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A Robust Assay for Assessing the Effects of Compounds on the Cell Cycle

White Paper

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Introduction

Proper functioning of the cell cycle is crucial for cell survival and replication. Cell cycle checkpoints control the progression of the cell through the different stages of interphase (G1, S and G2), determining whether to proceed with cell division based on a number of internal and external cues (figure 1).

The effect of compounds on the cell cycle during interphase can be measured by staining the cells with Propidium Iodide (PI), a fluorescent dye that binds double stranded DNA, and detection by flow cytometry. Those cells currently in G1 (preparing for DNA replication) will have half the level of PI fluorescence intensity than those in G2 (DNA replication complete, cell preparing for division).

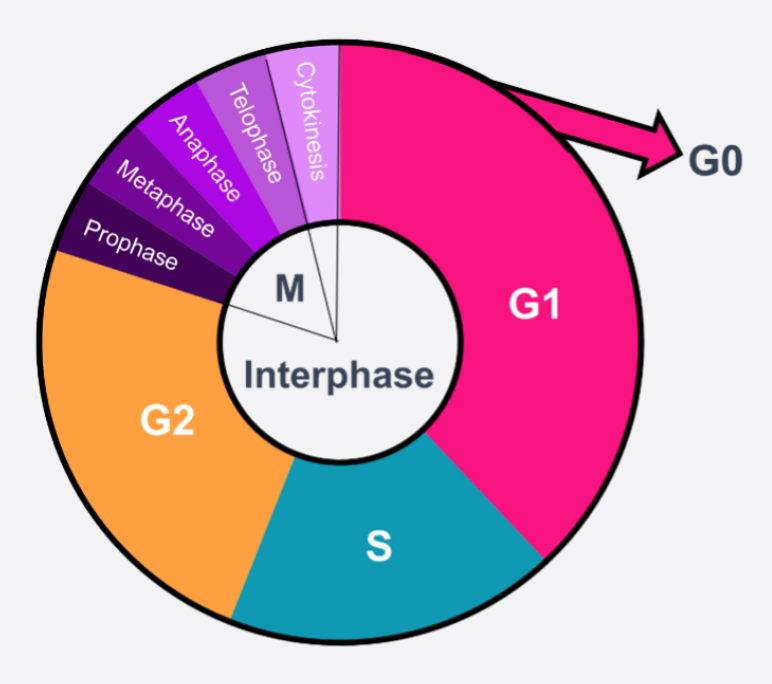

Figure 1: Overview of cell cycle. During interphase, cells move through 3 different stages; G1 where cells are metabolically active and increasing in size, S where DNA replication takes place, and G2 where the cell continues to grow and prepares for mitosis. At the junction of each of these stages are checkpoints which may prevent the cell from progressing if it is not ready for division. Following G2, cells undergo mitosis (M) followed by cytokinesis. Cells then start the process again or rest (G0).

Assay Procedure

THP-1 cells are plated into 96 well plates and incubated with test compounds, dispensed by a Multidrop™ Pico 8 (Thermo Scientific™), for 24 hours (37°C, 5% CO_2). Following washing, cells are fixed and permeabilized. Cells are then treated with RNase and stained with PI prior to sample acquisition on the MACSQuantX with PI intensity determination.

Data Analysis

Analysis is performed using FlowJo software. Cells are gated initially on total cell population using a forward scatter area (FSC-A) vs side scatter area (SSC-A) dot plot (to exclude dead cells/debris), and then gated on the single cell population using FSC-A vs forward scatter height (FSC-H). Gated single cells are subsequently viewed in a histogram plot for PI (B3 channel), with the cell cycle analysis FlowJo plugin applied, and percentage of cells in G1, S or G2/M analysed.

Plate statistics are calculated using positive (G1 or G2 arresting compounds) and negative (DMSO vehicle) controls to determine assay quality (Z'-factor ≥ 0.5), with IC_{50} values of the control compounds also examined if in CRC assay format.

Assay Throughput

The cell cycle assay can be performed either in a concentration-response curve (CRC) assay format with a maximum of 10 test compounds assessed in duplicate per batch, or in a single-point assay format with a maximum of 32 compounds assessed in duplicate per batch.

Example Data

Alisertib, an Aurora A Kinase inhibitor, and Cytochalasin D, an actin polymerization inhibitor, were tested alongside Palbociclib, Ribociclib and Abemaciclib, CDK4/6 inhibitors, in the cell cycle assay to determine G1 or G2 arrest.

Single Point Assay Format

Cells were treated in duplicate at a single concentration of compound; 1 µM (Alisertib) and 10 µM (Cytochalasin D, Palbociclib, Ribociclib and Abemaciclib) concentrations (figure 2).

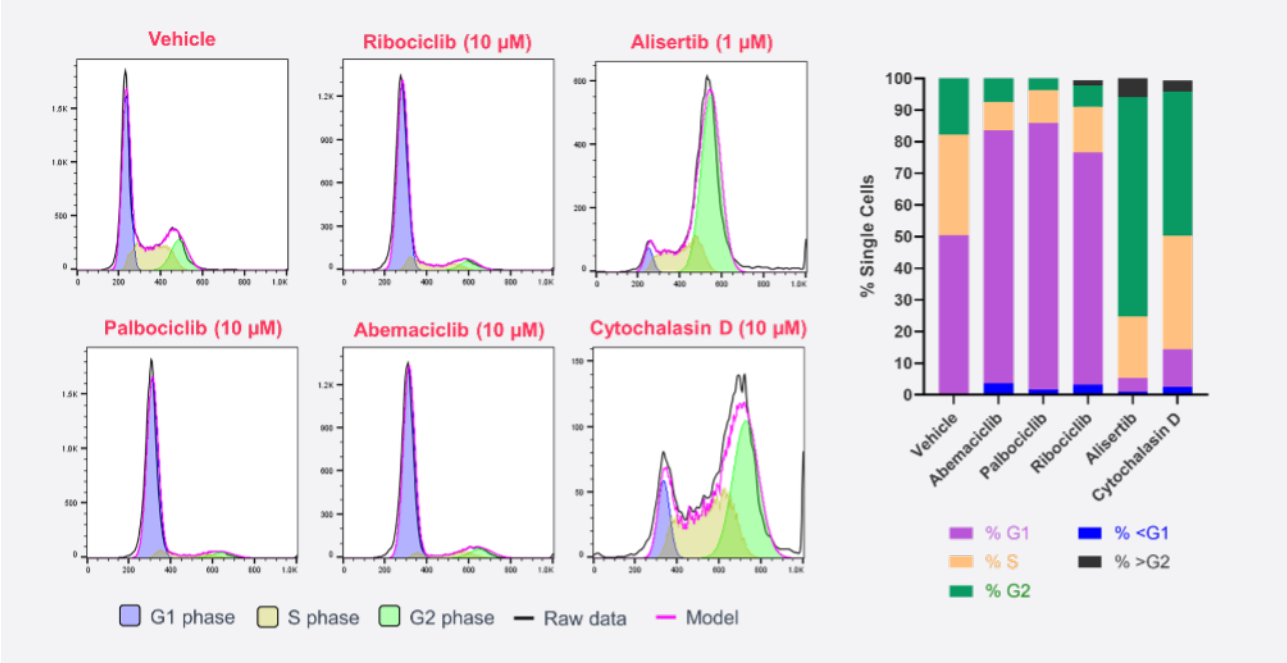

Figure 2: A range of cell cycle arresting compounds were tested alongside a vehicle control (DMSO) in the cell cycle assay in single point assay format. Histograms show fitted cell cycle model (pink line) used to determine % of cells in each of the cell cycle phases; G1 (purple), S (yellow) and G2 (green). Bar chart shows Abemaciclib, Palbociclib and Ribociclib arrest cells in the G1 phase (increase in %G1 compared to vehicle control – purple section of bar), whereas Alisertib and Cytochalasin D arrest cells in G2 (increase in %G2 compared to vehicle control – green section of bar).

CRC Assay Format

Cells were treated in duplicate with an 8-point, 3-fold dilutions series of each compound, with 1 µM (Alisertib) and 10 µM (Cytochalasin D, Palbociclib and Ribociclib) top assay concentrations (figures 3, 4 and 5).

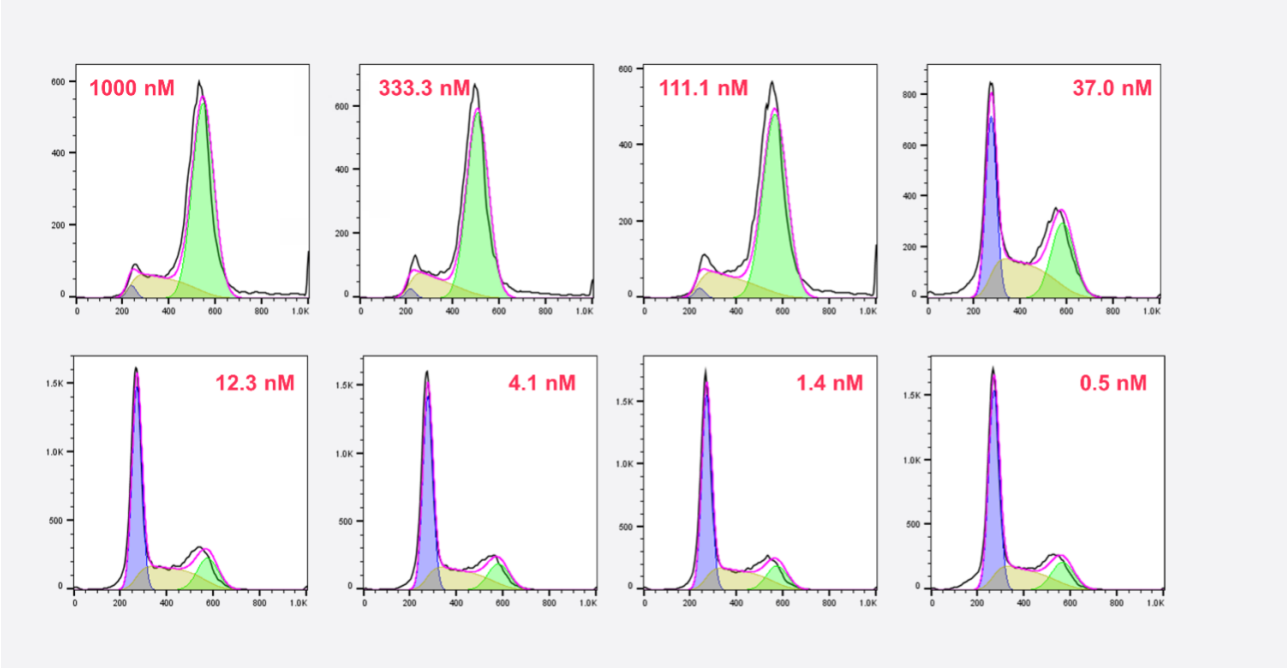

Figure 3: Example cell cycle histogram data for Alisertib (a G2 arresting compound) tested in CRC assay format; 8-point, 3-fold dilution series, 1 µM top concentration. G1 = purple, S = yellow, G2 = green, black line = raw data, pink line = fitted model. Concentration of Alisertib for each histogram indicated in pink.

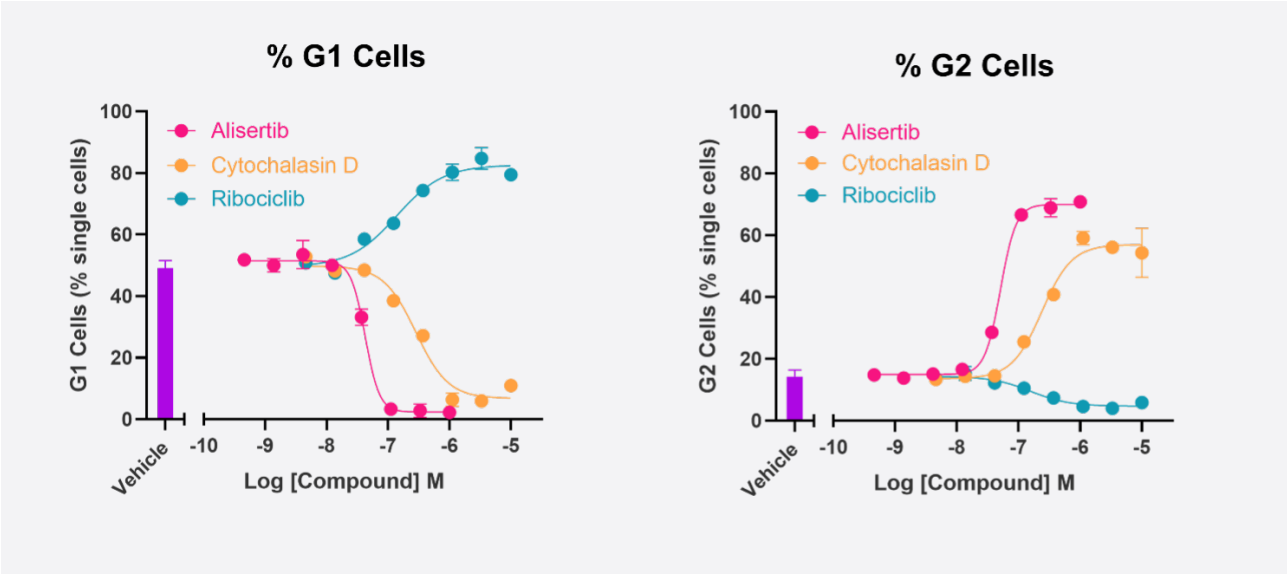

Figure 4: Concentration-response curves for Alisertib, Cytochalasin D (G2 arresting compounds) and Ribociclib (G1 arresting compound) tested in the cell cycle assay. Compounds were tested in duplicate in 8-point, 3-fold dilution series, at 10 µM (Cytochalasin D and Ribociclib) and 1 µM (Alisertib) top concentrations. IC50 values were determined as 46.7 nM (Alisertib), 261.7 nM (Cytochalasin D) and 147.3 nM (Ribociclib).

Figure 5: Plate statistics (Z'-factor) used to determine assay quality. The Z'-factor is calculated using the mean (μ) and standard deviation (σ) of the positive and negative controls; Z'-factor = 1 – $[(3\sigma_{pos} + 3\sigma_{neg})/(\mu_{pos} - \mu_{neg})]$. An assay Z'-factor > 0.5 (indicated by dotted line) is considered excellent. Using Alisertib as a control, the average Z'-factor of the cell cycle assay is 0.78.

Deliverables

If the assay is performed in single point format, the percentage of cells in each of G1, S and G2 phase will be reported per compound at the tested concentration. If the assay is performed in CRC format, curves for each compound will be provided with IC_{50} values. Data turn around expected to be within 1-2 weeks depending on the batch size.

Conclusion

Domainex has established a robust assay for assessing the effects of compounds on the cell cycle in THP-1 cells. This assay is available for routine screening. Domainex can develop the assay in further cell lines, on behalf of clients, as required.

Key Information	Details
Test compound concentration	Typically, 10 μM top concentration
Final DMSO concentration	0.1% DMSO (assuming 10 μM top concentration)
Positive controls	Ribociclib (G1 arresting compound) Alisertib (G2 arresting compound)
Incubation Duration	24 hours
Cell Line	THP-1 (assay can be developed in other cell lines on request)
Analysis	FlowJo Cell Cycle Analysis module
Compound Requirement	10 μL of 10 mM stock in DMSO (or 1mg of solid material)
Deliverables	% cells in each cell cycle phase (%G1, %S, %G2), IC_{50} values if tested in CRC assay format
Assay Format	Single point or CRC

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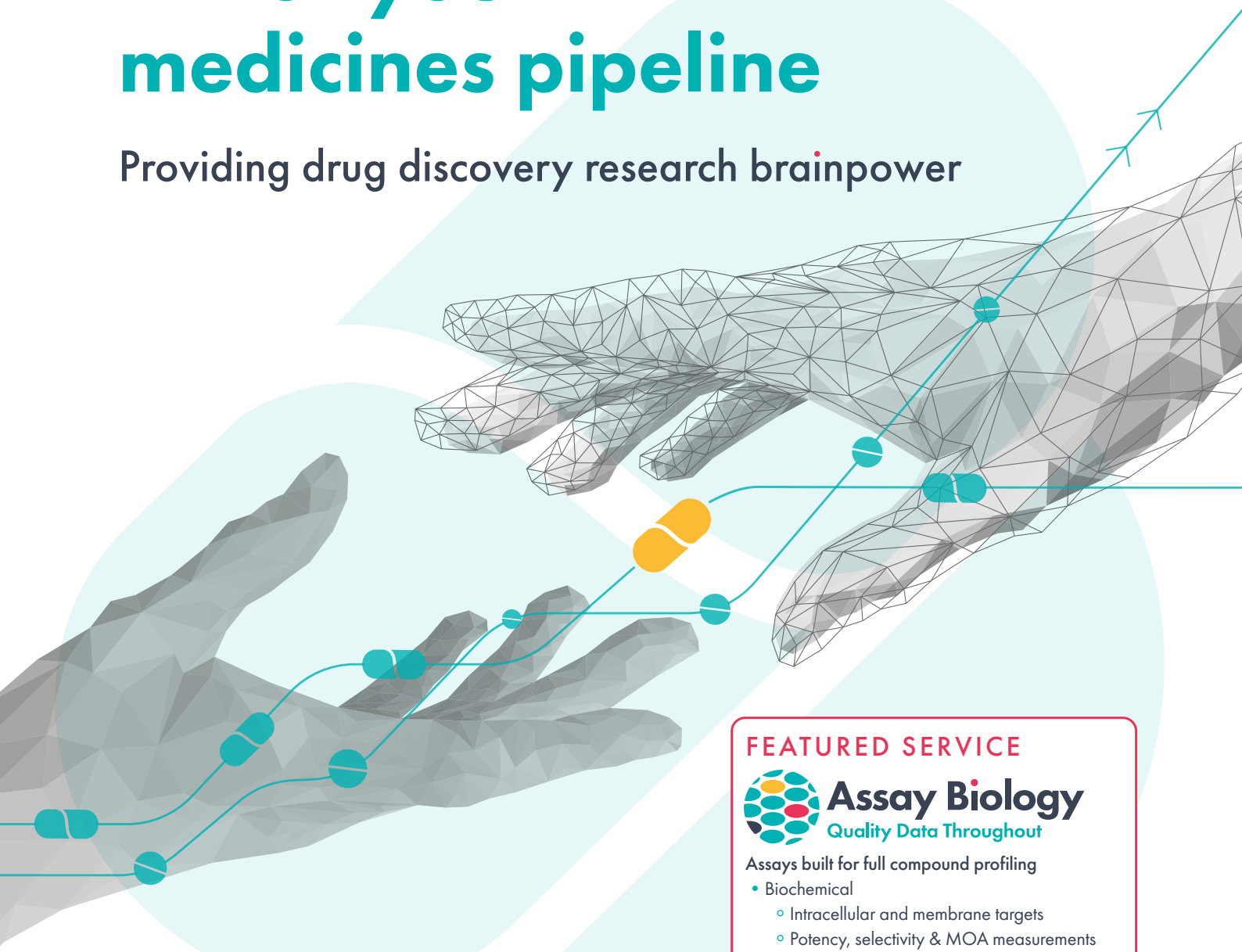

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