Supporting Information

1. Synthesis of non-isotope labeled DiART reagent

![Scheme 1](image)

**Compound 2**

The synthesis of compound 2 was previously reported\(^1\). It was synthesized from compound 1 here by using a different method.

Compound 1 (528 mg, 1.5 mmol)\(^2\), Boc-Leu-NHS (493 mg, 1.5 mmol)\(^3\), and TEA (700 µL, 5.0 mmol) were added into 20 mL DMF. After the mixture was stirred for 5 h at room temperature, the solvent was removed in vacuo. The residue was dissolved in 60 mL DCM, washed with 10% K\(_2\)CO\(_3\) (2x60 mL) and 1M KHSO\(_4\) (2x60 mL), then dried over anhydrous Na\(_2\)SO\(_4\). After the solvent was removed, the residue was purified by silica gel chromatography to give compound 2 (447 mg, 76% yield) as colorless oil.

\(^1\)H-NMR (CDCl\(_3\), 500 MHz) \(\delta = 7.35-7.23\) (m, 5H), 5.11 (s, 2H), 4.20 (m, 1H), 3.50 (t, \(J = 6.1\) Hz, 2H), 2.56 (t, \(J = 6.0\) Hz, 2H), 1.70-1.64 (m, 2H), 1.53-1.46 (m, 1H), 1.41 (s, 9H), 0.92 (d, \(J = 5.5\) Hz, 3H), 0.89 (d, \(J = 5.6\) Hz, 3H). MS (M+H)\(^+\) = 393.31, Cal. (M+H)\(^+\) = 393.23.

**Compound 3**

Compound 2 (392 mg, 1.0 mmol) was stirred in 10 mL deprotection cocktail (47.5% TFA, 2.5% H\(_2\)O, 50% DCM) for 1 hr, then the solvent was removed in vacuo and the residue was dried in a lyophilizer. To the reaction flask, 30 mL CH\(_3\)CN and 1.0 mL HOAc were added, followed by addition of aqueous HCHO (40%, 400 µL). After the solution was stirred for 5 min, NaCNBH\(_3\) (124mg, 2.0 mmol) was added. The reaction mixture was stirred for another 4 h at room temperature. After the solvent was removed, the residue was dissolved in 80 mL ethyl acetate, washed with 10% K\(_2\)CO\(_3\) (2x60 mL), and dried over anhydrous Na\(_2\)SO\(_4\). After the organic solvent was removed, the crude product was dissolved in 20 mL methanol, and then 30 mg Pd/C catalyst was added to the solution. The hydrogenation was carried out with a 200 mL hydrogen balloon. After the reaction mixture was stirred overnight, the solid catalyst was filtered out and the filtrate was evaporated. The residue was purified by Dowex 50 (H\(^+\) form) chromatography to give compound 3 (163 mg, 71% yield) as white solid.

\(^1\)H-NMR (D\(_2\)O, 500 MHz) \(\delta = 3.56\) (m, 1H), 3.28 (t, \(J = 6.5\) Hz, 2H), 2.67 (S, 6H), 2.22 (t, \(J = 6.3\) Hz, 2H), 1.64-1.51 (m, 2H), 1.35-1.24 (m, 1H), 0.72 (d, \(J = 6.7\) Hz, 3H), 0.70
(d, J = 6.7 Hz, 3H); $^{13}$C-NMR (D$_2$O, 500 MHz) $\delta$ = 177.0, 167.9, 67.7, 41.4, 36.9, 36.0, 33.1, 25.1, 22.8, 20.4; MS (M+H)$^+$ = 231.10, Cal. (M+H)$^+$ = 231.16

### Compound 4

Compound 3 (115 mg, 0.50 mmol) was added to the mixture of NHS (59 mg, 0.51 mmol) and DCC (113 mg, 0.55 mmol) in 10 mL DCM. The reaction mixture was stirred for 2 days at room temperature and insoluble solid was filtered out. After the solvent was evaporated, the residue was recrystallized from diisopropyl ether to afford compound 4 (106 mg, 65% yield) as white solid.

$^1$H-NMR (CDCl$_3$, 500 MHz) $\delta$ = 3.61 (m, 1H), 3.27 (t, J = 6.5 Hz, 2H), 2.82 (t, J = 6.3 Hz, 2H), 2.66 (S, 6H), 2.61 (S, 4H), 1.74-1.61 (m, 2H), 1.43-1.37 (m, 1H), 0.94 (d, J = 6.7 Hz, 3H), 0.92 (d, J = 6.7 Hz, 3H). $^{13}$C-NMR (CDCl$_3$, 500 MHz) $\delta$ = 174.0, 169.1, 168.0, 66.8, 41.8, 36.4, 34.8, 31.4, 25.6, 25.0, 23.5, 21.7. MS (M+H)$^+$ = 328.11, Cal. (M+H)$^+$ = 328.18.

### Table 1. Isotope-labeled starting materials for DiART reagents

<table>
<thead>
<tr>
<th></th>
<th>DiART-114</th>
<th>DiART-115</th>
<th>DiART-116</th>
<th>DiART-117</th>
<th>DiART-118</th>
<th>DiART-119</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-OH</td>
<td>$^{13}$C</td>
<td>$^{15}$N</td>
<td>$^{13}$C</td>
<td>$^{15}$N</td>
<td>$^{13}$C</td>
<td>$^{15}$N</td>
</tr>
<tr>
<td>HCHO</td>
<td>unlabeled</td>
<td>unlabeled</td>
<td>unlabeled</td>
<td>unlabeled</td>
<td>DCDO</td>
<td>DCDO</td>
</tr>
<tr>
<td>NaCNBH$_3$</td>
<td>unlabeled</td>
<td>unlabeled</td>
<td>NaCNBD$_3$</td>
<td>NaCNBD$_3$</td>
<td>unlabeled</td>
<td>unlabeled</td>
</tr>
<tr>
<td>$\beta$-Ala-OH</td>
<td>$^{13}$C</td>
<td>$^{15}$N</td>
<td>$^{13}$C</td>
<td>$^{15}$N</td>
<td>$^{13}$C</td>
<td>$^{15}$N</td>
</tr>
</tbody>
</table>

1-$^{13}$C and $^{15}$N labeled leucine were purchased from Cambridge Isotope Laboratory, Inc. DCDO and NaCNBD$_3$ were purchased from Sigma. D$_4$-$\beta$-alanine was purchased from C/D/N Isotopes Inc. D$_2$-$\beta$-alanine was synthesized in house$^4$.

### 2. HPLC of DiART-labeled phenylalanine

Six different amount of phenylalanine were reacted with each excess DiART reagent in a mixture (NaHCO$_3$, 50 mM, and 1,4-dioxane, 50%), respectively, mixed, and then purified with C$_{18}$-HPLC (Buffer A: water, 0.1% TFA; Buffer B: acetonitrile, 0.1% TFA; gradient: 0-6 mins 10% B, 6-8 mins 10% to 20% B, 8-48 mins 20% B to 60% B; flow rate: 1 mL/min) (see Figure 1 in this supporting information). The purified product was re-injected into HPLC using the same program. A single broad peak of the product was observed. Three time fractions of this peak were collected and analyzed with MALDI-MS/MS (see Figure 2a in paper).

### 3. Protein quantitation with DiART reagents and Mascot software

3.1 Detailed procedure

BSA (20 µg), bovine catalase (20 µg), and chicken ovalbumin (10 µg) were dissolved in 100 µL denaturing/reducing solution (8 M urea, 50 mM Sodium borate buffer, pH = 8.3, 5 mM TCEP) and incubated at 37 °C for 30 min. Then, 20 mM 2-bromoacetamide was added to alkylate free cysteine residues. The proteins were
precipitated with acetone, dissolved again in 100 μL buffer (200 mM Sodium borate buffer, pH = 8.3, 0.8 M urea), and then digested with trypsin (10 μg) at 37 °C overnight. Six fractions of samples (10 μL) were mixed with 20 μL DiART reagents (2 mg/mL in ethanol), respectively and the reaction was incubated at room temperature for 4 h. All of six samples were then mixed together, dried in a SpeedVac, then dissolved again in a SCX (strong ion exchange) loading buffer (10 mM KH₂PO₄, pH = 3.0, 25% acetonitrile). This sample was loaded onto a SCX column, washed, and eluted with 500 μL elution buffer (10 mM KH₂PO₄, pH = 3.0, 400 mM KCl, 25% acetonitrile). The elution was dried in SpeedVac and dissolved in 100 μL 5% acetonitrile. 4 μL of labeled peptide sample was injected into a capillary reverse phase HPLC (gradient, 5% acetonitrile, 0.1% TFA to 50% acetonitrile, 0.1% TFA in 60 min; column, Agilent Zorbax C18, 5 μm, 150×0.5 mm; flow rate, 15 μL/min) and each fraction was analyzed with ABI-4700 MALDI-MS/MS.

3.2. Isotope purity of DiART reagents

The isotope purity of DiART reagents was determined as described previously⁵. These numbers were included in a Mascot server configuration file in order to get correct quantitation.

Table-2. Isotope purity

<table>
<thead>
<tr>
<th>Δ(M/Z)</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiART-114</td>
<td>0.000</td>
<td>0.000</td>
<td>0.925</td>
<td>0.075</td>
<td>0.000</td>
</tr>
<tr>
<td>DiART-115</td>
<td>0.000</td>
<td>0.065</td>
<td>0.842</td>
<td>0.093</td>
<td>0.000</td>
</tr>
<tr>
<td>DiART-116</td>
<td>0.000</td>
<td>0.076</td>
<td>0.840</td>
<td>0.084</td>
<td>0.000</td>
</tr>
<tr>
<td>DiART-117</td>
<td>0.000</td>
<td>0.116</td>
<td>0.815</td>
<td>0.069</td>
<td>0.000</td>
</tr>
<tr>
<td>DiART-118</td>
<td>0.000</td>
<td>0.000</td>
<td>0.914</td>
<td>0.086</td>
<td>0.000</td>
</tr>
<tr>
<td>DiART-119</td>
<td>0.000</td>
<td>0.092</td>
<td>0.821</td>
<td>0.087</td>
<td>0.000</td>
</tr>
</tbody>
</table>

3.3. Compatibility of DiART reagents with custom-configured Mascot server

Only two configuration files in Mascot server (version 2.2 only) need to be updated to make the server compatible with DiART-based protein identification and quantitation. These two files (unimod.xml and quantitation.xml) will be distributed freely upon request. A result of Mascot searching is attached as an example.

4. References

Figure 1
Peptide Summary Report (Submitted from DiART-New by Mascot Daemon on LIW-XP)

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology (p<0.1).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Peptide Summary Report

Format As Peptide Summary Help
Significance threshold p< 0.1 Max. number of hits AUTO
Standard scoring MudPIT scoring Ions score or expect cut-off 0
Show sub-sets 0 Show pop-ups Suppress pop-ups Sort unassigned Decreasing Score Require bold red

[Graph showing the distribution of probability based Mowse scores]

Protein hits: 115/114 116/114 117/114 118/114 119/114

CATA_BOVIN Catalase (EC 1.11.1.6).- Bos taurus (Bovine).
OACH ovalbumin [validated] - chicken
ABBOS serum albumin precursor [validated] - bovine
Peptide Summary Report (Submitted from DiART-New by Mascot Daemon on LIW-XP)

### Proteins matching the same set of peptides:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass</th>
<th>Score</th>
<th>Queries matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATA BOVIN</td>
<td>66273</td>
<td>77</td>
<td>3</td>
</tr>
<tr>
<td>Catalase (EC 1.11.1.6).- Bos taurus (Bovine).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | 115/114 | 116/114 | 117/114 | 118/114 | 119/114 | Peptide |
|-------|----------|----------|----------|-------|------|-------|--------|------|---------|---------|---------|---------|---------|---------|---------|
| 1     | 12173.7300 | 1172.7227 | 1172.7260 | -0.0033 | 0 | 31 | 1.8 | 1 | 0.901 | 0.950 | 1.170 | 1.024 | 0.818 | K.LNSLTVGPR.G |
| 2     | 1263.6600 | 1262.6527 | 1262.6751 | -0.0223 | 0 | 18 | 48 | 1 | 1.076 | 1.066 | 1.000 | 1.162 | 0.973 | R.THFSDVQR.F |
| 4     | 1502.8000 | 1501.7927 | 1501.7908 | 0.0019 | 0 | 30 | 1.7 | 1 | 0.889 | 1.015 | 1.000 | 0.977 | 0.875 | R.LAHEDPDYGLR.D |

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass</th>
<th>Score</th>
<th>Queries matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>OACH</td>
<td>47756</td>
<td>75</td>
<td>1</td>
</tr>
<tr>
<td>Ovalbumin [validated].- chicken</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Query</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Miss</th>
<th>Score</th>
<th>Expect</th>
<th>Rank</th>
<th>115/114</th>
<th>116/114</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12173.7300</td>
<td>1172.7227</td>
<td>1172.7260</td>
<td>-0.0033</td>
<td>0</td>
<td>31</td>
<td>1.8</td>
<td>1</td>
<td>0.901</td>
<td>0.950</td>
</tr>
<tr>
<td>2</td>
<td>1263.6600</td>
<td>1262.6527</td>
<td>1262.6751</td>
<td>-0.0223</td>
<td>0</td>
<td>18</td>
<td>48</td>
<td>1</td>
<td>1.076</td>
<td>1.066</td>
</tr>
<tr>
<td>4</td>
<td>1502.8000</td>
<td>1501.7927</td>
<td>1501.7908</td>
<td>0.0019</td>
<td>0</td>
<td>30</td>
<td>1.7</td>
<td>1</td>
<td>0.889</td>
<td>1.015</td>
</tr>
</tbody>
</table>
Proteins matching the same set of peptides:

**OVAL\_CHICK** Mass: 47625 Score: 75 Queries matched: 1

Ovalbumin (Plakalbumin) (Allergen Gal d 2) (Gal d II).- Gallus gallus (Chicken).

**1OVAB2** Mass: 37887 Score: 75 Queries matched: 1

ovalbumin, chain B, fragment 2 - chicken

**1OVAC1** Mass: 42648 Score: 75 Queries matched: 1

ovalbumin, chain C, fragment 1 - chicken

**CAA23681** Mass: 19424 Score: 75 Queries matched: 1

**GGALB1** NID: - Gallus gallus

**Q3I349\_BOSIN** Mass: 65911 Score: 73 Queries matched: 2

Check to include this hit in error tolerant search or archive report

3. **ABBOS** Mass: 84469 Score: 73 Queries matched: 2

serum albumin precursor [validated] - bovine

Quantitation: Ratio Weighted N SD(geo)

<table>
<thead>
<tr>
<th>Query</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta Miss</th>
<th>Score</th>
<th>Expect</th>
<th>Rank</th>
<th>115/114</th>
<th>116/114</th>
<th>117/114</th>
<th>118/114</th>
<th>119/114</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1356.8200</td>
<td>1355.8127</td>
<td>1355.8426</td>
<td>-0.0299</td>
<td>0</td>
<td>0.25</td>
<td>1</td>
<td>0.973</td>
<td>0.860</td>
<td>0.802</td>
<td>0.905</td>
<td>1.078</td>
<td>K_AEFEVTK_LK</td>
</tr>
<tr>
<td>3</td>
<td>1502.8200</td>
<td>1501.8127</td>
<td>1501.7961</td>
<td>0.0166</td>
<td>0</td>
<td>0.71</td>
<td>1</td>
<td>1.038</td>
<td>1.145</td>
<td>1.220</td>
<td>1.254</td>
<td>1.028</td>
<td>K_QNCDFQEEK_LK</td>
</tr>
</tbody>
</table>

Proteins matching the same set of peptides:

**Q3I349\_BOSIN** Mass: 65911 Score: 73 Queries matched: 2
Serum albumin (Fragment).- Bos indicus (Zebu).

AA101062 Mass: 84434 Score: 73 Queries matched: 2
BC102742 NID: - Bos taurus

AA17824 Mass: 84492 Score: 73 Queries matched: 2
AF542068 NID: - Bos taurus

AAA51411 Mass: 84492 Score: 73 Queries matched: 2
BOVALBUMIN NID: - Bos taurus

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C),DiART6plex (K),DiART6plex (N-term),DiART6plex (K),DiART6plex (N-term)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 0.8 Da
Fragment Mass Tolerance : ± 0.5 Da
Max Missed Cleavages : 0
Instrument type : Default
Number of queries : 6

Mascot: http://www.matrixscience.com/