**SOP for Glycated albumin quantification using Opentrons OT-2**

**Labware requirements**

1. Grenier 300 µL 96 well plate (REF no: 650201)
2. Eppendorf 150 µL 96 well PCR plate (catalogue no. 0030129512)
3. Mikrolab Reservoir with 4 40 mL well (POR 390107)
4. OT-2 300 µL tips
5. OT-2 20 µL tips
6. OT-2 PCR 96 well aluminium block

**Reagents required:**

1. Plasma or serum sample. Primary calibrator: JCCRM-611, QC: Seronorm™ Immunoassay Liq L-1, Secondary calibrator: Seronorm™ Immunoassay Liq L-2
2. 1 M Tris buffer (pH 8.0)
3. 20 µg trypsin (V5111 Promega) per 80 samples
4. 4 % formic acid in LC-MS grade water (Milli Q)
5. 100 % LC-MS grade Acetonitrile
6. LC-MS grade Water

**Buffer A: 50 mM Tris-Formate buffer (pH 7.6)**

For preparing 500 mL of 50 mM Tris-Formate buffer

1. 25 mL of 1M Tris buffer (pH 8.0)
2. Initially add 400 mL of Milli Q or LC-MS grade water.
3. Adjust pH to 7.6 using 100 % formic acid.
4. Make up the volume to 500 mL with Milli Q or LC-MS grade water.

**Buffer B: LC-MS grade or MilliQ water**

**Buffer C: 100% Acetonitrile (LC-MS grade)**

**Buffer D: 4% formic acid in LC-MS grade or MilliQ water**

50 mL of 4% formic acid is prepared in a falcon tube.

2 mL 100% formic acid (handle in the fume hood !!) + 48 mL MilliQ water

**Glycated albumin quantification protocol:**

1. Dilute 2.5 µL (150-200 µg) of Serum/plasma in 300 µL of 50 mM TrisBase Formate buffer (0.66 µg/L).
2. 20 µL (13.2 µg) in 90 µL of 70% Acetonitrile.
3. 10 µL 0.025 µg/µL trypsin (52.5% Acetonitrile (final)).
4. Incubate it in RT or 37°C for 70 min.
5. Quench the reaction using 20 µL 4 % FA.
6. Dry them using a speed vacuum for 105 minutes at 45°C.
7. Resuspend the samples 300 µL 0.1% FA.
8. Load 20µL of the sample in Evo Tip (approx. load on tip 0.8 µg) using the recommended protocol (manual or automated).
9. Run the samples with 300 SPD or 500 SPD using Evosep LC. (Use EV1107

4 cm × 150 µm, 1.9 µm).

**MS method to be used can be found in the following file path:**

**C:\Xcalibur\data\Bharath\Method\300SPD\_gAlb\_p5\_tr15\_unsch\_LVT\_CEoptimized.meth**

**Or**

**C:\Xcalibur\data\Bharath\Method\500SPD\_gAlb\_p5\_tr15\_unsch\_LVT\_CEoptimized.meth**

**OT-2 Deck layout for glycated albumin quantification:**

**A screenshot of a computer screen

Description automatically generated with low confidence**

**Buffers reservoir:**

**A picture containing text, screenshot, font, rectangle

Description automatically generated**

**Trypsin Plate:**

**A picture containing screenshot, rectangle, text, square

Description automatically generated**

0.025 µg/µL of Promega sequencing grade trypsin was prepared as follows.

20 µg Promega trypsin vial + 80 µL of 50 mM Tris-formate buffer + 720 µL of MilliQ water (LC-MS grade) = 800 µL (0.025 µg/µL)

130 µL of trypsin aliquoted per well on to the trypsin plate (final volume can be adjusted according to the number of samples)

**Running the protocol in Opentrons for 96 samples:**

1. Aliquot 20 µL of plasma/serum into respective sample wells.
2. Place all the labware in their respective slots as shown in the deck layout.
3. Use the Glycated albumin\_BKR\_p20\_col\_96 protocol in the Opentrons app.
4. Run labware position check.
5. If satisfied save and apply the labware offsets
6. Run the protocol.
7. After the 579th step the digestion plate is closed with a plate cover to prevent the evaporation of the acetonitrile.
8. The plate cover is removed after 50 min (for a 96 well plate).
9. Once the protocol is complete, the samples are vacuum evaporated at 45°C for 105 min.
10. Store the dried samples at -20°C.
11. Resuspend the samples using 300 µL of 0.1% formic acid in water and load 20µL of samples onto Evotip using the recommended protocol (manual or automatic)
12. Run the samples with 300 SPD or 500 SPD using Evosep LC.

(Use EV1107 4 cm × 150 µm, 1.9 µm).

**MS method to be used can be found in the following file path:**

**C:\Xcalibur\data\Bharath\Method\300SPD\_gAlb\_p5\_tr15\_unsch\_LVT\_CEoptimized.meth**

**Or**

**C:\Xcalibur\data\Bharath\Method\500SPD\_gAlb\_p5\_tr15\_unsch\_LVT\_CEoptimized.meth**

**Data analysis using skyline:**

1. Raw files were imported on the skyline template file **C:\Xcalibur\data\Bharath\Method** \gAlb\_p5\_tr15\_BKR\_sch\_CEopt\_final.sky
2. Adjust integration boundaries if needed.
3. Export the report template gAlb\_p5\_t15\_sch\_optCE.
4. The %GA values can be calculated using the GA clean code python script that can be downloaded from https://github.com/rbharathkumar91/GA\_from\_skyline\_export

**Calculation of %GA**

%GA = Total peak area of Glycated peptide/Total peak area glycated + Total peak area unglycated