

3B5H10 F_{ab} purification:*Overview:*

- I) Day 1: Digest IgG (o/n)
- II) Day 2: Dialyze into ion-exchange binding buffer (o/n)
- III) Day 3: Equilibrate, run ion exchange column (2 1/2 hrs)
- IV) Day 3: Coomassie collected fractions from ion exchange
- V) Day 3: Run Gel Filtration column (o/n)
- VI) Day 4: Coomassie collected fractions from gel filtration
- VII) Day 4: Concentrate collected Fab
- VIII) Day 5: Dialyze Fab
- IX) Day 5: Bradford Protein Assay

Note: The following method is for 1 batch of a 40 mg 3B5H10 IgG which will yield about 5 mg Fab. If more Fab needed, 2 batch can be run at parallel. The Q Fab column should be run back to back 4x, while we have 2 Superdex 200 column that can be run at parallel at the same time.

(Day 1)

I) Digestion of intact IgG (affinity purified)

Digest buffer: 0.1M TrisHCl pH 8.0, 2mM EDTA

Use papain at 8.39 U papain/40 mg 3B5H10. Note: it also better if the papain stock activity is about 20 U/mg. Higher activity unit will give overdigestion that in turn will give poor yield.

- A) Activate papain in 10mM L-cysteine
 - i. Make 1M L-cysteine stock fresh (0.061g in 500ul of digest buffer)
 - ii. Example for papain stock 23.8 U/mg, 36.5 mg/ml. For 40mg IgG, add 9.6 μ l papain to 1ml tris/EDTA + 10.2 μ l 1M L-cysteine stock
 - iii. Incubate at 37C for 15 minutes

Notes:

- Actual volume of papain depends on concentration of papain in bottle, which varies from batch to batch. So check concentration, and add papain at ratio of unit papain to amount of antibody to be digested.
- Keep papain on ice throughout, and be consistent when pipetting the latex solution (wipe excess with Kimwipe if necessary).
- Mix papain thoroughly by pipetting up and down.
- Final L-cysteine concentration is 10mM

B) Prepare antibody

- i. Transfer desired amount of Ab to fresh tube (40mg)
- ii. Immediately before adding papain, supplement with L-cysteine (L-cysteine functions as a reducing agent to maintain a thiol group in the active site of papain)

Notes:

- As of 9/07, 3B5H10 volumes for above steps are 6mL. Thus 23 μ l L-cysteine stock (1M) is added to IgG. Use 50mL conical tube.

- Mix L-cysteine in thoroughly.
- Final L-cysteine concentration of antibody and papain should be 5mM, so calculate what the L-cysteine concentration of the antibody alone should be to produce the 5mM final. With the above volumes, the antibody L-cysteine concentration should be 4.5mM.
- If digest appears incomplete or the Fab2 band remains prominent, raise the final L-cysteine concentration (try 7.5mM or even 10mM)
- If troubleshooting on small scale digests, the L-cysteine must be pHed to ~7 before using or else it won't work. Generally, the small scale digests don't always faithfully mimic the large scale ones.

- C) Add papain to IgG
- Add the entire volume (~1ml) of activated papain
 - mix, parafilm and incubate at 37C for **18 hrs.**

Notes:

- Final L-cysteine concentration will be 5mM.
- Mix thoroughly.

- D) Kill digest
- Centrifuge tube briefly
 - Add iodoacetamine to 20mM final, incubate 30 min at 4C
 - add 1:25 stock of 0.5M (0.058g in 0.625mL water, powder located in cold room dessicator) light sensitive (liquid at rt, powder at 4C)
 - if brown, make fresh stock solution

Notes:

- For the above stated volumes, add 240 µl of iodoacetamide.

(Day 2)

II) Dialysis into ion exchange (Q) column binding buffer

Binding Buffer: 5mM Tris (base) pH 8 (stock at 10x = 50mM)

- Transfer digested protein to Slide-a-lyzer cassette
 - Use syringe for transfer, remove all air
 - Mark corner used, float that corner at top and don't reuse
- Place cassette in 4L of buffer
 - Incubate overnight, with stirring rod turning slowly
 - Change buffer twice (total of 3x4L), evening and morning at minimum

(Day 3)

III) Ion-exchange Column Chromatography (Hi-trap Q sepharose FF)

Binding Buffer/Buffer A: 5mM Tris (base) pH 8

Elution Buffer/Buffer B: 5mM Tris (base) pH 8 + 1M NaCl

Max flow for column: 1ml/min

1 ml bed volume

Washes/equilibration done manually – want to wash with 5 column volumes minimum

Run Biocad program under Method/Kartika/QFab

- Equilibration

Run 2 column volume of Buffer A

B) Sample Loading

- a. SAVE 5ul digested material for Coomassie
- b. Filter the sample with 0.2 μ M syringe filter
- c. Inject sample 2 x 3000 μ l with 5 ml sample loop.

C) Ion-exchange separation

- a. Run 1 column volume of Buffer A
- b. In 35 column volume, set gradient to 40% Buffer B
- c. In 2 column volume, set gradient to 90% buffer B
- d. In 2 column volume, maintain the gradient at 90% buffer B
- e. In 2 column volume, set gradient to 100% Buffer A
- f. In 10 column volume, maintain the gradient at 100% Buffer A

(Day 3)

IV) Coomassie for ion-exchange column fractions

A) 10% acrylamide gels, load 5ul add 5ul 2x LSB *with NO BME*, Coomassie stain: ~10 min is usually adequate

- a. at minimum, load:
 - i. starting Fab peak fractions
 - ii. one max peak fraction
 - iii. ending Fab peak fractions (to look for overlap with degraded F_c)
 - iv. digested material (input)
- b. optional:
 - i. initial peak that comes off

B) Pool collected fractions to load on size exclusion column

- a. exclude fractions with degraded F_c stringently
- b. optional: re-run fractions with both F_{ab} and F_c on Q column (combined with fractions containing high MW species and Fab from size exclusion column – see below)

Equilibrate: Day (3)

V) Size-exclusion Column Chromatography

Column: Superdex S-200 (GE Healthcare)

Running buffer: same as binding buffer for Q column: 10 mM Tris pH 7.4, 0.25 mM EDTA, 0.15M NaCl, 0.05% Na azide. Prepare at least 1.8 L binding buffer. Degas the buffer.

Max flow rate: 1.0 ml/min

Matrix volume: 400mls

Suggested sample loading volume: 6-7 ml. Concentrated the sample with Amicon Ultra-4 centrifugal filter device, 10,000 MW cutoff.

A) Equilibration

- a. Equilibrate the column with at least 3 column of binding buffer. Since the column is stored in this buffer too, the column should be equilibrated. But the column should be cleaned with 1 M NaOH periodically to avoid bacterial contamination.

B) Loading sample / Size Separation

- a. Connect the column to peristaltic pump, the UV detector, and fraction collector, i.e. connect the buffer to peristaltic pump input, peristaltic pump output to column input, column output to UV detector and fraction collector.
- b. Collect 5 ml/fraction, set up to collect 120 tubes during the overnight run. Set the blue pin to 20 mV and red pin to 50mV. Set the peristaltic pump output speed to be 1 ml/min.
- c. Inject sample into loop at 1 ml/min, followed by binding buffer
- d. At end of run, transfer fractions to 4C, save chart

VI) Day 4: Coomassie for Size-exclusion Column Fractions

A) 10% acrylamide gels, load 8ul + 8ul 2x LSB (*NO BME*), Coomassie stain: ~10 min is usually adequate

- a. Most important are peak starting fractions (to separate high MW specie – Fab2)

VII) Day 4: Concentrate Size-exclusion Column Fractions

Pool fractions, concentrate using Amicon Ultra; max volume 15mls; concentrates to 2-3ml ,max 2500 rpm. The final yield for 40 mg starting 3B5H10 is about 4-5 mg Fab.

VIII) Day 5: Dialysis

Dialyze (at least 1x 4L) the concentrated Fab to 5 mM Tris pH 8. Note: This is important because Fab used for crystallization need to be salt free. Store at 4⁰C.

IX) Bradford Protein Assay

A) 2, 5 ul of purified, concentrated protein added to 2 ml 1x Bradford reagent

- a. Usually obtain protein between 2-3 mg/ml
- b. Bradford Reagent – BioRad (5x stock at 4C)

B) Standard curve for BSA (2.5-20mg/ml in 2.5 mg/ml increments; 2x samples at each concentration)

C) Read at 595nm

APPENDIX: Notes about running separation columns:

- Two things ruin columns:
 - 1) Air in the column (prevent bubbles from entering column, degas buffers)
 - 2) Aggregated material in the column (clear lysates)

- Changing columns:

When moving inputs, stop pump

 - 1) Disconnecting column
 - a. Disconnect output of connected column first
 - b. Clamp and parafilm the end of the tubing to avoid bacterial contamination.
 - 2) Connecting column
 - a. Connect top first – open top
 - b. Allow enough fluid to drip in from input so that top is full
 - c. Connect top connection, remove bottom tubing connection
 - d. Again, when liquid is coming out, connect to output

- Storing and Equilibrating Columns:
 - Store column in binding buffer containing 0.05% NaN₃
 - Equilibration should be with 5x matrix volume of column at minimum