LAUR #05-9067

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An Inexpensive and Simple Nucleic Acid Dipstick for Rapid Pathogen Detection

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Systems Integration in Biodefense
Washington, D.C.

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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

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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**

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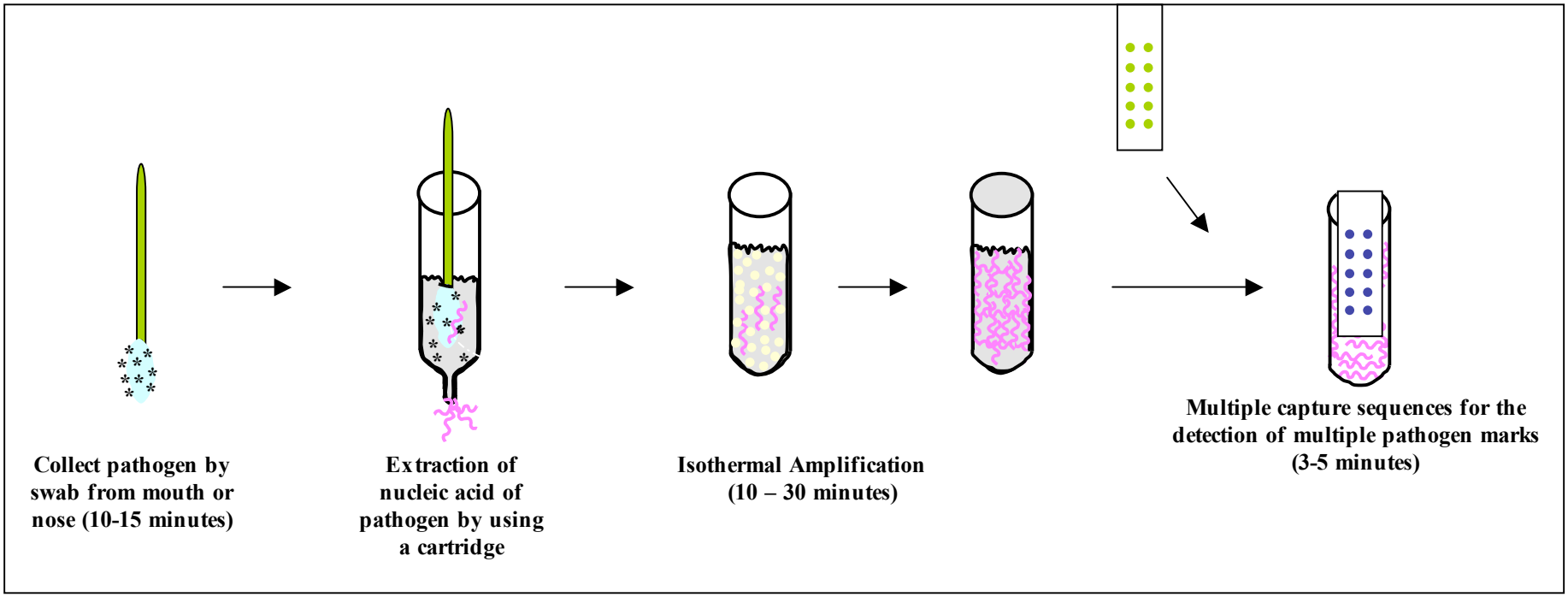
Nucleic Acid-based Dipstick Assays

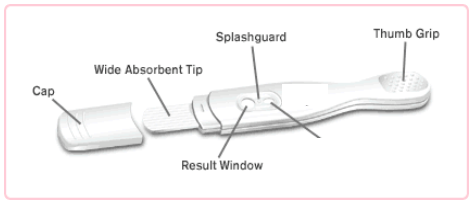
1. Extraction of nucleic acid
2. Isothermal amplification of pathogen sequence
3. Amplification product detection

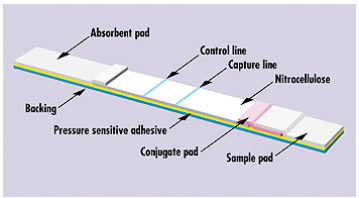


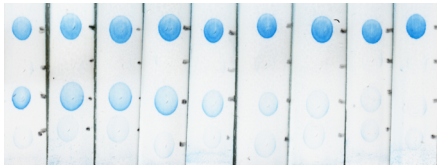
Simple Nucleic Acid Dipstick for Rapid and Specific Pathogen Detection

Clinical sample	→	DNA/RNA extraction	→	Pathogen NA amplification	→	Signature detection
swabs, wipes, air filters						

Conventional approach	vacuum or centrifugation	PCR cycler and micro pipettor	fluorescence detector
LANL approach	simple cartridge no vacuum or centrifugation !	isothermal amplification no cycling or special pipettor !	colorimetric by eye or simple scanner
Advantages of our approach	self-contained disposable	simple, inexpensive no electrical power	simple, inexpensive low and high density

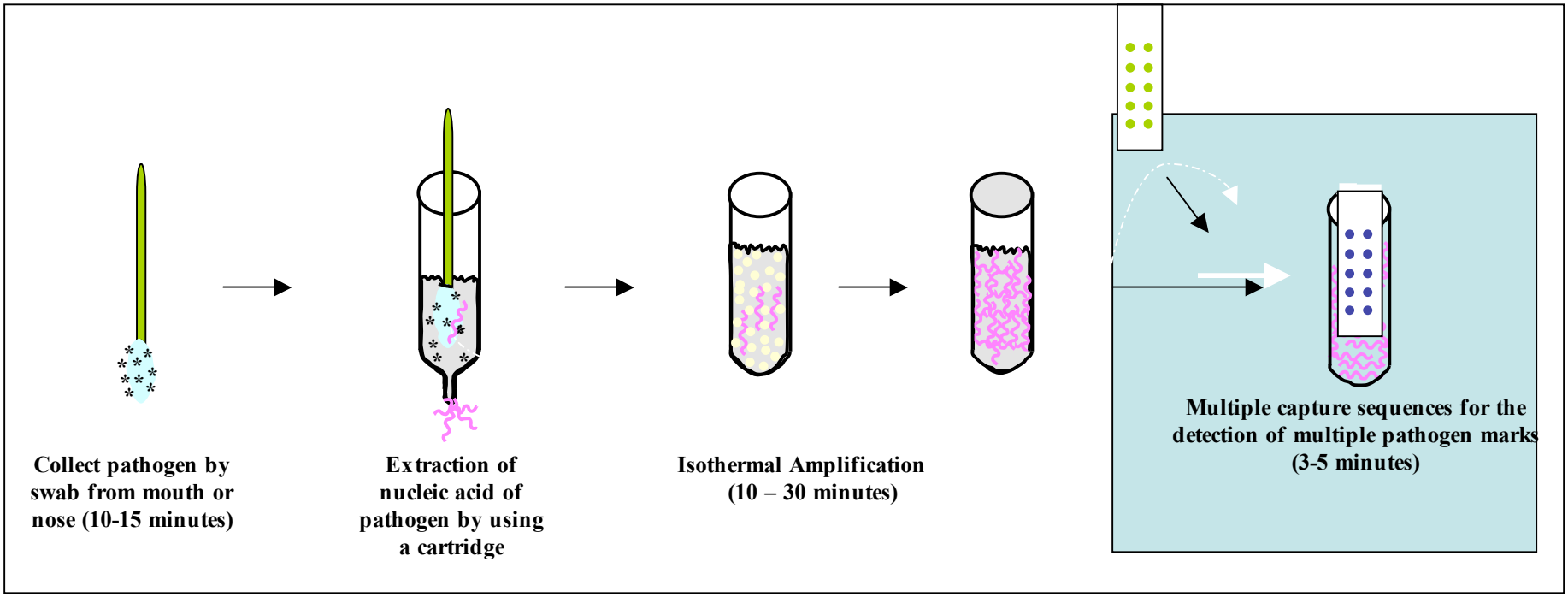




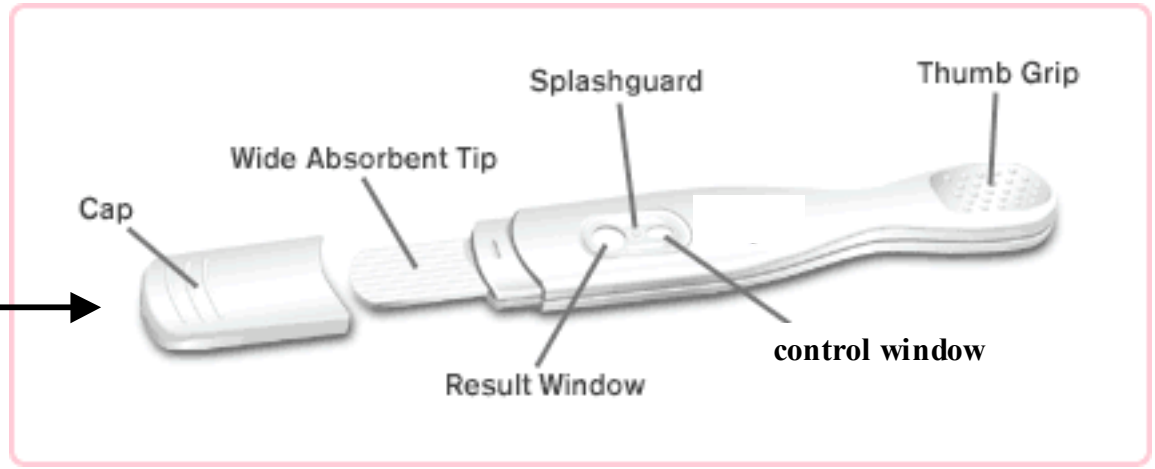


Nucleic Acid-based Dipstick Assays

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



Conventional Antibody-based Dipstick



Result readout

Test result

Positive control

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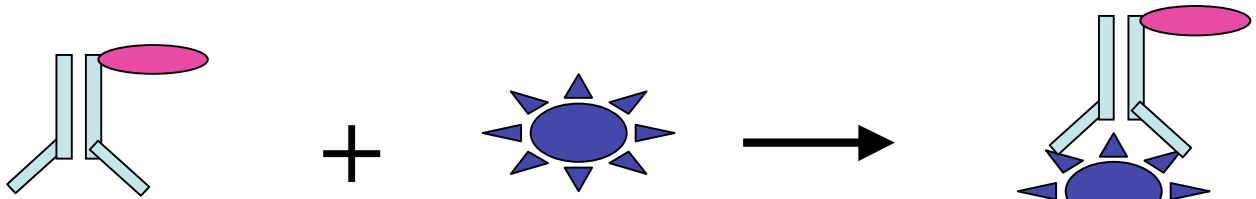
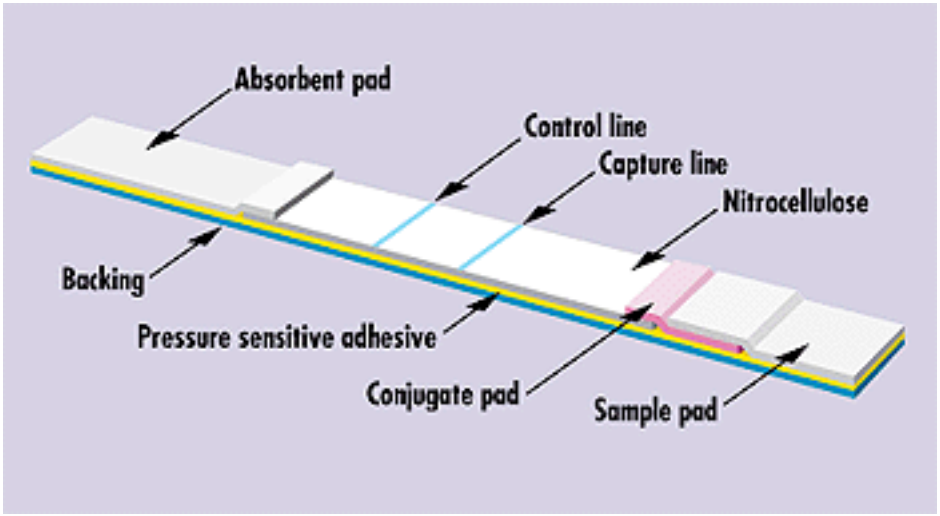
Typical Lateral-flow Dipstick Test Strip Design

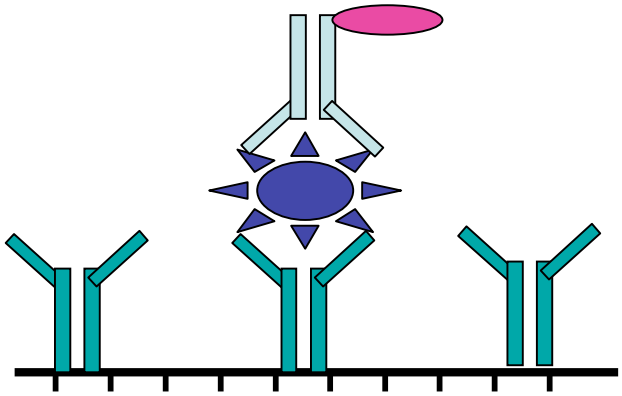


Antibody with gold label

Bacteria

capture





Membrane with capture Ab

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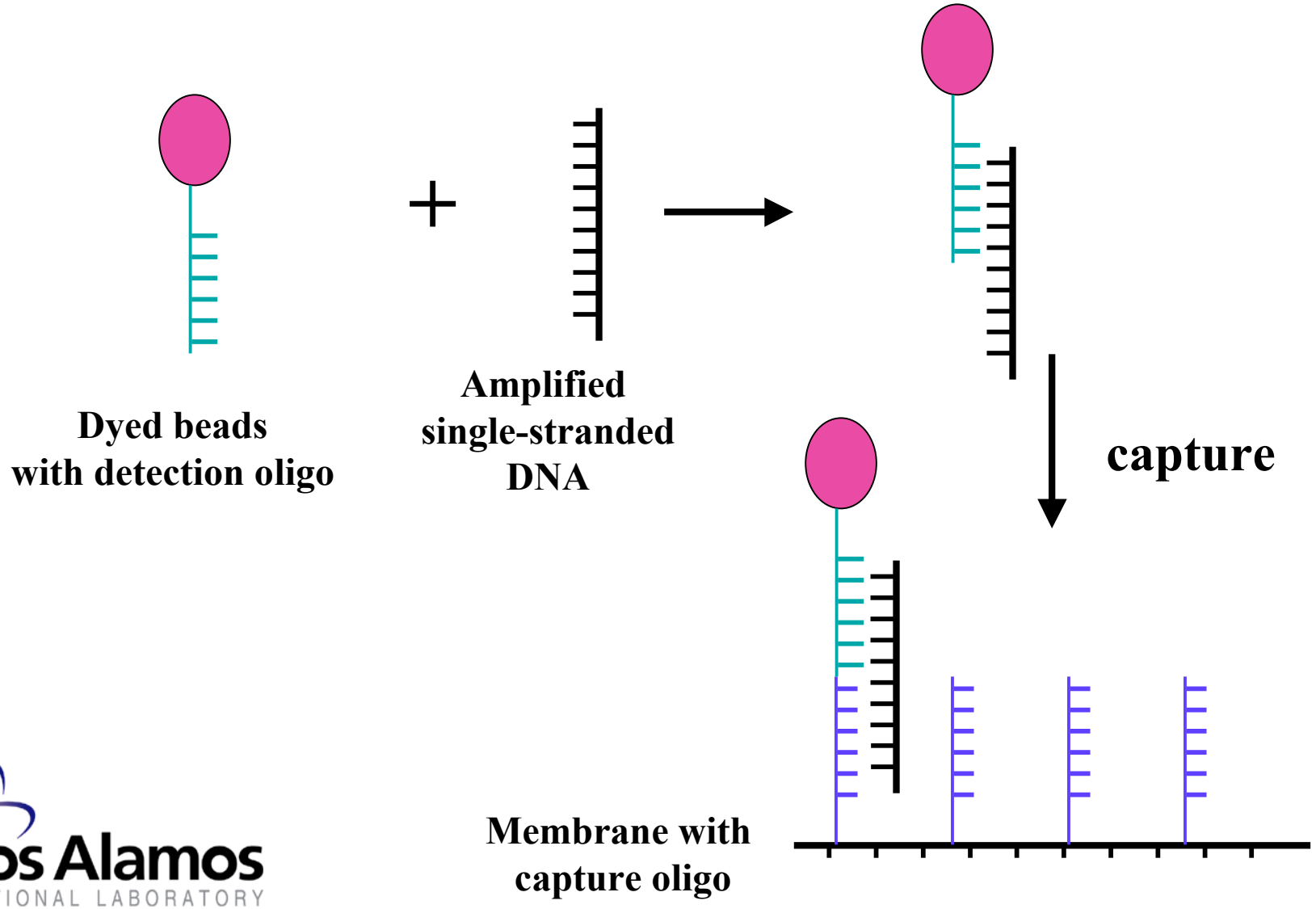
Nucleic Acid-based Dipstick

(LANL Sandwich Assay)



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Production of Nucleic Acid Dipstick

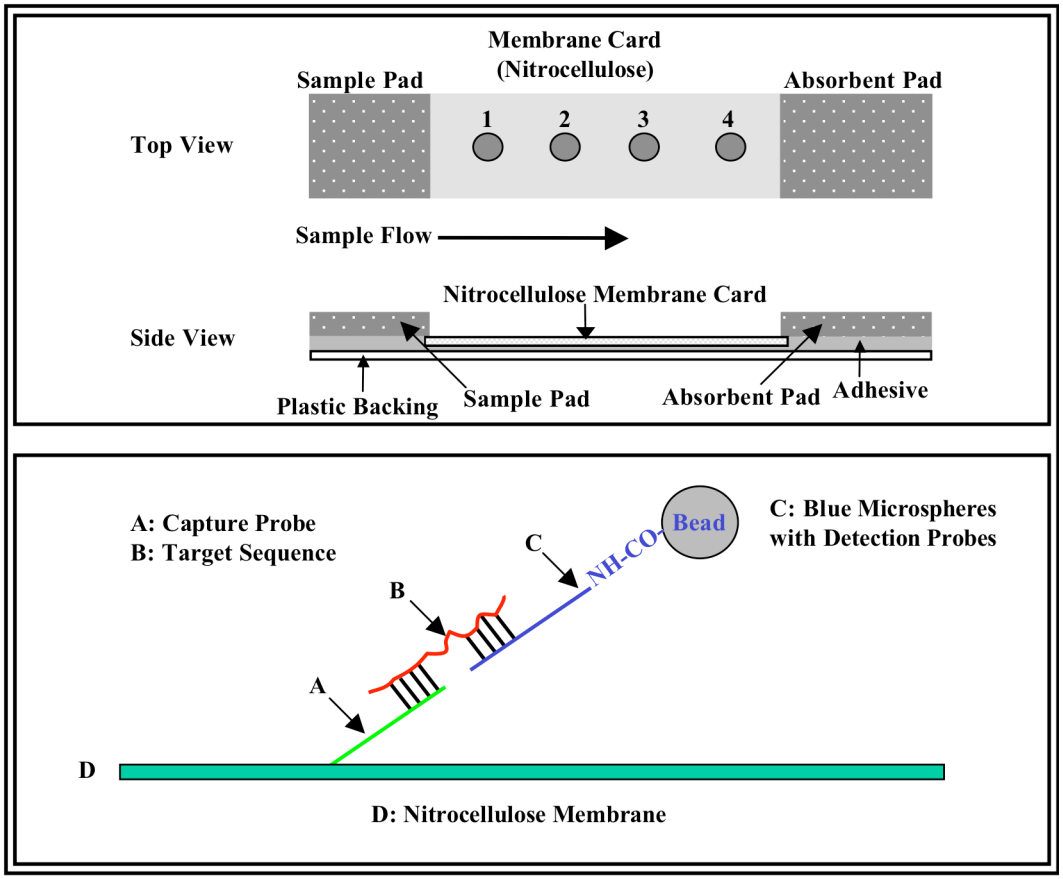
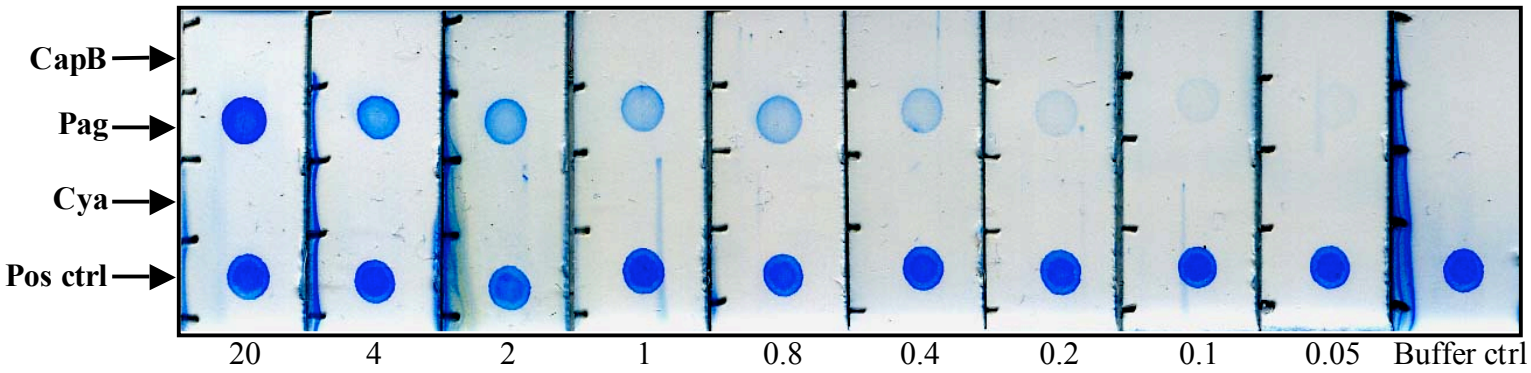


Figure 1. Schematic diagram of the developed sandwich-based NA lateral flow assay. Top panel: Top and side views of the assay assembly. Bottom panel: depicts detection of a target sequence. Detection of the single-stranded pathogen amplification products is achieved with two target-specific oligonucleotide 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.

Sandwich Nucleic Acid Dipstick Membrane Assay

Detection Sensitivity of Pag gene target.



[Pag target] in nM

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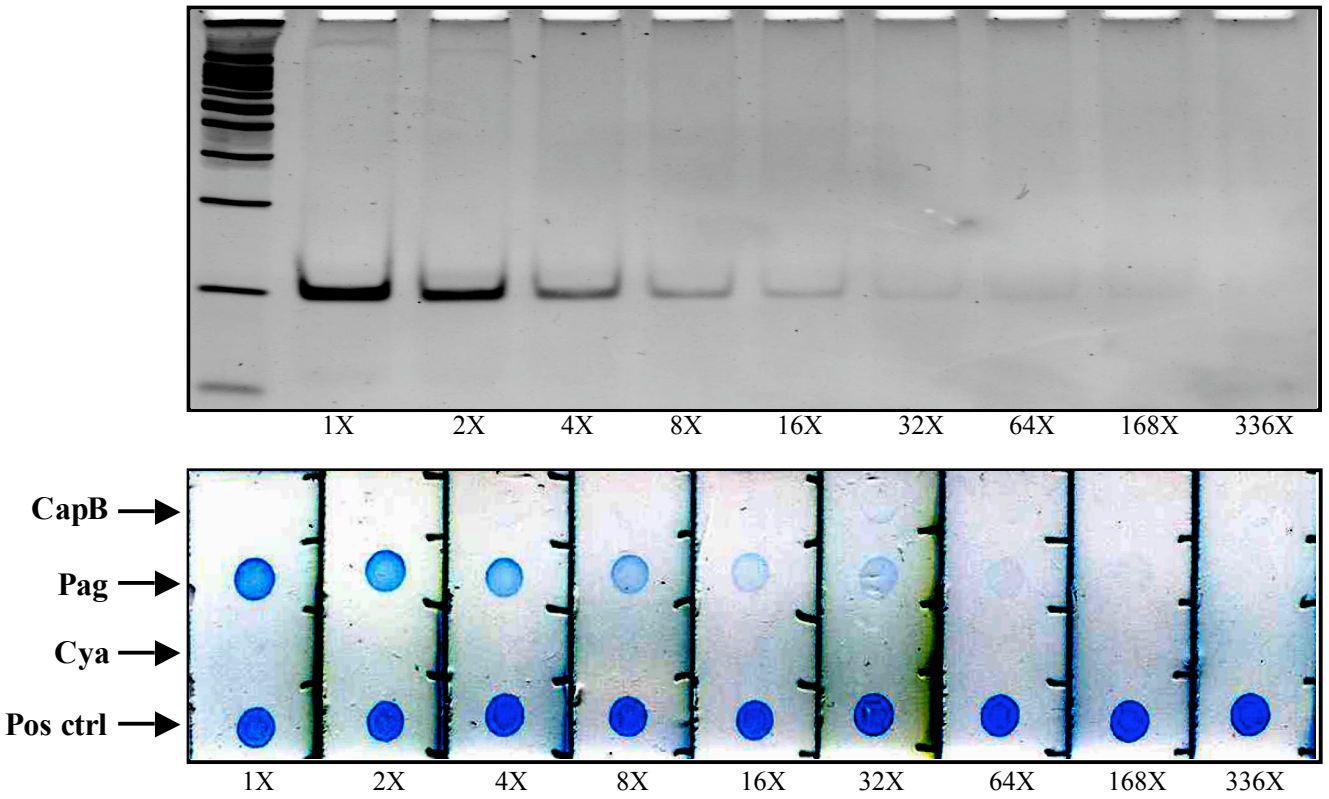
Dipstick Detection of Isothermal Amplification Product



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

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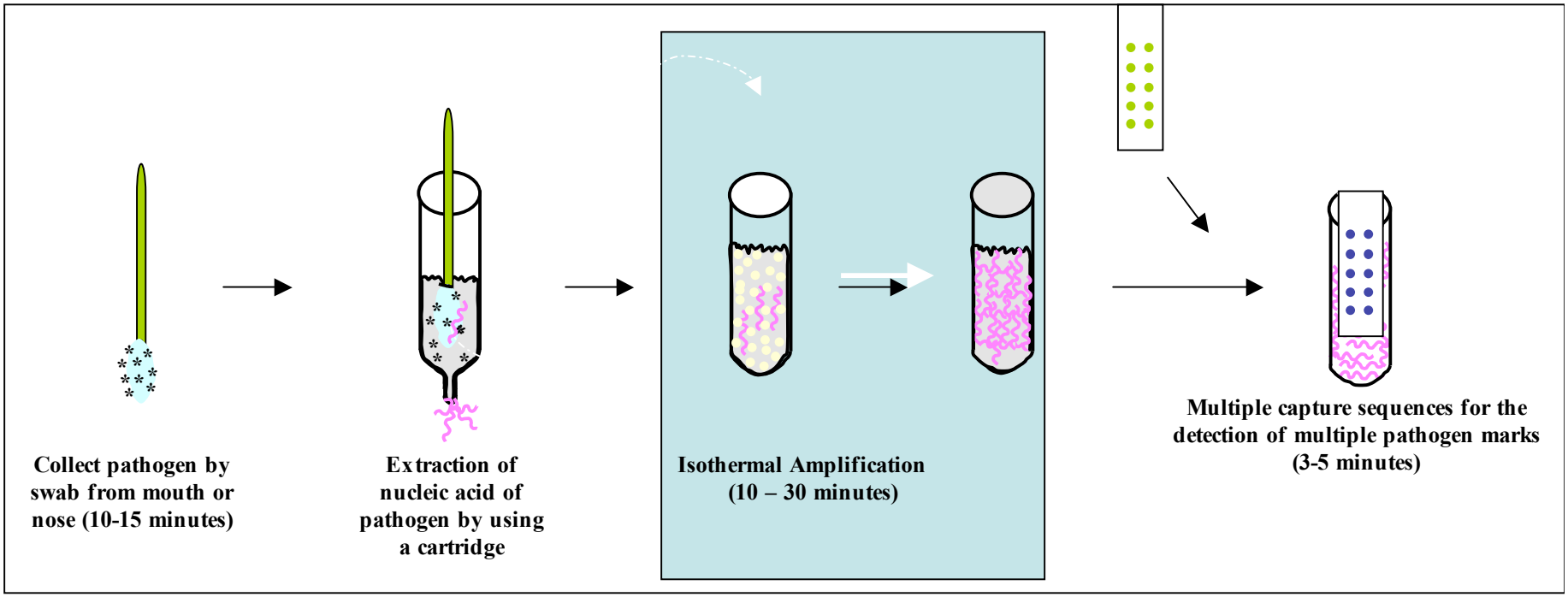
Nucleic Acid-based Dipstick Assays

1. Extraction of nucleic acid

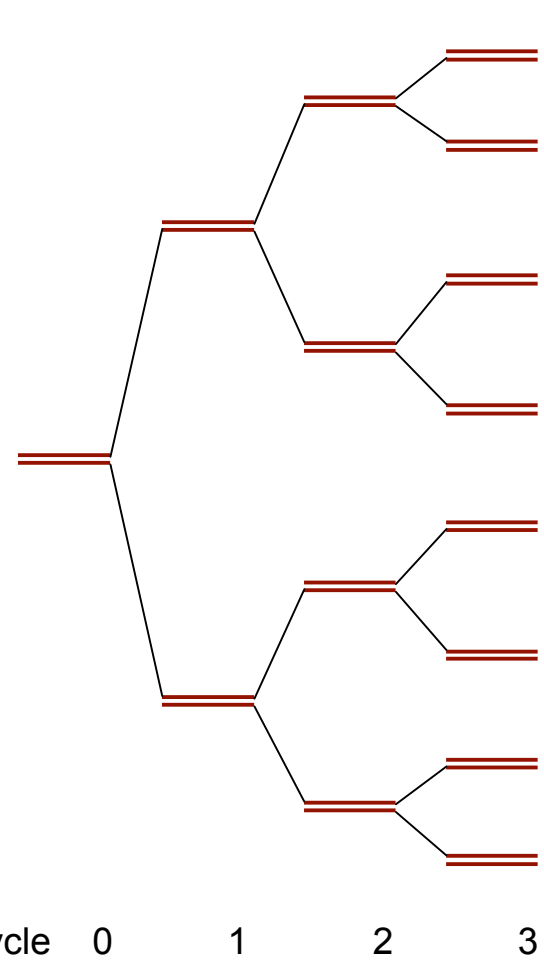
2. *Isothermal amplification of pathogen sequence*

3. Amplification product detection



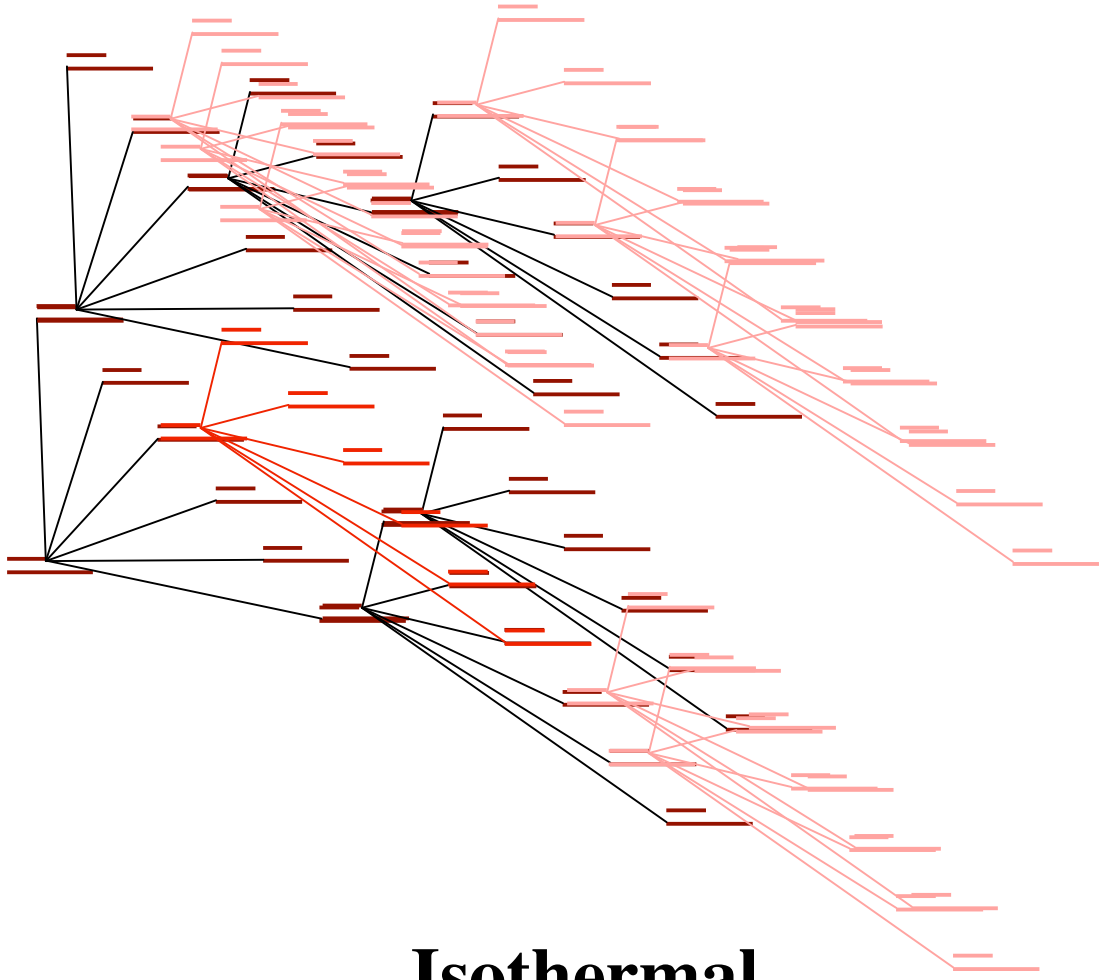
Exponential Amplification

chain reaction



PCR

30 sec/cycle



Isothermal Amplification

~2 sec/cycle

Comparison of Isothermal Amplification Methods

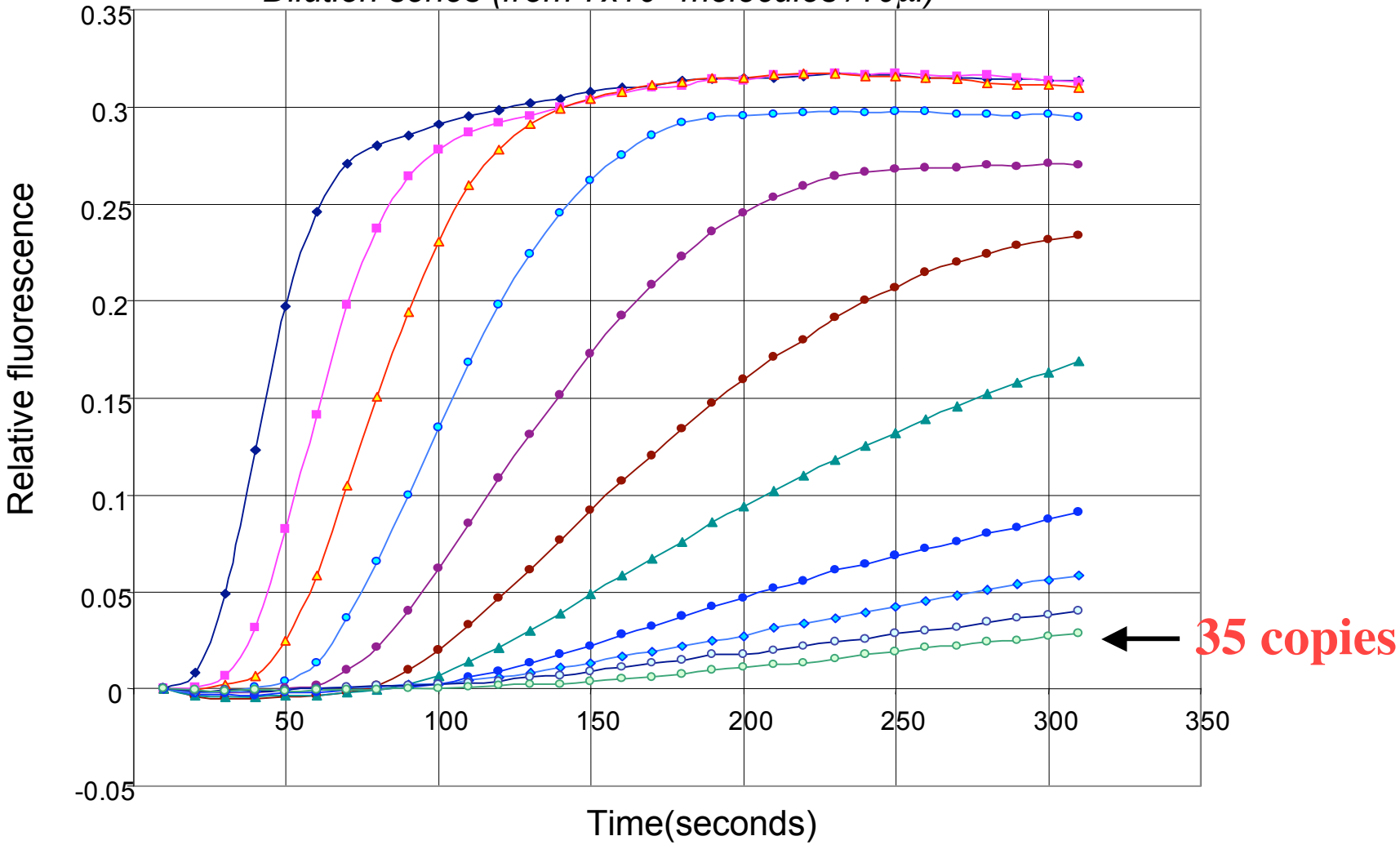
Parameter/method	RCA	SPIA	EXPAR	Invader	Thermo SDA
Numbers of primers required	**2**	**2 or more (for exponential)**	**2 or more (for exponential)**	**2 or more**	**4 for exponential**
Enzyme activity involved	**Ligase+ Phi 29 polymerase**	**RNase H + pol (rBst large fragment)**	**Restriction enzyme**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

Real-time Amplification

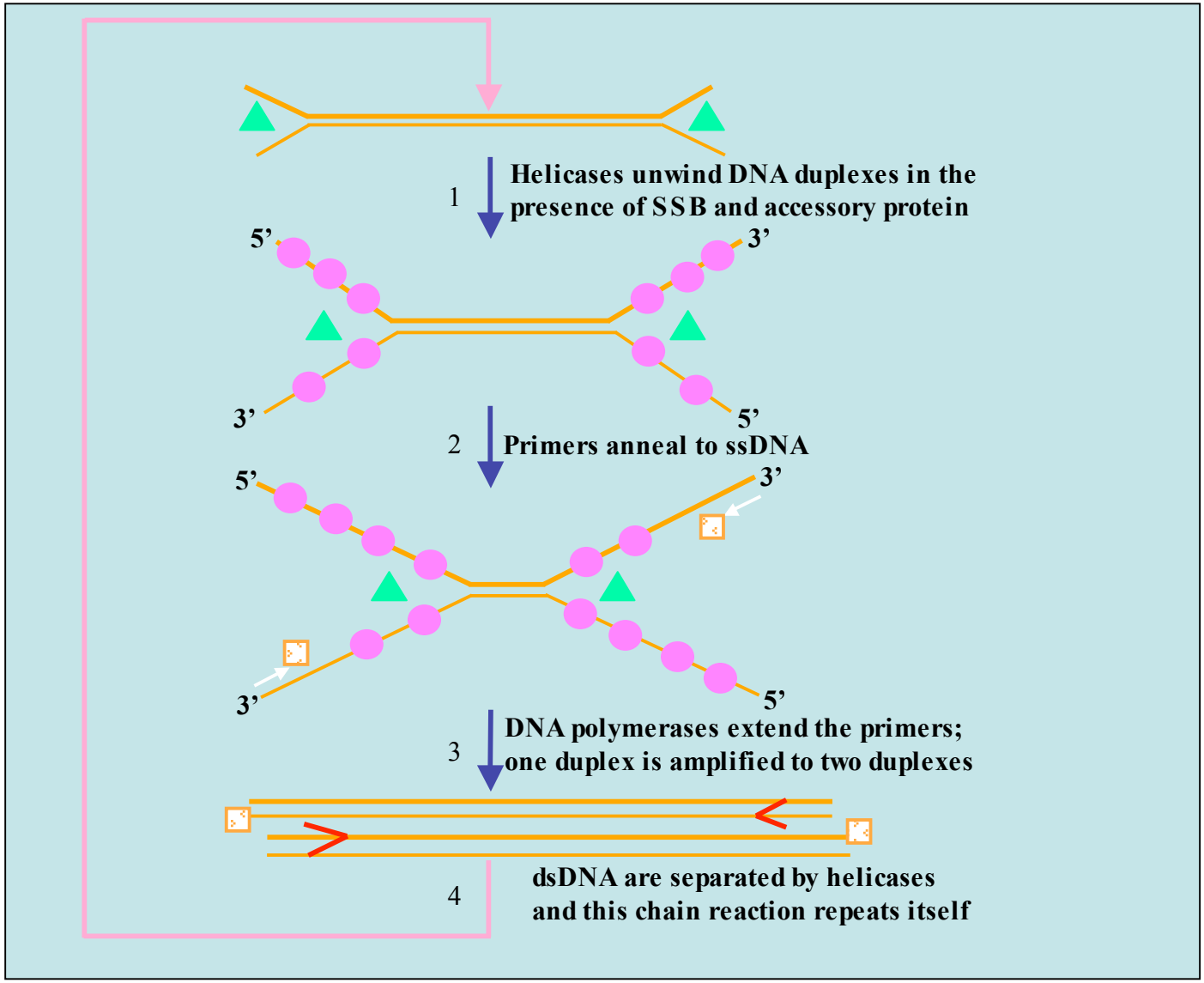
Ionian Technologies

MJ Opticon at 60º, SybrGreen dye

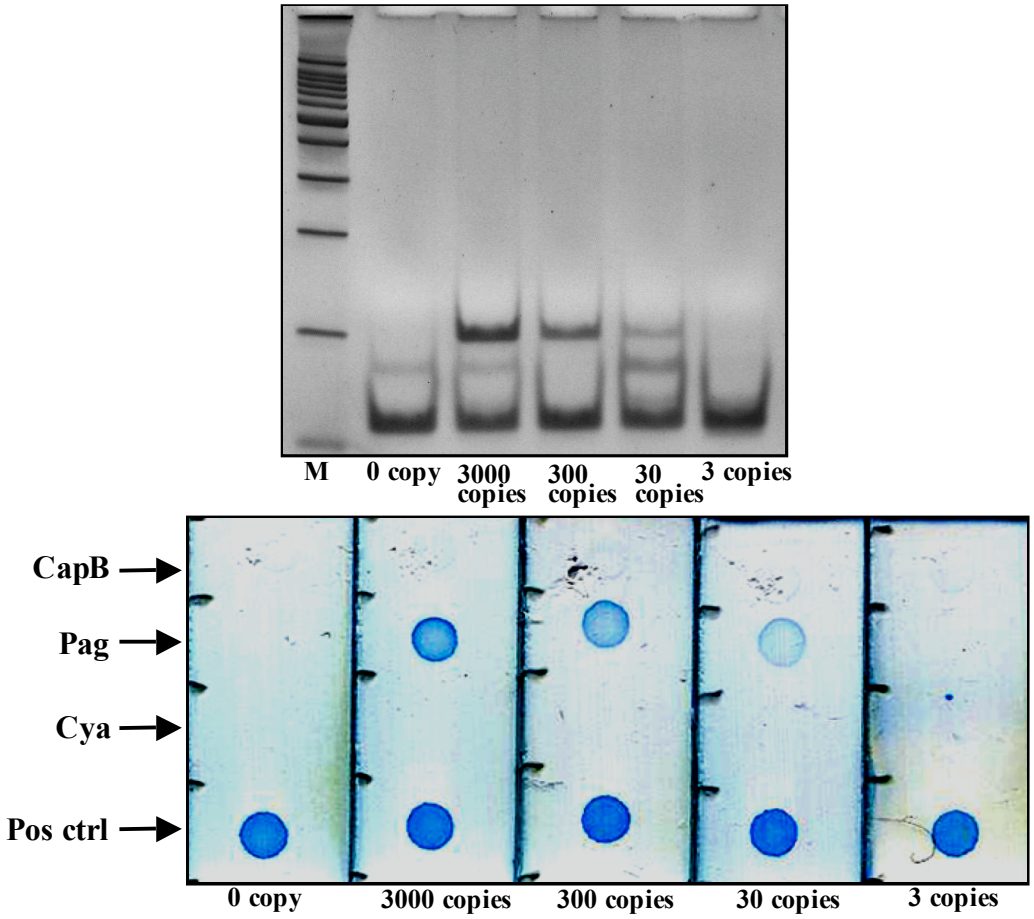
Dilution series (from 7x10^6 molecules /10μl)



Schematic Diagram of HDA (Helicase Mediated Amplification)
Biohelix Inc. 2004



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)

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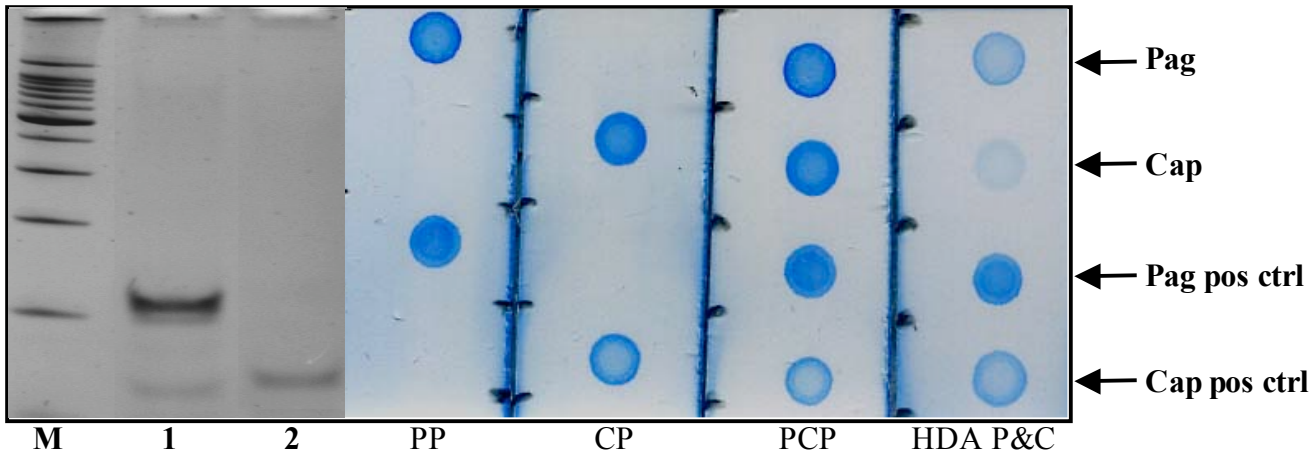
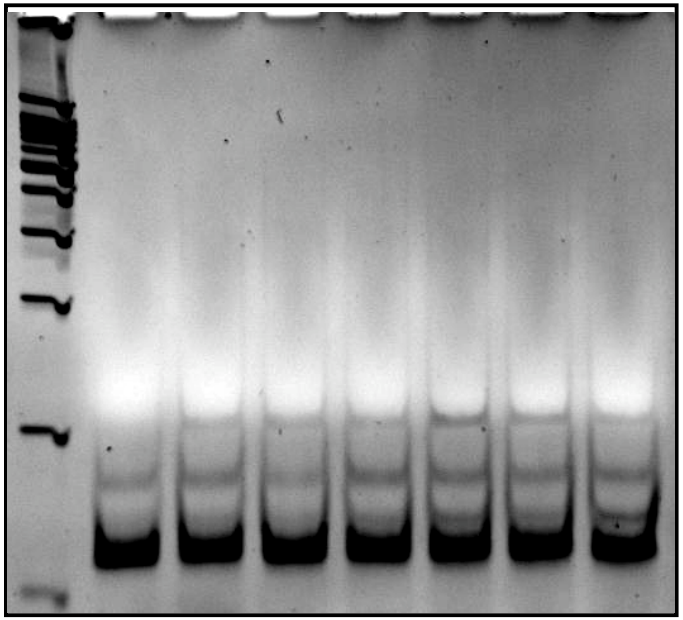
Multiple Pathogen Targets Detection Using Isothermal Amplification and Sandwich Lateral Flow Dipstick Assay

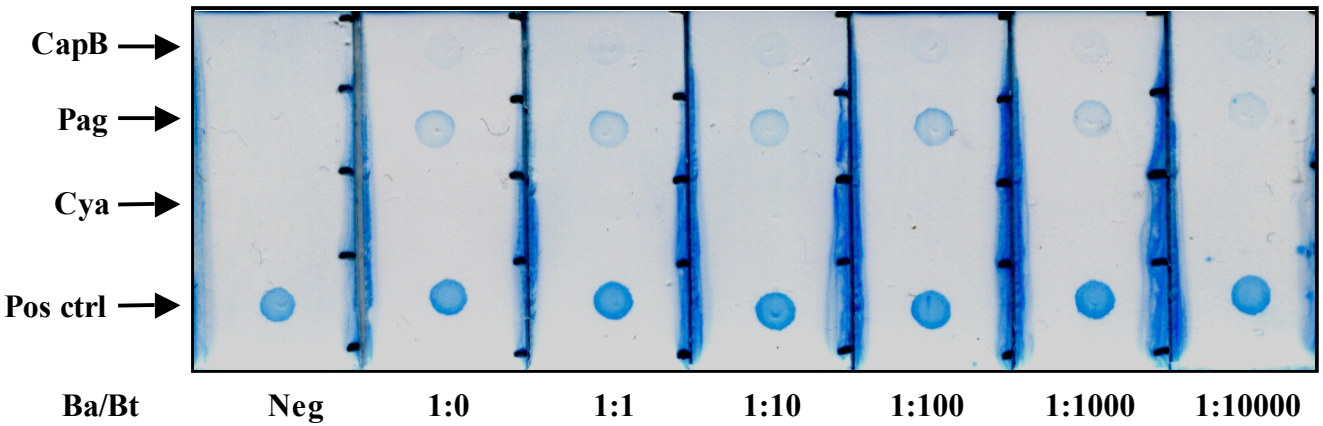


Electrophoresis of HDA Products amplified from genomic DNA and dipstick detection.
M: 100 bp DNA ladder (Promega); 1: multiplex amplification product of *pag* and *cap*; 2: Negative Control; PP: Dipstick test positive control Pag; CP: Dipstick test positive control Cap; PCP: Dipstick test positive control 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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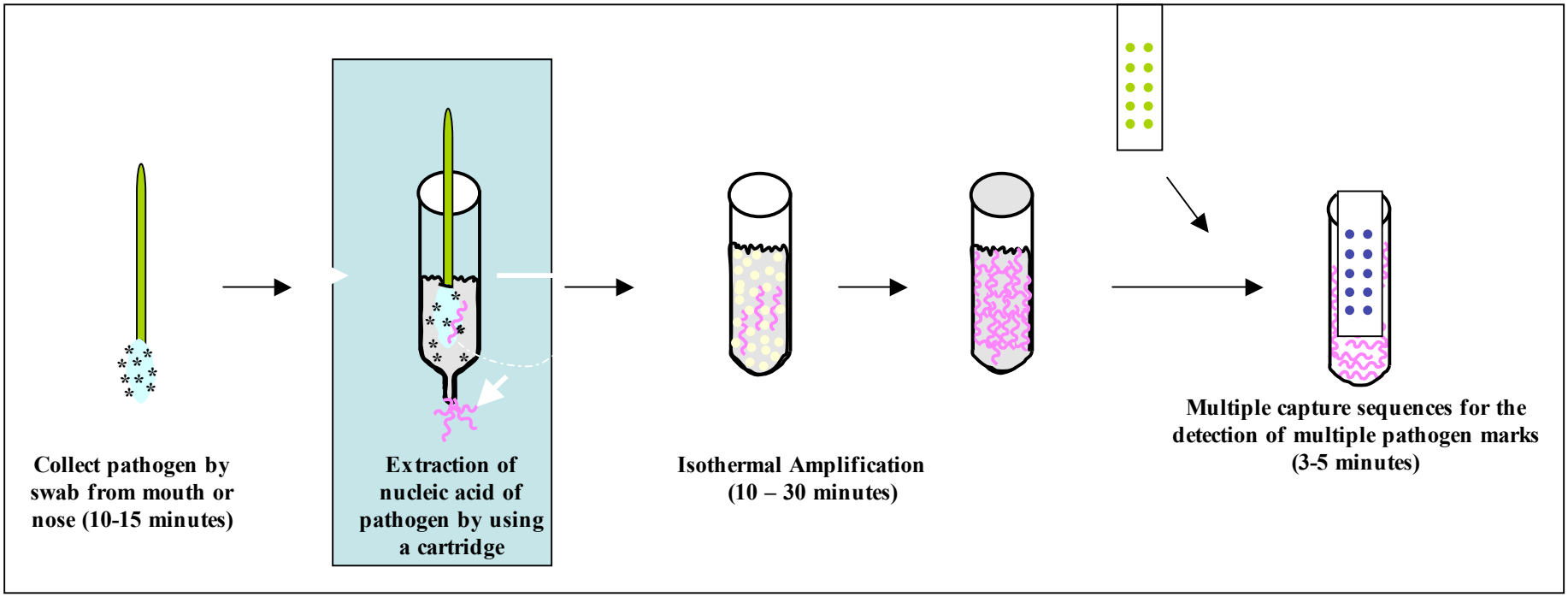

Conventional Nucleic Acid Extraction Methods Require Centrifugation or Vacuum

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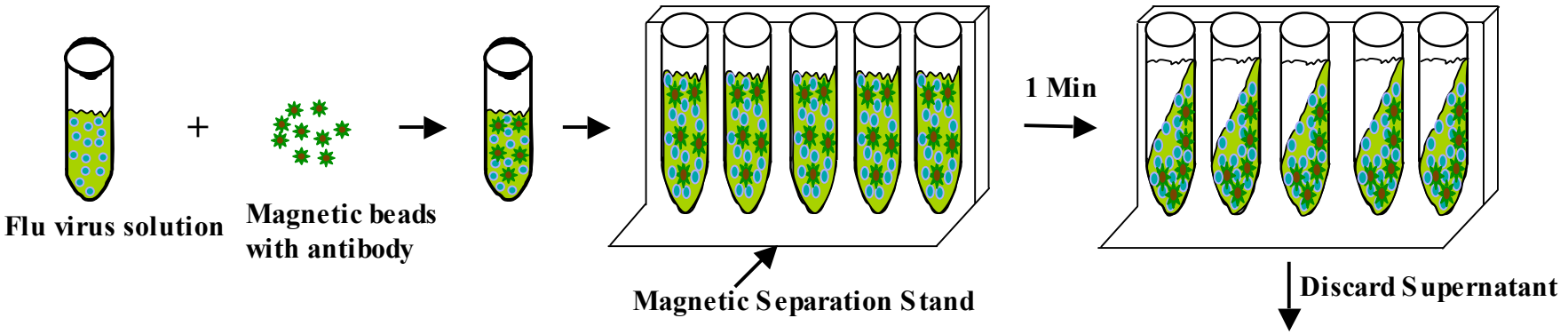
- **Alkaline Lysis: NaOH + SDS, neutralization, ethanol ppt**
- **Phenol and chloroform extraction for protein removal, ethanol ppt (TRIZOL)**
- **Silica-based NA absorption in the presence of chaotropic salt and affinity chromatography (Qiagen)**
- **Combinations of above**
- **Combination plus Magnetic affinity-based NA extraction**

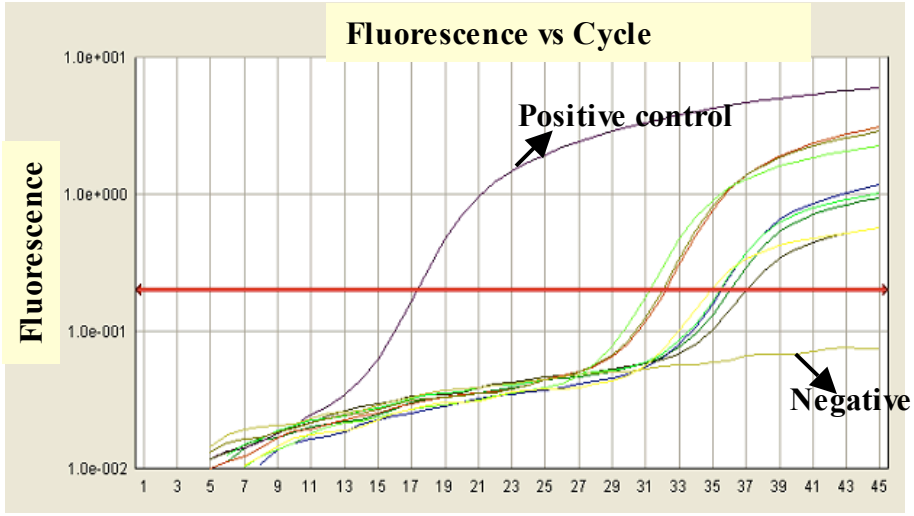
Magnetic Affinity-based Nucleic Acid Extraction without Centrifugation and Organic Solvent



Flu virus particle

Magnetic bead

Magnetic bead with flu antibodies



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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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LANL Cai's Team

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Dr. Shannon Eaker
Dr. Jun Chen
Dr. Jennifer F. Harris
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LANL Collaborators

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Dr. Tony Beugelsdijk, Chemistry Division
Dr. Torsten Staab, Engineering Division

External Collaborators

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Dr. Huiming Kong, BioHelix Inc.

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