TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

AlphaLISA Cry1Ab/1Ac Assay Kit

Product number: AL401 HV/C/F

Lot number: sample

Manufacturing date:

Caution: For Laboratory Use. A research product for research purposes only.

Contents

Product Information

Application: This kit is designed for the quantitative determination of Cry1Ab or Cry1Ac in plant seed and leaf extracts using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with other Cry proteins.

Sensitivity Cry1Ab: Lower Detection Limit (LDL): 0.6 ng/mL

Lower Limit of Quantification (LLOQ): 1.8 ng/mL

EC_{50}: 545 ng/mL

Dynamic range Cry1Ab: 0.6 – 3000 ng/mL (Figure 1, left).

Sensitivity Cry1Ac: Lower Detection Limit (LDL): 0.13 ng/mL

Lower Limit of Quantification (LLOQ): 0.41 ng/mL

EC_{50}: 272 ng/mL

Dynamic range Cry1Ac: 0.13 – 3000 ng/mL (Figure 1, right).

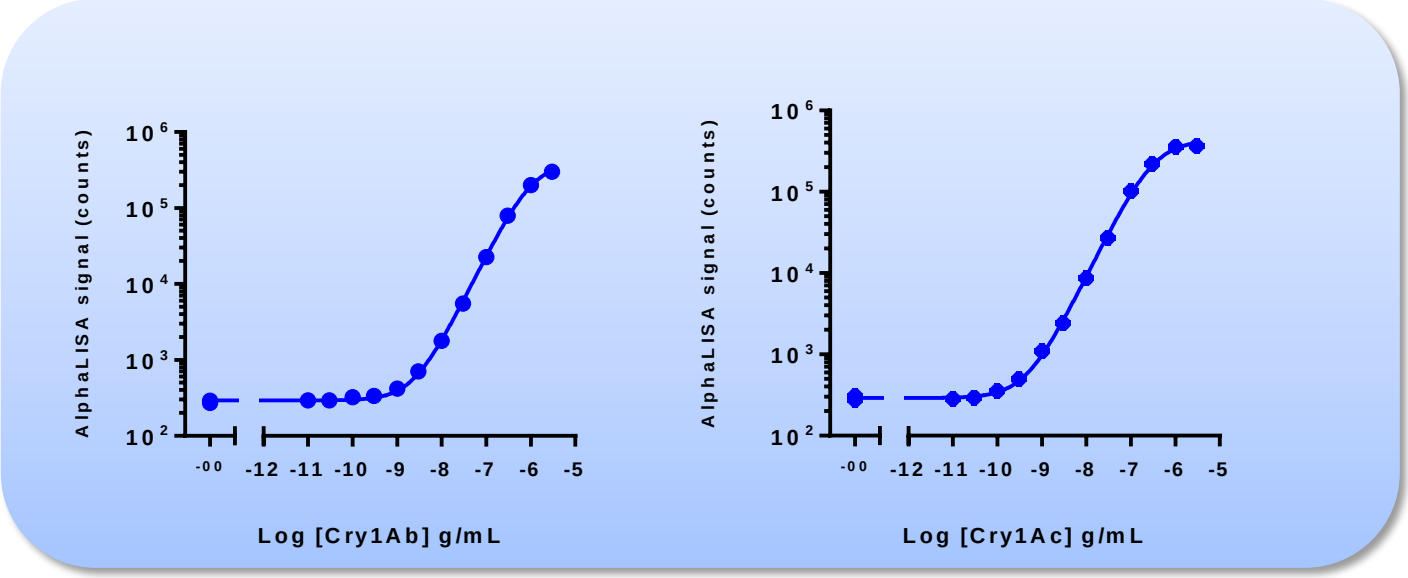


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer (Left, Cry1Ab and Right, Cry 1Ac). The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC_{50} and LDL were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and the instrument used, with no impact on LDL measurement.

Cry1Ab

EC_{50}:	729.2 ng/mL
LDL:	0.173 ng/mL
LLOQ:	0.556 ng/mL
Min Counts:	277
Max Counts:	360595

Cry1Ac

EC_{50}:	532.9 ng/mL
LDL:	0.149 ng/mL
LLOQ:	0.480 ng/mL
Min Counts:	264
Max Counts:	364130

Analytes of Interest

Cry1Ab and Cry1Ac are insecticidal proteins produced by the naturally occurring soil bacterium *Bacillus thuringiensis subsp. kurstaki* (Btk). The genes encoding Cry1Ab and Cry1Ac have been widely introduced to genomes of many crops (e.g. corn, cotton, and soybean) to create genetically modified organisms (GMO). During plant growth, genetically modified crops produce the introduced Cry proteins that confer protection against certain insect pests. The present kit allows the detection of Cry1Ab and Cry1Ac (i.e. analyte) in GMO seeds, leaves, and plant extracts.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

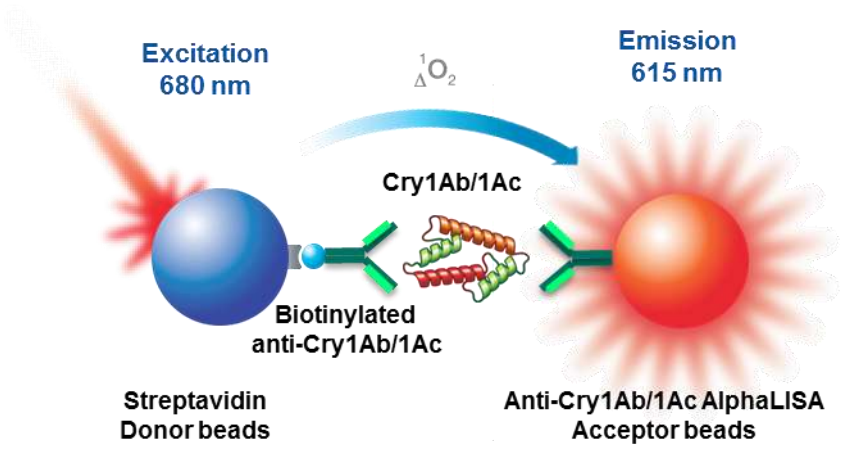


Figure 2. AlphaLISA Assay principle.

Precautions

- The AlphaScreen® Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All components and biological materials should be handled as potentially hazardous. The analytes included in this kit are produced by recombinant technology.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL401HV (100 assay points***)	AL401C (500 assay points***)	AL401F (5000 assay points***)
AlphaLISA Anti-Cry1Ab/1Ac Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, white cap)	100 µL @ 5 mg/mL (1 brown tube, white cap)	1 mL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	100 µL @ 5 mg/mL (1 brown tube, black cap)	1 mL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated Anti-Cry1Ab/1Ac Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Cry1Ab Analyte *	1.5 µg; lyophilized solid (1 tube, clear cap)	1.5 µg; lyophilized solid (1 tube, clear cap)	1.5 µg; lyophilized solid (1 tube, clear cap)
Cry1Ac Analyte *	1.5 µg; lyophilized solid (1 tube, clear cap)	1.5 µg; lyophilized solid (1 tube, clear cap)	1.5 µg; lyophilized solid (1 tube, clear cap)
AlphaLISA Immunoassay Buffer ** (10X)	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Two separate analytes are provided in this kit, allowing for the generation of separate standard curves with either Cry1Ab or Cry1Ac. The reconstituted analytes should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. Each vial contains enough Cry1Ab or Cry1Ac for 5 standard curves. Additional vials can be ordered separately (cat # AL401S).

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the Biotinylated Antibody stock solution will not

interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend all reagents by vortexing before use.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in the AlphaLISA Immunoassay Buffer for plant extracts.
- Chlorophyll interferes with the assay. It is recommended to dilute chlorophyll-rich samples such as leaf extracts at least 10 fold.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.

- The standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

		Volume					
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA Acceptor beads**	**Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL401HV	100	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL401C	250	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	10 µL	10 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	4 µL	4 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	2 µL	2 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL401F	5 000	50 µL	5 µL	10 µL	10 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	4 µL	4 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	2 µL	2 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

Protocol for Cry1Ab or Cry 1Ac AlphaLISA Assay

3-step high sensitivity protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
   a. Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL Milli-Q H_2O.

2) Preparation of Cry1Ab or Cry1Ac analyte standard dilutions:
   a. Reconstitute lyophilized Cry1Ab or Cry1Ac (1.5 µg) in 50 µL Milli-Q H_2O.
   b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of Cry1Ab or Cry1Ac (µL)	Vol. of diluent (µL) *	[Cry1Ab or Cry1Ac] in standard curve	
			(g/mL in 5 µL)	(ng/mL in 5 µL)
A	10 µL of reconstituted Cry1Ab or Cry1Ac	90	3.00E-06	3000
B	60 µL of tube A	120	1.00E-06	1000
C	60 µL of tube B	140	3.00E-07	300
D	60 µL of tube C	120	1.00E-07	100
E	60 µL of tube D	140	3.00E-08	30
F	60 µL of tube E	120	1.00E-08	10
G	60 µL of tube F	140	3.00E-09	3
H	60 µL of tube G	120	1.00E-09	1
I	60 µL of tube H	140	3.00E-10	0.3
J	60 µL of tube I	120	1.00E-10	0.1
K	60 µL of tube J	140	3.00E-11	0.03
L	60 µL of tube K	120	1.00E-11	0.01
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

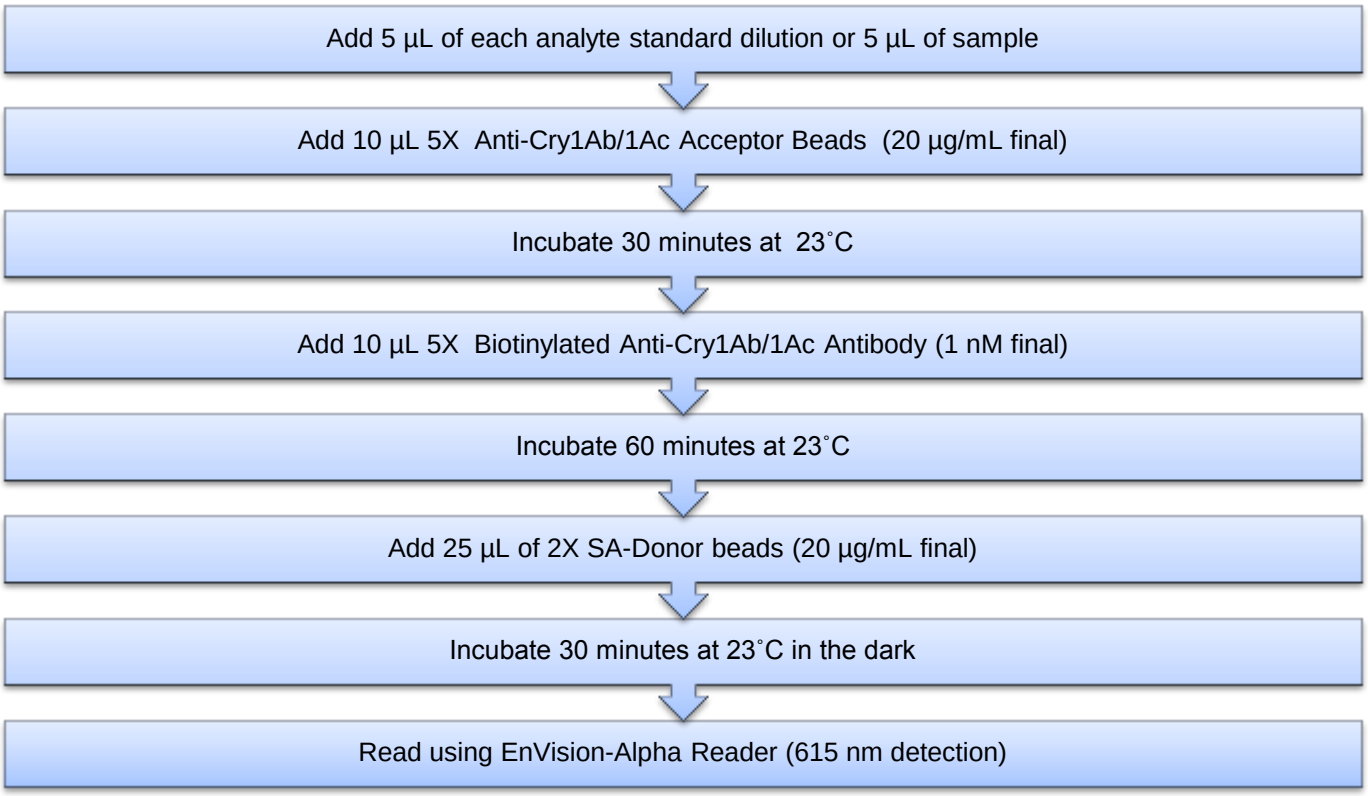
3) Preparation of 5X Anti-Cry1Ab/1Ac Acceptor Beads (100 µg/mL):
   a. Prepare just before use
   b. Add 100 µL of 5 mg/mL AlphaLISA anti- Cry1Ab/1Ac acceptor beads to 4900 µL of AlphaLISA Immunoassay Buffer.

4) Preparation of 5X Biotinylated Anti-Cry1Ab/1Ac antibody (5 nM):
   a. Prepare just before use
   b. Add 50 µL of 500nM biotinylated anti- Cry1Ab/1Ac antibody to 4950 µL of AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (40 µg/mL):
   a. Prepare just before use.

b. Keep the beads under subdued laboratory lighting.
c. Add 100 µL of 5 mg/mL SA-Donor beads to 12 400 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a white Optiplate (384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- EC_{50} is calculated using the medium counts. The medium counts = (Ave Maximum counts- Ave Minimum counts) ÷ 2. The medium counts is than interpolated to the standard curve to obtain the EC_{50}
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the 2 step protocol.

- Assay Sensitivity:

The LDL and LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a 5 µL sample volume using the recommended assay conditions. Average of 15 independent experiments is shown in the table.

Cry Proteins	LDL (pg/mL)	LLOQ (pg/mL)
Cry1Ab	0.6	1.8
Cry1Ac	0.13	0.4

* The standard was prepared in AlphaLISA Immunoassay Buffer

Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the standard curve was prepared in AlphaLISA Immunoassay Buffer. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

CV(%)	Immunoassay Buffer
Cry1Ab	5.7
Cry1Ac	4.7

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 10 ng/mL sample. Shown as CV%.

CV (%)	Immunoassay Buffer
Cry1Ab	7.3
Cry 1Ac	7.9

- Spike Recovery:

Three known concentrations of Cry1Ab or Cry1Ac analyte were spiked in AlphaLISA Immunoassay Buffer and the assays were performed along a standard curve. The average recovery from three independent measurements is reported.

Spiked (ng/mL)	Cry1Ab Recovery (%)	Cry1Ac Recovery (%)
100	101	105
10	95	98
1	102	98

- Specificity:

Cross-reactivity of the AlphaLISA Cry1Ab/1Ac Immunoassay Kit was tested using the following proteins at 100 ng/mL. To test cross-reactivity, samples we run alongside each standard curve (i.e. using the Cry1Ab standard curve, Cry1Ac levels are over-estimated). It is hence important to run the standard corresponding to the analyte of interest.

Cry Proteins	Cry1Ab Cross-reactivity (%)	Cry1Ac Cross-reactivity (%)
Cry1Ab	100	110
Cry1Ac	67	100
Cry2A	0	0
Cry1F	0	0
Cry3B	0	0
Cry9C	0	0

Testing Cry-positive and -negative samples

Lyophilized Cry1Ab/1Ac positive and negative control proteins from corn seed extracts with unknown concentrations of Cry proteins were purchased from Agdia Inc. The control samples (5 uL each sample volume) were tested along with Cry1Ab or Cry 1Ac standards. Positive/Negative Control samples contain corn seed extract expressing/ or not expressing Cry proteins.

Control Sample	Cry1Ab Detected (µg/mL)	Cry1Ac Detected (µg/mL)
Cry 1Ab/Ac Positive Control	1.1	1.0
Cry 1Ab/Ac Negative control	0	0
Immunoassay Buffer only	0	0

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at:

http://www.perkinelmer.com/in/resources/technicalresources/applicationsupportknowledgebase/alphalisa-alphascreen-no-washassays/alpha_troubleshoot.xhtml

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