

## Comparative Analysis of Screening Results from Various ELISA Formats Used for Detection of Anti-Erythropoietin Antibodies in Korean Patients

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**Abstract** – Clinical cases of pure red cell aplasia (PRCA) have been reported during the recombinant human erythropoietin (EPO) therapy for the anemia patients. PRCA is a rare hematological disorder leading to a severe anemia due to an almost complete stop of red blood cell production. Antibody (Ab)-associated PRCA is caused by the EPO-neutralizing Abs that eliminate the biological activity of EPO. In order to detect anti-EPO Abs in human sera, we performed conventional ELISA, directly coated bridging ELISA, and streptavidin coated bridging ELISA, and compared their sensitivity and specificity. Some false positive results were obtained in the conventional ELISA. One positive sample was detected successfully by streptavidin coated bridging ELISA, which was not appeared in the directly coated bridging ELISA. In conclusion, streptavidin coated bridging ELISA was substantially sensitive and specific format and one out of sixty-eight serum samples was proved to be anti-EPO positive.

**Keywords:** Erythropoietin, Pure red-cell aplasia, Bridging ELISA, Immunogenicity

### INTRODUCTION

Human erythropoietin (EPO) produced in the kidney is a heavily glycosylated protein that enhancing red blood cell production in the bone marrow. Chronic kidney disease (CKD) patients suffer from anemia caused by reduced EPO production. To treat anemia of CKD patients, recombinant EPO protein has been used successfully since 1988 (Erslev and Besarab, 1997; Bennett *et al.*, 2004). However, neutralizing anti-EPO Abs and pure red cell aplasia (PRCA) caused by these Abs have been reported in some EPO-treated patients (Casadevall *et al.*, 2002). PRCA is a rare hematological disorder leading to a severe

anemia due to an extremely decreased production of red blood cell. EPO-associated PRCA is occurred by anti-EPO neutralizing Abs depriving of the activity of endogenous and exogenous EPO (Casadevall *et al.*, 1996; Casadevall *et al.*, 2002) and this cases have recently been increased in Europe (Casadevall *et al.*, 2002; Gershon *et al.*, 2002; Bennett *et al.*, 2004; Tolman *et al.*, 2004).

The identification of anti-EPO Abs is the key to diagnose EPO-associated PRCA and several assays have been developed and used for this. Enzyme-linked immunosorbent assay (ELISA) (Kientsch-Engel *et al.*, 1989; Urra *et al.*, 1997; Hoesel *et al.*, 2004; Swanson *et al.*, 2004), radio-immunoprecipitation (RIP) (Casadevall *et al.*, 2002; Tacey *et al.*, 2003), and surface plasmon resonance (SPR) (Swanson, 2003) have been used to detect the presence of anti-EPO Abs and in vitro bioassays are used to measure the neutralizing activity of anti-EPO Abs. However, currently, there are no standard assays to compare data from

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different worldwide labs owing to absence of appropriate biological standards for assay validation and calibration.

Conventional ELISA for detection of anti-EPO Abs was developed (Urrea *et al.*, 1997). Plates were coated with EPO at 10  $\mu\text{g/ml}$  and added with diluted serum from a EPO-resistant hemodialysis patient. Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-human IgG or IgM was added and color was developed by tetramethylbenzidine substrate. Anti-EPO IgG Abs were detected from the single patient and no Abs from normal controls. This ELISA was rapid, easy to use, and inexpensive. However, it has some disadvantages such as non-specific matrix effects, alteration of EPO conformation and epitope masking by immobilization process (Thorpe and Swanson, 2005a).

Directly coated bridging ELISA was developed (Swanson *et al.*, 2004). Anti-EPO Abs in patient serum bridge biotinylated EPO to EPO immobilized to a plate well. Subsequently, streptavidin-HRP was added and color was developed by tetramethylbenzidine substrate. This ELISA was highly specific due to dual arm binding, however, immobilization process may alter the EPO conformation and biotin labeling of EPO is required (Thorpe and Swanson, 2005a).

Streptavidin coated bridging ELISA was developed and validated (Hoesel *et al.*, 2004; Gross *et al.*, 2006; Gross *et al.*, 2008). Anti-EPO Abs in patient serum bridge digoxigenylated EPO to biotinylated EPO immobilized to a streptavidin coated-plate well. Subsequently, anti-digoxigenin (DIG) Fab-HRP conjugate was added and color was developed by ABTS substrate. This ELISA was very sensitive (a lower limit of detection is 1  $\text{ng/ml}$ ), specific (no backgrounds in normal serum samples) and suitable for screening large numbers of samples. Epitope masking or conformation change of EPO by immobilization to plate was overcome by the use of streptavidin to capture biotinylated EPO in this ELISA.

As described, there are some ELISA formats to detect anti-EPO Abs and they have some advantages and disadvantages. In this study, we applied three kinds of ELISAs in order to adopt the most suitable assay to screen anti-EPO Abs in the human serum. Sixty-eight numbers of samples from CKD patients were analyzed and one sample was proved to be positive for anti-EPO Abs. Interaction between EPO and Abs in positive sample was confirmed by protein A/G pull down and western blot.

Our results suggest that among ELISA methods, streptavidin coated bridging ELISA is currently the most suitable assay for detection of anti-EPO Abs in human sera.

## MATERIALS AND METHODS

### Conventional ELISA

ELISA plates (Maxisorp, Nunc Inc., Copenhagen, Denmark) were coated with 1  $\mu\text{g/ml}$  of EPO in phosphate buffered saline (PBS, Amresco Inc., Solon, OH, USA). After incubation overnight at 4°C, the plates were blocked with PBSA (PBS, 1% BSA) for 1 h at room temperature (RT). After washing with PBST (PBS, 0.05% Tween-20), serum samples were diluted at 1:50 with PBSAT (PBS, 1% BSA, 0.05% Tween-20) and added to each well and incubated for 1 h at RT. Bound Ab was detected with HRP conjugated sheep anti-human IgG (Millipore Inc., Billerica, MA, USA). After washing, ABTS solution (Roche, Mannheim, Germany) was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm) by Versamax microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

### Biotinylation of EPO

Biotin was labeled to EPO with EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotinylation Kit (Pierce Inc., Rockford, IL, USA) according to the manufacturer's protocol. In brief, 10 mM Sulfo-NHS-LC-Biotin was dissolved in PBS and mixed with 1 mg of EPO for 2 hours on ice. A Zeba<sup>™</sup> desalt spin column was used to exchange buffer and remove excess biotin reagent. The level of biotin incorporation was measured by HABA assay. Protein concentration was determined by DC Protein Assay (Biorad Inc., Hercules, CA, USA).

### Directly coated bridging ELISA

ELISA plates were coated with 1  $\mu\text{g/ml}$  of EPO in PBS. After incubation overnight at 4°C, the plates were blocked with PBSA for 1 h at RT. After washing with PBST, 1:5 diluted serum samples were mixed with an equal volume of 300  $\text{ng/ml}$  of biotinylated-EPO (BIO-EPO) in PBSAT and this mixture was added to wells and incubated for 2 hours at RT. After incubation, plates were washed 3 times and streptavidin-HRP conjugate (Thermo scientific Inc., Rockford, IL, USA) diluted in PBSAT was added to each well and then incubated for 1 hour. After washing, ABTS solution was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm). Rabbit polyclonal anti-EPO Ab (H-162, Santa Cruz biotechnology, Santa Cruz, CA, USA) was used as positive control.

### Digoxigenylation of EPO

Digoxigenin-3-O-methylcarbonyl-aminocaproic acid-N-hydroxysuccinimide ester (DIG, Roche, Mannheim, Germany)

was labeled to EPO according to the manufacturer's protocol. In brief, 5 mg of DIG was dissolved in DMSO and incubated with 0.5 mg of EPO dissolved in PBS, pH 8.5, for 2 hours at room temperature (RT). A Microcon<sup>®</sup> Centrifugal Filter Units (Millipore, Billerica, MA, USA) was used to exchange buffer and remove excess DIG reagent. Protein concentration was determined by DC Protein Assay (Biorad Inc., Hercules, CA, USA).

### Streptavidin coated bridging ELISA

Streptavidin-coated 96-well microplates or 8-well strips (Pierce, Rockford, IL, USA) were washed twice with PBST. After washing, 1 µg/ml of BIO-EPO was added to each well and incubated for 1 hour at RT. After incubation, plates were washed 3 times in a microplate washer (Molecular Devices, Inc., Sunnyvale, CA, USA) or manually. 1:5 diluted serum samples were mixed with an equal volume of 40 ng/ml of digoxigenylated-EPO (DIG-EPO) in PBSAT and this mixture was added to wells and incubated for 2 hours at RT. After incubation, plates were washed 3 times and anti-DIG Fab-HRP conjugate (Roche, Mannheim, Germany) diluted in PBSAT was added to each well and then incubated for 1 hour. After washing, ABTS solution was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm).

### Immunoprecipitation and immunoblotting

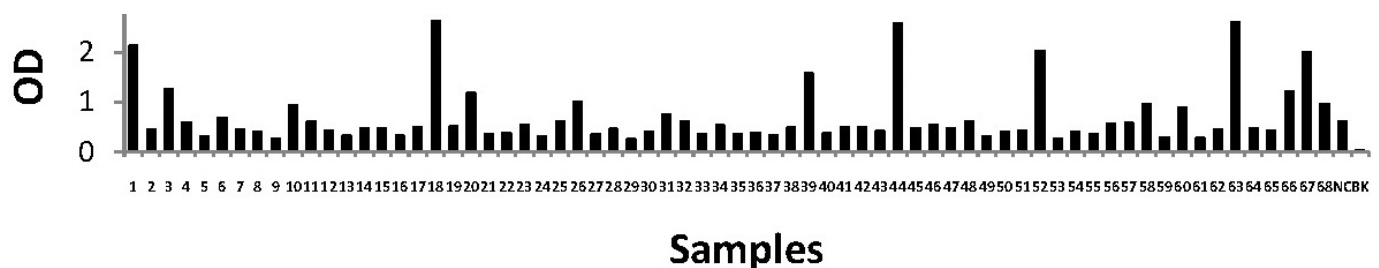
For immunoblotting, serum was incubated with 2 µg/ml of recombinant EPO together with 20 µl of protein A/G PLUS agarose (Santa Cruz biotechnology, Santa Cruz, CA, USA) in a total volume of 1 ml PBSAT for overnight with continuous rocking at 4°C. Agarose beads were pelleted by centrifugation, washed three times in PBSAT, and reconstituted in 20 µl of PBSAT. EPO were removed from the beads by boiling in a 6x sample loading buffer and electrophoresed under reducing conditions in 12% polyacrylamide gels and then transferred to a nitrocellulose

membrane. Nonspecific binding was inhibited by incubation in blocking buffer (20 mM Tris, pH 7.4-buffered saline with 0.1% Tween 20 containing 5% nonfat dried milk) for 1 h. Rabbit polyclonal antibody (Epo (H-162), Santa Cruz biotechnology, Santa Cruz, CA, USA) were exposed to membranes for 90 min at a dilution of 1:200. After washing, the blots were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG at a dilution of 1:10,000 and detected with the chemiluminescent substrate (Pierce, Rockford, IL, USA) for 1 min according to the manufacturer's instructions and visualized with X-ray film. Densitometric analysis was performed with TINA software (Raytest, Straubenhardt, Germany).

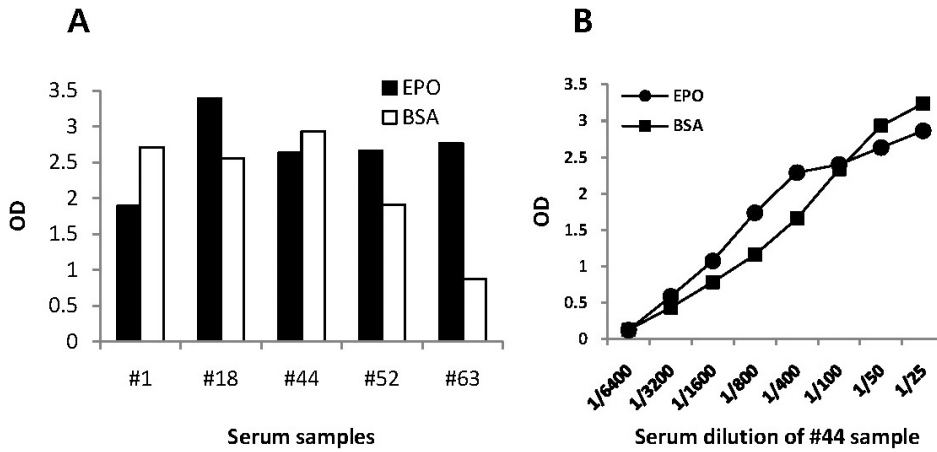
## RESULTS

### False positives were occurred frequently in conventional ELISA which was developed for the detection of anti-EPO binding Abs

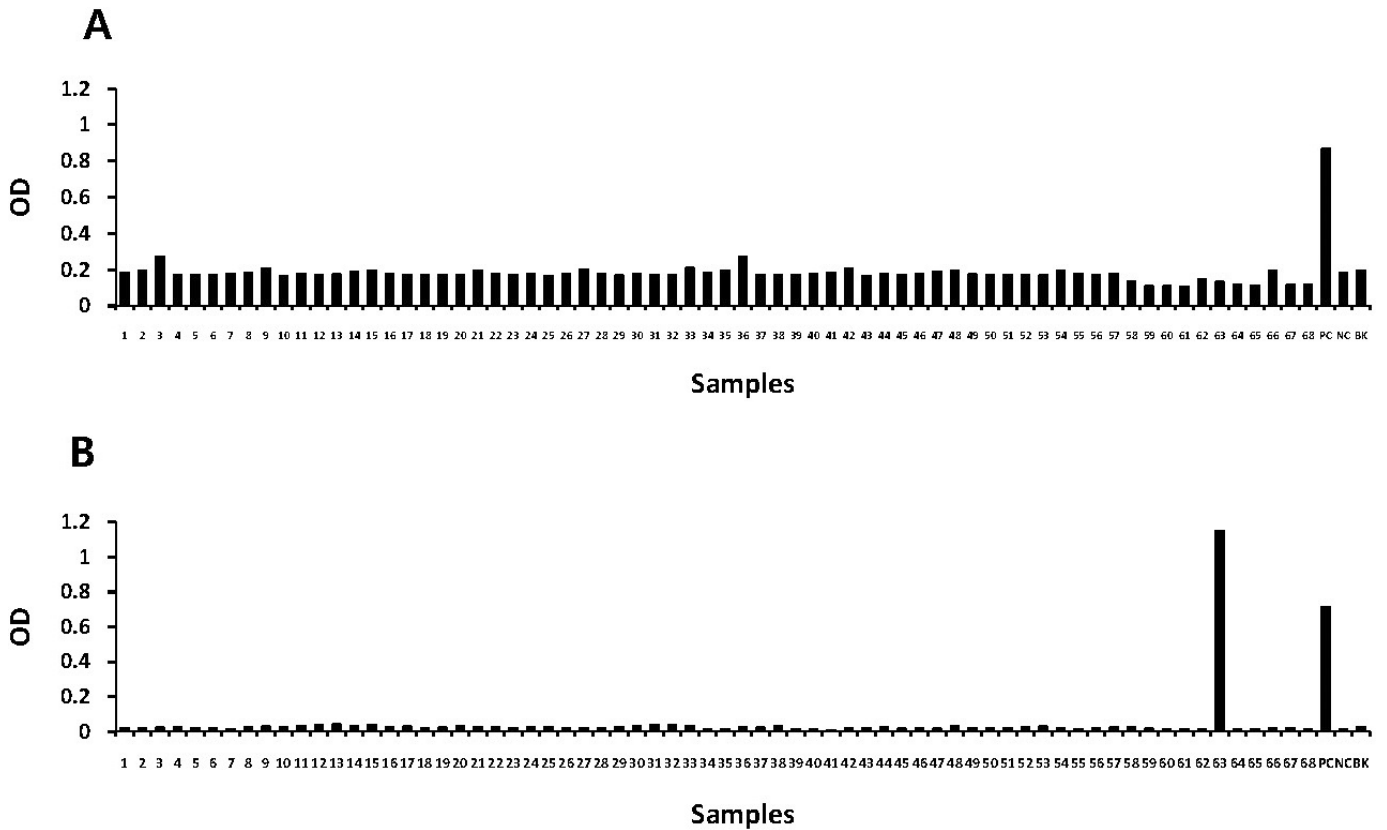
Because conventional ELISA is relatively easy to set up and has been used for long time, we initially tried to setup this assay for the purpose of detecting anti-EPO Abs in human sera. Anti-EPO Abs in serum samples were bound to recombinant human EPO which was directly immobilized to plate wells and detected by enzyme-conjugated secondary Ab. Sixty-eight serum samples were applied to this assay and the data were shown in Fig. 1. Throughout the whole samples, background level was generally high and the mean OD value of normal control serum was 0.6 after incubation for 30 minutes. Six samples were well above the cut-off value (mean OD of normal control  $\times 2$ ) and initially regarded as positives for anti-EPO Abs. However, the binding was turned out to be non-specific because the Abs interacted with the control antigen of bovine serum albumin (BSA) as well as to EPO (Fig. 2A). Single dilutions (1/50) of six serum samples were compared in EPO or BSA binding. The OD values of the binding of two samples



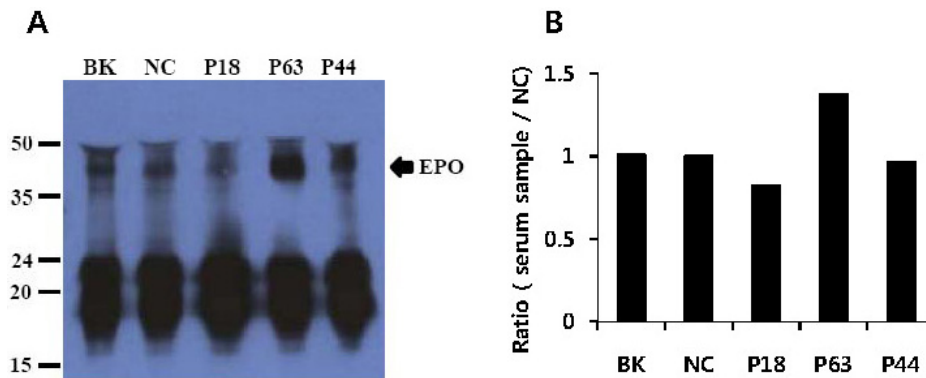
**Fig. 1.** Screening of serum samples with conventional ELISA. ELISA plates were coated with 1 µg/ml of EPO. The plates were blocked and serum samples diluted at 1:50 were added. Bound Ab was detected with HRP conjugated sheep anti-human IgG and ABTS solution was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm). NC is for normal control and BK is for blank.



**Fig. 2.** Non-specific binding of human serum samples. ELISA plates were coated with 1  $\mu\text{g/ml}$  of EPO or BSA. The plates were blocked and serum samples (#1, 18, 44, 52, and 63) diluted at 1:50 (A) or serial dilution of sample P44 (B) were added. Following procedure was described in Fig. 1.



**Fig. 3.** Screening of serum samples with bridging ELISA. For performing directly coated bridging ELISA (A), plates were coated with 1  $\mu\text{g/ml}$  of EPO and blocked with PBSA. 1:5 diluted serum samples were mixed with an equal volume of 300 ng/ml of BIO-EPO and this mixture was added to wells. After incubation, streptavidin-HRP conjugate was added to each well. After washing, ABTS solution was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm). Rabbit polyclonal anti-EPO Ab was used as positive control. PC is for positive control, NC is for normal control, and BK is for blank. For performing streptavidin coated bridging ELISA (B), streptavidin-coated 96-well microplates or 8-well strips were added by 1  $\mu\text{g/ml}$  of BIO-EPO. After incubation, 1:5 diluted serum samples were mixed with an equal volume of 40 ng/ml of DIG-EPO and this mixture was added to wells. After incubation, anti-DIG Fab-HRP conjugate was added to each well. After washing, ABTS solution was added to each well and incubated. Optical density was measured at 405 nm (reference wavelength: 492 nm). PC is for positive control and BK is for blank.



**Fig. 4.** Immunoprecipitation and immunoblot analysis of interaction between #63 and EPO. Blank (BK, without serum), normal control serum (NC), patients' sera (#18, #63, #44) were bound to EPO and immunoprecipitated with protein A/G. EPO was recovered from the beads and electrophoresed under reducing conditions in 12% polyacrylamide gels and then transferred to a nitrocellulose membrane. EPO was detected by using rabbit polyclonal antibody and horseradish peroxidase-conjugated anti-rabbit IgG (A). Densitometric analysis of EPO band was shown on the right side of images (B).

(#1, #44) to BSA were even higher than to EPO. To confirm the non specificity of serum samples more closely, serially diluted samples were applied to EPO and BSA coated microplates (Fig. 2B). The binding of #44 to BSA was comparable to EPO and dose-dependent.

#### All the samples were negative for EPO binding in directly coated bridging ELISA

To reduce the background signals obtained from conventional ELISA, we used the directly coated bridging ELISA to overcome this problem. Anti-EPO Abs in serum samples bridged BIO-EPO to EPO which directly immobilized to plate wells. Serum samples were applied to this assay and data were shown in Fig. 3A. Clearly, almost OD values were under 0.2 after incubation for 30 minutes. Even six samples which showed high activity of EPO binding in the conventional ELISA were not positive at all. 2  $\mu$ g/ml of rabbit polyclonal anti-EPO Ab was used as positive control.

#### One sample was positive for EPO binding in streptavidin coated bridging ELISA

Very sensitive and selective method of streptavidin coated bridging ELISA was reported (Hoesel *et al.*, 2004). In order to confirm the results of directly coated bridging ELISA, we screened all the samples again with this assay. To setup streptavidin coated bridging ELISA, additional labeling of EPO, DIG conjugation to EPO (DIG-EPO), was required. Anti-EPO Abs in serum samples bridged DIG-EPO to BIO-EPO which bound to streptavidin coated plate wells. Serum samples were applied to this assay and the data were shown in Fig. 3B. Only OD value of #63 sample was significantly higher than the other samples. Reference

material was mouse monoclonal anti-EPO Ab and standard curve was made with this. The concentration of anti-EPO Abs in #63 sample was 384.1 ng/ml all the other samples were 0 ng/ml.

#### Reactivity of #63 sample to EPO was confirmed by pull-down assay

The presence of anti-EPO Abs in #63 serum sample was further demonstrated by pull-down assay. Human sample #18 and #44 which showed high nonspecific signals in conventional ELISA were chosen as controls. Each serum sample was incubated with recombinant EPO and precipitated by protein A/G beads. Then EPO was eluted from beads and separated by SDS-PAGE and detected by western blotting. EPO protein band was only appeared in #63 sample (Fig. 4A). Densitometric analysis of EPO blot was showed in Fig.4B. Non-specific antibody bands also appeared in all samples.

## DISCUSSION

Ab-associated PRCA is a rare but life threatening adverse event occurred in CKD patients undergoing EPO administration. Therefore universal standardized assay is required for detection of anti-EPO Abs in patient serum. ELISA method has been widely used due to its properties of easiness and high throughput. However, it has been indicated that some ELISAs may not have some level of sensitivity and specificity for detection of low levels of EPO specific Abs (Thorpe and Swanson, 2005a).

Initially, by using conventional ELISA, clinical serum samples from CKD patients were screened to detect the presence of anti-EPO Abs. This assay was convenient to

perform because no labeling of EPO was required and experienced previously in other cases. However, we obtained false positive results with high background (Fig. 1). This phenomenon is probably due to some interfering serum factors such as rheumatic factors (Hennig *et al.*, 2000) and unknown sticky antibodies. Some samples showed slightly higher EPO-binding signals than BSA-binding and others showed significantly higher signals (Fig. 2A). Dose-dependent and comparable response to BSA as well as EPO and no substantial decrease of background despite multiple dilutions indicated that this signal is non-specific rather than EPO-specific (Fig. 2B). However, in case of #63, it showed much higher EPO-binding signals than BSA, therefore, further assays were needed to demonstrate their specificity. In addition, considering the PRCA statistics of incidence rates of 0.02 to 0.03 per 10,000 patient-years (McKoy *et al.*, 2008), this high rates (six per sixty eight) were certainly abnormal. Quantitative analysis was impossible with this assay because human anti-EPO standards are essential to this ELISA format but there are no available human standards.

To overcome the non-specificity problem of conventional ELISA, bridging format of ELISA was introduced. Bridging ELISA adopted dual recognition system, which ensures high specificity (Thorpe and Swanson, 2005b). EPO should be labeled with biotin to develop a directly coated bridging ELISA. Contrary to the data of conventional ELISA, we found no positives for anti-EPO Abs and low backgrounds in serum samples (Fig. 3A). However, when we made a standard curve with rabbit polyclonal anti-EPO Ab, we found this assay is not so sensitive (data not shown). Selectivity problem was solved but sensitivity problem still existed. So we searched more sensitive and selective one.

Highly sensitive and selective method of streptavidin coated bridging ELISA was reported (Hoesel *et al.*, 2004). They found that the method of EPO immobilization on plates is a critical determinant for the sensitivity of ELISA used for measuring anti-EPO Abs and streptavidin coated bridging ELISA results in a sensitivity of up to two orders magnitude higher than directly coated bridging ELISA (Gross *et al.*, 2006). In addition, this assay has been shown to have very low backgrounds compared to the non-bridging conventional ELISA (Thorpe and Swanson, 2005b). We finally applied our serum samples to this assay and found only one outstanding anti-EPO positive sample (#63). Actually, #63 sample was one of the false positives with #1, 18, 44, and 52 in conventional ELISA.

After performing the three kinds of ELISAs to measure the anti-EPO Abs in serum, we have found one case of an-

ti-EPO positive, #63. To test the interaction between EPO and #63 serum in solution state, EPO protein was mixed with #63 serum and protein A/G beads and incubated overnight. Precipitated EPO protein was revealed by SDS-PAGE and western blotting. Only #63 serum precipitated EPO protein and the other selected samples, which showed nonspecific signals in conventional ELISA, did not.

Although anti-EPO Ab was confirmed in patient #63, neutralizing assay and bone marrow biopsy will be required to diagnose PRCA. In addition, further kinetic monitoring of anti-EPO Abs will be helpful to diagnose and treat this condition.

In conclusion, we have found that streptavidin coated bridging ELISA was the most reliable assays among the popular three kinds of ELISA methods in terms of selectivity and sensitivity as for the purpose of screening anti-EPO Ab in human serum.

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