

Supplementary Materials for
**Combinatorially restricted computational design of protein-protein interfaces
to produce IgG heterodimers**

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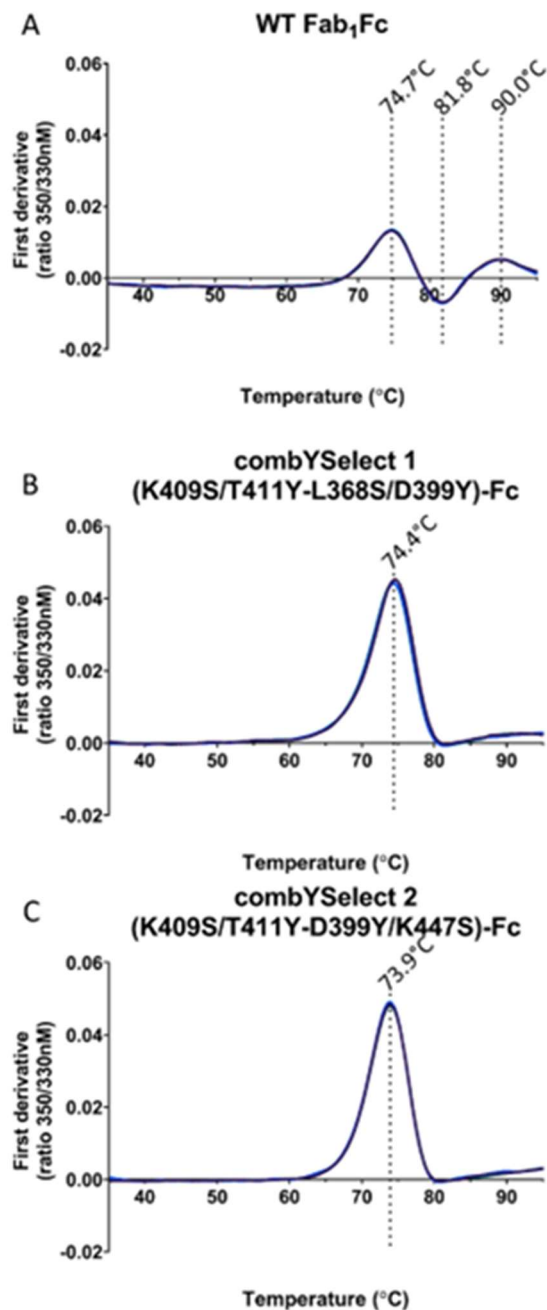
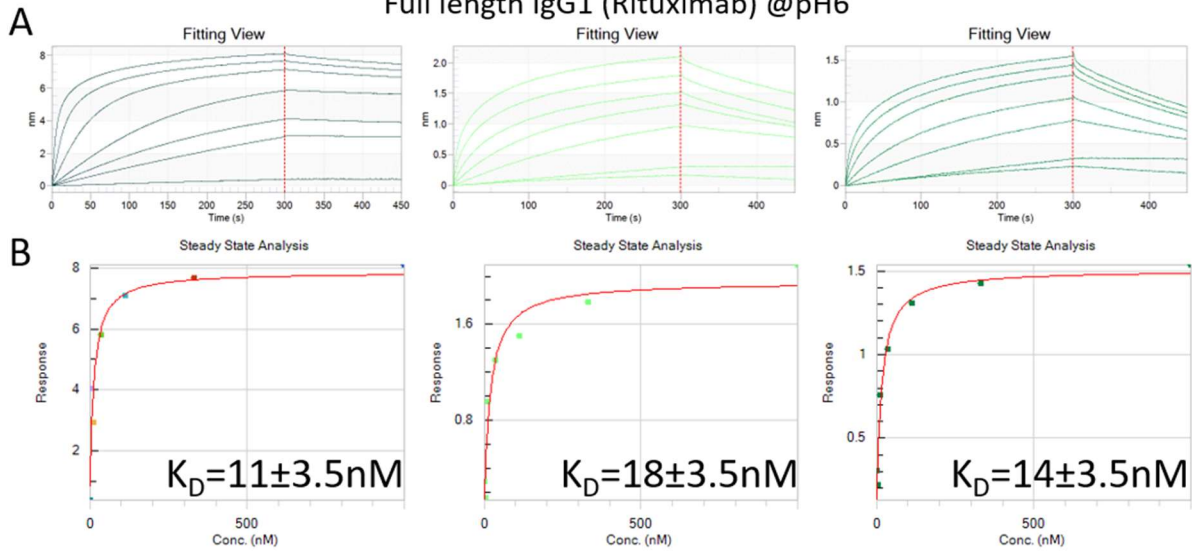


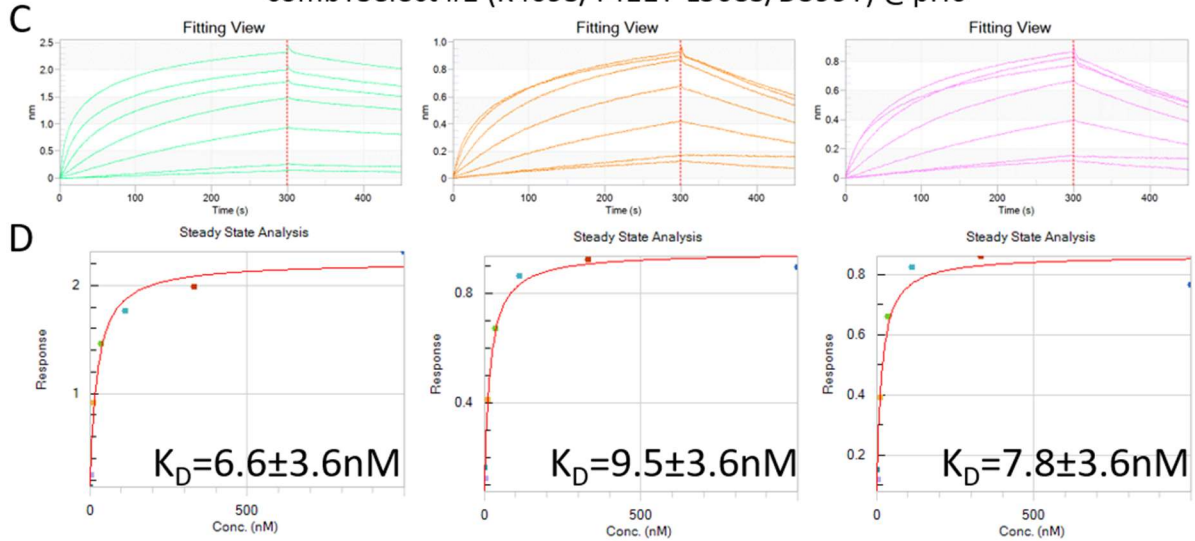
Fig. S1. Stability of IgG heterodimers.

First derivative plots of the melting temperatures of the Fab1Fc for WT (A), combYSelect #1 (L368S/D399Y-K409S/T411Y) Fc (B), and combYSelect #2 (D399Y/K447S-K409S/T411Y) Fc (C). The labelled temperatures are the mean of 6 total replicates, 3 for each orientation in which the mutations per chain are on the Fc fragment only, or on the Fc and Fab fragment.

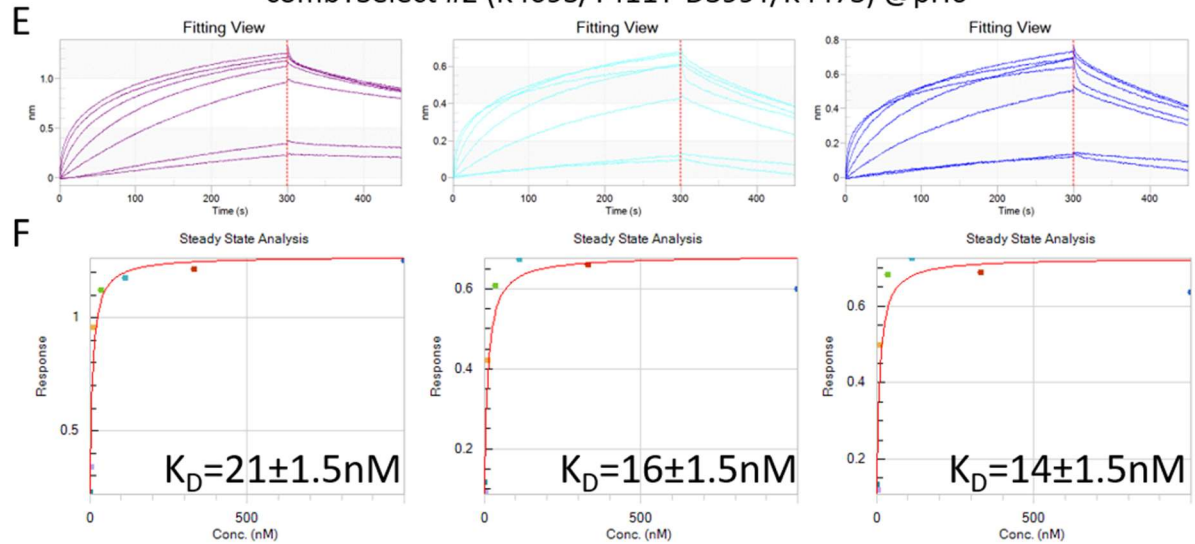
Full length IgG1 (Rituximab) @pH6



combYSelect #1 (K409S/T411Y-L368S/D399Y) @pH6



combYSelect #2 (K409S/T411Y-D399Y/K447S) @pH6



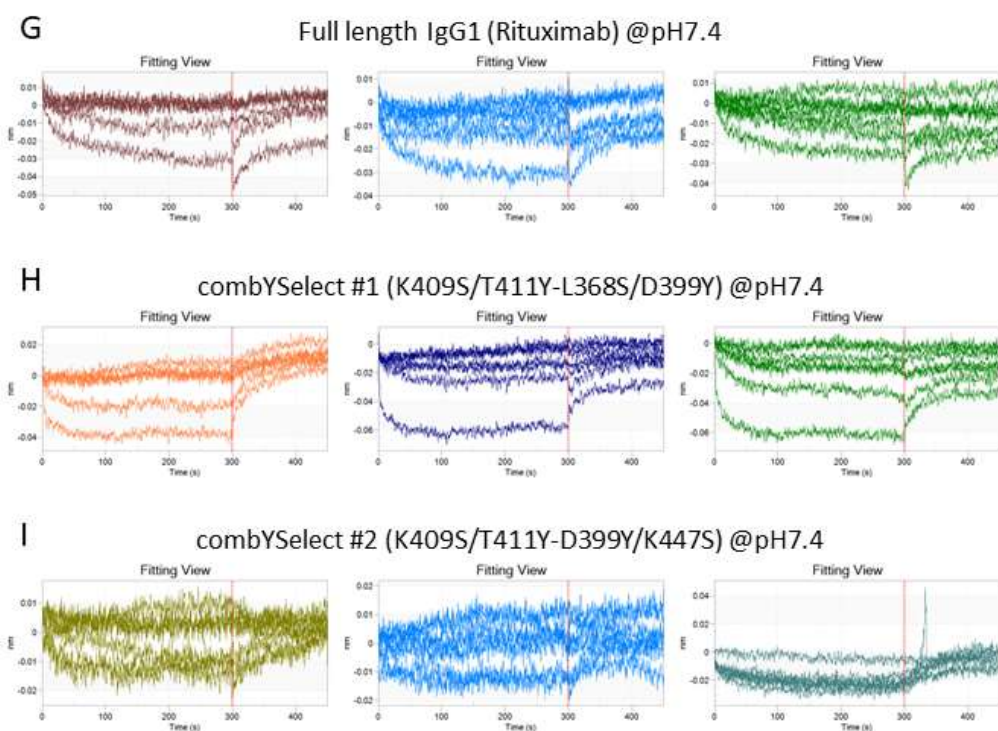


Fig. S2. Function of IgG heterodimers.

Sensograms and steady state plots at pH6 for WT-IgG (**A-B**), combYSelect #1 (L368S/D399Y-K409S/T411Y) (**C-D**), combYSelect #2 (D399Y/K447S-K409S/T411Y) (**E-F**) and at pH7.4 for WT-IgG (**G**), combYSelect #1 (L368S/D399Y-K409S/T411Y) (**H**) and combYSelect #2 (D399Y/K447S-K409S/T411Y) (**I**).

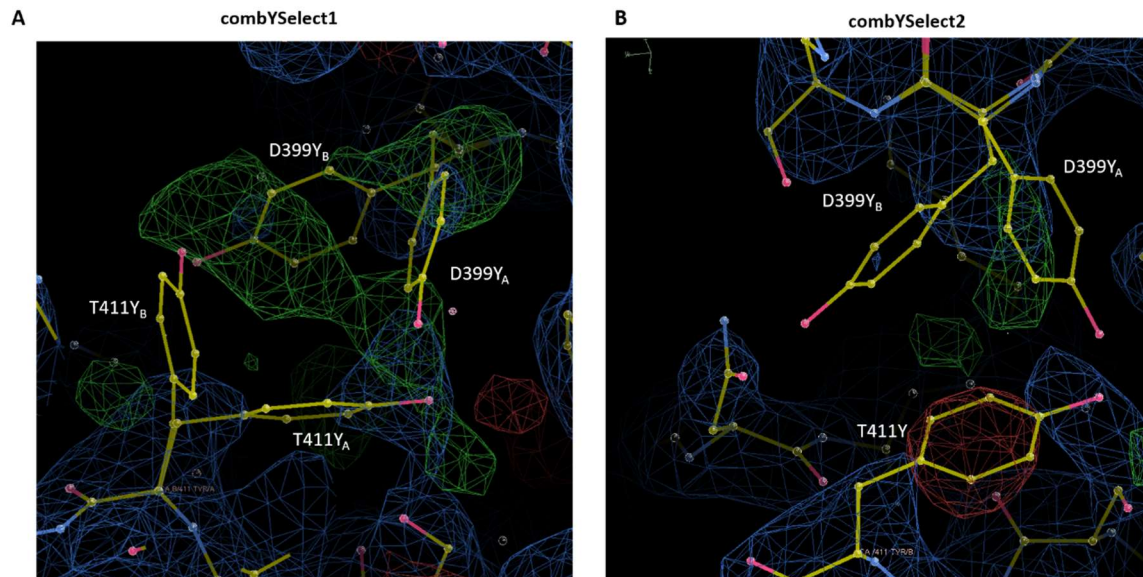


Fig. S3. Electron density maps for T411Y and D399Y mutations of combYSelect1 and 2. Electron density maps of the D399Y and T411Y mutations from the final refined structural models of combYSelect1 (**A**) and combYSelect2 (**B**). 2Fo-Fc map is in blue contoured to 1σ and the Fo-Fc difference map is in green and red indicating positive and negative density respectively.

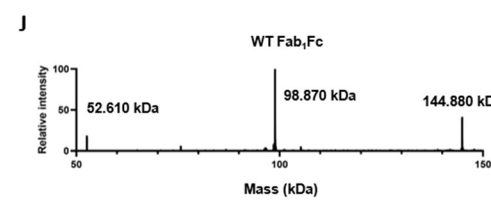
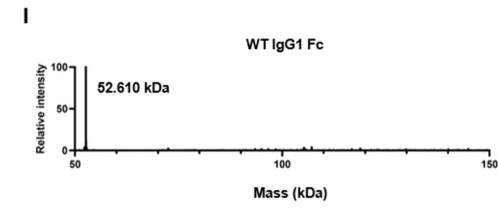
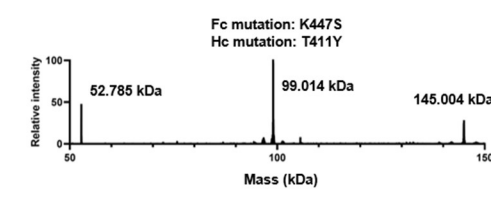
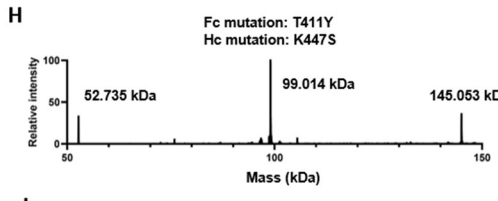
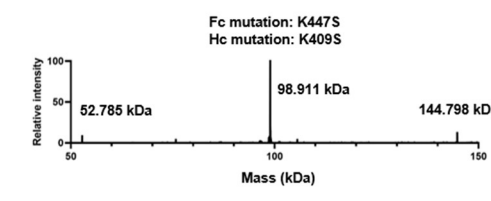
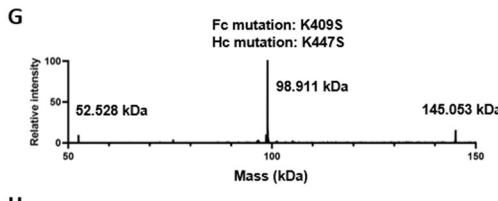
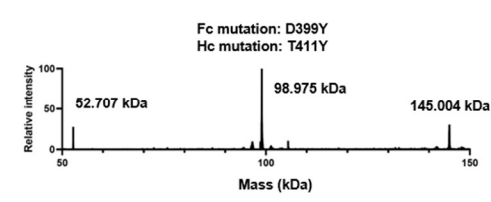
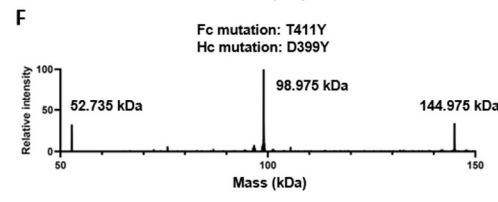
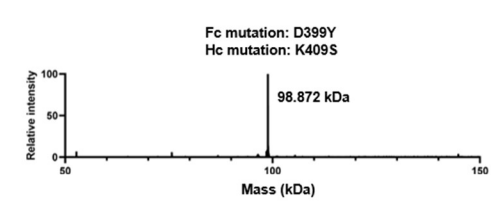
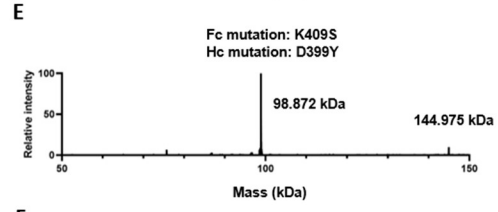
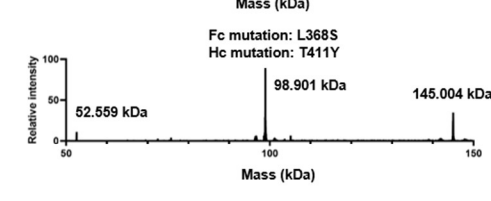
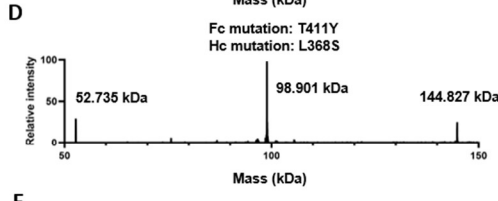
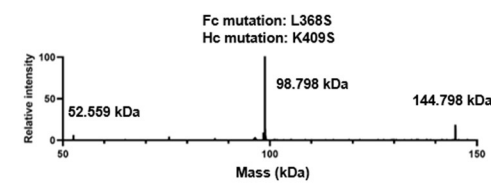
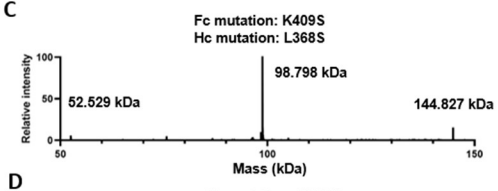
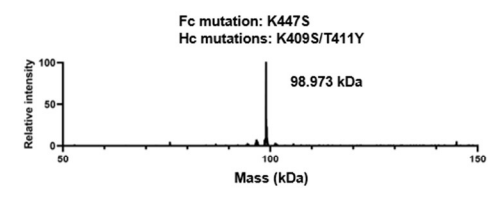
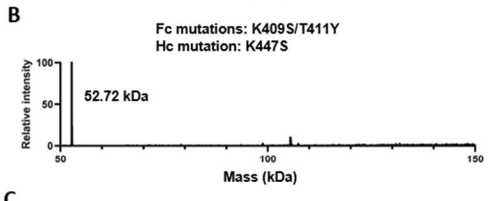
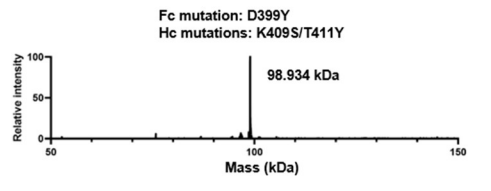
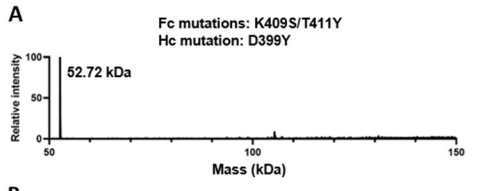


Figure S4: Intact LC/MS Heterodimer Assessment.

MS spectra depicting homodimer (Fc or IgG) and heterodimer formation Fab1Fc for electrostatic steering mutations D399Y-K409S/T411Y (**A**), K447S-K409S/T411Y (**B**), L368S-K409S (**C**), L368S-T411Y (**D**), D399Y-K409S (**E**), D399Y-T411Y (**F**), K447S-K409S (**G**), K447S-T411Y (**H**), WT-IgG1 Fc (**I**), WT-Fab1Fc (**J**), Percentages of each of the three peaks are determined relative to each other. Mutation(s) listed first are contained in the heavy chain and those listed second are in the Fc (Hc-Fc). All analyses were performed in triplicate.

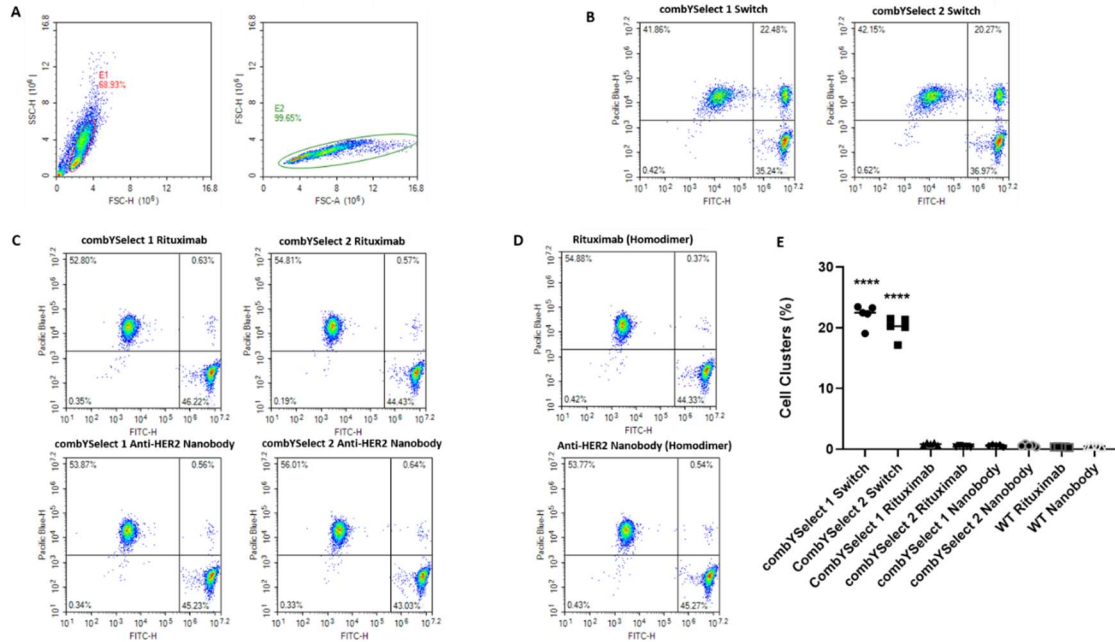


Figure S5: Gating strategy and controls for the cell-bridging heterodimer application.

(A) Density plots showing the gating strategy of the Raji cells stained with CFSE and BT474 stained with Calcein-Violet. (B-D) Flow cytometry density plots depicting cell cluster formations of Raji and BT474 cells when combYSelect 1 in which protomer A mutations are on the chain attached to the nanobody and protomer B mutations are on the chain attached to the Fab and combYSelect 2 in the switched orientation (B), monospecific combYSelect 1 and 2 (C), and homodimeric WT Rituximab and 5F7 nanobody (D) were added to the cell mixture. (E) Scatter plot quantifying the percentage of cell cluster formation for all of the tested antibody constructs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. The significance of combYSelect 1 and 2 switch is in comparison to all other listed constructs.

	combYSelect1	combYSelect2
Resolution range	40.85 - 2.51 (2.6 - 2.51)	40.63 - 3.001 (3.108 - 3.001)
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell	49.782 79.394 142.902 90 90 90	50.014 79.685 139.308 90 90 90
Total reflections	19866	11398
Unique reflections	19700 (1814)	11350 (993)
Multiplicity	10.2 (8.3)	11.5 (10.7)
Completeness (%)	98.13 (91.34)	96.87 (86.27)
Mean I/sigma(I)	30.3 (2.25)	18.5 (2.7)
Wilson B-factor	60.22	50.24
R-merge	0.240 (0.961)	0.453 (3.074)
R-meas	0.254 (1.020)	0.474 (3.221)
R-pim	0.079 (0.336)	0.137 (0.949)
CC1/2	0.993 (0.852)	0.964 (0.628)
Reflections used in refinement	19700 (1814)	11344 (993)
Reflections used for R-free	1970 (183)	1135 (100)
R-work	0.2019 (0.3633)	0.1921 (0.2578)
R-free	0.2425 (0.3945)	0.2816 (0.4279)
Number of non-hydrogen atoms	3547	3535
macromolecules	3343	3334
ligands	198	198
solvent	6	3
Protein residues	414	414
RMS(bonds)	0.014	0.011
RMS(angles)	1.51	1.19
Ramachandran favored (%)	98.54	93.66
Ramachandran allowed (%)	1.46	5.37
Ramachandran outliers (%)	0.00	0.98
Rotamer outliers (%)	3.09	4.39
Clashscore	6.74	10.92
Average B-factor	42.75	57.72
macromolecules	39.15	55.93
ligands	103.33	88.07
solvent	51.73	36.01
Number of TLS groups	2	2
PDB Code	8TTM	8TUD

Table S1. Data collection and refinement statistics