CellMosaic™

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KLH-Peptide Conjugation Kit (CM52411x1 and CM52411x3) 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 labeling using this kit may be affected by many different variables, including but not limited to: purity and complexity of the peptide, differences in preparation techniques, operator abilities, 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 perform KLH labeling of one (CM52411x1) or three peptide samples (CM52411x3).

⚠ 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 (CM52411x1)	Quantity (CM52411x3)	Storage condition
Box 1	Activated KLH (red label)	CM52109	1x2 mg	3x2 mg	-20°C
Box 2	Solution A (green label)	CM01003	1.5 mL	3 mL	2–8°C
	Solution B (purple label)	CM01007	0.5 mL	0.5 mL	
	1xPBS buffer	CM02045	6 mL	20 mL	
	Centrifuge filter device	CM03CD100A	2	6	
	Collection tubes	N/A	4	6	
	1.5 mL centrifuge tube	N/A	1	3	
	0.5 mL centrifuge tube	N/A	1	3	
User Material	Cys-peptide	N/A	NOT PROVIDED (User Supplied Material. **0.10–0.51 µmol** for each reaction)		

Cys-peptide amount: This kit uses KLH modified with a high number of maleimide groups (50–100 average maleimides per KLH. The exact number of maleimide per KLH for each individual batch were not determined). 0.10–0.51 µmole peptide will correspond to 20–100 equivalents of peptide over KLH.

Peptide to KLH ratio optimization: Peptide loading will be determined primarily by the amount of peptide added during the labeling if the equivalents of peptide over KLH is less than the maleimide groups per KLH. By balancing the amount of activated KLH and peptide added, the loading can be optimized to your target value (See Note in **Step A4**). You can experimentally measure the peptide loading by checking the % of peptide consumed during the labeling reaction (See HPLC or UV sampling and calculation in **Step A3** and **B6**). For very hydrophobic peptide, please target lower peptide to KLH ratio (20–40) to prevent the conjugate from precipitation.

Safety Information

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

Labeling Chemistry

Keyhole limpet hemocyanin (KLH) is used extensively as a carrier protein for antibody production. KLH is a large, oxygen-carrying, multi-subunit protein that contains chelated copper of non-heme origin.

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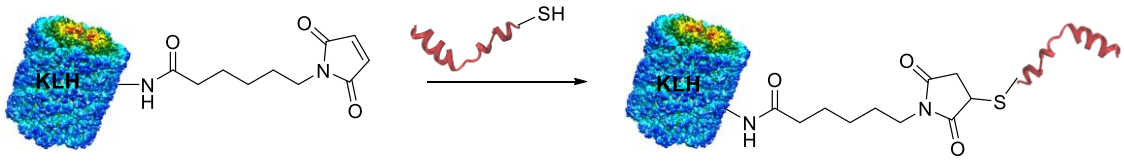
CellMosaic has designed this personalized KLH conjugation kit to work with any peptide containing a reactive Cys group. The user supplies their own peptide. Using the kit components, the customer prepares the KLH-peptide conjugate by reacting the peptide (customer supplied) with activated KLH. One-step purification typically provides the resulting KLH-peptide at greater than 95% purity.

This kit provides materials to perform KLH labeling of one to three peptides. The total amount of activated KLH included in a reaction: 2 mg.

Key features of this KLH-peptide conjugation kit:

- High quality maleimide-activated KLH for the conjugation: >99% purity
- High maleimide groups per KLH for peptide loading: 50–100
- A single purification affords over 95% of the KLH-peptide conjugates
- Fast preparation: less than 1 h hands-on time
- All reagents and consumables are included, from preparation to purification
- Options to choose tailored custom services at CellMosaic prior to and after conjugation in your lab:
    - Prior to conjugation, you can supply your peptide information when you place your order and CellMosaic will give recommendations for the conjugation if your peptide has special features (complementary service);
    - After conjugation you can choose to send your samples to CellMosaic for HPLC analysis of the conjugate or peptide samples for determination of the peptide loading.



Requirement for Cys-peptide:

1. Amount: 0.10–0.51 µmol (20–100 equivalents of peptide over KLH)
2. HPLC purified and lyophilized: please ensure no reducing reagents, such as DTT, are present
3. The Cys-peptide should be stored at -80°C
4. HPLC purity: >85% for *N*-terminal Cys peptide and >90% for *C*-terminal Cys peptide

Potential interfering compounds for labeling and conjugation reactions:
Thiols: e.g., DTT and mercaptoethanol

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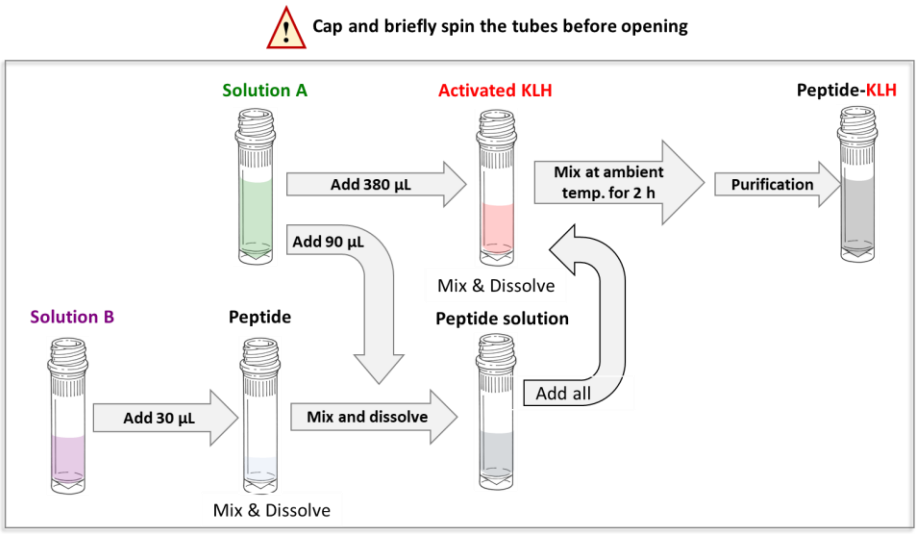
Protocol



Scheme 1. Schematic diagram of the workflow for preparing peptide-KLH 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. KLH Conjugation with Peptide

A1. Add 380 µL of **Solution A** (green label) to a tube containing **Activated KLH** (red label). Vortex for 30 seconds to 1 minute to dissolve the KLH.

> The following steps (**A2, A3, and A4**) are to be performed without any break. Cys peptide, once dissolved in solution, tend to oxidize quickly, and should be used immediately. Do not store any Cys peptide solution for later usage.

A2. Weigh 0.10-0.51 µmol of **Cys-peptide** into a 0.5 mL microcentrifuge tube. **Note:** the peptide in general is static charged. Use the tip of a glass Pasteur pipet to weigh the peptide if possible. It may be difficult to obtain the exact weight. Any amount between 0.10-0.51 µmol is acceptable (no need to adjust the volume of the dissolving solution).

Calculation for 0.10 µmol (target 25 peptides per KLH if all the peptides are labeled):

Amount of Cys-peptide (mg) = Molecular weight of Cys-peptide x 0.0001

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Calculation for 0.51 µmol (target 50-100 peptides per KLH):

Amount of Cys-peptide (mg) = Molecular weight of Cys-peptide x 0.00051

A3. Add 30 µL of **Solution B** (purple label) to the centrifuge tube containing Cys-peptide from **Step A2.** Vortex for 30 seconds and centrifuge the tube to get all of the liquid down to the bottom. Open the cap and add 90 µL of **Solution A** (green label) to the tube. Vortex for 30 seconds or sonicate for a few minutes to ensure all of the solid is dissolved. Discard any unused **Solution B** as hazardous chemical waste **until the experiments are done**.

Tip for solubility check (Step A3): It may take a while for your peptide to fully dissolve. In general, most of the peptide should be able to dissolve in this mixed solution system. Check the bottom of the micro-centrifuge tube to ensure the solution is clear and free of any solid residue. If some solid remains after a few minutes, centrifuge the tube and pipette the supernatant for the next step.

Tip for opening centrifuge tube after vortex: Always centrifuge the tube to ensure no liquid is in the cap.

HPLC or UV Sample (A3) for peptide loading measurement (Optional): If you are planning to experimentally determine the peptide loading, please follow the steps below:

- Transfer 6 µL of the peptide solution from **Step A3** (5% of the total amount) to a new centrifuge tube and label it as **sample A3.**
- For UV measurement, dilute the sample 10-50 times in 50% MeOH/water. Record the dilution factor DF(A3) and UV absorbance at 280 nm (A(A3)). Remember to use 50% MeOH/water as a blank for subtraction.
- For HPLC analysis, dilute the sample 5-20 times in 50% MeOH/water (detect at 205 and 280 nm). Record the dilution factor DF(A3) and HPLC Area (A(A3)).

A4. Transfer the solution from **Step A3** to the tube containing **Activated KLH** from **Step A1**. Pipette the solution up and down in the tube three times to mix. Incubate at room temperature for 2 hours (**Note:** if you use the decreased amount of **Activated KLH**, please pipette out the desired volume of **Activated KLH** from **Step A1** to the peptide solution from **Step A3** instead).

Peptide Loading Optimization: The desired peptide loading is specific to the project. Recommended target peptide loading is within 20–100 peptides per KLH. Your target loading can be adjusted by either changing the moles of peptide added in Step A2 or the volume of activated KLH from **Step A1**. You can also change both. For very hydrophobic peptide, please target lower peptide to KLH ratio (20–40) to prevent the conjugate from precipitation.

Calculation of the theoretical loading if 100% peptide are labeled:

$$Peptide\ per\ KLH\ (Loading) = \frac{Peptide\ (umole) * 74100}{Activated\ KLH\ (volume)}$$

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3. Purification to Remove Excess Peptide

B1. Place the centrifuge tube from **Step A4** containing the reaction mixture into the centrifuge rotor and counterbalance with a similar device. Spin **the centrifuge tube** at 10,000 x g for 2 minutes.

B2. Insert the **Filter Device** into one of the provided collection tubes (micro-centrifuge tube with the cap attached). Transfer and divide the supernatant from **Step B1** into two **Centrifuge Filter Devices**. Add 250 µL of **PBS Buffer** to make up the total volume to 500 µL in each filter device and cap it.

B3. Place the assembled **Filter Devices** into the centrifuge rotor, aligning the cap straps toward the center of the rotor. Spin the assembled **Filter Devices** at 14,000 x g for 8 minutes to concentrate down to < 100 µL (Spin time depends on many factors. A typical spin time for a 500 µL sample is approximately 8 to 20 minutes. The typical volume is ~40 µL of residual fluid in each filter device after spinning for 8 minutes on an Eppendorf 5417R at 4°C).

B4. Remove the assembled devices from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tubes 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 µL of **PBS Buffer** to make up the total volume to 500 µL in each filter device and cap it. Place the two assembled **Filter Devices** into the centrifuge rotor, aligning the cap strap toward the center of the rotor. Spin the assembled **Filter Devices** at 14,000 x g for 8 minutes.

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

HPLC or UV Sample (B6) for peptide loading measurement (Optional): If you are planning to experimentally determine the peptide loading, please follow these steps:

- Combine the three purification filtrates from **Steps B4–B6** and label it as **sample B6.** Record the total volume of the filtrate (~2.4 mL).
- For UV measurement, record the absorbance of the sample at 280 nm without dilution (A(B6)). Remember to use PBS buffer as a blank for subtraction.
- For HPLC analysis, inject the sample without dilution and detect the peak at 205 and 280 nm. Record the HPLC area (A(B6)).

Calculation of the peptide to KLH ratio:

$$Peptide\ per\ Ova = \frac{P}{K} \times \left(1 - \frac{A(B6) \times V(B6)}{A(A3) \times DF(A3) \times 6}\right) \times 70395$$

A(A3): UV absorbance at 280 nm or HPLC area of sample A3 (blank subtracted)
A(B6): UV absorbance at 280 nm or HPLC area of sample B6 (blank subtracted)
DF(A3): dilution factor of sample A3; **V**: total volume of filtrate B6
P: total amount of peptide added in **Step A2** (µmole)
K: total volume of activated KLH added in **Step A4** (µL)

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B7. To recover the conjugates, place the **Filter Device** upside down in a clean **Collection Tube**. Place in the centrifuge, aligning the open cap towards the center of the rotor. Spin for 2 minutes at 1,000 x g to transfer the conjugates from the **Filter Devices** to the **Collection Tubes.**

B8. Transfer the conjugates from the two **Collection Tubes** to a 1.5 mL micro-centrifuge tube.

B9. Rinse each **Collection Tube** with 50 µL of **PBS Buffer** and transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B8**.

B10. Add 100 µL of **PBS Buffer** to each **Filter Device** to rinse. Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B8**. Add **PBS Buffer** to make the total volume of the sample 400 µL and cap it (use the pipetman to measure the total volume).

B11. Vortex the combined protein sample for 30 seconds and then centrifuge to ensure no liquid is in the cap.

KLH-Peptide is Ready for Your Experiment

Tip: The approximate concentration of **the KLH-peptide conjugate** is 10.3 µM in 400 µL of PBS buffer (assuming 80% recovery). You can also determine the concentration using a UV/Vis spectrophotometer. For downstream applications, avoid using any buffer that contains free thiol (e.g., Cys).

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Other Considerations

1. Concentration Determination

To determine the concentration, dilute your KLH-peptide from **Step B11** with 1x PBS buffer. Measure the UV absorbance of the KLH-peptide at 280 nm (A_{280}) using a UV spectrometer and calculate the concentration using the following formula:

Concentration (µM) of the dilute sample = (A_{280})/(L x ε)

Where A_{280} is the absorbance of KLH-peptide conjugates at 280 nm; L is the UV cell path length (cm); and ε is the extinction coefficient of the KLH-peptide conjugate at 280 nm.

If you know the sequence of the peptide and the average molar ratio of peptide per KLH, you can calculate ε as described in the following equation (Gill, S.C. and von Hippel, P. H. *Anal. Biochem.* **1989***, 182***,** 319–326; Pace, C.N., *et al. Protein Sci.* **1995***, 4,* 2411–2423):

ε = m x ((nW x 5500)+(nY x 1490)+(nC x 125))+612300

Where m is the average molar ratio of peptide per KLH. If you do not know the molar ratio, you can use the amount of peptide added.

Where n is the number of corresponding residues present in the peptide: tryptophan (W), tyrosine (Y), and cysteine (C).

If you are using a 1 cm UV cell, you can dilute the KLH-peptide 10-20 times to get a good reading.

2. MW Calculation

Calculation of the MW of the conjugate:

MW(conjugate) = m x MW(peptide) + 390000

Where m is the average molar ratio of peptide per KLH.

3. Recommended Storage Conditions

For long-term storage, KLH-peptide conjugates can be stored frozen at -20°C.

4. Peptide-to-KLH Ratio

Peptide loading can be measured experimentally by checking the % of peptide consumed during the labeling reaction. Please see HPLC or UV sampling and calculation of peptide-to-KLH ratio in **Step A3** and **B6**.

5. Sample Submission for HPLC Analysis

If you are submitting samples to CellMosaic for C18 HPLC analysis for sample loading and SEC HPLC for conjugate analysis, please follow these instructions:

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1) Go online: https://www.cellmosaic.com/hplc-analysis/, select C18 HPLC Analysis (Product# AS0021) and SEC HPLC Analysis (Product# AS0023), choose the quantity (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) For peptide loading determination by C18 HPLC, transfer sample **A3** and **B6** to a micro-centrifuge tube with screw cap and label the vial properly.
3) For conjugate purity analysis by SEC HPLC, mix 10 µL of your conjugate from **Step B11** with 40 µL of 1x PBS buffer into a micro-centrifuge tube with screw cap. Label the vial properly.
4) Ship your samples with a cold pack for overnight or 2nd day delivery.