Chemicals needed:

- DNA Digestion Mix (per ml; ~20 samples): 50 U Benzonase, 60 mU phosphodiesterase I (P-3243, Sigma), and 40 U alkaline phosphatase (P-7923, Sigma) in Tris–HCl buffer (pH 7.9, 20 mM) containing 100 mM NaCl and 20 mM $MgCl_2$.
- Mobile phases, 10 mmol/l ammonium acetate + 0.1% (v/v) acetic acid and Methanol with 0.1% Formic Acid LC/MS grade
- deoxycytidine and methyldeoxycytidine Standards
- [U-15N]Nucleoside Standard Mix

Materials needed:

- Labeled 0.3 mL microcentrifuge tubes
- Repeater Pipette
- Calibrated Micropipettes in various volumes (see table below)
- Appropriate Micropipette tips (see table below)
- Incubator
- Autosampler tubes (with inserts) and caps
- LC-MS/MS
- 3 µm Luna C18(2) column 50 × 2 mm column.
- Personal Protective Equipment
-

Type	Volumes (µL)	Tip color
P10	0.5 – 10	white
P20	2 – 20	yellow
P200	20 – 200	yellow
P1000	200 – 1000	blue

Precise Micropipette Volume and Transfer capabilities

Instrumentation:

HPLC, Thermo Scientific- Accela: Check the lines for air bubbles and purge line if required.

Mass Spectrometer, Thermo Scientific- TSQ Quantum Ultra: The HESI II probe should be installed at position C.

Digestion Procedure:

1- Add [U-15N]Nucleoside Standard Mix (equivalent to ~2 µg/ml DNA) to 1ml of DNA Digestion Mix
2- Add ~500ng of DNA sample to labeled, clean autosampler tube (with inserts) using a P10 micropipette.
3- Make the volume in the microcentrifuge tube up to 50µl with water using a P10 micropipette.
4- Add 50µl DNA Digestion Mix to each sample, cap and vortex.
5- Incubate overnight at 37°C.
6- Load samples into autosampler.

Data Collection:

1- 3 µm Luna C18(2) column 50 × 2 mm column.
2- Set up sequence starting with
   - 2 x Blank
   - Unknown Samples
   - Blank
   - Standard Curve (low to high concentration).
3- Injection volume is 10 uL.

Gradient Information

- Flow rate is 300 uL/min.
- Duration of run is 5 min
- Initial conditions are 92% Solvent A (10 mmol/l ammonium acetate + 0.1% (v/v) acetic acid): 8% MeOH
- MeOH is linearly increases to 12% by 3.5 min, where it is held until 4.4 min. The gradient is returned to initial conditions (92% Solvent A) by 4.5 min.

Instrument Parameters	
HESI Probe	Positive (+)
Probe Temperature	300°C
Spray Voltage	2500 V
Capillary Temperature	300°C

Sheath Gas	50
Auxillary Gas	20
Sweep gas	1
Nitrogen Collision Gas Pressure	1.5 mTorr
Tube Lens	88
Collision Energy	60 eV
SRM: Deoxycytidine	228.1 →112.1
SRM: [15N3]Deoxycytidine	231.1 → 115.1
SRM: Methyldeoxycytidine	242.1 → 126.1
SRM: [15N3]Methyldeoxycytidine	245.1 → 129.1

Data Processing:

1- Integrate peak areas and calculate the Unlabeled: [15N3]Labeled peak ratio for each nucleoside.
2- Plot the Standard Curve by graphing deoxynucleoside concentrations against Peak Area Ratios (M+0/M+3).*
3- Calculate the nucleoside concentrations for each Unknown Sample from the Standard Curve using reverse prediction.
4- Percentage DNA methylation =

[methyldeoxycytidine]/([methyldeoxycytidine] + [deoxycytidine]) x 100

* See APPENDIX A for preparation of Standard Curves.

Created By:	Eoin Quinlivan	Date: 12/4/15
Reviewed By:	Tim Garrett	Date:
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Revision Number	Name	Reason for Revision	Effective Date
01	Eoin Quinlivan	Creation of SOP	12/4/15

APPENDIX A - Preparation of Nucleoside Standard Curves

Stock solutions of deoxycytidine and methyldeoxycytidine were prepared in water. The concentration of the stock solutions was determined by diluting 1 in 10 with 0.1 M HCl and measuring their absorbance. The molar absorptivity coefficients (Є) used were: deoxycytidine Є = 13 400 $M^{-1}cm^{-1}$ at 280 nm and methyldeoxycytidine Є = 12 200 $M^{-1}cm^{-1}$ at 286 nm.

80X Standard Solution: Combine and accurately dilute the two nucleosides so that their known final concentrations are approximately 800 µmol/L deoxycytidine and 32 µmol/L methyldeoxycytidine. Aliquot and store at -80°C

Standard Solution: Thaw a 80X Standard Solution and dilute 1 in 80 with water.

Standard Curve: Dilute the Standard Solution in water to give a 6-point linear curve (dilution of 0, 0.2, 0.4, 0.6, 0.8 and 1.0).

Process the Standard Curves in the same manner as the Unknown DNA Samples.