Characterization of Test Substance ECRY3.1AB-0208
and
Certificate of Analysis

Data requirement:	Not applicable
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Date:	Study completed on September 4, 2008
Syngenta Study No.:	5307-08-13
Report No.:	SSB-010-08
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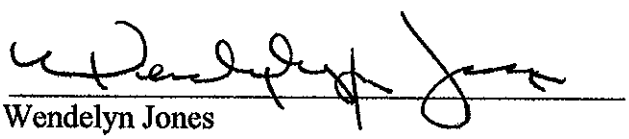
Company Representative:



Wendelyn Jones
Regulatory Affairs Manager

9/2/08
Date

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

With the exceptions noted below, this study was conducted in compliance with the relevant provisions of Good Laboratory Practice (GLP) Standards, 40 CFR Part 160 (U.S. EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act and subsequent revisions. The mass analysis conducted by Syngenta Analytical Sciences was not conducted under GLP.

Study Director

Andrea Nelson

September 4, 2008

Andrea Nelson, Scientist
Biochemical Assessment Team
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QUALITY ASSURANCE STATEMENT

Study Title: Characterization of Test Substance ECRY3.1AB-0208 and Certificate of Analysis

Study Director: Andrea Nelson

Study Number: 5307-08-13

Pursuant to Good Laboratory Practice Regulations (40 CFR Part 160), this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below.

Inspection/Audit Type	Inspection/Audit Dates	Reporting Date
Audit Protocol	June 20, 2008	June 20, 2008
In-Progress Inspection	July 3, 2008	July 3, 2008
In-Progress Data Audit	July 10, 2008	July 10, 2008
Initial Final Report	August 21, 2008	August 21, 2008
Second Final Report	September 3, 2008	September 3, 2008

Prepared by: Kimberly W. Hill Date: Sept. 3, 2008

Kimberly W. Hill
Sr. Quality Assurance Auditor
Quality Assurance Unit
Syngenta Crop Protection, Inc.

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Bis-Tris	bis[2-hydroxymethyl]imino-tris[hydroxymethyl]methane
Da	Daltons
EDTA	ethylenediaminetetraacetic acid
kDa	kiloDaltons
LDS	lithium dodecylsulfate
MES	morpholinoethanesulfonic acid
Milli-Q	Millipore system Ultrapure Organex Cartridge
MW	molecular weight
Q-TOF	quadrupole time-of-flight mass spectrometer
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl) aminomethane
Tween 20	polyoxyethylene sorbitanmonolaurate

SUMMARY

The microbially produced test substance ECRY3.1AB-0208 containing the eCry3.1Ab protein was characterized with respect to solubility and purity. Additionally the identity of eCry3.1Ab as contained in test substance ECRY3.1AB-0208 was confirmed by examining the apparent molecular weight, immunoreactivity and total mass of the protein.

The test substance was previously prepared from a recombinant *Escherichia coli* overexpression system. The resulting lyophilized protein preparation, designated as test substance ECRY3.1AB-0208 was soluble at 10 mg/ml in 10 mM ammonium bicarbonate buffer pH 10.0, water, 10% ethanol and 10 mM Tris buffer containing 0.4mM EDTA and 0.1% Tween 20. The test substance was determined by spectrophotometric analysis to contain 92.4% protein, and densitometric analysis indicated that 97.0% of the total protein in the test substance was eCry3.1Ab, thus the purity of test substance ECRY3.1AB-0208 was calculated to be 89.6% eCry3.1Ab by weight. The predicted eCry3.1Ab molecular weight of 74,833 Da was confirmed by mass spectrometry. Western blot analysis of test substance ECRY3.1AB-0208 revealed a dominant immunoreactive band corresponding to the expected eCry3.1Ab molecular weight.

The results presented in this study confirmed the identity of eCry3.1Ab in test substance ECRY3.1AB-0208.

INTRODUCTION

The purpose of this study was to characterize the microbially produced test substance ECRY3.1AB-0208, containing eCry3.1Ab protein, intended for use in a number of safety and characterization studies. The *ecry3.1Ab* gene in the recombinant *Escherichia coli* overexpression system used to produce the test substance encodes the same eCry3.1Ab protein expected to be produced by Event 5307 derived maize plants except that it contains one additional methionine and six additional histidine residues at the N-terminus. The eCry3.1Ab protein is an engineered chimera of mCry3A and Cry1Ab proteins and has insecticidal activity against significant plant pests including corn rootworm (*Diabrotica*) and Colorado potato beetle (CPB; *Leptinotarsa decemlineata*). In this study, test substance ECRY3.1AB-0208 was characterized with respect to solubility, purity and protein identity.

MATERIALS AND METHODS

Test Substance ECRY3.1AB-0208

Test substance ECRY3.1AB-0208 was prepared from an *E. coli* overexpression system. eCry3.1Ab in test substance ECRY3.1AB-0208 is expected to be identical to that expressed in Syngenta's transgenic maize, Event 5307 except that it contains one additional methionine and six additional histidine residues at the N-terminus. The modified *ecry3.1Ab* gene used for the microbial expression in the *E. coli* overexpression system was linked to the bacterial *tac* promoter in a vector derived from pET24a (Novagen Cat. No. 69749-3) and then transformed into *E. coli* strain DH5α (New England Biolabs Cat. No. C2988).

Prior to this study, test substance ECRY3.1AB-0208 was prepared from pooled batches of *E. coli* cell paste by Syngenta Protein Science (Jealott's Hill International Research Centre, Bracknell, UK) as described by Thompson (2008). Briefly, *E. coli* cells were ruptured and the cell debris removed by centrifugation. The soluble material was filtered, applied to an immobilized metal affinity column (GE Healthcare Nickel Sepharose Fast Flow column), and eluted using an imidazole step gradient. Fractions containing eCry3.1Ab were then further purified via anion exchange chromatography and eCry3.1Ab was eluted with a sodium chloride gradient. The eluted eCry3.1Ab-containing fractions were pooled, concentrated and the buffer was exchanged. The solution was lyophilized and designated test substance ECRY3.1AB-0208. The test substance was sent on dry ice to Syngenta Biotechnology, Inc. (Research Triangle Park, NC, USA), where it was stored at -20°C ± 8°C until further use.

Solubility Determination

The solubility of test substance ECRY3.1AB-0208 was determined in 10 mM ammonium bicarbonate buffer pH 10.0, 10% ethanol, 10 mM Tris buffer containing 0.4mM EDTA and 0.1% Tween 20 and purified water (Milli-Q Ultrapure Academic bench-integrated water purification system, Millipore Cat. No. ZMQP60001). To determine the solubility, defined volumes of aqueous solutions were added to the test substance and its solubility (e.g. the concentration at which the test substance is completely dissolved) was determined by visual observation.

Protein Quantification

Total protein in test substance ECRY3.1AB-0208 was quantified spectrophotometrically by determination of the absorbance at 280 nm (Gill and von Hippel 1989) using a Genesys 6 UV-visible spectrophotometer (Thermo Scientific Cat. No. 335908000). Invitrogen Vector NTI Advance software, version 9.1, was used to translate the *ecry3.1Ab* sequence and to calculate the extinction coefficient at 280 nm. The

absorbance at 280 nm was multiplied by the correlation factor for the extinction coefficient to calculate the total protein concentration.

$$A_{280} \times 1A[280] \text{ correlation coefficient} = \text{mg/ml protein}$$

A_{280} = absorbance measurement at 280 nm

1A[280] correlation coefficient = the concentration (mg/ml) of protein that yields an absorbance measurement of 1.00 at 280 nm

Densitometric Analysis

For purity determination, five aliquots of test substance ECRY3.1AB-0208 (2 to 10 µg of test substance ECRY3.1AB-0208 per lane) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a NuPAGE 4-12% Bis-Tris gel (Invitrogen Cat. No. NP0322) and MES SDS running buffer (Invitrogen Cat. No. NP0002). The molecular-weight standard was SeeBlue Plus2 pre-stained standard (Invitrogen Cat. No. LC5925). The gels were stained with Coomassie blue (Pierce Cat. No. 24592), and the distribution of the visible protein bands was estimated by scanning with the Personal Densitometer SI (GE Healthcare Life Sciences Cat. No. 63-0034-92) and densitometric analysis with GE Healthcare ImageQuant software, version 5.2.

Purity Determination

The purity of test substance ECRY3.1AB-0208 was calculated from the percentage of the total protein in relation to the sample weight, as determined by absorbance at 280 nm, and the percentage of eCry3.1Ab in the total protein fraction as determined by densitometry.

$$\text{Purity [\%]} = \text{total protein of ECRY3.1AB-0208 [\%]} \times \text{eCry3.1Ab of total protein [\%]}$$

Immunoreactivity and Molecular Weight Determination

Western blot analysis was used to investigate the integrity of eCry3.1Ab in test substance ECRY3.1AB-0208. Aliquots of dilutions prepared in NuPAGE LDS sample buffer (Invitrogen Cat. No. NP0007) containing 1, 5, 10 and 20ng of eCry3.1Ab were subjected to SDS-PAGE with a NuPAGE 4-12% Bis-Tris gel and MES SDS running buffer. The molecular-weight standard was SeeBlue Plus2 pre-stained standard. After electroblotting, the membrane was probed with immunoaffinity-purified polyclonal rabbit antibodies generated against mCry3a protein. The antibody generated against the mCry3A protein reacts with the mCry3A domain of the chimeric eCry3.1Ab protein. Alkaline-phosphatase-conjugated donkey anti-rabbit immunoglobin G (Jackson ImmunoResearch Cat. No. 711-055-152) diluted to 1:3,000 in Tris-buffered saline with Tween 20 (Sigma-Aldrich Cat. No. T9039) was used to bind to the primary antibody and was visualized by development with alkaline phosphatase substrate solution (Sigma Cat.

No. B1911). The Western blot was visually examined for the presence of intact immunoreactive eCry3.1Ab and immunoreactive eCry3.1Ab-derived fragments.

Total Mass

Intact mass measurements were performed using a quadrupole time-of-flight mass spectrometer (Q-TOF Premier, Waters/Micromass, UK) by Syngenta Analytical Sciences, Jealott's Hill International Research Centre, Bracknell, UK. The mass spectrometer was operated in ion positive mode using an electrospray ionization source. Samples and standards were delivered to the mass spectrometer with an Agilent HPLC (HP 1100). Separation of the samples was achieved using a Jupiter C4 column. A detailed description of the methods used is included in Syngenta Report No. J7474-047-JH-AUG07 (Green 2008).

RESULTS AND DISCUSSION

No circumstances occurred during conduct of this study that would have adversely affected the quality or integrity of the data generated.

Solubility

Test substance ECRY3.1AB-0208 was determined to be soluble as indicated in Table 1.

Table 1. Results of the solubility determination for test substance ECRY3.1AB-0208.

Buffer	Solubility of ECRY3.1AB-0208 (mg/ml)
10 mM NH_4HCO_3 buffer pH 10.0	10
Purified Water	10
10% Ethanol	10
10 mM Tris buffer containing 0.4 mM EDTA and 0.1% Tween 20	10

Quantification and Test Substance Purity

Test substance ECRY3.1AB-0208 was determined to contain 92.4% protein as estimated by absorbance at 280 nm, 97.0% of which was eCry3.1Ab as determined by densitometry analysis of a Coomassie blue stained SDS-gel (see Figure 1). The overall purity of test substance ECRY3.1AB-0208 (eCry3.1Ab content) was therefore calculated to be 89.6% eCry3.1Ab (w/w).

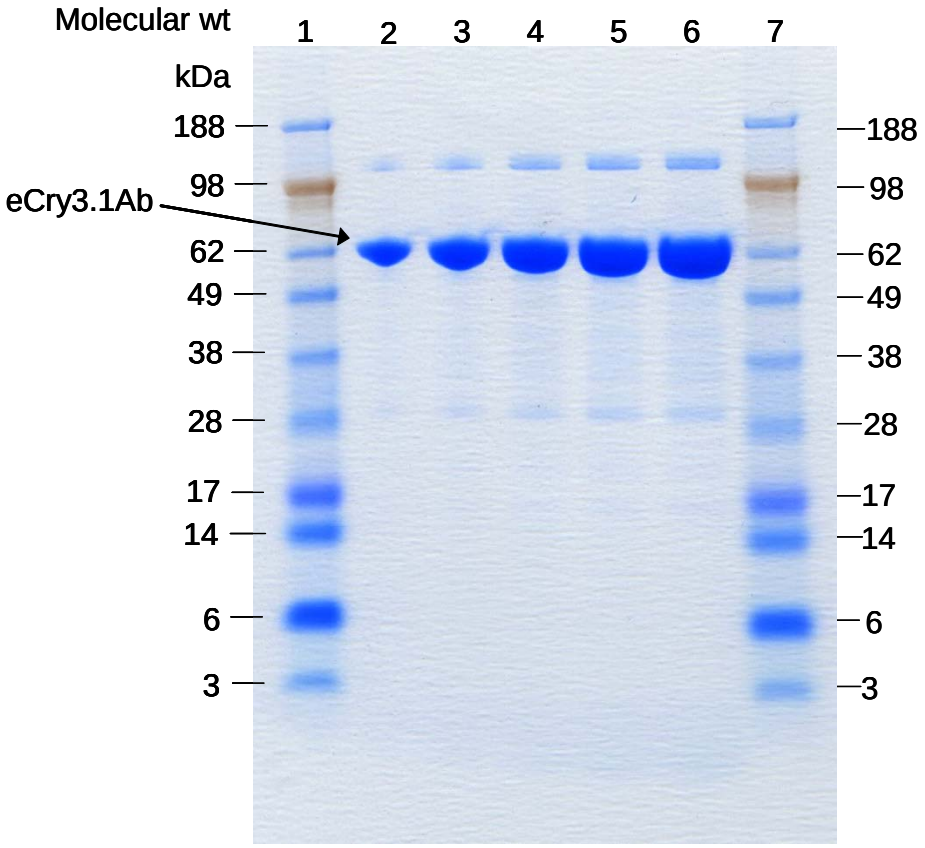


Figure 1. SDS-PAGE analysis of eCry3.1Ab in test substance ECRY3.1AB-0208.

Lanes 1 and 7: Molecular weight standard SeeBlue Plus 2
Lane 2 to 6: 2, 4, 6, 8 and 10 µg ECRY3.1AB-0208

The molecular weight of eCry3.1Ab in ECRY3.1AB-0208 is approximately 74.8 kDa.

Immunoreactivity and Molecular Weight

Western blot analysis of eCry3.1Ab in test substance ECRY3.1AB-0208 revealed a dominant immunoreactive band (74.8 kDa) consistent with the predicted molecular weight of eCry3.1Ab (see Figure 2). The Western blot analysis also revealed a protein band with a molecular weight of approximately 150 kDa (Figure 2). Since this protein cross reacted with the anti-mCry3A antibody and the fact that this protein band is showing the mobility of the molecular weight of two eCry3.1Ab proteins (150 kDa), this protein most likely represents a dimer of eCry3.1Ab.

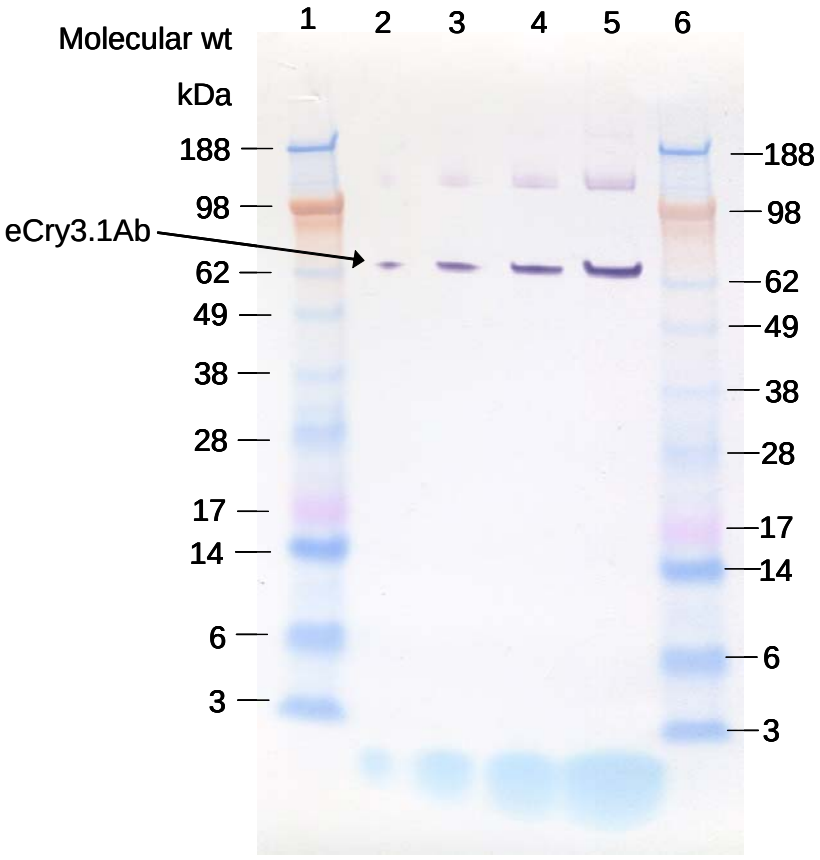


Figure 2. Western blot analysis of eCry3.1Ab in test substance ECRY3.1AB-0208.

Lane 1 and 6: Molecular weight standard See Blue Plus2.
Lane 2 to 5: 1, 5, 10 and 20 ng eCry3.1Ab from ECRY3.1AB-0208.

The molecular weight of eCry3.1Ab in ECRY3.1AB-0208 is approximately 74.8 kDa.

Total Mass

The mass of the eCry3.1Ab protein contained in ECRY3.1AB-0208, measured by mass spectrometry, was 74832.80 Da (74.8 kDa) confirming the theoretical mass based on amino acid sequence analysis (74832.66 Da).

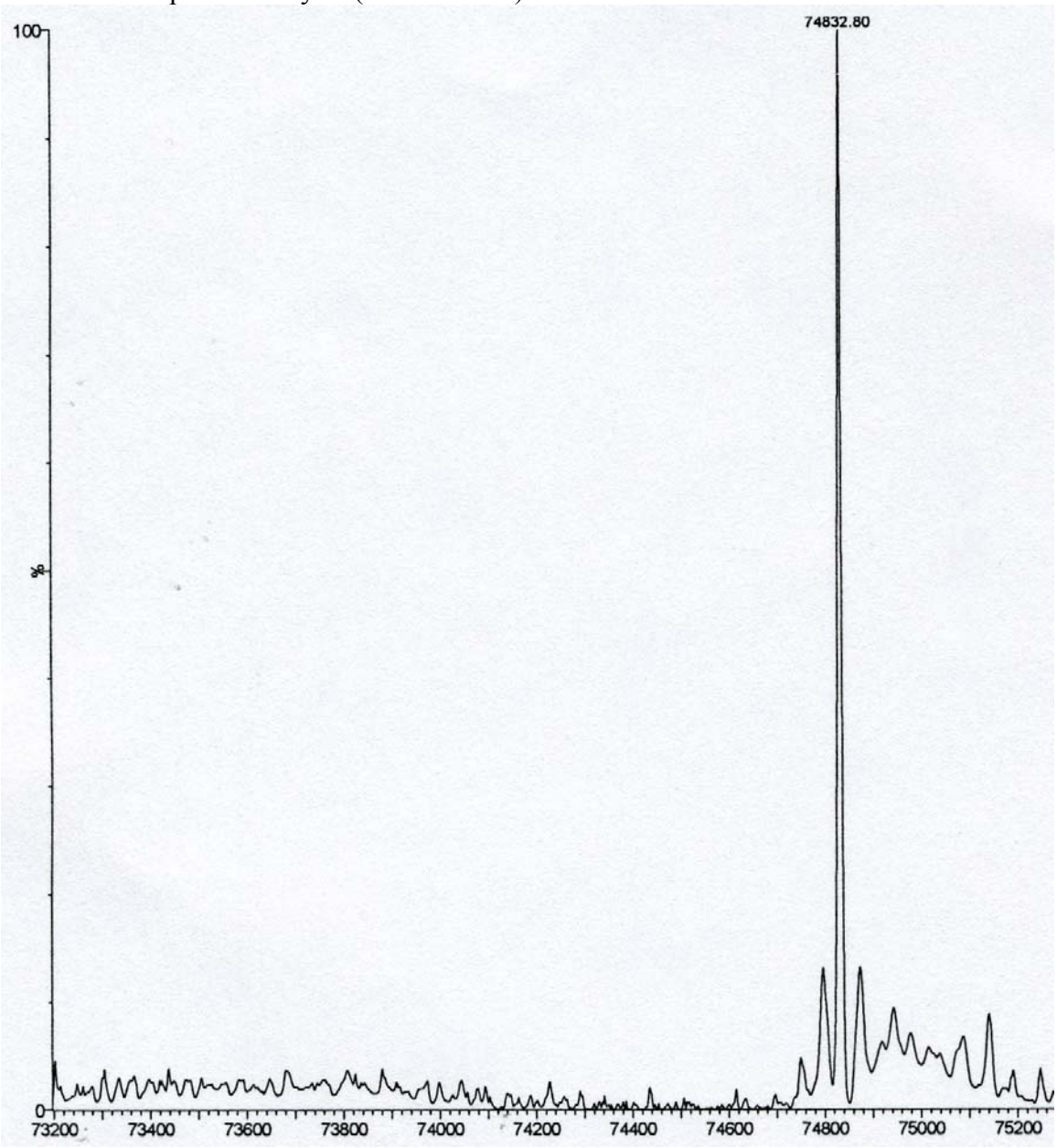


Figure 3. Deconvoluted mass spectrum for ECRY3.1AB-0208.

The measured mass of eCry3.1Ab from ECRY3.1AB-0208 is 74832.80 Da.

CONCLUSIONS

The results presented in this study confirmed the identity of eCry3.1Ab in test substance ECRY3.1AB-0208.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at Syngenta Biotechnology, Inc., 3054 East Cornwallis Rd., Research Triangle Park, NC 27709-2257, USA.

CONTRIBUTING SCIENTISTS

The analytical work reported herein was conducted by Andrea Nelson B.S. and was conducted at Syngenta Biotechnology, Inc., except for the mass analysis, which was conducted by Syngenta Analytical Sciences, Jealott's Hill, Bracknell UK.

Reported by: Andrea Nelson — September 4, 2008

Andrea Nelson
Study Director
Regulatory Science
Syngenta Biotechnology, Inc.

Date

Approved by: Scott Rabe — September 04, 2008

Scott Rabe
Group Leader, Regulatory Science
Syngenta Biotechnology, Inc.

Date

CRITICAL DATES

Study initiation date:	June 30, 2008
Experimental start date:	June 30, 2008
Experimental end date:	July 11, 2008

REFERENCES

Gill, S. and von Hippel, P. 1989. Calculation of Protein Extinction Coefficient from Amino Acid sequences. Analytical Biochemistry 182: 319-326.

Green, P. 2008. Accurate intact mass measurement of eCry3.1Ab protein as contained in test substance ECRY3.1AB-0208 using liquid chromatography / mass spectrometry (LC/MS). Bracknell, UK: Syngenta Analytical Sciences Jealott's Hill International Research Centre. Submitted to Syngenta Biotechnology, Research Triangle Park, NC.

Thompson, P. 2008. Production of ECRY3.1AB-0208 test substance from *E.coli*. Report No. PS-ECRY3.1AB-2008-02 (unpublished). Bracknell, UK: Syngenta Protein Science, Jealott's Hill International Research Centre. Submitted to Syngenta Biotechnology, Research Triangle Park, NC.

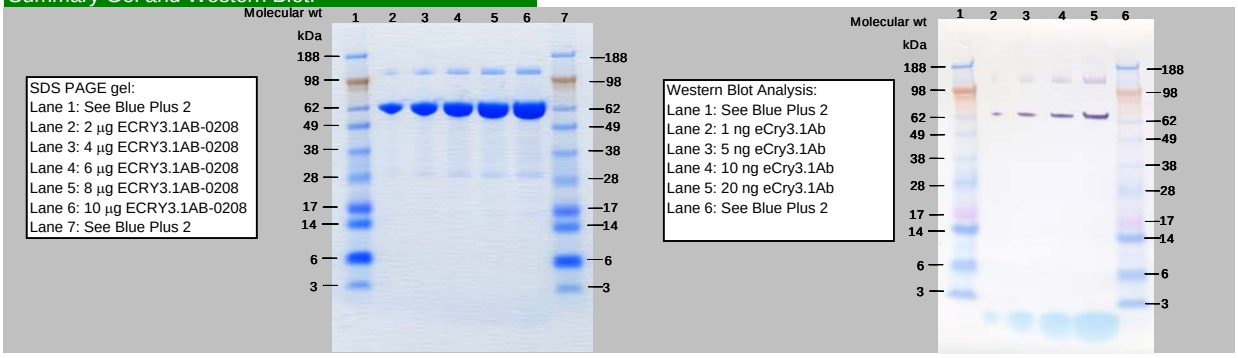
U.S. EPA. 1989. Good Laboratory Practice Standards. 40 CFR Part 160.

Certificate of Analysis

Syngenta Biotechnology, Inc.
Regulatory Science
Research Triangle Park, North Carolina USA

Test Substance	ECRY3.1AB-0208
Date Received/Prepared	6/2008
Study Number	5307-08-13
Active Ingredient	eCry3.1Ab
Event/Production Strain	DH5α
Lab Notebook Reference	SY2207
Solubility	10 mg/ml in 10 mM ammonium bicarbonate buffer pH 10.0, purified water, 10% Ethanol and 10 mM Tris buffer containing 0.4 mM EDTA and 0.1% Tween 20
Working Buffer	10 mM ammonium bicarbonate buffer pH 10.0
Total Protein	92.4%
Densitometry	97.0%
Purity	89.6% eCry3.1Ab in ECRY3.1AB-0208
Glycosylation Analysis	Not determined
Activity	Not determined
Molecular Weight	measured 74832.80 Da; theoretical 74832.66 Da
N-terminal Sequence	Not determined
Storage Conditions	-20 degrees Celsius +/- 8 degrees Celsius
Expiration Date	6/2018

Summary Gel and Western Blot:



General Comments:

This Certificate of Analysis is summarizing data from a study that was performed in compliance with Good Laboratory Practices per 40 CFR Part 160. Raw data, documentation, protocols, protocol ammendments, or reports pertaining to this study are maintained in the Syngenta Biotechnology, Inc. Archives, 3054 Cornwallis Rd., Research Triangle Park, NC USA 27709 in accordance with SOP 1.6.

Study Director: Print Andrea Nelson Signature Andrea Nelson Date Sept. 4, 2008