Rapid and Portable Genetic Identification Kits for World Health Applications

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Abstract We motivate the need for a rapid and portable genetic identification kit whose design must be fundamentally different from the DNA micro-arrays used in laboratories because of speed, detection sensitivity and facility concerns. A micro/nano bead platform developed in our laboratory with modified PCR and hybridization steps and with tailor-designed microfluidic fabrication techniques is then introduced as a first single-target prototype for rapid field-use genetic diagnostic kit.

1. Introduction. New infectious pathogens have introduced new threats to world health. Avian influenza and severe acute respiratory syndrome (SARS) are particularly relevant to China. Additional examples of infectious diseases in Asia and elsewhere in the third world include hemolytic uremic syndrome and bloody diarrhea (Escherichia coli O157:H7), tuberculosis (Mycobacterium tuberculosis), anthrax (Bacillus anthracis), pneumonia (Streptococcus pneumoniae), malaria (Plasmodium), hepatitis (Hepatitis A, B, C, D, and E virus), and hemorrhagic fever (Ebola virus) amongst many others. All told more than 50 new, or newly discovered, pathogens have been identified over the past 30 years. In addition to these new discoveries, there exists the constant threat of re-emerging pathogens with antibiotic resistance such as pneumococci, enterococci, staphylococci, Plasmodium falciparum, and Mycobacterium tuberculosis.

The ability to detect infectious pathogens is hence vital to mounting a quick and effective response to outbreaks of pathogen-caused disease. The clinical microbiologist, nurse, or technician is thus often tasked with answering the question, "Are there pathogens present in my sample (be it a clinical sample, food sample, environmental sample, etc), and if so, what is/are their identity/identities, and in what number are they present?" In general, the answer to the first part (what identities) may vary from just a few (< 5) to hundreds, and the number of each type may vary over several orders of magnitude (from a few to millions of colony forming units (CFU) per ml of sample).

The traditional, and most general, technique designed to answer this catch-all question, is the plate-culture method with bacteria screening agents but its sensitivity and slow response time have made it ineffective for effective pathogen detection. These inherent limitations of culture-based detection methodologies has resulted in interest directed at, and effort devoted to, developing alternate pathogen detection and quantification methods. One popular new detection method is standard micro-well plate enzyme-linked immunosorbent assay (ELISA). Genetic nucleic acid identification techniques have also become increasingly common. It is the belief of this reviewer that such DNA and RNA identification will be the key diagnostic techniques that can meet the stringent demands of infectious pathogen detection in the field. Genetic identification can detect less than 100 CFU per ml of pathogen by using PCR amplification. It is extremely species sensitive, much more sensitive than bacteria screening by culturing or antibody-based assays. However, genetic identification techniques still cannot be used in the field. The DNA micro-array, which is the leading genetic identification product, remains inadequate for this task, as it requires a lab-bound confocal facility and hour-long hybridization time.

The fundamental methods developed for nucleic acid diagnostics are enzymatic DNA restriction, nucleic acid hybridization, polymerase chain reaction (PCR), and fluorescence-based

techniques. Of these techniques, DNA amplification procedures utilizing PCR and PCR-based methods (real-time PCR, stand displacement amplification, nested PCR, etc) are of particular interest given that DNA is present at a low concentration within a typical biological sample. Consequently genetic identification requires microfluidic PCR-based technologies. Several excellent reviews of pathogen identification through diverse nucleic acid based assays can be found [1,2].

What is needed is an integrated microfluidic system containing both the PCR and fluorescent detection system. PCR chips such as those first reported by Mortola are now commercially available. They include GeneXpert® system produced by Cepheid (Sunnyvale, CA) and BioMark™ 48.48 Dynamic Array developed by the Fluidigm® Corporation (San Francisco, CA). However, the detection of the amplified target DNA remains a technical challenge. The standard technique is to use hybridization surface assays with fluorescent detection. As the hybridization reaction is mass-transfer limited, the small dimensions of a microfluidic chamber reduce the response time to minutes, compared to hours for the DNA array. Fluorescent detection without a confocal facility has become the final technical bottleneck.

Efforts to miniaturize optical detection platforms are currently underway, and are discussed in detail elsewhere. Light emitting diodes (LEDs) coupled with optical filters and silicon photodiodes offer the potential for developing a miniaturized optical detection platform [3]. In producing a hand-held device designed for real-time PCR, Higgins et al [4] developed a miniature optical platform employing two LEDs, at wavelengths of 490 nm and 525 nm, along with a miniaturized PMT. These optical sensors, however, remain insensitive and expensive.

2. The Micro/Nanobead Platform. One simple solution to the sensitivity issue is to use a micro-bead platform such that the oligos are functionalized onto beads [5]. Due to the large surface area

per unit volume of such beads, a micro-reservoir mm in dimension can capture all the target DNAs in a cc-volume sample with picomolar sensitivity. Yet, because all these fluourescent molecules are concentrated within a micro-liter volume, the fluorescent intensity is extremely high. As a reference, a single pixel on a DNA microarray has an area of 10^{-2} cm^2 and that in a microliter of microbeads (50% by volume) is four orders of magnitude higher, with a proportionally larger fluorescent intensity. With this enhancement, laser excitation and confocal detection can be eliminated. A simple optical filter with a digital camera, a diode sensor or a CCD camera can be adequate for positive-negative identification. Quantification of the target DNAs may not be possible but rapid and portable positive-negative diagnostics have many important field-use applications as a preliminary screening step: epidemic control at ports/airports, avian flu monitoring of poultry imports, environmental monitoring etc.

We have successfully developed the bead based hybridization platform for genetic identification of a single target. This is achieved by trapping the probe functionalized silica beads within a small chamber in a microfluidic channel and then passing biotinylated ssDNA (100 microlitre) through the packed bead chamber at 50˚C at a flow rate of 0.5 ml/h. The passing of DNA through packed bead chamber largely reduces the separation distance between target DNA and oligomer probes on the surface of the beads that reduces hybridization time. Additionally, this bead system offers a much greater surface area for hybridization, thus enhancing the detection sensitivity. After washing the unhybridized DNA from bead chamber, hybridization detection was accomplished through the addition of streptadivin bound fluorescent dye, thus taking advantage of the strong streptadivin-biotin binding reaction. Excess dye was then washed from the channel and fluorescence was measured using a confocal microscope. If the passing of DNA through the channel is considered the starting point for detection, then the detection time is 2-3 h and the detection sensitivity is in the range of 100 pM-nM. This bead based hybridization on a microfluidic chip is schematically represented in Figure 1.

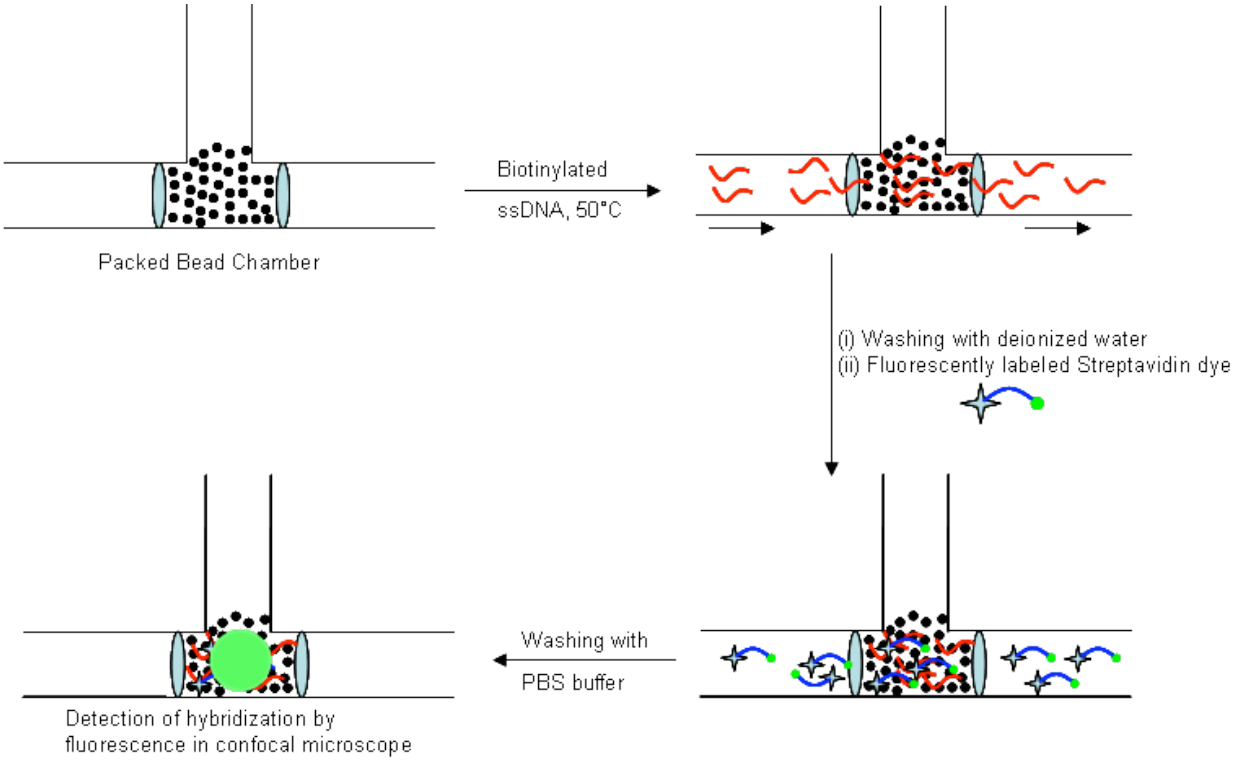


Figure 1. A single-target bead based genetic assay with large fluorescent signal intensity.

This bead based detection technique is comprised of number of steps, such as (i) Fabrication of microchannel on a glass slide using commercially available Loctite 363 UV curable glue, (ii) Fabrication of filters within the microchannel using a mixture of methacrylate photopolymers, (iii) Functionalization of oligomer probe on silica beads, (iv) asymmetric PCR, and finally the successful execution of hybridization by passing the target DNA solution through the packed bead microchannel within a chip. On-chip filter fabrication represents a new technology and is described in more detail below. The filter needs to be strongly bonded with the micro-channels and we employ an integrated UV curing polymerization process for both the micro-channel fabrication and the micro-filter fabrication such that the two are strongly bonded.

3. Microfluidic Fabrication. The fabrication of microchannel and bead trap is achieved by using a glass slide, a cover slip, UV curable Loctile 363 glue, a tap used as a spacer, a UV source and the solvents, acetone and methanol, as shown in Figure 2.

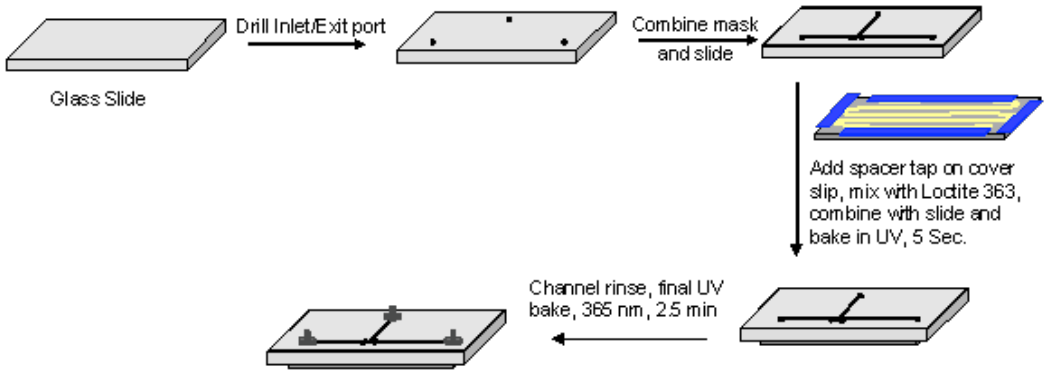


Figure 2 Micro-channel fabrication using UV polymerization curing.

The fabrication of filter is done inside the microchannel by using a mixture of methacrylate photopolymers (monomer) along with a mixture of toluene and isobutanol (porogen). By changing the ratio of monomer and porogen, the pore diameter of the filter can be manipulated. We are making the filter of pore size of ~2 micron and using 10 micron bead for hybridization experiment. The steps involving the fabrication of filter within the microchannel is schematically depicted in the following figure.

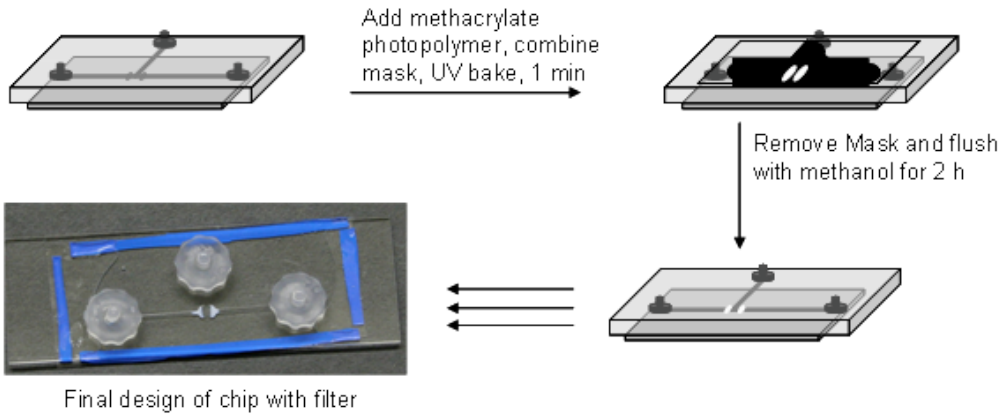
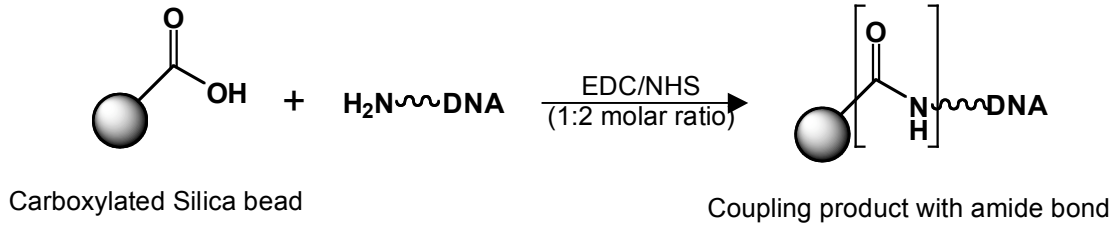


Figure 3 Micro-filter fabrication with the same technique.

4. Bead Functionalization, PCR Design and Hybridization Detection. Amine conjugated 27 mer oligo probe are functionalized to the carboxylated silica beads by coupling with water soluble carbodiimide (EDC) and N-hydroxysuccinimide (NHS).



Carboxylated Silica bead

Coupling product with amide bond

To make the bead based hybridization platform simpler and straightforward and also to avoid the denaturation of target DNA step in hybridization experiment, as the denatured ssDNA would recombine together before it reaches and interacts with probe functionalized silica bead trapped within the microchannel, asymmetric PCR is performed to produce single stranded DNA. In this approach, an unequal concentration of primers are used otherwise it is similar to normal, symmetric PCR. Initially, amplification starts exponentially, but as the lower concentrated primer is exhausted, the higher concentrated primer continues to amplify to produce single stranded DNA.

To carry out the hybridization experiment, the first step is to pass 2% Serum bovine albumin (BSA) solution through the microchannel to prevent any non-specific binding of target DNA and fluorescent dye to the filters and the surfaces of the channels. Then, the probe functionalized silica beads are trapped and packed properly by passing 4X SSC, hybridization buffer solution. After successful packing of beads, 100 µl biotinylated ssDNA are flown through it at 50˚C at a very slow flow rate of 0.5 ml/h for hybridization with the complementary oligoes functionalized on silica surfaces. E. Pure water is then passed to wash all non-specific DNA

bound either to the surface of the beads or to the filter. Finally, the fluorescently labelled streptavidin solution is passed through it and excess fluorescent dye is washed from the chamber by passing PBS buffer solution.

5. An Integrated Prototype. In collaboration with Professor Weijia Wen's group at the Hong Kong University of Science and Technology, we have recently developed an integrated and portable PCR-detection kit with the nanobead and microbead platform for single-target detection in 30 minutes, with most of the time used for the PCR cycles. As seen in Figure 4, the fluorescent signal from the trapped beads can be easily picked up with a portable digital camera without a confocal facility. This disposable chip contains on-chip pumping and valving, as well as diode sensor. It is hence the first self-contained portable genetic identification kit. The first product is expected to be on the market in 3 years.

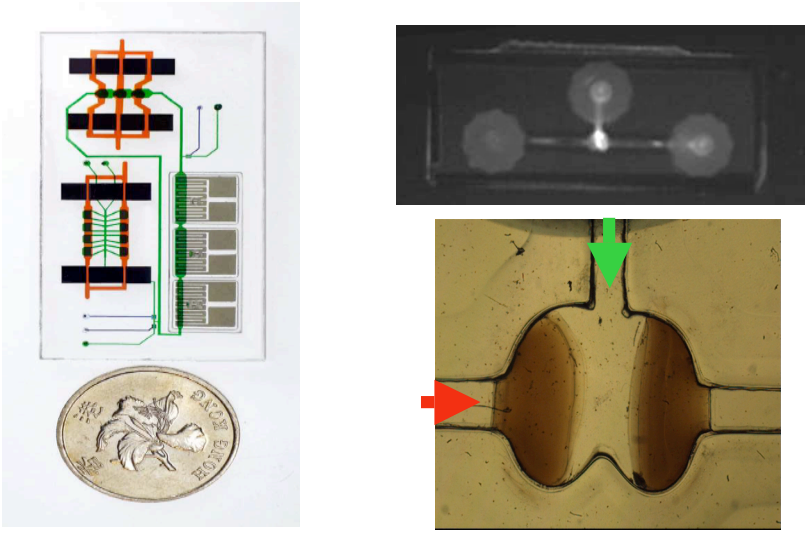


Figure 4. The size of the integrated PCR-Detection chip compared to a Hong Kong coin. The beads are confined within a microliter nanoporous polymer trap at the top of the chip. The

PCR unit and heater-controlling electrodes are at the bottom half of the chip. Blowups of the microliter trap and the fluorescent image by a digital camera with an optical filter are also shown.

[1] Barken, K. B., Haagensen, J. A. J., Tolker-Nielsen, T., Clin. Chim. Acta 2007, 384 (1-2), 1-11.

[2] Mothershed, E. A., Whitney, A. M., Clin. Chim. Acta 2006, 363 (1-2), 206-220.

[3] Meldrum, D. R., Science 2002, 297 (5584), 1197-1198.

[4] Higgins, J. A., Biosensors & Bioelectronics 2003, 18 (9), 1115-1123.

[5] Chang, H.-C., AIChE J 2007, 53, 2486-2490.

About the Author: Professor Hsueh-Chia Chang grew up in several diaspora Chinese communities in Taiwan, Singapore, Malaysia and Southern California. He received his BS from Caltech in 1976 and his PhD from Princeton University in 1980. He is currently the Director of the Center for Microfluidics and Medical Diagnostis at Notre Dame and the founding editor-in-chief of Biomicrofluidics, an American Institute of Physics journal (http://bmf.aip.org). His awards include the Frankiel Award from the American Physical Society and the Presidential Young Investigator Award from NSF. He is also a fellow of APS. He is popular on the lecture circuit, having delivered numerous named lectures, keynote lectures and plenary talks over three continents. Professor Chang has more than 200 publications and over 3000 citations. He has placed more than a dozen students in academic institutions throughout the world, including Howard, Michigan State, Florida, Mississippi State, Tennessee, Missouri, UC San Diego in the

US; Cheng Kung, Tung Hai and Chong Cheng in Taiwan; Imperial College in the UK, Wuhan in China (a Luojia Chair holder) and Monash in Australia. He travels to Asia constantly and collaborates with several groups in Taiwan, Singapore, Hong Kong and China. His group website is www.nd.edu/~changlab/.