Bayer CropScience

HPPD W336 PROTEIN

HEAT STABILITY STUDY

DATA REQUIREMENT
No applicable guidelines

REPORT OF STUDY SA 09053
Sponsor identification number: Lynx-PSI N° TX99L064

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: AUGUST 26, 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 test item solution, which was not 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).

- 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: August 26, 2009

J.B. RASCLE

Sponsor Representative:

Date: August 26, 2009

A. CAPT

Study Submitter:

Date:

FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by US EPA.

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	April 17, 2009	April 17, 2009	April 21, 2009
Process-based	Western blot	April 30, 2009	April 30, 2009	May 05, 2009
Study-based	Final report	August 25, 2009	August 25, 2009	August 25, 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: August 26, 2009

G. ODAGLIA

SIGNATURE

I, the undersigned, hereby declare that the work was performed under my 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.

Author / Study Director:

Date: August 26, 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
RESPONSIBLE TECHNICIAN	:	A. ARNAUD
REPORT UNIT ASSISTANT	:	P. ALMERAS

TABLE OF CONTENTS

SUMMARY

The HPPD W336 protein (produced in *E. coli*) was tested for heat stability at temperatures of 60, 75 and 90°C for periods of 10, 30 and 60 minutes.

The protein was examined with Coomassie blue stained-SDS-PAGE or western blot using a specific polyclonal rabbit anti-HPPD W336 protein antibody.

The HPPD W336 protein was heat-stable up to 60 minutes at 90°C.

INTRODUCTION

This *in vitro* heat stability study has been conducted to provide a GLP study to establish if heat treatment causes structural changes to the HPPD W336 protein, which can be detected by gel electrophoresis followed by Coomassie blue or immunological stainings.

In this study the test item was the HPPD W336 protein (produced in *Escherichia coli*).

The study protocol and amendments are presented in Attachment 1.

The study schedule was as follows:

Study initiation date*	April 17, 2009
Sponsor representative protocol approval date	April 17, 2009
Experimental starting date	April 20, 2009
Experimental completion date	June 25, 2009

* Date of protocol approval by Study Director

MATERIAL AND METHODS

1 - TEST ITEM

The test item HPPD W336 protein was supplied by BioAnalytics (Bayer CropScience NV, Ghent, Belgium).

Identification ..... : HPPD W336 protein (produced in *Escherichia coli*)
Batch N° ..... : LB020309
Description ..... : Lyophilized powder
Purity ..... : 96% ±2%
Storage ..... : –74 + 10°C *
Certified through .... : June 2009
*: Storage condition at Sophia-Antipolis (SOP MTR00962)

The certificate of analysis is presented in Attachment 2.

2 - TEST ITEM SOLUTION

The test protein was solubilized in 50 mM Tris pH 7.5 at the final concentration of 0.25 mg HPPD W336 protein/ml.

3 - TEST SYSTEM

The protein incubation was made in 1.5 ml microcentrifuge tubes containing 40 µl of test protein solution in a temperature-controlled hot-block at 60, 75 or 90 °C for 10, 30 or 60 minutes.

At the end of the incubation period, the reaction was immediately terminated by placing the sample tubes on ice, and adding 14 µl MilliQ H_2O.

Additional control samples were prepared:

- a sample of a zero minute incubation of protein (kept at + 4°C);
- a dilution of protein solution at 1/10 in 50 mM Tris pH 7.5 ("10% loading" control; kept at +4°C)
- the buffer solution without protein heated at 60°C for 60 minutes;
- the buffer solution without protein heated at 90°C for 60 minutes.

An aliquot of 25 µl of the samples was used for analysis on SDS-PAGE gels and the remaining was frozen at -20°C.

4 - SDS-PAGE ANALYSIS

The gel electrophoresis was carried out following the method of Laemmli (1) using a Bio-Rad Mini-Protean III cell (Bio-Rad, France). Prior to running SDS-PAGE, 5 µl of 5X Laemmli solution (supplemented with few grains of sucrose) was added to 20 µl of digestion samples. Samples of 15 µl were added to wells of an SDS-PAGE gel (15 well, 1 mm 10-20% gradient polyacrylamide Tris/Tricine) (Bio-Rad, France).

A suitable marker solution (2.5-200 kDa) was used to provide reference points of known molecular weights on the gel (Mark 12, Invitrogen, France). In addition, the Kaleidoscope, prestained standard molecular weight marker (Bio-Rad, France) was included on the gel.

The electrophoresis was carried out with a constant voltage set at 100 volts until the bromophenol line has reached the bottom of the gel. The gels were stained by with the Coomassie blue method dye (Invitrogen) based on the work of Neuhoff *et al.* (2). After rinsing, the gels were scanned using a GS800 scanner (Bio-Rad). The gels are not stable for more than two weeks, so the raw data was the scanned image.

In addition, a second SDS-PAGE-SDS was used for the western blot analysis. This gel was loaded with approximately 2000 times less protein in quantity compared to the Coomassie blue-stained gel, to take into account the higher sensitivity of the western blot technique.

5 - WESTERN BLOT ANALYSIS

A polyvinylidene fluoride (PVDF) membrane was placed on the SDS-PAGE gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the HPPD W336 protein bands and/or its potential fragments, the membrane was incubated in the presence of a specific rabbit polyclonal anti-HPPD W336 protein antibody.

The hybridization of the antibody with the proteins immobilized on the membrane was revealed using a goat anti-rabbit polyclonal antibody coupled with a peroxidase. The hybridization bands were visualized using enhanced chemiluminescent (ECL) detection system (Amersham ECL detection System, GE Healthcare Life Sciences, France).

The autoradiographs were scanned using a GS800 scanner (Biorad) and resulting image was retained in the raw data and reported in the final report.

6 - DATA STORAGE

All raw data, supporting documents as well as protocol, protocol amendments, aliquot of the test substance solution and final report are maintained in the archive room. 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 AND DISCUSSION

1 - SDS-PAGE ANALYSIS (Fig. 1)

The SDS-PAGE analysis of the HPPD W336 protein solution showed one major band located above the 36.5 kDa molecular weight marker, in line with the molecular weight of the HPPD W336 protein. The 10% HPPD W336 protein control was also visible with lower intensity than the undiluted protein band.

After heat treatments, there were no visible changes to the HPPD W336 band at 60, 75 or 90°C from 10 to 60 minutes with intensities similar to the unheated sample.

In the lanes with only buffer solution, there were no bands visible, as expected.

2 - WESTERN BLOT ANALYSIS (Fig. 2)

The western blot analysis of the HPPD W336 protein revealed one major band located above the 36.5 kDa molecular weight marker, in accordance with the HPPD W336 protein molecular weight.

Upon heat treatments, similar results are obtained either by the western blot or the Coomassie blue stained-SDS-PAGE.

In the lanes with only buffer solution, there were no bands visible, as expected.

CONCLUSION

The HPPD W336 protein was heat-stable up to 60 minutes at 90°C.

PROTOCOL DEVIATION

There were no protocol deviations during the study.

Author / Study Director:

Date: August 26, 2009

J.B. RASCLE

REFERENCES

DART Numbers

1 M-223866-01-1 LAEMMLI U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970, 227, pp. 680-685.

2 M-273056-01-1 NEUHOFF V., AROLD N., TAUBE D., EHRHARDT W. (1988): Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, 9, pp. 255-62.

ABBREVIATIONS

%	Percentage
°C	Degree (s) Celcius
µg	Microgram (s)
µl	Microliter (s)
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamineteraacetic acid
$FeCl_3$	Iron chloride
g	Gram (s)
g/ml	Gram (s)/milliliter
GLP	Good Laboratory Practice
ILSI	International Life Science Institute
kDa	Kilodalton
kg	Kilogram (s)
M	Molar
mg	Milligram (s)
mg/ml	Milligram (s)/milliliter
min	Minute (s)
ml	Milliliter (s)
mm	Millimeter(s)
mM	Millimolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
pH	Potential of hydrogen
SDS	Sodium dodecyl sulfate
Tris	Tris hydroxymethyl aminomethane
US OR USA	United States of America
w/v	Weight/volume

FIGURES

FIGURE 1 - COOMASSIE BLUE STAINED SDS-PAGE ANALYSIS OF HPPD W336 PROTEIN AFTER HEAT TREATMENT

Note: For clarity reasons, only the molecular weight maker 12 is presented in the following figures. The kaleidoscope molecular weight maker lane can be seen on the raw data file pictures.

Fig 1: Coomassie blue stained SDS-PAGE gel of HPPD W336 protein after heat treatment

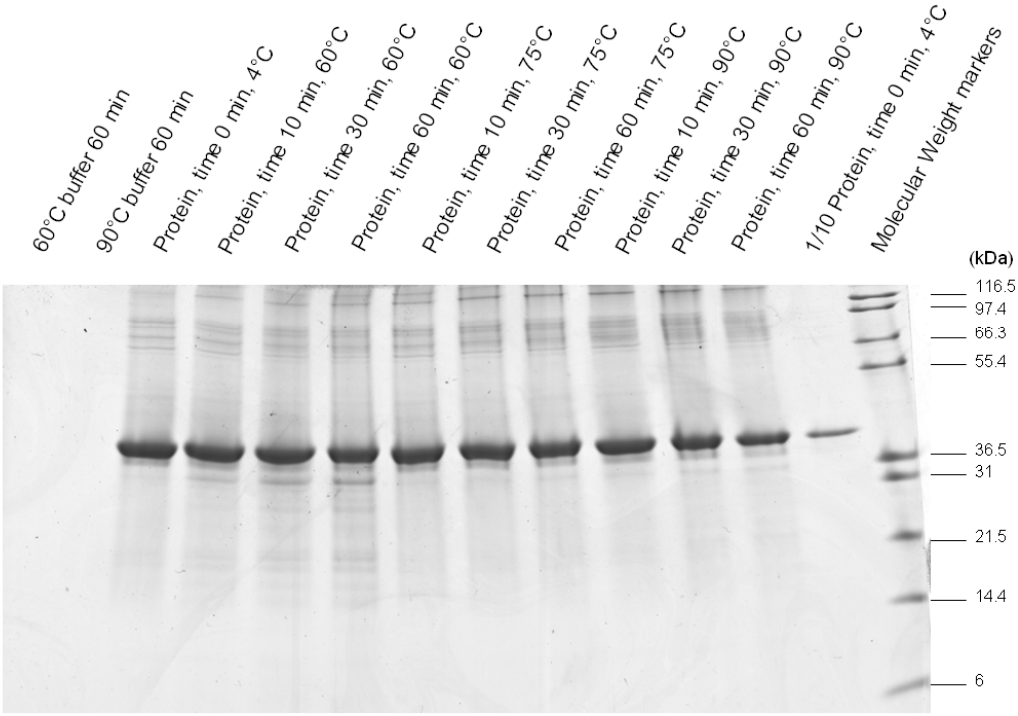
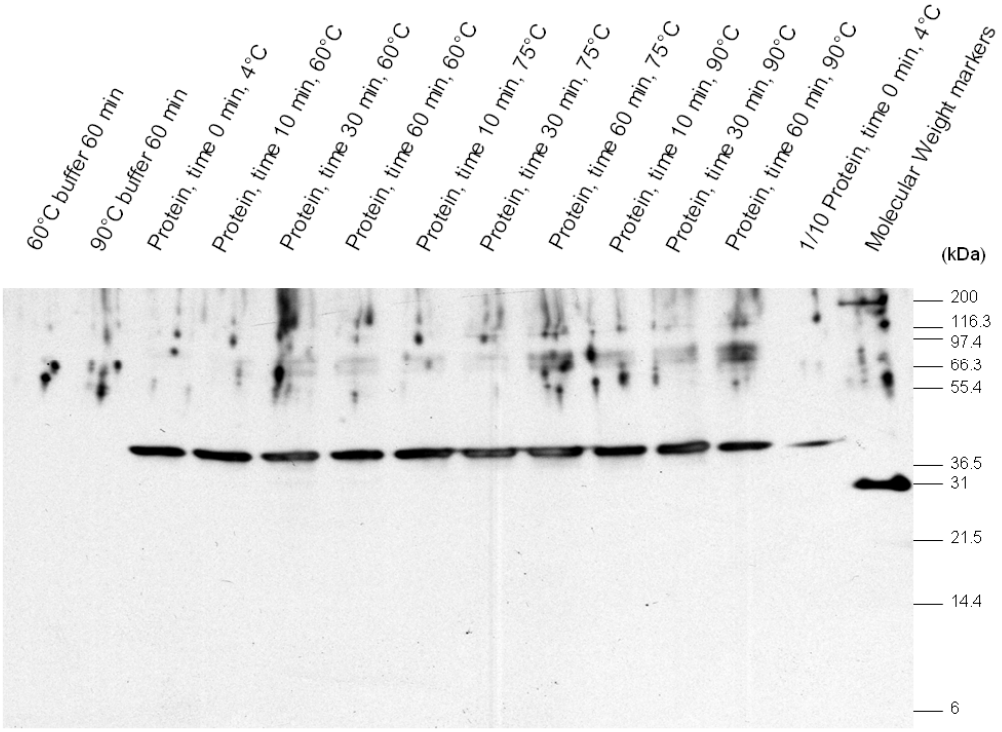


FIGURE 2 - WESTERN BLOT ANALYSIS OF HPPD W336 PROTEIN AFTER HEAT TREATMENT

Note: For clarity reasons, only the molecular weight maker 12 is presented in the following figures. The kaleidoscope molecular weight maker lane can be seen on the raw data file pictures.

Fig 2: Western blot of HPPD W336 protein after heat treatment



ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL AND AMENDMENTS**

HPPD W336 PROTEIN
HEAT STABILITY STUDY

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 present study is designated to determine the heat stability of the HPPD W336 protein (produced in *E. coli*). The potential *in vitro* protein degradation after heat treatment will be observed by SDS-PAGE and western-blot analyses.

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

No data requirement for this study.

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: April 17, 2009 J.B. RASCLE

2.2 SPONSOR REPRESENTATIVE:

Date: April 17, 2009 p.o. A. CAPT CORINNE HERLUET-GLUCKENEY

2.3 OTHER STUDY PERSONNEL

Responsibility		Name
Replacement Study Director	:	D. ROUQUIE
Responsible Technician	:	A. ARNAUD

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

3 PROPOSED DATES

Experimental starting date	:	April 20, 2009	
Experimental completion date	:	May 07, 2009	(estimated)

4 OVERVIEW OF STUDY DESIGN

A solution of the test protein will be heated to temperatures of 60, 75 or 90°C and samples will be taken for analysis at time-points of 0, 10, 30 and 60 minutes. The resultant protein solutions will be analysed for presence of the test protein and its potential stable protein fragments. The methods of analysis will be SDS-PAGE coupled with a Coomassie blue staining and a western blot analysis using a polyclonal antibody directed against the HPPD W336 protein. A "10% protein" control will be loaded on the gels to verify the sensitivity of the staining procedure.

5 MATERIALS AND METHODS

5.1 TEST ITEM

The test item HPPD W336 protein will be supplied by BioAnalytics (Bayer BioScience NV, Gent, Belgium).

Test item identification	:	HPPD W336 protein (produced in *E. coli*)
Batch number	:	LB020309
Purity	:	96% ±2%
Storage	:	–74 + 10°C *
Certified through	:	Will be defined in the study report

*: Storage condition at Sophia-Antipolis (SOP MTR00962)

The certificate of analysis will be attached to the study report.

5.2 PROTEIN SOLUTION

The test protein will be received lyophilized and will be solubilized in 50mM Tris pH 7.5 at the final concentration of 0.25 mg HPPD W336 protein /ml.

Once the test protein is solubilised, it may be stored frozen at at +5°C (±3°C) or colder.

Protein dissolution will be evaluated by visual inspection.

5.3 HEATING

Nine 1.5 ml microcentrifuge tubes of 40 µl protein solution will be warmed to 60, 75 or 90°C in a hot-block for 10, 30 or 60 minutes.

Forty µl of an additional protein sample (to act as the "time zero" control) and of a dilution of protein solution at 1/10 in 50mM Tris pH 7.5 (to act as the "10% loading" control) will be kept at approximately 4°C.

Two tubes of 40 µl of 50mM Tris pH 7.5 without test protein will be incubated as above for 60 minutes at 60 or 90°C.

5.4 SAMPLES

The heating must be stopped as soon as samples are taken, by placing the sample tubes on ice and by adding 14 µl MilliQ H_2O.

14 µL of Laemmli solution (possibly supplemented with sucrose) will be added to all samples and heated for 10 minutes at >90°C.

Samples may be directly used for analysis on SDS-PAGE gels or frozen at approximately -20°C. When frozen, an aliquot of approximately 25µL may be prepared prior to freezing.

Samples may be directly used for analysis on SDS-PAGE gels or frozen at -20°C.

5.5 5X LAEMMLI BUFFER

5X Laemmli buffer contains: 40% glycerol, 5% β-mercaptoethanol (BME), 10% SDS, 0.33 M Tris-HCl pH 6.8, 0.05% bromophenol blue.

5.6 SDS-PAGE ANALYSIS

The method will be based on Laemmli's method (1970) using a Mini-Protean III cell (BioRad, France). Samples of 15 µl will be added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris-Tricine, BioRad).

The unheated samples and the control without protein will be treated the same way.

Suitable molecular weight markers will be used to provide reference points of known molecular weights on the gel (Kaleidoscope, prestained standard, Biorad and Mark 12, Invitrogen, France). Prior to running, 10 µl of 5X Laemmli will be added to 5 µl of Markers.

Loading order on gel:

Lane	Sample
1	Kaleidoscope
2	60°C buffer 60 min
3	90°C buffer 60 min
4	Time zero 4°C
5	60°C 10 min
6	60°C 30 min
7	60°C 60 min
8	75°C 10 min
9	75°C 30 min
10	75°C 60 min
11	90°C 10 min
12	90°C 30 min
13	90°C 60 min
14	Protein 1/10 time zero 4°C
15	Marker 12

Electrophoresis will be carried out with a constant voltage set at 100 volts until the bromophenol line has reached the bottom of the gel.

Gels will be stained by the Coomassie blue method (Colloidal blue staining kit, Invitrogen, USA) and the gels will be scanned. The gels are not stable for more than a week, so the raw data will be the scanned image.

In addition, a second SDS-PAGE will be performed for the western blot analysis.

This gel will be loaded with approximately 200 times less protein in quantity to take into account the better level of detection of the western-blot technique compared to the one of Coomassie blue staining. The dilution will be performed in 1X Laemmli solution (possibly supplemented with sucrose) prepared in H_2O MilliQ.

5.7 WESTERN BLOT ANALYSIS

A polyvinylidene fluoride (PVDF) membrane will be placed on the gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the HPPD W336 protein bands and/or its potential fragments recognized by a specific anti-HPPD W336 protein antibody, the membrane will be incubated in the presence of a specific rabbit polyclonal anti-HPPD W336 protein antibody.

The hybridization of the antibody with the proteins immobilized on the membrane will be revealed using a goat anti-rabbit polyclonal antibody coupled with a Horseradish Peroxydase. The hybridization bands will be visualized using chemoluminescent (ECL) detection system (Amersham, France).

The autoradiographs will be scanned (Bio-Rad) and resulting image will be retained in the raw data and reported in the final report.

5.8 DATA ANALYSIS

The results of a detailed visual inspection of the scans will be reported. If stable fragments of the test protein are visible on the gel or on the blot, then their number and time-course will be reported.

The gel will be considered to be valid if:

- the protein band is clearly visible at time zero (lane 4) and is not visible in the lanes without protein (lanes 2 and 3).
- at least one marker lane is clearly visible to allow a molecular weight evaluation.

6 REPORTING

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 in the final report. The final report will include the information and data required by current internationally recognized regulations.

7 ARCHIVING

All raw data, supporting documents as well as protocol, protocol amendments and 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 proteins.

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

8 REFERENCES

LAEMMLI, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 227, pp. 680-5.

PROTOCOL AMENDMENT

Protocol SA 09053

HPPD W336 PROTEIN
HEAT STABILITY STUDY

Protocol amendment: N°1

Reason: **Typing error in section 5.4 of the study protocol.**

The following sentence:

"Samples may be directly used for analysis on SDS-PAGE gels or frozen at -20°C."

is repeated by error on two occasions in section 5.4 of the study protocol.

Study Director:

Date: April 22, 2009

J.B. RASCLE

PROTOCOL AMENDMENT

Protocol SA 09053

HPPD W336 PROTEIN
HEAT STABILITY STUDY

Protocol amendment: N°2

Reason: **Modification of the sample dilution factor for the western blot analysis.**

For the second SDS-PAGE run for the western blot analysis, an additional dilution factor of the sample will be applied in order to take into account the better level of detection of the western-blot technique compared to one of Coomassie blue staining.

The samples will now be loaded with approximately 2000 time less protein, and not 200 time less, as originally described in the study protocol.

Study Director:

Date: April 28, 2009

J.B. RASCLE

ATTACHMENT 2 - **CERTIFICATE OF ANALYSIS**

Bayer CropScience

Title

Certificate of analysis for the HPPD W336 protein produced in *E.coli* batch n°LB020309

Author

Veerle Habex

Completed on

April 24st, 2009

Testing Facility

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

Study number

BBS09-001

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

Veerle Habex
Molecular Characterization
BioAnalytics

24/4/09
Date

STUDY IDENTIFICATION PAGE

Study Initiation date:	March 3rd, 2009
Experimental start date:	March 9th, 2009
Experimental Termination date:	April 6th, 2009
Study Completion date:	April 24st, 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:	Dirk Nennstiel Address see Test Facility Tel: +32 9-243 04 39 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 Personnel:	Luc Beurms Address see Test Facility Tel: +32 9-243 04 51 Fax: +32 9-224 06 94 e-mail: luc.beurms@bayercropscience.com Kathleen De Pestel Address see Test Facility Tel: +32 9-243 04 36 Fax: +32 9-224 06 94 e-mail: kathleen.depestel@bayercropscience.com Ann De Wulf Address see Test Facility Tel: +32 9-243 04 24 Fax: +32 9-224 06 94 e-mail: ann.dewulf@bayercropscience.com Ann Wierckx Address see Test Facility Tel: +32 9-243 06 37 Fax: +32 9-224 06 94 e-mail: ann.wierckx@bayercropscience.com

Bayer CropScience

Sponsor Representative:

Nicolas De Schrijver
Global Regulatory Affairs Manager
Regulatory Affairs
Address see Test Facility
Tel: +32 9-243 04 18
Fax: +32 9-233 19 83
e-mail: nicolas.deschrijver@bayercropscience.com

Bayer CropScience

QUALITY ASSURANCE STATEMENT

Report **BBS09-001** Date: 24 APR 2009

Quality Assurance (GLP)

Quality Assurance Statement

Title: **Certificate of analysis for the HPPD W336 protein produced in E. *coli* batch n° LB020309**

Study: BBS09-001

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

Phase of Study	Inspection date	Reporting date
Study plan	05 MAR 2009	06 MAR 2009
Study conduct	09 MAR 2009	09 MAR 2009
Study conduct	10 MAR 2009 - 13 MAR 2009	17 MAR 2009
Study plan amendment	30 MAR 2009	31 MAR 2009
Study plan amendment	07 APR 2009	07 APR 2009
Draft report	16 APR 2009 - 17 APR 2009	20 APR 2009
Final report	22 APR 2009	24 APR 2009

L. Hottin
GLP Quality Assurance

24/04/09

Bayer BioScience N.V. - BioAnalytics

Bayer CropScience

APPROVALS PAGE

Study Director / Author:	Veerle Habex	24/4/09 Date
Test facility management/ Molecular characterization manager:	Dirk Nennstiel	24/04/09 Date
Sponsor Representative:	Nicolas De Schrijver	30/04/09 Date

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LIST OF FIGURES

LIST OF APPENDICES

SUMMARY

Bayer CropScience has introduced a *hppdPfW336* gene construct, conferring tolerance to isoxaflutole 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 extremely 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 LB020309 was confirmed by means of a molecular weight determination using SDS-PAGE, an immunoreactivity analysis using western blotting and an activity assay. The purity was determined by means of SDS-PAGE to be 96 ± 2%.

1. OBJECTIVE

In this study, the characterization of the HPPD W336 protein produced in *E. coli* batch n° LB020309 was performed by means of a concentration determination, a purity determination and a confirmation of the identity by means of a molecular weight determination, an immunoreactivity determination and an activity assay.

2. OVERVIEW OF EXPERIMENTAL DESIGN

In this study five analyses were performed on the resuspended test item T34-01. 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 T34-01, was lyophilized HPPD W336 protein purified from *E. coli* batch LB020309. The test item was produced by Bayer BioScience N.V., Ghent, Belgium, as described in the study report 'Production of the HPPD W336 protein in *Escherichia coli* – Batches LB020309 and LB090309'.
Lyophilisation of the dialysed HPPD W336 protein was done in batches of ca. 1 mg (0.2 ml at 5 mg/ml).
Before the resuspension of the test item, the protein was stored in the ultrafreezer; after resuspension, the test item was stored in the refrigerator.

Test Item ID:	T34-01
Test Item Identity:	HPPD W336 protein
Origin:	*Escherichia coli*
Batch n°:	LB020309
Expiry date:	The stability of the test item will be assessed in a quality management study, by means of a concentration determination, a molecular weight determination, a western blot analysis and an activity assay. The dates of the quality management studies for the HPPD W336 protein are June 2009, September 2009, December 2009, March 2010, September 2010, March 2011, September 2011, September 2012 and September 2013. The expiry date of the test item is guaranteed until the next quality management study. Based on the results obtained, the expiry date can be extended. 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. The work solution was during the study conduct stored in refrigerator 91RF.

4. EXPERIMENTAL DESIGN

4.1. Resuspension of the test item

The content of 5 tubes each containing ca. 1 mg of the test-item T34-01, was resuspended individually in 1 ml of 50 mM Tris pH 7.5. The 5 mixtures were thoroughly vortexed, kept on ice for more than 15 minutes and vortexed again. The tubes were shortly spinned to collect all material at the bottom of the tube. The different suspensions were pooled and vortexed again. After resuspension, the sample was kept on ice or in the refrigerator. The redissolved protein was discarded after study completion.

4.2. Quantification of the test item

The concentration of test item T34-01 was determined by means of OD_{280} measurement according to SOP BBS 07/80/00. Ten independent 1/2 dilutions of the test item in 50 mM Tris pH 7.5 were prepared. This dilution gave the appropriate OD_{280} value between 0.2 and 0.8.
The 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 1 OD_{280} corresponds to a HPPD W336 concentration of 1.15 mg/ml (molar extinction coefficient = 35110; molecular weight = 40312 Da).

4.3. Molecular weight determination of the test item

To determine the molecular weight, the resuspended 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 G1-09-001:

Lane 1: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 2: 0.5 µg of resolved HPPD W336
Lane 3: 0.5 µg of resolved HPPD W336
Lane 4: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 5: 0.5 µg of resolved HPPD W336
Lane 6: 0.5 µg of resolved HPPD W336
Lane 7: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 8: 0.5 µg of resolved HPPD W336
Lane 9: 0.5 µg of resolved 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 approximately 55 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 07/42/01. This scan received the ID G1-09-001-F1 and 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 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 G1-09-001-F1. In order to obtain a linear regression curve, the molecular weight proteins of 250 kDa, 150 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 resuspended 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 G2-09-001:

Lane 1: /
Lane 2: 2 µg of resolved HPPD W336
Lane 3: 2 µg of resolved HPPD W336
Lane 4: 2 µg of resolved 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 resolved HPPD W336
Lane 8: 0.01 µg of resolved HPPD W336
Lane 9: 0.003 µg of resolved HPPD W336
Lane 10: /

The gel was run at a constant voltage of 180 V during approximately 55 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. A picture of the stained gel was made using the G-BOX (SOP BBS 04/77/01). This scan received the ID G2-09-001-F1 and is shown in Figure 4 – Panel A. The proteins in lanes 6 to 10 were transferred to a ProBlott membrane according to SOP BBS 07/64/03. This membrane received ID M2-09-001

4.4.1.Purity analysis

Picture G2-09-001-F1 was analysed using the Genetools software to determine the protein purity of the test item (SOP BBS 07/42/01). 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 M2-09-001 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 electronical copy of the membrane was made according to SOP BBS 04/70/02. This scan (ID M2-09-001-F1) is presented in Figure 4 – Panel B. 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 1.01 ± 0.01 mg/ml (Figure 1).
Based on the volumes of the HPPD W336 protein subjected to lyophilisation and the amount described above, the amounts of HPPD W336 in the eppendorf tubes, the 15 ml and the 50 ml tubes were calculated to be either 1.01 ± 0.01 mg for the eppendorf tubes; 5.05 ± 0.05 mg or 25.25 ± 0.25 mg for the 15 ml tubes and 75.75 ± 0.75 mg for the 50 ml falcon tubes.

5.2. Molecular weight determination

A picture of gel G1-09-001 is shown in Figure 2. The calculation of the molecular weight is showed in Figure 3.

The molecular weight was calculated to be 40.7 ± 2.5 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 G2-09-001 (Figure 4 – panel A). 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 96 % and 2 %.

5.4. Immunoreactivity analysis

The western blot analysis (Figure 4 – panel B) 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 T34-01. Based on the analyses the T34-01 test item was identified as HPPD W336 protein. The concentration of the protein in the test item T34-01 was determined at 1.01 ± 0.01 mg/ml with a purity of 96 ± 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 BBS09-001 at the BBS N.V. GLP test facility document archive at the test facility address.

One vial containing 1.01 mg of test item T34-01 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.	- - - - - - -	BIOX-013_ProtProd_243	Beurms, L., Habex, V. – 2009 - Production of the HPPD W336 protein in *Escherichia coli* – Batch LB020309 and batch LB090309

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	0.0452	0.0013
	samples	0.2825	0.0037
OD280	blanks	0.0408	0.0010
	samples	0.4119	0.0038
Blank corrected OD280		0.3712	0.0040

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

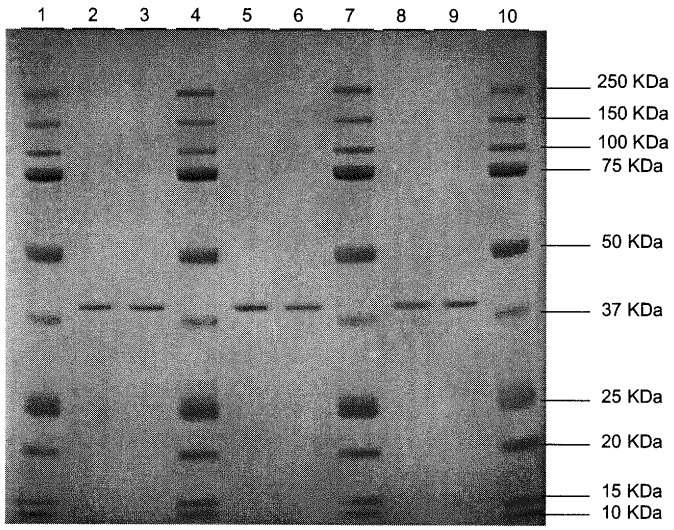
Protein :	HPPD
Test item ID :	T34-01
Dilution factor (1/x) :	2
Dilution buffer :	50 mM Tris-HCl pH 7,5
Batch N° dilution buffer :	50 mM Tris pH 7,5 - 01
Magellan workspace ID :	09032009-001.wsp

Molecular Weight of HPPD :	40312 Da
Molar extinction ratio of HPPD :	35110
Path length (300 µl):	0.84 cm

	Calculated concentration	Standard deviation	Relative 95% confidence interval	**95% confidence interval**
Diluted sample	0.51 mg/ml	0.01 mg/ml	0.66%	0.51 ± 0 mg/ml
Undiluted sample	1.01 mg/ml	0.01 mg/ml	0.66%	**1.01 ± 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 T34-01 to determine the molecular weight (Gel ID G1-09-001)



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

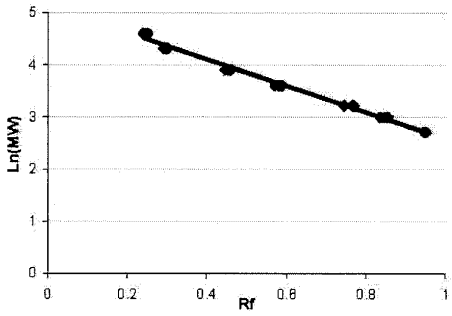
Marker: Precision Plus Protein Standards

Molecular weight of bands (kDa)	Migration distance of bands (mm), measurement number			
	1	2	3	4
250 kDa				
150 kDa				
100 kDa	25.0	24.5	24.0	24.0
75 kDa	30.0	29.5	29.0	29.0
50 kDa	45.5	45.5	45.5	44.5
37 kDa	58.0	58.5	58.0	57.0
25 kDa	76.5	76.5	76.0	74.5
20 kDa	84.5	85.0	84.5	83.5
15 kDa	94.5	94.5	94.0	94.5
10 kDa				
Total migration distance (mm)	100.0	99.5	99.0	100.0

Sample: T34-01

Measurement number	Migration distance (mm)	Total migration distance (mm)
1	56.0	100.0
2	55.5	99.5
3	55.5	99.0
4	55.5	99.0
5	55.0	99.5
6	55.0	99.5

Regression



$y = -2.5446x + 5.1255$

$R^2 = 0.9925$

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	89.1	89.9	90.8	91.4
75 kDa	78.4	79.1	79.9	80.4
50 kDa	52.9	52.6	52.3	54.2
37 kDa	38.5	37.7	37.9	39.5
25 kDa	24.0	23.8	23.9	25.3
20 kDa	19.8	19.1	19.2	20.1
15 kDa	15.2	15.0	15.0	15.2
10 kDa				

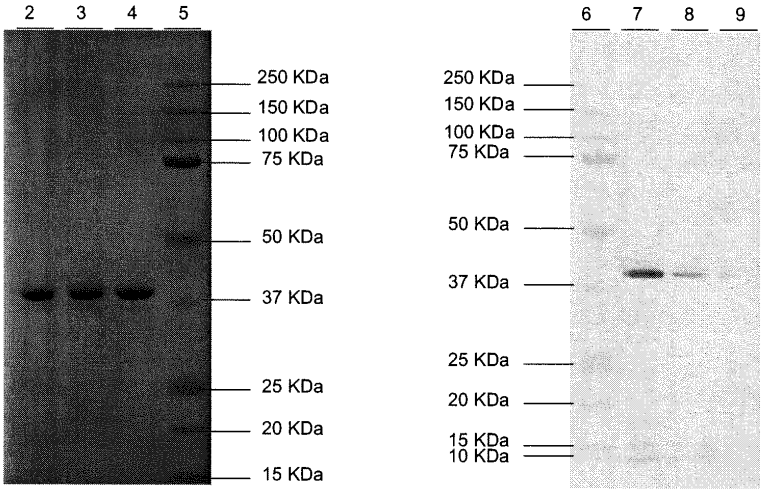
For calculations all available number of digits are taken into account
The values displayed are rounded values, in order to improve readability of data

Sample: T34-01

Measurement number	Predicted Molecular Weight (kDa)
1	40.5
2	40.7
3	40.4
4	40.4
5	41.2
6	41.2

Predicted Molecular Weight :	40.7 ± 2.5 kDa
Relative 95% confidence interval :	6.1%

Figure 4: SDS-PAGE gel of test item T34-01 to determine the purity (Gel ID G2-09-001) and Western blot of test item T34-01 (Membrane ID M2-09-001)



Panel A: Coomassie stained part of gel G2-09-001 **Panel B: Western blot M2-09-002**

Gel G2-09-001 was after electrophoresis divided in 2 parts:

- The first 5 lines were coloured with Coomassie Blue (SOP BBS 07/66/02). This part of the gel was used to determine the purity of the test item.
- The last 5 lines were blotted (SOP BBS 07/64/03) and developed with as primary antibody mouse anti-HPPD and as secondary antibody Rabbit anti-mouse-Alkaline Phosphatase (SOP BBS 07/65/01). In this western blot, the immunoreactivity of the HPPD W336 protein was demonstrated.

Lane 1: /
Lane 2: 2 µg of the resolved HPPD W336
Lane 3: 2 µg of the resolved HPPD W336
Lane 4: 2 µg of the resolved 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: 0.3 µg of the resolved HPPD W336
Lane 8: 0.1 µg of the resolved HPPD W336
Lane 9: 0.03 µg of the resolved HPPD W336
Lane 10: /

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

SOP	Title	Reference
BBS 07/42/01 **BBS 07/42/02**	Fragment analysis using the gene tools software	Genetools user manual - Syngene
BBS 07/62/00	HPPD activity assay	
BBS 07/64/03	Electro transfer 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	Coomassie Brilliant Blue Staining of gels	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.

FINAL REPORT AMENDMENT

There is no final report amendment at this time.

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