Evaluation of Nanobody Conjugates and Protein Fusions as Bioanalytical Reagents

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Materials and Instrumentation

Materials

All chemicals obtained from Sigma-Aldrich unless specified
LB Miller Broth – Fisher
Phosphate buffered saline (PBS) – Corning Cell Grow
5 – alpha chemical competent *E. coli* - NEB
BL21 (DE3) chemically competent *E. coli* – NEB
Agarose A – Bio Basic Inc.
Carbenicillin – GoldBio Technology
Restriction Enzymes – NEB
Isopropyl-β-D-1-thiogalactopyranoside (IPTG) – GoldBio Technology
cOmplete ULTRA Tablets, Mini, EDTA-free – Roche
Quick Ligation Kit – NEB
Vent Polymerase – NEB
Oligonucleotides – IDT
Miniprep Kits – OMEGA
All antibodies obtained from Abcam
iBlot gel transfer stack kit, Novex
PageRuler Prestained Protein Ladder – Thermo Scientific
12% Ready Gel Precast Gels – BioRad
Snakeskin Dialysis Tubing 10K MWCO – Thermo Scientific
BirA-Biotin Ligase Kit – Avidity
Casamino Acids – Fisher
Yeast Nitrogen Base without Amin Acids – BD
Yeast Extract – Alfa Aesar
Dextrose – Fisher
D-Galactose – GoldBio Technology
Peptone – Fisher
Penicillin – Streptomycin – Fisher
Gene Pulser/Micro Pulser Cuvettes 1 mm and 2 mm – BioRad
Odyssey Blocking Buffer – LI-COR
TMB One Substrate – Promega
L- (+)- Arabinose – Sigma Aldrich
Chloramphenicol – GoldBio Technologies
Sulfo-Cyanine5 Maleimide – Lumiprobe
IRDye 800 CW Maleimide – LI-COR
Albumin, Bovine Fraction V (BSA) – RPI
Streptavidin Coated 96-well Plates (clear and black) – Pierce
EZ-Link Maleimade Activated Horseradish Peroxidase – ThermoFisher Scientific
NanoGlo reagent – Promega
All water obtained from a Milli-Q water purification system

Instrumentation

CyAn – ADP flow cytometry cell analyzer
Sonifer W – 350 cell disrupter – Branson
MJ mini gradient thermal cycler – BioRad
Molecular imager gel doc XR+ system – BioRad
iBlot Apparatus – Invitrogen
Odyssey Classic Infrared Imager – LI-COR
Synergy Mx Microplate Reader – BioTek
NanoDrop 200 UV-Vis Spectrophotometer – Thermo Scientific

Experimental Data

Experimental Procedures

Cloning

Purified proteins: All plasmids were constructed on a pETDuet-1 backbone. All proteins were assembled from a set of overlapping oligonucleotides or purchased g-block. Constructs were amplified using vent and then ligated into NcoI and KpnI restriction enzyme cleavage sites in the pETDuet-1 plasmid.

Display vectors: EBY100 yeast (trp-, leu-, with the Aga1p gene stably integrated) and pCTCON2 plasmid were generously provided by the Wittrup lab (MIT). The gene coding for mSA2 flagged with C-terminal BC2T were PCR amplified using vent and the constructs were ligated into NheI and BamHI restriction enzyme cleavage sites in the pCTCON2 plasmid.

MC1061 bacteria electrocompetent cells and pB33-eCPX plasmid were generously provided by the Daugherty lab (UCSB). The gene coding for BC2T-mSA2-myc were PCR amplified using vent and the constructs were ligated into NdeI and XhoI restriction enzyme cleavage sites in the pB33-eCPX plasmid.

Protein purification

Plasmids were transformed into BL21s (DE3). Cells were grown in either 2500 or 500 mL LB cultures containing carbenicillin at 37 °C to OD_{600} = ~0.5 and induced with 1 mM IPTG at 20 °C overnight. Cells were then collected by centrifugation and resuspended in phosphate buffer with 2 M NaCl (20 mM Sodium Phosphate, pH 7.4) and stored at -20 °C. Frozen pellets were thawed and incubated with cOmplete ULTRA protease inhibitors tablets then sonicated for 2 minutes. The lysate was cleared by centrifugation (8000 rpm, 20 minutes) and the supernatant was mixed with 1 mL Ni-NTA resin for 30 minutes. The resin was collected by centrifugation (4750 rpm, 10 minutes). The resin was washed with 50 mL buffer and 20 mM imidazole then 10 mL buffer and 50 mM imidazole. The protein was then eluted with 7 mL buffer containing 200 mM imidazole. The proteins were dialyzed against buffer with 150 mM NaCl and analyzed for purity by SDS-PAGE. Purified proteins were quantified using absorbance at 280 nm.

Protein Conjugation

Nanobody Dye Conjugation/HRP: Purified BC2 nanobodies with a C-terminal Cysteine residue were reacted with maleimide dye conjugates or maleimide HRP as described by manufacturers' instructions. Briefly, ~10-20-fold molar excess of dye over protein was added to nanobody solution in PBS, mixed and incubated at room temperature for 2 hours to overnight. Final product was separated from unreacted materials via 30,000 MWCO centrifugal columns. The dye labeled nanobody or HRP labeled nanobody was then purified by dialysis and analyzed via SDS-PAGE. It was stored, protected from light, at 4 °C until ready for use.

Protein – Biotin conjugation: GFP was conjugated to biotin using Avidity BioMix protocols and purified BirA Protein Ligase at 1.0 mg/mL.

ELISA binding assay

HRP: ELISA assays were performed using clear, streptavidin coated, 96-well plates (Pierce). The plate was washed 3 times with wash buffer (20 mM phosphate, 150 mM NaCl, 0.05% Tween-20, and 0.1 mg/mL BSA, pH = 7.4). Following washing, 100 μL of biotinylated GFP at 10 μg/mL was incubated for 2 hours at RT. Wells were washed three times with 200 μL of wash buffer shaking for 5 minutes. Subsequently, wells containing GFP were then incubated for 1 hour at RT with 100 μL of buffer containing one of three different proteins, all at 50 nM: (1) a BC2 tagged protein that has no appreciable affinity for GFP (zinc finger protein HRX, referred to as HRX); (2) a GFP binding nanobody-His6 that tightly binds GFP (K_D ~1nM), but lacks the BC2T epitope, or (3) GFP binding nanobody fused to a C-terminal BC2T or *myc* tag, then washed three times with 200 μL wash buffer. Following this, a 1:10,000 dilution of HRP-conjugated anti-His6X or anti-myc antibody were incubated in 100 μL Odyssey Blocking Buffer separately for all samples and ~50nM solution of BC2nb-HRP in 100 μL Odyssey Blocking Buffer for a separate set of all constructs for 1 hour at RT, and washed 3 times with 200 μL wash buffer. Colorimetry was developed for 30 minutes using 100 μL of TMB-One substrate. Absorbance was measured at 655 nm on a plate reader.

NanoLuciferase: ELISA assays were performed using black, streptavidin coated, 96-well plates (Pierce). The plate was washed 3 times with wash buffer (20 mM phosphate, 150 mM NaCl, 0.05% Tween-20, and 0.1 mg/mL BSA, pH = 7.4). Following washing, 100 μL of biotinylated GFP at 10 μg/mL was incubated for 2 hours at RT. Wells were washed 3 times with 200 μL wash buffer, shaking for 5 minutes. Subsequently, wells containing GFP were then incubated for 1 hour at RT with 100 μL of buffer containing one of three different proteins, all at 50 nM: (1) a BC2 tagged protein that has no appreciable affinity for GFP (zinc finger protein HRX, referred to as HRX); (2) a GFP binding nanobody-His6 that tightly binds GFP (K_D ~10 nM), but lacks the BC2T epitope, or (3) GFP binding nanobody fused to a C-terminal BC2T, then washed three times with 200 μL wash buffer. Following this, 50 nM of BC2-nanobody-nLuc fusion protein in wash buffer was incubated for 1 hour, and washed four times with 200 μL wash buffer. Finally, 100 μL of NanoGlo reagent substrate diluted 1:50 in wash buffer was incubated with samples and allowed to shake for ~10 min minutes at RT. Luminescence was measured on a plate reader.

Flow cytometry analysis

Bacteria: 50 mL culture of bacteria displaying mSA2 with BC2T and *myc* were grown in a 250 mL baffled flask containing chloramphenicol at 37 C with shaking (250 rpm) until an OD_{600} =~0.5 and induced with a final concentration of 0.02% (w/v) L- (+)- Arabinose at 20 °C overnight with shaking (250 rpm). Approximately 10^8 cells were pelleted and washed with 500 μL of 4 °C PBS-BSA. Bacteria were subsequently incubated with either BC2 nanobody – Cy5 (~10μg/mL), FITC-conjugated anti-*myc* antibody (1:10,000 dilution), or both BC2 nanobody-GFP alone in 500 μL PBS-BSA and rotated at RT for 1 hour. After incubation, two final washes with cold PBS-BSA were made to remove any unbound material and samples taken to flow cytometry analysis.

Yeast: 50 mL culture of yeast displaying mSA2 with BC2T were grown in a 250 mL baffled flask containing SD-CAA for 2-3 days at 30 °C with shaking. After 2-3 days of growth in SD-CAA, the samples were subcultured in SD-CAA at an initial density of $1X10^7$ cells/mL and grown to a density of 2-5 $X10^7$ cells/mL. Yeast were subcultured again to a concentration of $1.0X10^7$ cells/mL in SG-CAA (Galactose containing induction media) and grown for 2 days shaking at 250 RPM at a temperature of 20 °C. Approximately 10^8 cells were pelleted and washed with 500 μL of 4 °C PBS-BSA. Yeast were subsequently incubated with either BC2 nanobody – Cy5 (~10 μg/mL), FITC-conjugated anti-*myc* antibody / FITC conjugated anti-HA antibody (1:10,000 dilution), both the nanobody and one antibody, or BC2 nanobody – GFP (50 nM) alone in 500 μL PBS-BSA and rotated at room temperature for 1 hour. After incubation, two final washes with cold PBS-BSA were made to remove any unbound material and samples were taken to flow cytometry analysis.

Western blot analysis

Using commercially available antibodies: Purified proteins were separated by SDS-PAGE and transferred to a PVDF membrane via an iBlot Western blot apparatus. The membrane was blocked with 1X PBS, 5% milk and 0.1% Tween-20 for 1 hour at RT. Primary antibodies for *myc* and HA tag were incubated separately with the appropriate membranes overnight at a 1:10,000 dilution in 10 mL of 1X PBS, 5% BSA, and 0.1% Tween-20 at 4 °C. Membranes were washed 3X with 1X PBS containing 0.1% Tween-20 and then incubated with Anti-Rabbit (Alexa Fluor 790) at a 1:10,000 dilution in 10 mL PBS, 5% milk and 0.1% Tween-20 for 1 hour at RT. The membranes were then washed 3X with 1X PBS containing 0.1% Tween-20 and imaged in 1X PBS using the Odyssey Classic Infrared Imager.

Using 'in-house' prepared nanobody-IR800 dye: Purified proteins were separated by SDS-PAGE and transferred to a PVDF membrane via an iBlot Western blot apparatus. The membrane was incubated with 1X PBS, 5% milk, and 0.1% Tween-20 for 1 hour at RT. The BC2 nanobody-IR800 dye conjugate was then incubated overnight at ~0.10 μM concentration in 10 mL of 1X PBS, 5% BSA, and 0.1% Tween-20 at 4 °C. The membrane was then washed 3X with 1X PBS containing 0.1% Tween-20 and imaged in 1X PBS using the Odyssey Classic Infrared Imager.

Figure S-1. ELISA – HRP.

ELISA data using HRP conjugated antibodies or nanobodies for analysis. All wells were without anything immobilized on the plate's surface. To test off-target binding of anti-His6 antibody-HRP, anti-*myc* antibody-HRP, and BC2nb-HRP to non-immobilized wells, wells were incubated with just buffer (NT), HRX-BC2T, the GFP nanobody with different tags depending on which antibody was used (anti-His6, colored black, anti-*myc*, colored grey, and BC2 nanobody-HRP, colored white) and washed to remove unbound material. After a 30-minute incubation with TMB-one substrate, plate was read at 655nm. Signal is the observed absorbance at 655 nm. All experiments were performed in triplicate. Error bars represent standard deviation of three experiments. NT = no treatment.

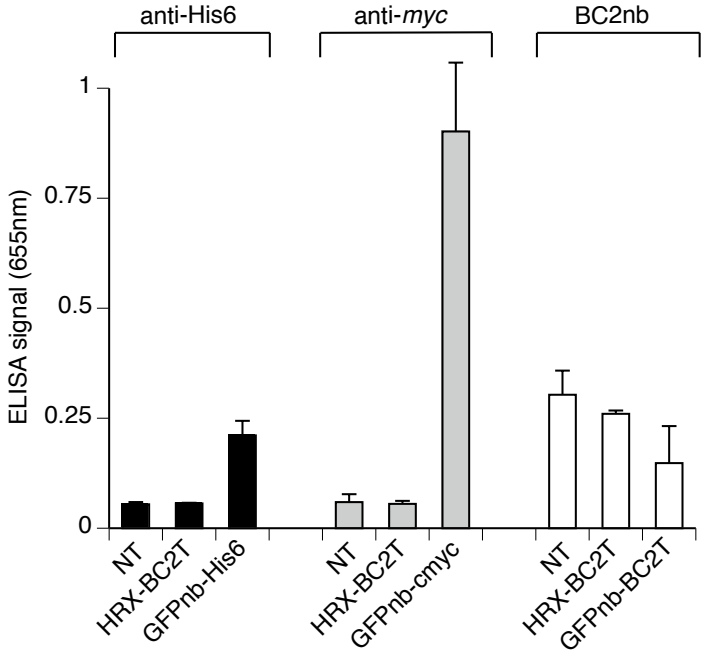


Figure S-2. ELISA – nLuc.

ELISA data using fusion protein BC2nb-nLuc for analysis. All wells were without anything immobilized on the plate's surface. To test off-target binding of BC2nb-nLuc to non-immobilized wells, wells were incubated with just buffer (NT), HRX-BC2T, the GFP nanobody with either a His6X tag or BC2T and washed to remove all unbound material. Luminescence was read after a 10 minute incubation with NanoGlo substrate. All experiments were performed in triplicate. Error bars represent standard deviation of three experiments. NT = no treatment. RLU = relative luminescence units.

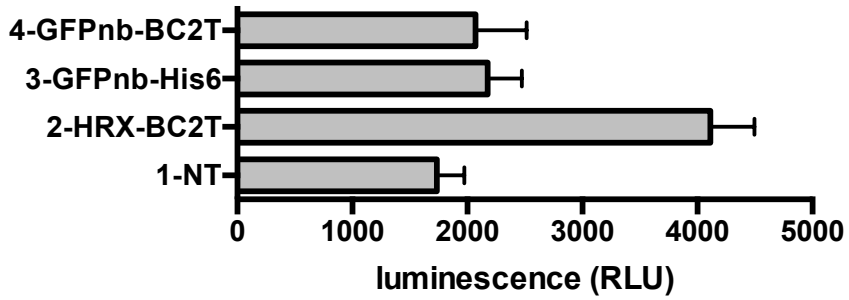


Table S-1. Flow cytometry data – bacteria.

Cy5, FITC, and GFP detected by flow cytometry to indicate display. All experiments were completed in triplicate. Values represent the mean of those experiments.

	construct	induced	incubated with	Cy5 (+)	FITC or GFP (+)
1	Bacteria - mSA2	-	-	0.87	1.73
2		Yes	-	1.72	1.23
3		-	Myc-ab-FITC	1.17	0.82
4		Yes	Myc-ab-FITC	3.11	94
5		-	BC2-nb Cy5	9.2	0.86
16		Yes	BC2-nb Cy5	98.6	1.9
7		-	BC2-nb-GFP	1.09	11.5
8		Yes	BC2-nb-GFP	3.68	98.1
9		Yes	BC2-nb Cy5 + Myc-ab-FITC	98.7	93.1

Table S-2. Flow cytometry data – yeast

Cy5, FITC, and GFP detected by flow cytometry to indicate display. All experiments were performed in triplicate. Values represent the mean of those experiments.

	construct	induced	incubated with	Cy5 (+)	FITC or GFP (+)
1	Yeast - mSA2	-	-	0.63	6.11
2		Yes	-	0.17	1.1
3		-	Myc-ab-FITC	0.27	0.67
4		Yes	Myc-ab-FITC	0.53	71.7
5		-	HA-ab-FITC	0.28	0.71
6		Yes	HA-ab-FITC	0.58	69.3
7		-	BC2-nb Cy5	1.01	0.81
8		Yes	BC2-nb Cy5	71.1	1.61
9		-	BC2-nb-GFP	0.28	0.82
10		Yes	BC2-nb-GFP	0.57	59.6
11		Yes	BC2-nb Cy5 + Myc-ab-FITC	70.9	71
12		Yes	BC2-nb Cy5 + HA-ab-FITC	71.2	69.8

Figure S-3. Representative flow cytometry histograms.

Representative histogram of flow cytometry data for display of mSA2 on (a) bacteria and (b) yeast. In all cases, bacteria or yeast displaying mSA2 were detected after incubation with anti-HA antibody-FITC, anti-*myc* antibody-FITC, BC2 nanobody – Cy5, or BC2 nanobody-GFP. Non-induced samples are shown as dashed lines and induced samples as solid lines.

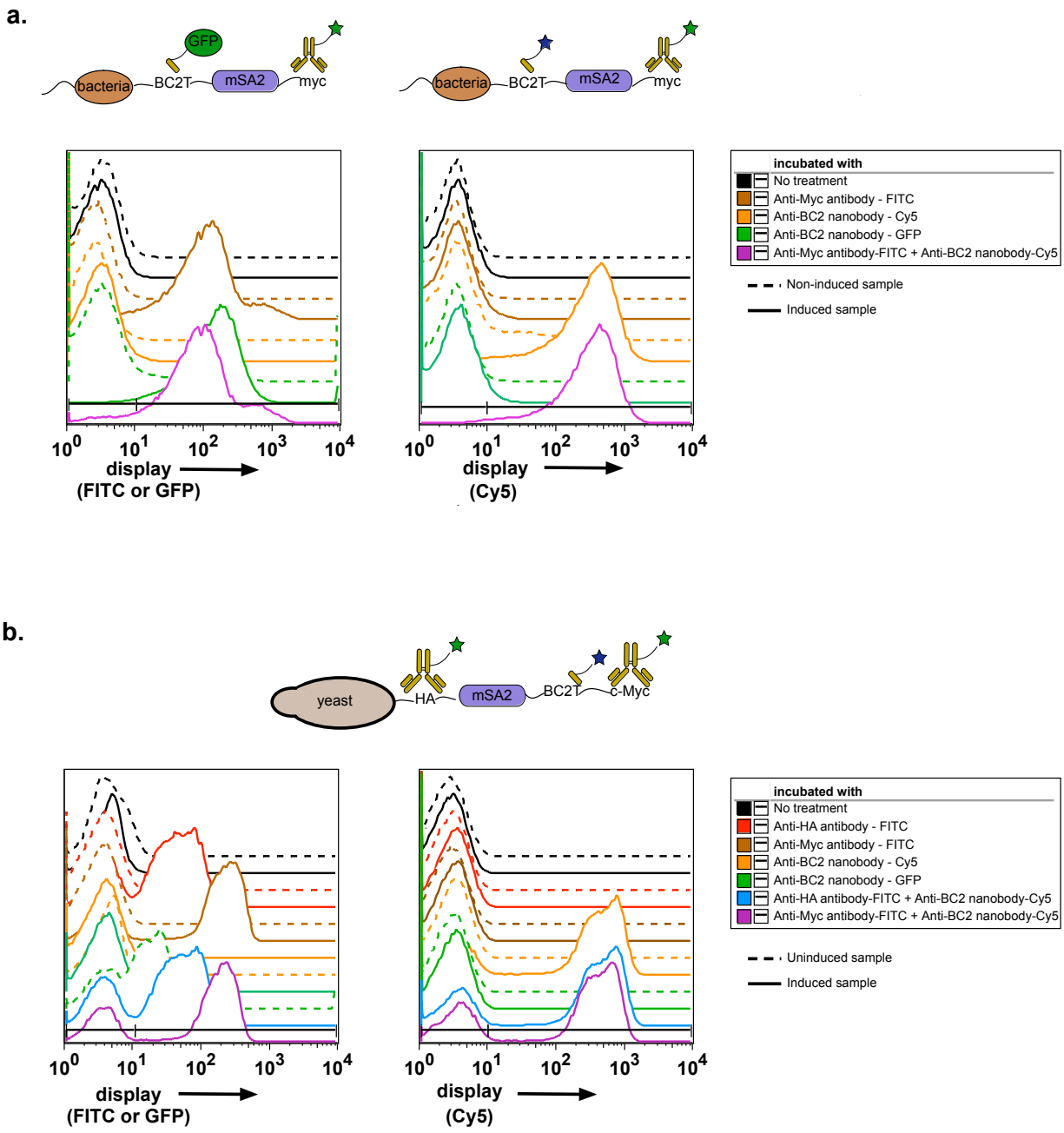


Figure S-4. Selectivity for epitope validated via Western blot.
(a) 5 μM coomassie stained gel and Western blot analysis of GFP-BC2T and GFP. Western blot analysis used BC2 nanobody-IRdye800. (b) 5 μM coomassie stained gel and Western blot analysis of GFP-HA and GFP. Western blot analysis used anti-HA antibody and was visualized with Donkey Anti-Rabbit IgG Alexa Fluor 790.

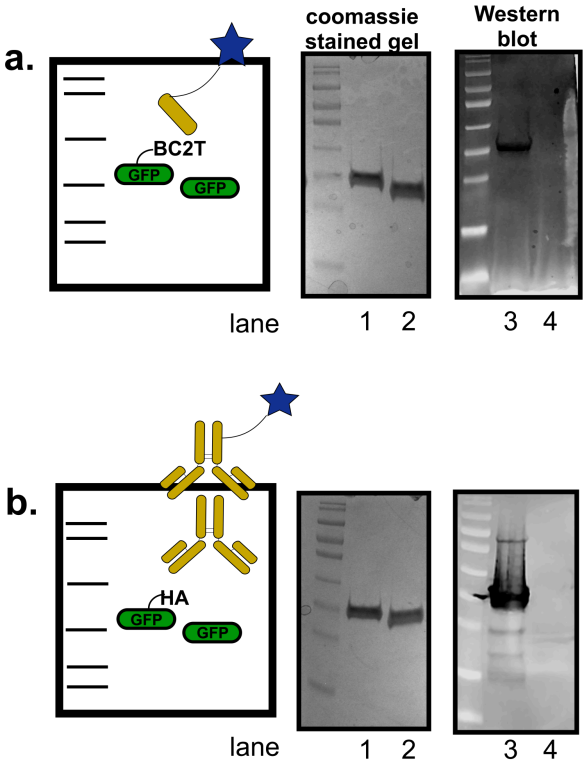
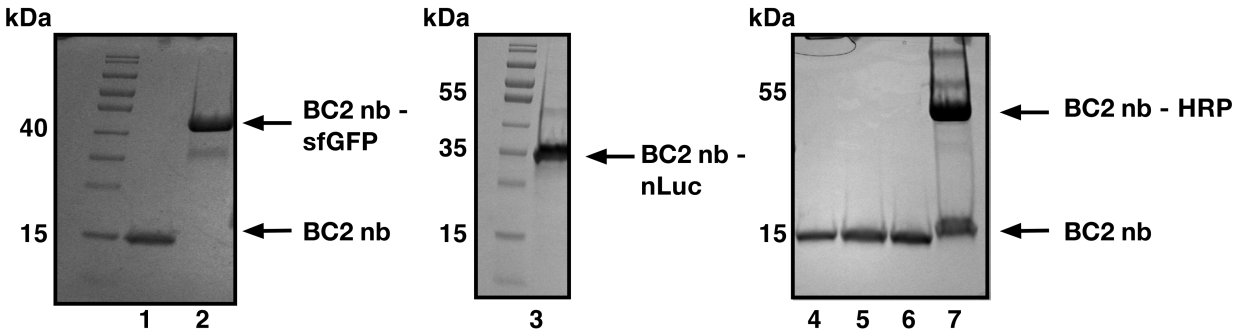


Figure S-5. SDS-PAGE of BC2 nanobodies utilized in this work.

SDS-PAGE of purified BC2 nanobody and its fusion proteins and conjugates used in this work. BC2nb-HRP is the only construct to not receive complete conjugation, however because the assay is done in excess of reagent a significantly larger signal when compared to off-target controls was still achieved.



Lane

1. **BC2nb (15.0 kDa)**
2. **BC2nb-sfGFP (42.2 kDa)**
3. **BC2nb-nLuc (34.3 kDa)**
4. **BC2nb-Cys (15.1 kDa)**
5. **BC2nb conjugated to IRdye800 (16.1 kDa)**
6. **BC2nb conjugated to Cy5 (15.9 kDa)**
7. **BC2nb conjugated to HRP (53.9 kDa)**