Conjugate, clean-up, & characterize ADCs on Unagi & Stunner

Introduction

Antibody-drug conjugates (ADCs) are a class of molecule that consist of 3 chief components: a monoclonal antibody (mAb), a linker, and a small molecule drug. Creating and optimizing ADCs involves careful sample preparation and characterization as you test different combinations and reaction conditions. Purifying these newly synthesized ADCs and exchanging them from their reaction buffers into their storage buffers adds extra steps to this process. Throughout the entire procedure, it's critical to keep an eye on the antibody concentration, check the drug-antibody ratio (DAR), and watch out for aggregation.

Removing drug-linker-related impurities from ADCs can be a hassle, especially when testing lots of different components and reaction conditions. Resin-based approaches, like chromatography or desalting, require multiple washes for buffer equilibration and will always dilute the samples, adding time to an already lengthy protocol. Centrifugal filters can cause dead-end filtration and concentrate samples unevenly at the membrane, leading to aggregation. TFF works well for large experiments but is a low-throughput method that's difficult for smaller-scale pilot and benchtop studies. Unagi (Figure 1A) fills in where other methods fall short, handling up 8 samples at a time with hands-off automation that prevents dilution and dead-end filtration to run tightly controlled clean-up, buffer exchange, and concentration steps

Characterizing ADCs once they're made usually involves a combination of several HPLC methods. However, HPLC often takes too long, requires too much sample, or needs too much optimization to work with every candidate. Stunner (Figure 1B) uses UV/Vis absorbance on just 2 µL of sample to determine the concentration and DAR of up to 96 samples at-a-time.[1]

A

B

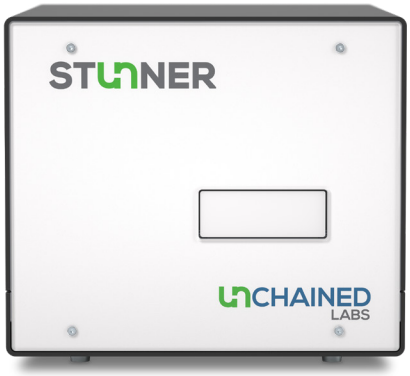

Figure 1: Unagi (**A**) is your automated benchtop buffer exchange solution. Stunner (**B**) is the only plate-based system that pulls together UV/Vis and DLS data on the same 2 µL sample.

Stunner simultaneously detects aggregates by looking at the size and size distribution of the ADCs with dynamic light scattering (DLS).[2]

In this study, we conjugated a mAb with 3 payload/linker combinations at 2 molar ratios to create 6 model ADCs. Payload-related impurities were removed by automated buffer exchange and filtration on Unagi (Figure 2) , and all ADCs were characterized on Stunner.

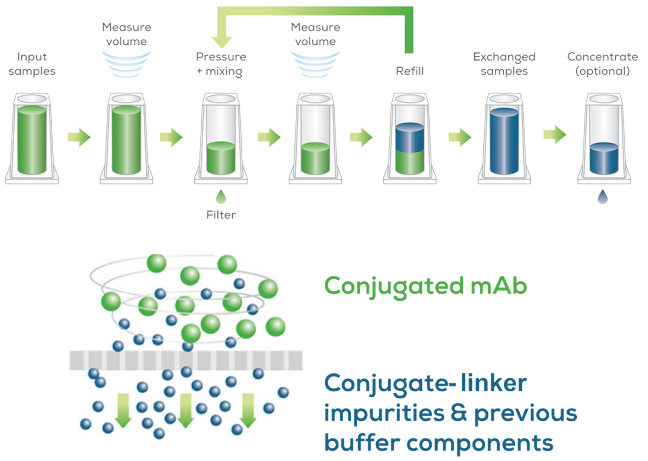

Figure 2: Unagi's automated pressure-based UF/DF technology uses ultrasonic volume measurement to monitor the flow rate and adapt the pressure cycle time for each sample. The sample volume measurement enables precise target exchange percentages and concentration values, since the amount of retentate is known at every step. Conjugated mAbs or ADCs are retained while excess, unreacted conjugate molecules are filtered out, along with the previous buffer components. New buffer is added to the well at each step of the buffer exchange.

Stunner uses Unmix algorithms and user-stored reference spectra to deconvolute the total UV/Vis absorbance spectrum into its component parts (Figure 3) . From these deconvoluted spectra, Stunner can quantify the total amount of protein and drug payload present and use them to calculate the DAR of a conjugate.

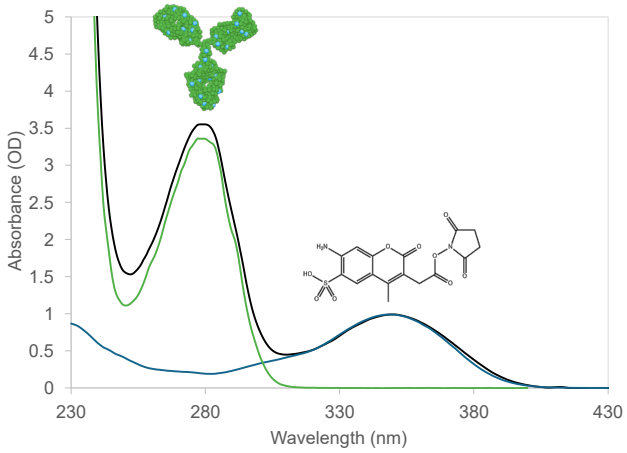

Figure 3: Unmix algorithms in Stunner let you use your own measured spectra to deconvolute the overall absorbance spectrum of your ADC (black line) into the contribution coming from the mAb (green line) and the drug or conjugate molecule (blue line). In this example we show the total and deconvoluted absorbance spectra from a mAb conjugated with Alexa Fluor™ 350 NHS ester.

Methods

Monoclonal antibody preparation

A human mAb at 26.6 mg/mL in 5 mM sodium succinate, 60 mM trehalose, pH 5.0 was diluted to 10 mg/mL and centrifuged at 14,000xg. The supernatant was filtered through a 0.1 µm syringe filter. 1.1 mL aliquots were exchanged into 50 mM borate buffer, pH 8.5, 100 mM bicarbonate buffer, pH 8.3, or 1X phosphate-buffered saline (PBS). Key buffer exchange parameters are shown in Table 1. Initial and final protein concentration, size, and polydispersity index (PDI) were checked in quadruplicate on Stunner.

Parameter	Setting
Mixing speed	700 rpm
Pressure	60 psi
Target % exchange	96%
Target volume removed per cycle	50%
Initial concentration	10 mg/mL
Initial well volume	1.1 mL
Target final concentration	10 mg/mL
Target final well volume	1.1 mL

Table 1: Key buffer exchange parameters used for mAb buffer exchange in 10 kDa molecular weight cut-off (MWCO) Unas.

Antibody conjugation and cleanup

Fluorescein isothiocyanate (FITC, Thermo Fisher 46425), Alexa Fluor™ 350 (AF350) NHS ester (Thermo Fisher A10168), and AF350 C5 maleimide (Thermo Fisher A30505) were dissolved to 10 mg/mL in dimethyl sulfoxide (DMSO). The dyes were added to 0.5 mL of mAb solution in the buffers indicated in Table 2 at dye-to-protein molar ratios of 2:1 or 10:1.

100 µg of tris-(2-carboxyethyl)phosphine (TCEP, Thermo Fisher T2556) was added to the

maleimide reaction as a reducing agent. Reaction mixes were incubated for 1 hour at room temperature, protected from light.

Buffer	Conjugate	Molar ratios
50 mM borate, pH 8.5	Fluorescein isothiocyanate (FITC)	2:1 and 10:1
100 mM bicarbonate, pH 8.3	Alexa Fluor™ 350 NHS Ester	2:1 and 10:1
PBS	Alexa Fluor™ 350 C5 maleimide	2:1 and 10:1

Table 2: Dye/buffer combinations and molar ratios for mAb conjugation reactions.

After incubation, the 6 reaction mixes were diluted to 1 mL and exchanged into PBS with Unagi. Key buffer exchange parameters are shown in Table 3. Initial and final protein concentrations, drug-antibody ratio (DAR), size, and PDI were checked in quadruplicate on Stunner.

Parameter	Setting
Mixing speed	700 rpm
Pressure	60 psi
Target % exchange	96%
Target volume removed per cycle	50%
Initial concentration	5 mg/mL
Initial well volume	1 mL
Diluent volume	1 mL
Target final concentration	10 mg/mL
Target final well volume	0.5 mL

Table 3: Key buffer exchange parameters used for ADC clean-up & buffer exchange in 10 kDa MWCO Unas. Unagi's pre-dilute and concentration functions were used to automatically dilute each sample to 2 mL at the beginning of the buffer exchange and concentrate them to 0.5 mL at the end.

ADC quantification and sizing

Solutions of 10 mg/mL of each dye in DMSO were diluted 1:9 into PBS, then a 2X dilution series was made in 90% PBS, 10% DMSO. The absorbance of the dilution series of each dye was measured on Stunner using the Store Analyte app and the spectra were saved. A spectrum of the mAb was stored the same way. Extinction coefficients (E1%s) were found from either the manufacturer's documentation or the concentration of the stock. The saved dye and mAb spectra were used with the ADC + Sizing app to determine concentration, DAR, size, and polydispersity index (PDI) of each sample. DLS acquisition settings of 5 acquisitions at 1 second each were used with the software's automatic outlier exclusion.

Results

Conjugating ADCs with different drug-linkers often involves first exchanging the precursor mAb into various reaction buffers, depending on the exact conjugation chemistry. For example, FITC and NHS esters require alkaline pH reaction buffers to label lysine residues and the N-terminus of proteins. In Unagi this buffer exchange took only a few minutes of hands-on time and less than 1 hour of hands-off time. The concentrations at the end were within 5% of the target value of 10 mg/mL and >90% sample recovery (Figure 4).

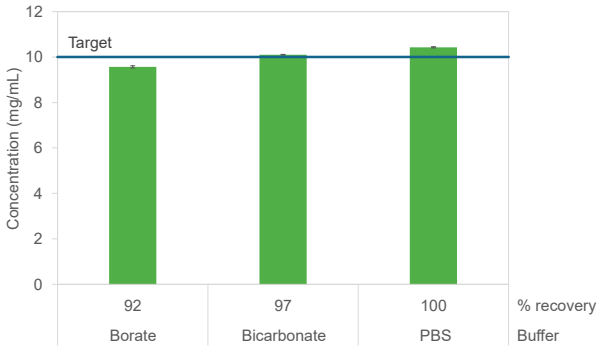

Figure 4: Protein concentration by A280 and percent recovery of the mAb in each of the 3 reaction buffers after Unagi buffer exchange. Error bars represent 1 standard deviation.

After exchanging a mAb into a new buffer it's important to check the quality of the mAb, especially to see if it aggregated. The size and PDI of the mAb in each of the reaction buffers indicated no aggregation occurred (Figure 5). In each case, the hydrodynamic diameter of 10–12 nm was consistent with the expected size of an antibody. PDI values <0.1 also indicate that all the samples were monodisperse.

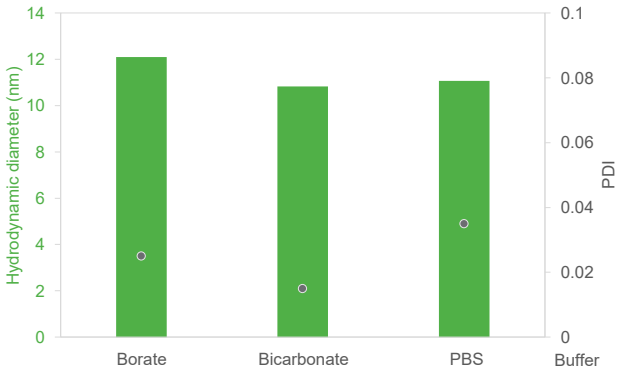

Figure 5: Z-average diameter (green bars, left y-axis) and PDI (grey dots, right y-axis) of the mAb in the 3 reaction buffers post Unagi buffer exchange.

In order to analyze ADC spectra by UV/Vis, you must distinguish the antibody's absorbance spectrum from the conjugated drug.[1] However, drug spectra can vary significantly, so instruments need to be smart enough to keep up. Stunner can learn a compound's absorbance spectrum from a dilution series of the drug via its Store Analyte feature. To illustrate this, we stored a spectrum of FITC (Figure 6) on Stunner and used it, along with a stored mAb spectrum, on a mAb sample reacted at a 10:1 FITC-to-protein molar ratio post clean-up on Unagi. Stunner used the stored spectra to Unmix the absorbance from the antibody and the conjugated FITC. It used the results, along with the E1% of the mAb and FITC, to determine the concentration of each. The ratio of these concentrations is the DAR. Applying this method to different drugs, linkers, and molar ratios eases optimizing reaction conditions for any ADC.

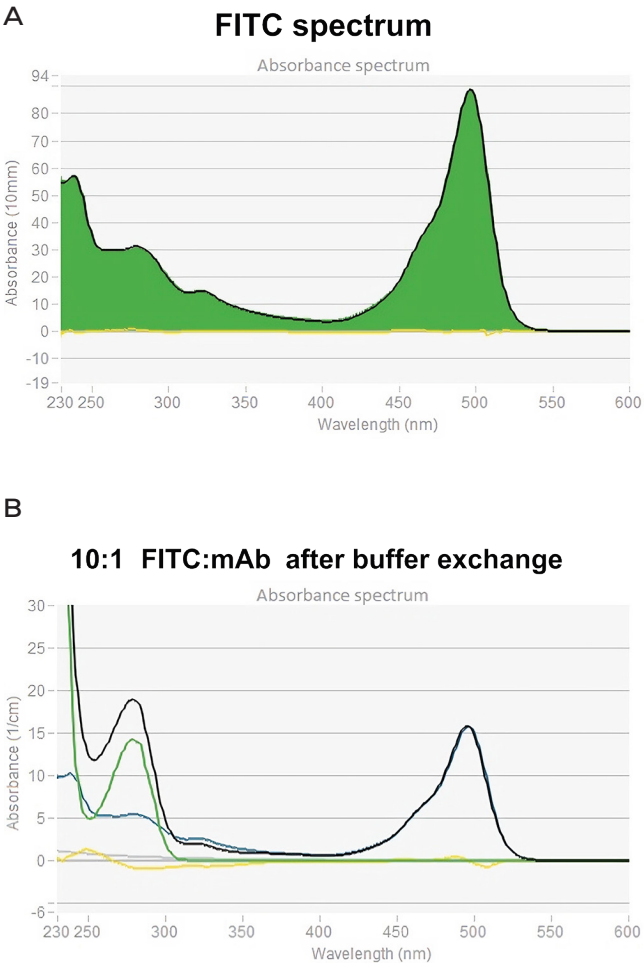

Figure 6: UV spectrum of FITC gathered from a dilution series on Stunner's Store Analyte feature (**A**). When running an ADC on Stunner, you can deconvolute the total absorbance (black) into the absorbance from the antibody (green), from FITC (blue), and any residual error at each wavelength (yellow) (**B**).

Changing the molar ratio of the drug-linker to the mAb affects the DAR of an ADC, but so do the reaction rates and abundance of target residues. Optimizing conjugation reaction conditions of ADCs to achieve the target DAR is a big part of ADC process development. By analyzing the reaction mixtures on Stunner before and after clean-up by Unagi, we can see how well the clean-up step worked, gauge the reaction efficiencies, and find the correct molar ratios for a target DAR for each dye (Figure 7).

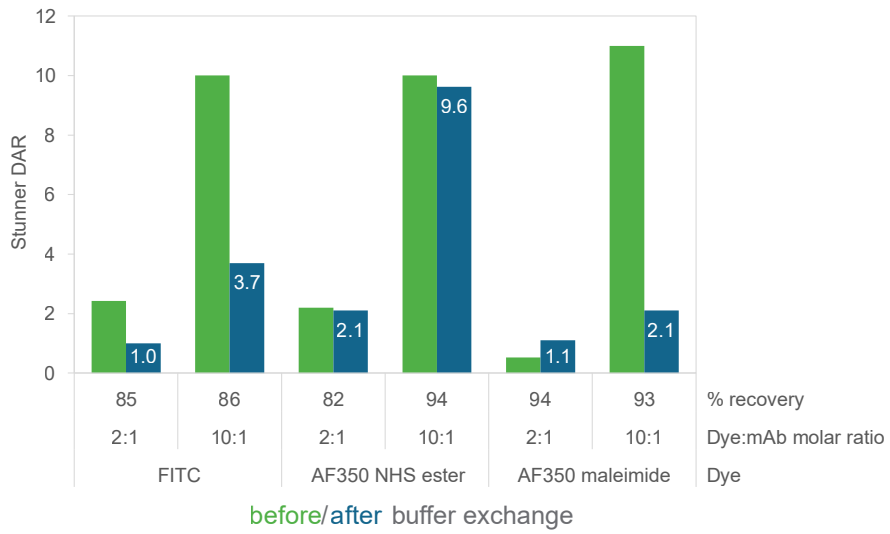

Figure 7: DAR of mAb labeled with FITC, AF350 NHS ester, or AF350 maleimide at 2:1 and 10:1 dye-to-protein molar ratios before (green) and after (blue with white labels) purification and buffer exchange into PBS by Unagi. Percent recovery of the conjugated mAbs after Unagi purification and buffer exchange is also shown.

Comparing the DAR before (Figure 7 green bars) and after (blue bars) buffer exchange allows us to see if the reactions occurred at all and how well the clean-up step worked. If none of the dyes had reacted with the mAb, 96% of the dye would have been removed from the solution along with the other buffer components. If that were the case, the DAR reported by Stunner would have been ~4% of the initial value. Generally, the DAR after buffer exchange was higher than that threshold, but still lower than the value measured pre-Unagi, evidence that the labeling worked and that Unagi removed just the unreacted dye. The DAR increased after buffer exchange only in the 2:1 maleimide sample, but that was most likely due to insufficient mixing of the sample in the pre-Unagi measurement. Unagi exchange is quite gentle and completely user-controlled, so the end product is at the expected concentration, not diluted like with resin-based methods.

Different linkers and conjugation chemistries inevitably give rise to differences in reaction efficiencies, even when using the same drug. For example, the AF350 NHS ester-labeled mAb had a higher DAR after clean-up with both molar ratios than the other samples, indicating the reaction was more efficient. The efficiency difference in the AF350 ester and maleimide reactions is likely due to their different chemistries: the ester reacts with amine groups while the maleimide reacts with thiols. One of the goals of testing different linkers is to find a combination of drug, linker, and mAb that hit a target DAR, concentration, and recovery.

Achieving the target DAR and good recovery is important, but meaningless if the ADC aggregates. DLS can be used to check biomolecules for aggregates, regardless if they're protein- or drug-linker-related.[2] The hydrodynamic diameters of the ADC models were either equal to or slightly higher than the unconjugated mAb (Figure 8). Samples with 10:1 molar ratios had larger diameters than 2:1 samples with the same drug-linker. Together, these observations suggest that conjugation increased the size of the ADC molecules. Sample clean-up on the Unagi generally decreased the PDI of the ADCs because the samples were more homogeneous after removal of the drug-linker impurities. It's possible that the 10:1 FITC:mAb sample aggregated, based on the PDI of 0.15, but the PDIs and hydrodynamic diameters of the other samples were both low enough to indicate that the ADCs did not aggregate during conjugation or buffer exchange.

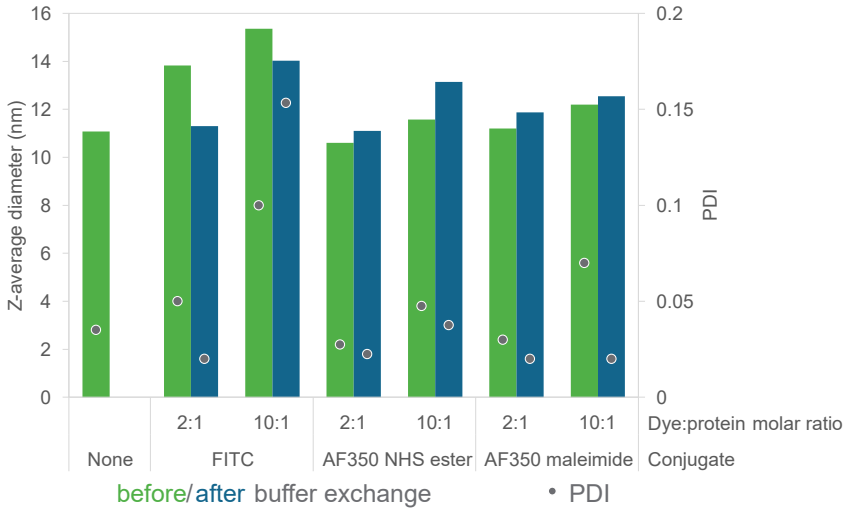

Figure 8: Z-average diameter (bars, left y-axis) and PDI (dots, right y-axis) of mAbs labeled with FITC, AF350 NHS ester, or AF350 maleimide reacted at 2:1 and 10:1 dye-to-protein molar ratios before (green) and after (blue) purification and buffer exchange into PBS by Unagi. Results for unreacted mAb (None) in PBS are provided for comparison.

Conclusion

ADC preparation and characterization are challenges that can be as complex as the structure of ADCs themselves. Stunner can rapidly quantify antibody concentration, DAR, size, and size distribution in a rapid, low-volume, high-throughput format. Unagi is ready for any ADCs that need to be buffer exchanged for sample prep, concentrated for conjugation reactions, or cleaned-up afterwards – with hands-off automation that frees up scientists for other labwork.

References

1. Drug-to-Antibody Ratio (DAR) by UV/Vis Spectroscopy. Y Chen. In: Ducry L, ed. Antibody-Drug Conjugates. Totowa, NJ: Humana Press; 2013:267–273.

2. Photoinduced Aggregation of a Model Antibody–Drug Conjugate. GM Cockrell, et al. Molecular Pharmaceutics. 2015; 12(6):1784–1797.

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