



Accelerate ADC Characterization with the New DAR Calculator

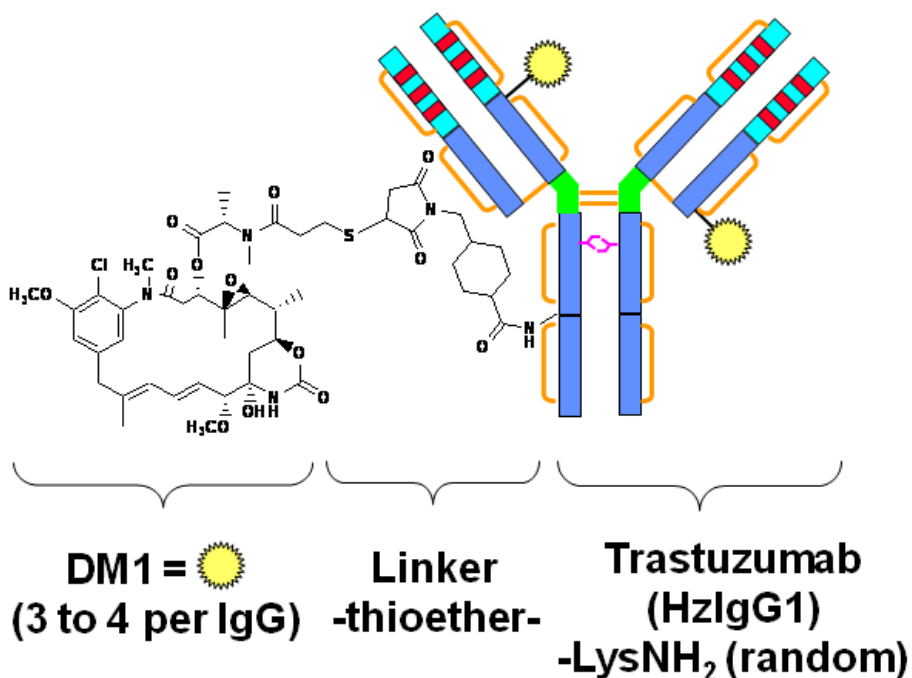
Ning Tang, Ph.D.

Agilent Technologies
Santa Clara, California, USA

Antibody-Drug Conjugates (ADCs)

Design

T-DM1 (Genentech)



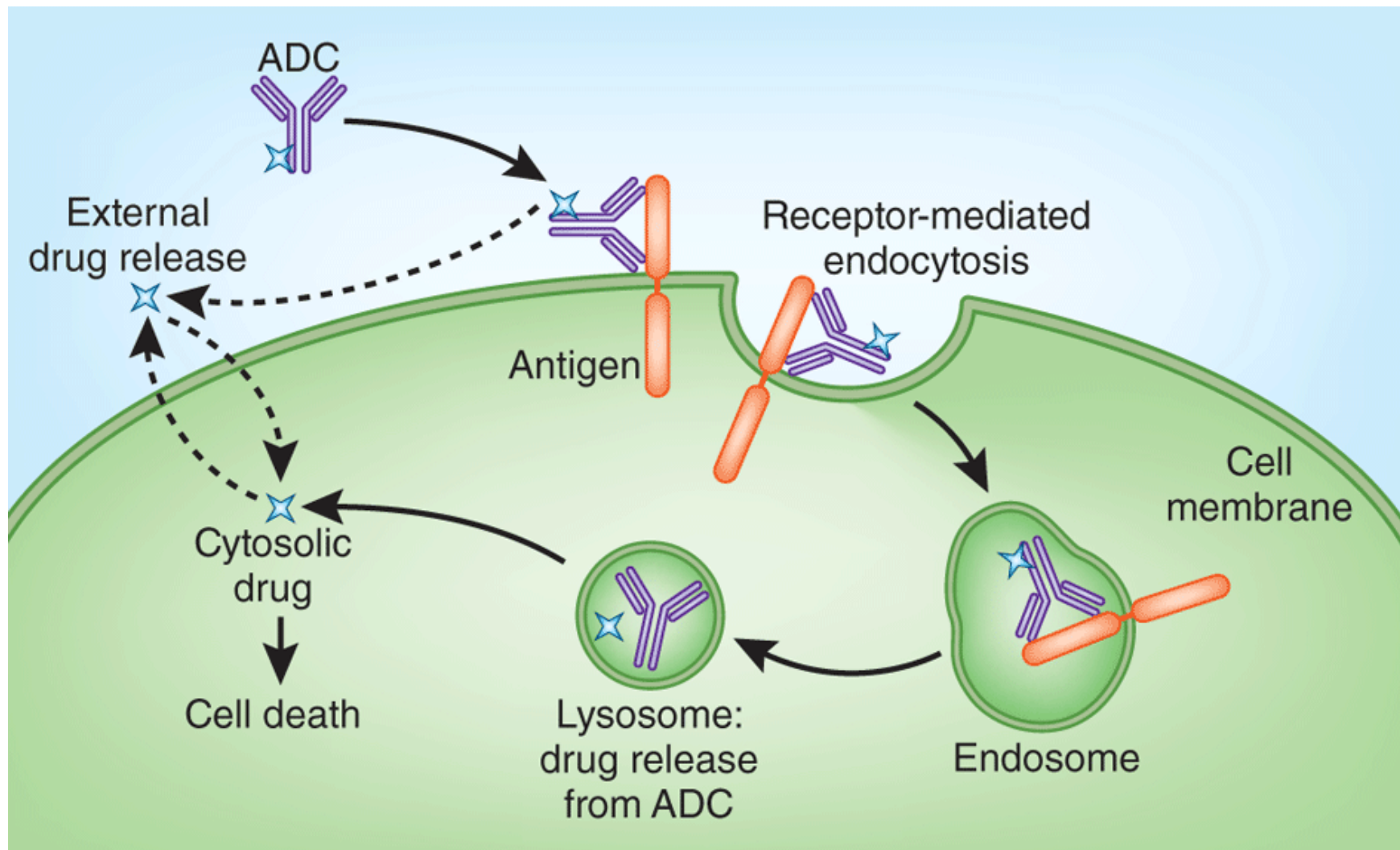
Components:

- Antibody
 - Targeted recognition
 - Abundant target expression & internalization
- Drug
 - Highly potent
 - Verified mechanism of action (microtubule inhibition, DNA damage)
- Linker
 - Stable in plasma
 - Labile upon internalization to release drug



Antibody-Drug Conjugates (ADCs)

Targeted therapy



Rationale for Antibody-Drug Conjugates (ADCs)

- Some small molecule drugs have high systemic toxicity, e.g. chemotherapy drugs used for cancer treatment
- Antibodies can target particular cells (e.g. antigen positive tumor cells) quite selectively
- Covalently linking antibodies to small molecule drugs can target the drug and reduce systemic toxicity
- Drug-to-Antibody Ratio (DAR) is one of the key attributes to be characterized

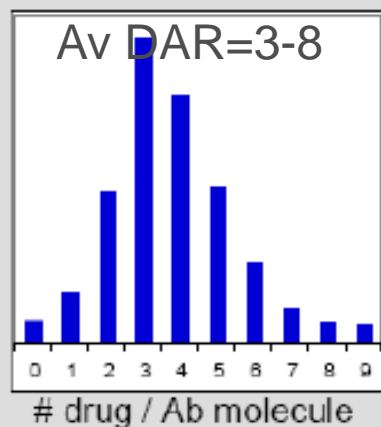
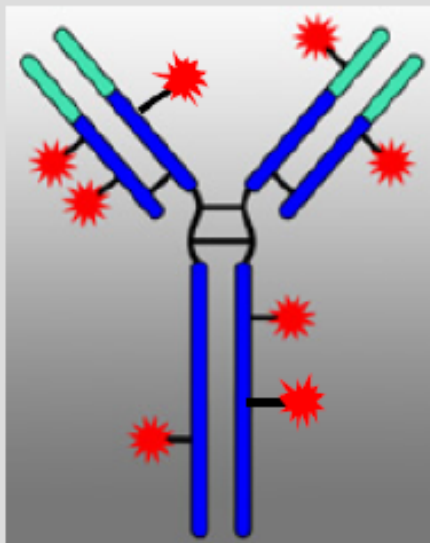
MAbs. 2009 May-Jun; 1(3): 247–253.



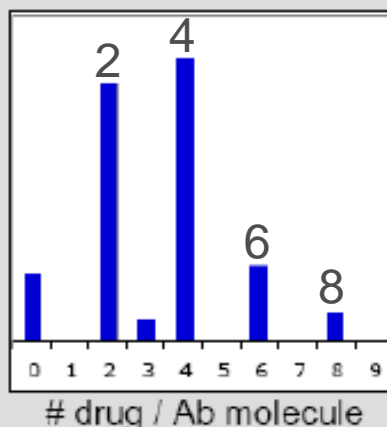
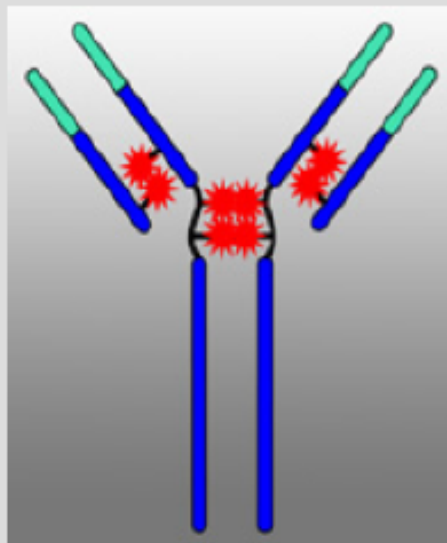
Heterogeneities in ADCs from Conjugation

Dependent on conjugation chemistry

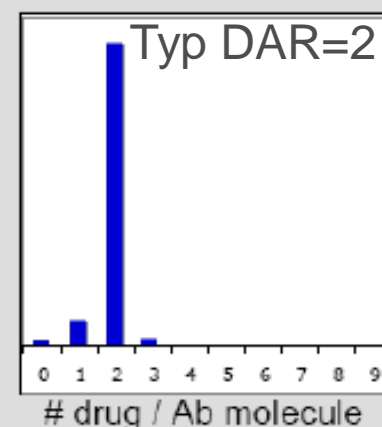
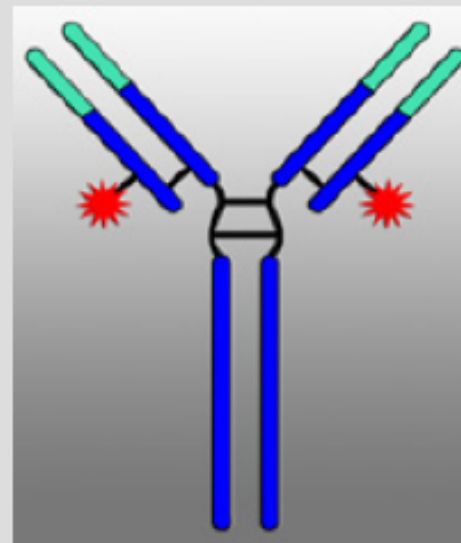
Lysine conjugation



Interchain disulfide conjugation



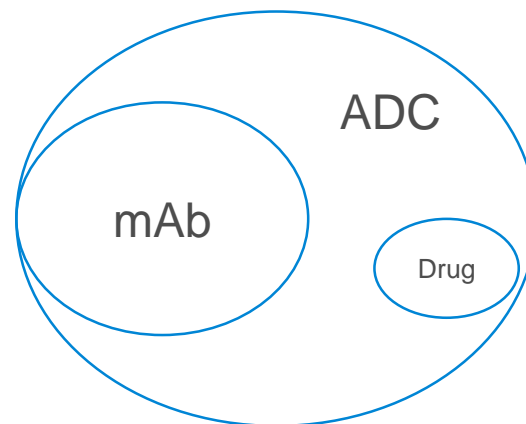
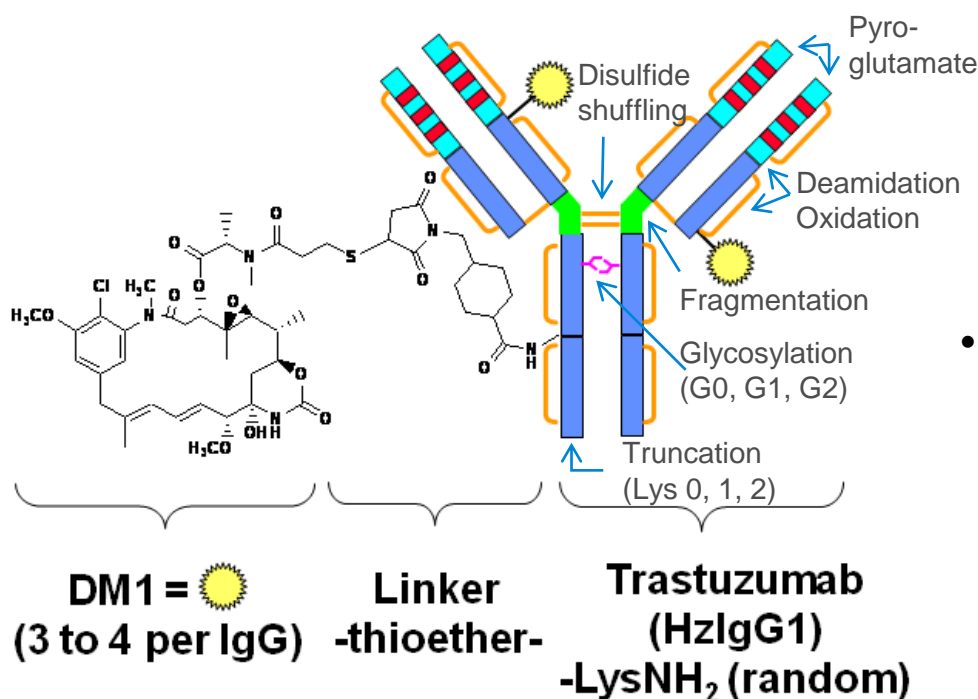
Site-directed conjugation



Heterogeneity in ADCs

Complexity

T-DM1 (Genentech)

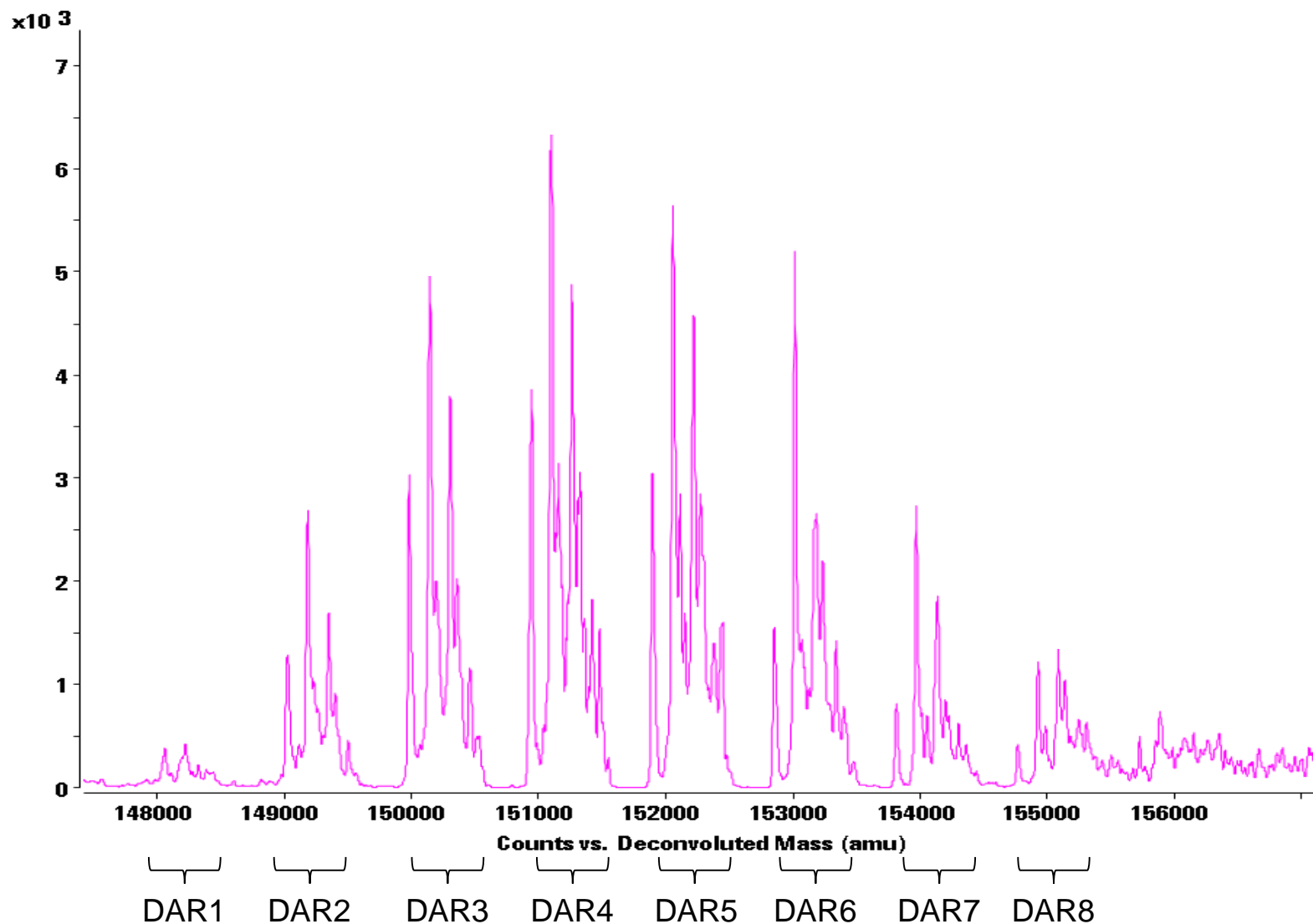


- Heterogeneity in ADC

- More complex than mAbs alone
- Dependent on linker & payload stability (hydrolysis, degradation, etc.)
- Dependent on the conjugation chemistry



Conjugation Adds Heterogeneity

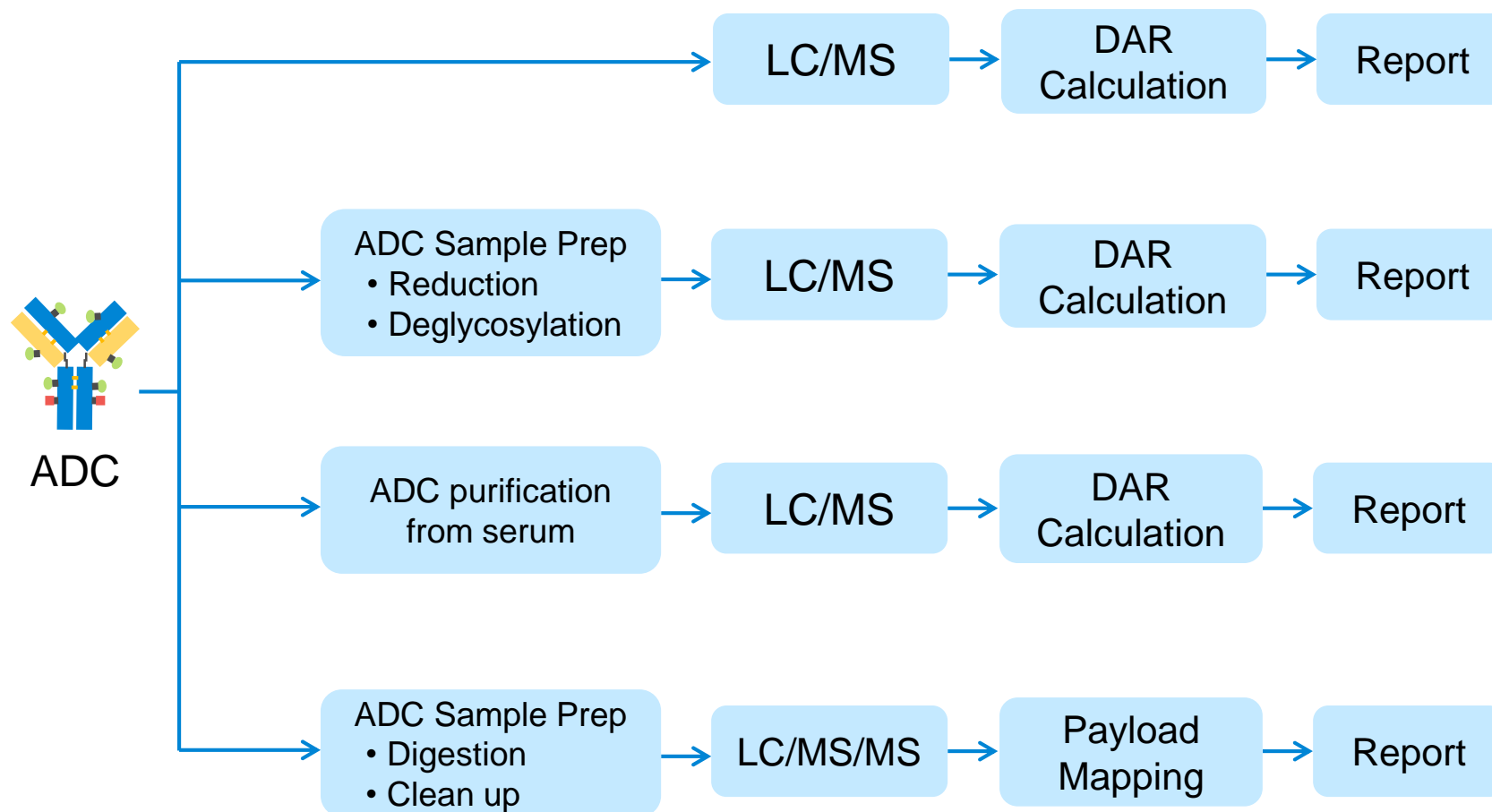


Key Quality Attributes & Methods for ADC

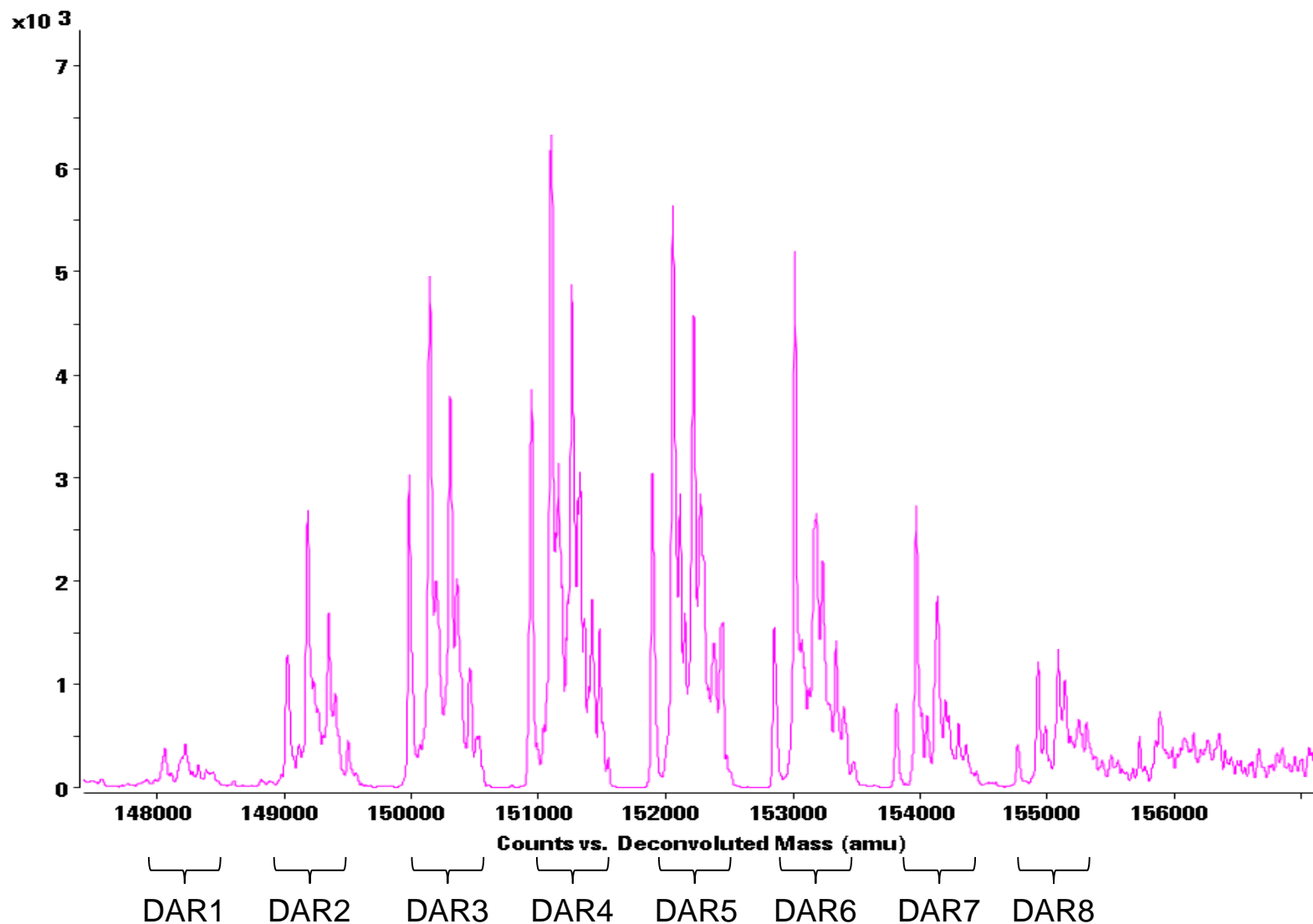
Quality attributes	Assays	
Identity	Intact Mass, Peptide Mapping, Sequence	mAb + ADC
Size heterogeneity (aggregates)	SDS-PAGE, SEC, MALS, MS	mAb + ADC
Charge heterogeneity	IEF, CEX	mAb + ADC
PTMs	LC/MS/MS	mAb + ADC
Drug load (DAR)	UV, HIC, HPLC, MS	ADC
Drug load distribution	HIC, MS	ADC
Residual drug	ELISA, HPLC	ADC
Potency (for drug)	Cytotoxicity	ADC
Potency (for mAb)	Antigen binding ELISA	mAb + ADC



Agilent DAR Characterization Workflow



Conjugation Adds Heterogeneity



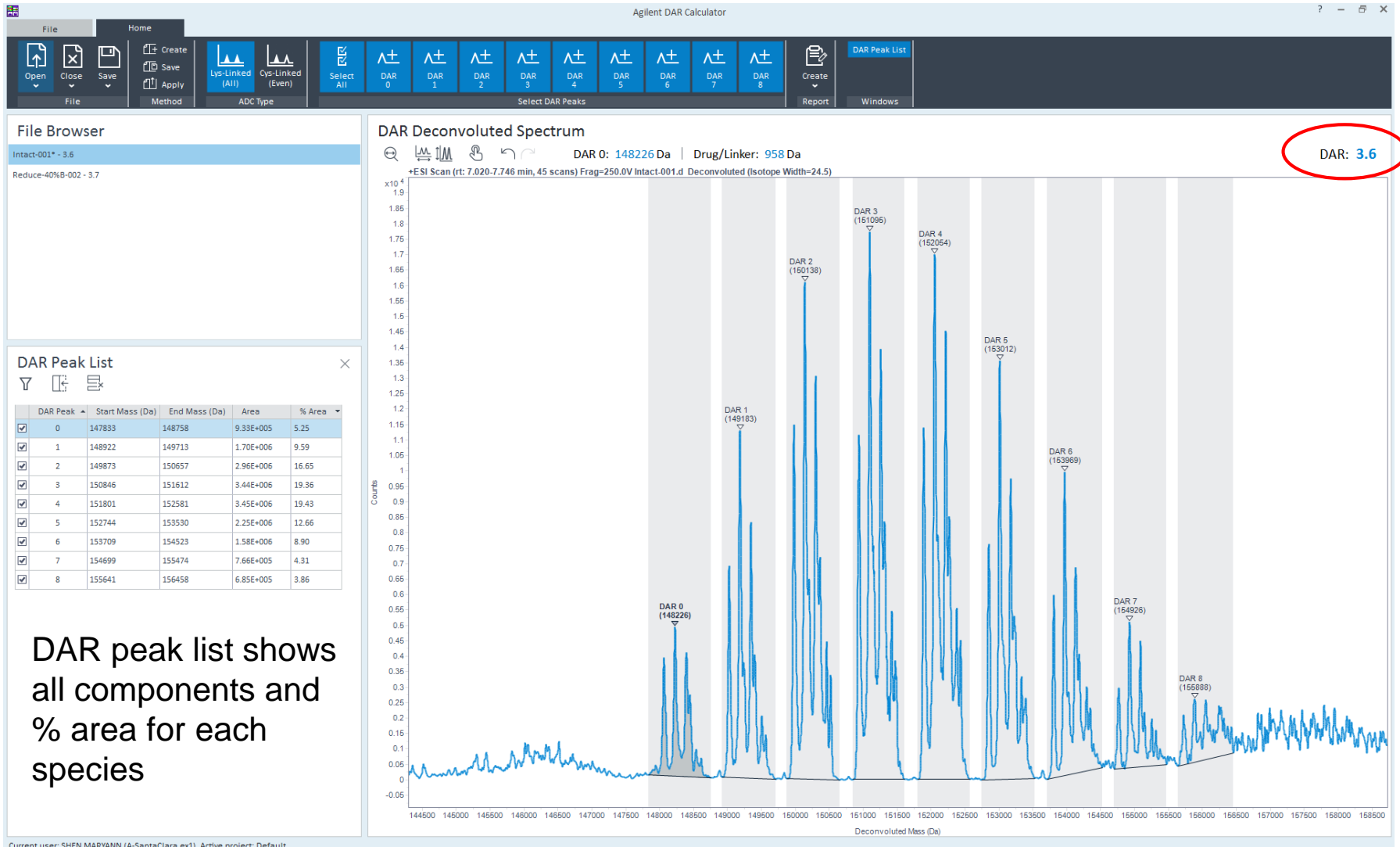
Introducing the MassHunter ADC DAR Calculator

Automatic calculation of DAR value

- Intuitive peak integration
- Built-in support for intact and reduced ADCs
- Easy analysis of different types of ADC molecules (Lys-linked, Cys-linked and site-specific ADCs)
- Flexible control to define peaks of interest
- Dedicated ADC DAR report in PDF and MS Word



Intact ADC DAR Calculation



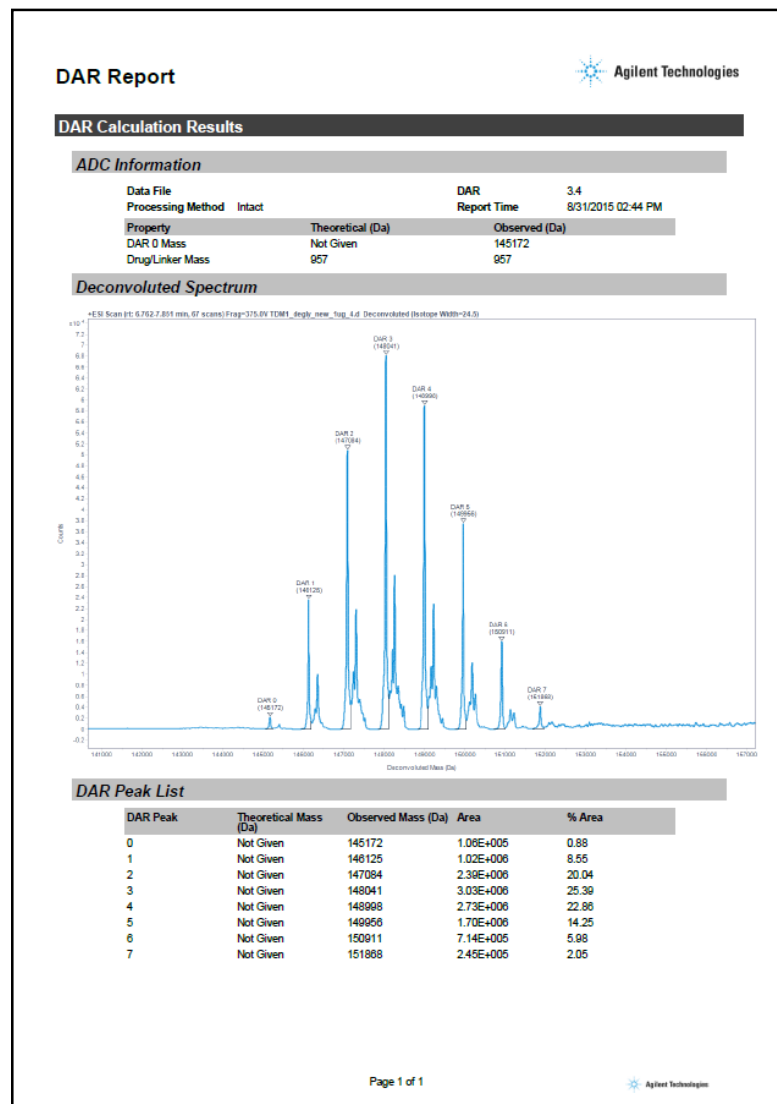
DAR peak list shows all components and % area for each species



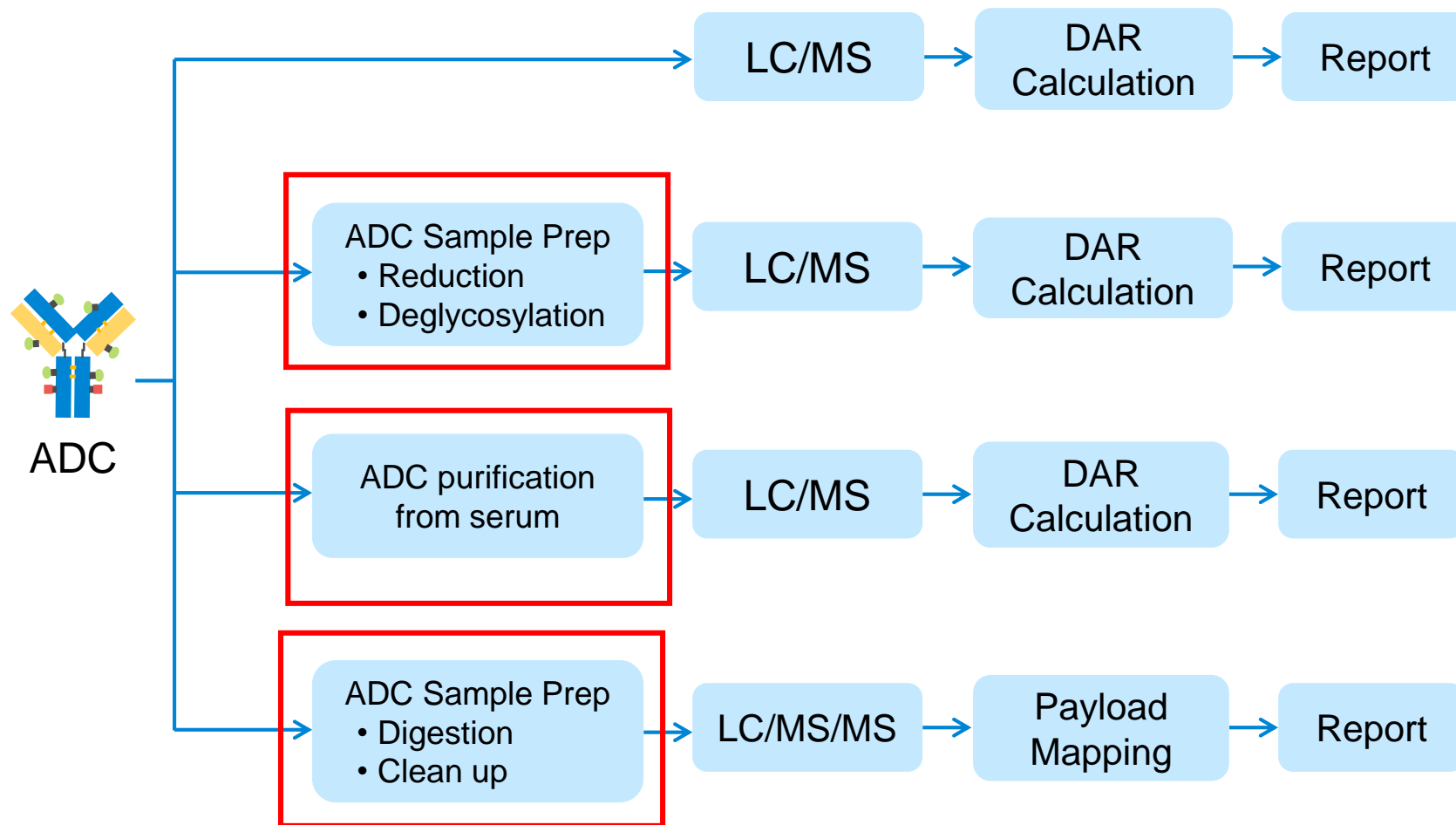
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Dedicated ADC DAR Report

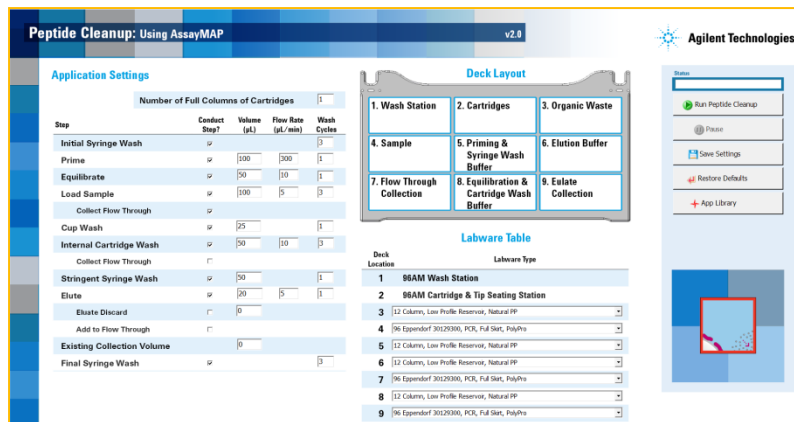


Agilent DAR Characterization Workflow



AssayMAP Technology Components

Automated workflows designed for analytical research chemists



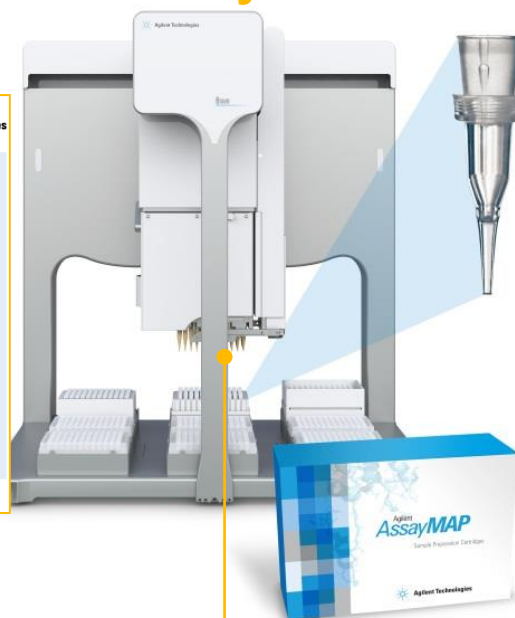
Simple User Interface

Uses customer language - not automation language

- Affinity (protein) Purification
- In-Solution Digestion
- Peptide Cleanup (desalting)
- Protein Cleanup (desalting)
- Phosphopeptide Enrichment
- IMAC Cartridge Customization
- Fractionation
- Sample Normalization
- Liquid handling utilities

Positive Displacement Pipetting

Syringes interface directly with cartridges and enable precise, controlled liquid flow through cartridges with no air bubbles to disrupt binding



Microchromatography Cartridges

Quantitative binding & elution

Protein purification

- PA-W (protein A)
- PG-W (protein G)
- SA-W (streptavidin)

Reversed-phase cleanup:

- C18 (peptide)
- RP-S (peptide)
- RP-W (denatured mAbs)

Peptide Fractionation:

- SCX
- RP-S
- C18

Phosphopeptide enrichment:

- TiO₂
- Fe(III)-NTA



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Workflow Library

Protein Sample Prep Workbench: WORKFLOW LIBRARY

v2.0



General Workflows



Affinity Purification Workflow

Create custom affinity cartridges and use them to enrich for target molecules. Using AssayMAP Bravo and Cartridges.

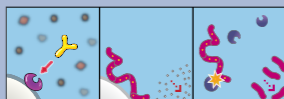
Open



Peptide Sample Prep Workflow

Digest proteins, desalt, and optionally fractionate peptides. Using AssayMAP Bravo and Cartridges.

Open



Rapid Antibody Digestion Workflow

Purify, denature, cleanup, and digest antibodies. Using AssayMAP Bravo.

Open

SISCAPA
assay technologies

SISCAPA® Workflow

Addition-only trypsin digest and immunocapture of target peptides and internal standards. Using SISCAPA® antibodies and Bravo 96LT Head.

Open

Post-Translational Modification Workflows



Phosphopeptide Enrichment Workflow

Digest proteins, desalt and enrich for phosphopeptides. Using AssayMAP Bravo and Cartridges.

Open

ProZyme

N-Glycan Sample Prep Workflow

Denature glycoproteins, then release, label and cleanup glycans. Using AssayMAP Bravo and ProZyme GlykoPrep-plus® Chemistry Kits.

Open

Workflow Library

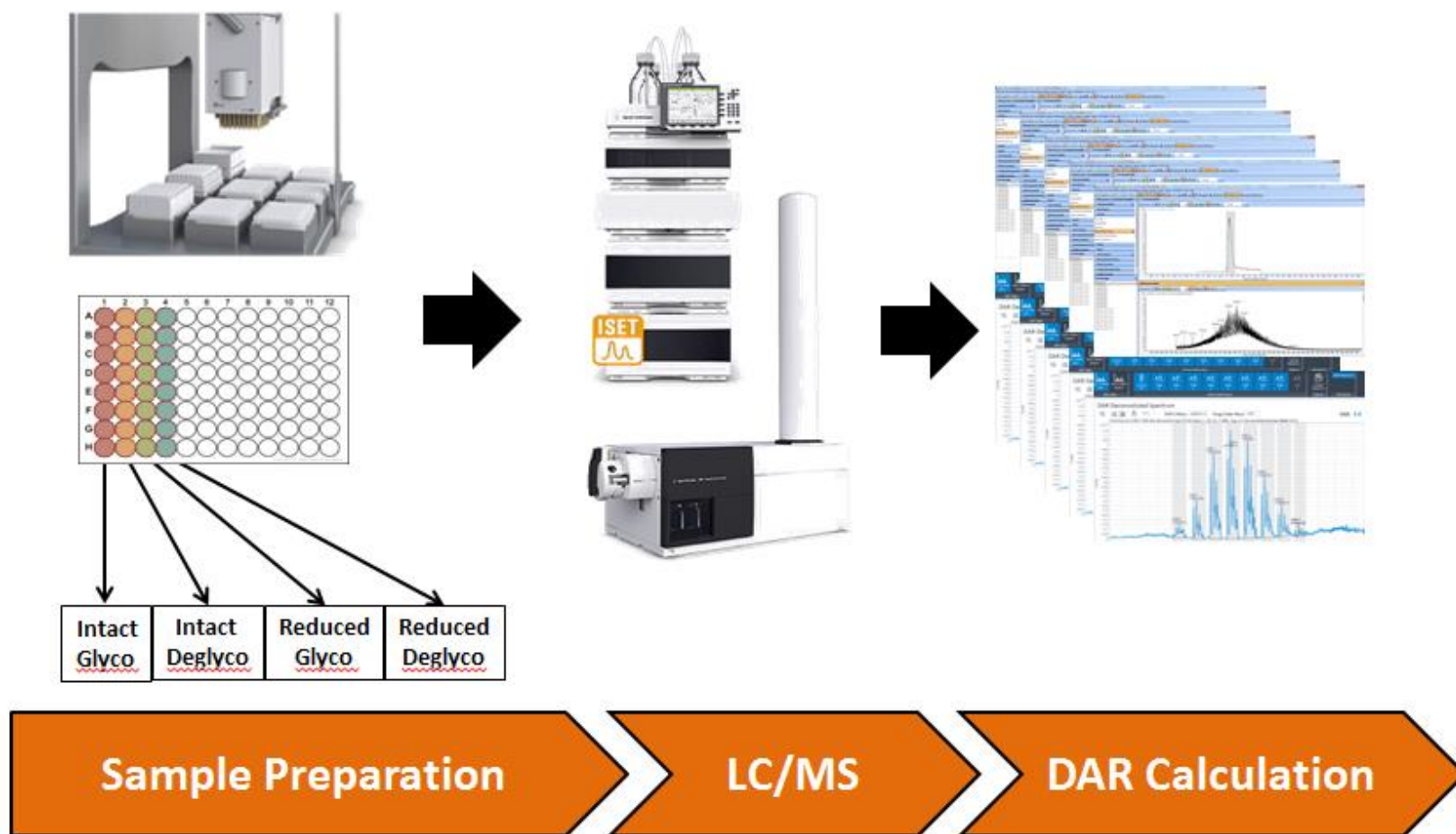
App Library

Utility Library

Literature Library

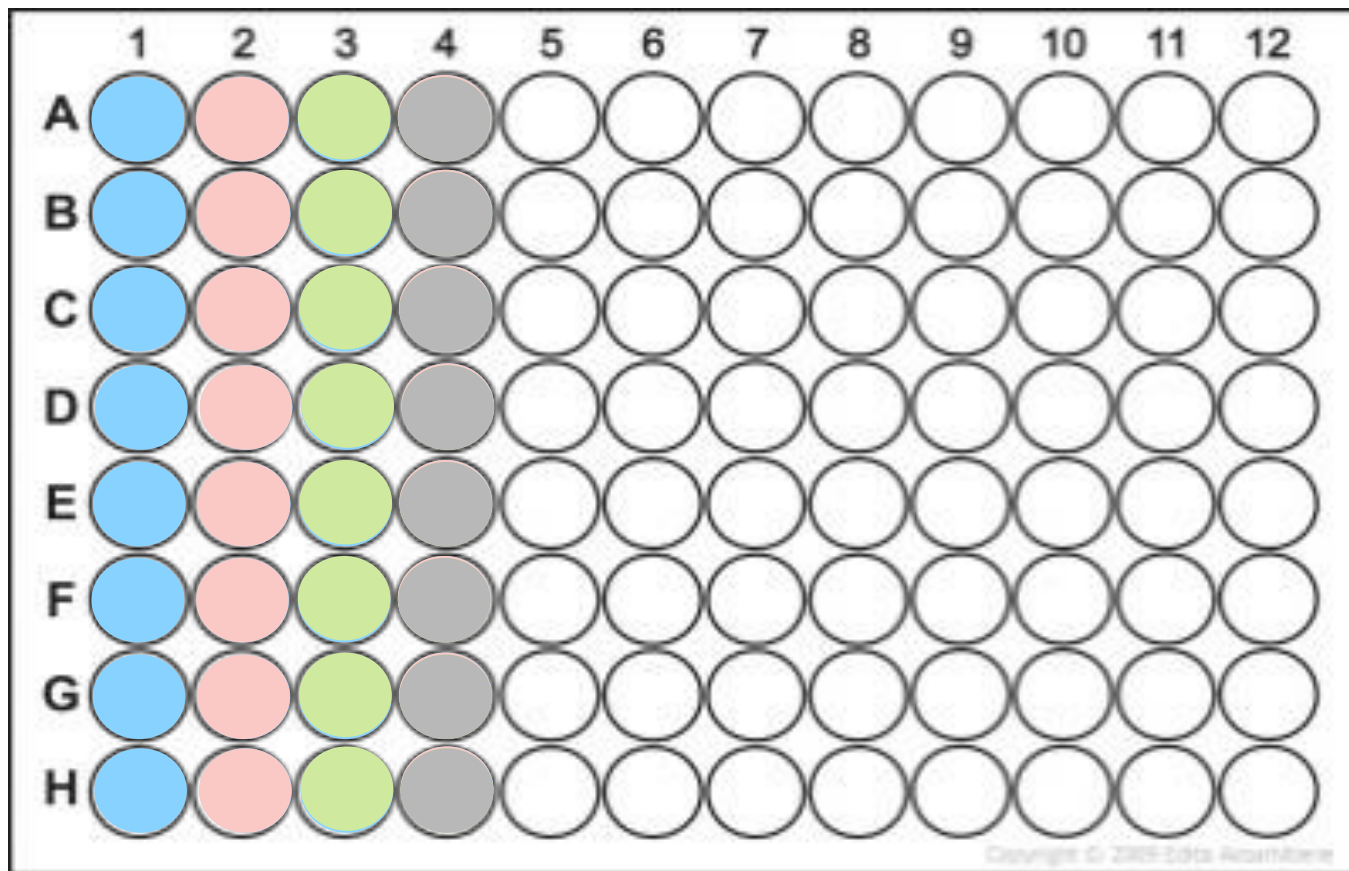


Automated Workflow for Deglycosylation & Reduction in DAR Characterization

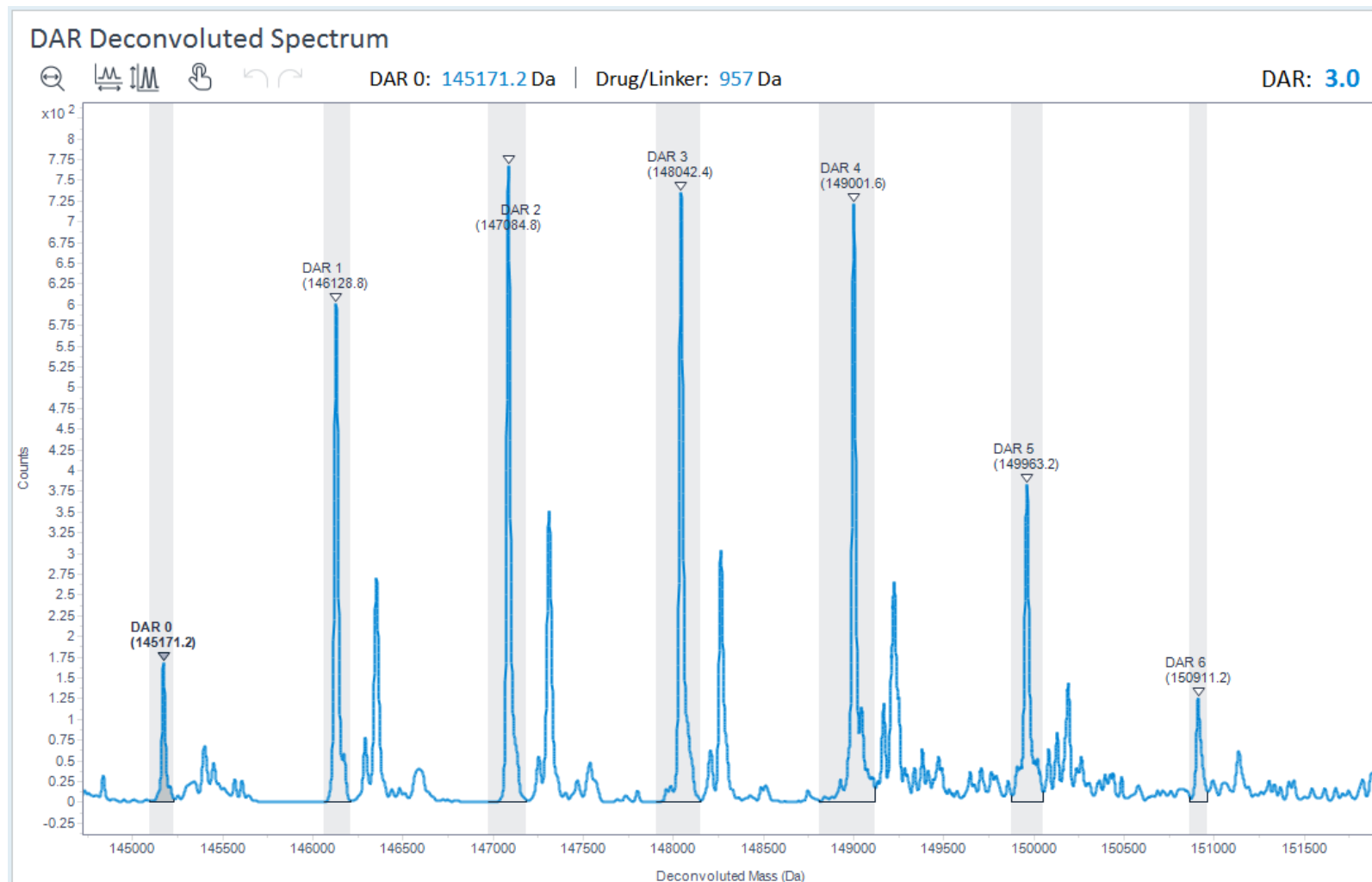


Sample Preparation: Using AssayMAP for DAR Calculation

Deglycosylation Reduction



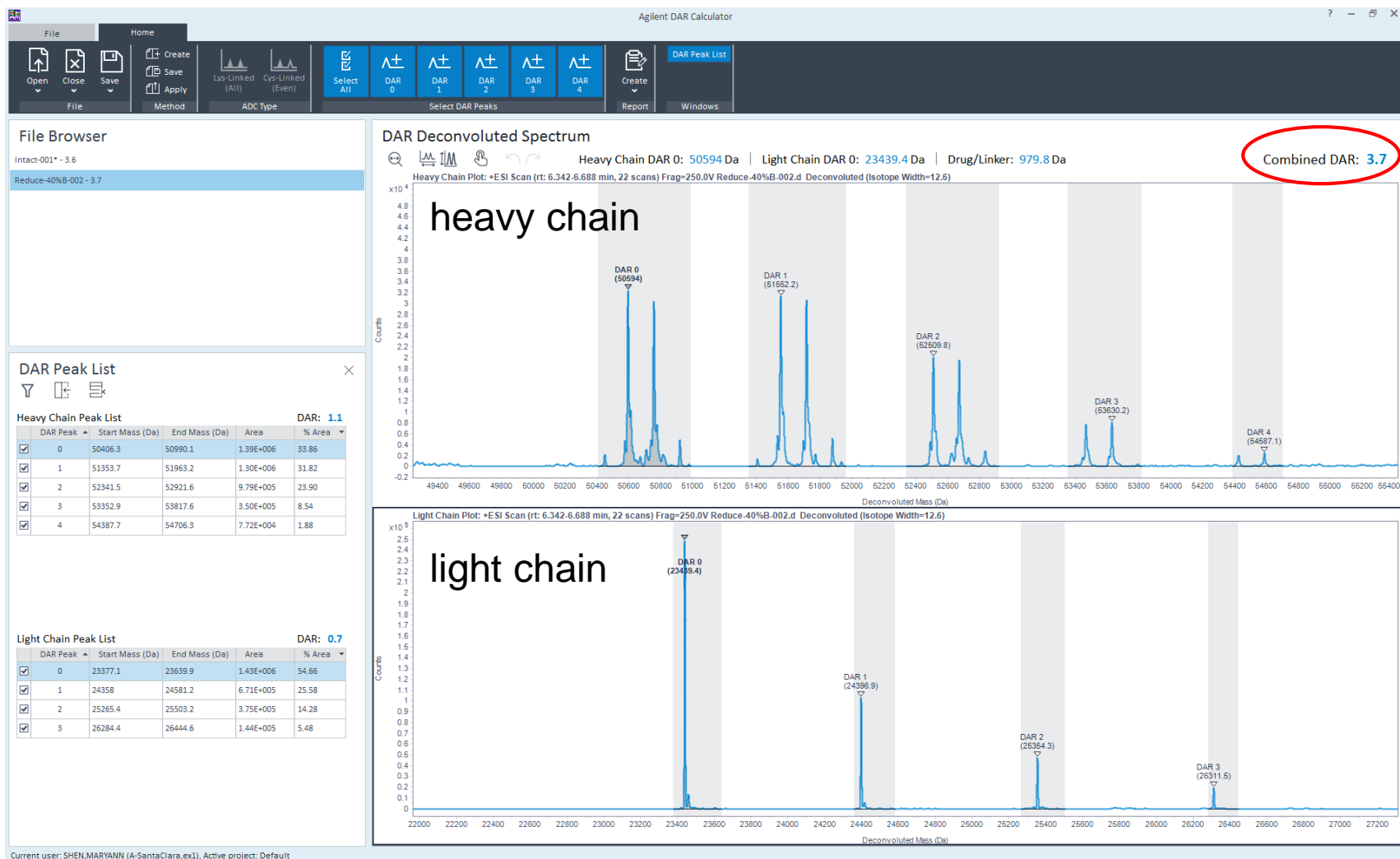
Deglycosylated ADC



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Reduced ADC DAR Calculation

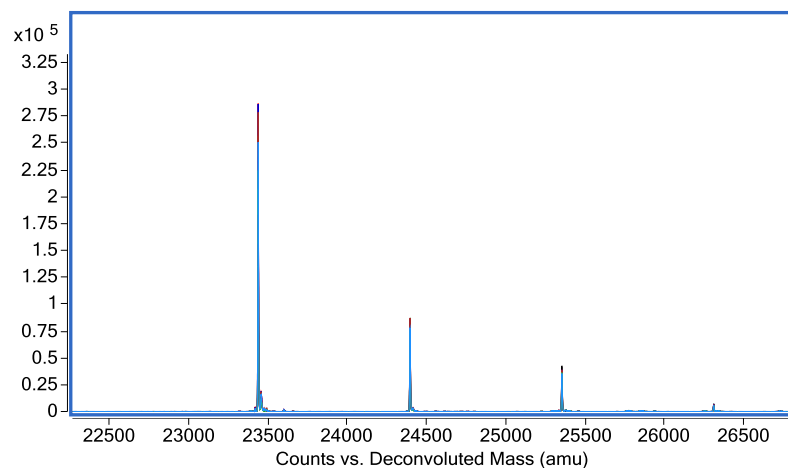


Agilent Technologies

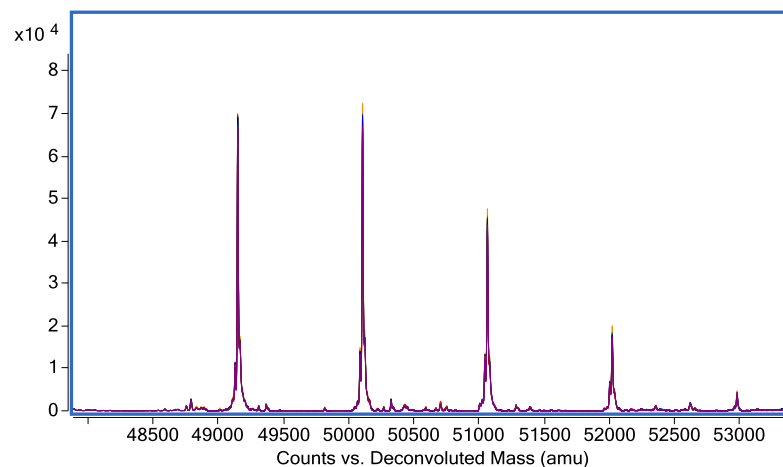
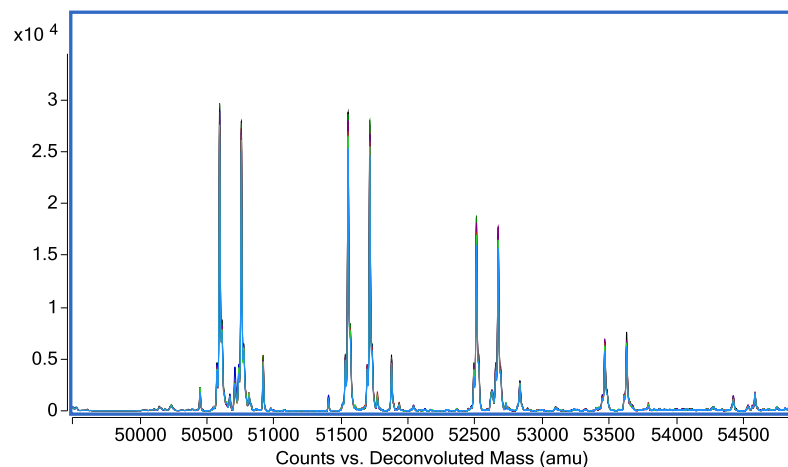
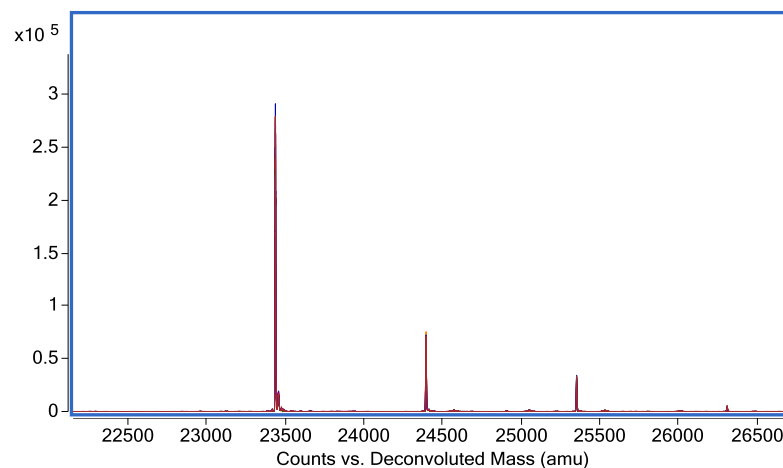
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Deglycosylated and Reduced ADC Analysis

Reduced



Deglycosylated and Reduced



DAR Report

DAR Report



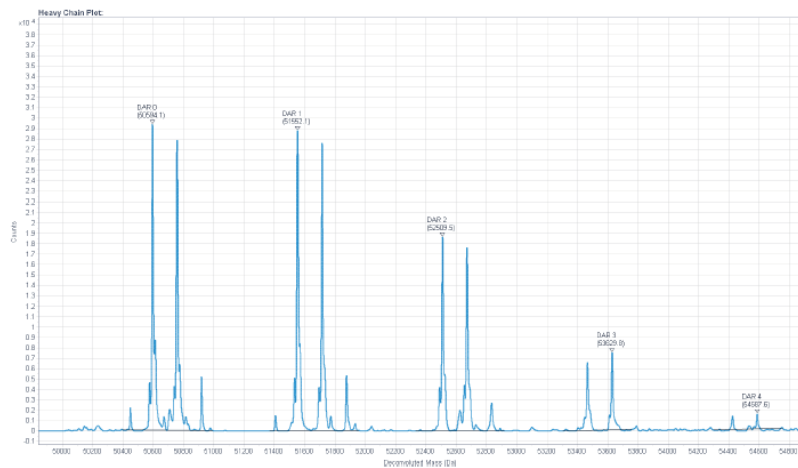
DAR Calculation Results

ADC Information

Data File	Reduce-20-75%Bin1min-001 - Copy	DAR	3.2
Processing Method	Reduced	Heavy Chain DAR	1.1
Report Time	9/10/2015 03:27 PM	Light Chain DAR	0.5
Property	Theoretical (Da)	Observed (Da)	
Heavy Chain DAR 0 Mass	Not Given	50594.1	
Light Chain DAR 0 Mass	Not Given	23439.3	
Drug/Linker Mass	Not Given	979.8	

Heavy Chain Results

Deconvoluted Spectrum

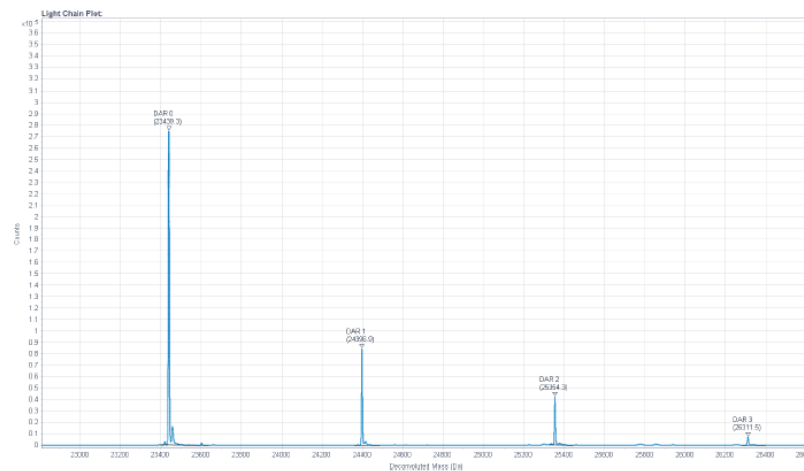


DAR Peak List

DAR Peak	Theoretical Mass (Da)	Observed Mass (Da)	Area	% Area
0	Not Given	50594.1	1.16E+008	34.36
1	Not Given	51552.1	1.13E+008	33.45
2	Not Given	52509.5	8.20E+005	24.17
3	Not Given	53629.8	2.66E+005	7.85
4	Not Given	54587.6	5.80E+003	0.17

Light Chain Results

Deconvoluted Spectrum



DAR Peak List

DAR Peak	Theoretical Mass (Da)	Observed Mass (Da)	Area	% Area
0	Not Given	23439.3	1.61E+006	64.54
1	Not Given	24398.9	5.31E+005	21.24
2	Not Given	25354.3	3.01E+005	12.05
3	Not Given	26311.5	5.44E+004	2.18



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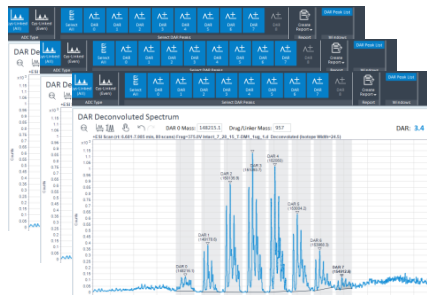
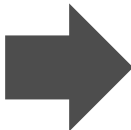
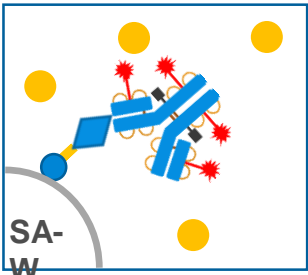
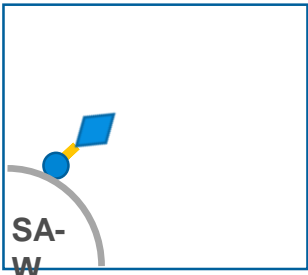
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Reproducibility of DAR Results with AssayMAP Enabled Sample Preparation

	Intact	Intact-deglyco	Reduced-glycosylated	Reduced-Deglycosylated
#1	3.6	3.9	3.7	3.8
#2	3.6	3.9	3.8	3.6
#3	3.6	3.9	3.8	3.8
#4	3.6	3.9	3.8	3.7
#5	3.6	3.9	3.7	3.8
#6	3.6	3.9	3.8	3.8
#7	3.6	3.8	3.7	3.8
#8	3.6	3.8	3.8	3.8
CV%	0	1.19	1.38	1.98
Mean	3.6	3.88	3.76	3.76



ADC DAR Determination Workflow for Serum Samples



Sample Preparation

LC/MS

**DAR
Calculation**

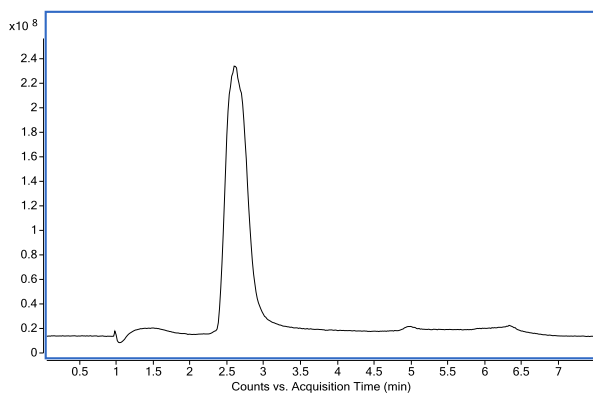


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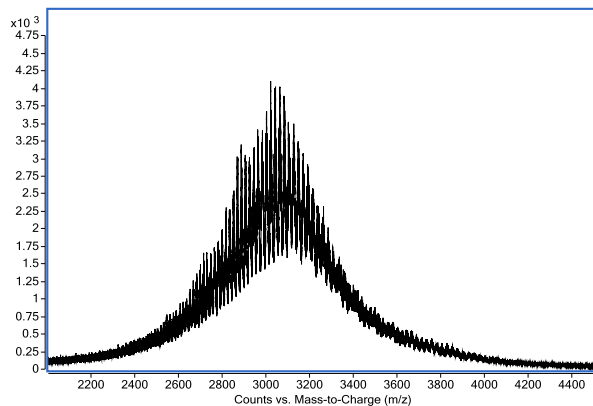
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Pure ADC Analysis and DAR Value

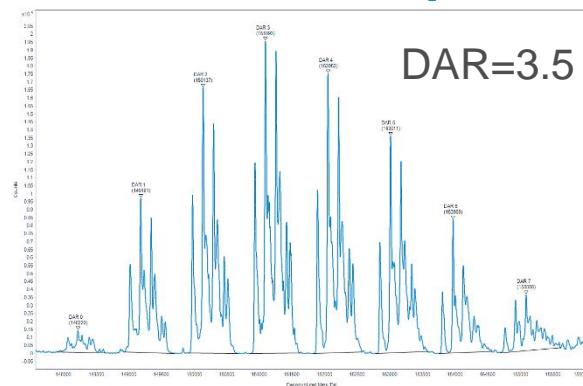
TIC



Extracted Spectra



Deconvoluted Spectra

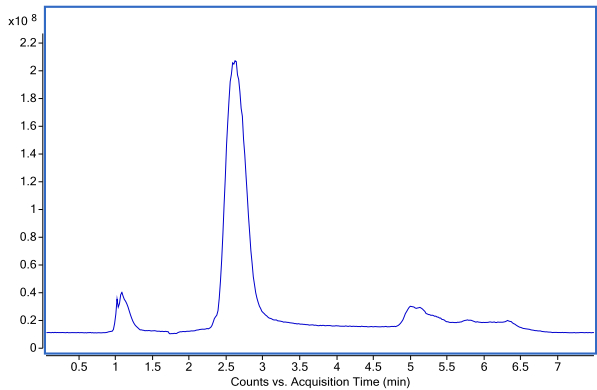


Sample	DAR	Percentage of Each DAR Species (%)							
		DAR 0	DAR 1	DAR 2	DAR 3	DAR 4	DAR 5	DAR 6	DAR 7
Directly Injected (n=4)	3.50±0.02	2.11±0.75	9.9±1.08	17.35±0.33	22.82±1.69	19.88±1.81	15.35±0.47	7.97±0.93	4.65±0.77

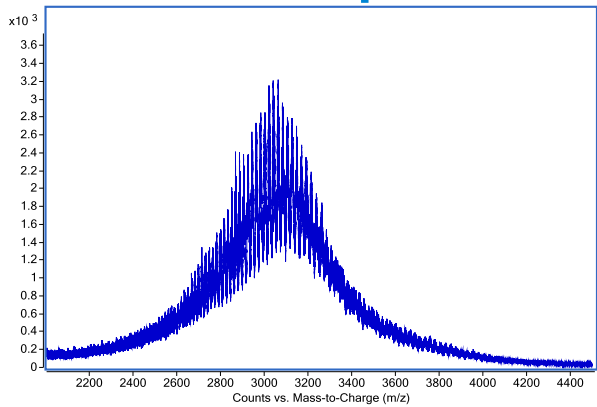


Purified ADC from Serum and DAR Value

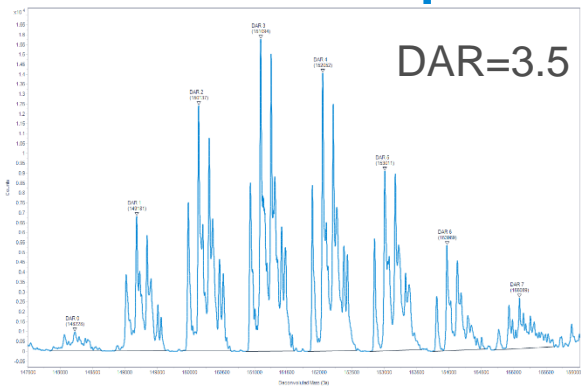
TIC



Extracted Spectra



Deconvoluted Spectra

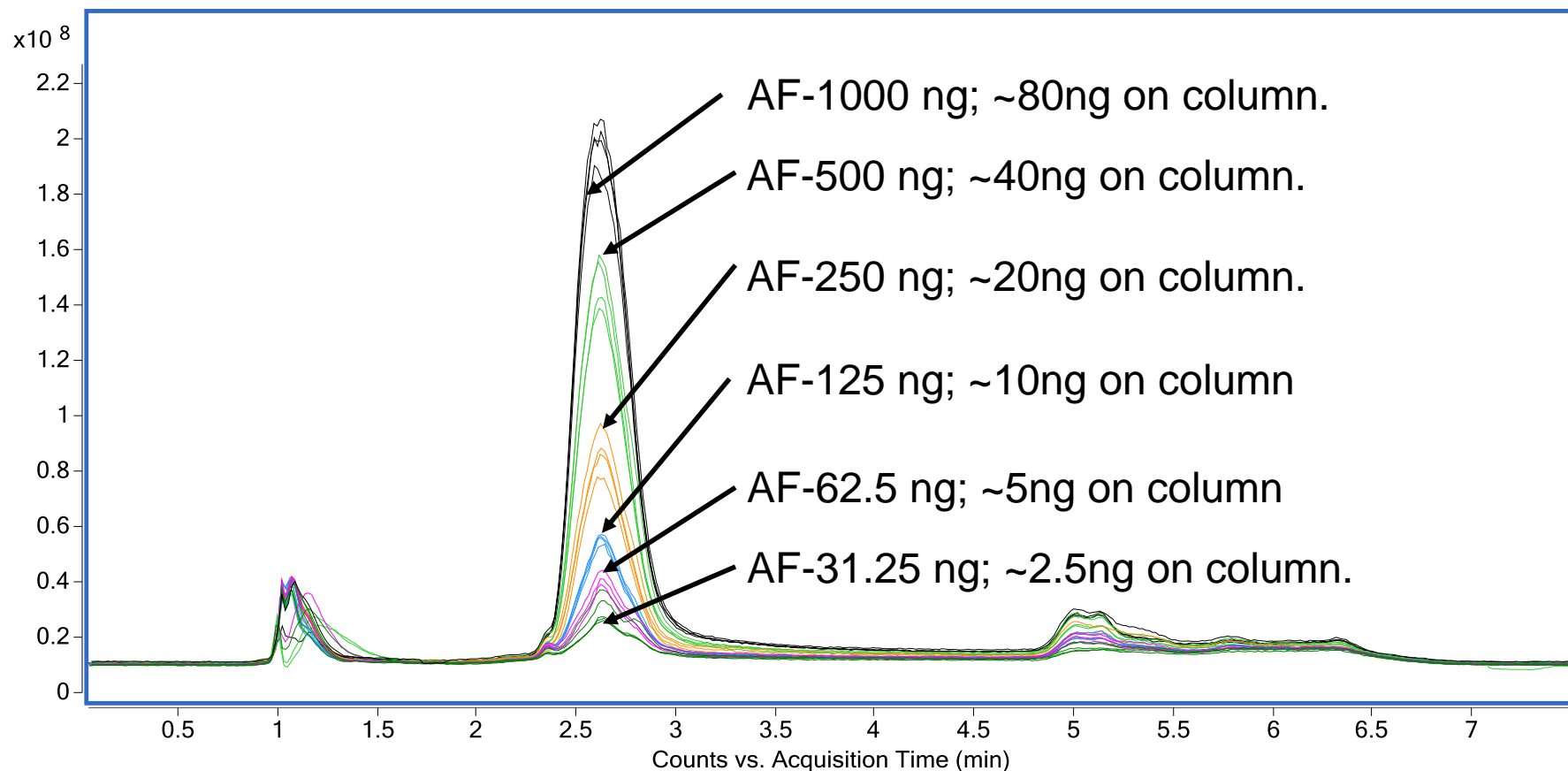


Sample	DAR	Percentage of Each DAR Species (%)							
		DAR 0	DAR 1	DAR 2	DAR 3	DAR 4	DAR 5	DAR 6	DAR 7
Affinity Purified 20 µg/mL in Rat Serum (n=4)	3.49±0.02	2.4±0.68	9.45±1.05	17.32±0.25	23.32±1.45	20.17±1.63	14.97±0.59	7.58±0.69	4.8±0.88



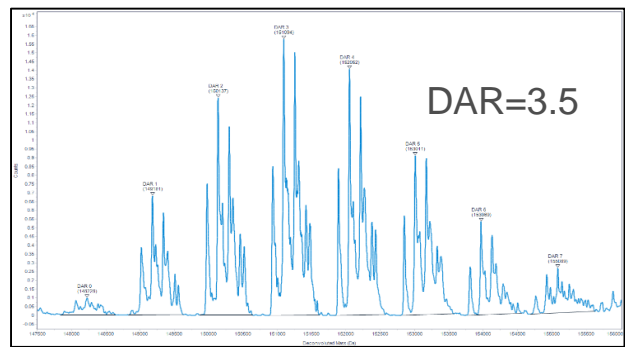
Reproducibility of Affinity Purified T-DM1 at 6 Different Loading Amounts

AF: Affinity purified

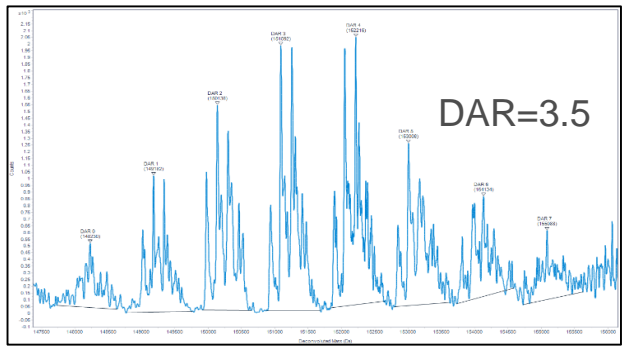


Affinity Purified T-DM1 at 6 Different Loading Amounts

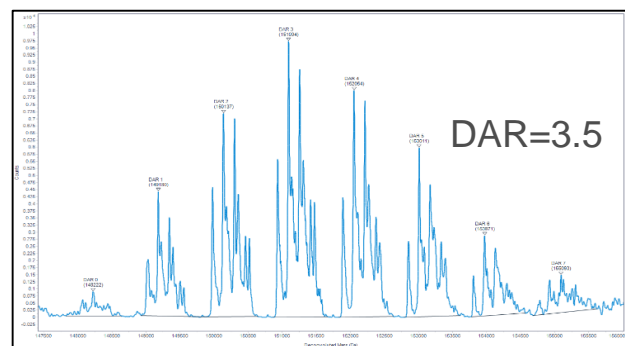
AF-1000 ng; ~80ng on column.



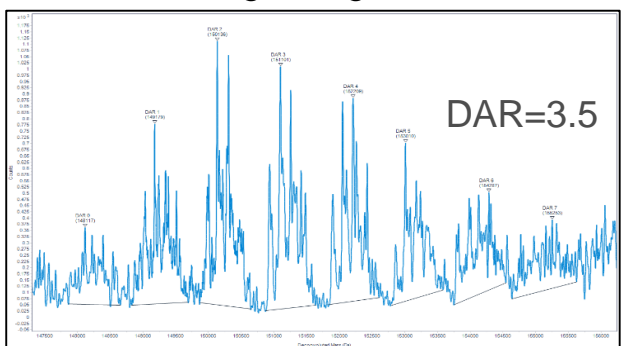
AF-125 ng; ~10ng on column



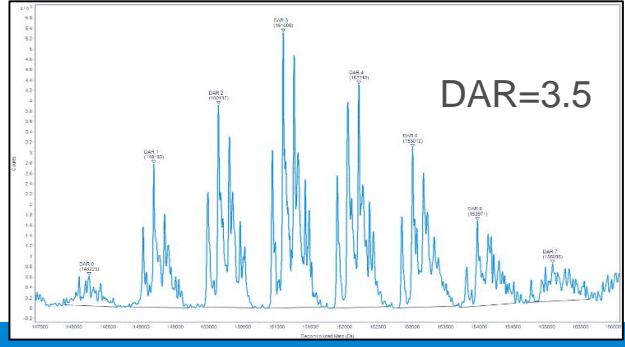
AF-500 ng; ~40ng on column.



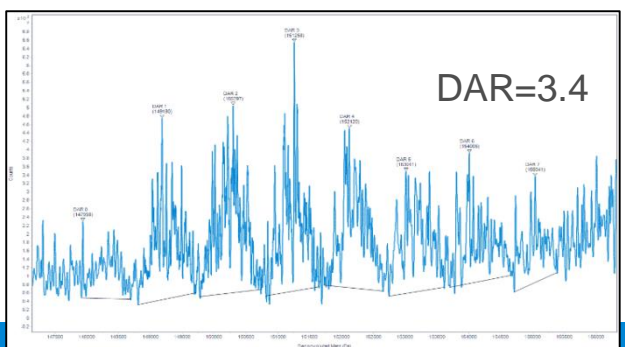
AF-62.5 ng; ~5ng on column



AF-250 ng; ~20ng on column.



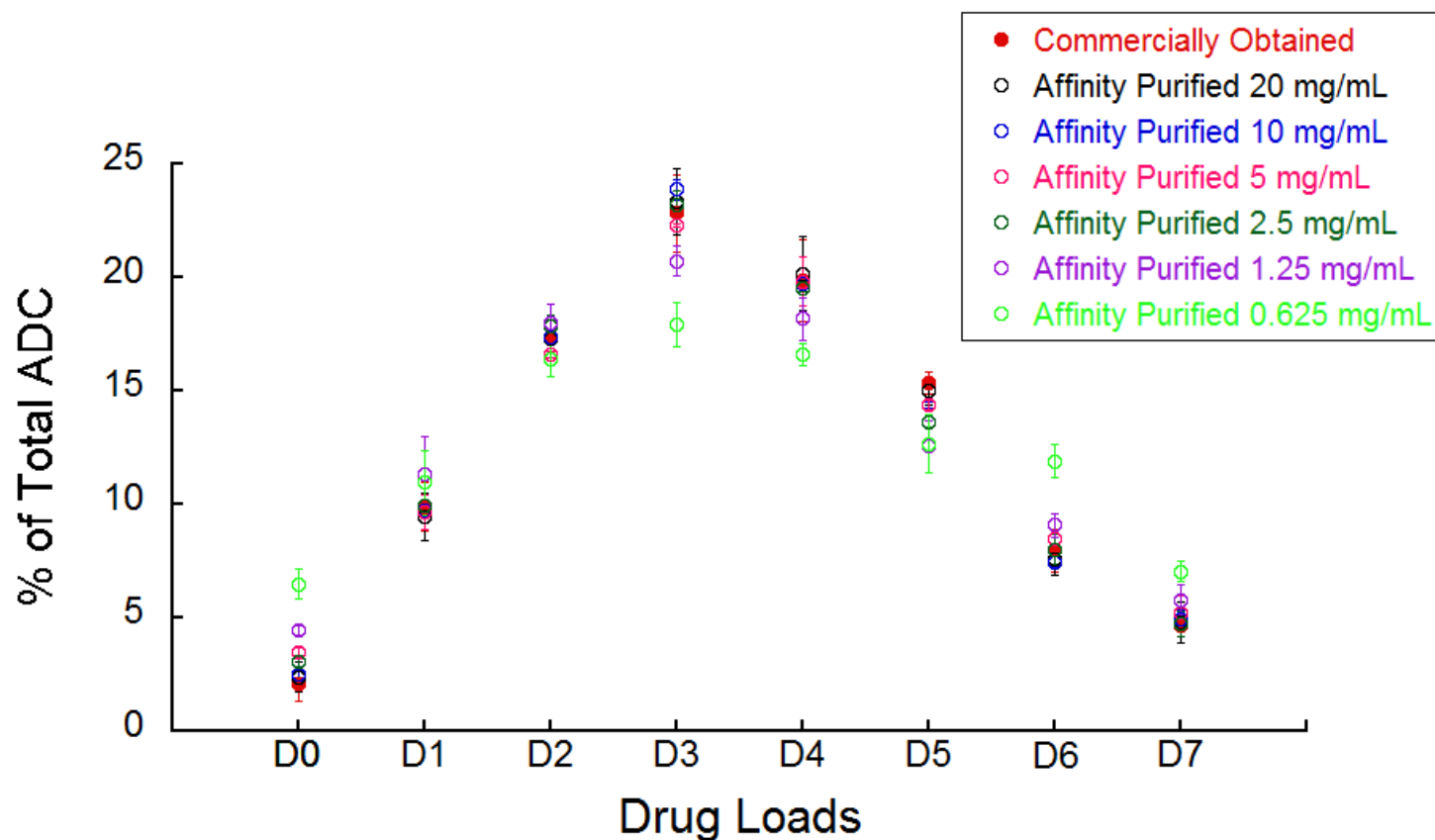
AF-31.25 ng; ~2.5ng on column.



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Percentage of ADC for Each Drug Load Analyzed at Different Concentrations



ADC Peptide Mapping Workflow Using AssayMAP Bravo and LC/MS/MS



Sample Preparation

LC/MS

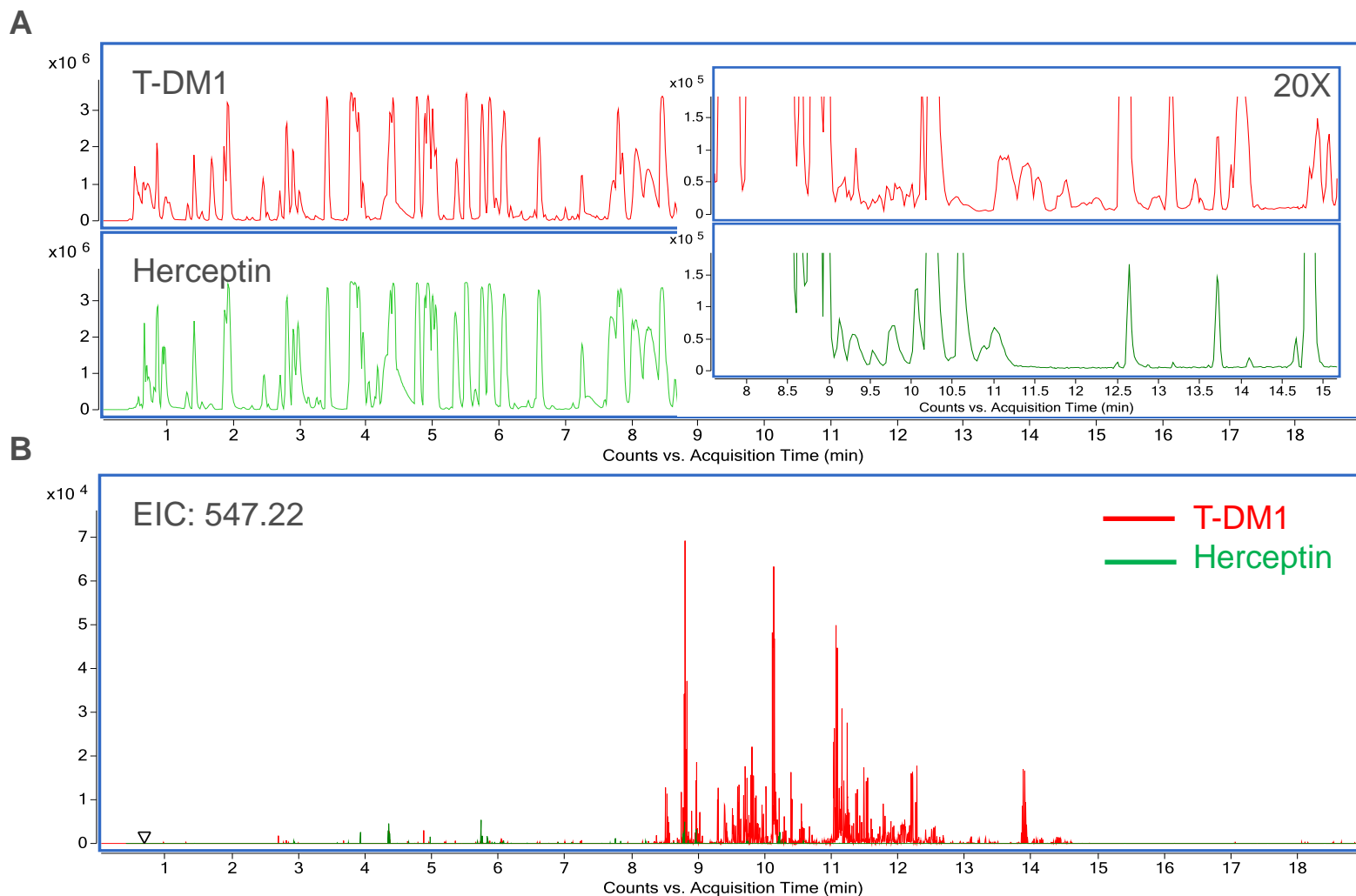
BioConfirm



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Identification of Conjugated Peptides



Using BioConfirm Qualitative Comparison to Compare Sample and Reference One-to-One

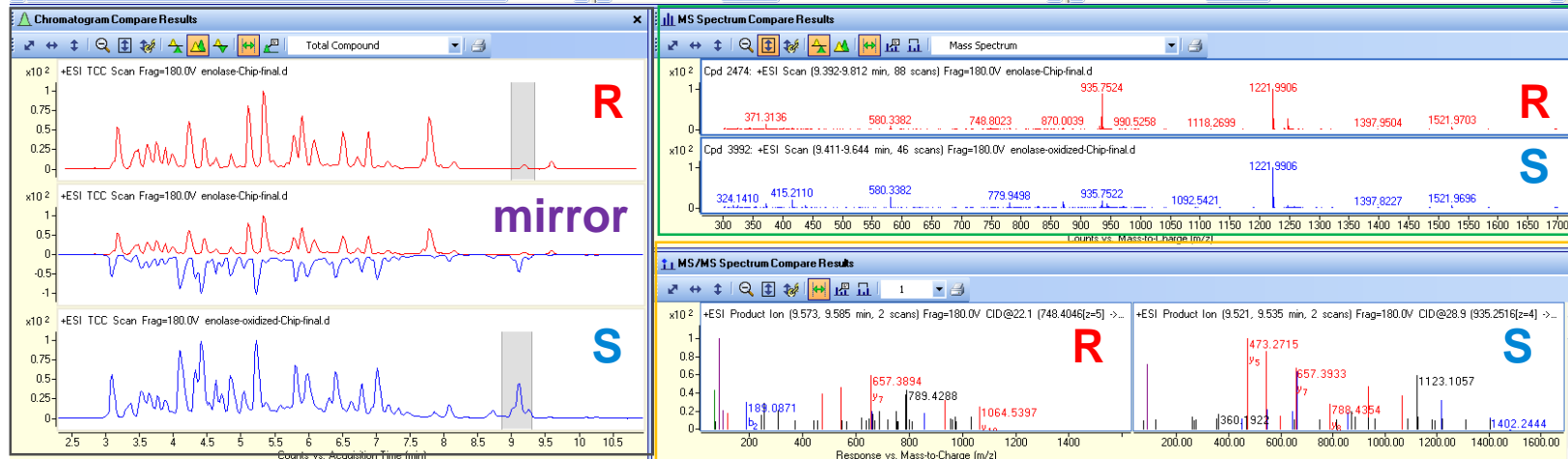
Agilent MassHunter Qualitative Comparison B.04.00 : enolase-Chip-final.d vs. enolase-oxidized-Chip-final.d

Compound Compare List

Found	Abundance Ra	Average Ma	Average RT	Fold Change Dir	RT Diff (D	Diff (Tgt. pp	Sequence	Pred Mods	Tgt Seq Mass	Vol	Overall Score
R, S	22.1	1855.921	4.497	Up Regulated	-0.152	2.49	SIVPSGASTGVHEALE...	1 st Oxidation[...]	1855.9098	1090160	68.08
R, S	1.2	1754.952	5.863	Up Regulated	-0.091	-1.04	TAGIQIVADDLTVTNPK		1754.9414	15905758	64.45
R, S	1.6	1415.7224	4.189	Up Regulated	-0.131	-0.71	GNPTVEVELTTEK		1415.7144	11299450	71.87
R, S	9.7	1887.9653	7.088	Up Regulated	-0.133	2.97	WLTGPQLADLYHSLMK	1 st Oxidation[...]	1887.9553	1766941	47.34
R, S	1.3	1577.8035	6.459	Up Regulated	-0.102	-0.4	AVDDFLSLDGTANK		1577.7937	13217935	12.62
R, S	0.9	2440.0193	5.289	Down Regulated	-0.118	-0.41				18232404	
R, S					-0.125	-0.91					63.94
R, S					-0.077	0.03	TSPYVL				66.16
R, S					-0.096	-0.58	LGAN				74.46
R, S					-0.09	-0.19					53.12
R, S					-0.094	-0.05					
R, S	1.5	813.5015	4.164	Up Regulated	-0.127	-0.07	AADALLK		813.496	6828317	46.74
R, S	1.2	806.4339	3.575	Up Regulated	-0.086	-0.41	TFAEALR		806.4287	8313948	49.77
R, S		1871.918	4.631								
R, S	0.5	2629.2177	6.837	Down Regulated	-0.096	0.28				12135837	
R, S	1.5	2327.0571	4.789	Up Regulated	-0.136	0.46	IEEELGDNVAFAGENF...		2327.0455	4085887	78.87
R, S	159	1754.8189	3.651	Up Regulated	0.001	2.2	DGKYLDLDFKNPNSDK		1754.8112	36408	52.5
R, S	3.2	1820.9232	8.122	Up Regulated	-0.084	0.84	SGFTFDTRIADIWGLR		1820.9156	1434780	84.24

Sequence	Pred Mods	Tgt Seq Mass	Vol	Overall Score
SIVPSGASTGVHEALEMR	1 st Oxidation(+15...	1855.9098	24073320	55.39
TAGIQIVADDLTVTNPK		1754.9414	18523838	72.77
GNPTVEVELTTEK		1415.7144	17893788	72.32
WLTGPQLADLYHSLMK	1 st Oxidation(+15...	1887.9553	17185340	40.87
AVDDFLSLDGTANK		1577.7937	16932412	65.46
			16926196	
VNIGI				65.25
TSPYVLPVFFLN...				76.35
LGANAILGV				80.85
AAGDSFAAGWGV				62.94
IGSE				50.55
AADALLK		813.496	9941130	55.17
TFAEALR		806.4287	9624650	51.61
SIVPSGASTGVHEALEMR	2 nd Oxidation(+15...	1871.9047	8378229	61.18
			6617986	
IEEELGDNVAFAGENFH...		2327.0455	6008103	75.38
DGKYLDLDFKNPNSDK		1754.8112	5788839	64.71
SGFTFDTRIADIWGLR		1820.9156	4588195	78.2

Table



MS spectra

Chromatograms

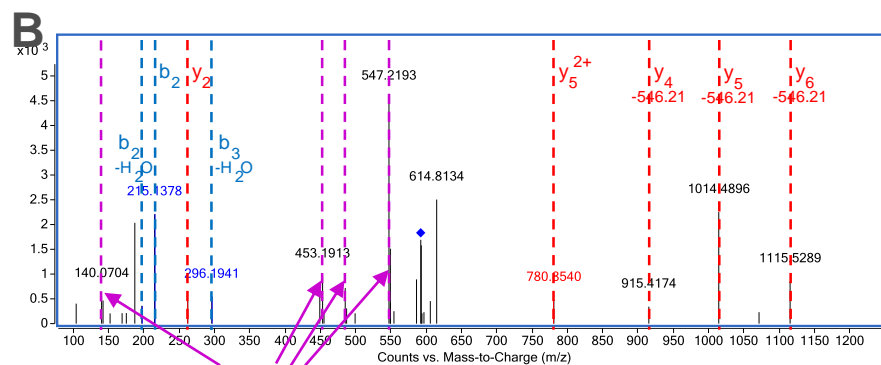
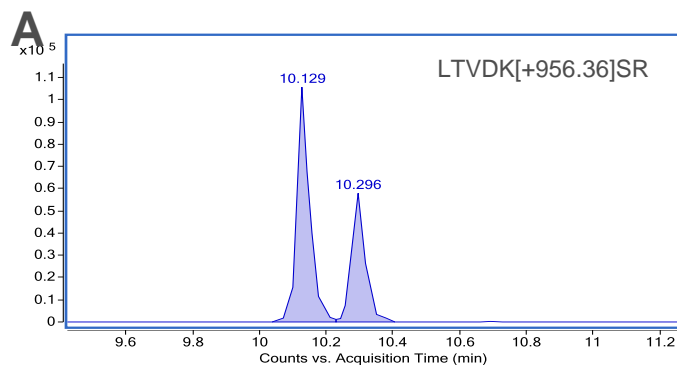
MS/MS spectra



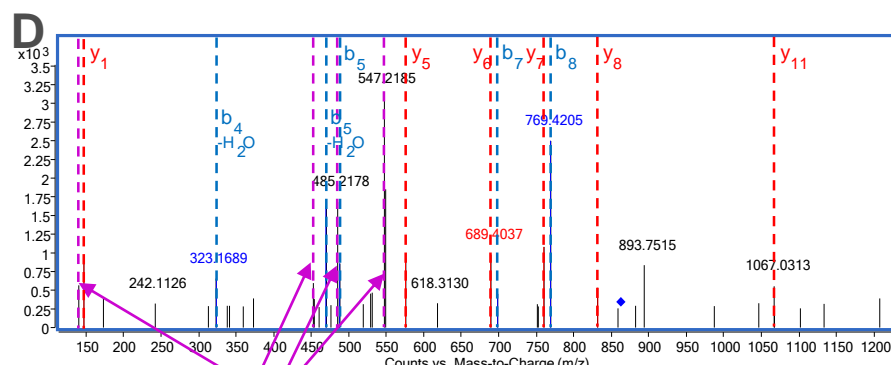
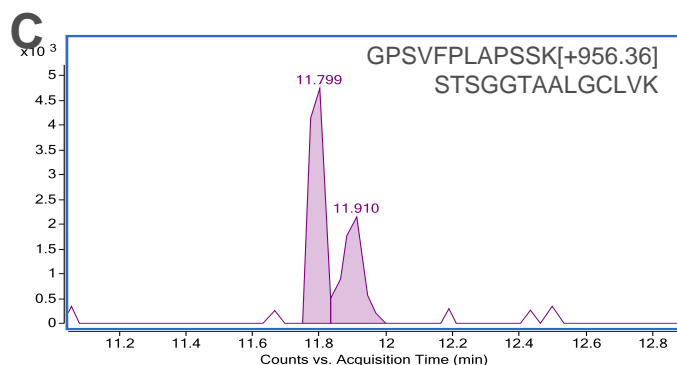
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Signature Elution Pattern and Fragment Ions



Drug Fragments



Drug Fragments

◆ Precursor
--- Drug
Fragment

-- y ion
--- b ion



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Sequence Coverage and Conjugation Sites Identification

Light Chain

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGS
RSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
VVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLSSTLTLSKADYEKHKVYACE
VTHQGLSSPVTKSFNRGEC

Heavy Chain

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHVVROAPGKGLEWVARIYPTNGYTRYADS
VKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGEYAMDYWGQGTIVTVSSASTKG
PSVFELAPSSKSTSGGTAALGCLVKDYFEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSSV
TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL
NGKEYKCKVSNKALPAPIEKTISKAGQPREPQVTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSEFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPG

K:	Confirmed drug conjugation site	---	29 (Light-9; Heavy 20)
K:	Potential drug conjugation site	---	5 (Heavy 5)
K:	Unconjugated site identified	---	10 (Light—4; Heavy 6)
Total		---	44 (Light-13; Heavy31)



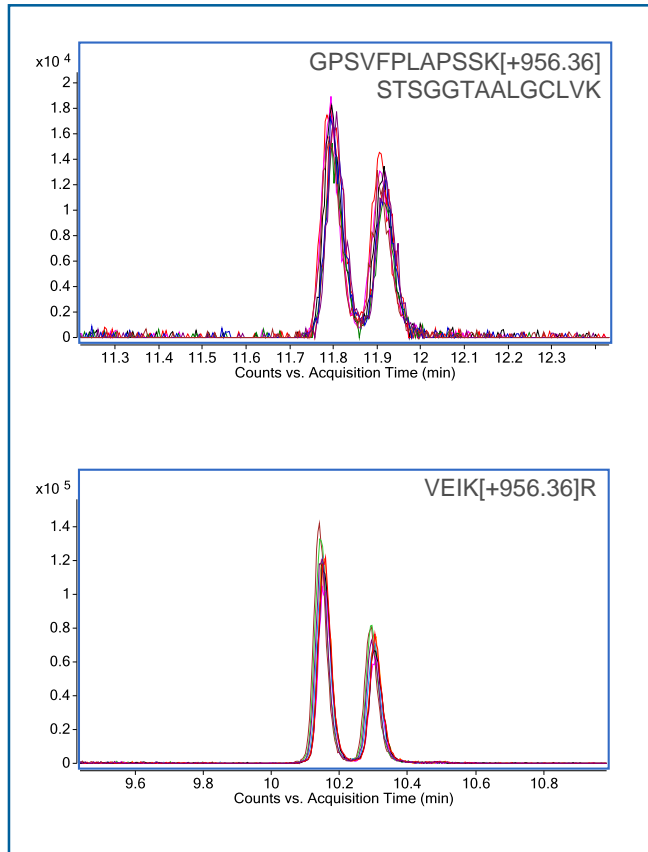
Conjugated Peptides Identification

No.	Chain	Seq Loc	Sequence	Modifications	Mass	Diff (Bio.ppm)	MS/MS Count
1	Light	25-45	ASQDVNTAVAWYQQKPGKAPK	1*Drug(+956.36444)A41	3242.5245	-4.82	1
2	Light	43-61	APKLLIYSASFLYSGVPSR	1*Drug(+956.36444)A44	3024.4894	-3.55	1
3	Light	104-108	VEIKR	1*Drug(+956.36444)A106	1599.7626	-2.24	3
4	Light	109-142	TVAAPSVFIFPPSDEQLKSGTASVCLNNFYPR	1*Drug(+956.36444); 1*Alkylation (iodoacetamide)(+57.021464)A125A133	4680.2396	-4.69	2
5	Light	143-149	EAKVQWK	1*Drug(+956.36444)A144	1843.8448	-3.31	1
6	Light	150-183	VDNALQSGNSQESVTEQDSKDSYLSSTLTLSK	1*Drug(+956.36444)A168	4575.0418	-5.4	1
7	Light	184-190	ADYEKHK	1*Drug(+956.36444)A187	1845.7912	-1.44	2
8	Light	189-207	HKVYACEVTHQGLSSPVTK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)A193A189	3096.4260	-3.87	1
9	Light	191-211	VYACEVTHQGLSSPVTKSFNR	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)A193A206	3335.5169	-3.51	1
10	Heavy	20-38	LSCAASGFNIKDTYIHWVR	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B22B30	3193.4581	-3.59	1
11	Heavy	39-50	QAPGKGLEWVAR	1*Drug(+956.36444)B43	2267.0663	-3.37	1
12	Heavy	60-67	YADSVKGR	1*Drug(+956.36444)B65	1850.814	-3.43	2
13	Heavy	99-136	WGGDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSK	1*Drug(+956.36444)B124	4907.1999	-2.29	1
14	Heavy	125-136	GPSVFPLAPSSK	1*Drug(+956.36444)B136	2142.0028	-0.46	3
15	Heavy	125-150	GPSVFPLAPSSKSTSGGTAAALGCLVK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B147B136	3444.6523	-3.41	1
16	Heavy	151-213	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTPVSSSLGTQT	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B203B208	7668.634	-4.9	6
17	Heavy	214-217	VDKK	1*Drug(+956.36444)B216	1444.6554	-3.4	1
18	Heavy	214-221	VDKKVEPK	1*Drug(+956.36444)(B216orB217orB221)	1897.9142	-2.56	1
19	Heavy	217-221	KVEPK	1*Drug(+956.36444)B217	1555.7243	-2.83	1
20	Heavy	222-251	SCDKTHTCPPCPAPPELLGGPSVFLFPPKPK	3*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B232B229B223B225	4289.9795	-4.61	3
21	Heavy	226-251	THTCPPCPAPPELLGGPSVFLFPPKPK	2*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B232B229B249	3799.799	-4.19	2
22	Heavy	226-258	THTCPPCPAPPELLGGPSVFLFPPKPKDTLMISR	2*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B232B229(B249orB251)	4616.2141	-3.68	2
23	Heavy	259-291	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B264B291	4753.1541	-3.07	3
24	Heavy	278-295	FNWYVDGVEVHNAKTQPR	1*Drug(+956.36444)(B291orB293)	3115.441	-4.72	1
25	Heavy	292-295	TKPR	1*Drug(+956.36444)B293	1456.6701	-0.95	1
26	Heavy	305-323	VSVLTVLHQDWLNGKEYK	1*Drug(+956.36444)B314B320	3183.5605	-1.27	2
27	Heavy	305-325	VSVLTVLHQDWLNGKEYKCK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B324(B320orB323orB325)	3471.6801	-2.89	3
28	Heavy	321-325	EYKCK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B324B323	1682.6981	-2.05	1
29	Heavy	324-329	CKVSNK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B324B325	1690.7356	-1.99	1
30	Heavy	326-337	VSNKALPAPIEK	1*Drug(+956.36444)B329	2222.0921	-2.98	1
31	Heavy	330-341	ALPAPIEKTISK	1*Drug(+956.36444)B337	2223.111	-3.67	3
32	Heavy	342-347	AKGQPR	1*Drug(+956.36444)B343	1611.7385	-1.53	1
33	Heavy	348-373	EPQVYTLPPSREEMTKNQVSLTCLVK	1*Alkylation (iodoacetamide)(+57.021464); 1*Drug(+956.36444)B370(B363orB373)	4002.899	0.75	1
34	Heavy	374-412	GFYPDSIAVEWESNGQPENNYKTPPVLDSDGSFFLYSK	1*Drug(+956.36444)B395	5354.3867	-1.08	1
35	Heavy	413-419	LTVDKSR	1*Drug(+956.36444)B417	1773.8267	-1.98	1

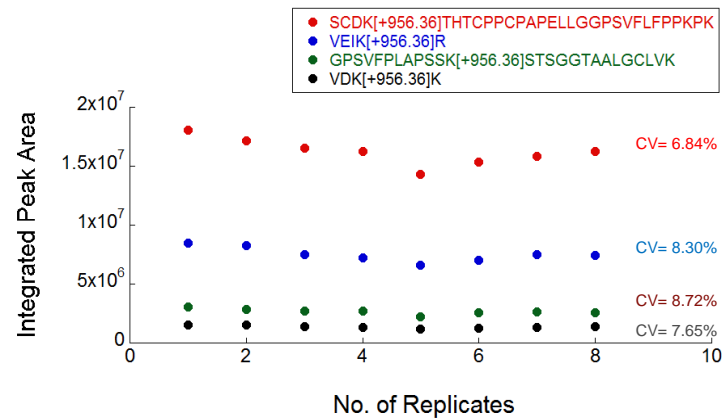


Reproducibility of Peptide Mapping Workflow

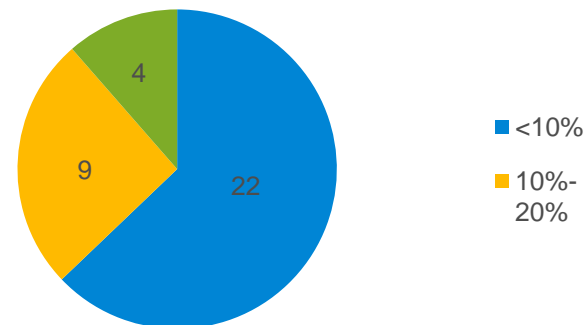
A



B



C



Summary

Determining ADC DAR is an important aspect of ADC characterization

- Workflow can be enabled in various ways
- New DAR calculator makes obtaining DAR value easy and straight forward
- Sample preparation can be streamlined and automated using the AssayMAP Bravo platform for reproducibility and scalability
- Localization of conjugation sites can be achieved by digesting the protein and analyzing by LC/MS/MS



Thank you for your attention!



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