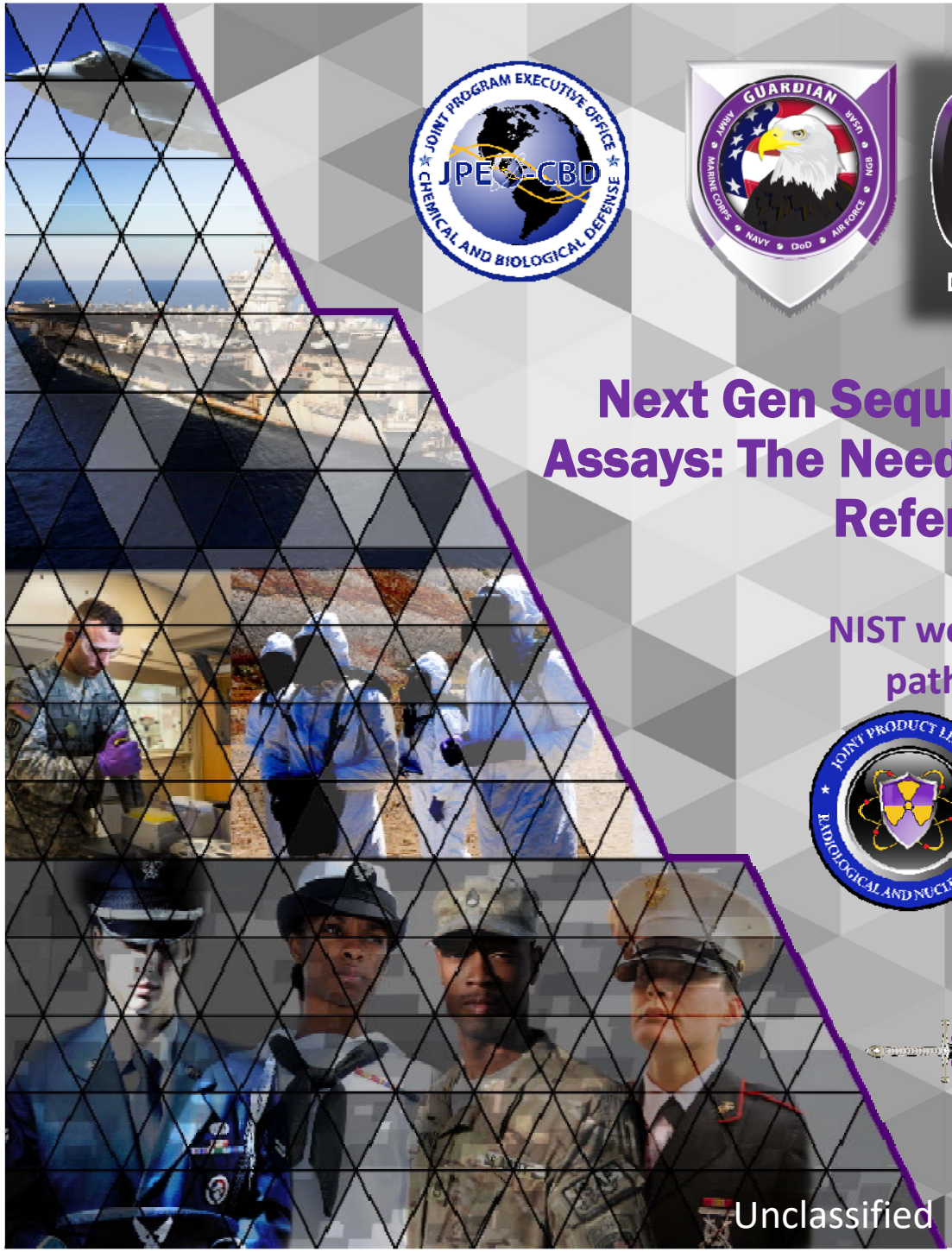

JOINT PROJECT MANAGER GUARDIAN

Discover, Identify, Characterize, & Eliminate CBRN Threats Globally

# Next Gen Sequencing based Biodefense Assays: The Need for Standardized Alternate Reference Materials

*Presented to:*

NIST workshop on standards for pathogen detection-2017


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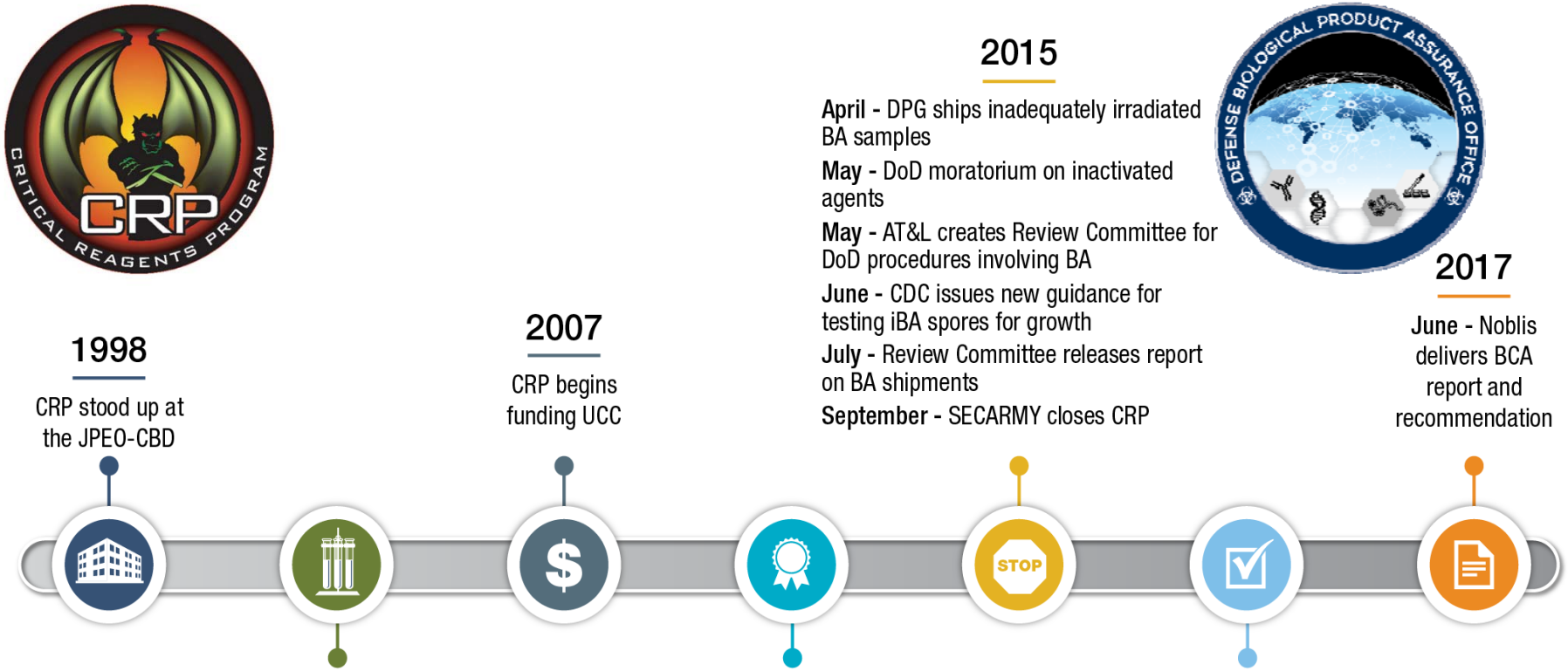
Unclassified

# Disclaimer

- The views expressed in this presentation are those of the author and do not necessarily represent the views or official position of Defense Biological Product Assurance Office (DBPAO), Joint Project Manager for Guardian (JPM G), Joint Program Executive Office (JPEO), U.S. Department of Defense (DoD)

# Who are we?


1998
CRP stood up at the JPEO-CBD
2003
CRP opens antigen repository at Dugway
2007
CRP begins funding UCC
2010
CRP obtains ISO 9001:2008 accreditation
2015
April - DPG ships inadequately irradiated BA samples
May - DoD moratorium on inactivated agents
May - AT&L creates Review Committee for DoD procedures involving BA
June - CDC issues new guidance for testing iBA spores for growth
July - Review Committee releases report on BA shipments
September - SECARMY closes CRP
2016
March - JPEO-CBD renamed CRP to DBPAO, aligns it to JPM-Guardian
July - SECARMY describes new policies/procedures for BSAT-related materials
July - SECARMY describes DBPAO BSAT-related requirements
2017
June - Noblis delivers BCA report and recommendation
STOP

# DBPAO: CAR and TARMAC

## Material Products

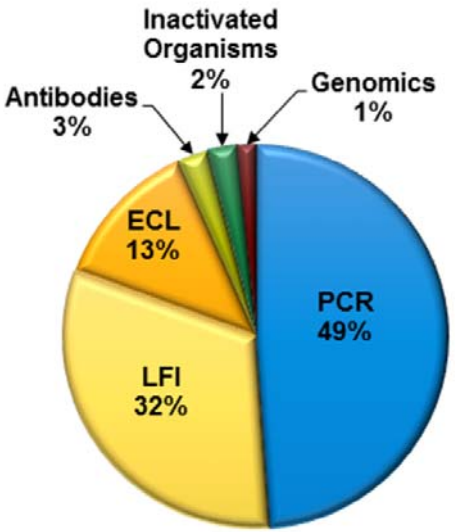
### CAR

**OSCAR**
ORDERING SYSTEM FOR CRITICAL ASSAYS AND REAGENTS

- Lateral Flow Immunoassays
- Electrochemiluminescence Assays
- Polymerase Chain Reaction Assays
- Antibodies
- Surrogates (Non-BSAT)

### EARO

- JIBS/Defense Business System
- Inactivated Organisms (BSAT)
- Genomic Material




## Knowledge Products

### TARMAC

**TARMAC Repository**

- UCC Pathogen Data
- Microbial Data Index
- Microbial Metadata
- Characterization Data
- TARMAC Data
- CAR Product Data
- Assay Data
- DHIS 2 Data

# Outline - modifications to standards

- Standards for Reference Materials
  - Surrogates as alternate reference materials for Select Agents
- Standards for Strain Characterization
  - End to end characterization
- Standards for Assay Development
  - Incorporation of extensive *in silico* analyses of assay signatures and minimize wet lab testing with risky reference materials

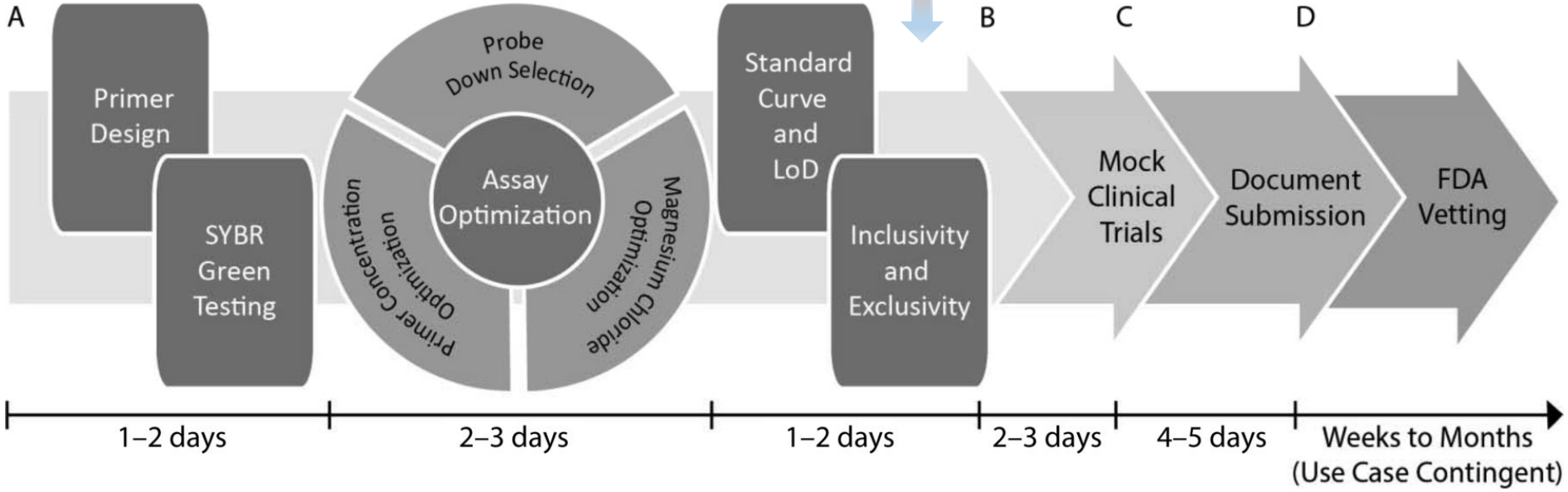
# The problems with select agent reference materials or derivatives for Biodefense Assay Development

- 2015 DPG debacle and the ensuing moratorium on working with select agents and shipping
- New samples/strains availability (e.g., Ebola Zaire Makona, Lassa)
  - Potential signature erosion with new sequences and the need for assay redesign
- Time line for optimization of assay for new sequences?
- Is there a need to reevaluate all steps in assay development and FDA approval?

# Traditional pathway for development of a nucleic acid based molecular assay

**Reference Materials: live or inactivated organisms or genomic materials**

**1 prototype strain** **Multiple strains**




Courtesy:

Hartman et al 2015. Demonstration of the Pre–Emergency Use Authorization Path Using 3 Minor Groove Binder–Hydrolysis Probe Assays to Detect *Escherichia coli* O104:H4. Clinical Chemistry 61:11 1391-1398. [Tim Minogue's group]

# New Standards for Select Agent Reference Materials

# Why were these products needed?

- What is the purpose for the inactivated Spores and other inactivated Select Agents?
  - The inactivated agent materials serve several critical needs
    - Positive controls in assays used to detect these pathogens in suspected samples
    - Improvement of currently existing detection methods or development of new methods/platforms for detection of Ba (develop and validate assays)
    - Quality assurance and Proficiency testing and Training activities
    - End to End Validation of systems

# DoD Solutions to the Problem

a) **Ba Spore Surrogate to Replace Inactivated Virulent Spores**

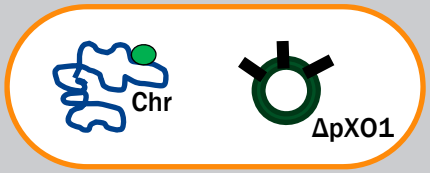
a) Ba Spore Inactivation Studies

# Alternate Risk Mitigated Reference Materials for Ba

**‘Genetically inactivated/modified (attenuated)’ ‘Non-pathogenic’ Ba Strains with Assay Targets**

**Construction, Testing and Production**

# Technical Aspects - What are we trying to do?

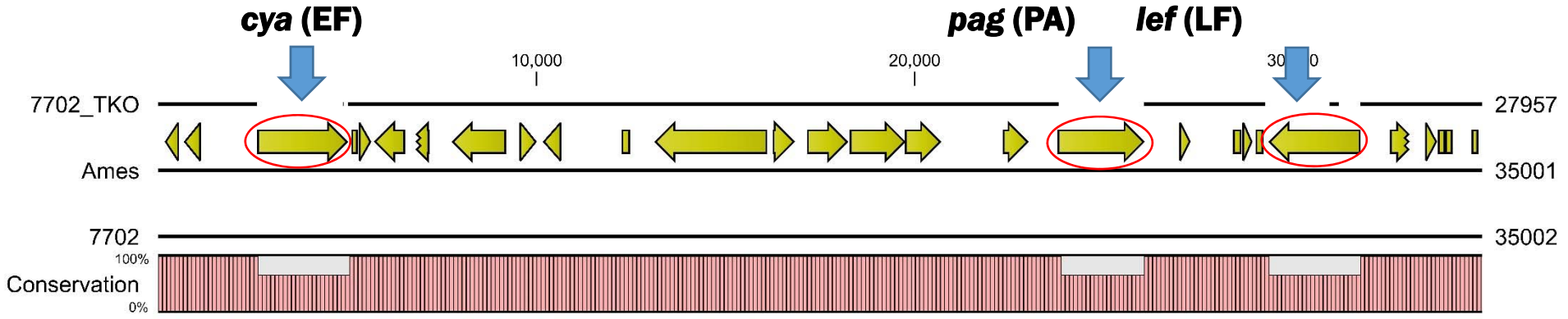
| Ba Strain Name | Genetic make up | Status/Risk | Pathogenicity: Toxin Genes | Pathogenicity: Capsule Genes | All Assay Targets? |
|---|---|---|---|---|---|
| *Bacillus anthracis* Ames (exists) |   | Select Agent | X | X | Yes (3) |
| *Bacillus anthracis* Sterne (exists) |   | Exempt (pathogenic for animals) | X | - | No (2) |
| *Bacillus anthracis* Sterne ΔpXO1 (aka TKO exists) |   | Exempt (non-pathogenic) | - | - | No (1) |
| *Bacillus anthracis* Sterne ΔpXO1 plus (to be constructed rBaSwAT) |   | Exempt (non-pathogenic) | - | - | **Yes (3)** |

Assay target sequences: ● ● ●  Gene deletion: ▮

**TKO- Triple Knock Out**

# Safety Aspects of the Parent Strain Sterne (TKO)

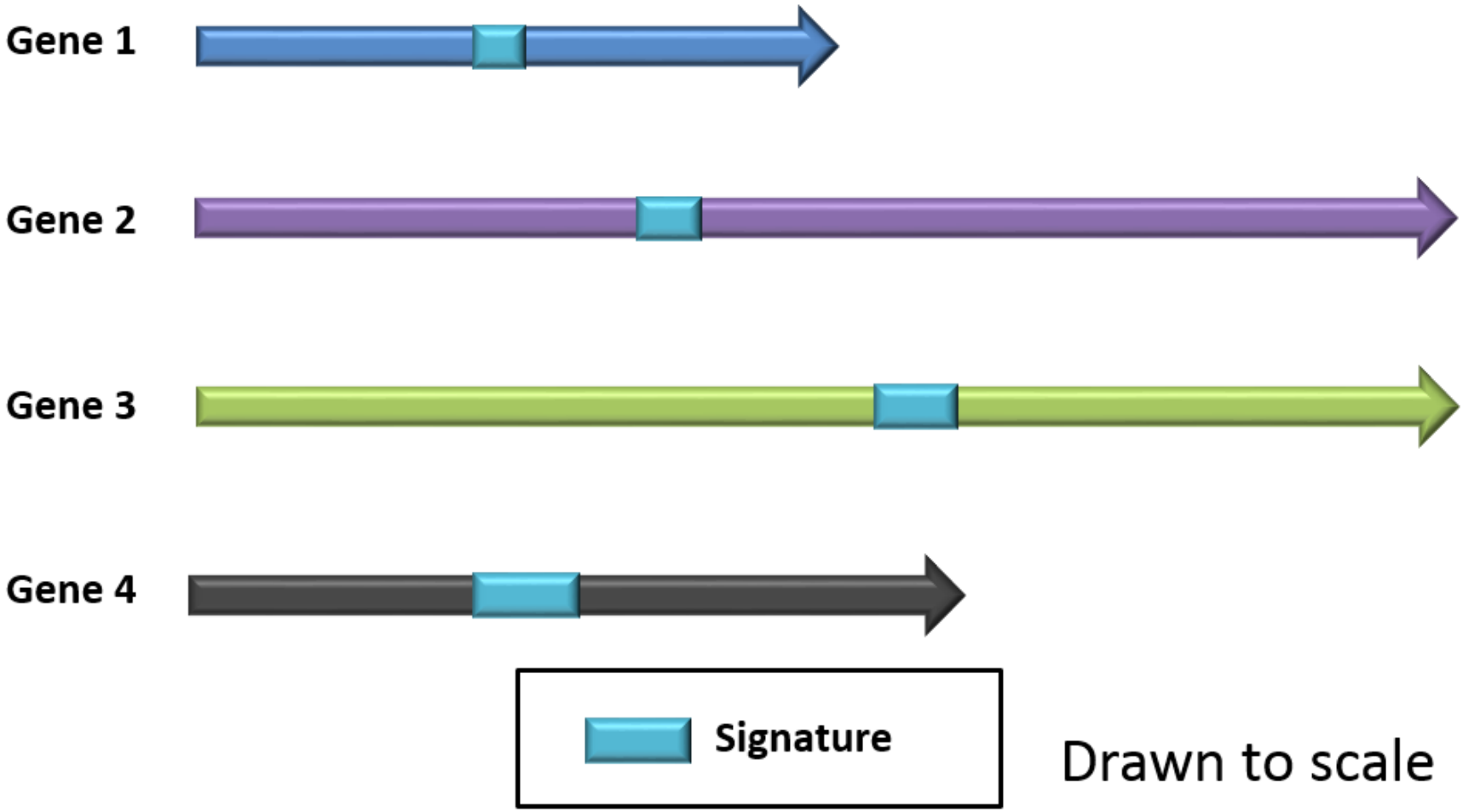
**TKO- Triple Knock Out**




**Toxin gene region of parent strains**

Ref: Janes BK, Stibitz S. Routine markerless gene replacement in *Bacillus anthracis*. Infect Immun. 2006 Mar; 74(3):1949-53.

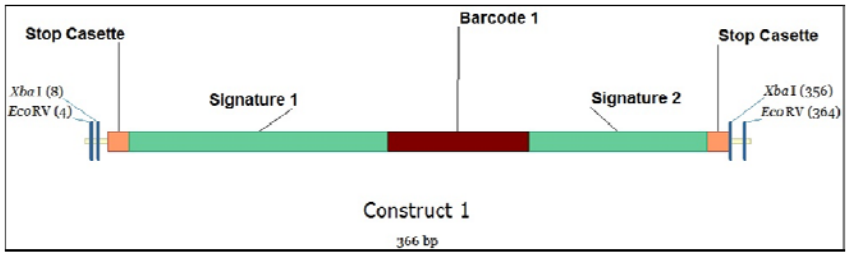
# Gene to Signature Fragment Ratio



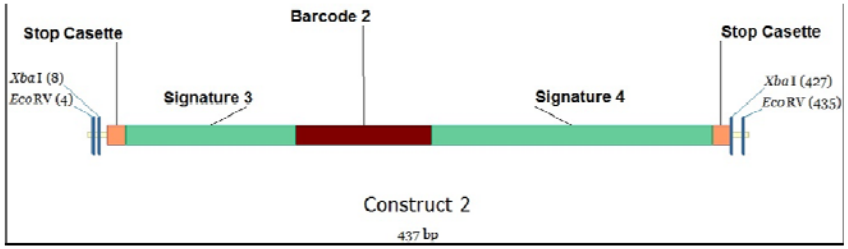
# Schematic of the introduced DNA

**Status**

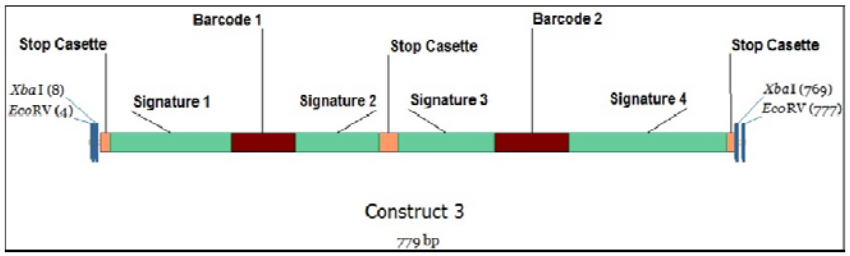
**In Ba**



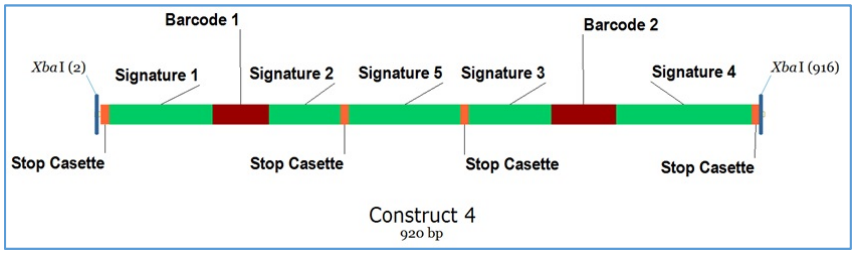

**In Ba**




**In Ba**




**In Ba**



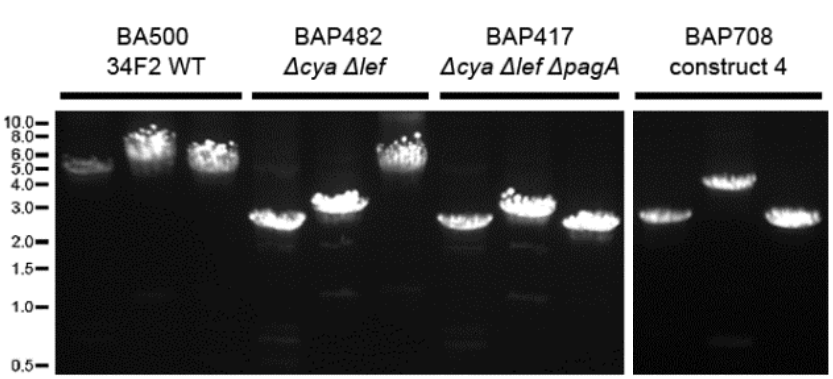

- **Different constructs for different assays**
- **Barcodes for complete traceability and bio forensics**
- **"Stop Cassette" as an extra measure to prevent fortuitous translation of insert from neighboring transcriptional read through**

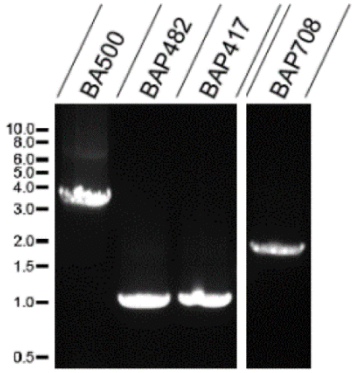
**Courtesy: Dr. Mark Munson-NMRC**

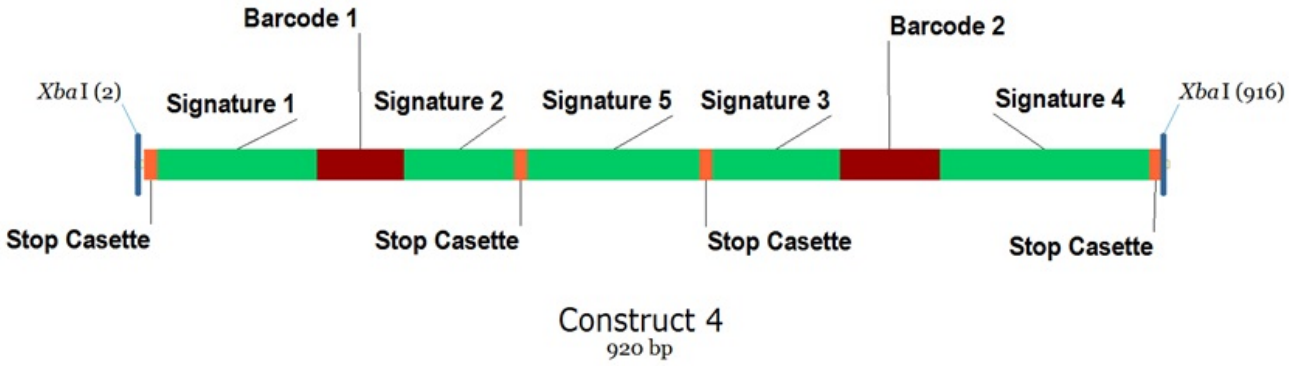
# End to End Characterization of rBaSwAT

- Vegetative cells
  - Phenotypic assays
    - ✓ Microbiological tests
    - ✓ Phage sensitivity ($\gamma$ and AP50c)
    - ✓ Antimicrobial resistance
    - ✓ Spore formation (@NSWC)
    - ✓ Immunological tests (LFI)
  - Genotypic assays
    - ✓ Whole genome sequencing
    - ✓ Molecular tests for signatures (PCR)
- Spores
  - Production, purification (@NSWC) and irradiation inactivation (using new protocol established by the working group @NMRC)
    - ✓ Molecular and Immuno assays and bridging studies (@NSWC)
    - ✓ Animal studies

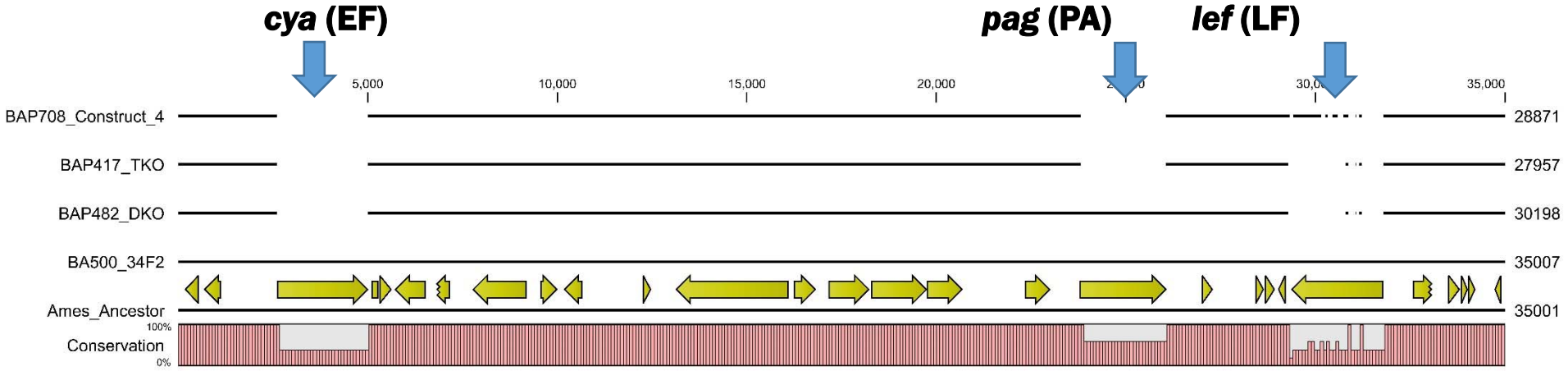
# PCR verification of toxin gene deletions in rBaSwAT



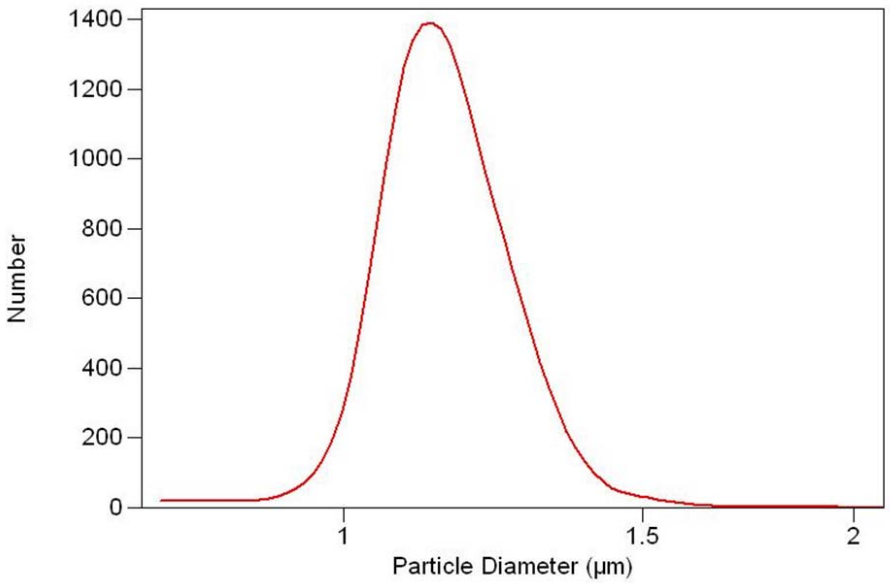



# Whole Genome Sequencing of rBaSwAT




**Toxin region in rBaSwAT strain compared to grant parent (34F2, 34F2 DKO, 34F2 TKO strains**

# Spore Characteristics

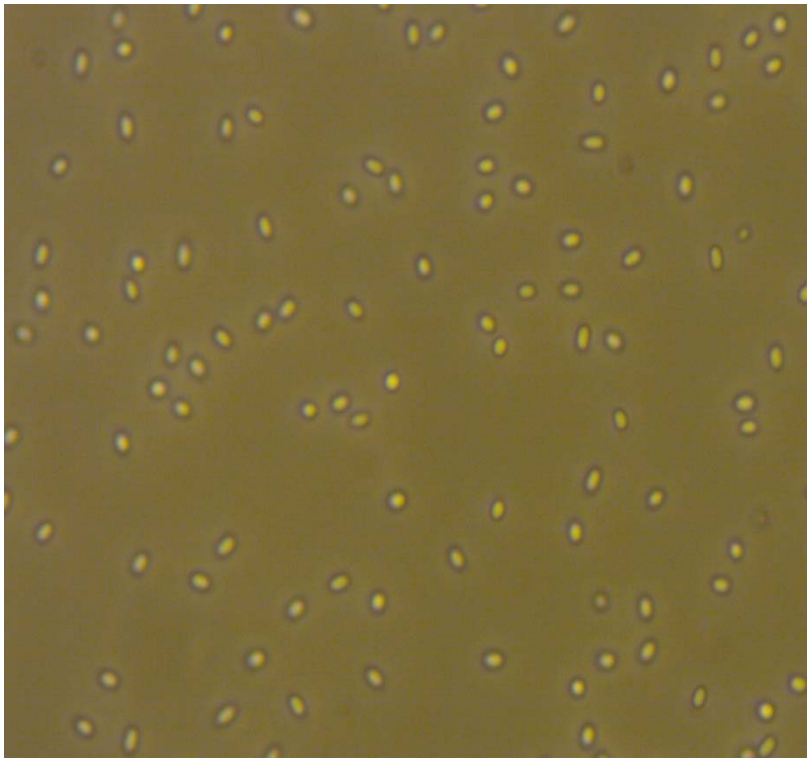



Coulter Counter

Mean (µm) ± S.D. (µm)

1.153 ± 0.122

Phase Contrast Microscopy

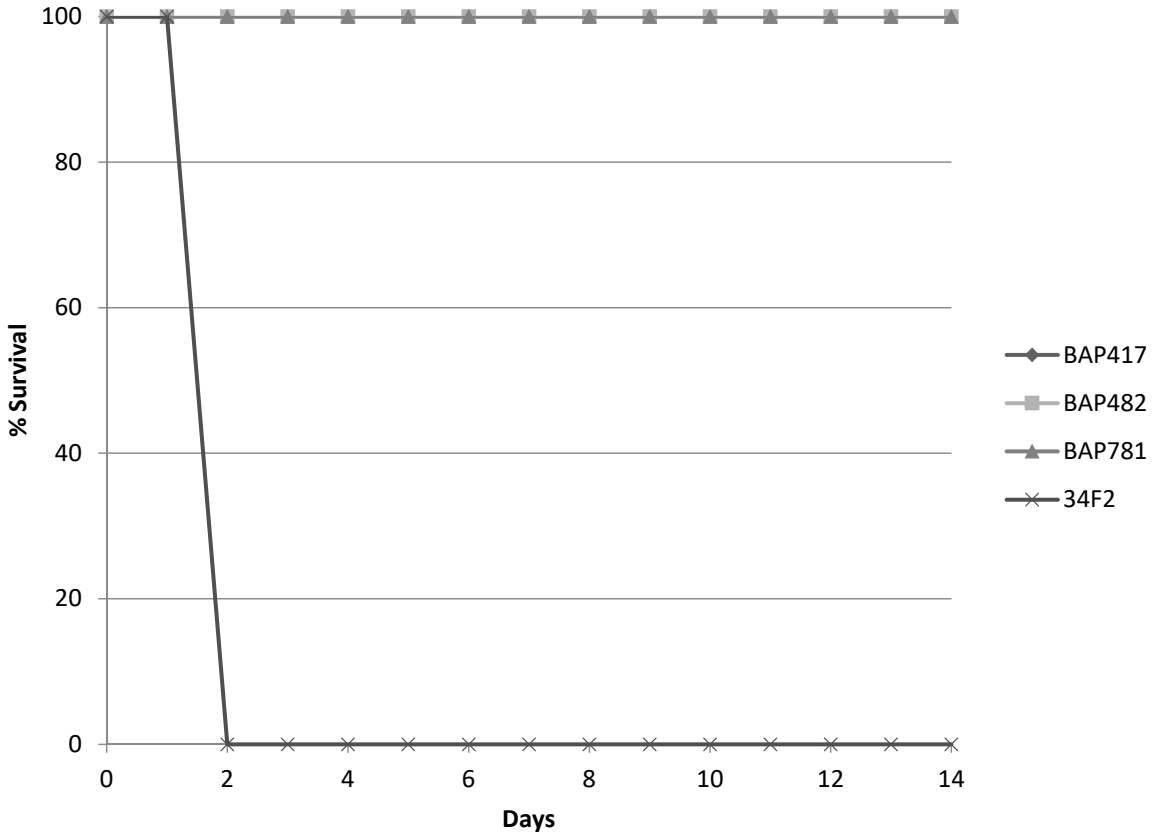


Spore titer: Plate counts: 1.5e10; Coulter Counter: 1.9e10; GE: TBD

# Signature PCR Evaluation

| ASSAY | STRAIN | | | | | |
|---|---|---|---|---|---|---|
| | Sterne | TKO | rBaSwAT1 | rBaSwAT2 | rBaSwAT3 | rBaSwAT4 |
| | BA663 | BA781 | BAP696 | BAP684 | BAP686 | BAP710 |
| | Grand Parent | Parent | Construct 1 | Construct 2 | Construct 3 | Construct 4 |
| Chr | + | + | + | + | + | + |
| Sig1 | - | - | + | - | + | + |
| Sig2 | + | - | + | - | + | + |
| Sig3 | + | - | - | + | + | + |
| Sig4 | - | - | - | + | + | + |
| Sig5 | + | - | - | - | - | + |

# Demonstration of non-lethality of constructs in A/J mice




Based upon a One-sided Fisher exact test P=0.0003 for groups compared to 34F2 (N=5 for that control)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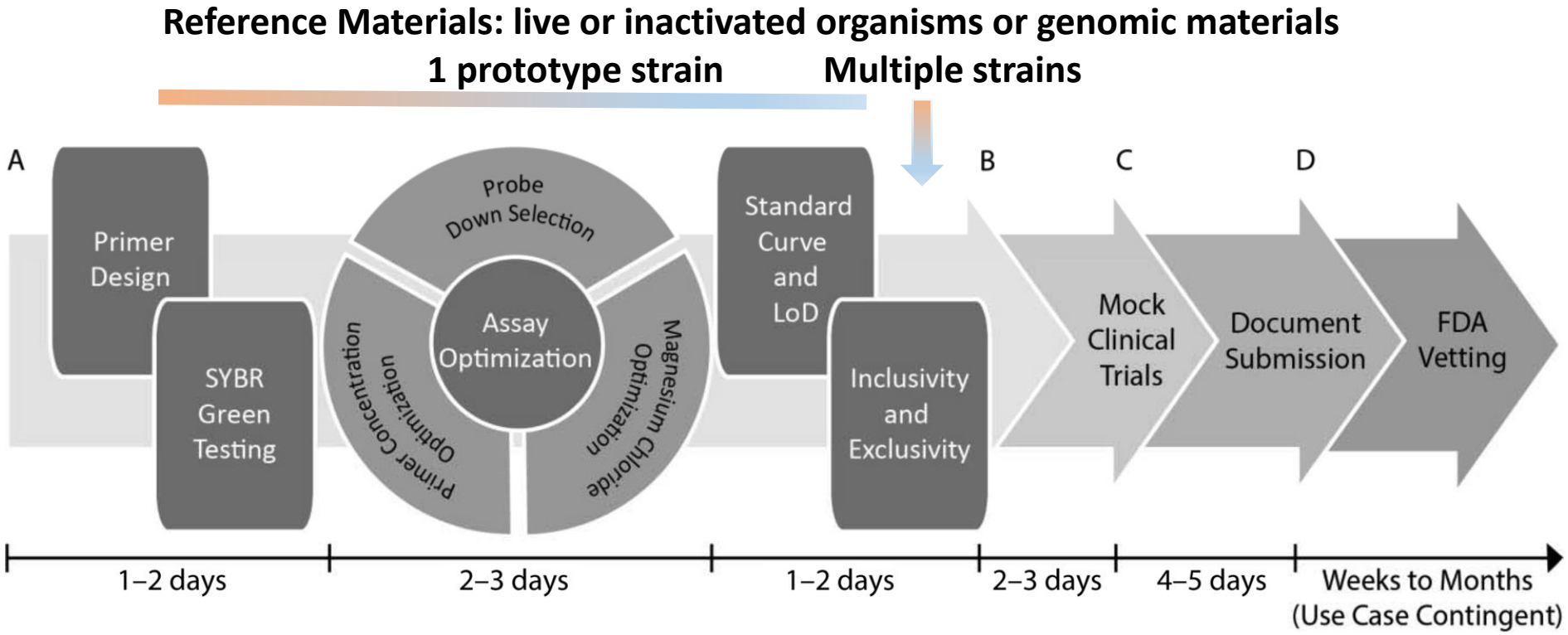
# Partners

- DBPAO Performers
  - Drs. Joan Gebhardt and Mark Munson
    - Naval Medical Research Center, Ft. Detrick, MD
  - Drs. Chris Cote, Dave Rozak and Terry Abshire
    - USAMRIID, Fort Detrick, MD
  - Drs. Cory Bernhards and Nicole Rosenzweig, Rebecca Rossmaier and Tracey Biggs
    - ECBC, Edgewood, MD
  - Drs. Tony Buhr, Linda Beck and Andrea Staab
    - NSWC, Dahlgren, VA
- FDA Collaborators
  - Drs. Roger Plaut and Scott Stibitz
    - FDA, Silver Spring, MD

# Traditional pathway for development of a molecular assay




Courtesy:

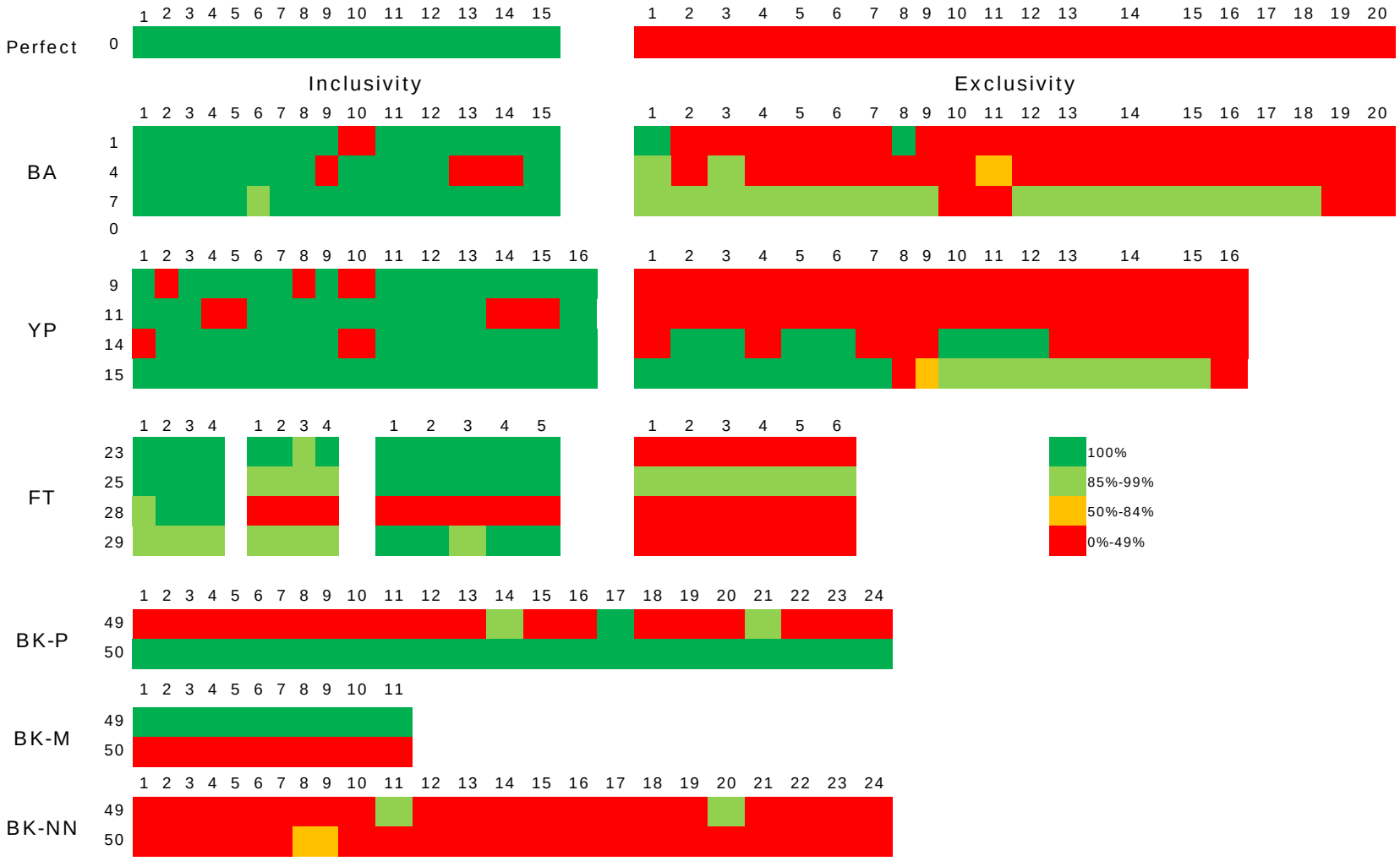
Hartman et al 2015. Demonstration of the Pre–Emergency Use Authorization Path Using 3 Minor Groove Binder–Hydrolysis Probe Assays to Detect *Escherichia coli* O104:H4. Clinical Chemistry 61:11 1391-1398. [Tim Minogue's group]

# Stakeholder Panel on Agent Detection Assays (SPADA)

- List of Inclusivity and Exclusivity Panel strains decided by SMEs for each organism
  - Ba
  - Yp
  - Ft
  - Burk
  - Brucella
  - Toxins (Various toxins)
- Any assay needs to be wet lab tested against this panel- expectation- the assay will hit all inclusivity panel and will not hit exclusivity panel

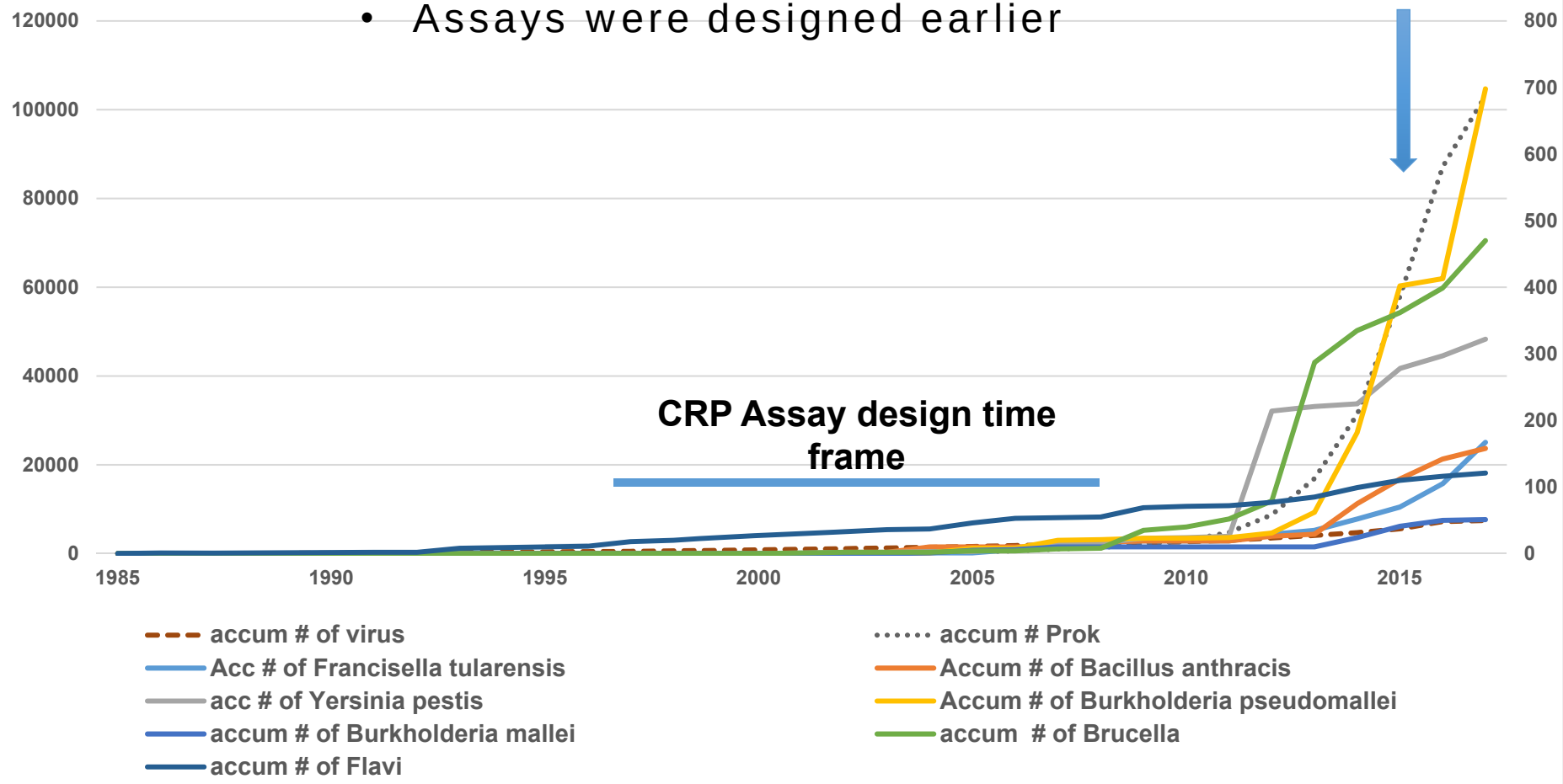
# On the need for *in silico* analyses to replace inclusivity/exclusivity testing

Heat map of signature sequence hits in various genomic sequences



# Genome Sequence Explosion with the advent of Next Gen Sequencing

- Sequences of SPADA panels were published in 2015
- Assays were designed earlier



# Standards for *in silico* analyses of assay signatures (rapidly evolving pathogen)

***In silico*** **signature evaluation of 2014 EBOV outbreak strains before we obtained samples**

# How do we make the call?

- Parameters
  - Assay Hit- A positive match between the assay primer/probe set sequences and sequences from the NCBI databases @ > **90% identity over 90% of the primer length** (2 mismatches allowed for a 20 nt primer)
  - Amplicon Hit- A positive match between an amplicon sequence from an assay and sequences from the NCBI databases @ > **85% identity over 90% of the amplicon** length (15 mismatches allowed for a 100 bp amplicon)

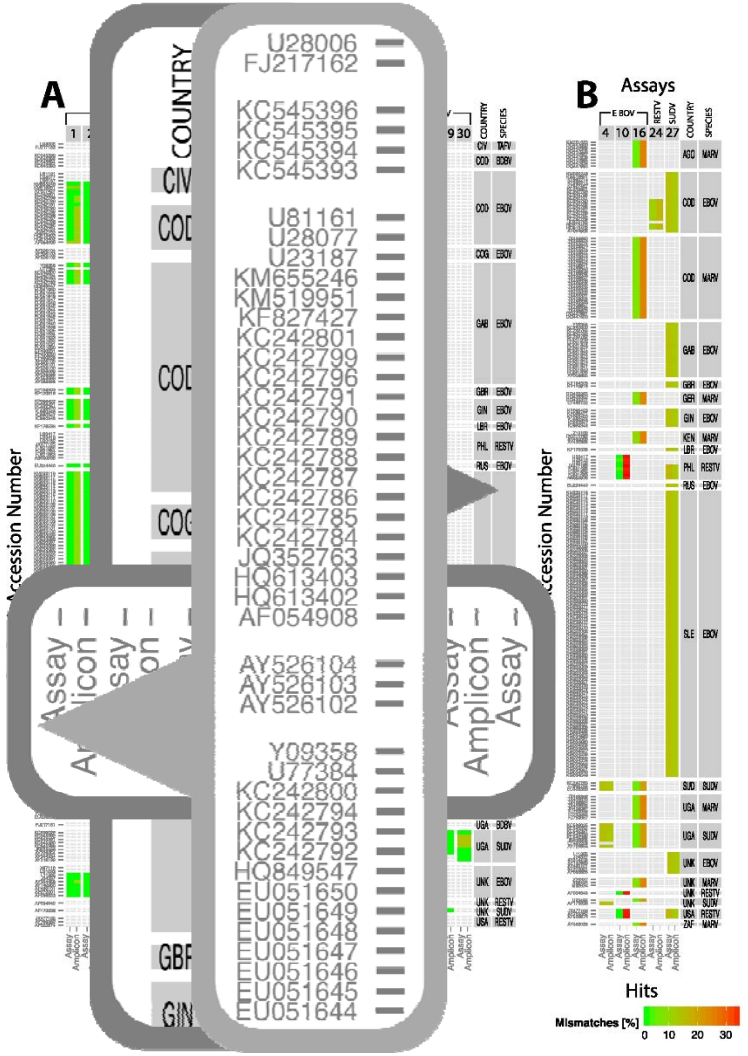
# PSET Results (Ebola Assays)

| Assay # | Assay ID | Intended Target Species | Gene Target | Species/Strain of hits | Amplicon length (bps) | Assay Hit | Perfect Assay Hits | Percentage of perfect | Amplicon hits | Perefect amplicon hit | Percentage of perfect | True-positive | False-negative | False-positive | True-negative | Report* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Sig 1 | EBOV | NP | EBOV | 124 | 135 | 132 | 97.8 | 135 | 11 | 8.1 | 135 | 0 | 0 | 0 | Pass |
| 2 | EbolaZaire-MGB | EBOV | NP | EBOV | 76 | 135 | 134 | 99.3 | 135 | 124 | 91.9 | 135 | 0 | 0 | 0 | Pass |
| 3 | Sig 3 | EBOV | NP | EBOV | 49 | 135 | 135 | 100.0 | 135 | 125 | 92.6 | 135 | 0 | 0 | 0 | Pass |
| 4 | EboZNP | EBOV | NP | EBOV | 80 | 136 | 22 | 16.2 | 136 | 19 | 14.0 | 136 | 0 | 0 | 0 | Pass |
| 4 | EboZNP | EBOV | NP | SUDV | 80 | 0 | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 0 | 11 | |
| 5 | ZAI-NP | EBOV | NP | EBOV | 268 | 148 | 123 | 83.1 | 148 | 10 | 6.8 | 148 | 0 | 0 | 0 | Pass |
| 6 | Ebola MGB-EBOV | EBOV | NP | EBOV | 79 | 148 | 23 | 15.5 | 148 | 11 | 7.4 | 148 | 0 | 0 | 0 | Pass |
| 7 | ENZ | EBOV | NP | EBOV | 70 | 148 | 32 | 21.6 | 148 | 32 | 21.6 | 148 | 0 | 0 | 0 | Pass |
| 10 | EBO-GP-1 | EBOV | GP | EBOV | 579 | 152 | 0 | 0 | 152 | 5 | 3.3 | 152 | 0 | 0 | 0 | Fail |
| 10 | EBO-GP-1 | EBOV | GP | RESTV | 579 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 0 | |
| 12 | ZebovGP | EBOV | GP | EBOV | 64 | 153 | 13 | 8.5 | 153 | 13 | 8.5 | 153 | 0 | 0 | 0 | Pass |
| 13 | Ebola Zaire-TM | EBOV | GP | EBOV | 80 | 144 | 13 | 9.0 | 153 | 13 | 8.5 | 144 | 9 | 0 | 0 | Fail |
| 16 | Filo AB | pan-Filo | L | EBOV | 419 | 135 | 0 | 0 | 135 | 12 | 8.9 | 135 | 0 | 0 | 0 | Pass |
| 16 | Filo AB | pan-Filo | L | MARV | 419 | 55 | 0 | 0 | 0 | 0 | 0 | 55 | 0 | 0 | 0 | |
| 16 | Filo AB | pan-Filo | L | SUDV | 419 | 12 | 0 | 0 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | |
| 17 | GAB-1 | EBOV | L | EBOV | 353 | 135 | 17 | 12.6 | 135 | 12 | 8.9 | 135 | 0 | 0 | 0 | Pass |
| 19 | Ebola BDBV-MGB | BDBV | NP | BDBV | 74 | 5 | 1 | 20.0 | 5 | 1 | 20.0 | 5 | 0 | 0 | 0 | Pass |
| 20 | Ebola BDBV-TM | BDBV | NP | BDBV | 74 | 5 | 1 | 20.0 | 5 | 1 | 20.0 | 5 | 0 | 0 | 0 | Pass |
| 21 | Ebola TAFV-MGB | TAFV | GP | TAFV | 64 | 2 | 1 | 50.0 | 2 | 1 | 50.0 | 2 | 0 | 0 | 0 | Pass |
| 22 | Ebola TAFV-TM | TAFV | GP | TAFV | 79 | 2 | 2 | 100.0 | 2 | 2 | 100.0 | 2 | 0 | 0 | 0 | Pass |
| 23 | Reston | RESTV | NP | RESTV | 337 | 8 | 8 | 100.0 | 8 | 3 | 37.5 | 8 | 0 | 0 | 0 | Pass |
| 24 | Ebola-MGB-RESTV | RESTV | GP | RESTV | 97 | 8 | 2 | 25.0 | 8 | 2 | 25.0 | 8 | 0 | 0 | 0 | Pass |
| 24 | Ebola-MGB-RESTV | RESTV | GP | EBOV | 97 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 9 | |
| 25 | Ebola Reston-TM | RESTV | VP40 | RESTV | 80 | 8 | 2 | 25.0 | 8 | 2 | 25.0 | 8 | 0 | 0 | 0 | Pass |
| 26 | Ebola Reston-MGB | RESTV | GP | RESTV | 55 | 11 | 10 | 90.9 | 12 | 10 | 83.3 | 11 | 1 | 0 | 0 | Fail |
| 27 | Ebola Sudan-MGB | SUDV | NP | SUDV | 80 | 11 | 10 | 90.9 | 11 | 10 | 90.9 | 11 | 0 | 0 | 0 | Pass |
| 27 | Ebola Sudan-MGB | SUDV | NP | RESTV | 80 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 8 | |
| 27 | Ebola Sudan-MGB | SUDV | NP | EBOV | 80 | 0 | 0 | 0 | 148 | 0 | 0 | 0 | 0 | 0 | 148 | |
| 28 | Ebola MGB-SUDV | SUDV | NP | SUDV | 81 | 11 | 7 | 63.6 | 11 | 2 | 18.2 | 11 | 0 | 0 | 0 | Pass |
| 29 | Sudan | SUDV | NP | SUDV | 89 | 11 | 10 | 90.9 | 11 | 10 | 90.9 | 11 | 0 | 0 | 0 | Pass |
| 30 | Ebola Sudan-TM | SUDV | GP | SUDV | 77 | 14 | 9 | 64.3 | 14 | 9 | 64.3 | 14 | 0 | 0 | 0 | Pass |

- **Most of the assays passed with the relaxed criteria (90/90; 85/90 rule) (3 failed-false positive or false negative)**
- **Only 3 passed 100/100 rule**

# Heat map of assay hits to GenBank entries

- Most of the EBOV assays have less than perfect matches to many GenBank entries
- Species specific assays are mostly specific with relaxed criteria
- Cross reactivity is seen with pan assays and specific assays
- Assay positive but amplicon negative hits are due to extensive variation and pan assays
- Amplicon positive but assay negative hits are usually genetic drift



# Summary

- **Assessed the performance of the existing EBOV assays using *in vitro* and *in silico* (PCR Signature Erosion Tool) approach-most assays work despite mismatches between signatures and target**
- **Periodic monitoring of assay performance *in silico* will facilitate better assay designs or improvements**
- **PSET can be a valuable tool to determine whether a wet lab testing of new sequences is needed or not**
- **FDA reevaluation?**

# Publication

*Viruses* **2015**, *7*, 3130-3154; doi:10.3390/v7062763

OPEN ACCESS

*viruses*

ISSN 1999-4915

www.mdpi.com/journal/viruses

*Article*

## Evaluation of Signature Erosion in Ebola Virus Due to Genomic Drift and Its Impact on the Performance of Diagnostic Assays

**Shanmuga Sozhamannan [1,2,*], Mitchell Y. Holland [3], Adrienne T. Hall [4], Daniel A. Negrón [3], Mychal Ivancich [3], Jeffrey W. Koehler [4], Timothy D. Minogue [4], Catherine E. Campbell [5], Walter J. Berger [3], George W. Christopher [6], Bruce G. Goodwin [1] and Michael A. Smith [1]**

# Summary

- Limited wet lab testing using risk mitigated recombinant surrogates, or synthetic constructs, or VLPs for viral agents
- New pathway for assay design using *in silico* approaches
- Well characterized reference materials
- Well defined assays
- Move existing panels of PCR assays to amplicon sequencing based assays with increased genomic content as a first step to “Microseq”
- Eventually move to UHTP sequencing later (pathogen discovery)

# Contact Us

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