APPLICATION NOTE

AlphaLISA Technology

Authors:

Lindsay Nelson

Steve Hurt

Vincent Dupriez

Seth Cohen

PerkinElmer, Inc.
Hopkinton, MA

Homogeneous No-Wash AlphaLISA Assays for Genetically-Modified (GMO) Protein Detection in Seed Extracts

Introduction

Genetically modified (GM) crops are widely used in the agricultural industry. These crops are manipulated to possess traits which can make them less susceptible to pests, more resistant to herbicides, allow for the production of more nutritionally dense food, and even produce pharmaceutical drugs. As the use of GM crops becomes more prevalent, it is imperative that rapid and sensitive tools exist for the detection of GM proteins from plants. Traditional methods for detection include labor-intensive multi-wash ELISA kits or strip-based tests which are not quantitative. Here, we present two assays using AlphaLISA® Technology that allow for the detection and quantitation of transgenic proteins from seed extracts in under two hours, without any wash steps.

PerkinElmer®
For the Better

As a model, we tested cotton seeds expressing two forms of Cry toxins, Cry1A (Cry1Ab and Cry1Ac--two variants nearly identical in sequence homology) and Cry2A, using AlphaLISA. Cry proteins are a class of insecticidal endotoxins from the bacterium Bacillus thuringiensis (Bt) that are non-toxic to mammals, making them ideal for conferring insect resistance to plants.

AlphaLISA Technology allows for the detection of molecules of interest in a homogeneous, no-wash format. As shown in Figure 1, a biotinylated anti-analyte antibody (specific for Cry1Ab/Ac or Cry2A) binds to streptavidin-coated Donor Beads while another anti-Cry antibody is conjugated directly to AlphaLISA Acceptor Beads. Both antibodies bind to the analyte, when present, bringing the Donor and Acceptor Beads in close proximity of each other. Upon excitation at 680 nm, the Donor Beads emit singlet oxygen molecules that travel in solution to activate the Acceptor Beads which then emit a sharp peak of light at 615 nm. This light emission can then be detected on an Alpha-enabled reader, such as the EnSpire® Multimode or EnVision® Multilabel Plate Readers. AlphaLISA assays can easily be automated using the JANUS® Automated Workstation to increase assay throughput.

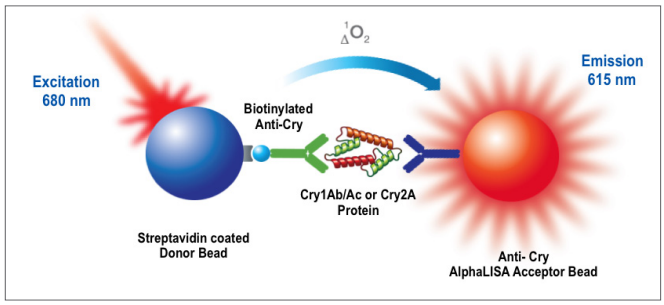

Figure 1. AlphaLISA assay principle.

Materials and Methods

Reagents

Cry1Ab/Ac Detection Antibodies	Fitzgerald # 10-1398, 10-1401
Cry2A Detection Antibodies	Fitzgerald# 70R-BR002 and Abraxis #490052
Cry1Ab Purified Recombinant	Abraxis, Inc # 300005
Cry1Ac Purified Recombinant	Abraxis, Inc # 250020
Cry2A Purified Recombinant	Abraxis, Inc # 250040
AlphaLISA Acceptor Beads	PerkinElmer # 6772002
AlphaLISA Streptavidin-coated Donor Beads (SA-Donor)	PerkinElmer # 6760002
ChromaLink™ Biotinylating Reagent	SoluLink™ # B1001-105
Zeba Spin Desalting Columns	Thermo Scientific # 89883
OptiPlate™-384 White Opaque Microplate	PerkinElmer # 6007290
TopSeal-A	PerkinElmer # 6050195
AlphaLISA Immunoassay Buffer	PerkinElmer # AL000C
96-well StorPlate™	PerkinElmer # 6008190
96-well Seed Crusher	PerkinElmer # HSC-200

Instrumentation

All AlphaLISA measurements were performed on the PerkinElmer EnSpire Multimode Plate Reader.

Antibody Biotinylation and AlphaLISA Acceptor Bead Conjugation

Biotinylation of antibodies (Fizgerald #10-1398 for Cry1Ab/Ac, and #70R-BR002 for Cry2A) was performed with the ChromaLink™ biotinylating reagent using standard biotinylation and purification procedures. Briefly, 0.1 mg of antibody and 3.8 µL of biotinylating reagent (2 mg/mL) were mixed together at a 30:1 molar biotin/antibody ratio. The reaction volume was completed to 0.2 mL with PBS pH 7.4 and the reaction was incubated for 2 hrs at 23 °C. Purification of the biotinylated antibody was performed using a Zeba 0.5 mL desalting column. The ratio of biotinylation of the final product and the protein recovery were determined from absorbance readings at 354 nm and 280 nm, respectively.

For AlphaLISA Acceptor Bead conjugations, 0.05 mg of antibody (Fitzgerald #10-1401 for Cry1Ab/1Ac, Abraxis #490052 for Cry2A), 0.0625% of Tween-20, 2.5 mg of AlphaLISA beads and 1.25 mg/mL of NaBH3CN were mixed together. The reaction volume was adjusted to 0.1 mL with 50 mM Hepes pH 7.4 (final) and the reaction was incubated for 18 hrs at 37 °C. The reaction was stopped by the addition of 5 µL of a 65 mg/mL CMO solution and incubation for 1 hr at 37 °C. Beads were then washed twice by centrifugation for 15 min (14,000 rpm/4 °C) and the bead pellet was resuspended in 0.5 mL of 100 mM Tris pH 8.0. After a third centrifugation step, the beads were resuspended at 5 mg/mL in PBS pH 7.2 containing 0.05% Proclin-300.

Seed Extract Preparation

Wt (non-Cry-protein expressing) or Bt (Cry toxin expressing) cotton seeds were crushed with a 96-well seed crusher in a 96-well StorPlate. Crushed seeds of the same type were pooled, weighed, and then extraction buffer was added for a final seed mass concentration of 90 mg/mL using 1x AlphaLISA immunoassay buffer. Extracts were vortexed vigorously for 1 min and centrifuged for 10 min at 14,000 rpm. Finally, 50% of each supernatant was removed for dilution and analysis, avoiding any seed debris.

AlphaLISA Assays

As depicted in Figure 2, samples of recombinant protein standards or seed extract were added to a 384-well OptiPlate in a 50 uL total reaction volume containing 20 µg/mL (final) SA-donor and Cry-specific AlphaLISA Acceptor Beads. Final biotin-anti-Cry specific antibody concentration was 1 nM. AlphaLISA immunoassay buffer (diluted to 1X) was used for all dilutions.

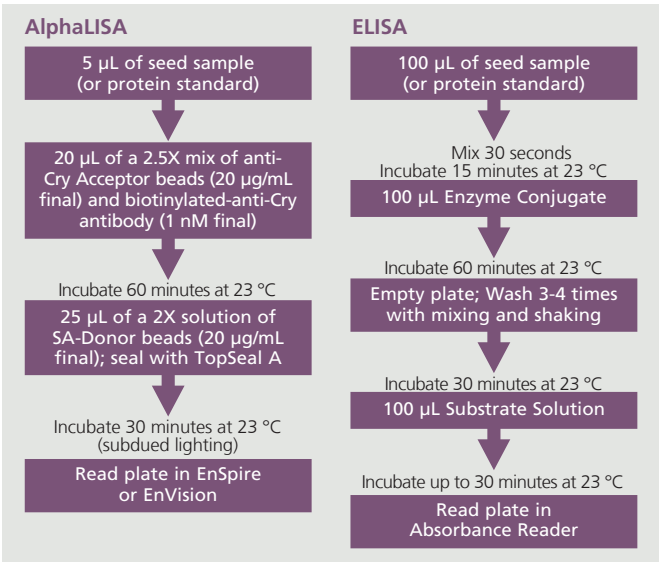

Figure 2. AlphaLISA assay protocol (left) compared to a standard wash-based ELISA protocol (right).

Results

Antibody sensitivity for either Cry1Ab and Cry1Ac or Cry2A was tested using recombinant proteins (Figure 3 upper and lower panels, respectively). In Figure 3 (upper panel), the antibody pairing selected for Cry1Ab/Ac has a similar sensitivity and dynamic range for the 1Ab and 1Ac variants, but with slightly more sensitive detection of Cry1Ab. The calculated EC_{50} for Cry1Ab detection was 419 ng/mL, while for Cry1Ac the EC_{50} was 907.9 ng/mL. Lower detection limit (LDL) values, calculated by taking three times the standard deviation of twelve background replicates and adding it to the background average, was determined to be 79.7 pg/mL for Cry1Ab and 197.5 pg/mL for Cry1Ac. For Cry2A detection, (Figure 3, lower panel) the EC_{50} was 155.6 ng/mL and the LDL was 40.7 pg/mL.

Cotton seed extracts were then tested for the presence of Cry1Ab/Ac or Cry2A using AlphaLISA. Results are shown in Figure 4 for Cry1Ab/Ac (upper panel) and Cry2A (lower panel). Extracts for Cry1Ab/Ac detection show a very robust Alpha signal at 90 mg/mL seed extract which decreases with serial dilutions. As most transgenic seeds contain both forms of Cry1Ab and Cry1Ac, it is not surprising the Alpha signal, compared to WT seeds, is so robust.

For Cry2A detection in cotton seeds, we see an effect by which the 90 mg/mL undiluted seed extract shows lower signal than the serial dilutions. This may be due to a "hook" effect which occurs when the concentration of analyte (Cry2A) is above a threshold by which Donor and Acceptor Beads become saturated with analyte, inhibiting the ability of beads to be in close enough proximity to general signal and reducing the overall counts.

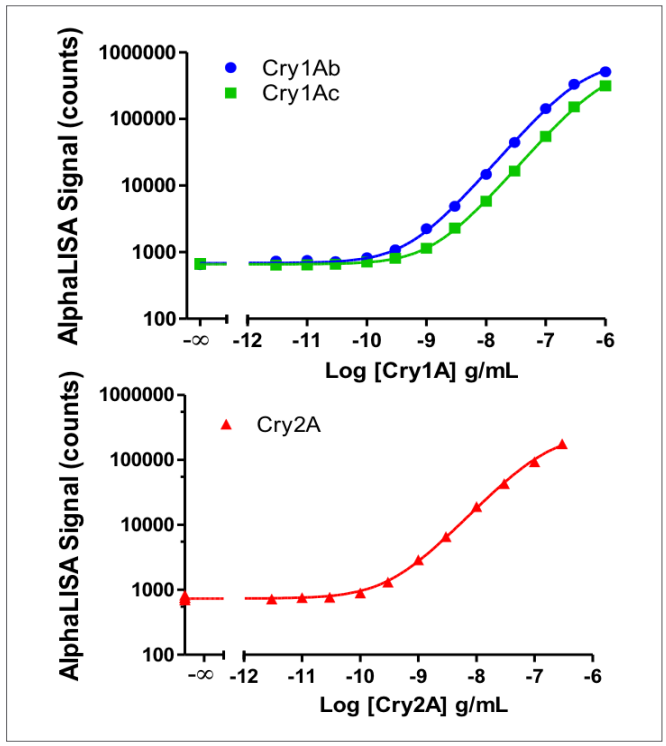

Figure 3. Cry protein detection using AlphaLISA.

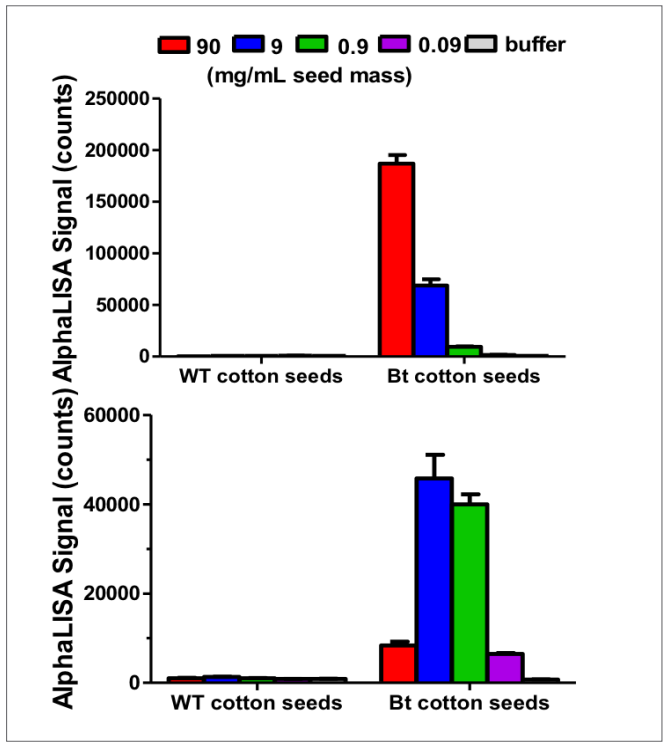

Figure 4. Cry protein detection from seed extracts using AlphaLISA.

It is important to mention that seed extractions performed for Figure 4 were prepared in AlphaLISA immunoassay buffer. As the efficiency of extraction using this buffer was not known, we felt it necessary to compare the results to an extraction buffer commonly used for seed extractions (from ELISA Company X, composition unknown). Results are shown in Figure 5, which demonstrate that the extraction efficiency, as measured by AlphaLISA signal of Bt seeds compared to that of WT seeds, are identical between the two buffers.

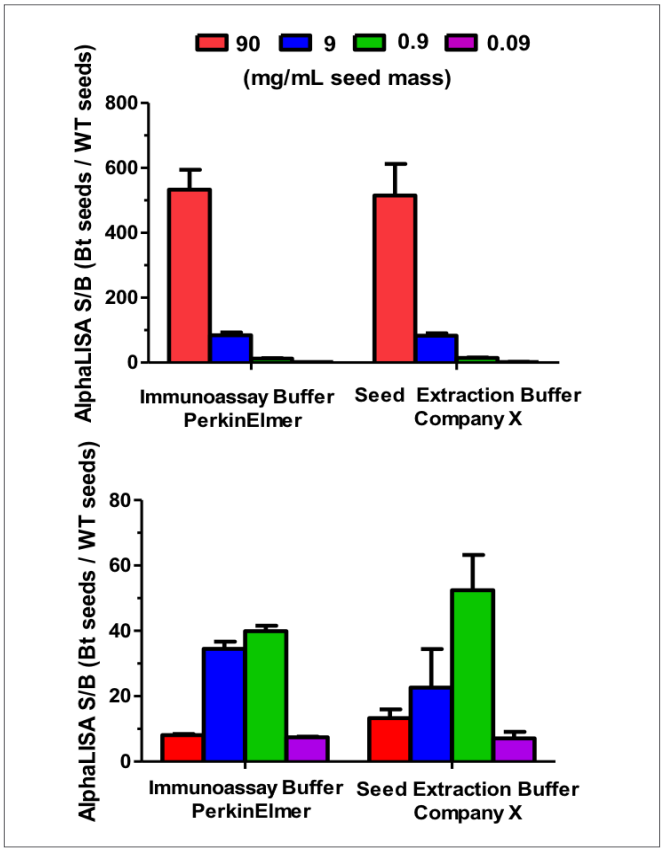

Figure 5. Seed extraction buffer comparison.

Finally, quantitation of Cry2A protein in seed extracts was calculated by comparing the AlphaLISA counts of Bt cotton seed extracts diluted at 0.9 mg/mL in immunoassay buffer, to the standard curve generated for recombinant Cry2A protein. Results are shown in Figure 6.

Figure 6 shows that seed extracts prepared at 0.9 mg/mL contain 34.5 ng/mL of Cry2A protein. WT seeds tested at the same concentration of seed mass generated counts below the calculated LDL of the assay.

Calculating the amount of Cry2A in Figure 6 led us to reanalyze the initial seed extract data generated for Cry2A (Figure 4, lower panel). If in 0.9 mg/mL of seed extract there are 34.5 ng/mL Cry2A protein (green bar, Figure 4 lower), 9 mg/mL of seed extract must therefore contain 345 ng/mL Cry2A (blue bar, Figure 4 lower), and 90 mg/mL seed extract (red bar, Figure 4 lower)

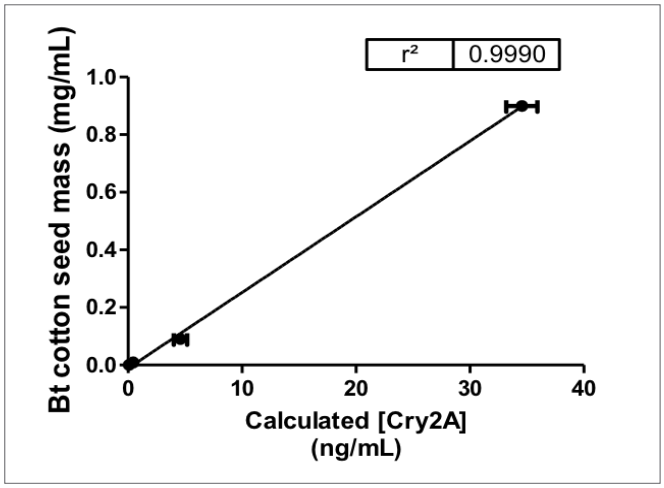

Figure 6. Quantitation of Cry2A protein in cotton seeds using the Cry2A AlphaLISA assay.

must contain 3450 ng/mL Cry2A, which is outside of the dynamic range for assay, resulting in the "hook" effect described earlier. However, the 9 mg/mL sample does not give significantly higher counts than the 0.9 mg/mL sample. This is most likely due to color quenching by components within the seeds themselves at this concentration of extract. Since AlphaLISA emission occurs at 615 nm, compounds that absorb in this region act as inner filters, lowering the amount of signal generated.

Due to the potential for color quenching, it is important to test multiple dilutions of seed preparations (or other plant material) to be sure any quenching effect is minimal using AlphaLISA. Fortunately, we were able to determine over the course of this study that 0.9 mg/mL of seed mass results in minimal interference while still allowing for robust detection of transgenic GM proteins from seed samples.

Conclusions

We have developed two AlphaLISA assays which can be used to detect the presence of protein toxins Cry1Ab/Ac and Cry2A from transgenic cotton seed extracts quickly and with great sensitivity. Over the course of this study, we have also validated the use of AlphaLISA immunoassay buffer for GM protein extraction from seeds.

References

1. AlphaLISA Assay Development Guide, PerkinElmer

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

011688A_01 May 2014