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PerKit™ F(ab')2 MMAE Conjugation Kit (CM11416x1 and CM11416x3) 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 0.73 to 2.2 mg of one (CM11416x1) or three (CM11416x3) F(ab')2 samples (from **IgG**) with monomethyl auristatin E (MMAE) using valine-citruline p-aminobenzylcarbamate (VC-PAB) linker.

⚠ 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 (CM11416x1)	Quantity (CM11416x3)	Storage condition
Box 1	MC-VC-PAB-MMAE (**red label**)	CM11001	0.11 mL	3 x 0.11 mL	-20°C
	Reagent A (**blue label**)	CM13004	1 unit	3 units	
Box 2	Solution A (**green label**)	CM01003	1.5 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	20 mL	60 mL	
	Centrifugal Filter Devices	CM03CD050 A	3	9	
	Desalting Column	CM03SG10	1	3	
	Collection Tubes	CM03CT0	6	18	
	1.5 mL Centrifuge Tubes	CM03CT2	2	6	
	2.0 mL Centrifuge Tube(s)	CM03CT3	1	3	
	Hazardous Waste Bag(s)	CM03HZ1	1	3	
User Material	IgG Antibody	N/A	NOT PROVIDED (User Supplied Material, **0.73-2.2 mg F(ab')2** needed per reaction)		

Reaction Scale: The protocol is optimized for conjugating 2.2 mg of IgG F(ab')2. If you have less than 2.2 mg of F(ab')2, use the calculations in **Steps B10**, **C3**, **D9**, **E2**, **F5,** and **F6** to obtain the correct volumes to be added in each step.

Drug-to-Antibody Ratio (DAR) Optimization: The reducing protocol is optimized for F(ab')2 fragmented from monoclonal IgG1 subtype to obtain an average 2-4 thiols per antibody. For F(ab')2 fragmented from other IgG subtypes or polyclonal antibodies, the DAR may vary. For the best performance of the ADC and to obtain the desired DAR, you can purchase the Thiol Assay Kit with Purification (Product #: CM90005) separately and use it to perform an in-process thiol assay after the antibody reduction (**Step C5 Note Section**). The amount of reducing reagent can be adjusted based on the data to obtain your desired DAR.

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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.

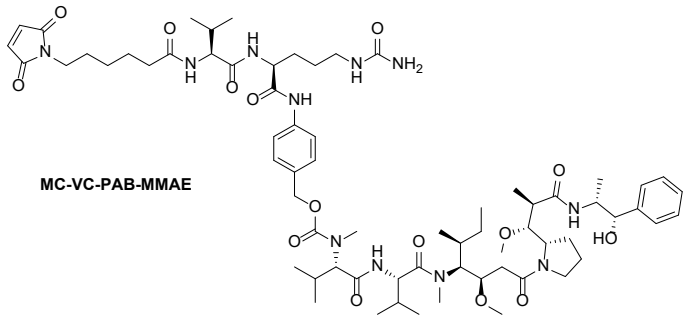
Labeling Chemistry

The kit is designed to label any F(ab')2 with monomethyl auristatin E (MMAE) using a valine-citruline p-aminobenzylcarbamate (VC-PAB) linker. The user supplies the F(ab')2. The kit includes maleimide-activated VC-PAB-MMAE, which can be coupled directly to the F(ab')2 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 F(ab')2 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
- Easy to control the DAR if used together with the Thiol Assay Kit with Purification (Product #: CM90005)
- Over 95% conjugated products (free of unreacted drug and less than 5% of unreacted F(ab')2)

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

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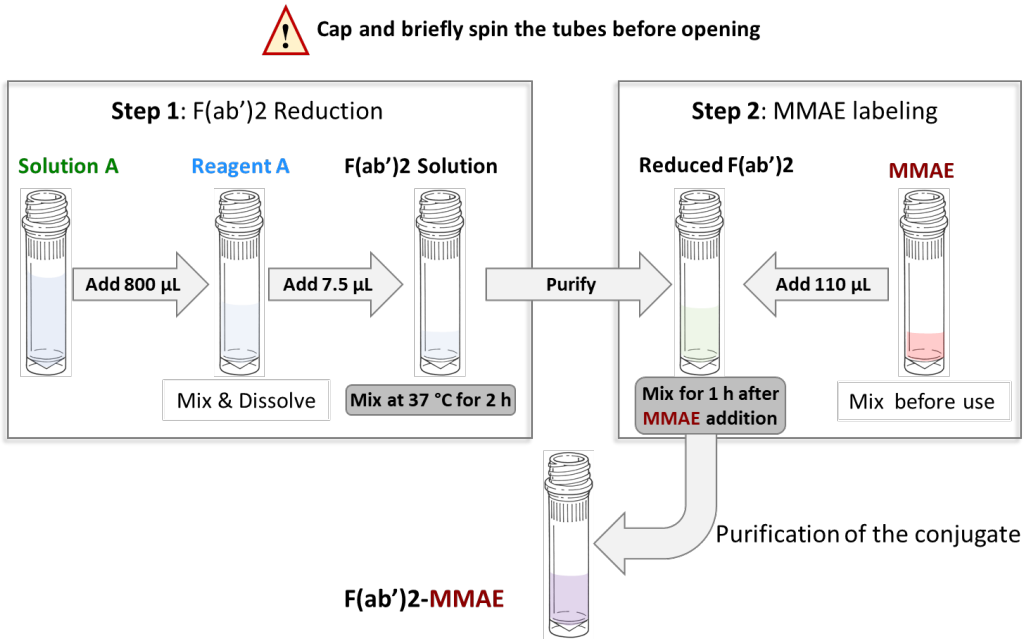
- **Mechanism of action**: Inhibits cell division by blocking the polymerization of tubulin. The VC-PAB linker is stable in extracellular fluid but cleaved by cathepsin B once inside the tumor cell, activating the antimitotic mechanism.
- **Activities**: Antioxidant, anti-inflammatory, anticancer, and insecticidal activities.

Requirement for F(ab')2:
1. Preferably > 90% pure by gel electrophoresis
2. Total amount: 0.73-2.2 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

A customer can request a recommendation for the conjugation if the F(ab')2 has a special feature or less than 0.73 mg of F(ab')2 to be labeled. CellMosaic provides other accessory tools, such as buffers, standards, and reagents for ADC research. CellMosaic also provides fee-based support services to customers who need help analyzing the final conjugates by HPLC and determining the DAR.

Protocol



Scheme 1. Schematic diagram of the workflow for preparing F(ab')2-MMAE conjugates starting with 2.2 mg of F(ab')2 (volume of reagents varies if the amount of F(ab')2 is < 2.2 mg).

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, 14,000 g capable), mini-centrifuge
- Pipettes and tips

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- 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. F(ab')2-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.

A3. Check if the frozen liquid is thawed inside the **MMAE** tube. Briefly mix & spin the centrifuge tube containing **MMAE**. 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 tubes after mixing: Always spin the tubes to ensure no liquid is in the cap.

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

3. Preparation of F(ab')2 Samples for Conjugation

Items needed: Filter Devices (CM03CD050A), Collection Tube, Reducing Buffer (CM02001, Orange label), 1.5 mL Centrifuge Tube (CM03CT2), Clean Centrifuge Tubes (not provided in the kit).

Total amount of F(ab')2 used for the conjugation is 2.2 mg per reaction (protein content measured by UV). The protocol is optimized for F(ab')2 fragmented from the monoclonal IgG1 subtype antibody to obtain 4 drugs per antibody.

Reaction Scale: If you have less than 2.2 mg of antibody, use the calculations in **Steps B10**, **C3**, **D9**, **E2**, **F5,** and **F6** to obtain the correct volumes to be added in each step.

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DAR Optimization: If you have F(ab')2 fragmented from non-IgG1 subtype or polyclonal antibody and would like to adjust the loading, follow **Step C5 Note Section** for optimization.

B1. 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 F(ab')2 is supplied as a lyophilized solid, dissolve the F(ab')2 in 500 µL of **deionized water** and then transfer the entire contents to the **Filter Device**.
- ✓ If your F(ab')2 is supplied in < 500 µL buffer, transfer your F(ab')2 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 F(ab')2 sample is between 500 and 1000 µL, divide the volume into two **Centrifugal Filter Devices** and add the F(ab')2 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 F(ab')2 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 F(ab')2 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 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.

B7. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube (use the pipetman to measure the approximate volume of the concentrated sample).

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B8. Add 20-100 µL of **Reducing Buffer** to the **Filter Device** to rinse (actual volume of **Reducing Buffer** added will depend upon the calculated total volume in **Step B10**). Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B7.**

B9. Repeat **Step B8** once.

B10. Add **Reducing Buffer** to the 1. 5 mL micro-centrifuge tube from **Step B9** to make up the total volume of the sample to **300 ± 5 µL** and cap it.

Calculation 1 for Less F(ab')2:

$$Total\ volume\ of\ the\ F(ab')2\ in\ Step\ \boldsymbol{B10}\ (\mu L) = F(ab')2\ in\ mg\ \times 137$$

B11. Vortex the combined F(ab')2 sample for 30 seconds and then spin down.

4. F(ab')2 Reduction (Step 1 in Scheme 1)

Items needed: Reagent A (CM13004, blue label), Solution A (CM01003, green label), Antibody Solution from **Step B11**, Ice Bath.

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

C2. Spin **Solution A** (green label) before opening it. Add 800 µL 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.

C3. Add **7.5 µL** of **Reagent A solution** from **Step C2** to the centrifuge tube containing antibody from **Step B11** (Discard any unused **Reagent A** as hazardous chemical waste **after completion of all experiments**).

Calculation 2 for Less F(ab')2:

$$Volume\ of\ Reagent\ A\ solution\ to\ be\ transferred\ in\ Step\ \boldsymbol{C3}\ (\mu L) = F(ab')2\ in\ mg\ \times 3.42$$

C4. Vortex the solution for 30 seconds, and then spin 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 the tube from **step C4** is securely capped. If you don't have any of this equipment, you can let the tube sit on the bench with manual mixing by pipetting every 20 minutes.

C5. Cool the reduced antibody solution to approximately 4°C by placing the tube on ice or keeping it inside a refrigerator at 2-8°C for 5 minutes.

Note: Optimization of Thiol Content for F(ab')2 fragmented from non-IgG1 Subtype Antibody or Polyclonal Antibody

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For F(ab')2 from the monoclonal IgG1 subtype, the average free thiol groups per antibody is 2-4 after reduction. If you have F(ab')2 from a polyclonal or other IgG subtype, you can purchase the Thiol Assay Kit with Purification (Product Number: **CM90005**) separately from CellMosaic to measure the free thiols while letting the reducing solution sit at 4°C in **Step C5**. Use 6 µL antibody solution from **Step C5** and follow the protocol of CM90005. To calculate the number of thiols, please use the antibody concentration of 13 µM.

The assay will take 30 minutes. The number of thiols per antibody (**n**) is satisfactory within 2-4. If n is lower (i.e., <2.0), you can add additional Reagent A solution from **Step C2** based on the following calculation. Repeat **Step C4,** but mixing at 37°C for 30 minutes will be sufficient, and then cool the antibody reducing solution to approximately 4°C for 5 minutes before moving to the next purification step.

Calculation for Additional Reagent A Solution for Targeting Total 4 Thiols per F(ab')2:

$$Volume\ of\ Additional\ Reagent\ A\ solution\ to\ be\ transferred\ from\ Step\ \boldsymbol{C2}\ (\mu L) = Ab\ in\ mg\ \times 3.42\ \times \left(\frac{4-n}{n}\right)$$

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 Tubes (CM03CT0), Labeling Buffer (CM02005, indigo label), 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 (micro-centrifuge tube with the cap attached). Transfer the reduced F(ab')2 solution from **Step C5** into the **Filter Device** directly. Wash the centrifuge tube once with 200 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. Insert the **Filter Device** back into the collection tube. Add 400-450 µL of **Labeling Buffer** to make up the total volume to 500 µL. Next, 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.**

D5. Repeat **Step D4** once.

Work quickly

D6. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube (use the pipetman to measure the approximate volume of the concentrated sample).

D7. Add 50-200 µL of **Labeling Buffer** to the **Filter Device** to rinse (actual volume of **Labeling Buffer** added will depend upon the calculated total volume in **Step D9**). Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step D6**.

D8. Repeat **Step D7** once.

D9. Add **Labeling Buffer** to make up the total volume of the sample to **640 ± 10 µL**.

Calculation 3 for Less F(ab')2:

$$Volume\ of\ Reduced\ F(ab')2\ in\ Step\ \boldsymbol{D9}\ (\mu L) = F(ab')2\ in\ mg\ \times 292$$

D10. Vortex the combined antibody sample for 30 seconds and then spin down.

6. MMAE Labeling (Step 2 in Scheme 1)

Items needed: MMAE solution from **step A3**, Hazardous Waste Bag (CM03HZ1), Antibody Solution from **step D10**.

E1. With personal protection equipment on, carefully open the centrifuge tube containing MMAE from **Step A3**.

E2. Transfer the entire solution (**110 µL** total) to the centrifuge tube containing F(ab')2 from **Step D10**. When you add the MMAE solution, place the pipette tip inside the antibody solution and then dispense the MMAE slowly while swirling the pipette tip. **Dispose of the pipette tip and MMAE tube in the hazardous waste bag**.

Calculation 4 for Less F(ab')2:

$$Volume\ of\ MMAE\ Solution\ to\ be\ Transferred\ in\ Step\ \boldsymbol{E2}\ (\mu L) = F(ab')2\ in\ mg\ \times 36.7$$

Dispose of the remainder of the MMAE solution in the hazardous waste bag.

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

Time-saving tip: While waiting for the reaction to complete, you can move on to **Step F1** and equilibrate the column for purification.

7. Purification of Conjugate

Items needed: Desalting Column, Storage Buffer (1x PBS), 2.0 mL Centrifuge Tube (CM03CT3), Hazardous Waste Bag (CM03HZ1), Antibody Solution from **Step E3**.

F1. In a chemical hood, securely attach the **Desalting Column** to a support stand, 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.

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F2. Add 5 mL of **Storage Buffer** and allow the buffer to completely enter the gel bed by gravity flow.

F3. Repeat **Step F2** twice.

F4. Spin the MMAE-labeled F(ab')2 solution from **Step E3** before opening it. Add the entire antibody solution to the column. **Dispose of the centrifuge tube in the hazardous waste bag.**

F5. Add 250 µL of **Storage Buffer** and allow the liquid to enter the gel bed completely (**Note:** this elution buffer does not contain any of your product, you can let it drain to the waste).

Calculation 5 for Less F(ab')2:

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{F5}\ (\mu L) = 1000 - F(ab')2\ in\ mg\ \times 342$$

F6. Place a 2 mL centrifuge tube under the column. Add 1.25 mL of **Storage Buffer** to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.

Calculation 6 for Less Antibody (Ab):

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{F6}\ (\mu L) = 500 + F(ab')2\ in\ mg\ \times 342$$

F7. Label the tube as your product. Store your conjugate at 4°C. **Dispose of the Desalting Column in the hazardous 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 F(ab')2 with an average drug-to-F(ab')2 ratio (DAR) of 2-4. The actual DAR will depend very much on the type of F(ab')2 you are using. A typical batch contains over 95% conjugated products by SEC (size exclusion chromatography) with less than 5% of unreacted F(ab')2 and is free of any unreacted drug. The approximate concentration of the conjugate is 0.88 mg/mL in PBS buffer assuming 50% recovery. You can determine the concentration and the estimated DAR of the conjugate by UV/vis spectrophotometry (see other considerations).

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Other Considerations

1. Concentration Determination for F(ab')2 (Unlabeled)

The accuracy of the F(ab')2 amount is important for obtaining an optimized DAR in this protocol. The simplest assay method for determining F(ab')2 concentration in solution is to measure the absorbance of the F(ab')2 at 280 nm (UV range) ($A_{1\ mg/mL}$ = 1.4).

If your F(ab')2 comes with a buffer that has no UV absorbance at 280 nm, you can measure the UV absorbance prior to starting an experiment.

$$Concentration\ (mg/mL)\ of\ F(ab')2 = \frac{(A280)}{1.4}$$

If your F(ab')2 comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **step B11** after exchanging it with Buffer A and assuming **95%** recovery of the F(ab')2 after buffer exchange. Buffer A does not contain any substances that will interfere with the UV measurement at 280 nm. The total volume of Buffer A added in **Step B10** can be estimated based on the initially estimated amount of F(ab')2 and will not affect the conjugation too much if the volume is off to some extent.

$$Concentration\ (mg/mL)\ of\ Starting\ F(ab')2 = \frac{(A280)}{1.4 \times 0.95}$$

After calculating the total amount, follow the calculations in **Steps B10, C3**, **D10**, **E3**, **F5,** and **F6** to obtain the correct volumes to be added in each step.

2. Concentration Determination for Conjugate

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:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) \times 6.4935}{L}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 0.974}{L}$$

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 F(ab')2 with MW of 110,000, the molar extinction coefficient is 154,000 $M^{-1}cm^{-1}$.

3. MW Calculation

Calculation of the MW of the conjugate:

MW(Conjugate) = n x 1317+ 110000

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Where n is the average molar ratio of MMAE per F(ab')2. Use 2.0 if you do not have the hydrophobic interaction chromatography (HIC) profile of your conjugates.

4. Drug-to-F(ab')2 Ratio (DAR) and Characterization by UV and HPLC

In this kit, the target DAR is 2-4. The actual DAR will depend very much on the type of F(ab')2 you have.

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

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

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

$$DAR = \frac{(15.4 \times R - 6.6)}{(1.615 - 0.1425 \times R)}$$

Note: The UV absorbance of the MMAE in an F(ab')2 conjugate can vary greatly depending on many factors, such as aggregation and stacking. Therefore, the **R** value for an F(ab')2 conjugate can differ greatly for different F(ab')2 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 conjugate, 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, 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

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solid precipitate out or aggregation during the reaction. The precipitate will be removed during purification. Depending on the properties of your F(ab')2, 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 (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.

7. If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis. 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 F(ab')2 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)

F(ab')2 information: F(ab')2 was prepared using CellMosaic's PerKit™ F(ab')2 preparation kit (Cat#: CM51408) from a mouse IgG1 antibody.

Kit Lot number: 5525.S13.032919

Scale of the reaction: 0.5 mg

DAR of the final conjugates: 2 calculated based on the UV absorbance ratio (R) of the conjugate at 248 nm and 280 nm.

Figure 1: HIC HPLC analysis of F(ab')2 and purified conjugates

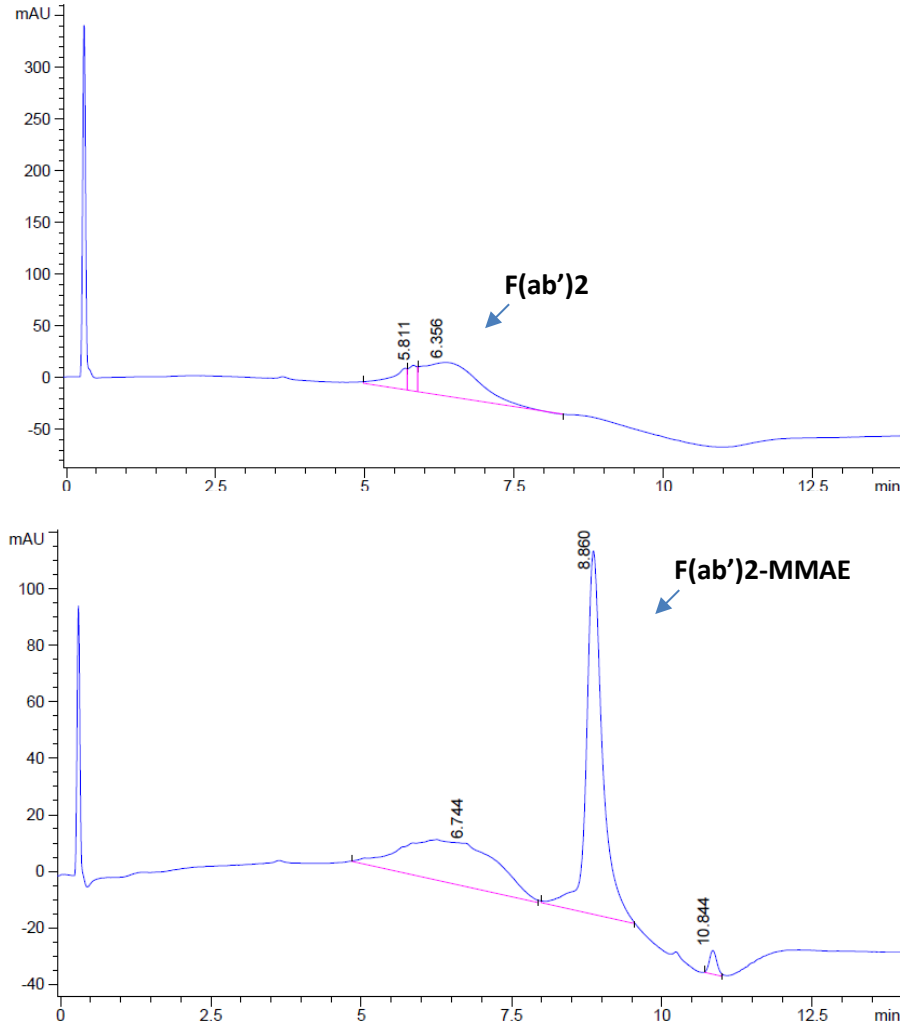

Figure 2: SEC HPLC analysis of F(ab')2 and purified conjugates

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