Bayer CropScience

HPPD W336 PROTEIN

ACUTE TOXICITY BY ORAL GAVAGE

IN FEMALE MICE

DATA REQUIREMENTS
Based on the U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100 adopted in 2002
and on the O.E.C.D. Test Guideline 425 adopted in 2001

REPORT OF STUDY SA 09201
Sponsor identification number: Lynx-PSI N° TX99L088

AUTHOR / STUDY DIRECTOR: J.B. RASCLE

TESTING FACILITY:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:

Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

STUDY COMPLETED ON: NOVEMBER 10, 2009

STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10 (d) (1) (A), (B) or (C).

Company Name:

Company Agent:

Title:

Signature:

Date: _____

These data are the property of Bayer CropScience, and as such, are considered to be confidential for all purposes other than compliance with FIFRA § 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality which may exist under any other statute or in any other country.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study here reported was performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issue, with the exception of the dosing suspensions that were not analyzed for concentration, homogeneity or stability.

- Organization for Economic Cooperation and Development (O.E.C.D.) Principles of Good Laboratory Practice, 1997 (January 26, 1998).

- European Directive 2004/10/EC (February 11, 2004).

- French Decree N°2006-1523, regarding Good Laboratory Practice (December 04, 2006).

- U.S. Environmental Protection Agency (E.P.A.)
  40 CFR part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards: Final Rule, August 17, 1989.

- Good Laboratory Practice Standards for Toxicology studies on Agricultural Chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by: notification 12 Nousan n°8628, December 06, 2000.

Author / Study Director:

Date: November 10, 2009

J.B. RASCLE

Sponsor Representative:

Date: November 10, 2009

A. CAPT

Study Submitter:

Date:

FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by E.P.A.

QUALITY ASSURANCE STATEMENT

The conduct of the study has been subjected to periodic inspections by the Bayer CropScience Sophia Antipolis Quality Assurance Unit. The types and dates of inspections and dates of reporting to Study Director and management are given below:

Type of Q.A. inspection	Study phases inspected	Date of Q.A. inspection	Date of reporting to Study Director	Date of reporting to Management
Study-based	Study plan	August 20, 2009	August 20, 2009	September 04, 2009
Study-based	Body weight treatment	September 02, 2009	September 02, 2009	September 04, 2009
Study-based	Final report	November 03, 2009	November 05, 2009	November 09, 2009

This report has been audited by Quality Assurance personnel in accordance with the appropriate standardized operating methods. The reported results accurately reflect the original data of the study.

Quality Assurance Group Leader:

Date: November 10, 2009

G. ODAGLIA

SIGNATURES

We, the undersigned, hereby declare that the work was performed under our supervision according to the procedures described and that this report provides a correct and faithful record of the results obtained.

There were no circumstances which affected the quality and integrity of the data.

Pathologist:

Date: November 10, 2009

F. SCHORSCH

Author / Study Director:

Date: November 10, 2009

J.B. RASCLE

STUDY PROFESSIONALS

The following professionals were involved in the conduct of this study:

STUDY DIRECTOR	:	J.B. RASCLE
REPLACEMENT STUDY DIRECTOR	:	D. ROUQUIE (From August 31, 2009 to September 01, 2009)
LABORATORY ANIMAL RESOURCES	:	J.P. KOCWIN
TOXICOLOGY SUPERVISOR	:	B. BONNAFOUS
RESPONSIBLE TECHNICIAN	:	M.P. MONIMEAU
PATHOLOGIST	:	F. SCHORSCH
REPORT UNIT ASSISTANT	:	P. ALMERAS

TABLE OF CONTENTS

SUMMARY

The objective of this study was to assess the acute oral toxicity of the HPPD W336 protein (produced in *Escherichia coli*) in OF1 mice. This study was conducted in accordance with the U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100 adopted in 2002 (1) and the O.E.C.D. Test Guideline 420 adopted in 2001(2).

A group of 5 female OF1 mice were administered a single dose of the HPPD W336 protein by oral gavage at the dose level of 2000 mg/kg body weight. A similarly constituted group of 5 female mice received bovine serum albumin (BSA) at the same dose level and acted as a control. The test or reference proteins were administered in two doses of 1000 mg/kg body weight administered within a 4 hours period on the day of treatment. All animals were observed for clinical signs daily for fifteen days whilst their body weights were measured weekly. At termination of the study period, all animals were subjected to a necropsy including macroscopic examination, and the spleen, liver, kidney and brain were weighted. Microscopic examination of the spleen was performed.

There were no mortalities, no clinical signs or treatment-related effects on body weight, body weight gain, organ weights, gross and microscopic examinations.

In conclusion, an acute oral dose of 2000 mg/kg body weight of the HPPD W336 protein did not induce any evidence of systemic toxicity in the OF1 female mouse.

INTRODUCTION

The objective of this study was to assess the potential for acute oral toxicity of HPPD W336 protein in the OF1 mouse.

This study was designed to meet the following guidelines:
The U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100 adopted in 2002 (1) and the O.E.C.D. Test Guideline 420 adopted in 2001(2).

The study protocol and its amendments are presented in Attachment 1.

The study time schedule was as follows:

Study initiation date *	August 18, 2009
Sponsor protocol approval date	August 18, 2009
Animal arrival date	August 19, 2009
Experimental starting date	August 19, 2009
Randomization date (Day -1)	September 01, 2009
Dosing date	September 02, 2009
Final sacrifice date	September 17, 2009
Experimental completion date	October 10, 2009

* Date of protocol approval by Study Director.

MATERIAL AND METHODS

1 - REFERENCE AND TEST ITEMS FORMULATION

The test item HPPD W336 protein (batch number VMLV968, 97±2% purity) and the reference item BSA (batch number VMLV968BSA, 98% purity) were used in this study. Information on the biochemical characterization of the proteins was documented by BioAnalytics (Bayer BioScience NV, Gent, Belgium) and is presented in Attachment 2. The test items were stored in an air-tight, light resistant container at approximately -74°C.
The formulations were prepared by dissolving the reference and the test item proteins in 50 mM Tris solution at pH 7.5 as described in the protocol to produce a suspension at the final concentration of 50 mg/ml. The formulations were prepared shortly prior to dosing, and placed on ice until use in air-tight plastic tubes.

2 - ANIMALS, HOUSING, DIET AND WATER

2.1 Animals

The mouse was chosen because of its recommendation by regulatory authorities as an appropriate test species to assess acute oral toxicity. The Crl:OF1 strain was used since sufficient background toxicity data exist to support interpretation of results. A total of 15 female Crl:OF1 mice were obtained from Charles River Laboratories, Saint Germain sur l'Arbresle, France. Animals were acclimatized to laboratory conditions for 14 days prior to treatment and were 8 weeks old at the start of treatment.

a/Selection and randomization

All animals were examined for mortality and clinical signs during the acclimatization phase. The day before test item administration, all animals were weighed. An automatic randomization procedure (XMS Path/Tox Version 4.2.2) was used to select animals for the study from the middle of the weight range of the available animals, ensuring a similar body weight distribution among groups. Ten female mice were selected for the study. They were within ±20% of the mean body weight at randomization. Selected animals were in a weight range from 20.5 to 22.3 g on the day of treatment. The animals not used in the study were maintained as stock animals within the animal facility.

b/Identification

Following randomization, animals were assigned permanent identification numbers within groups. Each animal was identified by a stainless steel ear tag bearing a unique animal number.

2.2 Housing

Mice were housed individually in suspended, stainless steel, wire-mesh cages. The temperature, humidity and lighting in the animal room were constantly monitored by an automatic system.

The target specifications were:

- temperature: 20°C- 24°C
- humidity: 40-70%
- lighting: 12-hour light, 12-hour dark cycles (7 am - 7 pm)

The ventilation system in the animal room was maintained to ensure adequate ventilation, with the performance of the system regularly checked for a target specification of 10 to 15 air changes per hour.

There were no deviations from target specifications which could have compromised the study. Housing data are placed in the study file.

2.3 Diet and water

Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animals Food and Engineering, Augy, France) and filtered and softened tap water from the municipal water supply were available *ad libitum,* except before treatment and final necropsy when animals were diet fasted overnight. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

3 - EXPERIMENTAL DESIGN

A limit dose level of 2000 mg HPPD W336 protein/kg body weight was selected after discussion with the Sponsor Representative.

This choice was based on the preliminary safety assessment of the test item which led to the conclusion with a high degree of certainty of the lack of harmful effects caused by the HPPD after oral administration to mammals. This conclusion is based on the following reasons:

1- HPPD proteins are ubiquitous in nature across all kingdoms, and are therefore present in food from plant, fungal or animal origin, with good safety records. Indeed, the organism source of the gene, *Pseudomonas fluorescens*, is ubiquitous in the environment, including soil, water and food. It has many beneficial uses in agriculture, human health and bioremediation.

2- HPPD proteins are not known to be toxic to healthy humans and animals.

3- HPPD protein has been reported to be rapidly and completely degraded in human simulated gastric fluids (3, 4). This minimizes the likelihood that the HPPD W336 protein could survive in the human digestive tract and then to be potentially toxic.

The oral route was selected as it is considered as one of the most important portals of entry into the digestive tract. Historically, the oral route of exposure has been used to investigate the toxicity of many novel proteins.

Groups of 5 female mice were given the test or reference protein by oral gavage in two doses, each dose at 1000 mg/kg body weight. Both doses were administered within a 4 hours period on the day of treatment. The test or reference protein were orally administered in solution in storage buffer at a volume of 20 ml/kg (based on body weight on Study Day 1). Therefore, a dose of test or reference protein at 2000 mg/kg body weight was administered to each mouse during the same day of treatment. After the second dosing, food was withheld for approximately a further 3 hours.

Details of group sizes and treatments:

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Clinical signs were recorded daily from Study Day 1 through Study Day 15. They were recorded on regular intervals on Study Day 1 (shortly after each dosing, and on two other occasions) and at least once each day thereafter. The nature, onset, severity, reversibility and duration of all clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose feces. In addition, animals were checked twice daily for mortality, except on weekends and public holidays when they were checked once daily.

4 - BODY WEIGHT

Each animal was weighed at least weekly during the acclimatization period, on the first day of test item administration, then at weekly intervals throughout the treatment period.
Additionally, animals were weighed before scheduled necropsy (Terminal body weight).

5 - POST MORTEM PROCEDURES

At final sacrifice on Study Day 16, all animals were anesthetized by Isoflurane inhalation (Baxter, Maurepas, France), then exsanguinated under deep anesthesia before necropsy.
All animals were necropsied. Necropsy included macroscopic examination of abdominal and thoracic cavities, major organs and tissues. Significant macroscopic abnormalities were recorded.
Brain, liver, spleen and kidney were weighted fresh at scheduled sacrifice only and sampled. These samples were preserved in 10% neutral buffered formalin for possible histological examination.
Spleen only was processed and embedded in paraffin was. Histological slides were prepared for all animals and stained with hematoxylin and esosin. They were submitted to histopathological examination.

6 - CALCULATIONS AND STATISTICAL ANALYSES

6.1 Variables analyzed

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters

6.2 Statistical methods

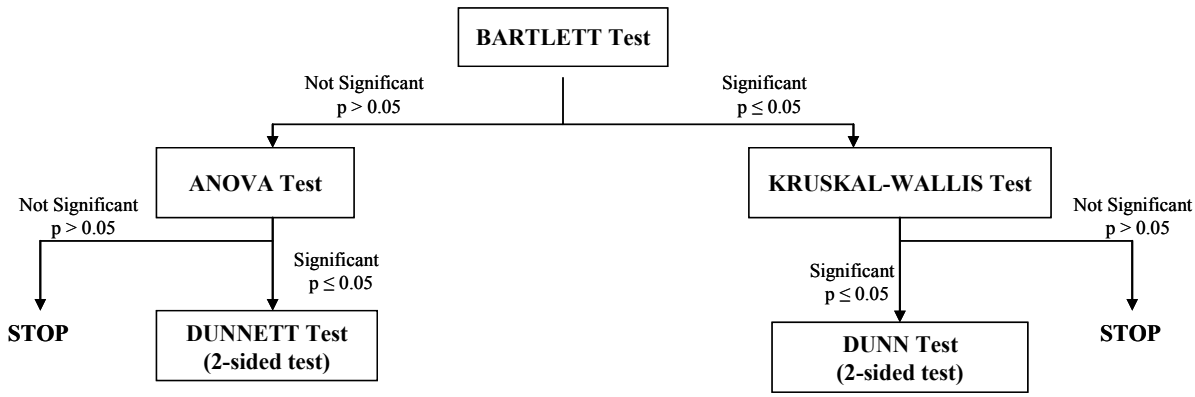
Mean and standard deviation were calculated for each group.

Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

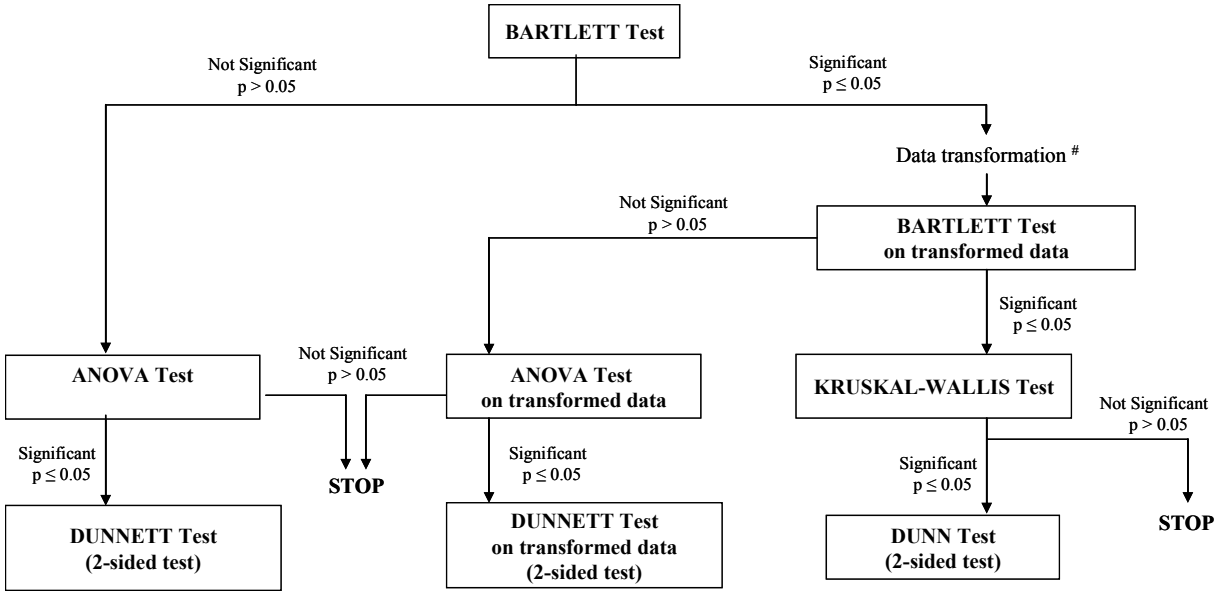
- - Body weight change parameters,
  - Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.



- - Body weight and average food consumption/day parameters

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



Data were transformed using the log transformation for body weight and food consumption parameters.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

7 - DATA STORAGE

All raw data, supporting documents, as well as protocol, protocol amendments and final report are maintained in the document archive room. A test item reference sample is retained in the area of the products storeroom defined for the archiving of test items. All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

RESULTS

1 - MORTALITY (Tab. 1)

There was no mortality during the course of the study.

2 - DAILY OBSERVATIONS (Tab. 1)

No clinical signs were observed in BSA protein- or HPPD W336 protein-treated animals throughout the study period.

3 - BODY WEIGHT (Tab. 2, 3)

Mean body weight parameters were unchanged throughout the study between groups of animals that received the reference protein (BSA) or the HPPD W336 protein.

4 - TERMINAL BODY WEIGHT AND ORGAN WEIGHTS (Tab. 4, App. A, B, C)

There was no terminal body weight difference noted.

Mean absolute and relative spleen weight were statistically significantly lower in the HPPD W336 treated group, when compared to controls. This change was considered not to be treatment-related due to the low magnitude of the change, the high inter-individual variability and the absence of association with any treatment-related gross or microscopic finding.

5 - GROSS PATHOLOGY (Tab. 5, App. D)

At final necropsy, there was no treatment-related macroscopic observation recorded.

6 - MICROSCOPIC PATHOLOGY (Tab. 6, App. D)

The spleen from all animals was submitted to histopathological evaluation.

All findings were consistent with changes commonly noted in mice of this age kept under laboratory conditions and they were not different in the HPPD W336 treated group when compared to the control group.

CONCLUSION

There were no mortalities, no clinical signs or treatment-related effects on body weight in female OF1 mice after an acute oral administration of HPPD W336 protein at 2000 mg/kg body weight. At necropsy, mean terminal body weight were unchanged, no treatment-related findings were noted at the organ weight or the macroscopical examination. In addition, no treatment-related microscopic findings were noted at the histopathological examination.

In conclusion, oral treatment with HPPD W336 protein at 2000 mg/kg body weight did not induce any evidence of systemic toxicity in the OF1 female mouse.

PROTOCOL DEVIATIONS

Number of ordered animals:

Fifteen animals were ordered, instead of fourteen as originally described in the study protocol.

Study personnel:
The study pathologist was not nominated in error in the protocol amendment n°2. F. SCHORSCH performed all the microscopic examinations on the study.

It is the opinion of the Study Director that these deviations did not affect the quality and the integrity of the results of this study.

Author / Study Director:

Date: November 10, 2009

J.B. RASCLE

REFERENCES

GENERAL REFERENCES

1 : U.S. E.P.A. (United States Environmental Protection Agency), 1998. Prevention, Pesticides and Toxic Substances (7101), Health Effects Test Guidelines OPPTS 870.1100, Acute Oral Toxicology, EPA 712-C-98-190. December 2002, 35 pages.

2 : O.E.C.D. (Organization for Economic Co-operation and Development), 2001. O.E.C.D. Guideline for Testing of Chemicals, Test Guideline N°420: Acute Oral Toxicity – Fixed Dose Procedure. Adopted by December 17, 2001. 26 pages.

STUDY REPORT REFERENCE

3 : Goodman, R., Ariyarathna, H., Ofori-Anti, A. 2009. 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein: *In vitro* digestibility study in human simulated gastric fluid (pH 1.2). Study number: REG-2009-Pepsin HPPD 11465. Department of Food Sciences and Technology. University of Nebraska. February 17, 2009. 29 pages. DART number: M-343397-01-1.

4 : Rascle, J.B. 2009. HPPD W336 – *In vitro* digestibility study in human simulated gastric fluid. Bayer CropScience. Study number: SA 09051. September 15, 2009. 59 pages. DART number: M-356196-01-1.

STATISTICAL REFERENCES

5 : Bartlett test, in SOKAL R.R. and ROHLF F.J. (1981): *Biometry*, W.H. Freeman, New York, pp. 403-407.

6 : DUNN O.J. (1964): Multiple comparisons using rank sums, *Technometrics*, Vol. 6, pp. 241-252.

7 : DUNNETT C.W. (1955): A multiple comparison procedure for comparing several treatments with a control, *J. Amer. Statist. Ass*., 50, pp. 1096-1121.

8 : KRUSKAL W.H. and WALLIS W.A. (1952): Use of ranks in one criterion variance analysis, *J. Amer. Statist. Ass*., 47, pp. 583-621.

9 : Transformations (log transformation), in SOKAL R.R. and ROHLF F.J. (1981): *Biometry*, W.H. Freeman, New York, pp 417-428.

ABBREVIATIONS

am	*ante meridiem*
BSA	Bovine Serum Albumin
°C	Degree Celsius
E.E.C.	European Economic Communities
E.P.A.	Environmental Protection Agency
g	Gram
GLP	Good Laboratory Practice
HPPD	p-hydroxyphenylpyruvate dioxygenase
M.A.F.F.	Ministry of Agriculture, Forestry and Fisheries
mg/kg	Milligram/kilogram
ml/kg	Milliliter/kilogram
mM	Millimolar
O.E.C.D.	Organization for Economic Cooperation and Development
%	Percentage
pH	Potential hydrogen
pm	*post meridiem*
QA	Quality Assurance
SDEVS	Standard Deviation
Tab.	Table
US	United States
USA	United States of America
W336	Tryptophan at position 336

TABLES

Tab.1a **Individual clinical signs**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

INDIVIDUAL CLINICAL SIGNS TABLE
Study number: SA 09201
DATES 01-Sep-09 TO 16-Sep-09
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1

Acute Toxicity/Oral LD50

DOSAGE LEVEL IN: mg/kg

ANIMAL	OBSERVATION	DAYS OBSERVED
TT1F3901 2000.0	NORMAL THROUGHOUT INTERVAL	
TT1F3902 2000.0	NORMAL THROUGHOUT INTERVAL	
TT1F3903 2000.0	NORMAL THROUGHOUT INTERVAL	
TT1F3904 2000.0	NORMAL THROUGHOUT INTERVAL	
TT1F3905 2000.0	NORMAL THROUGHOUT INTERVAL	
TT2F3906 2000.0	NORMAL THROUGHOUT INTERVAL	
TT2F3907 2000.0	NORMAL THROUGHOUT INTERVAL	
TT2F3908 2000.0	NORMAL THROUGHOUT INTERVAL	
TT2F3909 2000.0	NORMAL THROUGHOUT INTERVAL	
TT2F3910 2000.0	NORMAL THROUGHOUT INTERVAL	

NOTE: ! = Pre-study phase; " = Dosing phase 1

Tab. 1b **Individual death status**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

Dead Animal Status List for All Animals
Study number: SA 09201
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1
Acute Toxicity/Oral LD50

Animal Number	Grp	Sex	Study Phase	Date and Time Data was Entered	Oper. No.	Date of Death	Phase Day	Death Typ	Death Status	Term. Body Wt. (g)	Ow	Grs
TT1F3901	1	F	Dosing phase 1	17-Sep-09 11:50	84	17-Sep-09	16	s	Final phase sacrifice	22.2	C	C
TT1F3902	1	F	Dosing phase 1	17-Sep-09 11:51	84	17-Sep-09	16	s	Final phase sacrifice	22.6	C	C
TT1F3903	1	F	Dosing phase 1	17-Sep-09 11:51	84	17-Sep-09	16	s	Final phase sacrifice	23.4	C	C
TT1F3904	1	F	Dosing phase 1	17-Sep-09 11:51	84	17-Sep-09	16	s	Final phase sacrifice	22.5	C	C
TT1F3905	1	F	Dosing phase 1	17-Sep-09 11:52	84	17-Sep-09	16	s	Final phase sacrifice	22.4	C	C
TT2F3906	2	F	Dosing phase 1	17-Sep-09 11:52	84	17-Sep-09	16	s	Final phase sacrifice	24.4	C	C
TT2F3907	2	F	Dosing phase 1	17-Sep-09 11:53	84	17-Sep-09	16	s	Final phase sacrifice	22.2	C	C
TT2F3908	2	F	Dosing phase 1	17-Sep-09 11:53	84	17-Sep-09	16	s	Final phase sacrifice	23.1	C	C
TT2F3909	2	F	Dosing phase 1	17-Sep-09 11:53	84	17-Sep-09	16	s	Final phase sacrifice	23.1	C	C
TT2F3910	2	F	Dosing phase 1	17-Sep-09 11:54	84	17-Sep-09	16	s	Final phase sacrifice	21.6	C	C
6690*	-	F	Pre-study phase	01-Sep-09 09:10	27	01-Sep-09	14	u	Study director request	-----	-	-
6692*	-	F	Pre-study phase	01-Sep-09 09:10	27	01-Sep-09	14	u	Study director request	-----	-	-
6693*	-	F	Pre-study phase	01-Sep-09 09:10	27	01-Sep-09	14	u	Study director request	-----	-	-

Note: * = pretest animal no. P = partial data. C = complete data. - = no data.

TABLE 2 - **MEAN AND INDIVIDUAL BODY WEIGHTS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

ANIMAL BODY WEIGHTS IN (G)
Study number: SA 09201
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1
Acute Toxicity/Oral LD50

DOSAGE IN mg/kg / ANIMAL	SEX	DAY OF PHASE 1	DAY OF PHASE 8	DAY OF PHASE 15
		FEMALE ANIMALS		
TT1F3901 2000.0	F	21.1	25.6	26.8
TT1F3902		21.6	25.9	27.8
TT1F3903		22.1	26.6	27.0
TT1F3904		20.5	25.2	27.9
TT1F3905		21.5	25.1	26.3
	(n)	5	5	5
	MEANS	21.4	25.7	27.2
	SDEVS	0.6	0.6	0.7
TT2F3906 2000.0	F	20.7	26.1	28.6
TT2F3907		20.8	28.0	26.2
TT2F3908		21.9	25.9	27.8
TT2F3909		22.3	26.2	27.3
TT2F3910		21.2	24.0	26.4
	(n)	5	5	5
	MEANS	21.4	26.0	27.3
	SDEVS	0.7	1.4	1.0

NOTE: DATA FOR Dosing phase 1

TABLE 3 - **MEAN AND INDIVIDUAL ABSOLUTE WEIGHT GAINS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

ANIMAL ABSOLUTE WEIGHT GAINS IN (G)
Study number: SA 09201
ABSOLUTE WEIGHT GAINS REFERENCED TO Dosing phase 1 (DAY 1)
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1
Acute Toxicity/Oral LD50

DOSAGE IN mg/kg / ANIMAL	SEX	DAY OF PHASE 8	DAY OF PHASE 15
		FEMALE ANIMALS	
TT1F3901 2000.0	F	4.5	5.7
TT1F3902		4.3	6.2
TT1F3903		4.5	4.9
TT1F3904		4.7	7.4
TT1F3905		3.6	4.8
	(n)	5	5
	MEANS	4.3	5.8
	SDEVS	0.4	1.1
TT2F3906 2000.0	F	5.4	7.9
TT2F3907		7.2	5.4
TT2F3908		4.0	5.9
TT2F3909		3.9	5.0
TT2F3910		2.8	5.2
	(n)	5	5
	MEANS	4.7	5.9
	SDEVS	1.7	1.2

NOTE: DATA FOR Dosing phase 1

TABLE 4 - **MEAN TERMINAL BODY WEIGHT AND MEAN ABSOLUTE AND RELATIVE ORGAN WEIGHTS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience | Study number: SA 09201 | Printed: 16-Oct-09
Center of Toxicology | Page: 1
Sophia-Antipolis | Acute toxicity by oral gavage in mice
Mouse
F e m a l e s MEAN ORGAN WEIGHTS (g), ORGAN/TERMINAL BODY WEIGHT (%) AND ORGAN/BRAIN WEIGHT (%) RATIOS

	TBW (g)a	BRAIN (g)	(g)a	BRAIN (%)a	(%)b	LIVER (g)a	(%)a	(%)a
GROUP 1 BSA 2000.0 mg/kg								
(N)	5	5	5	5	5	5	5	5
Mean	22.6	0.44	0.44	1.938	100.000	0.98	4.346	225.306
Std dev	0.5	0.02	0.02	0.116	0.000	0.09	0.322	26.307
GROUP 2 HPPD W336 2000.0 mg/kg								
(N)	5	5	5	5	5	5	5	5
Mean	22.9	0.42	0.42	1.838	100.000	1.00	4.363	238.108
Std dev	1.1	0.02	0.02	0.097	0.000	0.08	0.321	25.277

a Ftst;NSg-05/T-test;
b Mann-Whitney U-Test

F e m a l e s MEAN ORGAN WEIGHTS (g), ORGAN/TERMINAL BODY WEIGHT (%) AND ORGAN/BRAIN WEIGHT (%) RATIOS

	TBW (g)a	BRAIN (g)	(g)a	SPLEEN (%)a	(%)a	(g)a	KIDNEY(S) (%)a	(%)a
GROUP 1 BSA 2000.0 mg/kg								
(N)	5	5	5	5	5	5	5	5
Mean	22.6	0.44	0.094	0.4172	21.6116	0.32	1.414	73.164
Std dev	0.5	0.02	0.006	0.0201	1.9502	0.03	0.119	6.929
GROUP 2 HPPD W336 2000.0 mg/kg								
(N)	5	5	5	5	5	5	5	5
Mean	22.9	0.42	0.074*	0.3248+	17.7012*	0.32	1.390	75.657
Std dev	1.1	0.02	0.014	0.0568	3.2570	0.04	0.169	8.716

\+ The group mean is significantly different from the control at P < 0.01
* The group mean is significantly different from the control at P < 0.05
a Ftst;NSg-05/T-test;

TABLE 5 - **SUMMARY TABLE OF GROSS PATHOLOGY FINDINGS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Note: In the following table, the first column of data corresponds to the control group and the second column corresponds to the HPPD W336 treated group.

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

Incidence Summary Report for Gross Observations
Study number: SA 09201
All Sacrifices
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1
Acute Toxicity/Oral LD50

Controls from group(s): 1	Animal sex:	F E M A L E S
Dosing units: mg/kg	Group dosage level:	2000
	Number in group:	5

	2000
	5
LIVER	
Enlarged	1
GENERAL COMMENT	
All organs, no abnormality	4

	1
	1
	4

TABLE 6 - **SUMMARY TABLE OF MICROSCOPIC PATHOLOGY FINDINGS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Note: In the following table, the first column of data corresponds to the control group and the second column corresponds to the HPPD W336 treated group.

Bayer CropScience | Incidence summary of microscopic pathology findings
Center of Toxicology | SA 09201 | Printed :10-Oct-09
Sophia-Antipolis | | Page :1
Mouse/OF 1 | P.T.S.4.2.2 | Acute Toxicity/Oral LD50

		-- Animals Affected --
Controls from group(s): 1	Animal sex:	-- Females --
Dosing units: mg/kg	Group dosage level:	2000 2000
Tissues With Diagnoses	No. in group:	5 5
SPLEEN .....Number examined:		5 5
	Unremarkable:	0 0
Extramedullary erythropoiesis: diffuse		5 5
Intramacrophagic brown pigment		5 5

All Diagnoses; Phases: All; Death types: All; Date of death range: 01-Sep-09 To 17-Sep-09

APPENDICES

APPENDIX A - **INDIVIDUAL ABSOLUTE ORGAN WEIGHTS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

Individual Absolute Organ Weights (g)
Study number: SA 09201
All Sacrifices
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1

Acute Toxicity/Oral LD50

Animal No/sex	Dose mg/kg	Terminal Body wt. (g)	BRAIN	LIVER	SPLEEN	KIDNEY(S)
				F e m a l e A n i m a l s		
TT1F3901/F	2000.0	22.2	0.46	0.88	0.096	0.29
TT1F3902/F	2000.0	22.6	0.46	1.04	0.089	0.35
TT1F3903/F	2000.0	23.4	0.42	1.11	0.102	0.33
TT1F3904/F	2000.0	22.5	0.42	0.96	0.096	0.29
TT1F3905/F	2000.0	22.4	0.43	0.93	0.089	0.34
Number of observ. :		(5)	(5)	(5)	(5)	(5)
TT2F3906/F	2000.0	24.4	0.44	1.02	0.092	0.35
TT2F3907/F	2000.0	22.2	0.43	0.98	0.066	0.36
TT2F3908/F	2000.0	23.1	0.40	1.13	0.082	0.33
TT2F3909/F	2000.0	23.1	0.41	0.94	0.055	0.27
TT2F3910/F	2000.0	21.6	0.42	0.92	0.077	0.28
Number of observ. :		(5)	(5)	(5)	(5)	(5)

APPENDIX B - **INDIVIDUAL ORGAN TO BODY WEIGHT RATIOS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

Individual organ to Body Weight ratios (%)
Study number: SA 09201
All Sacrifices
Study start date: 02-Sep-09

Printed: 16-Oct-09
Page: 1
Acute Toxicity/Oral LD50

Animal No/sex	Dose mg/kg	Terminal Body wt. (g)	BRAIN	LIVER	SPLEEN	KIDNEY(S)
				F e m a l e	A n i m a l s	
TT1F3901/F	2000.0	22.2	2.07	3.96	0.432	1.31
TT1F3902/F	2000.0	22.6	2.04	4.60	0.394	1.55
TT1F3903/F	2000.0	23.4	1.79	4.74	0.436	1.41
TT1F3904/F	2000.0	22.5	1.87	4.27	0.427	1.29
TT1F3905/F	2000.0	22.4	1.92	4.15	0.397	1.52
Number of observ. :		(5)	(5)	(5)	(5)	(5)
TT2F3906/F	2000.0	24.4	1.80	4.18	0.377	1.43
TT2F3907/F	2000.0	22.2	1.94	4.41	0.297	1.62
TT2F3908/F	2000.0	23.1	1.73	4.89	0.355	1.43
TT2F3909/F	2000.0	23.1	1.77	4.07	0.238	1.17
TT2F3910/F	2000.0	21.6	1.94	4.26	0.356	1.30
Number of observ. :		(5)	(5)	(5)	(5)	(5)

APPENDIX C - **INDIVIDUAL ORGAN TO BRAIN WEIGHT RATIOS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

Individual organ to Brain Weight ratios (%)
Study number: SA 09201
All Sacrifices
Study start date: 02-Sep-09

Animal No/sex	Dose mg/kg	Terminal Body wt. (g)	BRAIN	LIVER	SPLEEN	KIDNEY(S)
				F e m a l e	A n i m a l s	
TT1F3901/F	2000.0	22.2	100.00	191.30	20.870	63.04
TT1F3902/F	2000.0	22.6	100.00	226.09	19.348	76.09
TT1F3903/F	2000.0	23.4	100.00	264.29	24.286	78.57
TT1F3904/F	2000.0	22.5	100.00	228.57	22.857	69.05
TT1F3905/F	2000.0	22.4	100.00	216.28	20.698	79.07
Number of observ. :		(5)	(5)	(5)	(5)	(5)
TT2F3906/F	2000.0	24.4	100.00	231.82	20.909	79.55
TT2F3907/F	2000.0	22.2	100.00	227.91	15.349	83.72
TT2F3908/F	2000.0	23.1	100.00	282.50	20.500	82.50
TT2F3909/F	2000.0	23.1	100.00	229.27	13.415	65.85
TT2F3910/F	2000.0	21.6	100.00	219.05	18.333	66.67
Number of observ. :		(5)	(5)	(5)	(5)	(5)

APPENDIX D - **INDIVIDUAL GROSS AND MICROSCOPIC PATHOLOGY FINDINGS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

ANIMAL: TT1F3901 SEX: Female GROUP: 1 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 22.2

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Moderate. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT1F3902 SEX: Female GROUP: 1 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 22.6

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Moderate. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT1F3903 SEX: Female GROUP: 1 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 23.4

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
LIVER	Enlarged, Minimal

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Slight. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT1F3904 SEX: Female GROUP: 1 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 22.5

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Slight. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT1F3905 SEX: Female GROUP: 1 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 22.4

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Slight. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT2F3906 SEX: Female GROUP: 2 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 24.4

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Moderate. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT2F3907 SEX: Female GROUP: 2 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 22.2

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Slight. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT2F3908 SEX: Female GROUP: 2 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 23.1

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
LIVER	Enlarged, Minimal

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Moderate. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT2F3909 SEX: Female GROUP: 2 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 23.1

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Slight. Intramacrophagic brown pigment, Minimal.

ANIMAL: TT2F3910 SEX: Female GROUP: 2 DOSE LEVEL: 2000.0 mg/kg
DAY/WEEK OF DEATH:16/3 Dosing phase 1 STATUS: Final phase sacrifice TERMINAL BODY WEIGHT (G): 21.6

<< G R O S S O B S E R V A T I O N S >>

TISSUE	GROSS OBSERVATIONS / COMMENTS
GENERAL COMMENT	All organs, no abnormality

<< P A T H O L O G Y O B S E R V A T I O N S >>

TISSUE	HISTOPATHOLOGIC DIAGNOSES / SPECIAL HISTOLOGICAL COMMENTS
SPLEEN	Required tissue. Extramedullary erythropoiesis: diffuse, Moderate. Intramacrophagic brown pigment, Minimal.

ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL AND AMENDMENTS**

HPPD W336 PROTEIN
ACUTE TOXICITY BY ORAL GAVAGE IN FEMALE MICE

TESTING FACILITY:
Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:
Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

1 GENERAL

1.1 PURPOSE OF STUDY

The objective of this study is to investigate the acute toxicity of the single mutated p-hydroxyphenyl pyruvate dioxygenase (HPPD) protein (HPPD W336, produced in *Escherichia coli*) after an oral administration in female mice.

1.2 GOOD LABORATORY PRACTICE COMPLIANCE

This study will be performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issues with the exception of the test item solution, which will not be analyzed for concentration, homogeneity and stability:

- Organization for Economic Cooperation and Development (O.E.C.D.) Principles of Good Laboratory Practice, 1997 (January 26, 1998).
- European directive 2004/10/EC (February 11, 2004).
- U.S. Environmental Protection Agency (E.P.A.)
  40 CFR Part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards : Final Rule, August 17, 1989.
- Good Laboratory Practice Standards for Toxicology studies on Agricultural chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by: 12 Nousan N°8628, December 06, 2000.
- French Decree N°2006-1523, regarding Good Laboratory Practice (December 04, 2006).

1.3 REGULATORY GUIDELINES

This study is based on the U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100, adopted in 2002 (1) and on the O.E.C.D. Test Guideline 420, adopted in 2001 (2).

1.4 QUALITY ASSURANCE

The Quality Assurance Unit of Bayer CropScience, 355 rue Dostoïevski, BP 153, 06903 Sophia-Antipolis Cedex, France, will undertake and document inspections while the study is in progress and will audit the study report.

2 STUDY PERSONNEL

2.1 STUDY DIRECTOR:

Date: August 18, 2009 J.B. RASCLE

2.2 SPONSOR REPRESENTATIVE:

Date: August 18, 2009 A. CAPT

2.3 OTHER STUDY PERSONNEL

Responsibility		Name
Replacement Study Director	:	D. ROUQUIE
In-life Supervisor	:	B. BONNAFOUS
Responsible Technician	:	M.P. MONIMEAU

Other study personnel will be identified as appropriate in the study file.

3 PROPOSED DATES

Arrival of animals	:	August 19, 2009
Experimental starting date	:	August 19, 2009
Randomization	:	September 01, 2009
Date of treatment	:	September 02, 2009
Final sacrifice date	:	September 17, 2009
Experimental completion date	:	September 17, 2009 (estimated)

4 OVERVIEW OF STUDY DESIGN

Five female mice will be treated by oral gavage at the limit dose level of 2000 mg/kg body weight (2 doses of 1000 mg/kg within 4 hours on Study Day 1) in order to identify any potential acute toxic effects of the HPPD W336 protein. A similarly constituted group of 5 female mice will receive the bovine serum albumin (BSA) at 2000 mg/kg body weight. BSA is known to be innocuous at this dose (control group).

Animals will be observed for clinical signs daily for 15 days. Body weight will be recorded at weekly intervals and on the day of necropsy. All mice, including those found dead or killed for humane reasons during the study, will be subjected to macroscopic observations and, when deemed appropriate by the Study Director or the Sponsor Representative, tissues will be retained for possible microscopic examinations. At final necropsy, brain, liver, spleen and kidney will be weighed fresh and retained for possible microscopic examinations.

5 TEST SYSTEM

5.1 SPECIES SPECIFICATIONS

5.1.1 Species and strain

Species: Mouse

Sex: Female

Strain: Crl:OF1

Estimated body weight at study start: 18-30 g

5.1.2 Animal supplier

Charles River France Laboratories (Saint Germain sur l'Arbresle, France).

5.1.3 Reason for selection of species and gender

The mouse has been chosen because of its acceptance by Regulatory Authorities as a test species to assess acute toxicity.

The OF1 strain has been used extensively in toxicity evaluation studies, hence sufficient background data exist to support interpretation of results.

Based on the HPPD W336 protein properties (i.e., good certainty of no harm in males and females- see chapter 7.1), only one gender was used for ethical reasons in order to reduce the number of animals tested. The O.E.C.D. Test Guideline 420 (2) normally recommends to used females. Indeed, literature surveys of conventional LD50 tests show that usually there is little difference in sensitivity between the genders. In addition, when differences are observed, females are generally slightly more sensitive.

5.1.4 Age range and total number

Fourteen female mice will be ordered. Animals will be approximately 8 weeks of age at the date of exposure to the test substance.

5.1.5 Acclimatization phase and randomization

The duration of the acclimatization phase will be at least 14 days.

Animals will be checked twice daily for moribundity and mortality except on weekends and public holidays when they will be checked once daily.

All animals will be weighed at least weekly, and subjected to detailed physical examination on the day of randomization.

The acceptable body weight range will be ±20% of the mean body weight on the day of randomization.

Any animal deemed unsuitable for the study based on weight or clinical signs will not be used in the study.

From the remaining animals, five mice will be allocated to the dosage group by using a computerized randomization procedure that ensures a similar body weight distribution within this group.

5.1.6 Identification

At the time of randomization each animal will be identified by a stainless steel ear tag bearing a unique animal number.

5.2 DIET INFORMATION

5.2.1 Food

Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animals Food and Engineering, Augy, France) will be available *ad libitum*.

Food will be stored in an identified room controlled for temperature and humidity. Diet will be used only until date of expiry.

5.2.2 Water

Filtered and softened tap water from the municipal water supply will be provided *ad libitum* using automatic watering system. Filters servicing the watering system are changed regularly and sterilization of the system will be performed if considered necessary.

5.2.3 Analyses

Analytical data will be provided by the manufacturer for each batch of diet including the size of pellets and concentration of key nutritional components, selected health compromising compounds and microorganisms. Batches of diet will be only released for use after confirmation they meet specifications.

Certificates of water analysis will be provided by the "Laboratoire de l'Environnement Nice Côte d'Azur" (France) and "Institut Scientifique d'Hygiène et d'Analyse" (France).

5.2.4 Records

Records of certificates of food and water analyses will be retained in the archives.

5.3 ENVIRONMENTAL CONDITIONS

5.3.1 Room

Animal room number: L8

The animal room is within a barrier maintained unit with restricted entry.

5.3.2 Housing

Animals will be housed individually in suspended, stainless steel, wire mesh cages. The cage of each animal will be identified by a card bearing a unique identification number.

5.3.3 General environment

Temperature, humidity and ventilation:
Laboratory conditions will be controlled to ideally achieve a temperature of 20°C - 24°C and a relative humidity of 40% - 70% with a target of 10 to 15 air changes per hour.

Lighting:
12-hour light/dark cycles will be provided by automatically controlled fluorescent-tube lighting (7am - 7pm).

Monitoring:
The temperature, humidity and lighting in the animal room are constantly monitored by an automatic system. The ventilation system in the animal room is maintained to ensure adequate ventilation, with the performance of the system regularly checked. Records of all deviations from specifications will be placed in the study file.

6 TEST ITEMS

6.1 TEST ITEMS CHARACTERISTICS

6.1.1 Identification

Test item:

Para-hydroxyphenylpyruvate dioxygenase (HPPD) from *Pseudomonas fluorescens*, carries a single glycine (G) to tryptophan (W) amino acid substitution at position 336 of the native enzyme, resulting in the HPPD W336 protein.

Test item	:	HPPD W336 (produced in *Escherichia coli*)
Batch number	:	VMLV968
Purity	:	97±2%
Storage	:	Approximately –70 °C
Certified through	:	January 27, 2010

The test item will be supplied by BioAnalytics (Bayer BioScience NV, Gent, Belgium).

Reference item:

Reference item bovine serum albumin (BSA) will be supplied by BioAnalytics (Bayer BioScience NV, Gent, Belgium).

Reference item	:	Bovine serum albumin (BSA)
Batch number	:	Will be provided in the final report
Purity	:	Will be provided in the final report
Storage	:	Approximately –70 °C
Certified through	:	Will be provided in the final report

6.1.2 Activity

Expression of HPPD W336 confers to the modified plant herbicide tolerance properties.

6.1.3 Storage

The test and reference items will be stored frozen in an air-tight, light-resistant container at approximately -70°C or according to the conditions described in the test substance specifications when available. The storage and stability informations will be defined in the final report for the reference and test items.

6.1.4 Safety handling and requirements

Information on the appropriate safety precautions when handling the test substance will be given by the supplier or the Sponsor Representative.

In the absence of information on the potential toxic effects of the test substance, safety precautions will be applied according to the relevant standard operating procedures.

6.1.5 Analyses

The confirmation of the identity, purity of the control and the test proteins will be provided by the Sponsor Representative. The certificate of analysis of the test and reference proteins will be placed in the final study report.

6.2 REFERENCE AND TEST ITEMS FORMULATION

6.2.1 Preparation, shipment and storage conditions

The reference and test items will be received as lyophilized powders. These lyophilized samples will be shipped under frozen conditions and will be stored at -74 +10°C (Storage condition at Sophia-Antipolis according to SOP MTR00962).

On the day of dosing, both test and reference proteins will be resuspended in 50 mM Tris solution (at pH 7.5) at the final concentration of 50 mg/ml.

The formulated test and reference items will be kept on ice until use.

The unused residue of the formulations will be stored at -20°C or colder at the end of the administration period.

6.2.2 Analyses

Stability of the test protein will be analyzed by BioAnalytics (Bayer BioScience NV, Gent, Belgium) at the same concentration and in the same buffer than in the present study, for a period that covers the duration of the treatment. Concentration of the test and reference proteins will not be analyzed. However, the amount of lyophilized proteins in the tubes is known, and final concentration is given by the volume of buffer in which the powder is resuspended. The homogeneity of the formulations for the reference and the test item will be checked by a visual inspection.

7 ROUTE OF ADMINISTRATION AND TREATMENT GROUPS

7.1 CHOICE OF DOSES

A limit dose level of 2000 mg HPPD W336 protein/kg body weight was selected after discussion with the Sponsor Representative.

This choice was based on the preliminary safety assessment of the test substance which led to the conclusion with a high degree of certainty of the lack of harmful effects caused by the HPPD after oral administration to mammals. This conclusion is based on the following reasons:

1- HPPD proteins are ubiquitous in nature across all kingdoms, and are therefore present in food from plant, fungal or animal origin, with good safety records. Indeed, the organism source of the gene, *Pseudomonas fluorescens*, is ubiquitous in the environment, including soil, water and food. It has many beneficial uses in agriculture, human health and bioremediation.

2- HPPD proteins are not known to be toxic to healthy humans and animals.

3- A highly similar HPPD protein has been reported to be rapidly and completely degraded in human simulated gastric fluids (3). This minimizes the likelihood that the HPPD W336 protein could survive in the human digestive tract and then to be potentially toxic.

7.2 CHOICE OF ROUTE OF ADMINISTRATION

HPPD W336 protein will be potentially present in food and feed and the oral route is one of the most important portals of entry of the digestive tract.

7.3 NUMBER OF ANIMALS

Five female mice will be administered with the test protein. Five additional females will be administered with the reference protein (control group).

7.4 CONDITIONS OF ADMINISTRATION

All animals used in the study will be diet fasted overnight prior to dosing. Due to the limitation of the solubility of the test material in 50 mM Tris pH 7.5, the groups of 5 female mice will be given the test protein or the reference protein by oral gavage in two doses, each dose at 1000 mg/kg body weight. Both doses will be administered within a 4 hours period on the day of treatment. The test or reference proteins will be administered at a volume of 20 ml/kg (based on body weight on Day 1). After dosing, food will be withheld for approximately a further 3 hours.

7.5 DOSES

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Reference item: BSA	2000	5	TT1F3901 to 3905
2	Test item: HPPD W336	2000	5	TT2F3906 to 3910

7.6 ANIMAL WELFARE

During the study, the care and use of animals will be conducted in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, n°86/609/CEE du 24 Novembre 1986".

8 LABORATORY DETERMINATIONS AND SCHEDULES

All animal data will be recorded using a dedicated computer system (Path/Tox System version 4.2.2, protocol number 0962).

8.1 CLINICAL EXAMINATION

8.1.1 Clinical signs and mortality

Animals will be observed for clinical signs individually starting on Study Day 1, at least once during the first 30 minutes after each dosing, periodically during the first 24 hours post-dosing, and every day thereafter through Study Day 15. Additional observations will be necessary if the animals continue to display signs of toxicity. The nature, onset, severity, reversibility and duration of clinical signs will be recorded.

Throughout the study, animals will be checked twice daily for moribundity and mortality (once daily except on weekends and public holidays). Any animal suffering from severe distress, in a moribund condition or considered unlikely to survive will be humanely killed, and will be considered in the interpretation of the test results in the same way as animals that died on test.

8.1.2 Body weight

Body weights will be measured on Study Day 1 (shortly before administration), and at least weekly thereafter. Additionally, animals will be weighed at final sacrifice or when killed for humane reasons

8.2 POST MORTEM EXAMINATION

8.2.1 Necropsy procedures

Animals found dead:

Any animal found dead during the study will be necropsied as soon as possible but within 24 hours of the time of discovery. If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen).

Scheduled sacrifice and moribund animals:

Animals surviving to the end of the study and animals sent to necropsy for humane reasons will be deeply anesthetized by Isoflurane (Baxter, Maurepas, France) inhalation, then exsanguinated before necropsy. All animals will be diet fasted prior to scheduled sacrifices.

8.2.2 Necropsy

The necropsy of animals will include the macroscopic examination of the external surface, all orifices and all major body cavities and organs.

Significant macroscopic findings will be recorded. Tissues may be sampled at the discretion of the Study Director or the Sponsor Representative.

In addition, brain, liver, spleen and kidney will be weighed fresh at scheduled sacrifice only and collected. These samples will be preserved in 10% neutral buffered formalin for possible future microscopic examination.

8.2.3 Microscopic evaluation

Microscopic examination of organs showing evidence of gross pathology in animals may be performed at the discretion of the Study Director or the Sponsor Representative.

9 CALCULATIONS

For body weights, group means and standard deviations will be calculated for animals surviving more than 1 day.

10 REPORTING

10.1 INTERIM REPORTS

Any unexpected findings during the course of the study will be reported to the Sponsor Representative.

10.2 FINAL REPORT

A copy of the draft report will be submitted to the Sponsor Representative and the Quality Assurance Unit for review. With the exception of the dated signature of scientists and other professional personnel, the draft report will contain all information and data to be included into the final report. The final report will include the information and data required by the referenced guidance documents (1, 2).

11 ARCHIVING

All raw data, supporting documents, as well as protocol, protocol amendments, protocol deviations and the final report will be maintained in the archive room. An aliquot of the test substance solution will be kept in the area of the products storeroom defined for the archiving of test substances.

All of the above will be saved for at least ten years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

12 REFERENCES

12.1 GENERAL REFERENCES

1 - U.S. E.P.A. (United States Environmental Protection Agency), 1998. Prevention, Pesticides and Toxic Substances (7101), Health Effects Test Guidelines OPPTS 870.1100, Acute Oral Toxicology, EPA 712-C-98-190. December 2002, 35 pages.

2 - O.E.C.D. (Organization for Economic Co-operation and Development), 2001. O.E.C.D. Guideline for Testing of Chemicals, Test Guideline N°420: Acute Oral Toxicity – Fixed Dose Procedure. Adopted by December 17, 2001. 26 pages.

12.2 STUDY REPORT REFERENCE

3 - Goodman, R., Ariyarathna, H., Ofori-Anti, A. 2009. 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein: *In vitro* digestibility study in human simulated gastric fluid (pH 1.2). Study number: REG-2009-Pepsin HPPD 11465. Department of Food Sciences and Technology. University of Nebraska. February 17, 2009. 29 pages. DART number: M-343397-01-1.

PROTOCOL AMENDMENT

Protocol SA 09201

HPPD W336 PROTEIN
ACUTE TOXICITY BY ORAL GAVAGE IN FEMALE MICE

Protocol amendment: N°1

Reason 1: **Change of the Study Director**
David Rouquié replaces Jean Baptiste Rascle from August 31, 2009 to September 01, 2009 included.

Reason 2: **Change in the randomization**
Three animals were used in an another test consequently they will be excluded from the study and from the randomization.

Study Director:

Date: August 31, 2009

D. ROUQUIE

PROTOCOL AMENDMENT

Protocol SA 09201

HPPD W336 PROTEIN
ACUTE TOXICITY BY ORAL GAVAGE IN FEMALE MICE

Protocol amendment: N°2

Reason: **Histological examination for the spleen**

At the request of the Sponsor Representative, histopathology will be conducted on the spleen in all animals.

The fixed tissue will be processed and embedded in paraffin wax. Histological slides will be prepared for all animals and stained with hematoxylin and eosin.

Study Director:

Date: October 06, 2009 J.B. RASCLE

PROTOCOL AMENDMENT

Protocol SA 09201

HPPD W336 PROTEIN
ACUTE TOXICITY BY ORAL GAVAGE IN FEMALE MICE

Protocol amendment: N°3

Reason: Additional Statistical analysis

The following statistical analysis will be performed on the study as described below:

VARIABLES ANALYZED

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters

STATISTICAL METHODS

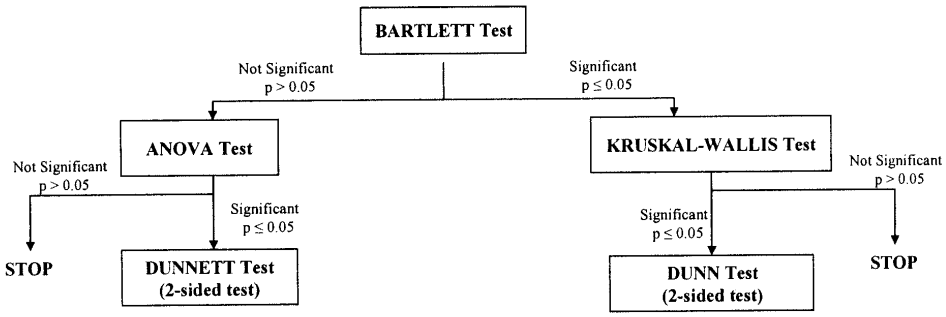
Mean and standard deviation will be calculated for each group.

Group means will be compared at the 5% and 1% levels of significance.

Statistical analyses will be carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

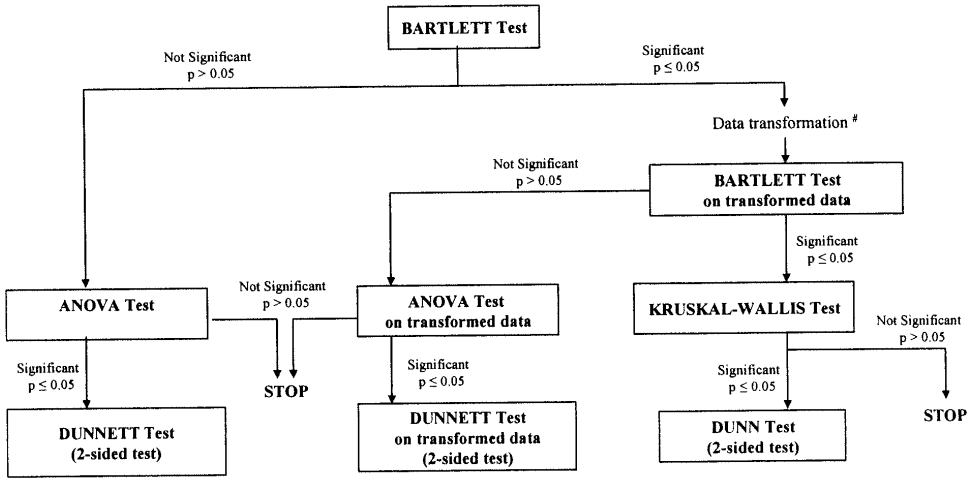
- - Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation will be calculated for each group and per time period for body weight change parameters.



- - Body weight and average food consumption/day parameters

Mean and standard deviation will be calculated for each group and per time period for average food consumption/day parameters.



Data will be transformed using the log transformation for body weight and food consumption parameters.

If one or more group variance(s) equal 0, means will be compared using non-parametric procedures.

STATISTICAL REFERENCES

Bartlett test, in SOKAL R.R. and ROHLF F.J. (1981): Biometry, W.H. Freeman, New York, pp. 403-407.

DUNN O.J. (1964): Multiple comparisons using rank sums, *Technometrics*, Vol. 6, pp. 241-252.

DUNNETT C.W. (1955): A multiple comparison procedure for comparing several treatments with a control, *J. Amer. Statist. Ass.*, 50, pp. 1096-1121.

KRUSKAL W.H. and WALLIS W.A. (1952): Use of ranks in one criterion variance analysis, *J. Amer. Statist. Ass.*, 47, pp. 583-621.

Transformations (log transformation), in SOKAL R.R. and ROHLF F.J. (1981): Biometry, W.H. Freeman, New York, pp 417-428

Study Director:

Date: November 03, 2009

J.B. RASCLE

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Bayer CropScience

Report N°: **BBS09-008**

Page: 1 (19)

Title

Certificate of analysis for the HPPD W336 protein produced in *E.coli* batch n°VMLV968

Authors

Veerle Habex
Ann Wierckx

Completed on

August 7 th, 2009

Testing Facility

BioAnalytics
Molecular Characterization
Bayer BioScience N.V.
Technologiepark 38
B-9052 Ghent
Belgium

Study number

BBS09-008

Bayer CropScience

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

This report is confidential. No part of the report or any information contained herein may be disclosed to any third party without the written prior authorisation of Bayer BioScience N.V.

Bayer CropScience

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The undersigned hereby declares that the work to which this report refers was performed according to the procedures herein described and this report provides an accurate record of the results obtained. The study was conducted in accordance with the Good Laboratory Practice Standards as specified in the OECD/EU principles of Good Laboratory Practice.

Study Director

Habex

Veerle Habex
Molecular Characterization
BioAnalytics

7/8/09
Date

STUDY IDENTIFICATION PAGE

Study Initiation date:	July 23th, 2009
Experimental start date:	July 29th, 2009
Experimental Termination date:	July 31th, 2009
Study Completion date:	August 7th, 2009
Test Facility Address:	Bayer BioScience N.V. BioAnalytics GLP Test Facility Technologiepark 38 9052 Ghent – Belgium Tel: +32 9-243 04 11 Fax: +32 9-224 06 94
Test Facility Manager:	Jean-Marc Ferullo Address see Test Facility Tel: +32 9-243 04 22 Fax: +32 9-224 06 94 e-mail: GLP_TFM@bayercropscience.com
Study Director:	Veerle Habex Address see Test Facility Tel: +32 9-243 05 84 Fax: +32 9-224 06 94 e-mail: veerle.habex@bayercropscience.com
Study Director in Training and Study Personnel:	Ann Wierckx Address see Test Facility Tel: +32 9-243 04 48 Fax: +32 9-224 06 94 e-mail: ann.wierckx@bayercropscience.com
Study Personnel:	Ine Criel Address see Test Facility
Sponsor Representative:	Donna Mitten Global Regulatory Affairs Manager Regulatory Affairs Bayer CropScience LP 2 T.W. Alexander Drive NC27709 Research Triangle Park – United States Tel: +1 530 277 0482 Fax: +1 919 549 3929 e-mail: donna.mitten@bayercropscience.com

Bayer CropScience

Report **BBS09-008** Print Date: 07 AUG 2009
Page

Quality Assurance (GLP)

Quality Assurance Statement

Title: **Certificate of analysis for the HPPD W336 protein produced in *E. coli* batch n°VMLV968**

Study: BBS09-008

This study was periodically inspected and properly signed records of these inspections were submitted to Test Facility Management and to the Study Director as listed below. This report has been audited by the GLP Quality Assurance Unit. The reported results accurately reflect the original data of the study.

Phase of Study	Inspection	Reporting
Study plan	23-24 JUL 2009	24 JUL 2009
Study plan amendment	29 JUL 2009	29 JUL 2009
Study conduct	29 JUL 2009	29 JUL 2009
Study conduct	30 JUL 2009	30 JUL 2009
Study conduct	04 AUG 2009	04 AUG 2009
Study plan amendment	06 AUG 2009	06 AUG 2009
Draft final report	06 AUG 2009	07 AUG 2009
Final report	07 AUG 2009	07 AUG 2009

S. Tanghe
GLP Quality Assurance

Tanghe 07/08/'09

Bayer CropScience

APPROVALS PAGE

Study Director / Author:	Veerle Habex	Date 7/8/09
Author:	Ann Wierckx	Date 7/7/09
Test Facility Management:	Jean-Marc Ferullo	Date 7/8/09
Sponsor Representative:	Donna Mitten	Date 25 Aug 2009

Bayer BioScience N.V. - BioAnalytics

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

SUMMARY

Bayer CropScience has introduced a *hppdPfW336* gene construct, conferring tolerance to isoxyflutole in *Glycine max* plants by means of particle bombardment. The explants were regenerated to whole plants and an elite event was selected on the basis of expression of the transgenic protein and agronomic performance.

Because the expression level of the HPPD W336 protein in transgenic plants is low, safety studies are conducted with the HPPD W336 protein produced in *E. coli*.
In this study, the identity of the HPPD W336 protein produced in *E. coli*, batch VMLV968 was confirmed by molecular weight determination using SDS-PAGE, immunoreactivity analysis using western blotting and enzymatic activity assay. The purity was determined to be 97 ± 2% by means of SDS-PAGE.

1. OBJECTIVE

In this study, the characterization of the HPPD W336 protein produced in *E. coli* batch n° VMLV968 was performed by concentration determination, purity determination and confirmation of the identity by molecular weight determination, immunoreactivity determination and enzymatic activity assay.

2. OVERVIEW OF EXPERIMENTAL DESIGN

In this study five analyses were performed on the resuspended test item T39-01. The concentration of the protein was determined by means of OD_{280} measurement; the molecular weight and the purity of the protein were analyzed after sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE); the identity of the protein was confirmed by western blotting and the activity of the protein was determined by means of the HPPD activity assay.

3. TEST ITEM, REFERENCE ITEM AND STANDARDS

3.1. Test item

The subject of this study, test item ID T39-01, was lyophilized HPPD W336 protein purified from *E. coli* batch VMLV968. The test item was produced by Bayer Technology Services GmbH, Leverkusen, Germany as described in the study report 'Purification of HPPD W336 batch VMLV968'.

Lyophilisation of the dialysed HPPD W336 protein was done in batches of ca. 1 mg (0.0375 mL at 26.75 mg/mL).
Before the resuspension of the test item, the protein was stored in ultrafreezer 112UF (-30°C to -90°C); after resuspension, the test item was stored in refrigerator 91RF (0°C to 10°C).

Test Item ID:	T39-01
Test Item Identity:	HPPD W336 protein
Origin:	*Escherichia coli*
Batch n°:	VMLV968
Storage conditions:	dry and ultra frozen (-30°C to -90°C) if lyophilized refrigerated (0°C to 10°C) after suspending
Expiry dates:	48 hours after resuspention of the lyophilized HPPD W336 protein 27/01/2010 for the lyophilized HPPD W336 protein. The stability of the lyophilized test item will be assessed in a quality management study, by means of concentration determination, molecular weight determination, western blot analysis and activity assay. The dates of the quality management studies for the HPPD W336 protein are October 2009, January 2010, April 2010, July 2010, January 2011, July 2011, July 2012, July 2013 and July 2014. The expiry date of the lyophilized test item is guaranteed until 27/01/2010. This expiry date can be extended based on the results of the quality management study. The latest updates can be obtained upon request.

3.2. Reference items

No reference items were used in this study.

3.3. Standards

As standard, the molecular weight marker 'Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)' was used to determine the molecular weight of the protein. The stock solution of this

standard was stored in freezer 90FZ (-5°C to -40°C). The work solution was during the study conduct stored in refrigerator 91RF (0°C to 10 °C).

4. EXPERIMENTAL DESIGN

4.1. Resuspension of the test item

The content of 5 tubes each containing ca. 1 mg of the test item T39-01, was dissolved individually in 1 mL of 50 mM Tris HCl pH 7.5. The mixtures were thoroughly vortexed, kept on ice for at least 15 minutes and vortexed again. The tubes were briefly centrifuged to collect all material at the bottom of the tube. Once dissolved, the tubes were kept on ice or in the refrigerator (0°C to 10°C).

4.2. Quantification of the test item

The concentration of test item T39-01 was determined by means of OD_{280} measurement according to SOP BBS 07/80/00. From each of the 5 tubes 2 independent 1/2 dilutions were made in 50 mM Tris HCl pH 7.5. This resulted in the required ten independent dilutions of the sample with the appropriate OD_{280} value between 0.2 and 0.8.
Calculation of the concentration was done using a validated excel sheet (Figure 1) as described in SOP BBS 07/80/00, knowing by the sequence of the protein that an OD_{280} of 1 corresponds to a HPPD W336 concentration of 1.15 mg/mL (molar extinction coefficient = 34990; molecular weight = 40312 Da).
After quantification, the different solutions were pooled and vortexed again.

4.3. Molecular weight determination of the test item

To determine the molecular weight, the dissolved test item was analyzed by SDS-PAGE according to the SOP BBS 07/77/00. A NuPAGE® NOVEX Bis-Tris 10% gel was used in combination with a NuPAGE® MOPS SDS gel running buffer (Invitrogen). The concentration determined in section 4.2 was used to determine the volume of HPPD W336 protein to be loaded.

Loading order of gel G1-09-008:

Lane 1: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 2: 0.5 µg of dissolved HPPD W336
Lane 3: 0.5 µg of dissolved HPPD W336
Lane 4: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 5: 0.5 µg of dissolved HPPD W336
Lane 6: 0.5 µg of dissolved HPPD W336
Lane 7: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 8: 0.5 µg of dissolved HPPD W336
Lane 9: 0.5 µg of dissolved HPPD W336
Lane 10: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)

The gel was run at a constant voltage of 180 V during 51 minutes. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue Staining according to SOP BBS 07/66/02. A photographic copy of the stained gel was made according to SOP BBS 04/77/01and is shown in Figure 2.

To dry the gel, the gel and 2 sheets of cellophane were soaked at least 2 min in Acrylamide gel drying solution. The gel was stretched between both cellophane sheets using the gel drying cassette of the DryEase Mini-Gel Drying system of Invitrogen. The gel was dried at least overnight and fixed to a white sheet of paper.

The molecular weight of the HPPD W336 protein was determined according to SOP BBS 07/42/02 using the scan with ID number G1-09-008-F2. In order to obtain a linear regression curve, the molecular weight proteins of 250 kDa, 150 kDa, 15 kDa and 10 kDa were not taken into account.
The molecular weight and the value of the 95% confidence interval of the HPPD W336 protein were determined automatically in a validated Excel sheet (Figure 3).

4.4. Purity determination and immunoreactivity of the test item

To determine the purity and the immunoreactivity of the test item, the dissolved test item was loaded on a NuPAGE® NOVEX Bis-Tris 10% gel in combination with a NuPAGE® MOPS SDS gel running buffer (Invitrogen). The concentration determined in section 4.2 was used to determine the volume of HPPD W336 protein to be loaded.

Loading order of gel G2-09-008:

Lane 1: /
Lane 2: 2 µg of dissolved HPPD W336
Lane 3: 2 µg of dissolved HPPD W336
Lane 4: 2 µg of dissolved HPPD W336
Lane 5: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 6: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 7: 0.03 µg of dissolved HPPD W336
Lane 8: 0.01 µg of dissolved HPPD W336
Lane 9: 0.003 µg of dissolved HPPD W336
Lane 10: /

The gel was run at a constant voltage of 180 V during 51 minutes. After electrophoresis, the gel was cut between positions 5 and 6. The proteins in lanes 1 to 5 were stained with Coomassie Brilliant Blue according to SOP BBS 07/66/02. The proteins in lanes 6 to 10 were transferred to a ProBlott membrane according to SOP BBS 07/64/03. This membrane received ID M2-09-008.
Since the first part of gel G2-09-008 (containing lane 1 to lane 5) was broken, these lanes were repeated on gel G3-09-008.

Loading order of gel G3-09-008:

Lane 1: /
Lane 2: 2 µg of dissolved HPPD W336
Lane 3: 2 µg of dissolved HPPD W336
Lane 4: 2 µg of dissolved HPPD W336
Lane 5: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 6: /
Lane 7: /
Lane 8: /
Lane 9: /
Lane 10: /

The gel was run at a constant voltage of 180 V during 52 minutes. After electrophoresis the proteins were stained with Coomassie Brilliant Blue according to SOP BBS 07/66/02. A picture of the stained gel was made using the G-BOX (SOP BBS 04/77/01) and is shown in Figure 4.

4.4.1.Purity analysis

Picture G3-09-008-F3 was analysed using the Genetools software to determine the protein purity of the test item (SOP BBS 07/42/02). The purity was calculated for the 3 lanes containing the HPPD W336 protein. The mean of these purity values and the standard deviation were calculated.

4.4.2.Immunoreactivity of the test item by means of western blot

The membrane M2-09-008 was developed according SOP BBS 07/65/01. In this technique two antibodies were used:

- Mouse anti HPPD W336 (batch A36440-3), supplied by MS Technologies and tested at Bayer BioScience N.V. The antibody was stored in refrigerator 91RF and used in a 1:1500 dilution.

- Rabbit anti mouse-Alkaline Phosphatase (Sigma cat # A1902). The antibody was stored in the refrigerator 91RF and used at a 1:1000 dilution.

An electronic image of the membrane was made according to SOP BBS 04/70/02 and is presented in Figure 5. The original membrane was fixed to a white sheet of paper by means of a self-adhesive acetate plate sealer sheet.

4.5. HPPD W336 activity assay

HPPD W336 catalyzes the transformation of 4-hydroxyphenylpyruvate (HPP) into homogentisate. This activity is measured in a colorimetric method by determining the amount of HPP remaining in the assay mixture at the end of the incubation period after derivatisation with 2,4-Dinitrophenylhydrazine (DNP) (SOP BBS 07/62/00).

5. RESULTS

5.1. Quantification of the test item

The protein concentration of the test item was determined to be 0.94 ± 0.01 mg/mL (Figure 1).
Based on the volumes of the HPPD W336 protein subjected to lyophilisation and the concentration of the test item described above, the amounts of HPPD W336 in the different tubes are

- 4.70 ± 0.05 mg for the eppendorf tubes;
- 18.80 ± 0.20 mg for the 2 mL HPLC glass vials and
- 263.20 ± 2.80 mg for the N20-25DIN glass vials.

5.2. Molecular weight determination

A picture of gel G1-09-008 is shown in Figure 2. The calculation of the molecular weight is showed in Figure 3.

The molecular weight was calculated to be 40.9 ± 2.8 kDa. The accuracy of the technique is 4 kDa. Taken both together, the determined molecular weight fits with the deduced molecular weight of the HPPD W336 protein of 40.3 KDa.

5.3. Purity determination

The purity of the test item was assessed using gel G3-09-008 (Figure 4). The purity of the test item was determined in all 3 lanes. The mean of the purity values and the standard deviation were calculated to be respectively 97 % and 2 %.

5.4. Immunoreactivity analysis

The western blot analysis (Figure 5) revealed in each sample the expected HPPD W336 band, confirming the identity of the test item.

5.5. HPPD W336 activity determination

The activity analysis showed the activity of the HPPD W336 protein.

6. CONCLUSION

Biochemical analyses were performed to confirm the identity of test item T39-01. Based on the analyses the T39-01 test item was identified as HPPD W336 protein. The concentration of the protein in the test item T39-01 was determined at 0.94 ± 0.01 mg/mL with a purity of 97 ± 2 %. The activity of the test item was demonstrated.

7. ARCHIVING

The study plan, amendments and deviations, other study data, and the original of the final report will be archived in study file BBS09-008 at the BBS N.V. GLP test facility document archive at the test facility address.

One vial containing 0.94 mg of test item T39-01 was stored in the GLP Test Facility test and reference item archive at the test facility address.

REFERENCES

No	Doc No	Report No	Author(s), year, title, source, edition, pages
1.	- - - - - - -	BTS-PT-AS-EFT 17-2009	Moehrle, V., 2009, Purification of HPPD W336 batch VMLV968

Figure 1: Determination of the concentration of the resuspended test item

Determination of Protein Concentration (OD280 method)

Average / Standard deviation OD values

		Average	Standard deviation
OD260	blanks	0.0500	0.0023
	samples	0.2573	0.0044
OD280	blanks	0.0429	0.0022
	samples	0.3844	0.0042
Blank corrected OD280		0.3416	0.0047

Acceptance criteria

Average OD280 samples > average OD260 samples ?	Yes
Average OD280 blanks < 0,2 * average OD280 samples ?	Yes
Average OD280 samples ≥ 0,2 and ≤ 0,8 ?	Yes
Average OD260 samples ≥ 0,2 and ≤ 0,8 ?	Yes
All acceptance criteria met?	Yes

Calculation Protein concentration

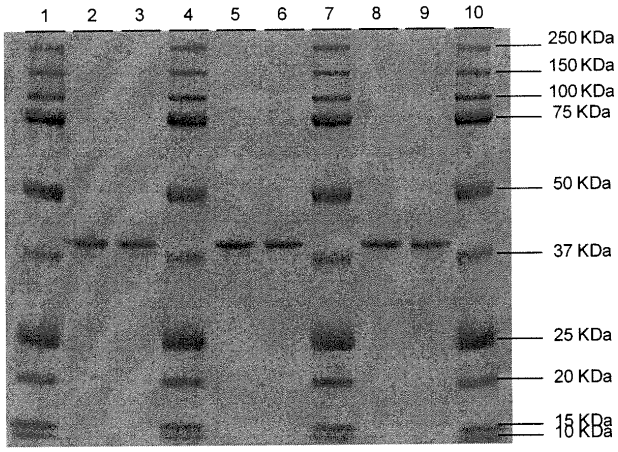
Protein :	HPPD W336
Test item ID :	T39-01
Dilution factor (1/x) :	2
Dilution buffer :	50 mM Tris pH 7,5
Batch N° dilution buffer :	50 mM Tris pH 7,5 -02
Magellan workspace ID :	29072009-001.wsp

Molecular Weight of HPPD W336 :	40312 Da
Molar extinction ratio of HPPD W336 :	34990
Path length (300 µl):	0.84 cm

	Calculated concentration	Standard deviation	Relative 95% confidence interval	**95% confidence interval**
Diluted sample	0.47 mg/ml	0.01 mg/ml	0.85%	0.47 ± 0 mg/ml
Undiluted sample	0.94 mg/ml	0.01 mg/ml	0.85%	**0.94 ± 0.01 mg/ml**

For calculations all available number of digits are taken into account.
The values displayed are rounded values, in order to improve readability of data.

Figure 2: SDS-PAGE gel of test item T39-01 to determine the molecular weight (Gel ID G1-09-008)



Loading order:
Lane 1: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 2: 0.5 µg of dissolved HPPD W336
Lane 3: 0.5 µg of dissolved HPPD W336
Lane 4: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 5: 0.5 µg of dissolved HPPD W336
Lane 6: 0.5 µg of dissolved HPPD W336
Lane 7: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 8: 0.5 µg of dissolved HPPD W336
Lane 9: 0.5 µg of dissolved HPPD W336
Lane 10: 5 µL of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)

Figure 3: Determination of the molecular weight of the test item

Determination of the molecular weights of proteins

Migration distances

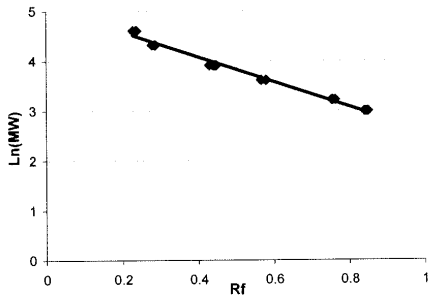
Marker: Precision Plus Protein Standards

Molecular weight of bands (kDa)	Migration distance of bands (mm), measurement number 1	2	3	4
250 kDa				
150 kDa				
100 kDa	26.0	26.0	26.0	25.0
75 kDa	31.5	31.5	31.5	30.5
50 kDa	48.5	49.0	48.5	47.0
37 kDa	64.0	63.5	63.5	62.0
25 kDa	83.5	83.5	83.5	82.5
20 kDa	93.0	93.0	92.5	92.0
15 kDa				
10 kDa				
Total migration distance (mm)	110.5	109.5	109.5	109.5

Sample : T39-01

Measurement number	Migration distance (mm)	Total migration distance (mm)
1	60.5	110.0
2	60.5	110.0
3	60.5	109.5
4	60.0	109.5
5	60.0	109.5
6	60.0	109.5

Regression



Predicted Molecular Weights

Marker : Precision Plus Protein Standards

Known Molecular Weight of bands (kDa)	Predicted Molecular Weight (kDa), measurement number 1	2	3	4
250 kDa				
150 kDa				
100 kDa	89.6	89.1	89.1	91.1
75 kDa	79.1	78.6	78.6	80.4
50 kDa	53.9	52.7	53.3	55.2
37 kDa	38.0	37.9	37.9	39.2
25 kDa	24.4	24.0	24.0	24.6
20 kDa	19.7	19.3	19.6	19.8
15 kDa				
10 kDa				

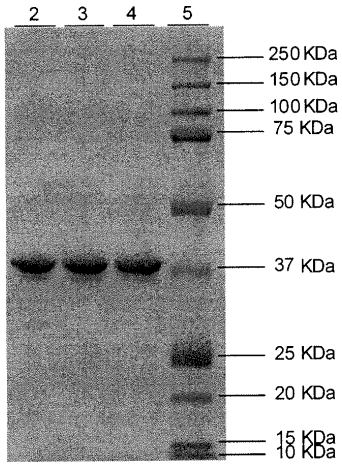
For calculations all available number of digits are taken into account.
The values displayed are rounded values, in order to improve readability of data.

Sample : T39-01

Measurement number	Predicted Molecular Weight (kDa)
1	40.8
2	40.8
3	40.6
4	41.0
5	41.0
6	41.0

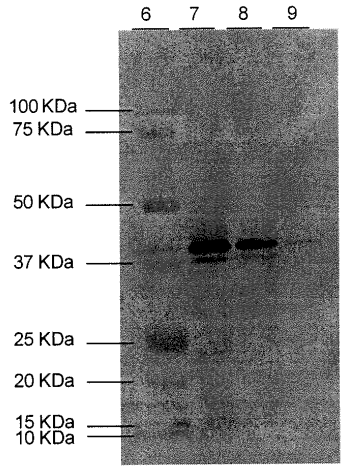
Predicted Molecular Weight :	40.9 ± 2.8 kDa
Relative 95% confidence interval :	6.8%

Figure 4: SDS-PAGE gel of test item T39-01 to determine the purity (Gel ID G3-09-008)



Loading order: Lane 2: 2 µg of the dissolved HPPD W336
Lane 3: 2 µg of the dissolved HPPD W336
Lane 4: 2 µg of the dissolved HPPD W336
Lane 5: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)

Figure 5: Western blot of test item T39-01 (Membrane ID M2-09-008)



Loading order: Lane 6: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 7: 0.3 µg of the dissolved HPPD W336
Lane 8: 0.1 µg of the dissolved HPPD W336
Lane 9: 0.03 µg of the dissolved HPPD W336

Bayer BioScience N.V. - BioAnalytics

Appendix 1: Overview of the analytical SOPs used in this study

SOP	Title	Reference
BBS 07/42/02	Fragment analysis using the genetools software	Genetools user manual - Syngene
BBS 07/62/00	HPPD activity assay	
BBS 07/64/03	Electrotransfer of proteins to membranes	Instruction manual Mini Trans-Blot® Electrophoretic Transfer Cell (Version M1703930 Rev.E) – BioRad Instruction manual immobilization membranes ProBlott® - Applied Biosystems
BBS 07/65/01	Western blotting	
BBS 07/66/02	Gelcode® blue staining for SDS-PAGE	Instructions Gelcode® Blue Stain Reagent, version 0714.2 – Pierce Biotechnology
BBS 07/77/00	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) using NuPAGE® Novex Bis-Tris gels	NuPAGE technical guide – Invitrogen
BBS 07/80/00	Determination of protein concentration by OD280 measurement (Tecan method)	How to measure and predict the molar absorption coefficient of a protein - Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. - Protein Sci. 1995 4:2411-2423.

Bayer CropScience

BioScience

CERTIFICATE OF ANALYSIS BA-10-09LB

Origin of the Certified Material
Bayer BioScience N.V.
BioAnalytics
Technologiepark 38
B-9052 Zwijnaarde, Belgium
Tel : +32 (0)9 243 0411
Fax : +32 (0)9 224 0694

Bayer BioScience N.V.
BioAnalytics
Technologiepark 38
B-9052 Zwijnaarde
Belgium

Date : 15 - 10 - 2009

General Protein Information

- Product name : Bovine serum albumin (BSA)
- Batch number : VMLV968BSA
- Produced by : Bayer Technology Services, Leverkusen, Germany as described by V. Moehrle in report BTS-PT-AS-EFT 17-2009.
  By means of SDS page, it was shown that the BSA protein before treatment (batch 058K1172, Sigma-Aldrich) did not change during the treatment. A picture of this SDS-PAGE gel containing BSA before treatment (058K1172) and BSA after treatment (VMLV958BSA) is shown in attachment 2. Therefore, the CoA of the BSA before treatment (attachment 1) stays valid.
- Concentration : The content 1 eppendorf tube of BSA batch VMLV958BSA was dissolved in 1 ml PBS. The concentration of this solution was determined by means of OD_{280} measurement to be 0.92 ± 0.01 mg/ml. Based on this concentration and the volumes of BSA solution that were added to the tubes before lyophilization, the amount of BSA in the different tubes was determined to be 0.92 ± 0.01 mg, 4.60 ± 0.05 mg, 18.40 ± 0.20 mg or 257.6± 2.8 mg
- Storage: at -70°C ± 20°C till use; 4 ± 4 °C or on ice after resolving
- Attachments : 2

THIS IS A CERTIFIED COPY
OF THE ORIGINAL
DOCUMENT

INIT.: LB DATE: 16/10/09

Bayer CropScience

BioScience

Attachment 1: Original Certificate of Analysis

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Albumin from bovine serum, powder, cell culture tested
Product Number	A1933
Product Brand	SIGMA
CAS Number	9048-46-8
Storage Temp	2-8°C

TEST	SPECIFICATION	LOT 058K1172 RESULTS
APPEARANCE	WHITE TO TAN POWDER	TAN POWDER WITH A GREEN CAST
SOLUBILITY	CLEAR FAINT YELLOW TO GREEN SOLUTION AT 40 MG/ML IN WATER	CLEAR FAINT YELLOW-GREEN SOLUTION
ELEMENTAL ANALYSIS *	NOT LESS THAN 15% NITROGEN	16%
IDENTITY	OF BOVINE ORIGIN	PASS
AGAROSE ELECTROPHORESIS	> OR = 98% ALBUMIN	98%
ENDOTOXIN LEVEL	< OR = 1 EU/MG	0.04 EU/MG
CELL CULTURE TEST	PASSES	PASS
CELL LINE		CHO, 3T3, BHK
NOTE	* ANHYDROUS BASIS	
RECOMMENDED RETEST	5 YEARS	JUNE 2013
QC RELEASE DATE		JUNE 2008

Rodney Burbach, Manager
Quality Control
St. Louis, Missouri USA

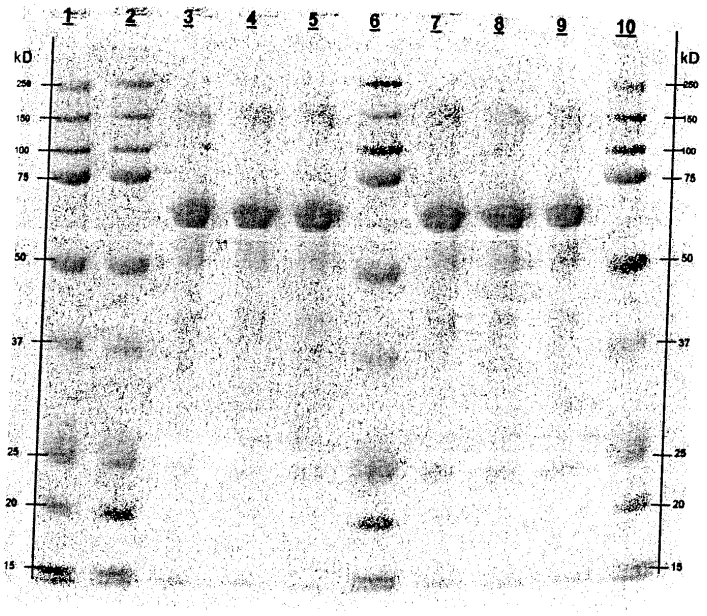
THIS IS A CERTIFIED COPY
OF THE ORIGINAL
DOCUMENT

INIT.: LB DATE: 16/10/09

Bayer CropScience

BioScience

Attachment 2: SDS-PAGE



Gel specifications:- 4-12 % acrylamide gel; MOPS buffer

Lane 1: Molecular weight marker
Lane 2: Molecular weight marker
Lane 3: 2.4 µg BSA batch 058K1172
Lane 4: 2.4 µg BSA batch 058K1172
Lane 5: 2.4 µg BSA batch 058K1172
Lane 6: Molecular weight marker
Lane 7: 2.4 µg BSA batch VMLV968BSA
Lane 8: 2.4 µg BSA batch VMLV968BSA
Lane 9: 2.4 µg BSA batch VMLV968BSA
Lane 10: Molecular weight marker

THIS IS A CERTIFIED COPY OF THE ORIGINAL DOCUMENT

INIT.: LB DATE: 16/10/09

Study Director

Luc Beurms, date 16/10/09

Molecular characterization manager

16/10/09

Dirk Nennstiel, date

FINAL REPORT AMENDMENT

There is no final report amendment at this time.

This page has been left blank intentionally