

WHERE
insights LEAD TO
answers



In Vitro Mammalian Cell Forward Mutation Assays

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Topics for Discussion

HPRT and Mouse Lymphoma Assay

Test System Theory

Exposure Methods

Study Design

Scoring

Criteria for a Valid Test

Evaluation Criteria

Clarifying Equivocal Responses

Sample Data

You make the Call

Summary

Definitions

HPRT: Hypoxanthine/Guanine Phosphoribosyl Transferase
(the gene/enzyme formerly known as HGPRT)

CHO: Chinese Hamster Ovary (cell line)

TG: 6-Thioguanine (selective agent for HPRT)

CE: Cloning efficiency (% surviving cells);

IMF: Induced Mutant frequency
(proportion of mutant cells over vehicle control)

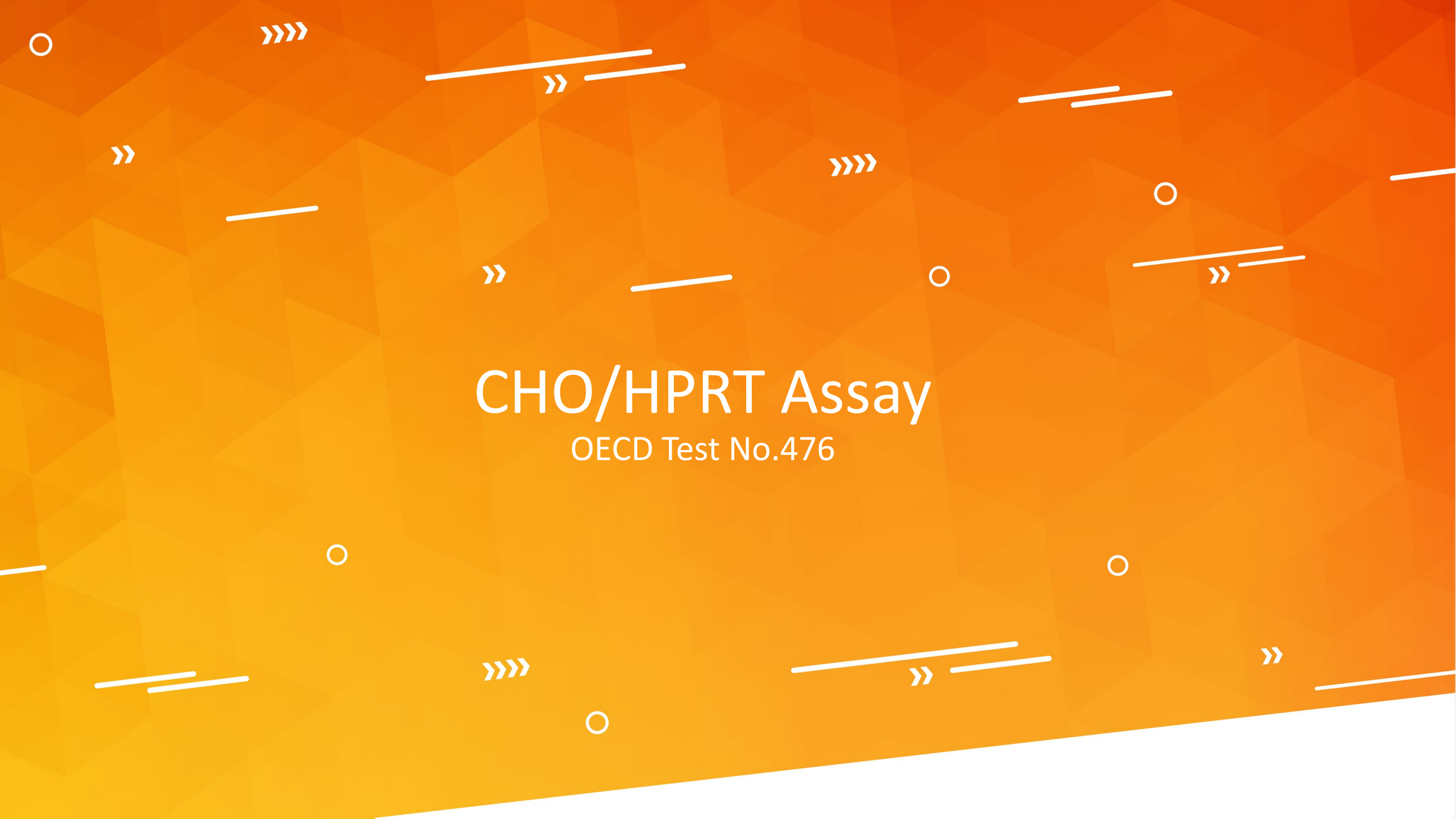
TK: Thymidine Kinase

TFT: Trifluorothymidine (selective agent for MLA)

MF: Mutant frequency (proportion of mutant cells)

Mammalian Cell Gene Mutation Assays

- Detection of gene mutations in mammalian cells (point mutations, chromosome rearrangements) not detected by Ames (base pair sub., frame-shift, insertion/deletion) or chromosome aberration assays
- TG476 was originally adopted in 1984. Revised in 1997
- New guidelines were put in place July 2015 and updated in 2016. MLA/TK assays were removed from TG476 and retain only HPRT and XPRT
- HPRT (TG476) and Mouse Lymphoma assays (TG490): evaluate forward mutations (any mutation which changes a formerly functional wild genotype or phenotype to nonfunctional)
- Current version of TG476 reflects nearly 40 years of experience with this test



CHO/HPRT Assay

OECD Test No.476

HPRT Assay Background

- HPRT – enzyme catalyzes salvage of the purine bases guanine and hypoxanthine into monophosphate nucleoside GMP and IMP DNA synthesis
- Measures forward mutations in reporter genes, specifically, the endogenous hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene
- HPRT gene is on the X chromosome of mammalian cells – has 1 functional copy (hemizygous)

Cell Types for HPRT

Cell types used for the HPRT tests should have a

- Demonstrated sensitivity to chemical mutagens
- High cloning efficiency
- Stable karyotype; Stable spontaneous mutant frequency

Most commonly used cells:

CHO, CHL and V79 lines of Chinese hamster cells, L5178Y mouse lymphoma cells, and TK6 human lymphoblastoid cells

Methodology:

PURPOSE: To evaluate a test substance for its ability to induce forward mutations at the HPRT locus (*Hprt*) in Chinese hamster ovary (CHO) cells, as assayed by colony growth in the presence of 6-thioguanine (TG resistance, TG^r).

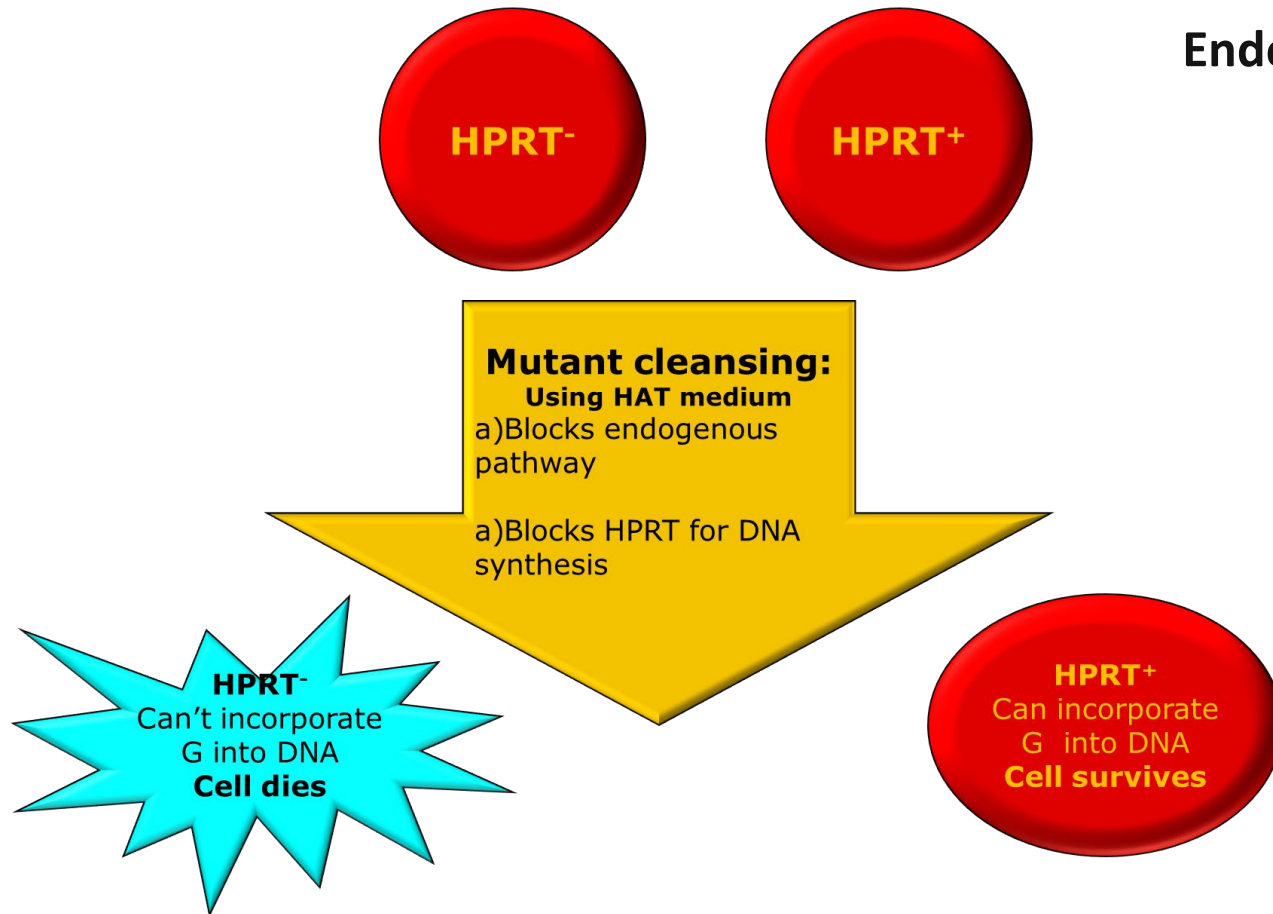
Mutations which destroy the functionality of the HPRT gene and or/protein are detected by positive selection using a toxic analogue, and HPRT mutants are seen as viable colonies

Mutants deficient in HGPRT grow in the presence of the purine analog, 6-thioguanine (6TG), but normal cells convert 6TG to its toxic nucleotides and die.

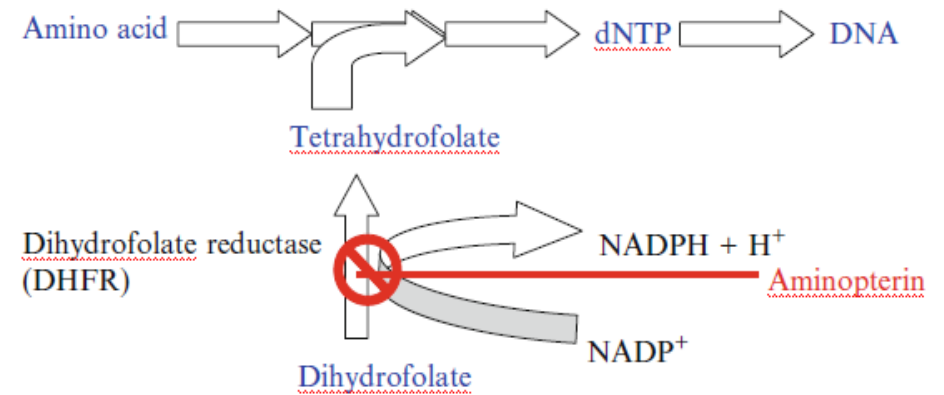
Formation of mutant colonies caused by result from either:

- 1) spontaneous mutation
- 2) induced mutation caused by a chemical agent

HAT Selection



Endogenous Pathway – direct synthesis of nucleotides



Study Design: Vehicle Selection

(HPRT and MLA)

- Does not impact the test system
- Does not react with culture vessel
- Does not impact the integrity of the TA
- Does not interrupt metabolic activity
- Is soluble or TA has reached the limit of solubility

Aqueous formulations
up to 5 or 50 mg/mL
(ICH or OECD)

- Water
- Culture media
- Saline

Organic formulations
up to 50 or 200 mg/mL
(ICH or OECD)

- DMSO
- Ethanol
- Acetone

Study Design: Dose Range-Finding Assay

(HPRT and MLA)

- Limit dose: for chemicals - 2000 μ g/mL (10mM) – whichever is lower for pharmaceuticals - 500 μ g/mL (1mM) - whichever is lower
- Test at least 9-10 concentrations, single cultures
- pH of the TA in treatment media: between 6.5 - 7.5
- Is the osmolality value >120% of solvent control? Yes? – then could result in false positive

Process Flow

SEED

- 1×10^6 cells seeded in 5 mL of Complete Ham's F12
- Using T-25 flasks, Grow overnight

RE-FEED

- Wash x 2 with HBSS
- (-S9) Re-feed with 5 mL Suppl. F12
- (+S9) Re-feed with 4 mL + 1 mL S9 mix

DOSE

- Dose with test article
- Incubate for 5 hours

Harvest

- Remove treatment media
- Wash x 2 with CMF-HBSS
- Trypsinize and Count

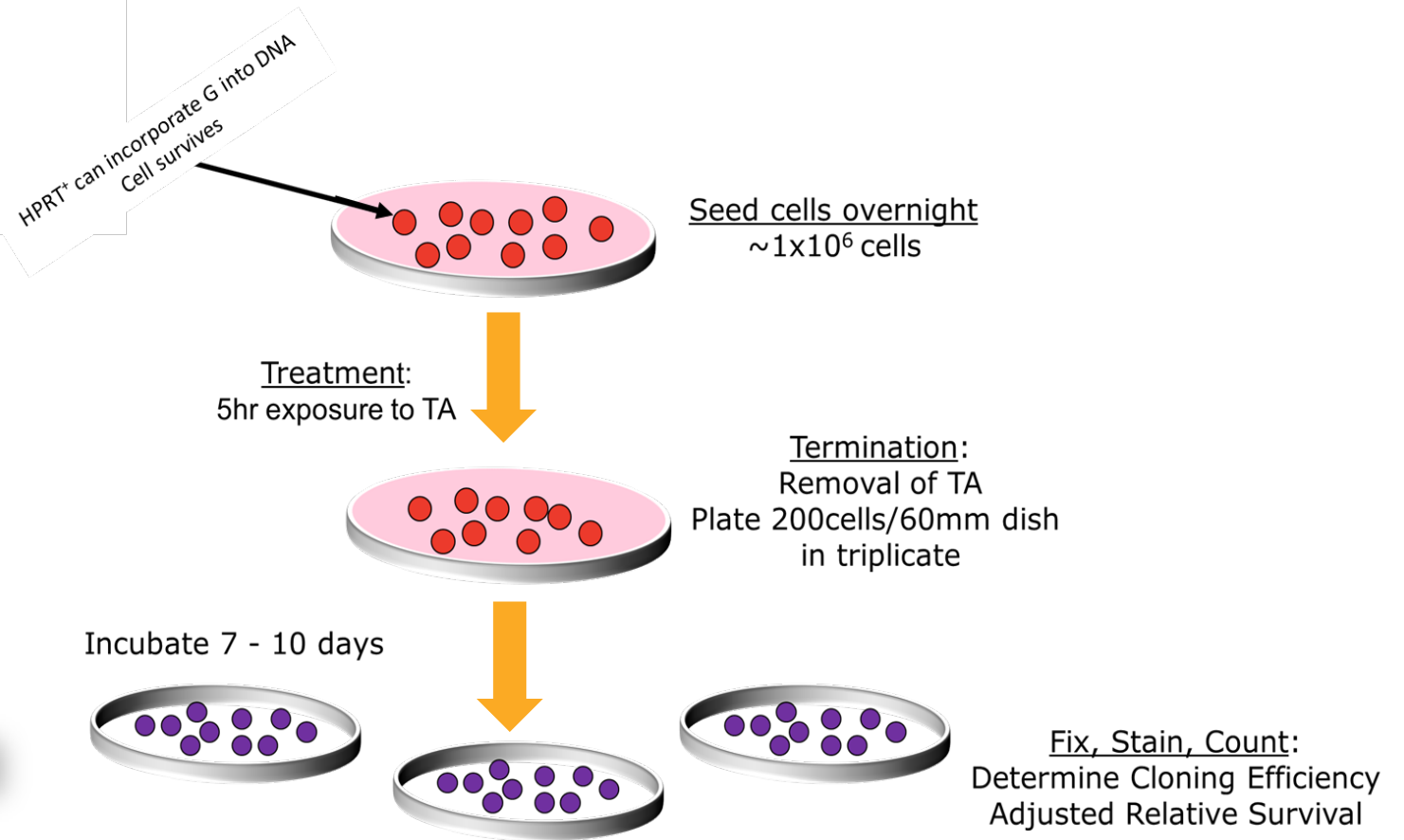
PC-Plate

- Plate 200 cells/60 mm dish x 3
- 5 mL of Complete Ham's F12
- Incubate 7-10 days

Fix / Stain / Count

Determine CE and Relative Survival (adjusted)

Preliminary Toxicity (Dose Range Finder)



Sample Data: Dose Range-Finding Assay

Treatment	Dose Level (µg/mL)	S9	Cloning Efficiency (Initial Survival)					Cell Density Cells/mL (x10 ⁶)	Adjusted Relative Survival (%)
			Plate Counts						
			1	2	3	Avg.	CE (%) ^a		
Vehicle	0.0 ^b	-	194	146	134	158.0	79.0	0.214	100.00
Test Article	3.91	-	141	156	163	153.3	76.7	0.231	104.74
Test Article	7.81	-	157	144	152	151.0	75.5	0.211	93.85
Test Article	15.6	-	157	140	137	144.7	72.3	0.219	93.49
Test Article	31.3	-	138	140	164	147.3	73.7	0.224	97.43
Test Article	62.5	-	148	153	168	156.3	78.2	0.209	96.37
Test Article	125	-	150	176	173	166.3	83.2	0.182	89.35
Test Article	250	-	126	134	118	126.0	63.0	0.215	79.93
Test Article	500	-	97	106	107	103.3	51.7	0.196	59.84
Test Article	1000	-	88	69	72	76.3	38.2	0.170	38.31
Test Article	2000	-	54	45	39	46.0	23.0	0.103	13.99

Process Flow Definitive Assay

- SEED
 - 5×10^6 or 8×10^6 cells
- RE-FEED
 - Wash x 2 with HBSS
 - Serum free media and/or S9
- DOSE/HARVEST
 - Dose with TA- Incubate for 5 hours
 - Remove treatment media, Wash x 2 with HBSS and trypsinize
- INITIAL SURVIVAL
 - Plate 200 cells / 60 mm dish x 3
 - Incubate 7-10 days
- SUBCULTURE
 - 2.4×10^6 cells
 - Every 2-3 days for 7-8 days

Mutagenicity (Definitive Assay)

1. Treatment
2. Expression
3. Selection
4. Scoring

TREATMENT

HPRT⁺ can incorporate G into DNA
Cell survives

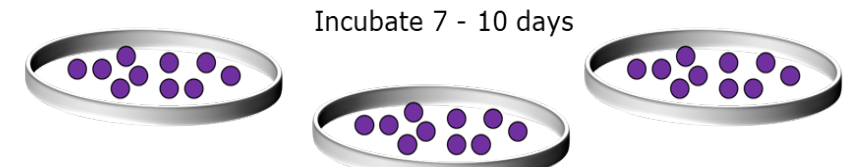
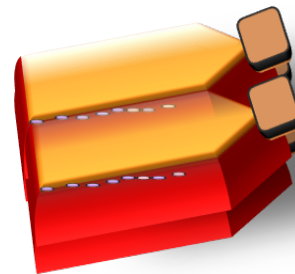
Seed cells overnight:
 5×10^6 cells

RE-FEED:

DOSE HARVEST:
Removal of TA
Wash with HBSS

Termination:
Removal of TA
Subculture (Phenotypic Expression)
Plate 200 cells/60mm dish (CC)

EXPRESSION



Incubate 7 - 10 days
Fix, Stain, Count (CC): Cloning Efficiency
Adjusted Relative Survival (ARS)

SUBCULTURE
 2.4×10^6 cells
every 2-3 days for 7-10days

Process Flow

Definitive Assay cont'd

CONCURRENT SURVIVAL

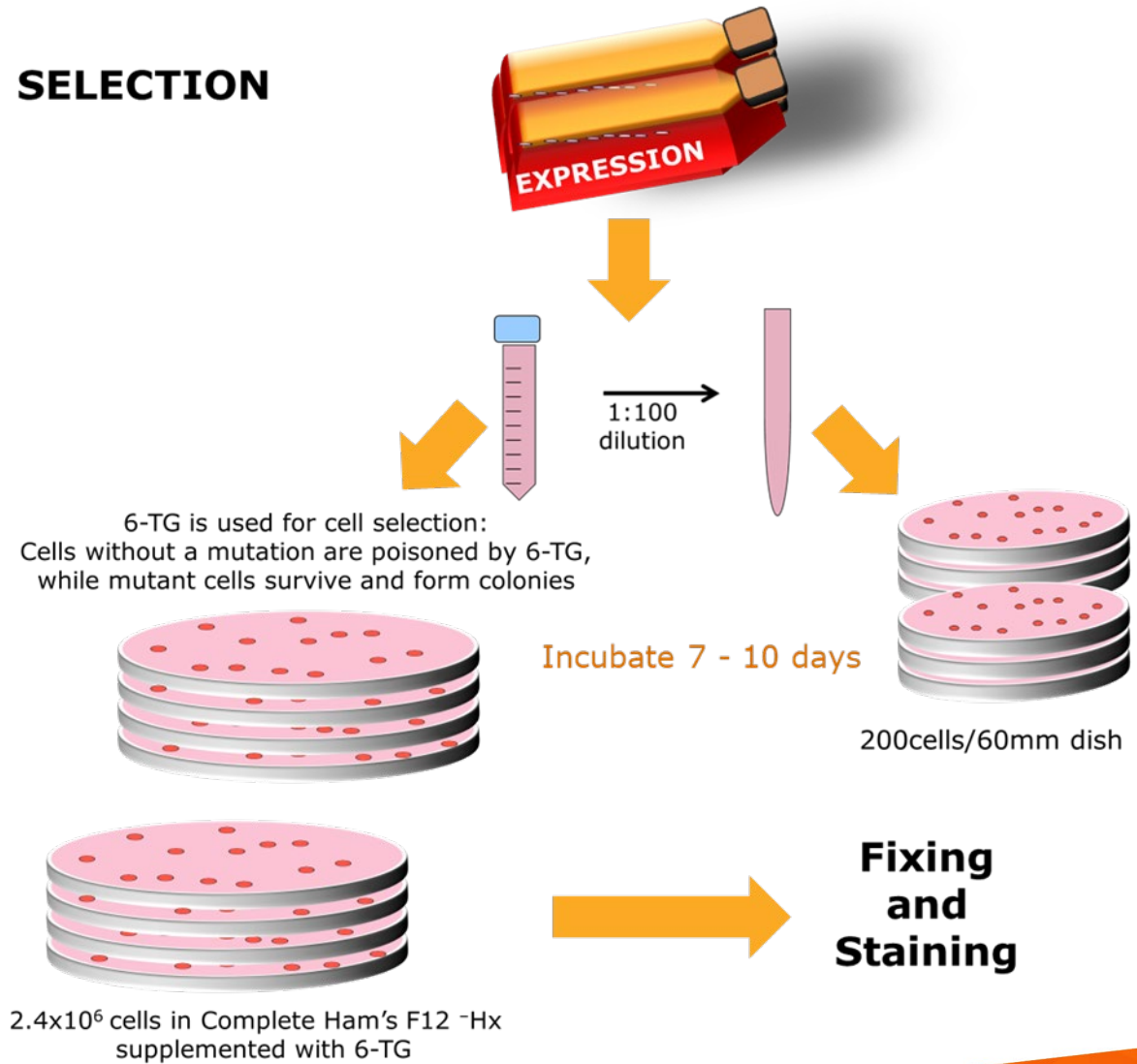
- Plate 200 cells / 60 mm dish x 3
- Incubate 7-10 days

SELECTION

- Plate 6×10^5 cells/150 mm dishes (4x TG/media dishes)
- Incubate 7-10 days

Fix / Stain / Count

SELECTION

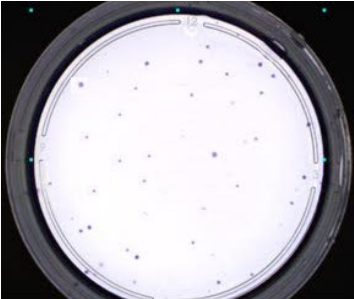


SCORING

Solvent Control



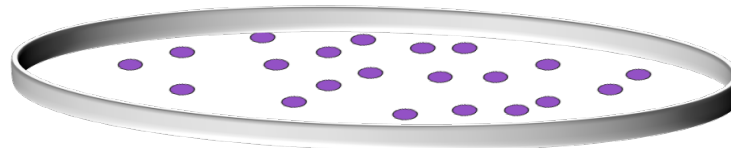
Positive Control



Mutation Plates



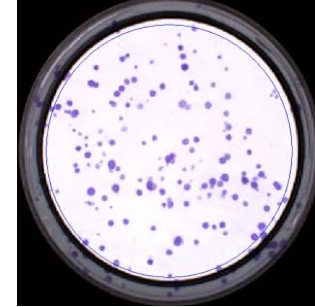
Solvent



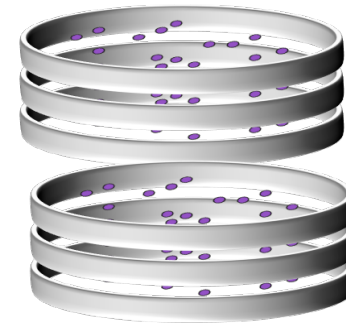
Positive Control



CE Plate



Fix, Stain, Count
(CE and M plates):
Cloning Efficiency



CE Plates

CE plates and number of
mutant colonies
determine the Mutant
Frequency

Inotiv's GLP vs non-GLP

GLP

- DRF assay with $\sim 1 \times 10^6$ cells/culture /treatment
- QA audits
- Duplicate cultures for each treatment
- Definitive assay with $\sim 5 \times 10^6$ or 8×10^6 cells/culture
- $\sim 2.4 \times 10^6$ cells/culture for expression and Mutation selection

Non-GLP screening

- No DRF assay
- No QA audit
- Single cultures for each treatment
- Definitive assay with $\sim 1 \times 10^6$ cells/culture for treatment expression and Mutation selection

Data Calculations/Endpoint readings

$$\text{Cloning Efficiency (CE)} = \frac{\text{number of colonies}}{\text{number of cells plated}} \times 100\%$$

$$\text{Adjusted Relative Survival (ARS)} = \frac{\text{CE (treated)} \times \text{cell density (treated)}}{\text{CE (control)} \times \text{cell density (control)}} \times 100\%$$

$$\text{Mutant Frequency} = \frac{\text{number of mutant colonies/number of cells selected}}{\text{CE}}$$

Criteria for a Valid Test

Vehicle Controls:

- Average absolute CE must be >60% at initial survival and selection
- Average spontaneous mutant frequency of the vehicle controls are within the 95% control limits of historical control data

Positive Controls:

- Must induce a significant increase in mutant frequency as compared to the concurrent vehicle controls ($p < 0.01$)

Test Concentrations:

- the limit dose (2000 μ g/mL or 10mM; 500 μ g/mL or 1mM)
- the highest concentration able to be prepared in the vehicle (whichever is lowest)
- induce 10 to 20% adjusted relative survival
- Minimum of four acceptable concentrations

Sample Data: Mutagenicity Assay

Treatment	Dose Level (µg/mL)	Cloning Efficiency (Initial Survival)				Cell Density [Cells/mL (x10 ⁶)]	ARS (%)	Selection Plate Counts										MF (x10 ⁶)	Avg. MF (x10 ⁶)
		Plate Counts						TGr Colonies/Dish					Cloning Efficiency						
		1	2	3	CE (%)			1	2	3	4	Sum	1	2	3	CE (%)			
Vehicle	0.0 ^a	197	204	183	97.3	0.214	101.0	1	1	1	6	9	171	179	198	91.3	4.11	3.83	
Vehicle	0.0 ^a	189	166	175	88.3	0.231	99.0	2	2	1	2	7	166	171	154	81.8	3.56		
Test Article	125	146	154	138	73.0	0.210	74.2	3	0	2	6	11	129	135	140	67.3	6.81	7.10	
Test Article	125	161	163	144	78.0	0.209	78.9	4	2	3	4	13	141	148	151	73.3	7.39		
Test Article	250	117	126	127	61.7	0.201	60.0	2	3	5	0	10	156	163	144	77.2	5.40	4.00	
Test Article	250	129	108	122	59.8	0.187	54.2	1	1	1	1	4	124	133	128	64.2	2.60		
Test Article	500	108	89	92	48.2	0.196	45.7	3	3	1	1	8	147	174	155	79.3	4.20	2.89	
Test Article	500	99	85	79	43.8	0.170	36.1	0	2	1	0	3	162	152	161	79.2	1.58		
Test Article	1000	88	82	77	41.2	0.144	28.7	8	0	0	2	10	119	177	148	74.0	5.63	2.82	
Test Article	1000	62	71	54	31.2	0.159	24.0	0	0	0	0	0	153	156	160	78.2	0.00		
Test Article	2000	75	65	58	33.0	0.121	19.3	0	1	0	0	1	187	181	189	92.8	0.45	2.57	
Test Article	2000	57	61	49	27.8	0.103	13.9	5	1	1	2	9	161	200	119	80.0	4.69		
EMS ^b	0.200 ^a	81	81	70	38.7	0.259	48.5	115	114	110	107	446	111	119	99	54.8	338.91	364.96 ^{**}	
EMS ^b	0.200 ^a	87	94	97	46.3	0.220	49.4	136	125	132	120	513	116	102	110	54.7	391.01		

^aDose level expressed in $\mu\text{L/mL}$, ^bEMS = Ethylmethanesulfonate (positive control)

**Significant increase in mutant frequency as compared to the concurrent vehicle control values ($p \leq 0.01$)

You Make the Call

Treatment	Dose Level (µg/mL)	Cloning Efficiency (Initial Survival)				Cell Density [Cells/mL (x10 ⁶)]	ARS (%)	Selection Plate Counts										MF (x10 ⁶)	Avg. MF (x10 ⁶)
		Plate Counts						TG ^r Colonies/Dish					Cloning Efficiency						
		1	2	3	CE (%)			1	2	3	4	Sum	1	2	3	CE (%)			
Vehicle	0.0 ^a	197	204	184	97.5	0.233	103.5	1	2	1	6	10	165	179	188	88.7	4.70		
Vehicle	0.0 ^a	189	166	174	88.2	0.240	96.5	2	2	1	2	7	176	171	164	85.2	3.42	4.06	
Test Article	125	146	154	138	73.0	0.219	72.9	3	0	2	6	11	130	155	142	71.2	6.44		
Test Article	125	161	163	144	78.0	0.228	81.1	4	2	3	4	13	140	158	161	76.5	7.08	6.76	
Test Article	250	117	126	127	61.7	0.230	64.6	2	3	5	0	10	155	162	154	78.5	5.31		
Test Article	250	129	108	122	59.8	0.226	61.6	1	1	1	1	4	162	141	139	73.7	2.26	3.79	
Test Article	500	108	89	92	48.2	0.195	42.8	3	3	1	1	8	155	176	139	78.3	4.26		
Test Article	500	99	85	79	43.8	0.189	37.8	0	2	1	0	3	151	155	158	77.3	1.62	2.94	
Test Article	1000	88	82	77	41.2	0.183	34.3	8	0	0	2	10	125	167	143	72.5	5.75		
Test Article	1000	62	71	54	31.2	0.178	25.3	0	0	0	0	0	149	166	151	77.7	0.00	2.87	
Test Article	2000	75	65	58	33.0	0.200	30.1	0	1	0	0	1	177	183	169	88.2	0.47		
Test Article	2000	57	61	49	27.8	0.221	28.0	5	1	1	2	9	162	159	144	77.5	4.84	2.66	
EMS ^b	0.200 ^a	81	81	70	38.7	0.209	36.8	115	114	110	107	446	118	129	103	58.3	318.57		
EMS ^b	0.200 ^a	87	94	97	46.3	0.201	42.5	136	125	132	120	513	131	122	137	65.0	328.85	323.71 ^{**}	

^aDose level is µL/mL

^bEMS = Ethylmethanesulfonate (positive control)

**Significant increase (p ≤ 0.01)

You Make the Call

Treatment	Dose Level (µg/mL)	Cloning Efficiency (Initial Survival)						Selection Plate Counts										MF (x10 ⁶)	Avg. MF (x10 ⁶)
		Plate Counts				Cell Density [Cells/mL (x10 ⁶)]	ARS (%)	TG ^r Colonies/Dish					Cloning Efficiency						
		1	2	3	CE (%)			1	2	3	4	Sum	1	2	3	CE (%)			
Vehicle	10.0 ^a	187	194	173	92.3	0.222	105.3	2	1	1	1	5	171	179	198	91.3	2.28	2.67	
Vehicle	10.0 ^a	179	156	165	83.3	0.221	94.7	3	0	1	2	6	166	171	154	81.8	3.05		
Test Article	99.5	166	174	158	83.0	0.210	89.6	0	1	2	0	3	129	135	140	67.3	1.86		
Test Article	99.5	181	183	164	88.0	0.219	99.0	1	2	3	2	8	141	148	151	73.3	4.55		
Test Article	199	167	190	185	90.3	0.221	102.6	1	0	5	3	9	156	163	144	77.2	4.86		
Test Article	199	183	216	172	95.2	0.217	106.1	5	4	1	6	16	124	133	128	64.2	10.39	7.62	*
Test Article	398	159	182	210	91.8	0.186	87.8	6	5	7	3	21	147	174	155	79.3	11.03	12.09	**
Test Article	398	188	203	212	100.5	0.180	93.0	9	6	6	4	25	162	152	161	79.2	13.16		
Test Article	796	150	184	189	87.2	0.174	78.0	10	8	9	7	34	119	177	148	74.0	19.14		
Test Article	796	165	173	156	82.3	0.169	71.5	9	12	11	8	40	153	156	160	78.2	21.32	20.23	**
Test Article	1592	195	167	160	87.0	0.191	85.4	14	11	12	9	46	187	181	189	92.8	20.65	24.39	**
Test Article	1592	179	183	149	85.2	0.212	92.8	12	16	15	11	54	161	200	119	80.0	28.13		
EMS ^b	0.200 ^a	101	101	91	48.8	0.249	62.5	112	129	112	116	469	111	119	99	54.8	356.38		
EMS ^b	0.200 ^a	108	115	118	56.8	0.210	61.3	106	115	102	112	435	116	102	110	54.7	331.55	343.97	**

^aDose level is µL/mL

^bEMS = Ethylmethanesulfonate (positive control)

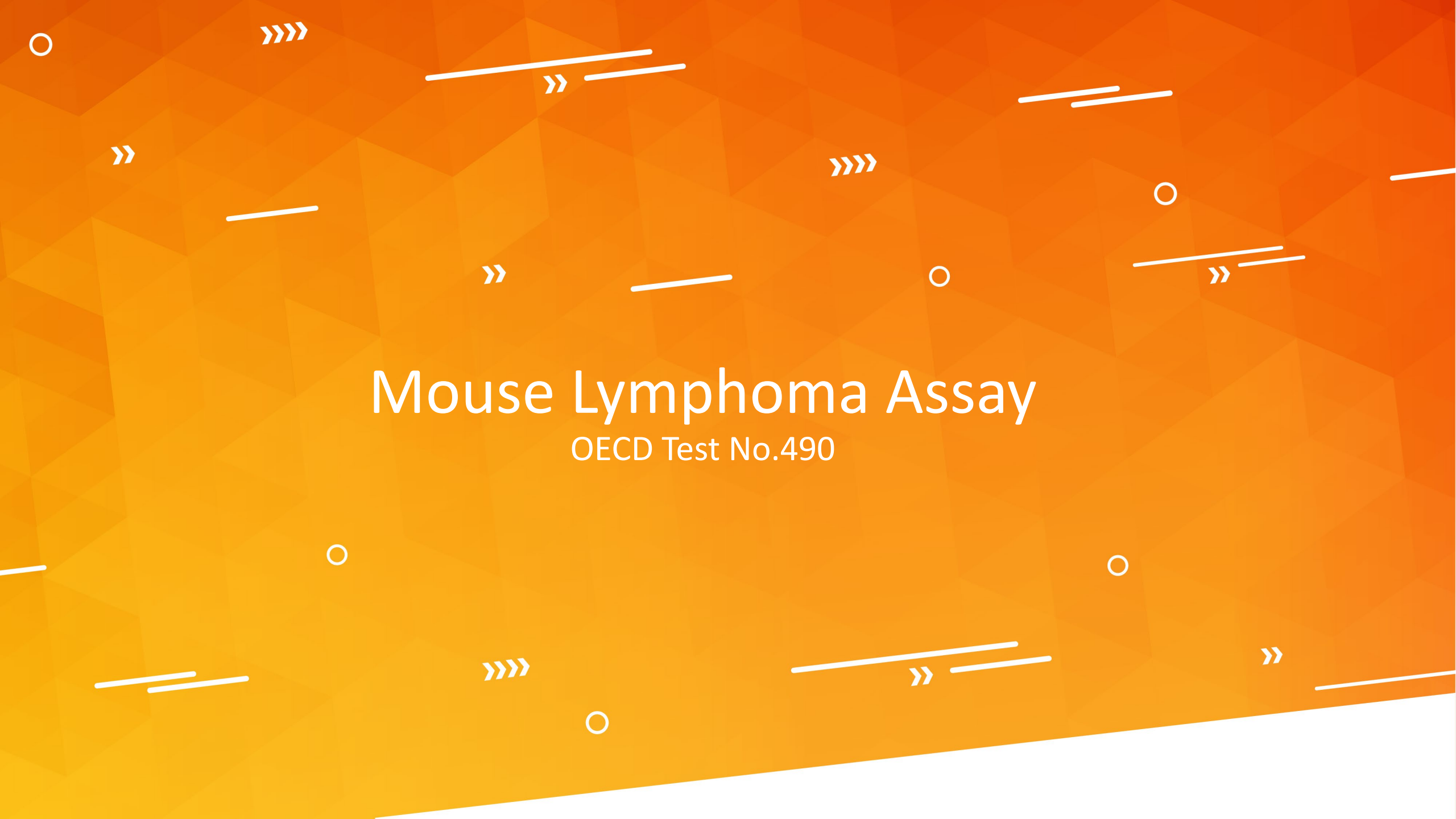
*Significant increase (p ≤ 0.05)

**Significant increase (p ≤ 0.01)

You Make the Call

Treatment	Dose Level (µg/mL)	Cloning Efficiency (Initial Survival)			Cell Density [Cells/mL (x10 ⁶)]	ARS (%)	Selection Plate Counts											Avg. MF (x10 ⁶)
		Plate Counts					TG ⁺ Colonies/Dish					Cloning Efficiency						
		1	2	3			CE (%)	1	2	3	4	Sum	1	2	3	CE (%)	MF (x10 ⁶)	
Vehicle	0.0 ^a	213	217	231	110.2	0.214	96.3	2	2	1	1	6	169	139	158	77.7	3.22	2.96
Vehicle	0.0 ^a	211	237	212	110.0	0.231	103.7	3	1	0	0	4	109	130	131	61.7	2.70	
Test Article	106	106	192	198	82.7	0.210	70.8	1	4	0	0	5	135	189	181	84.2	2.48	2.29
Test Article	106	205	217	196	103.0	0.209	87.7	2	1	0	1	4	137	163	177	79.5	2.10	
Test Article	212	211	208	217	106.0	0.201	86.8	2	4	3	2	11	153	118	182	75.5	6.07	4.04
Test Article	212	218	235	277	121.7	0.187	92.7	0	2	1	1	4	156	172	170	83.0	2.01	
Test Article	424	262	258	237	126.2	0.196	100.9	0	0	0	1	1	153	187	147	81.2	0.51	2.26
Test Article	424	167	190	185	90.3	0.170	62.6	2	2	1	3	8	170	158	170	83.0	4.02	
Test Article	848	163	216	153	88.7	0.144	52.0	1	0	0	0	1	169	162	160	81.8	0.51	3.21
Test Article	848	159	182	210	91.8	0.159	59.5	2	1	4	5	12	175	172	161	84.7	5.91	
Test Article	1696	158	203	212	95.5	0.121	47.1	0	3	3	5	11	121	146	147	69.0	6.64	8.53
Test Article	1696	150	184	189	87.2	0.103	36.6	6	9	2	3	20	174	154	152	80.0	10.42	
B(a)P ^b	4.0	105	111	111	54.5	0.259	57.5	3	1	1	2	7	111	119	99	54.8	5.32	5.33
B(a)P ^b	4.0	88	94	92	45.7	0.220	41.0	3	2	1	1	7	116	102	110	54.7	5.34	

^bB(a)P (positive control) = benzo(a)pyrene

The background is a vibrant orange with a subtle geometric pattern of overlapping triangles. Scattered across the surface are various white decorative elements: single lines, double lines, and groups of three or four chevron-like shapes pointing in different directions. Some small white circles are also present.

Mouse Lymphoma Assay

OECD Test No.490

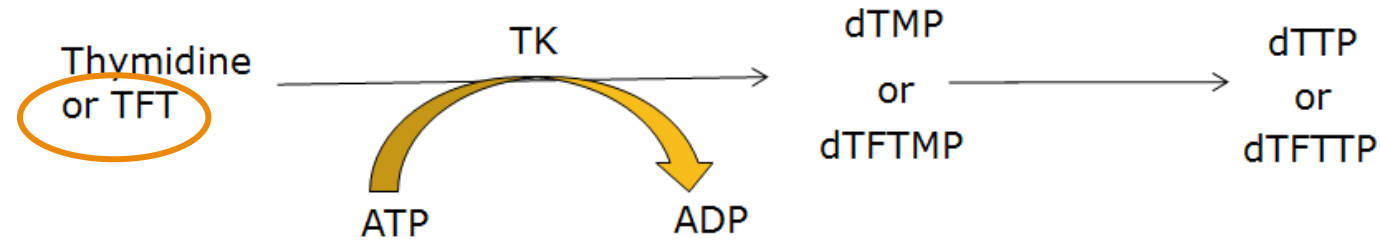
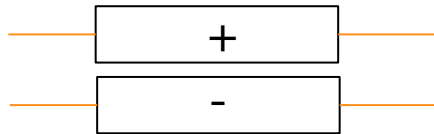
MOUSE LYMPHOMA ASSAY - Background

- TK an enzyme used in DNA replication and nucleotide synthesis
- Test system used to screen for possible gene/chromosome mutations induced by chemical compounds
- Measures forward mutations in the Thymidine Kinase (TK) reporter gene
- Mouse Lymphoma cell line L5178Y $TK^{+/-}$: heterozygous at TK locus

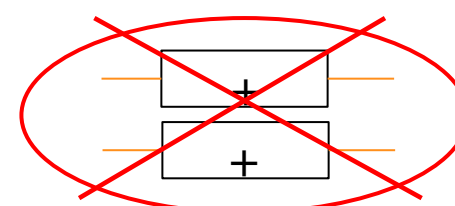
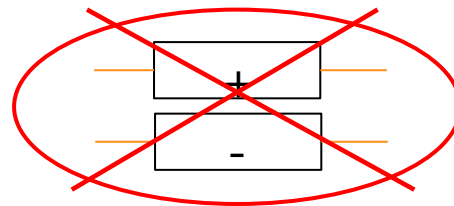
THMG Cleanse and TFT Selection

- THMG - Cells are cleansed within 7 days of use in the assay by treatment with THMG (Thymidine- Hypoxanthine-Methotrexate-Glycine).
- TFT -Thymidine Kinase (TK) -Salvage Pathway

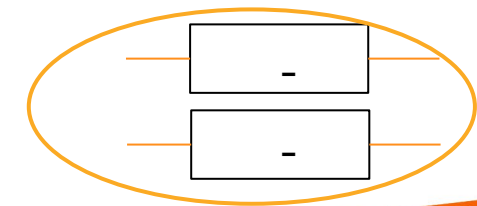
MLA (L5178Y TK^{+/-})
Functionally Heterozygous



Thymidine Kinase



Deficient in
Thymidine Kinase



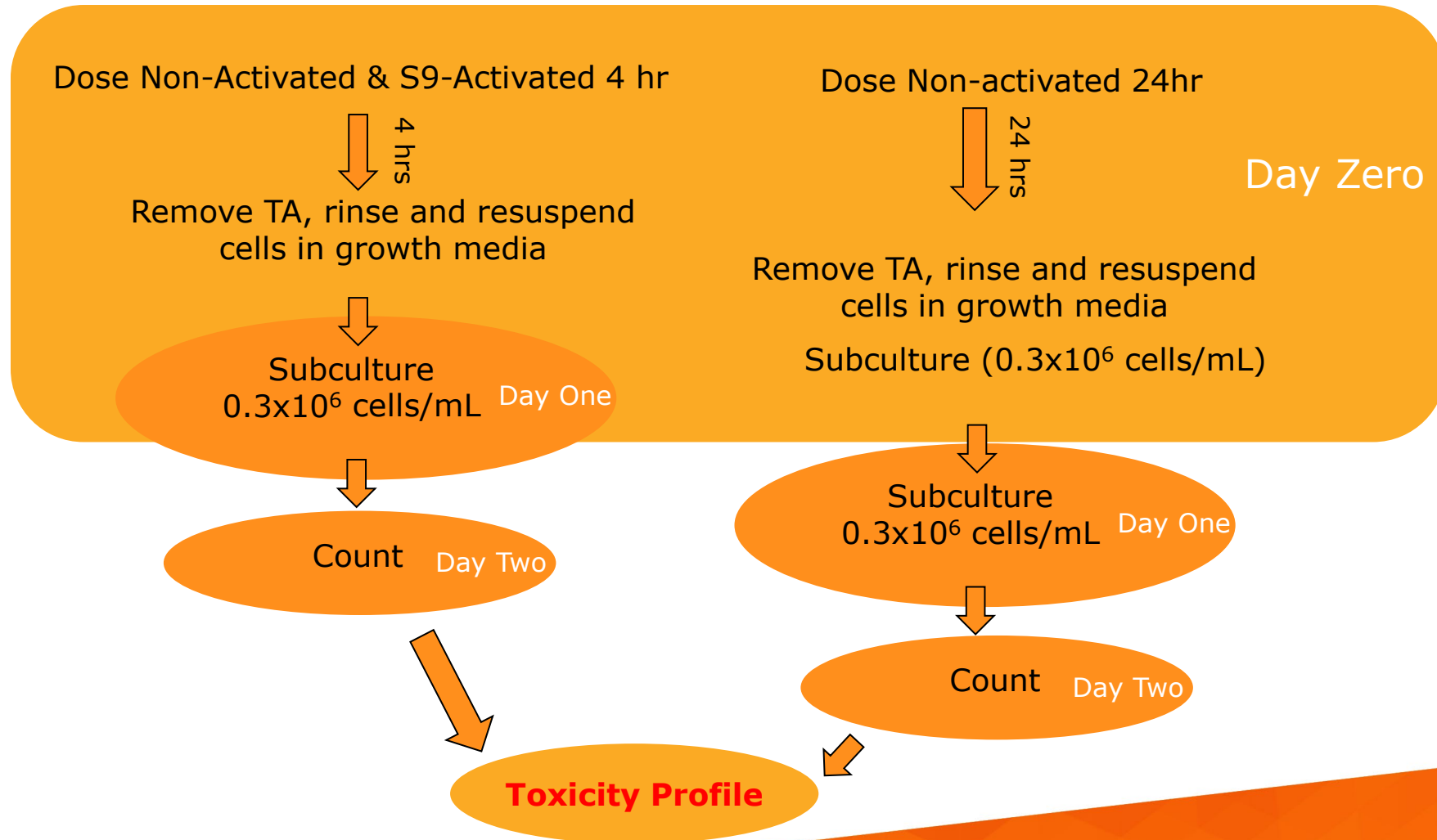
Study Design:

Vehicle Selection and Dose Range-Finding Assays are similar in HPRT and MLA

- Water
- Culture media
- Saline
- DMSO
- Ethanol
- Acetone
- Limit dose
- Number of concentrations (single cultures)
- pH and Osmolality

In MLA, cytotoxicity is based on relative suspension growth (RSG) over 48 hours (72 hrs for the 24-hr exposure)

Preliminary Toxicity (Dose Range Finder)



Pre-Tox Results

DOSE LEVEL (µg/mL)	T P D	CELL DENSITY (cells/mL x 10 ⁶)			SUSPENSION GROWTH	
		DAY 0	DAY 1	DAY 2	TOTAL	% OF CONTROL

4-HOUR –S9 CULTURES

SOLVENT A		Not applicable for 4-hour exposure	1.414	1.674	26.3	100
SOLVENT B			1.428	1.691	26.8	
0.150	N		1.385	1.799	27.7	104
0.500	N		1.243	1.571	21.7	82
1.50	N		1.511	1.590	26.7	100
5.00	N		1.532	1.556	26.5	100
15.0	N		1.484	1.519	25.0	94
50.0	N		1.499	1.663	27.7	104
150	N		1.354	1.516	22.8	86
500	N		1.490	1.554	25.7	97
1500	N		1.023	1.511	17.2	65

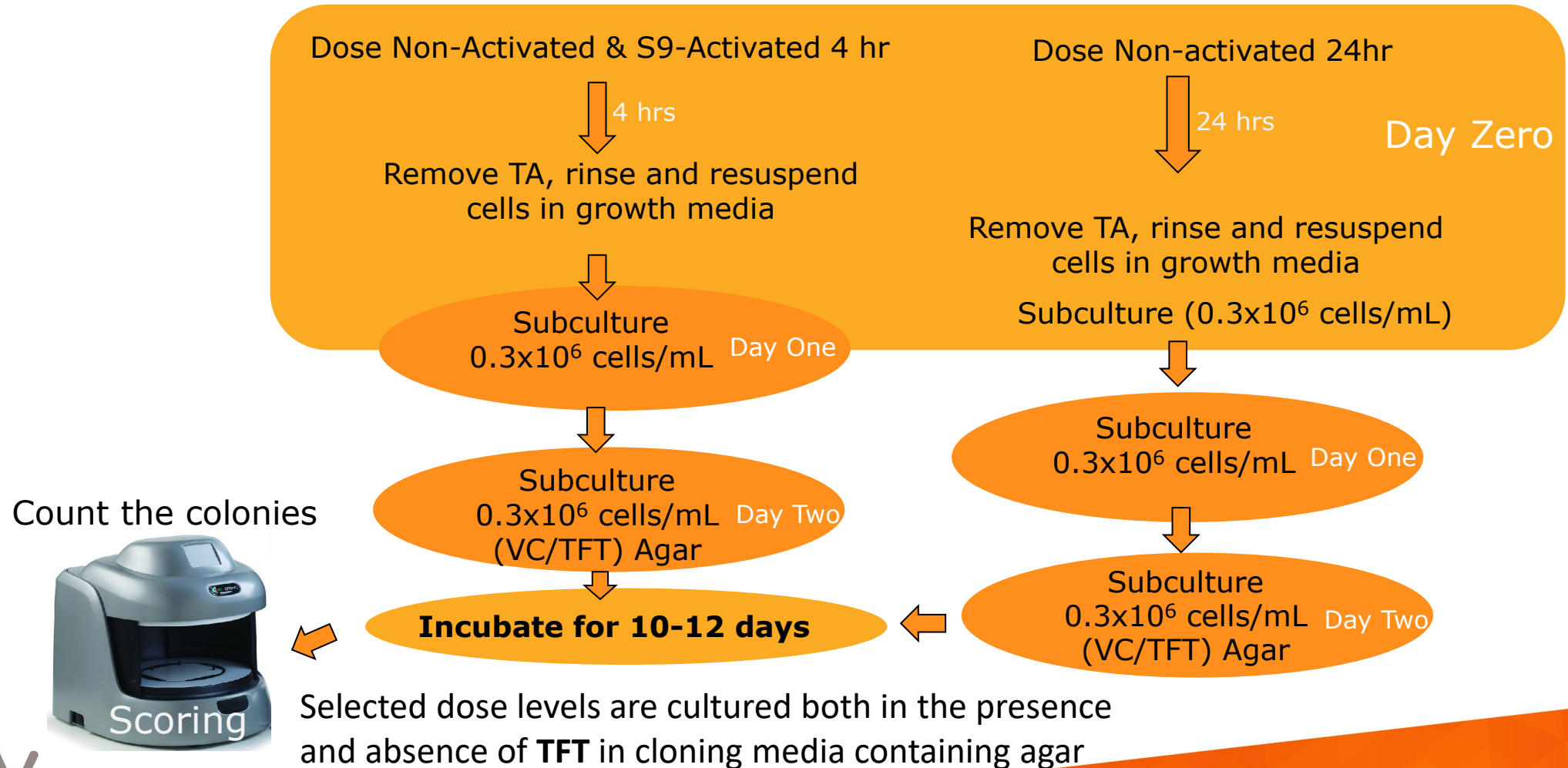
Mutagenicity Assay

- at least 5 doses, in duplicates
- positive and negative controls
- 4hr ±S9, 24hr –S9
- 6 million cells: to maintain 10-100 spontaneous mutants (all phases of assay)

Highest Concentration

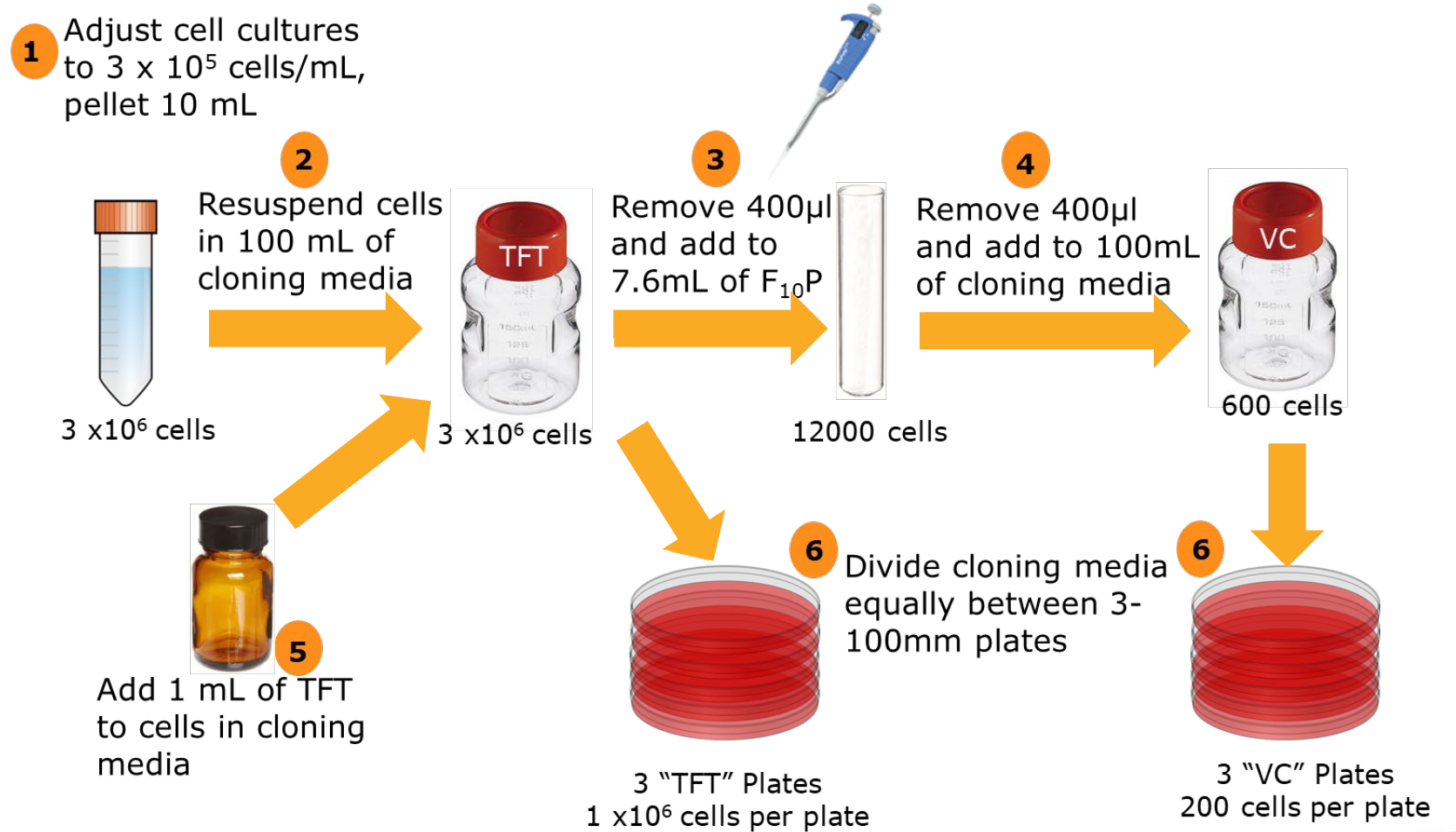
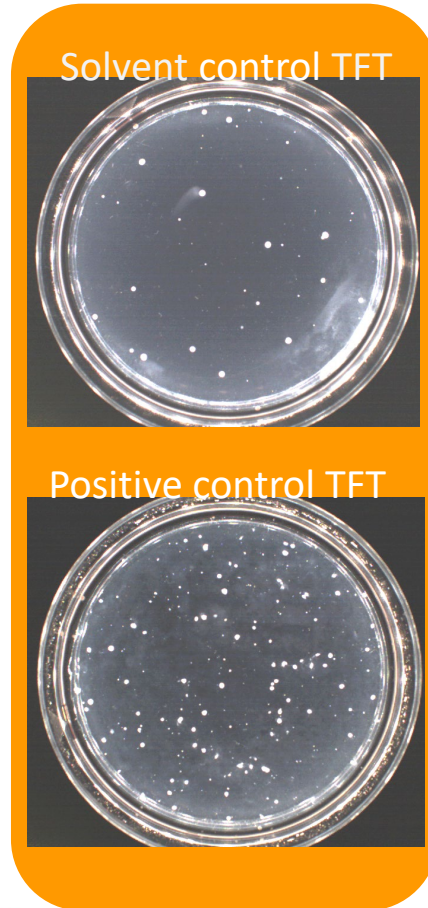
- between 10-90% Relative Survival
- limit dose 2000µg/mL (10mM) for chemicals, 500µg/mL (1mM) for pharmaceuticals–
whichever is lower

Mutagenicity Assay (Definitive Assay)



Study Design:

Selection



Inotiv's GLP vs non-GLP

GLP

- DRF assay with $\sim 6 \times 10^6$ cells/culture for treatment
- QA audits
- Duplicate cultures for each treatment
- Three treatment conditions (4-hour - S9; 4-hour +S9 and 24-hour -S9)
- Definitive assay with $\sim 6 \times 10^6$ cells/culture /treatment
- $\sim 3 \times 10^6$ cells/culture for expression and Mutation selection

Non-GLP screening

- No DRF assay
- No QA audit
- Two treatment conditions (4-hour - S9 and 4-hour +S9)
- Single cultures for each treatment
- Definitive assay with $\sim 6 \times 10^6$ cells/culture / treatment expression and Mutation selection

Criteria for a Valid Assay

- Solvent Controls:**
- Cloning efficiency: 65%-120%
 - Mutant frequency: 35-140 mutants per 10^6 cells (20-34 acceptable, if recovery of small colony mutants are demonstrated)
 - Suspension Growth: 8-32% (4-hr assays) or 32-180% (24-hr assays)
- Positive Controls:**
- At least one dose must exhibit ≥ 300 mutants per 10^6 IMF with 40% small colonies or, ≥ 150 mutants per 10^6 small colony IMF.
- Treated Cultures:**
- 4 analyzable TA-treated cultures required with-
 - Limit dose $2000\mu\text{g/mL}$ (10mM) for chemicals – whichever is lower $500\mu\text{g/mL}$ (1mM) for pharmaceuticals– whichever is lower
 - The limit of solubility is reached
 - At least one dose exhibits between 10% and 20% total growth

Results Analysis

- Exclude any cultures with <10% Relative Total Growth (RTG)
- A **Positive** Response will have:
 - Dose-dependent increase
 - IMF ≥ 90 mutants/ 10^6 cells
 - ≥ 2 -fold increase in MF
- A **Negative** Response will have:
 - None of the Above
- An **Equivocal** Response will have:
 - Some of the above
- Clarifying Equivocal Results
- Generally, requires additional testing:
 - Change in dose levels/ or activation conditions
 - Re-test using 24-hr continuous exposure (if not already done)
 - If re-test is positive, overall result is positive
 - If re-test is negative, overall result is negative
 - If re-test is still equivocal, overall result is equivocal

Assay Results (Solvent Controls)

Assay Results, 4hr +S9

DOSE LEVEL (µg/mL)		PRECIP.	% SUSP. GROWTH	VC COLONIES				TFT COLONIES				TOTAL MUTANT FREQUENCY (PER 10 ⁶ CELLS)	INDUCED MUTANT FREQUENCY (PER 10 ⁶ CELLS)	% RELATIVE TOTAL GROWTH	
				PLATE COUNTS				PLATE COUNTS							
				1	2	3	MEAN	1	2	3	MEAN				
SOLVENT A			100	180	169	165	171	45	45	46	45	53	N/A	100	
SOLVENT B				188	181	200	190	41	45	49	45	47			
125	A		113	122	116	126	121	45	45	53	48	79	28	76	
125	B		112	105	124	150	126	47	37	32	39	61	11	78	
250	A		87	98	121	104	108	41	51	44	45	84	34	52	
250	B		91	147	120	128	132	56	52	57	55	84	33	66	
500	A		50	180	171	153	168	76	77	83	79	94	43	46	
500	B		52	164	160	178	167	79	104	73	85	102	52	48	
1000	A		36	148	145	186	160	97	82	96	92	115	65	32	
1000	B		37	175	155	160	163	84	78	79	80	98	48	34	
2000	A		20	134	156	152	147	102	110	114	109	148	97	16	
2000	B		19	128	107	142	126	94	134	108	112	178	128	13	
POSITIVE CONTROL:			7,12-dimethylbenz(a)anthracene (DMBA) (µg/mL)												
1.5			23	77	71	75	74	149	169	132	150	404	353	10	
1			36	115	84	105	101	167	188	171	175	346	296	20	
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 12.5															
MEAN SOLVENT CLONING EFFICIENCY: 90%															
MEAN SOLVENT MUTANT FREQUENCY: 50												(PER 10 ⁶ CELLS)			

Solvent Control Criteria:

- Suspension Growth: 8-32 (4-hr assays) or 32-180 (24-hr assays)
- Cloning efficiency: 65%-120%
- Mutant frequency: 35-140 mutants per 10⁶ cells (20-34 acceptable if recovery of small colony mutants are demonstrated)

Assay Results (Positive Controls)

DOSE:	7,12-dimethylbenz(a)anthracene (DMBA)				1.5µg/mL	
INDUCED MUTANT FREQUENCY (per 10 ⁶ cells cloned):					353	404
% SMALL COLONIES:		78.6	SMALL COLONY IMF:		285	
SIZE (mm)	PLATE 1	PLATE 2	PLATE 3	MEAN	PERCENT	FREQ
0.2	48	59	49	52	41	166.6
0.4	26	24	15	22	17	70.5
0.52	14	17	14	15	12	48.0
0.63	13	9	9	10	8	32.0
0.71	13	16	8	12	10	38.4
0.79	4	13	3	7	6	22.4
0.86	5	1	6	4	3	12.8
0.93	2	3	4	3	2	9.6
0.99	1	1	0	1	1	3.2
1.05	0	0	1	0	0	0.0

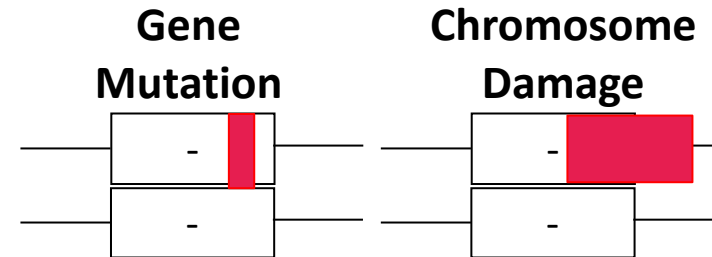
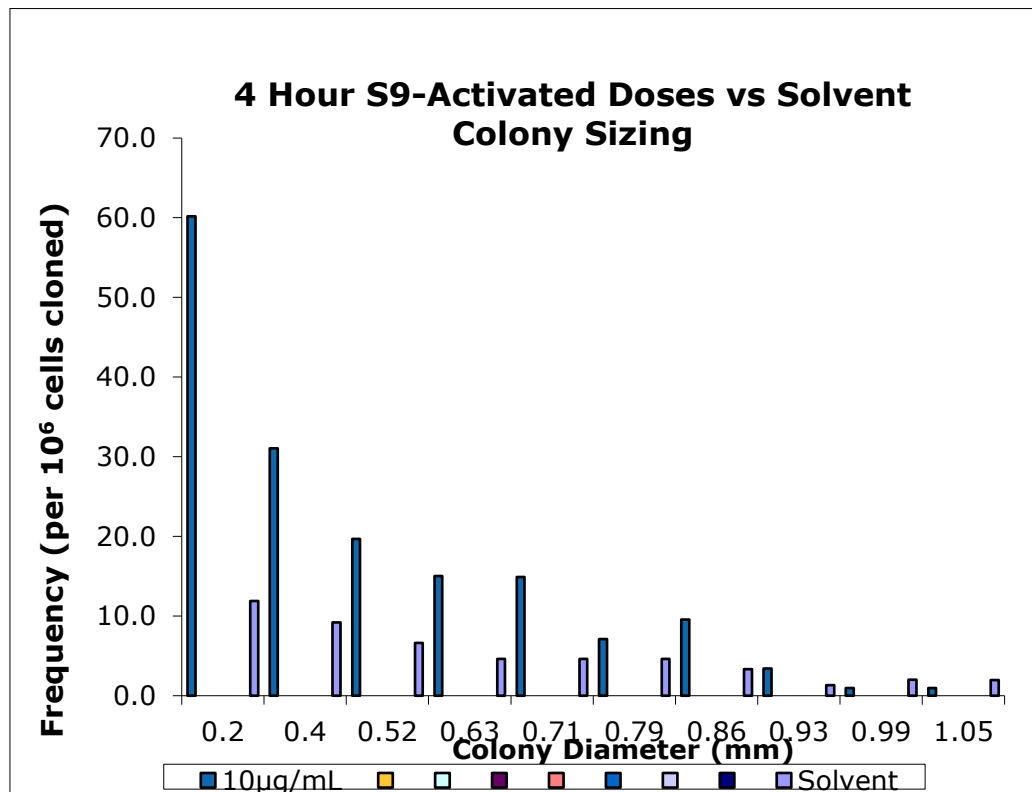
DOSE:		7,12-dimethylbenz(a)anthracene (DMBA)			1µg/mL	
INDUCED MUTANT FREQUENCY (per 10 ⁶ cells cloned):					296	346
% SMALL COLONIES:		82.8	SMALL COLONY IMF:			254
SIZE (mm)	PLATE 1	PLATE 2	PLATE 3	MEAN	PERCENT	FREQ
0.2	53	50	49	51	35	121.7
0.4	21	38	29	29	20	69.2
0.52	21	29	24	25	17	59.7
0.63	15	19	11	15	10	35.8
0.71	9	7	12	9	6	21.5
0.79	8	11	8	9	6	21.5
0.86	6	1	8	5	3	11.9
0.93	5	2	0	2	1	4.8
0.99	0	0	1	0	0	0.0
1.05	0	0	1	0	0	0.0

Positive Controls criteria:

- At least one dose must exhibit ≥ 300 mutants per 10⁶ IMF with 40% small colonies or, ≥ 150 mutants per 10⁶ small colony IMF.

Colony Sizing

MLA can detect mutations within a gene and chromosome.



Two classes of mutant colonies

- Large, arising from cells with localized damage (point mutations and small deletions)
- Small, arising from cells with more extensive damage (large deletions)

You Make the Call

Assay Results, 4hr +S9

DOSE LEVEL (µg/mL)		PRECIP.	% SUSP. GROWTH	VC COLONIES				TFT COLONIES				MF (x10 ⁶)	INDUCED MF (x10 ⁶)	RELATIVE TOTAL GROWTH (%)
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT A			100	196	201	181	193	68	44	44	52	54	N/A	100
SOLVENT B				206	206	184	199	50	54	45	50	50		
50	A	N	93	234	191	198	208	38	47	43	43	41	-11	99
50	B	N	95	196	200	178	191	34	56	50	47	49	-3	93
100	A	N	101	162	201	179	181	64	41	47	51	56	4	94
100	B	N	94	168	191	181	180	46	33	45	41	46	-6	86
250	A	N	97	203	225	196	208	43	43	44	43	42	-10	103
250	B	N	99	174	187	193	185	33	45	46	41	45	-7	94
500	A	N	101	214	236	228	226	55	50	41	49	43	-9	116
500	B	N	98	186	218	217	207	47	50	66	54	52	1	103
750	A	P	94	219	157	201	192	52	54	51	52	54	2	93
750	B	P	93	236	181	181	199	44	50	39	44	44	-8	95
POSITIVE CONTROL: 7,12-dimethylbenz(a)anthracene (DMBA) (µg/mL)														
1.5			15	106	105	68	93	253	256	223	244	525	473	7
1.25			19	119	113	112	115	220	247	276	248	432	380	11
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 22.0														
MEAN SOLVENT CLONING EFFICIENCY: 98%														
MEAN SOLVENT MUTANT FREQUENCY: 52 per 10 ⁶ clonable cells														

P = Precipitating dose level

You Make the Call

Assay Results, 24hr -S9

DOSE LEVEL (µg/mL)		PRECIP.	% SUSP. GROWTH	VC COLONIES				TFT COLONIES				MF (x10 ⁶)	INDUCED MF (x10 ⁶)	RELATIVE TOTAL GROWTH (%)
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT A			100	198	217	232	216	77	57	60	65	60	N/A	100
SOLVENT B				163	183	206	184	59	67	58	61	67		
62.5	A	N	99	182	198	193	191	47	60	52	53	55	-8	95
62.5	B	N	97	153	192	193	179	68	84	70	74	83	19	87
125	A	N	87	162	162	153	159	83	64	69	72	91	27	69
125	B	N	85	140	160	164	155	75	66	69	70	91	27	66
250	A	N	39	117	96	135	116	72	66	61	66	114	51	23
250	B	N	43	106	128	94	109	62	78	79	73	134	70	24
375	A	N	22	109	75	103	96	64	53	47	55	114	51	11
375	B	N	25	96	103	78	92	66	73	82	74	160	96	12
500	A	N	18	83	54	100	79	++	++	++				7
500	B	N	22	83	81	67	77	++	++	++				8
POSITIVE CONTROL: Methyl methanesulfonate (MMS) (µg/mL)														
7.5			15	116	115	108	113	242	259	232	244	432	369	8
5			23	129	123	138	130	149	180	176	168	259	196	15
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 32.3 (24-hour -S9)														
MEAN SOLVENT CLONING EFFICIENCY: 100%														
MEAN SOLVENT MUTANT FREQUENCY: 64 per 10 ⁶ clonable cells														

++ = Too toxic to clone

You Make the Call

Assay Results, 4hr +S9

DOSE LEVEL (µg/mL)		PRECIP.	% SUSP. GROWTH	VC COLONIES				TFT COLONIES				MF (x10 ⁶)	INDUCED MF (x10 ⁶)	% RELATIVE TOTAL GROWTH
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT A			100	121	126	106	118	43	19	19	27	46	N/A	100
SOLVENT B				131	131	109	124	25	29	20	25	40		
500	A	N	97	159	116	123	133	13	22	18	18	27	-17	99
500	B	N	93	121	125	103	116	9	31	25	22	37	-7	93
1000	A	N	96	87	126	104	106	39	16	22	26	49	5	94
1000	B	N	96	93	116	106	105	21	8	20	16	31	-13	86
2000	A	N	92	128	150	121	133	18	18	19	18	28	-16	103
2000	B	N	89	99	112	118	110	8	20	21	16	30	-14	94
3500	A	N	95	139	161	153	151	30	25	16	24	31	-13	116
3500	B	N	94	111	143	142	132	22	25	41	29	44	0	103
5000	A	N	92	144	82	126	117	27	29	26	27	47	3	93
5000	B	N	94	161	106	106	124	19	25	14	19	31	-13	95
POSITIVE CONTROL: 7,12-dimethylbenz(a)anthracene (DMBA) (µg/mL)														
1.5			14	31	30	37	33	114	128	112	118	721	677	7
1.25			21	44	38	37	40	110	124	138	124	624	580	11
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 15.9														
MEAN SOLVENT CLONING EFFICIENCY: 60%														
MEAN SOLVENT MUTANT FREQUENCY: 43 per 10 ⁶ clonable cells														

P = Precipitating dose level

Summary: Considerations

- Precipitation of test article in culture
- Certain solvents (ethanol and acetone) are incompatible with the 24hr exposure
- Screening assays available
- Extreme pH and high osmolality may induce adverse effects
- Gas exposure in closed systems

Summary: Comparisons

Mutational Event	Detected by	
	MLA	CHO/HPRT
Point mutation	yes	yes
Oligonucleotide (insertions, deletions)	yes	yes
Allele loss	yes	yes
Multi-locus mutations	yes	probably not
Small chromosome alterations	yes	no
Large chromosome alterations	yes	no

HPRT: Higher specificity, Lower false positive rate and cleaner interpretation

MLA: detects more types of genetic damage but Less specific for true gene mutations

Questions...



Thank you!