Characterization of a single reporter-gene potency assay for T-cell-dependent bispecific molecules

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ABSTRACT

T-cell-dependent bispecific antibodies (TDBs) are promising cancer immunotherapies that recruit patients’ T cells to kill cancer cells. There are many TDBs in clinical trials, demonstrating their widely recognized therapeutic potential. However, their complex, multi-step mechanism of action (MoA), which includes bispecific antigen binding, T-cell activation, and target-cell killing, presents unique challenges for biological characterization and potency assay selection. Here, we describe the development of a single reporter-gene potency assay for a TDB (TDB1) that is MoA reflective and sensitive to binding of both antigens. Our reporter-gene assay measures T-cell activation using Jurkat cells engineered to express luciferase under the control of an NFκB response element. The potencies of select samples were measured both by this assay and by a flow-cytometry-based cell-killing assay using human lymphocytes as effector cells. Correlating the two sets of potency results clearly establishes our reporter-gene assay as MoA reflective. Furthermore, correlating potencies for the same panel of samples against binding data measured by binding assays for each individual arm demonstrates that the reporter-gene potency assay reflects dual-antigen binding and can detect changes in affinity for either arm. This work demonstrates that one reporter-gene assay can be used to measure the potency of TDB1 while capturing key aspects of its MoA, thus serving as a useful case study of selection and justification of reporter-gene potency assays for TDBs. Furthermore, our strategy of correlating reporter-gene potency, target-cell killing, and antigen binding for each individual arm serves as a useful example of a thorough, holistic approach to biological characterization for TDBs that can be applied to other bispecific molecules.

Introduction

Bispecific antibodies (BsAbs) are a dynamic area of drug development, and currently, more than 100 BsAbs in a diverse array of formats are under development. A notable class of BsAbs are the T-cell-dependent bispecific antibodies (TDBs), which bind T cells (typically through an anti-CD3 arm) to target cells (through a cell-surface receptor-binding arm) (Figure 1(a)). Simultaneous ligation of target and effector cells induces T-cell activation, followed by killing of the target cell via the secretion of cytolytic enzymes across an immune synapse (Figure 1(b)). Dual-antigen binding is required for immune-synapse formation and cell-killing activity; in the absence of target cells, there is no cell-killing activity. While therapeutically effective, their complex mechanism of action (MoA), including simultaneous target- and effector-cell engagement, T-cell activation, and target-cell killing, presents challenges for the development and selection of potency assays and for biological characterization. A single potency assay that measures all key aspects of the MoA is desirable. However, the strategy for selecting such an assay and demonstrating how well it reflects the MoA is not straightforward, due to the complexity of the biology and the number of assays that need be carefully designed and executed to measure each aspect of the MoA.

Cell-killing assays, which directly quantify a molecule’s ability to induce cell death, are the most direct measure of a TDB’s biological activity. However, in addition to reflecting the MoA, a potency assay must be able to track changes in product quality that have the potential to affect the therapeutic molecule’s biological activity, in order to ensure patient safety and product efficacy via a robust and consistent manufacturing process. Cell-killing assays are generally not suitable for this quality-control (QC) purpose due to high assay variability, in addition to labor- and time-intensive procedures, making them difficult to sustain over a product’s lifetime from development through commercialization.

Reporter-gene assays have emerged as an attractive alternative to cell-killing assays for QC purposes. They typically use cell lines engineered to express luciferase under the control of a biologically relevant response element for T-cell activation, such as nuclear factor of activated T cells (NFAT) or nuclear factor kappa B (NFκB), allowing the measurement of events upstream of cell killing. Reporter-gene assays are faster, easier to perform, and more reproducible than cell-killing assays, making them more QC suitable. However, it
must be shown that they are fit for the purpose, i.e., MoA reflective.\textsuperscript{21,26}

Here, we describe a novel biological characterization strategy for a TDB (TDB1), most notably the development and justification of a single reporter-gene assay as the potency assay. The assay measures T-cell activation using an in-house Jurkat T-cell line engineered to express luciferase upon T-cell activation under the control of an NFkB response element (Figure 1(c)). In the presence of target cells expressing Antigen A, TDB1 conjugates target cells (via its anti-A arm) to T cells (via its anti-CD3 arm) and stimulates T-cell activation, inducing luciferase expression in a dose-dependent manner and enabling rapid, accurate, precise, and stability-indicating quantitation of T-cell activation. In order to demonstrate that our reporter-gene assay reflects the MoA, we designed and executed a proof-of-concept study to directly compare potencies measured by our reporter-gene assay to potencies measured by a flow-cytometry-based cell-killing assay developed specifically for this study. We also developed and used individual CD3- and Target-A-binding assays to demonstrate that the reporter-gene potency assay can measure binding to both target and effector antigens, as well as to investigate how changes in individual arm binding affect potency. The study results support reporter-gene T-cell-activation assays for TDBs as scientifically sound, QC-suitable alternatives to cell-killing assays that can capture major components of the MoA in a single potency assay. The results also represent a thorough biological characterization strategy for TDB1 that can serve as a useful example for other TDBs.

Figure 1. Mechanism of action of TDB1 and the reporter-gene potency assay. (a). Illustrated representation of the structure of TDB1, consisting of anti-A- and anti-CD3 binding arms. (b). Illustrated representation of TDB1’s MoA, including bispecific target engagement and induction of immune response factors (ImRFs) leading to immune-synapse formation. (c). Illustrated representation of the reporter-gene potency assay, using a T cell engineered to express luciferase upon T-cell activation. (d). Representative mock recovery data demonstrating accurate quantitation and linearity over a range of 50–150% relative potency (RP).

Results

T-cell activation initiates a cascade of intracellular signaling events, culminating in the expression of cytokines and other immune response factors (ImRFs).\textsuperscript{33}–\textsuperscript{39} Expression of many of these ImRFs occurs via the well-characterized NFkB and NFAT pathways.\textsuperscript{34–39} Although the signaling events and regulatory mechanisms downstream of T-cell activation differ between the NFAT and NFkB pathways, both transcription factors localize to the nucleus and induce transcription of ImRFs by binding to their respective promoter in response to T-cell activation, and response elements from each have been used to engineer widely used reporter-gene cell lines that measure T-cell activation.\textsuperscript{29,31} We selected Jurkat cells, a well-studied, immortalized human T-cell line, and engineered them to express luciferase under the control of an NFkB response element. The cell line showed robust luciferase expression in under 4 h in the presence of TDB1 and target cells endogenously expressing Target A, leading us to proceed with assay development. We hypothesized that T-cell activation of Jurkat-NFkBLuc cells could serve as a biologically relevant, MoA-reflective potency assay for TDB1.

The reporter-gene assay presented in this manuscript has several advantages over a traditional cell-killing assay: 1) There is no need to distinguish target cells from effector
cells by labeling; 2) Assay incubation times are faster (several hours as opposed to overnight) as a result of T-cell activation preceding cell killing; and 3) Reporter-gene cell lines can be generated from easy-to-culture, commonly used immortalized cell lines. By contrast, there are limited immortalized cytolytic (CD8+) T-cell lines commercially available and most cell-killing assays use peripheral blood mononuclear cells (PBMCs), which are expensive and difficult to maintain in culture. In addition, donor-to-donor variability between PBMC batches increases overall assay variability, which undermines the utility of a potency assay intended for product quality control. 28, 40, 41

The advantages of the reporter-gene assay enabled our development of a fast, robust potency assay with low assay-to-assay variability relative to most cell-killing assays. The method is sensitive to product quality and is stability indicating, as demonstrated by measuring potencies of samples subjected to various stress conditions (Table 1). It can be reliably validated over a range of 60–140% potency relative to reference standard (Figure 1(d)).

With a robust, quantitative reporter-gene potency assay in hand, we devised a strategy to perform a full biological characterization of TDB1 that would also demonstrate the relevance of the proposed potency assay to the molecule’s intended biological activity. In addition to the reporter-gene potency assay, we developed four characterization assays to measure T-cell activation by surface marker expression, target-cell killing by PBMC human T cells, and individual binding to Target A and CD3. By comparing reporter-gene potency assay results with cluster of differentiation (CD) marker expression, target-cell killing, and single-arm binding to both Target A and CD3, we show that the proposed potency assay can fully represent TDB1’s complex MoA.

**Table 1.** Relative potency of TDB1 stressed samples.

| TDB1 Stress Condition | Relative Potency (%) |
|-----------------------|----------------------|
| Control (Unstressed)  | 99                   |
| Stress Condition X    | 64                   |
| Stress Condition Y    | >140                 |

Table 1: Potencies of stressed samples (and unstressed control) measured in the reporter-gene potency assay. Values are from N = 3 independent assays and reported as Relative Potency (measured against TDB1 reference standard).

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**Figure 2.** T cell activation CD markers expression in the reporter-gene potency assay. (a). Representative dot plots obtained via flow cytometry showing CD25 expression on the y-axis and CD69 expression on the x-axis with increasing concentrations of TDB1. Q1 shows CD25+ cells, Q2 shows CD25+/CD69+ cells, Q3 shows CD25-/CD69-cells, and Q4 shows CD69+ cells. (b). Plot of %CD25+/CD69+ cells vs. TDB1 concentration. (c). Plot of CD69+ cells vs. TDB1 concentration. (d). Co-plot of reporter-gene potency assay standard curve with %CD25+/CD69+ and %CD69+ curves from b and c. Each individual curve was normalized to its respective maximum signal to account for differences in assay output.

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**Reporter-gene assay results correlate with T-cell activation CD marker expression**

To verify that our assay read-out, luciferase expression, is a true indicator of T-cell activation, we investigated whether TDB1 dose-dependently increases expression of two CD markers characteristic of T-cell activation, CD25 and CD69. 42–44 Using flow cytometry, we measured the expression levels of CD25 and CD69 with increasing dose of TDB1 (Figure 2(a–c)) in the same assay condition used for the reporter-gene assay, except for a longer incubation time, and compared the results to luciferase expression in the reporter-gene potency assay. Luciferase expression and the CD marker expression are well correlated (Figure 2(d)), confirming that the luciferase signal from this assay is a true indication of T-cell activation. It should be noted that the kinetics of upregulation of CD69 and CD25 are known to differ, with expression of CD69 appearing earlier than CD25. 45 The difference in expression level on the timescale of the assay likely...
accounts for the lower assay sensitivity observed for CD25+ and CD25+/CD69+ cells (Figure 2(a, b)).

Reporter-gene assay results correlate with direct cell-killing assay results

An orthogonal cell-killing assay was required to conclusively determine whether T-cell activation in the engineered Jurkat cell line is relevant to TDB1’s intended biological activity of killing target cells in humans. To this end, we developed a cell-killing assay using PBMCs as a source of cytotoxic T cells and flow cytometry analysis for quantitation (Figure 3(a)). The assay procedure is similar to that of the reporter-gene potency assay; the same target cells are used, but the target cells and PBMCs are incubated at a higher ratio (1:10) for a longer period of time (20–26 h). The increase in assay incubation time and target-to-effector cell ratio is logical, given that T-cell activation precedes cell killing and PBMCs are 45–70% CD3+ T cells, depending on the individual donor. Following incubation with TDB1, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Receptor Y (a cell-surface receptor expressed by the target cells) to track target cells and with PI to track dead cells. The percentage of live target cells (anti-Receptor Y/PE cells) was measured by flow cytometry. Titrating the amount of TDB1 incubated with both target and effector cells resulted in a clear dose-dependent decrease in live target cells, showing that the assay can quantify target-cell killing by TDB1 (Figure 3(a)). Importantly, removing CD3+ cells from PBMCs prior to performing the assay resulted in no cell killing at any dose of TDB1, strongly indicating that the measured cell killing is from CD3+ T cells and T-cell receptor binding rather than from any effector function such as antibody-dependent cell-mediated cytotoxicity (Figure 3(b)).

After assay development, we measured potencies for selected degraded, stability, and lot release TDB1 samples using the cell-killing method. The correlation plot between cell killing and reporter-gene potencies for the same set of samples demonstrates that the two methods are similarly stability indicating and sensitive to changes in product quality (Figure 3(c–d)). This result provides a clear justification for using the reporter-gene assay for control-system activities as an appropriate surrogate for cell killing by human T cells.

Reporter-gene assay results reflect binding to each antigen

To enable a thorough biological characterization strategy that addresses how antigen binding to each arm affects TDB1’s potency, individual binding assays were developed for Target A and CD3.

The Target A-binding assay measures binding to native receptor A on the surface of target-cell line A, a suspension cell line. The cells are immobilized on 96-well poly-D-lysine plates by crosslinking with paraformaldehyde (PFA), and binding is detected via an enzyme-linked immunosorbent assay (ELISA) readout (Figure 4(a)). PFA fixation is required to ensure assay reproducibility because the target cells do not adhere well to the poly-D-lysine-coated plates and binding of TDB1 to Target A is weak compared to a traditional bivalent mAb. Fixing the cells created a robust, reproducible monolayer of cells displaying Target A that remained adhered to the plate throughout the ELISA procedure, which includes multiple-automated wash steps.

The CD3-binding assay measures binding to the CD3e peptide immobilized on a Biacore SA chip via surface plasmon resonance (SPR) (Figure 4(c)). The highest point of the sensorgram is compared to that of a reference standard to produce a relative binding value. Both binding assays showed suitably low assay variability with coefficients of variation (CV) below 10% and were able to...
reproducibly quantitate antigen binding of samples prepared at 50%-150% potency relative to reference standard, suggesting that each is capable of detecting and quantifying changes in anti-A or anti-CD3 binding by TDB1 (data not shown).

To confirm the intended biological activity of TDB1, which involves binding to both antigens at the same time, BsAbs made with either the anti-A arm or the anti-CD3 arm of TDB1 paired with a mechanistically inert half-Ab arm (anti-X) were tested in the reporter-gene assay (Figure 5(a, c)). As expected, the BsAbs with a null arm were unable to activate T cells, suggesting that T-cell activation is dependent upon dual-antigen binding in the reporter-gene assay (Figure 5(b, d)). To confirm that the BsAbs exhibits relevant single-arm binding, we assayed each BsAb (anti-X/CD3 and anti-A/X) in both binding assays. Both BsAbs showed binding comparable to TDB1 in the binding assay corresponding to its TDB1 arm, but no binding in the other antigen-binding assay (all values listed in Figure 5(e)). The binding data for each individual arm of the BsAbs combined with the potency data clearly demonstrate that binding to either Target A or CD3 alone is not sufficient to induce T-cell activation in the reporter-gene assay, providing further supporting that the assay reflects the MoA.

Given our ability to measure binding to Target A and CD3 independently, we were interested in characterizing how changes in binding influence potency. To address this question, we assembled a panel of minor-variant, degraded, and control samples of TDB1 and assayed each set in the Target A-binding, CD3-binding, and potency assays. Comparing the relative binding and potency values for each binding assay revealed that stress-induced changes in binding for each arm correlate with potency. Plotting binding against potency shows the degree to which binding to each antigen correlates with potency (Figure 4(b, d)). We observed a strong correlation between Target A binding and the reporter-gene potency assay results. Most samples tested showed a limited effect on CD3 binding. Peptide map data (not shown) revealed that TDB1’s Target-A arm has high levels of chemical modification (such as deamidation and isomerization), while the CD3 arm has minimum modification, suggesting that the Target-A arm is more prone to chemical modification under certain stress conditions covered in this study than the relatively robust CD3 arm.

**Discussion**

Reporter-gene assays measuring T cell activation by luciferase expression under the control of either NFkB or NFAT have benefits of shorter assay incubation time, technically easier assay procedure, and better accuracy and precision over conventional cell-killing assays. However, demonstrating the relevancy of a reporter-gene assay to reflect a therapeutic antibody’s MoA is challenging, especially for molecules like TDBs, which have relatively complex, multi-step MoAs that include dual-antigen binding, T-cell activation, and eventually cell killing.

We developed a reporter-gene potency assay that measures T-cell activation by TDB1 in the presence of target cells using engineered Jurkat cells expressing luciferase under the control of an NFkB response element. The reporter-gene potency assay is stability indicating, robust, accurate, precise, and linear over a reasonable range (50–150% potency relative to reference standard). In addition, it has a shorter assay incubation time and simplified assay and data analysis procedures compared to the PBMC-based cell-killing assay, making it a suitable QC assay. Notably, we developed a thorough characterization strategy for TDB1 using orthogonal assays specifically developed to measure individual arm binding, T-cell activation via CD marker expression, and target-cell killing. Collectively, these assays address the major aspects of TDB1’s biological activities, including antigen binding,
T-cell activation, and target-cell killing. Characterization data using the assays developed with a defined set of TDB1 minor-variant, stressed, and control samples support and reflect the relevance of the reporter-gene potency in several aspects. First, potencies measured by the cell-killing assay using PBMC demonstrates that T-cell activation in our engineered assay system is a suitable surrogate signal for target-cell killing by human T cells. Second, comparing the binding data for both Target A and CD3 to reporter-gene potency has allowed us to clearly demonstrate that potency is both dependent on dual-arm binding and correlated with binding affinity for each individual arm.

This work represents a thorough biological characterization of TDB1 and presents the first model case study for justifying the use of a single reporter-gene potency assay in the control system for a TDB, by demonstrating that the potency assay reflects TDB1’s complex MoA. The proposed biological characterization strategy can therefore serve as a useful example that should be broadly applicable to any bispecific molecule.

**Materials and methods**

**TDB1**

As previously described, TDB1 was produced and assembled into a full-length human IgG BsAb using knobs-into-holes technology.46

**Jurkat-NFKBluc cells**

Through standard molecular biology methods, an NFkB transcriptional response element was subcloned into a lentiviral expression vector upstream of a minimal CMV promoter and firefly luciferase gene. Jurkat cells were subjected to lentiviral transfection and individual clones were isolated and screened.
for inducible luciferase expression. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (HI FBS), 1x Pen-Strep, 1x GlutaMAX™, and 1 µg/mL puromycin.

**Reporter-gene potency assay**

Sample dilutions of TDB1 were prepared in assay medium consisting of RPMI 1640 supplemented with 10% HI FBS. Dilutions for the standard and assay control curves were prepared in the same manner as the TDB1 samples. Fifty microliter sample, standard, and assay control dilutions were pipetted into a 96-well tissue culture plate before the addition of cells. During sample pre-incubation, frozen aliquots of target-cell line A and Jurkat-NFkBLuc cells were thawed, washed, and adjusted to 4.0 × 10^5 and 1.6 × 10^6 cells/mL, respectively, before being mixed together and added to the assay plate in a volume of 50µL (final cell ratio 1:4 Target: Jurkat). Plates were gently agitated before being placed in a 37°C incubator supplemented with 5% CO2 for approximately 4 h. After incubation, 100 µL SteadyGlo™ reagent was added to each well, and plates were shaken for 15–25 min at room temperature before measuring luminescence (in relative luminescence units [RLU]) using a suitable plate reader. The results for the standard curve, assay control, and samples were plotted as RLU versus concentration of TDB1. The potencies of the assay control and each sample relative to the standard were calculated using parallel-line analysis and reported as percent relative potency.

**SPR-based CD3-binding assay**

A Biacore T200 instrument (GE Healthcare) was used for SPR analysis. A Biacore sensor SA chip (GE Healthcare) was washed with 1 M NaCl in 50 mM NaOH. A biotinylated CD3e peptide prepared in house was diluted to 5 µg/mL and immobilized at 200 resonance units (RU) as the target immobilization level. Samples prepared at 1 µg/mL antibody concentration in assay running buffer (phosphate-buffered saline [PBS] with 0.05% Tween 20) were injected for 3 min at 50 µL/min for CD3e binding. The dissociation phase was achieved by passing the same assay running buffer through the chamber for 2 min. The regeneration was performed with a single injection of 10 mM glycine-HCl, pH 1.5 for 30 s at 50 µL/min. All experiments were performed at 25°C. Duplicate injections of each sample and a buffer blank were flowed over the two surfaces (a reference flow cell and a testing flow cell). Data were collected at a rate of 1 Hz. The readout was the maximum binding response during association phase, 5 s before the end of the sample injection. To negate the effects of non-specific binding, a reference flow cell was run in conjunction with the testing flow cell. In addition, injections of blank running buffer were included on experimental flow cells. Signals from the reference flow cell and blank buffer injections were subtracted from the absolute response of sample injections on experimental flow cells (double subtraction method). Data were analyzed using Biacore T200 evaluation software and JMP software.

**Target a-binding assay**

**PFA fixation and quenching**

Cultured target cells were resuspended to approximately 2 × 10^6 cells/mL in PBS + 0.5% bovine serum albumin (BSA), and 100 µL were pipetted into each well of a 96-well tissue culture plate coated with poly-D-lysine. Plates were centrifuged for approximately 3 min at 300 x g before the addition of 100 µL 8% PFA in PBS. Plates were centrifuged once more before being incubated overnight at 2–8°C. The next day, PFA was washed away using an automatic plate washer, and 100 µL of Tris-buffered saline (TBS) + 0.5% BSA was added to each well to quench residual PFA. Plates were quenched for times ranging from 20 to 60 min at 2–8°C while samples were prepared.

**Sample preparation and ELISA**

Sample incubations were performed on ice, and buffers were equilibrated to 2–8°C before use unless otherwise noted. Sample dilutions of TDB1 were prepared in PBS + 0.5% BSA. Samples and assay control dilutions were prepared in the same manner as the TDB1 samples. Plates were washed using an automated plate washer to remove quench buffer before the assay control, standard, and sample dilutions were pipetted onto the plates. Plates were incubated with dilutions for approximately 1 h. Plates were then washed again, and goat-anti human-Fcγ γG-horseradish peroxidase (HRP) (Jackson ImmunoResearch, cat#: 109–036-098) was added to each well. Plates were incubated with the HRP-conjugated secondary antibody for approximately 1 h on ice with shaking before being washed twice to remove unbound HRP. One hundred microliter room-temperature SureBlue Reserve™ was added to each well and color change was monitored (typically 10–20 min with shaking at room temperature). The reaction was quenched with room-temperature 100 µL 0.6N H2SO4 for approximately 3–5 min with shaking at room temperature before absorbance at 450 nm was measured using an appropriate plate reader. The results for the standard curve, assay control, and samples were plotted as absorbance versus concentration of TDB1. The results for the assay control and each sample relative to the standard were calculated using parallel-line analysis and reported as percent relative binding.

**Quantitation of CD69 and CD25 expression by flow cytometry**

Dilutions of TDB1 were prepared in assay medium consisting of RPMI 1640 supplemented with 10% HI FBS (Gibco, cat #: 10,082,139). 0.8 mL of each dilution was transferred to individual wells of 12-well tissue culture plates and pre-incubated at 37°C for 1 h. After pre-incubation, 0.8 mL of Jurkat-NFkBLuc and target cells were added to each well. Plates were incubated overnight in a 37°C incubator with 5% CO2. Cells were harvested by centrifugation (300 x g, 5 min) and washed twice with cold PBS lacking Ca^2+ and Mg^2+ (Gibco, cat #: 14,190–144) before being resuspended in cold PBS at a concentration of about 2 × 10^6 cells/mL and labeled with PE-aCD25 (BioLegend, 302,606) and FITC-aCD69 (BioLegend, 310,904) for 30 min on ice. Labeled cells were
analyzed by flow cytometry using a FACScalibur instrument, and data were processed using FlowJo software.

**Cell-killing potency assay**

**Cell and sample preparation**

The day before the assay was performed, a vial of human PBMCs (Stemcell Technologies, cat. #: 70,025) were thawed, washed, and resuspended to ~1 x 10^6 cells/mL in RPMI 1640 supplemented with 10% HI FBS. PBMCs were allowed to recover overnight in a 37°C incubator supplemented with 5% CO_2_. Dilutions of TDB1 reference material were prepared in assay medium consisting of RPMI 1640 supplemented with 10% HI FBS. On the day of the assay, dilutions for the standard and assay control curves were prepared in the same manner as the TDB1 samples. Sample, standard, and assay control dilutions were pipetted into a 96-well tissue culture plate and allowed to equilibrate to 37°C before the addition of cells. During sample pre-incubation, cultured target cells and PBMCs were adjusted to plating concentrations before 80 μL of each was added to the plate (final cell ratio 1:10 target:PBMC, ~40,000 and ~400,000 cells/well). Plates were placed in a 37°C incubator supplemented with 5% CO_2_ overnight (20–26 h).

**Flow cytometry and data analysis**

Following incubation of TDB1 with target-cell line A and PBMCs, assay plates were centrifuged for approximately 5 min at 300 x g. The assay medium was carefully aspirated, and each well was rinsed with 200 μL cold PBS lacking MgCl_2_ and CaCl_2_. The plates were centrifuged for approximately 3 min at 300 x g, and PBS was aspirated before propidium iodide (PI, ThermoFisher; cat. #: P3566) and anti-Receptor Y-FITC in PBS (70 μL/well, 5 μg/mL PI and 120 ng/mL anti-Receptor Y-FITC). Cells were labeled for at least 30 min in 2–8°C before analysis by flow cytometry using a FACScalibur instrument. Data analysis was performed using FlowJo Software, and Receptor Y+/PI-cells (live target cells) were gated to assess the level of cell killing. The potencies of the assay control and each sample relative to the standard were calculated using parallel-line analysis and reported as percent relative potency.

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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