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PerKit™ Antibody UM171 Conjugation Kit (CM11433x1 and CM11433x3) User Reference Guide

Contents

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Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

The information and methods included in this document are provided for information purposes only. CellMosaic provides no warranty regarding performance or suitability for the purpose described. The performance of this kit during labeling may be affected by various factors, including, but not limited to, the purity and complexity of the starting materials, differences in preparation techniques, operator proficiency, and environmental conditions.

Sample data if provided, is provided solely for illustrative purposes and as examples of a small dataset used to verify kit performance within the CellMosaic laboratory. Information regarding the chemicals and reagents used in the kit is included where necessary.

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Kit Components

UM171 is a small molecule first discovered in Canada and published in 2014, known for its ability to enhance hematopoietic stem cell renewal (Fares I. *et al. Science* **2014**, *345*, 1509–1512). UM171 activates the CULLIN3^{KBTBD4} ubiquitin ligase, which specifically targets the LSD1-CoREST repressor complex for proteasomal degradation (Chagraoui J. *et al. Cell Stem Cell*. **2021**, *28*, 48–62). A phase 1-2 trial of UM171-expanded cord blood (CB) transplants demonstrated safety and favorable preliminary efficacy (Cohen S. *et al. Blood Adv.* **2023**, *7*, 5717-5726).

This kit provides the materials to conjugate 1 to 3 mg of one antibody sample (CM11433x1) or three antibody samples (CM11433x3) of IgG with UM171, using a stable amide bond.

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

	Name	Part #	Quantity (CM11433x1)	Quantity (CM11433x3)	Storage condition
Box 1	UM171 NHS (**red label**)	CM11025.1	1 unit	3 units	-20 °C, dry
Box 2	Buffer A (**orange label**)	CM02001	4 mL	12 mL	2-8 °C
	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 Devices	CM03CD030A	2	6	
	Desalting Column(s)	CM03SG10	1	3	
	Collection Tubes	CM03CT0	4	12	
	1.5 mL Centrifuge Tube(s)	CM03CT2	1	3	
	2.0 mL Centrifuge Tube(s)	CM03CT3	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, refer to the calculations in **Steps B10**, **C2**, **D5, D6** and **D9** to determine the correct volumes to add in each step.

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 storing, handling, or using any of the materials.

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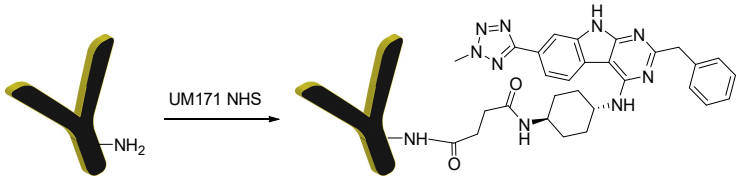
Labeling Chemistry

The kit is designed to label any antibody (IgG type) with UM171 through a stable amide bond. The user provides the antibody, while the kit includes UM171 *N*-hydroxysuccinimide (NHS) ester, which couples directly to the antibody's surface amines in a single step. The final product is then purified to remove unreacted UM171.



Key features of this conjugation kit:

- Provides a simple and efficient method to label IgG with UM171.
- Ensures a stable linkage.
- Quick and easy preparation: 4 h process with less than 1 hour of hands-on time.
- Includes all necessary reagents and supplies for both preparation and purification.
- Optimized Drug-to-Antibody Ratio (DAR): Average of 3–5 UM171 molecules per antibody.
- Features a stabilizing buffer for long-term storage.
- Delivers over 99% conjugated product, free of unreacted UM171.

Drug Information:

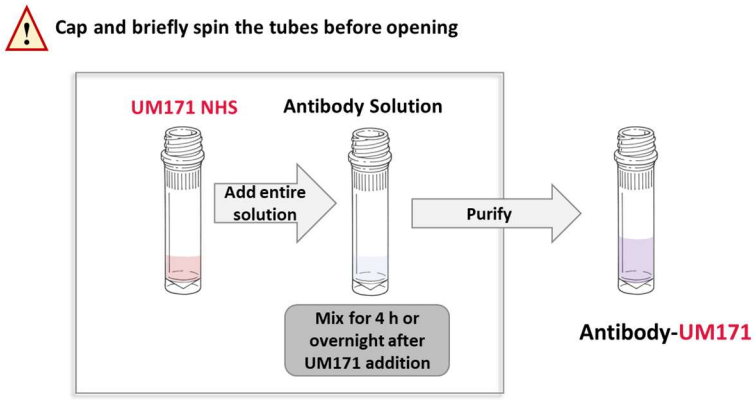
- **Name**: UM171
- **Synonym**: 4-N-[2-benzyl-7-(2-methyltetrazol-5-yl)-9H-pyrimido[4,5-b]indol-4-yl]cyclohexane-1,4-diamine, trans-N1-(7-(2-Methyl-2H-tetrazol-5-yl)-2-(phenylmethyl)-9H-pyrimido(4,5-b)indol-4-yl)-1,4-cyclohexanediamine.
- **CAS number**: 1448724-09-1
- **Chemical formula**: $C_{33}H_{34}N_{10}O_5$
- **MW**: 453.54
- **Mechanism of action** (list only few): Activates the CULLIN3^{KBTBD4} ubiquitin ligase, which specifically targets the LSD1-CoREST repressor complex for proteasomal degradation.
- **Medical usage**: Enhance hematopoietic stem cell renewal.

Requirement for antibody (IgG):
1. Preferably > 90% pure by gel electrophoresis
2. Total amount: 1-3 mg 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.

Support

A customer may request recommendations for the conjugation if their IgG has unique features or if they need to label less than 1 mg of IgG. CellMosaic provides additional accessory tools, such as buffers, standards, and reagents for ADC research. We also offer fee-based support services to customers who need assistance with final conjugate analysis by HPLC and determining the DAR.

Protocol



Scheme 1. Schematic workflow diagram for preparing antibody-UM171 conjugates, starting with 3 mg of IgG (Reagent volumes will vary if the amount of IgG is less than 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)

2. Prepare Site and Reagents for Labeling Experiment

UM171 is highly hydrophobic, and antibody-drug conjugates with UM171 tend to aggregate and precipitate out from solution. Therefore, it is recommended to perform the labeling experiment just a few days prior to your subsequent experiments. If immediate use is not possible, please store the conjugates using the stabilization PBS buffer under the recommended conditions.

Always use personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves) when handling UM171. Ensure you are working in a clean space inside a chemical fume hood.

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A1. Remove **Box 1** containing **UM171 NHS** (red label, CM11025.1) from the -20°C freezer and allow it to warm to room temperature before opening the bag.
A2. Remove **Box 2** from the refrigerator. Bring the rest of the items to the lab bench.
A3. Briefly mix and spin the centrifuge tube containing **UM171 NHS**. Place the **UM171 NHS** 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 briefly to ensure no liquid remains in the cap before opening.

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

3. Preparation of Antibody Samples for Conjugation

Items needed: Filter Devices (CM03CD030A), Collection Tubes (CM03CT0), Buffer A (CM02001, Orange label), 1.5 mL Centrifuge Tube (CM03CT2), Clean Centrifuge Tubes (not provided in the kit).

Total amount of antibody used for the conjugation is 3 mg per reaction (protein content as measured by UV). The protocol is optimized for the IgG antibody with a molecule weight of 150 KDa to obtain an average of 3-5 drugs per antibody.

Reaction Scale: If you have less than 3 mg of antibody, refer to the calculations in **Steps B10**, **C2**, **D5, D6** and **D9** to determine the correct volumes to add in each step.
Preparation of Antibody Containing His or Other Amine Containing Buffers: please check PAGE 10 (other considerations, Section 1) for any special treatment required prior to Step B1.

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 **Buffer A** 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 **Buffer A** 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 **Buffer A** 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 aligned toward the center of the rotor. Counterbalance with a similar device.

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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 5417R 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 **Buffer A** 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 x g 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.

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

B8. Add 50-100 µL of **Buffer A** to the **Filter Device** for rinsing (the actual volume of **Buffer A** will depend on the total volume calculated in **Step B10**). Stir gently with a pipet tip, then transfer the 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 bring the total sample volume to **600 ± 5 µL** and cap it.

Calculation 1 for Less Antibody (Ab):

$$Total\ volume\ of\ the\ antibody\ in\ Step\ \boldsymbol{B10}\ (\mu L) = Ab\ in\ mg\ \times 200$$

B11. Vortex the combined antibody sample for 30 seconds, then briefly spin it down.

4. UM171 Labeling

Items needed: UM171 NHS (CM11025.1, red label), Antibody Solution from **Step B11**.

C1. Spin the centrifuge tube containing **UM171 NHS** (red label) to ensure that no liquid remains in the cap before opening.

C2. Transfer the entire **UM171 NHS solution** from **Step C1** into the antibody solution from **Step B11**. When adding the UM171 NHS solution, insert the pipette tip into the antibody solution and dispense the UM171 NHS slowly while gently swirling the pipette tip.

Note: UM171 can cause significant ADC aggregation, leading to precipitation. To minimize aggregation and precipitation, reduce the amount of UM171 NHS ester added according to the table below.

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Calculation 2 for Less Antibody (Ab) and Various DAR:	
	Volume of UM171 NHS solution to be transferred in Step C2 (µL)
Target DAR: 3	$Ab\ in\ mg\ \times 32$
Target DAR: 4	$Ab\ in\ mg\ \times 42.6$
Target DAR: 5	$Ab\ in\ mg\ \times 53.3$

C3. Cap the centrifuge tube and mix at 37 °C for 1 hour.

Tip for Mixing: You can use a nutator, shaker, vortex, or incubator shaker for mixing. If using end-to-end nutation, ensure the centrifuge tube is properly capped. If none of this equipment is available, you can manually mix by pipetting every 20 minutes.

Time-saving tip: While waiting for the reaction to complete, you can move on to **Step D1** and equilibrium 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 (CM03CT3), Hazardous Waste Bag (CM03HZ1), Labeled antibody Solution from **Step C3**.

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, allowing the excess liquid to flow through by gravity. Collect the liquid in a flask.
D2. Add 5 mL of **PBS buffer** to the column and allow it to fully enter the gel bed by gravity flow.
D3. Repeat **Step D2** twice.
D4. Spin the UM171-labeled antibody solution from **Step C3** before opening the tube. Transfer the entire antibody solution to the column and allow it to fully enter the gel bed.
D5. Add 250 µL of **PBS buffer** to the column, allowing the liquid to fully enter the gel bed (***Note*:** this elution buffer does not contain any of your products and can be discarded as waste).

Calculation 3 for Less Antibody (Ab):

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

D6. Place a 2.0 mL centrifuge tube under the column. Add 1.25 mL of **PBS buffer** to the column and collect the eluent by gravity. Allow the buffer to fully enter the gel bed.

Calculation 4 for Less Antibody (Ab):

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

D7. Label the tube as your product and store the conjugate at 4 °C.
D8. Determine the concentration and estimate the DAR using UV/Vis spectrophotometry (refer to Other Considerations).

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D9. If the ADC will not be used within a few days, add **Stabilization PBS buffer (5x)** (pink label) to the ADC from **Step D7**. Aliquot the conjugate and store it in a freezer at < -20 °C, or lyophilize to dryness for long-term storage. **Dispose of all materials as solid waste, following the waste disposal regulations applicable to your area.**

Calculation 5 for ADC Stabilizing Buffer:

$$Volume\ of\ ADC\ Stabilizing\ Buffer\ in\ Step\ \boldsymbol{D9} = Total\ Vol.of\ ADC\ \times 0.25$$

Conjugate is Ready for Your Experiment

- **Specifications of your product:** UM171-labeled antibodies with an average drug-to-antibody ratio (DAR) of 3-5. A typical batch contains over 99% conjugated products 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. Preparation of Antibody Containing His or Amine Buffer

If your antibody is purified using His Tag purification and contains high levels of His, or if it is in an amine buffer, perform the following desalting purification prior to step B1 on page 6.

Catalog Number: CM03BP3

Items needed: Desalting Columns (CM03SG10), Buffer A (CM02001, Orange label), 2.0 mL Centrifuge Tube

Steps:

O1. In a chemical hood, securely attach the **Desalting Columns** to a support stand, lab frame, or support rod. Remove the top and bottom caps, allowing excess liquid to flow through by gravity. Collect the liquid in a flask.

O2. Add 5 mL of **Buffer A** to each column. Let the buffer completely enter the gel bed by gravity flow.

O3. Repeat **Step O2** two more times.

O4. Add up to 1 mL antibody solution to each column, allowing it fully enter the gel bed.

O5. Add the calculated volume of **Buffer A** to each column, allowing it to fully enter the gel bed. (***Note:*** this elution buffer does not contain your product and can be discarded as waste.)

Calculation O5 for Low Volume of Antibody (<1 mL):

$$Volume\ of\ Buffer\ A\ in\ Step\ \mathbf{O5}\ (\mu L) = 1000 - Ab\ volume \text{ in } \mu L$$

O6. Place a 2.0 mL centrifuge tube under the column. Add 1.5 mL or the calculated amount of **Buffer A** to the column. Collect the eluent by gravity, allowing the buffer to completely enter the gel bed.

Calculation O6 for Low Volume of Antibody (<1 mL):

$$Volume\ of\ Buffer\ A\ in\ Step\ O\mathbf{6}\ (\mu L) = 500 + Ab\ volume\ in\ \mu L$$

O7. If using multiple columns, combine the fractions. Proceed to **Step B1** on Page 6.

2. 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.

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

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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 B10** after buffer exchange with Reducing Buffer, assuming **95%** recovery of the IgG. Reducing Buffer does not interfere with UV measurement at 280 nm.

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

After calculating the total amount of IgG, follow the calculations in **Steps B10, C2**, **D5**, and **D6** to obtain the correct volumes are used in each step.

3. Concentration Determination for ADC

To determine the concentration of the ADC, dilute your conjugate from **Step D7** 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 formula:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) * 1000000}{L\ (210000 + n * 10109)}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280) \times 150000}{L(210000 + n * 10109)}$$

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

Where *n* is the average molar ratio of UM171 per antibody. Use a value of 4.0 if you do not have the experimental value.

For a typical IgG with a molecule weight (MW) of 150,000, the molar extinction coefficient at 280 nm is 210,000 $M^{-1}cm^{-1}$.

The molar extinction coefficient at 280 nm for UM171 is 10,109 $M^{-1}cm^{-1}$ based on CellMosaic's experimental data.

4. MW Calculation for ADC

Calculation of the MW of the conjugate:

MW(ADC) = n x 474.5+ 150000

Where *n* is the average molar ratio of UM171 per antibody. Use a value of **4.0** if you do not have the experimental value of your conjugates.

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

In this kit, the target DAR is 3-5.

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To estimate the DAR, measure the UV absorbance ratio (R) of your conjugate at 325 nm and 280 nm.

$$R = {(A325)}/{(A280)}$$

Unlabeled antibodies will have no absorbance at 325 nm. A UM171 -ADC with DOR of 3 – 5 will have an R value between 0.30 – 0.46.

For a more precise estimate of the DAR (for reference only), use the formula:

$$DAR = \frac{20.774 \times R}{(2.365 - R)}$$

UM171 Extinction Coefficients: $E_{280\ nm}$ = 10109 $M^{-1}cm^{-1}$, $E_{325\ nm}$ = 23907 $M^{-1}cm^{-1}$ (experimental value determined at CellMosaic).
Antibody Extinction Coefficients: $E_{280\ nm}$ = 210,000 $M^{-1}cm^{-1}$, no absorbance at 325 nm

6. Characterization of ADC by HIC HPLC

For ADCs prepared via surface amines of the antibody, hydrophobic interaction chromatography (HIC) HPLC can be used to determine if the antibody is labeled. However, due to the highly heterogeneous nature of surface amine labeling, antibodies with the same drug-to-antibody ratio (DAR) may exhibit slightly different hydrophobicity. For a typical UM171 ADC, this results in a broad peak on the HIC chromatogram, without clear peak separation.

CellMosaic offers an HIC buffer set (Product #: CM02025) that can be used with any HIC column. The CM02025 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 CellMosaic for analysis.

7. Aggregation and Precipitation Issue for UM171 Labeling and Characterization by SEC HPLC

UM171 is very hydrophobic. This kit is designed to minimize the aggregation and precipitation issues typically encountered with UM171 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 IgG to that of the ADC, you can estimate the level of aggregation in the ADC.

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CellMosaic offers two SEC standards (Product #: CM92004 and CM92005) for use with any SEC column. The CM92004 product sheet provides all the necessary information and methodology for running an SEC HPLC analysis.

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

8. ADC Stabilizing Buffer

CellMosaic's proprietary ADC stabilizing PBS buffer (5x) (Product #: CM02022) contains 5x PBS and additional stabilizers to prevent hydrophobic drugs from interacting during storage, which can lead to ADC precipitation. The stabilization buffer also helps maintain the structure of ADCs during lyophilization. This biocompatible buffer is suitable for direct use both *in vitro* and *in vivo* studies. For more information about stabilization buffers, please check our website.

9. Recommended Storage Conditions

UM171 is linked to an antibody via a stable linker. The main concern is aggregation and precipitation. It is recommended to use the ADC within a few days if stored at 2-8°C. Based on our preliminary data, conjugate prepared with this kit remains stable in PBS buffer for a few days at 2-8°C, though no long-term stability data is available. The stability of your conjugate may vary depending on your antibody and should be evaluated using HPLC or UV analysis. For long-term storage, dilute the ADC in Stabilization PBS buffer (5x) (included in this kit), aliquot, and store it in a freezer at < -20°C, or lyophilize it to dryness. Avoid repeated freeze-thaw cycles.

10. Submit Samples for HPLC Analysis

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

1) Visit CellMosaic's HPLC analysis page (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 your order. Alternatively, you can email info@cellmosaic.com for a quote and to place the order.
2) Dilute your un-conjugated antibody in PBS buffer to a concentration of 1 mg/mL. Transfer 50 µL of the diluted solution into a 500 µL micro-centrifuge tube and label the vial properly.
3) Transfer 50 µL of ADC (non-diluted solution) into a 500 µL micro-centrifuge tube and label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.

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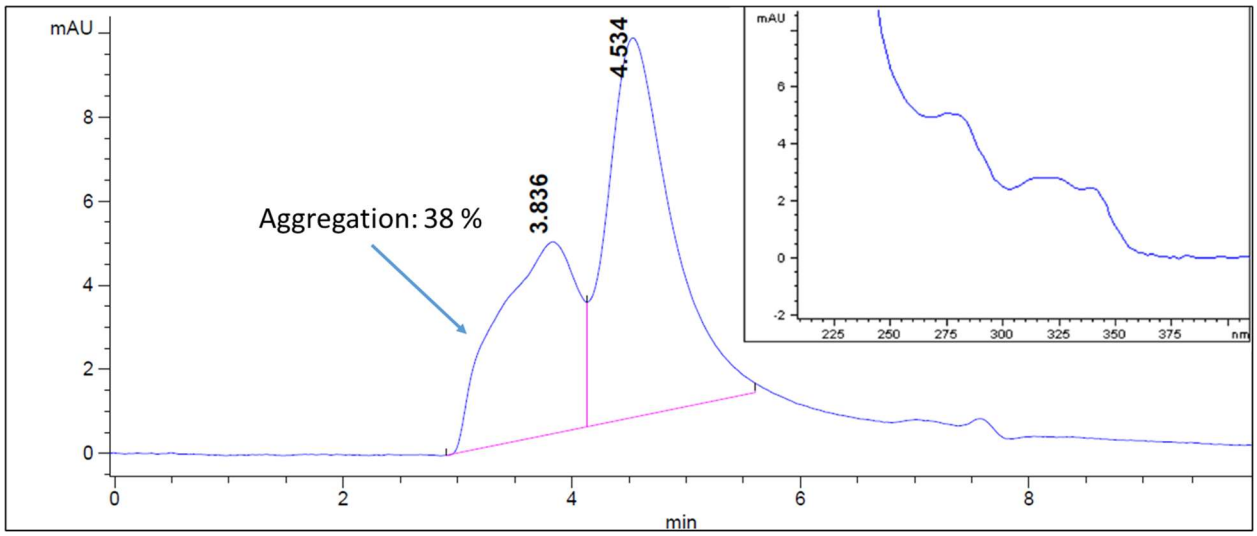
Appendix: Typical Kit Performance Data (LC analysis, CellMosaic)

Antibody information: A therapeutic antibody (human IgG1 subtype)

Kit Lot numbers: S538.S7.1203D and S538.S8.1203D

Figure 1: SEC HPLC analysis of purified UM171-ADC following the procedure of DCM11433, using the full amount of UM171 NHS ester (Inset: UV/Vis spectra of UM171-ADC). Scale of the reaction: 1 mg of antibody.



Summary of the results:

R value (considering the total peaks)	0.476
Average DAR based on R value	5.2
Extent of antibody aggregation (%)	38
Unreacted antibody (%)	<1
Unreacted UM171 (%)	0
Recovery (%): ***Note:*** *The recovery is very low for this antibody. The recovery of your antibody may vary. Reduce the amount of UM171 NHS ester added in Step C2 if the recovery is low.*	18