Section A 6.6.2-01 **Annex Point IIA VI.6.6.2**		**In-vitro cytogenicity in mammalian cells** *In vitro Mammalian Chromosome Aberration Test with Human Lymphocytes.*	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Mishra KR, 2002, Cytotoxicity Report - In vitro Mammalian Chromosome Aberration Test of ACTICIDE OIT 100 % with Human Lymphocytes, Jai Research Foundation, [redacted], unpublished	
1.2	**Data protection**	Yes	
1.2.1	Data owner	THOR GmbH, Germany	
1.2.2	Companies with letter of access		
1.2.3	Criteria for data protection	Data submitted on existing A.S. for the purpose of its entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes. EPA 8705375, OECD 473.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2 of dossier.	
3.1.1	Lot/Batch number	ACTICIDE OIT 100% [redacted]	
3.1.2	Specification	technical grade	
3.1.2.1	Description	Clear yellow-brown liquid	
3.1.2.2	Purity	[redacted] As given in section 2 of dossier.	
3.1.2.3	Stability	[redacted] Dosing solutions were freshly prepared on the day of application.	
3.2	**Study Type**		
3.2.1	Organism/cell type	Periphere Human Lymphocytes Primary Culture	
3.2.2	Metabolic activation system	S9Mix: S9 fraction [redacted] supplemented with cofactors and buffer	
3.2.3	Positive control	Mitomycin-C (without S9mix) and Cyclophosphamide (with S9mix)	
3.3	**Administration / Exposure; Application of test substance**		
3.3.1	Concentrations	0.0005, 0.001and 0.002 OIT mg/ml culture (main study)	
3.3.2	Way of application	OIT stock solution in DMSO.	
3.3.3	Pre-incubation time	48 hours before plating	
3.3.4	Other modifications		
3.4	**Examinations**		

Section A 6.6.2-01 **Annex Point IIA VI.6.6.2**		**In-vitro cytogenicity in mammalian cells** *In vitro Mammalian Chromosome Aberration Test with Human Lymphocytes.*	
3.4.1	Number of cells evaluated	The cells were harvested at 1.5 cell cycles after the beginning of treatment. A minimum of 1000 cells per slide (culture) were counted. Hundred well spread metaphases per culture were scored for structural and numerical changes.	
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**		
4.1.1	without metabolic activation	Phase I In phase 1 of the experiment, Acticide ® OIT 100% was exposed for a short period of time (3 hours) without and with metabolic activation system (5% v/v S9 mix). The results did not reveal any significant variation in the mitotic index as well as percent aberrant cells at dose levels of 0.0005, 0.001 and 0.002 mg of Acticide OIT 100%/mL of culture both in the absence and presence of metabolic activation system (5% v/v S9 mix) when compared with control. Phase II In phase II of the experiment, Acticide ® OIT. 100% was exposed for a prolonged period of time (30 h continuous exposure) without metabolic actvation system. The results did not reveal any significant variation in mitotic index and percent aberrant cells at dose levels of 0.0005, 0.001 and 0.002 mg of Acticide ® OIT 100%/mL of culture when compared with the control. Phase III In phase III of the experiment, Acticide ® OIT. 100% was exposed for a short period of time (3 hours) with an increased concentration of metabolic activation system (10% v/v S9 mix). Statistically significant decrease ($p < 0.05$) in mitotic index was observed at low dose level (0.0005) during the III phase of the experiment. However, no significant variation was observed at the dose levels of 0.001 and 0.002 mg of Acticide ® OIT 100%/mL of culture when compared with the control. The data on per cent aberrant cells did not reveal any significant variation in the tested dose levels. Positive Controls The results of the positive controls (Mitomycin - C and Cyclophosphamide) in the three phases showed chromosomal breaks, chromatid breaks, chromatid exchange, fragments, dicentric, trident (triradial) and deletions. The positive control group showed an increase in frequency of aberrant cells and thus demonstrates the sensitivity of the test system.	
4.1.2	with metabolic activation		
4.2	**Cytotoxicity**	Based on the results of cytotoxicity and solubility in the preliminary experiment (not reported) a dose level of 0.002 mg of ACTICIDE OIT 100% /ml was selected as the highest exposure concentration for the main study.	

Section A 6.6.2-01 **Annex Point IIA VI.6.6.2**	**In-vitro cytogenicity in mammalian cells** *In vitro Mammalian Chromosome Aberration Test with Human Lymphocytes.*	
	5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1 Materials and methods	*Per-guideline study with technical grade test item (OIT [redacted]).*	
5.2 Results and discussion	See 4.	
5.3 Conclusion	From the results of this study, it is concluded that Acticide OIT 100% at all tested dose levels viz., 0.0005, 0.001 and 0.002 mg Acticide ® OIT 100%/mL of culture does not posses chromosomal aberration induction potential in human lymphocytes both without and with metabolic system (5% v/v and 10 % v/v S9 mix).	
5.3.1 Reliability	1	
5.3.2 Deficiencies	no	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*08/04/2009*	
Materials and Methods	*In agreement with Applicant's assessment*	
Results and discussion	*In agreement with Applicant's assessment*	
Conclusion	*We agree that this is a negative study, but because of shortcomings mentioned below the study is considered not to be a rigorous investigation of the potential of OIT to induce chromosome aberrations.*	
Reliability	*3*	
Acceptability	*Used as supportining information*	
Remarks	*A significant shortcoming of this study is that it was conducted using exposure concentrations that did not elicit toxicity, or were not shown to be at the limits of solubility in the test medium.*	
	COMMENTS FROM ...	
Date		
Materials and Methods		
Results and discussion		
Conclusion		
Reliability		
Acceptability		
Remarks		

Table A 6.6.2-1: Table for cytogenetic in-vitro test: chromosomal analysis without S9 (Pooled results from duplicate cultures, cells fixed after 20 hours)

JRF [redacted]

In vitro Mammalian Chromosome Aberration Test of Acticide ® OIT 100%

TABLE 1

Summary of Mitotic Index and Chromosomal Aberrations in Positive Controls

Phases	Positive Controls	Mean Mitotic Index		Percentage of Aberrated Cells	
		-S9	+S9	-S9	+S9
I	Mitomycin-C (9×10^{-7}M)	4.160 ↑↑ (0.107)	-	9.500 (3.536)	-
	Cyclophosphamide (2×10^{-3}M)	-	3.583 (0.854)	-	11.000 ↑ (1.414)
II	Mitomycin-C (9×10^{-7}M)	5.012 (1.675)	-	15.000 ↑↑ (1.414)	-
III	Cyclophosphamide (2×10^{-3}M)	-	4.393 ↓ (0.571)	-	20.000 ↑↑ (1.414)

Values in parenthesis indicate standard deviation

Key : ↓ = Significantly lower than control group ($p \leq 0.05$),
↑ = Significantly higher than control group ($p \leq 0.05$),
↑↑ = Significantly higher than control group ($p \leq 0.01$)

In vitro Mammalian Chromosome Aberration Test of Acticide ® OIT 100%

TABLE 2

Summary of Mitotic Index and Chromosomal Aberrations in Control and Treatment Groups

PHASE - I

Dose Levels	Metabolic Activation System	Mean Mitotic Index	Percentage of Aberrated Cells
Control (DMSO)	-S9	3.262 (0.016)	0.000 (0.000)
	+ 5% v/v S9	2.872 (0.277)	0.500 (0.707)
0.0005 mg/ml	-S9	3.199 (0.740)	0.000 (0.000)
	+ 5% v/v S9	3.524 (0.363)	1.000 (1.414)
0.001 mg/ml	-S9	3.294 (0.264)	0.000 (0.000)
	+ 5% v/v S9	3.785 (0.411)	1.500 (0.707)
0.002 mg/ml	-S9	3.897 (0.217)	2.000 (0.000)
	+ 5% v/v S9	3.090 (0.185)	0.500 (0.707)

Values in parenthesis indicate standard deviation

JRF [redacted]

TABLE 2 (continued)

PHASE – II [Without Metabolic Activation System (-S9 mix)]

Dose Levels	Mean Mitotic Index	Percentage of Aberrated Cells
Control (DMSO)	6.053 (1.211)	0.500 (0.707)
0.0005 mg/mL	6.041 (1.662)	0.000 (0.000)
0.001 mg/mL	6.319 (2.322)	0.500 (0.707)
0.002 mg/mL	4.145 (0.662)	0.500 (0.707)

PHASE – III [With Metabolic Activation System (+ 10% v/v S9 mix)]

Dose Levels	Mean Mitotic Index	Percentage of Aberrated Cells
Control (DMSO)	8.395 (0.122)	0.500 (0.707)
0.0005 mg/ml	7.889 ↓ (0.095)	1.000 (1.414)
0.001 mg/ml	10.317 (3.977)	1.000 (0.000)
0.002 mg/ml	10.991 (1.839)	0.500 (0.707)

Values in parenthesis indicate standard deviation

Key : ↓ = Significantly lower than control group ($p \leq 0.05$)

In vitro Mammalian Chromosome Aberration Test of Acticide ® OIT 100%

APPENDIX 1

Chromosomal Aberration Data

Phase I

Dose Levels	Metabolic Activation System	Repli-cate	Mitotic Index	Frequencies of Aberration		Others	Total N° of Aberration	Percentage of Aberrated Cells
				Chromatid Break	Chromosomal Break			
Control (DMSO)	-S9	R1	3.251	0	0	0	0	0
		R2	3.273	0	0	0	0	0
	+5 % v/vS9	R1	2.676	1	0	0	1	1
		R2	3.068	0	0	0	0	0
0.0005 mg/ml	-S9	R1	2.676	0	0	0	0	0
		R2	3.722	0	0	0	0	0
	+5 % v/vS9	R1	3.781	0	0	2F	2	2
		R2	3.267	0	0	0	0	0
0.001 mg/ml	-S9	R1	3.107	0	0	0	0	0
		R2	3.481	0	0	0	0	0
	+5 % v/vS9	R1	3.494	1	0	0	1	1
		R2	4.075	2	0	0	2	2
0.002 mg/ml	-S9	R1	4.050	2	0	0	2	2
		R2	3.743	1	0	1F	2	2
	+5 % v/vS9	R1	3.220	0	0	0	0	0
		R2	2.959	0	0	1F	1	1
MMC (9×10^{-7}M)	-S9	R1	4.084	6	0	1F, 2E	9	7
		R2	4.236	8	1	1F, 3E, 1DL	14	12
CYP (2×10^{-3}M)	+5 % v/vS9	R1	2.979	9	0	3F, 2DL, 1E	15	12
		R2	4.187	10	1	6F, 1DL	18	10

Key: MMC = Mitomycin-C, CYP = Cyclophosphamide, DMSO = Dimethyl sulphoxide, F = Fragment, E = Exchange, DL = Deletion

JRF [redacted]

APPENDIX 1 (continued)

Phase II [Without Metabolic Activation System (-S9 mix)]

Dose Levels	Replicate	Mitotic Index	Frequencies of Aberration		Others	Total N° of Aberration	Percentage of Aberrated Cells
			Chromatid Break	Chromosomal Break			
Control (DMSO)	R1	6.909	0	0	1DL	1	1
	R2	5.196	0	0	0	0	0
0.0005 mg/ml	R1	7.216	0	0	0	0	0
	R2	4.866	0	0	0	0	0
0.001 mg/ml	R1	7.961	0	0	0	0	0
	R2	4.677	1	0	0	1	1
0.002 mg/ml	R1	4.613	0	0	0	0	0
	R2	3.677	0	0	1F	1	1
MMC (9×10^{-7}M)	R1	3.827	19	5	16F, 5DL, 13E	58	16
	R2	6.196	16	4	9F, 7E	36	14

Key: MMC = Mitomycin-C, DMSO = Dimethyl sulphoxide, F = Fragment, DL= Deletion, E = Exchange

APPENDIX 1 (continued)

Phase III [With Metabolic Activation System (+ 10% v/v S9 mix)]

Dose Levels	Replicate	Mitotic Index	Frequencies of Aberration		Others	Total N° of Aberration	Percentage of Aberrated Cells
			Chromatid Break	Chromosomal Break			
Control (DMSO)	R1	8.308	1	0	0	1	1
	R2	8.481	0	0	0	0	0
0.0005 mg/ml	R1	7.956	0	0	0	0	0
	R2	7.822	2	0	1DL	3	2
0.001 mg/ml	R1	7.504	1	0	0	1	1
	R2	13.129	0	0	1F, 2DL	3	1
0.002 mg/ml	R1	12.291	0	0	0	0	0
	R2	9.690	1	0	0	1	1
CYP (2×10^{-3}M)	R1	3.989	26	2	6F, 1D, 3DL, 7E	45	19
	R2	4.796	45	6	14F, 6DL, 6E	77	21

Key: CYP = Cyclophosphamide, DMSO = Dimethyl sulphoxide, E = Exchange, D = Dicentric, F = Fragment, DL = Deletion

Section A 6.6.3 Genotoxicity in vitro

Annex Point IIA 6.6.3 *Mammalian cell gene mutation (mouse lymphoma, tk) assay*

Official use only

		6 REFERENCE
6.1	**Reference**	*Clements J, 1995, N-Octylisothiazolinone (OIT) [redacted]: Mutation at the Thymidine Kinase (tk) Locus of Mouse Lymphoma L5178Y Cells using the Microtitre Fluctuation Technique, Corning HAZLETON, [redacted], unpublished*
6.2	**Data protection**	Yes
6.2.1	Data owner	THOR GmbH, Germany
6.2.2		
6.2.3	Criteria for data protection	Data submitted on existing A.S. for the purpose of its entry into Annex I.
		7 GUIDELINES AND QUALITY ASSURANCE
7.1	**Guideline study**	Yes. EPA 84-2, OECD 476
7.2	**GLP**	Yes
7.3	**Deviations**	No
		8 MATERIALS AND METHODS
8.1	**Test material**	As given in section 2
8.1.1	Lot/Batch number	*ACTICIDE OIT,* [redacted]
8.1.2	Specification	Technical grade; As given in section 2
8.1.2.1	Description	*White, beige crystals*
8.1.2.2	Purity	[redacted] *As given in section 2*
8.1.2.3	Stability	*Expiry date: Dec 2002*
8.2	**Study Type**	
8.2.1	Organism/cell type	L5178Y TK +/- mouse lymphoma cells
8.2.2	Deficiencies / Proficiencies	Purged for TK^- mutants
8.2.3	Metabolic activation system	S9 mix *Post-mitochondrial fraction (S9) prepared from Sprage Dawley rats, induced with Phenobarbital/ß-naphthoflavone.*
8.2.4	Positive control	*NQO, 4-Nitroquinoline-1-oxid, (without metabolic activation)* *BP, benoz(a)pyrene (with metabolic activation)*
8.3	**Administration / Exposure; Application of test substance**	

Section A 6.6.3 Genotoxicity in vitro

Annex Point IIA 6.6.3 *Mammalian cell gene mutation (mouse lymphoma, tk) assay*

8.3.1	Concentrations	*The study was performed in two independent experiments, using identical experimental procedures. Cell cultures were evaluated at the following concentrations:* *Experiment 1:* *without S9 mix: 0.1563; 0.3125; 0.625; 1.25; 2.5, 5, 10, 20 µg/ml* *with S9 mix: 0.1563; 0.3125; 0.625; 1.25; 2.5, 5, 10, 20 µg/ml* *Experiment II:* *without S9 mix: 0.125; 0.25; 0.5; 0.75; 1, 1.25 µg/ml* *with S9 mix: 0.125; 0.25; 0.5; 1, 1.5, 2, 2.5 µg/ml* *No precipitation of the test item was observed up to the maximal concentration in all experiments.*
8.3.2	Way of application	*Test substance was dissolved in DMSO.*
8.3.3	Pre-incubation time	*Well growing stock cultures were sub-cultivated (washing). Tthe medium were replaced with serum free medium containing the test item, either with or without S9 mix.*
8.3.4	Other modifications	
8.4	**Examinations**	
8.4.1	Number of cells evaluated	*Plates were incubated until scorable (12 days) and wells containing clones were identified and counted. In addition, the number of wells containing large colonies and the number containing small colonies were scored for the negative and positive controls.*

9 RESULTS AND DISCUSSION

9.1 Genotoxicity

Section A 6.6.3 Genotoxicity in vitro

Annex Point IIA 6.6.3

Mammalian cell gene mutation (mouse lymphoma, tk) assay

9.1.1	without metabolic activation	*In the absence of S-9, no statistically significant increases in mutant frequency were obtained at any dose level tested in Experiment 1 or 2. A linear trend was observed in both experiments but in Experiment 1 particularly the linear trend was not considered to reflect a true dose-relationship. The linear trend observed in Experiment 1 is likely to be attributable to the low mutant frequency obtained at the dose of 0.1563 µg/mL. Hence, a reproducible dose-relationship was not obtained and in view of the fact that no statistically significant increases in mutant frequency were observed in either experiment, the linear trends observed are not considered to be of biological significance.* *In the presence S-9, no statistically significant increases in mutant frequency were obtained at any dose level tested in Experiments 1 or 2. In Experiment 2 there was an indication of a weak linear trend but this was not considered to represent a true dose-relationship. Furthermore, a linear trend was not observed in Experiment 1. Hence, the weak linear trend indicated in Experiment 2 was considered to be attributable to a chance event of no biological significance.* *It may be noted that in Experiment 1 in the absence of S-9, the data for the lower dose of positive control (NQO 0.05 µg/mL) have not been reported. No colonies were obtained on the viability plates for this dose and this was attributed to technical error.* *In addition, for the negative and positive controls the number of wells containing small colonies and the number containing large colonies were scored. Thus the small and large colony mutant frequencies could be estimated and the proportion*
9.1.2	with metabolic activation	*of small mutant colonies could be calculated (Appendix 6). For the negative controls, the proportion of small colony mutants in the absence and presence of S-9 ranged from 53% to 57% in Experiment 1 and from 60% to 68% in Experiment 2. Good recovery of small colony mutants was observed following treatment with the positive control chemicals NQ4 and BP.*

Section A 6.6.3 Genotoxicity in vitro

Annex Point IIA 6.6.3 *Mammalian cell gene mutation (mouse lymphoma, tk) assay*

9.2	**Cytotoxicity**	*In the cytotoxicity range-finder, 6 doses of N-Octylisothiazolone (OIT) [redacted] were tested, separated by 2-fold intervals and ranging from 31.25 to 1000 µg/mL. Both upon addition of the test article to the cultures and after the 3 hour treatment incubation period, precipitate was observed at the top 2 doses tested (500 and 1000 µg/mL). Complete toxicity was observed at all dose levels tested in the absence and presence of S-9 (data not shown).* *Hence, to ensure an appropriate dose range was tested in Experiment 1, eight doses were selected, separated by 2-fold intervals and ranging from 0.1563 to 20 µg/mL. Three days after treatment the 4 highest doses tested in the absence of S-9 and the 3 highest doses tested in the presence of S-9 were considered to be too toxic to be selected for determination of viability and 5-trifluorothymidine resistance. All other doses were selected. However, 1.25 µg/mL in the absence of S-9 and 2.5 ig/mL in the presence of S-9 were later rejected from analysis due to excessive toxicity, yielding 7.1% and 9.4% relative survival respectively. The top doses analysed were 0.625 and 1.25 µg/mL which yielded 27.4% and 15.4% relative survival in the absence and presence of S-9 respectively.* *In Experiment 2, the dose range was modifled to take account of the toxicity observed in Experiment 1. In this experiment 6 doses were tested in the absence of S-9 (ranging from 0.125 to 1.25 µg/mL) and 7 doses were tested in the presence of S-9 (ranging from 0.125 to 2.5 µg/mL). Three days after treatment the 5 highest doses tested, in both the absence and presence of S-9, were selected to determine viability and 5-trifluorothymidine resistance. The top doses selected were 1.25 and 2.5 gg/mL, which yielded 11.2% and 14.6% relative survival in the absence and presence of S-9 respectively.*
		10 APPLICANT'S SUMMARY AND CONCLUSION
10.1	**Materials and methods**	*Per-guideline study with technical grade test item (OIT [redacted]).*
10.2	**Results and discussion**	*When tested up to toxic concentrations, N-Octylisothiazolone (OIT) did not induce mutation at the tk locus of L5178Y mouse lymphoma cells in 2 independent experiments in the absence or presence of S-9.*
10.3	**Conclusion**	*It is concluded that, under the conditions employed in this study, N-Octylisothiazolone (OIT) [redacted] is not mutagenic in this test System.*
10.3.1	Reliability	*1*
10.3.2	Deficiencies	*No*

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*08/04/2008*
Materials and Methods	
Results and discussion	
Conclusion	

Section A 6.6.3 Genotoxicity in vitro

Annex Point IIA 6.6.3 *Mammalian cell gene mutation (mouse lymphoma, tk) assay*

Reliability	*1*
Acceptability	*Acceptable*
Remarks	*In agreement with the applicant's assessment.*
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_6_1-1. Table for Gene Mutation Assay (modify if necessary)

TABLE 1

Summary table of results

Experiment 1

Treatment (µg/mL)	-S-9 %RS	-S-9 Mutant frequency#	Treatment (µg/mL)	+S-9 %RS	+S-9 Mutant frequency#
0	100.0	121.17	0	100.0	91.51
0.156	100.7	74.55 NS	0.156	120.3	103.42 NS
0.313	70.0	142.34 NS	0.313	84.9	103.12 NS
0.625	27.4	141.97 NS	0.625	41.4	89.64 NS
1.25 X	7.1		1.25	15.4	74.73 NS
2.5 $	3.6		2.5 X	9.4	
5 $	0.0		5 $	7.5	
10 $	0.0		10 $	0.0	
20 $	0.0		20 $	0.0	
Linear trend		**	Linear trend		NS
NQO			BP		
0.05 $,X	89.8		2	108.3	437.14
0.1	73.6	505.40	3	71.6	637.02

Experiment 2

Treatment (µg/mL)	-S-9 %RS	-S-9 Mutant frequency#	Treatment (µg/mL)	+S-9 %RS	+S-9 Mutant frequency#
0	100.0	111.46	0	100.0	111.27
0.125 $	95.7		0.125 $	95.8	
0.25	82.9	91.55 NS	0.25 $	93.7	
0.5	49.5	90.17 NS	0.5	64.7	83.55 NS
0.75	16.0	104.68 NS	1	28.5	80.84 NS
1	14.1	122.89 NS	1.5	15.7	110.31 NS
1.25	11.2	155.28 NS	2	10.2	120.68 NS
			2.5	14.6	116.23 NS
Linear trend		**	Linear trend		*
NQO			BP		
0.05	72.5	333.21	2	96.5	385.06
0.1	73.5	532.62	3	81.3	414.24

\# Per 10^6 viable cells
$ Not plated for viability / 5-TFT resistance
X Treatment excluded from test statistics
NS Not significant
*,**,*** Significant at 5%, 1% and 0.1% level respectively

For the purposes of statistical analysis doses have been expressed to 3 decimal places.

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 *Mammalian bone marrow micronucleus test*

Official use only

11 REFERENCE

11.1 Reference [redacted] *1995, n-Octylisothiazolinone (OIT)* [redacted] *Induction of*

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 *Mammalian bone marrow micronucleus test*

		Micronuclei in the Bone Marrow of Treated Mice. [redacted] *unpublished*
11.2	**Data protection**	*Yes.*
11.2.1	Data owner	*THOR GmbH*
11.2.2		
11.2.3	Criteria for data protection	Data submitted on existing A.S. for the purpose of its entry into Annex I.
		12 GUIDELINES AND QUALITY ASSURANCE
12.1	**Guideline study**	Yes. EPA guideline 84-2
12.2	**GLP**	Yes
12.3	**Deviations**	No
		13 MATERIALS AND METHODS
13.1	**Test material**	*As given in section 2 of dossier.*
13.1.1	Lot/Batch number	n-Octylisothiazolinone (OIT) [redacted]
13.1.2	Specification	*Technical grade*
13.1.2.1	Description	*Clear amber liquide*
13.1.2.2	Purity	[redacted]
13.1.2.3	Stability	*Dosing solutions were freshly prepared on the day of application.*
13.1.2.4	Maximum tolerable dose	*Oral LD_{50} value(rat) is more than 500 mg/kg body weight (OECD 423)*
13.2	**Test Animals**	
13.2.1	Species	mouse
13.2.2	Strain	[redacted]
13.2.3	Source	[redacted]
13.2.4	Sex	*m and f (see 13.2.6)*
13.2.5	Age/weight at study initiation	Age: 4-7 weeks Weight: 19-30 g
13.2.6	Number of animals per group	5m + 5f per group
13.2.7	Control animals	Yes. Vehicle control (group I)as well as positive control (group V): Mytomycin C.
13.3	**Administration/ Exposure**	
13.3.1	Number of applications	*1 on two consecutive days*
13.3.2	Interval between applications	24 hours
13.3.3	Postexposure period	24 or 48 hours after the second treatment (24/48h samples)
13.3.4	**Oral**	

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 *Mammalian bone marrow micronucleus test*

13.3.4.1	Type	gavage
13.3.4.2	Concentration	162.5, 325, 650 mg/kg bw
13.3.4.3	Vehicle	corn oil BP
13.3.4.4	Concentration in vehicle	8.125, 16.25, 32,5 mg/ml dosing preparation
13.3.4.5	Total volume applied	20 ml/kg bw
13.3.4.6	Controls	Vehicle: corn oil
13.3.4.7	Substance used as positive control	Cyclophosphamide (CPR) in physiological saline (4 mg/ml) *dose level: 80 mg/kg bw/day (single administration)*
13.4	**Examinations**	
13.4.1	Clinical signs	Yes, twice daily
13.4.2	Tissue	bone marrow
	Number of animals:	all animals
	Number of cells:	Phase I - ratio: 1000 cells of polychromatic erythrocytes (PCE) plus normochromatic erythrocytes (NCE) Phase II – 2000 PCE , only
	Time points:	12, 18, 24, 36, 48, 72 h after treatment or other
	Type of cells	bone marrow cells
	Parameters:	PCE/NCE-ratio micronuclei
13.5	**Further remarks**	Body weights

14 RESULTS AND DISCUSSION

14.1	**Clinical signs**	*Clinical signs observed in animals dosed with 650 mg/kg/day N-Octylisothiazolone (OIT) [redacted] included hunched gait, greasy fur (not observed with any of the other groups), sunken eyes, and 2 deaths. These signs indicate that it would not have been practicable to administer the test article at an appreciably higher dose.*
14.2	**Haematology / Tissue examination**	*No effects* *Groups of mice treated with the highest dose (650 mg/kg/day) of N-Octylisothiazolone (OIT) [redacted] exhibited PCE/NCE ratios which were decreased in relation to ratios observed in vehicle controls at both sampling times. This could be taken as evidence of bone marrow toxicity.* *Group mean frequencies of micronucleated PCE were also similar to or lower than those seen in vehicle control groups and were not significantly increased by Chi2 analysis.*
14.3	**Genotoxicity**	*It is concluded that N-Octylisothiazolone (0IT) [redacted] did not induce micronuclei in the polychromatic erythrocytes of the Bone marrow of mice treated up to 650 mg/kg/day, a dose found to be the maximum acceptable dose.*
14.4	**Other**	

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 *Mammalian bone marrow micronucleus test*

		15 APPLICANT'S SUMMARY AND CONCLUSION
15.1	**Materials and methods**	Per-guideline study with technical grade test item (OIT [redacted]).
15.2	**Results and discussion**	*See 4.*
15.3	**Conclusion**	*Two days oral administration of OIT up to a dose level of 650 mg/kg body weight dose not have a potential to induce micronuclei in mice.*
15.3.1	Reliability	*1*
15.3.2	Deficiencies	*No*

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*08/04/2009*
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	*1*
Acceptability	*Acceptable*
Remarks	*In agreement with the applicant's assessment.*
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Summary of group mean data

Data for N-Octylisothiazolone (OIT) [redacted]

Treatment group (mg/kg/day)	Kill time (hours)	Sex	Mean ratio PCE/NCE	Group mean frequency of micronucleated PCE (per 1000)	
				per sex	per treatment group
Vehicle control	24	♂	1.32	0.8	0.8
		♀	1.20	0.8	
	48	♂	0.85	0.8	0.85
		♀	0.94	0.9	
162.5	24	♂	1.17	1.0	0.8
		♀	1.11	0.6	
	48	♂	1.10	0.4	0.6
		♀	1.24	0.8	
325	24	♂	1.09	0.2	0.35
		♀	1.37	0.5	
	48	♂	1.03	0.6	0.45
		♀	0.84	0.3	
650	24	♂	0.94	0.6	0.55
		♀	0.85	0.5	
	48	♂	0.72	0.3	0.3
		♀	0.83	0.3	
CPA, 80+	24	♂	1.03	29.49	34.12
		♀	1.06	38.75	

+ Administered as a single dose

Total group weights

Data for N-Octylisothiazolone (OIT) [redacted]

Treatment (mg/kg/day)		Kill time after treatment (hours)			
		24		48	
		♂	♀	♂	♀
Vehicle control	Group number	1	11	6	16
	Total group weight (g)	130	105	133	108
162.5	Group number	2	12	7	17
	Total group weight (g)	131	109	134	105
325	Group number	3	13	8	18
	Total group weight (g)	135	111	134	108
650	Group number	4	14	9	19
	Total group weight (g)	136	109	135	104
				10*	20*
				129	105
CPA, 80+	Group number	5	15		
	Total group weight (g)	133	105		

Group weights checked on the first day of dosing to ensure weights of all groups differ from mean by no more than 5%

+ Administered as a single dose

* These animals included to be analysed only in the event of mortality among other animals receiving 650 mg/kg/day N-Octylisothiazolone (OIT) [redacted]

Mean group weight: males = 133 g; females = 107 g

Section A 6.6.5 Genotoxicity in vivo

Annex Point IIA 6.6.5 *UDS in vivo*

Official use only

16 REFERENCE

16.1 Reference [redacted] *2002, In Vivo Unscheduled DNA Synthesis in Rat Hepatocytes with Acticide OIT.* [redacted] *unpublished*

16.2 Data protection Yes

Section A 6.6.5 Genotoxicity in vivo

Annex Point IIA 6.6.5

UDS in vivo

16.2.1	Data owner	THOR GmbH, Germany
16.2.2		
16.2.3	Criteria for data protection	Data submitted on existing A.S. for the purpose of its entry into Annex I.
		17 GUIDELINES AND QUALITY ASSURANCE
17.1	**Guideline study**	Yes. OECD 486 (1997)
17.2	**GLP**	Yes
17.3	**Deviations**	No
		18 MATERIALS AND METHODS
18.1	**Test material**	As given in section 2
18.1.1	Lot/Batch number	*ACTICIDE OIT-*[redacted]
18.1.2	Specification	Technical grade; As given in section 2
18.1.2.1	Description	*Yellow-brown liquide*
18.1.2.2	Purity	[redacted] *As given in section 2*
18.1.2.3	Stability	[redacted]
18.1.2.4	Maximum tolerable dose	*500 ppm (estimated by pre-experiments in this study)* *LD50 (rat, oral) > 500 mg/kg bw*
18.2	**Test Animals**	
18.2.1	Species	rat
18.2.2	Strain	[redacted]
18.2.3	Source	[redacted]
18.2.4	Sex	*males*
18.2.5	Age/weight at study initiation	Age: 6-10 weeks Mean Weight (SD): 172.2 g (11.4 g)
18.2.6	Number of animals per group	4 males per dose
18.2.7	Control animals	Yes
18.3	**Administration/ Exposure**	Oral
18.3.1	Number of applications	*once*
18.3.2	Interval between applications	
18.3.3	Postexposure period	2 and 16 h after treatment
		Oral
18.3.4	Type	gavage

Section A 6.6.5 Genotoxicity in vivo

Annex Point IIA 6.6.5 *UDS in vivo*

18.3.5	Concentration	500 mg/kg bw (high dose) 250 *mg/kg bw (low dose)*
18.3.6	Vehicle	corn oil
18.3.7	Concentration in vehicle	
18.3.8	Total volume applied	10 ml/kg bw
18.3.9	Controls	Negative/vehicle control 2h and 16 h preparation interval: Positive control: 2 h preparation interval: DMH; N,N'-dimethylhydranzinedihydrochloride, 40 mg/kg b.w. 16 h preparation interval: 2-AAF, 2-acetylaminofluorene, 100 mg/kg b.w.
18.4	**Further remarks**	

19 RESULTS AND DISCUSSION

19.1	**Clinical signs**	*Reduction of spontaneous activity, abdominal position, ruffled fur, apathy.*
19.2	**Haematology / Tissue examination**	*The viability of the hepatocytes was not substantially affected due to the in vivo treatment with the test item.*
19.3	**Genotoxicity**	*No dose of the test item revealed UDS induction in the hepatocytes of the treated animals as compared to the current vehicle controls. Neither the nuclear grains nor the resulting net grains were distinctly enhanced due to the in vivo treatment of the animals with the test item for 2 hours or 16 hours, respectively. Therefore, the net grain values obtained after treatment with the test item were consistently negative.* *In addition, no substantial shift to higher values was obtained in the percentage distribution of the nuclear grain counts.* *Appropriate reference mutagens (DMH, 40 mg/kg b.w. and 2-AAF, 100 mg/kg b.w.) were used as positive controls. Treatment with the positive control substances revealed distinct increases in the number of nuclear and net grain counts.*
19.4	**Other**	

Section A 6.6.5 Genotoxicity in vivo

Annex Point IIA 6.6.5 *UDS in vivo*

		20 APPLICANT'S SUMMARY AND CONCLUSION
20.1	**Materials and methods**	Per-guideline study with technical grade test item ([redacted]OIT[redacted]).
20.2	**Results and discussion**	*The test item did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats.*
20.3	**Conclusion**	*ACTICIDE OIT [redacted] did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats.*
20.3.1	Reliability	*1*
20.3.2	Deficiencies	*No*

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*08/04/2009*
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	*1*
Acceptability	*Acceptable*
Remarks	*In agreement with the applicant's assessment.*
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6.6.5-1 Table for Micronucleus Test In Vivo (modify if necessary)

13.3. Viability and Number of the Hepatocytes

Test item: ACTICIDE OIT

Treatment	Period	Animal no.	Viability* [%]	Number of isolated cells [$\times 10^5$]
corn oil	2 h	2	73	175
		3	74	72
		4	72	189
250 mg/kg b.w. ACTICIDE OIT	2 h	5	74	426
		7	70	305
		8	72	254
500 mg/kg b.w. ACTICIDE OIT	2 h	9	77	460
		10	72	293
		11	73	410
40 mg/kg b.w. DMH	2 h	13	73	302
		14	75	277
		15	71	264
corn oil	16 h	17	84	263
		18	70	175
		19	76	323
250 mg/kg b.w. ACTICIDE OIT	16 h	21	71	261
		22	70	254
		23	72	209
500 mg/kg b.w. ACTICIDE OIT	16 h	25	74	165
		26	71	175
		27	72	187
100 mg/kg b.w. 2-AAF	16 h	29	74	339
		30	73	229
		31	73	310

* Viability determined by means of trypan blue dye exclusion assay

13.4. Mean Nucleus, Cytoplasmic Area, and Net Grains

Test item: Acticide OIT

Treatment	Period	Grains per nucleus		Grains per cytoplasmic area		Net grains per nucleus	
		Mean*	SD**	Mean*	SD**	Mean*	SD**
Corn oil	2 h	9.72	± 4.09	17.95	± 7.19	-8.24	± 6.56
250 mg/kg b.w. Acticide OIT	2 h	10.02	± 5.33	14.87	± 6.53	-4.84	± 5.55
500 mg/kg b.w. Acticide OIT	2 h	10.81	± 5.22	15.33	± 6.82	-4.51	± 5.84
40 mg/kg b.w. DMH	2 h	33.11	± 11.59	15.16	± 5.94	17.95	± 11.41
Corn oil	16 h	7.48	± 3.70	11.32	± 4.71	-3.84	± 4.50
250 mg/kg b.w. Acticide OIT	16 h	10.80	± 4.67	20.05	± 6.16	-9.25	± 5.79
500 mg/kg b.w. Acticide OIT	16 h	11.41	± 5.21	20.82	± 7.34	-9.41	± 6.01
100 mg/kg b.w. 2-AAF	16 h	29.43	± 12.36	15.49	± 6.69	13.95	± 10.72

* Mean of 3 animals
** Standard deviation

13.5. Percentage Distribution of the Nuclear Grain Counts

Test item: Acticide OIT

Treatment	Period	Mean*	>0	>1	>5	>10	>20	>30	>40	>50	>60	>70	>80	>90	>100
Corn oil	2 h	9.72	100	98.7	83.7	40.3	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
250 mg/kg b.w. Acticide OIT	2 h	10.02	100	99.7	80.0	39.7	3.3	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
500 mg/kg b.w. Acticide OIT	2 h	10.81	100	98.7	85.7	49.7	5.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
40 mg/kg b.w. DMH	2 h	33.11	100	100	100	98.0	87.0	59.7	23.0	6.7	1.7	0.7	0.0	0.0	0.0
Corn oil	16 h	7.48	100	99.7	62.7	23.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
250 mg/kg b.w. Acticide OIT	16 h	10.80	100	99.7	91.7	43.7	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
500 mg/kg b.w. Acticide OIT	16 h	11.41	100	99.7	87.7	54.3	6.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
100 mg/kg b.w. 2-AAF	16 h	29.43	100	100	98.7	93.7	75.3	44.0	19.3	5.0	0.3	0.0	0.0	0.0	0.0

* Mean of 3 animals