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APPLICATION NOTE

ELISA Method Validation Procedures for Quantitation of Expressed Plant Proteins in GMO Products

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ABSTRACT

Validation of expressed proteins in genetically modied organisms (GMO) by enzyme linked immunosorbant assay (ELISA) should follow established bioanalytical procedures. Validation of the analytical method should be subjected to the following experimental checks in the appropriate bio-matrix to ensure it is suitable for its intended use: accuracy, precision, specificity, sensitivity, reproducibility and stability. Validation relies on analysis of reference materials or other suitable plant control material for use as quality control (QC) standards and calibration curve standards. Since well-characterized reference materials are not always available, best efforts are needed to create suitable control materials to support method validation and subsequent field testing. Quality controls are prepared in triplicate at known concentrations (low, mid and high) and calibration curve standards prepared in duplicate at a minimum of six non-zero calibrator concentrations spanning the intended range of analysis. Due to the inherent non-linear behavior of immunoassays, a four- or five-parameter logistic model is used with anchoring points at the asymptotic high and low-concentrations. Intra-assay and Inter-assay precision and accuracy are evaluated using three sets of QC samples prepared in the appropriate bio-matrix. The limit of detection (LOD) are calculated from a standard curve using the standard deviation () derived from the absorbance values for the lowest concentration value established for range. Range is determined using results generated from interand intra-assay precision and accuracy. Specificity is evaluated using positive and negative controls prepared in triplicate. Present endogenous compounds that may interfere with the ELISA method are examined in multiple sources of the bio-matrix. Standard stability is assessed in both stock dilution preparations as well in bio-matrix for appropriate period of time and condition. To support subsequent routine testing, frozen stability is assessed by analysis of QC samples subjected to three freeze-thaw cycles as well as long term freezer stability for a suitable period of time. Generally accepted acceptance criteria and testing procedures for ELISA methods are described in this presentation.

OBJECTIVE

Provide practical guidance on conduct of method validation studies for ELISA methods in support of expressed proteins in GMO plant products.

BACKGROUND

Well established validation guidelines for bioanalytical methods ranging from LC-MS/MS to Immunoassays, to include ELISA, exist in support of preclinical GLP studies for pharmaceuticals. In this presentation we will describe the use of these established procedures for method validation and subsequent bioanalytical testing of expressed proteins in GMOs using ELISA methods.

METHODS

Principle of the ELISA ECL Method: Though many ELISA formats exist for quantitation of proteins in complex bio-matrices, in this presentation a sandwich ELISA using electrochemiluminescene (ECL) detection is used as a model method for description of validation procedures though other ELISA detection methods such as horse radish peroxidase (HRP) reporting methods are equivalent. The assays employ a sandwich immunoassay format where capture antibodies are coated on the bottom of the wells of a specialized ECL compatible 96-well plate. The sample and a solution containing the labeled detection antibody, an anti-GMO protein antibody labeled with a Meso Scale Discovery (MSD) SULFO-TAG™ containing an electrochemiluminescent Ru^{2+} compound, are incubated over a selected period of time. The GMO protein of interest in the prepared plant tissue sample binds to capture antibody immobilized on the working electrode surface followed by recruitment of the labeled detection antibody by the bound analyte to complete the sandwich. An MSD read buer that provides the appropriate chemical environment for electrochemiluminescence is added and the plate is then loaded into an MSD SECTOR® instrument for analysis. A voltage is applied to the plate electrodes causing the labels bound to the electrode surface to emit light. The instrument measured intensity of emitted light to provide a quantitative measure of GMO protein present in the sample. Quality Control Sample concentrations are

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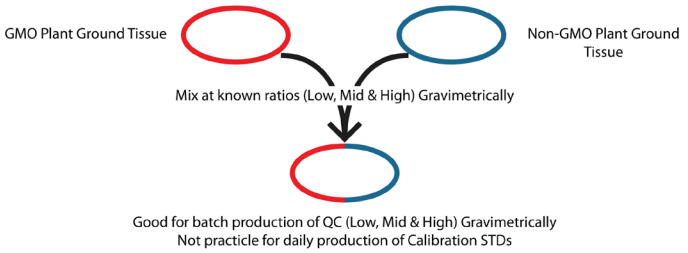

Figure 1. Graimetric approach for batch production of QC standard (Low, Mid and High)

extrapolated from a standard curve prepared in the appropriate bio-matrix using puried GMO protein as the reference standard.

GMO Reference Materials: Conduct of validation and subsequent eld sample testing require use of reference materials containing a known amount of GMO expressed protein of interest in an appropriate plant tissue bio-matrix. This reference material is needed to produce two independent standards typically needed for ELISA methods. First, calibration standards are needed to generate the non-linear curve for quantitation of all QCs and field samples. This requires a minimum of six calibration points with anchor points just outside the lower and upper limits of the curve. Calibration standards are typically prepared fresh on the day of analysis in duplicate.

They should range from blank matrix to the matrix containing known amounts of the GMO protein of interest from just below the lower level of quantitation (LLOQ) to the upper level of quantitation (ULOQ) for a minimum of 6 intermediate concentrations with anchor points just outside the calibration lower and upper range of the method. Second, QC standards are typically prepared well ahead of time and stored frozen until needed for analysis. They should be made at low (~3 x the LLOQ), mid and high (ULOQ) concentration in triplicate within the range of the intended method. Sufficient amounts of QC standards are required for multiple days of analysis. Once produced they should be characterized and shown to be stable upon long term storage under the appropriate conditions. Several approaches are possible for generation and characterization of such reference materials as shown below:

1. Purchase of commercial reference materials when available.
2. For QC samples, GMO tissue containing protein at well-established expression levels may be useful. However, likely not appropriate for use as daily calibration standards.
3. If commercial reference materials are not available, creation of reference materials could be performed in a number of ways. It is typical practice to prepare calibration curve standards for each day of analysis and independently of QC samples due to differences in concentration and range. The two approaches to product both QC and daily calibration standards are as follows:
   a. Creation of GMO reference materials by gravimetrically mixing ground GMO materials of known amount with non-GMO materials, preferably using the same parent plant strain as the GMO variant as depicted in Figure 1. Such materials should be prepared at varying amounts of the expressed protein in the same plant tissue matrix for use as QC standards. This approach may not be advantageous for generation of daily calibration standards due to the manually intensive nature of the process and the large number of concentrations levels needed for calibration standards.

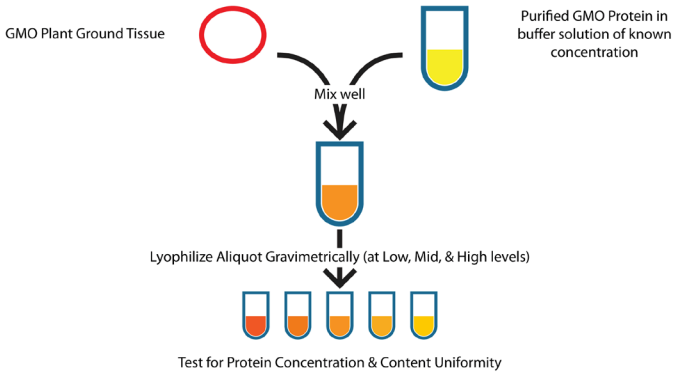

Figure 2. Wet addition for batch production of QC standards

   b. Creation of GMO reference materials by spiking in known amounts of purified GMO protein solutions into non-GMO plant tissue of the same parent strain as the recombinant GMO. This could be achieved in several ways:
      i. Preparation of large amounts of ground non-GMO plant tissue spiked with the GMO protein solution in a suitable buffer solution so that the material can be well mixed to form a homogeneous spiked QC material at low, mid and high concentrations (Figure 2). Then dry the material by lyophilization and aliquot into small reference vials for subsequent characterization and testing. To ensure the intended concentration and uniformity are satisfactory, 5 to 10% of the aliquots should be tested for protein concentration and content uniformity under a priori acceptance criteria prior to use.
         - **Advantage:** large scale preparations of QC standards would be simplied and should provide a homogeneous standard prepared under gentle conditions.
         - **Disadvantage:** very manually intensive procedures for preparation of calibration standards which are typically prepared daily and at more concentration levels than QCs.
      ii. Overcoming this obstacle for creation of daily calibration standards may involve wet spike addition of non-GMO ground tissue with protein solutions of known concentration in an extraction buffer during sample preparation just prior to analysis (Figure 3). Commonly, GMO plant materials are extracted using finely ground plant tissue with an ELISA compatible extraction buffer. Following extraction, the materials are centrifuged to

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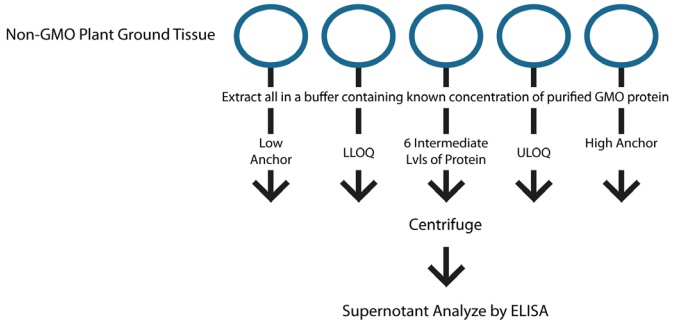

Figure 3. Production of daily QC standards

remove particulates prior to ELISA analysis. Test and QC samples are extracted in the same manner but using a protein free extraction buffer.

- **Advantage:** an easy approach for preparation of daily calibration standards. Therefore one could prepare this extraction buffer with known amounts of purified GMO protein for preparation of daily calibration standards in non GMO ground plant tissue.

Regardless of the approach for obtaining reference materials it is important to ensure that the concentration of the GMO protein of interest is known, the appropriate plant tissue matrix is used and the reference materials are characterized. Sufficient quantity of such reference materials are needed to support not only validation but subsequent field testing. Lastly, the stability profile of such reference materials needs to be understood to ensure subsequent field testing results are accurate, precise and reproducible over extended periods of time.

Validation Procedures: Method validation consists of several controlled experiments to ensure the method is suitable for its intended use in the appropriate plant bio-matrix. The validation experiments are designed to ensure the method is accurate, precise (intra and inter), specific, sensitive, reproducible and stabile within the intended range of the method. All validation work should be conducted under a formal validation protocol and any changes documented as protocol amendments. Validation experiments should be addressed versus a priori approved acceptance criteria. In Table 1 a listing of such validation tests with typical acceptance criteria are provided.

VALIDATION EXPERIMENTS

Selectivity/Specificity: Selectivity/Specificity should be evaluated for the GMO using multiple sources of the GMO bio-matrix. Samples of GMO bio-matrix from six to 12 different individual sources unfortified and fortified with the Reference Standard at the LLOQ and ULOQ should be evaluated. If non-GMO ground plant tissue is not available and only GMO plant tissue is used then evaluation of endogenous levels of GMO protein should be evaluated in unfortified samples.

Fortified samples are also to be evaluated for Inter- and intra-run Accuracy, Precision and Total Error. Of the lots evaluated, at least 2/3 of those fortified with Reference Standard should be within 75-125% Recovery Mean of the nominal concentration with a CV

Parameter	Procedure	Acceptance Criteria
Selectivity/Specificity	• Plant matrix samples prepared using at least 6 (six) different individual sources of plant tissue will be analyzed both unfortified and fortified with the Reference Standard at the LLOQ and ULOQ • Inter and intra-run Accuracy and Precision and the Total Error+ will be evaluated.	• Fortified samples should be within ±25% RE of nominal concentration for 2/3 of the lots tested with CV ≤25%. • Inter-run should be ±25% RE with CV ≤25%. • Total error should be ≤40% • Unfortified sample results will be reported.
Accuracy and Precision for Calibration Standards	• Spike plant tissue matrix at known concentrations of the Reference protein standard. • Inter and intra-run Accuracy and Precision will be evaluated. • Linearity will be evaluated.	• Intra-run: 75% of non-zero Standards must be ±20% RE, ±25% at LLOQ/ULOQ and ≤20% CV, ≤25% at LLOQ/ULOQ • Inter-run: ±20% RE, ±25% at LLOQ/ULOQ and ≤20% CV, ≤25% at LLOQ/ULOQ • Report Correlation Coefficient
Accuracy and Precision for QC Samples	• Plant tissue matrix fortified at 3 concentrations, QC-L (low), QC-M (medium) and QC-H (high) are analyzed, 3 sets per plate • Inter and intra-run Accuracy and Precision and the Total Error+ will be evaluated.	• %RE for at least 2/3 QCs with at least one at each level: ±20% and ≤20% CV • Inter-run %RE must be ±20% with %CV ≤20% • Total error should be ≤30%
Freeze/Thaw Stability	• Matrix fortified at QC-L and QC-H (minimum of three freezing/ambient temperature[1] thawing cycles); Analyze 3 sets at each level. • Freezing cycles should be at least overnight. • Thawing cycles should last a minimum of 2 hours	• Mean intra-run accuracy ±20% from theoretical and ≤20% CV
Bench Top Stability	• Matrix fortified at QC-L and QC-H assayed after storing for approximately 24 hours at ambient temperature[1]. Analyze 3 sets at each level	• Mean intra-run accuracy ±20% from theoretical and ≤20% CV
Freezer Storage Stability	• Short and long[2]-term sample storage stability will be tested by analyzing QC-L and QC-H stored at -20 °C ± 10 °C for up to 1 year (0, 0.5, 1, 2, 3, 6 and 12 months or more). Analyze 3 sets at each level.	• Mean intra-run accuracy ±20% from theoretical and ≤20% CV

+ Total error = The sum of the absolute %RE and %CV (evaluated using the primary validation plates)
[1] Thawing on wet ice may be evaluated if needed
[2] Long term storage stability time points not completed by the finalization of the initial validation report will be added by final report amendment.

Table 1. Representative validation tests with acceptance criteria for an ELISA method

25%. Inter-run data should be 75-125% Recovery Mean with CV 25% and the Total Error should be 40%.

Accuracy and Precision for Calibration Standards: Accuracy and Precision for calibration standards should be evaluated for the GMO protein of interest using the appropriate plant bio-matrix. Calibration standards, ranging from the LLOQ to ULOQ to include matrix blanks, are typically prepared fresh on the day of analysis by fortifying the plant bio-matrix with purified protein. Each calibrator is to be run in duplicate wells and analyzed for % Recovery Mean (% RM) and the coefficient of variation (% CV) using mean back-calculated concentrations. A four or five-parameter curve regression model is typically used to fit the calibrators to a standard curve.

Accuracy and Precision for calibration standards should be evaluated. The Coefficient of Determination (R2) for the non-linear calibration curve should be >0.980. The % Recovery Mean (% RM) and % CV for 75% of non-zero calibrators should be 80-120% Recovery Mean (75-125% Recovery Mean at LLOQ) and 20% CV (25% CV at LLOQ) for each plate.

Determination of 214:280 nm Ratio: Accuracy and Precision for QC samples are to be evaluated for the GMO protein in the plant tissue matrix. QC samples at the QC-H, QC-M, and QC-L concentrations are to be prepared fresh on the day of analysis by fortifying plant tissue matrix with the GMO protein. Each QC sample is run in duplicate wells and analyzed for % Recovery Mean (% RM) and the coefficient of variation (% CV) using mean back-calculated concentrations. Accuracy and Precision for QC samples are to be evaluated by determination of the % Recovery

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Mean and % CV. Typical acceptance criteria (Table 1) indicate 2/3 of the QC samples are required to be 80-120% Recovery Mean and 20% CV. Inter-run data are typically required to be 80-120% Recovery Mean with CV 20% and the Total Error was required to be 30%. Inter day analysis of the QC samples should indicated that the Inter-run total error data are within the acceptable criteria (Table 1).

Stability: The analyte is considered stable if the mean Recovery Mean is within 80-120% from theoretical and CV 20%.

Bench Top Stability: Bench Top Stability of the spiked QCs should be evaluated with two concentration levels (QC-H and QC-L) in triplicate. Bench top stability should be assayed after storing for approximately 12- to 18-hours on the bench top under conditions stipulated in the sample handling in the method or cold using cold packs or wet ice. Desired results should indicate that the test material is stable during the test period under the appropriate conditions. Mean recovery should be within 80-120% of the theoretical concentration and CV 20%.

Freeze/Thaw Stability: Freeze-thaw stability of the Fortified assay QC samples should be evaluated at a minimum of two concentration levels (QC-H and QC-L) in triplicate. Freeze-thaw stability samples should be subjected to minimum of three freeze-thaw cycles. There should be approximately a 24-hour interval or longer at the first freezing, and at least 12-hour intervals between freezing cycles thereafter. Thawing cycles should last a minimum of 2 hours on wet ice or other appropriate condition. Desirable results should indicate that the test material is stable through two or more freeze-thaw cycles as demonstrated by recovery within the required 80-120% of the theoretical concentration and 20% CV.

Freezer Storage Stability: The long term storage stability of the test material should be evaluated with fortified QC samples at a minimum of two concentration levels (QC-H and QC-L) after storage at ~-60°C or at ~-20°C. The long-term freezer storage stability should be tested in triplicate for both of these storage conditions for 0, 0.5, 1, 2, 3, 6 and 12 months (or more). Desired results should indicate that the test material was stable minimally for 1 month and optimally at 12 months when stored under the appropriate frozen condition. The target storage condition for Lyophilized QCs may be refrigerated. Freezer stability will be demonstrated by recovery within the required 80-120% from theoretical concentration and 20% CV.

DISCUSSION

Proper validation of an ELISA method for analysis of expressed proteins in plant GMOs ensures the results from subsequent field testing are of high quality. Ensuring the method is suitable for its intended use by demonstrating the method is accurate, precise, specific, sensitive, reproducible and stabile provides a strong foundation for subsequent field testing and regulatory submissions. In comparison to most bioanalytical procedures, analysis of transgenic proteins in plant tissue does present some technical obstacles. First, reference materials are not commonly available for non-European GMO products. Therefore, production of the 2 primary standards used in GLP compliant bioanalytical methods, calibration standards and quality control (QC) standards, are crucial. Two proposed procedures are presented: mixtures of GMO and non-GMO plant matrices by gravimetric means or spike addition of plant matrix material with purified protein solutions. The later, wet spike addition can be performed in two different ways. For production of large batches of QC standards, non-GMO matrix can be fortified by wet addition of purified protein solutions, mixed then lyophilized in order to produce homogeneous QC materials. Such QC batches would require testing to ensure they are accurate, homogeneous and stable upon long term frozen storage prior to routine use. Calibration standards could be produced daily also by wet addition, but by means of an extraction buffer, allowing for rapid and ease of production needed for daily use. Last, validation procedures are presented in detail to allow the reader a framework they could use for future method validation and subsequent GLP compliant analysis by ELISA.