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

PerKit™ BSA Small Molecule Acid Conjugation Kit (CM52403) 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.

For Research Use Only. Not for Use in Diagnostic Procedures.

The information in this document is subject to change without notice. CellMosaic assumes no responsibility for any errors that may appear in this document. In no event shall CellMosaic be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple, or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

NOTICE TO PURCHASER: LIMITED LICENSE

The purchase of this product includes a limited, non-transferable license to use this product to practice the labeling methods using the reagents solely for the purchaser's research activities. The license granted herein is personal to the original purchaser and may not be transferred to any other party outside the purchaser's company. No other right or license is conveyed or granted either expressly, by implication or by estoppel, to resell or repackage this product. Further information can be obtained by contacting:

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

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

Kit Components

This kit provides materials to conjugate 5 mg of BSA 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)	
	Reagent B (purple color)	CM10002	1 unit	
	Solution A (blue label)	CM01008	0.5 mL	
Plastic Bag 2	BSA (dark red label)	CM14001	5 mg (protein content)	
	Reaction Buffer (orange label)	CM02001	1 mL	
	1 x PBS buffer (grey label)	CM02013	20 mL	
Plastic Bag 3	Desalting Column	CM03SG10	1	
	2 mL Centrifuge Tube	CM03CT3	1	
	0.5 mL Centrifuge Tubes	CM03CT1	2	
Small Molecule Acid	Small Molecule Acid	N/A	NOT PROVIDED (User Supplied Material, 20 µmol)	

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

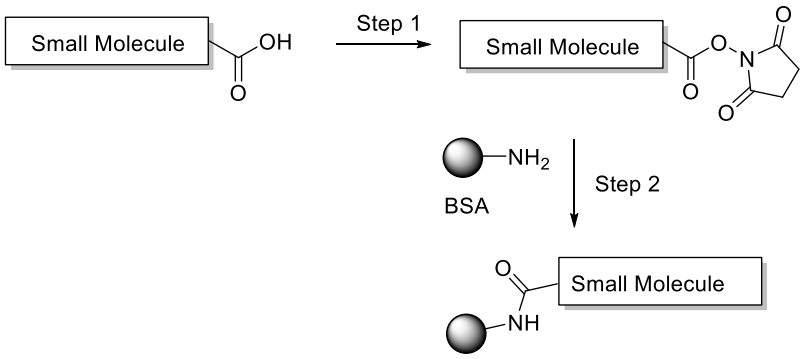
The kit is designed to work with small molecules containing one carboxylic acid functional group. The user supplies the small molecule. Using the kit components, the user converts the carboxylic acid to an activated *N*-hydroxysuccinimide ester (NHS ester), followed by reaction with the surface amino groups of BSA 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 BSA with small molecules containing carboxylic acid
- Fast and easy preparation: 4 h preparation and less than 30 minutes hands-on time
- Target average degree of loading: 3-6
- All reagents and supplies included for preparation and purification
- Over 90% pure conjugated products by SEC

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

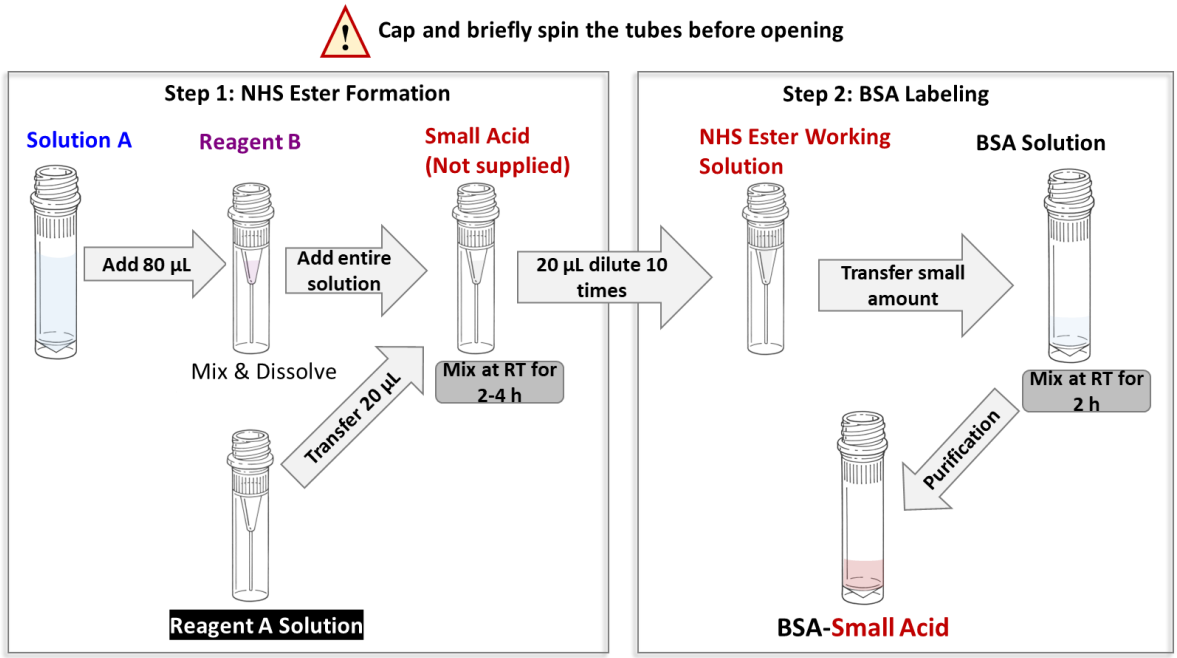


Requirement for small molecule:
1. Preferably > 90% pure
2. Total amount: 20 µmol
3. Absence of primary or secondary amine groups
4. Non-hindered aliphatic carboxylic acid
5. For molecule containing aromatic carboxylic acid, hindered aliphatic carboxylic acid, or hydroxyl groups, please consult CellMosaic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

Support

Customer can request a PerKit™ sheet containing the calculation, chemical structure, MW of the customer's final conjugate, and a recommendation for the conjugation if the molecule has a special feature or a low amount of small molecule is available. CellMosaic also provides additional support services to customers who need help analyzing the intermediates and final conjugates.

Protocol



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

Scheme 1. Schematic diagram of the work flow for preparing BSA-small molecule conjugates

1. Lab Instrumentation Needed

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

2. NHS Ester Formation (20 µmol scale)

Items needed: Reagent A Solution (CM10001, white label), Reagent B (CM100002, purple color insert), Solution A (CM01008, blue label), 0.5 mL Centrifuge Tubes (CM03CT1).

A1. 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

A2. 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.

A3. Transfer **20 µL** of **Reagent A solution** to the tube containing small molecule from **Step A1**. Vortex for 30 seconds or sonicate for a few minutes to ensure most of the solid is dissolved (**Note:** if there are some solids left, that is fine for this step). Centrifuge the tube to get all of the liquid down to the bottom.

A4. 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. Transfer the entire solution to the tube containing small molecules from **Step A3**.

A5. Vortex for 30 seconds or sonicate for a few minutes to ensure all of the solid is dissolved. Centrifuge the tube to get all of the liquid down to the bottom. (**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 A3, A4, & A5): 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.

Tip for precipitation check (Step A7): 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.

A6. Incubate the mixture at RT for **2 h**.

A7. 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. If not, leave the centrifuge tube in the incubator for another 2 h. (**Note**: If there is no solid precipitated out after 4 h of reaction, please consult with CellMosaic for an alternative method).

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

A8. 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**.

A9. Spin the centrifuge tube from **Step A7** to ensure there is no liquid in the cap 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 A8**.

A10. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap.

3. Conjugation with BSA

Items needed: NHS Ester solution from **step A10**, BSA (CM14001, dark red label), Reaction Buffer (CM02001, orange label), Solution A (CM01008, blue color insert)

B1. Briefly spin the tube containing **BSA** (dark red label). Add 0.66 mL of **Reaction Buffer** (orange label**)** to the tube. Vortex for 30 seconds to 1 minute to dissolve the reagent, and then centrifuge to ensure no liquid is in the cap.

B2. Add NHS solution from **Step A10** and **Reaction Buffer** to the 1.5 mL micro-centrifuge tube containing the solution of **BSA**. 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 with constant stirring by pipette tip. Make sure the NHS ester is mixed properly before adding the next drop. If your compound is very hydrophobic and you 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 after adding Solution A, move on to step **B3**. 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 A10 (µL)	Reaction Buffer or Solution A (µL)
Target DOL: 1-3	22.5	67.5
Target DOL: 3-6	**45**	**45**
Target DOL: 4-8	67.5	22.5
Target DOL: 6-11	90	0

B3. Incubate the solution from **Step B2** at RT for 2 h (overnight reaction is OK for extended labeling).

4. Purification of Conjugate

Items needed: Desalting Column (CM03SG10), Labeled BSA from **Step B3**, 1x PBS buffer (CM02013, grey label), 2.0 mL Centrifuge Tube (CM03CT3)

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

C1. In a chemical hood, securely attach the **Desalting Column** to support stands, lab frames, 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.
C2. Add 5 mL of **PBS buffer** and allow the buffer to completely enter the gel bed by gravity flow.
C3. Repeat **Step C2** twice.
C4: Add the sample from **step B3** to the column. Allow the sample to enter the gel bed completely.
C5. Add 250 µL of **PBS buffer** and allow the liquid to enter the gel bed completely.
C6: Place a 2 mL micro-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.
C6: Label the tube as your product. Determine the concentration by UV/Vis spectroscopy. Store your conjugate at 4°C. For long term storage, you can freeze your product.

Conjugate is Ready for Your Experiment

Specification for your product:

Small molecule labeled BSA and free or less than 5% of unreacted small molecules. The approximate concentration of the BSA conjugate is 3.2 mg/mL in PBS buffer assuming 80% recovery. You can determine the concentration by UV/Vis spectroscopy and the loading by HPLC or MS.

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

Other Considerations

1. Concentration Determination for BSA Conjugate

To determine the concentration of the BSA conjugate, dilute your conjugate from **Step C6** 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:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) * 1000000}{L\ (43824 + n * \varepsilon s)}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 66400}{L(43824 + n * \varepsilon s)}$$

Where **L** is the UV cell path length (cm); **n** is the average loading of small molecule; and **Ɛs** 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. The extinction coefficient at 280 nm for BSA is 43,824 $M^{-1}cm^{-1}$.

2. MW Calculation

Calculation of the MW of the conjugate:

$$MW(Conjugate) = n \times (MWs - 18) + 66400$$

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

3. 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 BSA, 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 BSA, you can calculate the DOL. If you do not have access to a MS facility, please contact CellMosaic for analysis.

4. Characterization of Conjugate by HIC HPLC

Hydrophobic interaction chromatography (HIC) HPLC can be used to check if BSA is labeled or not. However, due to the highly heterogeneous nature of surface amine labeling, BSA loaded with the same number of small molecules (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.

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

5. 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 BSA 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.

6. 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.

7. Recommended Storage Conditions

Recommend storing your conjugate at 2-8°C. Depending on the hydrophobicity of your small molecule, you might be able to store the conjugate without any stabilizer at < -20 °C for long term. However, it is preferrable to dilute your conjugate in CellMosaic's ADC stabilizing PBS buffer (5x) (not included in this kit), aliquot, and store in a < -20°C freezer or lyophilize to dryness. Avoid repeated freeze and thaw cycles.

8. 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) Transfer 50 µL of conjugate (non-diluted solution) to a 500 µL micro-centrifuge tube and label the vial properly (You do not need to submit a separate sample for unconjugated BSA).
3) Ship your samples with a cold pack for overnight delivery.

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

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.

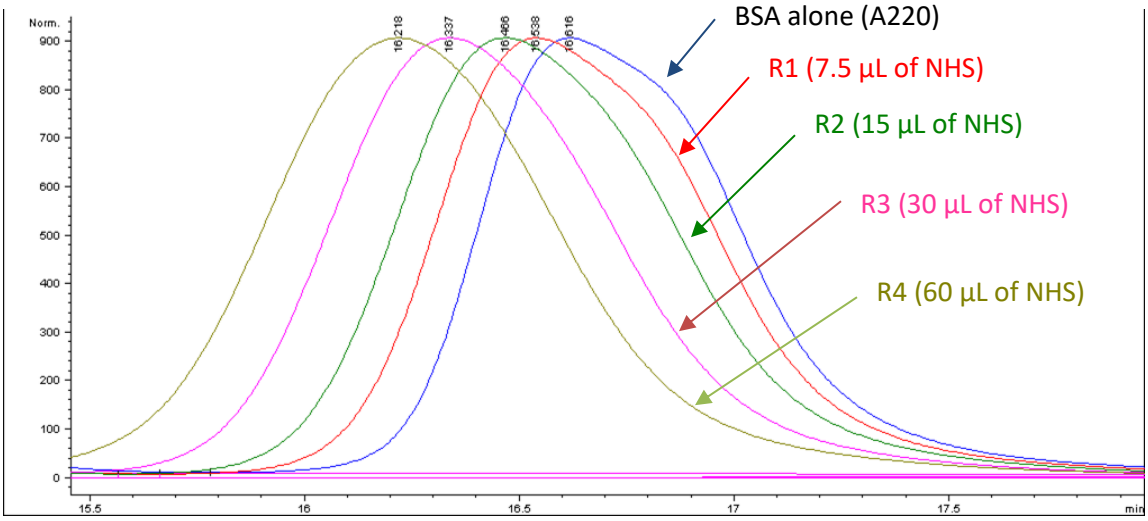
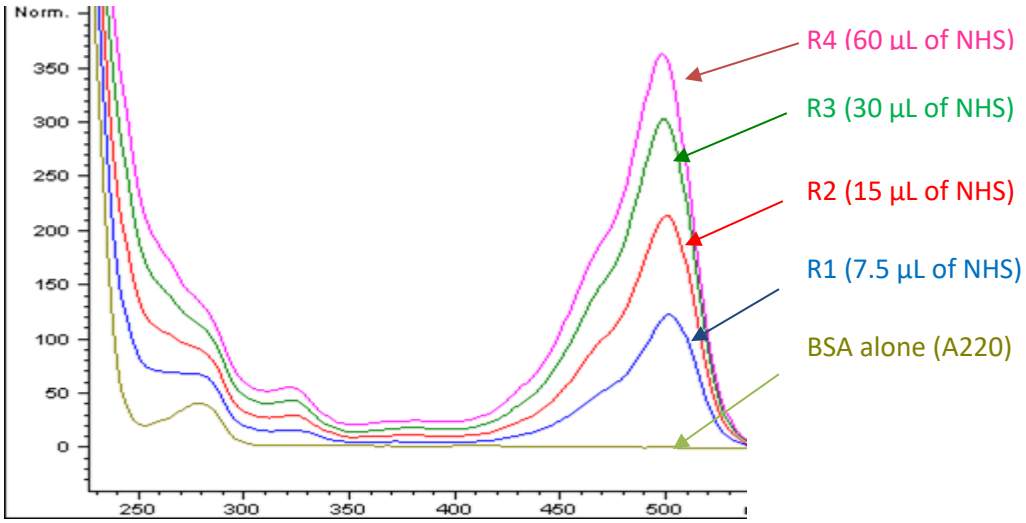


Figure 2: UV/Vis spectrum of the purified fluorescein labeled BSA from Step D8 using various amounts of Fluorescein NHS ester.



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

Table 1: Summary results of the purity and the average DOL of the fluorescein labeled BSA from Step D8 (**Note**: Fluorescein is a very hydrophobic compound. A lower DOL was obtained.)

Sample	NHS ester solution from Step B10 (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