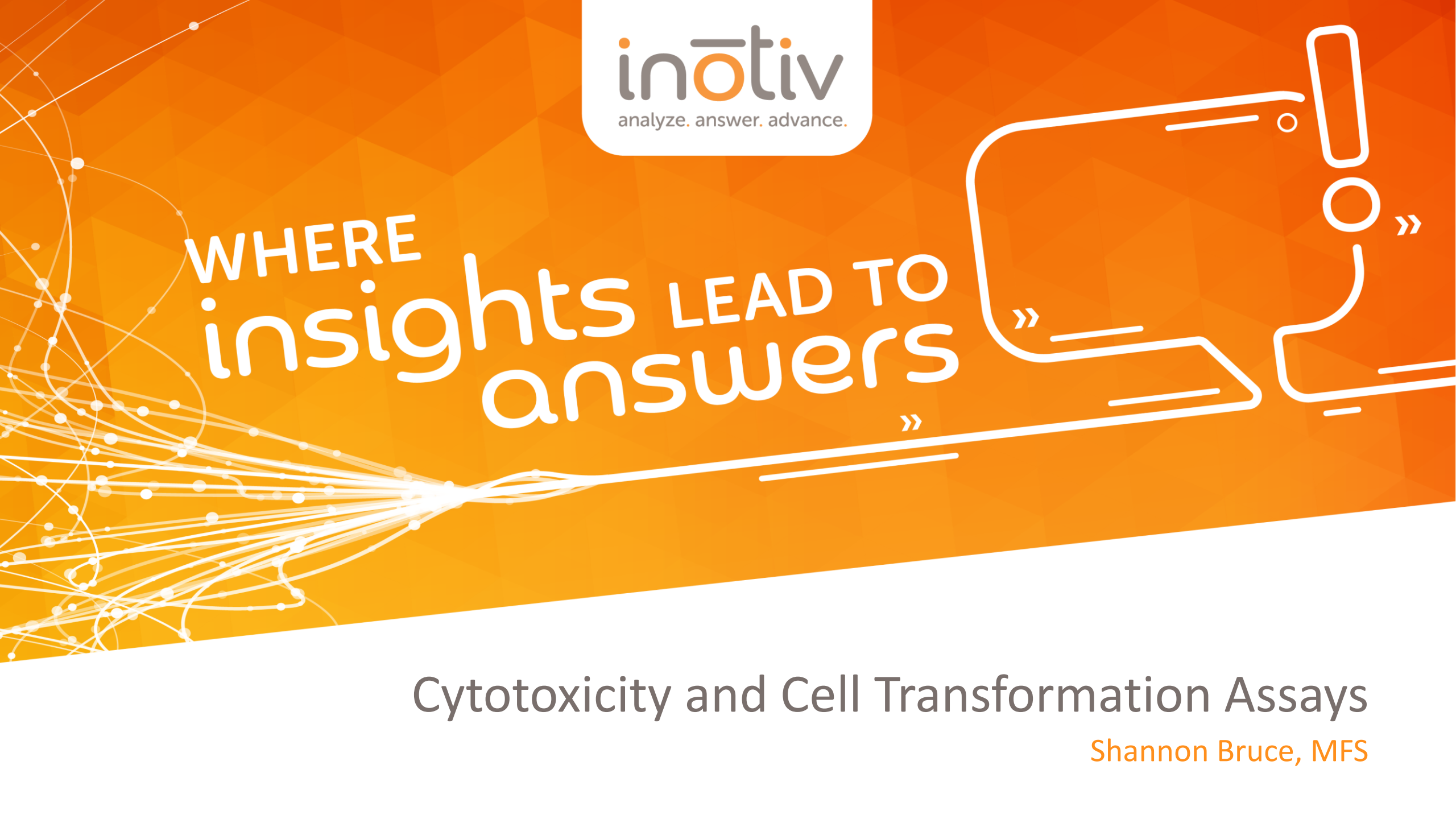




WHERE
insights LEAD TO
answers

Cytotoxicity and Cell Transformation Assays

Shannon Bruce, MFS



Neutral Red Uptake (NRU) Assay

Cytotoxicity Assay Topics for Discussion

- Neutral Red Uptake Assay

- > Assay History
- > Assay Foundation
- > Cell Lines
- > Experimental Design
- > Staining
- > Data Analysis
- > Evaluation

- Neutral Red Uptake Phototoxicity Assay

- > Assay History
- > Assay Foundation
- > Cell Lines
- > Experimental Design
- > Staining
- > Data Analysis
- > Evaluation

- Thiazole Blue (MTT) Cytotoxicity Assay

- > Assay Foundation
- > Cell Lines
- > Experimental Design
- > Staining
- > Data Analysis
- > Evaluation

Assay History

- Originally published as Appendix in Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Test Method Evaluation Report: In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests NIH Publication No. 07-4519 – Published 2006
- Organisation for Economic Co-operation and Development (OECD) document exists for the use of the assay
 - > OECD Guidance Document 129 entitled Guidance Document on using Cytotoxicity Test to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests (2010)

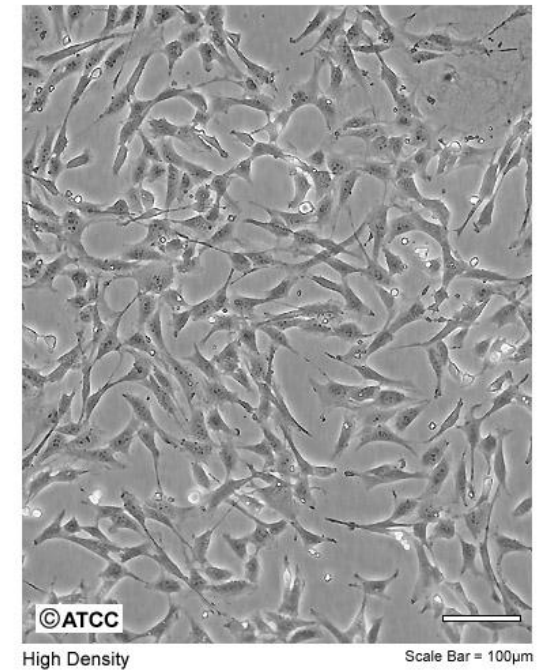
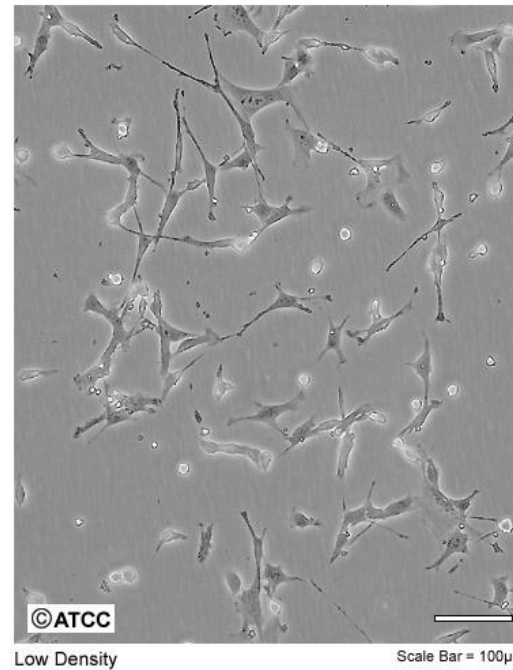
Assay Foundation

- The assay is based on the ability of viable cells to incorporate and bind neutral red , a supravital dye.
 - > Neutral Red is a weak cationic dye that readily penetrates the live cell membrane and accumulates intracellularly in lysosomes.
 - > Neutral red electrostatically binds to the anionic lysosomal matrix.
- Due to the cytotoxic effect of a test chemical, cellular membrane property changes will lead to cell death or a change in lysosomal structure and function resulting in decreased uptake and binding of neutral red dye.
 - > Thus, making it possible to distinguish between viable, damaged, and/or dead cells which is the basis of the assay.

Cell Lines

- BALB/c 3T3
 - > Mouse fibroblast cell line
 - > Obtained from ATCC (CCL-163)
 - May also be obtained from Japanese Collection of Research Bioresources Cell Bank (JCRB 9005)
 - > Adherent culture
 - Epithelial-like

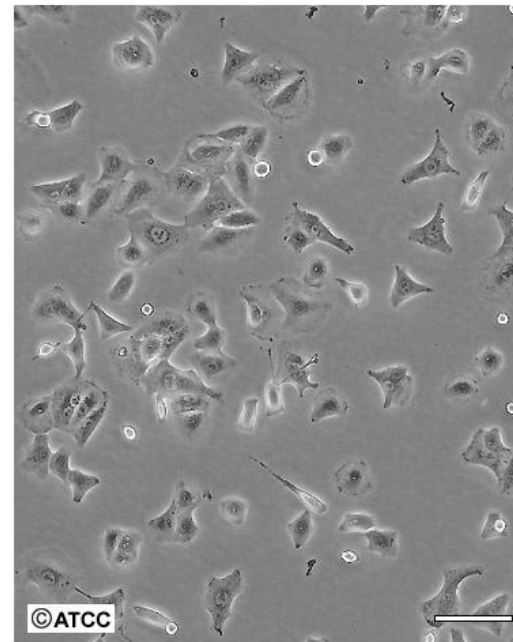
ATCC Number: **CCL-163**
Designation: **BALB/3T3 clone A31**



Cell Lines

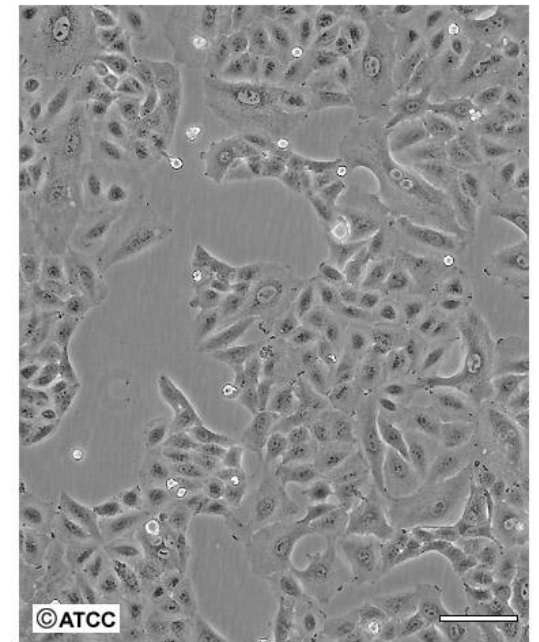
- A549
 - > Human Lung Carcinoma cell line
 - > Obtained from ATCC (CCL-185)
 - May also be obtained from Japanese Collection of Research Bioresources Cell Bank (JCRB 0076)
 - > Adherent culture
 - Epithelial-like

ATCC Number: **CCL-185**
Designation: **A-549**



Low Density

Scale Bar = 100µm



High Density

Scale Bar = 100µm

Experimental Design

- Assay is a three- to four-day experiment performed in 96-well plates
 - > Performed in two phases
 - Preliminary Toxicity Assay – singlet plate, 8 test chemical concentrations
 - Definitive Cytotoxicity Assay – duplicate plate, 8 test chemical concentrations
 - > Test chemical treatment time varies from 24 to 48 hours.
 - 48-hour treatment is standard
 - 24-hour treatment requires increase in cell number seeded
 - > Number of cells seeded varies by cell line
 - BALB/c 3T3 cells range from 2.0 to 3.0×10^4 cells/mL (2.0 to 3.0×10^3 cells/well)
 - A549 cells range from 5.0 to 8.0×10^4 cells/mL (5.0 to 8.0×10^3 cells/well)

Assay Design

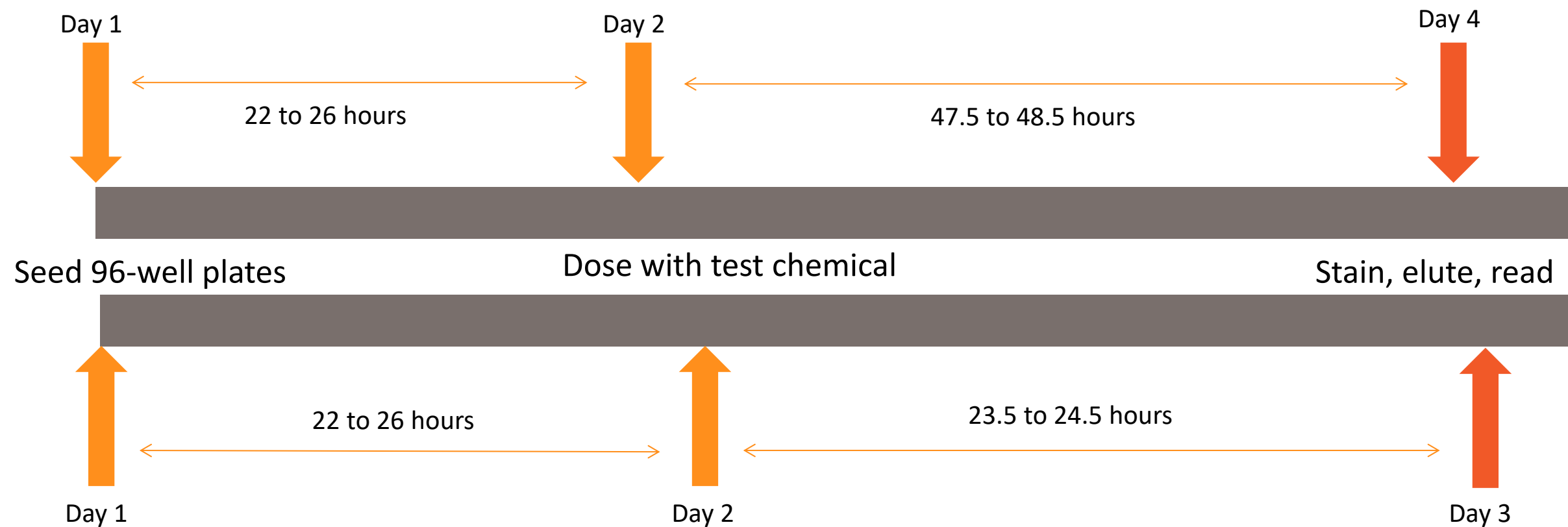


Plate Design

	1	2	3	4	5	6	7	8	9	10	11	12
A	VC _b	VC _b	C1 _b	C2 _b	C3 _b	C4 _b	C5 _b	C6 _b	C7 _b	C8 _b	VC _b	VC _b
B	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
C	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
D	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
E	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
F	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
G	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
H	VC _b	VC _b	C1 _b	C2 _b	C3 _b	C4 _b	C5 _b	C6 _b	C7 _b	C8 _b	VC _b	VC _b

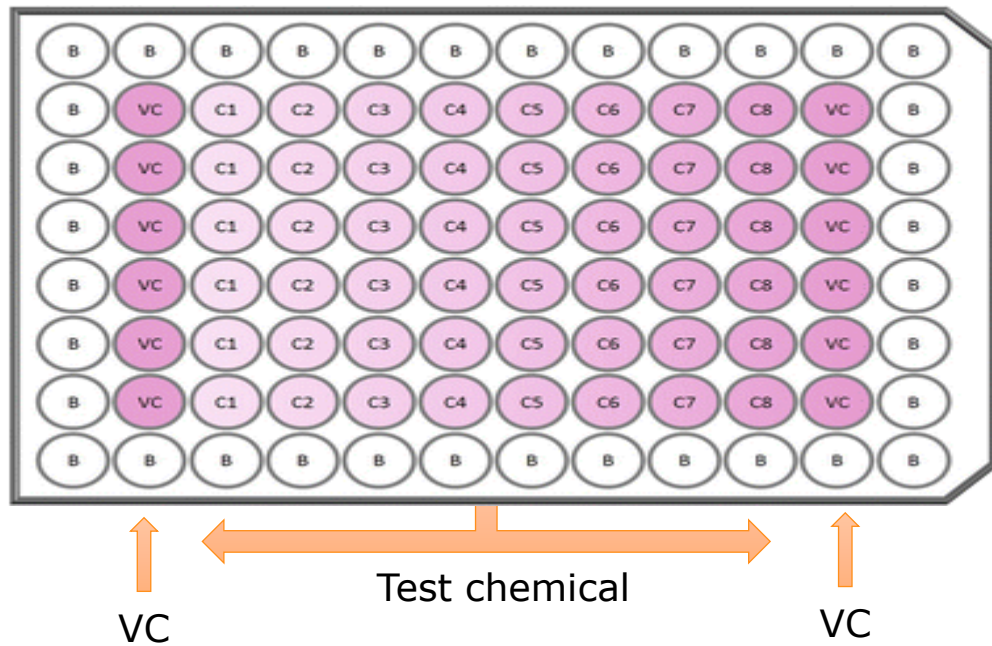
VC₁ and VC₂ = Vehicle controls. C1 – C8 = Test chemical or PC (SLS) at eight concentrations (C1 = highest, C8 = lowest)

Cx_b = Blanks (Test chemical or PC, but contain no cells)

VC_b = VC blanks (contain no cells)

Staining and Plate Reading

The lighter the color the fewer cells that have neutral red dye uptake

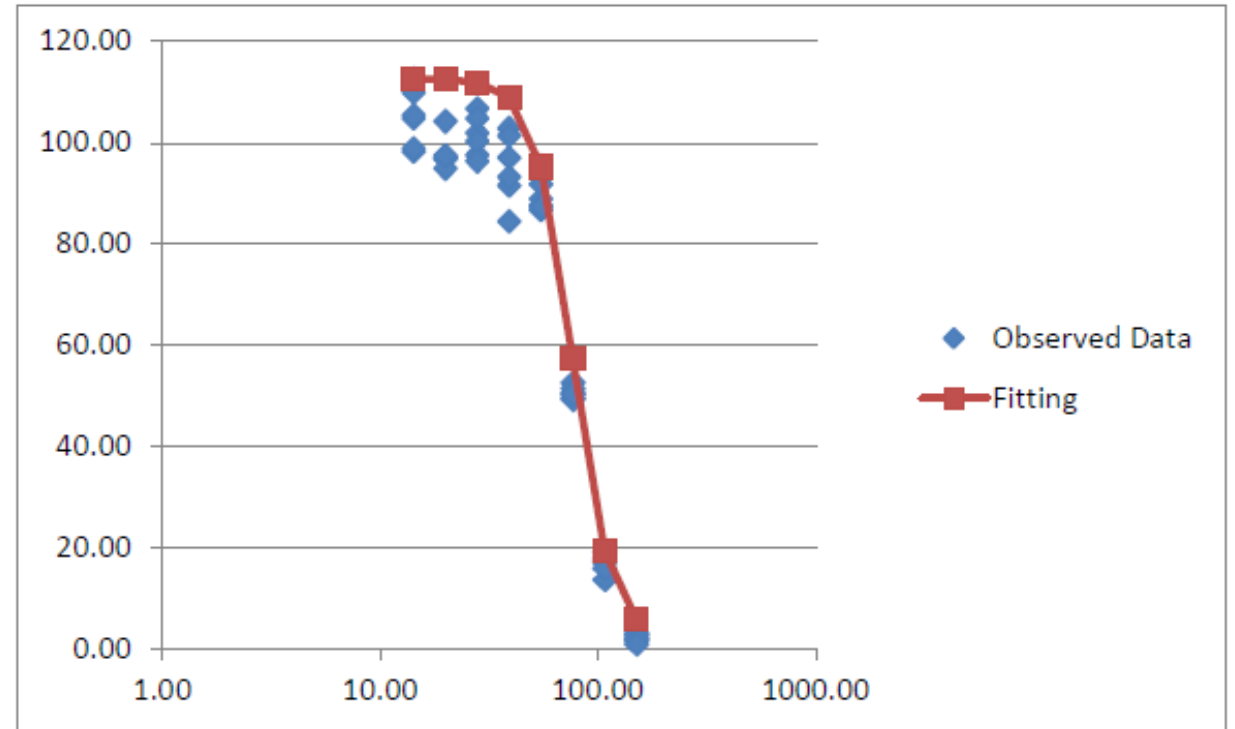


↑
Plate reader

$$\% \text{ Viability} = \frac{\text{OD of sample}}{\text{OD of Solvent}}$$

Analysis

- Spectrophotometer data is analyzed using a Microsoft Excel program to calculate a 50% inhibitory concentration (IC_{50}) using the Hillslope Equation
 - > Also known as 50% effective concentration (EC_{50})
 - > $\text{Log}IC_{50} = \text{Log} EC_{50} - (\log(\text{Top-Bottom})Y - \text{Bottom}) - 1 / \text{Hillslope}$
 - Hillslope is a four-parameter logistic mathematical model relating the concentration of test substance to the response being measure in a sigmoidal shape.

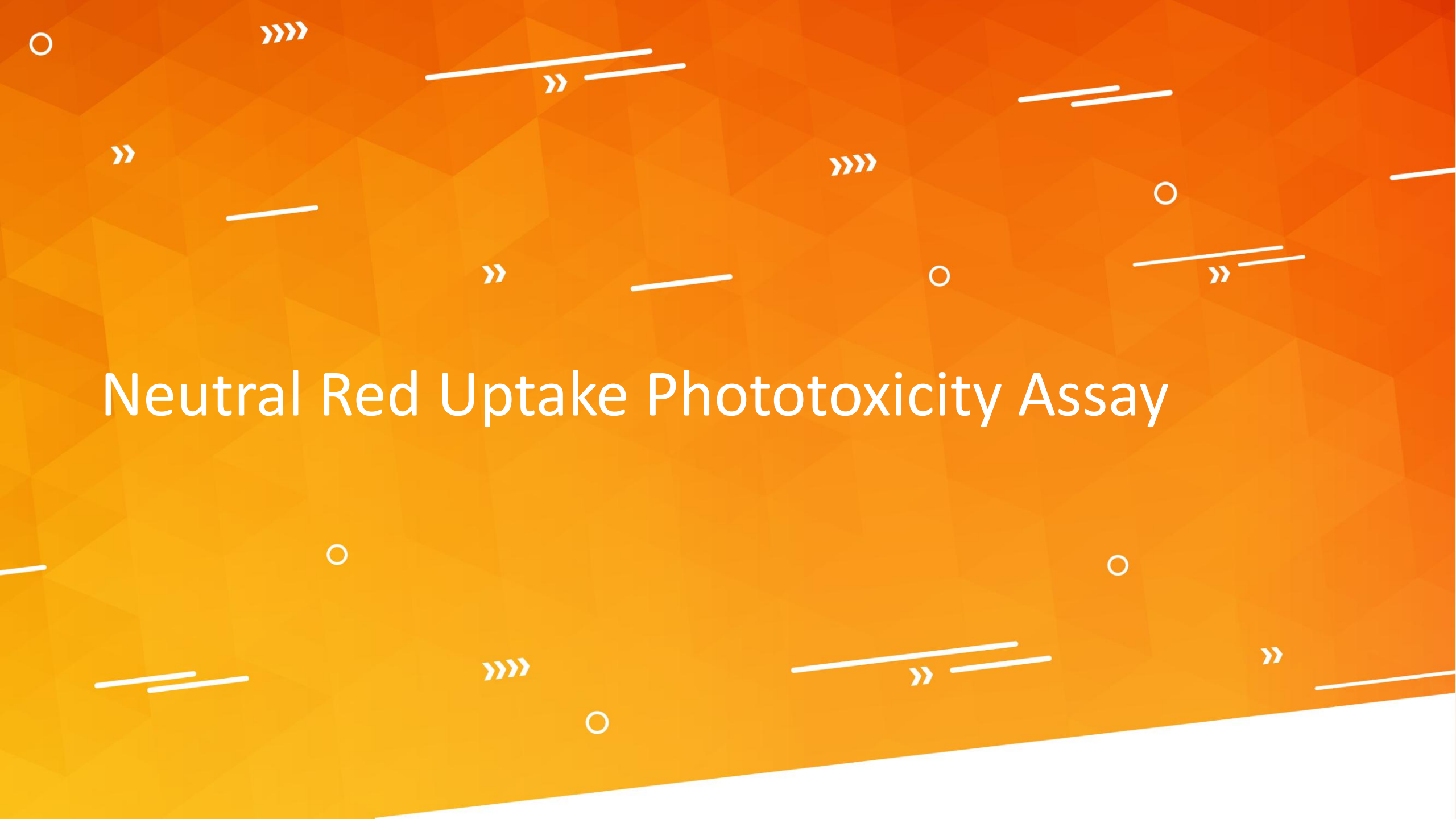


RSQ	0.89
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	IC20	IC50	IC80
Log	1.79	1.91	2.03
µg/mL	61.19	80.74	106.53

Criteria for a Valid Test

- Vehicle controls
 - > The mean of the OD values in the left and right vehicle control columns should not differ by more than 15% for the mean of all vehicle controls.
- Positive Control
 - > At least one positive control concentration (dose) with a calculated cytotoxicity value $> 0\%$ and $\leq 50\%$ viability and at least one calculated cytotoxicity value $> 50\%$ and $< 100\%$ viability must be present.
 - > The positive control IC_{50} value must be within ± 2.5 standard deviations of the historical mean established by the Testing Facility and
 - > Must have an R^2 value (coefficient of determination) ≥ 0.85 .
- Test Articles
 - > At least one positive control concentration (dose) with a calculated cytotoxicity value $> 0\%$ and $\leq 50\%$ viability and at least one calculated cytotoxicity value $> 50\%$ and $< 100\%$ viability must be present , if possible.

The background is a vibrant orange with a subtle geometric pattern of overlapping triangles. Scattered across the surface are various white decorative elements: single circles, double circles, single lines, double lines, and groups of three or four chevron-like shapes pointing in different directions. These elements are distributed across the entire frame, creating a modern, abstract aesthetic.

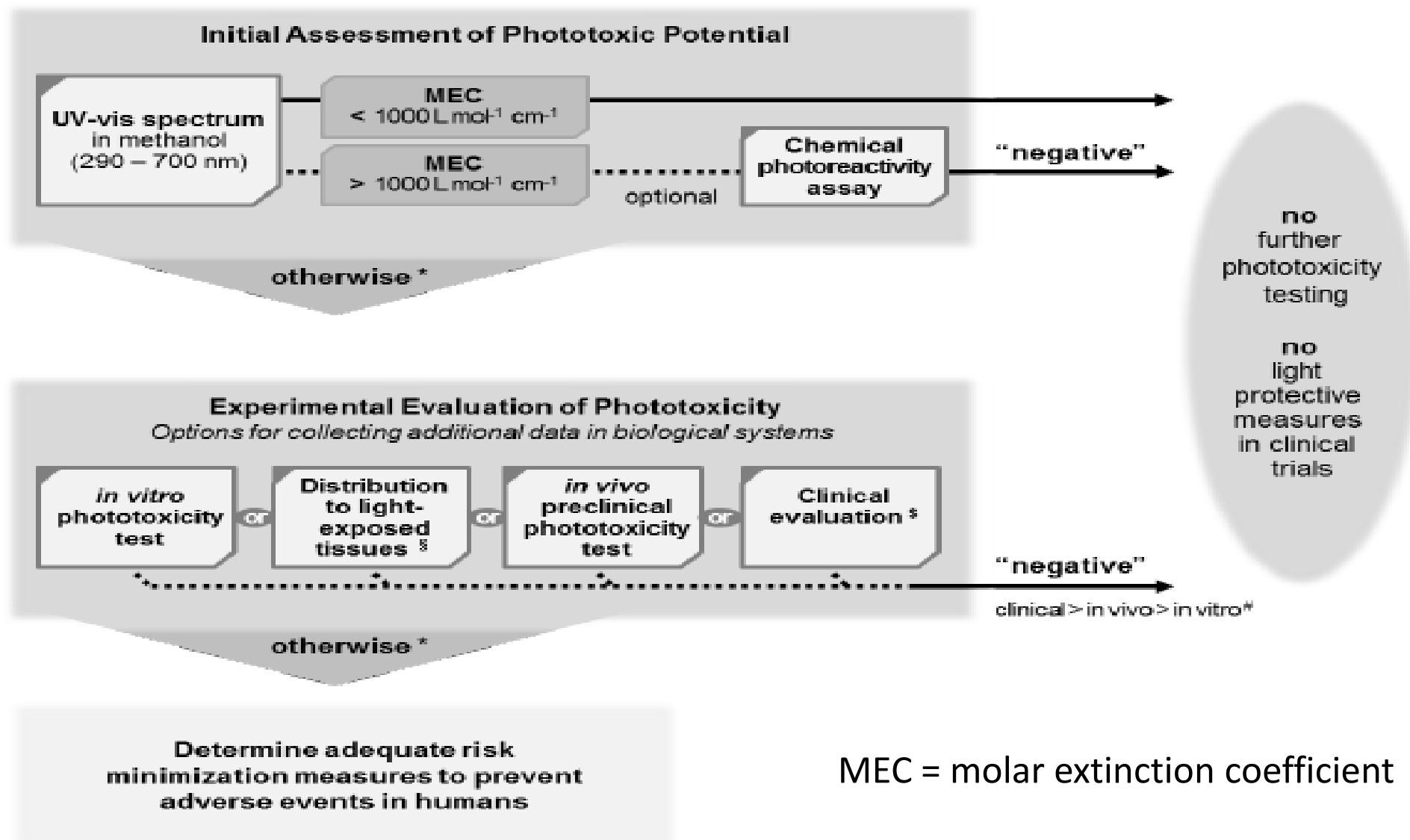
Neutral Red Uptake Phototoxicity Assay

Assay History

- Two ICH guidelines are relevant for the performance of the assay.
 - > ICH M3 (R2) “Nonclinical Safety Studies:”, chapter 14 “Photosafety Testing” (step 4, 2009)
 - > ICH S10 “Photosafety Evaluation of Pharmaceuticals” (step 4, 2013)
- One OECD guideline exists for the use of the assay
 - > OECD Test Guideline 432 entitled *In Vitro* 3T3 NRU Phototoxicity Test (2019)
- Performed prior to entering late-stage clinical trials (phase 3)
 - > e.g., for first-in-human up to phase 2
 - > If not completed, early clinical trials must have protective measures

When to Perform?

- Applies to new active pharmaceutical ingredients, new excipients, clinical formulations for dermal applicate (including dermal patches), photodynamic therapy products, chemicals, cosmetics/personal care products and ingredients.
 - > Does not apply to peptides, proteins, antibody drug conjugates, or oligonucleotides
 - > Does not apply to components of marketed products unless there is a cause for concern for either the API or an excipient (e.g., a reformulation from tablet to topical cream)
- If a chemical absorbs UV or visible light, it needs to be determined if it is likely to cause adverse phototoxic effects when intended for human use.
- Metabolic activation is not required as there is no evidence at this time that any phototoxicants would be missed in the absence of a metabolic activation toxicants.

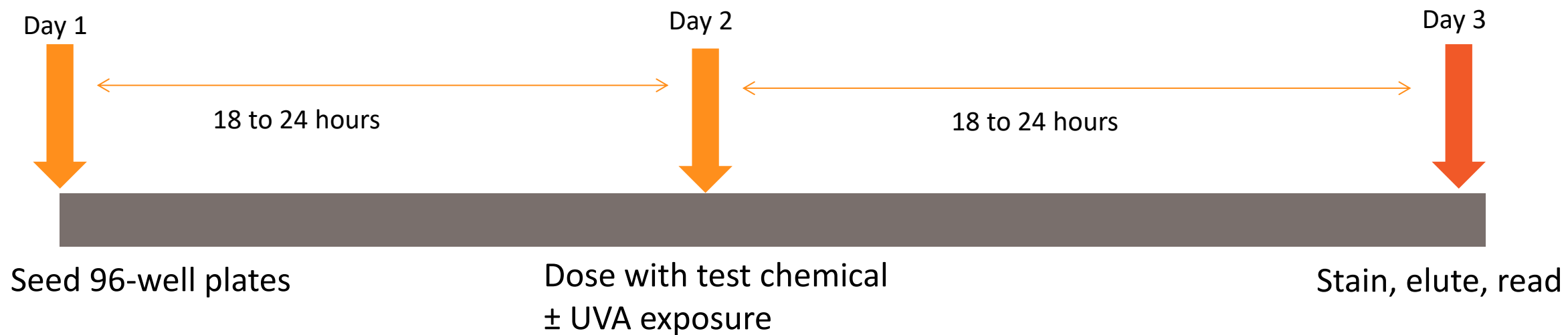


MEC = molar extinction coefficient

Experimental Design

- Assay is a three-day experiment performed in 96-well plates
 - > Performed in two phases
 - Preliminary Toxicity Assay – duplicate plates, 8 test chemical concentrations
 - One plate each for irradiation and non-irradiation
 - Definitive Cytotoxicity Assay – duplicate plates, 8 test chemical concentrations
 - One plate each for irradiation and non-irradiation
 - > Test chemical treatment time is 50 minutes
 - Irradiation to solar simulated light (1.6 to 1.8 mW/cm² for a total of 5 J/ cm²) on ice.
 - Non-irradiated placed in dark at room temperature,
 - > 48-hour recovery from solar simulated light treatment before staining with neutral red.
 - > BALB/c 3T3 cells are seeded at 1.0×10^5 cells/mL (1.0×10^4 cells/well)

Assay Design



Data Analysis

- Analysis is performed in Phototox software (freeware from OECD)
 - > Calculated IC50 values for both irradiated and non-irradiated plates, if possible.
 - > Calculates the Photo Irritation Factor (PIF) and Mean Photo Effect (MPE) from the calculated IC50 values, if possible.

- $PIF = IC50 \text{ (non-irradiated, -Irr)} / IC50 \text{ (irradiated, +Irr)}$

- $MPE = \frac{\sum_{i=1}^n w_i PE_{c_i}}{\sum_{i=1}^n w_i}$

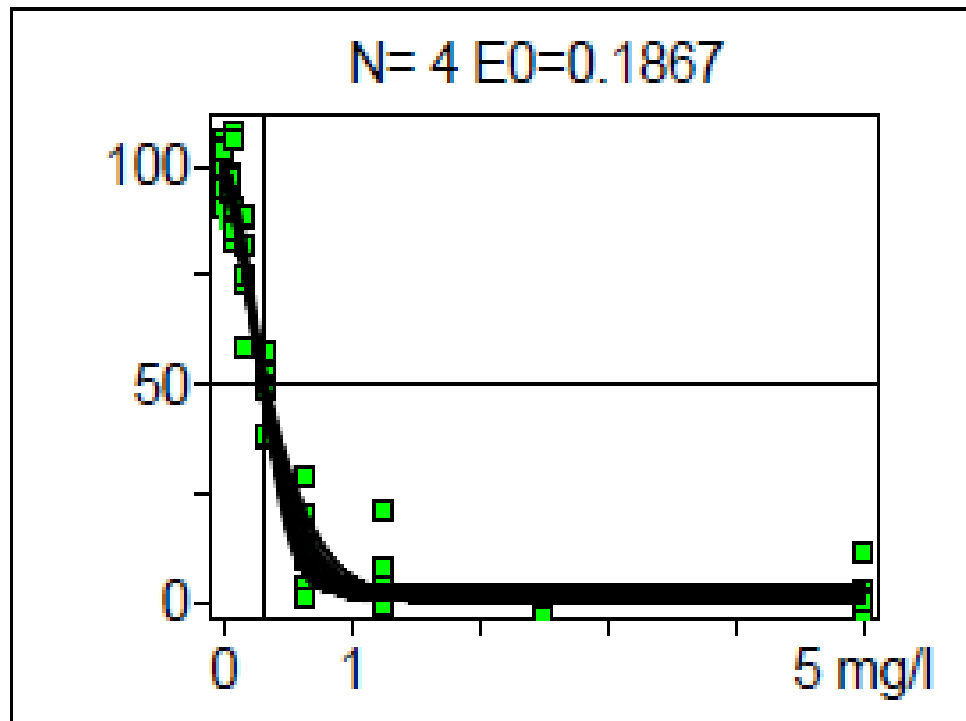
The photo effect (PE_c) at any concentration (C) is defined as the product of the response effect (RE_c) and the dose effect (DE_c) i.e., $PE_c = RE_c \times DE_c$. The response effect (RE_c) is the difference between the responses observed in the absence and presence of light, i.e., $RE_c = R_c(-Irr) - R_c(+Irr)$. The dose-effect is given by

$$DE_c = \left| \frac{C/C^* - 1}{C/C^* + 1} \right|$$

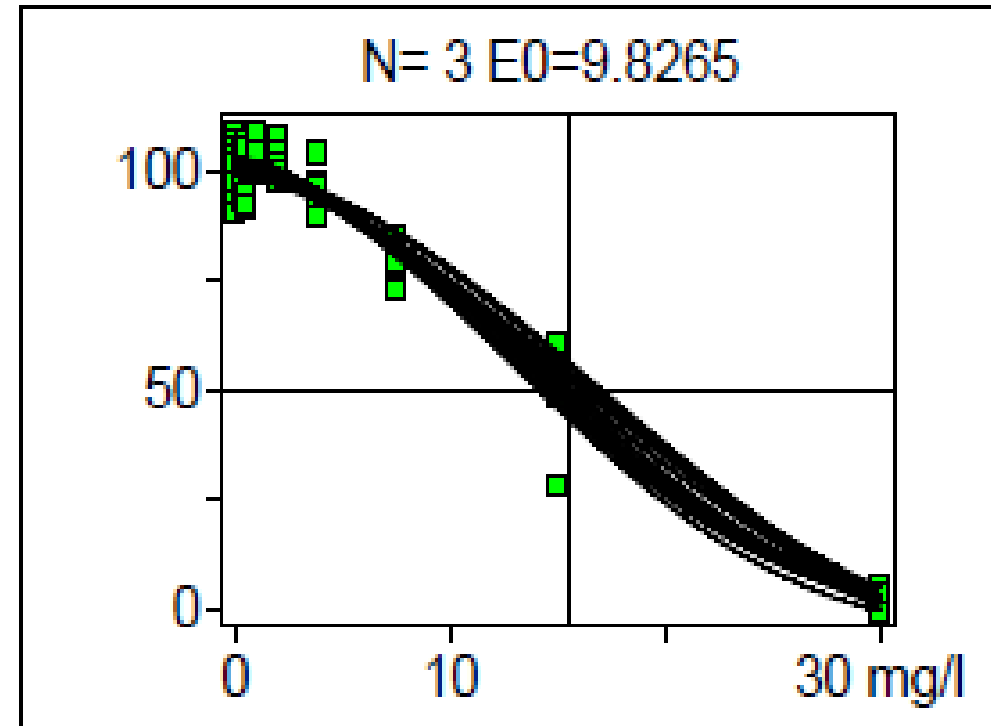
where C^* represents the equivalence concentration, i.e., the concentration at which the +Irr response equals the -Irr response at concentration C . If C^* cannot be determined because the response values of the +Irr curve are systematically higher or lower than $R_c(-Irr)$ the dose effect is set to 1. The

Phototoxicity – Sample Data

Irradiated



Non-Irradiated



PIF = 50.732; MPE = 0.598

Criteria for a Valid Test

- **Vehicle controls**
 - > The mean OD540 of the solvent control should ideally be ≥ 0.4 . The irradiated solvent control should ideally not be less than 80% of the non-irradiated solvent control.
- **Positive Control**
 - > At least one positive control concentration (dose) with a calculated cytotoxicity value $> 0\%$ and $\leq 50\%$ viability and at least one calculated cytotoxicity value $> 50\%$ and $< 100\%$ viability must be present.
 - > The positive control IC_{50} value must be within 0.1 to 2.0 $\mu\text{g/mL}$ (irradiated) and 7.0 to 90.0 $\mu\text{g/mL}$ (non-irradiated).
- **Test Articles**
 - > At least one positive control concentration (dose) with a calculated cytotoxicity value $> 0\%$ and $\leq 50\%$ viability and at least one calculated cytotoxicity value $> 50\%$ and $< 100\%$ viability must be present , if possible.

Evaluation Criteria

Prediction	PIF		MPE
No phototoxicity	$\text{PIF} < 2$	or	$\text{MPE} < 0.1$
Equivocal phototoxicity ^a	$\text{PIF} \geq 2 \text{ and } < 5$	or	$\text{MPE} \geq 0.1 \text{ and } < 0.15$
Phototoxicity	$\text{PIF} \geq 5$	or	$\text{MPE} \geq 0.15$

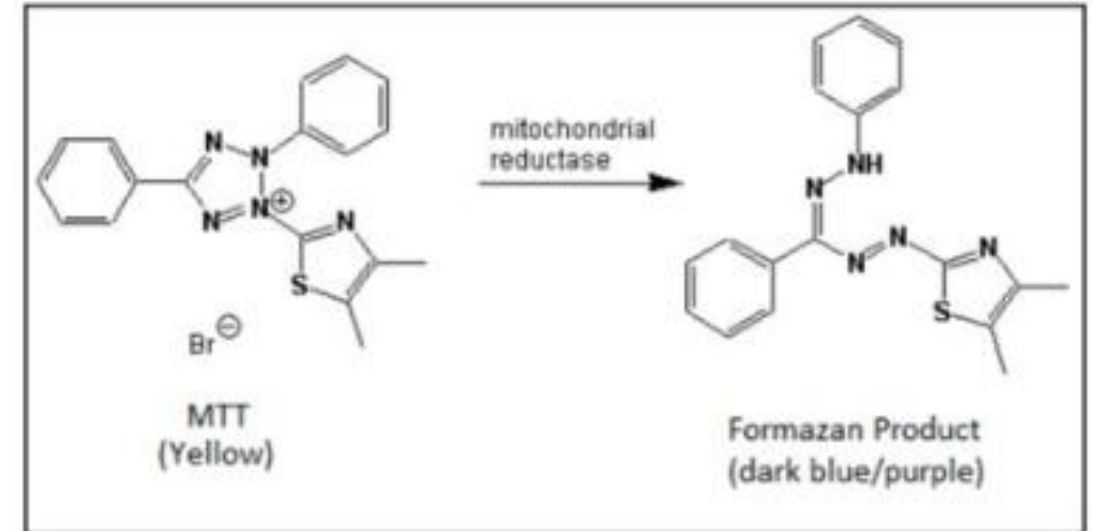
^a for pharmaceuticals, chemicals in this category are of questionable relevance for systemic drugs and generally do not warrant further photosafety evaluation.

The background is white with various light gray geometric elements scattered across it, including circles, double chevrons (»), and parallel lines. An orange geometric pattern is visible in the bottom right corner.

Thiazole Blue (MTT) Assay

Assay Foundation

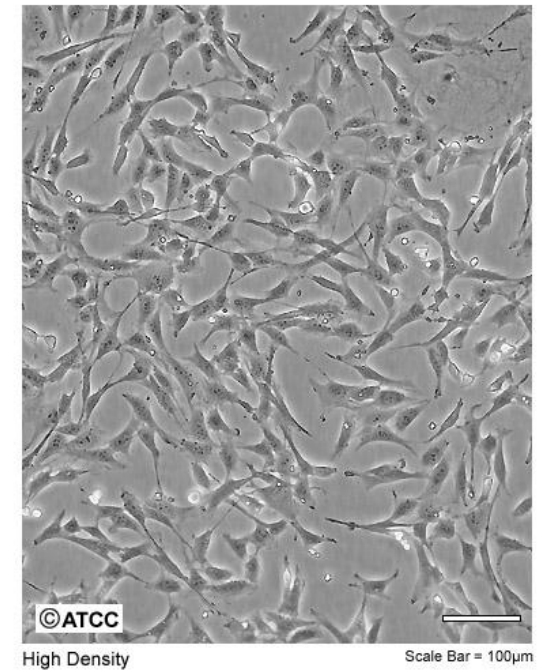
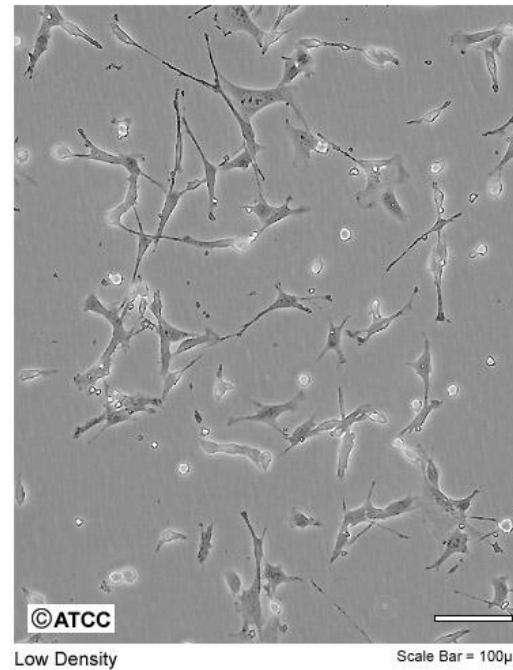
- The assay is based on the ability of viable cells to cleave yellow tetrazolium salt (MTT) to form a soluble blue formazan.
 - > Thiazole blue (MTT) is cleaved by mitochondrial reductase enzyme.
- Due to the cytotoxic effect of a test chemical, cellular membrane property changes will lead to cell death or a change in cell structure and function resulting in decreased cleavage of tetrazolium salt.
 - > Thus, making it possible to distinguish between viable, damaged, and/or dead cells which is the basis of the assay.



Cell Lines

- BALB/c 3T3
 - > Mouse fibroblast cell line
 - > Obtained from ATCC (CCL-163)
 - May also be obtained from Japanese Collection of Research Bioresources Cell Bank (JCRB 9005)
 - > Adherent culture
 - Epithelial-like

ATCC Number: **CCL-163**
Designation: **BALB/3T3 clone A31**



Experimental Design

- Assay is a four-day experiment performed in 96-well plates
 - > Performed in two phases
 - Preliminary Toxicity Assay – singlet plate, 8 test chemical concentrations
 - Definitive Cytotoxicity Assay – duplicate/triplicate plates, 8 test chemical concentrations
 - > Test chemical treatment time varies from 48 hours.
 - 48-hour treatment is standard
 - > Number of cells seeded varies by cell line
 - BALB/c 3T3 cells range from 2.0 to 3.0×10^4 cells/mL (2.0 to 3.0×10^3 cells/well)

Assay Design

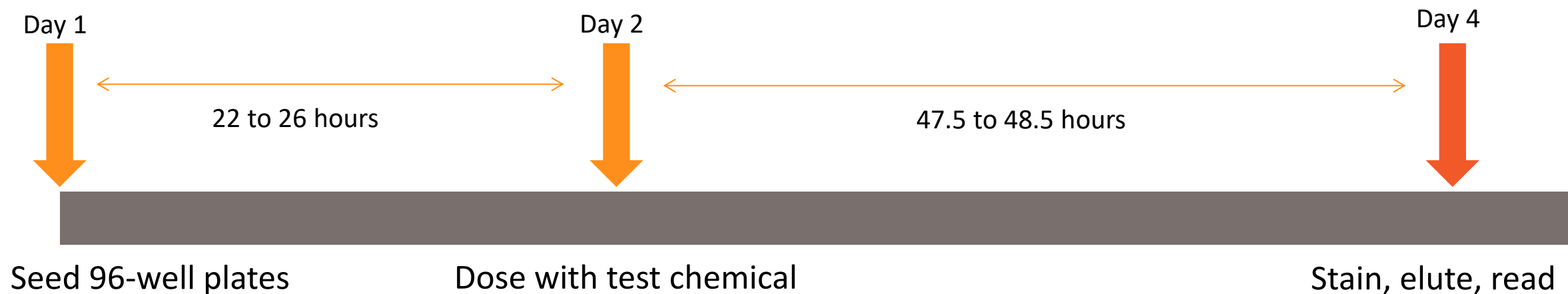


Plate Design

	1	2	3	4	5	6	7	8	9	10	11	12
A	VC _b	VC _b	C1 _b	C2 _b	C3 _b	C4 _b	C5 _b	C6 _b	C7 _b	C8 _b	VC _b	VC _b
B	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
C	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
D	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
E	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
F	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
G	VC _b	VC ₁	C1	C2	C3	C4	C5	C6	C7	C8	VC ₂	VC _b
H	VC _b	VC _b	C1 _b	C2 _b	C3 _b	C4 _b	C5 _b	C6 _b	C7 _b	C8 _b	VC _b	VC _b

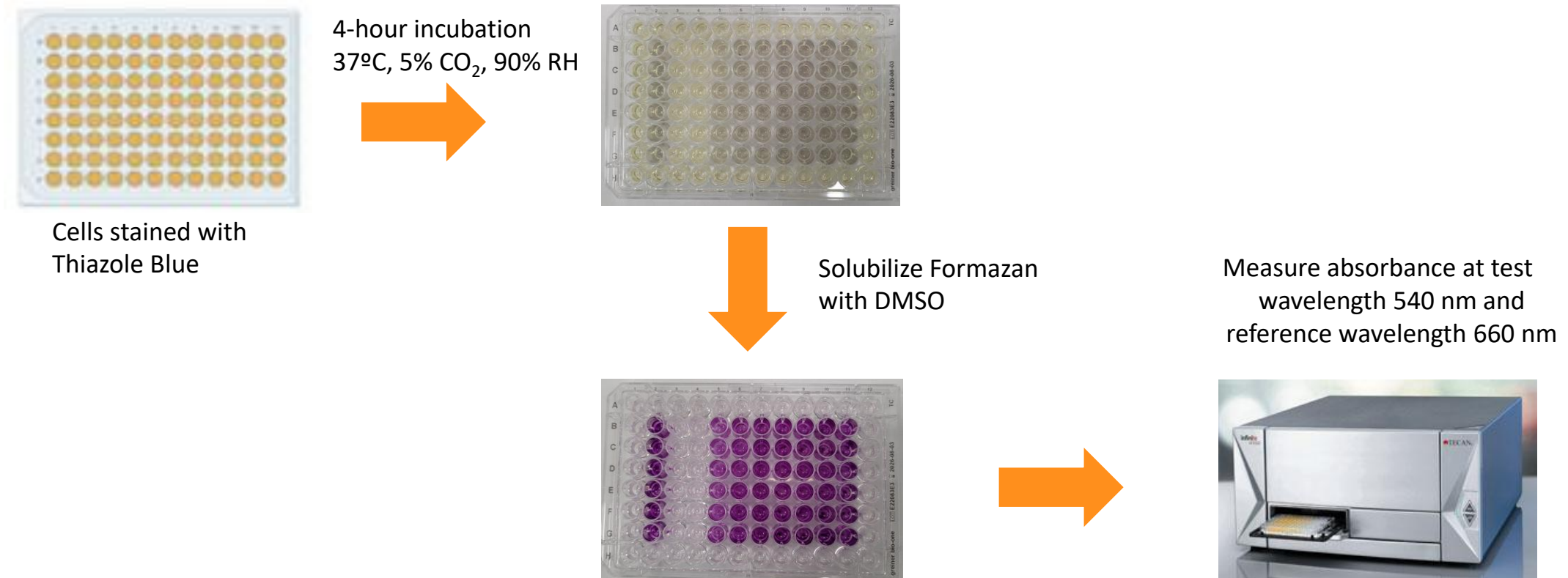
VC₁ and VC₂ = Vehicle controls. C1 – C8 = Test chemical or PC (SLS) at eight concentrations (C1 = highest, C8 = lowest)

Cx_b = Blanks (Test chemical or PC, but contain no cells)

VC_b = VC blanks (contain no cells)

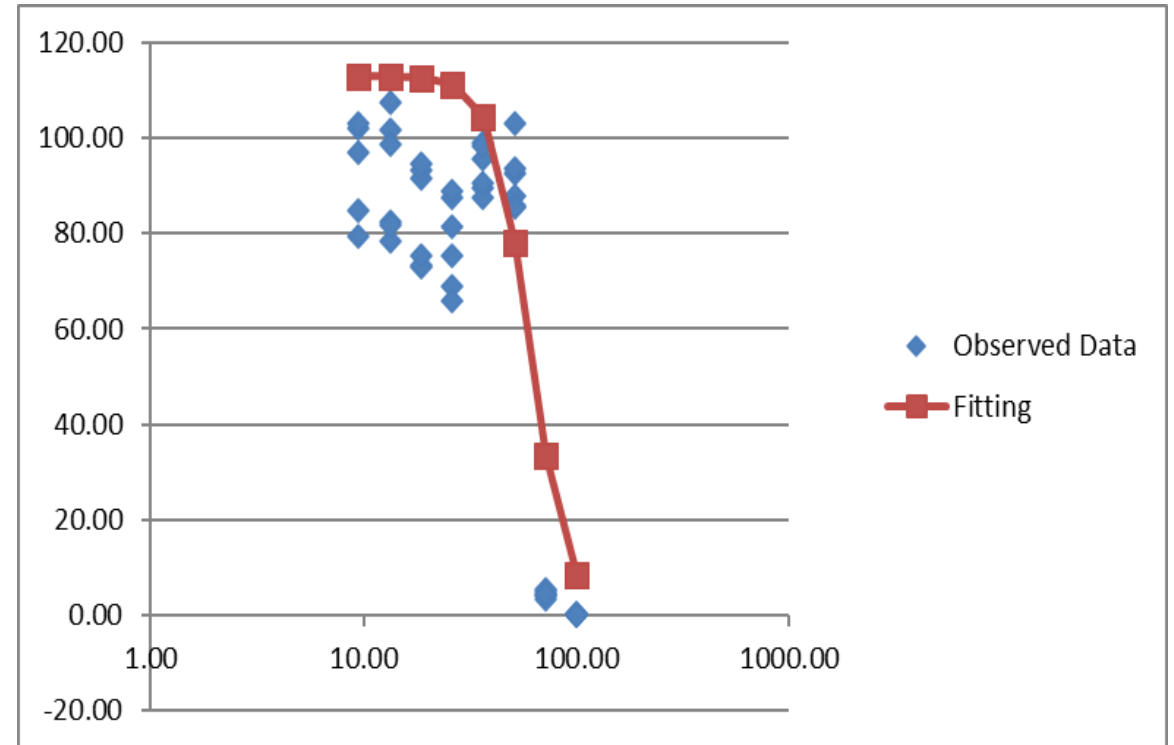
Staining and Plate Reading

The lighter the color the fewer cells that have formazan formation.



Analysis

- Analysis is performed using normalized difference (test wavelength – reference wavelength) optical density data
- Spectrophotometer data is analyzed using a Microsoft Excel program to calculate a 50% inhibitory concentration (IC_{50})
 - > Also known as 50% effective concentration (EC_{50})



RSQ	0.90
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	IC20	IC50	IC80
Log	1.68	1.80	1.92
µg/mL	47.64	62.86	82.95

Criteria for a Valid Test

- Vehicle controls
 - > The mean of the OD values in the left and right vehicle control columns should not differ by more than 15% for the mean of all vehicle controls.
- Positive Control
 - > At least one positive control concentration (dose) with a calculated cytotoxicity value $>0\%$ and $\leq 50\%$ viability and at least one calculated cytotoxicity value $> 50\%$ and $<100\%$ viability must be present , if possible.
 - > The positive control IC_{50} value must be within ± 2.5 standard deviations of the historical mean established by the Testing Facility and must have an R^2 value (coefficient of determination) ≥ 0.85 .
- Test Articles
 - > At least one positive control concentration (dose) with a calculated cytotoxicity value $>0\%$ and $\leq 50\%$ viability and at least one calculated cytotoxicity value $> 50\%$ and $<100\%$ viability must be present , if possible.

The background is a vibrant orange with a subtle geometric pattern of overlapping triangles. Scattered across the surface are various white symbols: single circles, double chevrons (»), and groups of three chevrons (»»). Some of these symbols are accompanied by short, parallel white lines, creating a sense of motion or data points. The overall aesthetic is modern and minimalist.

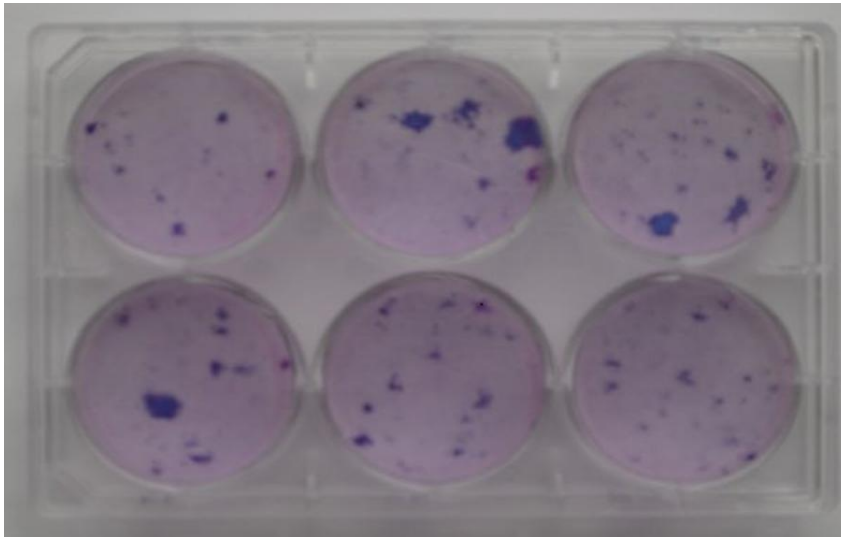
Bhas 42 Cell Transformation Assay

Topics for Discussion

- Cell Transformation Assay
 - > Background Information
 - > Cell Line Information
 - > Experimental Design
 - > Study Design
 - > Scoring
 - > Evaluation Criteria
 - > Sample Data

Assay History

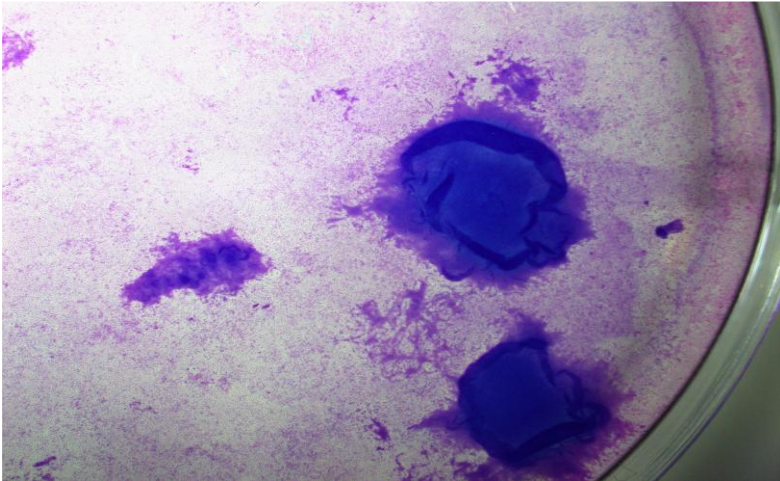
- Assay started in Japan and funded through a New Energy and Industrial Technology Development Organization (NEDO) project.
- Performed under Organisation for Economic Co-operation and Development (OECD) guidance document 231 entitled Guidance Document on the *In Vitro* Bhas 42 Cell Transformation Assay (2017)



Example Promoter Positive Control Plate

Assay Foundation

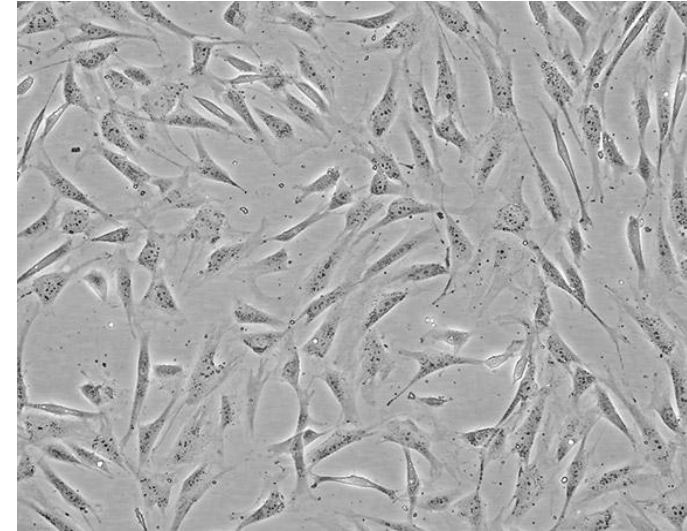
- Untransformed Bhas 42 cells grow to confluence forming a density-dependent contact-inhibited monolayer.
- After exposure to carcinogenic stimuli, such cells become morphologically altered and form discrete anchorage-independent altered colonies, referred to as transformed foci, atop the confluent monolayer.
 - > Not considered a true genetic toxicology assay as it measures carcinogenic potential



Example foci in
confluent well

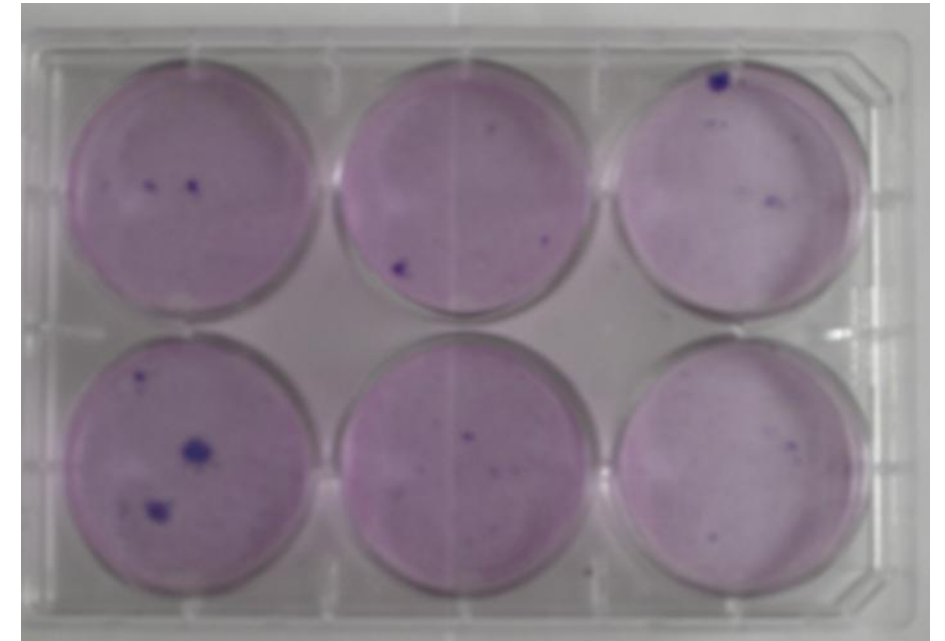
Cell Line

- Bhas 42 cells
 - > a clone formed by the stable transfection of the v-Ha-ras oncogene into the BALB/3T3 A31-1-1 cell line
 - Mouse fibroblast, epithelial-like
 - Forms contact-inhibited monolayers
 - > Considered an initiated cell line as no pretreatment with a tumor initiator is needed
 - > Grown for use to keep spontaneous transformation and passage number low
 - > Maintained between 40 and 70% confluency



Experimental Design

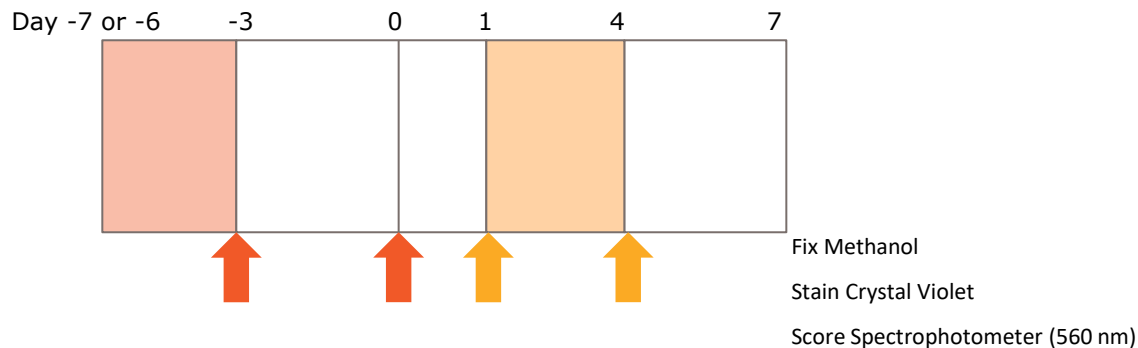
- Assay is performed in 6-well plates
 - > 96-well plate version also possible
- Assay is comprised of two components
 - > Initiating (Initiator) – used to detect tumor initiators, direct acting
 - > Promoting (Promoter) – used to detect tumor promoters, indirect acting
 - Should detect non-genotoxic carcinogens
- Each component is tested in two phases
 - > Preliminary Toxicity (Initial Cell Growth) Assay
 - Cell Growth Assay
 - > Definitive Assay
 - Transformation Assay
 - Parallel Cell Growth Assay (concurrent toxicity measurement)



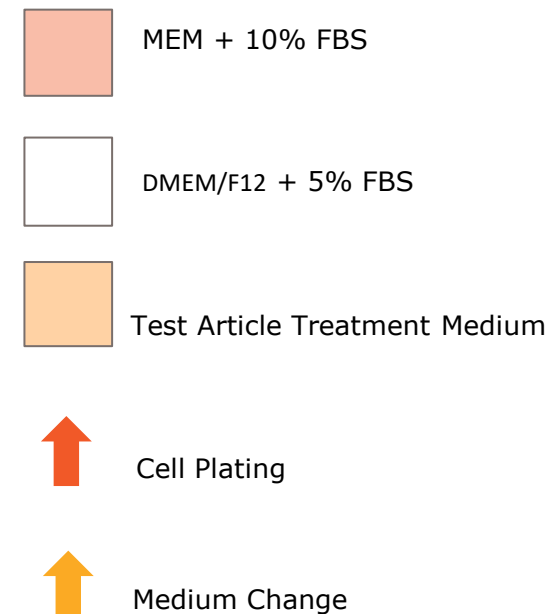
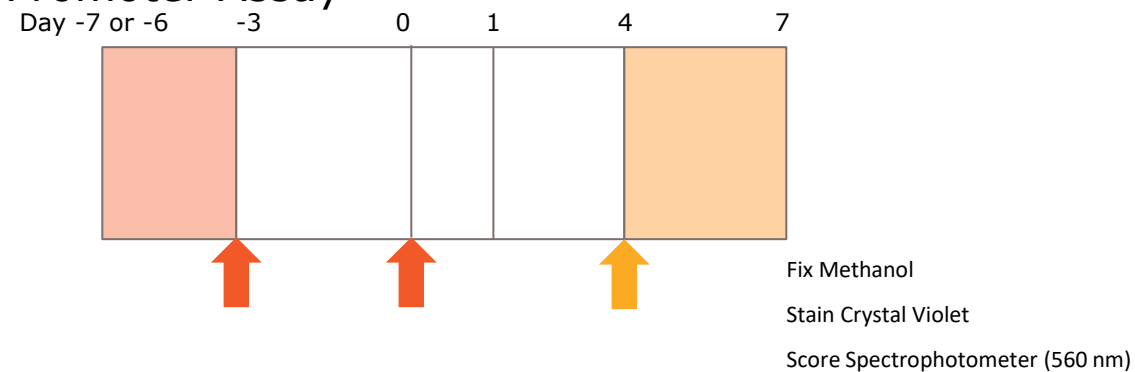
Example Initiator Vehicle Control Plate

Preliminary Toxicity Assay Design

Initiator Assay



Promoter Assay

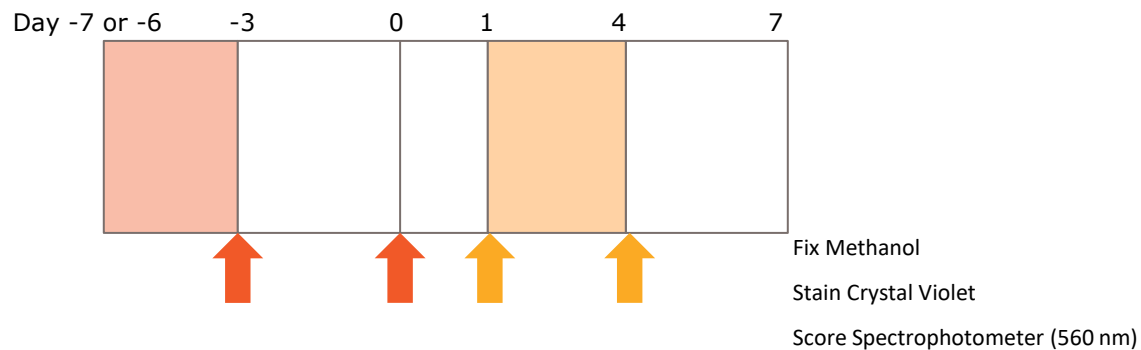


Days -7 to 0: Mother Culture of Cells

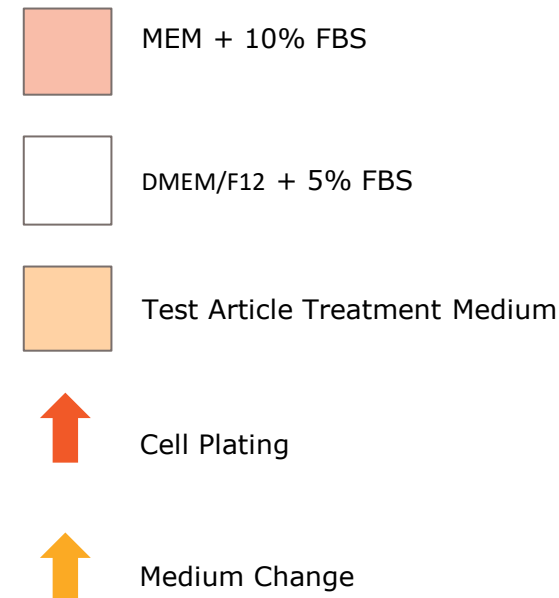
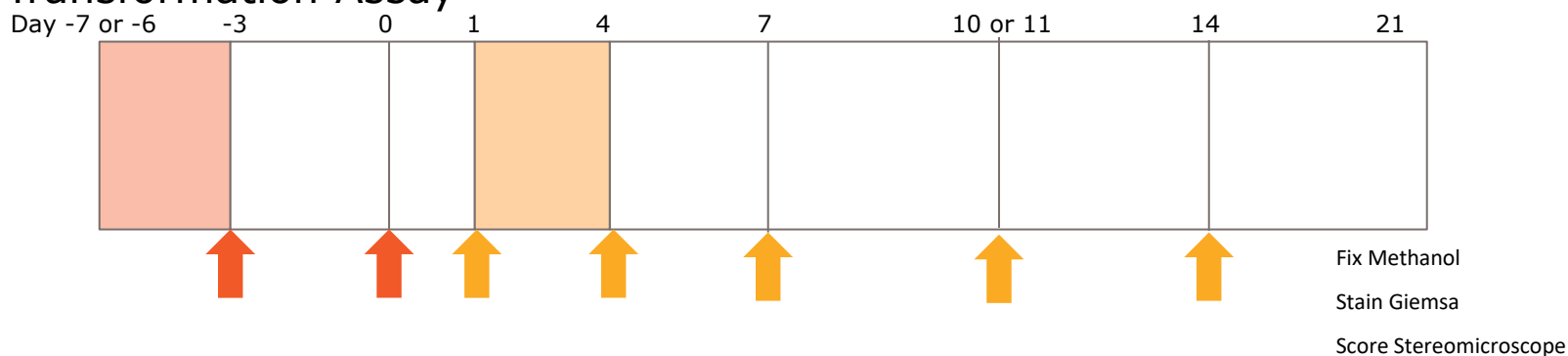
Days 1 to 7: Cell Growth Assay

Definitive Initiator Assay Design

Parallel Cell Growth Assay



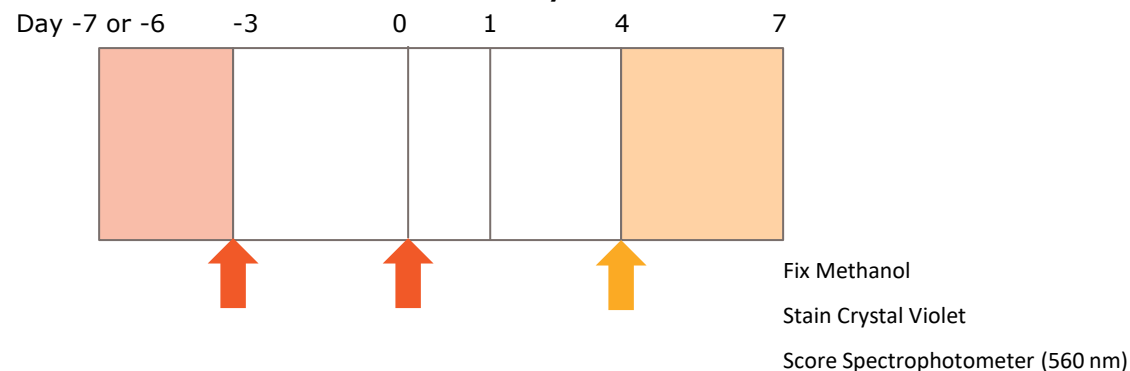
Transformation Assay



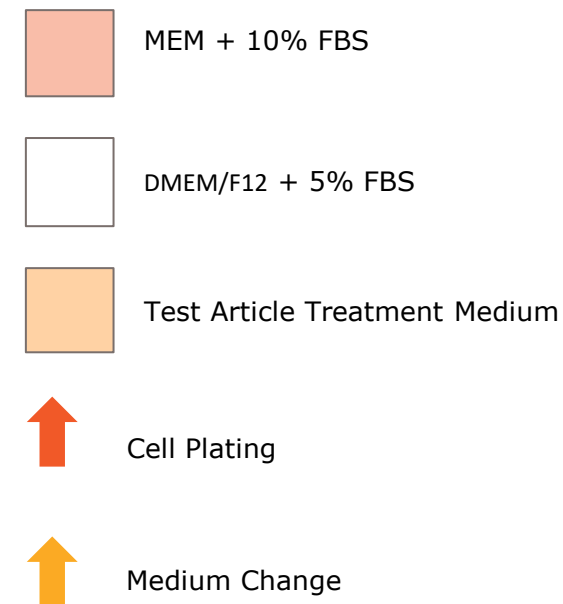
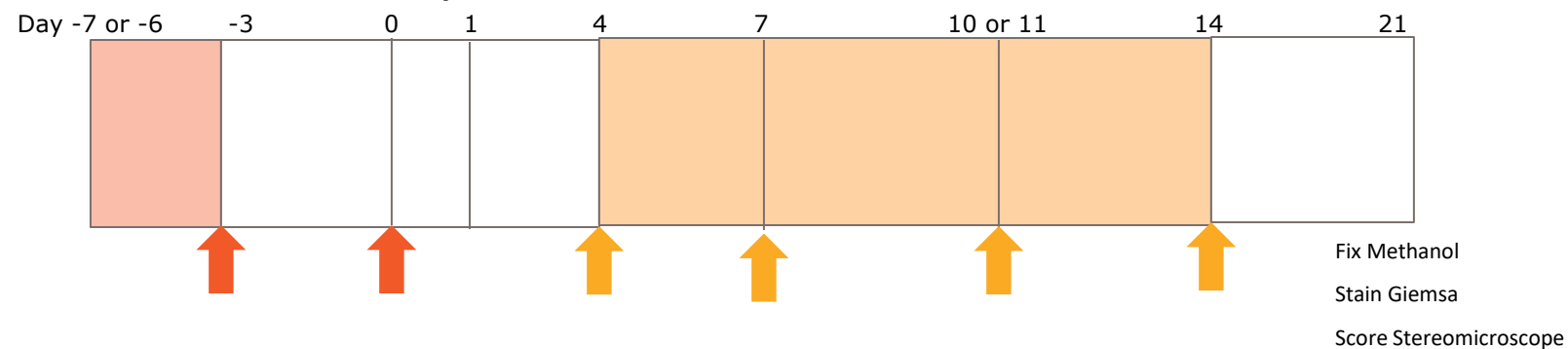
Days -7 to 0: Mother Culture of Cells
Days 1 to 7: Parallel Cell Growth Assay
Days 1 to 21: Transformation Assay

Definitive Promoter Assay Design

Parallel Cell Growth Assay



Transformation Assay



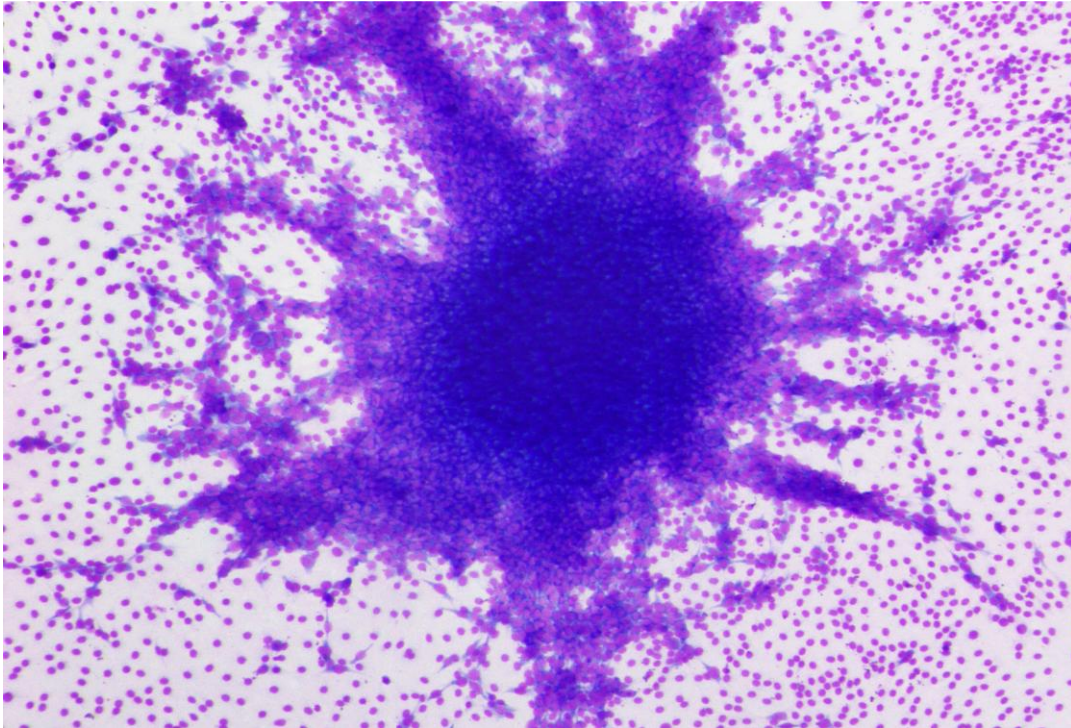
Days -7 to 0: Mother Culture of Cells
Days 1 to 7: Parallel Cell Growth Assay
Days 1 to 21: Transformation Assay

Transformation Assay Endpoint

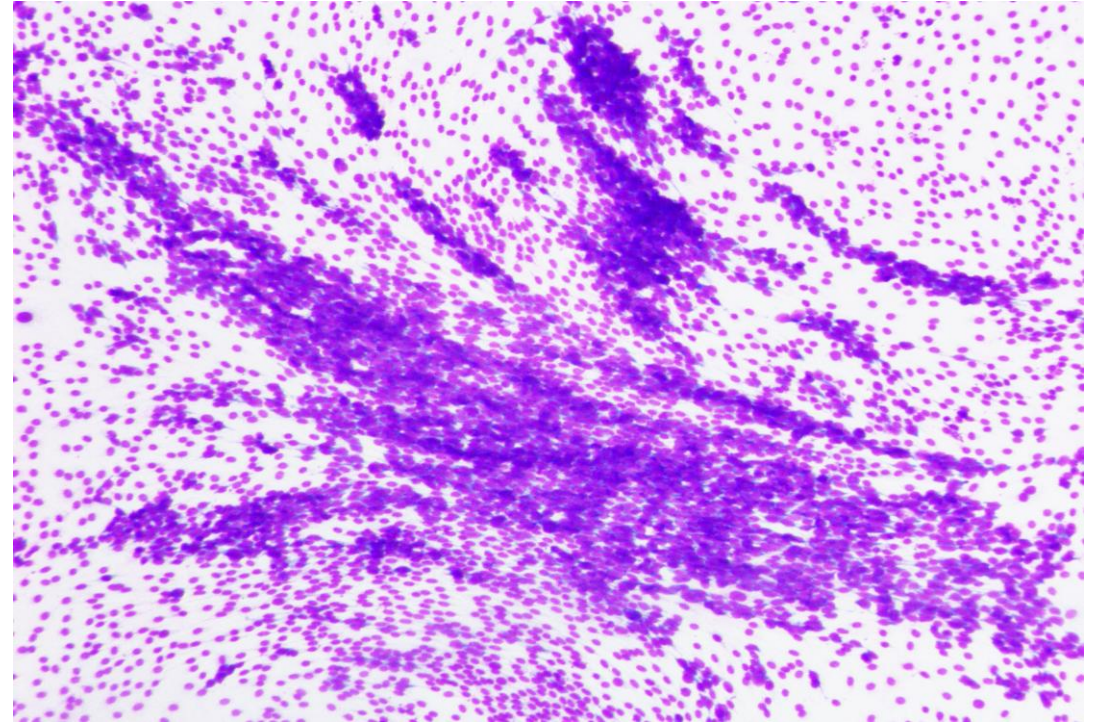
- Growth of foci within monolayer of cells
 - > Scored by eye with stereomicroscope
 - > Two evaluations of foci
 - Transformed
 - Non-Transformed
- Evaluation Criteria
 1. At least 100 cells in foci
 2. Spindle-shaped cells differing from the contact-inhibited monolayer cells
 3. Deep basophilic staining
 4. Random orientation of cells at edge of foci (criss-crossing)
 5. Dense multilayering of cells (piling up)
 6. Invasive growth into the monolayer of surrounding contact-inhibited cells

Transformation Evaluation Criteria

- At least 100 cells in foci

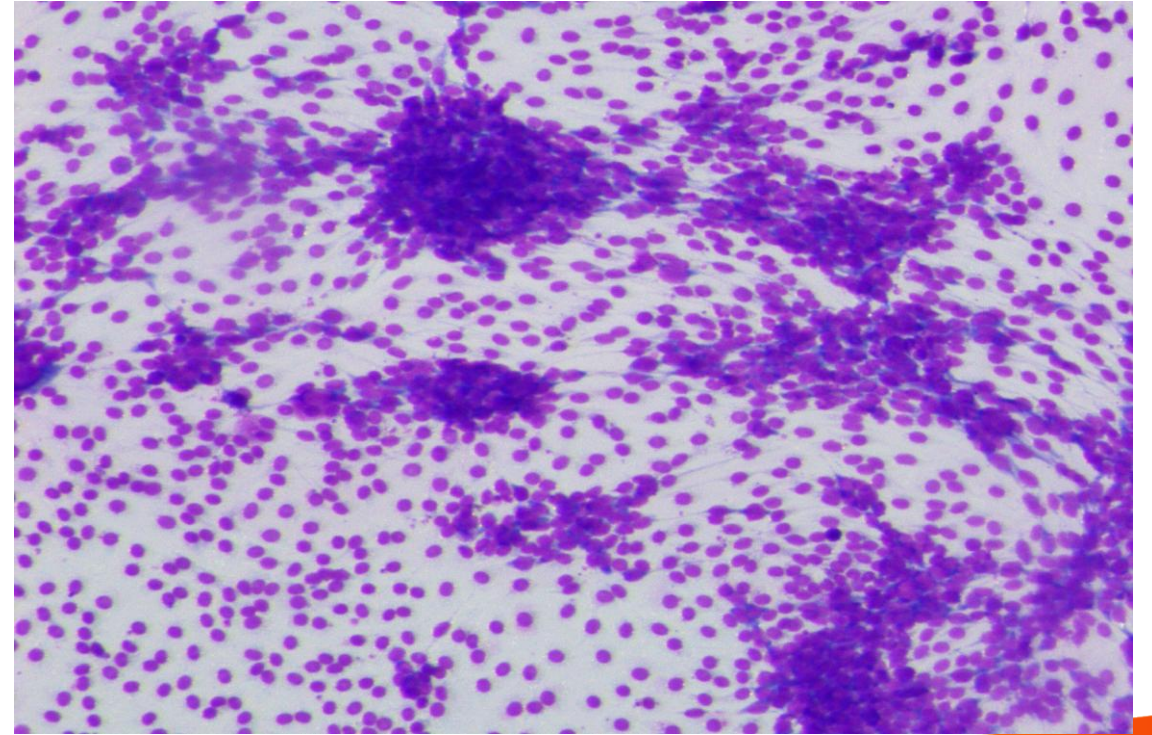
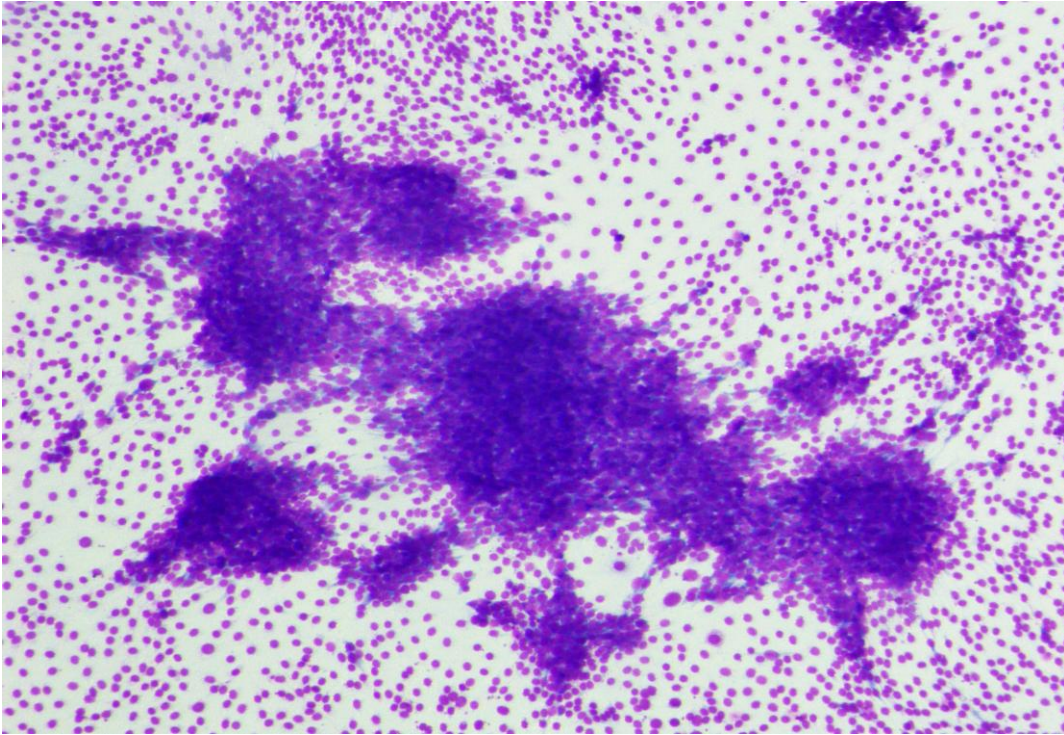


- Spindle-shaped cells differing from contact-inhibited monolayer cells



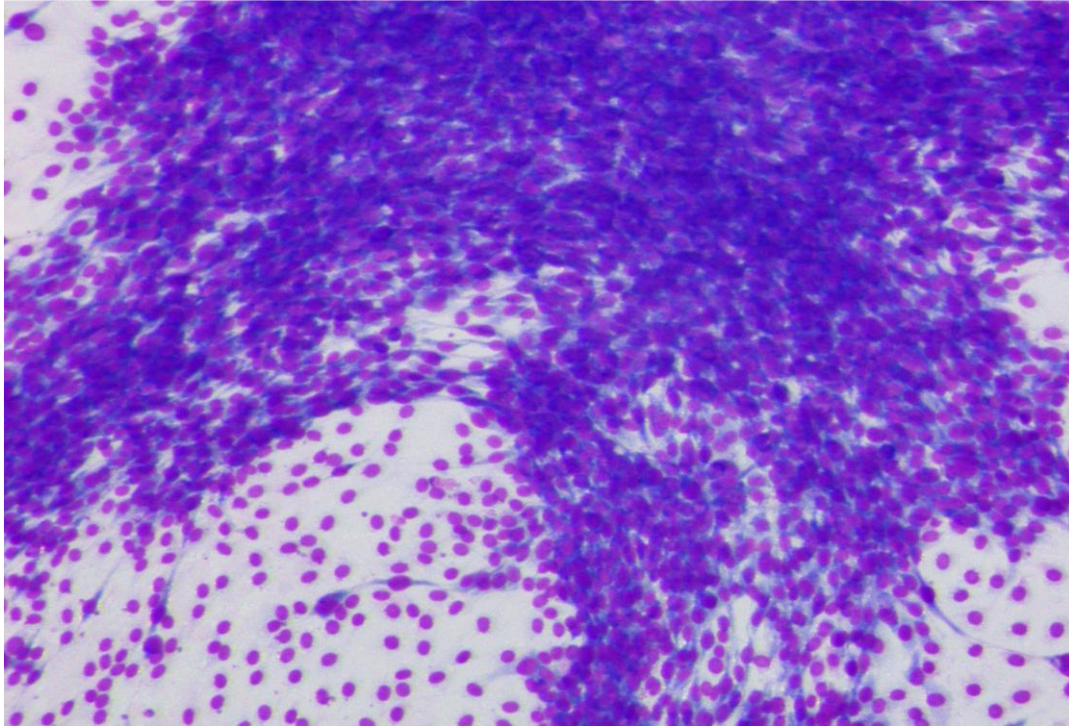
Transformation Evaluation Criteria

- Deep basophilic staining
- Random orientation of cells at the edge of foci (criss-crossing)

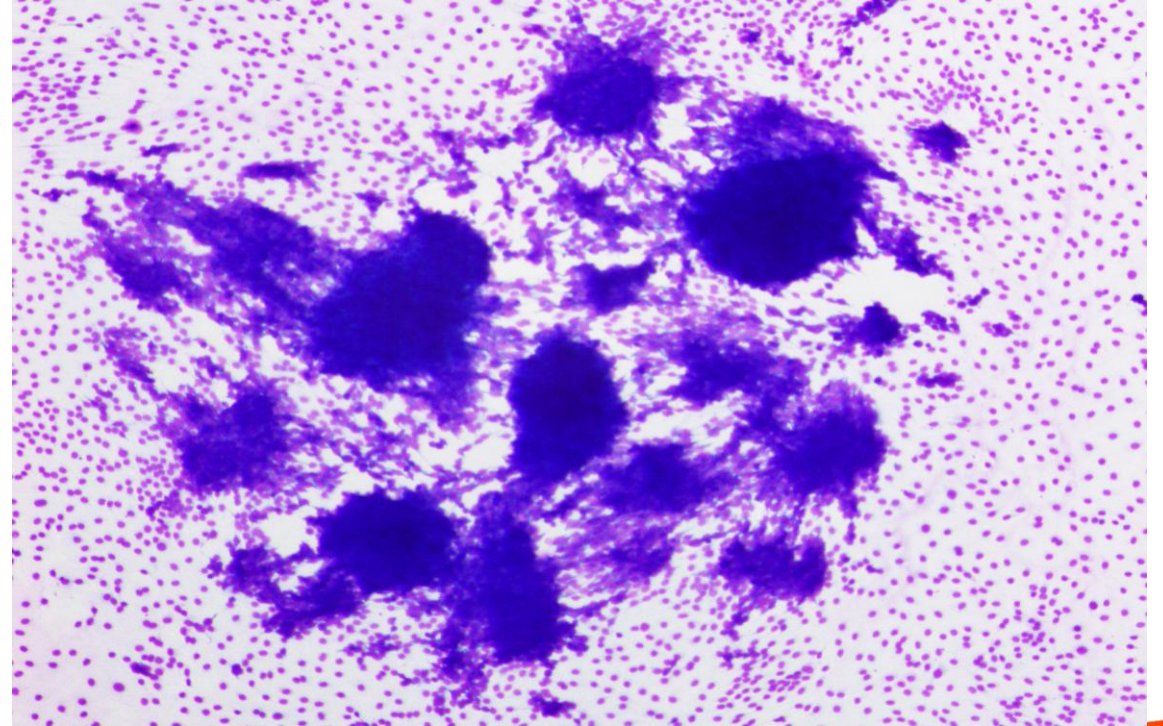


Transformation Evaluation Criteria continued

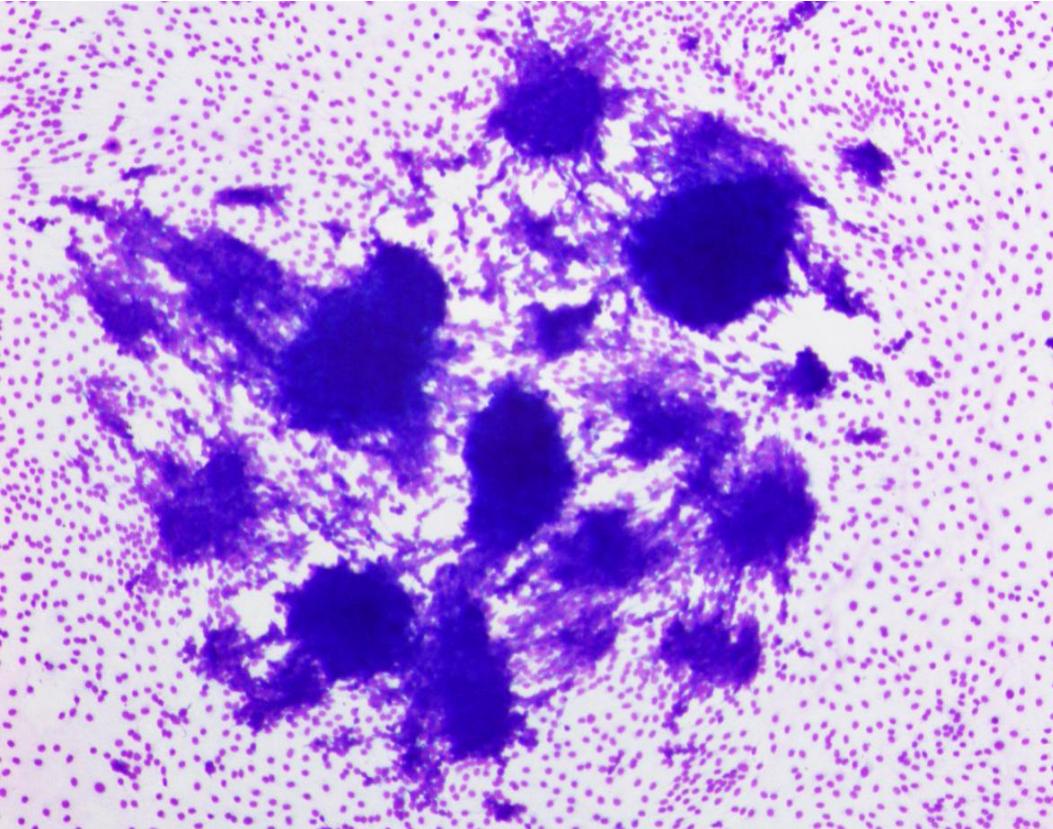
- Dense multilayering of cells (piling up)



- Invasive growth into the monolayer of surrounding contact-inhibited cells



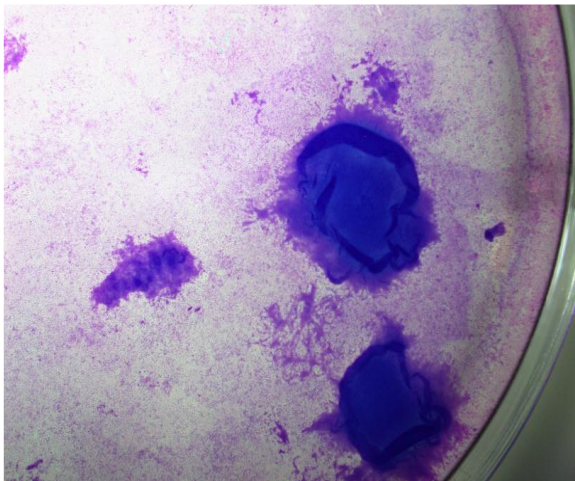
Satellite or Daughter Foci



- Formed from a foci that has broken apart during the growth period of the assay.
 - > Usually have connecting pieces between the foci
 - May be separated but in general vicinity of the original foci
 - Counted as 1 foci
 - Usually circled to show that they are same foci

Assay Acceptance Criteria

1. A minimum of four (4) scorable treatment concentrations (each with at least 5 confluent wells of a 6-well plates) are required.
2. When contamination or technical problems are observed and the number of affected/damaged wells is ≥ 2 in the parallel cell growth assay, the concentration is deemed invalid and therefore excluded from the growth rate calculation.
3. Positive control: The number of transformed foci observed should be statistically significant compared to the number of transformed foci for the appropriate vehicle control.
4. Vehicle Control: The average number of transformed observed foci should be ≤ 10 foci/well in the initiator portion and ≤ 12 foci/well for promoter portion.
 - Higher allowed spontaneous revertant in promoter portion due to a greater cell density of plated cells is used in assay.



Example foci in
confluent well

Evaluation Criteria

- **Positive Evaluation**

- > The initiation or promotion assays are judged positive if there are two or more sequential dose levels that induce statistically significant increase in the number of transformed foci per well relative to the corresponding solvent/vehicle control.
- > When the initiation assay is determined to be positive, the test article is considered to have carcinogenic initiating activity.
- > When the promotion assay is determined to be positive, the test article is considered to have carcinogenic promoting activity.
- > A positive result in either the initiation or promotion assay is considered to mean that the test article possesses potential carcinogenic activity, regardless of which endpoint is positive.

- **Negative Evaluation**

- > The initiation or promotion assays are judged negative if there is no dose level showing a statistically significant increase in the number of transformed foci per well.

Parallel Toxicity Assay – Sample Data

Initiator Assay

Sample ID	Average Absorbance	Relative Viability (%)
DMSO (0.2%)	0.205	100.0%
DMSO (0.5%)	0.181	88.5%
Ethanol (0.1%)	0.196	95.7%
Phosphate Buffered Saline (5%)	0.184	89.7%
3-MCA (1.0 µg/mL)	0.026	12.7%

3-MCA = 3-Methylcholanthrene

Promoter Assay

Sample ID	Average Absorbance	Relative Viability
DMSO (0.2%)	0.152	100.0%
DMSO (0.5%)	0.146	96.2%
Ethanol (0.1%)	0.136	89.7%
Phosphate Buffered Saline (5%)	0.147	96.5%
TPA (50 ng/mL)	0.195	128.1%

TPA = 12-O-Tetradecanoylphorbol 13-acetate

Morphological Transformation Assay – Sample Data

Initiator Assay

Sample ID	Sum of Foci	Mean of Foci $\pm$ SD
DMSO (0.2%)	15	2.50 $\pm$ 1.05
DMSO (0.5%)	16	2.67 $\pm$ 1.21
Ethanol (0.1%)	11	1.83 $\pm$ 1.33
Phosphate Buffered Saline (5%)	15	2.50 $\pm$ 1.87
3-MCA (1.0 μ g/mL)	164	27.33 $\pm$ 3.67**

Promoter Assay

Sample ID	Sum of Foci	Mean of Foci $\pm$ SD
DMSO (0.2%)	31	5.17 $\pm$ 2.23
DMSO (0.5%)	43	7.17 $\pm$ 1.33
Ethanol (0.1%)	28	4.67 $\pm$ 2.34
Phosphate Buffered Saline (5%)	27	4.50 $\pm$ 1.38
TPA (50 ng/mL)	167	27.83 $\pm$ 1.83**

3-MCA = 3-Methylcholanthrene; TPA = 12-O-Tetradecanoylphorbol 13-acetate

** statistically significant increase compared to vehicle control (t-test, $p < 0.05$)

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