An Inexpensive and Simple Nucleic Acid Dipstick for Rapid Pathogen Detection

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Systems Integration in Biodefense
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Requirement of Rapid Responder/Identifier
(suitable for POC and field use)

- Rapid response
- Highly sensitive
- Highly specific
- Low false positive rate
- Inexpensive!
- Simple and easy to use
- Portable
- Disposable!
- Long shelf life
- Easy to scale up production!
Limitation of two current methods

**Antibody-based detection: e.g. dipstick assay**
- Rapid response, <5 min
- Easy to operate and portable
- Inexpensive (<$1)
- Low sensitivity
- Low specificity
- Labor intensive antibody screening

**Nucleic acid detection: e.g. PCR Taqman assay**
- Highly sensitive
- Highly specific
- Low false positives
- Expensive due to the cost of PCR cycler (~$30K), centrifuge
- Heavy and needs power to operate
- ~40 minute response time (15-30 minute amplification, 10 minute DNA extraction)
Nucleic Acid Pathogen Dipstick Based on Isothermal Amplification and Lateral Flow Dipstick Detection

- **Fast response** (<30 min for DNA)
- **Highly specific with low false positives** (nucleic acid detection)
- **Highly sensitive** *(isothermal nucleic acid amplification)*
- **Capable to process a larger volume of sample** (~2 ml v.s. 20 µl of PCR sample)
- **Portable and simple**
- **Inexpensive** (<$10 price tag, due to the elimination of PCR and centrifugation)
- **Easy to operate**
- **Multiplexed pathogen analysis** (imprinting multiple strip lines)
- **Optional strip reader module**
Nucleic Acid-based Dipstick Assays

1. Extraction of nucleic acid
2. Isothermal amplification of pathogen sequence
3. Amplification product detection

Collect pathogen by swab from mouth or nose (10-15 minutes)

Extraction of nucleic acid of pathogen by using a cartridge

Isothermal Amplification (10 – 30 minutes)

Multiple capture sequences for the detection of multiple pathogen marks (3-5 minutes)
Simple Nucleic Acid Dipstick for Rapid and Specific Pathogen Detection

Clinical sample → DNA/RNA extraction → Pathogen NA amplification → Signature detection

Swabs, wipes, air filters

Conventional approach: vacuum or centrifugation
LANL approach: simple cartridge, no vacuum or centrifugation!

PCR cycler and micro pipettor:
- Isothermal amplification
- No cycling or special pipettor!

Fluorescence detector:
- Colorimetric by eye or simple scanner

Advantages of our approach:
- Self-contained
- Disposable
- Simple, inexpensive
- No electrical power
- Simple, inexpensive
- Low and high density
Nucleic Acid-based Dipstick Assays

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Conventional Antibody-based Dipstick

Reaction reservoir

Cap

Wide Absorbent Tip

Result Window

Splashguard

Thumb Grip

control window

Result readout

Test result

Positive control
Typical Lateral-flow Dipstick Test Strip Design

Antibody with gold label + Bacteria → capture

Membrane with capture Ab
Nucleic Acid-based Dipstick
(LANL Sandwich Assay)

Dyed beads with detection oligo

Amplified single-stranded DNA

Membrane with capture oligo
Production of Nucleic Acid Dipstick

Figure 1. Schematic diagram of the developed sandwich-based NA lateral flow assay. Top panel: Top and side views of the assay assembly. Bottom panel: depicts detection of a target sequence. Detection of the single-stranded pathogen amplification products is achieved with two target-specific oligonucleotide probes. A: a capture probe is immobilized on a nitrocellulose membrane through UV crosslinking. C: a labeling/detecting probe is conjugated to the surface of blue microsphere. When a specific target sequence (B) is present, a sandwich complex is formed among the capture probe, target sequence, and labeling/detecting probe resulting in a visible blue spot on the membrane.
Detection Sensitivity of Pag gene target.

[Image showing a sandwich nucleic acid dipstick membrane assay with concentrations of Pag target in nM ranging from 20 to 0.05 nM.]

CapB → Pag → Cya → Pos ctrl → Buffer ctrl

[Pag target] in nM
Dipstick Detection of Isothermal Amplification Product

20 ul of serial dilution of amplified DNA were applied to Dipsticks.
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Exponential Amplification

chain reaction

<table>
<thead>
<tr>
<th>Cycle</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
</table>

**PCR**
30 sec/cycle

**Isothermal Amplification**
~2 sec/cycle
## Comparison of Isothermal Amplification Methods

<table>
<thead>
<tr>
<th>Parameter/method</th>
<th>RCA</th>
<th>SPIA</th>
<th>EXPAR</th>
<th>Invader</th>
<th>Thermo SDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers of primers required</td>
<td>2</td>
<td>2 or more (for exponential)</td>
<td>2 or more (for exponential)</td>
<td>2 or more</td>
<td>4 for exponential</td>
</tr>
<tr>
<td>Enzyme activity involved</td>
<td>Ligase+ Phi 29 polymerase</td>
<td>RNase H + pol (rBst large fragment)</td>
<td>Restriction enzyme N BstNB + polymerase Vent exo-</td>
<td>Cleavase (flap endonuclease)</td>
<td>Restriction enzyme (BsoBI) + polymerase ExoBca</td>
</tr>
<tr>
<td>Required repeated primary primer binding to target</td>
<td>No. (Once, locked onto target for continuous polymerization)</td>
<td>Yes. (Repeated primer cleavage &amp; binding for each round)</td>
<td>No. (Binding once and continuous cleavage &amp; Polymerization)</td>
<td>Yes. (reporter oligo repeated disassociation &amp; association)</td>
<td>No. (Binding once and continuous nicking &amp; polymerization)</td>
</tr>
<tr>
<td>Probe modifications</td>
<td>Ligatable and circular probe</td>
<td>Chimeric RNA/DNA hybrid</td>
<td>3’ phosphorylation</td>
<td>Oligo with flap sequence</td>
<td>primer with BsoBI recognition site.</td>
</tr>
<tr>
<td>Amplification products</td>
<td>Large fragment &gt;20 kb</td>
<td>~1 kb</td>
<td>~8-16 bases</td>
<td>None</td>
<td>~100 bases</td>
</tr>
<tr>
<td>Extra requirement</td>
<td>restriction enzyme digestion</td>
<td>None</td>
<td>N BstNB recognition site on or near target</td>
<td>None</td>
<td>dCTPαS</td>
</tr>
<tr>
<td>Reaction temperature</td>
<td>30 deg</td>
<td>55-60 deg</td>
<td>60 deg</td>
<td>63 deg</td>
<td>60 deg</td>
</tr>
<tr>
<td>Specificity</td>
<td>Very good (two primers + ligase)</td>
<td>Good (two primers)</td>
<td>Good-Very good (two primers or three primers)</td>
<td>Very good (two matched primers + cleavage)</td>
<td>Very Good (four primers)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>~1000 copy</td>
<td>1000 copy</td>
<td>&lt;200 copy</td>
<td>~2000 copy</td>
<td>10 -1000 copies</td>
</tr>
<tr>
<td>Amplification speed</td>
<td>10^9 folds &gt; 1 hour</td>
<td>potentially &gt;10^10 folds in 1 hour</td>
<td>&gt;10^9 folds in less than 5 minutes</td>
<td>10^7 folds &lt; 1 hour</td>
<td>10^10 folds &lt;15 minutes or 10 copies in 30 minutes</td>
</tr>
<tr>
<td>Compatibility with rapid sensor</td>
<td>No. (extra restriction step and instability of polymerase)</td>
<td>Yes.</td>
<td>Yes.</td>
<td>No. (primer has to be around Tm and repeated hybridization)</td>
<td>Yes.</td>
</tr>
</tbody>
</table>
Real-time Amplification

Ionian Technologies

MJ Opticon at 60°, SybrGreen dye

Dilution series (from 7x10^6 molecules /10µl)

Relative fluorescence

Time (seconds)

35 copies
Helicases unwind DNA duplexes in the presence of SSB and accessory protein. Primers anneal to ssDNA and DNA polymerases extend the primers; one duplex is amplified to two duplexes. dsDNA are separated by helicases and this chain reaction repeats itself.
Dipstick Detection Limit: 30 Ba Genomic DNA Copies

Electrophoresis and dipstick detection of amplification products from genomic Ba DNA
M: 100 bp DNA ladder (Promega)
Multiple Pathogen Targets Detection Using Isothermal Amplification and Sandwich Lateral Flow Dipstick Assay

Electrophoresis of HDA Products amplified from genomic DNA and dipstick detection.
M: 100 bp DNA ladder (Promega); 1: multiplex amplification product of pag and cap; 2: Negative Control; PP: Dipstick test positive control Pag; CP: Dipstick test positive control Cap; PCP: Dipstick test positive control pag+Cap. HDA P&C: Dipstick test of HAD product of lane 1. Cap and pag are capture probes for pag and cap, respectively.

For multiplexed analysis!
Detection of Ba Target from a Highly Heterogeneous Mixture
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Conventional Nucleic Acid Extraction Methods
Require Centrifugation or Vacuum

- Alkaline Lysis: NaOH + SDS, neutralization, ethanol ppt

- Phenol and chloroform extraction for protein removal, ethanol ppt (TRIZOL)

- Silica-based NA absorption in the presence of chaotropic salt and affinity chromatography (Qiagen)

- Combinations of above

- Combination plus Magnetic affinity-based NA extraction
Magnetic Affinity-based Nucleic Acid Extraction without Centrifugation and Organic Solvent

Flu virus solution + Magnetic beads with antibody → Magnetic Separation Stand → 1 Min
Discard Supernatant
Add 0.1M NaOH
Add 0.1M HCl
Take 2 µl for RT at 48°C

Fluorescence vs Cycle

PCR Cycle Number

Positive control
Negative Control

Los Alamos National Laboratory
We have to prepare for the next pandemic!!!
Flu-like Symptom-Based Influenza Diagnosis Approach

- Nucleic acid dipstick < 1 hour,
- Inexpensive, < $10/unit
- Detect multiple flu-like bacteria and viruses including Type A, Type B, Avian H5N1 influenza viruses, SARS, Adenovirus, Parainfluenza, RSV, Rhino viruses
- Integrated immunological and nucleic acid detection in one simple dipstick platform
- Sensitivity ~100 copies of viruses
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