A General Approach for Receptor and Antibody-Targeted Detection of Native Proteins Utilizing Split-Luciferase Reassembly

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Supporting Information

Supporting Methods

Association between VEGF and Flt-1. Following successful expression and refolding of VEGF and Flt-1, we established correct folding by monitoring association. VEGF dimer and Flt-1 were mixed in a 1:2 molar ratio and equilibrated at 4 °C for 2 hours. As controls for this experiment, VEGF and Flt-1 were separately injected into a size-exclusion column at the same concentration, which resulted in specific retention volumes on the column (Figure S2). Injection of the mixture of VEGF and Flt-1 into the column resulted in a peak corresponding to a retention volume that is distinct from either VEGF or Flt-1 alone. To ascertain the molecular weight of the complex, protein standards were run separately to establish a calibration curve (Figure S3). As expected, the VEGF-Flt-1 complex peak corresponds to a 1:2 complex where two copies of Flt-1 bind a single VEGF homodimer.

Effect of protease inhibitors and detergent on VEGF detection. We analyzed the effect of (1) Protease Inhibitor Cocktail (Sigma, product P8849) containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), bestatin, pepstatin A, E-64, and phosphoramidon as well as (2) M-PER® Mammalian Protein Extraction Reagent (Pierce, product 78503), which utilizes a proprietary detergent in 25 mM Bicine buffer at pH 7.6 for mammalian cell lysis. To perform the experiment, translations of CLuciferase-Flt-1 and Flt-1-NLuciferase were carried out, followed by addition of 1.25 μL of 1.5 μM VEGF dimer or PBS and 1.25 μL of 1x Protease Inhibitor Cocktail, M-PER or water. The reactions were incubated at room temperature for one hour, followed by luminescence readings consisting of addition of 10 μL of the reaction to 40 μL of Steady-Glo Luciferase Assay System. Results are presented as the luminescence of the sample in the presence of VEGF relative to the sample containing PBS (Figure S4).

Initial gp120 Sandwich Assay. Translations using Flexi-Rabbit Reticulocyte Lysate were carried out according to the manufactures procedure using 2 pmols of each of the mRNAs encoding the CD4-NFluc and CFluc-17b fusions, 0.5 μL RNasin, 70 mM KCl, 20 μM of each amino acid, 66% Lysate, and either 100 nM B_{aL} gp120 or an equivalent

volume of PBS in a 25 μL reaction. Reactions were incubated at 30 °C for 90 min after which luminescence was monitored on a Turner TD20e luminometer by mixing 20 μL of translation with 80 μL of Steady-Glo Luciferase Assay System giving a final concentration of 20 nM B_{aL} gp120. Reactions were performed in duplicate and averaged (Figure S5).

gp120 Titration. Translations using Flexi-Rabbit Reticulocyte Lysate were carried out according to the manufactures procedure using 2 pmols of each of the mRNAs encoding the CD4-NFluc and CFluc-17b fusions, 90 μg mL^{-1} PDI, 70 mM KCl, 20 μM of each amino acid, 66% Lysate, and decreasing concentrations of B_{aL} gp120 or an equivalent volume of PBS in a 25 μL reaction. Reactions were incubated at 30 °C for 90 min after which luminescence was monitored on a Turner TD20e luminometer by mixing 20 μL of translation with 80 μL of Steady-Glo Luciferase Assay System. Luminescence was monitored 1 min after mixing with a 10 sec integration. Reactions were performed in duplicate, background subtracted (using samples containing no gp120), and averaged. Concentrations of gp120 after rapid dilution are shown (Figure S6)

HER2 Sandwich Assay after Storage at –80 °C. Two 25 μL translations were carried out as described above. Reactions were incubated at 30 ^{o}C for 90 min, flash frozen, and stored at –80 °C for 7 days. Solutions were thawed and purified HER2 ECD or an equivalent volume of storage buffer (10 mM Tris-HCl at pH = 7.5, 50 mM NaCl, and 500 mM imidazole) was added to the vials. These solutions were allowed to equilibrate at room temperature for 30 min. Luminescence was monitored on a Turner 20/20^{n} luminometer by mixing 20 μL of translation with 80 μL of Steady-Glo Luciferase Assay System. Luminescence was monitored 1 min after mixing with a 10 sec integration. Readings are compared to previous translations described above; HER2 ECD concentrations after rapid dilution are shown (Figure S7).

Figures and Legends

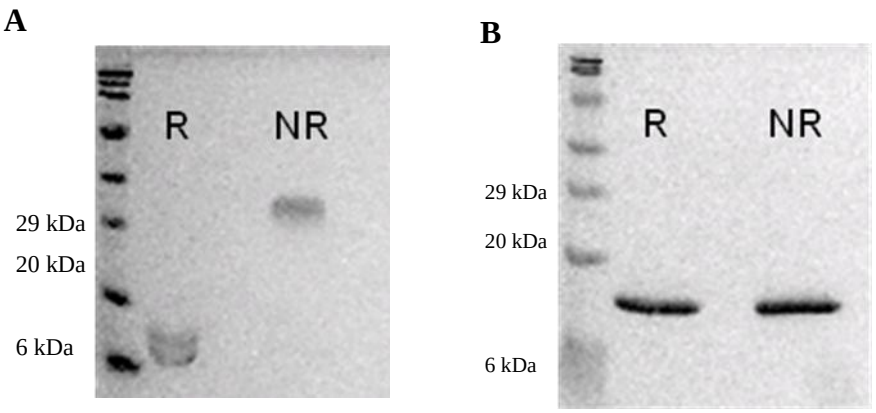

Figure S1. SDS-PAGE gels depicting refolding of proteins seen under reducing (R) and non-reducing (NR) conditions. (**A**) VEGF protein showing its native dimeric state seen under non-reducing conditions and (**B**) the monomeric receptor Flt-1 showing the same molecular weight under reducing and non-reducing conditions.

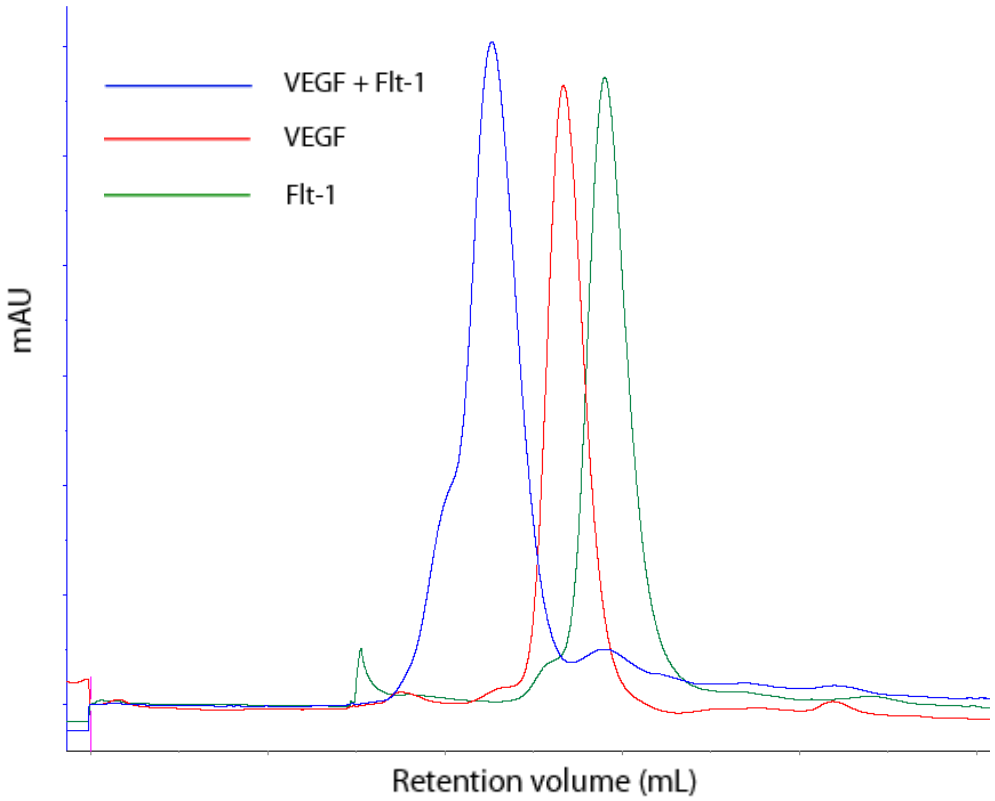

Figure S2. FPLC binding experiment between VEGF and its receptor, Flt-1. The VEGF protein (shown in red) and the Flt-1 protein (green) were run separately on the column. The mixture containing VEGF and Flt-1 (blue) was injected after a 2 h incubation time on the column. The distinct difference in retention volume of the mixture peak suggests that VEGF and Flt-1 associate in solution.

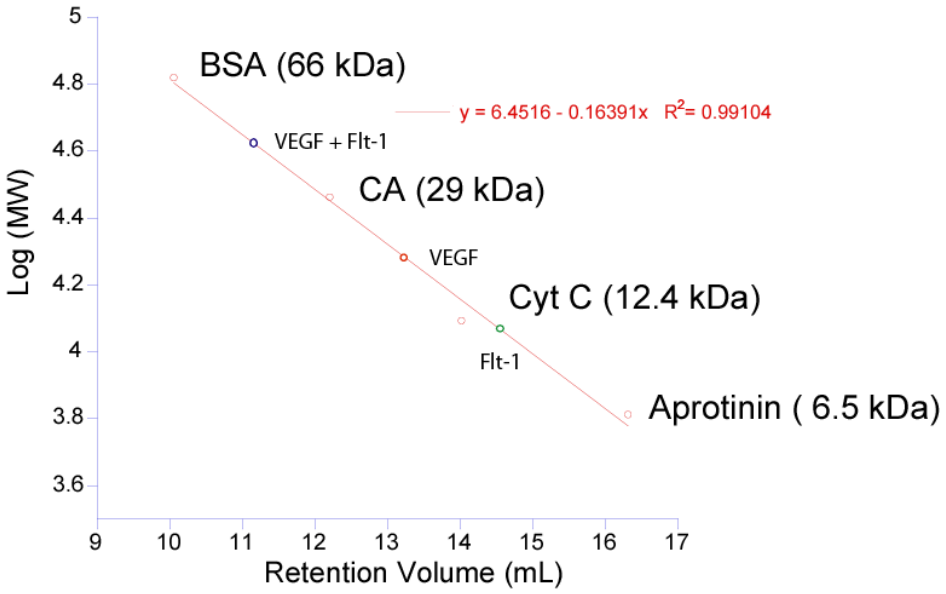

Figure S3. Protein standards run on the size-exclusion column overlaid with the experimental results of the VEGF-Flt-1 binding experiment. On deconvolution of the retention volumes to molecular weights, the results obtained were as follows: **1.** Flt-1 (Theoretical MW = 13.6 kDa; Experimental MW = 11.152 kDa). **2.** VEGF homodimer (Theretical MW = 27 kDa; Experimental MW = 18.33 kDa). **3.** Complex between VEGF and Flt-1 (Experimental MW = 39.4 kDa). These results suggest a 1:2 binding between VEGF and Flt-1 where two copies of Flt-1 bind a single VEGF homodimer.

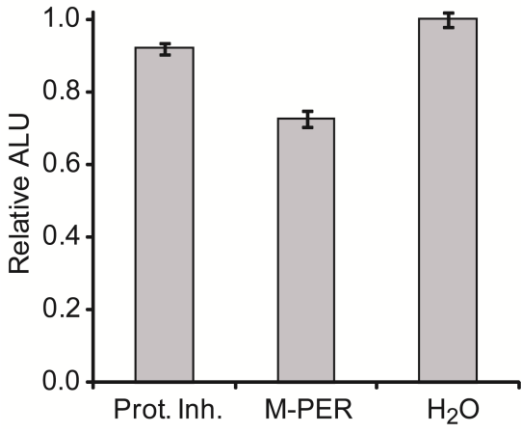

Figure S4. Effect of protease inhibitors and detergents on luciferase reassembly. Translations of CLuciferase-Flt-1 and Flt-1-NLuciferase were incubated with 15 nM VEGF or an equivalent volume of PBS in the presence of a protease inhibitor cocktail (Prot. Inh.), mammalian protein extraction reagent (M-PER), or water (H_2O). Luminescence readings are presented as the signal in the presence of VEGF relative to PBS.

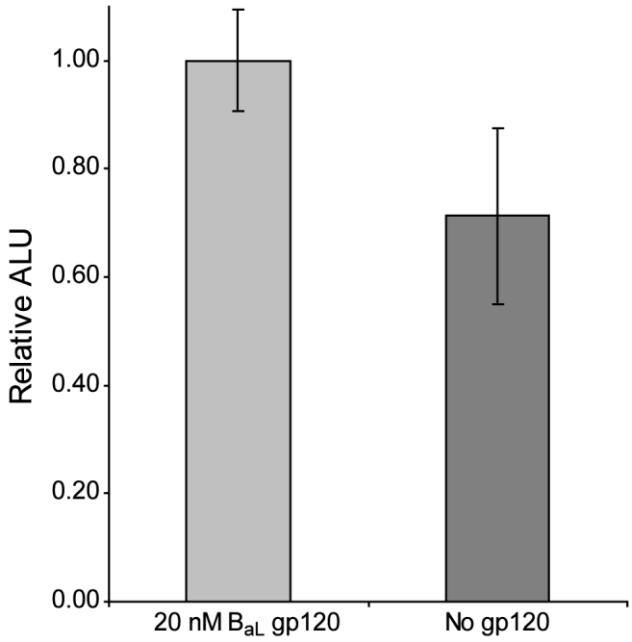

Figure S5. Luminescence in the presence and absence of B_{aL} gp120 for the initial gp120 sandwich assay.

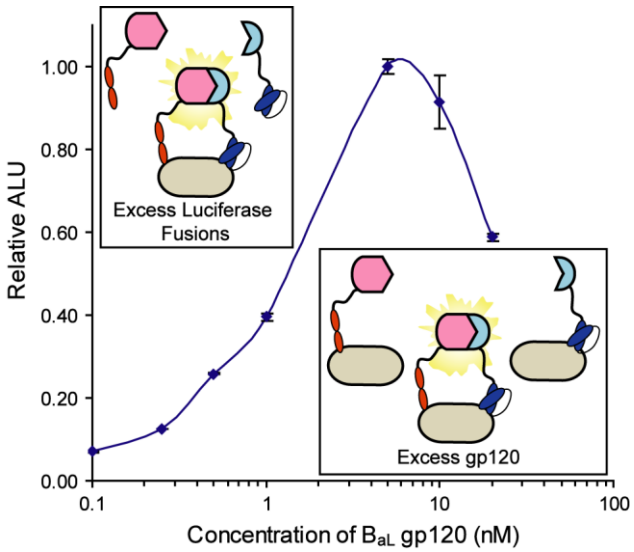

Figure S6. Luminescence from reassembled luciferase was monitored as a function of the concentration of gp120. Initially luciferase fusions are in excess however, as the concentration of gp120 increases, a maximum is reached were the concentration of luciferase fusions capable of forming a functional complex is equivalent to that of gp120. As the gp120 concentration is increased further, luminescence decreases due to localization of the luciferase fusions to different gp120s.

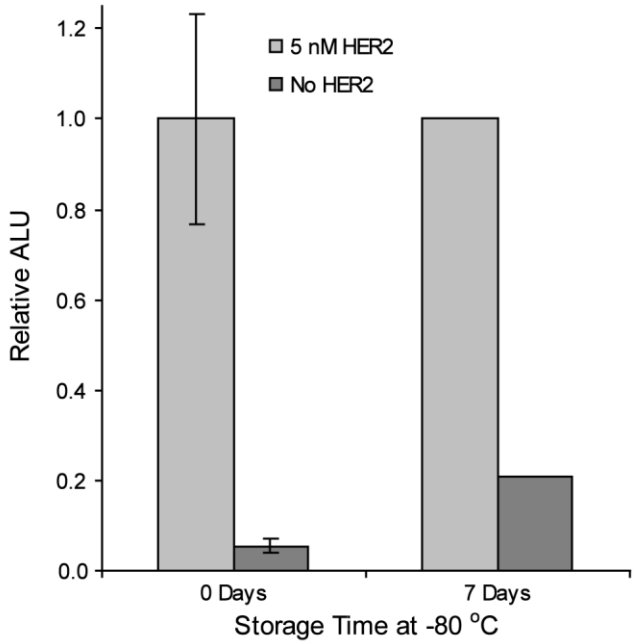

Figure S7. Cell-free translations of the HER2 sandwich assay were flash frozen and stored at –80 °C for 7 days; after which purified HER2 ECD was added and luminescence was monitored. Comparable HER2-dependent luciferase activity is still observed after storage for 7 days at –80 °C.

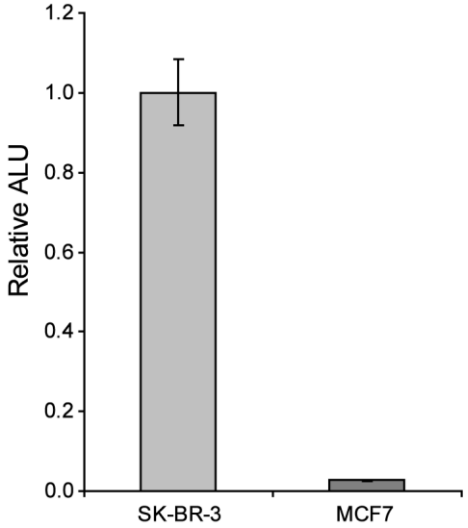

Figure S8. Luminescence as observed from the HER2 sandwich assay performed on 2,600 cells from the indicated human breast cancer cell lines.