

Table S1 - Construct design, expression parameters and associated binding kinetic and purification yield outcomes for each of the constructs tested as part of this study.

| Construct number | Expression vector | Signal peptide ^a | N-terminal (antibody clone and arrangement) | C-terminal (antibody clone and arrangement) | Inter-variable chain linker | Intra-variable chain linker | Purification tag | Codon optimization | Equilibrium dissociation constant (K _D) for EGFRvIII | Equilibrium dissociation constant (K _D) for CD3-epsilon | Equilibrium dissociation constant (K _D) for EGFRwt | Purification yield |
|------------------|-------------------|-----------------------------------|---|---|-----------------------------|--------------------------------------|------------------|--------------------|--|---|--|--------------------|
| 1 | pcDNA3.3 (+) | Murine Ig kappa light chain | 139 (VH-VL) | 28F11 (VL-VH) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | No | No binding detected | No binding detected | No binding detected | Not assessed |
| 2 | pcDNA3.3 (+) | Murine Ig kappa light chain | 139 (VL-VH) | 28F11 (VL-VH) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | No | No binding detected | No binding detected | No binding detected | Not assessed |
| 3 | pcDNA3.3 (+) | Murine Ig kappa light chain | 139 (VH-VL) | 28F11 (VH-VL) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | No | 3.48 x 10 ⁻⁶ M | 5.72 x 10 ⁻⁶ M | No binding detected | Not assessed |
| 4 | pcDNA3.3 (+) | Murine Ig kappa light chain | 139 (VL-VH) | 28F11 (VH-VL) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | No | 2.78 x 10 ⁻⁸ M | 1.56 x 10 ⁻⁸ M | No binding detected | Not assessed |
| 5 | pcDNA3.1 (+) | Murine Ig kappa light chain | 139 (VH-VL) | 28F11 (VH-VL) | [Gly ₄ -Ser] | 218 ^b | None | No | 8.68 x 10 ⁻⁶ M | 9.45 x 10 ⁻⁶ M | No binding detected | 11 mg/L |
| 6 | pcDNA3.1 (+) | Murine Ig kappa light chain | 139 (VL-VH) | 28F11 (VH-VL) | [Gly ₄ -Ser] | 218 ^b | None | No | 5.94 x 10 ⁻⁷ M | 4.05 x 10 ⁻⁷ M | No binding detected | 13 mg/L |
| 7 | pcDNA3.1 (+) | Murine Ig kappa light chain | 139 (VL-VH) | 28F11 (VH-VL) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | Yes | 2.78 x 10 ⁻⁸ M | 1.56 x 10 ⁻⁸ M | No binding detected | 19 mg/L |
| 8 | pcDNA3.1 (+) | Human immunoglobulin light chain | 139 (VL-VH) | 28F11 (VH-VL) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | Yes | 2.78 x 10 ⁻⁸ M | 1.56 x 10 ⁻⁸ M | No binding detected | 22 mg/L |
| 9 | pcDNA3.1 (+) | Human serum albumin preproprotein | 139 (VL-VH) | 28F11 (VH-VL) | [Gly ₄ -Ser] | [Gly ₄ -Ser] ₃ | None | Yes | 2.78 x 10 ⁻⁸ M | 1.56 x 10 ⁻⁸ M | No binding detected | 25 mg/L |

^aAmino acid sequence for signal peptides: Murine Ig kappa light chain - MDMRAPAGIFGFLLVLPFGYRS; Human immunoglobulin light chain - MAWSPLFLTLTHCAGSWA; Human serum albumin preproprotein signal peptide - MKWVTFISLLFLFSSAYS.

^bAmino acid sequence for: 218 Linker – GSTSGSGKPGSGEGSTKG.