High Throughput Analysis of Amplified Nucleic Acids with Mass Spectrometry: The Ibis Platform

Steven A. Hofstadler, Ph.D.
Ibis Biosciences, Inc.
Outline

• The challenge of broad pathogen detection
• The Ibis approach
  – Principle of operation
• Bacterial detection and strain typing
  – Group A strep - direct throat swab analysis
• Viral detection and strain typing
  – Influenza
    ▪ Pan-influenza detection and strain typing
• Integrated platform
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The Pathogen Detection Arena

- Biological weapons defense is not just about anthrax
- Food safety is not just about E. coli 0157
- Hospital associated infections are not just due to Staph. aureus
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The Pathogen Detection Arena

- There are *numerous* naturally occurring infectious diseases
- Over 1000 agents known to infect humans*
  - 217 virus species
  - 538 bacterial species
  - 307 fungi
  - 66 parasitic protozoa
- Additional plant and animal pathogens not counted
- Numerous strain variations
- Potential bio-engineered organisms

Mainstream Bioagent Detection

Today

• Culture techniques
  – Detects a subset of all pathogens
  – Can take multiple days (weeks)

• Single agent nucleic acid tests
  – One test for each agent (smallpox, anthrax, plague, etc.)
  – Need too many tests
  – Fail to detect newly emergent pathogens

• There is currently no good method to detect organisms that have never been seen before
### Bacterial Threat Symbols

<table>
<thead>
<tr>
<th>Category A Priority Pathogen</th>
<th>Globally Important Human Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category B Priority Pathogen</td>
<td>Medically Important Human Pathogen</td>
</tr>
<tr>
<td>Category C Priority Pathogen</td>
<td>Important Animal Pathogen</td>
</tr>
<tr>
<td>HHS Select Agent</td>
<td>Important Plant Pathogen</td>
</tr>
<tr>
<td>USDA High Consequence Animal Pathogen</td>
<td>High Potential For Bioengineering</td>
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<tr>
<td>USDA High Consequence Plant Pathogen</td>
<td>Zoonotic Agent</td>
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<tr>
<td>Validated Biological Weapon</td>
<td>CDC Notifiable Agent</td>
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<tr>
<td>Potential Biological Weapon</td>
<td>Principal Foodborne Pathogen</td>
</tr>
<tr>
<td>Validated Biocrime Agent</td>
<td>Emerging Infectious Agent</td>
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</table>

*Table courtesy of Christian Massire, Ph.D.*
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Bacteria

Cytophagales
- Chlamydia
- Chlamydiaceae
- Chlamydia trachomatis
- Chlamydia pneumoniae
- Chlamydia psittaci
- Pasteurellaceae

Spirochetes
- Spirochaeta
- Spirochaetaceae
- Treponema pallidum
- Borrelia burgdorferi
- Borrelia spielmanii
- Rickettsia prowazekii

Actinobacteria
- Corynebacterium diphtheriae
- Mycobacterium tuberculosis
- Mycobacterium avium
- Mycobacterium bovis
- Mycobacterium leprae
- Mycobacterium tuberculosis
- Mycobacterium Hansenii
- Mycobacterium genavense
- Mycobacterium fortuitum
- Mycobacterium avium
- Mycobacterium leprae
- Mycobacterium abscessus
- Mycobacterium kansasii
- Mycobacterium xenopi
- Mycobacterium gordonae
- Mycobacterium fortuitum
- Mycobacterium chelonae
- Mycobacterium smegmatis
- Mycobacterium avium
- Mycobacterium bovis
- Mycobacterium kansasii
- Mycobacterium fortuitum
- Mycobacterium avium
- Mycobacterium abscessus
- Mycobacterium leprae
- Mycobacterium xenopi
- Mycobacterium gordonae
- Mycobacterium chelonae
- Mycobacterium smegmatis

Fusobacteria
- Fusobacterium nucleatum
- Fusobacterium mortiferum
- Fusobacterium nucleatum
- Fusobacterium varium
- Fusobacterium necrophorum
- Fusobacterium periodonticum

Mollicutes
- Mycoplasma pneumoniae
- Mycoplasma mycoides
- Mycoplasma fermentans
- Mycoplasma autotrophicum

Bacilli
- Clostridium
- Clostridium tetani
- Clostridium tetani
- Clostridium perfringens
- Clostridium botulinum
- Clostridium difficile
- Clostridium novyi

Firmicutes
- Bacillus
- Bacillus subtilis
- Bacillus cereus
- Bacillus anthracis
- Bacillus thuringiensis
- Bacillus megaterium
- Bacillus subtilis
- Bacillus subtilis
- Bacillus lentus
- Bacillus anthracis
- Bacillus cereus
- Bacillus thuringiensis

IMAGE COURTESY OF CHRISTIAN MASSIRE, PH.D.
Why Detect and/or Type Microorganisms via Nucleic Acids?

- All living things rely on DNA and/or RNA to propagate
  - All infectious agents* contain DNA and/or RNA
  - Bacteria, viruses, fungi, protozoa

- DNA and RNA are unique among biomarkers in that they can be amplified (e.g. PCR, WGA, NASBA, etc.)
  - From trace amounts of sample
  - From highly degraded samples
  - From samples in complex backgrounds

- NO CULTURE REQUIRED

* Except those nasty prions!
Why Detect and/or Type Microorganisms via Nucleic Acids?

- Some genetic differences do not result in phenotypic differences
  - e.g. rRNA, VNTRs, SNPs

- Range of specificity can be “tuned” for different applications
  - “Name That Bug”: broad range primers
  - “Genotype/Strain - Type That Bug”: species specific primers
  - “Profile That Bug”: drug resistance, virulence markers, etc.
Interrogation of Amplified Nucleic Acids

- Sequencing
  - “Gold Standard”
- Fluorescent intercalating dye
- Hybridization
  - Specific probe with FRET pair
- DNA microarray
- Melting profiles
- Electrophoresis
  - Slab gels
  - Capillary gel electrophoresis

WHAT ABOUT MASS?

IMAGES COURTESY OF STEVEN A. HOFSTADLER, PH.D.
Identification and Strain Typing of Bacterial and Viral Pathogens Using High Performance Mass Spectrometry: The Ibis Concept

Defense Advanced Research Projects (DARPA)
Centers for Disease Control (CDC)
National Institute of Allergy and Infectious Diseases (NIAID)
Department of Homeland Security (DHS)
The Ibis Approach to Pathogen ID and Strain-Typing

**STEP 1** Identify genomic regions for identification:
Variable DNA sequences flanked by conserved sequences

**STEP 2** Amplify nucleic acids to measure:
Use broad-range, unbiased PCR primers

**STEP 3** Measure nucleic acid:
ESI-TOF

**STEP 4** Identify the organisms:
Base-composition fingerprints

Images courtesy of Steven A. Hofstadler, Ph.D.
Primers bind to conserved regions present in ALL (or groups of) bacteria

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Cox. burnettii</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
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<tr>
<td>Leg. pneumophila</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
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<tr>
<td>Ricket. prowazekii</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
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<tr>
<td>Mycb. tuberculosis</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Trep. pallidum</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Staph. epidermidis</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Strep. agalactiae</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
<tr>
<td>Strep. mutans</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
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<tr>
<td>Strep. pneumoniae</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
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<tr>
<td>Strep. pyogenes</td>
<td>ACGCCGTAACACGGTTCAACCTGGCCCTGAGTCCAGTAACCGCGGTACTAAGCGAC</td>
</tr>
</tbody>
</table>

Region varies in different kinds of bacteria

\[ \Delta [A_w G_x C_y T_z] \]

Image courtesy of Steven A. Hofstadler, Ph.D.
The Polymerase Chain Reaction (PCR)

- Performed using primers designed for broad coverage
- PCR cycling conditions tolerate mismatches on initial cycles
- All primer pairs designed to work under identical PCR conditions
- Each well contains an internal calibrant
- Generally don’t multiplex broad range primers (e.g. 16S and 23S rDNA)
- Multiplexing of more specific primers common (e.g. strain typing, drug resistance, virulence)
Measure nucleic acid:
Electrospray Ionization (ESI)
Time-of-flight (TOF) Mass Spectrometry

(M-30H\(^+\))\(^{30-}\)

MW = 32,588.90

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
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Masses to Base Composition

STEP 4

Penny = 2.500 g
Nickel = 3.950 g
Dime = 2.268 g
Quarter = 5.670 g

Weight = 4.6 grams
∴ 2 dimes

A = 313.0576 amu
G = 329.0526 amu
C = 289.0464 amu
T = 304.0461 amu

MW = 32,588.90 amu
∴ A28 G29 C25 T24

Requires 25 ppm mass measurement error
Math takes into account Watson-Crick base pairing

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
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Derived and Expected Base Compositions

Primer 356 (RplB) Expected Products

Identify the organisms:
Base-composition fingerprints

Streptococcus pyogenes
Streptococcus pneumoniae
Streptococcus equi
Streptococcus gordonii
Staphylococcus epidermidis
Staphylococcus aureus
Bacillus anthracis

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
Broad Pathogen Detection

Instead of asking; “Is pathogen X in my sample?” we ask: “Which pathogen, or pathogens, are in my sample?”
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Group A Streptococcus (GAS) Outbreaks in Military Settings

- Outbreaks of Group A strep at MCRC 2002/2003
  - Highly virulent strain
  - One death, 160 hospitalized
  - Training activities suspended

- Initial analysis of post-culture samples
  - 80 samples sent from NHRC, Dr. Kevin Russell, December 20, 2002
  - “Hijacked” some BW air surveillance plates

- Follow up surveillance at multiple military bases

- Direct analysis of throat swabs without culture

All primers of all samples consistent with S. pyogenes
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Direct Analysis of Throat Swab*

*Repeat swab positive on culture for *Streptococcus pyogenes*
<table>
<thead>
<tr>
<th>Organism</th>
<th>Cumulative Estimate of Genomes/Swab</th>
<th>Relative Abundances</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>7.38E+05</td>
<td>1.00</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>3.77E+05</td>
<td>0.51</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>1.89E+05</td>
<td>0.26</td>
</tr>
</tbody>
</table>
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**Haemophilus influenzae, Neisseria meningitidis, Streptococcus pyogenes** (Ratio 4/2/1, $1.5 \times 10^6$ genomes/swab)

<table>
<thead>
<tr>
<th>Primer #1</th>
<th>Mass</th>
<th>Base Count</th>
</tr>
</thead>
<tbody>
<tr>
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<td>17948.926</td>
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<td>Blue</td>
<td>18610.017</td>
<td>$A_{11}G_{19}C_{15}T_{15}$</td>
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<td>Blue</td>
<td>17936.912</td>
<td>$A_{11}G_{17}C_{16}T_{14}$</td>
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<tr>
<td>Blue</td>
<td>18877.118</td>
<td>$A_{18}G_{15}C_{15}T_{13}$</td>
</tr>
</tbody>
</table>

**IMAGE COURTESY OF CHRISTIAN MASSIRE, PH.D.**
Conclusions of Pneumonia Study*

• Primary pathogen
  – *Streptococcus pyogenes* (GAS)
  – Known virulent strain

• Secondary pathogens
  – Haemophilus influenzae
  – Neisseria meningitidis

• 5 other military facilities
  – Determined these sites had a mixture of strain types

• Throughput
  – >200/samples per day

Virus Identification and Typing
Identification and typing of Influenza virus species
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Influenza Virus Surveillance: Project Collaborators

CMDR Kevin Russell M.D., Naval Health Research Center, San Diego, CA

Kirsten St.George, MAppSc, PhD, New York State Department of Health, Slingerlands, NY

Charlotte Gaydos, Dr.P.H. and Rich Rothman, M.D. Johns Hopkins University, Baltimore, MD

Stan Lemon M.D., University of Texas Medical Branch, Galveston, TX

Wendy Sessions, M, SV (ASCP), Texas Department of State Health Services

Dave Stallknecht and Ginger Goekjian, College of Veterinary Medicine, University of Georgia
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Ibis T5000™ Influenza Virus Assay

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
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Pan-influenza Primer Polymerase PB1 Primer

PP2798 - Base Composition

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
Influenza Virus Assay Results Example

**Conclusion**

Samples 1 & 3: Influenza A virus (H3N2: A/New York/.../2003)

Samples 2 & 4: Influenza B virus

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
### Human Influenza Trial - Blinded Samples

<table>
<thead>
<tr>
<th>Source</th>
<th>Location</th>
<th>Collection Dates</th>
<th>Sample type</th>
<th>No. of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johns Hopkins University Medical Center</td>
<td>Baltimore, MD</td>
<td>2003-2005</td>
<td>Nasal aspirates</td>
<td>229</td>
</tr>
<tr>
<td>NY State Dept. of Health</td>
<td>Throughout NY</td>
<td>1999-2005</td>
<td>Nasal aspirates, BAL, tracheal aspirates, throat swabs</td>
<td>100</td>
</tr>
<tr>
<td>TX State Dept. of Health</td>
<td>Throughout TX</td>
<td>2005-2006</td>
<td>Throat swabs, nasal washes</td>
<td>10</td>
</tr>
</tbody>
</table>

**Total** 656

- Correctly identified all Influenza A types
  - 149 H3N2
  - 34 H1N1
- 67 Influenza B

<table>
<thead>
<tr>
<th>Influenza</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>96.8%</td>
<td>97.5%</td>
<td>96.0%</td>
<td>98.0%</td>
</tr>
</tbody>
</table>
Detection of Mixed Infections

Pan-Influenza PB1 Primer

Influenza A virus A40G31C24T33

Influenza B virus A37G31C20T34

Calibrant A36G28C27T32

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
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Validation Study
Test Isolates from Diverse Sources

- 24 human influenza isolates
  - 18 influenza A
  - 6 influenza B

- 63 avian influenza isolates
  - 16 different avian species
    - chicken, duck, goose, egret, teal, ...
  - 28 distinct H/N types
    - 29 HIGHLY PATHOGENIC H5N1 isolates
  - 8 worldwide geographic locations
    - North America, Africa, Asia

- 4 swine influenza isolates

- 1 equine influenza isolate
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Avian Flu Detection:
No Change in Assay or Primers

Influenza species, subtype, clade type
determined by comparison with database

IMAGE COURTESY OF STEVEN A. HOFSTADLER, PH.D.
Avian Influenza Virus Detection: University of Georgia Samples

24 avian influenza virus isolates collected over a six-year period

- Different host species: mallard, seagull, teal,…
- Different combinations of H and N subtypes: H12N4, H3N8…

<table>
<thead>
<tr>
<th>SCWDS ID#</th>
<th>Serotype</th>
<th>Species</th>
<th>Location</th>
<th>Date</th>
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<tbody>
<tr>
<td>AI00-1412</td>
<td>H6N1</td>
<td>REKN</td>
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<tr>
<td>AI00-1794</td>
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</tr>
<tr>
<td>H6N1</td>
<td>REKN</td>
<td>Bower's Beach, DE</td>
<td>5/25/00</td>
<td>No Detection</td>
</tr>
</tbody>
</table>

*Same panel was used for human Influenza detection*
Conclusions

• By “weighing” DNA with mass spectrometry, unambiguous base compositions can be derived
  – Remember coins and scale analogy!

• Base compositions derived from broad range primers can be used to triangulate to microbial identification

• Ibis platform enables broad range bacterial and viral detection
  – Broad bacterial coverage using broad range primers
    • Example: Direct analysis of throat swabs
  – All influenza (human and avian) in same assay
    • Example: Human clinical specimens and avian isolates

• Respiratory Virus Surveillance Assay provides a single test platform for 6 families of RNA and DNA respiratory viruses

• Demonstrated for bacteria and viruses without culture
  – Also applicable to fungi, protozoa, and humans (not shown)

Instead of asking; “Is pathogen X in my sample?”, Ibis approach asks: “Which pathogen(s) are in my sample.”
Hardware Overview
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System History

TIGER 1.0
2000-2003

TIGER 2.0
2006-2009

TIGER 2.0'
2004-2006

Ibis T6000
2009-
Primary mission is pathogen characterization & bioforensics

- Spatially isolated enclosures
  - A Deck: genome isolation and PCR setup
  - B Deck: PCR, desalt, ESI-TOF, GenX

- Magnetic bead desalting
- Technician transports samples from A Deck to B Deck
- Footprint (A + B) = 54 ft²
Change in Instrument Format

- **Rationale** – Motivated by discussions with Johns Hopkins and CDC
  - Space is premium in hospital/diagnostic labs
  - Much of “A Deck” function already present in many labs
  - Many different groups/applications use different sample prep protocols
    - CDC core lab model
    - Non-integrated “A Deck” components can be used for other lab functions
    - Lower cost for hardware

- **Deployments:**
  - CDC in Atlanta, GA
  - FBI in Quantico, VA (2)
• Federal Bureau of Investigation
• Two Ibis T5000™ systems installed in DNA Unit II
• mtDNA forensics analysis
  – Replaces existing sequence-based methods (details to follow)
    • Cost
    • Throughput
    • Heteroplasmy
    • Mixtures
• FBI and Ibis finishing validation package
Technology Transition Workshop

Ibis T5000™

• Amplicon purification
• Automated ESI-TOF analysis
  – Robotic arm moves plates for unattended operation
• Data analysis
• Other functions performed off-line
  – DNA/RNA extraction
  – Plate set up into pre-kitted plates
  – PCR
• High throughput
  – 1 well/minute
  – 46 sec spray
  – 14 sec rinse
Ibis T5000™ Components

- Bruker Daltonics micrOTOF™
- Thermo CRS robotic arm
  - 3 x 15 plate storage
- LEAP autosampler
- Custom fluidics module
  - Programmable Cavro® pumps
- Heat sealer
- Ibis magnetic bead cleanup module
  - Modified LEAP
  - 8-channel head
  - Shaker
  - Magnetic plate
- Bar code reader
- Computers
• Key features
  – Remove dependence on complex, high cost 3rd party components
  – Compatibility with existing PCR plates
  – Compatibility with existing cleanup chemistry
  – Accommodate priority “stat” sample
  – Bottom up design with IVD market in mind
    • Rigorously controlled design criteria from the start
  – Support 30 second/well throughput
  – Design and build prototypes such that clinical device manufacturer can build system under FDA-compliant design/manufacturing control
Ibis T6000™ Design

- Same magnetic bead chemistry as T5000
- Spin cuvettes (22) aligned in carousel
- Magnetic beads aliquoted from bead reservoir
  - No mag bead plate
  - No elution plate
- No robotic arms, heat sealers, LEAPS, etc
- Accommodate “stat” sample priority interrupt
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