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PerKit™ Antibody MMAE Conjugation Kit (CM11409.01 and CM11409.01x3)) User Reference Guide

Contents

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Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

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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 (CM11409.01) or three (CM11409.01x3) antibody samples (**IgG1 subtype only**). Scale of each reaction: 0.1 mg (protein content). Upon receipt, please remove the plastic bag containing MC-VC-PAB-MMAE and Reagent A and store in a freezer below -20°C. Store the rest of the items and box in a refrigerator at 2-8°C.

This kit is optimized for IgG1 subtype. If you have other IgG subtypes, please contact CellMosaic for any suggestion.

Name	Part #	Quantity (CM11409.01)	Quantity (CM11409.01x3)	Storage condition
MC-VC-PAB-MMAE (red label)	CM11001	5 µL	3 x 5 µL	-20°C, dry
Reagent A (blue label)	CM13004	1 unit	3 units	-20°C
Solution A (green label)	CM01003	2 mL	6 mL	2-8°C
Reducing Buffer (orange label)	CM02001	4 mL	12 mL	2-8°C
Labeling Buffer (indigo label)	CM02005	4 mL	12 mL	2-8°C
Storage Buffer (1 x PBS buffer) (grey label)	CM02013	5 mL	20 mL	2-8°C
Centrifugal Filter Device	CM03CD050A	3	9	2-8°C
Desalting Column	CM03SG02	1	3	2-8°C
Collection Tubes	N/A	6	18	2-8°C
1.5 mL Centrifuge Tube	N/A	1	9	2-8°C
0.5 mL Eppendorf Tube	N/A	2	6	2-8°C
Hazardous Waste Bag	N/A	1	3	2-8°C
Antibody (IgG1 subtype)	N/A	NOT PROVIDED (User Supplied Material, 0.1 mg antibody 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 IgG1 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.

Key features of this conjugation kit:

- Offers a simple and easy way to label IgG1 with MMAE with minimum exposure to the toxin

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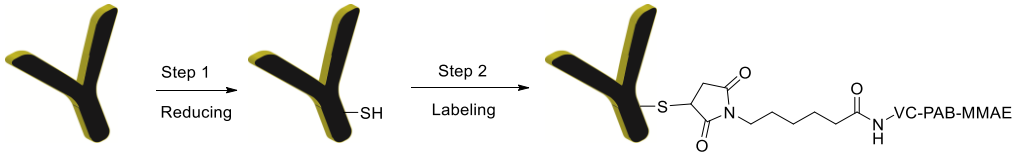
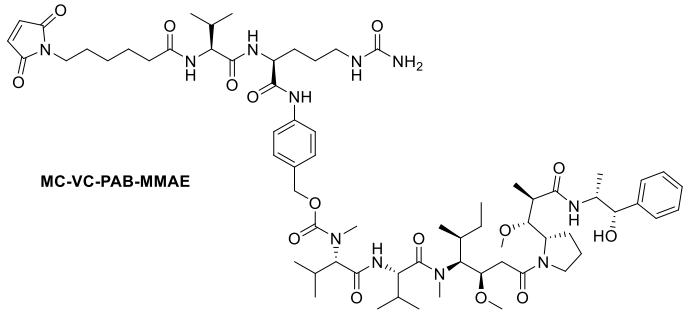
- Cathepsin B cleavable VC-PAB (Ref. Doronina *et al*. **2008**, *Bioconjugate Chem*. 19, 1960-1963)
- Fast and easy preparation: 6 h preparation and <2 h hands-on time
- All reagents and supplies included for preparation and purification
- Over 95% conjugated products (free of unreacted drug and less 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)

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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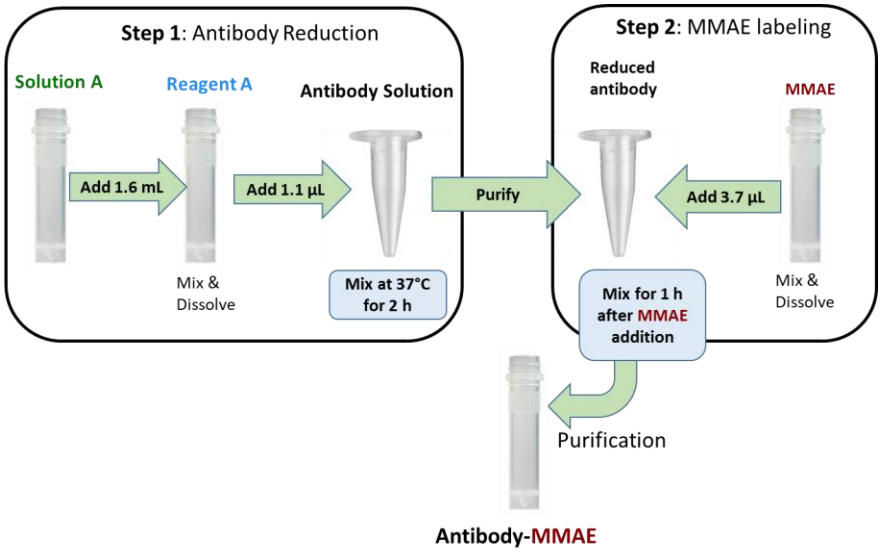
Protocol



Scheme 1. Schematic diagram of the work flow for preparing antibody-MMAE conjugates

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated)
- 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 have 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 the plastic bag containing **MMAE** (red label) and **Reagent A** (blue label) from the -20°C freezer and warm to RT before opening the bag.

A2. Remove the box 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 centrifuge the tube to make sure 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

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 of 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 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

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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 centrifuge to ensure no liquid is in the cap.

4. Antibody Reduction (Step 1 in Scheme 1)

C1. Spin the centrifuge tubes containing **Reagent A** (blue label) to ensure all the solid is at the bottom of the tube before opening it.

C2. Spin **Solution A** (green label) to ensure there is no liquid in the cap 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 centrifuge to ensure no liquid is in the cap.

C3. Add **1.1 µL** of **Reagent A solution** from **Step C2** to the centrifuge tube containing antibody from **Step B10.** (Discard any unused **Reagent A** as hazardous chemical waste **until the experiments are done**)

C4. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. 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.

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5. Purification to Remove Excess Reagent A

Note: The following steps need to be done without any break. Reduced thiols tend to oxidize quickly. Make sure **step A3** is completed prior to the following steps. Move quickly in **steps D6-D8**.

D1. Insert the **Filter Device** into one of the provided collection tube (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**.

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 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)

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

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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

F1. In a chemical hood, securely attach the **Desalting Column** to a support stand, a lab frame, or any support rod. Remove the top and bottom caps from the column and allow the excess liquid to flow through by gravity. Collect the liquid in a flask.

F2. Add 1 mL of **PBS buffer** and allow the buffer to completely enter the gel bed by gravity flow.

F3. Repeat **Step F2** three times.

F4. Spin the MMAE labeled antibody solution from **Step E3** to ensure there is no liquid in the cap before opening it. Add the entire antibody solution to the column. Allow the sample to enter the gel bed completely.

F5. Add 40 µL of PBS into the DM1 labeled antibody tube once to rinse and transfer the entire solution to the column. Allow the sample to enter the gel bed completely. **Dispose of the centrifuge tube in the solid waste bag.**

F6. Add 120 µL of **PBS buffer** and allow the liquid to enter the gel bed completely.

F7. Place a 1.5 mL centrifuge tube under the column. Add 0.25 mL of PBS buffer to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.

F8. Label the tube as your product. Store your conjugate at 4°C. **Dispose of the Desalting Column 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 0.2 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

To determine the concentration, dilute your conjugate from **Step F7** 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 $M^{-1}cm^{-1}$.

2. 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.

3. 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.

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.

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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.

4. Aggregation and Precipitation Issue for MMAE Labeling

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

5. Recommended Storage Conditions

Recommend storage at 2-8°C. Do not freeze.

Based on our preliminary data, the conjugate made with this kit can remain stable in PBS buffer for one month at 2-8°C. The stability of your conjugate may be different due to your antibody and should be checked either by HPLC or UV.

6. Submit Samples for HPLC Analysis

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

1) Dilute your un-conjugated antibody to 1 mg/mL in PBS buffer, then transfer 50 µL of the diluted solution to a 500 µL microcentrifuge tube. Label the vial properly.
2) Transfer 50 µL of ADC (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)

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%