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Perspectives

Isotype Controls—Time to Let Go?

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Keeney et al. (1) provide a graphic example of where isotype controls don't work and can in fact lead to erroneous estimations of the target subpopulation. We would agree that for the accurate enumeration of CD34 positive stem cells, isotype controls are not always helpful and indeed there are other examples of rare event analysis where the isotype controls don't work. We recognize therefore that it is appropriate for some individuals in some laboratories to eliminate isotype controls for some procedures. Admittedly isotype controls have limitations, many of which were expanded upon in the Keeney perspective. In fact we could probably be convinced that for every example where isotype controls are currently being used there is some other reagent, tool or advanced gating strategy which could replace them. However in response to the perspectives suggesting that isotypes controls be let go, we will provide examples where isotype controls are useful and indeed recommended.

Basically, the isotype control provides a ''negative control.'' Cells stained with these products serve to indicate the amount of fluorescence that is emitted by cells labeled with a monoclonal (in most cases) antibody that is not specific for any protein on those cells. This ''negative control'' is sometimes called background or non-specific fluorescence. In an ideal world, a sample stained with an isotype control reagent allows the operator to set a cursor or a discriminator at a point where any event which generates a signal above this point is specifically stained with the antibody of interest. Unfortunately isotype controls are not perfect negative controls. Right off monoclonal antibodies are proteins secreted by transformed hybridoma cells, therefore although the immunoglobulins may be matched for subclass, there may be subtle differences because these hybridomas are not normal cells. Furthermore, although procedures for purifying, conjugating and repurifying monoclonal antibodies have improved significantly, the procedures are not entirely consistent from lot to lot or from company to company. In the ideal situation of matched isotype and subclass, matched F/P ratio and concentration, there may still be differences in background binding characteristics of the negative controls. These are limitations inherent in the use of isotype controls. We argue however that if their use is approached with an understanding of these limitations then they can be particularly useful and there will be no unrealistic expectations as to the information they can provide. In addition to these limitations, the argument is made (correctly) that in many cases a separate isotype control is not required because you often have a negative control right in the tube of interest. This is correct when the positively stained population of interest is clearly separated from the ''negatively staining'' population. In fact when you have a clear positive and a clear negative population, you can move the positive/negative discriminator around quite a bit and you will not affect the percent positive cells of interest. With these points taken into consideration, where would you use an isotype control antibody?

Isotype controls are particularly useful for a new technologist, a new laboratory, evaluating a new monoclonal antibody reagent and/or in the establishment of establishing a new procedure. The isotype control generates what should be in most cases baseline or background fluorescence and will allow for the optimization of the cytometer and a reference point for negativity of monoclonal antibody-stained cells. For example, if a new laboratory with little experience in flow cytometry wanted to measure a marker, which is present on all leukocytes, for example CD45 or CD11b, it might be difficult to optimize the settings without a negative reference point. Would you adjust the gains to have the dimmest populations in the first decade, second decade or third decade? The use of unstained cells as your control for background fluorescence would not be appropriate because monoclonal antibodies bind non-specifically in different amounts on different subsets (e.g., lymphocytes vs. monocytes vs. granulocytes). Staining with a non-specific isotype control antibody allows the user to determine the level of negativity in the subset of interest. As an example, the absence or very low expression of CD11b on granulocytes is used in our laboratory to screen for the Leukocyte Adhesion Deficiency Type-1(2). Since neutrophils express receptors which bind the Fc portion of immunoglobulin they bind significantly more antibody simply by virtue of these receptors. Therefore comparing antigen expression on granulocytes vs. lymphocytes is problematic without first acknowledging that the background staining will be significantly higher on granulocytes. It is imperative to have some indication of the amount of ''background'' staining generated by the anti-

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body of interest. A matched isotype control (with its inherent limitations) provides such a tool. In this case, this is not in fact ''non-specific binding'' since the receptors are specific for the Fc portion of the immunoglobulin molecule, i.e. the receptor is specific for the antibody but the antibody is not specific for the receptor. In order to evaluate the staining patterns on granulocytes, we adjust the cytometer using granulocytes stained with an isotype control reagent such that the level of fluorescence generated by negative control granulocytes matches the level of fluorescence generated by the negative control lymphocytes.

Isotype controls are particularly useful when you are assessing the up or down-regulation of a specific surface marker after a particular in vitro manipulation. We already alluded to the use of isotype controls to ascertain ''background'' or the negative reference point for non-conventional leukocyte subsets. What if you wanted to assess the appearance of or level of expression of a neo-antigen following in-vitro stimulation? Without an isotype control would you be confident that the de-novo appearance of the neo-antigen was real? What if the in vitro manipulation caused a significant increase in the non-specific binding with the monoclonal of interest such that it appears that the antigen is now expressed? The isotype control serves to answer and control for this situation. This question can also be asked when you are trying to assess an increase in the level of expression of an antigen which is already constitutively expressed. Is the increase in expression due to an increase in the level of the antigen or once again did the in vitro manipulation alter the cells in such a way that any antibody would stick non-specifically? Without an isotype control it might not be possible to answer this question. Current examples of antigens, which are not present on resting cells, but which will appear following in vitro activation, include CD69 and the CD40 ligand, CD154. Our laboratory measures the ability of lymphocytes to up-regulate CD154 as a screen for the Hyper IgM syndrome (3). The T helper cells of patients with this syndrome are unable to up-regulate this antigen. It is important to know how much of the increase in the expression of CD154 is due to non-specific binding as a result of stimulating cells with phorbol ester and a calcium ionophore. The isotype control is used for this purpose. An example of an antigen, which is constitutively expressed, but the ability to up-regulate the antigen is of interest, is CD11b. Patients with the moderate phenotype of the leukocyte adhesion deficiency may express elevated baseline levels of CD11b due to infection, making their baseline level of CD11b appear normal. Upon activation, however, the level of expression should not increase significantly unless the stimulation protocol caused an increase in non-specific binding. The procedure for activation itself can lead to non-specific stickiness of the granulocytes and to an apparent increase in CD11b. This could not be ascertained without an appropriate negative staining control i.e. the isotype control. The isotype control, with its inherent limitations, provides an effective tool for assessing the extent to which the activation procedure alone might generate an increase in non-specific binding.

The inclusion of isotype controls can be particularly useful when included in the immunophenotyping panels used in leukemia/lymphoma analyses. They can provide an indication of the sample integrity before analysis of any of the specific markers. For example, the light scatter parameters may be acceptable, but the isotype controls suggest that the identification of specific subsets may be problematic due to a high level of non-specific staining. We also find isotype controls to be useful when you are evaluating low density antigen expression and there is not a clearly distinct separation of the positive population and the negative population, not a rare occurrence in leukemia/lymphomas immunophenotyping. The observation of a shoulder or apparent positive population not present in the isotype control tube gives the cytometrist confidence that in fact this is a specifically stained population. Will this give you an exact estimate of positivity? Absolutely not, but it will help you decide if the antigen is expressed or not. Given the complex routines involved in optimizing the clinical cytometers today, the isotype control tube also generates a very recognizable pattern, which allows us to ascertain that we have properly adjusted the cytometer. Isotype control samples can also help one evaluate unusual or unexpected staining patterns in specific antibody tubes. Similar atypical staining in the isotype control and specific antibody tubes allows one to assume that the pattern is not related to specific antigen staining. For example, when gating on a specific population of cells, one may see the entire population shift in fluorescent intensity for a certain antigen without knowing whether this cell population's staining is specific or not. The staining characteristics generated by the isotype control on this specific subset may help you ascertain whether the staining is specific for the antigen. Isotype controls can also give valuable information when dealing with samples that are burdened with poor viability and large amounts of cellular debris. The staining patterns generated by the isotype controls are instrumental in interpreting samples stained for specific antigens. It is sometimes possible by reviewing the dot plots of light scatter vs. isotype control to ascertain where the non-specific staining is coming from and these events can be acknowledged and, if appropriate, gated out. In fact, many of the automated analysis programs utilize this type of information from isotype control samples as part of their algorithms in identifying such artifacts.

Saying goodbye to isotype controls is premature. The use of isotype specific controls should be left up to the discretion of the individual responsible for the laboratory. With an understanding of their limitations, an informed decision of whether or not to adopt their routine inclusion into specific protocols can be made. In the perspective proffered by Keeney et al., we saw an example of an advanced gating protocol which obviates the requirement for an isotype control. There are other examples where a negative control is present due to the inclusion in the

sample of cells not expressing the antigen of interest. Both of these examples stem from procedures, which would not have evolved to where they are today without the original use of isotype controls in their development. We believe that the isotype controls provide a very useful, if imperfect, negative control in our analytical flow cytometry repertoire. At the very least the isotype control reagents help us to define a starting point. I believe that the value of the isotype control is under-appreciated, especially in the more advanced labs, which have forgotten that it was the isotype control reagent that provided the original comparator from which we ascertained whether a specific reagent labeled the antigen of interest or not.

LITERATURE CITED

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