

Detection of avian influenza virus by fluorescent DNA barcode-based immunoassay with sensitivity comparable to PCR†

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In this paper, a coupling of fluorophore-DNA barcode and bead-based immunoassay for detecting avian influenza virus (AIV) with PCR-like sensitivity is reported. The assay is based on the use of sandwich immunoassay and fluorophore-tagged oligonucleotides as representative barcodes. The detection involves the sandwiching of the target AIV between magnetic immunoprobes and barcode-carrying immunoprobes. Because each barcode-carrying immunoprobe is functionalized with a multitude of fluorophore-DNA barcode strands, many DNA barcodes are released for each positive binding event resulting in amplification of the signal. Using an inactivated H1N3 AIV as a model, a linear response over five orders of magnitude was obtained, and the sensitivity of the detection was comparable to conventional RT-PCR. Moreover, the entire detection required less than 2 hr. The results indicate that the method has great potential as an alternative for surveillance of epidemic outbreaks caused by AIV, other viruses and microorganisms.

Introduction

Avian influenza viruses (AIV), belonging to the *Orthomyxoviridae* family, have attracted global concern due to the potential pandemic threat for human health and enormous economic losses. It has killed millions of poultry and hundreds of people not only in Asia but also throughout Europe and Africa.^{1–3} To control the epidemic diseases, there has been a surge of interest in sensitive, specific and rapid detection of AIV.

Several decades ago, traditional viral detection methods such as Madin-Darby canine kidney (MDCK) cell culture,⁴ complement fixation (CF),⁵ or hemagglutinin-inhibition (HI)⁶ were mostly used. These approaches are laborious and time-consuming (e.g., up to 4–10 days for the viral culture). Furthermore, in some cases the techniques are insufficient due to lack of specificity. Recently, reverse transcriptase PCR (RT-PCR) has emerged as the most sensitive method for AIV detection and pathotyping.^{3,7} RT-PCR involves an extraction of viral nucleic acids (RNA), an *in vitro* reverse transcription process to synthesize cDNA from the viral RNA, followed by an enzymatic amplification and detection of the cDNA. Although very sensitive, specific, and much faster than the aforementioned procedures, RT-PCR also has some drawbacks such as a complicated procedure, high cost, and high false positive rate arising from cross contaminations between samples.

Furthermore, it still requires a day and experienced personnel to obtain results.

Immunoassays could be an alternative for detection of AIV *via* immunoreactions between surface antigens (nucleoproteins, matrix proteins) of the AIV and their developed antibodies. Many studies have been devoted to the development of enzyme-linked immunosorbent assay (ELISA),^{7–10} immunochromatographic strip test,¹¹ or microsphere immunoassay (MIA) for detection of AIV.¹² Although these immunoassays have been shown to be rapid, inexpensive to perform and possible to automate, they have lower sensitivity compared to those obtained from RT-PCR.⁸ Bio-barcode immunoassay, proposed by Nam *et al.*, is the only approach for detection of biological targets that can obtain PCR-like sensitivity without the enzymatic amplification.^{13,14} Typically, the bio-barcode immunoassay utilizes two types of particles: (1) a magnetic microparticle (MMP) functionalized with antibody (primary antibody) which is to capture and isolate the target analyte from the sample solution, and (2) another particle (gold nanoparticle, polystyrene or silica microparticle) anchored with secondary antibodies, which is specific to the same target, and double-stranded DNA.^{13–16} Only one strand of the double-stranded DNA is covalently immobilized onto the secondary particle probe, and after the sandwiching immunoreaction, the complementary DNA strand can easily be released by increasing the temperature. The DNA surrogates for the target of interest and is therefore called a DNA bio-barcode. The surrogate DNA bio-barcode can subsequently be detected by PCR, DNA microarrays, colorimetric assays, or fluorophore-based assays. Unlike other conventional sandwich immunoassays, where the signal intensity is limited by number of antigenic valences for specific binding of reporter-tagged antibody, each particle is functionalized with a multitude of DNA strands and thus many DNA barcodes are released for each positive binding event resulting in amplification of the assay. Although the bio-barcode amplification assays have

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† Electronic Supplementary Information (ESI) available: Compared images before and after dissociating Cy5-biobarcode from the immunocomplexes; and high resolution image of the Cy5-DNA barcode arrays. See DOI: 10.1039/b916821b

been the focus of many studies for detection of oligonucleotides and proteins, there have been no publications dedicated to barcodes for detection of pathogenic organisms. In this paper, we will address a detection strategy that incorporates the benefits of fluorophore-DNA barcodes as a representatively optical reporter with the use of bead-based immunoassay for the detection of AIV. The method has not only a wide dynamic range of detection but also a sensitivity equivalent to that of RT-PCR which outperforms all detection limits of immune-based tests so far. This, in combination with the shorter detection time and possibility of adapting the method for detection of other viruses or microorganisms, make it a potential tool for surveillance of infectious diseases.

Materials and methods

Virus strain

An inactivated AIV virus subtype H16N3 was used as a model for developing the method. The AIV strain was propagated in SPF chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) and harvested from the allantoic fluid. The virus sample was prepared and titrated by Haemagglutinin (HA) test at a concentration of 1:128 according to the guidelines of the European Union (EU) Council Directive 92/40/EEC.¹⁷ A Newcastle Diseases Virus (NDV) Ulter strain was used to test the specificity of the assay. Both of the virus strains were kindly prepared by Poultry Virus Laboratory, National Veterinary Institute, Technical University of Denmark (DTU-VET).

Chemicals

Magnetic microparticle (Dynabeads[®] M-270 Amine, diameter 2.8 μm) was purchased from Invitrogen Dynal AS (Oslo, Norway). Polystyrene microparticle (Polybead[®] amino microsphere, diameter 1 μm), glutaraldehyde kit, and BioMag[®]Plus amine protein coupling kit were supplied by Polysciences Europe GmbH, Germany. Influenza type A virus nucleo-protein monoclonal antibody (NP mAb) and influenza type A virus matrix-protein monoclonal antibody (MP mAb) were purchased from Statens Serum Institut, Denmark. Oligonucleotide sequences were obtained from TAG Copenhagen A/S, Denmark; primers used in RT-PCR were obtained from DNA Technology, Aarhus, Denmark (Table 1). Other essential chemical reagents were of analytical grade and supplied by Pierce, Sigma-Aldrich, or Fluka unless otherwise stated. Ultrapure water (18.2 $\text{m}\Omega/\text{cm}$) produced by a Millipore Milli-Q system was used to prepare the chemical solutions when needed.

Table 1 List of primers and oligonucleotide sequences used in this study

Oligonucleotides	Sequence (5' → 3')
Oligo 1	Amino-C6-AAAAAAAAAA AGG AAG GTG TGG ACG ACG TCA AGT CAT CAT GGC C
Oligo 2	Cy5-C GCC ATG ATG ACT TGA CGT CGT CCA CAC CTT CCT
Forward primer	AGA TGA GTC TTC TAA CCG AGG TCG
Reverse primer	TGC AAA AAC ATC TTC AAG TCT CTG

Preparation of magnetic microparticle immunoprobe

The amino-functionalized magnetic microparticle (MMP) was coated covalently with the NP mAb by using a BioMag[®]Plus amine protein coupling kit according to the manufacturer's protocol and a procedure reported previously with some modifications.¹⁸ Briefly, 100 μl of MMP solution containing 2×10^8 beads (approximately 3 mg) was equilibrated and washed intensively 2 times with a mixture of 3 ml EDTA (0.05 mM) and 6 ml pyridine wash buffer (0.01 M, pH 6). The MMPs were separated after each washing step by a MultiSep magnetic separator (Polysciences Inc.). Then, 3 ml of 5% glutaraldehyde was added to activate the MMPs for about 3 h at room temperature while shaking vigorously. After the activation, the MMPs were washed two times, and suspended in 6 ml pyridine wash buffer. 1 ml of NP mAb (80 $\mu\text{g}/\text{ml}$) was added to the glutaraldehyde-activated MMP suspension; the mixture was gently mixed and incubated for about 10 hr at room temperature to ensure covalent coupling, followed by the washing step with 6 ml pyridine wash buffer. Afterwards, 3 ml BSA (0.2 mg/ml) was added to the solution for an additional 5 hr to block the non-specific adsorption of other proteins. The unreacted aldehyde sites were subsequently deactivated by 10 ml of 1 M glycine solution (pH 8) for 1 hr at room temperature. It should be noted that the washing step with pyridine buffer was repeated after each reaction to eliminate any remaining free reactants. The resulting MMP immunoprobes were then suspended in 1 ml of 0.15 M PBS solution and stored at 4 $^{\circ}\text{C}$ for up to 1 month without loss of activity.

Preparation of polystyrene microbead immunoprobe (PMP)

The experimental steps for the preparation of PMP immunoprobes were carried out by covalently immobilizing MP mAb and barcode DNA complementary strand (Oligo 1) onto the Polybead[®] amino microsphere by using the glutaraldehyde kit.¹⁹ In brief, after washing and normalizing 1 ml of PMPs (approximately 2.28×10^{10} beads/ml) by PBS buffer, the PMPs were separated from the supernatant by centrifuging at 10 000 rpm for 10 min. Then, the PMP pellet was resuspended in 1 ml PBS buffer and reacted with 1 ml of 8% glutaraldehyde for 5 hr at room temperature. The reaction was performed in a 5 ml vial and stirred gently by a magnet bar to avoid settlement of the PMPs. The activated PMPs were centrifuged, washed, and resuspended in 1 ml PBS as described above. 10 μl of MP mAb (1 mg/ml) was added to the solution to conjugate with PMPs and incubated for 2 hr at room temperature with gentle stirring. Afterwards, 200 μl of 100 μM barcode DNA complementary strand (Oligo 1) was added continuously while stirring gently, and left to react overnight. The beads were washed 2 times with PBS buffer and unreacted glutaraldehyde sites of the PMP immunoprobes were deactivated by mixing with 1 ml of 0.2 M ethanolamine. The supernatant was removed by centrifuging at 13 000 rpm for 10 min, and 1 ml of 10% BSA was added to further block the unbound regions of the particle surface (30 min incubation). After washing with PBS and centrifugation, the resulting particle pellet was resuspended in 2 ml PBS buffer. Finally, 200 μl of 100 μM DNA barcode (Oligo 2) was added and incubated with the PMP immunoprobe for 1 hr. The barcode PMP

immunoprobe was washed and collected by centrifugation and resuspended in 2 ml of storage buffer. The final PMP probe was stored at 4 °C in a dark container to reduce photobleaching of the fluorophore. By using this method, the average numbers of mAb molecules and barcode DNA strands were respectively determined at about 1457 and 1.45×10^4 per polystyrene particle which are slightly lower than those reported previously.¹⁹

Immunoreaction and optical detection

In a typical experiment, a sandwiched immunoassay of MMP immunoprobe/AIV/PMP immunoprobe was carried out, and then the DNA bio-barcodes were collected by a magnetic separation and thermal dehybridization. Briefly, 30 μ l of MMP immunoprobe was incubated with 40 μ l of various 10-fold dilutions of the inactivated H1N3 with a HA titre of 1:128 in an Eppendorf tube. The reaction was kept for 30 min at room temperature on a rotating mixer. 30 μ l of PMP immunoprobe was added and incubated for 30 min. When the immunoreaction had finished, the MMP immunoprobe/AIV/PMP immunoprobe complexes were separated in the magnetic separator, and the supernatant was removed. The reacted complexes were washed 3 times with 0.15 M PBS buffer, and 30 μ l of pure and DNA-free water was added to the magnetically collected pellet. Finally, to release the surrogate DNA barcodes, the complexes were heated to 75 °C for 15 min, immediately followed by magnetic separation. The upper aqueous solution containing the released

surrogate DNA barcode strands was collected and spotted onto a microscope slide by using a non-contact inkjet Nano-plotter 2.1 (GeSiM, Germany). Fluorescence measurement was carried out by a scanner ScanArray Lite (Packard Bioscience, USA) with appropriate settings for the Cy5 fluorophore.

RT-PCR

Detection of AIV by RT-PCR for matrix gene (M1) was performed according to a procedure published elsewhere with some modifications.^{7,20} 25 μ l RT-PCR reaction contained 12 μ l of RNase free water, 5 μ l RT-PCR buffer, 10 pmol of each primer (Table 1),²⁰ 1 μ l of dNTP, 1 μ l of enzyme mix and 5 μ l of viral sample. RT-PCR was performed in a thermal cycler (Biometra, Germany) with reverse transcription at 56 °C for 30 min and enzyme activation at 95 °C for 15 min followed by 40 cycles with denaturation at 94 °C for 30 s, annealing 58 °C for 1 min, and extension at 72 °C for 1 min. The final extension step was carried out at 72 °C for 7 min. The obtained RT-PCR products were analysed using 1.5% agarose gel electrophoresis.

Results and discussion

The experimental procedure of the fluorescence-DNA barcode-based immunoassay is depicted in Fig. 1. The detection is initiated by sandwiching the target virus between the MMP immunoprobe and the PMP immunoprobe. After separation of the sandwich immunocomplex by a magnetic field, the surrogate

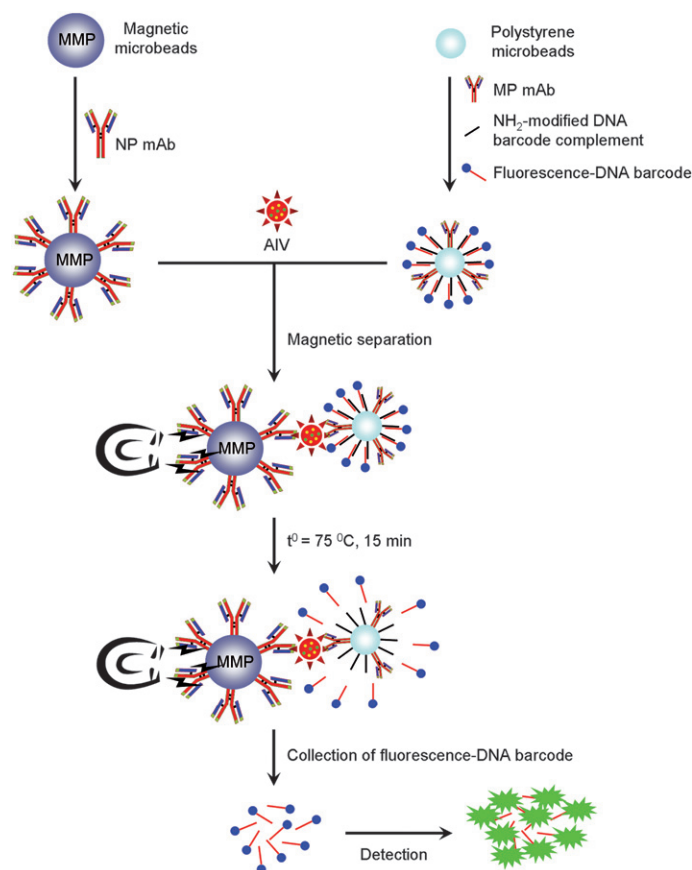


Fig. 1 Overall scheme illustrating procedure of the fluorophore-DNA barcode-based immunoassay for detection of AIV.

fluorophore-DNA barcodes are released by heating the immunocomplex at 75 °C for 15 min, and collected. Because the barcode immunoprobe particle can carry thousands of the representative barcodes, a single antibody-antigen binding event is translated to a multitudinous number of surrogate DNA barcodes leading to amplification of the assay. The fluorophore-DNA barcodes are then detected by any fluorescence readout available. In our lab, the most convenient way to detect the liberated DNA barcode strands was to spot them onto a microscope slide as microarrays following by a scanometric analysis, the entire detection required less than 2 hr to complete. It should be noticed that the Cy5-DNA barcode can be printed and measured directly without the dissociation step from the immunocomplex; however, due to the micro-sized nature of the microbeads the fluorescence was not dispersed leading to the fluctuation of the final results. Furthermore, the 3-D shape of the microbeads could hinder the Cy5-DNA barcodes located underneath leading to deterioration of the signal. To obtain more uniform fluorescent spot, the Cy5-barcode was released from the particles by heating and the results showed that the spot homogeneity was much better (See Fig. S1, ESI†). Therefore, the dissociation step is required in the protocol.

There are many factors that may limit performance of the DNA barcode-based immunoassay. These factors include the type of particles or antibodies, attachment chemistry, effectiveness of removal and washing steps, *etc.* Among them, immunological affinity of the immunoprobes towards their target analyte plays the most important role; it could be a key factor to determine the success or failure of the whole assay and their immunological activity could be deteriorated after the chemical immobilization. It is therefore necessary to ensure that the immunoprobes are able to capture the virus after enduring the chemical conjugation process. To determine this, conventional RT-PCR was exploited as a straightforward approach to validate the usefulness of the immunoprobes: The MMP and PMP immunoprobes were successively incubated with 10^{-3} dilution of the HA-titrated H16N3, and the captured viruses were collected by magnetic separation or centrifugation (in case of the PMP immunoprobes) as described in the method section. After the incubation period, the samples were washed several times with 1 ml of PBS buffer with 0.05% Tween 20 (pH 7.4). Finally, 50 μ l DNA-free water was added to dissolve the collected complexes for the RT-PCR analysis. False-positive or false-negative results could be obtained if the washing step is not performed adequately in any immunoassays. For this reason, the washing supernatants were also collected in 1 ml aliquots to be analyzed with RT-PCR. Fig. 2A shows the gel electrophoresis image of the RT-PCR products. As seen, both of the MMP and PMP immunoprobes were PCR positive, and neither secondary product nor contamination was observed. Moreover, both washing solutions were virus-free after 4 times of washing as shown by PCR negative signals in lane 3 and 5, indicating that the positive bands (lane 2 and 4) resulted solely from specific interactions between the virus and the immunoprobes. A number of viruses were still observed in the washing solution until the third washing step (data not shown). This could be because of blocking effect of the microbeads or non-specific interactions of the beads and the viruses, and this might contribute to a false-positive result of the assay if the washing step is not carried out

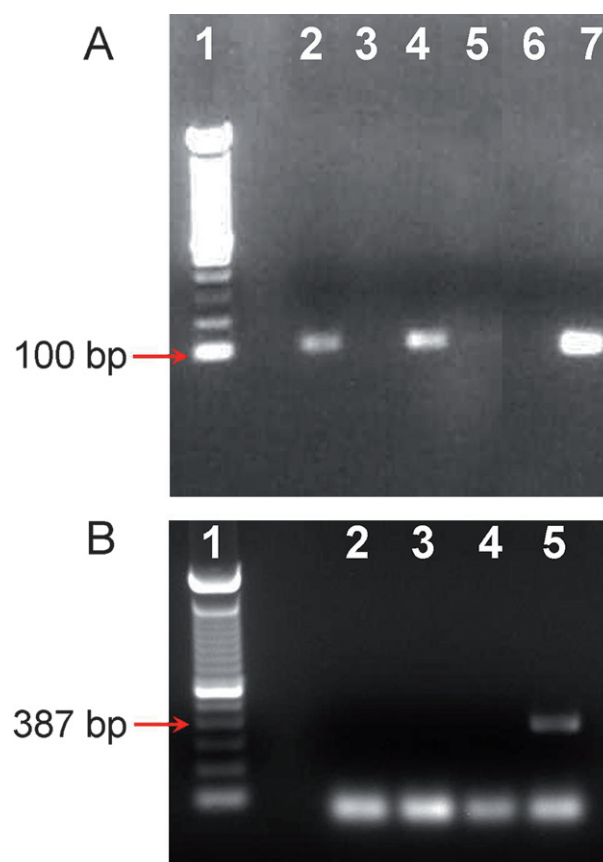


Fig. 2 (A) Agarose gel electrophoresis image illustrating validation of the immunoprobes by RT-PCR for H16N3 AIV (HA titers = 1:128). On gel, lane 1: 100 bp DNA ladder (Qiagen); lane 2: MMP immunoprobe sample (virus dilution 10^{-3}); lane 3: Washing solution of MMP immunoprobe sample (4^{th}); lane 4: PMP immunoprobe sample (virus dilution 10^{-3}); lane 5: Washing solution of PMP immunoprobe sample (4^{th}); lane 6: Negative control (virus free); lane 7: Positive control (virus dilution 10^{-3}). (B) Investigation of non-specific binding of Newcastle Disease Virus (NDV, HA titers = 1:128) on the immunoprobes. On gel, lane 1: DNA ladder; lane 2: NP mAb coated magnetic microbead sample (virus dilution 10^{-2}); lane 3: MP mAb coated magnetic microbead sample (virus dilution 10^{-2}); lane 4: Negative control (virus free); lane 5: Positive control (virus dilution 10^{-2}).

carefully. Non-specific interaction of the immunoprobes with other viruses is also important information that should be elucidated to evaluate the specificity of the immunoprobes. Newcastle Disease Virus (NDV) was used as the control virus because NDV is the viral infectious disease that is closely related to AIV in poultry. Briefly, NP mAb and MP mAb were conjugated to the magnetic beads. The immunoreactions with NDV were performed as described above. Subsequently, the immunocomplex was collected by a magnetic force; the captured NDV was then determined by RT-PCR and gel electrophoresis that were well-established at DTU-VET.²¹ As shown in Fig. 2B, both of the PCR products obtained from NP mAb and MP mAb coated microbeads were negative indicating that the non-specific binding of NDV was negligible. Overall, this result shows how many times the washing step is required, and indicates that the immunoprobes were prepared successfully; they are sufficient and specific for catching the AIV in the next experiments.

To realize the principle illustrated in Fig. 1, a practical experiment was implemented to detect the AIV subtype H16N3. A series of 10-fold dilutions of the virus were prepared and incubated with the immunoprobes. Fig. 3A illustrates that the spot intensities were intuitively differentiated and proportional to a certain range of viral dilutions (see Fig. S2 (ESI) for high resolution image of the arrays†). The proportional relation was observed on all 3 replicates of the experiment. More quantitatively, Fig. 3B shows the generalized logarithmic correlation between the fluorescence values and the diluted concentrations of the virus. As seen, this method is able to reliably detect AIV with a very wide dynamic range of virus titres spanning from 1:128 up to $1:128 \times 10^{-5}$ (five orders of magnitude) in which the fluorescence intensities were proportional to a log scale of the viral dilutions. The spot intensities at the dilutions higher than $1:128 \times 10^{-5}$ were poorly discriminated because their signals did not exceed 2 or 3 times of standard deviation from the blank

measurement (negative control sample, horizontal line); the limit of detection (L.O.D) was therefore proposed to be approximately $1:128 \times 10^{-5}$ dilutions of the HA titre of the virus.

To further evaluate the effectiveness of the strategy, the same 10-fold dilutions of the virus samples were analyzed by conventional RT-PCR assay. The results of the fluorophore-DNA barcode-based detection of H16N3 AIV agreed remarkably well with the RT-PCR analysis. A proportional relationship was also obtained for the concentration range of 1:128 to $1:128 \times 10^{-5}$ by RT-PCR (Fig. 4). Viral concentrations lower than 10^{-5} of dilution could also not be detected by RT-PCR owing to the extremely low number of virus particles. Therefore, the results suggest that the fluorophore-DNA barcode-based immunoassay described in this study is as sensitive as RT-PCR, it could be an alternative for detection of AIV where RT-PCR is not available.

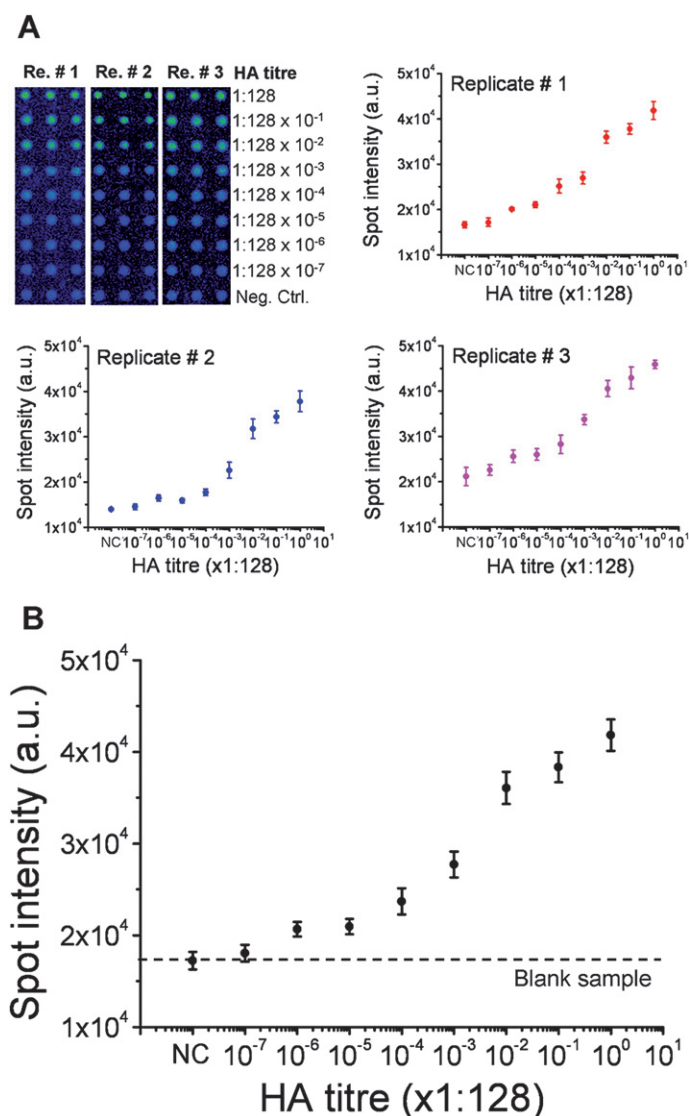


Fig. 3 Detection of fluorescent DNA barcodes. Scanometric image showing three replicated microarrays of surrogate Cy5-DNA barcode strands and their corresponding fluorescence intensity to the diluted concentrations of H16N3 AIV (A); and (B) generalized correlation of the average intensity and the diluted concentrations for the detection of H16N3 AIV (HA titre 1:128). NC negative control signal.

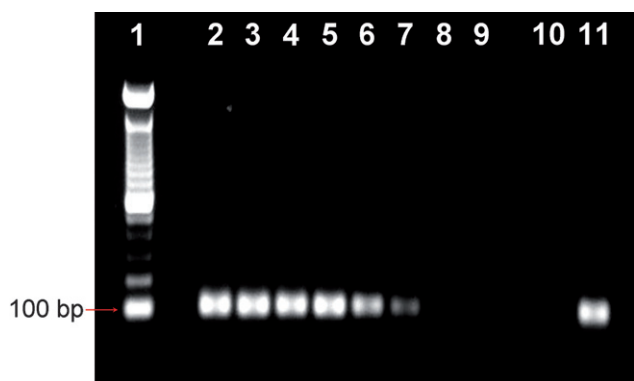


Fig. 4 Detection of H16N3 inactivated AIV (HA titers, 1:128) by RT-PCR. On gel, Lane 1: 100 bp DNA ladder (Qiagen); Lane 2–9: 10-fold dilutions of the virus pool ranging from the original HA titre 1:128 to 1:128 $\times 10^{-7}$, respectively; Lane 10: Negative control (virus free); Lane 11: Positive control. The virus strain was not recognized at dilutions higher than 1:128 $\times 10^{-5}$.

Conclusion

An effective method using fluorophore-DNA barcode combined with bead-based immunoassay was described for the first time to detect AIV. The single immuno-recognition events were translated into multitude numbers of the Cy5-tagged DNA barcodes which could be quantitatively measured by a fluorescence readout device. The H16N3 AIV strain could be detected with PCR-like sensitivity and a wide dynamic range of up to five orders of magnitude. Although simple in design and concept, the method introduced has PCR-comparably sensitive, is less time-consuming (less than 2 hr for the entire detection), highly applicable, and can be carried out using simple laboratory equipment. For these reasons, the assay deserves attention as an alternative for the surveillance and clinical detection of AIV outbreaks.

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Notes and references

- 1 S. Cui, C. Chen and G. Tong, *J. Virol. Methods*, 2008, **152**, 102–105.
- 2 D. J. Alexander, *Zoonoses Public Health*, 2008, **55**, 16–23.
- 3 J. Pasick, *Transboundary and Emerging Diseases*, 2008, **55**, 329–338.
- 4 S. R. Shih, K. C. Tsao, H. C. Ning, Y. C. Huang and T. Y. Lin, *J. Virol. Methods*, 1999, **81**, 77–81.
- 5 S. Sato, H. Ochiai and S. Niwayama, *J. Med. Virol.*, 1988, **24**, 395–404.
- 6 R. T. Schwarz and H. D. Klenk, *J. Virol.*, 1974, **14**, 1023–1034.
- 7 M. Munch, L. P. Nielsen, K. J. Handberg and P. H. Jorgensen, *Arch. Virol.*, 2001, **146**, 87–97.
- 8 S. Velumani, Q. Du, B. J. Fenner, M. Prabhakaran, L. C. Wee, L. Y. Nuo and J. Kwang, *J. Virol. Methods*, 2008, **147**, 219–225.
- 9 Q. He, S. Velumani, Q. Du, C. W. Lim, F. K. Ng, R. Donis and J. Kwang, *Clin. Vaccine Immunol.*, 2007, **14**, 617–623.
- 10 G. Sala, P. Cordioli, A. Moreno-Martin, M. Tollis, E. Brocchi, A. Piccirillo and A. Lavazza, *Avian Dis.*, 2003, **47**, 1057–1059.
- 11 S. Cui and G. Tong, *Journal of Veterinary Diagnostic Investigation*, 2008, **20**, 567–571.
- 12 D. Deregt, T. L. Furukawa-Stoffer, K. L. Tokaryk, J. Pasick, K. M. B. Hughes, K. Hooper-McGrevy, S. Baxi and M. K. Baxi, *J. Virol. Methods*, 2006, **137**, 88–94.
- 13 J. M. Nam, S. I. Stoeva and C. A. Mirkin, *J. Am. Chem. Soc.*, 2004, **126**, 5932–5933.
- 14 J. M. Nam, C. S. Thaxton and C. A. Mirkin, *Science*, 2003, **301**, 1884–1886.
- 15 J. M. Nam, S. J. Park and C. A. Mirkin, *J. Am. Chem. Soc.*, 2002, **124**, 3820–3821.
- 16 Y. P. Bao, T. F. Wei, P. A. Lefebvre, H. An, L. X. He, G. T. Kunkel and U. R. Muller, *Anal. Chem.*, 2006, **78**, 2055–2059.
- 17 CEC, *Official Journal of the European Commission*, 1992, **L167**, 1–15, The Hemagglutination Assay (HA) is a quantification of viruses by hemagglutination, *i.e.* agglutination (binding together) of red blood cells. The highest dilution of virus causing complete agglutination of the red blood cells is defined as a HA titre unit.
- 18 J. M. Nam, K. J. Jang and J. T. Groves, *Nat. Protoc.*, 2007, **2**, 1438–1444.
- 19 B. K. Oh, J. M. Nam, S. W. Lee and C. A. Mirkin, *Small*, 2006, **2**, 103–108.
- 20 E. Spackman, D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez, *J. Clin. Microbiol.*, 2002, **40**, 3256–3260.
- 21 P. H. Jørgensen, K. J. Handberg, P. Ahrens, H. C. Hansen, R. J. Manvell and D. J. Alexander, *J. Vet. Med. B*, 1999, **46**, 381–387.