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Antibody Purification and Labeling V.1

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Protocol status: Working

We use this protocol and it's working

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Abstract

This protocol describes the process for antibody purification and subsequent labeling for direct immunofluorescence.



Materials

MATERIALS

⊗ 1X PBS (Phosphate-buffered saline)

⊗ Amicon Pro Purification System MXCO 100 KDa **Fisher Scientific Catalog #ACS510012**

Other Reagents (Antibody > 100 ug)

- Sodium Bicarbonate (Sigma-Aldrich, S5761)
- Invitrogen NHS Ester (Succinimidyl Ester)
 - Alexa Fluor 488 (Thermo Fisher, A20000)
 - Alexa Fluor 594 (Thermo Fisher, A20004)
 - Alexa Fluor 647 (Thermo Fisher, A20006)
- Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, D8418)
- Antibody Conjugate Purification Kit, 50-100 µg conjugate (Thermo Fisher, A33088)

Other Reagents (Antibody ≤ 100 ug)

- Invitrogen Antibody Labeling Kit
 - Alexa Fluor 488 (Thermo Fisher, A20181)
 - Alexa Fluor 594 (Thermo Fisher, A20185)
 - Alexa Fluor 647 (Thermo Fisher, A20186)

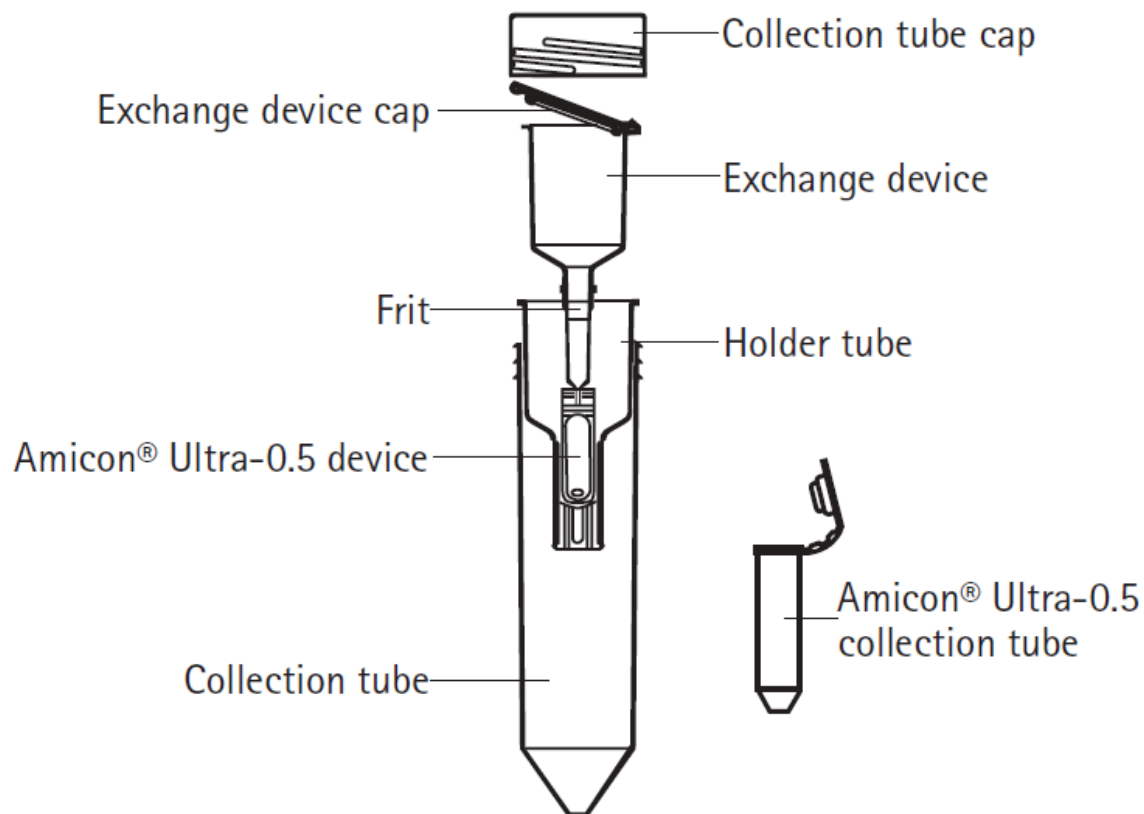
***All reagents necessary for labeling are included in labeling kit.**

Equipment:

- Centrifuge (swinging-bucket and fixed-angle rotors)
- Thermo Scientific Nanodrop 1000 Spectrophotometer (OR Nanodrop that reads the absorbance at 280 nm to give the concentration of purified proteins; contaminants/ buffers that absorb around 280 nm will affect protein concentration)¶

Antibody Purification

- 1 **For all preparations:** this is to remove BSA, azide, or glycine that are often added by the manufacturer. If the antibody has nothing added, skip the purification step.
- 2 Assemble Amicon Pro Affinity Concentrator by carefully attaching the Amicon Ultra device to the exchange device.



Amicon Pro Purification System.

- 3 Once the concentrator is assembled, remove the collection tube cap, lift the exchange device cap, and add 1 mL of 1X PBS to moisten the cellulose membrane of the Amicon Ultra device.
 - 3.1 This wash will ensure that the antibody does not stick to the membrane upon its addition.
- 4 Centrifuge at 4000 x g for 3 minutes.

- 5 Add desired amount of antibody to the exchange device.
- 6 Centrifuge for 10 minutes at 4000 x g in a swinging-bucket rotor.
- 7 Add 1 mL of PBS to the device and centrifuge again at 4000 x g for 10 minutes twice, discarding the flow through each time.
- 8 Collect purified antibody from the device by reverse spin.
 - 8.1 Place a collection tube on top of the Amicon Ultra-0.5 device. Make sure flow through has been removed from bottom tube.
 - 8.2 Invert the assembly and centrifuge in a fixed-angle rotor at 1000 x g for 2 minutes.
- 9 Use a nanodrop to measure the purified antibody at an absorbance of 280 nm.
 - 9.1 Make sure that the sampling arm on the nanodrop is up.
 - 9.2 Using a P10 pipette, add 1 μ L of the sample onto the lower measurement pedestal.
 - 9.3 Lower the sampling arm and measure the absorbance of the antibody at 280 nm (A280). The concentration should be given in mg/mL.
- 10 **If labeling 100 ug or less of antibody, stop here and follow procedure from Antibody Labeling Kit.**
- 11 **If you have more than 100 ug of antibody, continue to the next section.**

Labeling Preparation- Dye



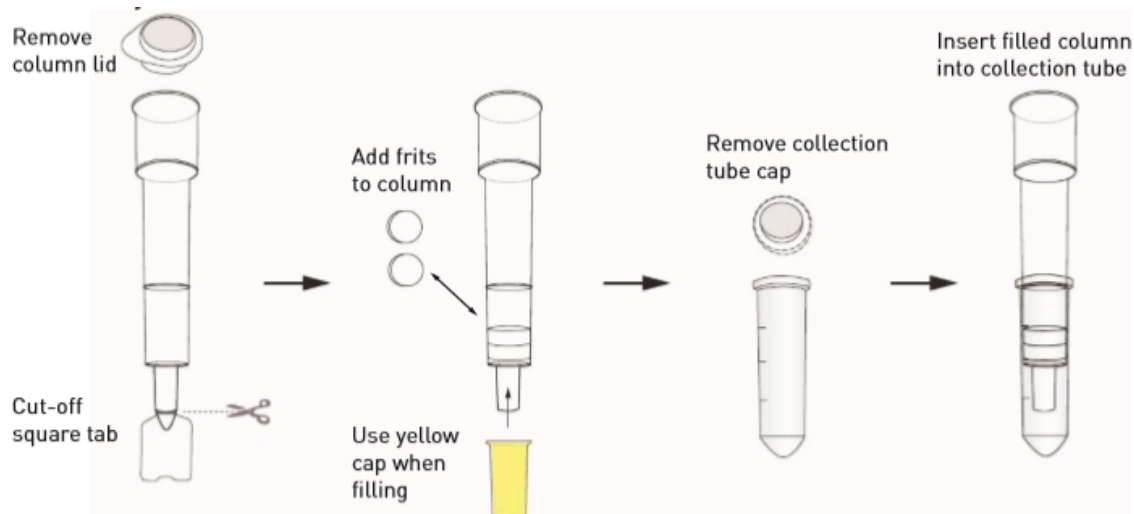
- 12 Dissolve amine-reactive compound (NHS ester) in DMSO or DMF at 10 mg/mL (i.e. 100 μ L of DMSO is added to 1 mg compound).
- 13 Mix the solution by vortex.
- 14 Aliquot solution into small Eppendorf tubes and store at -80C if all dye is not used during reaction.

Antibody Labeling

- 15 **For successful labeling, protein concentration should definitely not be less than 2mg/ml, ideally 5-20 mg/mL.**
- 16 **Antibodies should be in a buffer free of any amine-containing compounds (glycine, Tris, or ammonium ions) and stabilizing proteins (bovine serum albumin). These compounds will interfere with the labeling reaction.**
- 17 Add sodium bicarbonate to the antibody at a concentration of 0.1 M (i.e. 20 μ L of sodium bicarbonate per 200 μ g antibody) to make a slightly basic pH for the conjugation to occur.
- 18 Add 1 μ L of the chosen fluorophore NHS ester for each 200 μ g of antibody.
- 18.1 Labeling of antibody occurs by the Alexa Fluor dye linking to the primary amine (R-NH₂) in peptides and proteins.
- 19 Invert tube five times.
- 20 Wrap the tube in foil and incubate at room temperature for 60 minutes on a shaker.
- 20.1 Prepare the column for the purification of the labeled antibody during incubation step.

Purifying the Labeled Antibody

- 21 Assemble the spin column from the Antibody Conjugate Purification Kit.



Conjugate Purification Column



- 21.1 Remove the column lid.
- 21.2 Cut-off square tab at the bottom of the column.
- 21.3 Add both frits to the column and push them to the bottom of the column using a stir rod or P1000 pipette tip.
- 21.4 Use the yellow cap only when filling the column.
- 22 Stir the purification resin, then add 1 mL of the suspension into the column and allow it to settle by gravity.
- 23 Continue to add more of the suspension until the resin bed volume is about 1.5 mL.
- 24 Place the spin column in a collection tube, and place both in a 15 mL conical tube. Centrifuge at 1100 x g for 3 minutes.
- 25 Empty collection tube containing column buffer.



- 26 Add the sample to the center of the column, dropwise. Allow the solution to absorb into the resin bed.
- 27 Place the spin column into the empty collection tube and 15 mL conical tube.
- 28 Centrifuge at 1100 x g for 5 minutes.
- 29 Collect labeled antibody from the collection tube. Aliquot then store at 4°C short term, and -20°C long term.
- 29.1 **The conjugates can survive freeze thawing but you will need to evaluate each new antibody you use to make sure. We always evaluate one freeze/thaw cycle by repeating staining.**