Comparison of the Performance of the Colorimetric Ames MPF Assay with the Agar Plate Method

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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
### Ames Microplate Assay Procedure

#### Bacterial stock

- Store at -80°C

#### Overnight culture

- Grow at 37°C, 12-15 h, 250 rpm
- OD_{600} is measured

#### Assay preparation

- a) Test sample dilutions, controls
- b) Exposure medium

#### Exposure cultures

- 37°C, 90 min, 250 rpm
- (20 min E.coli + S9)
- C-, D1-D3, C+ D4-D6

#### 384-well plates

- OD_{600} measured after 48 h at 37°C

#### Indicator medium

- pH is monitored

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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

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Evaluation of Results
Agar Plate Test vs Ames MPF

Colony counting of individual plates
Automation possible

Laborious

Colorimetry
Counting sections of 48 Wells
Automation possible

Easy

Negative Control
Positive Control

Negative Control
Positive Control
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

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

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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

<table>
<thead>
<tr>
<th>Ames Agar Plate:</th>
<th>Ames MPF:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top dose: 5 mg/plate</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>Test sample: 220 mg</td>
<td>55 mg</td>
</tr>
</tbody>
</table>

Ames MPF:

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

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## S9 Consumption

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

<table>
<thead>
<tr>
<th></th>
<th>Ames Agar Plate:</th>
<th>Ames MPF:</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9 30%:</td>
<td>15.57 ml</td>
<td>1.35 ml - usually applied in Ames MPF</td>
</tr>
<tr>
<td>S9 10%:</td>
<td>5.25 ml - usually applied in agar plate test</td>
<td>0.45 ml</td>
</tr>
</tbody>
</table>

**Ames MPF:**

- 4-fold up to 11-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 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

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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)

<table>
<thead>
<tr>
<th>Addition</th>
<th>Stock</th>
<th>Final Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPF</td>
<td>10 µl</td>
<td>125 mg/ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Pre-incubation</td>
<td>100 µl</td>
<td>50 mg/ml</td>
<td>0.70 ml</td>
</tr>
</tbody>
</table>
48 Well Limit

- No limits of revertants for strong mutagens in agar test, continuous 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 dosage
- Low number of spontaneous revertants
Historical Solvent Control

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

Fold Increase over Baseline

- **Pass/Fail criteria for spontaneous revertants in Ames MPF**
- **Low spontaneous 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
ICH guideline M7 on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk

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.
High concordance with agar plate test

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%

The ability of a TA7000 series of Salmonella his- mutant tester strains to detect mutagens as classified by the traditional strains (TA100, TA1535, TA1537, TA97, TA102, and TA104) is evaluated using 25 chemicals in the 5-8
Assessment of the performance of the Ames II™ assay: a collaborative study with 19 coded compounds


Overall agreement standard Ames (all strains) - Ames II (TA98, TAMix): 84.2% (16/19)
Inter-laboratory consistency of 89.5% (17/19).
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
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

- 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%

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Direct Comparison
Ames MPF - Ames Pre-incubation

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

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

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....
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

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Chapter 5
Perspectives in genotoxicity screening
“Ames II (Xenometrix, Switzerland) is a microplate-based fluctuation test version of the Ames test and probably the best Ames predictor.”

Chapter 9: Mutation Research

New Paradigms for the Environmental Assessment, p. 26
“...thus, the all-liquid format of the Ames II/MPF assay, which requires less test compound and allows for the use of multichannel pipettes, thus automating the pipetting steps, makes this procedure an attractive method to evaluate mutagenicity of a large number of samples at the same time - a common situation in environmental monitoring.”

Chapter 10: Ames II and Ames Liquid Format Mutagenicity Screening Assays

...it has been proposed by the European Union-funded REBECA project as a screening tool to determine whether fungal biological control agents produce genotoxic/mutagenic metabolites which require further attention in the regulatory risk assessment.
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
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……”