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PerKit™ Protein Small Molecule Acid Conjugation Kit (CM52408) User Reference Guide

Contents

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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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Kit Components

This kit provides materials to conjugate 15 to 50 nmole of one protein sample (≥20 KDa) 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 (Part#: CM13002)	Reagent A solution (white label)	CM10001	1 unit (40 µL)	2-8 °C
	Reagent B (purple color insert)	CM10002	1 unit	
	Solution A (blue color insert)	CM01006	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 Device	CM03CD010A	2	
	Desalting Column	CM03SG10	1	
	Collection Tubes	N/A	2	
	1.5 mL Centrifuge Tube	N/A	2	
	0.5 mL Centrifuge Tube	N/A	2	
User Materials	Small Molecule Acid	N/A	NOT PROVIDED (User Supplied Material, 20 µmol)	
	Protein (protein)	N/A	NOT PROVIDED (User Supplied Material, 15-50 nmole)	

Reaction Scale: The protocol is optimized for conjugating 50 nmole of protein. If you have less than 50 nmole of protein, 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 protein (protein). Using the kit components, the user converts the carboxylic acid to an activated *N*-hydroxysuccinimide ester (NHS ester), followed by reaction with

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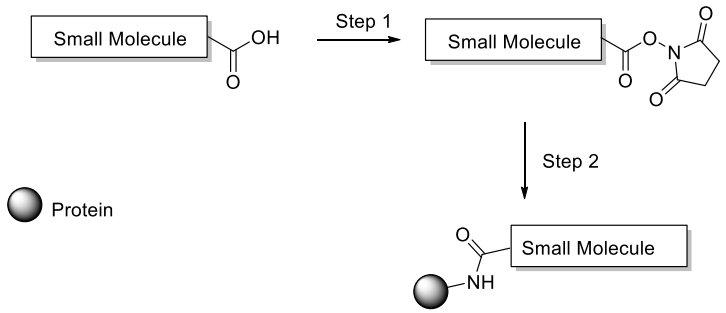
the surface amino groups of the protein to form a stable amide bond. The final product is desalted to remove any unreacted small molecule acid.

Key features of this conjugation kit:

- Offers a simple and easy way to label protein 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
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 protein:
1. Preferably > 90% pure by gel electrophoresis
2. Total amount: 15-50 nmole protein content as measured by UV. Note: the accuracy of your protein amount is the single most important factor to obtaining an optimized DAR. Please refer to the section Other Considerations in this manual to measure the protein amount.

Support

Customer can request a recommendation for the conjugation if the molecule has a special feature or a low amount of protein 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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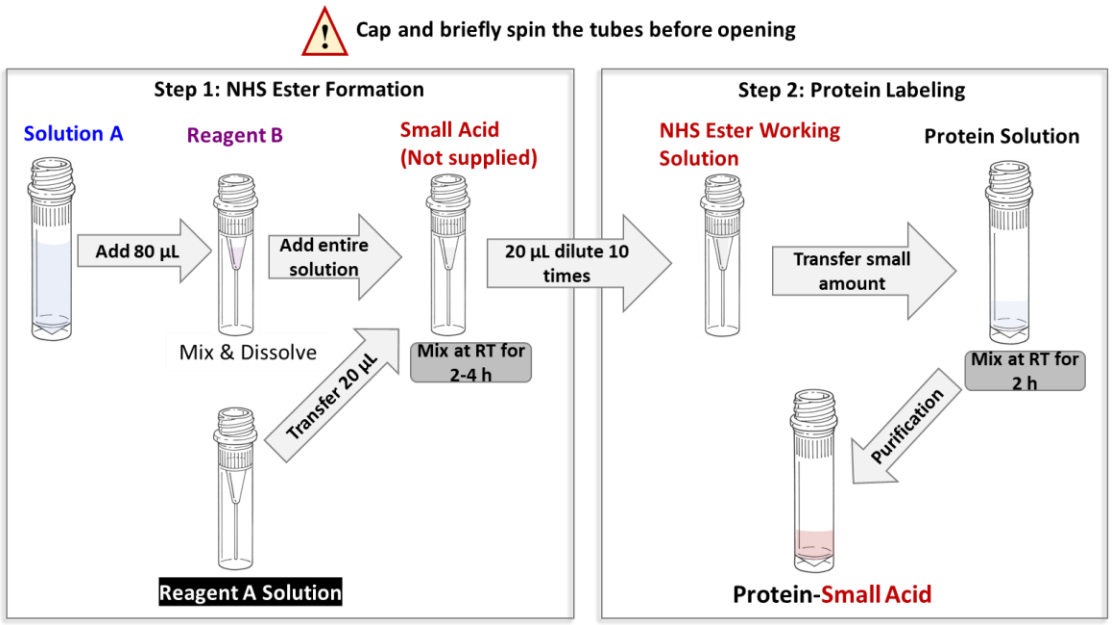
Protocol



Scheme 1. Schematic diagram of the workflow for preparing protein-small molecule conjugates starting with 50 nmole of protein (volume of reagents varies if the amount of protein is < 50 nmole).

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 Protein Samples for Conjugation

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

Total amount of protein used for the conjugation is 50 nmole per reaction (protein content measured by UV).

Calculation: Amount of protein (mg) = Molecular Weight (MW) of protein x 0.00005

> **Reaction Scale:** If you have less than 50 nmole of protein, 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 protein is supplied as a lyophilized solid, dissolve the protein in 500 µL of **deionized water** and then transfer the entire contents to the **Filter Device**.
- ✓ If your protein is supplied in < 500 µL buffer, transfer your protein 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 protein sample is between 500 and 1000 µL, divide the volume into two **Centrifugal Filter Devices** and add the protein 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 protein 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 protein 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 10 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 10 to 20 minutes. The typical volume is ~40 µL after spinning for 10 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 **440 ± 5 µL** and cap it.

Calculation 1 for Less Protein:

$$Total\ volume\ of\ the\ protein\ solution\ in\ Step\ \mathbf{A10}\ (\mu L) = protein\ in\ nmole\ \times 8.8$$

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A11. Vortex the combined protein 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: NHS Ester Formation Kit (CM13002), 0.5 mL Centrifuge Tubes

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. Open the **NHS Ester Formation Kit** package. Spin the centrifuge tubes containing **Reagent A solution** (white label), **Reagent B** (purple color insert), and **Solution A** (blue color insert) before opening it.

B3. Transfer **20 µL** of **Reagent A solution** (white label) 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** (blue color insert) to **Reagent B** (purple color insert). 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**.

> **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.

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).

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.
> **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**.

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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. Protein Labeling with NHS Ester (Step 2)

Items needed: NHS Ester solution from **step B10**, Protein Solution from **step A11**, Reaction Buffer (CM02001, orange label), Solution A (CM01006, blue color insert)

C1. Add NHS solution from **Step B10** and **Reaction Buffer** (orange label) to the tube containing **Protein** 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	7.5	52.5
Target DOL: 2-3	15	45
Target DOL: 3-6	**30**	**30**
Target DOL: 4-8	60	0

DOL: The target DOL will depend on the number of Lys groups in your protein and the hydrophobicity of your small molecule. If your protein 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 30 µL of NHS ester solution and target DOL 3-6.**

Calculation 2 for Less Protein (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	$Protein\ in\ nmole \times 0.15$	$Protein\ in\ nmole \times 1.05$
Target DOL: 2-3	$Protein\ in\ nmole \times 0.3$	$Protein\ in\ nmole \times 0.9$
Target DOL: 3-6	$Protein\ in\ nmole \times 0.6$	$Protein\ in\ nmole \times 0.6$
Target DOL: 4-8	$Protein\ in\ nmole \times 1.2$	0

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

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5. Purification of Conjugate

Items needed: Desalting Column (CM03SG10), Labeled Protein from **Step C2**, Storage Buffer (1x PBS), ADC Stabilizing PBS Buffer (5x), 1.5 mL Centrifuge Tube

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 times.
D4. Spin the labeled protein solution from **Step C2** before opening it. Add the entire protein solution to the column. Allow the sample to enter the gel bed completely. **Dispose of the centrifuge tube as solid waste.**
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 Protein:

$$Volume\ of\ Storage\ buffer\ for\ washing\ in\ Step\ \boldsymbol{D5}\ (\mu L) = 600 - Protein\ in\ nmole \times 10$$

D6. Add 400 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 1.1 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).
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 protein small molecule conjugate is 22.7 µM 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 Protein (Unlabeled)

The accuracy of the protein amount is important for obtaining an optimized DOL in this protocol. The simplest assay method for determining protein concentration in solution is to measure the absorbance of the protein at 280 nm (UV range) if you know the extinction coefficient of your protein. Otherwise you can use a protein assay such as BCA to determine the concentration.

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

$$Concentration\ (M)\ of\ protein = \frac{(A280)}{\varepsilon \times L}$$

Where **L** is the UV cell path length (cm) and **Ɛ** is the extinction coefficient of your protein ($cm^{-1}M^{-1}$)

If your protein comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **step B11** after exchanging it with Buffer A and assuming **95%** recovery of the protein after buffer exchange. Buffer A does not contain any substances that will interfere with the UV measurement at 280 nm. The total volume of Buffer A added in **Step B10** can be estimated based on the initially estimated amount of protein and will not affect the conjugation too much if the volume is off to some extent.

$$Concentration\ (M)\ of\ Starting\ Protein = \frac{(A280)}{\varepsilon \times L \times 0.95}$$

After calculating the total amount, follow the calculations in **Steps A10, C2**, 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 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:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) * 1000000}{L\ (\varepsilon p + n * \varepsilon s)}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 150000}{L(\varepsilon p + n * \varepsilon s)}$$

Where **L** is the UV cell path length (cm); **n** is the average loading of small molecule; **Ɛp** is the molar extinction coefficient of your small molecule ($cm^{-1}M^{-1}$); and **Ɛs** is the molar extinction coefficient of your small molecule ($cm^{-1}M^{-1}$). If the small molecule has only

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weak or no UV absorbance at 280 nm, you can use 0. For a typical protein 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:

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

Where **n** is the average loading of the small molecule; MWs is the MW of small molecule; and MWp is the MW of protein

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 protein, 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 protein, 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 protein is labeled or not. However, due to the highly heterogeneous nature of surface amine labeling, protein 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 protein 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.

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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.

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 protein 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 conjugate (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.

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Appendix: Typical Kit Performance Data (CellMosaic)

SM acid information: 6-carboxyfluorescein (20 µmol)
Protein information: Bovine Serum Albumin (BSA) (50 nmol)
Kit Lot number: 5506.S51207

Figure 1: Size-exclusion HPLC analysis of the purified fluorescein labeled BSA from **Step D8** using various amounts of Fluorescein NHS ester.

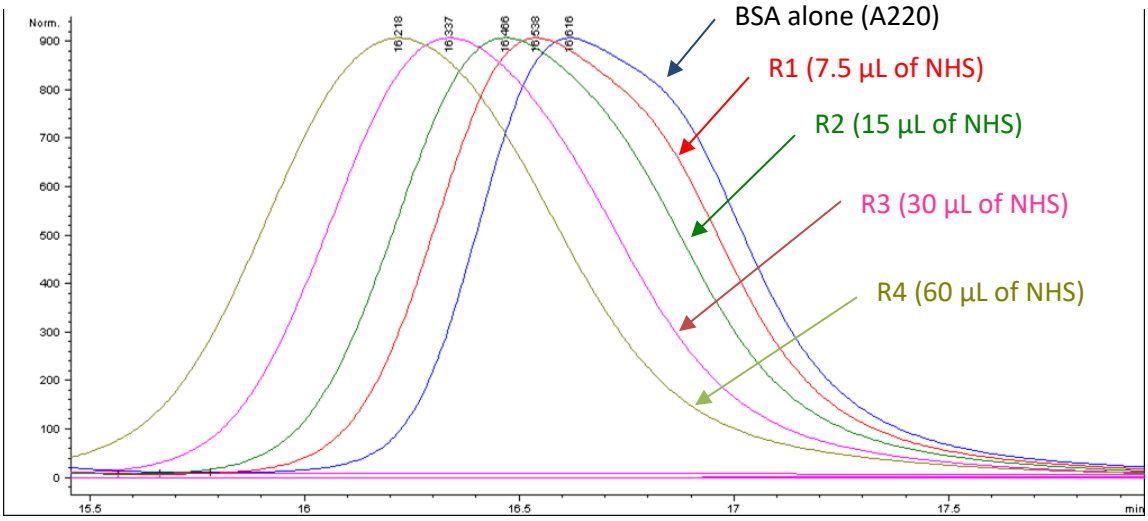

Figure 2: UV/Vis spectrum of the purified fluorescein labeled BSA from Step D6 using various amounts of Fluorescein NHS ester.

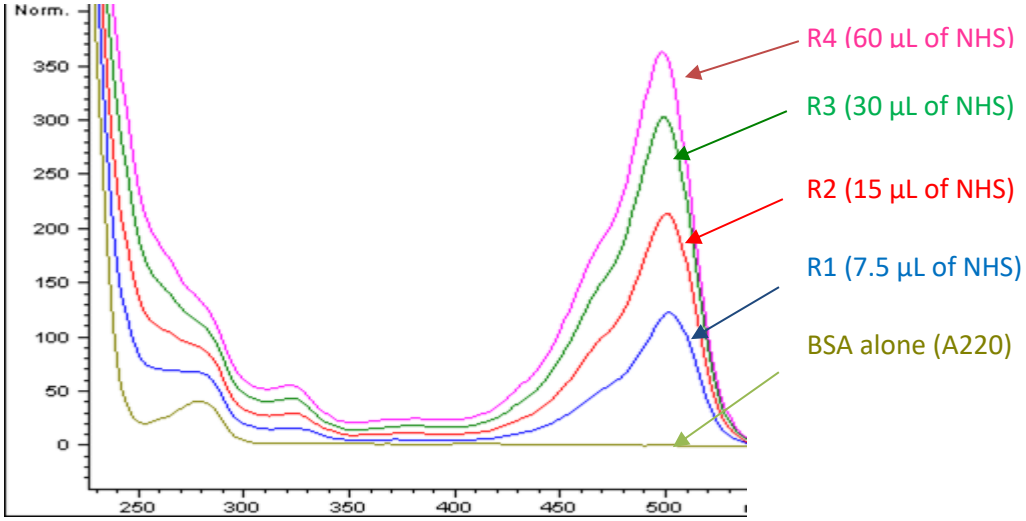

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Table 1: Summary results of the purity and the average DOL of the fluorescein labeled BSA from Step D6 (**Note**: Fluorescein is a very hydrophobic compound. A lower DOL was obtained.)

Sample	NHS ester solution from Step B7 (μL)	SEC HPLC Rt (min) (220 nm)	ΔRt	% of small molecule after purification	Calc. DOL based on UV/Vis
BSA	N/A	16.616	N/A	N/A	N/A
R1	7.5	16.538	0.078	0	1
R2	15	16.466	0.150	0	1.8
R3	30	16.337	0.279	1.3	2.7
R4	60	16.218	0.398	2.2	3.3