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PerKit™ Antibody Small Molecule Acid Conjugation Kit (CM51403) User Reference Guide

Contents

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Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

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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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Kit Components

This kit provides materials to conjugate 1 to 3 mg of one antibody sample (**IgG**) with a small molecule containing a carboxylic acid functional group. Upon receipt, please store box in a refrigerator at 2-8°C.

Container	Name	Part #	Quantity	Storage condition
Plastic Bag 1: NHS Ester Formation Kit	Reagent A solution (white label)	CM10001	1 unit (40 µL)	2-8 °C
	Reagent B (purple color insert)	CM10002	1 unit	
	Solution A (blue label)	CM01008	0.5 mL	
Plastic Bag 2	Reaction Buffer (orange label)	CM02001	8 mL	
	Storage Buffer (1 x PBS buffer) (grey label)	CM02013	20 mL	
	ADC Stabilizing PBS Buffer (5x) (pink label)	CM02022	0.5 mL	
Plastic Bag 3	Centrifugal Filter Devices	CM03CD050A	2	
	Desalting Column	CM03SG10	1	
	Collection Tubes	CM03CT0	2	
	1.5 mL Centrifuge Tubes	CM03CT2	2	
	0.5 mL Centrifuge Tubes	CM03CT1	2	
User Materials	Small Molecule Acid	N/A	NOT PROVIDED (User Supplied Material, 20 µmol)	
	Antibody (IgG)	N/A	NOT PROVIDED (User Supplied Material, 1-3 mg)	

Reaction Scale: The protocol is optimized for conjugating 3 mg of IgG antibody. If you have less than 3 mg of IgG, use the calculations in **Steps A10**, **C1**, and **D5** to obtain the correct volumes to be added in each step.

Safety Information

Warning: some of the chemicals used can be potentially hazardous and can 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

The kit is designed to work with small molecules containing one carboxylic acid functional group. The user supplies the small molecule and the antibody (IgG). Using the kit components, the user converts the carboxylic acid to an activated *N*-hydroxysuccinimide ester (NHS ester), followed by reaction with the surface amino groups of the antibody to form a stable amide bond. The final product is desalted to remove any unreacted small molecule acid.

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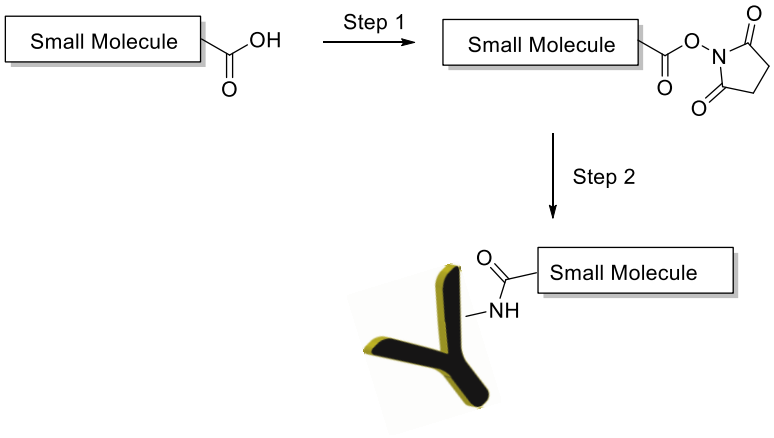
Key features of this conjugation kit:

- Offers a simple and easy way to label antibody with small molecules containing carboxylic acid
- Fast and easy preparation: 4 h preparation and less than 30 minutes hands-on time
- Offers in-process optimization of the degree of loading during the conjugation
- All reagents and supplies included for preparation and purification
- Over 90% pure conjugated products by SEC



Requirement for small molecule:
1. Preferably > 90% pure by C18 HPLC
2. Total amount: 20 µmol
3. Absence of primary or secondary amine groups
4. Non-hindered aliphatic carboxylic acid
5. For molecule containing aromatic carboxylic acid, hindered aliphatic carboxylic acid, or hydroxyl groups, please consult CellMosaic for custom conjugation.

Requirement for antibody (IgG):
1. Preferably > 90% pure by gel electrophoresis
2. Total amount: 1-3 mg protein content as measured by UV. Note: the accuracy of your antibody amount is the single most important factor to obtaining an optimized DOL. Please refer to the section Other Considerations in this manual to measure the antibody amount.

Support

Customer can request a recommendation for the conjugation if the molecule has a special feature or a low amount of antibody and small molecule is available. CellMosaic provides other accessory tools, such as buffers, standards, and reagents for bioconjugation research. CellMosaic also provides fee-based support services to customers who need help analyzing the final conjugates by HPLC and determining the DOL.

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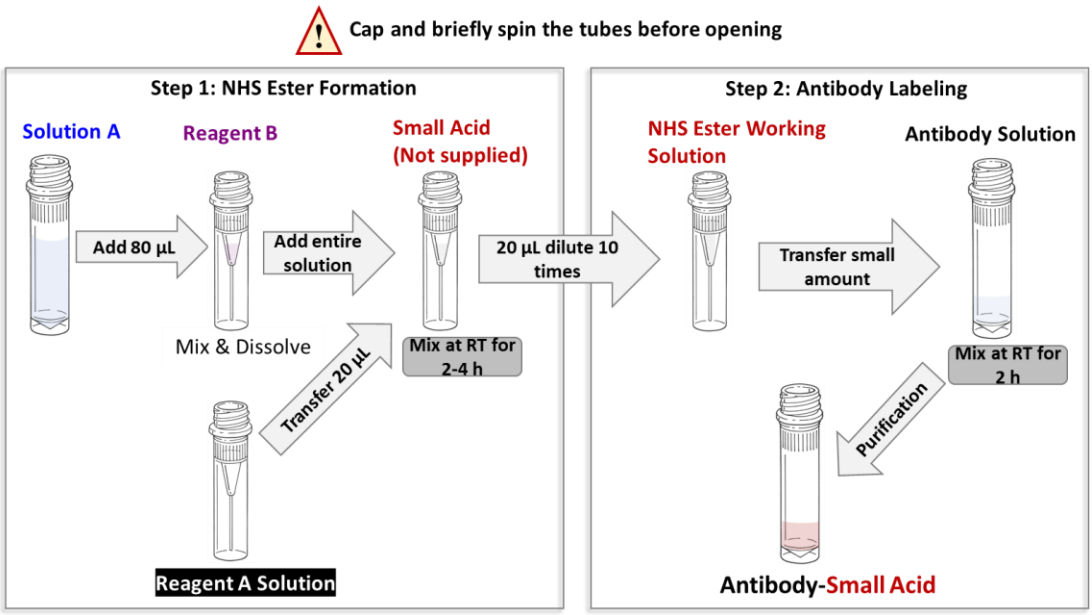
Protocol



Scheme 1. Schematic diagram of the workflow for preparing antibody-small molecule conjugates starting with 3 mg of IgG (volume of reagents varies if the amount of IgG is < 3 mg).

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, 14,000 g capable), mini-centrifuge
- Pipettes and tips
- Timer
- Incubator or shaker set at 25°C or RT
- Balance

2. Preparation of Antibody Samples for Conjugation

Items needed: Filter Devices (CM03CD050A), Collection Tube (CM03CT0), Reaction Buffer (CM02001, Orange label), 1.5 mL Centrifuge Tube (CM03CT2), Clean Centrifuge Tubes (not provided in the kit).

Total amount of antibody used for the conjugation is 3 mg per reaction (protein content measured by UV).

> **Reaction Scale:** If you have less than 3 mg of antibody, use the calculations in **Steps A10**, **C1**, and **D5** to obtain the correct volumes to be added in each step.

A1. Insert the **Filter Device** into one of the provided collection tubes (micro-centrifuge tube with the cap attached). Perform the step based on the following conditions.

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- ✓ If your antibody is supplied as a lyophilized solid, dissolve the antibody in 500 µL of **deionized water** and then transfer the entire contents to the **Filter Device**.
- ✓ If your antibody is supplied in < 500 µL buffer, transfer your antibody sample to the **Filter Device** directly. Add **Reaction Buffer** to make up the total volume to 500 µL and cap it.
- ✓ If the volume of your antibody sample is between 500 and 1000 µL, divide the volume into two **Centrifugal Filter Devices** and add the antibody sample to the filter device. Add **Reaction Buffer** to make up the total volume to 500 µL in each device and cap them.
- ✓ If the volume of your antibody sample is >1000 µL, add up to 500 µL of sample to the two **Filter Devices** and cap them. Repeat Steps **A1-A4** until all of the antibody sample is transferred into the **Filter Device**. Move on to Step **A5**.

A2. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

A3. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably cooled to 4°C) to concentrate to **< 100 µL** (Spin time depends on many factors. The typical spin time for a 500 µL sample is approximately 8 to 20 minutes. The typical volume is ~40 µL after spinning for 8 minutes on an Eppendorf 5417R at 4°C.)

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 centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

A5. Insert the **Filter Device** back to the collection tube. Add 400-450 µL of **Reaction Buffer** to make up the total volume to 500 µL. Next, place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the device at 14,000 x g to concentrate to **< 100 µL**. 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 centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

A6. Repeat **Step A5** two times.

A7. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube (use the pipetman to measure the approximate volume of the concentrated sample).

A8. Add 20-100 µL of **Reaction Buffer** to the **Filter Device** to rinse (actual volume of **Reaction Buffer** added will depend upon the calculated total volume in **Step A10**). Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step A7.**

A9. Repeat **Step A8** once.

A10. Add **Reaction Buffer** to the 1.5 mL micro-centrifuge tube from **Step A7** to make up the total volume of the sample to **340 ± 5 µL** and cap it.

Calculation 1 for Less Antibody (Ab):

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$$Total\ volume\ of\ the\ antibody\ in\ Step\ \mathbf{A10}\ (\mu L) = Ab\ in\ mg\ \times 113.3$$

A11. Vortex the combined antibody sample for 30 seconds and then spin down.

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

3. NHS Ester Formation (20 µmol scale) (Step 1)

Items needed: Reagent A Solution (CM10001, white label), Reagent B (CM10002, purple color insert), Solution A (CM01008, blue label), 0.5 mL Centrifuge Tubes (CM03CT1).

B1. Weigh 20 µmol of **Small Molecule** into a clean 0.5 mL micro-centrifuge tube.

Calculation: Amount of small molecule (mg) = Molecular Weight (MW) of small molecule x 0.02

B2. Take out the **Plastic Bag 1** from the box. Spin the centrifuge tubes containing **Reagent A solution** (white label), **Reagent B** (purple color insert), and **Solution A** (blue label) before opening it.

B3. Transfer **20 µL** of **Reagent A solution** to the tube containing small molecule from **Step B1**. Vortex for 30 seconds or sonicate for a few minutes to dissolve and spin down (**Note:** if there are some solids left, that is fine for this step). **Dispose of the leftover solution as hazardous waste after the experiment is done.**

B4. Add **80 µL** of **Solution A** to **Reagent B**. Vortex for 30 seconds or sonicate for a few minutes to ensure all of the solid is dissolved and spin down.

Transfer the entire solution to the tube containing **Small Molecule Solution** from **Step B3. Dispose of the leftover solution as hazardous waste after the experiment is done**.

B5. Vortex for 30 seconds or sonicate for a few minutes to dissolve and spin down. (**Note:** some NHS ester formations are very fast, you might notice colorless solid precipitate out immediately after you mix up the solution).

Tip for solubility check (Step B3, B4, & B5): It may take a while for your compound to fully dissolve. In general, most of the compound should be able to dissolve. Check the bottom of the micro-centrifuge tube to ensure the solution is clear and free of any solid residue.

B6. Incubate the mixture at RT for **2 h**.

B7. Remove the centrifuge tube from the incubator to check if there is any clear solid precipitated out. If there is solid precipitated out, move on to the next step (**Step B8**). If not, leave the centrifuge tube in the incubator for another 2 h and check again if there is any clear solid precipitated out.

Tip for precipitation check (Step B7): Place the tube at a 45-degree angle and see if the solution can flow freely. Remove the tape label if necessary.

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Note: If there is no solid precipitated out after 4 h of reaction, please consult with CellMosaic for an alternative method.

B8. Add **180 µL** of **Solution A** to a clean 0.5 mL micro-centrifuge tube. Discard any unused **Solution A** as hazardous chemical waste **until the experiments are done**.

B9. Spin the centrifuge tube from **Step B7** before opening it. Pipette **20 µL** of solution using a very fine pipette tip (gel loading tip works great) and ensure there is no solid on the side of the tip. Transfer the liquid to the centrifuge tube containing the Solution **A** from **Step B8**.

B10. Vortex the solution for 30 seconds and spin down.

4. Antibody Labeling with NHS Ester (Step 2)

Items needed: NHS Ester solution from **step B10**, Antibody Solution from **step A11**, Reaction Buffer (CM02001, orange label), Solution A (CM01008, blue color insert)

C1. Add NHS ester solution from **Step B10** and **Reaction Buffer** (orange label) to the tube containing **Antibody** solution from **Step A11**. Set up the reaction as in the following table based on your target degree of labeling (DOL). Add the NHS ester solution first. When you add the NHS ester solution, place the pipette tip inside the sample solution and then dispense the NHS ester slowly while swirling the pipette tip. If your compound is very hydrophobic, you might also notice some solid precipitate out after you finish adding all the NHS ester solution. Add **Solution A** instead of **Reaction Buffer** afterwards to bring the precipitate back to solution. If the solution is not clear, move on to step **C2**. The precipitate will be removed during the purification. **Dispose of all the leftover NHS ester solution as hazardous waste after the experiment is done.**

	NHS ester solution from Step B10 (µL)	Reaction Buffer or Solution A (µL)
Target DOL: 1-2	3	57
Target DOL: 2-3	6	54
Target DOL: 3-6	**12**	**48**
Target DOL: 4-8	24	36

DOL: The target DOL will depend on the number of Lys groups in your antibody and the hydrophobicity of your small molecule. If your antibody has limited Lys groups, the average DOL may be much lower. If your small molecule is very hydrophobic, you might also get lower DOL. **Recommended to use 12 µL of NHS ester solution and target DOL 3-6.**

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Calculation 2 for Less Antibody (Ab):

Volume of NHS Ester Solution to be Transferred in Step C1 (µL)

	NHS ester solution from Step B10 (µL)	Reaction Buffer or Solution A (µL)
Target DOL: 1-2	$Ab\ in\ mg\ \times 1$	$Ab\ in\ mg\ \times 19$
Target DOL: 2-3	$Ab\ in\ mg\ \times 2$	$Ab\ in\ mg\ \times 18$
Target DOL: 3-6	$Ab\ in\ mg\ \times 4$	$Ab\ in\ mg\ \times 16$
Target DOL: 4-8	$Ab\ in\ mg\ \times 8$	$Ab\ in\ mg\ \times 12$

C2. Incubate the solution from **Step C1** at RT for 2 h (overnight reaction is OK for extended labeling).

5. Purification of Conjugate

Items needed: Desalting Column (CM03SG10), Labeled Antibody from **Step C2**, Storage Buffer (1x PBS, CM02013, grey label), ADC Stabilizing PBS Buffer (5x) (CM02022, pink label), 1.5 mL Centrifuge Tube (CM03CT2)

D1. In a chemical hood, securely attach the **Desalting Column** to a support stand, lab frame, or any support rod. Remove the top and bottom caps from the column and allow the excess liquid to flow through by gravity. Collect the liquid in a flask.
D2. Add 5 mL of **Storage Buffer** and allow the buffer to completely enter the gel bed by gravity flow.
D3. Repeat **Step D2** twice.
D4. Spin the labeled antibody solution from **Step C2** before opening it. Add the entire antibody solution to the column. Allow the sample to enter the gel bed completely.
D5. Wash the centrifuge tube from **Step D4** once with 100 µL of **Storage Buffer**. Transfer the entire washing solution from the centrifuge tube to the column. Allow the sample to enter the gel bed completely. **Dispose of the centrifuge tube as solid waste.**

Calculation 3 for Less Antibody (Ab):

$$Volume\ of\ Storage\ buffer\ for\ washing\ in\ Step\ \boldsymbol{D5}\ (\mu L) = 500 - Ab\ in\ mg\ \times 133.3$$

D6. Add 500 µL of **Storage Buffer** and allow the liquid to enter the gel bed completely (**Note:** this elution buffer does not contain any of your product, you can let it drain to the waste).
D7. Place a 1.5 mL centrifuge tube under the column. Add 1.0 mL of **Storage Buffer** to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.
D8. Label the tube as your product. Store your conjugate at 4°C. **Dispose of the Desalting Column as solid waste. Dispose of all waste following regulations appropriate for your area.**
D9. Determine the concentration and the estimated DOL by UV/Vis spectrophotometry (see Other Considerations).

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D10. If the conjugate is not used immediately for the experiment, add **Stabilization PBS buffer (5x)** (pink label) to the conjugate from **Step D8**. Aliquot and store the conjugate in a < -20°C freezer or lyophilize to dryness for long-term storage.

Conjugate is Ready for Your Experiment

Specification for your product

The approximate concentration of the antibody small molecule conjugate is 1.5 mg/mL in PBS buffer assuming 50% recovery. You can determine the concentration by UV/vis spectrophotometry and the loading by HPLC or MS.

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Other Considerations

1. Concentration Determination for IgG Antibody (Unlabeled)

The accuracy of the IgG amount is important for obtaining an optimized DOL in this protocol. The simplest assay method for determining IgG concentration in solution is to measure the absorbance of the IgG at 280 nm (UV range) ($A_{1\ mg/mL}$ = 1.4).

If your antibody comes with a buffer that has no UV absorbance at 280 nm, you can measure the UV absorbance prior to starting an experiment.

$$Concentration\ (mg/mL)\ of\ IgG = \frac{(A280)}{1.4}$$

If your antibody comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **Step A11** after exchanging it with Reaction Buffer and assuming **95%** recovery of the IgG after buffer exchange. Reaction Buffer does not contain any substances that will interfere with the UV measurement at 280 nm. The total volume of Reaction Buffer added in **Step A10** can be estimated based on the initially estimated amount of antibody and will not affect the conjugation too much if the volume is off to some extent.

$$Concentration\ (mg/mL)\ of\ Starting\ IgG = \frac{(A280)}{1.4 \times 0.95}$$

After calculating the total amount, follow the calculations in **Steps A10, C1**, and **D5** to obtain the correct volumes to be added in each step.

2. Concentration Determination for Conjugate

To determine the concentration of the conjugate, dilute your conjugate from **Step D8** with **Storage Buffer (**1x PBS buffer). Measure the UV absorbance of the conjugate at 280 nm (A280) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) * 1000000}{L\ (210000 + n * \ \text{Ɛ})}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 150000}{L(210000 + n * \ \text{Ɛ})}$$

Where **L** is the UV cell path length (cm); **n** is the average loading of small molecule; and Ɛ is the molar extinction coefficient of your small molecule ($cm^{-1}M^{-1}$). If the small molecule has only weak or no UV absorbance at 280 nm, you can use 0. For a typical IgG with MW of 150,000, the molar extinction coefficient is 210,000 $M^{-1}cm^{-1}$.

3. MW Calculation

Calculation of the MW of the conjugate:

$$MW(Conjugate) = n \times (MWs - 18) + 150000$$

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Where **n** is the average loading of the small molecule and MWs is the MW of small molecule.

4. Degree of Labeling (DOL) calculation and Characterization by UV and MS

If your small molecule has a characteristic UV absorbance that is not overlapping with the UV absorbance of antibody, you can use it for the calculation of the DOL. Otherwise, we recommend sending your sample for a MS analysis (either MALDI-TOF MS or LC-MS will be fine). By comparing MS data of the labeled and unlabeled antibody, you can calculate the DOL. If you do not have access to a MS facility, please contact CellMosaic for analysis.

5. Characterization of Conjugate by HIC HPLC

Hydrophobic interaction chromatography (HIC) HPLC can be used to check if an antibody is labeled or not. However, due to the highly heterogeneous nature of surface amine labeling, antibody loaded with the same number of drugs (same DOL) may have slightly different hydrophobicity. CellMosaic offers a high quality and sterilized HIC buffer set (Product #: CM02025) for our customers to use with any HIC column. The CM02025 product sheet contains all of the information and methodology needed to run an HIC HPLC analysis. If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

6. Characterization of Conjugate by SEC HPLC

If you are concerned with the aggregation, you can use size exclusion chromatography (SEC) to check the extent of aggregation. SEC separates the conjugates by apparent molecular weight (MW) or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes. By comparing the SEC profile of unlabeled IgG and the conjugate, you can estimate how much aggregation is in the conjugate. CellMosaic offers two SEC standards (Product #: CM92004 and CM92005) for our customers to use with any SEC column. The CM92004 product sheet contains all of the information and methodology you need to run an SEC HPLC analysis. If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

7. ADC Stabilizing Buffer

CellMosaic's proprietary ADC stabilizing PBS buffer (5x) (Product #: CM02022) contains 5x PBS buffer and other stabilizers to prevent the hydrophobic drugs from interacting with each other during storage, which would cause the conjugate to precipitate out. Stabilization buffer also helps preserve the structure of the conjugate during lyophilization. The buffer is biocompatible and can be used directly for any *in vitro* and *in vivo* studies. For more information on the stabilization buffers, please check our website.

8. Recommended Storage Conditions

Recommend store your conjugate at 2-8°C. Do not freeze.

If you need to store the conjugate for a longer period of time, dilute your conjugate in Stabilization PBS buffer (5x) (included in this kit). Aliquot and store the conjugate in a < -20°C freezer or lyophilize to dryness. Avoid repeated freeze and thaw cycles.

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9. Submit Samples for HPLC Analysis

If you are submitting samples to CellMosaic for SEC and HIC analysis, please follow these instructions:

1) Go online: https://www.cellmosaic.com/hplc-analysis/, select SEC HPLC Analysis (Product# AS0023) and HIC HPLC Analysis (Product#: AS0025), choose the quantity (number of samples. Bulk discounts available for multiple samples) and submit the order. Alternatively, you can email info@cellmosaic.com for a quote and to place the order.
2) Dilute your un-conjugated antibody in PBS buffer to 1 mg/mL, and then transfer 50 µL of the diluted solution to a 500 µL micro-centrifuge tube. Label the vial properly.
3) Transfer 50 µL of ADC (non-diluted solution) to a 500 µL micro-centrifuge tube and label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.