SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Method Validation for the Determination of Phosphinotricin Acetyltransferase (PAT) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

DATA REQUIREMENTS

NA

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STUDY COMPLETED ON

2-June-2009

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Regulatory Sciences and Government Affairs
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081022

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- The efficiency of the tissue extraction process was determined by comparison of five sequential extractions. The apparent extraction efficiency was based on the amount of PAT protein in the first extract relative to the total PAT in all five extracts. The mean extraction efficiency for soybean tissues ranged from 87.6% to 98.9%.

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- Equivalence of standard and test substance response in the PAT ELISA was evaluated using serial dilutions of extracts of forage (whole plant), root, V5 leaf, and seed positive tissues. For each tissue extract, six to seven of the eight dilutions fell within the quantitative range of the standard curve, and the %CV of the quantified results was less than 10%.

- False-positive and false-negative rates of the ELISA method were tested using unfortified control samples (matrix blanks) and samples fortified at 0.025 ng/mg. There were no false positives from the unfortified control samples and no false negatives from the 0.025 ng/mg fortified samples analyzed during this study.

Based on the results of this study, it is concluded that the PAT ELISA method is suitable for quantitative measurement of the PAT protein in soybean tissue.

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DATA REQUIREMENTS

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Phosphinotricin Acetyltransferase

Title: Method Validation for the Determination of Phosphinotricin Acetyltransferase (PAT) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

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

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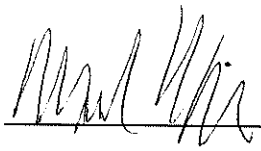
Signature: 

Date: 31 March 2009

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

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

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Method Validation for the Determination of Phosphinotricin Acetyltransferase (PAT) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

Study Initiation Date: 03/25/2008

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.

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Dow AgroSciences Quality Assurance Unit
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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.

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Method Validation for the Determination of Phosphinotricin Acetyltransferase (PAT) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

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The objective of this study was to validate the Dow AgroSciences Method GRM 08.05; a direct, double-antibody sandwich enzyme linked immunosorbent assay (ELISA) for measurement of the PAT protein as expressed in the tissues of transgenic soybean plants. The validation included the evaluation of assay sensitivity, specificity, extraction efficiency, accuracy, precision and ruggedness. Soybean tissues included in the validation were as follows: V5 leaf, V10 leaf, forage (whole plant) at R3 growth stage, root at R3 growth stage, and seed.

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Based on the results of this study, it is concluded that the PAT ELISA method is suitable for quantitative measurement of the PAT protein in soybean tissue.

SCIENTIFIC TERMS AND ABBREVIATIONS

AAD	aryloxyalkanoate dioxygenase
°C	degrees Celsius
µg	microgram (10^{-6} g)
µL	microliter (10^{-6} L)
CV	coefficient of variation
DAS	Dow AgroSciences LLC
DW	dry weight
EE	extraction efficiency
ELISA	enzyme linked immunosorbent assay
g	gram
LOD	limit of detection
LOQ	limit of quantitation
mg	milligram (10^{-3} g)
mL	milliliter (10^{-3} L)
M	molar
mM	millimolar
ng	nanogram (10^{-9} g)
OD	optical density
PAT	Phosphinothricin Acetyltransferase
PBST	phosphate buffered saline with 0.05% Tween 20
QC	quality control
R3 Stage	soybean growth stage- reproductive growth stage where pod is 5 mm long at one of the four uppermost nodes on the main stem with a full developed leaf.
RSD	relative standard deviation (equivalent to CV)
SGN	sample group number
STD	standard

STDEV	standard deviation
TSN	test substance number
V5	soybean growth stages – vegetative growth stage where plant has six nodes with unfolded leaflets
V10	soybean growth stages – vegetative growth stage where plant has ten nodes with unfolded leaflets
WP	whole plant; stem and leaf together at the R3 stage; also called forage

INTRODUCTION

Soybean plants have been modified by the insertion of an herbicide tolerant gene (pat) from *Streptomyces viridochromogenes* which encodes for the phosphinotricin acetyltransferase (PAT) protein. When expressed in soybean plants, this protein confers tolerance to glufosinate ammonium herbicides.

A rapid, selective, and sensitive method for monitoring protein levels in plant and related products is of significant importance for registration, quality control, and other relevant studies. Immunoassay has been shown to be a useful tool for such a need (1).

An enzyme-linked immunosorbent assay (ELISA) has been developed for the determination of PAT protein expressed in various soybean tissues. A validation study is needed to define important method parameters such as accuracy, precision, specificity, quantitative range, and limit of detection. The method will be used to analyze soybean tissue samples from Dow AgroSciences (DAS) Regulatory Laboratories and will support registrations.

MATERIALS AND METHODS

Materials

Test substances

The test substances were representative soybean tissue samples which were genetically modified to express the AAD-12 and PAT proteins, and non-transgenic control soybean of the variety Maverick. The tissues, listed below, were collected from the greenhouse. The presence or absence of the PAT protein in the transgenic or control plants was confirmed with specific immunoassay or bioassay.

List of non-transgenic soybean samples:

SGN	Tissue	Sample Description
081008-001-0001	Forage (Whole plant; leaf and stem; R3)	Maverick
081008-004-0001	Root (R3)	Maverick
081008-009-0001	Seed	Maverick
081008-010-0001	Leaves (V5)	Maverick
081008-011-0001	Leaves (V10)	Maverick

List of transgenic soybean samples:

SGN	Tissue	Description
081008-003-0001	Forage (Whole Plant; leaf and stem; R3)	PAT
081008-006-0001	Root	PAT
081008-007-0001	Leaves (R7)	PAT
081008-012-0001	Seed	PAT
081008-013-0001	Leaves (V5)	PAT
081008-014-0001	Leaves (V10)	PAT (QC)

Reference Substances

The reference substances employed in this study were a purified PAT protein used as a calibration standard and as fortification material in the ELISA analysis, a purified AAD-1 protein, and a purified AAD-12 protein used to test for cross reactivity.

Characterization of the reference standards and documentation of the source is located on file at Dow AgroSciences, LLC archives.

Protein	TSN or lot #	Purity or Concentration	Reference
PAT	105742	0.3 mg/mL	BIOT 063302
AAD-1	105930	36.1%	BIOT 08-162945
AAD-12	030732	35.3%	BIOT 08-161536

All test and reference substances were stored in temperature monitored freezers, and removed only for sample preparation and analysis.

Methods

Test System

The test system for this study was the PAT protein ELISA. A specific sandwich ELISA kit (Catalog Number AP 014 NW V10) developed by Envirologix Inc. (Portland, ME) was used to quantify levels of PAT protein in genetically modified soybean tissues. A method, GRM 08.05, was developed specifically for the quantitation of the PAT protein in the tissues of transgenic soybean plants (see Appendix A). Briefly, PAT protein was extracted from soybean tissues with a phosphate buffered saline solution containing a detergent Tween-20 and polyvinylpyrrolidone (PBST/PVP). The extract was centrifuged and the aqueous supernatant was collected. It was then diluted for assay using the PAT enzyme-linked immunosorbent assay (ELISA) kit. An aliquot of the diluted sample was incubated with the enzyme-conjugated anti-PAT monoclonal antibodies in the wells of a 96-well plate coated with anti-PAT protein polyclonal antibodies in a sandwich ELISA format. At the end of an incubation period, the unbound reagents were removed from the plate by washing with PBST. The presence of PAT was detected by the addition of an enzyme substrate, generating a colored product. The resulting color intensity measured as optical density (OD) is relative to the concentration of PAT in the sample. (i.e., lower protein concentration results in lower color development).

Sensitivity and Quantitative Range

The preliminary quantitative range for the method was established independently during method development and a pre-validation study at DAS Biotechnology Regulatory Science laboratory. The standard concentrations provided the lowest mean percent errors for the given concentration points.

The limit of detection (LOD) and limit of quantitation (LOQ) for the determination of PAT in each tissue were empirically defined on the basis of assay parameters (absorbance, background, and linear range), matrix interferences and/or doses constituting the standard curve. They were also supported by statistical approaches following the method of Keith et al. (2) and by testing each control sample fortified with 0.6 ng/mL (0.06 ng/mg) of PAT protein.

Specificity

Cross-reactivity. The cross-reactivity of this PAT ELISA to non-target proteins Cry1Ab, Cry1Ac, Cry1F, Cry34Ab1, Cry35Ab1, and BAR has been reported in previous study (3). Additional cross-reactivity against non-target protein AAD-1 and AAD-12 was tested in this study. These proteins were prepared at a concentration range from 0 ng/mL to 10,000 ng/mL in PBST/PVP. On the same plate, a PAT standard curve was generated as a reference. The OD responses for the non-target proteins was interpolated from the PAT standard curve and percent cross-reactivity was calculated using the following formula:

% cross-reactivity = 100 x (measured conc. by PAT std curve/theoretical conc. of target protein).

RC_{50} (concentration of analyte giving 50% response) was calculated by plotting absorbance against the logarithm of analyte concentration, which was fitted to a four-parameter logistic equation: $y = \{(A-D)/[1+(x/C)B]\} + D$ where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the RC50, and D is the minimum absorbance with no analyte.

Matrix Testing

Sample extracts (matrix) for each soybean tissue (1X, 3X and 9X dilutions) of negative control were spiked with different concentrations to create standard curves. The matrix-spiked standard curves were interpolated from a non-spiked standard curve run on the same plate. A difference of greater than 15% between the observed (a non-spiked standard curve used to interpolate the matrix-spiked standard concentrations) and theoretical (concentration of the matrix-spiked standard curve) means for each standard concentration level was considered indicative of a potential matrix effect.

Extraction Efficiency

A series of five extractions were performed on transgenic soybean tissues known to express PAT. Briefly, 1.5 mL of buffer was added to the tissue sample (15 mg) and extracted as described in the method (Appendix A). Following extraction and centrifugation, the extracted

solution was removed by pipette. After the first extract, an aliquot of 200 µL of buffer was added and mixed with the sample, centrifuged and the supernatant removed and added to the first extraction solution. Another 1.5 mL of buffer was added to the tissue, and the extraction process was repeated. This procedure was repeated three more times to obtain 5 consecutive extractions. The concentration of PAT in each extraction was determined using the PAT ELISA test kit. At least five replicates were studied for each tissue sample. The apparent efficiency of the tissue extraction process was determined by comparison of the PAT protein in the first extract relative to the total PAT protein in all five extracts.

Accuracy

The accuracy of the method was determined by measuring the recovery of the PAT protein from negative control matrices spiked with low (0.06 ng/mg DW), midpoint (0.12 and 0.36 ng/mg DW) and high (0.96 ng/mg DW) levels of PAT protein. A minimum of five replications for each concentration was analyzed. The accuracy of the assay was indicated as percent of recovery. Recoveries between 70-120% were considered acceptable (1).

Precision

The precision of the method was determined using the results of fortified soybean control samples analyzed by two analysts on multiple days. The control sample extracts were fortified with three levels of PAT standard (0.06 ng/mg, 0.12 ng/mg, and 0.36 ng/mg). Each level of fortified extract was run in triplicate on each ELISA plate. The mean recovery concentration, standard deviation (stdev), and percent coefficient of variation (%CV) were calculated for each of the samples.

Positive samples (forage (whole plant) and root) were tested for precision as well. The mean predicted concentration, standard deviation (stdev), and percent coefficient of variation (%CV) were calculated for each sample. Within and across day precision were calculated.

Equivalence of Standard and Test Substance Response in the ELISA

The purpose of this experiment was to verify that the PAT protein standard and the PAT protein in plant extracts exhibited a similar overall response in the ELISA. This was done for all transgenic tissues by assessing the agreement of the results from the dilution of a single extract interpolated from the quantitative range of the standard curve. The coefficient of variation for the interpolated results from all quantifiable dilutions was calculated for each tissue type.

False Positive and False Negative

Seed, leaf, forage (whole plant) and root tissues were tested for false-positive and false-negative occurrences. Fifteen unfortified control samples and fifteen samples fortified at 0.025 ng/mg were analyzed for each tissue to determine false-positive and false-negative rates. A false-positive result occurs when residue at or above the established LOD is found in a sample known to be free of analytes. A false negative occurs when no residue is detected in a sample fortified at the LOD.

Data Analysis

ELISA readings were recorded from a MAXline Vmax Microplate Reader using SOFTmax PRO software program. Concentration data were transferred to SAS, JMP or Microsoft Excel for calculations of mean, percent error, statistical mean, standard deviation, and %CV. Example calculations are provided in Appendix A.

RESULTS AND DISCUSSION

Calculated Limits of Quantitation and Detection

The limit of detection (LOD) of an immunoassay is defined as the analyte concentration that gives a response which has a statistically significant difference from the response of a zero analyte sample. Limits of quantitation (LOQ), or the working range of an assay, are generally defined as the highest and lowest concentrations which can be determined with an acceptable

degree of precision. In this study, the targeted LOD and LOQ for the determination of PAT in each tissue were empirically defined on the basis of assay parameters (such as absorbance, background, signal-to-noise ratio, and linear range), matrix interferences, and the standard curve concentrations. The LODs and LOQs were also determined by statistical approaches (2). Following established guidelines, the LOD and LOQ were calculated using the standard deviation from the 0.60 ng/mL recovery results. The LOQ was calculated as ten times the standard deviation (10*s*), and the LOD was calculated as three times the standard deviation (3*s*) of the results of the analysis of a minimum of 5 samples per matrix. The calculated results and target LODs and LOQs for each tissue are summarized in Appendix A-Table 1. The target LOD is 0.06 ng/mg (0.6 ng/mL) for all soybean matrices. The target LOQ is 0.12 ng/mg (1.2 ng/mL) for all soybean matrices.

Specificity

Cross-reactivity and Interference

Several relevant proteins such as Cry1Ab, Cry1Ac, Cry1F, Cry34Ab1, Cry35Ab1 and BAR were tested for cross reactivity using the Envirologix Inc. ELISA plate assay under study 021335 (3). Additionally, AAD-12 and AAD-1 were tested for cross reactivity. No cross reactivity was observed at the concentrations tested for these proteins (10,000 ng/mL) except the BAR protein, which had a slight cross reactivity of 1.3% (Table 1).

Matrix Effect

The results of the matrix tests are summarized in Table 2. A difference of greater than 15% between the observed and theoretical means for any of the seven standard concentration levels was considered indicative of a matrix effect. No matrix effects were found at the 3X and 9X spiked-matrix levels for V5 leaf, V10 leaf, forage (whole plant), root and seed. However, matrix effects were found in V5 and forage (whole plant) tissues at the 1X level. A 2X dilution is recommended for all matrices.

Extraction Efficiency

Determining total PAT protein levels in a sample is critical for examining extraction efficiency. Positive samples were extracted with extraction buffer five consecutive times and the PAT protein concentration in each extract was determined by ELISA. The apparent extraction efficiency was based on the amount of PAT protein in the first extraction relative to the total amount of PAT in all five extractions. The extraction efficiencies of the PAT protein from soybean tissues are shown in Appendix A-Table 2. The extraction efficiencies for forage (whole plant), root, seed, V5 and V10 leaf ranged from 83.1-99.6%%.

Accuracy

The mean recovery levels of PAT from all tissues when fortified at levels equating to the LOQ, mid- and high-points of the standard curve are shown in Appendix A-Table 3. Spiked at the LOQ level or above, all tissues were within the 70-120% specification for the mean recovery with percent coefficient of variances (%CVs) at or below 17.1% (Appendix A-Tables 3-8).

Ruggedness/Precision

The precision data from tissue extract fortified at four levels are shown in Appendix A-Tables 9-10. The intra-day precision of the assay was less than or equal to 12.1%, 6.7% and 10.0% %CV for the forage (whole plant) tissue fortified with 0.36, 0.12 and 0.06 ng/mg, respectively. Positive forage (whole plant) and root samples were also tested for assay ruggedness. The intra-day precision of the assay was less than or equal to 5.9% and 15.2% for the forage (whole plant) and root, respectively.

The inter-assay precision across all days and analysts was 13.0%, 11.7%, and 8.3% for the extracts fortified at 0.36, 0.12 and 0.06 ng/mg, respectively. The inter-assay ruggedness across days and analysts was 4.7% and 11.2% for forage (whole plant) and root, respectively.

Equivalency

Equivalence of standard and test substance response in the PAT ELISA was demonstrated using up to eight serial dilutions of extracts from PAT positive tissues. For each tissue extract, six to seven of the dilutions fell with in the quantitative range of the standard curve, and the %CV of the quantified results was less than 10% (Appendix A Table 11).

False-Positive/False-Negative Rate

Unfortified control samples (matrix blanks) and samples fortified at 0.25 ng/mg (LOD=0.06 ng/mg) were analyzed to determine the false-positive and false-negative rate. There were no false positives from the unfortified control samples and no false negatives reported from the LOD fortified samples analyzed in this study.

CONCULSIONS

Dow AgroSciences LLC analytical method GRM 08.05, "Method Validation for the Determination of Phosphinotricin Acetyltransferase (PAT) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)", has been demonstrated to be suitable for its intended purpose. The method was validated over the concentration range of 0.06 to 0.96 ng/mg dry weight (DW) and has a validated limit of quantitation (LOQ) in all soybean tissues of 0.12 ng/mg DW. The PAT protein was recovered at acceptable levels from all tissues. The validated assay is specific for PAT protein when compared to the non-target proteins tested in previous studies. No significant matrix effects were detected for all soybean tissues at a 3X dilution, and a 2X dilution is recommended to eliminate potential matrix effects. In addition, PAT protein was efficiently extracted from all soybean tissues. The assay was shown to have acceptable accuracy and precision, and no false-positive or false-negative results were seen below the target LOD. This PAT ELISA method has been demonstrated to be suitable for quantitative measurements of the PAT protein in soybean tissue.

ARCHIVING

The protocol, raw data, and the original version of the final report are all filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, Indiana 46268-1054.

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Table 1. Summary of Cross-reactivity for PAT Assay

Protein	Lot# or TSN	RC_{50}[a] (ng/mL)	Cross-reactivity (%)[c]
PAT[b]	TSN 101850	9.4	--
BAR[b]	TSN 102718	735.2	1.3
Cry35Ab1[b]	TSN 104066	> 10,000	0
Cry1F[b]	TSN 103881	> 10,000	0
Cry1Ab[b]	MR818 970214	>10,000	0
Cry1Ac[b]	TSN 102337	> 10,000	0
Cry34Ab1[b]	TSN 104097	> 10,000	0
AAD1	TSN 105930	> 10,000	0
AAD12	TSN 030732	> 10,000	0

[a] RC_{50}: The concentration of analyte giving a 50% ODmax response
[b] Data from Study 021335
[c] Cross-reactivity: (RC_{50} of PAT/RC_{50} of target protein) X 100

Table 2. Summary of Matrix Effects

Tissue	SGN#	Matrix Dilution[a]			Lowest dilution w/o matrix effect
		1X	3X	9X	
V5 Leaf	081008-010-0001	Yes	No	No	1:2
V10-12 Leaf	081008-011-0001	No	No	No	1:2
R3 Forage (whole plant)	081008-001-0001	Yes	No	No	1:2
R3 Root	081008-004-0001	No	No	No	1:2
R8 Seed	081008-009-0001	No	No	No	1:2

[a] "Yes" represents a standard curve is affected by matrix when the mean percent error between the observed and theoretical values for all the seven standard concentration levels is greater than 15%. "No" represents no matrix effects or the mean percent error between the observed and theoretical values for all the seven standard concentration levels is less than 15%.

APPENDIX A – GRM 08.05

GRM: 08.05
EFFECTIVE: 2-June-2009
SUPERSEDES: NEW

Determination of Phosphinothricin Acetyltransferase (PAT) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay

J. Smith-Drake, M. Sosa, and G. Shan

1. SCOPE

This method is applicable for the quantitative determination of Phosphinothricin Acetyltransferase (PAT) protein expressed in soybean tissues using an enzyme-linked immunosorbent assay (ELISA) kit. The calibration standard curve quantitative range is from 0.25 ng/mL to 6.0 ng/mL in buffer. The PAT protein level in soybean seed, V5 leaf, V10 leaf, root and forage (whole plant) at R3 stages can be determined with a validated limit of quantitation (LOQ) of 0.12 ng/mg and a limit of detection (LOD) of 0.06 ng/mg.

2. PRINCIPLE

An analytical method has been developed for the determination of PAT protein expressed in soybean plants. The PAT protein is extracted from soybean samples with a phosphate buffered saline solution containing 0.05% Tween 20 and 1% polyvinylpyrrolidone (PBST/PVP). The extract is centrifuged; the aqueous supernatant is collected, diluted and assayed using a specific PAT ELISA kit. An aliquot of the diluted sample is incubated with enzyme-conjugated anti-PAT protein monoclonal antibody in the wells of an anti-PAT polyclonal antibody coated plate in a sandwich ELISA format. Both antibodies in the sandwich pair capture the PAT protein in the sample. At the end of the incubation period, the unbound reagents are removed from the plate by washing with PBST. The presence of PAT is detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate, generating a colored product. Since the PAT is bound in the antibody sandwich, the level of color development is proportional to the concentration of PAT in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm minus absorbance at 650 nm is measured using a plate reader. A calibration curve is estimated from the 7 standard concentrations using a quadratic regression equation.

3. SAFETY PRECAUTIONS

3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

3.2. Avoid contact of Stopping Solution (0.5% sulfuric acid or 1N hydrochloride acid) with skin and mucous membranes. Wear protective clothing and proper eye protection when working with this material. If this reagent comes in contact with skin, flush the affected area with water.

3.3. It is imperative that proper eye and personal protective equipment be worn when handling these reagents.

4. EQUIPMENT AND MATERIALS (Note 12.1)

4.1. Equipment

4.1.1. Balance, analytical, Model AE50, Mettler Instrument Corporation, Hightstown, NJ 08520.

4.1.2. Balance, analytical, Model AB54-S, Mettler Instrument Corporation.

4.1.3. Centrifuge, capable of holding 96-well plates, Model GR422, catalog number 11176916, Jouan, Inc., Winchester, VA 22602.

4.1.4. Centrifuge, rotor, RTR M4 Hrz 4 Place, catalog number 11175338, Jouan, Inc.

4.1.5. Centrifuge, capable of holding 2 mL Eppendorf tubes, Eppendorf–5417C, Brinkmann Instruments. Inc., Westbury, NY 11590.

4.1.6. Freezer, capable of maintaining –20 °C, Model 75F, U-Line Corporation, Milwaukee, WI 53223.

4.1.7. Freezer, capable of maintaining –80 °C, Model ULT2586, catalog number 13-989-233, Fisher Scientific, Pittsburgh, PA 15205.

4.1.8. Incubator, Precision, Economy, catalog number 51221087, Jouan, Inc.

4.1.9. Mortar, porcelain, Coors 60316, catalog number 12-961A, Fisher Scientific.

4.1.10. Pestle, porcelain, Coors 60317, catalog number 12-961-5A, Fisher Scientific.

4.1.11. Pipettor, 0.5-10 µL, catalog number p 10, Rainin, Woburn, MA 01888.

4.1.12. Pipettor, 10-100 µL, catalog number p 100, Rainin.

4.1.13. Pipettor, 10-200 µL, catalog number p 200, Rainin.

4.1.14. Pipettor, 100-1000 µL, catalog number p 1000, Rainin.

4.1.15. Pipettor, 5-50 µL, 12-channel, Finnpipette, catalog number 21-377-201, Fisher Scientific.

4.1.16. Pipettor, 50-300 µL, 12-channel, Finnpipette, catalog number 21-377-202, Fisher Scientific.

4.1.17. Pipettor, Eppendorf Maxipetter, catalog number 21-278-43C, Fisher Scientific.

4.1.18. Pipettor, Eppendorf 0.5-10 µL, catalog number 05-402-45, Fisher Scientific.

4.1.19. Pipettor, Eppendorf 10-100 µL, catalog number 05-402-48, Fisher Scientific.

4.1.20. Pipettor, Eppendorf 100-1000 µL, catalog number 05-402-50, Fisher Scientific.

4.1.21. Pipettor, Eppendorf 500-5000 µL, catalog number 05-402-91, Fisher Scientific.

4.1.22. Pipettor, Eppendorf 8 channel, 30-300 µL, catalog number 13-688-502, Fisher Scientific.

4.1.23. Pipettor, Eppendorf 12 channel, 10-100 µL, catalog number 13-688-504, Fisher Scientific.

4.1.24. Pipettor, Eppendorf 12 channel, 30-300 µL, catalog number 13-388-505, Fisher Scientific.

4.1.25. Pipet Aid, portable, catalog number 13-681-19, Fisher Scientific.

4.1.26. Plate reader, MAXline Vmax microplate reader with SOFTmax PRO software, capable of reading 450 and 650 nm, catalog number 0200-2018, Molecular Devices, Sunnyvale, CA 94089

4.1.27. Refrigerator, capable of maintaining 4 °C, catalog number 13-991-86, Fisher Scientific.

4.1.28. Shaker/Grinder, Model Geno/Grinder, catalog number 2000-115, Certiprep, Metuchen, New Jersey 08840.

4.1.29. Stir plate, Model 220T, catalog number 14-493-220T, Fisher Scientific.

4.1.30. Vortex, Genie-2 Model, catalog number 12-812, Fisher Scientific.

4.1.31. Washer, 96-well microplate, Model Elx 405, Bio-Tek Instruments, Inc., Winooski, VT 05404.

4.1.32. Water purification system, Model Milli-Q UV Plus, Millipore Corporation, Milford, MA 01757.

4.1.33. Wiley Mill, Intermediate Thomas Wiley Cutting Mill, catalog number 08-338, Fisher Scientific.

4.2. Materials

4.2.1. Basin, Reagent, non-sterile, catalog number 13-681-100, Fisher Scientific.

4.2.2. Bead, 1/8" chrome steel, catalog number 039347, Small Parts Inc., Miami Lakes, FL 33014-0650.

4.2.3. Cap, for 2.0-mL conical tube, catalog number 02-681-361, Fisher Scientific.

4.2.4. Pipet, 10-mL disposable serological, catalog number 13-678-11E, Fisher Scientific.

4.2.5. Pipet tip, 10-µL, Continental Lab Products, catalog number 21-102-8, Fisher Scientific.

4.2.6. Pipet tip, 200-µL, Costar, catalog number 07-200-300, Fisher Scientific.

4.2.7. Pipet tip, 200-µL, Eppendorf, catalog number 21-371-3, Fisher Scientific.

4.2.8. Pipet tip, 1000-µL, Eppendorf, catalog number 21-372-4, Fisher Scientific.

4.2.9. Pipet tip, 1000-µL, Fisher Scientific, catalog number 21-197-8A, Fisher Scientific.

4.2.10. Pipet tip, 5000-µL, Maxitip, Eppendorf, catalog number 21-379-50, Fisher Scientific.

4.2.11. Plate, 96-well, non-binding for sample dilution, catalog number 14-245-145, Fisher Scientific.

4.2.12. Plate cover, 96-well, catalog number 07-200-375, Fisher Scientific.

4.2.13. Plate stand, 96-well, catalog number Z36, 335-9, Sigma, St. Louis, MO 63178.

4.2.14. Tubes, 1.2-mL polypropylene cluster, 96 tubes per rack, catalog number 7200320, Fisher Scientific.

4.2.15. Tube, 2.0-mL conical polypropylene Eppendorf micro-centrifuge, catalog number 02-681-344, Fisher Scientific.

4.2.16. Tube, 5-mL polypropylene centrifuge with cap, catalog number 14-959-11A, Fisher Scientific.

4.2.17. Tube, 15-mL polypropylene centrifuge with cap, catalog number 05-538-59A, Fisher Scientific.

4.2.18. Tube, 50-mL polypropylene centrifuge with cap, catalog number 05-526B, Fisher Scientific.

4.2.19. Weigh dish, small, catalog number 02-204A, Fisher Scientific.

5. REAGENTS, STANDARDS AND PREPARATION

5.1. Reagents (Note 12.1)

5.1.1. PAT Microtiter Plate ELISA Test Kit, catalog number AP 014 NW V10, Envirologix Inc., Portland, Maine 04103 (Note 12.2). Store at 2-8 °C. Contents:

a. Antibody coated 96-well microtiter plates
b. PAT Antibody Conjugate (1x solution)
c. Substrate Solution (120 mL)
d. Stop Solution (120 mL)
e. PAT Microtiter Plate ELISA Assay User's Guide (Reference 13.1)

5.1.2. PBST, pH 7.4, packets for making 1L, catalog number P-3563, Sigma. Store at 2-8 °C.

5.1.3. Polyvinylpyrrolidone (PVP), molecular weight 40,000, catalog number PVP-40, Sigma.

5.2. Standards

5.2.1. Obtain PAT microbial protein from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268.

5.2.2. If needed, quality control samples (positive and negative) may be obtained from Dow AgroSciences LLC, 9330 Zionsville Road, Building 306, Indianapolis, IN 46268.

5.3. Reagent and Standard Preparations

5.3.1. Phosphate Buffered Saline, pH 7.4, with 0.05% Tween 20 (PBST) (Note 12.3)

a. Add one packet to 1.0 liter of de-ionized water.
b. Add a stir bar and mix to dissolve on the stir plate.
c. Store at 20-25 ºC for up to 2-3 months or at 2-8 ºC for a maximum of 6 months. Discard if any visible contamination is observed.

5.3.2. Phosphate Buffered Saline, pH 7.4, with 0.05% Tween 20 plus 1% PVP (w/v) (PBST/PVP):

a. Add 10 g PVP to 1.0 liter PBST in the container (Section 5.3.1).
b. Add a stir bar and mix on stir plate to dissolve.
c. Store at 2-8 ºC for a maximum of 3 months. Discard if any visible contamination is observed.

5.3.3. PAT Stock Solution 3,000 ng/mL and 100 ng/mL

a. The starting PAT standard may be lyophilized powder or aliquoted liquid stock solutions. For example, one stock solution used in DAS Regulatory Laboratories is a 0.3-mg/mL solution.
b. Vortex the stock solution and then add 10 µL of the 0.3-mg/mL PAT stock solution into 990 µL of PBST/PVP and mix well to make the 3000-ng/mL stock solution. Similarly, add 40 µL of the 1000-ng/mL stock solution into 1160 µL of PBST/PVP and mix well to make the 100-ng/mL stock solution. Keep them on ice and use within 2 hours. Discard if any visible contamination is observed.

5.3.4 Fortification Solutions

Dilute appropriate aliquots of the 100 ng/mL stock solution to volume with PBST/PVP in 15-mL tubes to obtain the desired concentrations for the fortification of recovery samples, as shown in the table below[a]:

Initial Stock Soln. Conc.	Aliquot of Stock Soln.	Buffer Vol. Added	Final Soln. Volume	Spiking Soln. Final Conc.	Equivalent Sample Conc[b]
ng/mL	mL	mL	mL	ng/mL	ng/mg
100	0.96	9.04	10.0	9.60	0.96
100	0.36	9.64	10.0	3.60	0.36
100	0.12	9.88	10.0	1.20	0.12
100	0.06	9.94	10.0	0.60	0.06
0	0	10.00	10.0	0.00	0

[a] Spiking solutions can be stored on ice for up to 2 hours after preparation.
[b] The equivalent sample concentrations are based on fortifying the 15-mg samples with 1.5 mL of spiking solutions.

6. INSTRUMENT SETTINGS

To obtain results from the PAT ELISA kit, use the following parameter settings on Microplate Reader:

Parameter	Reader Abbreviation	Setting
Read Mode	Read Mode	Endpoint
Data Reduction	Data Reduction	quadratic
Number of Standard Replicate		3
Number of Standards		7
Control	Ctl	0
Standard #1 Concentration	Std01	6
Standard #2 Concentration	Std02	4.8
Standard #3 Concentration	Std03	3.6
Standard #4 Concentration	Std04	2.4
Standard #5 Concentration	Std05	1.2
Standard #6 Concentration	Std06	0.6
Standard #7 Concentration	Std07	0.25
Minimum Correlation	Correlation Flag	0.990
Wavelength	Dual Wavelength	450 nm-650 nm
Data Mode	Data Mode	Absorbance
Units	Units	ng/mL
Precision of Standards	Rep %CV Flag	15

7. DETERMINATION OF RECOVERY OF PAT PROTEIN IN SOYBEAN TISSUES

7.1. Preparation of Recovery Samples

7.1.1. Extract the recovery samples at the same time and manner as the unknowns. Store the recovery extracts in the same manner as the unknown samples.

7.1.2. Soybean tissues are stored frozen at –80 ºC until lyophilized. After lyophilization, samples are ground and then stored in a –80 ºC freezer until weighed for analysis.

7.1.3. For all sample types, weigh 15-mg portions of the prepared control soybean tissue samples and dispense into 2-mL polypropylene tubes. Add two or three metal beads to each tube. For laboratory recovery samples, add 1.5 mL of the appropriate spiking/extraction solution from Step 5.3.4. A reagent blank and a control should be carried through the method with each sample set. The reagent blank contains 1.5 mL of PBST/PVP extraction solution.

7.1.4. Cap all of the tubes. Extract the samples using the Geno/Grinder automatic shaker/grinder at a dial setting of 500 and the toggle switch at the 1X setting (approximately 1500 strokes per minute) for 3 minutes as one cycle.

7.1.5. Centrifuge the samples at 3,000 (or greater) rpm for 5 minutes or until separated (no visible particles in the supernatant). The supernatant can be transferred to a separate tube or aliquoted for analysis as described in Section 7.2. Keep the extract on ice and assay it within 4 hours.

7.1.6. Assay each sample according to the procedure described in Section 7.2.

7.2. Assay Procedure

7.2.1. PAT ELISA Kit Preparation (See Note 12.2 and Reference 13.1)

Allow the ELISA microtiter plate to equilibrate to 20-25 °C by removing from the refrigerator at least 30 minutes prior to performing the assay. Keep reagents on ice or in refrigerator.

7.2.2. Standard Calibration

Prepare standard calibration solutions in 5-mL polypropylene tubes by diluting the 100 ng/mL stock solution from Section 5.3.4 with PBST/PVP as follows. The following example preparation provides enough standard for 1 plate. Adjust volumes as necessary for additional plates. Store tubes on the benchtop on ice and use within 2 hours of preparation.

Conc. of Stock Soln. (ng/mL)	Aliquot of Stock Soln (µL)	Starting Buffer Volume (µL)	Final Soln. Volume (µL)	Final Standard Conc. (ng/mL)	Remaining Volume after Aliquot (µL)
100	105	1645	1750	6.00	550
6.00	1200	300	1500	4.80	600
4.80	900	300	1200	3.60	500
3.60	700	350	1050	2.40	600
2.40	450	450	900	1.20	500
1.20	400	400	800	0.60	550
0.60	250	350	600	0.25	600
0	0	500	500	0	500

7.2.3. ELISA Analysis

7.2.3.1. Conduct each test in an individual microtiter plate. The average of duplicate analyses of a sample or standard constitutes a single result. A calibration curve and the appropriate control must be included in each plate.

7.2.3.2. Transfer the ELISA standard calibration solutions from Step 7.2.2 to a non-binding 96-well microtiter plate (100 µL/well) and record the location on the 96-well assay template sheet (Figure 1).

7.2.3.3. Prepare sample dilutions as needed and transfer diluted samples to the non-binding 96-well microtiter plate (100 µL/well) containing the standard calibration solutions and record the location on the 96-well assay template sheet (Figure 1).

7.2.3.4. Dispense approximate 6 mL of the PAT antibody conjugate per plate into a reagent basin.

7.2.3.5. Pipet 50 µL of the PAT antibody conjugate from the reagent basin to each well of the antibody coated 96-well microtiter plate. **Discard any unused PAT antibody conjugate solution.**

7.2.3.6. Add 50 µL of the ELISA standard solutions and diluted samples from the non-binding 96-well microtiter plate from Steps 7.2.3.2 and 7.2.3.3 to the antibody coated 96-well microtiter plate, keeping the same orientation as the 96-well assay template. Change pipet tips with each sample.

7.2.3.7. Cover the plate with an adhesive plate sealer. Gently swirl the ELISA plate on the benchtop or on a plate shaker for approximately ten seconds to mix the reference standards and diluted samples with the PAT antibody conjugate.

7.2.3.8. Allow the microtiter plate to incubate at room temperature (20-30 °C) for 60 minutes either on the benchtop or in a room temperature incubator.

7.2.3.9. Wash the plate five times with 350 µL/well PBST using an automatic plate washer. Tap out excess liquid on a paper towel.

7.2.3.10. Dispense approximately 12 mL of the color reagent (Substrate Solution) per plate into a reagent basin.

7.2.3.11. Pipet 100 µL of the color reagent from the reagent basin into each well of the antibody coated 96-well microtiter plate. Cover the plate and gently mix. **Discard any unused color reagent solution.**

7.2.3.12. Allow the microtiter plate to incubate at room temperature (20-30 °C) for 15-30 minutes either on the benchtop or in a room temperature incubator.

7.2.3.13. Dispense approximately 12 mL per plate of the Stop Solution into a reagent basin.

7.2.3.14. Add 100 µL of Stop Solution to each well to stop the reaction. Mix the plate gently. The addition of stop solution should be completed without interruption. Protect the microtiter plate from sunlight; otherwise, color intensity is influenced.

7.2.3.15. Read the absorbance at 450 nm minus 650 nm using a 96-well microtiter plate reader. All readings should be completed within 30 minutes of adding the stop solution.

8. DETERMINATION OF PAT PROTEIN IN SOYBEAN TISSUES

8.1. Prepare the samples as described in Step 7.1.2.

8.2. For all sample types, weigh 15-mg portions of the prepared unknown soybean samples and dispense into 2-mL polypropylene tubes. Add two or three metal beads to each tube. Add 1.5 mL of PBST/PVP extraction solution.

8.3. Extract the samples as described in Steps 7.1.4 and 7.1.5 substituting, PBST/PVP extraction solution for the spiking/extraction solution.

8.4. Assay each sample according to the procedure described in Section 7.2. If the sample contains more than 10 ng/mL of PAT protein, perform an additional dilution of the sample from Step 7.2.3.3 prior to assay (e.g., for a 1:10 dilution, pipet 125 µL of PBST/PVP onto a non-binding dilution plate, add 25 µL of sample from the Step 8.3 plate, and mix with the pipettor). Assay the diluted aliquot as described in Section 7.2.3.

9. DATA ANALYSIS AND CALCULATIONS

9.1. Calibration Curve

9.1.1. SOFTmax PRO software is available for use 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 is constructed using a quadratic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance (optical density).

9.1.2. 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

An example of a calibration curve is presented in Figure 2.

9.2. Calculation of PAT in Unknown Samples

9.2.1. The SOFTmax PRO software will calculate the concentration of PAT in each sample as noted in Section 9.1 above. 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.

9.3. Example Calculations

$$\text{Method Factor (MF)} = \frac{\text{weight of tissue (mg)}}{\text{extraction volume (mL)}}$$

Final concentration calculation:

$$\underset{\text{(ng/mg)}}{\text{PAT Concentration}} = \underset{\text{(ng/mL)}}{\text{Mean Result}} / \text{MF}$$

Example: For a 15.0-mg forage (whole plant) sample extracted with 1.5 mL of buffer, the results of two dilutions of the sample were 0.925 and 0.751 ng/mL.

$$\text{PAT concentration} = \frac{[(0.925+ 0.751)/2] \text{ ng/mL}}{15.0 \text{ mg}/1.5\text{mL}}$$

$$= 0.084 \text{ ng/mg}$$

9.4. Calculation of Percent Recovery

The percent recovery is calculated as the average of all replicate (well) concentrations divided by the fortification concentration.

$$\text{Mean \% recovery} = \frac{\text{the average of protein concentration found}}{\text{fortification concentration}} \times 100\%$$

$$\text{Recovery} = \frac{0.084 \text{ ng/mg}}{0.100 \text{ ng/mg}} \times 100\%$$

$$\text{Recovery} = 84\ \%$$

9.5. Predicted Concentration (predconc)

9.5.1. The predicted concentration is the basis for the mean percent error calculation. The predicted concentration is determined using the coefficients of the curve and optical density (OD) readings in the quadratic formula. The regression equation was applied as follows:

$$y = C_1x^2 + C_2x + C_3$$

(where x = predicted concentration and y = OD)

$$\text{predicted concentration} = \frac{-C_2 + \sqrt{C_2^{\,2} - 4C_1 * (C_3 - OD)}}{2C_1}$$

For example, given equation parameters of C_1 = -0.007, C_2 = 0.307, C_3 = 0.087, and OD = 0.388

$$\text{Predconc} = \frac{-0.307 + \sqrt{0.307^2 - 4\ (-0.007)\ (0.087 - 0.388)}}{2\ (-0.007)} = 1.005$$

9.6. Mean Percent Errors

9.6.1. Mean percent errors are determined for each standard concentration of the database curves. The percent error is calculated from the predicted concentration and the theoretical concentration (tconc).

$$\text{Mean percent error} = \left|\frac{\text{predconc} - \text{tconc}}{\text{tconc}}\right| \text{x}\,100$$

For example, given the predicted concentration from one of standard curve in method validation for the 1.00-ng/mL standard

Predconc = 1.005 ng/mL

$$\text{Mean \% error} = \left|\frac{1.005 - 1.00}{1.00}\right| \text{x}\,100 = 0.5\% \text{ error}$$

10. QUALITY CONTROL

10.1. Analytical Batch Definition

An analytical batch of samples is defined as a group of 96 wells. The size of the batch is based on the capacity of the unit (1 solid microplate) of the Envirologix PAT ELISA test kit. An analytical batch of less than 96 wells can be analyzed. The first 24 wells (well positions in columns 1, 2, and 3: A1-H1, A2-H2, A3-H3) are used for triplicate analysis of the seven concentrations of the standard. The Quality Control (QC) Sample should be included in each batch if available. Following the Quality Control Sample, up to 35 samples may be analyzed in duplicate (two wells). If more samples are to be analyzed than can be accommodated in one plate, the remaining samples should be analyzed as a different analytical batch with a new standard curve.

10.2. Study Samples

All study samples should be assayed in duplicate. If the concentration of PAT in the sample exceeds the range of the assay, dilute the sample with Assay Buffer PBST/PVP (typically a 5- or 10-fold dilution is performed) and then assay the diluted sample aliquot. Multiply the result by the appropriate method factor and dilution factor to obtain the final result.

10.3. Criteria for Acceptance of an Analytical Batch

Each run shall meet the accept criteria in the procedure to be valid as listed below. If the data fail to meet these performance criteria, the analyst should evaluate the results; determine the potential source of the variation, and repeat the analysis if necessary.

Assay Buffer Blank	Absorbance (450 nm-650 nm) < 0.120
0 ng/mL standard	Absorbance (450 nm-650 nm) < 0.120
6 ng/mL standard	Absorbance (450 nm-650 nm) ≥ 0.900
Calibration curve	r^2 (Correlation of determination) > 0.990
All positive reference standard, OD	CV (OD) of triplicates ≤ 15%
Unknown or QC samples, solution	CV (OD) of replicates ≤ 20%
Quality control samples, solution (if applicable)	Measured value ≤ ±20% expected value

10.4 Specificity

This PAT ELISA method is specific to PAT and shows no cross reactivity with other transgenic proteins such as AAD-12, AAD-1, Cry34Ab1, Cry35Ab1, Cry1Ac, Cry1Ab, and Cry1F at concentrations up to 10,000 ng/mL.

10.5 Matrix Effect

Tissue matrix effects have been evaluated by comparing standard curves that had not been fortified with matrix to those that have been fortified. Three different matrix dilutions, 1X, 3X, and 9X were tested representing dilution levels commonly used in the ELISA. A difference of greater than 15% between the observed and theoretical means for any of the seven standard concentration levels was considered indicative of a matrix effect. No matrix effects were found at the 3X and 9X spiked-matrix levels for V5 leaf, V10 leaf, forage (whole plant), root and seed. However, matrix effects were found in V5 and forage (whole plant) tissues at the 1X level. A 2X dilution or greater is recommended for all matrixes.

10.6 Modifications and Uses

Modifications to the assay are not recommended. This procedure is for use with soybean tissue samples (forage (whole plant), leaf, root and seed). Validation of method for analysis of other sample matrices should be performed prior to implementing this method for sample analysis.

11.0 RESULTS AND DISCUSSION

11.1 Method Validation

11.1.1 Calculated Limits of Detection and Quantitation

The targeted LOD and lower LOQ for the determination of PAT in each tissue were empirically defined on the basis of individual assay, matrix properties and/or concentrations of the standard curve. These LODs and LOQs were further determined by statistical approaches. Following established guidelines (13.2), the theoretical LOQ and LOD for the determination of PAT protein were calculated using the standard deviation from the results of the recovery samples fortified at 0.06 ng/mg. The theoretical LOQ was calculated as ten times the standard deviation (10s), and the theoretical LOD was calculated as three times the standard deviation (3s), of the results of a minimum of 5 samples. The results are listed in Table 1. The target LOD is 0.06 ng/mg dry weight (DW) for all soybean matrices. The target LOQ is 0.12 ng/mg DW for all soybean matrices. In all cases the target LOD's and LOQ's were higher than the values calculated using the standard deviation. In actual plant samples, numerical results should be reported and note it as less than the LOQ for samples with the PAT protein levels that are above the LOD, but less than the validated LOQ. For results less than the LOD, it should be reported as not detected.

11.1.2 Extraction Efficiency

The efficiency of the sample extraction process was determined by comparison of five serial extractions. The apparent extraction efficiency was based on the amount of PAT protein in the first extraction relative to the total amount of PAT in all five extractions. The extraction efficiencies of the PAT protein from the soybean tissues are shown in Table 2. The average extraction efficiency for soybean tissue ranged from 87.1-98.9%.

11.1.3 Standard Curve

The coefficient of determination (r^2) values for the quadratic regression equations describing the absorbance as a function of standard concentration ranged from 0.992 to 1.000 for analytical batches or plates analyzed during the method validation.

11.1.4 Accuracy

The mean recovery levels of PAT protein from sample extracts when spiked at levels equal to the upper (0.96 ng/mg DW), low or LOQ (0.12 ng/mg DW) and midpoint (0.36 ng/mg DW) are summarized in Table 3. Individual accuracy results for each matrix are listed from Table 4 thru Table 8. For results spiked at the LOQ level or above, all matrices were within the 70-120% specification for the mean recovery with a %CV ranging from 4.4% to 17.1%.

11.1.5 Ruggedness/Precision

The assay precision and ruggedness were examined using forage (whole plant) extracts containing three levels of PAT protein. The levels were 0.36 ng/mg, 0.12 ng/mg and 0.06 ng/mg. The intra-day precision of the assay was less than or equal to 12.1%, 6.7% and 10.0% for the forage (whole plant) extract fortified at 0.36, 0.12 and 0.06 ng/mg, respectively (Table 9). Positive forage (whole plant) and root samples were also tested for assay ruggedness. The intra-day precision of the assay was less than or equal to 5.9% and 15.2% for the forage (whole plant) and root, respectively (Table 9).
The inter-assay precision across all days and analysts was 13.0%, 11.7% and 8.3% for the extracts fortified at 0.36, 0.12 and 0.06 ng/mg, respectively (Table 10). The inter-assay ruggedness across days and analysts was 4.7% and 11.2% for forage (whole plant) and root, respectively (Table 10).

11.1.6 Equivalency

Equivalence of standard and test substance response in the PAT ELISA was demonstrated using up to eight serial dilutions of extracts from PAT positive tissues. For each tissue extract, six to seven of the dilutions fell within the quantitative range of the standard curve, and the CV of the quantified results was less than 10% (Table 11).

11.1.7 False-Positive/False-Negative

Non-fortified control samples (15 samples per matrix) and fifteen samples (per matrix) fortified at 0.025 ng/mg (LOD=0.06 ng/mg) were analyzed during the study to verify the false-positive and false-negative levels. A false-positive result occurs when a residue (OD value) at or above the established LOD is found in a control sample known to be free of analyte. A false-negative occurs when no residue is detected in a fortified sample. There were no false positives from the non-fortified control samples and no false negative observed from the samples fortified below the LOD.

11.2 Confirmatory Method

If needed, the detection of the PAT protein in soybean tissue samples may be confirmed by western blotting, using Dow AgroSciences, LLC, SOP ECL-27 “SDS-Page and Western Blotting”.

11.3 Assay Time

The time required to analyze a typical batch (35 samples or recoveries in duplicate, 8 standards in triplicate), including the sample extraction, is 4-5 hours.

11.4 Limitation of the Method

This ELISA method is limited to samples where the amount of PAT protein can be correlated with the level of PAT present in the microbial standard or reference material used.

12 NOTES

12.1 Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are, therefore, not listed.

12.2 The QualiPlate Kit for LibertyLink PAT/pat by EnviroLogix, Inc. was used for performing this method validation. An equivalent kit may be used as well.

12.3 PBST solution may be made from individual ingredients to achieve the same concentrations.

13 REFERENCES

13.1 EnviroLogix, Inc., QualiPlate Kit for LibertyLink PAT/pat Instructional Insert, Catalog Number AP 014 NW V10.

13.2 Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. 1983, Principals of Environmental Analysis, *Anal. Chem.*, *55*, 2210-2218.

Table 1. Summary of LOD and LOQ Calculation of PAT ELISA in Soybean Tissue

Tissue	Spiked Level ng/mg	Average Recovery ng/mg	Standard Deviation *s*	3 x *s*	Target LOD ng/mg	10 x *s*	Target LOQ ng/mg
Forage (Whole Plant)	0.06	0.056	0.003	0.009	0.06	0.03	0.12
Root	0.06	0.072	0.005	0.015	0.06	0.05	0.12
V5 Leaf	0.06	0.043	0.008	0.024	0.06	0.08	0.12
Seed	0.06	0.049	0.004	0.012	0.06	0.04	0.12
V10 Leaf	0.06	0.059	0.004	0.012	0.06	0.04	0.12

Table 2. Summary of Extraction Efficiency

Sample	SGN#	Mean Extraction Efficiency	Standard Deviation	CV%	%EE Range
Forage (Whole Plant)	081008-003-0001	94.2	1.7	1.8	92.0-96.2
Root	081008-006-0001	87.6	3.1	3.5	83.1-91.3
V5 Leaf	081008-013-0001	98.9	0.9	0.9	97.5-99.6
Seed	081008-012-0001	91.3	1.6	1.8	88.5-92.8
V10 Leaf	081008-014-0001	96.3	0.5	0.5	95.6-96.8

Table 3. Summary of Accuracy Results

Matrix	Fortification Level		Recovery Rate (%)		CV%	n
	ng/mg	ng/mL	Mean	Range		
Forage (Whole Plant)	9.6	0.96	89	80-96	7.7	5
	3.6	0.36	93	86-109	10.2	5
	1.2	0.12	94	89-106	7.2	5
	1.2-9.6	0.12-0.96	92	80-109	8.6	15
Root	9.6	0.96	103	91-113	9.7	5
	3.6	0.36	109	98-123	9.0	5
	1.2	0.12	115	103-126	7.9	5
	1.2-9.6	0.12-0.96	109	91-126	9.3	15
Leaf V5	9.6	0.96	70	58-82	12.8	5
	3.6	0.36	78	58-92	17.1	5
	1.2	0.12	80	70-94	12.0	5
	1.2-9.6	0.12-0.96	76	58-94	15.4	15
Seed	9.6	0.96	75	70-80	5.8	5
	3.6	0.36	81	68-92	11.8	5
	1.2	0.12	83	74-92	10.2	5
	1.2-9.6	0.12-0.96	82	68-92	9.0	15
Leaf V10	9.6	0.96	97	90-105	7.3	5
	3.6	0.36	93	89-99	4.4	5
	1.2	0.12	89	64-106	17.1	5
	1.2-9.6	0.12-0.96	98	64-106	9.8	15

Table 4. Recovery of PAT Protein from Forage (Whole Plant) (SGN 081008-001-0001)

Sample Number	Date of Analysis	PAT (ng/mg) Spike	PAT (ng/mg) Recovery	Percent Recovery	Statistical Calculations[a]
Control A	11 Sep 08	0.00	ND[b]		
Control B	11 Sep 08	0.00	ND		
Control C	12 Sep 08	0.00	ND		
Control D	12 Sep 08	0.00	ND		
Control E	10 Sep 08	0.00	ND		
LOD A	11 Sep 08	0.06	0.0609[c]	102	$\bar{x}$ = 0.056
LOD B	11 Sep 08	0.06	0.0580	97	s = 0.003
LOD C	12 Sep 08	0.06	0.0553	92	CV= 6.5 %
LOD D	12 Sep 08	0.06	0.0527	88	LOD(3s)[d] = 0.009
LOD E	10 Sep 08	0.06	0.0523	87	LOQ(10s)[e] = 0.03
1.2 A (LOQ)	11 Sep 08	0.12	0.1266	106	
1.2 B	11 Sep 08	0.12	0.1154[c]	96	
1.2 C	12 Sep 08	0.12	0.1080	90	$\bar{x}$ = 0.113
1.2 D	12 Sep 08	0.12	0.1072	89	s = 0.004
1.2 E	10 Sep 08	0.12	0.1090	91	CV= 7.2 %
3.6 A	11 Sep 08	0.36	0.3935	109	
3.6 B	11 Sep 08	0.36	0.3279	91	
3.6 C	12 Sep 08	0.36	0.3192	89	$\bar{x}$ = 0.334
3.6 D	12 Sep 08	0.36	0.3179	88	s = 0.008
3.6 E	10 Sep 08	0.36	0.3095	86	CV = 10.2 %
9.6 A	11 Sep 08	0.96	0.9208	96	
9.6 B	11 Sep 08	0.96	0.9087	95	
9.6 C	12 Sep 08	0.96	0.8731	91	$\bar{x}$ = 0.856
9.6 D	12 Sep 08	0.96	0.7661	80	s = 0.064
9.6 E	10 Sep 08	0.96	0.8109	84	CV = 7.7 %
			$\bar{x}$ =	92[f]	
			s =	7.9	
			CV =	8.6	
			n =	15	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.06 ng/mg.

[c] Region of less certainty (positive above limit of detection (0.06 ng/mg), but below limit of quantification (0.12 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with PAT protein at LOQ or above.

Table 5. Recovery of PAT Protein from Root (SGN 081008-004-0001)

Sample Number	Date of Analysis	PAT ng/mg		Percent Recovery	Statistical Calculations[a]
		Spike	Recovery		
Control A	11 Sep 08	0.00	ND[b]		
Control B	11 Sep 08	0.00	ND		
Control C	12 Sep 08	0.00	ND		
Control D	12 Sep 08	0.00	ND		
Control E	10 Sep 08	0.00	ND		
LOD A	11 Sep 08	0.06	0.0807[c]	135	$\bar{x}$=0.0.072
LOD B	11 Sep 08	0.06	0.0759	127	s = 0.005
LOD C	12 Sep 08	0.06	0.0712	119	(3s)[d] = 0.015
LOD D	12 Sep 08	0.06	0.0653	109	(10s)[e] = 0.05
LOD E	10 Sep 08	0.06	0.0670	112	CV = 8.8 %
1.2 A (LOQ)	11 Sep 08	0.12	0.1510	126	
1.2 B	11 Sep 08	0.12	0.1393	116	
1.2 C	12 Sep 08	0.12	0.1436	120	$\bar{x}$=0.138
1.2 D	12 Sep 08	0.12	0.1233	103	s = 0.009
1.2 E	10 Sep 08	0.12	0.1305	109	CV = 7.9 %
3.6 A	11 Sep 08	0.36	0.4412	123	
3.6 B	11 Sep 08	0.36	0.4014	112	
3.6 C	12 Sep 08	0.36	0.4000	111	$\bar{x}$ = 0.392
3.6 D	12 Sep 08	0.36	0.3512	98	s = 0.025
3.6 E	10 Sep 08	0.36	0.3651	101	CV = 9.0 %
9.6 A	11 Sep 08	0.96	1.0497	109	
9.6 B	11 Sep 08	0.96	1.0831	113	
9.6 C	12 Sep 08	0.96	1.0281	107	$\bar{x}$ = 0.986
9.6 D	12 Sep 08	0.96	0.8709	91	s = 0.102
9.6 E	10 Sep 08	0.96	0.8970	93	CV = 9.7 %
			$\bar{x}$ =	109[f]	
			s =	10.2	
			CV =	9.3	
			n =	15	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.06 ng/mg.

[c] Region of less certainty (positive above limit of detection (o.o6 ng/mg), but below limit of quantification (0.12 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with PAT protein at LOQ or above.

Table 6. Recovery of PAT Protein from V5 Leaf (SGN 081008-010-0001)

Sample Number	Date of Analysis	PAT ng/mg Spike	PAT ng/mg Recovery	Percent Recovery	Statistical Calculations
Control A	21 Oct 08	0.00	ND[b]		
Control B	21 Oct 08	0.00	ND		
Control C	21 Oct 08	0.00	ND		
Control D	21 Oct 08	0.00	ND		
Control E	23 Oct 08	0.00	ND		
LOD A	21 Oct 08	0.06	0.0403	67	$\bar{x}$=0.043
LOD B	21 Oct 08	0.06	0.0344	57	*s* = 0.008
LOD C	21 Oct 08	0.06	0.0400	67	(3s)[d] = 0.016
LOD D	21 Oct 08	0.06	0.0535	89	(10s)[e] = 0.08
LOD E	23 Oct 08	0.06	0.0469	78	CV = 17.1%
1.2 A	21 Oct 08	0.12	0.0976[c]	81	
1.2 B	21 Oct 08	0.12	0.0864	72	
1.2 C	21 Oct 08	0.12	0.0835	70	$\bar{x}$=0.096
1.2 D	21 Oct 08	0.12	0.1125	94	*s* = 0.013
1.2 E	23 Oct 08	0.12	0.0978	82	CV = 12.0%
3.6 A	21 Oct 08	0.36	0.2960	82	
3.6 B	21 Oct 08	0.36	0.2601	72	
3.6 C	21 Oct 08	0.36	0.2077	58	$\bar{x}$=0.280
3.6 D	21 Oct 08	0.36	0.3312	92	*s* = 0.054
3.6 E	23 Oct 08	0.36	0.3048	85	CV = 17.1%
9.6 A	21 Oct 08	0.96	0.7116	74	
9.6 B	21 Oct 08	0.96	0.6316	66	
9.6 C	21 Oct 08	0.96	0.5575	58	$\bar{x}$=0.673
9.6 D	21 Oct 08	0.96	0.7875	82	*s* = 0.096
9.6 E	23 Oct 08	0.96	0.6778	71	CV = 12.8%
			$\bar{x}$ =	76[f]	
			s =	11.7	
			CV =	15.4	
			n =	15	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.06 ng/mg.

[c] Region of less certainty (positive above limit of detection (o.o6 ng/mg), but below limit of quantification (0.12 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with PAT protein at LOQ or above

Table 7. Recovery of PAT Protein from Seed (SGN 081008-009-0001)

Sample Number	Date of Analysis	PAT ng/mg Spike	PAT ng/mg Recovery	Percent Recovery	Statistical Calculations
Control A	21 Oct 08	0.00	ND[b]		
Control B	21 Oct 08	0.00	ND		
Control C	21 Oct 08	0.00	ND		
Control D	21 Oct 08	0.00	ND		
Control E	23 Oct 08	0.00	ND		
LOD A	21 Oct 08	0.06	0.0416	69	$\bar{x}$=0.049
LOD B	21 Oct 08	0.06	0.0472	79	*s* = 0.004
LOD C	21 Oct 08	0.06	0.0481	80	(3s)[d] = 0.016
LOD D	21 Oct 08	0.06	0.0516	86	(10s)[e] = 0.08
LOD E	23 Oct 08	0.06	0.0569	95	CV = 11.5%
1.2 A	21 Oct 08	0.12	0.0886[c]	74	
1.2 B	21 Oct 08	0.12	0.1038	87	
1.2 C	21 Oct 08	0.12	0.0897	75	$\bar{x}$=0.100
1.2 D	21 Oct 08	0.12	0.1068	89	*s* = 0.009
1.2 E	23 Oct 08	0.12	0.1109	92	CV = 10.2%
3.6 A	21 Oct 08	0.36	0.2782	77	
3.6 B	21 Oct 08	0.36	0.2868	80	
3.6 C	21 Oct 08	0.36	0.2447	68	$\bar{x}$=0.292
3.6 D	21 Oct 08	0.36	0.3193	89	*s* = 0.039
3.6 E	23 Oct 08	0.36	0.3316	92	CV = 11.8%
9.6 A	21 Oct 08	0.96	0.6783	71	
9.6 B	21 Oct 08	0.96	0.7279	76	
9.6 C	21 Oct 08	0.96	0.6689	70	$\bar{x}$=0.717
9.6 D	21 Oct 08	0.96	0.7439	77	*s* = 0.041
9.6 E	23 Oct 08	0.96	0.7646	80	CV = 5.8%
			$\bar{x}$ =	80[f]	
			s =	7.2	
			CV =	9.0	
			n =	15	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.06 ng/mg.

[c] Region of less certainty (positive above limit of detection (o.o6 ng/mg), but below limit of quantification (0.12 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with PAT protein at LOQ or above.

Table 8. Recovery of PAT Protein from V10 Leaf (SGN 081008-011-0001)

Sample Number	Date of Analysis	PAT ng/mg Spike	PAT ng/mg Recovery	Percent Recovery	Statistical Calculations
Control A	3 Mar 09	0.00	ND[b]		
Control B	3 Mar 09	0.00	ND		
Control C	3 Mar 09	0.00	ND		
Control D	3 Mar 09	0.00	ND		
Control E	4 Mar 09	0.00	ND		
LOD A	3 Mar 09	0.06	0.0612	102	$\bar{x}$=0.059
LOD B	3 Mar 09	0.06	0.0604	101	*s* = 0.004
LOD C	3 Mar 09	0.06	0.0613	102	(3s)[d] = 0.016
LOD D	3 Mar 09	0.06	0.0578	96	(10s)[e] = 0.08
LOD E	4 Mar 09	0.06	0.0527	88	CV = 6.2%
1.2 A	3 Mar 09	0.12	0.1109[c]	92	
1.2 B	3 Mar 09	0.12	0.1107	92	
1.2 C	3 Mar 09	0.12	0.0773	64	$\bar{x}$=0.107
1.2 D	3 Mar 09	0.12	0.1088	91	*s* = 0.021
1.2 E	4 Mar 09	0.12	0.1275	106	CV = 17.1%
3.6 A	3 Mar 09	0.36	0.3328	92	
3.6 B	3 Mar 09	0.36	0.3195	89	
3.6 C	3 Mar 09	0.36	0.3439	96	$\bar{x}$=0.335
3.6 D	3 Mar 09	0.36	0.3238	90	*s* = 0.017
3.6 E	4 Mar 09	0.36	0.3555	99	CV = 4.4%
9.6 A	3 Mar 09	0.96	0.8643	90	
9.6 B	3 Mar 09	0.96	0.9364	98	
9.6 C	3 Mar 09	0.96	0.8594	90	$\bar{x}$=0.931
9.6 D	3 Mar 09	0.96	0.9848	103	*s* = 0.066
9.6 E	4 Mar 09	0.96	1.0090	105	CV = 7.3%
			$\bar{x}$ =	93[f]	
			s =	9.1	
			CV =	9.8	
			n =	15	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.06 ng/mg.

[c] Region of less certainty (positive above limit of detection (o.o6 ng/mg), but below limit of quantification (0.12 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with PAT protein at LOQ or above.

Table 9. Summary of Precision Results (Intra-day, Inter-analyst)

Spiked Concentration or Sample		Day 1	Day 2
0.36 ng/mg	Mean	0.282	0.343
	Stdev (ng/mg)	0.034	0.010
	CV%	12.1	3.0
	Range (ng/mg)	0.2577-0.3060	0.3497-0.3530
0.12 ng/mg	Mean	0.096	0.115
	Stdev (ng/mg)	0.007	0.008
	CV%	6.7	6.7
	Range (ng/mg)	0.0918-0.1010	0.1099-0.1208
0.06 ng/mg	Mean	0.048	0.054
	Stdev (ng/mg)	0.005	0.001
	CV%	10.0	2.2
	Range (ng/mg)	0.0448-0.0516	0.0527-0.0544
Forage (Whole Plant) (SGN 081008-003-0001)	Mean	14.6262	14.6989
	Stdev (ng/mg)	0.861	0.842
	CV%	5.9	5.7
	Range (ng/mg)	14.0174-15.2349	14.1036-15.2941
Root (SGN 081008-006-0001)	Mean	2.6937	2.7398
	Stdev (ng/mg)	0.409	0.329
	CV%	15.2	12.0
	Range (ng/mg)	2.4046-2.9827	2.5072-2.9724

Table 10. Summary of Precision Results (Inter-day, Inter-analyst)

Spiked Concentration or Samples	Mean ng/mg	Standard Deviation ng/mg	CV%	Range ng/mg
0.36	0.312	0.041	13.0	0.2577-0.3497
0.12	0.106	0.012	11.7	0.0918-0.1208
0.06	0.051	0.004	8.3	0.0448-0.0544
Forage (Whole Plant)	14.6625	0.696	4.7	14.0174-15.2941
Root	2.717	0.304	11.2	2.4046-2.9724

Table 11. Equivalence of Standard and Positive Tissue Sample response in the ELISA

Sample	SGN#	Number of Quantifiable Dilutions	Mean ng/mg	Standard Deviation ng/mg	CV%	Range ng/mg
Forage (Whole Plant)	081008-003-0001	6	13.853	0.69	5.00	13.1984-15.0806
Root	081008-006-0001	7	2.257	0.22	9.53	2.0637-2.6806
V5 Leaf	081008-013-0001	6	13.446	1.03	7.66	12.1021-15.1387
Seed	081008-012-0001	6	2.895	0.28	9.52	2.6170-3.3384

Figure 1 Example Template Sheet

Template Sheet

Analyst: _____ Protocol Number: _____ **ELISA Kit Information**

Date: _____ Experimental Purpose: _____ Lot #

Protein Assay: _____ Kit: _____

Standard TSN: _____ Plate: _____

Standard Lot: _____ Conjugate: _____

Sample Matrix: _____ STDs Buffer: _____ Substrate: _____

Sample ID: _____ Sample Buffer/Extraction: _____ Stop Solution: _____

DSTAR (yes/no): _____ Sample Buffer/Dilution: _____ Buffer: _____

Plate Identification: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Comments: _____

Figure 2 Title