SOP: Purification of cNISTmAb from NISTCHO Cell Clarified Media Using Gravity Flow Protein A Chromatography

Approvals:

Preparer: Dr. Maggie Bryans Date: 01NOV2023
Reviewer: Hetal Doshi Date: 02NOV2023

1. **Purpose:**
   1.1. The isolation/purification of cNIST monoclonal antibody from NISTCHO conditioned media using gravity flow protein A chromatography.

2. **Scope:** This SOP can be used to isolate cNIST mAb in NISTCHO conditioned media for further analysis.

3. **Summary of Method:**
   cNIST monoclonal antibody is purified by affinity chromatography using a protein A-agarose column run on gravity flow. The protein A column is equilibrated and cNIST mAb-containing conditioned media is loaded onto the column. The column is washed, then eluted using a low pH buffer. Antibody containing fractions are collected and the column resin regenerated for further use.

4. **Responsibilities:**
   4.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   4.2. It is the responsibility of the students/technician to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

5. **References:**
   5.1. SOP: NISTCHO Shake Flask Batch Culture for Monoclonal Antibody Production
   5.2. Protein A IgG Purification Kit, User Guide, Thermo Scientific,
   5.3. Tech Tip #7: Remove air bubbles from columns, Thermo Scientific, Thermo Fisher.com

6. **Precautions:**
   6.1. Routine care should be exercised in the handling of buffers and samples of biological materials, which may have harmful biological activity in the case of accidental ingestion, needle stick etc.
   6.2. Gloves, a lab coat and protective eyewear should be worn when handling buffers and samples.

7. **Materials:**
   7.1. 1ml protein A gravity column (Protein A IgG Purification Kit, Thermo Scientific Catalog # 44667
   7.2. 50 ml of Neutralization Buffer, 1M Tris, pH 9

7.3. 20% Ethanol for column storage
7.4. Sterile Filtered Milli Q water
7.5. 5ml microfiltered NISTCHO Conditioned Media, from a culture with a cell density of 8-14 x10^6 cells/ml
7.6. A ring stand and clamp to support the column
7.7. 2 ml microfuge tubes and rack
7.8. 2 – 15 ml conical tubes
7.9. P1000 pipettor and tips or transfer pipettes.
7.10. 0.2µm syringe filter and syringe
7.11. Spectrophotometer for measuring Absorbances at 280nm
7.12. pH meter
7.13. Binding Buffer
7.14. Wash Buffer
7.15. Elution Buffer

8. Procedure:

Notes:

- Always remove the top screw cap from the column first and then the remove the bottom cap. This is to prevent an air bubble from being drawn in to the column resin.
- Each column has a disc above the resin bed. This disc will help prevent the column from drying out. The column flow will stop when the buffer reaches the disc. Do not leave the column without buffer above the disc for more than a few minutes.

8.1. Preparation of Neutralization buffer
8.1.1. Prepare 50ml of a 1M Tris solution and adjust the pH to 9.0
8.1.1.1.Weigh 6.057g of Tris and dissolve in 35 ml of DI water.
8.1.1.2. Adjust the pH to 9.0 using 5M HCl.
8.1.1.3. Adjust the volume to 50 ml with DI water.
8.2. **Equilibrate the protein A column and all buffers to room temperature.**
8.3. Prepare the sample
8.3.1. Collect 5ml of pre filtered NISTCHO conditioned media (see appropriate SOP). If the sample was stored after harvest it should be clarified either by centrifugation, (several minutes at 10,000 x g) or filtration, (using a 0.2µm filter).
8.4. Equilibrate the protein A column
8.4.1. Remove the top cap from the column and using p1000 to discard the storage solution into a suitable waste container, the filter disc above the resin will prevent the resin from being discarded.
8.4.2. Equilibrate the column by adding 4ml of binding buffer to the top of the column and allowing it to flow through. Collect the buffer volume added in a multiple 1.5 ml microfuge tubes labeled equilibration.
8.4.3. Add 1 ml of binding buffer to the column. Replace cap and stopper
8.5. Apply the prepared conditioned media to the column
8.5.1. Once the level of binding buffer used to equilibrate the column reaches the level of the disc at the top of the column begin adding the prepared sample to the top of the column.

8.5.2. Continue adding sample to the top of the column and collecting 2 ml fractions until all 5ml of sample has been added and 5 separate 2 ml fractions have been collected. Label these tubes Flow Through 1, 2, 3, 4, and 5, save these fractions for further analysis.

8.6. Wash the column

8.6.1. Begin adding, 6 ml of binding buffer to the top of the column 2-3mls at a time and collect the flow through in 2 ml fractions.

8.6.2. Continue adding binding buffer to the column and collecting 2 ml fractions until all 6 ml of wash has been added to the column and the liquid level in the column is just above the disc at the top of the resin. Label these fractions as Wash 1, 2, and 3. Collect any additional wash volume in an additional tube Wash 4 so that the level of buffer in the column is just above the disc at the top of the resin. Save the wash fractions for additional analysis.

8.7. Elute the bound IgG from the column

8.7.1. Begin adding 5 ml, (5 column volumes), of elution buffer to the top of the column.

8.7.2. Collect the eluate in 5 - **1ml fractions**. Label these fractions as Elution 1, 2, 3, 4, and 5.

8.7.3. As each of the elution fractions is collected add 50µl of neutralization buffer to the fraction tube and flick the tube to mix.

8.7.4. Once 5 elution fractions have been collected carefully stop the flow by capping the bottom of the column. Allow the bottom cap to fill with a drop of buffer from the column and then gently slide the cap onto the column. Make sure there is elution buffer above the level of the disc at the top of the resin, if needed add 1ml of elution buffer to the top of the column. Place the top cap on the column.

8.8. Determine which Eluate fractions contain IgG

8.8.1. Measure the Absorbance at 280nm of each of the flow through, wash and elution fractions.

8.8.2. Prepare a blank by transferring 1ml of elution buffer mixed with 50µl of neutralization buffer.

8.8.3. Read 2ul of each fraction on the Nanodrop instrument.

8.8.4. Record the absorbance of each fraction at 280nm and the concentration by selecting the IgG function

8.8.5. The absorbance value of the final elution fraction should be the in line with that of the buffer blank

8.8.6. Place the elution fractions on ice or store at 4˚C.

8.8.7. Measure the Absorbance at 280nm of each of the flow through and wash fractions using the Nanodrop using binding buffer as the blank for these fractions

8.8.8. Record the absorbance of these fractions and determine the concentration using the A of 1 = 1mg/ml, general protein function

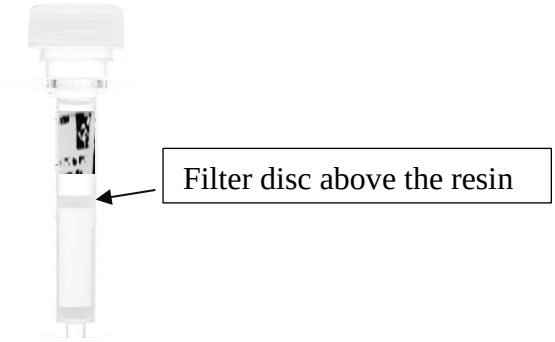
8.9. Regenerate the column

8.9.1. Remove the top cap and then the bottom cap from the column.

8.9.2. Wash the column with an additional 5ml of elution buffer. Collect this wash in one tube and discard.

8.9.3. To store the column, add 5ml 20% Ethanol in sterile filtered DI water to the column and stop the flow when there is about 2ml above the resin. Cap the bottom of the column and then cap the top of the column. Store the columns upright at 4˚C

9. Attachment



10. History:

Name	Date	Amendment
Maggie Bryans	01NOV2023	Initial release