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PerKit™ KLH Universal SM Acid Conjugation Kit (CM52426) 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

Keyhole limpet hemocyanin (KLH) is used extensively as a carrier protein for antibody production. KLH is a large, oxygen-carrying, and multi-subunit protein that contains chelated copper of non-heme origin. CellMosaic® has designed this personalized KLH conjugation kit to work with any small molecule containing a carboxylic acid (-COOH) functional group. The kit provides materials to conjugate 5 mg of KLH.

⚠ 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	Storage condition
Box 1	Reagent A (yellow label)	CM10004.2	1 unit	-20°C
	Reagent B (green label)	CM10003.1	1 unit	
Box 2	Keyhole Limpet Hemocyanin (KLH) Solution (red label)	CM52003.1	5 mg (protein content)	2-8°C
	Solution A (blue label)	CM01006	0.5 mL	
	Buffer A (brown label)	CM02069	4 mL	
	Buffer B (sky blue label)	CM02006	1 mL	
	Storage Buffer (1 x PBS buffer with stabilizers) (grey label)	CM02045	30 mL	
	Centrifugal Filter Device	CM03CD100A	1	
	Collection Tubes	CM03CT0	2	
	Desalting Column	CM03SG25	1	
	1.5 mL Centrifuge Tube	CM03CT2	2	
	2.0 mL Centrifuge Tube	CM03CT3	1	
	5 mL Centrifuge Tube	CM03CT10	1	
User Material	Small Molecule Acid	N/A	NOT PROVIDED (User Supplied Material, **≥1.8 µmol**)	

Safety Information

Warning: Some of the chemicals used can be potentially hazardous and 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 sulfo *N*-hydroxysuccinimide ester (NHS ester), followed by reaction with the surface amino groups of KLH to form a stable amide bond. The product is then purified to remove any unreacted small molecule acid.

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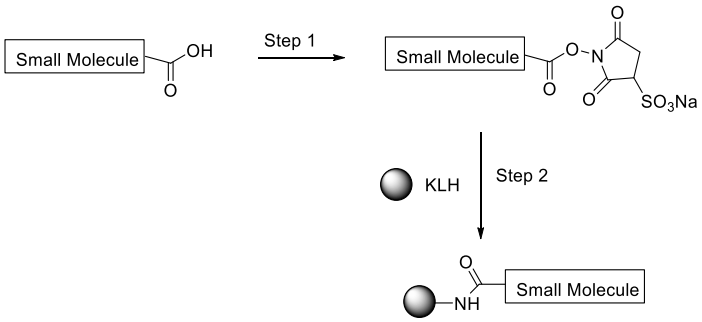
Key features of this conjugation kit:

- Offers a simple and easy way to label KLH with any small molecule containing a carboxylic acid group
- Stable linkage
- Fast and easy preparation: 4 h preparation and <1 h hands-on time
- All reagents and supplies included for preparation and purification
- More than 99% conjugated product (free of any unreacted small molecules)



Requirement for small molecule:
1. Preferably > 90% pure
2. Total amount: ≥ 1.8 µmol
3. Preferably contains only one aliphatic carboxylic acid (-COOH)
4. Absence of primary or secondary amine groups

Note: the presence of a hydroxide (-OH) group will not affect the labeling.

Support

Customer can request a recommendation for the conjugation if the small molecule has a special feature. CellMosaic also provides additional support services to customers who need help analyzing the final conjugates by HPLC.

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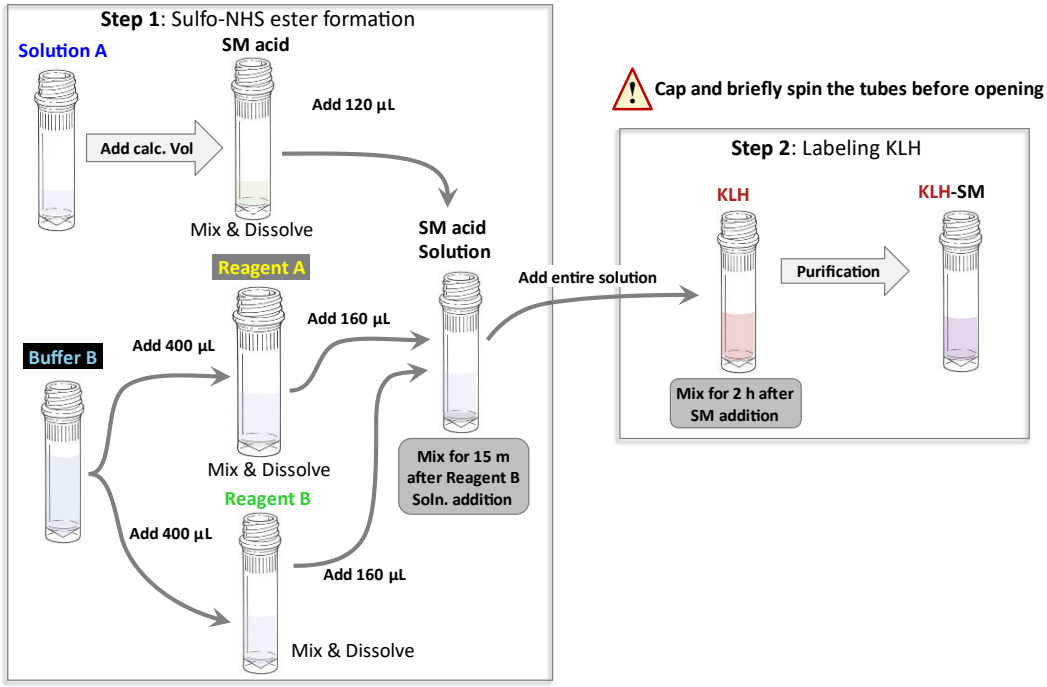
Protocol



Scheme 1. Schematic diagram of the workflow for preparing KLH small molecule conjugates.

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

A1. Remove Box 1 containing **Reagent A** (yellow label) and **Reagent B** (green label) from the freezer and warm to RT.

A2. Remove Box 2 from the refrigerator.

A3. Set the temperature of the incubator or shaker to 25°C.

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3. Preparation of KLH Samples for Conjugation

Items needed: Centrifugal Filter Device (CM03CD100A), Collection Tubes, Buffer A (CM02069, brown label), 2.0 mL Centrifuge Tube, Clean Centrifuge Tubes (not provided in the kit).

B1. Insert the Filter Device into one of the provided collection tubes (microcentrifuge tube with the cap attached). Briefly spin the centrifuge tube containing KLH solution (red label). Transfer up to 500 µL of KLH solution to the Filter Device and cap it.

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

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 8-15 minutes (preferably cooled to 4°C) to concentrate to < 100 µL. (Spin time depends on many factors. The typical volume is ~40 µL after spinning for 8 minutes in 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.** Repeat Steps **B1-B4** once to get all of the KLH solution into the Filter Device and then move on to Step **B5**.

B5. Insert the Filter Device back into the collection tube. Add 400 µL of Buffer A to make up the total volume to 500 µL. Spin the device at 14,000 x g to concentrate to < 100 µL. Then 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 times.

B7. Transfer the concentrated sample from the **Filter Device** to a 2.0 mL micro-centrifuge tube (use the pipetman to estimate the approximate volume of the concentrated sample).

B8. Add 200 µL of **Buffer A** to the **Filter Device** to rinse. Stir gently with a pipet tip, and then transfer the entire contents to the 2.0 mL micro-centrifuge tube from **Step B7.**

B9. Repeat **Step B8** once.

B10. Add **Buffer A** to the 2.0 mL micro-centrifuge tube from **Step B9** to make up the total volume of the sample to **808 ± 5 µL** and cap it.

B11. Vortex the combined KLH solution for 30 seconds and then spin down.

4. Sulfo-NHS Ester Formation and KLH Labeling

Items needed: Small Molecule Acid (user supplied), Reagent A (CM10004.2, yellow label), Reagent B (CM1003.1, green label), Solution A (CM01006, blue label), Buffer B (CM02006, sky blue label), KLH Solution from **Step B11**.

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C1. Weigh at least 1.8 µmol (but no more than 6 µmol) of the small molecule (SM) acid into a clean 1.5 mL micro-centrifuge tube. Try to weigh at least 1 to 2 mg to obtain an accurate reading. Record the weight.

Calculation 1 for SM Acid Amount:

$$Amount\ of\ the\ minimum\ SM\ (mg)\ needed = MW\ \times 0.0018$$

C2. Spin the centrifuge tubes containing **Reagent A** (yellow label), **Reagent B** (green label), **Solution A** (blue label), and **Buffer B** (sky blue label) before opening them.

C3. Transfer calculated amount of **Solution A** to the centrifuge tube containing SM acid from **Step C1**. Vortex for 30 seconds to ensure all of the solid is dissolved and then spin down.

Calculation 2 for Solution A Volume:

$$Vol.\ of\ Solution\ A\ (\mu L) = \frac{Amt\ SM\ (in\ mg)}{MW} \times 75188$$

C4. Transfer **120 µL** of SM acid solution from **Step C3** to a clean 1.5 mL centrifuge tube.

C5. Transfer **400 µL** of **Buffer B** (sky blue label) to the tube containing **Reagent A** (yellow label). Vortex for 30 seconds to ensure all of the solid is dissolved and then spin down.

C6. Transfer **160 µL** of **Reagent A** solution from **Step C5** to the tube containing SM acid from **Step C4.** Vortex for 30 seconds to mix and then spin down.

Tip for solubility check (Steps C3, C5, & C7): Check the bottom of the micro-centrifuge tube to ensure the solution is clear and free of any solid residue.

C7. Transfer **400 µL** of Buffer B (sky blue label) to the tube containing **Reagent B** (green label). Vortex for 30 seconds to ensure all of the solid is dissolved and then spin down.

C8. Transfer **160 µL** of **Reagent B** solution from **Step C7** to the tube containing SM acid and **Reagent A** from **Step C6.** Vortex for 30 seconds to mix and then spin down.

C9. Let the tube remain at RT for exactly 15 minutes (no need to mix).

Start Time: _____ End Time:_____

C10. Transfer the entire solution from **Step C9** to the KLH solution from **Step B11**. When you add the **SM acid solution**, place the pipette tip inside the KLH solution and then dispense the small molecule solution slowly while swirling the pipette tip.

Degree of SM Acid labeling (DOL): If you add the entire volume of the activated SM acid solution, you will obtain an average 10-20 SM acid per KLH. If the SM acid is very hydrophobic, you can decrease the volume of SM acid solution that is added to avoid precipitation of the conjugate.

C11. Cap the centrifuge tube. Mix at 25°C or RT for 2 h.

Start Time: _____ End Time:_____

Tip for mixing: You can use a nutator, shaker, vortex, or incubator shaker for mixing. If you are using end to end nutating, make sure the centrifuge is capped properly. If you do not have any of this equipment, you can let the centrifuge tube sit on 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 equilibrate the column for purification.

5. Purification of Conjugate

Items needed: Desalting Column (CM03SG25), Storage Buffer (1x PBS with stabilizers, CM02045, grey label), 5.0 mL Centrifuge Tube, SM-labeled KLH Solution from **Step C11**.

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.

Storage Buffer: PBS storage buffer does not contain any preservatives, protease inhibitors, reducing agents, metal chelators (e.g., EDTA), or other carrier proteins. Stabilizers are biocompatible and will not interfere with any in vitro or in vivo studies.
If used immediately, you can substitute the storage buffer with your own buffer for downstream studies.

D3. Repeat **Step D2** five times.
D4. Spin the SM-labeled KLH solution from **Step C11** to ensure there is no liquid in the cap before opening it. Add the entire KLH solution to the column. Allow the sample to enter the gel bed completely.
D5. Add 1250 µL of Storage Buffer and allow the liquid to enter the gel bed completely.
D6. Place a 5.0 mL centrifuge tube under the column. Add 2.25 mL of Storage Buffer to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.
D7. Label the tube as your product.
D8. Determine the concentration by UV/Vis spectrophotometry (see Other Considerations).
D9. Store the conjugate at 2−8°C for immediate usage. Aliquot and store the conjugate in a freezer at < -20°C for long-term storage.

Conjugate is Ready for Your Experiment

- **Specification for your product:** SM-labeled KLH with an average DOL of 10−30. The actual DOL will depend on the activities of the carboxylic acid of your SM. A typical batch contains over 99%

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conjugated product by SEC and is free of any unreacted SM. The approximate concentration of the KLH is 1.78 mg/mL in PBS buffer assuming 80% recovery.

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Other Considerations

1. Concentration Determination for Small Molecule Labeled KLH

To determine the concentration of the conjugate, dilute your conjugate from **Step D7** with 1x PBS buffer. Measure the UV absorbance of the conjugate at 279 nm (A279) and/or 347 nm (A347) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (mg/mL)\ of\ the\ dilute\ sample = \frac{(A279)}{L(1.4 + n \times \varepsilon(279nm))}$$

$$Concentration\ (mg/mL)\ of\ the\ dilute\ sample = \frac{(A347)}{L(0.4 + n \times \varepsilon(347nm))}$$

Where **L** is the UV cell path length (cm); **n** is the average loading of small molecule; and **ε** is the extinction coefficient of your small molecule ($cm^{-1}mg^{-1}mL$) at 279 nm or 347 nm as appropriate. If the small molecule has only weak or no UV absorbance, you can use 0.

2. MW Calculation

Calculation of the MW of the conjugate:

$$MW(Conjugate) = n \times (MWs - 18) + 390000$$

Where **n** is the average loading of the small molecule (use 15 as average) and MWs is the MW of the small molecule.

3. Degree of Labeling (DOL) Calculation and Characterization by UV and MS

Due to the large MW of KLH, it is difficult to obtain the DOL by mass spectrum analysis. If the small molecule has a characteristic UV absorbance that does not overlap with the UV absorbance of KLH or has a considerably higher extinction coefficient than KLH at the same wavelength, you can use it for the calculation of the DOL if you are familiar with . Otherwise, you can assume 10-20 small molecules per KLH for your conjugate if you add the standard amount of small molecule in the protocol during the labeling reaction.

4. Characterization of Conjugate by HIC HPLC

Hydrophobic interaction chromatography (HIC) HPLC can be used to check whether KLH is labeled. However, due to the highly heterogeneous nature of surface amine labeling, KLH 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.

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5. Characterization of Conjugate by SEC HPLC

If you are concerned with aggregation, you can use size exclusion chromatography (SEC) to check the extent of aggregation. SEC separates the conjugates by apparent MW or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes. By comparing the SEC profile of the unlabeled protein and 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. Recommended Storage Conditions

Recommended storage of the conjugate is at 2-8°C for short-term usage.

If you need to store the conjugate for a longer period, aliquot and store the conjugate in a freezer at < -20°C or lyophilize to dryness. Avoid repeated freeze and thaw cycles.

7. 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 (i.e., number of samples - Bulk discounts are 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 antibody to 1 mg/mL in PBS buffer, and then transfer 50 µL of the diluted solution to a 500 µL microcentrifuge tube. Label the vial properly.
3) Transfer 50 µL of ADC (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.