## Overview and Considerations:

This procedure can be used to purify all of the antibodies that recognize GlcNAc. This procedure is inappropriate for antibodies which recognize GlcNAc and the underlying peptide backbone, such as RL2.

## Reagents:

1. GlcNAc-agarose or GlcNAc-sepharose (EY labs, CA, USA, Catalog # CG-003-5; Sigma-Aldrich, Catalog # 01143; *This is typically kept in the deli case with the other resins*).
2. Column housings, such as BIORAD (Hercules, CA) ECONO-columns or disposable ECONO-PAC columns.
3. Phosphate buffered saline (PBS): 10mM phosphate buffer, 136mM sodium chloride, 2.6mM potassium chloride.
4. 0.45uMfilters (non-sterile), this will either be a syringe filter or a bottletop filter. Which filter will depend on the volume of starting material..
5. 1M Tris-HCl pH 8.0.
6. CTD110.6 ascites or cell culture supernatant
7. Elution buffer: 1M GlcNAc in PBS.
8. 0.05% w/v sodium azide in PBS.
9. Dialysis equipment.
10. 80% v/v glycerol in water, autoclaved.

## Protocol:

1. Prepare antibody, note the method will depend on whether you are using ascites or cell culture supernatant.
   * **Ascites:** Defrost on ice, dilute 10 fold with PBS, centrifuge at >10 000xg for 20min at 4oC, decant supernatant and filter through a 0.45uM filter. Adjust the pH to pH7-8 with 1M Tris pH8.
   * **Cell Culture Supernatant:** Pellet hybridoma cells at 3000xg. Decant supernatant, add sodium azide to 0.01% and adjust the pH to pH7-8 with 1M Tris pH8. Filter though a 0.45uM filter into a sterile bottle.
2. Pack 16mL of GlcNAc-sepharose slurry (50% v/v) in a column housing (BIORAD econocolumn), resulting in a column with a 8mL bed volume. This should be sufficeint to bind 4mg of antibody, which is the approximate yield from 1ml of ascites or 250mls of cell culture supernatant. To avoid developing air bubbles in the column bed, fill the column with water before applying the resin.
3. Typically we don't run out columns as an open gravity system, we close the system and use syphoning to draw the buffers/antibody over the column. Place a two way valave on the bottom of the column, and a three way valve on the top of the column. Run a line to a bottle of buffer on a shelf above the column.
4. Wash the column with a minimum of 10 column volumes (~200mL) of PBS, with a flow rate of 0.5-1mL/min.
5. Apply the antibody to the column with a flow rate of 0.2-0.5mL/min. Typically, we do this in an organce capped conical centrifuge tube (volume 250mls). Collect the material that flows through the column, designate this fraction as the “flowthrough”.
   * For ascites, Reapply the “flowthrough” to the column, save the material that flows through the column.
6. Wash the column with at least 10 column volumes of PBS (~200mL).
7. Elute the bound antibody from the column by adding 15x2mL of GlcNAc elution buffer.
8. Collect 2mL fractions, designated “eluant 1-15”.
   * Before proceeding to the next step, measure the protein concentration to ensure that all of the antibody has eluted. If not, elute with 5x2ml of GlcNAc.
9. Wash the column with at least 5 column volumes (~10mL) of PBS.
10. The column should be stored in PBS with 0.05% w/v sodium azide.

### What do I do with my antibody fractions?

1. Combine fractions containing antibody
2. Dialyze against buffer, which buffer depends on your application:
   * PBS: Antibody for westerns and coupling to fluorescent ligands
   * 200mM Sodium Bicarbonate, 0.5M NaCl, pH8.3: Coupling to CNBR sepharose
   * Remember, the volume of buffer to sample needs to be at least 100:1
3. Dialyze in the cold room, with mixinfg, for 2-3h.
4. Change the buffer, and dialyze overnight.
5. Recover the antibody from the dialysis tubing, and concentrate by spin filtration.

### Testing the antibody fractions

1. In a 96 well plate (see attached file)
   * 50ul of positive control antibody
   * 50ul of start material
   * 50ul of flowthrough
   * 50ul of wash
   * 50ul of each fraction
2. In the remaining wells, place 40ul of PBS
3. Use the 12 well multichannel to make 1/5 serial dilutions, by adding 10ul to the next well
4. Dot blot the dilutions onto a piece of nitrocelluloase
5. Dry the nitrocellulose for ~10min
6. Wash in TBS for 10min
7. Block in 3% Milk in TBST, 20min
8. Wash 3x5min in TBST
9. Apply secondary antibody, for CTD anti-mouse IgM, 1/5000 in 3% Milk in TBST, for 30 minutes
10. Wash 3x5min in TBST
11. Wash 1x5min in TBS
12. Perform ECL