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PerKit™ HRP–Protein Conjugation Kit (HMW) (CM52414) 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 Protein, 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 HRP labeling of one protein sample (CM52414). Scale of each reaction: 8 nmol protein.

- 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.

Box No. (Store Temp.)	Name	Part #	Quantity
Box 1 (≤-20°C)	Activated HRP (**red label**)	CM53214	1 unit
	Reagent A (**cyan label**)	CM12101	1 unit
	Reagent B Solution (**yellow label**)	CM12004.1	1 unit
Box 2 (2–8°C)	Buffer A (**orange label**)	CM02001	4 mL
	Buffer B (**indigo label**)	CM02005	4 mL
	PBS Buffer (**grey label**)	CM02013	4 mL
	Filter Device for Protein	CM03CD010A	2
	Filter Device for Conjugate	CM03CD100A (default) or CM03CD050A (customized)	1
	Collection Tubes	CM03CT0	6
	1.5 mL Centrifuge Tubes	CM03CT2	2
User Supplied Material	Protein (MW≥20KDa)	N/A	NOT PROVIDED (**8 nmol** per reaction)

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

CellMosaic® has designed this personalized HRP–protein conjugation kit to work with any protein with a MW over 20KDa. The customer supplies its own unmodified protein. Using the kit components, the customer converts some of the surface amines of the protein to free thiol groups (Step 1 in **Scheme 1**), followed by reaction of the thiol-protein with activated HRP to generate the HRP–protein conjugates (Step 2 in **Scheme 1**). If there are any unreacted thiols on the protein, they are subsequently capped (Step 3 in **Scheme 1**). One step purification typically provides the resulting HRP–Protein at greater than

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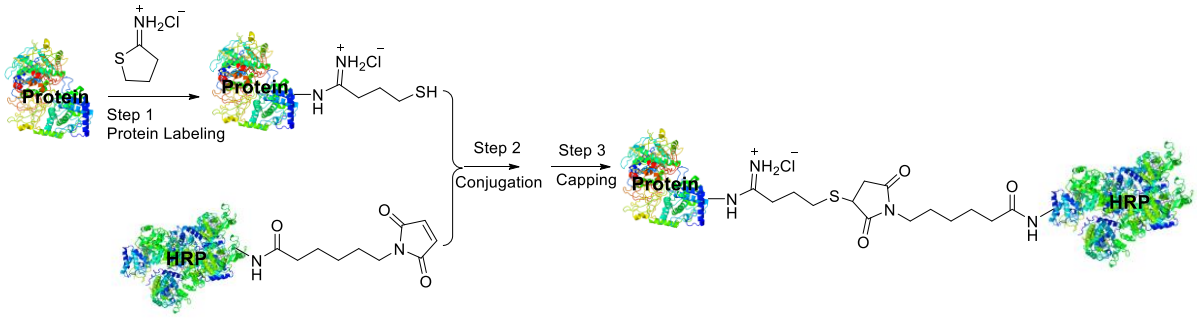
90% content.



Scheme 1: Synthetic route to HRP–protein conjugate.

Key features of this HRP-Protein conjugation kit:

- Offers a convenient way to prepare HRP−protein conjugate with heterobifunctional crosslinking reagents.
- High quality maleimide activated HRP for the conjugation: >99% purity and >200 units/mg protein activity.
- Target 1−3 HRP per protein (actual loading will depend on your protein).
- ≥90% HRP−Protein conjugates if the quality of the protein is at par.
- Preparation can be done in a day with less than 3 h hands-on time.
- All reagents included, from preparation to purification.
- Options to choose tailored services at CellMosaic after conjugation:
    - You can choose to send your conjugates to CellMosaic for HPLC analysis of the sample or complete removal of HRP and/or unreacted protein.

Requirement for Protein (IgG):
1. Preferably, the Protein should be >90% pure by gel electrophoresis
2. Total amount: 8 nmol total

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

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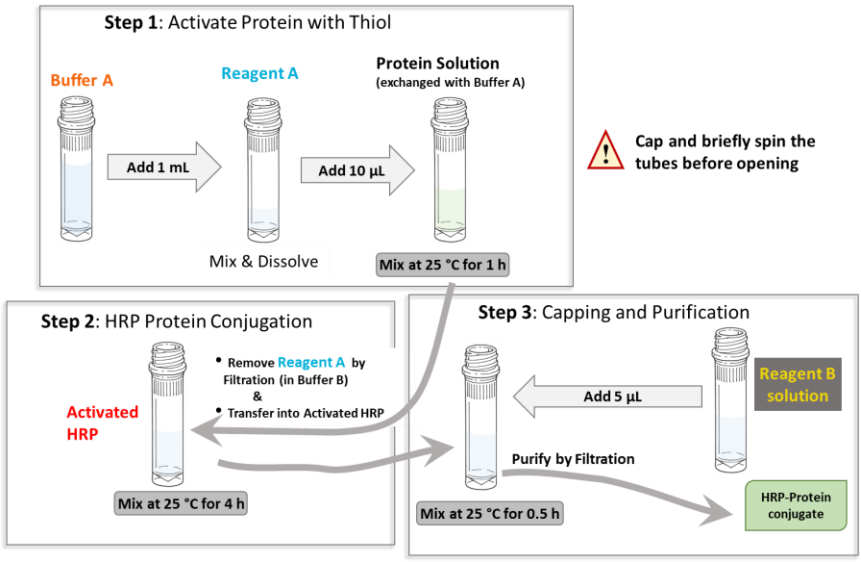
Protocol



Scheme 2. Schematic diagram of the workflow for preparing HRP–protein conjugates starting with 8 nmol of protein.

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated for example Eppendorf 5417R)
- Pipettes and tips
- Timer
- Incubator or shaker set at 25°C (room temperature between 20–27°C is acceptable)
- Personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves)
- UV spectrophotometer (optional)

2. Preparation of Protein Samples for Conjugation

Items needed: Protein (user supplied), Filter Device for Protein (CM03CD010A), Collection Tube (CM03CT0), Buffer A (CM02001, orange label), 1.5 mL Centrifuge Tube (CM03CT1), Clean Centrifuge Tubes (not provided in the kit).

Total amount of Protein used for the conjugation is 8 nmol.

Calculation: Amount of protein (mg) = Molecular Weight (MW) of protein x 0.000008

A1. Insert one of the Filter Device for Protein (CM03CD010A) into one of the provided collection tubes. Perform the step based on the following conditions.

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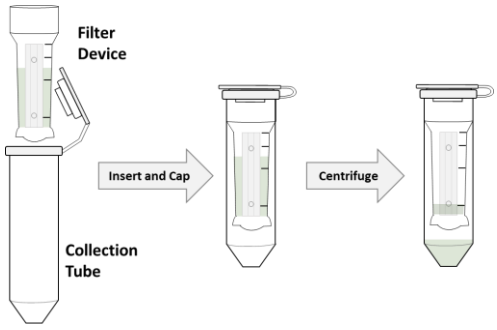
- ✓ If your protein is supplied as a lyophilized solid, dissolve the Protein in 500 µL of deionized water and then transfer the entire contents to the Filter Device 1.
- ✓ If your protein is supplied in < 500 µL buffer, transfer your protein to the Filter Device directly. Add Buffer A to make up the total volume to 500 µL and cap it.



- ✓ If the volume of your protein solution is >500 µL, add up to 500 µL of sample to the Filter Device. Repeat Step **A1-A4** until all of the protein solution goes into the Filter Device. Move on to Step **A5**. Add Buffer A to make up the total volume to 500 µL for the last refill.

A2. Place the capped Filter Device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

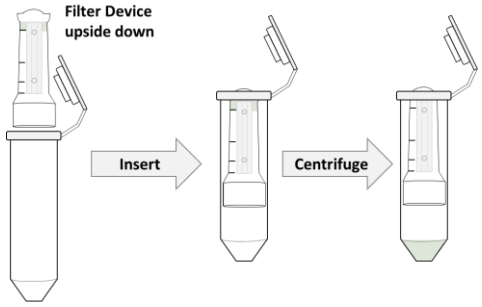
A3. Spin the Filter Device at 14,000 x g for 10 minutes to concentrate to concentrate to < 50 µL. (Spin time depends on many factors. The typical spin time for a 500 µL sample in this Filter Device is approximately 10 to 15 minutes. The typical volume is ~42 µL after spinning for 10 minutes on an Eppendorf 5417R at 4°C).

A4. 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.**

A5. 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 for 10 minutes to concentrate to < 50 µL. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done**.

A6. Repeat **Step A5** one time.

A7. To recover the protein, place the Filter Device upside down in a clean Collection Tube. Place the Filter Device in the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 x *g* to transfer the protein from the Filter Device to the Collection Tube.



A8. Transfer the concentrated protein from the Collection Tube from **Step A7** to a clean 1.5 mL micro-centrifuge tube (use the pipetman to measure the volume of the concentrated sample. The typical volume is around 50 µL after concentration).

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A9. Wash the Collection Tube once with 50 µL of Buffer A and transfer the wash to the tube from **Step A8**.

A10. Wash the filter once with 50 µL of Buffer A and transfer the wash to the tube from **Step A8**. Add Buffer A to make up the total volume of the sample to ~240 µL and cap it.

(Note: Wash = Add buffer, aspirate with pipette 2-3 times.)

A11. Vortex the combined protein solution for 30 seconds and then centrifuge to ensure no liquid is in the cap.

3. Activate Protein with Thiol

Items needed: Reagent A (CM12101, cyan label), Buffer A (CM02001, orange label), Protein solution from **Step A11**.

B1. Briefly spin the tube containing Reagent A (cyan label). Add 1 mL of Buffer A to the tube with Reagent A. Vortex for 10−30 seconds to dissolve the reagent.

Tip for solubility check: Check the bottom of the micro-centrifuge tube to see if the solution is clear and free of any solid residue.

B2. Transfer 10 µL Reagent A solution from **Step B1** to the tube containing protein solution from **Step A11**.

B3. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Mix the reaction mixture at 25°C (RT between 20 to 27°C is acceptable) for 1 hour.

Start Time: _____ End Time:_____

4. Purification to Remove Excess Reagent A and Conjugation with HRP

Items needed: Filter Device for Protein (CM03CD010A), Collection Tubes, Buffer B (CM02005, indigo label), Activated HRP (CM53214), Clean Centrifuge Tubes (not provided in the kit), Thiol Protein Solution from **Step B3**.

Steps C2 to C**7** are to be performed without any break. Reduced thiols tend to oxidize quickly. Work quickly through **Steps E3-E7**.

C1. Insert one of the Filter Device for Protein (CM03CD010A) into one of the provided collection tubes. Spin the thiol-modified protein from **Step B3** to ensure there is no liquid in the cap before opening it. Transfer the protein solution into the Filter Device. Wash the tube once with 200 µL of Buffer B (indigo label) and transfer the wash to the Filter Device. Add Buffer B to make up the total volume to 500 µL and cap it.

C2. Spin the Filter Device at 14,000 x *g* for 10 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.

C3. Remove the assembled device from the centrifuge. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

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C4. Insert the Filter Device back into the collection tube. Add 400 µL of Buffer B to the Filter Device. Spin the device at 14,000 x *g* for 10 minutes to concentrate to **< 50 µL**. Remove the assembled device from the centrifuge. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

C5. Spin the centrifuge tube containing Activated HRP (red label) to make sure no solid is in the cap before opening it.

C6. Pipet the concentrated protein from the Filter Device directly to the tube containing Activated HRP from **Step C6**.

C7. Wash the filter twice with 50 µL of Buffer B and transfer the wash to the HRP tube.

(Note: Wash = Add buffer, aspirate with pipette 2-3 times.)

C8. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Mix the reaction mixture at 25°C (RT between 20 to 27°C is acceptable) in the dark for 4 h.

Start Time: _____ End Time:_____

Reaction Time: Conjugation is usually done within 4 h. However, you can leave the reaction for a longer time or place it at 4°C overnight after 4 h at RT.

5. Capping Unreacted Thiol Groups of Protein

Items needed: Reagent B solution (CM12004.1, yellow label), Conjugate Solution from **Step C8**.

D1. Briefly spin the tube containing Reagent B solution (yellow label). Transfer 5 µL Reagent B solution to the reaction mixture from **Step C8**.

D2. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Mix the reaction mixture at 25°C (RT between 20 to 27°C is acceptable) for 30 minutes.

Start Time: _____ End Time:_____

6. Purification to Remove Excess Reagent B and Unreacted HRP

Items needed: Filter Device for Conjugate (CM03CD100A or CM03CD050A), Collection Tubes, PBS buffer (CM02013, grey label), Conjugate Solution from **Step D2**, 1.5 mL Centrifuge Tube (CM03CT2), Clean Centrifuge Tubes (not provided in the kit).

E1. Insert one of the **Filter Devices for Conjugate** (CM03CD100A or CM03CD050A) into one of the provided collection tubes. Spin the conjugate solution from **Step D2** to ensure there is no liquid in the cap before opening it. Transfer the conjugate solution into the Filter Device. Wash the tube once with 250 µL of PBS buffer and transfer the wash to the Filter Device. Add PBS buffer to make up the total volume to 500 µL and cap it.

E2. Spin the Filter Device at 14,000 x *g* for 5 minutes (preferably cooled to 4°C) to concentrate to < 50 µL ((Spin time depends on many factors. The typical spin time for a 500 µL sample in this

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Filter Device is approximately 5 to 8 minutes. The typical volume is ~30 µL after spinning for 5 minutes on an Eppendorf 5417R at 4°C).

E3. Remove the assembled device from the centrifuge. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

E4. Insert the Filter Device back into the collection tube. Add 400 µL of PBS buffer to make up the total volume to 500 µL. Then place the capped Filter Device into the centrifuge rotor, spin the device at 14,000 x g for 5 minutes.

E5. Repeat **Step E4** one time.

E6. To recover the conjugate, place the Filter Device upside down in a clean Collection Tube. Spin for 2 minutes at 1,000 x g to transfer the concentrated sample from the Filter Device to the Collection Tube.

E7. Transfer the conjugate from the Collection Tube to a clean 1.5 mL centrifuge tube**.**

E8. Wash the Collection Tube with 100 µL of PBS Buffer and transfer the wash to the centrifuge tube from **Step E7**.

E9. Wash the filter once with 100 µL of PBS buffer and transfer the wash to the centrifuge tube from **Step E7**.

(Note: Wash = Add buffer, aspirate with pipette 2-3 times.)

E10. Vortex the combined conjugates for 30 seconds and then centrifuge to ensure no liquid is in the cap. Label your conjugate.

HRP–Protein is Ready for Your Experiment

Tip: Pay attention to the color of your sample; if the conjugation reaction is successful, the solution of your product will be light brown. The number of HRP per Protein is between 1–3 depending on the properties of your protein. Estimate concentration of the HRP–protein is 25.6 µM if the total volume is 250 µL assuming 80% recovery. Conjugate recovery may be lower or higher depending on the MW of your protein. The higher MW of your protein, the higher your recovery.

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Other Considerations

1. Concentration Determination

To determine the concentration, dilute your HRP−Protein from **Step E10** with PBS buffer. Measure the UV Absorbance of HRP−Protein at 403 nm (A_{403}) using a UV spectrometer and calculate the concentration based on the following formula:

Concentration (µM of HRP) = (A_{403}) * 10 / (L x 1.02)

L: UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute HRP-Protein 5 to 10 times to get a good reading.

2. Analyze the Conjugate and Determine the Degree of the HRP Loading by HPLC

The purity of the conjugate as well as the average number of the HRP loaded onto the Protein can be analyzed by Size exclusion HPLC. If you are familiar with HPLC, you can analyze them yourself. You will need to obtain the standard curves of the Protein and HRP to determine the degree of the loading. Alternatively, you can also send the samples to CellMosaic for analysis on your behalf.

HPLC conditions

Buffer A: PBS buffer
Method: Isocratic
Flow rate: will be determined by your column (usually 1 mL/min).
Injection amount for the conjugate: Dilute the sample 5 times with PBS buffer, then inject 10 µL

3. Recommended Storage Conditions

Depending on the stability of your Protein, HRP−Protein conjugate solution is recommended to store at 2−8 °C and should be used as soon as possible. Some HRP−Protein conjugates may be lyophilized for long-term storage.