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Antibody Purification and Labeling V.1

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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:

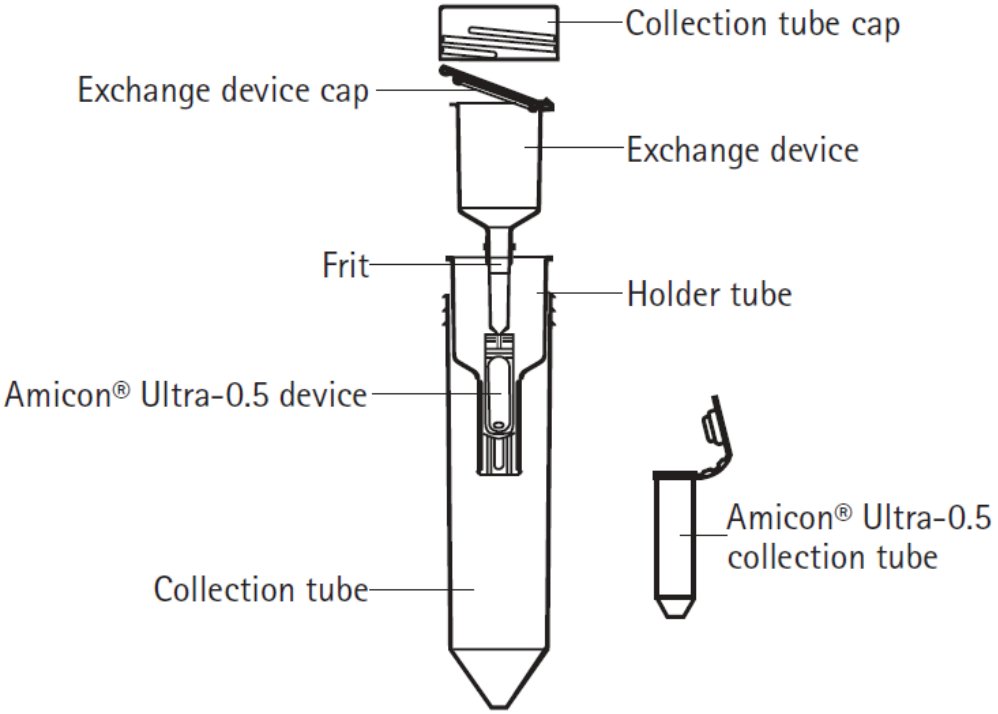
- Centrifuge (swinging-bucket and fixed-angle rotors)
- Thermo Scientific Nanodrop 1000 Spectrophotometer (OR Nanodrop that reads the absorbance at 280 nm to give the concentration of purified proteins; contaminants/ buffers that absorb around 280 nm will affect protein concentration)

Troubleshooting

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 µL 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 µL 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 µL of sodium bicarbonate per 200 µg antibody) to make a slightly basic pH for the conjugation to occur.

18 Add 1 µL of the chosen fluorophore NHS ester for each 200 µg of antibody.

18.1 Labeling of antibody occurs by the Alexa Fluor dye linking to the primary amine ($R\text{-}NH_2$) in peptides and proteins.

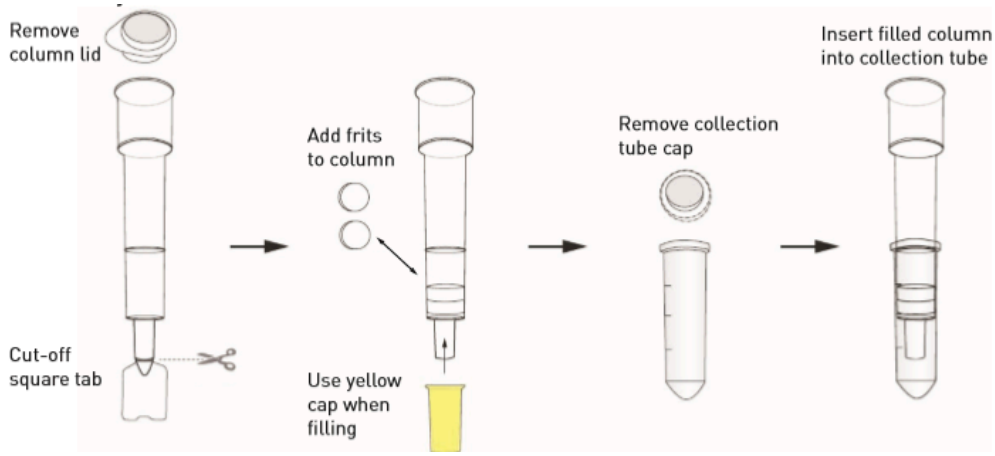
19 Invert tube five times.

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.**