Critical Reagent Characterization of Biotinylated Antibody Conjugates in a Flow Cytometry-Based Receptor Occupancy Assay

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INTRODUCTION

Development and validation of robust and reproducible bioanalytical assays relies heavily on well-defined critical reagents. Careful characterization and monitoring of critical reagents throughout development, validation, and clinical sample analysis of flow cytometry-based assays is especially crucial, as readouts can be susceptible to fluctuations in data quality and long-term performance due to the inherent nature of high-dimensional datasets.

Receptor occupancy (RO) assays can generate pharmacodynamic (PD) biomarker data and, when combined with pharmacokinetic (PK) profiles, provide insight into PKPD relationships in early-phase clinical studies. Unique challenges can arise when establishing an optimal receptor occupancy (RO) assay that reflects the drug's mechanism of action while maintaining sensitivity and continuity over time in fresh whole blood samples. Here, we describe the characterization of an in-house generated biotinylated antibody conjugate which provides the central readout for a validated flow cytometry-based 6-parameter RO assay.

METHODS

Occupied Receptor Format RO Assay

In this RO assay approach, the bound receptor is directly assessed through a biotinylated anti-idiotype (anti-Id) antibody. This binds the receptor-bound drug antibody, thereby causing increased signal with accumulating levels of bound receptor when detected with a streptavidin-tagged PE fluorophore (Figure 1).

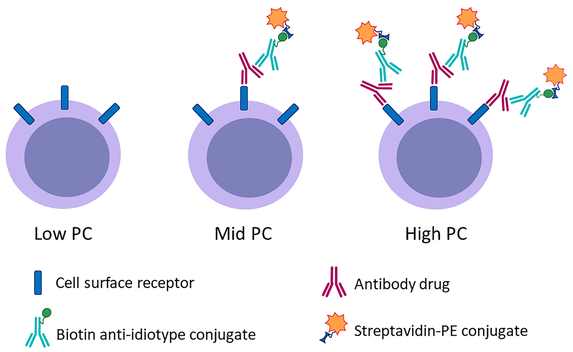
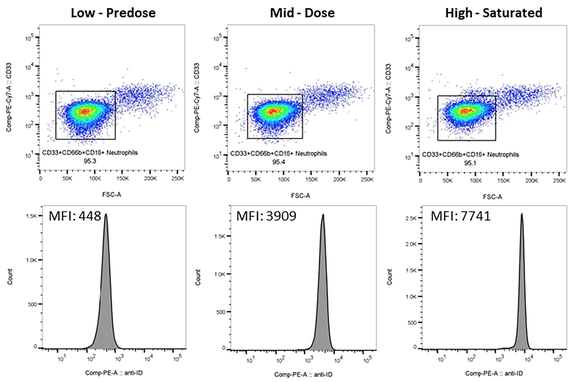


Figure 1: RO Format scheme depicting assessment of drug-bound receptors at 3 positive control levels.

For validation purposes, human whole blood is spiked with two concentrations of antibody drug, creating a non-saturating and saturating condition (Mid PC and High PC, respectively). These are assayed in conjunction with an unspiked replicate sample (Low PC). The samples are stained with an immunophenotyping panel of antibodies against CD45, CD66b, CD14, CD16, and CD33 to detect myeloid cells, including neutrophils and monocytes. The samples are lysed, fixed, and washed. The samples are then incubated with the anti-Id reagent, washed, and incubated with streptavidin-PE detection reagent. Samples are resuspended in staining buffer and assessed on a BD LSRFortessa™ Cell Analyzer (Figure 2). Raw data was analyzed in FlowJo™ v10.7.2 Software.



$$\frac{MESF_{Dose} - MESF_{Predose}}{MESF_{Saturated} - MESF_{Predose}} \times 100 = \%RO$$

Figure 2: Example flow cytometry data from CD66b+CD16+CD33+ neutrophil population with resulting PE MFI histograms. MESF is calculated from PE MFI. The calculation for % Receptor Occupancy (RO) is shown.

RESULTS

Biotin Conjugation

The biotinylated anti-idiotype conjugate was prepared according to the following method. Briefly, Zeba™ desalting columns were equilibrated and unconjugated anti-idiotype antibody solution was added to the resin beds. The columns were centrifuged and the resulting solution was incubated for 30 minutes with EZ-Link Sulfo NHS-LC-Biotin solution at a challenge ratio of 10. The reaction solution was added to new desalting columns equilibrated with MSD® Conjugate Storage Buffer and centrifuged. The resulting anti-Id biotin conjugate was assessed for total protein concentration and biotin incorporation ratio, and subsequently used for functional RO validation assessments.

Biotin-Conjugate Protein Characterization

To characterize the new reagent preparation, biotin incorporation and total protein quantitation assays were performed. The new lot of biotinylated anti-Id was determined to be less concentrated than the original conjugated lot; however, the biotin incorporation was more efficient, resulting in a 4-fold difference in biotin conjugates per antibody.

While this information was helpful to assess differences in conjugation efficiency, functional assessments of lot performance were completed to ensure that raw response values (e.g. median fluorescence intensity (MFI), molecules of equivalent soluble fluorochrome (MESF)) were comparable to the validated RO method using the original lot.

Biotin Anti-Id Preparation	Protein Concentration	Biotin Concentration	Incorporation Ratio
	mg/mL	µM	Biotin/Protein
New Biotin-Anti-Id Lot	0.438	24.8	6.8
Original Biotin-Anti-Id Lot	0.565	8.2	1.7

Table 1: Total protein concentration and biotin concentration/incorporation results.

Conjugate Performance in RO Assay

To test the newly prepared biotinylated anti-Id reagent, three supporting validation runs of the RO assay were performed on human whole blood in parallel with the original reagent lot. Briefly, whole blood was spiked at two concentrations of the drug antibody (High and Mid processing controls (PCs)) and compared to a naïve sample (Low PC) to mimic a non-saturated, fully saturated, and placebo condition, respectively. The samples were then processed according to the validated method procedure and analyzed by flow cytometry. Results from the initial functional test indicated that the new reagent lot produced significantly higher raw MFI values at each PC level in the three target cell populations compared to the original lot (Figure 3. A).

For the second functional assessment, a dilution series of the new reagent lot was prepared at 1X, 0.75X, 0.5X, and 0.25X, using the unconjugated anti-idiotype antibody as the diluent to maintain total protein concentration between lots. The RO processing procedure and analysis was performed in human whole blood from a single donor according to the validated method.

Following gating analysis and data export, a curve fit assessment of the MESF values was performed for each PC level (Figure 3. B–C.). This data supported the identification of a theoretically optimal dilution of new biotin anti-Id to match the raw MFI response of the original lot, where a dilution of roughly 0.61X of the new reagent lot was expected to perform similarly to the original undiluted biotin anti-Id lot.

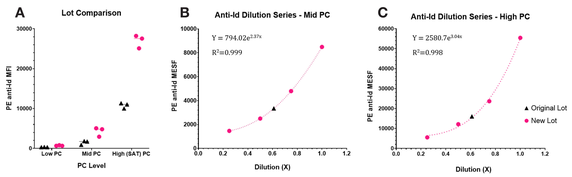


Figure 3: (A) Assessment of biotin anti-Id lots in a one-to-one comparison at 3 positive control (PC) levels. (B–C) Calculated MESF values of new biotin anti-Id lot at 4 dilutions; Mid PC (B) and High PC (C) are shown. The plotted theoretical dilution was calculated for each PC level using the MESF values of the sample prepared with the original biotin anti-Id lot. The MESF values for the dilution series of the new biotin anti-Id reagent is shown in magenta. The MESF value for the original undiluted biotin anti-Id reagent lot is shown in black.

Confirmation RO Assay

A third confirmation assessment tested a smaller range of dilutions at 0.61X, 0.66X, and 0.74X compared to the original undiluted lot. Data from this run showed that the 0.66X dilution of the new reagent lot performed most similarly to the original lot when comparing MESF values at each PC level (Figure 4. A–B) and their resulting %RO calculations for both low PC and Mid PC levels (Figure 4. C–E); High PC MESF values are always set at 100% RO as this is a fully-saturating condition. When taking all data comparisons into account, the 0.66X dilution of the new reagent lot performed most similarly to the original lot, which was then cleared for use in clinical sample analysis.

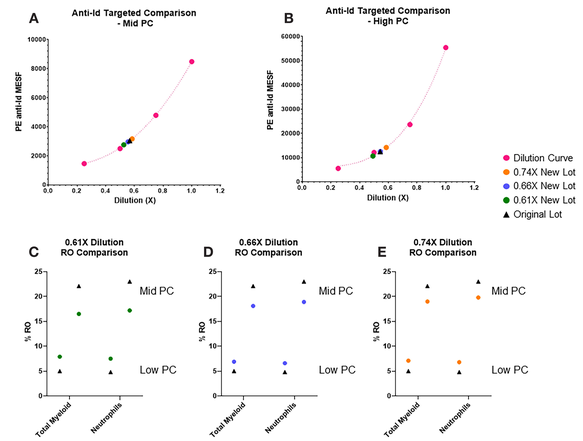


Figure 4: (A–B) Assessment of new reagent lot biotin anti-Id dilution MESF values compared to the original undiluted lot. Mid PC (A) and High PC (B) are shown. Each sample was fitted to the dilution curve shown in Figure 3, shown in magenta. The MESF value for the original undiluted biotin anti-Id reagent lot is shown in black. (C–E) Calculated %RO values for both Low and Mid PC Levels of new biotin anti-Id lot dilutions are compared to the original undiluted lot, shown in black.

DISCUSSION AND CONCLUSIONS

Multiparametric flow cytometry assays can be particularly sensitive to changes in critical reagent lots and therefore must be monitored and evaluated accordingly. New reagent lots, especially those not commercially available , should be thoroughly characterized and validated before they are released for use in clinical sample analysis.

In this study, although our initial study plan included preparation of ample biotinylated material, additional validation studies consumed more than expected, and it was pertinent that we monitor and identify a potential shortage in the amount of material needed to complete the study. Once this shortage was identified, we exacted a plan to bridge the two reagents to minimize fluctuations in assay performance. The new reagent preparation was found to be comparable to the original lot at a dilution of 66% while maintaining the same total protein concentration.

Ultimately, we used a systematic approach and overcame several hurdles to prepare and characterize a new lot of biotinylated anti-idiotype reagent, which ensured the reproducibility and robustness of this technically challenging flow cytometry-based RO assay.

REFERENCES

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