Reference Number	Catalog Number	Substrate Sequence
1	**PEPDAB011**	**Dabcyl-Gly-Pro-Leu-Gly-Met-Arg-Gly-Lys(5-FAM)-NH2**
2	PEPDAB014	Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Lys(5-FAM)-NH2
3	**PEPDAB021**	**Dabcyl-Val-Pro-Val-Asn-Norleu-Thr-Val-Lys(5-FAM)-NH2**
4	PEPDAB015	Dabcyl-Val-Asp-Leu-Phe-Tyr-Leu-Gln-Gln-Pro-Lys(5-FAM)-NH2
5	**PEPDAB008**	**Dabcyl-Pro-Cha-Gly-Cys(Me)His-Ala-Lys(5-FAM)-NH2**
6	PEPDAB022	Dabcyl-Leu-Arg-Ala-Glu-Gln-Gln-Arg-Leu-Lys-Ser-Lys(5-FAM)-NH2
7	**PEPDAB005**	**Dabcyl-Leu-Ala-Gln-Ala-Homophenylalanine-Arg-Ser-Lys(5-FAM)-NH2**
8	PEPDAB017	Dabcyl-Ala-Pro-Arg-Trp-Ile-Gln-Asp-Lys(5-FAM)-NH2
9	**PEPDAB010**	**Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(5-FAM)-NH2**
10	PEPDAB052	Dabcyl-Ala-Pro-Phe-Glu-Met-Ser-Ala-Lys(5-FAM)-NH2
11	**PEPDAB016**	**Dabcyl-Ser-Asn-Leu-Ala-Tyr-Tyr-Thr-Ala-Lys(5-FAM)-NH2**
12	PEPDAB059	Dabcyl-Ala-Pro-Arg-Trp-Leu-Thr-Thr-Ala-Lys(5-FLU)-NH2
13	**PEPDAB053**	**Dabcyl-Ala-Pro-Phe-Glu-Phe-Ser-Ala-Cys(5-FLU)-NH2**
14	PEPDAB012	Dabcyl-Val-Pro-Phe-Glu-Phe-Thr-Val-Lys(5-FAM)-NH2
15	**PEPDAB013**	**Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(5-FAM)-NH2**
16	PEPDAB020	Dabcyl-Val-Pro-Thr-Trp-Ile-Gln-Asp-Lys(5-FAM)-NH2
17	**PEPDAB200**	**Dabcyl-GABA-Arg-Pro-Lys-Pro-Val-Glu-Norvaline-Ala-Arg-Cys(5-FLU)-Gly-CONH2**
18	PEPDAB201	Dabcyl-GABA-Pro-Gln-Gly-Leu-Cys(5-FLU)-Ala-Lys-CONH2

Table S1: Synthetic polypeptide FRET-substrate sequences. Reference numbers denote substrate indices used throughout the paper and in the online supplemental data.

Text S1: Comments regarding substrate depletion. Substrate depletion often occurs in our experiments involving purified recombinant enzyme. For example, roughly 70% of the initial substrate is degraded in the reaction shown in Fig. 1A. Accounting for substrate depletion extends the quantitative range of the assay and has no negative impact on inference when actual depletion is negligible (Fig. 1B). Lastly, substrates can become depleted in live-cell applications. For example, basal proteolytic activity in mouse embryonic fibroblasts results in roughly 25% of substrate 6 being degraded within three hours (Fig. S17). After three hours, raw fluorescence for that sample is ~6000 FLU. Peak fluorescence of the corresponding positive control is roughly ~25000 FLU (data not shown).

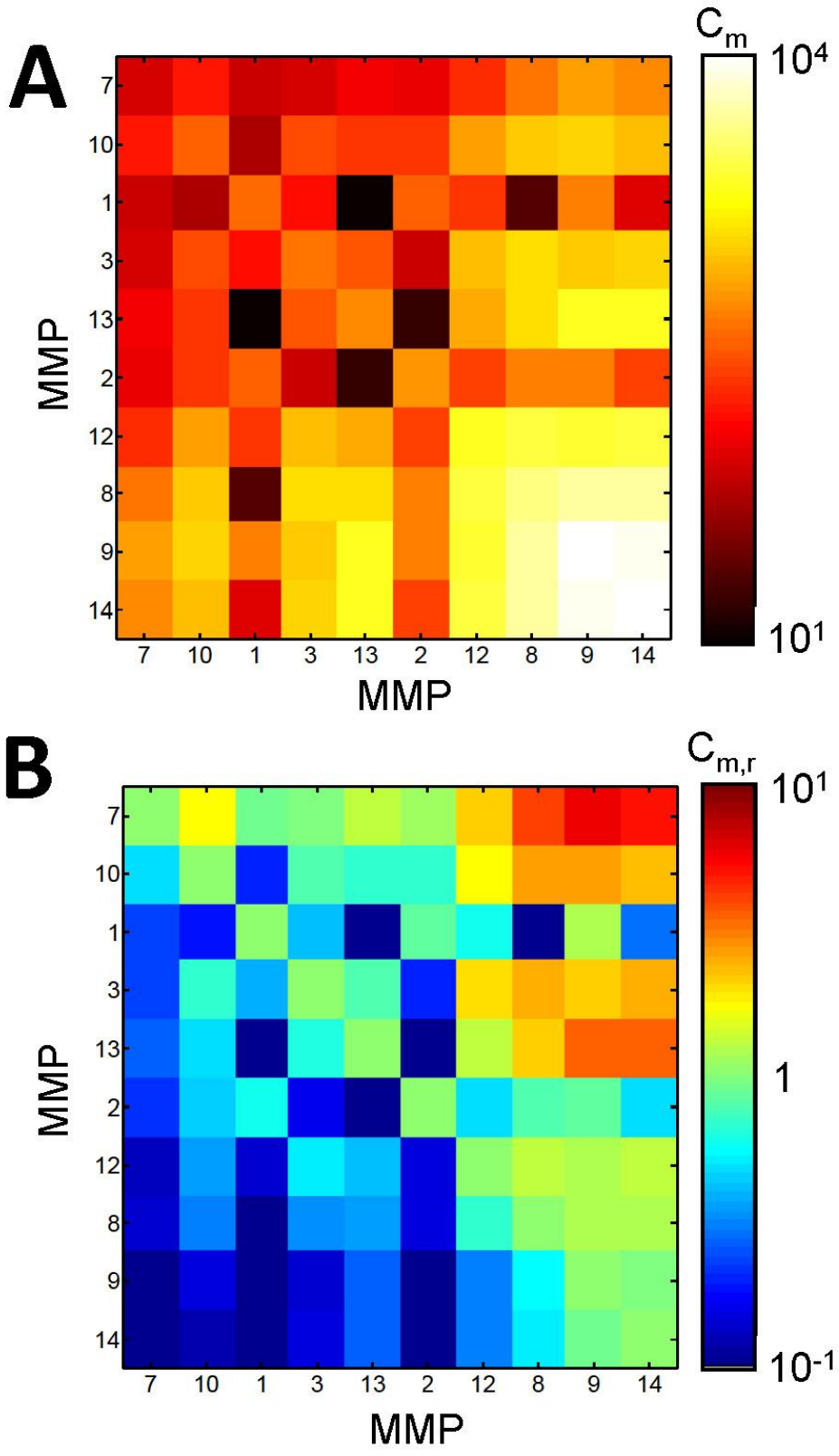

Figure S1: R_M transformed to emphasize additive rather than multiplicative error. Columns of the parameter matrix **C** were normalized by their Euclidean norm prior to calculation of R_M.

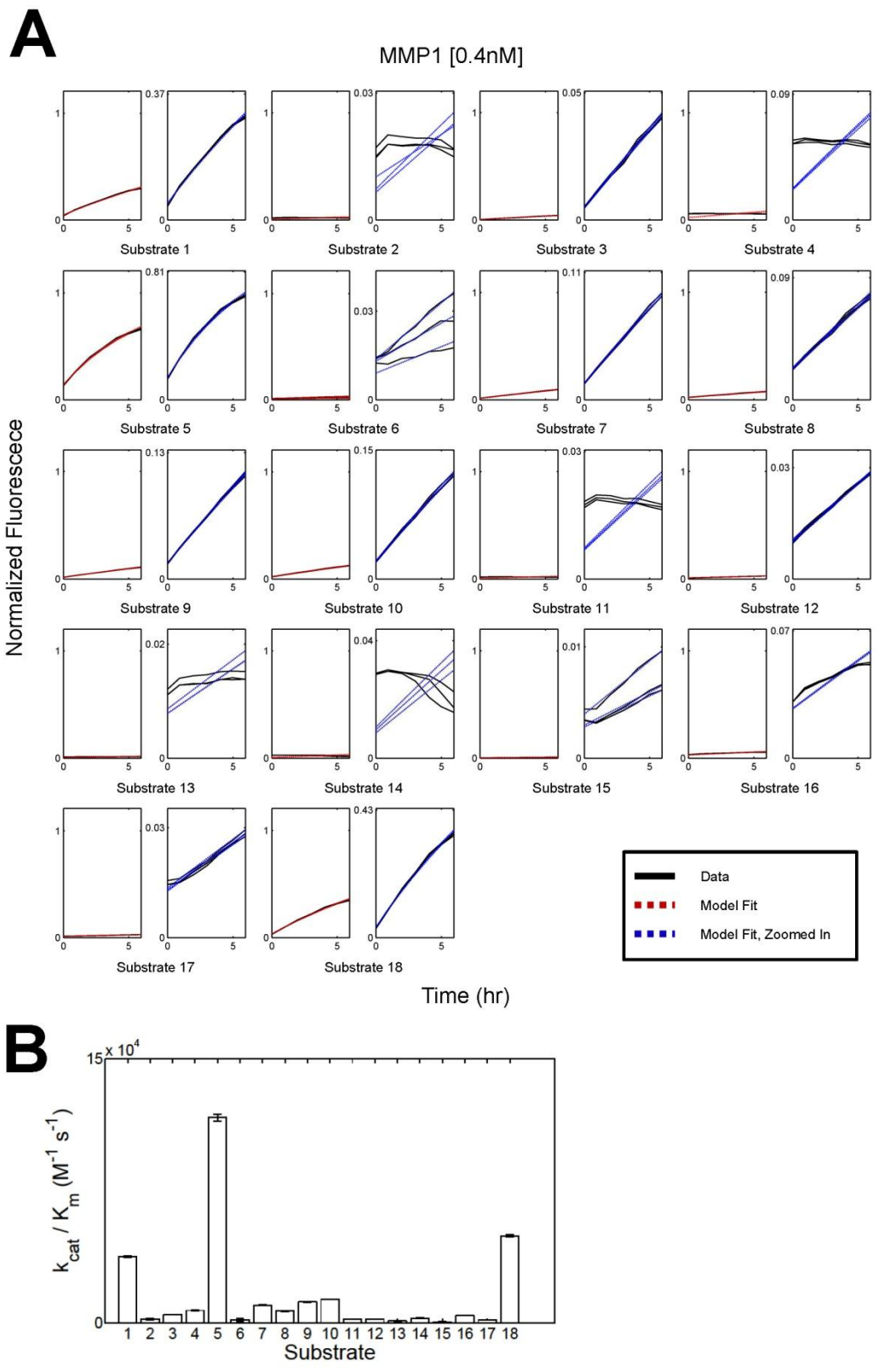

Figure S2: Example Cleavage Signature of MMP1. k_{cat}/K_m values were determined using MMP1 at 0.4nM and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) k_{cat}/K_m values are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

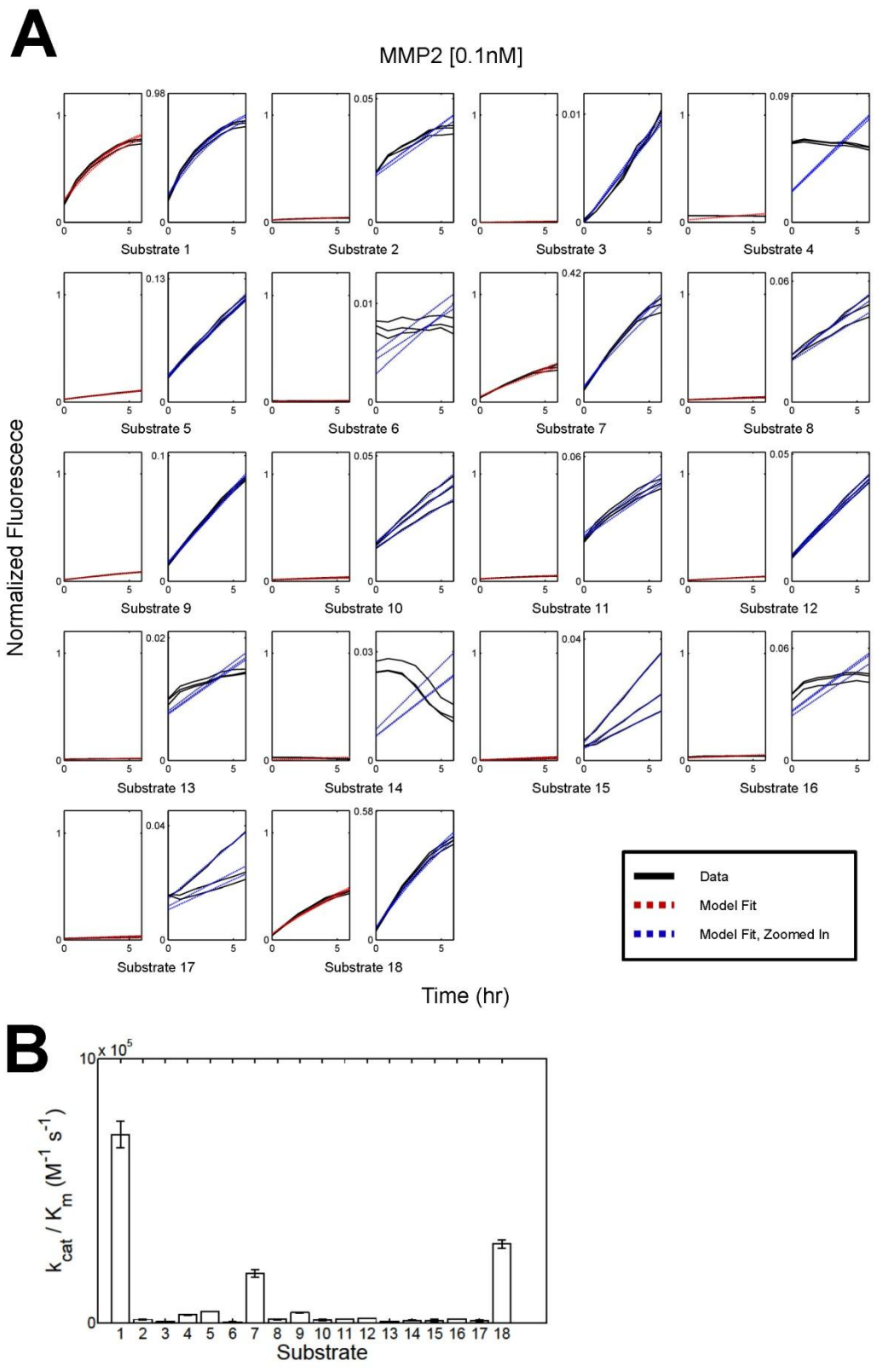

Figure S3: Example Cleavage Signature of MMP2. k_{cat}/K_m values were determined using MMP2 at 0.1nM and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) k_{cat}/K_m values are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

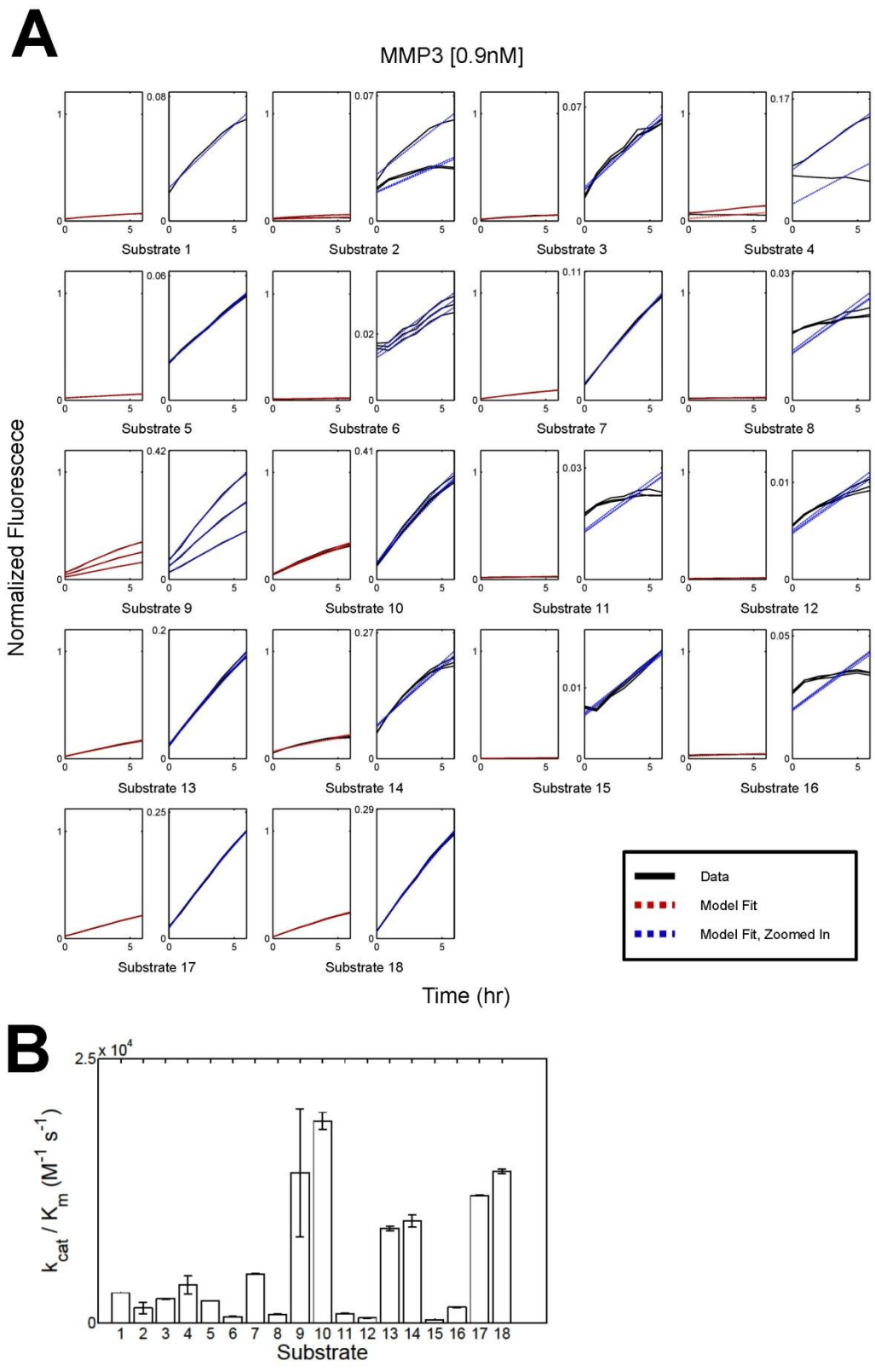

Figure S4: Example Cleavage Signature of MMP3. k_{cat}/K_m values were determined using MMP3 at 0.9nM and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) k_{cat}/K_m values are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

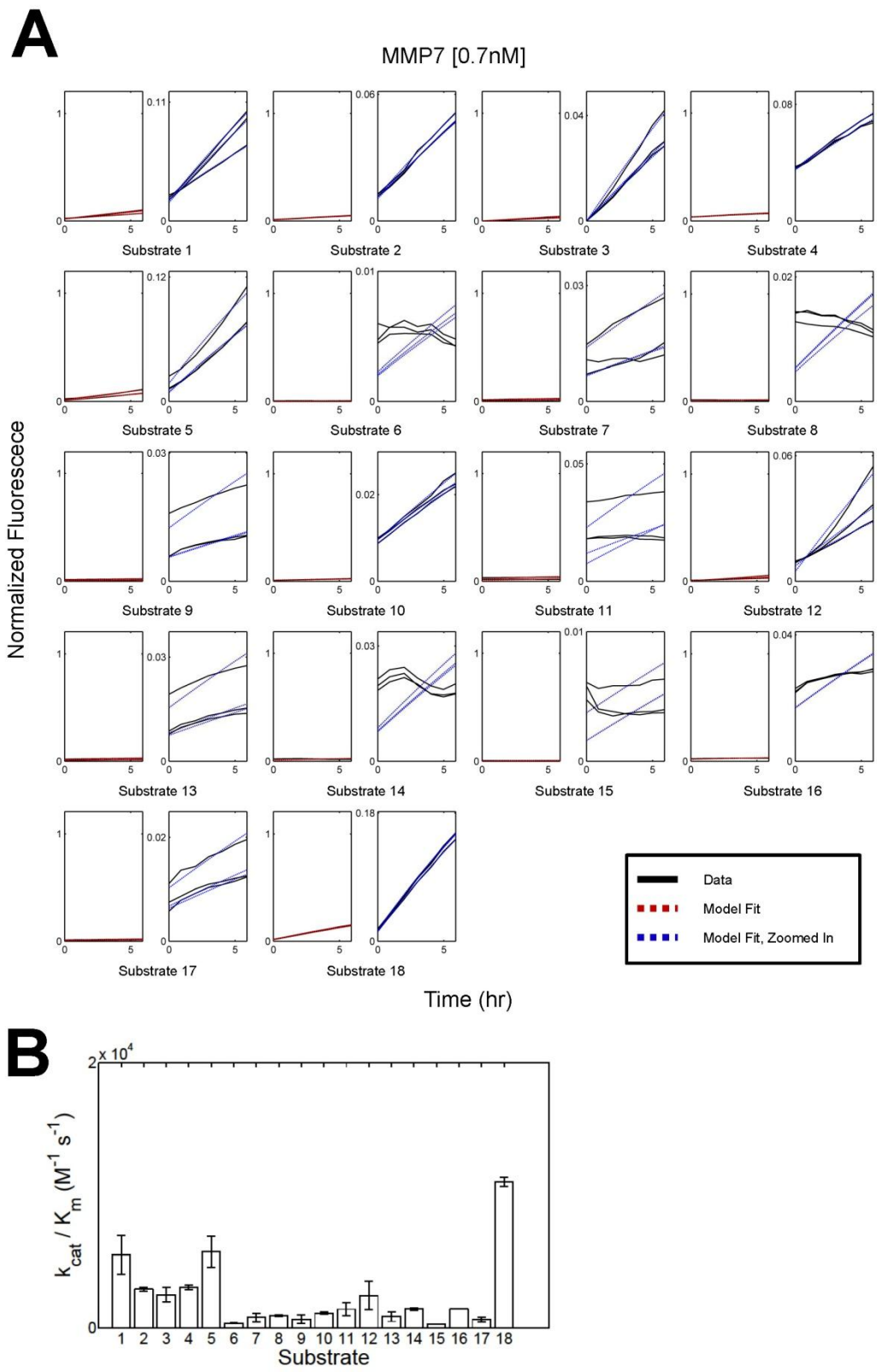

Figure S5: Example Cleavage Signature of MMP7. k_{cat}/K_m values were determined using MMP7 at 0.7nM and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) k_{cat}/K_m values are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

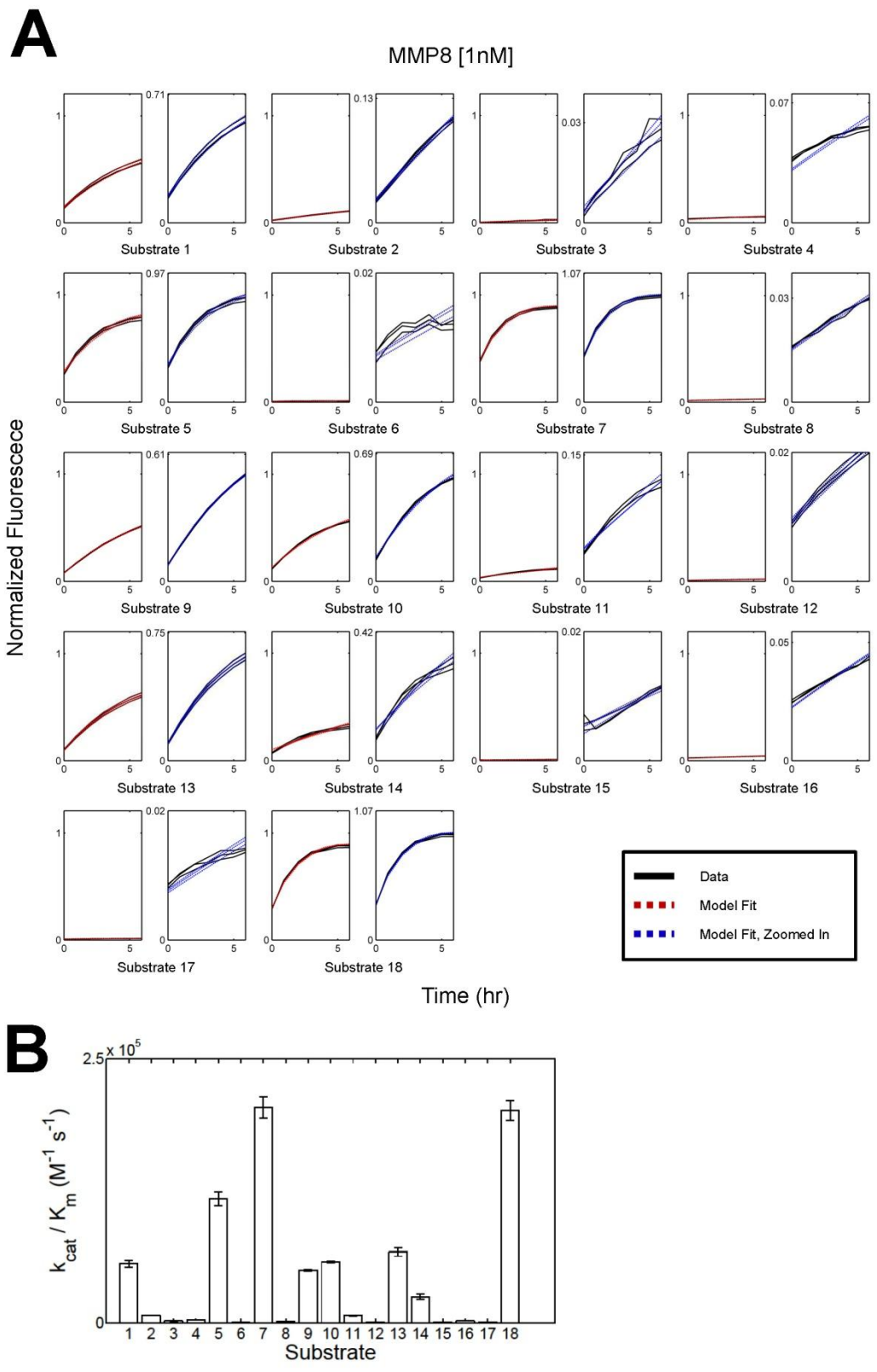

Figure S6: Example Cleavage Signature of MMP8. k_{cat}/K_m values were determined using MMP8 at 1nM and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) k_{cat}/K_m values are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

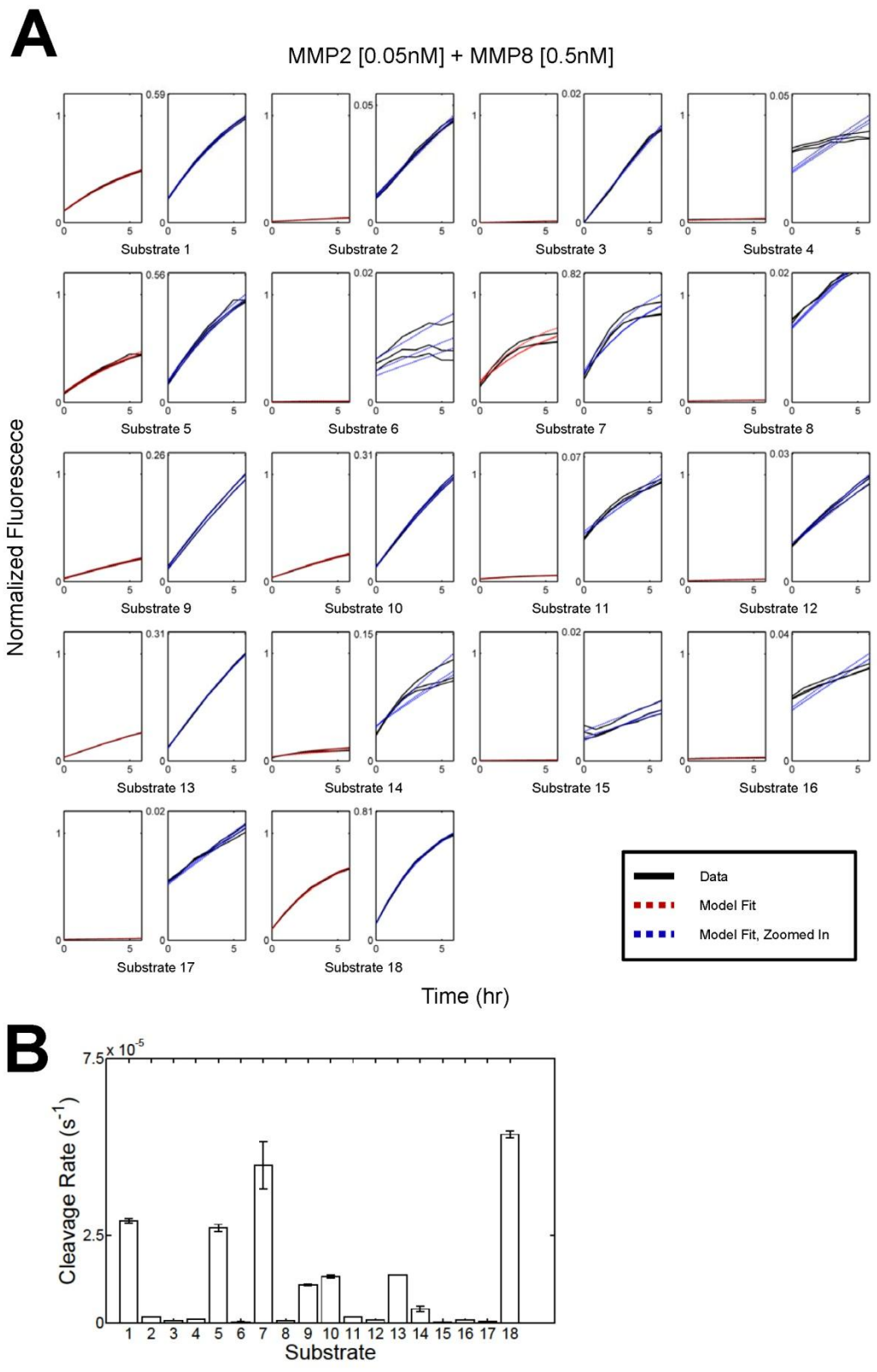

Figure S7: Example Cleavage Signature of MMP mixture. k_{cat}/K_m values were determined using 0.05nM MMP2, 0.5nM MMP8, and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) Cleavage rates are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

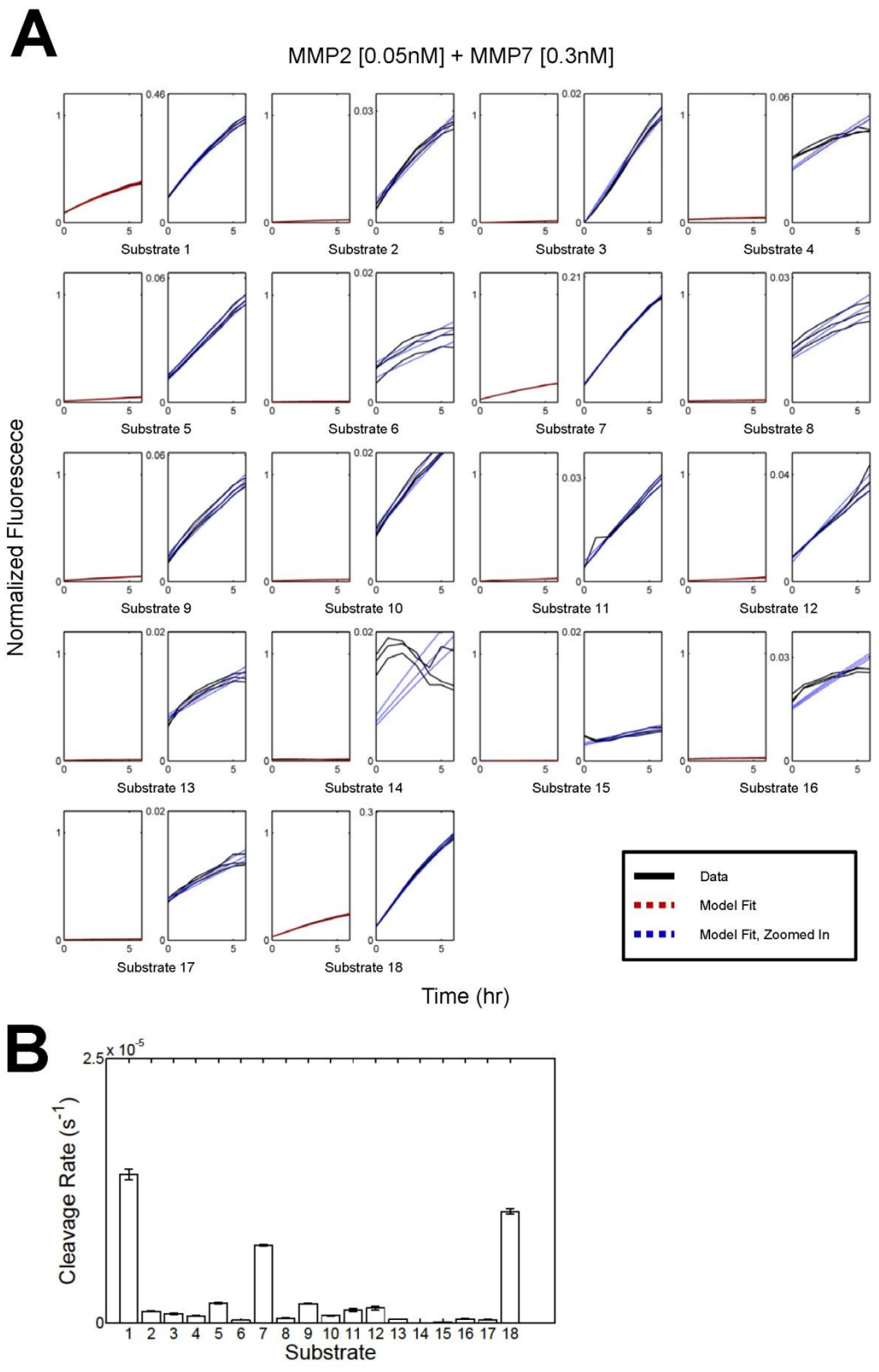

Figure S8: Example Cleavage Signature of MMP mixture. k_{cat}/K_m values were determined using 0.05nM MMP2, 0.3nM MMP7, and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) Cleavage rates are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

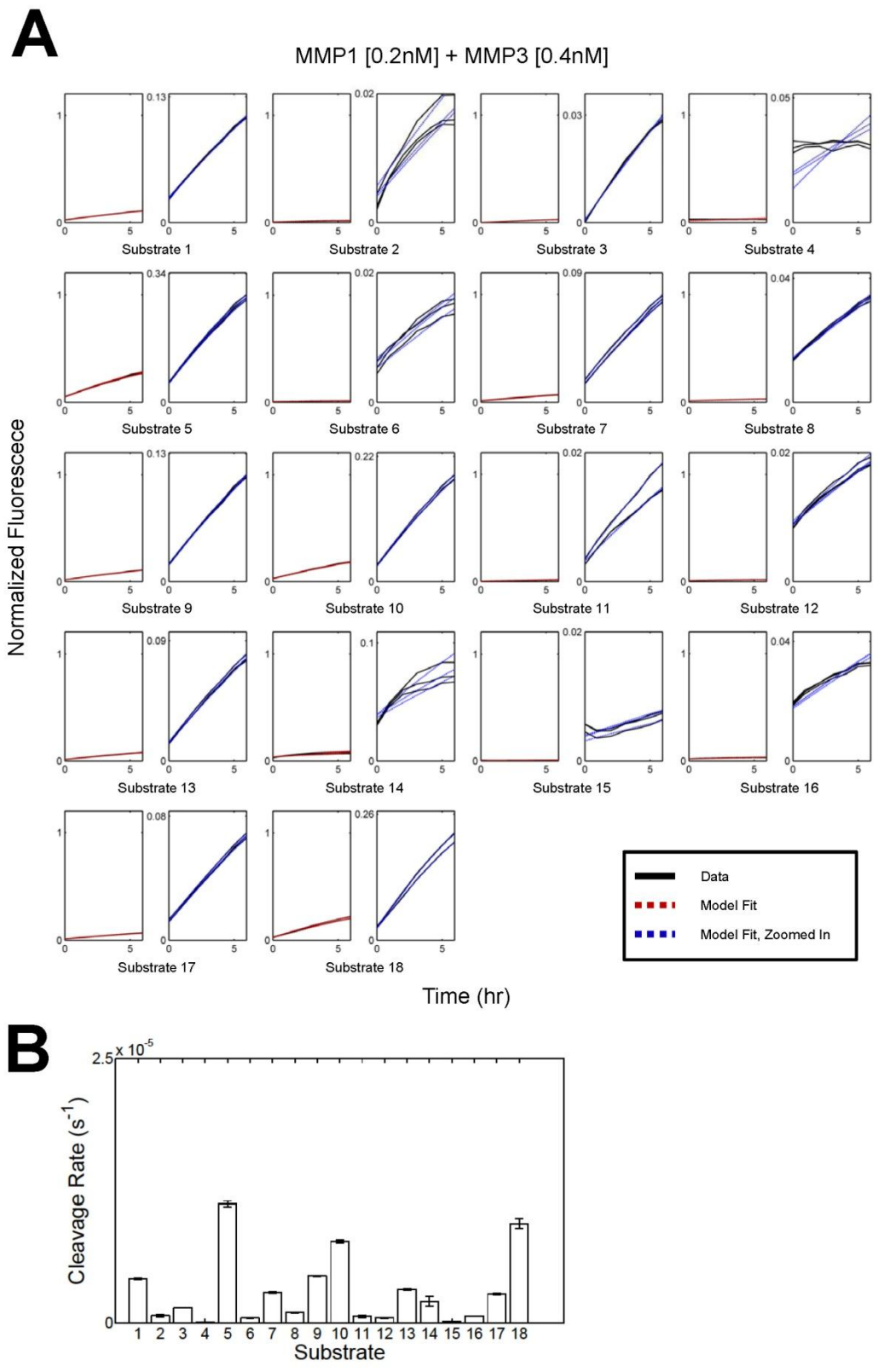

Figure S9: Example Cleavage Signature of MMP mixture. k_{cat}/K_m values were determined using 0.2nM MMP1, 0.4nM MMP3, and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) Cleavage rates are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

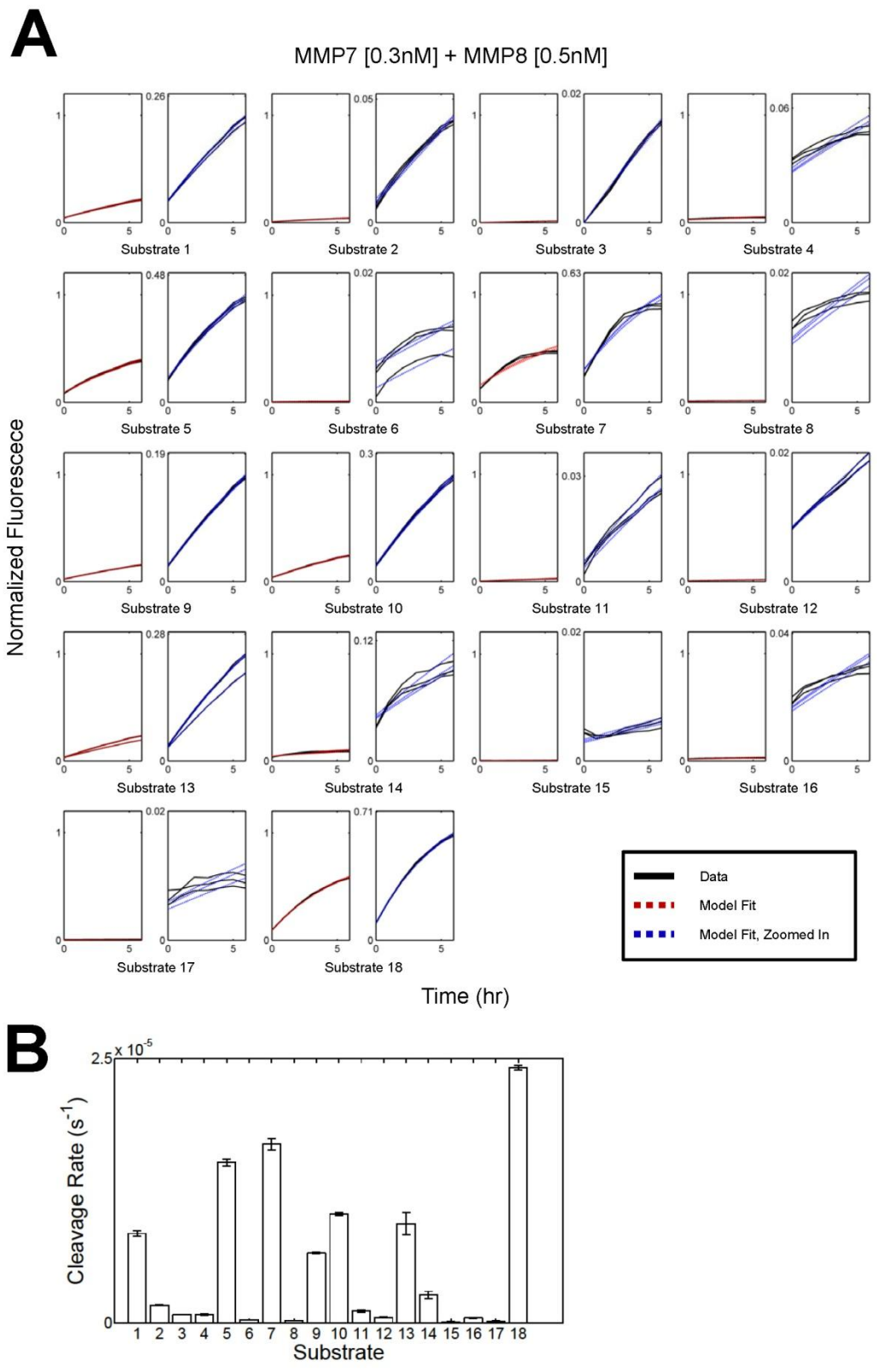

Figure S10: Example Cleavage Signature of MMP mixture. k_{cat}/K_m values were determined using 0.3nM MMP7, 0.5nM MMP8, and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) Cleavage rates are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

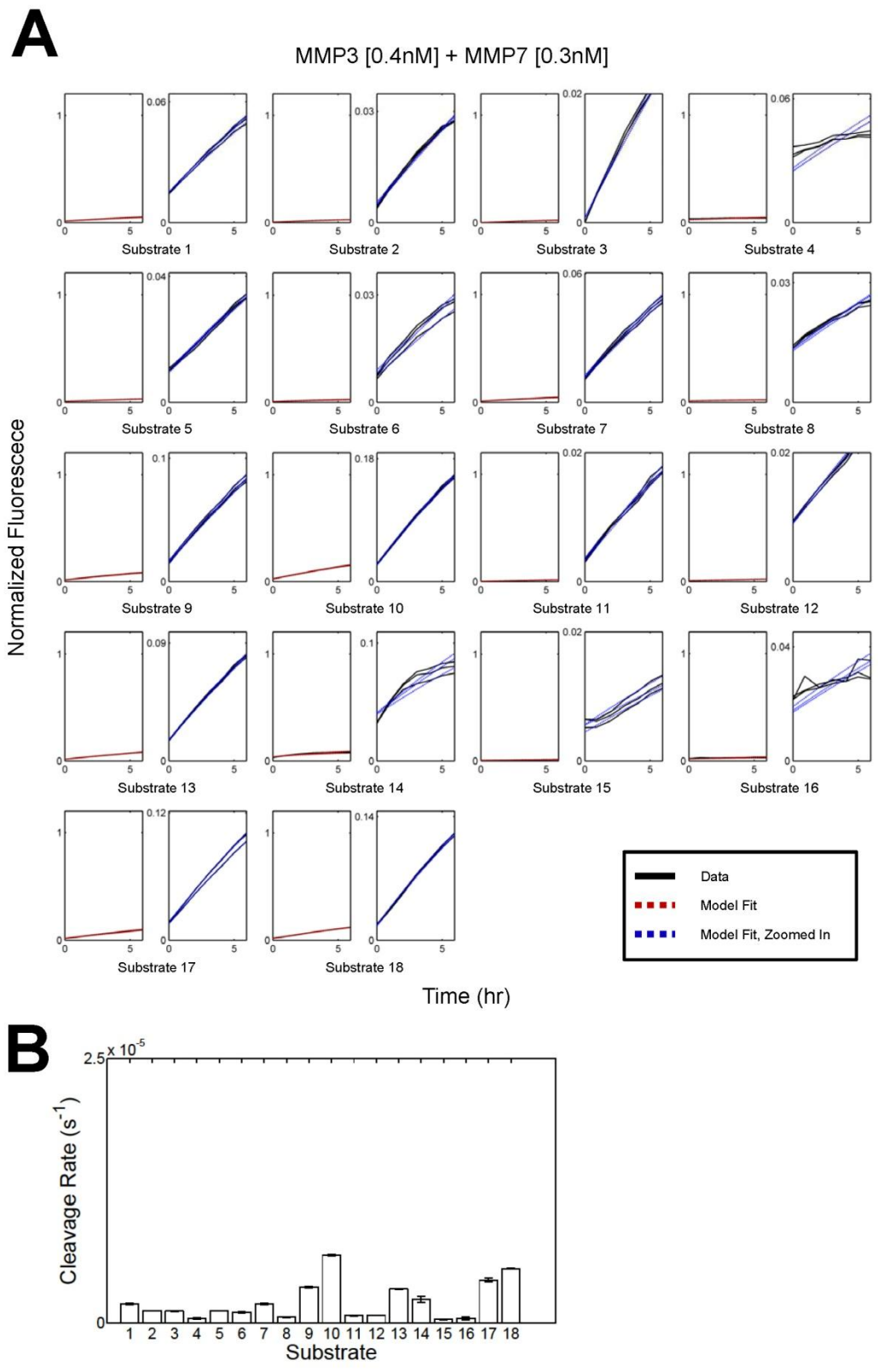

Figure S11: Example Cleavage Signature of MMP mixture. k_{cat}/K_m values were determined using 0.4nM MMP3, 0.3nM MMP7, and individual substrates at 10uM in MMP minimal buffer (see text). (A) Time-lapse fluorimetry data, normalized to the positive control signal, $F_{0,S}$. Blue and red plots are paired, such that blue plots show the identical data but at a zoomed in scale on the y-axis. Both blue and red lines indicate fit to the non-linear kinetic model. Individual lines indicate technical replicates. (B) Cleavage rates are inferred from each of the time-courses shown in A. Error bars indicate standard deviation.

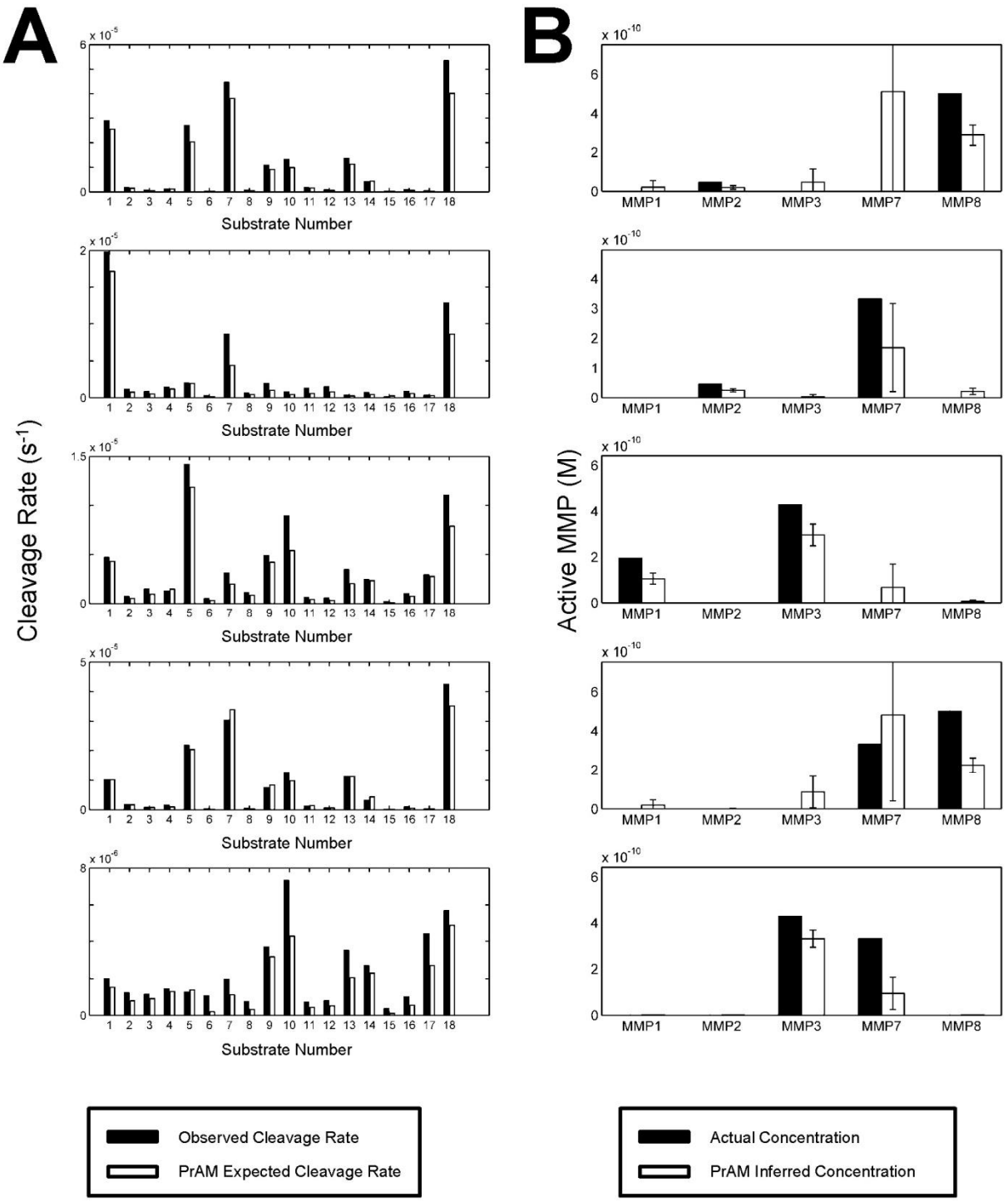

Figure S12: Summarized Cleavage Signatures of MMP mixtures and PrAM Inference Results. (A) Cleavage rates were observed for various mixtures of MMPs (black, Figs. S6-S10) and compared to rates expected by PrAM based on the known mixture composition and observed cleavage rates for single enzyme mixtures (white, Figs. S1-S5). (B) Actual mixture compositions (black) and PrAM inference results (white) correspond to the cleavage signatures in *A*. Error bars indicate std. dev. of inference, using 20% synthetic sampling error.

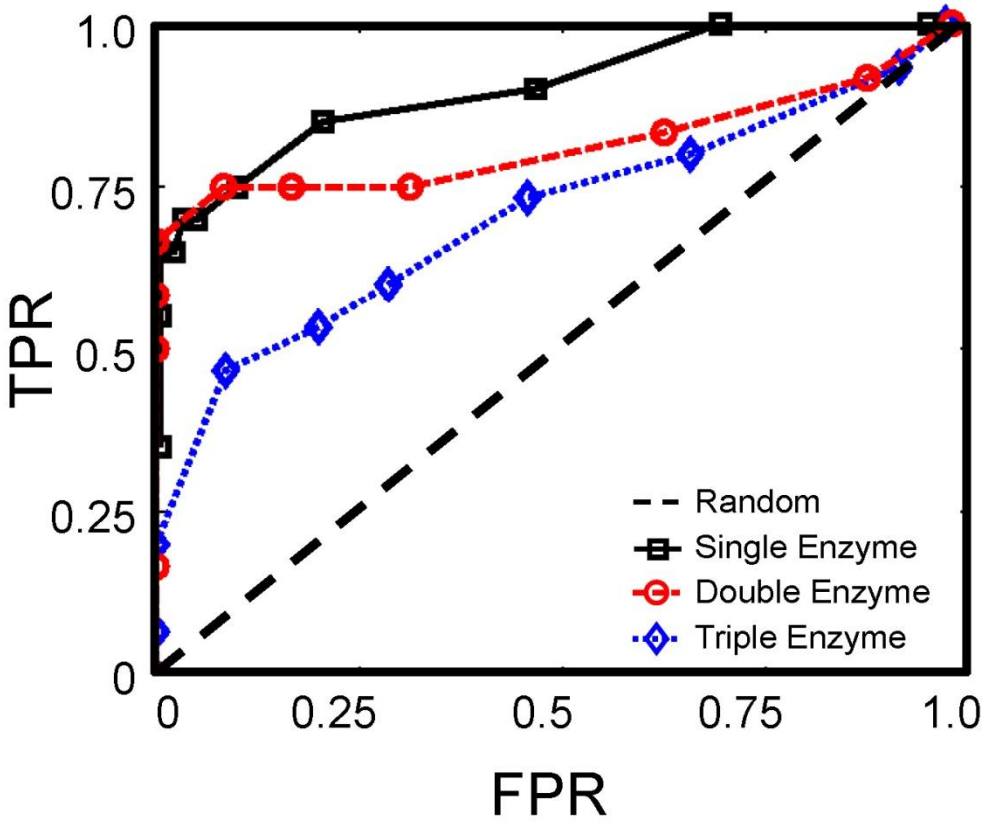

Figure S13: PrAM inference using alternative bootstrapping scheme. PrAM inference was conducted on enzyme mixtures containing MMPs 1--8, corresponding to Fig. 5A, but using a modified bootstrapping scheme that more directly considers uncertainty in the parameter matrix **C**. Max accuracy is roughly 95% for single and double enzyme mixtures. For triple enzyme mixtures, max accuracy is roughly 80%.

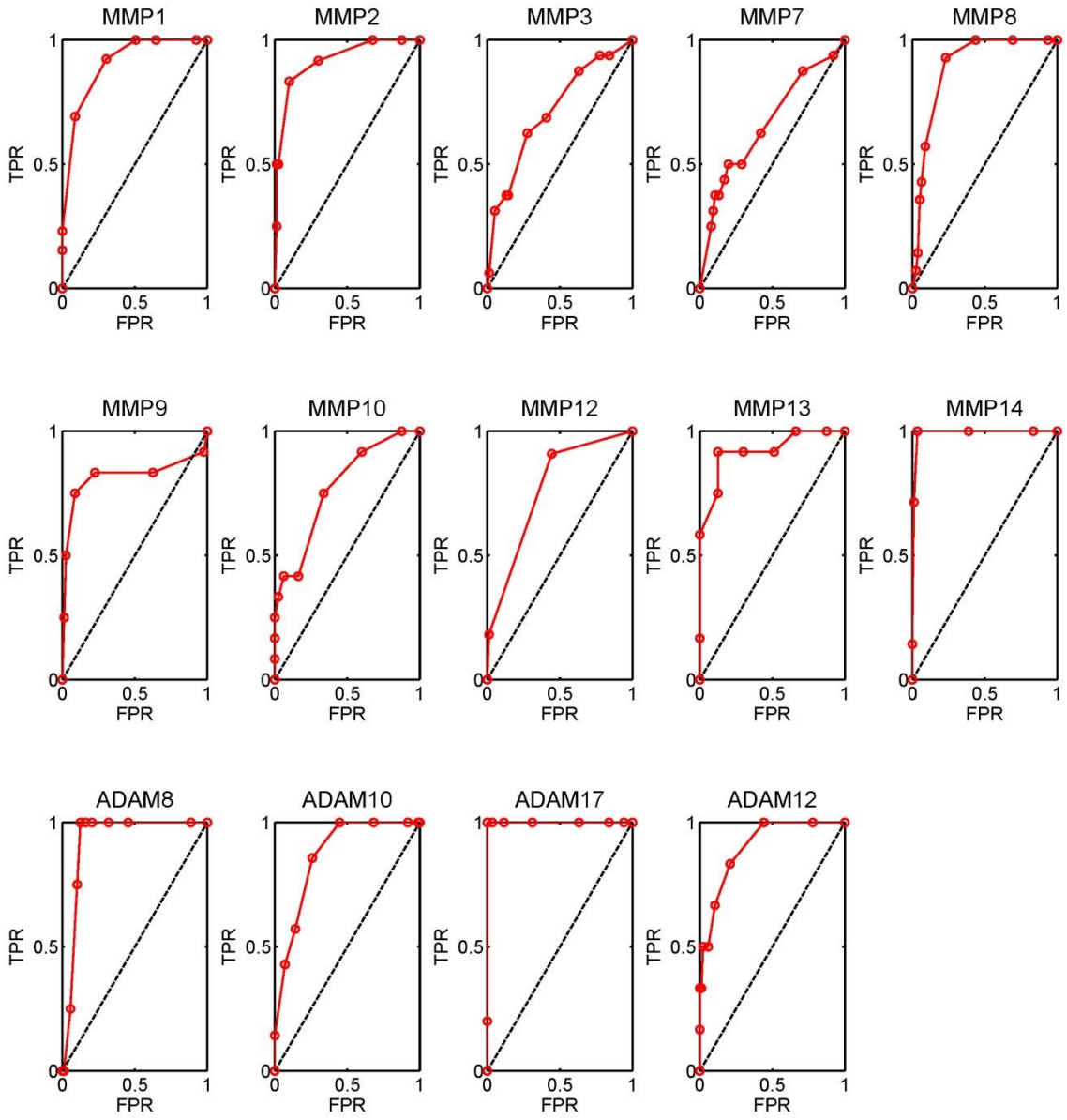

Figure S14: ROC Curves describing inference accuracies of individual enzymes. PrAMA inference corresponds to results shown in Fig. 6A,B,E. True positive rate (TPR) and false positive rate (FPR) refer to inference results for each individual enzyme, for all mixtures tested and using a 16 substrate x 14 enzyme parameter matrix **C** (see Fig. 6A).

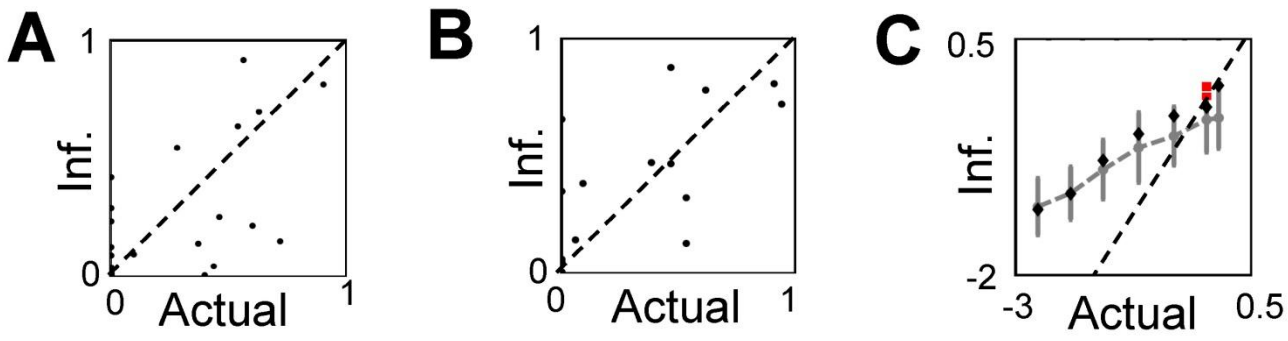

Figure S15: PrAM quantitative inference. PrAM inference was performed on all double enzyme mixtures involving MMPs 1-8 (A) and ADAMs 10 & 17 (B). These plots show actual concentrations vs. PrAM inferred concentrations, where enzyme levels were first normalized such that total enzyme concentration in a given mixture sums to unity. R^2 values for (A) and (B) are both roughly 0.5. (C) Black diamonds indicate PrAM inference (ordinate) of MMP7 at various actual concentrations (abscissa), based on the cleavage signature $\mathbf{V_0}$ from substrates 1-16. Red points show PrAM inference of MMP7 in conditioned media from breast cancer cells. Axes are $\log_{10}$-transformed and scaled such that the maximum actual concentration = 1. All MMPs were considered in the parameter matrix **C.** The grey line indicates inferred $V_{0,i}$, averaged over all 16 substrates, with standard deviation shown by error bars. The dashed line indicates linear inference.

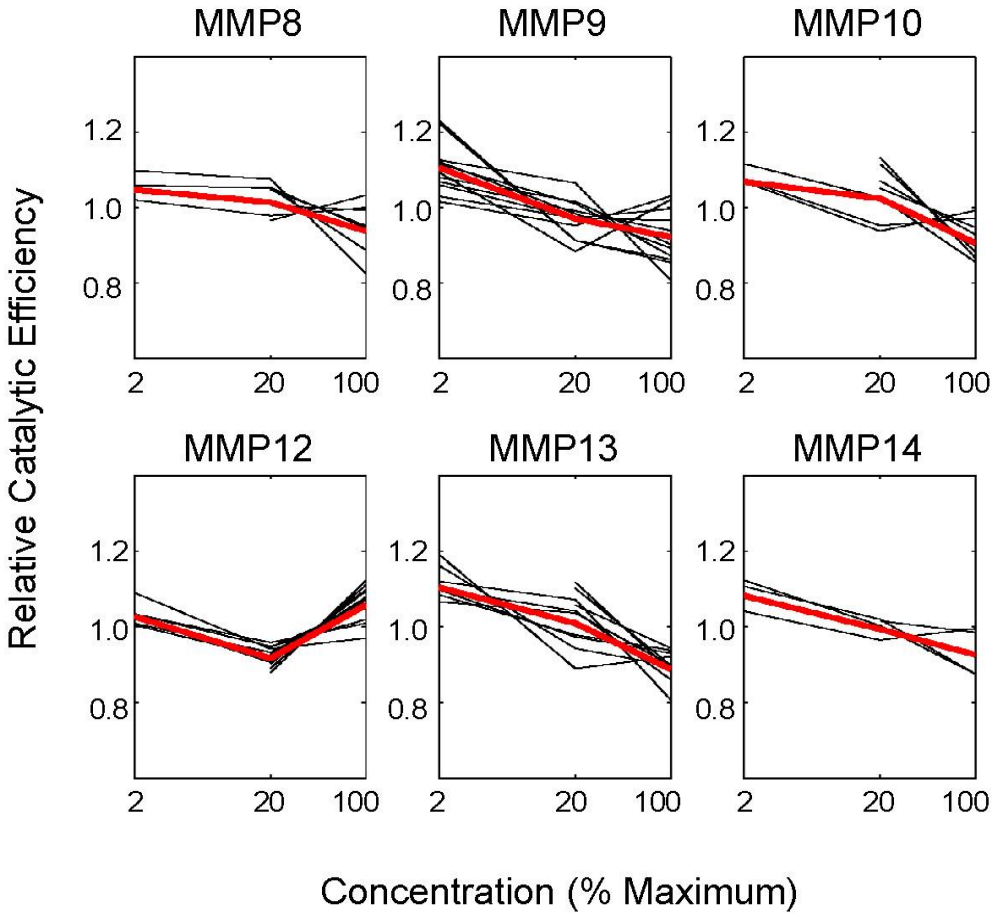

Figure S16: Concentration effects on enzyme catalytic efficiency. Substrate catalytic efficiencies $C_{i,j}$ were calculated at three different concentrations of MMPs (see Fig. 3) using 16 substrates. We define relative catalytic efficiency as the log-transformed $C_{i,j}$ divided by the average log-transformed $C_{i,j}$ from all MMP concentrations. Each black line corresponds to the relative catalytic efficiencies observed from individual substrates, and the red line denotes the average over all substrates. Experiments with the lowest two concentrations used MMP buffer, and the highest concentration experiments used MEBM. We only considered C_{ij} measurements detected significantly above background signal (see supplemental data).

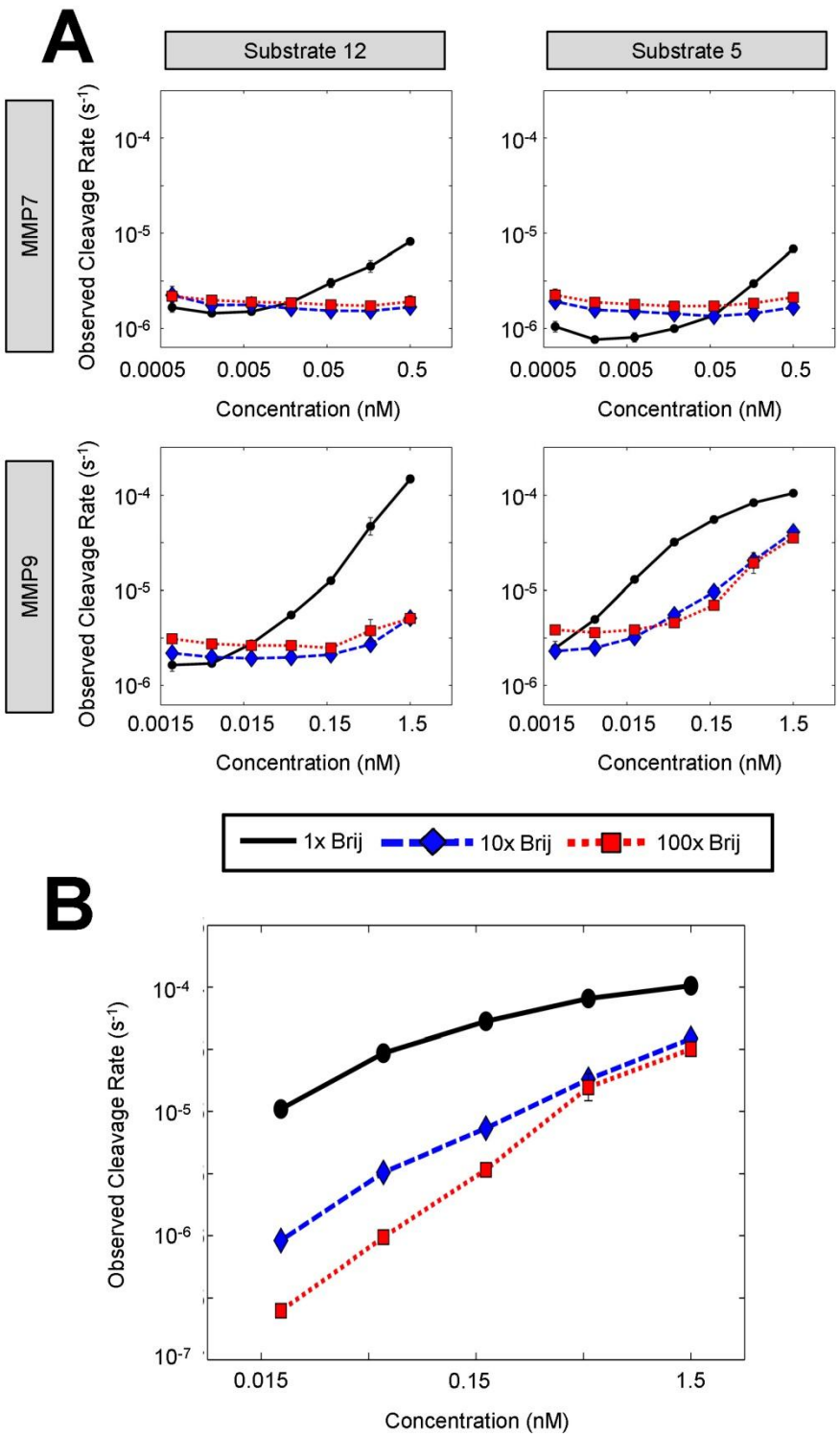

Figure S17: The impact of Brij-35 on MMP proteolytic activity. MMP7 and MMP9 proteolytic activities were observed in MMP buffer with increasing amounts of Brij-35 (10x and 100x the concentration for MMP buffer). (A) Cleavage rates ($\mathbf{V_0}$ / [S]) were calculated from time-lapse fluorimetry data. To emphasize Brij effects on background proteolysis, background cleavage rates were not subtracted in this case. (B) MMP9 cleavage of substrate 5, corresponding to the lower right panel of *A*. In this case, the background cleavage rates for each Brij concentration were subtracted from the total observed rates. The slopes for the 1x, 10x, and 100x Brij lines on log-log axes are {0.50, 0.82, and 1.0}. A slope of 1.0 indicates assay linearity.

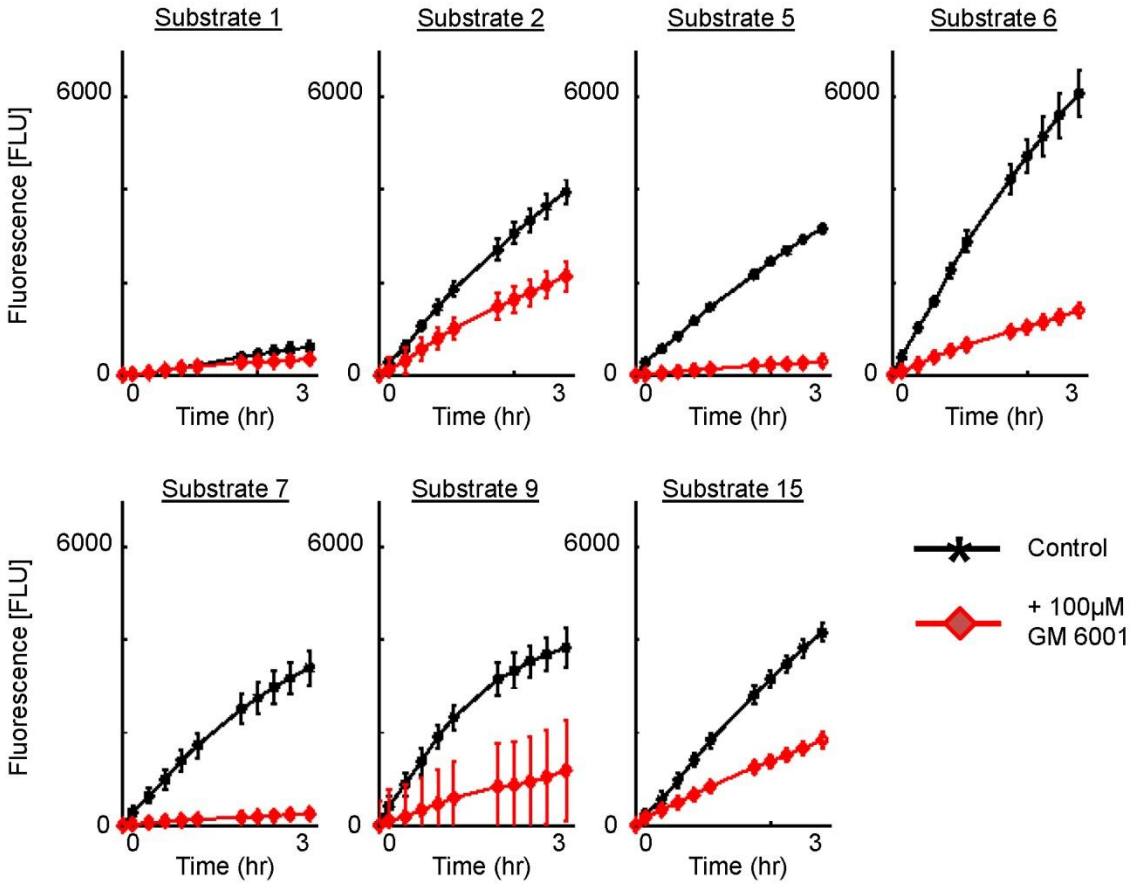

Figure S18: Impact of GM6001 treatment on observed protease activity. WT MEFs were seeded at 30,000 cells per well in a 384-well plate. The following day, media was replaced with fresh media +/- 100μM GM6001. After an hour incubation with the inhibitor, cells were treated with fresh 100μM GM6001 and one of seven FRET substrates, used at a final concentration of 10μM. Cells were imaged for 3hrs at 37^{o}C using a plate reader. Error bars indicate standard deviation of biological quadruplicates.