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PerKit™ AP Oligo Conjugation Kit (CM53403) 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 oligomer, 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

This kit provides materials to perform Alkaline Phosphatase (AP) labeling of one or three oligo samples. Scale of the reaction: 2.5 nmol of oligo.

If you purchased this kit previously, the current one is an upgraded and improved kit with slightly different components and workflow as the first-generation kit. This kit has multiple configurations for various lengths of oligos and can be used to conjugate 2.5 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™ AP Oligo conjugation kit (CM53403)

Configuration	No. of Reactions	Catalog No.
1. Labeling for short oligo (5–30 bases)	1	CM53403.1x1
	3	CM53403.1x3
2. Labeling for long oligo (≥31 bases)	1	CM53403.2x1
	3	CM53403.2x3

How to use this protocol: The protocol in this user manual is written for two different configurations: 5–30 bases oligo (CM53403.1) and ≥31 bases oligo (CM53403.2). 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 amine-oligo:

1. Total amount: 2.5 nmol oligo content as measured by UV. **Note:** the accuracy of your oligo amount is important for obtaining optimized loading. Please refer to the section Other Considerations in this manual to measure the oligo amount.
2. HPLC purified with amino-oligo content ≥90% by C18 HPLC.
3. ≥5-mer (oligos longer than 60-mer may result in lower loading and high impurities).

Note: You can purchase HPLC purified C6 amino-modified oligo from standard oligo manufacturers up to 90-mer length 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™ AP Oligo conjugation kit. All kits share the same components except for 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)	Activated AP (red label)	CM53212	Any	1 unit	3 units
	Reagent A (silver label)	CM12002.1		1 unit	3 units
	Reagent B (**blue label**)	CM13001		1 unit	3 units
Box 2 (2–8°C)	Buffer A (Orange label)	CM02001	Any	4 mL	12 mL
	Buffer B (Cyan label)	CM02033		1.5 mL	5 mL
	Buffer C (Green label)	CM02032		3 mL	9 mL
	Buffer D (purple label)	CM02031		1.5 mL	5 mL
	0.5 mL Eppendorf Tube	CM03CT7		1	3
	Centrifugal Filter Devices (Only one part# will be supplied, 3 or 9 units)	CM03CD003A	CM53403.1	3	9
		CM03CD010A	CM53403.2		
	Collection Tubes for Filter	CM03CT0	Any	6	18
	Column Q	CM03SC5		1	3
	Collection Tubes for Column Q	CM03CT6		2	6
User Supplied Material	Amine Oligo (≥5 bases)	N/A	NOT PROVIDED (User Supplied Material, 2.5 nmol 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 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 AP-oligo conjugate with linkers and conjugation chemistry that retains the activity of AP. The same configuration and chemistry have been tested for AP-oligos with different oligo sequences from a few diagnostic companies since 2011.

The kit includes purified maleimide activated AP that prepared from highly active and EIA grade AP from Calf intestine (>3000 U/mg). The user supplies the amine-modified oligos, which are readily available from many commercial oligo suppliers. Using the kit components, the user converts the amine-oligo to a thiol-oligo, followed by reaction of the thiol-oligo with activated AP to generate the AP-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 AP-oligo at greater than 80% purity.

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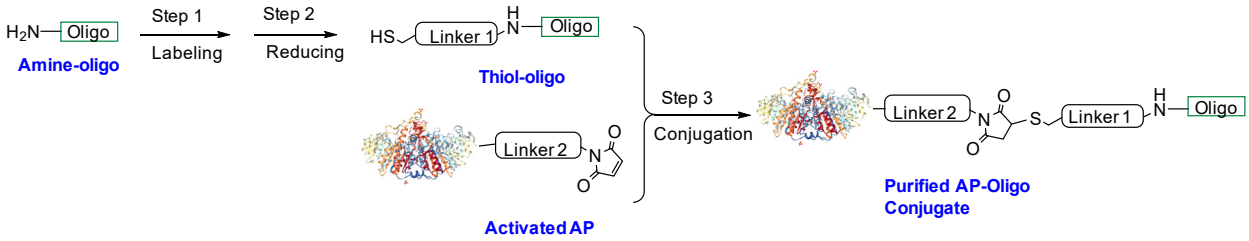

Scheme 1. Synthetic route to AP Oligo conjugate.

An HPLC purified oligo is required for this conjugation. If the content of the amine oligo is >90%, the purity of the final conjugate will generally be >90% pure with an average of 1 oligo per AP (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 AP-oligo conjugation kit:

- High quality activated alkaline phosphatase (>95% pure).
- Fast preparation: less than 5 h hands-on time.
- Minimum or no AP activity loss during the conjugation and purification.
- A single purification affords 85-90% single-labeled HRP-oligo conjugates if the quality of the oligo is at par.
- Stable linkage with optimal spacer to ensure no interference between oligo binding and AP 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 AP.

Support

Customer can request a recommendation for the conjugation if the oligo has a special feature 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 three reactions in this protocol. Keep in mind that thiol-labeled oligo should be purified quickly once the reaction is done and used immediately. AP-oligo conjugation may be done within 2 h at room temperature, recommend leaving it at 2−8°C overnight afterwards. The Q-column purification will take less than 10 minutes.

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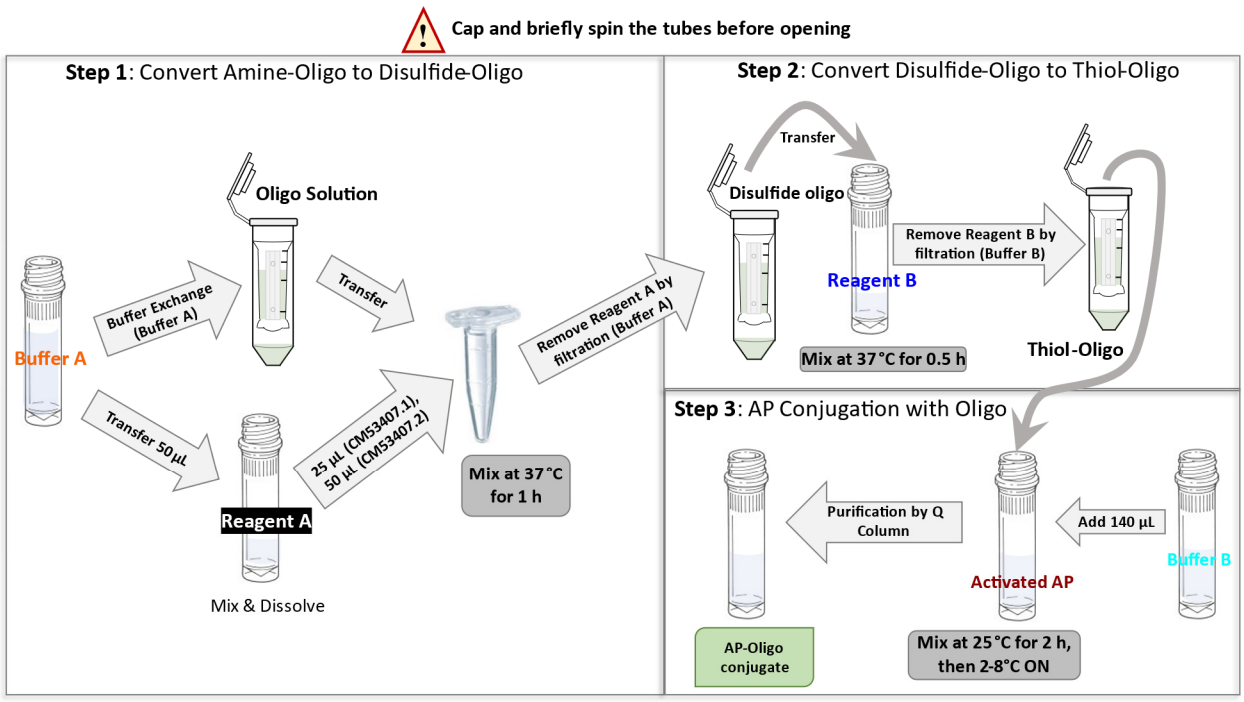

Scheme 2. Schematic diagram of the workflow for preparing 1:1 AP-oligo conjugates

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. Preparation of Oligo Sample for Labeling

Items needed: Amine-Oligo (user supplied), 1 Filter Device (CM03CD003A or CM03CD010A), 2 Collection Tubes for Filter (CM03CT0), Buffer A (CM02001, orange label), clean centrifuge tubes (not provided in the kit).

Total amount of oligo used for the conjugation is **2.5 nano-mole**.

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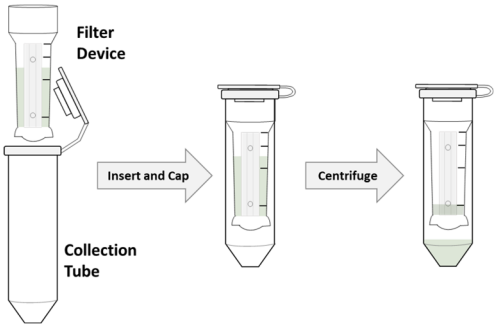
A1. Insert a new Filter Device into one of the provided Collection Tubes for filter. Perform the step based on the following conditions.

- ✓ If your oligo is supplied as a lyophilized solid, dissolve the oligo in deionized water to a concentration of 25 µM and then transfer up to 100 µL to the Filter Device. Add Buffer A (orange label) to make up the total volume to 500 µL and cap it.
- ✓ If your oligo is supplied as liquid, transfer up to **2.5 nano-mole** to the Filter Device directly. Add Buffer A to make up the total volume to 500 µL and cap it.



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 (preferably cooled to 4°C) 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.
CM53403.1(x1, x3)	5-30 bases	15	80 µL
CM53403.2(x1, x3)	≥31 bases	8	35 µL
Typical leftover volume is obtained using an Eppendorf 5417R and centrifuge 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 the Filter Device. Spin the device at 14,000 x g for 8 to 15 minutes to concentrate to < 100 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.**

3. Convert Amine-Oligo to Disulfide-Oligo (Step 1)

Items needed: Amine-Oligo from Step A5, Reagent A (CM12002.1, white label), Buffer A (CM02001, orange label), 0.5 mL Eppendorf Tube (CM03CT7), 1 Filter Device (CM03CD003A or CM03CD010A), 2 Collection Tubes for Filter (CM03CT0).

B1. Transfer the Amine-Oligo sample from the Filter Device from **Step A5** to a 0.5 mL Eppendorf 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.

B2. Wash the Filter Device twice with 10 µL of Buffer A and transfer the wash to the same Eppendorf Tube from **Step B1 (Note: Wash = add buffer, aspirate with pipette 2-3 times).**

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B3. Briefly spin the tube containing Reagent A (white label). Make sure you can see a tiny droplet at the bottom of the tube. Add 50 µL of Buffer A (orange label) to the tube. Vortex for 30 seconds to 1 minute to dissolve Reagent A.

Tip for solubility check: Check the bottom of the tube to see if the solution is clear.
Tip for opening centrifuge tube after vortex: Always centrifuge the tube to ensure no liquid is in the cap.

B4. Add Reagent A solution from **Step B3** to amine-oligo solution tube from **Step B2**. Pipette the solution up and down in the tube three times to mix.

Catalog No# (1 rxn, 3 rxn)	Oligo Length	Volume of Reagent A Solution	Check Mark
CM53403.1(x1, x3)	5-30 bases	25 µL	
CM53403.2(x1, x3)	≥31 bases	50 µL	

B5. Incubate at 37°C for 1 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 tube from **step B4** is securely capped. If you don't have any of this equipment, you can let the tube sit on the bench with manual mixing by pipetting every 10 minutes.

B6. Remove the tube from **Step B5** from the incubator and spin to ensure no liquid is in the cap.

B7. Insert a new Filter Device into one of the provided Collection Tubes for filter. Transfer the reaction mixture from **Step B6** into the Filter Device directly. Wash the tube twice with 170-210 µL of Buffer A, transfer the solution to the Filter Device (total volume should be ≤500 µL), and cap it. Spin the Filter Device at 14,000 x g for 8 to 15 minutes to concentrate to < 100 µL (refer to **Step A3** for spin time).

B8. 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.**

B9. Insert the Filter Device back to the collection tube. Add 400-450 µL of Buffer A 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 A3** for spin time).

B10. Repeat **steps B8** and **B9** one time.

4. Convert Disulfide-Oligo to Thiol-Oligo (Step 2)

Items needed: Disulfide-Oligo from Step B10, Reagent B (CM13001, blue label), Buffer A (CM02001, orange label), Buffer B (CM02005, indigo label), 1 Filter Device (CM03CD003A or CM03CD010A), 2 Collection Tubes for Filter (CM03CT0).

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C1. Centrifuge the tube containing 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.

C2. Transfer the Disulfide-Oligo sample from the Filter Device from **Step B10** to the Reagent B 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.

C3. Wash the Filter Device twice with 10 µL of Buffer A and transfer the wash to the Reagent B tube from **Step C2 (Note: Wash = add buffer, aspirate with pipette 2-3 times).**

C4. 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

C5. Insert a new Filter Device into a new provided Collection Tube. Transfer the reaction mixture from **Step C4** 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. Spin the Filter Device at 14,000 x g for 8 to 15 minutes to concentrate to < 100 µL (refer to **Step A3** for spin time).

C6. 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.**

C7. 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 A3** for spin time).

Note: Just few minutes before the centrifugation (**Step C7**) is about to complete, please move on to the next step (**Step C1)** and prepare the activated AP for conjugation.

5. AP Conjugation with Oligo (Step 3)

Items needed: Activated AP (CM53212, red label), Thiol Oligo from Step C7, Buffer B (CM02033, cyan label).

Work quickly

D1. Add 140 µL of Buffer B (cyan label) to the tube containing Activated AP (red label). Vortex for 30 seconds to 1 minute to dissolve the HRP.

D2. Transfer the Thiol-Oligo sample from the Filter Device from **Step C7** to the Activated AP 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.

D3. Wash the Filter Device twice with 20 µL of Buffer B and transfer the wash to the Activated AP tube from **Step D2 (Note: Wash = add buffer, aspirate with pipette 2-3 times).**

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D4. Pipette the solution up and down in the tube three times to mix. Incubate at room temperature for 2 hours, then at 2-8°C for overnight.

Start Time: _____ End Time:_____

6. Purification to Remove Excess AP

Items needed: AP-Oligo Reaction Mixture from Step D4, Buffer C (CM02032, green label), Buffer D (CM02031, purple label), 1 Column Q (CM03SC5), 2 Collection Tubes for Column Q (CM03CT6).

E1. Sample Preparation: When **Step D4** is complete, add 100 µL of Buffer C (equilibration and washing buffer, green label). Pipette the solution up and down in the tube three times to mix.

E2. Column Equilibration: Insert the Column Q into one of provided Collection Tubes for Column Q, add 400 µL of Buffer C and centrifuge at 2000 x g for 2 minutes. Discard the flow-through. Repeat this step once more.

E3. Sample Application: Add the sample solution from **Step E1** 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.

E4. Washing: Add 400 µL of Buffer C to Column Q and centrifuge at 2000 x g for 2 minutes. Repeat twice more. Make sure the final wash is colorless. Discard the flow-through.

E5. Elution: Place Column Q on a clean Collection Tube for Column Q. Add 400 µL of Buffer D (elution buffer, purple 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 AP-oligo conjugates. However, conjugating longer than 50mer oligos may require more volume to elute. Repeat **Step D5** to elute and collect another fraction (E2).

AP-Oligo is Ready for Your Experiment

Tip: The approximate concentration of **Elution 1** is 3.13 µM in 400 µL of 50 mM Tris buffer, pH 8.0, 1M NaCl, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ assuming 50% recovery. The elution can be diluted for a 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 AP-Oligo Conjugate

To determine the concentration, dilute your AP-oligo from **Step** E**5** with water. Measure the UV absorbance of AP-oligo at 260 nm (A_{260}) using a UV spectrometer and calculate the concentration based on the following formula:

$$\boldsymbol{Concentration\ of\ the\ diluted\ sample}\ (\mu\boldsymbol{M}) = \frac{\mathrm{A260}}{\mathrm{L\ x\ (\varepsilon o + 55900)}}$$

L: UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute AP-oligo 2 to 4 times to get a good reading.

Ɛo: extinction coefficient of oligo at 260 nm.

3. MW Calculation

Calculation of the MW of the conjugate:

Mw(conjugate) = Mw(oligo) + 116000

4. Recommended Storage Conditions

The recommended storage is 2-8°C. Do not freeze. You can add up to 50% glycerol in the buffer solution if you want to store the sample below -20°C in the freezer.

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5. Characterization of AP–Oligo by HPLC

AP–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 AP. 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. Submit Samples for HPLC Analysis

If you are submitting samples to CellMosaic for anion exchange chromatographic (AEX) and SEC analysis, please follow these instructions:

1) Dilute your un-conjugated oligo to 10 µM in water, then transfer 50 µL of the diluted solution to a 500 µL microcentrifuge tube. Label the vial properly.
2) Transfer 50 µL of AP-oligo (non-diluted solution) to a 500 µL microcentrifuge tube and label the vial properly.
3) Ship your samples with a cold pack for overnight delivery.

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Appendix: Typical Kit Performance Data (LC analysis, CellMosaic)

Oligo information: HPLC-purified 23mer oligo with 5' NH_2-C6 modification
Kit Lot number: 5527.s12.070819

Figure 1: SEC HPLC profiles of AP (blue trace), oligo (green trace), and purified product (Elution 1) (red Trace) detected at 220 nm. SEC separates the conjugates by apparent molecular weight (MW) or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes.

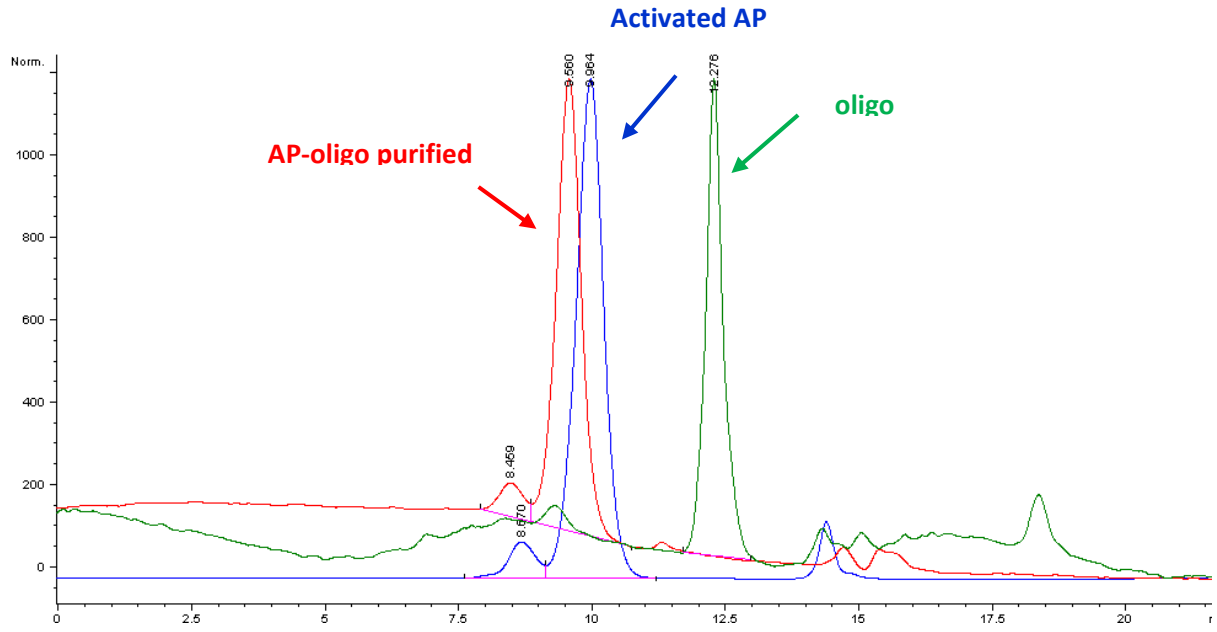

Figure 2: Anion exchange chromatographic HPLC profiles of the AP (blue trace), oligo (green trace), and AP oligo conjugation reaction mixture (red Trace) detected at 220 nm

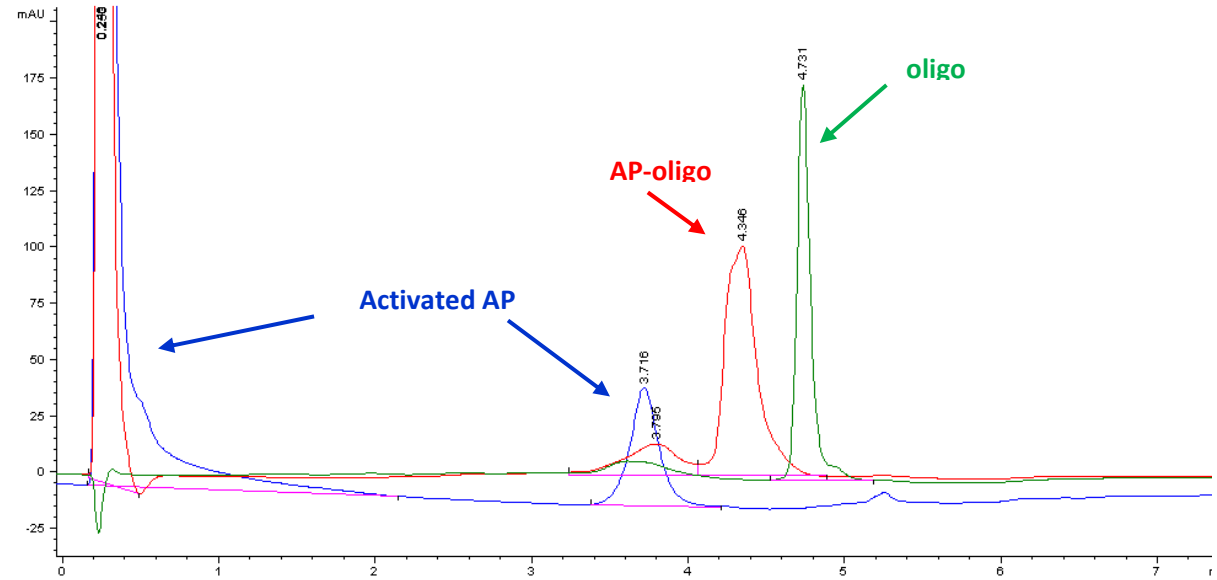

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Table 1: Summary results

Sample	Volume (µL)	% free AP	% free oligo	% of oligo/AP (1:1)
Elution 1	400	Not detected	Not detected	93%
Recovery: 52% of the conjugates from 2.5 nmol of starting oligo.				