

SUMMARY

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for public release after registration)

STUDY TITLE

Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from
Transgenic Soybean Event DAS-444Ø6-6

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

B. W. Schafer, A. A. Juba

STUDY COMPLETED ON

11 – May – 2011

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

102098

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Transgenic Soybean Event DAS-444Ø6-6

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The purpose of this study was to characterize the recombinant PAT protein derived from both *Escherichia coli* and transgenic soybean plants (Event DAS-444Ø6-6). Biochemical analyses were performed to characterize the PAT protein derived from microbial and transgenic plant test materials. The analyses performed were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and ELISA analysis. SDS-PAGE confirmed the microbe-derived PAT protein migrated at the expected molecular weight. Western blotting confirmed the plant-derived extracts, from event DAS-444Ø6-6, contained protein immunoreactive to antibodies specific to the PAT protein at the expected molecular weight. A commercially available ELISA assay provided additional evidence the transgenic soybean leaf extracts contained the PAT protein in its proper conformation. Non-transgenic soybean extracts did not contain immunoreactive proteins in either western blot or ELISA assay analyses.

Together, these biochemical tests indicate the microbe- and plant-derived (Event DAS-444Ø6-6) proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies.

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AUTHOR(S)

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Phosphinothricin Acetyltransferase (PAT)

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-44406-6

- STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA sec. 10(g).

Company: Dow AgroSciences LLC

Company Agent: M. S. Krieger

Title: Regulatory Manager

Signature: 

Date: 4 Feb 2011

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived
from Transgenic Soybean Event DAS-44406-6

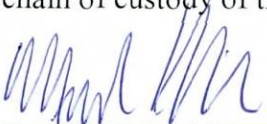
Study Initiation Date: 17-Dec-2010

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

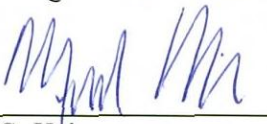
Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions. The GLP status of the commercially available protein molecular markers and bovine serum albumin was unknown. The chain of custody of the standards was not monitored



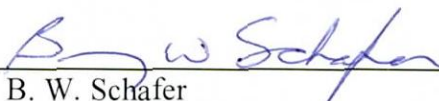
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B. W. Schafer
Study Director/Author
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11-may-2011
Study Completion Date

**Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page**

Study ID: 102098

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-44406-6

Study Initiation Date: 17-Dec-2010


Study Completion Date: 11-May-2011

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
17-Dec-2010	17-Dec-2010	Protocol Review
28-Dec-2010	29-Dec-2010	SDS-PAGE & Western Blot
26, 28, 29-Apr-2011	2-May-2011	Report and Raw Data Review; Test Substance Container Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

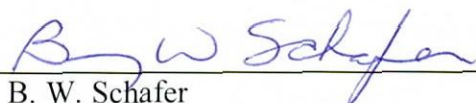


Tracey Froggatt
Dow AgroSciences, Quality Assurance

11-May-2011

Date

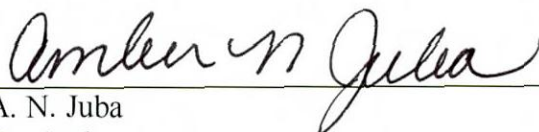
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Peer Reviewer
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11-Feb-2011

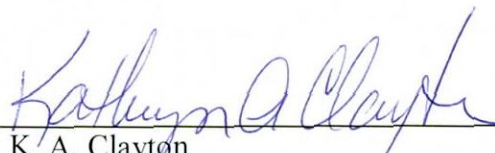
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10 Feb 2011

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STUDY PERSONNEL

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-444Ø6-6

Study Director: Barry W. Schafer

Analysts: Amber A. Juba
Michelle R. Mayes

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Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-444Ø6-6

The purpose of this study was to characterize the recombinant PAT protein derived from both *Escherichia coli* and transgenic soybean plants (Event DAS-444Ø6-6). Biochemical analyses were performed to characterize the PAT protein derived from microbial and transgenic plant test materials. The analyses performed were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and ELISA analysis. SDS-PAGE confirmed the microbe-derived PAT protein migrated at the expected molecular weight. Western blotting confirmed the plant-derived extracts, from event DAS-444Ø6-6, contained protein immunoreactive to antibodies specific to the PAT protein at the expected molecular weight. A commercially available ELISA assay provided additional evidence the transgenic soybean leaf extracts contained the PAT protein in its proper conformation. Non-transgenic soybean extracts did not contain immunoreactive proteins in either western blot or ELISA assay analyses.

Together, these biochemical tests indicate the microbe- and plant-derived (Event DAS-444Ø6-6) proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies.

INTRODUCTION

To perform various toxicology, eco-toxicology, and biochemical characterization studies, large quantities of the PAT protein are required. Because it is technically infeasible to extract and purify sufficient amounts of PAT protein from transgenic plants, the protein was produced in an *Escherichia coli* (*E. coli*) expression system. The purpose of this study was to characterize the recombinant PAT protein derived from both *E. coli* and transgenic soybean plants (Event DAS-444Ø6-6).

The biochemical and immunological methods employed in this study are among those that have been well established for protein analysis. SDS-PAGE separates proteins based on the apparent molecular weight (mass). Western blotting of proteins to a nitrocellulose membrane following SDS-PAGE, and immunodetection with a protein specific antibody is widely used to identify the authenticity of a molecule in a crude preparation. Commercially available ELISA plate assays provide additional evidence of the authenticity of the PAT protein by reacting immunochemically with antibodies that are sensitive and specific for the PAT protein.

MATERIALS AND METHODS

Test Substance/Test System:

The test substance was the PAT protein expressed and extracted from tissues grown from the T4 seeds of transgenic soybean event DAS-444Ø6-6 (Source ID: YX09KX590371.018). The seeds were planted on 23-June and 13-July-2010 and harvested on 09-September-2010. After harvest, the tissues were frozen, lyophilized, and ground for short term storage at -80 °C. The presence of PAT protein in the soybean tissue was confirmed by a commercially available ELISA assay kit (Product #: AP-014) from EnviroLogix Inc. (Portland, ME).

Control Substances:

1. The control substance used in this study was a non-transgenic soybean plant extract (*Glycine max* cv Maverick). Seeds of the Maverick soybean line (Source ID: YX09KX540002) were planted, grown, harvested and processed under the same conditions as the transgenic plants described above. The absence of PAT protein in the non-transgenic soybean tissue was confirmed by a commercially available ELISA assay kit as described above.
2. Recombinant PAT microbial protein, (Lot #: 55238-1A), molecular weight: 20.6 kDa. The sample contained 810 µg/mL of PAT protein (Embrey, 2009). The microbial preparation was produced in recombinant *E. coli* at the Dow AgroSciences Core Biotech R&D facility in Indianapolis, IN and purified by the GeneScript Corporation (Piscataway, NJ). An aliquot of the purified sample was sent to the Test Substance Coordinator at Dow AgroSciences located in Indianapolis and the material was designated TSN031116-0001.

Reference Substances:

The commercially available reference substances used in this study are listed in the following table:

Reference Substance	Product Name	Lot Number	Assay	Reference
Bovine Serum Albumin Fraction V (BSA)	Pre-diluted BSA protein assay standard set	KA136690C	SDS-PAGE	Pierce Cat #: 23208
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	764840	SDS-PAGE and Western Blot	Invitrogen Cat #: LC5800, Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa

ELISA Plate Assay

The soybean leaf tissues of the transgenic and non-transgenic events were harvested fresh on 09-September-2010 and were frozen, lyophilized, ground, and stored at approximately -80°C until use. To confirm the presence/absence of the PAT protein in the pooled tissues, approximately 16 mg of the lyophilized tissues (Event DAS-44406-6 and Maverick) were weighed into 2.0-mL microfuge tubes and tested by ELISA assay as described by EnviroLogix (Portland, ME). Briefly, the soluble proteins were extracted by adding 2 metal beads to the extraction buffer (1.5 mL PBST) and grinding in a Geno-Grinder (Spex, Metuchen, NJ - Model #: 2010) for 3 minutes at 1500 strokes per minute. The resulting supernatants were clarified by centrifuging the samples for 5 minutes at 20,000xg. Serial dilutions of the supernatants were then incubated on the ELISA plate and assayed according to the manufactures' protocol.

SDS-PAGE and Western Blot

SDS-PAGE analysis of the transgenic (DAS-44406-6) and non-transgenic Maverick soybean extracts was performed with Bio-Rad Criterion gels (Cat #: 345-0123) fitted in a Criterion Cell gel module (Bio-Rad Cat #: 165-6001) with MES running buffer (Bio-Rad Cat #: 161-0789). Extracts were prepared by grinding ~34 mg of tissue for 3 minutes in a Geno-Grinder with steel ball bearings in a PBST based buffer (Table 1). The supernatants were clarified by centrifuging for 5 minutes at 20,000xg, and then 120 μL of each extract was mixed with 30 μL of 5x Laemmli sample buffer [LSB, 2% SDS, 50 mM Tris pH 6.8, 0.2 mg/mL bromophenol blue, 50% (w/w) glycerol containing 10% freshly added 2-mercaptoethanol (Bio-Rad, Cat #: 161-0710)]. Samples were heated for 5 minutes at $\sim 95^{\circ}\text{C}$, and after a brief centrifugation, 40 μL of the supernatant was loaded directly on the gel. The reference standard, microbe-derived PAT (TSN031116-0001), and control standard, BSA (Pierce Cat #: 23208), were diluted with Bio-Rad 2x LSB (Bio-Rad Cat #: 161-0737 containing 5% 2-mercaptoethanol) and processed as described earlier. The electrophoresis was conducted at a constant voltage of 150 V for ~65 minutes using MES running buffer (Bio-Rad Cat #: 161-0789). After separation, the gel was cut in half and

one half was stained with Thermo Scientific GelCode Blue protein stain (Cat #: 24592) and scanned with a densitometer to obtain a permanent record of the image. The remaining half of the gel was electro-blotted to a nitrocellulose membrane (Bio-Rad, Cat #:162-0233) with a Criterion trans-blot electrophoretic transfer cell (Bio-Rad Cat#: 170-4070) for ~60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad (Cat #: 161-0771). After transfer, the membrane was cut in half and one half was probed with a PAT specific polyclonal rabbit antibody (Lot #: C98420) and the remaining half was probed with a PAT specific monoclonal antibody (Lot #: 200.624-2-5). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase (Pierce, Cat #: 31460), and goat anti-mouse IgG (H+L) and horseradish peroxidase (Pierce, Cat#: 31430) were used as the secondary antibodies, respectively. GE Healthcare chemiluminescent substrate (Cat #: RPN2132) was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to Thermo Scientific (Cat #: 34091) CL-XPosure detection film for various time points and subsequently developed with a Radiation Services film developer (Model #: All-Pro 100 Plus).

Data Analysis and Calculations

SOFTmax PRO software was used with the MAXline Vmax plate reader. SOFTmax PRO allows the creation of computer generated data files containing all of the parameters required for acquiring and analyzing data from any MAXline instrument. The calibration curve for the PAT ELISA kit was constructed using a quadratic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance (optical density).

The equation fits the best parabola to the standard curve based on the equation:

$$y = A + Bx + Cx^2$$

Where:

y = mean absorbance value (OD)

x = reference standard concentration

The SOFTmax PRO software was used determine the presence or absence of the PAT protein in each sample. The absorbance value and calculated concentration, as well as individual well results, mean sample result, standard deviation and the percent coefficient of variation are reported on the SOFTmax PRO data report but were not used in the final report.

Statistical Treatment of Data

No statistical analyses were conducted during this study.

RESULTS AND DISCUSSION

ELISA Assay

The presence of the PAT protein in the pooled T4 leaf tissue of DAS-444Ø6-6 was confirmed using a commercially prepared ELISA test kit from EnviroLogix. The ELISA assay easily discriminated between transgenic and non-transgenic plants as the non-transgenic extracts of Maverick did not contain detectable amounts immunoreactive protein. This result was also confirmed by western blot analysis.

SDS-PAGE and Western Blot Analysis

In the toxicology-lot preparation of *E. coli*-produced PAT protein (TSN031116-0001), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, was approximately 20.5 kDa (Figure 1). As expected, the corresponding soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal antibodies at an identical size to the microbe-expressed proteins (Figure 2). In the PAT western blot analysis, no immunoreactive proteins, consistent with the PAT protein, were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the transgenic samples (Figure

2). The monoclonal antibody did detect a small amount of a protein dimer in the microbe-derived PAT protein preparation. These results add to the evidence that the PAT protein expressed in soybean is not post-translationally modified or processed which would have added to or subtracted from the overall protein molecular weight.

CONCLUSIONS

The results of this study demonstrated that both the transgenic soybean-plant extract and the microbe-derived PAT toxicological lot contained the intact, full-length PAT protein. This was confirmed by SDS-PAGE molecular-weight approximation, western blot analysis and immunoreactivity using a commercially available ELISA kit assay. Together, these biochemical tests indicate that the plant- and microbe-derived proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies.

ARCHIVING

The final report, protocol, and all raw data including verified and signed copies associated with this study will be filed in the Dow AgroSciences facility archives, Indianapolis, Indiana upon issuing the final report.

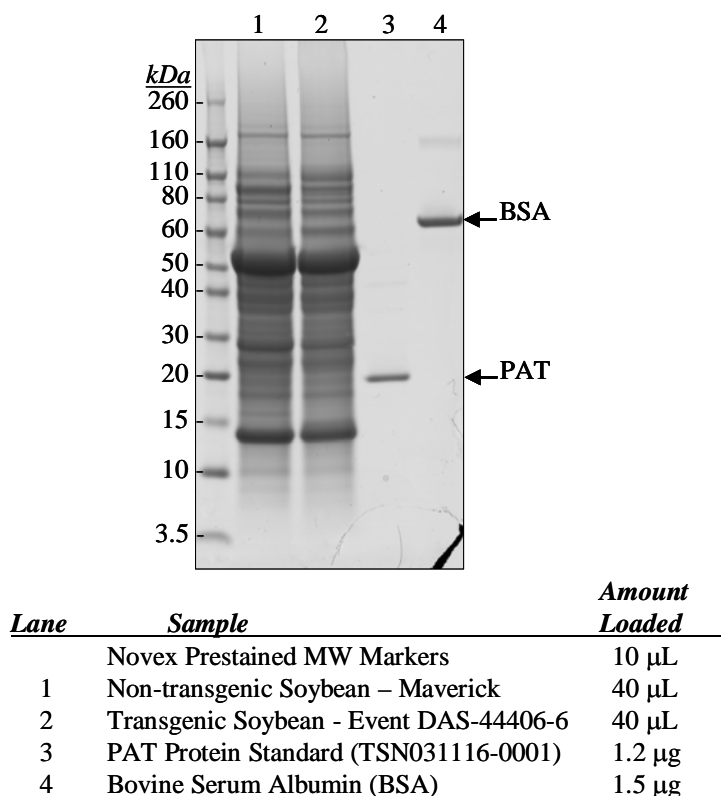
REFERENCES

- 1) Embrey, S. K. (2009) Certificate of Analysis for Test/Reference/Control Substance: Phosphinothricin Acetyltransferase (TSN031116-0001). Unpublished report of Dow AgroSciences, Study #: BIOT09-203839.

Table 1. Soybean-Derived PAT Extraction Buffer Composition

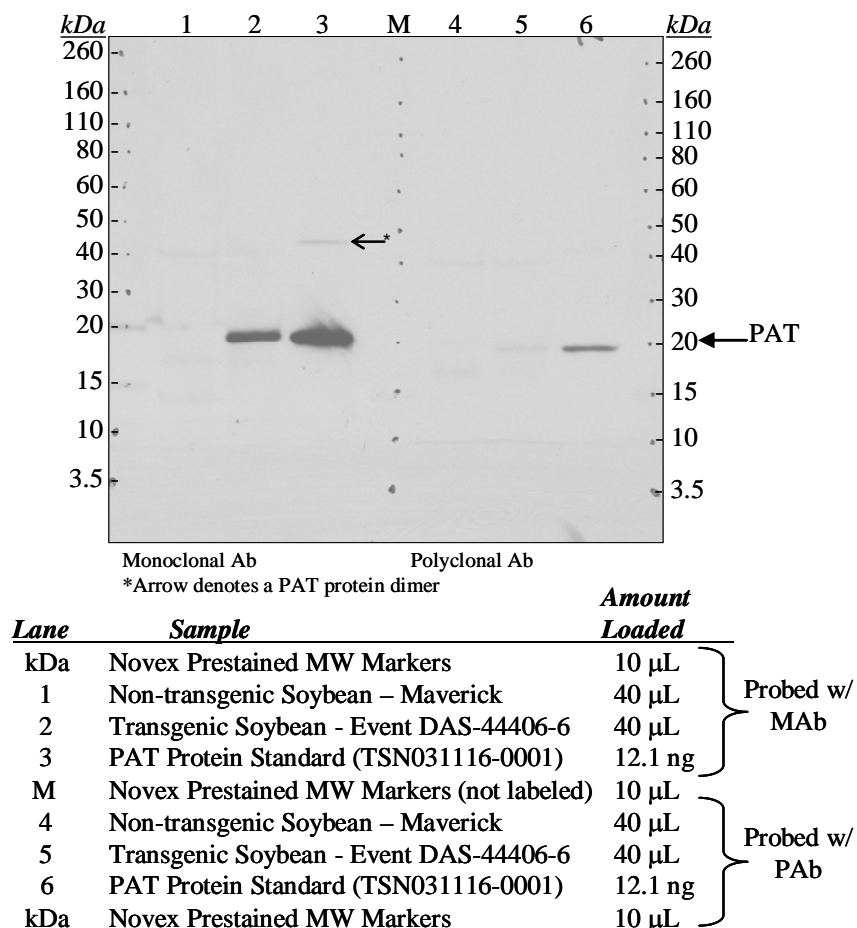
Amount	Ingredient	Catalog Numbers
5.83 mL	Phosphate Buffered Saline with 0.5% Tween ^a 20, pH 7.4	Sigma Cat #: P3563
60 µL	0.5 M EDTA	AccuGENE Cat #: 51234
100 µL	Protease inhibitor cocktail	Sigma Cat #: P8849
10 µL	β-mercaptoethanol	Bio-Rad Cat#: 161-0710

Note: the extraction buffer was prepared immediately before use.



Note: SDS-PAGE analysis of the transgenic (DAS-44406-6) and non-transgenic Maverick soybean extracts was performed with Bio-Rad Criterion gels fitted in a Criterion Cell gel module. Extracts were prepared by grinding ~34 mg of tissue for 3 minutes in a Geno-Grinder with steel ball bearings in a PBST based buffer. The supernatants were clarified by centrifuging for 5 minutes at 20,000 \times g, and then 120 μ L of each extract was mixed with 30 μ L of 5x Laemmli sample buffer (LSB). Samples were heated for 5 minutes at ~95 $^{\circ}$ C, and after a brief centrifugation, 40 μ L of the supernatant was loaded directly on the gel. The reference standard, microbe-derived PAT (TSN031116-0001), and control standard, BSA, were diluted with Bio-Rad 2x LSB containing 5% 2-mercaptoethanol and processed as described earlier. The electrophoresis was conducted at a constant voltage of 150 V for ~65 minutes using MES running buffer. After separation, the gel was stained with Thermo Scientific GelCode Blue protein stain and scanned with a densitometer to obtain a permanent record of the image.

Figure 1. SDS-PAGE of transgenic soybean Event DAS-44406-6 and non-transgenic Maverick



Note: After the proteins were separated by SDS-PAGE as described in Figure 1, the gel was electro-blotted to a nitrocellulose membrane with a Criterion trans-blot transfer cell for ~60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was cut in half and one half was probed with a PAT specific polyclonal rabbit antibody (Lot #: C98420) and the remaining half was probed with a PAT specific monoclonal antibody (Lot #: 200.624-2-5). A conjugate of goat anti-rabbit IgG (H+L) horseradish peroxidase and goat anti-mouse IgG (H+L) horseradish peroxidase were used as the secondary antibodies, respectively. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to Thermo Scientific CL-XPosure detection film for various time points and subsequently developed with a Radiation Services film developer (Model #: All-Pro 100 Plus).