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Microflow LC-MS/MS analysis of monoclonal antibody in human plasma at ng/mL level with nSMOL proteolysis.

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Overview

- nSMOL proteolysis (Ref. 1-7) has been reported as the novel technology to standardize the sample preparation workflow and to improve the sensitivity of mass spectrometric assay of therapeutic monoclonal antibodies in human serum or plasma.
- We developed highly sensitive bioanalysis method to achieve low ng/ml sensitivity of Trastuzumab in human plasma with the combination of the nSMOL proteolysis and the newly developed Nexera Mikros system.

Introduction

Mass spectrometric (LC-MS/MS) determination of therapeutic monoclonal antibodies in serum or plasma is increasingly used for pharmacokinetic studies in the preclinical, clinical, and therapeutic phases. One major advantage of this approach over conventional ligand binding assay (LBA) is high specificity for the target antibodies that can be achieved by selecting tryptic peptides derived from the complementarity-determining region (CDR) as the antibody signature peptide and subjecting it to LC-MS/MS quantitation. Moreover, LC-MS/MS approach requires much less assay developmental work than LBA, which completes within days rather than several months (Table 1). Our recent advancement of sample preparation strategy, namely **nano-surface and molecular-orientation limited (nSMOL) proteolysis** (Fig 1), have further simplified the method development process. nSMOL proteolysis yields extremely clean CDR peptide mixture thereby alleviating the need to address interference from biological matrix.

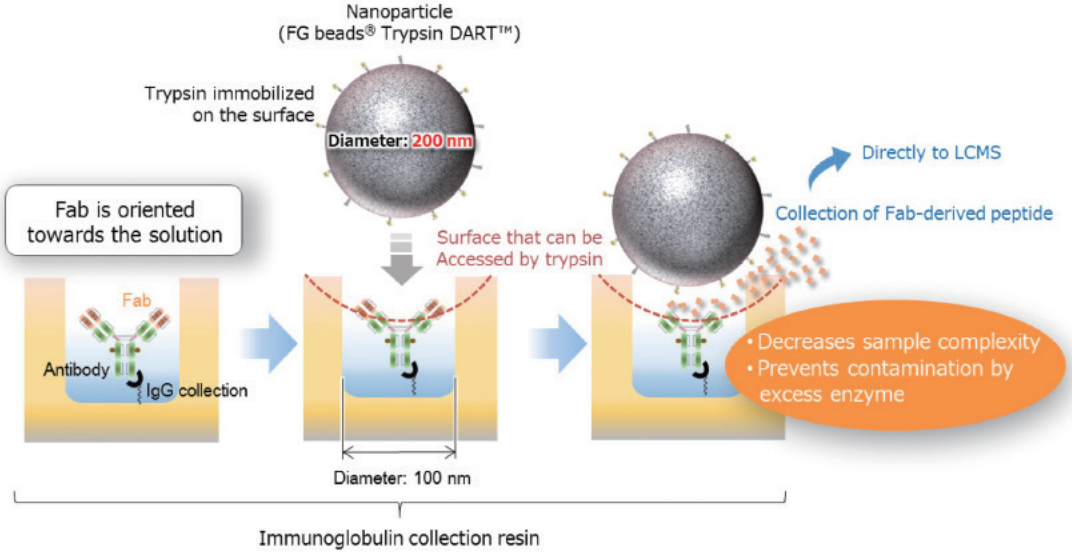

Figure 1. The working principle of Fab-selective reaction of IgG by nSMOL proteolysis.

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Despite various advantages, one drawback of LC-MS/MS assays is that the level of sensitivity depends on the mass spectrometric response (efficiency of ionization and fragmentation) of the signature peptide, which is essentially unpredictable and uncontrollable. For example, recently reported bioanalyses of therapeutic mAb [ref.1-8] showed varying LLOQ levels ranging from 0.06-0.58 µg/mL in plasma. Currently there is risk that a newly-developed assay might not fulfil the sensitivity requirement for pre-clinical trials.
Here we aim to overcome this issue by implementation of a robust microflow LC-MS/MS system to measure signature peptides at increased sensitivity than conventional semi-microflow systems, while maintaining the same level of robustness, analysis turnaround time and ease of system configuration.

Table 1. Comparison of nSMOL+LCMS and LBA

	nSMOL+LCMS	LBA
Ab for Collection/Detection	Not needed	6++ months to develop
Cross-reactivity test	Not needed	Mandatory and tricky
Pre-validation	1 - 3 days	2 - 3 weeks
Full validation	3 - 4 w	3 - 4 w
Sample prep.	3 - 5 h	2 - 4 h
Data features	Highly selective and reliable, wide dynamic range, easy to multiplex, independent of antibodies	Highly dependent on quality of detection Ab.

Development of LCMS bioanalysis in combination with nSMOL proteolysis is much faster, and can dramatically accelerate the total R&D workflow period of biologics by alleviating the bottleneck that typically occur when entering the preclinical and clinical phase.

Methods

Sample and Pretreatment

Pooled human plasma sample was purchased from Kohjin Bio (Saitama, Japan). Trastuzumab was spiked at various concentrations (0, 0.00763, 0.0153, 0.0305, 0.0610, 0.122, 0.0244, 0.488, 0.977, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5 µg/mL) for calibration curve and independently at four concentration set for QC samples. QC set 1 and 2 were prepared and ran on two separate days.
Spiked and blank plasma samples were pretreated after keeping at -80°C for 24 h or longer using the nSMOL™ Antibody BA Kit (Shimadzu Corporation, Japan) in accordance with the instruction manual.

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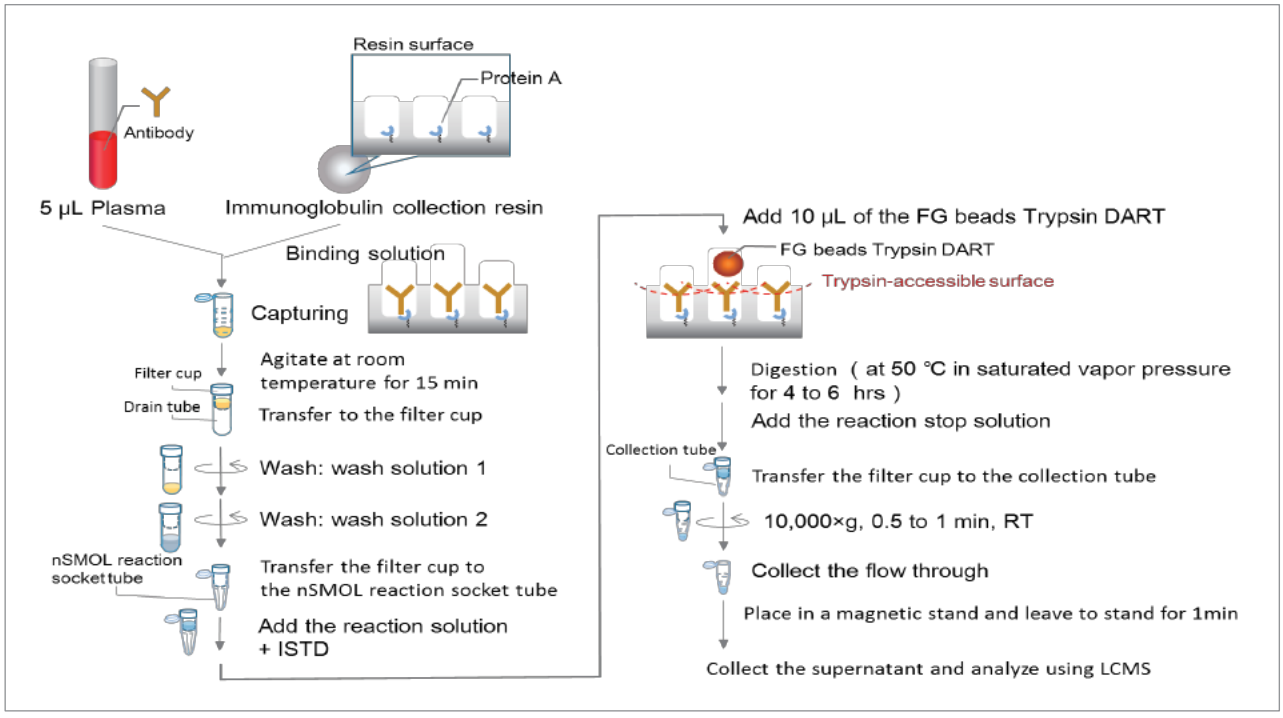

Figure 2 Standard protocols of nSMOL workflow.

System Configuration

The newly developed micro-LC system by Shimadzu Corporation, **Nexera Mikros** (Fig. 2), consists of

(1) **LC-Mikros**, the 'micro to semi-micro flow' pump with 1-500 µL/min range and 800 bar pressure tolerance,

(2) **CTO-Mikros**, the new-design column oven that couples any analytical column directly to the ion source by the UF-Link™ technology (Fig. 3) to minimize post-column void volume,

(1) **Micro ESI-8060**, the camera-equipped and X-Y adjustable ESI ion source for maximum ionization efficiency and usability.

Figure 3. Nexera Mikros system, equipped with additional modular pumps for Trap & Elute

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[LC] Nexera Mikros	
Analytical Column	: Shim-Pack MC C18 (0.175 mm I.D. x 50 mm L.)
Trap column	: CERI L-column2 Micro (0.3 mm I.D. x 50 mm L.)
Oven Temp.	: (Analytical) 50 deg.C, (Trap) 40 deg.C
Solvent A	: 0.1% Formic Acid in water
Solvent B	: 0.1% Formic Acid in Acetonitrile
Gradient	: 0.00-0.50 min 5%B → 4.50 min 22%B → 4.51 min 95%B → 5.50 min 95%B → 5.60 min 5%B → 11.00 min STOP
Analytical flow Rate	: 4 µL/min
Inj. Volume	**: 10 µL**

[MS] LCMS-8060 with Micro ESI-8060	
Ionization	: ESI Positive
DL Temp.	: 250 deg.C
Heat Block Temp.	: 400 deg.C
ESI Temp.	: 100 deg.C
Nebulizer Gas	: 2 L/min.
Drying Gas	: OFF
Heating Gas	: 3 L/min.

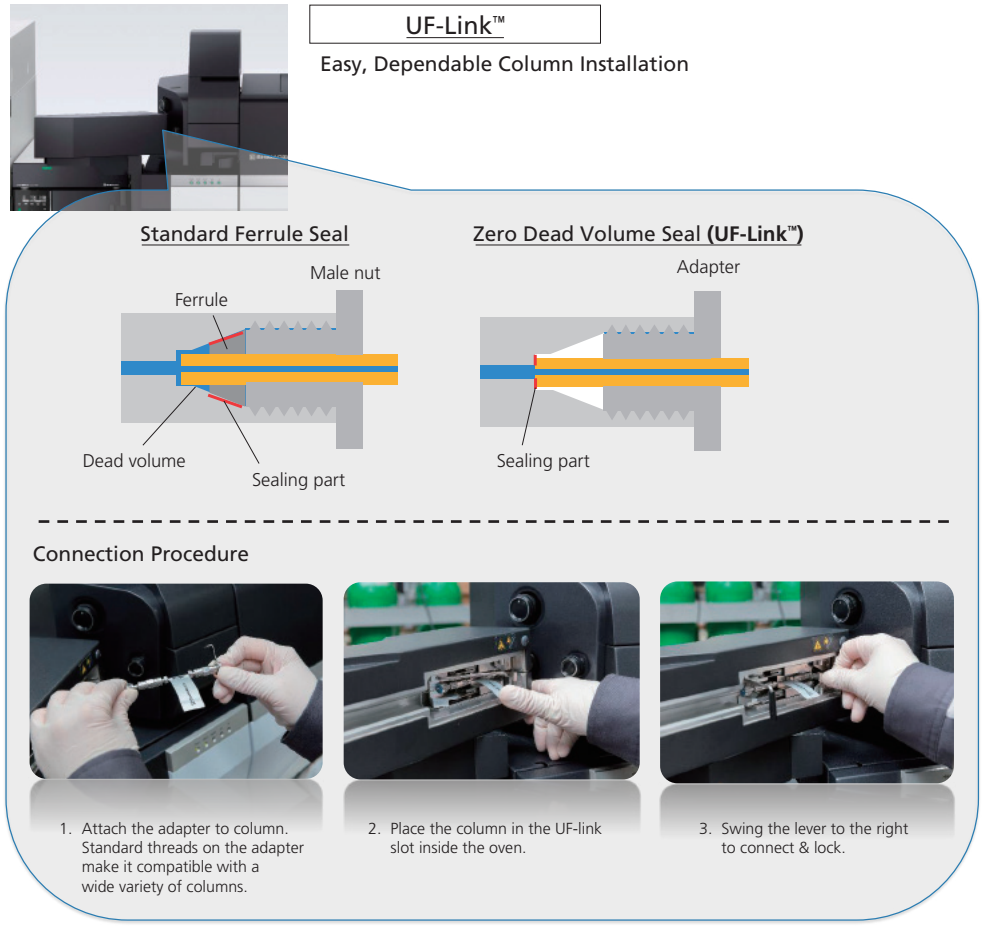

Figure 4. The sealing mechanism of UF-Link and its facile attachment

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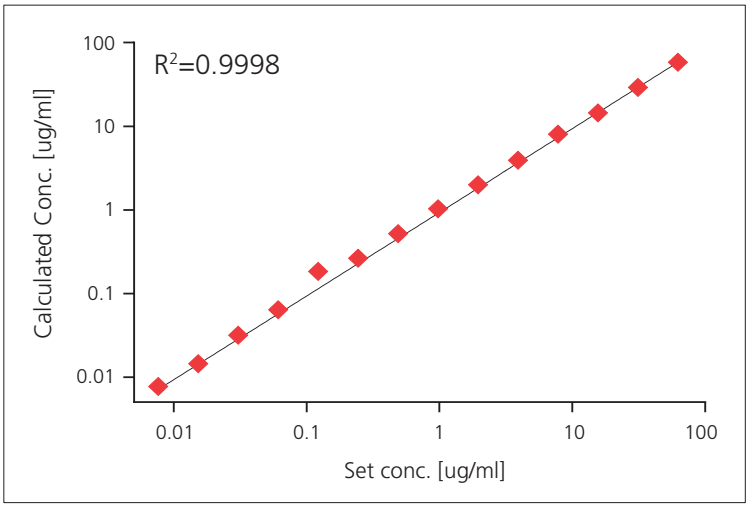
Results

Calibration curve in plasma matrix showed good linear response in the range 7.6 ng/mL to 62.5 µg/mL (Fig 4). Compared to the LLOQ of 0.06 µg/mL as previously reported for Trastuzumab (also using nSMOL proteolysis and LCMS-8060), switching to the Nexera Mikros system contributed to sensitivity improvement by nearly one order of magnitude. Notably, the chromatographic peak shape and elution band was equivalent to UHPLC system with average W0.5h of 3.7 seconds, most likely due to near-zero post-column dead volume achieved by the UF-Link.

Quantitation range in human plasma : 0.00763 to 62.5 µg/mL, Averaged accuracy : 101 %



Peptide	MRM transition	Objectives
IYPTNGYTR	542.8>404.7 (y7++)	Quantifier
	542.8>808.4 (y7+)	Qualifier
	542.8>610.3 (y5+)	Qualifier

Peptide	MRM transition	Objectives
$P_{14}R$ (IS)	512.1>292.3 (b3+)	Quantifier
	512.1>389.3 (b4+)	Qualifier
	512.1>660.4 (b6+)	Qualifier

Figure 5. Calibration curve for Trastuzumab bioanalysis.

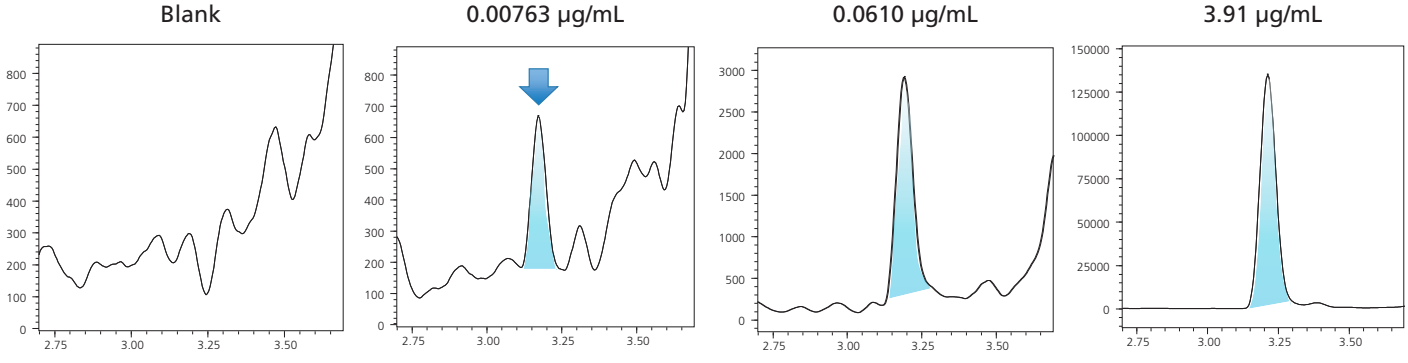


Figure 6. Representative MRM chromatograms

As part of assay validation, intra-day repeatability (%RSD) was evaluated using two sets of QC samples. The results are shown in Table 2. Good repeatability was observed (<20% for LLOQ and otherwise well under 15%) and accuracies fell under 85-115% range, which are the commonly accepted criteria for quantitative adequacy from FDA Bioanalytical Method Validation.

Table 2. Results of assay repeatability evaluation using QC samples.

Set conc. (µg/mL)	QC set 1 (N=5 for each level)			QC set 2 (N=5 for each level)		
	Determined	Accuracy	Repeatability	Determined	Accuracy	Repeatability
0.00763	0.00741	97.1%	5.69%	0.00762	100%	11.3%
0.0229	0.0234	102%	6.68%	0.0232	101%	2.84%
5.86	6.19	106%	2.67%	5.83	99.4%	3.12%
50.0	46.9	94%	6.36%	45.8	91.7%	7.23%

Conclusion

- Combination of Nexera Mikos™r and nSMOL™ Antibody BA Kit achieved single digit ng/mL LLOQ in the bioassay of Trastuzumab in 11 minutes of analysis runtime.
- Enhancement in sensitivity may be attributed to increased ionization efficiency at lower flow rate, while peak shape was maintained by the UF-Link column connection at ion source. The system is also suitable for routine analysis without the use of specialized tubings that typically suffer from clogging.
- Assuming same level of sensitivity enhancement for other signature peptides of therapeutic mAbs, it now became highly probably that a developed LC-MS/MS assay will satisfy the sensitivity required for both preclinical and clinical studies.

Acknowledgement

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Disclaimer

nSMOL™ Antibody BA Kit, Nexera Mikros™ and LCMS-8060 is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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