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An Integrated Quantum Dot Barcode Smartphone Optical Device for Wireless Multiplexed Diagnostics

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KEYWORDS

quantum dot barcode, smartphone, point-of-care, infectious disease, diagnostics

5 ABSTRACT

Inorganic nanoparticles are ideal precursors for engineering barcodes for rapidly detecting diseases. Despite advances in the chemical design of these barcodes, they have not advanced to clinical use because they lack sensitivity and are not cost-effective due to requirement of a large read-out system. Here we combined recent advances in quantum dot barcode technology with
10 smartphones to engineer a simple and low-cost chip-based wireless multiplex diagnostic device. We characterized the analytical performance of this device and demonstrated the device is capable of detecting down to 1000 viral genetic copies per mL and this enabled the diagnosis of patients infected with HIV or hepatitis B. More importantly, the barcoding enabled us to detect multiple infectious pathogens (e.g., HIV and hepatitis B) simultaneously, in a single test, in less
15 than 1 hour. This multiplexing capability of the device enables the diagnosis of infections that are difficult to differentiate clinically due to common symptoms such as a fever or rash. The integration of quantum dot barcoding technology with a smartphone reader provides a capacity for global surveillance of infectious diseases and the potential to accelerate knowledge exchange-transfer of emerging or exigent disease threats with healthcare and military organizations in real-
20 time.

TEXT

The detection of diseases requires the development of technologies that can rapidly analyze genetic and proteomic targets with a single drop of biological fluid. The detection assays must be cost-effective and detect multiple targets simultaneously which would increase accessibility and improve the speed of analysis, respectively. Ultimately, this leads to improvements in the diagnostic sensitivity and specificity and would contain the spread of diseases, especially for infectious pathogens. In the last decade, researchers have developed a wide-array of barcoding structures and have demonstrated the detection of multiple biological targets in buffer. The barcodes comprise of graphical,^{1,2} optical,^{3,4} or magnetic^{5,6} structures with unique patterns that identify surface coated bio-recognition molecules which can selectively recognize biological targets of interest (e.g. whole virus, antigen, or genetic sequence). Detection occurs when a fluorescently labelled secondary targeting agent is added to the barcode, forming a sandwich structure. The code identifies the coated recognition molecule, which identifies the target; and the secondary probe confirms the presence of the disease and the quantity. The advantage of using barcode in a diagnostic setting is that multiple targets from patient samples can be detected simultaneously which increase the speed of analysis and improve the precision and accuracy of diagnosis. Despite advances with the chemical design of barcodes, nanotechnology-based barcoding technologies have not advanced to patient care. The read-out device (e.g. spectrophotometer/fluorometer, fluorescent microscope, or flow cytometer) remains expensive, and the poor sensitivity (limit of detection of femto to attomole) limits their utility for detecting most clinically relevant samples. While this can be overcome with amplification steps, current approaches are often too complex or expensive to be practical outside of research laboratories. Here, we have combined recent advances in quantum dot barcoding technology,

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smartphones, amplification, and software design to engineer a diagnostic device capable of detecting multiple pathogens (Figure 1) in patient samples. This work is, to the best of our knowledge, the first to demonstrate the use of a portable, smartphone-based read-out of quantum dot barcodes, which enhances their ability and practicality for use in point-of-care diagnostics.

Barcodes can be engineered by infusing polystyrene beads with different emitting quantum dots.^{7,8} The quantum dots provide significant advantages over organic fluorophores for barcoding. They are brighter, have wider excitation spectra, narrower emission spectra, and are more resistant to photobleaching.⁹⁻¹¹ From a device perspective, these properties are attractive because they reduce the costs of manufacturing the diagnostic device and simplify the design of the read-out system while giving high precision in identifying the optical code. This strategy also provides quantum dots with greater multiplexing capabilities, as the use of quantum dot in a non-barcoded detection system can reasonably differentiate 5-6 unique signals^{10,11} versus the potentially millions in a barcode format.⁷ Furthermore, the optical signals of the quantum dots are protected in the microbead from fluorescence fluctuations as the polystyrene reduces the interaction of the quantum dots with ions, proteins, and other biological molecules.^{12,13} The microbeads themselves are also versatile in molecular detection and can detect both genomic or proteomic targets.^{7,14,15} We have previously optimized the methods to prepare quantum dot barcodes with hundreds of distinct optical patterns and demonstrated their utility as a universal platform for detecting different panels of infectious targets using model synthetic genomic sequences.¹⁶ A critical next step is then to improve the analytical sensitivity to the zeptomole range, to design a cost-effective read out system, and to clinically validate this technology. This is important next step for the translation of barcoding technologies to the clinic.

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We expect the barcoded device to enable clinicians to better manage the spread of diseases by improving the diagnosis of infectious pathogens, which can spread rapidly leading to economic burden, morbidity, and mortality. The integration of mobile-cellular devices with state-of-the-art multiplexing molecular diagnostic devices would help alleviate these problems by permitting clinical diagnosis in the absence of large laboratory infrastructure leading to more appropriate treatment, as well as enable real-time global surveillance of infectious transmission events and predict temporal infection trends through crowd-sourced data collection. While the concepts of smartphones have been proposed for diagnostic applications, they have primarily been used in direct imaging applications from identifying bacteria or viruses labeled with a fluorophore,^{17,18} cell counting,^{19–21} to imaging the test lines on lateral flow immunoassays²² and results from custom-made lab-on-chips assays,^{23–25} among many other approaches.^{26,27} Many of these techniques often cannot diagnose the early stages of infection because of poor analytical sensitivity. More importantly they are incapable of detecting different strains or pathogens in a high-throughput manner due to their inability to detect multiple biomarkers simultaneously. To validate our technology, we demonstrate the ability to detect patients infected with the multiple chronic blood-borne infections such as HIV and hepatitis B because these co-infections can accelerate disease progression. The diagnostic device can be extended to develop molecular testing panels for other important pathogens underlying sexually transmitted infections, malaria, influenzas A and B, and tuberculosis by simply modifying the barcode recognition molecules to the target of choice.

RESULTS AND DISCUSSION

Integration of quantum dot barcoding with smartphone technology

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A multiplex-chip platform that is simple to use and can be easily transported were two of the main design foci of our diagnostic device. To this end, we arrayed the quantum dot barcodes on microfabricated slides with controlled number of microbeads per unit area for detecting genetic samples (Figure 1a). Barcodes in a chip format are easier to transport than when they are in solution. And compared to microarrays where the assay is performed on a flat surface, a barcoded chip is cheaper to produce due to cheaper microbead production, offers faster reaction kinetics due to the increased surface area, and allows higher tunability of microbead size and conjugation chemistry.²⁸ Microbead arrays on a chip are currently used in sequencing analysis but the cost of the final chip is high because the microbeads are arrayed on the ends of optical fibers.²⁹ We developed a simple method to array quantum dot barcodes on the surface of a chip. Glass slides were microfabricated with 3.0 μm -diameter wells (Figure 1b). A solution of ~ 3.0 μm sized microbead barcodes containing different combinations of fluorescence emitting CdSeS alloyed-ZnS quantum dots were added to the chip. They spontaneously settled into each well, reducing overlapping and aggregation that may confound analysis of their fluorescence in later steps (Figure 1c). Once bound, these microbeads do not desorb from the surface as they are held in place by non-covalent forces. The concentration and size of the barcodes determine the filling efficiency (Supporting Information Figure S1). While minor variations of microbead numbers from chip to chip were expected, they would constitute only a fraction of the thousands of microbeads functionalized on each chip, and thus not have a significant effect on detection outcomes. The deposition of barcodes on the chip, compared to those stored in solution, enables higher portability of barcodes and reduces the number of steps in the quantum dot barcode assay process. The device itself is also portable and easy to use (Figure 1d). The components – batteries, switch, laser diodes, lenses, and filters – and 3D-printed plastic chassis are all

lightweight and able to fit in one hand. The two laser diodes are switched on independently via a
115 manual switch: Excitation Laser 1 (405 nm) excites the barcodes and Excitation Laser 2 (650
nm) excites the secondary label with a filter ($\lambda_{\text{ex}} = 655/15$) that controls the wavelength
excitation observed by the chip. The eyepiece and movable objective lens magnifies and focuses
barcodes on the chip to allow them to be viewable clearly by the naked eye on the smartphone
display. The smartphone camera, in this case Apple's iPhone 4S, then captures that view. A total
120 of five images are acquired for each sample, corresponding to each of the five emission filters
($\lambda_{\text{em}} = 430\text{LP}$, 530/10, 580/10, 640/10, and 692/40). Specifically, the 430LP filter image is used
for extracting the location and size of the barcodes in the camera field-of-view; the 530/10,
580/10, and 640/10 filter images for isolating quantum dot signals to determine barcode
signatures; and the 692/40 filter image for isolating the secondary label signal to determine the
125 presence of target analyte bound to the barcode surface. While quantum dot barcode
differentiation using just one color image (i.e. $\lambda_{\text{em}} = 430\text{LP}$ filter image) is possible when the
barcode colors are few and distinct (e.g. 'green', 'yellow', 'red'), it becomes increasingly
difficult and prone to errors when higher multiplexing is desired, which requires barcodes to
contain varying mixtures of the same quantum dots. This causes some barcodes to share similar
130 color channel intensities (Supporting Information Figure S2), increasing the possibility of false
identifications. Our approach of using multiple emission filters, on the other hand, allows
differentiation with high accuracy while remaining easily scalable for greater barcode
combinations. Finally, a custom-written algorithm analyzes these images and produces the
results. The algorithm develops a histogram of the optical signal from the secondary probe of all
135 barcodes within the sample. A threshold is established in a measurement based on the highest
signal from barcodes that do not contain any of the target molecules of interest (i.e. the negative

control in the experiments). A graph of barcode numbers above this threshold is used to determine a positive or negative detection (Supporting Information Figure S3). We expect samples that have the target molecule of interest to have more barcodes above this threshold. We
140 chose this method of analysis rather than using absolute intensity values because we found this method to obtain greater consistency in the analysis due to reduction in skewing of the measurements from a small population of microbeads. Our measurement strategy is adapted from flow cytometry, where the quantification is based on single cell counts instead of a population average. The entire imaging and analytical process takes less than ten minutes and
145 can be performed with minimal training.

Use of quantum dots over traditional fluorophores

The unique optical properties of the quantum dots for barcoding are critical for engineering a low-cost multiplex device. Here we compared the optical properties of two microbeads encapsulated with organic fluorophores ('Yellow' with $\lambda_{em} = 480$ nm, and 'Nile
150 Blue' with $\lambda_{em} = 630$ nm) versus quantum dots ('QD540' with $\lambda_{em} = 530$ nm and 'QD640' with $\lambda_{em} = 640$ nm). As shown in Figure 2, the quantum dots have a continuous absorption profile while the organic fluorophore has a peak-like profile (Figure 2 'Spectra'). The absorbance profile presents a significant advantage for quantum dots for engineering point-of-care device. This would reduce the size of the final device and reduce costs as a quantum dot-barcode device will
155 only require a single energy source to excite all of the barcodes (in this case, we used a 405 nm diode laser) while the organic fluorophore-barcodes will require multiple emitting diodes or lasers to maximally excite different barcodes (Figure 2 'Image'). The quantum dot barcodes also have narrower emission profiles (e.g. full-width half-max of ~ 35 nm versus 60 nm for 'QD540')

and ‘Yellow’, respectively) and retain their emission peaks despite being excited by different
160 wavelengths (e.g. ‘QD640’ versus ‘Nile Blue’) (Figure 2 ‘Varying Excitation’). The narrower
emission profiles allow more quantum dots, compared to broad-emission fluorophores, to be
used within a range of wavelengths (e.g. the 400-700 nm visible spectrum) without spectral
overlap, enabling greater number of barcodes to be engineered for multiplexing analysis. This
and the emission peak retention property also reduce the likelihood of false barcode
165 identification. Finally, the quantum dot barcodes are much more resistant to photobleaching
compared to the organic fluorophore encapsulated barcodes (Figure 2 ‘Photobleaching’). This is
important for accurate barcode identification as bleaching of the coded microbeads can lead to
mis-detection. Using quantum dots for barcoding simplifies the read-out device and reduces the
costs of the final device so that it could be broadly used in the future in both resource-rich and
170 resource-limited settings.

Synthetic targets to assess device sensitivity and multiplexing capability

The current detection platforms for identifying quantum dot barcodes require expensive
instruments and detectors.^{14,15} Here we evaluated whether our device can differentiate the optical
signals between barcodes as well as the secondary fluorescent probe used in our genetic assays.
175 Figures 3a and b demonstrate that an iPhone camera is able to capture the distinct optical
emissions of each barcode on the microwell chip and proper filtering can differentiate the
barcode optical signal from the secondary probe’s signal. These studies confirmed that an iPhone
camera can image barcodes on the chip surface and be used as a detector for biological assays.

We first determine the analytical performance of the microbead-based sandwich assay
180 using our engineered smartphone reader. We designed seven barcodes for detecting seven

infectious disease biomarker targets as shown in Table 1 plus two barcodes for the positive and negative control samples. When the target is absent (i.e. negative detection), the optical signal from the microbead comprises only the quantum dot signal of the barcode. When the target is present (i.e. positive detection), the microbead optical signal consists of emissions from both the quantum dots and Alexa Fluor 647 dye secondary probe. The limit of detection and linear dynamic range for each of the targets for HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV), and the influenza type A targets H1N1, H3N2, H5N1, and influenza type B (Flu B) (Figure 3c and 3d) is between 10 to 50 fmol (6×10^9 to 3×10^{10} copies) and up to 40-fold, respectively, in a final hybridization sample volume of 10 μ L. This suggests that analytical performance is independent of the infectious disease targets. Note that while the dye does photobleach under continuous excitation in the device, it still retains > 85% of its intensity within the first minute of excitation (Supporting Information Figure S4), the longest possible image acquisition time during our experiments, and thus should not have a significant impact on the sensitivity of detection.

Next, we demonstrated the ability to simultaneously detect multiple synthetic genetic targets from bloodborne virus panel (Figure 4). We prepared six different mock genetic samples by mixing various combinations of the genetic target sequences for each of the three pathogens of interest – HIV, HBV, and HCV – plus a positive control sequence to ensure that the barcodes are working as designed, and the secondary fluorescent probe sequence. For example, we would prepare solutions that were spiked with the target sequences for HIV and positive control sequence in one combination (Figure 4b), then HIV, HBV, HCV and positive control sequence in another combination (Figure 4f). A final hybridization sample of 20 μ L was added to the chip and incubated at 37 °C for 20 minutes, rinsed with a washing buffer, dried, imaged, and analyzed

using the algorithm. In all cases the target sequences were correctly identified by the assay. For
205 example, in our solution containing the sequences for HCV and positive control (Figure 4d), the
bar graph shows our technique can discriminate between barcodes bound with secondary probes
(i.e. HCV and positive control) versus those not bound (i.e. HIV, HBV and negative control).
All of the probe recognition sequences for the bloodborne viral panels were carefully designed *in*
silico to minimize cross-reactivity with closely related viruses using subtypes against the gag
210 gene of HIV, core protein gene of HBV and nucleocapsid protein gene of HCV, as per gold
standard testing standards.

Clinical validation of diagnostic device

We determined whether the device was capable of detecting and differentiating samples
from patients with HIV and HBV from those without (i.e., HIV- or HBV-negative subjects). The
215 experiment done using synthetic targets showed that the device has a limit of detection of 10^{-15}
mol but to use the device for diagnosing patient samples, the limit of detection needed to be
lowered by a factor of 10^6 . We cannot incorporate conventional PCR amplification methods to
our device between these PCRs are bulky and expensive. To address this issue, we optimized an
isothermal amplification step, which can be performed in 10 – 30 minutes, is simple, and does
220 not require precise temperature control.

Patient samples were collected using standard protocols and we extracted the hepatitis B
viral DNA and HIV viral RNA using magnetic microbeads. The cell membrane was first
disrupted by the addition of lysis buffer, and the surface functionalized magnetic microbeads
captured viral nucleic acid. The sample was then placed in a magnetic separator to collect
225 magnetic microbeads. Before use in our assay, the HIV RNA was reverse-transcribed into
cDNA. All samples were then amplified using recombinase polymerase amplification. The

recombinase proteins were added to the isolated genetic targets to form nucleoprotein complex, which facilitates strand-transfer at the homologous sequence of the template DNA. Single-stranded binding proteins then stabilize the displaced strand of the template DNA, and the DNA polymerase extends the complimentary strand.³⁰ Of the final amplified sample, 20 μ L of the double-stranded DNAs were denatured and added to the chip, incubated at 37 °C, rinsed with a washing buffer, dried, imaged, and analyzed using the algorithm in a manner that is similar to detection of the synthetic targets. Figure 5a and 5b demonstrate the successful diagnosis of individual patient samples with HIV and HBV, respectively, of varying viral loads before amplification (Supporting Information Table S1). These viral loads are reported as per a clinical setting, and represent a viral load range from treatment-naïve patients. They do not necessarily indicate the clinical limit of detection. The HBV-infected samples were comprised of multiple genotypes to ensure our test would be widely applicable. In order to determine whether the measurement results were significant, we combined the measurements from all samples of non-infected subjects, and then compared them to the combined measurements from the infected patients with either HIV or HBV (Figure 5c and 5d, respectively). Our results showed a significant difference with a p-value of 0.05 and 0.01, respectively. We further confirmed the results using flow cytometry (Supporting Information Figure S5) and showed comparable outcomes. Our diagnostic device is capable of detecting viral loads in the range of 10^3 to 10^9 copies/mL and different genotypes (Supporting Information Table S1). To confirm our device is capable of multiplex detection of patient samples, we mixed 10 μ L of each amplified patient samples containing HIV and/or HBV. We performed four-plex assays (HIV, HBV, as well as positive and negative controls), and the results of which clearly demonstrated that the device is capable of differentiating between the two viruses (Figure 5e-h using smartphone device,

250 Supporting Information Figure S6 using flow cytometry). We further investigated the level of
specificity with our genetic assay by sequencing 7 HBV and 10 HIV patient samples (Supporting
Information Figure S7 and S8, respectively). We found that there are 5 to 9 and 3 to 4
mismatches within HBV and HIV probe-binding regions used for the assay, respectively.
Although we acknowledge that we have only used wild-type probes to detect patient samples in
255 this study, we believe that the level of specificity can be further improved to detect single base
pair mismatch by following Liong, M. et al's approach of using mutant probes that are designed
specifically to match mutated region and therefore bind stronger with mutated target than the
wild-type target.³¹ The development of our device for diagnosing single-nucleotide
polymorphisms was not a focus but such studies will be a focus in future studies. Our clinical
260 validation with real-world HBV- and HIV-infected patient samples demonstrates the
effectiveness of the proposed detection platform for diagnosing infectious diseases.

All of the pathogen targets used as test panels in this study represent major infectious
threats to the global community, necessitating the development of effective and innovative
means for detection and informatics to identify infected individuals and accelerate clinical
265 management. HIV, HBV and HCV are prevalent in resource-limited settings and pose major
threats to populations, often related to unknown transmission through sexual contact, drug use
and contaminated blood products.^{32,33} For more rapidly spreading pathogens, immediate
implementation of infection control measures and enhanced surveillance to curb the spread of
disease will be critical. Here we have shown that the integration of quantum dot barcodes with
270 smartphone technology can be used for multiplex molecular diagnosis of these infectious
diseases with wireless transmission. The steps in our assay, excluding the standard blood
collection/separation processes, there are four-steps (1 through 4) and two-steps (only 3 and 4)

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the genetic and proteomic detection process respectively. They are (1) extraction of the genetic target, (2) amplification of the target, (3) recognition and hybridization to barcodes on chip and secondary probe, and (4) read-out of the chip optical signal with the smartphone followed by analysis. We believe these two or four steps could be automated in black box system. We envision a final blackbox device to all of these components, with specific chambers for each of these steps and their overall dimensions (Figure 6). Each compartment would contain disposable lyophilized samples that can be dissolved by buffers, and transferred from one compartment to the next using capillaries and electrically-driven flow. Building a single unit device is conventionally done as technologies advance from academic development to commercial use once each step in the process has been confirmed to work as design. The device may also be custom-designed for different types of target molecules. For example, in detection of a protein target, we would not need steps 1 and 2. The number of device chambers in the device can be engineered according to the target molecule. This diagnostic device should be both easy-to-use and transportable and enable wireless transmission of diagnostics for interpretation, thus allowing the mapping, surveillance, and potentially prediction of diseases in real-time. The chip is highly versatile for proteomic, genomic, or whole pathogen targets.^{7,16} It may also be designed to detect different pathogen strains, co-infections, markers of drug resistance, or diagnose infections that are difficult to differentiate clinically due shared symptoms such as a fever or rash. The only requirement will be to design panels of barcodes for each specific infectious disease agent. Future work will focus on integrating the extraction and amplification components with the existing device into a singular unit, field-testing of the device, and to conduct a clinical trial with greater sample size to evaluate clinical sensitivity and specificity in both the developed and developing worlds. Although we have conducted our studies using a specific brand of

smartphone, the concept lends itself to further device engineering such that it can physically accommodate any cellphone or smartphone. This device can be assembled into a point-of-care unit that will enable hospitals, disease control centers, mobile units and the military to monitor and potentially predict the onset, spread, and resistance of infectious diseases.

300 **METHODS**

Quantum Dot Synthesis

Quantum dots (CdSeS alloyed-ZnS capped) of peak emission wavelength 540 nm (“QD540”) were purchased from CytoDiagnostics and used as instructed. Quantum dots of peak emission wavelengths 515 nm (“QD515”), 547 nm (“QD547”), 560 nm (“QD560”), 589 nm
305 (“QD589”), 596 nm (“QD596”), 615 nm (“QD615”), and 640 nm (“QD640”) were synthesized and characterized according to published procedures^{34,35} and stored in chloroform at room temperature until later use.

Quantum Dot Barcode Synthesis

Quantum dot barcodes were prepared by mixing together the quantum dots (QD515,
310 QD540, QD547, QD560, QD589, QD596, QD615 and QD640) in different ratios with a polymer-based solution (Supporting Information Table S2 and S3). The polymer solution consisted of poly(styrene-co-maleic anhydride) (32%, cumene terminated) from Sigma-Aldrich dissolved in chloroform, with the polymer concentration at 4-wt%. The resultant quantum dot polymer solution was then introduced into a nozzle system from Ingeniatics using a syringe
315 pump from Harvard Apparatus at a rate of 0.9 mL/hour, as well as double-distilled (DD) water as the focusing fluid at a rate of 180 mL/hour. The nozzle system was then submerged inside a

beaker partially filled with double distilled water. The polymeric barcode microbeads were synthesized *in situ*, and the microbeads formed a white colloidal suspension in the water. After synthesis, the valve was closed and the microbeads were stabilized by overnight stirring and then collected. The microbeads were filtered using 35 μm BD Falcon nylon mesh strainer cap, and characterized using an automated Beckman Coulter Vi-Cell counter, and stored in DD water at 4 °C until use. The quantum dot concentrations required for preparing the barcodes are presented in Supporting Information Table S2 and S3.

Excitation, Absorption, and Emission Spectra Measurement

The excitation and emission spectra of the Yellow and Nile Blue microbeads were measured using the Excitation and Emission Acquisition modes, respectively, on Horiba Jobin Yvon FluoroMax-3 fluorometer. The quantum dot absorption spectra were measured using Shimadzu UV-1601PC UV-Visible spectrophotometer. The quantum dot barcode emission spectra were measured using the Emission Acquisition mode on Horiba Jobin Yvon FluoroMax-3 fluorometer.

Sensitivity Assay

Sensitivity assays (Figure 3c and 3d) were performed directly on the microwell chips for all infectious disease DNA target strands (T_H1N1, T_H3N2, T_H5N1, T_FluB, T_HIV, T_HBV, and T_HCV) and their respective conjugated barcode microbeads (B_H1N1, B_H3N2, B_H5N1, B_FluB, B_HIV, B_HBV, and B_HCV). DNA target strands from Bio Basic Inc., purchased HPLC-purified and used without further purification, were prepared in increasing concentrations of 0, 5, 10, 50, 100, 500, 1000, and 2000 fmol/ μL in TE buffer. DNA detection strand from IDT DNA Technologies with Alexa647 fluorophore on the 5' end, purchased HPLC-

purified and used without further purification, were prepared with concentration of 100 pmol/ μ L
340 in TE buffer. Both DNA target and detection strand samples were stored at 4 °C until further use.
To perform the assay, 1 μ L of the conjugated microbead sample, corresponding to approximately
10⁴ conjugated microbeads, was deposited on a microwell chip for each assay condition and let
dry for 1 hour. Then, 1 μ L of each DNA target strand sample was mixed with 5 μ L of
hybridization buffer (10x SSC, 0.1% SDS, heated to 60 °C), 3 μ L of DD water, and 1 μ L of
345 DNA detection strands or DD water (for the blank condition). This resulted in a total
hybridization volume of 10 μ L for each assay condition, which include blank, 0, 10, 50, 100,
300, 500, 1000, and 2000 fmol target DNA. The hybridization solution for each assay condition
was deposited over the conjugated microbead spots on the microwell chips and incubated at 37
°C for 20 minutes. The microwell chips were then submerged in 10 mL of washing buffer (0.5x
350 SSC, 0.1% SDS, heated to 37 °C), washed by agitation for 20 s, then let dry for 5 minutes before
being imaged. Note that care must be taken so that the washing buffer does not dry and
crystallize over the sample spots.

Multiplexing Assay

Cross reactivity between the bloodborne virus DNA target strands (T_HIV, T_HBV, and
355 T_HCV) and their corresponding conjugated barcodes (B_HIV, B_HBV, and B_HCV), as well
as positive and negative control cases (B_Pos and T_Pos, and B_Neg and T_Neg, respectively),
was studied (Figure 4). First, 6 μ L of each conjugated barcode sample, corresponding to
approximately 6x10⁴ barcodes each, were mixed together with 90 μ L of DD water to produce a
4x dilution factor of the original. The dilution was to reduce microbead aggregation after
360 deposition on chip, which may confound barcode resolution during analysis. To perform the

assay, 8 μL of the diluted conjugated barcode mixture, corresponding to approximately 2×10^4 conjugated microbeads, was deposited on a microwell chip for each multiplexing case and let dry for 4 hours. Then, 4 μL of each target case (DD water for the negative conditions, and corresponding DNA target strand sample with concentration of 2 pmol/ μL for the positive conditions) was mixed with 40 μL of hybridization buffer (10x SSC, 0.1% SDS, heated to 60 °C) and 20 μL of the detection strand (concentration of 100 pmol/ μL). This resulted in a total hybridization volume of 80 μL for each multiplexing case. From this, 20 μL of the hybridization solution for each multiplexing case was deposited over the conjugated barcode spots on the microwell chip and incubated at 37 °C for 20 minutes. The microwell chip was then submerged in 10 mL of washing buffer (0.5x SSC, 0.1% SDS, heated to 37 °C), washed by agitation for 20 s, washed again in another 10 mL of washing buffer to further reduce non-specific binding, and then let dry for 5 minutes before being imaged. Note that care must be taken so that the washing buffer does not dry and crystallize over the sample spots.

Whole Blood Collection/Separation, Viral DNA/RNA Extraction, and Reverse Transcription

Whole blood was collected by venipuncture in either a Vacutainer (serum) or anticoagulant-treated tubes (plasma). Tubes were inverted several times, and stood upright for 30 – 60 minutes (for serum collection). Samples were then spun in a refrigerated centrifuge, and serum or plasma was aliquoted and stored at -80 °C. HBV or HIV nucleic acid was extracted using the Chemagic Viral DNA/RNA Kit (PerkinElmer), and HIV RNA was then reverse transcribed as per the iScript cDNA Synthesis kit (Bio-Rad).

Recombinase Polymerase Amplification (RPA) and Purification

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RPA was performed using either extracted DNA or reverse-transcribed cDNA using the TwistAmp Basic kit (TwistDx, UK). For HBV detection, a premix solution containing 0.48
385 pmol/μL of each forward and reverse primers (5'-GGCATGGACATTGACCCTTATAAAGAATTTGG-3', 5'-TGTCGAGAAGGTCCCGAATAGACGGAAAGA-3'), 9.2 μL of nuclease-free water, 29.5 μL of rehydration buffer, and 4 μL of the either extracted non-infected or infected DNA was prepared in a volume of 47.5 μL. This solution was then transferred to a tube containing the
390 reaction pellet and mixed. The reaction was initiated by the addition of 2.5 μL of 280 mM magnesium acetate, and incubated at 37 °C for 10 minutes to produce 100 base-pair amplicon.

For HIV detection, a premix solution containing 0.48 pmol/μL of each forward and reverse primers (5'-GAAAGGTGAAGGGGCAGTAGTAATACAAGACA-3', 5'-CCACACAATCATCACCT GCCATCTGTTTTCCA-3'), 11.2 μL of nuclease-free water, 29.5
395 μL of rehydration buffer, and 2 μL of the either extracted and reverse-transcribed non-infected or infected cDNA was prepared for a total volume of 47.5 μL. This solution was then transferred to a tube containing the reaction pellet and mixed. The reaction was initiated by the addition of 2.5 μL of 280 mM magnesium acetate, and incubated at 37 °C for 30 minutes to produce 116 base-pair amplicon.

400 RPA products were purified using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic), and eluted into 50 μL for detection. Purified DNA was visualized by gel electrophoresis, and kept at 4 °C until later use.

Mono-Infection Assays using Amplified Clinical Samples

Clinical mono-infection assays (Figure 5a-d) were performed directly on the microwell
405 chips using HIV- and HBV-negative, as well as HIV- and HBV-positive samples after
amplification. DNA detection strands from IDT DNA Technologies with Alexa647 fluorophore
on either 5' end (CD_HIV) or 3' end (CD_HBV), purchased HPLC-purified and used without
further purification, for the HIV and HBV target sequences, were prepared with concentration of
100 pmol/ μ L in TE buffer and stored at 4 °C until further use. To perform the assay, 1 μ L of the
410 conjugated microbead sample, corresponding to approximately 10^4 conjugated microbeads, was
deposited on a microwell chip for each assay condition and let dry for 1 hour. During this time
20 μ L of the amplified sample was mixed with 5 μ L of the corresponding detection strand and
denatured at 100 °C for 15 minutes. Then, the 25- μ L denaturation solution was mixed with 25
 μ L of hybridization buffer (10x SSC, 0.1% SDS, heated to 60 °C). The 50- μ L hybridization
415 solution was deposited over the dried conjugated microbead spot on the microwell chip and
incubated at 37 °C for 60 minutes, and let cool at room temperature for 5 minutes. The microwell
chip was then submerged in 200 mL of washing buffer (0.5x SSC, 0.1% SDS, heated to 37 °C),
washed by agitation for 10 s, washed again in another 200 mL of washing buffer to further
reduce non-specific binding, and let dry for 5 minutes before being imaged. Note that care must
420 be taken so that the washing buffer does not dry and crystallize over the sample spots.

Co-Infection Assays using Amplified Clinical Samples

Cross-reactivity between the amplified HIV and HBV clinical samples (CT_HIV and
CT_HBV) and their corresponding conjugated barcodes (CB_HIV and CB_HBV), as well as
positive and negative control cases (CB_Pos and CT_Pos, and CB_Neg and CT_Neg,
425 respectively), was studied (Fig 5e-h). First, 5 μ L of each conjugated barcode sample,

corresponding to approximately 5×10^4 barcodes each, were mixed together with 20 μL of DD water to produce a 2x dilution factor of the original. The dilution was to reduce microbead aggregation after deposition on chip, which may confound barcode resolution during analysis. To perform the assay, 1 μL of the diluted conjugated barcode mixture, corresponding to approximately 5×10^3 conjugated microbeads, was deposited on a microwell chip for each multiplexing case and let dry for 1 hour. During this time 10 μL of each amplified sample was mixed with 5 μL of each of the corresponding detection strands (5 μL of CD_HIV, 5 μL CD_HBV, and 10 μL of CD for both CT_Pos and CT_Neg) and denatured at 100 °C for 15 minutes. Then, the 60- μL denaturation solution was mixed with 60 μL of hybridization buffer (10x SSC, 0.1% SDS, heated to 60 °C). The 120- μL hybridization solution was deposited over the dried conjugated microbead spot on the microwell chip and incubated at 37 °C for 60 minutes, and let cool at room temperature for 5 minutes. The microwell chip was then submerged in 400 mL of washing buffer (0.5x SSC, 0.1% SDS, heated to 37 °C), washed by agitation for 10 s, washed again in another 400 mL of washing buffer to further reduce non-specific binding, and let dry for 5 minutes before being imaged. Note that care must be taken so that the washing buffer does not dry and crystallize over the sample spots.

Device Design and Construction

The device was designed using SolidWorks 2012 and 3D printed commercially (ReproDux, North York, Ontario, Canada). Laser diode excitation sources of 405 nm 50 mW (<http://www.ebay.com/itm/170719374707>), and 650 nm 50 mW (http://www.ebay.com/itm/1pcs-650nm-50mw-Red-Laser-Diode-Dot-Module-/370650098149?pt=LH_DefaultDomain_0&hash=item564c77a9e5) were purchased online and

secured into the device as delivered. The device was designed such that both lasers could excite the same spot on the chip. An excitation filter $\lambda_{\text{ex}} = 655/15$ nm (Edmund Optics) was fixed in front of the 650 nm laser diode source to reduce background signal. Both laser diodes were electrically connected to 2x AA batteries via a battery holder and single-pole triple-throw switch (both purchased from a local electronics shop) that switches between the two sources as well as an OFF state. A generic 160x – 200x pocket microscope was purchased online (<http://www.gadgetplus.ca/science/Microscope160-200x.html>). It was disassembled to extract the eyepiece and objective lenses, and installed into the device manually. The eyepiece was fixed in place but the objective was made to be movable along a track to allow focusing on the sample.

Sample Imaging

All images were acquired using the iPhone 4S from Apple (unless otherwise specified) mounted in our device. Quantum dot barcodes and Alexa647 fluorophore were excited using laser diodes of wavelengths 405 nm and 650 nm, respectively. Emission filters $\lambda_{\text{em}} = 430\text{LP}$ (Thorlabs), $\lambda_{\text{em}} = 530/10$ (Thorlabs), $\lambda_{\text{em}} = 580/10$ (Thorlabs), $\lambda_{\text{em}} = 640/10$ (Thorlabs), and $\lambda_{\text{em}} = 692/40$ (Semrock, Brightline Cy5-4040A) were placed in the device's emission filter slot one at a time during imaging. The emission filter $\lambda_{\text{em}} = 430\text{LP}$ was used in conjunction with a neutral density filter OD = 1.3 (Thorlabs) to image all barcodes to determine their size and location, while avoiding intensity saturation. The emission filters $\lambda_{\text{em}} = 530/10$, $\lambda_{\text{em}} = 580/10$, and $\lambda_{\text{em}} = 640/10$ corresponded with quantum dots QD540, QD589, and QD640, respectively, and were used to isolate for their fluorescence for resolving barcodes. The emission filter $\lambda_{\text{em}} = 692/40$ was used to isolate for the detection strand Alexa647 secondary label fluorescence as a means to measure the amount of analyte that hybridized with its corresponding capture strand. Image

exposure times, made adjustable with the use of the NightCap app from Apple's App Store, was maintained at 1 s for all filters. In the case of fluorophore particles, they were excited using only the 405 nm laser diode source and imaged using only the emission filter $\lambda_{em} = 430LP$, the images of which were used for subsequence intensity analysis.

Image Analysis

A custom-made algorithm was written in MathWork's MATLAB for all image analysis (see supplement section for the code). The algorithm accepts as inputs five emission filter images ($\lambda_{em} = 430LP$, $\lambda_{em} = 530/10$, $\lambda_{em} = 580/10$, $\lambda_{em} = 640/10$, and $\lambda_{em} = 692/40$) of a sample. The images were cropped to include microbeads of interest based on user selection. The cropped filter images were aligned with the $\lambda_{em} = 430LP$ filter image through the use of the discrete Fourier transform registration.^{36,37} The algorithm then identified the size and location of each microbead, based on its appearances in the $\lambda_{em} = 430LP$ filter image, using the Hough transform.^{38,39} Erroneously identified microbeads (e.g. debris, imaging artefacts, overlapping microbeads) were excluded based on user input. Each microbead was then associated with the mean pixel intensity across its area at each of the four remaining filter images. For each microbead, the $\lambda_{em} = 530/10$, $\lambda_{em} = 580/10$, and $\lambda_{em} = 640/10$ filter image intensities comprised its intensity profile, while the $\lambda_{em} = 692/40$ filter image intensity indicated the fluorescent secondary probe intensity. In order to identify the microbeads on the chip, known barcode intensity profiles were first established (Supporting Information Figure S9). These profiles were obtained by imaging all the barcodes – B_H1N1, B_H3N2, B_H5N1, B_FluB, B_HIV, B_HBV, B_HCV, B_Pos, B_Neg, CB_HIV, CB_HBV, CB_Pos, and CB_Neg – alone (Table 1) and calculating the mean filter intensity across all microbeads for each filter. A microbead's intensity profile was

then compared against each known barcode's intensity profile to identify the barcode of interest.

Specifically, a barcode was classified according to its type (i.e. synthetic or clinical sample) and

highest to lowest intensities among the filters $\lambda_{em} = 530/10$, $\lambda_{em} = 580/10$, and $\lambda_{em} = 640/10$. This

495 narrowed the selection down to either one barcode, in which case the barcode of interest was

identified, or two barcodes. Between the two possibilities B_{high} (with higher mean intensities) and

B_{low} (with lower mean intensities) a threshold was defined for B_{low} :

$$I_{barcode} = I_{mean} + I_{STD} \quad [1]$$

$I_{barcode}$ = Intensity threshold for B_{low} .

500 I_{mean} = Mean intensity of B_{low} .

I_{STD} = Intensity standard deviation of B_{low} .

This threshold was calculated for the highest intensity amongst the three filters $\lambda_{em} = 530/10$, λ_{em}

= $580/10$, and $\lambda_{em} = 640/10$ for B_{low} . If the highest intensities were similar in value between B_{low}

and B_{high} , the second highest filter intensity was used. With this, if the microbead's

505 corresponding filter intensity was equal to or lower than $I_{barcode}$, the barcode of interest was B_{low} ,

otherwise B_{high} was chosen. An example to illustrate the microbead identification process

(Supporting Information Figure S10):

A microbead B is imaged during a synthetic sample multiplexing test – thus classified as

'synthetic sample' – has an intensity profile of $[\lambda_{em} = 530/10, \lambda_{em} = 580/10, \lambda_{em} = 640/10] = [4,$

510 $40, 80]$, so $[\lambda_{em} = 640/10] > [\lambda_{em} = 580/10] > [\lambda_{em} = 530/10]$. On examination the set of barcodes

used for synthetic multiplexing (Supporting Information Figure S9a), B has a similar intensity

profile (i.e. same order of highest to lowest filter intensities) as B_{Pos} and B_{Neg} . Between

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them, B_Neg has the lower mean intensities and thus chosen as B_{low} , with the its highest filter intensity at $[\lambda_{em} = 640/10] = 30.50$. And because it is much lower and thus distinguishable from

515 B_Pos's $[\lambda_{em} = 640/10] = 80.35$, the intensity threshold is calculated using B_Neg's $[\lambda_{em} = 640/10]$:

$$I_{barcode} = I_{mean} + I_{STD} = 30.50 + 6.26 = 36.76.$$

Since B's $[\lambda_{em} = 640/10] = 80$ is greater than $I_{barcode} = 36.76$ from B_Neg, B is identified as B_Pos.

520 We determined whether the analyte of interest is present by using the intensity values from the $\lambda_{em} = 692/40$ filter, which isolates for the Alexa Fluor 647 secondary probe signal. For the synthetic sample sensitivity assays the intensities were used directly to establish the limit of detection and dynamic range for the device. However, we used a micobead-counting method to determine the optical detection of multiplex samples and clinical samples because we achieve
525 greater accuracy in the measurement when the measurement is based on a comparison to negative controls. Negative and positive controls are always required in analyzing complex samples, as these control samples confirm whether a technique is working as designed. When we conduct the measurements, we develop a histogram of the fluorescence intensity from the secondary probe and compare that signal to those of the negative sample. Equation 2 describes
530 this analysis.

$$Q = (\text{population of barcode whose } \lambda_{em}=692/40 \text{ filter intensity} \geq I_{assay}) / (\text{total barcode population})$$

[2]

That is, the barcodes whose secondary probe intensities were equal to or above the threshold I_{assay} , defined empirically, were counted and a percentage, relative to the barcode's total population, was calculated. In the case of multiplexed detection of synthetic bloodborne viral targets (Figure 4), a detection was considered positive if $Q > 30\%$ (i.e. over 30% of said barcode had secondary probe signals above the threshold). In the case of amplified mono- and co-infected clinical samples (Figure 5), a detection was instead considered positive if $Q > 3\%$ due to their overall lower signals.

Statistics and General Methods

All data represent analysis from at least 100 barcodes to ensure they are representative of the experimental conditions studied. The variability in the number of barcodes analyzed is due to the field of view. In some cases, there are more microbeads per field of view than others. Once we analyze > 100 barcodes, the measurements were relatively consistent and did not influence the statistics. For the detection of mono-infected patient samples, we used infection-negative subject samples as a negative control, which is procedurally and clinically more accurate as negative controls than any other types of samples. To ensure clinical sample blindness, they were prepared and provided unlabeled by the collaborators (MJB, MO, and JJF) to the investigators (KM, JK, and WCWC), who then performed the experiments without knowledge of the samples' identities. For where statistical tests were necessary (i.e. comparing the infection-negative subject groups with the HIV- or HBV-infected subject groups), the two-sided t -test was used because the data exhibits normal distribution (e.g. [Supporting Information Figure S3](#)).

Human Subjects

The de-identified clinical samples were obtained from the Toronto Western Hospital
555 Liver Clinic and St. Michael's Hospital biobank repository. The protocol was approved by the
Research Ethics Board of the University Health Network and St. Michael's Hospital, both
affiliates of the University of Toronto. All patients provided written informed consent for storage
and use of their specimens for research.

560 FIGURE CAPTIONS

Figure 1. Overview of the smartphone device utilizing quantum dot barcodes. (a) The assay involves the addition of patient samples to a chip coated with microbeads, which are optically barcoded by quantum dots and are coated with molecules that recognize a target analyte. This target analyte joins the barcode to the secondary probe. Since each barcode is conjugated with a known bio-recognition molecule for a specific pathogen target, the imaging of the optical signal from the barcode would allow for the identification of the pathogen and whether it is present in a patient sample (i.e. lack of secondary probe signal indicates no pathogen present, in this case the yellow microbead). (b) A typical microwell chip containing different barcodes in each well. In a biological assay, we add a 20- μ L (for multiplexing synthetic targets), or a 50- μ L (for mono-infection patient samples) total hybridization sample on the chip (see black arrow), incubate at 37 °C for 20 – 60 minutes, rinse, and image. (c) A smartphone camera captures the image of four different quantum dot barcodes arrayed on the surface of the chip. These barcodes are excited with a violet laser source (λ_{ex} = 405 nm, 50 mW), optical signals collected by a set of lenses, filtered with 430 nm long-pass filter, and imaged using an Apple iPhone 4S smartphone with an exposure time of 0.05 s. (d) Two excitation sources excite the quantum dot barcoded chip independently. The optical emission is collected by a set of objective and eyepiece lenses, imaged using a smartphone camera, and interpreted as positive or negative detection using a custom-designed algorithm. The images may be sent wirelessly to a centralized facility for further evaluation or for the mapping and tracking of infectious diseases. (e) Image of the smartphone device.

Figure 2. Comparison of the optical properties between organic dye molecules and quantum dot inside polystyrene microbeads. Polymeric particles impregnated with fluorophores (‘Yellow’ and ‘Nile Blue’) are compared with quantum dot barcodes (‘QD540’ and ‘QD640’): excitation/absorption and emission spectra; visual images captured by our device when excited using a 405 nm laser excitation source; emission spectra under varying excitation wavelengths; and photobleaching under continuous excitation in the device, representing the average intensities of 591, 642, 1198, and 1145 barcodes analyzed, over the 180 s duration, for ‘Yellow’, ‘Nile Blue’, ‘QD540’, and ‘QD640’, respectively.

Figure 3. Visual demonstration of a barcode assay and device assay sensitivity. (a) Yellow, green, and red barcodes (identified as B_HBV, B_HCV, and B_Pos in Table 1, respectively) are deposited on the chip and imaged using the device (λ_{ex} = 405 nm, λ_{em} = 430LP, exposure time = 1 s). (b) After the assay, the device-acquired fluorescence image of the microbeads bound with the target analyte and secondary probe (λ_{ex} = 655/15, λ_{em} = 692/40, exposure time = 1 s). Both green and red microbeads had positive signals. This demonstrates that their respective genomic targets are present in the sample but not for the yellow barcode. (c) Sensitivity curves for genetic biomarkers for the bloodborne viruses human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), representing 8017, 7324, 10334 barcodes analyzed, respectively. (d) Sensitivity curves for genetic biomarkers for the influenza A viruses H1N1, H3N5, and H5N1; and influenza B virus (FluB), representing 8491, 5967, 10182, and 4114 barcodes analyzed, respectively. All values represent the average secondary probe intensity, and

error bars were calculated based on the standard deviation from three replications of each condition.

Figure 4. Multiplexed detection for synthetic target strands for the bloodborne viruses HIV, HBV, HCV with positive and negative controls. (a) Only positive control was present during hybridization, representing 1039 barcodes analyzed. (b) Targets for HIV and positive control were present during hybridization, representing 1581 barcodes analyzed. (c) Targets for HBV and positive control were present during hybridization, representing 1749 barcodes analyzed. (d) Targets for HCV and positive control were present during hybridization, representing 1202 barcodes analyzed. (e) Targets for HIV, HBV, and positive control were present during hybridization, representing 2401 barcodes analyzed. (f) All targets except for negative control were present during hybridization, representing 1343 barcodes analyzed. Results represent data from three experimental replications of each condition combined into a single data set. Note that samples above the dashed 30% line are considered positive detection, otherwise they are considered negative detection.

Figure 5. Detection of HIV and HBV in patient samples. (a) Detection of mono-infected amplified samples from 3 HIV-negative subjects and 10 HIV-positive patients, representing 1306, 728, 321, 1573, 626, 297, 1268, 429, 468, 499, 332, 375, and 287 barcodes analyzed, respectively, for each subject. (b) Detection of mono-infected amplified samples from 3 HBV-negative subjects and 7 HBV-positive patients, representing 361, 207, 1345, 371, 221, 1310, 806, 665, 778, and 674 barcodes analyzed, respectively, for each subject. (c) Comparison between the average combined statistics of all subjects of the HIV-negative group (3 subjects) and HIV-positive group (10 patients) from (a); error bars represent standard deviation, with statistical significance ($P < 0.05$) indicated and determined using two-sided t -test. (d) Comparison between the average combined statistics of all subjects of the HBV-negative group (3 subjects) and HBV-positive group (7 patients) from (b); error bars represent standard deviation, with statistical significance ($P < 0.01$) indicated and determined using two-sided t -test. (e-h) Detection of co-infection assays simulated with amplified HIV- and HBV-negative, as well as HIV- and HBV-positive patient samples. (e) Only positive control was present during hybridization, representing 384 barcodes analyzed. (f) HIV-positive patient sample and positive control were present during hybridization, representing 866 barcodes analyzed. (g) HBV-positive patient sample and positive control were present during hybridization, representing 1888 barcodes analyzed. (h) HIV- and HBV-positive patient samples, and positive control, were present during hybridization, representing 1019 barcodes analyzed. Results represent data from three replications of each condition combined into a single data set. Note that samples above the dashed 3% line are considered positive detection, otherwise they are considered negative detection. All samples were amplified. Additionally, all samples were blinded during the experiment to reduce bias.

Figure 6. Integrated assembled device. (a) A visual representation of the envisioned final device, with the different compartments colored for clarity, capillary tubes to show the flow of solutions between them, and a smartphone for scale. (b) Schematics of the envisioned final device with colors matching the various compartments in (a) and numbers indicating the movement of sample in these compartments. (c) A flow diagram illustrating the steps required for detecting pathogen targets using the envisioned final device, from patient sample to imaging and analysis,

with colors and numbers matching the compartments in (a) and (b). The liquid can be moved from one compartment to the next using electrically-driven flow.

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