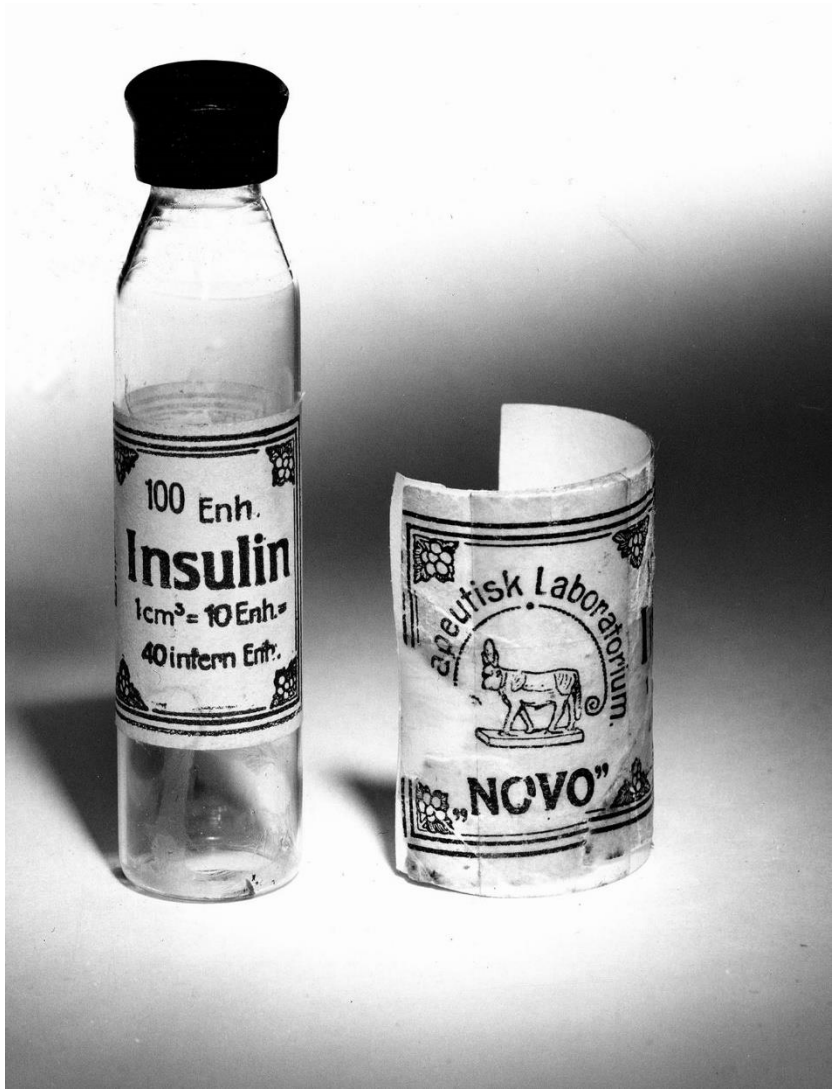


Novel HCP analysis and characterisation tools provides freedom of operation for efficient process development

Brian Kåre Kristensen on behalf of the HCP2.0 team

October 18 2022

Our core technology platforms

Proteins & peptides

Our core expertise is discovering and developing therapeutic proteins and peptides with world-leading capabilities in protein expression, engineering and formulation.

Cell therapy

By using pluripotent stem cells to replace absent or damaged cells, we aim to develop specialised cells for regenerative or curative cell therapies.

RNAi

RNA interference (RNAi) therapies harness biological processes to selectively silence genes that cause or contribute to disease.

Gene editing

Inserting, deleting, modifying or replacing DNA in the human genome holds the promise of delivering a true cure for genetic diseases.

Delivery platforms

Injection devices

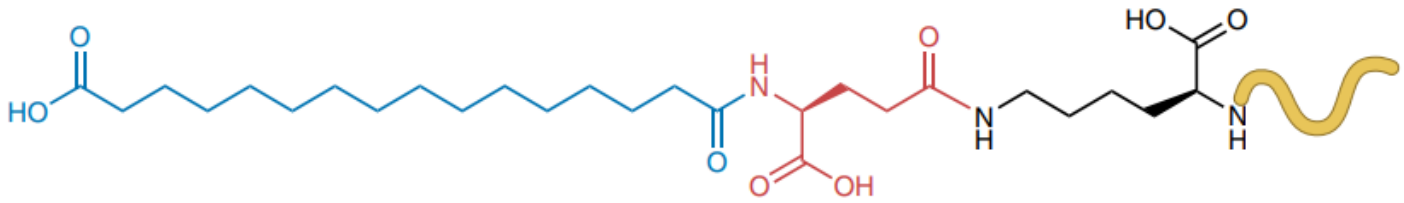
Our innovative devices and connected solutions combine patient insight with engineering excellence to make drug delivery as simple as possible.

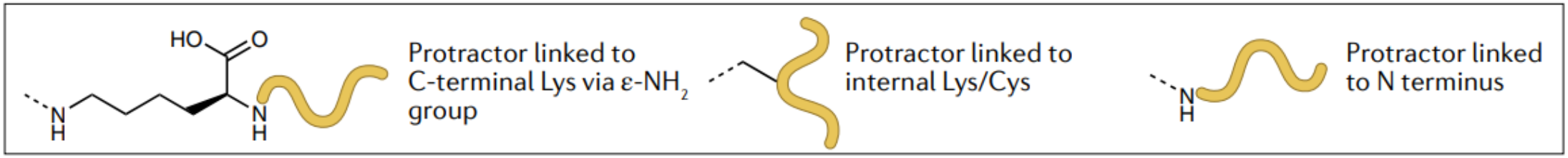
Oral delivery

Most of our therapeutic peptides and proteins are injectable, but we aim to make the latest innovations more accessible to more patients through oral products.

Protraction of protein drugs by derivatisation with fatty acids

- Reversible binding to human serum albumin increases the half-life *in vivo*





Review Article | Published: 24 August 2022

Derivatization with fatty acids in peptide and protein drug discovery

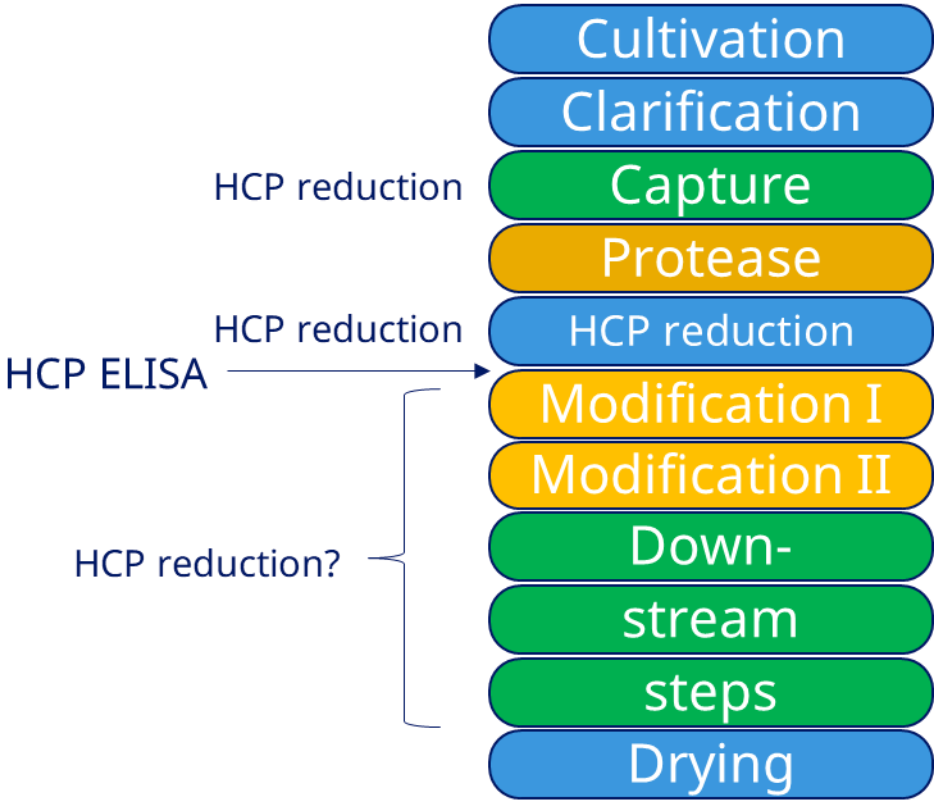
Peter Kurtzhals, Søren Østergaard, Erica Nishimura & Thomas Kjeldsen

Nature Reviews Drug Discovery (2022) | Cite this article

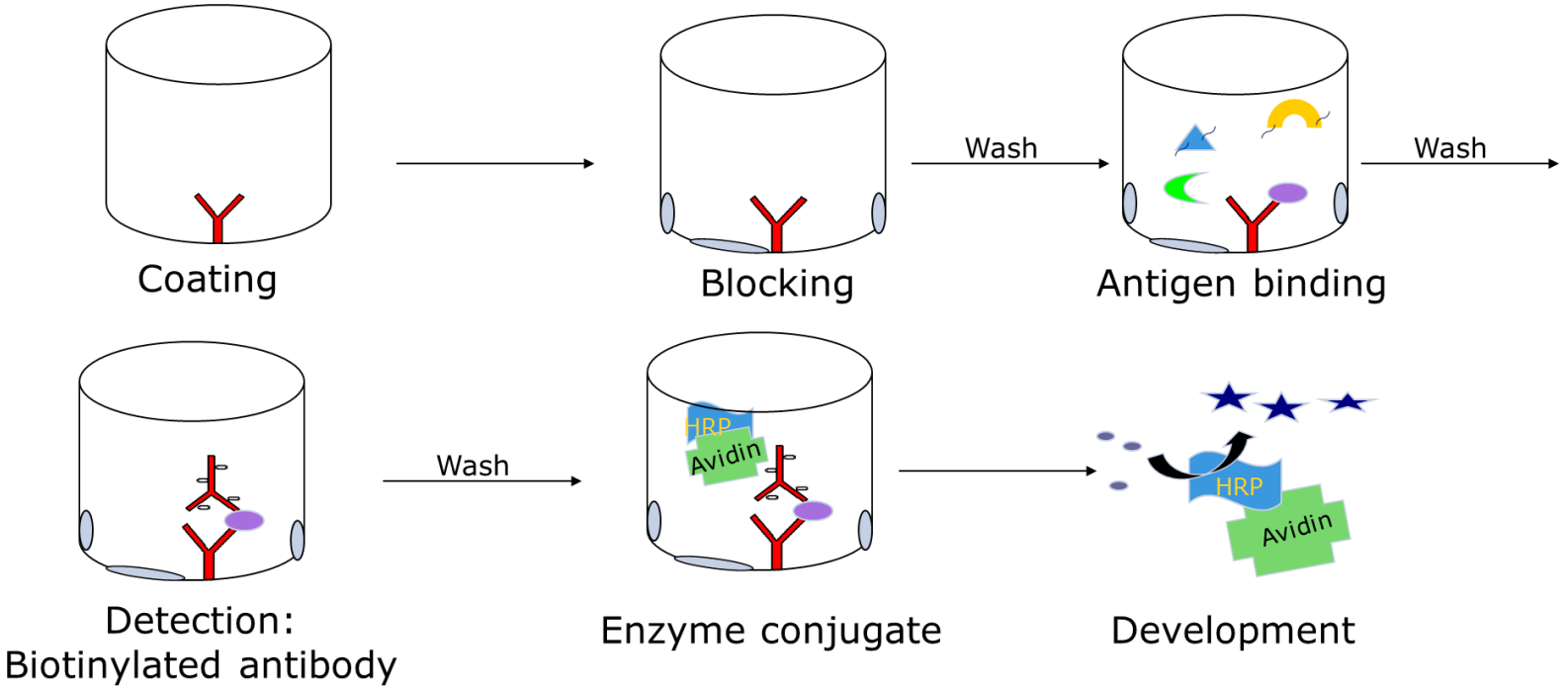
https://doi.org/10.1038/s41573-022-00529-w

Control strategy for HCP restrain process development

- Reduction of HCP to adequate levels required for safety and efficacy
- Modification steps reduce HCP detectability by ELISA – extensive epitope shielding (to ~30%)
- Potential reduction of HCP of downstream steps not accounted for



HCP ELISA: the QC workhorse - step by step



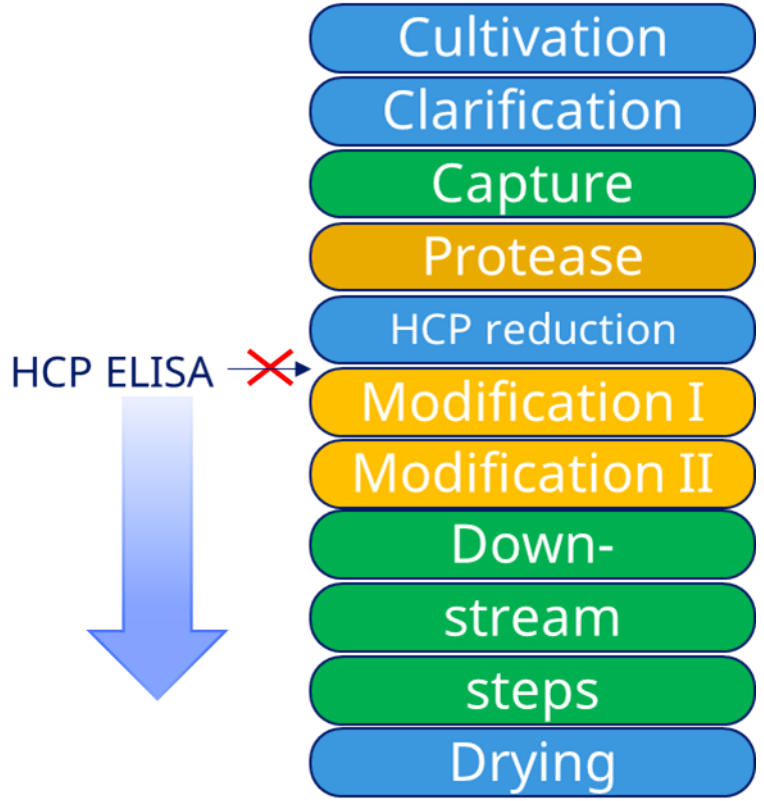
HCP ELISA – the QC workhorse

- Platform assay is preferred
  - LCM, accomodate optimisation/changes
- Produce representative antigen
  - Dummy strain, multiple dummy strains
- Immunise e.g. 20 rabbits
- Develop assay
  - Antigen is the standard
- Justify
  - Coverage - 2D gels and western blot
  - Dilutional linearity
  - Limit of quantification (sensitivity)
  - LC MS/MS, similarity & antibody recognition

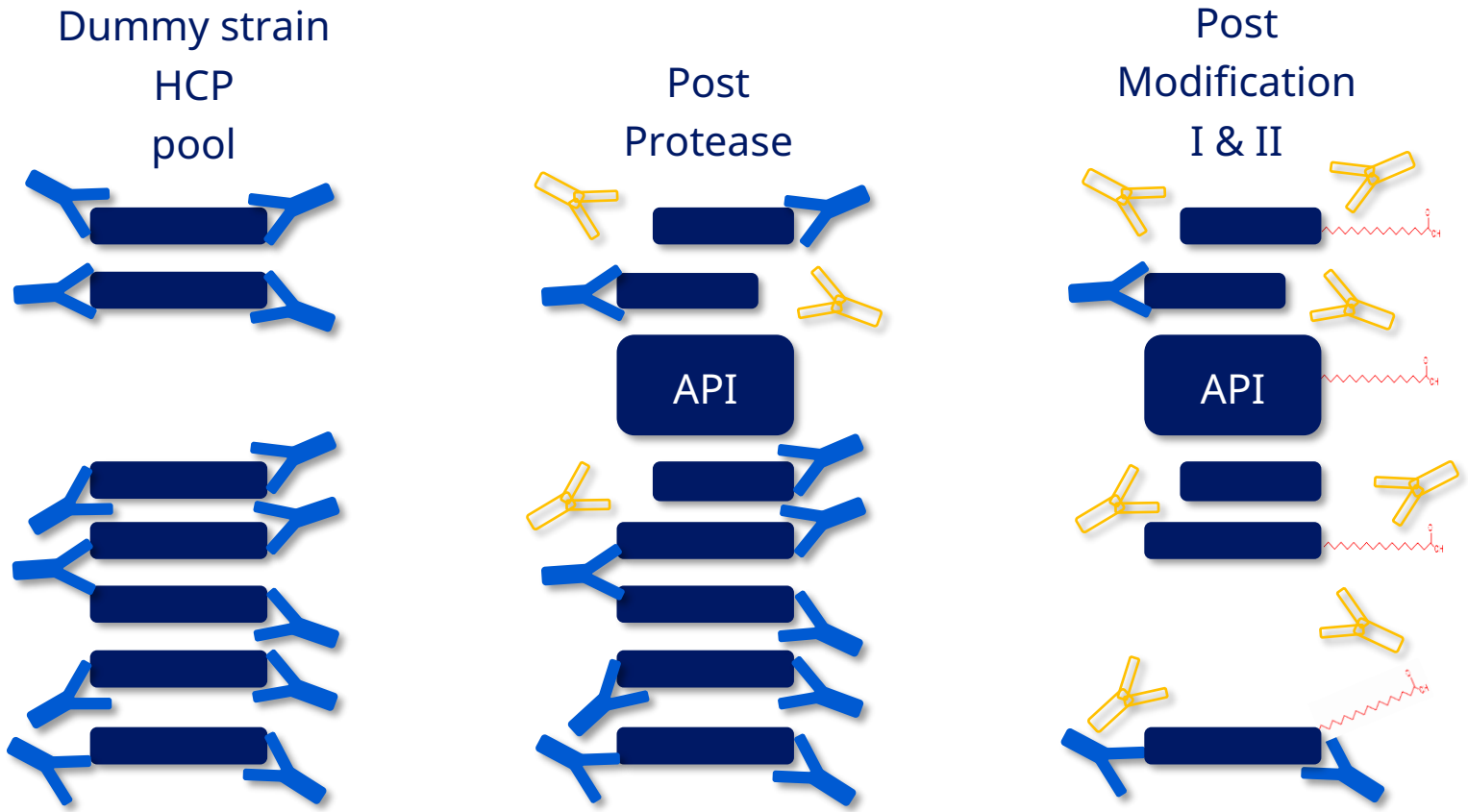
Challenge: HCP monitoring and control must not dictate process design and development

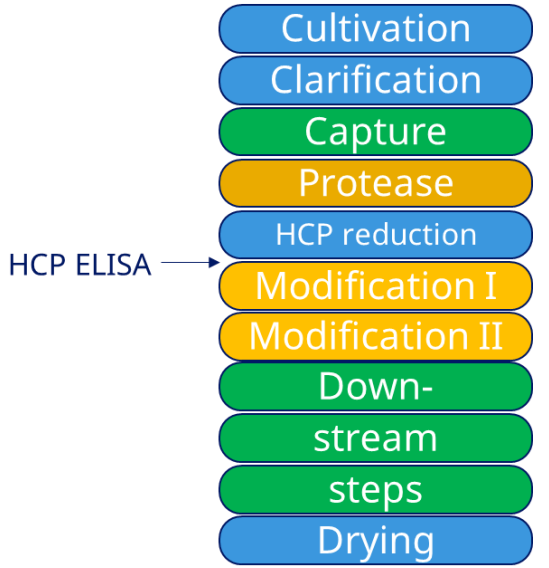
- “Move” HCP analysis down-stream
  - Gain full flexibility in process design
  - Gain full HCP reduction potential for all relevant process steps

- How?
  - ELISA preferred in QC
  - Obtain scientific understanding of HCP fate by HCP-MS
  - Development of HCP ELISA that can measure modified epitope



What causes epitope shielding?

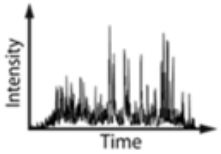


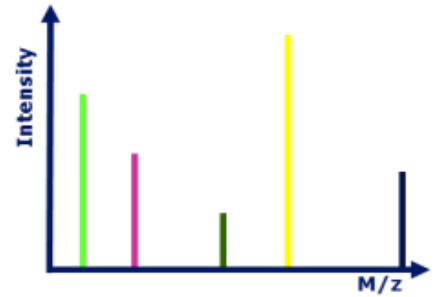


Classical strategy for HCP-MS analysis

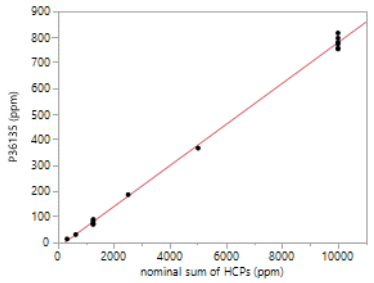
Biological samples → **Enzymatic digestion** → **LC separation** → **MS**

Cell lines
Process samples

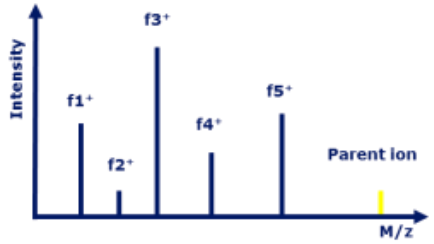




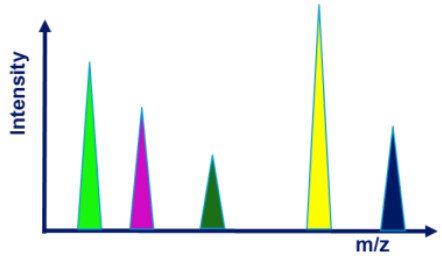
Spike-in Dummy HCP pool in peptide DS
LOD = 5 ppm
LOQ = 10 ppm



Fragmentation MS/MS



Quantification



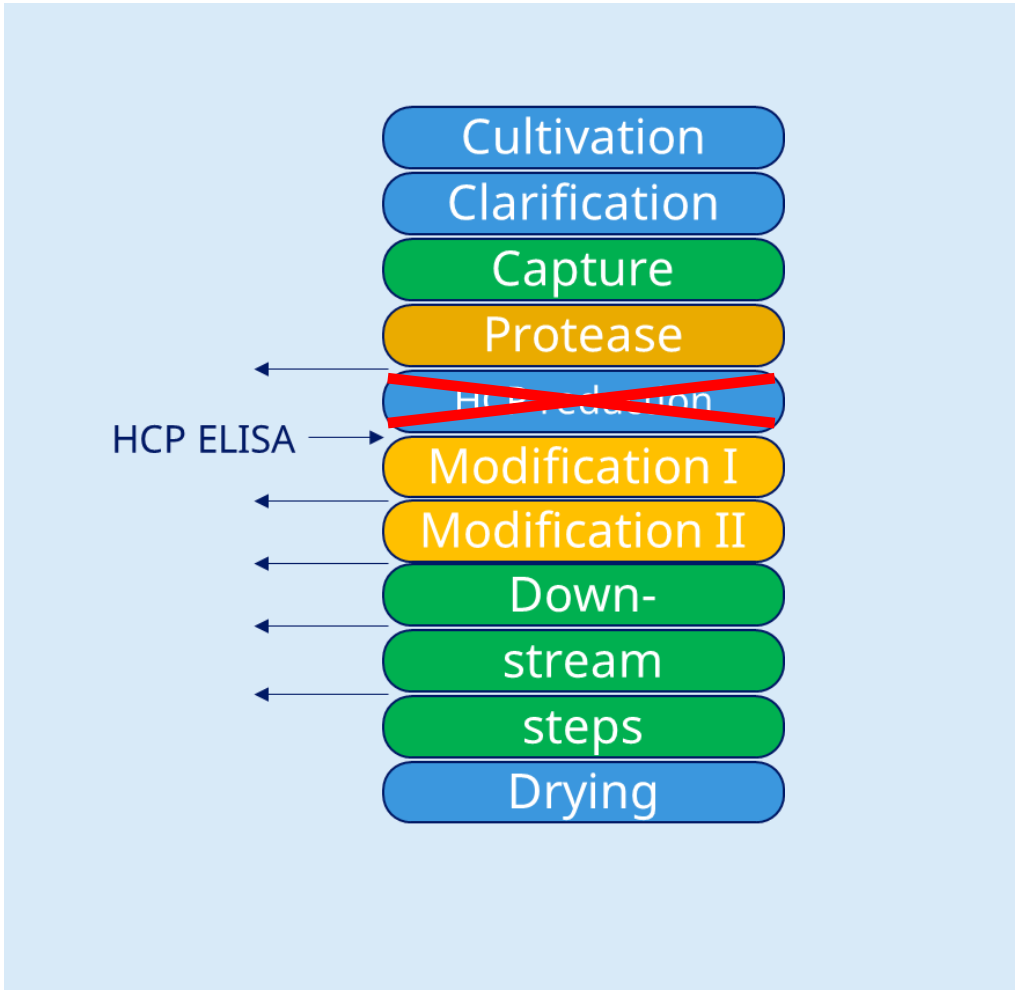
Area under curve based quantification for each HCP

MS/MS based ID

Protein	Quantification
DnaK	233 ppm
Flagellin	178 ppm
PLBL-2	62 ppm
PDI	11 ppm
GST	2 ppm

HCP reduction step reduces HCP to below LOQ for HCP ELISA

- ”Mock” process without HCP precipitation step
  - Enable characterisation of HCP fate during modification steps and downstream column steps
  - Generate antigen for modification-tolerant HCP ELISA
    - Will this process give representative antigen? Mass balance/stoichiometry
    - Is the fatty acid moiety immunogenic?
    - Cross reaction to drug?

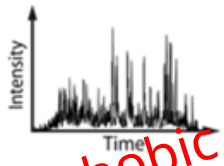


Classical strategy for HCP-MS analysis – Affected?

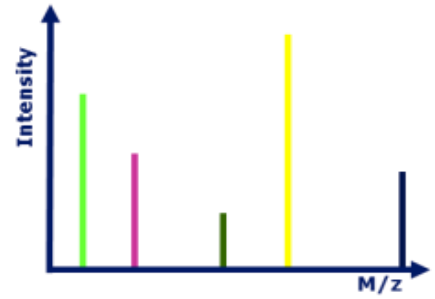
Biological samples → **Enzymatic digestion** → **LC separation** → **MS**

Cell lines
Process samples

Modified lysines!



Hydrophobic modification!



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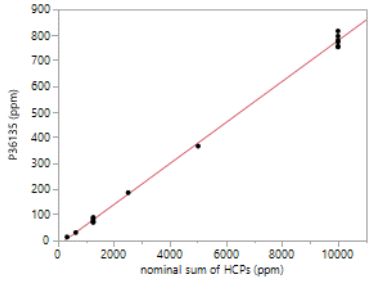
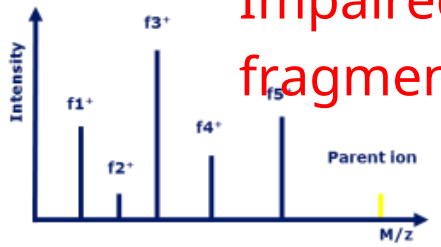
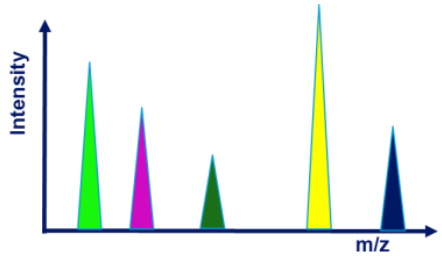


Fragmentation MS/MS

Impaired fragmentation?



Quantification



Area under curve based quantification for each HCP

MS/MS based ID

Protein	Quantification
DnaK	233 ppm
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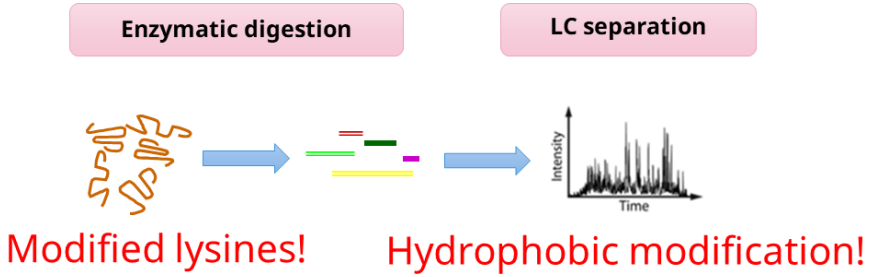
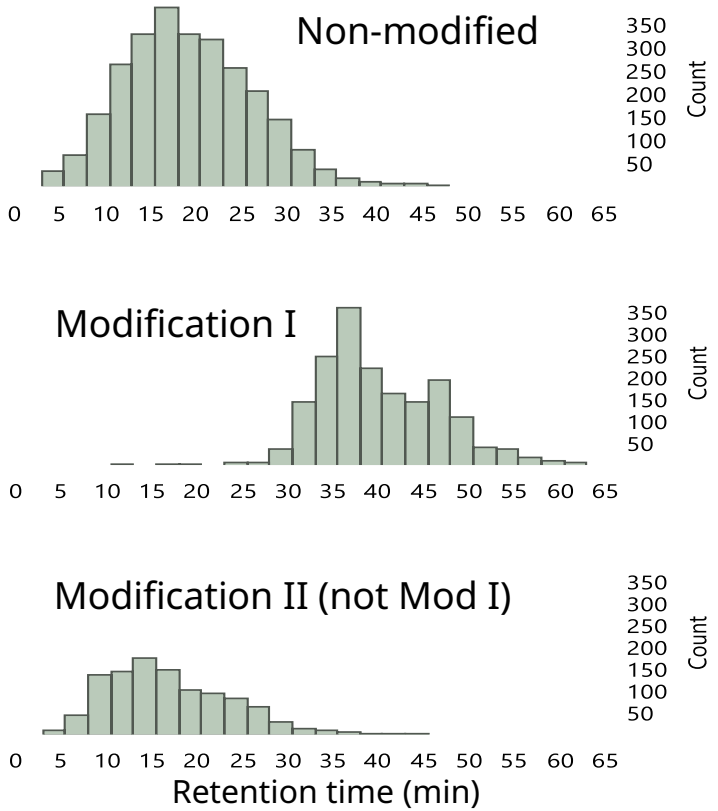
PLBL-2	62 ppm
PDI	11 ppm
GST	2 ppm

Modified vs. non-modified peptide versions!

Optimization of LC-MS for discovery of modified HCPs

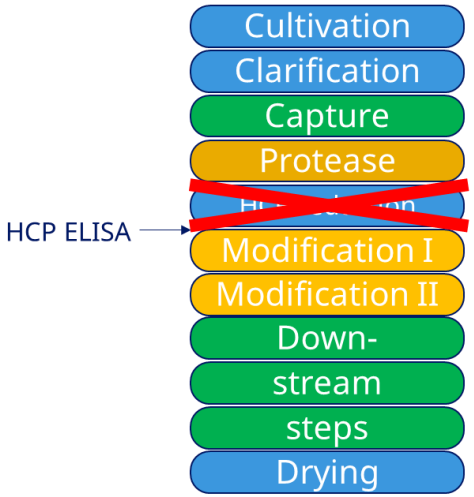


- Sample preparation optimisation
- Gradient optimisation
- Search parameter optimisation

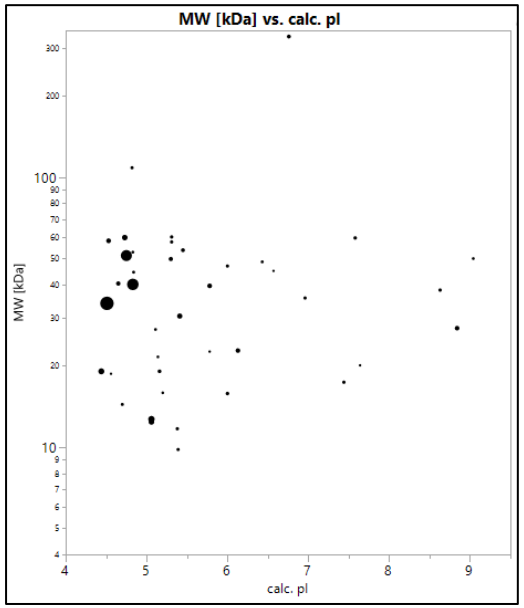


HCP peptide with	Sum
Modification I	2177
Modification II	1437
Modification I+II	342
Mod I or Mod II	3272
No modification	2648

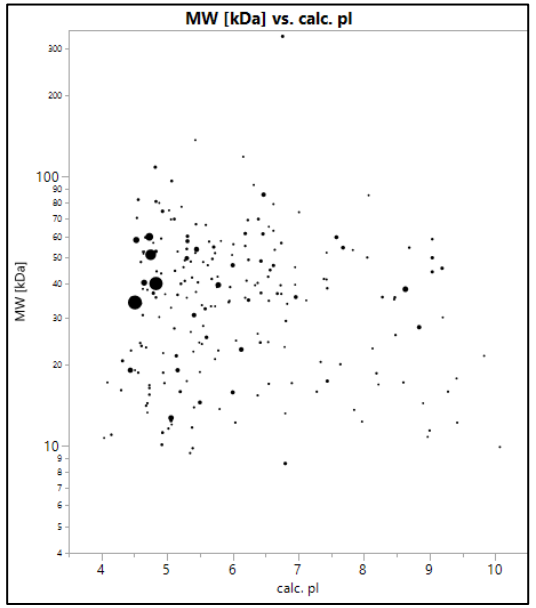
Compare full process and mock process



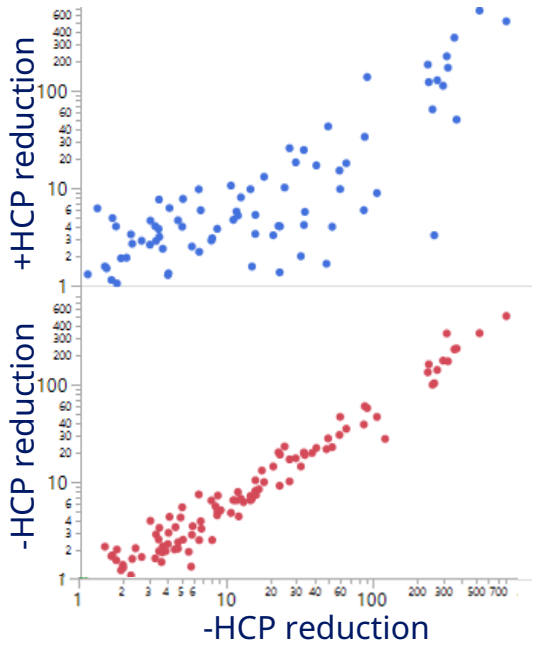
+ Partial HCP reduction
MS/MS identified



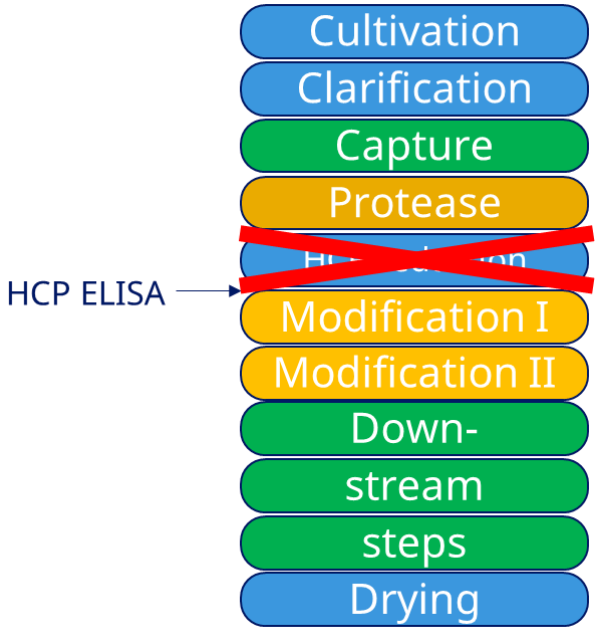
- HCP reduction
MS/MS identified

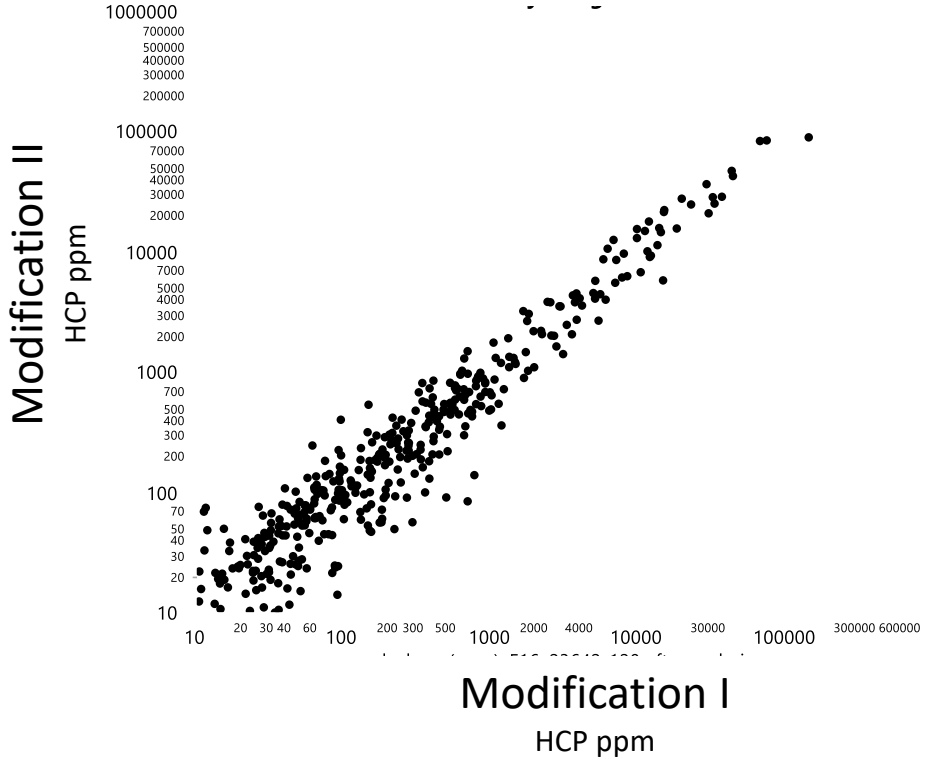


HCP levels
+/- reduction step



HCP levels following modification I and II steps





HCP reduction by downstream steps demonstrated

Strategy for HCP-ELISA antibody development

Cultivation
Clarification
Capture
Protease
HCP reduction
Modification I
Modification II
Down-
stream
steps
Drying

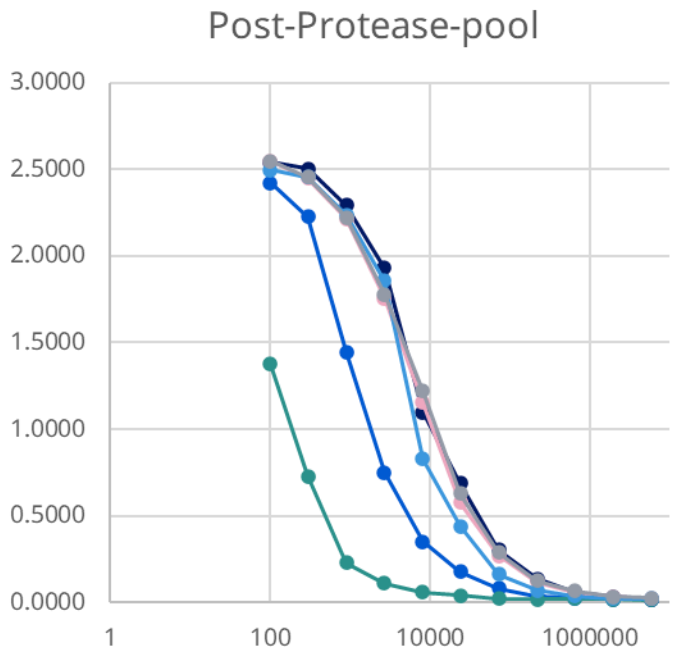
3 antigen pools

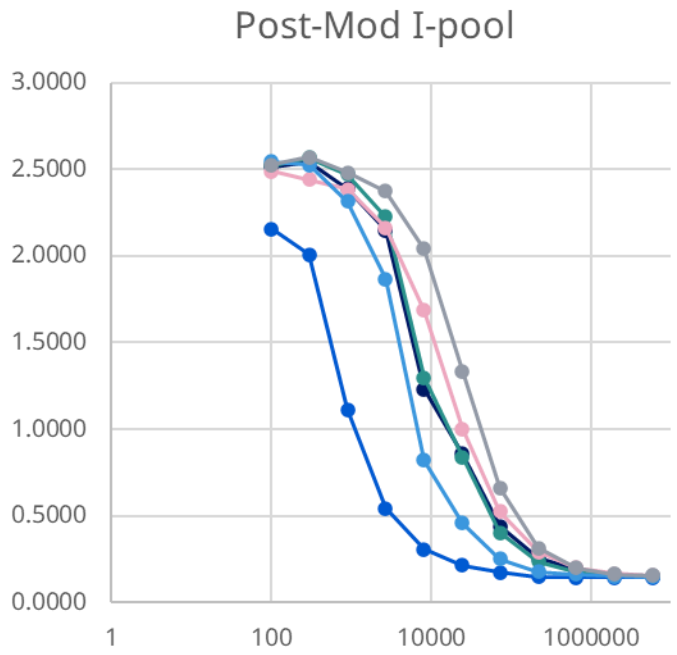
6

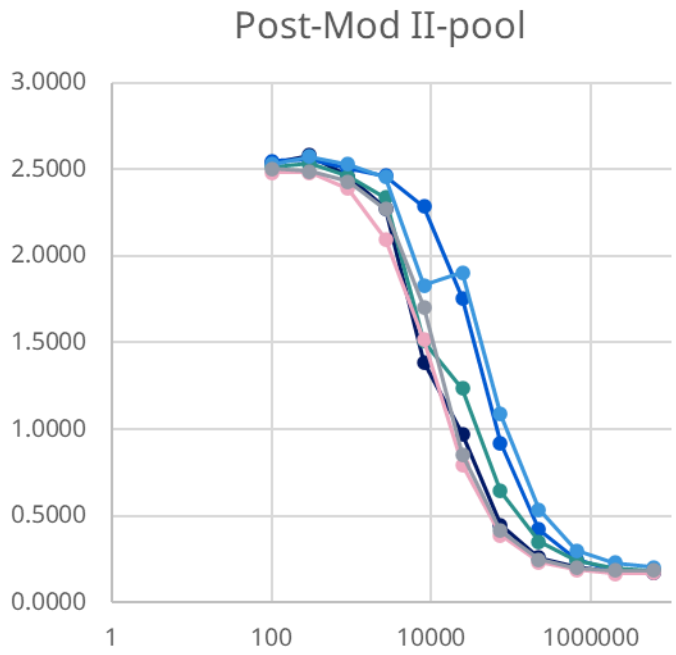
2

2

ELISA Titer - biotinylated rabbit pAb pools



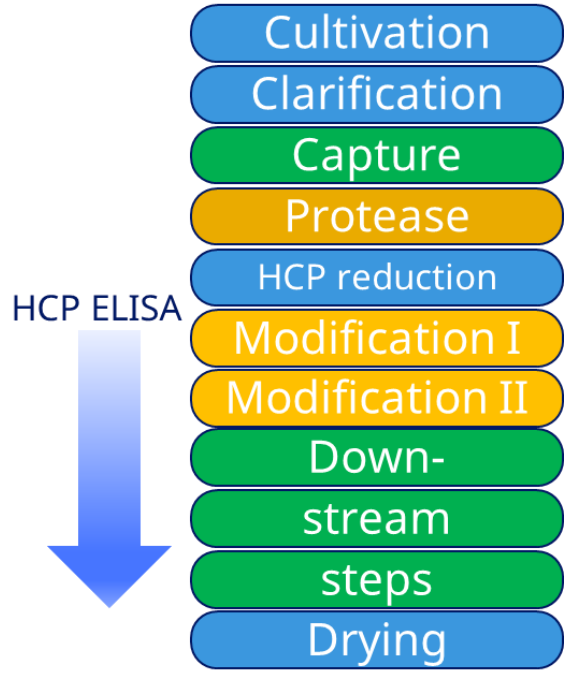




Conclusion & Perspectives

- Proof-of-concept for HCP ELISA monitoring after modification was established
- New paradigm for HCP ELISA reagent development
- Improved LC-MS analysis for modified HCPs
- HCP proteome conserved over modification steps
- High level of HCP modification by the process
- Efficient HCP reduction by downstream steps was demonstrated

- Next steps
  - Validation of novel highly modification-tolerant HCP ELISA
  - Feasibility of novel HCP ELISA as platform assay to be tested
  - Challenged reagent characterisation – HCP-MS required



Acknowledgements

- Lise Leonardsen – HCP ELISA
- Vibeke Mortensen – HCP ELISA
- Jonas Borch Jensen – HCP-MS
- Lars Sejersgaard – Mock DS Process
- Niels Krogsgaard-Larsen – Mock DS Process

- Jan Amstrup, Morten Hach, Sune Kobberup, Brian Kåre Kristensen

Team Novo Nordisk
Professional cycling team

Questions

Extra slides

Lysine occupancy

- Modification I and II inhibits tryptic cleavage
- A substantial number of lysines are occupied by Modification I or II
- Careful reporter peptide selection for targeted assays