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AqT™ Biotin Active Ester Antibody Labeling Kit (T2A15) (CM86140 Group) 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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If you are interested in using AqT™ for commercial usage or partnering/collaboration in the development of AqT™ therapeutics, please contact:

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Kit Configuration and Components

AqT™ Biotin Active Ester Antibody Labeling kit (T2A15) is a proprietary biotinylation kit developed at CellMosaic for obtaining high quality and high loading of biotinylated antibody with minimal or no aggregation. The kit uses a biotin modified with a super-hydrophilic, water-soluble, and charge neutral AqueaTether™ (AqT™) T2A15 linker to improve its water solubility and decrease its non-specific interaction with other proteins or plastic surfaces. The kit has multiple configurations for labeling various amounts of antibody (10 µg to 3 mg) with either one (x1) or three (x3) antibody samples. **Table 1** provides the catalog numbers for various kit configurations. **Table 2** lists the kit components.

Table 1: Configurations for the AqT™ Biotin Active Ester Antibody Labeling Kit (T2A15)

Catalog Number	Number of Reactions in Kit	Antibody Amount per Reaction
CM86140.1x1	1	10 µg
CM86140.1x3	3	
CM86140.2x1	1	50 µg
CM86140.2x3	3	
CM86140.3x1	1	100 µg
CM86140.3x3	3	
CM86140.4x1	1	1 mg
CM86140.4x3	3	
CM86140.5x1	1	3 mg
CM86140.5x3	3	

How to use this protocol: The protocol in this user manual is written for five reaction scales: 10 µg (CM86104.1), 50 µg (CM86104.2), 100 µg (CM86104.3), 1 mg (CM86104.4), and 3 mg (CM86104.5) of Antibody. Most of the steps are common to all reaction scales. However, some steps are specific to each reaction scale.

⚠ Please follow the specific instructions for individual reaction scale.

Quick reference protocol: A one-page quick reference protocol for each reaction scale is included at the end of this user manual and recommended for those who have performed the labeling at least once following the extensive protocol.

Biotinylation level (see Other Considerations): The biotinylation level is easily tunable by simply increasing the temperature. The recommended biotinylation level is 26.7 to 40 nmole biotin per mg of antibody. This translates to an average of 4 to 6 biotins per antibody for IgG (MW 150 KDa). If your antibody has a different MW, the biotinylation level per mg is still the same. However, the molar ratio will be proportionally different. The kit includes a 10-minute biotin assay reagent and protocol to estimate the biotinylation level immediately after labeling.

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Table 2: Components and Storage Temperatures for AqT™ Biotin Active Ester Antibody Labeling Kits (T2A15). All kits share the same components except the amount of activated AqT™ Biotin (CM86123), which varies with the reaction scale.

⚠ 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 #	Antibody Amount	Kit Cat#	Quantity (x1) (1 Rxn)	Quantity (x3) (3 Rxn)
Box 1 (store in ≤-20°C freezer)	Activated AqT™ Biotin (red label) (Only one part# will be supplied, 1 or 3 units)	CM86123.1	10 µg	CM86140.1	1 unit (One part # will be supplied)	3 units (Same part# will be supplied)
		CM86123.2	50 µg	CM86140.2		
		CM86123.3	100 µg	CM86140.3		
		CM86123.4	1 mg	CM86140.4		
		CM86123.5	3 mg	CM86140.5		
Box 2 (store at 2-8°C. Do not freeze)	Labeling Buffer (orange label)	CM02001	Any	Any	4 mL	12 mL
	PBS Buffer (grey label)	CM02013			4 mL	12 mL
	Biotin Assay Reagent	CM61006			2 units	6 units
	Biotin Assay Buffer (green label)	CM01003			0.3 mL	0.3 mL x 3
	Centrifugal Filter Device	CM03CD050A			2	6
	Collection Tubes	CM03CT0			4	12
User Material	Antibody (≥100 KDa)	N/A	NOT PROVIDED (User Supplied Material)			

Requirements for antibody:

- **MW:** ≥ 100 KDa (if MW is lower, please use an alternative kit)
- **Purity**: preferably > 90% pure by gel electrophoresis
- **Amount:** 10 µg to 3 mg protein content as measured by UV (the accuracy of the protein amount is important factor to obtaining an optimized biotinylation level – please refer to the section '**Other Considerations**' in this manual to measure the protein amount)

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.

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AqT™ Biotinylation Chemistry

AqT™ linkers are novel proprietary biomaterials invented at CellMosaic that are chemically assembled from a class of natural and edible sugar alcohol compounds with properties by design. AqT™ linkers can be designed for labeling and conjugating biomolecules with very hydrophobic small molecules to improve the overall performance of the bioconjugates in their downstream applications.

Biotin is a very hydrophobic molecule. Antibodies and proteins labeled with biotin tend to aggregate and precipitate out from solution over time. We have modified biotin with AqT™ linker to increase its hydrophilicity and water solubility. The hydroxy (-OH) groups of AqT™ form a network of hydrogen bonds (H-bond) with neighboring water and create a microenvironment that shields neighboring biotins from stacking or interacting with one another (**Figure 1**). An antibody can be labeled with a high amount of AqT™-biotin with no change to the properties of the antibody (see **Other Considerations HPLC profiles**). This AqT™ enhanced H-bond network also protects the antibody from enzymatic degradation and retains its activities.

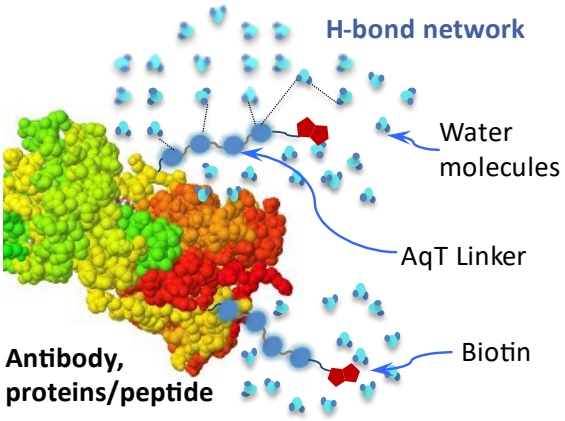

Figure 1. AqT™ enhanced H-bond network

Biotin is sparsely water soluble (0.19 mg/mL, Saturated solution measured at CellMosaic). All other biotinylation kits currently commercialized require an organic solvent, such as DMSO, in the solution to dissolve the biotin or activated biotin. The use of organic solvent usually induces aggregation of the antibody and promotes precipitation. AqT™ linker makes biotin water soluble. AqT™ biotinylation is performed in 100% aqueous buffer.

The CM86140 kit is designed to biotinylate any antibody with an AqT™ T2A15 linker composed of two threitols and 15 atom lengths (**Scheme 1**) via the surface amines of the antibody. This atom length is equivalent to dPEG4 (16 atom lengths) and, based on our experience, can largely keep the bivalent molecule function independent. The conjugates we have tried with this atom length include antibody oligo, streptavidin oligo, and HRP oligo, among others. For more information regarding the properties of AqT™ T2A15, please see **Other Considerations** in this manual.

The user supplies the antibody. Using the kit components, the user biotinylates their antibody in one simple step, followed by filtration-type purification to remove any unreacted AqT™-biotin, exchanging it with PBS or other buffer for downstream application. A quick 10-minute biotin assay (protocol and reagent) is also included in the kit to obtain the biotinylation level immediately after labeling. The biotinylation level can easily be tuned by increasing or decreasing the reaction temperature. The hands-on time is less than 30 minutes, and the whole preparation time varies from 3 h to overnight depending on the reaction time.

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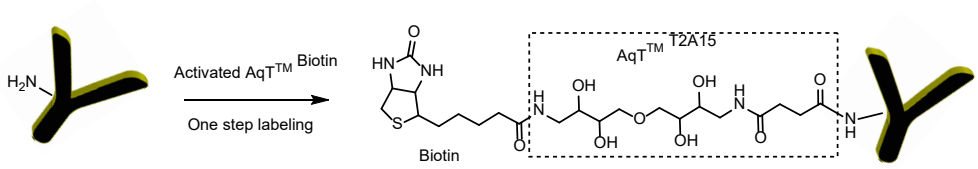

Scheme 1: Biotinylation with pre-activated AqT™ Biotin linker.

Table 3: CM86140 and Its Product Compared to Other Commercial Antibody Biotinylation Kits.

		AqT™ Biotin Labeling Kit	Other Commercial Kits
Biotinylated Product Features	Chemical structure	**Fully Disclosed** • Complete knowledge of your product	Varies by vendor, mostly **not disclosed.**
	Properties	**Retain antibody properties** • No changes in hydrophobicity • No increase of apparent MW (hydrodynamic volume) • No increase in aggregation • Minimal heterogeneity after labeling	**Change antibody properties** • Increased hydrophobicity (non-specific interaction) • Increased apparent MW (hydrodynamic volume) • May increase aggregation • Highly heterogeneous product
	Stability	**Stable even with high biotin loading** Can be easily stored frozen and in lyophilized form without any stabilizer	**Decreased stability** High biotin loading may cause aggregation and precipitation
Kit Features	Reagent	Proprietary **water-soluble pre-activated biotin** with 15-atom spacer	Biotin (zero length) or biotin with hydrophobic ethylene- or polyethylene glycol-type linker
	Labeling buffer	**100% aqueous buffer**	Some percentage of **organic solvent** needed, such as DMSO
	Biotinylation level	**Predictable** • Tunable loading, from low to very high, per your need	**Unpredictable** Usually target low labeling to avoid aggregation (difficult to tune)
	Usability	**Easy to use** • One step labeling • <30 minutes hands-on time	Comparable quick labeling may use EDC chemistry with antibody **crosslinking** to each other causing large aggregates
	Purification method & components	**Included** Product is free of any unreacted AqT™ biotin after purification.	Varies by vendor, usually **not included**
	Biotin Assay	**Included** A quick 10-minute biotin assay protocol with reagents and buffer	**Not included**

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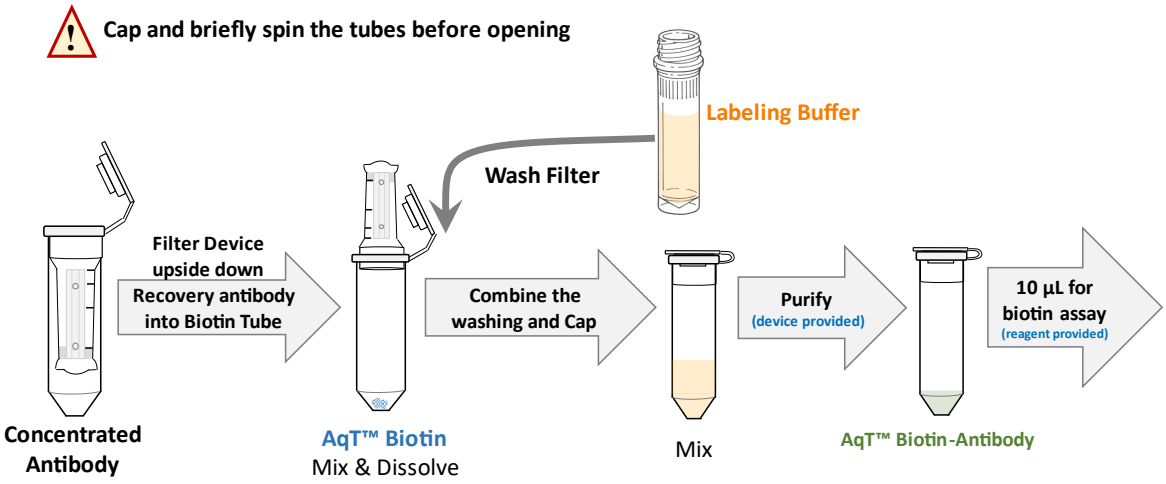
Support

CellMosaic provides support services to customers who may need help analyzing the final conjugates by HPLC.

Protocol



Scheme 2. Overview of the workflow for preparing AqT™ antibody-biotin conjugates.

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, 14,000 *g* capable), mini-centrifuge
- Pipettes and tips
- Timer
- Incubator or shaker (e.g., Eppendorf® Thermomixer 5350)
- Personal protection equipment (lab coat, safety glasses, and chemical-resistant nitrile gloves)

2. Preparation of Antibody Samples for Labeling

Items needed: Filter Devices (CM03CD050A), Collection Tubes, Labeling Buffer (CM02001, Orange label), 1.5 mL Centrifuge Tube, Clean Centrifuge Tubes (not provided in the kit).

A1. Insert the Filter Device into one of the provided collection tubes (microcentrifuge tube with the cap attached). Perform the step based on the following conditions.

- If your antibody is supplied as a lyophilized solid, dissolve the antibody in 500 µL of deionized water and then transfer the entire contents to the Filter Device.

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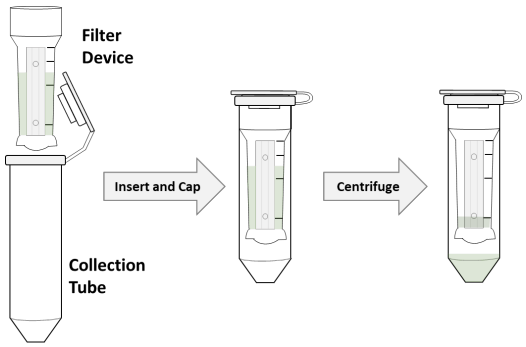
- If your antibody is supplied in < 500 µL buffer, transfer your antibody sample to the Filter Device directly. Add Labeling Buffer to make up the total volume to 500 µL and cap it.



- If the volume of your antibody sample is >500 µL, add up to 500 µL of sample to the Filter Device. Repeat Step **A1-A4** until all of the antibody sample goes into the Filter Device. Move on to Step A5. Add Labeling Buffer 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 8 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 is approximately 8 to 10 minutes. The typical volume is 28 µL for ≤100 µg of antibody and 40 µL for ≥1 mg of antibody after spinning for 8 minutes in 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 Labeling Buffer into the Filter Device. Then place the capped Filter Device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the device at 14,000 x *g* to concentrate to < 50 µL. 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.**

A6. Follow the instructions below for individual kit configurations:

- If the total volume of your antibody added in **Step A1** for buffer exchange is <25 µL for 10 µg (CM86140.1), 50 µg (CM86140.2), and 100 µg CM86140.3), go directly to **Step B1**. Otherwise, repeat **Step A5** one time.
- Repeat **Step A5** two times for 1 mg (CM86140.4) and 3 mg (CM86140.5).

3. Antibody Labeling

Items needed: Activated AqT™ Biotin (red label) (CM86123 group, red label), Labeling Buffer (CM02001, Orange label), Antibody Solution in the Filter from **Step A6**.

B1. Spin the centrifuge tubes containing activated AqT™ Biotin (red label) and remove the parafilm wrap around the cap.

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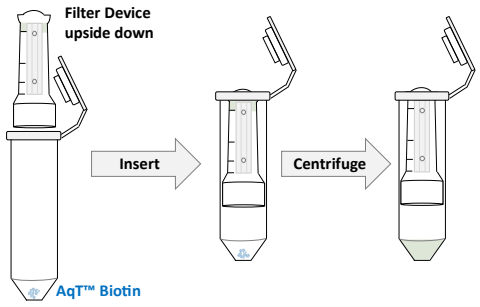
B2. Place the Filter Device containing concentrated antibody from **Step A6** upside down in the activated AqT™ Biotin tube. Spin for 2 minutes at 1,000 x *g* to transfer the antibody from the Filter Device to the activated AqT™ Biotin tube.



B3. Follow the instructions below for individual kit configurations.

- For 10 µg (CM86140.1), go directly to **Step B4.**
- For 50 µg (CM86140.2) and 100 µg (CM86140.3), wash the Filter Device once with **25 µL** of Labeling Buffer and transfer the wash to the activated AqT™ Biotin tube.
- For 1 mg (CM86140.4), wash the filter two times with **100 µL** of Labeling Buffer and transfer the wash to the activated AqT™ Biotin tube.
- For 3 mg (CM86140.5), wash the filter two times with **300 µL** of Labeling Buffer and transfer the wash to the activated AqT™ Biotin tube.

  Wash = Add buffer, aspirate with pipette 2-3 times.

B4. Make sure the cap is tight. Use parafilm to wrap around the cap. Vortex the mixture of activated AqT™ Biotin and antibody for 10 seconds to mix and then spin down.

B5. Mix at 37 °C for 2 h for high loading. If you have very sensitive antibody, you can mix at 4 °C for overnight. Use the following table as a guideline to choose the appropriate temperature for optimized biotinylation level.

Ab Stability	Temp.	Time	Biotinylation level (nmole per mg of Ab)	Biotinylation level (Number per IgG)
Very stable	37 °C	2 h	33.3–46.7	5–7
Stable	30 °C	2 h	26.7–40	4–6
Stable	25 °C	2 h	13.3–26.7	2–4
	25 °C	4–24 h (ON)	13.3–33.3	2–5
Not stable	4 °C	16–24 h (ON)	13.3–26.7	2–4

Note: your biotinylation level may not fit in this range. Please follow **Steps D1–D5** to experimentally determine the biotinylation level. Once you have the initial biotinylation level, you can optimize your biotinylation level with confidence. ON = Overnight

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 your centrifuge is capped properly. If you do not have any of this equipment, you can let the centrifuge tube sit at the bench with manual mixing by pipetting every 20 minutes.

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4. Purification of Biotinylated Antibody

Items needed: Storage Buffer (1x PBS, CM02013, grey label) (you can substitute PBS with your buffer of choice), 2.0 mL Centrifuge Tube, Antibody Solution from **Step B5**.

C1. Insert the Filter Device into one of the provided collection tubes. Transfer the antibody solution from **Step B5** to the Filter Device. Follow the instructions below for individual kit configurations:

- For 10 µg (CM86140.1), 50 µg (CM86140.2), and 100 µg (CM86140.3), wash the collection tube twice with **200 µL** of PBS and transfer the wash to the Filter Device.
- For 1 mg (CM86140.4), wash the collection tube once with **200 µL** of PBS and transfer the wash to the Filter Device.
- For 3 mg (CM86140.5), go directly to **Step C2 and C3** and repeat Steps **C1-C3** until all of the antibody sample goes into the Filter Device.
  Wash = Add buffer, aspirate with pipette 2-3 times.

C2. Place the capped Filter Device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.

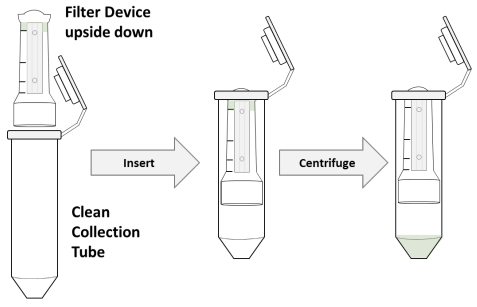
C3. Remove the assembled device from the centrifuge and separate the Filter Device from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

C4. Insert the Filter Device back into the collection tube. Add 400 µL of PBS to the Filter Device. Spin the device at 14,000 x *g* to concentrate to < 50 µL. 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.**

C5. Follow the instructions below for individual kit configurations:

- Repeat **Step C4** one time for 10 µg (CM86140.1), 50 µg (CM86140.2), and 100 µg (CM86140.3).
- Repeat **Step C4** two times for 1 mg (CM86140.4) and 3 mg (CM86140.5).

C6. To recover the sample, place the Filter Device upside down in a new collection tube. Spin for 2 minutes at 1,000 x *g* (preferably cooled to 4°C) to transfer the labeled sample from the Filter Device to the collection tube.



C7. Follow the instructions below for individual kit configurations:

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- For 10 µg (CM86140.1) and 50 µg (CM86140.2), if you need to do a biotin assay, go directly to **Step C8**. Otherwise, wash the Filter Device once with **22 µL** of PBS and transfer the wash to the collection tube.
- For 100 µg (CM86140.3), wash the Filter Device once with **50 µL** of PBS and transfer the wash to the collection tube.
- For 1 mg (CM86140.4), wash the Filter Device once with **200 µL** of PBS and transfer the wash to the collection tube.
- For 3 mg (CM86140.5), wash the Filter Device twice with **200 µL** of PBS and transfer the wash to the collection tube.

Wash = Add buffer, aspirate with pipette 2-3 times.

C8. Determine the concentration using a UV/Vis spectrophotometer (see **Other Considerations**).

Recovery: The lower the reaction scale, the higher the loss during handling. Here is a summary of yield (product recovery) for various scale reactions.

Antibody Amount (Cat#)	Product Recovery
10 µg (CM86140.1)	40–60%
50 µg (CM86140.2)	60–70%
100 µg (CM86140.3)	70–80%
1 mg (CM86140.4)	≥85%
3 mg (CM86140.5)	≥85%

C9. Store the AqT™ biotinylated antibody at 2–8°C. For long-term, aliquot and store the conjugate at or below -20°C or lyophilize to dryness.

5. Biotin Assay (Optional, see Other Considerations for assay information)

Items needed: Biotinylated Antibody Solution from **Step C7,** Biotin Assay Buffer (CM01003, green label), Biotin Assay Reagent (HABA/Avidin Mixture, CM61006, purple label).

Equipment needed (not provided): UV-Vis spectrophotometer set at A500 reading mode and zero, Ultra-micro UV transparent cuvette with 1 cm path length (100 µL volume)

Note: Due to sample limitations of the final product obtained from 10 µg (CM86140.1) and 50 µg (CM86140.2) labeling, it may not be practical to perform a biotin assay unless it is for a test run. Alternatively, smaller volume UV cuvette or UV/Vis spectrophotometers for smaller volume may be used.

Assay Sensitivity: This method is very sensitive for 1 mg (CM86140.4) and 3 mg (CM86140.5). However, for 10 µg (CM86140.1), 50 µg (CM86140.2), and 100 µg (CM86140.3) synthesis, it should be used just for qualitative purpose since the difference UV absorbance after the displacement of HABA from HBAB/Avidin is very small (0.01–0.1).

D1. Briefly spin the tube containing Biotin Assay Reagent (purple label) before opening it. Add

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100 µL of Biotin Assay Buffer (green label). Vortex for 10 seconds to mix and then spin it down.

D2. Transfer 90 µL of biotin assay solution to a 100 µL UV cuvette. UV absorbance at 500 nm is temperature sensitive, wait 1−2 minutes until the reading stabilizes to record the UV absorbance at 500 nm (A0).

D3. Transfer 10 µL of biotinylated antibody sample from **Step C7** to the UV cuvette. Use 100 µL pipette tip, aspirate with pipette 2−3 times to mix. Wait until the reading stabilizes to record the final UV absorbance at 500 nm (As) (2−4 minutes in general).

D4. Calculate the biotin content as follows:

$$\mu\text{M (Biotin)} = \frac{(0.9 \times \text{A0} - \text{As})}{34} \times 1000 \times 10 = \_\_\_\_\_$$

D5. Calculate the degree of labeling (DOL) based on the following formula:

$$DOL = \frac{\mu M\ (Biotin)}{\mu M\ (Antibody)} = \_\_\_\_\_$$

Where µM (Antibody) is the concentration of antibody before dilution in **Step C7**.

The Biotinylated Antibody is Ready for Your Experiment

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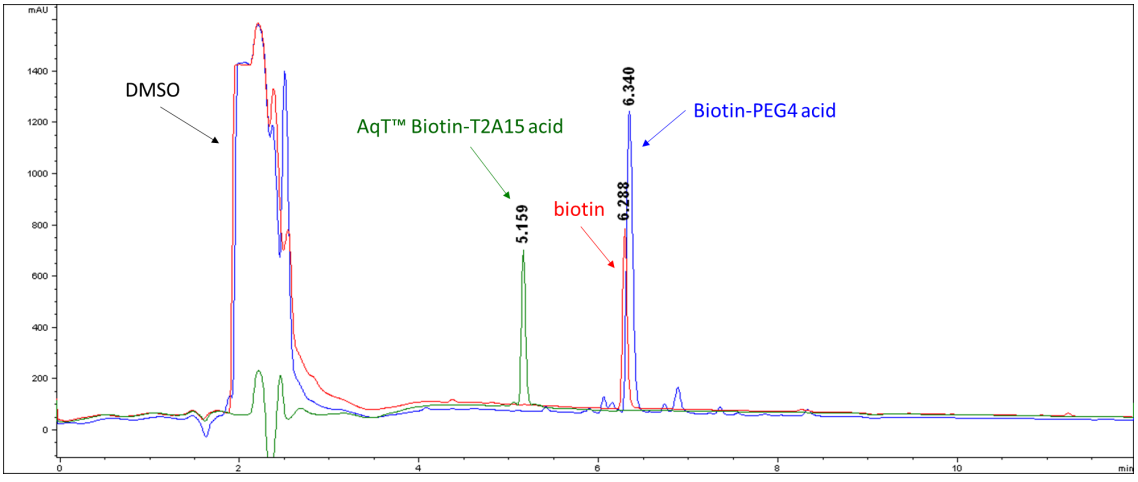
Other Considerations

1. AqT™ T2A15 Linker

Biotin is sparsely water soluble (0.19 mg/mL, saturated solution measured at CellMosaic). All other biotinylation kits currently commercialized require an organic solvent, such as DMSO, in the solution to dissolve the biotin or activated biotin. The use of organic solvent usually induces aggregation of the antibody and promotes precipitation. AqT™ T2A15 linker increases the water solubility of biotin dramatically. This solubility enhancement extends to the activated AqT™ biotin. Essentially, all biotinylations are performed at 100% aqueous solution.

AqT™ T2A15 linker is composed of two threitols and 15 atom lengths. This atom length is equivalent to four polyethylene glycol modified biotin (PEG4) (16 atom lengths) except PEG4 does not increases any hydrophilicity of the biotin. Hydrophobicity of biotin (acid), AqT™ biotin-T2A15-acid, and Biotin-PEG4-acid can be easily analyzed and compared by C18 reversed phase HPLC. **Figure 2** shows overlaid HPLC traces of these three compounds. The red trace is biotin (6.288 min peak) in DMSO (front solvent peak); The green trace is AqT™ T2A15 linker modified biotin in water, which elutes much earlier (5.159 min; 10% less acetonitrile needed to elute out); the blue trace is PEG4 linker modified biotin in DMSO, which elutes a slightly later (6.340 min, 0.5% more acetonitrile needed to elute out). These data confirm that AqT™ T2A15 linker increases the hydrophilicity dramatically while PEG4 stays similar.

Figure 2: Overlay of C18 reversed phase HPLC analysis of biotin (red, in DMSO), AqT™ biotin-T2A15 acid (green, in water), and biotin-PEG4 acid (blue, in DMSO).



2. Concentration Determination for IgG Antibody (Unlabeled)

The accuracy of the amount of IgG is important for achieving optimized biotinylation in this protocol. The simplest assay method for determining the IgG concentration in solution is to measure the absorbance of the IgG at 280 nm (UV range) ($A_{1\ mg/mL}$ = 1.4).

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If your IgG comes with a buffer that has no UV absorbance at 280 nm, you can measure the UV absorbance prior to starting an experiment.

$$Concentration\ (mg/mL)\ of\ IgG = \frac{(A280)}{L \times 1.4}$$

Where **L** is the UV cell path length (cm).

If your IgG comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **Step A6** after exchanging it for Labeling Buffer and assuming **95%** recovery of the IgG after buffer exchange. Labeling Buffer does not contain any substances that will interfere with the UV measurement at 280 nm.

$$Concentration\ (mg/mL)\ of\ Starting\ IgG = \frac{(A280)}{L \times 1.4 \times 0.95}$$

Where **L** is the UV cell path length (cm).

For antibody other than standard IgG, please substitute the IgG extinction coefficient with your antibody extinction coefficient.

3. Concentration Determination for Biotinylated Antibody (IgG)

Biotin does not contribute too much to the UV absorbance of the biotinylated antibody at 280 nm. The concentration will be determined similarly to unlabeled antibody. To determine the concentration of the biotinylated antibody, dilute your product from **Step C8** with 1x PBS buffer. Measure the UV absorbance of the biotinylated antibody at 280 nm (A280) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (\mu M)\ of\ the\ dilute\ sample = \frac{(A280) \times 1000000}{L \times 210000}$$

$$Concentration\ (mg/mL)\ of\ the\ dilute\ sample = \frac{(A280)}{L \times 1.4}$$

Where **L** is the UV cell path length (cm). If using a 1 cm UV cell, you can dilute the labeled antibody 10 times (for 10, 50, and 100 µg scale) or 20 times (for 1 mg and 3 mg scale) to obtain a good reading.

For antibody other than standard IgG, please substitute the IgG extinction coefficient with your antibody extinction coefficient.

4. MW Calculation

Calculation of the MW of the biotinylated antibody:

MW = n x 533.5 + MW (Ab)

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Where **n** is the average molar ratio of biotin per antibody. Use $n = \frac{MW(Ab)}{37500}$ if you do not have the experimental value of your conjugates.

5. Estimation of Biotinylation Level

Depending on your experimental requirement for biotinylation and the stability of your antibody, this kit allows you to optimize the biotinylation level by simply increasing or decreasing the reaction temperature. Regardless of the MW of your antibody, the biotinylation level per mg is the same for any antibody. However, the molar ratio will be proportionally different and can be experimentally determined by the biotin assay.

All kits include two pre-mixed biotin assay reagent tubes for quick estimation of the biotinylation level. You will only need one tube for the assay, with the other as back up or repeat assay. Please determine the concentration of the biotinylated antibody (**Step C9**) first.

The assay is based on the ability of biotin to displace HABA from the HABA/Avidin complex in stoichiometric proportions. This displacement is accompanied by a change in the absorbance at A500 (Extinction Coefficient 34,000 $cm^{-1}M^{-1}$ for HABA/Avidin complex in pH 7.0 buffer) (Ref.: Green, N. M. *Methods, Enzymol.,* **1970**, *Vol 18A*, 418).

6. Recommended Storage Conditions

AqT™ biotinylated antibody is very stable at ambient temperature. We have not observed additional aggregation after biotinylation of the antibody we tested. However, the long-term stabilization effect of AqT™ biotinylation on an antibody has not been studied thoroughly. We recommend using AqT™ biotinylated antibodies within a few weeks if stored at 2-8°C. For long-term storage, we recommend storing aliquots of the conjugate ≤ -20°C or lyophilizing to dryness. We checked the antibody before and after lyophilization in PBS buffer and did not see any difference in the SEC HPLC profiles.

The stability of your conjugate may be different due to your antibody and should be checked by SEC HPLC. We will update the recommendations when long-term stability studies are available.

7. Characterization of AqT™ Biotinylated Antibody by HIC HPLC and Heterogeneity

For biopolymers labeled with very hydrophobic small molecules, such as biotin, hydrophobic interaction chromatography (HIC) HPLC may be used to check the extent of the biotin labeling and the heterogeneity of the labeled molecules. Regular biotin labeled antibody is highly hydrophobic. After biotinylation with regular biotin, the peak retention time increases 1.5 minutes (0.27M more salt to elute out) (**Figure 3**). Regular biotin labeled antibody is also highly heterogeneous with an HIC peak spans more than 2 minutes. However, for AqT™ biotinylated antibody, there is only slight increase of the hydrophobicity (0.15 minutes). High level biotinylation does not add additional hydrophobicity. AqT™ biotinylated antibody is also not very heterogeneous with only slight increase of the peak width comparing to unlabeled antibody.

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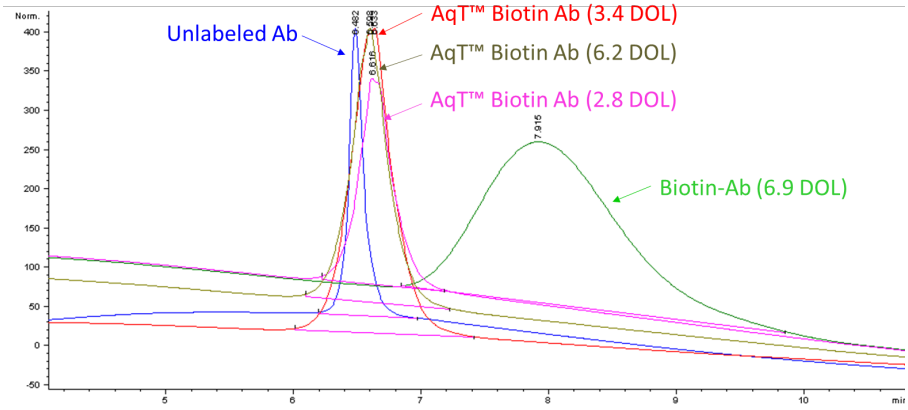
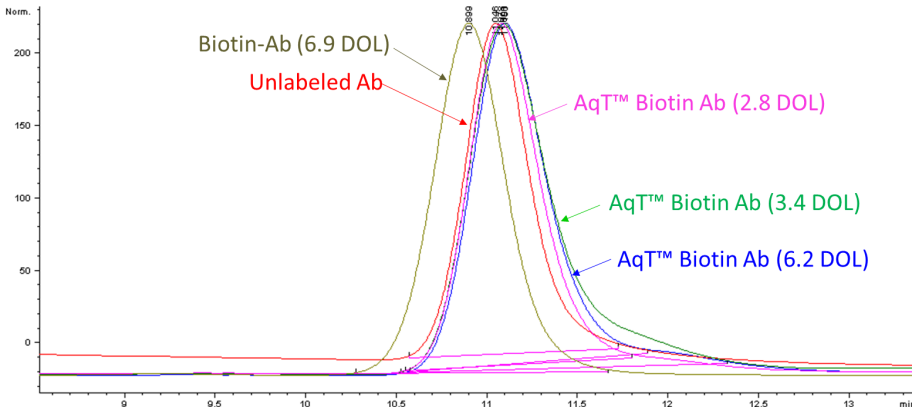
Figure 3: Overlay of HIC HPLC profiles of antibody, biotin-antibody (zero length, DOL: 6.9), and AqT™ biotinylated antibodies with various loading.



8. Characterization of AqT™ Biotinylated Antibody by SEC HPLC and Hydrodynamic Volume

Size exclusion chromatography (SEC) separates the conjugates by apparent MW or size in aqueous solution. The larger MW of the conjugate, the earlier it elutes out. Although the actual MW change is negligible after biotinylation, you will see a sizable increase in the apparent MW for biotin or dPEG biotin labeled antibody (increase in the hydrodynamic volume due to the hydrophobicity of the compound). However, with AqT™ biotinylated antibody, there is hardly any shift of the apparent MW. AqT™ biotinylated antibody has similar hydrodynamic volume as the native antibody (**Figure 4**).

Figure 4: Overlay of SEC HPLC analysis of antibody, biotin-antibody (zero length, DOL: 6.9), and AqT™ biotinylated antibodies with various loading.



SEC is also a good tool for checking the amount of aggregation of a biopolymer. By comparing the SEC profiles of unlabeled antibody and biotinylated antibody, you can estimate how much aggregation is in the biotinylated antibody. **Figure 5** shows a typical example of aggregation caused by regular biotinylation of antibody. AqT™ biotinylation in general will not add any additional aggregates.

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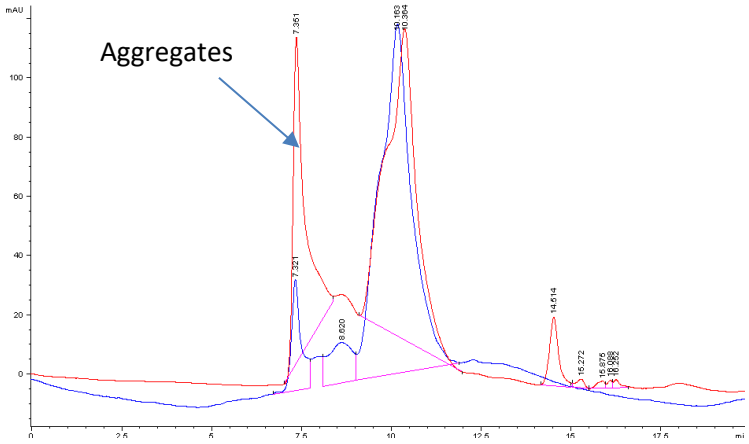
Figure 5: An example of aggregation caused by regular biotinylation (labeling and analysis done at CellMosaic).



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Appendix 1: Typical Kit Performance Data (LC analysis, CellMosaic)

Antibody information: A therapeutic antibody (human IgG1 subtype)
Lot number: S322.1.S9.030921

Summary of the results from various test reactions (note that the biotinylation level obtained for 10 µg, 50 µg and 100 µg may not as accurate as the 1 mg scale due to the limitation of the sample. The lower the reaction scale, the less reliable the data).

	Reaction Condition	Biotinylation Level	Recovery
10 µg (CM86140.1)	25°C, 2 h	4.3	43%
50 µg (CM86140.2)	25°C, 2 h	5.2	66%
100 µg (CM86140.3)	25°C, 2 h	2.8	65%
100 µg (CM86140.3)	37°C, 2 h	6.2	76%
100 µg (CM86140.3)	25°C, ON	2.9	68%
100 µg (CM86140.3)	4°C, ON	3.4	88%
1 mg (CM86140.4)	25°C, 2 h	3.4	90%

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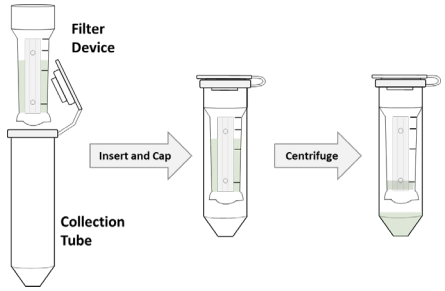
Appendix 2: Quick Reference Protocol for 10 µg Scale Antibody Labeling (CM86140.1)

A. Preparation of antibody samples for labeling

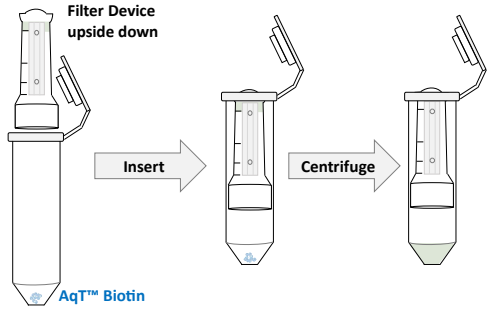
A1. Insert the Filter Device into one of the provided collection tubes. Transfer up to 500 µL of antibody samples (10 µg) to the Filter Device. If the total volume is ≤ 500 µL, add Labeling Buffer to make up the total volume to 500 µL and cap it.



A2. Place the capped Filter Device into the centrifuge rotor.
A3. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.
A4. 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.**
A5. Insert the Filter Device back into the collection tube. Add 400 µL of Labeling Buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL.
A6. If the total volume of your antibody added in **Step A1** for buffer exchange is ≤25 µL, go directly to **Step B1**. Otherwise, repeat **Step A5** one time.

B. Antibody Labeling

B1. Spin the centrifuge tubes containing activated AqT™ Biotin and remove the parafilm wrap around the cap.



B2. Place the Filter Device from **Step A6** upside down in the activated AqT™ Biotin tube. Spin at 1,000 x *g* for 2 minutes.
B3 & B4. Make sure the cap is tight. Use parafilm to wrap around the cap. Vortex the tube for 10 seconds to mix and then spin down.
B5. Mix at 37°C for 2h for high loading. Alternatively, use the table in your main protocol as a guideline to choose the temperature for optimized biotinylation.

C. Purification of Biotinylated Antibody

C1. Transfer the antibody solution from **Step B5** to the Filter Device. Wash the collection tube two times with 200 µL of PBS or your buffer and transfer the wash to the filter.
C2. Spin the Filter Device at 14,000 x *g* for 8 minutes to concentrate to < 50 µL.
C3. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C4. Add 400 µL of PBS or your buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C5. Repeat **Step C4** one time.
C6. To recover the sample, place the Filter Device upside down in a new collection tube. Spin for 2 minutes at 1,000 x *g*.
C7. If you need to perform a biotin assay, go directly to **Step C8**. Otherwise, add 22 µL of PBS or your buffer into the Filter Device, aspirate with pipette 3 times to mix, and transfer the wash to the collection tube.
C8. Determine the concentration by UV/Vis (see **Other considerations**).
C9. Store the AqT™ biotinylated antibody at 2–8 °C. For long-term, aliquot and store the conjugate ≤ -20°C or lyophilize to dryness.

D. Biotin Assay (Optional)

D1. Add 100 µL of Biotin Assay Buffer. Vortex for 10 seconds to mix and then spin it down.
D2. Transfer 90 µL of biotin assay solution to a 100 µL UV cuvette. Record the UV absorbance at 500 nm (A0) (wait 1−2 minutes to stabilize).
D3. Transfer 10 µL of biotinylated antibody samples from **Step C7** to the UV cuvette. Use a 100 µL pipette tip to pipette up and down three times to mix. Record the UV absorbance at 500 nm (As) (wait 2−4 minutes to stabilize).
D4. Calculate the biotin content:

$$\mu\text{M (Biotin)} = \frac{(0.9 \times \text{A0} - \text{As})}{34} \times 1000 \times 10 = \_\_\_\_$$

D5. Calculate the degree of labeling (DOL) based on the following formula:

$$DOL = \frac{\mu M\ (Biotin)}{\mu M\ (Antibody)} = \_\_\_\_$$

Where µM (Antibody) is the concentration of antibody before dilution in **Step C7**.

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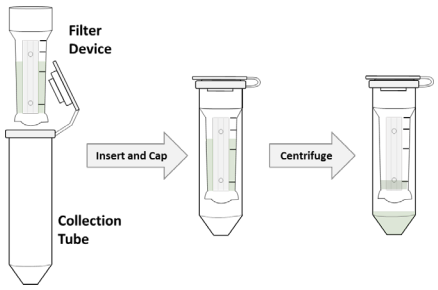
Appendix 3: Quick Reference Protocol for 50 µg Scale Antibody Labeling (CM86140.2)

A. Preparation of antibody samples for labeling

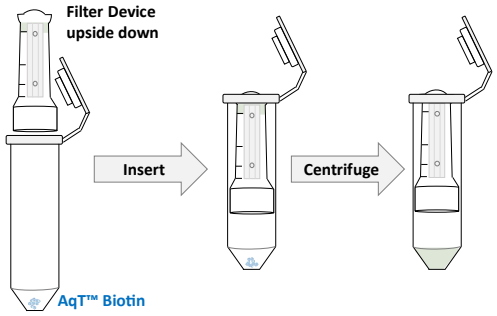
A1. Insert the Filter Device into one of the provided collection tubes. Transfer up to 500 µL of antibody samples (50 µg) to the Filter Device. If the total volume is ≤ 500 µL, add Labeling Buffer to make up the total volume to 500 µL and cap it.



A2. Place the capped Filter Device into the centrifuge rotor.
A3. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.
A4. 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.**
A5. Insert the Filter Device back into the collection tube. Add 400 µL of Labeling Buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL.
A6. If the total volume of your antibody added in **Step A1** for buffer exchange is ≤25 µL, go directly to **Step B1**. Otherwise, repeat **Step A5** one time.

B. Antibody Labeling

B1. Spin the centrifuge tubes containing activated AqT™ Biotin and remove the parafilm wrap around the cap.



B2. Place the Filter Device from **Step A6** upside down in the activated AqT™ Biotin tube. Spin for 2 minutes at 1,000 x *g*.
B3. Add 25 µL of Labeling Buffer into the Filter Device. Aspirate with pipette 3 times to mix and transfer the wash to the activated AqT™ Biotin tube.
B4. Make sure the cap is tight. Use parafilm to wrap around the cap. Vortex the tube for 10 seconds to mix and then spin down.
B5. Mix at 37°C for 2h for high loading. Alternatively, use the table in your main protocol as a guideline to choose the temperature for optimized biotinylation.

C. Purification of Biotinylated Antibody

C1. Transfer the antibody solution from **Step B5** to the Filter Device. Wash the collection tube two times with 200 µL of PBS or your buffer and transfer the wash to the filter.
C2. Spin the Filter Device at 14,000 x *g* for 8 minutes to concentrate to < 50 µL.
C3. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C4. Add 400 µL of PBS or your buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C5. Repeat **Step C4** one time.
C6. To recover the sample, place the Filter Device upside down in a new collection tube. Spin for 2 minutes at 1,000 x *g*.
C7 If you need to perform a biotin assay, go directly to **Step C8**. Otherwise, add 22 µL of PBS or your buffer into the Filter Device, aspirate with pipette 3 times to mix, and transfer the wash to the collection tube.
C8. Determine the concentration by UV/Vis (see **Other considerations**).
C9. Store the AqT™ biotinylated antibody at 2–8°C. For long-term, aliquot and store the conjugate ≤ -20°C or lyophilize to dryness.

D. Biotin Assay (Optional)

D1. Add 100 µL of Biotin Assay Buffer. Vortex for 10 seconds to mix and then spin it down.
D2. Transfer 90 µL of biotin assay solution to a 100 µL UV cuvette. Record the UV absorbance at 500 nm (A0) (wait 1–2 minutes to stabilize).
D3. Transfer 10 µL of biotinylated antibody samples from **Step C7** to the UV cuvette. Use a 100 µL pipette tip to pipette up and down three times to mix. Record the UV absorbance at 500 nm (As) (wait 2–4 minutes to stabilize).
D4. Calculate the biotin content:

$$\mu M\ (\text{Biotin}) = \frac{(0.9 \times A0 - As)}{34} \times 1000 \times 10 = \_\_\_\_$$

D5. Calculate the degree of labeling (DOL) based on the following formula:

$$DOL = \frac{\mu M\ (Biotin)}{\mu M\ (Antibody)} = \_\_\_\_\_$$

Where µM (Antibody) is the concentration of antibody before dilution in **Step C7**.

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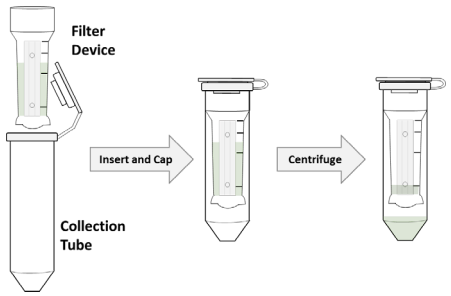
Appendix 4: Quick Reference Protocol for 100 µg Scale Antibody Labeling (CM86140.3)

A. Preparation of antibody samples for labeling

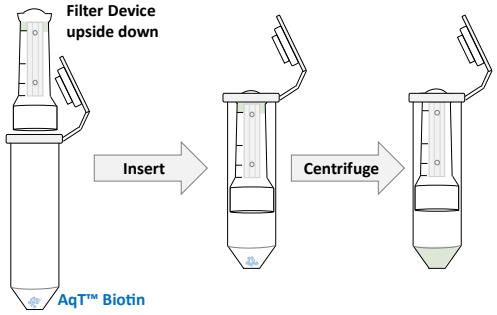
A1. Insert the Filter Device into one of the provided collection tubes. Transfer up to 500 µL of antibody samples (100 µg) to the Filter Device. If the total volume is ≤ 500 µL, add Labeling Buffer to make up the total volume to 500 µL and cap it.



A2. Place the capped Filter Device into the centrifuge rotor.
A3. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.
A4. 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.**
A5. Insert the Filter Device back into the collection tube. Add 400 µL of Labeling Buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL.
A6. If the total volume of your antibody added in **Step A1** for buffer exchange is ≤25 µL, go directly to **Step B1**. Otherwise, repeat **Step A5** one time.

B. Antibody Labeling

B1. Spin the centrifuge tubes containing activated AqT™ Biotin and remove the parafilm wrap around the cap.



B2. Place the Filter Device from **Step A6** upside down in the activated AqT™ Biotin tube. Spin for 2 minutes at 1,000 x *g*.
B3. Add 25 µL of Labeling Buffer into the Filter Device. Aspirate with pipette 3 times to mix and transfer the wash to the activated AqT™ Biotin tube.
B4. Make sure the cap is tight. Use parafilm to wrap around the cap. Vortex the tube for 10 seconds to mix and then spin down.
B5. Mix at 37°C for 2h for high loading. Alternatively, use the table in your main protocol as a guideline to choose the temperature for optimized biotinylation.

C. Purification of Biotinylated Antibody

C1. Transfer the antibody solution from **Step B5** to the Filter Device. Wash the collection tube two times with 200 µL of PBS or your buffer and transfer the wash to the filter.
C2. Spin the Filter Device at 14,000 x *g* for 8 minutes to concentrate to < 50 µL.
C3. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C4. Add 400 µL of PBS or your buffer**.** Spin the device at 14,000 x g to concentrate to < 50 µL. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C5. Repeat **Step C4** one time.
C6. To recover the sample, place the Filter Device upside down in a new collection tube. Spin for 2 minutes at 1,000 x *g*.
C7. Add 50 µL of PBS or your buffer into the Filter Device, aspirate with pipette 3 times to mix and transfer the wash to the collection tube.
C8. Determine the concentration by UV/Vis (see **Other considerations**).
C9. Store the AqT™ biotinylated antibody at 2–8°C. For long-term, aliquot and store the conjugate ≤ -20°C or lyophilize to dryness.

D. Biotin Assay (Optional)

D1. Briefly spin the tube containing Biotin Assay Reagent. Add 100 µL of Biotin Assay Buffer. Vortex for 30 seconds to mix and then spin it down.
D2. Transfer 90 µL of biotin assay solution to a 100 µL UV cuvette. Record the UV absorbance at 500 nm (A0) (wait 1−2 minutes to stabilize).
D3. Transfer 10 µL of biotinylated antibody samples from **Step C7** to the UV cuvette. Use a 100 µL pipette tip to pipette up and down three times to mix. Record the UV absorbance at 500 nm (As) (wait 2−4 minutes to stabilize).
D4. Calculate the biotin content:

$$\mu\text{M (Biotin)} = \frac{(0.9 \times \text{A0} - \text{As})}{34} \times 1000 \times 10 = \_\_\_\_$$

D5. Calculate the degree of labeling (DOL) based on the following formula:

$$DOL = \frac{\mu M\ (Biotin)}{\mu M\ (Antibody)} = \_\_\_\_$$

Where µM (Antibody) is the concentration of antibody before dilution in **Step C7**.

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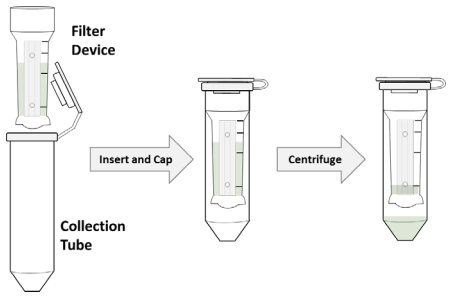
Appendix 5: Quick Reference Protocol for 1 mg Scale Antibody Labeling (CM86140.4)

A. Preparation of antibody samples for labeling

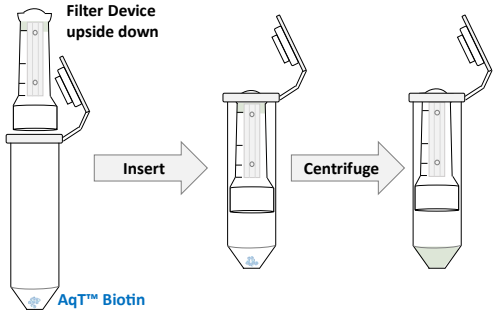
A1. Insert the Filter Device into one of the provided collection tubes. Transfer up to 500 µL of antibody samples (1 mg) to the Filter Device. If the total volume is ≤ 500 µL, add Labeling Buffer to make up the total volume to 500 µL and cap it.



A2. Place the capped Filter Device into the centrifuge rotor.
A3. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.
A4. 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.**
A5. Insert the Filter Device back into the collection tube. Add 400 µL of Labeling Buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL.
A6. Repeat **Step A5** two times.

B. Antibody Labeling

B1. Spin the centrifuge tubes containing activated AqT™ Biotin and remove the parafilm wrap around the cap.



B2. Place the Filter Device from **Step A6** upside down in the activated AqT™ Biotin tube. Spin for 2 minutes at 1,000 x *g*.
B3. Wash the Filter Device two times with 100 µL of Labeling Buffer and transfer the wash to the activated AqT™ Biotin tube.
B4. Make sure the cap is tight. Use parafilm to wrap around the cap. Vortex the tube for 10 seconds to mix and then spin down.
B5. Mix at 37°C for 2h for high loading. Alternatively, use the table in your main protocol as a guideline to choose the temperature for optimized biotinylation.

C. Purification of Biotinylated Antibody

C1. Transfer the antibody solution from **Step B5** to the Filter Device. Wash the collection tube once with 200 µL of PBS or your buffer and transfer the wash to the filter.
C2. Spin the Filter Device at 14,000 x *g* for 8 minutes to concentrate to < 50 µL.
C3. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C4. Add 400 µL of PBS or your buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C5. Repeat **Step C4** one time.
C6. To recover the sample, place the Filter Device upside down in a new collection tube. Spin for 2 minutes at 1,000 x *g*.
C7. Wash the Filter Device once with 200 µL of PBS or your buffer and transfer the wash to the collection tube.
C8. Determine the concentration by UV/Vis (see **Other considerations**).
C9. Store the AqT™ biotinylated antibody at 2–8°C. For long-term, aliquot and store the conjugate ≤ -20°C or lyophilize to dryness.

D. Biotin Assay (Optional)

D1. Add 100 µL of Biotin Assay Buffer. Vortex for 30 seconds to mix and then spin it down.
D2. Transfer 90 µL of biotin assay solution to a 100 µL UV cuvette. Record the UV absorbance at 500 nm (A0) (wait 1−2 minutes to stabilize).
D3. Transfer 10 µL of biotinylated antibody samples from **Step C7** to the UV cuvette. Use a 100 µL pipette tip to pipette up and down three times to mix. Record the UV absorbance at 500 nm (As) (wait 2−4 minutes to stabilize).
D4. Calculate the biotin content:

$$\mu M\ (\text{Biotin}) = \frac{(0.9 \times A0 - As)}{34} \times 1000 \times 10 = \_\_\_\_$$

D5. Calculate the degree of labeling (DOL) based on the following formula:

$$DOL = \frac{\mu M\ (Biotin)}{\mu M\ (Antibody)} = \_\_\_\_$$

Where µM (Antibody) is the concentration of antibody before dilution in **Step C7**.

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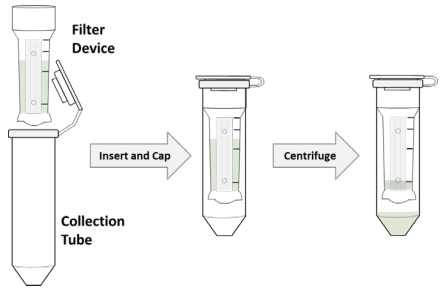
Appendix 6: Quick Reference Protocol for 3 mg Scale Antibody Labeling (CM86140.5)

A. Preparation of antibody samples for labeling

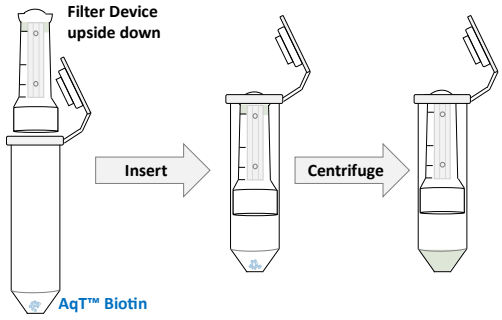
A1. Insert the Filter Device into one of the provided collection tubes. Transfer up to 500 µL of antibody samples (3 mg) to the Filter Device. If the total volume is ≤ 500 µL, add Labeling Buffer to make up the total volume to 500 µL and cap it.



A2. Place the capped Filter Device into the centrifuge rotor.
A3. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL.
A4. 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.**
A5. Insert the Filter Device back into the collection tube. Add 400 µL of Labeling Buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL.
A6. Repeat **Step A5** two times.

B. Antibody Labeling

B1. Spin the centrifuge tubes containing activated AqT™ Biotin and remove the parafilm wrap around the cap.



B2. Place the Filter Device from **Step A6** upside down in the activated AqT™ Biotin tube. Spin for 2 minutes at 1,000 x *g*.
B3. Wash the Filter Device two times with 300 µL of Labeling Buffer and transfer the wash to the activated AqT™ Biotin tube.
B4. Make sure the cap is tight. Use parafilm to wrap around the cap. Vortex the tube for 10 seconds to mix and then spin down.
B5. Mix at 37°C for 2h for high loading. Alternatively, use the table in your main protocol as a guideline to choose the temperature for optimized biotinylation.

C. Purification of Biotinylated Antibody

C1. Transfer up to 500 µL of antibody solution from **Step B5** to the Filter Device. Repeat **Steps C1–C3** until all of the antibody sample goes into the filter.
C2. Spin the Filter Device at 14,000 x g for 8 minutes to concentrate to < 50 µL.
C3. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C4. Add 400 µL of PBS or your buffer. Spin the device at 14,000 x *g* to concentrate to < 50 µL. Transfer the filtrate from the collection tube to a clean centrifuge tube. **Save the filtrate.**
C5. Repeat **Step C4** two times.
C6. To recover the sample, place the Filter Device upside down in a new collection tube. Spin for 2 minutes at 1,000 x *g*.
C7. Wash the Filter Device twice with 200 µL of PBS or your buffer and transfer the wash to the collection tube.
C8. Determine the concentration by UV/Vis (see **Other considerations**).
C9. Store the AqT™ biotinylated antibody at 2–8°C. For long-term, aliquot and store the conjugate ≤ -20°C or lyophilize to dryness.

D. Biotin Assay (Optional)

D1. Briefly spin the tube containing Biotin Assay Reagent. Add 100 µL of Biotin Assay Buffer. Vortex for 30 seconds to mix and then spin it down.
D2. Transfer 90 µL of biotin assay solution to a 100 µL UV cuvette. Record the UV absorbance at 500 nm (A0).
D3. Transfer 10 µL of biotinylated antibody sample from **Step C7** to the UV cuvette. Pipette up and down three times to mix. Then wait until the reading stabilizes to record the final UV absorbance at 500 nm (As) (5 minutes in general).
D4. Calculate the biotin content:

$$\mu\text{M (Biotin)} = \frac{(0.9 \times \text{A0} - \text{As})}{34} \times 1000 \times 10 = \_\_\_\_$$

D5. Calculate the degree of labeling (DOL) based on the following formula:

$$DOL = \frac{\mu M\ (Biotin)}{\mu M\ (Antibody)} = \_\_\_\_$$

Where µM (Antibody) is the concentration of antibody before dilution in **Step C7**.