Protocol MSU_MSMC_013

Measurement of nucleotides ppGpp and pGpG, using c-di-GMPF as an internal standard – reversed phase ion-pairing UPLC/MS/MS for Waters Xevo TQ-S instrument

Last modified July 15, 2020

Measurement of target compounds:

1. Guanosine-3′,5′ -Bisdiphosphate (ppGpp)
2. 5′-Phosphoguanylyl-(3′→5′)-guanosine (pGpG)

Refer to mass spectrometer method file:	*ppGpp_cdiGMPF_pGpG_MRM_2 transition*
Refer to inlet method file:	*BEHC18_5cm_15min_BC_column2*
Refer to tune page file:	*Phosphate nucleotide tune method*

Reversed-Phase ion-pairing UPLC separation

Sample prepared using protocol MSU_MSMC_009

Note: For best results, extracts should be evaporated to dryness and then dissolved in solutions approaching the initial chromatographic mobile phase. The initial chromatographic mobile phase (mobile phase A) is [8 mM DMHA (*N*,*N*-dimethylhexylamine) + 2.8 mM acetic acid] in water, pH~9), the final extracts contain methanol-acetonitrile-water (40:40:20) with 0.1 M formic acid+ 1/25 volume NH_4HCO_3, the extraction solvent is too strong for good chromatographic separation. It is recommended that the extracts be evaporated to dryness (e.g. using a SpeedVac concentrator) followed by dissolving the residue in 50 µL (or a proper volume) of mobile phase A ([8 mM DMHA (*N*,*N*-dimethylhexylamine) + 2.8 mM acetic acid] in water) immediately before analysis.

Protocol for preparing mobile phase solvent A: Add 1.387 mL of DMHA (density=0.744 g/mL, from Sigma-Aldrich), and 0.1647 mL of acetic acid (17.4 M) into 1L Milli-Q water. This yields pH~9.

Preparing internal standard c-di-GMPF at 50 nM:

1. Add 0.5 mL of 1 µM stock IS c-di-GMPF to 9.5 mL of MP-A in a 15-mL polypropylene Falcon centrifuge tube to make 50 nM of IS solution.
2. Divide the stock solution into 10 1-mL aliquots in polypropylene microcentrifuge tubes.
3. Store each tube at -20˚ C (or -80˚ C) until ready to use by LC/MS/MS

Preparing standard curve:

1. From 1 µM standard (ppGpp and pGpG) stock solution, dilute into MP-A to make a set of serial dilutions with concentrations (nM): 500, 250, 100, 50, 20, 10, 5, 2.

2. Then make 1/2 dilutions of each solution by mixing the solutions from Step 1 with an equal volume of with 50 nM internal standard c-di-GMPF solution.

3. The final concentration of internal standard c-di-GMPF is then 25 nM, and the ppGpp and pGpG solutions will be:

500 nM ——>	250 nM
250 nM ——>	125 nM
100 nM ——>	50 nM
50 nM ——>	25 nM
20 nM ——>	10 nM
10 nM ——>	5 nM
5 nM ——>	2.5 nM
2 nM ——>	1 nM

Add IS to sample:

1. Make 1/2 dilutions of each sample extract by mixing with an equal volume (~ 50 µL) of 50 nM internal standard c-di-GMPF solution.
2. The final concentration of internal standard in each sample is 25 nM.

LC/MS/MS Method

HPLC column: Acquity UPLC BEH C18, 2.1 x 50 mm, 1.7 µm particle size. (Waters part # 186002350). Use with 0.2 µm precolumn filter [(Waters part # 205000343 (Kit, Acquity column In-line Filter)]

Mobile phase solvents: A) [8 mM DMHA +2.8 mM Acetic acid] in water, pH ~ 9.

B) methanol

LC gradient:

Time (min)	Flow rate (ml/min)	%A	%B
0.0	0.300	100	0
10.0	0.300	60	40
10.5	0.300	100	0
15.0	0.300	100	0

Column Temp: 40˚C

Autosampler Temp: 10˚C

Injection volume: 5 µL

Tune Page parameters: (For Xevo TQ-S and Quattro Premier)

- Ionization method: electrospray ionization; standard ESI probe
- Capillary voltage: 1.0 kV (Negative ion mode)
- Capillary voltage: 3.0 kV (Positive ion mode)

- Source Temp: 150˚C
- Desolvation Temp: 400˚C
- Desolvation gas: 700 L/hr
- Cone gas: 120 L/hr

MS/MS parameters

List of MRM channels

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision voltage	Approx. retention time (min)	ESI mode
Function #1 (0-5.25 min)						
pGpG	709	152	83	35	4.97	positive
pGpG	709	558	83	18	4.97	positive
Function #2 (5.26-15 min)						
ppGpp	602	159	5	44	7.24	negative
ppGpp	602	504	5	18	7.24	negative
c-di-GMPF (Internal standard)	693	346	108	33	5.56	negative

Notes

Multiple chromatographic peaks may be detected for individual metabolites when multiple isomers are present in the extracts. To distinguish the target metabolite in such cases, it is recommended to spike standard (for ppGpp/pGpG) into such samples to achieve an added concentration of about 50-100 nM.