SUPPORTING INFORMATION

Clostridium difficile ClpP Homologs are Capable of Uncoupled Activity and Exhibit Different Levels of Susceptibility to Acyldepsipeptide Modulation

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Table S1. qRTPCR Primers. Integrated DNA Technologies (IDT) PrimerQuest tool was used to pick primer pairs for *clpP1* and *clpP2*. *rpoB*, the gene that encodes the β-subunit of bacterial RNA polymerase, was used as the reference gene.

Primer ID	**Primer Sequence (5'-3')**
rpoB_F	AACTAGGGCCAGAGGAAATAAC
rpoB_R	CTGAGTCTACTTCTGCACCTATTC
clpP1_F	GCTGAAGACCCAGACAAAGATA
clpP1_R	CTCCCATAGAAGCAGCCATAC
clpP2_F	GGCTCTGCTACATCAGGATTTG
clpP2_R	CATGTGTTCCTCCTGCAAGTAA

Table S2. Ct counts for qRT-PCR

	clpP1	***clpP2***	***rpoB***
Exp	14.80 ± 0.02	20.88 ± 0.02	16.81 ± 0.00
Stat	17.34 ± 0.01	22.68 ± 0.02	19.89 ± 0.02

```
A0A125YDM9_CLODI MSLVPHVIEQTGQGERSYDIYSKLLKDRIIFIGEEINDAIASLVVAQLLFLESEDPDSDI 60
U3V0G5_CLODI     MALVPVVVEQTGRGERSYDIFSRLLKDRIIFLGDQVNDATAGLIVAQLLFLEAEDPDKDI 60
                 *:*** *:****:*******:*:********:*:::*** *.*:********:****.**

A0A125YDM9_CLODI IIYINSPGGSATSGFAIYDTMNYVRCDVSTICIGMAASMSAFLLAGGTHGKRCALPNSEI 120
U3V0G5_CLODI     HLYINSPGGSITSGMAIYDTMQYIKPDVSTICIGMAASMGAFLLAAGAKGKRLALPNSEI 120
                  :******** ***:******:*:: ************.*****.*::*** *******

A0A125YDM9_CLODI MIHQPMGGAKGQATDVKIAVDNILKIKERLDKILSENTGKSIEDIRRDTDRDNFMTALEA 180
U3V0G5_CLODI     MIHQPLGGAQGQATDIEIHAKRILKIKETLNEILSERTGQPLEKIKMDTERDNFMSALEA 180
                 *****:***:*****::* ...****** *::****.**: :*.*: **:*****:****

A0A125YDM9_CLODI KEYGLIDYIMNRNE 194
U3V0G5_CLODI     KEYGLIDEVFTKRP 194
                 ******* ::.:.
```

Figure S1. HR-ESIMS peptide digest and sequencing results. Combined sequence coverage of ClpP1 (bottom) and ClpP2 (top) prepared from Δ*Ec*ClpP. Coverage obtained following data merging after trypsin and chymotrypsin digest. Bold underline: identified sequence; green: amino acids differing between the two isoforms.

ClpP1 Δ*Ec*ClpP

Date:	3/7/2018 5:06:44 PM	**Temperature:**	4°C	**Data Filter:**	Default
Solvent:	Glycerol	**Solute:**	-	**Laser:**	100 %
Intensity:	286,121 counts/s	**Intercept:**	0.878	**Attenuator:**	51 %
Z - Av. Diameter:	15.87nm	**Std. Deviation:**	2.55nm	**MW model:**	Globular Proteins
Polydispersity:	16.05 %	**Pd. Index:**	0.026	**Remark:**	-

Peak #	Mean Dh (nm)	Mode Dh (nm)	Std. Dev. (nm)	Polydisp. (%)	Est. MW. (kDa)	Intensity (%)	Mass (%)	Volume (%)	Number (%)
1	12.23	13.49	3.36	27.50	319.05	87.84	99.99	100.00	100.00
2	202.86	223.97	55.84	27.53	Out of Range	12.16	0.01	0.00	0.00

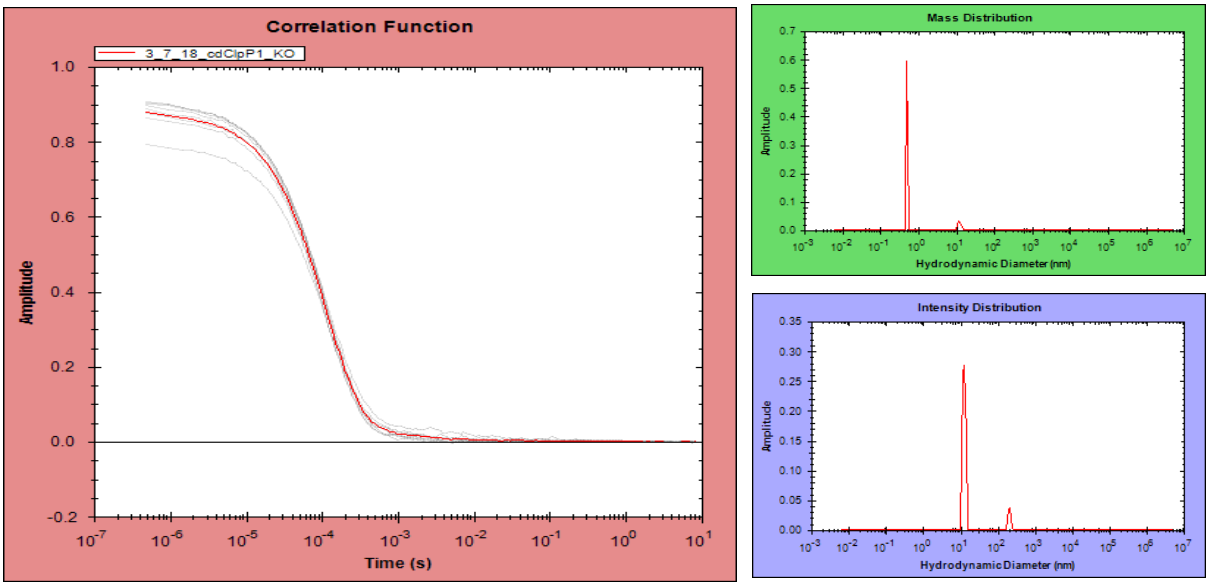


Figure S2. pUNK DLS results for ClpP1 prepared from Δ*Ec*ClpP cells.

ClpP2 Δ*Ec*ClpP

Date:	3/7/2018 5:02:24 PM	**Temperature:**	4.02°C	**Data Filter:**	Default
Solvent:	Glycerol	**Solute:**	-	**Laser:**	100 %
Intensity:	293,245 counts/s	**Intercept:**	0.923	**Attenuator:**	57 %
Z - Av. Diameter:	14.78nm	**Std. Deviation:**	5.24nm	**MW model:**	Globular Proteins
Polydispersity:	35.50 %	**Pd. Index:**	0.126	**Remark:**	-

Peak #	Mean Dh (nm)	Mode Dh (nm)	Std. Dev. (nm)	Polydisp. (%)	Est. MW. (kDa)	Intensity (%)	Mass (%)	Volume (%)	Number (%)
1	12.87	13.50	2.47	19.21	319.58	86.96	99.97	99.99	100.00
2	152.42	162.57	29.05	19.06	Out of Range	13.04	0.03	0.01	0.00

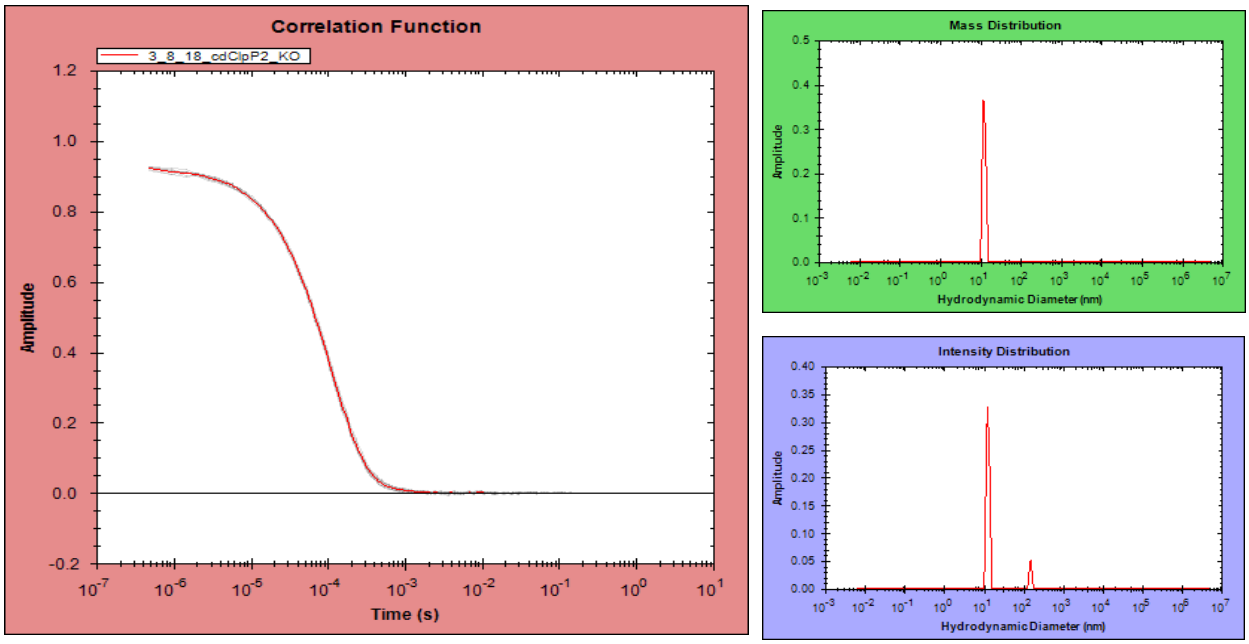


Figure S3. pUNK DLS results for ClpP2 prepared from Δ*Ec*ClpP cells.

Sequence **inputSeq** Length **196** residues

Cysteines in this sequence: **3**

Disulfide Connectivity prediction

Step 1: Running PSI-BLAST with input sequence; click here to see the output

Step 2: Predicting secondary structure using PSIPRED; click here to see the output

Step 3: Disulfide Oxidation State Prediction; click here to see the results

Warning! The number of predicted half-*cystines* is lower than 2.

Step 4: Disulfide Bonds Prediction using a trained Neural Network

Disulfide bond scores

Cysteine sequence position	Distance	Bond	Score
86 - 92	6	MNYVRCDVSTI-DVSTICIGMAA	0.0104
86 - 113	27	MNYVRCDVSTI-THGKRCALPNS	0.47017
92 - 113	21	DVSTICIGMAA-THGKRCALPNS	0.99116

Figure S4. Predicted ClpP2 disulfides predicted by DiANNA.

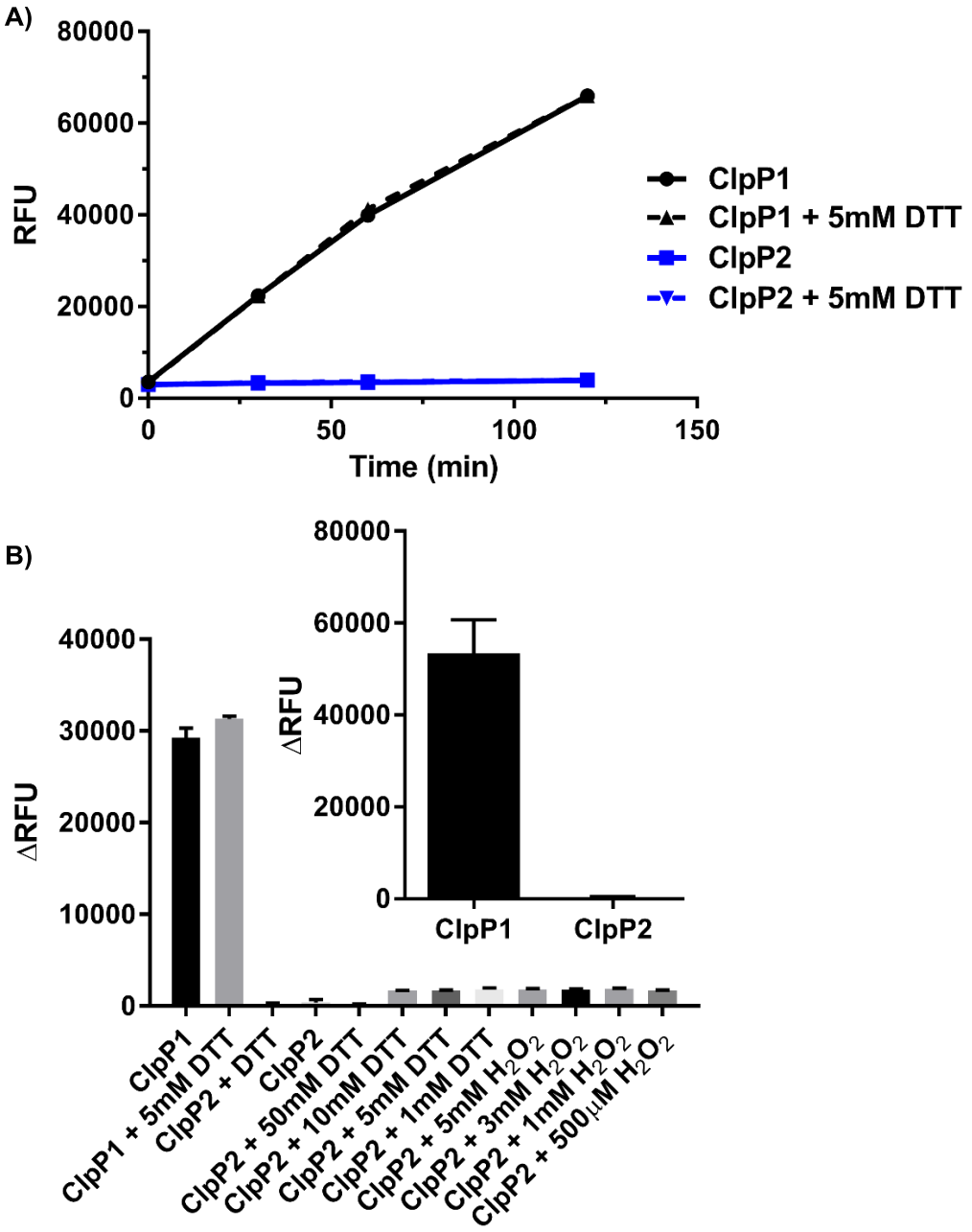
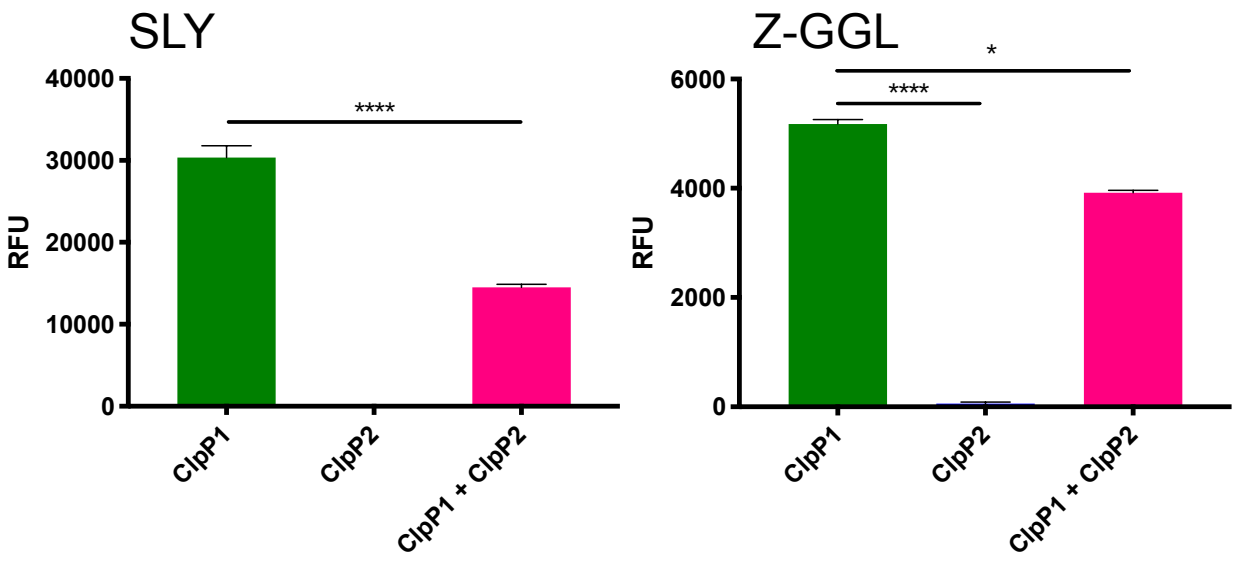


Figure S5. Reduction or oxidation of ClpP2 has no consequences on proteolytic activity. A) SLY-AMC peptidolytic assay over 2h, fluorescence measurements taken 30 m, 60 m, and 120 m with and without 5 mM DTT. B) SLY-AMC peptidolytic assay with reducing (DTT) and oxidizing (H_2O_2) conditions had no effect on proteolytic activity of ClpP2. Inset, Aliquots from GFP-ssrA degradation assays were diluted 10-fold into 100 µL activity buffer containing 5 mM DTT, and pipetted in triplicate into a black 96-well flat bottom plate. SLY-AMC was added, and the reaction was allowed to proceed for ~48h before reading.



Rate of Degradation (ΔRFU, min^-1)

	SLY	Rel. ClpP1	Z-GGL	Rel. ClpP1
ClpP1	1944	n/a	540.6	n/a
ClpP2	21.1	1.1	8.0	1.5
ClpP1 + ClpP2	899.8	46.3	306	56.6

Figure S6. Peptidolytic activity of mixed solutions of ClpP1 and ClpP2 compared to homogenous solutions.

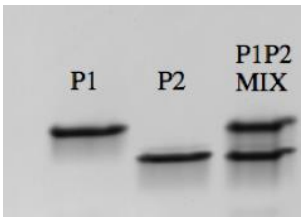
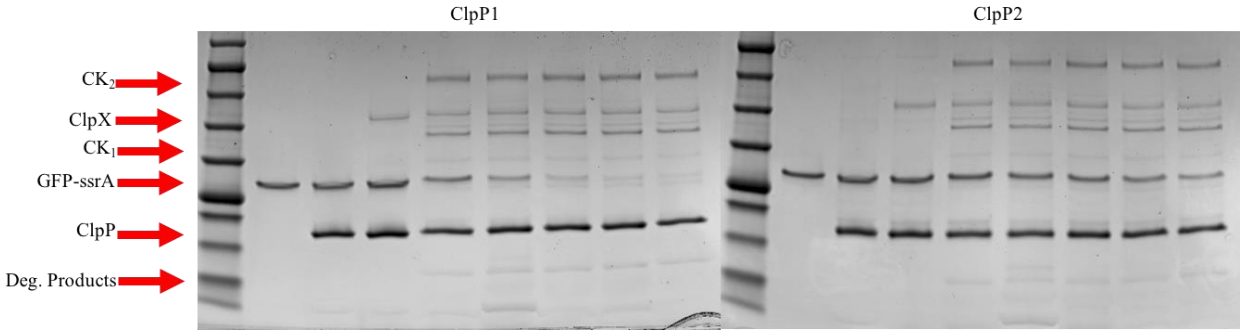


Figure S7. Non-denaturing PAGE gel (7-15%) of mixed ClpP1 and ClpP2 homotetradecamers after 2 h.



Lane	Contents
1	GoldBio BlueStain Protein Marker
2	ssrA-GFP
3	ssrA-GFP + ClpP
4	ssrA-GFP, ClpP, ClpX, no ATP 5 h
5	ssrA-GFP, ClpP, ClpX, + 4 mM ATP 1 h
6	ssrA-GFP, ClpP, ClpX, + 4 mM ATP 2 h
7	ssrA-GFP, ClpP, ClpX, + 4 mM ATP 3 h
8	ssrA-GFP, ClpP, ClpX, + 4 mM ATP 4 h
9	ssrA-GFP, ClpP, ClpX, + 4 mM ATP 5 h

Figure S8. SDS-PAGE visualization of ssrA-GFP degradation over time via ClpXP in the presence of 4 mM ATP.

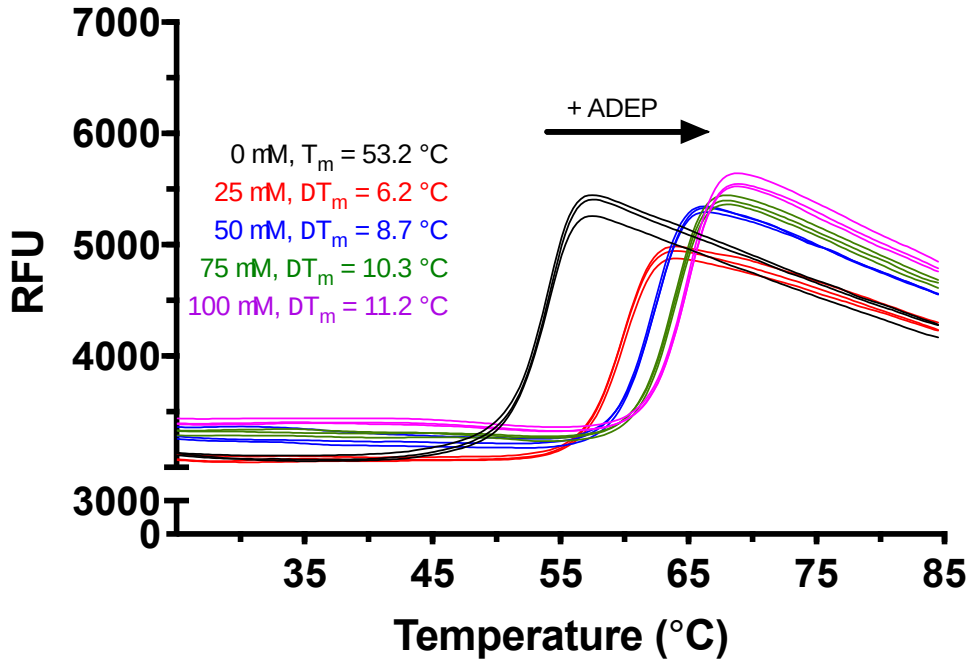


Figure S9. Raw data replicates of ClpP1 + ADEP thermalshift experiments.

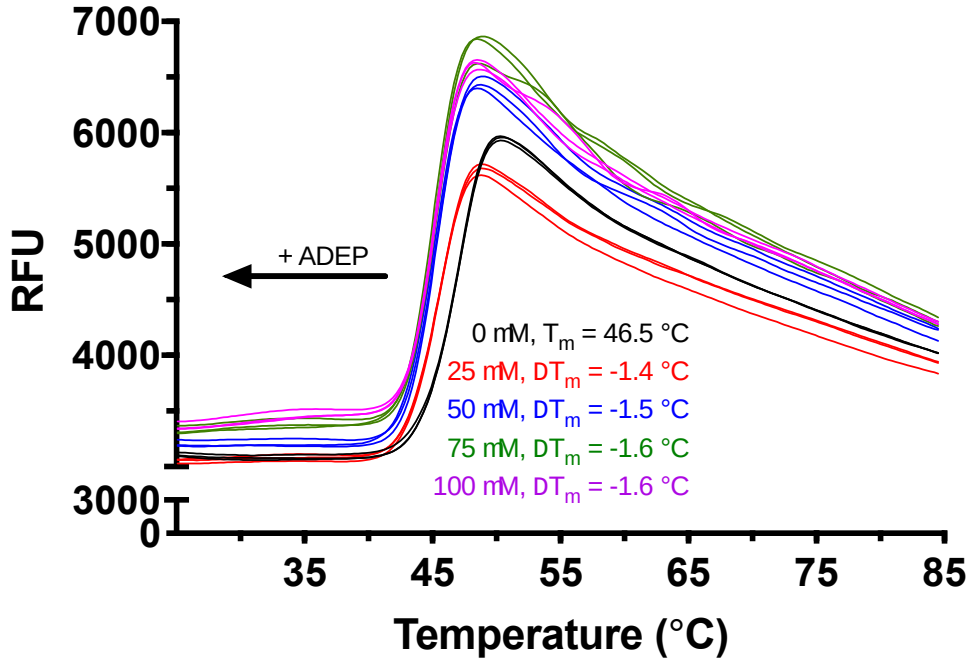


Figure S10. Raw data replicates of ClpP2 + ADEP thermal shift experiments.