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Phage-mediated competitive chemiluminescent immunoassay for detecting Cry1Ab toxin by using an anti-idiotypic camel nanobody

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Abstract

Cry toxins have been widely used in genetically modified organisms for pest control, raising public concern regarding their effects on natural environment and food safety. In this work, a phage-mediated competitive chemiluminescent immunoassay (c-CLIA) was developed for determination of Cry1Ab toxin using anti-idiotypic camel nanobodies. By extracting RNA from camels' peripheral blood lymphocytes, a naive phage-displayed nanobody library was established. Using anti-Cry1Ab toxin monoclonal antibodies (MAbs) against the library for anti-idiotypic antibody screening, four anti-idiotypic nanobodies were selected and confirmed to be specific for anti-Cry1Ab MAb binding. Thereafter, a c-CLIA was developed for detection of Cry1Ab toxin based on anti-idiotypic camel nanobodies and employed for sample testing. The results revealed that a half-inhibition concentration of developed assay to be 42.68 ± 2.54 ng/mL, in the linear range of 10.49–307.1 ng/mL. The established method is highly specific for Cry1Ab recognition, with negligible cross-reactivity for other Cry toxins. For spiked cereal samples, the recoveries of Cry1Ab toxin ranged from 77.4 % to 127 %, with coefficient of variation of less than 9 %. This study demonstrated that the competitive format based on phage-displayed anti-idiotypic nanobodies can provide an alternative strategy for Cry toxin detection.

Graphical Abstract

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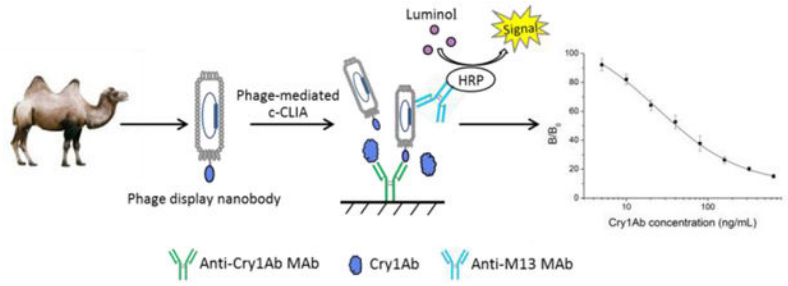
CONFLICT OF INTEREST

The authors declare no conflict of interest.



Keywords

Cry1Ab toxin; anti-idiotypic camel nanobody; competitive chemiluminescent immunoassay

INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive soil bacterium that produces Cry toxins (e.g., Cry1Ab, Cry1F, Cry2A, and Cry3B) in the form of inclusion bodies during its sporulation phase.[1–3] Cry toxins are widely used for developing genetically modified organisms (GMOs) because of their high toxicity to lepidopteran pests. Toxic effects are based on disruption of midgut cells of insect pests, resulting in pore formation and ultimately leading to death.[4–6] Since the toxins are highly specific to insects, they are considered non-toxic to humans and animals. However, serious debates and increasing concern arise regarding potential and perceived environmental and public health safety issues resulting from the use of GMOs. Several countries have implemented appropriate labeling regulations for GMO-derived products.[7, 8] Therefore, it is necessary to develop rapid and reliable methods for detecting Cry toxins. Besides, Cry toxins are still the most widely used biological insecticides, a reliable assay could also be used as quality control for production, distribution, and persistence of the toxin produced by Bt.

At present, various methodologies have been applied for GMO analysis. Among these approaches, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) are the two most frequently used formats.[9–16] PCR methods are highly sensitive and accurate but cannot identify the level of expression of transgenic proteins; and the requirements of skilled operator and well-equipped laboratory are non-negligible factors that limit their application.[17, 18] By contrast, ELISA methods are suitable for detecting transgenic proteins expressed in GMOs with the advantages of simplicity, cost-effective and high-throughput.[19] To date, the most commonly used ELISA method for Cry proteins is double antibody sandwich ELISA (DAS-ELISA).[20–22] However, the main drawback of DAS-ELISA is the difficulty of matching between two antibodies and complicated procedures of the assay. Compared with DAS-ELISA, competitive ELISA (c-ELISA) holds the advantages of labor-saving, ease of operation and shortened assay time. As for competitive immunoassays, an appropriate competitive antigen or antigen mimetic is necessary. Several competitive immunoassays have been reported for detecting Cry toxins, whereas these approaches commonly use Cry toxin standard both as coating antigen and competitive antigen, thus possibly incurring high costs.[23, 24]

In recent years, anti-idiotypic nanobodies have been used as antigen mimetics in immunoassays for detection.[25–27] Nanobodies, which are derived from sera of camelids, are the smallest functional antigen-binding fragments completely devoid of light chains.[28, 29] Compared with conventional antibodies, nanobodies contain a longer complementarity determining region 3 (CDR3), which can present unique epitopes and bind to the cavities of target antibodies, and they have been proven as valuable tools for antigen mimicry.[30, 31] In addition, nanobodies have the advantages of high solubility, high thermal stability and ease of production, which make nanobody valuable involved in rapid method development. Until now, no study reported the use of anti-idiotypic nanobodies for detecting Cry toxins in c-ELISA.

In the present study, anti-idiotypic nanobodies that specifically bind to anti-Cry1Ab monoclonal antibodies (MAbs) were successfully selected from a naive phage-displayed nanobody library. Subsequently, a phage-mediated competitive chemiluminescent immunoassay (c-CLIA) based on anti-idiotypic nanobody was established for the determination of Cry1Ab toxin. Accuracy and precision of the assay were evaluated by determining cereal samples spiked with Cry1Ab toxin. With anti-idiotypic nanobody as competitive antigen mimetic, the proposed c-CLIA may provide an alternative strategy for Cry1Ab toxin analysis.

MATERIALS AND METHODS

Materials and reagents

The anti-Cry1Ab MAb used was previously produced in our laboratory.[32] Cry1Ab, Cry1Ac, Cry1F, Cry2A, and Cry3B toxins were purchased from You Long Bio. Co. Ltd. (Shanghai, China). SuperScript III First-Strand Synthesis SuperMix was purchased from Invitrogen (Carlsbad, CA, USA). Restriction enzymes and T4 DNA ligase were purchased from NEB (Ipswich, MA, USA). *Escherichia coli* strain TG1 and helper phage M13K07 were obtained from MRC (Cambridge, England). Horseradish peroxidase (HRP) conjugated anti-M13 antibody was purchased from GE Healthcare (Beijing, China). Tetramethylbenzidine was purchased from Sigma (St. Louis, MO, USA). SuperSignal ELISA Pico chemiluminescent substrate was obtained from Thermo Scientific (Waltham, MA, USA). All other reagents used were of analytical grade.

Construction of a naive Bactrian camel nanobody library

The schematic diagram of the construction of a naive nanobody library is presented in Figure 1. Briefly, total RNA was extracted from peripheral blood lymphocytes by using the TRIZOL reagent (Invitrogen) and used as template for cDNA synthesis via SuperScript III First-Strand Synthesis SuperMix Kit. Nanobody genes were amplified by two-step nested PCR. In the first PCR, the VH regions were amplified with the specific primers CALL001 (5’-GTCCTGGCTCTCTTCTACAAGG-3’) and CALL002 (5’-GGTACGTGCTGTTGAACTGTTCC-3’). After the first PCR fragments of around 700 bp were gel-purified, the nanobody genes were re-amplified with VHH-for (5’-ACTGGCCCAGGCGGCCGAGGTGCAGCTGSWGSAKTCKG-3’) and VHH-back (5’-ACTGGCCGGCCTGGCCTGAGGAGACGGTGACCWGGGTC-3’) nested primers

containing the restriction site *sfi* I (underlined) in the secondary PCR.[33, 34] Final PCR products around 400 bp were purified and sub-cloned into the phagemid pComb3x. Then, recombinant phagemids were transformed into electrocompetent *E. coli* TG1 cells, and these cells were plated on 2×YT plates containing 100 μg/mL ampicillin. The library diversity was measured by calculating the number of colonies and DNA sequencing.

Biopanning and identifying anti-idiotypic nanobodies

In the first round of biopanning, 100 μL of anti-Cry1Ab MAb (100 μg/mL) was coated on microplate wells at 4 °C overnight. After washing with phosphate-buffered saline with 0.1 % Tween-20 (PBST), wells were blocked with 3 % bovine serum albumin (BSA)-PBS at 37 °C for 2 h. Then, 100 μL of naive phage-displayed nanobody library (2×10^{11} pfu) was added to the wells and incubated for 1 h at 37 °C. Unbound phages were discarded, and wells were washed 10 times with PBST. Bound phages were eluted with 100 μL of elution buffer [0.2 M glycine-HCl (pH 2.2), and 1 mg/mL BSA] for 8 min at 37 °C with gentle shaking and immediately neutralized with 15 μL of 1 M Tris-HCl (pH 9.0). Eluted phages were titered and amplified for subsequent round of panning. For the next three rounds of panning, the number of input phage libraries remained the same (2×10^{11} pfu), whereas concentrations of coated anti-Cry1Ab MAbs gradually reduced to 50, 25, and 10 μg/mL. Meanwhile, the elution buffer was replaced with 100 μL of Cry1Ab standard diluted in PBS at 10, 5, and 1 μg/mL. After four rounds of panning, 95 individual clones from the fourth round of elution were randomly picked and identified by phage-ELISA. Subsequently, the coding DNA of positive phages was sent for sequencing.

Preparation of phage-displayed nanobodies

The selected clone was cultured in 2×YT medium containing 2 % (v/v) glucose and 100 μg/mL ampicillin at 37 °C with shaking until optical density at 600 nm reached approximately 0.4. The culture was then superinfected with helper phage M13K07 at 37 °C for 15 min and incubated at 37 °C with shaking at 250 rpm for 45 min. Cells were harvested by centrifugation at 1000 *g* for 10 min and transferred to fresh 2×YT medium (containing 100 μg/mL ampicillin and 50 μg/mL kanamycin). Phage-displayed nanobodies were produced by growing the culture at 30 °C with shaking (250 rpm) overnight. After the culture was centrifuged at 10,000 *g* for 15 min, supernatant of amplified phages was precipitated with 1/6 volume of 20 % (w/v) PEG/NaCl, and the phages were collected by centrifugation at 10,000 *g* for 15 min. Finally, purified phages were suspended in PBS and titered by determining colony-forming units.

Phage-mediated competitive chemiluminescent ELISA

A competitive chemiluminescent ELISA for Cry1Ab toxin detection was developed using an anti-idiotypic nanobody isolated from the naive nanobody library. First, the wells of a 96-well microplate were coated with 100 μL of anti-Cry1Ab MAbs overnight at 4 °C and blocked with 5 % BSA in PBST for 2 h at 37 °C. Then, 50 μL of selected phage-displayed nanobodies and 50 μL of Cry1Ab standard at various concentrations (from 0 ng/mL to 625 ng/mL) or sample extracts were added to the wells. After incubation at 37 °C for 1 h and washing six times with PBST, 100 μL of HRP-conjugated anti-M13 phage antibodies (1:5000 diluted in blocking buffer) was added and incubated in wells for 1 h at 37 °C.

Finally, 100 μL of chemiluminescent substrate was added to the washed wells and incubated for 3 min. Chemiluminescence intensity was detected on a luminescence reader Mithras LB 940 (Berthold, Germany). To measure the optimized dilution of immunoassay reagents, a checkerboard assay was conducted using different dilutions of phages and anti-Cry1Ab MAbs in advance.

Assessment of competitive chemiluminescent ELISA

The method was validated by evaluating the cross-reactivity (CR) with other Cry toxins and by determination of spiked samples. To evaluate CR, various concentrations (from 5 ng/mL to 312.5 ng/mL) of other Cry toxins (Cry1Ac, Cry1F, Cry2A, and Cry3B) were applied to chemiluminescent ELISA. To measure recovery of the assay, Cry1Ab-free cereal samples (rice, wheat, and corn) which collected from our own farm station were spiked with a known concentration of Cry1Ab at 500, 1000, 2000, and 5000 μg/kg. Sample extracts and dilution methods were performed as follows: 1 g of ground samples were mixed with 1 mL of extraction solution (10 mM PBS, pH 7.4, containing 0.05 % Tween-20 and 0.1 % BSA) and extracted by gentle shaking at room temperature for 2 h. After centrifugation at 10,000 *g* for 15 min, the supernatant was diluted by tenfold PBS for assay analysis.

RESULTS AND DISCUSSION

Construction of a naive phage-displayed nanobody library

Peripheral blood lymphocytes were isolated from 10 non-immunized Bactrian camels. Nanobody genes were amplified from lymphocyte cDNA by nested PCR. The first PCR fragments displayed evident bands at 700 bp, and these 700 bp fragments were purified as templates for re-amplifying nanobody genes (400 bp) in secondary PCR (Figure 2B). After digestion with *sfi* I restriction enzyme, nanobody genes were inserted into phagemid pComb3x. The recombinant phagemid was transformed into *E. coli* TG1 competent cells by electroporation transformation to generate the nanobody library. By counting the number of colonies in gradient dilution plates, library size calculated was 1.7×10^8, this value can be expected to generate sufficient antibody diversity. Twenty clones were randomly selected to perform colony PCR, and results revealed 100 % correct insertion rate as shown in Figure 2C. The results demonstrated that a high quality naive nanobody library was successfully constructed, which can be used for selecting anti-idiotypic nanobodies specific to Cry1Ab.

Selection of specific phage-displayed nanobodies

After four rounds of biopanning, output phages increased from 6.0×10^4 pfu to 2.3×10^7 pfu; this result indicated effective enrichment of specific clones binding to target antibody (Figure 3A). A total of 95 phage clones randomly selected from the fourth round of panning were analyzed by phage ELISA. Among these phage clones, four (P-22, P-24, P-32, and P-48) were identified to specifically bind to anti-Cry1Ab MAbs competing with free Cry1Ab. The phage clone P-48 exhibited the highest sensitivity, and was hence selected for further research.

The DNA sequencing results showed the presence of four different sequences. As presented in Figure 3C, P-22 and P-24 only involved a single amino acid difference, and showed

similar sensitivities in immunoassay. Besides, framework regions were conserved among the four sequences, whereas the amino acids in CDRs were considerably distinct. Interestingly, two amino acids 'GR' coexisted in the CDR3 of the four sequences, which may play an important role in binding with anti-Cry1Ab MAbs.

Optimization of phage-displayed nanobody based c-CLIA

The purified phage-displayed nanobody P-48 was evaluated for its characterization by competitive chemiluminescent immunoassay. To select an appropriate blocking buffer, 5 % skim milk, 5 % BSA, 5 % OVA, and 2 % gelatin in 0.05 % PBST, which are commonly used as blocking reagents in immunoassay, were each tested to avoid non-specific signals. Results showed that 5 % BSA in 0.05 % PBST presented a relatively lower signal than the others and was thus used as blocking reagent in development of the assay. The optimal concentrations of anti-Cry1Ab MAbs (0.1 μg/mL) and phage-displayed nanobodies (2.5×10^7 pfu/mL) were then determined by a checkerboard procedure.

Considering that ionic strength and pH can affect the performance of immunoassay, the influence of ionic strength (from 5 mM to 40 mM) and pH (from 5.0 to 9.0) were evaluated. Maximum relative light unit (RLUmax) showed a slight difference for 5, 10, and 20 mM ionic strengths, but the signal decreased when ionic strengths reached 40 mM (Figure 4A). The lowest half-inhibition concentration (IC_{50}) value was obtained at a concentration of 10 mM. At optimal ionic strength, the effect of pH was also examined. The IC_{50} values showed no significant differences within pH 6.0–8.0, and the lowest IC_{50} was observed at pH 7.4 (Figure 4B). Therefore, 10 mM ionic strength and pH 7.4 were selected as the optimal conditions for the assay.

Development and Evaluation of c-CLIA

Under the optimal conditions, as shown in Figure 5, IC_{50} of the assay established with P-48 was 42.68 ± 2.54 ng/mL. The linear range (IC_{20}-IC_{80}) of the assay was 10.49–307.1 ng/mL. The limit of detection (LOD) in the immunoassay was estimated from the mean (plus three standard deviations) of 10 blank samples and measured 6.45 ng/mL.

Recently, several studies have been conducted on Cry1Ab detection; the LODs in these studies ranged from 0.01 ng/g to 0.56 ng/g.[35–40] The developed phage-mediated c-CLIA exhibits lower sensitivity than those methods, including our previously developed DAS-ELISA (with LOD of 0.47 ng/mL).[32] As is well known, non-competitive ELISA is commonly more sensitive than competitive ELISA. In the present work, taking advantage of phage-displayed system, thousands of pVIII copies of phage capsid and HRP-conjugated anti-M13 pVIII MAb were helpful for the signal amplification. In addition, a chemiluminescence system was applied to improve the sensitivity of the assay. To further improve sensitivity, molecular modeling and directed evolution technology may be applied in further studies. The majority of the reported ELISAs for detecting Cry1Ab toxin were DAS-ELISAs, which are based on monoclonal antibodies and polyclonal antibodies. However, polyclonal antibodies can bind to multiple epitopes on a protein, resulting in false-positive results and cross-reactivity. Conversely, excluding the use of polyclonal antibodies, the developed competitive assay exhibits a lower blank signal and higher specificity. In

addition, compared with conventional DAS-ELISA, the proposed method not only avoids a complicated sandwich pairing process but also requires a relatively short assay time. Furthermore, phage-displayed anti-idiotypic nanobodies can be easily produced at a large scale and may reduce the use of Cry1Ab toxin to conserve costs and to ensure environmental safety.

Soluble nanobodies have also been expressed by pET26b plasmid in *E. coli* and evaluated for their performance by c-CLIA. However, comparing with phage-displayed nanobodies, less sensitivity and limited linear range for the determination of Cry1Ab toxin have been obtained based on these soluble nanobodies (data not shown). Several studies also reported similar results, which indicated that sensitivity can be improved by using phage-displayed antibodies instead of soluble antibodies[41, 42]. This result may be explained by the functional structure of nanobodies, which cannot be represented well by prokaryotic expression, whereas eukaryotic expression may improve the process in future studies; meanwhile, the phage-mediated format can favor the performance of nanobodies with functional structure, and provide signal amplification over the soluble nanobodies[43–45]. In this study, phage-displayed nanobodies, rather than soluble nanobodies, were used in the development of immunoassay for Cry1Ab detection. The antigen was intended to be replaced by anti-idiotypic antibody (nanobody) for detecting Cry1Ab with competition, and required the hapten-inhibitable or competitive characteristics of the anti-idiotypic nanobody. Therefore, selecting Ab2β anti-idiotypic antibodies with the antigen internal image will be the best choice, which rigidly targets the CDR of Ab1 for antigenic determinant recognition[46–48]. Thus, another possibility could be the binding site of selected anti-idiotypic nanobody P-48 combined with anti-Cry1Ab MAb may not be identical to that of Cry1Ab combined with anti-Cry1Ab MAb. Therefore, due to its small size, nanobody P-48 cannot completely occupy the binding site of anti-Cry1Ab MAb in the Cry1Ab toxin. By contrast, the large size of phage-displayed nanobody simplified inhibition of Cry1Ab binding to anti-Cry1Ab MAb. Therefore, using Fab or ScFv for anti-idiotypic nanobodies selection is preferred for improving competitive reaction, which would fit in the epitope and completely inhibit the binding of MAb to Cry1Ab toxin. Several immunoassay formats have been assembled based on the selected anti-idiotypic nanobody, the immunoassay presented here achieved the highest sensitivity, which can be explained as described above.

Cross-reactivity

To determine the specificity of the assay, the cross-reactivity against Cry1Ac, Cry1F, Cry2A, and Cry3B was evaluated. Cross-reactivity was calculated using the formula CR %= IC_{50} of Cry1Ab/IC_{50} of other Cry toxins × 100 %. As shown in Figure 6, negligible cross-reactivity was observed with other Cry toxins.

Assay validation

Matrix interference is a common problem in immunoassays. Before sample analysis, three GMO-free cereal samples (rice, wheat, and corn) were selected to evaluate the matrix effect. Diluted sample extract was used to prepare serial concentrations of Cry1Ab solution as described above. No significant matrix effect was observed in the assays with each sample matrix (data not shown). Then, accuracy and precision of the anti-idiotypic-nanobody-based

c-CLIA were evaluated by spike and recovery analysis. Rice, wheat, and corn samples were spiked with different concentration of Cry1Ab (500, 1000, 2000, and 5000 μg/kg), and each sample was measured twice for repeatability. As presented in Table 1, the average recovery ranged from 81.5 % to 96.6 % and coefficient of variation (CV) ranged from 2.8 % to 6.6 % in the rice sample. By contrast, recovery ranged from 77.4 % to 105 % and CV ranged from 3.2 % to 5.9 % in wheat. Finally, recovery ranged from 86.4 % to 127 % with a CV of 2.6 %–8.8 % in the corn sample. These results demonstrated that adequate recovery and efficiency from cereals were achieved through anti-idiotypic-nanobody-based c-CLIA for Cry1Ab detection. In addition, thirty cereal samples randomly collected from local markets were analyzed by the nanobody based c-CLIA. All the samples were found negative for Cry1Ab by the developed method. These results are in accordance with the results obtained from commercial ELISA kit.

In conclusion, we constructed a naive phage-displayed nanobody library. Four anti-idiotypic nanobodies specific to Cry1Ab were selected after four rounds of biopanning. The phage-displayed nanobody P-48, which exhibited the highest sensitivity, was further applied to anti-idiotypic-nanobody-based competitive chemiluminescent ELISA for Cry1Ab detection. The developed assay showed a working range of 10.49–307.1 ng/mL and LOD of 6.45 ng/mL. To date, it has not been reported that application of anti-idiotypic nanobodies in competitive immunoassay for Cry1Ab. The results of this work indicate that competitive immunoassay based on anti-idiotypic nanobodies may provide an alternative strategy for detecting Cry and other proteins.

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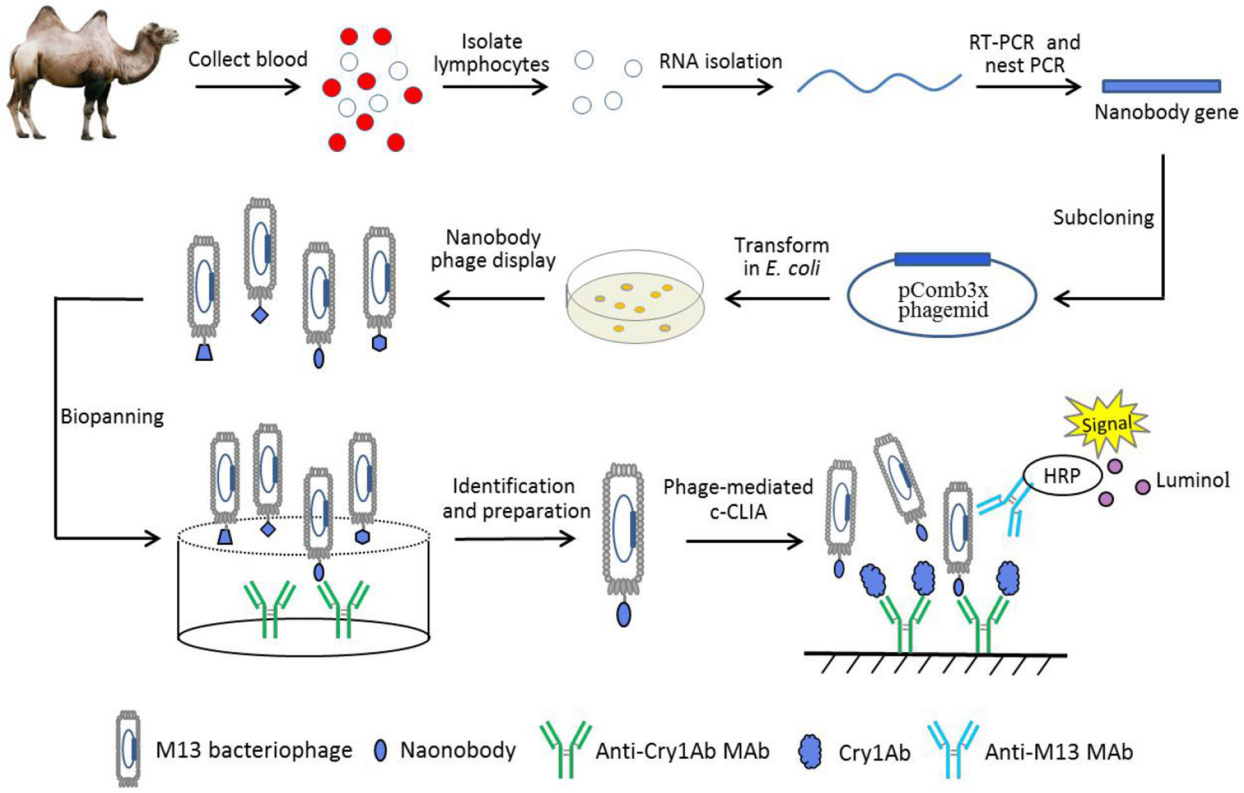

Figure 1.

Outline of strategies to select the anti-idiotypic nanobodies from a naive nanobody library and to develop a c-CLIA for Cry1Ab.

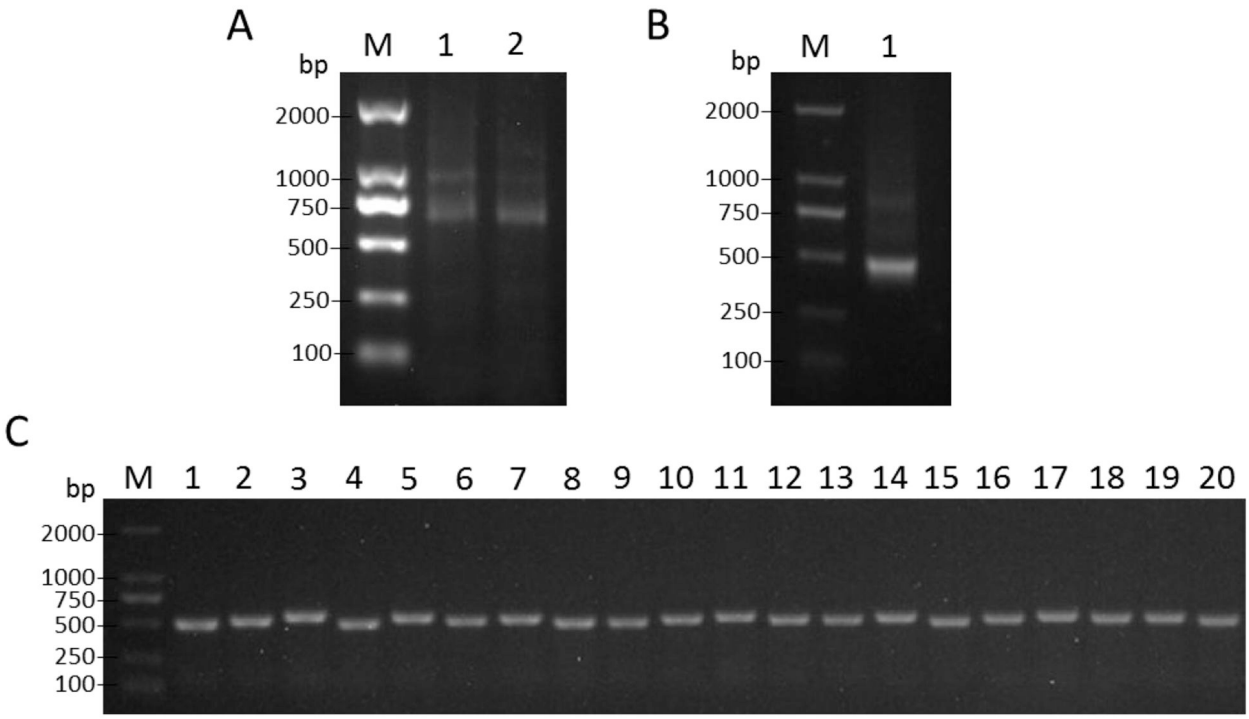

Figure 2.

Construction of the naive nanobody library. (A) The first PCR fragments displayed a band around 700 bp. (B) The nanobody genes were amplified by secondary PCR with a band of 400 bp. (C) The correct insertion rate of the library detected by PCR of 20 individual clones.

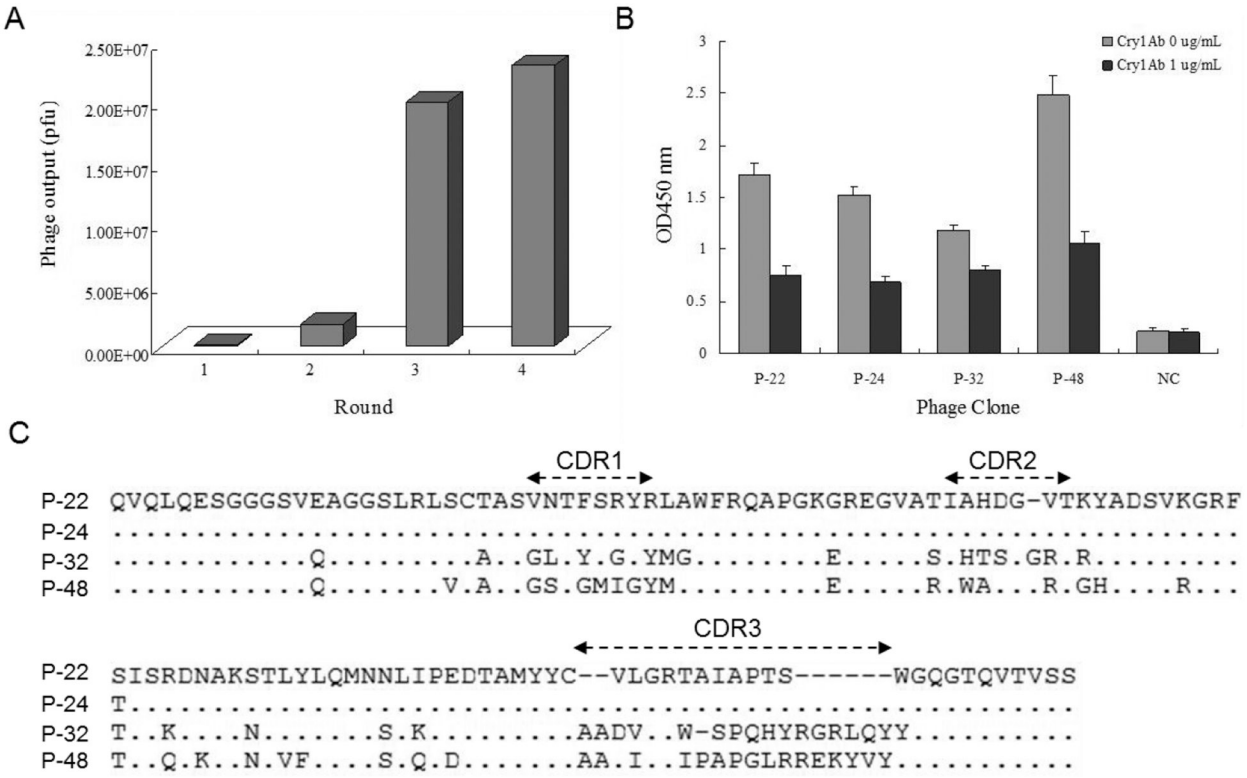

Figure 3.

(A) Number of phage output in each round of panning. (B) Four positive clones selected from phage-ELISA. (C) The amino acid sequences of the positive clones.

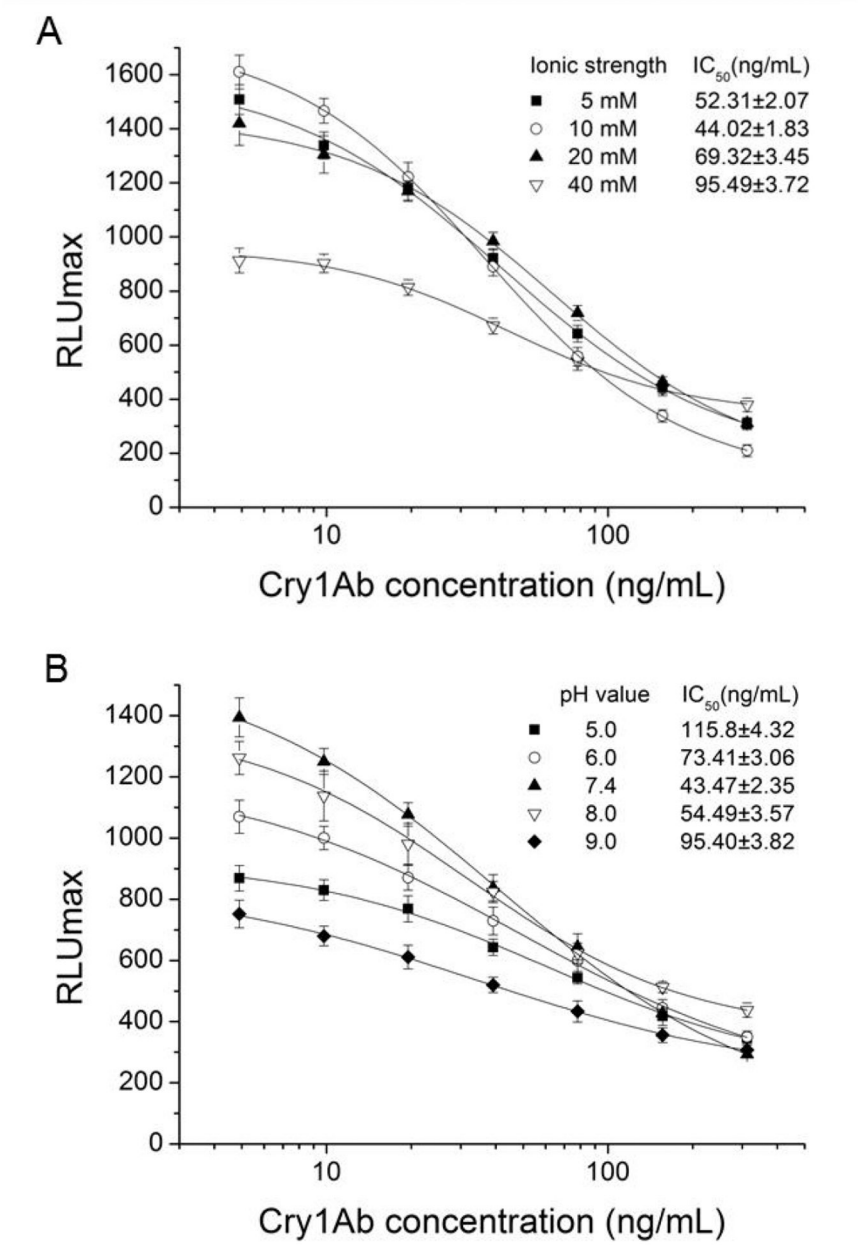

Figure 4.
Effects of (A) ionic strength and (B) pH on the performance of phage-mediated c-CLIA based on anti-idiotypic nanobody.

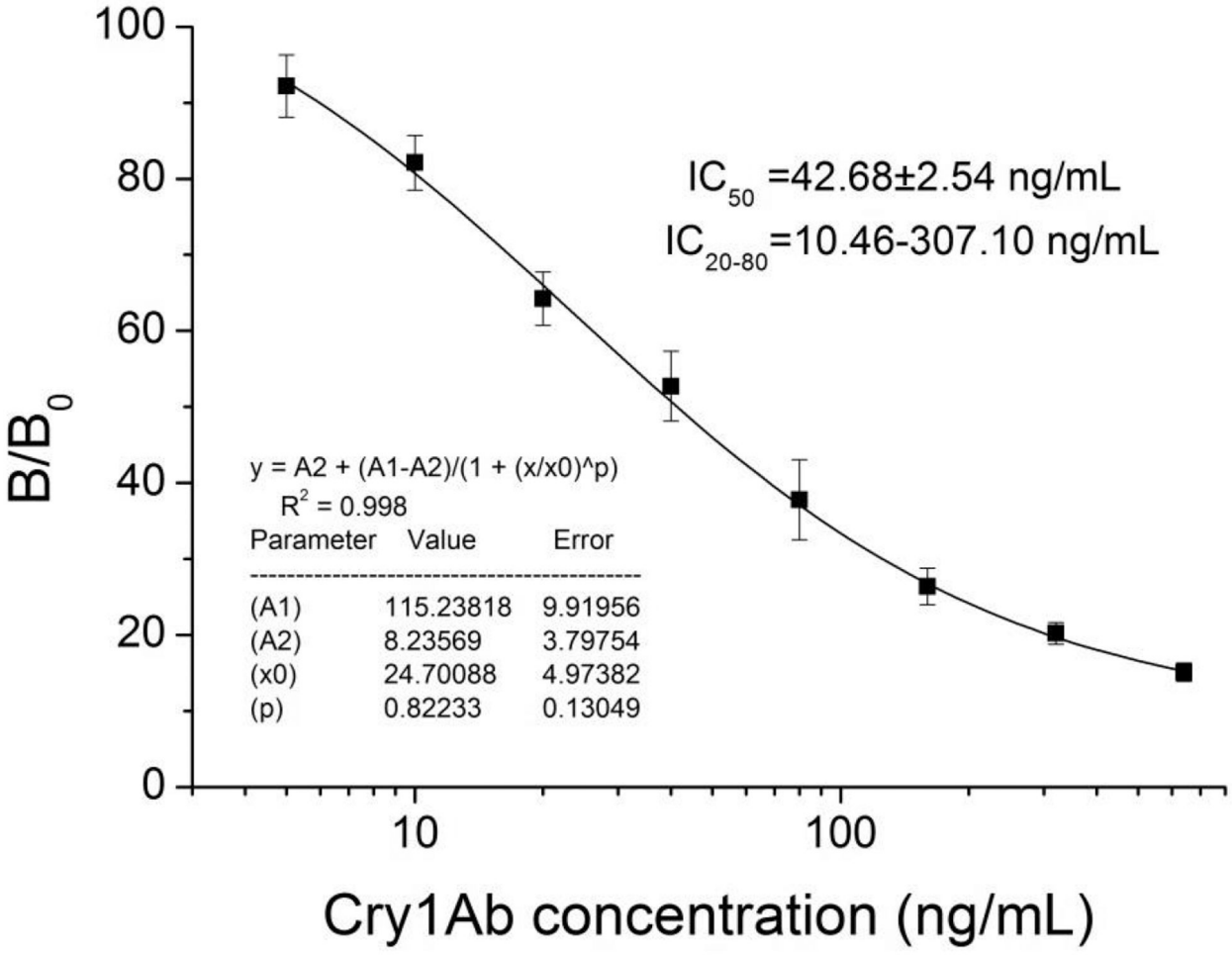

Figure 5.

Standard competitive inhibition curve for Cry1Ab analysis under the optimized conditions. The error bars represent the standard deviation (n =5).

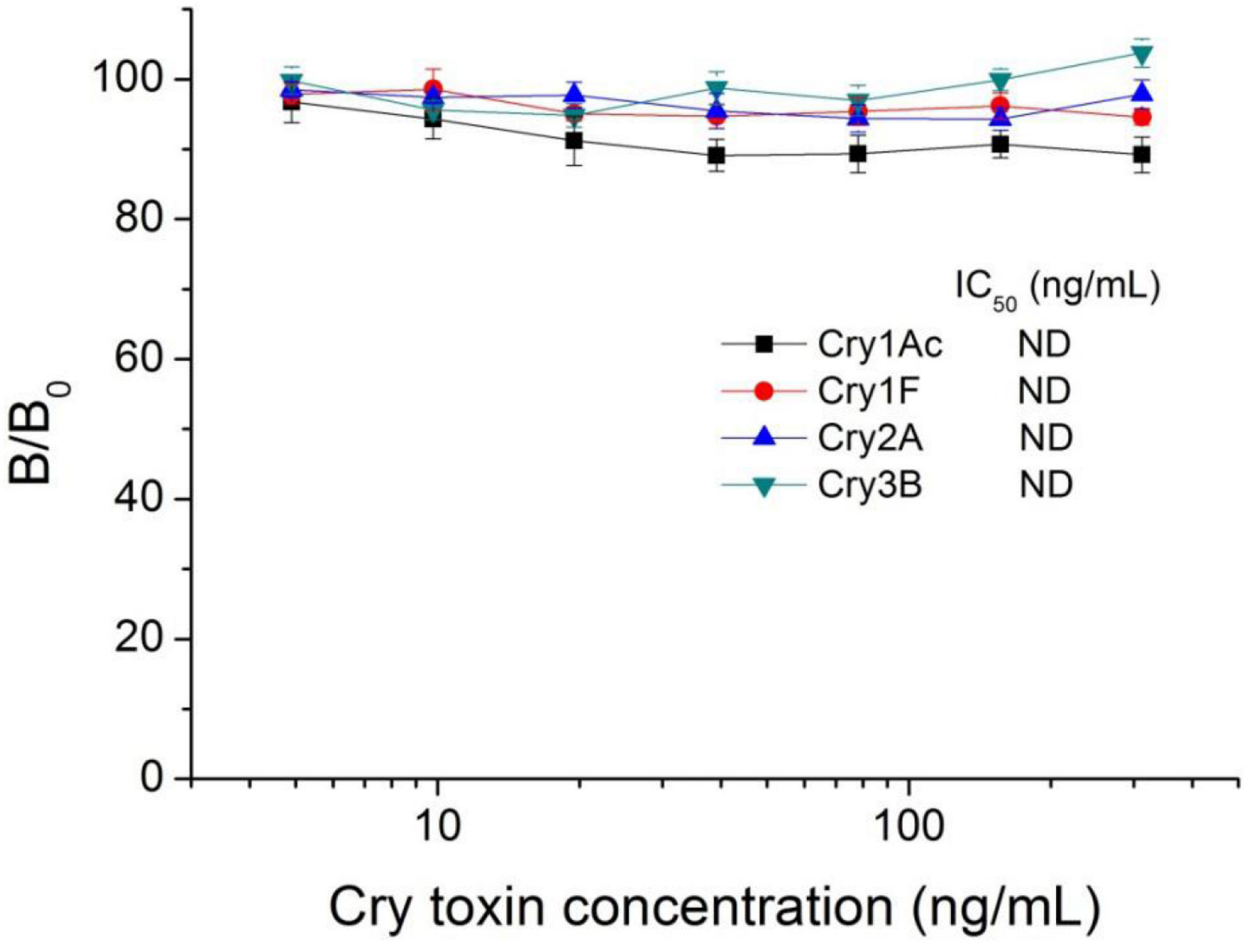

Figure 6.
Cross-reactivity of the c-CLIA with other Cry toxins.

Table 1.

Recovery analysis of Cry1Ab in rice, wheat, and corn samples by c-CLIA

Matrix	Spike level (μg/kg)	c-CLIA (n=3[a])		
		Mean±SD (μg/kg)	Recovery (%)	CV (%)
Rice	500	483.2±32.1	96.6	6.6
	1000	903.8±37.5	90.4	4.1
	2000	1686±46.7	84.3	2.8
	5000	4075±120	81.5	2.9
Wheat	500	427.1±25.4	85.4	5.9
	1000	774.3±32.0	77.4	4.1
	2000	2102±89.1	105	4.2
	5000	4504±142	90.1	3.2
Corn	500	635.2±23.2	127	3.6
	1000	1114±98.3	111	8.8
	2000	1984±51.6	99.2	2.6
	5000	4320±305	86.4	7.1

[a]Each assay was performed thrice on the same day, and each reading was taken by the same person.