Qualitative Bt-Cry2Ab &Bt-Cry3Bb1 Dual Trait Testing

2016 SCST Superworkshop Immunoassay Day
Iowa State Seed Science Center
Dave Rambow

agdia®

Overview

- Contents of kit
- Additional required materials
- Storing the reagents
- Intended use
- Test principle
- Limitations
- Preparation for the test
- Test procedure
- Test validity
- Interpreting Results

Contents of kit

- Antibody-coated 96 well microtiter plates
- Alkaline phosphatase & peroxidase enzyme conjugate mix, concentrated
- RUB6 enzyme conjugate diluent
- pNPP Substrate solution
- TMB Substrate solution
- Positive control
- PBST wash buffer

agdia®

Additional required materials

- Distilled water
- Paper towels
- Micropipette and micropipette tips
- Seed & leaf extraction equipment
- Humid box for incubation
- Negative control
- Plate reader with 405nm & 650 nm filter

Storing the reagents

- Store all kit components @ 4^{o}C to assure full shelf life
- Keep ELISA plate pouch sealed and desicated between uses
- Allow the components of the kit to warm to room temperature prior to use

Intended use

- This dual trait ELISA is validated to detect the presence or absence of Bt-Cry2Ab & Bt-Cry3Bb1 proteins expressed in corn seed containing events MON 89034 and / or MON 88017
- Shows no cross-reaction with other GMO proteins currently on the market

Test principle

- Direct Double Antibody Sandwich (DAS) ELISA
- Antibodies specific to Bt-Cry2Ab & Bt-Cry3Bb1 have been coated to the testwells of the microplate
- An enzyme conjugate containing monoclonal antibodies specific to Bt-Cry3Bb1 protein conjugated to alkaline phosphatase & monoclonal antibodies specific to Bt-Cry2Ab conjugated to horseradish peroxidase

agdia®

Test principle

- Enzyme conjugate is added to the testwells followed by sample extracts
- If either protein is present in the sample, they are bound by the appropriate antibody and captured on the microplate
- After an incubation period, the plate is washed to remove any unbound enzyme conjugate and sample

Test principle

- pNPP substrate is added to the microplate
- If alkaline phosphatase conjugate is present, a yellow color will be produced signifying the presence of Bt-Cry3Bb1
- After a second wash step, the TMB substrate is added
- If peroxidase conjugate is present, a blue color will be produced signifying the presence of Bt-Cry2Ab
- The color reactions can be measured with a spectrophotometer or observed visually

agdia®

Limitations

- Recommend for use with single seed
- For optimal results, 1X PBST must be used for sample extraction
- Do NOT use sample extraction buffer supplied with other ELISA kits
- Assay performance is very dependent on the proper sample to buffer ratio
- Protect substrate solutions from light, could cause background color in negative wells

agdia®

Preparation for the test

- Familiarize yourself with the kit components and read the instructions carefully before performing the test
- Prepare buffers
- Prepare controls
- Prepare testwells (strip plates)
- Prepare humid box

Preparation for the test

- Prepare samples
  - Seed samples: Thoroughly crush seeds into a uniform powder (Dilute crushed seed samples in 1XPBST buffer at 1:10, typically 1 seed in 3ml buffer)
  - Let stand at least 5 minutes at room temperature
  - Use only the supernatant (top liquid layer) when adding sample extracts to testwells

agdia®

Preparation for the test

- Prepare enzyme conjugate
  - The EC is concentrated (100x) and must be diluted with RUB6 enzyme conjugate diluent before use
  - Prior to use gently shake each vial 10 deconds or vortex for 5 seconds
  - Add 110µL of concentrated EC to 11ml of RUB6 diluent, this will be sufficient for one plate
  - Add 1.1ml of concentrated EC to 110 ml of RUB6 diluent, this will be sufficient for ten plates
  - Mix the EC thoroughly before adding it to the plate

agdia®

Test Procedure

- Add enzyme conjugate
  - Dispense 100µL of EC per well
- Dispense samples and controls
  - Following your loading diagram, dispense 100µL of each prepared sample into the appropriate wells of the ELISA plate
  - Mix the contents of the wells by gently swirling the plate on the bench-top
- Incubate plate
  - Set the plate inside the humid box and incubate one hours

agdia®

Test Procedure

- Wash plate
  - Empty testwells into a sink without allowing the contents of one testwell to mix with the contents of another testwell
  - Fill all testwells completely with 1x PBST and quickly empty, repeat seven times
  - It is very important all testwells are thoroughly washed
  - After washing, hold the plate upside down and tap firmly on a paper towel to remove any excess liquid

* If using an automated plate washer, be sure the machine is at the appropriate setting for flat bottom plates

agdia®

Test Procedure

- Add pNPP substrate solution
  - Add 100µL pNPP substrate to each testwell
  - Let the plate incubate for 30 minutes, keep testwells away from strong light
- Read at 405nm
  - Measure the optical density (O.D.) of the testwell on a plate reader at 405nm
  - Do NOT use a stop solution or make an subtractions from the O.D. values

agdia®

Test Procedure

- Wash plate
  - Wash the plate 8 times with 1x PBST
  - After washing, hold plate upside down and tap firmly on a paper towel to remove any excess liquid
- Add TMB substrate solution
  - Add 100µL of the TMB substrate solution into each well of the plate
  - Let the plate incubate for 30 minutes, keeping test wells away from strong light
- Read at 650nm
  - Measure the O.D. of the test wells on a plate reader at 650nm
  - Do NOT use a stop solution or make any subtractions from the O.D. values

agdia®

Test Validity

- For the test to be valid the following criteria must be met:
  - The positive control O.D. value must be greater than or equal to 1.0
  - The buffer O.D. value must be less than or equal to 0.110
  - The negative control O.D. value must be less than or equal to 0.15
  - O. D. values are based on raw data with no blanking or subtraction of negative values
  - Stop solutions cannot be used with this test and O. D. values obtained from stopping the reaction cannot be applied to this criterion

agdia®

Interpreting results

Optical Density of Samples	Test Result
Greater than or equal to 0.5	Positive
Less than 0..110	Negative

Trouble shooting

- Edge Effects: unexpected absorbance value variances from outside to inside. Most typically seen when plates are stacked or incubated at uneven temperatures
    - Positive effect: increase in OD values
    - Negative effects: decrease in OD values
- Main causes:
    - Incubation environment (various inconsistencies)
    - Cold plates or reagents (outside wells will be warmer)
    - Color developed in strong light

Trouble shooting

- Preventing Edge Effects
  - Plate sealers - prevent evaporation
  - Strive for consistent temperature of plates, reagents and incubating plates, reagents and incubating environment – allow everything to warm up to the same temperature each time
  - Avoid harsh lighting
  - Avoid stacking plates if possible
  - Consistent plate washing

Trouble shooting

- Proper Handling of Substrates
  - Do not pipette directly from the substrate bottle
  - Never pour left over substrate back into the substrate bottle
  - Dispense substrate into ultra clean reagent reservoirs, and use immediately. Avoid re-using containers for substrates
  - Avoid intense light
  - Use caution if re-using pipette tips
  - Store at 4 to 5C

agdia®