Enhanced Ames Test Conditions for Nitrosamines

The Organisation for Economic Co-operation and Development (OECD) Test Guideline No. 471 "Bacterial Reverse Mutation Test" provides standard recommendations for the conduct of the bacterial reverse mutation test (also known as the Ames assay) to assess the mutagenic potential of a test compound. For N-nitrosamines, enhanced testing conditions for the Ames assay are recommended due to the reported reduced sensitivity of the assay under standard conditions for some N-nitrosamines such as N-nitroso-dimethylamine (NDMA). Moreover, little is known about the sensitivity of the Ames assay to N-nitrosamine drug substance related impurities (NDSRIs), which are a recently recognized class of N-nitrosamine impurities structurally related to the drug substance. NDSRIs generally have a wider variety of functional groups present than typically found in low molecular weight N-nitrosamines (such as NDMA) historically studied.

If a standard Ames assay is conducted and produces a positive result, there is no need to conduct an additional assay using enhanced testing conditions.

The enhanced Ames assay test conditions presented below are informed by work conducted by FDA's National Center for Toxicological Research (NCTR) (Li et. al., 2023), as well as other groups, and have been evaluated for a variety of N-nitrosamines including NDSRIs. Evaluation of Ames assay test conditions for N-nitrosamines is ongoing with a goal to identify the most robust Ames testing conditions. The enhanced Ames assay test conditions described below will be updated as warranted. Deviations from the recommended conditions should be justified.

Tester strains: *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2 uvrA (pKM101) tester strains should be included.

Type of assay and preincubation time: The pre-incubation, and not plate incorporation, method should be used. The recommended pre-incubation time is 30 minutes.

Species and concentration of S9: Ames assays should be conducted in the absence of a post-mitochondrial fraction (S9), and in the presence of 30% rat liver S9, as well as 30% hamster liver S9. The rat and hamster post-mitochondrial fractions (S9s) should be prepared from rodents treated with inducers of cytochrome P450 enzymes (e.g., a combination of phenobarbital and β-naphthoflavone).

Negative (solvent/vehicle) control: Solvents need to be compatible with the Ames assay as per the OECD 471 guideline. Solvents can include, but are not limited to:

- water
- organic solvents such as acetone, methanol and DMSO

  When an organic solvent is used, the lowest possible volume should be included in the pre-incubation mixture with justification to indicate that the volume of solvent does not interfere with metabolic activation of the N-nitrosamine.

Positive controls: Concurrent strain-specific positive controls should be included per the OECD 471 guideline.

Two N-nitrosamines that are known to be mutagenic in the presence of S9 should also be included as positive controls.

The choice of the N-nitrosamine positive controls needs to be justified based on the anticipated metabolism of the N-nitrosamine and the cytochrome P450 enzymes most likely involved. In addition, if an organic solvent is used to dissolve the test compound, it is recommended that the volume of organic solvent employed to dissolve the N-nitrosamine positive controls results in a similar concentration as for the test compound in the pre-incubation mix.

N-Nitrosamine positive controls to consider include:

1. NDMA (CAS # 62-75-9)
2. 1-Cyclopentyl-4-nitrosopiperazine (CAS # 61379-66-6)
3. An NDSRI

All other recommendations for the Ames assay should follow the OECD 471 guideline.

A checklist of the information required for the Enhanced Ames Test (EAT) is presented below and can be used to provide a summary of the conditions used and results obtained in the report for the EAT.

Test substance:

Sponsor:

Test facility:

GLP compliance: Y/N

Study initiation date:

Experiment start date:

Experiment completion date:

EAT requirements		Conditions used in EAT	Comments
Strains: (e.g., *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101))			
Preincubation method, 30 min			
S9	rat (30%)		
	hamster (30%)		
Solvent used and volume[1] (µL) (a)			
Volume of preincubation mix (µL) (b)			
Solvent concentration in preincubation (% v/v)[1]			

Concentrations tested (µg/plate)		
Nitrosamine positive control 1 (µg/plate), including solvent concentration (% v/v)[2, 3]		
NDSRI positive control 2 (µg/plate), including solvent concentration (% v/v)[2, 3]		
Non-nitrosamine positive controls[2,4]		
Negative controls[2]		
Result (positive/negative)		

[1] *When an organic solvent is used, the lowest possible volume should be included in the preincubation mixture with justification to indicate that the volume of solvent does not interfere with metabolic activation of the N-nitrosamine. The organic solvent concentration (% v/v) should be calculated by dividing the applied solvent volume containing the test substance in the preincubation mix by the total volume of the preincubation mix (i.e., (a/b)*100). For example, if the test substance is applied in 100 µL organic solvent and the total preincubation mix volume is 700 µL, the solvent concentration is 14.3% (v/v).*

[2] *Concurrent controls*

[3] *Solvent used for test substance and positive control should ideally be the same or justified if not.*

[4] *Positive control reference substance(s) (e.g., as suggested by OECD 471 for assays employing a metabolic activation system).*

References:

OECD Test Guideline No. 471 “Bacterial Reverse Mutation Test”. 2020

Li et al. Revisiting the mutagenicity and genotoxicity of N-nitroso propranolol in bacterial and human in vitro assays. Regulatory Pharmacology and Toxicology. 2023

Source: EMA’s Appendix 3 to Questions and answers for marketing authorisation holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products