Challenging Hybrid Affinity Approach: Streamlined Total Antibody Quantitation with Uncompromised Sensitivity

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INTRODUCTION

- Antibody–drug conjugates (ADCs) consist of a payload covalently bound to a monoclonal antibody via a chemical linker. By merging the specific binding properties of antibodies to the potency of the payload, ADCs are designed to selectively deliver cell-killing agents while limiting systemic toxicity[1].
- Ligand-Binding Assays (LBA) and Hybrid Ligand-Binding Assay–Mass Spectrometry (LBA-LC/MS) are two prominent techniques for analyzing antibody-drug conjugates (ADCs). LBAs, particularly ELISA, offer high sensitivity and a broad quantification range without extraction steps. LBA/LC-MS combines the strengths of both technologies, providing sensitive quantitative bioanalysis with multiple analyte detection and high specificity within a single sample thanks to the physical detection of one or multiple surrogate peptides. Both technologies can utilize various capture reagents, including generic anti-human antibodies or more specific anti-idiotype antibodies, for total antibody quantification. However, since both methods rely on capture reagents they can be negatively affected by their availability (too early into the development stage), lack of access to the epitope with the payload of ADCs hindering capture, and potential cross-reactivity with pre-clinical IgGs.
- To address these challenges, we describe a mass spectrometry-based workflow utilizing a direct digestion quantitative method, implemented with an automated spiking procedure using the Bravo AssayMap. By eliminating affinity capture steps, this simplified protocol offers a sensitive and straightforward method that is robust, scalable, and easy to implement, ensuring reliable quantification of ADCs across a broad dynamic range.

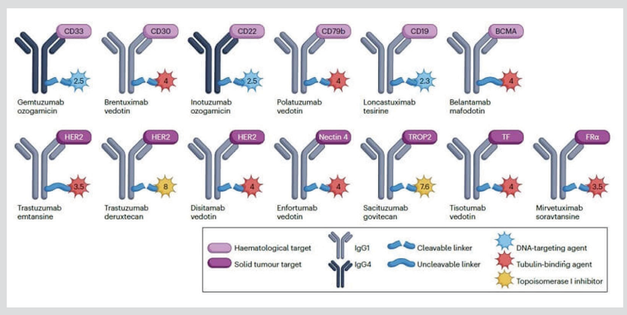
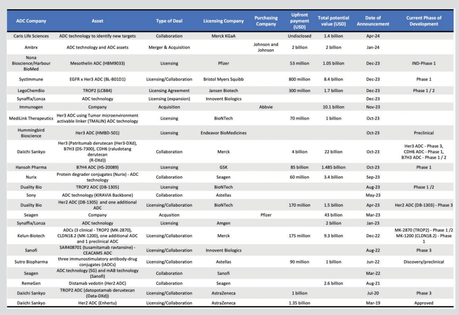


Figure 1. FDA Approved ADC[2]



ADC Company	Asset	Type of Deal	Licensing Company	Purchasing Company	Upfront payment (US$)	Total potential value (US$)	Date of Announcement	Current Phase of Development
Curis Life Sciences	ADC technology to identify new targets	Collaboration	Merck KGaA	Undisclosed	1.4 billion	Apr-24		
Ambrx	ADC technology and ADC assets	Merger & Acquisition		Johnson and Johnson	2 billion	3 billion	Jan-24	
Nona Biosciences/Harbour BioMed	Mesothelin ADC (HBM9033)	Licensing	Pfizer		53 million	1.05 billion	Dec-23	IND-Phase 1
Spitfireone	EGFR x Her3 ADC (BL-B01D1)	Licensing/Collaboration	Bristol Myers Squibb		800 million	8.4 billion	Dec-23	Phase 1
LegoChemBio	TROP2 (LCB84)	Licensing Agreement	Jansen Biotech		300 million	1.7 billion	Dec-23	Phase 1 / 2
Synaffix/Lonza	ADC technology	Licensing (expansion)	Innovent Biologics				Dec-23	
Immunogen	Company	Acquisition		Abbvie		10.1 billion	Nov-23	
MedLink Therapeutics	Her3 ADC using Tumor microenvironment activable linker (TMAL/AL) ADC technology	Licensing	BioNTech		70 million	1 billion	Oct-23	
Hummingbird Bioscience	Her3 ADC (HMBD-501)	Licensing	Endeavor BioMedicines				Oct-23	Preclinical
Daiichi Sankyo	Her3 (Patritumab deruxtecan (Her3-DXd), B7H3 (DS-7300), CDH6 (raludotatug deruxtecan (R-DXd))	Collaboration	Merck		4 billion	22 billion	Oct-23	Her3 ADC - Phase 3, CDH6 ADC - Phase 1, B7H3 ADC - Phase 1 / 2
Hanosh Pharma	B7H4 ADC (HS-20089)	Licensing	GSK		85 billion	1.485 billion	Oct-23	Phase 1
Nuvia	Protein degrader conjugates (Nuvia) - ADC technology	Collaboration	Seagen		60 million	3.4 billion	Sep-23	
Duality Bio	TROP2 ADC (DB-1305)	Licensing	BioNTech				Aug-23	Phase 1 /2
Sony	ADC technology (XRAY4 Backbone)	Collaboration	Astellas				May-23	
Duality Bio	Her2 ADC (DB-1303) and one additional ADC	Licensing/Collaboration	BioNTech		170 million	1.5 billion	Apr-23	Her2 ADC (DB-1303) - Phase 3
Seagen	Company	Acquisition		Pfizer		43 billion	Mar-23	
Synaffix/Lonza	ADC technology	Licensing	Amgen			2 billion	Jan-23	
Kelun-Biotech	ADCs (3 clinical - TROP2 (MK-2870), CLDN18.2 (MK-1200), one additional ADC target) - ADC technology	Licensing/Collaboration	Merck		175 million	9.3 billion	Dec-22	MK-2870 (TROP2) - Phase 1 /2 MK-1200 (CLDN18.2) - Phase 1
Sanofi	SAR408701 (tusamitamab ravtansine) - CEACAM5 ADC	Licensing/Collaboration	Innovent Biologics				Aug-22	Phase 3
Sutro Biopharma	three immunostimulatory antibody-drug conjugates (ADCs)	Licensing/Collaboration	Astellas		90 million	1 billion	Jun-22	Discovery/preclinical
Seagen	ADC technology (Glycotoxin and mAB technology (GlycoMab))	Collaboration	Sanofi				Mar-22	
ReineGen	Distamab vedotin (Her2 ADC)	Collaboration	Seagen			2.6 billion	Aug-21	
Daiichi Sankyo	TROP2 ADC (datopotamab deruxtecan (Dato-DXd))	Licensing/Collaboration	AstraZeneca	1 billion			Jul-20	Phase 3
Daiichi Sankyo	Her2 ADC (Enhertu)	Licensing/Collaboration	AstraZeneca	1.35 billion			Mar-19	Approved

Figure 2. Top ADC Deals[3]

AFFINITY-FREE METHOD DEVELOPMENT

The initial goal of this study was to find the limits of the direct digestion approach by using a common workflow that eliminates affinity purification while maintaining sensitivity comparable to generic or specific affinity capture methods. Initial efforts were made to reduce the amount of plasma used compared to the standard affinity protocol, using sub-10 µL samples, while aiming for a 1-day turnaround time, if possible.

Figure 3. A) Digestion buffer optimization; **B)** Enzyme ratio selection; **C)** Digestion time optimization; **D)** Sample processing optimization.

EARLY PERFORMANCE EVALUATION

The final analytical workflows include aliquoting 5µL of mouse or rat plasma, followed by a simple organic solvent denaturation and direct digestion of the samples. Filtration post digestion is added to ensure additional instrument robustness.

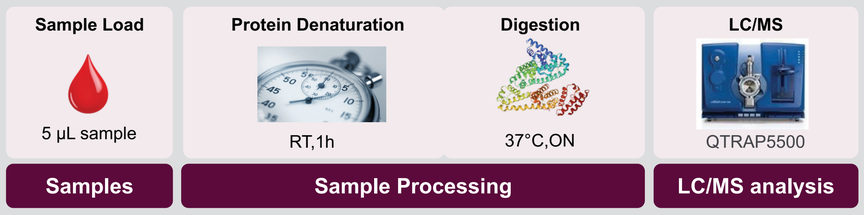


Figure 4. Optimized Analytical Workflow

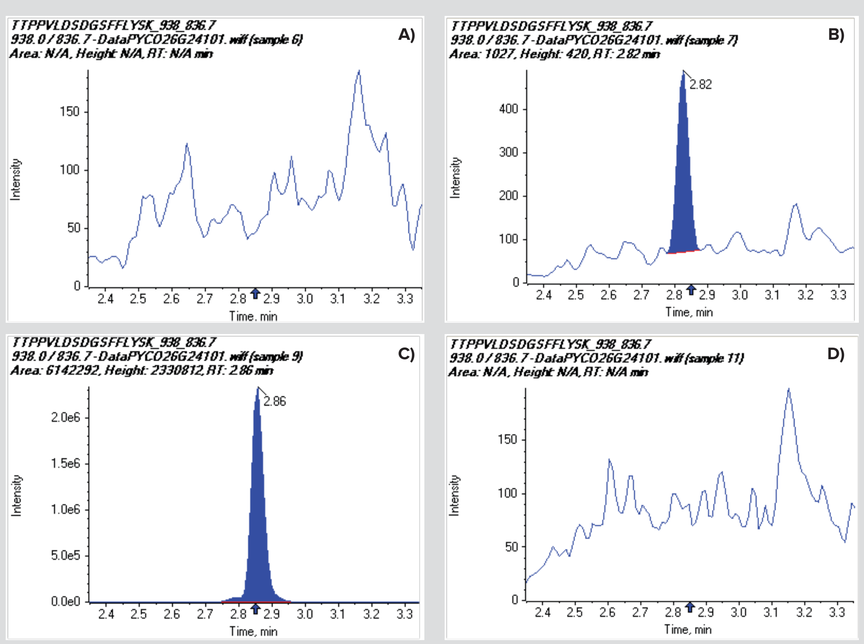


Figure 5. A) Mouse plasma, double blank; **B)** LLOQ spiked at 100ng/mL; **C)** ULOQ spiked at 500µg/mL; **D)** 1st carryover blank after 3 ULOQ injected.

ASSAY OPTIMIZATION AND AUTOMATION

Building on our implementation of a generic and sensitive assay for ADC analysis by direct digestion, we have further enhanced our workflow by incorporating the Agilent AssayMAP Bravo liquid handler from Agilent. Using the built-in reformatting software, standard and QC samples were prepared independently, with volumes easily adjusted based on the number of plates to be extracted.

Figure 6. Agilent Bravo Assay Map standard configuration and software[4].

The integration of the AssayMAP Bravo was shown to improve overall efficiency by reducing manual errors and ensuring consistent sample treatment. To evaluate performance, the same calibration curve was spiked manually and using the AssayMAP Bravo, then extracted with the developed protocol. Results were compared for precision and accuracy.

PRECISION & ACCURACY: MANUAL VS. AUTOMATED

Table 1. Precision and accuracy run, manual spiking, Peak area only.

Manual Spiking	Standards (µg/mL)										Quality Controls matrix (µg/mL)				
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	QC-LLOQ	QC-1	QC-2	QC-3	QC-4
	0.100	**0.200**	**0.500**	**1.00**	**4.00**	**12.50**	**50.00**	**150.00**	**400.00**	**500.00**	**0.100**	**0.300**	**1.50**	**10.00**	**375.00**
FC-Peptide	0.107	0.182	0.435	1.03	4.10	12.29	56.97	147.52	416.39	469.94	0.106	0.260	1.52	10.98	355.75
											0.096	0.293	1.55	10.47	361.11
											0.102	0.307	1.52	10.29	362.48
n	1	1	1	1	1	1	1	1	1	1	3	3	3	3	3
Mean	0.107	0.182	0.435	1.030	4.100	12.290	56.970	147.52	416.390	469.94	0.101	0.287	1.530	10.580	359.78
SD	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	0.005	0.024	0.017	0.358	3.557
% C.V.	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	5.0	8.4	1.1	3.4	1.0
% bias	7.0	-9.0	-13.0	3.0	2.5	-1.7	13.9	-1.7	4.1	-6.0	1.3	-4.4	2.0	5.8	-4.1

Table 2. Precision and accuracy run, Assay Map Spiking, Peak area only.

Assay Map Spiking	Standards (µg/mL)										Quality Controls matrix (µg/mL)				
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	QC-LLOQ	QC-1	QC-2	QC-3	QC-4
	0.100	**0.200**	**0.500**	**1.00**	**4.00**	**12.50**	**50.00**	**150.00**	**400.00**	**500.00**	**0.100**	**0.300**	**1.50**	**10.00**	**375.00**
FC-Peptide	0.108	0.172	0.503	0.938	3.88	11.98	48.69	158.19	437.72	533.56	0.114	0.306	1.64	10.30	376.01
											0.103	0.279	1.68	9.96	406.64
											0.110	0.279	1.33	9.66	381.51
n	1	1	1	1	1	1	1	1	1	1	3	3	3	3	3
Mean	0.108	0.172	0.503	0.938	3.88	11.98	48.69	158.19	437.72	533.56	0.109	0.288	1.546	9.981	388.057
SD	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	0.006	0.015	0.189	0.320	16.327
% C.V.	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	5.3	5.3	12.2	3.2	4.2
% bias	7.5	-13.8	0.6	-6.2	-3.0	-4.2	-2.6	5.5	9.4	6.7	9.0	-4.0	3.1	-0.2	3.5

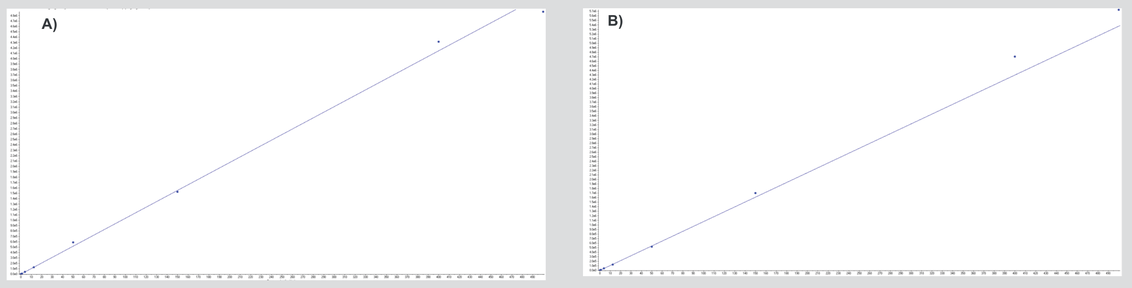


Figure 7. A) Standard curve generated by manually spiking a test molecule into mouse plasma at different concentrations; **B)** Standard curve generated by spiking a test molecule into mouse plasma at different concentrations using Bravo Assay Map platform.

ASSAY OPTIMIZATIONS

In mass spectrometry, physical divert valves have traditionally been used to prevent contamination, but they face significant challenges in capillary HPLC (capLC) systems. These valves introduce dead volume, causing peak dispersion and reduced resolution, and disrupt low-flow stability, risking electrospray ionization instability. Moreover, the valves are prone to failure due to high backpressure, require frequent cleaning, and are often incompatible with narrow capillaries. To improve method robustness, making this method suitable for back-to-back analysis, despite having a sample processing with minimal clean-up, we have introduced a virtual divert valve, based on the principle of voltage modulation. This solution offers an elegant approach, by temporarily disabling the ionization voltage, preventing contamination without introducing additional hardware. This technique preserves chromatographic integrity, maintains stable flow, and is independent of backpressure constraints. Software-controlled voltage modulation significantly reduces source fouling, extending maintenance intervals and enhancing long-term robustness, making it a highly effective and efficient contamination control method for capLC-MS systems.

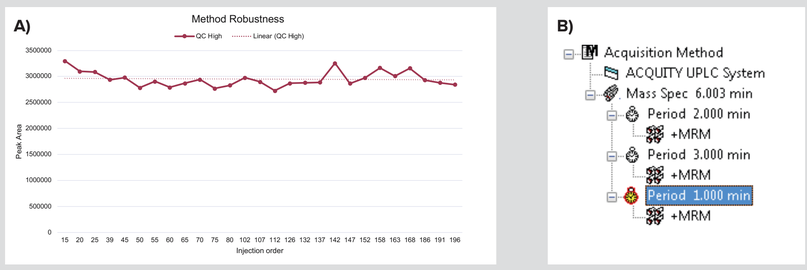


Figure 8. A) Peak area of 27 QC-High samples independently extracted and continuously injected with the virtual divert valve; **B)** Structure of the virtual divert valve.

CONCLUSION

- The developed method proved to be fit for purpose, achieving a sensitivity in line with affinity capture assays of 100 ng/mL from just 5 µL of plasma, with good robustness, precision, and accuracy (small molecule criteria when spiking using the AssayMap).
- The current analytical range spans from 0.1 to 500 µg/mL with just a 6-minute injection, demonstrating broad applicability for ADC and therapeutic antibody quantification. Access to state-of-the-art triple quadrupole instruments could push even further the current level of sensitivity obtained on a QTRAP5500, matching results obtainable by Hybrid LBA/LC-MS or LBA assay.
- Due to the minimal sample preparation steps (no reduction, alkylation, or affinity capture), automated spiking, and low reagent costs, this approach represents a low-cost, high-efficiency, and easily deployable solution for preclinical bioanalysis, that is unaffected by the increased complexity of ADC's.

REFERENCES

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