

Assessment of Receptor Occupancy via Flow Cytometry: Benefits and Pitfalls of Two Common Approaches

ABSTRACT

Receptor occupancy assays can be a powerful tool in the determination of drug dosage. Recently, flow cytometry has been the method of choice to assess receptor occupancy due to its ability to measure drug-target binding interactions on multiple cell populations simultaneously. Receptor occupancy assays using flow cytometry involve measurement of target receptors bound by the drug, number of receptors not bound to the drug, and the total number of receptors present. The methods for measuring these parameters, particularly for antibody-based therapeutics, can be categorized into two approaches based on the detection antibodies used. Here, we discuss the benefits and pitfalls of these methods.

In one approach, fluorescently labelled competing and non-competing antibodies to the drug are used to detect Free and Total Receptors, respectively. In this approach, the competing antibody is often the labelled drug itself. One of the main obstacles in method development is to identify a non-competing antibody with comparable binding affinity to the target as the drug tested. We have observed that when competing and non-competing antibodies display different affinities to the target, the results can be difficult to interpret. In addition, it is imperative that in a multiplex assay, competing and non-competing antibodies do not interfere with each other's binding to the target.

The other approach utilizes a single, fluorescently labelled secondary antibody to detect both Bound and 2c. Total Receptors. Samples from animals treated in vivo are first incubated with excess drug ex vivo, to fully saturate all targets, and stained with secondary antibody to detect total receptors. Staining with a single detection antibody results in a direct comparison between bound and total receptors, removing any difficulties from different binding affinities between reagents. We have observed that identifying a suitable secondary antibody is crucial, especially when the targeted populations consist of sticky cells, such as granulocytes and monocytes. In addition, we have found that incubation of samples ex vivo with excess drug can lead to down regulation of the target, leading to underestimation of the Total Receptors and overestimation of receptor occupancy of the drug.

RO assay with secondary antibody to measure Bound and Total Receptor. Study samples from animals that have received TA were The technical considerations presented here, when incorporated early in the receptor occupancy design phase, split into two tubes. In Tube 1, samples were incubated with PBS in vitro followed by staining with secondary antibody to detect Bound will aid in determining the most suitable approach, and avoid common pitfalls in assay design. Receptor. In Tube 2, samples were incubated with saturating amounts of TA *in vitro* followed by staining with the same secondary antibody to detect Total Receptor.

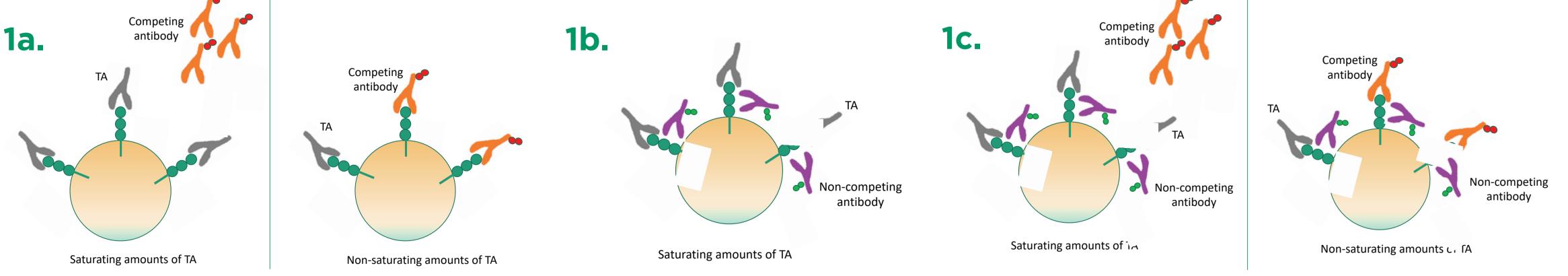
INTRODUCTION

RESULTS Flow cytometry has long been a preferred technique used by immunologists to immunophenotype various immune cell populations and understand a plethora of cell functions, including proliferation, activation, and Figures 3. Assessment of RO by TA to HLA-DR+CD20- antigen presenting cells from whole blood using competing and nonintracellular cytokine productions. With the breakthrough of immunotherapies as a new class of drug to treat cancers and other diseases, flow cytometry has emerged as the preferred method of evaluating receptor competing antibodies occupancy (RO) by monoclonal antibodies that target immune checkpoints or modulators. The failure of **3a. STEP 1 STEP 2 -** Multiplexing of competing and non-competing antibodies TGN1412 in its first-in-human clinical trials has further emphasized the need to assess RO, as a significantly Titration of non-competing antibody lower dose of TGN1412 would have been recommended in human trials had RO assessment been performed during preclinical safety assesment. Assessment of RO by these monoclonal antibodies at the preclinical phase not only provides invaluable information with regards to dosage in clinical trials, it also demonstrates that the relevant species has been selected for safety assessment through adequate target binding.

Assessment of RO by test article (TA) using flow cytometry involves the measurement of target receptors bound by the TA (Bound Receptor), number of receptors not bound to the TA (Free Receptor), and the total number of receptors present (Total Receptor). The methods to assess RO can also be categorized based on the detection reagents used such as 1) antibodies that compete and do not compete with the TA and 2) anti-TA secondary human IgG antibodies.

METHOD

Figures 1. RO assay with competing and non-competing antibodies

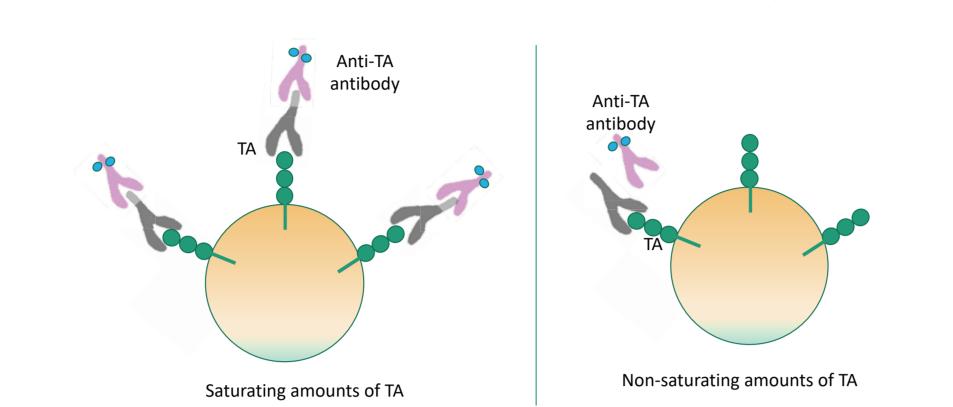


Competing saturating amounts of TA, the target. Non-competing antibody is used amounts of TA, only the non-competing no binding to the therapeutic target. In throughout the study across multiple target while the competing antibody contrast, under non-saturating amounts time points and enables the assessment will not. As TA concentration decreases, of TA, the competing antibody will bind of receptor modulation in response t to the therapeutic target and, therefore, TA administration. report on the level of Free Receptor.

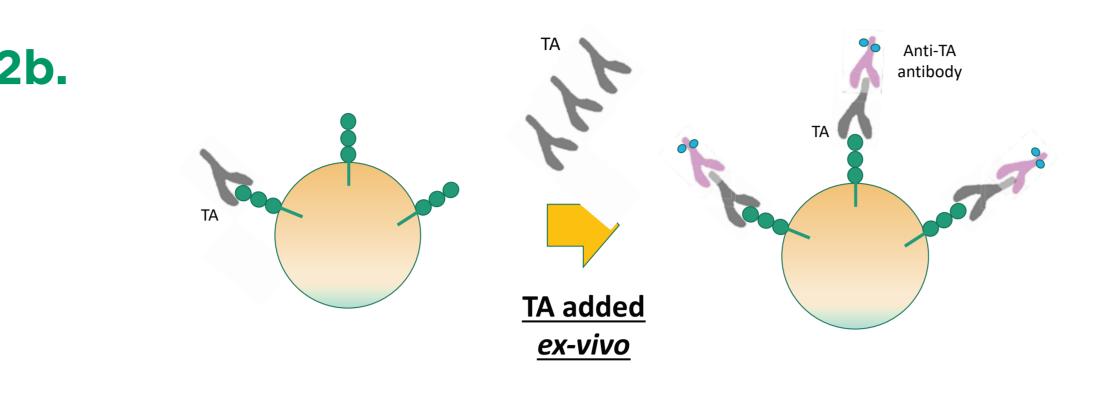
antibody measures Non-competing antibody measures RO assay with competing and non-Free Receptor and can either be a Total Receptor and is a fluorescently competing antibodies. Samples for fluorescently labelled test article or labelled antibody that binds to the animals dosed with TA were co-stained another fluorescently labeled antibody therapeutic target, and does not with competing and non-competing known to compete with the TA for interfere with the bind of TA or the antibodies to determine levels of Free the therapeutic target. Under competing antibody to the therapeutic and Total Receptor. Under saturating competing antibody will have little to to monitor Total Receptor expression antibody will bind to the therapeutic the Free Receptor or therapeutic target becomes available and binding of the competing antibody is observed.

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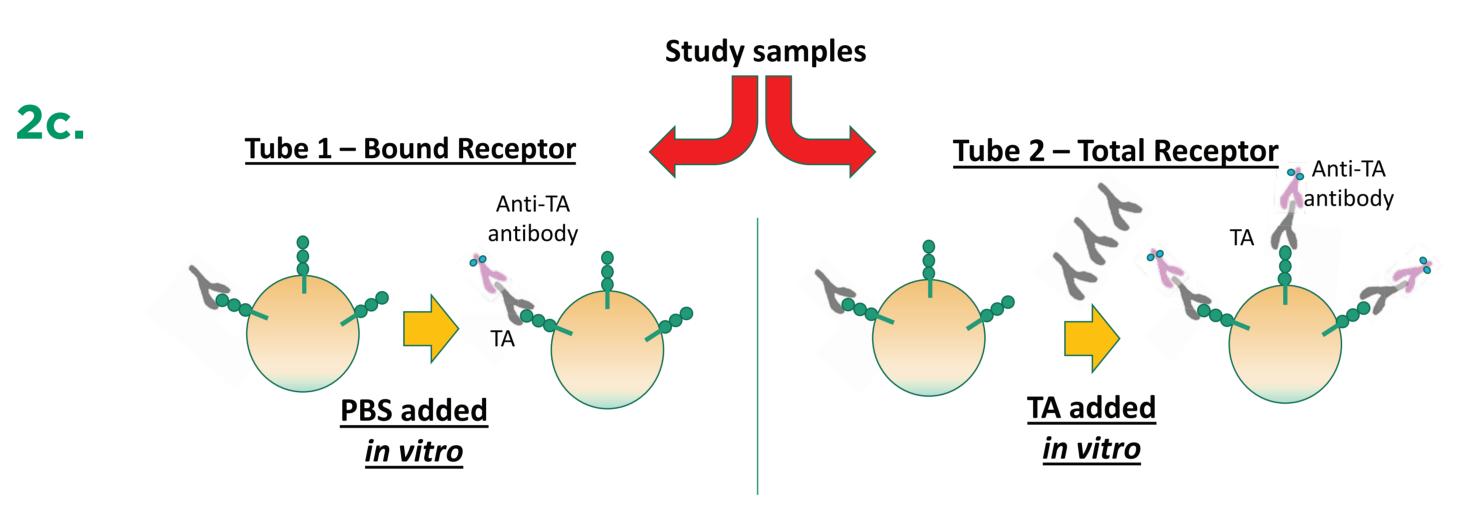
Figures 2. RO assay with single fluorescently labelled secondary antibody to detect both Bound and Total Receptors.



Use of secondary antibody to measure Bound Receptor. Commercially available anti-human IgG antibodies are the commonly used secondary antibody to detect TA bound on the therapeutic target.

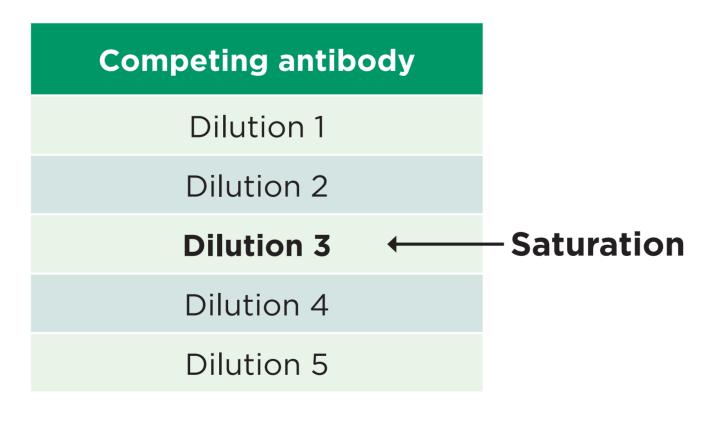


Use of secondary antibody to measure Total Receptor. The same anti-human IgG antibody used to detect Bound Receptor can be used to measure Total Receptor. To achieve this, samples will be incubated with TA ex vivo to saturate all available therapeutic targets, followed by staining with the secondary antibody.



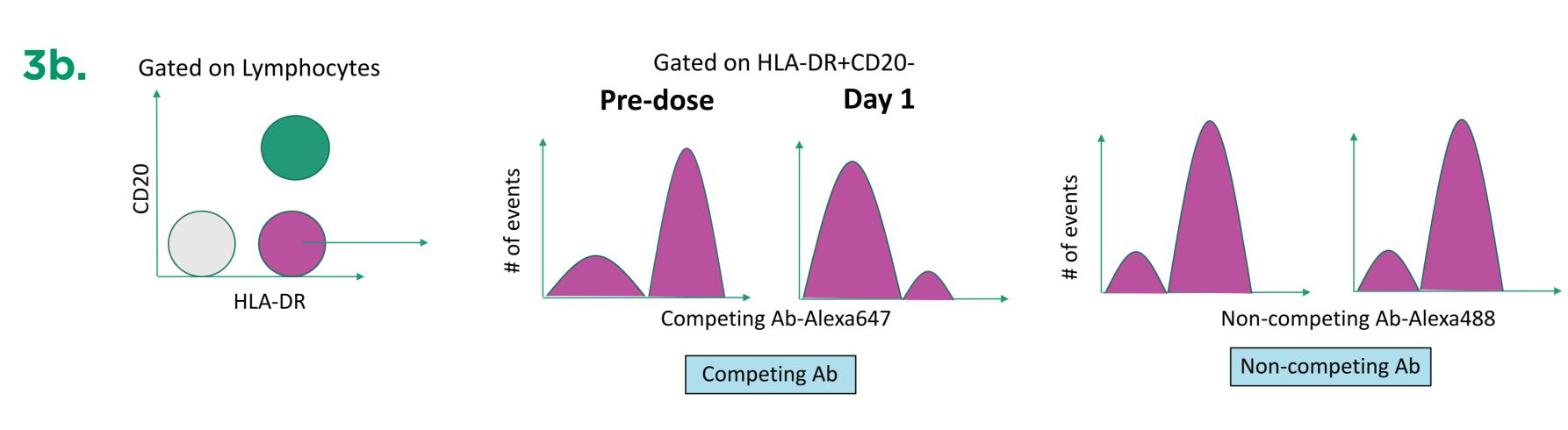
Non-competing and	tibody
Dilution 1	
Dilution 2	•
Dilution 3	
Dilution 4	
Dilution 5	

Titration of competing antibody



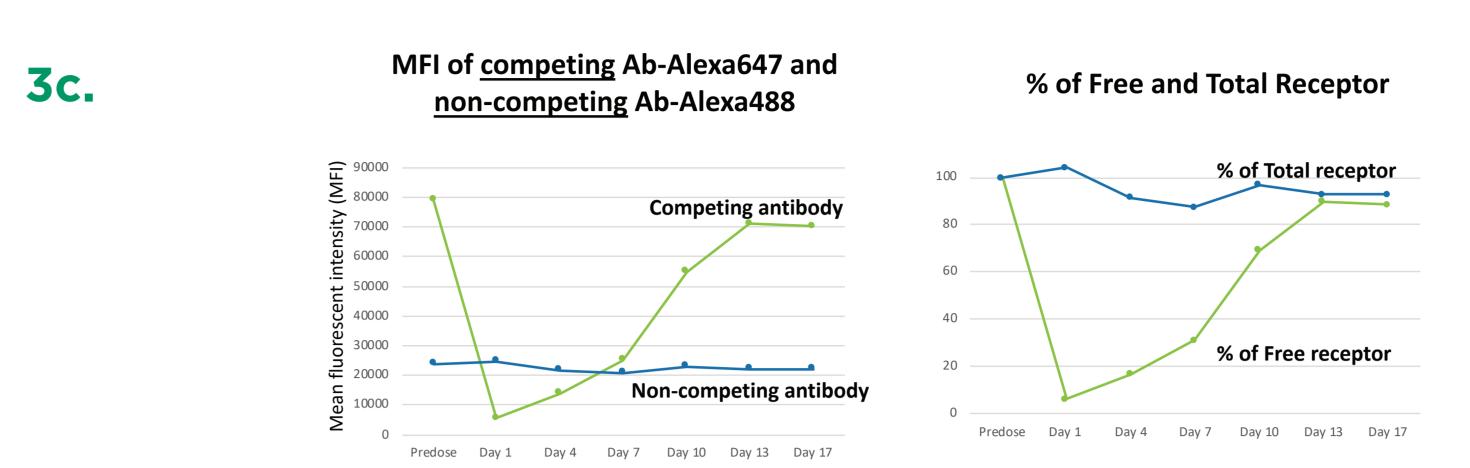
Concentration of TA	Non-competing antibody @ Dilution 2	Competing antibody @ Dilution 3	Non-competing + competing antibody
Dilution 1	Tube 1	Tube 2	Tube 3
Dilution 2	Tube 4	Tube 5	Tube 6
Dilution 3	Tube 7	Tube 8	Tube 9
Dilution 4	Tube 10	Tube 11	Tube 12
Dilution 5	Tube 13	Tube 14	Tube 15

Characterization of competing and non-competing antibodies. **Top left:** Titration of competing and non-competing antibodies to determine the minimal amount of antibodies needed to saturate entire therapeutic target. Bottom left: Next, samples were pre-incubated with varying amount of TA, and stained with saturating amount of 1) non-competing antibody only 2) competing antibody only 3) competing and non-competing antibodies. To multiplex competing and non-competing antibodies in one reaction, the staining pattern obtained in the multiplex should be comparable to staining pattern obtained when only one of the two antibodies is used.



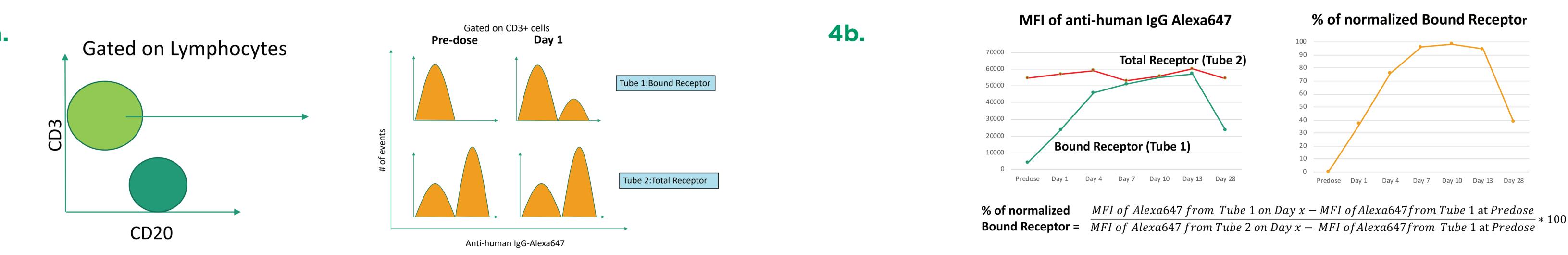
Detection of Free and Total receptor with competing and non-competing antibodies, respectively. Whole blood was collected from animals pre-dose and post-dose on Day 1 following TA treatment. Samples were stained with fluorescently labeled antibodies against HLA-DR, CD20, and known TA-competing antibody conjugated to Alexa647 and non-competing antibody conjugated to Alexa488. The therapeutic target was previously identified to be highly expressed on HLA-DR+CD20- antigen- presenting cells and was therefore chosen for RO analysis. Histograms showed lower binding of competing antibody on Day 1 when compared to pre-dose, indicating decreased Free Receptor or available therapeutic target. In contrast, binding of non-competing antibody remained comparable between pre-dose and Day 1.





Normalization of Free Receptor to Total Receptor mitigates effects Assessment of Free and Total receptor. Left: Whole blood from animal treated with TA was collected at multiple time points and analyzed using the method receptor modulation. It has been reported that treatment of TA can sometimes result in described in (A). Mean fluorescent intensity (MFI) of HLA-DR+CD20- cells in downregulation of the therapeutic target, leading to lower levels of available target for the Alexa647 and Alexa488 channels indicated binding of competing and nonoccupancy by TA. Normalizing the percentage of Free Receptor to the percentage of competing antibodies, respectively. Binding by non-competing antibody can Bound Receptor will likely produce a more accurate calculation of receptor occupancy of sometime produce a lower MFI when compared to competing antibody as shown here, therapeutic target by TA. likely due to differences in antibody-binding affinity and inherent brightness of the fluorochromes. Right: To adjust for these differences, calculations of percentage of Free and Total Receptor were normalized to pre-dose using the formula listed in Figure 3D.

Figures 4. Assessment of RO by TA to CD3+ T cells using secondary anti-human IgG antibody

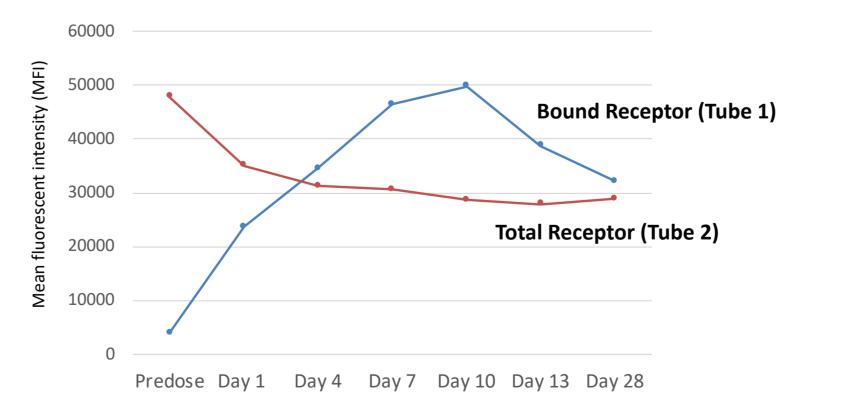


Detection of Bound and Total receptor with secondary anti-human IgG. Whole Assessment of Bound and Total receptor and calculating percentage of blood was collected from animals pre-dose and post-dose on Day 1 following TA normalized Bound Receptor. Left: Whole blood from animals treated with TA was treatment and divided into two tubes for incubation with phosphate buffered collected at multiple time points and analyzed using the method described in (A). Mean fluorescent intensity (MFI) of CD3+ T cells in the Alexa647 channel indicated binding of saline (PBS, Tube 1) or TA (Tube 2) in vitro. Following red blood cell lysis, cells were stained with fluorescently labeled antibodies against CD3, CD20, and anti-human the secondary anti-human IgG antibody to TA on therapeutic target (Bound Receptor, Tube 1) and the total available therapeutic target expressed on target cells (Total IgG conjugated to Alexa647. Receptor, Tube 2). Right: Normalization of Bound Receptor to Total Receptor will likely produce a more accurate calculation of receptor occupancy of therapeutic target by TA. The formula used to calculate percentage of normalized Bound Receptor also subtracts non-specific binding observed in the absence of TA (Tube 1 pre-dose).

Figures 5. Comparison of two secondary anti-human IgG (anti-TA) antibodies

Features	Antibody #1	Antibody
Product name	AffiniPure F(ab') ₂ Fragment Goat Anti-Human IgG, Fcγ fragment specific	Goat Anti-Human IgG, M
Manufacturer	Jackson Immunoresearch	Southern Bio
Catalogue #	109-136-098	2049-3
Pre-absorption	Bovine, Horse, Mouse Serum Proteins	Human IgM and IgA; rhesus (Macaca mulatta) a
Clonality	Polyclonal	Polyclon
Isotype	Goat IgG	Goat Ig0

Selection of an appropriate anti-human Ig antibody for detection of TA can have a significant impact on the results. When choosing between comparable secondary antibodies, one feature that should be considered is the pre-absorption step performed by the manufacturer to minimize cross reactivity to other species.

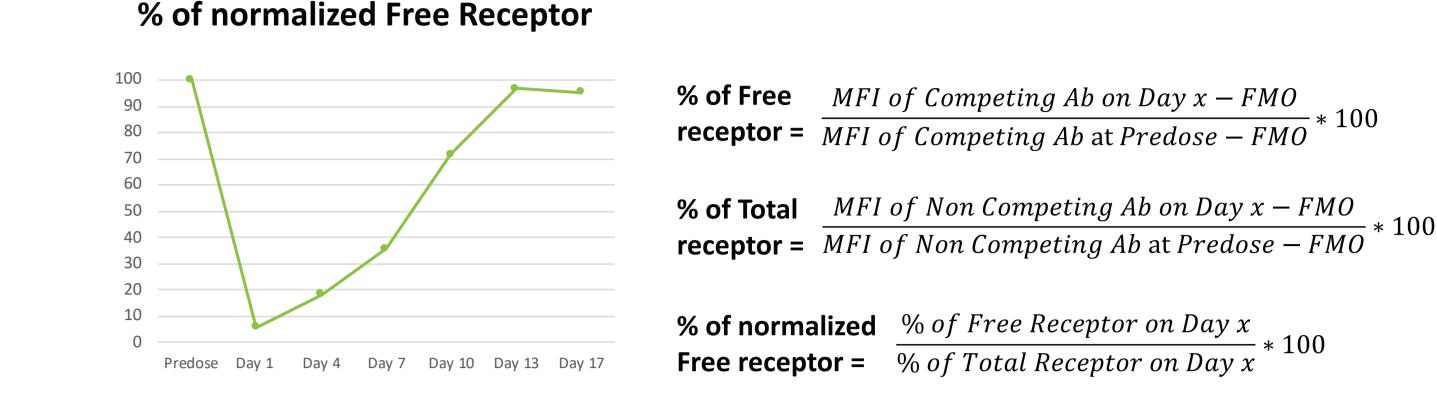


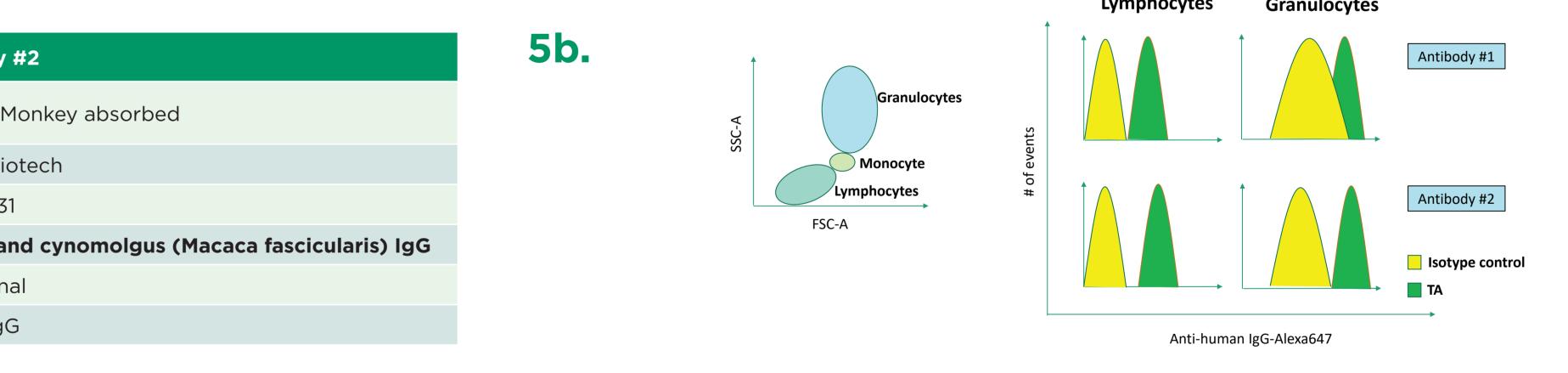
Figures 6. Receptor modulation following re-exposure to TA *in vitro* RO assessment using secondary antibody to assess Total Receptor expression by saturating all therapeutic targets with TA in vitro can sometimes lead to receptor modulation. This was evident when samples collected after dosing in vivo exhibited lower levels of Total Receptor when compared to samples collected from the same animal prior to dosing. This observation suggested that some therapeutic targets will undergo receptor modulation when samples are re-exposed to TA in vitro.

CONCLUSION

Here, we discussed two methods to assess RO via flow cytometry and pitfalls of both methods. Assessment of RO using competing and noncompeting antibodies to measure Free and Total Receptor enables multiplexing in one tube when sample volume is limited, but identification and characterization of both antibodies can be laborious and time consuming. In contrast, a RO assay using secondary anti-human IgG antibody can be set up quickly, but will require more samples for separate assessment of Bound and Total Receptor. In addition, identification of the appropriate secondary and blocking reagents are crucial and receptor downregulation may occur following re-exposure of TA *in vitro*. The technical considerations presented here, when incorporated early in the RO design phase, will aid in determining the most suitable approach.

Reference: https://www.ncbi.nlm.nih.gov/pubmed/26054054





Secondary antibody affects resolution of cell population. For cell types with low expression of Fc receptors such as T cells, the choice of secondary antibody can still have a minor impact on the resolution between positive and negative populations. For cell types such as monocytes and granulocytes, the secondary antibody can have a major impact on the resolution signal and noise.