis buffer to each pellet (+ 0.5 mg/mL lysozyme). Vortex ~~[illegible]~~ and shake for 10'
to resuspend pellet in each conical tube. Now rocking on ice for another 10'. Sonicate 6' total, 2' pulse, 1' rest at power 6. Kept on ice during sonication, though felt warm.
Spun at 19,000 rpm in JA 25.50 for 45'. Took sample for gel.

Column turned black w/ 2 mM DTT. Running 4 mL min⁻¹, ~~new~~ Histrap from Dinusha. Starting w/ CTD.

$60\ mL \cdot \frac{min}{4\ mL} = 15$ min gradient from 0-100% B.

Collecting 5 mL fractions

Result for CTD is below. I will pool A3-A6.



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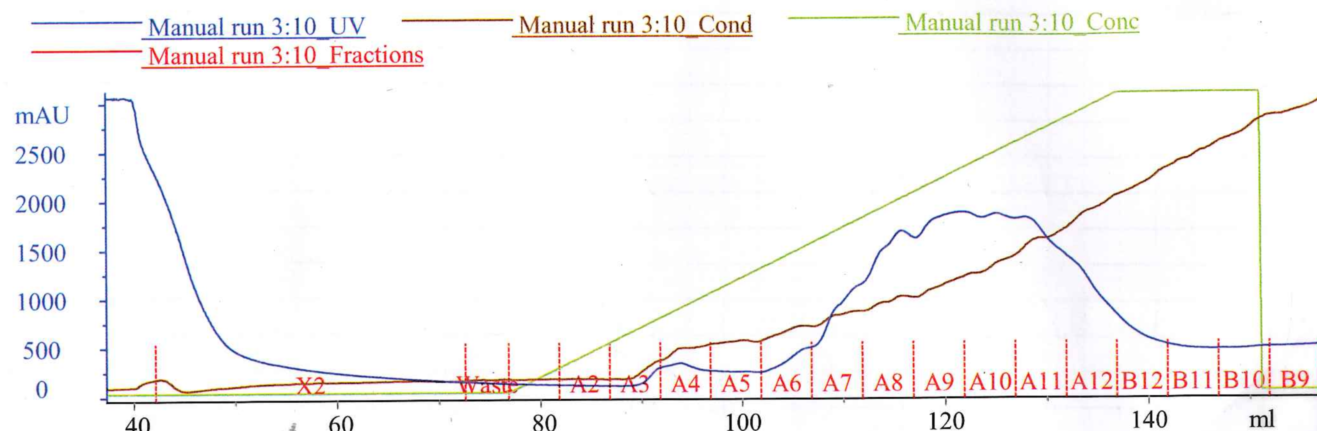
Running WT, same settings, different column. Shape of elution is odd. Bad column? Overloaded? I will pool A6-B12.



Need dialysis buffer. Will use 2L of

150 mM NaCl		60 mL
20 mM HEPES	→ pH 7.5 ~~adj pH to 6.5~~	40 mL
2 mM DTT		0.62 g

WT Pin1 pooled sample (1mm = l) A_{280} = 0.373 = 177 μM

CTD " " " " A_{280} = 0.277 = 396 μM

~~177 μM~~

$\frac{177\times10^{-6}\,mol}{L} \cdot \frac{20463\,g}{mol} \times \frac{1000\,mg}{g} \times 0.04\,L = 144\,mg$ WT Pin1

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$396\times10^{-6} \cdot \frac{15039\,g}{mol} \cdot \frac{1000\,mg}{g} \cdot 0.02\,L = 120\,mg$ CTD Pin1

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Prep recommends 1U/mg. Stock is 40 U/μL. 3.6 μL for WT Pin1 and 3 μL for CTD ~~Stir~~ Stirred O/N @ 4degC in emerson fridge (4:30 pm)

Ran 2 gels, one for each prep.

WT

M / Induced / Lysed / Supernatant / FT / Wash / Pool / Blank / M

CTD

M / Induced / Lysed / Supernatant / FT / Wash / Pool / M

Gels are asymmetric, so I should be able to tell them apart easily.

5/2/17

Removed from dialysis (looked good) and put in fridge @ 8:30 am.

~~Added 1 mL of~~ Took 2x1mL of benz. beads and added dialysis buffer to equilibrate. Rocked samples w/ beads ~5 min. Spinning down. 4k rcf for 5 min. Will filter in 0.45 μm filter.

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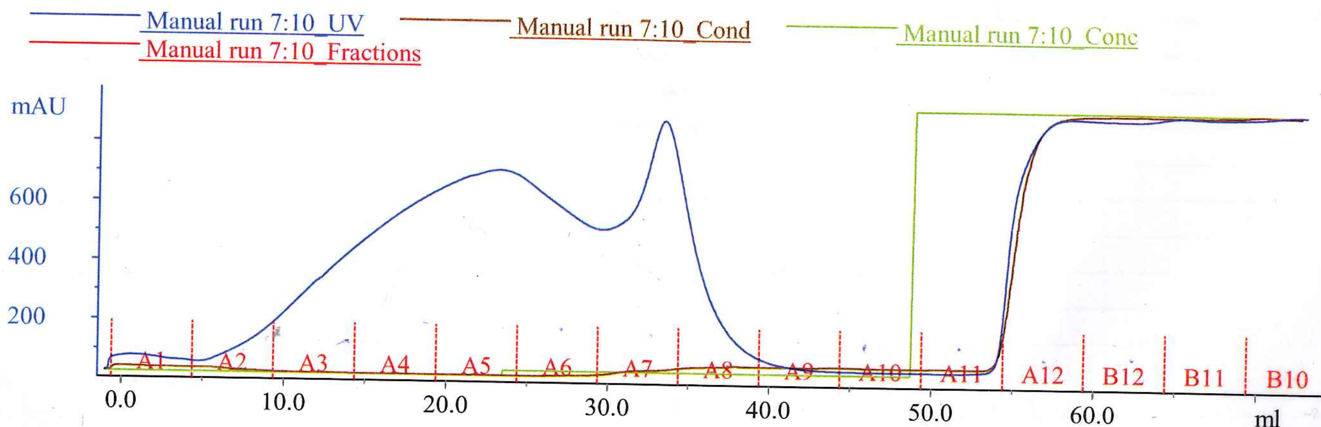
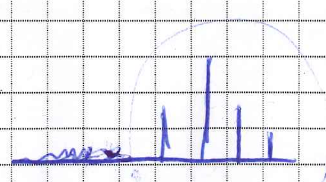
Eluting CTD through column. Running 5 mL/min, after load switch to 2% B to help w/ non-specific binding. That led to a 2nd peak, which I'll keep before pooling. Will pool A2-A9, run A8 separately (but still pool it)



No more NiNTA - I will add 10 mM DTT to pooled protein to protect it during conc, gel filt. (400 μL)

Same thing w/ WT. I will set aside some ~~A12~~ ~~A11~~ A12 B11 fraction & run separately. Will pool A2-~~B12~~. More volume here, will still add 10 mM DTT (600 μL)



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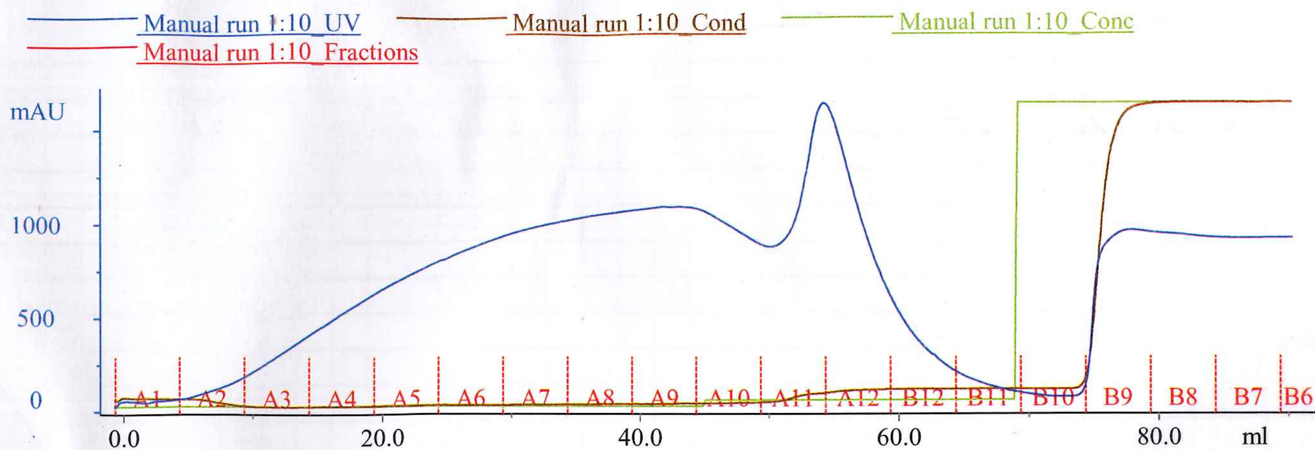

Concentrations

			volume	C (μM)	C mg/mL	μmol	mg
CTD	1mm	0.103	40mL	147	1.93	5.88	77
WT Pin1 #1 (A2-A7)	1mm	0.147	30 mL	70	1.30	2.1	40
WT Pin1 #2 (A8-B11	1mm	0.227	35 mL	108	2.00	3.78	70

$$\frac{x\ \mu mol}{L} \cdot \frac{1\ mol}{10^6\ \mu mol} \cdot \frac{M\ g}{mol} \cdot \frac{1\ mg}{0.001\ g} \cdot \frac{1\ L}{1000\ mL} = \frac{x \cdot M}{10^6} \frac{mg}{mL}$$

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Need to make gel filtration buffer. Given application (activity) I will use ~~a~~ the following

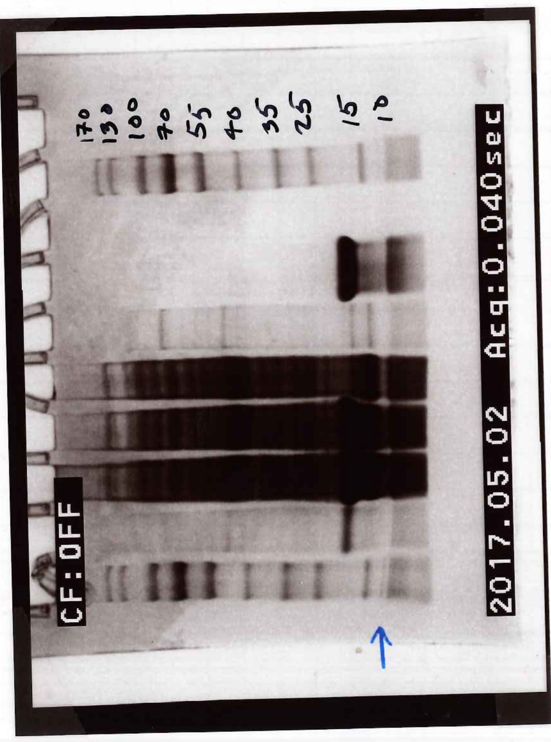
Total Vol: 1.5 L

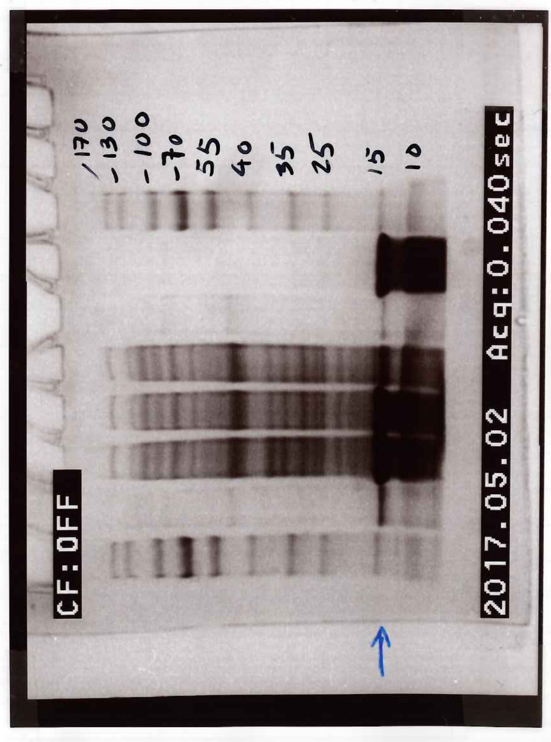
	Stock	Vol
50 mM NaCl	5 M	15 mL
10 mM Imidazole pH 6.5	3.5 M	4.3 mL
1 mM TCEP	0.5 M	3 mL
~~0.02 % (w/v) NaN_3~~	—	~~0.3 g~~

~~Forgot~~ will add later.

Gels from yesterday are below. See p. 46 for lanes.

WT Pin1 CTD Pin1





Note strong 15 kDa band in both WT ~~[illegible]~~ and CTD growths while the FT looks like CTD lost protein, the presence in WT means that it's not likely.

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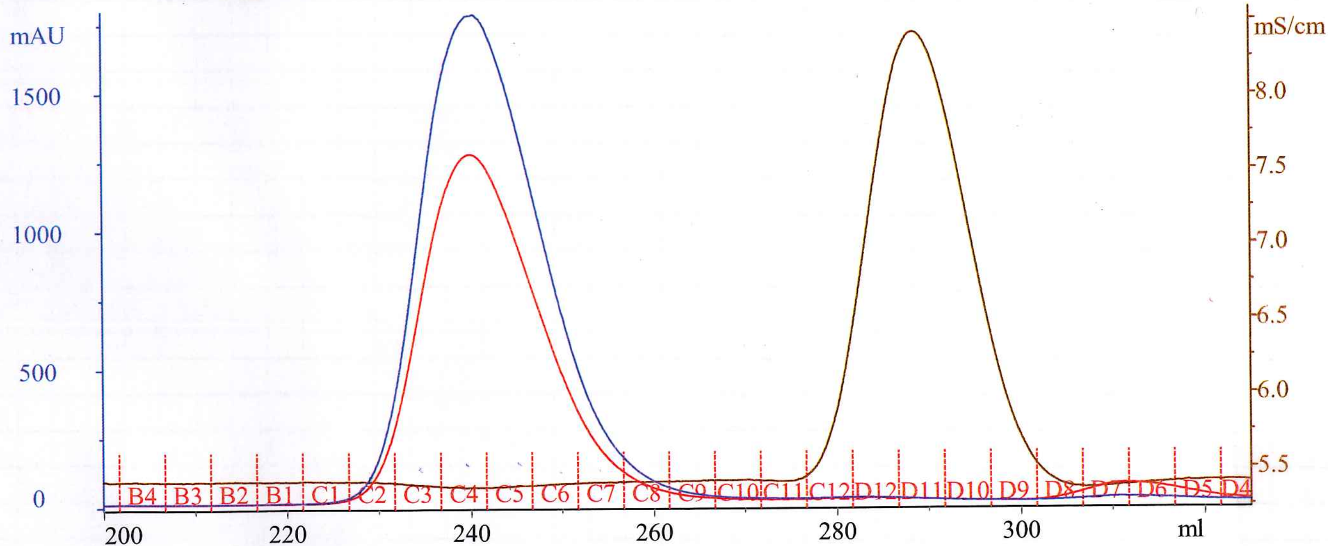
Ran concentration of CTD sample - 0.632 for 1mm. TKatis'
Volume is approx 5 mL. Conc is 904 μM.

Conc of WT is approx since UV is so high. Nanodrop registers 1.997 @ 1mm, but I think that's probably not reliable. As a working value, the c is approx. 952 μM
@ A 1:5 dilution (2:10 μL) gives A=0.453, so it's close.



I will pool C2-C8. Adding NaN_3 to 0.02%.

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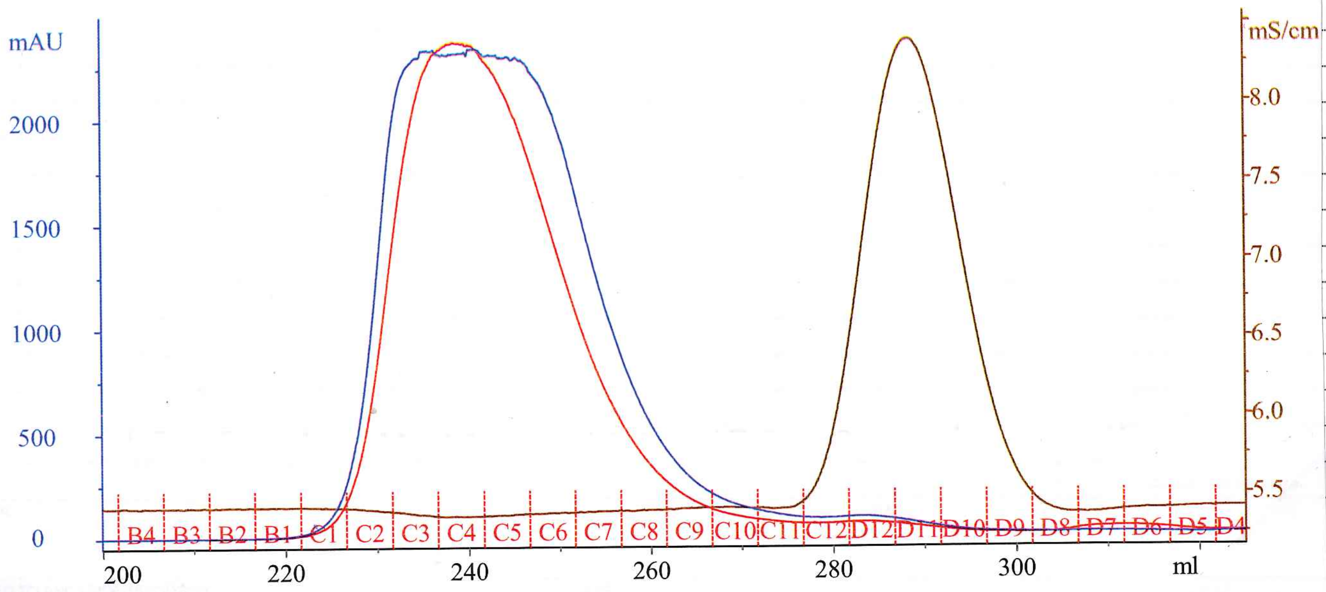
$10\mu L \frac{(35 \times 0.02)}{10\%} = 70\,\mu L$ of 10% NaN_3

A_{280} of CTD is 0.093 (1mm). Conc is 133 μM (V = 35 mL)

The retention time is much smaller than I would have predicted from our calibration. 13.5 kDa should appear at ~200 mL based on that. Will need to look into this, but gel will confirm size tomorrow.

5/3/2017.

Gel of FL WT Pin1 is below. Definitely something fishy w/ the column. Peak is nearly identical.



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I will fractions C1-C10. Adding 100 µL of 10% NaN_3.
A_{280} is 0.198 @ 1mm. Conc is 94 µM, w a volume of ~50 mL.

5/4/17 Mass Spec In Curie Lab

Basic Protocol

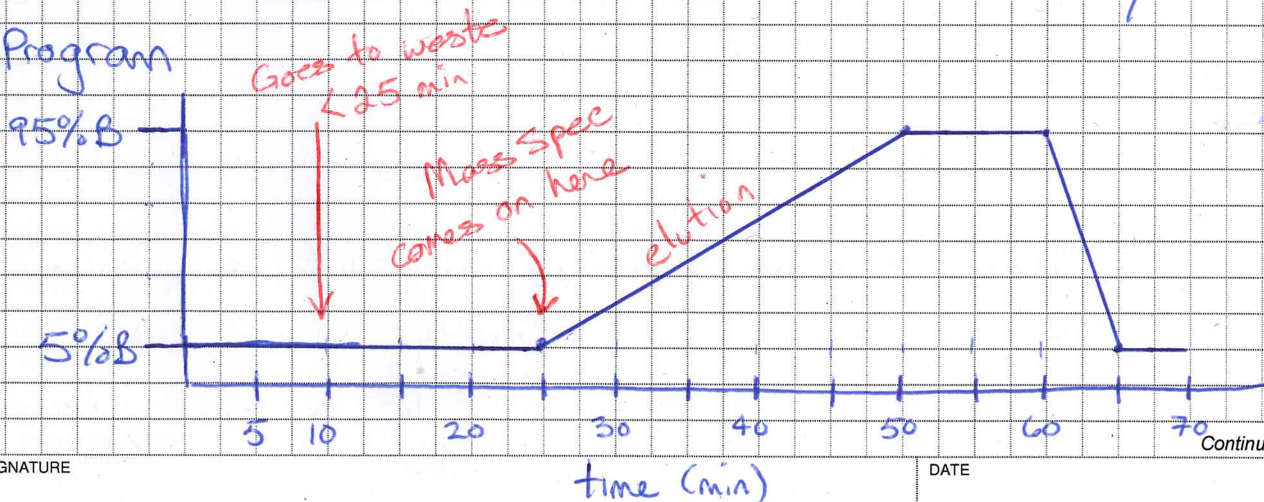
Buffer A - 5% Acetic Acid in H_2O
Buffer B - Acetonitrile

Column: We've been using a ResTek ~~10~~ 15 cm C18 column.
At NIH, they were using:
1) Zorbax 300SB-C3 4.6x150 (PN 883995-909), or
2) Poroshell 300SB-C3 2.1x75 (PN 660750-909) (newer)

Temp = 40°C

The C18 column probably doesnt resolve proteins as well as a C3 would, but our results have been okay.

Program



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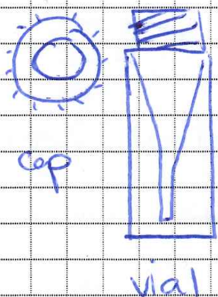
Eric Anderson recommends ~0.2 mL/min but we've had good results w/ ~~75~~ 0.5 mL/min. Pressure is ~100 bar (typically a bit less, goes down as Acetonitrile ↑)

Note that when the column is 1st ~~ava~~ connected it should probably be run through a blank (inject buffer) or equilibrated for ~30 min. The blank can also help determine if anything is stuck on the column.

UV-Vis should be set to monitor 280 nm if proteins have Trp. 205 will catch proteins, too but it will catch buffers and small organics as well.

Vials are Sun-Sri (PN 200410). Make sure there are no air bubbles when loading vials. I typically load > 30 μL into the vial and give them a good shake to eliminate air bubbles. These vials have a tapered insert and are disposable.

You may be able to ~~too~~ load less than 30 μL, I haven't tried.



These settings are generally ~~s~~ saved in the fitzkee protein method on the CPU desktop.

BUT you should **always** check everything to make sure.

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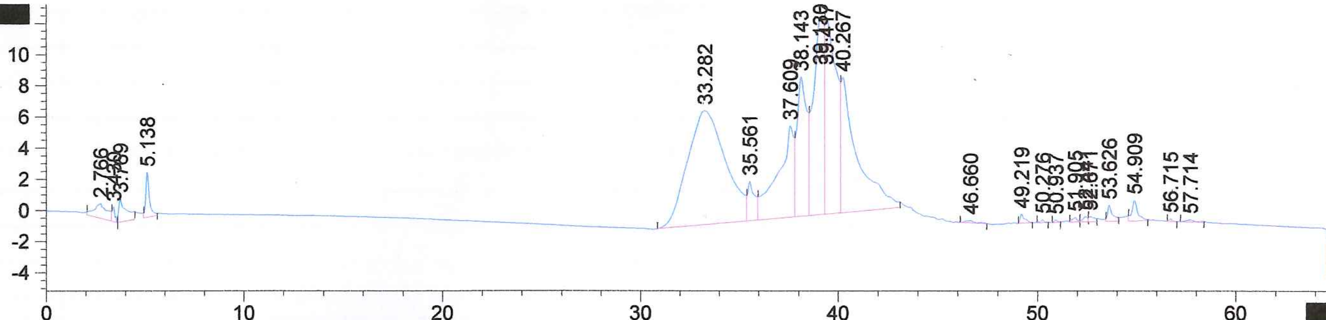
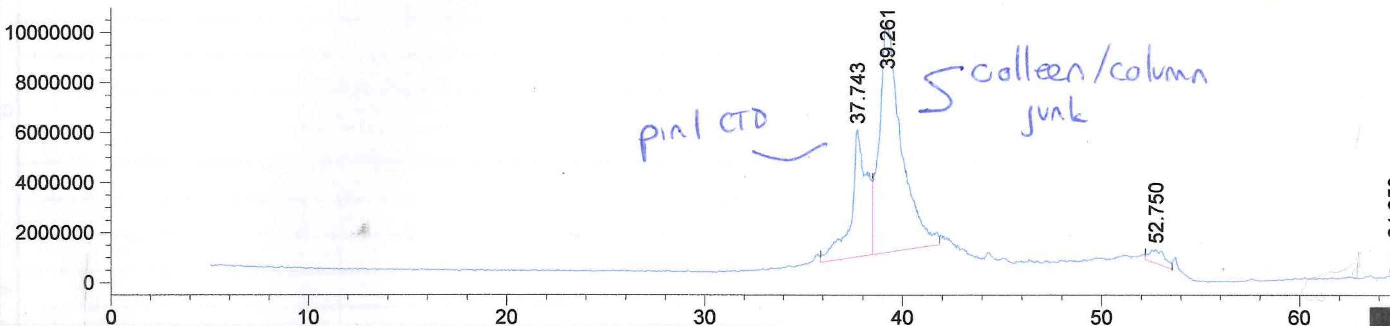
LCMS Data for Pin1 CTD:

I Injected 5 μL of sample (approx ~133 μM). This is plenty.

(modified after loading)





The column had contamination peaks near ~39 min. These diminished as we ran the column more, but and were present in the blank. The MS for these points was high MW but very messy, and I wonder if Colleen's polymers had contaminated the LC.

I intended to run 2 μL, and I believe that would have been enough. I recommend 1 μL when conc is ~100 μM, as one can see contamination of CTD in the pin1 sample I ran next. (see p. 56)

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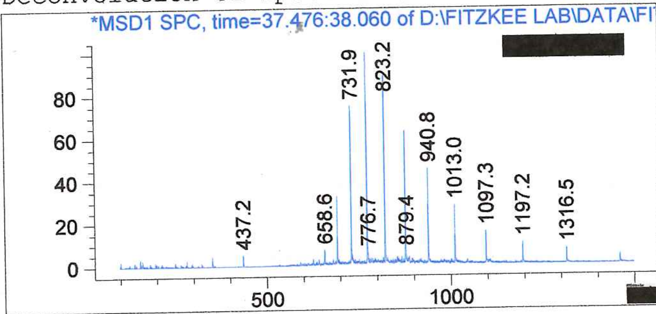
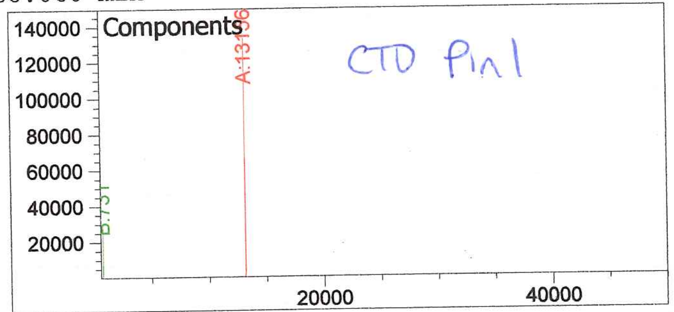
The deconvolution is below. Expected mass is on page 43 and is 13157.8

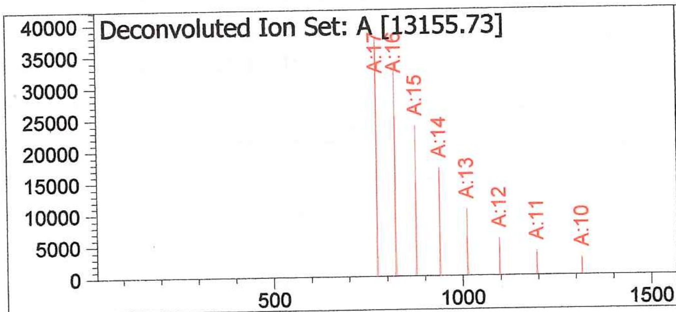
Deconvolution Parameters

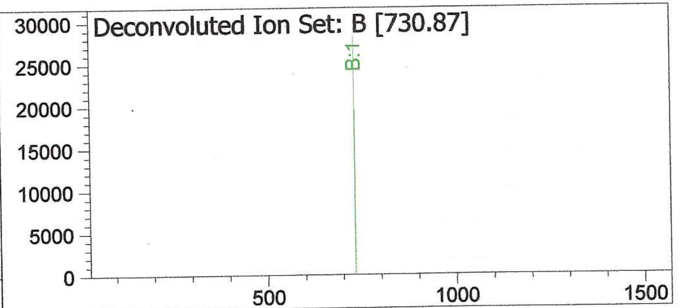
```
Adduct Ion(Positive): +H, 1.0079 Da
Adduct Ion(Negative): -H, -1.0079 Da
Low MW:               500
DeconvStartChgMaximum Charge:      50
Minimum Peaks in Set: 3
Retain Residual:      No
Ion PWHH:             0.6 Da
MW Agreement:         0.05 %
Noise Cutoff:         1000 counts
Abundance Cutoff:     10 %
MW Assign:            Curve fit
MW Assign Cutoff:     40 %
Envelope Cutoff:      50 %
```

Deconvolution of Spectrum # 1 @ 37.476 - 38.060 min









Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	13155.73	133703	100.00
B	730.87	28281	21.15

Instrument 1 5/9/2017 6:37:20 PM Min

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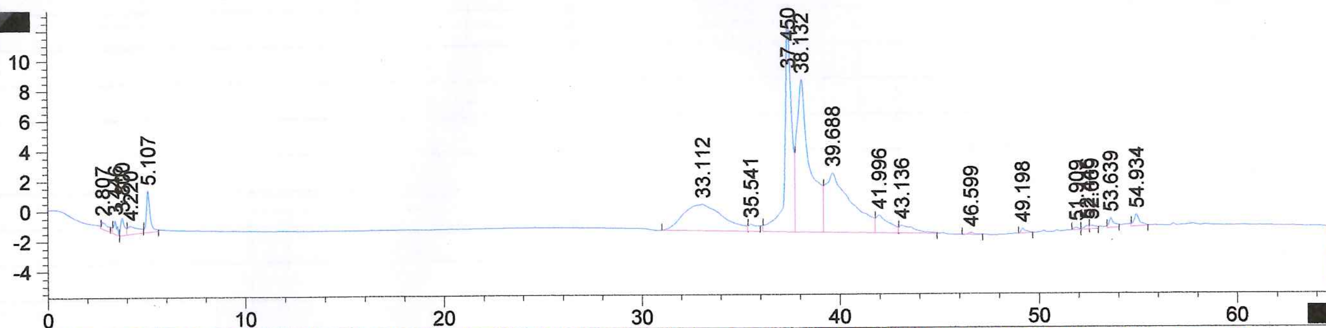
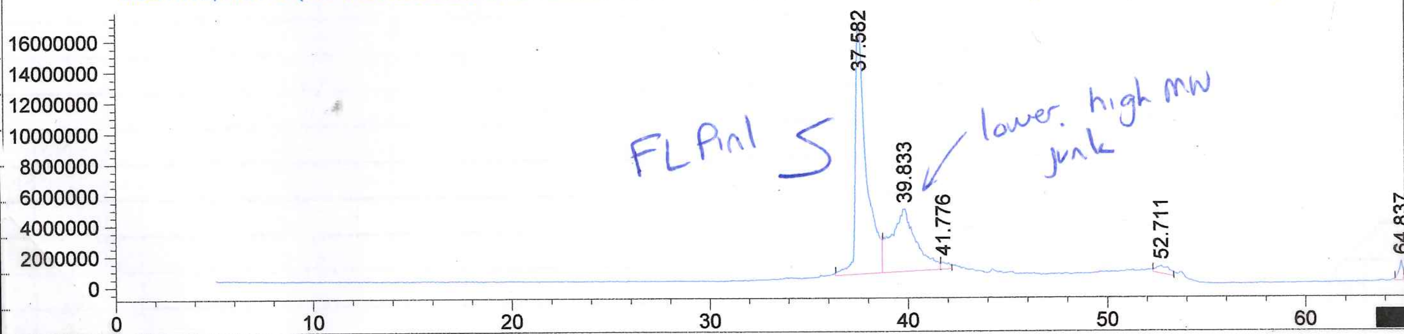
FL Pin1 construct! LCMS. Again, report says I injected 5 μL, 1 would have been enough. Note the 2nd set of peaks at 39 min is much less.

(modified after loading)





Note similarity between CTD and FL Pin1 (FL 37.6 min vs. CTD 37.7). This would be better on a C3 column I'm sure, as C3 would improve resolution.

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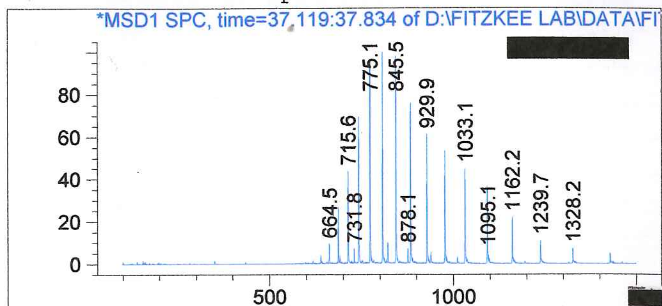
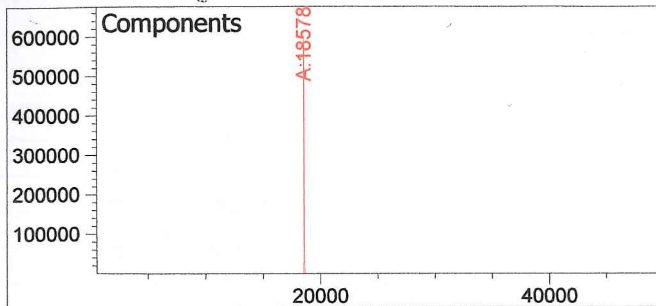
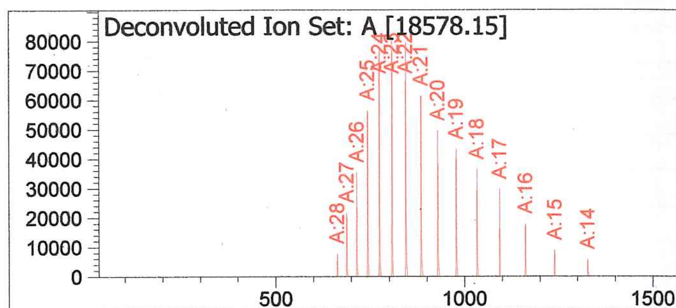
Deconvolution of Spectrum # 1 @ 37.119 - 37.834 min







(FL Pin1)

Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	18578.15	604016	100.00

*** End of Report ***

Instrument 1 5/9/2017 6:38:45 PM Min

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Deconvolution parameters identical to what was done before. Expected MW is 18581.6. Both MW are slightly less than expected (2-3 Da). ~~But~~ The MS may need calibration, but I'm not ~~so~~ concerned – the error is small enough that it ~~can't be~~ is most likely not meaningful.

Continued to page

SIGNATURE | DATE

DISCLOSED TO AND UNDERSTOOD BY | DATE

PROPRIETARY INFORMATION