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PerKit™ Protein SN38 Conjugation Kit (CM11430x1 & CM11430x3) 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 6.67–20 nano-mole (nmol) of one (CM11430x1) or three (CM11430x3) Protein samples (**MW ≥ 20KDa**) with SN38.

⚠ Upon receipt, please remove **Box 1** and store in a freezer at or below -20°C.
Store **Box 2** in a refrigerator at 2-8°C.

	Name	Part #	Quantity (CM11430x1)	Quantity (CM11430x3)	Storage condition
Box 1	*O*-succinyl SN38 NHS ester (red label)	CM11003.1	1 unit	3 units	-20°C, dry
Box 2	Solution A (blue label)	CM01008	0.5 mL	1.5 mL	2-8°C
	Buffer A (orange label)	CM02001	4 mL	12 mL	
	Storage Buffer (1 x PBS buffer) (grey label)	CM02013	20 mL	60 mL	
	ADC Stabilizing PBS Buffer (5x) (pink label)	CM02022	0.5 mL	1.5 mL	
	Centrifugal Filter Device	CM03CD010A	2	6	
	Desalting Column	CM03SG10	1	3	
	Collection Tubes	CM03CT0	4	12	
	1.5 mL Centrifuge Tube	CM03CT2	1	3	
	2.0 mL Centrifuge Tube	CM03CT3	1	3	
	Hazardous Waste Bag	CM03HZ1	1	3	
User Material	Protein (MW: ≥20 KDa)	N/A	NOT PROVIDED (User Supplied Material, **6.67–20 nmol** needed per reaction)		

Reaction Scale: The protocol is optimized for conjugating 20 nmol of Protein. If you have less than 20 nmol of protein, use the calculations in **Steps B10**, **C2**, **D5,** and **D6** 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 label any Protein (MW ≥ 20KDa) with SN38 via a releasable ester linker. The user supplies the Protein. This kit includes *O*-succinyl SN38 NHS ester, which can be coupled to the Protein directly via surface amines in a single step. The product is then purified to remove any unreacted drug.

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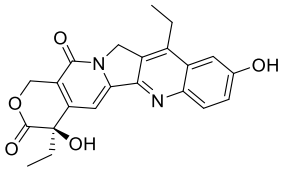
Key features of this conjugation kit:

- Offers a simple and easy way to label protein with SN38 with minimum exposure to the chemotherapeutic drug
- Releasable linkage
- Fast and easy preparation: 6 h preparation with <1 h hands-on time
- All reagents and supplies included for preparation and purification
- DPR (drug protein ratio) with average 3 SN38 labeling per Protein
- Included stabilizing buffer for long-term storage
- More than 95% conjugated products (free of any unreacted drugs)

Drug Information:



- **Name**: SN38 (7-ethyl-10-hydroxycamptothecin)
- **CAS number**: 86639-52-3
- **Chemical formula**: $C_{22}H_{20}N_2O_5$
- **MW**: 392.41
- **Mechanism of action**: Inhibition of topoisomerase I leads to inhibition of both DNA Replication and DNA transcriptions
- **Medical usage**: Pro-drug Irinotecan (brand name: Camptosar) is used for treatment of colon and small cell lung cancer.

Requirement for Protein:

1. Preferably > 90% pure by gel electrophoresis.
2. Total amount: 6.67−20 nmol protein content as measured by UV. Note: the accuracy of your protein amount is the single most important factor to obtaining an optimized DPR. Please refer to the section “Other Considerations” in this manual to measure the protein amount.
3. MW: ≥20 KDa (for smaller MW, contact CellMosaic for custom conjugation).

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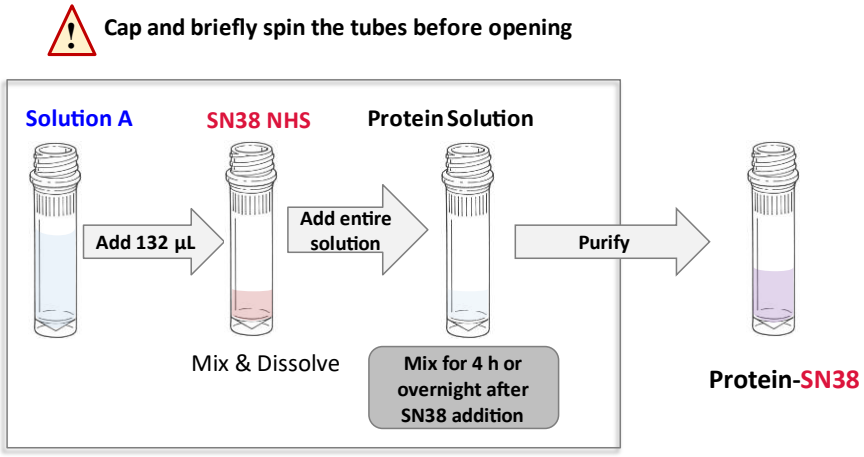
Support

Customer can request a recommendation for the conjugation if the Protein has a special feature or a less than 6.67 nmol of protein to be labeled. CellMosaic provides other accessory tools, such as buffers, standards, and reagents for PDC research. CellMosaic also provides fee-based support services to customers who need help analyzing the final conjugates by HPLC and determining the DPR.

Protocol



Scheme 1. Schematic diagram of the workflow for preparing Protein−SN38 conjugates starting with 20 nmol of Protein (volume of reagents varies if the amount of Protein is < 20 nmol and the MW is <60 KDa).

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
- Chemical hood
- Support stand, lab frame, or any support rod for desalting column
- Flask
- Personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves)

2. Prepare Site and Reagents for Labeling Experiment

> **Note:** SN38 is very hydrophobic. Protein−drug conjugates with average 3 SN38 per protein tend to aggregate and precipitate out from the solution over time. It is recommended that the labeling experiment be planned for only a few days or right before your other experiments. If not possible, then please use the stabilization PBS buffer to store under recommended conditions.

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Ensure you use personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves) while handling SN38. Locate a clean space inside a chemical hood.

A1. Remove **Box 1** containing ***O*-succinyl SN38 NHS ester** (red label) from the -20°C freezer and warm to RT before opening the bag.
A2. Remove **Box 2** from the refrigerator. Take the hazardous waste bag and place it inside the chemical hood for solid waste disposal. Bring the rest of the items to a lab bench.
A3. Briefly spin the centrifuge tube containing **SN38**. Place the **SN38** tube in a tube holder inside a chemical hood and wait until the Protein is ready for conjugation.

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

A4. Set the temperature of the incubator or shaker to 25 °C.

3. Preparation of Protein Samples for Conjugation

Items needed: Filter Devices (CM03CD010A), Collection Tubes (CM03CT0), Buffer A (CM02001, Orange label), 1.5 mL Centrifuge Tube (CM03CT2), Clean Centrifuge Tubes (not provided in the kit).

Total amount of protein used for the conjugation is 20 nmol (protein content measured by UV) per reaction.

Reaction Scale: If you have less than 20 nmol of Protein, use the calculations in **Steps B10**, **C2**, **D5,** and **D6** to obtain the correct volumes to be added in each step.

B1. Insert the **Filter Device** into one of the provided collection tube (microcentrifuge tube with the cap attached). Perform the step based on the following conditions.

- ✓ 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 **Buffer A** 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**. Add **Buffer A** to make up the total volume in each filter device to 500 µL and cap them.
- ✓ If the volume of your protein sample is >1000 µL, add up to 500 µL of sample to each of the two **Filter Devices** and cap them. Repeat Step **B1-B4** until all of the protein sample goes into the **Filter Device**. Move on to Step **B5**. Add **Buffer A** to make up the total volume to 500 µL in each device for the last refill.

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B2. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

B3. 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.)

B4. 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.**

B5. Insert the **Filter Device** back into the collection tube. Add 400-450 µL of **Buffer A** 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.**

B6. Repeat **Step B5** two more times.

B7. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube (use the pipetman to estimate the approximate volume of the concentrated sample).

B8. Add 50−100 µL of **Buffer A** to the **Filter Device** to rinse (actual volume of **Buffer A** added will depend upon the calculated total volume in **Step B10**). Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B7.**

B9. Repeat **Step B8** once.

B10. Add **Buffer A** to the 1. 5 mL micro-centrifuge tube from **Step B9** to make up the total volume of the sample to **618 ± 5 µL** and cap it.

Calculation 1 for Less Protein:

$$Total\ volume\ of\ the\ protein\ in\ Step\ \boldsymbol{B10}\ (\mu L) = protein\ in\ nmol\ \times 30.9$$

B11. Vortex the combined Protein sample for 30 seconds and then spin down.

4. SN38 Labeling

Items needed: *O*-succinyl SN38 NHS Ester (CM11003.1, red label), Solution A (CM01008, blue label), Protein Solution from **Step B11**.

C1. Spin **Solution A** (blue label) to ensure there is no liquid in the cap before opening it. Add **132 µL** of **Solution A** to the **SN38** tube from **Step A3**. Vortex for 30 seconds to 1 minute to dissolve the reagent and then spin down.

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Tip for solubility check: Check the bottom of the micro-centrifuge tube to see if the solution is clear of any solid residue.

C2. Transfer **SN38 solution** from **Step C1** to the centrifuge tube containing protein from **Step B11** according to the following list.

MW (≥60KDa): transfer **the entire SN38 solution** (target 3-5 SN38 per protein)

MW (20-60KDa): transfer **100 µL** (target 2-4 SN38 per protein)

(When you add the SN38 solution, place the pipette tip inside the Protein solution and then dispense the SN38 slowly while swirling the pipette tip. Discard any unused **Reagent A** as hazardous chemical waste **Dispose of the pipette tip and SN38 tube in the hazardous waste bag**.)

Note: The volume of **SN38 solution** being transferred is adjusted based on the MW of your protein so that PDC is not very aggregated. This volume is recommended for the first-time conjugation with a new protein. You can certainly add more volume of SN38 solution to increase the loading for a repeat conjugation of the same protein if the previously synthesized PDC is not very aggregated.

Calculation 2 for Less Protein:

MW (≥60KDa):

$$Volume\ of\ SN38\ solution\ to\ be\ transferred\ in\ Step\ \boldsymbol{C2}\ (\mu L) = Protein\ in\ nmol\ \times 6.6$$

MW (20–60KDa):

$$Volume\ of\ SN38\ solution\ to\ be\ transferred\ in\ Step\ \boldsymbol{C2}\ (\mu L) = Protein\ in\ nmol\ \times 5$$

C3. Cap the centrifuge tube. Mix at 25°C or ambient temperature for 4 h or overnight (≤ 16 h).

Tip for mixing: You can use a nutator, a shaker, vortex, or an incubator shaker for mixing. If you are using end to end nutating, make sure your centrifuge is capped properly. If you don't have any of this equipment, you can let the centrifuge tube sit at the bench with manual mixing by pipetting every 20 minutes.

Time-saving tip: While waiting for the reaction to complete, you can move on to **Step D1** and equilibrium the column for purification.

5. Purification of Conjugate

Items needed: Desalting Column (CM03SG10), Storage Buffer (1x PBS, CM02013, grey label), 2.0 mL Centrifuge Tube (CM03CT3), Hazardous Waste Bag (CM03HZ1), Protein Solution from **Step C3**.

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 **PBS buffer** and allow the buffer to completely enter the gel bed by gravity flow.

D3. Repeat **Step D2** twice.

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D4. Spin the SN38 labeled Protein solution from **Step C3** 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 in the solid waste bag.**

D5. Add 250 µL of **PBS 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).

Calculation 5 for Less Protein:

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{D5}\ (\mu L) = 1000 - protein\ in\ nmol\ \times 37.5$$

D6. Place a 2.0 mL centrifuge tube under the column. Add 1.25 mL of PBS buffer to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.

Calculation 6 for Less Protein:

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{D6}\ (\mu L) = 500 + protein\ in\ nmol\ \times 37.5$$

D7. Label the tube as your product. Store your conjugate at 4°C. **Dispose of the Desalting Column in the hazardous waste bag and seal the bag. Dispose of the waste following regulations appropriate for your area.**

D8. Determine the concentration and the estimated DPR by UV/Vis spectrophotometry (see Other Considerations).

D9. If the PDC is not used within 4 h for the experiment, add **ADC Stabilizing PBS buffer (5x)** (pink label) to the PDC from **Step D7**. If the total volume of your PDC is 1.25 mL, you need to add 312.5 µL of Stabilization PBS buffer. Aliquot and store the conjugate in a < -20°C freezer or lyophilize to dryness for long-term storage.

ADC Stabilizing PBS Buffer (5x): Stabilizing buffers are biocompatible and will not interfere with any *in vitro* or *in vivo* studies. The buffer does not contain preservative, protease inhibitors, reducing agents, metal chelators such as EDTA, or other carrier proteins. If needed, all buffer components can be removed by dialysis or desalting before use in downstream assays. For more information, see Other Considerations.

Conjugate is Ready for Your Experiment

- **Specification for your product:** SN38-labeled proteins with an average drug-to-protein ratio (DPR) of 3. A typical batch contains over 99% conjugated products by SEC and is free of any unreacted drug. The approximate concentration of the PDC is 9.6 µM in PBS buffer assuming 60% recovery (without the ADC stabilizing buffer).

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Other Considerations

1. Concentration Determination for Protein (Unlabeled)

The accuracy of the protein amount is important for obtaining an optimized drug loading in this protocol. The simplest assay method for determining protein concentration in solution is to measure the absorbance of the protein at 280 nm 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 **ε** 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 B10, C2**, **D5**, and **D6** to obtain the correct volumes to be added in each step.

2. Concentration Determination for PDC

To determine the concentration of the PDC, dilute your conjugate from **Step D7** 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:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) * 1000000}{L\ (\varepsilon + n * 6100)}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 150000}{L(\varepsilon + n * 6100)}$$

Where **L** is the UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute the conjugate 4 times to obtain a good reading.

Where **n** is the average molar ratio of SN38 per protein. use 2 for protein with MW of 20-60KDa, 3 for protein with MW ≥60KDa.

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The molar extinction coefficient for SN38 is 6100 $M^{-1}cm^{-1}$ based on CellMosaic's experimental data.

3. MW Calculation

Calculation of the MW of the conjugate:

MW(PDC) = n x 474.5+ MW (Protein)

Where **n** is the average molar ratio of SN38 per Protein. Use 2 for protein with MW of 20-60KDa, 3 for protein with MW ≥60KDa.

4. Drug-to-Protein Ratio (DPR) and Characterization by UV and HPLC

In this kit, the target DPR is 3.

A) If you know the extinction efficient of your protein at 280 and 380 nm, you can use UV to determine the DPR. To estimate the DPR, you first have to obtain the UV absorbance ratio (R) of your conjugate at 380 nm and 280 nm.

$$R = {(A380)}/{(A280)}$$

You can also use the following formula to calculate the estimated DPR (for reference only):

$$DPR = \frac{(E280nm\ of\ protein \times R - E380nm\ of\ protein)}{(20985 - 6100 \times R)}$$

SN38: $E_{280\ nm}$ = 6100 $M^{-1}cm^{-1}$ (data from CellMosaic) and $E_{380\ nm}$ = 20985 $M^{-1}cm^{-1}$ (Nakatsuji M. et al. Human Lipocalin-Type Prostaglandin D Synthase-Based Drug Delivery System for Poorly Water-Soluble Anti-Cancer Drug SN-38. *PLOS One* **2015***, 10(11):* e0142206).
Protein: most of the protein doesn't have any UV absorbance at 380 nm.

B) If you don't know the extinction efficient of your protein, we strongly recommend sending your sample for an intact MS analysis (either MALDI-TOF MS or LC-MS will be fine). By comparing the intact MS of PDC and unlabeled protein, you can calculate the average DPR. If you do not have access to a MS facility, please contact CellMosaic for analysis.

5. Characterization of PDC by HIC HPLC

For PDCs prepared via surface amines of the Protein, hydrophobic interaction chromatography (HIC) HPLC can be used to check if a 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 DPR) may have slightly different hydrophobicity. For a typical SN38 PDC, a broad peak will be seen without clear separation of the peaks.

CellMosaic offers an 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.

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If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

6. Aggregation and Precipitation Issue for SN38 Labeling and Characterization by SEC HPLC

SN38 is very hydrophobic. This kit is designed to minimize the aggregation and precipitation issues generally seen with SN38 labeling. However, you may still notice some solid precipitate out or PDC aggregation during the reaction. The precipitate will be removed during purification. Depending on the properties of your Protein, recovery will be 40-80%. 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 PDC, you can estimate how much aggregation is in the PDC.

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. Use of ADC Stabilizing Buffer for PDCs

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 PDCs to precipitate out. Stabilization buffer also helps preserve the structure of the PDCs 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

SN38 is linked to protein through a releasable linker. Recommended use within few days if you store SN38-PDC at 2-8°C. Based on our preliminary data, the conjugate made with this kit can remain stable in PBS buffer for few days at 2-8°C (no long-term stability data). The stability of your conjugate may be different due to your Protein and should be checked either by HPLC or UV. If you need to store the PDCs for longer term, please dilute your PDC 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 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

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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 to 1 mg/mL in PBS buffer, then transfer 50 µL of the diluted solution to a 500 µL microcentrifuge tube. Label the vial properly.
3) Transfer 50 µL of PDC (non-diluted solution) to a 500 µL microcentrifuge tube and label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.

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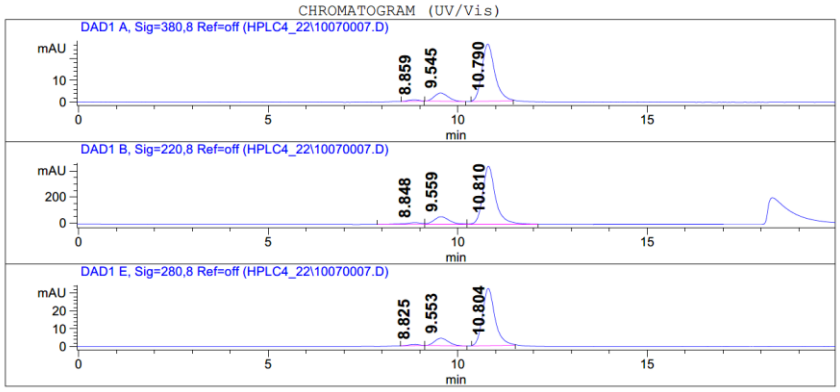
Appendix: Typical Kit Performance Data (LC analysis, CellMosaic)

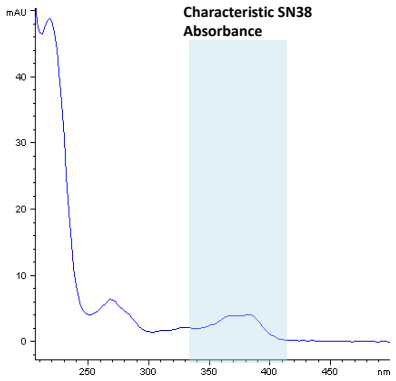
Protein information: BSA

Lot number: DCM11430.S2.D8.1006B

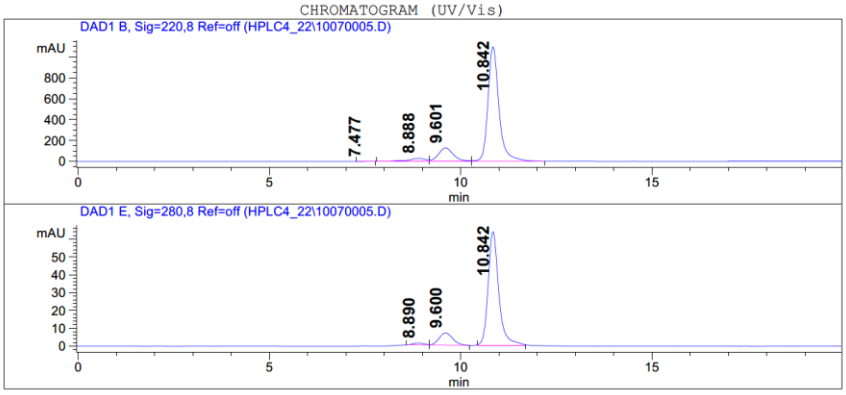
Figure 1: SEC HPLC analysis of purified SN38-BSA following the exact procedure of DCM11430 (Inset: UV/Vis spectra of SN38-BSA). Scale of the reaction: 1.4 mg of protein.



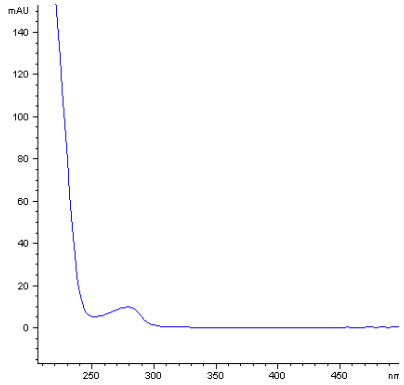
BSA-SN38 Conjugate



UV/vis spectra of BSA-SN38



BSA-Only



UV/vis spectra of BSA-only

Summary of the results:

R value (consider the total peaks)	0.884
Average DPR based on R value	2.48
Extent of Protein aggregation (%)	None detected
Unreacted Protein (%) after purification, step 5	0
Unreacted SN38 (%) after purification, step 5	0
Recovery (%)	58%