# Dual-antigen Bridging ELISAs to Validate Bispecific Molecules with CD3-directed T Cell Engager Function

C. Zhang, K. Zientara-Rytter, M. Samoudi, S. Green, K. Skuster, C. Chang, M. Awad, V. Baron, M. Kinbara, J. Mikolosko, P. Shashkin
BPS Bioscience, San Diego, CA 92121

Abstract 5457

## ABSTRACT

### Background

Bispecific antibodies (bsAbs), designed to recognize two distinct antigens or epitopes, represent a promising class of next-generation biological therapeutics and have gained significant interest over the past decade for their potential to harness the immune system against specific targets. With additive or synergistic mechanisms of action, bsAbs and other engineered dual-targeting molecules often exhibit greater potency than monoclonal antibodies or combination therapies. By simultaneously bridging two targets, bsAbs provide additional clinical benefits, such as enhanced target cell killing. Therefore, assessing their ability to bind both targets concurrently is essential for advancing their therapeutic potential.

CD3-bispecific antibodies (CD3-bsAbs) have emerged as a promising approach in cancer immunotherapy, particularly for hematological cancers. These bsAbs activate T cells by binding both a tumor-associated antigen on tumor cells and CD3 (cluster of differentiation 3) on T cells, leading to tumor cell destruction. Recent advancements in bsAb biology and technologies have enabled the development of diverse CD3-bsAb formats.

### Results

We have developed dual-target bridging ELISAs for bispecific molecules designed to engage T cells via CD3 binding. These assays are simple, cost-effective, and require no elaborate equipment, making them accessible to most laboratories. By leveraging the bridging capability of bispecific molecules, the method uses an immobilized capture recombinant antigen 1 and a CD3-containing detection reagent (antigen 2) to assess both binding events simultaneously, regardless of bispecific molecule format, isotype, or species of origin. Our assays feature low background, high signal-to-noise ratios, and compatibility with purified proteins, human serum, or cell culture media, making them ideal for evaluating bsAb bridging abilities in drug discovery and high-throughput screening (HTS). Additionally, the CD3-containing detection reagent is an all-in-one HRP-conjugated CD3 complex that simplifies the protocol by reducing steps, making these assays less time consuming and more cost effective when compared to surface plasmon resonance (SPR).

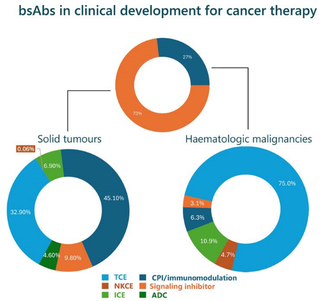


*Figure 1. Bispecific antibodies currently in clinical development. Adapted from C. Klein et al. Nat. Review Drug Discov. 2024, 23: 301-319. Abbreviations; TCE: T cell engager; NKCE: NK cell engager; ICE; innate cell engager, CPI: checkpoint inhibitor; ADC: antibody-drug conjugate.*

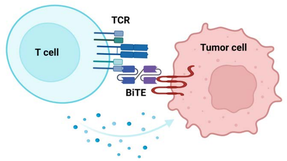


*Figure 2. Illustration of the mechanism of action of a CD3-directed T cell engager molecule*

## PRINCIPLE OF THE BRIDGING ELISA

### Principle

The Bispecific Bridging Chemiluminescence ELISA Kits are designed to analyze the ability of bispecific molecules to bridge a target protein, for example BCMA (B-cell maturation antigen) and CD3 (cluster of differentiation 3), for screening and profiling applications. This assay determines if a bispecific antibody binds to both targets simultaneously and is useful to validate the potential efficacy of a bispecific immune engager.

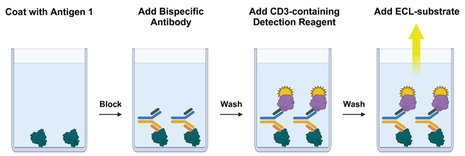


*Figure 3. Illustration of the assay principle.*

### Methods

A white 96-well plate is coated with 100 ng/well of a recombinant protein of interest (Protein 1), diluted in PBS (phosphate buffer saline) and incubated overnight at 4°C. The plate is blocked for 90 minutes at Room Temperature (RT) before being washed three times in PBST ( (PBS with 0.05% Tween-20). The bispecific antibody is serially diluted in PP-02 Assay Buffer and incubated with Protein 1 for 1 hour at RT. The plate is washed again, and the CD3-containing detection reagent is added for 1 hour at RT. The ECL substrate is added, and results are read immediately using a Bio-Tek microplate reader.

## DUAL TARGET BRIDGING ASSAYS

Known bispecific engagers were shown to bind BCMA × CD3, or claudin-18.2 × CD3, confirming that the assay functions as intended. Both colorimetric and chemiluminescent versions of the assay yielded highly comparable results. Both ScFv and IgG-type bispecific engagers were evaluated, demonstrating the assay versatility across different bispecific formats. Each target pair was validated using at least one known bispecific engager. In addition, dual antigen binding was confirmed using BLI (bio-layer interferometry).

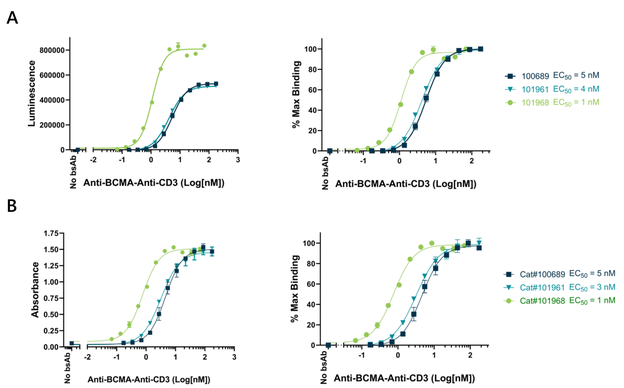


*Figure 4. Assessment of bispecific performance in bridging assays. Three anti-BCMA x anti-CD3 bispecifics (IgG-like antibody #101968, or ScFv-based molecules #100689 and #101961) were incubated with coated recombinant BCMA at increasing concentrations before washing and incubating with CD3-based detection reagent as per instructions provided with assay kit #82801. Chemiluminescence **(Panel A)** and colorimetric **(Panel B)** detection was performed by adding an ELISA ECL Substrate or an HRP (Horseradish Peroxidase) Colorimetric Substrate, respectively. Left panels show the raw data whereas the right panels show results normalized as percent of maximum binding for each bispecific molecule. $EC_{50}$ was determined by curve fitting using Prism software v 11.0.*

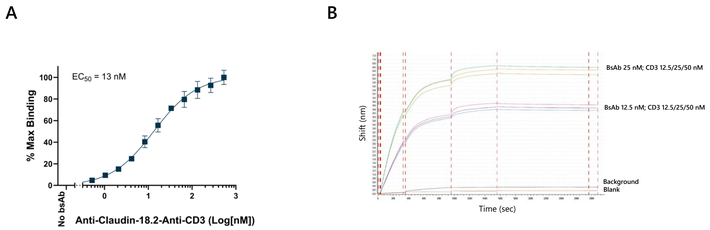


*Figure 5. Orthogonal validation of dual binding. **(Panel A):** Increasing concentrations of an anti-claudin-18.2 x anti-CD3 bispecific antibody (#101541) were incubated with recombinant claudin-18.2 protein pre-coated onto a 96-well plate. The plate was processed as described in the protocol for assay #82829 (colorimetric assay). Results are expressed as percent of maximum antibody binding. $EC_{50}$ was determined by curve fitting using Prism software v 11.0. **(Panel B): Dual epitope Binding of anti-claudin-18.2 x anti-CD3 antibody.** The anti-Claudin-18.2x anti-CD3 IgG Bispecific Antibody (#101541) was loaded at concentrations of 12.5 and 25 nM. The first association was performed with recombinant claudin-18.2 (#101570) at 50nM, and the second association was performed with the CD3-containing Detection Reagent (#82705) at concentrations of 12.5, 25 and 50 nM. Channel 1 used only buffer whereas channel 8 represents the non-specific binding (no antibody load, both antigen proteins added). BLI measurements were performed using a Gator Fc Generation II.*

## STABILITY & USE WITH MEDIUM OR HUMAN SERUM

Using cell culture medium such as DMEM (Dulbecco's modified Eagle medium) or human serum for antibody dilution did not affect antigen binding compared to an optimized buffer (Panels B and C). Thus, the assay is suitable for use with bispecific antibodies diluted in cell culture medium or in human serum.

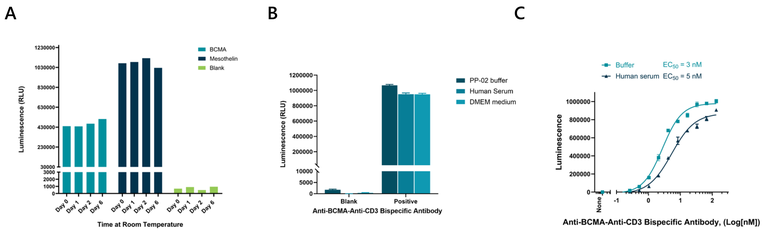


***Figure 6: Stability study and influence of culture medium or human serum on antibody binding. (Panel A):** Mesothelin (#100290) or BCMA (#82801) recombinant proteins were coated onto a 96-well plate (100 ng/well) overnight at 4°C. The plate was kept at RT for up to 6 days before addition of the antibodies and the CD3-containing detection reagent. Results are expressed as raw luminescence signal. **(Panels B and C)**: Anti-BCMA x anti-CD3 bispecific antibody (#101968) was assessed using assay kit #82801 in the presence of buffer only, DMEM, or human serum (Sigma #P2918). Luminescence was measured using a Bio-Tek microplate reader. Results are presented as total luminescence. $EC_{50}$ was determined by curve fitting using Prism software v 11.0.*

## CONCLUSION

Antibodies represent a cornerstone of modern therapeutics, demonstrating remarkable efficacy in oncology and immunology. Bispecific Immune Engager antibodies designed to bind simultaneously to a therapeutic target on cells of interest such as cancer cells and to a specific cell surface molecule on immune cells may be designed to recognize CD3 (cluster of differentiation 3), a co-receptor complex expressed almost exclusively in mature T cells and implicated in their activation.

Bispecific antibodies require rigorous characterization throughout their development, with one key aspect being target engagement. Our collection of assays are useful to validate the potential efficacy of a bispecific immune engager by assessing that it binds to both targets simultaneously.

These assays can be used to:

- Determine which antibody best recognizes both targets
- Optimize antibody/biologic construct and format
- Functional validation of a new molecule

### Advantages

- Agnostic to biologic isotype or species
- Versatile: accommodates various Biologic formats, no Fc required in the test molecule
- Requirement for two binding events increases the specificity of the assay
- Time-saving and cost effective
- Positive control antibodies available

| Assay Cat# | Detection | Target 1 | Target 2 | Name of Biologic | Format | Biologic Cat# |
|---|---|---|---|---|---|---|
| 82818 | Luminescence | POI | CD3 | - | - | - |
| 82801 | Luminescence | BCMA | CD3 | Anti-BCMA-Anti-CD3 Bispecific Molecule | BiTE™ | 100689 |
| 82807 | Colorimetric | BCMA | CD3 | Anti-BCMA-Anti-CD3 Bispecific Molecule | BiTE™ | 100689 |
| 82764 | Luminescence | CD19 | CD3 | Anti-CD19-Anti-CD3 Bispecific Molecule | BiTE™ | 100441 |
| 82764 | Luminescence | CD19 | CD3 | Anti-CD19-Anti-CD3 IgG format Bispecific Antibody | IgG | 101076 |
| 82828 | Luminescence | Claudin18.2 | CD3 | Anti-Claudin-18.2-Anti-CD3 IgG Bispecific Antibody | IgG | 101541 |
| 82829 | Colorimetric | Claudin18.2 | CD3 | Anti-Claudin-18.2-Anti-CD3 IgG Bispecific Antibody | IgG | 101541 |
| 82830 | Luminescence | Mesothelin | CD3 | Anti-Mesothelin-Anti-CD3 Bispecific Antibody | IgG | - |
| 82831 | Colorimetric | Mesothelin | CD3 | Anti-Mesothelin-Anti-CD3 Bispecific Antibody | IgG | - |
| Coming soon | Luminescence | VEGF165 | PD-1 | Ivonescimab | IgG | - |

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