Chapter 10

Cysteine Re-bridging Using Divinyl Pyrimidine Reagents for the Generation of Antibody-Drug Conjugates

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Abstract

Antibody-drug conjugates (ADCs) are a powerful form of targeted therapy that can deliver drugs with a high level of selectivity towards a specific cell type, reducing off-target effects and increasing the therapeutic window compared to small-molecule therapeutics. However, creating ADCs that are stable, homogeneous, and with a controlled drug-to-antibody ratio (DAR) remains a significant challenge. Divinyl pyrimidine (DVP) reagents were developed as a method to irreversibly conjugate linker-payloads onto native antibodies to generate stable ADCs and antibody-probe systems with a precise drug-to-antibody ratio (DAR) of 4. This protocol describes how to conjugate DVPs to antibodies and methods for ADC characterization.

Keywords Antibody-drug conjugates, Antibody characterization, Cysteine conjugation, Disulfide re-bridging, Drug-to-antibody ratio

1 Introduction

Antibody-drug conjugates (ADCs) have emerged as powerful therapeutics over the last three decades, with interest in these biologics continuing to grow [1, 2]. ADCs are typically composed of three components: a targeting antibody, a drug, and a linker system [2]. In the conjugate, the targeting nature of the antibody is utilized to bring the therapeutic to the site in the body where it is required. Once there, the drug then acts upon the desired target. Many ADC programs have been highly successful, with, as of 2024, 13 FDA-approved therapies and more than 350 currently in clinical trials [3, 4]. However, current ADC synthetic methods lead to reversible conjugation and highly heterogenous mixtures with widely varying drug-to-antibody ratios (DARs). This heterogeneity is widely recognized to cause poor pharmacokinetic and pharmacodynamic properties [1, 5]. Furthermore, the number of payload molecules appended to each antibody is crucial for its activity, binding, toxicity,

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and propensity for aggregation [6, 7]; therefore, methods to precisely attain a specific DAR are of high importance.

If a conjugating agent reacts with both cysteines of an interchain disulfide bridge at once, then the antibody is said to have been chemically "re-bridged" with the conjugator covalently holding together the light and heavy antibody chains. Disulfide re-bridging has shown to be an efficient bioconjugation strategy allowing for four conjugations to take place in a site-specific way without the need for genetic or enzymic engineering of the antibody [8–10].

To add to this toolbox, the Spring group developed divinyl pyrimidine (DVP) linkers that have been shown to exhibit efficient, site-selective, and irreversible disulfide re-bridging, allowing access to DAR 4 ADCs and antibody-probe constructs from native antibodies (Fig. 1) [11–13]. DVP scaffolds have also been used in generating DAR 1 ADCs [14–16] and to staple peptides with the aim of improving stability [17, 18]. Herein is described a guide to their utilization in generating DAR 4 ADCs and an explanation of various analytical techniques used for ADC characterization. Each characterization technique gives important information about the ADC: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the extent of conjugation; mass spectroscopy (MS), the presence of the desired DAR 4 conjugate; hydrophobic interaction chromatography (HIC), the ratio between different DAR species; and size exclusion chromatography (SEC), the presence of aggregation.

Carboxylic acid functionalized DVPs (Fig. 2) are recommended for general ADC generation as they exhibit high stability, efficient bioconjugation, and facile functionalization with a desired payload. The synthesis of these DVPs and other variants has been described previously [11–13, 16], and are therefore not covered in this chapter.

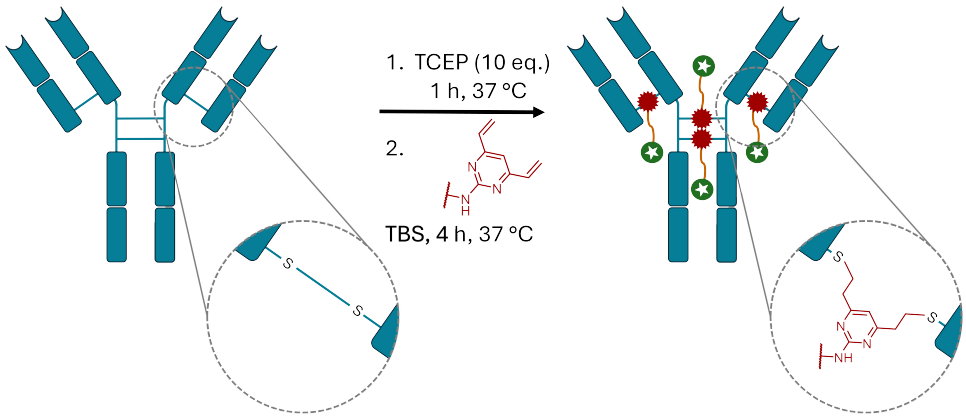

Fig. 1 Antibody schematic showing the relative locations of the interchain disulfide bridges and how DVP linkers conjugate to the antibody

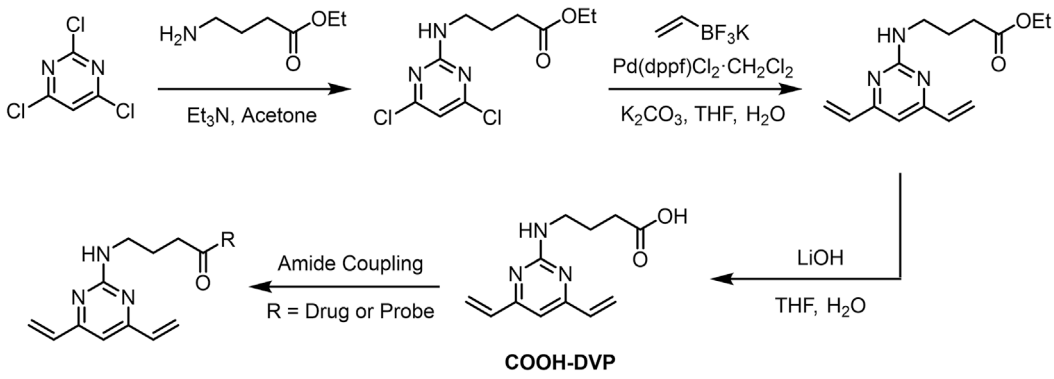

Fig. 2 Synthesis of a carboxylic acid DVP (COOH-DVP) and its functionalization [11, 12]

2 Materials

All buffers are made using ultrapure water (i.e., Milli-Q) unless otherwise stated. A selection of adjustable pipettes are required for transferring liquids between 0.4–1000 μL.

2.1 Antibody Preparation and Conjugation

1. IgG1 antibody (i.e., Trastuzumab; lyophilized solid) (*see* **Note 1**).
2. 500 μL Centrifuge tubes (*see* **Note 2**).
3. Tris-buffered saline (TBS) solution: 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 8.0 with 1 M aq. NaOH.
4. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).
5. Thermal shaker unit (such as a Grant-bio PCMT Thermo-Shaker) capable of agitating a sample whilst maintaining 37°C.
6. UV–Vis spectrometer (such as a ThermoFisher NanoDrop One) for antibody concentration determination.
7. DVP linker; 5 mM in DMSO (*see* **Note 3**) [12].
8. 0.5 mL 40k Molecular weight cut-off (MWCO) Zeba™Spin Desalting columns.
9. 0.5 mL 10k MWCO Amicon® Ultra 10k MWCO Diafiltration columns.
10. Centrifuge capable of holding 0.5–2 mL centrifuge tubes and running at up to 14,000 × *g* (such as VWR Micro Star 17).
11. Phosphate Buffer Saline (PBS) solution: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 (*see* **Note 4**).

2.2 SDS-PAGE Analysis

1. Heating block/sand bath capable of heating 0.5 mL vials to 90°C.
2. 0.5 mL vials.
3. PBS solution (as above).
4. Broad range unstained protein standard, molecular weight (MW) ladder (10–200 kDa).
5. Non-reducing sample stain: 0.125 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-HCl), 20% glycerol, 4% SDS, 0.02% bromophenol blue, adjusted to pH 6.8.
6. Reducing sample stain: 0.125 M Tris-HCl, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 1.25 mM β-mercaptoethanol, adjusted to pH 6.8.
7. Laemmli running buffer (LRB): For 1 L; 3 g 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), 14.4 g glycine, 1 g SDS.
8. 12% Acrylamide gel including 4% stacking gel (*see* **Note 5**).
9. Gel electrophoresis tank and power supply (such as Bio-Rad mini-PROTEAN™ Tetra System).
10. Coomassie brilliant blue (CBB) stain: For 500 mL, dissolve in order: 0.5 g CBB R250 dye, 50 mL EtOH, 400 mL water, then 50 mL AcOH.

2.3 Mass Spectrometry Analysis

1. PNGase F for deglycosylation.
2. Protein LC-MS system (such as Waters Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 × 50 mm)) with MS deconvolution software (such as Waters MaxEnt1 algorithm).
3. Mobile phase: A: 0.1% aq. formic acid. B: 95% MeCN with 5% aq. formic acid (0.1%).

2.4 HIC Analysis

1. HPLC (such as Agilent 1260 Infinity system) with analytical HIC column (such as Tosoh TSKgel Butyl-NPR column (3.5 cm × 4.6 mm, 2.5 μm)).
2. Mobile phase: A: 1.5 M ammonium sulfate, 25 mM Na_3PO_4, pH 7. B: 25% (v/v) isopropyl alcohol in 25 mM Na_3PO_4, pH 7.

2.5 SEC Analysis

1. HPLC (such as Agilent 1260 Infinity system) with analytical SEC column (such as Tosoh TSKgel G3000SWXL column (30 cm × 7.8 mm, 5 μm)).
2. Mobile phase: 50 mM Na_3PO_4, 100 mM NaCl, 0.2% (w/v) sodium azide, pH 7.

3 Methods

All procedures are carried out at ambient temperature and in air unless otherwise stated.

3.1 Antibody Preparation and Conjugation

1. Make up an antibody solution in TBS (100 μL, c. 10 μM) (*see* **Note 6**). Record the UV-vis absorbance at 280 nm (using TBS for the background reading) and calculate the concentration using the extinction coefficient of the antibody (*see* **Note 7**) and the Beer–Lambert law. For trastuzumab ε = 215,380 (*see* **Note 8**).
2. Make up a 5 mM solution of TCEP in TBS (*see* **Note 9**).
3. Add 10 eq. of TCEP (5 mM) to the antibody solution (*see* **Note 10**) and incubate on a thermal shaker with agitation (1 h, 37°C, 400 rpm).
4. Add 10 eq. of DVP (5 mM) to the antibody solution (*see* **Note 10**) and incubate on a thermal shakerwith agitation (4 h, 37°C, 400 rpm).
5. Equilibrate a 0.5 mL 40k MWCO desalting column into PBS (*see* **Note 11**) according to the manufacturer's specification.
6. Pipette the antibody solution into the desalting column and centrifuge according to the manufacturer's specifications.
7. Transfer the filtrate into a 0.5 mL 10k Amicon® diafiltration column and dilute with 300 μL PBS (*see* **Note 12**). Spin-concentrate the sample for 2 min at 14,000 × *g* using a centrifuge. Add a further 300 μL PBS and spin-concentrate again. Repeat this process of dilution and concentration four times. Dilute a fifth time and centrifuge for 10 min to concentrate the sample to ~30 μL.
8. Recover the ADC by inverting the filter section of the Amicon® into a clean tube and centrifuging for 2 min at 4000 × *g*.
9. Record the final concentration via UV–vis (*see* **Note 13**).

3.2 SDS-PAGE: Assessing Extent of Conversion

1. Preheat a heating block to 90°C.
2. Make up solutions (10 μL, 1 μM. *see* **Note 14**) of the analyte and any controls (e.g., unfunctionalized antibody) in PBS buffer in 0.5 mL vials.
3. Add 10 μL sample stain (reducing or nonreducing) to all samples.
4. Aliquot a 5 μL sample of MW Ladder into a 0.5 mL vial.
5. Heat all samples to 90°C for 5 min to denature the protein.
6. Prepare the gel electrophoresis equipment in LRB according to the manufacturer's specifications, placing the gel in the holder, and wetting the components and gaskets to ensure a good seal is made.

7. Load the individual wells of the acrylamide gel with the MW ladder followed by the analyte and control samples (*see* **Note 15**).
8. Run the gel at 200 V for 65 min.
9. Remove the gel from between the glass plates, rinse with deionized water, cut off the staking gel, and submerge in CBB stain for at least 2 h (*see* **Note 16**).
10. Holding the gel gently down with a finger, carefully decant off the CBB stain and rinse the gel gently with water. To destain, submerge the gel in 10% aq. AcOH. Replace the aqueous acid periodically until the main face of the gel is colorless (*see* **Note 17**).
11. Remove the gel from the aq. AcOH and photograph it (*see* **Note 18**).

Running the SDS-PAGE gel with unfunctionalized antibody as a control ensures that the method has been run correctly and that comparisons can be made. An efficiently re-bridged antibody will appear as high molecular weight bands in reducing conditions as there are no remaining disulfide bonds to cleave (Fig. 3, ADC 1 and ADC 2). Reduced unmodified antibody will split into the

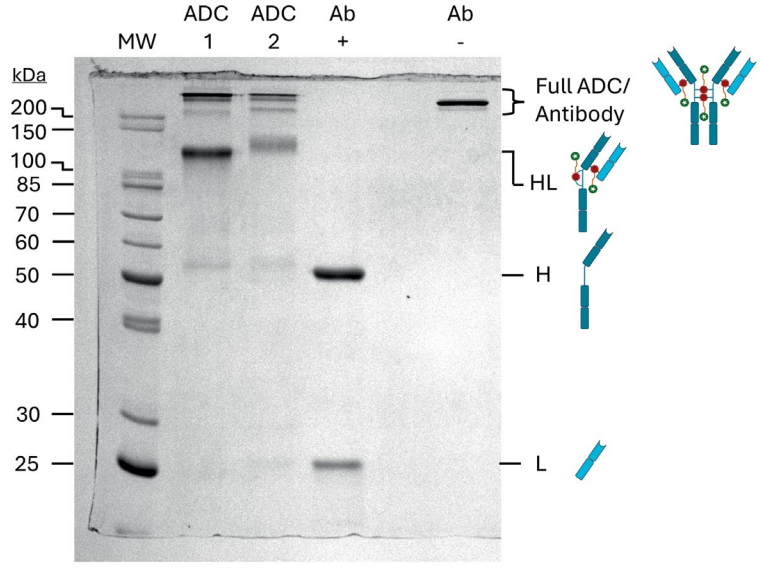

Fig. 3 SDS-PAGE gel showing the extent of conjugation for two ADCs. The ADC analytes were run in a reducing stain, therefore, bands of higher molecular weight show that the heavy and light chains have been chemically re-bridged. Ab +/− = Native antibody with/without reducing stain, MW = Molecular weight ladder, H = Heavy chain, L = Light chain

constituent heavy and light chains on the gel (Fig. 3, Ab +) whilst unreduced unmodified antibody will appear as a single high MW band on the gel (Fig. 3, Ab −). The presence of re-bridged heavy-light chain (Fig. 3, HL) is caused by intra-chain cysteine re-bridging competing with the inter-chain re-bridging. This is common for disulfide re-bridging systems and has no significant effect on ADC properties as the full ADC is held together via noncovalent interactions [8–12].

3.3 Mass Spectrometry: Assessing Most Prevalent ADC Species

1. Make up analytical samples (25 μL, 3–10 μM) in PBS (*see* **Note 19**).
2. Add 0.2 μL PNGase F and incubate the sample at 37°C for 2 h to remove sugars on the antibody surface that interfere with the MS.
3. Inject 3 μL into the MS. Run the analysis at a flow rate of 0.2 mL/min with gradient: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 min, then a gradient to 95% A over 1.04 min. Capillary voltage of 2.0 kV and a cone voltage of 190 V.
4. Combine the ion count of the raw spectra and run the spectra deconvolution algorithm for m/z ions between 1400 and 3000, resolving for masses between 40,000 and 160,000 (Fig. 4). Due to the high mass values being extrapolated, inaccuracies of up to +/− 5 Da are considered reasonable.

3.4 HIC Analysis: Calculating Conversion and Average DAR

1. Make up analytical samples (30 μL, 3–10 μM) in PBS.
2. Inject 15 μL into HPLC system. Run with a linear gradient of 100% solvent A for 3 min, then 0%–100% solvent B over 17 min at a flow rate of 0.6 mL/min.
3. Monitor UV absorbance at 280 nm, with conversion calculated by peak area compared to unmodified antibody. Coupled with MS, the DAR can be calculated via comparison of the peak positions (*see* **Note 20**) and sizes (see Fig. 5a).
4. Once all analyses are complete, flush the HPLC system and column thoroughly with deionized water to prevent the high salt concentration buffers blocking the system.

3.5 SEC Analysis: Checking for Aggregation

1. Make up analytical samples (30 μL, 3–10 μM) in PBS.
2. Inject 6 μL into HPLC system. Run for 30 min at a flow rate of 0.5 mL/min.
3. Monitor UV absorbance at 280 nm, with aggregation calculated by peak area compared to the antibody. Aggregation appears as a peak of higher molecular weight (Fig. 5b) where a relative integration of <1.5% is considered ideal.
4. Once all analyses are complete, flush the HPLC system and column thoroughly with deionized water to prevent the high salt concentration buffers blocking the system.

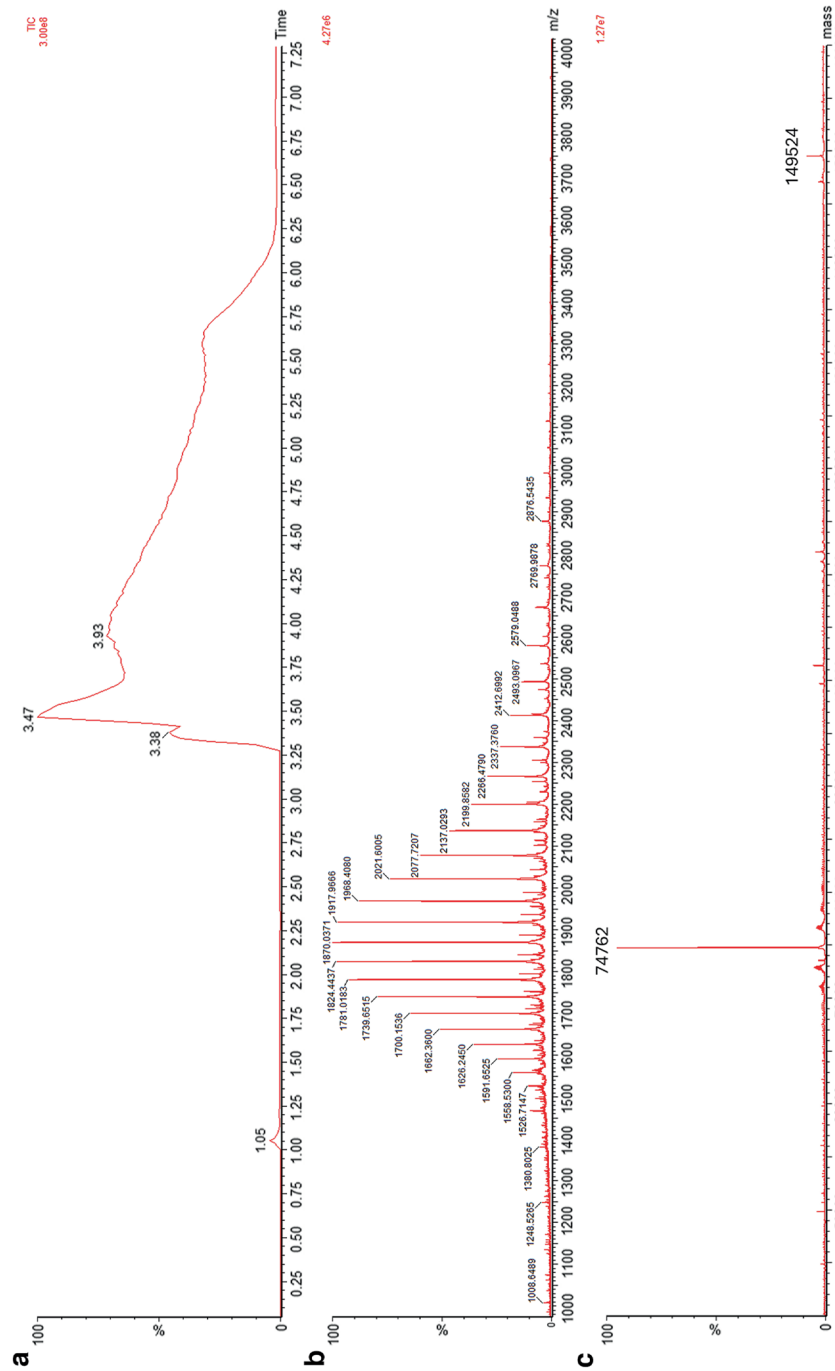

Fig. 4 (**a**) Raw total ion count, (**b**) raw *m/z* data from combining raw total ion count, (**c**) deconvoluted spectrum after running MaxEnt1 algorithm data between 1400 and 3000 *m/z* searching for masses between 40,000 and 160,000. Major peaks are the expected DAR 4 mass (exp. 149,522) and the half mass $[ADC+2H]^{+2}$ (exp. 74,762)

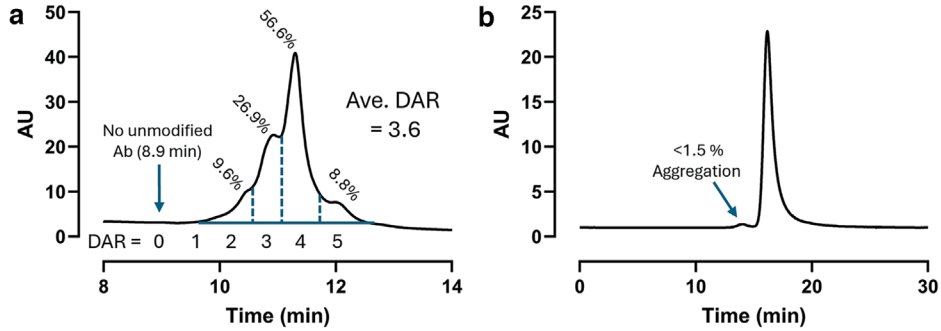

Fig. 5 (**a**) HIC spectrum showing how the peaks are assigned and integrated to calculate an average DAR. (**b**) SEC spectrum showing the presence of aggregation to the left of the antibody peak. Ab = Antibody, AU = Arbitrary units, Ave. = Average

4 Notes

1. Antibodies bought in solution can be used but require buffer exchange into TBS using a 10k MWCO Amicon® diafiltration column.
2. A 500 µL centrifuge tube is ideal for test reactions and reaction optimization. 2 mL Centrifuge tubes can be used for scale-up as long as the thermal shaker can accommodate their shape to provide even heating to the sample.
3. DVP solutions in DMSO should be stored at −4°C (or below) and can remain viable for up to 2 years.
4. PBS can be purchased as a concentrated solution or as dissolvable pellets for simple buffer preparation.
5. SDS-PAGE gels are commercially available but can be self-cast if relevant moulds are available; these should be used within 2 weeks of casting.
6. If using a lyophilized powder, about 1 mg is weighed out and diluted to 2.5 mg/mL with TBS. This gives a concentration of c. 10 µM. Antibodies acquired in solution must be buffer exchanged into TBS using an Amicon® diafiltration column via repeated dilution and concentration with TBS.
7. Concentrations between 5 µM and 20 µM are recommended. The exact concentration should be noted to allow accurate calculation of the TCEP and DVP reagents. Bioconjugation has been successfully carried out up to 2 mL in a single vial (20 µM). Due to size restraints on many thermal shakers, for larger scales multiple vials should be used.
8. If the amino acid sequence of the antibody is known then the extinction coefficient can be estimated using the Expasy ProtParam online tool [19]. If the amino acid sequence is

unknown, then use the extinction coefficient of a similar known antibody or trastuzumab (215,380). The differences in extinction coefficient for IgG1 antibodies tend to be less than 10% and so inaccuracies here are not critical and will be offset by the excess of TCEP and DVP used.

9. TCEP oxidizes in solution over time and so stocks must be made fresh directly before use to ensure complete disulfide reduction.
10. When adding TCEP or DVP to the antibody solution gently mix the solutions by repeatedly withdrawing and readmitting the solution with the pipette. This is especially key for the DVP solution to prevent the DMSO settling and creating a high local concentration, which can lead to antibody aggregation and precipitation.
11. Desalting columns help to remove excess salts (TCEP, Copper, etc.), preventing further reactivity. The need for desalting can be assessed on a case-by-case basis; whilst diafiltration should remove TCEP, experience has shown that copper salts (after CuAAC) are not removed and so desalting is required. The desalting column should be equilibrated into an appropriate buffer system for any post-purification analysis or further study. PBS is commonly used as it is compatible with copper-catalyzed "click" chemistry and biological studies.
12. Repeated PBS dilution and concentration through a 10k MWCO filter affords buffer exchange (reducing DMSO and TCEP concentration) whilst allowing excess DVP linker to be filtered off, purifying the sample. The cellulose filters are delicate so care must be taken not to pierce the membrane with the tip of a pipette. Once wetted, it is recommended not to let the membrane dry out for high sample recovery. Occasionally, agitate the solution in the filter with the tip of a pipette when adding further PBS to ensure the ADC is in solution and not stuck to the cellulose.
13. Concentrations far exceeding 100 μM are not recommended due to increased aggregation propensity. If above 100 μM, dilute with buffer.
14. The following equation can be used to easily generate 10 μL solutions at 1 μM:

$$\frac{10}{x} = y$$

where x = original sample concentration (in μM) and y = volume of sample (in μL) that needs to be diluted to 10 μL to afford a 1 μM solution (e.g., if your sample is 5 μM then 10/5 μM = 2 μL, diluted up to 10 μL to be a 1 μM solution).

15. Aiming to pipette 10% less volume than was originally in the vial (e.g., 18 µL of a 20 µL sample) will ensure good sample loading whilst preventing bubbles forming in the pipette tip; some of the solution will have evaporated during the denaturing step. It is recommended to leave an empty well between reducing and non-reducing stain samples as the mercaptoethanol can leach between wells causing partial reduction to other samples thus effecting band shape.
16. The stacking gel section can be removed at any stage before imaging but removing it early reduces the chance for parts of the stacking gel to fold over, effecting the efficiency of de/staining. Plastic pipette tip boxes are an ideal size for gel staining. A gel rocker (such as a Stuart SSM4 see-saw rocker) can be used to gently agitate the submerged gel and ensure that the gel is stained and destained evenly.
17. The gel is destained when only the protein bands are visibly blue. The protein will not be easily destained by the aqueous acid so it is fine to leave the gel in 10% aq. AcOH for several days.
18. Due to the staining, a standard camera can be used to record the bands on the gel. Gel imagers (such as Syngene ingenious 3 with GeneSys software) are useful if fluorescent components are bioconjugated to the antibody.
19. For ease, a 50 µL sample can be made up and used for HIC and SEC analyses. The remaining material can then by deglycosylated with PNGase F for MS analysis.
20. As increasing the number of payloads conjugated to an antibody usually increases the hydrophobicity of an ADC in an additive manner, different DAR species are identifiable by HIC retention time with fairly even spacing between species as the number of linkers increases. MS data is not quantitative but should show a DAR 4 species as the major product of a successful conjugation; therefore, the largest HIC peak is thus assigned "DAR 4" with the peaks around it assigned consecutively (e.g., 3, 2, 1).

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