Protocol MSU_MSMC_014

Procedure for preparation of nucleotide phosphate 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 OD_{600} = 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.
N.N-Dimethylhexylamine (Sigma-Aldrich cat# 308102-5G).
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) Fill 1000 mL of MilliQ water in a 1-liter HPLC solvent bottle.
   2) Add 1.387 mL of N.N-Dimethylhexylamine (DMHA).
   3) Add 164.7 µL of acetic acid; these ingredients give 8 mM DMHA, 2.8 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 (8 mM DMHA/2.8 mM acetic acid in water, pH~9).

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

1. Take 250 µL of 1 µM stock IS c-di-GMPF, add 4500 µL 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 standard solutions:

1. From 1 µM 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 µL of extraction solution per sample, store on ice or in a -20˚C explosion-proof or flammable storage freezer.
3. Transfer two replicate 1000-µL aliquots of OD_{600} = 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 µL from the original culture to a new tube and add 500 µL of LB medium; measure and record OD_{600}
    a. This OD_{600} 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-µL pipette.

7. Resuspend the pellet in 100 µL 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 µL of mobile phase solvent A.
13. Transfer to HPLC autosampler vials with 200-µL glass insert.

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