# In-Sample Calibration Curve (ISCC) Using Multiple Isotopologue Reaction Monitoring (MIRM) of a Stable Isotopically Labeled Analyte for Instant LC-MS/MS Bioanalysis and Quantitative Proteomics

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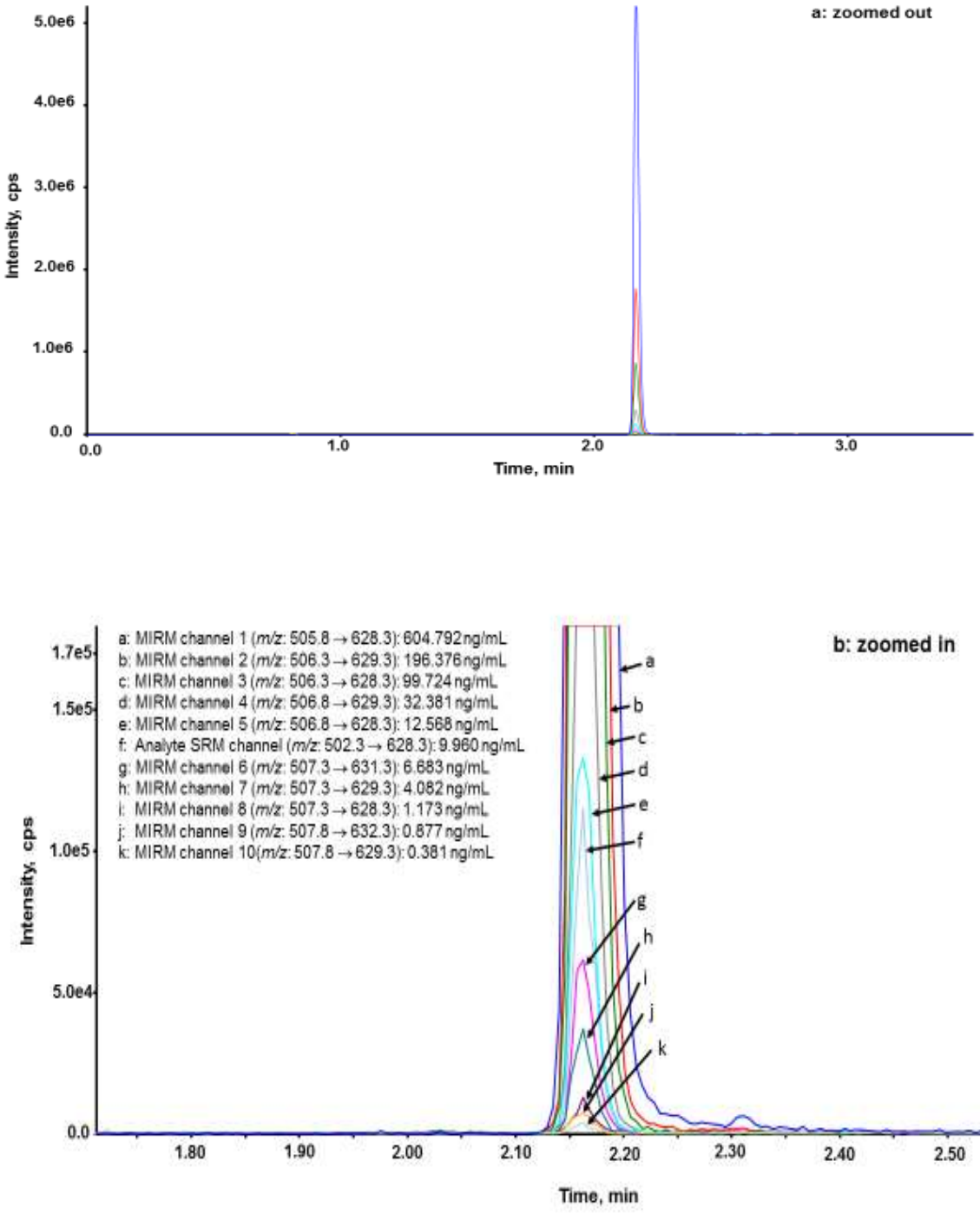
## Supporting Information A:

### Table SI-A1, Performances of ISCCs with $1/x^2$ weighted linear regression for the first three injections

| ISCC nominal concentration (ng/mL) | 1st injection | | | 2nd injection | | | 3rd injection | | |
|---|---|---|---|---|---|---|---|---|---|
| | Peak area | Measured concentration | %Dev | Peak area | Measured concentration | %Dev | Peak area | Measured concentration | %Dev |
| 604.792 | 12728343 | 593.664 | -1.8 | 12591027 | 611.535 | 1.1 | 13336529 | 609.439 | 0.8 |
| 196.376 | 4233869 | 197.466 | 0.6 | 4067840 | 197.559 | 0.6 | 4262965 | 194.766 | -0.8 |
| 99.724 | 2127881 | 99.239 | -0.5 | 1978622 | 96.084 | -3.6 | 2235632 | 102.115 | 2.4 |
| 32.381 | 696185 | 32.463 | 0.3 | 648848 | 31.497 | -2.7 | 720287 | 32.862 | 1.5 |
| 12.568 | 269720 | 12.571 | 0.0 | 246124 | 11.936 | -5.0 | 272520 | 12.398 | -1.4 |
| 6.683 | 145766 | 6.790 | 1.6 | 147531 | 7.147 | 6.9 | 149035 | 6.755 | 1.1 |
| 4.082 | 91382 | 4.253 | 4.2 | 85126 | 4.116 | 0.8 | 85889 | 3.869 | -5.2 |
| 1.173 | 25357 | 1.174 | 0.1 | 25394 | 1.215 | 3.6 | 29138 | 1.275 | 8.7 |
| 0.877 | 17703 | 0.817 | -6.9 | 18356 | 0.873 | -0.4 | 18833 | 0.805 | -8.3 |
| 0.381 | 8559 | 0.390 | 2.5 | 8122 | 0.376 | -1.3 | 9666 | 0.386 | 1.2 |

**Figure SI-A1, Representative chromatograms for all ten MIRM channels used for ISCC and one SRM channel for analyte**



**Supporting Information B:**

**Table SI-B1, SIL analyte isotope concentrations and analyte concentration equivalents in selected MIRM channels for MIRM-ISCC-LC-MS/MS quantitative determination of LAAFPEDR, LQDAGVYR and VIYPAVEGR in trypsin digested colon tissue homogenates**

| | LAAFPED[Arg($^{13}C_6$, $^{15}N_4$)]$^{++}$→y4 ion$^+$ | | | LQDAG[Val($^{13}C_5$, $^{15}N$)]YR$^{++}$→y6 ion$^+$ | | | | V[Ile($^{13}C_6$, $^{15}N$)]YPAVEGR$^{++}$→y6 ion$^+$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MIRM channel (m/z) | Calculated theoretical relative isotopic abundance (%) | ISCC SIL-LAAFPEDR isotope concentration (ng/mL) | ISCC LAAFPEDR concentration equivalent (ng/mL)[a] | MIRM channel (m/z) | Calculated theoretical relative isotopic abundance (%) | ISCC SIL-LQDAGVYR isotope concentration (ng/mL) | ISCC LQDAGVYR concentration equivalent (ng/mL)[b] | MIRM channel (m/z) | Calculated theoretical relative isotopic abundance (%) | ISCC SIL-VIYPAVEGR isotope concentration (ng/mL) | ISCC VIYPAVEGR concentration equivalent (ng/mL)[c] |
| 464.7→526.2 | 100 | 100 | 98.92 | 464.2→686.4 | 100 | 100 | 99.35 | 505.8→628.3 | 100 | 100 | 99.31 |
| 465.2→526.2 | 24.80 | 24.8 | 24.53 | 464.7→687.4 | 29.99 | 30.0 | 29.81 | 506.3→629.3 | 32.47 | 32.5 | 32.27 |
| 465.7→526.2 | 3.75 | 3.75 | 3.71 | 465.2→688.4 | 6.63 | 6.63 | 6.59 | 506.8→630.3 | 6.92 | 6.92 | 6.87 |
| 466.2→528.2 | 0.79 | 0.79 | 0.78 | 465.7→689.4 | 1.01 | 1.01 | 1.00 | 507.3→630.3 | 1.14 | 1.14 | 1.13 |
| 466.2→526.2 | 0.42 | 0.42 | 0.42 | 465.7→687.4 | 0.43 | 0.43 | 0.43 | 507.8→632.3 | 0.14 | 0.14 | 0.14 |
| 459.7→516.2 | SRM channel for analyte, LAAFPEDR | | | 461.2→680.4 | SRM channel for analyte, LQDAGVYR | | | 502.3→628.3 | SRM channel for analyte, VIYPAVEGR | | |

[a]: ISCC LAAFPEDR concentration equivalent = ISCC SIL-LAAFPEDR isotope concentration * (LAAFPEDR molecular weight of 918 / SIL-LAAFPEDR molecular weight of 928)

[b]: ISCC LQDAGVYR concentration equivalent = ISCC SIL-LQDAGVYR isotope concentration * (LQDAGVYR molecular weight of 921 / SIL-LQDAGVYR molecular weight of 927)

[c]: ISCC VIYPAVEGR concentration equivalent = ISCC SIL-VIYPAVEGR isotope concentration * (VIYPAVEGR molecular weight of 1003 / SIL-VIYPAVEGR molecular weight of 1010)

Note: The percentage differences for the measured relative isotopic abundances (%) from the calculated theoretical isotopic abundances are within 3.7%, 2.9% and 13.5% for LAAFPED[Arg($^{13}C_6$, $^{15}N_4$)], LQDAG[Val($^{13}C_5$, $^{15}N$)]YR (data not shown) and V[Ile($^{13}C_6$, $^{15}N$)]YPAVEGR (Table 2), respectively.

**Table SI-B2, Quantitative determination of surrogate peptides LAAFPEDR, LQDAGVYR and VIYPAVEGR in trypsin digested colon tissue homogenates using MIRM-ISCC-LC-MS/MS approach**

| Analyte | LAAFPEDR | | LQDAGVYR | | VIYPAVEGR | |
|---|---|---|---|---|---|---|
| Nominal concentration (ng/mL) | Measured concentration (ng/mL) | %Dev | Measured concentration (ng/mL) | %Dev | Measured concentration (ng/mL) | %Dev |
| Digested tissue homogenate blank | <0.42 | | <0.43 | | <0.14 | |
| | <0.42 | | <0.43 | | <0.14 | |
| | <0.42 | | <0.43 | | <0.14 | |
| 1.00 | 1.03 | 3.0 | 0.95 | -5.0 | 1.17 | 17.0 |
| | 1.06 | 6.0 | 1.02 | 2.0 | 1.15 | 15.0 |
| | 1.03 | 3.0 | 0.99 | -1.0 | 1.20 | 20.0 |
| 10.0 | 9.75 | -2.5 | 9.59 | -4.1 | 10.92 | 9.2 |
| | 10.09 | 0.9 | 10.03 | 0.3 | 10.92 | 9.2 |
| | 9.84 | -1.6 | 9.86 | -1.4 | 10.92 | 9.2 |
| 50.0 | 49.86 | -0.3 | 48.48 | -3.0 | 53.33 | 6.7 |
| | 47.78 | -4.4 | 48.88 | -2.2 | 52.24 | 4.5 |
| | 49.76 | -0.5 | 48.29 | -3.4 | 52.83 | 5.7 |

**Supporting Information C:**

***MIRM-ISCC-LC-MS/MS Quantitation of Small Molecule Drug Daclatasvir in Human and Rat Plasma***

Sometimes it is not as straightforward as peptides to figure out the fragmentations (daughter ion and neutral loss) for small molecules for the calculation of the isotopic abundances in its MIRM channels. However, after the daughter ion and neutral loss are determined, the same MIRM-ISCC-LC-MS/MS work flow can also be used for the measurement of small molecule analytes, including small molecule drugs and biomarkers. Here we show an example of instant quantitative analysis of a small molecule drug, daclatasvir, in human and rat plasma using MIRM-ISCC-LC-MS/MS approach. The molecular structure, the labeling positions for the SIL-daclatasvir, and the selected parent ion and daughter ion were described in our previous paper.[1]

A volume of 100 μL of human and rat plasma samples containing 10, 100, 500 and 1,000 ng/mL of daclatasvir were mixed with 20 μL of 5,000 ng/mL of SIL $^{13}C_2{}^{15}N_4$-daclatasvir in human, and rat plasma, respectively. The equivalent concentration of $^{13}C_2{}^{15}N_4$-daclatasvir in human and rat plasma samples was 1,000 ng/mL ([20 μLx5,000 ng/mL]/100 μL=1,000 ng/mL). The samples were extracted with liquid-liquid extraction using MTBE[2] and injected for MIRM-ISCC-LC-MS/MS analysis. The MIRM channels and their concentration equivalents used in ISCC for quantitation of daclatasvir are listed in Table SI-C1. All ISCCs were constructed using a weighted ($1/x^2$) least squares linear regression. The measured concentrations for all calibration points are well within the acceptance criteria for regulated LC-MS/MS bioanalysis (data not shown). Table SI-C2 shows the measured results for daclatasvir in human and rat plasma, indicating the MIRM-ISCC-LC-MS/MS measurement of daclatasvir was accurate.

**Table SI-C1, SIL daclatasvir isotope concentrations and daclatasvir concentration equivalents in selected MIRM channels for MIRM-ISCC-LC-MS/MS quantitative determination of daclatasvir in human and rat plasma**

| MIRM channel (m/z) $^{13}C_2{}^{15}N_4C_{38}H_{51}N_4O_6^+ \rightarrow$ $^{13}C_2{}^{15}N_4C_{31}H_{37}N_2O_3^+$ | Calculated theoretical relative isotopic abundance (%) | ISCC SIL-daclatasvir isotope concentration (ng/mL) | ISCC daclatasvir concentration equivalent (ng/mL)[a] |
|---|---|---|---|
| 745.4→571.3 | 100 | 1,000 | 991.9 |
| 746.4→572.3 | 34.96 | 349.6 | 346.8 |
| 746.4→571.3 | 8.64 | 86.4 | 85.7 |
| 747.4→572.3 | 3.02 | 30.2 | 30.0 |
| 747.4→571.3 | 0.93 | 9.30 | 9.23 |
| 748.4→573.3 | 0.56 | 5.60 | 5.55 |
| 739.4→565.3 | SRM channel for analyte, daclatasvir | | |

[a]: ISCC daclatasvir concentration equivalent = ISCC SIL-daclatasvir isotope concentration * (daclatasvir molecular weight of 739 / SIL-daclatasvir molecular weight of 745)

Note: The percentage difference for the measured relative isotopic abundance (%) from the calculated theoretical isotopic abundance is within 7.0% (data not shown).

**Table SI-C2, Quantitative determination of daclatasvir in human and rat plasma using MIRM-ISCC-LC-MS/MS approach**

| | Human plasma | | Rat plasma | |
|---|---|---|---|---|
| Nominal concentration (ng/mL) | Measured concentration (ng/mL) | %Dev | Measured concentration (ng/mL) | %Dev |
| 10.00 | 10.81 | 8.1 | 12.72 | 27.2* |
| | 11.69 | 16.9 | 10.72 | 7.2 |
| 100.0 | 107.7 | 7.7 | 110.2 | 10.2 |
| | 110.1 | 10.1 | 111.8 | 11.8 |
| 500.0 | 523.9 | 4.8 | 574.2 | 14.8 |
| | 550.7 | 10.1 | 567.4 | 13.5 |
| 1,000 | 1066 | 6.6 | 1045 | 4.5 |
| | 1087 | 8.7 | 1077 | 7.7 |

* Possible sample preparation error.

**Supporting Information D:**

***Step-by-Step Workflow for MIRM-ISCC-LC-MS/MS Methodology***

The MIRM-ISCC-LC-MS/MS step-by-step work flow is summarized below:

1. Identify the daughter ion and neutral loss for the SIL analyte
2. Obtain isotopic distributions for the daughter ion and neutral loss using an online isotopic distribution calculator.[3]
3. Calculate isotopic abundances in MIRM channels of the SIL analyte as described in the Theory and MIRM-ISCC-LC-MS/MS Methodology section.[1]
4. The assay ULOQ of (Mass/Volume)*($M_{analyte}/M_{SIL\text{-}analyte}$) (ng/mL) is defined by the total amount of the SIL analyte (Mass, ng) spiked into the sample (Volume, mL), adjusted by molecular weights for the analyte ($M_{analyte}$) and SIL analyte ($M_{SIL\text{-}analyte}$), respectively. The assay lower limit of quantitation (LLOQ) of $I_a$*ULOQ (ng/mL) is defined by the lowest isotopic abundance ($I_a$) among the selected MIRM channels and the assay ULOQ. This assay LLOQ needs to be within the instrument detection limit.
5. Spike appropriate amount of the SIL analyte into a neat solution and multiple lots of extracted matrix so that the MS/MS response in the most abundant MIRM channel is within the high limit of the instrument linear range.
6. Measure MS/MS peak areas in MIRM channels for the prepared samples.
7. Select MIRM channels to be used for the ISCC by comparing the measured results in (5) with the calculated results in (3). Please also refer to the example 1 in the paper for the considerations in selecting MIRM channels for ISCCs.
8. Spike a known amount of SIL analyte into samples to achieve the desired assay ULOQ and LLOQ.

9. Sample extraction. In some cases, the SIL analyte is spiked after sample extraction.
10. LC-MS/MS analysis by monitoring the selected MIRM channels for the SIL analyte and the SRM channel for the analyte.
11. ISCC regressions and concentration calculations for each sample.

**Supporting Information E:**

***Impact of Labeling Impurity on MIRM-ISCC-LC-MS/MS Assay Accuracy***

The SIL daclatasvir (Supporting Information C) is used to estimate the impact of labeling impurity on the MIRM-ISCC-LC-MS/MS assay accuracy. It is assumed that the labeling impurity is five-position labeled daclatasvir, while six-position labeled daclatasvir is the SIL analyte. Table SI-E1 shows that, with 3%, 5% and 10% of the 5-position labeled impurity, the calculated maximum contributions from the 5-position labeled impurity to the SIL analyte's MIRM channels are 1.1%, 1.9% and 3.9%, respectively. The calculated results indicate that the impact from the labeling impurity on the MIRM-ISCC-LC-MS/MS assay accuracy could be ignored if the impurity is less than 5%. It should be pointed out that the impact of the labeling impurity should be evaluated for each individual compound, especially when the labeling impurity is more than 5%.

**Table SI-E1, Impact of labeling impurity on MIRM-ISCC-LC-MS/MS assay accuracy**

| No | MIRM channels (*m/z*) | Calculated theoretical relative isotopic abundance (M%) of the SIL analyte (6-position labeled daclatasvir) | Calculated theoretical relative isotopic abundance (N%) of the impurity (5 position labeled daclatasvir) in the SIL analyte's MIRM channels | Contributions (C%)* from the 5-position labeled impurity (I%) to the SIL analyte's MIRM channels | | |
|---|---|---|---|---|---|---|
| | | | | 3% impurity | 5% impurity | 10% impurity |
| 1 | 745.4 → 571.3 | 100.0000 | 35.325 | 1.06 | 1.86 | 3.93 |
| 2 | 746.4 → 571.3 | 8.6379 | 3.0513 | 0.09 | 1.86 | 3.92 |
| 3 | 746.4 → 572.3 | 34.9637 | 6.6573 | 0.20 | 1.00 | 2.12 |
| 4 | 747.4 → 571.3 | 0.9322 | 0.3293 | 0.01 | 1.86 | 3.93 |
| 5 | 747.4 → 572.3 | 3.0200 | 0.5751 | 0.02 | 1.00 | 2.12 |
| 6 | 747.4 → 573.3 | 6.5310 | 0.8834 | 0.03 | 0.71 | 1.50 |
| 7 | 748.4 → 571.3 | 0.0590 | 0.0208 | 0.00 | 1.86 | 3.92 |
| 8 | 748.4 → 572.3 | 0.3259 | 0.0621 | 0.00 | 1.00 | 2.12 |
| 9 | 748.4 → 573.3 | 0.5641 | 0.0763 | 0.00 | 0.71 | 1.50 |
| 10 | 748.4 → 574.3 | 0.8598 | 0.0915 | 0.00 | 0.56 | 1.18 |
| 11 | 749.4 → 571.3 | 0.0031 | 0.0011 | 0.00 | 1.87 | 3.94 |
| 12 | 749.4 → 572.3 | 0.0206 | 0.0039 | 0.00 | 1.00 | 2.10 |
| 13 | 749.4 → 573.3 | 0.0609 | 0.0082 | 0.00 | 0.71 | 1.50 |
| 14 | 749.4 → 574.3 | 0.0743 | 0.0079 | 0.00 | 0.56 | 1.18 |
| 15 | 749.4 → 575.3 | 0.0883 | 0.0077 | 0.00 | 0.46 | 0.97 |

***** C% = 100*(N*I)/[M*(100-I)]

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