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PerKit™ HRP–Oligo Conjugation Kit (Thiol Oligo) (CM53408) 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 HRP, 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 Configuration and Components

CellMosaic® has designed this PerKit™ HRP–Oligo conjugation kit to prepare single-labeled HRP–oligo conjugates with linkers and conjugation chemistry that retains the activity of HRP. The same configuration and chemistry have been tested for HRP-oligos with different oligo sequences at a few diagnostic companies since 2011.

This kit is an upgraded and improved replacement kit with slightly different components and workflow as the first-generation kit (Cat# CM53402). This kit has multiple configurations for various lengths of oligos and can be used to conjugate 5 nano-mole (nmol) of oligo. **Table 1** provides the catalog numbers for various kit configurations and **Table 2** lists the kit components.

Table 1: Configurations of the PerKit™ HRP–Oligo conjugation kit (CM53408)

Configuration	No. of Reactions	Catalog No.
1. Labeling for short oligo (5–30 bases)	1	CM53408.1x1
	3	CM53408.1x3
2. Labeling for medium oligo (≥31 bases)	1	CM53408.2x1
	3	CM53408.2x3

How to use this protocol: The protocol in this user manual is written for two different configurations: 5–30 bases oligo (CM53408.1) and ≥31 bases oligo (CM53408.2). Steps are common to all configurations. However, some of the kit components are specific to each configuration.

⚠ Please follow the specific instructions for individual configurations.

Requirement for disulfide-oligo:

1. Total amount: 2 to 5 nano-mole oligo content as measured by UV.
2. HPLC purified with disulfide-oligo content ≥90% by C18 HPLC.
3. ≥5- bases (oligos longer than 60 bases may result in lower loading and high impurities).

Note: You can purchase HPLC purified 3' C6 disulfide linker or 5' C6 disulfide-modified oligo from standard oligo manufacturers, up to 90 bases for this kit preparation. It is highly recommended that customers analyze and quantify oligos prior to using them.

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Table 2: Components and storage temperatures for PerKit™ HRP–Oligo conjugation kit. All kits share the same components except for the number of tubes provided for Reagent A and the type of Filter Devices.

⚠
- 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. (Storage T)	Name	Cat#	Kit Cat#	Quantity (x1)	Quantity (x3)
Box 1 (≤-20°C)	C6 Maleimide Activated HRP (**red label**)	CM53214	Any	1 unit	3 units
	Reducing Reagent (**blue label**)	CM13001		1 unit	3 units
Box 2 (2–8°C)	Buffer A (**orange label**)	CM02001	Any	0.5 mL	1 mL
	Buffer B (**indigo label**)	CM02005		4 mL	12 mL
	Buffer C (equilibration and washing buffer, **lime label**)	CM02003		4 mL	10 mL
	Buffer D (elution buffer: 50 mM Tris buffer, pH 8.0, 1M NaCl, **navy label**)	CM02004		1.5 mL	5 mL
	Filter Devices (Only one part# will be supplied, 1 or 3 units)	CM03CD003A	CM53408.1	1	3
		CM03CD010A	CM53408.2		
	Collection Tubes for Filter	CM03CT0	Any	2	6
	Column Q	CM03SC5		1	3
	Collection Tubes for Column Q	CM03CT6		2	6
User Supplied Material	Disulfide-Oligo (≥5 bases)	N/A		NOT PROVIDED (2–**5 nano-mole)** for each reaction)	

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 www.CellMosaic.com before you store, handle, or use any of the materials.

Labeling Chemistry

The kit is designed to work with protected thiol-modified oligos (disulfide form). The user supplies the thiol-modified oligos containing 3' C6 disulfide linker or 5' C6 disulfide linker, which are readily available from many commercial oligo suppliers. Using the kit components, the user reduces the disulfide bond, followed by reaction of the thiol-oligo with activated HRP to generate the HRP-oligo conjugates. The total length of the linkage between the HRP and the amine-oligo is 29 atoms excluding the linker used for amine modification of the oligo. The Q-column purification step typically provides the resulting HRP-oligo at greater than 80% purity.

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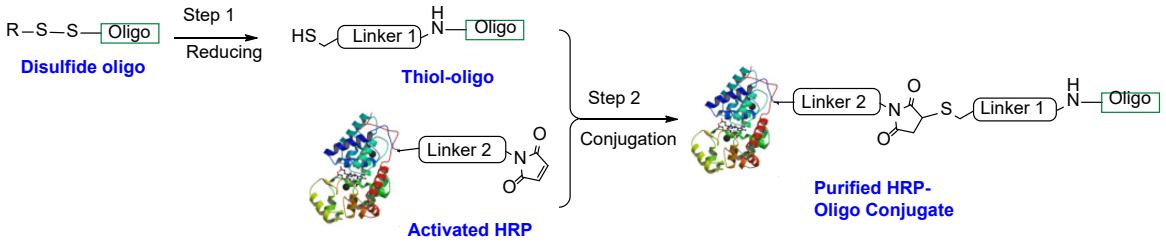

Scheme 1. Synthetic route to HRP−oligo conjugate.

An HPLC purified disulfide oligo is required for this conjugation. If the content of the disulfide oligo is >90%, the purity of the final conjugate will generally be >90% pure with an average of 1 oligo per HRP (majority are single labeled). This purity is sufficient for ELISA, and a trace amount of impurities will negligibly affect the signal intensity. For oligos longer than 60 bases or oligos modified with non-phosphate backbone or bases, the purity of the amine oligo may be lower. In addition, if there is a solubility issue in aqueous buffer, the labeling reaction may be inefficient, resulting in low loading and high impurities. For complete removal of oligo impurities, gel filtration chromatographic purification is recommended.

Key features of this HRP-oligo conjugation kit:

- High quality maleimide-activated HRP for the conjugation: >99% purity and >200 units/mg protein activity.
- A single purification affords 85-90% single-labeled HRP-oligo conjugates if the quality of the oligo is at par.
- Preparation can be done within a few hours.
- Stable linkage with optimal spacer to ensure no interference between oligo binding and HRP activation.
- 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 trace oligo impurities and unreacted HRP.

Support

Customer can request a recommendation for the conjugation if the oligo has some special features or solubility issues. CellMosaic also provides fee-based support services to customers who need help analyzing the final conjugates by HPLC and further purification to remove trace oligo impurities.

Protocol

There are a total of two reactions in this protocol. Keep in mind that thiol-labeled oligo should be purified quickly once the reaction is done and used immediately. HRP-oligo conjugation is generally done within 4 h, but you can take longer or leave it at 2−8°C overnight after the reaction. The Q-column purification will take less than 10 minutes.

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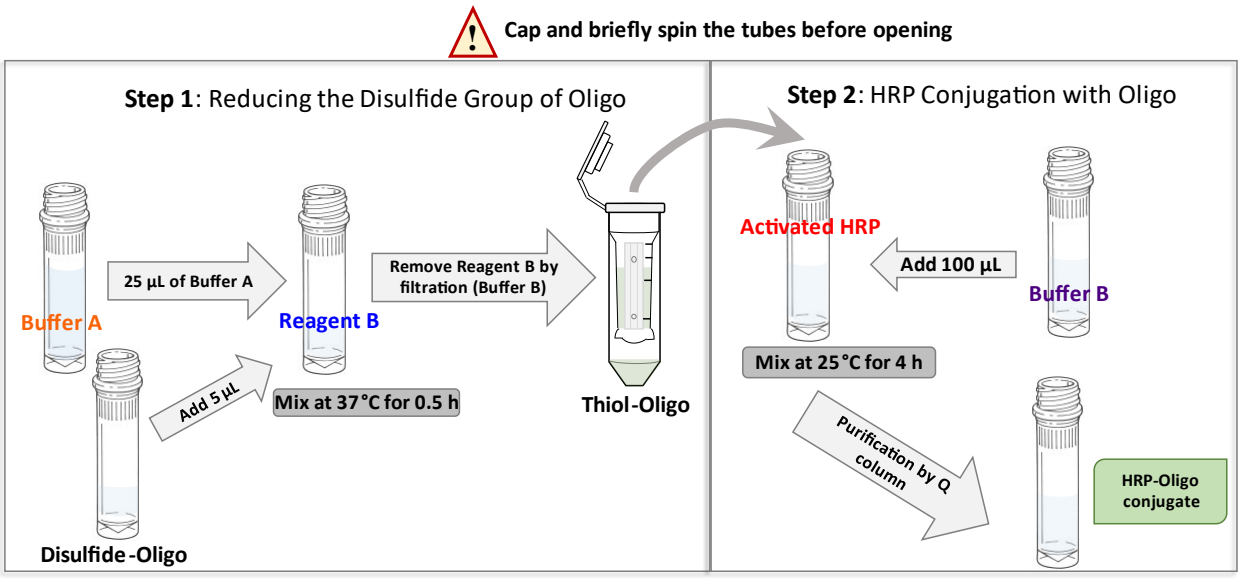


Scheme 2. Schematic diagram of the workflow for preparing HRP–oligo conjugates.

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, 14,000 g capable), mini-centrifuge
- Pipettes and tips
- Timer
- Incubator or shaker set at 37°C and RT
- Support stand, lab frame, or any support rod for desalting column
- Flask
- Personal protection equipment (lab coat, safety glasses, and chemical-resistant nitrile gloves)
- UV spectrophotometer (optional)

2. Reducing the Disulfide Group of Oligo (Step 1)

Items needed: Disulfide-Oligo (user supplied), Reducing Reagent (CM13001, blue label), Buffer A (CM02001, orange label), Buffer B (CM02005, indigo label), 1 Filter Device (CM03CD003A or CM03CD010A), 2 Collection Tubes (CM03CT0).

Total amount of oligo used for the conjugation is **2–5 nano-mole**.

A1. Re-suspend the disulfide-oligo in water to a concentration of 0.5 mM.

A2. Centrifuge the tube containing Reducing Reagent B (blue label) to spin down the solid. Make sure you can see a small amount of solid at the bottom of the tube. Add 25 µL of Buffer A to the tube. Vortex for 30 second to 1 minute to dissolve the reagent.

A3. Transfer 4 to 10 µl (2 to 5 nano-mol) of disulfide-oligo solution to the tube containing Reducing Reagent from **Step A2**. Pipette the solution up and down in the tube three times to mix.

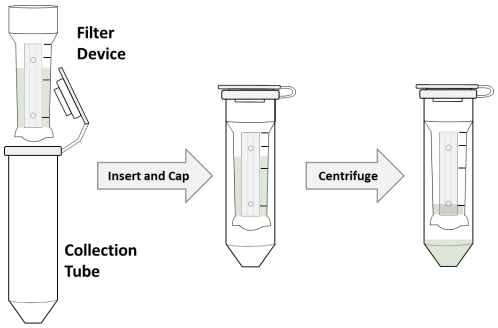
A4. Incubate at 37°C for 30 min.

Start Time: _____ End Time:_____

> The following steps are to be performed without any break. Reduced thiols tend to oxidize quickly. Work quickly through **Steps C5-C7 and D1-D3**.

Work quickly

A5. Insert a new Filter Device into one of the provided Collection Tubes for Fiter. Transfer the reaction mixture from **Step A4** into the Filter Device directly. Wash the centrifuge tube twice with ~200 µL Buffer B (indigo label), transfer the solution to the Filter Device (total volume should be ≤500 µL), and cap it.



A6. Spin the Filter Device at 14,000 x g for 8 to 15 minutes to concentrate to < 100 µL. Spin time will depend on the Filter Device supplied in the kit.

Catalog No# (1 rxn, 3 rxn)	Oligo Length	Spin Time	Typical Leftover Vol.
CM53407.1(x1, x3)	5-30 bases	15	80 µL
CM53407.2(x1, x3) CM53407.3(x1, x3)	≥31 bases	8	35 µL
Typical leftover volume is obtained using an Eppendorf 5417R and centrifuge at 4°C.			

A7. 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.**

A8. Insert the Filter Device back into the Collection Tube. Add 400-450 µL of Buffer B to make up the total volume to 500 µL. Spin the device at 14,000 x g for 8 to 15 minutes to concentrate to **< 100 µL** (refer to **Step A6** for spin time).

> **Note**: Just few minutes before the centrifugation (**Step A8**) is about to complete, please move on to the next step (**Step B1)** and prepare the activated HRP for conjugation.

3. HRP Conjugation with Thiol-Oligo (Step 2)

Items needed: Activated HRP (CM53211, red label), Thiol Oligo from Step C7, Solution A (CM01003, green label).

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Work quickly

B1. Add 125 µL of Solution A (green label) to the tube containing Activated HRP (red label). Vortex for 30 seconds to 1 minute to dissolve the HRP.

B2. Transfer the Thiol-Oligo sample from the Filter Device from **Step A8** to the Activated HRP tube by pipetting (preferably using a sterilized 100 µL pipette tip with filter). Make sure the pipette tip reaches the bottom of the Filter Device.

B3. Wash the Filter Device twice with 20 µL of Buffer B and transfer the wash to the Activated HRP tube from **Step B2 (Note: Wash = add buffer, aspirate with pipette 2-3 times).**

B4. Incubate at 25°C or room temperature **in the dark (wrap the tube in aluminum foil or place in a closed incubator)** for at least 4 h, preferably overnight.

Start Time: _____ End Time: _____

4. Purification to Remove Excess HRP

Items needed: HRP-Oligo Reaction Mixture from Step D4, Buffer C (CM02003, lime label), 1 Column Q (CM03SC5), Buffer D (CM02004, navy label), 2 Collection Tubes (CM03CT6).

C1. Sample Preparation: When **Step B4** is complete, add 175 µL of Buffer C (equilibration and washing buffer, lime label). Pipette the solution up and down in the tube three times to mix.

C2. Column Equilibration: Insert the Column Q into one of provided Collection Tubes for Column Q, Add 400 µL of Buffer C to a Column Q and centrifuge at 2000 x g for 2 minutes. Discard the flow-through. Repeat this step one more time.

C3. Sample Application: Add the sample solution from **Step C1** to the equilibrated Column Q at a maximum volume of 400 µL. Centrifuge at 2000 x g for 5 minutes. Set aside the flow-through.

> **Tip**: Pay attention to the color of Column Q; if the conjugation reaction is successful, the top of Column Q will be light brown. **If the top of Column Q is colorless, please store your remaining buffers/solvents/Column Q inside a 4°C refrigerator and contact CellMosaic.**

C4. Washing: Add 400 µL of Buffer C to Column Q and centrifuge at 2000 x g for 2 minutes. Repeat two more times. Make sure the final wash is colorless. Discard the flow-through.

C5. Elution: Place Column Q on a clean Collection Tube. Add 400 µL of Buffer D (elution buffer, navy label) to the column. Centrifuge at 2000 x g for 2 minutes. Collect the flow-through and label it as elution (**Elution 1**).

> **Tip for elution volume**: **Elution 1** contains the majority of your purified HRP-oligo conjugate. However, conjugating longer oligos may require a greater volume to elute. Pay attention to the color of Column Q; if it is still brown-colored, repeat **Step C5** to elute and collect more fractions.

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HRP-Oligo is Ready for Your Experiment

The approximate concentration of **Elution 1** is 6.25 µM in 400 µL of 50 mM Tris buffer, pH 8.0, 1 M NaCl assuming 50% recovery (5 nmol oligo scale reaction). Elution can be diluted (for lower concentration) or a desalting column (not included in kit) can be used to exchange the buffer.

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Other Considerations

1. Concentration Determination for Oligo (Unlabeled)

The accuracy of the amount of oligo is important for achieving optimized oligo labeling in this protocol. The oligo manufacturer will usually supply the oligo in lyophilized form with the amount measured prior to lyophilization. This quantitation is usually sufficient. For accuracy, you can re-measure the concentration of the oligo after re-suspending the oligo in deionized water in **Step A1**.

$$Concentration\ (M)\ of\ oligo\ before\ dilution = \frac{(A260) \times DF}{L \times \varepsilon o}$$

A260: UV absorbance of oligo at 260 nm.

L: UV cell path length (cm) - if you are using a 1 cm UV cell, dilute the oligo in water to a concentration of 1 to 2 µM.

DF: dilution factor.

Ɛo: extinction coefficient of oligo.

2. Concentration Determination for HRP–Oligo Conjugate

To determine the concentration of your conjugate, dilute your HRP–oligo from **Step E5** with 1x PBS buffer. Measure the UV absorbance of the HRP–oligo at 403 nm (A403) using a UV spectrometer and calculate the concentration using the following formula:

$$Concentration\ (\mu M)\ of\ the\ diluted\ sample = \frac{\mathrm{A403}\ \times 10}{\mathrm{L}\ \times 1.02}$$

$$Concentration\ (mg/mL)\ of\ the\ diluted\ sample = \frac{\mathrm{A403}\ \times 10\ \times (43000 + \mathrm{Mw(oligo)})}{\mathrm{L}\ \times 1.02}$$

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

Mw(oligo): molecular weight of oligo.

3. MW Calculation

Calculation of the MW of the conjugate:

Mw(conjugate) = Mw(oligo) + 43000

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4. Recommended Storage Conditions

For long-term storage, HRP-oligo conjugates can be lyophilized and stored as lyophilized powder at -80°C for 1 year.

5. Characterization of HRP–Oligo by HPLC

HRP–oligo conjugate can easily be characterized by size-exclusion chromatography (SEC) and anion exchange chromatography (AEX) HPLC. SEC separates the molecules by apparent molecular weight (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 HRP, unlabeled oligo, and conjugate, you can check whether a conjugate formed, the heterogeneity of the labeling, and the percentage of unlabeled oligo and HRP. AEX separates the molecules based on the net surface negative charge (total charge). After conjugation, the total negative charge of the conjugates may be different and result in separation. It may be difficult to obtain good data with AEX HPLC without optimization. We recommend customers use SEC HPLC as the main method for conjugate analysis.

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 needed 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. Sample Submission for HPLC Analysis

If you are submitting samples to CellMosaic for HPLC analysis, please follow these instructions:

1) Go online: https://www.cellmosaic.com/hplc-analysis/, select SEC HPLC Analysis (Product# AS0023) and/or AEX HPLC Analysis (Product# AS0026), 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) Dilute your un-conjugated oligo to 10-20 µM in PBS buffer, and then transfer 50 µL of the diluted solution to a 0.5 mL micro-centrifuge tube. Label the vial properly.
3) Dilute your conjugate to 0.1–0.5 mg/mL in PBS buffer, and then transfer 50 µL of the diluted solution to a 0.5 mL micro-centrifuge tube. Label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.