**Protocol CyTOF004.2: Metal Labeling 200μg IgG CyTOF Antibodies with Maleimide MaxPar Reagents - Pre-Loading Method (Adapted from S. Bendall)**

Indication: for preparing conjugated (metal-tagged) antibodies for CyTOF analysis (200μg)

The current generation of polymer has been functionalized with an –SH reactive maleimide group. In the absence of interfering agents (DTT, BME, low pH, -SH rich protein) maleimide chemistry is essentially 100% with reduced –SH groups when used in excess. Moreover, the nature of the lanthanide reporter metals used can induce precipitation of the IgG (and other proteins) as well as some buffer components (phosphate) at even relatively low concentrations (μM-mM). Therefore, the protocol herein focuses on preventing the exposure of the IgG to high metal concentration while still optimizing the timing for maximum labeling efficiency.

Also note that this protocol requires “carrier-free” IgG for labeling (meaning no BSA, hydrolyzed protein, gelatin, etc. for stabilization). Most IgGs stored in the presence of carrier contain BSA. Many companies offer custom preparation of carrier-free versions of antibodies normally containing BSA. While expensive, this option is very useful when no other alternatives are available. However, if the antibody cannot be obtained in the absence of carrier commercially, you can try the Melon clean-up procedure at the end of this protocol. For other/unknown carriers, a protein A/G affinity purification of antibody is recommended.

**Note:** This protocol is optimized for a multitude of IgG isotypes and also works well for affinity purified polyclonal preparations – **will not work for IgM mAbs**

**Reagents and Materials:**

* 200μg **carrier-free** IgG (MW ~180,000; 1.1x10-9 moles)
* 0.5M Neutral TCEP (Tris-carboxyethyl phosphine): 10μL aliquots
* MaxPAR antibody labeling reagent (DVS Sciences)
  + Use 0.4mg (2 polymer tubes) for 200μg of IgG
* Buffers:
  + R-Buffer
  + C-Buffer
  + L-Buffer
  + W-Buffer
* Trivalent Metal Lanthanide Solutions (50-100 mM stocks in L-Buffer – XCl3)
* TBS or PBS-based antibody stabilization solution (Candor) – *Note: this can be supplemented with 0.1% azide after purchase.*
* Millipore Centricon columns:
  + 3kDa MWCO Amicon Ultra 500μL V-bottom – Cat#UFC500396 (1 per labeling reaction)
  + 50kDa MWCO Amicon Ultra 500μL V-bottom – Cat#UFC505096 (3 per labeling reaction)
* Screw top Eppendorf tubes
* Filter Tips (to prevent cross-contamination between metal stocks and reagents)

**Before Starting:**

* Equilibrate all conjugation buffers to RT
* Pre-label MWCO tubes with isotope mass (3kDa) and antibody epitope (50kDa), and pre-label 2mL stock tubes with antibody epitope, metal, clone, and date
* Be careful throughout the procedure not to touch the filters in the MWCO tubes with pipette tips, as they are easily punctured

**Protocol and Timing:**

**0:00 *Pre-loading chelating polymer.***Before reconstituting the MaxPar chelating polymer, spin the tube for 1 minute in a microfuge to ensure the reagent is at the bottom of the tube. Reconstitute the prescribed amount of MaxPar reagent, i.e. 0.4mg (2 tubes) for 200μg of IgG, in 90μL of L-Buffer\* per labeling reaction by pipetting up and down 20 times. Transfer each reaction to a 1.5mL microcentrifuge tube. Use filter tips for all pipetting steps. Add a sufficient amount of the desired lanthanide metal solution to the MaxPar reagent to make an approximate final concentration of 5mM (i.e. 10μL of 0.05M stock). Vortex briefly to mix. Incubate at RT for 60 min and vortex every 20 min.

**\*IMPORTANT NOTE:** For 113In, use 1% HCl instead of L-buffer. For 209Bi, use 5% HNO3.

**0:40 *Buffer exchanging the antibody.***In a 50kDa MWCO micro-filter device, add 300μL of R-buffer then 100μg of antibody (Max Volume 500μL). If 100μg of antibody accounts for greater than 200μL of volume, either pre-concentrate it in the same 50kDa MWCO column, or reduce the volume of R-buffer accordingly. Centrifuge at 12,000xg at RT for 8 min. Discard flow-through (ideally the final volume should be 20μL or less).



**0:50 *Partial antibody reduction.***Mix 8μL of TCEP stock with 992μL of R-buffer (4mM final concentration). Remove the concentrated antibody (~20µL) from the bottom of the 50kDa MWCO tube and transfer to a new 1.5mL microcentrifuge tube. Rinse the interior portion of each filter in the 50kDa MWCO tube with 100μL of the diluted TCEP 3 times, then transfer to the antibody in the 1.5mL microcentrifuge tube. Pipette up and down 5 times to mix. Place microcentrifuge tube in a flotation device and incubate in a 37°C water bath for 30 min.

**1:00 *Clean-up pre-loaded MaxPar labeling reagent.*** Add 300μL of L-buffer\* to the metal loaded polymer tube and mix by pipetting up and down 3 times. Transfer the mixture to a 3kDa MWCO micro-centrifuge column. Reduce the volume by centrifugation at 12,000xg for 25 min at RT. Discard column flow-through. Repeat wash and spin with 400μL C-buffer and 25 min spin.

**\*IMPORTANT NOTE:** For 113In, use 1% HCl instead of L-buffer. For 209Bi, use 5% HNO3.

**1:20 *Clean-up partially reduced antibody.*** Collect the partially reduced antibodies from the 37°C water bath. Add 300μL of C-buffer to the partially reduced antibody and mix by pipetting up and down 5 times. Transfer mixture to a new 50kDa MWCO tube. Reduce the volume by centrifugation at 12,000xg for 8 min at RT. Discard flow-through and repeat wash and spin with an additional 400μL of C-buffer.

**1:50 *Coupling Antibody and Metal-Loaded Polymer.*** Remove all centrifugal concentration columns from the centrifuge. Remove the metal-loaded polymer (~40µL) from the bottom of the 3kDa MWCO tube and transfer to a new 1.5mL microcentrifuge tube. Rinse the interior portion of each of the filters in the 3kDa MWCO tube 3 times with 100μL of C-buffer and transfer to the microcentrifuge tube. Pipette up and down 5 times to mix. Remove the partially reduced antibody (~20µL) from the bottom of the 50kDa MWCO tube and transfer to the microcentrifuge tube. Rinse the interior portion of each of the filters in the 50kDa MWCO tube 3 times with 100μL of C-buffer and transfer to the microcentrifuge tube. Pipette up and down 5 times to mix. Incubate at 37°C for 60-120 min (typically 90 min).

**3:30** ***Washing metal conjugated antibody.*** Add 200μL of W-buffer to the antibody conjugation mixture. Transfer contents of the 1.5mL microcentrifuge tube to a new 50kDa MWCO tube. Centrifuge at 12,000xg for 8 min. Discard flow-through. Wash 3 more times with 400μL of W-buffer by adding W-Buffer to MWCO tube each time and pipetting up and down 3 times to mix, being careful not to touch the filters or generate excessive bubbles.

**4:15 *Recovery of metal conjugated antibody.***Remove the concentrated labeled antibody (~20µL) from the bottom of the 50kDa MWCO tube and transfer to a pre-labeled 2mL hinged-cap storage tube. Rinse the interior portion of each of the filters in the 50kDa MWCO tube 5 times with 200µL W-buffer and add to the microcentrifuge tube. Pipette up and down 5 times to mix.

**Post *Quantification.***Quantify antibody by measuring on the Qubit 4 fluorometer in the drawer under Bench 2.

1. Prepare reporter cocktail by diluting concentrated reporter (200X – small vial of orange liquid) in an appropriate volume of Qubit Protein Assay Buffer. Prepare enough for 200µL reporter cocktail per conjugated antibody, making extra to account for pipetting error. For instance, for 4 conjugated antibodies, add 995µL Protein Assay Buffer to 5µL 200X reporter in to make 1mL reporter cocktail.
2. Dispense 2µL conjugated antibody into the bottom of a 0.5mL Qubit Assay Tube (top shelf in plastic bag). Add 198µL reporter cocktail and pipette up and down 5 times to mix.
3. Incubate antibody with reporter cocktail at RT for 15 minutes.
4. Read tube on Qubit 4 Fluorometer using the Protein Quantification program, indicating 2µL used for assay.
5. Output units will be in mg/mL (µg/µL).

**Post *Titration.*** Titrate the antibody on positive and negative control cell populations over the range of 0.5-8μg/mL final concentrations in 100μL staining reactions.

**Post *Storage.*** Once titrated, dilute the antibody to a final concentration of 0.2mg/mL in antibody stabilization buffer (with NaN3) and store at 4°C.

**Troubleshooting**

If little or no specific signal is observed, check the antibody by fluorescence flow cytometry with a secondary antibody stain. Suggested concentrations: 3μg/mL for primary and 2μg/mL for secondary fluorophore conjugate. *If there is specific staining by fluorescence it is likely that the CyTof antibody was under-labeled; if there is an absence of specific staining by fluorescence it is likely that the CyToF labeling has inactivated/denatured the antibody. This is assuming this antibody has been previously qualified for flow cytometry.*

**Antibody BSA/Carrier Protein Removal Protocol**

This protocol is a mild purification of IgG by preferential removal of other components (namely albumin). As a consequence, an expected loss of up to 50% of starting antibody can be expected. Therefore, it is recommended that you start with 250-300μg of antibody in this case in order to ensure the recovery of at least 100μg for labeling.

**Adapted from:** Thermo Zeba Desalt Spin Columns

Thermo Melon Gel IgG Purification

**Buffer exchange**(Zeba Desalt Spin Columns- order #89889)**:**

1. Take 2mL Zeba Column,twist off the column’s bottom closure and loosen cap. Place the column in a 15mL Falcon tube for collection.

2. Centrifuge column at 1,000 × *g* for 2 min to remove storage solution. Place a mark on the side of the column where the compacted resin is slanted upward. Place the column in centrifuge with the slant mark facing outward in all subsequent steps.

3. Add 1mL Melon Gel Purification Buffer to the column.

4. Centrifuge at 1,000 × *g* for 2 min to remove buffer.

5. Repeat steps 3 and 4 two or three additional times, discarding buffer from the collection tube.

6. Place column in a new collection tube, remove cap and slowly apply sample (200-700μL) to the center of the compact resin bed.

7. Centrifuge at 1,000 × *g* for 2 min to collect the sample as the flow-through. Discard column after use.

**BSA removal**(Melon Gel IgG Purification – order #45206)**:**

1. Equilibrate the Melon Gel IgG Purification Support and Purification Buffer to room temperature.

2. Swirl bottle containing the Melon Purification Support (**do not vortex**) to obtain an even suspension. With apre-cut 1000μL pipette tip (to increase opening) dispense 250μL of slurry into a Mini-Spin Column placed in a micro-centrifuge tube.

3. Centrifuge the uncapped column/tube assembly for 1 min, then remove the spin column and discard flow-through. **Note:** Perform all centrifugations at 4000 × *g*. Note: Centrifugal force > 6,000 × *g* produces suboptimal results.

4. Add 300μL of Melon Purification Buffer to the column, pulse centrifuge for 10 seconds and discard flow-through. Repeat this wash once. Place the bottom cap on the column.

5. Add up to 500μL of buffer-exchanged Ab from the first part of the protocol. Cap the column (bottom and top) and incubate for 5 min at room temperature with end-over-end mixing.

6. Remove bottom cap from the column, loosen the top cap, and re-insert spin column in the collection tube.

7. Centrifuge at 4000× *g* for 1 min to collect the purified antibody in a new micro-centrifuge tube.

8. Move purified Ab into a storage tube, repeat steps 6-8 with the rest of the sample, recycling the same Melon resin, until the entire sample has been processed.

9. Quantify you antibody recovery by measuring the absorbance at 280nm as previously described. Again, 50% recovery of starting antibody is expected.

**TIMELINE**

0:00

4:30

1:00

2:00

3:00

4:00

Pre-load polymer

Ab buffer exchange & reduction

Wash polymer (2X)

Wash Ab (2X)

Conjugation

Wash (4X)