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PerKit™ Antibody Doxorubicin Conjugation Kit (CM11406 and CM11406x3) 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 1-3 mg of one (CM11406) or three (CM11406x3) antibody samples (**IgG**) with doxorubicin.

⚠ 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 (CM11406)	Quantity (CM11406x3)	Storage condition
Box 1	*N*-succinyl doxorubicin acid (red label)	CM11006.1	1 unit	3 units	-20°C, dry
	Reagent A (yellow label)	CM10004.1	1 unit	3 units	
	Reagent B (green label)	CM10003.1	1 unit	3 units	
Box 2	Solution A (blue label)	CM01006	0.5 mL	0.5 mL	2-8°C
	Buffer A (orange label)	CM02001	4 mL	12 mL	
	Buffer B (sky blue label)	CM02006	0.5 mL	1.5 mL	
	Storage Buffer (1 x PBS buffer) (grey label)	CM02013	20 mL	60 mL	
	ADC Stabilizing PBS Buffer (5x) (pink label)	CM02022	0.5 mL	1.5 mL	
	Centrifugal Filter Device	CM03CD050A	2	6	
	Desalting Column	CM03SG10	1	3	
	Collection Tubes	N/A	4	12	
	1.5 mL Centrifuge Tube	N/A	1	3	
	2.0 mL Centrifuge Tube	N/A	1	3	
	Hazardous Waste Bag	N/A	1	3	
User Material	IgG Antibody	N/A	NOT PROVIDED (User Supplied Material, **1-3 mg IgG** needed per reaction)		

Reaction Scale: The protocol is optimized for conjugating 3 mg of IgG antibody. If you have less than 3 mg of IgG, use the calculations in **Steps B10**, **C2**, **D5,** and **D6** to obtain the correct volumes to be added in each step.

Safety Information

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

Labeling Chemistry

The kit is designed to label any antibody (IgG type) with doxorubicin via succinic acid linker. The user supplies the antibody. Using the kit components, the user converts the carboxylic acid of doxorubicin to

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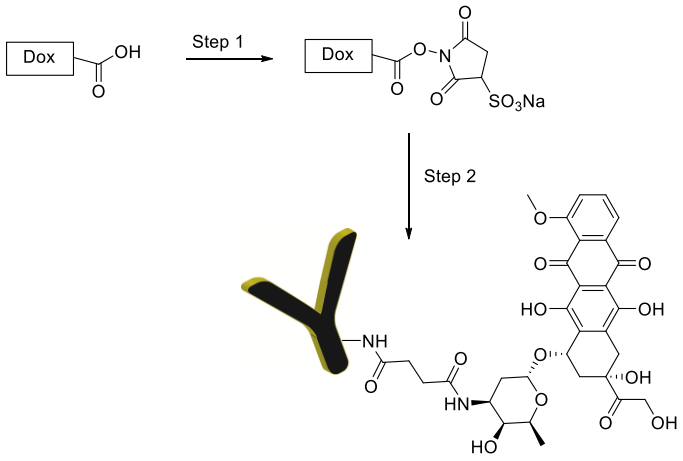
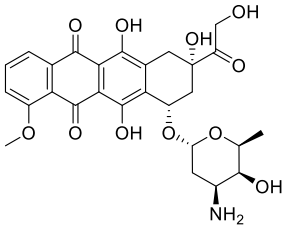
an activated sulfo *N*-hydroxysuccinimide ester (NHS ester), followed by reaction with the surface amines of the antibody. The product is then purified to remove any unreacted drug.

Key features of this conjugation kit:

- Offers a simple and easy way to label IgG with Dox with minimum exposure to the chemotherapeutic drug
- Stable linkage
- Fast and easy preparation: 4 h preparation and <1 h hands-on time
- All reagents and supplies included for preparation and purification
- DAR with average 4 Dox per antibody
- Included stabilization buffer for long-term storage
- More than 99% of conjugated products (free of any unreacted drug)



Drug Information:



- **Name**: Doxorubicin (trade name: Adriamycin)
- **CAS number**: 23214-92-8
- **Chemical Formula**: $C_{27}H_{29}NO_{11}$
- **MW**: 543.53
- **Mechanism of action**: Intercalation of DNA and inhibition of macromolecular biosynthesis

Requirement for antibody (IgG):

1. Preferably > 90% pure by gel electrophoresis

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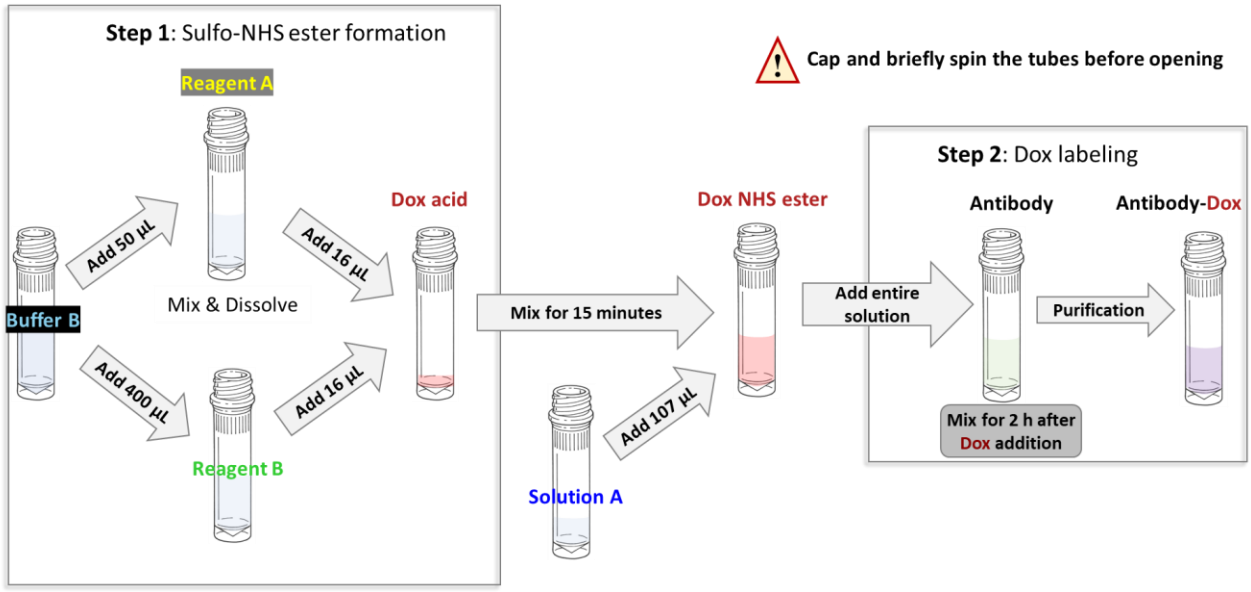
2. Total amount: 1-3 mg protein content as measured by UV. Note: the accuracy of your protein amount is the single most important factor to obtaining an optimized DAR. Please refer to the section Other Considerations in this manual to measure the protein amount

Support

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

Protocol



Scheme 1. Schematic diagram of the workflow for preparing antibody-Dox conjugates starting with 3 mg of IgG (volume of reagents varies if the amount of IgG is < 3 mg).

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, 14,000 g capable), mini-centrifuge
- Pipettes and tips
- Timer
- Incubator or shaker set at 25°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)

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2. Prepare Site and Reagents for Labeling Experiment

> **Note:** Dox is very hydrophobic. Antibody-drug conjugates (ADCs) with average 4 Dox per antibody tend to aggregate and precipitate out from the solution over time. It is recommended that the labeling experiment be planned right before your other experiments. If not possible, then please use the stabilization PBS buffer to store under recommended conditions.

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

A1. Remove box 1 containing **Dox** (red label), **Reagent A** (yellow label), and **Reagent B** (green label) from the -20°C freezer and warm to RT.
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. Briefly spin the centrifuge tube containing **Dox**. Place the **Dox** 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 25°C.

3. Preparation of Antibody Samples for Conjugation

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

Total amount of antibody used for the conjugation is 3 mg (protein content measured by UV) per reaction.

> **Reaction Scale:** If you have less than 3 mg of antibody, use the calculations in **Steps B10**, **C2**, **D5,** and **D6** to obtain the correct volumes to be added in each step.

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 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 **Buffer A** 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**. Add **Buffer A** to make up the total volume in each filter device to 500 µL and cap them.

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- ✓ If the volume of your antibody sample is >1000 µL, add up to 500 µL of sample to each of 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 **Buffer A** 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 in 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 into the collection tube. Add 400-450 µL of **Buffer A** 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 estimate the approximate volume of the concentrated sample).

B8. Add 50-100 µL of **Buffer A** to the **Filter Device** to rinse (actual volume of **Buffer A** 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 **Buffer A** to the 1. 5 mL micro-centrifuge tube from **Step B9** to make up the total volume of the sample to **605 ± 5 µL** and cap it.

Calculation 1 for Less Antibody (Ab):

$$Total\ volume\ of\ the\ antibody\ in\ Step\ \boldsymbol{B10}\ (\mu L) = Ab\ in\ mg\ \times 202$$

B11. Vortex the combined antibody sample for 30 seconds and then spin down.

4. Sulfo-NHS Ester Formation and Antibody Labeling

Items needed: *N*-succinyl Doxorubicin Acid (CM11006.1, red label), Reagent A (CM10004.1, yellow label), Reagent B (CM1003.1, green label), Solution A (CM01006, blue label), Buffer B (CM02006, sky blue label), Antibody Solution from **Step B11**.

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C1. Spin the centrifuge tubes containing **Reagent A** (yellow label), **Reagent B** (green label), **Solution A** (blue label), and **Buffer B** (sky blue label) before opening it.

C2. Transfer **50 µL** of **Buffer B** (sky blue label) to the tube containing **Reagent A** (yellow label). Vortex for 30 seconds to ensure all the solid is dissolved and then spin down.

Tip for solubility check (Step C2 & C4): Check the bottom of the micro-centrifuge tube to ensure the solution is clear and free of any solid residue.

C3. Transfer **16 µL** of **Reagent A solution** from **Step C2** to the tube containing **Dox** (red label) from **Step A3.** Vortex for 30 seconds to mix and then spin down.

C4. Transfer **400 µL** of **Buffer B** (sky blue label) to the tube containing **Reagent B** (green label). Vortex for 30 seconds to ensure all of the solid is dissolved and then spin down.

C5. Transfer **16 µL** of **Reagent B solution** from **Step C4** to the tube containing **Dox** and **Reagent A** from **Step C3.** Vortex for 30 seconds to mix and then spin down.

C6. Let the tube remain at RT for exactly 15 minutes (no need to mix).

C7. Add **107 µL** of **Solution A** (blue label) to the tube from **Step C6.** Pipette up and down three times, then transfer the entire solution to the antibody solution from **Step B11**. When you add the **Dox solution**, place the pipette tip inside the antibody solution and then dispense the Dox slowly while swirling the pipette tip. **Dispose of the pipette tip and Dox tube in the solid waste bag**.

Calculation 2 for Less Antibody (Ab):

$$Volume\ of\ Dox\ solution\ to\ be\ transferred\ in\ Step\ \boldsymbol{C7}\ (\mu L) = Ab\ in\ mg\ \times 46$$

Note for drug-to-antibody ratio (DAR): If you add the entire calculated volume of sulfo-NHS ester solution, you will obtain an average 4 Dox per antibody and ~25% aggregation. If your antibody is prone to aggregation, you can add 52% of the calculated volume to obtain 2-3 Dox per antibody.

C8. Cap the centrifuge tube. Mix at 25°C or RT for 2 h.

Tip for mixing: You can use a nutator, shaker, vortex, or incubator shaker for mixing. If you are using end to end nutating, make sure your centrifuge is capped properly. If you 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.

Time-saving tip: While waiting for the reaction to complete, you can move on to **Step D1** and equilibrate the column for purification.

5. Purification of Conjugate

Items needed: Desalting Column (CM03SG10), Storage Buffer (1x PBS, CM02013, grey label), 2.0 mL Centrifuge Tube, Hazardous Waste Bag, Antibody Solution from **Step C8**.

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D1. 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.
D2. Add 5 mL of **PBS buffer** and allow the buffer to completely enter the gel bed by gravity flow.
D3. Repeat **Step D2** twice.
D4. Spin the **Dox-labeled antibody solution** from **Step C8** 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. **Dispose of the centrifuge tube in the solid waste bag.**
D5. Add 250 µL of **PBS buffer** and allow the liquid to enter the gel bed completely.

Calculation 5 for Less Antibody (Ab):

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{D5}\ (\mu L) = 1000 - Ab\ in\ mg\ \times 250$$

D6. Place a 2.0 mL centrifuge tube under the column. Add 1.25 mL of **PBS 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):

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{D6}\ (\mu L) = 500 + Ab\ in\ mg\ \times 250$$

D7. Label the tube as your product. **Dispose of the Desalting Column in the solid waste bag and seal the bag. Dispose of the waste following regulations appropriate for your area.**
D8. Determine the concentration and the estimated DAR by UV/Vis spectrophotometry (see Other Considerations).
D9. If the ADC is not used immediately for the experiment, add **Stabilization PBS buffer (5x)** (pink label) to the ADC from **Step D7**. If the total volume of your ADC is 1.25 mL, you need to add 312.5 µL of Stabilization PBS buffer. Aliquot and store the conjugate in a < -20°C freezer or lyophilize to dryness for long-term storage.

Conjugate is Ready for Your Experiment

- **Specification for your product:** Dox-labeled antibodies with an average drug-to-antibody ratio (DAR) of 4. A typical batch contains over 99% conjugated product by SEC and is free of any unreacted drug. The approximate concentration of the ADC is 1.44 mg/mL in PBS buffer assuming 60% recovery (without the ADC stabilizing buffer).

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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 B11** after exchanging it with Buffer A and assuming **95%** recovery of the IgG 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 antibody and will not affect the conjugation too much if the volume is off to some extent.

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

After calculating the total amount, follow the calculations in **Steps B10, C3**, **D9**, **E2**, **F5,** and **F6** to obtain the correct volumes to be added in each step.

2. Concentration Determination for ADC

To determine the concentration of the ADC, dilute your conjugate from **Step E7** 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:

$$Concentration\ (\mu M)\ of\ the\ dilute\ sample = \frac{(A280) * 1000000}{L\ (210000 + n * 6940)}$$

$$Concentration\ (mg/mL)\ of\ the\ dilute\ sample = \frac{(A280) \times 150000}{L(210000 + n * 6940)}$$

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

Where **n** is the average molar ratio of Dox per antibody. Use 4.0 if you do not have the experimental value of your conjugates.

For a typical IgG with MW of 150,000, the molar extinction coefficient is 210,000 $M^{-1}cm^{-1}$. The molar extinction coefficient for Dox is estimated to be 6940 $M^{-1}cm^{-1}$ based on CellMosaic's experimental data.

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3. MW Calculation

Calculation of the MW of the conjugate:

$MW_{(ADC)}$ = n x 625.6 + 150,000

Where **n** is the average molar ratio of Dox per antibody. Use **3.0** if you do not have the experimental value of your conjugates.

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

In this kit, the target DAR is 4. To estimate the DAR, you can obtain the UV absorbance ratio (R) of your conjugate at 481 nm and 280 nm.

$$R = {}^{(A481)}\Big/_{(A280)}$$

The unlabeled antibody has no absorbance at 481 nm. A Dox-ADC with DAR of 4 will have an R value of 0.175.

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

$$DAR = \frac{30.26 \times R}{(1.5 - R)}$$

Dox: $E_{280\ nm}$ = 6940 $M^{-1}cm^{-1}$ (data from CellMosaic) and $E_{481\ nm}$ = 10410 $M^{-1}cm^{-1}$ (Tian, Y., Bromberg, L., Lin, S. N., Alan Hatton, T., and Tam, K. C. (2007) Titration microcalorimetry study: interaction of drug and ionic microgel system. *J. Contr. Rel.,* **121**, 137-145).
Antibody: $E_{280\ nm}$ = 210,000 $M^{-1}cm^{-1}$ and no absorbance at 481 nm

5. Characterization of ADC by HIC HPLC

For ADCs prepared via surface amines of the antibody, hydrophobic interaction chromatography (HIC) HPLC can be used to check if an antibody is labeled or not. However, due to the highly heterogeneous nature of surface amine labeling, antibody loaded with the same number of drugs (same DAR) may have slightly different hydrophobicity. For a typical Dox ADC, a broad peak will be seen without clear separation of the peaks.

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. Aggregation and Precipitation Issue for Dox Labeling

Dox is very hydrophobic. This kit is designed to minimize the aggregation and precipitation issues generally seen with Dox labeling. However, you may still notice some solid precipitate out or ADC aggregation during the reaction. The precipitate will be removed during purification.

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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 (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) contains 5x PBS buffer and other stabilizers to prevent the hydrophobic drugs from interacting with each other during storage and causing 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

Recommended use within 24 h and do not store Dox-ADC. If you need to store the ADCs, please dilute your ADC in Stabilization PBS buffer (5x). Aliquot and store the conjugate in a < -20°C freezer or lyophilize to dryness. You might still see some solid precipitate out during the storage using our stabilization buffer. Please centrifuge or filtrate before use. Avoid repeated freeze and thaw cycles. If the ADC is in a lyophilized powder, after dissolving, the solution should be used immediately within 24 h. The stability of your conjugate may be different due to your antibody and should be checked by SEC HPLC.

9. Submit Samples for HPLC Analysis

If you are submitting samples to CellMosaic for SEC 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 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.
3) Transfer 50 µL of ADC (non-diluted solution) to a 500 µL microcentrifuge 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)

Lot number: 5522.S11.011119

Figure 1: SEC HPLC analysis of purified Dox-ADC with average 4 Dox per antibody from test reaction 1 (Inset: UV/Vis spectra of Dox-ADC). Scale of the reaction: 1 mg of antibody.

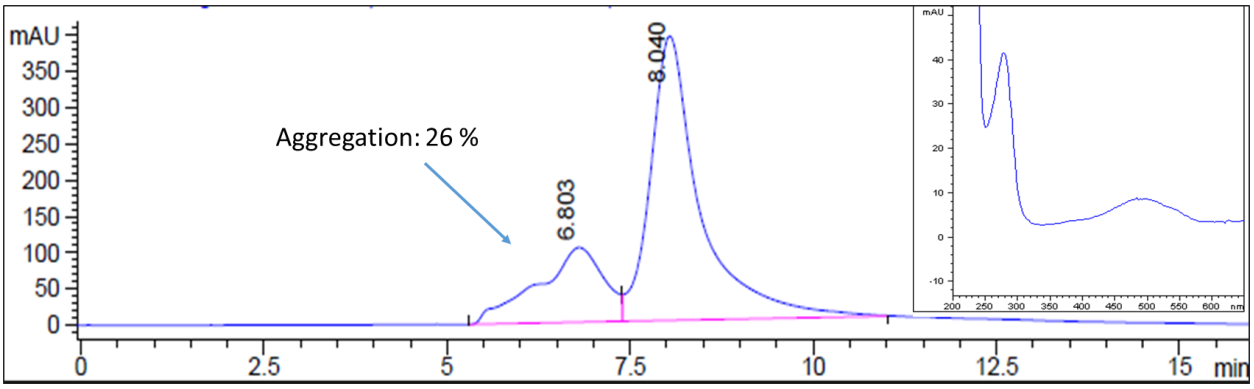
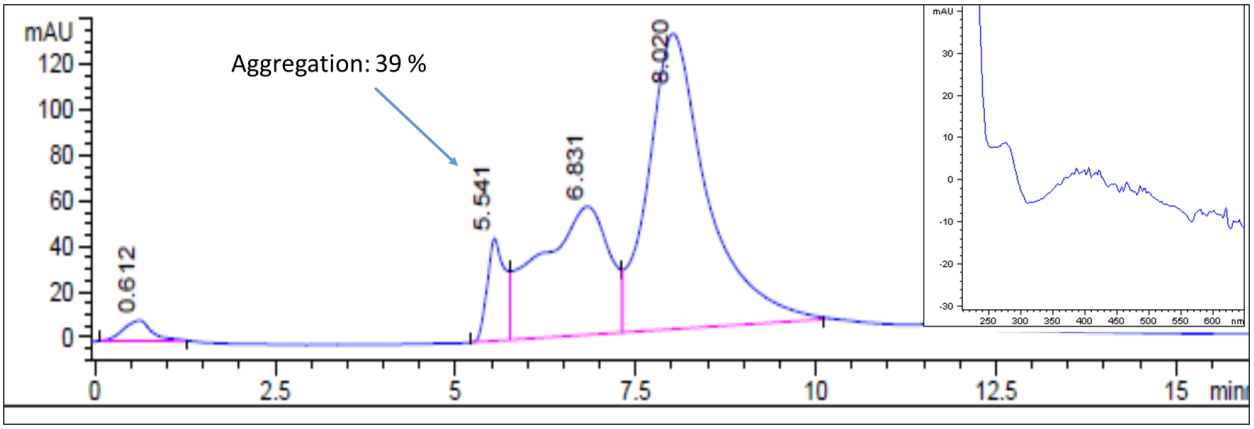


Figure 2: SEC HPLC analysis of purified Dox-ADC with average 7.5 Dox per antibody from test reaction 2 (Inset: UV/Vis spectra of Dox-ADC). Scale of the reaction: 1 mg of antibody. Use twice the amount of Dox sulfo-NHS ester.



Summary of the results:

	Reaction 1	**Reaction 2**
R value (consider the total peaks)	0.1844	0.297
Average DAR based on R value	4.2	7.5
Extent of antibody aggregation (%)	26	39
Unreacted antibody (%)	0	0
Unreacted Dox (%)	0	0
Recovery (%)	67	37