AGLink® ADCConjugation Kit(MMAE,DAR2&DAR4, 2mg)

Size: This kit is designed to label 2mg antibody Catalog Number：ADC-P002

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

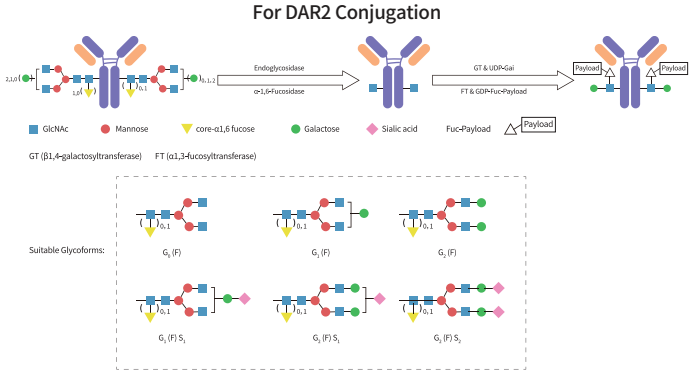
BoxA

Name	Details	Quantity	Storage temperature
Enzyme A	GT	1×150 μL	-20°C
Enzyme B	FT	1×60 μL	-20°C
Enzyme C	Endoglycosidase	1×15 μL	-20°C
Enzyme D	α-1,6-Fucosidase	1×45 μL	-20°C
Cofactor A	MnCl2 (0.1 M)	1×50 μL	-20°C
Cofactor B	MgCl2 (0.5 M)	1×50 μL	-20°C
20×Reaction buffer	20×Reaction buffer	1×50 μL	-20°C
Substrate A	UDP-Gal (0.1 M)	1×30 μL	-20°C
Substrate B	GDP-Fuc-vc-PAB-MMAE (50 mM)	1×24 μL	-20°C

BoxB

Name	Details	Quantity	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	500ul	4°C
Tris-HCl	250mM Tris-HCl buffer (pH7.5)	1.5mL	4°C
10xPBS	10xPBS	6ml	4°C
Desalting Spin column	Desalting Spin column,2 mL	2 pieces	4°C
Ultrafiltration concentrator	Ultrafiltration concentrator (include 2 collection tubes), 0.5 mL	2 pieces	4 °C to 25 °C
Pierce™ spin column	Pierce™ spin column (containing 100 μL protein A resin)	1 piece	4°C

[PRINCIPLE OF THE Kit]

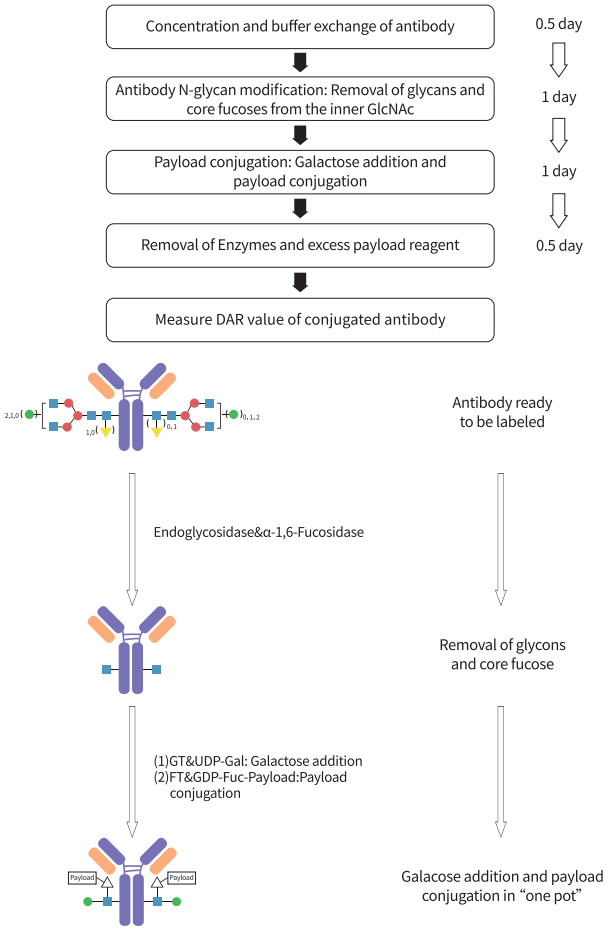


The principle of DAR2 ADC preparation with AGLink®

Almost all monoclonal antibodies are glycosylated at Asn-297 of Fc domain. While the glycans have different isoforms, the types which typically dominate are G0F, G1F&G2F (>90%)[1]. AGLink® ADC kit, which is based on the YTConju™ Platform of Glyco-therapy Biotechnology,employs the N-linked glycosylation site for site-specific toxin conjugation.The principle of conjugation is showed in Figure 1. Firstly, the glycans and core fucoses are removed from the inner GlcNAc catalyzed by Endoglycosidase and α-1,6-Fucosidase. Secondly,galactose is added to the GlcNAc catalyzed by β-1,4-galactosyltransferase (GT) to form LacNAc. Then, the α-1,3-fucosyltransferase (FT) recognizes LacNAc, and the toxin is transferred to the GlcNAc of LacNAc. So, the theoretical DAR value of the final conjugates is 2.

The conjugation procedure, which is enzyme catalyzed, is performed as one-pot by combining glycan modification and toxin conjugation simultaneously. The conjugation procedure , which is easy to operate, is performed under physiological conditions, and organic solvents are not introduced. The conjugates prepared through this method usually have high homogeneity and the binding activity of the conjugates to related antigens is not changed.

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)

Protocol for conjugation of 2 mg of antibody：

A. Concentration and buffer exchange of the antibody

• Concentration step (Optional)

Note：Only needed when the start concentration of the antibody is lower than 13 mg/mL.

1. Add 500 μL of ddH2O 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: If the antibody volume in the concentrator is not suitable after first concentration, centrifuge for an additional 2-6 min at 10000×g, until the appropriate volume is achieved.

6. Collect the solution in the concentrator.

•Buffer exchange with Desalting Spin column, 2 mL

1. Prepare 10 mL of 25mM Tris-HCl buffer (pH7.5) by adding 1 mL of Tris-HCl to 9 mL of ddH2O 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 (200-700 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).

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

1. Add appropriate concentrated antibody sample (2 mg) to a sterilized 1.5 mL tube.

Note：The concentration of start antibody should be more than 13 mg/mL. If not, add by the maximum volume (188 μL)。

2. Add 10 μL 20×Reaction buffer to the antibody solution.
3. Add 5 μL Endoglycosidase & 30 μL α-1,6-Fucosidase to the antibody solution.
4. Add appropriate ddH2O to the antibody solution to a whole volume of 200 μL.
5. Mix the Reaction solution by carefully pipetting up and down.
6. Incubate the Reaction solution at incubator or water bath protected from light for 24 h, at 30 °C.

•Toxin conjugation

7. Add 10 μL 20×Reaction buffer to the above Reaction solution.
8. Add 20 μL UDP-Gal & 16 μL GDP-Fuc-vc-PAB-MMAE to the above Reaction solution.
9. Add 100 μL GT & 40 μL FT to the above Reaction solution.
10. Add 8 μL MnCl2 & 8 μL MgCl2 to the above Reaction solution solution.
11. Mix the Reaction solution by carefully pipetting up and down.
12. Incubate the Reaction solution at incubator or water bath protected from light for another 24 h, at 30 °C.

C. Removal of Enzyme and excess toxin reagent

•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 (400 μL) with 200 μL 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

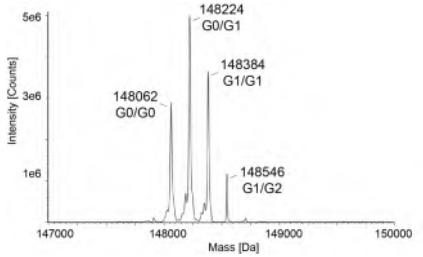
10. Remove the bottom caps and place the spin columns in 2 mL collection tubes. Loosen the top lids.

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 StepA.
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 ADC



The MS of DAR2 ADC= The MS of G1/G2(Herceptin)+ [(180.16)(Galactose)-18.02(H2O)]- 1422.5 (Glycan removed by Endoglycosidase)*2-[(164.16)(Fucose)-18.02(H2O)]*2+[(180.16)(Galactose)-18.02(H2O)]*2+[(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*2
The MS of DAR1 ADC= The MS of G1/G2(Herceptin) + [(180.16)(Galactose)-18.02(H2O)]- 1422.5 (Glycan removed by Endoglycosidase)*2-[(164.16)(Fucose)-18.02(H2O)]*2+[(180.16)(Galactose)-18.02(H2O)]*2+[(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*1
MS of DAR0 ADC= The MS of G1/G2(Herceptin) + [(180.16)(Galactose)-18.02(H2O)]- 1422.5(Glycan removed by Endoglycosidase)*2-[(164.16)(Fucose)-18.02(H2O)]*2+[(180.16)(Galactose)-18.02(H2O)]*2+[(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*0

2. Measure the DAR value of the conjugated antibody

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

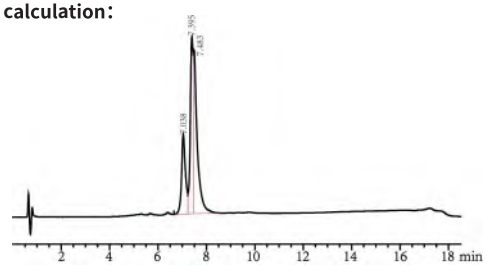
•Measure the DAR value of the conjugated antibody (HIC-HPLC based)

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:



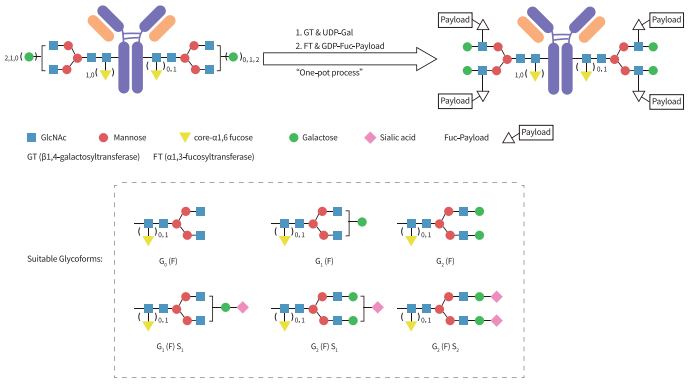
DAR value = {[DAR0 (7.034) peak area percent]*0+[DAR1 (7.395) peak area percent]*1+[DAR2 (7.483) peak area percent]*2}/100

Trouble shooting:

Problem	Possible Cause	Solution
DAR value ＜1.6	Low removal efficiency of core fucose.	Reduce the concentration of antibody, or increase the concentration of α-1,6-Fucosidase in the reaction system, or extend the reaction time.
DAR value ＞2.0	The antibody has more than one N-linked glycosylation sites.	---
Precipitation occurred immediately when MnCl2 is added to the reaction system.	The buffer exchange of the antibody is incomplete.	Buffer exchange of the antibody again.
Precipitation occurred during the reaction.	A small amount of the enzyme inactivates during the reaction.	It has no effect on the final results.

[PRINCIPLE OF THE Kit]

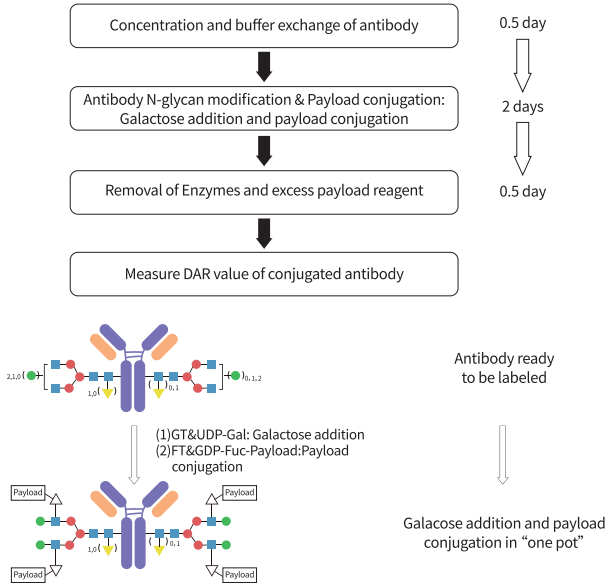
For DAR4 Conjugation



The principle of DAR4 ADC preparation with AGLink®

Almost all monoclonal antibodies are glycosylated at Asn-297 of Fc domain. While the glycans have different isoforms, the types which typically dominate are G0F, G1F&G2F (>90%)[1]. AGLink® ADC kit, which is based on the YTConju™ Platform of Glyco-therapy Biotechnology, employs the N-linked glycosylation site for site-specific toxin conjugation.
The principle of conjugation is showed in Figure 1. Firstly, galactose is added to the terminal GlcNAc catalyzed by β-1,4-galactosyltransferase (GT) to form LacNAc. Then, the α-1,3-fucosyltransferase (FT) recognize LacNAc, and the toxin is transferred to the GlcNAc of LacNAc. So, the theoretical DAR value of the final conjugates is 4.
The conjugation procedure, which is enzyme catalyzed, is performed as one-pot by combining glycan modification and toxin conjugation simultaneously. The conjugation procedure , which is easy to operate，is performed under physiological conditions, and organic solvents are not introduced. The conjugates prepared through this method usually have high homogeneity and the binding activity of the conjugates to related antigens is not changed.

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 2 mg of antibody:

A. Concentration and buffer exchange of the antibody

• Concentration step (Optional)
Note：Only needed when the start concentration of the antibody is lower than 11 mg/mL.
1. Add 500 µL of ddH2O 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: If the antibody volume in the concentrator is not suitable after first concentration, centrifuge for an additional 2-6 min at 10000×g, until the appropriate volume is achieved.

6. Collect the solution in the concentrator.

• Buffer exchange with Desalting Spin column, 2 mL

1. Prepare 10 mL of 25mM Tris-HCl buffer (pH7.5) by adding 1 mL of Tris-HCl to 9 mL of ddH2O 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 (200-700 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).

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

1. Add appropriate concentrated antibody sample (2 mg) to a sterilized 1.5 mL tube.
Note：The concentration of start antibody should be more than 11 mg/mL. If not, add by the maximum volume (188 µL)。
2. Add 20 µL 20×Reaction buffer to the antibody solution.
3. Add 20 µL UDP-Gal & 16 µL GDP-Fuc-vc-PAB-MMAE to the antibody solution.
4. Add 100 µL GT & 40 µL FT to the antibody solution.
5. Add 8 µL MnCl2 & 8 µL MgCl2 to the antibody solution.
6. Add appropriate ddH2O to the antibody solution to a whole volume of 400 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 48 h, at 30 ℃.

C. Removal of Enzyme and excess toxin reagent

• 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 (400 µL) with 200 µL 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.

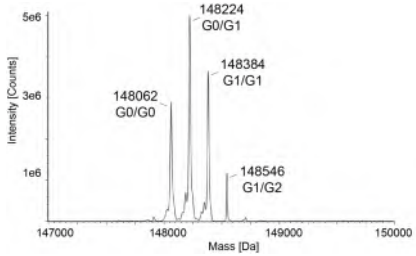
• Elution of purified, conjugated antibody

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 StepA.
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 ADC



MS of DAR4 ADC= The MS of G1/G2(Herceptin)+[(180.16)(Galactose)-18.02(H2O)]+ [(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*4
MS of DAR3 ADC= The MS of G1/G2(Herceptin)+[(180.16)(Galactose)-18.02(H2O)]+ [(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*3
MS of DAR2 ADC= The MS of G1/G2(Herceptin)+[(180.16)(Galactose)-18.02(H2O)]+ [(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*2
MS of DAR1 ADC= The MS of G1/G2(Herceptin)+[(180.16)(Galactose)-18.02(H2O)]+ [(1808.8)(GDP-Fuco-vc-PAB-MMAE)-443.20(GDP)]*1
MS of DAR0 ADC= The MS of G1/G2(Herceptin)+ [(180.16)(Galactose)-18.02(H2O)]+[(1808.8)(GDP-Fuco vc-PAB-MMAE)-443.20(GDP)]*0

2. Measure the DAR value of the conjugated antibody

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

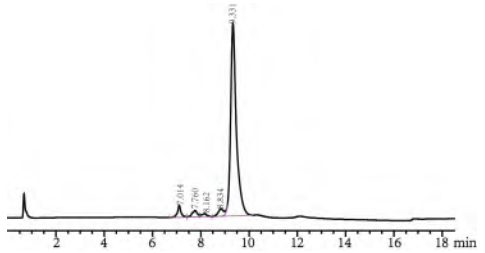
•Measure the DAR value of the conjugated antibody (HIC-HPLC based)

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 (7.014) peak area percent]*0+[DAR1 (7.760) peak area percent]*1+[DAR2 (8.162) peak area percent]*2+[DAR3 (8.834) peak area percent]*3+[DAR4 (9.331) peak area percent]*4}/100

Trouble shooting：

Problem	Possible Cause	Solution
DAR value ＜3.2	The reaction dos not reach the end point	Reduce the concentration of antibody, or increase the concentration of enzyme in the reaction system, or 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 of the antibody is incomplete.	Buffer exchange of the antibody again.
Precipitation occurred during the reaction.	A small amount of the enzyme inactivates during the reaction.	It has no effect on the final results.

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: