Characterization of Antibody-Drug Conjugates using Affinity Enrichment and High-Resolution Mass Spectrometry

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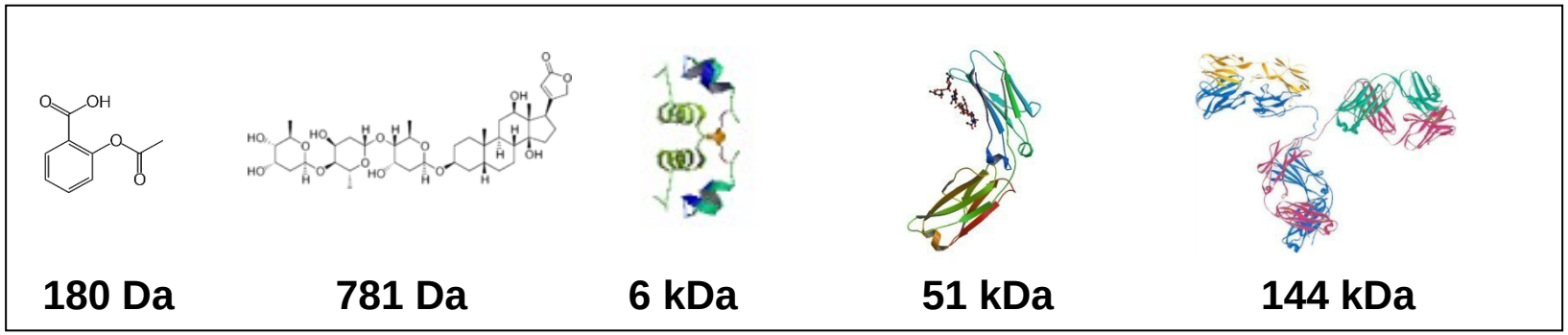
Different Types of Therapeutics

Small Molecules, Peptide and Protein Therapeutics

Acetylsalicylic acid | Digoxin | Insulin | Alefacept | Basiliximab



- Acetylsalicylic acid - 1899, Aspirin - Headache and pain
- Digoxin - 1785, Lanoxin - Atrial fibrillation, atrial flutter
- Insulin -1922 - Treating diabetes
- Alefacept - 2005, Amevive - Immunosuppressive function, psoriasis
- Basiliximab - 1998, Simulect - Immunosuppressive function, rejection in organ transplantation

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Small Molecule and Macromolecule Compounds

Comparison of Characteristics

Characteristics	Small Molecule	Macromolecules
Size	Small< 1000 Da	Large> 5000 Da
Structure	Organic molecules	Biopolymers, (amino acids)
Purity	Homogeneous	Heterogeneous
Solubility	Often hydrophobic	Often hydrophilic
Stability	Chemical	Chemical, physical and biological
Presence in matrix	Xenobiotic	Endogenous
Synthesis	Defined	Biological production
Metabolism	Defined	Defined

Antibody Drug Conjugate

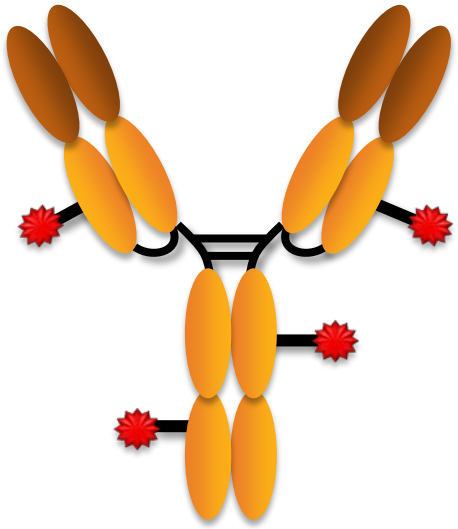
Structure and characteristics

Antibody

- The antibody maintains characteristics when linked to the requisite number of cytotoxic molecules.
- Targeted at an antigen found only on target cells, minimal non-specific binding.

Payload

- Highly potent cytotoxin,100-10 000 times more compared to standard chemotherapies.
- When conjugated to the antibody the cytotoxin is inactive.



Linker

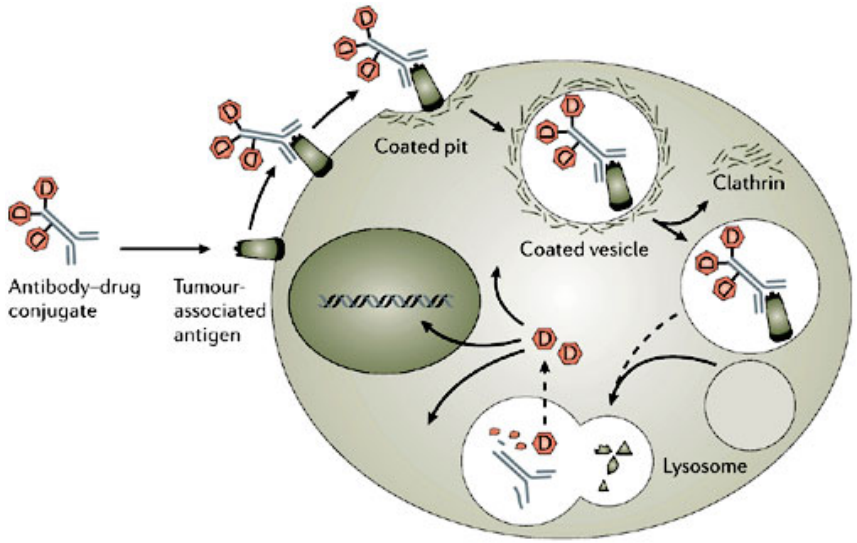
- Stabile until reaching the target to ensure intact ADC.
- Affects both safety and anti-tumor activity.

ADC pharmacokinetics are dependent on all three components

Antibody Drug Conjugate

Mode of action

- The mAb binds to the tumor associated antigen
- The ADC is internalized by receptor mediated endocytosis
- The cytotoxin is released in the lysosome
- Free cytotoxin acts via several mechanisms e.g. interactions with microtubuli, DNA, RNA and RNA polymerase)



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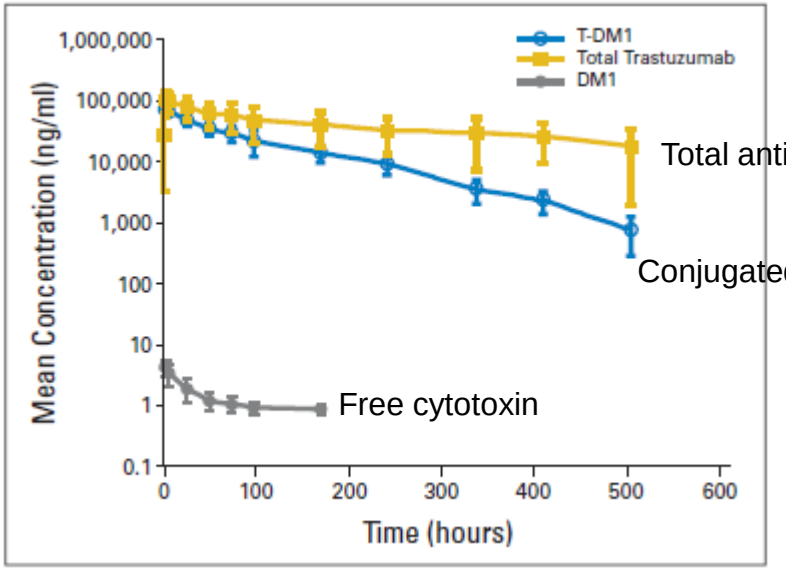
Bioanalytical Strategy for pre-clinical studies:

PK assays for ADCs

- Free cytotoxin
- Free total cytotoxin, (cytotoxin+catabolites)
- Total antibody – measures total amount of antibody, both conjugated and non-conjugated
- Conjugated antibody
- ADC characterization: DAR



Krop *et al* J Clin Oncol 2010 28:2698-2704.

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Total antibody determination in pre-clinical species

Humanized mAbs – universal peptides

GPSVFPLAPSSK
Universal peptide IgG1
(Law *et al*, in press)

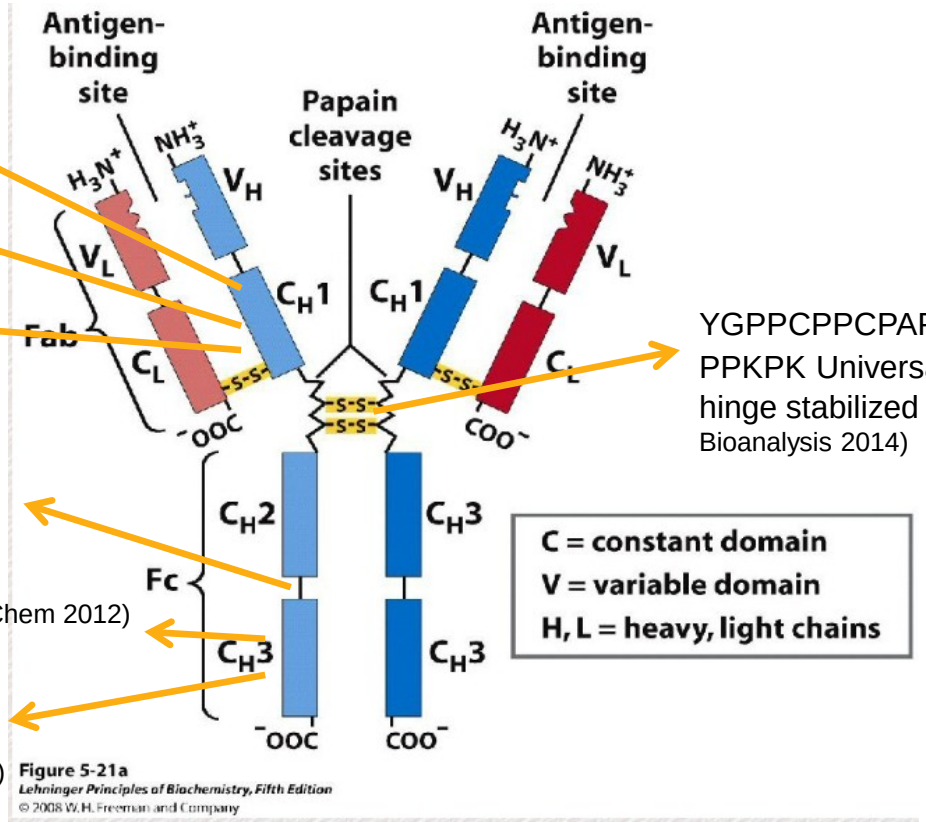
SGTASVVCLLNNFYPR
(Li *et al*, Anal Chem 2012)

TVAAPSVFIFPPSDEQLK
(Furlong *et al*, Bioanalysis 2013)

VVSVLTVLHQDWLNGK
Universal peptide
IgG1/IgG4
(Furlong *et al*, Bioanalysis 2012)

NQVSLTCLVK (Li *et al*, Anal Chem 2012)

TTPPVLDSDGSFFLYSK
Universal peptide IgG1/
(Furlong *et al*, Bioanalysis 2012)



YGPPCPPCPAPEFLGGPSVFLF
PPKPK Universal peptide for
hinge stabilized IgG4 (Furlong *et al*,
Bioanalysis 2014)

- For the total ADC assay and conjugated assay, peptide mapping must be performed to ensure no linker and payload is attached to the selected universal peptide.

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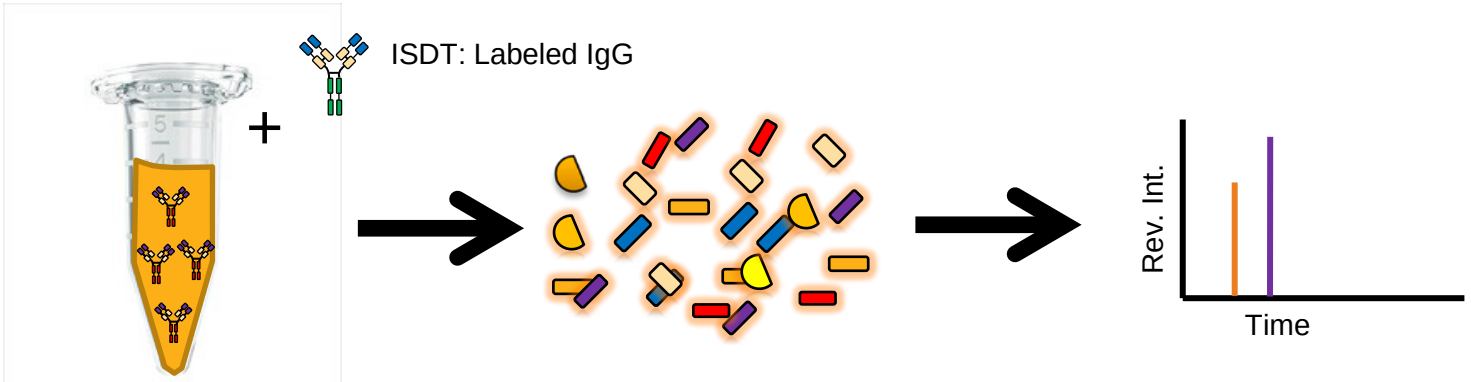
Total antibody determination

Total LC-MS/MS assay based on universal peptide quantification

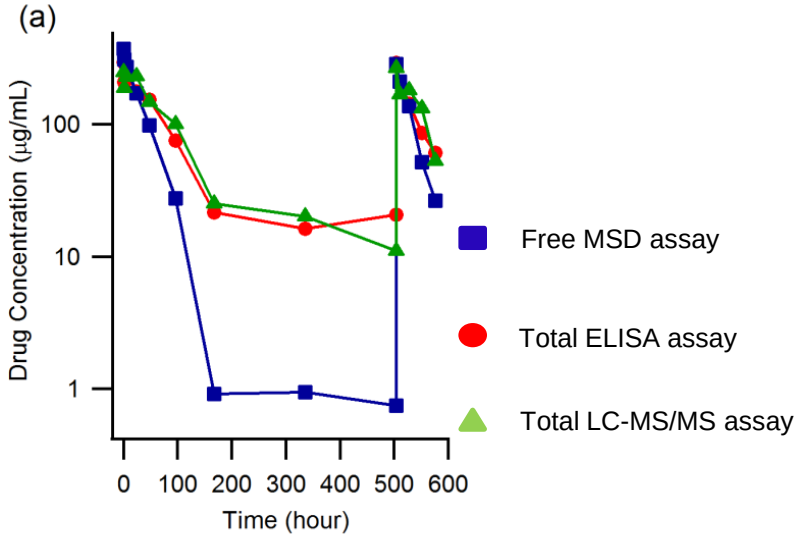


Sample from pre-clinical study

Tryptic digest of matrix+ ADC+ ISTD

Monitor quantifier and qualifier peptide

Total antibody assay: PK profile:



Law *et al*, in press

A generic total LC-MS/MS method gave similar results as for the ELISA method

In dose range finding studies, early tox studies, an LLOQ of 1-10 µg/mL of the ADC can be expected.

However, for follow-up studies lower LLOQ can be required to cover the entire PK profile.

	Total-LC-MS/MS
Dynamic range	10,000-150,0000 ng/mL
Sensitivity	10 µg/mL
Dilution linearity	20 mg/mL (1:20)

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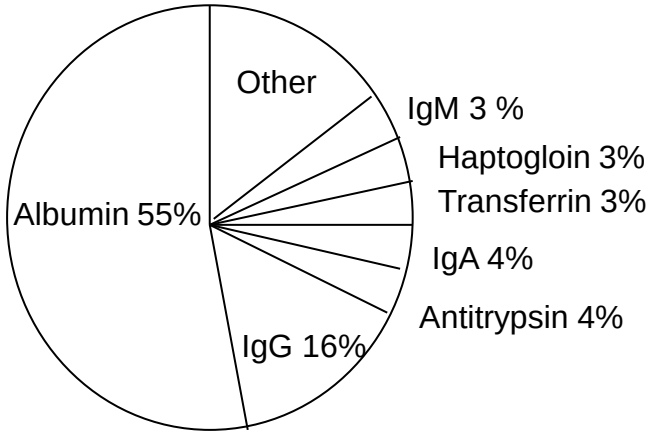
Dynamic Range and Composition of Serum

How to improve the LLOQ?

Serum protein and peptide concentration

Albumin, IgGs, Transferrin, IgA	mg/mL
C reactive peptide	µg/mL
Myelin basic protein	ng/mL
Interleukins, Interferons	pg/mL

Serum protein content



Improvement of LC-MS/MS assay sensitivity

- Targeted depletion of abundant proteins. Digestion and quantification of selected peptide.
- Two step sample clean up (orthogonal modes). Digestion and quantification of selected peptide.
- Targeted enrichment of target protein with immuno-precipitation. Digestion and quantification of selected peptide.

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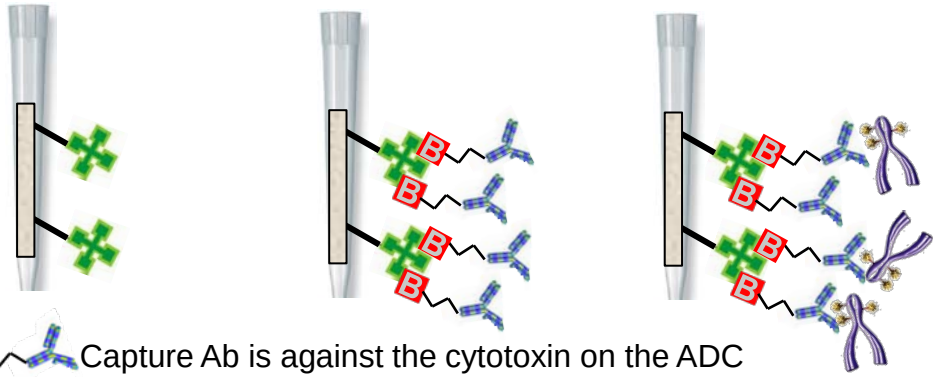
Conjugated Antibody determination

Assay: Immunoprecipitation LC-MS/MS and universal peptide quantification

MSIA Streptavidin Assay

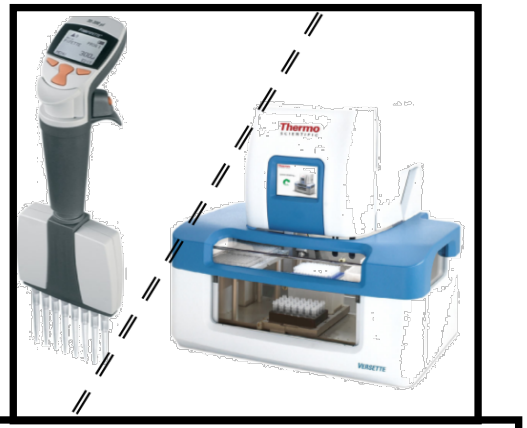
Start → Capture → Enrich → Elute → Digest → SPE → LC-MS/MS on universal peptide



ISDT: Spike labeled universal peptide

Capture Ab is against the cytotoxin on the ADC

ADC, target analyte



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	Immunoprecipitation LC-MS/MS
Dynamic range	25-2000 ng/mL
Sensitivity	25 ng/mL
Dilution linearity	2 mg/mL (1:5000)

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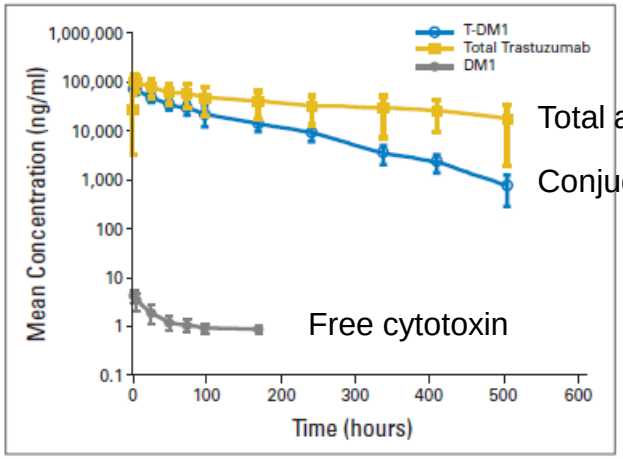
ADC characterization

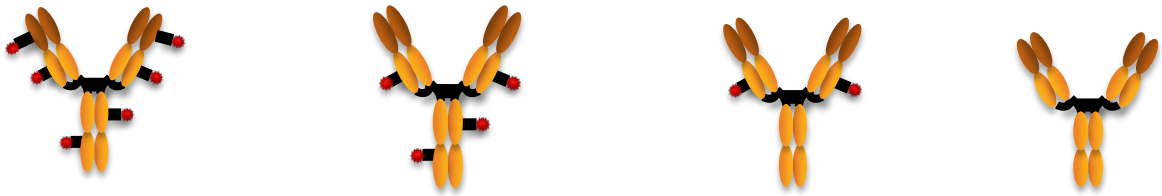
Measurement of the drug to antibody ratio (DAR)



Krop *et al* J Clin Oncol 2010 28:2698-2704.

- As the ADC releases the cytotoxin, the concentration of the conjugated antibody is decreasing.
- The release of cytotoxin changes the drug to antibody ratio (DAR).

Drug to antibody ratio (DAR)



D6 D4 D2 D0

Drug = 12
Antibody = 4
DAR = 3

Intact ADCs are measured by high resolution mass spectrometry to determine the DAR

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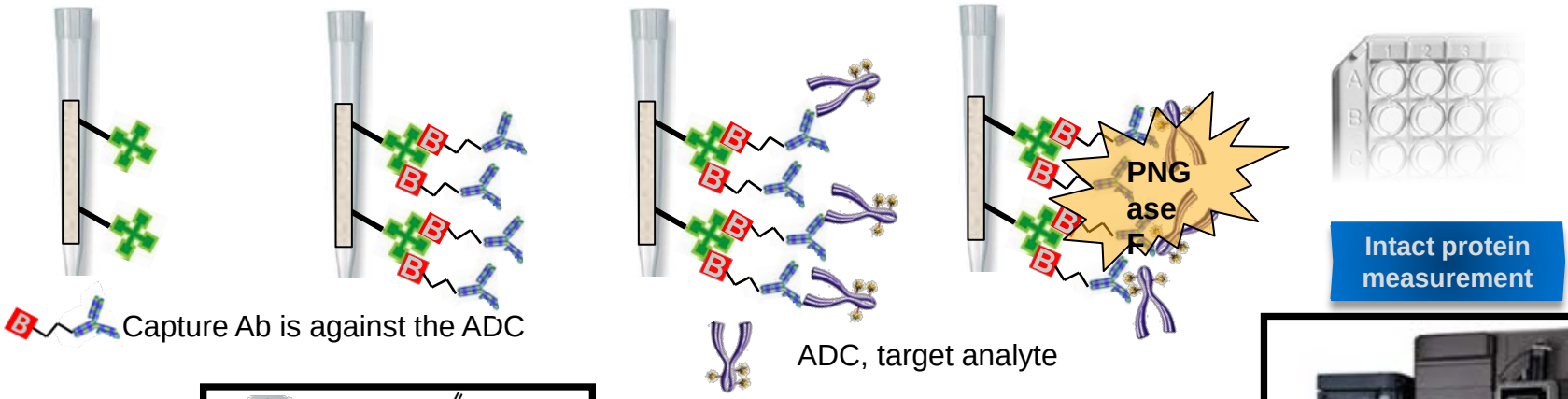
ADC characterization

Measurement of the drug to antibody ratio (DAR) - workflow

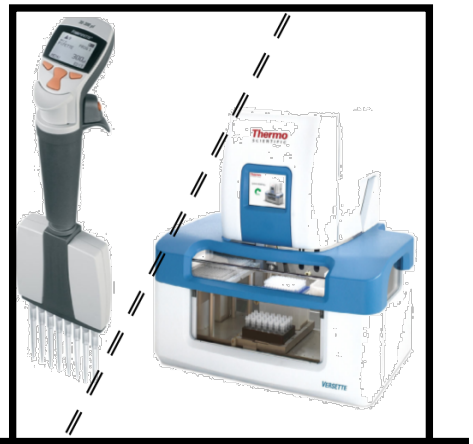
MSIA Streptavidin Assay

Start → Capture → Enrich → Deglycosylation → Elute



Capture Ab is against the ADC

ADC, target analyte



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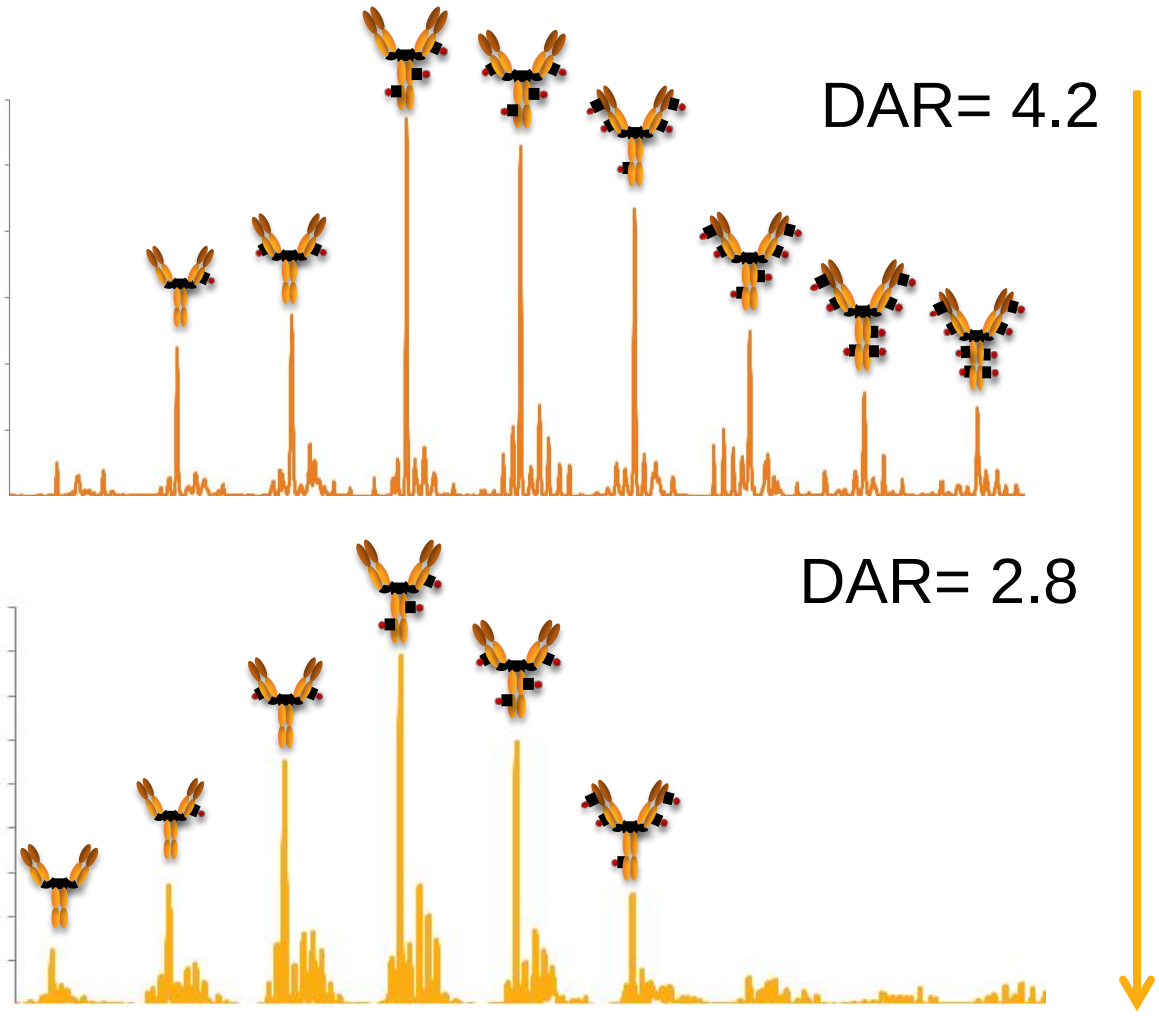
Drug to Antibody Ratio (DAR)

Determination of DAR

- After deconvoluting the obtained mass spectra, the DAR can be determined.
- The DAR can be determined using either the peak high or peak area.
- The drug release process can be monitored by change in the DAR



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Summary

- Antibody Drug Conjugates (ADC) consists of three components: the antibody, the linker and the payload.
- The pharmacokinetics of the ADCs is dependent on all three components, therefore several assays have to be developed to support a PK study.
- The total antibody assay is based on the quantification of a universal peptide from the constant region of the ADC.
- For the conjugated assay, immunoprecipitation of the toxin on the ADC is applied prior to digestion and quantification of the universal peptide.
- The release of the cytotoxin can be seen by a change in the drug antibody ratio (DAR). The DAR is determined by high resolution mass spectrometry.

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