



CHROMOSOMAL ALTERATIONS:

RECOMBINATION INDUCTION ASSAY:

Saccharomyces cerevisiae D7, Ade 1-119, ade 2-40

Test substance: FR-91

Submitted by: F. Chacon

Laboratory: Interlab Group



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GENOTOXICITY STUDIES: FR-91

I. INTRODUCTION

The substance FR-91 has been evaluated for its genotoxic potential, chromosomal aberrations and primary damage to eukaryotic DNA. The recombination induction assay using Saccharomyces cerevisiae as the indicator organism is of great use for the identification of genotoxic agents (Zimmermann 1975, 1981, 1984), as well as constituting a good complement to the Ames and other tests on eukaryotic cell cultures (Zimmermann 1984, Von Borstel 1984).

The molecular and/or structural changes induced in the genome should be considered as evidence of genotoxic activity. Alterations in DNA, as it is the universal carrier of genetic information, can have mutagenic, carcinogenic or cytotoxic effects in mammals.

The sensitivity and reliability of the test has been amply evaluated and it currently has a large base of toxicological data which validate it (see Annex III, reference 3).

The sensitivity of the assay is increased by the use of hepatic enzymes prepared in the laboratory from mammals pretreated with Aroclor 1254.

These in vitro systems can be used to determine whether or not:

- The substance being tested has mutagenic properties per se
- Those possible metabolites generated by biotransformation –mixed function oxidases– are genotoxically active.
- A direct mutagenic agent is detoxified by hepatic microsomal enzymes

This assay has been carried out according to the norms of the EU, including Directive 75/318, which establishes toxicological analytic methods and in function of its



most recent modification, Directive 87/19 which permits reference to Directive 79/831, part B, Toxicological Methods for Annex VIII and the method laid out in the Official Bulletin of the European Communities N° L251/143, -Other effects: Genotoxicity -- Mitotic Recombination Induction Assay in Saccharomyces cerevisiae.

II. ASSAY

The system of mitotic recombination induction in Saccharomyces cerevisiae permits the detection of an increase in the number of chromosome breakages during cellular mitosis, due to the recombination (crossing-over) of genetically marked chromosome fragments (mutation Ade 1-119; 2-40) and which, once recombined, are detected by a change in the color of the resulting colonies (sectors in a colony), when they are plated in adenine-deficient (5 mg/L) synthetic medium.

This determines the distinct types of colonies which can be obtained:

SECTORIZED: pink, red, white, and their combinations (pink-red, white-pink, etc.)

UNIFORM: pink, white, red

HAIRLINE: pink, red

MULTILOBED

In the strict sense, only sectorized colonies can be considered recombinant (Zimmermann 1975), given that the color change in a sector is subsequent to several cell divisions and thus depends in large degree on the recombination which can be produced during these divisions. The other types of genetically aberrant colonies can be due to mutations, genic-mitotic conversions, aneuploidias, etc. Given that these are also genetic alterations, the results are evaluated; however, they are not used in calculation of recombination frequency.

This assay also permits the determination of the damage produced on mitochondrial DNA, since the colonies which suffer this damage can show failures in the



cellular respiration system and they grow with an extremely reduced size (petite mutants).

III. MATERIAL AND METHODS

a) Strain

Saccharomyces cerevisiae D was used, a homozygote for the mutation of the isoleucine Ile 1-92 gene and which carries mutations for the adenine gene in two different loci of the same chromosome.

Mitotic recombinations in these sectors of this chromosome determine homozygosis in the mutant and the apparition of different-colored colonies in the selective medium.

These are red if the mitotic crossing over is produced between the centromere and the mutant locus proximal to it, and pink if the crossing over is produced between the two mutated loci.

It is a strain characterized and standardized for laboratory use. Its detailed genetic characteristics have been described by FK Zimmermann (1975).

b) Metabolic activation

Each of the experiments was carried out with metabolic fractions from rat livers. The preparation of these S9 metabolic fractions was carried out according to the following protocol.

Five days before the hepatic extraction, four adult Wistar rats were injected intraperitoneally with Aroclor 1254 diluted in sesame oil in doses of 500 mg/Kg. The livers were extracted, homogenized mechanically in 0.15 M KCl at a w/v ratio of



1:3.

The homogenates were centrifuged at 9000 x g for 15 minutes. The supernatants were filtered in Millipore filters and stored in liquid nitrogen (-196°C) until use. They were never stored in liquid nitrogen for over one month.

In accordance with Ames *et al.* (1975), the S9 mixture was the following:

10% S9

100 mM phosphate buffer, pH 7.4

5 mM glucose-6-phosphate

4 mM NADP

8 mM MgCl₂

33 mM KCl

c) Cultures

The cultures were incubated at 28°C in the dark and with agitation in YEP-glucose medium. The cells used were always in logarithmic growth phase, with a cellular density of 1×10^9 cells/ml. From the parent culture, a stock culture was prepared by dilution to a density of 2×10^7 cells/ml. The cells were counted in a hemocytometer at a dilution of 10^{-2} of the parent culture.

d) Positive and negative controls

The following reference substances were used to verify the response of the strain and to verify the activity of the S9 fraction used.

-Positive

-Methyl methane sulphonate 3 μ l/ml

-Cyclophosphamide 20 mg/ml

-Negative

-Distilled water



8) Prepare 20 plates for each dosage. At least 4000 colonies should be counted in spontaneous and test plates for statistically reliable results.

b) With metabolic activation

The protocol is as above, except that in step 2, 3.75 ml phosphate buffer + 0.5 S9 metabolic fraction + 0.25 test solutions are added.

The plates are incubated at 28°C in the dark for three days and then in the refrigerator at 4°C for two days, in order to intensify the color of the colonies. They are then counted.

The survival is evaluated by comparison with the number of colonies on control plates.

IV. RESULTS AND DISCUSSION

The results are presented in the tables in Annex I (individual values and means).

Survival always falls within the normal range of values for this assay in this laboratory.

The values for the negative controls (distilled water) were within the normal range found in this laboratory and mentioned in the scientific literature for this strain of Saccharomyces cerevisiae.

The values for the positive controls were, on the contrary, inducers of a substantial genotoxic response of the magnitude expected for this strain. Thus positive effects were observed for cyclophosphamide (CP) after its biotransformation by microsomal enzymes and direct positive genotoxic effects were seen with the methyl



methane sulphonate (MMS).

In conclusion, in the presence and absence of the S9 mixture, there was no significant increase in the total number of aberrations or recombinants in the concentration range tested; thus the solution technically known as FR-91 does not induce abnormal mitotic recombination in the conditions of this assay.

We declare that the results emitted in this report were obtained according to the protocol described.

LABORATORY DIRECTOR

A handwritten signature in black ink, appearing to read "José Miguel Sicilia Socias".

José Miguel Sicilia Socias

STUDY DIRECTOR

A handwritten signature in black ink, appearing to read "Luis de la Fuente Ramírez".

Luis de la Fuente Ramírez

QUALITY CONTROL TECHNICIAN

A handwritten signature in black ink, appearing to read "Angel Villanueva Río".

Angel Villanueva Río



MITOTIC RECOMBINATION INDUCTION ASSAY

PRODUCT: FR-91

METHOD: Preincubation in liquid medium

EVALUATION: Visual

STRAIN: Saccharomyces cerevisiae D7 ade 1-119, ade 2-40

CLASSIFICATION OF ABERRANTS

	Sectors		Colonies		Hairline	Total	Colonies counted
	Pink	Red	Pink	Red			
Negative control							
Dist. water	2	1	1	0	1	5	4437
Dist. water + S9	1	1	1	1	0	4	4532
Undiluted	2	1	0	1	1	5	4564
Undiluted + S9	1	1	2	2	1	7	4426
1/10	1	1	2	1	0	5	4216
1/10 + S9	1	2	1	2	1	7	4376
1/100	2	2	2	1	1	8	4653
1/100 + S9	2	0	0	2	1	5	4538
1/1000	1	1	1	1	1	5	4423
1/1000 + S9	1	1	0	1	0	3	4176
Positive controls							
-S9: MMS 3	38	64	19	39	5	165	2546
+S9: CP 20	45	58	32	44	8	187	2322

Date: 24/08/91

Conclusion: Assay negative



MITOTIC RECOMBINATION INDUCTION ASSAY
Saccharomyces cerevisiae D/ ade 1-119, ade 2-40

Negative control	Without S9			With S9		
	Total aberr	Colonies	Total %	Total aberr	Colonies	Total %
Dist. water	5	3337	0.11	4	4532	0.09
Undiluted	5	4564	0.11	7	4426	0.16
1/10	5	4216	0.12	7	4376	0.16
1/100	8	4653	0.17	5	4538	0.11
1/1000	5	4423	0.11	3	4176	0.07
Positive control	165	2546	6.48	187	2322	8.05





ANNEX II: BIBLIOGRAPHY

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DIRECTIVE 79/831/EEC Toxicological Methods for the Analysis of Chemical Substances, Annex VIII

DIRECTIVES 75/318/EEC and 87/19/EEC