INSTRUCTION FOR USE

ELISA for detection of Cry3Bb

Cat No. AID 053

GOLD STANDARD DIAGNOSTICS

1 INTENDED USE

Cry3Bb ELISA test kit is intended to be used for the qualitative detection of Cry3Bb protein present in transgenic Maize plant carrying Monsanto Yieldgard Root worm RW event 863. The test is used to detect the Cry3Bb protein in individual Maize seed or leaf.
It can also be used to detect event 863 in bulk grain at 0.2% (1 positive in 500 negative seeds)
The total incubation time of the assay is 60 minutes for leaf/seed, and 90 minutes for bulk grain.

2 PRINCIPLE OF THE TEST

An antibody specific to the Cry3Bb protein molecule is immobilized on the microwell plates and second antibody specific for Cry3Bb protein is conjugated with horse radish peroxidase (HRP). When the sample extracts are added to the microtiter wells, the Cry3Bb from sample binds to the antibody immobilized in the well. This binding is subsequently detected by addition of enzyme labeled antibody. After a washing step, substrate is added. The enzymatic reaction and development of color is proportional to the amount of Cry3Bb present in the sample. The reaction is terminated by addition of stopping solution. Absorbance is then measured on a plate reader. Light color indicates lower concentration while dark color indicates higher concentration of Cry3Bb protein in the sample.

3 CROSS-REACTIVITY

The Cry3Bb ELISA kit does not cross react with CP4EPSPS (Roundup Ready**), Cry1F, Cry1Ac, eCry3.1Ab (AgriSure Duracade), Cry3Bb, Cry1Ab, Cry2A, Vip3A, Cry34Ab1, Cry35Ab1 or PAT (LibertyLink***).

4 CONTENTS OF THE KIT:

Kits are provided in 1 plates format.

- One plate of 96 wells coated with Anti-Cry3Bb antibody, packed in a laminate bag with silica gel.
- Cry3Bb enzyme conjugate: One Ready-to-use bottle of 6 ml
- Extraction buffer: (10X Stock): 50 ml, One vial
- Wash solution: (10X Stock): 50 ml, One vial
- TMB Substrate, One Ready-to-use bottle of 12 ml
- Cry3Bb Negative control: One ready-to-use vial of 2 ml

Kits are provided in 10 plates format.

- Ten plates of 96 wells coated with Anti-Cry3Bb antibody, individually packed in a laminate bag with silica gel.
- Cry3Bb enzyme conjugate: One Ready- to- use bottle of 55 ml
- Extraction buffer concentrate: One packet of powder with a 25 ml vial of Tween-20.
- Wash solution concentrate: One packet of powder with a 2.5 ml vial of Tween-20
- TMB Substrate: One ready-

Kits are provided in 50 plates format.

- Fifty plates of 96 wells coated with Anti-Cry3Bb antibody, each packed individually in a laminate bag with silica gel
- Cry3Bb enzyme conjugate: One ready to use bottle with 275 ml.
- Extraction buffer concentrate: One packet of powder with a 25 ml vial of Tween-20
- Wash solution concentrate: One packet of powder with a 2.5 ml vial of Tween-20

to-use bottle of 110 ml
- Cry3Bb Negative control: One ready-to-use vial of 10 ml
- TMB Substrate: One ready-to-use bottle of 500 ml
- Negative control: One ready-to-use vial of 10 ml

5 MATERIAL AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- Pipette with disposable plastic tips
- Multichannel pipette with disposable pipette tips
- Plate ELISA reader with 450/620 nm filter
- Table top centrifuge
- Marking pen, Parafilm, Pipette tips, Timer and Paper towels
- Deionized or distilled water
- Graduated cylinders with one liter capacity
- Reagent troughs
- Plate shaker
- Plate ELISA washer or wash bottle

6 PRECAUTIONS

The Cry3Bb ELISA test is intended for in vitro use only. The reagents contain Thimerosal as preservative. Prevent direct skin and eye contact with kit components. Seek medical attention in case of accidental ingestion of kit components.

7 STORAGE OF THE KIT

1. The kit should be stored at 2 - 8° C. The unopened kit is stable till the expiry date printed on the kit label.
2. The expiry date of each unopened component is printed on the label of the component.
3. Bring all the components of the Kit to room temperature prior to use.

8 REAGENT PREPARATION

1. **Preparation of Working extraction buffer:**
   - For 1 plate kit: To 450 ml of distilled/deionized water, empty entire contents of the extraction buffer vial. Mix well before use.
   - For 10 or 50 plate kit: Dissolve the packet of extraction buffer concentrate powder in 500 ml of distilled/deionised water and add 25 ml of Tween-20 which is provided along with the powder. Mix it well before use. The resultant extraction solution is 10X concentrate.
   - This has to be further diluted with 4500 ml of water to have a working extraction buffer.
   - Store unused portion of working extraction buffer at 2 - 8° C. Thaw to room temperature before using the same again.
   - Store unused portion at 2 - 8° C.
2. **Preparation of Working wash buffer:**
   - For 1 plate kit: To 450 ml of distilled/deionized water, empty entire contents of the wash buffer vial. Mix well before use. Store unused portion at 2 - 8° C.
   - For 10 or 50 plate kit: Dissolve the packet of wash buffer concentrate powder in 500 ml of distilled/deionised water and add 2.5 ml of Tween-20 which is provided along with the powder. Mix it well before use. The resultant wash solution is 10X concentrate.
   - This needs to be further diluted with 4500 ml of water to have working wash buffer

- Store unused portion of working wash buffer at 2 - 8° C. Thaw to room temperature before using the same again.

3. **Preparation of stop solution:**
   - Add 27.0 ml of 98 % H_2SO_4 in 973 ml of deionized or distilled water. Work in fume hood while preparing stop solution. Store at room temperature for up to one year.
4. **Preparation of user positive control:**
   - The user is advised to prepare in-house positive control. Crush or grind one known positive seed into a uniform powder and add 1.0 ml of extraction buffer. Wait for 10 minutes before use. Prepare fresh before use.

9 SAMPLE PREPARATION

1. **Individual seed**
   - Crush or grind seed thoroughly into a uniform powder using mortar and grinder or seed crusher. Transfer it to a micro tube and add 1.0 ml of extraction buffer. Allow the mixture to stand for 30-60 minutes at room temperature (RT). Allow particles to settle and use only the supernatant to do the test. Wash and rinse grinding equipment carefully between samples to avoid cross contamination.

2. **Individual leaf**
   - Weigh 20 mg (about two leaf punch) and put it in a micro tube. Crush leaf with pestle in 0.5 ml working extraction buffer. Mix well and incubate for 30-60 minutes at RT. Allow particles to settle and use only the supernatant to do the test. Avoid cross contamination between samples.

   Avoid cross contamination between samples.

3. **Sample preparation for bulk seed testing**
   - Grind 125-250 gram corn seeds in a blender at high speed for 1-2 minute. Shake the jar and repeat grinding for one additional minute. Mix powder well. Weigh 10 gm grounded seed powder and add 20 ml water. Shake well and allow it to stand for 30 minutes at RT. Shake again and then centrifuge it at 5000g for 5 minutes. Use supernatant to do the ELISA test.

10 ASSAY PROCEDURE

Test protocol 1 for individual seed or leaf samples. Estimated procedure time is 60 minutes.

1. Allow all reagents to reach room temperature before use.
2. Add 50 µl enzyme conjugate to each well.
   Add 50 µl working extraction buffer to blank well.
   Add 50 µl of positive control in two wells and 50 µl of negative control in two wells. Add 50 µl of sample extracts to wells. Mix contents of the plate carefully, to avoid cross contamination.
3. Cover the plate and incubate it for 45 minutes at room temperature.
4. Remove content of the wells by decanting into a sink or a waste container. Add 300 µl/well wash solution to all wells and then empty wells by inverting the plate. Repeat washing three more times. Alternately, perform four washes by using microtiter plate washer. After last wash, tap the inverted microtiter plate on paper towel to remove as much liquid as possible.
5. Add 100 µl substrate per well.
6. Cover the plate and incubate for 15 minutes at room temperature.
7. Stop the reaction by adding 100 µl stop solution.
8. Measure the absorbance at 450 nm (primary filter) and 620/630 nm (secondary filter). Read

the plate within 15 minutes after addition of stop solution.

Test protocol 2 for bulk seed testing. Estimated procedure time is 90 minutes

1. Allow all reagents to reach room temperature before use.
2. Add 50 µl enzyme conjugate to each well.
   Add 50 µl working extraction buffer to blank well.
   Add 50 µl of positive control in two wells and 50 µl of negative control in two wells. Add 50 µl of sample extracts to wells. Mix contents of the plate carefully, to avoid cross contamination.
3. Cover the plate and incubate it for 60 minutes at room temperature.
4. Remove content of the wells by decanting into a sink or a waste container. Add 300 µl/well wash solution to all wells and then empty wells by inverting the plate. Repeat washing three more times. Alternately, perform four washes by using microtiter plate washer. After last wash, tap the inverted microtiter plate on paper towel to remove as much liquid as possible.
5. Add 100 µl substrate per well.
6. Cover the plate and incubate for 30 minutes at room temperature.
7. Stop the reaction by adding 100 µl stop solution.
8. Measure the absorbance at 450 nm (primary filter) and 620/630 nm (secondary filter). Read the plate within 15 minutes after addition of stop solution.

Notes on Technique:

1. Protect the plates from draught, strong light or direct sunlight during the test procedure.
2. Careful aspiration of the washing solution is essential for good assay precision.
3. Since the timing of the incubation steps is important to performance of the assay, use multi channel pipettes to dispense samples/reagents.
4. Plate readers measure absorbance vertically. Do not touch the bottom of the wells.

11 INTERPRETATION OF RESULTS

General test criteria:

1. Absorbance of the blank well should not exceed 0.15.
2. Absorbance value of blank should be subtracted from positive control, negative control and samples.
3. The coefficient of variance between the duplicate positive control should be less than 15 %
4. Mean positive control should have absorbance of at least 0.5.
5. Mean negative control should have absorbance below 0.2.
6. If above criteria are not met, the test is invalid and should be repeated.

Qualitative Test

1. For single corn/cotton leaf and seed samples:
   CUTOFF CALCULATION: for single corn/cotton leaf or seed sample Cutoff value: (Mean absorbance of negative control + 0.3)
2. **Positive sample:** If sample absorbance is more than the cutoff value.
3. **Negative sample:** If sample absorbance is less than the cutoff value.
4. For bulk seed testing:
   Sample is positive: If the sample absorbance value is above 0.5, ground corn sample contains at least 1 positive Cry3Bb seed in 500 negative seeds.
   Sample is negative: If the sample absorbance value is below 0.5, ground corn sample does not contain 1 positive Cry3Bb seed in 500 negative seeds.
   This is only a qualitative test.

5. Low level results normally indicate improper washing, cross contamination, or technique. In such cases, retesting is recommended.
6. Performance characteristics
   Sensitivity: 100 %
   Specificity: 100 %

12 WARRANTY

Gold Standard Diagnostics Hyderabad warrants that the products sold hereunder ("the Product") are defect- free in material and workmanship, provided they are used in accordance with the prescribed instructions before the expiry of the products as printed on the product label. The customer should notify Gold Standard Diagnostics Hyderabad in writing of Warranty defects during the warranty period, including an offer by the customer to return the Products to Gold Standard Diagnostics Hyderabad for evaluation. Gold Standard Diagnostics Hyderabad will repair or replace, at its sole option, any product or part thereof that proves defective in materials or workmanship within the warranty period.
This warranty also does not apply to Products to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Gold Standard Diagnostics Hyderabad.

13 THIS WARRANTY IS EXCLUSIVE

The sole and exclusive obligation of Gold Standard Diagnostics Hyderabad shall be to repair or replace the defective Products in the manner and for the period provided above.Gold Standard Diagnostics Hyderabad shall not have any other obligation or liability, whatsoever it may be, with respect to the Products or any part thereof. Under no circumstances, whatsoever the circumstances may be, shall Gold Standard Diagnostics Hyderabad be liable for incidental, special, or consequential damages.If any part of this Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.
*Parafilm is a registered trademark of American Can Corporation (now Pechinney Plastic Packaging).
**Bollgard & Roundup Ready are registered trademarks of the Monsanto Company.
***Liberty Link is a register trademark of Bayer Crop Science.

TECHNICAL SUPPORT SERVICE

For technical assistance and more information please contact the
Eurofins Amar Immunodiagnostics Pvt. Ltd.
Customer Service or your local distributor.

#1504, 15th Floor, Manjeera Trinity Corporate, eSeva Lane,
KPHB Phase 3, Kukatpally, Hyderabad, Telangana – 500072, India.
Tel: +91 7337480355
E-mail: technologies.amar@eurofins.com

www.goldstandarddiagnostics.com.