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

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First study

Avastin Project

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

Method development and Validation

Dosage 94 patient samples

Valorization: 1 publication 1 comm

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- **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
  - metastatic colorectal cancer
  - non-small cell lung cancer
  - renal cell carcinoma
  - ovarian cancer
  - glioblastoma

  *(cf. Han, AAPS journal, 2014)*
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

MS quantitative assay
After 7 cycles of treatment

Theoretical Avastin concentration in a 70kg patient with treatment by 7 repeat administrations of 10 mg/kg every two weeks
1- Reference Protocol – based on protein-A tips

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1- Reference Protocol – based on protein-A tips

Sample preparation → Protein A Immuno-enrichment

Antibodies capture

≈50µm

Tips ProtA

BRAVO Agilent

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

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

Monitored peptides by Mass Spectrometry

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLIYFTSSLHSGVPSR</td>
<td>[45,60] Light chain</td>
</tr>
<tr>
<td>GLEWVGWINTYGEPTYADFK</td>
<td>[45,64] Heavy chain</td>
</tr>
<tr>
<td>FFTSLDTSK</td>
<td>[67,75] Heavy chain</td>
</tr>
<tr>
<td>STAYLQMNSLR</td>
<td>[76,86] Heavy chain</td>
</tr>
</tbody>
</table>
2- Analysis by LCMS

Addition of internal standard

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

<table>
<thead>
<tr>
<th>Universal Peptide Sequence</th>
<th>Location</th>
<th>Isotype Overlap</th>
<th>Species Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DTLMISR</strong></td>
<td>Heavy Chain</td>
<td>IgG1, IgG2, IgG3, IgG4</td>
<td>Rhesus monkey, Cynomolgus monkey</td>
</tr>
<tr>
<td><strong>FNWYDGVEVHNAK</strong></td>
<td>Heavy Chain</td>
<td>IgG1</td>
<td></td>
</tr>
<tr>
<td><strong>VVSVLTVLHODWLNK</strong></td>
<td>Heavy Chain</td>
<td>IgG1, IgG3, IgG4</td>
<td></td>
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<tr>
<td><strong>NQVSLTCLVK</strong></td>
<td>Heavy Chain</td>
<td>IgG1, IgG2, IgG3, IgG4</td>
<td>Rhesus monkey, Cynomolgus monkey</td>
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<td><strong>GFYPSDIAVEWESNGOPENNYK</strong></td>
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<td>IgG1, IgG4</td>
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<tr>
<td><strong>AGVETTPSK</strong></td>
<td>Light Chain</td>
<td>lambda</td>
<td>Rhesus monkey, Cynomolgus monkey</td>
</tr>
<tr>
<td><strong>YAASSYLSLTPEQWK</strong></td>
<td>Light Chain</td>
<td>lambda</td>
<td></td>
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Sequence Information
SILuMab Heavy Chain:
EVQLVESGGGLVQPSGGSLRLSCLVASGFTLNNYDMH
WYRGIGKGLQEVSVKIGTERGDYAGSVVGRFTISR
ENAKDSLNLQMNLSRNVGDAVYVYCARAGRWAPLG
AFDIDWGGTMTVSAAASTKGPSVFLAPSSSTSSSSTSG
TAALGCLVKKDYPPEPVTYSVNSGALTSGSVIHTFFAVL
QSSGLYLSVSSVTVPSSSLGQTICYNVHPSNTKV
DKKVEPKSCDKTHCPCAPELLLGGPSVFLFPPPKP
**DTLMISR**TPEVTCVV/VDVEDPEVKNFWYDGVEVHNAKTPREEQNYSTRVVSVLTVLHODWLNKKEY
KCKVSNKALPAEIKTSAKQGPPRQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGOPENNYK
TTPPLDSGSGFSFVYSLKTVDKRQWGGNVSFCFSVM
HEALNHYTGKLSSLPSG

SILuMab Light Chain:
QSLAQRLPSGSGGQFSTISCTGTSSDIGGYNFSL
WYQQHGPKAPKLMIYDARKPSGVPDRFSGSKGNN
TASLTIGLQAEDAADYCCSYAGDYTPPGVPFGGTT
KLTQLVQKAPSVTLPPSSELOQANKATLVCLISDF
YPAGAVTVAWKADSPPVKA**GVTTPSK**QSNNKYAA
SSYLSLTPEQWK**SHRSYSCQVTHEGSTVEKTAPTECS**

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3 peptides well detected on our workflow. DTLMISR exhibit a lower CV and will use to normalize experiments
2- Analysis by LCMS

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.

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Blank serum was used as matrix.
+ 80 ng SiLu™MAb
+ 0; 10; 50; 100; 250; 500; 750 and 1000 ug/mL of Avastin

\[ y = 4.3662x + 0.0011 \]
\[ R^2 = 0.9729 \]
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
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4- Example of quantification during patient treatment

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 developed

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

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II- New technology introduction
nSMOL workflow

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....
II- New technology introduction

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

Protein A beads

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II- New technology introduction

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

Iwamoto N. et.al. Analyst, 2014
1- nSMOL Workflow

Step 1
Protein A Immuno-enrichment

Enrichment of antibody from plasma

1. Protein A beads
2. 5 µL of plasma
3. Rotation: 15 min at RT
4. Transfer to filter cup
5. Centrifugation

Waste
30min

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

From Shimadzu

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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

Centrifugation

5 hours at 50°C

From Shimadzu

For research use only. Not for use in diagnostic procedures.
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

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2- Step 3: LCMS method

Previous study:
30min LC run
4 different peptides were monitored and FTFSLDTSK peptide was selected (also on Shimadzu work)

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2- Step 3: LCMS method

- Global profiles were different.
- FTFSLDTSK peptide was still well detected.

ProtA tips

nSMOL
Trypsin digestion gave different yield (site specific), between in-solution and on nano-bead.
Limited digestion was investigated on pure avastin prepared by nSMOL, follow by HRMS analysis.
Remarks

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

nSMOL: limited proteolysis, mainly on Fc region.
Remarks

Comparison to sequence coverage obtain with in-solution trypsin digestion

>"Bevacizumab light chain"
DIQMTQSPSSLSASVGDRVTITCQDSDISNYLWYQQKPGKAPKVLIYFTSSLHSGVPS
RFSQSGSGTDFDLTISSLQPEDFATYYCQQYSTVPWFGQGTVIKEIKRRTVAAPSVEIFPP
SDEQLKSGTASVCLNFTYPREAKVQWKVDNALQGSNSQESVTEQDSK
DSTYSLSSTLT
LSKADYEKHKVYACEVTQHQLSSPVTSFNRCG

>"Bevacizumab heavy chain"
EVQLVESGGGLVQPGGLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY
AADIFRTFSLDTK-STAYLQMNSLRAEDTAVYYCAKYPHYGSHYFDWQGTLVT
VSSASTKGPSVFPLAPSSKSTSGTALGCLVQKYFPEPVTVSWNSGALTSTGVHTFPAVL
QSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDFKEKVEPKSCDKTCTCPCCPAPEL
LGGPSVFLFPPKPDSDMTEKVDSDVEDEKDPEVTFQIVYVVDGVEVHNAKTPFR
QYNSTYRVSVLTVLHQDWNKGYKCKVSNKLPAIEKTISKAKGQPREPQVYTLPS
REEMTKNQSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSGFLYSLTVDK
SRWQQGNVFSCEMVHEALHNHYTQKSLSPGK

MRM peptide

Identified in both digestion

Identified only in solution digestion

➢ In-solution trypsin digestion: as excepted, sequence coverage is higher

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3- Step 1 of nSMOL workflow

Reference Protocol

Enrichment of antibody from plasma

1. Protein A beads
2. 5 uL of plasma
3. Rotation: 15 min at RT
4. Transfer to filter cup
5. Centrifugation

Waste

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

Simplified Protocol

Enrichment of antibody from plasma

1. Protein A beads
2. 5 uL of plasma
3. Rotation: 15 min at RT
4. Transfer to filter cup
5. Centrifugation

Waste

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

All the steps were directly made on filter

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3- Step 1 of nSMOL workflow

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

+ 25% at 1 and 50 ug/mL
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
3- Step 1 of nSMOL workflow

-> 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*
4- Workflow summary

**nSMOL**

- **Step 1**: Protein A Immuno-enrichment
  - Duration: 30 min

- **Step 2**: Protein Digestion
  - Duration: 5 hours

- **Step 3**: LCMS
  - Duration: 10 minutes
  - 144 samples/day

**Protein-A tips Version 2.0**

- **Protein A Immuno-enrichment**: 0.5 Day
- **Trypsin Digestion**: Overnight
- **LCMS**: 10 minutes
  - 144 samples/day

<table>
<thead>
<tr>
<th></th>
<th>nSMOL</th>
<th>ProtA tips (v2.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration (Days)</strong></td>
<td>&lt; 1</td>
<td>&lt; 2</td>
</tr>
<tr>
<td><strong>Cost/point (euro)</strong></td>
<td>58</td>
<td>28</td>
</tr>
</tbody>
</table>

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5- Analytical validation

Similar performances on calibration curves except for the LLOQ
## 5- Analytical validation

Analytical performances:

<table>
<thead>
<tr>
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<th>LLOQ protA</th>
<th>LQC ProtA: 15 ug/mL</th>
<th>MQC ProtA: 100 ug/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>within-run accuracy (%)</td>
<td>115.8</td>
<td>101.4</td>
<td>89.2</td>
</tr>
<tr>
<td>Between-run accuracy (%)</td>
<td>103.9</td>
<td>106.1</td>
<td>86.6</td>
</tr>
<tr>
<td>within-run precision (%)</td>
<td>1.7</td>
<td>1.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Between-run precision (%)</td>
<td>8.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LLOQ nSMOL</th>
<th>LQC nSMOL: 15 ug/mL</th>
<th>MQC nSMOL: 100 ug/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>within-run accuracy (%)</td>
<td>109.5</td>
<td>98.1</td>
<td>102.8</td>
</tr>
<tr>
<td>Between-run accuracy (%)</td>
<td>101.2</td>
<td>92.1</td>
<td>101.1</td>
</tr>
<tr>
<td>within-run precision (%)</td>
<td>1.0</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Between-run precision (%)</td>
<td>9.1</td>
<td>4.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>

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

**Patient n°1**

- y-axis: Bevacizumab (ug/mL)
- x-axis: Cycle
- Legend:
  - Blue circles: nSMOL
  - Orange circles: VEGF Bioplex
  - Grey triangles: ProtA tips
  - Poly. (nSMOL)
  - Poly. (VEGF Bioplex)
  - Poly. (ProtA tips)

**Patient n°2**

- y-axis: Bevacizumab (ug/mL)
- x-axis: Cycle

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Close performances on patient samples.
Kinetic of available Bevacizumab on serum were equivalent
Levels were slightly different depending of the techniques
Conclusions

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### Conclusions

<table>
<thead>
<tr>
<th>Performance Category</th>
<th>Protein-A tips (AssayMap Bravo)</th>
<th>nSMOL</th>
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</thead>
<tbody>
<tr>
<td><strong>Performances on patient samples</strong></td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td><strong>Analytical Performances</strong></td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>1.9565 µg/mL</td>
<td>0.26986 µg/mL</td>
</tr>
<tr>
<td><strong>Number of samples</strong></td>
<td>96 samples in parallel</td>
<td>Limited number of samples (24 by experiment)</td>
</tr>
<tr>
<td><strong>Liquid handling</strong></td>
<td>Automated</td>
<td>Manual</td>
</tr>
<tr>
<td><strong>Workflow</strong></td>
<td>Training needed</td>
<td>user friendly</td>
</tr>
<tr>
<td><strong>Technician</strong></td>
<td>Dedicated and trained people</td>
<td>Open access</td>
</tr>
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<td><strong>Process duration</strong></td>
<td>&lt; 1 day</td>
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<td><strong>Process duration</strong></td>
<td>&lt; 1 day</td>
<td>&lt;2 days</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>28 euros</td>
<td>58 euros</td>
</tr>
</tbody>
</table>
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