Document Number:	**SASoM/METHOD/125.v1**
Title:	**Analysis of ATP, ADP, AMP and other nucleotides by uHPLC.**
Version:	**v1**
Author:	**Peter Mullen**

Effective from:	28/05/2020
Valid to:	27/05/2022

SOP History		
Number	Date	Reason for Change
v1	28/05/20	Original

1.0 Purpose –

This SOP describes the current procedures for measuring ATP, ADP, AMP and other nucleotides by uHPLC in Laboratory 248/249 at the St Andrews School of Medicine (SASoM).

2.0 Scope –

This SOP applies to all staff in the SASoM measuring ATP, ADP, AMP and other nucleotides by uHPLC in Laboratory 248 at the St Andrews School of Medicine (SASoM).

3.0 Responsibilities –

All staff measuring ATP, ADP, AMP and other nucleotides by uHPLC in this manner are responsible for ensuring that the methods are followed in accordance with this SOP. All staff must have read and signed the relevant risk assessment documents before performing this procedure.

4.0 Procedure –

This procedure describes the measurement of ATP, ADP, AMP and other nucleotides by uHPLC using either (i) cell lysates or (ii) 'spent' cell culture media. Given that ATP release can be a product of immunogenic cell death (ICD), this can be an important tool for cell culture studies following treatment with drugs (including the Nucana Protide compounds) in that the intracellular / extracellular balance can potentially be studied.

Materials and Reagents:

Adenine (Sigma; A8626-1G) – make up at 10mM in 0.1M HCl.
Adenosine (Sigma; A9251-5G) – make up at 10mM in 1M NaOH at 37°c.
Adenosine (Stratech; B1877-APE; 10 mM in 1mL DMSO).
Adenosine 5′-triphosphate - ATP (Sigma; A2383-1G)
Adenosine 5′-diphosphate – ADP (Sigma; A2754-1G)
Adenosine 5′-monophosphate – AMP (Sigma; 01930-5G)
Guanosine 5′-triphosphate sodium salt – GTP (Sigma; G8877-100MG)
Inosine 5′-monophosphate – IMP (Sigma; 57510-5G)
Perchloric acid solution 1.0M (Sigma; 34288).
XBridge BEH C18 Column, 130Å, 5 µm, 4.6 mm X 150 mm (Waters; 186003116;).
Column Guards
VanGuard Cartridge Holder (Waters; 186007949).

Buffers:

2M KOH

Weigh out 11.2g of Potassium Hydroxide and then make up to a final volume of 100mL with DW.

10M NaOH

Weigh out 80g of Sodium Hydroxide into a glass beaker and then slowly / carefully transfer to a glass beaker containing DW to a final volume of 200mL. Sodium Hydroxide pellets should however be avoided for LC-MS work.

2.5M NaOH

Add 50mL of 10M NaOH stock to 150mL of DW.

0.5M Na_2EDTA, pH8.0

Weigh out 9.3g of Na_2EDTA into a glass beaker containing approximately 35mL of DW. Adjust to pH8.0 using 10M NaOH and then make up to a final volume of 50mL in DW.

[A] Preparation and collection of Cell culture media samples:

Cells can be cultured and treated in various petri-dishes as per standard procedures already documented. In general, cells are plated into petri dishes or 6-well trays, left for 24/48hrs, treated with a given drug at a range of concentrations and then left for a further 3-4 days. At the end of the time course the media can be removed, transferred to amber-coloured glass vials (to prevent non=specific binding to plastic) and stored at -80°C until ready for analysis.

[B] Preparation and collection of cell culture lysates:

Once the 'spent' cell culture media has been removed at the end of the experiment, cell lysates can be separately prepared and stored as described below:

1. Make up Sample Extraction Buffer (for cellular lysates) as follows:
   Add 10mL of Milli Q water to a clean universal container
   Remove 20µL
   Remove 258 µL
   Add 20µL of 0.5M Na_2EDTA, pH8.0
   Add 258µL of Perchloric Acid (70%) – need to check concentration as our product appears to be 1M solution which equates to 10.64% v/v)

2. Remove media from petri dishes (do not rinse with PBS).
3. Aspirate off any residual media and place dishes on ice.
4. Add 475µL of Extraction Buffer to each petri dish.
5. Scrape the cells to one side of the petri dish and leave for 5mins on ice.
6. Transfer the cell lysate to a sterile microcentrifuge tube.
7. Neutralise with 85µL of 2M KOH and gently mix by inverting
8. Centrifuge at full speed (14,000rpm for 10 mins at 4°c)
9. Transfer the (pinkish) supernatant to fresh tubes and store at -80°c prior to analysis.

Note: When the KOH is added to neutralise the extraction buffer it causes the precipitation of Potassium Perchlorate (a low-solubility salt). The neutralised extraction buffer must therefore be briefly centrifuged to sediment the precipitate before transferring the clear solution to a fresh tube. Failure to do this may result in the column guard or the column itself being blocked.

[C] uHPLC Analysis of ATP, ADP, AMP and other nucleotides:

- All samples are analysed using a (Dionex) Thermo Fisher 'Ultimate 3000' uHPLC system controlled by Chromeleon v6.80 software.

- All samples are separated using an XBridge BEH C18 column, 130Å, 5 µm, (4.6 mm X 150 mm), regardless of the nucleotide being analysed. A column guard is fitted to the column at all times to prevent sample blockage.

- All samples are passed through a PTFE 13mm x 0.45uM Orange syringe filter device (Conex: FIL-S-PT-045-13-100) prior to running down the column.

[D] Standard Curve Preparation:

uHPC Standard Curves for each of the nucleotides (eg. ATP, ADP, AMP, IMP, GTP, Adenine and Adenosine) are made up from stock solutions (10mM) using Mobile Phase A as follows:

[A] 50µM = 5µL in 1000µL of Mobile Phase A
[B] 25µM = 2.5µL in 1000µL of Mobile Phase A
[C] 10µM = 1.5µL in 1500µL of Mobile Phase A
[D] 5µM = 100µL of [A] + 900µL of Mobile Phase A
[E] 2.5µM = 100µL of [B] + 900µL of Mobile Phase A
[F] 1µM = 100µL of [C] + 900µL of Mobile Phase A
[G] 0.5µM = 100µL of [D] + 900µL of Mobile Phase A
[H] 0.1µM = 100µL of [F] + 1000µL of Mobile Phase A

[E] HPLC Conditions for ATP, ADP, AMP and other nucleotides:

Mobile Phase A (0.1M Ammonium Dihydrogen Phosphate pH6.0 / 1% MeOH).

- Weigh out 11.5g of $NH_4H_2PO_4$ into a 2L glass beaker.
- Add approximately 800mL of Milli Q water.
- Adjust to pH6 with 10M NaOH.
- Add 10mL of Methanol
- Make up the volume to 1L with fresh Milli Q water.
- Filter into a clean 1L Duran Bottle prior to use.

Mobile Phase B (Methanol).

- All 500mL of Methanol to a clean Duran Bottle.

uHPLC Conditions:

Column:	Waters XBridge BEH C18, 130Å, 5µm, 4.6 x150mm.
Mobile phase A:	0.1M $NH_4H_2PO_4$ (pH6.0) / 1% MeOH
Mobile phase B:	Methanol
Diluent:	Methanol
Column temperature:	20.0°C
Sample temperature:	6.0°C
Flow rate:	0.8 mL/min
Injection volume:	5µL
Detection:	254 nm
Needle wash:	Acetonitrile

Solvent Profile:
The solvent profile (22mins) is shown below:

Time (mins)	% B
0.00	0%
10.00	0%
12.00	10%
18.00	10%
22.00	0%

[F] uHLC Shutdown Procedure:
At the end of the run, the system MUST BE SHUTDOWN in order to prevent precipitation of the Ammonium Dihydrogen Phosphate buffer (Mobile Phase A). Failure to go through this shutdown procedure correctly will result in blockage of the tubes or the piston seals within the pump modules.

Shutdown should be carried out as follows:

1. At the end of the experimental run, ensure that the column heater has been switched off and the column returned to ambient temperature.
2. Stop the flow to both pumps, remove the bottles containing 'Mobile Phase A' and 'B' and then replace each of them with fresh Milli Q water.
3. Separately purge Pumps 'A' and 'B' with Milli Q water.
4. Flush the column with Milli Q water for 15mins, checking the backpressure in case it should increase. This should be done with a 50:50 mix from pumps A/B.
5. Stop the flow to both pumps, remove the bottles containing 'Mobile Phase A' and 'B' and then replace each of them with fresh Acetonitrile.
6. Separately purge Pumps A and B with Acetonitrile.
7. Flush the column with Acetonitrile for 15mins, checking the backpressure in case it should increase. This should be done with a 50:50 mix from pumps A/B. This will equilibrate the column with acetonitrile (the default storage medium for the columns)
8. Stop the flow to both pumps, remove the bottles containing 'Mobile Phase A' and 'B' and then replace each of them with fresh Iso Propyl Alcohol / Propan-2-ol.
9. Separately purge Pumps A and B with Propan-2-ol.
10. Close the Purge Valve and turn off the instrument.

5.0 Personal protection –
A Howie coat must be worn at all times. Gloves as specified in the appropriate COSHH RA.

6.0 Spillages –
Always clean up any spills immediately after use, only you know what you have spilt and are aware of its hazard. Spillages should be mopped up with paper towel, disinfected with 70% ethanol and finally washed with detergent.

7.0 Training –
All staff should undergo training in this technique before performing the procedure.

8.0 Related documents –
8.1 Risk assessments –
RA20262: Analysis of ATP, ADP, AMP and other nucleotides by uHPLC

9.0 Approval and sign off –

Author:

Name: Peter Mullen

Position: Research Fellow

Signature: Date:

Management Approval:

Name: Peter Mullen

Position: SOP Administrator

Signature: Date:

QA release by:

Name: Alex MacLellan

Position: QA Manager

Signature: Date:

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE