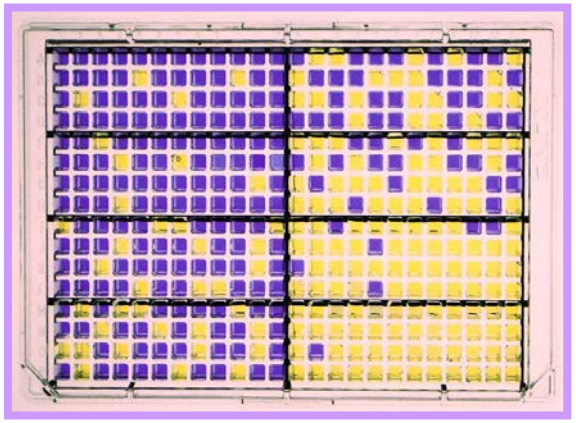
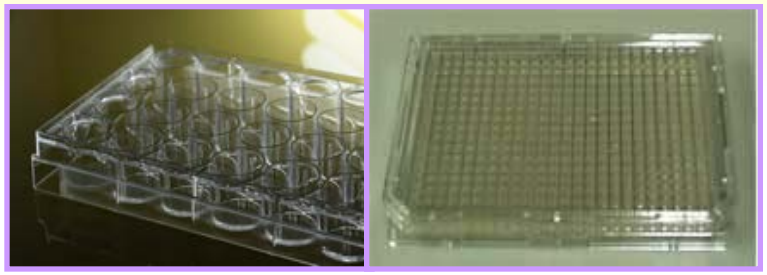
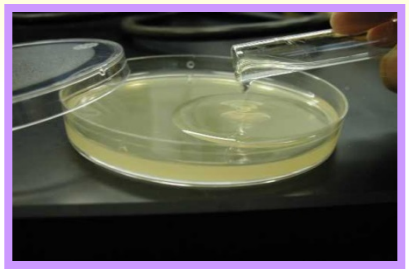
Comparison of the performance of the colorimetric Ames assay with the agar plate method



Ames MPF and Ames agar plate test





Ames MPF is based on same principle as agar plate test but

- Liquid low-volume format
- Use of microplates and multichannel pipettes
- Colorimetric read-out
- Less test sample - up to 4 fold
- Less S9 – up to 12 fold
- Higher throughput

Procedure Ames Microplate Assay

Bacterial stock -80ºC

Overnight culture

37ºC, 12-15 h
250 rpm

OD_{600}

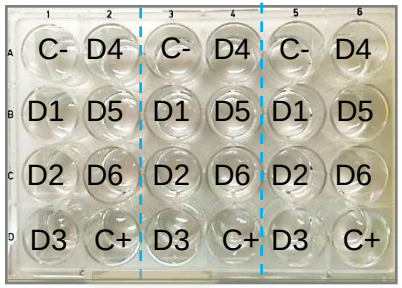
Assay preparation

a) Test sample dilutions, controls

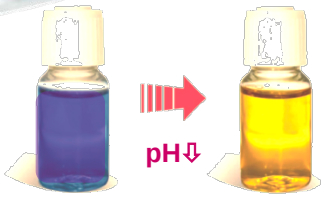
b)

Exposure cultures

replicates 1 replicates 2 replicates 3

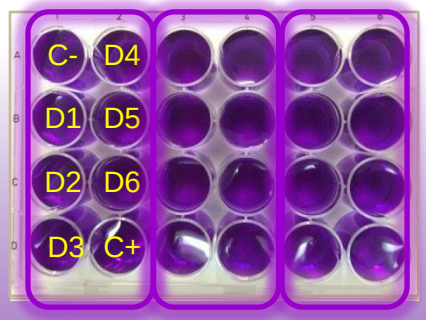


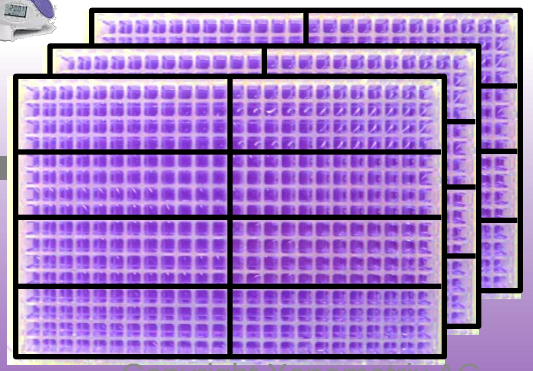
37ºC, 90 min, 250 rpm
(20 min E.coli +S9)



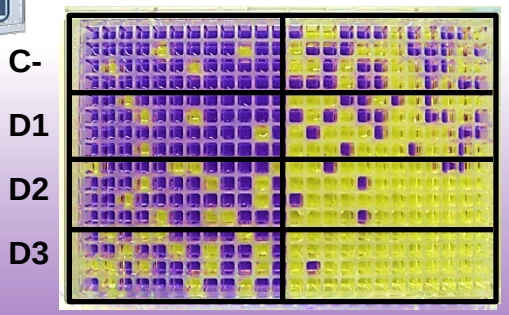
384-well plates

Indicator medium





48 h, 37ºC



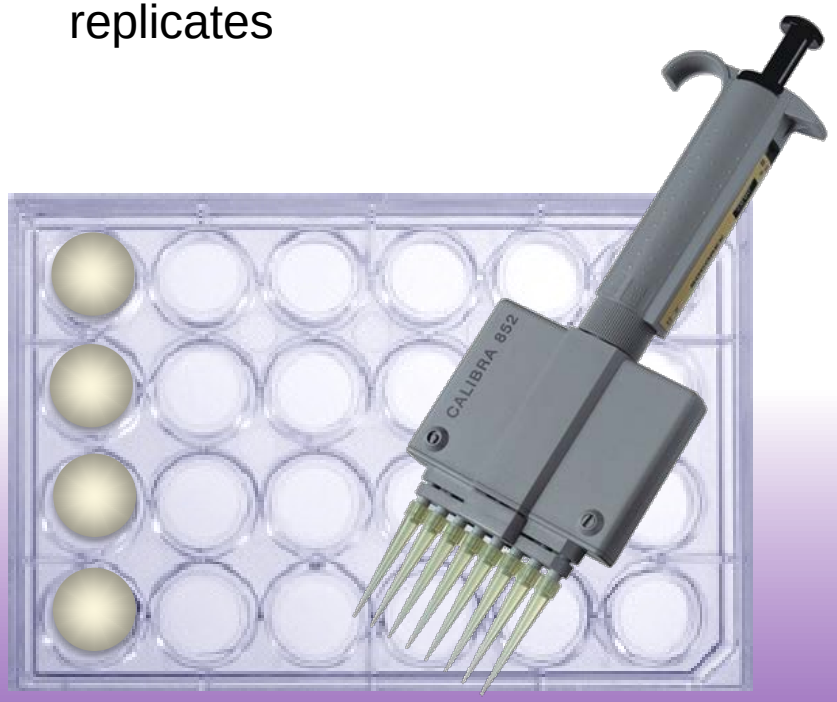
Measuring Points

Agar Plate test

- 1 plate - 1 measuring point
- Individual handling:
  1 plate requires mixing of
  1 compound, agar and plating

liquid culture Ames MPF

- 1 plate - 24 measuring points
- Simultaneous handling of several replicates

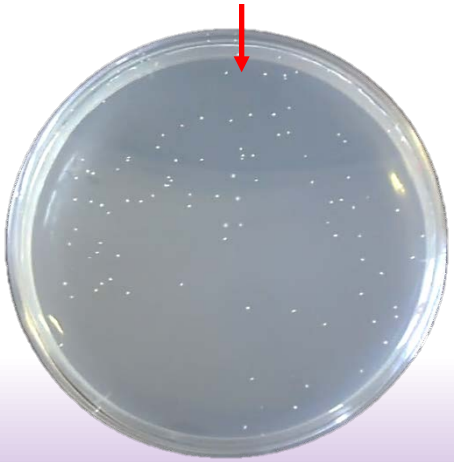


Evaluation of Results
Agar Plate Test vs Ames MPF

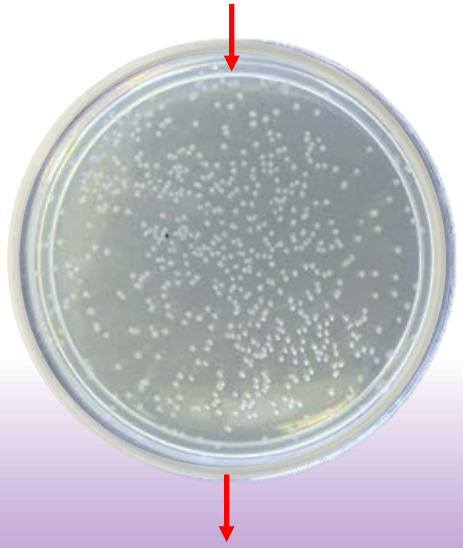
Colony counting of individual plates
Automation possible

Colorimetry
Counting sections of 48 Wells
Automation possible

Negative Control

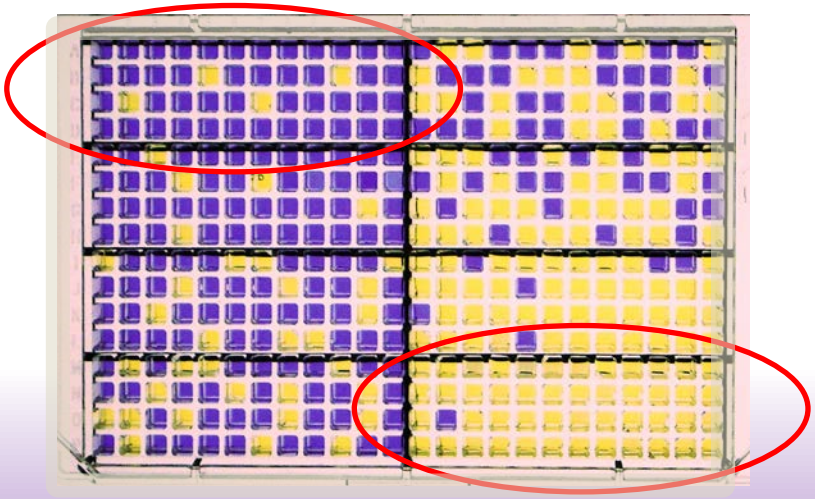


Positive Control



Laborious

Negative Control



Positive Control

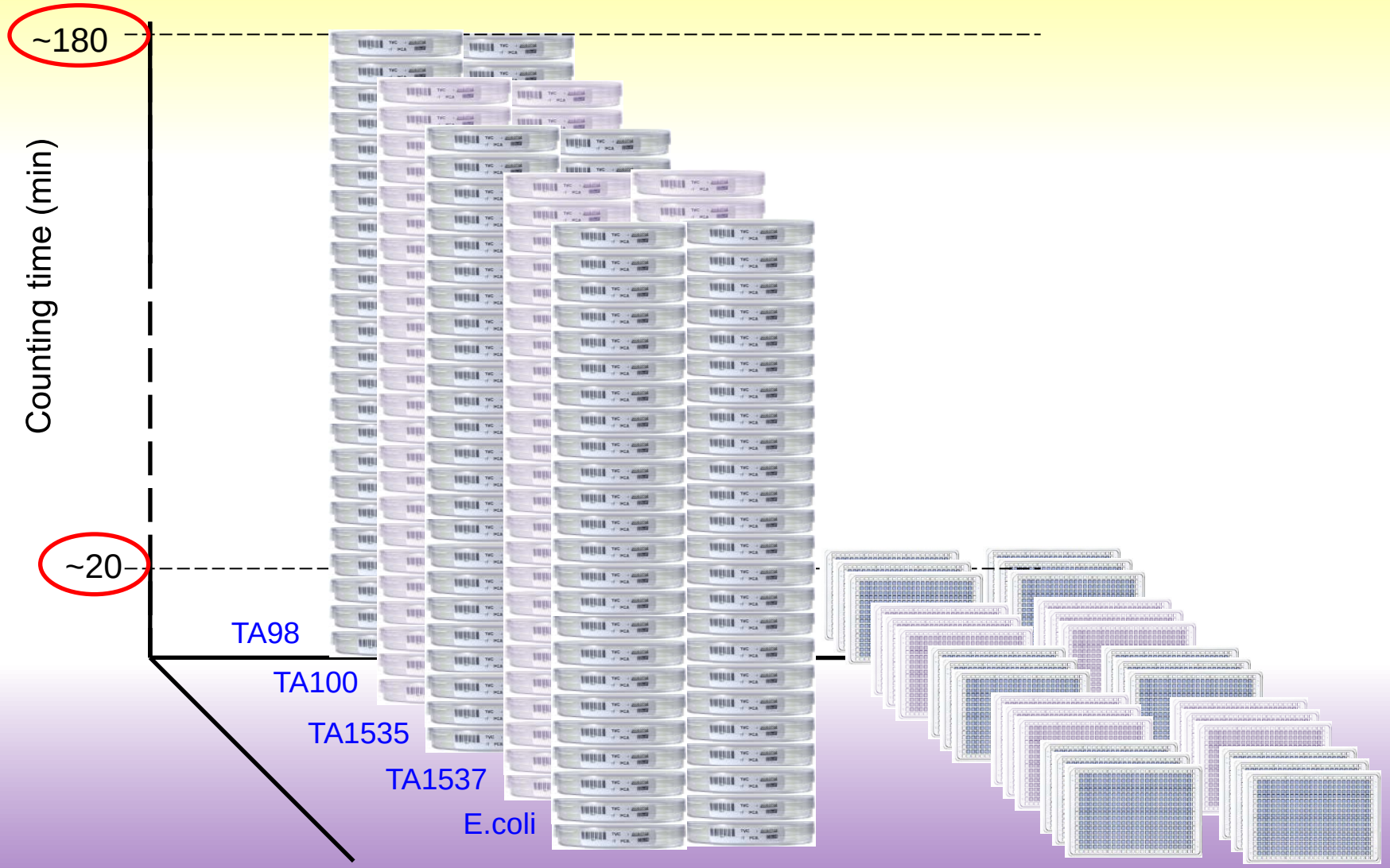
Easy

Throughput of compounds: Hands-on-time for 1 compound in 5 strains

1 sample, 5 concentrations, 5 strains (OECD), -/+ S9, controls, triplicates,
→ Conditions: manual handling, ready-made agar plates and top agar

	Agar Plate / 5 Conc.	MPF / 6 Conc.
Sample dilutions:	~5 min	~5 min
Top agar (preparation of tubes):	~35 min	-
Addition of sample, culture, S9:	~50 min	~25 min
Plating:	~40 min	-
Transfer to 384-well plates:	-	~40 min
Handling time:	**~130 min**	**~70 min**
Counting time:	**~180 min**	**~20 min**
Total time:	**~ 5 h**	**~1½ h**

Visualization of Plate Counting Time



210 plates

Test Sample Consumption

Minimum amount of sample needed: Agar plate test vs. Ames MPF

Setup: 5 strains (OECD 471), ½ log dilution steps, triplicates, -/+ S9

	Ames Agar Plate:	Ames MPF:
Top dose:	5 mg/plate	5 mg/ml
Test sample:	220 mg	55 mg

Ames MPF:

⇨ 4-fold less test sample
⇨ Very important when compound quantity is limited!
⇨ Genotoxic impurities

S9 Consumption

Setup: 5 strains (OECD 471), ½ log dilution steps, triplicates, S9

	Ames Agar Plate:	Ames MPF:
S9 fraction 30%:	18 ml	1.4 ml
S9 fraction 10%:	6 ml	0.5 ml

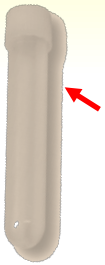
Ames MPF:

⇨ 12-fold less S9
⇨ Reduced number of sacrificed animals !
⇨ In line with 3Rs: Replace, Reduce, Refine !

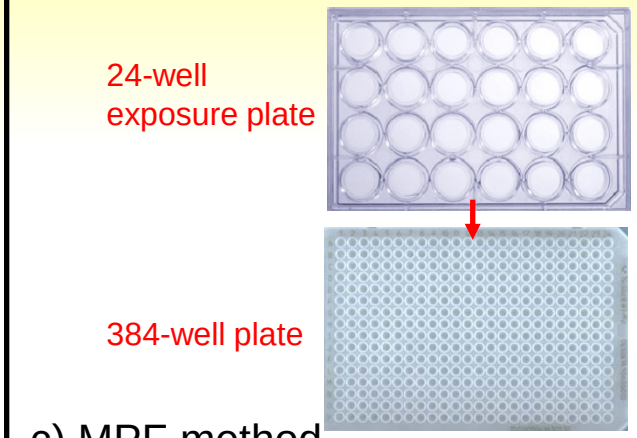
Critical Points of Ames MPF

- Comparability of concentrations used (mg/plate - mg/ml)?
- 48-well limit?
- Cytotoxicity?
- Colored compounds: Interference with colorimetric read-out?

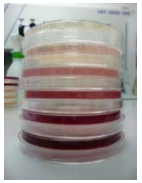
Concentrations used - Comparison



a) Plate incorp. method b) Pre-inc. method



c) MPF method



a) Plate incorporation: defined sample amount in top agar
→ immediate pouring
→ **possible diffusion of sample and cofactors into lower agar**
→ **volume not always clearly defined during exposure**

b) Pre incubation.: defined sample amount in defined volume
→ liquid pre-incubation/exposure → dilution with top agar → pouring
→ **defined volume during exposure**

c) Ames MPF: defined sample amount in defined volume
→ liquid exposure → dilution with indicator medium
→ **defined volume during exposure**

Sample Concentration - Comparison

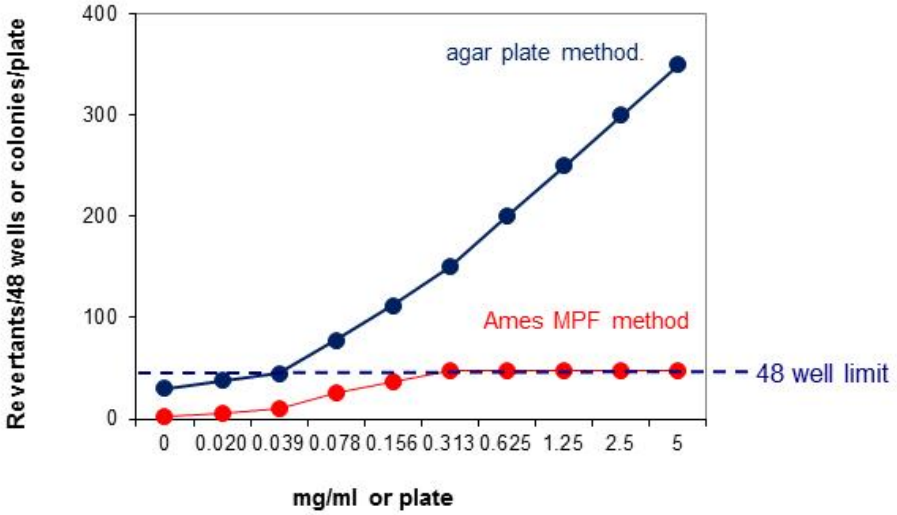
MPF method and Pre-incubation method: Both exposures performed in liquid media ⇨ Bacteria incubated with constant sample concentrations

Liquid exposure with 5 mg/ml (MPF) or 5 mg/plate (pre-incubation)

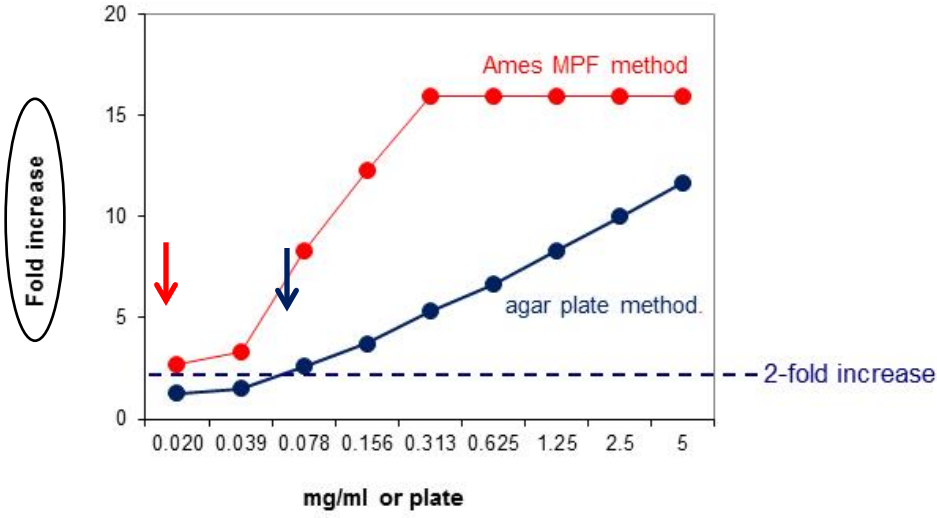
	Addition	Stock	Final Volume	Final concentration
MPF	10 µl	125 mg/ml	0.25 ml	5.0 mg/ml
Pre-incubation	100 µl	50 mg/ml	0.70 ml	7.1 mg/ml

48 Well Limit

Strong mutagens
Number of revertants



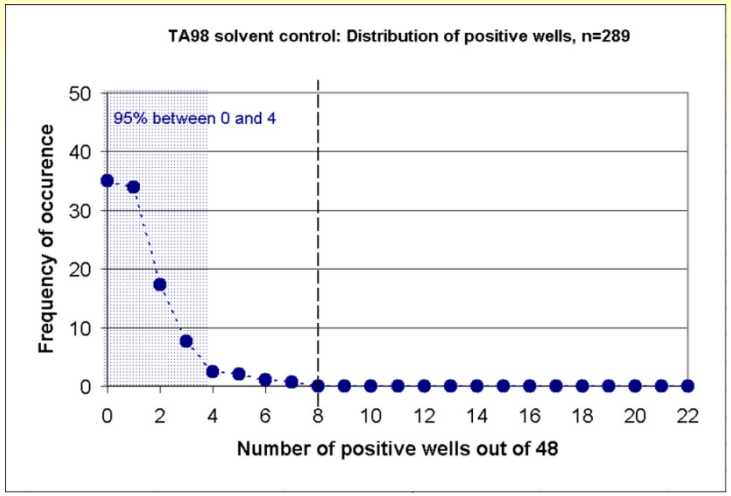
Lowest mutagenic
concentration

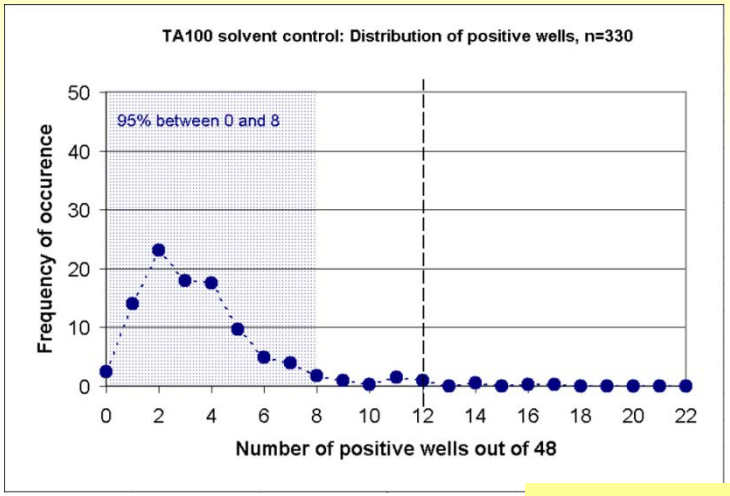


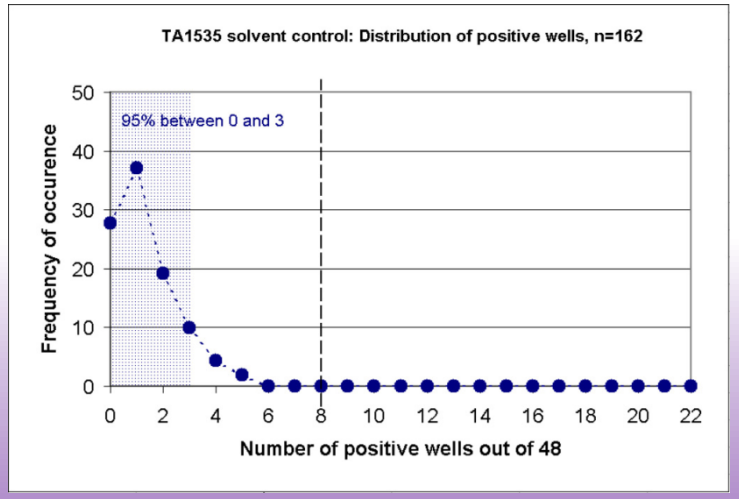
- No limits of revertants for strong mutagens in agar test, continous increase of revertants
- Plateau of 48 wells, but:
  Repeated 48 revertant wells = strong mutagen in Ames MPF

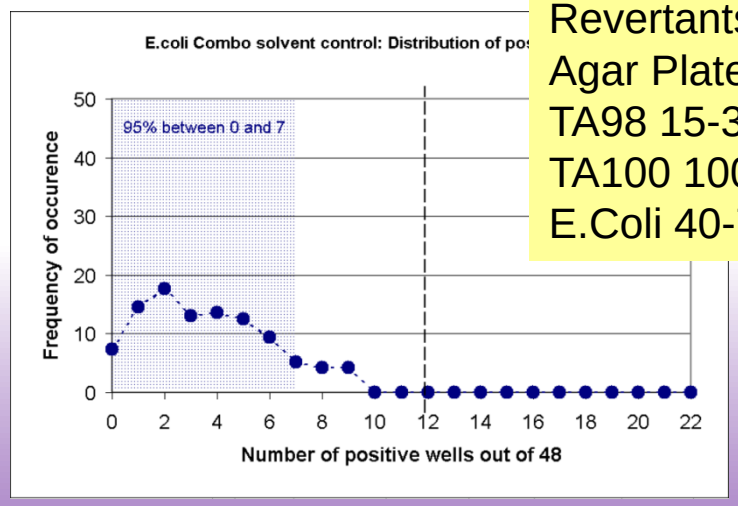
- Ames MPF detects lowest mutagenic concentration at lower dosis
- Low number of spontaneous revertants

Historical Solvent Control





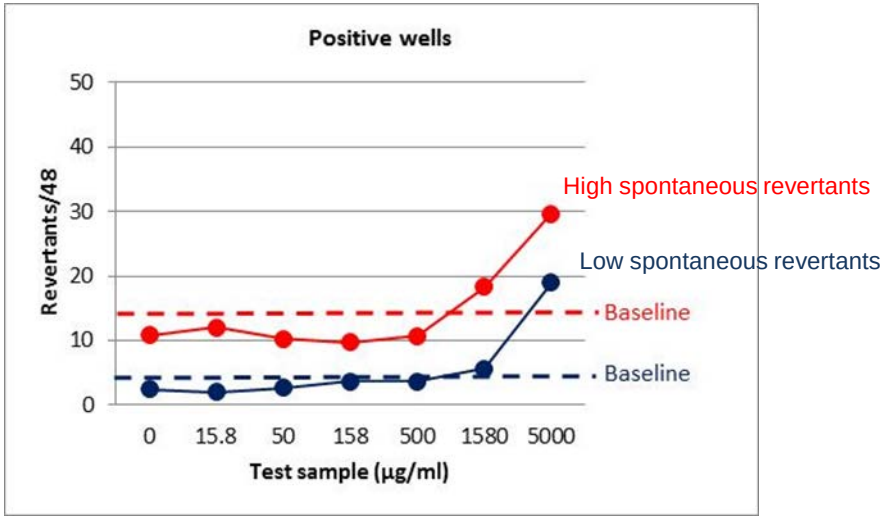




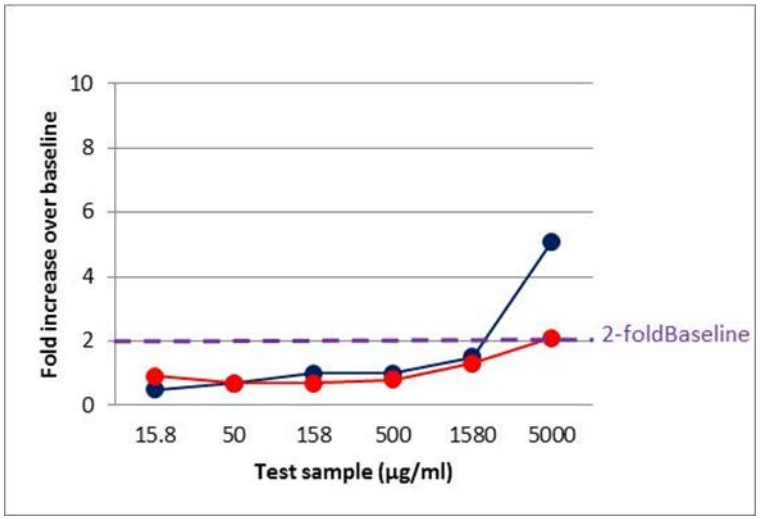
Spontaneous Revertants on Agar Plate Test
TA98 15-30
TA100 100-200
E.Coli 40-70

48 Well Limit in Ames MPF
"Low" and "High" Spontaneous Revertants

Positive Wells



Fold Increase over Baseline



- ⇨ **Pass/Fail criteria for spontaneous revertants in Ames MPF**
- ⇨ **Low spontanous revertants -> larger dynamic range**

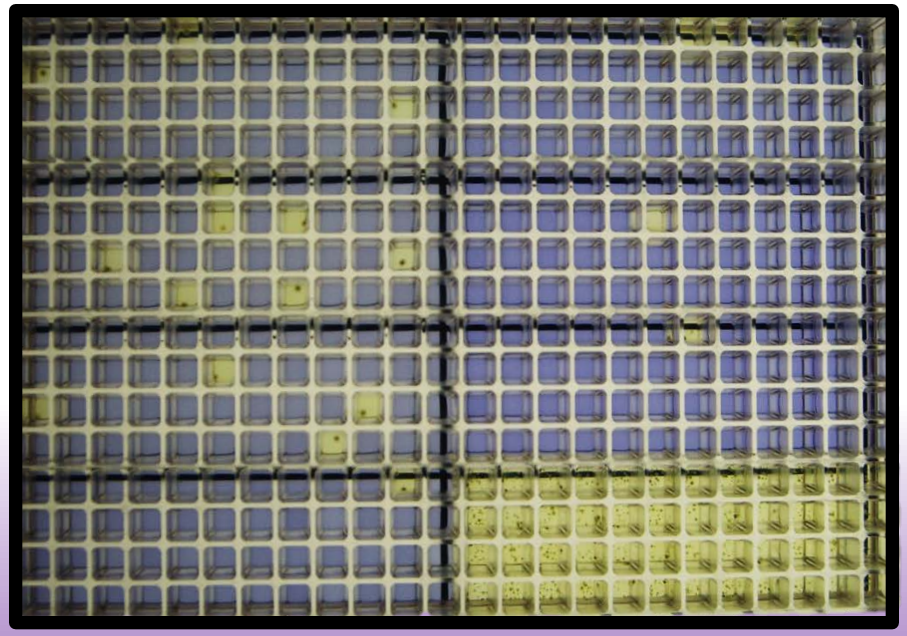
- ⇨ **Selection of cultures with low spontaneous revertant rate at Xenometrix, 2 quality controls after production**

Cytotoxicity in Ames MPF

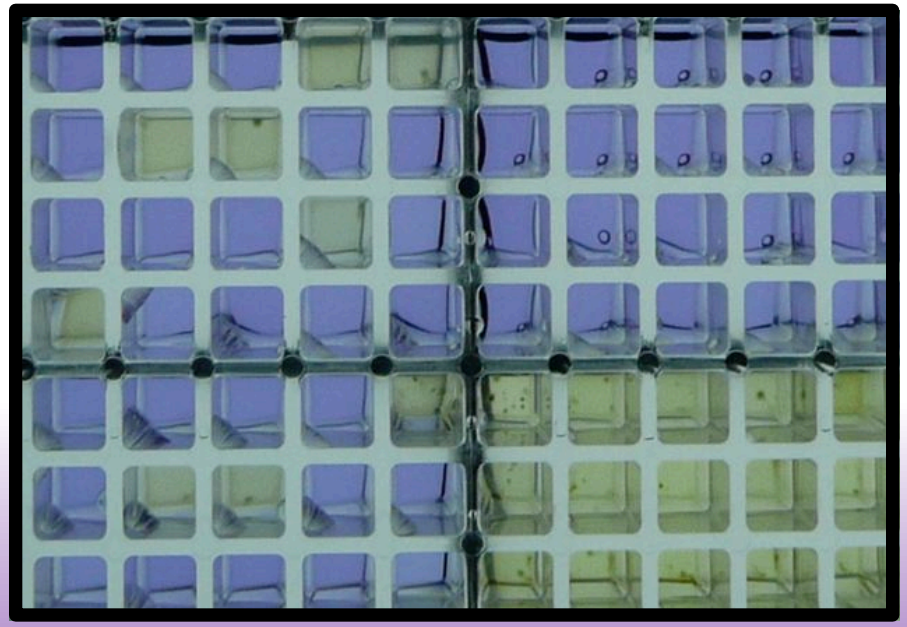
Cytotoxicity can be detected easily:

Reduction of revertant wells and

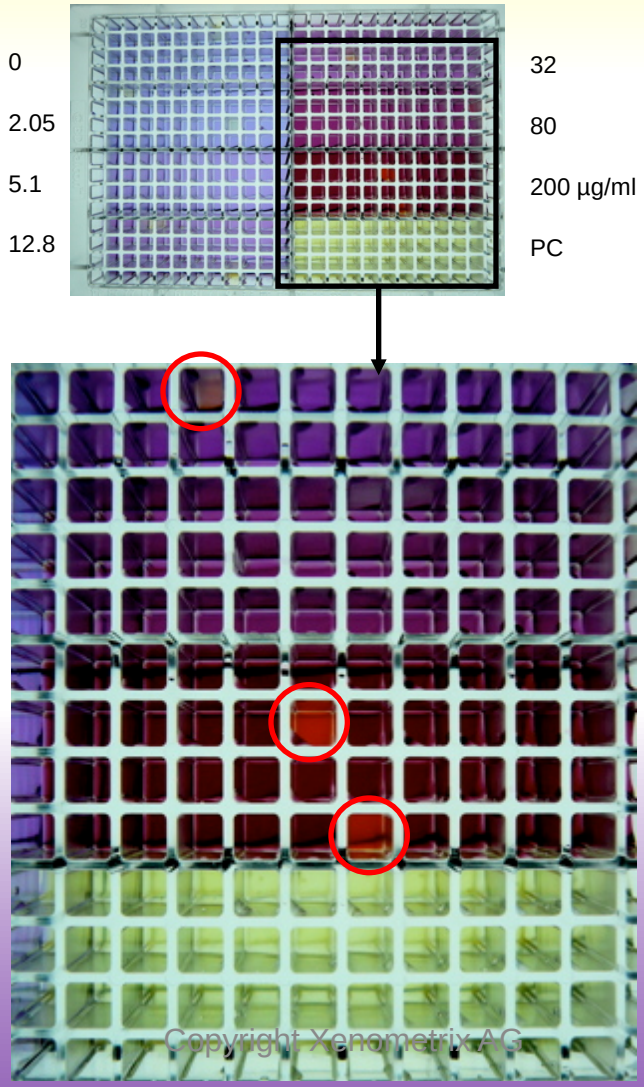
Increased brilliance of purple medium



Lipid droplets (bubbles) without S9



Colored compounds - colorimetric read-out



Orange instead of yellow wells

Easily detectable

High concordance with agar plate test

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Mutation Research 412 (1998) 115–130

Genetic Toxicology and Environmental Mutagenesis

Comparison of responses of base-specific Salmonella tester strains with the traditional strains for identifying mutagens: the results of a validation study

P. Gee [a,*], C.H. Sommers [a], A.S. Melick [a], X.M. Gidrol [a], M.D. Todd [a], R.B. Burris [a], M.E. Nelson [a], R.C. Klemm [a], E. Zeiger [b]

TA98, TA1537, TAMix compared with all strains NTP

25 chemicals tested

Overall agreement: 88%

Abstract

The ability of a TA7000 series of Salmonella *his*⁻ mutant tester strains to detect mutagens as classified by the traditional

High concordance with agar plate test

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Mutation Research 558 (2004) 181–197

Genetic Toxicology and Environmental Mutagenesis

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Assessment of the performance of the Ames II™ assay: a collaborative study with 19 coded compounds

S. Flückiger-Isler[a,*], M. Baumeister[b], K. Braun[c], V. Gervais[d], N. Hasler-Nguyen[e], R. Reimann[f], J. Van Gompel[g], H.-G. Wunderlich[h], G. Engelhardt[i]

[a] *Xenometrix by Endotell GmbH, CH-4125 Allschwil, Switzerland*

[b] *Boehringer Ingelheim, Department of Non-Clinical Drug Safety, Boehringer Ingelheim Pharma KG & Co. KG, D-88397 Biberach, Germany*

[c] *Aventis Pharma Deutschland GmbH, Drug Innovation & Approval, Lead Optimization, Drug Safety Evaluation, D-65795 Hattersheim, Germany*

[d] *Servier Group, Drug Safety Assessment, F-45403 Orléans-Gidy, France*

[e] *Novartis Consumer Health, Toxicology, CH-1260 Nyon, Switzerland*

[f] *Schering AG, Experimental Toxicology, D-13342 Berlin, Germany*

[g] *Johnson&Johnson Pharmaceutical Research & Development, Department of ADME/Tox, B-2340 Beerse, Belgium*

[h] *Federal Environmental Agency, Department for Hygiene of Drinking and Swimming Pool Water, D-08645 Bad Elster, Germany*

Overall agreement standard Ames (all strains) - Ames II (TA98, TAMix): 84.2% (16/19)

Inter-laboratory consistency of 89.5% (17/19).

High concordance with agar plate test

ASSESSMENT OF A SCREENING EXPERIENCE WITH THE AMES II™ TEST AND FUTURE PROSPECTS

V. GERVAIS[1], D. BIJOT[1] and N. CLAUDE[2]

[1] *Drug Safety Assessment, Servier, Orléans-Gidy, France,* [2] *IRIS, Servier, Coubervoie, France*

quid fluctuation version of the *Salmonella* mutagenicity assay, provided by Xenometrix GmbH, was used for an early compound selection in the discovery process. The ai
e Ames II compared to the standard Ames test and to explore a way to reduce the required compound quantity without lowering the predictability of the test.

METHODS

of a mixture of 6 *Salmonella* *TA7001, TA7002, TA7003,* *TA7006,* which revert to a specific base substitution n. This "mix" is used as a n, the frameshift tester strain mix and *TA98* strains are lium for growth overnight at t, performed in 24-wells ws partial automation and about 60-fold less compound lard Ames. After a 90 min- ith or without Aroclor-induced with solvent and positive medium lacking histidine is ch well is then aliquoted into

Tab.1: Results of a collaborative study by Xenometrix GmbH (March 2003)

Company	Compound Number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
C1	P	P	P	P	P	-	-	-	-	-	P	N	N	N	N	-	-	-	-	-
C1 (Robot)	P	P	P	P	P	-	-	-	-	-	P	N	N	N	N	-	-	-	-	-
C2	N	P	P	P	-	-	-	-	-	P	-	-	-	-	N	N	N	N	N	N
C3	-	-	P	P	P	P	P	-	-	-	-	-	N	N	N	N	N	-	-	-
C4 (Robot)	N	-	-	-	-	-	P	E	N	P	E	N	-	-	-	-	N	N	N	-
C5	-	-	-	P	N	P	P	P	-	-	-	-	-	N	N	N	N	N	-	-
C6	-	P	P	P	P	P	-	-	-	-	-	N	N	N	N	N	-	-	-	-
C7	P	P	-	-	-	-	-	P	N	P	P	N	N	-	-	-	-	N	N	-
C8	-	-	-	-	-	P	P	P	N	P	P	-	-	-	-	N	N	P	N	-
C9	P	-	-	-	P	P	P	P	N	-	P	-	-	N	N	-	N	E	-	-
Result	?	P	P	P	P	P	P	P	N	P	P	N	N	N	N	N	N	N	N	N
Annual	P	P	P	P	P	P	P	?	?	P	?	?	N	N	N	N	N	N	N	N

N=Negative, P=Positive, E=Equivocal Sensibility : 80 % Specificity : 100 % Concordance : 89%

RESULTS

350 compounds were tested, including molecu from our own research, known non- or genoto molecules producing equivocal resu concordance between the results achieve Ames II™ test and those reported in the liter the standard Ames test ranged from 79 (Ref (Tab.2). The concordance reached 89 collaborative study (Tab.1). No false positi were obtained with known non-mutagenic s False negative results may arise when chemi only specific strains like *TA1535* or *E. coli (pKM101)* which meet no equivalent in the "mi The positive responses were randomly among the strains or the concentration range 3). In contrast, only 11% of positive results specifically in the absence of S9 (Fig.4), wh

83% Concordance — Ames II vs. traditional Ames using 42 company-own chemicals (disagreement mainly with compounds that specifically revert E.coli, TA1535)

No false positive results

High concordance with agar plate test

Mutagenesis vol. 24 no. 4 pp. 359–366, 2009
Advance Access Publication 15 May 2009

doi:10.1093/mutage/gep017

Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity

Markus Kamber*, Sini Flückiger-Isler, Günter Engelhardt[1], Rudolf Jaeckh[2] and Errol Zeiger[3]

Xenometrix AG, Gewerbestrasse 25, CH-4123 Allschwil, Switzerland, [1]Experimental Toxicology and Ecology, BASF SE Product Safety, [2]Regulations, Toxicology and Ecology, BASF SE Product Safety, 67056 Ludwigshafen am Rhein, Germany and [3]Errol Zeiger Consulting, Chapel Hill, NC 27514, USA

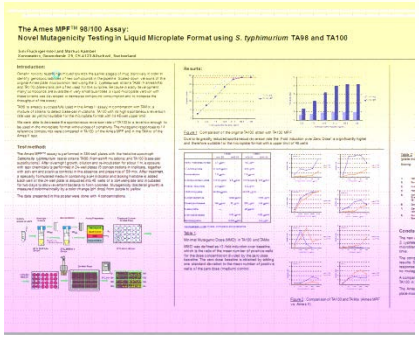
The Ames II *Salmonella* mutagenicity assay procedure was used to test 71 chemicals, and the results were compared with those from the traditional Ames *Salmonella* test using the NT perform format.

a different test method, including a simple, overall agreement or disagreement; agreement or disagreement with regard to the genetic endpoint, and whether metabolic activation is required for activity; comparisons of the active test chemical concentration ranges and with respect to the effect the test is designed to predict, i.e. cancer. Two previous studies (3,4) have compared the performance of the Ames II assay to that of the traditional Ames test procedure [i.e. the procedure with the traditional strains, as described in (5) and (6)] to validate its use as an alternative to the traditional Ames test procedure.

84% agreement between the two procedures in identifying mutagens and non-mutagens
Discordant results included chemicals requiring reductive metabolism using FMN, hamster liver S9

High concordance with agar plate test

Xenometrix Posters: Comparison with Correspondent Traditional Strains


The Ames MPF™ 98/100 Assay:
Novel Mutagenicity Testing in Liquid Microplate Format using *S. typhimurium* TA98 and TA100

- TAMix *vs*. TA100 MPF and TA100 published traditional Ames
- TA98, TA100, TA1535, TA1537 MPF *vs*. TA98, TA100, TA1535, TA1537 published traditional Ames
- Ames MPF PENTA I (strains as above plus EC Combo) *vs*. published traditional Ames

⇨Overall agreement: 89 - 100%

Mutagenic responses in the Xenometrix Ames MPF PENTA I assay compared to published Ames plate incorporation data

Compound	CAS Nr.	S9	Ames MPF					Ames plate incoroporation (published data)				
			TA98	TA1537	TA100	TA1535	E.coli Combo	TA98	TA1537	TA100	TA1535	E.coli
9-aminoacridine x HCl x H_2O	52417-22-8	-	neg	pos	neg	neg	neg	neg	pos	neg	neg	neg [a]
2-aminoanthracene	613-13-8	+	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos [b]
N4-aminocytidine [c]	57294-74-3	-	neg	neg	pos	pos	pos	neg	neg	pos	pos	pos
5-azacytidine	320-67-2	-	neg	neg	neg	neg	neg	neg	neg	neg/pos	w+	X
		+	pos	neg	neg	pos	?	neg	neg	neg/?/pos	pos	X
Benzo(a)pyrene	50-32-8	+	pos	pos	pos	neg	pos	pos	pos	pos	neg/?	
Cumene hydroperoxide	80-15-9	-	neg	pos	pos	neg	pos	neg	neg	neg/w+	neg	pos [d]
	80-15-9	+	neg	neg	w+	neg	pos	neg	neg/?/pos	neg/?/w+/pos	neg	pos [d]
Cyclophosphamide	6055-19-2	+	neg	neg	pos	pos	?	neg	neg	pos	pos	
Danthron	117-10-2	+	neg	pos	neg	neg	neg	neg [e]	pos [e]	neg [e]	neg [e]	neg [d]
Formaldehyde	50-00-0	-	pos	neg	pos	neg	pos	neg/?/pos	neg	neg/?/w+/pos	neg	pos [d,f]
Glutaraldehyde	111-30-8	-	w+	neg	pos	neg	pos	neg	neg	neg/?/pos	neg	pos [g]
		+	neg	neg	w+	neg	?	neg/?	neg	?/pos	neg	
ICR-191	17070-45-0	-	pos	pos	pos	?	pos	pos [h]	pos [h]	pos [h]	neg [h]	
6-mercaptopurine	6112-76-1	+	neg	neg	neg	pos	neg	neg	Z	neg	pos	
Methyl methanesulfonate	66-27-3	-	neg	neg	pos	pos	pos	neg/?	neg/pos	pos	?/pos	pos [b]
Pyrene	129-00-0	-	neg	w+	neg	neg	neg	neg	neg	neg	neg	neg
		+	pos	pos	?	neg	n.d.	neg/?/pos	?/w+/pos	neg/?pos	neg	neg/?/pos
2-nitrofluorene	607-57-8	-	pos	pos	w+	neg	neg	pos [a]	pos [a]	pos [a]	neg [a]	neg [i]
4-nitroquinoline-N-oxide	56-57-5	-	pos	pos	pos	pos	pos	pos [i]	pos [i]	pos [i]	pos [i]	pos [k]
		+	pos	pos	pos	pos	pos	pos [i]	pos [i]	pos [i]	pos [i]	pos [k]
Streptonigrin	3930-19-6	-	neg	neg	neg	neg	pos	neg [l]	neg [l]	neg [l]	neg [l]	pos [d,l]

neg = negative; pos = positive, ? = equivocal, w+ = weak positive, neg/pos = conflicting published results n.d. = not determined

High concordance with agar plate test

XENOMETRIX
Swiss Commitment for Bioassays

Direct Comparison
Ames MPF - Ames Pre-incubation

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Direct comparison of the Ames microplate format (MPF) test in liquid medium with the standard Ames pre-incubation assay on agar plates by use of equivocal to weakly positive test compounds

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- 15 equivocal to weakly positive chemicals
- Same overnight cultures, chemicals and S9 to exclude external variations, i.e. culture growth, chemical purity, weighing errors, S9 activity
- Parallel tests with most responsive strains of the NTP database (mg/plate *vs*. mg/ml)
- Each test was repeated at least once
- 87% concordance (13/15)
- Excellent concordance for equivocal to weak positive chemicals
- Confirms the high concordance with the ICH-compliant assay

Direct Comparison Ames MPF and Pre-incubation Method (see publication before)

XENOMETRIX
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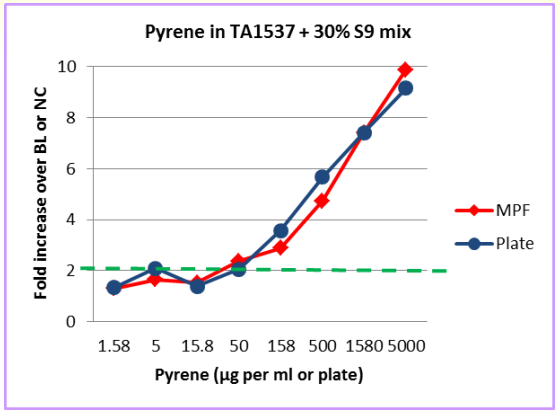
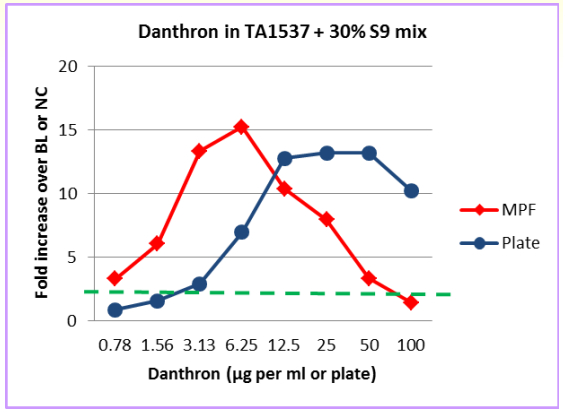
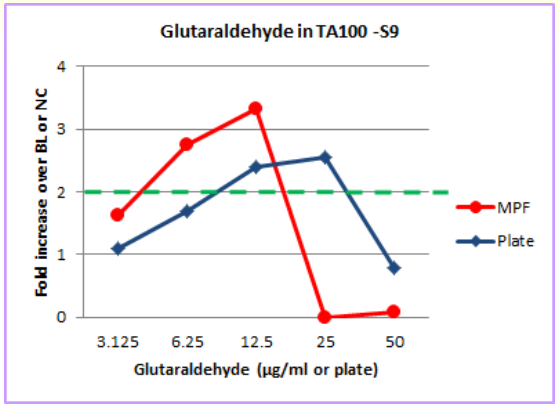
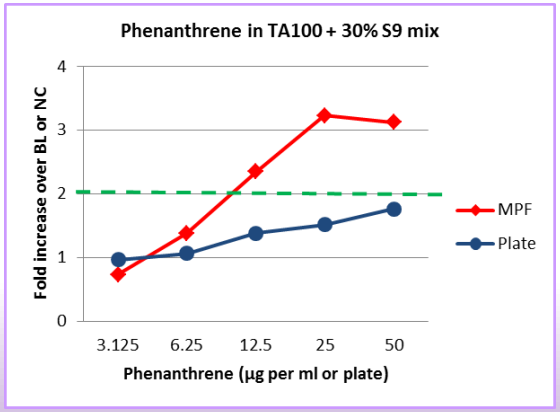
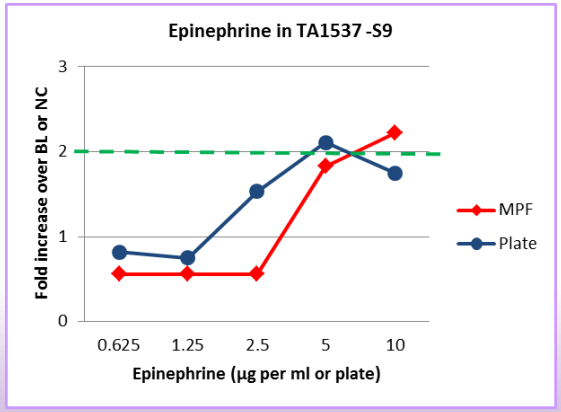
µg/ml vs. µg/plate

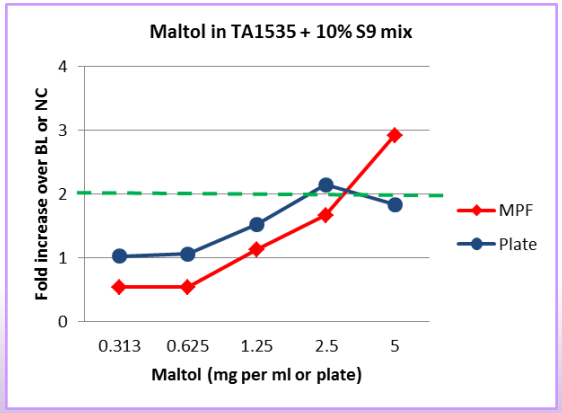












Higher sensitivity of Ames MPF with several compounds, such as Danthron, Glutaraldehyde, Phenanthrene

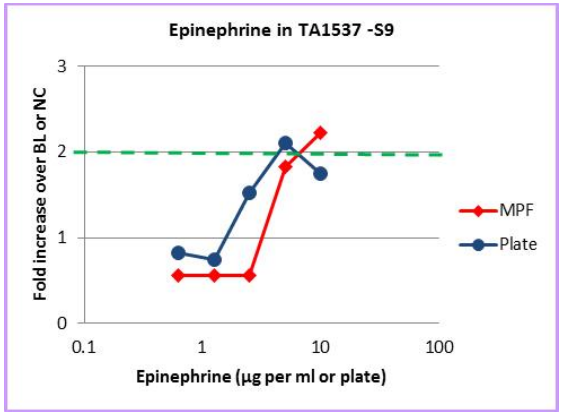
At first glance higher sensitivity of Pre Incubation Assay with Maltol and Epinephrine, but….

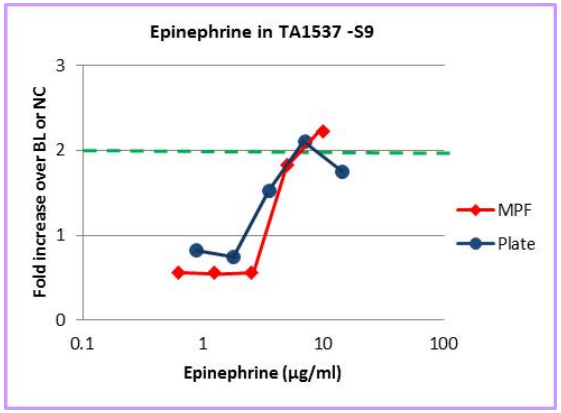
AG

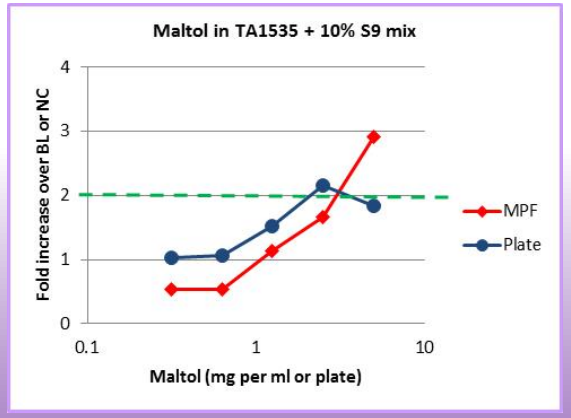
Direct Comparison Ames MPF and Pre-incubation Method – Epinephrine, Maltol

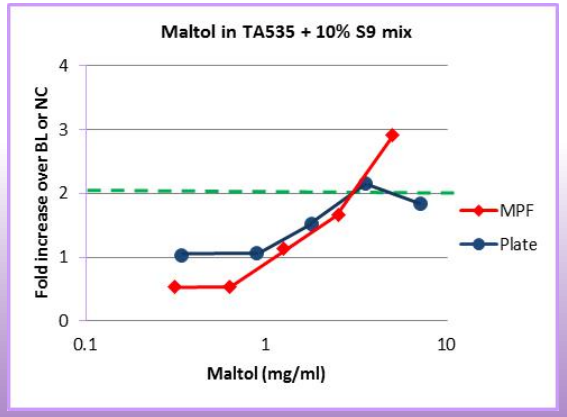
Correction for concentration in preincubation assay (5.0 mg vs 7.1 mg)

µg/ml *vs.* µg/plate → µg/ml *vs.* µg/ml (pre-incubation volume 0.7 ml)









Congruent curves

Ames MPF is a very sensitive assay

Note 2

To assess the mutagenic potential of impurities, a single bacterial mutagenicity assay can be carried out with a fully adequate protocol according to ICH S2(R1) and OECD 471 guidelines……..**For degradants that are not feasible to isolate or synthesize or when compound quantity is limited, …… bacterial mutagenicity testing could be carried out using a miniaturized assay format with proven high concordance to the ICH-compliant assay** to enable testing at higher concentrations with justification…..

Conclusion I - Test Performance

- Ames MPF – Ames agar test: same principle, same tester strains
- Comparative studies: mean concordance of ~87%
- Comparable to the intra- and inter-laboratory reproducibility of the agar plate Ames test procedure

Conclusion II

Advantages

- 4 x less test sample necessary
- Liquid microplate format allows for less hands-on-time, simultaneous processing of several replicates
- Higher throughput, partly automatable
- 12 fold less consumption of S9 – following 3Rs
- Quick, easy colorimetric read-out, less error prone
- Less plastic ware, reduced contaminated waste in environment
- Listed explicitly in ICH M7 Guideline
- **Higher Sensitivity – depending on compound**

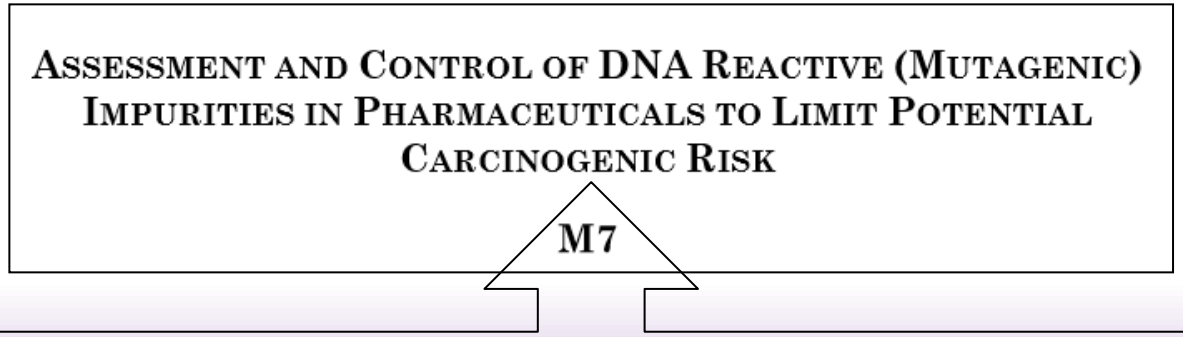
Disadvantages

- Not same large database as agar plate method
- Not listed explicitly in OECD 471

Conclusion III - ICH Guideline M7

- The Ames MPF features a miniaturized assay format with proven high concordance with the ICH-compliant assay.
- It is highly sensitive and allows testing compounds present in limited quantity.

⇨Ames MPF = Excellent tool for assessing mutagenic impurities



"For degradants that are not feasible to isolate or synthesize or when compound quantity is limited, it may not be possible to achieve the highest test concentrations recommended for an ICH compliant bacterial mutagenicity assay according to the current testing guidelines. In this case, bacterial mutagenicity testing could be carried out using a miniaturized assay format with proven high concordance to the ICH compliant assay to enable testing at higher concentrations with justification….. ."