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Teiko Bio's Panel & Custom Antibody Verification Process

February 2024

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At Teiko Bio, we develop customizable mass cytometry (Cytometry by Time of Flight, or CyTOF®) panels built upon pre-designed, verified backbone panels to address our clients' immune profiling needs. Each panel is carefully designed to map all the major immune lineages and cell subsets of interest in the blood and other tissues, including the tumor. They also allow for customization with numerous open channels for additional markers that suit specific project needs. Both the backbone panels and all additional custom antibodies are tested and verified in-house on Teiko Bio's CyTOFs in accordance with the highest standards of scientific rigor, to ensure the best quality of the resulting data. In this white paper, we describe the verification process for our antibody panels. We focus on the verification process for both the original backbone panels, as well as a representative selection of custom antibodies verified for individual projects. For the verification process, we primarily discuss CyTOF channel placement, signal and spillover assessment and antibody titration approach.

Teiko Bio Antibody Panels

Teiko Bio has several pre-designed and fully verified mass cytometry [CyTOF] backbone panels that facilitate the immune profiling or biomarker discovery workflow for any (pre)clinical project. Predesigned backbone panels are available for projects involving human, non-human primate and mouse samples. All panels currently available can be found at https://teiko.bio/panels/.

Immune cell lineages detectable by Teiko Bio's backbone panels

Each panel is carefully designed to detect all major immune cell lineages and their subsets that are ideal to detect in blood and tumor samples. The panels contain markers targeting both cell surface and intracellular antigens. **Figure 1** depicts a non-exhaustive list of the immune cell lineages that Teiko Bio's panels can detect. Identifiable immune cell lineages include T cells, B cells, Natural Killer (NK) cells and various myeloid populations, such as classical monocytes and plasmacytoid dendritic cells (pDCs). If whole blood is analyzed (instead of isolated peripheral blood mononuclear cells (PBMCs)), the panels can also detect various granulocyte populations.

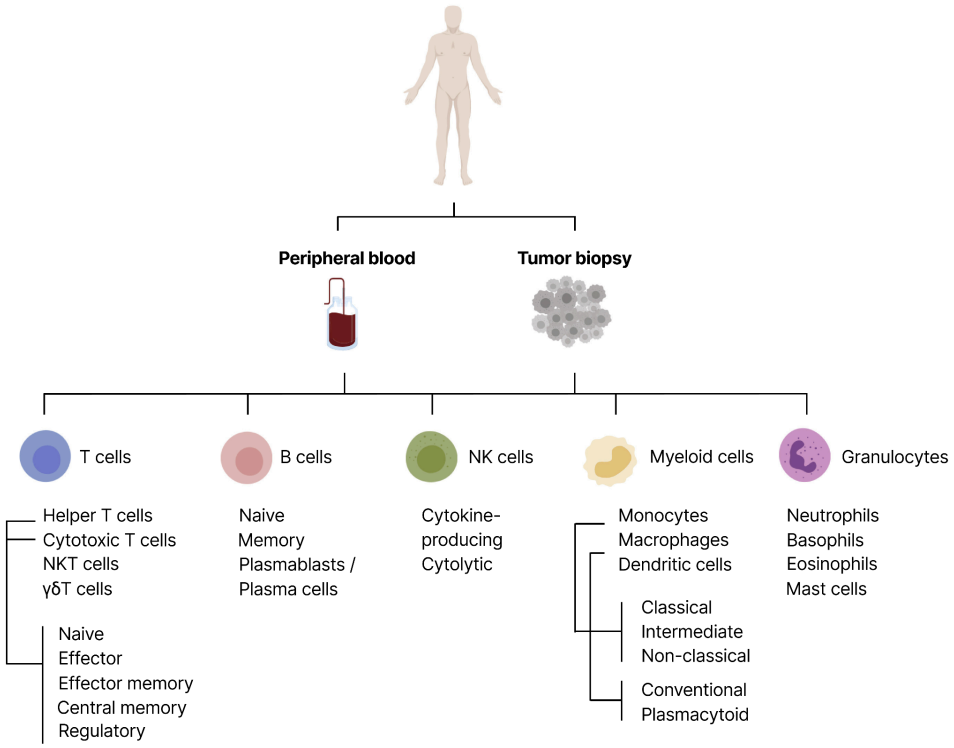

Figure 1) An overview of the major immune cell populations and their respective subsets that Teiko Bio's backbone panels can detect in both peripheral blood and tumor biopsies. Note that the list shown is not exhaustive, but serves as a reference set of interest for comprehensive immune phenotyping. Besides mapping these cell populations, the Teiko Bio panels can detect biomarkers and clinical targets. Image adapted from Hartmann et al. [1].

Recognizing the importance of T cells in tumor immunology, the panels cover all major functionally diverse T cell subsets, including CD4+ and CD8+ naive T cells, as well as different effector and memory T cell subsets. Through inclusion of FoxP3 (and CD25 and CD127 in the human panels), the panels are also able to identify regulatory T cells (Tregs), an important T cell subset associated with poor prognosis in oncology due to their suppression of anti-tumor T-cell responses [3]. Given the importance of checkpoint molecule inhibitors in cancer immunotherapy, e.g. nivolumab, pembrolizumab, ipilimumab, atezolizumab and others, the panels can also measure expression of various checkpoint-related molecules, such as CTLA-4, PD-1/PD-L1 and TIM-3.

Various other immune cell subsets express incredible heterogeneity in their phenotype and function that can affect antitumor immune responses. Various NK cell populations (e.g. $CD56^{high}CD16^{-}$ cells and $CD56^{low}CD16^{+}$ cells) and myeloid populations (e.g. $CD14^{+}CD16^{-}HLA\text{-}DR^{high}$ monocytes) have been correlated with clinical outcomes to immunotherapy [4-6]. To allow for the examination of cell population heterogeneity, each Teiko

Bio panel includes markers that delineate different B cell, NK cell and myeloid cell subsets. Even rare myeloid cell subsets, such as pDCs, can be detected.

Beyond markers that identify immune cell subsets, the panels include various markers that assess cell functional states, such as activation, inhibition, proliferation, maturation and migration. For example, CD38 is a marker of activation in T cells and NK cells [7], whereas CCR7 indicates cells that are migrating to lymph nodes [8]. By including these markers, Teiko Bio's panels can not only detect common and rare immune cell populations, but also assess their functional state and contribution to potential antitumor immune responses.

Teiko Bio's open channels allow for easy panel customization

Teiko Bio's antibody panels do not exhaust all available channels on a CyTOF. For example, if a project team has a particular interest in a rare cell subset not detectable with the standard markers on the backbone panel, or a project-specific hypothesis requires the addition of several cell state markers to the backbone panel, the backbone panels can accommodate that need.

All panels Teiko Bio uses have been carefully verified. The original Human and Mouse Antibody Panels are based on publications by the lab of Dr. Matthew Spitzer, a professor at University of California, San Francisco (UCSF) and co-founder of Teiko Bio. His research group has successfully used both panels to map the entire innate and adaptive immune systems, both cellular frequencies and functional states. Teiko Bio has modified both panels to allow for better customization and re-verified the updated panels in-house. The general verification process is outlined in **Figure 2**, and each step is explained in more detail in the next few sections.

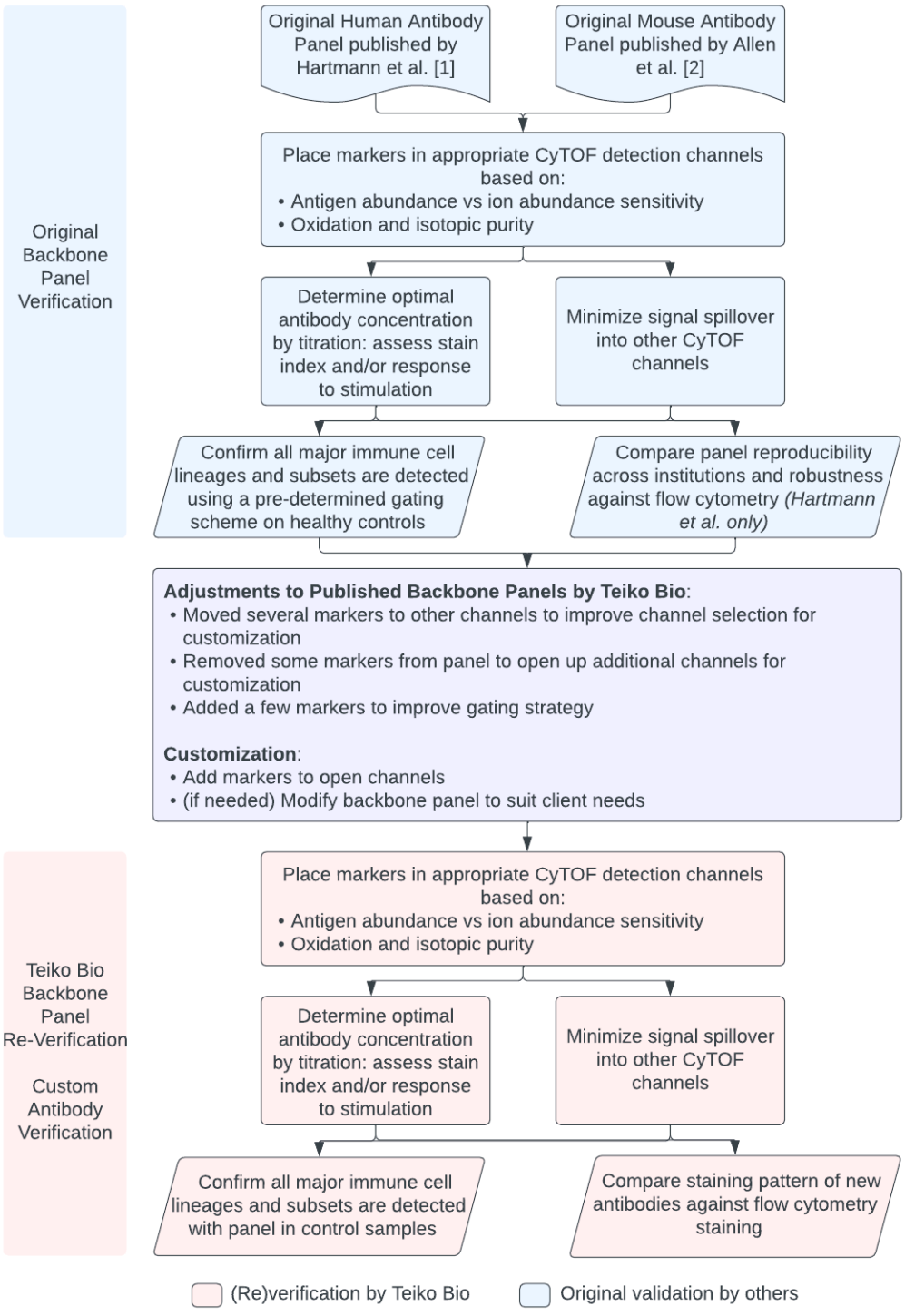

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Figure 2) An overview of the Antibody Panel verification process. The original Human Antibody Panel is based on Hartmann et al [1], whereas the original Mouse Antibody Panel is based on Allen et al [2]. Both used a similar verification process as Teiko Bio. First, the panels were designed through careful channel selection and antibody titration to ensure the best signal is detected with minimal spillover for each marker. Then, each panel was confirmed to detect all major immune cell lineages and subsets in healthy controls. Hartmann et al. also compared the robustness and reproducibility of the panel by comparing across research institutions and against flow cytometry. After Teiko Bio adjusted both panels, we reverified the panels in a similar fashion. For customizing antibodies, the verification process is similar, and the obtained staining patterns are compared to reported flow cytometry staining data.

Original verification of the Human Antibody Backbone Panel

The Teiko Bio Human Antibody Backbone Panel is an adaptation from a mass cytometry panel designed by the labs of Drs. Sean Bendall at Stanford University and Matthew Spitzer at UCSF. In a paper by Dr. Felix Hartmann et al., published in *Cell Reports* in July 2019, the authors describe the design of a mass cytometry workflow for standardized, systems-level biomarker discovery in immunotherapy trials [1].

To facilitate the discovery of immune-based biomarkers in clinical trials for new and existing immunotherapies, Hartmann et al. designed an original 33-marker mass cytometry panel that allowed one to comprehensively assess all the major immune cell populations one would expect to find in the blood and tumor tissue, and which is described above in more detail. In creating the panel itself, the authors evaluated the most appropriate placement of each of the 33 markers by balancing antigen abundance and channel detection sensitivity. They also made sure each antibody-metal conjugate was verified by careful titration, to maximize signal detection of both negative and positive cell populations while minimizing spillover into adjacent channels. Importantly, the Hartmann et al. panel did not exhaust all available channels in a standard CyTOF panel; it had 10 open channels that allowed for the flexibility to add markers necessary to test project-specific hypotheses [1].

After creating the 33 marker panel, Hartmann et al. set out to verify the panel by assessing its ability to assay the blood immune composition and activation state in PBMCs isolated from healthy donors. The team implemented a sequential gating analysis on pre-gated single, DNA+, live CD45+ non-platelet and non-erythrocyte cells to map the major immune populations within isolated PBMCs. Using this gating strategy, all major immune populations were readily identified, as well as multiple immune cell subpopulations. In fact, the authors were able to assign 98.4% ± 0.3% (median ± standard error of the mean (SEM)) of pre-gated cells to a specific immune lineage, and all calculated cell frequencies were within expected ranges (**Figure 3A**). T cells could be subdivided into naive, effector, effector memory and central memory states within CD4 and CD8 T cells, as well as regulatory T cells (Treg), natural killer T (NKT) and γδ T cells. In addition, the various stages of B cell maturation could be discriminated through CD27 and CD38 expression, monocytes were subdivided through CD14 and CD16 expression, and NK cells were delineated through combinatorial expression of CD16 and CD56. Hartmann et al. also confirmed that immune checkpointed-related molecules, such as PD-1, CTLA-4 and TIM-3 could be assessed on all relevant cell subsets, and that other markers indicating functional states (activation, proliferation, maturation, etc.) could be evaluated.

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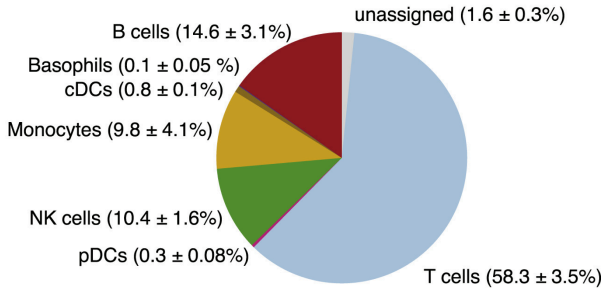

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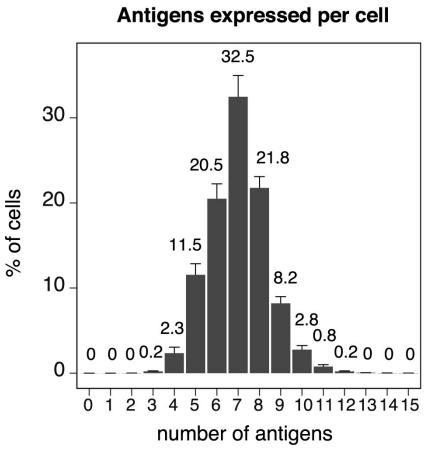

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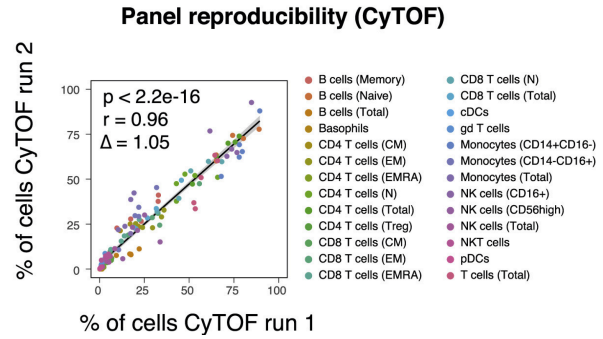

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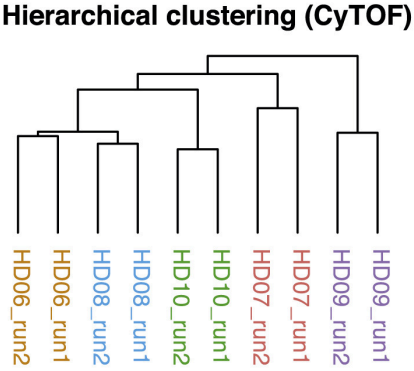

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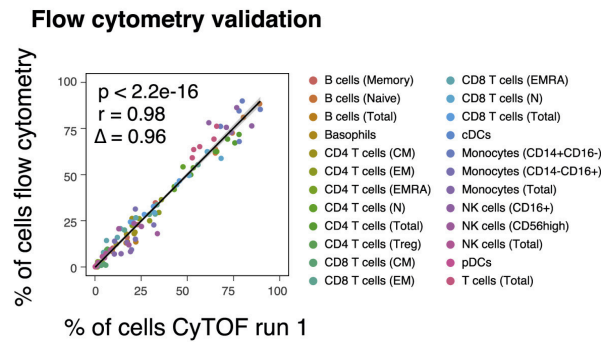

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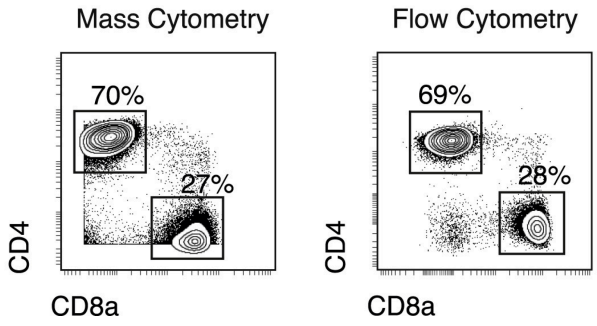

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Figure 3) All images adapted from Hartmann et al. [1]. A) PBMCs from healthy donors (n=5) were pre-gated on DNA+, live, CD45+, CD235ab/CD61-, non-neutrophils, and major immune lineages were identified through various gating steps. Image shows median frequencies ± SEM of major identified immune cell populations. B) Median number of antigens detected per cell, based on manual gates. Y-axis indicates median frequency of total pre-gated cells. Error bars represent SEM. C) PBMCs from healthy donors (n=5) were analyzed by mass cytometry in two different research centers, and immune cell lineages were identified as before. Linear regression line is shown in black with the 95% confidence intervals. Coefficients, p values and slope Δ were calculated based on data from all donors. D) Hierarchical clustering of samples from (C), based on cell frequencies. E-F) PBMC aliquots from the same healthy donors (n=5) as (A-D) were stained and acquired by flow cytometry using four separate staining panels. Frequencies of immune lineages were determined using the same gating strategy as used for mass cytometry. E) Linear regression line is shown in black with the 95% confidence intervals. Coefficients, p values and slope Δ were calculated based on data from all donors. F) Plots and frequencies of CD4+ and CD8+ T cell subsets within one healthy PBMC donor, as detected through mass cytometry (left) or flow cytometry (right).

Hartmann et al. also assessed the reliability and robustness of the immune profiling backbone panel. First, they calculated the number of antigens on each individual cell and found that 99.8% ± 0.1% (median ± SEM) of live cells, as well as individual immune cell lineages, were positive for ≥ 4 antigens in the panel (**Figure 3B**). The fact that differentiated cell subsets were also positive for at least 4 antigens is indicative of the broad expression range of functional state markers on human PBMCs, and shows the potential of mass cytometry to characterize these states. Next, they assessed panel robustness by comparing immune profiling performance between different research institutions. The same PBMC aliquots were stained in two separate research institutions and run on their respective CyTOFs. The authors found strong agreement (r=0.96) between the two independent experiments, both in comparing manually gated populations (**Figure 3C)** as well as hierarchical clustering of grouped aliquots from the same donor (**Figure 3D**). Finally, Hartmann et al. compared mass cytometry immune profiling results directly to the same immune cell populations obtained through standard flow cytometry, which required four independent flow cytometry antibody panels. They found strong agreement (n=0.98) between immune cell populations over a broad range of frequencies analyzed with either flow cytometry or CyTOF (**Figures 3E-F**).

The authors demonstrated the power of their mass cytometry backbone panel by identifying an immune signature for Graft versus Host Disease (GvHD) in bone marrow transplant recipients. They also use the open channels to build in additional markers to investigate both B cells as well as tissue-resident myeloid cell populations in more detail.

Teiko Bio has since replaced CD61 and CD235ab with the marker CD66b for gating out neutrophils. CD61 and CD235ab were used on the original panel to gate out thrombocytes and erythrocytes, but these cell populations usually are gated out when narrowing down on DNA+ live single cells. Our scientists have also moved some markers into different channels to improve panel customization options.

Original verification of the Mouse Antibody Backbone Panel

The Teiko Bio Mouse Antibody Panel is also an adaptation from a mass cytometry panel designed by the lab of Dr. Matthew Spitzer at UCSF. In a paper by Dr. Breanna Allen et al., published in *Nature Medicine* in May 2020, the authors define the systemic immune landscape in response to tumor development across five tissues in various mouse tumor models [2].

To map the murine immune landscape, the authors designed an original 41-marker mass cytometry panel. The main panel was designed to comprehensively assess all the major immune cell populations one would expect to find in the blood and tumor microenvironment, as well as the spleen, bone marrow and tumor-draining lymph nodes. Similar to the authors of the Hartmann et al. paper [1], Allen et al. evaluated the most appropriate placement of each of the 41 markers by balancing antigen abundance and channel detection sensitivity. Using titration on positive and negative control cell populations, the authors determined the antibody-staining concentration with the best positive and negative cell population signals. Instead of having open channels, the authors had 18 substitute markers for which they could swap a selection of the main panel markers out, if needed [2].

After creation of the 41 marker panel, Allen et al. verified the panel by assessing the immune landscape in murine blood, the tumor microenvironment, bone marrow, spleen and tumor-draining lymph nodes in eight commonly-used mouse tumor models and healthy controls. They used a sequential gating analysis on pre-gated single, live CD45+ leukocytes to identify major immune cell populations. Using this gating strategy, the authors were able to quantify the abundance and activity state of all the major immune populations and their related subpopulations across all different tissues. For example, they found that the tumor microenvironment showed distinct variation in the degree of immune infiltration and diversity across the different tumor models (**Figure 4A**), with some models showing higher levels of tumor-associated macrophages, whereas others had higher abundance of eosinophils. In general, they noted unique immune profiles across tumor types and murine tissues (**Figure 4B**). Allen et al. went on to study the impact of tumor growth and subsequent tumor resection on immune cell frequencies and states across immune organs. In addition, they assessed the ability to mount *de novo* T cell responses in response to a microbial challenge in each mouse tumor model. The paper shows that the murine mass cytometry panel is able to cast a broad net across the immune system, as well as focus on individual immune lineages for project-specific hypotheses.

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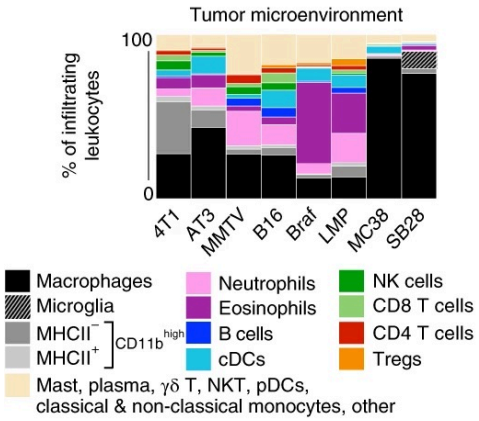

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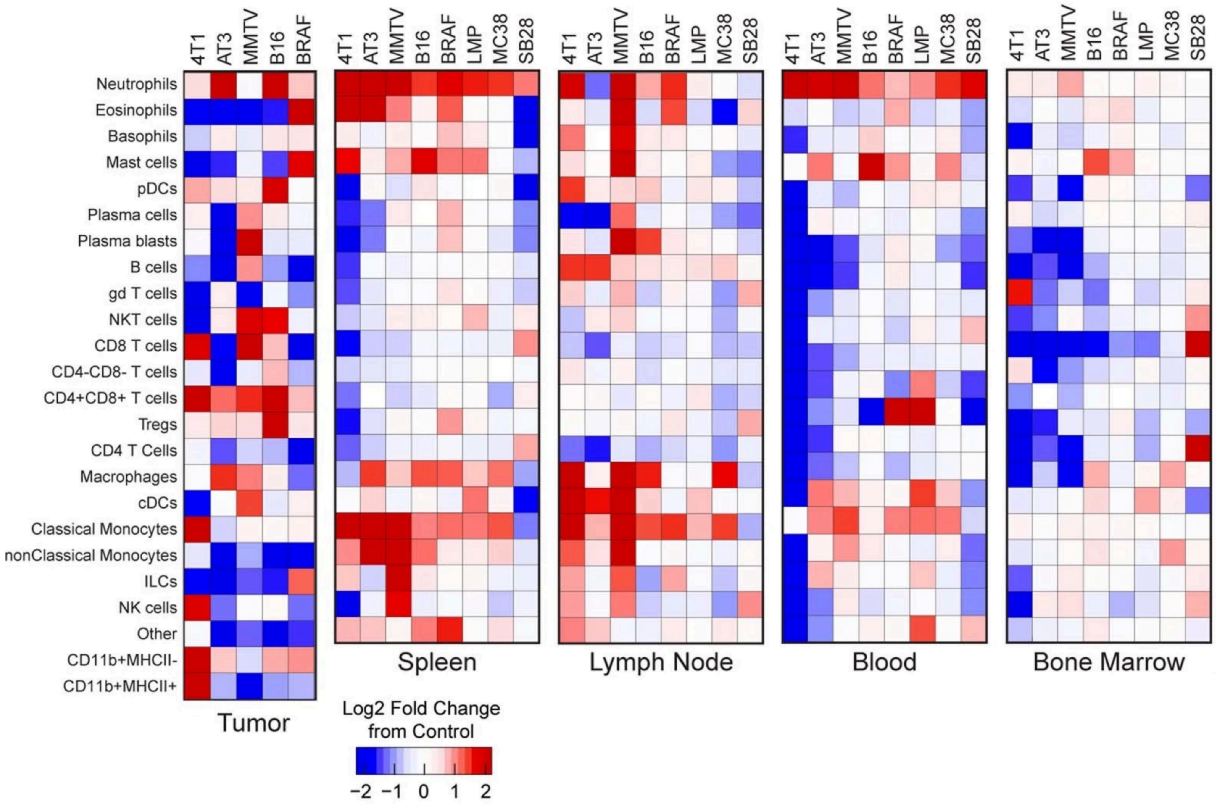


Figure 4) A) Composition of tumor immune infiltrates across late-stage mouse models, identified by manual gating. Breast cancer models 4T1 (n = 3 independent animals), AT3 (n=6) and MMTV-PyMT (n=7); Melanoma models B16 (n=6) and Braf/Pten (n=6); Pancreatic model LMP (n=4); Colorectal model MC38 (n=6); Glioblastoma model SB28 (n=1); n = 30 healthy controls. B) Heatmaps of the log2 adjusted fold change (compared to healthy control) in bulk immune cell frequencies across all five tissues, where relevant, across all models.

Teiko Bio has altered the original Allen et al. panel to allow for more customization. Our scientists have removed several markers from the panel, opening up several channels for customization. The following markers were removed from the Allen et al. panel: CD16/CD32, CD49d, CD90, cKit, FceR1a, GATA3, IgD, IgM, Ki67, RORγt and T-bet. These markers are mostly involved in finetuning immune subset phenotypes, such as CD4+ T helper cells, and are available as customizable antibodies. In exchange, Teiko Bio added Granzyme B and CD127 from the substitute panel, and added CD206 to better characterize macrophage subsets. In addition, we changed some of the channels for the existing markers to better distribute the open channels across the various detection ranges.

Channel selection

As discussed above, the Teiko Bio backbone panels were designed to capture all major cell subsets of the innate and adaptive immune system, and include many functional and signaling markers to capture cell states. Each marker was placed on an appropriate CyTOF channel where the relative antigen abundance is detected at a level where there is strong signal but minimal spillover into other channels. While Hartman et al. [1] and Allen et al. [2] both placed the markers on their panel in good detection channels, Teiko Bio modified some of the channel placements to allow for better customization. In doing so, Teiko Bio reverified the placement of each marker. In addition, the backbone panels allow for customization to slot in additional antibody-metal conjugates against targets of specific interest, without the need to redesign the overall panel. In designing the final panel for a project, Teiko Bio scientists work with the customer to optimize placement of each new marker into the most appropriate open channel. The most important factors to consider when placing a marker into an open channel are antigen abundance and ion detection sensitivity of the CyTOF system to certain probes [9]. To understand why, we need a closer look at how a CyTOF works.

A CyTOF is capable of detecting many metal isotopes separated by their time-of-flight using a specialized detector. The Helios™, the Standard Biotools CyTOF instrument that Teiko Bio uses, uses a single detector capable of detecting channels ranging from 75 to 209 atomic mass units (Da) [10]. Teiko Bio's backbone panels use channels in the 89 - 209 Da range.

Each metal isotope delivers a signal to the detector that matches a specific channel. However, the ion optics in the Helios instrument are tuned for optimal delivery of metals to the detector in the 159-169 Da range. For example, the Helios system delivers approximately three times as much 159Tb to the detector as the lowest mass in the lanthanide series, 139La, and 1.5 times as much 159Tb as the heaviest lanthanide isotype, 176Yb. Each machine has its own unique mass response curve, and **Figure 5** shows a representative mass response curve for Helios instruments (adapted from [10]).

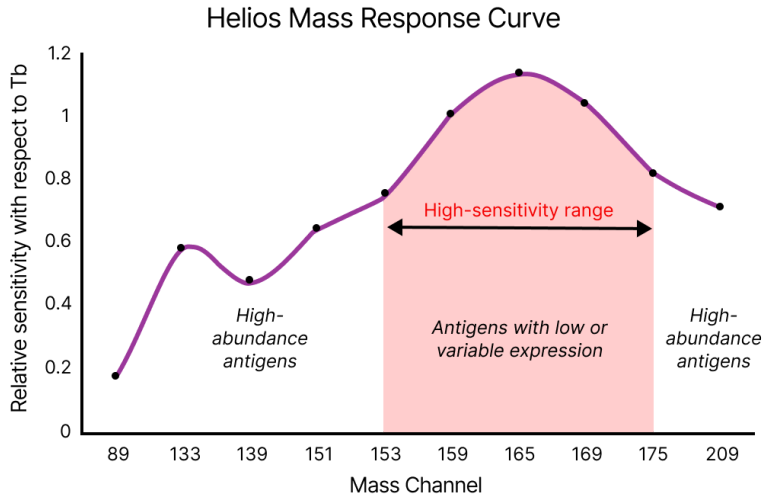


Figure 5) Representative mass response curve for Helios instruments. Image adapted from [10]. Indicated is the 153-176 Da high-sensitivity detection range suitable for antigens with low or variable expression, and the outside ranges suitable for high-abundance antigens. The pink arrows on the x-axis indicate the placement of the 12 open channels in the Teiko Bio Human backbone panel, and the blue arrows indicate the placement of the 10 open channels in the Teiko Bio Mouse backbone panel.

Because of this difference in detection sensitivity for the various metal isotopes, some CyTOF channels are not suited for low abundance antigens, whereas others are not suited for high abundance antigens. A low abundance antigen on a low sensitivity channel can result in low signal and difficulties in detection. Inversely, a high abundance antigen on a high sensitivity signal can result in signal spillover into adjacent channels through a process called abundance sensitivity. With that process, an antigen can have such a strong signal that the mass peak detected by the CyTOF reads as a detectable signal into adjacent channels.

Additional factors that need to be considered during mass cytometry panel design are metal oxidation and isotopic purity. Some metal isotopes are more sensitive to oxidation than others. When oxidized, a metal isotope gains an extra mass of 16 Da, which can lead to signal spillover into the wrong channel. For example, 139-Lanthanum (139La) can oxidize and be detected in the 155-Gadolinium (155Gd) channel. Isotope purity comes into play for metals that are difficult to purify; consequently, their preparations have trace contaminants of a similar metal with a different molecular mass. For example, the 113 and 115 CyTOF channels both detect Indium (113In and 115In), which has a challenging purification process. As a result, even the best metal preparation of 113In contains trace amounts of 115In, and vice versa.

In selecting the appropriate channel for each antibody, Teiko Bio follows Standard Biotools's guidelines for panel design [9]. In that way, we select the most optimal open channel for any marker that a client would like to add to the backbone panel.

1. Low abundance antigens are placed in high sensitivity channels, i.e. the 153-176 Da range.
2. High abundance antigens are put outside of the high sensitivity channels. In addition, we avoid placing high abundance antigens in a channel that is -16 Da or +/- 1 Da relative to the channel of a low abundance antigen. They are used at the lowest concentration possible to avoid signal spillover.
3. Antigens with variable expression are treated as low abundance.
4. For metals with issues with isotope purity or natural higher spillover signal, we consider markers that are typically mutually exclusive on the same cell population, e.g. CD3 (T cell marker) and CD19 (B cell marker). In that way, any cross-signal can be attributed to isotope impurity and not be mistaken for a true signal.

The open channels in Teiko Bio's panel are distributed across the detection range and incorporate several higher sensitivity as well as lower sensitivity channels. In this way, both antigens of low or variable abundance as well as high abundance antigens can be accommodated into the backbone panel. After the most appropriate channel is selected for the marker of interest, we proceed with antibody verification.

Antibody verification

To ensure all antibodies we use work well in our hands and generate high-quality data, Teiko Bio scientists verify each antibody in-house. We have done this for each antibody on our backbone panels, and do so for each new antibody that a client requests to be slotted into an open channel. Through the verification process, Teiko Bio verifies the optimal staining concentration at which each antibody demonstrates a detectable and accurate signal on the mass cytometer, while minimizing background signal and spillover into other channels. Teiko Bio's verification process incorporates several readouts.

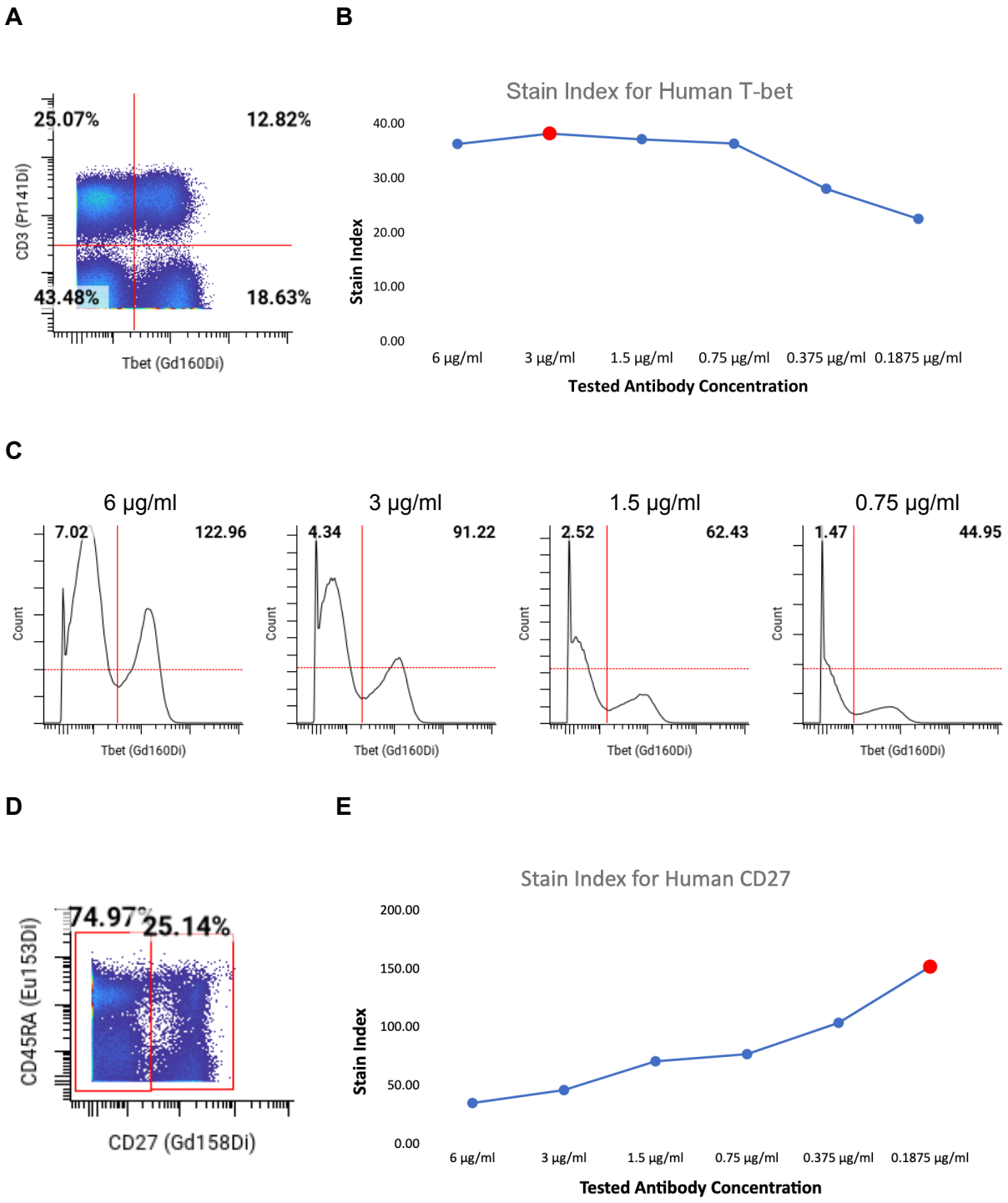
The first step in the verification process is dependent on the nature of the marker. For most immunophenotypical markers, we determine the correct antibody concentration using the stain index of the positive and negative cell populations. In contrast, analysis for most functional markers, such as signaling or metabolic markers, involves measuring the intensity of the marker in a cell population under a given stimulation condition. Therefore, in addition to the stain index, we determine the ideal antibody concentration using the arcsinh ratio of unstimulated and stimulated samples. For all antibody-metal conjugates, we assess spillover into other channels and select a concentration with minimal spillover contamination. All these processes are explained in more detail below. Once the optimal antibody concentration is established using these methods, Teiko Bio runs the customized antibody panel on control samples to ensure all markers perform well and the signals look appropriate before using it on client samples. In addition, obtained staining patterns are compared to reported flow cytometry data with the same antibody clone to ensure a similar pattern is detected.

Stain Index

The stain index is used for markers that yield a bimodal stain pattern, meaning both a negative and a positive population can be detected for the marker of interest (resulting in two peaks on a histogram plot, i.e. a bimodal distribution). Most markers are assessed using this method. The stain index identifies the optimal antibody concentration for staining 1 million cells. Targeting the correct stain index ensures that you minimize background shift while preserving an accurate signal. The stain index is the ratio of the separation between a defined cell population with the highest expression level (positive population) and a defined cell population with the lowest expression level (negative population) for a specific marker, divided by 2 * the standard deviation of the negative population (**Figure 6**). It is obtained by performing a serial dilution of the antibody cocktail of interest during cell staining and applying the following formula on each tested concentration, which yields a graph as seen in **Figures 6B** and **6E**:

$$Stain\ Index = (MCV^{positive\ population} - MCV^{negative\ population}) / (2 * \sigma^{MCV^{negative\ population}})$$

MCV: median channel value. σ : standard deviation.



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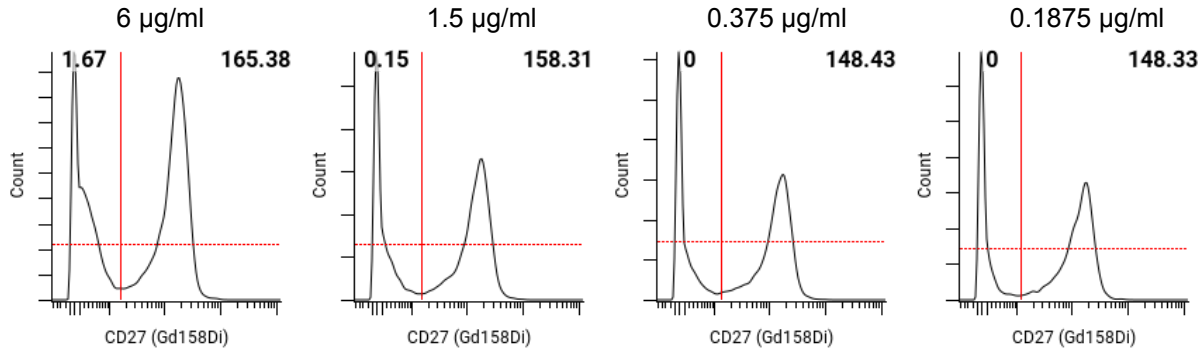

Figure 6) A) Staining pattern for human T-bet at 3 µg/ml in unstimulated human leukocytes. T-bet staining is mapped on the x-axis, CD3 staining is mapped on the y-axis. A clear T-bet-positive population and T-bet-negative population is seen (i.e. a bimodal distribution), making T-bet a good candidate for antibody concentration assessment by stain index. The percentages in the plot indicate the percentage of total parent population (live leukocytes). B) Stain indices for all tested antibody concentrations for human T-bet in unstimulated human leukocytes. The stain index of the optimal concentration is indicated in red. C) Histograms of the staining pattern for human T-bet in unstimulated human leukocytes under various tested concentrations. The optimal staining pattern is seen at 3 µg/ml. D) Staining pattern for human CD27 at 0.1875 µg/ml in unstimulated human leukocytes. CD27 staining is mapped on the x-axis, CD45RA staining is mapped on the y-axis. A bimodal distribution is also seen for human CD27 staining. The percentages in the plot indicate the percentage of total parent population (live leukocytes). E) Stain indices for all tested antibody concentrations for human CD27 in unstimulated human leukocytes. The stain index of the optimal concentration is indicated in red. F) Histograms of the staining pattern for human CD27 in unstimulated human leukocytes under various tested concentrations. The optimal staining pattern is seen at 0.1875 µg/ml.

Arcsinh Ratio

The arcsinh ratio is used for markers that yield a unimodal stain pattern, meaning there is no clear distinction between a negative and a positive population for the marker of interest even with an appropriate cell stimulation, resulting in one peak on a histogram plot (i.e. a unimodal distribution). Typically, the arcsinh ratio is used for functional markers, such as signaling or metabolic markers.

Because there is no clear positive vs negative population, the arcsinh ratio is obtained by comparing the antibody staining in a cell population before and after activation with a stimulation agent **(Figure 7)**. The optimal concentration is then calculated from the arcsinh-transformed median intensity of the cell population in stimulated sample minus unstimulated sample across various concentrations. For example, for the antibody in **Figure 7A-B**, the following calculation is made using the median channel values (MCV):

$$Arcsinh\, x = ln(x + \sqrt{x^2 + 1})$$

$$Arcsinh\, ratio = Arcsinh\,(MCV^{simulated}/5) - Arcsinh\,(MCV^{unstimulated}/5)$$

$$Arcsinh\, ratio = ln((13.25/5) + \sqrt{(13.25/5)^2 + 1}) - ln((1.59/5) + \sqrt{(1.59/5)^2 + 1})$$

$$Arcsinh\, ratio = 1.39$$

If the marker of interest is a molecule that is uniformly expressed across the general immune population, the arcsinh ratio is assessed in total leukocyte (non-granulocytes) populations. However, if the marker expression varies across different immune cell populations, or if the marker is only expressed in specific immune cells, response to stimulation is reviewed in the most appropriate immune cell subset instead.

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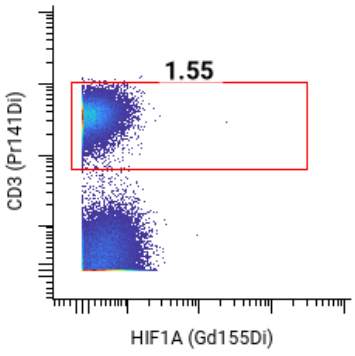

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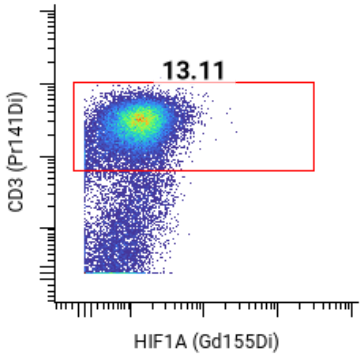

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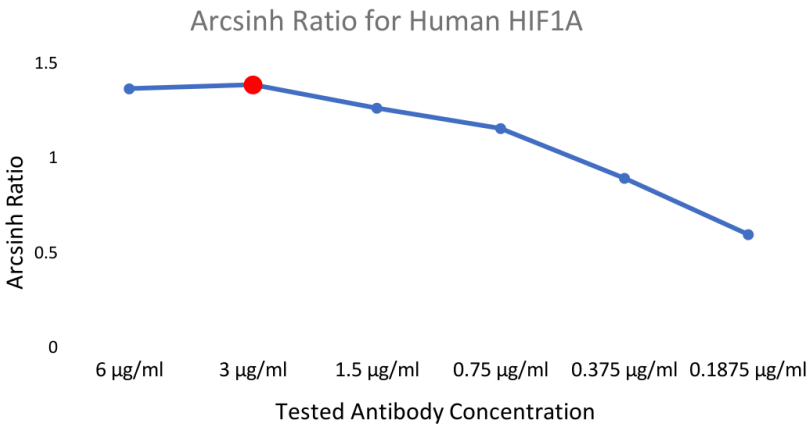

D

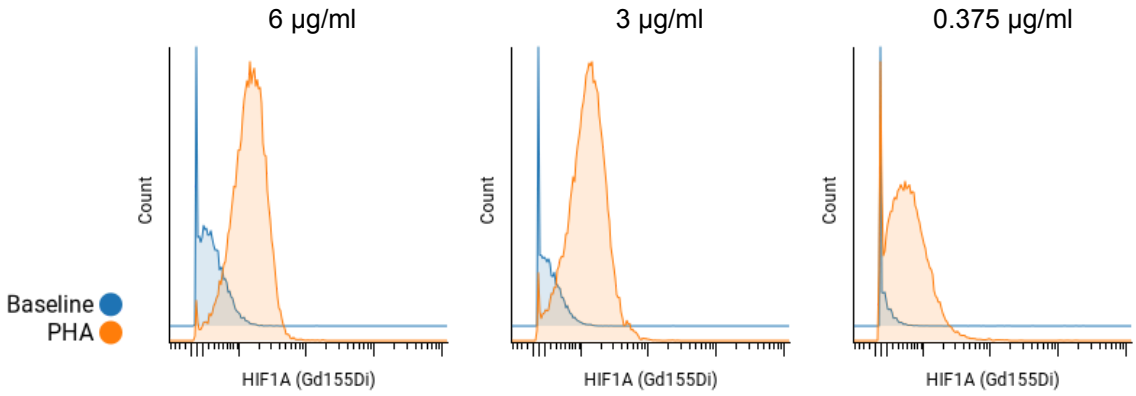

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Figure 7) A) Staining pattern in unstimulated leukocytes for human HIF1A at optimal concentration of 3 µg/ml. Value shown in the gate is the median channel value (MCV) of the x-axis. A unimodal staining pattern is seen. B) Staining pattern in phytohemagglutinin (PHA)-stimulated human leukocytes for human HIF1A at optimal concentration of 3 µg/ml. Value shown in the gate is the MCV of the x-axis. A unimodal staining pattern is seen also under stimulated conditions. C) Arcsinh ratios in unstimulated human leukocytes vs PHA-stimulated human leukocytes for each tested concentration of human HIF1A. The optimal concentration of 3 µg/ml and resulting arcsinh ratio are marked in red. D) Histogram overlay of the staining patterns of human HIF1A in T cells under unstimulated condition (baseline, blue) and stimulation with PHA (orange), stained at various antibody concentrations. Even though the arcsinh ratios in leukocytes for 6 µg/ml and 3 µg/ml are very similar, the latter was determined to be an optimal staining concentration due to reduced signal in unstimulated cells.

Background and spillover detection

For any marker, if background staining in other channels is observed, the optimal concentration for antibody staining is also determined through evaluation of spillover. If spillover is suspected, a full mass minus one (MMO) control [11] is used to compare the level of signal spillover of the marker of interest compared to the potential spillover marker **(Figure 8).** For example, if we observe potential spillover of the T cell marker CTLA-4 into the B cell marker CD20, we'd do a full panel stain without the CD20 marker and see if we detect CTLA-4 signal in that channel. Spillover is usually tested under unstimulated conditions, but for functional markers or signaling markers it is also assessed with an appropriate stimulation condition (e.g. phorbol 12-myristate 13-acetate (PMA) stimulation for T cells). We then select the antibody concentration where we see the least amount of spillover in other channels, but still detect a strong signal in the channel of interest.

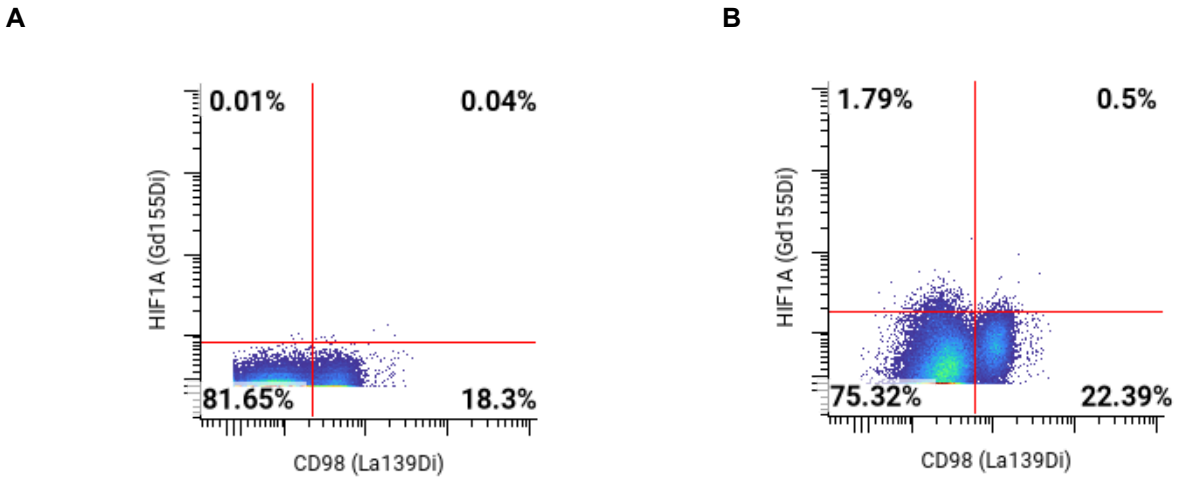

Figure 8) Spillover signal in leukocytes stained with human CD98-La139 (x-axis), evaluated against the Gd155 channel (y-axis). Signal shown for A) Unstimulated leukocytes stained with 0.1875 µg/mL human CD98-La139 antibody, which Teiko Bio determined to be the optimal concentration; B) Unstimulated leukocytes stained with 6 µg/mL human CD98-La139 antibody. Increased spillover signal into the Gd155 channel can be seen at this concentration. Gates show the percentage of parent population (live singlets).

Conclusion

To summarize, Teiko Bio's backbone panels have been carefully designed and verified to cover all the major immune cell populations that are important to detect in the blood and tumor. The verification process for the original backbone panels is described in more detail by the Hartmann et al. [1] *Cell Reports* publication for the human antibody panels, as well as by the Allen et al. [2] *Nature Medicine* article for the mouse antibody panel. Teiko Bio has made minor adjustments to both backbone panels and re-verified all antibodies in-house. Each antibody in the backbone panel has been assessed for optimal channel placement by evaluating the relative marker antigen abundance against the channel detection sensitivity, as well as isotopic purity and oxidation. In addition, we assessed possible spillover into other channels. Next, each antibody was titrated to find the optimal concentration where the signal separation between negative and positive cell populations (stain index), or unstimulated and stimulated cell populations (arcsinh ratio) is greatest. This process is done for any new antibody requested during the panel customization process, with additional comparison to any reported flow cytometry staining of the same antibody clone.

A list of all verified antibodies available for customization is available upon request. Verification data for individual antibodies, both those part of the backbone panels and those available for customization, are also available upon request.

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