Salmonella typhimurium Reverse Mutation Test

Salmonella typhimurium Reverse Mutation Test of E-55 compound

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April 11, 2011
Date

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1 Summary

The *Salmonella typhimurium* Reverse Mutation Test (Ames test) is a standard bacterial assay which measures histidine reversion (his^- to his^+) induced by chemicals causing base changes or frameshift mutations in the genome of this organism.

This assay employs strains of *Salmonella typhimurium* histidine auxotroph mutants which are deficient in the synthesis of a necessary for bacterial growth amino acid - histidine. The mutants can grow only in a medium containing sufficient histidine supplement. A reverse mutation in one of the genes involved in histidine biosynthesis leads to reversion to production of this amino acid. On agar media containing a small quantity of histidine only histidine prototrophs (his+ revertants) will grow and form visible colonies. The presence of these colonies indicates a reverse mutation occurs. Different types of mutations carried by bacterial strains make it possible to determine a mutation type caused by the examined substance. Introduction of the chemical with a mutagenic activity into the bacterial population will result in a higher number of revertants in comparison to an untreated control culture lacked of the investigated agent. The Ames test includes using an extract of liver enzymes to simulate mammalian metabolic activity which may activate non-mutagenic chemicals to their mutagenic derivatives.

The objective of this study was to evaluate E-55 compound for the ability to induce reverse mutations at the histidine locus in five strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) in the presence and absence of an exogenous metabolic activation system (S9) containing mammalian microsomal enzymes.

E-55 compound was evaluated in the mutagenicity assay in five tester strains with and without S9 activation system at doses of 10, 50, 100, 500 and 1000 µg/plate in TA100, TA98, TA102, TA1535 and TA1537 tester strains. All doses of the test compound, as well as concurrent positive and solvent controls were evaluated using three plates per single dose.

These results of the *Salmonella typhimurium* Reverse Mutation Test indicated that under the study conditions E-55 test compound did not cause positive increases in the mean value of revertants per plate in any of tester strains i.e. TA98, TA100, TA102, TA1535 or TA1537 in the presence and in the absence of S9 exogenous activation system.

2 Objective

The objective of this study was to evaluate the test compound and/or its metabolites for the ability to induce reverse mutations at the histidine locus in five strains of *Salmonella typhimurium* in the presence and absence of an exogenous metabolic activation system (S9) containing mammalian microsomal enzymes. This assay design was based on OECD Guideline 471, updated and adopted 21 July 1997.

3 Materials and methods

3.1 Reagents

Reagent name	Supplier	Cat. no
Davis Minimal Agar	Sigma	79332
D-(+)-Glucose	Sigma	G7021
Bacteriological agar	Sigma	A5306
Sodium Chloride	Sigma	S5629
Brain Heart Infusion Broth	Sigma	53286
Sodium phosphate, monobasic monohydrate	Sigma	S9638
Sodium phosphate, dibasic dodecahydrate	Sigma	71644
D-Glucose 6-phosphate sodium salt	Sigma	G7879
β-Nicotinamide adenine dinucleotide phosphate hydrate	Sigma	N5755
L-Histidine	Sigma	H6034
D-Biotin	Sigma	47868
Dimethyl sulfoxide	BioShop	DMS555.500
Salmonella typhimurium TA98 strain	Moltox	71-098L
Salmonella typhimurium TA100 strain	Moltox	71-100L
Salmonella typhimurium TA102 strain	Moltox	71-102L
Salmonella typhimurium TA1535 strain	Moltox	71-1535L
Salmonella typhimurium TA1537 strain	Moltox	71-1537L
2-Aminoanthracene	Sigma	A38800
Sodium azide	Sigma	S8032
Mitomycin C	Sigma	M0503
ICR 191 Acridine mutagen	Sigma	I3636
Ampicillin	Sigma	A9393
Tetracycline	Sigma	T3258
Crystal violet	Sigma	C3886
Magnesium chloride	Sigma	M4880
Potassium chloride	Sigma	P5405
S9,SD rat liver,frozen, Aroclor ind. KCl	Moltox	11-101.5

3.2 Buffers

Buffer name	Components	Amounts
Co-factors for S9 mix per 100 mL	D-Glucose-6-phosphate	188 mg
	NADP	330 mg
	$MgCl_2 \cdot 6H_2O$	180 mg
	KCl	270 mg
	$Na_2HPO_4 \cdot 12H_2O$	2.87 g
	$NaH_2PO4 \cdot H_2O$	280 mg
	S9	10 mL
	H_2O	Up to 90 mL
GM agar plates medium per 200 mL	Minimal Davis Agar	5.32 g
	10% glucose	10 mL
	H_2O	190 mL
Glucose solution (10% v/v) per 1 L	glucose	100 g
	H_2O	Up to 1 L
Histidine/biotin Solution (0.5 mM) per 1 L	D-Biotin	123
	L-Histidine	78
	H_2O	Up to 1 L
Top agar supplemented with histidine/biotin per 1 L	Agar	6 g
	NaCl	6 g
	His/Bio Solution (0.5 mM)	100 mL
	H_2O	Up till 1 L
Nutrient broth per 1 L	Oxoid nutrient broth #2	25 g
	H_2O	Up till 1 L
Nutrient agar plates per 1 L	Oxoid nutrient broth #2	25 g
	Agar	15 g
	H_2O	Up till 1 L
Sodium phosphate buffer, 0.1 mM, pH 7.4, per 900 mL	$Na_2HPO_4 \cdot 12H_2O$	29.01 g diluted in 500 mL of H_2O
	Titrate with NaH_2PO_4x H_2O up to pH 7.4	
	H_2O	Up till 1 L
Biotin solution (0.01% w/v) per 100 mL	D-Biotin	10 mg
	H_2O	Up till 100 mL
Histidine solution (0.5% w/v) per 500 mL	L-Histidine	500 mg
	H_2O	Up till 100 mL

Ampicillin (0.8%, w/v) per 100 mL	Ampicillin	800 mg
	H_2O	Up till 100 mL
Tetracycline (0.08%, w/v) per 100 mL	Tetracycline	80 mg
	H_2O	Up till 100 mL
Crystal violet (0.1%, w/v) per 100 mL	Crystal violet	100 mg
	H_2O	Up till 100 mL
GM agar plates enriched with biotin per 200 mL	GM Agar	200 mL
	Biotin solution (0.01% w/v)	1.6 mL
GM agar plates enriched with biotin and histidine per 200 mL	GM Agar	200 mL
	Biotin solution (0.01% w/v)	1.6 mL
	Histidine solution (0.5% w/v)	1.6 mL
GM agar plates enriched with histidine per 200 mL	GM Agar	200 mL
	Histidine solution (0.5% w/v)	1.6 mL
GM agar plates enriched with biotin, histidine and ampicillin per 200 mL	GM Agar	200 mL
	Biotin solution (0.01% w/v)	1.6 mL
	Histidine solution (0.5% w/v)	1.6 mL
	Ampicillin (0.8%, w/v)	0.6 mL
GM agar plates enriched with biotin, histidine, ampicillin and tetracycline per 200 mL	GM Agar	200 mL
	Biotin solution (0.01% w/v)	1.6 mL
	Histidine solution (0.5% w/v)	1.6 mL
	Ampicillin (0.8%, w/v)	0.6 mL
	Tetracycline (0.08%, w/v)	0.5 mL
GM agar plates enriched with biotin, histidine and tetracycline per 200 mL	GM Agar	200 mL
	Biotin solution (0.01% w/v)	1.6 mL
	Histidine solution (0.5% w/v)	1.6 mL
	Tetracycline (0.08%, w/v)	0.5 mL

3.3 Tester strains

Tester strains used were *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA102, TA1535 and TA1537. Specific genotypes of the strains are shown in Table 1.

Table 1. Characteristics of the strains used in the Ames test.

Strain	Allele	Reversion event	LPS defect	DNA repair mutation	Biotin requirement	Plasmid
S. typhimurium TA1535	*hisG*	Base-pair substitution	rfa	uvrB	bio^-	No plasmid
S. typhimurium TA1537	*hisC*	frameshift	rfa	uvrB	bio^-	No plasmid
S. typhimurium TA98	*hisD*	frameshift	rfa	uvrB	bio^-	pKM101
S. typhimurium TA100	*hisG*	Base-pair substitution	rfa	uvrB	bio^-	pKM101
S. typhimurium TA102	*hisG*	Base-pair substitution	rfa	–	–	pKM101, pAQ1

Frameshift and base-pair substitution defects in *Salmonella typhimurium* strains are represented to identify both types of mutation caused by the chemical. Additional genetic markers serve to make the strains more sensitive to certain types of mutagens. The *rfa* mutation changes one of the bacterial cell wall components – lipopolisacharide (LPS) increasing its permeability for certain compounds. The mutation is indicated by sensitivity to crystal violet. The DNA repair mutation results in gaining the gene for the enzymes that catalyze a repair pathway for DNA damage from UV light and certain mutagens. The R factor plasmid (pKM101) makes the strains more responsive to a variety of mutagens and carries an ampicillin resistance gene. The pAQ1 plasmid carries a tetracycline resistance gene and corresponds to the part of the histidine operon being deleted in the original genome.

3.4 Preparation of overnight cultures

Inoculation and harvest

Overnight cultures for use in all testing procedures were inoculated by transferring a volume of a glycerol stock from the appropriate working cultures to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator (shaking 100 rpm; incubation 37°C). When the overnight cultures were in late log phase optical density (OD) was measured. The cultures in the assay had a cell density 1-2 x 10^9 CFU/mL and had not overgrown. Cultures were removed

from incubation when target OD was reached and were kept at room temperature away from direct fluorescence light until used in the assay.

Medium

The broth used to grow overnight (11-13 hours) cultures of the tester strains was Oxoid Nutrient Broth No. 2.

Minimal bottom agar (20 mL per 14.2 x 90 mm Petri dish) was Davis Minimal Agar supplemented with 0.5% (w/v) glucose.

Top agar for selection of revertants consisted of 0.6% (w/v) agar and 0.6% (w/v) NaCl and was supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL agar for selection of histidine revertants. 2.0 mL of supplemented top agar was used in the overlay.

3.5 The test compound

The test substance described as TOSLab LTD, E-55 was delivered on 21.03.2011 in a plastic bottle. It was packed in the paper box and in the envelope. The test substance described as E-55 was solid and white. The package contained the sample (1x 5 g).

E-55 was dissolved in dimethylsulfoxide (DMSO) and diluted to required concentrations.

3.6 Test controls

Solvent Controls

Solvent controls were plated for five tester strains in the presence and absence of S9 using a 50 µL aliquot of dimethylsulfoxide (DMSO) (equal to the aliquot of test compound dilution plated), along with a 100 µL aliquot of the appropriate tester strain and a 500 µL aliquot of S9 mix or phosphate buffer on selective agar.

Positive Controls

Positive controls demonstrate the effective performance of each assay. Combinations of positive controls, activation conditions and tester strains used in the assay are shown in Table 2.

Table 2. Positive controls.

Strain	Positive controls (µg/plate)	
	Without activation	With activation (S9 mix)
TA98	2-Nitrofluorene (1)	2-Aminoanthracene (2.5)
TA100	Sodium azide (2)	2-Aminoanthracene (2.5)
TA102	Mitomycin C (0.5)	2-Aminoanthracene (5)
TA1535	Sodium azide (2)	2-Aminoanthracene (2.5)
TA1537	ICR 191 Acridine mutagen (50)	2-Aminoanthracene (2.5)

All positive controls compounds were purchased from Sigma-Aldrich Chemical Company.

3.7 S9 metabolic activation system

Bacteria were exposed to the test substance both in the presence and absence of a metabolic activation system which is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rats treated with enzyme-inducing agents. The post-mitochondrial fraction was used at concentrations of 10% v/v in the S9-mix. S9 homogenate were purchased from TRINOVA Biochem GmbH (33.4 mg/mL protein). The homogenate was prepared from male Sprague-Dawley rats. S9 mix was prepared immediately prior to use in all experimental procedure.

3.8 Mutagenicity assays

The assay was performed using five tester strains TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of S9. Tester strains were exposed to the test compound E-55 via the standard plate incorporation assay originally described by Ames *et al.* (1975) and Maron and Ames (1983). In the plate incorporation methodology the tester strains were exposed to the test compound directly on a minimal glucose agar plate. The components were added to test tube containing molten top agar and poured onto the minimal bottom agar. All doses of the test compound, solvent controls and positive controls were plated in triplicates.

3.9 Plating procedures

The S9 mix and dilutions of the test compound were prepared immediately prior to use.

When S9 was not required (assay without activation), 0.5 mL of phosphate buffer, 50 µL of test or control compound dilution and 100 µL of overnight culture of tester strain (1-2 x 10^8 bacteria per tube) were added to 2 mL of molten selective top agar (maintained at 43-48°C). When S9 was required (assay with metabolic activation) 0.5 mL of S9 mix, 50 µL of test or control compound dilution and 100 µL of tester strain were added to 2.0 mL of molten selective top agar. The contents of test tubes were mixed and poured onto the surface of 20 mL of minimal bottom agar in a 14.2 x 90 mm Petri dishes. When the top agar hardened, the plates were inverted and placed in a 37°C incubator for 48 or 72 h.

3.10 Counting of histidine revertant colonies

After the incubation period the revertant colonies were counted by hand. Condition of the bacterial background lawn was evaluated macroscopically and microscopically for indications of cytotoxicity of the test compound.

4 Data

4.1 Data presentation

For all replicate plates the mean value of revertants per plate and standard deviation were calculated. Results of these calculations are presented in tables in the Data Tables section of this report.

4.2 Assay criteria

The following criteria were used to determine a valid assay:

Number of spontaneous revertants for solvent control cultures was strain characteristic and demonstrates the requirement for histidine. Ranges for mean solvent controls are presented in Table 3.

Table 3. Spontaneous revertant control values.

Strain	Number of revertants	
	Without S-9	With S-9
TA98	8-60	8-60
TA100	60-240	60-240
TA102	100-300	200-400
TA1535	4-45	4-45
TA1537	2-25	2-25

Tester Strain Culture Density. Cell densities of all tester strain cultures were 1-2 x 10^9 bacteria/mL and the ODs of these cultures reached a target value demonstrated to produce cultures with 1-2 x 10^9 bacteria/mL.

Cytotoxicity. A minimum of three non-toxic doses was required to evaluate assay data. Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn. Compounds inhibiting bacteria growth by more than 60% of control level are considered as cytotoxic.

Assay Evaluation Criteria. For a test compound to be considered as positive, it had to produce at least a 2-fold increase in the mean value of revertants per plate of at least one of these tester strains over the mean value of revertants per plate of the appropriate solvent control. This increase in the mean value of revertants per plate

had to be accompanied by a dose response to increasing concentrations of the test compound or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system.

5 Results

5.1 The test compound

The test compound, i.e. E-55 was stored at 4°C in darkness. Dimethylsulfoxide (DMSO) was selected as a solvent. At the concentration of 100 mg/mL, the compound formed a transparent, colorless solution. It remained soluble at all lower concentrations.

5.2 Mutagenicity assays

E-55 was evaluated in the mutagenicity assay in five tester strains i.e. TA98, TA100, TA102, TA1535 and TA1537 at doses 10, 50, 100, 500 and 1000 µg/plate with and without S9 fraction. Results are presented in tables 4 and 5. Data is presented as individual plate counts (table 4) and as a mean value of revertants per plate ± standard deviation (table 5).

All doses of the test compound, as well as concurrent positive and solvent controls were evaluated using three plates per single dose.

Addition of the tested substance to the molten selective top agar did not cause any precipitations.

In all tester strains with and without S9 activation system (beside TA102 and TA1537 tester strains with S9 fraction) at the highest dose (1000 µg/plate) the bacterial growth was inhibited below 60% of control and thus was considered as cytotoxic. Additionally in TA1535 tester strain in the presence and in the absence of S9 exogenous activation system the cytotoxic effect was visible for the dose of 500 µg/plate. In TA102 and TA1537 tester strains S9 with metabolic activation no inhibition of bacteria growth was observed.

For E-55 tested substance normal growth was observed at doses of 10, 50, 100 and 500 µg/plate in TA100, TA98, TA102 and TA1537 tester strains without S9 mix and in TA100, TA98 tester strains with S9 exogenous activation system. Additionally normal growth was visible at doses 10, 50 and 100 µg/plate in TA1535 tester strain in the presence and in the absence of S9 fraction and at all doses of E-55 in TA102 and TA1537 tester strains with S9 fraction.

In TA100 and TA98 tester strains revertant frequencies for 10, 50, 100 and 500 µg/plate of E-in the presence and in the absence of S9 mix approximated those observed in the concurrent solvent control cultures, except the decrease, caused by the cytotoxic effect at the highest dose with and without S9 exogenous activation system. In TA100 tester strain at the dose of 1000 µg/plate in the absence and in the

presence of S9 mix the bacterial growth was inhibited respectively to 40% and 39% of control. In TA98 tester strain at the highest dose of E-55 without and with exogenous activation system the bacterial growth was inhibited respectively to 50% and 47% of control. Additionally under these cytotoxic conditions the thinning of background lawn and presence of pintpoint colonies were observed.

In TA102 and TA1537 tester strains revertant frequencies for doses of 10, 50, 100 and 500 µg/plate of E-55 without S9 fraction and for all doses with S9 activation system approximated those observed in the concurrent solvent control cultures except the decrease, caused by the cytotoxic effect of the highest dose without S9 metabolic activation system. In TA102 and TA1537 tester strains in the absence of S9 mix at the dose of 1000 µg/plate the bacterial growth was inhibited respectively to 43% and 28% of control. Additionally under these conditions the thinning of background lawn and presence of pintpoint colonies were observed.

In TA1535 tester strain revertant frequencies for 10, 50 and 100 µg/plate of E-55 in the presence and in the absence of S9 mix approximated those observed in the concurrent solvent control cultures, except the decrease, caused by the cytotoxic effect at doses of 500 and 1000 µg/plate with and without S9 exogenous activation system. In TA1535 tester strain at doses of 500 and 1000 µg/plate without S9 mix the bacterial growth was inhibited respectively to 44% and 24% of control. In this tester strain in the presence of S9 mix the bacterial growth was inhibited respectively to 54% and 12% of control. Additionally under these cytotoxic conditions the thinning of background lawn and presence of pintpoint colonies were observed.

Obtained results indicate that E-55 tested compound is considered as non-mutagenic because no mutagenicity effect under the conditions and according to the criteria of the test protocol in any of tester strain with and without S9 exogenous metabolic activation system.

All results are presented in tables 4 and 5. Data is presented as individual plate counts (table 4) and as the mean value of revertants per plate ± standard deviation (table 5).

6 Conclusions

E-55 tested compound did not produce any significant increases in the mean value of revertants per plate over the mean value of revertants per plate of the appropriate solvent control in any of tester strains with or without an extract of liver enzymes that simulates mammalian metabolic activity.

The Ames test utilizes an extract of liver enzymes to simulate mammalian metabolic activity. Obtained results may suggest that S9 activation system did not activate a non-mutagenic E-55 chemical to its mutagenic derivatives. These results indicate that tested compound nor its derivatives were positive in the *Salmonella typhimurium* Reverse Mutation Test under the conditions, and according to the criteria, of the test protocol.

The cytotoxic effect was observed in all tester strains with and without S9 exogenous activation system (beside TA102 and TA1537 tester strains with S9 fraction) at the highest dose (1000 µg/plate). Additionally the bacterial growth was inhibited below 60% of control in TA1535 tester strain without S9 exogenous metabolic activation system for the dose 500 µg/plate.

7 References

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Maron D.M., Ames B. (1983) Revised Methods for the Salmonella Mutagenicity Test. Mutat. Res. 113: 173-215.

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Mortelmans K., Zeiger E. (2000) The Ames Salmonella/microsome mutagenicity assay. Mutat. Res. 455: 29-60.

S2(R1) Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use; Version 5.3 (2008), ICH Steering Committee

8 Data tables

Table 4. Mutagenicity Assay Results – Individual Plate Counts for E-55

	Dose /Plate (µg)	Revertants per plate														
		TA100			TA98			TA102			TA1535			TA1537		
Without Metabolic Activation		**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Solvent Control		103	112	141	44	36	42	311	340	275	14	19	17	10	11	11
	10	91	103	119	29	27	36	329	301	274	13	11	17	10	8	13
	50	114	141	132	24	26	32	280	295	313	24	22	18	9	11	7
Test Article	100	112	90	121	33	24	27	319	326	277	12	14	15	8	9	13
	500	103	161	119	19	30	33	371	303	320	7	7	8	11	8	9
	1000	45	51	48	14	22	25	160	108	129	3	3	6	2	2	5
Positive Control		421	478	512	122	137	159	1424	1112	1224	688	704	752	1632	1984	2096
With Metabolic Activation (S9)																
Solvent Control		100	132	122	45	51	54	339	358	295	16	11	14	9	12	7
	10	134	121	132	35	40	33	329	324	374	13	13	15	10	5	9
	50	123	126	116	50	43	53	289	312	281	16	13	14	10	6	7
Test Article	100	154	145	137	43	44	44	305	340	282	9	15	13	14	13	9
	500	96	93	100	44	42	36	330	345	282	8	9	5	8	11	7
	1000	55	37	47	22	28	21	247	237	212	1	2	2	10	7	10
Positive Control		896	954	852	956	1036	988	966	998	1054	216	198	235	201	185	245

[a] TA98 2-Nitrofluorene (1 µg/plate)
[a]TA100 Sodium azide (2 µg/plate)
[a]TA102 Mitomycin C (0.5 µg/plate)
[a]TA1535 Sodium azide (2 µg/plate)
[a]TA1537 ICR 191 Acridine mutagen (50 µg/plate)

[b] TA98 2-Aminoanthracene (2.5 µg/plate)
[b]TA100 2-Aminoanthracene (2.5 µg/plate)
[b]TA102 2-Aminoanthracene (5 µg/plate)
[b]TA1535 2-Aminoanthracene (2.5 µg/plate)
[b]TA1537 2-Aminoanthracene (2.5 µg/plate)

Table 5. Mutagenicity Assay Results – Summary for E-55

	Dose/Plate (µg)	Revertants per plate TA100		TA98		TA102		TA1535		TA1537	
Without Metabolic Activation		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Solvent Control		119	20	41	4	309	33	17	3	11	1
	10	104	14	31	5	301	28	14	3	10	3
	50	129	14	27	4	296	17	21	3	9	2
Test Article	100	108	16	28	5	307	27	14	2	10	3
	500	128	30	27	7	331	35	7	1	9	2
	1000	48	3	20	6	132	26	4	2	3	2
Positive Control		470	46	139	19	1253	158	715	33	1904	242
With Metabolic Activation (S9)											
Solvent Control		118	16	50	5	331	32	14	3	9	3
	10	129	7	36	4	342	28	14	1	8	3
	50	122	5	49	5	294	16	14	2	8	2
Test Article	100	145	9	44	1	309	29	12	3	12	3
	500	96	4	41	4	319	33	7	2	9	2
	1000	46	9	24	4	232	18	2	1	9	2
Positive Control		901	51	993	40	1006	45	216	19	210	31

[a] TA98 2-Nitrofluorene (1 µg/plate)
[a] TA100 Sodium azide (2 µg/plate)
[a] TA102 Mitomycin C (0.5 µg/plate)
[a] TA1535 Sodium azide (2 µg/plate)
[a] TA1537 ICR 191 Acridine mutagen (50 µg/plate)

[b] TA98 2-Aminoanthracene (2.5 µg/plate)
[b] TA100 2-Aminoanthracene (2.5 µg/plate)
[b] TA102 2-Aminoanthracene (5 µg/plate)
[b] TA1535 2-Aminoanthracene (2.5 µg/plate)
[b] TA1537 2-Aminoanthracene (2.5 µg/plate)

9 Data storage

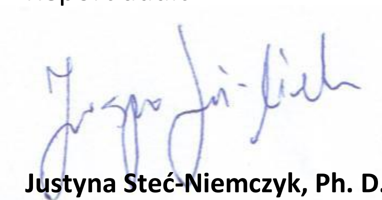
Raw data, all the documents connected with the study and the copy of the original report will be stored at BioCentrum sp. z o. o. for the period of 10 years after the study completion.

The original report is delivered to the study sponsor.

10 Quality assurance

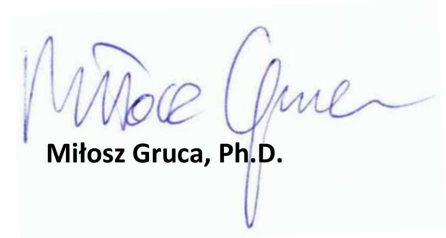
The report was audited:

Report audit Date: **11.04.2011**



Justyna Steć-Niemczyk, Ph. D.

Report was accepted:



Miłosz Gruca, Ph.D.