Choosing Modern Assay Technologies to Develop Test Guidelines

ICCVAM Public Forum
May 21, 2020

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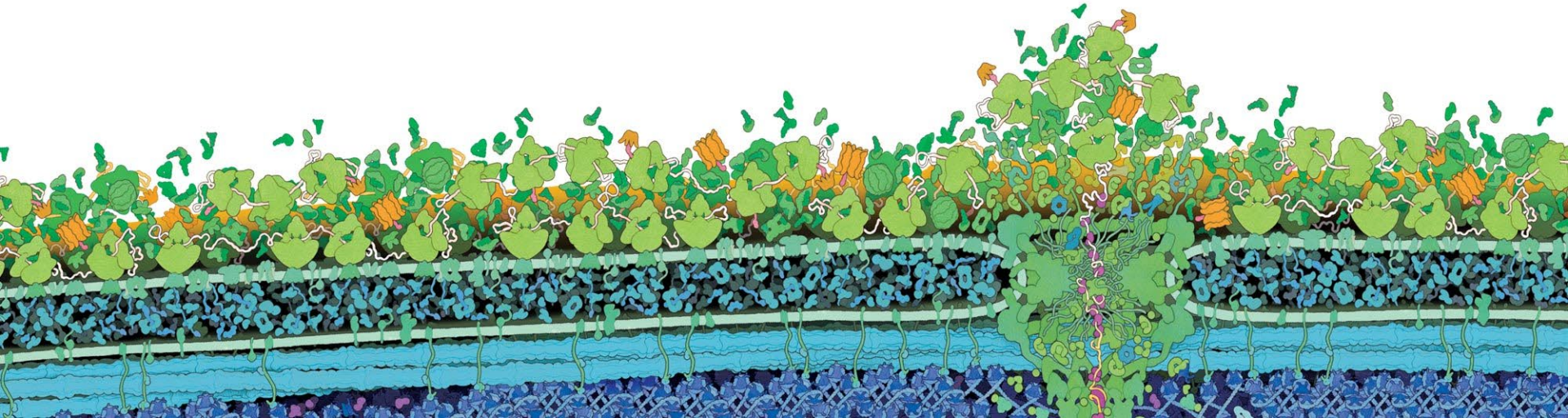


Goals:
Convince Assay Development Scientists to…

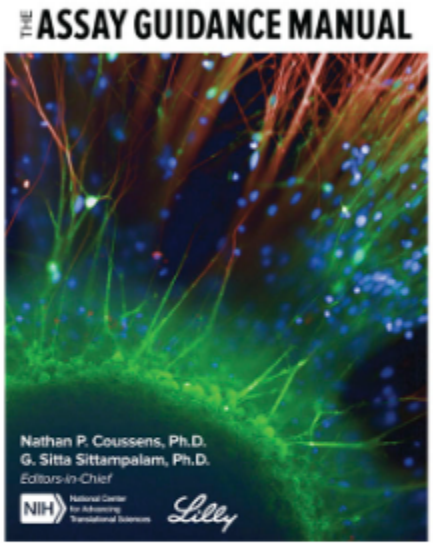
1. Become aware of available *in vitro* assay technologies to detect cell health…NIH Assay Guidance Manual

2. Take advantage of multiplexing orthogonal assays as efficient internal controls.

3. Reach out to scientific experts (including vendors) willing to consult on choosing the most appropriate assay chemistries.

4. Do these things before OECD guidelines are “set in stone”

Assay Guidance Manual



The **Assay Guidance Manual (AGM)** is a free, best-practices online resource devoted to the successful development of robust, early-stage drug discovery assays.

The manual was originally developed by Eli Lilly and Company to provide step-by-step guidance based on "tribal knowledge" from drug developers for planning and creating projects for high-throughput screening, lead optimization and early phases of regulated drug development. Tribal knowledge is any unwritten, well-tested information that is not commonly known by others within an institution. Well-tested methods outlined in the manual address appropriate statistical ways to analyze assay results and accommodate minor changes to assay protocols to ensure robustness.

https://ncats.nih.gov/expertise/preclinical/agm

U.S. National Library of Medicine
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NLM Citation: Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Sittampalam GS, Grossman A, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-.
Bookshelf URL: https://www.ncbi.nlm.nih.gov/books/

Cell Viability Assays

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Created: May 1, 2013; Updated: July 1, 2016.

Abstract

This chapter is an introductory overview of the most commonly used assay methods to estimate the number of viable cells in multi-well plates. This chapter describes assays where data are recorded using a plate-reader; it does not cover assay methods designed for flow cytometry or high content imaging. The assay methods covered include the use of different classes of colorimetric tetrazolium reagents, resazurin reduction and protease substrates generating a fluorescent signal, the luminogenic ATP assay, and a novel real-time assay to monitor live cells for days in culture. The assays described are based on measurement of a marker activity associated with viable cell number. These assays are used for measuring the results of cell proliferation, testing for cytotoxic effects of compounds, and for multiplexing as an internal control to determine viable cell number during other cell-based assays.

https://www.ncbi.nlm.nih.gov/books/NBK144065/pdf/Bookshelf_NBK144065.pdf

Missed Opportunities for Multiplexing

Examples:

- OECD Test No. 432: In Vitro 3T3 NRU Phototoxicity Test
- OECD Test No. 442D: In Vitro Skin Sensitization *ARE-Nrf2 Luciferase Test*

Example 1: In Vitro 3T3 NRU Phototoxicity Test

The phototoxicity test uses mouse fibroblast cells with an endpoint of viable cell number measured using the multistep Neutral Red uptake assay.

If you were designing a new *in vitro* phototoxicity test to predict effects in humans…

- Which cell line would you choose?
- Which cell viability assay method would you choose?
- Can you measure more than a marker of viable cells?

Why not choose a homogeneous assays to detect orthogonal endpoints using a multiplex approach?

Comparison of NR & ATP Assay Protocols

Set up Assay Plate and Treatments

↓

+/- Irradiation of cells

Multi-step Neutral Red

Wash cells warm buffer

↓

Remove by gentle tapping

↓

Add NR in serum-free medium

↓

Incubate 3 hours

↓

Remove NR medium

↓

Wash cells with buffer

↓

Decant & blot

↓

Add EtOH + acetic acid

↓

Shake 10min

↓

Record Abs 540nm

Homogeneous ATP

Equilibrate plate to RT

↓

Add 100µl CTG reagent

↓

Mix 2 min

↓

Incubate 10 min at RT

↓

Record RLU

Homogeneous single reagent addition no wash steps

Comparison of NR & MultiTox-Fluor Protocols

Set up Assay Plate and Treatments

↓

+/- Irradiation of cells

Multi-step Neutral Red

Wash cells warm buffer

↓

Remove by gentle tapping

↓

Add NR in serum-free medium

↓

Incubate 3 hours

↓

Remove NR medium

↓

Wash cells with buffer

↓

Decant & blot

↓

Add EtOH + acetic acid

↓

Shake 10min

↓

Record Abs 540nm

Homogeneous MultiTox-Fluor

Add MultiTox-Fluor reagent

↓

Mix

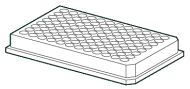
↓

Incubate 30 min

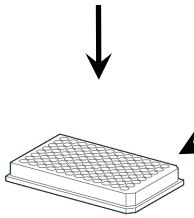
↓

Record fluorescence

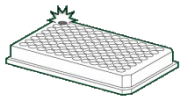
Measuring Viable Cells & Dead Cells Simultaneously



Subject cells to
various treatments

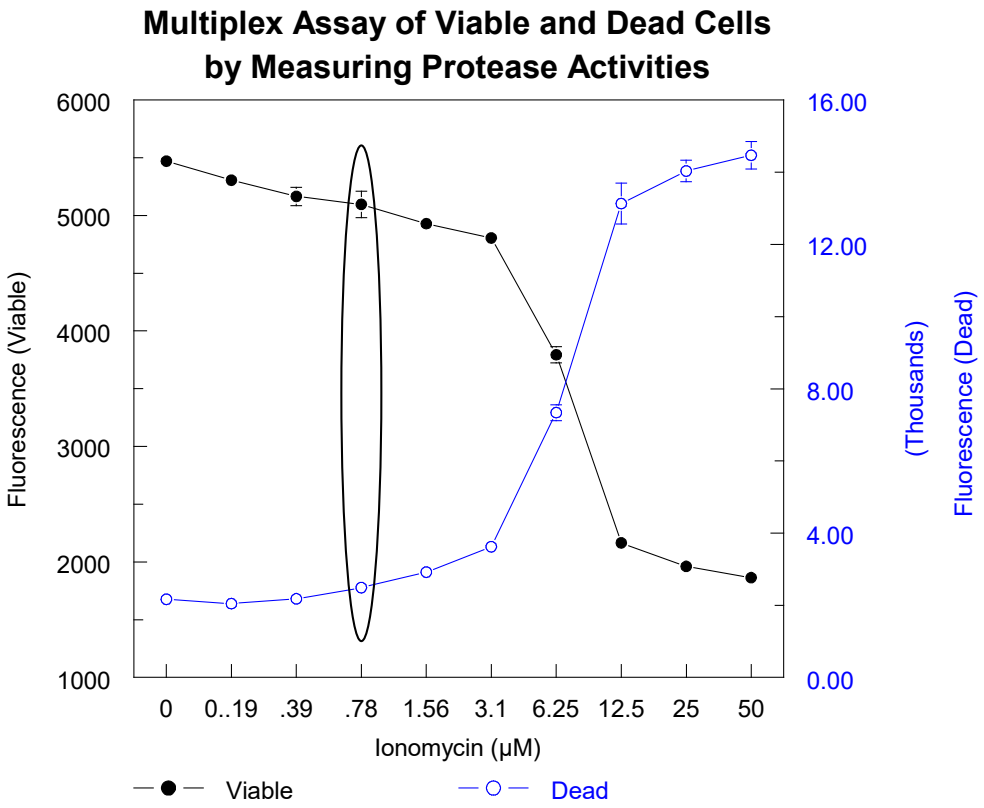


Add Reagent
with both Substrates



Incubate 15-30 min

Record Fluorescence
at 2 wavelengths



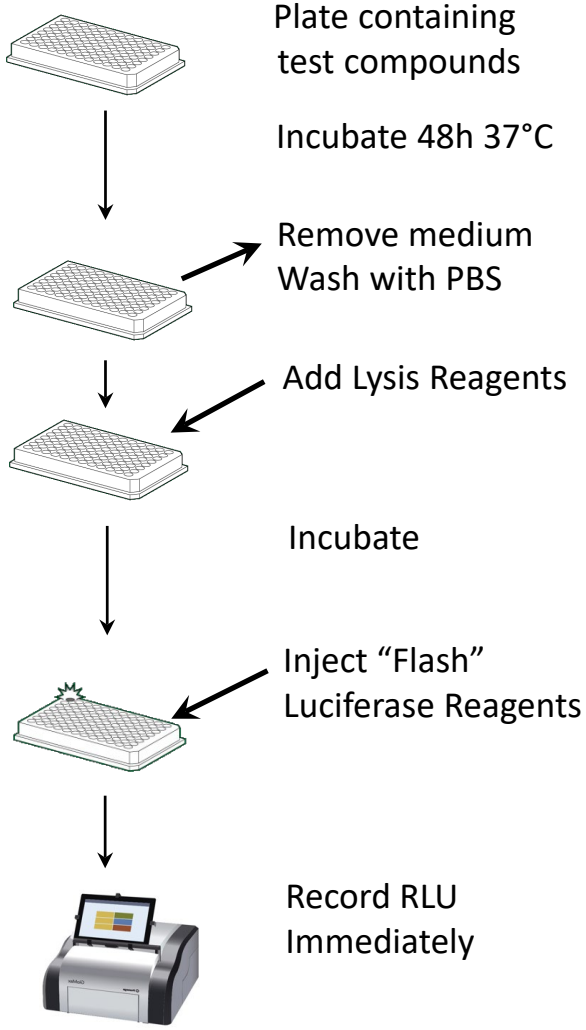
Example 2: Skin Sensitization

- KeratinoSens (and LuSens) In Vitro Skin Sensitisation assay uses a firefly luciferase reporter method to measure the expression of the ARE-Nrf2 gene.

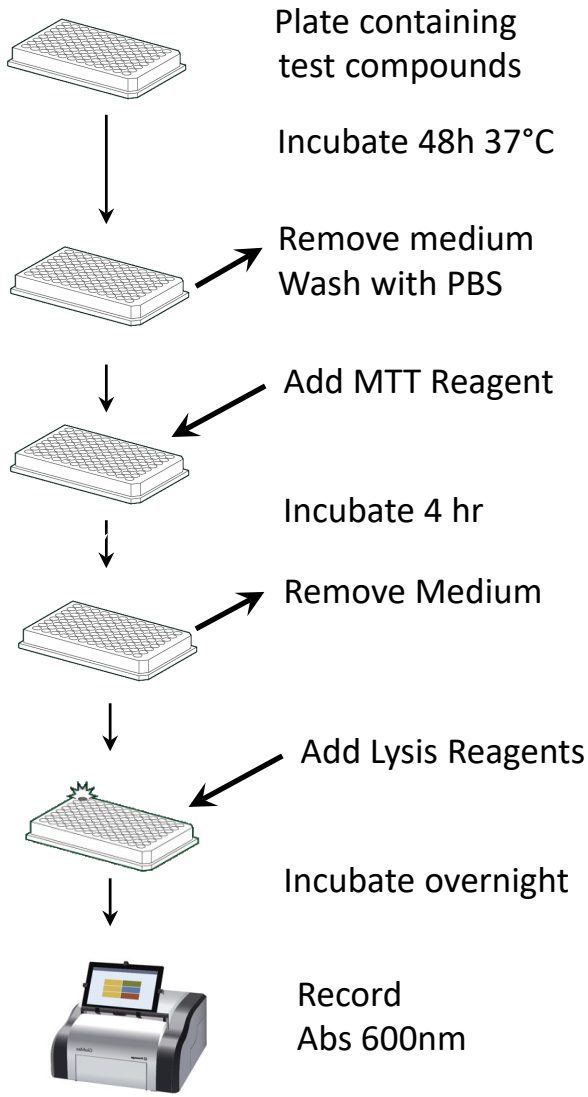
- The OECD protocol recommends using a separate assay plate as a control to test for cytotoxicity using an MTT assay.

KeratinoSens Assay in Parallel Plates

Reporter Assay

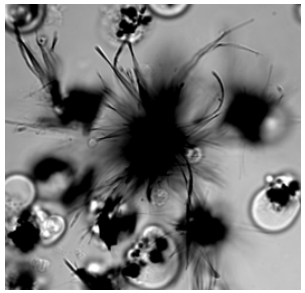


Viability Control



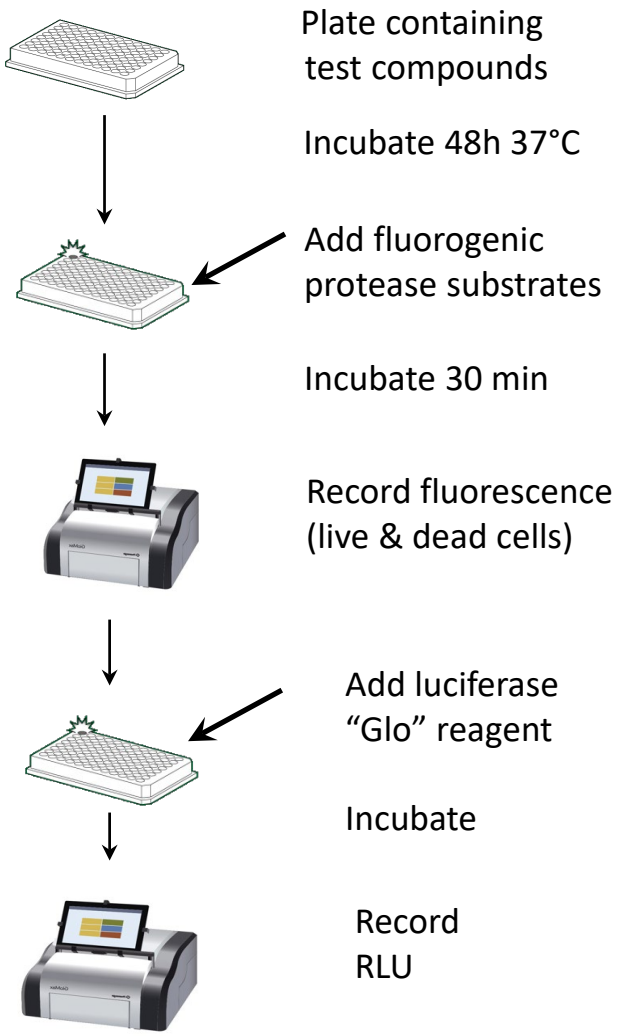
Why choose the MTT Assay?

- Why is the MTT assay done in a parallel plate?
- Why is the culture medium removed?
- Why is MTT solution removed?
- Do multiple protocol steps result in increased variability?



- It is possible to measure both viable and dead cells in the same sample used for the luciferase reporter assay.

Homogeneous Multiplex Live + Dead and Luciferase Reporter Assays



- **Multiplex protocol is "Add-Mix-Read"**
- **No medium removal or wash steps**
- **Reduced use of cells, culture medium, plates, etc.**
- **Statistical advantage of collecting control data from the same sample rather than parallel plates**

Benefits of Multiplexing

- More data per sample well
- Reduces costs (cells, media, plates)
- Confirming results with orthogonal method
- Normalization of data reduces error
  - Correct for plating errors
  - Differential growth of cells & edge effects
- Statistical advantage of assaying the same sample of cells instead of parallel plates

Recommendation

Scientists developing *in vitro* cell health assays that are proposed to become OECG guidelines should consider reaching out to the scientific staff of the vendors providing the assays to seek technical input regarding the choice of assays and potential for multiplexing.

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Questions Welcome

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