Development of a Double-Recombinant Antibody Sandwich ELISA for Quantitative Detection of Epsilon Toxoid Concentration in Inactivated Clostridium Perfringens Vaccines

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Research article

Keywords: Epsilon toxin, Clostridium perfringens, Recombinant antibody, Phage display, Sandwich ELISA

Posted Date: May 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-25433/v1

Version of Record: A version of this preprint was published on September 29th, 2020. See the published version at https://doi.org/10.1186/s12917-020-02572-4.

Abstract

Background: Epsilon toxin (ETX) causes a commonly fatal enterotoxemia in domestic animals and causing serious economic losses to animal husbandry. In this study, we selected several clones against ETX using repertoires displayed on filamentous phage. Anti-ETX specific clones were enriched by binding to immobilized antigen, followed by elution and re-propagation of phage. After multiple rounds of binding selection, we evidenced that most isolated clones had high affinity and specificity for the ETX by ELISA.

Results: We isolated two recombinant monoclonal antibodies against ETX by phage display technology and Using the B1 phage VH antibody isolated from DAb library as capture antibody and G2 soluble scFv antibody isolated from Tomlinson I + J libraries as the detector antibody, we developed a sandwich ELISA test.

Conclusions: This sandwich ELISA could be a good candidate for quantitative detection ETX with the detection range of 5 ~ 100000 ng/ml in inactivated commercial vaccines against enterotoxemia.

Background

Epsilon toxin (ETX) is a 33 kDa protein and one of the pore-forming toxins that synthesized by *Clostridium perfringens* type B and D strains [1]. ETX has high potency and the centers for disease control and prevention (CDC) categorized epsilon toxin into the second highest priority agents group (Category B) and can be a potential bioterrorism weapon [2]. This toxin plays a significant role in causing enterotoxemia in domestic ruminants, especially sheep and can cause sudden death and severe economic losses [3]. Enterotoxemia begins when *C. perfringens* type B or D strains secrete the ETX prototoxin into the intestinal lumen [4]. At first, the toxin is inactive, and it is activated after cleaving the 13 N-terminal and 22 C-terminal residues by proteases [5]. After activating, ETX forms the pores across the cell membrane of ruminant cells and alters the permeability of cell monolayers such as epithelium and endothelium and leads to necrotic lesions and perivascular edema in different tissues especially in kidney and brain cells [6, 7]. An effective manner to control ETX-induced enterotoxemia in domestic ruminants is vaccination. Commercially available vaccines are based on inactivated toxins that isolated from formaldehyde-treated bacterial culture filtrate. Unfortunately, these vaccines usually have variable immunogenicity and have many production defects, such as quality control steps, standardization that antigen concentration and potency of the vaccine is varies from batch to batch [8–11]. Therefore, it is essential to develop methods for detection and the quantitative measurement of antigen in each batch. One of the most appropriate methods for quantitative detection of a specific protein in a complex mixture is sandwich enzyme-linked immunosorbent assay (ELISA) that used as a common tool for clinical and research programs and have many benefits like sensitivity, specificity, simplicity, stability, rapid analysis and inexpensive assay [12–14].

The aim of this study was to isolate specific recombinant antibody fragments from Human Domain Antibody (DAb) and Tomlinson I + J libraries as capture and detector antibodies for developing a

sandwich ELISA for quantitative detection of ETX in inactivated commercial enterotoxemia vaccines.

Results

Confirmation of epsilon toxoid

To evaluate the molecular size and purity of the epsilon toxoid, ETX was analyzed by SDS-PAGE. The results showed that the toxoid is pure and exhibited the expected molecular weight (Fig. 1).

Biopanning of phage display libraries

To isolate specific phage antibodies against ETX, two selections in three rounds were performed using the Tomlinson I+J and DAb libraries. In each round of selection, the phages that had a binding affinity for ETX were isolated and after amplification were used for the next round of selection.

Polyclonal and monoclonal phage ELISA

The populations of phage recovered after each round of selection from each library were screened against ETX by polyclonal phage ELISA. The results of polyclonal ELISA tests showed the reactivity towards the epsilon toxoid increased from the first round to third round, respectively. These results indicated that phage antibodies reactive with epsilon toxoid were successfully isolated and enriched from both libraries (Fig. 2).

Also, phages were prepared individually from clones of second and third rounds of selection from each library (Tomlinson I + J and DAb) for analysis by monoclonal phage ELISA. As Fig. 3, displays, most of the isolated clones especially from Tomlinson I + J libraries had a high affinity toward antigen.

In the next steps, the best-isolated clone from monoclonal scFv ELISA of Tomlinson I + J libraries (Clone No. G_2) was used for expression soluble scFvs and the best-isolated phage from DAb Library (Clone No. B_1) was used for designing sandwich ELISA.

Expression and purification of G2 scFv

To improve the expression of recombinant anti-ETX G_2 scFv, the gene was subcloned into the pET26b(+) vector using *Not*I and *Nco*I restriction enzymes. Following subcloning and transformation, the presence of the full-length insert of a subcloned gene was analyzed by double digestion using the above restriction enzymes. Figure 4, Shows digested plasmid of clone G_2 contained a fragment of 708 bp corresponding full length of the insert.

After expression, soluble G_2 scFv was purified by nickel affinity chromatography and analyzed by SDS-PAGE and Western blotting using HRP-conjugated monoclonal anti-polyhistidine antibody (Fig. 5). This result implies that G_2 scFv successfully expressed in *E. coli* BL_{21} (DE_3) and purified.

Sandwich ELISA for quantitative detection of ETX

A sandwich ELISA test was designed to determine the concentration of ETX using the B_1 phage VH antibody that was isolated from the DAb library as capture antibody and G_2 soluble scFv antibody that was selected from Tomlinson I + J libraries as the detector antibody (Fig. 6). The results of titration showed that the optimal concentration of the capture B_1 phage VH antibody was 25 µg/ml (100 µl), and the detector G_2 scFv antibody was 50 µg/ml (100 µl). Also, 1:5000 HRP-conjugated anti-his-tag antibody was used as the conjugated antibody. The linear standard curve was obtained with the detection range of 5 ~ 100000 ng/ml. The linear equation was Y = 0/2843x + 0/0775 with R_2 = 0.9918 (Fig. 7).

To assess the efficacy of the designed sandwich ELISA, an inactivated tetravalent enterotoxemia vaccine produced by the razi vaccine and serum research institute (RVSRI) was diluted and tested by designed ELISA. As table 1 shows, the concentration of ETX in the diluted vaccine series could be easily calculated using the linear standard curve.

	25 µg/ml (coated) capture phage VH antibody isolated from DAb library (B1)	
Various dilutions of inactivated tetravalent Enterotoxemia (RVSRI) vaccine in PBS	50 µg/ml Detector scFv antibody isolated from Tomlinson I + J libraries(G2)	
	OD (vaccine)	Concentration epsilon toxoid in Razi commercial Enterotoxemia vaccine using the linear standard curve (Y = 0/2843X + 0/0775)
0.001	1.166	3.828702
0.0001	0.85	2.7172
0.00001	0.424	1.218783
0.000001	0.3145	0.833626

Table 1
The quantity of epsilon toxin in the commercial vaccine.

Discussion

Previous studies reported many problems with commercial enterotoxemia vaccines such as differences in antigen concentration and potency of the vaccine from batch to batch and this cause inequality of antigens level distributed in each injection vial [8, 11, 15, 16] so the potency of the vaccine varies from batch to batch [9, 10, 17]. For detection and the measurement of the *C. perfringens* epsilon toxoid in each batch and retaining the immunogenicity of vaccines prepared, we developed a sandwich ELISA test using specific recombinant antibodies against pure *C. perfringens* epsilon toxoid. We used recombinant antibodies because recombinant monoclonal antibody fragments have several advantages in comparison with conventional monoclonal antibodies and have been proposed as alternative tools for various diagnostic purposes [18, 19]. These proteins can be easily and economically produced in various expression systems, for example, *E. coli*, plant, yeast, and cell culture and their biological activities can be readily improved by *in vitro* techniques [19, 20].

To isolate specific antibodies against the important epsilon toxoid, we used the two DAb and Tomlinson I + J phage display libraries. These libraries have billions of different antibody fragments built simultaneously with high expression, proper folding and low toxicity for *E. coli* cells. After three rounds of selection, and evaluation by polyclonal and monoclonal phage ELISA, several clones from rounds two and three showed high reactivity against ETX toxoid. Nine of the best clones were chosen for further experiments. Finally, clone B_1 from DAb library for producing anti-ETX phage antibody and clone G_2 from Tomlinson I + J libraries for making anti-ETX scFv used to design the sandwich ELISA.

As indicated previously, the double antibody sandwich ELISA has higher sensitivity and specificity than indirect ELISA, which can accurately quantify antigens with simple operation [21–24]. We designed a sandwich ELISA for the measurement of the *C. perfringens* epsilon toxoid. Both purified anti-ETX scFv and purified anti-ETX phage antibody were evaluated as a capture antibody for coating to microtiter plates or as detector antibody, and their affinity and efficiency toward antigen were checked. Then the coated plates were tested for dose-dependent reactions against a dilution series of the ETX.

The results showed our phage antibody fragment can capture antigen much better than scFv antibody (data have not presented). Then, the optimum concentration of both recombinant anti-ETX antibodies were applied for testing dose-dependent reactions against a dilution series of the ETX and commercial vaccine. Fortunately, the sandwich ELISA test was able to determine the concentration of ETX in the commercial vaccine.

In summary, we isolated two recombinant monoclonal antibodies (B1 and G2) with a high affinity to ETX from two synthetic libraries by phage display technology. We established a sandwich ELISA using the B1 phage antibody for capture and G2 scFv antibody fragment as a detector of ETX. This sandwich ELISA could be used for quick detection ETX and monitoring vaccine quality of commercial enterotoxaemia vaccines production for farm animals.

Conclusion

The results of this study demonstrate that the sandwich ELISA developed is a rapid, sensitive, and reliable diagnosis method that has well performance in sensitivity and features so this sandwich ELISA can be used as a valuable tool for quantitative detection ETX and assessment of commercial vaccines against enterotoxemia.

Methods

The Tomlinson I + J human single fold synthetic naïve phage display single-chain antibody fragment (scFv) libraries constructed in pIT_2 (HIS MYC tag) vector with the size of 1.4×10^8 for each, and DAb human VH domain library constructed in PR_2 (MYC VSV tag) with the size of 3×10^9, helper phage KM_{13}, *E. coli* strains TG_1 and HB_{2151} for selection of specific antibody clones and production of phage and soluble single-chain Fvs antibodies, respectively, were purchased from GeneService (Cambridge, UK). *C. perfringens* epsilon toxoid was purchased from NIBSC, UK.

Biopanning of phage display libraries

The purity of the epsilon toxoid was initially confirmed by SDS-PAGE. The libraries and KM_{13} helper phage stocks were amplified to have enough quantities for use in several rounds of selection. Biopanning was performed using Tomlinson I + J and DAb libraries in parallel to ensure selecting the most epsilon toxoid binding clones. For selection, MaxiSorp® immunotubes (Nunc, Denmark) were coated with 5 ml pure *C. perfringens* epsilon toxoid (100 µg/ml) in carbonate buffer (pH 9.4) and incubated overnight at 4 °C, washed three times with PBS and blocked with 4% MPBS (4% Marvel in PBS) buffer overnight at 4 °C. The next day, 5×10^{12} purified phages from each library in 5 ml MPBS buffer were added to each immunotube and incubated for one hour with gentle agitation at room temperature. The unbound phages were removed by washing 10 times with PBS containing 0.1% Tween 20 and twice with PBS buffer. To recover bound phages, 5 ml trypsin solution (1 mg/10 ml) was added to each immunotube and incubated for one hour at room temperature with gentle agitation. Then eluted phages (5 ml) were used to infect exponentially growing *E. coli* TG_1 (30 ml), incubated at 37 °C for one hour in a water bath and plated onto TYE agar plates (100 µg/ml ampicillin, 4% glucose) and incubated at 30 °C overnight. The following day, cells were scraped from agar plates and diluted into 500 ml of 2xTY medium (100 µg/ml ampicillin, 4% glucose) and infected with KM_{13} phage. Then grown phages were precipitated and purified by cold PEG/NaCl solution (20% Polyethylene glycol 6000, 2.5 M NaCl). The purified phages were used in the next round of selection and repeated binding, elution and infection steps for 2 times.

Screening of clones by polyclonal phage ELISA

The screening was done with the entire population of phages eluted (fused to the antibody fragments) after each round of selection. ELISA plates (Nunc, Denmark) were coated with epsilon toxoid (100 µg/ml) for overnight at 4 °C. Next day plates were washed and blocked by 4% MPBS buffer per well. After

washing, 10 µl of PEG precipitated phages recovered from each round of selection were diluted in 100 µl MPBS buffer and added to each well. One hour later, plates were washed and the binding of phages were detected using a monoclonal anti-c-Myc antibody (Biolegend) and anti-mouse HRP conjugate (Sigma Aldrich, USA) and detected with TMB substrate (Biobasic, Canada). Sulfuric acid solution 1 M was used to stop the reaction and enzymatic activity of HRP–antibody conjugate and the absorbance was read at 450 nm and 620 nm with a microplate reader.

Monoclonal phage ELISA

For monoclonal phage ELISA, individual colonies were picked after second and third rounds of selection and grown into 2xTY medium containing 100 µg/ml ampicillin and 4% glucose in a 96-well plate and incubated at 37 °C, 250 rpm for 12 hours. Then the overnight culture for each clone diluted 100-fold into 200 µl 2xTY medium (100 µg/ml ampicillin and 0.1% glucose) and incubated at 37 °C, 250 rpm until OD600 = 0.4. The culture was infected with 4×10^8 KM_{13} helper phages for 30 min at 37 °C, the bacteria pelleted by centrifugation and resuspended in 150 µl of 2 × YT containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) before growth overnight at 25 °C, 250 rpm for 16 hours. Then the plate was centrifuged and 100 µl of culture supernatant of each well was used for ELISA plates that pre-coated with epsilon toxoid (100 µg/ml) and blocked with 4% MPBS buffer. Phage binding was detected with a monoclonal anti-c-Myc antibody (Biolegend) and anti-mouse HRP conjugate (Sigma).

scFv ELISA

Antibodies fused to the pIII coat protein of phage were converted to soluble scFv proteins by induction of bacteria with IPTG. scFv ELISAs were done similar to monoclonal phage ELISA. Soluble scFvs were produced by induction of culture of individual bacterial colonies at absorbance of about 0.9 at OD of 600 nm with 1 mM of IPTG and growth for 16 hour at 25 °C, 250 rpm in 2 × YT. For ELISA, the culture supernatants that contained soluble scFvs were transferred to the antigen-coated wells of ELISA plates. Bound scFvs were detected using 1:5000 monoclonal anti-Polyhistidine HRP conjugate (Sigma Aldrich, USA).

Expression and purification of scFvs

The best clone of scFv ELISA from Tomlinson I + J libraries (Clone No. G_2) was tested to confirm the presence of full-length insert by double digestion.

To improve the expression of anti-ETX scFv, the gene of selected clone (G_2) was subcloned between the *Nco*I and *Not*I restriction sites of the pET26b (+) vector. The pET26b (+) vector is containing the kanamycin resistance marker and carries an N-terminal pelB signal sequence for potential periplasmic localization in addition to an optional C-terminal His Tag sequence. $CaCl_2$-treated *E. coli* BL21 (DE3) was

used as the host strain and transformation was performed using the heat shock method. G_2 scFv antibody was expressed using 0.01 mM IPTG for 24 hour at 25 °C. To extract the antibody, one gram of pelleted cell was resuspended in 5 ml ice-cold protein extraction buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1% Triton X_{100} and 300 µg⁄ml lysozyme, pH 7.8) and it was incubated for 30 min on ice. Then sample was sonicated at 24–25% amplitude for 6 sec ON and 6 sec OFF cycle on ice and total sonication time was 10 min. Following centrifugation at 10,000 g for 10 minutes at 4 °C supernatant containing total protein was mixed with 500 mM NaCl and 20 mM imidazole to purify the scFv protein by Ni-NTA affinity chromatography. Samples were loaded at a speed of 1 ml ⁄min onto the affinity column that had previously been equilibrated with cold binding buffer (50 mM Tris–HCl, 500 mM NaCl and 20 mM imidazole, pH 7.4). Unrelated bacterial proteins were eluted with binding buffer containing 50 mM imidazole before recovery of the scFv with binding buffer containing 400 mM imidazole. The purified protein fractions containing anti-ETX scFv were analyzed by SDS-PAGE and western blotting.

Development a sandwich ELISA for quantitation of ETX

The sandwich ELISA was designed using B_1 (the best clone isolated from DAb library) phage antibody as capture antibody and soluble G_2 scFv (the best clone isolated from Tomlinson I + J libraries) as a detection antibody. The optimal concentrations of capture (100 µg/ml, 50 µg/ml, 25 µg/ml, 5 µg/ml, 0.5 µg/ml, 0.05 µg/ml and 0.005 µg/ml) and detection (100 µg/ml, 50 µg/ml, 25 µg/ml, 5 µg/ml, 0.5 µg/ml, 0.05 µg/ml and 0.005 µg/ml) antibody were determined by checkerboard titration. To obtain a standard curve of the sandwich ELISA, microtiter plates were coated with 100 µl (25 µg/ml) capture phage VH antibody (B_1) for overnight at 4 °C. After washing and blocking with 3% BSA/PBS (3% BSA in PBS), the 100 µl 10-fold serial dilutions of pure *C. perfringens* epsilon toxoid antigen (5 ~ 100000 ng/ml) were added into the wells and incubated at 37 °C for 1 hour. The wells were washed five times with PBST (0.1% Tween-20 in PBS) buffer and once with PBS buffer and then 100 µl (50 µg/ml) G_2 scFv antibody was added into the wells and incubated at 37 °C for 1 hour. Subsequently, 1:5000 dilution of HRP-conjugated monoclonal anti-Polyhistidine antibody as the conjugate antibody was added into all wells after washing. Subsequent addition of TMB substrate, the OD value was measured at 450 nm. The standard curve was calculated with the plotting OD450 nm against the epsilon toxoid concentration by using a scatter plot in Excel.

Finally, the antigen contents of serially diluted an inactivated enterotoxemia commercial vaccine (razi vaccine and serum research institute (RVSRI)) was determined by the above standard curve.

Abbreviations

CDC: Centers for disease control and prevention; ELISA: Enzyme-linked immunosorbent assay; ETX: Epsilon toxin; DAb: Human domain antibody; PEG: Polyethylene glycol; RVSRI: Razi vaccine and serum research institute; scFv: Single-chain antibody fragment.

Declarations

Ethics approval and consent to participate

The investigation was conducted according to the Declaration of Helsinki principles.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and supplementary information files.

Competing interests

The authors declare that they have no conflicts of interest associated with this study.

Funding

This work was supported by the Shiraz University and Ministry of Science, Research and Technology [grant number 9330154].

Author’s contributions

MA: Conceived and Performed the experiments; Analyzed and interpreted the data; Wrote the manuscript. MG: Developed the study protocol; Assisted with data interpretation, drafted and revised the manuscript, supervised the project. MT: Helped in planning the project; contributed to revision of the manuscript; supervised the project. All authors read, edited, and approved the final version of the manuscript.

Acknowledgements

We would like to acknowledge the Faculty of Veterinary Medicine, the Shahid Bahonar University of Kerman for their assistance in design and implementation of this study.

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Figures

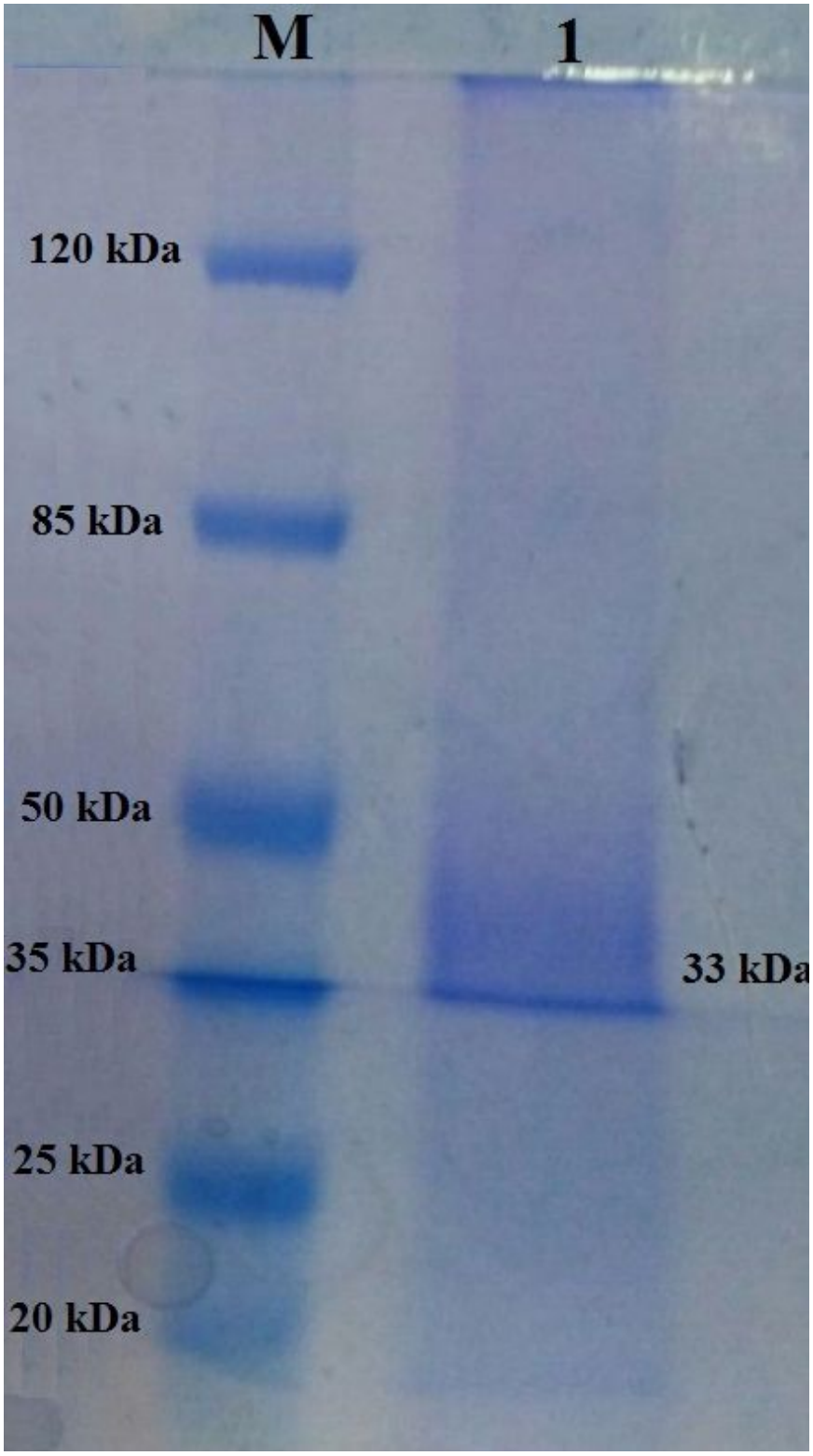

Figure 1

Confirmation of epsilon toxoid by SDS-PAGE. Lane M: 20 - 120 kDa prestained protein ladder (Bio Basic, Canada INC), lane 1: Epsilon toxoid (33 kDa).

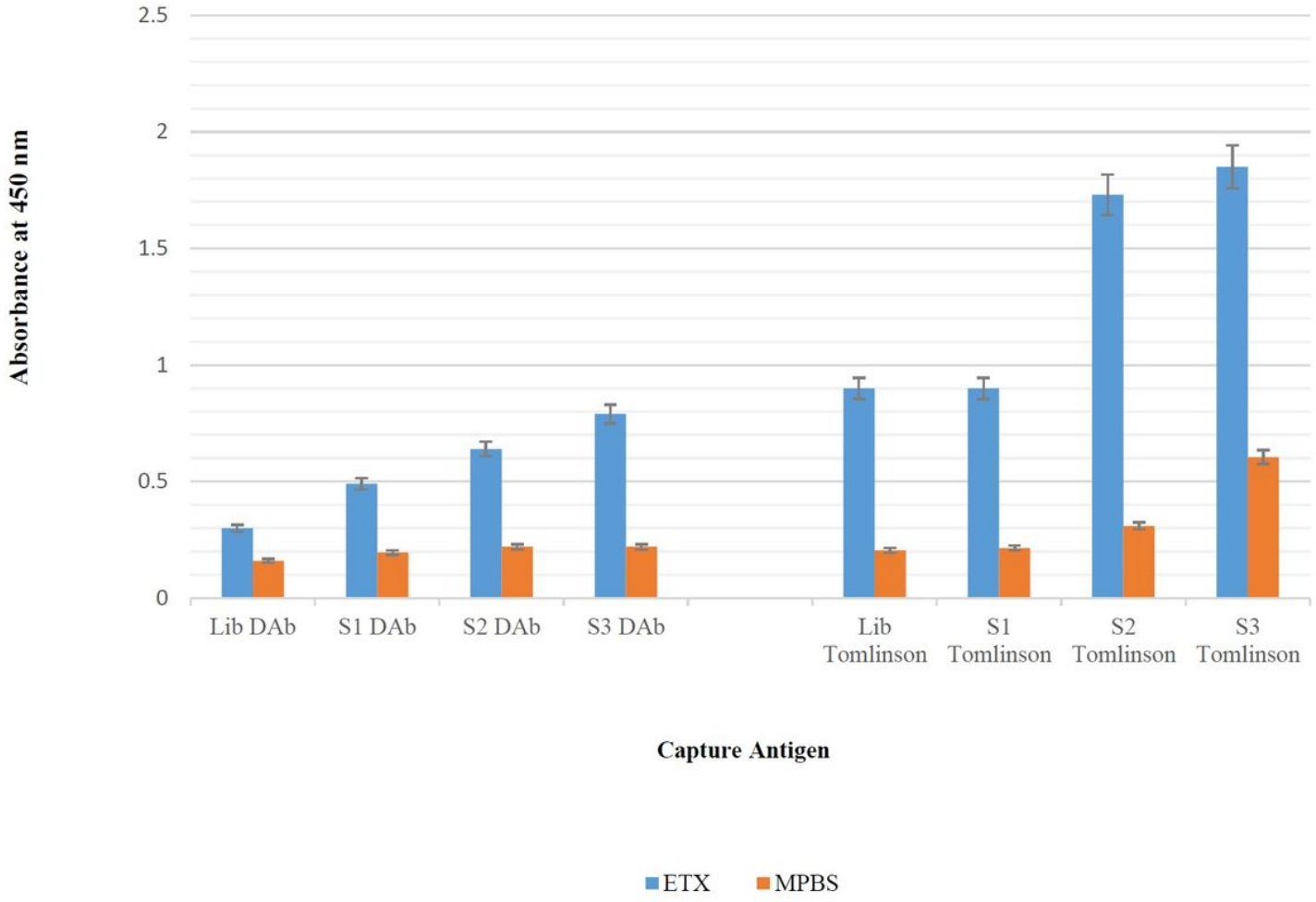

Figure 2

Screening of clones by polyclonal phage ELISA. Epsilon toxoid was coated to plastic and then detected with an anti-c-Myc antibody. The coating was carried out in duplicate; the mean value is presented, the error bars indicating the standard deviation of the two values. 5% MPBS buffer was coated as a negative control. S1, S2, S3= selection rounds 1, 2 and 3.

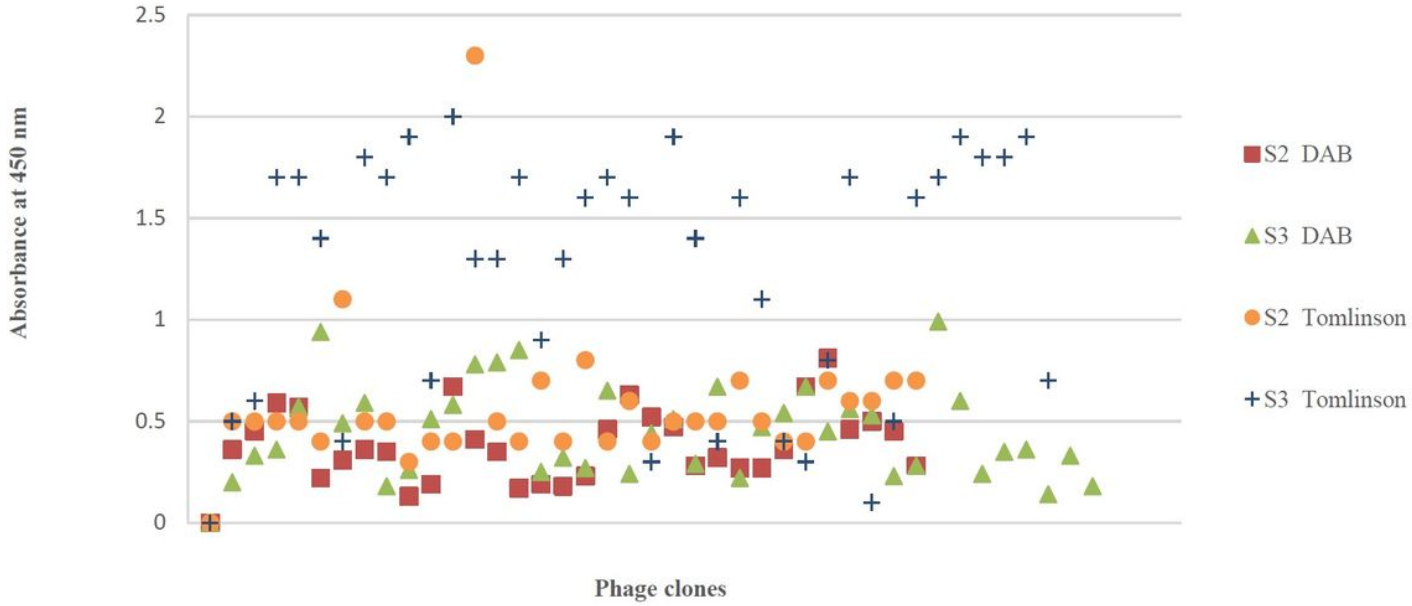

Figure 3

Screening of clones by monoclonal phage ELISA of Tomlinson I+J and DAb libraries. Individual phage clones from the second and third rounds of selection were tested by monoclonal phage ELISA against purified epsilon. Phages were applied as follows; S2: 32 clones picked at random after round 2 of selection; S3: 36 clones picked at random after round 3 of selection. The OD at 450 nm was measured after 10 minutes.

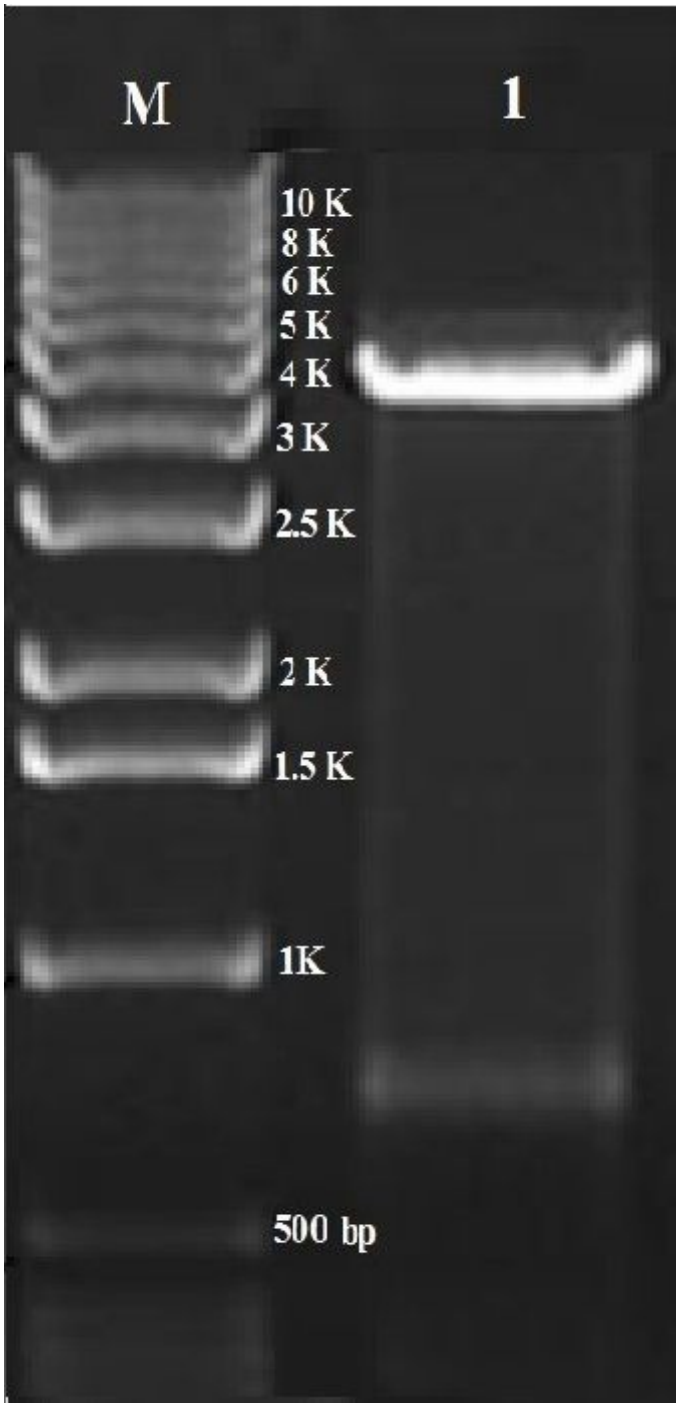

Figure 4

Restriction digestion of plasmid of clone G2. Plasmid DNA from clone G2 was digested with NcoI and NotI. Lane M: 1 kb DNA Ladder (Invitrogen, UK), Lane 1: The expected size of the small fragment released is 708bp.

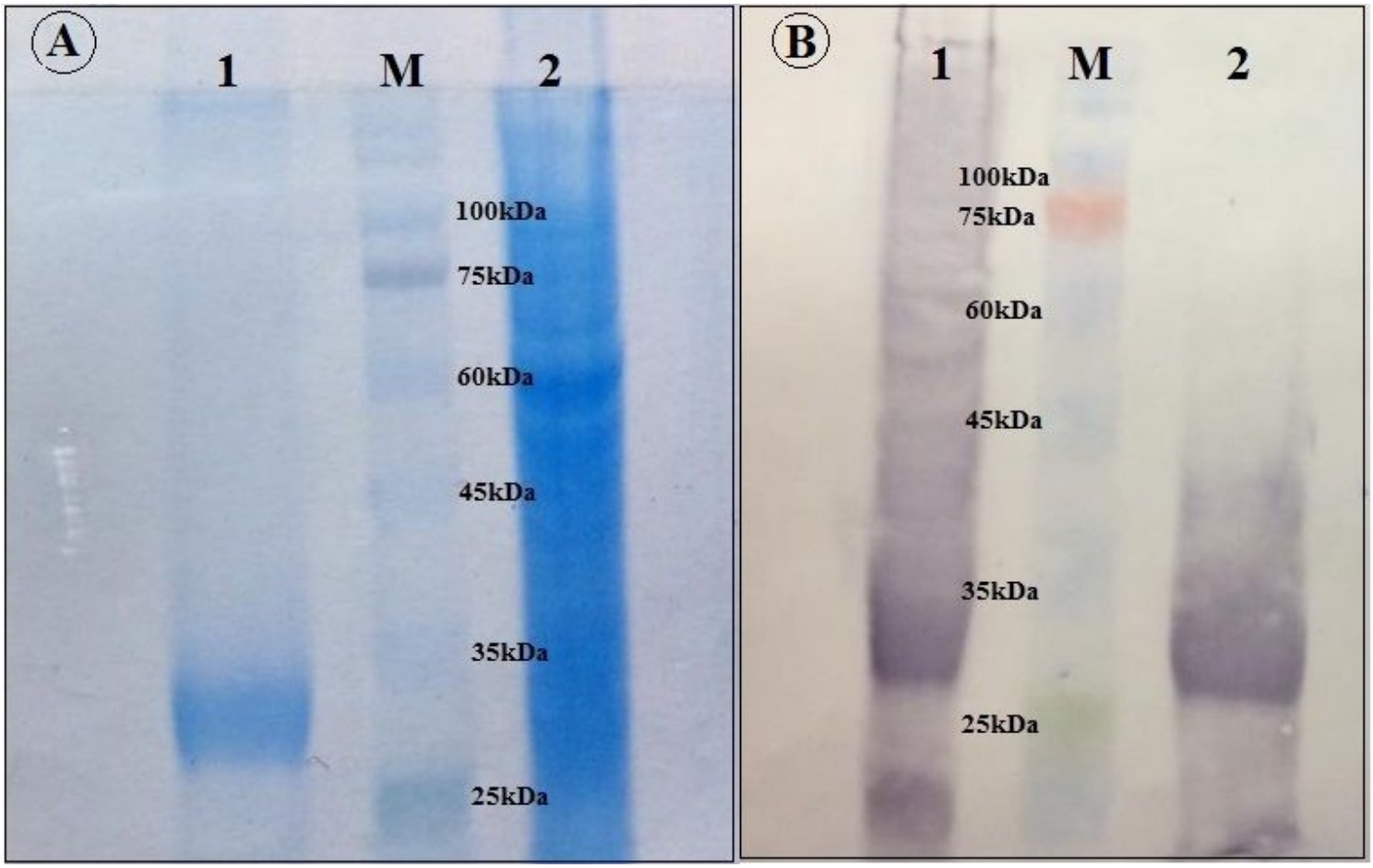

Figure 5

SDS-PAGE and Western blotting of purified and unpurified G2 anti-ETX scFv. Lane M: protein molecular weight marker (SMOBIO, 3.5-245 kDa). (a) Lane 1: SDS-PAGE of the purified G2 anti-ETX scFv, Lane 2: SDS-PAGE of total proteins extracted from G2 clone after induction with IPTG. (b) Lane 1: Western blotting of total extracted proteins of G2 clone, Lane 2: Western blotting of the purified anti-ETX scFv of clone G2. The molecular weight of the expressed scFv fragments is approximately 27 kDa.

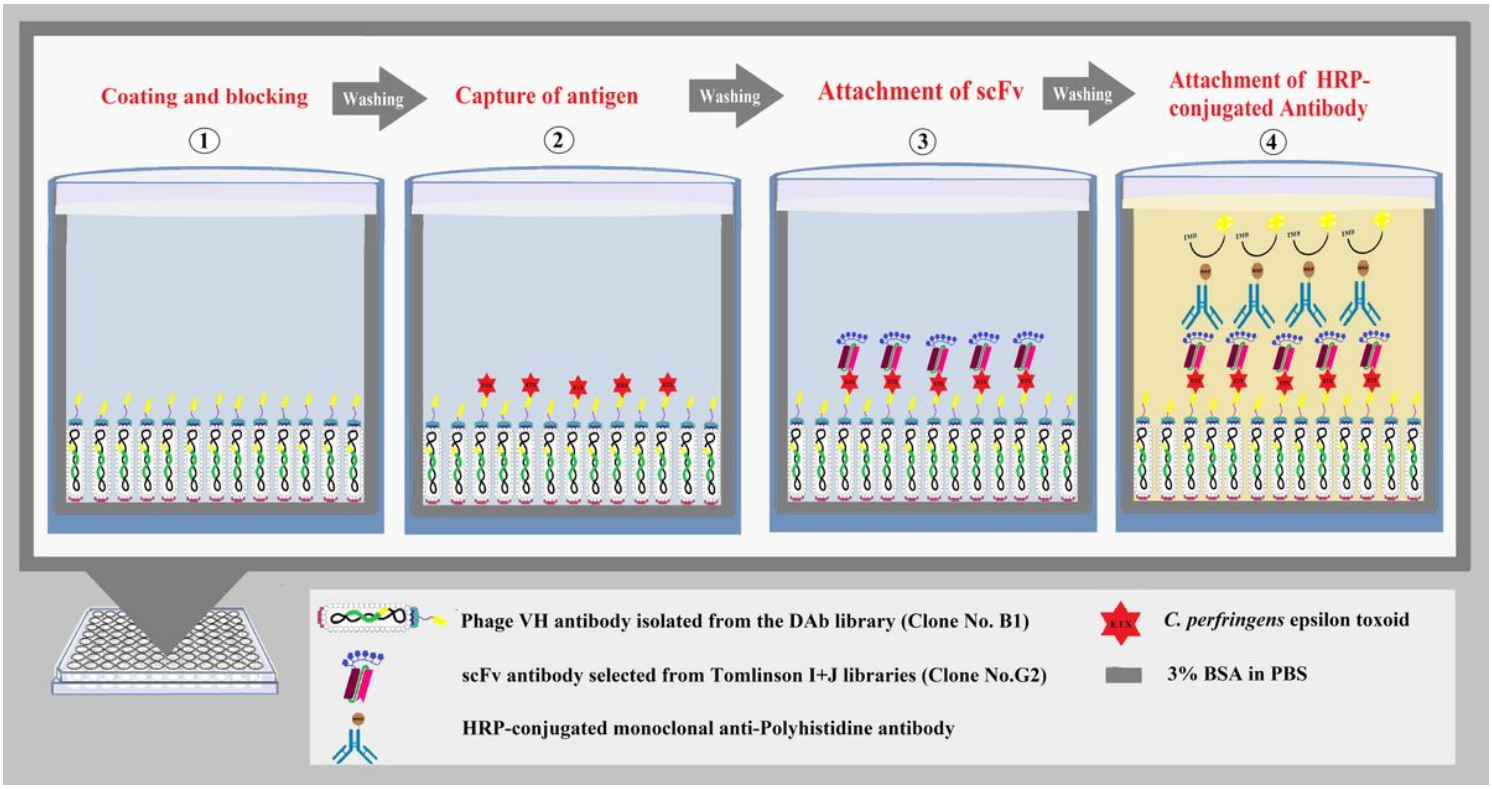

Figure 6

Schematic illustrations of double-recombinant antibody sandwich ELISA for quantitative measurement of ETX. (1) Well is coated with the B1 phage VH antibody isolated from the DAb library and the uncoated surface is blocked by 3% BSA/PBS. (2) The C. perfringens epsilon toxoid binds to the coated B1 phage VH antibody. (3) G2 soluble scFv antibody selected from Tomlinson I+J libraries as the detector antibody binds to epsilon toxoid. (4) HRP-conjugated monoclonal anti-Polyhistidine antibody as the conjugate antibody binds to hexahistidine tag fused to G2 scFv.

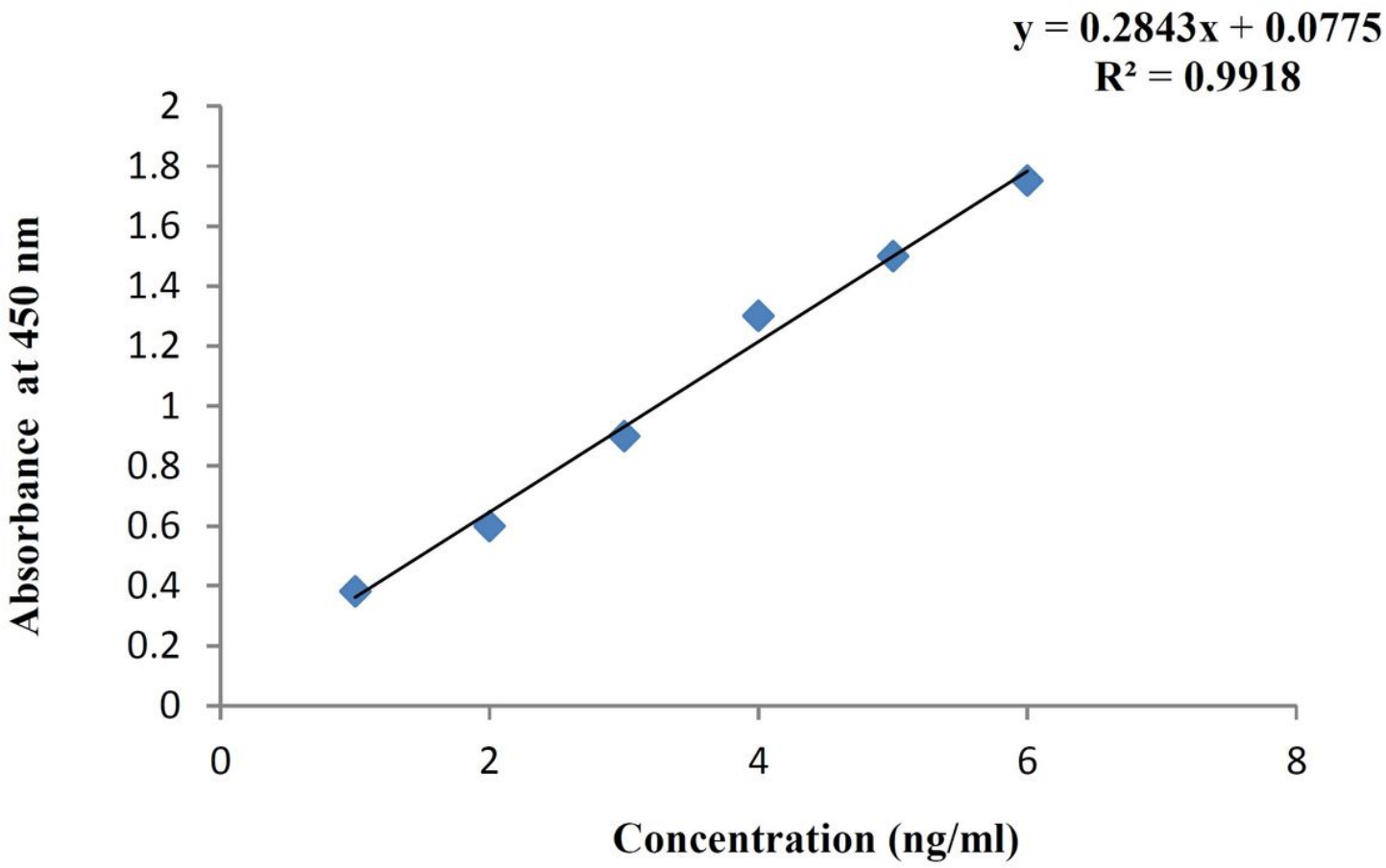

Figure 7

The linear standard curve of sandwich ELISA for the concentration measurement of ETX with the detection range of 5~100000 ng/ml. The linear equation: Y = 0.2843 X + 0.0775 with R2=0.9918.

Supplementary Files

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- GraphicalAbstracts.pdf