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On-chip detection of a single nucleotide polymorphism without polymerase amplification

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ABSTRACT

A nanoparticle-assembled photonic crystal (PC) array was used to detect single nucleotide polymorphism (SNP). The assay platform with PC nanostructure enhanced the fluorescent signal from nanoparticle-hybridized DNA complexes due to phase matching of excitation and emission. Nanoparticles coupled with probe DNA were trapped into nanowells in an array by using an electrophoretic particle entrapment system. The PC/DNA assay platform was able to identify a 1 base pair (bp) difference in synthesized nucleotide sequences that mimicked the mutation seen in a feline model of human autosomal dominant polycystic kidney disease (PKD) with a sensitivity of 0.9 fg/mL (50 aM)-sensitivity, which corresponds to 30 oligos/array. The reliability of the PC/DNA assay platform to detect SNP in a real sample was demonstrated by using genomic DNA (gDNA) extracted from the urine and blood of two PKD-wild type and three PKD positive cats. The standard curves for PKD positive (PKD^+) and negative (PKD^-) DNA were created using two feline-urine samples. An additional three urine samples were analyzed in a similar fashion and showed satisfactory agreement with the standard curve, confirming the presence of the mutation in affected urine. The limit of detection (LOD) was 0.005 ng/mL which corresponds to 6 fg per array for gDNA in urine and blood. The PC system demonstrated the ability to detect a number of genome equivalents for the PKD SNP that was very similar to the results reported with real time polymerase chain reaction (PCR). The favorable comparison with quantitative PCR suggests that the PC technology may find application well beyond the detection of the PKD SNP, into areas where a simple, cheap and portable nucleic acid analysis is desirable.

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1 Introduction

The rapid sequencing of genes, and in particular, the detection of single nucleotide polymorphisms (SNP), has attracted a great deal of attention over the past few decades. Real time polymerase chain reaction (PCR) has been the standard laboratory technique for the analysis of SNPs but it suffers from some disadvantages in terms of sample preparation and handling, cost and lack of portability. In addition, with the exception of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) which requires a new restriction site to occur from the SNP [1], it is difficult to detect SNPs using the standard SYBR Green real-time PCR that needs to use multiple sets of primers [2] or labeled probes [1] to isolate the SNP. In addition, the amount of DNA extracted from real samples is typically not sufficient for the real-time PCR. Alternatives to conventional real time quantitative PCR have been proposed and studied for the detection of SNP, some of them reviewed by Galvin [3] and Briones and Moreno [4]. The recent techniques that have been developed include electrochemical approaches [5], quartz microbalances [6], and a combination of surface plasmon resonances with a microbalance [7]. Lee et al. [8] investigated the use of a microarray where probe single stranded DNA was immobilized at the bottom of a non-fouling titanium well. Hybridization was detected via a fluorescence signal. Qavi et al. [9] detected SNP in a silicon micro-ring resonator that used a shift in the resonant wavelength of the ring to report hybridization to a specific capture probe. Gold nanoparticles that were functionalized with probe DNA were used by Oh and Lee [10] to detect a single-base mutation in the breast cancer gene BRCA1.

Despite some use of nanomaterials such as gold nanoparticles in other areas, it appears that recent advances in nanophotonics have not seen comparable applications in SNP detection up to this point. All the above-mentioned alternatives offer different ways to quantify SNPs, but lack the sensitivity to be an alternative to real-time PCR. Nanophotonic structures such as a photonic crystal may offer unique advantages in terms of ease of use and high sensitivity that arises from amplification of the optical signal.

A photonic crystal (PC) is a periodic nanostructure that manipulates light in novel ways, in this case to enhance fluorescence. With such characteristics, PCs have been used in various ways for improving biosensing [11–14]. An assay platform with a PC nanostructure can provide a great improvement in the signal to noise ratio. Previously, we developed a PC nanostructure that was created by the assembly of 40 nm-nanoparticles into wells in an array; the structure very effectively exploited phase matching of the nanostructure with the excitation light and the emitted fluorescence [15]. The performance of the nanoparticle-assembled PC-array showed great potential in antibody/protein-based bioassays [15, 16], wherein sensitive detection of clinically important biomarkers were achieved rapidly. The photonic crystal technology can be exploited for the analysis of DNA and genetic diseases, the topic of this study. We have chosen a particular disease which arises from a SNP to demonstrate the feasibility of the photonic array for the purpose.

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited disease of the kidney and a common cause of end-stage kidney disease in humans. ADPKD is characterized by the increasing prevalence of fluid-filled cyst in the kidney due to a non-hotspot mutation—hotspots are the nucleotide sequences where mutations are frequently concentrated [17]—in one of two genes, although 85% of cases in humans are due to mutations in the polycystic kidney disease 1 (PKD1) gene [18]. These cysts ultimately lead to destruction of the tissue renal tubules from which they arise and, in the long term, to fibrosis of the renal parenchyma; most patients require dialysis or renal transplant in their 6^{th} or 7^{th} decades of life. Genetic testing of the PKD1 gene has proved to be difficult because of the lack of a hotspot mutation so that restriction fragment length polymorphisms (RFLPs) are not useful and sequencing of the entire (quite large) gene is often required. Not only does this gene carry a large coding sequence, it is also found to have duplicated homologous regions that lie on the same locus, which requires advanced techniques for detection [19]. Recently, a domestic feline model has been promoted as a suitable substitute for detection of human ADPKD because felines and

humans shows biological similarity and share more than 30 homologous inherited diseases [20]. In addition, ADPKD is the dominant genetic disease of cats, with about 38% of Persian cats worldwide being positive.

Due to the ready availability of such a homologous model, we have utilized these cats in this proof-of-concept study. In addition, the feline PKD model allows us to directly compare our PC approach with a nanoparticle-based method [21] for PKD that we developed in the past, which used a typical plate reader for signal read-out, with a sensitivity that did not compare well with conventional PCR.

In this work we demonstrate the ability of the photonic crystal to detect nucleotides without complex hybridization protocols. A "drop–delay–detect" method with a photonic crystal platform is shown to be as effective as the standard hybridization (e.g. PCR) that requires thermal cycling. Our method is first demonstrated with synthetic DNA oligos that contain a SNP to ascertain the sensitivity of the platform; then its robustness is evaluated with real biofluid DNA samples taken from animals which exhibited the PKD mutation. Although there are variations of exon polymorphisms on the feline PKD1 mutation, we used an exon 29-SNP (transverse mutation) for designing synthetic DNA oligos due to its high relevance to the development of the disease [22]. Our method is akin to a DNA microarray technique, but with sensitivity that matches real-time PCR. Our results on the PC platform suggest that the amplification of the oligo copies can be substituted by optical enhancement of the fluorescence signal, resulting in quantification of few oligos. This opens up new avenues for simple and ultrasensitive nucleic acid detection that can be readily applied to the clinic for rapid, even possibly bedside, diagnosis of PKD and other hereditary diseases.

2 Experimental

2.1 Materials

The DNA oligo probe (5′-ATCCTTAAAAGGTGACA-3′), DNA targets with different complementary base pairs (3, 6, 9, 12 and control; 5′-CGCGCGCGAT-3′, 5′-CGCGAAGGAT-3′, 5′-CTTTAAGGAT-3′. 5′-CCTTTTAAGGAT-3′, and 5′-GCGCGCGCGC-3′), feline-exon 29-PKD positive (PKD^+) and negative (PKD^-) DNA samples (Genbank accession no. AY612847) conjugated with Alexa 532 (5′-CGGGCCACCTGTTGAGTCCTCCTCGTCTGCCTCTTCCTGGGCGCCAATGCTGTGTGG/Alexa 532/-3′ and 5′-CGGGCCACCTGTTGCGTCCTCCTCGTCTGCCTCTTCCTGGGCGCCAATGCTGTGTGG/Alexa 532/-3′), biotinylated DNA probe (the probe capturing target DNA oligos; 5′-/Biotin/CGAGGAGGACTCAACAGGTG-3′), and signal probe (the probe emitting fluorescent signal after binding to the target DNA; only used for detecting feline urine and blood samples; 5′-/Alexa532/ACACAGCATTGGCGCCCAGG-3′) were designed and commercially synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Persian feline PKD urine and blood were obtained from Dr. Leslies Lyon's laboratory in the School of Veterinary Medicine, University of California, Davis. The GeneJet PCR purification kit and QIAamp DNA mini kit were purchased from Thermo Scientific (Waltham, MA) and Qiagen (Venlo, Netherlands) respectively. DIG easy Hyb buffer used for detecting synthesized feline PKD^+ and PKD^- was purchased from Roche (Basel, Switzerland). Hybridization buffer used for SNPs detection of feline urine and blood sample was made with 30% formamide and 75 mmol/L NaCl. Forty-nm carboxylated polystyrene (PS)-nanoparticles (F-8789) were purchased from Invitrogen (Carlsbad, CA).

2.2 Preparation of DNA assay

Genomic DNA (gDNAs) from feline urine was extracted following the methods described in Deelman et al. [23]. Briefly, 100 μL of the urine was mixed with 500 μL of binding buffer and was centrifuged at 10,000 g for 5 min. Supernatant was then passed through a GeneJet purification column. The column was then washed with 750 μL of wash buffer and then the gDNA was eluted with elution buffer (10 mM Tris-HCl, pH 8.5). The gDNA from blood was extracted with the QIAamp DNA Mini Kit following the manufacturer's instructions.

10 μL of 0.05% carboxylated PS particles suspended in deionized (DI) water (990 μL), were mixed with streptavidin (128 μg) by gentle shaking for two hours at room temperature to achieve passive adsorption.

The nanoparticle–streptavidin complexes were then mixed with 3 μg of biotinylated-DNA probe for an hour. After trapping the nanoparticle–streptavidin–biotin–DNA probe into nanowells of the array by using an electrophoretic particle entrapment system (EPES) [15], 10 μL of target synthetic DNA oligos, PKD^+ or PKD^- DNA-Alexa 532 in DIG easy Hyb buffer were dropped onto the area with a total of nine arrays in a 3 × 3 format with the distance between the arrays of 250 μm. In the case of synthetic DNA oligos that were used to test the performance of the PC array, SYTOX Orange (intercalating dye, λ_{ex} = 547 nm, λ_{em} = 570 nm) was used after hybridization. The target DNA molecules in 10 μL were thereby shared among the individual arrays. The arrays were incubated for an hour at room temperature followed by removal of the solution in the EPES. In detecting gDNA extracted from feline urine and blood, an additional 10 μL of 10 μg/mL-signal DNA probe–Alexa 532 in the hybridization buffer (30% formamide and 75 mmol/L NaCl) was dropped on the arrays after hybridization with gDNA target DNA and probe DNA. Another one hour incubation and removal of the solution then followed. Before applying urine/blood target DNA, the samples were denatured by 5 min of ultrasonication at 95 ˚C. Denaturation of gDNA samples by using ultrasonication at 95 ˚C was needed to obtain better hybridization with the unfolded structure of super-coiled gDNA. The combination of 30% formamide and 75 nmol/L-NaCl in the hybridization buffer offered optimal conditions for SNP detection [21]. All hybridization was performed in our customized humid chamber (i.e. Tupperware) containing saturated NaCl solution.

The materials and detailed procedures for fabricating a PC nanostructured array with EPES for nanoparticle-in-well assembly, and a single photon counting detection system, have been described previously [15, 16].

3 Results and discussion

3.1 Detection of synthesized DNA oligos with different complementary bases

To assess the performance of the PC array in detecting mismatches in DNA sequences, a series of simple DNA oligos were designed with different numbers of base pairs complementary to the probe. The sequence of the probe was 5′-ATCCTTAAAAGGTGACA-3′. The target oligos with the number of matching bases (3, 6, 9, 12, and control) were respectively 5′-CGCGCGC GAT-3′, 5′-CGCGAAGGAT-3′, 5′-CTTTAAGGAT-3′. 5′-CCTTTTAAGGAT-3′, and 5′-GCGCGCGCGC-3′. The concentrations of the target and SYTOX Orange were 1 μg/mL and 50 nM respectively. Hybridization on the PC array was performed at room temperature for 1 h. After hybridization and intercalation of the dye to hybridized bases, the fluorescent signal from the hybridized complex showed linearity (R^2: 0.99) based on the number of complementary base pairs (Fig. 1).

For each datum point on the curve, the signal of negative control (17 photons/second) was subtracted. Real-time PCR, which is the most rapid PCR method, needs delicate thermal control during the cycles and is sequence dependent, taking up to an hour [24] until the signal is detected against the background. In comparison, our PC nanostructured array operates at room temperature, irrespective of the size of the DNA sequences, and has assay times (1 h) that are comparable to real-time PCR, with a signal that was highly sensitive to the small number of complementary

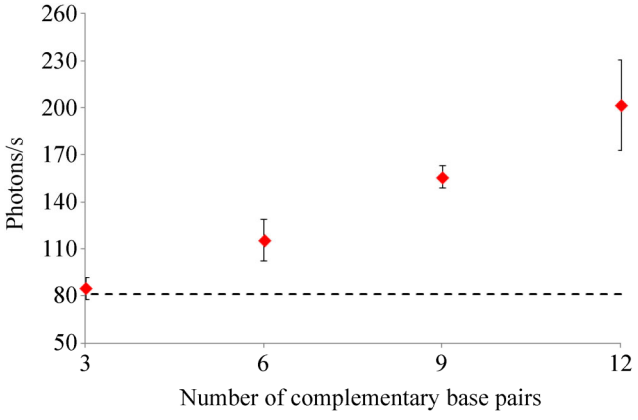

Figure 1 Detection of synthesized DNA oligos with systematically varied number of bases complementary to the probe oligo in the nanowells. The sequence of the probe, target DNA oligos with different number of complementary bases (3, 6, 9, and 12), and negative control were respectively 5'-ATCCTTAAAAGGTGACA-3', 5'-CGCGCGCGAT-3', 5'-CGCGAAGGAT-3', 5'-CTTTAAGGAT-3', 5'-CCTTTTAAGGAT-3', and 5'-GCGCGCGCGC-3'. Error bars are based on the standard deviations of four replicates. The dashed line represents the background signal.

base pairs. For proof-of concept, we employed intercalating dye to demonstrate the performance of the PC nanostructured array for simple DNA assay. However, due to the limited optical signal from the intercalating dye, the highest signal was ~200 photons/second at 1 μg/mL. Hence, Alexa 532 dye was identified as an alternative to demonstrate a sensitive PKD DNA assay on PC nanostructured array.

3.2 SNPs detection of synthesized feline PKD$^+$ and PKD$^-$ DNA sequences

Synthesized single-strand DNA (ssDNA), which had an adenine (A) in exon 29 of the sequences, and synthesized ssDNA with a cytosine (C), were used as PKD$^+$ and PKD$^-$ targets, respectively, to test the PC-DNA assay platform used for distinguishing one mutated base pair occurring in feline PKD (Fig. 2).

The sequences mimicked the exon 29 polymorphism (C→A transversion) in real PKD samples. The hybridization that created a complementary pair of target and probe DNA sequences was performed on the PC nanostructured array (Fig. 3). Six different concentrations of the target DNA (PKD$^+$/PKD$^-$) were tested: 0.9, 9, 90, 9×10^2, 9×10^3 and 9×10^4 fg/mL (5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-1} and 5 pM) (amount of oligo in PKD$^+$: 0.31 mg = 17 nmoles, PKD$^-$: 0.38 mg = 20.6 nmoles). A single photon counting detection system connected to an oscilloscope [15, 16] was used to collect and read the fluorescent signal emitted from hybridized DNA complexes in the array.

Figure 4 shows the measured fluorescent signals according to the different concentrations of the target DNA. The background noise that originated from the 532 nm-laser was 25 ± 3 photons/second which was measured by shining the laser on the arrays in the absence of particles and DNA. The signal difference between PKD$^+$ and PKD$^-$ at each concentration was significant, with more than two-fold change although the signals obtained from PKD$^-$ increased slightly beyond 9×10^3 fg/mL (0.5 pM). The difference between PKD$^+$ and PKD$^-$ was also statistically confirmed by a two-tailed t-test ($\alpha = 0.05$, p-value: 0.01). The measured limit of detection (LOD) was 0.9 fg/mL (50 aM) concentration where the LOD was defined as the concentration of target DNA that produced a signal that was three standard deviations higher than the background noise. This amounted to ~30 oligos/array and illustrates the ability of the device to detect a single mismatch of the nucleotide in very few copies without the amplification and thermal cycling of a conventional PCR method. The amplification in this method is achieved optically, rather than biochemically.

The enhanced excitation and emission of the fluorescent signal generated by the nanoparticle-assembled photonic crystal nanostructure contributed

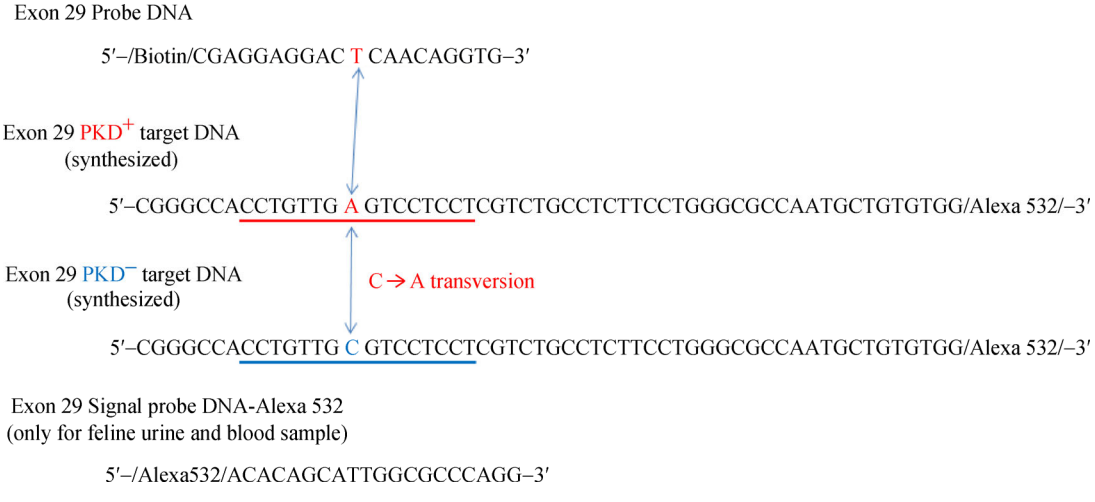

Figure 2 The sequences of PKD positive (PKD$^+$), negative (PKD$^-$), probe DNA and signal probe DNA. PKD$^+$ (57 bases) and PKD$^-$ (57 bases) DNA have a single polymorphism of cytosine (C) → adenine (A) transversion in exon 29. The synthesized target DNAs are conjugated with Alexa 532 for fluorescent detection. Probe DNA (20 bases) was designed and synthesized to discriminate a single mismatched base-pair (A and C) between PKD$^+$ and PKD$^-$. Signal probe DNA conjugated with Alexa 532 (20 bases) is used for second hybridization to emit the fluorescent signal.

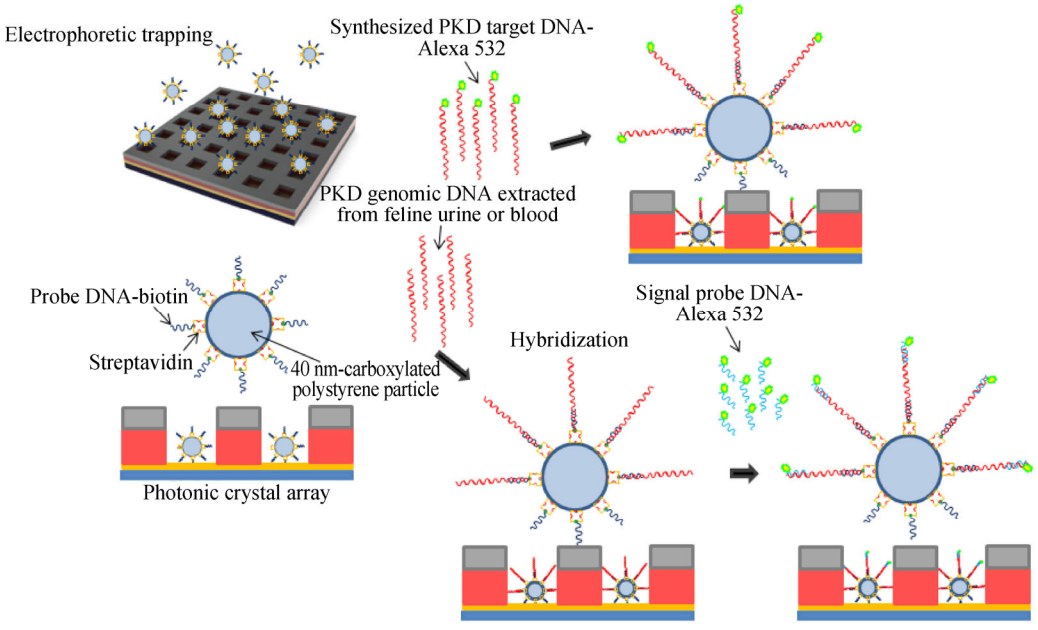

Figure 3 Schematic of PKD SNPs detection on the nanoparticle-assembled PC array. The 40 nm-nanoparticles are coated with streptavidin and then mixed with biotinylated probe DNA. Nanoparticles–probe DNA are trapped into the nanowells of the array by using a electrophoretic particle entrapment system. Synthesized PKD^+/PKD^- target DNAs are conjugated with Alexa 532 for rapid detection after hybridization with probe DNA. For detection of SNP of feline urine/blood gDNA, second hybridization of signal probe DNA-Alexa 532 with the target DNA provided a fluorescence signal.

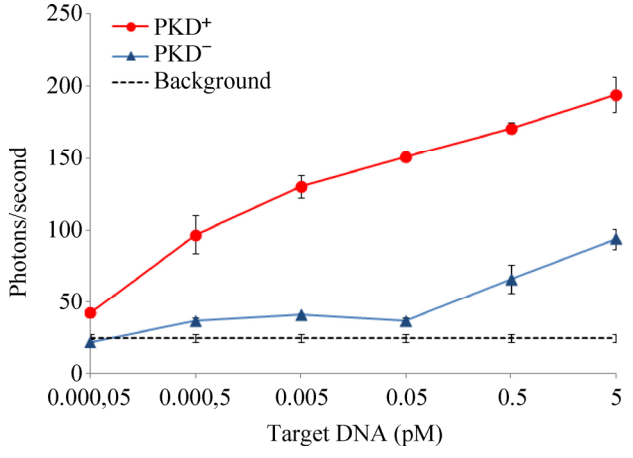

Figure 4 SNPs detection of the synthesized feline PKD^+ and PKD^- DNA on the PC array. Six different concentrations were detected: 0.9, 9, 90, 9×10^2, 9×10^3 and 9×10^4 fg/mL (5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-1} and 5 pM). LODs were determined from the mean plus three standard deviations of the background noise. Error bars are based on the standard deviations of four replicates.

to the significant signal difference between the positive and negative target DNA with high signal-to-noise ratio, which is the most important criterion in SNP detection. In addition, the linear dynamic range (R^2: 0.96; 0.9–9×10^4 fg/mL), starting from the attomolar concentration range, enabled accurate quantification of the SNP in exon 29 of the PKD. The results that we have obtained with the PC technology are far superior, in terms of sensitivity, to the nanoparticle-based method [21] that we reported some years ago for the same target—the LOD was 1 pM. In addition, considering comparison of sensitivity in general SNP detection with other targets, our measured LOD (0.9 fg/mL, 50 aM) is 10^7 fold better than those provided from using other methods such as quartz microbalance (0.3 nM) [6], surface plasmon resonance (30 nM) [7], and ring resonator (1.95 nM) [9].

3.3 SNPs detection of gDNA for feline urine and blood

The reliability of the PC-DNA assay platform was tested by using gDNA extracted from PKD feline urine and blood samples. The PKD^+ gDNA has a mutation (C→A transversion) in exon 29. For this experiment, five affected Persian cats were chosen. Three of them (names of the cats: Ken, Barbie, MJ) were PKD positive while two (Marcus, Polo) were PKD wild-type. With gDNA extracted from the urine of Ken and Marcus, the standard curve was first created with four different

concentrations via serial dilution: 5×10^{-4}, 5×10^{-3}, 5×10^{-1}, and 50 ng/mL. The signals of Ken (PKD^+) showed log-linearity (R^2: 0.99) with respect to concentration, indicating significant differences with those of Marcus (PKD^-) which were almost equivalent to the level of background noise plus three standard deviation. The LOD was 0.005 ng/mL. Three other urine-gDNA samples from MJ, Barbie and Polo followed the standard curves at 0.005, 0.5 and 50 ng/mL respectively. Each sample correctly corresponded to either as PKD^+ or PKD^- (Fig. 5). In addition, based on the result obtained from MJ which was used for a negative control, non-specific binding of the signal probe DNA-Alexa 532 to the PS nanoparticles or polymethylmethacrylate (PMMA) surface of the array was negligible. Blood-gDNA from the five cats was also measured at 0.005, 0.5 and 50 ng/mL. The data were then compared to those with urine-gDNA (Table 1). PKD^+ blood gDNA yielded the same LOD (0.005 ng/mL) as measured in the urine.

Although the signals from blood-gDNAs were generally lower than those of the urine-gDNAs, the two sets of data from urine and blood showed good agreement statistically (two-tailed *t*-test, *p*-value: 0.45, $\alpha = 0.05$). The two boundary concentrations (50 and 0.005 ng/mL) in urine and blood samples were specifically chosen to corroborate negativity for known PKD^- specimens (Marcus and Polo) and to compare the sensitivity for known PKD^+ specimens (Ken and MJ). The signal variation of the negative control PKD^- with concentration in real samples was barely above the background, an improvement in background level compared to the synthetic oligo, which showed a slight increase of the signal with increasing concentration (Fig. 4). This can be attributed to the application of the sandwich assay format using the additional signalling probe that provides another level of discrimination against non-specific binding.

A sample volume of 10 μL of the target DNA solution was shared among the nine arrays on the chip. Therefore, a single array was able to detect as little as 6 fg per array. This LOD is 7×10^4 fold lower than that the LOD that was obtained by using nanoparticle-based plate reader system (350 ng/mL) [21] with feline PKD^+-blood gDNA. In fact, the sensitivity of the PC-DNA assay is comparable to a

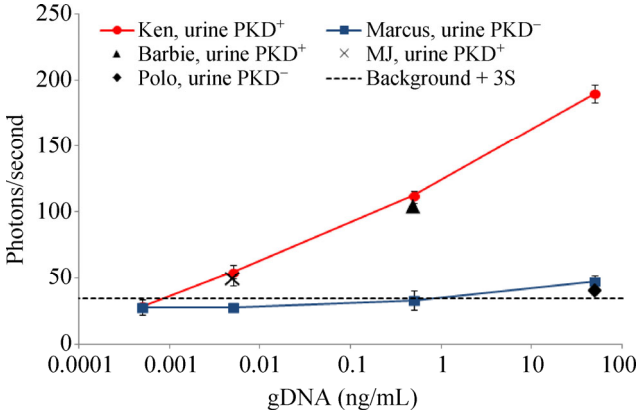

Figure 5 SNPs detection of the PKD^+/PKD^- gDNA extracted from feline urine. The samples were obtained from five Persian cats with known PKD positivity and negativity: Ken (+, ●), Marcus (–, ■), Barbie (+, ▲), MJ (+, ×) and Polo (–, ♦). The dashed line indicates the mean plus three standard deviations of the background noise. The concentrations used for the standard curve: 5×10^{-4}, 5×10^{-3}, 5×10^{-1}, and 50 ng/mL. The signal from target urine-gDNA of MJ, Barbie and Polo were compared to the standard curve created from the signals of Ken and Marcus. Error bar: standard deviation determined from three replicates.

Table 1 Comparison of the fluorescent signals measured from feline urine and blood samples

Cats	Target DNA concentration (ng/mL)					
	50		0.5		0.005	
	Urine	Blood	Urine	Blood	Urine	Blood
Ken (+)					55 ± 5	41 ± 1
Marcus (–)	47 ± 5	33 ± 1				
Barbie (+)			104 ± 3	85 ± 3		
MJ (+)					49 ± 5	42 ± 3
Polo (–)	40 ± 2	35 ± 2				

(+): PKD positive, (–): PKD negative.

Measured signal (photons/second): mean ± S.D. ($n = 3$).

real-time PCR method for the same PKD target–PCR analysis yielded a LOD of between 3 to 30 gene copies [25] in a similar volume. The real-time PCR method required 90 min for the analysis [25]; our results were obtained at a constant room temperature—without thermal cycling—over 120 min. Although the time comparison may appear to be unfavorable for the PC technique, the time and specificity for the assay were not optimized and could certainly be improved significantly if the assay were performed at a suitably elevated constant temperature. The approximate equivalency between the PC method and real time

PCR is very encouraging and suggests that the PC method may find much broader applicability in situations where simplicity, cheapness, robustness and portability are desirable attributes for an assay.

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