



Can 1+1 be 3?

**The combination of LBA and LC-MS
to look beyond the horizon of large
molecule quantification**

19 November 2014

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Small and large molecule analysis

different principles

different techniques

different people

more sensitive!

larger dynamic range!

golden standard!

structural information!

3D-integrity!

more accurate!

easier to use!

multiplexing!

*correlation with
biological activity!*

more selective!

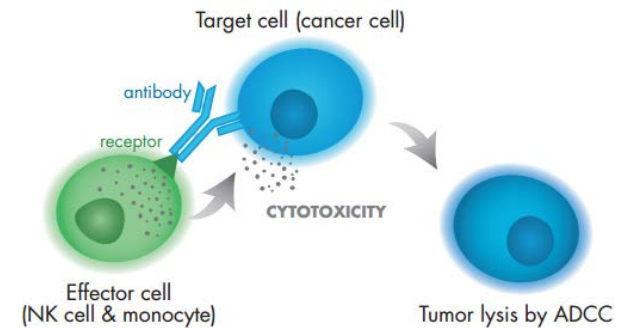
no critical reagents!



Trastuzumab

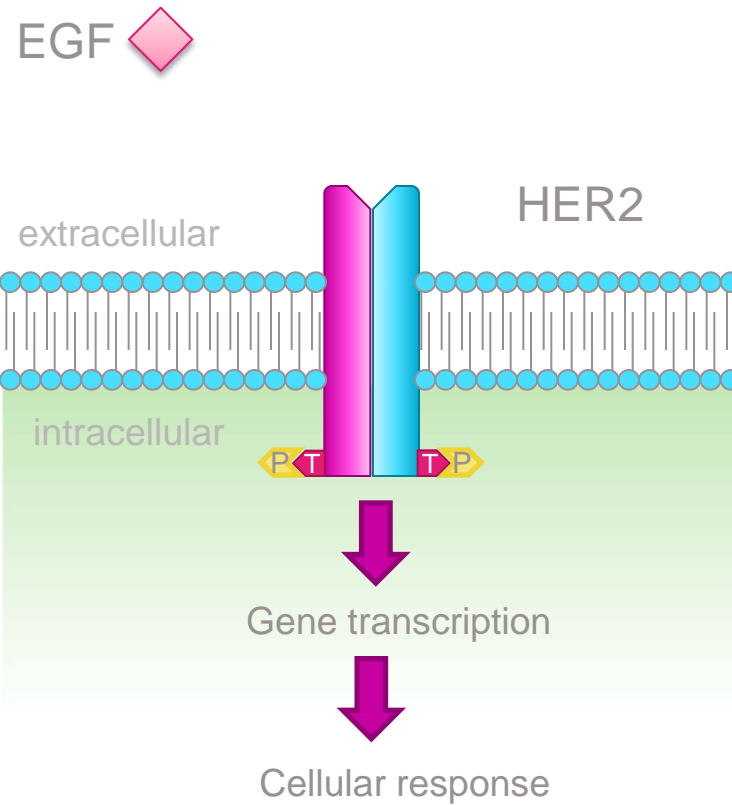
What it does – large molecule perspective:

- Humanized monoclonal antibody, IgG1 isotype
- Directed against HER-2 receptor (Human Epidermal Growth Factor-2)
- Treatment of breast cancer
- 20-30% of breast cancers have overexpression of HER-2
- Inhibits activation mechanism of HER-2 upon binding
- ADCC



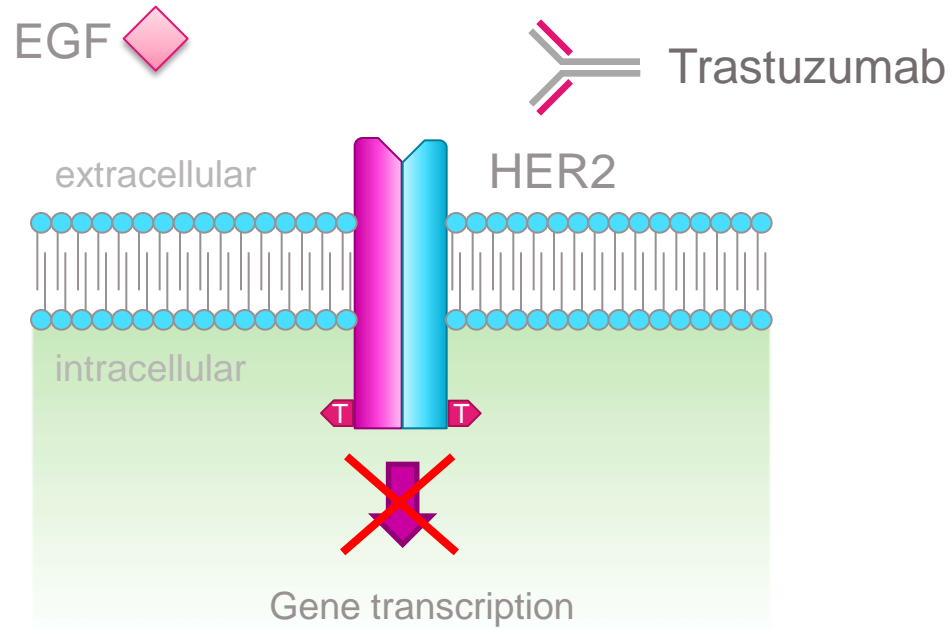


Trastuzumab





Trastuzumab





Trastuzumab

What it is – large molecule perspective:



Schematic overview



Full detail rendering of structure



Trastuzumab

What it does – small molecule perspective:

- Something against cancer



Trastuzumab

What it is – small molecule perspective:

- 1328 amino acids in four polypeptide chains (two light and two heavy) connected by four disulfide bonds, containing two N-linked oligosaccharides
- $C_{6470}H_{10012}N_{1726}O_{2013}S_{42}$; molecular mass: 145,531.5 Da
- Hydrophobicity: -0.4
- Isoelectric point: 8.5



Trastuzumab

Amino acid sequence

Light chain:

DIQMTQSPSS	LSASVGDRV T	ITCRASQDVN	TAVAWYQQKP	GKAPKLLIYS	ASFLYSGVPS
RFSGSRSGTD	FTLTSSLQP	EDFATYYC QQ	HYTTPPTFGQ	GTKVEIKRTV	AAPSVFIFPP
SDEQLKSGTA	SVVCLLNNFY	PREAKVQWKV	DNALQSGNSQ	ESVTEQDSKD	STYLSLSTLT
LSKADYEKHK	VYACEVTHQG	LSSPVTKSFN	RGEC		

Heavy chain:

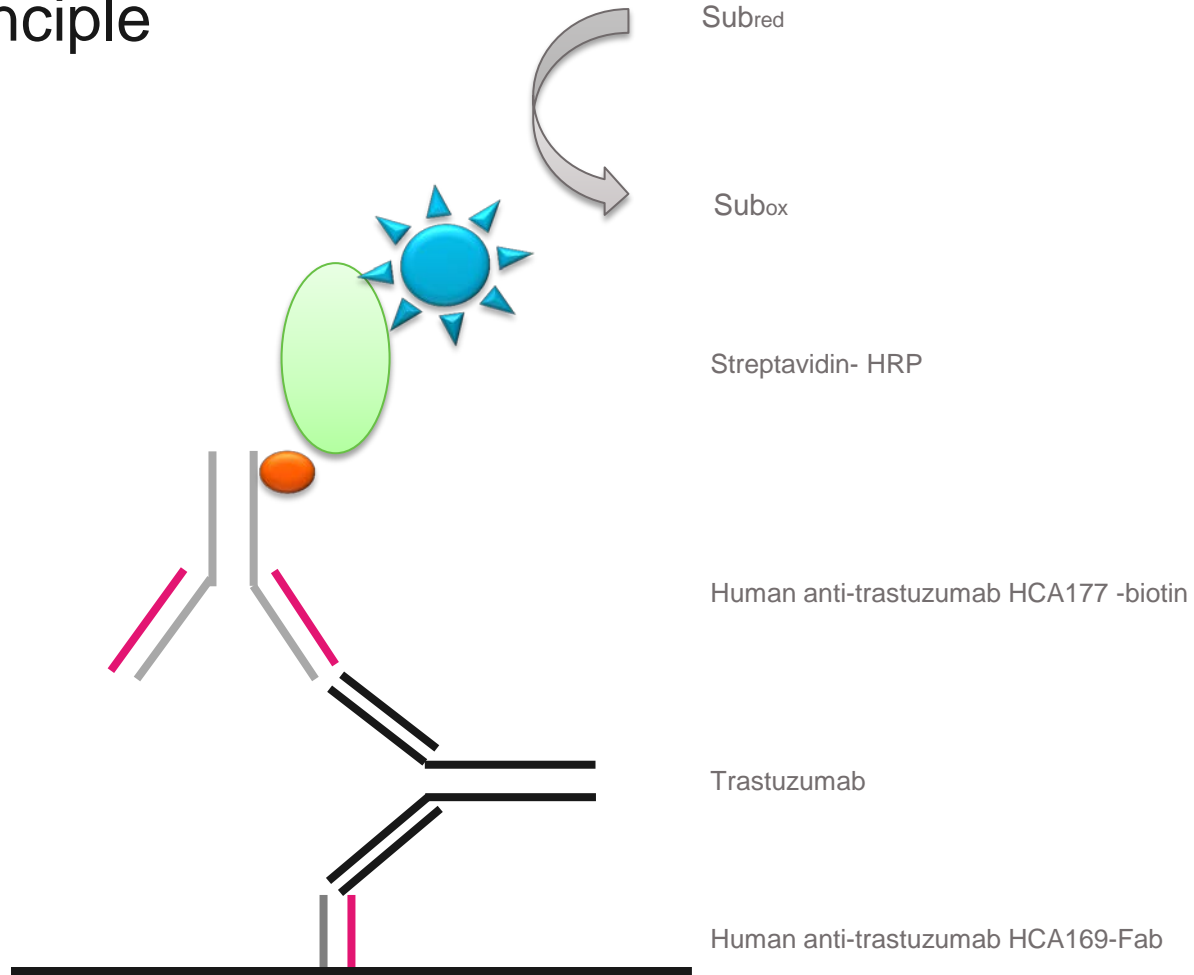
EVQLVESGGG	LVQPGGSLRL	SCAASGFNIK	DTYIHWVRQA	PGKGLEWVAR	IYPTNGYTRY
ADSVKGRFTI	SADTSKNTAY	LQMNSLRAED	TAVYYCSR WG	GDGFYAMDYW	GQGTLVTVSS
ASTKGPSVFP	LAPSSKSTSG	GTAALGCLVK	DYFPEPVTVS	WNSGALTSGV	HTFPAVLQSS
GLYSLSSVVT	VPSSSLGTQT	YICNVNHKPS	NTKVDKKVEP	KSCDKTHTCP	PCPAPELLGG
PSVFLFPPKP	KDTLMISRTP	EVTCVVDVVS	HEDPEVKFNW	YVDGVEVHNA	KTKPREEQY <u>N</u>
STYRVVSVLT	VLHQDWLNGK	EYKCKVSNKA	LPAPIEKTIS	KAKGQPREPQ	VYTLPPSREE
MTKNQVSLTC	LVKGFYPSDI	AVEWESNGQP	ENNYKTTTPV	LDSDGSFFLY	SKLTVDKSRW
QQGNVFSCSV	MHEALHNHYT	QKSLSLSPGK			

Bold = complementarity determining region (CDR)



ELISA

Assay principle



Adapted from Knappik et al, AbD serotec



ELISA

Validation results:

accuracy and precision

Level (ng/ml)	Total CV (%)	Mean bias (%)	Total error (%)
Val A: 102	5.4	0.0	5.4
Val B: 250	6.5	1.5	7.9
Val C:1000	4.7	-3.3	7.9
Val D: 3000	7.7	-4.9	12.7
Val E: 4000	9.0	-12.6	21.6
OC: 600,000 (200x dilution)	9.2	-4.8	14.0



Amino acid sequence

Light chain:

DIQMTQSPSS	LSASVGDRV T	IT CRASQDVN	TAVAWYQQKP	GKAPKLLIYS	ASFLYSGVPS
RFSGSRSGTD	FTLTISLQP	EDFATYYC QQ	HYTTPPTFGQ	GTKVEIKRTV	AAPSVFIFPP
SDEQLKSGTA	SVVCLLNNFY	PREAKVQWKV	DNALQSGNSQ	ESVTEQDSKD	STYLSLSTLT
LSKADYEKHK	VYACEVTHQG	LSSPVTKSFN	RGEC		

Heavy chain:

EVQLVESGGG	LVQPGGSLRL	SCAASGFNIK	DTYIHWVRQA	PGKGLEWVAR	IYPTNGYTRY
ADSVKGRFTI	SADTSKNTAY	LQMNSLRAED	TAVYYCSR WG	GDGFYAMDYW	GQGTLVTVSS
ASTKGPSVFP	LAPSSKSTSG	GTAALGCLVK	DYFPEPVTVS	WNSGALTSGV	HTFPAVLQSS
GLYSLSSVVT	VPSSSLGTQT	YICNVNHKPS	NTKVDKKVEP	KSCDKTHTCP	PCPAPELLGG
PSVFLFPPKP	KDTLMISRTP	EVTCVVVDVS	HEDPEVKFNW	YVDGVEVHNA	KTKPREEQY <u>N</u>
STYRVVSVLT	VLHQDWLNGK	EYKCKVSNKA	LPAPIEKTIS	KAKGQPREPQ	VYTLPPSREE
MTKNQVSLTC	LVKGFYPSDI	AVEWESNGQP	ENNYKTTTPV	LDSDGSFFLY	SKLTVDKSRW
QQGNVFSCSV	MHEALHNHYT	QKSLSLSPGK			

Bold = complementarity determining region (CDR) **Red** = selected tryptic signature peptides

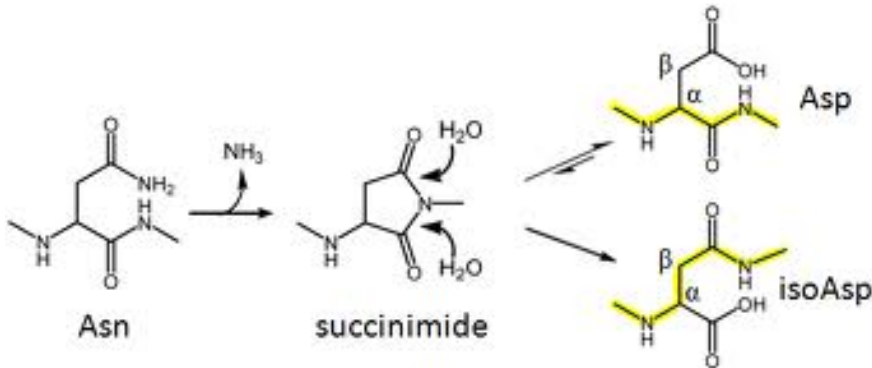


Assay principle

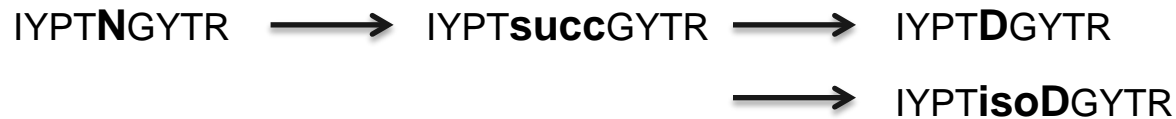
- Pellet digestion after protein precipitation with methanol
- 0.2 mg/mL trypsin, pH 7.0, 37°C, 3 hours, 2 SIL internal standards
- 4 µL-injection of 9-fold diluted digest
- C18 column (1.8 µm particles) at 40°C, 8-min gradient at 0.8 mL/min
- Positive electrospray MS/MS at 750°C, ISV: 5500V
- six MRM transitions:
 - signature peptide 1 (IYPTNGYTR) + IS
 - degradation products (IYPTDGYTR / IYPTisoDGYTR)
 - Intermediate (IYPTsuccGYTR)
 - signature peptide 2 (FTISADTSK) + IS



Deamidation of peptide 1



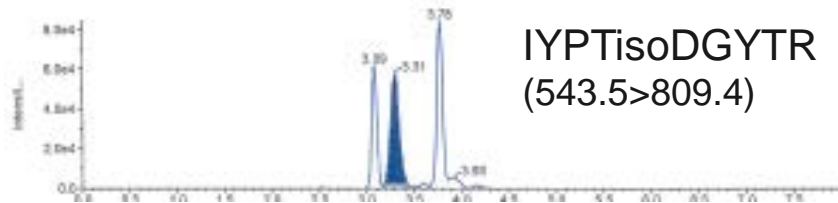
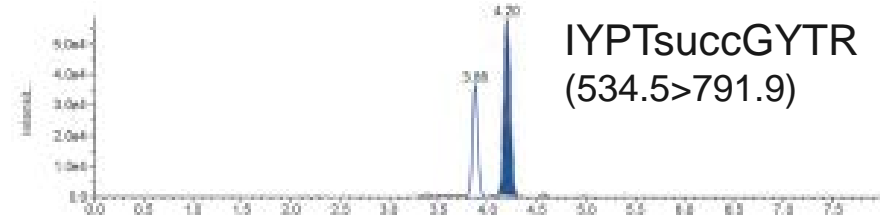
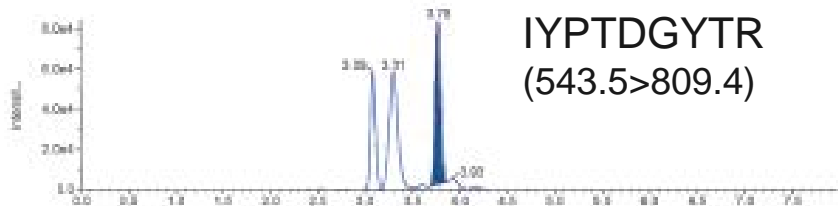
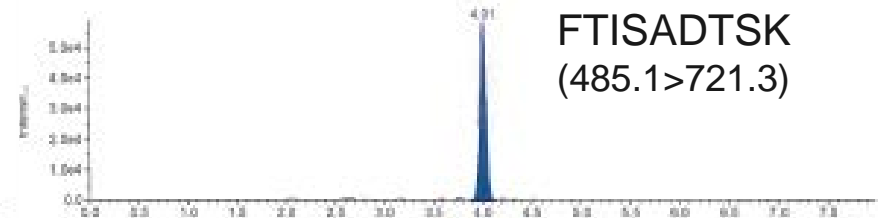
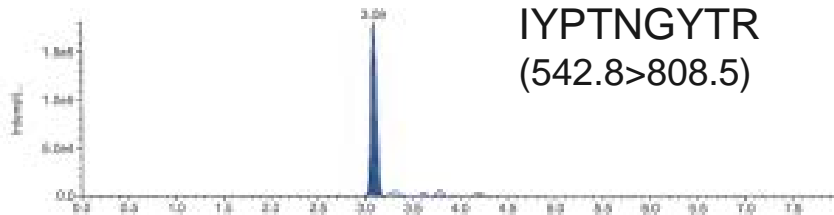
For trastuzumab:



Spontaneous reaction at alkaline conditions (e.g. during digestion)

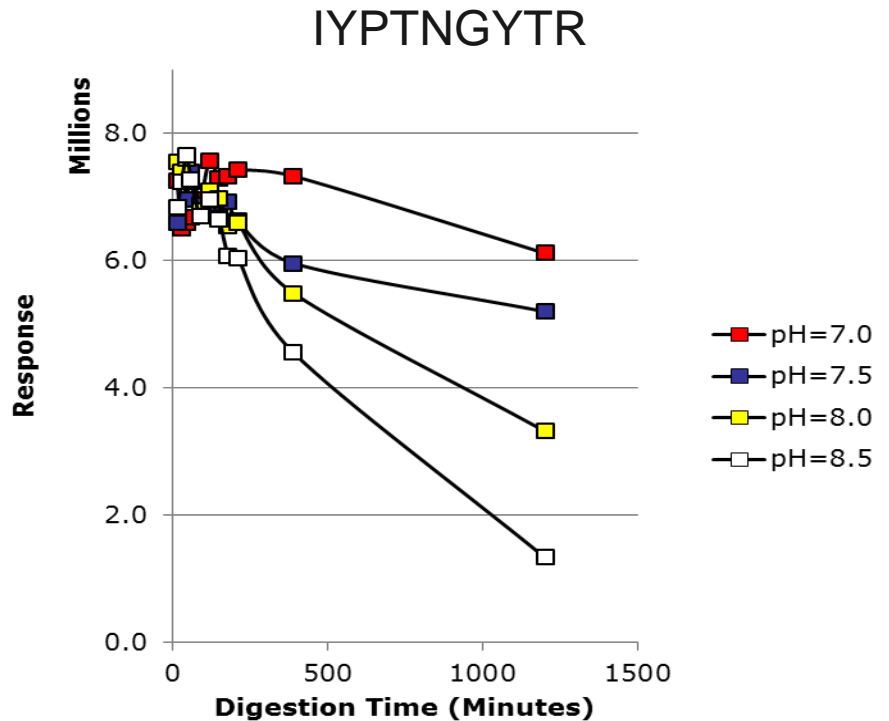
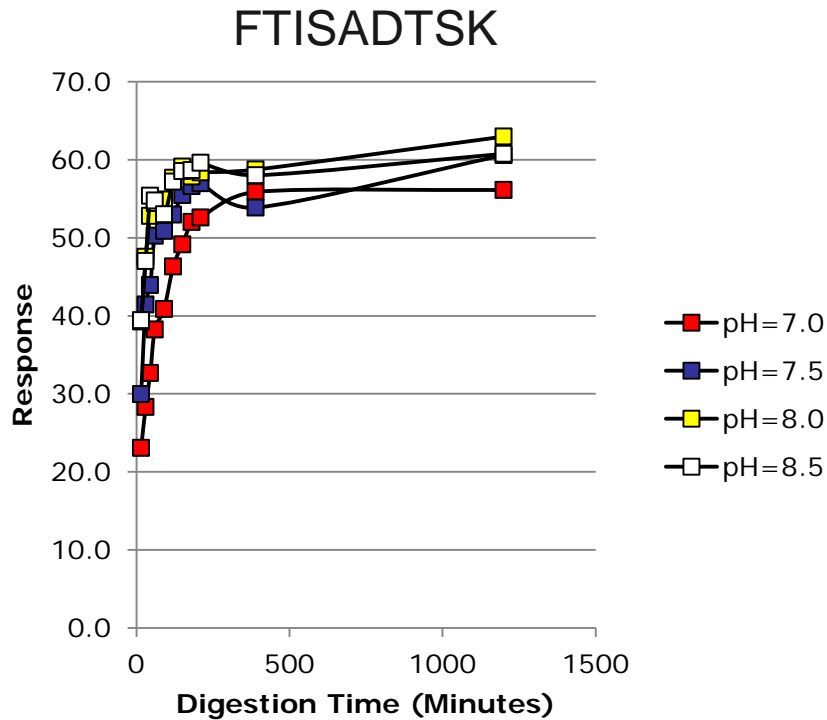


LC-MS/MS





LC-MS/MS



Digestion at pH 7.0 for 180 min:

>90% digestion efficiency

<2% formation of degradation products

suitable approach for
–NG– containing peptides



Validation results

Accuracy and precision

Level ($\mu\text{g/ml}$)	IYPTNGYTR		FTISADTSK	
	Total CV (%)	Mean bias (%)	Total CV (%)	Mean bias (%)
Val A: 0.500	12.1	-7.3	20.3	+11.7
Val B: 1.50	7.0	-0.7	7.7	+7.3
Val C: 25.0	5.0	-1.0	4.8	+0.7
Val D: 400	4.0	-2.8	2.8	-1.8



Comparison

Cross-validation results

Accuracy and precision of LC-MS/MS QCs on ELISA

Level (µg/ml)	dilution	CV (%)	bias (%)
QC A: 1.50	-	3.3	+5.2
QC B: 25.0	100x	2.9	+3.3
QC C: 400	200x	1.0	-0.9



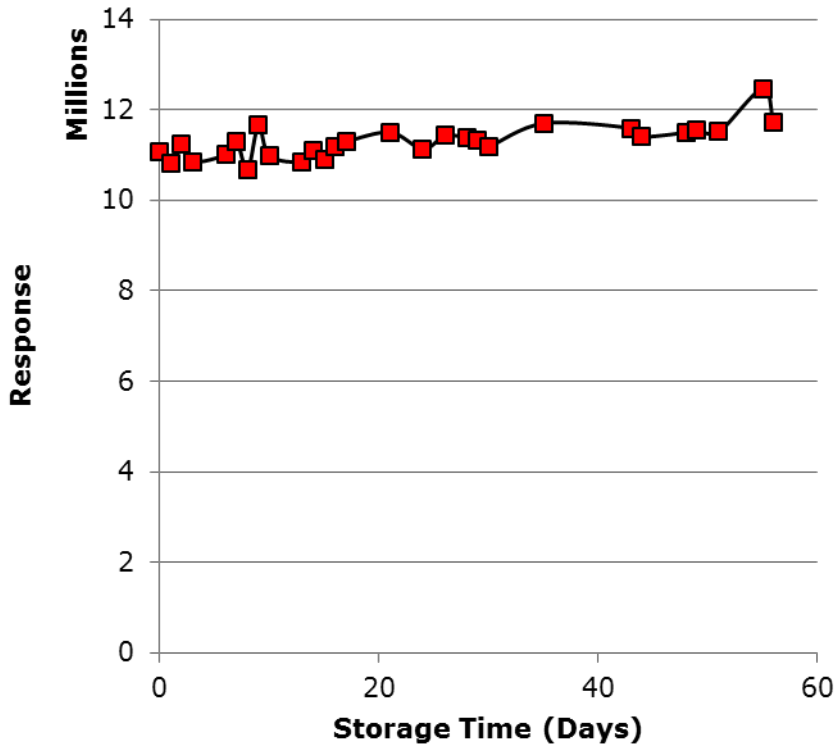
1+1=3?

In vitro stress test

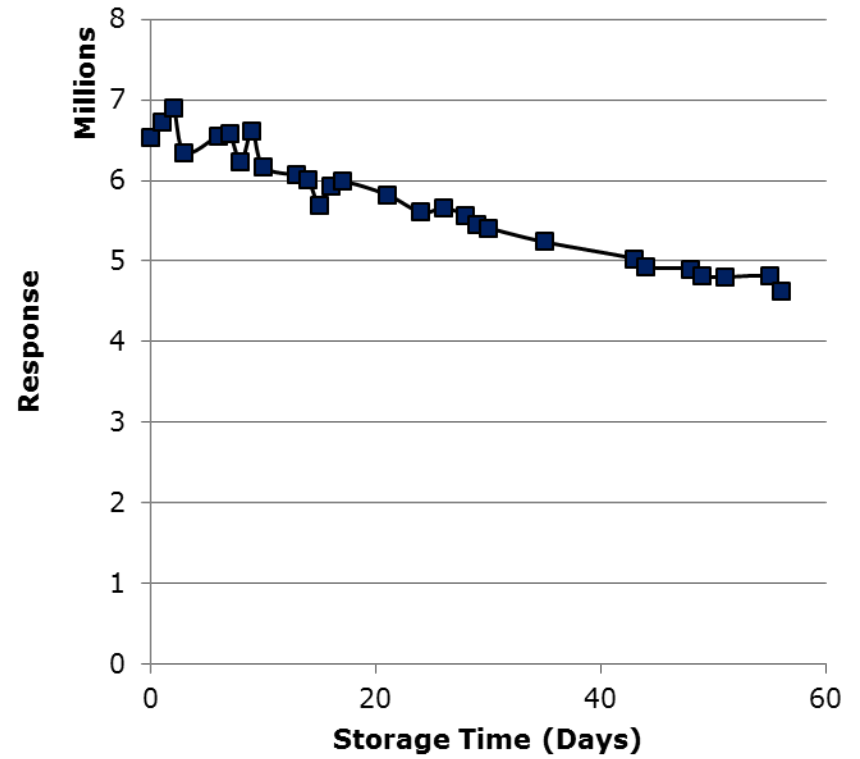
- Blank human plasma set at pH 8
- Trastuzumab added at 400 µg/mL
- Incubated at 37 °C
- Regular sampling for up to 56 days
- Analysis by ELISA and LC-MS/MS (2 signature peptides)



1+1=3?



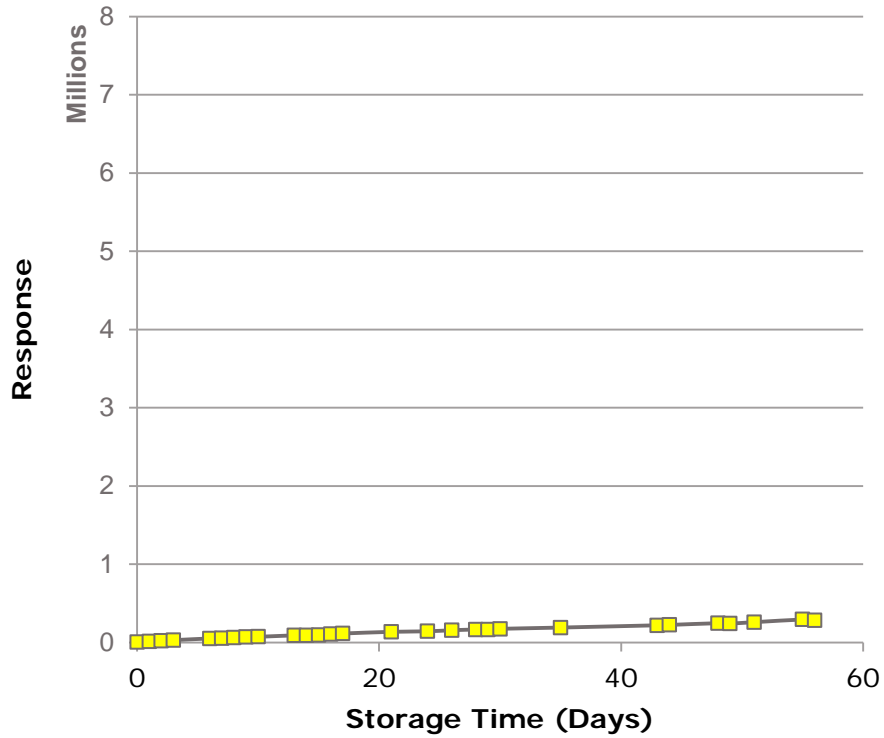
FTISADTSK



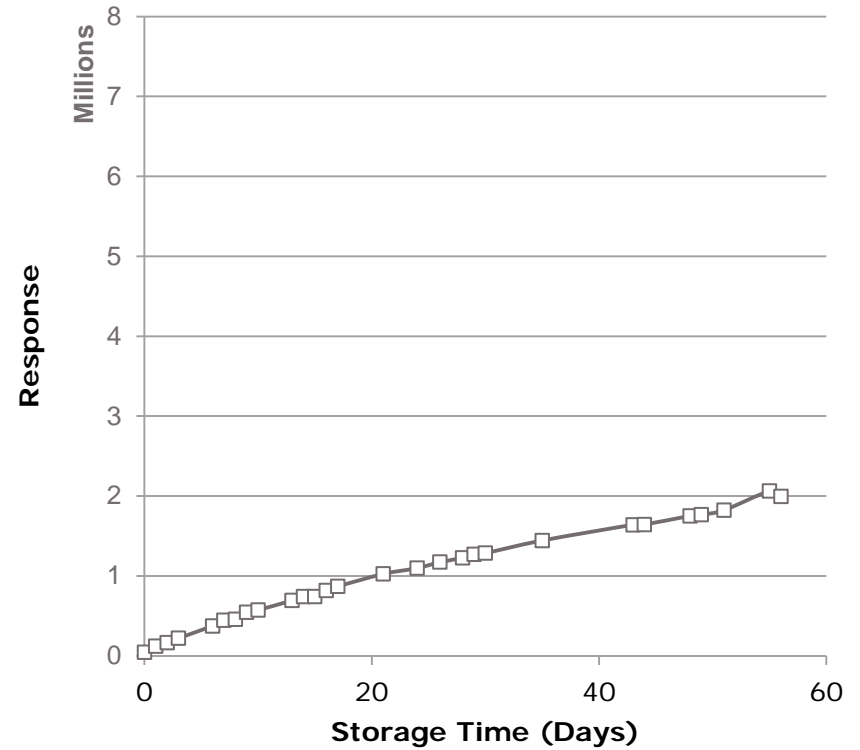
IYPTNGYTR



1+1=3?



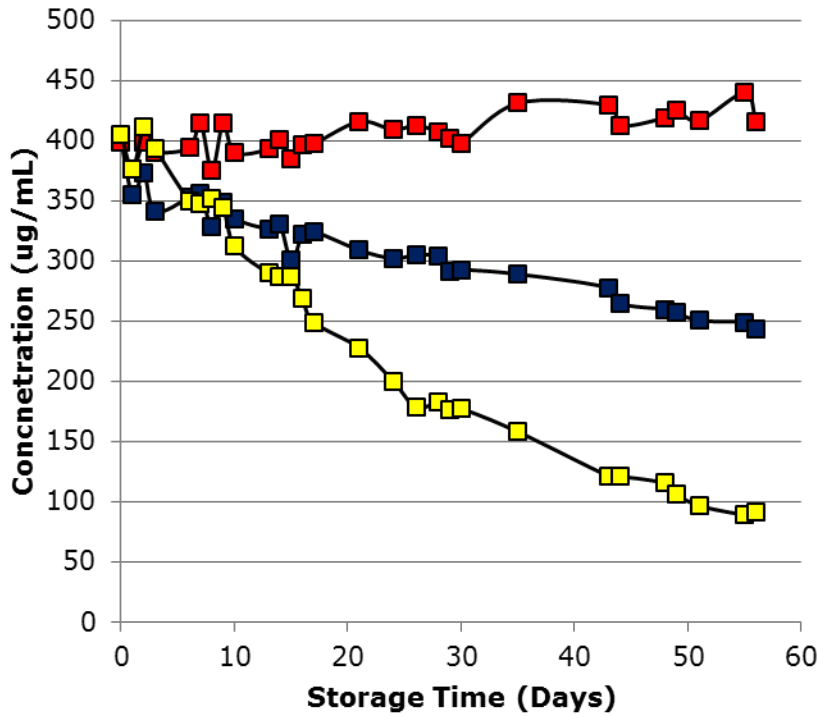
IYPTDGYTR



IYPTisoDGYTR



1+1=3?



LC-MS/MS: FTISADTSK

LC-MS/MS: IYPTNGYTR

ELISA

trastuzumab concentrations:

total

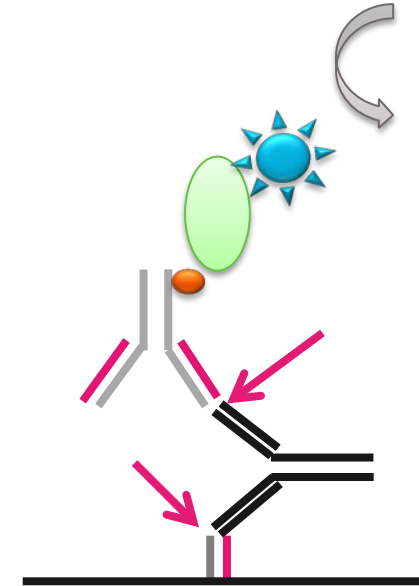
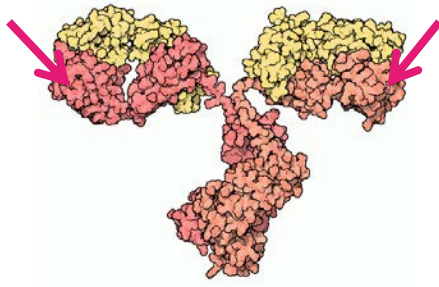
non-deamidated

immunoreactive



1+1=3?

Two deamidation sites per trastuzumab molecule



LC-MS/MS (YPTNGYTR):
Non-deamidated: full response
Singly deamidated: **half response**
Doubly deamidated: no response

ELISA:
Non-deamidated: full response
Singly deamidated: **no response**
Doubly deamidated: no response



Conclusion

- LBA and LC-MS are complementary techniques with different assay principles
- Therefore, they may and often will give different concentration results for proteins
- “The” concentration of a protein does not exist
- Both techniques help provide detailed answers to the complex questions in the field of protein bioanalysis
- Small and large molecule scientists: no competition but collaboration!



Acknowledgment

Peter Bults

Hilde Bakker

Rainer Bischoff

Samenwerkingsverband

Noord-Nederland



PRAHEALTHSCIENCES



rijksuniversiteit
 groningen



WE ARE DEDICATED TO
THE FUTURE OF CLINICAL DEVELOPMENT
AND TO EVERY LIFE IT SAVES.



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