Document Number: **SASoM/METHOD/101.v2**

Title: **BrdU Incorporation for Cell Cycle Analysis by Flow Cytometry.**

Version: **v2**

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SOP History		
Number	Date	Reason for Change
v1	24/08/2017	Original
v2	24/08/2019	Update
V3	09/09/2021	Update

1.0 Purpose –

This SOP describes the current procedure to measure 5'-Bromo-2'-dexyzuridine incorporation as a means of more accurately assessing cell cycle distribution in vitro.

2.0 Scope –

The scope of this document is to describe the step by step procedures for preparing cells prior to performing cell cycle analysis by flow cytometry means.

3.0 Responsibilities –

All staff preparing cells by this protocol 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 –

Make up the following Solutions (based on 6 samples):

BrdU (5-Bromo-2'-Deoxyuridine; 10µM)
(Invitrogen B23151; 10mg/mL stock solution ≈ 33mM).
Dilute the stock solution 1 in 3 to make a **Working Solution** of ≈10mM

Pepsin (0.4mg/mL in 100mM HCl)
Add 4mg Pepsin (Sigma: P6887) to 10mL of 0.1M HCL (final volume 10mL).

2N HCl / 0.5% Triton X-100
Add 50µL of Triton X-100 to 950µL of 2N HCl (final volume 10mL)

0.1M Sodium Tetraborate (Decahydrate) (mwt 381.4), pH8.5.
Add 494mg of $Na_2B_4O_7.10H_2O$ to 10mL of Dw, adjust pH and then make up to 13mL with DW.

0.1M HCL
Add 0.5mL of concentrated 2N HCl to 9.5mL of DW (1 in 20) to give a final concentration of 100mM in a volume of 10mL (final volume 10mL).

0.5% BSA / 0.5% Tween 20 in PBS
Combine 250mg of BSA and 250µL of Tween 20 in 50mL of PBS (final volume 50mL).

RNase A
(Qiagen 1031301; 10mg/mL stock solution to be diluted 1:100)

Propidium Iodide
(Sigma P4864; 50µg/ml stock solution to be diluted 1:20150

Procedure – Part 'A' (preparation of cells):

Grow cells in 60mmØ petri dishes and treat appropriately (eg with drugs) for the required amount of time.

Add BrdU at a final concentration of 10µM (4µL/4mL taken from the 10mM Working Solution - see above) and then incubate for 30mins at 37°C.

Spin down cells (trypsinized if adherent) at 1200rpm for 5 mins and discard the s/n.

Wash cells in 2mL PBS and centrifuge at 1200rpm for 5 mins. Discard the s/n.

Add (while gently vortexing) 1mL of ice-cold 70% Ethanol and leave for 20mins at room temperature (or store at 4°C until ready to stain and analyse on the flow cytometer).

Procedure – Part 'B' (approximately 3½ hours):

Add 1.5mL of pepsin (0.4mg/mL in 100mM HCl) to each sample and incubate at 37° for 45 mins with frequent mixing.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Add 2mL of 2N HCl / 0.5% Triton X-100 and incubate for 30 mins at room temperature.

Wash cells in 2mL PBS

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Wash cells in 2mL of 0.1M Sodium Tetraborate, pH8.5.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Wash cells in 2mL PBS.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Add 2.0mL of PBS / 0.5% BSA / 0.5% Tween 20 containing mouse anti-BrdU (Beckton Dickinson; clone B44; #347580) at a dilution of 1:100 (130µL in 13mL) and incubate at room temperature for 30mins.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Wash cells in 2mL of PBS.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Add 2.0mL of PBS / 0.5% BSA / 0.5% Tween 20 containing anti-mouse FITC (Alexa Fluor 488; Invitrogen #A11001) at a dilution of 1:200 (65µL in 13mL) and incubate at room temperature for 30 mins in the DARK.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Wash cells in 2mL of PBS.

Centrifuge at 1700rpm for 4 mins and carefully remove the s/n.

Make up RNAse A (0.1mg/mL) by adding 7µL of stock solution (10mg/mL) to 693µL of PBS (700µL-7µL). Add 100µL to each tube.

Make up Propidium Iodide (50µg/mL) by adding 150µL of stock solution (1mg/mL) to 2.85mL of PBS (3000µL-150µL). Add 400µL to each tube.

Wrap samples in tinfoil and store on ice (in the dark) until ready to analyse on the flow cytometer. Final sample volume = 0.5mL. Samples should ideally be run on the same day but certainly within 24hrs.

5.0 Personal protection -
A Howie laboratory coat and lab gloves must be worn at all times.

6.0 Spillages -
Always clean up and disinfect any spills immediately after use, only you know what you have spilt and are aware of its hazard.

7.0 Training -

8.0 Related documents –
8.1 Risk assessments –
CHARM_RA20270_Cell Cycle analysis by flow cytometry.
CHARM_RA22236_Cell viability using Calcein AM, PI and Hoescht 33342.

9.0 Approval and sign off –

Author:

Name: Peter Mullen

Position: Research Fellow

Signature:  Date: 09/09/2021

Management Approval:

Name: Peter Mullen

Position: Research Fellow

Signature:  Date: 09/09/2021

QA release by:

Name: John O' Connor

Position: QA Manager

Signature:  Date: 09/09/2021

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