Protocol MSU_MSMC_009a

Procedure for Cyclic nucleotide analysis, use c-di-GMPF as an internal standard – reversed phase ion-pairing UPLC/MS/MS for Waters Xevo TQ-S instrument

Last modified January 23, 2020

Measurement of target compounds:

Cyclic di-GMP(c-di-GMP)
Cyclic di-AMP (c-di-AMP)
Cyclic di-UMP (c-di-UMP)
Cyclic di-GMPF (c-diGMPF)
Cyclic (adenosine monophosphate-guanosine monophosphate) (cApGp; cGAMP; 3,3′-cGAMP)
Cyclic CMP (cCMP)
Cyclic UMP (cUMP)
Cyclic AMP (cAMP)
Cyclic GMP (cGMP)

Refer to mass spectrometer method file:	*Phosphate nucleotide MRM method*
Refer to inlet method file:	*Phosphate nucleotide UPLC inlet method*
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 [10 mM TBA (tributylamine) + 15 mM Acetic acid] in 3% aq. methanol, pH=4.94~4.95. Although in sample preparation protocol MSU_MSMC_Protocol 009), 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 of mobile phase A ([10 mM tributylamine +15 mM Acetic acid] in 97:3/water:methanol) immediately before analysis.

Protocol for preparing mobile phase solvent A: Add 2.39 mL of TBA (density=0.778 g/mL, from Sigma-Aldrich), and 0.862 mL of acetic acid (17.4 M) into a mixture of 30 mL HPLC-grade methanol and 970 mL Milli-Q water. This yields pH=4.94~4.95.

Preparing internal standard c-di-GMPF at 100nM:

1. Take 250uL of 1uM stock IS c-di-GMPF, add 4750uL of MP-A, to make 50nM of IS solution.
2. Store it at -20˚ C (or -80˚ C) until ready to use by LC/MS/MS

Preparing standard curve:

1. From 1 µM standard stock solution, use MP-A to make a serial concentrations (nM): 500, 250, 100, 50, 20, 10, 5, 2.
2. Then make 1/2 dilutions of each solution with 50 nM internal standard c-di-GMPF solution.
3. The final concentration of internal standards c-di-GMPF is then 25 nM.

500nM		250nM
250 nM		125 nM
100 nM	½ dilution with 100nM	50 nM
50 nM	IS solution	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 using 50 nM internal standard c-di-GMPF solution.
2. The final concentration of internal standards in each sample is 25 nM.

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) [10 mM TBA +15 mM Acetic acid] in 97:3 water: methanol (v/v).

B) methanol

Column Temp: 50˚C

Autosampler Temp: 10˚C

Injection volume: 5 µL

LC gradient:

Time (min)	Flow rate (ml/min)	%A	%B
0.0	0.300	90	10
1.0	0.300	90	10
2.5	0.300	80	20
3.0	0.300	80	20
6.0	0.300	35	65
6.5	0.300	5	95
8.0	0.300	5	95
8.01	0.300	90	10
10.0	0.300	90	10

Tune Page parameters:

- Ionization method: electrospray ionization; standard ESI probe
- Ionization mode: ESI Negative ion mode
- Capillary voltage: 1.0 kV
- Source Temp: 130˚C
- Desolvation Temp: 350˚C
- Desolvation gas: 700 L/hr
- Cone gas: 20 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)**
c-di-GMPF	693	346	108	33	5.71
c-di-AMP	657	134	70	57	5.59
c-di-GMP	689	344	83	31	5.40
c-di-UMP	611	305	20	35	5.34
cApGp (cGAMP)	673	150	70	59	5.60
cCMP	304	79	59	33	2.40
cUMP	305	111	45	33	2.74
cAMP	328	134	57	31	3.97
cGMP	344	133	50	46	2.94

Notes

Multiple isomers may be detected for individual metabolites. To distinguish the target metabolite in such cases, it is recommended to spike standards (for cyclic dinucleotides) into such samples to achieve an added concentration of about 50-100 nM.