Noncompetitive Phage Anti-Immunocomplex Real-Time Polymerase Chain Reaction for Sensitive Detection of Small Molecules

ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2011

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Published in final edited form as:
Anal Chem. 2011 January 1; 83(1): 246–253. doi:10.1021/ac102353z.

Noncompetitive Phage Anti-Immunocomplex Real-Time PCR (PHAIA-PCR) for Sensitive Detection of Small Molecules

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Abstract

Immuno-PCR (IPCR) is an analytical technology based on the excellent affinity and specificity of antibodies combined with the powerful signal amplification of PCR, providing superior sensitivity to classical immunoassays. Here we present a novel type of immuno PCR (IPCR) termed phage anti-immunocomplex real time PCR (PHAIA-PCR) for the detection of small molecules. Our method utilizes a phage anti-immunocomplex assay (PHAIA) technology in which a short peptide loop displayed on the surface of the M13 bacteriophage binds specifically to the antibody-analyte complex allowing the non-competitive detection of small analytes. The phagemid DNA encoding this peptide can be amplified by PCR, and thus, this method eliminates hapten functionalization or bio-conjugation of a DNA template while providing improved sensitivity. As a proof of concept, two PHAIA-PCRs were developed for the detection of 3-PBA, a major urinary metabolite of pyrethroid insecticide and for molinate, a herbicide implicated in fish kills. Our results demonstrate that phage DNA can be a versatile material for IPCR development, enabling universal amplification when the common element of the phagemid is targeted, or specific amplification when the real time PCR probe is designed to anneal the DNA encoding the peptide. Using magnetic beads for rapid separation of reactants, the PHAIA-PCRs proved to be 10-fold more sensitive than conventional PHAIA and significantly faster. The assay was validated with both agricultural drain water and human urine samples showing its robustness for rapid monitoring of human exposure or environmental contamination.

INTRODUCTION

Owing to an excellent sensitivity and specificity, immunoassays have been widely used for rapid high throughput assays of a variety of substances including viruses bacteria, disease-associated proteins, food toxins, and environmental contaminants.[1-5] Immunoassays can be categorized into two different assay formats, non-competitive sandwich and competitive format. The non-competitive sandwich type assays are mostly used for the detection of large molecules possessing more than two antibody-binding sites for which one antibody captures the target analyte and a second antibody conjugated to a signal producing molecule, binds to a second site on the analyte producing a quantitative readout. On the other hand, competitive assays are needed for the detection of small analytes because the small molecule bound to the surrogate antibody is unlikely to provide the recognizable portion for a secondary antibody. Thus, prior to the development of PHAIA, a non-competitive sandwich format

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was very difficult to apply to small molecules. Non-competitive assays are superior to competitive assays in terms of sensitivity, dynamic linear range, and easy adaptability into other formats, including immunochromatography and biosensors.[6] Due to their superior performance, there have been efforts to develop non-competitive assays for small molecules by producing anti-immune complex antibodies.[7,8] recombinant antibodies that form analyte-associated complexes[9-11], or employing modified assay procedures that convert competitive formats to non-competitive formats.[12, 13] However, these methods are cumbersome and laborious, and success has been mostly case specific, which may explain why almost all assays reported for small size analytes have a competitive format.

To overcome this shortcoming, we have recently introduced the PHAIA (phage anti-immunocomplex assay) in which we use small peptide loops displayed on phage M13 as innovative elements for the specific detection of immunocomplexes. The M13 bacteriophage is a filamentous virus with a diameter of 6 nm and length of 0.9 μm containing single strained DNA packed in a few thousands of major and minor coat proteins. By modification of the phage genome it is possible to efficiently express polypeptides fused to its coat proteins, and build libraries of enormous complexity. The physical linkage between the phage phenotype (displayed peptide) and its genotype (peptide encoding sequence) allows the efficient selection of polypeptide ligands virtually for any selector molecule, and constitutes the working principle of the phage display technology.[14] In order to generate more complex libraries and control the valence of the displayed peptide, phagemid libraries composed by hybrid viral particles have been introduced. Phagemids are plasmid vectors that encode the peptide fused to the gene of the coat protein used for display but lack all other phage proteins. These vectors can be propagated in bacteria and be packaged as single strand DNA in viral particles upon hyperinfection with a helper phage.15 In the PHAIA method, a phage borne cyclic peptide selected from phage display peptide libraries forms a trivalent antibody-analyte-peptide complex by specific recognition of the conformational change of the antibody binding pocket upon binding of the analyte.16-18 This method accelerates the development of non-competitive two site assays using a well known *in vitro* selection method, “biopanning”, resulting in dramatically improved assay sensitivity and increased specificity.

In this paper we demonstrate that it is possible to combine the advantageous characteristics of PHAIA with the power of amplification of IPCR to develop a highly sensitive detection method for small molecules. IPCR was first reported by Sano et al.[19] and has been used for ultrasensitive detection of virus and biomarkers.20-22 In the classical application, a DNA sequence is chemically conjugated to the detecting molecule. Upon binding, this tracer reagent can be detected with high sensitivity by PCR amplification.19 More recently, Guo et al.[23] demonstrated that the DNA tracer could be substituted by a detecting molecule displayed on the surface of the M13 phage. In their application, they used phage particles expressing single chain antibody fragments (scFv) that served simultaneously as detection reagent and DNA template, allowing the ultrasensitive detection of viral particles and prion proteins in two-site sandwich formats. In spite of its advantages, IPCR could not be applied to the analysis of a critical group of analytes that due to their small size can not be simultaneously detected by two antibodies. This group includes most drugs, environmental pollutants, explosives, hormones, food additives, toxins, metabolites, etc., for which there is a growing need of rapid yet highly sensitive detection methods.

As a proof of concept, two PHAIA-PCRs for 3-phenoxybenzoic acid (3-PBA), a major human urinary metabolite of pyrethroid insecticides and the herbicide molinate were developed. The assay conditions were optimized using magnetic beads to separate the reacted phage, with allowed a 10-fold increase in sensitivity. The robustness of the PHAIA-

PCR methods was validated with real samples, using agricultural drain water and human urine.

EXPERIMENTAL SECTION

Materials

The 3-PBA-anibody immunocomplex specific phage peptide (CFNGKDWLYC) and molinate-antibody immunocomplex specific phage peptide (CSTWDTTGWC) were selected using M13 bacteriophage displayed peptide libraries with diversity of 2.4-3 × 10^9 independent clones on the phagemid vector pAFF/MBP ($ASGSACX_8CGP_{6-}$) and p8V2 ($GGCX_8C(GGGGS)_{3-}$), respectively as previously described.16,17 The anti-3-PBA polyclonal antibody 294 (PAb 294) and anti-molinate monoclonal antibody 14D7 (MAb 14D7) were produced as previously described.24,25 3-PBA and molinate standard compounds, BSA, polyethyleneglycol 8000 (PEG 8000), Tween 20, and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO). Helper phage M13KO7 was purchased from New England Biolabs (Ipswich, MA). Mouse anti-M13 monoclonal antibody-horseradish peroxidase (HRP) was purchased from GE Health Care (Piscataway, NJ). TaqMan probes (5'-FAM and 5'-VIC), 7500 Fast RT-PCR system, and PCR master mix (TaqMan® Universal PCR Master Mix (2X), No Amperase® UNG) were obtained from Applied Biosystems (Carlsbad, CA). The sequence of TaqMan probes and primers were designed using a primer designing software, Primer Expression® v3.0 (Applied Biosystems, Carlsbad, CA). The sequences of the primers and TaqMan are shown in the table 1. Epoxy activated magnetic bead (2.8 μm diameter) was purchased from Invitrogen (Carlsbad, CA).

Buffers—PBS, 10 mM sodium phosphate buffer containing 137 mM NaCl and 2.68 mM KCl, pH 7.4. PBST, PBS containing 0.05 (v/v)% Tween 20.

Preparation of Phage-Displayed Peptides

The ARI 292 type cells of *E. coli* (Affymax Research Institute, Palo Alto, CA) containing the phagemid vectors PAFF/MBP or p8V2 encoding the 3-PBA and molinate immunocomplex specific peptides16, 17 were grown in 5 ml Luria-Bertoni (LB) medium containing 100 μg ampicillin per ml by overnight shaking at 37 °C. Four milliliter of the overnight culture was added into a 1-L flask containing 400 ml of SOP medium (LB media containing 0.25% K_2HPO_4, 0.1% $MgSO_4$, 0.1% glucose and 100 μg/ml ampicillin) and the flask was shaken until an Abs_{600nm} = 0.4 AU was reached. The cells were then super infected with M13K07 helper phage at a concentration of 1 × 10^{11} transducing units/ml by 30 min incubation without shaking at 37 °C. Arabinose and kanamycin were added to a final concentration of 0.02 % and 40 μg/ml, respectively and cultures were grown overnight with vigorous shaking at 37 °C. The next day, the cells were pelleted by centrifugation at 10,000 rpm for 15 min and the supernatant was mixed with 0.2 volume of 20% PEG 8000 in 2.5 M NaCl solution. After 1 h incubation on ice the phages were precipitated by 15 min centrifugation at 10,000 rpm. The phage pellet was resuspended with 100 ml of PBS and the phage was precipitated again as described above. The phage pellet was resuspnded with 5 ml of suspension buffer (PBS buffer containing protease inhibitor cocktail (Roche Applied Science), 0.02% sodium azide and 1% BSA). The aliquots were stored at -80 °C.

Phage Anti-Immune Complex Assay (PHAIA)

An ELISA plate (Maxisorop, Nunc) was coated with protein A purified PAb 294 or MAb 14D7 at 10 and 3 μg/ml, respectively in PBS by 2 h incubation at 37 °C. The plate was blocked with 350 μl of 1% BSA in PBST / well by 1 h incubation at 37 °C. The 3-PBA or molinate phage peptides were mixed with an equal volume of various concentrations of 3-

PBA or molinate standard diluted in PBST. One hundred μl of each mixture was added into the plate followed by 1 h incubation at room temperature. The plate was washed ten times with PBST and then the bound phages were captured by adding 100 μl of anti-M13 phage MAb-HRP with the plate incubated at room temperature for 1 h. After ten washings with PBST, 100 μl of substrate buffer (25 ml of 0.1 M citrate acetate buffer pH 5.5, 0.4 ml of 6 mg of TMB /ml DMSO and 0.1 ml of 1% H_2O_2) was added into each well and the enzyme reaction was stopped by adding 50 μl of 2 M sulfuric acid. The absorbance at 450 nm was obtained with a plate reader (Molecular device, Sunnyvale, USA).

PHAIA-PCR

The procedures are same as described in the section of PHAIA except that the bound phage were eluted by 15 min incubation with 100 μl of glycine buffer (0.2 M, pH 2.2) per well. The eluates were immediately neutralized with 5.8 μl of Tris-base (2 M, pH unadjusted). Five μl of eluted whole phage was then used as DNA template for PHAIA-PCR in total a PCR volume of 20 μl. The obtained Ct values were converted to relative Ct values by subtracting Ct values from 100 for the easy comparison of the sensitivity of the PHAIA-PCR to that of the PHAIA. For the RT-PCRs, minor grove binding TaqMan probes (5'-FAM and 5'-VIC) and 7500 Fast RT-PCR system (Applied Biosystems) were used through the study. The PCR premix consisted of 1x PCR buffer (TaqMan ® Universal PCR Master Mix (2X), No Amperase ® UNG, Applied Biosystems), 600 nM of each primer, 250 nM of TaqMan probe and pure water in final volume of 15 μl. The PCR parameters were as follows: 94 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s.

Covalent Conjugation of Antibody to Magnetic Beads and Magnetic Bead-Based PHAIA-PCR

Covalent conjugation of 3-PBA PAb 294 to the epoxy activated magnetic beads was carried out following the instructions in the manual. Briefly, 63 μg of 3-PBA PAb 294 (18 μl of 3.5 mg/ml of 3-PBA PAb 294 in 102 μl of 0.05 M borate buffer (pH 8.5)) was added to 100 μg of beads, followed by 60 μl of 3 M ammonium sulfate. The mixture was incubated overnight at 37 °C with gentle rocking. The following day, the beads were washed three times with PBST with the aid of a magnetic extractor, and were then resuspended with 300 μl of PBS containing 0.02% of sodium azide and 1% BSA. For the bead-based PHAIA-PCR, 5 μl of the conjugated beads (1.7 μg) were placed into wells of the 96-well plate. The mixture of phage peptide and 3-PBA at various concentrations prepared as described above was added and the plate was incubated for 1 h at room temperature with gentile shaking on an orbital plate shaker. The beads were then washed with PBST and suspended with 100 μl of sterilized dH_2O. Five μl of bead solution was added to the PCR plate containing 15 μl of PCR pre mix. The PCR was carried out as described above.

RESULTS

Principle of PHAIA-PCR

The schematic diagram of PHAIA-PCR is shown in Figure 1. The 3-PBA PAb 294 or molinate MAb14D7 is first immobilized on the surface of the ELISA plates. In the presence of target compounds and phage peptides, two-site sandwich trivalent complexes are formed. After washing, the bound phage peptides are dissociated and transferred to a PCR plate for amplification. Two sets of primers and TaqMan probes bind either to the region of the arabinose promoter, a common sequence of the phagemid vector, which allow universal amplification, or to the peptide encoding sequence, allowing sequence dependent amplification that can be used to design multiplex immunoassays.

PHAIA-PCR

To evaluate the performance of the PHAIA-PCR, the linear range of detection was estimated using ten-fold serial dilutions of 3-PBA phage particles (10^9 – 0 phage particles) diluted in distilled water and subjecting 5 μl of each dilution to the PCR using the primers and TaqMan probe that targets the arabinose promoter (universal amplification). The trend of fluorescent signals obtained by different number of phage particles is shown in figure 2.A. As expected, the Ct values defined as the number of PCR cycles generating the threshold intensity of fluorescence (0.2) gradually increased as the number of phage particles decreased. However, no change in Ct values was observed when the number of phage particles was less than 10^2. The plotting of the Ct values against the log number of phage particles revealed that the PCR is able to detect phage particles over seven orders of magnitude, ranging from 10^2 to 10^9 phage particles (Fig 2.B) with a R^2 value of 0.99.

Then we evaluated the number of phage particles that are bound to the analyte-antibody immunocomplex in the PHAIA assay. To this end a PHAIA assay for 3-PBA was set up as previously described[17] and four concentrations of 3-PBA (0, 1, 5, and 25 ng/ml) were assayed. In a colorimetric plate-based PHAIA the plateau of the maximum readout starts at approximately 2 ng/ml of 3-PBA. As shown in Figure 2.C., this corresponds to a number of particles of about 10^6 cfu/well, which is well below the maximum number of phage particles that can be detected by PCR. Considering that saturation of the colorimetric method may occurs due to the fast turn-over of substrate to products caused by multiple binding of anti-M13 antibody-HRP conjugate to phage particle, we speculate that the use of PCR could dramatically increase the linear range of the assay. Although the number of eluted phage particles significantly increased up to 5 ng/ml of 3-PBA, it showed a modest incremental increase when 25 ng/ml of 3-PBA was used, indicating that PCR may not substantially extend the detectable linear range under the assay condition optimized for the PHAIA.

PHAIA-PCR Dose-Response Curves

In order to explore the influence of the target template we designed two sets of primers and TapMan probes for universal or peptide encoding sequence dependent amplification as shown table 1. Our initial attempts showed that inconsistent results were obtained when the DNA of the bound phage was directly extracted by adding water and heating the plate, but we found that this could be corrected by previous elution of the phage. To optimize this process we first examined the effect of the acidic elution buffer (glycine buffer, pH 2.2) neutralized with basic buffer (2 M Tris, pH unadjusted) on PCR performance because addition of eluted phage in the buffer to the premix of PCR may deteriorate the activity of the DNA polymerase.[26] For this experiment, we performed PCR amplifications of equal numbers of phage in distilled water or neutralized elution buffer. Negligible differences in Ct values were found (not shown).

Under these conditions, the dose-response curves for 3-PBA and molinate were obtained using the universal amplification-based PHAIA-PCR (figure 3.A and 3.C) and the conventional PHAIA (figure 3.B and 3.D). In the case of 3-PBA, the limit of detection (LOD) by PHAIA-PCR was 20 pg/ml which is 10-fold lower than that of the conventional PHAIA. Similar enhancement in assay sensitivity (0.2 ng/ml of LOD) was obtained for the molinate PHAIA-PCR. As observed above, the maximum concentration that can be detected was similar for both methods, however due to the lower LOD attained by PHAIA-PCR, it provided extended detection range of an order of magnitude.

In order to study the feasibility of using PCR probes that would allow the unambiguous detection of analyte-specific phage, we performed a parallel PHAIA-PCR for 3-PBA and molinate analyte/phage systems using a probe that anneals with the anti-PBA peptide

encoding sequence of the 3-PBA phage. As shown in figure 4.A, the 3-PBA PHAIA-PCR performed in the same way that it did with the universal probe. On the other hand, and as expected, no amplification of the molinate phage was observed when the 3-PBA specific probe was used, not even at 40 PCR cycles (figure 4.B). This result demonstrates that in addition to provide higher sensitivity, PHAIA-PCR possess a high potential for adaptation into multiplex formats.

Magnetic Bead-Based PHAIA-PCR

In order to further simplify the method, we explored the use of magnetic beads as an advantageous solid phase for the PHAIA-PCR. To this end, we covalently immobilized the purified 3-PBA PAb 294 on the surface of epoxy-activated magnetic beads, Then, we first evaluated whether the use of different amounts of beads would exert an inhibitory effect on the amplification reaction. Two, 5, and 10 μl of antibody functionalized beads were incubated with phage and four concentrations of 3-PBA (0, 0.03, 0.25, and 2 ng/ml), washed as described and then used for PCR. As shown in the figure 5.A, the overall Ct values decreased when the amount of beads increased, although the Δ Ct values for the different amounts of beads remained the same. For practical reasons, we choose to use 5 μl of the magnetic bead suspension and performed the PHAIA-PCR for 3-PBA using magnetic bead separations. The LOD was approximately 60 pg/ml of 3-PBA (10% increase in relative Ct compared to Ct at zero concentration) (figure 5.B), which is 3-fold higher than that obtained with the plate-based PHAIA-PCR. In spite of its lower sensitivity the magnetic bead adaptation of PHAIA-PCR is still highly sensitive to trace amounts of the metabolite and due to its simplicity, it may still be an attractive option if assay automation is required.

Validation of PHAIA-PCR with Real Samples

We validated the plate-based PHAIA-PCR using an agricultural drain water collected from a local farming area with no record of molinate use and human urine from individuals with no known exposure to pyrethroid insecticides. Molinate and 3-PBA were spiked to samples at 4 different concentrations (2, 5, 20, and 50 ng/ml). The assays were carried out at different days and the recoveries were calculated based on the concentrations detected by the PHAIA-PCR. As shown in the table 2, the overall recoveries for the molinate assay ranged from 70 to 140% with inter-assay coefficient variations (CV) of 14.3-20%. The recoveries for the 3-PBA assay were 80-115% showing an inter-assay CV of 3.1-10%. These results indicate that the PHAIA-PCR method is suitable for highly sensitive yet rapid high throughput monitoring of human exposure to toxic compounds or environmental contamination.

DISCUSSION

Immunoassays are rapid and cost effective analytical methods to detect quantitatively or semi-quantitatively a large number of compounds. Although immunoassays have been predominantly used for clinical diagnosis, for the last decades, these methods have been successfully applied to the detection of environmental compounds that cause concerns of adverse effects on public health or environmental. To develop sensitive immunoassays for the detection of small molecules, the competitive format is the first choice due to the extremely difficult technical challenge to develop non-competitive assay even if a non-competitive format is desirable. In previous reports, we presented an innovative method which allows easy conversion of existing competitive assays to highly versatile non-competitive two-site assays. We took advantage of the huge diversity of phage-displayed peptide libraries to *in vitro* select phage peptides that can substitute for anti immune complex antibodies. In this paper, we demonstrated that the phage-displayed peptide is an excellent bio-nano material by developing a sensitive PHAIA-PCR which provides two advantages over a classical IPCR assay: (a) the DNA of the peptide bearing phage used for

detection is a “ready to use” template for PCR amplification, which eliminates the necessity of DNA bio-conjugation chemistry, and (b) the anti analyte/antibody specific phage carries a unique DNA sequence that can be used as an internal “bio-barcode” to develop multiplexed detection platforms. The conserved phage DNA can be used as a standard. These two properties make the PHAIA-PCR a highly versatile method. Indeed, the use of a specific probe for the common arabinose promoter sequence can be used for universal amplification of any PHAIA phage, while the design of individual TaqMan probes for the peptide-coding sequence allows specific detection of the target phage, even in the presence of other phage clones, making possible the simultaneous detection of multiple analytes.

In their studies on IPCR, Guo et al., [23] and Yu et al., [27] released the ssDNA of captured phages by heating the ELISA plate at 95 °C for 10-15 min and then added a few μl of this lysate to the PCR premix. At the beginning of this study, we followed their methods placing an ELISA plate on a water bath or in a DNA hybridization chamber to release the ssDNA, however we encountered two problems, firstly, we observed very high signal variations among replicates probably due to inefficient DNA exposure, and secondly, the Ct values at near zero concentrations were frequently lower than the zero value, a critical drawback to attain high sensitivity. These variations were corrected when the phage was previously eluted from the plate using acidic conditions. The combination of the PHAIA-PCR with magnetic beads further enhances the flexibility of the method, and this modification could be particularly useful for its instrumentation in automatic high throughput formats.

A major advantage of the PHAIA-PCR is the further increase in assay sensitivity provided by the amplification power of PCR. Although we demonstrate that no matrix interference was observed for any of the two analytes, in two matrices this may not be the case for other analyte/antibody pairs. In those cases, the low LOD of the PHAIA-PCR would be of great importance because it will allow avoiding matrix effects by simple sample dilution. Since the improve sensitivity of the PHAIA-PCR is accompanied by a wider assay linear range, the new method may be particularly useful addition to the toolbox of rapid, highly sensitive methods, particularly for the detection of small molecules.

CONCLUSIONS

With the phagemid DNA contained in M13 bacteriophage displaying peptides that bind to an analyte-antibody immune complex combined with powerful amplification by PCR, the novel noncompetitive PHAIA-PCRs were developed for the sensitive detection of small molecules, 3-PBA and molinate, showing 10-fold improved sensitivity and extended detection range compared to the PHAIA. The assays were successfully performed applying to human urine and agricultural drain water samples with good recoveries. To our knowledge, this work is the first report of developing a noncompetitive IPCR for small molecules. Since the PHAIA technology has bee proven to be readily performed in developing noncompetitive two-site assays for various small molecules and polyclonal or monoclonal antibodies, sensitive PHAIA-PCR can be easily developed omitting somewhat complicated bioconjugation of hapten or antibody with template DNA. The technology of course can be applied to the detection of pharmaceuticals and compounds of human health interest.

Acknowledgments

This work was supported in part by the following: The National Institute of Environmental Health Sciences (NIEHS) Superfund Basic Research Program [P42 ES004699]; the NIEHS Children’s Environmental Health Center [P01ES011269]; and the Fogarty International Center Grant [TW05718]. The support of the Western Center for Agricultural Health and Safety at the University of California Davis [PHS OH07550] is also acknowledged. BDH is a George and Judy Marcus Senior Follow of the American Asthma Foundation.

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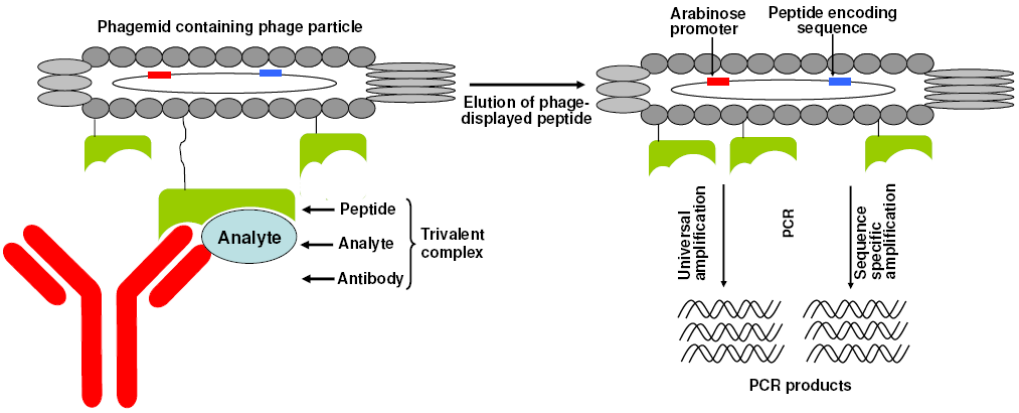

Figure 1.

Schematic diagram of the PHAIA-PCR. The phage peptide binding to an immunocomplex of an antibody-analyte was eluted in an acidic buffer (glycine, pH 2.2) and immediately neutralized with the basic buffer (2 M Tris, pH unadjusted). Five μl of phage eluate was subjected to the PCR detection.

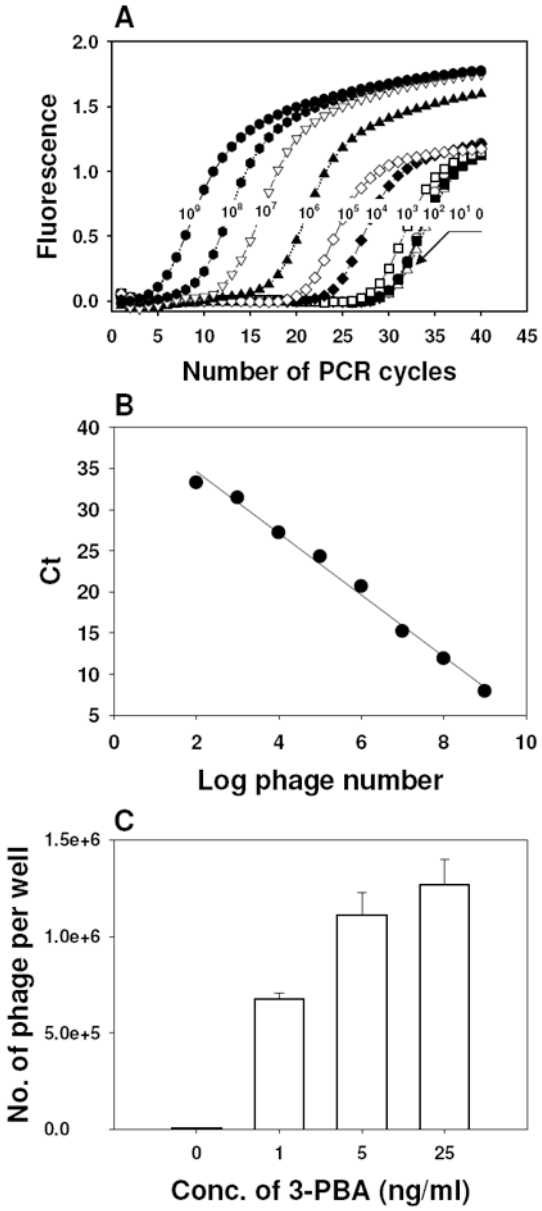

Figure 2.

Estimation of linear range of phage particles by phage PCR. (a) Real-time PCR amplification of 10-fold serial dilutions of phage particles in water. Five μl of each preparation was mixed with 15 μl of PCR pre mix containing primers specific to the arabinose promoter of the phagemid vector and the 5'-FAM probe. (b) Standard curve of the phage PCR obtained by plotting the threshold Ct value of each dilution against the log number of phage particles added to the PCR pre mix. Each data point refers to an average of two replicates (c) Titration of 3-PBA phage particles forming the antibody-alalyte-phage complex in the PHAIA plate. The phage particles were recovered from PHAIA wells incubated with 0, 1, 5, and 25 ng/ml of 3-PBA. Each column represents the mean value of three replicates and the error bar the standard deviation.

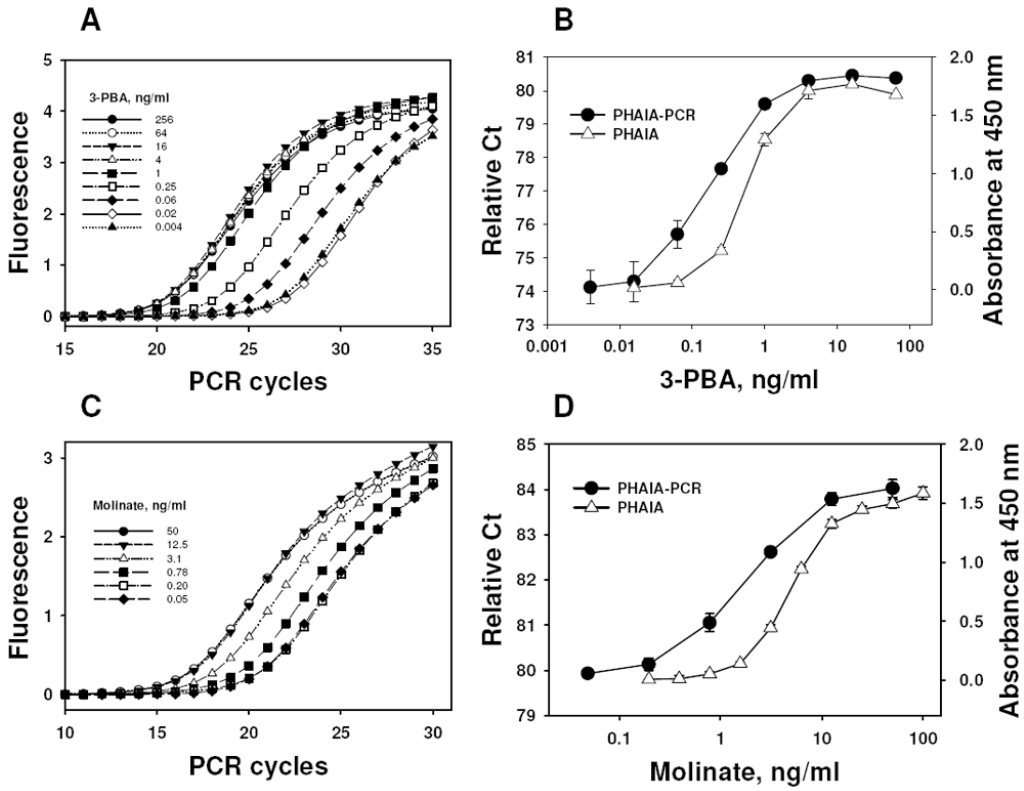

Figure 3.

PHAIA-PCR and conventional PHAIA for 3-PBA and molinate. Five μl of phage particles eluted after incubation at various concentrations of analytes were mixed with 15 μl of PCR pre mix containing primers and 5'-FAM probe designed for universal amplification. Assay conditions of the PHAIA-PCR for each analyte are the same as the PHAIAs. Each value represents the mean value of three replicates (a) Amplification of 3-PBA phage. (b) Dose-response curves for 3-PBA. (c) Amplification of molinate phage. (d) Dose-response curves for molinate.

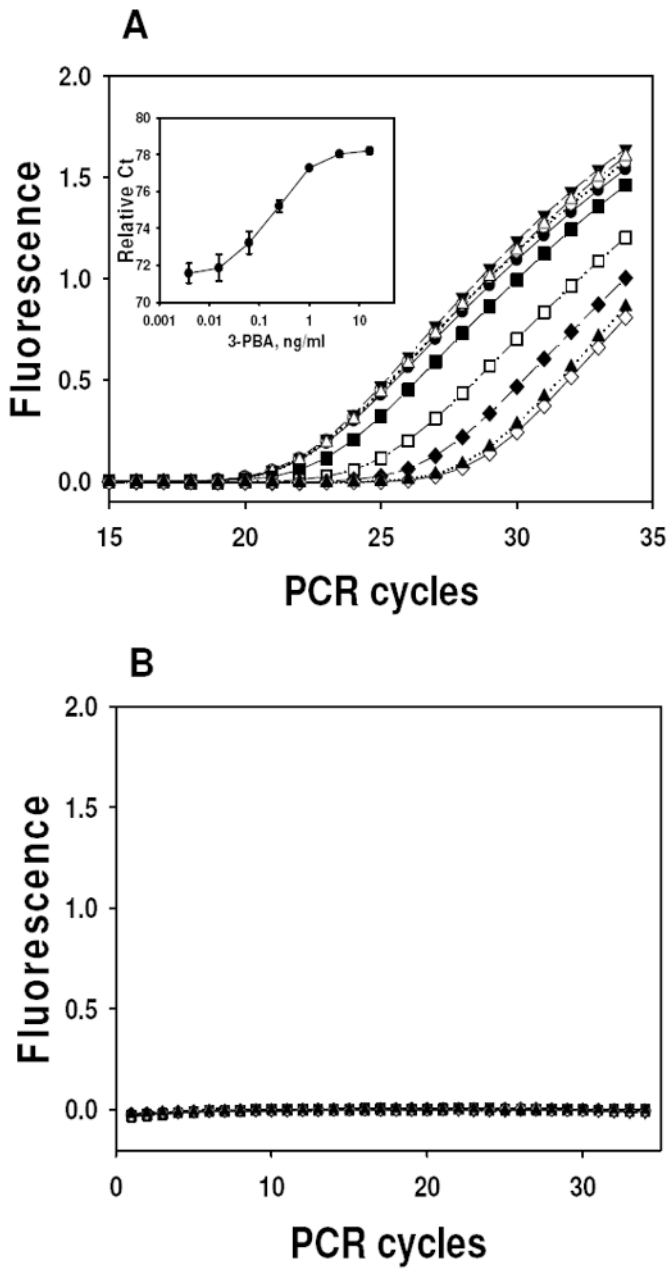

Figure 4.
PHAIA-PCR using the 3-PBA peptide specific probe. Primers and the 5'-VIC probe designed for specific amplification of the DNA sequence encoding the anti 3-PBA/PAb 294 immunocomplex were used for 3-PBA (A) and molinate (B) PHAIA-PCR amplification. The insert in figure A plots dose-response curve as the Ct threshold values versus the 3-PBA concentration, each point represents the mean value of three replicates.

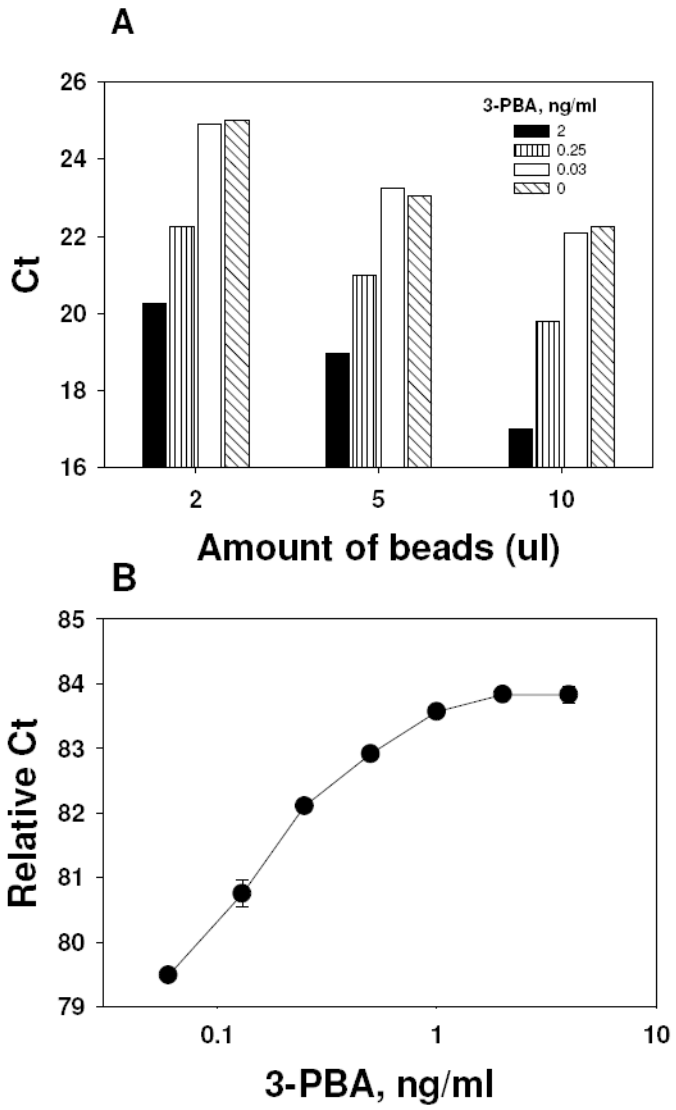

Figure 5.

PHAIA-PCR performed with magnetic beads. The Ct valued obtained with the use of different amount of magnetic beads were measure using different concentrations of 3-PBA (A). The dose-response curve of the PHAIA-PCR is depicted showing the relative value of Ct for each concentration of 3-PBA. Each value represents the mean value of three replicates.

Table 1

Sequences of primers, probe and target region of phagemid vector

Specific to a common sequences	**Specific to a peptide encoding sequenc**
	Primers
5’-ACTGCTGGCGGAAAAGATGT-3’	5’-GTCTGGGTCCGCGTGTTT-3’
5’-CGCACAGCATGTTTGCTTGT-3’	5’-CTGTATGAGGTTTTGCCAGACAA
	Probes
[a]5’FAM- ACAGACGCGACGGC-MGBNFQ 3’	[a]5’VIC-TGGTAAGGATTGGCTGTAT-
	[b]Target sequences
[c]CTTAAGACGCTAATCCCTAACTGCTGGCGGAAAAGATGTG**ACAGACGCGACGGC**GACAAGCAAACATGCTGTGCG	[d]GTCTGGGTCCGCGTGTTTTAA**TGC**

[a] 5’-FAM or VIC labeled DNA minor grove binding probe with non fluorescent quencher

[b] Forward and reverse primer annealing sites are underlined. Bold characters indicate the probe binding sites.

[c] Arabinose promoter sequences.

[d] Peptide encoding sequences.

Table 2

Assay validation of PHAIA-PCRs

Spiking (ng/ml)	[a]Expected detection after dilution	Run 1		Run 2		CV (%)
		Detection	Recovery (%)	Detection	Recovery (%)	
Recovery of molinate						
50	5	4.7 ± 0.5	94	3.5 ± 0.5	70	20
20	2	1.7 ± 0.3	85	2.1 ± 0.4	105	17.9
5	0.5	0.7 ± 0.1	140	0.7 ± 0.1	140	14.3
2	0.2	ND		ND		
Recovery of 3-PBA						
50	5	5.0 ± 0.2	100	5.1 ± 0.1	102	3.1
20	2	2.2 ± 0.1	110	2.3 ± 0.06	115	4.3
5	0.5	0.4 ± 0.05	80	0.5 ± 0.04	100	10
2	0.2	0.18 ± 0.02	90	0.2 ± 0.01	100	10

[a]detection level by the PHAIC-qPCR from diluted samples

ND: not detected