Protocol MSU_MSMC_009 v. 1.1
Preparation of mono- and di-nucleotide extracts for UHPLC/MS/MS quantification using ion-pairing separation on a reversed phase column

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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)

Materials
Extract of pelleted material from 1-mL of OD_{600} = 0.5 microbial culture
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
Acetic acid
Methanol, HPLC grade
Acetonitrile, HPLC grade
Water, MilliQ (> 18 MΩ)
Formic acid (88%)
LB media

Preparation of reagents and solvents
Preparation of Nucleotide Extraction Solution (0.1 M formic acid in acetonitrile/methanol/water (40:40:20 v/v/v)

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. Add formic acid (43 µL of 88% formic acid) and mix well
5. Label the tube as “Nucleotide Extraction Solution” and store it at -20˚C.

Preparation of 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)

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 15000 x *g* for 30 seconds.
5. Following centrifugation, transfer 500 µL from each tube and add 500 µL of LB medium; measure and record OD_{600} if desired for normalization to cell density
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 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.