Comparison of sample preparation for mAbs quantification by LC-MRM: Protein A cartridges vs nSMOL

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2: Institut Gustave Roussy, Service de Pharmacologie et UMS CNRS 3655 & INSERM US23 AMMICa, Laboratoire de Pharmacologie et d'Analyse (LPA), Villejuif, France

3: Grenoble Institut des Neurosciences (GIN) INSERM U836, Safra Chemin Fortuné Ferrini, La Tronche, France
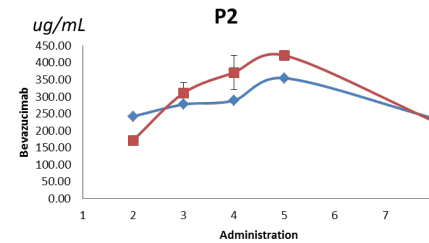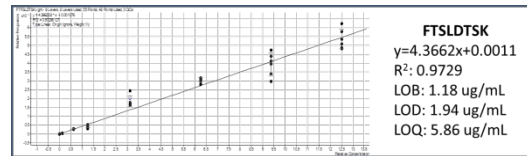
First study

Avastin Project

Method development and Validation

Dosage 94 patient samples

Valorization: 1 publication 1 comm

Service for IGR
Quantification on patient with brain cancer – Pharmacokinetic study

FTSLDTSK
y=4.3662x+0.0011
R^2: 0.9729
LOB: 1.18 ug/mL
LOD: 1.94 ug/mL
LOQ: 5.86 ug/mL

P2

Bevacizumab / **Avastin**

- **VEGF** (Vascular endothelial growth factor) is overexpressed in most human tumors and that increased VEGF expression is associated with tumor progression and/or risk of cancer recurrence (Ternant, 2010).

- **Bevacizumab** (Avastin®, Roche-Genentech) is a humanized monoclonal immunoglobulin G1 (IgG1) antibody that specifically binds circulating vascular endothelial growth factor (VEGF). It is used to limit tumor vascularization.
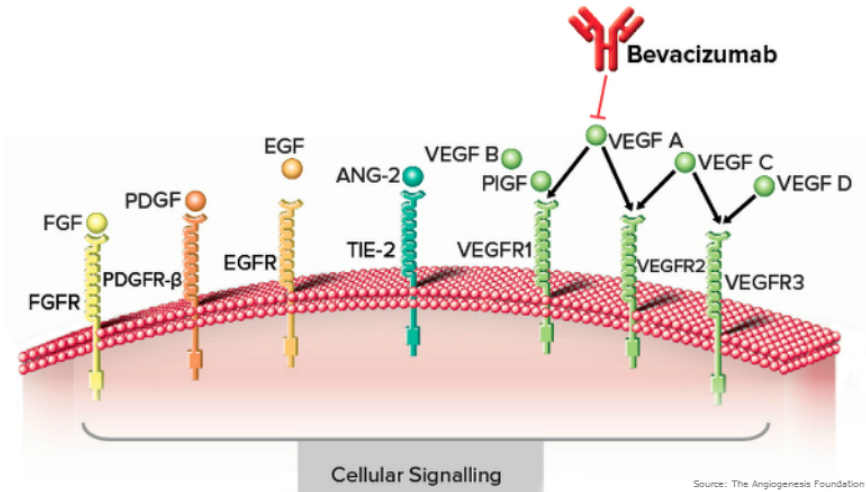
Bevacizumab is used in combination with standard chemotherapy, and is approved in
>   breast cancer   *(cf. Han, AAPS journal, 2014)*
>   metastatic colorectal cancer
>   non-small cell lung cancer
>   renal cell carcinoma
>   ovarian cancer
>   glioblastoma
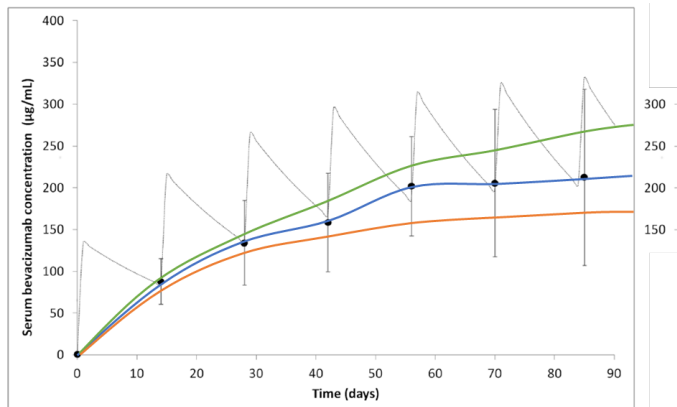
Case Study

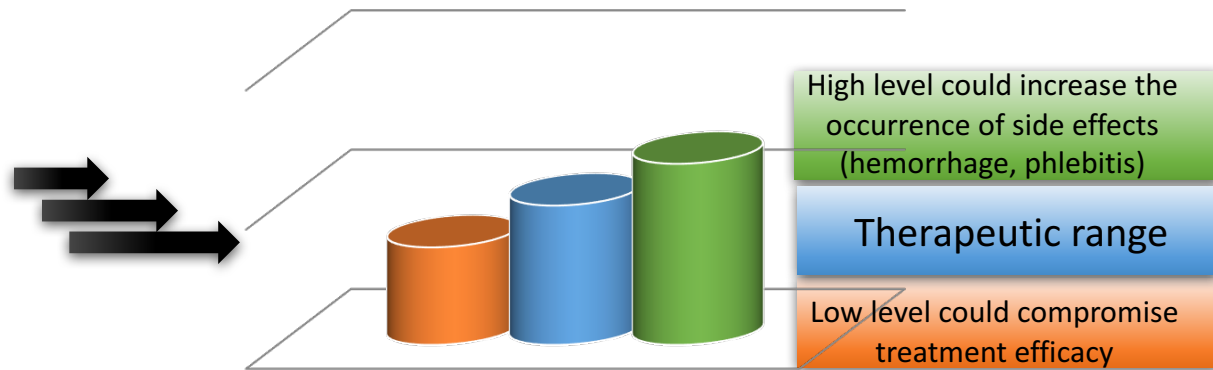**Brain cancer (n=15) with different treatment points -> 94 samples (duplicate on analysis)**

Avastin was intravenously infused at 10 mg/kg of body weight every two weeks. Blood samples were taken just before Avastin infusion.

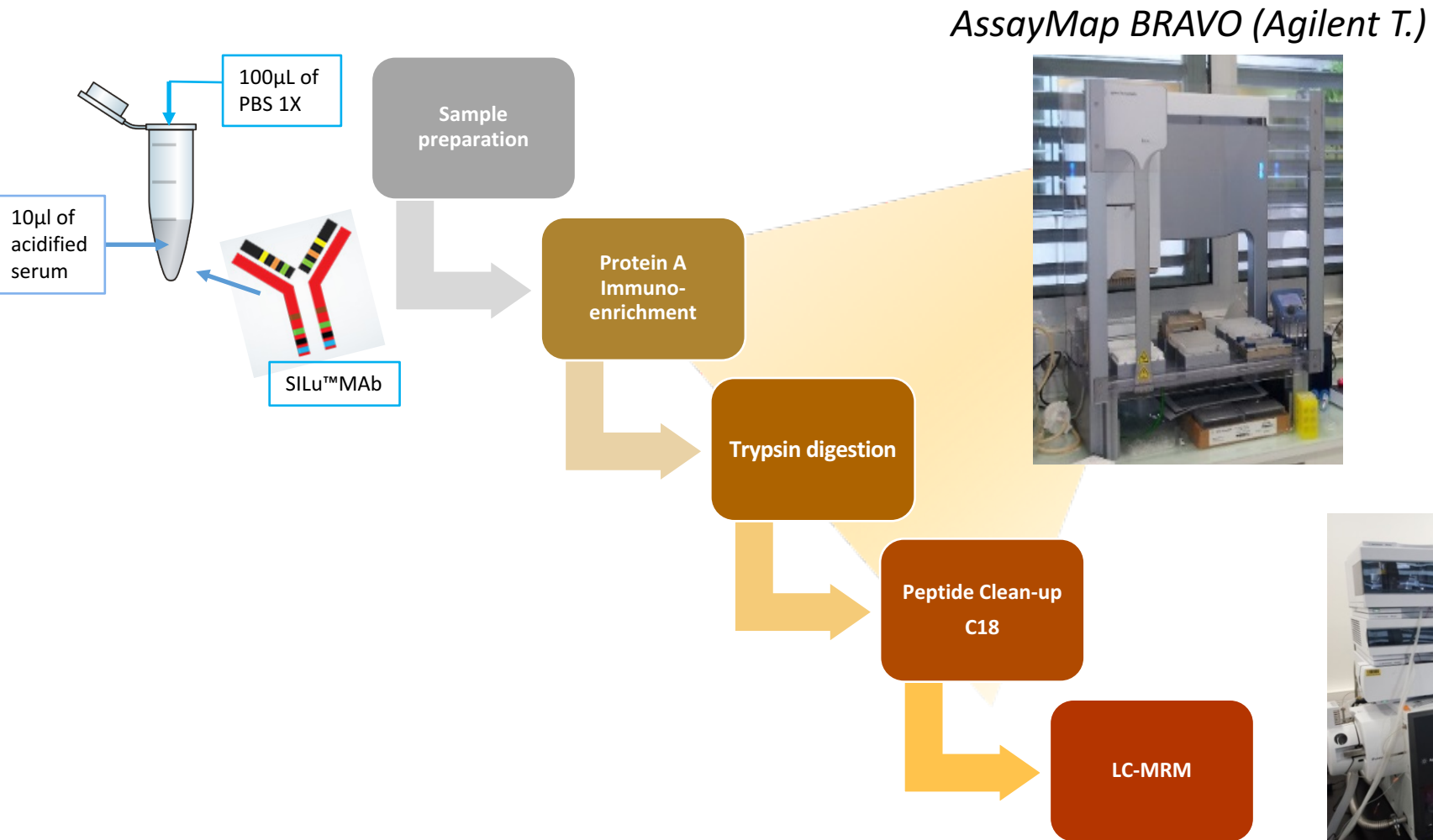10uL of serum was used to perform the assay

Theoretical Avastin concentration in a 70kg patient with treatment by 7 repeat administrations of 10 mg/kg every two weeks

$[Avastin]_{Blood}$

High level could increase the occurrence of side effects (hemorrhage, phlebitis)

Therapeutic range

Low level could compromise treatment efficacy

MS quantitative assay
After 7 cycles of treatment

1- Reference Protocol – based on protein-A tips

AssayMap BRAVO (Agilent T.)

100µL of PBS 1X

10µl of acidified serum

SILu™MAb

Sample preparation

Protein A Immuno-enrichment

Trypsin digestion

Peptide Clean-up C18

LC-MRM

Sample preparation

Protein A Immuno-enrichment

Antibodies capture

Prot-A

Tips ProtA

≈50µm

BRAVO Agilent

pH 7 – 8

1 | Prepare Cartridge

2 | Bind Target

3 | Wash & Repeat

pH 2

4 | Elute Target

Sample preparation

Protein A Immuno-enrichment

pH 7 – 8

1 | Prepare Cartridge

2 | Bind Target

3 | Wash & Repeat

pH 2

4 | Elute Target

Serum 1/100

Elution ProtA 1/10

191kDa

97kDa

71kDa

51kDa

39kDa

28kDa

19kDa
14kDa

Decomplexification of the sample by prot-A capture was very efficient

2- Analysis by LCMS

Avastin Structure

zoom
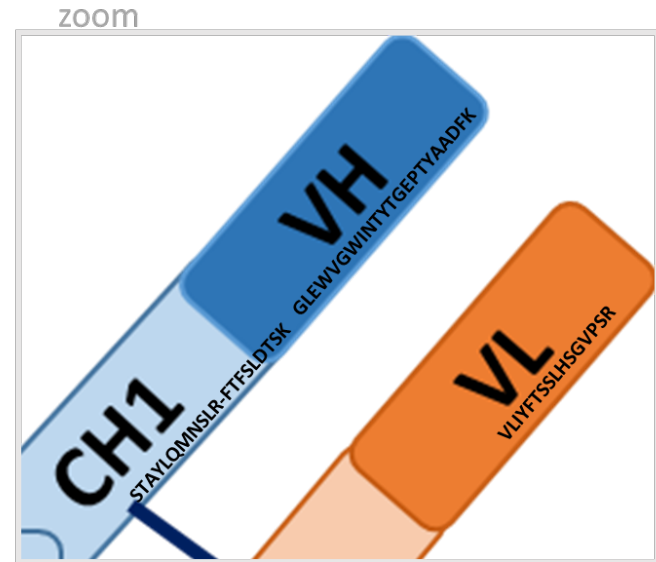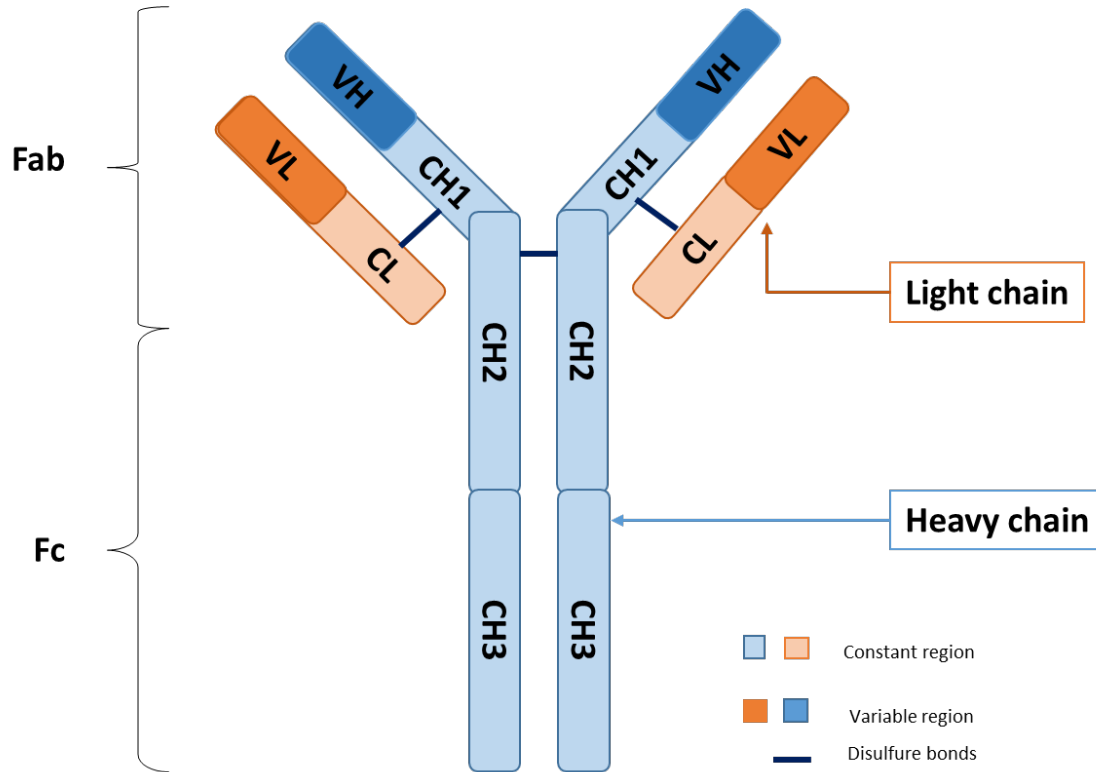
Monitored peptides by Mass Spectrometry	Localisation
VLIYFTSSLHSGVPSR	[45,60] Light chain
GLEWVGWINTYTGEPTYAADFK	[45,64] Heavy chain
FTFSLDTSK	[67,75] Heavy chain
STAYLQMNSLR	[76,86] Heavy chain

3 peptides from the heavy chain, and one from the light chain were monitored

2- Analysis by LCMS

100µL of PBS 1X

10µl of acidified serum

SILu™MAb 80ng

Sample preparation
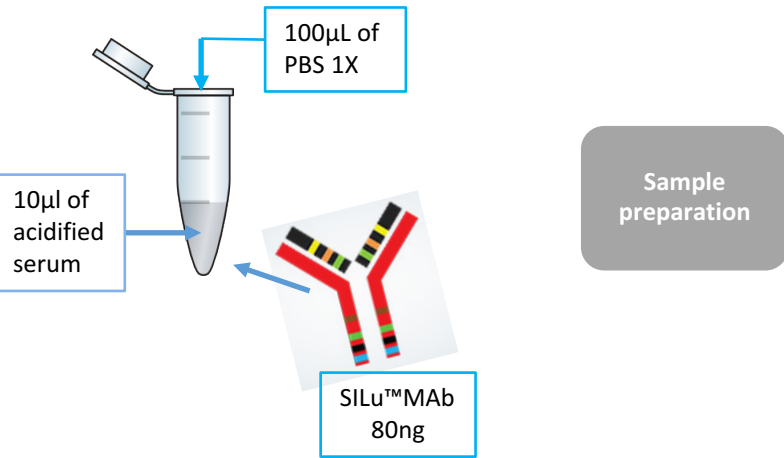
Addition of internal standard

Sequence Information
SILuMab Heavy Chain:
EVQLVESGGGLVQPGGSLRLSCVASGFTLNNYDMH
WVRQGIGKGLEWVSKIGTAGDRYYAGSVKGRFTISR
ENAKDSLYLQMNSLRVGDAAVYYCARGAGRWAPLG
AFDIWGQGTMVTVSSASTKGPSVFPLAPSSKSTSGG
TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV
DKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVK**FNWYVDGVEV
HNAK**TKPREEQYNSTYR**VVSVLTVLHQDWLNGK**EY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTK**NQVSLTCLVKGFYPSDIAVEWESNGQPENNYK**
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPG

SILuMab Light Chain:
QSALTQPRSVSGSPGQSVTISCTGTSSDIGGYNFVS
WYQQHPGKAPKLMIYDATKRPSGVPDRFSGSKSGN
TASLTISGLQAEDEADYYCCSYAGDYTPGVVFGGGT
KLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDF
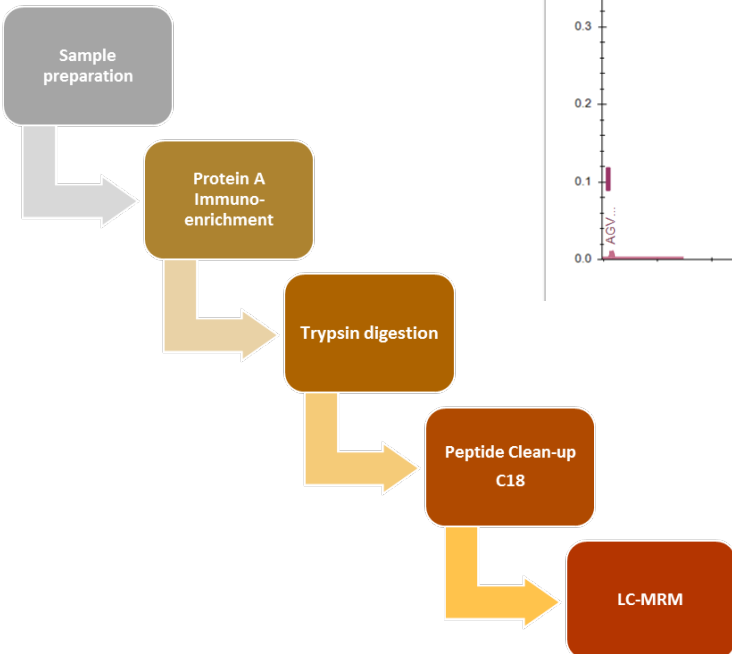YPGAVTVAWKADSSPVK**AGVETTTPSK**QSNNK**YAA
SSYLSLTPEQWK**SHRSYSCQVTHEGSTVEKTVAPTE
CS

Table 1.
Recommended Universal Peptide Sequences Liberated from SILuMab Tryptic Digest

Universal Peptide Sequence	Location	Isotype Overlap	Species Homology
DTLMISR	Heavy Chain	IgG1, IgG2, IgG3, IgG4	Rhesus monkey Cynomolgus monkey
FNWYVDGVEVHNAK	Heavy Chain	IgG1	
VVSVLTVLHQDWLNGK	Heavy Chain	IgG1, IgG3, IgG4	
NQVSLTCLVK	Heavy Chain	IgG1, IgG2, IgG3, IgG4	Rhesus monkey Cynomolgus monkey
GFYPSDIAVEWESNGQPENNYK	Heavy Chain	IgG1, IgG4	
AGVETTTPSK	Light Chain	lambda	Rhesus monkey Cynomolgus monkey
YAASSYLSLTPEQWK	Light Chain	lambda	

3 peptides well detected on our workflow. DTLMISR exhibit a lower CV and will use to normalize experiments

2- Analysis by LCMS

Bevacizumab
- K.VLIYFTSSLHSGVPSR.F [45, 60] — A
Bevacizumab
- K.GLEWVGWINTYTGEPTYAADFK.R [43, 64] — B
- R.RFTFSLDTSK.S [66, 75] (missed 1) — C
- R.FTFSLDTSK.S [67, 75] — D
- K.STAYLQMNSLR.A [76, 86] — E
- K.STAYLQMNSLR.A [76, 86] — F

SiluMab_HC
- DTLMISR — G
- FNWYVDGVEVHNAK — H

SiluMab_LC
- AGVETTTPSK — I

30min LC-MRM run

Sample preparation → **Protein A Immuno-enrichment** → **Trypsin digestion** → **Peptide Clean-up C18** → **LC-MRM**
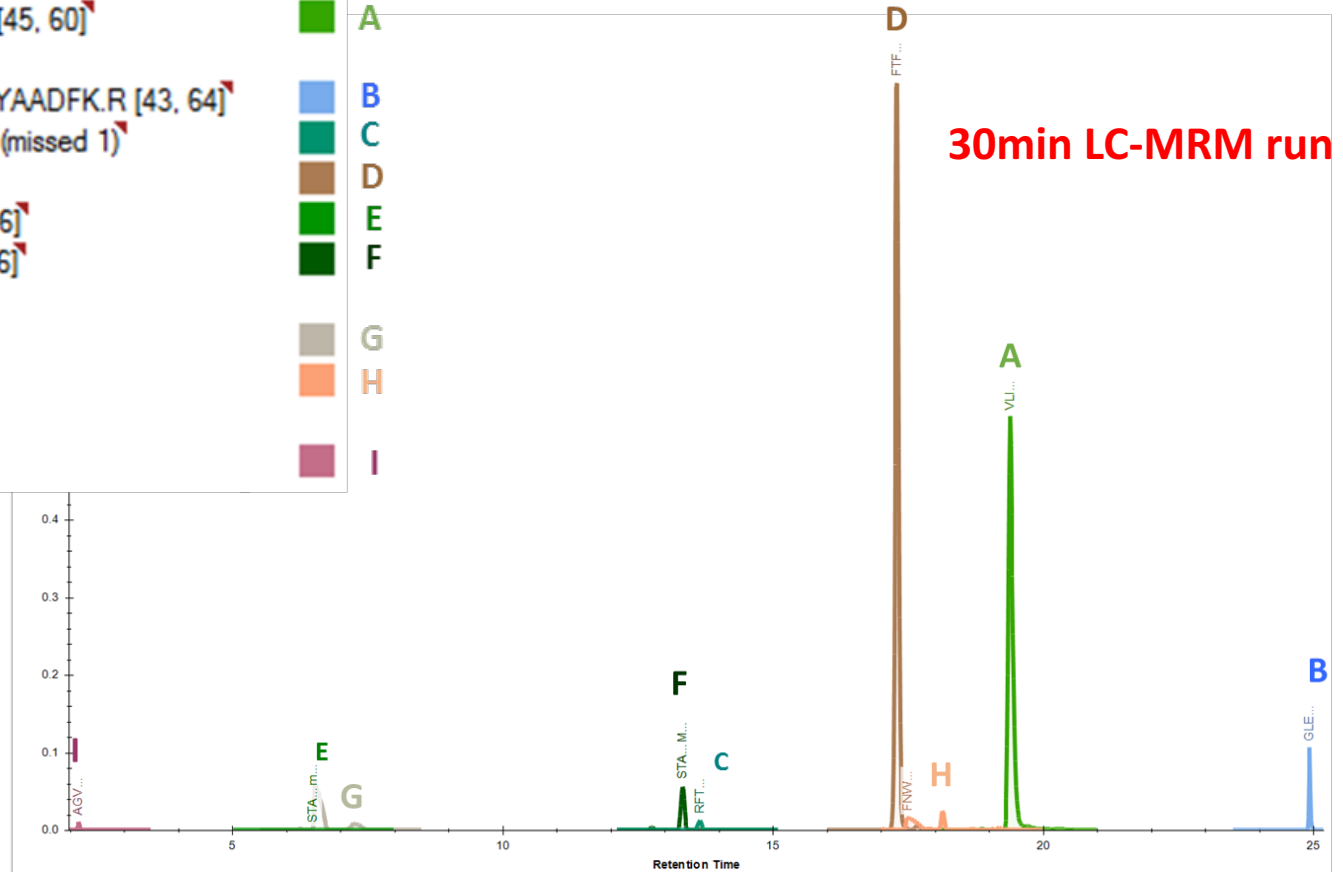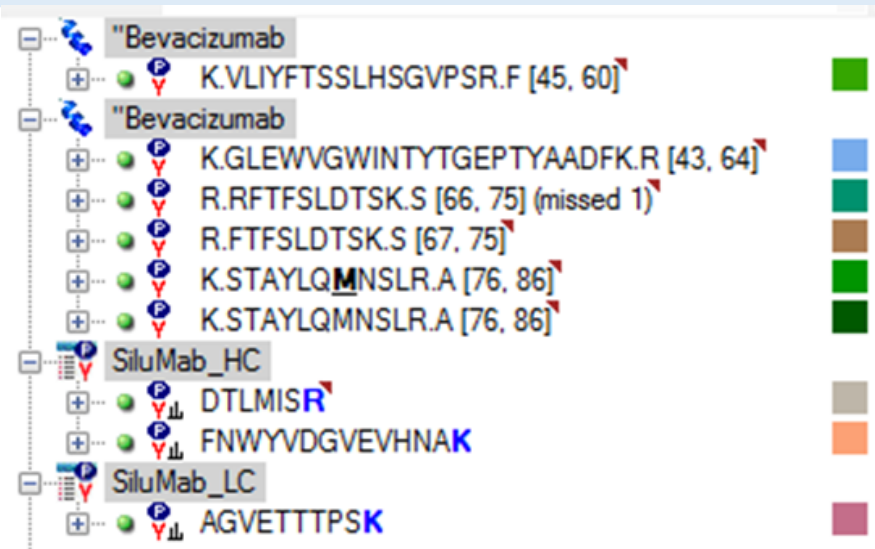
Reversed-phase column (RRHD Eclipse Plus C18, 2.1x150mm, 1.8um) at 400µL/min. 30 min multi-step gradient (B: 2.7% at 0min; 9.9% at 2min; 17.1% at 15min; 26.1% at 22min; 40.5% at 25min; then flush and re-equilibration).

Peptides analysis were carried out on a QqQ MS system (6490, Agilent technologies), equipped with an Agilent Jet-Stream ESI interface and performed in positive ion mode. The MS operated in dynamic MRM with a retention time window of 3 minutes and a maximum cycle time fixed at 800ms.

3- Analytical validation

Blank serum was used as matrix.

+ 80 ng SILu™MAb

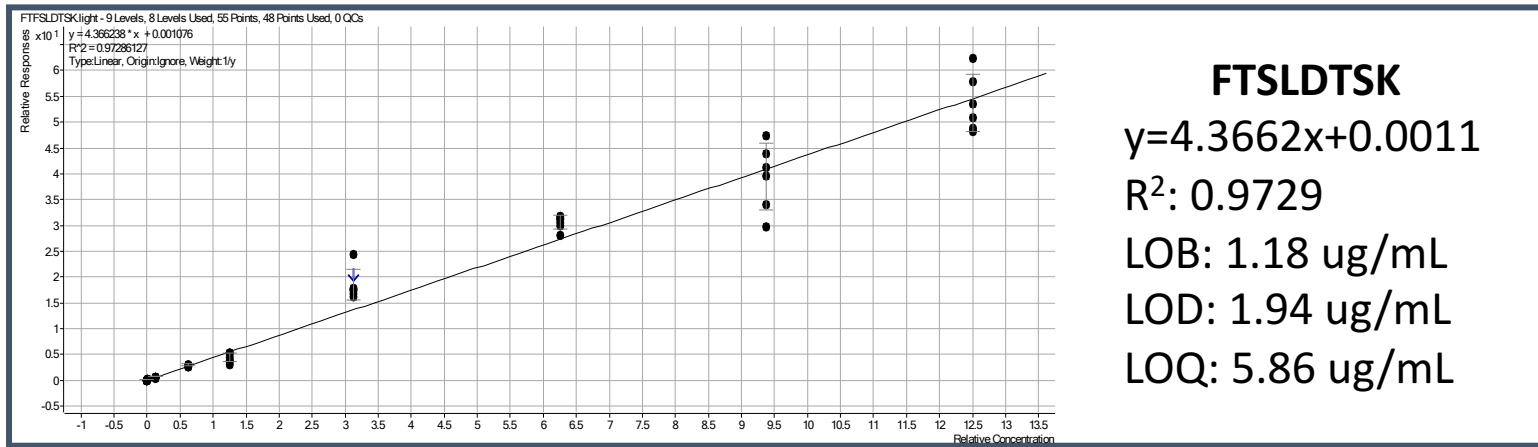+ 0; 10; 50; 100; 250; 500; 750 and 1000 ug/mL of Avastin

FTSLDTSK light - 9 Levels, 8 Levels Used, 55 Points, 48 Points Used, 0 QCs

y = 4.366238 * x + 0.001076
R^2 = 0.9728127
Type:Linear, Origin:Ignore, Weight:1/y

FTSLDTSK
$y=4.3662x+0.0011$
R^2: 0.9729
LOB: 1.18 ug/mL
LOD: 1.94 ug/mL
LOQ: 5.86 ug/mL

Figure: Calibration curves of FTSLDTSK
6 repetitions; 8 points calibration curves (0; 10; 50; 100; 250; 500; 750 and 1000 ug/mL)
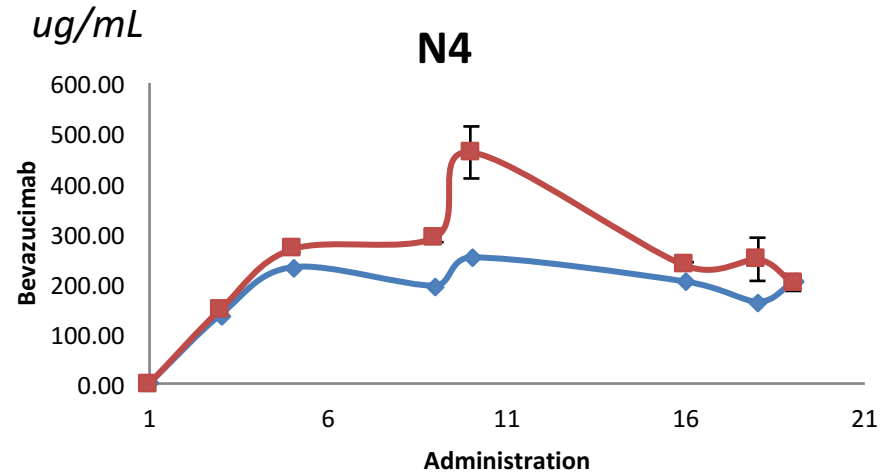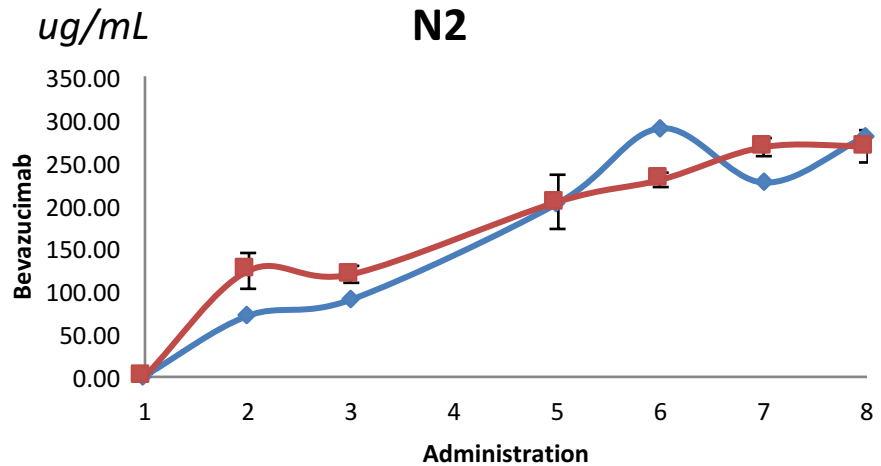
All the peptides shown a nice linear response, and a good correlation.
FTFSLDTSK peptide was used to quantify Avastin due to a superior analytical performances

4- Example of quantification during patient treatment
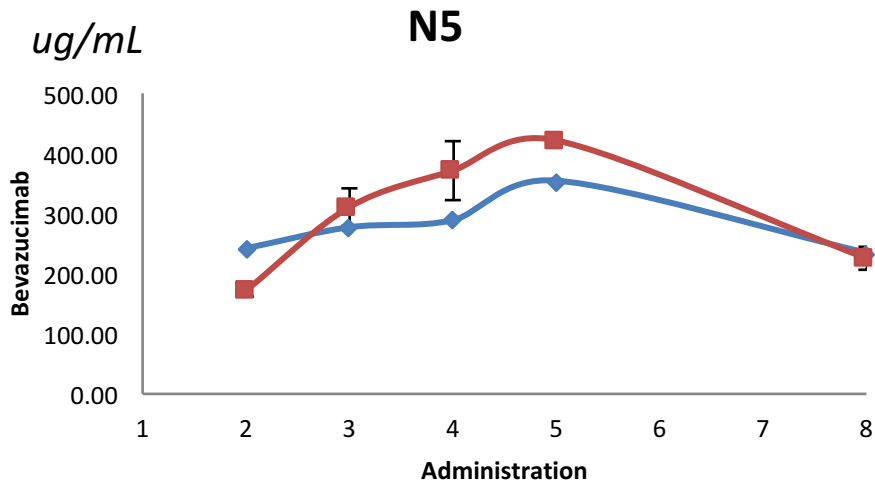
**3 patients
(20 samples in duplicats)**

- ◆ VEGF Bioplex
- ■ MRM

Equivalent Avastin pattern with BioPlex or MRM test on patients

Conclusions
Workflow based on protein-A tips

❑ Depending of pharmacokinetic parameters (ex: tumor burden) , dosing optimization regimen must be design individually

❑ MS assay was developed and validated to quantify Avastin
       - Quantifier peptide evaluation -> FTFSLDTSK peptide
       - Analytical validation of the method -> higher reproducibility CV
            Standardization with SILu™MAb was required but not perfect

❑ Assay was applied to monitore this drug during patient treatment
       - 15 patients -> 94 samples

❑ Correlation was found with previous Bioplex test developped

SCIENTIFIC
REP◯RTS

3rd Annual Conference and Exhibits
MSACL 2016 EU
September 12-15   Salzburg, Austria

Quantification of total Bevacizumab in human serum samples by targeted mass spectrometry. Method validation and applicability for therapeutic drug monitoring.
Sophie Broutin et al., Submitted, Scientific Reports

nSMOL Antibody BA Kit
nSMOL for nano-surface and molecular orientation limited proteolysis

nSMOL introduction to LBPC

- Platform comparison: AssayMap BRAVO vs nSMOL
- Improve our service list -> More client, more available assays on mAbs
- Improve our sensitivity
- Scientific publications
- And unexpected improvements….

nSMOL Antibody BA Kit
nSMOL for nano-surface and molecular orientation limited proteolysis

Resin surface — Immunoglobulin Collection

Protein A beads

Captured Antibody Fab Oriented toward Solution

Fab

Antibody

IgG collection

nSMOL Antibody BA Kit
nSMOL for nano-surface and molecular orientation limited proteolysis

Resin surface — Immunoglobulin Collection
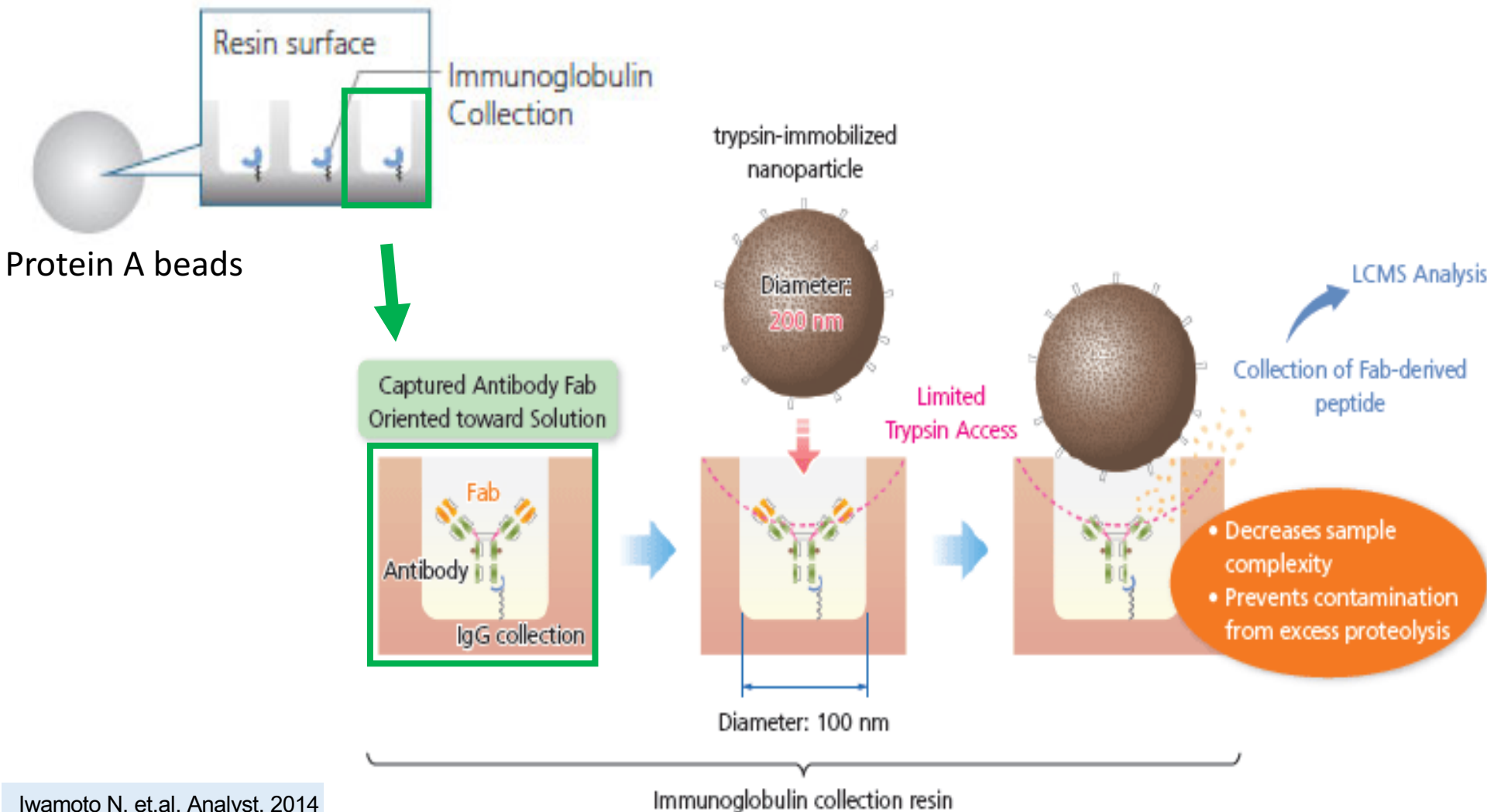
Protein A beads

Captured Antibody Fab Oriented toward Solution

Fab

Antibody

IgG collection

trypsin-immobilized nanoparticle

Diameter: 200 nm

Limited Trypsin Access

Diameter: 100 nm

LCMS Analysis

Collection of Fab-derived peptide

- Decreases sample complexity
- Prevents contamination from excess proteolysis

Immunoglobulin collection resin

Iwamoto N. et.al. Analyst, 2014
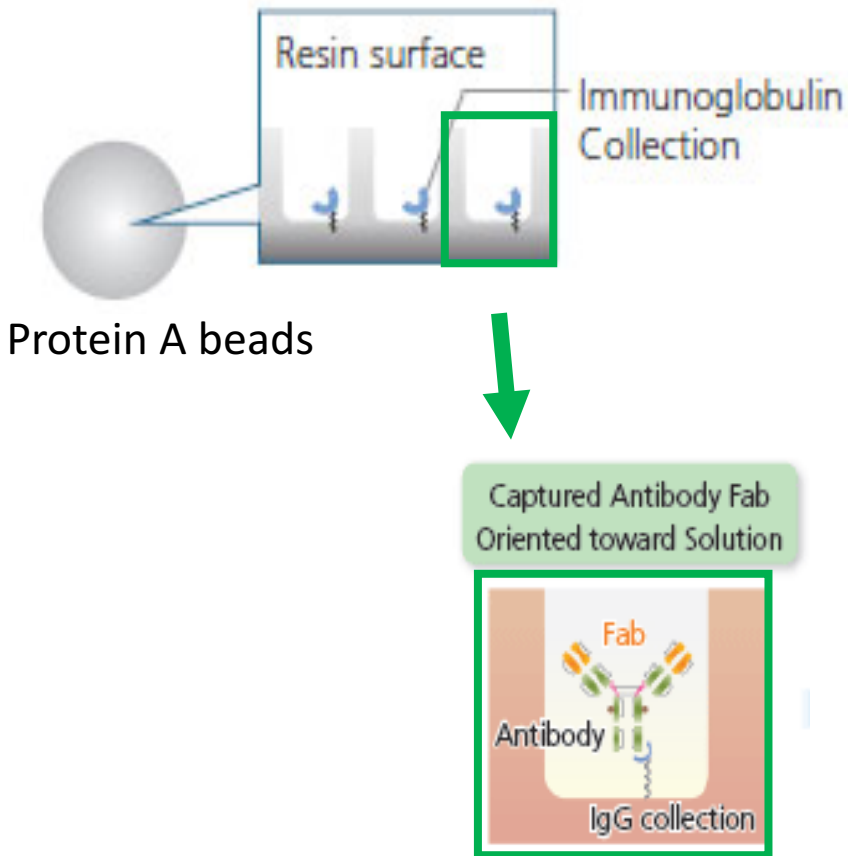
Step 1
Protein A Immuno-enrichment

Enrichment of antibody from plasma

1. Protein A beads

Add: Buffer A

2. 5 µL of plasma

3. Rotation: 15 min at RT

4. Transfer to filter cup

5. Centrifugation

Wash: Buffer A
Wash: Buffer B
Add: Buffer C

Waste

30min

From Shimadzu

1- nSMOL Workflow

Step 1
Protein A Immuno-enrichment

Step 2
Protein Digestion

Step 3
LCMS

Proteolysis
nSMOL
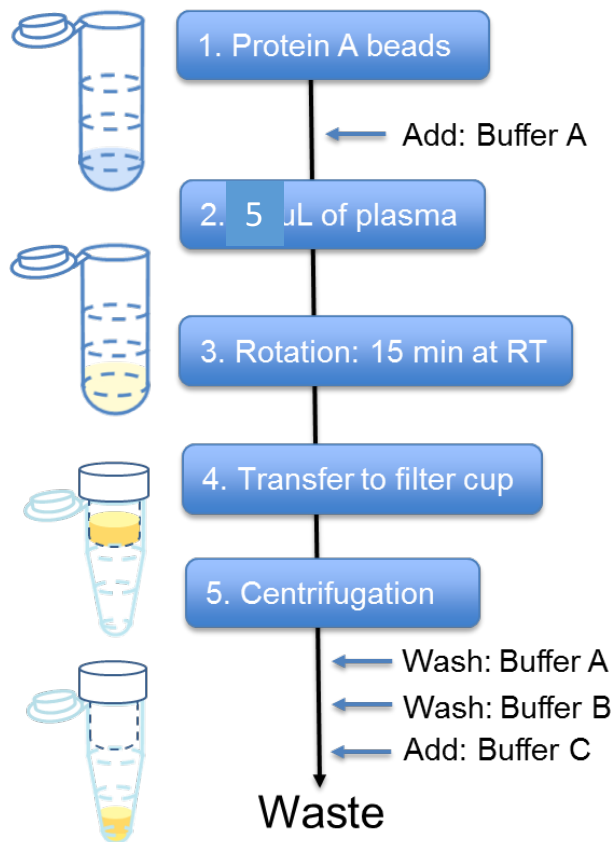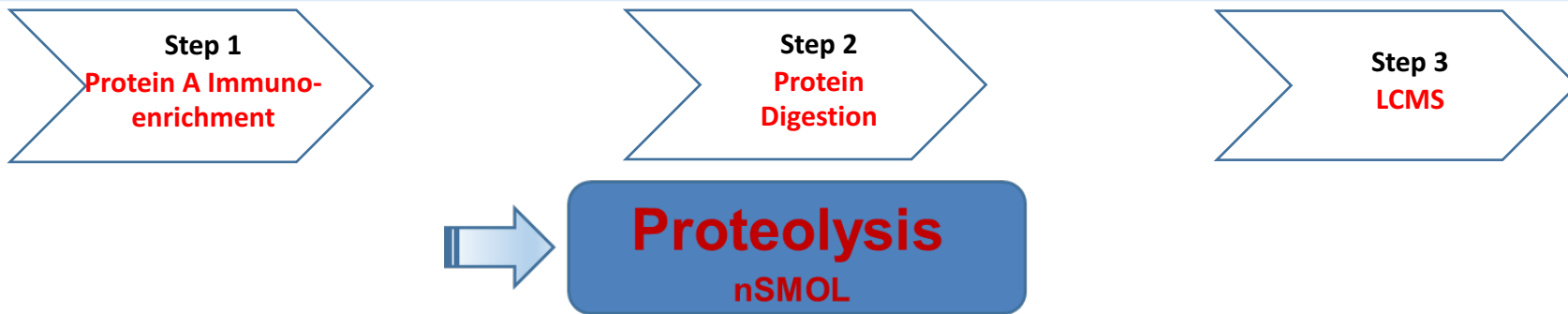
- Selective digestion of Fab region
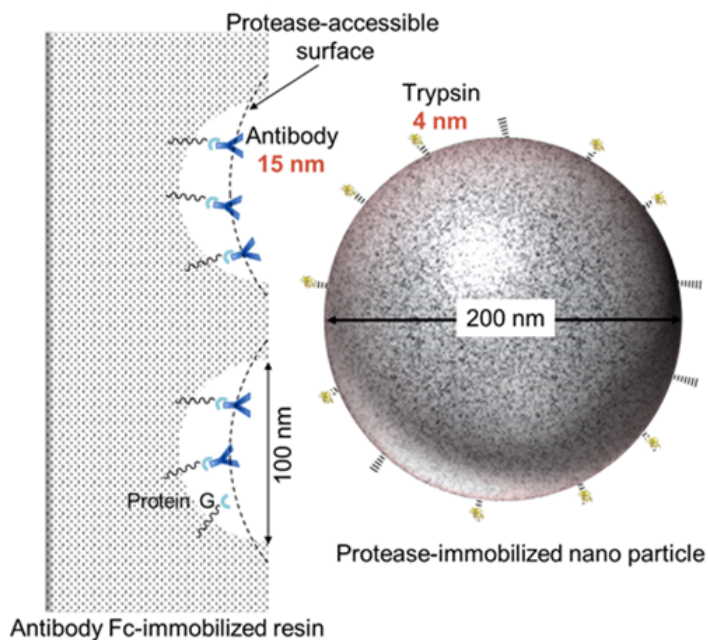
- Addition of trypsin-nanoparticle :
200 nm diameter

Protease-accessible surface

Antibody
15 nm

Trypsin
4 nm

200 nm

100 nm

Protein G

Protease-immobilized nano particle

Antibody Fc-immobilized resin

Centrifugation

Peptide solution

5 hours at 50°C

From Shimadzu

1- nSMOL Workflow

Step 1
Protein A Immuno-enrichment

Step 2
Protein Digestion
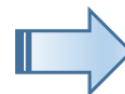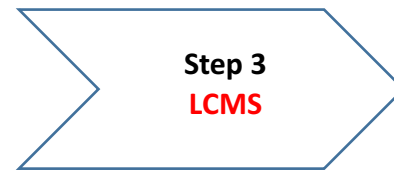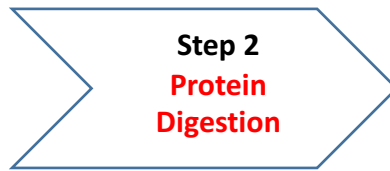
Step 3
LCMS

LCMS

8060 (Shimadzu)

10min LC run at 400uL/min, 50°C Column Shim-pack GISS-HP C18, 3um, 150 x 2.1mm

MS: Positive mode, DL: 150°C, Heat Block: 250°C, Interface: 400°C, Nebulizer gas: 2L/min, Drying gas: 5L/min, Heating gas: 15L/min, Dwell time: 636ms

From Shimadzu

FWHM: 0.07min
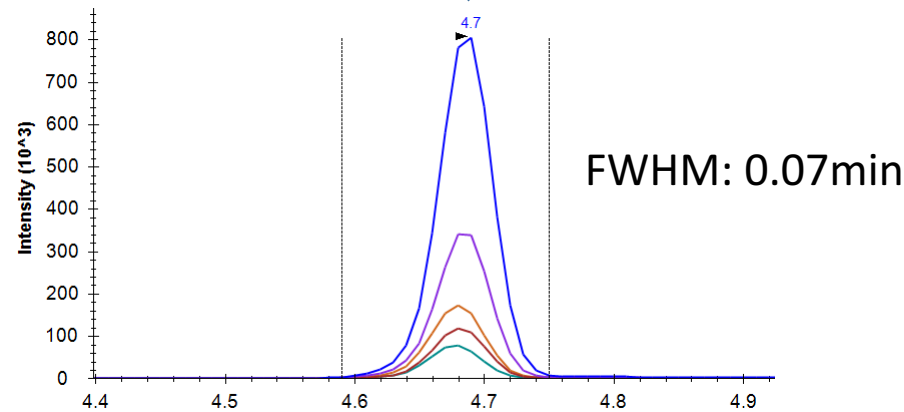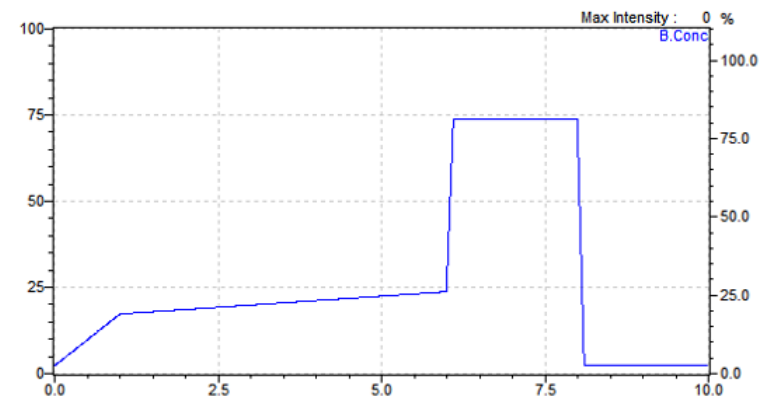
Previous study:

30min LC run

4 different peptides were monitored and FTFSLDTSK peptide was selected (also on Shimadzu work)

Fully validated LCMS bioanalysis of Bevacizumab in human plasma using nano-surface and molecular-orientation limited (nSMOL) proteolysis.

Iwamoto N[1], Umino Y[1], Aoki C[1], Yamane N[2], Hamada A[3], Shimada T[4].
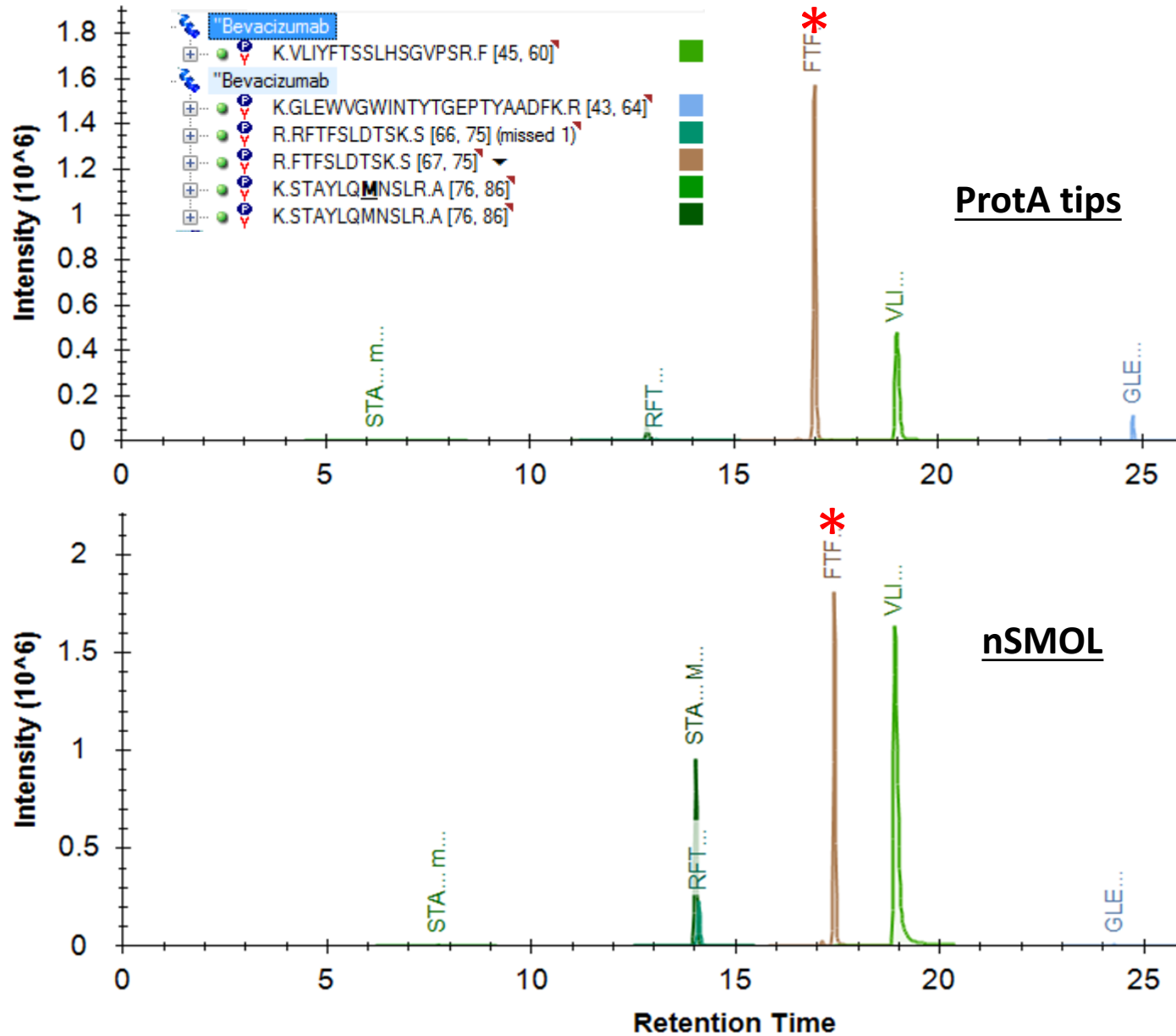
Chromatogram legend (ProtA tips panel):

"Bevacizumab
- K.VLIYFTSSLHSGVPSR.F [45, 60]

"Bevacizumab
- K.GLEWVGWINTYTGEPTYAADFK.R [43, 64]
- R.RFTFSLDTSK.S [66, 75] (missed 1)
- R.FTFSLDTSK.S [67, 75]
- K.STAYLQ**M**NSLR.A [76, 86]
- K.STAYLQMNSLR.A [76, 86]

ProtA tips

nSMOL

- **Global profiles were differents**

- **FTFSLDTSK peptide was still well detected**
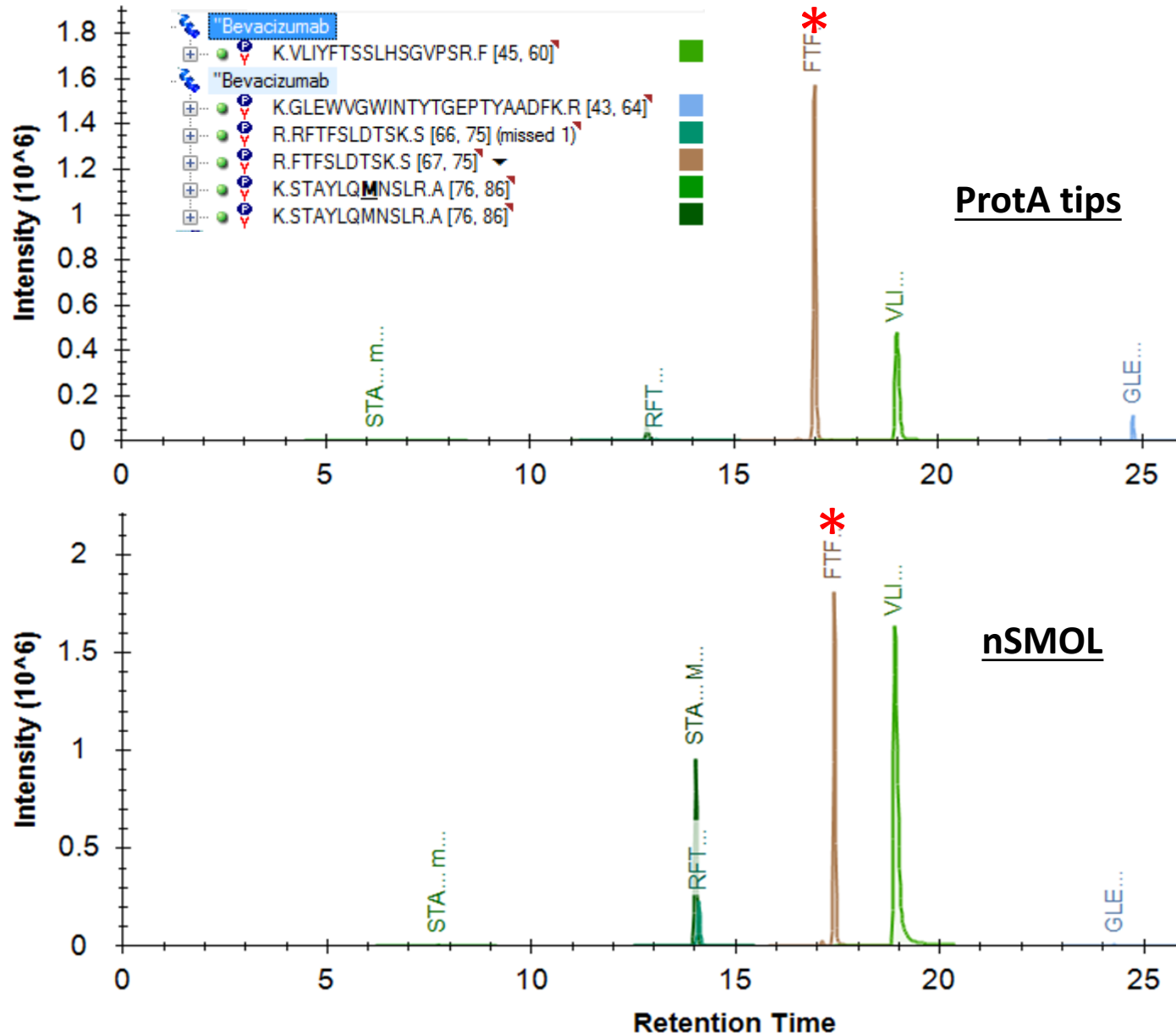
- **Global profiles were differents**

- **FTFSLDTSK peptide was still well detected**

Trypsin digestion gave different yield (site specific), between in-solution and on nano-bead

Remarks

Limited digestion was investigated on pure avastin prepared by nSMOL, follow by HRMS analysis
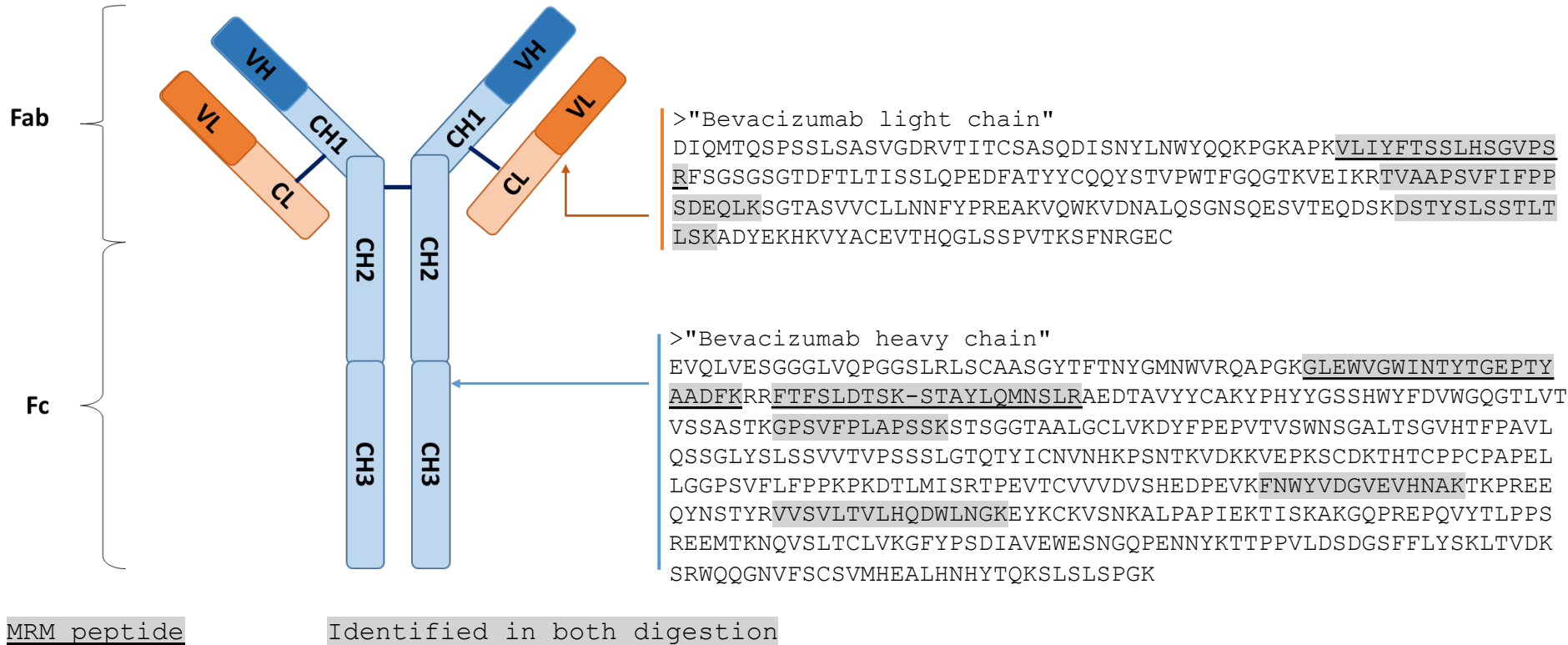
Limited digestion was investigated on pure avastin prepared by nSMOL, follow by HRMS analysis


```
>"Bevacizumab light chain"
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

```
>"Bevacizumab heavy chain"
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY
AADFKRRFTFSLDTSK–STAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVT
VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEL
LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
```

<u>MRM peptide</u>   Identified in both digestion

➤ **nSMOL: limited proteolysis, mainly on Fc region**

Remarks

Comparison to sequence coverage obtain with in-solution trypsin digestion

>"Bevacizumab light chain"
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

>"Bevacizumab heavy chain"
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY
AADFKRRFTFSLDTSK–STAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVT
VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEL
LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

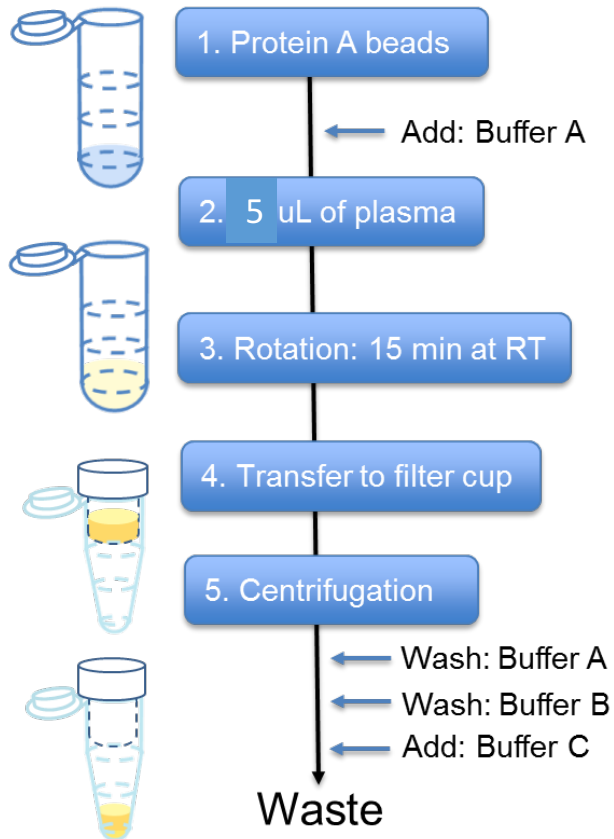MRM peptide | Identified in both digestion | Identified only in-solution digestion

➢ **In-solution trypsin digestion: as excepted, sequence coverage is higher**
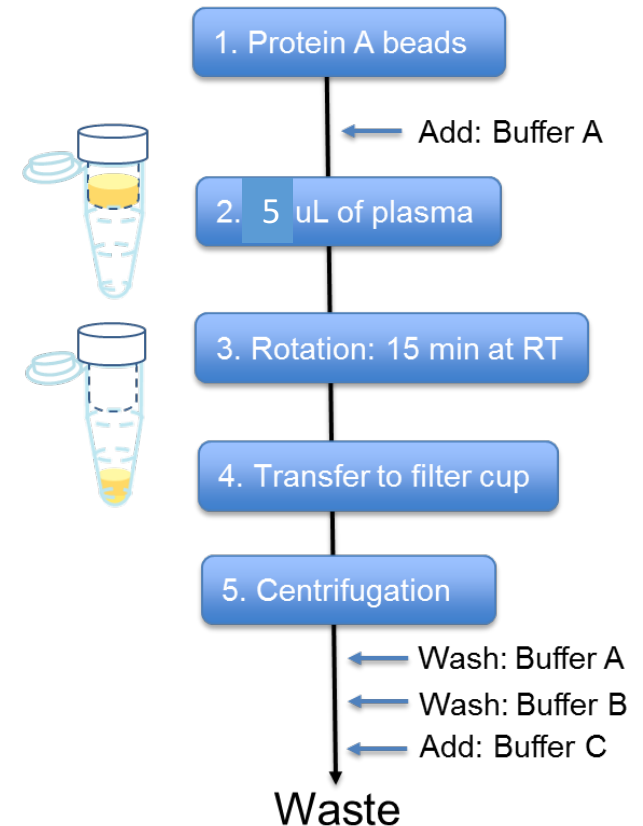
3- Step 1 of nSMOL workflow

Reference Protocol
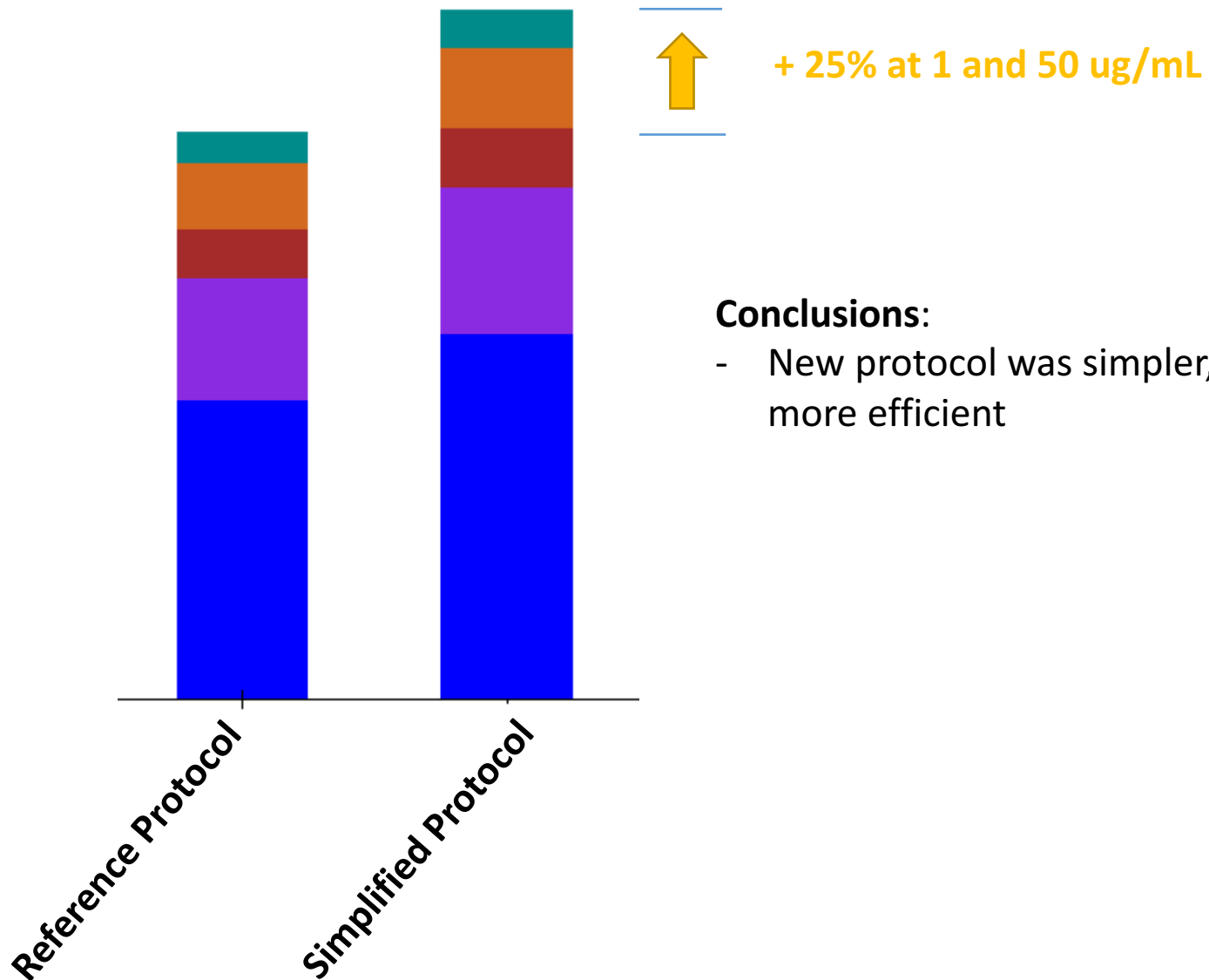
Enrichment of antibody from plasma

1. Protein A beads

Add: Buffer A

2. 5 uL of plasma

3. Rotation: 15 min at RT

4. Transfer to filter cup

5. Centrifugation

Wash: Buffer A

Wash: Buffer B

Add: Buffer C

Waste

Simplified Protocol
All the steps were directly made on filter

Enrichment of antibody from plasma

1. Protein A beads

Add: Buffer A

2. 5 uL of plasma

3. Rotation: 15 min at RT

4. Transfer to filter cup

5. Centrifugation

Wash: Buffer A

Wash: Buffer B

Add: Buffer C

Waste

+ 25% at 1 and 50 ug/mL

Conclusions:
- New protocol was simpler, faster and more efficient

Reference Protocol

Simplified Protocol

Sample preparation normalization:

-> nSMOL protocol recommend to spike peptide (P14R) at digestion step to compensate volume variation before the LCMS analysis
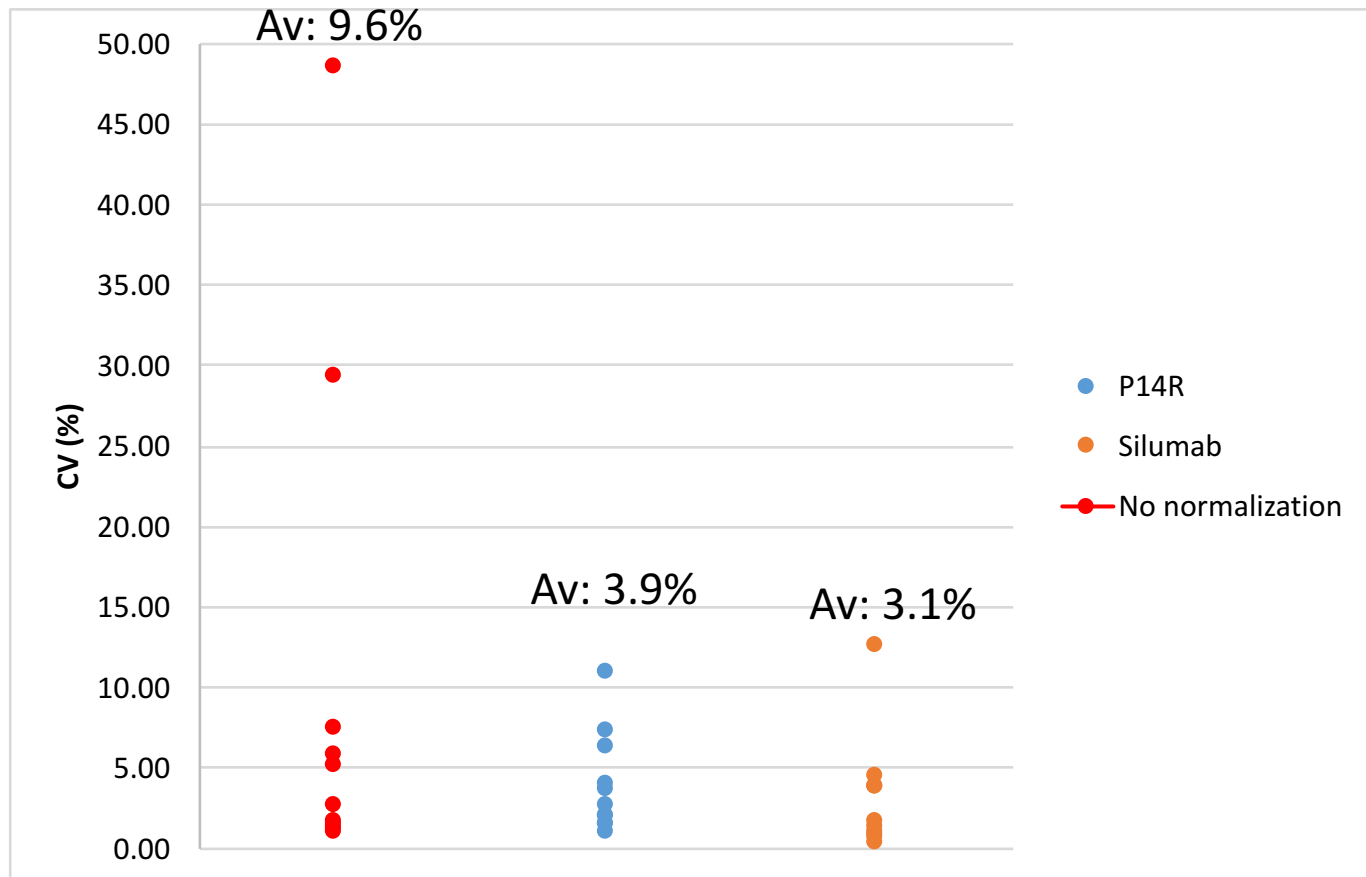
-> In our previous work, normalization with silumab was useful for our protA tips workflow

- SILu™MAb can be apply to nSMOL workflow also based on protein-A
- SILu™MAb was spiked directly on the serum
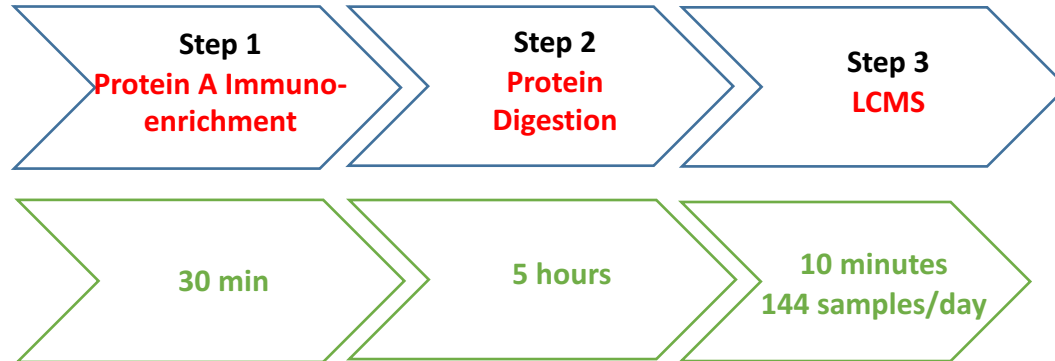- The use of same internal standard gave an easier method comparison

-> P14R and SILu™MAb were spiked in this study
CV were extracted for each point of the calibration curve

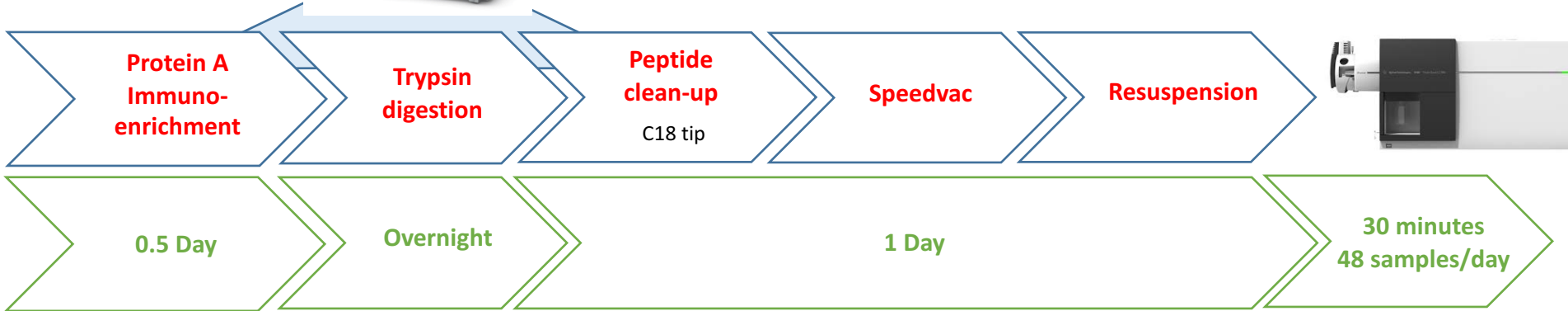-> Based on these results, normalization with P14R was less efficient than with SILu™MAb.

nSMOL

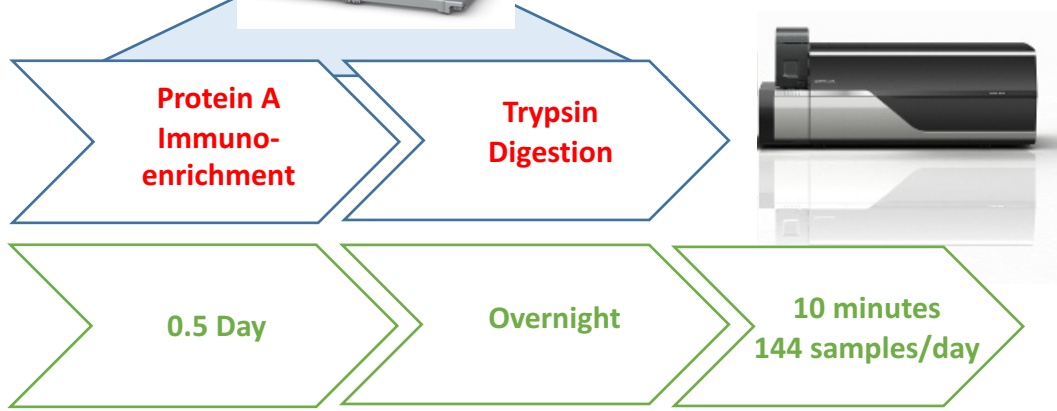|		
Step 1 **Protein A Immuno-** **enrichment**	**Step 2** **Protein** **Digestion**	**Step 3** **LCMS**
30 min	**5 hours**	**10 minutes** **144 samples/day**

4- Workflow summary

Protein-A tips Version 1.0

Protein A Immuno-enrichment	Trypsin digestion	Peptide clean-up C18 tip	Speedvac	Resuspension

0.5 Day	Overnight	1 Day	30 minutes 48 samples/day

Version 2.0

Protein A Immuno-enrichment	Trypsin Digestion

0.5 Day	Overnight	10 minutes 144 samples/day

New MS detector with higher MRM scan speed

nSMOL

Step 1 Protein A Immuno-enrichment	Step 2 Protein Digestion	Step 3 LCMS
30 min	5 hours	10 minutes 144 samples/day

Protein-A tips Version 2.0

	nSMOL	ProtA tips (v2.0)
Duration (Days)	< 1	< 2
Cost/point (euro)	58	28

Protein A Immuno-enrichment	Trypsin Digestion	LCMS
0.5 Day	Overnight	10 minutes 144 samples/day
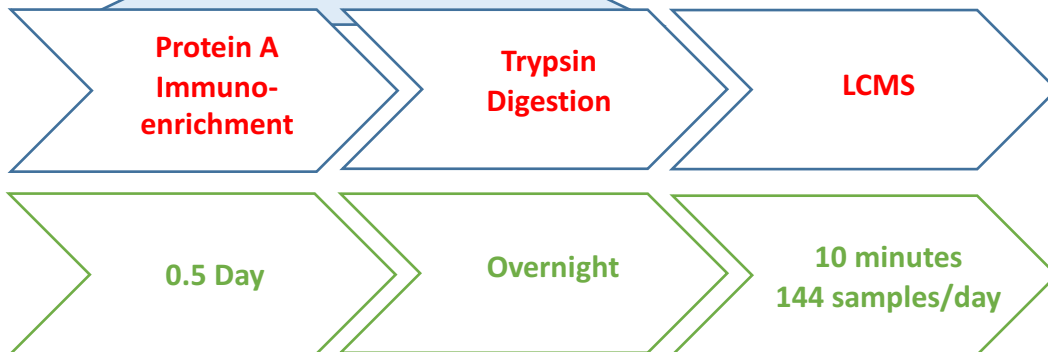
5- Analytical validation
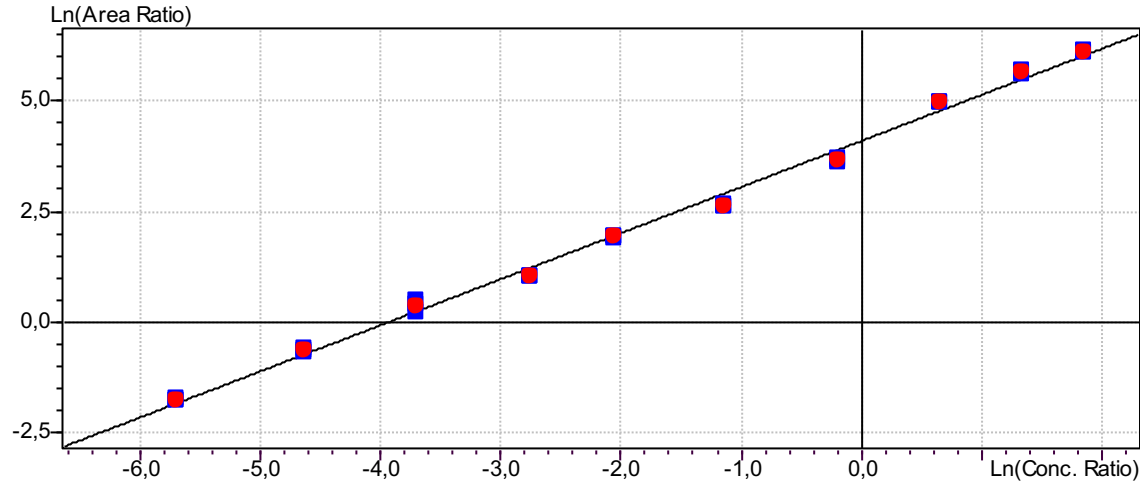
	Range (ug/mL)	Equation	R²	RSD (%)	LLOQ (ug/mL)	CV concentration (%)	Accuracy (%)
ProtA tips v2.0	1.9565 -766.667	y = 1.0918x+ 10.8395	0.9959	22.90	1.9565	2.60	100.60
nSMOL	0.26986 -766.667	y = 1.0439x+ 60.1451	0.9970	20.80	0.26986	3.1	101.6

Similar performances on calibration curves except for the LLOQ

Analytical performances:

	LLOQ protA	LQC ProtA: 15 ug/mL	MQC ProtA: 100 ug/mL
within-run accuracy (%)	115.8	101.4	89.2
Between-run accuracy (%)	103.9	106.1	86.6
within-run precision (%)	1.7	1.7	3.5
Between-run precision (%)	8.5	2.5	2.5

|	LLOQ nSMOL	LQC nSMOL: 15 ug/mL	MQC nSMOL: 100 ug/mL
within-run accuracy (%)	109.5	98.1	102.8
Between-run accuracy (%)	101.2	92.1	101.1
within-run precision (%)	1.0	4.5	4.5
Between-run precision (%)	9.1	4.9	2.9

Similar performances on QC samples.

within-run accuracy and precision: 5 LCMS replicats; Between-run accuracy and precision: 3 LCMS injection/3 days
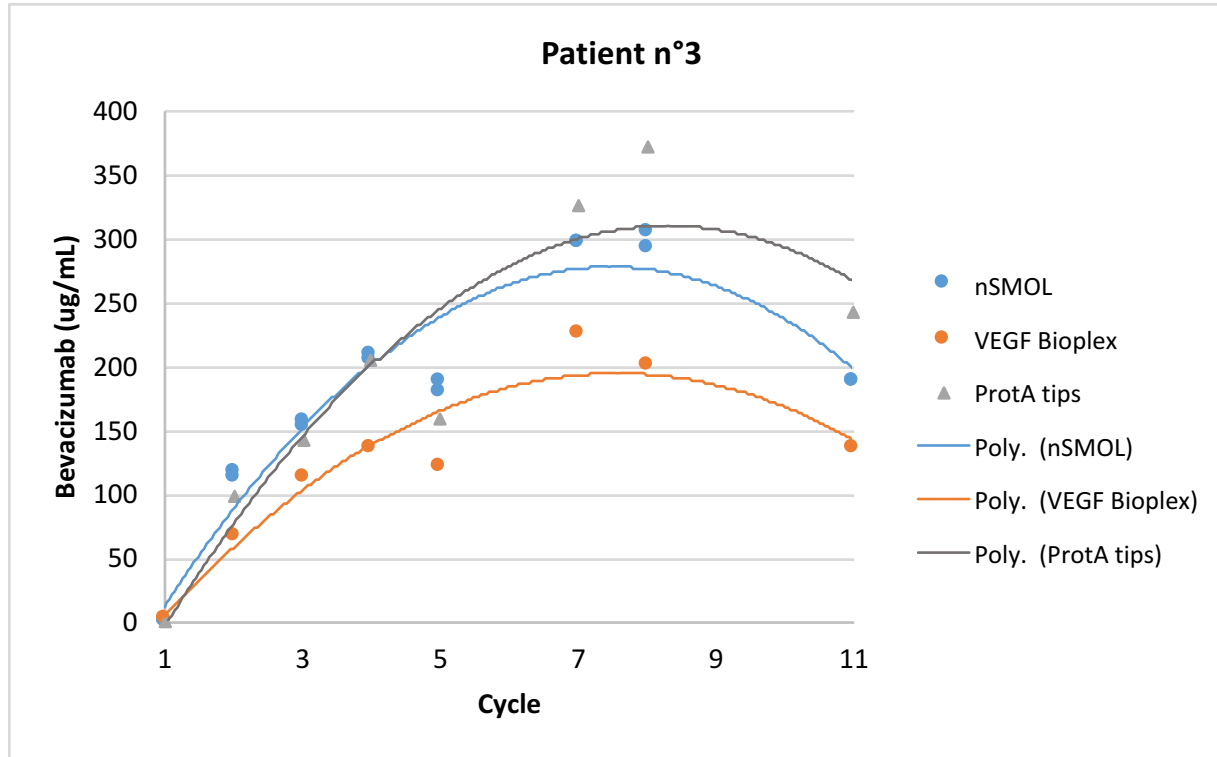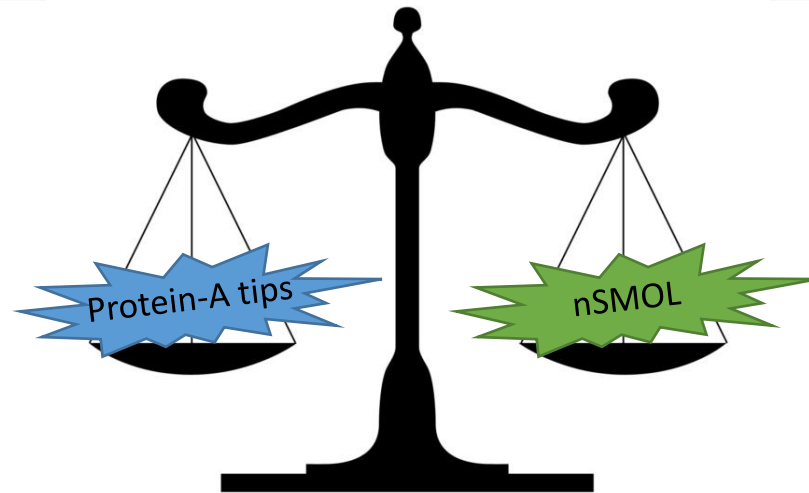
6- Patient samples
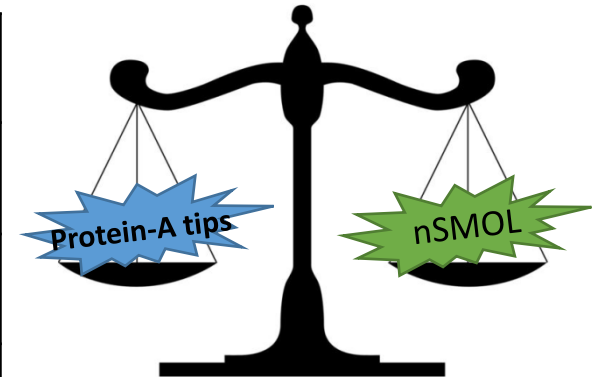
6- Patient samples

Patient n°3
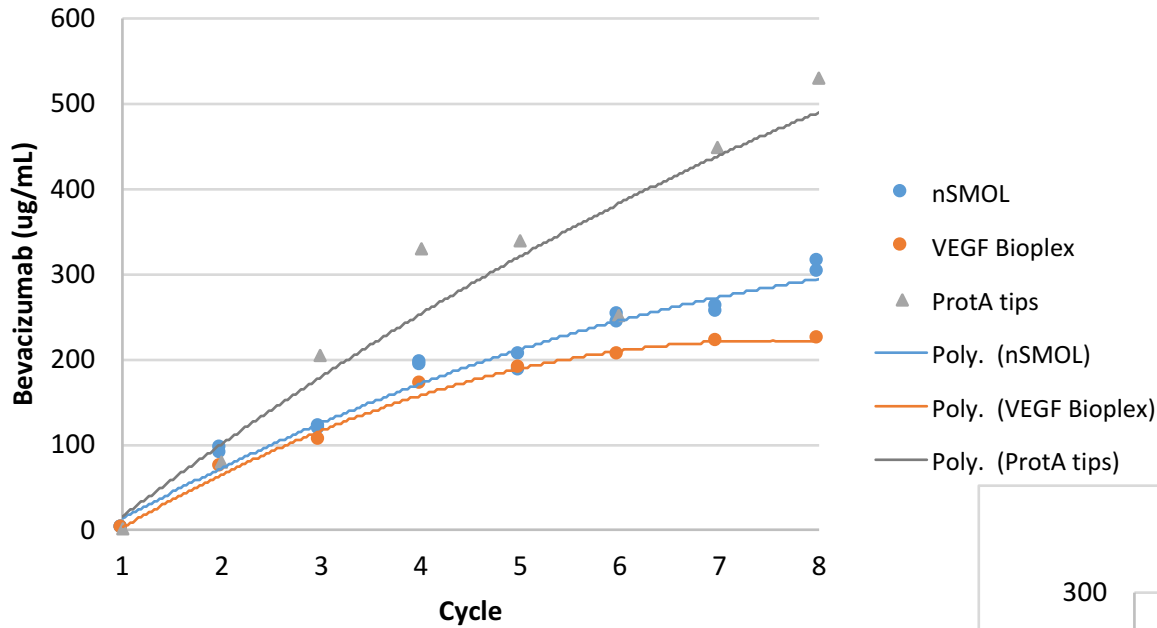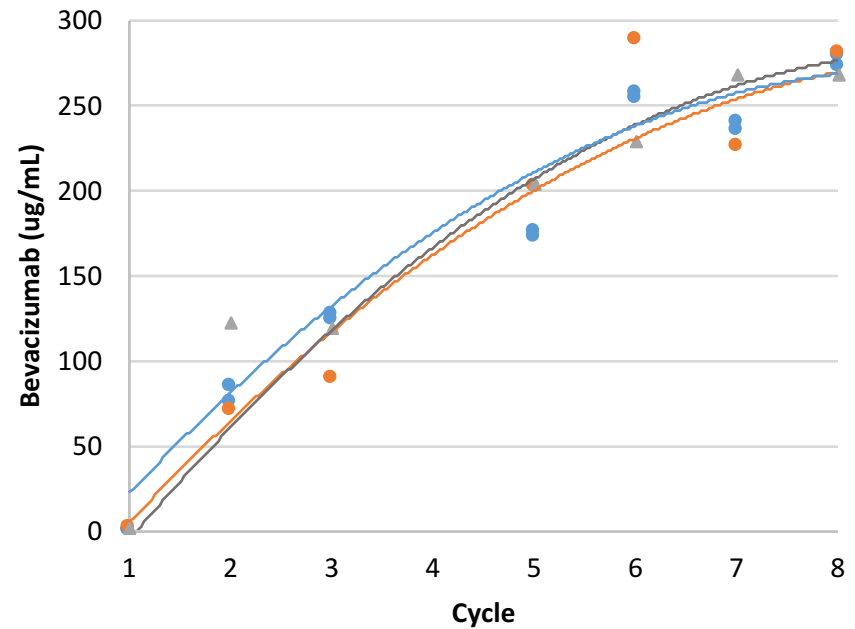
Close performances on patient samples.
Kinetic of available Bevacizumab on serum were equivalent
Levels were slighty different depending of the techniques
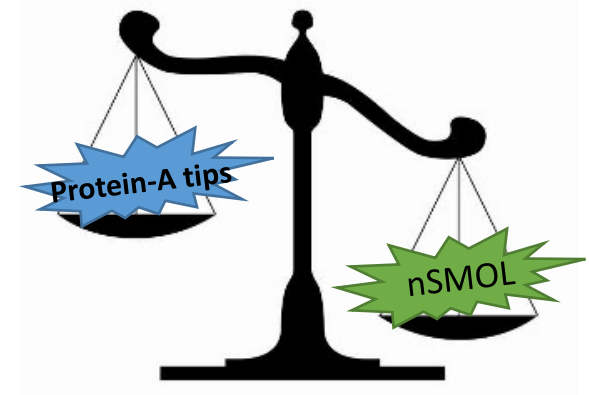
Conclusions

Conclusions

	Protein-A tips (AssayMap Bravo)	nSMOL
Performances on patient samples	Similar	Similar
Analytical Performances	Similar	Similar
Sensibility	1.9565 µg/mL	0.26986 µg/mL
Number of samples	96 samples in parallel	Limited number of samples (24 by experiment)
Liquid handling	Automated	Manual
Workflow	Training needed	user friendly
Technician	Dedicated and trained people	Open access
Process duration	< 1 day	<2 days
Cost	28 euros	58 euros

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Conclusions

Protein-A tips

nSMOL

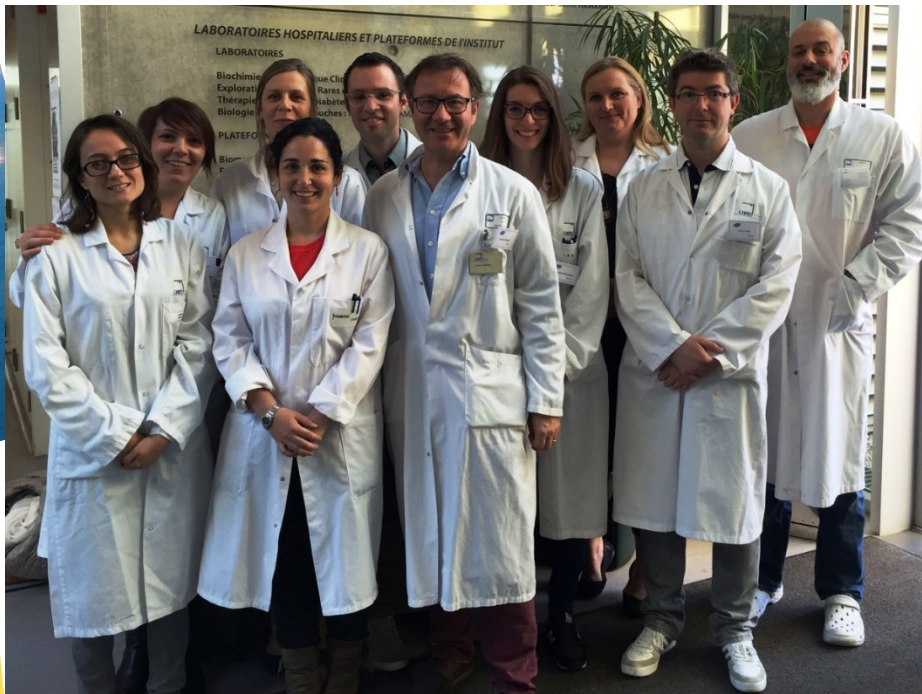To summarize our point of view:

nSMOL is most interesting
- for our customers
- when the sensitivity is an issue – but it has a cost

ProteinA tips workflow is most interesting
- for our internal project
- for larger cohort

Thank you!

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