STANDARD OPERATING PROCEDURE

Title: Lanthanide Antibody Labeling using Standard BioTools Maxpar Multiple Metal Labeling Kit

SOP #: M-147

Version #: 1.0

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1. PURPOSE

This SOP describes the procedure for lanthanide labeling of IgG antibodies for subsequent staining and downstream acquisition using Standard BioTools Imaging Mass Cytometry (IMC) Hyperion imaging system.

2. SCOPE

This SOP provides information for lanthanide labeling of IgG antibodies. It includes steps such as loading the polymer with lanthanide, buffer exchange and reduction of antibody, and conjugation.

3. RESPONSIBILITIES

It is the responsibility of the person(s) performing this procedure to be familiar with lab safety procedures and to have basic laboratory skills. It is the responsibility of the analyst to follow the procedure steps as written and to document any deviations, problems, and observations during an experiment in their laboratory notebook.

4. EQUIPMENT

4.1. 4°C Fridge, Fisher Scientific Model ISOTEMP 97-920-1, or equivalent

4.2. -20°C Freezer, Fisher Scientific Model ISOTEMP 97-926-1, or equivalent

4.3. Mini Centrifuge, Fisher Scientific Model SPROUT, or equivalent

4.4. Mini Centrifuge, VWR Model C0803, or equivalent

4.5. Microcentrifuges, Eppendorf Model 5425 or 5424, or equivalent

4.6. Nanodrop, Fisher Scientific Model Nanodrop One C, or equivalent

4.7. Pipettes, Rainin Pipet-Lite XLS series, or equivalent

4.8. 0.5 mL screw cap O-ring tube, or equivalent

4.9. Heat Block, Fisher Scientific Model Isotemp FS Drybath Standard 2 block 100-120V, or equivalent

4.10. Vortex, Fisher Scientific, Model Analog Vortex Mixer 02215365, or equivalent

4.11. pH meter, VWR Model SymPHony SR01, or equivalent

4.12. Water Unit, Millipore Sigma Model MilliQ IQ 7005 plus Q-Pod, or equivalent

Note: Two microcentrifuges are required for this assay, and they both must be able to reach 12,000 RCF.

5. CHEMICALS

5.1. Maxpar X8 Multimetal Antibody Labeling Kit (includes Polymer, lanthanides, buffers (C, L, R, and W)). Standard Biotools, Catalog # 201300

5.2. Centrifugal Filter unit 3 KDa Amicon Ultra – 500 µL V bottom. Millipore, Catalog UFC500396

5.3. Centrifugal Filter unit 50 KDa Amicon Ultra – 500 µL V bottom Millipore, Catalog # UFC505096

5.4. TCEP Bond Breaker Solution, 0.5M. Thermo Scientific, Catalog # 77720, or equivalent

5.4.1. Prepare 10µL aliquots of 0.5M TCEP and store at -20°C according to the manufacturer.

5.5. 10X PBS. Fisher Bioreagents, Catalog # BP399-500, or equivalent

5.6. Sodium azide 5% w/v. RICCA Chemical Company, Catalog # 7144.8-32, or equivalent

5.7. Antibody Stabilizer solution. Candor Bioscience, Catalog # 131050

5.7.1. 100-200µg of antibody (BSA and azide free) is required.

Note: It is best to perform this assay in even numbers for centrifuge balance (example: label 2, 4, or 6 antibodies at a time depending on the number you are comfortable with).

6. REAGENTS

6.1. 1X PBS pH 7.4 (± 0.02) from 10x PBS stock solution.

6.1.1. Combine 100 mL of 10x PBS with 900 mL of ultrapure water. Store at room temperature for up to one month.

6.2. 4mM Bond Breaker TCEP in R-buffer

6.2.1. Remove one 10 µL aliquot of 0.5M TCEP stored at -20°C.

6.2.2. Mix 8 µL of 0.5M TCEP + 992 µL R-buffer.

6.3. W-E buffer with 0.5% azide

6.3.1. 1 µL of 5% azide + 99 µL of W-buffer

Note: 100 µL is required for each antibody being labeled.

7. PROCEDURE

Note: This protocol has been optimized for a multitude of IgG isotypes and works well for affinity purified monoclonal and polyclonal preparations. This protocol will not work with IgM antibodies.

7.1. Remove kit buffers (C, L, R, and W), polymer, and antibodies out of 4°C refrigerator or -20°C and equilibrate to Room Temperature (RT).

Note: Buffers and lanthanides are stored at 4˚C. Polymers are stored at -20˚C with provided desiccant in a sealed container.

7.2. Spin down antibodies (short spin ~10 seconds).

7.3. Perform a Nanodrop reading on each antibody being labeled to determine initial concentration.

7.3.1. Use the Protein 280 IgG setting.

7.3.2. Use 1X PBS pH 7.4 (± 0.2) as blank.

7.3.3. Use 2 µL of sample (blank and antibody).

7.3.4. Record all values obtained in lab notebook.

7.4. Load polymer with lanthanide into Centrifuge 1. Add buffer exchange and reduction of antibody into Centrifuge 2.

Note: Steps 7.5 and 7.6 should be completed simultaneously.

7.5. **Centrifuge 1 – Load polymer with lanthanide**

7.5.1. Spin polymer tubes for 10 seconds in mini centrifuge.

Note: Use 1 polymer tube per antibody if labeling 100 µg.

Note: Use 2 polymer tubes per antibody if labeling 200 µg.

7.5.2. Resuspend first polymer tube with 90 µL L-buffer and mix by pipetting.

Note: Use filter tips for all pipetting steps to prevent cross-contamination between metal stocks and reagents.

7.5.3. Transfer contents to second polymer tube and mix by pipetting (if labeling 200 µg).

7.5.4. Add lanthanide to polymer tube and mix by pipetting.

7.5.4.1. Use 10 µL for 200 µg and 5 µL for 100 µg.

7.5.5. Incubate at 37°C for 40 minutes.

Note: When there is 15 minutes remaining on the 40-minute incubation, begin centrifuge 2 – buffer exchange and reduction of antibody (Step 7.6).

7.5.6. After the 40-minute incubation, add 200 µL L-buffer to a 3kDa spin filter.

7.5.7. Spin tubes for 10 seconds at 12,000 RCF then add metal loaded polymer (from Step 7.5.4) to the 3kDa spin filter.

Note: For all centrifugation steps, orient spin columns so filters are facing the sides.

7.5.8. Spin at 12,000 RCF for 25 minutes at RT.

7.5.9. Discard flow through.

7.5.10. Add 300 µL C-buffer to the 3kDa spin filter.

7.5.11. Spin at 12,000 RCF for 30 minutes at RT.

7.5.12. Discard flow through, the sample is retained on the column.

7.6. **Centrifuge 2 – Buffer exchange and reduction of antibody**

7.6.1. Add 100 µg or 200 µg of antibody to a 50kDa spin column. The maximum volume is 200 µL. If volume is greater than 200 µL, add the required volume to the 50kDa spin column and spin at 12,000 RCF for 5 minutes to get a final volume of < 200 µL in the filter spin column.

7.6.2. Add 300 µL R-buffer to the 50kDa spin filter.

7.6.3. Spin at 12,000 RCF for 10 minutes at RT.

7.6.4. Discard flow through, the antibody will be retained on the column.

7.6.5. Add 100 µL of 4mM Bond Breaker TCEP/R-buffer and mix by gentle pipetting.

7.6.6. Incubate for 30 minutes at 37°C. **Do not exceed 30 minutes or the experiment will be invalid.**

7.6.7. Add 300 µL C-buffer to the 50kDa spin filter.

7.6.8. Spin at 12,000 RCF for 10 minutes at RT.

7.6.9. Discard flow through, the antibody will be retained on the column.

7.6.10. Add 400 µL C-buffer.

7.6.11. Spin at 12,000 RCF for 10 minutes at RT.

7.6.12. Discard flow through, the antibody will be retained on the column.

7.7. Conjugation

Note: Always check for remaining volume in spin column after centrifugation to ensure minimal remaining. If larger volume remains, spin column again. Also, do not to touch or damage the filter with the pipette tips during addition and mixing steps.

7.7.1. Resuspend to elute the polymer/lanthanide (from Step 7.5.12) by adding 60 µL C-buffer.

7.7.2. Transfer all the polymer/lanthanide mixture to the 50kDa spin filter containing the partially reduced antibody (from Step 7.6.12) then mix by pipetting.

7.7.3. Incubate at 37°C for 2 hours.

7.7.4. After the 37°C incubation, add 300 µL W-buffer.

7.7.5. Spin at 12,000 RCF for 10 minutes at RT.

7.7.6. Discard flow through.

7.7.7. Add 400 µL W-buffer.

7.7.8. Spin at 12,000 RCF for 10 minutes at RT.

7.7.9. Discard flow through.

7.7.10. Add 400 µL W-buffer.

7.7.11. Spin at 12,000 RCF for 10 minutes at RT.

7.7.12. Discard flow through.

7.7.13. Add 50 µL of W-E buffer with 0.5% azide.

7.7.14. Pipette to mix and rinse walls of filter.

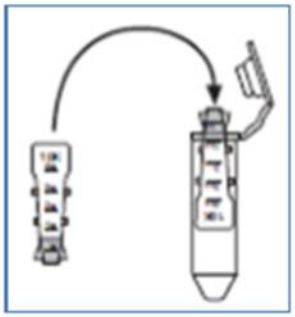
7.7.15. Invert the 50kDa filter over a clean collection tube.

7.7.16. Spin at 1,000 RCF for 2 minutes.

7.7.17. Remove the spin filter and wash the walls with 50 µL of W-E buffer with 0.5% azide.

Note: Dispense half of the volume down one side of the filter and the remaining volume down the other side of the filter. When removing the filter from the collection tube, only touch the sides of the filter with gloves.

7.7.18. Invert the filter over the same collection tube.



7.7.19. Spin at 1,000 RCF for 2 minutes at RT.

7.7.20. Measure volume in tube. It is expected the volume will be approximately 120 µL.

7.8. **Determine yield by Nanodrop using the protein 280 IgG setting**.

7.8.1. Use W-buffer as the blank.

7.8.2. Use 2 µL of sample (blank and antibody).

7.8.3. Record all values obtained in lab notebook.

7.8.4. Expected recovery is 60%.

Note: If the recovery is <60%, make note regarding low concentration.

7.9. **Final wash and storage in antibody stabilizer**

7.9.1. Using a new 50kDa filter and collection tube, add 250 µL of W-buffer + 120 µL sample from Step 7.7.20. **Do not invert filter over collection tube.**

7.9.2. Spin at 12,000 RCF for 10 minutes at RT.

7.9.3. Discard flow through.

7.9.4. Add half the calculated amount of antibody stabilizer (see Step 7.9.4.1), pipette to mix and rinse walls of filter.

7.9.4.1. The final concentration of antibody should be 0.5 mg/mL. Calculate the amount of antibody stabilizer needed using $C_1V_1=C_2V_2$.
C_1 = the concentration from the nanodrop reading in Step 7.8.
V_1 = 120 µL.
C_2 = 0.5 mg/mL.
Solve for
Once V_2 is obtained, subtract 20 µL from this value (to account for the volume of antibody) and then divide by 2 to get half of the calculated amount.

Note: If the final volume (V_2) is 20 µL or below, use 30 µL as half the calculated amount of antibody stabilizer.

7.9.5. Invert the 50kDa filter over a clean collection tube.

7.9.6. Spin at 1,000 RCF for 2 minutes at RT.

7.9.7. Remove the 50kDa spin filter and wash the walls with the other half of the antibody stabilizer.

7.9.8. Invert over the same collection tube.

7.9.9. Spin at 1,000 RCF for 2 minutes at RT.

7.9.10. Transfer antibody to 0.5 mL screw cap O-ring tube.

7.9.11. Label tube with antibody name, metal tag, concentration from final nanodrop read, date and technician initials.

7.9.12. Store antibody at 4°C for up to one year.

8. VERSION HISTORY

8.1. New SOP.