Day 2 – Breakout 1
Hybrid Assays – Application
18 Nov 2020

Therapeutic/biomarker protein quantification in tissues: method development strategies to overcome sensitivity & selectivity issues using hybrid LBA–LCMS

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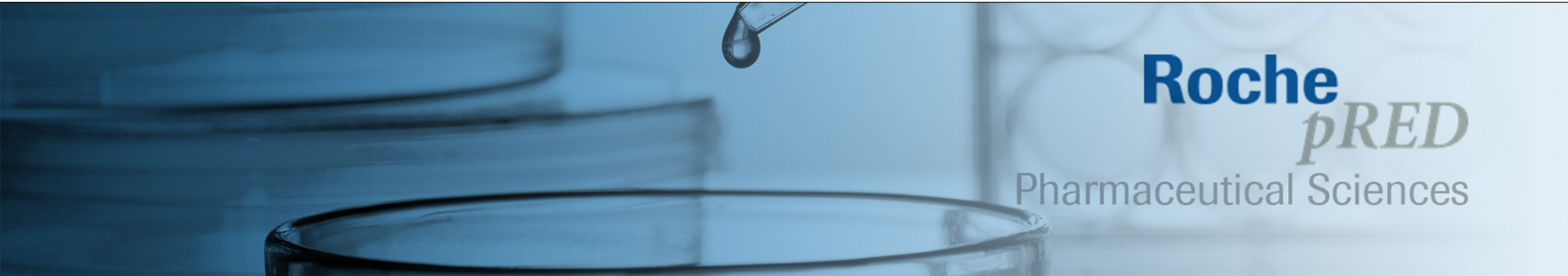


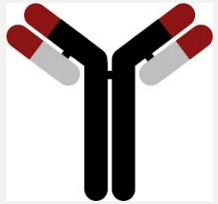
Outline

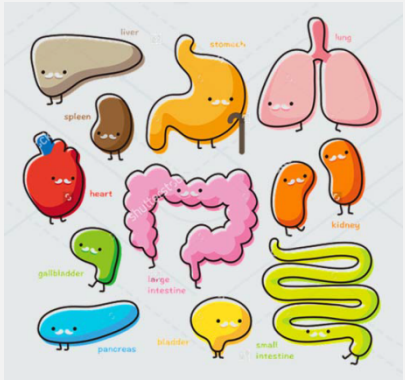
- Low-level therapeutic/biomarker protein quantification in tissues by LC-MS
- Sensitivity, selectivity and other analytical challenges
- A proposed reagent free, ultrasensitive quantification workflow
- Case studies

Low-level therapeutic/biomarker protein quantification in tissues by LC–MS

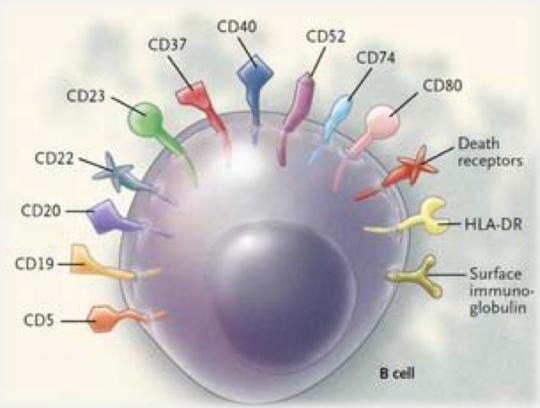
Ability to measure not only the drug, but also its target and/or biomarkers

PK, tissue distribution, tumor penetration, etc.

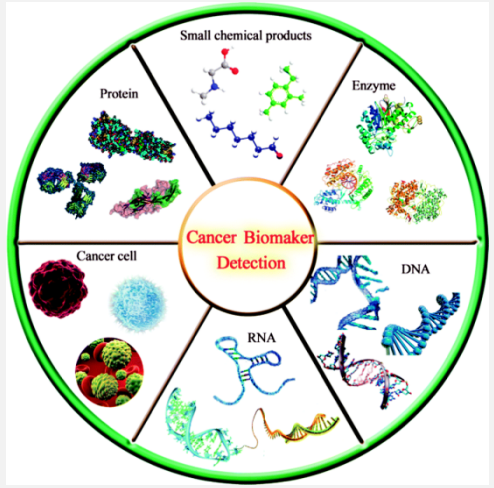




Antigen expression level in different tissues (e.g., CD20, CD40, PD1, CEA…)

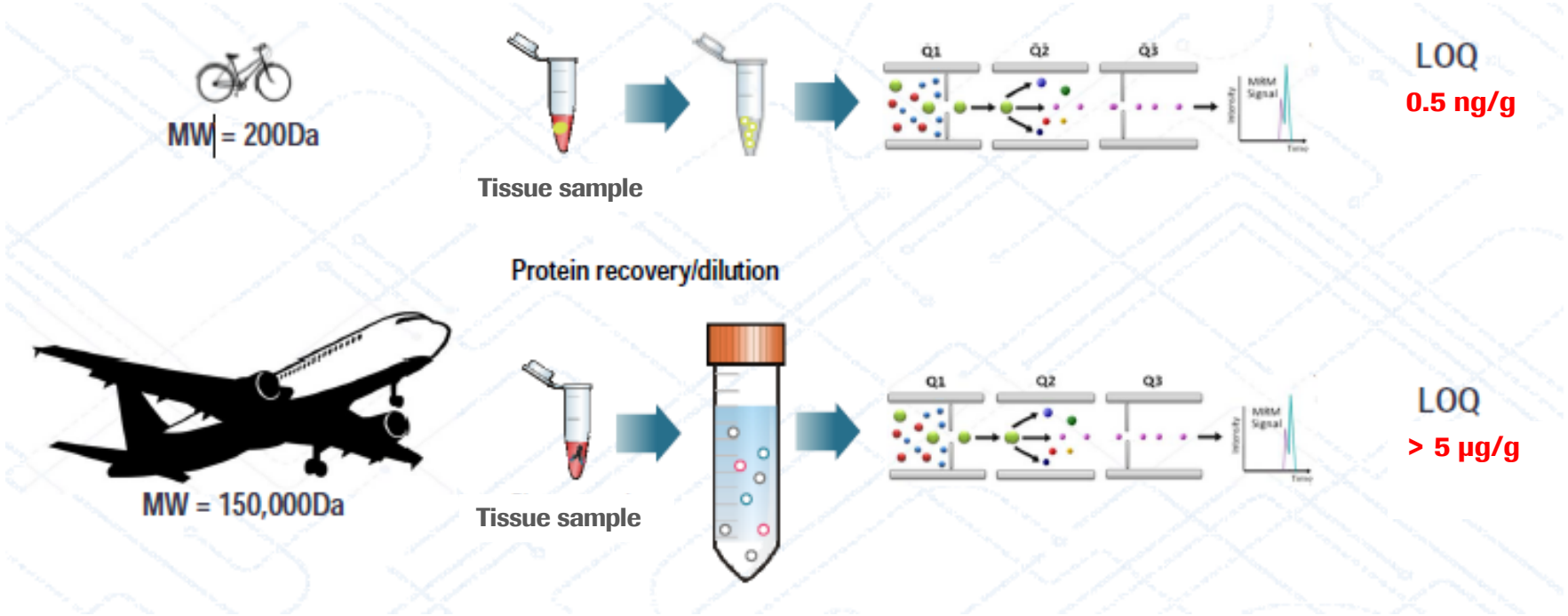


Biomarkers of drug effects, e.g. cell death or immune cell activation for cancer treatment



The sensitivity challenge

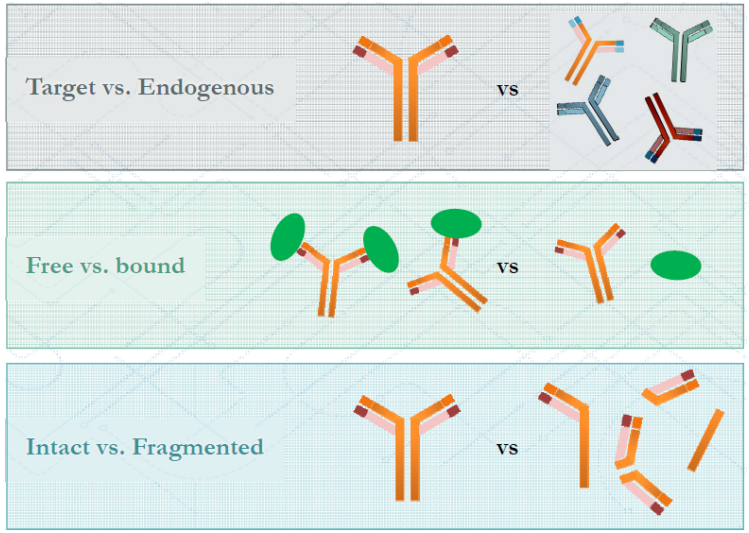
- low abundances of target analytes
- concentrations in tissues can be significantly lower than those found in serum.

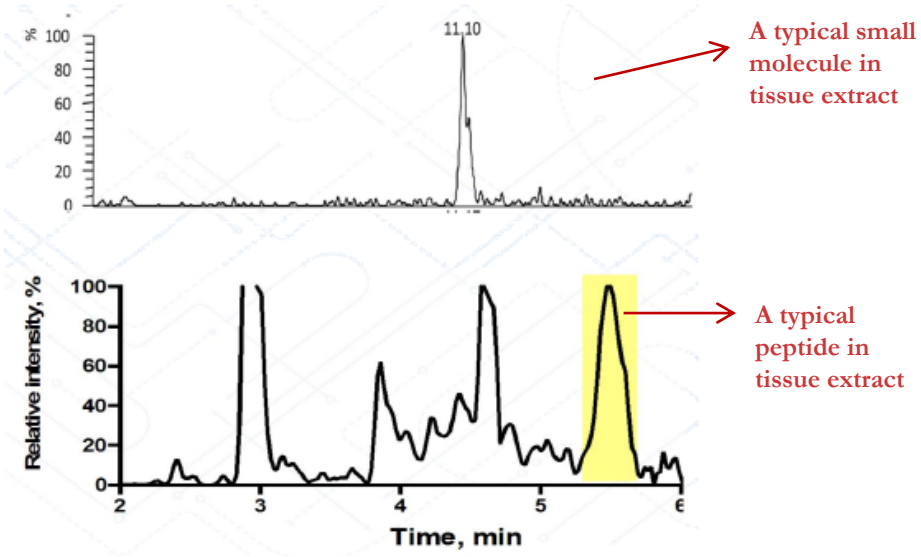


The sensitivity on molar scale is much lower for mAb quantification than for small molecules.

The selectivity challenge

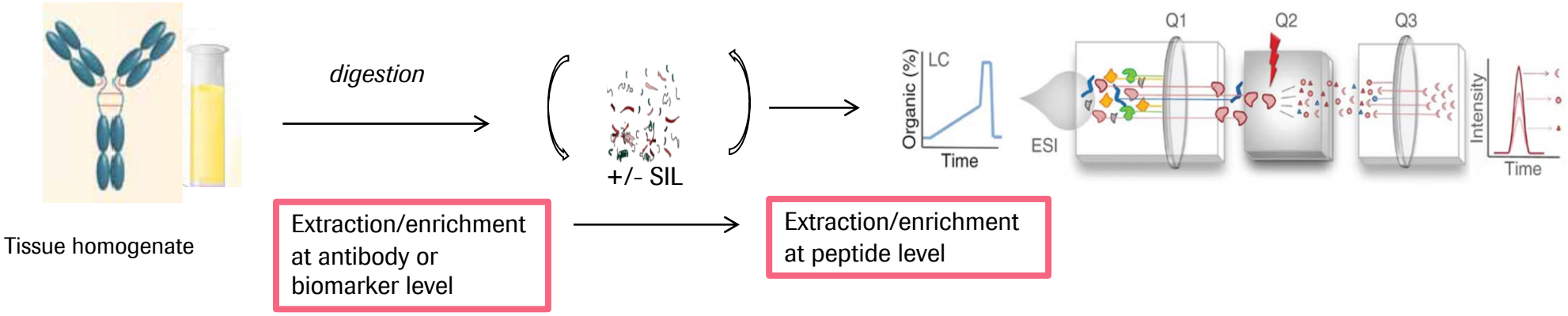
- complexity of tissue matrices
- heterogeneity of the analytes, which can be present in various forms.





Key factors affecting the sensitivity and selectivity of LM LC-MS methods in tissues

Sample preparation	e.g. tissue perfusion, homogenization, extraction, digestion
Chromatography	e.g. use of selective stationary phases, particle size, column diameters and flow rate, use of 2D-LC
MS parameters	e.g. ionization efficiency, SRM transitions optimization, HRMS



Use of immune based enrichment methods to overcome selectivity and sensitivity issues

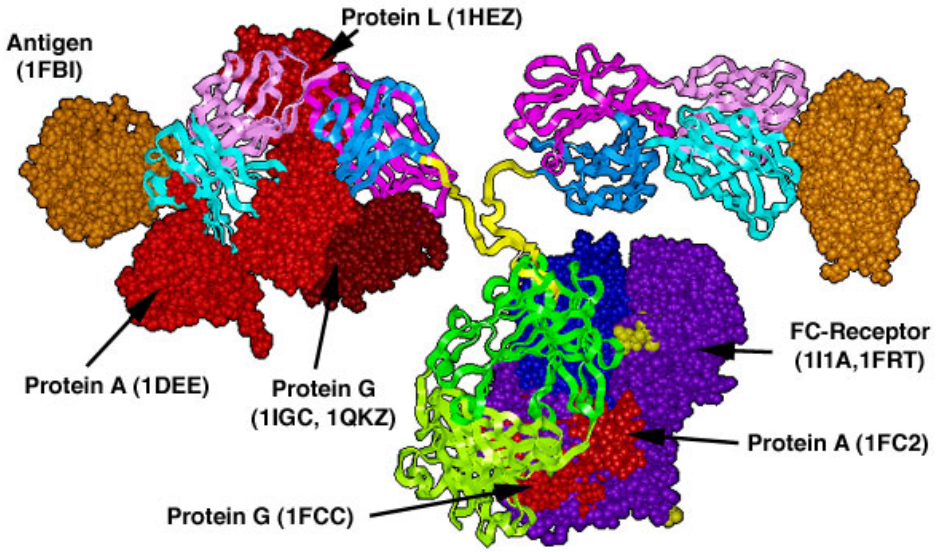
There are two types of affinity capture techniques:

- At protein level
- At peptide level

<u>These are based on:</u>

- immunoaffinity interactions with an immobilized target ligand/receptor or antibody → **specific**
- affinity interactions with a generic binding protein, such as protein A/G or anti-Fc → **non-specific**

Protein A, protein G enrichment

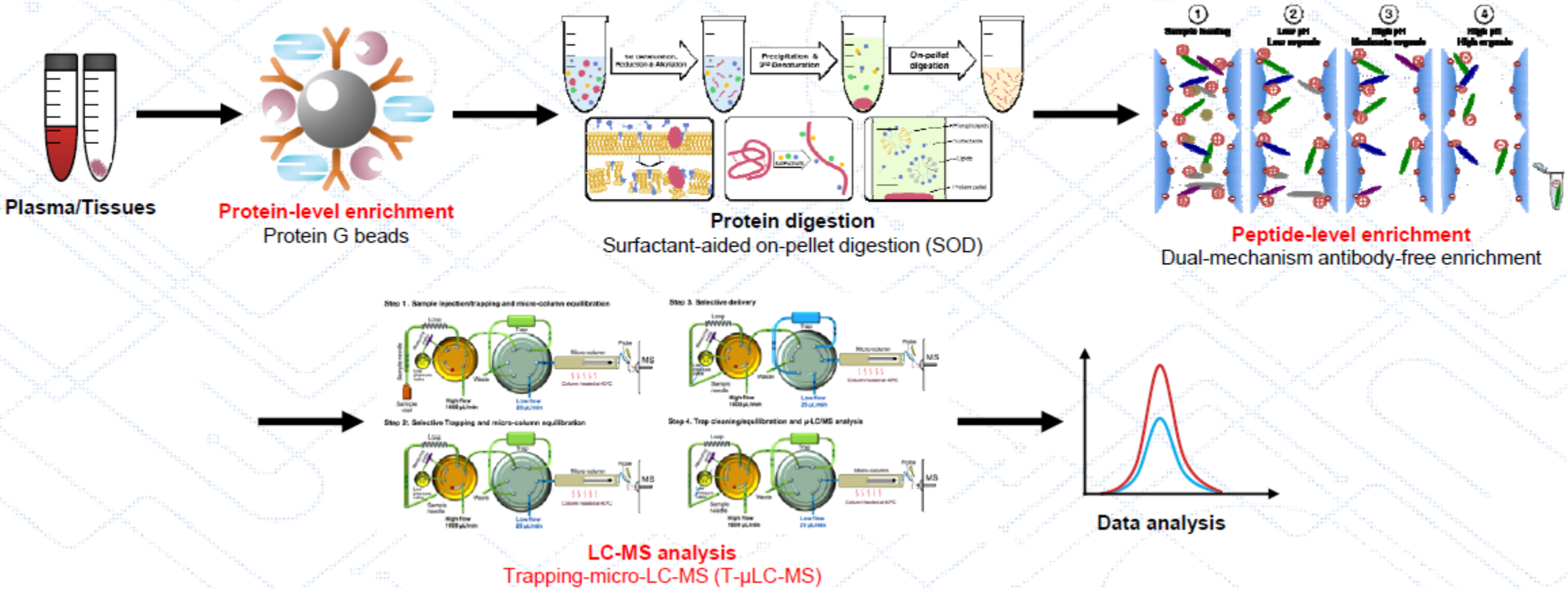


Interactions of Antibodies with Protein A, Protein G and Protein L

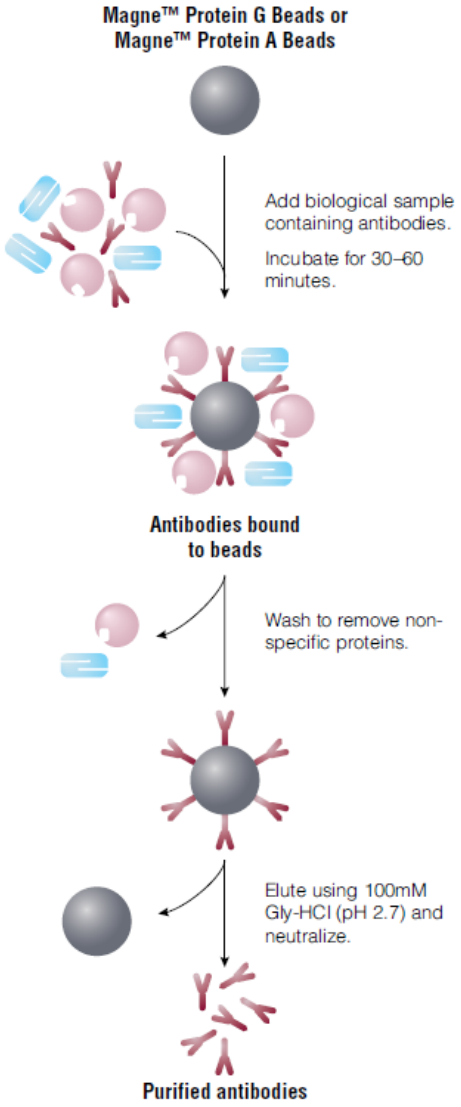
(courtesy of AAAAA, AHo's Amazing Atlas of Antibody Anatomy)

Species	Immunoglobulin	Binding to Protein A	Binding to Protein G
Human	IgG (normal)	++++	++++
	IgG1	++++	++++
	IgG2	++++	++++
	IgG3	-	++++
	IgG4	++++	++++
	IgM	-	-
	IgA	-	-
	IgE	-	-
Mouse	IgG1	+	++++
	IgG2a	++++	++++
	IgG2b	+++	+++
	IgG3	++	+++
Rat	IgG1	-	+
	IgG2a	-	++++
	IgG2b	-	++
	IgG2c	+	++
Goat	IgG	+/-	++
Rabbit	IgG	++++	+++
Sheep	IgG	+/-	++

Development of a reagent free, ultrasensitive quantification workflow



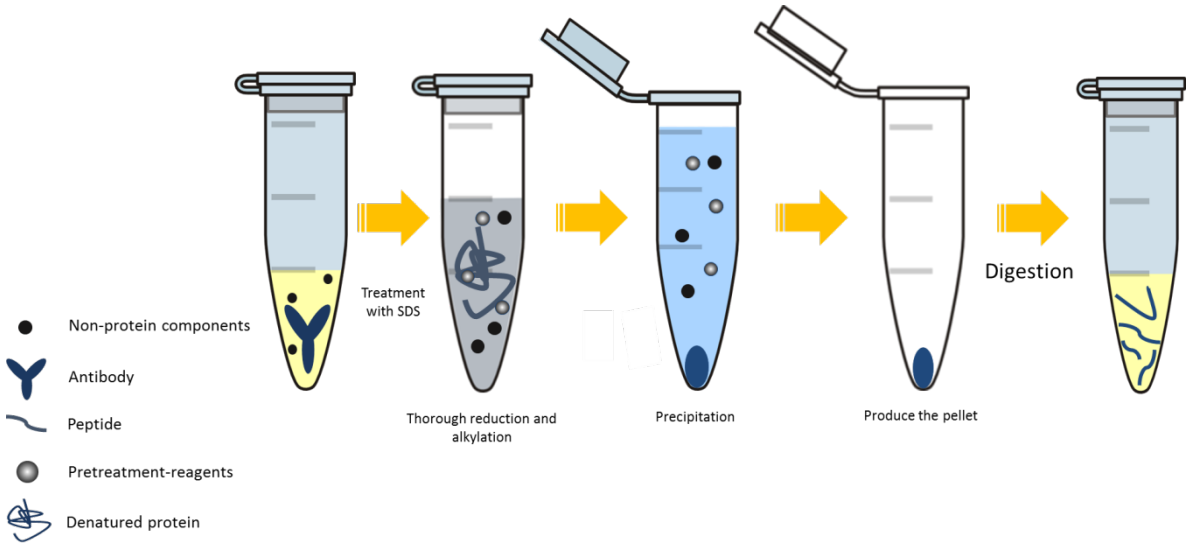
Protein G enrichment – an example



Courtesy of Promega

1. Gently vortex or invert the beads to obtain a uniform suspension. Keep the suspension uniform when aliqotting beads.
2. Add 50µl of bead slurry to a 1.5ml microcentrifuge tube. Place in the magnetic stand for 10 seconds.
3. Remove and discard the storage buffer.
4. Add 500µl of bind/wash buffer. Mix and place in the magnetic stand for 10 seconds. Remove and discard the bind/wash buffer.
5. Combine 50µl of bind/wash buffer and 50µl of plasma or 1000µl tissue extract (extracted with PBS + 0.1% Formic acid, neutralized after extraction), then add to the equilibrated beads.
6. Mix sample for 60 minutes at room temperature. Make sure the beads remain in suspension by using a tube shaker or end-over-end mixer.
7. Place tube in the magnetic stand for 10 seconds. Remove the supernatant.
8. Wash beads by adding 250µl of bind/wash buffer and mix for 5 minutes. Place in the magnetic stand for 10 seconds. Remove and discard bind/wash buffer.
9. Repeat Step 8.
10. Add 100µl of elution buffer [100mM glycine-HCl (pH 2.7)] to the beads.
11. Mix for 5 minutes at room temperature.
12. Place tube in the magnetic stand for 10 seconds. Remove eluted sample, and transfer to a new microcentrifuge tube. This is the first elution.
13. Repeat elution Steps 10–12. Combine the eluent.
14. SOD digestion and analysis.

Surfactant-aided on-pellet digestion (SOD)



Anal. Chem. 2015, 87, 4023–4029

Surfactant facilitates pretreatment, more thoroughly denaturation and alkylation, which will increase the digestion efficiency;

Surfactant helps to cleanup matrix components and deactivates protease inhibitor, such as alpha-1-anti trypsin;

Works well with cells, tissues and plasma (high yields of membrane proteins).

Acetone precipitation:

1) Take 10 µl tissue extract sample into the centrifuge tube, add 190 µl 1%SDS (or take 20 µl tissue extraction supernatant sample into the centrifuge tube, add 180 µl 1%SDS)
2) Add 15 µl DTT solution; vortex and spin; incubate at 56 ˚C for 30 min**;**
3) Add 30 µl IAM solution; vortex and spin; incubate at 37 ˚C for 30 min in darkness;
4) Add 200 µl (1x) of chilled acetone (-20 ˚C), then vortex for 1 min then add 1000 µl (5x) of chilled acetone (-20 ˚C), vortex until the solution turned clear with pellet precipitation; Incubate at -20 ˚C for 3h;
5) Centrifuge samples to pellet protein at 20,000 g for 30 min at 4 ˚C; then remove the supernatant carefully;
6) Expose the samples in air for 3-5 min to evaporate acetone.

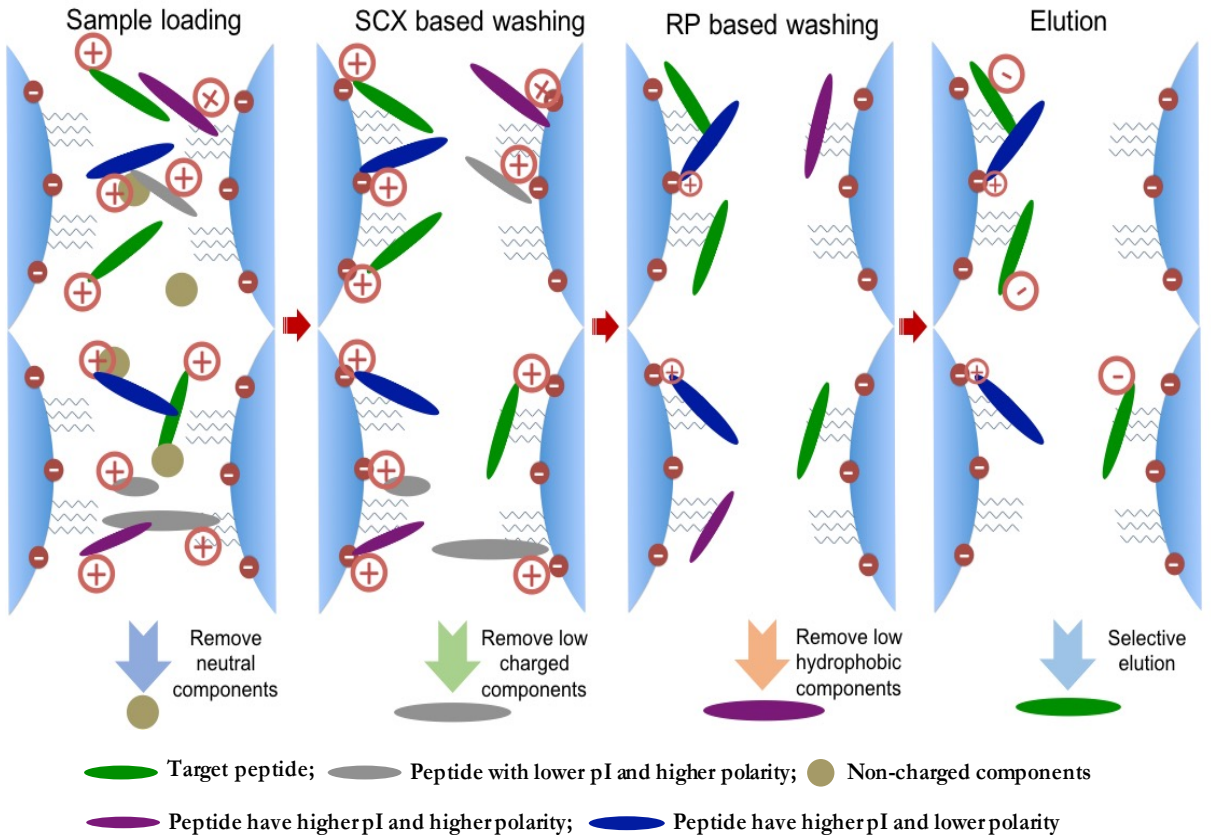
Digestion

1) Add 68 µl Tris-FA buffer (pH 8.5) (for trypsin) into the centrifuge tubes with protein pellet.
2) Activate trypsin: Thaw trypsin stock solution (1mg/ml). Make a 1:4 dilution with Tris-FA buffer, vortex and spin.
3) One step digestion: add 32 µl of activated trypsin to the vial, incubate 45min at 37 °C with vortex at 500 rpm in darkness.
4) Terminate digestion

Enrichment at peptide level

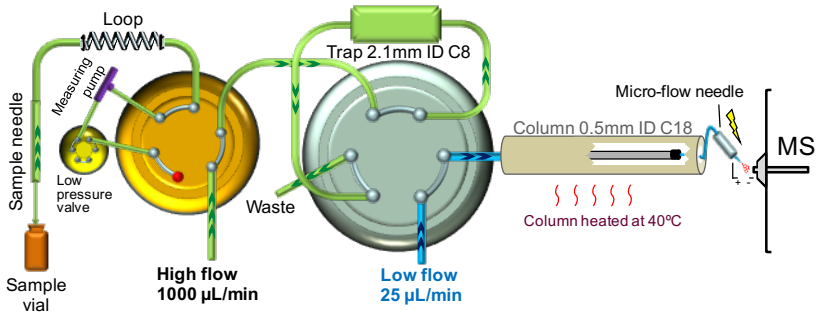
Off-line mixed-mode ion exchange solid phase extraction

Combining reversed-phase and ion-exchange retention mechanisms into a single protocol.

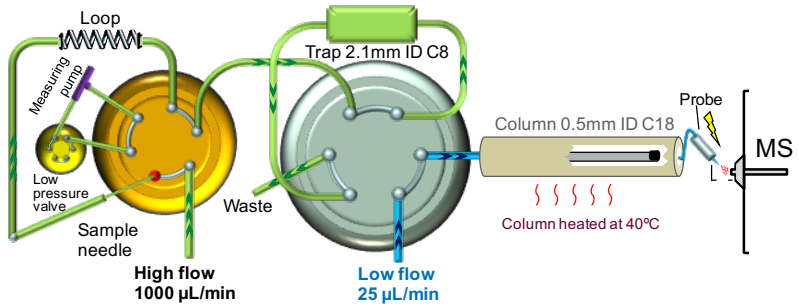


Selective trapping and delivery to improve sensitivity and selectivity

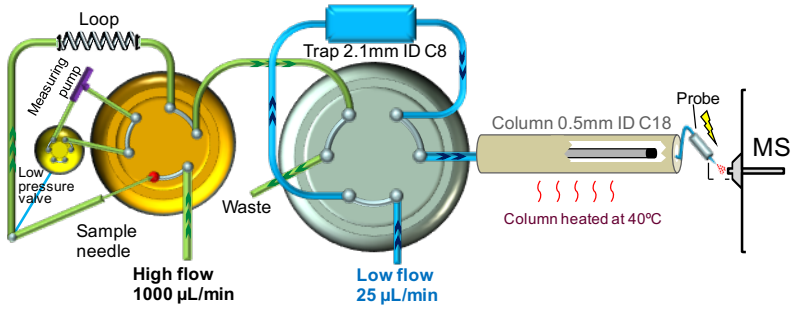
Step 1. Sample Injection and trap/column equilibration



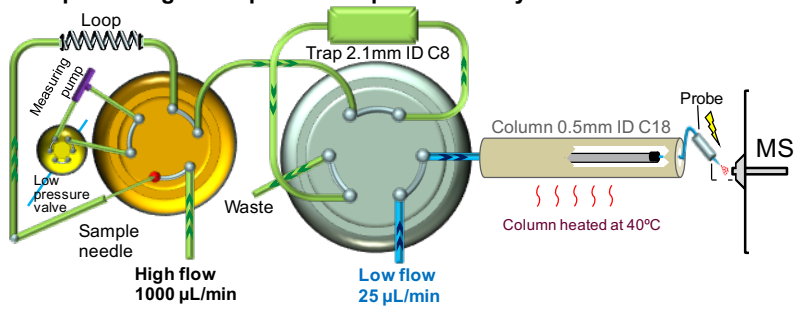
Step 2. Selective Trapping/u-column equilibration



Step 3. Selective delivery



Step 4. Trap cleaning and equilibration/µ-LC/MS analysis



Anal. Chem. 2018, 90, 1870−1880

1. Improved **sensitivity** comparing with high-flow rate LC-MS;
2. Improved **throughput** comparing with nano LC-MS;
3. Improved **capacity and robustness** comparing with micro LC-MS

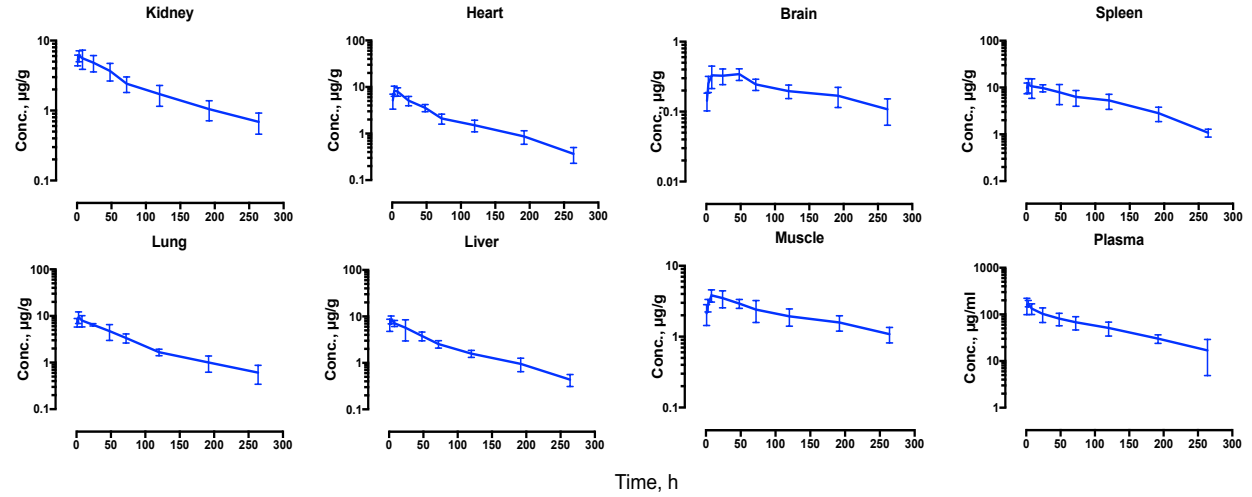
Rat PK study with Roche mAb

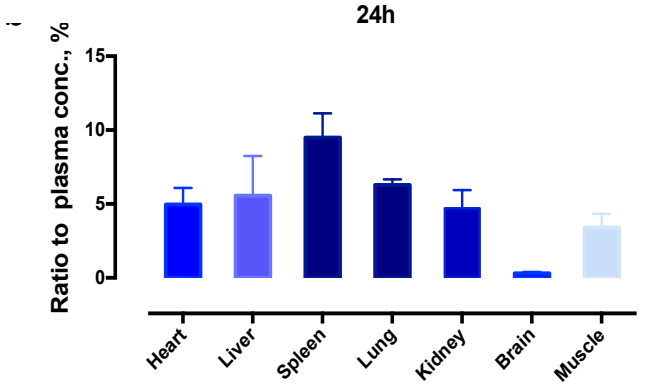
A PK study with a single intravenous injection of 10 mg/kg of Roche mAb to rats.

Calibration curve has been established over 0.1-200 µg/g for the target mAb.

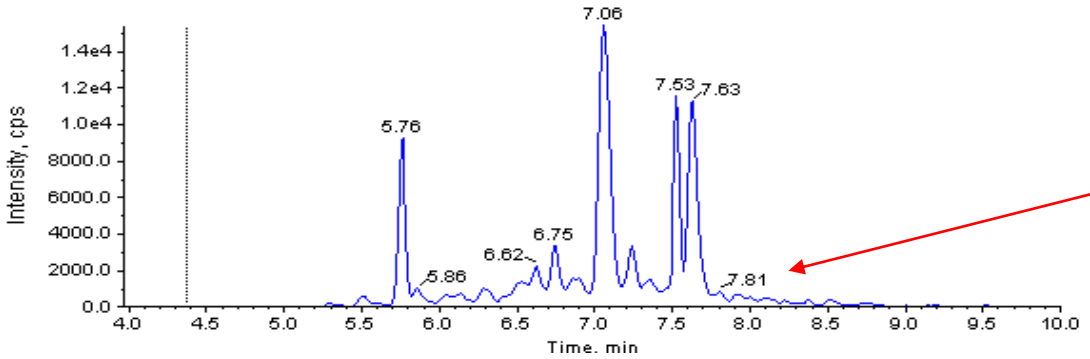
Qualification was done with QC samples at 0.2, 20, and 100 µg/g.

The accuracy was within 89.7-107.8%, and the CV% was within 15% at all levels.

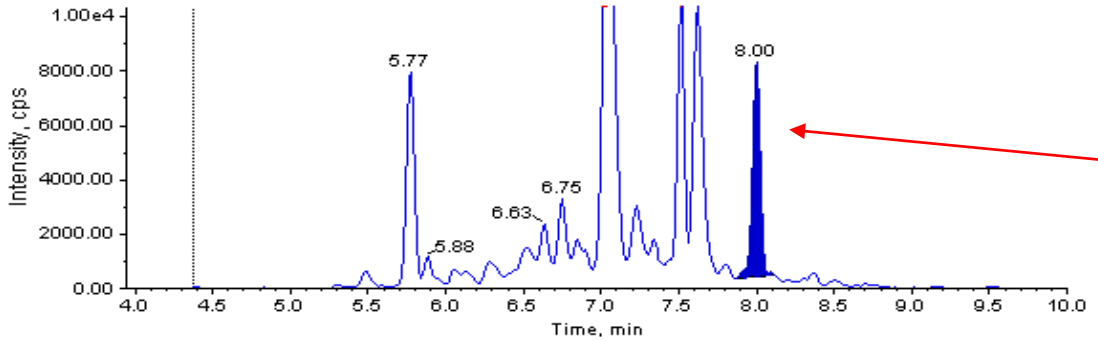




Selective trapping and delivery to improve sensitivity and selectivity

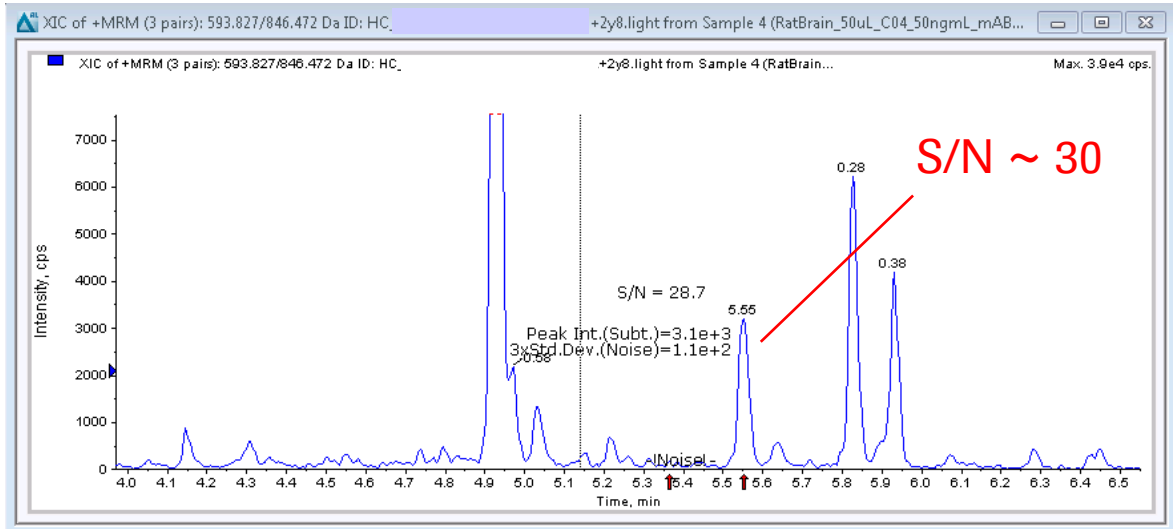


Representative T-µLC-MS chromatogram of a signature peptide in a blank tissue extract

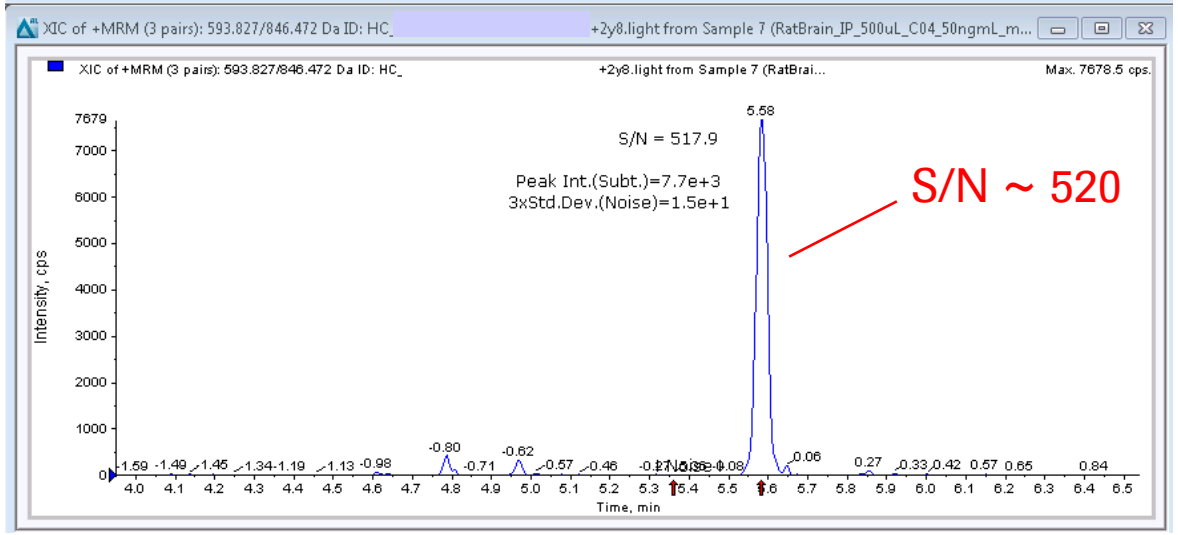


Representative T-µLC-MS/MS chromatogram of a signature peptide in a tissue extract (100 ng/g protein analyte)

Rat PK study with Roche mAb: effect of Protein G enrichment



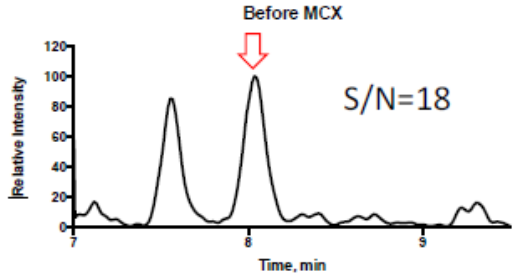
Representative tissue homogenate sample – without IAC



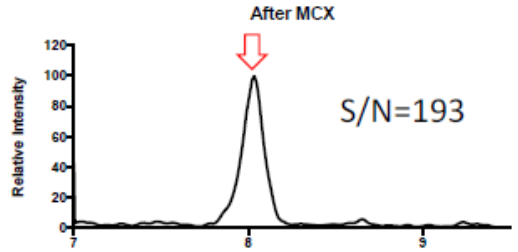
Representative tissue homogenate sample – following Protein G enrichment

Rat PK study with Roche mAb: effect of MCX enrichment

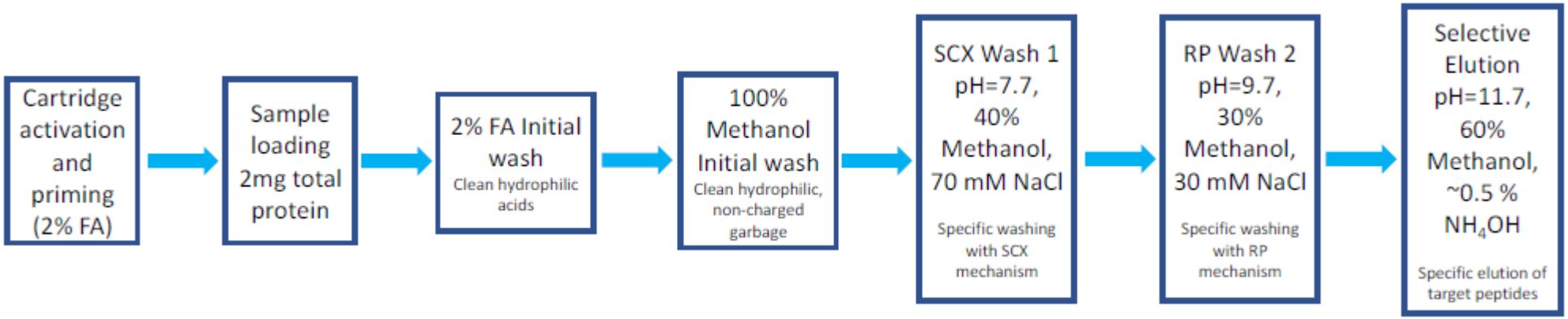
Before MCX



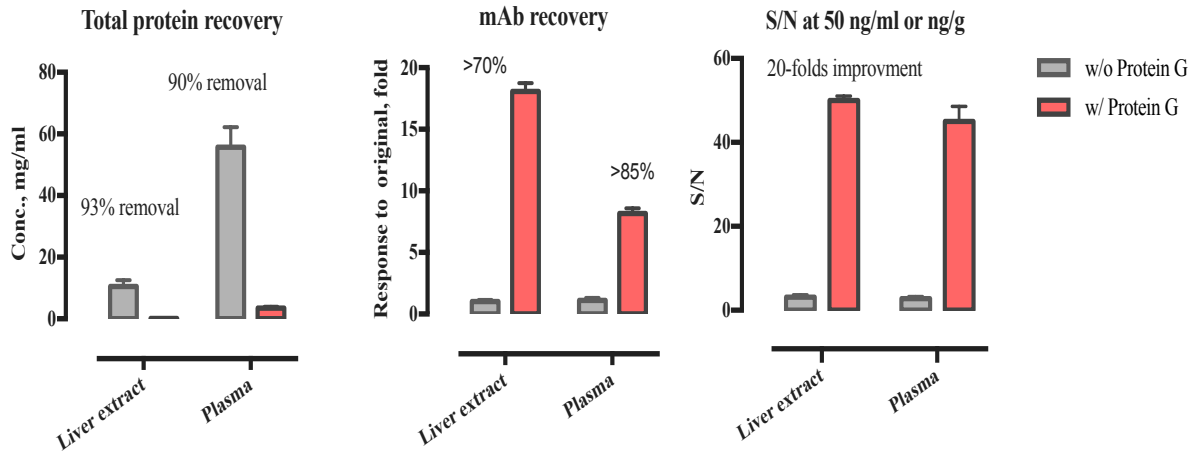
After MCX

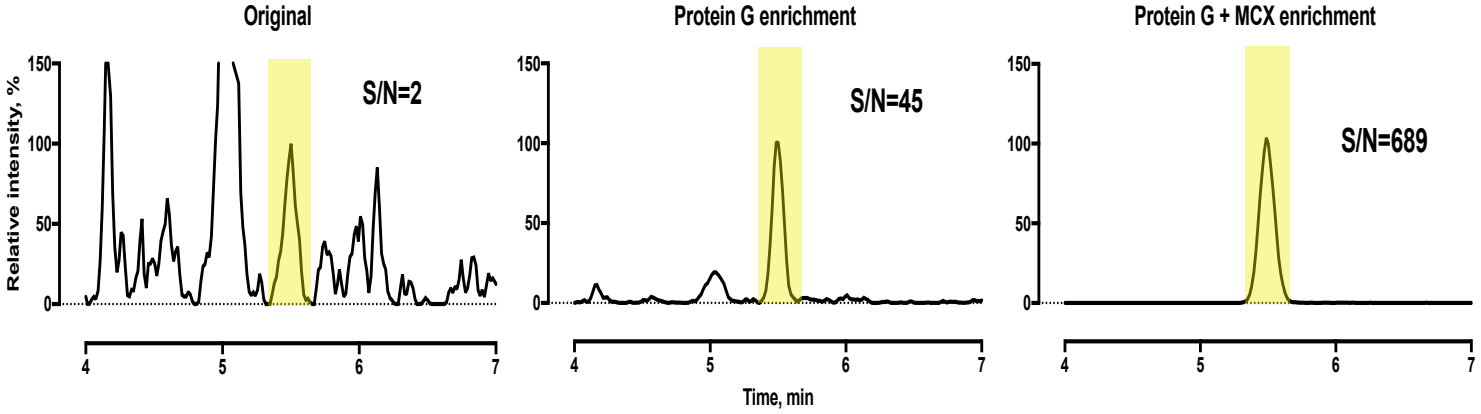


Performance of current MCX-based enrichment procedure for SP of Roche mAb in plasma or tissue homogenate



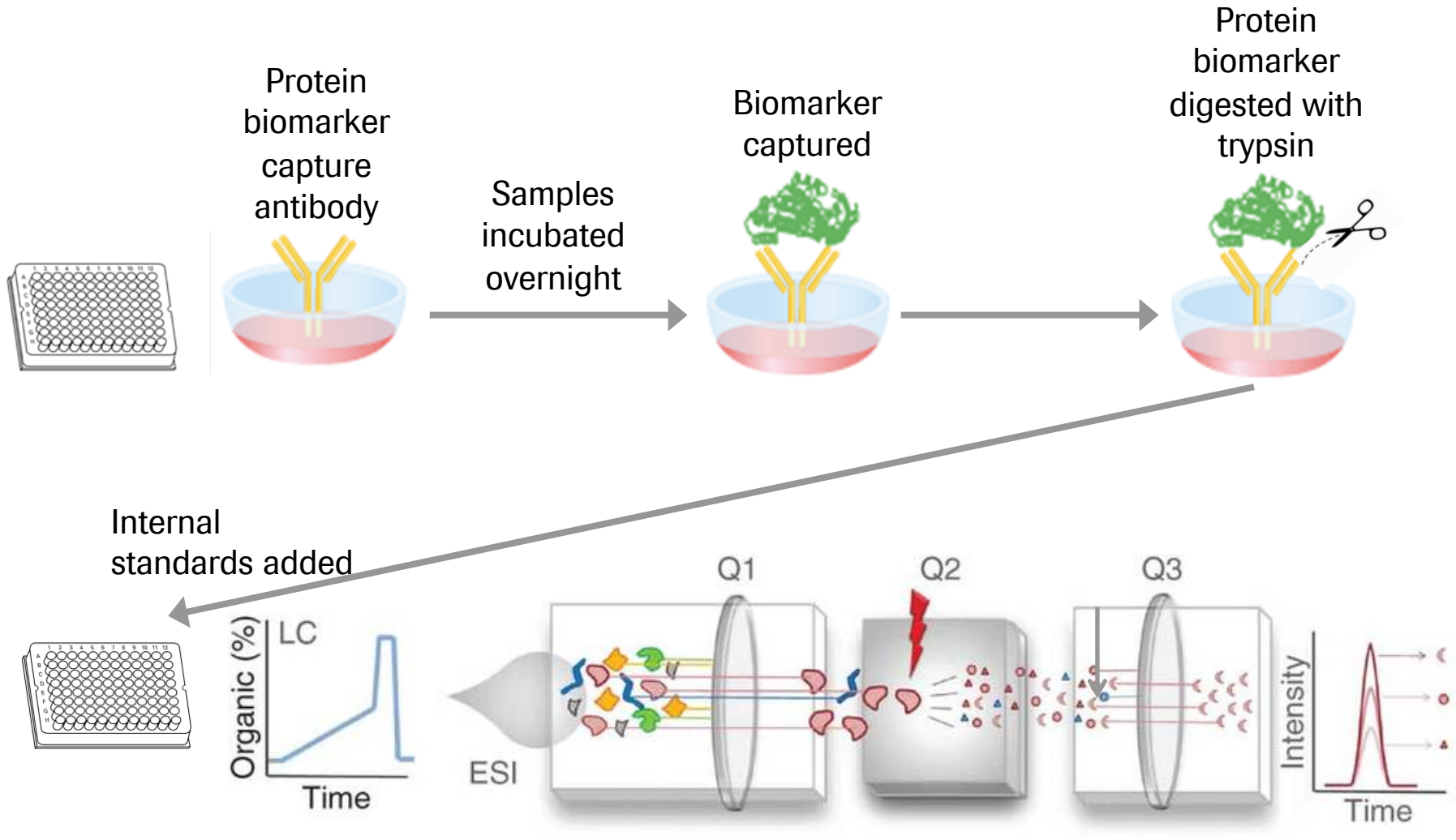
Rat PK study with Roche mAb: combined effect of Protein G and MCX enrichment





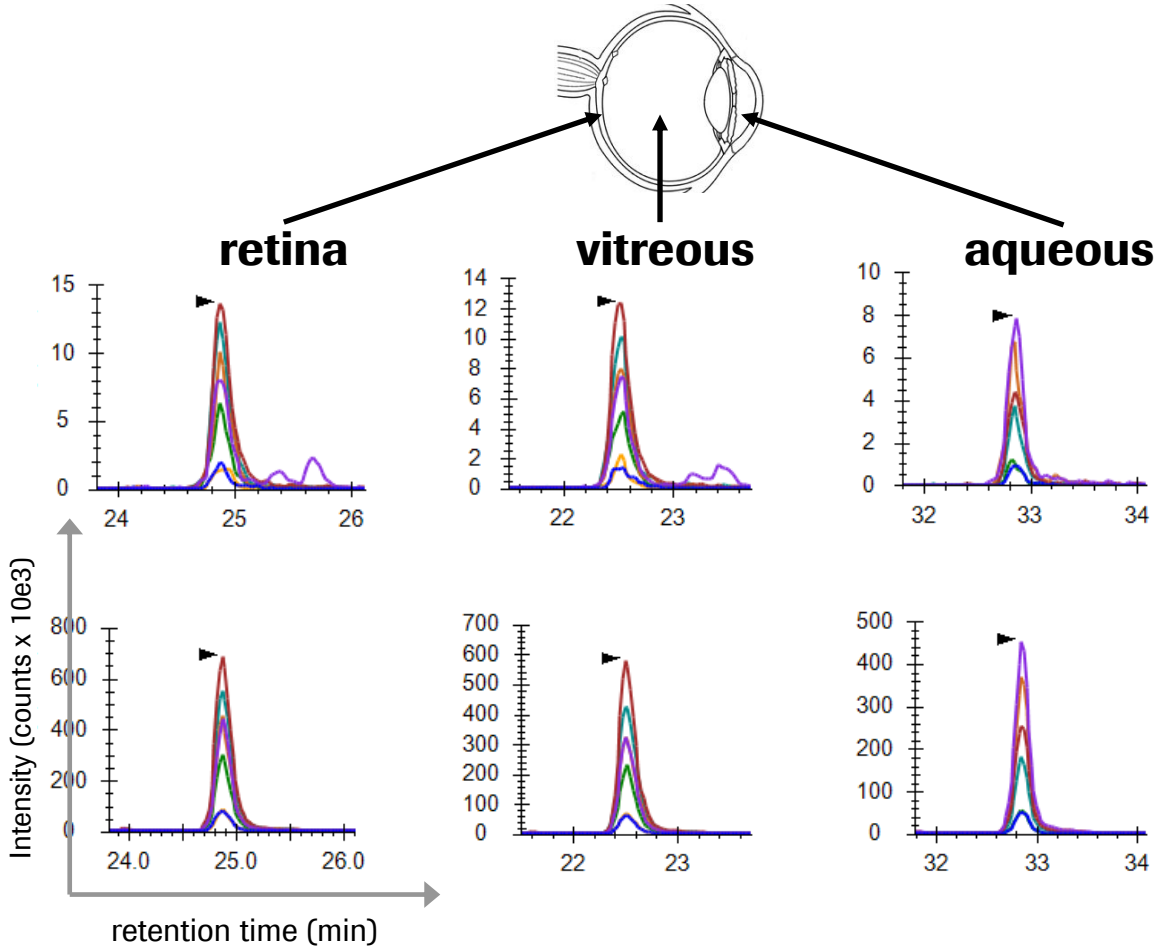
Performance of current sequential Protein G and MCX-based enrichment procedure for SP of Roche mAb in plasma or tissue homogenate (conc.=50 ng/mL in plasma, 500ng/g in tissue)

PD protein biomarker quantification based on immuno affinity capture



Protein biomarker quantification by LC-MS

PD biomarker quantification in preclinical eye tissues



Signature peptide

LH……QR

Internal standard

LH……QR(+10Da)

REFERENCES

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Antibody-free workflows for protein quantification by LC–MS/MS
Bioanalysis (2015) 7(6), 763–779

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  - Roland Staack
  - Julia Heinrich
  - Thomas Singer

Q&A

Doing now what patients need next