

Protocol MSU_MSMC_009

Procedure for preparation of mono- and di-nucleotide extracts for UHPLC/MS/MS quantification using ion-pairing separation on a reversed phase column

(following the protocol of Fernandez and Waters, Current Protocols in Microbiology, 52, e74. doi: 10.1002/cpmc.74)

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Materials

Microbial culture at OD₆₀₀ = 0.5

SpeedVac with cold trap

-20°C freezer

Refrigerated centrifuge with rotor for 1.5-mL microcentrifuge tubes

15-mL Falcon polypropylene centrifuge tubes

Crushed ice

Tributylamine (Sigma cat# 90781-10ML)

c-di-GMP fluorinated (InVivoGen STING Ligand - Difluoro 3'3'-c-di-GMP, cyclic [FdG(3',5')pFdG(3',5')p]; Catalog #tlrl-nacdgf); abbreviated below as c-di-GMPF

Acetic acid (glacial)

Methanol, HPLC grade

Acetonitrile, HPLC grade

Water, MilliQ (> 18 MΩ)

Formic acid (98%) (Fisher Scientific cat# 60-006-17)

LB media

Preparation of reagents and solvents

1. Nucleotide extraction solution (0.1 M formic acid in acetonitrile/methanol/water 40:40:20 v/v/v) containing 25 nM c-di-GMPF as internal standard:
 - 1) Add 4000 μL of HPLC grade acetonitrile to a 15-mL polypropylene centrifuge tube.
 - 2) Add 4000 μL of HPLC grade methanol to the 15-mL polypropylene centrifuge tube.
 - 3) Add 2000 μL of MilliQ water to the 15-mL polypropylene centrifuge tube.
 - 4) Label acetonitrile/methanol/water (40:40:20 v/v/v) as solvent A.
 - 5) Mix 25 μL of 10 μM c-di-GMPF and 38.5 μL of 98% formic acid and 9936.5 μL of solvent A in a 15-mL polypropylene centrifuge tube.
 - 6) Label the tube as “Nucleotide Extraction Solution with 25 nM IS” and store it at -20°C.
2. Mobile phase solvent A:
 - 1) Mix 970 mL of MilliQ water and 30 mL of HPLC grade methanol in a 1-liter HPLC solvent bottle
 - 2) Add 2.39 mL of tributylamine (TBA)
 - 3) Add 862 μL of acetic acid; these ingredients give 10 mM TBA, 15 mM acetic acid
 - 4) Cap the solvent bottle and place it in an ultrasound bath. Ultrasonicate for 15 minutes. Allow solution to stand overnight before use.

- 5) Label the solvent bottle (10 mM TBA/15 mM acetic acid in water/methanol (97:3 v/v), pH 5

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

1. Take 250 μ L of 1 μ M stock IS c-di-GMPF, add 4500 μ L of MP-A, to make 50 nM 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 μ M standard stock solution, use MP-A to make serial dilutions to obtain concentrations (nM): 500, 250, 100, 50, 20, 10, 5, 2.
2. Then dilute each standard stock solution with an equal volume of 50 nM internal standard c-di-GMPF solution.
3. The final concentration of internal standard c-di-GMPF is then 25 nM.

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

Sample preparation procedure

1. Chill centrifuge to 4° C.
2. Prepare 100 μ L of extraction solution per sample, store on ice or in a -20° C explosion-proof or flammable storage freezer.
3. Transfer two replicate 1000- μ L aliquots of OD₆₀₀ = 0.5 culture into separate 1.5-mL polypropylene microcentrifuge tubes.
4. Centrifuge tubes at 15,000 x g for 30 seconds.
5. During the above centrifugation step, transfer 500 μ L from the original culture to a new tube and add 500 μ L of LB medium; measure and record OD₆₀₀
 - a. This OD₆₀₀ measurement is to be used for normalizing the nucleotide concentrations to the number of cells extracted
 - b. Alternatively, concentrations can be normalized to protein content of extracted sample
 - c. More detailed information on normalization can be found in the Fernandez and Waters protocol.
6. From the original tubes (from step 4), quickly remove the supernatant using a 1000- μ L pipette.

7. Resuspend the pellet in 100 μ L of cold nucleotide extraction solution (with 25 nM IS) to lyse cells and quench metabolism.
8. Place the quenched pellet/extraction solution tubes in a -20°C freezer for 20 minutes.
9. Remove tubes from freezer and centrifuge for 15 minutes at 15,000 x g, 4°C .
10. Transfer supernatant to a new 1.5-mL microcentrifuge tube.
11. Evaporate extracts to dryness under vacuum (SpeedVac) without application of heat.
12. Redissolve in 100 μ L of mobile phase solvent A.
13. Transfer to HPLC autosampler vials with 200- μ L glass insert.

Note: Redissolving samples in mobile phase A containing tributylamine is critical for optimal ion-pairing and reduces the chances of split peaks during chromatography.