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PerKit™ Antibody MMAE Conjugation Kit (CM11409.01x1 and CM11409.01x3) 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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Kit Components

This kit provides materials to conjugate monomethyl auristatin E (MMAE) with valine-citruline p-aminobenzylcarbamate (VC-PAB) onto one (CM11409.01x1) or three (CM11409.01x3) antibody samples (**IgG**). Scale of each reaction: 0.1 mg (protein content). The loading is optimized for monoclonal IgG1 subtype to obtain an average of 3 to 5 MMAEs per antibody. For other IgG subtypes or polyclonal antibodies, the loading may vary.

⚠ Upon receipt, please remove **Box 1** and store in a freezer at or below -20°C.
Store **Box 2** in a refrigerator at 2-8°C.

	Name	Part #	Quantity (CM11409.01x1)	Quantity (CM11409.01x3)	Storage condition
Box 1	MC-VC-PAB-MMAE (**red label**)	CM11001.01	5 µL	3 x 5 µL	-20°C
	Reagent A (**blue label**)	CM13004	1 unit	3 units	
Box 2	Solution A (**green label**)	CM01003	2 mL	6 mL	2-8°C
	Reducing Buffer (**orange label**)	CM02001	4 mL	12 mL	
	Labeling Buffer (**indigo label**)	CM02005	4 mL	12 mL	
	Storage Buffer (1 x PBS buffer) (**grey label**)	CM02013	5 mL	20 mL	
	Centrifugal Filter Devices	CM03CD050 A	3	9	
	Collection Tubes for Filter	CM03CT0	6	18	
	Desalting Spin Column	CM03SG50	2	6	
	Collection Tubes for Spin Column	CM03CT9	2	6	
	0.5 mL Eppendorf Tubes	CM03CT7	2	6	
	1.5 mL Centrifuge Tube	CM03CT2	2	6	
	Hazardous Waste Bag(s)	CM03HZ1	1	3	
User Material	IgG Antibody	N/A	NOT PROVIDED (User Supplied Material, **0.1 mg IgG** needed per reaction)		

Safety Information

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

Labeling Chemistry

The kit is designed to label any IgG antibody with monomethyl auristatin E (MMAE) using a valine-citruline p-aminobenzylcarbamate (VC-PAB) linker. The user supplies the antibody. The kit includes maleimide-activated VC-PAB-MMAE, which can be coupled directly to the antibody after reduction through alkylation in a single step (a method developed by Seattle Genetics: Sun *et al*. **2005**, *Bioconjugate Chem*. 16, 1282-1290). The product is purified to remove any unreacted drugs.

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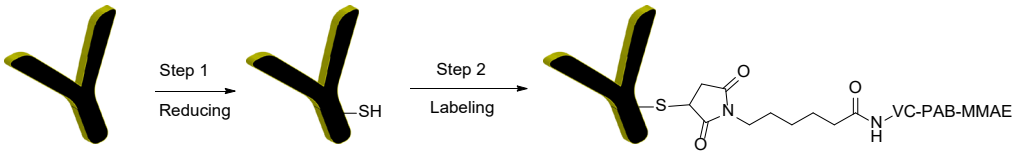
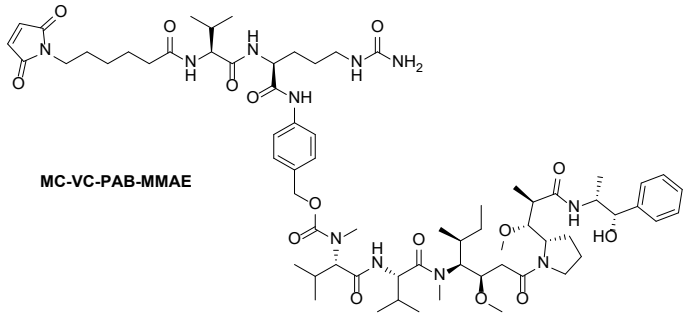
Key features of this conjugation kit:

- Offers a simple and easy way to label IgG1 with MMAE with minimum exposure to the toxin
- Cathepsin B cleavable VC-PAB (Ref. Doronina *et al*. **2008**, *Bioconjugate Chem*. 19, 1960-1963)
- Fast and easy preparation: 6 h preparation and <2 h hands-on time
- All reagents and supplies included for preparation and purification
- Over 95% conjugated products (free of unreacted drug and less than 5% of unreacted antibody)



Drug Information:



- **Name**: Monomethyl auristatin E (MMAE) with Mal-VC-PAB linkage
- **CAS number**: 646502-53-6
- **Chemical Formula**: $C_{68}H_{105}N_{11}O_{15}$
- **MW**: 1316.65
- **Mechanism of action**: Inhibits cell division by blocking the polymerization of tubulin, VC-PAB linker is stable in extracellular fluid but cleaved by cathepsin B once inside the tumor cell, activating the antimitotic mechanism
- **Activities**: Antioxidant, anti-inflammatory, anticancer, and insecticidal activities

Requirement for antibody (IgG1 subtype):
1. Preferably > 90% pure by gel electrophoresis.
2. Total amount: 0.1 mg protein content as measured by UV. Note: the accuracy of your protein amount is the single most important factor to obtaining an optimized DAR. Please refer to the section Other Considerations in this manual to measure the protein amount.

Support

Customer can request a recommendation for the conjugation if the molecule has a special feature or a low amount of antibody. CellMosaic also provides additional support services to customers who need help analyzing the final conjugates by HPLC and determination of the DOL.

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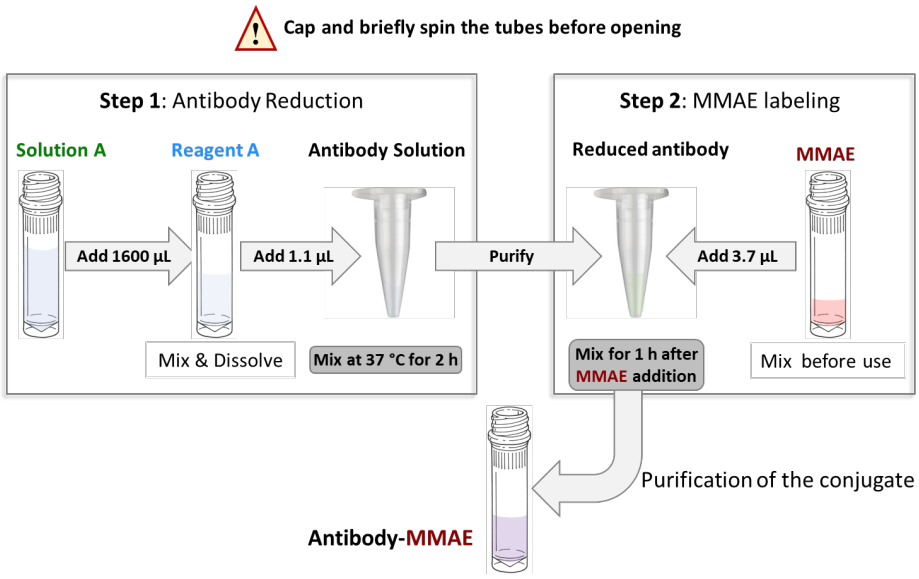
Protocol



Scheme 1. Schematic diagram of the workflow for preparing antibody-MMAE 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 or RT
- Chemical hood
- Support stand, lab frame, or any support rod for desalting column
- Flask
- Personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves)

2. Prepare Site and MMAE for Labeling Experiment

MMAE with VC-PAB is very hydrophobic. Antibody-drug conjugates with VC-PAB-MMAE tend to aggregate and precipitate out from the solution. It is recommended that the labeling experiment be planned only a few days before your other experiments.

Ensure you use personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves) while handling MMAE. Locate a clean space inside a chemical hood.

A1. Remove **Box 1** containing **MMAE** (red label) and **Reagent A** (blue label) from the -20°C freezer and warm to RT before opening the bag.

A2. Remove **Box 2** from the refrigerator. Take the hazardous waste bag and place it inside the chemical hood for solid waste disposal. Bring the rest of the items to a lab bench.

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A3. Check if the frozen liquid thawed inside the **MMAE** tube. Briefly spin the centrifuge tube containing **MMAE**. Make sure no liquid is in the cap. Place the **MMAE** tube in a tube holder inside a chemical hood and wait until the antibody is ready for conjugation.

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

A4. Set the temperature of the incubator or shake to 37 °C.

3. Preparation of Antibody Samples for Conjugation

Items needed: Filter Devices (CM03CD050A), Collection Tube (CM03CT0), Reducing Buffer (CM02001, Orange label), 0.5 mL Eppendorf Tube (CM03CT7), Clean Centrifuge Tubes (not provided in the kit).

Total amount of antibody used for the conjugation is 0.1 mg (protein content measured by UV) per reaction.

B1. Insert the **Filter Device** into one of the provided collection tube (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**.
- ✓ If your antibody is supplied in < 500 µL buffer, transfer your antibody sample to the **Filter Device** directly. Add **Reducing Buffer** to make up the total volume to 500 µL and cap it.
- ✓ If the volume of your antibody sample is between 500 and 1000 µL, divide the volume into two **Centrifugal Filter Devices** and add the antibody sample to the filter device. Add **Reducing Buffer** to make up the total volume to 500 µL in each device and cap them.
- ✓ If the volume of your antibody sample is >1000 µL, add up to 500 µL of sample to the two **Filter Devices** and cap them. Repeat Step **B1-B4** until all the antibody sample goes into the **Filter Device**. Move on to Step **B5**. Add **Reducing Buffer** to make up the total volume to 500 µL in each device for the last refill.

B2. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

B3. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably cooled to 4°C) to concentrate to **< 100 µL** (Spin time depends on many factors. The typical spin time for a 500 µL sample is approximately 8 to 20 minutes. The typical volume is ~40 µL after spinning for 8 minutes on an Eppendorf 5417R at 4°C.)

B4. 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.**

B5. Insert the **Filter Device** back to the collection tube. Add 400-450 µL of **Reducing Buffer** to make up the total volume to 500 µL. Then place the capped **Filter Device** into the centrifuge

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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 **< 100 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.**

B6. Repeat **Step B5** two more times. For the last repeat, spin the **Filter Device** at 14,000 x g to concentrate to **< 20 µL**.

> **Note**: If you divide your samples into two **Centrifugal Filter Devices,** you can combine the samples into one **Centrifugal Filter Device** during the last repeat of **Step B6**.

B7. Transfer the concentrated sample from the **Filter Device** to a 0.5 mL Eppendorf tube (Use the pipetman to estimate the approximate volume of the concentrated sample. Calculate the volume of **Reducing Buffer** needed for rinsing the **Filter Device** in **Step B8**. The total volume of the sample should be **~30 µL** after combining the concentrated sample from **Step B7** and the rinsing solution from **Step B8.**)

B8. Add 10-20 µL of **Reducing Buffer** to the **Filter Device** to rinse. Stir it gently with a pipet tip, then transfer the entire contents to the 0.5 mL Eppendorf tube from **Step B7.**

B9. Vortex the combined antibody sample for 30 seconds and then spin down the liquid.

4. Antibody Reduction (Step 1 in Scheme 1)

Items needed: Reagent A (CM13004, blue label), Solution A (CM01003, green label), Antibody Solution from **Step B9**, Ice Bath.

C1. Spin the centrifuge tubes containing **Reagent A** (blue label).

C2. Spin **Solution A** (green label) before opening it. Add 1.6 mL of **Solution A** to the tube with **Reagent A** from **Step C1**. Vortex for 30 seconds to 1 minute to dissolve the reagent and then spin down the liquid.

C3. Add **1.1 µL** of **Reagent A solution** from **Step C2** to the centrifuge tube containing antibody from **Step B9.** (Discard any unused **Reagent A** as hazardous chemical waste **until the experiments are done**)

C4. Vortex the solution for 30 seconds, and then spin down the liquid. Mix at 37°C for 2 h.

> **Tip for mixing**: You can use a nutator, a shaker, a vortex, or an incubator shaker for mixing. If you are using end to end nutating, make sure your centrifuge is capped properly. If you don't have any of this equipment, you can let the centrifuge tube sit at the bench with manual mixing by pipetting every 20 minutes.

C5. Cool the antibody reducing solution to 4°C either on ice or place it inside a refrigerator at 2-8°C for 5 minutes.

5. Purification to Remove Excess Reagent A

> ⚠ The following steps are to be performed without any break. Reduced thiols tend to oxidize quickly. Make sure **step A3** is completed prior to the following steps. Work quickly through **steps D6-D8**.

Items needed: Filter Device (CM03CD050A), Collection Tube (CM03CT0), Labeling Buffer (CM02005, indigo label), 0.5 mL Eppendorf Tube (CM03CT7), Clean Centrifuge Tubes (not provided in the kit), Antibody Solution from **Step C5**.

D1. Insert the **Filter Device** into one of the provided collection tubes (microcentrifuge tube with the cap attached). Transfer the reduced antibody solution from **Step C5** into the **Filter Device** directly. Wash the centrifuge tube once with 400 µL **Labeling Buffer**, transfer the solution to the **Filter Device** (total volume 500 µL), and cap it. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

D2. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably cooled to 4°C) to concentrate to < 100 µL

D3. 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.**

D4. Add 400-450 µL of **Labeling Buffer** to make up the total volume to 500 µL. 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 **< 100 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.**

D5. Repeat **Step D4** once. Spin the **Filter Device** at 14,000 x g to concentrate to **< 20 µL**.

Work quickly

D6. Transfer the concentrated sample from the **Filter Device** to a 0.5 mL Eppendorf tube (Use the pipetman to estimate the approximate volume of the concentrated sample. Calculate the volume of **Labeling Buffer** needed for rinsing the **Filter Device** in **Step D7**. The total volume of the sample should be **~30 µL** after combining the concentrated sample from **Step D6** and the rinsing solution from **Step D7.**)

D7. Add 10-20 µL of **Labeling Buffer** to the **Filter Device** to rinse. Stir it gently with a pipet tip, then transfer the entire contents to the 0.5 mL Eppendorf tube from **Step D6.**

D8. Vortex the combined antibody sample for 30 seconds and then centrifuge to ensure no liquid is in the cap.

6. MMAE Labeling (Step 2 in Scheme 1)

Items needed: MMAE solution from **step A3**, Hazardous Waste Bag (CM03HZ1), Antibody Solution from **step D8**.

E1. With personal protection equipment on, carefully open the centrifuge tube of MMAE from **Step A3**. Make sure all the liquid is thawed.

E2. Transfer **3.7 µL** of MMAE solution from **Step E1** to the centrifuge tube containing antibody from **Step D8**. When you add the MMAE solution, place the pipette tip inside the antibody solution and then dispense the MMAE slowly with stirring using the pipette tip. **Dispose of the pipette tip and MMAE tube in the solid waste bag**.

E3. Cap the centrifuge tube. Mix at 25 °C or RT for 1 h.

Tip for time saving: While waiting for the reaction to complete, you can move on to **Step F1** and equilibrium the column for purification.

7. Purification of Conjugate

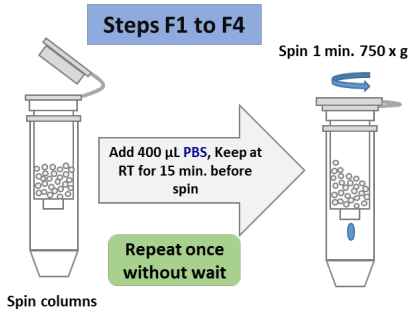
Items needed: Desalting Spin Column (CM03SG50), Storage Buffer (1x PBS) (CM02013, grey label), Collection Tubes for Spin Column (CM03CT9), 1.5 mL Centrifuge Tube (CM03CT2), Hazardous Waste Bag (CM03HZ1), MMAE−Antibody Solution from **Step E3**.

F1. Take out 2 desalting spin columns, remove the bottom red cap. Spin for 1 min at 750 x *g* before opening the top cap.

F2. Apply 400 µL of PBS buffer (grey label) to the top-center of the resin of each column. Let it remain at RT for 15 min to swell the resin.

F3. Spin for 1 min at 750 x *g* and discard the flow through.

F4. Repeat **Steps F2–F3** once. Spin immediately after applying PBS without wait and discard the flow through.

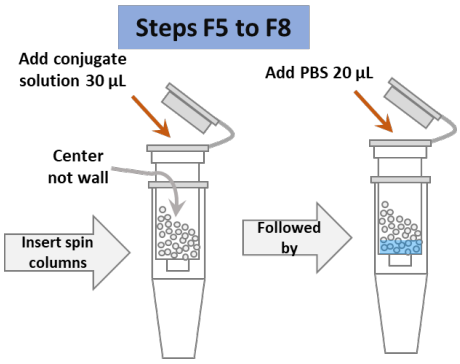


F5. Insert the spin columns into clean 1.5 mL collection tubes.

F6. Spin the MMAE−antibody solution from **Step E3** to ensure there is no liquid in the cap before opening it. Add 1xPBS buffer to make up the total volume of the MMAE solution to 60 µL.

F7. Slowly apply up to 30 µL of each conjugate solution from **Step E3** to the top-center of the resin of each spin column without disturbing the resin bed (2 x 30 µL).

F8. Washing the tube with 40 µL of PBS buffer, then apply 20 µL of PBS buffer to the top-center of the resin of each spin column to make up the volume to 50 µL in each spin column. **Dispose of the centrifuge tube in the solid waste bag.**



⚠ The resin may detach a little from the column to form a pillar with an unbalanced resin bed due to the centrifuge force. Make sure the sample and subsequent PBS buffer is applied slowly in the resin and not run down the sides of the resin bed. Wait for the

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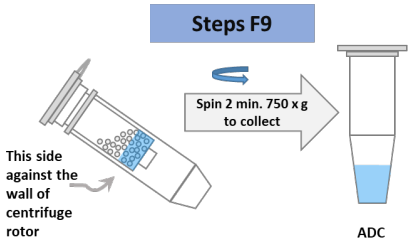
conjugate solution to enter the resin before applying PBS buffer. Avoid touching the resin bed with the pipette tip.

F9. Rotate/align the spin column so that the higher resin bed side is against the wall of the centrifuge rotor and the lower resin bed faces the center of the centrifuge (same centrifuge force). Spin for 2 min at 750 x *g* to collect the fractions.



F10. Transfer and combine the fractions from the two collection tubes into the provided 1.5 mL centrifuge tube and cap it. **Dispose of the Desalting Spin Columns, Collection Tubes in the solid waste bag and seal the bag. Dispose of the waste following regulations appropriate for your area.**

Conjugate is Ready for Your Experiment

- **Specification for your product:** MMAE-labeled antibodies with an average drug-to-antibody ratio (DAR) of 4. A typical batch contains over 95% conjugated products by SEC (size exclusion chromatography) with less than 5% of unreacted antibody and is free of any unreacted drug. The approximate concentration of the ADC is 0.5 mg/mL in PBS buffer assuming 50% recovery. You can determine the concentration and the estimated DAR of the ADC by UV/vis spectrophotometry (see other considerations).

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Other Considerations

1. Concentration Determination for IgG Antibody (Unlabeled)

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

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

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

If your antibody comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **step B9** after exchanging it with Reducing Buffer and assuming **95%** recovery of the IgG after buffer exchange.

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

2. Concentration Determination for ADC

To determine the concentration, dilute your conjugate from **Step F10** with 1x PBS buffer. Measure the UV absorbance of the conjugate at 280 nm (A280) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) \times 4.7619}{L}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 0.7143}{L}$$

Where **L** is the UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute the conjugate 4 times to obtain a good reading.

For a typical IgG with MW of 150,000, the molar extinction coefficient is 210,000 $M^{-1}cm^{-1}$.

3. MW Calculation

Calculation of the MW of the conjugate:

MW(ADC) = n x 1317+ 150000

Where n is the average molar ratio of MMAE per antibody. Use 4.0 if you do not have the hydrophobic interaction chromatography (HIC) profile of your conjugates.

4. Drug-to-Antibody Ratio (DAR) and Characterization by UV and HPLC

In this kit, the target DAR is 4. Depending on your antibody, you may achieve a lower DAR.

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To estimate the DAR, you can obtain the UV absorbance ratio (R) of your conjugate at 248 nm and 280 nm.

$$R = {(A248)}/{(A280)}$$

The unlabeled antibody will have an R value of 0.4 – 0.5. An MMAE-ADC with DOR of 3 – 5 will have an R value of 0.65 – 0.80.

You can also use the following formula to calculate the estimated DAR (only for reference):

$$DAR = \frac{(21 \times R - 9)}{(1.615 - 0.1425 \times R)}$$

Note: The UV absorbance of the MMAE in an ADC can vary greatly depending on many factors, such as aggregation and stacking. Therefore, the **R** value for an ADC can differ greatly for different antibodies and should be determined experimentally. The calculation for the DAR using this formula is only for reference only.

For more accurate DAR calculation and to check the homogeneity of the ADC, you can analyze it by hydrophobic interaction chromatography (HIC). If you do not have access to such a facility setup, you can send your sample to CellMosaic for analysis.

5. Characterization of ADC by HIC HPLC

For ADCs prepared via a reduced thiol of the antibody, hydrophobic interaction chromatography (HIC) HPLC is used to calculate the DAR and the heterogeneity of the ADCs. The conjugates are separated based on hydrophobicity. Antibodies loaded with the same number of drugs (same DAR) will have similar hydrophobicity and be eluted as a single peak. For a typical MMAE ADC, multiple peaks represent various amounts of drug-loaded ADCs. You will find examples of HIC HPLC profiles of MMAE ADCs of various antibodies in the Appendix.

CellMosaic offers an HIC buffer set (Product #: CM02025) for our customers to use with any HIC column. The CM02025 product sheet contains all of the information and methodology needed to run an HIC HPLC analysis.

If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

6. Characterization of ADC by SEC HPLC

VC-PAB-MMAE is very hydrophobic. This kit is designed to minimize the aggregation and precipitation issues generally seen with MMAE labeling. However, you may still notice some solid precipitate out or ADC aggregation during the reaction. The precipitate will be removed during purification. Depending on the properties of your antibody, recovery will be 40-80%. If you are concerned with the aggregation, you can use size exclusion chromatography (SEC) to check the extent of aggregation. SEC separates the conjugates by apparent molecular weight

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(MW) or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes. By comparing the SEC profile of unlabeled IgG and the ADC, you can estimate how much aggregation is in the ADC.

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 you need 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.

7. ADC Stabilizing Buffer

CellMosaic's proprietary ADC stabilizing PBS buffer (5x) (Product #: CM02022) contains 5x PBS buffer and other stabilizers to prevent the hydrophobic drugs from interacting with each other during storage, which would cause the ADCs to precipitate out. Stabilization buffer also helps preserve the structure of the ADCs during lyophilization. The buffer is biocompatible and can be used directly for any *in vitro* and *in vivo* studies. For more information on the stabilization buffers, please check our website.

8. Recommended Storage Conditions

Unlike other ADCs labeled with hydrophobic drug, ADC with MMAE is relatively stable. Based on our preliminary data, the conjugate made with this kit can remain stable in PBS buffer for several weeks at 2-8°C. Do not freeze MMAE ADC.

The stability of your conjugate may be different due to your antibody and should be checked by either HPLC or UV. If you need to store the ADCs for a longer period of time, you can purchase the ADC stabilization PBS buffer separately. Dilute your ADC in Stabilization PBS buffer (5x). Aliquot and store the conjugate in a < -20°C freezer or lyophilize to dryness. Avoid repeated freeze and thaw cycles.

9. Sample Submission for HPLC Analysis

If you are submitting samples to CellMosaic for SEC and HIC analysis, please follow these instructions:

1) Go online: https://www.cellmosaic.com/hplc-analysis/, select SEC HPLC Analysis (Product# AS0023) and HIC HPLC Analysis (Product#: AS0025), choose the quantity (number of samples. Bulk discounts 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 antibody in PBS buffer to 1 mg/mL, and then transfer 50 µL of the diluted solution to a 500 µL micro-centrifuge tube. Label the vial properly.
3) Transfer 50 µL of ADC (non-diluted solution) to a 500 µL micro-centrifuge tube and label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.

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

Antibody information: A therapeutic antibody (Human IgG1 subtype)

Kit Lot number: 5508.S9.020918

Figure 1: HIC HPLC analysis of antibody, Mal-VC-PAB-MMAE, and purified conjugates

Scale of the reaction: 3 mg (CM11409)

Specification of the final conjugates:

Calculated average DAR: 4.86 Percentage of unreacted antibodies: 2.6%;

Percentage of unreacted MMAE: 0% ADC recovery: 81%