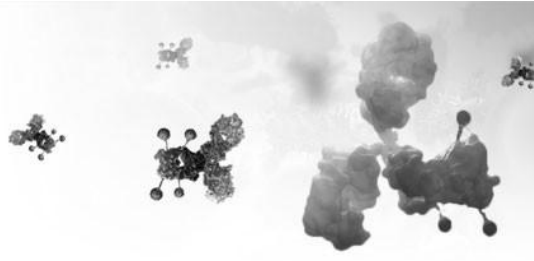


Magic Fast™ MMAE Conjugation Kit (With VC-PAB Linkage) CD4006 (100 μg) and CD4006 (300 μg)

(User Reference Guide- Product Overview)

Important Notes

The information provided in this document and the methods included in this package are for information purposes only. Creative Diagnostics provides no warranty of performance or suitability for the purpose described here in. The performance of labeling using this kit may be affected by antibodies. Sample data are provided for illustration and example purposes only. Information about the chemicals and reagents used in the kit are provided as necessary.
There agents provided are for research use only. Not for use in diagnostic procedure.

Product Overview

Creative Diagnostics has designed this Magic Fast™ MMAE Conjugation Kit to label any IgG antibody with monomethyl auristatin E (MMAE) using a valine-citruline p-aminobenzylcarbamate (VC-PAB) linker for early-stage screening of your antibody.

The kit includes maleimide-activated VC-PAB-MMAE, which can be coupled directly to the IgG following reduction and alkylation in a single step. The product is then purified to remove any unreacted drug.

Performance

- Labeling chemistry: Simple and efficient labeling of IgG1 with MMAE via cathepsin B cleavable VC-PAB linker by reduced thiols at heavy chains.
- DAR: Delivers an average of 4 MMAE molecules per antibody.
- Fast preparation: 4 hours total, with the whole process less than 1 hour of hands-on time.
- Includes all necessary reagents and supplies for preparation and purification.
- Yield: Achieves over 95% conjugation, free from unreacted MMAE.

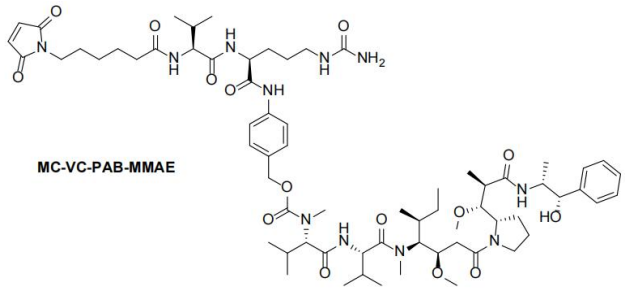
Requirement for antibody (IgG1):

1.Preferably >90% pure by gel electrophoresis.

2. Total amount: 100 μg or 300 μg protein content as measured by UV.

Note: The accuracy of your protein measurement is the single most important factor in obtaining an optimized DAR of 4. Please refer to the “Other Considerations" section in this manual for instructions on measuring the protein amount.

Drug Information:



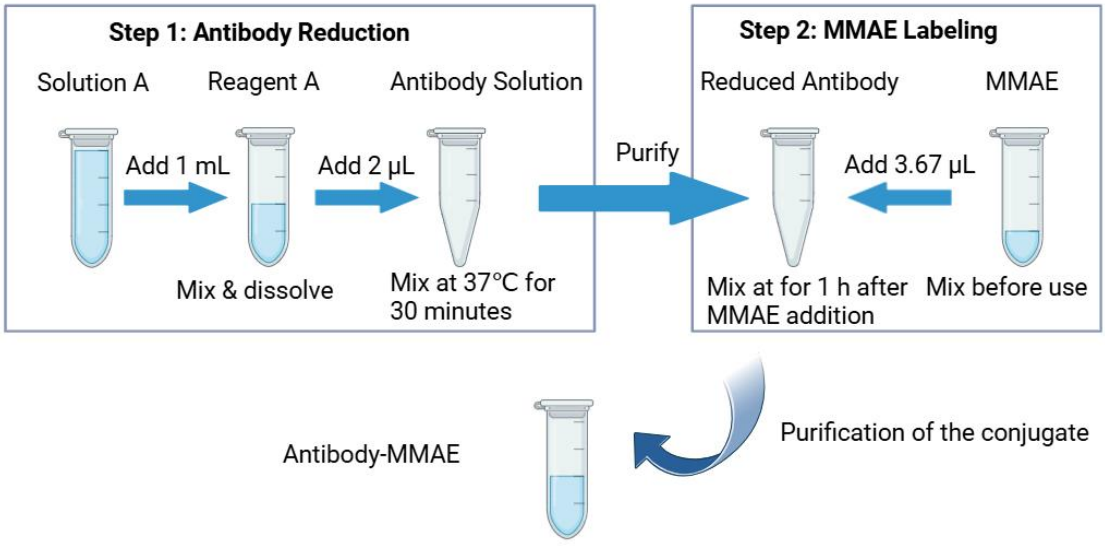
Name: Monomethyl auristatin E (MMAE) with Mal-VC-PAB linkage

CAS number: 646502-53-6

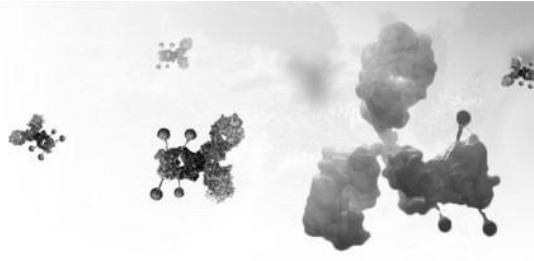
Chemical Formula: $C_{68}H_{105}N_{11}O_{15}$

MW: 1316.65

Workflow diagram:



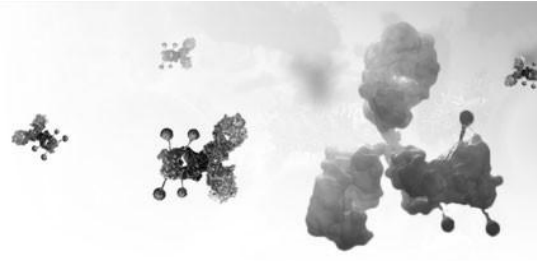
Scheme 1. Schematic workflow diagram for preparing antibody-MMAE conjugates



Kit Components

This kit provides materials to conjugate 100 μg of single antibody sample (CD4006-100μg) or 300 μg of single antibody sample (CD4006-300μg) (IgG) with MMAE using VC-PAB linker.

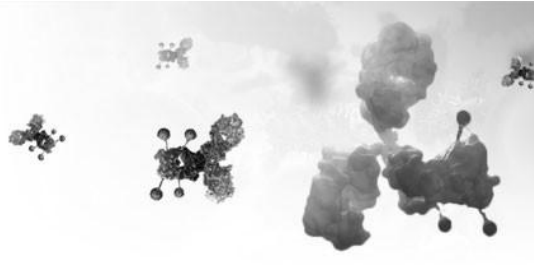
Kit Components	Name	Quantity (CD4006-100μg)	Quantity (CD4006-300μg)	Storage
Set 1	MC-VC-PAB-MMAE	5 μL	3 x 5 μL	-20°C
	Reagent A	1 unit	3 units	
Set 2	Solution A	2 mL	6 mL	2-8°C
	Reducing Buffer	4 mL	12 mL	
	Labeling Buffer	4 mL	12 mL	
	Storage Buffer (1 x PBS)	5 mL	20 mL	
	Centrifugal Filter Devices	3	9	
	Collection Tubes for Filter	6	18	
	Desalting Spin Column	2	6	
	Collection Tubes for Spin Column	2	6	
	0.5 mL Eppendorf Tubes	2	6	
	1.5 mL Centrifuge Tube(s)	2	6	
	Hazardous Waste Bag(s)	1	3	
User Material	IgG Antibody			



Magic Fast™ MMAE Conjugation Kit (With VC-PAB Linkage) CD4006 (100 μg) and CD4006 (300 μg)

(User Reference Guide- Protocol)

Content



1. Preparation

1.1 Preparation of MMAE

MMAE with VC-PAB is highly hydrophobic and antibody-drug conjugates with VC-PAB-MMAE tend to aggregate and precipitate out from solution. Therefore, it is recommended to perform the labeling experiment just a few days before your subsequent experiments.

Always use personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves).

A1. Remove **Set 1** containing **MMAE** and **Reagent A** from the -20°C freezer and allow it to warm to room temperature before opening the bag.

A2. Remove **Set 2** from the refrigerator. Place the hazardous waste bag inside the chemical hood for solid waste disposal and bring the remaining items to the lab bench.

A3. Check if the frozen liquid inside the **MMAE** tube has thawed. Briefly mix and spin the centrifuge tube containing **MMAE**. Place the **MMAE** tube in a tube holder inside the chemical hood and wait until the antibody is ready for conjugation.

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

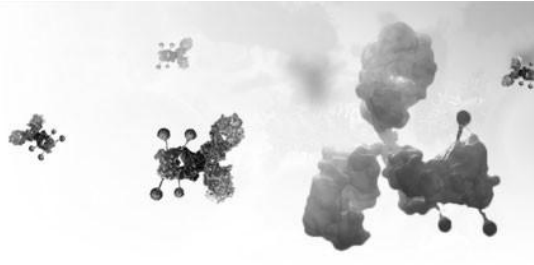
1.2 Preparation of Antibody Samples

Items needed: **Filter Devices**, **Collection Tube**, **Reducing Buffer**, **0.5 mL Eppendorf Tube**, Clean Centrifuge Tubes (not provided in the kit).

The total amount of antibody used for the conjugation is 0.1 mg (100 microgram) per reaction (protein content as measured by UV).

B1. Insert the **Filter Device** into one of the provided collection tubes (micro-centrifuge tube with the cap attached). Follow the appropriate step based on the condition of your antibody.

- ✓ **Lyophilized antibody**: Dissolve the antibody in 500 µL of **deionized water** and transfer the entire contents to the **Filter Device**.
- ✓ **Antibody in < 500 µL buffer**: Transfer the antibody sample directly to the **Filter Device**, then add **Reducing Buffer** to bring the total volume to 500 µL. Cap the device.
- ✓ **Antibody in 500-1000 µL buffer**: Split the sample between two **Centrifugal Filter Devices**, adding the antibody to each device. Add **Reducing Buffer** to bring the volume in each device to 500 µL and cap them.



- ✓ **Antibody in >1000 µL buffer**: Transfer up to 500 µL of the sample into two **Filter Devices**. Cap the devices and repeat Steps **B1-B4** until the entire antibody sample has been transferred. For the final refill (Step **B5**), add **Reducing Buffer** to bring the total volume to 500 µL in each device.

B2. Place the capped **Filter Device** into the centrifuge rotor, ensuring the cap strap is alignedtoward the center of the rotor. Counterbalance with a similar device.

B3. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably at 4°C) to concentrate the sample to < 100 µL. (Spin time may vary; typically, a 500 µL sample will concentrate to ~40 µL after 8 to 20 minutes of spinning. The typical time for an Eppendorf is 8 minutes).

B4. Remove the 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 all experiments are done.

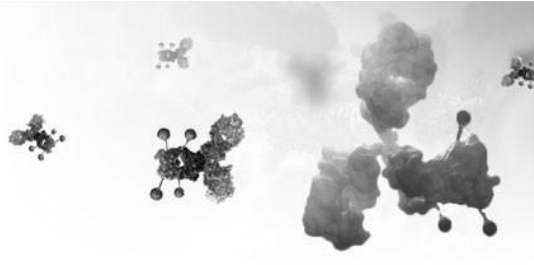
B5. Reinsert the **Filter Device** into the collection tube. Add 400-450 µL of **Reducing Buffer** to bring the total volume to 500 µL. Place the capped **Filter Device** back into the centrifuge rotor,align the cap strap toward the center, and spin at 14,000 xg to concentrate the sample to < 100 µL. Remove the device, transfer the filtrate to a clean centrifuge tube (not provided). Save the filtrate until all experiments are done.

B6. Repeat Step **B5** two more times. For the last repeat, if you start with two Filter Devices, combine the samples into one Filter Device and spin at 14,000 x g to concentrate the solution to less than 20 µL.

B7. Transfer the concentrated sample from the **Filter Device** to a **0.5 mL Eppendorf tube**. Use a 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**. After combining the concentrated sample from **Step B7** and the rinsing solution from **Step B8**, the total volume should be approximately 30 µL.

B8. Add 10-20 µL of **Reducing Buffer** to the **Filter Device** to rinse. Gently stir the buffer 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, then spin down the liquid.



2. Antibody Reduction (Step 1 in Scheme 1)

Items needed: **Reagent A**, **Solution A**, **Antibody Solution from Step B9**, Ice Bath.

C1. Spin the centrifuge tube containing **Reagent A**.

C2. Spin **Solution A** briefly before opening. Add 1 mL of **Solution A** to the tube containing **Reagent A** from **Step C1**. Vortex for 30 seconds to 1 minute to fully dissolve the reagent, then spin briefly.

C3. Add 2 µL of **Reagent A** solution from **Step C2** to the centrifuge tube containing the **antibody from Step B9**. (Discard of any unused **Reagent A** as hazardous chemical waste once all experiments are done)

C4. Vortex the solution for 30 seconds, then spin briefly to ensure no liquid remains in the cap. Incubate the mixture at 37°C for exactly 30 minutes.

Tip for mixing: You can use a nutator, shaker, vortex, or incubator shaker for mixing. If using end-to-end nutating, ensure the tube from **step C4** is securely capped. If you don't have access to this equipment, you can let the tube sit on the bench and manually mixing it by pipetting every 10 minutes.

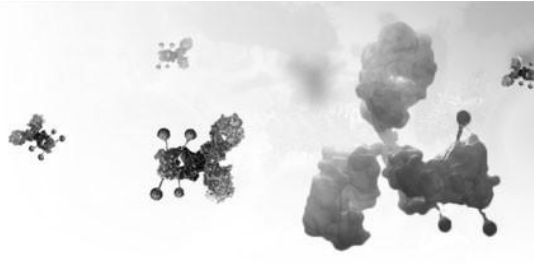
Purification to Remove Excess Reagent A

Note: The following steps should be performed consecutively without interruption, as reduced thiols oxidize quickly. Ensure **step A3** is completed before proceeding. Work quickly through **steps D6-D8**.

Items needed: **Filter Device**, **Collection Tube**, **Labeling Buffer**, **0.5 mL Eppendorf Tube**, 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 cap attached). Transfer the reduced antibody solution from **step C4** directly into the **Filter Device**. Rinse the centrifuge tube with 200 µL of **Labeling Buffer** and transfer this solution to the **Filter Device** (total volume 500 µL). Cap the device and place it 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 at 4°C) to concentrate the sample to < 100 µL.



D3. Remove the 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 all experiments are done**.

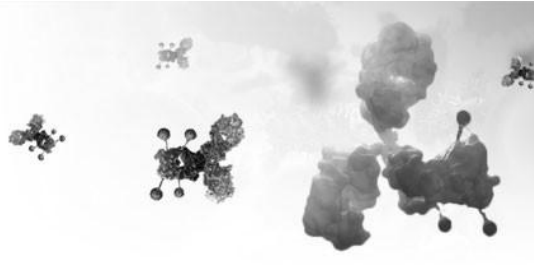
D4. Reinsert the **Filter Device** into the collection tube. Add 400-450 µL of **Labeling Buffer** to bring the total volume to 500 µL. Cap the device and place it back into the centrifuge rotor, aligning the cap strap toward the center of the rotor. Counterbalance with a similar device, and spin at 14,000 x g to concentrate to < 100 µL. Remove the device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate to a clean centrifuge tube (not provided). Save the filtrate until all experiments are done.

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

D6. Transfer the concentrated sample from the **Filter Device** to a **0.5 mL Eppendorf tube**. Use a 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**. After combining the concentrated sample from **Step D6** and the rinsing solution from **Step D7**, the total volume should be approximately **30 µL**.

D7. Add 10-20 µL of **Labeling Buffer** to the **Filter Device** to rinse. Gently stir with a pipet tip, then transfer the entire contents to the **0.5 mL Eppendorf tube** from **Step D**6.

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



3. MMAE Labeling (Step 2 in Scheme 1)

Items needed: **MMAE solution** from **step A3**, **Hazardous Waste Ba**g, Antibody Solution from **step D10**.

E1. While wearing personal protection equipment, carefully open the centrifuge tube containing MMAE from **step A3**.

E2. Transfer 3.67 µL of MMAE solution from **Step E1** to the **centrifuge tube** containing the antibody from **Step D10**. When adding the **MMAE solution**, insert the pipette tip into the antibody solution and slowly dispense the MMAE while gently swirling the pipette tip. Dispose of the pipette tip and MMAE tube in the **hazardous waste bag**.

E3. Cap the centrifuge tube and mix the solution at 25°C or room temperature for 1 hour.

Time-saving tip: While waiting for the reaction to complete, you can proceed to **step F1** and begin equilibrating the column for purification.

4. Purification

Items needed: **Desalting Spin Column**, **Storage Buffer (1x PBS)**, **Collection Tubes**, **2.0 mL Centrifuge Tube**, **Hazardous Waste Bag**, **MMAE-Antibody Solution** from **Step E3**.

F1. Take out two **Desalting Spin Columns** and remove the bottom red cap. Spin the columns for 1 minute at 750 xg before opening the top cap.

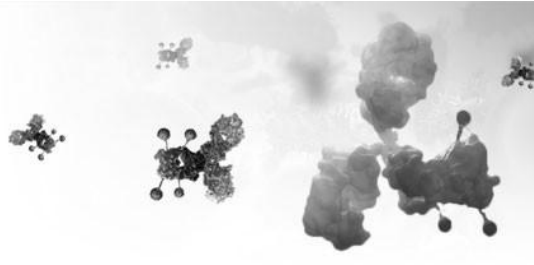
F2. Apply 400 µL of **PBS buffer** (grey label) to the top-center of the resin in each column. Allow the resin to swell at room temperature for 15 minutes.

F3. Spin the **columns** for 1 minute 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 no liquid remains in the cap before opening. Add 1xPBS buffer to bring the total volume of the MMAE solution to 60 µL.



F7. Slowly apply up to 30 µL of the conjugate solution from **Step E3** to the top-center of the resin in each spin column, taking care not to disturb 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 in each **spin column**, bringing the total volume in each column to 50 µL. **Dispose of the centrifuge tube in the solid waste bag.**

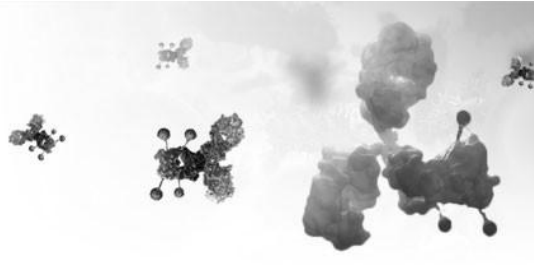
NOTE: The resin may slightly detach from the column to form, forming a pillar with an unbalanced resin bed due to centrifuge force. To prevent issues, ensure that both the sample and subsequent **PBS buffer** are applied slowly to the center of the resin bed, avoiding any runoff down the sides. Wait for the conjugate solution to fully enter the resin before applying the **PBS buffer**. Be careful not to touch the resin bed with the pipette tip.

F9. Rotate and align the spin column so that the higher side of the resin bed is positioned against the outer wall of the centrifuge rotor, while lower side faces the center. 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** and **Collection Tubes** in the solid waste bag, then seal the bag. Follow local regulations for proper waste disposal.

Conjugate is Ready for Your Experiment

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



Magic Fast™ MMAE Conjugation Kit (With VC-PAB Linkage) CD4006 (100 μg) and CD4006 (300 μg)

(User Reference Guide - Other Considerations)

1. Concentration Determination for IgG Antibody (Unlabeled)

Accurately determining the IgG concentration is crucial for obtaining DAR of 4 in this protocol. The simplest method for measuring IgG concentration in solution is to measure the absorbance at 280 nm (UV range), using the formula assuming that 1 mg/mL IgG has an absorbance of 1.4 at 280 nm.

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

If your antibody is in a buffer that does not absorb at 280 nm, you can measure the UV absorbance directly prior to starting an experiment.

If your antibody is in a buffer that absorbs at 280 nm, determine the concentration in **step B9** after buffer exchange with Reducing Buffer, assuming **95%** recovery of the IgG. Reducing Buffer does not interfere with UV measurement at 280 nm.

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

2. Concentration Determination for ADC

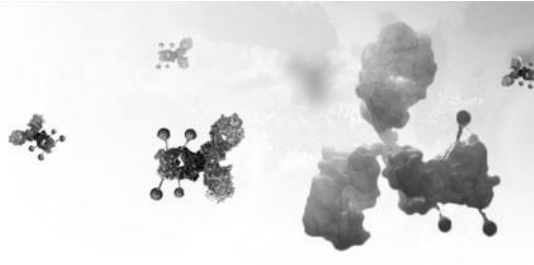
To determine the concentration of the ADC, dilute your conjugate from Step **F7** with 1x PBS buffer. Measure the UV absorbance of the conjugate at 280 nm (A280) using a UV spectrometer, and calculate the concentration using the following formulas:

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

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

Where **L** is the path length of UV cell in centimeters. If you are using a 1 cm UV cell, you may dilute the conjugate 4 times to obtain an accurate reading.

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



3. MW Calculation for ADC

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 a value of 4.0 if you do not have the experimental data.

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

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

To estimate the DAR, you can calculate the UV absorbance ratio (*R*) of your conjugate at 248 nm and 280 nm using the following formula.

$$R = \frac{(\mathrm{A248})}{(\mathrm{A280})}$$

Unlabeled antibody typically has an ***R*** value between 0.4 and 0.5.

MMAE-ADC with a DAR of 3 to 5 have an ***R*** value between 0.65-0.80.

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

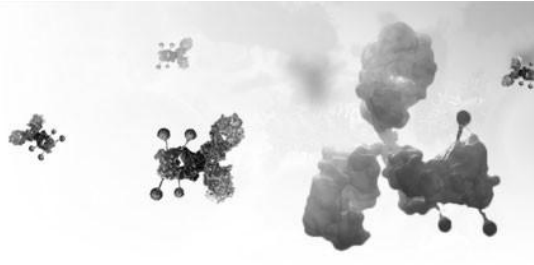
$$DAR = \frac{(21 \times R - 9)}{(1.615 - 0.1425 \times R)}$$

Note: The UV contribution of the VC-PAB-MMAE to the ADC is experimentally determined at Creative Diagnostics. The UV absorbance of the VC-PAB-MMAE in an ADC can vary significantly due tofactors like aggregation and stacking. Therefore, the ***R*** value for an ADC may difer greatly depending on the antibodies and should be determined experimentally. The DAR calculation using this formula is for reference purpose only.

5. Characterization of ADC by HIC HPLC

For ADCs prepared via the reduction of antibody thiols, hydrophobic interaction chromatography (HIC) HPLC is used to calculate the DAR and assess the heterogeneity of the ADCs. The conjugates are separated based on hydrophobicity. Antibodies with the same drug-to-antibody ratio (DAR) will have similar hydrophobicity and will elute as a single peak. For a typical MMAE ADC, multiple peaks indicate different levels of drug-loading.

Creative Diagnostics offers an HIC buffer set that can be used with any HIC column.The product sheet includes detailed information and methodology for running an HIC HPLC analysis.



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

6. Characterization of ADC by SEC HPLC

VC-PAB-MMAE is highly hydrophobic. This kit is designed to minimize the aggregation and precipitation issues typically encountered with MMAE labeling. However, you may still notice some solid precipitate or ADC aggregation during the reaction. The precipitate will be removed during purification. Depending on the properties of your antibody, recovery may range from 40-80%.

If you are concerned about aggregation, you can use size exclusion chromatography (SEC) to assess the extent of aggregation. SEC separates conjugates based on apparent molecular weight(MW) or size in aqueous solution. Larger MW conjugate elute earlier. By comparing the SEC profile of unlabeled lgG to that of the ADC, you can estimate the level of aggregation in the ADC

7. ADC Stabilizing Buffer

Creative Diagnostics' proprietary ADC Stabilizing PBS buffer (5x) contains 5x PBS buffer and other stabilizers designed to prevent hydrophobic drugs from interacting with each other during storage, which can lead to ADCs precipitation. The Stabilizing Buffer also helps preserve the structure of the ADCs during lyophilization. This biocompatible buffer can be used directly for both in vitro and in vivo studies. for more information on stabilization buffers, please visit our website,

8. Recommended Storage Conditions

Based on our preliminary data, the conjugates made with this kit can remain stable in PBS buffer for several weeks at 2-8°C. Freezing is not recommended.The stability of your conjugate may vary depending on your specific antibody and should be checked by either HPLC or UV analysis. If you need to store ADCs for an extended period, you can purchase the ADC stabilization PBS buffer separately. Dilute your ADC in Stabilization PBS Buffer (5x), aliquot the conjugate, and store it in a < -20°C freezer, or lyophilize to dryness. Avoid repeated freeze-thaw cycles.