TMC MM 3.4: Development of farmer friendly diagnostic kits for transgenic event seed purity

Objectives:

1. To develop farmer usable 'on-the-spot' rapid immunodiagnostic kits (5-min test) to detect specific transgenic products/events released for commercial cultivation in India.
2. To develop a ubiquitous test kit for the detection of any GMOs / LMOs at port of entry
3. To develop standard quantifiable parameters for regulatory testing purposes.
4. To develop methodology for detecting the presence of transgenes in a bulk sample

Scilient Findings:

Simple, single step, easy to use dip-strips were developed to detect genetically modified crops that express Cry1C, VIP3A, Cry1F, Cry2Ab2, gus (glucuronidase) & NPT-II *neomycin phospho transferase II (npt-II)* or *phosphinotricin acetyl transferase (pat)* genes.

Gus-detect:

A simple 5-minute test was developed to detect the reporter GUS which is tightly linked with Cry2Ab in Bollgard-II.

A widely used reporter gene in plants is the *uidA*, or *gusA*, gene that encodes the enzyme ~-glucuronidase (GUS)that can cleave the chromogenic substrate X-gluc (5-bromo-4-chloro-3-indolyl ~-D-glucuronic acid), resulting in the production of an insoluble blue color in those plant cells displaying GUS activity. Plant cells themselves do not contain any GUS activity, so the production of a blue color when stained with X-gluc in particular cells indicates the activity of the promoter that drives the transcription of the gusA-chimeric gene in that particular cell. The GUS assay is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual. However the conventional test takes 3-4 hours or even 12 hours with some tissues for colour to develop. Several developer solutions have been developed by CICR Nagpur, which expedite colour development within 5 minutes. One of the developer solutions has been commercialized and is being used to detect the reporter GUS which is tightly linked to Cry2Ab expression and therefore indicates the presence of Cry2Ab in Bollgard-II. The kit has become extremely popular with farmers, seed testing agencies and seed companies allover the country.

ELISAand strip tests to detect npt-n and pat markers

The two genes, *npt-II* and *pat* are the most commonly used selection markers in plant transformation for the development of GM crops, across the world. The *nptII* gene confers resistance to some aminoglycoside antibi,?tics including neomycin and kanamycin in bacteria and plant cells. NPTII phosphorylates and inactivates kanamycin, preventing kanamycin from binding to the 30S ribosomal subunit to inhibit protein synthesis, thus rendering cells resistant to the antibiotic. NPTII has been used routinely as a selection marker in the production of genetically engineered crops for probably the longest period in the history of GM crop development. The marker gene *bar/pat* was isolated originally from *Streptomyces hygroscopicus.* It encodes a small (21 kDa) protein PAT that belongs to the family of acetyltransferases, which acetylate and inactivate the glufosinate ammonium herbicides bialaphos (BASTA)and phosphinotricin PPT, thus conferring resistance to the herbicides in bacteria and plant cells expressing the gene.

Four sets of polyclonal antibodies

were used to prepare immuno-chromatographic dip-strips that are useful in detecting NPT-II and PAT. Two sets of antibodies developed in rabbits against each of the two antigens NPT-II and PATwere used. One antibody detected a C-terminal part of NPT-II another detected mid-region of the antigen. The two antibodies against PATdetected epitopes independently at the C-terminal and the N-terminal portions of the antigen. The purified immunoglobulin (IgG) was used for coating with colloidal gold particles (20 nm) or for immobilization of the membrane use for the preparation of detection strips. The anti-PAT (N terminal) and anti-N PT-II (C terminal) were coated with gold and immobilized on porous sample release pads. The affinity purified immunoglobulin (IgG) was conjugated to colloidal gold. The IgG of anti-PAT (C terminal) and anti-NPT-II (mid region) were immobilized as two separate lines on a nitrocellulose/nylon membrane that was assembled along with the sample release pad. To detect resistance, the leaf tissue or seed is crushed in a suitable buffer and the sample release pad end of the strip is dipped into the sample. The gold coated IgG captures the antigen and carries it along the membrane by capillary action until the free ends of the isozyme bind to the capture antibody line (polyclonal immunoglobin (IgG) immobilized/striped midway across the membrane. The IgG coated in Gold accumulates at the capture IgG line and generates a visible signal indicating the presence of the NPT-II or PATat the site of IgG immobilization. In the absence of the two test antigens (NPT-II or PAT)in the sample, the gold coated IgG travels along the membrane, binds with the goat-anti-rabbit antibody that is immobilized on the distal end of the nitrocellulose/nylon membrane to accumulate and generate a visible signal.

Testswith the strips are rapid (10 min to complete), simple to operate and enable the detection of GM crops that express NPT-II or PAT,without any equipment or technical assistance. The strips are highly sensitive and reliable and can assist in establishing prima-facie evidence of the presence of trans-genes in crop produce. The current method reported in this report is based on immuno-chromatography and is thus different from all the existing methods used to detect NPT-II or PAT.Immuno-chromatography has the additional advantage of being extremely simple, very rapid and highly reliable. The method enables officials at quarantine ports, farmers and extension workers to use the strips to detect GM crops.

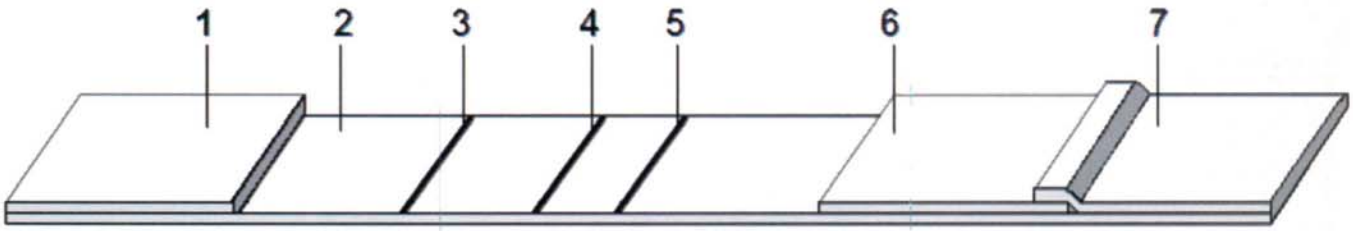
The following procedure was used to detect NPT-II or PATin leaf tissue or seeds of the test material. Seed kernels or leaf tissues are crushed individually in 0.5 ml 0.01 M, sodium phosphate buffer, pH 7.2 in a 1.5 ml microcentrifuge plastic vial, using a teflon pestle. The bottom end of the strip is dipped into the vial containing the crushed material. The solution flows up into the glass fibre pad and moves up the membrane through capillary action. In about 10-15 minutes the solution reaches the top end of the strip. One clear purple line appears at the upper portion 0.5 cm below the top end of the membrane region, indicating that the membrane is functional. This is called as a control line. If another purple coloured line appears at the central portion 1.25 cm from top end of the membrane, the test sample is considered to be positive for PAT and therefore considered positive for GM crop. Similarly if a line appears at 1.0 cm from the bottom of the membrane, the test sample is considered to contain NPT-II and therefore considered positive for NPT-II containing GM crop. If three lines (including the top control line) appear, then the sample is considered as a GM crop containing both NPT-II and PAT.Non-GM conventional plant tissues or seeds which are devoid of NPT-II or PAT, display only one purple line at 0.5 cm from the top of the membrane, thus indicating that the strip is functional and the sample is not genetically modified with respect to the presence of NPT-II or PAT.

The strips can be stored at 2-4°C in plastic bottles or aluminum pouches containing a small desiccant pack for at least one year without any loss of activity. The strips are sensitive, reliable, easy to use and interpret. Under field conditions, it take about 10-15 minutes to complete the test. The basic methods used in the development of the strips, can be utilized for the development of strips to detect GM crops incorporating any other marker genes.

EUSA and strip tests to detect CrylC, VIP3A, CryIF:

Antigens (Cry1C, VIP3A, CrylF & Cry2Ab2) were purified. Antisera was raised and ELISAdeveloped for the Cry toxins. The Cry2Ab ELISAwas developed as quantification Bt-Express-2 commercial kits and were sent to some state seed testing laboratories for spot validation. The kits were found to be accurate, sensitive and robust. The shelf life of the Cry2Ab2 and Cry1C detection kits were estimated for 6 months at 4° C and found to be sturdy, with no deterioration in the quality of the kits. Primer sets were developed for 6 genes (CrylF, Cry1C, Cry1C, VIP3A, Cry1Aa and Round-up Ready). The primers worked well and can be used to confirm gene presence. Real-Time PCRwas standardized for transgene events 'MON-531' and 'MON-15985'.

Fig. 1. Schematic diagram of a lateral flow assembly to detect NPT-II and PAT



1. sample absorption pad
2. Nitrocellulose/nylon membrane
3. The control line: The control line is goat anti-rabbit IgG.
4. The first sample line: The sample capture line is striped with anti-PAT IgG
S. The second sample line: The sample capture line is striped with anti-NPT-II IgG
6. The conjugate release pad contains colloidal gold coated with polyclonal antibody (affinity purified IgG of anti-PAT and anti-NPT-II).
7. Sample release pad

Fig. 2.schematic diagram of the steps involved in the use of lateral flow assembly to detect NPT-II or PATin putative GM test material

Step 1. Place a seed kernel or piece of a leaf in a plastic vial.
Step 2. Pour 0.5 ml buffer (provided with the kit)
Step 3. Crush the seed kernel or leaf tissue in buffer with a pestle.
Step 4. Place the dip-stick into the homogenate as per the instructions provided.
Step 5. Wait for 10 minutes until the strip is saturated with the capillary flow of the solution.
Step 6. Appearance of only one purple band at the upper portion indicates negative for NPT-II or PAT
Results as shown in figures A, B or C represent the following
Result A: Positive for PAT
Result B: Positive for NPT-II
Result C: Positive for both PATand NPT-II and therefore considered as GM crop