Developing LC-MS/MS methods for quantifying mAbs: Transitioning from pre-clinical to clinical matrices.

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Overview

- Existing methods for mAb extraction and analysis
- Improvements to existing methods
- Potential patent issues
- Alternative enzymes and surrogate peptides
- Moving into human

Methods for extracting mAb's

- Immunoprecipitation:
  - Protein A/G extraction
  - Anti-human IgG extraction
  - Anti-idiotypic
  - Target protein
- Pro's – selective extraction methods
- Con's – expensive reagents

- Chemical extraction
  - Protein precipitation
- Pro's – cheap
- Con's – non-specific

Protein A/G selectivity issues

Species IgG	Weak	Strong	Useful for mAb extraction?
Human		✓	Not really
Cyno		✓	Not really
Rat	✓		Yes
Mouse	✓		Yes
Rabbit		✓	Not really

- Protein A will extract IgG from monkey and human serum
- An issue with capacity…
  - IgG = ~ 15 mg/mL,
  - 25 µL of serum = ~ 375 µg of IgG
  - Protein A magnetic beads will capture 8 µg protein per mg of beads
  - Requires 46 mg beads per 25 µL….
  - Supplied at 20mg/mL = ~2.5 mL of beads per sample

Protein precipitation based method

- The pellet digestion method
  - Bioanalysis (2012) 4(1), 17–28

    Pellet digestion: a simple and efficient sample preparation technique for LC–MS/MS quantification of large therapeutic proteins in plasma

Zheng Ouyang[1], Michael T Furlong[2], Steven Wu[1], Bogdan Sleczka[1], James Tamura[3], Haiqing Wang[4], Suzanne Suchard[5], Anish Suri[5], Timothy Olah[1], Adrienne Tymiak[1] & Mohammed Jemal*[1]

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- Precipitate all large proteins and digest pellet
  - **Discard** supernatant from protein crash

- Referenced a paper from my PhD:
  - Analysis of IGF-I after GH administration to athletes
  - **Keep** supernatant from protein crash

16 Kay RG, Barton C, Velloso CP *et al.* High-throughput ultra-high-performance liquid chromatography–tandem mass spectrometry quantitation of insulin-like growth factor-I and leucine-rich a-2-glycoprotein in serum as biomarkers of recombinant human growth hormone administration. *Rapid Commun. Mass Spectrom.* 23(19), 3173–3182 (2009).

Pellet digestion method

- 25 µL of plasma (~2.5 mg of protein)
- Add 75 µL of methanol (ACN was better, but gave tight pellet)
- Mix and centrifuge
- **Discard** supernatant
- Digest pellet (Sigma trypsin, 8mg/mL)
- Analyse by LC-MS/MS (2.6 µg/mL LLOQ using 25 µL)

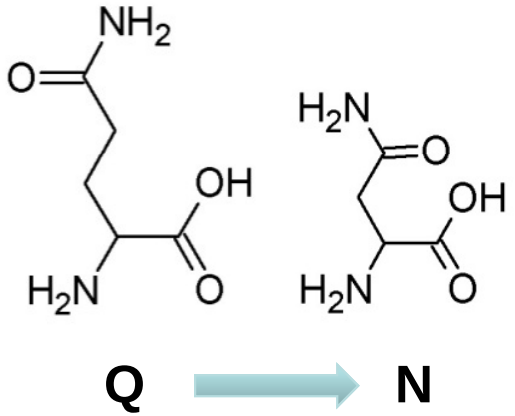
- Can we improve on this?

- Can we reduce the albumin level whilst improving mAb recovery?
  - Monitor an Albumin peptide along with a mAb peptide

Albumin peptide (rodent Vs. primate)

Rodent albumin mascot hit

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	114.0913	57.5493					L							10
2	213.1598	107.0835					V	1036.5310	518.7691	1019.5044	510.2558	1018.5204	509.7638	9
3	341.2183	171.1128	324.1918	162.5995			Q	*937.4625*	469.2349	920.4360	460.7216	919.4520	460.2296	8
4	470.2609	235.6341	453.2344	227.1208	452.2504	226.6288	E	*809.4040*	405.2056	792.3774	396.6923	791.3934	396.2003	7
5	569.3293	285.1683	552.3028	276.6550	551.3188	276.1630	V	*680.3614*	340.6843	663.3348	332.1710	662.3508	331.6790	6
6	670.3770	335.6921	653.3505	327.1789	652.3665	326.6869	T	*581.2930*	291.1501	564.2664	282.6368	563.2824	282.1448	5
7	785.4040	393.2056	768.3774	384.6923	767.3934	384.2003	D	*480.2453*	240.6263	463.2187	232.1130	462.2347	231.6210	4
8	932.4724	466.7398	915.4458	458.2266	914.4618	457.7345	F	*365.2183*	183.1128	348.1918	174.5995			3
9	1003.5095	502.2584	986.4829	493.7451	985.4989	493.2531	A	218.1499	109.5786	201.1234	101.0653			2
10							K	147.1128	74.0600	130.0863	65.5468			1



LVQEVTDFAK Q-N=+14 Da 575.3 / 809.4

Generic cross species albumin peptide transition = 575.3 / 937.5

LVNEVTEFAK D-E=-14 Da 575.3 / 823.4

Primate albumin mascot hit

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	114.0913	57.5493					L							10
2	213.1598	107.0835					V	1036.5310	518.7691	1019.5044	510.2558	1018.5204	509.7638	9
3	327.2027	164.1050	310.1761	155.5917			N	*937.4625*	469.2349	920.4360	460.7216	919.4520	460.2296	8
4	456.2453	228.6263	439.2187	220.1130	438.2347	219.6210	E	*823.4196*	412.2134	806.3931	403.7002	805.4090	403.2082	7
5	555.3137	278.1605	538.2871	269.6472	537.3031	269.1552	V	*694.3770*	347.6921	677.3505	339.1789	676.3665	338.6869	6
6	656.3614	328.6843	639.3348	320.1710	638.3508	319.6790	T	*595.3086*	298.1579	578.2821	289.6447	577.2980	289.1527	5
7	785.4040	393.2056	768.3774	384.6923	767.3934	384.2003	E	*494.2609*	247.6341	477.2344	239.1208	476.2504	238.6288	4
8	932.4724	466.7398	915.4458	458.2266	914.4618	457.7345	F	*365.2183*	183.1128	348.1918	174.5995			3
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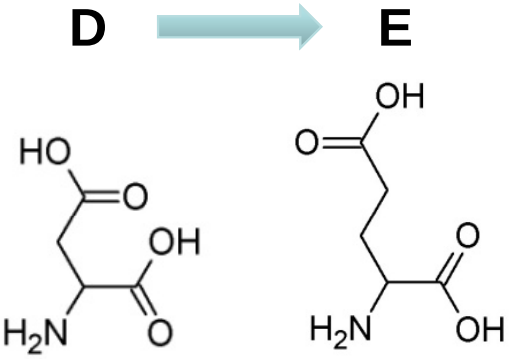
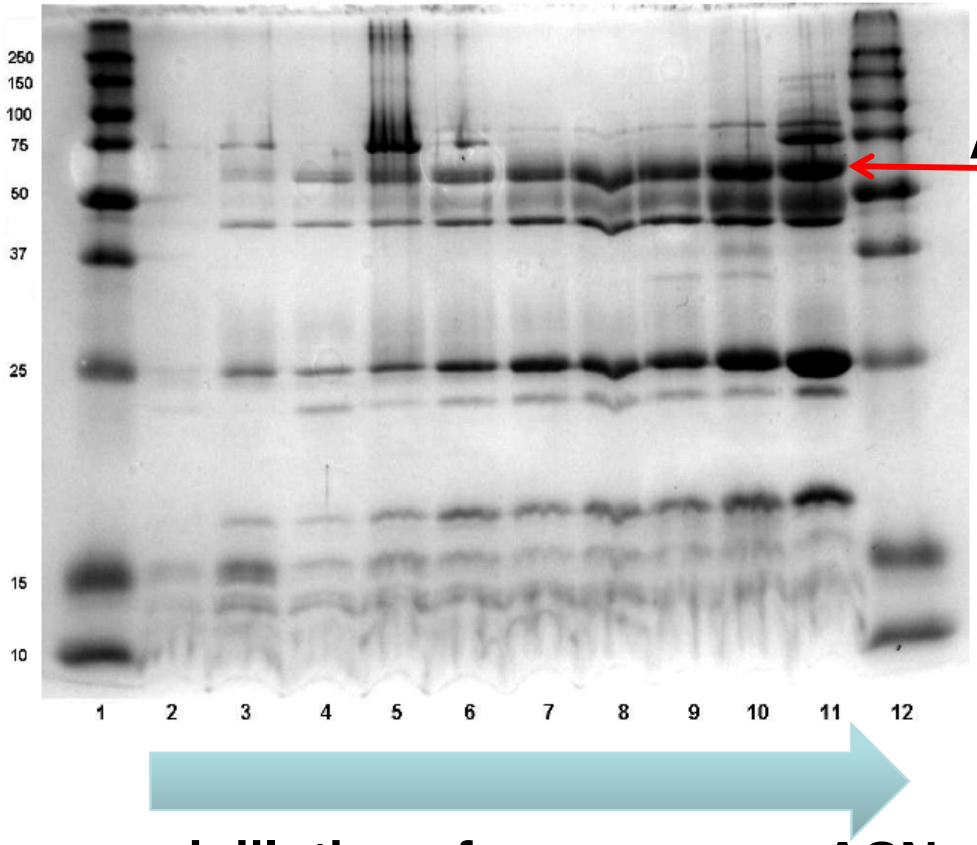
10							K	147.1128	74.0600	130.0863	65.5468			1



Protein crash solvent selection

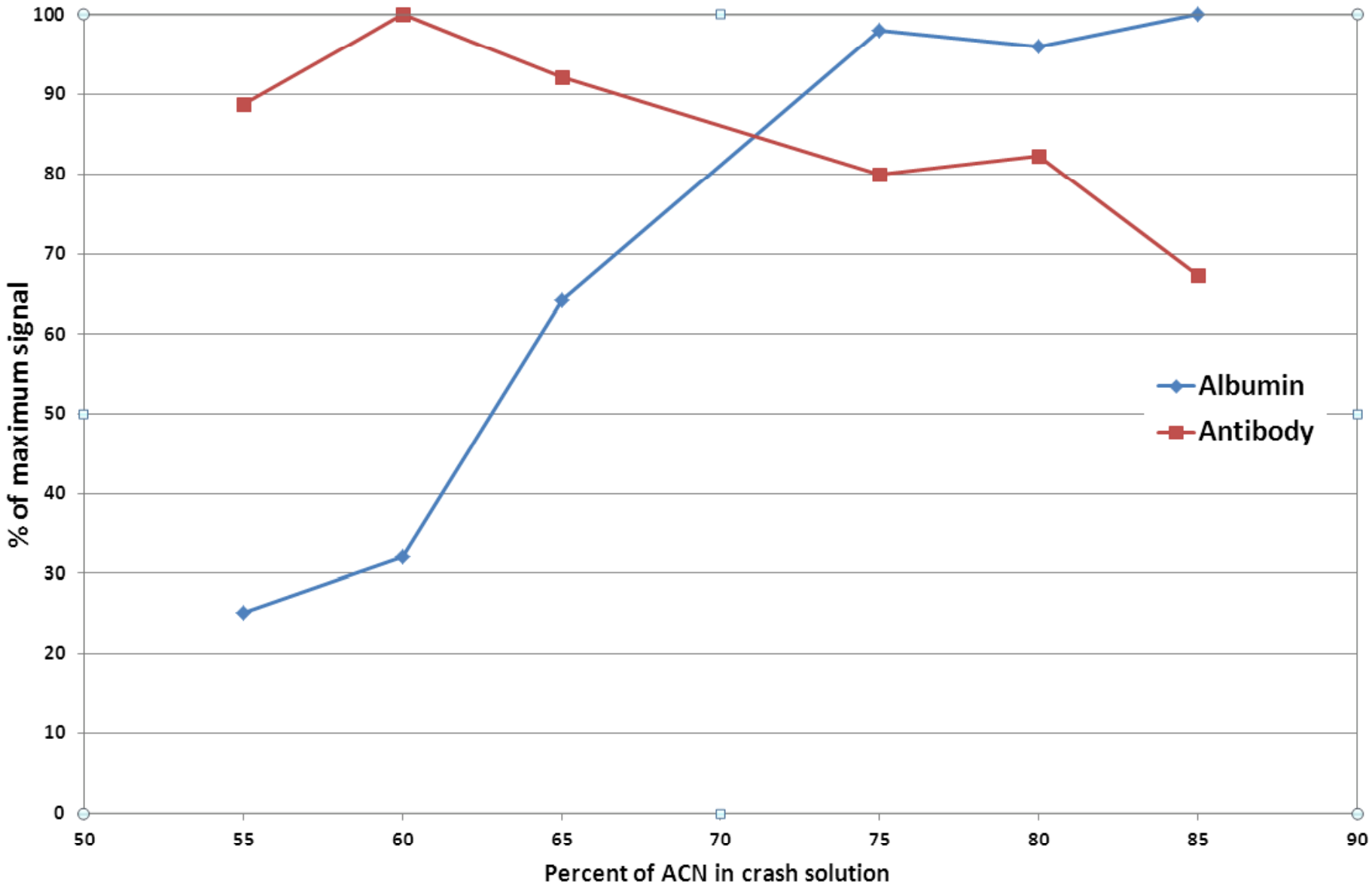
- Adding water to serum prior to crash gave better recovery of proteins
- Take supernatant after protein crash and run on SDS-PAGE gel



Increased dilution of serum, same ACN ratio:diluted serum (1.5 volumes ACN)

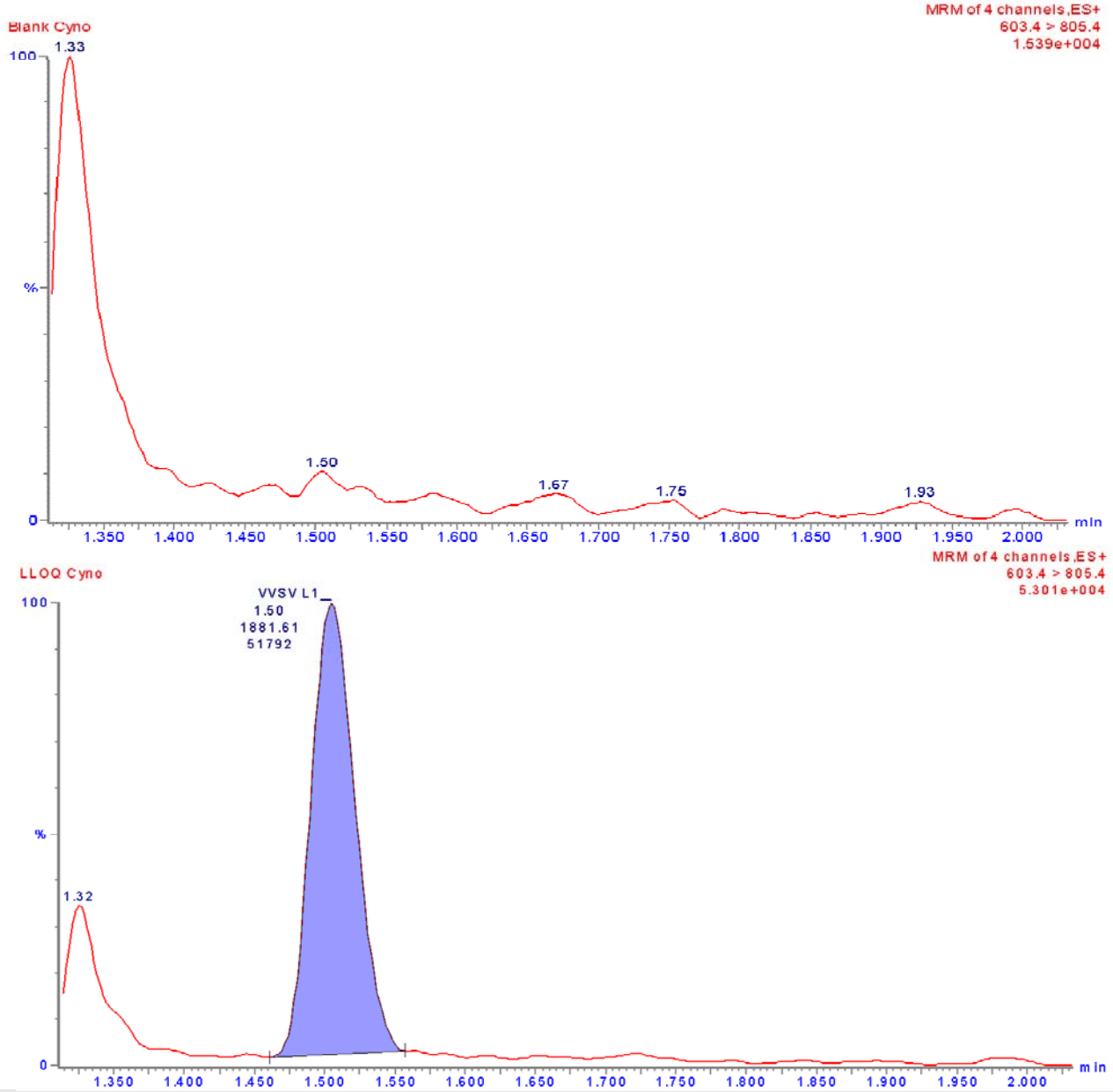
1= markers
2= no dilution
3= 1:0.5 serum:water
4= 1:1 serum:water
5= 1:1.5 serum:water
6= 1:2 serum:water
7= 1:2.5 serum:water
8= 1:3 serum:water
9= 1:3.5 serum:water
10=1:4 serum:water
11=1:9 serum:water
12= markers

Optimisation of crash solvent

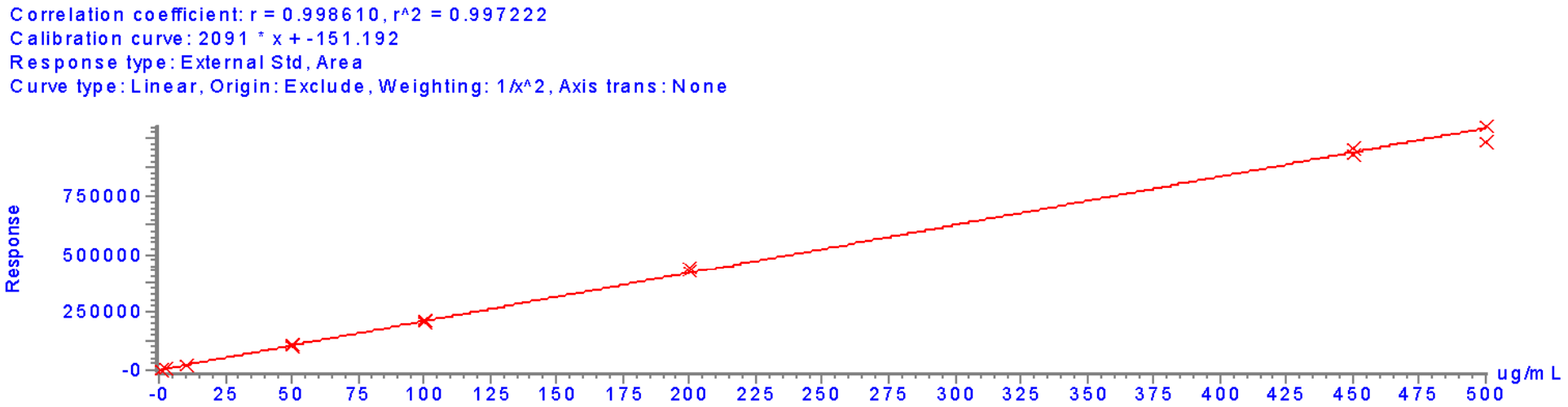


LC-MS/MS analysis of mAb in preclinical matrices

mAb in cyno serum at 1 µg/mL
10 µL of serum extracted, 200 nL injected



Cyno extraction results



QC	Conc	Mean	%CV	%RE
LLOQ	1.00	0.954	3.0	-4.6
LOW	4.00	3.75	4.0	-6.3
MED	40.0	37.7	4.2	-5.8
HIGH	400	375	4.7	-6.3

Patent issues…

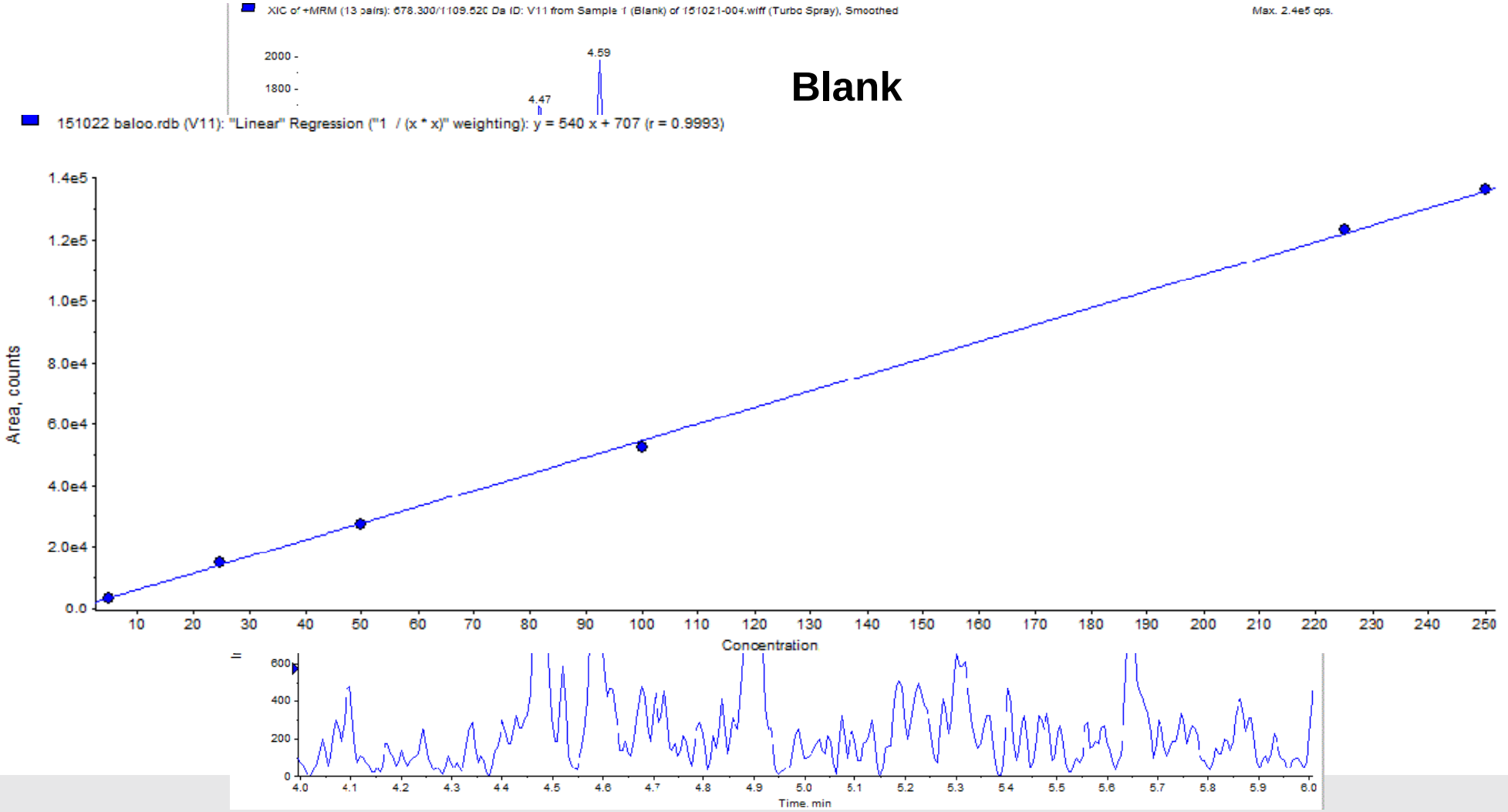
- Patent applied for using human mAb peptides in pre-clinical matrix
  - Filed in May 2012
  - Granted in the US, currently being considered in EU
- Application is specific for 8 framework specific peptides “FSP”

GPSVFPLAPSSK	SEQ ID NO:1
STSGGTAALGCLVK	SEQ ID NO:2
TPEVTCVVVDVSHEDPEVK	SEQ ID NO:3
FNWYVDGVEVHNAK	SEQ ID NO:4
VVSVLTVLHQDWLNGK	SEQ ID NO:5
ALPAPIEK	SEQ ID NO:6
GFYPSDIAVEWESNGQPENNYK	SEQ ID NO:7
TTPPVLDSDGSFFLYSK	SEQ ID NO:8

- Application specifies trypsin as the enzyme
- Glu C may be potentially used instead
  - VKFNWYVDGVE - heavy chain constant region peptide
  - We have achieved 0.25 µg/mL using Protein A extraction on 25 µL plasma
  - Performed in 2013

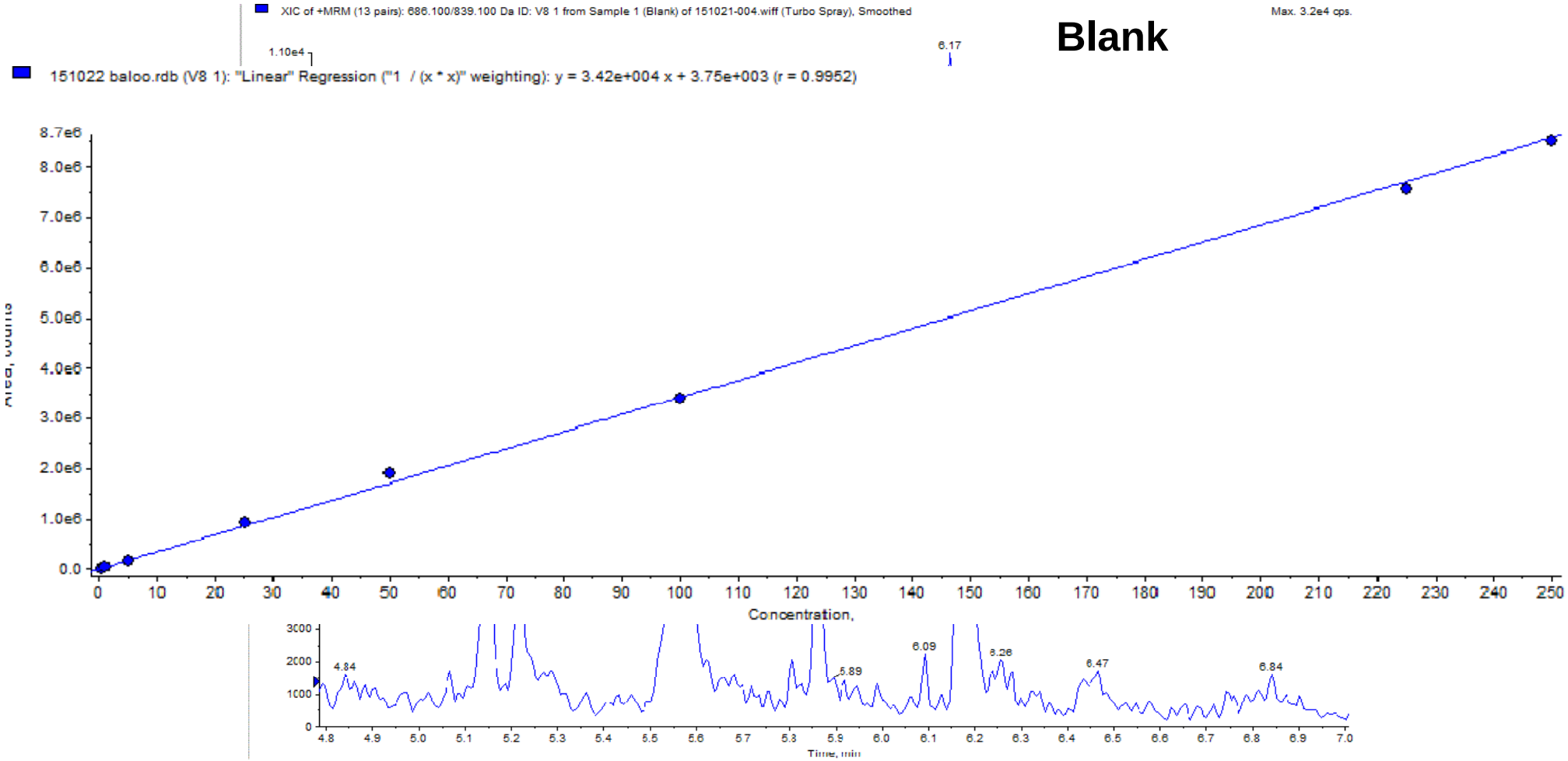
Protein crash and Glu C peptides

- Generic peptide sequence (VKFNWYVDGVE)
  - Not present in cyno or rodent protein
- Currently at 5 µg/mL LLOQ with 10 µL of plasma



Protein crash and Glu C peptides

- LLGGPSVFLFPPKPKDTLMISRTPE
- Common with monkey but OK in rodents
- Currently at 0.5 µg/mL LLOQ with 10 µL of plasma

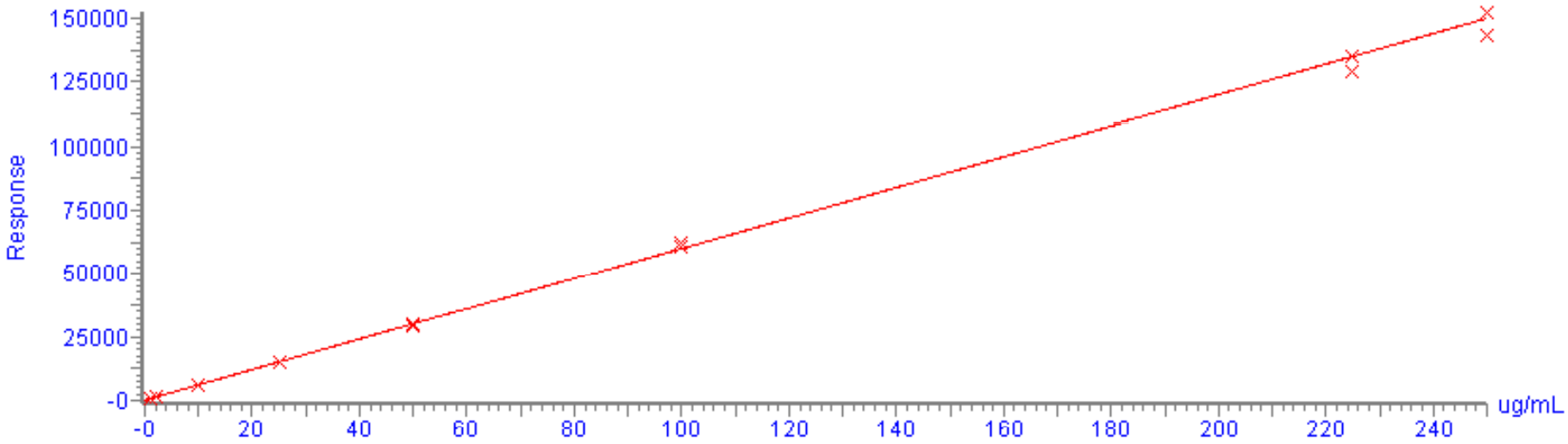


Human mAb in human serum

Method development

- LC-MS/MS approach had to target a peptide from the variable region
- Found 2 that were selective and sensitive
  - 1 that was sufficient for LLOQ in question (1 µg/mL)
- Same solvent assessment as previously performed in pre-clinical
  - Gave same results – 60% ACN in water
- Very quick extraction method development
  - Method was established within 5 working days

Linearity and precision and accuracy



QC	Conc	Mean	%CV	%RE
LLOQ	1.00	1.10	8.3	9.6
LOW	3.00	3.13	10.1	4.3
MED	20.0	19.6	2.6	-1.9
HIGH	200	182	2.5	-8.9

Assessment of selectivity

- 20 individual serum samples taken for analysis

Selectivity sample	% of LLOQ	Selectivity sample	% of LLOQ
Sample 1	0	Sample 11	10
Sample 2	0	Sample 12	10
Sample 3	0	Sample 13	30
Sample 4	10	Sample 14	0
Sample 5	10	Sample 15	10
Sample 6	20	Sample 16	0
Sample 7	0	Sample 17	0
Sample 8	40	Sample 18	0
Sample 9	0	Sample 19	0
Sample 10	40	Sample 20	0

Conclusions

- A modified pellet digestion method was developed
  - Gave good sensitivity for trypsin approach
- Pellet digestion approach is species and matrix agnostic
  - Good for a fast generic mAb extraction method
- Potential patent issues
  - May potentially be circumvented in EU by using Glu C enzyme instead
- Sensitivity is not far off tryptic peptides
  - Still in development..

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NovImmune

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Thank you for listening

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