Objective.
LC-MS analysis of nucleosides from DNA and or RNA.

Chemicals and Tools	**Vendor**	**Part#**	**Hazards/Notes**
• LC-MS grade methanol (MeOH)	Fisher	A456-4	(HPLC grade MeOH can be used)
• Nucleoside Digestion Mix	NEB	M0649S	
• Eppendorf tubes	Eppendorf	022431081	LoBind tubes
• Vortexer			

Targeted analysis of nucleosides
Nucleosides are measured using targeted LC-MS methods. The metabolites are separated using hydrophilic interaction chromatography (HILIC) and the mass to charge ratio (m/z) is measured in both positive and negative ionization modes– typically with mass accuracies ≤2 ppm. A library of >30 nucleoside standards (Refer to **Table 1**) are used for retention time validation. The PRC staff can measure other nucleosides if an authentic standard is provided by the user.

We recommend that 3 biological replicates (n≥3) are prepared per condition. The recommended amount of RNA/DNA for sample preparation is 0.5-1 ug per sample – though this is dependent on the abundance of the nucleoside of interest.

	Procedure.	*Examples, Tricks & Comments*
1	Cleanup DNA/RNA sample using phenol-chloroform extraction with ethanol precipitation, or a spin-column based cleanup kit like; Qiagen DNeasy Blood and tissue kit (e.g. 69504) or the Zymo Quick-DNA micro-prep kit (e.g. D3020).	*Step 1 is a general guideline. Prepare the samples as you see fit.*

<table>
<tr><td>2</td><td><u>Digestion of (deoxy)ribonucleic acids:</u>
(Optional): Spike in a known concentration of isotope-labeled internal standard (ISTD) to the RNA/DNA sample.
Digest 0.5-1ug RNA/DNA for 1 hour at 37°C using the NEB Nucleoside Digestion Mix [I].
[I] You can digest the RNA using the NEB digest kit which can be found here: https://www.neb.com/products/m0649-nucleoside-digestion-mix#Product%20Information . The NEB kit suggests a 1 hour digestion time but we have had users incubate overnight with no issues.</td><td>Step 2 is also a guideline- other digestion methods can be used. We prefer this NEB kit due to the low% of glycerol and added salts.

Isotopically labeled nucleosides can be ordered from Cambridge Isotopes and other vendors. These can be used for relative quantitation if a known concentration is spiked into the bio sample.</td></tr>
<tr><td>3</td><td><u>Extraction from digested nucleic acids:</u>

Pre-chill the MeOH at -20°C prior to extraction.
Cool the reaction mixture on ice and add cold MeOH (10X the reaction volume).
Vortex vigorously for 5-10 minutes followed by centrifugation (>10,000 rpm or max speed) for 10 minutes.
Carefully remove supernatant into a pre-chilled Eppendorf tube.</td><td>Note. You can save the protein pellet to measure protein concentration for sample normalization or proteomic analysis.

All steps must be performed on ice or at 4°C.</td></tr>
<tr><td>4</td><td>You can evaporate the methanol extracts using a temperature-controlled evaporator or speed vac and submit the dry pellet to the PRC. Note: You will likely not observe a pellet post digestion/extraction. The tube will look empty and that is okay. The dry pellet can be stored at -20°C or -80°C till submission.</td><td></td></tr>
<tr><td>5</td><td>Fill out the metabolomics/lipidomics submission form.

https://www.rockefeller.edu/proteomics/uploads/www.rockefeller.edu/sites/216/2020/07/Metabolomics_submission_form_FY21.xlsx

Please list which method/kits were used for oligonucleotide cleanup and digestion in the submission form.</td><td>Required information:
Sample type/volume
List of specific metabolites (or full profiling)
ISTD composition/ concentration
Cell treatment (e.g. labels, inhibitors, etc.)</td></tr>
</table>

Table 1.

Nucleoside	Formula	HMDB ID	KEGG #
2-deoxyAdenosine	$C_{10}H_{13}N_5O_3$	HMDB0000101	C00559
5-deoxy-5-(methylthio)Adenosine	$C_{11}H_{15}N_5O_3S$	HMDB0001173	C00170
N6-methyl-deoxyAdenosine	$C_{11}H_{15}N_5O_3$		
2-deoxyGuanosine	$C_{10}H_{13}N_5O_4$	HMDB0000085	C00330
N2-ethyl-deoxyGuanosine	$C_{12}H_{17}N_5O_4$		
2-deoxyCytidine	$C_9H_{13}N_3O_4$	HMDB0000014	C00881
5-carboxy-deoxyCytidine	$C_{10}H_{13}N_3O_6$		
5-formyl-deoxyCytidine	$C_{10}H_{13}N_3O_5$		
5-methyl-deoxyCytidine	$C_{10}H_{15}N_3O_4$	HMDB0002224	C03592
5-hydroxy-methyl-deoxyCytidine	$C_{10}H_{15}N_3O_5$		
2-deoxyUridine	$C_9H_{12}N_2O_5$	HMDB0000012	C00526
2-deoxyInosine	$C_{10}H_{12}N_4O_4$	HMDB0000071	C05512
Thymidine	$C_{10}H_{14}N_2O_5$	HMDB0000273	C00214
1-methylAdenosine	$C_{11}H_{15}N_5O_4$	HMDB0003331	C02494
N6-methylAdenosine	$C_{11}H_{15}N_5O_4$	HMDB0004044	
Adenosine	$C_{10}H_{13}N_5O_4$	HMDB0000050	C00212
Guanosine	$C_{10}H_{13}N_5O_5$	HMDB0000133	C00387
1-methylGuanosine	$C_{11}H_{15}N_5O_5$	HMDB0001563	C04545
7-methylGuanosine	$C_{11}H_{15}N_5O_5$	HMDB0001107	
N2-methylGuanosine	$C_{11}H_{15}N_5O_5$	HMDB0005862	
2-O-methylGuanosine	$C_{11}H_{15}N_5O_5$		
8-hydroxyGuanosine	$C_{10}H_{13}N_5O_6$	HMDB0002044	
N2-N2-dimethylGuanosine	$C_{12}H_{17}N_5O_5$	HMDB0004824	
Cytidine	$C_9H_{13}N_3O_5$	HMDB0000089	C00475
3-methylCytidine	$C_{10}H_{15}N_3O_5$	HMDB0240577	
5-methylCytidine	$C_{10}H_{15}N_3O_5$	HMDB0000982	
2-O-methylCytidine	$C_{10}H_{15}N_3O_5$		
Uridine	$C_9H_{12}N_2O_6$	HMDB0000296	C00299
5-methylUridine	$C_{10}H_{14}N_2O_6$	HMDB0000884	
2-O-methylUridine	$C_{10}H_{14}N_2O_6$	HMDB0240328	
pseudoUridine	$C_9H_{12}N_2O_6$	HMDB0000767	C02067
Inosine	$C_{10}H_{12}N_4O_5$	HMDB0000195	C00294
Xanthosine	$C_{10}H_{12}N_4O_6$	HMDB0000299	C01762