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SepSphere™ Small Molecule Alcohol Immobilization Kit (CM71007) 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 immobilize a small molecule containing a hydroxy functional group onto amine-modified agarose beads.

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

	Name	Part #	Quantity	Storage condition
Box 1	Reagent A (brown label)	CM12003	1 unit	-20°C, dry
	Reagent B (cyan label)	CM12101.1	1 unit	
	Reagent C (green label)	CM12004	1 unit	
Box 2	Amine-modified 4% agarose beads	CM71603	5 mL	2-8 °C
	Solution A (blue label)	CM01006	3.5 mL	
	Buffer A (orange label)	CM02001	20 mL	
	Buffer B (blue violet label)	CM02017	50 mL	
	Storage buffer (0.02% NaN_3 in PBS, grey label)	CM02008	10 mL	
	Snap cap for Reagent A	CM03PT2	1	
	1.5 mL centrifuge tube	CM03CT2	1	
	Column set	CM03SC7	1	
	Centrifuge tube 15 mL	CM03CT4	1	
	Stirrer	CM03PT1	4	
	Airtight syringe 20 mL	CM03SR1	1	
User Material	Small molecule alcohol	N/A	NOT PROVIDED (User Supplied Material, 25 µmol)	
	Deionized water and HPLC vials	N/A	Not provided	

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 hydroxyl group. The customer supplies the small molecule. Using the kit components, the customer converts the hydroxyl group of the small molecule to an activated chloroacetyl group and the amine-modified agarose beads to a thiol-modified agarose, followed by the reaction of both components.

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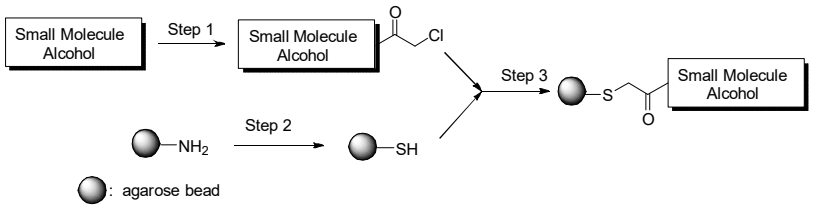
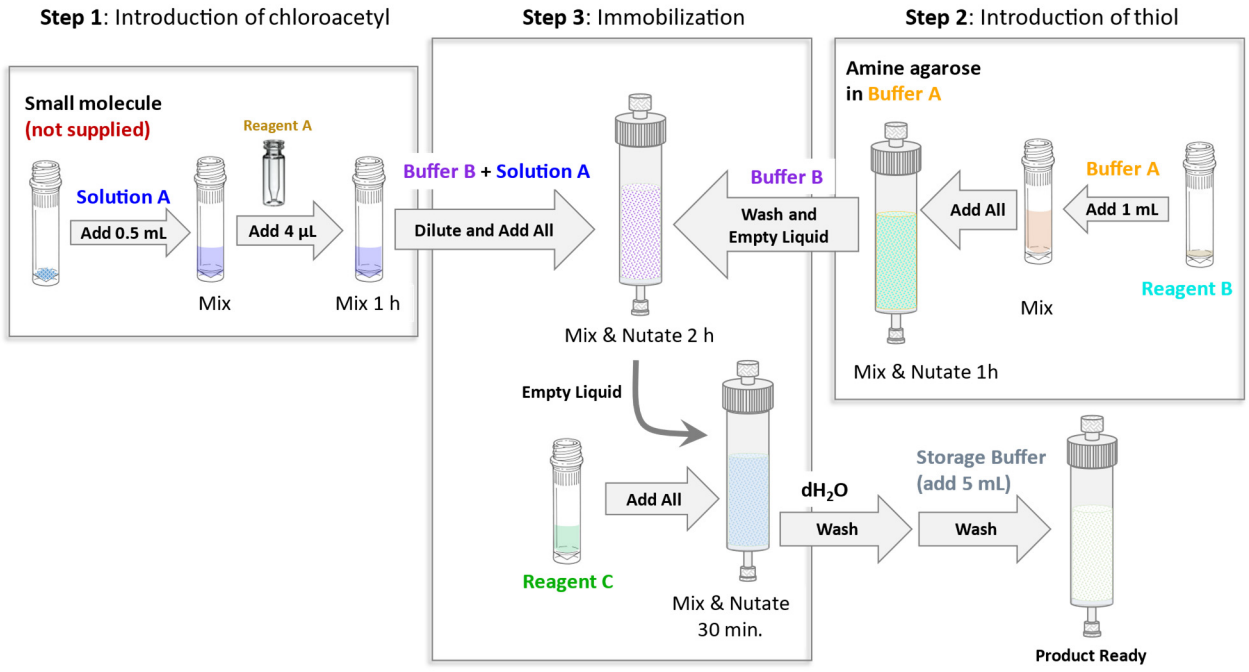
Key features of this SepSphere™ immobilization kit:

- Offers a simple and easy way to label small molecules with a hydroxyl group
- Includes all reagents and supplies needed for preparation
- Uses agarose beads with an optimized amount of amine functional groups for loading to preserve the nature of the agarose beads
- Minimum requirement for immobilization set-up
- Easy preparation with air push mechanism for washing: 6 h preparation time and less than 2 h hands-on time



Requirement for small molecule:
1. Preferably > 90% pure
2. Absence of other reactive functional groups, such as amine and thiol groups
3. For molecules containing a phenol group, multiple hydroxyl groups, or a secondary hydroxy group, please consult CellMosaic prior to conducting the experiment



Scheme 1. Workflow for small molecule alcohol immobilization with 5 mL of amine agarose beads.

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1. Lab Instrumentation Needed

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

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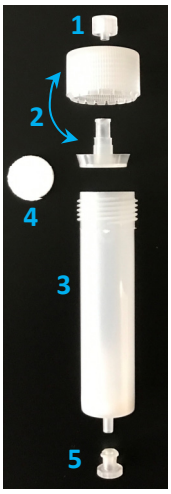
2. Immobilization Lab Techniques at CellMosaic (for reference)

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



Column design and setup:

Design: Column comprises five pieces (see left picture)

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

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

Use of buffer(s) with resin in column

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

Procedure:

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

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Mixing resin in column

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

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

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

Washing resin after stirring

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

Procedure for washing:

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

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

Column setup for immobilization:

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

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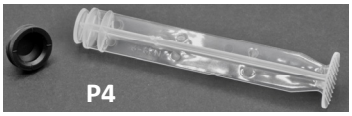
Storage buffer and others:

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

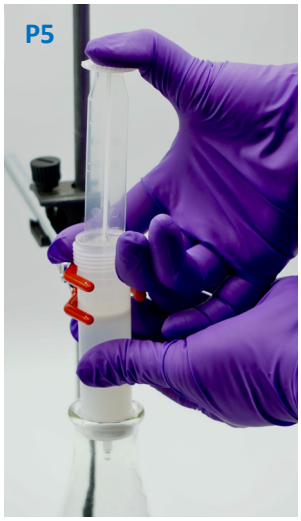
Column packing:

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

Procedure:



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



3. Wet the frit with deionized water before placing the frit inside the column.
4. Remove the black rubber cap in the plunger of the 20 mL syringe.
5. Use the plunger to push the frit inside the column and all the way down to the top of the resin. Make sure the pressure is even and the frit is flat and not tilted. There should be no air in between the frit and the resin before pushing the frit all of the way down to the top of the resin.
6. Once the frit reaches the top of the resin, use slight pressure to make the frit tight.
7. Securely attach the column top with the Luer attachment and cap the top.

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

Protocol for immobilization starts on next page

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3. Preparation of Small Molecule (25 µmol scale)

Items needed: Small molecule (user supplied), Solution A (CM01006, blue label), Reagent A (CM12003, brown label), Snap cap for reagent A (CM03PT2).

A1. Weigh 25 µmol of **Small Molecule** into a 1.5 mL centrifuge tube and add 0.5 mL of **Solution A** (blue label). Vortex for 30 seconds or sonicate for a few minutes to ensure that all of the solid is dissolved.

Calculation: Amount of small molecule (mg) = Molecular weight (MW) of small molecule x 0.025

Amount of small molecule: If your small molecule is very hydrophobic or the cost of the small molecule is very high, you can decrease the amount of small molecule to as low as 5 µmol per reaction. Scale down the amount of **Solution A** and **Reagent A** used in **Step A2.** Keep the final total volume 1 mL in **Step A5.**

A2. Locate the HPLC vial containing **Reagent A** (brown label, hazardous chemical, see SDS). Centrifuge to make sure the liquid is in the bottom of the vial before opening (~ 50 µL total). In a chemical hood, use a decrimper to remove the crimp seal from the vial. Alternatively, you can use scissors or pliers to remove the aluminum seal first. Transfer 4 µL of **Reagent A** (supernatant solution only, may contain some solid in the bottom of the vial) into the 1.5 mL centrifuge tube from **Step A1**. Cap the vial containing **Reagent A** with the replacement cap and set it aside until all experiments are done.

A3. Vortex the reaction mixture from **Step A2** for 30 seconds and centrifuge the tube to get all of the liquid into the bottom.

A4. Incubate the mixture at room temperature for 1 h (while waiting for the reaction to complete, you can proceed to **Step B1**).

HPLC Sample A4 (Optional). If you are planning to remove the sample for HPLC analysis, please follow these steps: in an HPLC vial, transfer 5 µL of small molecule solution from **Step A4** into the HPLC vial and add 95 µL of **Solution A**. Pipette up and down three times to mix. Label the HPLC vial as **Sample A4.**

Reaction time: 1 h to overnight at RT will be fine for **Step A4**. If your OH is an aromatic or secondary OH group, running the reaction for a minimum 2 h is recommended. The unreacted starting material will not interfere with the immobilization.

A5. After 1 h (or overnight), centrifuge the tube to get all of the liquid into the bottom and then add 0.5 mL of **Solution A** to the tube to make up the total volume to 1 mL. Mix for 30 seconds, then centrifuge briefly to ensure no liquid is in the cap.

Disposal of Reagent A: Add 150 µL ethanol to the vial. You will see air bubbles come up. When it stops bubbling, you can dispose of the liquid as normal chemical waste. Wash the vial with deionized water. The vial can be disposed of as normal waste.

Small Molecule is Ready for Immobilization

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4. Preparation of Agarose Beads

Items needed: Amine-modified 4% agarose beads (CM71603), Column set (CM03SC7), Airtight syringe 20 mL (CM03SR1), Stirrer (CM03PT1), Buffer A (CM02001, orange label), Reagent B (CM12101.1, cyan label).

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

B2. Slightly centrifuge the tube containing agarose beads to ensure no beads are in the cap before opening. Pour all of the beads into the **Column**. Use deionized water to aid the transfer of the agarose beads.

B3. Pour 8-10 mL of **deionized water** into the column. Stir the resin with a clean stirrer for 5 seconds. Set aside the stirrer for later use.

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

B5. Remove the column top and repeat the washing (**Steps B3 and B4**) two times.

B6. Locate **Buffer A** (orange label) and pour 5 mL into the column. Stir the resin with the stirrer from **Step B3** for 5 seconds. Set aside the stirrer for later use.

B7. Attach the column top. Use the 20 mL syringe to push the liquid out.

B8. Securely plug the end of the column with the bottom plug. Add 9 mL of **Buffer A**.

B9. Centrifuge the tube containing **Reagent B** (cyan label) to spin down the solid**.** Add 1 mL of **Buffer A** to the tube. Vortex for 30 seconds to 1 minute to dissolve the reagent.

B10. Add the entire contents of the tube containing the **Reagent B** solution into the column containing agarose beads from **Step B8.** Stir the resin solution for 10-30 seconds with the stirrer from **Step B6** until there are few air bubbles. Securely cap the column with top cap. Dispose of the stirrer.

B11. Nutate the column at room temperature for 1 h.

Thiol Agarose Bead is Ready for Immobilization

5. Immobilization

Items needed: Buffer B (CM02017, violet label), Small molecule from Step A5), Solution A (CM01006, blue label), Column set (CM03SC7), Airtight syringe 20 mL (CM03SR1), Stirrer (CM03PT1).

C1. To a 15 mL centrifuge tube, add 7 mL of **Buffer B** and 2 mL of **Solution A**. Vortex for 10 seconds to mix and then then transfer the entire contents of the small molecule solution from **Step A5**. Vortex for 30 seconds and then centrifuge at 14,000 x g for 1 minute.

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HPLC Sample C1 (Optional). If you are planning to remove the sample for HPLC analysis, please follow these steps: Transfer 100 µL of the supernatant to an HPLC vial and label it as **Sample C1**.

C2. Attach the column from **Step B11** to the lab stand. Open the column end plug and push the liquid out of the column using the syringe. Wash the end plug with deionized water for reuse.
C3. Remove the column top and pour in 8-10 mL of **Buffer B.** Wash any residual beads in the top cap of the column with **Buffer B**. Stir the resin solution for 10-30 seconds with a new stirrer, then put the column top back and push the liquid out of the column using the syringe. Set aside the stirrer for later use.

C4. Repeat **Step C3** once. For the second washing, try to remove the residual liquid as much as possible using the syringe.
C5. Securely plug the end of the column. Unscrew the column top.

C6. Transfer the supernatant from **Step C1** into the column. Stir the resin with the stirrer from **Step C3** to mix until there are few air bubbles. Securely cap the column with top cap. Dispose of the stirrer.
C7. Nutate the column at room temperature for 2 h (2 h to overnight at RT will be fine).

HPLC Sample C7 (Optional). If you are planning to remove the sample for HPLC analysis, please follow these steps: Place the column in an upright position for a few minutes before removing the column top. Transfer 200 µL of liquid to a 1.5 mL centrifuge tube. Centrifuge at 14,000 x g for 5 minutes, then transfer 50-100 µL of the supernatant to an HPLC vial and label it as **Sample C7**.

6. Capping

Items needed: Buffer B (CM02017, violet label), Reagent C (CM12004, blue label), Storage buffer (CM02008, grey label), Column set (CM03SC7), Airtight syringe 20 mL (CM03SR1), Stirrer (CM03PT1).

D1. Attach the column from **Step C7** to the lab stand. Open the column end plug and push the liquid out of the column using the syringe. Wash the end plug with deionized water for reuse.

D2. Securely plug the end of the column. Unscrew the column top and add 9.75 mL of **Buffer B.**

D3. Centrifuge the tube containing **Reagent C** solution (green label). Add the entire contents of the tube containing **Reagent C** solution to the column. Stir the resin solution for 10-30 seconds with a new stirrer. Replace the column top and securely cap the column. Dispose of the stirrer.

D4. Nutate the column at room temperature for 30 minutes.

D5. Place the column upside down and open the end cap. Attach the column to a vacuum filtration device. Open the top cap and let the liquid drain. Wash the top cap with deionized water to transfer the residual beads to the column. Wash the end cap with deionized water and set it aside for reuse.

D6. Remove the column top and pour in 8-10 mL of **deionized water.** Wash any residual beads in the top cap of the column with **deionized water**. Stir the resin solution for 10-30 seconds with

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a new stirrer. Replace the column top and push the liquid out of the column using the syringe. Set aside the stirrer for later use.

D7. Repeat **Step D6** four times.

D8. Securely plug the end of the column and add 5-10 mL of deionized water or your buffer. Screw the column top back on and securely cap the column. Store at 2-8°C in a refrigerator. Do not freeze. For long-term storage, add 5 mL of **Storage Buffer** (grey label) instead.

Agarose Beads are Ready for Your Experiment

Loading capacity: In general, the immobilization reaction is very fast. You do not have to analyze the reaction if you performed a binding capacity assay. Your loading capacity will be 1-5 µmol per mL of settled beads (5 µmol per mL of settled beads if all of the small molecules are loaded).

Stability and Storage: The immobilized product is relatively stable and storage in a neutral pH buffer is recommended. Avoid storing the compound at very low (< 4.0) or high pH (>10).

Specification for your product

Matrix: 4% beaded agarose (source: GE Life Sciences, Sepharose® 4B)
Particle size: 45 – 165 µm
Ligand concentration: ≤5 µmol per mL of settled beads

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Other Considerations

1. Determine the Loading Capacity by HPLC

The amount of the small molecule loaded onto the resin can be calculated based on the amount of the small molecule consumed during the reaction. If you are familiar with HPLC, you can remove two samples and analyze them by HPLC. Alternatively, you can send the samples to CellMosaic for analysis on your behalf.

HPLC conditions

Buffer A: 0.1% TFA in water **Buffer B**: 0.1% TFA in acetonitrile
Method: Linear gradient of AB solvent (5% B to 95% B in 12 minutes, then held at 95% B for another 3 minutes)
Flow rate: Determined by your column (usually 1 mL/min).
Injection amount: 5 µL

Loading capacity

µmol per mL: ([HPLC area of **Sample C1**]-[HPLC area of **Sample C7**]×1.05) x 5 /[HPLC area of **Sample A4**]

2. Determine the Loading Capacity by UV-Vis Spectrophotometry

The amount of the small molecule loaded onto the resin can be calculated based on the amount of the small molecule consumed during the reaction using a UV-Vis spectrophotometer if your small molecule has high extinction efficiency at certain UV or visible wavelengths (>250 nm).

Sample preparation
If the extinction coefficient of the small molecule is between 10^4 and 10^5 and you are using a 1 cm path length UV cell, dilute your HPLC samples 100 times to get a good reading.

If the extinction coefficient of the small molecule is between 10^5 and 10^6 and you are using a 1 cm path length UV cell, dilute your HPLC samples 100 times to get a good reading.

Prepare buffer blanks accordingly for each sample.

Loading capacity

µmol per mL: [**A**(sample C1)-**A**(sample C7)×1.05] x 5 /**A**(sample A4)
A: UV/Vis absorbance reading after subtracting blank

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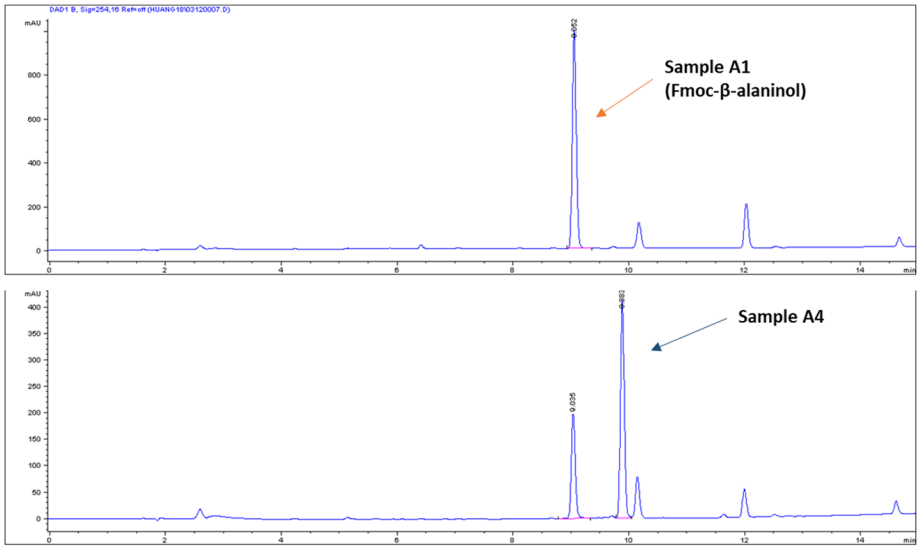
Appendix: Typical Kit Performance Data (LC Analysis, CellMosaic)

Lot#: 5509.030818

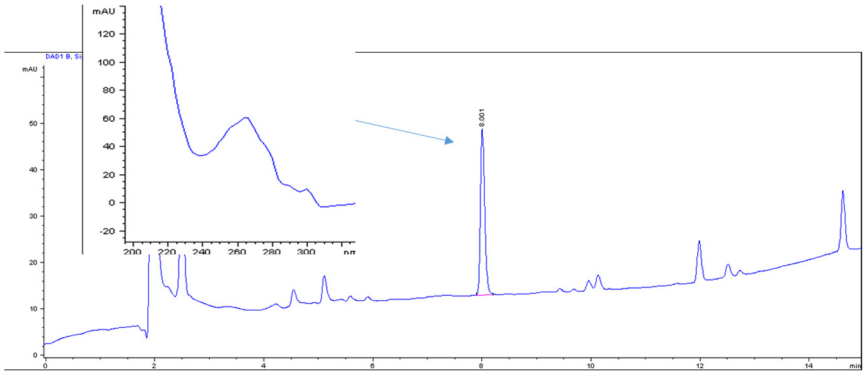
Small molecule information: Fmoc-β-alaninol

Figure 1: C18 HPLC/UV analysis of Fmoc-β-alaninol before (**Sample A1**) and after modification (**Sample A4** after 1 h reaction) as detected at 254 nm. 66% of alaninol is converted after 1 h at RT.



Experiment for analyzing the Fmoc group loaded onto the resin: After immobilization of Fmoc-β-alaninol, ~500 µL of beads was removed and added to 500 µL of 20% piperidine in NMP. After mixing for 15 minutes, 5 µL was removed for HPLC analysis.

Figure 2: C18 HPLC/UV analysis of deprotected resin. Inset: UV spectrum of Fmoc-piperidine adduct at 8.0 min confirming that Fmoc-β-alaninol was loaded onto the resin.



Calculate loading for Fmoc-β-alaninol: (1020.1-549.3x1.05) x 5/1909.9 = 1.16 µmol per mL (some of the Fmoc-β-alaninol precipitated out in **Step C1**)