MAY 22, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.eq2ly7qpqlx9/v1

Protocol Citation: Rolando DZ Lyles, M Julia Martinez, Benjamin Sherman, Kerry Burnstein 2023. 96-well plate cell growth optimization for integrated live-cell and endpoint viability drug screening assay. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2ly7qpqlx9/v1

Protocol status: Working

Created: Mar 01, 2023

Last Modified: May 22, 2023

PROTOCOL integer ID: 77847

96-well plate cell growth optimization for integrated live-cell and endpoint viability drug screening assay

In 1 collection

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ABSTRACT

This protocol details the cell growth optimization for integrated live-cell and endpoint viablility drug screening assay on a 96-well plate.

ATTACHMENTS

S1.pdf

GUIDELINES

To accurately integrate live-cell imaging and endpoint viability, the user must define conditions which promote cell growth, prevent over-confluence, and fall within the detectable linear range of the endpoint cell viability assay. This protocol is designed to identify the optimal cell seeding density and media conditions to be used for integrated live-cell imaging and endpoint metabolic ATP viability assays for 72-hour drug screens using 96-well plates.

Note

*This protocol may be iteratively repeated and adjusted to obtain the user's desired 72-hour cell growth rate and final confluence.

This protocol is designed to identify optimized seeding conditions for a single cell line so that it is compatible for sequential live-cell imaging and endpoint viability assays in a 72-hour drug screen. Successfully optimized seeding densities and conditions meet the following basic criteria:

Keywords: Prepare cell lines for seeding, Seeding cells, IncuCyte ZOOM live-cell imaging

1. **Live-cell imaging displays healthy cells which are growing throughout 72-hours.**
2. **Cells aren't overconfluent at 72-hour endpoint. (We recommend a final confluence ≤ 60%, however, some slow growing cell lines may need to be seeded more densely and, in these cases, avoid conditions where cells are growing in clumps at experimental endpoint).**
3. **The range of seeding densities (at each condition) fall within the detectable linear range of the endpoint viability assay.**

The user may find a range of options that fit these criteria. In these cases, it is to the user's discretion to select the conditions that align best with their study objective.

Tips for data analysis:

- **Track confluence throughout live-cell imaging steps- at endpoint export confluence data for all wells as an average of each of the images. This will allow you to calculate growth rate as well as final average endpoint confluence.**
- **To determine if seeding densities are within the detectable linear range of the endpoint viability assay, Create an X-Y scatterplot where raw luminescence values averaged for each replicate is graphed at each seeding density. Perform a linear regression or linear curve fit to calculate the R^2 (a value which denotes how well the data "fits" the linear curve). Note: R^2 values range from 0-1 where ≥ 0.9 is considered a good fit.**
- **If users are planning to optimize multiple cell lines for comparative drug screens, then it is recommended to select conditions which allow the most similarities across cell lines. I.e. if one cell line is only within an optimized range when 2% FBS is used but another is in an optimized ranged at either 2% or 5% FBS, proceed with both cell lines using 2% FBS to limit variables.**

MATERIALS

Required Materials:

End live-cell imaging procedure and perform CellTiter-glo endpoint viability assay

- White opaque tape
- Standard 8-channel p200 multi-channel pipette
- Standard 25 mL reagent reservoirs (VWR, Cat#89094-662)
- CellTiter-glo (Promega, Cat# G7572) Luminescence Viability Assay or comparable endpoint cell viability assay
- Luminometer compatible with the Promega CellTiter-Glo assay. This protocol features the Promega GloMax (Promega, Cat# GM3500) Explorer Multimode Microplate Reader.

Note

*Pre-mixed CellTiter-glo reagent is stored at -80 °C (pre-aliquot 10 mL into 15mL conical tubes) so begin thawing (in a dark environment) to Room temperature upon arrival to lab. It will take approximately 3-4 hours to equilibrate to Room temperature.

HyClone Dulbeccos Phosphate Buffered Saline: Liquid **Cytiva Catalog #SH30028.02**

Corning® 100 mL Trypsin EDTA 1X **Corning Catalog #25-052-CI**

Defined Trypsin Inhibitor **Thermo Fisher Catalog #R007100**

Reagent reservoirs **VWR International Catalog #89094-662**

Greiner CELLSTAR® 96 well plates **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M0437-32EA**

Greiner bio one cell culture microplate (#655098) **greiner bio-one Catalog #655098**

CellTiter-Glo® Luminescent Cell Viability Assay **Promega Catalog #G7570**

Equipment

GloMax Explorer	NAME
multi-mode plate reader	TYPE
Promega	BRAND
GM3500	SKU

BEFORE START INSTRUCTIONS

Before starting:

- Determine the cell line to be optimized and expand cell line to ~50-60% confluence in any standard tissue t75-Flask or 15cm tissue culture dish.
- Define cell seeding density range to be tested. This protocol uses 4 seeding densities. Always set initial seeding density to the cell line-specific lab standard or literature recommended seeding density for 96-well plate assays. Then set a range using 50% increments (one below and two above) of initial seeding density.

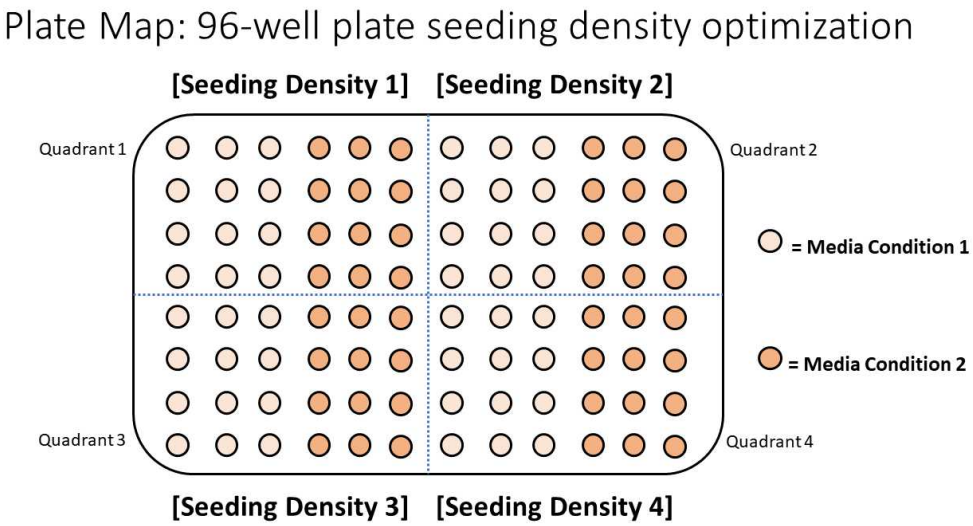
Note

Example: If 1000 cells/well is recommended, then the range should be set based on (%50 of 1000) 500 cells/well. The initial 4 seeding increments would be 500 cells/well, 1000 cells/well, 1500 cells/well, and 2000 cells/well.

- Determine desired cell culture media conditions to be tested. This protocol 2 media conditions with varying serum concentrations. As a reference point, this protocol suggests preparing cell culture media with cell specific recommended serum concentrations and preparing cell culture with between 50-80% less serum to regulate cell growth rates.

Note

Example: If 10% Fetal Bovine Serum (FBS) is recommended, then the second concentration could be set at 5% or 2% FBS. See plate map below:



Day 1: Prepare cell lines for seeding into 96-well plates for...

1 In a sterile tissue culture hood, aspirate media from t75-flask.

2 Perform a wash with 5 mL Phosphate Buffered Saline (PBS) (Hyclone, Cat# SH30028.02).

3 To detach cells from tissue culture flasks, add 3 mL of trypsin (Corning, Cat# 25-052-cl) directly on to the cells. Gently tilt the flask until the surface is equally covered with trypsin then place cells into tissue culture incubator for 00:03:00. 3m

4 After incubation, gently tap the sides of the flask to insure complete detachment of adherent cells. Return to incubator for 00:01:00 increments if needed. 1m

Note

*Use a microscope at 5-10x magnification to verify detachment.

5 Add 3 mL of trypsin inhibitor (Thermofisher, Cat# R007100) (or equal volume to trypsin used) to deactivate the trypsin enzyme, collect all the cell suspension, and then transfer into a sterile 15mL conical tube.

6 Pellet cells via centrifugation at 100-300 x g, 00:05:00. 5m

Note

It is recommended that you prepare your media at the pre-determined optimization conditions during centrifugation. See suggestions below:

- *Media condition 1*: General cell-specific base culture medium supplemented with antibiotics and full-serum (dependent on recommended cell line specific culture conditions). ~ 12 mL.
- *Media condition 2*: General cell-specific base culture medium supplemented with antibiotics and 50-80% less serum than condition 1. ~ 12 mL
- You will also need to prepare general cell-specific base culture medium supplemented with antibiotics but SERUM FREE. ~ 5 mL.

7 After centrifugation, aspirate all supernatant while being careful to not disrupt the cell pellet.

8 Uniformly resuspend the cell pellet in 1mL of SERUM FREE media using a p1000 pipette.

Note

*Be gentle so to not shear the cells.

*Then add an additional 1 mL of SERUM FREE media and slowly vortex to uniformly mix.

9 Using the well-mixed cell suspension, accurately count cells via your preferred hemocytometry method to a final unit of [cells/mL].

Day 1: Seeding cells into 96-well plates for optimization

10

Note

Cells will be seeded into 96-well plates at a volume of 200 µL /well. Thus, the concentrations that were selected should be adjusted so that there are X#ofCells/ 200 µL of appropriate media.
Example: If the desired cell density range was 500 cells/well, 1000 cells/well, 1500 cells/well, and 2000 cells/well, then the adjusted concentrations should be 500 cells/200µL etc. This would result in 2500 cells/mL, 5000 cells/mL, 7500 cells/mL, and 10,000 cells/mL respectively.

After counting, calculate the volume of cells suspended in SERUM FREE media needed to prepare 12 mL at the adjusted seeding density concentration range previously determined.

Note

Note: In this protocol, each seeding density and seeding density are tested in replicates of 12. Thus, at 200 µL /well, 2.4 mL of each media condition will be the minimum required volume per seeding density (See plate map above). To find the requisite volume of cell suspension, use the formula:

$$V_1= \frac{(C_2 V_2)}{C_1}$$

where V_1= Desired volume of cell suspension, C_1= Concentration [cells/mL] of cell suspension, C_2=Desired final concentration [cells/mL] of new cell preparation, & V_2= Desired final volume of new cell preparation.

11 Using the two media conditions prepared between steps 6 & 7, prepare 3 mL of each seeding density/ media condition combination in separate sterile 15 mL conical tubes.

Example table:

	A	B	C	D
	Cell Line	**Seeding Density**	**Media Condition**	**Final Volume**
	Cell X	500 cells/well	Condition 1	3 mL
	Cell X	1000 cells/well	Condition 1	3 mL
	Cell X	1500 cells/well	Condition 1	3 mL
	Cell X	2000 cells/well	Condition 1	3 mL
	Cell X	500 cells/well	Condition 2	3 mL
	Cell X	1000 cells/well	Condition 2	3 mL
	Cell X	1500 cells/well	Condition 2	3 mL
	Cell X	2000 cells/well	Condition 2	3 mL

12 One at a time, gently vortex each tube and transfer into a standard sterile standard 25 mL reagent reservoir (VWR, Cat# 89094-662). Then using an 8-channel p200 multi-channel pipette, transfer the cell suspensions into a cell culture 96-well microplate with white opaque walls and a clear bottom (Sigma Aldrich, M0437-32E) at their proper locations as specified by the above plate map.

Note

***Note**: These types of plates are required for the execution of the integrated live-cell and endpoint viability drug screen as live cell imaging requires clear bottoms for imaging and endpoint viability assays work best with wells isolated by opaque walls.

13 Place 96-well plate into standard tissue culture incubator Overnight . 5m

Day 2: Verify cell attachment to plates and begin IncuCyte ...

14 Observe cells with a microscope at 10x magnification to verify that cells are settled and adhered to the inner surface of the 96-well plate. Verify that there is a reasonable difference in expected

seeding densities.

Note

*__Ask the question__: “Do cells seeded at 2000 cells/well appear ~4 times more confluent than cells seeded at 500 cells/well?”

15 At this stage, the plate can be transferred to the preferred live-cell imaging platform. This protocol uses the IncuCyte Zoom platform. The plate will remain in the imaging platform for 72:00:00 (the duration of the drug screens in the integrated live-cell and endpoint viability drug screen protocol). 3d

16 The instructions below apply exclusively to the IncuCyte Zoom platform which this protocol features.

Note

__Note__: basic user instructions for the IncuCyte Zoom can be found in publicly available online videos.

Begin IncuCyte Zoom set up:

16.1 Open IncuCyte Zoom software on computer desktop.

16.2 Connect to device.

16.3 Under the “Task List” panel on the left-hand side, select “Schedule Scans”.

16.4 Click one of the “Empty” slots on the live representative plate map for the hardware then click “Add Vessel”.

16.5 Once prompted, search from the vessel (96-well plate) by catalog number. This protocol features the Greiner bio one cell culture microplate (#655098). Once selected proceed to setting up experimental parameters:

16.6 On the bottom panel on the left-hand side, select “Edit Scan Pattern” and select all wells and set the scan pattern to 4 images/well. Save this scan pattern.

16.7 In the Channel Selection section in the center, click “Phase” (no colored acquisition channel is need for this protocol).

16.8 In the “Scan Mode” section in on the top-right side, toggle to the scan pattern that was previously created and saved.

16.9 In the “Analysis Job Setup” section on the right-hand side, toggle the “Job Type” and select “Basic Analyzer.” Toggle the “Processing Definition” tab and select a pre-determined processing definition with masks optimized for your specific cell line. (If this hasn’t been created, the “DEMO Phase” processing definition can be used.

16.10 Click the “Properties” tab and label the plate as desired.

16.11 Click “Apply” on the bottom right corner to save changes and register the plate to the IncuCyte Zoom hardware.

Note

Aside: Set the scan intervals based on desired timepoints. This protocol recommends a scan every 4 hours.

17 Transfer the 96-well plate into the same IncuCyte Zoom slot selected during the software setup and begin real-time image capture.

Note

*THIS PORTION OF THE PROTOCOL WILL LAST FOR ~72 HOURS. THE LIVE-CELL IMAGING PROCESS SHOULD NOT REQUIRE ANY ADDITIONAL ADJUSTMENTS DURING THIS TIME; HOWEVER, THE USER CAN CHECK IMAGES DAILY TO TRACK PROGRESS AND VERIFY IMAGE FOCUS AND SCAN QUALITY. *

Day 5: End live-cell imaging procedure and perform CellTite..

20m

18 After 72-hours have passed, end the experiment on the live-cell imagining platform software then remove the 96-well plate and allow and allow it to equilibrate to Room temperature ~ 00:20:00 . 20m

Note

This can be done in a non-sterile environment such as a bench top or isolated drawer.

19

Note

*Use the following instructions to end the experiment on the IncuCyte ZOOM software.

End IncuCyte Zoom Experiment:

19.1 Open IncuCyte Zoom software on computer desktop.

19.2 Connect to device.

19.3 Under the “Task List” panel on the left-hand side, select “Schedule Scans”.

19.4 Click on the slot housing the plate being tested on the live representative plate map for the hardware then click “Remove Vessel”.

19.5 Click “Apply” on the bottom right corner to save changes.

19.6 Manually remove the corresponding 96-well plate to the IncuCyte Zoom hardware.

20 Cover the bottom of the 96-well plate with white opaque lab tape.

Note

***Note**: Cover the bottom of each plate with opaque white tape (as recommended by Promega) to prevent any luminescence decrease or “cross talk” when performing the CellTiter-glo assay as the luminometer reads the each well from the top of the plate.

See example below:

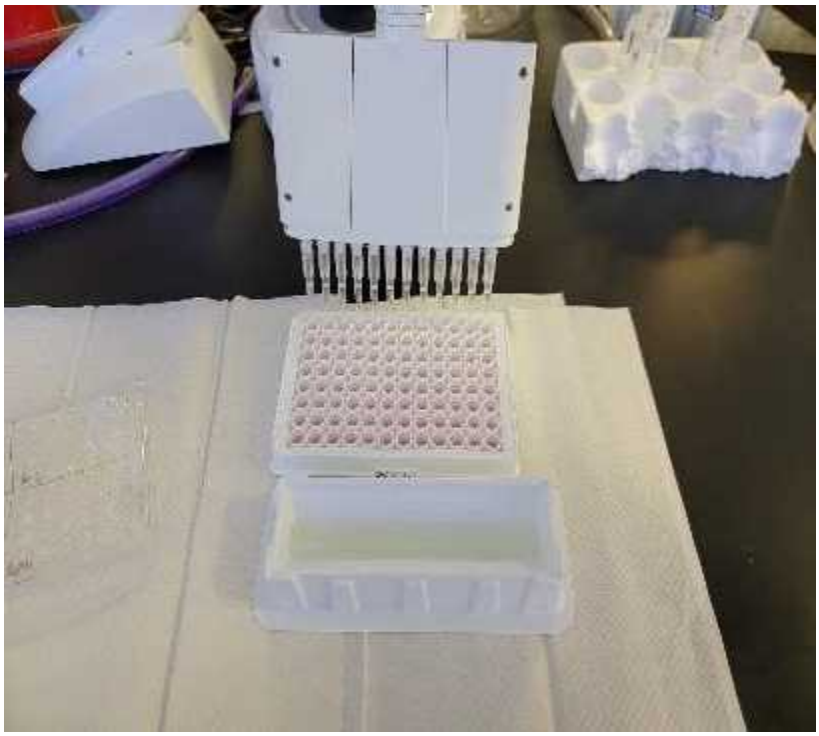
21 Transfer 10 mL (per plate) of the thawed CellTiter-Glo reaction reagent into a standard 25 mL reagent reservoir.

22 Use an 8-channel p200 multi-channel pipette (or comparable multi-channel pipette) to transfer 100 µL of CellTiter-Glo reaction reagent into each well of the 96-well plate.

23 Leave the plate cover off and transfer into a luminometer compatible with CellTiter-glo.

> **Note**
>
> Be mindful of the plate orientation and alignment to insure proper placement into the device.

See example below:



Note

* This protocol features the Promega GloMax (Promega, Cat# GM3500) Explorer Multimode Microplate Reader.

24 12m

Recommended parameters for the Luminometer are as follows:

1. Shake in an orbital shaker for 00:02:00 at 500 cycles/minute with a 1mm shaking diameter (cell lysis).
2. Incubate for 00:10:00 in a dark environment (reaction).
3. Read luminescence of each well at an integration of 400ms (data acquisition).

Note

*It will take ~ 00:13:00 to read each plate.

25

Export data and remove plate from luminometer.