AGLink® ADC Conjugation Kit(MMAE,DAR2&DAR4, 5mg)

Size: This kit is designed to label 5mg antibody Catalog Number：ADC-P009

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedures

[INTENDED USE]

ADC conjugation kit is designed to conjugate antibody and payload through site-specific enzymatic reaction.

The kit is for research use only.

Content and Storage

BoxA

Name	Details	Size	Storage Temperature
20×Reaction buffer	20×Reaction buffer	105ul	-20 °C
Cofactor A	$MnCl_2$	55ul	-20 °C
Cofactor B	$MgCl_2$	55ul	-20 °C
Substrate A	UDP-Gal	125ul	-20 °C
Substrate B	GDP-Fuc-vc-PAB-MMAE	125ul	-20 °C
Enzyme A	GT	125ul	-20 °C
Enzyme B	FT	125ul	-20 °C
Enzyme C	Endoglycosidase	45ul	-20 °C

BoxB

Components	Details	Size	Storage Temperature
Binding buffer	25 mM Tris-HCl, 100 mM NaCl, pH 7.5	15ml	4°C
Elution buffer	0.1 M Glycine, pH 2.7	1.5ml	4°C
Neutralization buffer	1 M Tris-HCl, pH 8.0	0.5ml	4°C
250 mM Tris-HCl buffer (pH7.5)	250mM Tris-HCl buffer (pH7.5)	1.5ml	4°C
10×PBS (pH7.2- 7.4)	10xPBS (pH7.2- 7.4)	6ml	4°C
Desalting column	Desalting Spin column, 2 mL, 40 K	2per	4°C
Concentrator tube	Ultrafiltration concentrator (include 2 collection tubes),0.5 mL, 50 K	2per	4°C
Protein A Resin	Pierce™ spin column (containing 100 μL protein A resin)	100ul×5	4°C

[PRINCIPLE OF THE Kit]

For DAR2 Conjugation

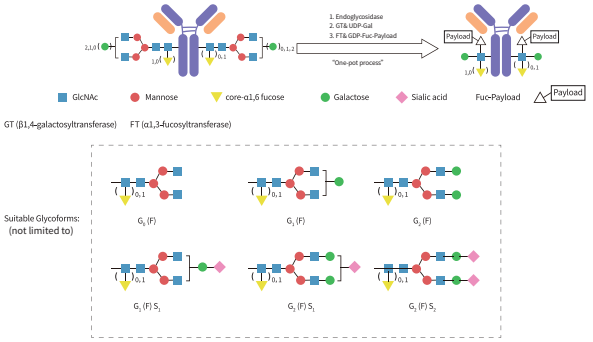


Figure 1 The principle of DAR2 ADC preparation with AGLink®

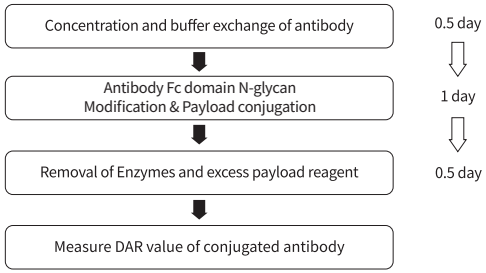
Almost all monoclonal antibodies are glycosylated at (or around) Asn-297 of the Fc domain. While the glycans have different isoforms, the types that typically dominate are G0F, G1F&G2F (>90%)[1]. The AGLink® Antibody Conjugation Kit utilizes the YTConju™ platform, a glycan remodeling strategy developed by Glyco-therapy Biotechnology Co., Ltd. for the construction of site-specific antibody conjugates[2].

The AGLink® DAR2&4 Antibody Conjugation Kit integrates the procedure for constructing DAR2 conjugates and DAR4 conjugates in one kit, providing flexibility in DAR design.

To construct the DAR2 conjugates, glycans are first removed from the core GlcNAc catalyzed by endoglycosidase. Galactose is then added to the GlcNAc catalyzed by β-1,4-galactosyltransferase (GT) to form core LacNAc. Finally, α-1,3-fucosyltransferase (FT) recognizes LacNAc and transfers the Fuc-Payload to the GlcNAc of the LacNAc to form a conjugate with a theoretical DAR of 2 (Figure 1).

The DAR4 and DAR2 conjugation procedures share the same FT, GT and GDP-Fuc-Payload. The main difference between these two procedures is the addition of an endoglycosidase. These procedures are performed in a “one-pot” manner with only one purification step, which is highly convenient and efficient. Antibody conjugates produced with this kit generally exhibit high homogeneity, high stability and high hydrophilicity.

Overview of the protocol for constructing DAR2 ADC：



Equipment required：

•Centrifuge for 1.5-2 mL&15-50 mLtubes (With refrigeration function)

•Incubator or water bath for 30 °C

•Rotary mixer

•UV/VIS Spectrophotometer (Thermo NanoDrop one)

Additional Materials Required：

•Centrifuge tubes: 1.5-2 mL and 15 mL

•ddH_2O

5 mg antibody conjugation can be performed in a 5*1 mg form.

Protocol for conjugation of 1 mg of antibody：

A. Concentration and buffer exchange of the antibody

Note 1: This step is the same for DAR 2 and DAR 4 conjugation procedures.

•Concentration step（Optional)

Note 2: This step is recommended to ensure that the antibody concentration after the buffer exchange step is higher than 10.3 mg/ml, to avoid the final reaction volume exceeding 200 μL. If you choose to proceed with a lower concentration, see Note 5 in Part B for instructions and details.

1. Add 500 μL of ddH_2O to the concentrator and cap the device.
2. Centrifuge at 10000×g for 5 min.
3. Discard the solution in the concentrator and the flow-through.
4. Add the antibody solution to the concentrator.
5. Centrifuge at 10000×g for 2-6 min.

Note 3: If the antibody volume or concentration in the concentrator is not suitable after first concentration, centrifuge for an additional 2-6 min at 10000×g, until the appropriate volume or concentration is achieved.

6. Collect the solution in the concentrator.

•Buffer exchange with Desalting Spin column, 2 mL

1. Prepare 10 mL of 25 mM Tris-HCl buffer (pH7.5) by adding 250 μL of 1 M Tris-HCl buffer (pH7.5) to 9.75 mL of ddH_2O in a 15 mL tube. Vortex briefly to mix.
2. Break off the bottom closure of the Desalting Spin column. Loosen the lid (do not remove the lid).
3. Place the column in a collection tube (15 mL) and centrifuge at 1000×g for 2 min to remove the storage solution.
4. Discard the flow-through and place the column in the collection tube.
5. Add 1 mL of 25 mM Tris-HCl buffer (pH7.5) on top of the resin. Centrifuge the column at 1000×g for 2 min and discard the flow-through.
6. Repeat 25 mM Tris-HCl buffer (pH7.5) wash in step 5 two more times. Last spin for 3 min to remove excess liquid. Place the column in a new collection tube (15 mL).
7. Apply the antibody solution on top of the resin (100-400 μL).
8. Centrifuge at 1000×g for 3 min and collect the flow-through containing the antibody in 25 mM Tris-HCl buffer (pH7.5).

Note 4: It is recommended that the antibody concentration after the buffer exchange step be higher than 10.3 mg/ml, to ensure that the final reaction volume in the next step does not exceed 200 μL. A low antibody concentration after the buffer exchange step (e.g. 2 mg/ml) would result in a larger reaction volume and a lower final enzyme and GDP-Fuc-Payload concentration, possibly resulting in a lower DAR value. In this case, a longer incubation time may be performed to achieve a high DAR. If you choose to proceed with low antibody concentration, please see Note 5 in part B for instructions and details.

B. Antibody Fc domain N-glycan Modification & Payload conjugation.

1. Add appropriate concentrated antibody sample（1 mg）to a sterilized 1.5 mL tube.
2. Add 10 μL 20×Reaction buffer to the antibody solution.
3. Add 20 μL UDP-Gal & 20 μL GDP-Fuc-vc-PAB-MMAE to the antibody solution.
4. Add 5 μL Endoglycosidase、20 μL GT & 20 μL FT to the antibody solution.
5. Add 4 μL $MnCl_2$ & 4 μL $MgCl_2$ to the antibody solution.
6. Add appropriate ddH_2O to the antibody solution to a whole volume of 200 μL.
7. Mix the Reaction solution by carefully pipetting up and down.
8. Incubate the Reaction solution at incubator or water bath protected from light for 24 h, at 30 °C.

Note 5: If the antibody concentration after the buffer exchange step is less than 10.3 mg/mL, the final reaction volume would exceed 200 μL and the final enzyme concentration would be lower than expected, which in some cases may result in a lower DAR. Proceeding with a lower concentration may require a longer incubation time to ensure a high DAR.

Taking trastuzumab as an example:

#1 If the antibody concentration after the buffer exchange step is 5 mg/mL, the final reaction volume is 303 μL and the final antibody concentration is 3.3 mg/mL. In this case, the final DAR value after 24 hours of reaction is 1.93 and after 40 hours of reaction is 2.0 (Measured by LC-MS).

#2 If the antibody concentration after the buffer exchange step is 2 mg/mL, the final reaction volume is 603 μL and the final antibody concentration is 1.7 mg/mL. In this case, the final DAR value after 24 hours of reaction is 1.79 and after 40 hours of reaction is 1.94 (Measured by LC-MS).

C. Removal of Enzyme and excess toxin reagent

Note 6: This step is the same for DAR 2 and DAR 4 conjugation procedures.

•Equilibration

1. Place one Pierce™ spin column containing 100 μL protein A resin in 2 mL centrifuge tube.
2. Remove the cap in the bottom of the column，Centrifuge 1 minute at 1000×g to remove the storage solution.
3. Discard the liquid in the collection tube.
4. Add 500 μL of Binding Buffer to the column, Centrifuge 1 minute at 1000×g.
5. Discard the liquid in the collection tube.
6. Repeat steps 4 and 5 twice for a total of three washes.

•Binding of the antibody conjugate

7. Dilute the Reaction solution above (200 μL) with 400 μL Binding Buffer.

Note 7: If the reaction volume is greater than 200 μL, add suitable binding buffer to the solution to make a total volume of 600 μL. If the reaction volume is greater than 600 μL, do not add binding buffer.

8. Add the diluted sample to the resin in the spin column. Cap top and bottom of the spin column.
9. Incubate the spin column assembly on a Rotary Mixer, and mix slightly at room temperature for 60 minutes.

•Wash

10. Remove the bottom caps and place the spin columns in 2 mL collection tubes. Loosen the top lids.
11. Centrifuge 1 minute at 1000×g; do not discard the collected solution.
12. Place the spin columns in a new 2 mL collection tubes.
13. Add 500 μL of Binding Buffer to the spin columns, centrifuge 1 minute at 1000×g.
14. Discard the liquid in the collection tube.
15. Repeat steps 13 and 14 twice for a total of three washes.

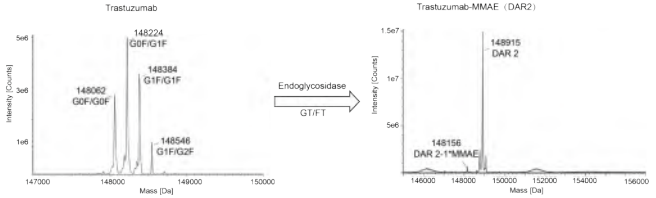
•Elution of purified, conjugated antibody

16. Cap the bottom and place the spin columns in a new 2 mL sterilized collection tubes
17. Add 300 μL Elution buffer to the resin in the spin column. Cap top of the spin column.
18. Incubate the spin column assembly on a Rotary Mixer, and mix slightly at room temperature for 10 minutes.
19. Remove the bottom caps and loosen the top lids.
20. Centrifuge the spin column assembly 1 min at 1000×g to elute the conjugated antibody.

21. Add 60 µL Neutralization buffer immediately to the elution to adjust the pH to neutral.
22. Repeat elution (steps16-21) one more time.
23. Pool the eluted fractions.
24. Concentration and buffer exchange of the conjugated antibody to 1xPBS(Prepare 10 mL of 1xPSB(pH7.2-7.5) by adding 1 mL of 10xPBS to 9 mL of ddH2O in a 15 mL tube. Vortex briefly to mix.) according to PartA.
25. Store the conjugated antibody at -80°C for long preservation.

D. Measure the DAR value of the conjugated antibody (Herceptin as example)

•Measure the DAR value of the conjugated antibody (LC-MS based)



1.Calculation the MS of different peaks

The MS of DAR2 peak= The MS of Trastuzumab-G0F/G0F(148062)- 1113.99 ( )*2+180.16(Galactose)*2+[1808.8(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*2

The MS of DAR1 peak= The MS of Trastuzumab-G0F/G0F(148062)- 1113.99 ( )*2+180.16(Galactose)*2+[1808.8(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*1

2. Measure the DAR value of the conjugated antibody

DAR value = [Intensity (DAR0 peak)*0+ Intensity (DAR1 peak)*1+ Intensity (DAR2 peak)*2]/[Intensity (DAR0 peak)+ Intensity (DAR1 peak)+ Intensity (DAR2 peak)]

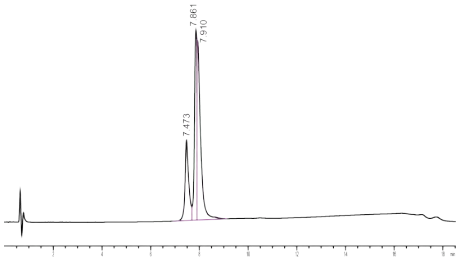
•Measure the DAR value of the conjugated antibody (HIC-HPLC based)

Column：TSKgel Butyl-NPR column (4.6 mm × 35 mm, 2.5 µm; TOSOH)
Mobile phase A：20 mM sodium phosphate, 1.5 M ammonium sulfate (pH 6.9)
Mobile phase B：75% (v/v) 20 mM sodium phosphate, 25% (v/v) isopropanol (pH 6.9)
Flow rate: 0.4 mL/min
Column temperature: 30 °C
Detection wavelength：280nm
Loading Amount:10 µg (5-10 µL)

Gradient：

Time	A (%)	B (%)
0.00	100	0
1.00	100	0
13.00	0	100
13.50	0	100
14.50	100	0
18.50	100	0

DAR value calculation：



DAR value = {[DAR0 (7.473) peak area percent]*0+[DAR1 (7.861) peak area percent]*1+[DAR2 (7.910) peak area percent]*2}/100

Trouble shooting：

Problem	Possible Cause	Solution
DAR value ＜1.6	The reaction dos not reach the end point.	Extend the reaction time.
DAR value ＞2.0	The antibody has more than one N-linked glycosylation sites.	---
Precipitation occurred immediately when $MnCl_2$ is added to the reaction system.	The buffer exchange is incomplete. Some phosphate remains in the buffer.	Repeat the Buffer exchange steps
A small amount of precipitate occurred after long incubation	Small amount of protein precipitates	DAR value generally not affected

[PRINCIPLE OF THE Kit]

For DAR4 Conjugation

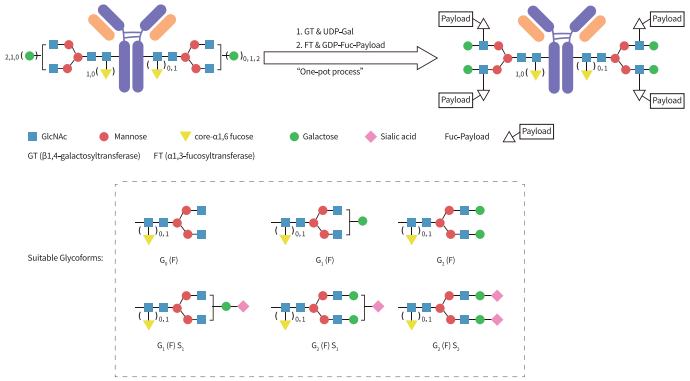
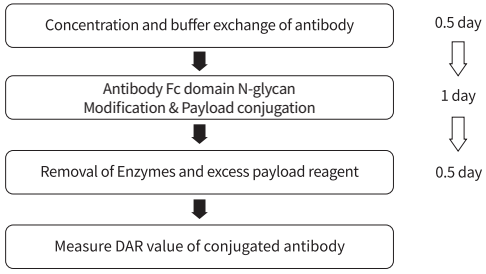


Figure 2 The principle of DAR4 ADC preparation with AGLink®

To construct the DAR4 conjugates, galactose is first added to the terminal GlcNAc catalyzed by β-1,4-galactosyltransferase (GT) to form LacNAc. Then, α-1,3-fucosyltransferase (FT) recognizes LacNAc and transfers the Fuc-Payload to the GlcNAc of the LacNAc to form a conjugate with a theoretical DAR of 4 (Figure 2).

Overview of the protocol for constructing DAR4 ADC：



Equipment required：

- Centrifuge for 1.5-2 mL&15-50 mLtubes (With refrigeration function)
- Incubator or water bath for 30 °C
- Rotary mixer
- UV/VIS Spectrophotometer (Thermo NanoDrop one)

Protocol for conjugation of 1 mg of antibody：

A. Concentration and buffer exchange of the antibody

Note 1: This step is the same for DAR 2 and DAR 4 conjugation procedures.
•Concentration step（Optional）
Note 2: This step is recommended to ensure that the antibody concentration after the buffer exchange step is higher than 9.8 mg/ml, to avoid the final reaction volume exceeding 200 µL. If you choose to proceed with a lower concentration, see Note 5 in Part B for instructions and details.
1. Add 500 µL of ddH_2O to the concentrator and cap the device.
2. Centrifuge at 10000×g for 5 min.
3. Discard the solution in the concentrator and the flow-through.
4. Add the antibody solution to the concentrator.
5. Centrifuge at 10000×g for 2-6 min.
Note 3: If the antibody volume or concentration in the concentrator is not suitable after first concentration, centrifuge for an additional 2-6 min at 10000×g, until the appropriate volume or concentration is achieved.
6. Collect the solution in the concentrator.
•Buffer exchange with Desalting Spin column, 2 mL
1. Prepare 10 mL of 25 mM Tris-HCl buffer (pH7.5) by adding 250 µL of 1 M Tris-HCl buffer (pH7.5) to 9.75 mL of ddH_2O in a 15 mL tube. Vortex briefly to mix.
2. Break off the bottom closure of the Desalting Spin column. Loosen the lid (do not remove the lid).
3. Place the column in a collection tube (15 mL) and centrifuge at 1000×g for 2 min to remove the storage solution.
4. Discard the flow-through and place the column in the collection tube.
5. Add 1 mL of 25 mM Tris-HCl buffer (pH7.5) on top of the resin. Centrifuge the column at 1000×g for 2 min and discard the flow-through.
6. Repeat 25 mM Tris-HCl buffer (pH7.5) wash in step 5 two more times. Last spin for 3 min to remove excess liquid. Place the column in a new collection tube (15 mL).
7. Apply the antibody solution on top of the resin (100-400 µL).
8. Centrifuge at 1000×g for 3 min and collect the flow-through containing the antibody in 25 mM Tris-HCl buffer (pH7.5).
Note 4: It is recommended that the antibody concentration after the buffer exchange step be higher than 9.8 mg/ml, to ensure that the final reaction volume in the next step does not exceed 200 µL. A low antibody concentration after the buffer exchange step (e.g. 2 mg/ml) would result in a larger reaction volume and a lower final enzyme and GDP-Fuc-Payload concentration, possibly resulting in a lower DAR value. In this case, a longer incubation time may be performed to achieve a high DAR. If you choose to proceed with low antibody concentration, please see Note 5 in part B for instructions and details.

B. Antibody Fc domain N-glycan Modification & Payload conjugation.

1. Add appropriate concentrated antibody sample (1 mg) to a sterilized 1.5 mL tube.
2. Add 10 µL 20×Reaction buffer to the antibody solution.
3. Add 20 µL UDP-Gal & 20 µL GDP-Fuc-vc-PAB-MMAE to the antibody solution.
4. Add 20 µL GT & 20 µL FT to the antibody solution.
5. Add 4 µL $MnCl_2$ & 4 µL $MgCl_2$ to the antibody solution.
6. Add appropriate ddH_2O to the antibody solution to a whole volume of 200 µL.
7. Mix the Reaction solution by carefully pipetting up and down.
8. Incubate the Reaction solution at incubator or water bath protected from light for 24 h, at 30 °C.
Note 5: If the antibody concentration after the buffer exchange step is less than 9.8 mg/mL, the final reaction volume would exceed 200 µL and the final enzyme concentration would be lower than expected, which in some cases may result in a lower DAR. Proceeding with a lower concentration may require a longer incubation time to ensure a high DAR.

Taking trastuzumab as an example:
#1 If the antibody concentration after the buffer exchange step is 5 mg/mL, the final reaction volume is 298 µL and the final antibody concentration is 3.4 mg/mL. In this case, the final DAR value after 24 hours of reaction is 4.0 (Measured by LC-MS).
#2 If the antibody concentration after the buffer exchange step is 2 mg/mL, the final reaction volume is 598 µL and the final antibody concentration is 1.7 mg/mL. In this case, the final DAR value after 24 hours of reaction is 4.0 (Measured by LC-MS).

C. Removal of Enzyme and excess toxin reagent

Note 6: This step is the same for DAR 2 and DAR 4 conjugation procedures.
•Equilibration
1. Place one Pierce™ spin column containing 100 µL protein A resin in 2 mL centrifuge tube.
2. Remove the cap in the bottom of the column，Centrifuge 1 minute at 1000×g to rem the storage solution.

3. Discard the liquid in the collection tube.
4. Add 500 µL of Binding Buffer to the column, Centrifuge 1 minute at 1000×g.
5. Discard the liquid in the collection tube.
6. Repeat steps 4 and 5 twice for a total of three washes.

•Binding of the antibody conjugate

7. Dilute the Reaction solution above (200 µL) with 400 µL Binding Buffer.

Note 7: If the reaction volume is greater than 200 µL, add suitable binding buffer to the solution to make a total volume of 600 µL. If the reaction volume is greater than 600 µL, do not add binding buffer.

8. Add the diluted sample to the resin in the spin column. Cap top and bottom of the spin column.
9. Incubate the spin column assembly on a Rotary Mixer, and mix slightly at room temperature for 60 minutes.

•Wash

10. Remove the bottom caps and place the spin columns in 2 mL collection tubes. Loosen the top lids.
11. Centrifuge 1 minute at 1000×g; do not discard the collected solution.
12. Place the spin columns in a new 2 mL collection tubes.
13. Add 500 µL of Binding Buffer to the spin columns, centrifuge 1 minute at 1000×g.
14. Discard the liquid in the collection tube.
15. Repeat steps 13 and 14 twice for a total of three washes.

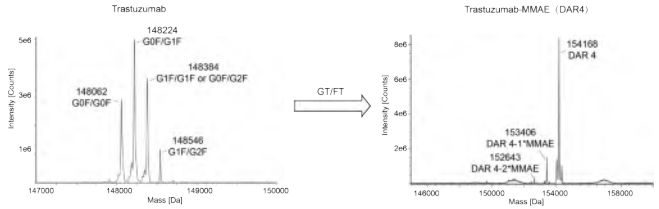
•Elution of purified, conjugated antibody

16. Cap the bottom and place the spin columns in a new 2 mL sterilized collection tubes
17. Add 300 µL Elution buffer to the resin in the spin column. Cap top of the spin column.
18. Incubate the spin column assembly on a Rotary Mixer, and mix slightly at room temperature for 10 minutes.
19. Remove the bottom caps and loosen the top lids.
20. Centrifuge the spin column assembly 1 min at 1000×g to elute the conjugated antibody.
21. Add 60 µL Neutralization buffer immediately to the elution to adjust the pH to neutral.
22. Repeat elution (steps16-21) one more time.
23. Pool the eluted fractions.
24. Concentration and buffer exchange of the conjugated antibody to 1xPBS(Prepare 10 mL of 1xPSB(pH7.2-7.5) by adding 1 mL of 10xPBS to 9 mL of ddH2O in a 15 mL tube. Vortex briefly to mix.) according to PartA.
25. Store the conjugated antibody at -80°C for long preservation.

D. Measure the DAR value of the conjugated antibody (Herceptin as example)

•Measure the DAR value of the conjugated antibody (LC-MS based)

1.Calculation the MS of different peaks



The MS of DAR4 peak= The MS of Trastuzumab-G0F/G0F(148062)+ [180.16(Galactose)-18.02(H2O)]*4+[1808.8 (GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*4

The MS of DAR3 peak= The MS of Trastuzumab-G0F/G0F(148062)+ [180.16(Galactose)-18.02(H2O)]*4+[1808.8 (GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*3

The MS of DAR2 peak= The MS of Trastuzumab-G0F/G0F(148062)+ [180.16(Galactose)-18.02(H2O)]*4+[1808.8 (GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*2

The MS of DAR1 peak= The MS of Trastuzumab-G0F/G0F(148062)+ [180.16(Galactose)-18.02(H2O)]*4+[1808.8 (GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*1

2. Measure the DAR value of the conjugated antibody

DAR value=[Intensity (DAR0 peak)*0+ Intensity (DAR1 peak)*1+ Intensity (DAR2 peak)*2+ Intensity (DAR3 peak)*3+ Intensity (DAR4 peak)*4] /[Intensity (DAR0 peak)+ Intensity (DAR1 peak)+ Intensity (DAR2 peak)+ Intensity (DAR3 peak)+ Intensity (DAR4 peak)]

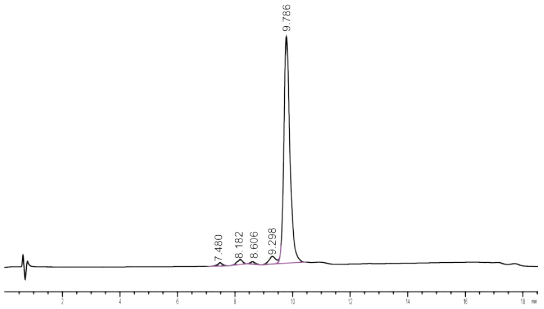
•Measure the DAR value of the conjugated antibody (HIC-HPLC based)

Column: TSKgel Butyl-NPR column (4.6 mm × 35 mm, 2.5 µm; TOSOH)
Mobile phase A: 20 mM sodium phosphate, 1.5 M ammonium sulfate (pH 6.9)
Mobile phase B: 75% (v/v) 20 mM sodium phosphate, 25% (v/v) isopropanol (pH 6.9)
Flow rate: 0.4 mL/min
Column temperature: 30 °C
Detection wavelength: 280nm
Loading Amount: 10 µg (5-10 µL)

Gradient:

Time	A (%)	B (%)
0.00	100	0
1.00	100	0
13.00	0	100
13.50	0	100
14.50	100	0
18.50	100	0

DAR value calculation:



DAR value = {[DAR0 (7.480) peak area percent]*0+[DAR1 (8.182) peak area percent]*1+[DAR2 (8.606) peak area percent]*2+[DAR3 (9.298) peak area percent]*3+[DAR4 (9.786) peak area percent]*4}/100

Trouble shooting:

Problem	Possible Cause	Solution
DAR value ＜3.6	The reaction dos not reach the end point	Extend the reaction time.
	The antibody has some glycan types, which can not be modified, such as high mannose type	---
DAR value ＞4.0	The antibody has more than one N-linked glycosylation sites.	---
Precipitation occurred immediately when MnCl2 is added to the reaction system.	The buffer exchange is incomplete. Some phosphate remains in the buffer.	Repeat the Buffer exchange steps.
A small amount of precipitate occurred after a long incubation	Small amount of protein precipitates	DAR value generally not affected

Reference:

[1] Van Geel R, Wijdeven M A, Heesbeen R, Heesbeen R, *et al*. Chemoenzymatic conjugation of toxic payloads to the globally conserved N-glycan of native mAbs provids homogeneous and highly efficacious antibody-drug conjugates *J. Bioconjugate Chemistry*, 2015 , 26(11):2233-2242.
[2] Yang Y, Zhentao S, Tian T, *et al*. Reducing the Complexity of Fc Glycan Enables the Construction of Antibody onjugates with Unexpected High Efficiency and Payload Capacity via Gycoengineering; *bioRxiv* preprint doi: https://doi.org/10.1101/2022.09.04.506510.

Statement of Rights:

Note of application: