

SepSphere™ Small Molecule Ketone Immobilization Kit (CM71010) User Reference Guide

Contents

Important Notes & Contact Information	2
Kit Components.....	3
Safety Information	3
Labeling Chemistry.....	3
1. Lab Instrumentation Needed.....	4
2. Immobilization Lab Techniques at CellMosaic (for reference)	5
3. Preparation of Small Molecule (25 µmol scale).....	8
4. Preparation of Agarose Beads	8
5. Immobilization	9
Other Considerations.....	10
1. Determine the Loading Capacity by HPLC.....	10
2. Determine the Loading Capacity by UV-Vis Spectrophotometry	10
Appendix: Determining Resin Loading Capacity (LC Analysis, CellMosaic).....	11



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

Kit Components

This kit provides materials to immobilize a small molecule containing a ketone or aldehyde functional group onto aminoxy-modified agarose beads.

 Upon receipt, store box in a refrigerator at 2-8°C. Do not freeze.

	Name	Part #	Quantity	Storage condition
Supplied Materials	SepSphere™ Aminoxy Agarose Beads	CM71620	5 mL	2-8°C
	Solution A (blue label)	CM01006	3.5 mL	
	Labeling Buffer (violet label)	CM02066	20 mL	
	Storage Buffer (0.02% NaN ₃ in PBS buffer) (grey label)	CM02008	10 mL	
	Column Set	CM03SC7	1	
	Centrifuge Tube 15 mL	CM03CT4	1	
	Stirrer	CM03PT1	4	
	Airtight Syringe 20 mL	CM03SR1	1	
User Material	Small Molecule	N/A	NOT PROVIDED (User Supplied Material, 25 μmol)	
	Deionized Water and HPLC Vials	N/A		

Safety Information

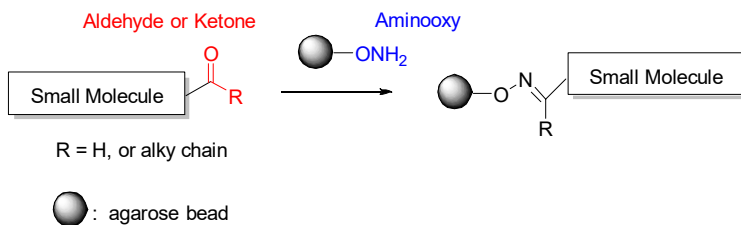
Warning: some of the chemicals used can be potentially hazardous and can cause injury or illness. Please read and understand the Safety Data Sheets (SDS) available at CellMosaic.com before you store, handle, or use any of the materials.

Labeling Chemistry

The kit is designed to work with small molecules containing ketone or aldehyde functional groups. The customer supplies the small molecule. Using the kit components, the customer immobilizes the small molecule onto SepSphere™ aminoxy agarose beads via oxime bond formation in one step.

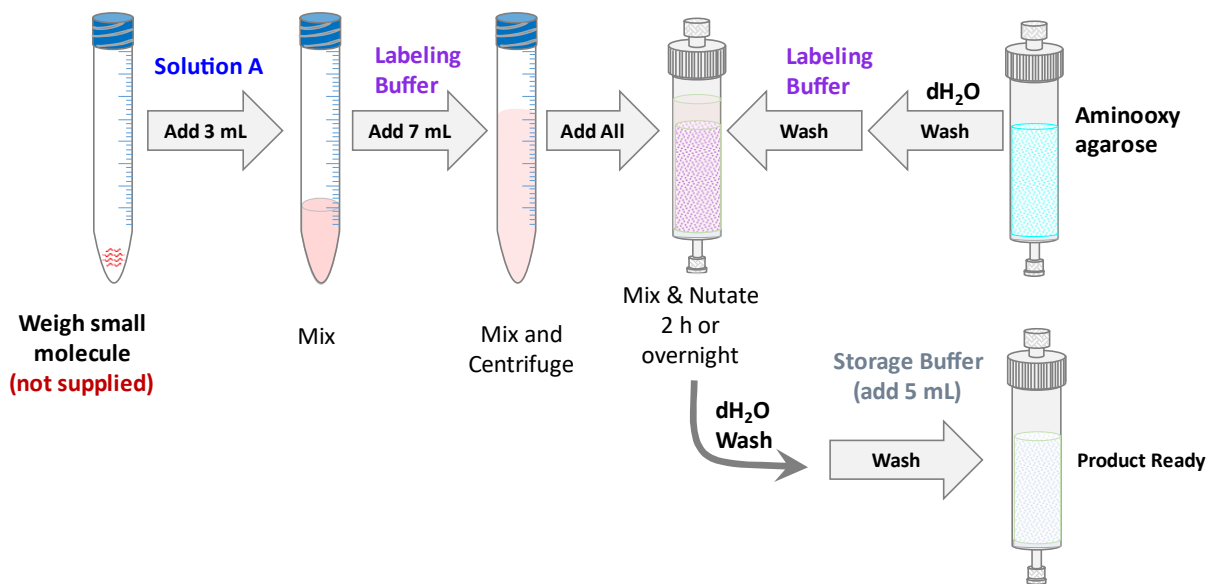
Key features of this SepSphere™ immobilization kit:

- Offers a simple and easy way to label small molecules with ketone or aldehyde groups
- Includes all reagents and supplies needed for preparation
- Uses agarose beads with an optimized amount of aminoxy 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. Stable under slightly acidic aqueous conditions (pH 4.5)



Scheme 1. Workflow for small molecule ketone immobilization with 5 mL of aminoxy agarose beads.

1. Lab Instrumentation Needed

- Vortex mixer
- Centrifuge for 15 mL and 50 mL
- 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

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).



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 (nutating).



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 $\leq 750 \times g$ 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.



Storage buffer and others:

The kit comes with standard storage buffer (1x PBS with 0.02% NaN₃). 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 peristaltic pump with Luer lock adapters.

Protocol for immobilization starts on next page

3. Preparation of Small Molecule (25 µmol scale)

Items needed: Small molecule (user supplied), Centrifuge Tube 15 mL (CM03CT4), Solution A (CM01006, blue label), Labeling Buffer (CM02066, violet label).

A1. Remove **Box 1** containing **Solution A** (blue label) and **Labeling Buffer** (violet label) from the 2-8°C refrigerator.

A2. Weigh 25 µmol of **Small Molecule** into a 15 mL centrifuge tube.

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 to 5 µmol per reaction. Keep the volume of **Solution A** and **Labeling Buffer** used in **Step A3** and **A4** the same.

A3. Add 3 mL of **Solution A** to the centrifuge tube. Vortex for 30 seconds or sonicate for a few minutes to dissolve.

HPLC Sample A3 (Optional). If you are planning to remove the sample for HPLC analysis, please follow these steps: Transfer 30 µL of small molecule solution from **Step A3** into an HPLC vial and add 70 µL of **Solution A**. Pipette up and down three times to mix. Label the HPLC vial as **Sample A3**.

A4. Add 7 mL of **Labeling Buffer** to the centrifuge tube. Vortex for 30 seconds or sonicate for a few minutes to dissolve. Not all small molecules are soluble in this mixed solvent system. Centrifuge at 14,000 x g for 1 minute. **Discard any unused Solution A as chemical waste when the experiment is done.**

HPLC Sample A4 (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 A4**.

4. Preparation of Agarose Beads

Items needed: Aminoxy Agarose Beads (CM71620), Column Set (CM03SC7), Airtight Syringe 20 mL (CM03SR1), Stirrer (CM03PT1), Labeling Buffer (CM02066, violet label), Deionized water.

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 the **Labeling Buffer** (violet 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.

5. Immobilization

Items needed: Small molecule from Step A2, Stirrer (CM03PT1), Deionized water, Storage Buffer (grey label).

C1. Transfer the supernatant from **Step A2** into the column. Stir the resin with a new stirrer to mix until there are few air bubbles. Securely cap the column with top cap. Dispose of the stirrer.

C2. Nutate the column at room temperature for 2 h (2 h to overnight at RT will be fine).

HPLC Sample C2 (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 C2**.

C3. 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.

C4. 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 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.

C5. Repeat **Step C4** four times.

C6. 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.

Small Molecule Ketone Immobilized 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 immobilized molecule on resin 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

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 μ L

Loading capacity

μ mol per mL: $([\text{HPLC area of Sample A4}] - [\text{HPLC area of Sample C2}] \times 1.05) \times 5 / [\text{HPLC area of Sample A3}]$

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

μ mol per mL: $[A(\text{sample A4}) - A(\text{sample C2}) \times 1.05] \times 5 / A(\text{sample A3})$

A: UV/Vis absorbance reading after subtracting blank

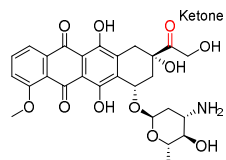
Appendix: Determining Resin Loading Capacity (LC Analysis, CellMosaic)

Two examples of the loading capacity determination for aminoxy agarose resin following the protocol in CM71010 are shown below. A large excess of small molecules was used with smaller amount of resin to determine the maximum loading capacity. The actual amount of small molecules used in this kit manual is below the maximum loading capacity to guarantee efficient loading.

Example 1: Test the loading capacity of the aminoxy agarose resin using Doxorubicin

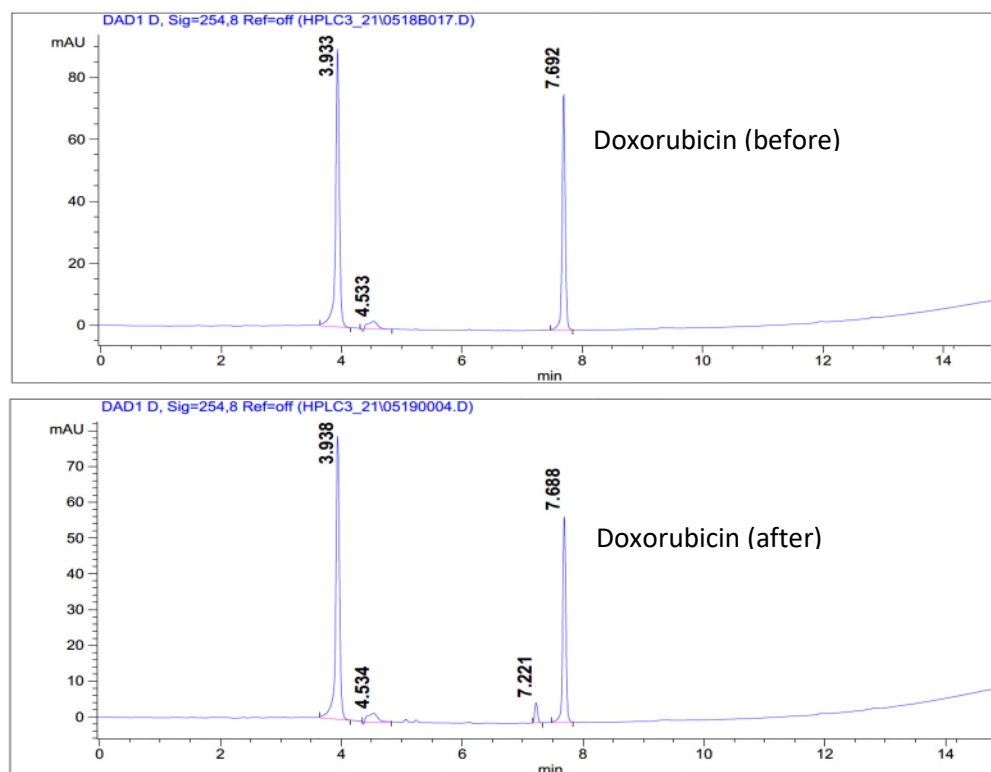
Aminoxy Agarose Lot#: NB0340541, **Amount:** 0.95 mL

Small Molecule: Doxorubicin, Amount used for immobilization: 1.2 mg



Calculate Loading: = 6.7 μ mol per mL of agarose resin. Color of resin after wash = Pink (inset picture) indicating presence of immobilized doxorubicin on resin.

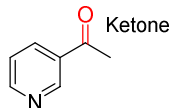
Figure 1: C18 HPLC/UV analysis of doxorubicin samples before and after immobilization (overnight reaction, detected at 254 nm).



Example 2: Test the loading capacity of the aminoxy agarose resin using 3-Acetyl Pyridine

Aminoxy Agarose Lot#: NB0340541, Amount: 1.0 mL

Small Molecule: 3-Acetyl Pyridine, Amount used for immobilization 3 mg.



Calculate Loading: = 7.0 μ mol per mL of agarose resin

Figure 2: C18 HPLC/UV analysis of 3-acetyl pyridine before and after immobilization (after overnight reaction, detected at 254 nm).

