PP062.1
Modified 6.27.23 by KCB

Purification of Native Rv1411c

Materials and Reagents:

1. GlycoLink™ Immobilization Kit (ThermoFisher Catalog number: 88941)
    a. GlycoLink column (10x1mL, Ultralink hydrazide resin in 50% slurry, 0.05% sodium azide)
    b. GlycoLink coupling buffer (0.1M sodium acetate, 0.15M sodium chloride, pH 5.5)
    c. GlycoLink wash buffer (1M sodium chloride, 0.05% sodium azide)
    d. BupH™ Phosphate Buffered Saline Pack, 1 pack, yields 0.1M phosphate, 0.15M sodium chloride; pH 7.2, when reconstituted with 500mL of water
    e. Aniline (Note 1)
    f. Sodium meta-periodate
    g. Zeba™ Spin desalting column (10x5mL, 7k MWCO)
    h. Column accessories, caps and lids
2. Culture filtrate Protein, 70% ammonium sulfate cut or Pooled ConA Elution from PP024 (Note 2)
3. Purified α-Rv1411c monoclonal antibody (Note 3)
4. Binding Buffer (phosphate buffered saline, pH 7.2, note 4)
5. Elution buffer (citric acid, sodium citrate buffer, 0.3M glycine, Note 4)
6. Neuralization buffer (1M Tris-HCl, pH 8.5, Note 4)
7. 10 mM Ammonium Bicarbonate
8. 3kDa Amicon 15 spin column
9. 15mL Falcon tubes
10. Eppendorf spin tubes
11. Aluminum foil

Protocol:
Preparing the antibody for coupling (oxidizing carbohydrate groups)- All spins unless further specified at performed at 1000g for 2 minutes

1._____ Allow the components of the GlycoLink Immobilization Kit to come to room temperature before proceeding, about 30 minutes.

2._____ Dilute or dissolve 1-10mg of antibody in Glycolink coupling buffer. Dilute samples at least three-fold into in coupling buffer to a final volume of 1 mL and a pH of less than 6. (Note 5)

3._____ Weigh 2.1 mg sodium meta-periodate in a small Eppendorf tube and add the 1 mL of purified antibody in GlycoLink coupling buffer.

4._____ Gently pipette until the meta-periodate is completely in solution

5._____ Wrap with aluminum foil to protect the solution from light and incubate at room temperature for 30 minutes. Do not exceed 30 minutes, longer incubation times may result in an over-oxidation of the antibody. At the end of 30 minutes of incubation, this tube is the oxidized antibody.

6._____ Break the bottom tab off of the 5 mL Zeba desalting column, remove the lid, and centrifuge to remove storage buffer.

7._____ Equilibrate the desalting column by adding 2mL of GlycoLink Coupling Buffer and centrifuge. Repeat this step two times.

8._____ Once the desalting column is equilibrated and spun, slowly apply the oxidized antibody solution to the center of the compact resin bed of the desalting column. Centrifuge and collect the oxidized antibody in a clean 15 mL falcon tube.

9._____ In a fume hood, prepare 0.2M aniline coupling catalyst in GlycoLink buffer by adding 18µL of aniline to 1mL of GlycoLink Coupling Buffer. Vortex GlycoLink Coupling Catalyst for 10 seconds. (Note 1)

10._____ Add the 1 mL volume of aniline coupling catalyst to the oxidized glycoprotein sample, resulting in a final concentration of 0.1M aniline. Gently mix by pipetting up and down. This 2 mL solution is the oxidized antibody in conjunction with the aniline catalyst that will bind the resin and antibody. At this point, a small 0.1 mL aliquot can be saved for determining coupling efficiency. (optional)

Coupling the oxidized antibody to the GlycoLink column- All spins are performed at 1000g for 2 minutes unless otherwise specified.

11._____ Suspend the resin in the GlycoLink Column by end-over-end mixing. To avoid drawing air into the column, sequentially remove the top cap and then the bottom tab.

12._____ Centrifuge the column to remove the storage buffer

13._____ Wash the column by adding 2mL of GlycoLink Coupling Buffer and centrifuge. Repeat this step once. Replace the bottom cap before adding antibody.

14._____ Add the oxidized antibody and catalyst in 2 mLs of GlycoLink Buffer as prepared in step 9.

15._____ Replace the top and bottom cap and mix gently with end-over-end mixing until the resin is completely suspended.

16._____ Place the column on a rocker and incubate for 4 hours at room temperature. When rocking, make sure that the resin is suspended and moving.

17._____ Remove the top and bottom cap of the column and centrifuge to collect the non-bound antibody. Save this flowthrough to determine the coupling efficiency with the oxidized antibody from step 9.

18._____ Wash the column with 2 mLs of GlycoLink coupling buffer and centrifuge, repeating this step for a total of 3 washes.

19._____ Wash the column with 2 mLs of Wash Buffer and centrifuge, repeating for a total of 3 washes.

20._____ Wash the column with 2 mLs of Elution buffer and centrifuge, repeating for a total of 3 washes.

21._____ Equilibrate the column for storage or binding by adding 2 mLs of PBS/Binding buffer and centrifuge. Repeat this step for a total of three washes.

22._____ If proceeding with binding and elution, proceed to step 25, making sure not to add any buffer after the last spin.

23._____ Store the column in 2 mLs of PBS/Binding buffer at 4°C or proceed with protein binding. If storing the column for longer than a week, add 0.05% sodium azide to the PBS used to store the column.

Binding and Eluting Rv1411c from Culture Filtrate Protein

24._____ Resuspend 50% ammonium sulfate cut CFP in binding buffer to a concentration of 5 mg/mL

25._____ If taking the column from storage at 4°C, allow the column to come to room temperature and centrifuge to remove the storage buffer. If removing buffer with sodium azide, wash, equilibrate, and centrifuge the column 3 times with binding buffer.

26._____ Add 2 mLs of the CFP in binding buffer to the resin bed.

27._____ Recap the bottom and top of the column and gently suspend the resin with the sample with end-over-end mixing.

28._____ Incubate the column with sample for 1 hour at room temperature while rocking.

29._____ Centrifuge the column, making sure to save the unbound protein for further purification. (Note 6)

30._____ Wash the column with binding buffer and centrifuge for a total of 3 washes.

31._____ In a fresh 15 mL Falcon tube, add 0.5 mL of neutralization buffer (Note 7)

32._____ Elute the bound protein by adding 1 mL of Elution Buffer to the top of the column and centrifuging.

33._____ Repeat the elution two more times for a total of 3 elution spins, collecting the flowthroughs together and into the neutralization buffer.

34._____ Concentrate and perform a buffer exchange into 10 mM ammonium bicarbonate with an Amicon 15, 3kDa

35._____ Quantify the purified protein by BCA (see SOP SP003).

36._____ Based on the resulting concentration, run 2 µg of protein on an SDS page gel (SOP SP007) and visualize by silver stain (SOP SP012) and Western blot (SOP SP011).

37._____ Aliquot or bulk freeze the purified protein and lyophilize for storage.

Notes:

1. Aniline is ***highly*** toxic, handle with care and use a fume hood.

2. See SOP PP024, the 70% supernatant cut is also used for the purification of native 38kDa. After eluting protein from the ConA column, pick fractions to pool for clean 38kDa and pool the rest, and buffer exchange into PBS for Rv1411c affinity purification. Either one can be done first, (though 38kDa done first is easier) make sure to <u>save all unbound protein before elution as it can be used to purify 38kDa.</u> If doing affinity purification first, take care to keep unpurified protein cold and in appropriate 10mM ammonium bicarbonate buffer whenever possible.

3. Purified monoclonal antibody is obtained from SOP AB100.6 followed by AB108.1. Hybridomas of the clone of choice are taken from liquid nitrogen, grown and scaled, and supernatant is harvested and purified.

4. Phosphate buffered saline- PBS is included in the GlycoLink kit, but separately bought phosphate buffered saline sufficient:
   0.1M phosphate
   0.15M sodium chloride
   pH 7.2

   Elution Buffer:
   46.5 mL of 0.1M citric acid
   3.5 mL of 0.1M sodium citrate
   2.25 g glycine (0.3M)
   Qs to 100 mL with MilliQ water
   pH 3

   Neutralization Buffer:
   1M Tris-HCl
   pH 8.5-9.0

5. For example, dilute 0.3 mL of purified antibody in PBS, TBS, or Melon Gel purification buffer into at least 0.7 mL of GlycoLink coupling buffer. The pH should always be below 6 or the coupling process

could be compromised. Alternatively, desalt to perform a buffer exchange into coupling buffer with a final volume of 1 mL.

6. This flowthrough should be kept, as it contains other proteins for further purification. This flowthrough can also be passed over the column again as many times as needed to extract and purify as much Mpt64 as possible. After Mpt64 purification is done, the flowthrough used in purification can be concentrated, buffer exchanged back into ammonium bicarbonate, and lyophilized for the starting material of other protocols.

7. Because the elution buffer has a pH 3, it is very important to bring the pH back up with neutralizing buffer so that the Mpt64 does not begin to degrade or denature. Always make sure there is neutralizing buffer at the bottom of your falcon tube before adding elution buffer to the column and spinning.

8. Adapted from ThermoFisher GlycoLink™ Protocol, Pierce Biotechnology, Number 88941