



Validation of a biologic with ligand-binding-LC-MS/MS assay in monkey and mouse serum

C. Hunger

R&D, DMPK FF

Introduction and Overview

- Validation of a Fab in mouse and monkey serum



Monoclonal
Antibody: mAb



Single arm Ab



Modified Fab



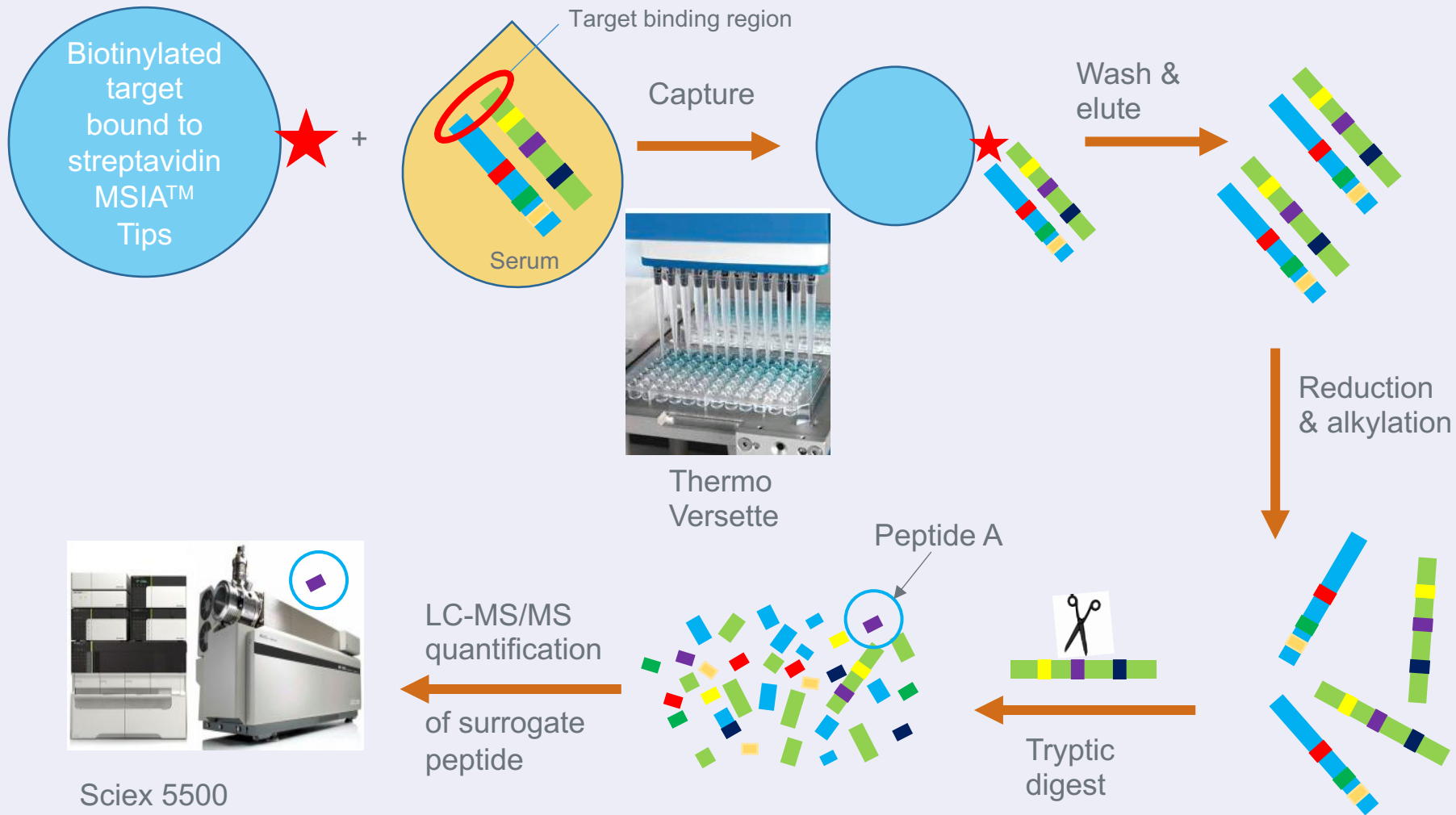
Fab

150 kDa

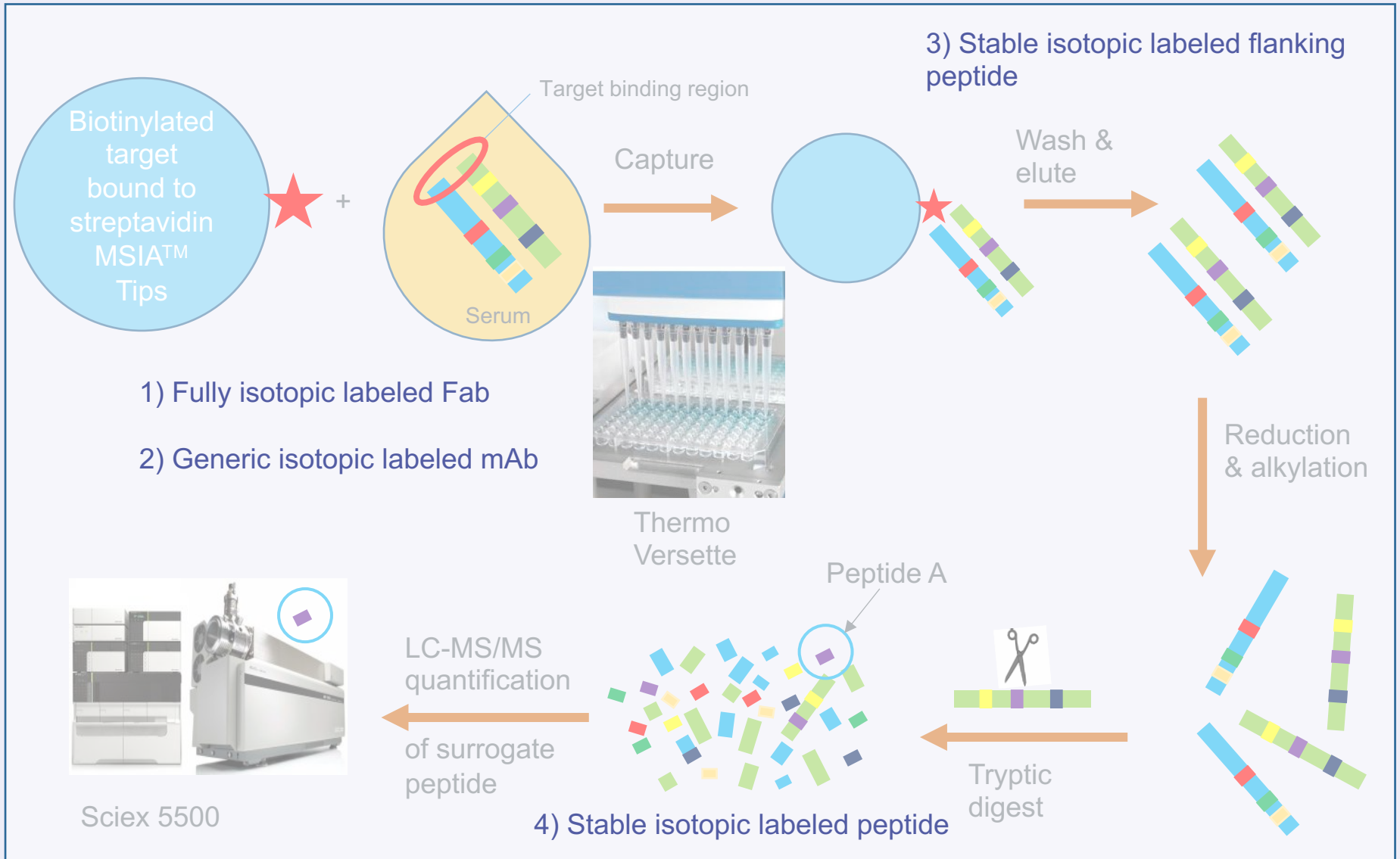
50 kDa

- Introduction of assay format
- „Thoughts“ about the internal standard
- Presentation of validation results
- Let's have a closer look on the acceptance criteria!
- Conclusions

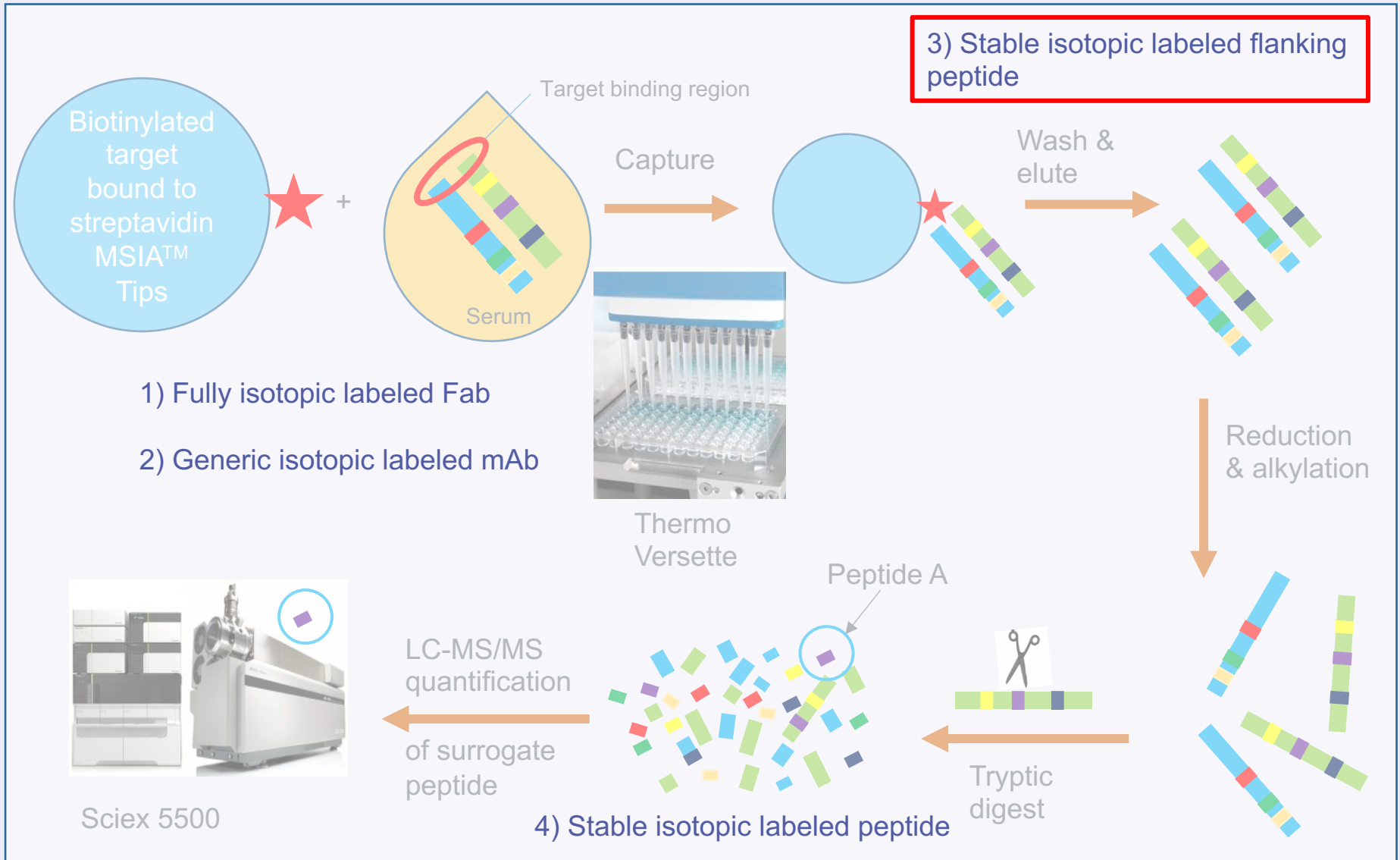
Assay format



Assay format

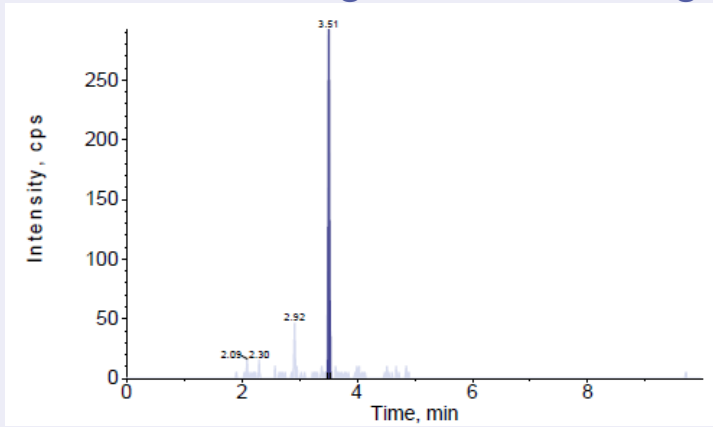


Assay format

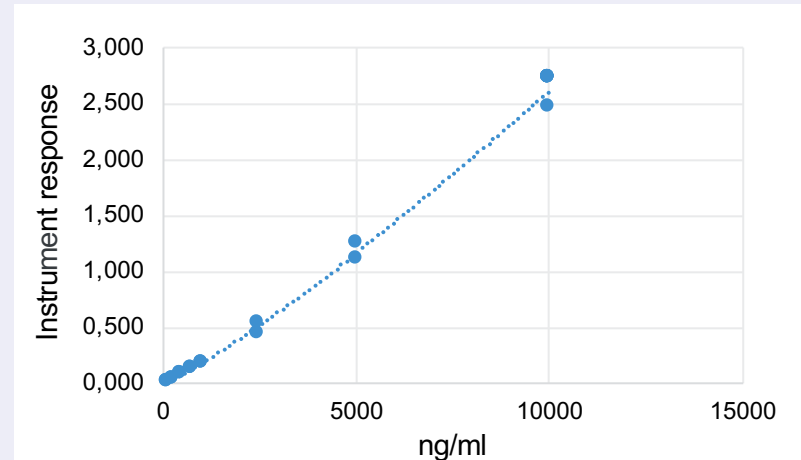


LC-MS/MS assay, example mouse serum

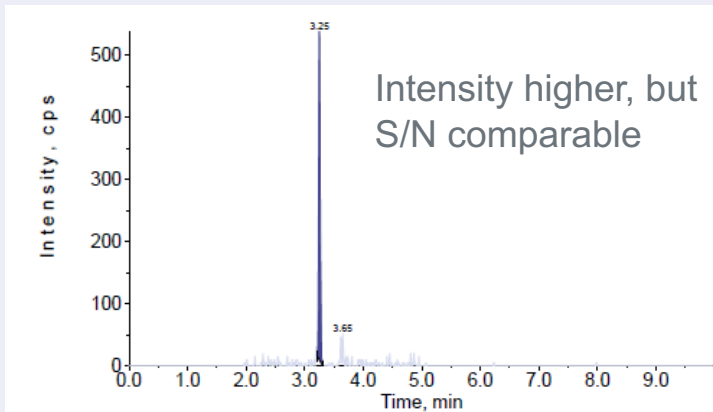
- Calibration range 100-10000 ng/ml, quadratic calibration $1/x^2$



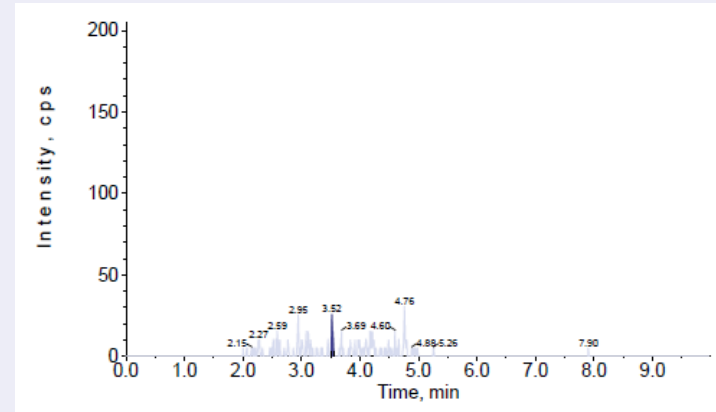
LLOQ Peptide A (Quantifier)
100 ng/ml Fab (~ 3 ng/ml peptide)



Calibration



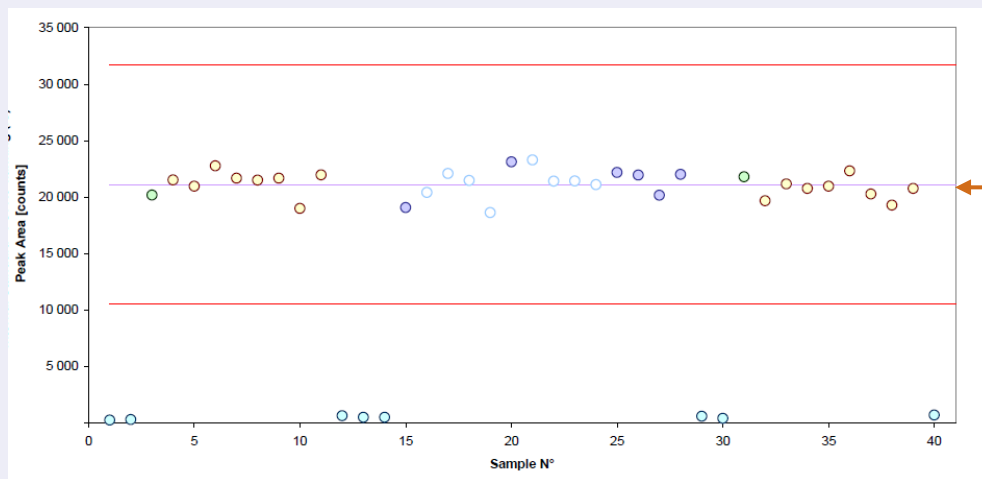
LLOQ Peptide B



Blank mouse serum

LC-MS/MS assay: role of internal standard (ISTD)

- Internal standard plot: Validation run, assay variability

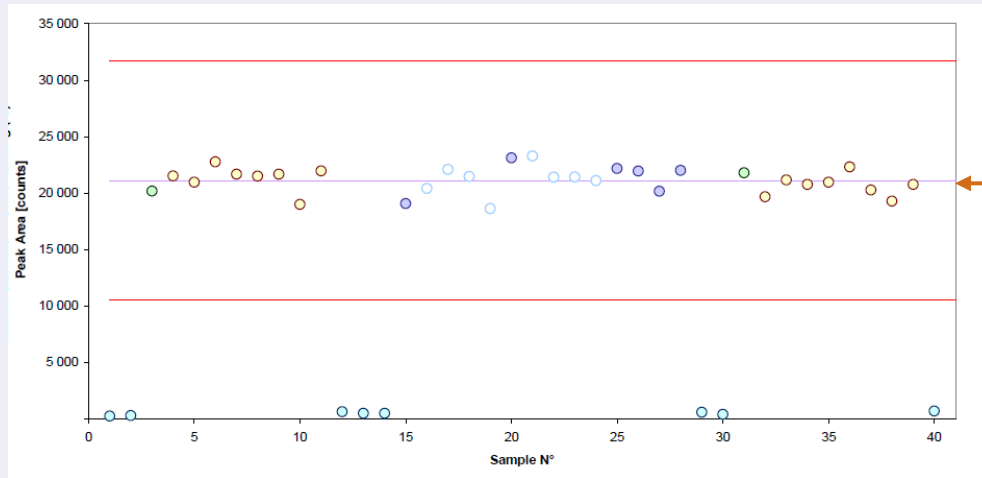


- Blank with ISTD
- Calibration Standard
- Quality control
- Validation sample

- Blank without ISTD

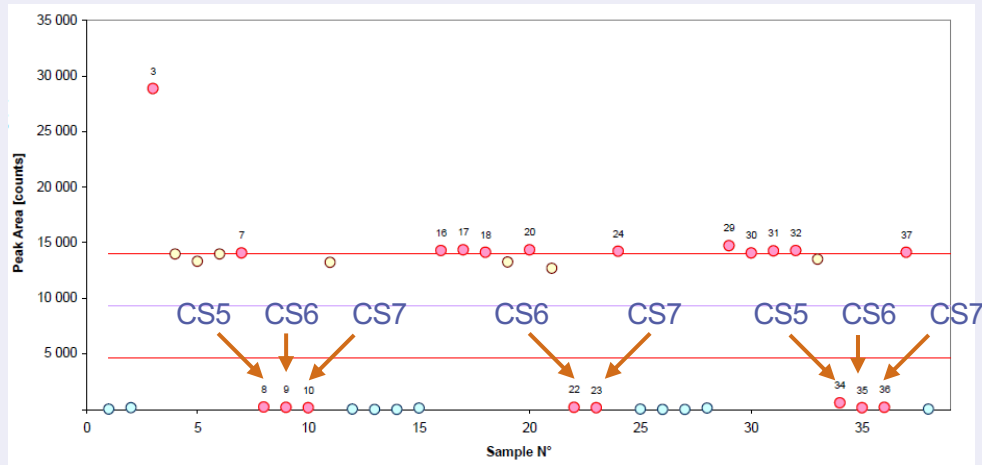
LC-MS/MS assay: role of internal standard (ISTD)

- Internal standard plot: Validation run, assay variability



- Blank with ISTD
- Calibration Standard
- Quality control
- Validation sample
- Blank without ISTD

- Internal standard plot: 3 sets of calibration



- Internal standard outlier
>± 50 % mean peak area

Sequential sample digestion of calibration standards (CS) on a 96 well plate: 3 x CS1, 3 x CS2, 3 x CS3 etc...

Sample preparation error

Assay variability, mouse and monkey serum

- Assay variability in mouse serum, three independent batches

Validation Level: Nominal Concentration (ng/mL)	Mean Calculated Concentration	Mean % difference estimate	Within-run precision CV %	Between-run precision CV %	Total precision %
100 (n=18)	117	17.0	13.4	0.00	13.4
300 (n=18)	281	-6.35	9.40	7.28	11.9
800 (n=18)	780	-2.48	8.73	3.29	9.33
8000 (n=18)	8090	1.08	6.45	2.15	6.80

- Assay variability in monkey serum, three independent batches

Validation Level: Nominal Concentration (ng/mL)	Mean Calculated Concentration	Mean % difference estimate	Within-run precision CV %	Between-run precision CV %	Total precision %
100 (n=18)	107	7.04	14.9	6.64	16.3
300 (n=18)	280	-6.65	12.3	1.16	12.4
800 (n=18)	789	-1.33	10.2	0.00	10.2
8000 (n=18)	7960	-0.528	8.50	4.63	9.68

Matrix variability

- Matrix variability

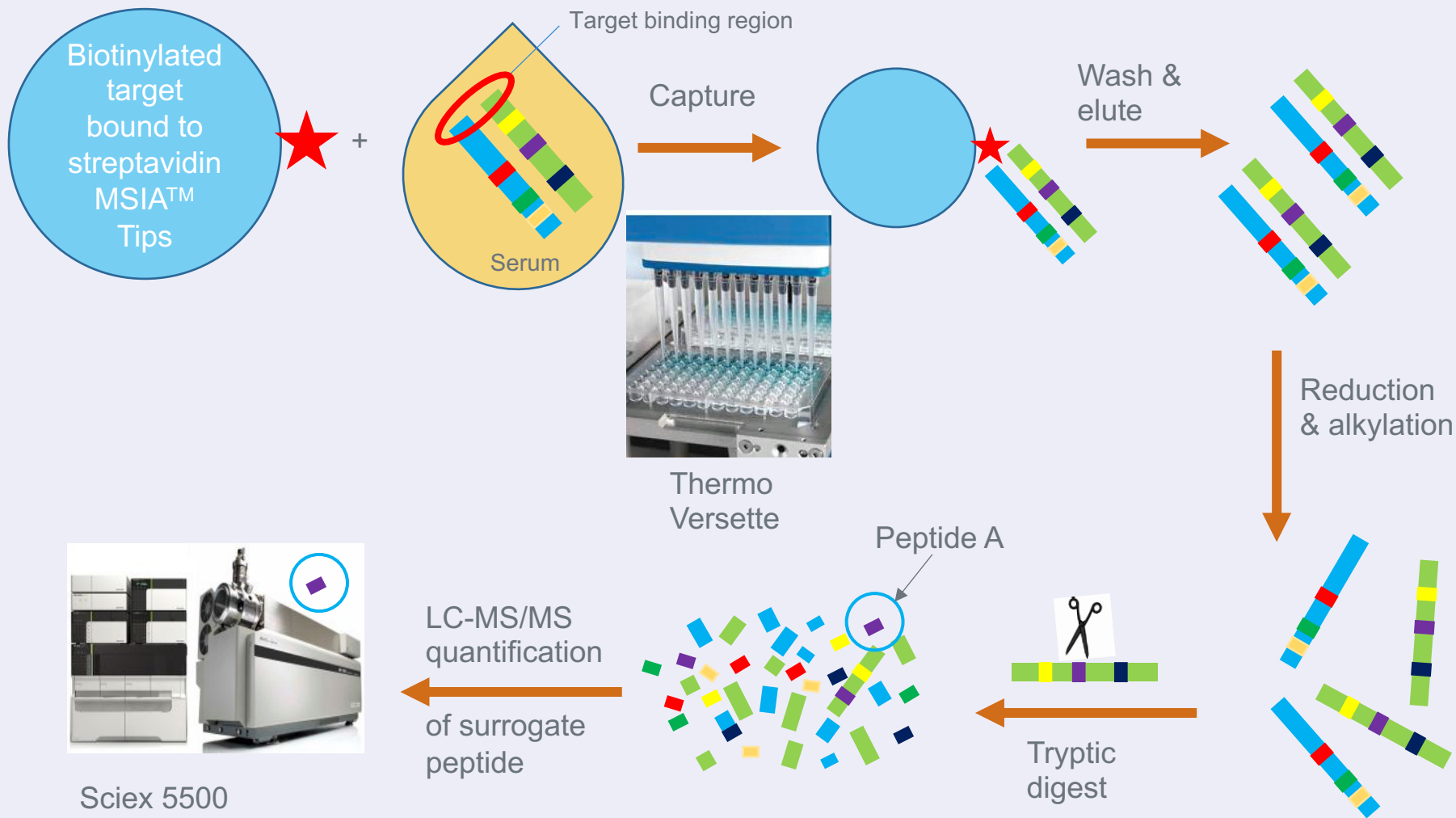
	Male 1	Male 2	Male 3	Female 1	Female 2	Female 3
	Mean concentration found for LLOQ (100 ng/ml) in mouse serum					
Mean	89	102	90	115	109	113
CV %	8.2	12.6	9.3	17.3	6.3	7.7
	Mean concentration found for LLOQ (100 ng/ml) in monkey serum					
Mean	109	113	119	111	122	102
CV %	10.4	8.0	9.6	15.0	11.1	7.3

	Male 1	Male 2	Male 3	Female 1	Female 2	Female 3
Mean peak area in blank samples / Mean peak area in LLOQ samples (%) Acceptance criteria < 20%	Mouse serum					
	3.73	4.14	0.00	1.97	1.67	1.99
	Monkey serum					
	4.26	3.88	2.75	3.03	5.49	5.05

Validation results

	Mouse	Monkey
Specificity and selectivity	✓	✓
Assay variability	✓	✓
Matrix variability	✓	✓
Container binding	✓	✓
Matrix effect and recovery	✓	✓
Autosampler carry over	~ 1 %	~ 1 %
Freeze-thaw stability	3 times	3 times
Stability of ITSD in solution	91 days	
Stability of stock solution	82 days	
Stability of samples at -80°C	166 days	181 days
Stability of samples at RT	24 h	24 h
Stability of samples at 37°C	6 h	3 h
Autosampler stability	7 days	7 days
Blood stability	3 h	
Hemolyzed blood (3%)	No effect observed	

Assay format



Acceptance criteria: 15 % versus 20 %

- Three GLP-Studies supported

Acceptable batches	Study 1 (mouse)	Study 2 (mouse)	Study 3 (monkey)
20 % / 25 %	5 of 5 passed	10 of 11	11 of 14 (11 of 11)
15 % / 20 %	4 of 5	7 of 11	10 of 14 (10 of 11)

Overall statistic %CV	Study 1 (mouse)	Study 2 (mouse)	Study 3 (monkey)
CV % QC LOW	12.15	15.36	16.13
CV % QC MID	7.60	14.19	9.19
CV % QC HIGH	5.96	8.66	9.01

- → Acceptance criteria used 20 % / 25 %
- → Definition of the acceptance criteria in PRIOR to validation.

Conclusions

- The LC-MS/MS validation of biologics is still a challenging field, but LC-MS/MS is a good alternative and/or an orthogonal Method for LBA only assays.
- Some validation test require adaption when used for biologics.
- MSIA™ microcolumn tips in combination with the Versette are suitable for target-specific LBA purification from biologic samples also under GLP-Environment.
- Isotopic labeled flanking surrogate peptide as internal standard is suitable for validation of a hybrid assay and able to correct tryptic digesting step.
- Still some variability in assay precision due to ligand-binding purification step.
- Acceptance criteria of $\pm 20\%$ (25 % at LLOQ) are suitable to validate ligand-binding-LC-MS/MS hybrid assay.

Acknowledgment




- Project Team and BA Group Frankfurt



BACK-UP

- **Thermo Versette Automated Liquid Handling System**

- **Streptavidine MSIA™ Tips**

- Conditioning step (buffer) 5-20 times
- Loading step with target  >100 times
- Washing step (buffer) 5-20 times
- Conditioning step (buffer) 5-20 times
- Loading step (serum in buffer)  >100 times
- 2 Washing steps (buffer) 5-20 times
- 2 Washing steps (water) 5-20 times
- Elution step (Elution solution with internal standard)  10-20 times