Protocol MSU_MSMC_014a

Measurement of nucleotide tri- and mono-phosphates and dimeric nucleotides by UPLC/MS/MS

Note: method uses c-di-GMPF as an internal standard – reversed phase ion-pairing UPLC/MS/MS for Waters Xevo TQ-S instrument

Last modified September 22, 2020

Measurement of target compounds:

1. Deoxyadenosine triphosphate (dATP)
2. Deoxycytidine triphosphate (dCTP)
3. Deoxyguanosine triphosphate (dGTP)
4. 2′-Deoxythymidine-5′-triphosphate (dTTP)
5. 2′-Deoxyuridine, 5′-triphosphate (dUTP)
6. Deoxyuridine monophosphate (dUMP)
7. Guanosine-5′-triphosphate (GTP)
8. 5′-Phosphoguanylyl-(3′ → 5′)-guanosine (pGpG)
9. Cyclic diguanosine-5′-monophosphate (c-di-GMP)
10. Deoxycytidine monophosphate (dCMP)

Refer to mass spectrometer method file:	*9 comps_MRM_IS*
Refer to inlet method file:	*Premier BEH C18_10cm_DMHA*
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 (9 compounds mixture) 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 9 compounds mixture concentrations 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 (for sample which has not added IS during sample prep.):

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 Premier BEH C18, 2.1 x 100 mm, 1.7 µm particle size, VanGuard FIT (Waters part # 186009457).

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)

- 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 (5-7 min)						
pGpG	709	152	70	34	5.57	positive
pGpG	709	558	70	22	5.57	positive
Function #2 (0-15 min)						
dCMP	306	97	43	22	4.06	negative
dCMP	306	79	43	22	4.06	negative
dUMP	307	79	22	22	4.17	negative
dUMP	307	111	22	22	4.17	negative
dCTP	466	79	34	58	6.19	negative
dCTP	466	156	34	34	6.19	negative
dUTP	467	79	25	34	6.39	negative
dUTP	467	159	25	34	6.39	negative
dTTP	481	79	25	58	6.46	negative
dTTP	481	159	25	34	6.46	negative
dATP	490	79	34	65	6.61	negative

dATP	490	159	34	34	6.61	negative
dGTP	506	79	15	70	6.43	negative
dGTP	506	159	15	46	6.43	negative
GTP	522	159	15	34	6.33	negative
GTP	522	424	15	22	6.33	negative
c-di-GMP	689	150	20	46	5.00	negative
c-di-GMP	689	344	20	22	5.00	negative
c-di-GMPF (Internal standard)	693	346	108	33	5.26	negative
c-di-GMPF (Internal standard)	693	426	98	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 into such samples to achieve an added concentration of about 50-100 nM.