Journal of Immunological Methods 474 (2019) 112642

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Addressing soluble target interference in the development of a functional assay for the detection of neutralizing antibodies against a BCMA-CD3 bispecific antibody

Ying Wang*, Michael Luong, Crisanto Guadiz, Minlei Zhang, Boris Gorovits

BioMedicine Design, Pfizer, Andover, MA, United States of America

ARTICLE INFO

Keywords:
CD3 Bispecific antibody biotherapeutics
Cell based antibody neutralizing assay
Sample pretreatment procedures
Soluble B cell maturation antigen level
Tumor-associated antigen
Jurkat T cells and Multiple Myeloma cells

ABSTRACT

Proper evaluation of immunogenicity during clinical development of biotherapeutics is a major challenge to bioanalytical scientists, in part due to matrix interference in anti-drug antibody (ADA) and neutralizing antibody (NAB) assays. If not addressed, matrix interference could confound the immunogenicity assessment of a given biotherapeutic in clinical development. To support clinical development of a B cell maturation antigen (BCMA)-CD3 bispecific antibody, a cell-based NAB assay was developed as part of a tiered approach to evaluating the immunogenicity of the drug. The assay endpoint (T cell activation) was chosen based on its strong association with the mechanism of action of the drug. The BCMA-CD3 bispecific antibody activates T cells through simultaneous binding of CD3 on T cells and BCMA on target cells. In this system, T cell activation was assessed through the measurement of luciferase activity in an engineered Jurkat cell line. In the presence of NAB, the degree of T cell activation measured by the amount of luciferase activity can be reduced. During method development, soluble BCMA (sBCMA) interference in the NAB assay was apparent. The binding of sBCMA to the anti-BCMA domain of the bispecific drug led to reduced T cell activation, which caused false positive results in NAB testing. To mitigate this interference, several strategies to eliminate sBCMA were investigated. Among the procedures tested, a bead-based approach proved most effective in depleting sBCMA, while maintaining robust assay performance and achieving fit-for-purpose sensitivity. Using this sample pretreatment procedure, the NAB assay tolerated sBCMA up to 2 μg/mL, or approximately four times the estimated median sBCMA concentration in serum samples from patients with active multiple myeloma.

1. Introduction

Significant efforts have been made in the pharmaceutical industry to harness the potent tumor killing capacity of T cells in antibody drug design. CD3-Tumor Associated Antigen (TAA) bispecific antibodies seek to redirect T cell cytotoxicity to cancer cells through their binding to CD3 on T cells and a specific TAA on target cells. By bringing tumor cells and T cells within proximity of each other, bispecific antibodies trigger T cell proliferation and activation, resulting in the targeted release of cytotoxic molecules such as granzyme and perforin to lyse tumor cells (Huehls et al., 2015; Zhukovsky et al., 2016; Zhang et al., 2017). PF-06863135, a humanized full-length bispecific antibody comprised of one anti-BCMA binding domain and one anti-CD3 binding domain, is in clinical development for the treatment of relapse/refractory multiple myeloma (ClinicalTrials.gov Identifier: NCT03269136; Lesokhin et al., 2018,60th ASH Annual Meeting). To support immunogenicity assessment of this drug in clinical development, we developed a cell-based neutralizing antibody (NAB) assay as part of a tiered approach to characterizing anti-drug antibodies (ADA). In this NAB assay, T cell (Jurkat cell) activation is triggered by the drug simultaneously binding to CD3 on Jurkat cells carrying a luciferase reporter gene and BCMA on multiple myeloma cells (MM cells). The degree of T cell activation achieved is entirely dependent on the drug's ability to bridge the Jurkat and MM cells and is reflected in the level of luciferase activity measured. The presence of domain specific NAB against the drug could interfere with the bridging of Jurkat and MM cells and, therefore, diminish T cell activation. One of the major challenges encountered in the development of this assay was interference

Abbreviations: ADA, antidrug antibodies; ECD, extra- cellular domain; FBS, fatal bovine serum; MAB, Monoclonal Antibody; MM, Multiple Myeloma; NAB, neutralizing antibodies; NFAT, Nuclear Factor of Activated T cells; NCT#, Clinical Trial Registration number; PC, positive control; NC, negative control; sBCMA, soluble B cell Maturation antigen; PAB, Polyclonal Antibody; TAA, Tumor associated antigen

* Corresponding author.
E-mail address: ying.wang9@pfizer.com (Y. Wang).

https://doi.org/10.1016/j.jim.2019.112642
Received 1 June 2019; Received in revised form 28 July 2019; Accepted 6 August 2019
Available online 07 August 2019

from soluble BCMA (sBCMA). BCMA is a type-I transmembrane glycoprotein of the tumor necrosis factor receptor superfamily. It consists of 184 amino acids, including a 54-amino acid extracellular domain (ECD) that can be shed into circulation by gamma-secretase (Laurent et al., 2015). sBCMA level is increased with the progression of multiple myeloma and the amount of sBCMA in circulation correlates with disease status and response to treatment (Ghermezi et al., 2017; Cho et al., 2018). While the estimated median level of sBCMA is approximately 0.5 μg/mL in multiple myeloma patients, the concentration could be as high as 2 μg/mL in subjects with active, untreated disease (Ghermezi et al., 2017). At these levels, sBCMA has the potential to interfere in ADA and NAB assays due to its interaction with the anti-BCMA domain of the drug. By competing with cell-associated BCMA for drug binding, sBCMA can diminish T cell activation, causing false positive results in the NAB assay. Matrix interference in general can be overcome by solid phase extraction and acid dissociation (SPEAD) procedures to capture ADA out of serum matrix (Smith et al., 2007). However, these procedures are not intended for the elimination of drug targets, because drug targets would also be enriched and, in the absence of additional procedures, cause interference. In contrast, the use of anti-target antibodies or target-specific ligands as scavengers of the target has proven effective, as complexes between the drug target and the scavenger can be eliminated through washing procedures (Zhong et al., 2017). In practice, this type of approach is best implemented as a pretreatment protocol in which samples are depleted of drug target prior to NAB detection in the cell-based portion of the assay. Herein, we describe a simple pretreatment method involving the use of polyclonal anti-sBCMA antibodies as scavengers to mitigate target interference. Using this approach, we were successful in depleting sBCMA at a concentration four times greater than the median level of this TAA in the multiple myeloma patient population.

2. Materials and methods

2.1. Reagents

2.1.1. Critical reagents

PF-06963135, a fully humanized IgG CD3 bispecific antibody targeting BCMA, was produced by Pfizer (Panowski et al., 58th ASH Annual Meeting). The sBCMA protein used in this study was generated from mammalian cells and contained a 57-amino acid sequence that encompassed the entire extracellular domain of BCMA (Pfizer). Domain specific anti-PF-06963135 positive controls (PC) against the anti-CD3 domain and anti-BCMA domain were anti-idiotype monoclonal antibodies produced and characterized by Pfizer (with the affinities for the drug from M^{-9} to M^{-12}). The rabbit anti-BCMA polyclonal antibody (PAB) was purchased from R&D systems (cat. # AF193). Per vendor's specification, this antibody was made using extracellular domain (ECD) of BCMA as the immunogen, and it was shown to block BCMA binding to its ligands (APRIL and BAFF proteins). The biotinylated version of this antibody was produced by Pfizer. Streptavidin conjugated magnetic beads were purchased from GE Healthcare (cat. # 66152104010150). The luminescent substrate (ONE-Glo® Luciferase) was purchased from Promega (cat. # E6110). Drug naïve normal and disease human serum (multiple myeloma) were purchased from Bio-IVT and informed consent was obtained from all donors.

2.1.2. Cell lines and cell culture materials

The Nuclear Factor of Activated T cells (NFAT).B3-Luc Jurkat T cells were from Pfizer and a master cell bank was generated at Pfizer. MM.1S Multiple myeloma cells (MM cells) were purchased from ATCC (cat. # CRL-2974) and a master cell bank was subsequently established at Pfizer. Cells from each master cell bank were further expanded to working cell banks for this study. All cell culture media and related components and chemicals were purchased from ThermoFisher; these included RPMI-1640 Medium (Dutch modification, cat. # 22409), RPMI-1640 Medium (ATCC modification, cat. # A10491), heat inactivated fetal bovine serum (HI FBS, cat. # 10082-147), GlutaMAX™ (cat. # 35050-061), Puromycin (cat. # A111380-03), and Penicillin/Streptomycin (10,000 U/mL, cat. # 15140-122). Dimethyl sulfoxide (DMSO, cat. # D2650) and Trypan Blue (cat. # T8154) were purchased from Sigma Aldrich.

2.1.3. Chemicals for assay buffers and sample diluents

Phosphate Buffered Saline (DPBS) was purchased from VWR (cat. # 16777–250). Calcium and Magnesium Free PBS (PBS-CMF, cat. # 28352) and 20× PBS Tween 20 (PBST) Buffer (cat. # 10010072, Pierce brand) were purchased from ThermoFisher. Bovine Serum Albumin (BSA) was purchased from Sigma Aldrich (cat. # A7030).

2.2. Cell growth and cell banking

MM cells were maintained in RPMI 1640 Medium (ATCC modification in the presence of 10% heat inactivated FBS). Jurkat cells were maintained in RPMI-1640 Medium (Dutch modification) with 10% heat inactivated FBS, 1% GlutaMAX™ (stock is 100 x), 1% PenStrep (stock is 10,000 U/mL) and 0.005% of Puromycin (stock is 10 mg/mL) to maintain expression of the luciferase reporter gene. Both cell lines were cultured in either 75 cm^2 or 175 cm^2 cell culture vessels in a 37 °C incubator with CO_2 set at 5% and humidity set at 95%. Both cell banks were established with cell viability > 90%. These suspension cells were slightly attached during culture and no reagent was needed to dislodge them during subculture or assay setup.

Working cell banks for Jurkat cells and MM cells were established at 4×10^6/mL and 8×10^6/mL, respectively. Cell banks were characterized for their ability to trigger T cell activation in the presence of drug (elevated luciferase activity compared to absence of drug). The assay response window (ratio of luciferase signal in presence vs absence of drug) was expected to be ≥4×, and the viability of the both cell lines was expected to be ≥80% when used in the assay. Both cell banks were free of microbial contamination (mycoplasma and sterility tests were performed at IDEXX Bioresearch). One vial of each working cell bank (Jurkat cells and MM cells) was thawed and cultured in their respective culture medium for 24 to 48 h in a 37 °C CO_2 incubator prior to use in the assay. To minimize fluctuations in cell response levels to system drug and, therefore, maintain robust assay response windows for NAB detection, cells were not continuously cultured between assays. Rather, unused cells were discarded on the day of assay, and fresh vials were introduced into culture on an as-needed basis.

2.3. Assay buffers and assay medium

Assay buffers used for sample pretreatment included PBS-CMF (Phosphate-buffered saline, calcium and magnesium free), PBST (Phosphate Buffered Saline, Calcium and Magnesium Free with 0.05% Tween 20), and 1% BSA in PBST (Phosphate Buffered Saline with 1% Bovine Serum Albumin, 0.05% Tween 20).

The components of assay medium were RPMI-1640 with 10% FBS.

2.4. Preparation of system drug and assay controls

The system drug was a single concentration of PF-06963135 used in the assay that yielded 50 to 70% T cell activation as measured by luciferase activity in genetically engineered Jurkat cells in the presence of target cells (MM cells). The system drug was prepared in assay medium and incubated with samples, positive and negative controls. The mixtures of system drug with samples or controls were subsequently added to cells. During method development, PF-06963135 was titrated to

Table 1
Neutralizing assay controls (System suitability controls for each assay run).

System suitability controls	Composition of each System Suitability Control in a Assay Plate (Assay System: System drug + MM/Jurkat cells)
Drug Control (DC)	Negative Matrix Control (NC) added to Assay System
Positive Controls (PCs)	Positive matrix control (spiked with respective domain specific anti-drug antibody) added to Assay System
sBCMA Assay Control	sBCMA matrix control added to Assay System
Assay Blank	Negative matrix control (NC) Added to Jurkat/MM cells only, no System Drug added): serves as basal level activity control and to calculate the assay response window (ratio of DC /Assay Blank)

select an appropriate system drug concentration that produced the desired level of T cell activation (reflected by 50 to 70% luciferase activity). The system drug concentration was finalized at 1 nM (150 ng/mL) at the end of method development.

Assay performance was monitored by domain specific PC, negative control (also called drug control or DC) and sBCMA controls. The negative matrix control was established using pooled drug naïve serum lots from healthy volunteers (normal human serum pool) and serum from multiple myeloma patients (disease serum pool); the positive matrix controls were prepared by spiking affinity purified domain specific anti-PF-06963135 anti-idiotype antibodies in the drug naïve normal human serum pool. The positive matrix controls for anti-CD3 domain and anti-BCMA domain were implemented at three levels: HPC (5 μg /mL), MPC (2.5 μg /mL) and LPC (870 ng/mL and 850 ng/mL for anti-CD3 domain and anti-BCMA domain, respectively). The BCMA matrix control was prepared at 2 μg/mL in a drug-naïve normal human serum pool. All matrix controls were prepared in bulk, aliquoted for single use and stored in a − 70 °C freezer.

In the NAB assay, the positive and negative matrix controls were mixed with system drug (or assay medium) and target (MM) cells/ reporter(Jurkat) cells to generate neutralizing assay controls as described in Table 1.

2.5. NAB assay procedures

2.5.1. Assay principle

This cell-based NAB assay was established based on the mechanism of action of PF-06863135 and was comprised of a single concentration of system drug (PF-06863135) and a mixture of reporter T cells and target MM cells. The T cells used (Jurkat) were derived from acute T cell leukemia and were genetically engineered to express the luciferase reporter gene driven by the Nuclear Factor of Activated T cell (NFAT) response element. The MM cells expressed the BCMA receptor (ATCC). T cell activation through the NFAT pathway was triggered by the drug simultaneously binding to CD3 on Jurkat cells and BCMA on MM cells, effectively bridging the two cell types and resulting in elevated luciferase activity. In the presence of NAB, either against the anti-CD3 domain or anti-BCMA domain of the drug, the binding of PF-06863135 to its targets could be disrupted, which in turn led to the down-regulation of the NFAT pathway and subsequently a decrease in luciferase activity. The decrease in luciferase activity is proportional to the degree of NAB activity. The assay principle is illustrated in Fig. 1.

2.5.2. General assay procedures

This assay was performed over a two-day period, starting with sample pretreatment after samples and controls (Fig. 2) had been diluted at a Minimum Required Dilution (MRD) 1/5 in assay medium containing anti-BCMA antibody and incubated before added to Streptavidin coated magnetic beads. The pretreated samples were then incubated with system drug (PF-06863135 at 1 nM or 150 ng/mL) for approximately 30 min in 96-well polypropylene plates, which constituted an additional 1.2-fold dilution. During sample incubation, cells were prepared by mixing equal volumes of Jurkat and MM cells at a reporter to target ratio of 2:1, using cell numbers established during assay development. The cell mixture was then added at 50 μL per well to an assay plate, followed by addition of 50 μL of sample. The assay plate was incubated for 4 to 4.5 h. At the end of the incubation period, 100 μL of luciferase substrate was added to each well and assay responses were acquired using the SpectraMax M5 microplate reader (Molecular Devices).

2.5.3. General sample pretreatment procedures

A summary of the established sample pretreatment procedure is illustrated in Fig. 2.

In the final method, on day 1, the samples and controls were mixed with assay medium containing 10 μg/mL of biotinylated anti-BCMA polyclonal antibody (anti-BCMA PAB from R&D Systems) in a deep-well 96-well polypropylene plate (ThermoFisher, cat. # 260251 or equivalent) and incubated for approximately 2 h at room temperature (RT) on a shaker to ensure continued, thorough mixing of the samples. Following incubation, streptavidin magnetic beads prewashed with PBST and subsequently PBS were added to the controls and samples and these mixtures were incubated for an additional 2 h at RT with vigorous shaking to ensure continued suspension of the beads, followed by a similar overnight incubation at 2 to 8 °C. The total volume of the mixture was 200 μL for each sample and control. On day 2, the 96-well deep-well plate containing controls or samples was removed from the plate shaker and placed on 96-well format magnet to pellet the beads. For each sample, 150 μL of supernatant was transferred from the deep-well plate (containing pretreated controls or samples) to the 96-well polypropylene plate containing 30 uL of system drug (1 nM or 150 ng/mL). The assay was performed as described in section 2.5.2: “General assay procedures.”

2.6. Results acquisition and assay acceptance criteria

In this NAB assay, samples were tested in duplicate and raw responses (luminescent signals) were acquired using a microplate reader as relative light units (RLU). The targeted acceptance criteria for intra-assay CV of replicates was ≤25%. Multiple DC (NAB negative drug control) was tested in a typical assay run and the mean response of DC was used to normalize the mean response of PC and samples. All controls and samples were converted to Signal to DC response ratios (S/DC) unless otherwise indicated. The S/DC ratios of PCs were expected to be below the Cut Point Factor (CPF) and were expected to correspond with concentrations of PCs in an inverse relationship: the higher the concentration of PC, the lower the S/DC ratio obtained. The samples with S/DC values equal to or below the CPF were reported as NAB positive. The S/DC value of the sBCMA control was expected to be consistently above the CPF to demonstrate that this assay was able to tolerate sBCMA interference at the expected 2 μg/mL level.

2.7. Statistical analysis

JMP 812.0.1 (SAS Institute) was used to perform outlier and normality analyses prior to calculating CPF. Briefly, the S/DC values from all cut point analysis runs were grouped as one set of data and imported into JMP for normality analysis using the Shapiro–Wilk test after one

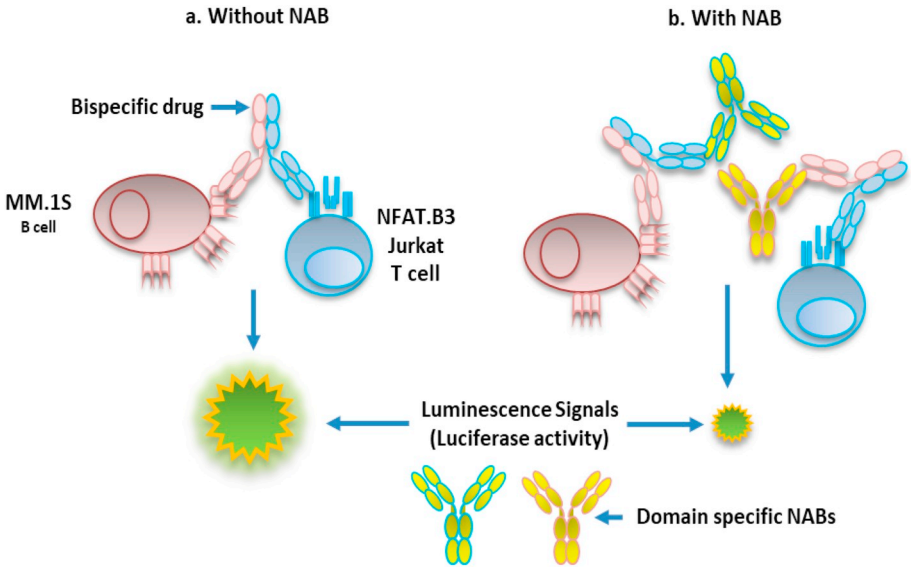


Fig. 1. Assay principle. The assay system is based on the mode of action of PF-06963135 and includes target multiple myeloma (MM) cells and reporter Jurkat T cells. The results are measured as relative light units (RLU) and the level of T cell activation is proportional to the signals obtained, as indicated in scenario "a" (without NAB). In the presence of domain specific neutralizing antibodies (NABs), the luciferase activity will be decreased and the decrease in signal is correlated to the level of NAB (scenario b, with NAB).

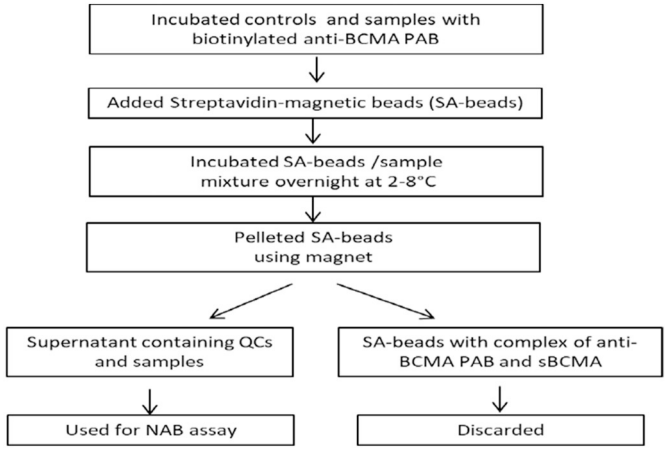


Fig. 2. Schematic illustration of sample pretreatment procedure (reference text for details).

round of outlier removal (using 1.5 IQR). The CPF was established per industry practice with a 1% false positive rate (Shankar et al., 2008) using drug naïve multiple myeloma patient samples (total of 30 samples were used for cut point analysis in method validation). The assay sensitivity was calculated using mean concentrations + $t_{0.05\ xdf}$ x SD, assuming a 5% false positive rate, while the LPC concentration was calculated using mean concentration + $t_{0.01xdf}$ x SD, assuming a 1% failure rate.

3. Results

3.1. Demonstration of suitability of assay endpoint

To demonstrate that the assay endpoint was relevant to the mechanism of action of the drug, we first performed drug titration experiments to evaluate drug concentration versus T cell activation levels. PF-06863135 was prepared in a range of concentrations in assay medium containing 10% drug naïve human serum and subsequently incubated with a mixture of Jurkat and MM cells overnight. T cell activation was measured by the addition of Bright-Glo™ luciferase substrate. The results indicated a typical dose-dependent drug response curve (Fig. 3a). To further demonstrate the suitability of this assay system, we tested the requirement for simultaneous binding of targets on both cell types to produce robust T cell activation. This evaluation was performed by incubating a single drug concentration with either a mixture of MM and Jurkat cells, or with Jurkat cells alone. The results shown in Fig. 3b indicated that PF-06863135 was able to induce T cell activation only in the presence of both cell types. The inability of the drug to induce T cell activation in the absence of the MM cells underscores the necessity of the drug to bind targets on both cell types.

3.2. Optimization of assay parameters

To establish a robust assay system for the detection anti-PF-06863135 neutralizing antibodies, assay parameters were further optimized. The assay window, which was defined as the assay signal in the presence vs absence of drug (signal/noise, S/N), was targeted at ≥4. This assay window was achieved by choosing an appropriate system drug concentration (the lowest concentration that yielded a ≥ 50% cellular response in the linear portion of the drug titration curve) in the context of other assay parameters, including absolute cell numbers, cell ratios and appropriate MRD. The system drug (PF-06863135) concentration was evaluated from 0.001 nM (150 pg/mL) to 3 nM (450 ng/mL) under various conditions of cell numbers and ratios. The data indicated that a 2:1 ratio of Jurkat to MM cells was optimal for T cell activation and that the assay window was robust when reporter cells were used at 60×10^3 and target cells at 30×10^3 per well. System drug concentration at 1 nM (150 ng/mL) did provide expected assay response window (Fig. 4).

The appropriate MRD was determined using domain specific positive controls, which were monoclonal antibodies developed using the hybridoma platform. A total of eight anti-CD3 domain specific monoclonal antibodies (MAB) and one anti-BCMA domain specific MAB were screened for neutralizing activity in the NAB assay. The MABs were individually spiked into 100% drug naïve human serum pool at 10 µg/mL and were then diluted at MRD 1/2.5, 1/5 and 1/10 in assay medium. The system drug concentration used was 3 nM (450 ng/mL). As shown in Fig. 5a, seven of the eight anti-CD3 domain MAB and the sole anti-BCMA domain MAB neutralized system drug induced T cell activation. Similar levels of neutralization were observed for MAB-3 (anti-CD3 domain PC) and anti-BCMA domain MAB. Of the MRD tested, similar neutralizing activity by PCs was observed at MRD 1/2.5 and 1/5 (Fig. 5a). The validity of the MRD at 1/5 and system drug concentration at 1 nM (150 ng/mL) was further confirmed by matrix selectivity

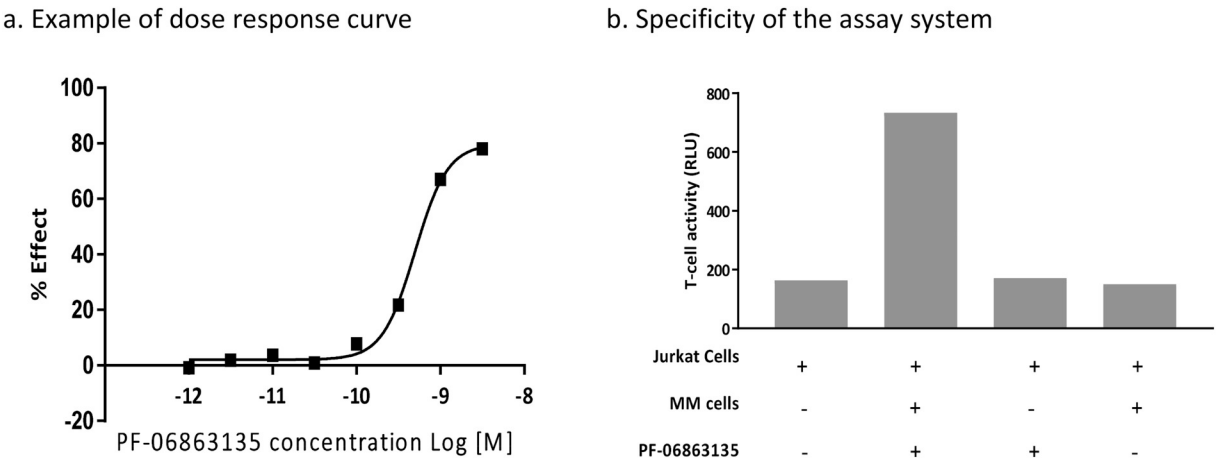


Fig. 3. Demonstration of specificity of assay endpoint.
a. Dose-dependent T cell activation. PF-06863135 was prepared in a range of concentrations in assay medium with 10% human serum and then incubated with a mixture of MM and Jurkat cells prior to measuring T cell activation. The estimated drug activity at EC_{50} was 0.5 nM (75 ng/mL) and estimated EC70 was 1 nM (150 ng/mL).
b. Specificity of T cell activation. This assay was performed in several conditions as indicated in the figure with system drug concentration at 3 nM (450 ng/mL). An increase in luciferase activity of approximately 4-fold was observed only when the drug was exposed to both cell types. Elevated levels of luciferase activity were not observed under other conditions (Jurkat cells alone, Jurkat cells with drug, or both cell types in the absence of drug).

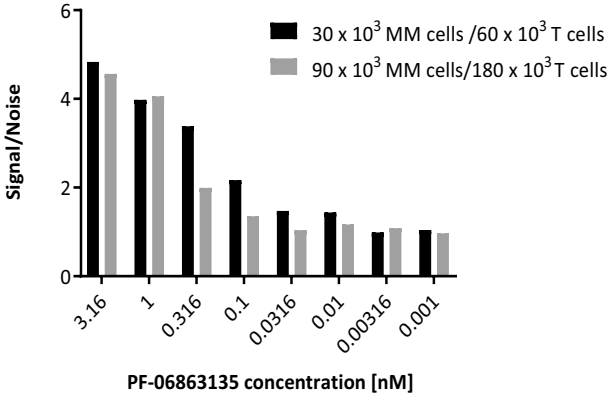


Fig. 4. Comparison of the number of cells versus responses.
Cell numbers used in the assay were evaluated under a 2:1 ratio of Jurkat to MM cells in the presence of serially diluted PF-06863135. The absolute cell number in each well was tested at either 30×10^3 MM cells and 60×10^3 Jurkat cells, or 90×10^3 MM cells and 180×10^3 Jurkat cells. The results were evaluated by assay window (response of each sample with drug / response assay blank control, or S/N). Increases in absolute cell numbers did not enhance assay windows. The 30×10^3 MM/60×10^3 T cell condition in combination with system drug concentrations between 1 nM (150 ng/mL) and 3 nM (450 ng/mL) produced assay window (S/N) ≥ 4.

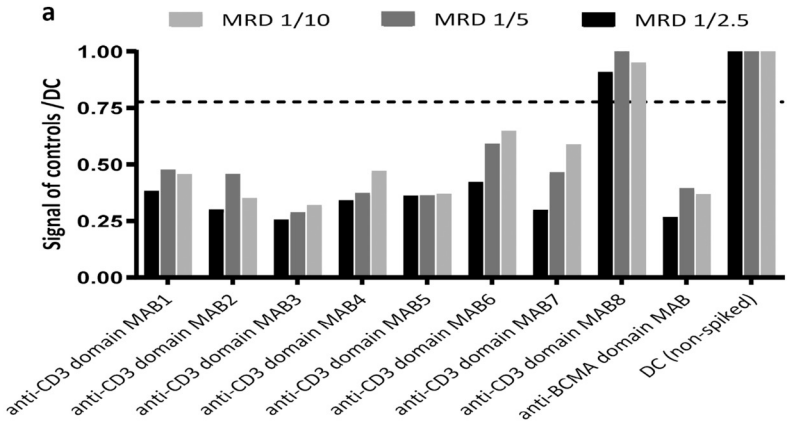
assessment in drug naïve individual serum lots from healthy donors and from multiple myeloma patients (Fig. 5 b-1 and b-2).

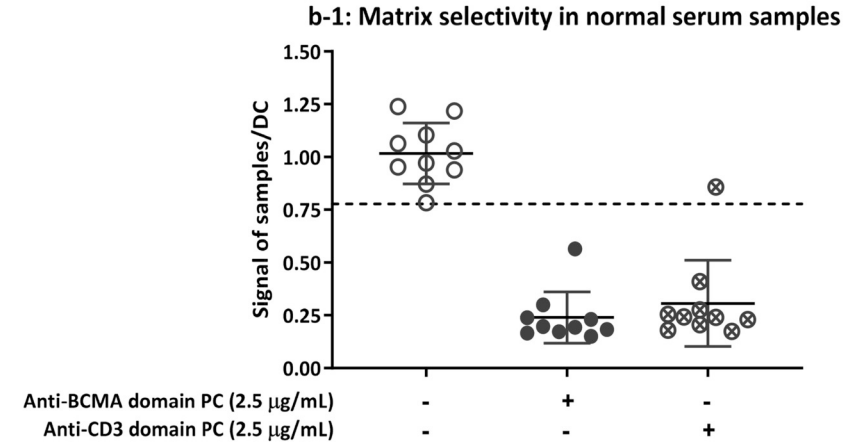
3.3. Investigation of sBCMA interference

Studies have reported the median level of sBCMA in multiple myeloma patients at approximately 500 ng/mL, while in some subjects the level could be as high as 2 μg/mL (Ghermezi et al., 2017). Our investigation was conducted by using both MM patient serum samples from commercial sources and using human recombinant BCMA (extra cellular domain of BCMA, ECD-BCMA) spiked in pooled human serum samples. MM serum samples from commercial sources generally lacked complete medical history, including information on prior treatment and disease stage. Therefore, it was essential to thoroughly investigate sBCMA interference using BCMA-ECD as a surrogate in the NAB assay in addition to examining the signal response levels of samples from MM patients.

To investigate sBCMA interference in the NAB assay prior to development of the sample pretreatment protocol, we tested 24 individual serum samples from MM patients obtained from a commercial source. Among the samples, two were listed as in MM remission and the treatment history for other samples was not provided. sBCMA concentrations from these MM serum samples were quantified using a commercial enzyme linked immunosorbent assay kit with the procedures established by vendor (ELISA, R&D Systems, DY193E). The range of sBCMA in these MM serum samples was from 8.4 ng/mL to 550 ng/mL (median of 48.55 ng/mL) with 1 sample over 550 ng/mL. Significant signal reduction was observed in 1 of 24 samples in the NAB assay (Fig. 6). Surprisingly, the sBCMA concentration of the sample that caused a signal reduction was 95.8 ng/mL, while the samples with sBCMA at 550 ng/mL did not appear to interfere in the assay (patients listed as in remission in the information provided by the vendor). In addition, we also observed that the responses induced by system drug in MM serum samples were more variable than the responses in normal human serum samples. These results indicated that, while system drug induced responses were similar between MM and normal serum, the variable responses within the MM samples might be associated with nonspecific matrix interference and/or sBCMA levels.

Because virtually all MM serum samples contained sBCMA at about or below 500 ng/mL, we also investigated sBCMA interference by testing serum from healthy donors spiked with much higher concentrations of a recombinant form of the extracellular domain of BCMA (BCMA-ECD). Results presented in Fig. 7 indicated that T cell activation signals were diminished in the presence of BCMA-ECD in a dose-dependent manner. Indeed, concentrations of sBCMA ≥700 ng/mL reduced T cell activation with signal/DC below the tentative CPF. The signal reduction in the presence of sBCMA was similar to the signal reductions observed in the presence of NAB. These results confirmed that sBCMA at its estimated median level or higher interfered in the NAB assay to cause false positive results. To achieve sBCMA tolerance up to 2 μg/mL in the NAB assay, it was imperative that we establish an effective sample pretreatment procedure to remove or otherwise





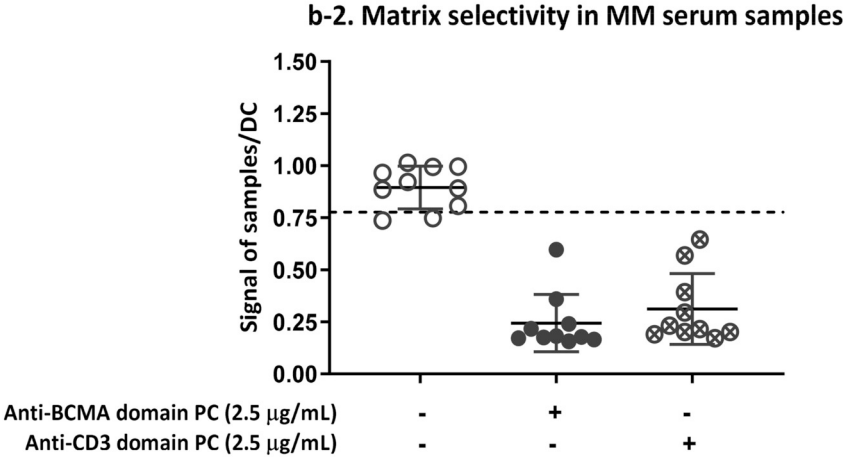


Fig. 5. Optimization of Minimum Required Dilution (MRD).
a: Examining appropriate MRD in pooled human serum: Domain specific anti-PF-06863135 PCs were prepared by spiking 10 μg/mL each ADA in normal human serum pool. Samples were subsequently diluted at the indicated MRD in assay medium. The NAB samples and negative control were incubated briefly with system drug (PF-06863135, 3 nM or 450 ng/mL) prior to their addition to a mixture of Jurkat and MM cells. The samples and cells were incubated overnight in a 37 °C incubator with CO_2 set at 5%. Luciferase activity was measured the following day. Results are presented as ratios: mean response of each domain specific control / mean response of DC (Drug Control with NC at proper MRD, the ratio of DC itself is 1 as indicated in the far right of the figure). The dotted line indicates a tentative Cut Point Factor (CPF, 0.777) using multiple myeloma (MM) patient samples during early method development, as discussed in section-3.3.
b: Examining validity of MRD established by matrix selectivity assessment: This assessment was performed using optimized assay conditions as described in section 2.5. Matrix selectivity was evaluated with 10 individual normal serum samples and 10 MM serum samples, unspiked and spiked with domain specific antibodies at 2.5 μg/mL. The signal/DC ratio at the dotted line indicates a tentative CPF, which was 0.777. The signal/DC ratios of domain specific PC spiked samples in both normal serum samples and in MM serum samples were very similar to each other and remained positive with PC spiked at 2.5 μg/mL, while all non-spiked samples were negative. These results indicated that sample dilution at MRD 1/5 was appropriate.

neutralize sBCMA.

3.4. Development of sample pretreatment procedures for sBCMA depletion

Several sBCMA depletion procedures were explored. We initially attempted to perform acid dissociation followed by sBCMA adsorption using biotinylated anti-BCMA antibodies immobilized on a streptavidin-coated plate. However, these efforts were not successful for mainly two reasons. First, the acid-treated samples caused nonspecific reduction in T cell activation/luciferase activity, resulting in assay windows below our expectations for a robust method (data not shown). We suspected that T cell activation in this system was highly susceptible to changes in assay medium composition, including fluctuations in pH. Second, the plate-based sBCMA depletion procedure did not sufficiently deplete sBCMA (Fig. 8a), even though NAB detection was not affected in the presence or absence of spiked drug at 2 μg/mL (which is relevant to the trough level of the drug concentration in study support). Based on these findings, we explored a streptavidin magnetic beads-based procedure. Using this approach, an anti-BCMA polyclonal antibody was biotinylated and added to assay medium. Samples and controls were then

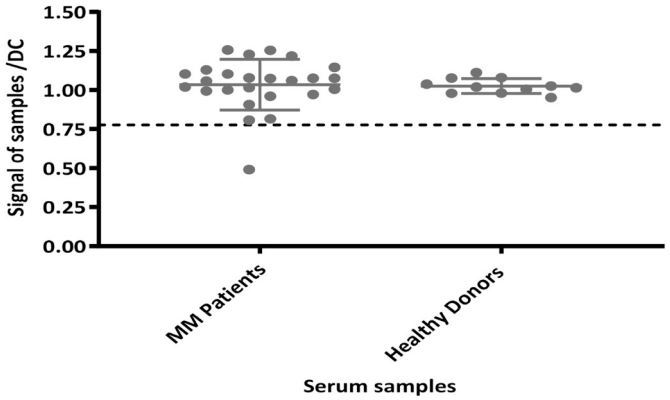


Fig. 6. Investigation of sBCMA interference in multiple myeloma patient serum. Serum samples, either from healthy donors or from multiple myeloma patients, were diluted in assay medium at MRD 1/5 then tested in the NAB assay. The system drug concentration used in this assay was 3 nM (450 ng/mL). The solid lines in each sample group indicate the mean ratio of sample signals / DC signals ± SD from total samples tested. The result from MM serum lots was 1.034 ± 0.1625, and the result from normal serum lots was 1.026 ± 0.048. The tentative CPF calculated with S/DC of MM serum samples (1% false positive) was 0.777 (calculated using S/DC ratio for 1% false positive).

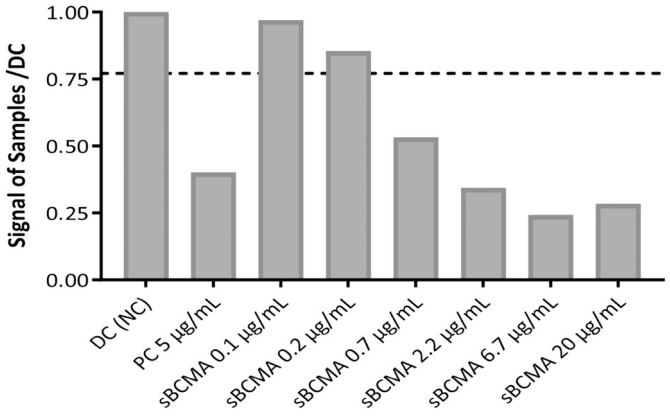


Fig. 7. Investigating sBCMA interference with BCMA-ECD.
Human recombinant BCMA-ECD at concentrations of 0.1, 0.2, 0.7, 2.2, 6.7 and 20 µg/mL were spiked into human serum and then diluted at MRD 1/5 in assay medium prior to testing in the NAB assay. Anti-CD3 domain specific MAB (anti-CD3 domain specific) was used as a positive control and normal human serum was used as a NAB negative control (DC). The dotted line represents the tentative CPF (0.777) from MM disease population.

diluted at MRD 1/5 in assay medium containing the biotinylated anti-BCMA antibody and incubated at ambient for two hours to form BCMA-anti-BCMA antibody complexes. After incubation, streptavidin coated magnetic beads were added to controls and samples with an additional incubation overnight at 2 to 8 °C with vigorous shaking. On the following day, the magnetic beads were pelleted and supernatants from controls and samples were removed and tested in the NAB assay (procedure summarized in Fig. 2). As shown in Fig. 8b, this beads-based procedure improved sBCMA interference when the biotinylated anti-BCMA antibodies were used at 5 µg/mL in the absence and presence of drug in serum samples. Subsequently, several concentrations of biotinylated anti-BCMA antibodies were tested to examine the specificity and capacity of the beads-based procedure to deplete sBCMA. As shown in Fig. 8c, the reduction of sBCMA interference level was dependent on the concentration of biotinylated anti-BCMA antibody used. The targeted tolerability of sBCMA interference at 2 µg/mL was achieved when 10 µg/mL of biotinylated anti-BCMA antibody was used. Importantly, the addition of biotinylated anti-BCMA antibody to samples did not have a significant impact on NAB detection either in the presence or absence of residual drug. Therefore, the bead-based sample pretreatment procedure was integrated into the NAB assay, using a biotinylated anti-BCMA antibody concentration of 10 µg/mL.

3.5. Summary of assay performance in pre-study method validation

Once the sample pretreatment procedure was implemented and remaining assay parameters were optimized, the anti-PF-06863135 NAB assay was successfully validated according to best industry practices for NAB assay validations (Gupta et al., 2011). All raw data from samples and positive controls were normalized to the response of the NAB negative control (drug control, DC) as a ratio of mean values (signals of sample or PC/mean signal of DC). The NAB negative control was established by myeloma drug naïve serum to better suit the purpose of this NAB assay. The CPF of this NAB assay was established using drug naïve myeloma patient serum samples, which was at 0.861 (1% false positive rate). The assay sensitivity was between 738 ng/mL and 682 ng/mL in 100% human serum using domain specific NAB positive controls (anti-CD3 domain and anti-BCMA domain, respectively). The LPC concentrations of domain specific NAB controls were at 870 ng/mL and 850 ng/mL, with acceptable inter- and intra-assay precision (CV < 20%) and short-term stability. In addition, matrix selectivity evaluated in drug naïve myeloma patient serum samples (either non-hemolyzed or hemolyzed up to 5%) was acceptable and free drug tolerance of the assay was up to 2 µg/mL with both domain specific PC at 2.5 µg/mL, which met expectations for clinical study support. To ensure that the sBCMA tolerance level met the targeted concentration (2 µg/mL) and that the assay was specific for NAB detection, method specificity was evaluated by spiking sBCMA into NC and PCs (HPC, MPC and LPC of each domain specific control) at several concentrations. The results in Fig. 9 demonstrated that this assay tolerated sBCMA up to 2 µg/mL.

Additionally, in the design of system suitability controls, a sBCMA tolerance control was prepared along with domain specific PC controls and negative controls. The sBCMA tolerance control was generated by spiking BCMA-ECD into a normal human serum pool at a concentration of 2 µg/mL and tested in all validation assay runs. This control consistently exhibited negative response levels (S/DC > assay cut point) across all validation runs (Fig. 10). These results indicated that sBCMA interference at targeted tolerability levels was addressed successfully using the bead-based sample treatment procedure described.

4. Discussion

The evaluation of immunogenicity during clinical development of biotherapeutics is of critical importance to patient safety and drug efficacy. As an accompaniment to traditional ADA assays, the functional cell-based NAB assay has become an increasingly common regulatory expectation (Gouty et al., 2017). Due to the complexity of NAB assays, bioanalytical scientists often face challenges not typically encountered in the development of ADA assays. In the case of NAB assays for CD3 bispecific antibodies, many of the challenges are attributable to the mechanism of action of this class of molecules. For example, due to the bispecific nature of the antibody, two-cell systems are often utilized to measure T cell activation in the effector cell or cytotoxicity in the target cell (Trinklein et al., 2019). Furthermore, the binding of the bispecific antibody to targets on both cell types in the assay can be susceptible to interference from several sources, including serum components, residual drug, and circulating drug target.

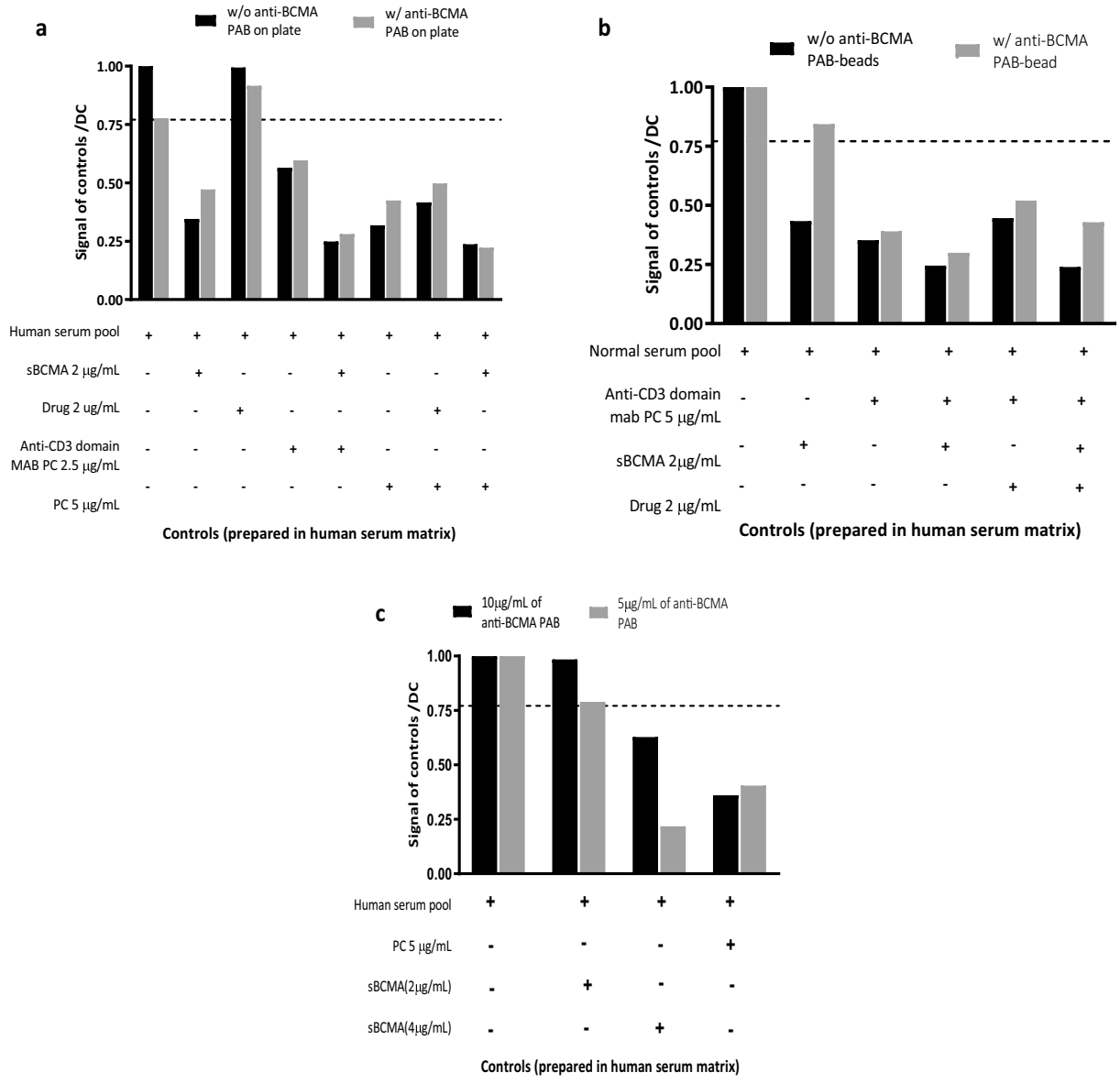


Fig. 8. Depletion of sBCMA using plate-based and bead-based procedures.
a: sBCMA depletion using plate-based sample pretreatment procedure: The plate was coated with 200 μL of 2 μg/mL (capacity of plate binding: 1.5 μg/mL of biotinylated IgG) biotinylated anti-BCMA PAB using standard capture procedure and then washed three times using PBST washing buffer. 200 μL of diluted samples and controls (MRD 1/5) were added to the plate and incubated for overnight. Subsequently the supernatant from wells were tested in the NAB assay. b: sBCMA depletion using bead-based sample pre-treatment procedures (25 μL streptavidin beads at 10 mg/mL were added to each sample). The biotinylated anti-BCMA PAB at 5 μg/mL was spiked into assay diluent and then incubated with controls and samples (sample final dilution at MRD 1/5) using the procedures described in section-2.5.3. In both cases, the system drug was at 3 nM (450 ng/mL). c: sBCMA depletion using bead-based procedures with anti-BCMA PAB at 5 and 10 μg/mL. The dotted line represents the CPF (0.777). The ratio of signals of PC / signal of DC would be < 0.777 as NAB positive.

To support clinical development of a BCMA-CD3 bispecific antibody, a functional cell-based assay was developed for the detection of NAB against the drug. During method development, it became apparent that sBCMA interference would present one of the most difficult challenges, in part due to the relatively high concentration of circulating drug target in the relevant patient population (MM). Indeed, using a two-cell system to measure drug-induced T cell activation, we found the assay response to be more variable among MM patient samples, as compared to healthy donor samples. Additionally, healthy donor serum spiked with recombinant sBCMA at concentrations that could be encountered in a subset of patient samples (up to 2 μg/mL) produced false positive results in the NAB assay. These findings prompted us to develop and implement a sample pretreatment protocol for the depletion of sBCMA. Others in the field have shown that circulating target interference can be mitigated through the use of scavengers (Zhong et al., 2017). Our initial efforts were focused on an approach in which sBCMA/anti-BCMA biotin complexes would be adsorbed on streptavidin-coated plates. However, the plate-based approach proved unsuccessful, perhaps due to the limited surface area of each well of the microplate. In contrast, using an alternative approach in which sBCMA/

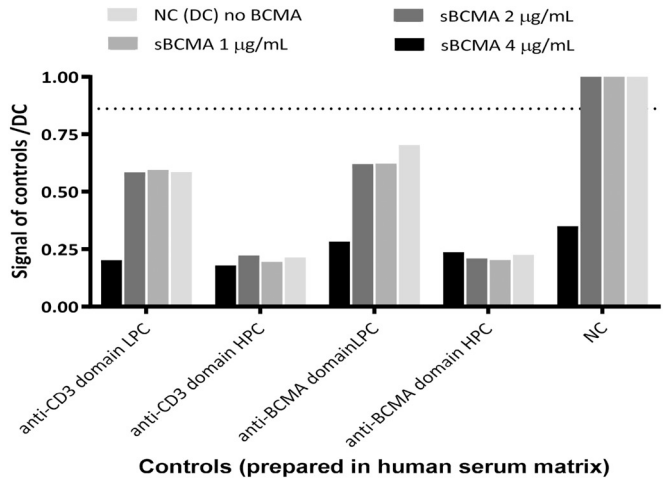


Fig. 9. Effectiveness of sBCMA depletion demonstrated in pre-study method validation.
BCMA-ECD was spiked into normal serum pool (NC) and domain specific positive controls (PCs) at the concentrations indicated. These samples were prepared, incubated at RT for 45 min and stored frozen. On the day of assay, the samples were thawed and tested using the assay method. High and low positive controls concentrations: LPC for anti-CD3 domain of drug was 870 ng/mL and LPC for anti-BCMA domain of drug was 850 ng/mL. The high PC concentration for both domain specific positive controls was 5 μg/mL. The dotted line indicated CPF established in method validation (CPF was 0.861).

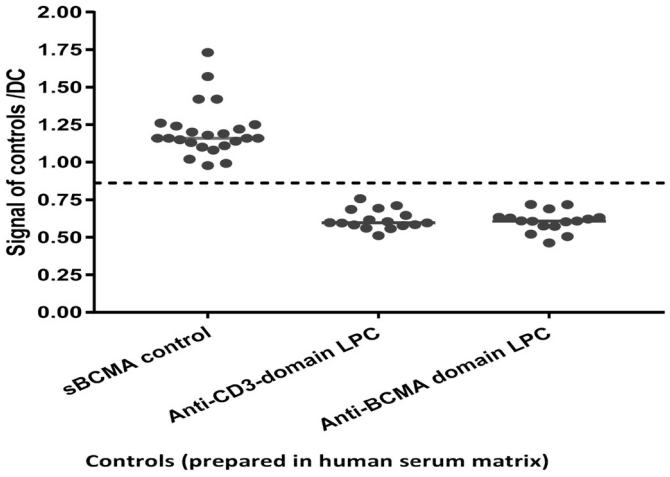


Fig. 10. Performance of sBCMA tolerability control and LPCs.
sBCMA tolerability control was prepared by spiking BCMA-ECD at 2 μg/mL into NC and stored as aliquots. For each assay run, this control was used as part of a system suitability control along with other controls. The acceptance criterion for this control was established as the signal of this control / signal of mean DC and must be above the established CPF. The range of this control was established at the end of method validation. The dotted line indicates the validated CPF with multiple myeloma patient population (0.861). The solid line in each control represents the median.

anti-BCMA biotin complexes are adsorbed on streptavidin-coated beads, which have a combined higher surface area, we were able to deplete sBCMA at concentrations that would enable accurate NAB detection in MM patient samples. Importantly, the implementation of our sample pretreatment protocol did not have a negative effect on the ability of the assay to detect NAB in the presence or absence of residual drug.

Acknowledgment

We thank current and past Pfizer colleagues who supported this work: Teresa Caiazzo and Renee Ramsey (BMD Reagent Group) supported characterization of the NAB positive controls; Edward Pascua and Sindy Liao Chan provided purified soluble BCMA (ECD of BCMA) with characterization data; Dr. Heike Iris Krupka and Dr. Alison Joyce provided critical review of the manuscript. Lastly, we thank Jeffrey S Thomas at Pfizer's CAG group who coordinated method implement and pre-study method validation at CRO and CRO colleagues who participated pre-study method validation and study support.

Funding

This work was exclusively supported by Pfizer Inc.

Declaration of Competing Interest

This study was funded by Pfizer. All the authors listed are employees of the BioMedicine Design group within Pfizer. Preclinical and clinical studies of PF-06863135 have been disclosed (58th and 60th ASH Annual Meeting). Part of the work described in this manuscript was presented in 13th WRIB 2019 as a poster presentation.

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