The Importance of Using an Isotype Control

White paper

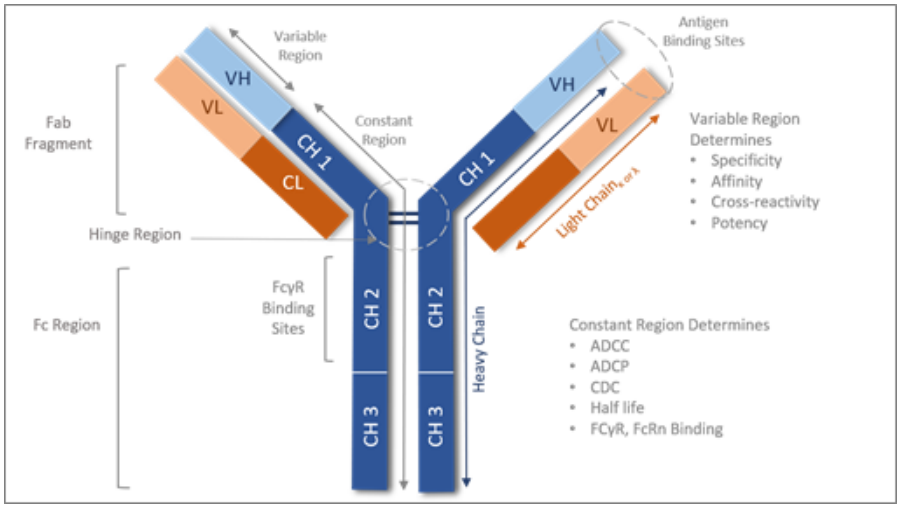
What are Isotype Controls?

Isotype control antibodies are essential negative controls for in vitro and *in vivo* studies and can also play an important role in standard immunoassays, such as flow cytometry and immunohistochemistry (IHC). They match the characteristics of the assay's primary antibody, but are raised against antigens known not to be present in common preclinical species such as Hen Egg Lysozyme (HEL), Keyhole Limpet Hemocyanin (KLH), etc. Therefore, they lack any relevant target antigen specificity.

When choosing an appropriate isotype control, matching the characteristics of the primary antibody is key for accurate interpretation of study results. The main factors to consider when choosing the appropriate isotype control antibody for a study are host, isotype, conjugation, light chain, and concentration.

Structurally antibodies are very similar with only a small variable region determining antigen specific binding. The rest of the antibody molecule consists of the constant region, where sequences are often shared by antibodies of the same isotype (Figure 1).

Figure 1: Antibody Structure



The constant regions help mediate antibody mechanism of action by coordinating binding to the Fc receptors (FcRs). FcRs are expressed on a large number of different cell types in the immune system and interact with an antibody differently based on antibody isotype (Table 1). This makes certain isotypes are better as drug candidates than others.

Table 1: Binding Preference of Mouses, Rat, and Human IgG Isotypes(1)

Mouse	mFcγRI	mFcγRII	mFcγRIII	mFcγRIV	
IgG1	No Bind	++	++	No Bind	
IgG2a	+++	+	++	+++	
IgG2b	ND	++	+	+++	
IgG3	ND	No Bind	No Bind	No Bind	
Rat	**mFcγRI**	**mFcγRII**	**mFcγRIII**	**mFcγRIV**	
IgG2a	No Bind	++	+	+	
Human	**FcγRI**	**FcγRIIa**	**FcγRIIb/c**	**FcγRIIIa**	**FcγRIIIa**
IgG1	+++	++	+	++	+
IgG2	No Bind	+	+	+	No Bind
IgG3	+++	+	+	++	++
IgG4	+++	+	+	+	No Bind
Affinity (K_A)					

Choosing an Appropriate Negative Control

The goal of good experimental design is to control for all variables that may have an unintended impact on the evaluation of the variable of interest.

As discussed above, all antibodies are structurally very similar except for the variable region determining antigen binding. In an *in vivo* efficacy study of a therapeutic monoclonal antibody (mAb), the binding of the variable region of the mAb to its specific antigen is the first part of having a therapeutic effect.

Secondly, efficacy of some therapeutics is also dependent upon engagement of immune cells via the Fc region of the antibody[2]. The potential for the host immune response to impact study outcomes and confound the data interpretation highlights additional variables that must be accounted for in the experimental design and is why simple vehicle controls such as PBS are often not sufficient.

Why PBS is not a Suitable Subsitute for an Isotype Control

While the use of PBS instead of an isotype control antibody as a negative control is not ideal, it does occur in published research. Using this non-optimal control can result in a range of issues during efficacy studies and immunoassays.

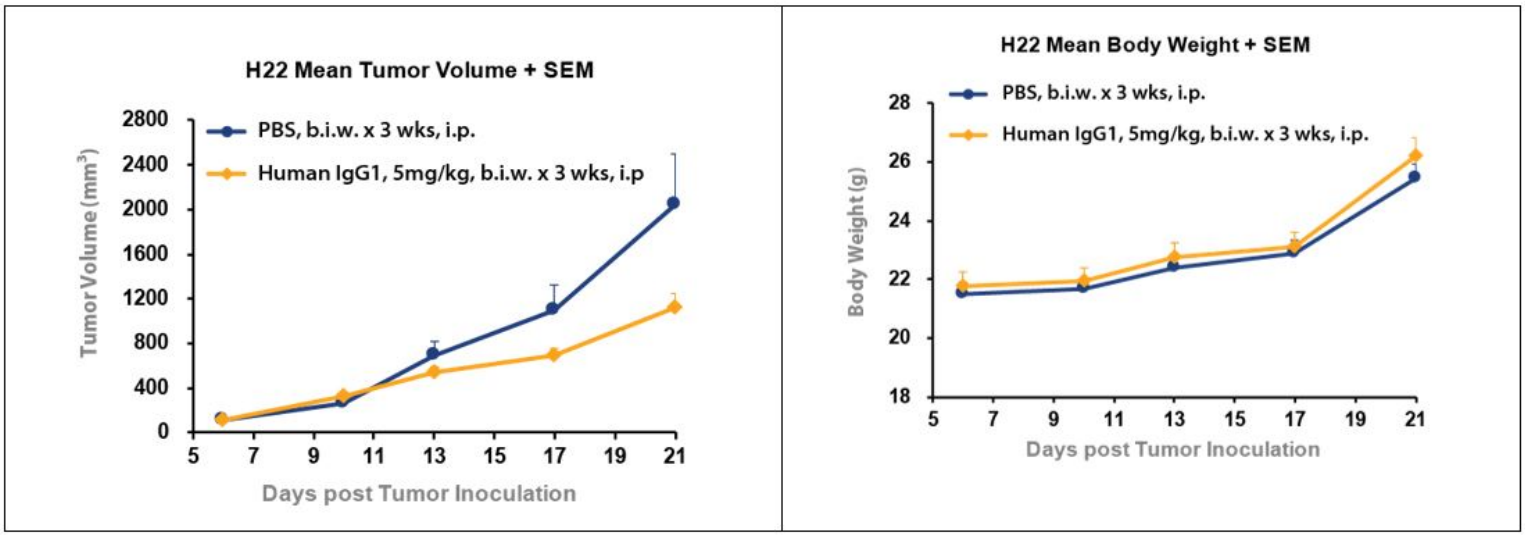
1. Use of PBS in Efficacy Experiments Fails to Mimic the Effects Caused by Widespread FcR Engagement

Using PBS as a simplified negative control in a range of *in vivo* efficacy assays can result in inaccurate interpretation of results. The degree of impact on outcomes can vary in significance based on the test article and test model used.

In Figure 2, the effect of a therapeutic mab on tumor growth was observed in the H22 syngeneic liver cancer model. Significant tumor inhibition can be observed with hIgG1 isotype control compared to PBS in this experimental run. The cause of the observed growth inhibition in this experiment is unknown, but appears to be specific to the addition of hIgG1 antibody when compared to the PBS control.

In this case, correct calculation of tumor growth inhibition and a more accurate measurement of drug efficacy would be to compare the test antibody with that of the hIgG1 isotype control, not PBS, or risk over estimation of efficacy of the therapeutic.

Figure 2: Tumor Growth Inhibition Induced by hIgG1 compared with PBS

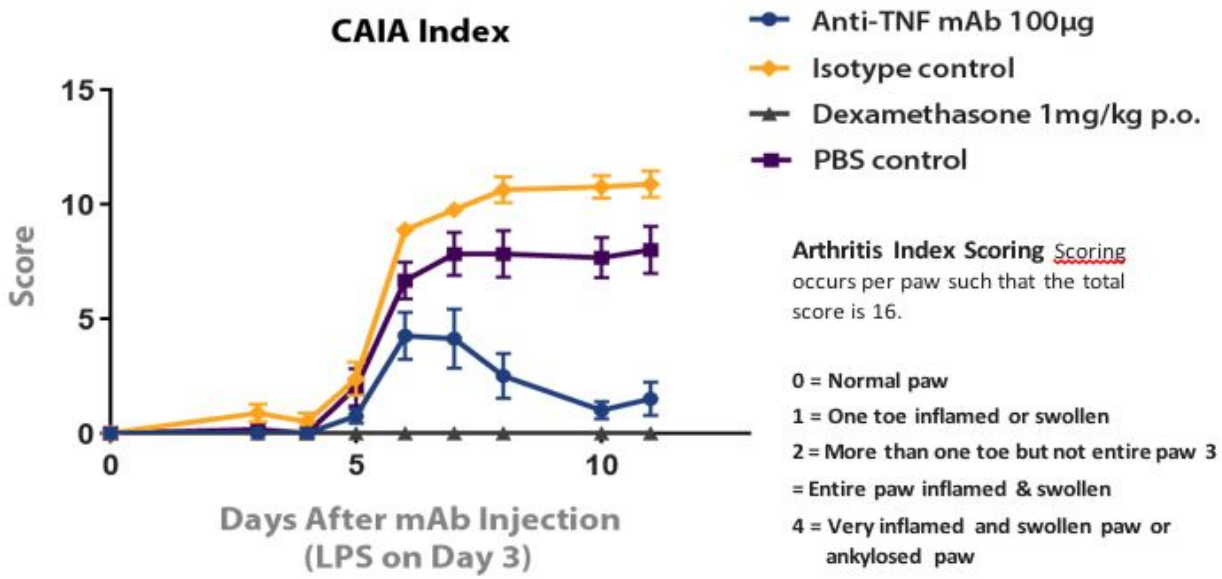


The importance of comparison with the correct control agent is also exemplified in the Collagen Antibody-Induced Arthritis (CAIA) model shown in Figure 3.

In this model, rheumatoid arthritis is induced in BALB/c mice through the administration of an athrogenic antibody followed by LPS, which can then be treated with agents designed to reduce inflammatory effects and assess therapeutic response.

When treated with dexamethasone (a corticosteroid) or an anti-TNF mAb there is a clear reduction in the CAIA index, a measure of disease severity. Interestingly, in this experiment the score of animals treated with a huIgG1 isotype control is significantly higher than that of animals treated with PBS resulting in a higher score in CAIA index. Selection of the comparator arm can alter the magnitude of effect observed with the therapeutic agents and should be considered carefully.

Figure 3: Performance Difference between PBS and Isotype Negative Controls in CAIA Model



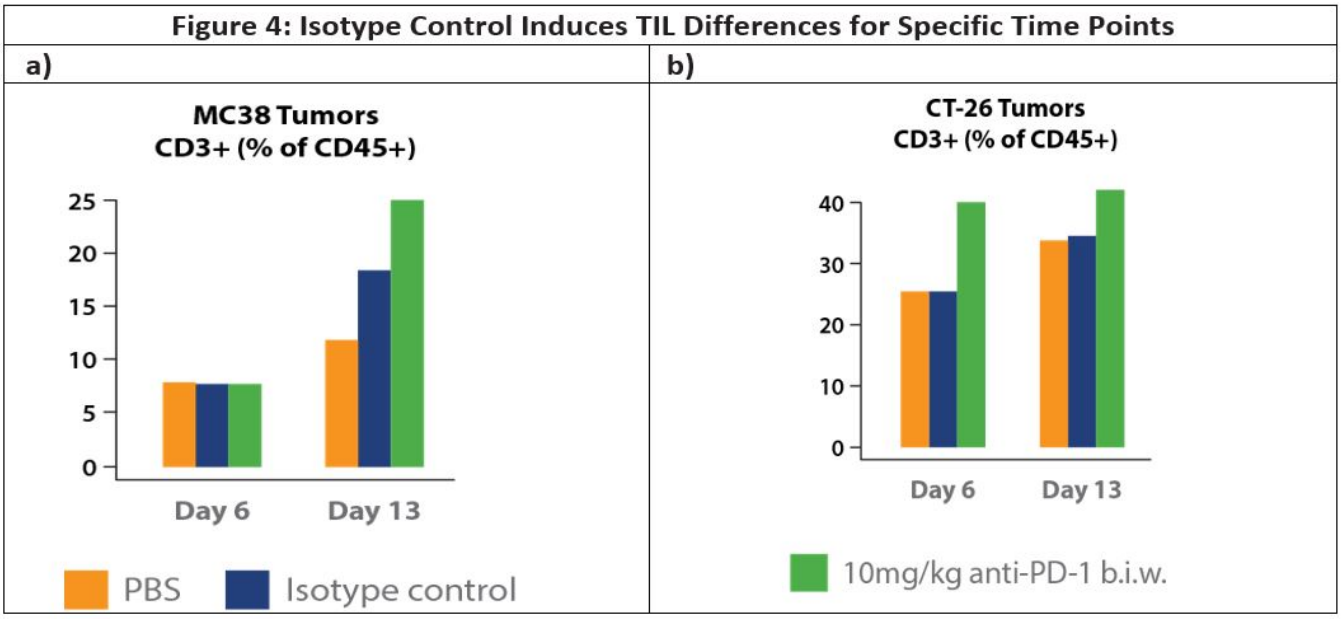
Antibody Isotype Can Induce Changes in Tumor Infiltrating Lymphocyte Populations in Some Models

Treating a negative control group with an isotype control can result in differences in tumor infiltrating lymphocyte populations compared to a group treated with PBS. However, the degree of impact is model dependent.

Figures 4a and 4b show the impact on two different anti-PD-1 treated models. Figure 4a displays this occurrence for the MC38 syngeneic colon cancer model. By Day 13, treatment with isotype control (hIgG1, 10mg/kg twice per week) causes an increase in CD3+ T cells found within the tumor infiltrate. This was not observed in the PBS treatment group.

In contrast, this effect was not observed in CT-26.WT syngeneic tumors using the same isotype control, suggesting it is highly model dependent. These data show how utilizing the correct control is critical for understanding the true response of these anti-PD-1 treated tumors.

Figure 4: Isotype Control Induces TIL Differences for Specific Time Points



2. PBS Negative Controls Do Not Mimic FcR or Protein Staining in Flow Cytometry Experiments

In flow cytometry experiments, antibody binding to FcRs or other proteins can cause non-specific staining in cell types dependent upon FcR expression. This can appear deceptively specific due to the variation in FcR expression in different tissues. Therefore, it may be useful to have matched isotype controls which can mimic this staining, rather than use a non-binding PBS control.

As a result of this, in order to be useful in flow cytometry, isotype controls should ideally be the same species, heavy chain (IgA, IgG, IgD, IgE, or IgM), and light chain (kappa or lambda) class. For directly conjugated antibodies, isotype controls should be conjugated to the same fluorophore at the same labelling ratio as the target specific staining antibody, such that an identical number of fluorescent molecules is conjugated to each antibody. This is also referred to as the fluorphore to protein (F:P) ratio.

Due to this level of complexity, researchers have different preferences regarding inclusion of isotype controls for flow cytometry. Since differentially conjugated isotype controls can lead to misleading results, it is important to fully characterize isotype control comparator molecules in flow cytometry experiments.

3. Immunohistochemistry Experiments Require Appropriate Isotype Controls

Isotype controls are also an important negative control when performing IHC assays. One of two alternate options is generally used if isotype controls are not included in an experiment:

1. Direct staining with the secondary antibody used
2. Staining with a polyclonal pool from the same species as the primary antibody

Using a secondary antibody alone as a control instead of an isotype control antibody may not be appropriate as the secondary antibody can bind directly to the slide section in the absence of preferential binding to primary antibody or isotype control. The secondary could also have altered antigen independent binding properties in isolation compared to non-specific binding when complexed with a primary antibody. Depending upon the tissue, the antibody isotype could also cause a noticeable difference due to the potentially significant interaction with resident FcRs present in different tissue sections.

Likewise, using a polyclonal mixture of immunoglobulins may lead to increased background or non-specific staining as the multiple isotypes contained in polyclonal mixtures may bind to additional targets. Therefore, the polyclonal pool and the secondary antibody are unlikely to match the isotype of the primary antibody and may not replicate primary antibody binding patterns in tissue as well as an isotype control.

Ideally, the negative control used for IHC should mimic the exact concentration and isotype of the primary antibody, which is why an isotype control is the most appropriate choice to control for primary antibody staining.

Isotype control antibodies can also serve as a blocking or coating reagent in other immunoassays including, which may help to minimize non-specific FcR mediated binding with a primary antibody. Other assays where an isotype control antibody could be used as a blocking agent include Western blots and ELISAs.

Isotype Controls are an Ideal Reagent Choice for your Studies

MBL International provides a range of isotype controls, suitable for *in vivo* studies and immunoassays. Our controls have been carefully developed, providing a range of features, including:

- Primary antibody concentration (our isotype controls are supplied at 1-5mg/ml to match antibody drug concentrations
- No carriers or additives - supplied in PBS only
- No cross reactivity with the species you are working with
- Very low endotoxin and high purity to ensure animal safety.

Summary

Isotype controls are essential tools in a range of *in vivo* efficacy and immunoassays and are appropriate negative controls for accurate measurement of antibody drug effects. The substitution of other agents such as PBS for isotype controls causes a range of issues which can hinder study performance and contribute to erroneous interpretation of results. The use of high quality isotype controls provides a reliable method to accurately differentiate specificity versus background for precision drug development assays.

References

[1]Vonderheide and Glennie. Agonistic CD40 antibodies and cancer therapy. Clinical Cancer Research 2013;19(5): 1035-43.

[2]Yun Shi, et al. Engagement of immune effector cells by transtuzumab induces HER2/ERB2 downregulation in cancer cells through STAT1 activation. Breast Cancer Research 2014.