Nucleic-Acid Signatures at Three Levels of Readiness for Biowatch

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This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.
I am co-inventor on a patent with M. Allen Northrup of a sleeve-based thermal cycler, licensed by Cepheid, Northrop-Grumman, and MFSI. Patent will expire in 2016.
Raymond P. Mariella, Jr. will discuss nucleic-acid signatures (PCR, microarrays, and other probe-based systems) at three levels of readiness for BioWatch:

**Tier 1:** fully automated bio-detection system, capable of 24/7/365 unattended outdoor and indoor operation, that will be at a Technology Readiness Level of **TRL 6 plus by 2016**.

**Tier 2:** similar requirements but will not reach a **TRL 6 plus level until sometime between 2016 and 2020**.

**Tier 3:** technologies that have the potential of meeting or exceeding the BioWatch requirements, but a fully automated, **TRL 6 plus system would take us beyond the 2020 time frame**.
Phased approach

**Tier 1: Deploy by 2016**
- Essentially off-the-shelf systems have no sample preparation, *per se*, no enrichment techniques

**Tier 2: Deploy by 2020**
- Could add one more level of ID, with targeted amplification of known virulence regions; known science, needs years of Engineering and Instrumentation/Integration for autonomous operation

**Tier 3: Deploy after 2020**
- Requires years of scientific research to create and validate assays, as well as develop the Informatics software and interface, along with extensive Engineering and Instrumentation/Integration
Notional: General System Overview

(A) High-Volume Aerosol Collection into Aqueous Medium

1. Light scattering unit could log the ambient particle count, size distribution, fluorescence, etc. [aerosol flow cytometer]
2. Virtual Impactor determines particle-size distribution of particles that get collected
3. High-volume particle collector [wetted-wall cyclone or other]
4. Divide collected solutions for archive, immunoassay, and nucleic-acid-based assays

(B) Sample Preparation

5. Enrichment Chemistry via surface molecular recognition
6. Introduce buffers for immunoassays
7. Lysis
8. Enrichment Chemistry for nucleic-acid target sequences
9. Amplification of targeted nucleic-acid sequences

(C) Primary Detection Assays

10. For nucleic acids: Divide sample into sub-samples for single-target, real-time PCR versus multiplex PCR and capture probes
11. For toxins: Immunoassays [antibodies, aptamers, ...]

(D) Slower, Higher-Information-Content Assays

12. Microarray or liquid array capture [and release?]
13. 400-base-read sequencing?

Steps 2, 3, 4, 6, 7, 10, 11 are already included in fully-autonomous systems that are at TRL 8 or 9, today

Steps 1, 8, 9, 13 could be included by 2020

Steps 5, 12 are probably after 2020

- Informatics – Resident Expert System? Remote Number Crunching?
- 2-way communications with Monitoring & Control Center
APDS and M-BANDS are mature systems

Steps 2, 3, 4, 6, 7, 10, 11 are already included in fully-autonomous systems that are at TRL 8 or 9, today


1. light-scattering particle characterization – before 2020

- Simple Aerodynamic Particle Sizer [APS]
- FLAPS or Biological Agent Warning Sensor [BAWS]
- Bioaerosol Flow Cytometer with Mass Spectrometer [BAMS]
5. Selective capture of pathogenic bacteria & viruses

- Antibodies
- Aptamers™ or SOMAmer™
- Synthetic peptides
- Nanolipoproteins
- ?

8. Selective capture of bacterial DNA

Looxster is advertised to remove human DNA – are there similar reagents and techniques that can be automated that enrich for virulent regions of bacteria and viruses?
9. Selective NA amplification of virulence regions?

One does not want to perform Metagenomic analysis of every collected sample

- The Ion AmpliSeq™ technology allows ultra-high multiplex PCR combined with targeted sequencing library construction in just 16 hours for over 400 genes. Utilizing only 10 ng of input DNA per primer pool for a total of 40 ng of input DNA.

Higher-performance, more complicated PCR?

Emulsion or Digital PCR offers improvement to both single-plex and multiplex amplification


- Intrinsic quantification of starting copy #
- If CCD imaging is performed, real-time, detection in fewer cycles [both single-plex and multiplex]
- If multiplex is used, there is less amplification bias

Amplification bias:

Fewer cycles, more instrumentation complexity

20,000, 5-nL PCR droplets, either fixed planar array or flowing, serially

100-µL PCR tube

Suppose starting copy number = 1 and 20 Taqman cycles have been done, then ideally, \(2^{20} \approx 10^6\) reporter fluorophores have been released into solution [1.7 aMoles].

For bulk PCR, it is effectively impossible to image the entire 100 µL; only a few µL can be interrogated and weak fluorescence must be detected over background fluorescence from the solution and walls and over detector dark current.

For digital PCR, all \(10^6\) fluorophores are in a 5-nL volume, producing 20,000X higher intensity of emission, which is added to easier discrimination against background fluorescence [spatial filters/”confocal” techniques; and smaller detector area has less dark current]
12. Capture and release

- Microarrays with long oligomers can capture matches and minor variants of sequences of interest and potentially release them for library construction and sequencing.

DuBose, et al., “Use of microarray hybrid capture and next-generation sequencing to identify the anatomy of a transgene” *Nucleic Acids Research* 2013 41, 1
Extra Viewgraphs
Poisson limits for random distributions

Probability of actually having “n” targets in a sample that contains an average of “s” targets

\[ P(n,s) = \frac{s^n e^{-s}}{n!} \]

\( n! = 1 \) for \( n = 0 \) and \( s^0 = 1 \)

Thus, \[ P(0,s) = e^{-s} \]

Experimentally demonstrated by Sykes, not long after the multi-stage amplification of PCR became known

2. Virtual Impactor rejects particles > 10-µm diameter

- Sampler Virtual Impactor collects <10-µm diameter in 4 mL of buffer Regan, Dzenitis, & co-workers, “Environmental Monitoring for Biological Threat Agents Using the Autonomous Pathogen Detection System with Multiplexed Polymerase Chain Reaction” Anal. Chem. 2008, 80, 7422–7429

NASA has defined Technology Readiness Levels

TRL 6

• System/sub-system model or prototype demonstration in an operational environment.

• A high fidelity system/component prototype that adequately addresses all critical scaling issues is built and operated in a relevant environment to demonstrate operations under critical environmental conditions.

• Prototype implementations of the software demonstrated on full-scale realistic problems. Partially integrate with existing hardware/software systems. Limited documentation available. Engineering feasibility fully demonstrated.

• Documented test performance demonstrating agreement with analytical predictions.
family Togaviridae (formerly the Group A arboviruses)

- GGTGCCAGCT
- ATGACCATGCTAA
- GCCCAGTTGG
- GGTGTGTGTGC
- TACCAGTGC
- CAGCCGTAAGAG
- GAAGATTGATGC
- GCCCAGTTTGT
- GCAGAAGACCC
- GGTTGAGCCC
- CGGTGTGAGAC
- TTTGTTACTATTCAG
- CCGAAAGACCA
- TGTCCGTTTCC
- GATGAGAGAGCT
- TGCCTAAATGC
- CAGTGCGAAGA
- AACCCAATCCAAT
- GACCATGCTAATG
- TGCCAGTTGAAA
- CTACCCAGATCC
- GCCCTTTTCTAATAA
- GTGTACCCCGTTT