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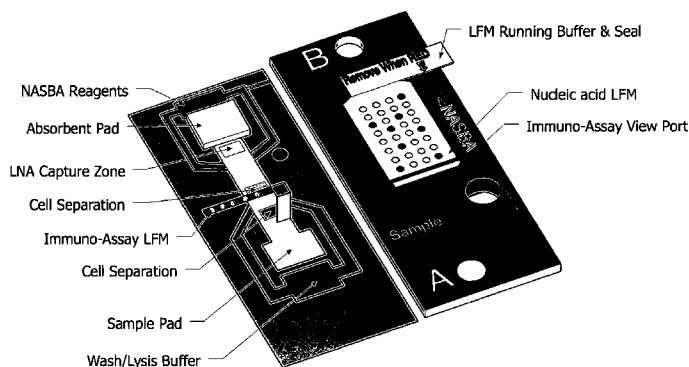
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(54) **Title:** HIGHLY SIMPLIFIED LATERAL FLOW-BASED NUCLEIC ACID SAMPLE PREPARATION AND PASSIVE FLUID FLOW CONTROL

FIG. 15



(57) **Abstract:** Highly simplified lateral flow chromatographic nucleic acid sample preparation methods, devices, and integrated systems are provided for the efficient concentration of trace samples and the removal of nucleic acid amplification inhibitors. Methods for capturing and reducing inhibitors of nucleic acid amplification reactions, such as humic acid, using polyvinylpyrrolidone treated elements of the lateral flow device are also provided. Further provided are passive fluid control methods and systems for use in lateral flow assays.

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## HIGHLY SIMPLIFIED LATERAL FLOW-BASED NUCLEIC ACID SAMPLE PREPARATION AND PASSIVE FLUID FLOW CONTROL

### 5 STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Contract No. DE-AC52-06NA25396, awarded by the United States Department of Energy. The  
10 government has certain rights in this invention.

### RELATED APPLICATIONS

This application claims priority to United States Provisional Application No.  
15 61/126,645 filed May 5, 2008.

### BACKGROUND OF THE INVENTION

The diverse nature of biological sample matrices present a need for robust yet  
20 general front-end sample processing methods that enable the collection of trace analytes even when present in complex mixtures of non-probative sample constituents [5]. These challenges are often compounded by the presence of materials confounding to effective immunological or molecular analytical techniques. For example, samples derived from human tissue are likely to contain  
25 complex polysaccharides, hemoglobin, iron and other substances known to be inhibitory to DNA polymerases employed for polymerase chain reaction (PCR). Similarly, environmental samples or trace samples contaminated with environmental constituents, such as soil or plant material, can also contain organic materials, such as humic acids, that are strongly inhibitory to PCR and  
30 other enzymatic reactions critical to thorough nucleic acid analysis.

Although reliable nucleic acid isolation methods applicable to diverse biological samples have been reported for both DNA and RNA [5-11], such methods are labor intensive, dependent upon laboratory instrumentation and require hours to

complete resulting in limited sample throughput and significant sample backlogs [12]. Down-stream enzymatic manipulations, such as polymerase chain reaction (PCR), can be adversely impacted by the presence of matrix constituents inhibitory to enzymatic activity rendering reliable sample preparation indispensable [5]. Hemoglobin, iron and complex polysaccharides are commonly encountered in biological samples while additional inhibitory compounds such as humic acids often accompany environmentally collected samples containing soil, plant material or decaying mater [13-15]. Additionally, the trace nature of many analytes in diagnostic and forensic samples as well as the abundance of closely related but non-probative constituents contribute significantly to analytical challenges [16-20].

Lateral flow immuno-chromatography is well established and has been used for the detection of proteins and small molecules for many years [21, 22]. Indeed, immuno-capture during lateral flow is the basis for rapid hand-held immuno-assays that have found widespread use in the point-of-care (e.g. group A Streptococcal antigen) and in the home (e.g. pregnancy tests). While these assays make use of immuno-capture during lateral flow as a detection end-point, we propose the use of the same principle as a means of attaining rapid and efficient immuno-capture as a first step in a sample preparation strategy designed to enable the recovery of scarce targets (cells, viruses, spores) from mixed samples. Once captured in the stationary phase, these targets can then be subjected to further processing for nucleic acid isolation or collected for other analyses.

Nucleic acid-based assays for pathogen detection and identification offer sensitivity, specificity and resolution. These characteristics render nucleic acid analysis a powerful diagnostic and forensic technique. Nonetheless, many technologies for nucleic acid preparation have focused on isolation from relatively abundant samples such as clinical blood specimens. Many applications, however, often must address the need to isolate and identify trace constituents in complex mixed samples of diverse origin. In contrast to DNA-based assays, immunoassays have found widespread acceptance in low cost, easily used formats, perhaps

most notable of which is the chromatographic lateral flow immunoassay [23]. Lateral flow assays, also known as hand-held assays or dipstick assays, are used for a broad range of applications where rapid antigen detection is required in an easily used, low cost format. Lateral flow immunoassays have been successfully employed for pathogen identification, diagnostics, and environmental and agriculture surveillance [4]. Several chromatographic lateral flow assays have been described for the detection of nucleic acid sequences using a variety of detection techniques [24-28]. Early work made use of cumbersome enzymatic detection strategies that relied on time consuming manipulations of dipsticks following introduction of the sample [25, 26] and detection schemes poorly suited for multiplexed applications [24, 27].

More recently described, the Lateral Flow Microarray (LFM) is a miniaturized lateral flow-based method for multiplexed nucleic acid detection [1]. The approach makes use of DNA microarray-like patterning of a small lateral flow chromatography strip allowing multiple nucleic acid sequences to be detected in a single assay. The reduced surface area of the device confers several advantages over traditional lateral flow device form factors. Sample volumes are reduced to 10  $\mu$ L resulting in reduced reagent consumption as well as reduced sample transport times. Moreover, hybridization times exhibited by the lateral flow microarray (LFM) are significantly reduced compared to standard glass substrate microarrays, which typically are allowed to hybridize with sample for several hours, as well as more complex microarray implementations that make use of microfluidic systems to facilitate more rapid hybridization [1, 29, 30]. The convective fluid movement through the lateral flow substrate as well as the open-ended pores of the membrane substrates employed result in superior chromatography performance compared to bead-based column chromatography [31-34]. These factors result in hybridization-based detection of < 250 amol of analyte in 2 minutes [1]. LFM is further described in United States Patent Application No. 11/894,910 and PCT International Application No. PCT/US2007/018537.

The LFM platform has been used to develop a rapid assay for *Bacillus anthracis*, the causative agent of anthrax, and has been shown to detect RNA from as few as 2-3 *B. anthracis* cells when present in a complex nucleic acid background consisting of 1 µg of total human RNA [1]. The reported LFM approach made use of standard laboratory methods for RNA isolation and an isothermal RNA amplification scheme known as nucleic acid sequence based amplification (NASBA) [35-37]. Perhaps most significantly, the miniaturization of lateral flow exemplified by the LFM, offers a physical configuration amenable to integration with fluidic or microfluidic systems for sample preparation support.

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Integration of LFM-based protein and nucleic acid detection with simplified sample processing methods would offer several potential advantages for processing and screening of a broad range of sample types, and is desirable. Similarly, more robust sample preparation methods applicable to trace and/or dilute analytes would greatly facilitate nucleic acid amplification and detection in point of care and field deployed assays.

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### SUMMARY OF THE INVENTION

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The invention provides highly simplified lateral flow chromatographic nucleic acid sample preparation methods, devices, and integrated systems for the efficient concentration of trace samples and the removal of nucleic acid amplification inhibitors. The invention makes use of LFM technology and passive fluid flow control systems, enabling a combination of (a) rapid immuno-affinity capture of analytes, including eukaryotic and prokaryotic cells, viruses and plant cells and material, and (b) hybridization-based affinity capture of specific DNA or RNA sequences to be concentrated and washed free of contaminating proteins and other matrix derived constituents after cell/virus lysis. The invention also provides means for removing inhibitors of nucleic acid amplification reactions (i.e., PCR) within the context of a lateral flow device.

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The invention also provides lateral flow structures for passively controlling the flow of different and multiple solutions used in and the reaction sequences of a lateral flow assay. In one embodiment, at least the sample receiving zone of the LFSP device comprises a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of at least one fluid flow therein, such as nitrocellulose. In one embodiment, the invention provides a device comprising a lateral flow matrix which defines a flow path and which comprises a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of at least one fluid flow therein. In other embodiments described herein, a geometrically-defined nitrocellulose strip has a configuration essentially as shown in FIGS. 5, 6, 7, 8, 9 or 14A.

The devices and systems of the invention are easily fabricated and use lateral capillary flow chromatographic substrates as a support for efficient low volume affinity purification of nucleic acids from biological samples. The invention's approach to sample preparation enables highly efficient capture of trace analytes at spatially defined regions in a lateral flow sample preparation (LFSP) device. This results in a high local concentration of target analyte immobilized on the substrate, thus greatly facilitating extensive washing and additional manipulations including amplification with no user intervention or additional sample handling. These features, together with their compatibility with established molecular analytical techniques such as real-time PCR, MLVA, genotyping and other nucleic acid-based methods, render lateral flow sample concentration and processing an attractive means of obtaining suitable nucleic acids for environmental surveillance, disease diagnostics and bioforensic investigations.

In one embodiment, a LFSP device comprises a lateral flow matrix which defines a flow path and which comprises in series the following elements: (a) a sample receiving zone for receiving an aliquot of a fluid sample; and (b) an immuno-capture zone in lateral flow contact with said sample receiving zone, which contains immobilized antibody reactive with a ligand present on a biological particle or cell of interest. Another embodiment further comprises, in series: (c) a

lysis zone, in lateral flow contact with said immuno-capture zone, wherein lysis of the biological particle or cell of interest is achieved, thereby liberating nucleic acid therefrom. In still another embodiment, a LFSP device further comprises, in series: (d) one or more assay zones, in lateral flow contact with the lysis zone, which together form the nucleic acid and labeling constituents for a sandwich nucleic acid hybridization assay. In yet another embodiment, a LFSP device further comprises a nucleic acid amplification zone downstream of and in lateral flow contact with the lysis zone, and upstream and in lateral flow contact with the assay zone(s). This aspect of the invention is further described by way of the Examples, *infra*.

LFSP devices of the invention may also incorporate the invention's methods for capturing and reducing inhibitors of nucleic acid amplification reactions, such as humic acid, using polyvinylpyrrolidone treated elements of the lateral flow device. Thus, referring to the above embodiments, a further embodiment of a LFSP device further comprises a pre-treatment zone comprising polyvinylpyrrolidone, in lateral flow contact with the element or elements of the device to which it is adjacent. For example, a treatment zone may be placed upstream of and in lateral flow contact with the immuno-capture zone, and downstream and in lateral flow contact with the sample zone. In related embodiments, substances other than or in addition to polyvinylpyrrolidone may be incorporated into the pre-treatment zone in order to capture or reduce unwanted inhibitors or other contaminants from the sample matrix prior to amplification and/or assay. This aspect of the invention is described further in the Examples, *infra*.

LFSP devices of the invention may also incorporate the invention's passive fluid control methods and systems. Briefly, passive solution or buffer flow control is achieved by cutting lateral flow membranes (e.g., nitrocellulose membranes) or other bibulous material, such as chromatography paper capable of supporting capillary flow/fluid wicking, into distinct geometries, such that individual flow paths, of varying lengths and/or widths, are defined for each of the buffers to be employed in an assay conducted using the device, yet are integrated within a

single membrane. This aspect of the invention is described further in the Examples, *infra*.

5 LFSP devices of the invention may be used in any LF format, but may be particularly suited to use with LFM methods, devices and systems. Fully integrated, sample-to-answer assay devices comprising LFSP integrated with LFM are envisioned.

## 10 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Lateral flow microarrays (LFMs) were challenged with NASBA reactions programmed with crude lysate from the indicated number of *E. coli* cells. Positive hybridization controls mark each row of spots on the LFM (column of five spots on the left side of the LFMs) and a set of duplicate spots indicate positive detection of *E. coli* (second row from the bottom, right side). "Negative" is a no template control. "Positive" contains 6 ng of *E. coli* RNA isolated using a Qiagen RNeasy kit. As few as 2000 cells could be detected using a crude lysate prepared by heating cells in cells-to-cDNA buffer (Ambion). See Example 1, *infra*.

20 FIG. 2. Lateral flow lateral flow facilitated immuno-capture. (A) Agdia TMV immuno-assay strips were run using 200  $\mu$ L of the indicated dilution of dried cure tobacco leaf extract (DCTLE) in SEB1 extract buffer (Agdia, Inc.). DCTLE was generated by crushing 100 mg of dried cured tobacco leaf in 3 ml SEB1 extract buffer (Agdia, Inc.) in a plastic bag containing an abrasive mesh (Agdia, Inc.). Dilutions of 1:200 and greater were negative by immuno-assay. (B) Real-time reverse-transcriptase PCR (RT-PCR) was used to examine regions below, at and above the TMV capture zone (CZ). 200  $\mu$ L of undiluted extract was subjected to lateral flow and subsequent real-time RT-PCR of strip regions below, at and above the capture zone. A strip region from below the CZ revealed little or no detectable amplification with a cycle threshold (Ct) value of 29.7. A sample taken from the CZ generated a strongly positive signal for TMV with a Ct value of 26.3.



A region above the capture zone also resulted in positive detection with a Ct value of 27.1. Thus, neat extract generated clearly positive PCR reactions only at and above the CZ while the region below the CZ inhibited PCR amplification. These data demonstrate that simple lateral flow immuno-capture without washes or further manipulation can alleviate PCR inhibition both through concentration of target particles and through physical sequestration of inhibitory matrix constituents. Significantly, the region above the CZ in the neat extract generates a positive PCR reaction apparently as a result of viral particle bleed-through from the CZ and an apparent concomitant depletion of inhibitors. (C) Real-time reverse-transcriptase PCR (RT-PCR) was used to examine regions below, at and above the TMV capture zone (CZ) of TMV immuno-assay test strips following challenge with 200  $\mu$ L of a 1:2000 dilution of DCTLE in sample buffer. A strip region from below the CZ revealed only weak amplification suggesting at this dilution inhibitors became sufficiently dilute to allow some amplification to take place (Ct = 28.9). A sample taken from the CZ generated a strongly positive signal for TMV with a Ct value of 22.4 suggesting that the combined effect of inhibitor dilution and immuno-capture mediated virus concentration conspire to enable more robust amplification relative to neat extract experiments (compare with part A). A region above the capture zone also resulted in positive detection with a Ct value of 28.0.

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FIG 3. PVP sample pads alleviate PCR inhibition resulting from exogenously added humic acid. DCTLE was generated as for FIG 2. 200  $\mu$ L aliquots of extract were spiked with 0, 12, 25 ng of humic acid and subjected to lateral flow immuno-capture of TMV using either a standard sample pad (Untreated Pad) or a sample pad treated with 10% polyvinylpyrrolidone (molecular weight 360,000) (PVP Samp Pad). Capture zones were collected and subjected to RT-PCR. Although 12 ng and 25 ng humic acid supplemented extracts failed to generate detectable PCR products following TMV capture using lateral flow strips with untreated sample pads, all samples exhibited detectable PCR products when subjected to immuno-capture using PVP treated sample pads. Interestingly, 0, 12 and 25 ng humic acid samples run on PVP treated sample pads exhibited improved PCR amplification relative to 0 ng controls processed with the untreated pads. Lane labeled "RNA" is

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a positive control making use of total tobacco RNA preparations made using RNeasy (Qiagen).

FIG. 4. Passive buffer flow control over a prototype lateral flow substrate: (A) Sample (purple) and wash buffer (pink) have been introduced to the device but are not yet visible on the substrate. (B) Sample solution reaches the capture zone of the device (located at the top of the frame). (C) Sample continues to flow over the capture zone as wash buffer migrates, by capillary flow through a path longer and more narrow than used for the sample path, to the main strip junction. (D) As sample is exhausted, wash buffer begins to displace sample buffer. (E) Sample has now completely traversed the capture zone and wash buffer begins to flood the capture zone. (F) Within 5 minutes wash buffer has completely replaced sample buffer.

FIG. 5. Examples of buffer exchange structures which can be cut using a vinyl cutter or laser cutter from absorbant materials such as nitrocellulose or chromatography paper. The depicted structures support a system making use of two fluids. (A) Two fluid flow channels support a buffer exchange or reagent introduction. Sample may be introduced by employing a fluidic device such as described in Example 8. The central tab descends into a reservoir chamber to accomodate uptake of the primary solution. A second solution is introduced via the left most absorbant region of the structure. A larger volume of solution in the second reservoir assures that the second fluid replaces the first in the downstream regions of the substrate. (B) An example of a geometry used to accomodate circular punches of affinity matrices for nucleic acid or protein capture. (C) An example of a geometry with an extended second fluid path allowing the supporting fluidic system to accomodate larger fluid volumes.

FIG. 6. Examples of buffer exchange structures which can be cut using a vinyl cutter or laser cutter from absorbant materials such as nitrocellulose or chromatography paper. The depicted structures support a system making use of three fluids. (A) A structure with three fluid input pads. The right most pad is used

for sample application, the middle for a first buffer exchange e.g. a staining or wash buffer and the left most pad a final buffer exchange to accommodate, for example, a wash buffer or amplification reagent. This structure was been employed for immuno-assays making use of colloidal gold conjugated antibody as the first exchange buffer and a wash to reduce background as a second exchange buffer in a compact fluidic system (see also FIG. 14). (B) A structure with three fluid input tabs spaced to allow introduction to solutions in a 96 well plate. (C) A structure with three fluid inputs suitable for integration with simple polycarbonate fluidic system.

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FIG. 7. Laser ablation of the nitrocellulose layer of backed nitrocellulose can be used to generate buffer exchange structures similar to those fabricated by cutting methods. (A) A pattern used for generating a two fluid input exchanger by means of ablation. (B) Another of example of a two solution exchanger pattern. (C) Backed nitrocellulose (HF-135, Millipore, Inc.) was laminated, using a double-side adhesive tape, to polycarbonate sheet and subject to laser ablation using the pattern shown in part (B).

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FIG. 8. Passive buffer flow control over a refined microfluidic nitrocellulose membrane substrate. (A) To demonstrate the utility of microfluidic nitrocellulose structures for accomplishing passive yet rapid and complete buffer exchanges, buffers carrying easily visualized dyes representing sample (blue), lysis buffer (red) and amplification buffer (yellow) were introduced to the device. (B) Sample flows over the immuno-capture zone through a wide membrane path, displacing lysis and amplification buffer to the membrane region proximal to the substrate walls until sample flow is exhausted. (C) As sample is exhausted, lysis buffer invades the immuno-capture zone disrupting captured particles and liberating nucleic acids for hybridization-based capture on LNA probes immobilized at the down-stream "LNA-Capture Zone" (indicated in part A). (D) Following exhaustion of the lysis buffer, buffer compatible with NASBA amplification washes the LNA-capture zone removing residual lysis buffer and facilitating hybridization of primers. Within 3 minutes three buffer exchanges are accomplished using 10  $\mu$ L

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sample, lysis and amplification buffer volumes. The calculated bed volume of immuno- and LNA-capture zones is approximately 250 nL, thus each buffer exchange washes the capture zones with approximately 40 bed volumes. Further fluid flow modulation could be realized using additional flow paths of varying lengths and widths to allow further buffer washes and exchanges. Similarly, modulating the viscosity of the buffers could be used to further refine such assay parameters as incubation times in lysis buffer. Most significantly, device size can be altered to accommodate the processing of larger sample volumes. Ruler divisions at the right of each panel are 1 mm. (E) A breadboard three fluid buffer exchange system showing integration of buffer exchange nitrocellulose structure with an immuno-assay strip. This device was employed for immuno-capture and wash experiments described in Example 5.

FIG. 9. Passive buffer exchange structures designed for use with 384 well titer plates. (A) A three fluid exchanger that makes use of five wells of a 384 well titer plate. The central fluid input occupies one well while second and third fluids are placed in well pairs flanking the central axis of symmetry. This structure was employed for nucleic acid capture from guanidinium isothiocyanate lysates as described in Example 7. (B) Another example of a 384 well compatible buffer exchange structure. (C) The buffer exchange structure used for experiments described in Example 6.

FIG. 10. LFM detection of TMV following dilution of tobacco extract in SEB1 sample buffer. DCTLE (100 mg tobacco in 3 mL SEB1) was diluted into additional SEB1 as indicated. (A) 100  $\mu$ L sample volumes were subjected to lateral flow immuno-capture and passive buffer exchange was used to rinse residual SEB1 from the nitrocellulose matrix using 25  $\mu$ L of H<sub>2</sub>O (No Wash). Capture zones were harvested and subjected to NASBA amplification and LFM mediated colorimetric detection. Neat tobacco extract under these conditions generated a false negative LFM result due to the high concentration of inhibitors in the sample. Dilutions of 1:2000 to 1:8000 sufficiently reduced the concentration of inhibitor to allow detection under these conditions. 1:16,000 dilution did not generate a

detectable LFM signal presumably due to low viral titer together with inhibition resulting from residual SEB1 buffer in the chromatography matrix. A no template negative control (NTC) is shown. TMV-2 capture probes provided the most sensitive detection of TMV derived amplicon. (B) 100  $\mu$ L sample volumes were subjected to lateral flow immuno-capture and passive buffer exchange was used to wash captured virus with 50  $\mu$ L NME buffer (50 mM MOPS, pH 7, 0.5M NaCl, 15% ethanol) and to rinse residual buffer from the nitrocellulose matrix with a final rinse of 25  $\mu$ L of H<sub>2</sub>O. Capture zones were harvested and subjected to NASBA amplification and LFM mediated colorimetric detection. Neat tobacco extract under these conditions generated a robust positive result by LFM. Dilutions of 1:2000 to 1:16,000 were also positive under these conditions. The increased detection limit relative to the no wash treatment presented in part A is likely a result of the more complete removal of residual SEB1 buffer from the nitrocellulose substrate. Some SEB1 mediated inhibition has been noted in other studies. A no template negative control (NTC) is shown.

FIG. 11. Real-time RT-PCR of immuno-captured and passive buffer exchange washed TMV. Soil extracts spiked with DCTLE at a final dilution of 1:2000 or 1:4000 were subjected to immuno-capture and wash using TMV immuno-assay strips modified to carry a buffer exchange nitrocellulose structure designed to accommodate sample, wash buffer, and a final H<sub>2</sub>O rinse in a 384 well plate format (see FIG. 8E and FIG. 9C). Soil extracts were generated using 3g of soil in 30 mL of SEB1 extract buffer. Sample volume was 100  $\mu$ L. Washes were conducted using 50  $\mu$ L of NME buffer and were followed by a 25  $\mu$ L H<sub>2</sub>O equilibration. Soil RNA isolated using a Qiagen RNeasy kit was included as a negative control. Without the wash step, but with the 25  $\mu$ L H<sub>2</sub>O rinse, 1:2000 and 1:4000 dilutions generated high Ct values of 28.2 and 28.3 respectively. Inclusion of a 75  $\mu$ L NME buffer wash generated positive detection of TMV with 1:2000 sample exhibiting a Ct value of 26.2 and 1:4000 samples a Ct of 27.2.

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FIG. 12. Alleviation of PCR inhibition following passive buffer exchange washes of TMV immuno-captured from a highly inhibitory soil extract. Soil extract was spiked

with DCTLE to give a final dilution of 1:2000. The volume of sample was 100  $\mu$ L while NME washes were 50  $\mu$ L and the final H<sub>2</sub>O rinse was 25  $\mu$ L. The final H<sub>2</sub>O equilibration reduced the potential for carry-over of residual wash buffer into subsequent PCR reactions. Washes using 50  $\mu$ L of water followed by a 25  $\mu$ L water rinse (labeled as 75  $\mu$ L H<sub>2</sub>O) generated a faint PCR product. The use of NME wash buffer generated robust PCR amplification (NME). Washes with RLT, a Qiagen guanidinium-based lysis buffer, failed to generate detectable PCR amplicon (RLT). Eliminating the 50  $\mu$ L wash but retaining the 25  $\mu$ L rinse failed to sufficiently reduce inhibitor concentrations as evidenced by the lack of detectable amplicon in these samples (25  $\mu$ L H<sub>2</sub>O). Soil extract sample without added TMV subjected to NME wash did not generate detectable TMV amplicon (Soil Only (NME wash)). SEB1 extraction buffer alone was assayed as a further negative control (SEB1). Additionally, negative control immuno-capture experiments were conducted using virgin soil extract and a NME buffer wash to further establish the absence of TMV from the soil used. The TMV lane is a positive control PCR reaction programmed with RNA isolated from tobacco using the Qiagen RNeasy kit.

FIG. 13. Real-time RT-PCR analysis of an RNA binding matrix challenged with dried cured tobacco leaf lysate generated by crushing 22  $\mu$ g/ $\mu$ L of tobacco in RLT guanidinium isothiocyanate-based lysis buffer (Qiagen, Inc.). Tobacco leaf lysate was subjected to lateral flow mediated nucleic acid capture using a nitrocellulose buffer exchange device of the structure shown in FIG. 9A laminated to a 3 mm biopsy punch of an RNeasy column silica RNA binding matrix. 10  $\mu$ L of RLT tobacco extract was used as the sample. 40  $\mu$ L washes were conducted using NME buffer for all treatments except the no wash control. The initial NME wash was followed by 80  $\mu$ L of NME (NME Only), or NaCl at concentrations varying from 0-1M as indicated. This aspect of the invention is described further in Example 7.

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FIG. 14. Components of a self contained passive buffer exchange device. (A) An example three fluid exchanger suitable for integration with a simple supporting

fluidic system. (B) Pattern for cutting polycarbonate to accommodate the fluid input tabs of the exchanger depicted in part (A). (C) fluid reservoir forming pattern for cutting polycarbonate sheet. Lamination to layer shown in part (B) carrying inserted exchange structure and to bottom layer shown in part (D) forms a  
5 integrated buffer exchange device allowing three solutions to be introduced via solution input ports. (D) Pattern for cutting bottom piece of device. (E) Scan of an assembled device shown here making use of 3MM chromatography paper for the buffer exchange component interfaced to an immuno-assay strip. In the depicted device, immuno-assays are conducted by introducing sample to the right most  
10 port, staining reagent (antibody conjugated colloidal gold) to the middle port and a final wash buffer to reduce background is added to the left most port. All solutions are added at the time of assay initiation. The size of the device is 25 mm x 75 mm, similar to a standard microscope slide.

15 FIG. 15. Artists rendering of one possible embodiment of the proposed integrated sample preparation device. The different subsystems of the device's disposable component are depicted here as indicated. Fabricated from an inexpensive plastic housing and supported large pore nitrocellulose, the system will make use of capillary lateral flow and passive flow regulation to enable analyte affinity and  
20 hybridization-based capture as well as subsequent buffer exchanges required for lysis, washing and isothermal amplification by NASBA. 10% of the sample volume is interrogated immediately following sample addition using an immuno-assay LFM. Subsequent cell separation and lysis results in the liberation of cellular RNAs and stabilization by a guanidinium-based buffer. RNA sequences of interest  
25 are collected by hybridization in guanidinium buffer to LNA capture oligonucleotides. Any extracellular RNAs present in the sample should also be captured on the LNA oligonucleotides. Swab elution buffers may also be optimized for elution efficacy, immuno-assay compatibility and RNA stabilizing properties. For off-device analysis, a simple punch-out system could be  
30 incorporated to enable facile collection of cell separation or LNA capture zones (not shown).

FIG. 16. Schematic of an alternative integrated device layout, which utilizes anion/cation exchange. A lateral flow strip accepts sample either in native buffer for immuno-affinity purification of cell and viral targets using an immobilized antibody ligand (Cell/Particle Capture) or as a lysate for affinity purification of nucleic acids by anion exchange (Anion Exchange Ligand). Depending on the input sample, a wash or lysis buffer is introduced at the time of protocol initiation to the Wash/Lysis zone where it flows from its absorbent pad (not shown) to the main substrate via a narrow nitrocellulose path reaching the main strip only after complete transport of the sample solution. Sample path treatments such as PVP, PVPP or cation exchange ligands may be included for amplification inhibitor removal. High ionic strength wash buffer elutes nucleic acids from the anion exchange ligand and provides an electrostatic environment supportive of efficient binding to the silica matrix. The resulting purified nucleic acids may be recovered by elution or collection of the silica matrix into a microcentrifuge tube carrying a frit where the nucleic acids may eluted using at least 2-bed volumes ( $> 0.8 \mu\text{L}$ ) of low ionic strength buffer (water, TE, etc).

## DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains, unless otherwise defined. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodologies by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and *Current Protocols*



in Molecular Biology (Ausbel et al., eds., John Wiley & Sons, Inc. 2001. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

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### **LATERAL FLOW SAMPLE PREPARATION METHODS AND SYSTEMS**

The invention provides highly simplified lateral flow chromatographic nucleic acid sample preparation methods, devices, and integrated systems for the efficient  
10 concentration of trace samples and the removal of nucleic acid amplification inhibitors. LFSP devices may consist of various elements of the invention disclosed herein, including lateral flow immuno-capture of biological particles or cells, lysis directly within the lateral flow matrix, the various elements constituting a sandwich hybridization assay, passive fluid/buffer control systems, and  
15 pretreatment with compositions capable of sequestering or reducing the activity of nucleic acid amplification inhibitors.

For example, a LFSP device may comprises a sample receiving zone for receiving an aliquot of a fluid sample, together with an immuno-capture zone in lateral flow  
20 contact with said sample receiving zone, which contains immobilized antibody reactive with a ligand present on a biological particle or cell of interest. Such a device may be used to capture a biological particle or cell of interest, and may also contain means for lysing the particle or cell, and for amplifying the nucleic acid liberated therefrom. The device may be coupled to or integrated with a lateral  
25 flow assay, such as a sandwich hybridization nucleic acid assay. In preferred embodiments, the LFSP devices are coupled to or integrated with an LFM device or assay. LFM devices and assays are described in United States Patent Application No. 11/894,192.

30 Immuno-capture zones may be prepared, for example, as follows. A lateral flow substrate (i.e., nitrocellulose) is treated such that a ligand (i.e., antibody) is immobilized to form an immuno-capture zone on the substrate. Specifically,

antibody solutions are prepared in a physiological ionic strength buffer at a concentration found empirically to provide specific binding to the antigen (typically 0.01 mg/ml to 1 mg/ml). Antibody deposition onto a large pore nitrocellulose membrane can be accomplished by any of a number of means including but not limited to manual application, airbrush deposition, robotic fluid handling systems or similar methods that deposit controlled and reproducible volumes of ligand onto the substrate. Suitable substrates include HiFlow 135 (Millipore, Inc) and similar products available from a variety of commercial providers. Once deposited onto the substrate the ligand is immobilized by drying (in the case of proteinaceous ligands) and/or by UV irradiation at a dose of 5000 microjoules (in the case of nucleic acid/LNA immobilization).

The lateral flow immuno-capture aspect of the invention provides the capacity to concentrate target analytes from a wide range of dilute sample volumes. Once immobilized at the device's capture zone, the targets may be detected as well as subsequently washed, lysed and any liberated nucleic acids amplified. Incorporating multiple capture zones, each carrying ligands to different analytes, would enable the separation and collection of multiple sample constituents of interest for subsequent on- or off- device analyses. The multiplex capacity of this approach would allow multiple proteinaceous and nucleic acid analytes to be collected (and if desired detected) rapidly with minimal user intervention, requiring less than 2 minutes to obtain immuno-assay results and less than 60 minutes to obtain sensitive sequence specific nucleic acid amplification and detection.

In a simplified sample preparation scheme, based upon immuno-capture and subsequent chemical and/or heat-mediated lysis, several factors must be considered including the removal of potential enzyme inhibitors and the impact of residual sample materials on amplification efficiency. Though crude cell lysate appropriately prepared can be used for NASBA (see Example 1, *infra*), applicant hypothesized that improved sensitivity could be achieved through the use of methods devised to further reduce cellular and matrix contaminants while simultaneously concentrating analyte particles from a complex mixture by means

of immuno-affinity capture. This led to the development of a LFSP method and device which is capable of sequestering virus particles from a complex sample matrix, resulting in a cleansed viral sample that could be lysed to provide nucleic acids suitable for subsequent amplification without further purification. This aspect of the invention is described in more detail in Example 2, *infra*, wherein TMV particles were sequestered from crude macerated dried tobacco leaf by immuno-affinity chromatography within a nitrocellulose membrane context. The studies described in Example 2 demonstrate that lateral flow can be used to not only concentrate dilute analytes to a spatially defined capture zone but that regions of the device downstream of the capture zone are depleted with respect to the captured species. These data support the hypothesis that simple lateral flow immuno-assay methods can form the basis for a rapid and cost effective immuno-affinity purification system for separation and preparation of complex biological samples as well as the assertion that appropriately treated substrates can be used to deplete samples of unwanted constituents at downstream capture zones.

The samples utilized in the experiments described in Example 2 represent a very challenging matrix owing to the presence of complex polysaccharides, organic matter and other constituents strongly inhibitory to enzymatic manipulations, such as PCR and NASBA amplification, as well as potentially confounding non-probative nucleic acids (plant derived DNA and RNA). Therefore, the results described in Example 2 demonstrate the utility of a preceding lateral flow mediated immuno-capture step in the analysis of complex biological samples wherein the target analyte is a minority species and PCR and NASBA inhibitors are present that preclude direct amplification of the target without preparatory processing. Further, the results obtained in the experiments of Example 2 support the hypothesis that simple lateral flow immuno-assay methods can form the basis for a rapid and cost effective immuno-affinity purification system for separation and preparation of complex biological samples as well as the assertion that appropriately treated substrates can be used to deplete samples of unwanted constituents at downstream capture zones.

### **PASSIVE LF BUFFER EXCHANGE SYSTEMS**

To eliminate the need for electronic control systems, valves, and other fluid flow control schemes that require moving parts, applicants have developed various  
5 nitrocellulose structures to mediate passive control of buffer and sample flow over a lateral flow substrate. Through the use of geometrically-defined flow paths in a single integrated lateral flow membrane, such as nitrocellulose or chromatography paper, the flow rate of multiple solutions/buffers may be passively controlled. In one methodology, described further by way of the examples presented in Example  
10 4, *infra*, nitrocellulose membranes are cut to form individual flow paths for different solutions, which flow paths vary by length and or width of the membrane (see, for example, FIGS. 4-9). In addition to the geometric shapes exemplified by the prototype devices shown in FIGS. 7, 8 and 14, one skilled in the art will readily appreciate that many other shapes can achieve the desired modulation of the  
15 multiple solution flow paths required by the assay it is intended to facilitate. In addition, although this aspect of the invention is exemplified by single nitrocellulose membranes in which all flow paths are seamlessly integrated, other systems will also be apparent, including but not limited to those in which flow paths are modulated not only by membrane flow path length and/or width, but also  
20 by interrupting sequences of materials other than the membrane material, such as cellulose esters, glass fiber, polyethersulfone, cotton, dehydrated polyacrylamide, silica gel, and polyethylene glycols. It will be appreciated that defining precisely controlled fluid paths and reaction sequences will vary according to the assay type and complexity. However, based on the teachings provided herein, one will be  
25 able to empirically derive the necessary control for a specific assay with ordinary experimentation.

### **INTEGRATED SYSTEMS:**

30 Also envisioned are fully integrated, sample-to-answer lateral flow assay devices that incorporate one or more elements of the present invention. Example integrated systems are schematically represented in FIGS. 15 and 16. For

example, in one embodiment, a LFSP device is integrated with both immuno-assay screening and NASBA amplification followed by a downstream lateral flow sandwich hybridization nucleic acid assay. A schematic representation of such a device is shown in FIG. 15.

5

Such devices may comprise a lateral flow compatible chromatography support, such as HiFlow 135 large pore supported nitrocellulose (Millipore). This substrate is patterned using a fluid deposition system such as a piezo-actuated picoliter deposition system (NanoPlotter 2.0, GeSim) to confer specific properties at  
10 desired locations in the sample flow. For example, regions up-stream of an immuno-capture zone will be treated to confer modifications capable of removing or reducing the activity of amplification inhibitors or other undesirable sample matrix contaminants. Antibodies to pathogens of interest are located such that target particles are captured from sample solutions during capillary lateral flow.  
15 Further, by incorporating passive fluid flow control, captured cells or viruses can be lysed to liberate nucleic acids for affinity capture and purification at downstream substrate zones. Some embodiments may be equipped with a heating element powered by a USB interface or internal or external power source or battery, to incubate sample in lysis buffer at an elevated temperature [49]. Nucleic  
20 acids liberated during lysis then flow to down-stream affinity capture zones for immobilization, washing and collection. The capture zone of the device may incorporate PCR, RT-PCR, NASBA or isothermal nucleic acid amplification reactions.

25 To allow the incorporation of more stringent washing conditions without significant increases in protocol complexity, the invention also supplies an approach that exploits the increased stability of LNA duplexes [39]. In this scheme, the sample preparation device carries immobilized LNA oligonucleotides. These immobilized probes are predicted to allow sequence specific hybridization-mediated capture of  
30 target RNAs under the denaturing conditions imposed by a guanidinium-based lysis buffer. Prior work by others has shown that an LNA oligonucleotide can be used to capture, by hybridization, RNA molecules present in crude cell lysates

containing 4M guanidinium [40]. LNA capture probes will be designed to hybridize to regions near but not overlapping with NASBA amplification primer binding sites.

5 One advantage of a supported nitrocellulose system is the ease with which chemical modifications can be made to the membrane. Prior reports have detailed methods for covalent and adsorptive modification of nitrocellulose to introduce immobilized functional groups for both cation and anion exchange chromatography [17]. Treatments that result in an immobilized anion exchange  
10 ligand at a defined zone or component membrane of a multiple membrane system may also be included for nucleic acid binding and purification, including without limitation polyethyleneimine (PEI) as well as diethyl aminoethyl (DEAE) functional groups, both of which have been used for membrane based ion exchange chromatography [17]. Additionally, regions up-stream of nucleic acid affinity  
15 ligand can be treated to reduce or preclude the transport of common nucleic acid sample contaminants. Such modifications could include, for example, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP) [31-33], novel inhibitor sequestering agents (*e.g.* PIR, J. Dunbar, LANL, pers. comm.) and cation exchange ligands.

## EXAMPLES

### 5 EXAMPLE 1: NASBA AMPLIFICATION OF RNA FROM CRUDE BACTERIAL CELL LYSATES

In order to evaluate the feasibility of using crude lysates to supply template RNA for amplification, the efficacy of NASBA amplification from *E. coli* lysates was examined. Lysates were prepared by adding varying quantities of *E. coli* liquid culture to cells-to-cDNA buffer (Ambion) and heating to 75 °C for 10 minutes. This method was reported to generate suitable template for RT-PCR (reverse transcriptase-polymerase chain reaction) from crude *L. monocytogenes* lysates [49]. Lysates were diluted 1:5 and 2 µL of the resulting material used in a 10 µL NASBA reaction.

15

A constitutively expressed mRNA, *rplV*, was used as the NASBA target [50]. NASBA P1 and P2 primer sequences were as follows:

EC-*rplV*-P1: 5'-  
20 aattctaatacgactcactatagggagaaggCCATCGTTGTGTTTCAGCGTTA-3'  
[SEQ ID NO: 1]

and

EC-*rplV*-P2: 5'-gatgca aggtcg cat atg agAACTATCGCTAAACATCGCCA-3' [SEQ  
ID NO: 2].

25

Lower case characters in the P1 sequence denote the T7 RNA polymerase promoter sequence. Lower case characters in the P2 sequence represent the tag sequence used for hybridization sandwich assay mediated detection. The sequences used for *rplV* capture and detection on LFMs were the capture probe:

30

*rplV*-cap: 5'-CTGCTCAGAAGGTTTCGCCTT-3' [SEQ ID NO: 3]

and the detection probe:

UNI-det-5Tbio: 5'-TT-U-biotin-TTTT-U-biotin-TTTT-U-biotin-TTTTTTT gat gca agg  
tcg cat atg ag -3' [SEQ ID NO: 4].

- 5 NASBA reactions were allowed to proceed for 60 minutes at 41 °C after which 4  
μL was removed and assayed for *rpIV* amplicon by LFM using colorimetric  
detection mediated by dyed polystyrene microspheres conjugated to streptavidin.

The results presented in FIG. 1 show the LFM membranes following exposure to  
10 NASBA reactions containing crude lysate from the indicated number of cells. As  
few as 2000 cells could be detected by LFM, following NASBA amplification, using  
crude whole cell lysate to supply template. This experiment demonstrates that a  
crude lysate prepared under denaturing conditions in the presence of guanidinium  
can be used successfully as NASBA template. Given that one proposed lateral  
15 flow method for sample preparation would allow a sequence specific capture of  
the target RNA and stringent washes in guanidinium buffer, it is likely that the  
detection limit of 2000 cells obtained with crude lysate can be significantly  
improved.

20

**EXAMPLE 2: LATERAL FLOW CONCENTRATION OF ANALYTE AND  
SUBSEQUENT AMPLIFICATION FROM TMV PARTICLES  
CONTAINED WITHIN LEAF TISSUE**

- 25 In this Example, the utility of lateral flow facilitated immuno-capture as a means of  
concentrating analyte prior to nucleic acid isolation or amplification was  
investigated with tobacco mosaic virus (TMV).

FIG. 2A-C depicts the results of immuno-affinity capture and concentration of  
30 tobacco mosaic virus (TMV) particles during lateral flow of 200 μL of crude  
macerated tobacco and subsequent amplification (reverse-transcriptase-PCR)  
reactions programmed with regions of the lateral flow substrate below (proximal to  
the sample pad), at and above (distal to the sample pad) the immuno-capture



zone (as shown in FIG. 2A). The capture zone is greatly enriched in virus particles while the relative concentration of inhibitory constituents is reduced. The 1000-fold reduction of sample volume from 200  $\mu$ L to 200 nL, based on the calculated bed volume of the capture zone, exhibited here will also facilitate subsequent washing to further reduce inhibitor concentrations.

These data demonstrate that lateral flow can be used to not only concentrate dilute analytes to a spatially defined capture zone but that regions of the device downstream of the capture zone are depleted with respect to the captured species. These data support applicant's hypothesis that simple lateral flow immunoassay methods can form the basis for a rapid and cost effective immuno-affinity purification system for separation and preparation of complex biological samples, as well as the assertion that appropriately treated substrates can be used to deplete samples of unwanted constituents at downstream capture zones.

15

### **EXAMPLE 3: LATERAL FLOW REDUCTION OF AMPLIFICATION INHIBITORS USING POLYVINYLPIRROLIDONE**

20

This Example shows that polyvinylpyrrolidone treated sample pads can deplete PCR inhibitors via lateral flow.

The observation, made during the course of applicant's preliminary studies, that crude sample constituents inhibitory to PCR can be depleted during lateral flow, suggested that specific substrate treatments may further facilitate amplification of nucleic acids from captured target cells or particles. To test this hypothesis, absorbent sample pads were treated with a 10% solution of polyvinylpyrrolidone (MW = 360,000) [31-33] and RT-PCR amplification of TMV concentrated from macerated leaf tissue following lateral flow immuno-capture (Example 2, *supra*) on devices carrying either PVP treated or untreated sample pads was evaluated.

30

These reactions made use of previously reported primer sets for TMV detection [35]. Neat tobacco extract added directly to RT-PCR reactions was negative for TMV without prior immuno-capture to deplete inhibitors. Consistent with this interpretation, 1:50 dilutions of extract were positive by PCR presumably due to lower inhibitor concentrations.

As shown in FIG. 3, PVP sample pads alleviated inhibition from exogenously added humic acid, a potent PCR inhibitor [9, 34], while untreated sample pads did not. Perhaps most significantly, the use of PVP treated sample pads resulted in significantly improved amplification relative to untreated sample pads even in the absence of exogenously added inhibitor. Although 12 ng and 25 ng humic acid supplemented extracts failed to generate detectable PCR products following TMV capture using lateral flow strips with untreated sample pads, all samples exhibited detectable PCR products when subjected to immuno-capture using PVP treated sample pads (FIG. 3). Interestingly, 0, 12 and 25 ng humic acid samples run on PVP treated sample pads exhibited improved PCR amplification relative to 0 ng controls processed with the untreated pads (FIG. 3).

#### **EXAMPLE 4: GEOMETRIC ARCHITECTURES FOR PASSIVELY CONTROLLING MULTIPLE SOLUTION FLOWS IN LATERAL FLOW SYSTEMS**

This Example shows prototypes of passive lateral flow buffer control systems utilizing attributes of geometrically-defined nitrocellulose strips to control the speed and volume of fluid flow. In order to rapidly generate prototypes of nitrocellulose or other adsorbent material-based devices, methods were developed for cutting miniaturized structures from sheets of absorbent material using either a vinyl cutter (Roland GX-24 CAMM-1) or a laser cutting/engraving system (VersaLaser VL-300 equipped with a 30W CO<sub>2</sub> laser (Universal Laser Systems, Inc.)) (FIGS. 4-9). Additionally, it was found that laser ablation of nitrocellulose from backed nitrocellulose sheets allows the fabrication of planar nitrocellulose structures suitable for use as fluid flow control and buffer exchange

components (FIG. 7). A number of different shaped nitrocellulose structures were evaluated for their utility in exchanging two (FIG. 4, FIG. 5 and FIG. 7) and three fluids (FIG. 6, 8 and 9) by means of passive capillary wicking in lateral flow mode.

- 5 Fluid flow control using the prototype device shown in FIG. 4. In this crude prototype, two different buffer solutions migrate at different rates by capillary flow through variable paths defined by differing geometric attributes cut into the nitrocellulose strips.
- 10 A more refined prototype device was fabricated using the vinyl cutter, and is shown in FIG. 8A-D. By varying the length and widths of the nitrocellulose paths defined for different solutions, this device was able to manage the temporal control of three different solutions relative to defined reaction zones on the device. As shown in FIG. 8, sample, lysis buffer, and amplification buffer are introduced to
- 15 the device (FIG. 8A). Sample buffer flows over the immuno-capture zone through a wide membrane path, displacing the lysis and amplification buffers to the membrane region proximal to the substrate walls until sample flow is exhausted (FIG. 8B). As sample is exhausted, lysis buffer invades the immuno-capture zone disrupting captured particles and liberating nucleic acids for hybridization-based
- 20 capture on LNA probes immobilized at the down-stream "LNA-Capture Zone" (indicated in FIG. 8A). Following exhaustion of the lysis buffer, buffer compatible with NASBA amplification washes the LNA-capture zone removing residual lysis buffer and facilitating hybridization of primers. Within 3 minutes, three buffer exchanges are accomplished using 10  $\mu$ L sample, lysis and amplification buffer
- 25 volumes. The calculated bed volume of immuno- and LNA-capture zones is approximately 250 nL, thus each buffer exchange washes the capture zones with approximately 40 bed volumes. Further fluid flow modulation could be realized using additional flow paths of varying lengths and widths to allow further buffer washes and exchanges. Similarly, modulating the viscosity of the buffers could be
- 30 used to further refine such assay parameters as incubation times in lysis buffer. Most significantly, device size can be altered to accommodate the processing of larger sample volumes. Ruler divisions at the right of each panel are 1 mm.

**EXAMPLE 5: GEOMETRIC ARCHITECTURES FOR PASSIVELY WASHING  
IMMUNOCAPTURED TARGETS TO INCREASE  
AMPLIFICATION EFFICIENCY**

5

Results obtained from experiments employing real-time detection of RT-PCR amplicons as well as LFM detection of NASBA reaction products indicated that residual inhibitory constituents of the sample matrix remain associated with immuno-captured virus or remain in the included bed volume of the chromatography substrate. These data suggested that a buffer wash following lateral flow immuno-capture may provide a simple but effective means of increasing amplification efficacy from complex sample matrices. To test this hypothesis, lateral flow strips were subjected to a buffer wash prior to capture zone harvest and nucleic acid amplification by laminating a nitrocellulose structure designed to mediate passive buffer exchange onto a nitrocellulose immunoassay strip carrying an anti-TMV antibody at the capture zone line and a control antibody capable of binding colloidal gold conjugated detection antibody at a control line.

10

15

To minimize the level of user intervention required to accomplish buffer washes of lateral flow substrates, lateral flow strips were laminated to backed nitrocellulose cut into shapes designed to accomplish passive buffer exchange. These devices allowed rapid exchange of sample with wash buffer and a final equilibration in ultrapure H<sub>2</sub>O prior to PCR to reduce the potential impact of residual wash buffer on PCR performance. The device used is shown in FIG. 8E. A 100  $\mu$ L sample volume was used. Wash buffer volume was 50  $\mu$ L followed by a final rinse using 25  $\mu$ L of H<sub>2</sub>O to remove residual buffer constituents. Sample, wash buffer and H<sub>2</sub>O were added to the corresponding wells of a 384 well plate at the time of assay initiation. Following completion of capillary transport, capture zones were harvested and subjected to analysis by real-time RT-PCR.

20

25

30

To identify buffers with a composition compatible with immuno-affinity immobilization of virus at the capture zone of lateral flow strips, the effect of

various wash buffers on TMV immuno-strip capture zone signals generated using neat tobacco extract were evaluated. These studies revealed no visually detectable elution of sequestered gold particles from the capture zone following capillary wicking of 200  $\mu$ L of an ethanol containing wash buffer, referred to here as NME (0.5M NaCl, 50 mM MOPS pH 7.0, 15% ethanol). In contrast to the effect of NME on capture zone signals, the guanidinium isothiocyanate containing buffer RLT (Qiagen, Valencia, CA) rapidly cleared the capture zone of detection particles.

10 Using a 50  $\mu$ L NME wash following challenge of TMV immuno-assay strips laminated to the passive buffer exchange structure with varying dilutions of DCTLE in SEB1, capture zones were harvested and subjected to NASBA amplification and amplicon detection by LFM. These studies revealed an alleviation of NASBA amplification inhibition previously observed in reactions  
15 programmed with capture zone material harvested from strips exposed to undiluted DCTLE (compare FIG. 10A with FIG. 10B). Additionally, the NME buffer wash resulted in detection of TMV at DCTLE dilutions of at least 1:16,000 suggesting that the more thorough removal of the SEB1 extract buffer employed for DCTLE dilutions from the chromatography substrate further increased  
20 amplification efficiency.

LFM detection of TMV amplicons made use of capture probes TMV-1 and TMV-2 immobilized on the LFM substrate:

25

TMV-1:

5' TTATGCTATAACCCACCCAGG 3' [SEQ ID NO: 5]

TMV-2:

30 5' TTATGCTATAACCCACCCAGGACGCGATGAAAAACGTCTGGCAA 3' [SEQ ID NO: 6]

and a detection probe:

UNI-det-5Tbio:

5'-TT-U-biotin-TTTT-U-biotin-TTTT-U-biotin-TTTTTTT gat gca agg tcg cat atg ag -  
3' [SEQ ID NO: 7]

5

visualized by streptavidin conjugated dyed polystyrene microsphere (Spherotech)  
capture.

NASBA amplification of TMV diagnostic sequence was accomplished using:

10

TMV-P1: 5' aat tct aat acg act cac tat agg g aga GAA AGC GGA CAG AAA CCC  
GCT Ga 3' [SEQ ID NO: 8]

TMV-P2: 5' gat gca agg tcg cat atg ag GAC CTG ACA AAA ATG GAG AAG ATC

15

T 3' [SEQ ID NO: 9]

The TMV-P2 primer incorporates a tag sequence into the NASBA product that is  
capable of hybridizing to the UNI-det-5Tbio oligonucleotide to mediate detection.

20

#### **EXAMPLE 6: USE OF PASSIVE BUFFER EXCHANGE TO DETECT TRACE VIRUS IN A COMPLEX AND INHIBITORY SAMPLE MATRIX**

25 Studies making use of tobacco extract revealed that the high viral titer in these  
samples allows PCR-based detection of TMV to be accomplished by simply  
diluting extract to a sufficient extent that inhibitors in the crude lysate fall below a  
critical concentration. To better evaluate the utility of a lateral flow immuno-  
capture step to render inhibitor laden samples containing low titers of target virus  
amenable to amplification, samples making use of soil extracts were devised to  
30 contain sufficient enzymatic inhibitor concentrations to completely abrogate PCR  
amplification of TMV diagnostic sequences in the absence of an intervening  
sample processing procedure. These samples allow an assessment of the impact  
of lateral flow immuno-capture on PCR-based detection schemes and provide an

approach to evaluate the impact of a buffer wash on the level of amplification inhibition. To provide a challenging inhibitor laden sample, DCTLE was diluted 1:2000 or 1:4000, as indicted in FIG. 11, into a PCR inhibitory soil extract prepared using 3 g locally gathered soil measured into 50 mL polypropylene centrifuge tubes and vortexed vigorously in 30 mL SEB 1 extraction buffer (Agdia, Inc.) and allowed to rotate overnight at room temperature. The resulting soil slurry was allowed to settle for 3 minutes prior to the collection of aliquots for RNA isolation, PCR testing for TMV or preparation of soil extracts spiked with DCTLE.

10 Capture zones were collected following completion of sample and wash buffer transport and introduced to reverse-transcriptase reactions followed by real-time PCR using 1  $\mu$ L of the RT reaction as template. To characterize soil extracts, soil extract total RNA was isolated using Qiagen RNeasy protocols and assayed for TMV by real-time RT-PCR to confirm the matrix was negative for TMV.

15 Additionally, DCTLE spiked soil derived sample matrix generated false negative real-time RT-PCR results using capture zones harvested from TMV assay strips challenged with 100  $\mu$ L of soil extract containing either 1:2000 or 1:4000 DCTLE (FIG. 11). Passive capillary flow mediated wash of TMV assay strips challenged with soil extract containing tobacco extract at a final dilution of 1:2000 using 50  $\mu$ L of NME buffer generated a positive real-time RT-PCR result (Ct = 26.2) (FIG. 11) as well as a clearly identifiable band in agarose gels (FIG. 12, NME). Washes with 50  $\mu$ L of RLT buffer did not result in successful TMV detection by either RT-PCR and gel analysis (FIG. 12, RLT) or by real-time RT-PCR (not shown). Additionally, a more dilute solution of DCTLE in soil extract, 1:4000, also generated positive

25 TMV detection by real-time RT-PCR (FIG. 11) with a Ct value of 27.2 when a 50  $\mu$ L NME buffer wash was employed prior to capture zone harvest.

#### 30 **EXAMPLE 7: GEOMETRIC ARCHITECTURES FOR PASSIVELY WASHING NUCLEIC ACIDS BOUND TO AN AFFINITY MATRIX**

To evaluate the capacity of the passive buffer exchange approach to allow nucleic acids to be directly captured from a guanidinium lysate, tobacco lysate was

prepared by maceration of dried cured tobacco leaf in Qiagen RLT guanidinium isothiocyanate lysis buffer using 22  $\mu\text{g}$  of tobacco per  $\mu\text{L}$  RLT. A device for lateral flow chromatography and buffer exchange was fabricated using a laser cutter (VersaLaser VL-300, 30W CO<sub>2</sub> laser, Universal Laser Systems). The device was  
5 designed such that the input tails of the buffer exchange component were spaced at 4.5 mm intervals to allow sample and buffer to be absorbed from the wells of a 384 well plate. The distal end of the buffer exchanger was laminated to a 3 mm diameter punch taken from a Qiagen RNeasy column. This silica RNA binding matrix was used to evaluate the suitability of capillary lateral flow mediated buffer  
10 exchange to support viral RNA capture. Other materials, such as glass fiber filter material, may also be employed. Similarly, using other buffer systems, DEAE membranes could be incorporated into a similar system.

10  $\mu\text{L}$  of RLT tobacco extract was used as the sample. 40  $\mu\text{L}$  washes were  
15 conducted using NME buffer for all treatments except the no wash control followed by 80  $\mu\text{L}$  of NME, or NaCl at concentrations varying from 0-1M as indicated in FIG. 13. These data show that the NME wash followed by a 0.5M NaCl wash provided the best amplification of the conditions tested. Samples that were not washed failed to generate detectable real-time RT-PCR products.

20

**EXAMPLE 8: INTEGRATION OF PASSIVE BUFFER EXCHANGE GEOMETRIC ARCHITECTURES INTO A FLUIDIC SYSTEM TO SUPPORT FACILE TARGET ENRICHMENT AND WASHING PRIOR TO AMPLIFICATION AND DETECTION**

25

To incorporate the lateral flow capture and buffer exchange systems into a compact and self-contained housing, a supporting fluidic system was devised. The fluidic system was fabricated to accommodate a nitrocellulose or chromatography  
30 paper buffer exchanger such as that shown in FIG. 14A by cutting polycarbonate sheet using a laser cutter into the geometries shown in FIG. 14B-D. Appropriately cut polycarbonate sheet was laminated to form sample and buffer wells using UV cured adhesive or water proof double-sided tape (ACE double-sided carpet tape



50106). The resulting devices allowed sample and wash buffers to be introduced at the time of assay initiation and supported sample chromatography and washing without further user intervention.

5

10 All publications, patents, and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

15 The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any which are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall  
20 within the scope of the invention. Such modifications or other embodiments can be practiced without departing from the true scope and spirit of the invention.

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**WHAT IS CLAIMED IS:**

1. A lateral flow sample preparation (LFSP) device comprising a lateral flow matrix which defines a flow path and which comprises in series the following elements:
  - 5 (a) a sample receiving zone for receiving an aliquot of a fluid sample; and,
  - (b) an immuno-capture zone in lateral flow contact with said sample receiving zone, which contains immobilized antibody reactive with a ligand present on a biological particle or cell of interest.
  
- 10 2. A LFSP device comprising a lateral flow matrix which defines a flow path and which comprises in series the following elements:
  - (a) a sample receiving zone for receiving an aliquot of a fluid sample;
  - (b) an immuno-capture zone in lateral flow contact with said sample receiving zone, which contains immobilized antibody reactive with a ligand present
  - 15 on a biological particle or cell of interest; and,
  - (c) a lysis zone, in lateral flow contact with said immuno-capture zone, which is capable of achieving the lysis of the membrane(s) of a biological particle or cell and the liberation of nucleic acid therefrom.
  
- 20 3. A LFSP device comprising a lateral flow matrix which defines a flow path and which comprises in series the following elements:
  - (a) a sample receiving zone for receiving an aliquot of a fluid sample;
  - (b) an immuno-capture zone in lateral flow contact with said sample receiving zone, which contains immobilized antibody reactive with a ligand present
  - 25 on a biological particle or cell of interest;
  - (c) a lysis zone, in lateral flow contact with said immuno-capture zone, which is capable of achieving the lysis of the membrane(s) of a biological particle or cell and the liberation of nucleic acid therefrom; and,
  - (d) one or more assay zones, in lateral flow contact with the lysis zone,
  - 30 which together form the nucleic acid and labeling constituents for a sandwich nucleic acid hybridization assay.

4. The LFSP device according to claim 2 or 3, further comprising a nucleic acid amplification zone downstream of and in lateral flow contact with the lysis zone, and upstream and in lateral flow contact with the assay zone(s).
- 5 5. The LFSP device according to any one of claims 1-4, wherein the immuno-capture zone comprises a microporous membrane.
6. The LFSP device according to any of claims 2-5, wherein the lysis zone comprises a microporous membrane.
- 10 7. The LFSP device according to any of claims 3-6, wherein the assay zone(s) comprise(s) a microporous membrane.
8. The LFSP device according to any one of claim 5-7, wherein the microporous  
15 membrane is nitrocellulose.
9. The LFSP device according to any one of claims 1-8, wherein at least the sample receiving zone comprises a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of at least one fluid  
20 flow therein.
10. The LFSP device according to claim 9, wherein at least the sample receiving and immuno-capture zones comprise a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of at least one fluid  
25 flow therein.
11. The LFSP device according to claim 10, wherein at least the sample receiving, immuno-capture and lysis zones comprise a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of  
30 at least one fluid flow therein.

12. The LFSP device according to any one of claims 2-11, further comprising a nucleic acid binding matrix in lateral flow contact with the lysis zone.
13. The LFSP device according to any one of claims 3-12, further comprising a  
5 nucleic acid binding matrix in between and in lateral flow contact with both the lysis zone and the assay zone(s).
14. The LFSP device according to any one of claims 12-13, wherein the nucleic acid binding matrix is selected from the group consisting of silica, glass fiber and  
10 DEAE membrane.
15. A device comprising a lateral flow matrix which defines a flow path and which comprises a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of at least one fluid flow therein.  
15
16. The device according to claim 15, wherein the geometrically-defined absorbent material allows for the passive control of multiple fluid flows therein.
17. The device according to claims 15-16 further comprising a nucleic acid  
20 binding matrix in lateral flow contact with the geometrically-defined absorbent material.
18. The device according to claim 17, wherein the nucleic acid binding matrix is selected from the group consisting of silica, glass fiber and DEAE membrane.  
25
19. A geometrically-defined nitrocellulose strip having a configuration essentially as shown in FIGS. 5, 6, 7, 8, 9 or 14A.
20. The LFSP device according to any one of claims 9-11 and 15-18, wherein the  
30 absorbent material comprises nitrocellulose.



FIG. 1

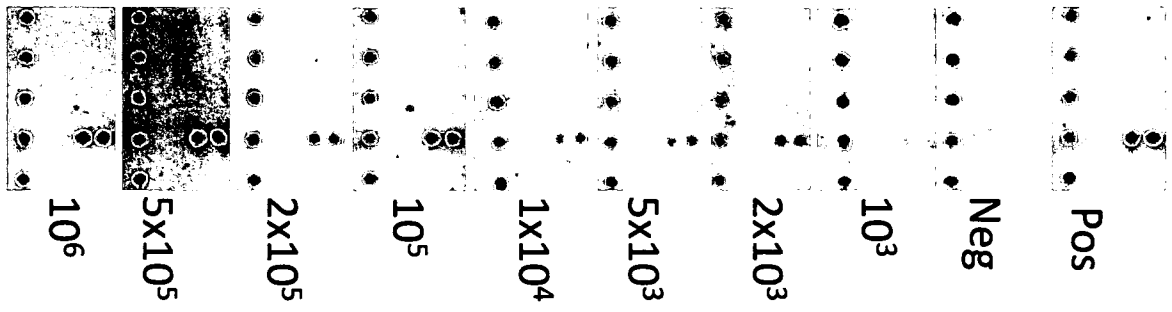


FIG. 2

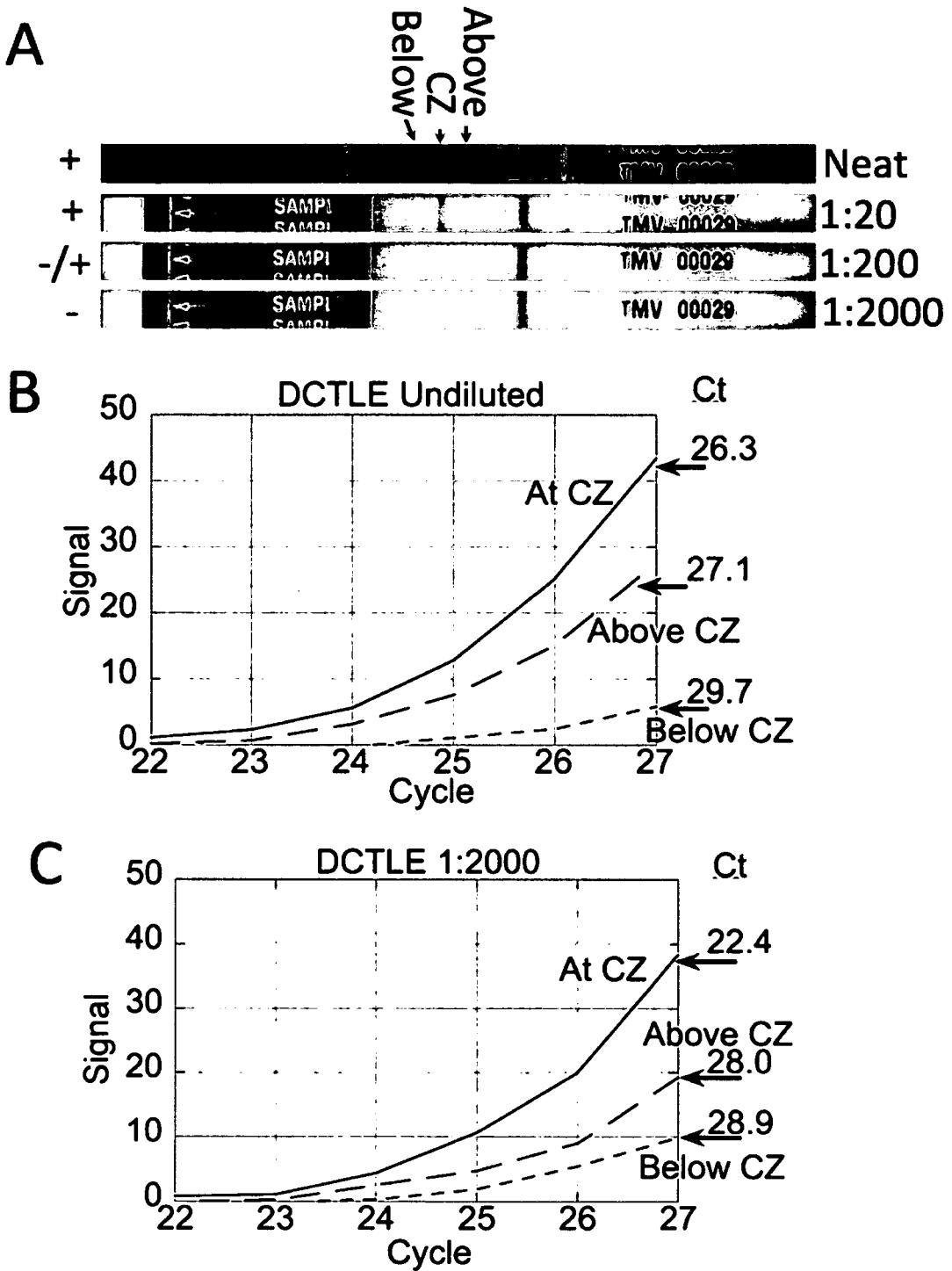


FIG. 3

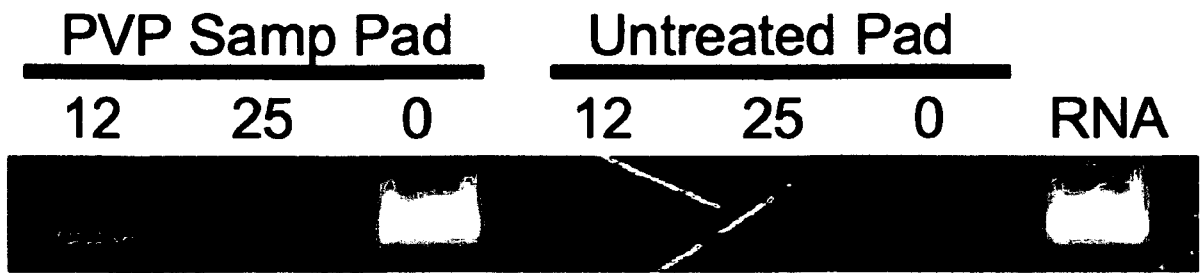


FIG. 4

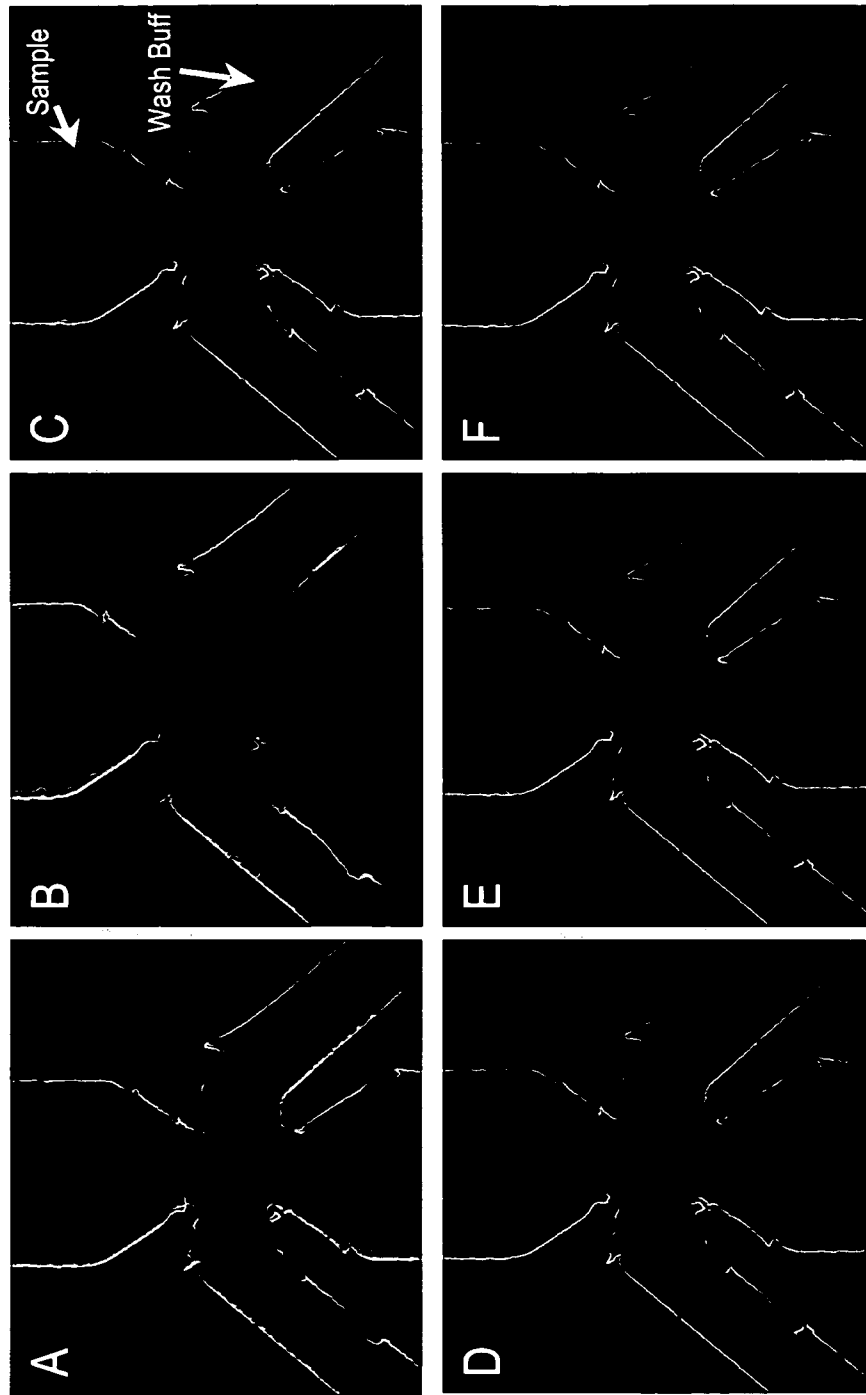


FIG. 5

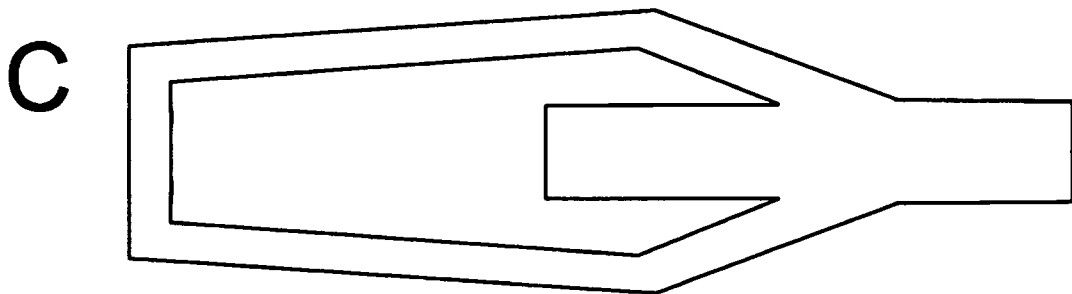
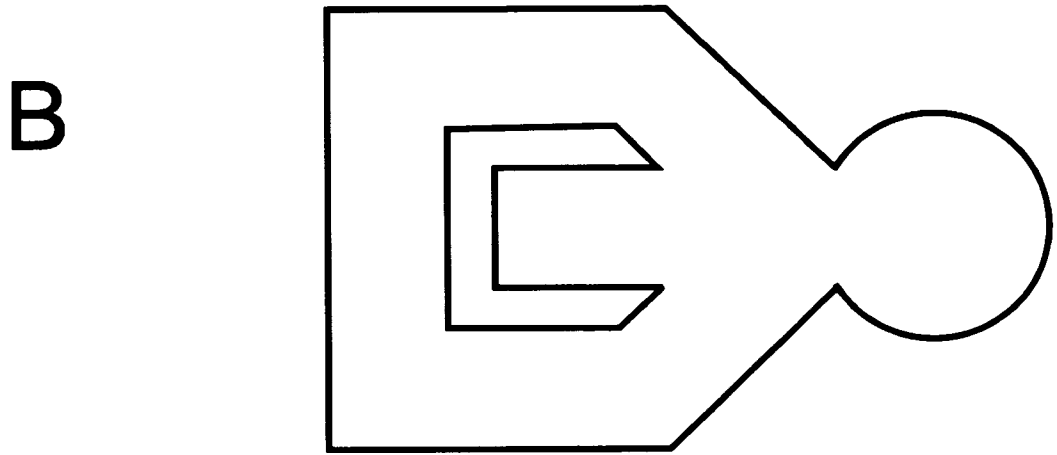
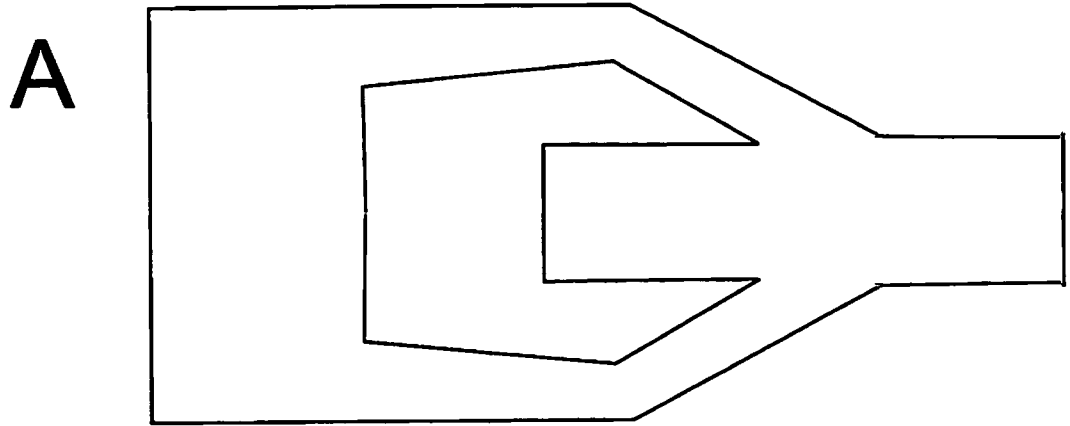


FIG. 6

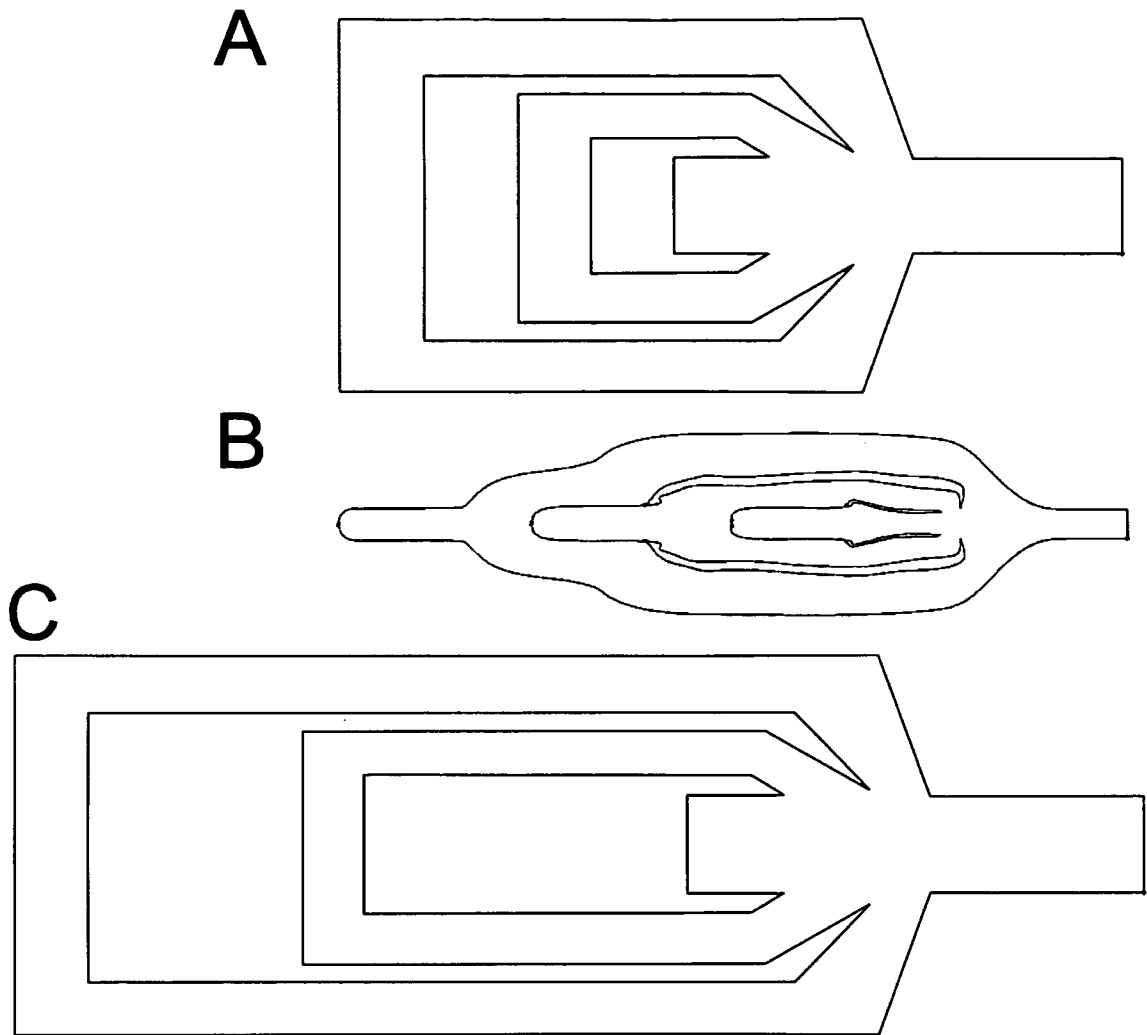


FIG. 7

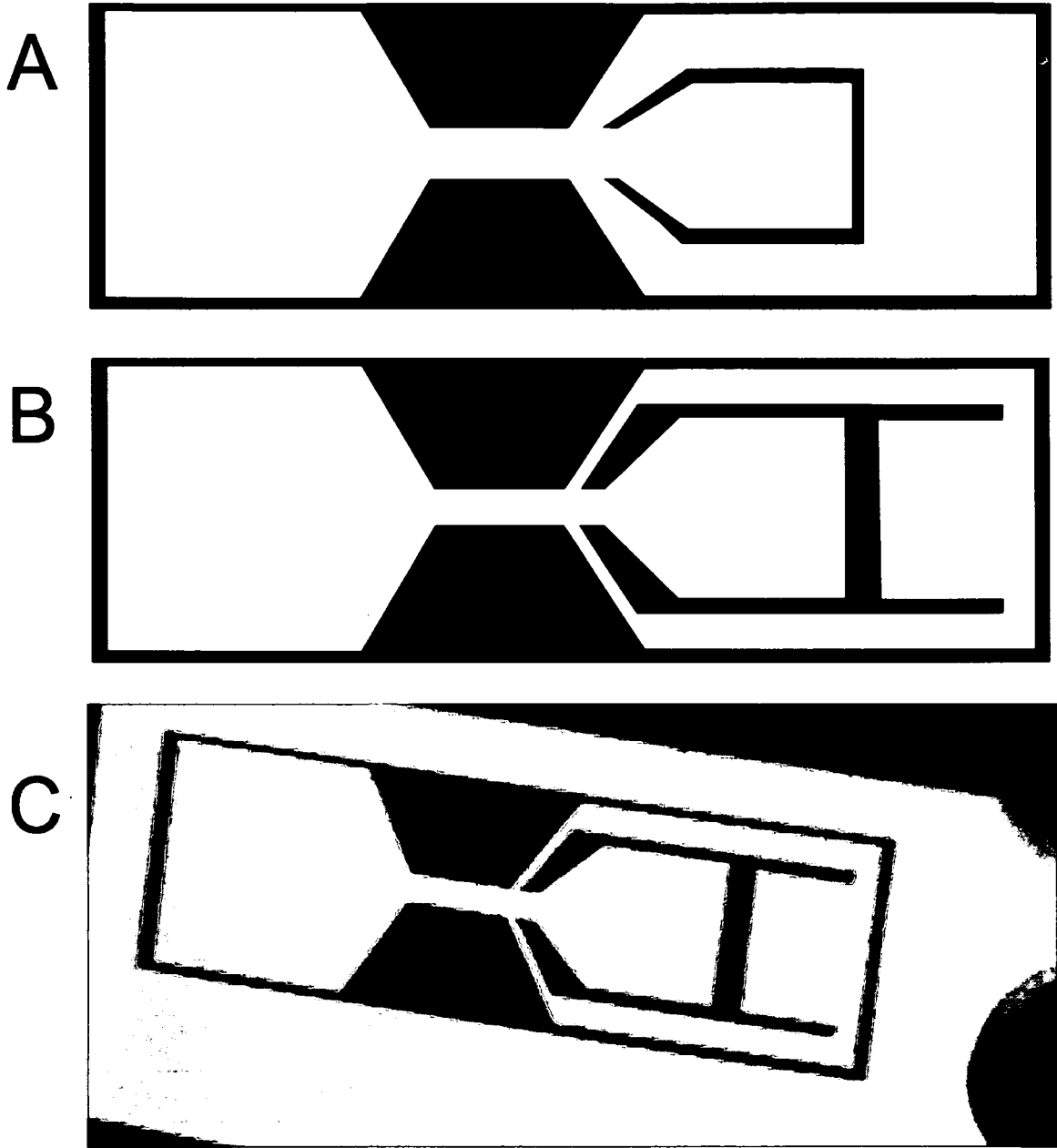


FIG. 8

Immuno  
Capture  
LNA-  
Captu

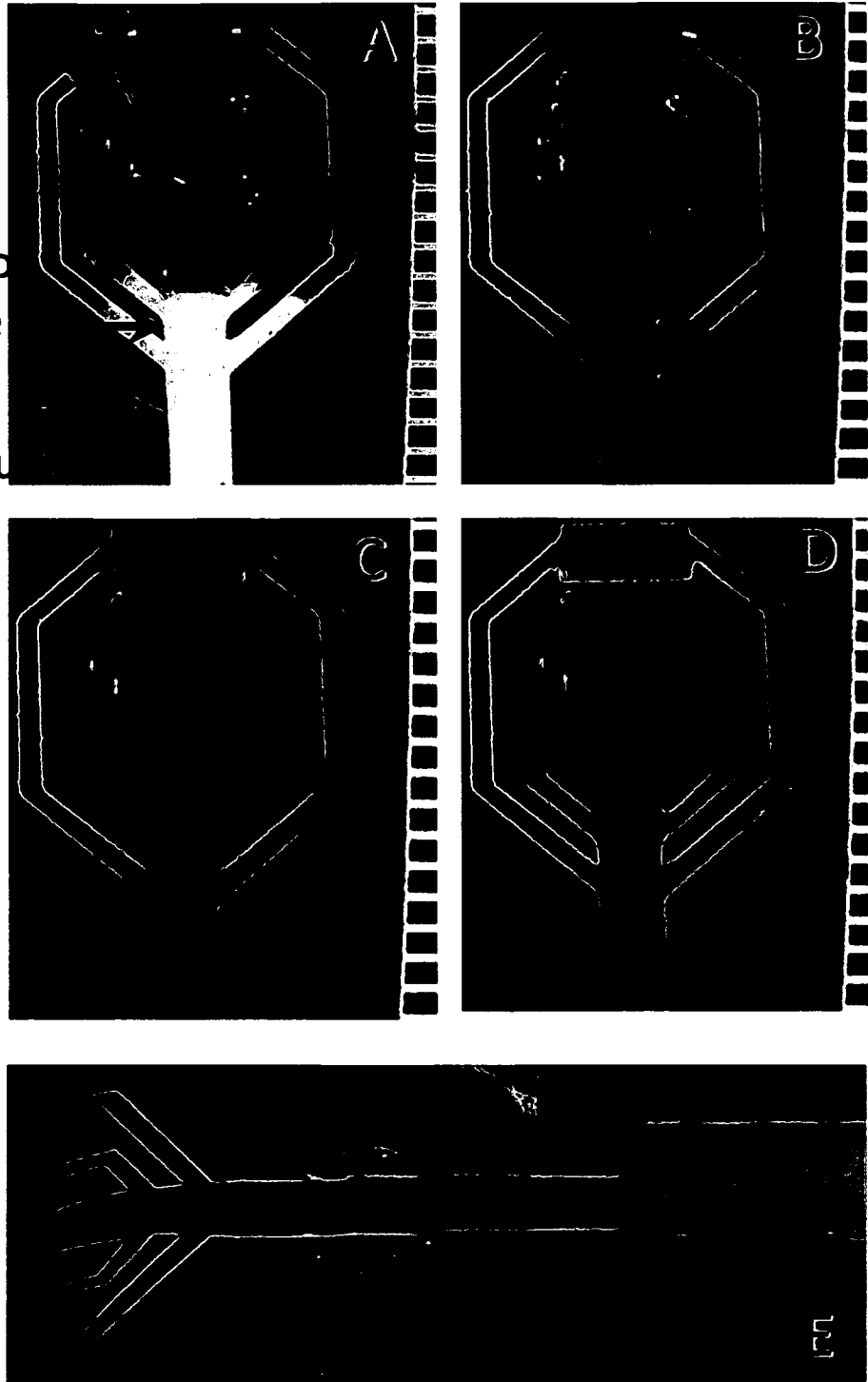




FIG. 9

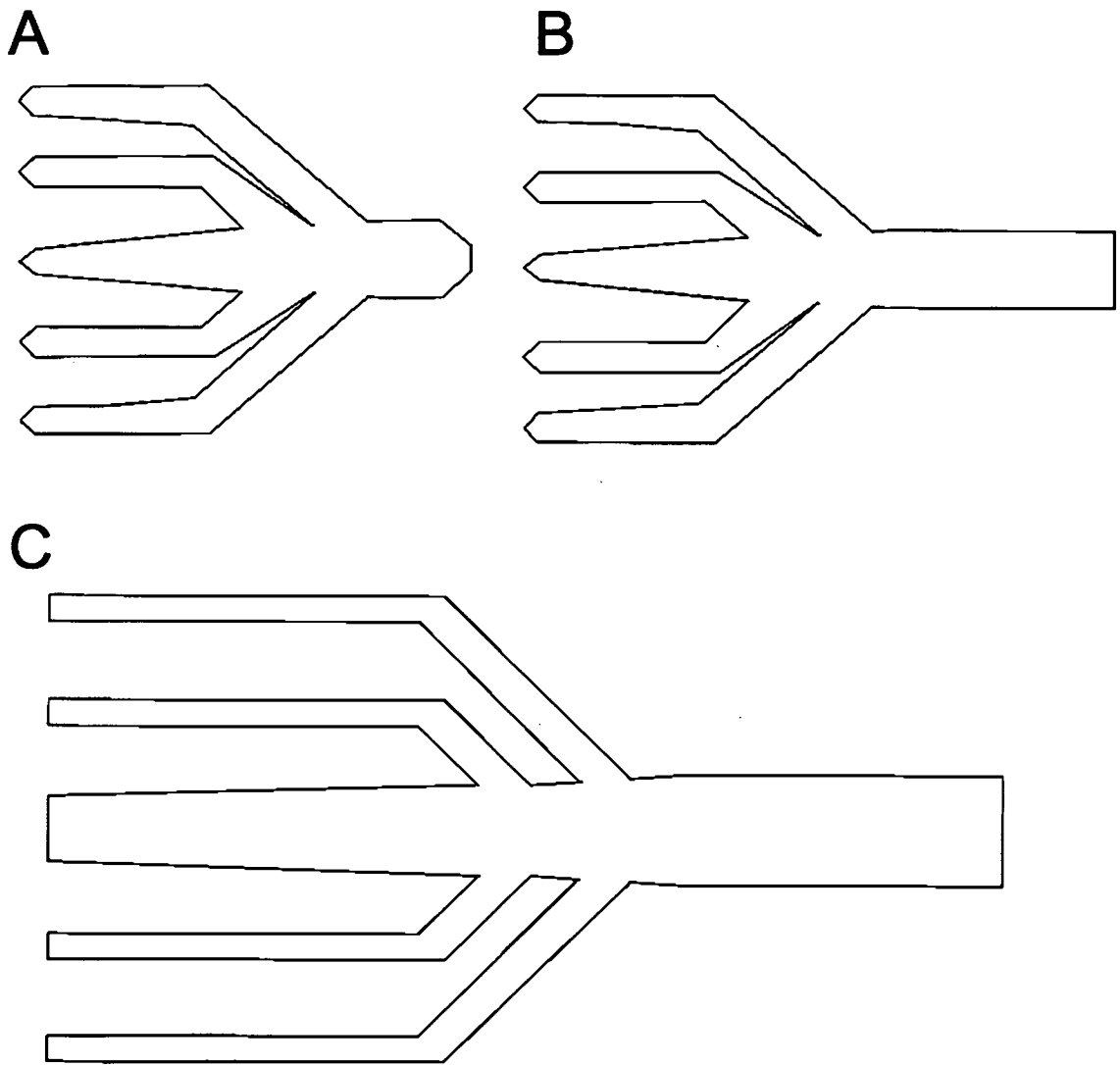


FIG. 10

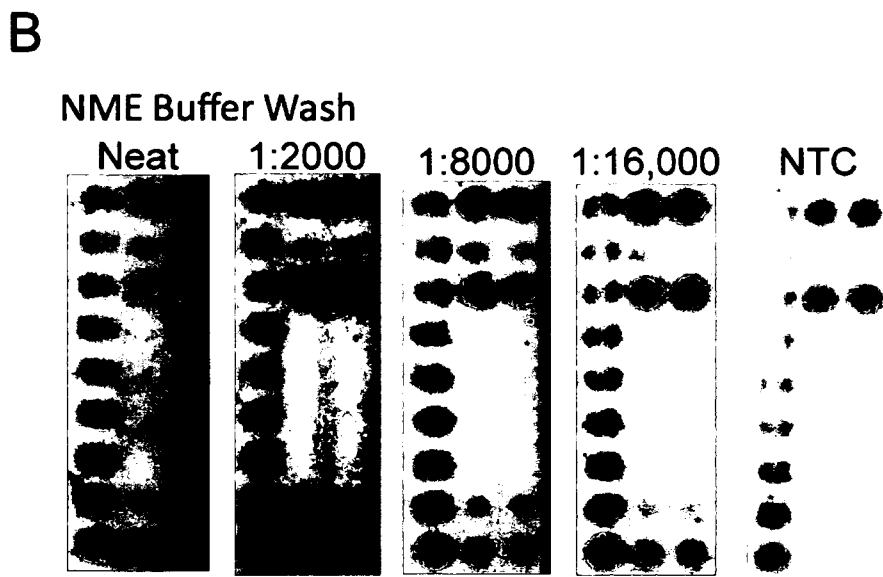
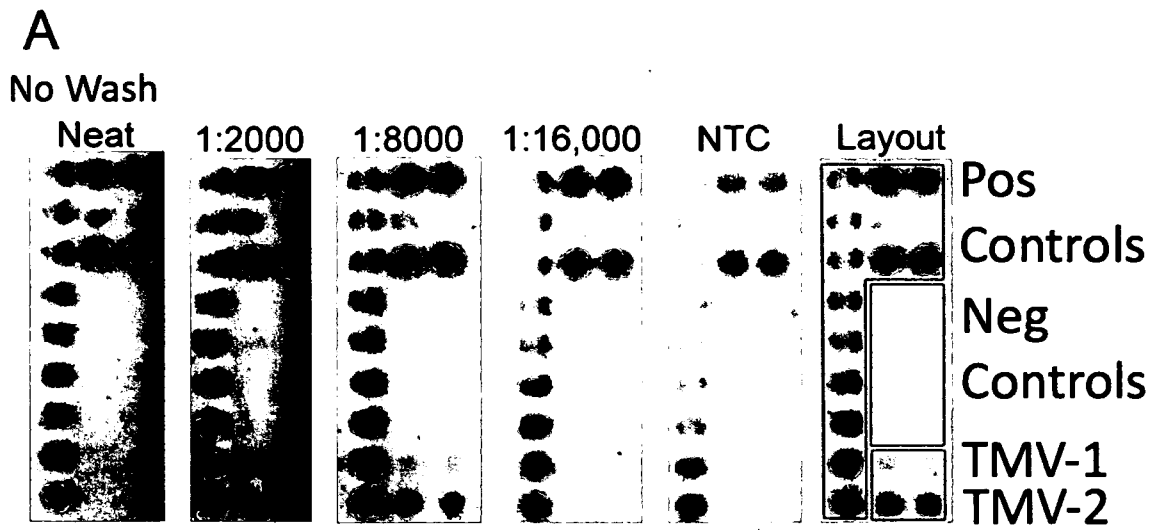


FIG. 11

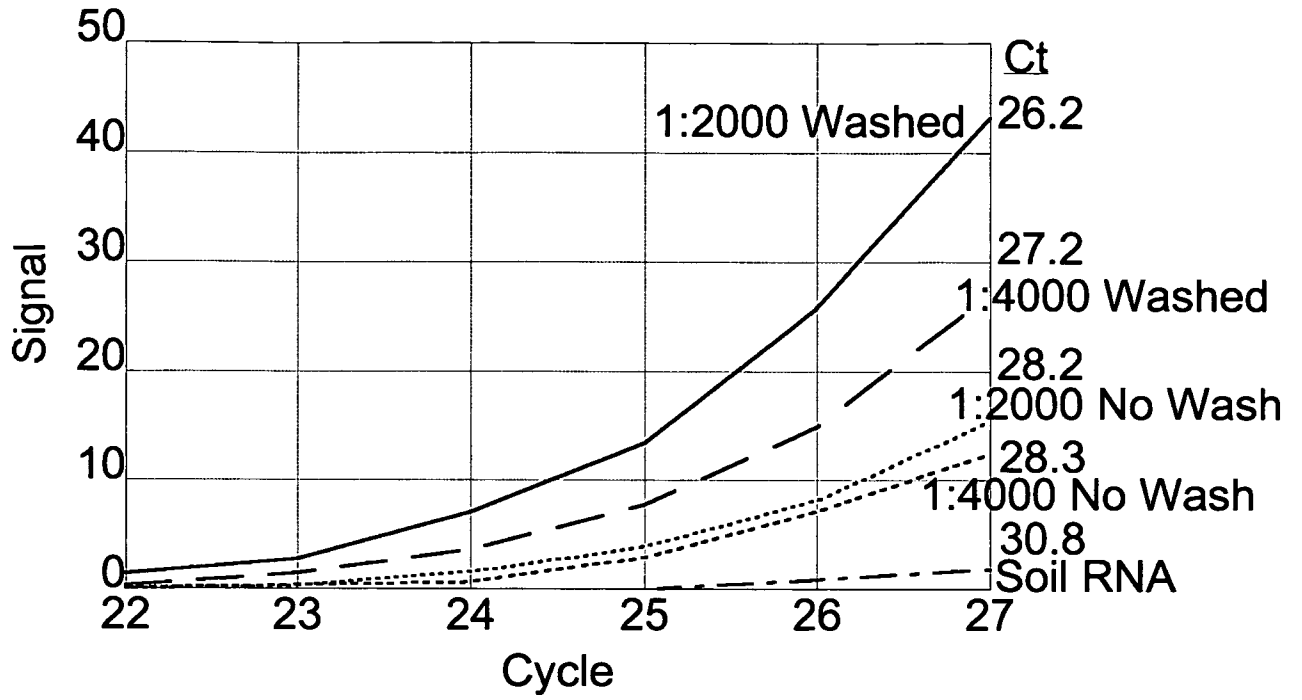


FIG. 12

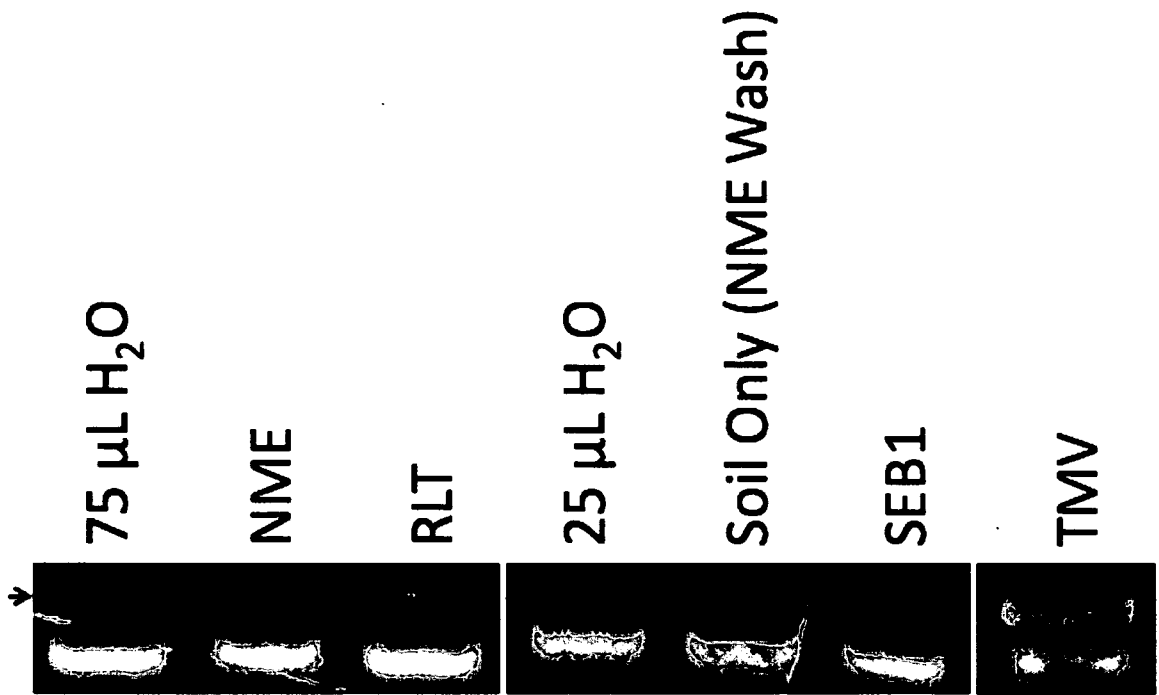


FIG. 13

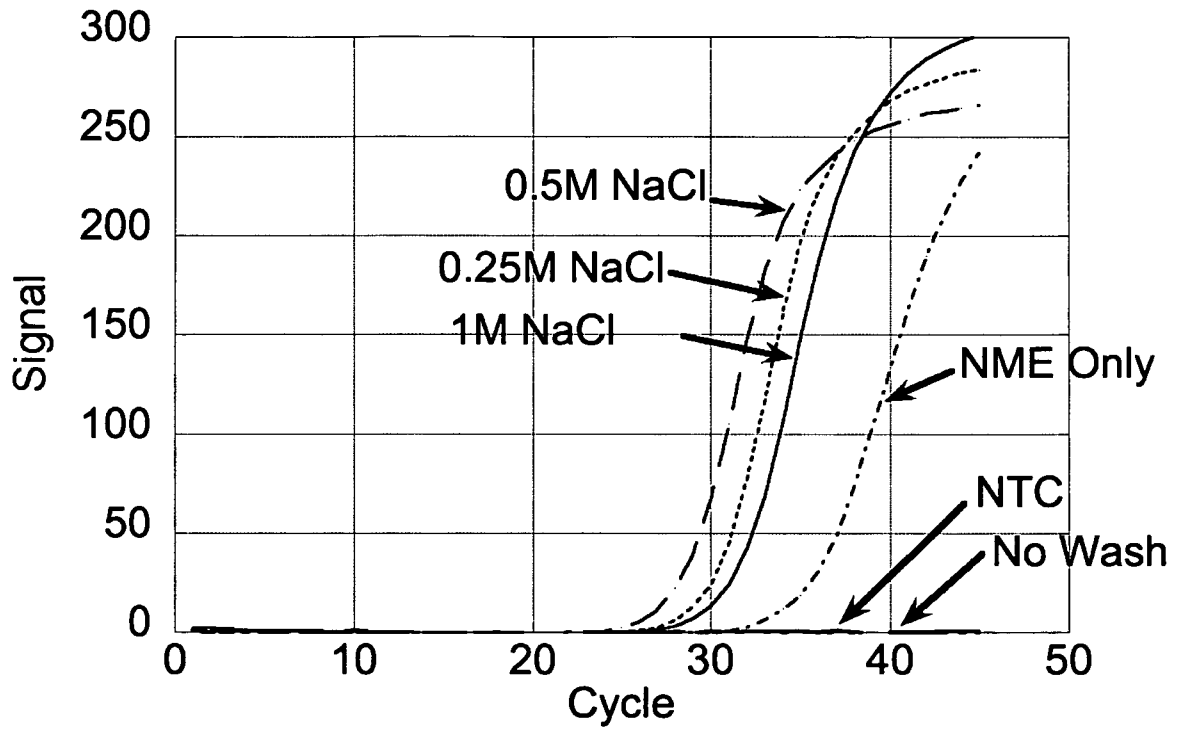


FIG. 14

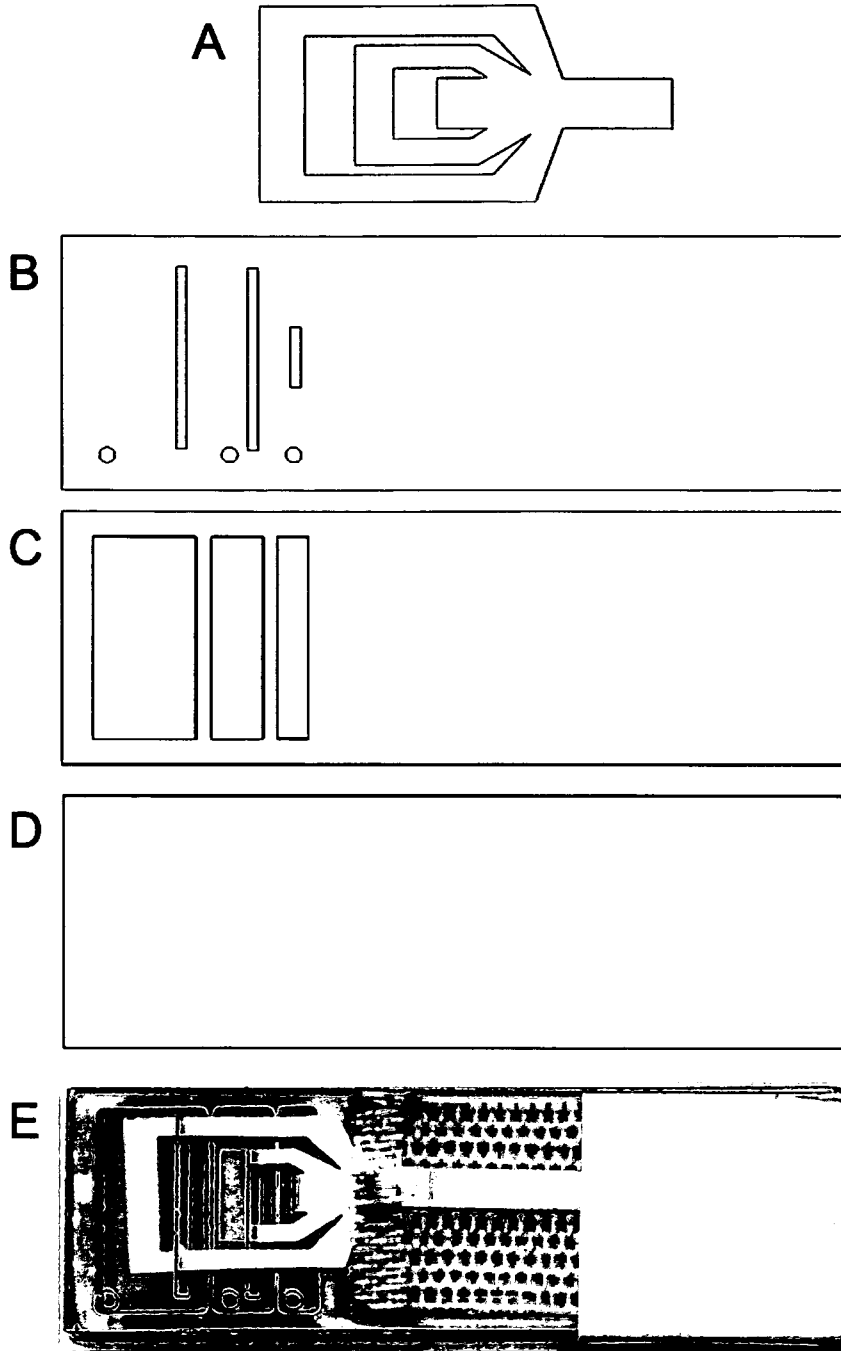


FIG. 15

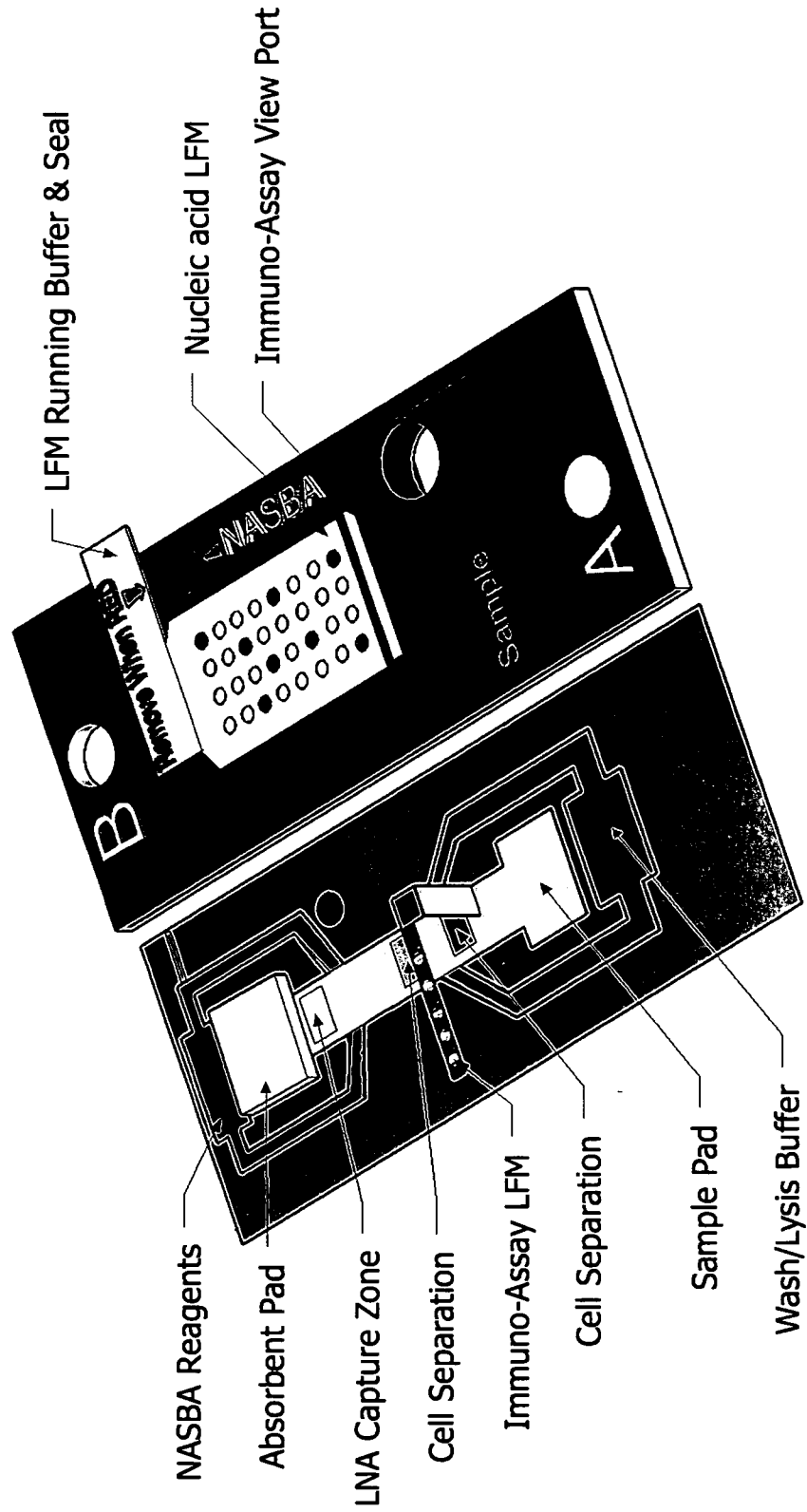
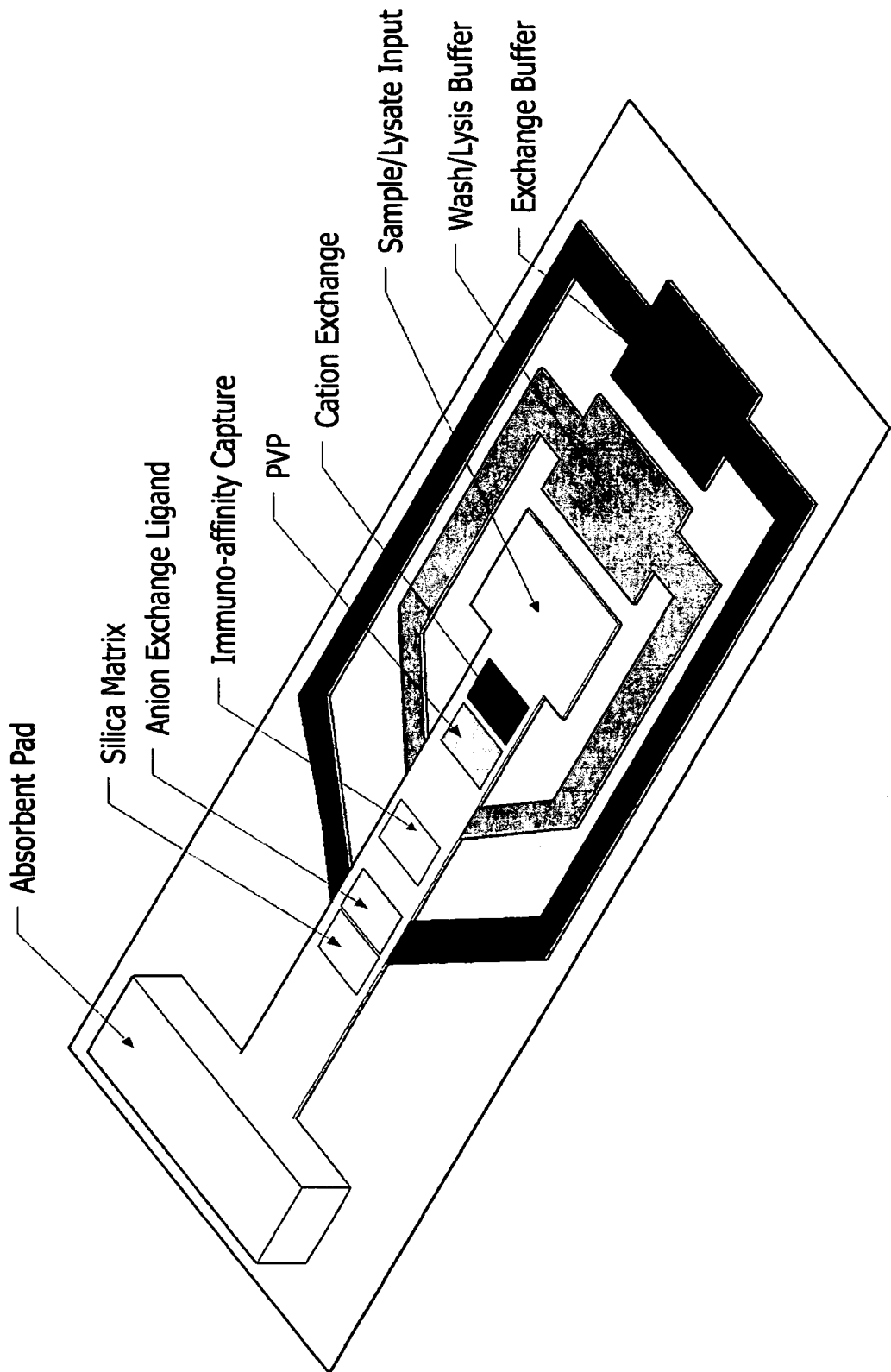


FIG. 16





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/02809

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 15/06; C12Q 1/68 (2009.01)

USPC - 435/6; 73/61.71

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - G01N 15/06; C12Q 1/68 (2009.01)

USPC - 435/6; 73/61.71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
IPC(8) - G01N 15/06; C12Q 1/68 (2009.01), USPC - 435/6; 73/61.71, 61.73, 53.04, 863.03: keyword search, as belowElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
USPTO PubWest (databases: PGPB, USPT, USOC, EPAB, JPAB), Google Scholar - Search Terms: lateral, horizontal, flow, sandwich, hybridize, hybridization, anneal, assay, lysis, lyse, disrupt, cell, bacterium, antibody, immunoglobulin, immobilize, bound, anchored, matrix, channel, fluidic, microfluidic, RNA, DNA, polynucleotide, nucleic acid

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/0076825 A1 (CHENG et al.) 20 June 2002 (20.06.2002) para [0058], [0083], [0146], [0188], [0012], [0023], [0211], [0253], [0141]	1-4
A	US 2003/0190608 A1 (BLACKBURN) 9 October 2003 (09.10.2003)	1-4
A	US 2006/0024813 A1 (WARTHOE) 2 February 2006 (2.02.2006)	1-4

 Further documents are listed in the continuation of Box C.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

13 October 2009 (13.10.2009)

Date of mailing of the international search report

22 OCT 2009

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/02809

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: 19  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
Claim 19 fails to meet PCT Rule 6.2(a)-(b) as it expressly refers to the figures.
  
3.  Claims Nos.: 5-14, 17, 18, 20  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-4, directed to a lateral flow sample preparation device comprising a lateral flow matrix comprising at least: (a) a sample receiving zone, and (b) an immuno-capture zone for a biological particle in lateral flow contact with said sample.

Group II: claims 15 and 16, directed to a device comprising a lateral flow matrix which defines a flow path and which comprises a geometrically-defined absorbent material capable of supporting fluid wicking and the passive control of at least one fluid flow.

- Please see extra sheet for continuation -

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-4

- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/02809

## Continuation of Box III: Lack of Unity of Invention

The inventions listed as Groups I - II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I does not require the absorbent material, as required for Group II.

Group II does not require an immuno-capture zone for a biological particle in lateral flow contact with said sample, as required by group I.

The only common technical element shared by the above groups is that they are related to a matrix or material which supports passive lateral flow. This common technical element, however, does not represent an improvement over the prior art of US 2006/0246513 A1 to Bohannon (see para [0006], [0011], [0015], [0027], [0042], [0044], [0051], [0065], [0076], [0087], [0093], [0115], [0119], [0127], [0137], [0138]).

Groups I to II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.