Supplementary information

Balance-directed protein engineering of *Is*PETase enhances both PET hydrolysis activity and thermostability

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[#]These authors contributed equally to this work.

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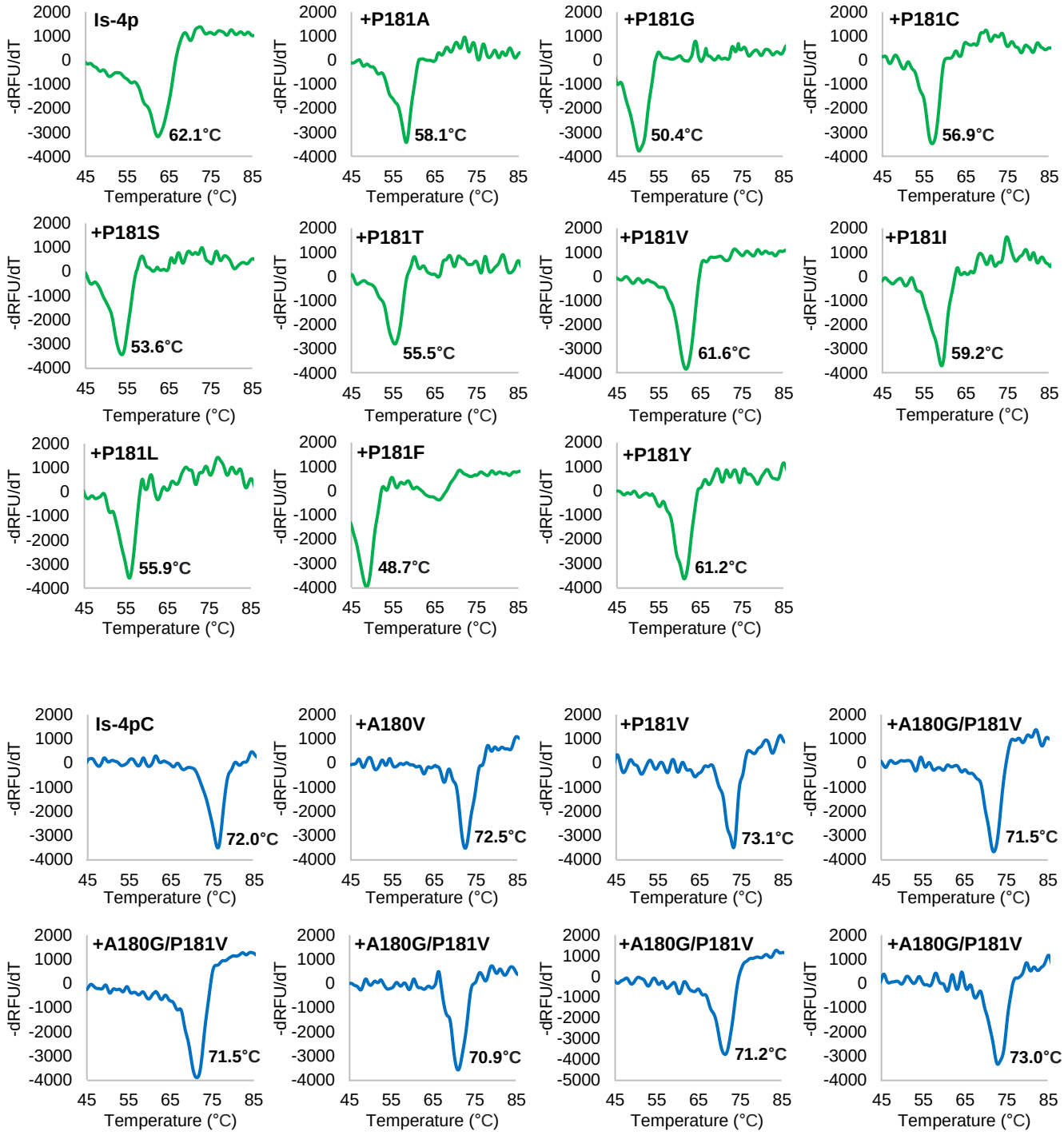
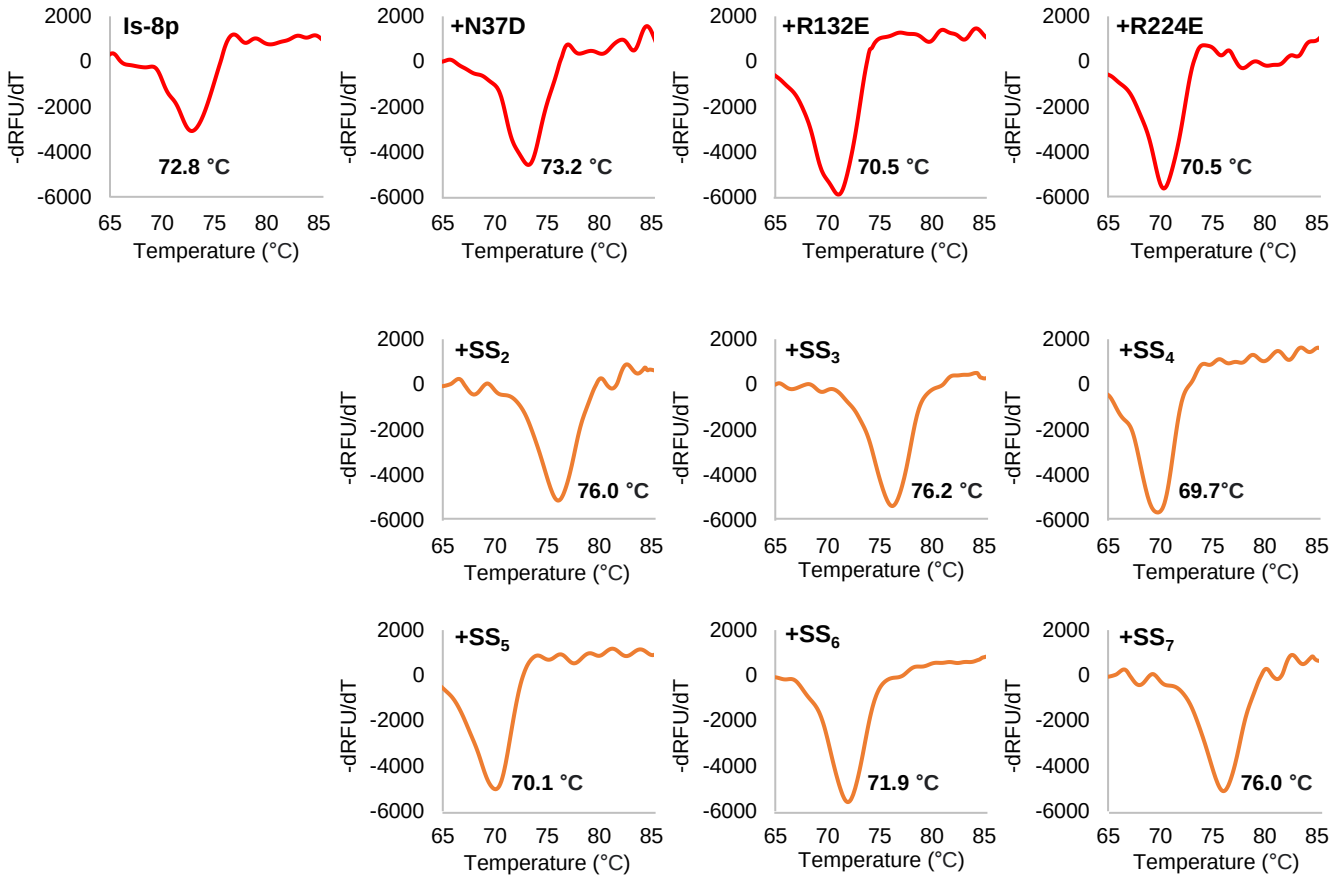
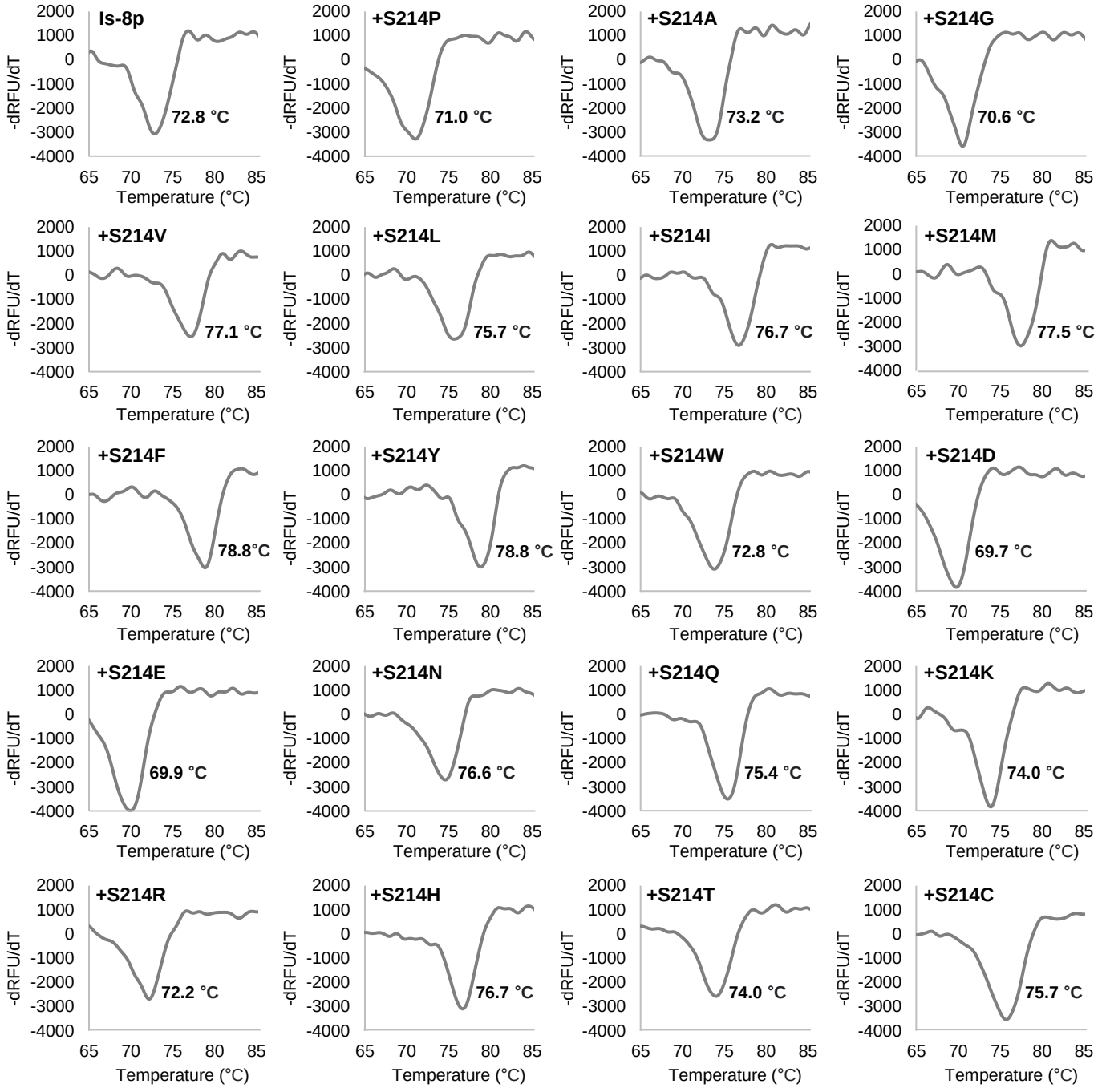
This PDF file includes: Supplementary Figures 1 to 12.
Supplementary Tables 1 to 3



Supplementary Figure 1. Differential scanning fluorimetry of the *Is*PETase variants used in this study.



Supplementary Figure 1. Differential scanning fluorimetry of the *Is*PETase variants used in this study. (continued)



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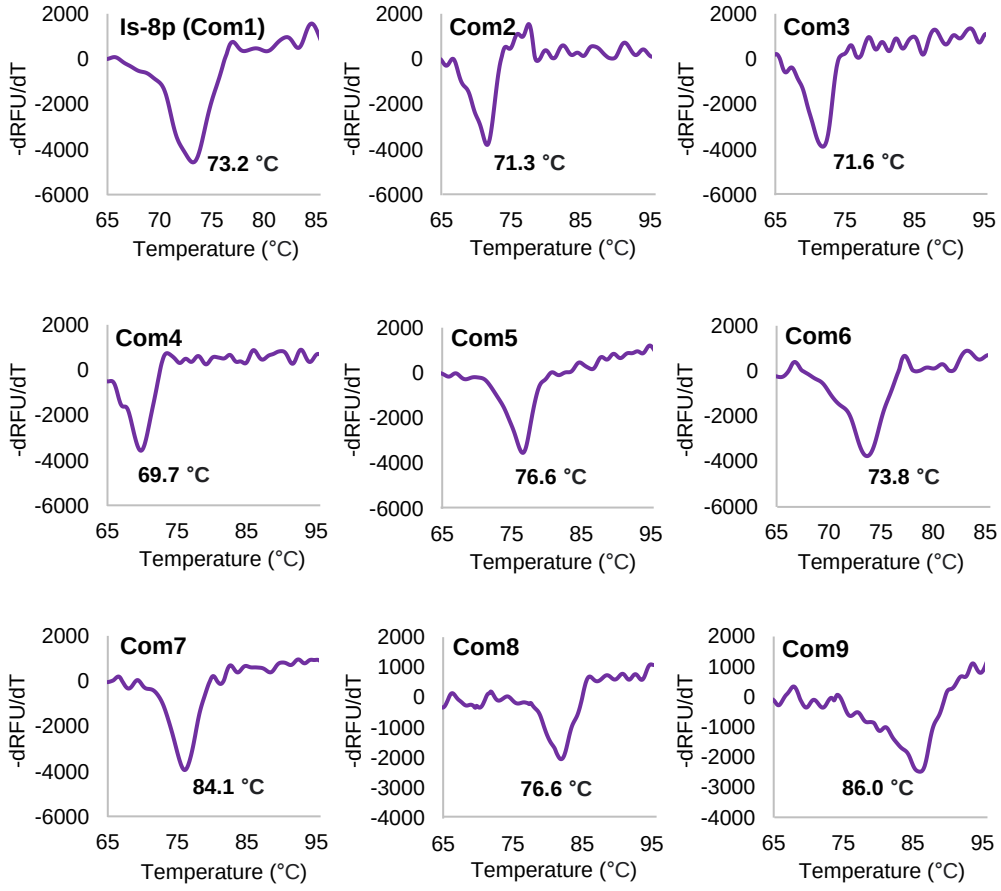
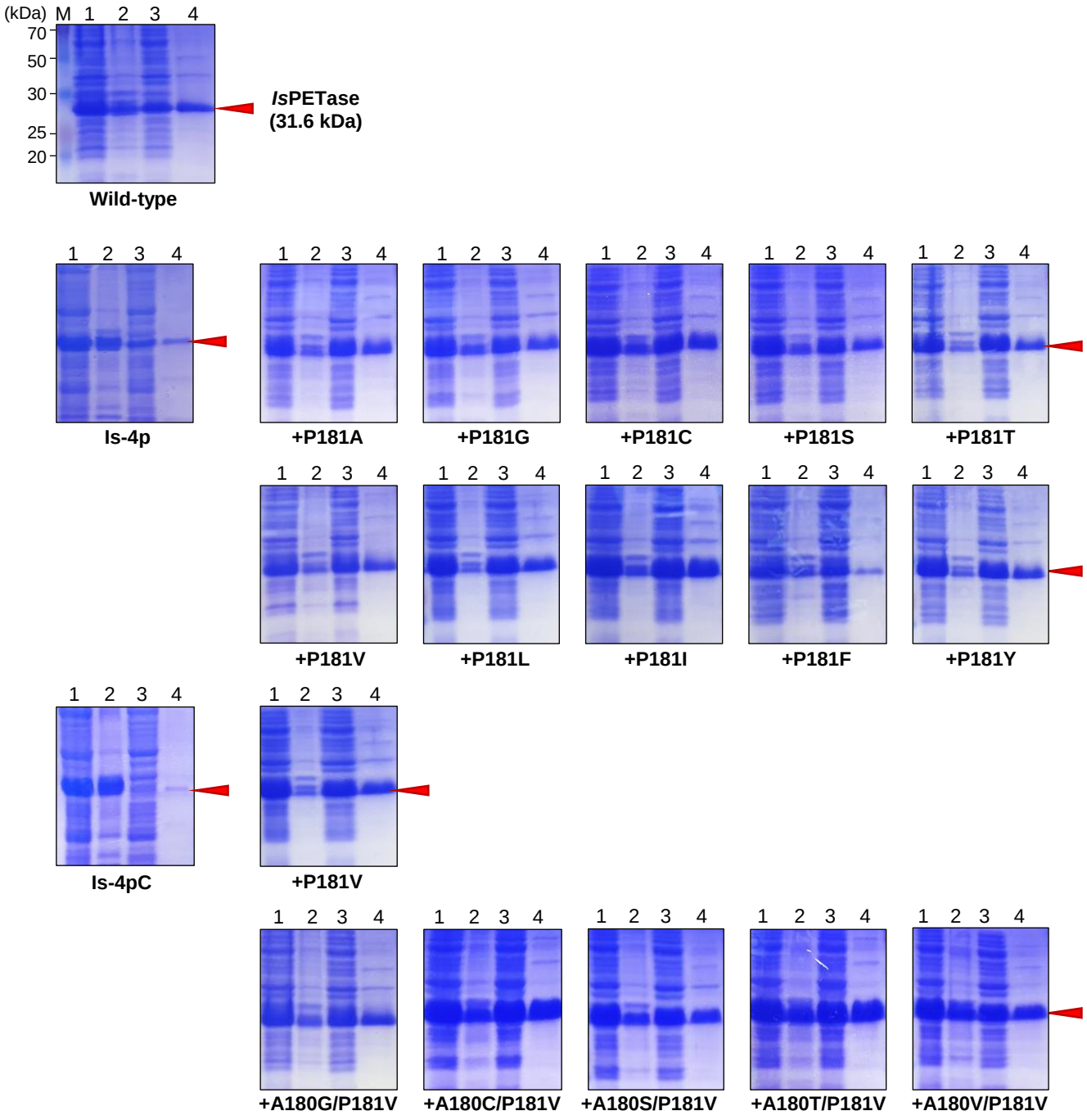
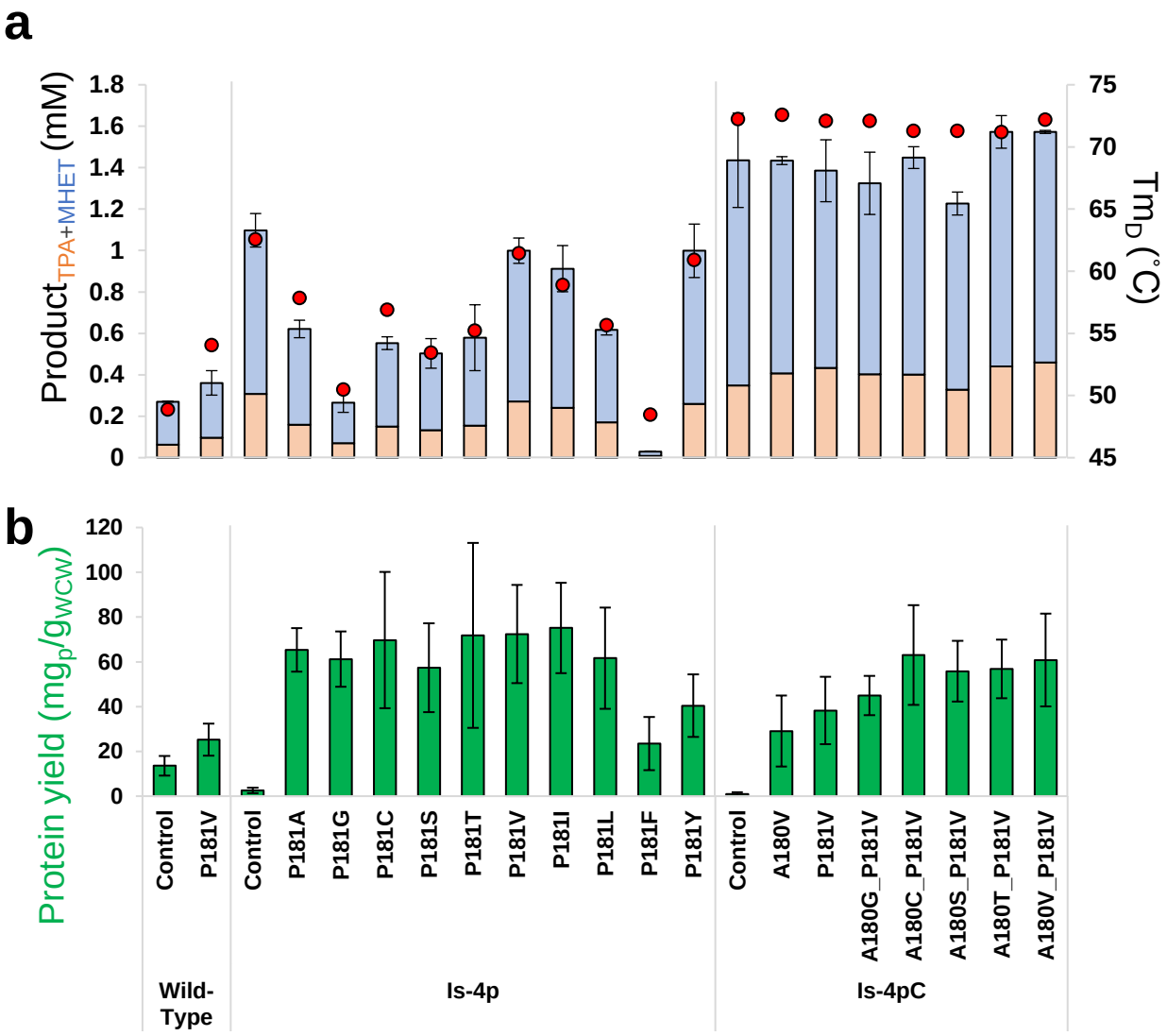


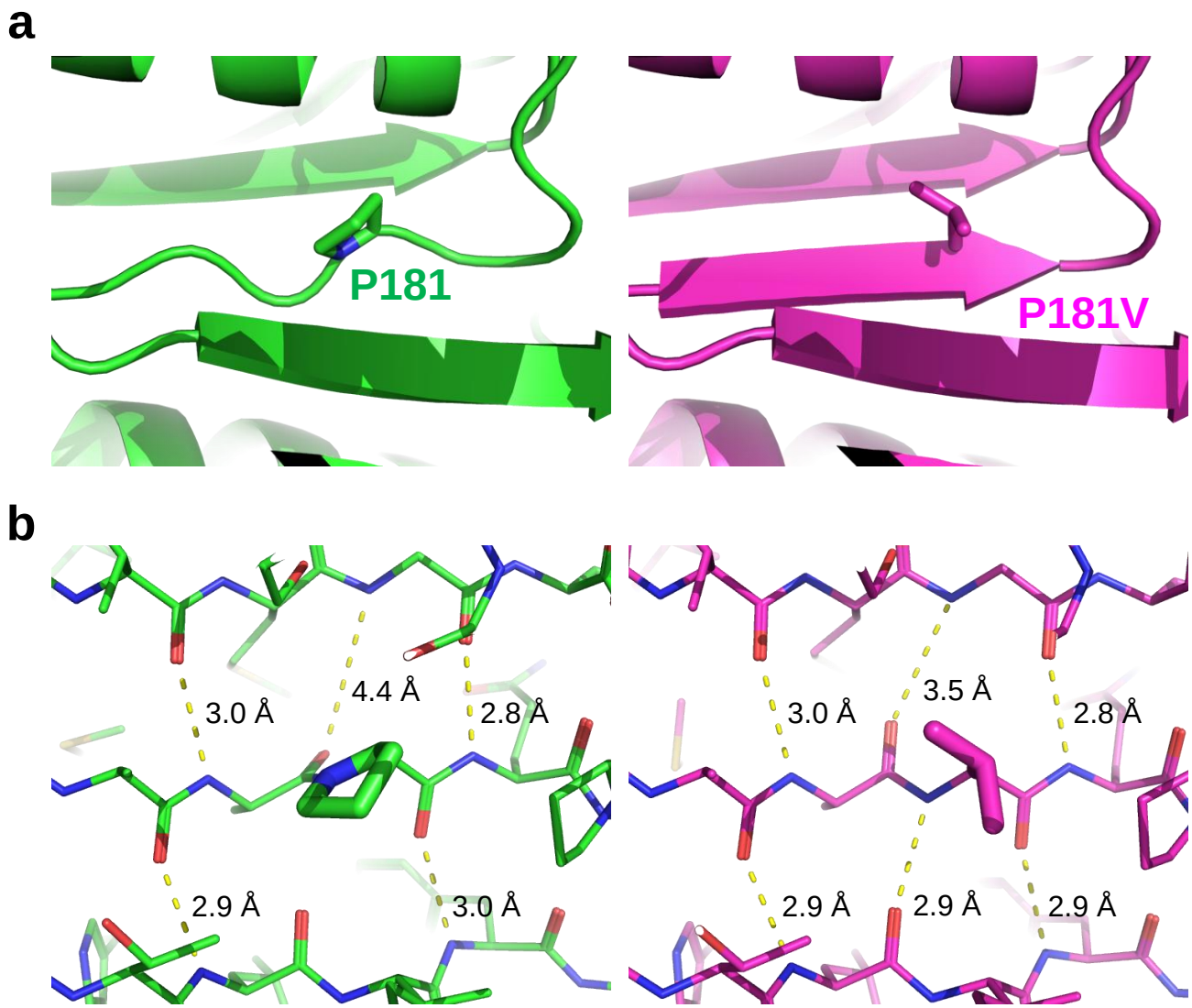
Supplementary Figure 1. Differential scanning fluorimetry of the *Is*PETase variants used in this study. (continued)



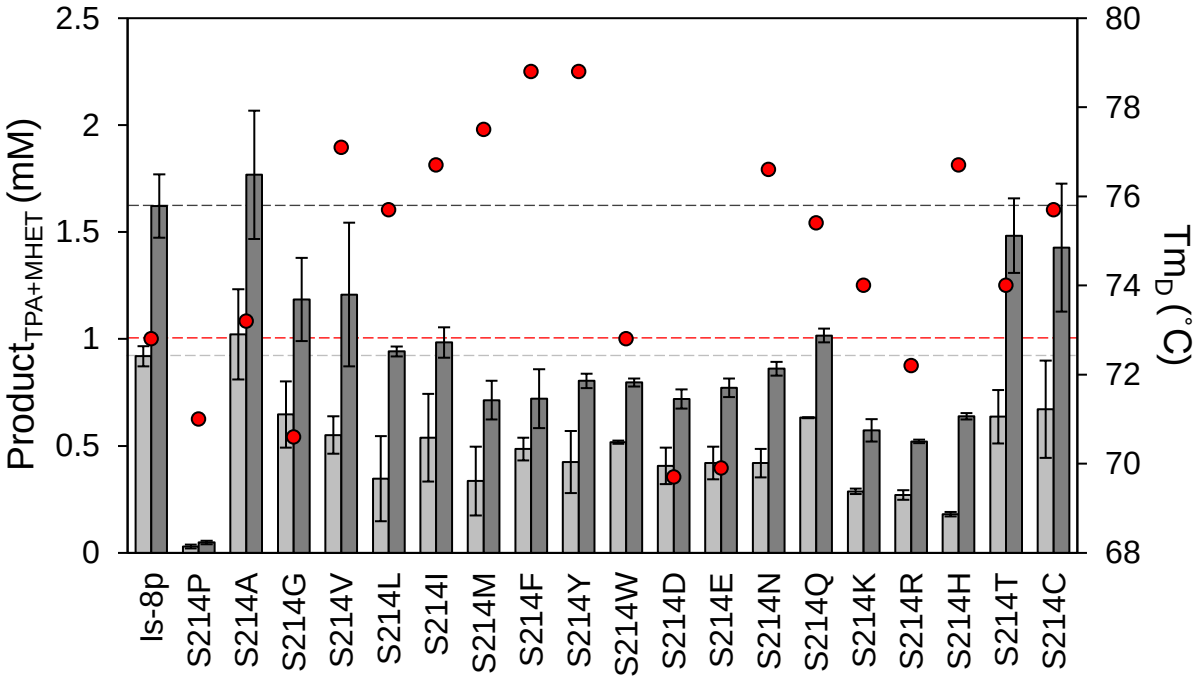
Supplementary Figure 2. SDS-page analysis of overexpression of Is-4p+P181X and Is-4pC+A180X/P181V variants. The purified *Is*PETase variants using the *E. coli* Rosetta-gami™ 2(DE3) expression system are loaded on SDS-PAGE. The molecular weight of the protein is 31.6 kDa. Lane M represents the ladder (molecular marker). Lanes 1, 2, 3, and 4 indicate cell lysate, cell pellets, and supernatant, and eluted protein, respectively.



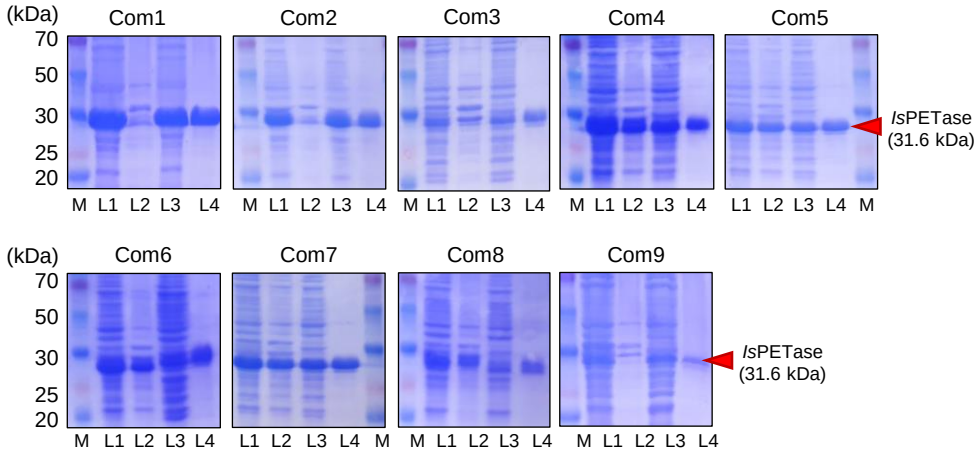
Supplementary Figure 3. PET degradation activity, thermal stability (Tm_D), and protein yield of 4p+P181X and 4pC+A180X/P181V variants. a, PET hydrolysis activity and thermal stability of Is-4p+P181X and Is-4pC+A180X+P181V. The PET hydrolysis activity is measured at 6 hours of incubation using 15 gL^{-1} of bottle-derivative PET powder in 50 mM glycine-NaOH pH 9.0 at 50 °C. All the data is reacted in triplicate via HPLC. **b,** Protein yields of Is-4p+P181X and Is-4pC+A180X+P181V. The protein yields are detected from soluble protein expression in the elution buffer. The unit is protein weight (mg_p) of 1g of wet cell weight (g_{wcw}). All the experiments were performed in triplicate, and the error bars of measuring samples represent the sampling s.d. (n=3).



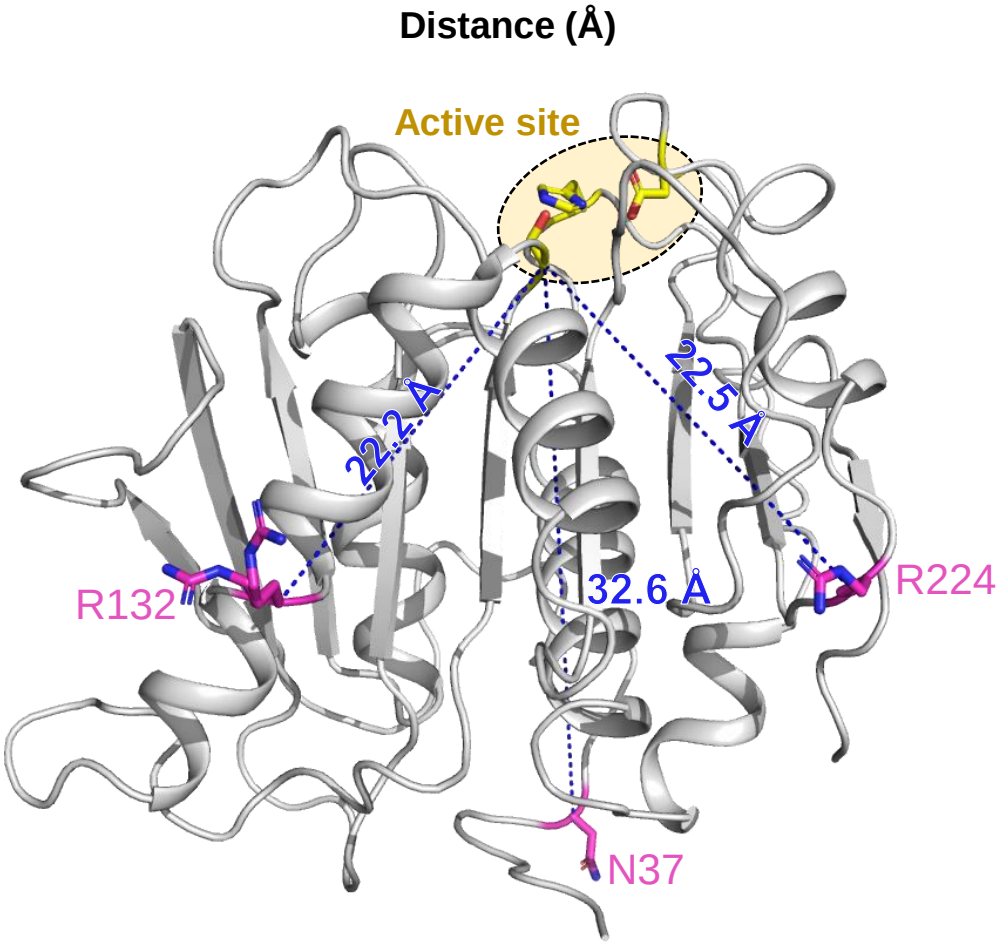
Supplementary Figure 4. β-strand reconstruction in the *Is*PETase+P181V variant. a, Comparison of β-strand of Is-4pC and Is-4pC+P181V. The structures of Is-4pC and Is-4pC+P181V are colored as green and magenta, respectively. The P181 and P181A residue are shown as a stick. **b,** Comparison of hydrogen bond length. The hydrogen bonds are shown as a yellow-colored dotted- lines and their length are labeled.



Supplementary Figure 5. Site saturation mutagenesis of Ser214. The PET hydrolysis activity is measured at 3 and 6 hours of incubation using bottle-derivative PET at 50 °C colored as light gray and gray, respectively. All experiments were performed in triplicate and detected via HPLC, and error bars of measuring samples represent the sampling s.d. (n=3).

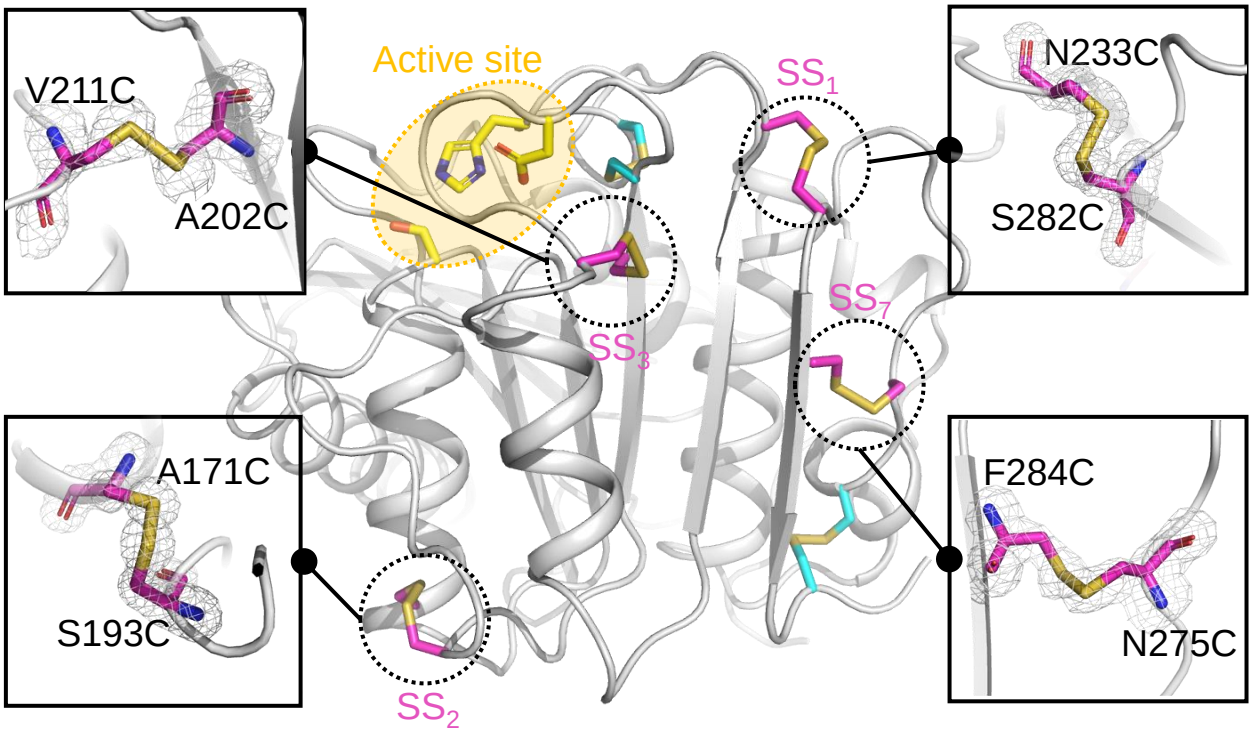


Supplementary Figure 6. Analysis of overexpression of combinatorial variants on the 8p variants. The purified *Is*PETase combinatorial mutants using the *E. coli* Rosetta-gami™ 2(DE3) expression system are loaded on SDS-PAGE. The molecular weight of the protein is 31.6 kDa. Lane M represents the ladder (molecular marker). Lanes 1, 2, 3, and 4 are cell lysate, cell pellets, supernatant, and eluted protein, respectively.



Supplementary Figure 7. The distance between the catalytic site and the residues of N37D, R132E, and R224E. The Is-4pC variant structure is shown as a cartoon diagram colored gray. The catalytic and surface charge mutation residues are shown as a stick with yellow and magenta colores, respectively.

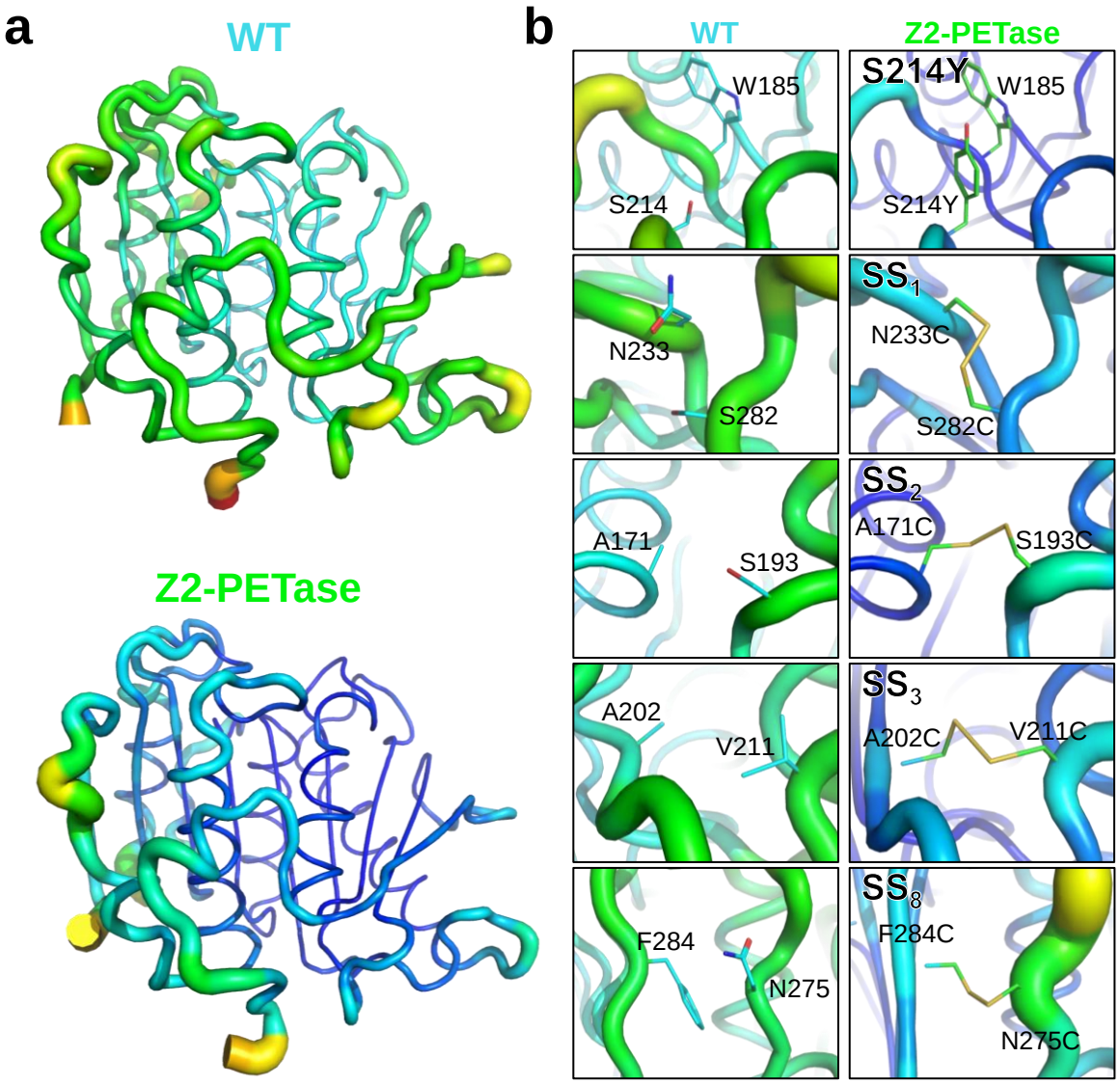
a



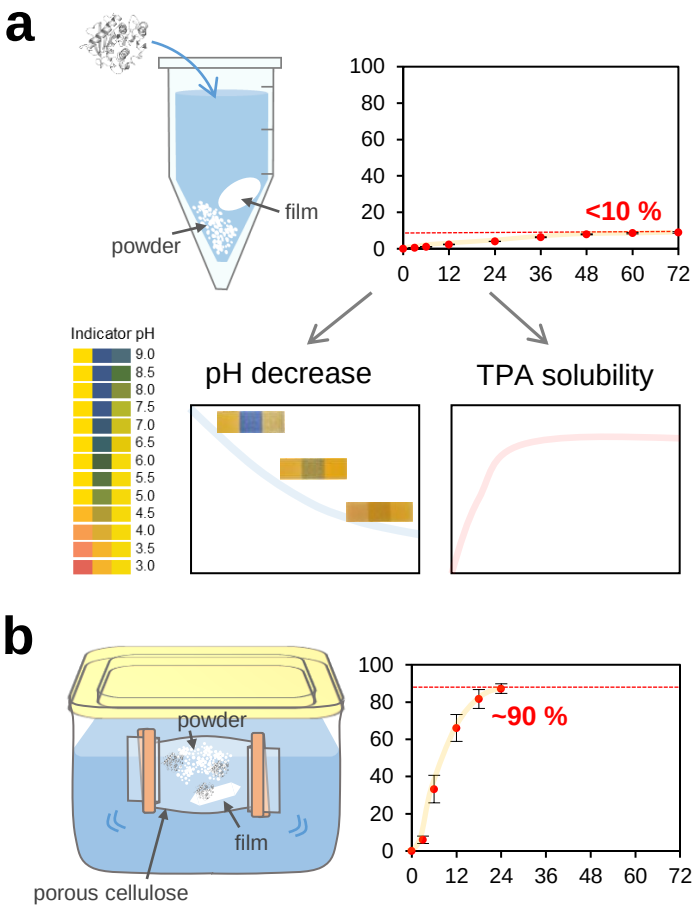
b

Pair of Cysteine		Chi1(X1)	Chi2(X2)	Chi2'(X2')	Chi1'(X1')	$C^{\beta}-S^{\gamma}-S^{\gamma}-C^{\beta}$ (°)	Distance (Å)	Disulfide Strain Energy (kJ/mol)
A171C	S193C	-70.88 (±1.67)	-129.36 (±2.85)	86.32 (±1.12)	53.20 (±1.07)	66.34 (±0.87)	2.12 (±0.04)	17.95 (±0.61)
A202C	V211C	77.47 (±1.93)	105.96 (±2.87)	-90.56 (±4.02)	-65.52 (±2.92)	111.37 (±1.30)	2.30 (±0.02)	23.91 (±0.58)
N233C	S282C	-61.63 (±3.30)	-55.92 (±3.21)	62.20 (±4.09)	-91.95 (±6.90)	110.60 (±2.53)	2.06 (±0.03)	17.88 (±2.53)
N275C	F284C	-74.05 (±0.31)	-62.53 (±3.21)	-115.12 (±2.32)	-178.83 (±0.90)	-79.89 (±1.60)	2.10 (±0.02)	12.63 (±0.31)

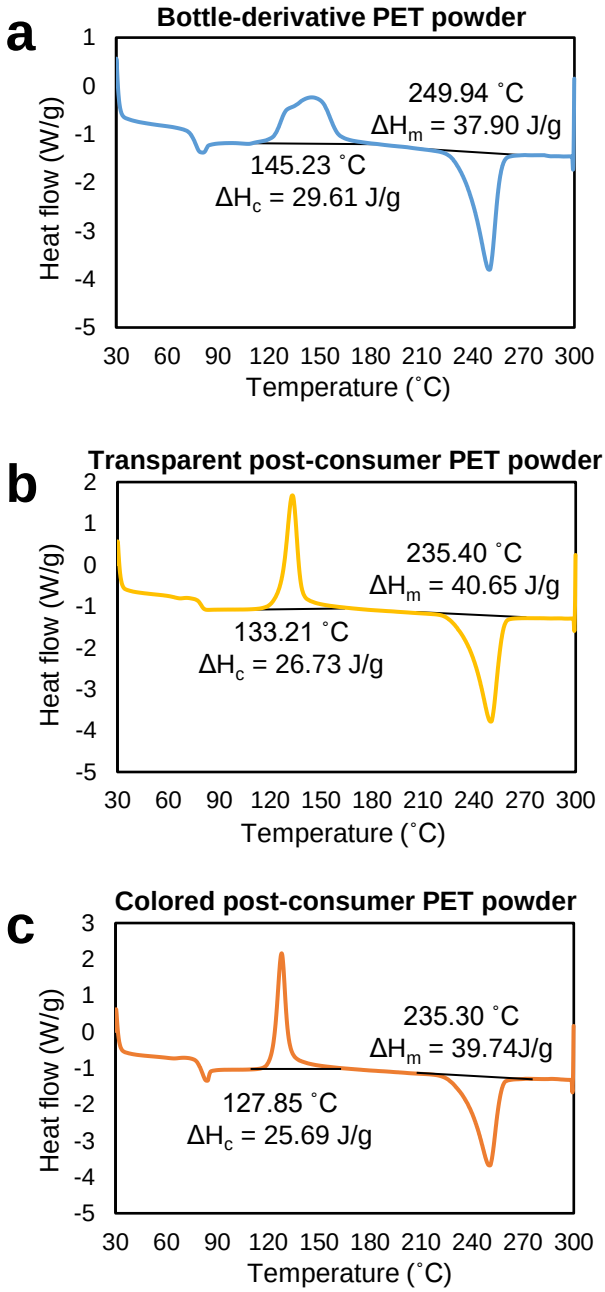
Supplementary Figure 8. Disulfide bond formation analysis of Z2-PETase. The Z2-PETase structure is shown as a cartoon diagram colored gray. Disulfide bonds from the *Is*PETase$^{\text{Wild-type}}$ and constructed in this study are shown as a stick with cyan and magenta colored, respectively. The electron density (Fo-Fc) maps of the introduced disulfide bonds are shown with a 1.5 σ contour.



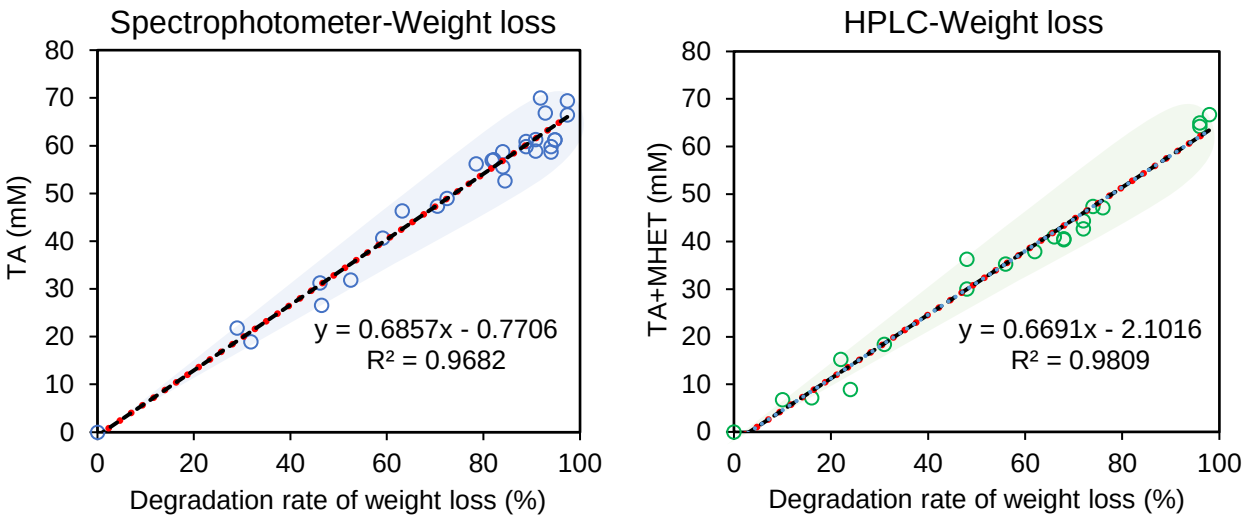
Supplementary Figure 9. Comparison of b-factor putty of *Is*PETase$^{\text{Wild-type}}$ and Z2-PETase.



Supplementary Figure 10. Schematic diagram of a product-deficient dialysis system. a, The limitation to measuring real PET hydrolysis activity at the closed vial system. **b,** Designed product deficient *in-situ* dialysis system.



Supplementary Figure 11. A DSC plot for polyethylene terephthalate (PET) sample. The crystallinity of bottle-derivative PET, Transparent post-consumer PET, and Colored post-consumer PET powder were calculated using DSC experiments. The crystallinity of bottle-derivative PET, Transparent post-consumer PET, and Colored post-consumer PET powder were calculated using DSC experiments. The bottle-derivative PET has an enthalpy of cold crystallization (ΔH_c) of 29.61 J/g, a melting enthalpy (ΔH_m) of 37.90 J/g, and a percent crystallinity of 7.8 %. The Transparent post-consumer PET has an enthalpy of cold crystallization (ΔH_c) of 26.73 J/g, a melting enthalpy (ΔH_m) of 40.65 J/g, and a percent crystallinity of 10.1 %. The colored post-consumer PET powder has an enthalpy of cold crystallization (ΔH_c) of 25.69 J/g, a melting enthalpy (ΔH_m) of 39.74 J/g, and a percent crystallinity of 10.04 %.



Supplementary Figure 12. PET hydrolysis rate calculation comparison between HPLC and spectrophotometer detection method with weight loss of the PET powder. The total amount of each sample was starting from 50 mg. The x-slope of the spectrophotometer and the HPLC is within error range.

Supplementary Table 1. List of primers used in this study.

Site-directed mutagenesis	Forward sequence (5'-3')	Reverse sequence (5'-3')
N37D	CATATGCGCGGCCCGGATCCGACAGCCGCCAGT	ACTGGCGGCTGTCGGATCCGGGCCGCGCATATG
R132E	CAAATGGCCGCGCTGGAACAGGTGGCGTCGTTA	TAACGACGCCACCTGTTCCAGCGCGGCCATTTG
A171C	TCCCTGATCTCTGCTTGCAACAACCCTTCGCTG	CAGCGAAGGGTTGTTGCAAGCAGAGATCAGGGA
A180V	TCGCTGAAAGCAGCGGTGCCTCAAGCACCATGG	CCATGGTGCTTGAGGCACCGCTGCTTTCAGCGA
A180G+P181V	TCGCTGAAAGCAGCGGCGGTGCAAGCACCATGG	TCGCTGAAAGCAGCGGTGCCTCAAGCACCATGG
A180C+P181V	TCGCTGAAAGCAGCGTGCGTGCAAGCACCATGG	TCGCTGAAAGCAGCGGTGCCTCAAGCACCATGG
A180S+P181V	TCGCTGAAAGCAGCGAGCGTGCAAGCACCATGG	TCGCTGAAAGCAGCGGTGCCTCAAGCACCATGG
A180T+P181V	TCGCTGAAAGCAGCGACCGTGCAAGCACCATGG	TCGCTGAAAGCAGCGGTGCCTCAAGCACCATGG
A180V+P181V	TCGCTGAAAGCAGCGGTGGTGCAAGCACCATGG	TCGCTGAAAGCAGCGGTGCCTCAAGCACCATGG
P181A	CTGAAAGCAGCGGCGGCGCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181G	CTGAAAGCAGCGGCGGGCCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181C	CTGAAAGCAGCGGCGTGCCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181S	CTGAAAGCAGCGGCGAGCCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181T	CTGAAAGCAGCGGCGACCCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181V	CTGAAAGCAGCGGCGGTCCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181I	CTGAAAGCAGCGGCGATTCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181L	CTGAAAGCAGCGGCGCTGCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181F	CTGAAAGCAGCGGCGTTTCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
P181Y	CTGAAAGCAGCGGCGTATCAAGCACCATGGGAT	ATCCCATGGTGCTTGGACCGCCGCTGCTTTCAG
S193C	TCGACAAATTTTAGTTGCGTAACTGTGCCCACG	CGTGGGCACAGTTACGCAACTAAAATTTGTCGA
A202C	CCCACGCTGATCTTCTGCTGTGAAAACGATAGT	ACTATCGTTTTCACAGCAGAAGATCAGCGTGGG
V211C	GATAGTATAGCCCCGTGCAACTCTTCAGCACTT	AAGTGCTGAAGAGTTGCACGGGGCTATACTATC
S214Y	GCCCCGGTCAACTCTTATGCACTTCCTATCTAT	ATAGATAGGAAGTGCATAAGAGTTGACCGGGGC
R224E	TATGATTCTATGTCAGAAAACGCTAAGCAGTTT	AAACTGCTTAGCGTTTTCTGACATAGAATCATA
N233C	CAGTTTCTCGAAATTTGCGGTGGCTCACATTCC	GGAATGTGAGCCACCGCAAATTTCGAGAAACTG
N275C	ACTTTTGCCTGCGAGTGCCCGAATAGCACCAGA	TCTGGTGCTATTCGGGCACTCGCAGGCAAAAGT
S282C	AATAGCACCAGAGTGTGCGATTTTCGTACAGCG	CGCTGTACGAAAATCGCACACTCTGGTGCTATT
F284C	ACCAGAGTGAGCGATTGCCGTACAGCGAATTGC	GCAATTCGCTGTACGGCAATCGCTCACTCTGGT
S282C+F284C	ACCAGAGTGTGCGATTGCCGTACAGCGAATTGC	GCAATTCGCTGTACGGCAATCGCACACTCTGGT

Supplementary Table 2. DNA sequence of the *Is*PETase variant.

Enzyme	Nucleotide sequence
4p-PETase	ATGCGCGGTCCGAATCCGACAGCCGCCAGTTTGGAAGCGAGCGCTGGTCCATTCACCGTTCGCTCC TTTACCGTGAGTAGACCGAGCGGTTATGGCGCTGGCACCGTTTACTATCCAACAAATGCTGGGGGT ACCGTGGGCGCCATAGCCATAGTTCCCGGGTATACGGCACGGCAGTCATCAATTAAATGGTGGGGA CCGCGTCTGGCATCCCACGGTTTCGTAGTAATTACAATTGACACAAATTCCACGTTAGACCAGCCA GAAAGTCGGAGTTCGCAACAAATGGCCGCGCTGCGCCAGGTGGCGTCGTTAAACGGTACAAGTAG CAGCCCGATTTACGGAAAGGTCGATACCGCTCGTATGGGTGTTATGGGGTGGAGTATGGGAGGTGG AGGCTCCCTGATCTCTGCTGCTAACAACCCTTCGCTGAAAGCAGCGGCGCCTCAAGCACCATGGC ATTCTTCGACAAATTTTAGTTCTGTAACTGTGCCCACGCTGATCTTCGCATGTGAAAACGATAGTAT AGCCCCGGTCAACTCTTCAGCACTTCCTATCTATGATTCTATGTCACGCAACGCTAAGCAGTTTCTC GAAATTAACGGTGGCTCACATTCCTGTGCGAATACCGGCAATTCTGACCAAGCATTAATCGGAAAA AAAGGCGTTGCATGGATGAAACGTTTTATGGACAATGATACTAGGTATTCTACTTTTGCCTGCGAGA ACCCGAATAGCACCAGAGTGAGCGATTTTCGTACAGCGAATTGCAGC
4pC- PETase	ATGCGCGGTCCGAATCCGACAGCCGCCAGTTTGGAAGCGAGCGCTGGTCCATTCACCGTTCGCTCC TTTACCGTGAGTAGACCGAGCGGTTATGGCGCTGGCACCGTTTACTATCCAACAAATGCTGGGGGT ACCGTGGGCGCCATAGCCATAGTTCCCGGGTATACGGCACGGCAGTCATCAATTAAATGGTGGGGA CCGCGTCTGGCATCCCACGGTTTCGTAGTAATTACAATTGACACAAATTCCACGTTAGACCAGCCA GAAAGTCGGAGTTCGCAACAAATGGCCGCGCTGCGCCAGGTGGCGTCGTTAAACGGTACAAGTAG CAGCCCGATTTACGGAAAGGTCGATACCGCTCGTATGGGTGTTATGGGGTGGAGTATGGGAGGTGG AGGCTCCCTGATCTCTGCTGCTAACAACCCTTCGCTGAAAGCAGCGGCGCCTCAAGCACCATGGC ATTCTTCGACAAATTTTAGTTCTGTAACTGTGCCCACGCTGATCTTCGCATGTGAAAACGATAGTAT AGCCCCGGTCAACTCTTCAGCACTTCCTATCTATGATTCTATGTCACGCAACGCTAAGCAGTTTCTC GAAATTTGCGGTGGCTCACATTCCTGTGCGAATACCGGCAATTCTGACCAAGCATTAATCGGAAAA AAAGGCGTTGCATGGATGAAACGTTTTATGGACAATGATACTAGGTATTCTACTTTTGCCTGCGAGA ACCCGAATAGCACCAGAGTGTGCGATTTTCGTACAGCGAATTGCAGC
Z1-PETase	ATGCGCGGCCCGGATCCGACAGCCGCCAGTTTGGAAGCGAGCGCTGGTCCATTCACCGTTCGCTC CTTTACCGTGAGTAGACCGAGCGGTTATGGCGCTGGCACCGTTTACTATCCAACAAATGCTGGGGG TACCGTGGGCGCCATAGCCATAGTTCCCGGGTATACGGCACGGCAGTCATCAATTAAATGGTGGGG ACCGCGTCTGGCATCCCACGGTTTCGTAGTAATTACAATTGACACAAATTCCACGTTAGACCAGCC AGAAAGTCGGAGTTCGCAACAAATGGCCGCGCTGGAACAGGTGGCGTCGTTAAACGGTACAAGT AGCAGCCCGATTTACGGAAAGGTCGATACCGCTCGTATGGGTGTTATGGGGTGGAGTATGGGAGGT GGAGGCTCCCTGATCTCTGCTTGCAACAACCCTTCGCTGAAAGCAGCGGTGGTGCAAGCACCATG GCATTCTTCGACAAATTTTAGTTGCGTAACTGTGCCCACGCTGATCTTCGCATGTGAAAACGATAGT ATAGCCCCGGTCAACTCTTCAGCACTTCCTATCTATGATTCTATGTCAGAAAACGCTAAGCAGTTTC TCGAAATTTGCGGTGGCTCACATTCCTGTGCGAATACCGGCAATTCTGACCAAGCATTAATCGGAA AAAAAGGCGTTGCATGGATGAAACGTTTTATGGACAATGATACTAGGTATTCTACTTTTGCCTGCGA GAACCCGAATAGCACCAGAGTGTGCGATTTTCGTACAGCGAATTGCAGC
Z2-PETase	ATGCGCGGCCCGGATCCGACAGCCGCCAGTTTGGAAGCGAGCGCTGGTCCATTCACCGTTCGCTC CTTTACCGTGAGTAGACCGAGCGGTTATGGCGCTGGCACCGTTTACTATCCAACAAATGCTGGGGG TACCGTGGGCGCCATAGCCATAGTTCCCGGGTATACGGCACGGCAGTCATCAATTAAATGGTGGGG ACCGCGTCTGGCATCCCACGGTTTCGTAGTAATTACAATTGACACAAATTCCACGTTAGACCAGCC AGAAAGTCGGAGTTCGCAACAAATGGCCGCGCTGGAACAGGTGGCGTCGTTAAACGGTACAAGT AGCAGCCCGATTTACGGAAAGGTCGATACCGCTCGTATGGGTGTTATGGGGTGGAGTATGGGAGGT GGAGGCTCCCTGATCTCTGCTTGCAACAACCCTTCGCTGAAAGCAGCGGTGGTGCAAGCACCATG GCATTCTTCGACAAATTTTAGTTGCGTAACTGTGCCCACGCTGATCTTCTGCTGTGAAAACGATAGT ATAGCCCCGTGCAACTCTTATGCACTTCCTATCTATGATTCTATGTCAGAAAACGCTAAGCAGTTTCT CGAAATTTGCGGTGGCTCACATTCCTGTGCGAATACCGGCAATTCTGACCAAGCATTAATCGGAAA AAAAGGCGTTGCATGGATGAAACGTTTTATGGACAATGATACTAGGTATTCTACTTTTGCCTGCGAG TGCCCGAATAGCACCAGAGTGTGCGATTGCCGTACAGCGAATTGCAGC

Supplementary Table 3. Data Collection and Refinement Statistics

Protein	4pC	4pC+P181V	8p-PETase	Z1-PETase	Z2-PETase
PDB deposit	8H5M	8H5O	8H5J	8H5K	8H5L
Molecules	1	1	2	1	3
Space group	$P2_12_12_1$	$P2_12_12_1$	C_12_1	$P2_12_12_1$	$P12_11$
Unit cell					
a, b, c (Å)	50.7, 67.8, 78.5	50.9.52.6,95.2	139.2, 50.7, 80.9	51.0, 51.7, 65.6	100.2, 55.6, 100.4
r (˚)	90.00	90.00	90.0, 124.6, 90.0	90.00	90.0, 119.1, 90.0
X-ray source	PAL-7A	PAL-7A	PAL-7A	PAL-7A	PAL-7A
Wavelength (Å)	0.97934	0.97934	0.97934	0.97934	0.97934
Energy (keV)	12.660	12.660	12.660	12.660	12.660
Resolution (Å)	28.23-1.90	29.01-2.10	28.65-1.40	25.05-1.20	27.97-1.70
Reflections					
Observed	1213143	597332	828222	1391065	1181822
Unique	22366	15494	90308	63488	99271
Completeness[1]	94.6 (90.6)	100.0 (99.8)	98.3 (96.8)	99.9 (99.2)	97.1 (92.3)
$< I / \sigma(I) >^1$	22.79 (4.74)	45.07 (16.61)	30.00 (2.88)	37.55 (2.78)	18.55 (3.45)
R_{merge} (%)[1]	9.3 (67.6)	11.4 (30.0)	9.8 (51.3)	6.4 (58.2)	15.9 (80.0)
CC1/2[1]	1.00 (0.62)	0.99 (0.95)	0.98 (0.74)	1.00 (0.84)	0.98 (0.73)
Protein atoms	1932	1948	4153	1998	5977
Ligands	1	0	13	12	10
Water	222	148	534	330	1068
R.m.s. deviations					
Bond lengths (Å)	0.0120	0.0112	0.0139	0.0132	0.0164
Bond angles (˚)	1.6611	1.6969	1.8883	1.7874	1.8167
R_{free} (%)	18.85	18.67	19.02	18.80	18.12
R_{factor} (%)	15.15	14.15	15.67	15.86	15.19
Mean B ($Å^2$)[2]	14.08	21.39	17.28	13.98	13.24