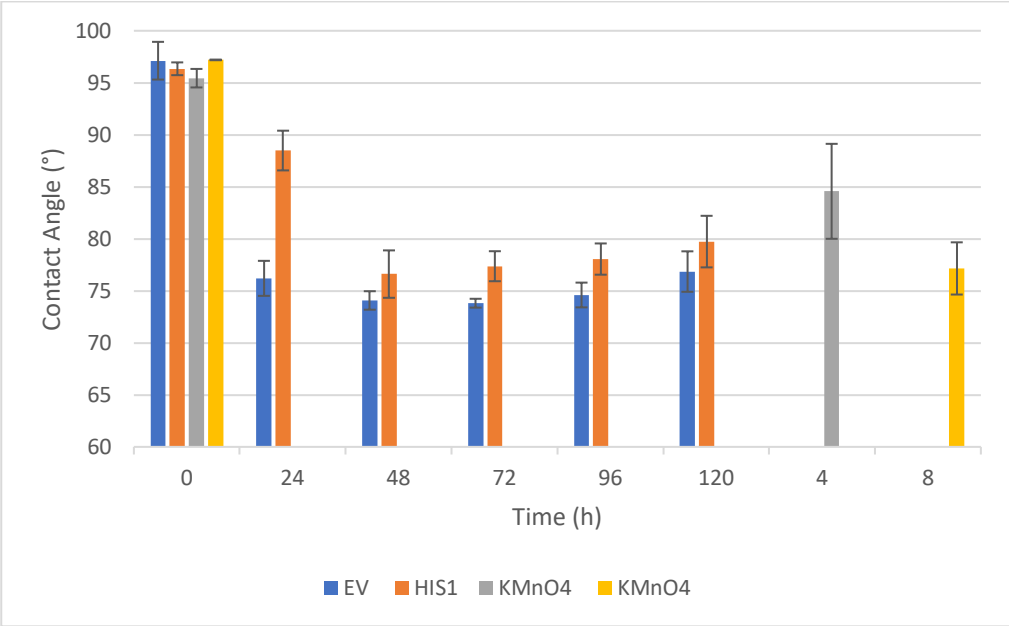
Development of a High-Throughput Assay for Directed Evolution of Polypropylene-Degrading Enzyme HIS1

Supplementary Materials

Surface Contact Angle

Contact angle measurements showed distinct outcomes for chemical (KMnO4) vs biological (EV + HIS1) treatments (Fig. S1). EV and HIS1 treatments showed an initial decrease from original angle ~95° after 24h of treatment, though with EV angles showing a larger decrease. By 48h and beyond, EV and HIS1 treatments converged to similar contact angle values (~74-76°) that were consistent across the following 96h however HIS1 values were consistently higher than EV. $KMnO_4$ chemical oxidation demonstrated effective surface modification. The 8-hour treatment produced significantly lower contact angles compared to untreated controls (77.15± 2.05° vs 97.2 ± 0.02°, $p < 0.01$, paired t-test), representing a 20° decrease in contact angle. The 4-hour treatment showed intermediate values at 84.6 ± 3.73°, indicating time dependent oxidation effects.



Supplementary Figure 1. Surface Contact Angle Measurements over Time for Empty Vector (EV), HIS1 and KMnO4 Oxidation Treatments. Data represents means ± SD (n=3 plastic replicates per treatment, with 3 drops per plastic and 60 measurements per drop). Error bars denote the population Standard deviation. Separate plastics underwent the 4 hour and 8 hour KMnO4 treatments.

The higher contact angles observed on the HIS1-treated plastics indicate greater surface hydrophobicity compared to the EV-treated counterparts aross all time points. Since the only difference between the lysates was the presence of HIS1 (which was hypothesised to hydroxylate polypropylene and increase hydrophilicity) this result contradicts the initial hypothesis. This pattern suggests that HIS1 and EV treatment may deposit hydrophobic materials from the lysate onto the plastic surface, which ultrasonic cleaning failed to remove completely. This inference is supported by the KMnO4 positive control treatment which included no biological material and with thorough cleaning (via a concentrated HCl wash followed by ultrasonication) demonstrated the expected increase in hydrophilicity. The 20° decrease seen between the 0h and 8h time points closely align with the literature where a 28.6° difference was observed under the same conditions indicating 70% of the expected outcome was achieved (Fávaro et al., 2007)While the cleaning protocol could be optimised further, even trace amounts of deposited material are likely to significantly affect the angles measured. This elucidates surface contact's inability to distinguish between actual structural changes to the plastic and residual contamination which is a primary limitation when used in the context of enzymatic degradation.

TBO Assay in 96-well Plate

Supplementary Table 1.

TBO Absorbance (630 nm) Measurements in 96-Well Polypropylene Plates.

Time	0h	0h	0h	2h	2h	2h	4h	4h	4h	6h	6h	6h	8h	8h	8h
0:00:00	0.039	0.04	0.044	0.043	0.042	0.043	0.041	0.04	0.041	0.046	0.041	0.043	0.045	0.04	0.04
0:01:00	0.039	0.04	0.043	0.043	0.042	0.042	0.041	0.04	0.041	0.046	0.042	0.043	0.044	0.04	0.04
0:02:00	0.039	0.04	0.043	0.043	0.042	0.043	0.041	0.04	0.041	0.046	0.042	0.043	0.043	0.04	0.04
0:03:00	0.039	0.04	0.042	0.043	0.042	0.043	0.041	0.04	0.041	0.046	0.042	0.043	0.042	0.04	0.04
0:04:00	0.039	0.04	0.041	0.043	0.042	0.043	0.041	0.04	0.041	0.046	0.042	0.043	0.042	0.041	0.04
0:05:00	0.039	0.04	0.04	0.043	0.042	0.043	0.041	0.04	0.041	0.046	0.042	0.043	0.042	0.04	0.04

Columns denote the length of KMnO4 treatment. Absorbance taken every minute for 5 minutes by plate reader. n=3.

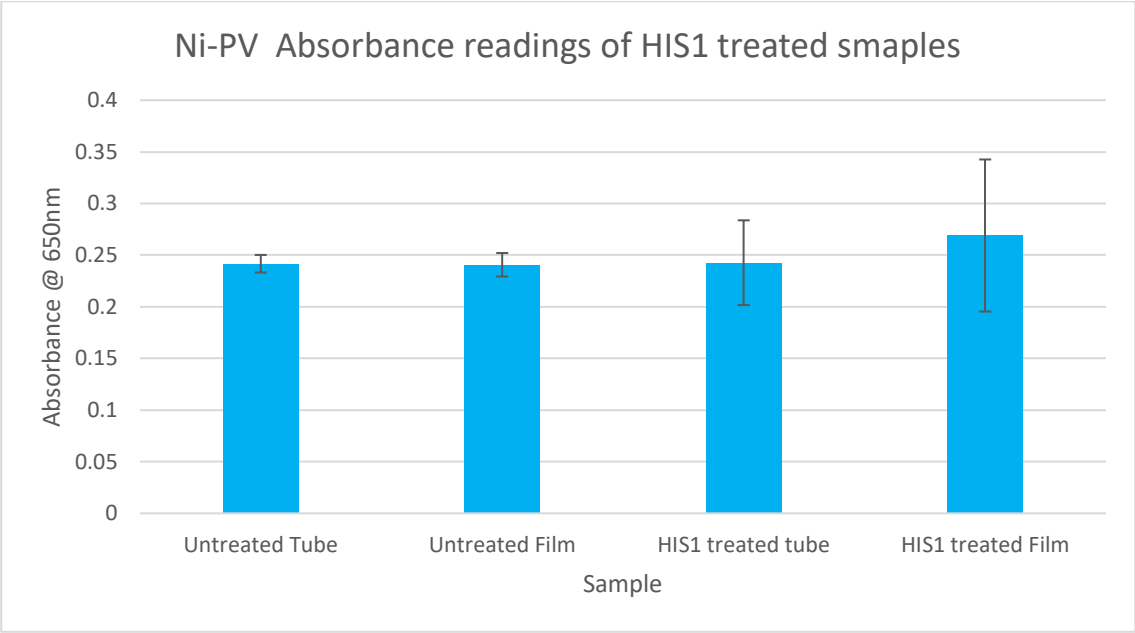
Nickel Pyrocatechol Violet Colorimetric Assay in 96 Well Plate

Supplementary Table 2.

96-well plate absorbance readings on the Nickle-Pyrocatechol violet colorimetric assay detecting surface carboxy groups on PP.

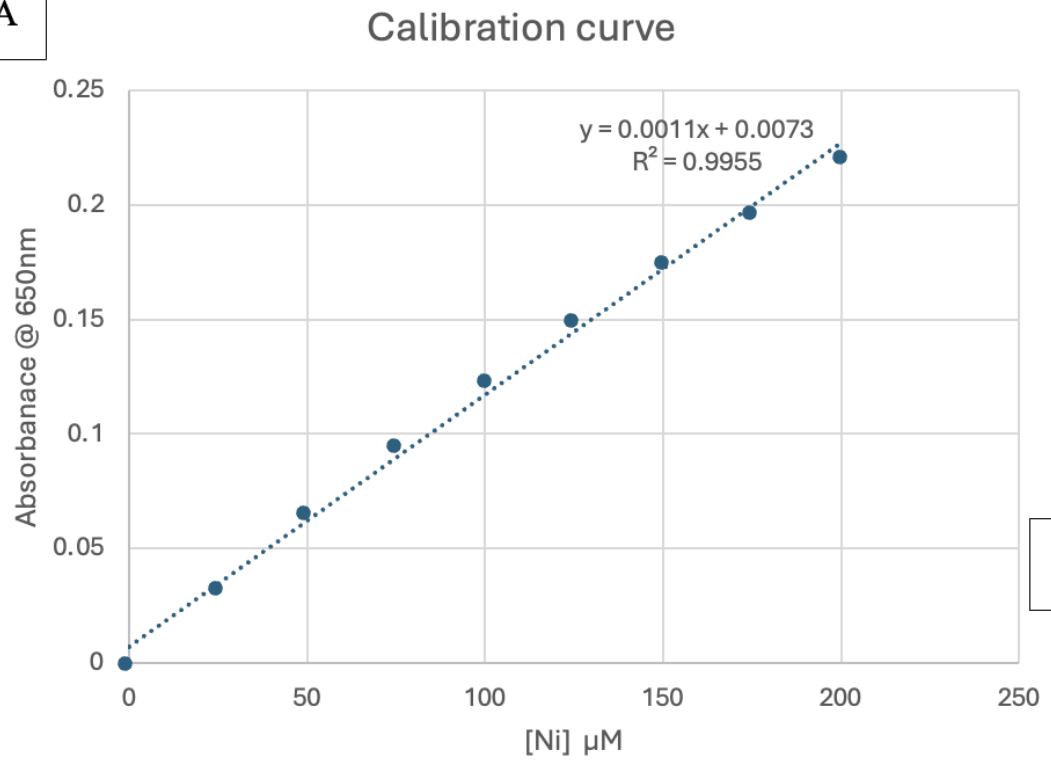
Time	T° 650	2hr	2hr	2hr	4hr	4hr	4hr	6hr	6hr	6hr	8hr	8hr	8hr	NC	NC	NC
00:00:00	20.8	0.294	0.292	0.288	0.307	0.272	0.31	0.268	0.303	0.306	0.3	0.308	0.288	0.21	0.312	0.34
00:01:00	20.8	0.294	0.296	0.285	0.307	0.278	0.308	0.272	0.3	0.309	0.302	0.305	0.287	0.212	0.311	0.344
00:02:00	20.8	0.296	0.301	0.284	0.307	0.281	0.307	0.277	0.3	0.306	0.303	0.303	0.287	0.211	0.31	0.338
00:03:00	20.9	0.295	0.304	0.282	0.307	0.282	0.306	0.275	0.299	0.304	0.301	0.302	0.286	0.211	0.309	0.333
00:04:00	20.9	0.294	0.299	0.279	0.304	0.282	0.304	0.271	0.298	0.302	0.298	0.3	0.285	0.211	0.307	0.329
00:05:00	20.9	0.292	0.295	0.277	0.302	0.281	0.302	0.268	0.296	0.3	0.296	0.298	0.283	0.21	0.305	0.326
00:06:00	21	0.289	0.293	0.275	0.3	0.28	0.3	0.266	0.295	0.298	0.294	0.297	0.281	0.21	0.303	0.324
00:07:00	21	0.286	0.291	0.274	0.297	0.278	0.298	0.265	0.293	0.296	0.293	0.295	0.28	0.21	0.301	0.321
00:08:00	21	0.284	0.289	0.272	0.295	0.277	0.295	0.264	0.291	0.294	0.291	0.293	0.278	0.209	0.299	0.319
00:09:00	21	0.282	0.287	0.27	0.293	0.276	0.293	0.262	0.29	0.292	0.289	0.292	0.276	0.209	0.297	0.317
00:10:00	21.1	0.28	0.285	0.269	0.291	0.274	0.291	0.261	0.288	0.29	0.287	0.29	0.274	0.208	0.295	0.314

NC: negative control. Columns denote the length of $KMnO_4$ treatment. Absorbance taken every minute for 5 minutes by plate reader. n=3.



Supplementary Figure 2. Mean absorbance values of the Ni-PV assay applied to HIS1 treated samples. Error bars denote standard deviation. HIS1 treated tube: 1.5ml microtube treated with the lysate of HIS1 expressing *E. coli,* Mean absorbance = 0.243 ± 0.0410, n=3. HIS1 treated Film: 5mg of PP film treated with the lysate of HIS1 expressing *E. coli*, Mean absorbance = 0.269 ± 0.0736, n=3. Untreated Tube: negative control 1.5ml microtube, Mean absorbance = 0.241 ± 0.00850, n=3. Untreated Film: negative control 5mg of PP film, Mean absorbance = 0.241 ± 0.0114, n=3.

A



B

Calibration curve: concentrations used and absorbance readings.

[Ni] µM	[PV] µM	Absorbance @ 650nm
200	40	0.221
175	40	0.196
150	40	0.175
125	40	0.149
100	40	0.123
75	40	0.095
50	40	0.065
25	40	0.032
0	40	0

C

Mean absorbance readings of the negative control (maximum absorbance) and standard deviation, for calculations of assay sensitivity.

Blank (negative control) absorbance readings Mean (n=9)	standard deviation
0.24	0.004555217

Supplementary figure 3. **(A)** Calibration curve for the Ni^{2+}-PV colorimetric assay**.** Absorbance at 650nm plotted against Ni^{2+} concentration (0-200 µM) with 40 µM PV. Linear regression gave the equation y = 0.0011x + 0.0073 (R^2 = 0.995). Data points represent single measurements without replicates (Data found in **Table B**). **Table C)** Mean absorbance of the negative control (200 µM Ni^{2+} with 40 µM PV), n=9, and the standard deviation, these were calculated to then decipher the analytical LOD.

1) LOD and LOQ using negative control noise (analytical sensitivity)

Standard definitions used:

$$m = gradient\ of\ calibration\ curve,\ s_{blank} = SD\ of\ Blank\ (negative\ control)\ absorbance\ readings$$

$$LOD_{[Ni]} = \frac{3.3 \times 0.00456}{0.0011} = 13.7\ \mu M$$

$$LOQ_{[Ni]} = \frac{10 \times 0.00456}{0.0011} = 41.4 \mu M$$

2) Converting concentration LOD into the amount of Ni (in incubation)

$$LOD_{\mu mol\ Ni} = 13.7\ \mu M \times 6.00 \times 10^{-4} L = 0.00820\ \mu mol\ Ni$$

$$LOQ_{\mu mol\ Ni} = 41.4 \mu M \times 6.00 \times 10^{-4} L = 0.0248\ \mu mol\ Ni$$

3) Converting Ni amount into carboxyl groups (using n = 2.65 carboxyl/Ni)

$$\mu mol\ carboxyl = n \times \mu mol\ Ni$$

$$LOD_{\mu mol\ carboxyl} = 2.65 \times 0.00820 = 0.0217\ \mu mol\ carboxyl\ (total)$$

$$LOQ_{\mu mol\ carboxyl} = 2.65 \times 0.0248 = 0.0658\ \mu mol\ carboxyl\ (total)$$

4) Per-area estimate

$$Estimated\ area\ of\ eppendorf\ treated\ with\ KMnO_4 \approx 1.979 cm^2$$

$$LOD_{\mu mol \cdot cm^{-1}} = \frac{0.0217\ \mu mol\ of\ carboxyl}{1.98\ cm^2} = 0.0110\ \mu mol \cdot cm^{-2}$$

$$LOQ_{\mu mol \cdot cm^{-1}} = \frac{0.0658\ \mu mol\ of\ carboxyl}{1.98\ cm^2} = 0.0333\ \mu mol \cdot cm^{-2}$$

5) Practical detection limit using $KMnO_4$ PP microtube sample variability

SD = 0.0266

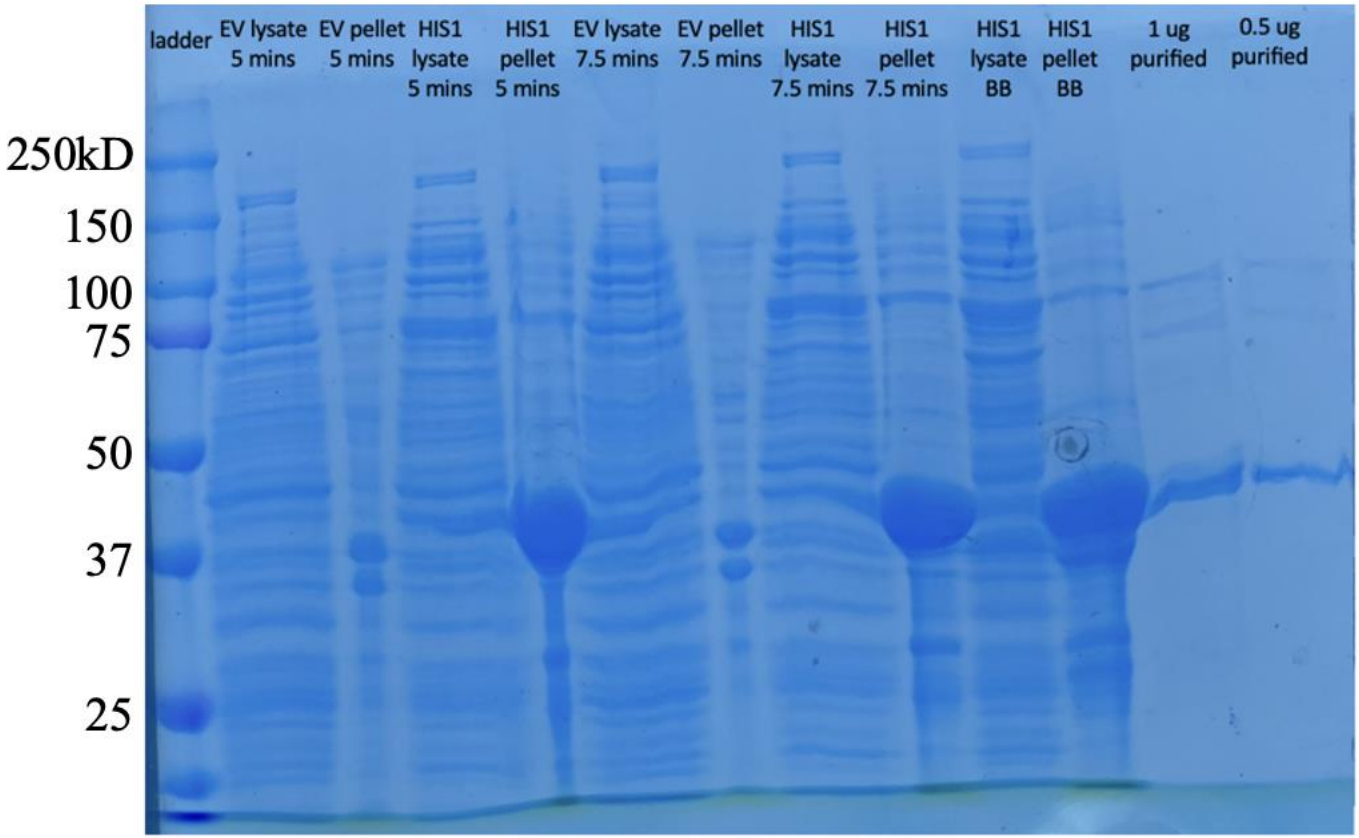
$$LOD_{[Ni]} = \frac{3.3 \times 0.0266}{0.0011} = 79.9\mu M$$

$$LOD_{\mu mol\ carboxyl,\ sample} \approx 0.127 \mu mol\ total$$

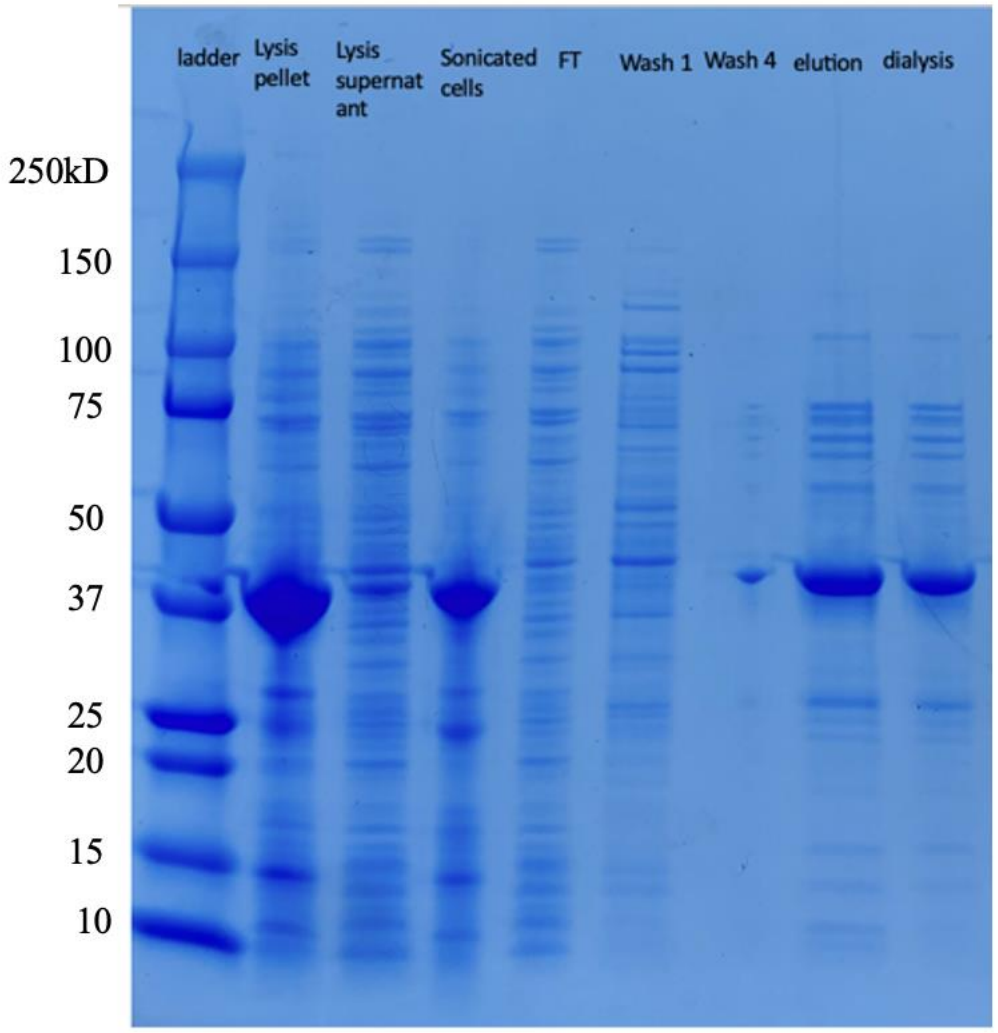
$$LOD_{\mu mol.cm^{-2}} \approx\ 0.0641 \mu mol \cdot cm^{-2}$$

Supplementary Figure 4: Calculating the sensitivity of the Ni^{2+}-PV assay using data from Fig. S3

Purification

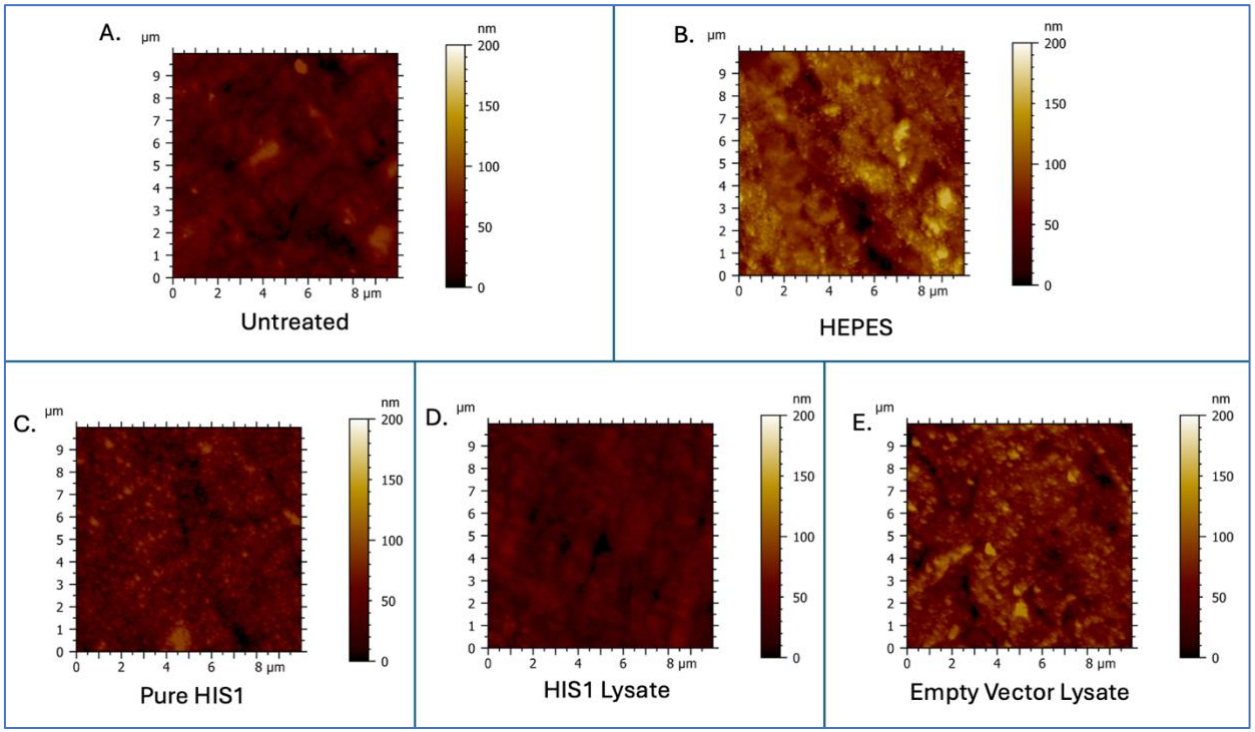


Supplementary Figure 4. SDS-PAGE gel testing methods of lysis by comparing the pellet and supernatant of each sample. (HIS1: 42kDa). EV = Empty Vector. BB = Bugbuster. 5/7.5 mins denotes the length of sonication. HIS1 pellet = insoluble fraction. HIS1 lysate = soluble fraction.



Supplementary Figure 5. SDS-PAGE of purification of HIS1 (his-tagged) from supernatant of the cell lysate using a Ni-IMAC column*. Dialysis: the purified sample after buffer exchange using a viva spin column.*

AFM



Supplementary Figure 6. 10 µm AFM Scans. Images of the scans provided by the microscope are compiled by Dr. Laura Charlton. The scale to the right of each scan indicates the height of each coloured area displayed in the picture, giving an overview of terrain in the 10 µm x 10 µm scan area. **(A)** 'Untreated' is the negative control sample of just the PP film. **(B)**'HEPES' is the PP film treated in HEPES buffer. **(C)** 'Pure HIS1' is the PP film treated with the purified HIS1 enzyme. **(D)** 'HIS1 Lysate' is the PP film treated with the HIS1 lysate. **(E)** 'Empty Vector Lysate' is the PP film treated with the (EV) lysate

Supplementary Table 3.
Table of Root Mean Square (RMS) Roughness Values on 10 µm Scans

Treatment Type:	Untreated	HEPES	Pure His1	HIS1 Lysate	EV Lysate
#1 RMS	12.16772	20.8295309	27.3887735	17.7024421	11.09201558
#2 RMS	16.4923105	18.7979344	24.0668666	20.7892183	17.77275388
#3 RMS	15.401059	26.7417713	26.437474	16.0446716	14.93862178

Average of RMS	14.6870298	22.1230789	25.9643713	18.1787773	14.60113041

Supplementary Table 4.
Table of P-value of Unpaired T-Test on Root Mean Square (RMS) Roughness Values on 10 µm Scans

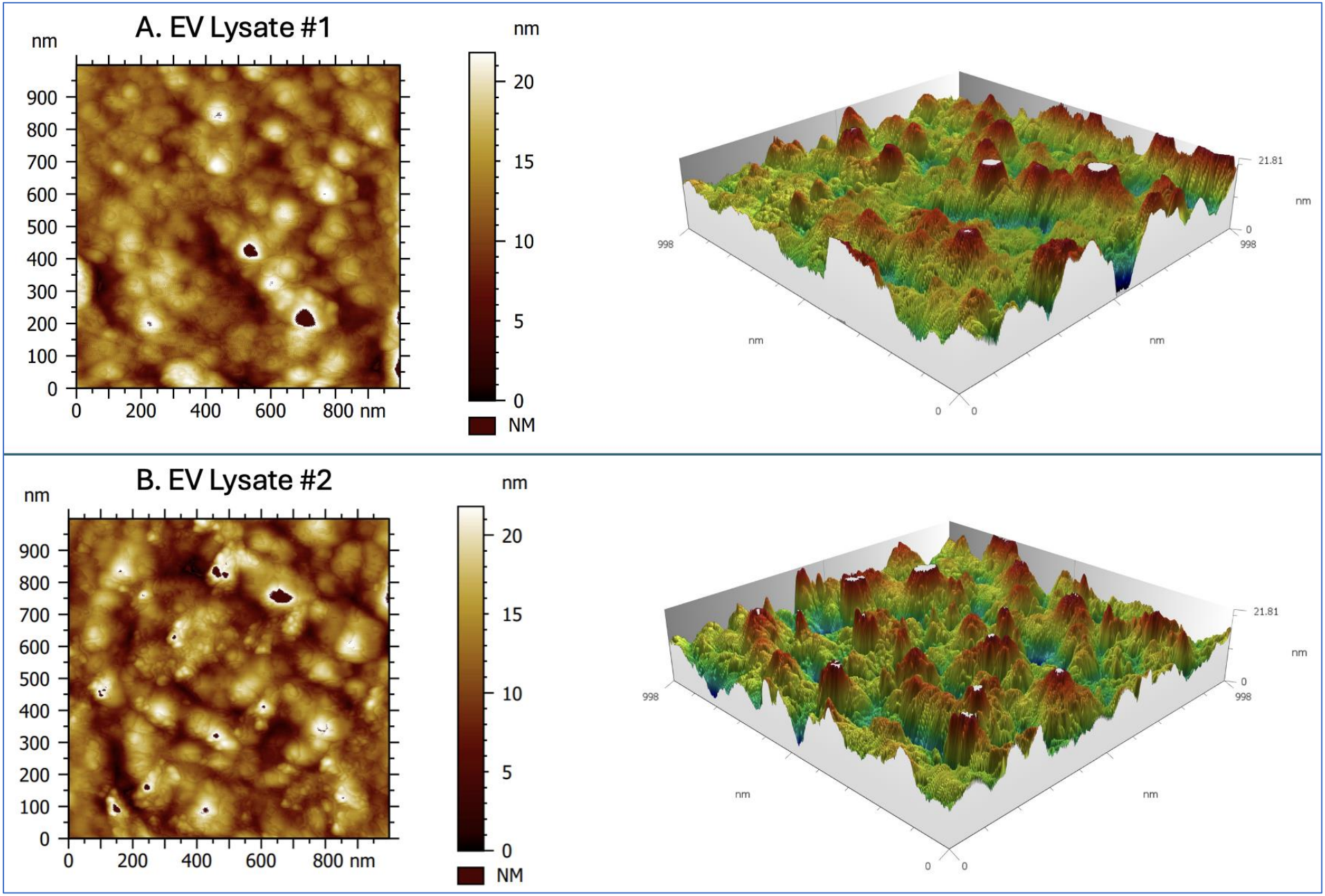
Test Samples	p-value
Untreated vs. Pure HIS1	**0.00294907**
HEPES vs. Untreated	0.0689704
HIS1 Lysate vs. Untreated	0.14064842
EV Lysate vs. Untreated	0.97261015
EV Lysate vs. HIS1 Lysate	0.21477407

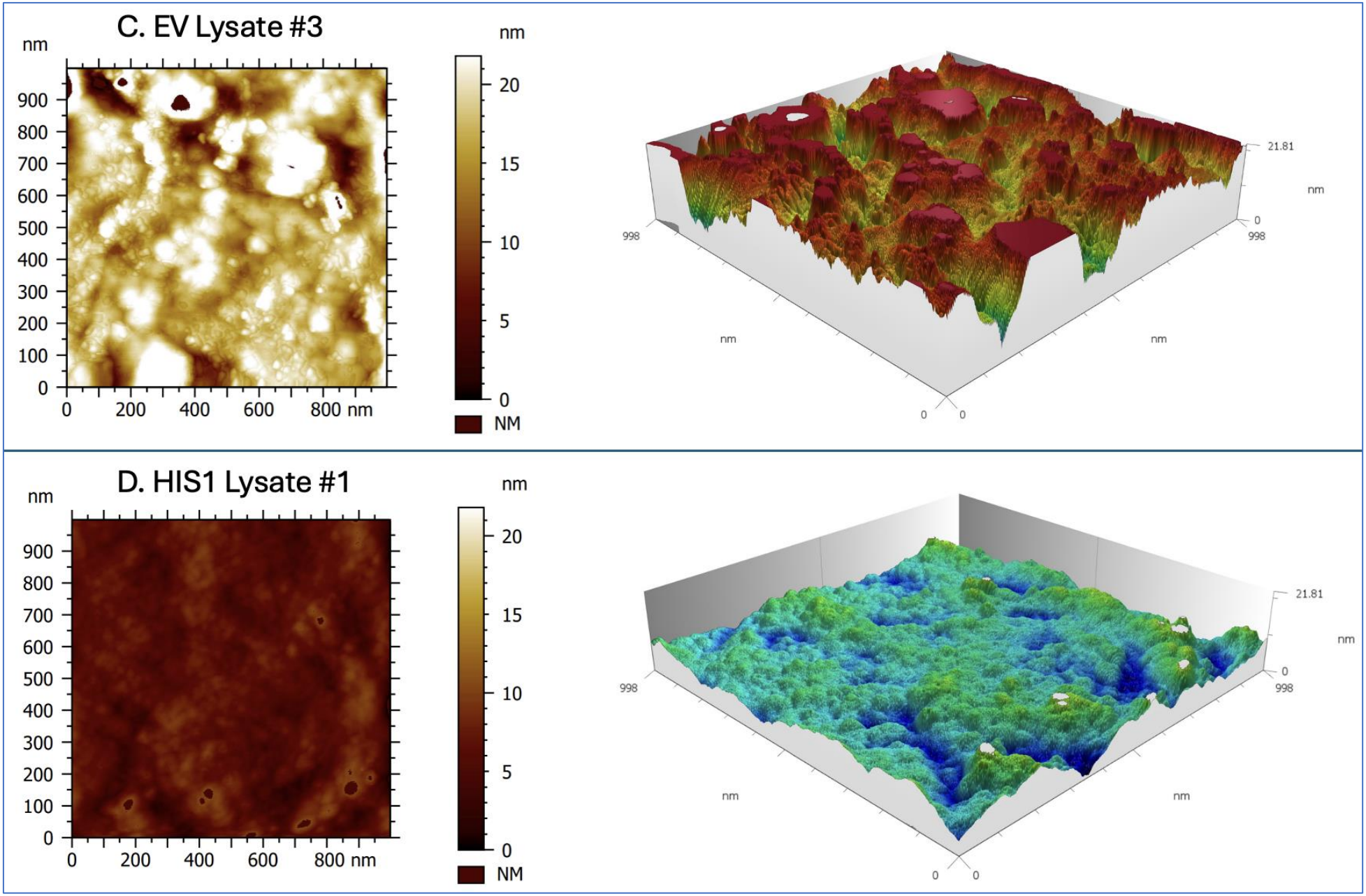
Supplementary Table 5.
Table of Root Mean Square (RMS) Roughness Values of 1 µm Scans

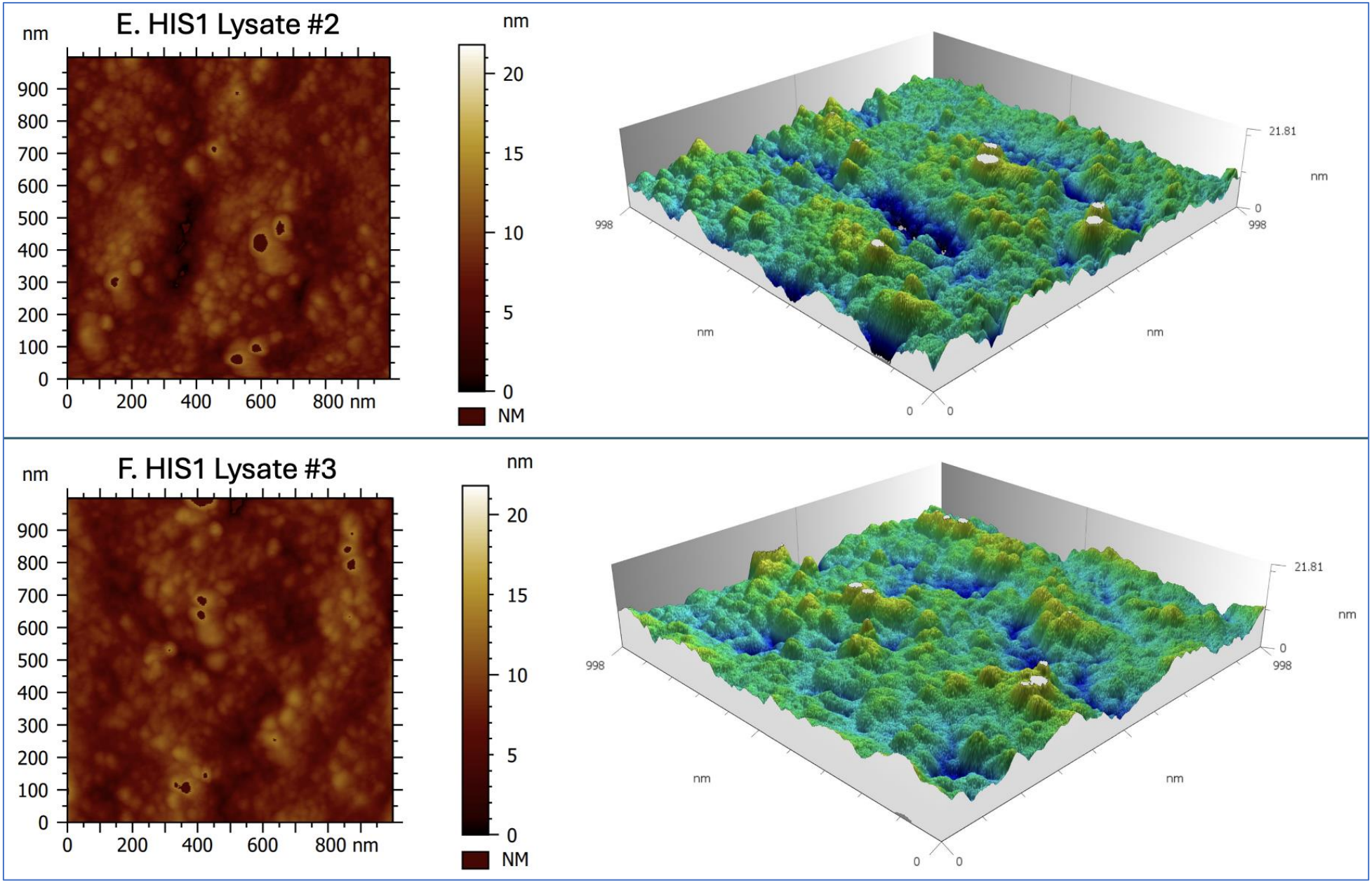
Treatment Type:	Untreated	HEPES	Pure HIS1	HIS1 Lysate	EV Lysate
#1 RMS	1.79267434	2.82960483	5.35696273	1.58956938	3.520480955
#2 RMS	2.16565491	2.23260149	5.54795521	1.93402887	3.755920225
#3 RMS	2.15791943	1.80224681	4.7728881	1.94612472	4.419176899
Average of RMS	2.03874956	2.28815104	5.22593535	1.82324099	3.898526026

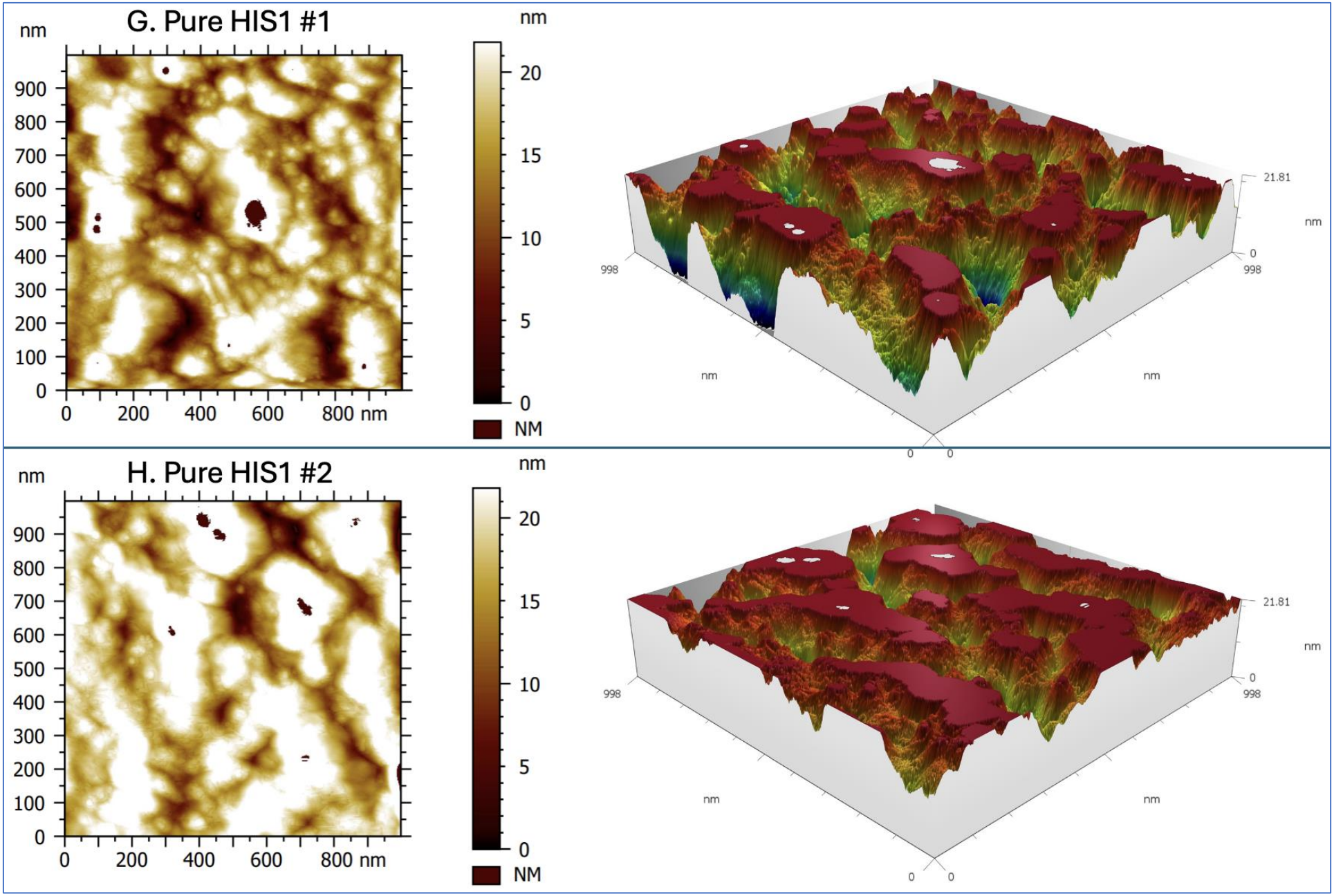
Supplementary Table 6.
Table of P-value of Unpaired T-Test on Root Mean Square (RMS) Roughness Values on 1 µm Scans

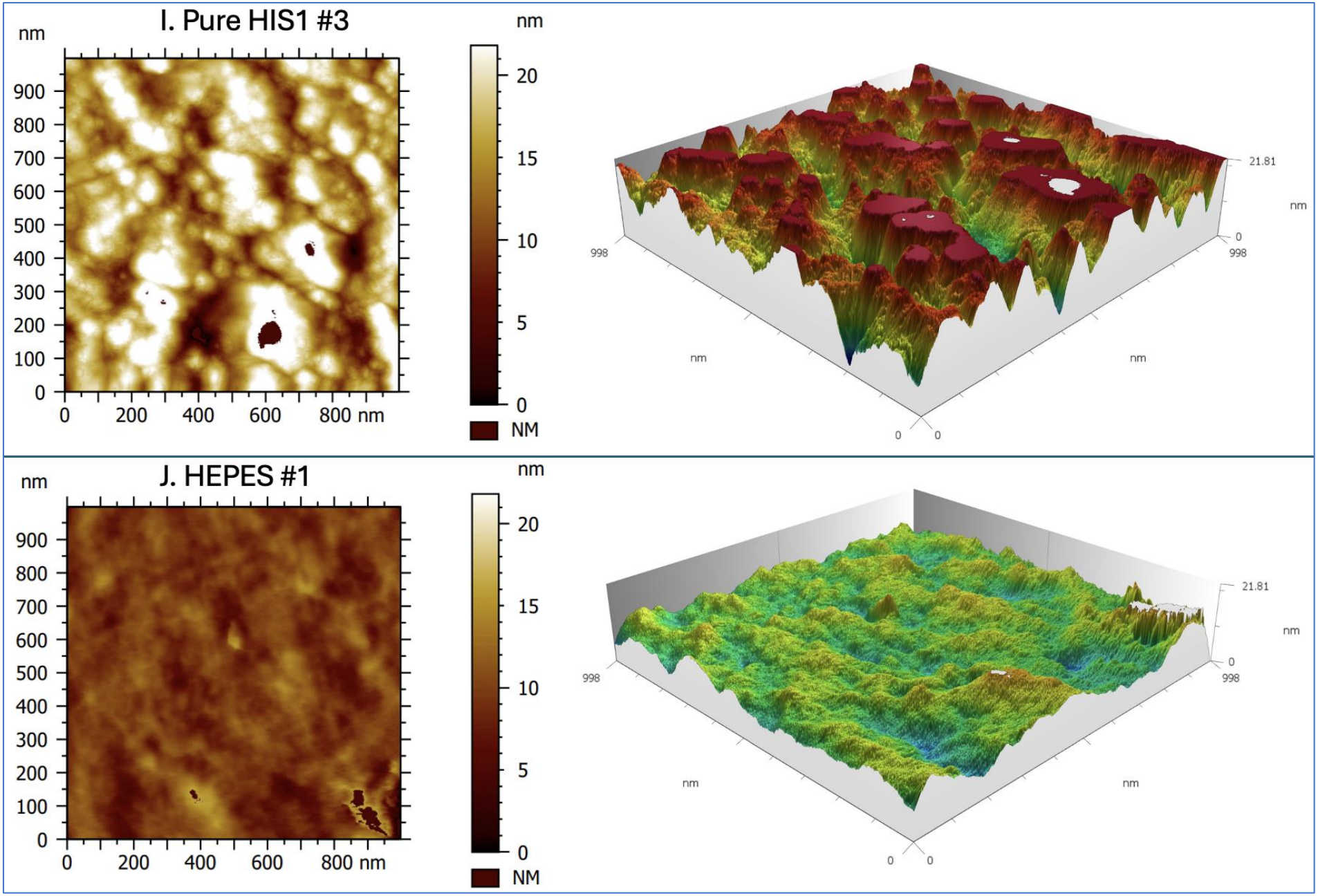
Test Samples	p-value
Untreated vs. Pure HIS1	**0.00115317**
HEPES vs. Untreated	0.50184625
HIS1 Lysate vs. Untreated	0.27318011
EV Lysate vs. Untreated	**0.00993342**
EV Lysate vs. HIS1 Lysate	**0.00787381**

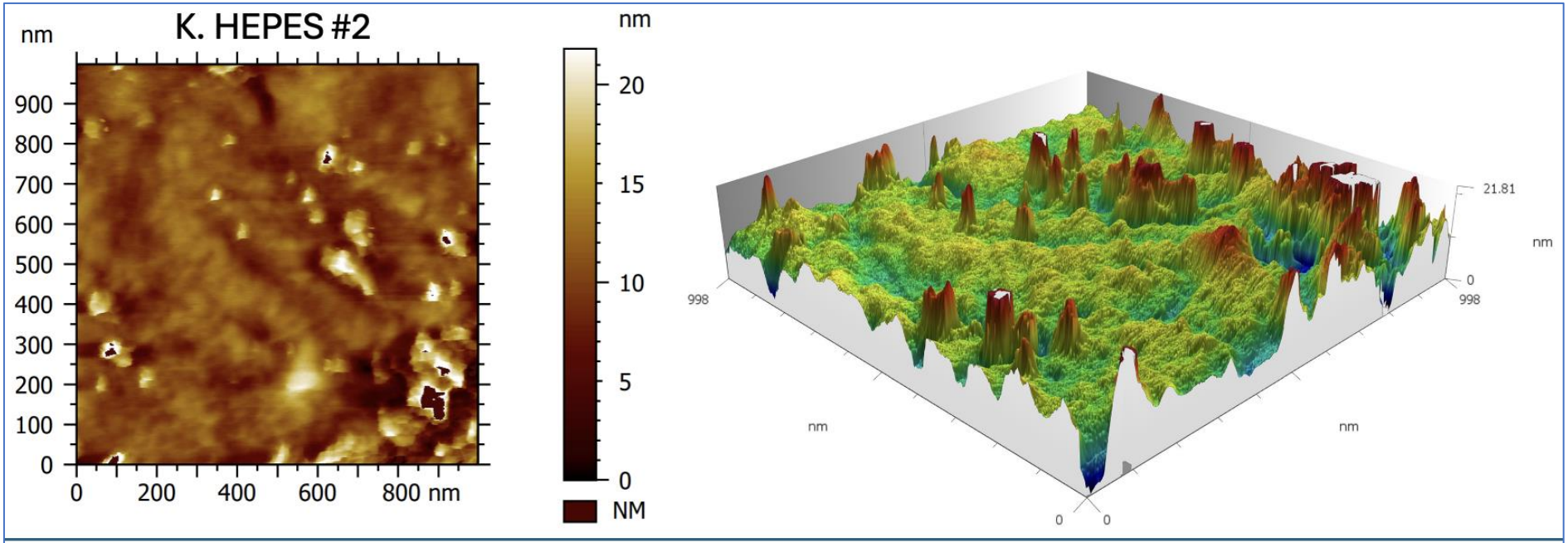

A. EV Lysate #1
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0
B. EV Lysate #2
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0

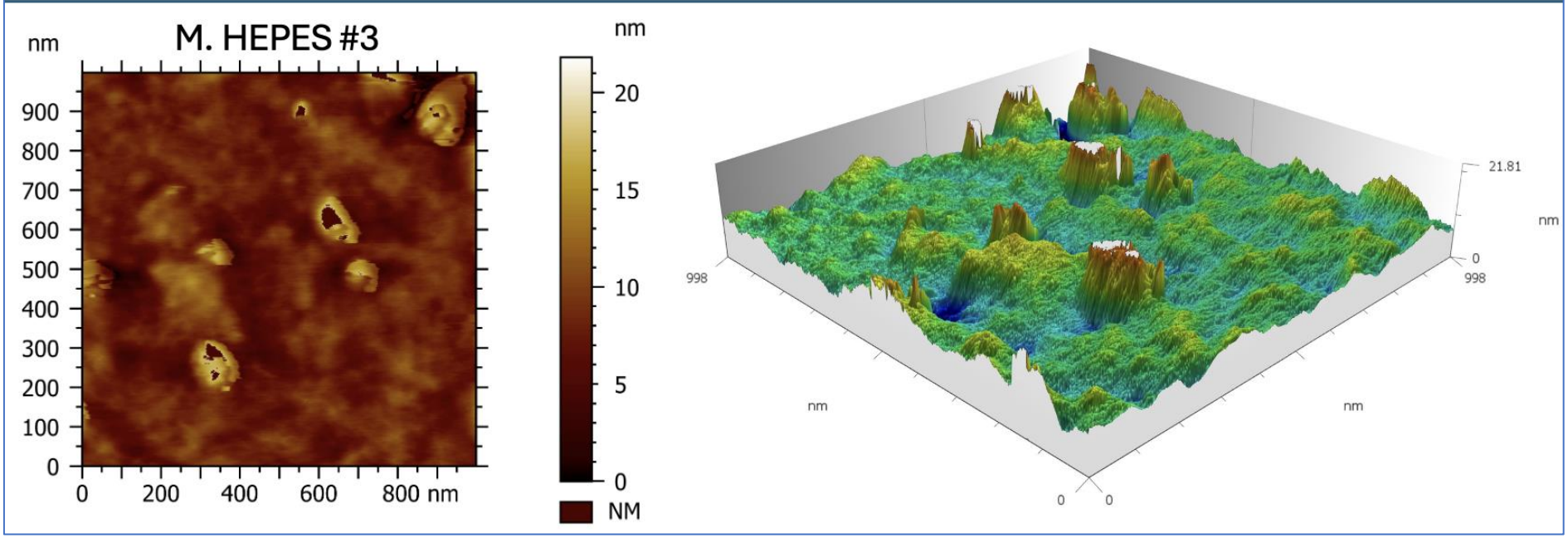

C. EV Lysate #3
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0
D. HIS1 Lysate #1
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0

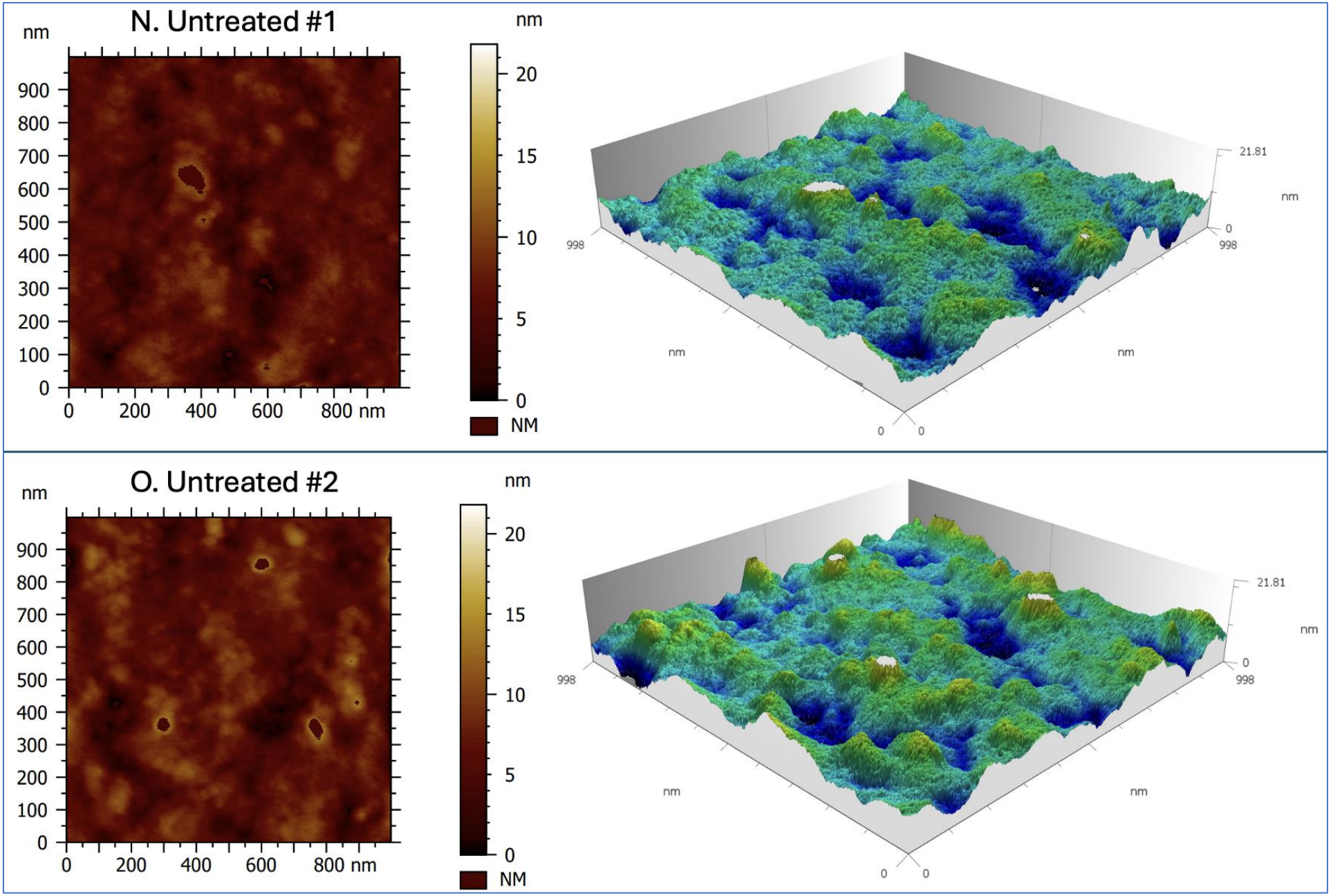

E. HIS1 Lysate #2
nm
900
800
700
600
500
400
300
200
100
0
0 200 400 600 800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0
F. HIS1 Lysate #3
nm
900
800
700
600
500
400
300
200
100
0
0 200 400 600 800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0

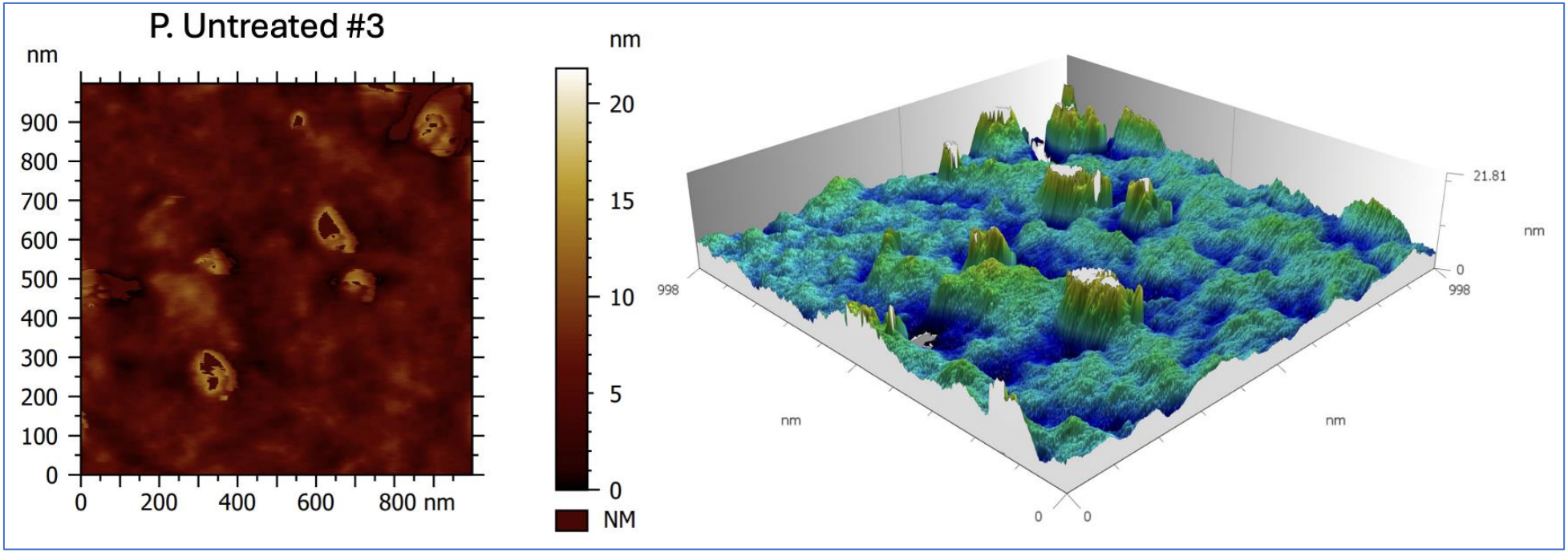

G. Pure HIS1 #1
nm
900
800
700
600
500
400
300
200
100
0
0 200 400 600 800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0
H. Pure HIS1 #2
nm
900
800
700
600
500
400
300
200
100
0
0 200 400 600 800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0


I. Pure HIS1 #3
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0
J. HEPES #1
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0


K. HEPES #2
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0


M. HEPES #3
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0


N. Untreated #1
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0
O. Untreated #2
nm
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800 nm
nm
20
15
10
5
0
NM
21.81
nm
0
998
998
nm
nm
0
0



Supplementary Figure 7. 1 µm AFM Scans and 3D surface topography of treatment sample. *Triplicate samples of all treatment utilised on PP films. Height scales are cut off at approximately 21.81 nm. All scans are done in a 1 µm x 1 µm size.* ***(A-C****) EV Lysate refers to samples treated with the EV lysate.* ***(D-F)*** *HIS1 lysate refers to samples treated with the HIS1 lysate.* ***(G-I)*** *Pure HIS1 refers to samples treated with the purified HIS1 enzyme.* ***(J-M)*** *HEPES refers to samples treated with the HEPES negative control.* ***(N-P)*** *Untreated refers to untreated plastic samples.*

FTIR

Supplementary Table 7. FTIR Spectra Peaks and their annotation

Frequency Range (cm-1)	Functional Group
2950	CH3 (asymmetric)
2875	CH3 (symmetric)
1375	CH3 (symmetric)
2917	CH2 (asymmetric)
2839	CH2 (symmetric)
3200-3400	O-H
1550-1600	COO-

Mutagenic Library Synthesis

Supplementary Table 8: Asymmetric PCR Setup

	Concentration	Reaction Concentration	Volume for 50µL PCR (µL)
Non-mutagenic reverse primer	10µM	2500nM	12.5
Mutagenic forward primer	1µM	25nM	1.25
Template	40ng/µL	0.8ng/µL	1
dNTPs	2mM	200µM	5
Buffer	5x	1x	10
Q5 DNA polymerase	-	-	1
Nuclease-free water	-	-	19.25

Supplementary Table 9: Symmetric Control PCR Setup

	Concentration	Reaction Concentration	Volume for 50µL PCR (µL)
Non-mutagenic reverse primer	10µM	1µM	5
Mutagenic forward primer	10µM	1µM	5
Template	40ng/µL	0.8ng/µL	1
dNTPs	2mM	200µM	5
Buffer	5x	1x	10
Q5 DNA polymerase	-	-	1
Nuclease-free water	-	-	23

Supplementary Table 10. Full-length PCR setup

	Concentration	Reaction Concentration	Volume for 50µL PCR (µL)
Non-mutagenic forward primer	10µM	500nM	2.5

Mutagenic reverse megaprimer	63ng/µL	50nM	9
Template	40ng/µL	0.8ng/µL	1
dNTPs	2mM	200µM	5
Buffer	5x	1x	10
Q5 DNA polymerase	-	-	1
Nuclease-free water	-	-	21.5

Supplementary Table 11: Asymmetric, Symmetric Control, and Full-length PCR Thermocycle

Step	Temperature (℃)	Time	Cycle
Denaturation	98	2 min	1x
Denaturation	98	30 sec	35x
Anneal	65	20 sec	
Extension	72	60 sec	

*Supplementary Table 8, 9, 10, 11 is adapted from Sadler et al., 2018.

Supplementary Table 12: T4 Ligation Setup

			Concentration	Volume for 20µL PCR (µL)
+L+I	T4 DNA Ligase		-	1
	T4 DNA Ligase Buffer		10x	2
	Insert		4.6ng/µL	4.6
	Vector		33ng/µL	1
	Nuclease-free water		-	11.4
+L-I	T4 DNA Ligase	-	1	
	T4 DNA Ligase Buffer	10x	2	
	Vector	33ng/µL	1	
	Nuclease-free water	-	16	
-L-I	T4 DNA Ligase Buffer	10x	2	
	Vector	33ng/µL	1	
	Nuclease-free water	-	17	

*Supplmentary Table 12 is adapted from New England Biolabs 2021. Ligation Protocol with T4 DNA Ligase (M0202). protocols.io https://dx.doi.org/10.17504/p

Rotocols.io.bcchist6

Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay for purified HIS1 activity

Supplementary Table 13. ***Succinate-Glo™ Assay analysis of purified HIS1 activity***

	1	2	3	4	5	6	7	8	9	10	11	12
A	3628	1864	762	591	362	244	167	133	122	103	Succinate Calibration Curve 15µM-0µM	
B	3031	1427	1181	639	378	235	186	147	118	112		
C	15µM	7.5µM	3.75µM	1.88µM	0.94µM	0.47µM	0.23µM	0.12µM	0.06µM	0µM		
D												
E	59	58	65	71	64	65	58	59	62			
F	Pure HIS1 + HPP			Pure HIS1 + PP			Pure HIS1					
G	66	66	96	63	73	78	99	72	90			
H	HEPES + HPP			HEPES + PP			HEPES					

This assay was used to assess the activity of pure HIS1 on 4-hydroxyphenypyruvate (HPP) and polypropylene (PP), using appropriate reaction mixtures that were incubated at 30°C for 24hrs. A standard curve for succinate was generated following the protocol (Promega Corporation, 2017). Standards were prepared in assay buffer (HEPES with 2µM FeSO4 and 6µM 2-OG) by a serial twofold dilution yielding a range of 15µM to 0µM. Reaction mixtures were diluted (2µM FeSO4 and 6µM of 2-OG). The Succinate-Glo™ assay was carried out following the manufacturer's protocol provided. Luminescence was measured with a 96-well plate reader. Results suggested no HIS1 activity on either substrate.

REFERENCES

FáVARO, S. L., RUBIRA, A. F., MUNIZ, E. C. & RADOVANOVIC, E. 2007. Surface modification of HDPE, PP, and PET films with KMnO4/HCl solutions. *Polymer Degradation and Stability,* 92**,** 1219-1226.Available at: https://doi.org/10.1016/j.polymdegradstab.2007.04.005

Promega Corporation Measuring JumonjiC Demethylase and Fe(II)/alpha-KG-Dependent Hydroxylase Activities Using the Succinate-Glo™ Assay and GloMax® Discover System. [Internet] June 2017. [cited: 2025, 10, 01]. Available from: https://www.promega.co.uk/resources/pubhub/applications-notes/measure-jmjcdemethylasefeii-alpha-kg-hydroxylase-activitysuccinate-glo-assay-and-glomax-discover/

New England Biolabs 2021. Ligation Protocol with T4 DNA Ligase (M0202). protocols.io https://dx.doi.org/10.17504/p