CellMosaic, Inc.
10A Roessler Road
Woburn, MA 01801
USA

Phone: 781-463-0002
Fax: 781-998-4694
Email: info@cellmosaic.com
Website: www.cellmosaic.com

SepSphere™ Antibody/Protein/Enzyme Immobilization Kit via Glyoxyl (CM71542) User Reference Guide

Contents

CellMosaic, Inc.
10A Roessler Road
Woburn, MA 01801
USA

Phone: 781-463-0002
Fax: 781-998-4694
Email: info@cellmosaic.com
Website: www.cellmosaic.com

Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

The information provided in this document and the methods included in this package are for information purposes only. CellMosaic provides no warranty of performance or suitability for the purpose described herein. The performance of this kit in labeling may be affected by many different variables, including but not limited to the purity and complexity of the starting materials, differences in preparation techniques, operator ability, and environmental conditions.

Sample data are provided for illustration and example purposes only and represent a small dataset used to verify kit performance in the CellMosaic laboratory. Information about the chemicals and reagents used in the kit are provided as necessary.

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Director of Licensing
c/o CellMosaic, Inc.
10-A Roessler Road, Woburn, MA 01801.
Phone： 781-463-0002
Fax： 781-998-4694
E-mail: info@cellmosaic.com

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Kit Components

This kit provides materials to immobilize any protein (including antibody and enzyme) onto glyoxyl agarose beads.

⚠ Upon receipt, please remove **Box 1** and store in a refrigerator at 2-8°C.
Store **Box 2** at room temperature.

	Name	Part #	Quantity	Storage condition
Box 1	Glyoxyl Agarose Beads in a 15 mL centrifuge tube (CM03SC7)	CM71602	5 mL settle down beads	2-8°C
	Buffer A (Reaction Buffer, **blue label**)	CM02010	80 mL	
	Buffer B (Reducing Buffer: 4.5M NaBH4 in 2M NaOH, **red label**)	CM02016	0.6 mL	
	Buffer C (Neutralization Buffer: 2N HCl, **cyan label**)	CM02015	0.7 mL	
	Buffer D (Washing Buffer, **green label**)	CM01003	60 mL	
	Storage Buffer (0.02% NaN_3 in PBS buffer) (**grey label**)	CM02008	20 mL	
Box 2	Centrifugal Filter Device	CM03CD010A2	2	RT
	Column Set	CM03SC7	1	
	Centrifuge Tube 15 mL	N/A	1	
	Centrifuge Tube 50 mL	N/A	1	
	Stirrer	CM03PT1	4	
	Airtight Syringe 20 mL	CM03SR1	1	
User Material	Protein (including antibody and enzyme)		NOT PROVIDED (User Supplied Material, **1 to 25 mg**)	

Loading and Protein Amount:

Protein immobilization via glyoxyl-activated agarose beads is very efficient and offers very high protein loading. CellMosaic's kit can result in a maximum of 0.2 µmol of protein per mL of settled beads (1 nmol of total protein per 5 mL) based on the protein A immobilization test. If you are targeting maximum loading, you will need 1 nmol of total protein assuming 100% of your protein is immobilized. This translates to 50 mg of a 50 KDa protein and 150 mg of antibody IgG. Unreacted protein can be recovered.

However, depending on your application requirements for loading and the amount of protein you can supply, you can decide how much protein you will use for immobilization. The recommended loading target for CM71542 is 0.2-5mg/mL of settled beads (1-25 mg of protein).

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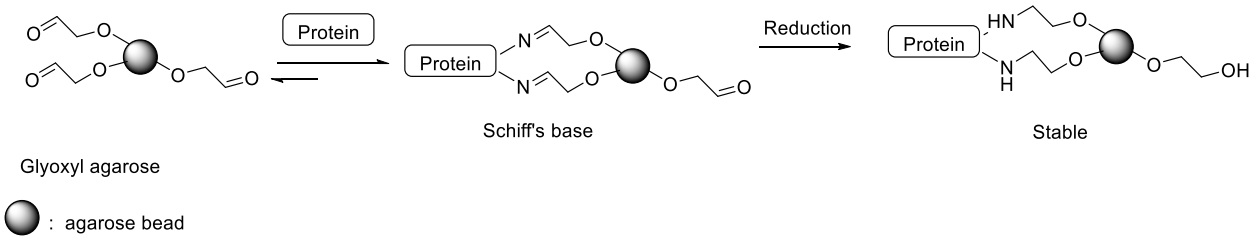
Safety Information

Warning: some of the chemicals used can be potentially hazardous and cause injury or illness. Please read and understand the Safety Data Sheets (SDS) available at CellMosaic.com before you store, handle, or use any of the materials.

Labeling Chemistry

Glyoxyl agarose has been frequently used for multipoint covalent attachment of protein (including antibody and enzyme) and is particularly suited for enzyme immobilization when the stability of the immobilized enzyme is a concern (Selected review ref.: Zucca P. *et. al.* Molecules **2016**, *21*, 1577).

CellMosaic designed this kit to work directly with glyoxyl agarose. Glyoxyl groups are aliphatic aldehyde groups with low steric hindrance towards the immobilization reaction and high stability at alkaline pH. Glyoxyl reacts with the amino group, forming Schiff's base (imino bond). The imino bond is not very stable and can be reversible, so only proteins that form several imino bonds will be able to stay. For this reason, glyoxyl agarose permits the immobilization of the protein through the area with the highest density of lysine groups, where the highest likelihood of multipoint covalent attachment may be achieved. This multiple covalent attachment also allows appropriate alignment of the protein on the surface. After immobilization, the double bond of the Schiff's base is finally reduced to form a stable secondary amino bond. The remaining aldehyde groups are converted into inert hydroxyl groups. However, protein such as antibody containing disulfide bridges or enzyme bearing a metal ion in the active center may be affected by the final reducing step and should be used cautiously with this method.



Scheme 1. Immobilization of protein on glyoxyl agarose beads via reductive amination

Key features of this SepSphere™ Immobilization Kit:

- Minimum requirement for immobilization set-up
- All reagents, buffers, and plasticware are included
- Highly activated glyoxyl agarose: ~200 µmol glyoxyl per mL agarose
- Maximum loading assessed by Protein A immobilization: ~0.2 µmol protein A per mL agarose
- Stable and multipoint covalent attachment
- Easy preparation with air push mechanism for washing: less than 2 h hands-on time

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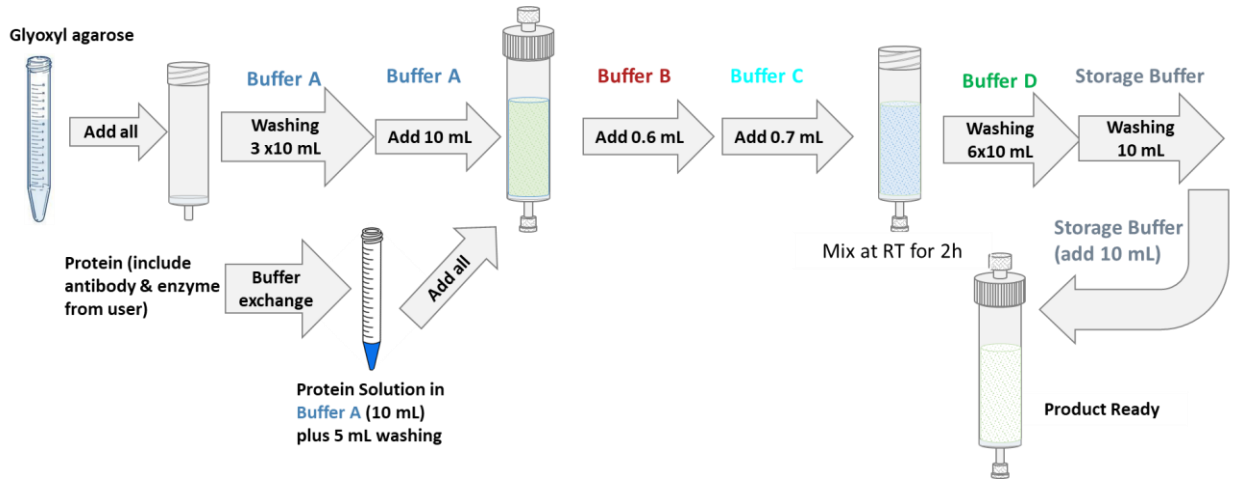
Requirement for protein (including antibody and enzyme):
1. Preferably > 90% pure by gel electrophoresis
2. Total amount: 1-25 mg protein content as measured by UV (>25 mg is fine)
3. If protein is not stable at pH 10.0, it should not be used
4. Protein with a disulfide bridge may be affected by the reducing condition
5. Enzyme with a metal ion in the active center may be affected by the reducing condition

Protocol



Scheme 2. Schematic diagram of the workflow for protein immobilization starting with 5 mL of glyoxyl agarose beads.

1. Lab Instrumentation Needed

- Vortex mixer
- Centrifuge for 15 mL and 50 mL (preferably refrigerated)
- IEC clinical centrifuge for quick spin (if possible)
- Pipettes and tips
- Timer
- Nutating mixer or 3-D rocker
- Support, clamp, and beaker for waste collection

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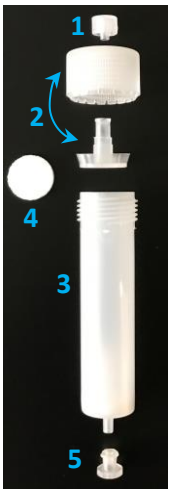
2. Immobilization Lab Techniques at CellMosaic (for reference)

CellMosaic has a universal workflow and kit design that allow immobilization of small molecules and large biomolecules, washing of unreacted starting materials, and column packing all in one column with simple set-up in any lab. The immobilization efficiency in the column is the same as if performed in a regular reaction vessel. The washing is performed with a simple manual air push mechanism using a syringe and requires no gas/air flow or vacuum. The following table outlines the kit component design and key techniques used at CellMosaic for immobilization. Please refer to these key techniques while performing the immobilization according to the user manual.



Column design and setup:

Design: Column comprises five pieces (see left picture)

1. **Male Luer lock cap** (referred to as **top cap** in the protocol and is used during the mixing/nutation).
2. **Column top with Luer attachment** (referred to as **column top** in the protocol). The Luer attachment is for a tight seal and for attachment of the syringe for washing. Column top is removed for addition of buffer and stirring.
3. **Column body containing one polypropylene frit at the bottom** (referred to as **column** in the protocol).
4. **One extra frit for column packing after immobilization** (referred to as **frit** in the protocol).
5. **Bottom female Luer lock plug** (referred to as **bottom plug** and is used during the mixing/nutation and storage).

Setup (see right picture): Securely attach the column to a support stand, lab frame, or any support rod and place a beaker or a flask under the column for waste collection.

Use of buffer(s) with resin in column

Design: All of CellMosaic's buffers are supplied in small tubes and are easy to pour directly into the column. Washing buffers generally come with the exact amount needed for optimal washes. The combined wash volume is fixed, regardless of how much you pour each time.

Procedure:

1. Remove the top cap.
2. Unscrew the column top and bottom plug.
3. Place them on a clean surface for reuse later.
4. Pour 8-10 mL of **any buffer** into the column containing resin for immobilization (~2 volumes of the resin bed).

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Mixing resin in column

Design: The kit comes with one or a few blue polypropylene stirrers of the right length for individual columns. The stirrer is resistant to general solvents, and resin in general will not stick to the stirrer.

Procedure for mixing resin with washing buffer: Mix the resin with buffer using a clean stirrer for ~5-10 seconds. Make sure the stirrer goes into the bottom of the column and the resin is mixed well. Then remove the stirrer without any resin adhering to it. Set aside the stirrer for repeated washing.

Procedure for mixing resin with reaction buffer: Mix the resin with buffer using a clean stirrer for 10-30 seconds. Make sure the stirrer goes into the bottom of the column and the resin is mixed well. Then remove the stirrer without any resin adhering to it. Dispose of the stirrer as solid waste. **Note:** For some reactions, gas/air bubbles may be generated. Stir slowly and continuously until most of the air bubbles are gone before capping it for mixing (nutation).

Washing resin after stirring

Design: The kit comes with one 20 mL airtight syringe with slip tip that can be used for pushing air through the column.

Procedure for washing:

1. Attach the column top to the Luer attachment after stirring.
2. Draw 20 mL of air into the syringe and attach the syringe to the column top.
3. Push the air through the column.
4. Once the plunger reaches the bottom of the syringe, detach the syringe. Repeat the air drawing and purging process. In general, there will be approximately 1.2 mL of residual liquid left after several pushes. It is OK to go forward with the next washing step.
5. Remove the column top, fill with buffer, and repeat the washing and purging process.

Procedure to remove residual liquid: If residual liquid is to be removed for certain reactions (see individual protocol), use this additional step after air purging with the syringe. Place the column into a 50 mL centrifuge tube with the column top attached without the top cap. Create a counterbalance with a 50 mL centrifuge tube with water in it. Place both tubes into a quick spin centrifuge, such as ICE clinical centrifuge. Set the spin to the maximum setting and spin for 15-20 seconds. Alternatively, you can use the conventional centrifuge equipment and spin at ≤750 x g for 1 minute.

Column setup for immobilization:

After adding reagents per protocol, attach the column top and securely cap/plug the column top and bottom. Check to make sure everything is tight and there is no leak before putting the column in a nutator. If you do not have a mixer or nutator, leave the column open and stirrer in place. Stir the resin every 5-10 minutes for the specified time.

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Storage buffer and others:

The kit comes with standard storage buffer (1x PBS with 0.02% NaN_3). If you need to use your own buffer, substitute your buffer during the last washing.

Column packing:

Design: You can pack the resin in the column once the immobilization is done. A separate polypropylene frit is included in the kit for column packing. Otherwise, you can store the resin in the column and pipette out as needed for usage.

Procedure:

1. Remove the top cap. Unscrew the column top and bottom plug.
2. Fill the column with deionized water or buffer up to the top and wait a few minutes so that most of the resin settles down.

3. Wet the frit with deionized water before placing the frit inside.
4. Remove the black rubber cap in the plunger of the 20 mL syringe.
5. Use the plunger to push the frit inside the column and all the way down to the top of the resin. Make sure the pressure is even and the frit is flat and not tilted. There should be no air between the frit and the resin before pushing the frit all the way down to the top of the resin.

6. Once the frit reaches the top of the resin, use slight pressure to make the frit tight.
7. Attach the column top with Luer attachment securely and cap the top.

The column is ready to use. You can use gravity flow or attach the column to a peristatic pump with Luer lock adapters.

Protocol for immobilization starts on next page

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3. Preparation of Protein Samples for Conjugation

Items needed: Centrifugal Filter Devices (CM03CD010A2), Buffer A (CM02010, blue label), 15 mL centrifuge tube, clean flask for collection (not provided in the kit).

Total amount of protein used for the immobilization is 1-25 mg to target 0.1-5 mg protein per mL of settled resin. For loading >5 mg/mL, you can use more protein.

A1. Take out the two **Centrifugal Filter Devices** and perform the step based on the following conditions.

- ✓ If your protein is supplied as a lyophilized solid and the amount is <25 mg, dissolve the protein in 3.5 mL of **Buffer A** and then transfer to one of the **Centrifugal Filter Devices**.
- ✓ If your protein is supplied as a lyophilized solid and the amount is >25 mg, dissolve the protein in 7 mL of **Buffer A** and then divide the volume into two **Centrifugal Filter Devices**.
- ✓ If your protein is supplied in < 7 mL buffer, divide the volume into two **Centrifugal Filter Devices**. Add **Buffer A** to make up the total volume to 3.5 mL in each device and cap them.
- ✓ If the volume of your protein sample is >7 mL, add up to 7 mL of sample to the two **Filter Devices** and cap them. Repeat Steps **A1-A4** until all of the protein sample is transferred into the **Filter Device**. Move on to Step **B5**. Add **Buffer A** to make up the total volume to 3.5 mL in each device for the last refill.

A2. Place the capped **Filter Devices** into the centrifuge rotor. Make sure the devices are balanced.

A3. If a swinging-bucket rotor is used, spin the **Filter Device** at 4,000 x g maximum for 15-25 minutes (preferably cooled to 4°C) to concentrate to **< 0.5 mL**. If a fixed-angle rotor is used, orient the device with the membrane panel facing up and spin the **Filter Device** at 7,500 x g maximum for 10-15 minutes (preferably cooled to 4°C) to concentrate to **< 0.5 mL**.

A4. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean flask or a 50 mL centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

A5. Insert the **Filter Device** back into the collection tube. Add 2-3 mL of **Buffer A** to make up the total volume to 3.5 mL. Next, place the capped **Filter Device** into the centrifuge rotor. If a swinging-bucket rotor is used, spin the **Filter Device** at 4,000 x g maximum for 15-25 minutes (preferably cooled to 4°C) to concentrate to **< 0.5 mL**. If a fixed-angle rotor is used, orient the device with the membrane panel facing up and spin the **Filter Device** at 7,500 x g maximum for 10-15 minutes (preferably cooled to 4°C) to concentrate to **< 0.5 mL**. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to the flask or 50 mL centrifuge tube. **Save the filtrate until the experiments are done.**

A6. Repeat **Step A5** one more time.

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A7. Transfer the concentrated sample from the **Filter Device** to a 15 mL centrifuge tube.

A8. Add 1 mL of **Buffer A** to each **Filter Device** to rinse. Stir it gently with a pipet tip, then transfer the entire contents to the 15 mL centrifuge tube from **Step A7.**

A9. Repeat **Step** A**8** once.

A10. Add **Buffer A** to the 15 mL centrifuge tube from **Step A9** to make up the total volume of the sample to **10 mL** and cap it. Vortex the combined protein sample for 30 seconds and spin down.

Tip for opening centrifuge tubes after mixing: Always spin the tubes to ensure no liquid is in the cap.

4. **Preparation of Agarose Beads and Immobilization**

Items needed: Glyoxyl Agarose Beads (CM71609)), Column Set (CM03SC7), Airtight Syringe 20 mL (CM03SR1), Stirrer (CM03PT1), Buffer A (CM02010, blue label), Buffer B (CM02016, red label), buffer C (CM02015, cyan label), 50 mL centrifuge tubes.

B1. Securely attach the **Column** to a support stand, lab frame, or any support rod and place a beaker or a flask under the column for collection. Remove the top cap. Unscrew the column top and bottom plug. Place them on a clean surface for reuse later.

B2. Slightly centrifuge the tube containing agarose beads to ensure no bead is in the cap before opening. Stir the resin with a clean stirrer to mix up the beads with the buffer. Set aside the stirrer for later use. Pour all the beads into the **Column**. Attach the column top and use the 20 mL syringe to push the liquid out.

B3. Unscrew the column top. Locate **Buffer A** (orange label) and pour 8-10 mL of **Buffer A** into the centrifuge tube from **Step B2** to wash the tube. Pour all the washing solution into the column. Stir the resin for 5 seconds. Dispose of the 15 mL centrifuge tube.

B4. Attach the column top. Use the 20 mL syringe to push the liquid out.

B5. Remove the column top and repeat the washing (**Steps B3 and B4**) by pouring 8-10 mL of **Buffer A** directly into the column two times. Dispose of the stirrer.

B6. Securely plug the end of the column with the bottom plug, add the protein solution from **Step A10.** Then wash the protein tube with 5 mL of **Buffer A** and add the washing to the column. Total volume is 15 mL solution**.**

B7. Stir the resin solution for 10-30 seconds with a new stirrer until there are few air bubbles. Securely cap the column with top cap. Dispose of the stirrer.

UV/Vis sample from Step B7 for loading calculation (Optional). Pipette 50-200 µL of liquid from **Step B7** into a 1.5 mL centrifuge tube (let the resin slightly settle before removing the liquid). Centrifuge at 14,000 x g for 1 minute. Remove the supernatant and dilute 2-5 times in **Buffer A** for UV measurement at 280 nm. The volume taken depends on the protein concentration and UV

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cell volume. The dilution factor will depend on the extinction coefficient of the protein and the concentration of the protein.

B8. Nutate the column (end-to-end mixing) at RT for 2-4 h or overnight at 4°C (note: the resin may clump together).

In-process sample analysis by UV/Vis (Optional): Pipette 50-200 µL of liquid from **Step B8** into a 1.5 mL centrifuge tube (let the resin slightly settle before removing the liquid). Centrifuge at 14,000 x g for 1 minute. Remove the supernatant and dilute in **Buffer A** the same factor as in **Step B7** for UV measurement at 280 nm.

Percentage of the protein consumed:

$$\% = \left[1 - \frac{A(sB8)}{A(sB7)}\right]$$

A(sB8): UV absorbance of supernatant sample from **Step B8** at 280 nm
A(sB7): UV absorbance of the sample from **Step B7** at 280 nm
Both samples are measured under the same condition using the same dilution and UV cell

Reaction time: In general, after 4 h at RT or overnight at 4°C, 70-80% of the protein will be consumed depending on your initial protein concentration. If your protein is very stable, you can continue letting the resin nutate at RT until most of the protein is consumed. If your protein is not very stable, you can cut down your reaction time to 2 h. If you want to pause at this step, you can store the resin temporarily at 4°C. Remember to take a new sample for UV reading before moving on to the next step for calculating the loading.

Calculation for Loading (mg per mL of resin):

$$Protein\ loading = Protein\ amt\ (mg) \times 0.2 \times \left[1 - \frac{A(sB11)}{A(sB7)}\right]$$

Safe Handling Chemical Notice: Buffer B contains 4.5M NaBH4 in 2M NaOH. **Buffer C** contains 2N HCl. Both are very corrosive hazardous materials. Wear proper personal protection equipment (nitrile gloves, lab coat, and eye protection) to handle these chemicals. Read the SDS prior to the experiment.

B9. In a fume hood, securely attach the **Column** from **Step B8** to a support stand. Remove the top cap. Unscrew the column top and add the entire contents of **Buffer B** (0.6 mL of reducing buffer, 12% NaBH4 in 14M NaOH) into the column. Mix with a clean stirrer for 5 seconds.
B10. Add the entire contents of **Buffer C** (0.7 mL of neutralization buffer, 6N HCl) drop by drop into the column. Use the same stirrer to stir the solution. A lot of gas will be generated during the stirring.
B11. Let the column sit at RT with the top open for 2 h. Use the same stirrer to stir the solution occasionally to mix the resin (every 10-15 minutes).

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5. Washing and Storage

Items needed: Stirrer (CM03PT1), Buffer D (CM01003, green label), Storage Buffer (CM02008, grey label).

C1. Place a clean 50 mL centrifuge tube under the column for collecting unreacted protein solution. Open the bottom plug. Push the liquid out of the column using the syringe.
C2. Remove the column top and pour in 8-10 mL of **Buffer D** (green label). Stir the resin solution for 10-30 seconds with a new stirrer, then put the column top back and push the liquid out of the column using the syringe.
C3. Repeat washing 5 times.
C4. Remove the column top and pour in 8-10 mL of **Storage Buffer.** Stir the resin solution for 10-30 seconds with the same stirrer, then screw the column top back on and push the liquid out of the column using the syringe.
C5. Repeat washing 2 times. Dispose of the stirrer.
C6. Securely plug the end of the column, add 5-10 mL of Storage Buffer. Screw the column top back on and securely cap the column. Store at 2-8°C in a refrigerator. Do not freeze.

The Agarose Beads are Ready for Your Experiment

Specification for your product

Matrix: 4% beaded agarose (source: GE Life Sciences, Sepharose® 4B)
Particle size: 45 – 165 µm
Ligand concentration: calculated in **Step B8**. If you do not perform the UV measurements, assume 70-80% of protein consumed and use that value for the calculation.