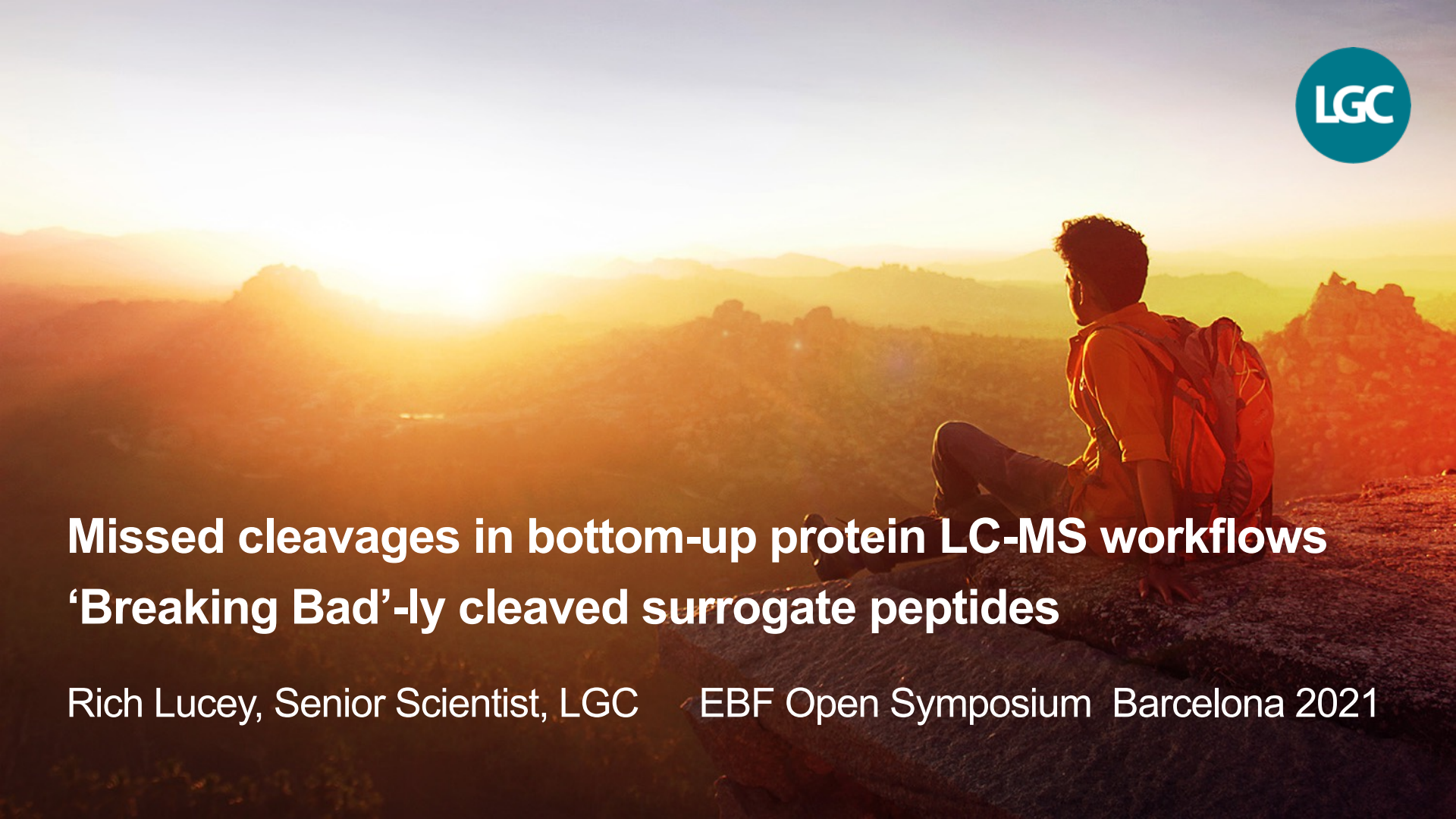


Missed cleavages in bottom-up protein LC-MS workflows

‘Breaking Bad’-ly cleaved surrogate peptides

Rich Lucey, Senior Scientist, LGC

EBF Open Symposium Barcelona 2021

Brief intro to protein structure

- 4 levels of protein structure

Quaternary

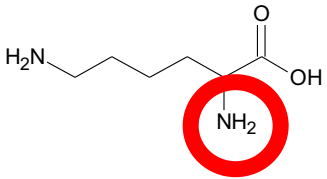
Tertiary

Secondary

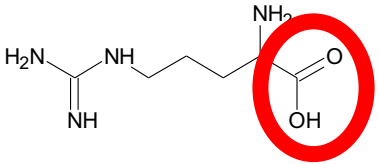
Primary

Brief intro to protein structure

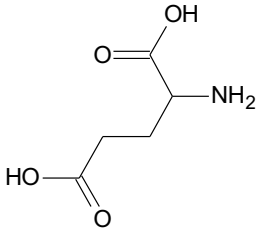
Primary



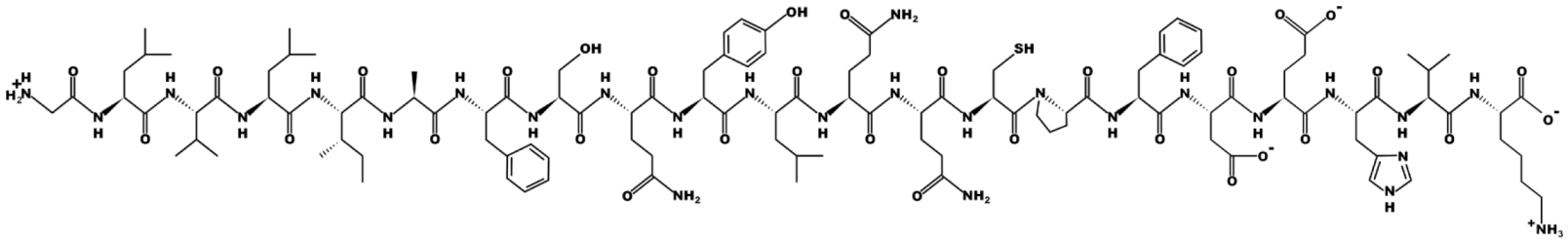
Lysine (K)



Arginine (R)

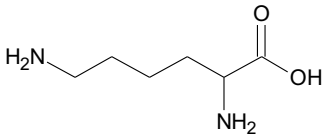


Glutamic acid (E)

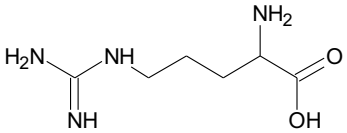


Brief intro to protein structure

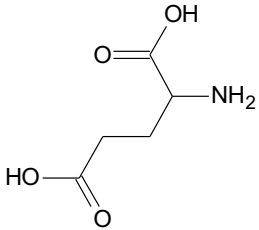
Primary



Lysine (K)



Arginine (R)



Glutamic acid (E)

M-P-S-S-V-S-W-G-I-L-L-L-A-G-L-C-C-L-V-P-V-S-L-A

Brief intro to protein structure

Secondary

Secondary structures formed by hydrogen bonding

α-helix

β-sheet

Primary

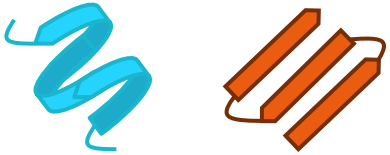
M-P-S-S-V-S-W-G-I-L-L-L-A-G-L-C-C-L-V-P-V-S-L-A

Brief intro to protein structure

Tertiary

3D structure of bound Primary and secondary structures

Secondary



Primary

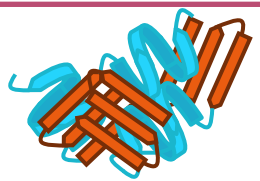
M-P-S-S-V-S-W-G-I-L-L-L-A-G-L-C-C-L-V-P-V-S-L-A

Brief intro to protein structure

Quaternary

Multiple amino acid chains bound together

Tertiary



Secondary

Primary

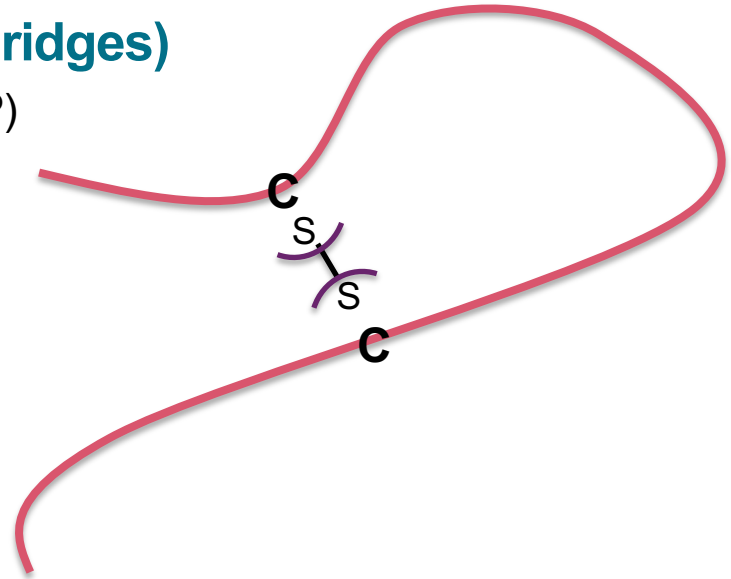
M-P-S-S-V-S-W-G-I-L-L-L-A-G-L-C-C-L-V-P-V-S-L-A

Brief intro to “bottom-up” approach

- **“Denature” (unfold protein)**
  - Chemically: Urea / Guanidine / Sodium deoxycholate (DOC)
  - Physically: Heat

Brief intro to “bottom-up” approach

- **“Denature” (unfold protein)**
  - Chemically: Urea / Guanidine / Sodium deoxycholate (DOC)
  - Physically: Heat
- **“Reduce” (break di-sulphide bridges)**
  - Tris(2-carboxyethyl)phosphine (TCEP)
  - Dithiothreitol (DTT)
- **“Alkylate” (cap)**
  - Iodoacetamide (IAA)



Brief intro to “bottom-up” approach

- **“Digest” (specific surrogate peptides)**
  - Peptides: Short chains of amino acids
  - Start with theoretical

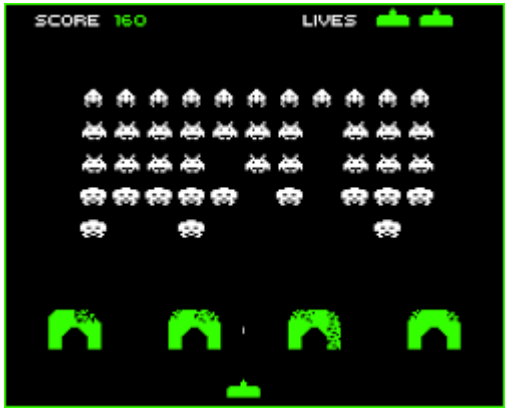
Brief intro to “bottom-up” approach

- **“Digest” (specific surrogate peptides)**
  - Peptides: Short chains of amino acids
  - Start with theoretical

H-R-L-T-I-D-E-K-G-T-E-A

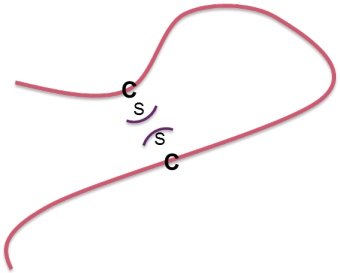
  - Blast search

  - Denaturant needs to be diluted (e.g. Urea <1M for trypsin)
  - Clean up sample before or after digestion (immuno-capture / SPE)



Brief intro to “bottom-up” approach

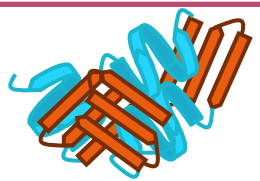
- **“Denature” (unfold protein)**
  - Urea / Guanidine / sodium deoxycholate (DOC)
  - Heat
- **“Reduce” (break sulphide bridges)**
  - TCEP / DTT
- **“Alkylate” (cap)**
  - Iodoacetamide (IAA)
- **“Digest” (specific surrogate peptides)**
  - Trypsin / Glu-C / Lys-C



Brief intro to protein structure

Quaternary

Tertiary



Secondary

Primary

M-P-S-S-V-S-W-G-I-L-L-L-A-G-L-C-C-L-V-P-V-S-L-A

The Challenge: Alpha-1-antitrypsin (A1AT)

- **Wild-type**
  - A protease inhibitor which protects tissues from enzymes of inflammatory cells
- **Mutant (Z type)**
  - A single mistake in the DNA encoding the protein causes A1AT deficiency leading to both liver and lung disease
  - To support safety and efficacy study for a novel compound

The Challenge: Alpha-1 antitrypsin

- **Wild-type**

MPSSVSWGILLLAGLCCLVPVSLAEDPQ
GDAAQKTDTSHHDQDHPTFNKITPNLAE
FAFSLYRQLAHQSNSTNIFFSPVSIATAFA
MLSLGTKADTHDEILEGLNFNLTEIPEAQI
HEGFQELLRTLNQPDSQLQLTTGNGLFL
SEGLKLVDKFLEDVKKLYHSEAFTVNFGD
TEEAKKQINDYVEKGTQGKIVDLVKELDR
DTVFALVNYIFFKGKWERPFEVKDTEEED
FHVDQVTTVKVPMMKRLGMFNIQHCKKL
SSWVLLMKYLGNATAIFFLPDEGKLQHLE
NELTHDIITKFLENEDRRSASLHLPKLSIT
GTYDLKSVLGQLGITKVFSNGADLSGVTE
EAPLKLSKAVHKAVLTIDEKGTEAAGAMF
LEAIPMSIPPEVKFNKPFVFLMIEQNTKSP
LFMGKVVNPTQK

- **Mutant (Z type)**

MPSSVSWGILLLAGLCCLVPVSLAEDPQ
GDAAQKTDTSHHDQDHPTFNKITPNLAE
FAFSLYRQLAHQSNSTNIFFSPVSIATAFA
MLSLGTKADTHDEILEGLNFNLTEIPEAQI
HEGFQELLRTLNQPDSQLQLTTGNGLFL
SEGLKLVDKFLEDVKKLYHSEAFTVNFGD
TEEAKKQINDYVEKGTQGKIVDLVKELDR
DTVFALVNYIFFKGKWERPFEVKDTEEED
FHVDQVTTVKVPMMKRLGMFNIQHCKKL
SSWVLLMKYLGNATAIFFLPDEGKLQHLE
NELTHDIITKFLENEDRRSASLHLPKLSIT
GTYDLKSVLGQLGITKVFSNGADLSGVTE
EAPLKLSKAVHKAVLTIDKKGTEAAGAMF
LEAIPMSIPPEVKFNKPFVFLMIEQNTKSP
LFMGKVVNPTQK

Missed Cleavage (Ragged End)

	Cleaves	Wild type	Mutant
Trypsin	After R and K	AVLTID**E**K	AVLTID**K**

Missed Cleavage (Ragged End)

	Cleaves	Wild type	Mutant
Trypsin	After R and K	AVLTID**E**K	AVLTID**K** *AVLTID**K**K*

- Trypsin method produces “ragged end” missed cleavage for the mutant protein
- Missed cleaved peptides can cause variation in protein quantitation

Missed Cleavage (Ragged End)

	Cleaves	Wild type	Mutant
Trypsin	After R and K	AVLTID**E**K	AVLTID**K** *AVLTID**K**K*
Glu-C	After E	APLKLSKAVHKAVLTID**E**	APLKLSKAVHKAVLTID**K**KGTE

Method/results

- **Sample prep**
  - Wild type or mutant protein in PBS buffer
  - 30min denature and reduce (urea & TCEP)
  - 30min alklation (IAA)
  - Overnight digest Glu-C (37°C)
  - Reaction quenched with formic acid

- **How did we measure?**
  - Acquity UPLC - generic slow gradient
  - Sciex 6600 QToF HRMS - Swath acquisition (data independent analysis)
  - Peptide ID using BioPharmaView™ software

Results

	Cleaves	Wild type	Mutant
Glu-C	After E	APLKLSKAVHKAVLTID**E**	APLKLSKAVHKAVLTID**K**KGTE

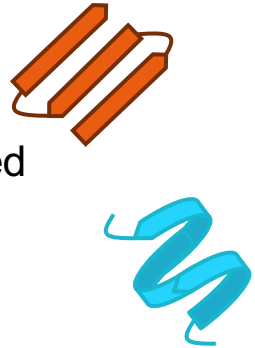
Results

- **Only missed cleaved peptides detected**

	Cleaves	Wild type	Mutant
Glu-C	After E	APLKLSKAVHKAVLTID**E**	APLKLSKAVHKAVLTID**K**KGTE
		*E*APLKLSKAVHKAVLTID**E**	*E*APLKLSKAVHKAVLTID**K**KGTE

Why? – Lit search

- **Denature**
  - Not fully denatured unfolded, and reduced
    - Structures may remain!



M-P-S-S-V-S-W-G-I-L-L

  - Denature with heat?
  - Denature with guanidine?
  - Denature with DOC?

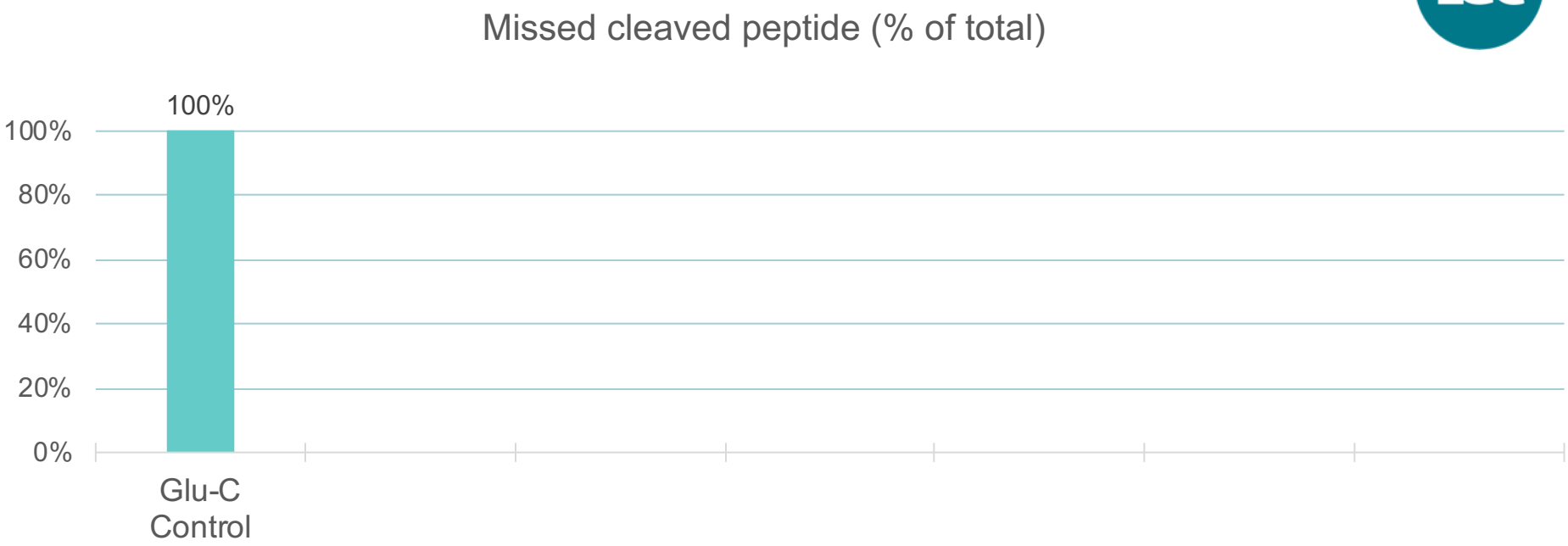
Why? – Lit search

- **Digestion**
  - Increase enzyme to protein ratio
  - Digest in denaturing conditions (Lys-C – special Glu-C)
  - Digest in acidic conditions (pH4) (Glu-C)
  - Rapid digest trypsin (heat stable)

	Cleaves	Wild type	Mutant
Trypsin	After R and K	AVLTID**E**K	AVLTID**K** *AVLTID**K**K*
Glu-C	After E	APLKLSKAVHKAVLTID**E**	APLKLSKAVHKAVLTID**K**KGTE
Lys-C	After K	AVLTID**E**K	AVLTID**K** *AVLTID**K**K*

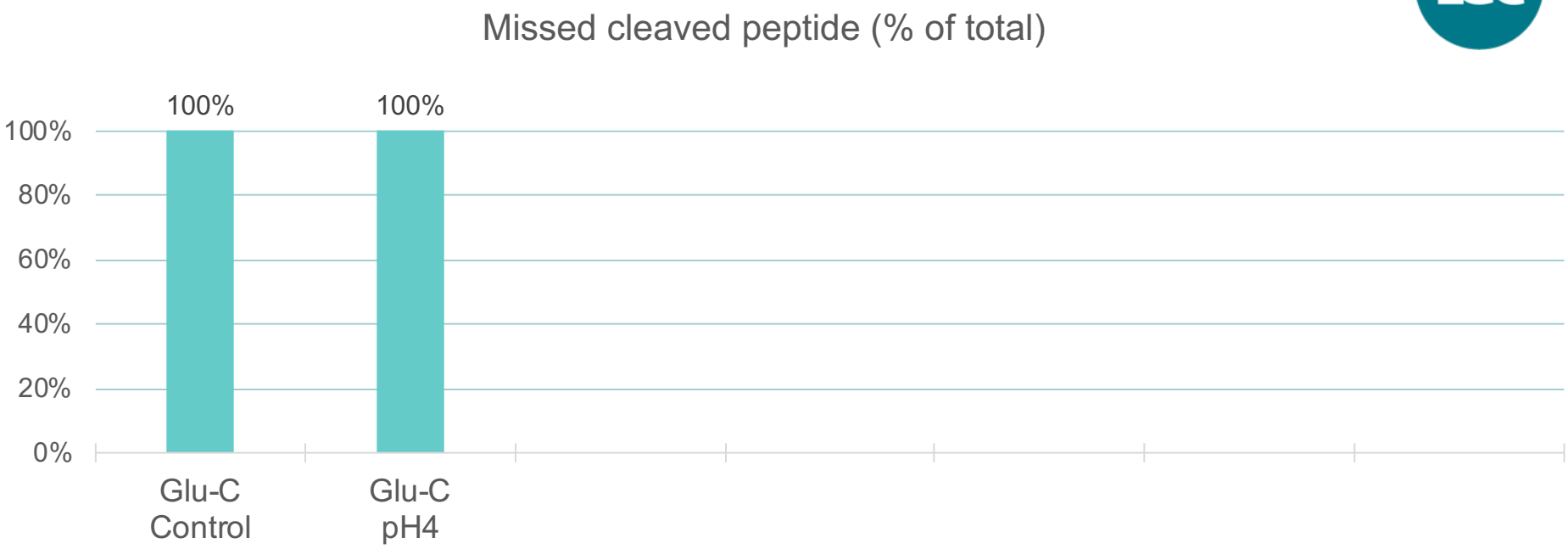
  - Trypsin and Lys-C mix can reduce missed cleavages

Results



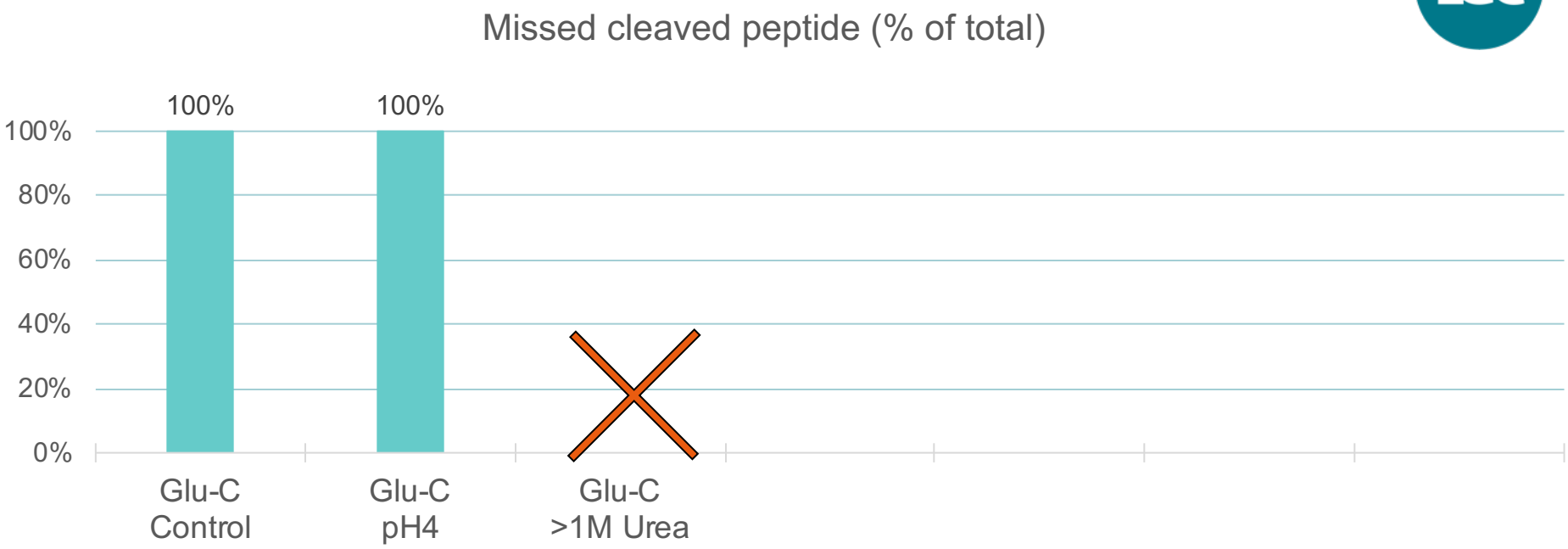
- 1 missed cleave: EAPLKLSKAVHKAVLTIDKKGTE
- No fully cleaved peptide detected

Results



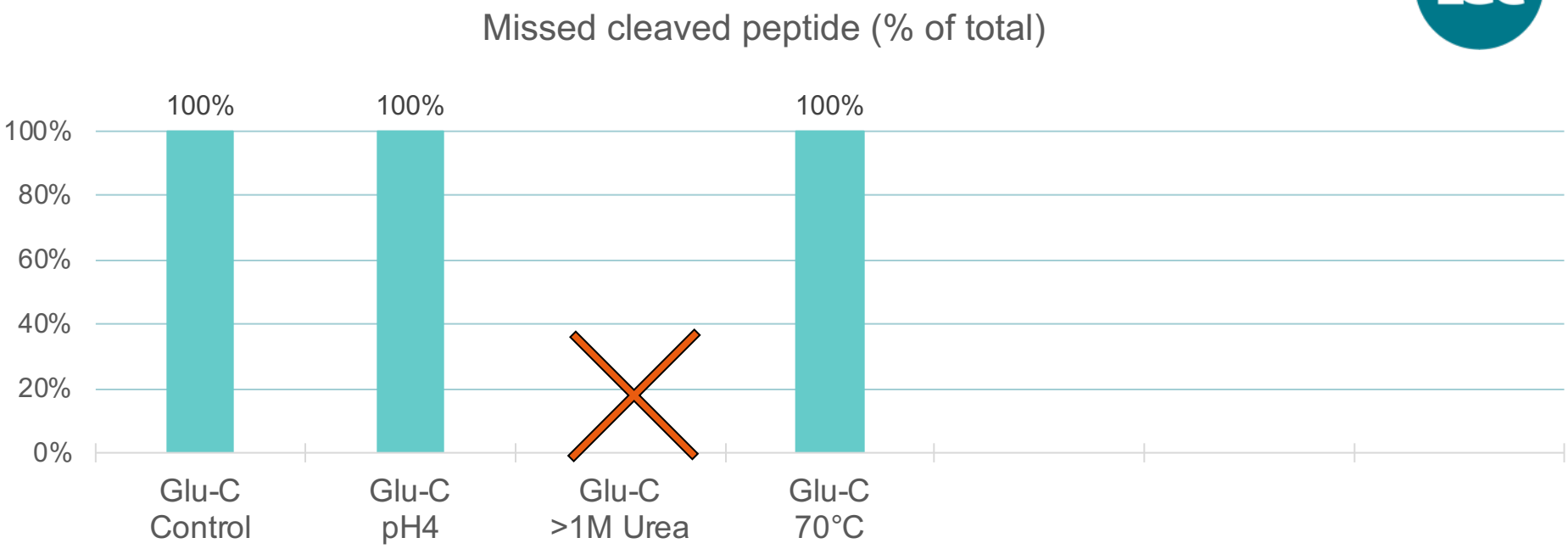
- 2 missed cleaves: ***E***APLKLSKAVHKAVLTIDKKGTE***AAGAMFLE***
- No fully cleaved peptide detected

Results



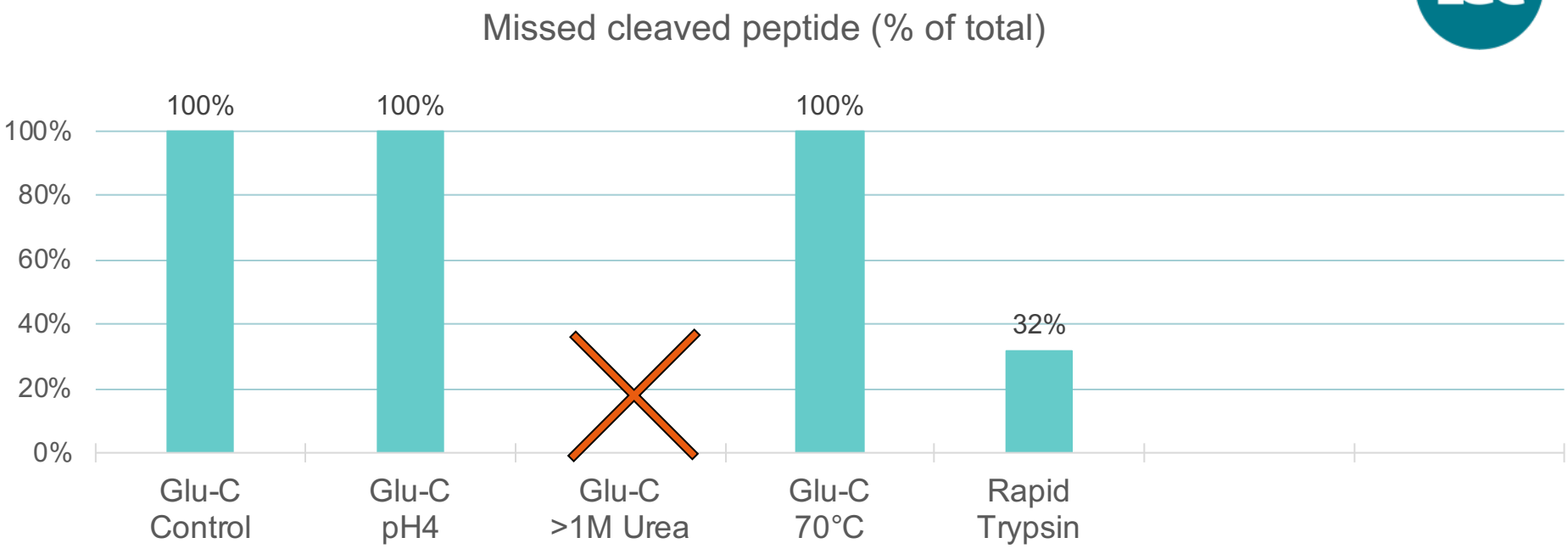
- 2M Urea, 4M Urea
- No peptides detected in target region

Results



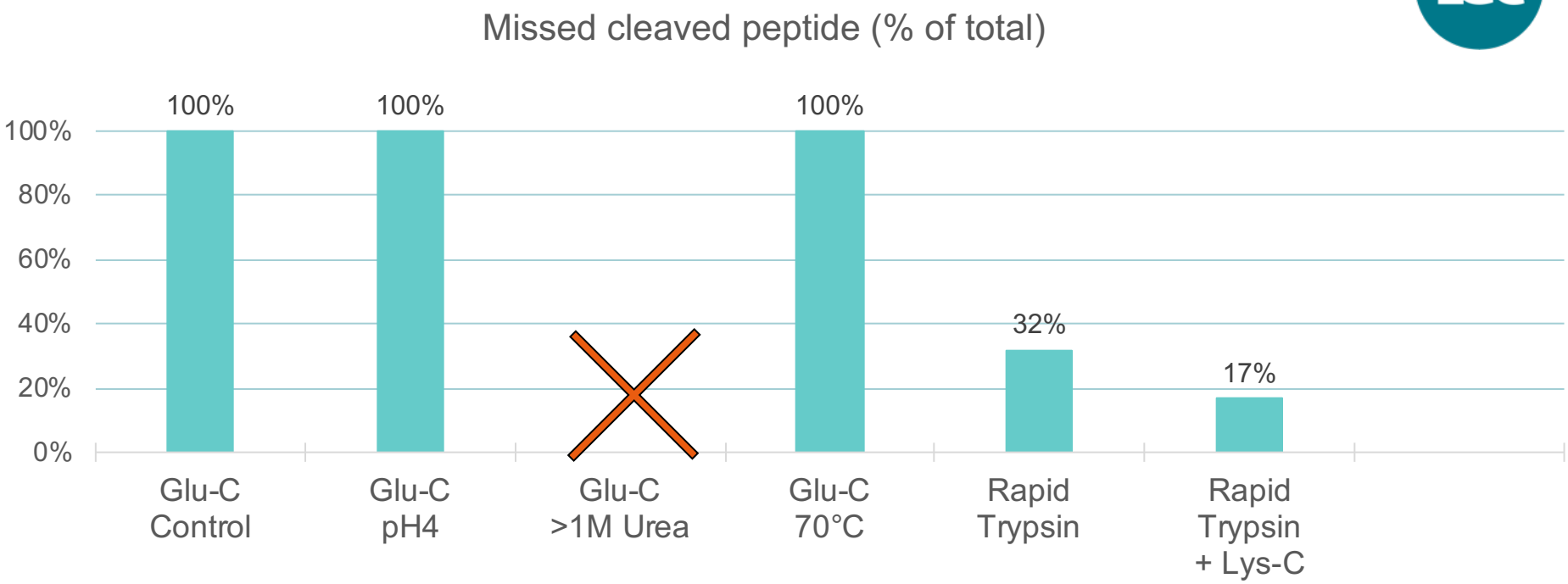
- 1 missed cleave: EAPLKLSKAVHKAVLTIDEKGTE
- No fully cleaved peptide detected
- Wild type used for this experiment

Results



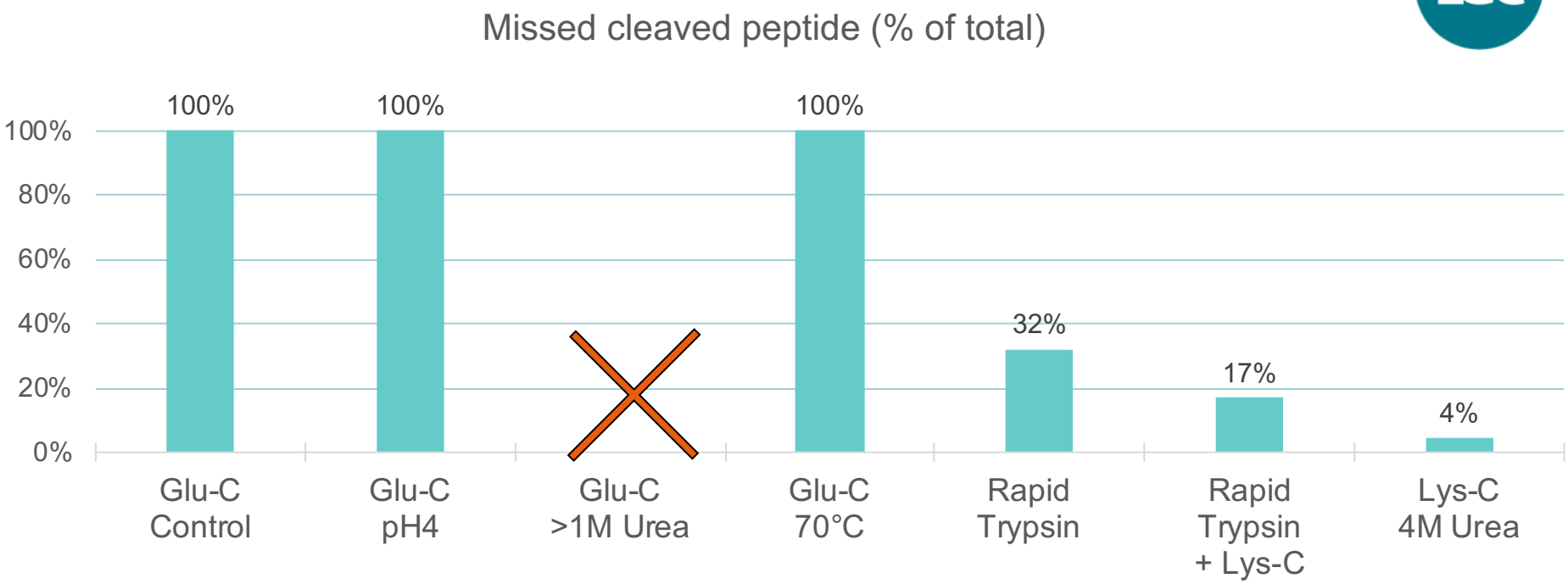
- 3 hour digestion at 70°C, no urea, rapid buffer
- 1 missed cleave: AVLTIDK***K***
- Fully cleaved peptide also detected: AVLTIDK

Results



- 3 hour digestion at 70°C, no urea, rapid buffer
- 1 missed cleave: AVLTIDK***K***
- Fully cleaved peptide also detected: AVLTIDK

Results



- Overnight digestion in 4M urea
- 1 missed cleave: AVLTIDK***K***
- Fully cleaved peptide also detected: AVLTIDK

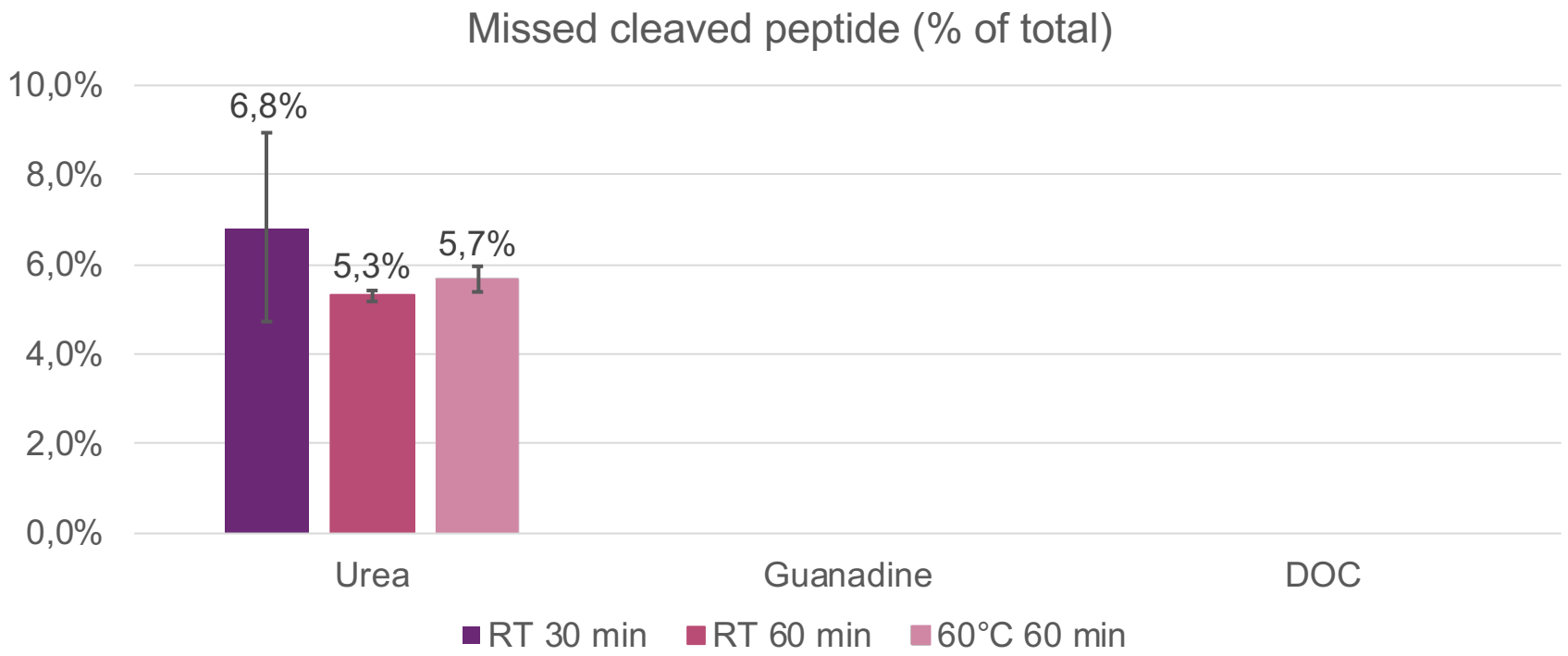
Further Lys-C optimisations

- **Denaturing conditions in matrix (serum)**
  - Denaturant: (urea / guanidine / DOC)
  - Denaturing temperature
  - Time 30/60 minutes

- **Digestion conditions in matrix (serum)**
  - Enzyme : protein ratio
  - Digestion time (1 hour/ 2 hour/ overnight)

Results

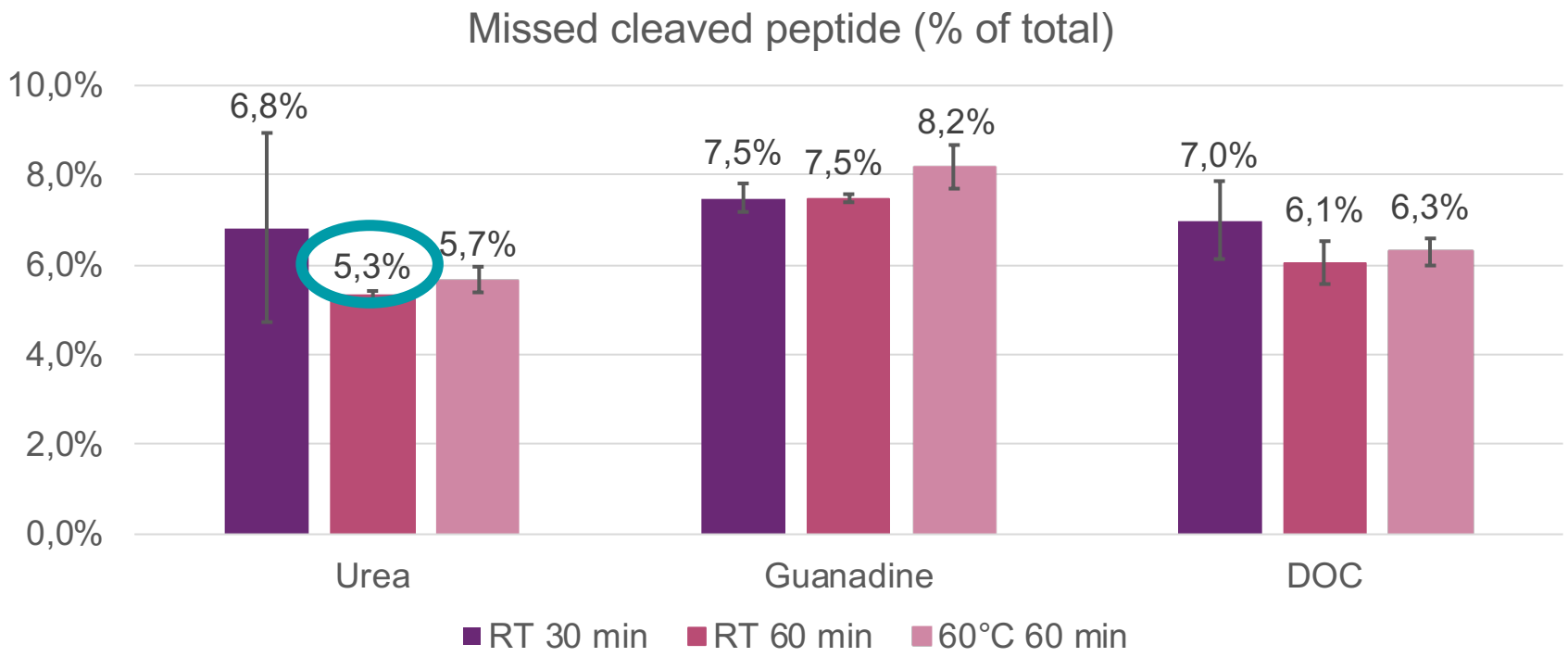
- **Denaturing conditions in human serum**



Average of 2 concentrations of mutant A1AT digested in singlicate

Results

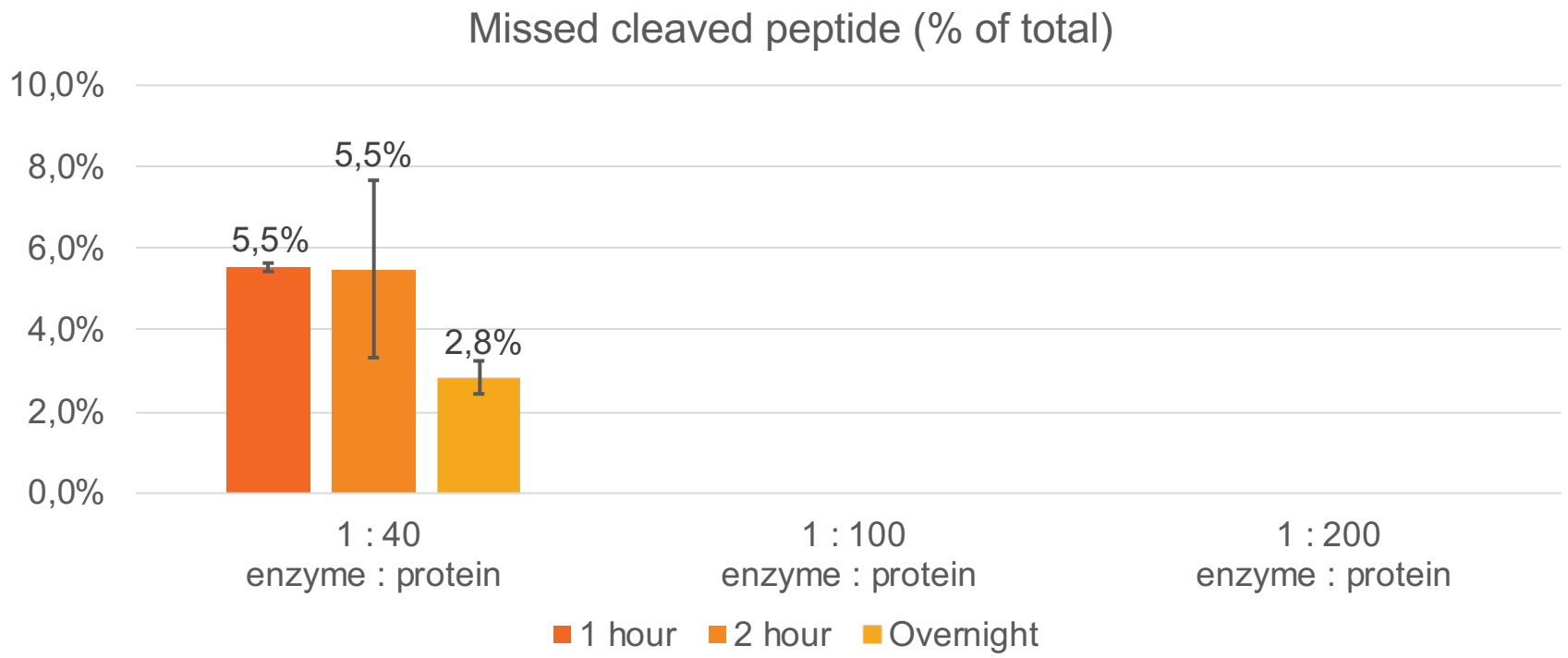
- **Denaturing conditions in human serum**



Average of 2 concentrations of mutant A1AT digested in singlicate

Results

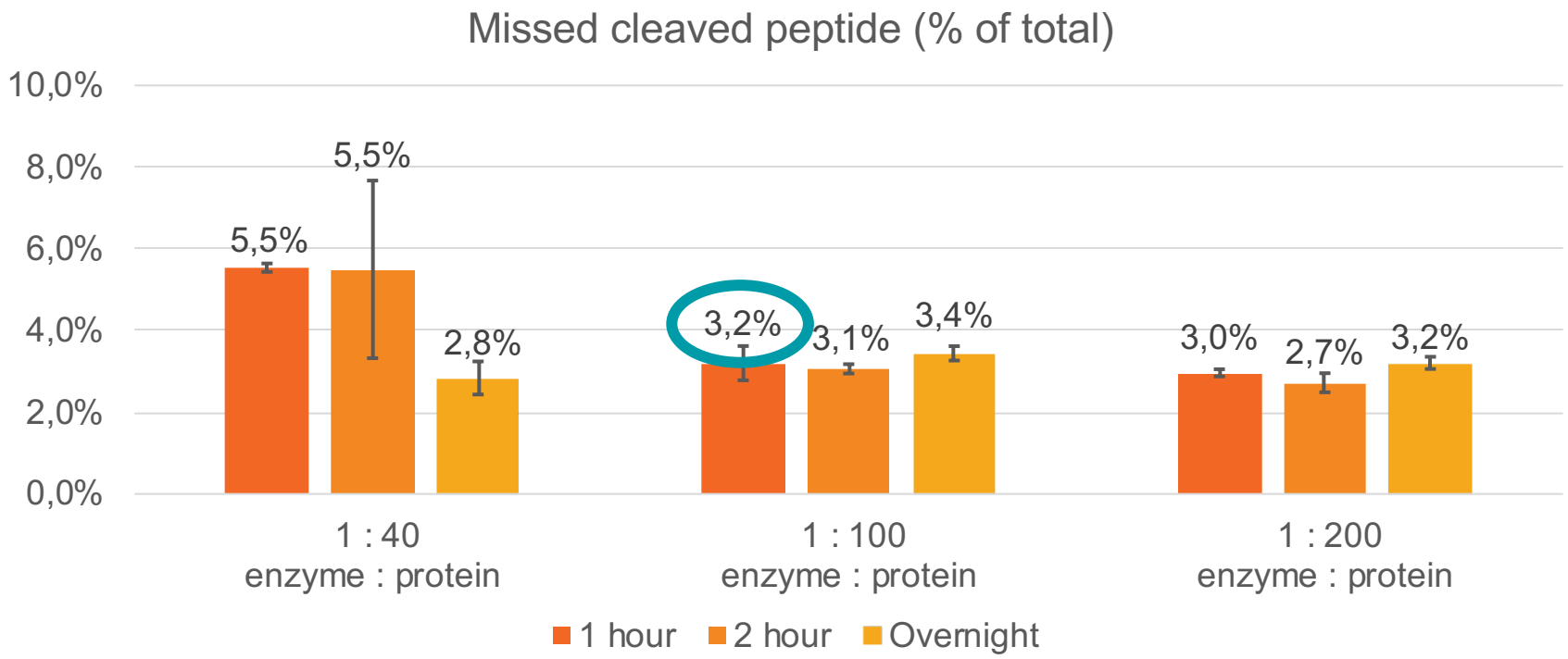
- **Digestion conditions in human serum**



Average of 2 concentrations of mutant A1AT digested in singlicate

Results

- **Digestion conditions in human serum**



Average of 2 concentrations of mutant A1AT digested in singlicate

Final method

- **Final method**
  - Denaturation and Reduction: 7M Urea and 5mM TCEP 60 minutes
  - Alkylation: 10mM IAA
  - Digestion: 100:1 enzyme : protein ratio
    1 hour 37°C
    Digested in 4M urea

- **Test in 6 individuals**
  - Ranged from **1 - 2.5%** missed cleavage
    - (Caveat: recombinant z mutant A1AT used)

Conclusions – “Bottom Up” approach

- **Suggested approach to avoid missed cleaved peptides**
  - Digestion under denaturing conditions
  - Enzyme selection
  - Optimise enzyme to protein ratio
  - Choice of denaturant

- **What worked for us**
  - Lys-C in 4M Urea was optimal
  - 1:100 enzyme protein ratio suitable

- **Qualified**
  - Successfully validated method for biomarker assay
  - Missed cleaved peptide monitored

Thank you
for listening!

Questions?

Thank you:
Jason Pembroke
Szabolcs Szarka
Sponsor

Etymology - Bottoms up!

The legend is that during the 18th and 19th Century, if men accepted a King's Shilling then they had consented to join the English Navy.

Recruiters ran a scam, discreetly dropping a shilling into a drunk man's beer. When they'd finished their drink it would be too late and they'd be hauled off to sea the next morning.

Bar owners used to warn sailors "Bottoms up!" before patrons took a sip or shot.

It's now used as a toast or to tell people to finish their drinks.

Richard.Lucey@LGCGroup.com