

## **Site-specific antibody conjugation to engineered double cysteine residues**

(Supplementary files)

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Figure S1

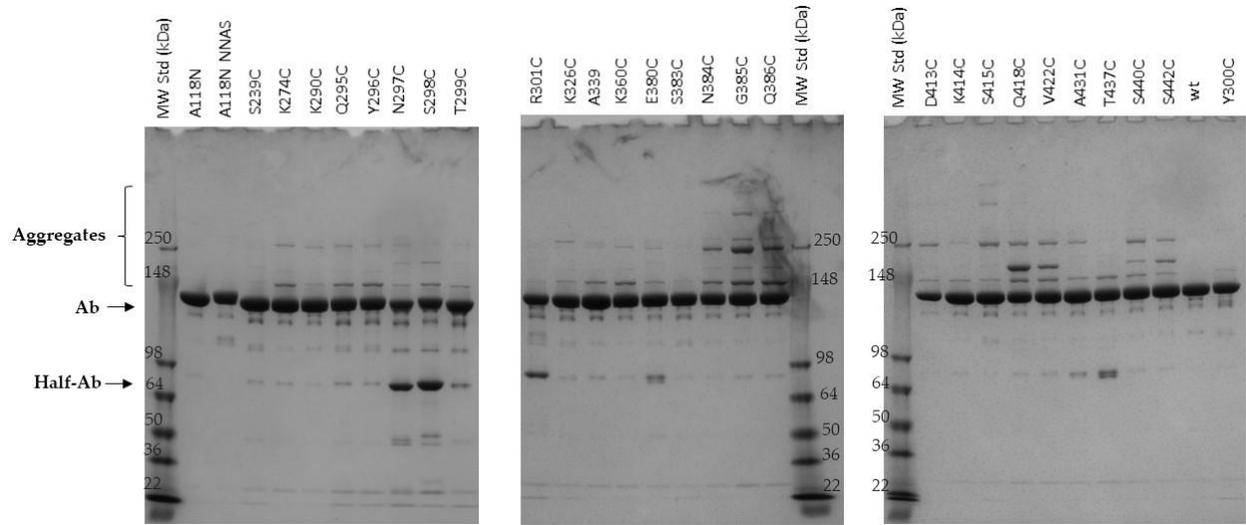


Figure S2

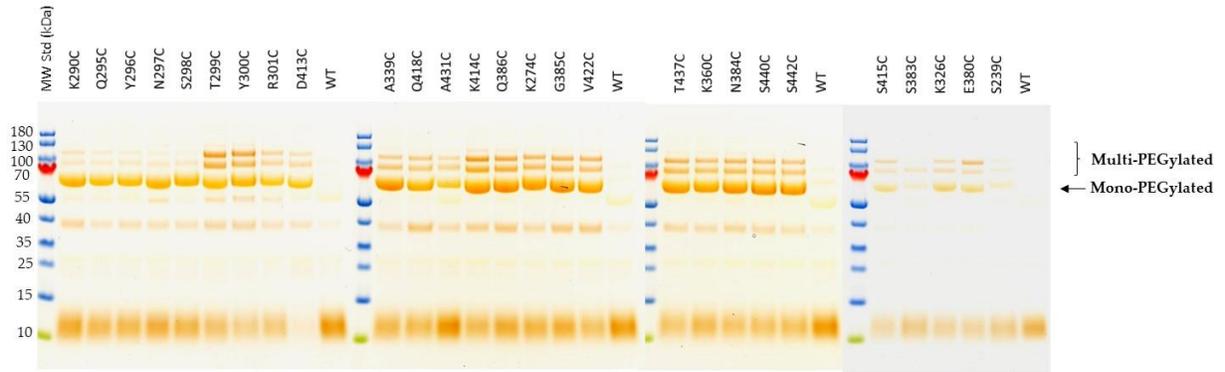


Figure S3

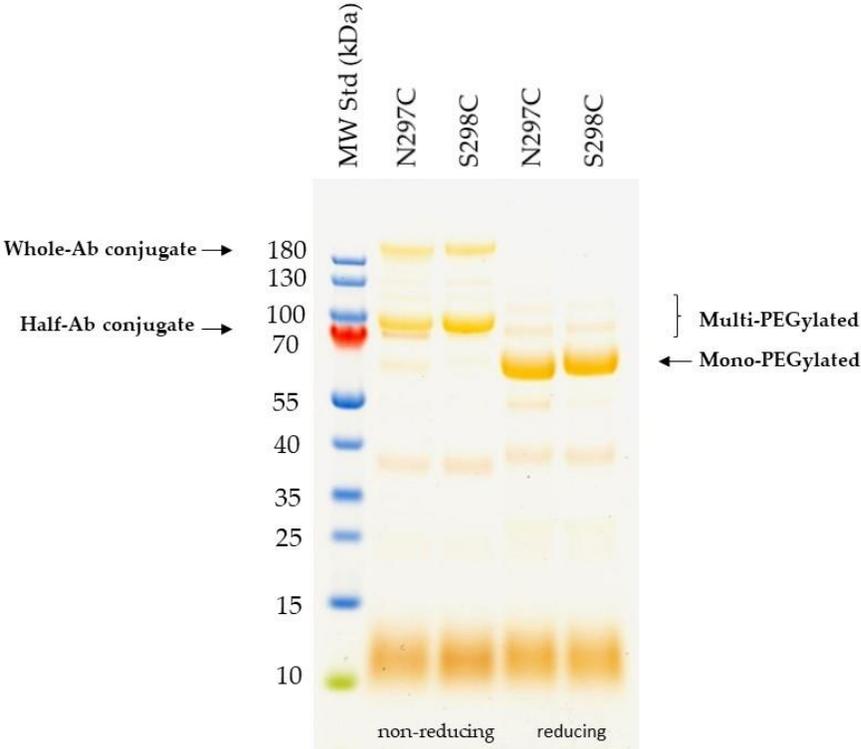


Figure S4

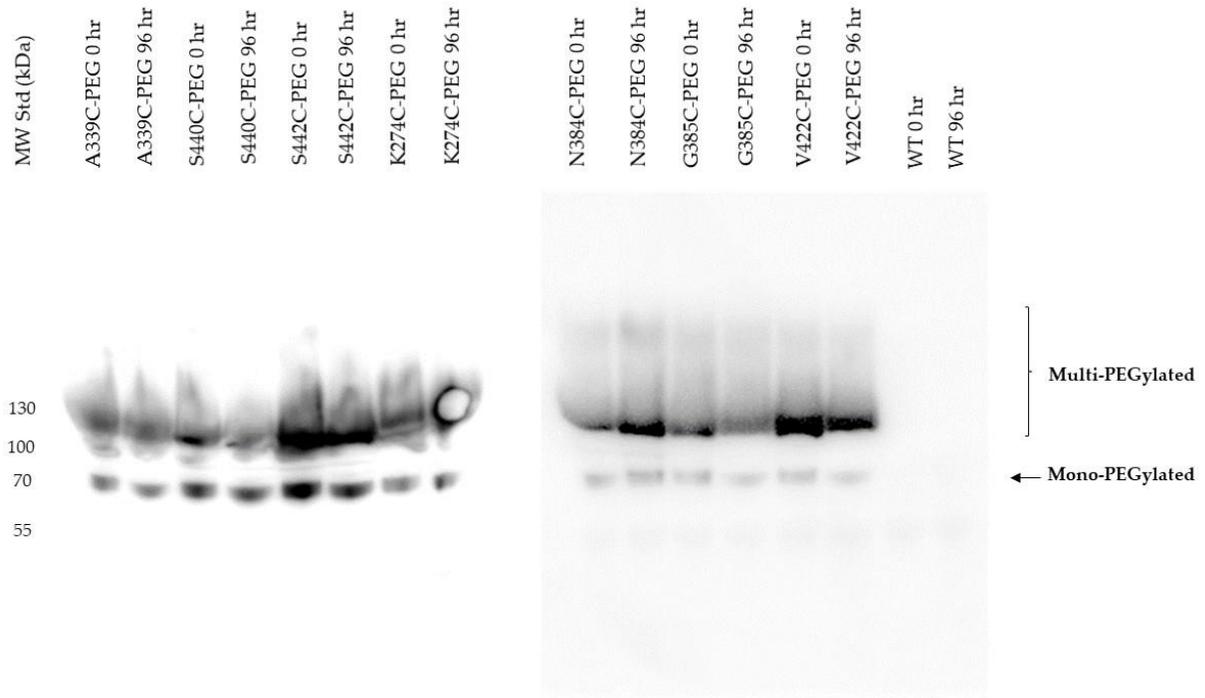
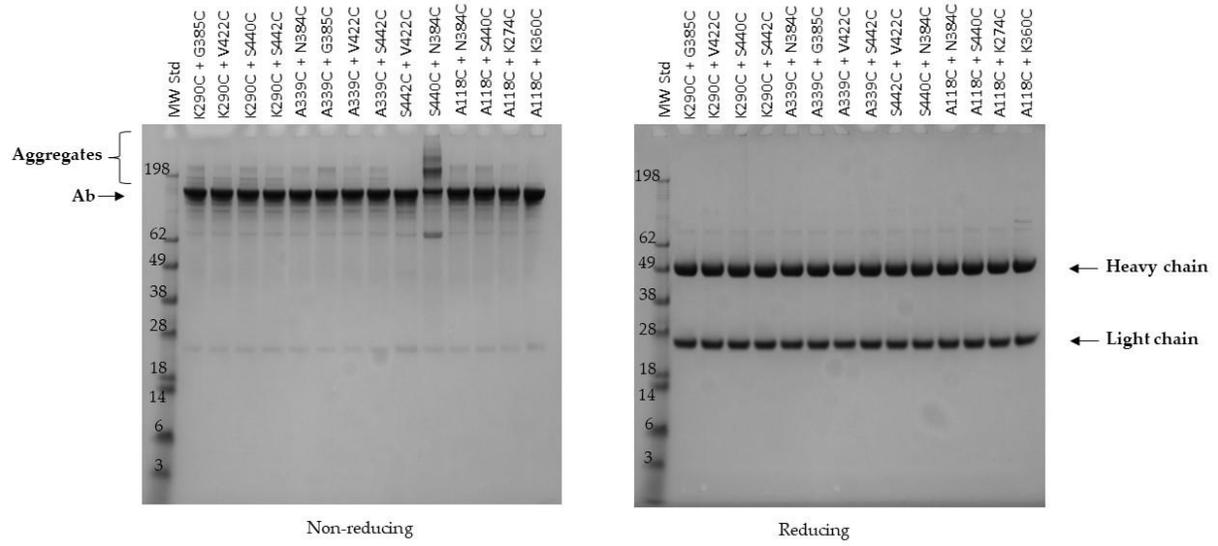


Figure S5



## Supplementary Figure Legends:

**Figure S1: SDS-PAGE analysis of purified single cysteine mutants.** 27 single cysteine mutants were purified using protein A and analyzed using 4-12% non-reducing Tris-Glycine SDS-PAGE. Wild-type antibody and hyperglycosylated mutants, A118N and NNAS, were used as controls. SeeBlue Plus 2 was used as protein molecular weight standards (MW Std) with different kDa as shown on the left of the gel.

**Figure S2: SDS-PAGE and PEG staining of single cysteine mutant PEGylated.** 27 single cysteine mutants PEGylated (13  $\mu$ g each) were analyzed using 4-12% Bis-Tris NuPAGE under reducing conditions. The gels were stained using PEG staining as described in the Materials and Method. The wild-type antibody (WT), which was also PEGylated, was used as a control. PageRuler pre-stained protein ladder was used as protein molecular weight standards (MW Std) with different kDa as shown on left of the gel.

**Figure S3: SDS-PAGE and PEG staining of two single cysteine mutants, N297C and S298C, after PEGylation.** Two single cysteine mutants, N297C and S298C, were PEGylated and analyzed using 4-12% Bis-Tris NuPAGE under non-reducing and reducing conditions. The gel was stained using PEG staining as described in the Materials and Method. Significant amounts of half antibody conjugates were detected in the gel under non-reducing condition. PageRuler pre-stained protein ladder was used as protein molecular weight standards (MW Std) with different kDa as shown on left of the gel.

**Figure S4: Western blot of PEGylated single cysteine mutants incubated with plasma.** The samples (0.1  $\mu$ g) from PEGylated antibodies and wild-type antibody incubated with mouse plasma were applied to SDS-PAGE under reducing condition and were analyzed using western

blot with anti-PEG antibody. The mono-PEGylated antibody bands, as shown by arrow, were analyzed using AlphaView software to determine the amount of PEG remained after incubation in plasma for 96 hours. The diffuse bands above the mono-PEGylated antibody bands are multi-PEGylated species which reacts strongly to the anti-PEG antibody due to the presence of additional PEG.

**Figure S5: SDS-PAGE analysis of purified double cysteine mutants.** The double cysteine mutants were analyzed using 4-12% Tris-Glycine SDS-PAGE under non-reducing and reducing conditions. SeeBlue Plus 2 was used as protein molecular weight standards (MW Std) with different kDa as shown on left of the gels.