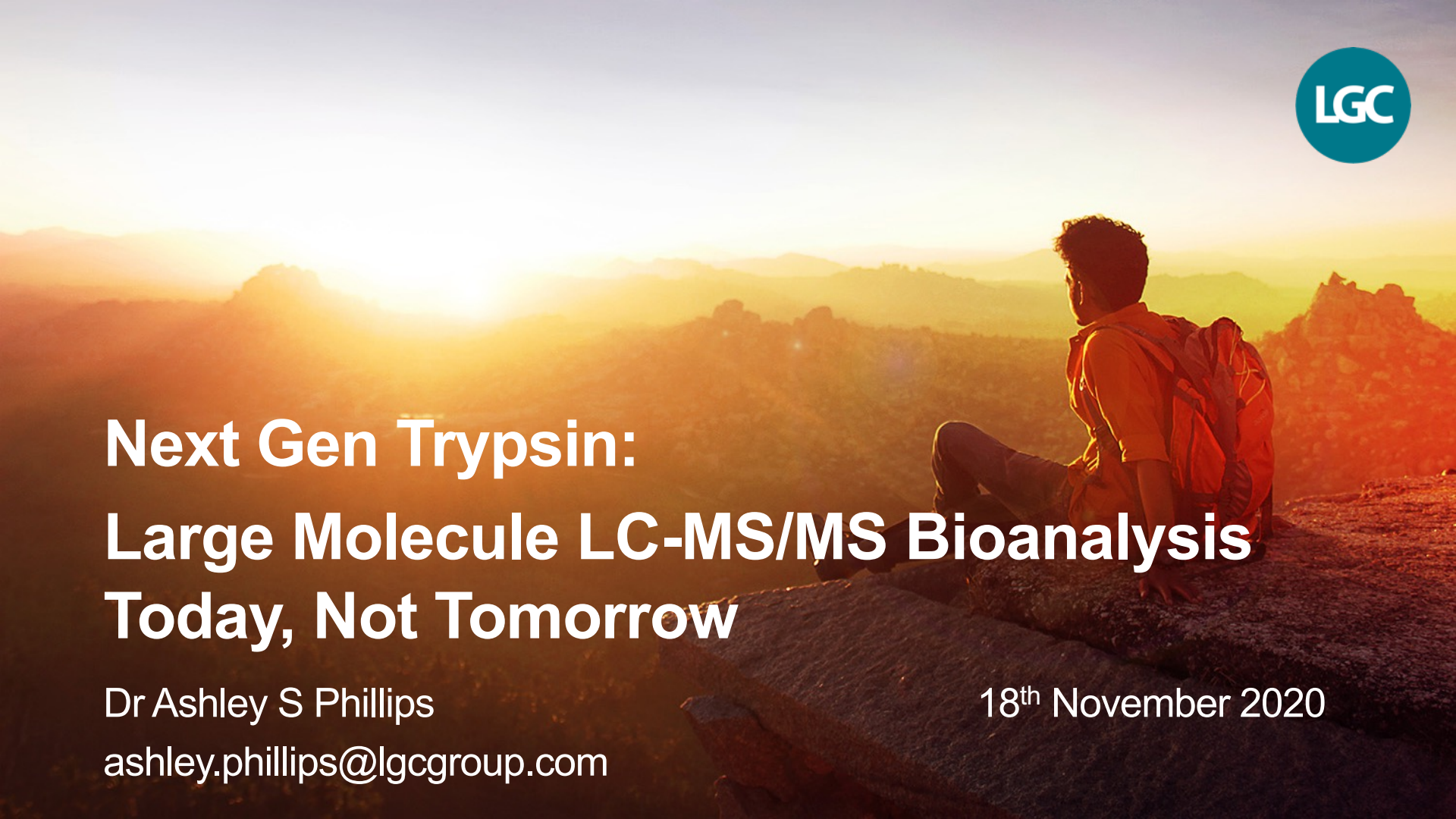

Next Gen Trypsin:

Large Molecule LC-MS/MS Bioanalysis Today, Not Tomorrow

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Large Molecule Bioanalysis by LC-MS/MS

We are always in search of lower LLOQs

which pushes us towards long, complicated protocols

leading us to:

- **Overnight digestion steps**
- **Immunoaffinity enrichment**
- **Solid Phase Extraction**

What is Next Generation Trypsin?

- **Next Generation Trypsin reagents aim to speed up the digestion process:**

- **They are either:**
  - Heat Stable Variants.
  - Bioreactor Style.
  - All in One Kits.

Fast… but is it any good?

The aim is to assess the next gen trypsin reagents for use in a quantitative bioanalytical method within a regulated laboratory environment.

- Stringent Criteria:
  - No miscleavages.
  - No deamidation or oxidation modifications.
  - Confident assignment.
  - Reproducible!

The Master Plan

Phase 1

Assess trypsins by digestion of protein standards in buffer.

Phase 2

Top performing trypsins evaluated using test sample.

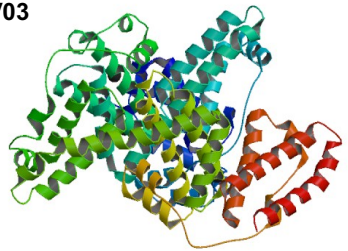
Our Candidates

	Promega Trypsin Gold	**Thermo Smart Digest Soluble**	**Thermo Magnetic Smart Digest**	**PreOmics iST Kit**	**Promega Rapid Digestion Trypsin**
Type	Traditional	Heat Stable	Heat Stable	All in One	Heat stable
Digest	Overnight	1 hour	1 hour	3 hour	1 hour
Flexibility					
Cost	€	€€	€€	€€€	€€

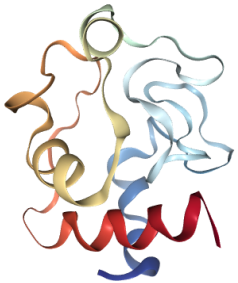
Phase 1 Overview

Protein standards selected to represent potential future biotherapeutics or biomarkers and a range of structural complexities.

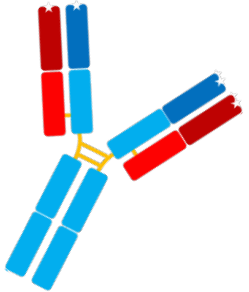
BSA
3V03



Cytochrome C Horse Heart
1AKK



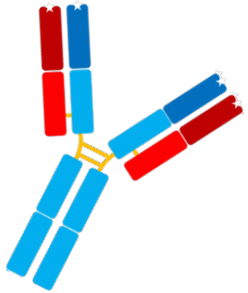
SILuLite SigmaMAb IgG1



Phase 1 Overview

Protein standards selected to represent potential future biotherapeutics or biomarkers and a range of structural complexities.

SILuLite
SigmaMAb
IgG1



The Method

Standard Peptide Mapping Approach used for Phase 1 and Phase 2

> Gradient: 2-50% organic over 50 minutes
> Phases: 0.1% FA in ACN and 0.1% FA (aq)
> Column: Waters Acquity HSS T3 2.1x100 mm

Instruments:

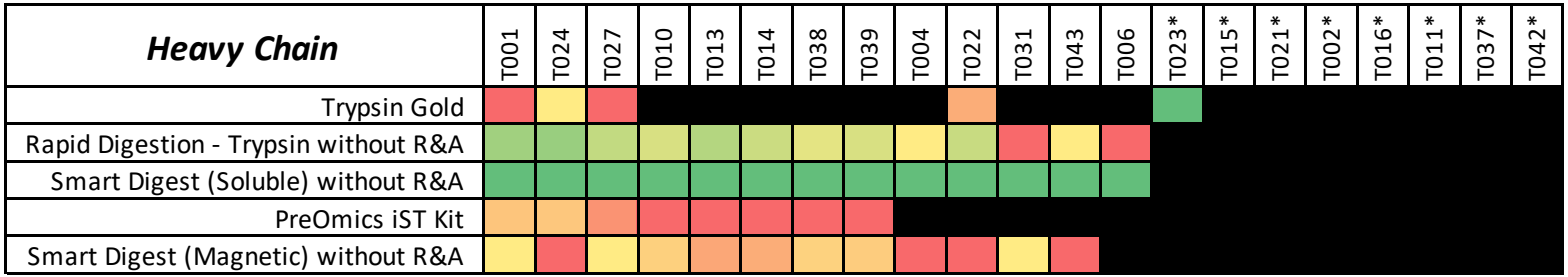
- **Phase 1:**
  > Waters Synapt G2 equipped with a Waters Acquity H-Class Bio
  > Acquisition Type: MS^e
  > Analysis Software: Biopharmalynx

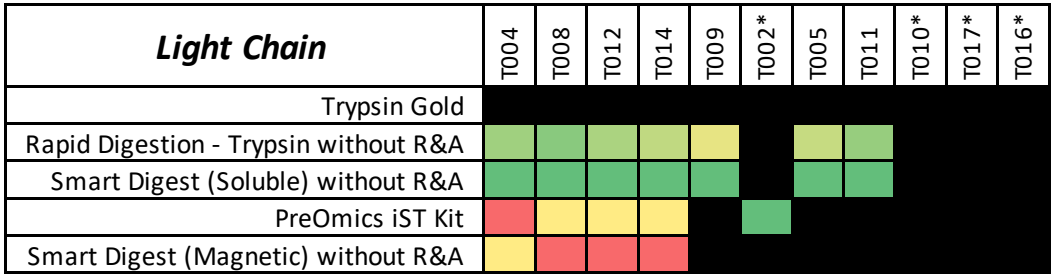
- **Phase 2:**
  > Sciex 6600 equipped with a Waters Classic Acquity
  > Acquisition Type: SWATH
  > Analysis Software: Biopharmaview

Phase 1

Digestion in buffer

SILuLite SigmaMAb





* denotes carbamidomethyl modification

n/o - not observed

Smart Digest Soluble and Rapid Digestion – Trypsin are the best performing candidates across all protein standards

Reduction and Alkylation: a thing of the past?

Heat Stable Trypsins:

☑ Heat denaturation

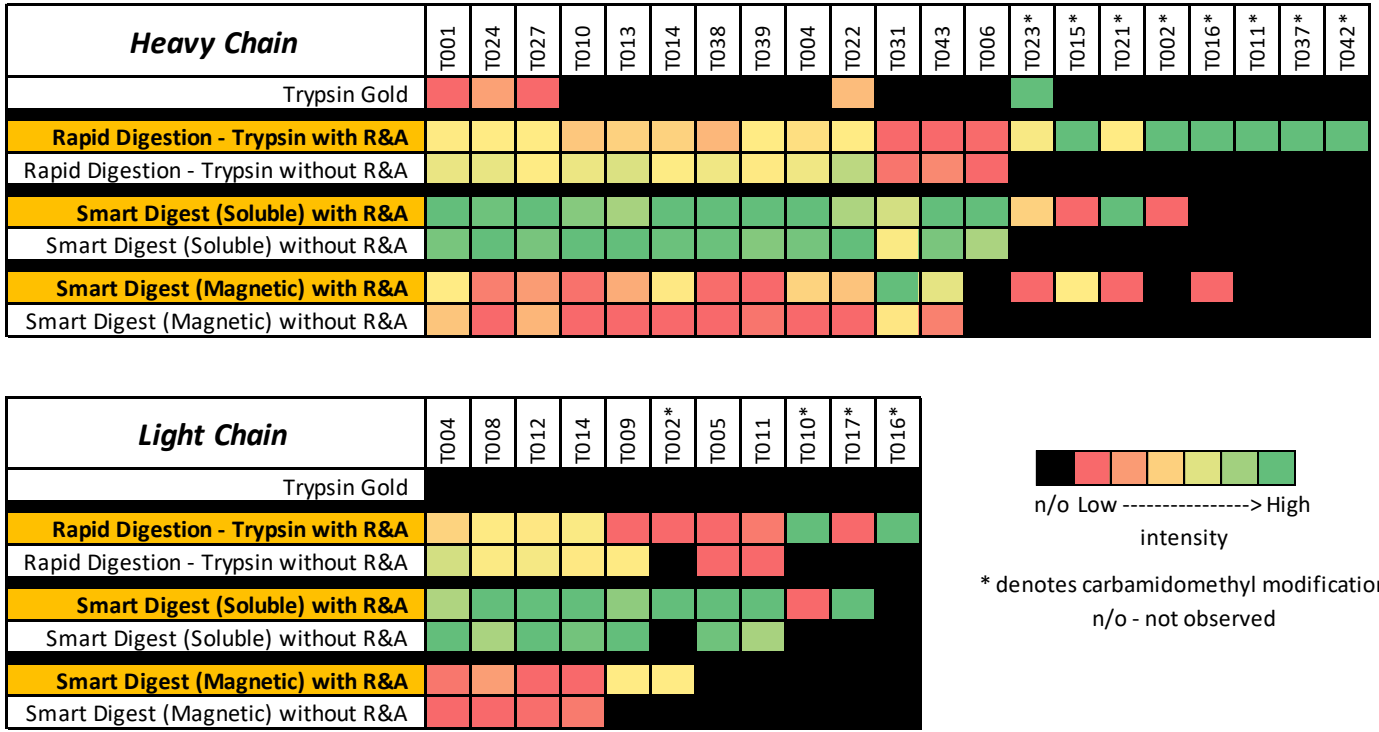
vs.

☒ Chemical denaturation

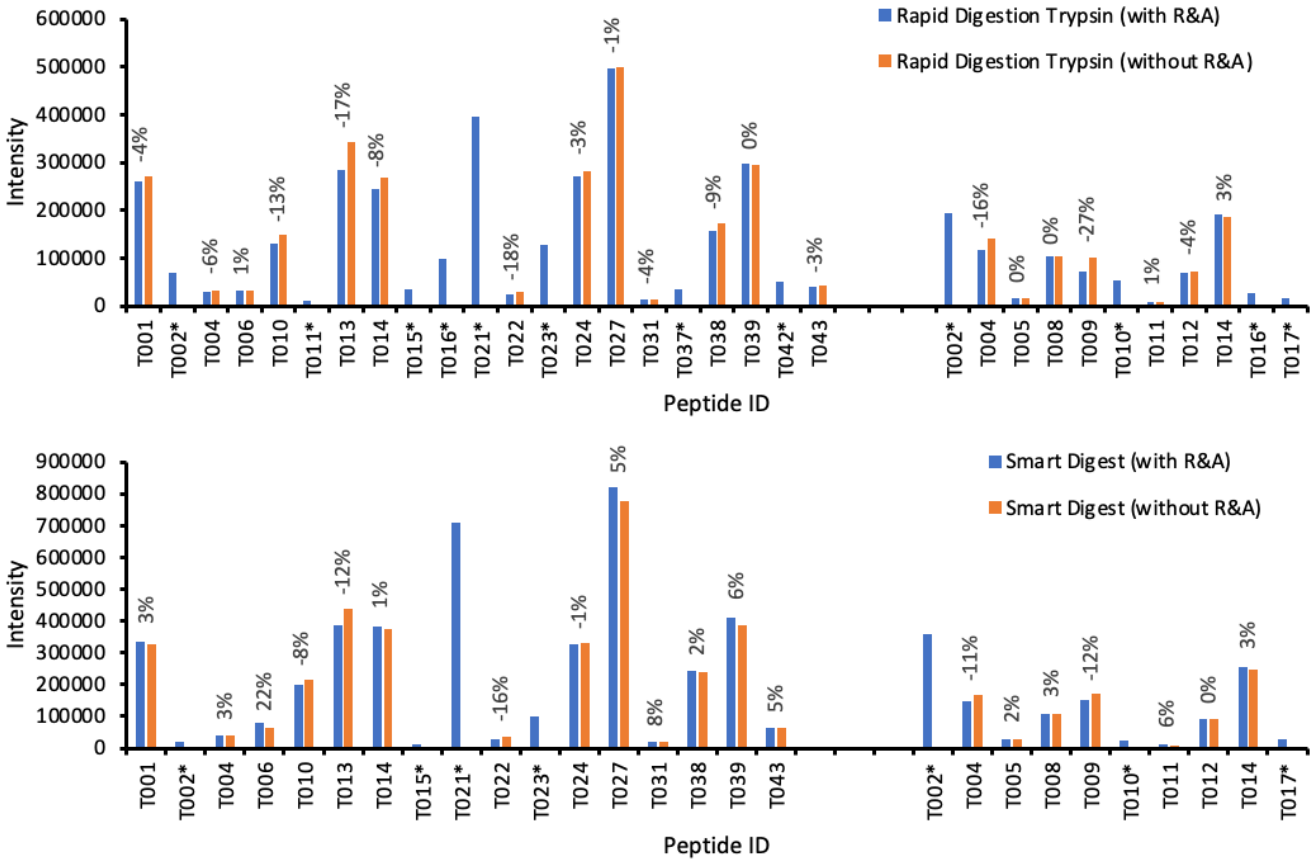
But why?

I'll do it anyway

The impact of reduction and alkylation

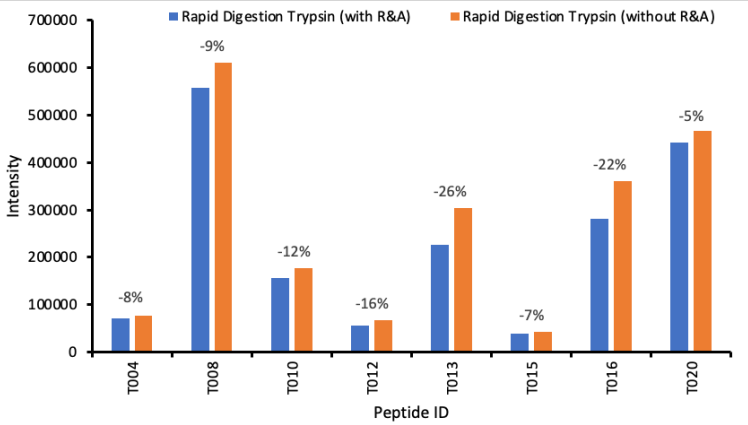


SILuLite SigmaMAb ± Reduction & Alkylation



A word of warning: *its subject specific*

Cytochrome C	T016	T008	T004	T010	T013	T020	T015	T012
Trypsin Gold								
Rapid Digestion - Trypsin with R&A								
Rapid Digestion - Trypsin without R&A								
Smart Digest (Soluble) with R&A								
Smart Digest (Soluble) without R&A								
Smart Digest (Magnetic) with R&A								
Smart Digest (Magnetic) without R&A								



★ *assess during development*

Our Candidates

★ *Weigh up requirements*

Phase 2

Digestion in matrix

Phase 2 Overview

- **Test sample: SILuLite SigmaMAb was reconstituted in Rat K2 EDTA plasma.**
  - Samples run in triplicate.
  - Reduction & Alkylation performed prior to digestion

	Promega Rapid Digestion - Trypsin	**Promega Trypsin Gold**	**Thermo Smart Digest Soluble**
Time	1, 2, 3 hours	Overnight	1, 2, 3 hours
Ratio	50:1, 100:1, 500:1	100:1	50:1, 100:1, 700:1 (MRR)

Overview statistics do not tell the whole story…

A Bottom Up Protein LC-MS/MS Bioanalytical Assay will typically focus on a single surrogate peptide for quantitation.

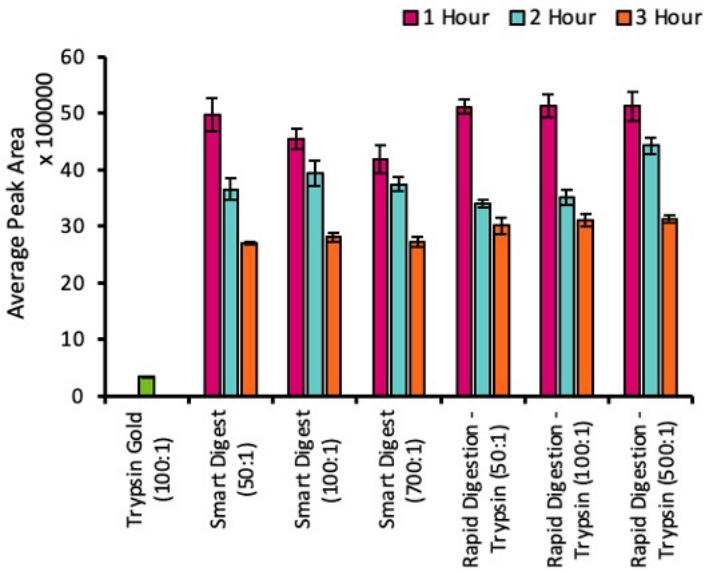
Selection of Surrogate Peptides

- The most intense charge state for each peptide was selected and averaged amongst replicates to enable comparison.

- **Heavy Chain**
  - STS GGTAALGCLV K
    - Does not feature any residues susceptible to modification (M,N,Q)
  - T PEVTCVVVDV SHEDPEVK
    - Does not feature any residues susceptible to modification (M,N,Q)

- **Light Chain**
  - TVAPTECS
    - Observed in all reagents including Trypsin Gold

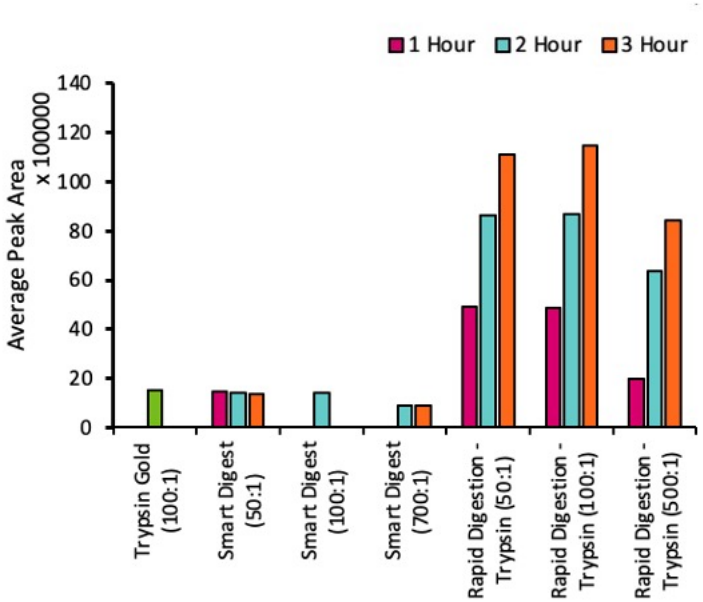
TVAPTECS Surrogate Peptide



Next Gen Trypsins perform much better than traditional overnight digestion

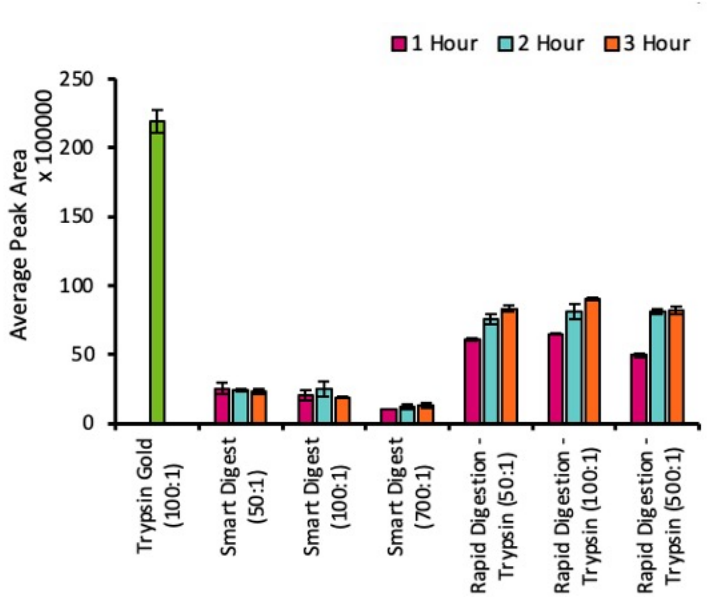
- Potential issues with peptide degradation.
- Rapid Digestion suggested protein:enzyme ratios are overkill
  - *Higher protein:enzyme ratios give comparable data*

STSG Surrogate Peptide



Rapid Digestion Trypsin generates a far greater peptide intensity in a fraction of the time!

TPEV Surrogate Peptide



The traditional overnight digest far outperforms the next gen trypsins

Phase 2 Outcomes

- **Promega Rapid Digestion – Trypsin is our preferred next generation trypsin**
  - It performs as comparably or better than Trypsin gold, whilst requiring a shorter digestion time.
  - Although TPEV is an exception to the rule – sensitivity demand will decide whether next gen trypsins are a viable option.

Is this the end of two day protein LC-MS/MS bioanalysis?

But what are the benefits?

- Improved Sensitivity*

*not guaranteed

- Quicker Study Turnaround
- Faster Method Development
- Rapid Troubleshooting

Is this the end of two day protein LC-MS/MS?

Almost…

Will a 1 hour digest actually save me any time?

Limited selection of enzymes

Sometimes trypsin just isn’t suitable

Optimisation is Key!

Protein:Enzyme Ratio,

Incubation Time and Pre-Digestion Processing all impact performance.

Acknowledgments

- **Curiosity Grant Scheme**
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  - Rob Wheller
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