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Phage display mediated immuno-PCR

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ABSTRACT

Immuno-PCR (IPCR) is a powerful detection technology in immunological study and clinical diagnosis due to its ultrasensitivity. Here we introduce a new strategy termed phage display mediated immuno-PCR (PD-IPCR). Instead of utilization of monoclonal antibody (mAb) and chemically bond DNA that required in the conventional IPCR, a recombinant phage particle is applied as a ready reagent for IPCR experiment. The surface displayed single chain variable fragment (scFv) and phage DNA themselves can directly serve as detection antibody and PCR template, respectively. The aim of the design is to overcome shortcoming of low detection sensitivity of scFv so as to largely facilitate the real application of scFv in immunoassay. The idea has been demonstrated by applying hantaan virus nucleocapsid protein (NP) and prion protein (PrP) as detection targets in three experimental protocols (indirect, sandwich and real-time PD-IPCR assays). The detection sensitivity was increased 1000- to 10 000-folds compared with conventional enzyme-linked immunosorbent assays (ELISAs). This proof-of-concept study may serve as a new model to develop an easy to operate, low cost and ultrasensitive immunoassay method for broad applications.

INTRODUCTION

Immuno-PCR (IPCR), first described by Sano *et al*. (1), is a highly promising technique for the ultrasensitive analysis of antigens. General IPCR approach involves initial capture of a target analyte with a capture antibody coated on a surface and subsequent detection with a detection antibody attached with a DNA marker that can be amplified by PCR with appropriate primers. IPCR combines the versatility of enzyme-linked immunosorbent assays (ELISAs) with the amplification power and sensitivity of PCR. As a consequence, IPCR not only leads to an about 1000- to 10 000-fold gain in sensitivity, as compared to conventional ELISA, but it also reveals a very broad linear dynamic range of up to six orders of magnitude (2). Due to the high sensitivity, IPCR has been widely used in detection of antigens in immunological study and clinical diagnosis (3–5). Successful application of IPCR relies upon the availability of monoclonal antibody (mAb) (6). Although production technology of mAbs is mature, it remains to be a labor-intensive and high-cost process (7,8). Further, preparation of mAb–DNA conjugate involves complex covalent coupling chemistry (9), or stepwise addition of biotinylated antibody, streptavidin and biotinylated DNA (10).

With the development of antibody technology, engineered antibody fragments have gained increased interest during last decade (11). Single chain variable fragment (scFv) (12) is one of the most popular formats of engineered antibody. A scFv is the variable domains of a heavy and a light chain (V_H and V_L) of its parent antibody, joined with a flexible polypeptide linker (13). In comparison with mAbs, scFvs have a number of attractive characteristics. First, they are easy to produce in large quantity with low cost. Second, their small sizes permit them to penetrate tissues and solid tumors more rapidly, showing improved targeting performance (14). Third, a scFv can be easily fused with another labeling protein, such as enzyme (15) or fluorescent protein (16), to form a ready reagent for immunoassay. However, scFv molecules normally have an apparent affinity that is typically 10- to 1000-fold lower than their parent mAbs (17), which greatly limits the real application of scFvs in immunoassays.

To avoid these problems, here we introduce a new immunoassay protocol with the aid of phage displayed scFv. Phage display technology was first introduced in 1985 by George Smith (18) and has been widely used to build peptide and antibody libraries, from which the desired scFvs or peptides with specificity to almost any target are to be screened by biopanning (19,20). We aim to develop a more reliable,

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easy to operate, low cost and ultrasensitive immunoassay method by subtly combining main features of scFv, phage display and IPCR. The new design is termed PD-IPCR, denoting for phage display mediated IPCR. As proof-of-concept, nucleocapsid protein (NP) of Hantaan virus and prion protein (PrP), which have high pathogenicity and cause serious or fatal human diseases (21–24), were employed as both detection targets and demonstrative examples for PD-IPCR. The results are presented herein.

MATERIALS AND METHODS

Materials

The single chain antibody L13, mAb L13 and purified NP were from Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Single chain antibody 163 and PrP expression plasmid were constructed by Dr Ji-Bin Zhang (reported elsewhere), and mAb KG9 was donated by TSE Resource Centre (UK).

Preparation of phages

The recombinant TG1 containing pCANTAB5E-scFv was inoculated into 2× YT culture broth, which contained 2% glucose and 100 μg/ml ampicillin, and then incubated at 37°C with shaking (250 r.p.m.) until OD_{600} of the medium reached 0.5. After being infected with helper phage M13K07 (10^9 p.f.u./ml, Amersham Pharmacia Biotech), the culture was gently shaken for an additional 1 h at 37°C. The cells were harvested by centrifugation and transferred to 2× YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Phages were produced by growing the culture overnight with shaking at 30°C and then titred by determining the colony forming unit (CFU).

ELISA

In indirect ELISA, the polystyrene microtitre plate (Maxisorp, 96-wells, Nunc) was coated with 100 μl of 10-fold serial dilutions of purified NP in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. The negative control well was coated with carbonate buffer without NP. After blocking the wells with TBS (Tris Buffered Saline) containing 5% (w/v) skimmed milk (TBSM), phage in TBSM was applied to the wells and incubated for 2 h at 37°C. After washing with TBS, bound phage was detected using horseradish peroxidase (HRP)-labeled anti-M13 antibody (Amersham Pharmacia Biotech). Color development was produced by using tetramethylbenzidine (TMB). The reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm by a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). Sandwich ELISA was carried out with 100 μl of mAb in 0.05 M carbonate buffer (pH 9.6) coated on microtitre plate, following by addition of 10-fold serial dilutions of target protein. Phage incubation, washing condition and signal detection was similar to that of indirect ELISA.

PD-IPCR

The indirect PD-IPCR assays were carried out with the same steps as described for the ELISA except that the plate was subjected to PCR, instead of enzyme reaction. After phage incubation, the plate was washed with TBSET (TBS buffer containing 5 mM EDTA and 0.1% Tween-20) and distilled H_2O to remove unbound phages. After washing, 50 μl of distilled H_2O was added into the microtitre wells, and the plate was then treated with water bath (95°C for 10 min) to lyse the bound phages. The PCR mixture contained 5 μl of phage lysate as template, 1× PCR buffer (Takara), 0.4 μM each primer (Supplementary Table 1), 0.25 mM dNTP each, 0.01% BSA, 2.5 U r*Taq* DNA polymerase (Takara) and distilled H_2O in a total volume of 50 μl. The amplification reaction conditions included denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 30 s. The final extension was done at 72°C for 5 min. PCR amplification was performed in a T1 Thermocycler (Biometra). The 220 bp PCR products were electrophoresed on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Band density was determined by densitometry using a computer assisted image analyzer (Alpha Imager 2200, Alpha Innotech Corporation). Immunological process and PCR condition of sandwich PD-IPCR were similar to that of sandwich ELISA and indirect PD-IPCR, respectively.

Real-time PD-IPCR

All the real-time PCRs were carried out with TaqMan probe using the Opticon PCR machine of MJ Research. The PCR premix consisted of 5 μl of phage lysate as template, 1× PCR buffer (Takara), 4.5 mM $MgCl_2$, 0.2 μM each primer (Supplementary Table 1), 0.8 μM TaqMan probe, 0.25 mM dNTP each, 0.01% BSA, 2.5 U r*Taq* DNA polymerase (Takara) and distilled H_2O in a final volume of 50 μl. The step program for PCR was as follows: 94°C for 5 min, followed by 50 cycles of 95°C for 45 s, 55°C for 70 s. In addition, negative control containing no template DNA was included in each batch of PCR tests. The threshold level was determined to be above the background signals and the threshold cycles (Cts) values were set as the cycle at which the measured fluorescence intersected the cycle threshold line. Subsequent analysis was accomplished with Excel software (Microsoft).

RESULTS

Principle of PD-IPCR

The principle of PD-IPCR is depicted in Figure 1. Capture antibody is first coated on solid surface to provide a reaction platform. Target antigen in sample is then captured by the immobilized capture antibody. Recombinant phage particle can thus be anchored through the interaction between the displayed scFv and the bound target antigen. The phage DNA is released by heat lysing and serves as template for PCR. As a consequence, the existence of the target antigen is determined by detection of the PCR products.

ScFv L13 (25) displayed on M13 phage is specific to NP of Hantaan virus (Supplementary Figure 1). The L13 recombinant phages were produced by growing the *Escherichia coli* TG1 harboring the recombinant vector pCANTAB5E-L13 and rescued by M13K07 helper phage. After incubation overnight, the L13 recombinant phage particles were obtained

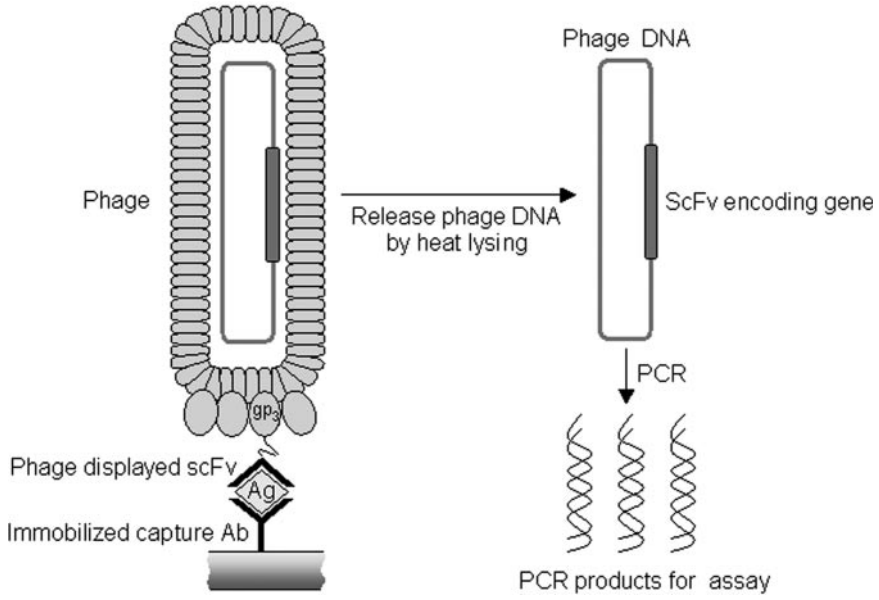

Figure 1. Schematic diagram of phage display mediated IPCR.

by centrifugating the *E.coli* culture and then used in PD-IPCR experiment for detection of Hantaan virus NP.

Indirect PD-IPCR for purified NP

The sensitivity of PD-IPCR for the detection of NP (~60 kDa) was determined in indirect format. The microtitre plate was coated with 10-fold serial dilutions of purified NP, followed by addition of recombinant phages L13. The bound phages were lysed by heating and then subjected to PCR with primers specific to partial V_H gene of L13 scFv (Supplementary Table 1). The presence of 220 bp amplification products were detected by agarose electrophoresis (Figure 2a). The amplification band of PD-IPCR was quantified by a computer assisted image analyzer. Band intensity of the PCR products increased proportionally with the amount of antigen in the sample. The results of electrophoresis and intensity analysis indicated that the lowest level of NP detected by indirect PD-IPCR was 10 pg/ml (~0.16 fmol/ml) (Figure 2). No DNA band was observed in negative control samples without NP coating (Figure 2a, lane 8). This sensitivity is about four orders of magnitude higher than that of ELISA dose-response assay using the same phages (Figure 2b).

To further compare the detection limit of conventional ELISA and PD-IPCR, the CFU assay was performed. The amount of NP in plates receiving phages was determined by infecting *E.coli* TG1 with the phages bound to each well and counting the CFU by standard CFU assays (26). The results suggested that about 10^5 CFU in ELISAs was needed for color development, while, in PCR tests, a detection limit of 10^2 CFU was observed (Supplementary Figure 2). The high amplification power gives PD-IPCR the potential for higher detection sensitivity than ELISA.

Real-time indirect PD-IPCR for purified NP

Real-time PCR detection (27,28) was employed in indirect PD-IPCR experiments using TaqMan probe (Supplementary Table 1). Figure 3a shows mean threshold cycles (Cts) of 22.23, 22.65, 23.47, 25.26, 26.61, 28.46, for 10-fold serial dilutions of the purified NP from 1 μg/ml to 10 pg/ml, while 31.68 for the control without NP coating. This result indicated that the Ct values increase with the decrease of

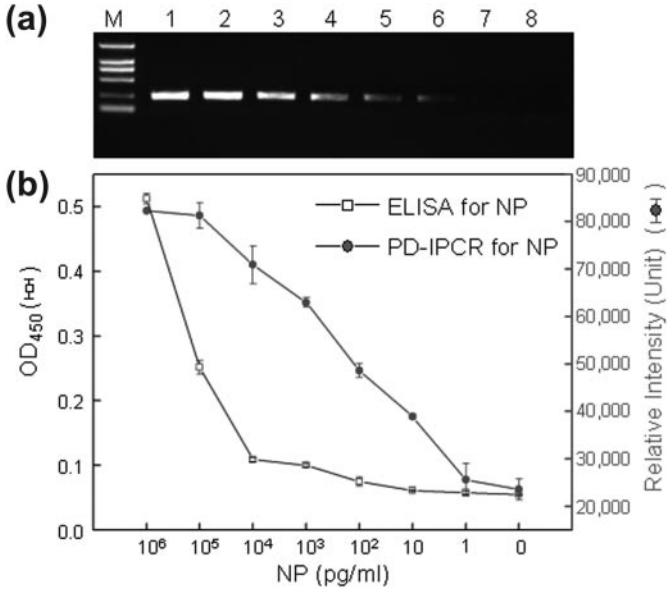

Figure 2. Detection sensitivity of indirect PD-IPCR and ELISA for NP. (**a**) Indirect PD-IPCR experiment. The polystyrene microtitre plate was coated with 100 μl of 10-fold serial dilutions of NP. Bound phages were used for PCR and amplification products were run on a 1.5% agarose gel. Lane 1 to 7 represent amplification products from 10^6 to 1 pg/ml. Lane 8 was negative control without NP coating. Lane M is DNA marker (DL2000) with 2000, 1000, 750, 500, 250 and 100 bp. (**b**) Sensitivity comparison between PD-IPCR and ELISA. The amplification band of PD-IPCR was quantified by a computer assisted image analyzer. The color development in ELISA was achieved with HRP-labeled anti-M13 monoclonal conjugate secondary antibody and TMB substrate. Average values of three independent experiments are given.

antigen concentration. The detection limit referred to 10 pg (NP)/ml (~0.16 fmol/ml), which corresponded to an about 10 000-fold increase in sensitivity as compared to the ELISA under analogous conditions. A linear regression of the signals obtained for the spiked purified NP against the logarithmic concentrations from 200 ng/ml to 0.8 ng/ml is well ($R^2 = 0.99$) (Figure 3b).Use of this regression curve for the quantification of the NP with 50 and 1 ng/ml yields a recovery of 72.6 and 126.0%, respectively. These results evidenced that real-time PD-IPCR is a sensitive detection method with high reproducibility.

Sandwich PD-IPCR and real-time sandwich PD-IPCR for purified NP

Due to the multimerization in nature (29,30), Hantaan virus NP offers multiple unique epitopes for certain antibody. Thus, it is possible to perform sandwich PD-IPCR assay of NP using phage displayed L13 scFv as detection antibody and its parent mAb L13 as the capture antibody. The result of sandwich PD-IPCR is shown in Figure 4, a detection limit of 10 pg (NP)/ml was achieved, corresponding to an about 10 000-fold increase in sensitivity as compared to the sandwich ELISA. When real-time PCR was used as the read-out method of sandwich PD-IPCR, similar detection sensitivity was achieved (Figure 3c), and the linear regression was good ($R^2 = 0.96$) for the signals of spiked samples against the logarithmic concentrations from 2000 to 2 ng/ml (Figure 3d).

To study accuracy and reproducibility of PD-IPCR, the intra-assay precision was determined with three duplicates for real-time sandwich PD-IPCR, and inter-assay variation was determined from the results of three independent experiments in three days. Both intra-assay and inter-assay were carried out using two different concentrations of NP (4 ng/ml and 400 ng/ml). As shown in Table 1, variation coefficients (CV) were 0.12–2.46% and 4.77–10.90% for the intra-assay and inter-assay, respectively. CV for intra-assay was calculated with the Ct variation from the Ct mean value, while CV for inter-assay was based on the concentration variation of the spiked purified NP from the concentration mean value.

Sandwich PD-IPCR for PrP

As the second demonstrative example, PrP was detected by the sandwich PD-IPCR. The experiment protocol was similar to that of NP detection process, but using mAb

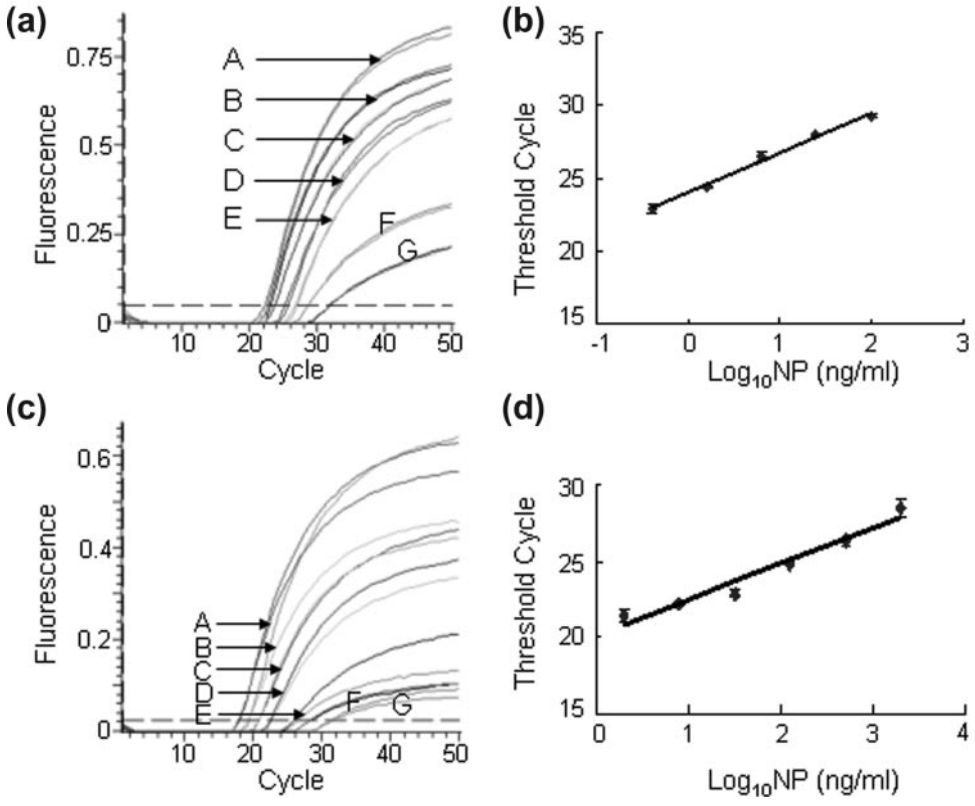

Figure 3. Real-time quantitative PD-IPCR assay of NP. (**a**) Quantitative curve of indirect PD-IPCR for purified NP. Curves A–F represent serial 10-fold dilutions of NP from 10^6 pg/ml to 10 pg/ml. G stands for the negative control I that had no NP antigen coating. No fluorescent signal was observed for negative control II that had no any template. Note the high similarity of the duplicate determinations, the regular intervals between the signals of the 10-fold dilutions and the difference between the control I and the control II. (**b**) Calibration plot of log purified NP concentration versus threshold cycles (Cts) in indirect PD-IPCR. NP coating was 100 µl of 4-fold serial dilutions from 200 to 0.8 ng/ml. The results from three individual experiments were averaged, and the error bars give the SD. (**c**) Quantitative curve of sandwich PD-IPCR for purified NP. The plate was coated with 100 µl of L13 mAb. Curves A–F represent serial 10-fold dilutions of NP from 10^6 pg/ml to 10 pg/ml, each had two duplicates. G stands for the negative control I that had no NP coating. No fluorescent signal was observed for negative control II that had no any template. (**d**) Calibration plot of log purified NP concentration versus Ct in sandwich PD-IPCR. The plate was coated with 100 µl of L13 mAb, followed by the addition of 4-fold serial dilutions of NP from 2000 to 2 ng/ml. Again, the results from three individual experiments were averaged, and the error bars give the SD.

Table 1. Intra-assay and inter-assay precision for real-time sandwich PD-IPCR of hantaan virus NP

Concentration spiked (NP*)	Run1 Recovery*	CV (%)[a]	Run2 Recovery*	CV (%)[a]	Run3 Recovery*	CV (%)[a]	CV (%)[b]
4	3.85	1.16	3.65	1.13	3.50	2.46	4.77
400	358.01	0.55	405.82	0.12	445.76	1.14	10.90

[a]intra-assay coefficient of variation (CV).
[b]inter-assay coefficient of variation (CV).
*ng/ml.

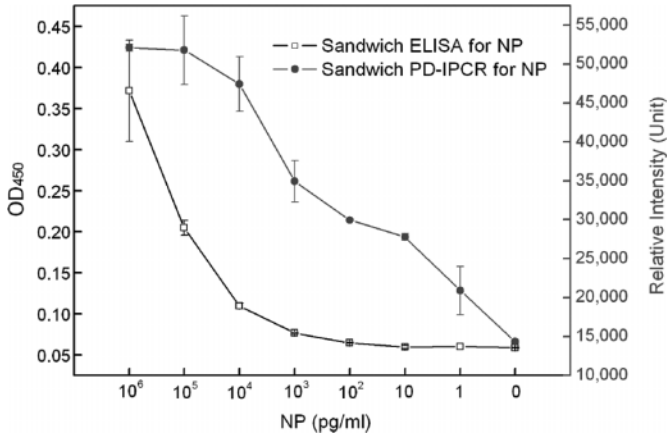

Figure 4. Detection sensitivity of sandwich PD-IPCR and sandwich ELISA for NP. The amplification band of sandwich PD-IPCR was quantified by a computer assisted image analyzer. The color development in sandwich ELISA was achieved with HRP-labeled anti-M13 monoclonal conjugate secondary antibody and TMB substrate. Average values of three independent experiments are given.

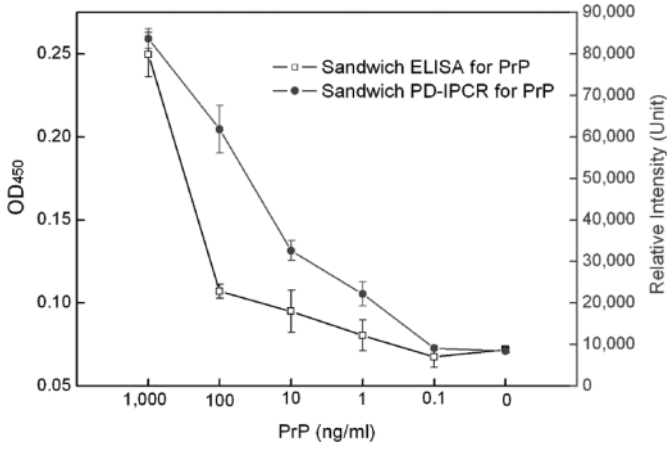

Figure 5. Detection sensitivity of sandwich PD-IPCR and sandwich ELISA for PrP. The amplification band of PD-IPCR was quantified by a computer assisted image analyzer. The color development in ELISA was achieved with HRP-labeled anti-M13 monoclonal conjugate secondary antibody and TMB substrate. Average values of three independent experiments are given.

KG9 as capture antibody and recombinant phage scFv 163 as the detection antibody. The results suggested that 1000 increased sensitivity was achieved compared with ELISA (Figure 5).

DISCUSSION

It is known that low affinity and avidity of scFv to specific antigen have a negative impact on its detection sensitivity. To increase the affinity and avidity of scFv, affinity maturation (31) and multimerization (32) of antibody fragments have been applied. Nevertheless, the improvement by these approaches has been limited. Whereas, the amplification power of the proposed method based on phage displayed scFv and PCR protocol greatly improved the detection sensitivity. For example, 10 pg/ml (~0.16 fmol/ml) of hantaan virus NP could be detectable with acceptable reproducibility. In addition, expression efficiency of scFvs is strongly influenced by their amino acid sequences (33), so far there is no universal high expression system for all kinds of scFvs yet. In fact, in many cases, it is difficult to obtain adequate high pure scFvs, which has become another obstacle for real application of scFv *in vitro* immunological analysis. In PD-IPCR, the scFvs are displayed on the surface of the phage particles that themselves as a whole can serve as the detection antibody carrying DNA, so that additional production and purification of scFv are not necessary. Indeed, phage display is a mature technique that can display a broad range of scFvs without captiousness. Further more, preparation of phage particles through *E.coli* cultivation is a rather simple and reliable process.

The PD-IPCR method offers two merits over the conventional IPCR. First, the recombinant phage particle is almost as a 'ready to use' reagent because the phage particles carry both scFv and DNA to be amplified and they can be obtained by simply centrifugating the overnight *E.coli* culture broth. These features obviate the need for complicated conjugation chemistry for attaching DNA to the antibody in conventional IPCR, while avoid the production of mAb that needs both hybridoma cell culture and affinity chromatography, which take several days and are expensive. Second, the approach should be general for almost any target with the fact that countless antibodies and peptides have been selected from phage display libraries.

However, although markedly increase in sensitivity as compared to ELISA the sensitivity of PD-IPCR is not as high as that of mAb-based IPCR. This is mainly because of the application of the scFv, whose affinity to antigen is normally lower than its parent mAb. Screening and using the higher affinity scFv or peptide are always expected to improve the detection sensitivity further. Another reason is the nonspecific adsorption of recombinant phages to the microplate surface. As seen in Figure 3a, weak fluorescence signal was observed with the negative control I that had no NP coating (curve G). Working conditions were investigated, TBSET and

non-fat milk were found to be suitable blocking and washing reagents. Under these conditions, the background noise was largely reduced (Supplementary Figure 3).

CONCLUSIONS

In summary, the experiment data show that the PD-IPCR principle shares main advantages of scFv, IPCR and phage display, greatly facilitating the real application of scFv in immunoassay. Another point, the protocol completely avoids complex processes of antibody production and antibody-DNA conjugation in conventional IPCR. Sensitivity of PD-IPCR could be further improved through optimization experiments. We believe the proposed method has broad sense in immunoassay of wide variety of antigens.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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