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A microwell plate-based multiplex immunoassay for simultaneous quantitation of antibodies to infectious viruses

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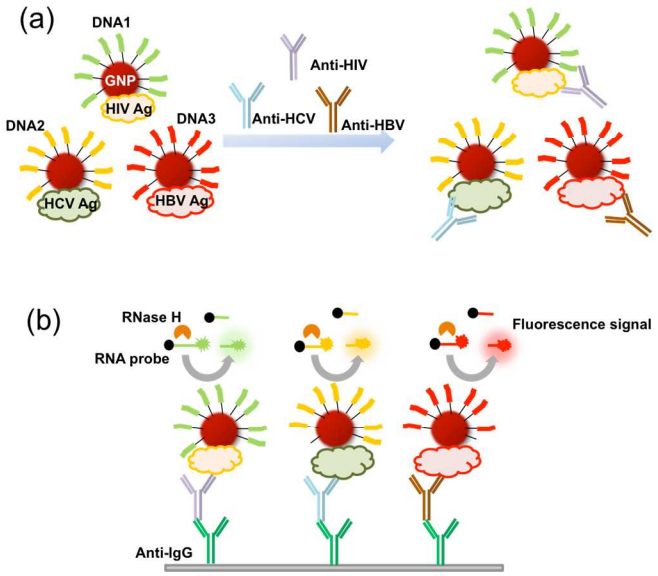
Antibodies (Abs) to disease-causing viruses in human blood are important indicators of infection status. While ELISA has been widely used to detect these Abs, a multiplex assay system for simultaneous detection of multiple Abs is still a desirable alternative method for more efficient screening process because of lack of multiplexing ability in ELISA. However, since all antibodies are based on immunoglobulin and recognized commonly by the same secondary antibody, it is impossible to multiplex the conventional indirect ELISA in a 96-microwell plate-based platform. Overcoming this hurdle, we here designed an assay consisting of two step: capturing target Abs by specific antigens on DNA-encoded gold nanoparticle and quantifying the target Abs by producing RNase H-mediated detection signal based on the DNA and additionally added RNA probes. With this newly designed method, we could successfully analyze three infectious disease-related Abs such as anti-HIV Ab, anti-HCV Ab, and anti-HBV Ab at the same time on the microwell-based platform. The assay performance was evaluated by comparison with ELISA. Further, accuracy and precision of the assay in a practical condition was also estimated by determining amount of target Abs in human serum solutions.

Introduction

Detection of virus infection on every unit of donated blood is critical for preserving reliable blood donation chain. Nucleic acid amplification testing methods are regarded as sensitive detection platform for analysis of viral nucleic acids, such as the branched-chain DNA signal amplification assay[1-2] and transcription mediated amplification (TMA)-based assay,[3-5] and PCR-based nucleic acid amplification assay.[6-9] However, these techniques require DNA preparation steps before amplification and expensive instruments and well-trained operators. Moreover, multiplex detection of the target viral genes using such nucleic acid amplification testing (NAT) should be performed after very careful design of primers to prevent undesired off-target amplification. In particular, since donated blood samples to be controlled are in general too many to be screened using NAT in a limited time, pre-screening of the samples using ELISA is recommended in countries such as Japan and Korea.[10-11] While ELISA is a robust assay system for detection of the target molecules, detection of more than one target cannot be achieved by using the conventional ELISA system. Three ELISA assays are required, for example, to detect three kinds of antibodies (Abs), anti-HIV, anti-HCV, and anti-HBV in a serum sample. More efficient and improved screening processes would be realized by using a multiplex immunoassay system. Most of previously known multiplex immunoassays for Abs are based on multiplex microbead immunoassay (MMIA) in which each bead coated with a specific antigen to each Ab contains a fluorescence barcode with a ratiometric fluorescence signal from two dyes. Abs captured by the multiplex beads are covered by a fluorescence-labeled secondary antibody and then quantitated using a flow cytometric instrument with identification of the barcode of each bead. MMIA has been used for the detection of serum Abs to multiple peptide epitope,[12] auto-antigens,[13] antigens from microorganisms,[14-15] and simian viruses from nonhuman primates.[16] In spite of its powerful multiplexing ability, however, MMIA requires also a sophisticated and expensive instrument. A multiplex assay method based on user-friendly conventional 96-microwell plates and a relatively cost-effective plate reader would be a good alternative way particularly in a small-sized clinics. Although several multiplex immunoassay have been developed for analysis of multiple biomarkers,[17-19] the devise of an indirect immunoassay for detection of multi-

Abs against various viruses at the same time is still challenging in microwell plate-based platform because those Abs all produced in a human body will bind to the same secondary Ab, anti-human IgG, which makes target specific signal generation impossible.



Scheme 1. The work-flow of multiplex-rGNP-OLISA is composed of two steps: (a) capturing target Abs by Ab-GNP-DNA complexes and (b) producing fluorescence detection signals on microwells using RNase H and RNA probes to quantify amount of the captured Abs.

Recently, we have developed a microwell plate-based multiplex immunoassay called gold nanoparticle-enhanced oligonucleotide-linked immunosorbent assay (GNP-OLISA).[20] GNP-OLISA utilizes detection Abs immobilized on DNA-encoded gold nanoparticles of which the DNA strands mediate the cleavage of fluorogenic RNA probes by RNase H for generation of the fluorescence detection signal. Multiplex detection could be achieved in GNP-OLISA, since the DNA can play a dual role as the signal generator and the barcode for a detection Ab. Here, we designed and performed the multiplex immunoassay to detect the human Abs using GNP-OLISA (Scheme 1). In the assay, viral antigens (Ags) binding to model three Abs (anti-HIV, anti-HCV, and anti-HBV) instead of detection antibodies were coated on gold nanoparticles with specific DNA barcode strands. The GNP conjugates then were incubated to capture target Abs and recognized by a secondary antibody (anti-IgG) coated on the bottom of microwells (Scheme 1a). The amount of the captured Abs was measured by using the GNP-OLISA signalling procedure after removing unbound materials by washing (Scheme 1b). This assay type is a kind of reverse-indirect ELISA similar to indirect ELISA except the signalling part is conjugated to Ags instead of the secondary antibody. Fluorescence emission specific for each antigen was finally measured to examine the assay performance. In addition, the developed reverse-indirect GNP-OLISA (rGNP-OLISA) was also used to analyze Abs spiked in human sera to demonstrate practical utility of the method.

Experimental

Materials

Anti-HIV Ab, anti-HCV Ab, anti-mouse IgG, biotinylated anti-mouse IgG and streptavidin-HRP were purchased from Abcam (Cambridge, UK). HIV Ag was purchased from BioLink International Inc. (IL, USA). HCV Ag, HBV Ag and anti-HBV Ab were from Meridian Life Science (Memphis, TN, USA). TMB substrate solution and stop solution were from Sigma-Aldrich (St. Louis, MO, USA), Protector RNase Inhibitor (PRI) was from Roche (Mannheim, Germany), gold nanoparticles (15 nm) were from British Biocell International (Cardiff, UK), and RNase H was from Takara (Otsu, Japan). DNA oligonucleotides (thiol-ethylene glycol$_6$-AACCACAGTC for HIV, thiol-ethylene glycol$_6$-ACTCTATGGG for HCV and thiol-ethylene glycol$_6$-AGCGTTGTAG for HBV) and RNA probes (RNA1: FAM-CACUGUGGUU-BHQ1 for HIV, RNA2: TAMRA-CCCAUAGAGU-BHQ2 for HCV and RNA3: Cy5-CUACAACGCU-BHQ2 for HBV) were synthesized by Bioneer (Daejeon, Korea). The optical characteristics of these fluorogenic RNA probes such as relative quantum yield (QY) and full width at half maximum (FWHM) were measured (Fig. S2, Table S1, and Table S2)

ELISA

The 100 μL of Ags were diluted to a concentration of 2 μg mL^{-1} for HIV and HCV, 4 μg mL^{-1} for HBV with PBS buffer and were coated in the transparent Maxisorp 96-well plate (Thermo scientific) by incubation overnight at 4 °C, followed by washes with PBS (3×300 μL). The Ag coated-wells were blocked with 200 μL of Blocking Buffer (3% BSA in PBS), incubated for 1 h at room temperature, and rinsed with PBST (0.05% Tween-20 in PBS, 3×300 μL). Ab solutions of varying concentrations (0, 62.5, 125, 250, 500, and 1000 pg mL^{-1}) in 100 μL of Assay Buffer (1% BSA in PBST) were added to the wells, followed by incubation for 1 h at room temperature. The plate was then washed with PBST (3×300 μL) before addition of the biotinylated anti-mouse IgG (100 μL, 0.2 μg mL^{-1} for HIV and HCV, 0.1 μg mL^{-1} for HBV in Assay Buffer) and incubation for 1 h at room temperature. After rinsing with PBST (3×300 μL), streptavidin-HRP (1 μg/mL) in Assay Buffer (100 μL) was added and incubated for 1 h at room temperature. The plate was further washed with PBST (3×300 μL), and each well was incubated in TMB substrate solution (100 μL) for 10 min at room temperature. After addition of TMB stop solution (100 μL) to the each well, optical density at 450 nm was immediately measured by means of a 96-well microplate reader (SpectraMax Plus™, Molecular Devices, Sunnyvale, CA, USA).

Ag-GNP-DNA complex

1 mL of 15 nm gold colloid was incubated for 30 min at room temperature with Ag (2 μg of HIV multi-epitope Ag, 100 nM of HCV core Ag and HBV core Ag) with gentle shakes. After adding Tween 20 and phosphate buffer (pH 8.0) at a final concentration of 0.05% and 10 mM, respectively, the particles were then incubated with 5'-thiolated DNA oligonucleotide (40 μL, 100 μM) overnight at 4°C. After incubation, the solution was further salted by adding NaCl (30 μL, 5 M), followed by gently shaking for 1h at 25 °C. GNP conjugates were stabilized for 1 h by adding Blocking Buffer (500 μL). Next, the solution was centrifuged under 18,000 g for 25 min at 4°C, and the supernatant was removed. This centrifugation procedure was repeated twice and the final pellet was resuspended in phosphate buffer (pH 8.0, 1 mL, 10 mM) containing 0.1% BSA. The concentration of GNP conjugates was determined by

measuring absorbance at 522 nm (ε=3.64 x 10^8 $M^{-1}cm^{-1}$). The wavelength of absorbance maximum and extinction coefficient were 522 nm and, respectively.

Determination of the number of DNA oligonucleotides per GNP

To determine the quantity of DNA oligonucleotides in an Ag-GNP-DNA complex, RNase H reaction solutions (100 μL, 100 nM F-RNA-Q probe, 0.4 U of PRI, 6 U of RNase H, 40 mM Tris-HCl, 4 mM MgCl2, 10 mM DTT, 0.003% BSA, pH 7.7) containing serially diluted free DNA strands or Ag-GNP-DNA conjugates were incubated for 15 min at 37°C, and then fluorescence intensities of the reaction mixtures were measured by Appliskan (Thermoscientific, Walthan, MA, USA) with excitation/emission filter set of 485/535, 544/589 and 633/675 nm for FAM, TAMRA and Cy5, respectively. The concentrations of the released DNA oligonucleotides from the conjugates were determined by interpolation from a standard linear calibration curve obtained from reactions with free DNA. The average number of oligonucleotides per GNP was calculated by dividing the concentration of oligonucleotides by the concentration of GNP.

Single rGNP-OLISA

Maxisorp Black 96-well microplates (Thermo scientific) were coated with anti-mouse IgG (4 μg mL^{-1}) in PBS (100 μL/well) by incubation for 1 h at 25 °C. Each well was then washed with PBS (3×300 μL), blocked with Blocking Buffer (200 μL), and incubated for 1 h at 25 °C. The Ag-GNP-DNA complex solution was prepared by diluting Assay Buffer to a final concentration of 1/10 with assay buffer and incubated at varying concentrations of each target Ab. After incubation for 1 h at room temperature, the precipitated Ag-GNP-DNA/Ab complex by centrifugation was suspended in Assay Buffer (100 μL) and the solutions were added to the wells pre-treated with Blocking Buffer. After further incubation for 1 h at 25 °C, the mixture solutions were rinses with PBST (3×300 μL) and the RNase H reaction solutions (100 μL) were added and incubated for 30 min at 37°C. The fluorescence intensities of the reaction mixtures were measured by Appliskan (Thermoscientific, Walthan, MA, USA) with excitation/emission filter sets of 485/535 , 544/589 and 633/675 nm. All assays were performed in triplicates.

Multiplex rGNP-OLISA

Multiplex rGNP-OLISA procedure is similar to the single rGNP-OLISA except for the steps of coating with anti-mouse IgG (8 μg mL^{-1}). Assays were carried out using mixed solutions of three target Abs (anti-HIV Ab, anti-HCV Ab, anti-HBV Ab) at varying concentrations, a mixed solution of three Ag-GNP-DNA conjugates (Ag_{HIV}-GNP-DNA1, Ag_{HCV}-GNP-DNA2 and Ag_{HBV}-GNP-DNA3) specific to the markers, and a mixed solution of three RNA probes in the same manner as described in the rGNP-OLISA for a single analyte.

Serum spiking test

To evaluate the analytical performance of the multiplex GNP-OLISA in clinical applications, the precision (%CV) and accuracy (%Recovery) were determined spiked with a mixture of 3 targets in human sera. The serum samples were prepared by diluting the healthy human serum 1/20 with an Assay buffer and target spike concentrations were 2, 4, 8 ng mL^{-1}. The spiked samples were analyzed via the developed multiplex rGNP-OLISA and calculations of CV and recovery.

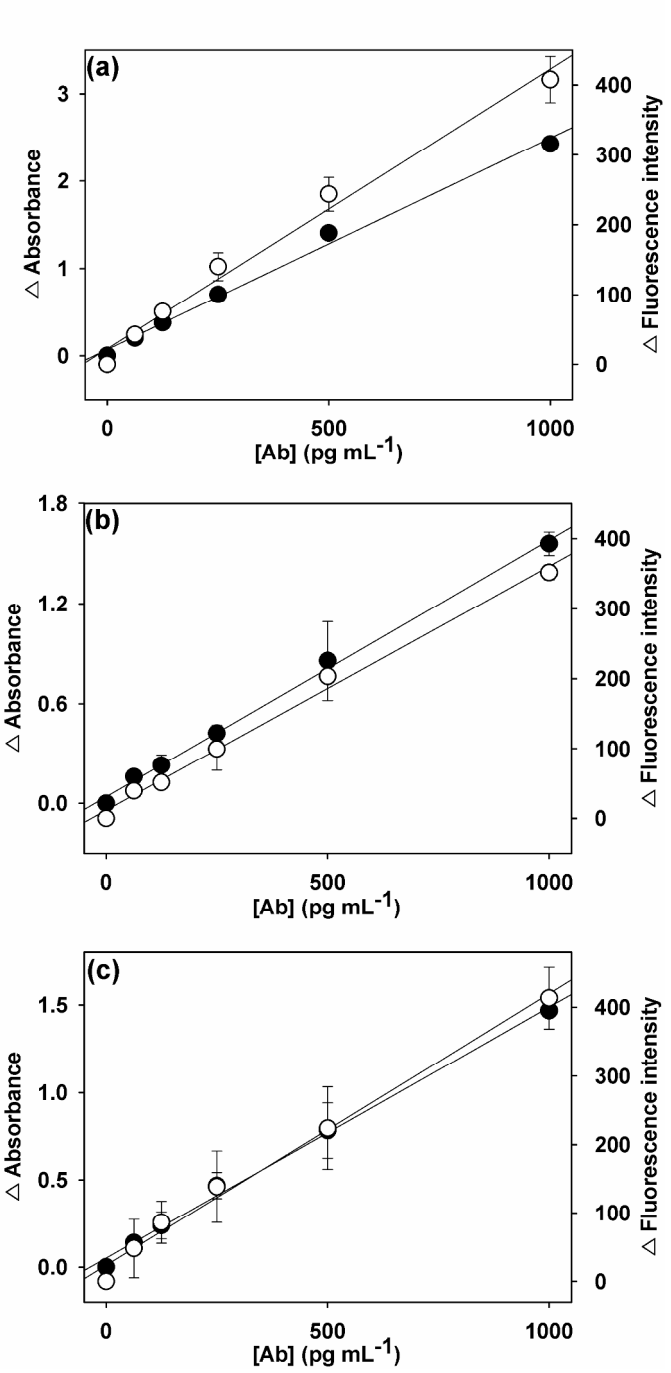


Figure 1. Comparison of single rGNP-OLISA with indirect ELISA for (a) anti-HIV Ab, (b) anti-HCV Ab, and (c) anti-HBV Ab. Each Ab was independently quantified by using single rGNP-OLISA (open circles) based on fluorescence intensity and indirect ELISA (closed circles) based on absorbance, respectively.

Results and discussion

Single rGNP-OLISA

Before executing rGNP-OLISA, Ag-GNP-DNA complexes should be prepared properly for stable signal production upon detection of the target Abs by Ags on GNP. For the preparation of the Ag-GNP-DNA complex, 15 nm GNPs were sequentially incubated with each Ag and 5-thiolated DNA, and then blocked by BSA. The Ag-GNP-DNA complex was then washed twice with 10 mM phosphate buffer (pH 8.0) containing 0.1% BSA. Coating GNPs with protein and DNA did not cause considerable aggregation as examined by absorption values of the nanoparticles before and after complexation (†ESI, Fig. S1). The number of DNA strands per GNP in the complex was calculated by dividing the concentration of bound DNA by the GNP concentration. The concentration of bound DNA strands was estimated on the basis of the standard curve obtained from RNase H reactions at various DNA concentrations. The number of DNA molecules per GNP was determined to be 24, 242 and 229 for HIV Ag, HCV Ag, and HBV Ag, respectively. After preparation of Ag-GNP-DNA complexes, rGNP-OLISA was carried out for a single kind of Ab to examine whether the method could be feasibly used to detect the target molecules. Anti-IgG was coated on wells of an opaque microplate, and then serially diluted biomarker solutions were added into each well after the blocking step. The Ag-GNP-DNA complex solution prepared in advance was incubated at varying concentrations of each target Ab. After removing unbound molecules by centrifugation, the precipitated Ag-GNP-DNA complex was resuspended in Assay Buffer and added in to each microplate well followed by the RNase H-mediated signal generation step.

As shown in Fig. 1a (open circles), the fluorescence intensity produced by the RNase H reaction was linearly proportional to each Ab concentration in the range from 62.5 to 1000 pg mL^{-1}. LOD values determined on the curves were 102 pg mL^{-1} for anti-HIV Ab, 122 pg mL^{-1} for anti-HCV Ab, and 88 pg mL^{-1} for anti-HBV Ab, respectively. To compare these results obtained by single rGNP-OLISA with those by indirect ELISA, the conventional assay was also performed to construct the quantitation curves, and LOD values were determined for the three Abs (closed circles in Fig. 1b and Table 1) based on the curves. The LOD values obtained in single rGNP-OLISA were very closes to those in indirect ELISA, indicating that rGNP-OLISA could be used for quantitative analysis of the target Abs.

Table 1. LOD values for Abs determined in single rGNP-OLISA and ELISA.

Target	Limit of detecton (LOD, pg mL^{-1})	
	rGNP-OLISA	ELISA
Anti-HIV Ab	102	102
Anti-HCV Ab	122	70
Anti-HBV Ab	88	88

Multiplex rGNP-OLISA

After observing successful performance of rGNP-OLISA for quantitative detection of each single kind Ab, a multiplex assay was examined for simultaneous detection of the three different Abs in a buffer solution. For the multiplex assay, a mixture of three kinds GNP complexes in which each complex labelled with a different DNA barcode (Ag_{HIV}-GNP-DNA1, Ag_{HCV}-GNP-DNA2, and Ag_{HBV}-GNP-DNA3) was used to capture target Abs. The same assay protocol used for single rGNP-OLISA was again adopted for the multiplex version. Since distinguished signals for each Ab were required, we employed a mixture of three fluorogenic RNA probes, of which each probe was labeled with a fluorophore having different excitation and emission wavelengths for multiplex detection. As the result, multiplex rGNP-OLISA for three Abs was in good agreement with the data obtained in the single Ab detection assay, providing the quantitation curves with good linearity (Fig. 2). The LOD values were 114 for anti-HIV, 110 for anti-HCV, and 135 for HBV, respectively (Table 2), which were comparable to LODs found in single rGNP-OLISA and ELISA (Table 1). These results illustrate that multiplexing of the assay could be achieved with preserving detection sensitivity of the assay. The LOD values of rGNP-OLISA were slightly better than those of other multiplex assay systems based on microarray providing 1 – 3 ng/mL of LOD for detection of various IgG antibodies.[21-22]

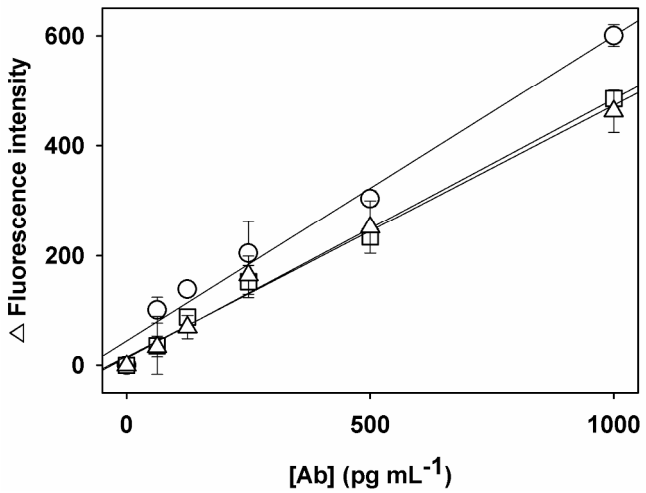


Figure 2. Quantitation curve obtained for (a) anti-HIV Ab (circles), (b) anti-HCV Ab (squares), and (c) anti-HBV Ab (triangles) by using rGNP-OLISA.

Table 2. Limit of detection values of multiplex rGNP-OLISA for Abs.

Target	Anti-HIV Ab	Anti-HCV Ab	Anti-HBV Ab
LOD (pg mL^{-1})	114	110	135

Specific signal increase should be accomplished for development of a viable multiplex immunoassay method. When specificity of the detection signal for each target Ab was examined by monitoring whether a false-positive signal in the absence of the target Abs appears, the fluorescence signal was produced only by the presence of the corresponding target Ab. The other fluorescence emission signals at different wavelengths for indication of the presence of the other Abs were negligible. The LOD values determined were also nearly unaffected by the absence of Abs other than the target. These results suggest that multiplex rGNP-OLISA system provides reliable specificity of detection signal.

To further examine whether the performance of rGNP-OLISA is suitable for clinical analysis, we determined the precision (%CV) and the accuracy (%Recovery) values of the method for Abs spiked in human sera. Spiked serum samples were prepared by addition of the three Abs into healthy human serum and analyzed by means of multiplex rGNP-OLISA. As

summarized in Table 3, the %CV values were less than 10% in all three Abs at tested concentrations, indicating that rGNP-OLISA is a potentially applicable method for clinical uses. The accuracy values of multiplex GNP-OLISA as expressed in %Recovery ranged from 94.7 to 104.7% and also showed reasonable accuracy for practical applications.

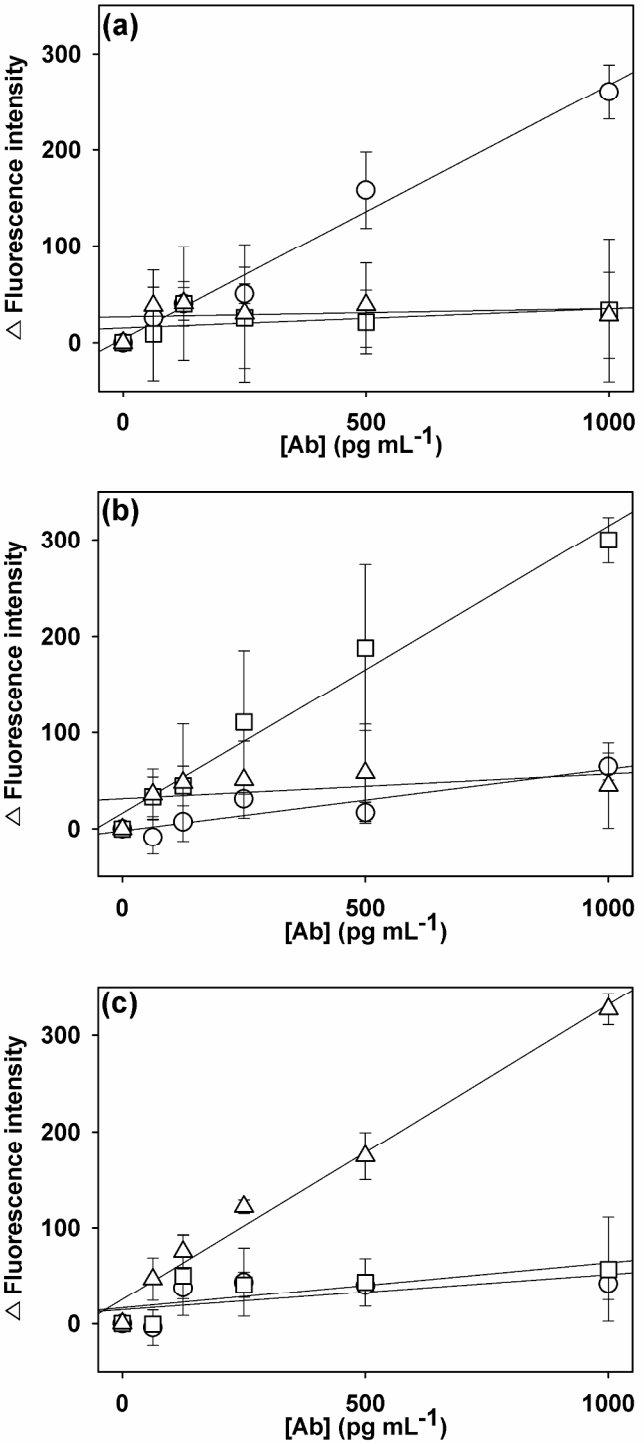


Figure 3. Specific generation of detection signal for each target Ab in rGNP-OLISA. Quantitation curve obtained for (a) anti-HIV Ab (circles), (b) anti-HCV Ab (squares), and (c) anti-HBV Ab (triangles) by using rGNP-OLISA.

Table 3. Precision (%CV) and accuracy (%Recovery) of multiplex rGNP-OLISA.

Norminal concentration (ng mL^{-1})	2	4	8	2	4	8
	%CV			%Recovery		
Anti-HIVAb	3.8	3.5	3.9	94.7	103.5	96.5
Anti-HCVAb	7.8	4.9	2.2	104.7	101.2	94.9
Anti-HBVAb	6.0	5.6	3.7	95.3	101.8	99.3

Conclusions

In this study, multiplex detection of Abs to three viruses has been successfully demonstrated in a microwell plate-based immunoassay method, called rGNP-OLISA. The detection sensitivity of single rGNP-OLISA was comparable to that of ELISA. When multiplex detection of three Abs against virus Ags was attempted with the method, quantitative detection profiles of the three targets could be acquired without sacrificing the sensitivity, the accuracy and the reproducibility of the single assay to detect each Ab. In addition to convenient protocol for conjugation of Ags with signal producers, GNPs provide simple procedures for separation of target Ab-bound GNP complexes by centrifugation, which is a critical step for generation of a multiplexed signal. Further, reliability and robustness of the method have been successfully demonstrated for analysis of Abs spiked in human serum. We therefore expect that multiplex rGNP-OLISA presented here can be potentially useful for identifying individuals infected with various pathogenic organisms more efficiently.

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Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1. F. Fabrizi, A.F. de Vecchi, G. Como, G. Lunghi and P. Martin, *Aliment. Pharmacol. Ther.*., 2005, **21**, 861-869.
2. M.E. Roland, T.A. Elbeik, J.O. Kahn, J.D. Bamberger, T.J. Coates, M.R. Krone, M.H. Katz, M.P. Busch and J.N. Martin, *J. infect. Dis*., 2004, **190**, 598-604.
3. E. Munson, D. Wenten, P. Phipps, R. Gremminger, M.K. Schuknecht, M. Napierala, D. Hamer, R. Olson, R.F. Schell and J.E. Hryciuk, *J. Clin. Microbiol*., 2013, **51**, 1855-1860.
4. H. Altunay, E. Kosan, I. Birinci, A. Aksoy, K. Kirali, S. Saribas, M. Aslan, P. Yuksel, E. Alan, O.S. Yenen and B. Kocazeybek, *Transfus. Apher. Sci*., 2010, **43**, 265-268.
5. V. Rao, F. Fabrizi, P. Pennell, E. Schiff, M. de Medina, J.R. Lane, P. Martin and L. Ivor, *Ren. Fail*., 2010, **32**, 721-726.
6. L.F. Botelho-Souza, Ade. O. dos Santos, L.M. Borzacov, E.R. Honda, J.M. Villalobos-Salcedo and D.S. Vieira, *J. Virol. Methods*, 2014, **197**, 19-24.
7. H. Nuriya, K. Inoue, T. Tanaka, Y. Hayashi, T. Hishima, N. Funata, K. Kaji, S. Hayashi, S. Kaneko and M. Kohara, *J. Clin. Microbiol*., 2010, **48**, 3843-3851.
8. S. Nakamoto, T. Kanda, Y. Yonemitsu, M. Arai, K. Fujiwara, K. Fukai, F. Kanai, F. Imazeki and O. Yokosuka, *Scand. J. Gastroenterol*., 2009, **44**, 872-877.
9. C. Sarrazin, A. Dragan, B.C. Gärtner, M.S. Forman, S. Traver, S. Zeuzem and A. Valsamakis, *J. Clin. Virol*., 2008, **43**, 162-168.
10. V.P. Le, Y.C. Jung, K.S. Kang, I. Lim, S.C. Myung and W. Kim, *J. Med. Virol*., 2010, **82**, 1065-1070.
11. H. Mine, H. Emura, M. Miyamoto, T. Tomono, K. Minegishi, H. Murokawa, R. Yamanaka, A. Yoshikawa, K. Nishioka and Japanese Red Cross NAT Research Group, *J. Virol. Methods*, 2003, **112**, 145-151.
12. N. Komatsu, S. Shichijo, M. Nakagawa and K. Itoh, *Scand. J. Clin. Lab. Invest.,* 2004, **64**, 535-546.
13. B. Gilburd, M. Abu-Shakra, Y. Shoenfeld, A. Giordano, E.B. Bocci, F. delle Monache and R. Gerli, *Clin. Dev. Immunol*., 2004, **11**, 53-56.
14. J.W. Pickering, T.B. Martins, R.W. Greer, M.C. Schroder, M.E. Astill, C.M. Litwin, S.W. Hildreth and H.R. Hill, *Am. J. Clin. Pathol*., 2002, **117**, 589-596.
15. J.W. Pickering, T.B. Martins, M.C. Schroder and H.R. Hill, *Clin. Diagn. Lab. Immunol*., 2002, **9**, 872-876.
16. I.H. Khan, S. Mendoza, J. Yee, M. Deane, K. Venkateswaran, S.S. Zhou, P.A. Barry, N.W. Lerche and P.A. Luciw, *Clin. Vaccine Immunol*., 2006, **13**, 45-52
17. T. Inoue, T. Kawaji and H. Tanihara, *Invest. Ophthalmol. Vis. Sci..,* 2013, **54**, 5353-5358.
18. Z. Wang, N. Liu and Z. Ma, *Biosens. Bioelectron*., 2014, **53**, 324-329.
19. K.C. Han, D.R. Ahn and E.G. Yang, *Bioconj. Chem*., 2010, **21**, 2190-2196.
20. K.C. Han, E.G. Yang and D.R. Ahn, *Chem. Commun.*, 2012, **48**, 5895-5897.
21. B.B. Haab, M.J. Dunham and P.O. Brown, *Genome. Biol.,* 2001, **2**, research0004.1-0004.13.
22. L. Duan, Y. Wang, S.S. Li, Z. Wan and J. Zhai. *BMC Infec.. Dis.*, 2005, **5**, 53.

Graphical Abstract