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Cell DIVE™ Platform | Antibody Purification Chemistry

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1 Works for me dx.doi.org/10.17504/protocols.io.bpx9mpr6

Human BioMolecular Atlas Program (HuBMAP) Method Development Community | GE Research

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ABSTRACT

The purpose of the protocol is to purify antibodies that will be conjugated to Cy dyes as per the Cell DIVE™ technology. Affinity chromatography will be used to remove impurities from the vendor antibody to enable conjugation.

ATTACHMENTS

Cell_DIVE-manual-Ab-purification_chemistry_final_version.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

antibody, antibody purification, affinity chromatography

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GUIDELINES

Definitions

A	B
Definition/Acronym	**Definitions**
BSA	Bovine Serum Albumin
PBS	Phosphate Buffered Saline
dH20	Distilled water
nm	nanometers

Descriptions

This procedure is to purify an antibody using HiTrap columns. Purified antibody in PBS buffer is essential for conjugation, hence any additives that may interfere with conjugation have to be removed prior to conjugation following the guidelines in this document.

MATERIALS TEXT

Required Materials

A	B
Material	**Definitions**
Ring stand	Holds HiTrap column for purification
20G Needle	Used to aid in drawing solutions into syringe
20G Blunt Cannula	Used to aid in drawing solutions into syringe
5 mL Syringe	Used to apply solutions to Hitrap column
Pipettes (2-1000uL)	Used to measure liquids
Pipette Tips (0.5-1000uL)	Used for pipettes
Nitrile Gloves	PPE
Amicon Ultra-4 30K centrifugal filtration devices	Used for Buffer Exchange and Concentrations of Antibodies
15 mL Conical Tube	Used for collecting flow through of washes
50 mL Conical Tube	Used to mix working solutions
Eppendorf Tubes	Used for collecting wash, elution, and purified antibody fractions
Kimwipe	Used to wipe Nanodrop pedesta

Required Reagents

Please follow the proper disposal for all reagents.

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A	B
Reagent/Media/Controls/Probes	**Definitions**
HiTrap Protein A HP Column (GEHC, 17-0402-01)	• Quantity: Pack of 5 • Composition: Recombinant Protein A in 6% agarose • Storage: 4°C • Expiration date: Do not use after date stamped on manufacturer's label
HiTrap Protein G HP Column (GEHC, 17-0404-01)	• Quantity: Pack of 5 • Composition: Recombinant Protein G in 6% agarose • Storage: 4°C • Expiration date: Do not use after date stamped on manufacturer's label
Antibody Buffer Kit (GEHC, 28-9030-59)	• Quantity: 50mL 10x Binding Buffer, 15mL 10X Elution Buffer,25mL Ready To Use Neutralizing Buffer • Composition: o 10x Binding Buffer – 0.2 M sodium phosphate, pH 7.0 o 10x Elution Buffer -1 M glycine-HCl, pH 2.7 (liquid) o 1x Neutralizing Buffer –1 M Tris-HCl, pH 9 • Storage: 4°C • Expiration date: Do not use after date stamped on manufacturer's label
10X PBS (Sigma, P7059)	• Quantity: 1L • Composition: 10X concentrate phosphate buffered saline • Storage: Room Temperature (liquid) • Expiration date: one year from date of receipt
Sodium Azide 0.65% (Sigma, P5493)	• Quantity: 1mL • Composition: 0.1M Sodium Azide in aqueous buffer (liquid) • Storage: 4°C • Expiration date: Do not use after date stamped on manufacturer's label
CF-1 (K2Cr2O7) NanoDrop 2000C Calibration Kit (Thermo Fisher,(CF1)	• Quantity: 6 vials per pkg, 1ul each • Composition: Potassium dichromate .07-.10%, perchloric acid .02-.04%, water 99.8% • Storage: Room Temperature • Expiration date: Do not use after date stamped on manufacturer's label
PR-1 Pedestal Reconditioning Kit (Thermo Fisher, PR-1)	• Quantity: 25 test swaps and 10ml of paste • Composition: reconditioning compound & 25 convenient swabs • Storage: Room Temperature • Expiration date: Do not use after date stamped on manufacturer's label
Ethanol 200 Proof (ordered as Doe & Ingalls, Catalog#: P111000200CSGL, labeled as Pharmco)	• Quantity: 1 gallon • Composition: Pure, 200 Proof Ethanol • Storage: Room temperature in a flammables cabinet • Expiration date: Do not use after date stamped on manufacture's label.

Required Equipment

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A	B
Equipment	**Definitions**
NanoDrop	Measure protein absorbance at A280
Microcentrifuge	Used for spinning antibodies down prior to purification
Centrifuge	Used for buffer exchange and concentration of purified antibodies
Vortex	Used for mixing of solutions
NanoDrop Computer	Drives Nanodrop program

Reagent Prep

Please follow the proper disposal for all reagents. Also, label all reagents with the appropriate name, date made, expiration date, and safety label.

A	B
Reagents	**Definitions**
1X Binding Buffer	• Quantity: 50mL • Composition: 5 mL 10X Binding Buffer; 45 mL dH2O • Storage: Room Temperature • Expiration date: one month from date of preparation • Prior to making, remove 10X buffer from 4°C and allow to equilibrate at room temperature for approximately 30 minutes. Mix solutions in 50 mL conical tube. • Complete Reagent Prep Record (FORM-0634) • NOTE: The 10X binding buffer can develop a precipitate upon storage at 4 °C. Ensure that the buffer has warmed to room temperature and all the solids have been dissolved before diluting with water.
1X Elution Buffer	• Quantity: 50mL • Composition: 5 mL 10x Elution Buffer; 45 mL dH2O • Storage: Store at 4°C • Expiration date: One month from date of preparation • Mix in a 50 mL conical tube • Complete Reagent Prep Record (FORM-0634)
1X Neutralization Buffer	• Quantity: 25mL • Composition: 1 M Tris-HCl, pH 9 • Storage: Store at 4°C • Expiration date: One month from date of preparation
Neutralized Elution Buffer	• Quantity: 1000ul • Composition: 60uL 1X Neutralization Buffer; 940uL Elution Buffer • Storage: Store at Room Temperature • Expiration date: One month from date of preparation • Mix in a 1.5 mL eppendorf tube
Column Storage Solution	• Quantity: 50 mL • Composition: 10 mL Ethanol; 40 mL dH2O • Storage: Store at Room Temperature • Expiration date: One month from date of preparation • Mix solutions in a 50 mL conical tube

SAFETY WARNINGS

Warning: For research use only.

Cell DIVE software and workflows are for internal research use only and not for third party service use or clinical

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diagnosis. Do not use internally or externally in humans or animals. All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.

BEFORE STARTING

Please take particular note of the following instructions regarding critical steps:

- It is essential to read the complete instruction booklet before starting work.
- Unless noted, it is essential to allow reagents discussed to reach room temperature prior to use.
- Mix samples and all reagents thoroughly before use.
- Avoid extensive exposure of fluorescent reagents to ambient light.

Evaluate Need for Purification as per Formulation

1 To determine the formulation of the vendor antibody solution refer to the vendor data sheet for product specifications.

Refer to Table 7.1, for additives that require the antibody to be purified.

A	B	C	D
Formulation	**Additive**	**Buffer**	**Needs purification**
Tissue culture supernatant	N/A	PBS	Yes
Ascites Fluid	N/A	PBS	Yes
Purified	Stabilizer e.g. BSA	PBS	Yes
Purified	Contains both BSA and Glycerol	PBS	Dilute glycerol content to 10% (5% preferred) before purification
Purified	Stabilizer e.g. Trehalose	PBS	No
Purified	Stabilizer e.g. Gelatin	PBS	Yes
Purified	Any amine e.g. glycine etc.	PBS	If amine is low molecular weight - Buffer exchange using Amicon filters
Purified	N/A	TRIS	Buffer exchange using Amicon filters
Purified	Glycerol	PBS	Buffer exchange using Amicon filters
Purified	Azide (up to 0.1%)	PBS	No
Purified	N/A	PBS	If concentration below 0.5 mg/mL, concentrate using Amicon filters

Table 7.1: Additives in Vendor Antibody Formulations

Column Equilibration

2 Remove the Protein A or Protein G column from **4 °C** and allow it to equilibrate to room temperature (~1/2hr).

Protein A is used to purify rabbit antibodies. Protein G is used to purify all mouse IgG. For purification of antibodies from other species, please consult the product insert.

3 Screw on the red luer lock adapter in to the top of the column and break off the bottom tab.
- If necessary, open up the hole with a clean, sharp blade by cutting off the tip.

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4

Wash the column with **10 mL dH2O** at a flow of 1-2 drops/second.

- Using a syringe, draw **10 mL dH2O** .
- Attach syringe to the top of the column.
- Gently push down on the syringe to deliver at flow rate of 1-2 drops/second.
- Collect flow through in a waste beaker.
  o Discard any flow through liquid

5

In order to equilibrate the column, wash with **5 mL 1X Binding Buffer** at a flow of 1-2 drops/second.

- Using a syringe draw up **5 mL 1X Binding Buffer** .
- Attach syringe to the top of the column.
- Gently push down on the syringe to deliver 1-2 drops/second flow rate.
- Collect flow through in a waste beaker.
  o Discard any flow through liquid

Antibody Application to the HiTrap Column

6 Remove the unpurified antibody from the **4 °C** .

7 Retain **5 µg unpurified antibody** for troubleshooting purposes.

8 Use a 5mL syringe to remove the remaining volume of un-purified antibody from the antibody vial.

- Use a small gauge needle (23g, usually) to reach the bottom of the container if needed.
- Retain antibody solution in the syringe.

9 To remove the residual antibody, rinse the antibody vial with **500 µl 1X Binding Buffer** .

- This step is only done if you are not returning the retained 5µg of unpurified antibody (step 7) to the original vial.
- If you are returning the retained 5µg of unpurified antibody (step 7) to the original vial, use a separate Eppendorf vial to draw up the 1X binding buffer from - so as to not contaminate the stock vial.

10 Using the same 5 mL syringe, remove the wash volume from the antibody vial.

- See notes on the preceding step.

11 Remove the needle, invert the syringe and remove any air bubbles by gently flicking the sides of the syringe.

12 Apply the antibody sample to the column at a rate of 1-2 drop/second, collecting flow through liquid in a 15 mL conical tube.

- Minimize the amount of air pushed through the column

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- Save the flow through for potential troubleshooting.
- Discard after 24 hours - typically discarded after adequate recovery of the antibody is shown.

Wash Unbound proteins from the HiTrap column

13 Prepare five 1.5 mL clear eppendorf tubes by labeling them W1-W5 (Wash 1-Wash 5).

14 Measure **5 mL fresh 1X Binding Buffer** in a new 5 mL syringe.

15 Apply the 1X Binding Buffer to the column at a flow rate of 1-2 drop/second.

16 Collect **1 mL fractions of the wash** in the labeled tubes starting with W1 and proceed in sequential order.

17 Vortex each wash for **00:00:05** . Briefly centrifuge to remove any liquid from the underside of the cap and upper 5s parts of the tube.
- An alternate to vortexing is to invert the tube several times, flicking the bottom to ensure good mixing. This is more gentle on the antibody.

Use the NanoDrop to confirm the complete removal of unbound protein

18 Open the Nanodrop program from the computer connected to the Nanodrop.

19 From the main screen select “Proteins A280” program.

20 Within the “Protein A280” program, select Type BSA, refer to Figure 7.5.1

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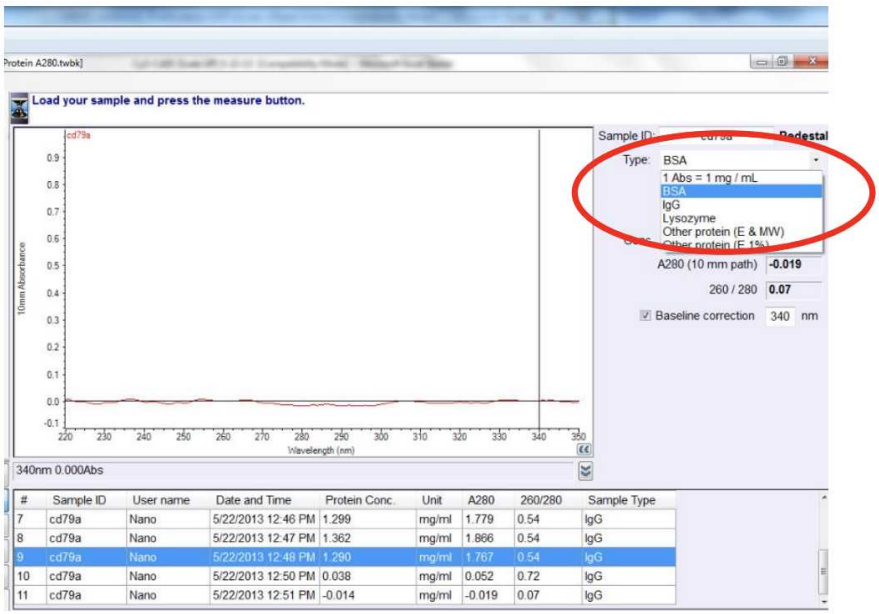

#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280	260/280	Sample Type
7	cd79a	Nano	5/22/2013 12:46 PM	1.299	mg/ml	1.779	0.54	IgG
8	cd79a	Nano	5/22/2013 12:47 PM	1.362	mg/ml	1.866	0.54	IgG
9	cd79a	Nano	5/22/2013 12:48 PM	1.290	mg/ml	1.767	0.54	IgG
10	cd79a	Nano	5/22/2013 12:50 PM	0.038	mg/ml	0.052	0.72	IgG
11	cd79a	Nano	5/22/2013 12:51 PM	-0.014	mg/ml	-0.019	0.07	IgG

Figure 7.5.1: Selecting Protein Type in Nanodrop 2000c

21 To blank the system, add **2 µl fresh 1X Binding Buffer** to the pedestal.

22 Select “Blank.”

23 After blanking is completed, wipe pedestal with a Kimwipe or other low lint wipe.

24 To ensure a stable baseline add **2 µl fresh 1X Binding Buffer** to the pedestal.

25 Select “Measure”
- Refer to Figure 7.5.2
- A stable baseline (right) shows a level line at zero with little signal noise.
- An unstable unacceptable baseline appears tilted.

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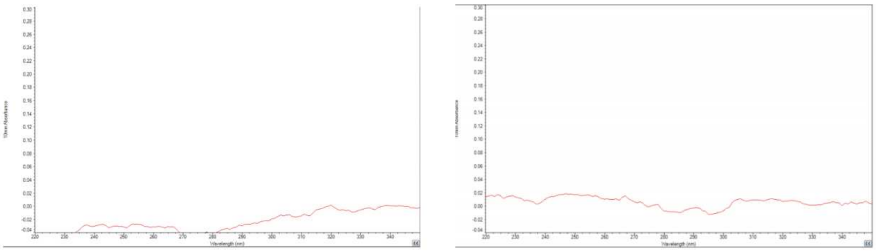

Not acceptable-tilted baseline

Acceptable – Stable Baseline

Figure 7.5.2: Ensuring a Stable Baseline on the NanoDrop 2000c

26 After measuring, wipe pedestal with a Kimwipe.
- If the baseline is unacceptable, return to step 21.

27 Once a stable baseline is achieved measure the absorbance of each wash step in triplicate
- Measurements will automatically save on the NanoDrop Program.

28 After each measurement, wipe pedestal with a kimwipe.

29 The Nanodrop will calculate the protein concentration of each reading.
- If a protein concentration at >0.03 mg/mL is present in Wash 5, continue collecting **1 mL wash fractions** until the concentration in the last wash reads <0.03 mg/mL (as was done in step 16) or until the concentration reading remains constant (this may be a sign your antibody is leaching off).
- Refer to Figure 7.5.3.

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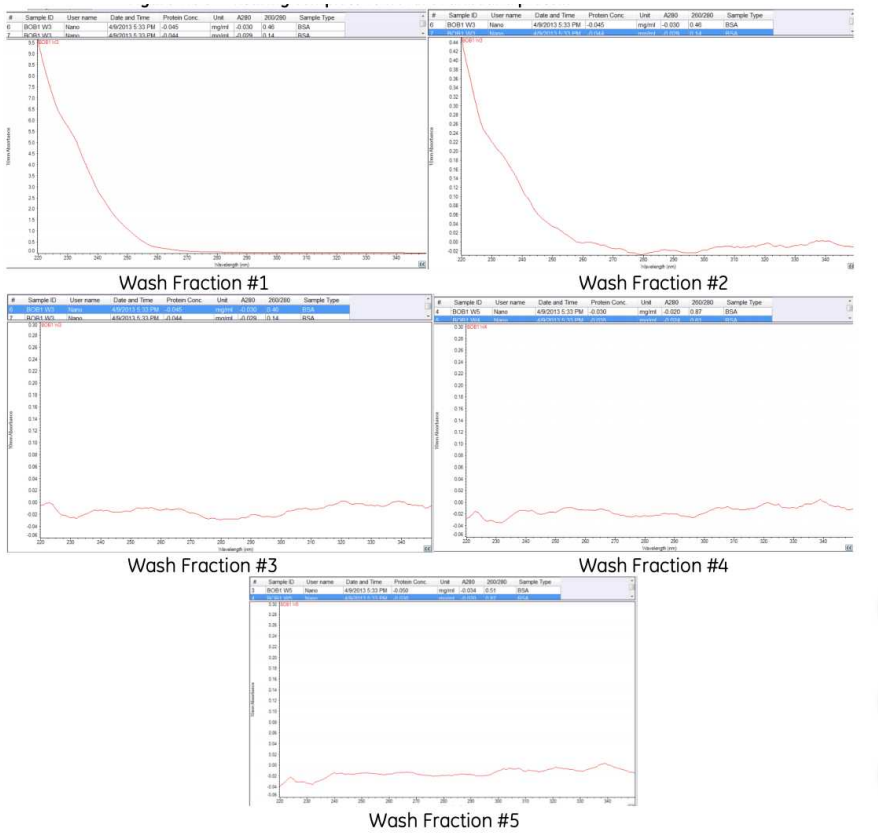

Figure 7.5.3: Ensuring complete removal of unbound protein

Preparing Amicon 30K filtration device

30 Remove Amicon 30K filter from its packaging.

31 Fill the filter to the top with dH_2O and cap.

32 Gently invert the filter 5 times.

33

Spin the filter at **3000 rpm, 00:10:00** in the centrifuge.

- While spinning proceed to the next section ("Eluting the purified antibody from the HiTrap Column") to begin antibody elution in parallel

34 After **00:10:00** discard the flow through liquid. 10m

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35 Repeat steps 31-34 one more time. **go to step #31**

Eluting the purified antibody from the HiTrap Column

36 Prepare five 1.5 mL eppendorf tubes by labeling them E1-E5 (Elution1-Elution5).

37 Measure **60 µl fresh 1X Neutralizing buffer** and pipette into tubes E2-E5.

38 Draw **5 mL fresh 1X Elution Buffer** into a 5 mL syringe.

39 Apply the **5 mL 1X Elution Buffer** to the HiTrap Column at a flow rate of 1-2 drops/second.

40 Collect **1 mL fractions of the eluted antibody** in the labeled tubes starting with E1 and proceed in sequential order.

41 Vortex each fraction for **00:00:05** . 5s

- Alternately, invert tube and flick so as to mix.
- Centrifuge briefly so as to remove solution from the underside of the cap and the upper parts of the tube.

Confirming the complete removal of purified antibody via NanoDrop

42 From the opened Nanodrop program, select “Proteins A280” program.

43 Within the “Protein A280” program, select protein Type: IgG. Refer to Figure 7.8.1.

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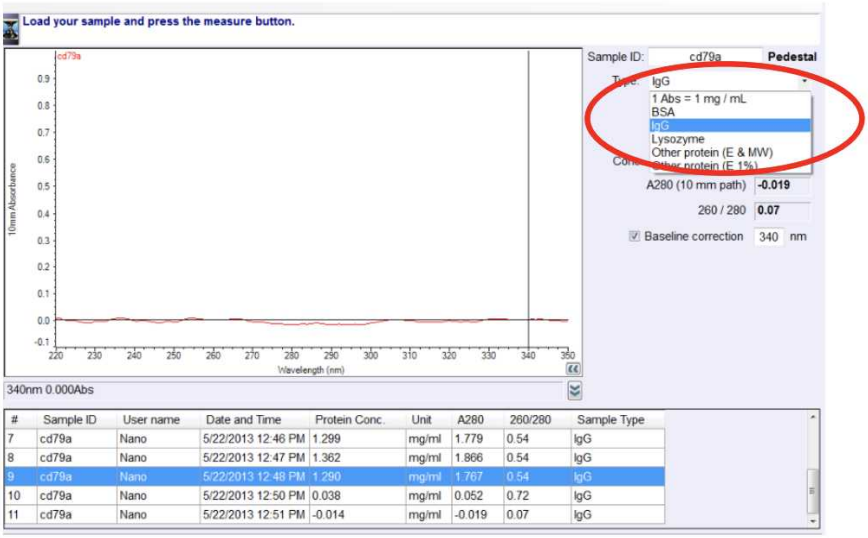

Figure 7.8.1: Selecting Protein Type: IgG

44 To blank the system add **2 µl Neutralized Elution Buffer** to the pedestal.

45 Select “Blank.”

46 After blanking has completed, wipe pedestal with a Kimwipe.

47 To ensure a stable baseline add **2 µl Neutralized Elution Buffer** to the pedestal.

48 Select “Measure”
- After measuring, wipe pedestal with a Kimwipe. If a satisfactory baseline isn't achieved, return to step 41.
- Refer to Figure 7.5.2.

49 In subsequent steps, recover the antibody after each measurement by drawing it back in to the pipette and placing it back into the assigned eppendorf tube.
- After each measurement, wipe pedestal with a Kimwipe.

50 Measure each fraction in triplicate, generally the first fraction will be free of any protein, elution 2 and 3 will contain pure antibody, and elution 4 and 5 will be free of any protein.
- Refer to Figure 7.8.2.
- If fraction 5 contains a protein concentration > 0.03 mg/mL continue collecting **1 mL fractions** until no more protein is eluted.

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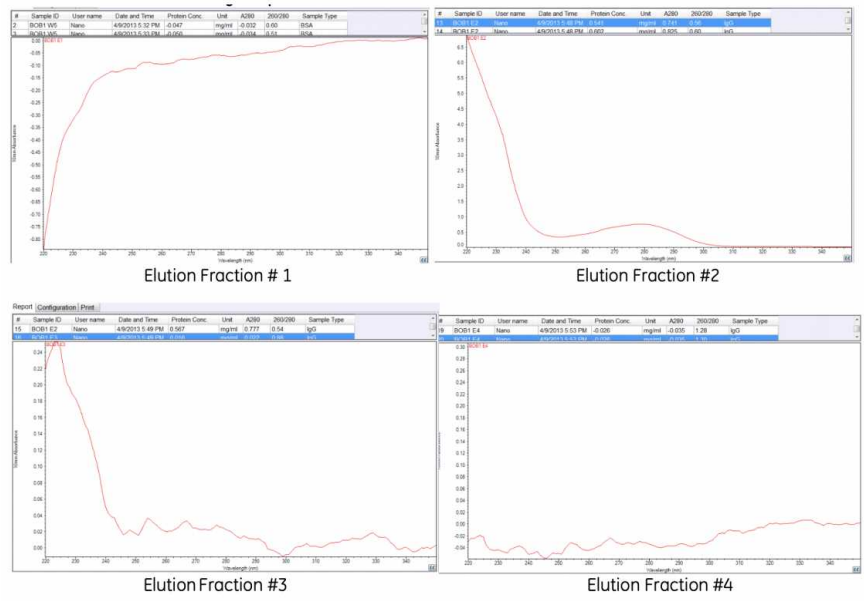

Figure 7.8.2: Ensuring Complete Removal of Purified Protein

Concentration and Buffer Exchange of the purified antibody

51 Combine all elution fractions with a protein concentration of >0.03 mg/mL into the prepared Amicon 30K filter.

52 Fill the filter to the manufacturer's recommended level with fresh 1X PBS.

53

Apply the cap and invert the filter 5 times to completely mix the PBS and antibody solution.

54

Place the solution in the centrifuge and spin at **3000 rpm, 00:10:00** .

55 11m

After **00:10:00** , check the filter to ensure the volume will lead to a calculated concentration of **1 mg/ml** or higher.

- **Example**: If you purify **800 µg** , the maximum volume to spin down to would be **800 µl** to get a concentration of **1 mg/ml** .
- If it has not, continue spinning at **3000 rpm** for **00:01:00** intervals until the appropriate volume is achieved.

56 When the solution is at or above a calculated concentration of **1 mg/ml** , remove the bottom tube and transfer the flow through liquid to a separately labeled 15 mL conical tube.

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57 Repeat steps 52-55, three more times for a total of 4 spins. **go to step #52**

58 Following the 4th spin, remove the filter from the 15 mL tube.

59 

Remove the purified antibody from the filter with a 200uL pipette.

60 Transfer to a clean 1.5 mL eppendorf tube labeled with antibody name.

61 

Wash the filter with **20 µl fresh 1X PBS** and then repeatedly pipette to wash each side of the filter.

62 Remove the wash and combine with purified antibody in 1.5 mL Eppendorf tube from step 60.

63 Close the lid of the Eppendorf tube and mix the solution gently but well, centrifuging briefly to remove liquid from the under-side of the cap.

Determining final yield via NanoDrop

64 From the opened Nanodrop program, select “Proteins A280” program.

65 Within the “Protein A280” program, select protein Type: IgG, Selecting protein Type: IgG.

66 To blank the system, add **2 µl fresh 1X PBS** to the pedestal.

67 Select “Blank.”

68 After blanking, wipe pedestal with a kimwipe.

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69 Select “Measure.”
- After measuring, wipe pedestal with a kimwipe.
- If the baseline is poor, return to step 66.
- Refer to Figure 7.5.2.

70 Measure the purified antibody 5 times.
- After each measurement, wipe pedestal with a kimwipe.

71 Take a volumetric reading of the final purified antibody with a pipette and record volume.

72 Calculate %Yield ((Ab concentration (µg/µL)*Volume (µL))/Initial Ab amount (µg)). If the initial antibody amount is not known, the %yield cannot be calculated.

73 Dilute purified antibody with 0.65% Sodium Azide (Volume of purified antibody/12) to a final concentration of 0.05% Sodium Azide.

74 Label the vial of purified antibody with the biomarker name, vendor name, catalog number, lot number, date purified and final concentration.

Stabilizing the HiTrap Column with Column Storage Solution

75

The HiTrap columns can be re-used for up to 1 year; however, only antibodies from the same vendor and with the same clone can be re-applied to a previously used column.

76 Measure **5 mL water** with a 5mL syringe.

77 Apply water to HiTrap column at a rate of 1-2 drops/second and discard flow through liquid.

78 Measure **5 mL Column Storage solution** , typically 20% ethanol in water, with a 5mL syringe.

79 Apply solution to HiTrap column at a rate of 1-2 drops/second and discard flow through liquid.

80 Label the column using a tough tag labeled with the antibody name, clone, vendor, catalog number, protein type (A or G), and date opened.

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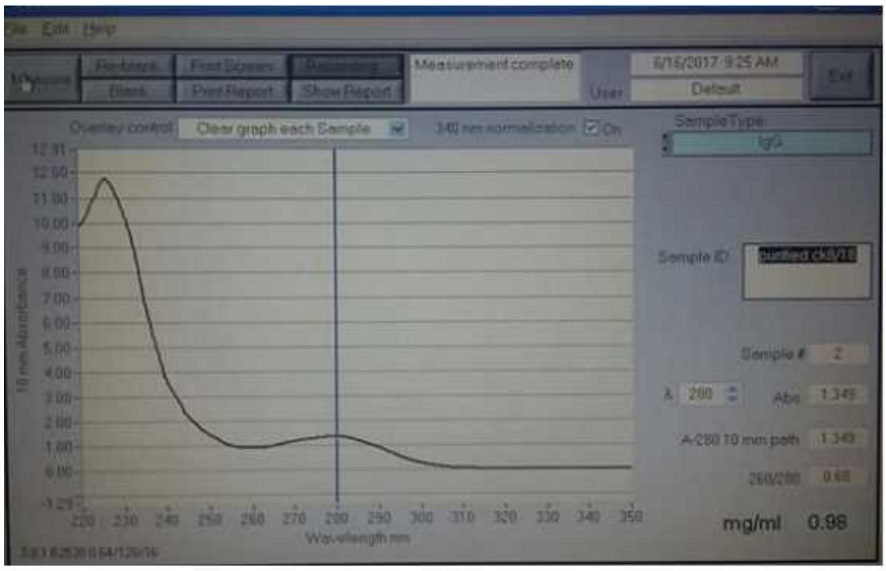
81 Re-attach the top and bottom enclosures and store at 4 °C for up to 1 year.

Troubleshooting

82 If antibody yield is <10% please check whether correct column is used for purification if yes contact vendor for support.

83 Also, when there is question about stabilizer such as BSA present in the antibody supplied from vendor. Please record UV spectrum of the antibody and make sure it looks like below with A260/A280=~0.70. If BSA is present A280 will go up and the ratio will drop down from 0.70 to any number depending on the amount of BSA present in the sample.



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