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## Ultrasensitive immuno-detection using viral nanoparticles with modular assembly using genetically-directed biotinylation

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### Abstract

We report a novel, modular approach to immuno-detection based on antibody recognition and PCR read-out that employs antibody-conjugated bacteriophage, easily-manipulated nonpathogenic viruses, as affinity agents. Our platform employs phage genetically tagged for *in vivo* biotinylation during phage maturation that can easily be linked, through avidin, to any biotinylatable affinity agent, including full-length antibodies, peptides, lectins or aptamers. The presence of analyte is reported with high sensitivity through real-time PCR. This approach avoids the need to clone

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antibody-encoding DNA fragments, allows the use of full-length, high affinity antibodies and, by having DNA reporters naturally encapsulated inside the bacteriophage, greatly reduces nonspecific binding of DNA. We validate the efficacy of this new approach through the detection of VEGF (Vascular Endothelial Growth Factor), a known angiogenic cancer biomarker protein, at attomolar concentrations in bronchoalveolar lavage (BAL) fluid.

## Keywords

Bacteriophage; biomarker; ELISA; immuno-PCR; immuno-phage assay

## Introduction

Over the last 30 years, improvements in the ultra-sensitive detection of nucleic acids through DNA amplification (e.g., PCR) have revolutionized diagnosis and research, but there is still no direct equivalent for equally-sensitive protein detection. Immuno-PCR reagents have been constructed from nanoparticles decorated with antibodies and DNA oligonucleotides, resulting in assays that require fewer steps to detection, and show a lower detection limit (Nam et al. 2003; Barletta et al. 2009; Thaxton et al. 2009; Malou et al. 2011).

Recently, a sensitive immunoassay for small molecule detection using phage-displayed peptides, which bind to antibody-analyte complexes in either ELISA-based (Kim et al. 2009; Kim et al. 2010) or real-time PCR-based (Kim et al. 2011) detection has been published. Here we report an improved modular approach to immuno-detection based on PCR that employs intact antibodies, (rather than single-chain variable fragment antibodies (scFvs), which must be cloned and which usually have lower affinity than the parent antibody), avidin-linked to biotinylated bacteriophage as affinity agents. These SAM-AviTag phage are derivatives of the *Escherichia coli* phage M13 where the N-terminus of the phage tail protein III contains the enzymatically-biotinylatable AviTag peptide (GLNDIFEAQKIEWHE) (Scholle et al. 2006). The lysine residue (K) in the AviTag is a substrate for biotinylation by the *E. coli* biotin ligase (*birA*) enzyme. Using streptavidin or NeutrAvidin, any biotinylated affinity agent (including full-length uncloned antibodies, aptamers, lectins, etc.) can then easily be linked to these enzymatically-biotinylated phage particles. We have recently demonstrated the great utility of these antibody-functionalized AviTag phage in a lateral-flow immuno-chromatographic assay (Adhikari et al. 2013) and here demonstrate their use in detecting Vascular Endothelial Growth Factor (VEGF) at attomolar concentrations in bronchoalveolar lavage (BAL) fluid in an assay based on PCR amplification of the phage DNA.

## Materials and Methods

### Materials

AviTag bacteriophage were a gift from Prof. Brian Kay at the University of Illinois at Chicago. De-identified bronchoalveolar lavage (BAL) fluid samples were provided through an Institutional Review Board (IRB) approved protocol at The Houston Methodist Research Institute, Houston, TX.

### Preparation of antibody reagents

Biotinylation of the antibody used in this study, Ranibizumab, a recombinant G1 $\kappa$  isotype antibody F<sub>ab</sub> fragment, MW: 48 kDa, (Chen et al. 1999), was performed using the EZ-Link Sulfo-NHS-SS-Biotin Kit as described in the manufacturer's protocol. The reaction was allowed to proceed for 1 h before unincorporated biotin was removed using 7 kDa Zeba spin desalting columns. The degree of biotinylation was estimated to be 1.5 biotins per antibody using 4'-hydroxyazobenzene-2-carboxylic acid (HABA).

### Capture particle functionalization

Magnetic capture particles (60  $\mu$ l,  $\sim 6 \times 10^9$  Dynabeads® MyOne (1  $\mu$ m), Tosyl activated, Life Technologies) were antibody-modified according to the manufacturer's protocol. The particles were washed, resuspended in coating buffer (1  $\times 10^7$  particles in 1 ml 0.1 M sodium borate, pH 9.5) and mixed with 240  $\mu$ l polyclonal anti-human Vascular Endothelial Growth Factor (VEGF) antibody (1 mg/ml, AB-293-NA, R&D Systems) and 240  $\mu$ l 3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. After 24 h incubation at 37 °C with slow tilt rotation, the particles were collected and resuspended in 500  $\mu$ l phosphate-buffered saline (PBS) containing 0.5% (w/v) Bovine Serum Albumin (BSA) and 0.05% (v/v) Tween 20, and incubated for 24 h at 37 °C. The particles were then washed three times with 1 ml PBS containing 0.1% (w/v) BSA and 0.05% (v/v) Tween 20, and finally resuspended in 200  $\mu$ l PBS containing 0.1% (w/v) BSA.

### Preparation of immuno-phage reagents

Growth and titer estimation of M13 phage were performed as previously described (Lee et al. 2007). Phage were found to be roughly 50% biotinylated during growth in *E. coli* BL21. This could be raised to nearly 100% by additional *in vitro* biotinylation using recombinant biotin ligase. The enzyme was expressed in inclusion bodies from cells (4 l) grown in the *Overnight Express Autoinduction System 1* (Novagen). The cells were harvested by centrifugation (30 min, 3,000 x g, 4 °C), washed in 50 ml TBS (20 mM Tris-HCl, pH 8, 150 mM NaCl), and resuspended in 150 ml resuspension buffer (20 mM Tris-HCl, pH 8, 10 mM  $\beta$ -mercaptoethanol). After a 1 h incubation at 4 °C with 0.1 mg/ml lysozyme and 1.25% (v/v) *Serratia* nuclease solution (Miller et al. 1991) the cells were sonicated for 5 min, and 2 ml 70% NP-40 solution, and 40 ml B-PER were added. After 1 h at 4 °C, the cell lysate was centrifuged (30 min, 16,000 x g, 4 °C), and the pellet containing the inclusion bodies was denatured using 7.5 M urea, 0.4 M L-arginine, 10 mM DTT, 50 mM Tris-HCl, pH 8, 500 mM NaCl. The inclusion bodies were refolded in 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 % (v/v) glycerol, 5% (v/v) sucrose, and applied to a chelating Sepharose column charged with 0.1 M NiSO<sub>4</sub>. The hexahistidine-tagged biotin ligase was eluted using an imidazole gradient (0.02-0.25 M). Peak fractions were checked for homogeneity using SDS-PAGE.

For *in vitro* biotinylation, 100  $\mu$ l of  $1 \times 10^{11}$  phage/ml were mixed with 14.3  $\mu$ l Bicine (0.5 M, pH 8.3), 14.3  $\mu$ l of a solution containing 100 mM ATP, 100 mM MgO(Ac)<sub>2</sub> and 500  $\mu$ M Biotin, 10  $\mu$ l D-biotin (500  $\mu$ M), and 10  $\mu$ l biotin ligase (2 mg/ml) in Tris-HCl, pH 8 and incubated for 1 h at 25°C. Phage were precipitated from the reaction by adding 250  $\mu$ l of 20% PEG in 2.5 M NaCl to 1 ml of biotinylated phage, followed by 1 h incubation on ice and centrifugation at 11,000 x g for 20 min. The phage pellet was resuspended in 1 ml PBS.

To prepare the NeutrAvidin/biotinylated phage construct, NeutrAvidin (A-2666, Life Technologies) and biotinylated phage were incubated at a molar ratio of 100:1 in 500  $\mu$ l PBS, pH 7.7 on a rotary mixer for 40 min at 25 °C. Unincorporated NeutrAvidin was removed using an Amicon 100 kDa centrifugal filter according to the manufacturer's instructions. Phage were collected in a final volume of 20  $\mu$ l, and were stored at 4 °C.

To prepare the antibody/NeutrAvidin/biotinylated phage construct, the biotinylated Ranibizumab and NeutrAvidin/biotinylated phage were mixed at a molar ratio of 10:1 in 500  $\mu$ l of PBS, pH 7.7, and incubated for 24 h at 25°C with continuous rotation. Free antibodies were removed using an Amicon 100 kDa filter as described above. The concentrated antibody/NeutrAvidin/biotinylated phage construct ( $1 \times 10^{11}$  phage constructs/ml) was stored at 4°C, and was diluted just prior to use.

### Immuno-phage assay format

Antibody-functionalized magnetic capture particles are added to the target solution, and, after a single wash step, the integrated immuno-phage reagent is added for detection (Fig. 1). 100  $\mu$ l VEGF at concentrations from 26 aM to 2.6 pM in 50% BAL fluid (in PBS) were mixed with magnetic particles (0.5  $\mu$ g,  $\sim 5 \times 10^6$ ) functionalized with the polyclonal anti-VEGF antibody, incubated on a shaker for 2 h at 25 °C and then washed three times with PBS. The reaction was blocked with 100  $\mu$ l 3% BSA in PBS. The antibody/NeutrAvidin/biotinylated phage construct was added to the reaction at 60 pM, and the reaction was allowed to incubate for 2 h at 25 °C on a shaker. After two washes with 0.3% Tween 20 in PBS to remove unbound phage and two washes with PBS, the particles were analyzed by PCR.

After the final wash of the captured immuno-phage constructs, the magnetic particles were resuspended in 20  $\mu$ l PBS. 15  $\mu$ l PCR master mix (0.1  $\mu$ l of 10  $\mu$ M forward primer, 0.1  $\mu$ l of 10  $\mu$ M reverse primer, 10  $\mu$ l of 2xPCR mix (Brilliant III Ultra-Fast SYBR mix, Agilent) and 4.8  $\mu$ l of RNase- and DNase-free DI water) were added to 5  $\mu$ l of each sample to achieve 20  $\mu$ l total PCR volume.

The number of retained phage particles was determined by PCR against a standard curve derived from a dilution series ( $10^{11}$  to  $10^5$  phage/mL). The AviTag-targeted PCR primers were as following: *Forward*: 5'-GTTGTTTCTTTCTATTCTCACT-3', and *Reverse*: 5'-CAGACGTTAGTAAATGAATTTT-3'. The PCR conditions were: 10 min at 95 °C, 40 cycles of 30 sec at 62 °C and 30 sec at 72 °C, followed by a dissociation step (1 min at 95 °C, 30 sec at 55 °C, and 30 sec at 95 °C).

The presence of the magnetic particles did not affect the PCR (at the levels used here); results were indistinguishable if analyte- and phage-loaded particles were added to the PCR reaction, or if the phage DNA was liberated from the particles by boiling and then added to the PCR.

## Results and Discussion

Here we present a novel approach to ultrasensitive immuno-detection using non-pathogenic bacteriophage, easily linkable to common affinity agents such as antibodies, and even to non-proteins such as aptamers or DNA probes. The principle of the method is shown in Fig. 1. Initially, antibody-functionalized magnetic capture particles are used to collect and concentrate the analyte from solution. M13 phage engineered to express the AviTag peptide as a *gene III* fusion are enzymatically biotinylated, thus allowing the attachment of chemically biotinylated antibodies using NeutrAvidin as a linker. This approach yields affinity agents where the binding of an antibody to its target can be ultra-sensitively reported through real-time amplification of the phage genome, with very low non-specific binding. We have demonstrated the use of these phage immuno-detection reagents using Vascular Endothelial Growth Factor (VEGF) as a model analyte in bronchoalveolar lavage (BAL) fluid.

The assay protocol uses a pre-assembled antibody/NeutrAvidin/biotinylated “antibody-phage” construct. Micrometer-sized magnetic particles, widely used in sample cleanup and concentration (Mani et al. 2011), were used as capture media. The use of magnetic particles and optimized reaction and washing conditions help improve the sensitivity but we believe that the high detection sensitivity is also likely attributable to the low non-specific binding of the immuno-phage reagent; 500-6,000 phage bound non-specifically to the magnetic particles for each million phage offered, with the exact number depending on the matrix and washing conditions used. To validate the analytical sensitivity of the assay in complex samples VEGF was spiked into BAL fluid (50% in PBS), a potential sample source for diagnostic assays, at concentrations ranging from 26 aM to 2.6 pM, Fig. 2. The BAL was not expected to contain any VEGF and, from the linear relationship between Ct values and concentration of spiked VEGF, the level of endogenous VEGF in the BAL was determined to be below the limit of detection (LOD) of this assay. The LOD was calculated to be 200 aM by subtracting three standard deviations from the mean Ct value of the no-VEGF control and calculating the corresponding analyte concentration from the Ct vs. VEGF concentration curve. The  $r^2$  value for this curve was 0.97, showing a good representation of the dependence. Operation below the half-saturation concentration of an affinity agent is a general concern for all ultrasensitive measurements, which often employ affinity reagents with lower affinities than those used here. Such operation is feasible because binding is non-zero even at concentrations below the half-saturation concentration. Since the assay can succeed with only a small minority of potential binding sites occupied (due to the great amplification power of PCR), this is sufficient. Moreover, in a recent review of magnetic bead-based assays (Tekin et al. 2013) it is noted that immuno-magnetic particles used in non-competitive sandwich assays similar to ours, enhance immunoassay sensitivity; particles carry an excess of capture antibodies that kinetically drive the reaction towards protein binding (Chang et al. 2012). These numbers are in good accordance with the numbers measured in PBS (LOD 1 fM, data not shown), and also suggest low inherent non-specific binding of the modified phage reagent even in more complex backgrounds; only 0.6 % of the phage offered was found to bind non-specifically in 50 % BAL. This LOD is approximately 100-fold lower than the LOD established for well-optimized commercial

ELISAs, (e.g. VEGF ELISA from R&D Systems) emphasizing the improved sensitivity gained through the use of real-time PCR as the read-out. The use of phage greatly reduces the non-specific binding of bare DNA which often troubles immuno-PCR (Malou et al. 2011).

We believe that the M13 phage used here pose sufficient steric hindrance to give a relatively shallow slope of immuno-phage PCR Ct as a function of analyte. While in its current state of development the assay could not reliably detect small changes in concentration at very low concentrations, it can provide ultrasensitive Yes/No detection of analytes such as toxins, agricultural and industrial chemicals, or the circulating TRPs (Tandem Repeat Protein) of the difficult-to-detect intracellular pathogen *Ehrlichia chaffeensis* (Luo et al. 2009). The approach might also be useful with rapidly-mutating RNA viruses, in which PCR detection is hindered by the rapid appearance of escape mutants, and the need for RNA reverse transcription before PCR. The versatility of the assay can be extended by using polyclonal antibodies, or non-antibody detection agents, such as lectins and aptamers, avidin-coupled to the biotinylated AviTag phage. Finally, we note that for future applications, our approach lends itself to the detection of multiple analytes in the same sample, using engineered phage populations, each carrying a different antibody, and each containing a unique DNA sequence recognizable by a distinct TaqMan probe.

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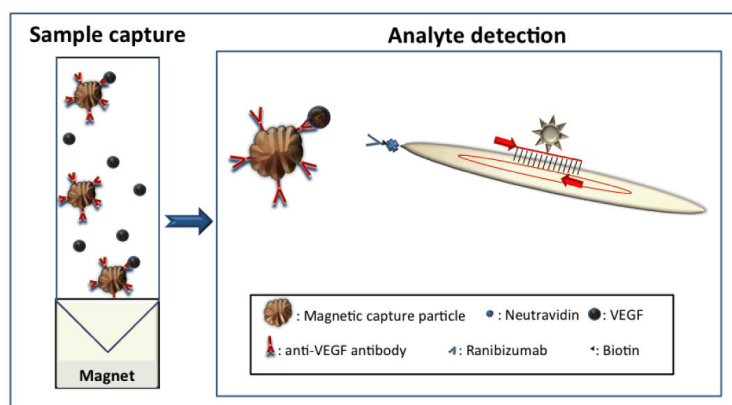
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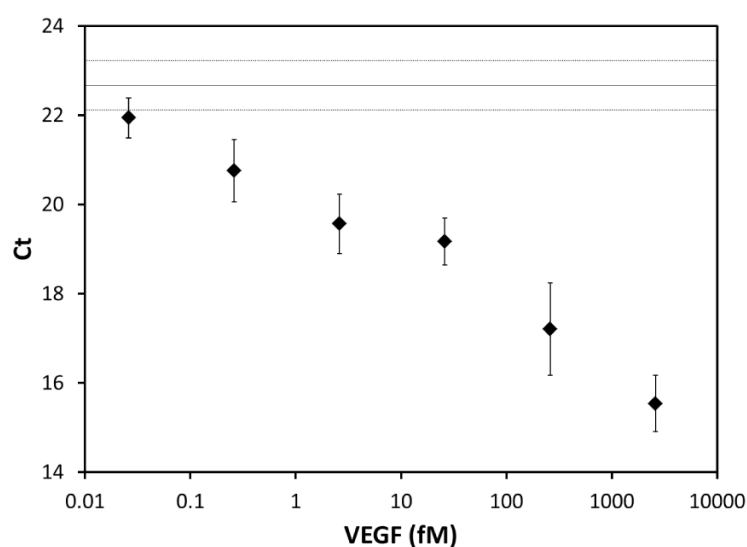
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**Fig. 1. Analyte detection using immuno-phage particles**

Left panel: Paramagnetic capture particles functionalized with analyte-specific antibodies used to concentrate analyte from solution. Right panel: Pre-made antibody/NeutrAvidin/biotinylated phage affinity agents are detected by real-time PCR of phage DNA; not to scale.





**Fig. 2. EGF detection using the immuno-phage assay in 50% BAL fluid**

VEGF (26 aM - 2.6 pM) was spiked into 50% BAL fluid (in PBS), and incubated with polyclonal anti-VEGF antibody functionalized magnetic particles. The biotinylated antibody/Neutravidin/phage affinity reagent was added, followed by washing. The sample was then analyzed using real-time PCR with phage-specific primers ( $n=6$ , error bars =  $\pm 1$  SD, Non-template control  $C_t = 34$ ). The No-VEGF control ( $C_t = 22.67 \pm 0.55$ ) is shown as a solid line with  $\pm$  one standard deviation represented by dotted lines.