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Technology Transition Workshop| *Steven A. Hofstadler, Ph.D.*

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

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**
    - **Pandemic 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**

- **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**

*Taylor et al, Phil. Trans. R. Soc. Lond. B (2001) 356, 983-989

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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**

Nucleic acid tests (NAT's)

- **There is currently no good method to detect organisms that have never been seen before**

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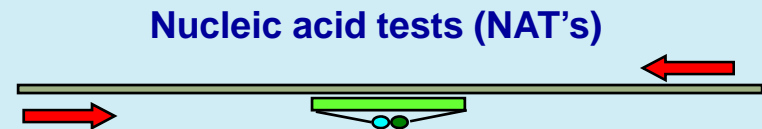
Bacterial Threat Symbols
Microbial Rosetta Stone Database

- NIAID Category A Priority Pathogen
- NIAID Category B Priority Pathogen
- NIAID Category C Priority Pathogen

- HHS Select Agent
- USDA High Consequence Animal Pathogen
- USDA High Consequence Plant Pathogen

- Validated Biological Weapon
- Potential Biological Weapon
- Validated Biocrime Agent

- Globally Important Human Pathogen
- Medically Important Human Pathogen
- Important Animal Pathogen
- Important Plant Pathogen
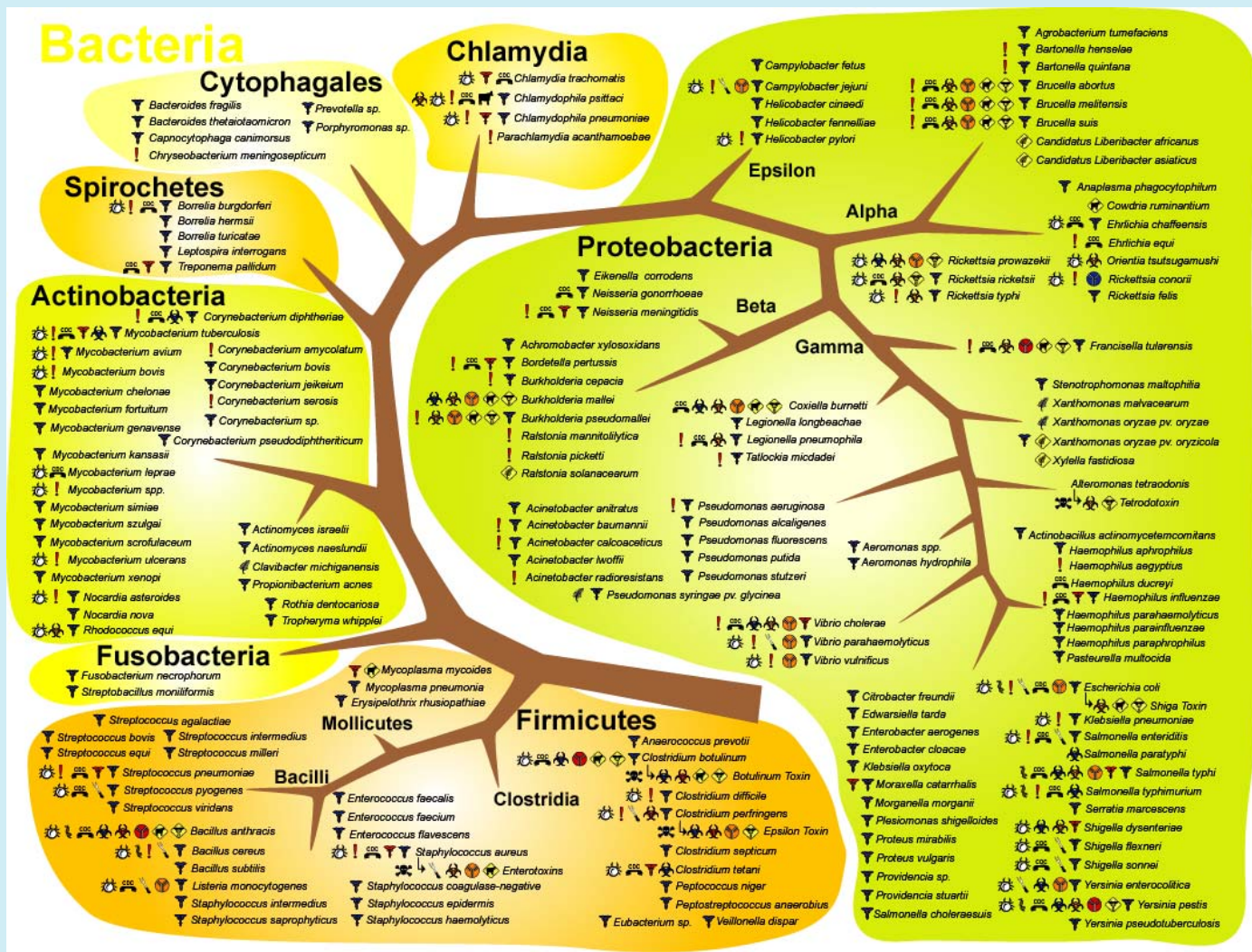- High Potential For Bioengineering
- Zoonotic Agent
- Toxin
- CDC Notifiable Agent
- Principal Foodborne Pathogen
- Emerging Infectious Agent

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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**
- **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.**

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* Except those nasty prions!

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?**

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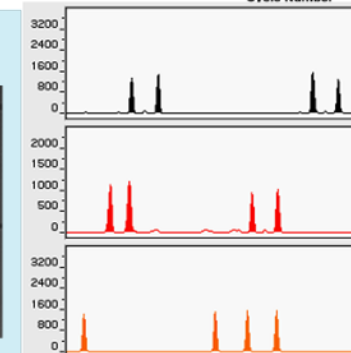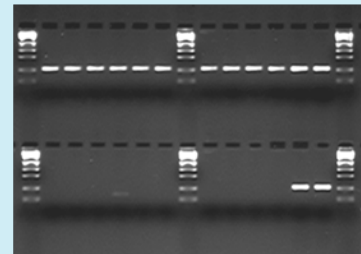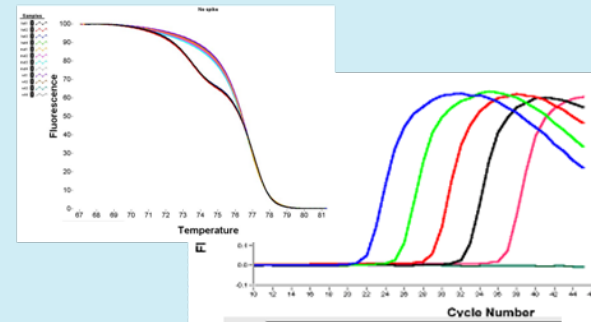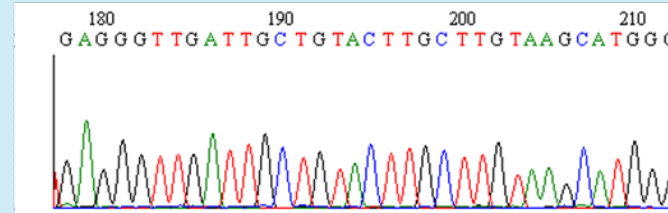
- **Identification and Strain Typing of Bacterial and Viral Pathogens using High Performance Mass Spectrometry: The Ibis™ Concept**
  - **DARPA**
  - **CDC**
  - **NIAID**
  - **Department of Homeland Security**

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The Ibis™ Approach to Pathogen ID and Strain-Typing

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

Conserved DNA   Variable DNA   Conserved DNA

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

Broad range primer   PCR   Broad range primer

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

Sample → ESI needle

Capillary

Ion Store

Reflectron

Electron Multiplier

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

As: 17
Gs: 30
Cs: 11
Ts: 61

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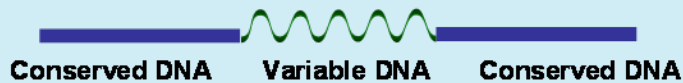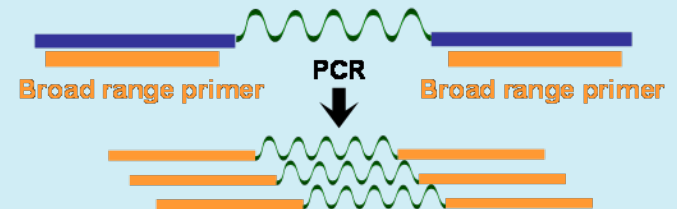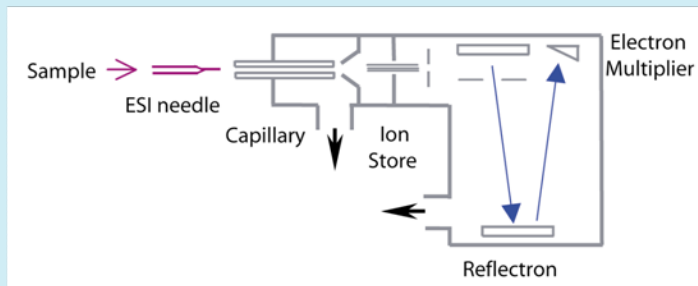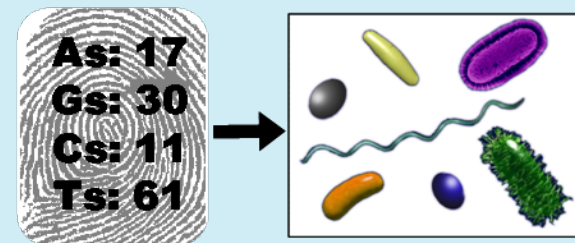
Broad Range Priming in Bacteria

Primers bind to conserved regions present in ALL (or groups of) bacteria
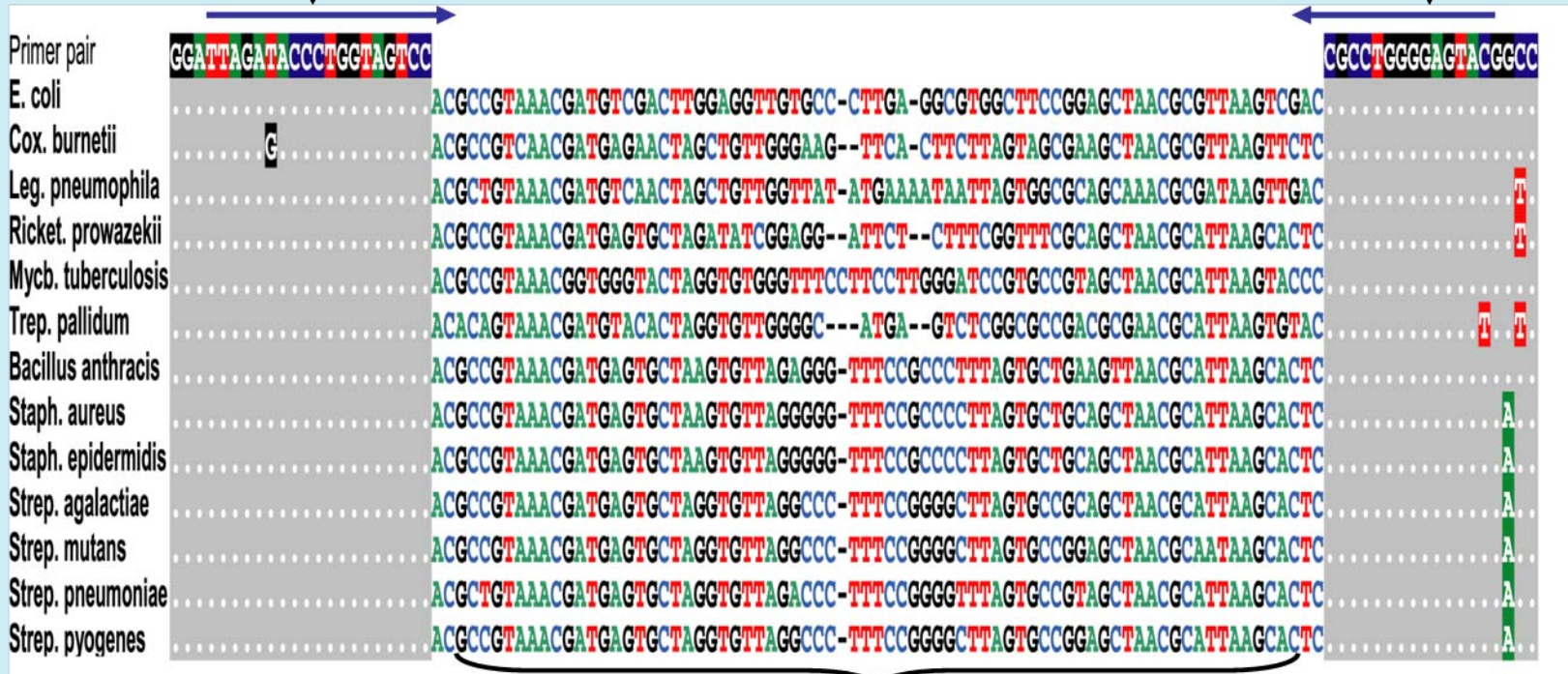
Region varies in different kinds of bacteria $\Rightarrow \Delta\ [A_wG_xC_yT_z]$

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The Polymerase Chain Reaction (PCR)

STEP 2
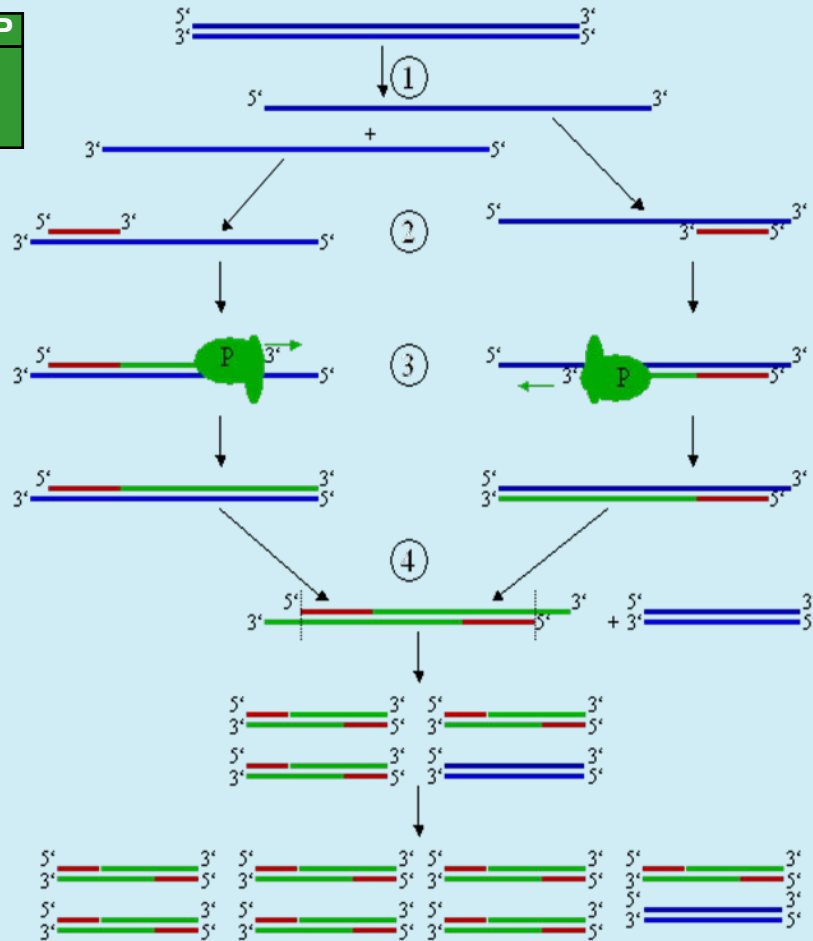
- **Performed using primers designed for broad coverage**
- **PCR cycling conditions tolerate mismatches on initial cycles**
- **All primer pairs designed to work under identical PCR conditions**
- **Each well contains an internal calibrant**
- **Generally don't multiplex broad range primers (e.g. 16S and 23S rDNA)**
- **Multiplexing of more specific primers common (e.g. strain typing, drug resistance, virulence)**

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Automated ESI-TOF of PCR Amplicons on T5000
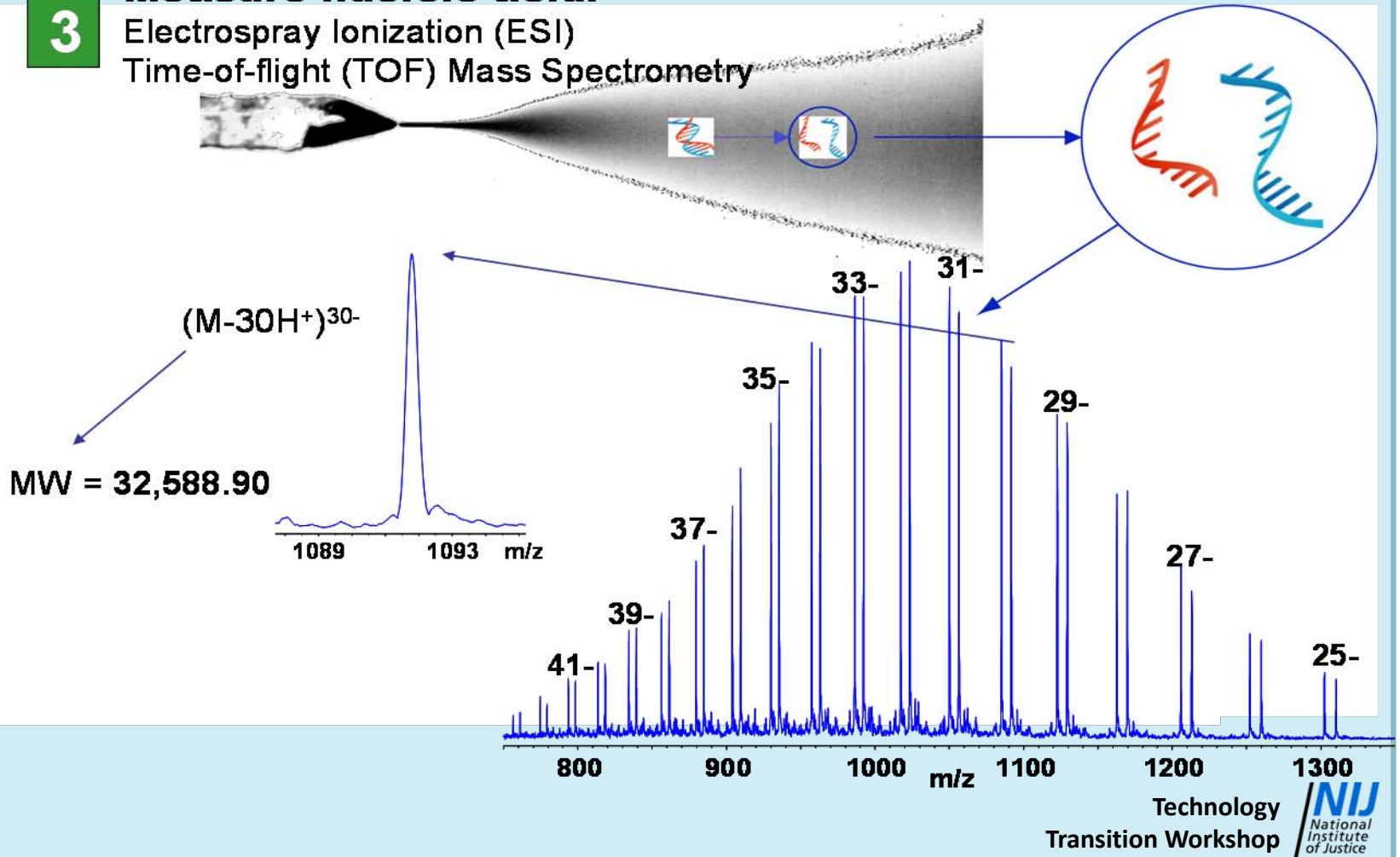
Masses to Base Composition

STEP 4

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

Scale

Weight = 4.6 grams
∴ 2 dimes

A = 313.0576 amu
G = 329.0526 amu
C = 289.0464 amu
T = 304.0461 amu
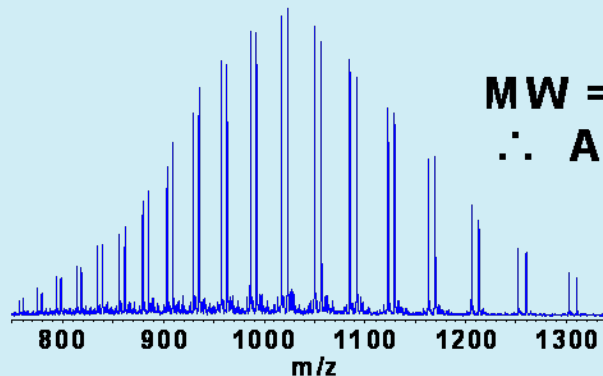
MW = 32,588.90 amu
∴ A28 G29 C25 T24

**Requires 25 ppm
mass measurement error**

**Math takes into account
Watson-Crick base pairing**

Mass spectrum

m/z

800   900   1000   1100   1200   1300

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Derived and Expected Base Compositions
Primer 356 (RplB) Expected Products

STEP 4 Identify the organisms:

Base-composition fingerprints

Streptococcus pyogenes

Streptococcus pneumoniae

A

30 28 29 30 31

31

38

38

Streptococcus equi

37

37

Staphylococcus epidermidis

36

36

35

Streptococcus gordonii

35

34

34

Staphylococcus aureus

C

30

33

G 28

33

Bacillus anthracis

A34 G31 C29 (T27)

29

30 31

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Forensic SNP Analysis   High-Throughput Analysis of Amplified Nucleic Acids with Mass Spectrometry: The Ibis™ Platform   15

Broad Pathogen Detection

Instead of asking; "Is pathogen X in my sample?", we ask: "Which pathogen, or pathogens, are in my sample?"

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**

Ecker et al. *Proc. Nat. Acad. Sci. USA,* (2005) 102(22), 8012-17

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Primer 17 (a 23S Primer) Observed Products (from Culture)

Strep Gordonii

Strep mutans

Strep pyogenes

NHRC Test Samples

Strep pneumonia

Staph epidermidis

Staph aureus

Bacillus anthracis

A

G

C
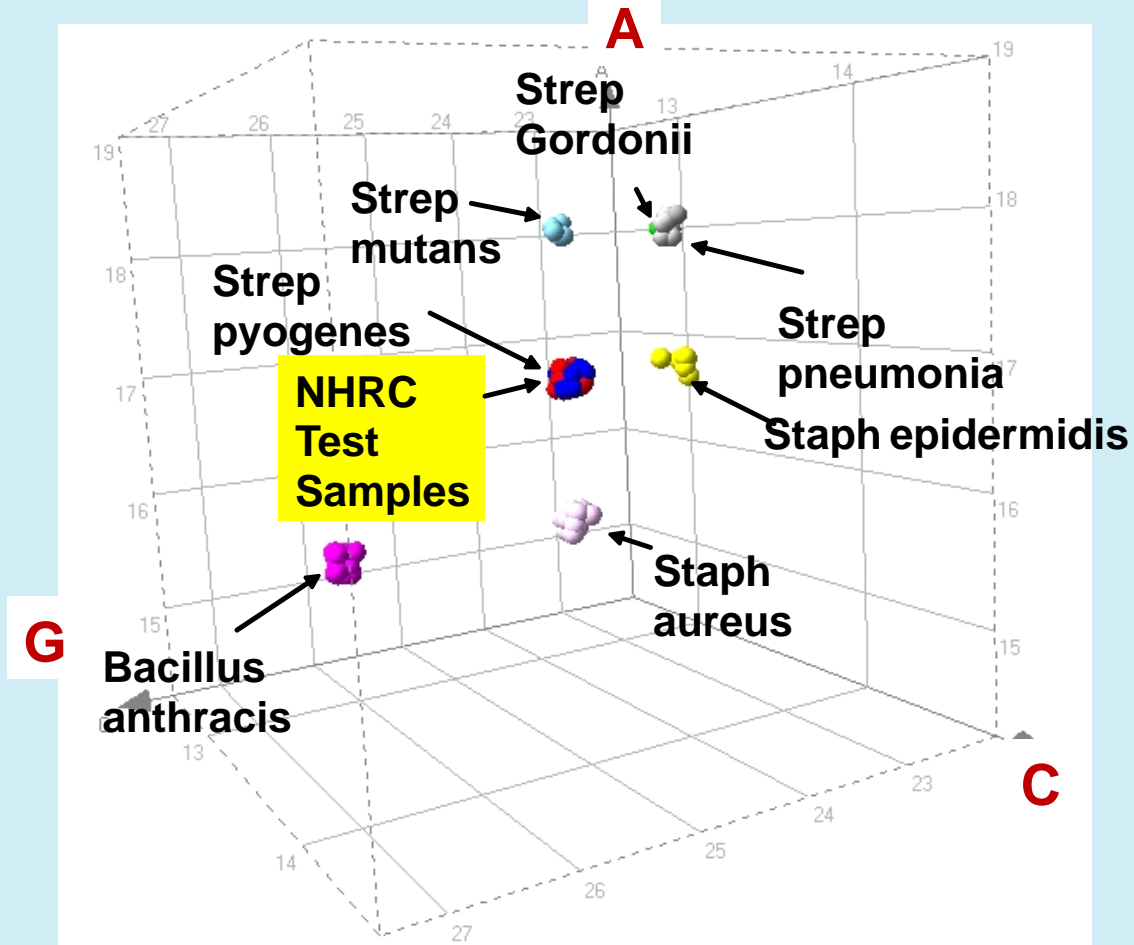
All primers of all samples consistent with S. pyogenes

*Direct Analysis of Throat Swab**

*Repeat swab positive on culture for *Streptococcus pyogenes*

Primer 349: 23S rDNA 1826-1924

From an Interesting Throat Swab

Ecker et al. *Proc. Nat. Acad. Sci. USA,* (2005) 102(22), 8012-17

Multi-primer Triangulation

Ecker et al. *Proc. Nat. Acad. Sci. USA,* (2005) 102(22), 8012-17

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Haemophilus influenzae, Neisseria meningitidis, Streptococcus pyogenes (Ratio 4/2/1, 1.5 X 10^6 genomes/swab)

Primer #1	Mass	Base Count
Blue	18234.970	$A_{12}G_{17}C_{17}T_{13}$
Blue	17948.926	$A_{14}G_{14}C_{12}T_{18}$
Blue	18610.017	$A_{11}G_{19}C_{15}T_{15}$
Blue	17936.912	$A_{11}G_{17}C_{16}T_{14}$
Blue	18877.118	$A_{18}G_{15}C_{15}T_{13}$

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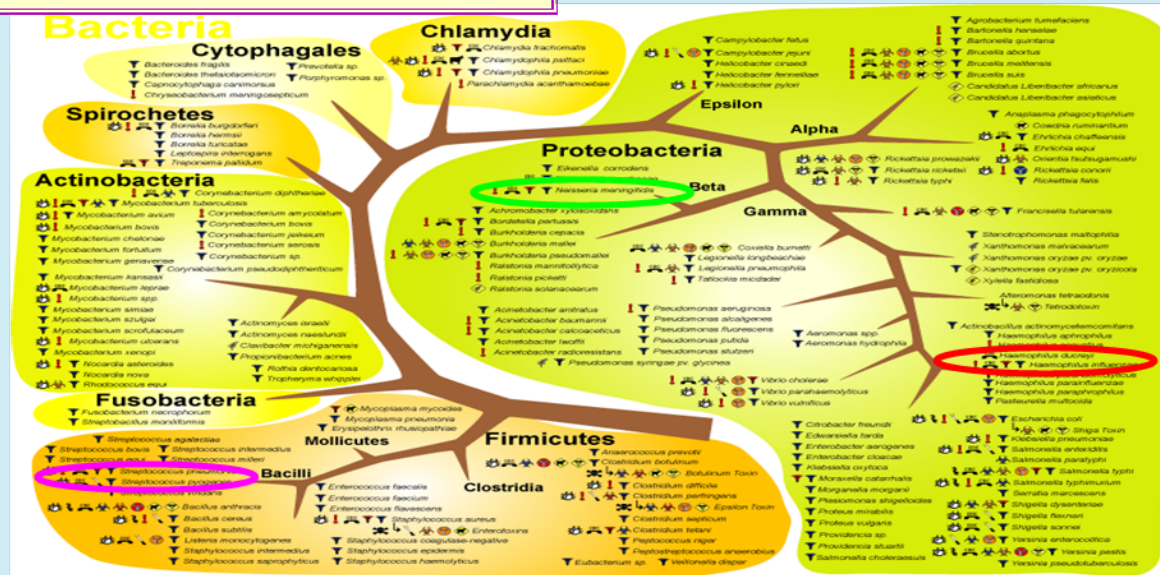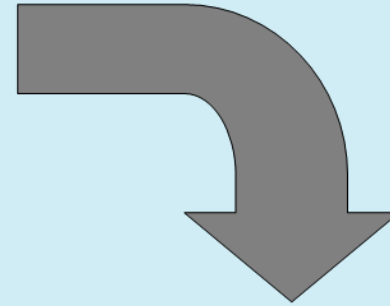
*Conclusions of Pneumonia Study**

- **Primary pathogen:**
  - **Streptococcus pyogenes (GAS)**
  - **Known virulent strain**

- **Secondary pathogens:**
  - **Haemophilus influenzae**
  - **Neisseria meningitidis**

- **Five other military facilities**
  - **Determined these sites had a mixture of strain types**

- **Throughput**
  - **>200 / samples per day**

*Ecker et al. (2005) Proc. Natl. Acad. Sci. Vol. 102: p8012-8017

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Virus Identification and Typing

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Viral Coverage

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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™ Influenza Virus Assay
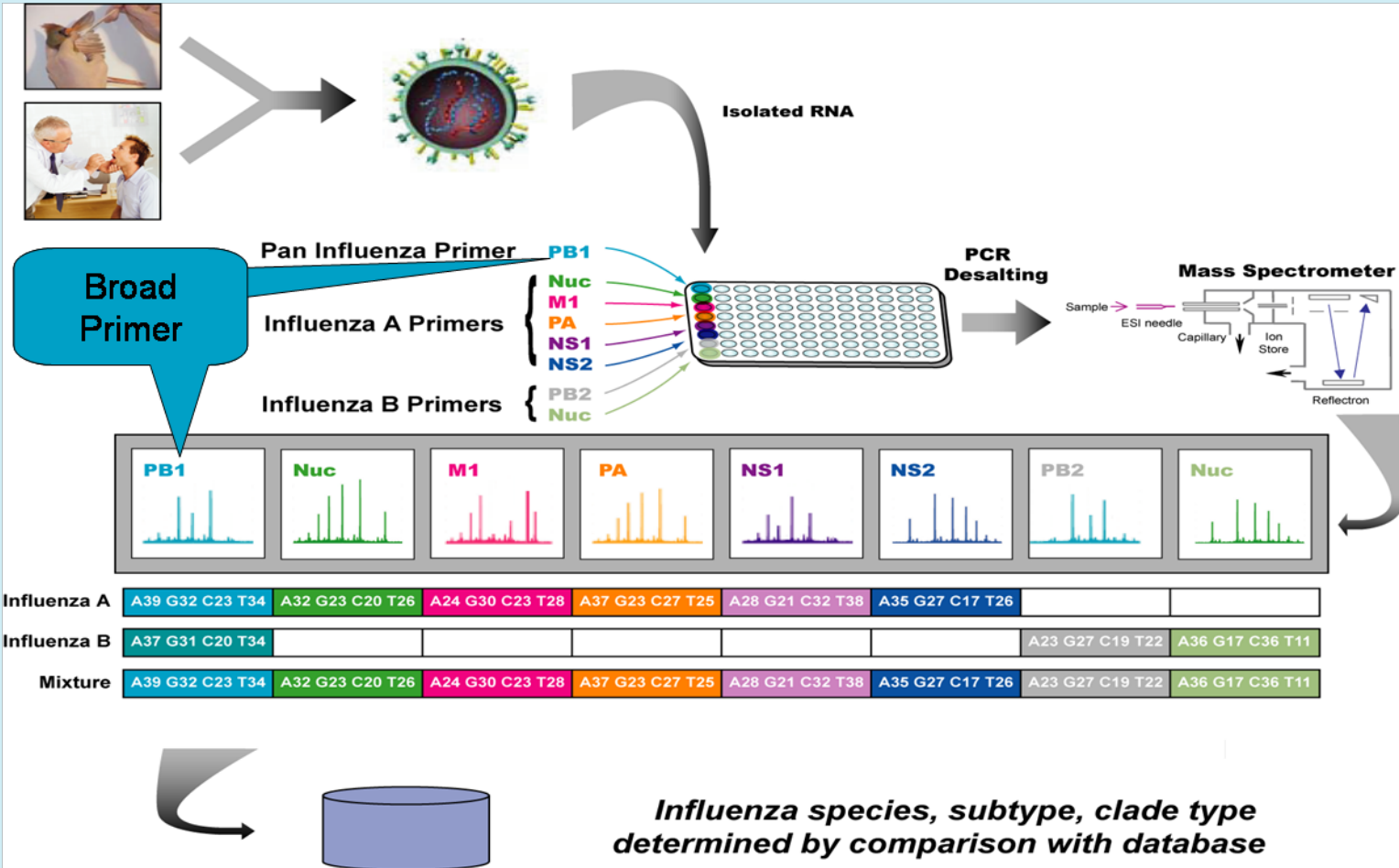
Pan Influenza Primer Polymerase PB1 Primer

Human and Swine H1N1, H2N2, H3N2

Canine, Equine H3N8

Human, Avian H5N1

Other Avian, Swine

Influenza B and C
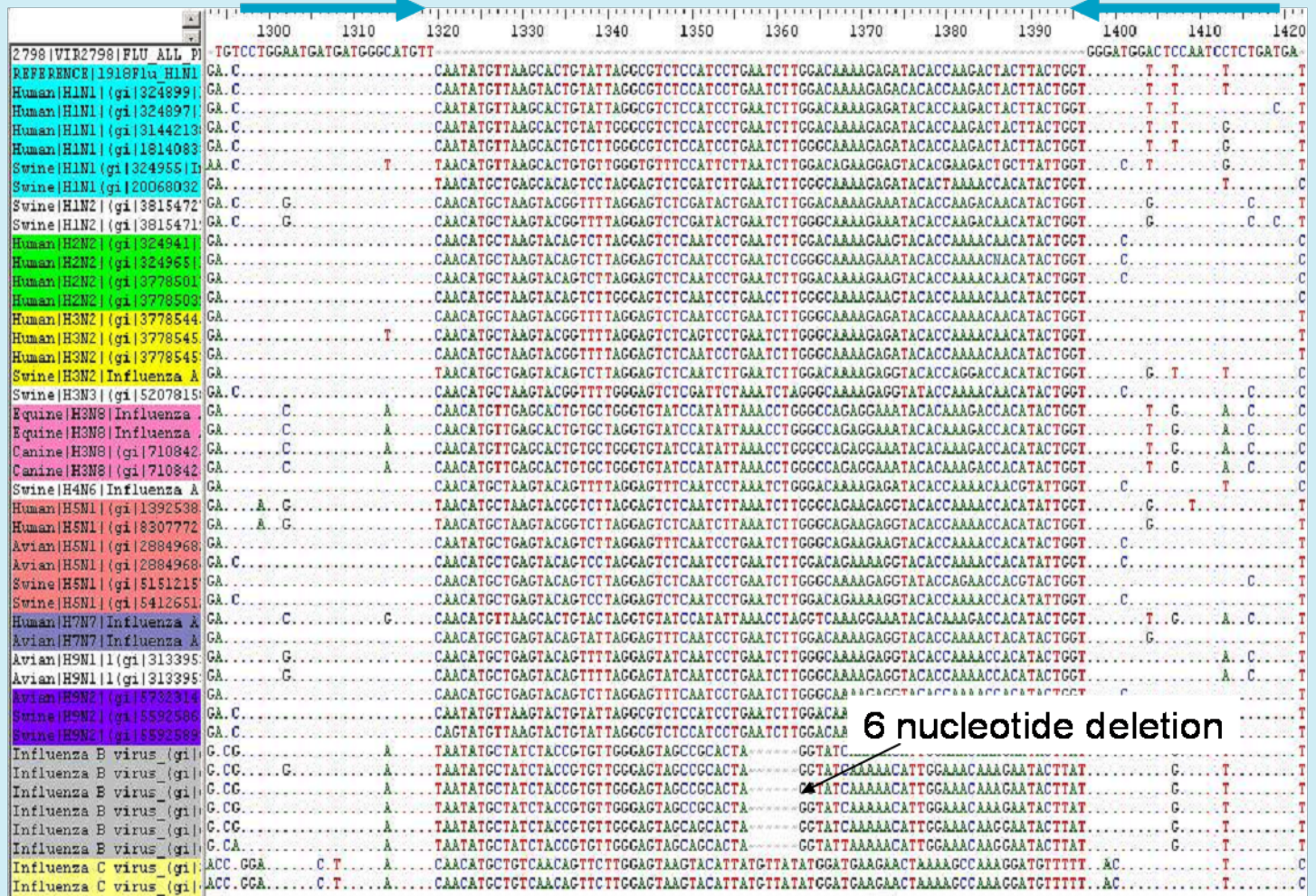
6 nucleotide deletion

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Pan Influenza Primer Polymerase PB1 Primer PP2798 - Base Composition
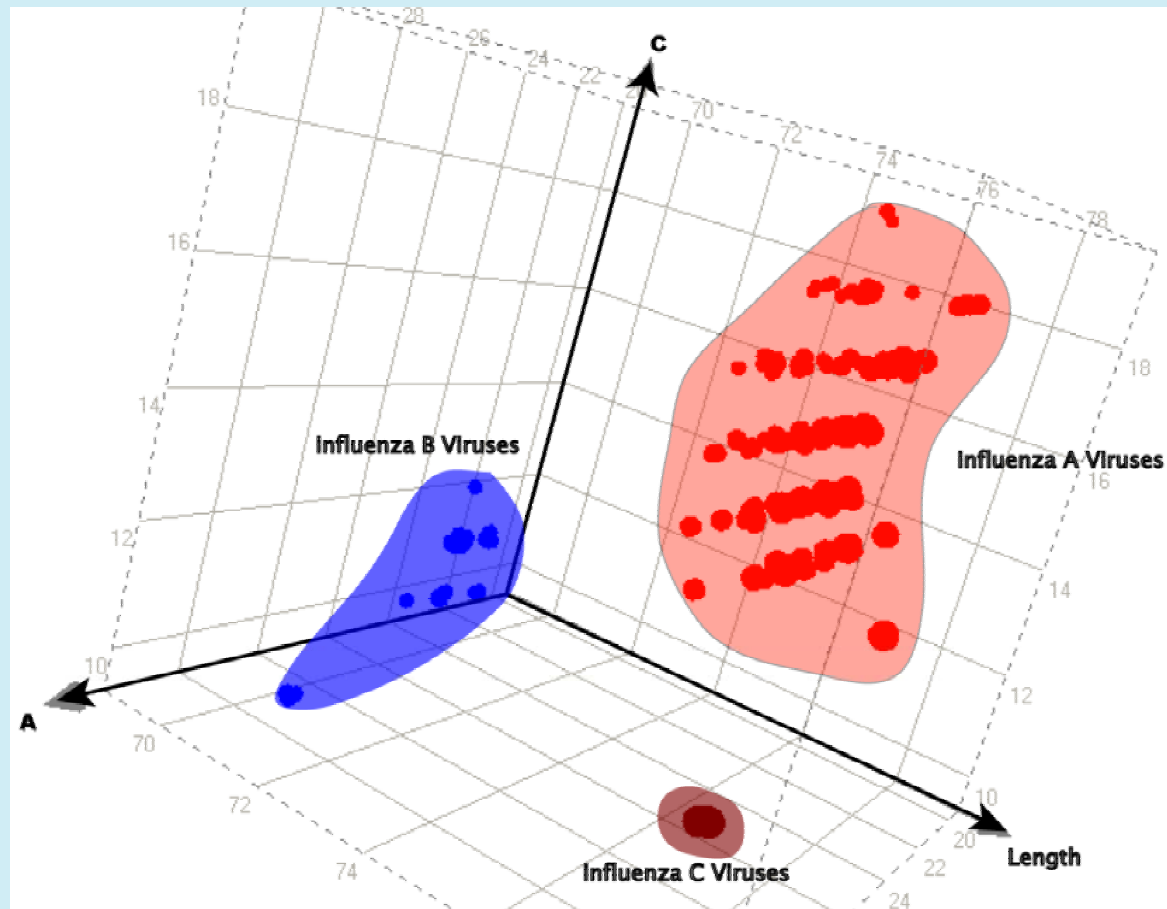
Influenza Virus Assay Results Example

Tiger Analysis Plate Viewer – Plate P00001450

Influenza A virus

Influenza B virus   -ve control

Score Cutoff: 0.821

New Plate

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

Samples 2 & 4: Influenza B virus

Well 15, Sample 3
Primer VIR1266
FLUANUC_J02147_118_218

Organism	Sample Score	Basecount	Peak1 Mass	Peak1 Abundance	Peak1 Fit Error	Peak2 Mass	Peak2 Abun
Influenza A virus|A/NEW YORK/14/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/15/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/16/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/21/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/22/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/24/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/25/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/26/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/268/2003(H3N2)	0.96539	A26G20C23T32	31051.6 / 31364.1	3030.0 / 396.0	0.0 / 0.0	31225.7 / 31225.7	3440.0 / 3440.0
Influenza A virus|A/NEW YORK/27/2003(H3N2)	0.96539	A26G20C23T32	31051.6	3030.0	0.0	31225.7	3440.0

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Human Influenza – Blinded Samples

Source	Location	Collection Dates	Sample type	No. of Samples
Naval Health Research Center	MCRD, San Diego Ft. Leonard Wood, Ft. Sill, Ft. Benning, Lackland AFB	1999-2005	Throat swabs, nasal swabs, nasal washes	317
Johns Hopkins University Medical Center	Baltimore, MD	2003-2005	Nasal aspirates	229
NY State Dept. of Health	Throughout NY	1999-2005	Nasal aspirates, BAL, tracheal aspirates, throat swabs	100
TX State Dept. of Health	Throughout TX	2005-2006	Throat swabs, nasal washes	10
Total				656

–**Correctly identified all Influenza A types**
  ➢149 H3N2
  ➢34 H1N1
–**67 Influenza B**

	Influenza
Sensitivity	96.8%
Specificity	97.5%
PPV	96.0%
NPV	98.0%

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Detection of Mixed Infections

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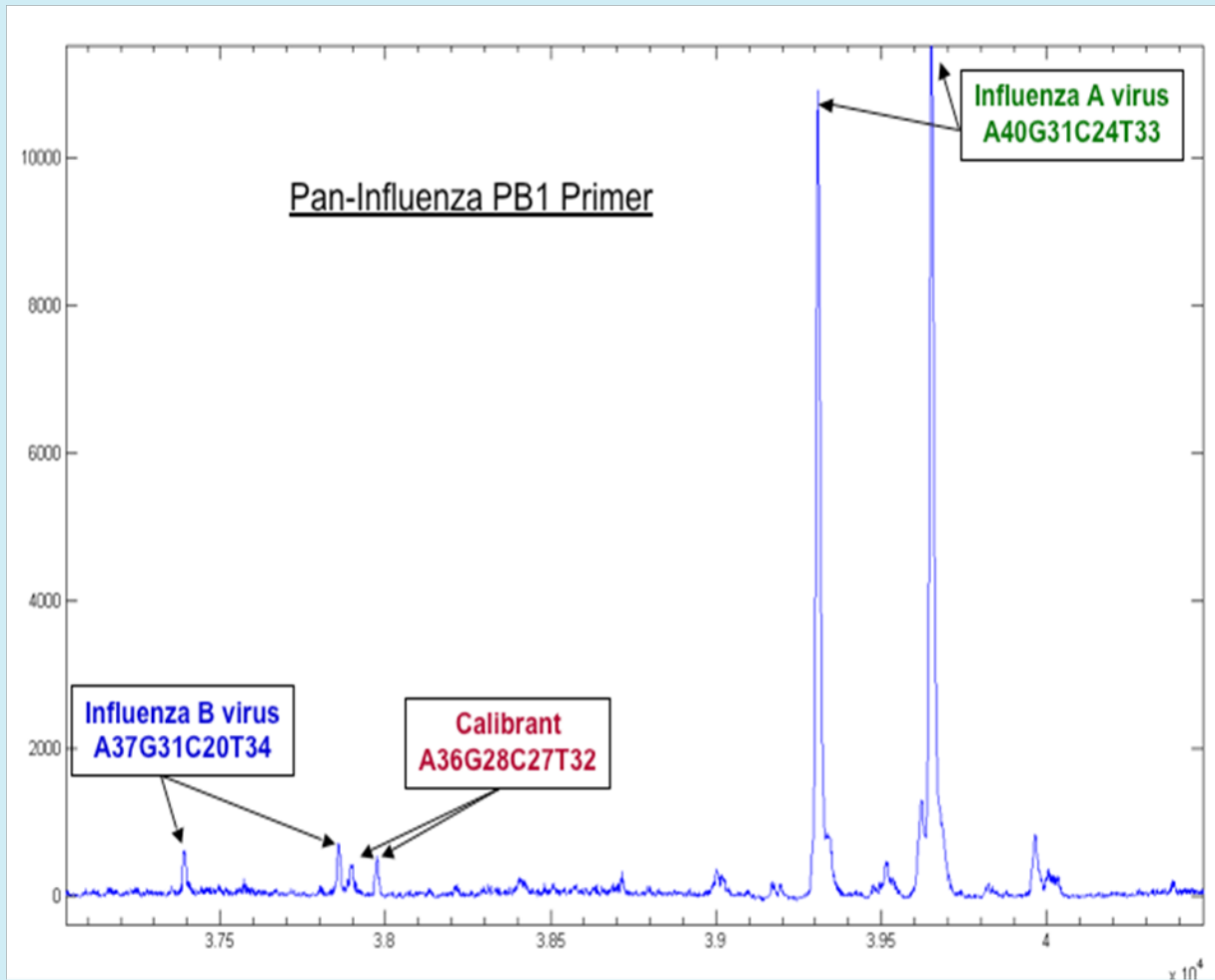
Validation Study Test Isolates from Diverse Sources

- **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

Isolated RNA

Pan Influenza Primer — PB1

Influenza A Primers — Nuc, M1, PA, NS1, NS2

Influenza B Primers — PB2, Nuc

PCR Desalting

Mass Spectrometer

Sample → ESI needle, Capillary, Ion Store, Reflectron

	PB1	Nuc	M1	PA	NS1	NS2	PB2	Nuc
Influenza A	A39 G32 C23 T34	A32 G23 C20 T26	A24 G30 C23 T28	A37 G23 C27 T25	A28 G21 C32 T38	A35 G27 C17 T26		
Influenza B	A37 G31 C20 T34						A23 G27 C19 T22	A36 G17 C36 T11
Mixture	A39 G32 C23 T34	A32 G23 C20 T26	A24 G30 C23 T28	A37 G23 C27 T25	A28 G21 C32 T38	A35 G27 C17 T26	A23 G27 C19 T22	A36 G17 C36 T11

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

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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...**

Avian Influenza Virus Detection: University of Georgia Samples

SCWDS ID#	Serotype	Species	Location	Date
AI00-1412	H6N1	REKN	Bower's Beach, DE	5/25/00
AI00-1794	H12N4	RUTU	Bower's Beach, DE	5/20/96
AI00-2150	H12N5	RUTU	Villas, NJ	5/15/96
AI00-629	H7N9	RUTU	Port Mahon, DE	5/19/96
AI02-262	H2N4	RUTU	Mispillion Harbor,DE	5/22/98
AI02-690	H2N9	RUTU	Reed's Beach, NJ	5/22/98
AI03-128	H9N7	RUTU	Reed's Beach, NJ	5/20/99
AI03-128	H9N7	RUTU	Reed's Beach, NJ	5/20/99
AI03-755	H9N5	RUTU	Mispillion Harbor,DE	5/20/99
AI04-127	H10N7	RUTU	Bower's Beach, DE	5/19/00
AI05-415	H3N8	RUTU	Fortescue Beach, NJ	5/21/01
AI05-415	H3N8	RUTU	Fortescue Beach, NJ	5/21/01
AI05-669	H11N8	RUTU	Reed's Beach, NJ	5/25/01
AI05-784	H11N6	RUTU	Reed's Beach, NJ	5/25/01
MN00-283	H5N2	MALL	Thief Lake, MN	9/10/96
MN00-382	H5N3	MALL	Thief Lake, MN	9/10/96
MN98-115	H4N8	MALL	Roseau Co., MN	09/ /1998
MN98-115	H4N8	MALL	Roseau Co., MN	09/ /1998
MN98-66	H6N5	MALL	Roseau Co., MN	09/ /1998
MN99-160	H4N6	MALL	Roseau Co., MN	/ /1999
MN99-17	H7N7	MALL	Roseau Co., MN	/ /1999
NC6412-009	H10N7	MALL	JM Futch, NC	12/20/00
NC675-075	H3N2	ABDU	Mattamuskeet, NC	12/21/00
TX01-32	H8N4	CITE	Brazoria Co., TX	2/11/97
TX01-7	H8N4	AGWT	Brazoria Co., TX	2/11/97
TX02-27	H1N4	BWTE	Brazoria Co., TX	2/18/98
TX02-75	H1N3	BWTE	Brazoria Co., TX	2/18/98

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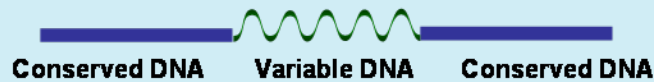
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Ibis™ T5000 Discrimination of Diverse Avian Influenza Virus Isolates

Serotype	Species	Location	Date	2798	1266	1279	1287	2775	2777
H1N3	BWTE	Brazoria Co., TX	2/18/98	A37G33C24T34	A35G22C22T22	A23G30C25T27	A35G27C28T22	A40G30C20T29	A35G27C16T27
H9N7	RUTU	Reed's Beach, NJ	5/20/99	A37G33C25T33	A37G21C20T23	A22G30C26T27	A35G26C28T23	A37G32C22T28	A34G27C18T26
H3N8	RUTU	Fortescue Beach, NJ	5/21/01	A37G34C23T34	A34G23C23T21	A23G30C24T28	A34G27C28T23	A37G32C22T28	A35G27C16T27
H3N8	RUTU	Fortescue Beach, NJ	5/21/01	A37G34C23T34	A34G24C20T23	A23G30C24T28	A34G27C28T23	A37G32C22T28	A34G27C18T26
H3N2	ABDU	Mattamuskeet, NC	12/21/00	A37G34C23T34	A34G24C21T22	A21G32C24T28	A35G26C29T22	A37G32C22T28	A34G27C18T26
H11N8	RUTU	Reed's Beach, NJ	5/25/01	A38G33C22T35	A35G22C22T22	A22G31C25T27	A34G27C28T23	A40G30C20T29	A35G27C16T27
H11N6	RUTU	Reed's Beach, NJ	5/25/01	A38G33C22T35	A35G22C22T22	A23G30C24T28	A34G27C28T23	A40G30C20T29	A35G27C16T27
H9N5	RUTU	Mispillion Harbor, DE	5/20/99	A38G33C23T34	A37G21C20T23	A24G28C23T30	A35G26C28T23	A37G32C22T28	A34G27C18T26
H9N7	RUTU	Reed's Beach, NJ	5/20/99	A38G33C24T33	A34G24C21T22	A22G30C26T27	A35G26C28T23	A37G32C22T28	A34G27C18T26
H6N5	MALL	Roseau Co., MN	09/ /1998	A39G32C22T35	A36G21C22T22	A21G32C25T27	A34G27C28T23	A40G30C20T29	A35G27C16T27
H4N6	MALL	Roseau Co., MN	/ /1999	A39G32C23T34	A35G22C23T21	A22G31C27T25	A34G27C28T23	A37G32C22T28	A34G27C18T26
H5N2	MALL	Thief Lake, MN	9/10/96	A39G32C23T34	A36G21C22T22	A23G31C26T25	A35G26C27T24	A37G32C22T28	A34G27C18T26
H8N4	AGWT	Brazoria Co., TX	2/11/97	A39G32C23T34	A36G22C21T22	A24G28C25T28	A34G27C28T23	A37G32C22T28	A34G27C18T26
H7N7	MALL	Roseau Co., MN	/ /1999	A39G32C23T34	A36G22C22T21	A20G32C27T26	A34G27C29T22	A37G32C22T28	A34G27C18T26
H5N3	MALL	Thief Lake, MN	9/10/96	A39G32C24T33	A34G24C21T22	A21G32C25T27	A34G27C28T23	A37G32C22T28	A34G27C18T26
H2N4	RUTU	Mispillion Harbor, DE	5/22/98	A39G32C24T33	A35G22C22T22	A22G31C25T27	A35G26C29T22	A37G32C22T28	A34G27C18T26
H12N5	RUTU	Villas, NJ	5/15/96	A39G32C24T33	A36G21C22T22	A21G32C24T28	A35G26C29T22	A37G32C22T28	A34G27C18T26
H7N9	RUTU	Port Mahon, DE	5/19/96	A39G32C24T33	A36G21C22T22	A21G32C24T28	A34G27C29T22	A37G32C22T28	A34G27C18T26
H2N9	RUTU	Reed's Beach, NJ	5/22/98	A39G32C24T33	A36G21C22T22	A23G30C26T26	A35G26C29T22	A37G32C22T28	A34G27C18T26
H4N8	MALL	Roseau Co., MN	09/ /1998	A40G31C22T35	A29G24C19T29	A25G28C25T27	A34G27C28T23	A37G32C22T28	A34G27C18T26
H4N8	MALL	Roseau Co., MN	09/ /1998	A40G31C22T35	A34G24C21T22	A25G28C25T27	A34G27C28T23	A37G32C22T28	A34G27C18T26
H12N4	RUTU	Bower's Beach, DE	5/20/96	A40G31C23T34	A35G23C22T21	A23G30C26T26	A35G26C29T22	A37G32C21T29	A34G27C17T27
H10N7	RUTU	Bower's Beach, DE	5/19/00	A40G31C24T33	A33G25C21T22	A23G30C25T27	A35G27C27T23	A38G31C22T28	A34G27C18T26
H10N7	MALL	JM Futch, NC	12/20/00	A40G31C24T33	A35G22C21T23	A23G30C26T26	A34G27C29T22	A37G32C22T28	A34G27C18T26
H8N4	CITE	Brazoria Co., TX	2/11/97	A40G31C24T33	A35G22C22T22	A22G30C27T26	A34G27C29T22	A40G30C20T29	A35G27C16T27
H1N4	BWTE	Brazoria Co., TX	2/18/98	A40G31C24T33	A35G22C22T22	A24G28C25T28	A35G26C28T23	A40G30C20T29	A35G27C16T27

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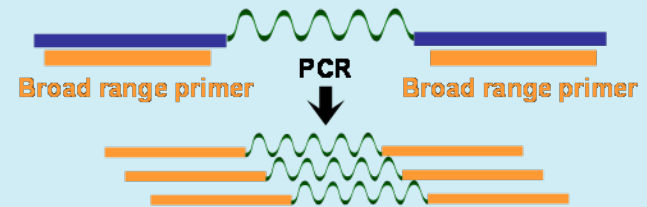**NIJ** National Institute of Justice

*Same panel was used for human Influenza detection

The Ibis™ Approach to Pathogen ID and Strain-Typing

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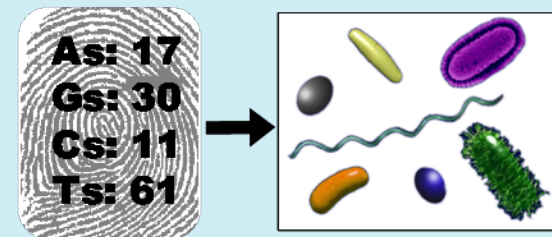
Conserved DNA — Variable DNA — Conserved DNA

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

Broad range primer — PCR — Broad range primer

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

Sample → ESI needle → Capillary → Ion Store → Reflectron → Electron Multiplier

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

As: 17
Gs: 30
Cs: 11
Ts: 61

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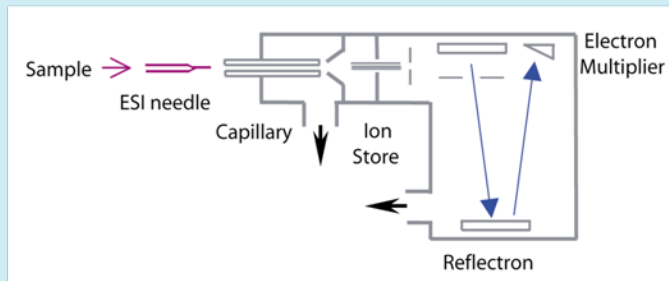
PCR Primer Design for Forensic Markers
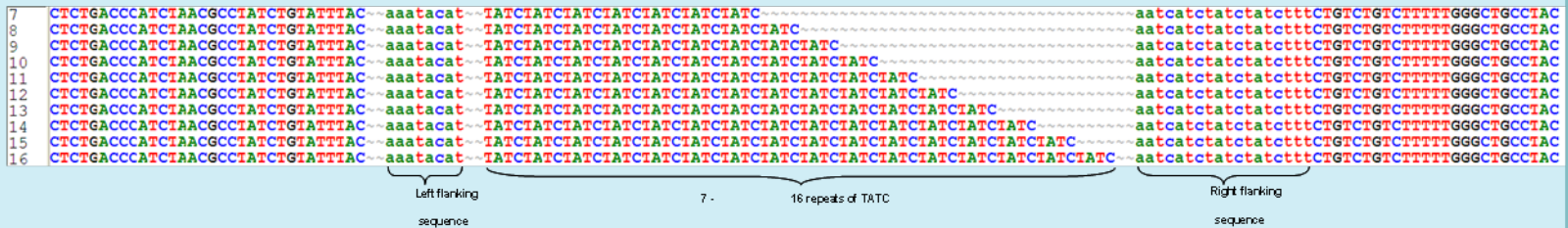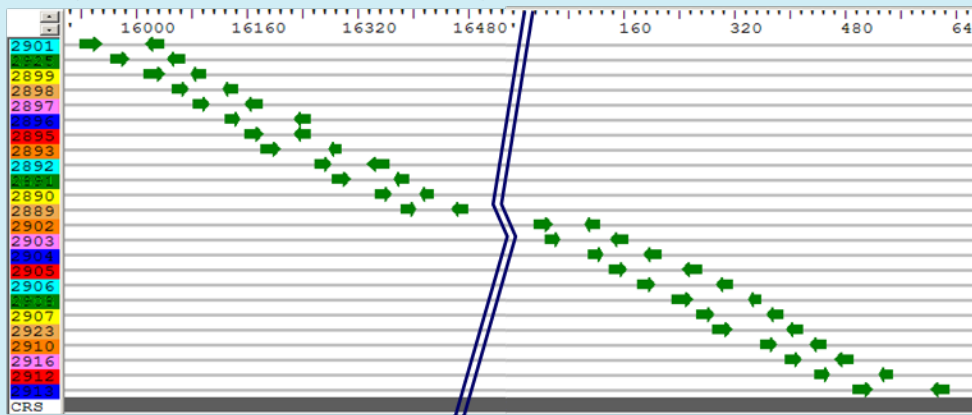
Conclusions

- **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**

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Conclusions

- **Respiratory Virus Surveillance Assay (RVSA) provides a single test platform for six 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?"**

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Questions?

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