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Production of Diagnostic Kit to Detect Cry 2B Antigen by Use of scFv Monoclonal Antibody

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The single chain fragment antibody (scFv) antibodies were raised against CRY2B antigen by phage display technology. In antigen-antibody affinity selection the scFv antibody displaying phages concentration was increased (4.54×10^{10} to 2.3×10^{11}) and fourth round phages were higher in concentration and affinity (1.06) to antigen revealed by ELISA. The ELISA reading of randomly selected forty-five scFv monoclonal antibodies were almost similar except two clones *viz*., pscFvCry2B15 and pscFvCry2B29. The ELISA reading of clone pcFvCry2B15 was 1.40 and for pscFvCry2B29 was 1.260. The sequence result had indicated both the clones were identical and size was about 448 bp. Both the sequences were shown homology to *Homo sapiens* Ig kappa variable chain. The developed scFv gene was cloned in to pQUANTa body vector by *sfi*I and *Not*I restriction enzymes to produce the secondary antibody conjugated scFv antibody. The raised antibody conjugates were cross checked with other than *Cry*2B proteins and found no cross reactivity ensuring the developed antibodies were specific to CRY2B protein. The minimum concentration of antigen to detect by developed scFv antibody was checked by spotting the different concentration antibody on NC membrane and found 0.8 µg/ml of antigen is enough to detect by conjugated scFv antibody.

Introduction

Bt is a gram-positive spore forming soil dwelling bacteria with entomopathogenic properties. They produce insecticidal proteins during the sporulation phase as parasporal crystals. These crystals are predominantly comprised of *Cry* proteins, also called δ-endotoxins (regarded as environmental friendly) which are highly selective against target molecules (Bravo *et al.,* 2007). These toxins are highly specific to their target insect, are innocuous to humans, vertebrates and plants and are completely biodegradable. Therefore, *Bt* spores are viable alternative to synthetic chemical pesticides for the control of insect pests in agriculture (Bravo et al., 2005).

In order to protect the plants from insect attack, *Cry* proteins were used as *Bt* spore formulations over several decades. However, spores have short shelf life and sensitivity to the environmental factors. Hence, genes

encoding *Cry* proteins have been transferred to crop plants through transgenic approach. Detection of expression of *Cry* protein both quantitatively and qualitatively is important at developmental stage and during post release into field. Molecular approaches such as polymerase chain reaction (PCR) and DNA hybridization are used to detect the presence of transgene. Immunological methods are very convenient and have the potential to be developed into a routine method for the detection of presence of transgenes in plants and animals. Immuno based diagnostic methods against *cry* gene is simple, rapid and user friendly.

The immunological method includes the production of monoclonal antibody against an antigen that can be done by hybridoma technology, which involves immunization of an animal, sacrifying and isolation of spleen cells and their subsequent mixing with myeloma cells for continuous production of antibody. The spleen cell isolation is time consuming, laborious, each time it requires the sacrifying an animal. Therefore, a new technology called phage display technology was developed. The term indicates, display of foreign peptide on phage particle (Smith, 1985). Phage circumvents the need for production of large-scale cell culture by generating a large natural display library from V-gene repertoires. It is even possible to completely bypass both immunization and hybridoma development and isolate antibodies with high affinity against any antigens (Marks *et al.,* 1991). This technique produces antibody quickly in bacterial culture, facilitate genetic manipulation of their structure, and improve their performance. Since the antibodies can be generated *de novo*, phage display technology does not require scarifying any animals.

Phage display technology mainly employs filamentous phages such as M13, fd and f1. The M13 filamentous phage is cylindrical rod shaped and circular single stranded DNA about 6407 nucleotide long, consists of eleven genes. The technology employs fusion of foreign peptide fusion with one of coat protein. The technology makes use of a comprehensive library of amplified immunoglobulin genes or gene fragments that are cloned into phage expression vector, where expressed antibody is fused to M13 capsid protein (gIIIp) so that the antibody displayed on phage surface. This phage library is screened for its ability to bind to an antigen of interest, which is artificially mounted on a solid support. Phage expressing a specific antibody against a given antigen is then selected and enriched by biopanning. These engineered antibodies make them suitable for developing diagnostic kits. Hence, the present study was taken to produce the antibody and its diagnostic kit to detect the *Cry*2B protein.

Materials and Methods

The phage display technology was carried out to isolate scFv antibody fragment against *Cry*2B protein. The antibody library was gifted from MRC, Gene Service, (Cambridge, UK) and for its mass multiplication a helper phage strain, M13K07 was purchased from New England Biolab, UK. The affinity selection between antibody and antigen conducted in the Maxisorp Nunc immunotubes and its quantification for affinity conducted in Nunc ELISA plates, purchased from Nunc, (Denmark). The interaction was detected by HRP (Horse radish peroxidase) conjugated anti M13 secondary antibody, purchased from Pharmacia, UK, The other chemicals, adjuvant and detergents like polyethylene glycol (PEG) - 6000, bovine serum albumin (BSA), bacterial media components, antibiotics, components of PBS and PBST (Phosphate buffer saline + 0.05 % Tween-20)

were purchased from Hi Media, (Mumbai, India). The all cultures were stored at 4º until there next use.

Multiplication of Tomlinson library

The library was amplified as per the user manual of supplier. The entire library was inoculated in 500ml 2X TY containing ampicillin (100mg/ml) and 1% Glucose and culture was grown at 37°C until the OD at 600nm reached 0.5. The 25 ml of culture was mixed with M13K07 helper phage ($1x10^{10}$ concentration) and incubated in water bath for 30 min at 37 ^{0}C. Infected cells were spun at 3,300 g for 10 min and to the supernatant $1/5^{th}$ volume of it the PEG-NaCl (20% polyethylene glycol 6000: 2.5M NaCl) was added and incubated in ice for 1.5 h in order to precipitate the phage particles by centrifuging it at 3300 g for 10 min. The formed pellet was dissolved in 5 ml 1X phosphate buffer saline (PBS) and PEG-NaCl precipitation was carried out for one more time and finally, the phages were dissolved in 5ml of 3% bovine serum albumin (BSA) containing 1X PBS and filtered through 0.45 µm. The phages were stored at 4 ^{0}C until next use.

Amplification of M13K07 helper strain

M13K07 phages were serially diluted up to 15 concentrations and 10 µl of each concentration was used to infect 200 µl of *E. coli* K12 cells. After incubation of 30 min in 37 ^{0}C the infected culture was mixed with molten 6ml Top agar and poured on TYE hard agar plate and incubated for overnight at 37 ^{0}C. Next day, single plaque was isolated from plate and incubated in 3ml of an exponentially growing *E. coli* K12. The culture was incubated for about 2 h shaking at 37 ^{0}C to get an OD of 0.4 at 600 nm. 3 ml of K12 cells were inoculated into 500 ml of 2X TY in 2L flask and grown for 1h. Then Kanamycin was added to final concentration of 50mg/ml and grown overnight. Next day the culture was spun down at 10,800g for 15 min to remove the bacterial cells. 1/5th volume PEG-NaCl (20% polyethylene glycol 6000-2.5M NaCl) was added to the supernatant and incubated for 1 h on ice. Supernatant was spun at 10,800g for 15 min. The pellet was resuspended in 2 ml of 1X PBS and again added 1/5 volume PEG-NaCl and incubated for 1 h on ice. Tube with 1X PBS containing phages was spun at 10,800 rpm for 15 min. The pellet was dissolved in 4 ml of 1X PBS and filter sterilized using 0.45µm filter. Then phage yield was estimated by absorption spectroscopic method.

Affinity selection

The purified *Cry*2B protein at the concentration of 100 µg/ml was coated in immuno tube using coating buffer (prepared in 4ml of carbonate buffer). Next day, after washing the tube with 1X PBS, those were filled with blocking solution (3% BSA made in 1X PBS) and incubated at 37°C for 2 h. Later, tube was rinsed with 1X PBS. The Tomlinsion library stock ($1x10^{12}$) was mixed with blocking solution and filled the immunotubes up to brim level. The tube was incubated on an under-and-over turnable shaker for 30 min at room temperature and later kept in standing position for 60 min. the loosely bound phage particles were washed off by rinsing the tube with 1X PBS containing 0.05% tween-20 for ten times then the residues of detergent was removed by washing with 1X PBS. Later, the bound phages were eluted by adding 1ml of 100mM triethylamine and tube was inverted for couple of times for 5-10 min. The phages were quick neutralized by adding 0.5 ml of 1M Tris, pH 7.4. The eluted phages were transferred in to *E. coli* K12 strain by their infection process, later these phages were

amplified with the aid of helper strain M13K07. The amplified phage particles were isolated by PEG-NaCl method and isolated phages were used for second round of affinity selection (biopanning).

Total four round of selection was carried out and they differ from each other mainly b two parameters *viz.,* stringency of washing and blocking solution. First, The detergent washing step was increased 10 times compared to their previous round of detergent washing step and second difference, for blocking 2% marvel skim milk powder or 3% BSA prepared in 1X PBS was used in an alternative fashion.

Polyclonal ELISA

A population of phage produced at each round of selection was screened for its binding ability to the *Cry*2B by polyclone ELISA. The reaction was ran for all four rounds of biopan in two eplica along with two negative control *viz.,* one with antigen without antibody; other with no antigen but with antibody. After overnight incubation the washing was carried out with 1X PBS buffer and wells were blocked with 300µl of 2 per cent skim milk powder blocking solution and incubated for 2 h at 37°C. Later, along with 3% BSA in 1X PBS solution the all four biopan rounds phages were added to their respective wells at $1x10^{12}$ concentration. After, 90 min incubation wells were washed with 1X PBS-0.05%, Tween-20, followed by 3 washes of 1X PBS. Later, three hundred microliter of anti M13-HRP conjugate (diluted 1:5000 times in 3% BSA made in 1X PBS) was added to each well and incubated for 90 min at room temperature. Subsequently, the wells were washed thrice with 1X PBS containing 0.05% Tween-20 and thrice with 1X PBS alone. The 1X concentration of TMB (tetra-methyl benzidene) substrate was added to each well and incubated in a dark room for 30 min. The reaction was stopped by 50µl 1M sulphuric acid. The ELISA plate was later read at 450 nm.

Production of monoclones

The biopan round with highest ELISA reading was taken for monoclones production. The phage particles of this round was infected to *E. coli* K12 strain and formed colonies were individually picked and maintained on separate plate. From these colonies, phage particles were produced by helper phage particle and it is mentioned in Tomlinson library amplification section.

Monoclonal ELISA

Total 45 clones were selected for monoclonal ELISA and each in two replications. The experiment had negative control like antigen control and antibody control. The produced $1x10^{12}$ monoclones were allowed to interact with 100 µg of of immobilized antigen in ELISA plate. The rest protocol is mentioned in polyclonal ELISA section.

Sequencing and analysis of clones

The confirmed monoclones (pscFvCry2B15 and pscFvCry2B29) were sequenced at Dr. Swamy labs. Pvt. Ltd. The sequence results of both the monoclones were analyzed using bioinformatics tools. The forward primer and reverse primer sequences of pscFvCry2B15 were aligned by using vector NTI. The assembled sequence contains *Sfi*I and *Not*I restriction sites. The fragment between these sites is responsible for monoclonal pscFvCry2B15 gene and the full length pscFvCry2B15 homology search was made at NCBI through BLASTn algorithm and nucleotide translated homology search was made at BLASTx algorithm. The similar methodology was practiced with pscFvCry2B29 clone.

Cloning of monoclonal antibody gene pscFvCry2B in pQUANTabody vector

This vector contain highly active bacterial alkaline phosphate (PhoA) gene hence, it will be quick to identify the target antigen without addition of secondary antibody. The monoclonal antibody pscFvCry2B15 and pscFvCry2B29 genes where restricted from its native vector pIT2 with *Not*I and *Sfi*I and ligated in to pQUANTabody vector and transformed in to *E. coli* DH5α as per Sambrook and Russel, 2001 with few modification where, for spreading of the transformed colonies were done on LA plate amended with ampicillin (100µg/ml), 20µl of BCIP (20mg/ml) and 20µl of NBT (20mg/ml). Later, the plates were incubated at 37 ^{0}C incubator for overnight. The transformed colonies were confirmed through PCR by use of Pho forward and reverse set of primers and positive colonies were resubjected for restriction analysis with *Sfi*I and *Not*I enzymes. This makes the construct as anti-cry2B-ALP (alkalin phosphatase).

Expression and extraction of pscFvCry2B antibody gene from transformed clones

The pscFvCry2B clones inoculated in LB along with the 0.5mM of IPTG (Sambrook and Russell, 2001). The induced cells were lysed (0.1 mg/ml) with lysozyme and followed by sonication for 10 times at 10 sec interval. The suspension was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was stored at -20 ^{0}C.

One step detection of Cry2B using fusion antibodies

The extracted protein, which contained Anti-Cry2B-ALP conjugate was used to detect the Cry2B by ELISA, it gave a confirmation that the extracted protein contains Cry2B-ALP conjugate.

Determination of sensitivity of anti-Cry2B-ALP

The purified *Cry*2B protein was dotted onto nitrocellulose strip at different concentrations like 1µg to 10µg. A negative control without antigen was used for comparison. The NC strip was then reacted with scFv antibodies. Visible purple dots developed with lowest concentration of the *Cry*2B protein recorded.

Results and Discussion

Amplification of M13K07 helper phage

A plaque formed by M13K07 up on infecting to *E. coli* K12 isolate was picked and re-infected to fresh *E. coli* K12 isolate. The phage particle released from freshly infected cells was ranged from 0.8 x 10^{10} virion /µl. The phage obtained was further used for amplification of library.

Biopanning

The concentration of *Cry*2B bound scFv phages were ranged from 4.54 × 10^{10} to 2.3 ×10^{11}. The phages population eluted in each biopan round contains strong and weak binders. After third round of biopanning approximately hundred times of phage concentration was increased compared to second round of biopanning. However, minimal increase was observed after the fourth round of biopanning which indicated that the library was already enriched with specific binders against *Cry*2B.

Phage ELISA for Cry2B

The ELISA reading of polyclonal antibody against immobilized *Cry*2B was ranged from 0.169 to 1.06 from biopan I to biopan IV (Table 1). Hence, fourth round of biopan was selected for monoclonal antibody production.

The ELISA reading of raised forty-five monoclonal antibodies were almost similar except two clones *viz*., pscFvCry2B15 and pscFvCry2B29. The ELISA reading of clone pcFvCry2B15 was 1.40 and for pscFvCry2B29 was 1.260 (Fig. 1).

PCR amplification of pscFvCry2B15 and pscFvCry2B29 and characterization

The amplified scFv region of pscFvCry2B15 and pscFvCry2B29 with LMB3 forward and pHEN reverse primers was approximately ~500 bp. The sequence result had indicated both the clones were identical and size was about 448 bp. The sequence of clones were shown 99% identity to *Homo sapiens* Ig kappa variable chain with an E value of $5e^{-107}$.

Cloning of scFv gene from pscFvCry2B15 and pscFvCry2B29 into PQUANTabody

The pscFvCry2B15 and pscFvCry2B29 phagmids were subjected to restriction digestion. In both the cases fragment of approximately 450bp was released (Fig. 2A). These restricted products along with pQUANTabody vector were ligated, transformed and screened via blue white selection method on specific medium.

The initial the 450 bp region of scFv fragment from both clones were moved in to pQUANTabody and putative transferred colonies were selected by blue/white colony.

The putative transferred colonies were further confirmed after getting the amplification of 550 bp from phoA forward and phoA reverse primer pairs of pQUANTabody.

In restriction analysis of pQUANTabody with *sfi*I and *Not*I enzymes, a 450 bp fragment was released it confirmed the ligated vector (Fig. 2B).

One step detection of Cry2B using fusion antibodies

The scFv antibody protein with alkaline phosphate fusion protein was expressed from scFv ligated pQUANTabody. The ELISA reading of fusion protein expressed from pscFvCry2B15-ALP and pscFvCry2B29-ALP was 1.236 and 1.08 respectively (Table 2). The level of detection of *Cry*2B was almost 20 fold high compared to control value (0.056).

Diagnostic kit for cry2B

There was visible color observed on cassettes except in negative control (Fig. 3A). The antibodies produced were used for cross testing with other *Cry* protein like *Cry*1Ac and lectin protein samples (Fig. 3B). The raised antibody conjugates were reacted only with *Cry*2B protein samples not with other proteins. The pscFvCry2B15-ALP and pscFvCry2B29-ALP clones were used as antibody conjugate for the detection of *Cry*2B antigen and at the 1:10 fold diluted 10µg of crude *Cry*2B protein was detected by NBT-BCIP substrate.

Sensitivity of diagnostic kit

The antigen coated on cassettes at different concentration was allowed to reacts with antibody-ALP conjugates. In ELISA it was revealed that at 4 µg/ml concentration of *Cry*2B, the antibody conjugate was able to detect the antigen *Cry*2B. But when antigen was spotted on NC membrane it was revealed that at concentration of 0.8 µg/ml, the antibody conjugate was able to detect. In case of ELISA the antibody conjugate was able to detect antigen at the concentration of 4 µg/ml. At this concentration, there was significant colour development, the ELISA reading was 0.071 and control value was 0.055 (Table 3).

Table.1 Polyclonal ELISA readings

	Only antigen	Only antibody	BP-I	BP-II	BP-III	BP-IV
Mean value 450 nm	0.036	0.038	0.169	0.517	0.908	1.06

Table.2 Direct ELISA readings

ELISA plate wells	Mean readings
Only antigen	0.056
Only antibody	0.058
pSNMALP15	1.236
PSNMALP29	1.08

Table.3 Sensitivity test readings

ELISA plate wells	Mean readings at 415 nm	ELISA plate wells	Mean readings at 415 nm
A1B1C1	0.055	A7B7C7-5 µg	0.080
A2B2C2	0.055	A8B8C8-6 µg	0.081
A3B3C3-1 µg	0.056	A9B9C9-7 µg	0.098
A4B4C4-2 µg	0.057	A10B10C10-8 µg	0.102
A5B5C5-3 µg	0.060	A11B11C11-9 µg	0.112
A6B6C6- 4 µg	0.071	A12B12C12-10 µg	0.110

Fig.1 Monoclonal ELISA of 45 scFv clones from fourth biopan round. C1- only antigen, C2- only antibody and numerical numbers are scFv clones

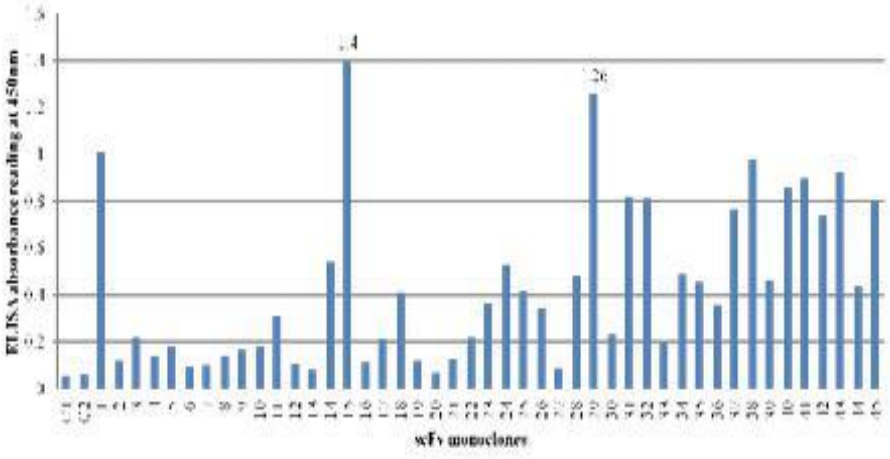


Fig.2 A. The release of scFv (450bp) from pIT2 vector after restriction digestion with *Not*1 and *Sfi*1 enzymes. B. The scFv fragment was cloned in pQuantabody and it is confirmed by restriction digestion with *Not*1 and *Sfi*1 enzymes

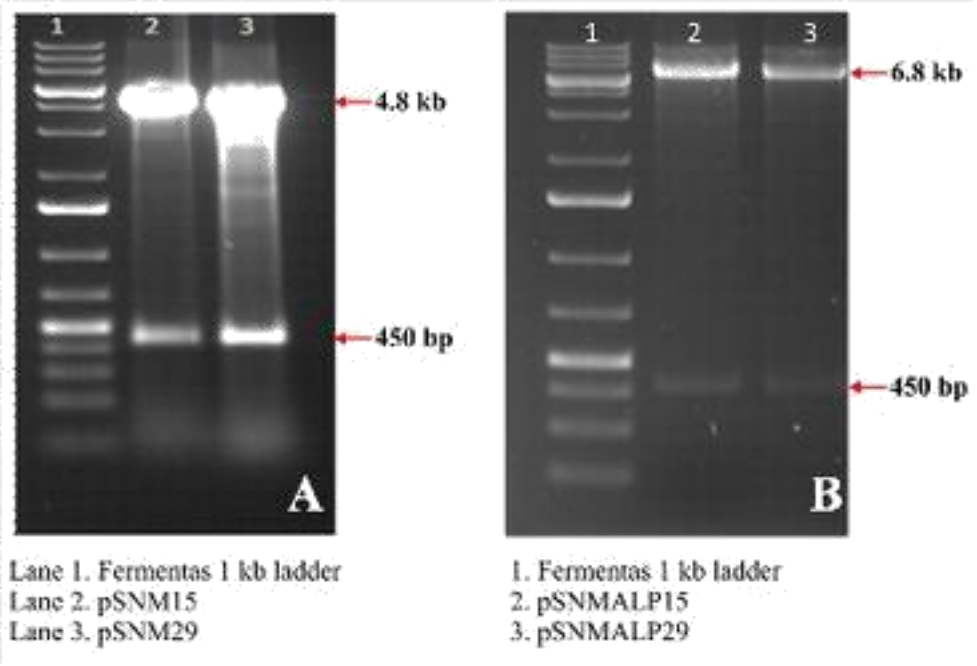
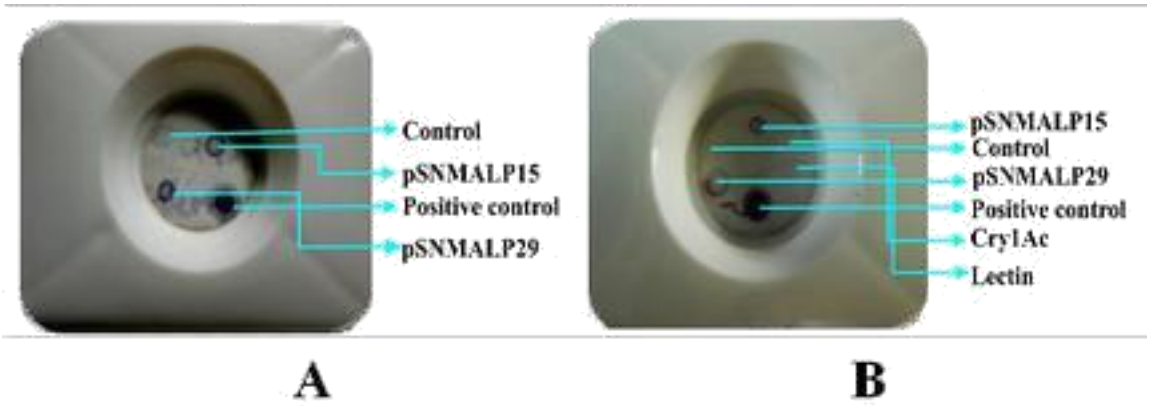


Fig.3 A. Detection of *Cry*2B antigen through rapidot on NC-membrane cassette, B. Cross reactivity test of developed scFv antibodies against different different antigens



Phage display technique has been successfully attempted in producing monoclonal antibodies against toxins, hormones, growth factors, chemical, protein, enzyme, viruses, fungi and bacteria (Geuijen *et al.*, 2005). The success rate of monoclonal antibodies production is hundred per cent by using phage display technique depicted in many research reports (Griffiths *et al.*, 1994; Francoise et al., 2005 and Anderson *et al.*, 2007). In this study, panel of single chain fragment variable monoclones against *Cry*2B was produced and monoclonal antibody gene responsible against *Cry*2B was characterized. These monoclonal antibodies were used for developing diagnostic kit for detection of *Cry*2B.

Increase in the number of antigen specific scFv phages were identified by steady increase in absorbance after each round of

panning. There was increase in phage recovery after third round of panning when compared to second round of biopanning and minimal increase in fourth round of panning over the third round. The phage population indicated that the fourth round of panning was almost equivalent population size as compared to third round because number of strong binders reached the highest population, which means that the scFv antibodies already enriched with specific antigen. These enriched scFv antibodies were confirmed by polyclonal phage ELISA in which the fourth panning showed higher value than previous panning process even when same concentration of phages ($1x10^{12}$/well) were used. Similar result was reported by Griep, Van Twisk, Beckhoven et al., (1998) for selected scFv antibody against lipopolysaccharide of *Ralstonia solanacearum* Race-3. The phages obtained from IV panning were used to generate the monoclones. The phages obtained at the end of fourth round of biopanning of *Cry*2B were subsequently used to generate the monoclonal antibodies by infecting to suitable host (*E. coli* K12). Forty-five clones were randomly picked from fourth biopan and the phage particle from each clone was isolated. Out of forty-five, two clones namely pSNM15 and pSNM29 had showed the highest ELISA value of about 1.4 and 1.26 respectively. This suggests that these two clones carrying a scFv gene which is highly specific to *Cry*2B than remaining other clones. The similar result was observed by Francoise *et al.,* (2005), who screened monoclonal antibodies against carrageenan which is a polysaccharide. The phage isolated from 120 clones was subjected to monoclonal ELISA and each scFv showed different absorbance value against the carrageenan. Lower reading value has indicated the less affinity to the antigen whereas higher absorbance value showed the stronger binding to target molecule (carrageenan). About 25 scFv clones (42 per cent) were showed absorbance values greater than 1.5 and values greater than 2.0 were observed in 12 clones (20 per cent).

The two clones namely pSNM15 and pSNM29 were sequenced and characterized. The clone pSNM15 was of 448bp long and clone pSNM29 had same length base pairs. The assembled sequence results indicated 98% homology with anti-TREM-like transcript-1 antibody [synthetic construct]. Single chain antibody fragments (scFv) genes of pSNM15 and pSNM29 were subcloned into pQUANTabody expression vector which enabled to transcriptionally fuse with scFv monoclonal antibody genes and the gene coding for bacterial alkaline phosphatase (PhoA) enzyme. Upon expression of clone, it has produced 17 kDa fusion protein of ALP along with pSNM15 and pSNM29 monoclonal antibody. After digesting the pQUANTabody vector, the pSNM15 and pSNM29 clones with *Sfi*I and *Not*I enzymes, the digested sample was ligated and transformed into competent *E. coli* DH5□. The transformed colonies grown on selection medium containing ampicillin 100 □g/ml and NBT-BCIP, the blue colonies were picked and tested by PCR using phoA forward and phoA reverse primers for the insert.

The advantage of using the transcriptionally fused antibody is to save time and cost. We have developed anti-Cry2B-ALP conjugate antibody for *Cry*2B. Single chain antibodies developed here are advantageous over traditional monoclones because the production costs are less and stability of single chain antibodies during storage is high due to its small size. The monoclonal antibody conjugates prepared as crude extract readily detected *Cry*2B even at low concentrations (0.8 □g/ml). This was similar to the finding of other workers (Mousli et al., 1998) who used crude preparations containing the conjugate in rapid visual immunoassay for

the specific detection of *A. australis* hemocyanin.

The Rapidot kit have developed using monoclonal antibody (pSNM15 and pSNALP29 as capture and pSNMALP15 and pSNMALP29 conjugates as detection) proved to be efficient and reliable for *Cry*2B detection. The antibody conjugates able to detect the antigen *Cry*2B in the span of 20-25 minutes. Similar results were obtained (Gautam et al., 1999) against *Phytophthora nicotianae* spores. The assay does not require any sophisticated equipment nor can highly trained technical staff for carrying out this experiment.

The cost of production of kit is reasonable. There are two types of kit we have developed. One is with membrane cassettes and other one is with NC membrane strips (Plate 16). The components of the kit are extraction buffer, wash solution, blocking solution and conjugate antibody solution and substrate buffer. Except substrate solution remaining components are considerably cheap

In conclusion, we developed the two monoclonal antibody scFv specific to antigen *Cry*2B protein through the phage display technology without scarifying the animals. The both clones were highly similar and they produced the same type of interaction with the antigen *Cry*2B. The scFv antibodies were able to detect the protein even at low 0.8 □g/ml concentration. Therefore, the developer kit can be used directly in the field to detect the *Cry*2B protein in plant samples.

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