# Supporting information

# Development and validation of a LC-MS-based quantification assay for new therapeutic antibodies: application to a novel therapy against herpes simplex virus

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## I. Liquid chromatography and mass spectrometry parameters for method development and optimization

The surrogate peptide discovery analyses (micro-flow liquid chromatography – high resolution tandem mass spectrometry (µLC-HR-MS/MS)) were performed on a Waters Acquity M-class µLC system coupled to a Waters Xevo G2-XS Q-TOF mass spectrometer featured with an IonKey/MS™ separation device (Waters). The parameters used for the µLC-HR-MS/MS analyses are detailed in the **Table S1**.
The final choice of HDIT101 surrogate peptides and further workflow optimizations using µLC-MS/MS in multiple reaction mode (MRM) (µLC-MRM/MS) were performed on a Waters Xevo TQ-S mass spectrometer equipped with a Waters Acquity M-class µLC system and an IonKey/MS™ separation device. As for the µLC-HR-MS/MS analyses, the parameters used for the µLC-MRM/MS analyses are detailed in the **Table S1**.

Table S1. µLC-MS/MS parameters used for the method development steps.

| | **µLC-HR-MS/MS** | **µLC-MRM/MS** | | |
|---|---|---|---|---|
| **LC eluent** | A: $H_20$ 0.1% FA<br>B: ACN 0.1% FA | A: $H_20$ 0.1% FA<br>B: ACN 0.1% FA | | |
| **Trapping conditions** | C18, 300 µm x 50 mm, 100 Å, 5 µm<br>30 µL/min, 2% B | C18, 300 µm x 50 mm, 100 Å, 5 µm<br>20 µL/min, 2% B | | |
| **Analytical separation** | CSH C18 iKey, 150 µm x 50 mm, 130 Å, 1.7 µm<br>3 µL/min, 2% B, 60 °C on column<br>2% B for 4 min<br>2 to 8% B in 1 min<br>8 to 40% B in 15 min<br>40 to 85% B in 1 min<br>85% B for 4 min<br>85 to 2% in 0.5 min<br>2% B for 6.5 min | CSH C18 iKey, 150 µm x 50 mm, 130 Å, 1.7 µm<br>3 µL/min, 2% B, 60 °C on column<br>2% B for 0.5 min<br>2 to 52% B in 14.5 min<br>52 to 90% B in 1 min<br>90% B for 1.9 min<br>90 to 2% in 0.1 min<br>2% B for 2 min | | |
| **ESI parameters** | ESI positive mode<br>Capillary voltage: +3 kV, cone voltage: 40 V<br>Source at 110 °C, desolvation at 250 °C.<br>Cone gas flow at 50 L/h, iKey gas at 0.3 bar and desolvation gas at 600 L/h. | ESI positive mode<br>Capillary voltage: +2.7 kV, cone voltage: 50 V<br>Source at 110 °C.<br>Cone gas flow at 10 L/h, desolvation gas at 1.5 bar and nebulizer gas at 3 bar. | | |
| **Acquisition parameters** | Data independent acquisition $MS^e$ mode<br>Collision energy from 15 to 35 V<br>Continuum mode<br>MS: m/z 350-2000, 0.1 sec/scan<br>MS/MS: m/z 100-1500, 1 sec/scan | | MRM mode transitions [m/z] | Collision energy |
| | | HC3 | 1027.17 > 780.65 | 26 V |
| | | | 1027.17 > 879.77 | 28 V |
| | | LC1 | 896.06 > 807.55 | 20 V |
| | | | 896.06 > 982.26 | 22 V |
| | | SIL-HC3 | 1071.57 > 786.76 | 26 V |
| | | | 1071.57 > 885.94 | 26 V |
| | | SIL-LC1 | 899.60 > 814.44 | 22 V |
| | | | 899.60 > 989.27 | 22 V |
| **Data analyses** | Biopharmalynx targeted on HDIT101 sequence<br>MS and MS/MS tolerances: 10 and 20 ppm<br>1 allowed miscleavage. Carbamidomethylation of Cys (fixe), deamidation of Gln and Asn (variable)<br>Acceptance criteria on MS/MS: 5 b/y ions | Targetlynx v. 4.1 | | |

ACN: Acetonitrile; ESI: electrospray ionization; HC: high chain; LC: light chain; µLC-HR-MS/MS: micro-flow liquid chromatography high resolution tandem mass spectrometry; MRM: multiple reaction monitoring; SIL: stable isotopically labeled.

## II. Investigation of LC1 and SIL-LC1 signal instability after MCX µElution.

During analytical method development, a signal instability was observed for the selected surrogate peptide from HIDT101 light chain, LC1, and its stable isotopically labelled internal standard (SIL-IS), SIL-LC1, when the processed samples were cleaned up with MCX µElution (Waters, mixed-mode polymeric cation exchange sorbent, particle size 30 µm, pore size 80 Å, sorbent weight 2 mg) at basic pH using 100 µL of 25% ACN in 2% $NH_4OH$ as eluent. Over a full analytical run (at least 50 samples) and with sample reinjection after 24-h storage in the autosampler (10 °C), a signal modification could be observed with a shift from a three-peak pattern (1 : 0.15 : 1, peak maxima at 8.27, 8.48, and 8.75 min) to a two-peak pattern (0.2 : 1, peak maxima at 8.48 and 8.75 min) as illustrated in **Figure S1A**. Further µElution workflows were then evaluated and a final alternative workflow was established using HLB µElution (Waters, polymeric reversed-phase sorbent, particle size 30 µm, pore size 80 Å, sorbent weight 2 mg) at acidic pH using 100 µL of $H_2O$ 0.1% FA as eluent. With this alternative clean-up workflow, no signal modification was observed over a full analytical run or with sample reinjection after 24-h storage in the autosampler, as illustrated in **Figure S1B**.

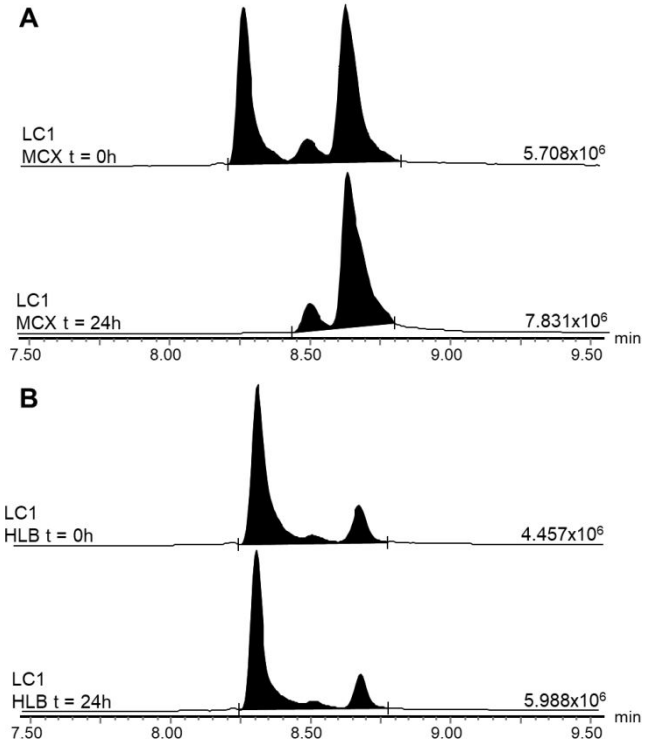


Figure S1. MRM traces of HDIT101 surrogate peptide, LC1, after sample processing with MCX µElution clean-up at basic pH (A) showing a compound instability with signal modification over 24 hours. MRM traces of HDIT101 surrogate peptide, LC1, after sample processing with HLB µElution clean-up at acidic pH (B) showing no signal modification over 24 hours, and thus compound stability. Black areas highlight the peaks attributed to the targeted peptide.

We hypothesized that the signal instability was caused by the basic pH conditions used for the MCX µElution and resulted from deamidation of glutamine (Q) or asparagine (N) amino acids within the targeted peptide, which comprises six of these residues. To prove this assumption, LC1 synthetic peptide was resuspended in digestion buffer in order to mimic buffer conditions used during the complete digestion workflow and was subsequently transferred either to MCX µElution plates and eluted with 100 µL of 25% ACN in 2% $NH_4OH$, pH 10, or to HLB µElution plates and eluted with 100 µL of 50% ACN in 0.1% FA. Samples were then diluted with 100 µL $H_2O$ 0.1% FA and analyzed by micro-flow liquid chromatography-tandem mass spectrometry (µLC-MS/MS) in data-independent acquisition mode. MCX and HLB samples were analyzed directly after processing (t = 0 h), and then again after storage for 1, 2, 3, 4, and 24 h in the autosampler (10 °C) to determine at which time point the modification occurs. Acquisition data were subsequently interpreted using Biopharmalynx software v. 1.3.5 (Waters) by targeting HDIT101 amino acid sequence in-silico digested with trypsin. MS and MS/MS tolerances were set to 10 and 20 ppm, respectively, deamidation of Q and N residues was set as variable modification and matched $MS^2$ spectra were validated only when more than 5 b/y ions could be clearly identified. The corresponding results are summarized in **Table S2.** The validated spectra of LC1 after MCX µElution showed the appearance of one asparagine deamidation (ninth amino acid of LC1 sequence) between 4 and 24 h after sample processing and storage in the autosampler at 10 °C. In LC1 samples cleaned-up with HLB µElution, no deamidation was detected after 24 h.

Table S2. Result summary for the deamination investigation on LC1 peptide.

| Time | MCX µElution | | | | HLB µElution | | | |
|---|---|---|---|---|---|---|---|---|
| | **Matched[1] LC1 MS/MS spectra** | **Validated[2] LC1 MS/MS spectra** | **Unmodified matched spectra[3]** | **Deamidated matched spectra[3]** | **Matched[1] LC1 MS/MS spectra** | **Validated[2] LC1 MS/MS spectra** | **Unmodified matched spectra[3]** | **Deamidated matched spectra[3]** |
| 0 h | 3 | 1 | 31 b/y ions | n.d. | 5 | 2 | 28 b/y ions | n.d. |
| 1 h | 8 | 1 | 33 b/y ions | 1 b/y ion | 6 | 2 | 33 b/y ions | 2 b/y ions |
| 2 h | 5 | 1 | 24 b/y ions | n.d. | 4 | 2 | 32 b/y ions | n.d. |
| 3 h | 4 | 1 | 21 b/y ions | 2 b/y ions | 8 | 2 | 25 b/y ions | n.d. |
| 4 h | 6 | 1 | 28 b/y ions | 1 b/y ion | 8 | 2 | 38 b/y ions | n.d. |
| 24 h | 3 | 1 | n.d. | 32 b/y ions | 9 | 2 | 31 b/y ions | 1 b/y ion |

[1]An experimental MS/MS spectrum is defined as matched if the software could associate it with a theoretical spectrum of one of the *in silico* HDIT101 peptides.

[2]A matched MS/MS spectrum must have ≥ 5 b/y ions to be validated.

[3]Mean number of detected b/y ions in validated spectra, or highest number of detected b/y ions in matched spectra if no spectrum could be validated.

MS/MS: tandem mass spectrometry; n.d.: not detected.

## III. HDIT101 quantification final calibration range.

Before method validation and analysis of clinical samples, the dynamic range and LOQ of the method were confirmed using further analyses of calibration standards processed in plasma at ten non-zero calibration levels (10, 20, 40, 75, 150, 315, 625, 1250, 2500, and 5000 µg/mL). These analyses revealed response linearity from 20 to 5000 µg/mL (**Supporting information part III**, **Figure S2**). LLOQ was set to 20 µg/mL, which was the lowest concentration level fulfilling the < 20% CV precision limit between duplicates, and the ULOQ was set to 5000 µg/mL to avoid saturation of the IP resin. Response linearity in this concentration range was proven with an $R^2$ coefficient of 0.998 and 100% of back calculated concentrations within the ± 15% bias accuracy limits or ± 20% bias at the LLOQ (**Figure S2** and **Table S3**).

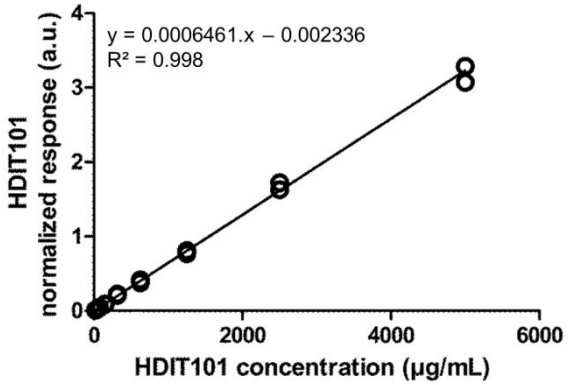


Figure S2. Calibration curve obtained on final calibration range from 20 to 5000 µg/mL for HDIT101 quantification assay.

Table S3. Summary of calibration results for the determination of the dynamic range of HDIT101 quantification assay.

| HDIT101 concentration (µg/mL) | Mean accuracy (%) | Precision (% CV) |
|---|---|---|
| 10 | -7.4 | 28.3 |
| 20 | -6.0 | 12.9 |
| 40 | -9.4 | 9.4 |
| 75 | -4.1 | 7.8 |
| 150 | -6.0 | 1.6 |
| 315 | 7.3 | 5.2 |
| 625 | -1.5 | 6.6 |
| 1250 | -2.2 | 3.6 |
| 2500 | 3.9 | 4.0 |
| 5000 | -1.4 | 4.8 |