

# Guidelines for Mass Cytometry Panel Design

Mass cytometry combines the advantages of single-cell high speed analysis common to conventional flow cytometry with the ability to resolve over 90 metal probes with minimal signal overlap common to atomic mass spectroscopy, thereby providing researchers with an unparalleled ability to generate high resolution phenotypic and functional profiles of cells from normal and diseased states.

Multi-parametric cytometry analysis platforms have sufficient sensitivity, dynamic range and resolution to independently measure a wide number of targets simultaneously. These targets, such as cell surface markers, signaling proteins, cytokines and nucleic acids, are identified with probes conjugated to agents detectable by the platform. For mass cytometry, these probes are typically target-specific antibodies conjugated to rare earth metals, and the array of metal-conjugated antibodies (MCAs) used to define a system is referred to as a panel. When designing a panel, one must consider several things, including antigen abundance, sensitivity of the system to certain probes, sources of background signal, and measurement of probe signal in undesired channels (crosstalk). CyTOF<sup>®</sup> has several advantages that enable simplified high dimensional panel design, including lack of endogenous cellular signal, large number of available probes with extremely low signal crosstalk, and relative uniformity of detection across all channels. This document discusses the important considerations and provides guidelines for high dimensional mass cytometry panel design.

When designing a panel, it is important to prioritize a strategy that provides maximum signal detection with minimum background in the channels for your lowest abundance targets.

## Signal

Detected signal is dependent on the metal content of the probe and on the efficiency of detection of the metal isotope by the detector.

## Isotope detection

The CyTOF employs one detector capable of simultaneously detecting over 90 isotopes separated by their time-of-flight. Each isotope delivers a similar signal to the detector. However, the ion optics within the CyTOF are tuned for optimal delivery of metals in the 153–176 Da range to the detector. As a result, the system delivers to the detector approximately 3-fold more <sup>159</sup>Tb (for example) than the lowest mass in the lanthanide series (<sup>139</sup>La) and 1.5-fold more <sup>159</sup>Tb than

the heaviest lanthanide isotope (Figure 1). Therefore, it is recommended that low abundance targets are probed with metals in the 153-176 Da range.

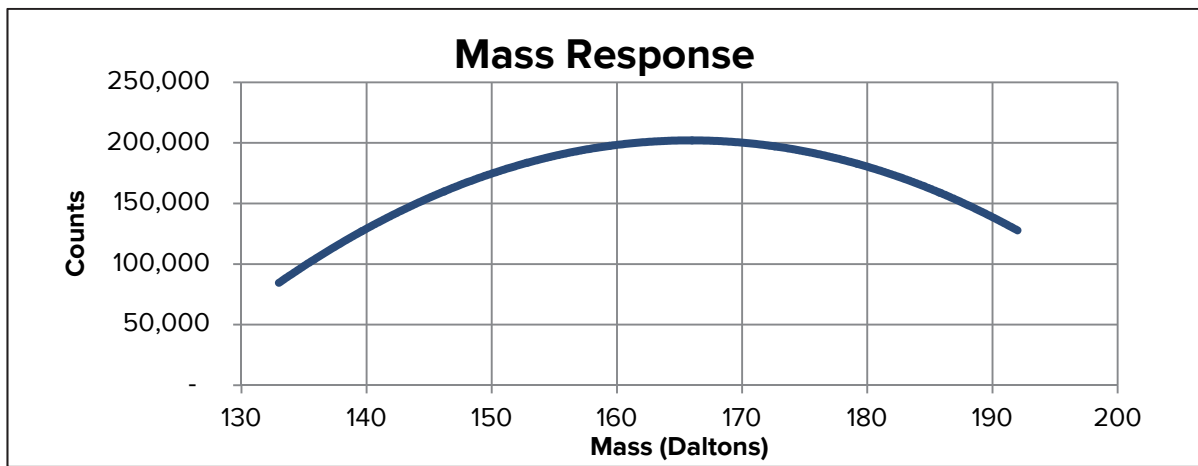


Figure 1: Mass response curve example

Relative counts (y-axis) detected in each mass channel (x-axis) of a typical CyTOF instrument. The relative count measured in a given channel is directly proportional to the transmission efficiency of the corresponding isotope through the ion optics of the mass cytometer. The mass range shown covers all isotopes in the lanthanide series. The actual mass response curve for any given instrument is empirically determined; the data shown here is a typical example.

## Probe Signal

Metal-conjugated antibody (MCA) probes are created by conjugating metal-loaded polymers (mass tags) via maleimide linkage to antibodies under partial reducing conditions. Probes that deliver optimal signal have not only a high metal content but also retain high avidity for the antigen. This latter point is very important because binding may be impaired with over-reduction of the antibody and/or steric hindrance if too many metal-loaded polymers are conjugated to the antibody.

If labeling with  $^{159}\text{Tb}$  (or isotopes close to  $^{159}\text{Tb}$ ) yields no signal for cells of known positivity, either: (1) the antibody is insufficiently labeled; (2) the antibody has lost binding avidity upon labeling; or (3) the target molecule is not expressed in sufficient density. To troubleshoot, determine the number of metals conjugated per antibody. If the metal content is reasonable ( $\geq 80$  metals per antibody), then check binding by staining the cells with the MCA followed by fluorochrome-conjugated species-specific secondary antibody to resolve the second possibility. If the MCA has sufficient metal content and binding avidity but you cannot resolve the population, consider amplifying the signal. One method is to use metal conjugated anti-biotin or anti-fluorochrome secondary

antibodies to amplify signal from biotin or fluorochrome-conjugated primary antibodies to the target. A second method is to use more than one antibody, each directed against a different target epitope, all conjugated to the same isotope.

## Background

Background is defined as detected signal that is unrelated to the measured probe, and it is important to minimize any sources of background in channels designated for detection of low abundance markers. Because the isotopes analyzed in mass cytometry have masses greater than 80 Da and are particularly rare in nature, there is very little endogenous signal derived from the cells themselves. Thus, the principal sources of background are derived from the environment or from the probes themselves, as discussed below.

### Environmental

Some heavy metals detected in the CyTOF mass range can be found in products and/or processes used to prepare mass cytometry samples. In particular, barium (130–138 Da) contamination results in measurement of barium oxides, contributing to undesired background in the M+16 (154) mass channel (see below for information on oxide formation). Barium is one of the more abundant elements in the earth's crust, and may contaminate samples left exposed in the normal laboratory environment (due to dust fallout). Also, laboratory glassware soaps and reagent bottles from non-Fluidigm commercial sources may contain high levels of barium, and use of such containers should be avoided in later steps of the staining protocol. In addition, low levels of barium, mercury, lead, iodine and tin can be found in some lab buffers. To avoid these, be sure to prepare lab buffers using water purified by reverse osmosis (i.e., Milli Q water), or use certified metal-free buffers and plastics from Fluidigm or other commercial vendors. If lab containers are used to store mass cytometry buffers, make sure they are new and rinsed with distilled water. It is recommended that each laboratory test every wash solution in the workflow for environmental contamination before running the first set of samples on the CyTOF.

### Crosstalk

Sources of background intrinsic to the CyTOF and the metal-conjugated probes used in mass cytometry experiments are referred to as crosstalk. The three

sources of crosstalk in mass cytometry, abundance sensitivity, oxides and isotopic purity, are described below.

## Abundance Sensitivity

In time-of-flight (TOF) mass spectrometry, ions are resolved according to their relative velocities, which are determined by their mass and kinetic energy. In the incoming beam, ions of the same kind have small differences in initial position and velocity from each other, and these position and velocity spreads lead to broadening of the apparent mass peak with some signal measurable in adjacent mass channels. Abundance sensitivity is defined as the ratio of the signal observed at the  $M\pm 1$  masses from a dominant signal at mass  $M$ . It is a fixed value for a specific instrument under specified operating conditions, and is typically 1-2% at  $M+1$  and a little less at  $M-1$ .

## Oxides

Some of the lanthanide ions form strong oxides that survive at low levels in the ionizing plasma and appear in the  $M+16$  channels (Figure 2,  $M+16$  boxes). The abundance of lanthanide oxide ions at the high temperature of the plasma ionization source is determined by the atomic ion-oxygen bond strength. Thus, La, Ce, Pr and Nd isotopes produce the largest  $M+16$  signal (typically 2–3%), while the Eu isotopes form the weakest bonds (typically  $< 0.1\%$   $M+16$  signal). Reducing the level of oxides to a minimum is dependent on optimizing the plasma temperature, which is part of the normal tuning procedure.

## Isotopic Purity

Naturally occurring elements are a mixture of isotopes which can be commercially obtained in enriched form. The enrichment that is possible for a candidate mass tag is related to the natural abundance of the isotopes of that element, and when the isotope is naturally greater than 20% abundant, an enrichment of better than 98% is often achieved. The purity of each isotope is determined at the time of its production and can be described by a fixed purity matrix (Figure 2) that is independent of the number of parameters measured, the instrument used, or the operating conditions. Values in each mass tag row indicate the relative amount of signal expected in each Mass Channel and are a reflection of the isotopic composition of the purified tag. (See Figure 2 legend for color-code explanation.) Note that we do not offer  $^{155}\text{Gd}$ ,  $^{157}\text{Gd}$ ,  $^{161}\text{Dy}$ ,  $^{163}\text{Dy}$ , and  $^{173}\text{Yb}$  (white boxes) because they are not available in highly pure form.

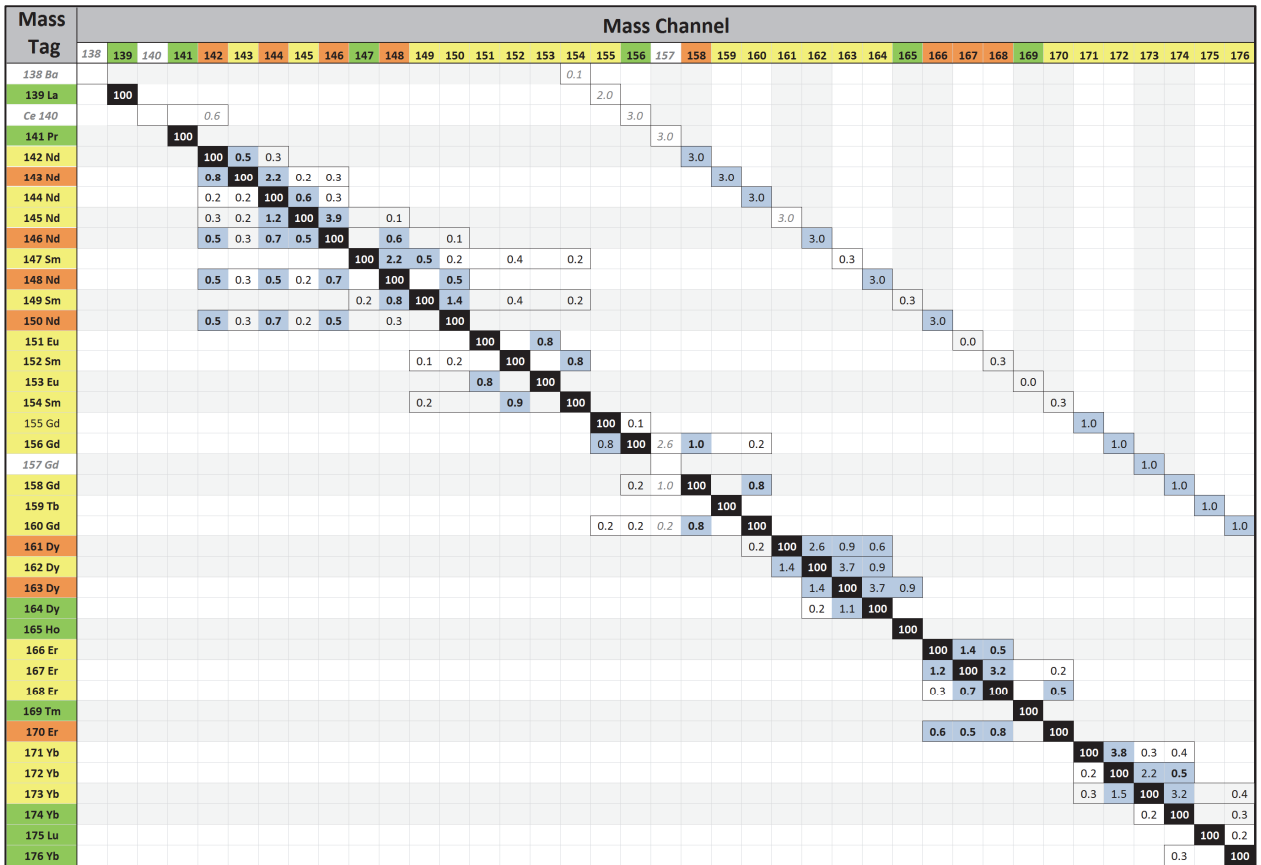


Figure 2: Lanthanide isotope purity and oxide matrix

The amount of signal derived from mass tags (rows) in each mass channel (columns) is indicated as a percent of maximum signal in the matrix. Off-channel signals derived from impurity or oxides (M+16 channels) are indicated in black font, and those of magnitude greater than 0.5% are in blue boxes. Note that oxide values are approximates only, and are influenced by instrument performance. Crosstalk into unused channels is indicated in grey italic font with a white background. Mass tags are color coded to indicate probes that contribute  $\geq 0.5\%$  crosstalk into no channels (green), one or two channels (yellow), or more than two channels (orange). Mass channel labels are color coded to indicate channels that receive  $\geq 0.5\%$  crosstalk from no probes (green), one or two probes (yellow), or more than two probes (orange).

<b>Mass Channels that Receive Crosstalk from:</b>		
<b>0 mass tags</b>	<b>1-2 mass tags</b>	<b>&gt; 2 mass tags</b>
139 La	143 Nd	142 Nd
141 Pr	145 Nd	144 Nd
147 Sm	149 Sm	146 Nd
156 Gd	150 Nd	148 Nd
161 Dy	151 Eu	158 Gd
165 Ho	152 Sm	166 Er
169 Tm	153 Eu	167 Er
171 Yb	154 Sm	168 Er
191 Ir	155 Gd	
193 Ir	159 Tb	
	160 Gd	
	162 Dy	
	163 Dy	
	164 Dy	
	170 Er	
	172 Yb	
	173 Yb	
	174 Yb	
	175 Lu	
	176 Yb	

Table I: Mass channels categorized by number of mass tags from which impurities and/or oxides

(of  $\geq 0.5\%$ ) are detected.

<b>Mass Tags that Contribute Crosstalk to:</b>		
<b>0 channels</b>	<b>1-2 channels</b>	<b>&gt; 2 channels</b>
139 La	142 Nd	143 Nd
141 Pr	144 Nd	146 Nd
155 Gd	145 Nd	148 Nd
164 Dy	147 Sm	150 Nd
165 Ho	149 Sm	161 Dy
169 Tm	151 Eu	163 Dy
174 Yb	152 Sm	170 Er
175 Lu	153 Eu	
176 Yb	155 Gd	
191 Ir	156 Gd	
193 Ir	158 Gd	
	154 Sm	
	159 Tb	
	160 Gd	
	162 Dy	
	166 Er	
	167 Er	
	168 Er	
	171 Yb	
	172 Yb	
	173 Yb	

Table II: Mass tags categorized by number of channels into which impurities and/or oxide

(of  $\geq 0.5\%$ ) are contributed.



# Panel Design

Here are guidelines for designing mass cytometry panels. Use the companion Application Guide (APG13-01) to develop your panel:

- 1** Low-abundance antigens:
  - a Choose high sensitivity channels. The CyTOF is most sensitive to metal tags in the 153-176 Da range (Figure 1).
  - b Choose mass tags in channels which receive little or no crosstalk from other metals in the panel (see Table I, green mass tag column and Figure 2, green channels).
  - c Consider amplifying low abundance signal:
    - Use metal-conjugated anti-biotin or anti-FITC secondary antibodies.
    - Stain a low abundance antigen simultaneously with more than one metal-conjugated antibody, each directed against a different epitope, all conjugated to the same isotope.
  
- 2** High-abundance antigens:
  - a Choose mass tags which contribute little or no crosstalk signals to the mass channels used for low-abundance antigens (see Table II, green mass tag column and Figure 2, green mass tags).
  - b Do not place a high abundance antigen in a channel that is -16 Da or +/-1 Da relative to the channel of a low abundance antigen.
  - c Titrate antibody and use at the lowest concentration possible so as to minimize crosstalk impact to other mass channels.
  
- 3** Antigens with variable expression:
  - a Treat as low abundance and follow guidelines for mass tag choice as outlined above.
  - b Perform pilot experiments to determine the biological dynamic range of abundance levels.
  - c Titrate staining reagents such that condition of lowest abundance is detectable.
  
- 4** Identify targets that are mutually exclusive of one another (CD4 and CD20, for example). It is acceptable if two such antibodies are bound to metals that crosstalk if the identified populations can be gated independently.

## References

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Leipold M, Maecker HT. *Multiparameter phenotyping of human PBMCs using mass cytometry*. Methods Mol Biol. 2014 (in press).

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