MICHIGAN STATE UNIVERSITY

Methods for Detection of Microorganisms False and False (Specificity and sensitivity)

Joan B. Rose

Methods and Microbes

- No method for concentration and detection is 100% efficient
- Microbes are individual particulates not solutes, and are not necessarily evenly distributed in a given media (air,water,soil)
- Concentrate
- Purify
- Separate
- Detect & Quantify (culture, microscopic, indirect)
- Determine viability (CULTURE)
- Determine Hazard (carrying virulence genes)

Issues in Detection

- Sensitivity – Detection Volume and presence of interfering substances that reduce assay sensitivity
- Specificity – is it the right organism or group of organisms
- Quantification – precision
- TWO ways to test new approaches
  - Compare to golden standard
  - Seeded recovery methods (ratio of recovered concentration to a known concentration added to the matrix)

Methods and Microbes

- False positive: A test result that indicates a positive status when the true status is negative
- False negative: A test result that indicates a negative status when the true status is positive
- A variety of probability estimates can be computed from the information organized in a two-way table given in Table 1.
- *Sensitivity*: Probability of a positive test result (or presence of the symptom) given the presence of the disease
  - *Specificity*: Probability of a negative test result (or absence of the symptom) given the absence of the disease
  -
- *Predictive value positive*: Probability that a subject has the disease given that a subject has a positive screaming test result (or has the symptom)
  -
- *Predictive value negative*: Probability that a subject does not have the disease, given that the subject has a negative screening test result (or does not have the symptom)

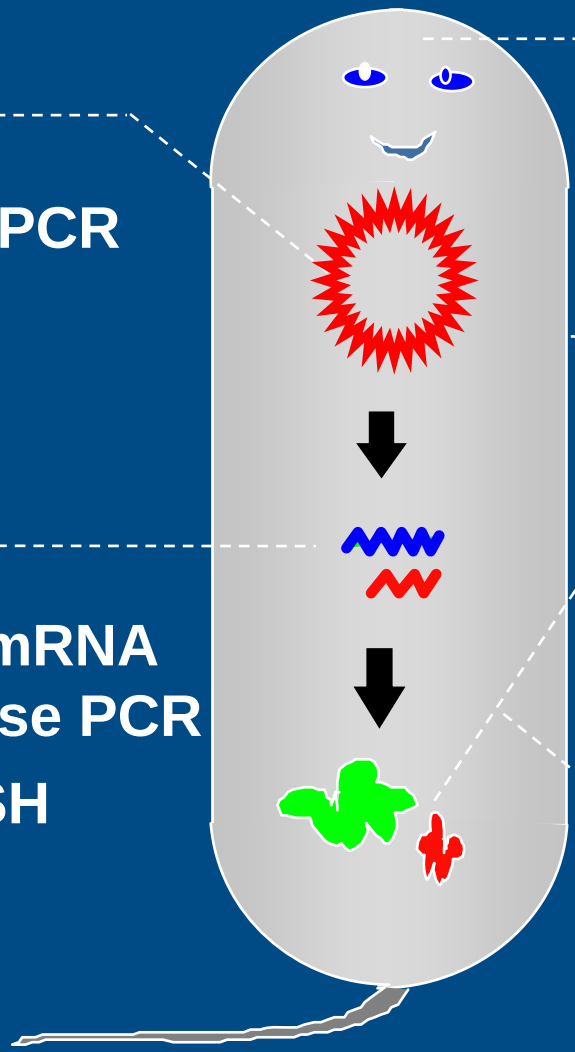
Cultural/Standard Methods for the Detection of Pathogens in the Environment

- Virus
  - Concentration/separation
  - Cell culture (one cell line does not detect all)
  - Serology (identification)
- Bacteria
  - Concentration/separation
  - Enrichment media
  - Selective media
  - Biochemical tests, serology, immunochemical

Methods for the Detection of Pathogens

- Protozoa
  - Concentration or elution
  - Purification
    - Differential centrifugation
    - Immune Magnetic Separation
  - Stain with monoclonal antibodies
  - Observe under UV light
  - Examine for characteristics
    - Shape and size
    - Internal structures

Detection Targets



DNA

PCR, Real Time PCR

RNA

16S rRNA probes, mRNA

Reverse transcriptase PCR

Flow cytometry, FISH

Morphology

Microscopy/
Image analysis

Cell wall

Antibody

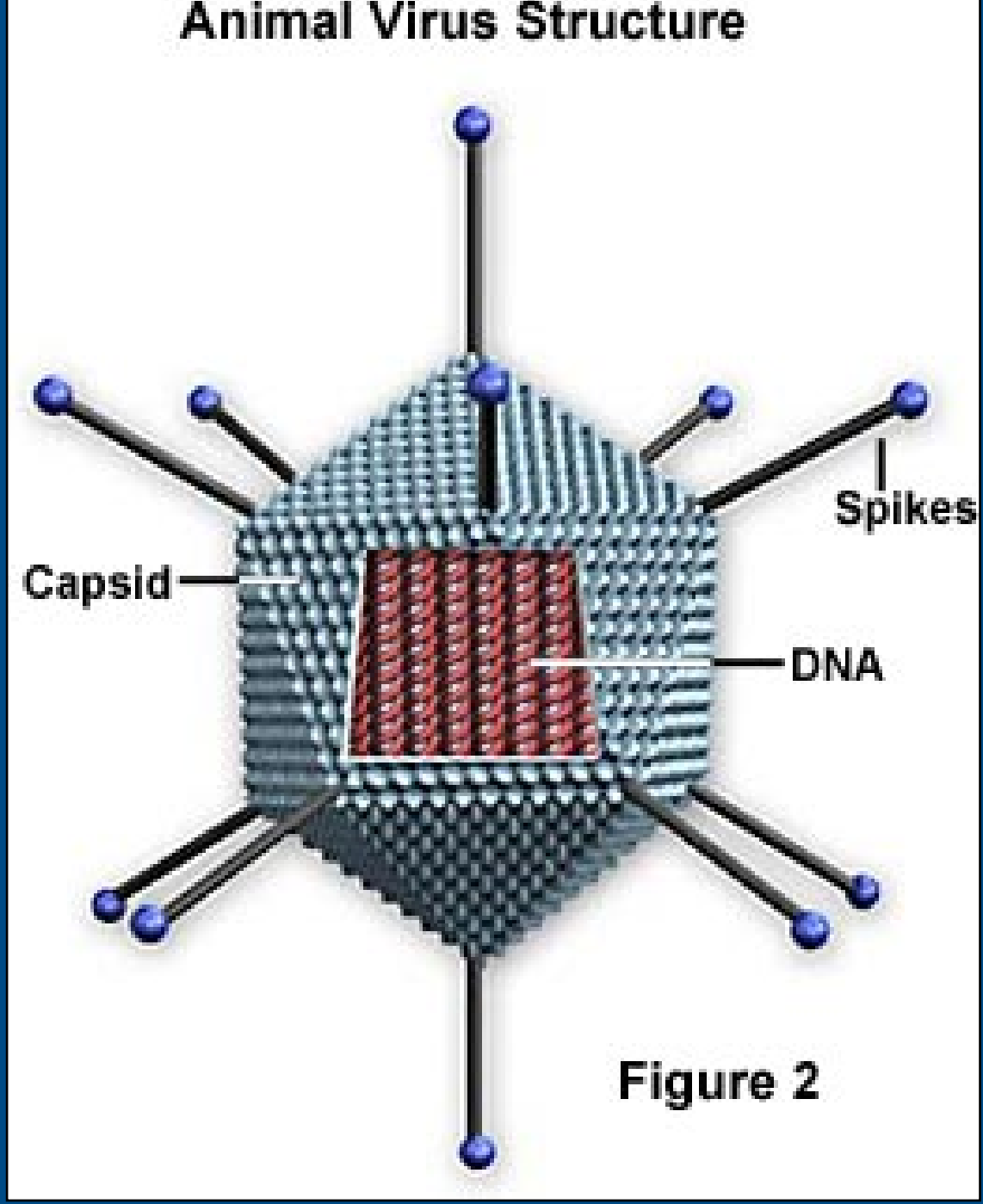
Protein

MALDI-TOF

Function

Activity assay,
Biolog

The cell: Isolation and culture techniques


Animal Virus Structure
Spikes
Capsid
DNA
Figure 2

Typical Sample Volumes for Water

- Bacteria
  - 100 ml
- Viruses
  - Raw Sewage 1-5 liters
  - Treated sewage 40 liters
  - Surface waters 400 liters
  - Drinking water 400 to 2000 liters
- Protozoa
  - Treated sewage 4 liters
  - Surface waters 10 to 100 liters
  - Drinking water 10 to 100 liters

Time and Cost for Assay of Enteric Pathogens in Water

- Bacteria
  - 2 to 5 days; $40 to $200
- Virus
  - 14 to 60 days; $500 to $1,200
- Protozoa
  - 2 to 3 days; $250 to $450

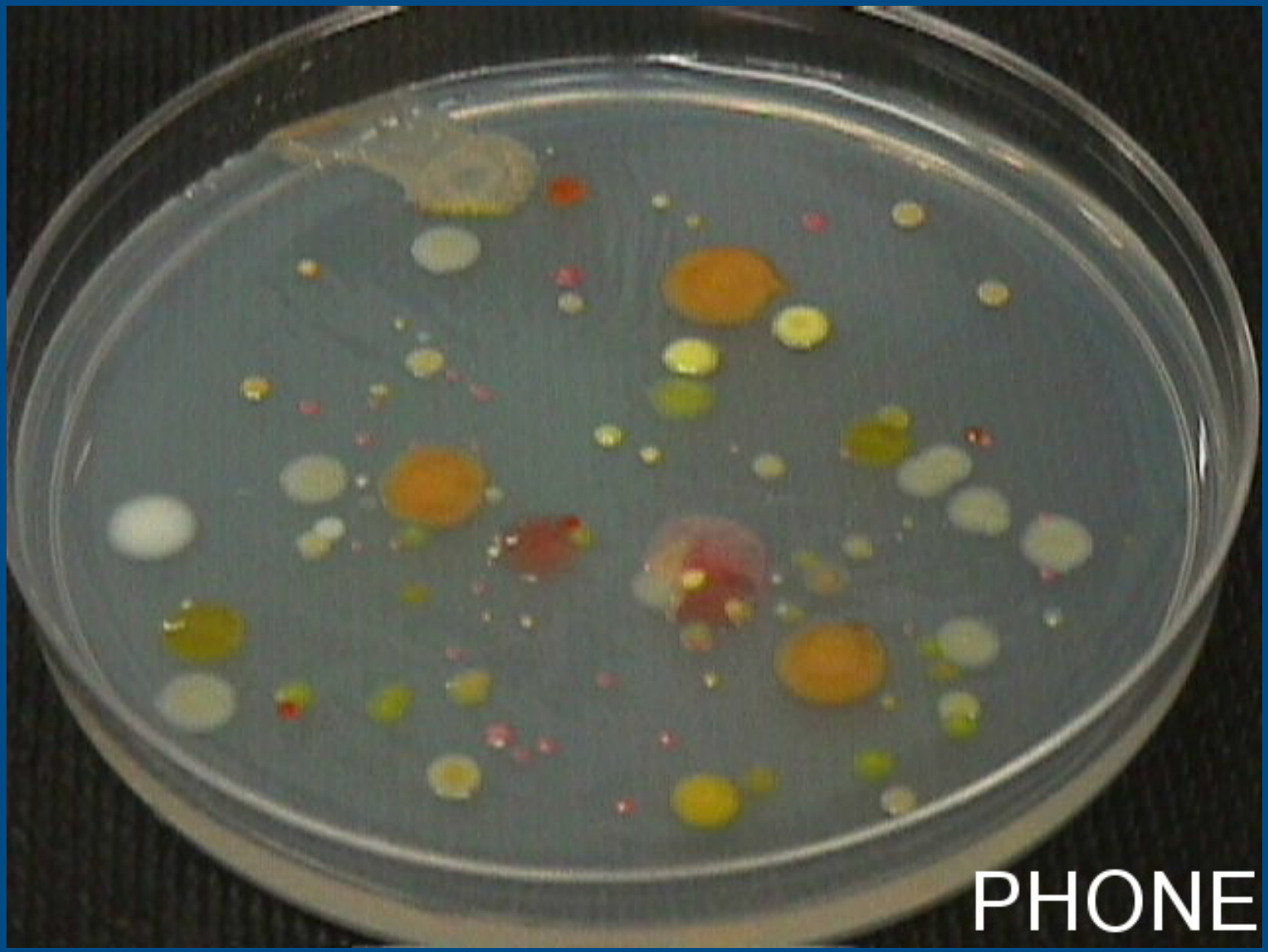
Enumeration of Microbes

- Quantitative measurements
- Quantal measurements (presence/absence)

- CFU Colony Forming Unit

- PFU Plaque Forming Unit

- MPN Most Probable Number (statistical method to estimate concentrations with multiple assays at dilutions to extinction [0])


PHONE

Steps Involved in the Detection of Waterborne Enteric Viruses and Parasites

Sample Collection

↓

Concentration

↓

Purification

↓

Replication

↓

Identification

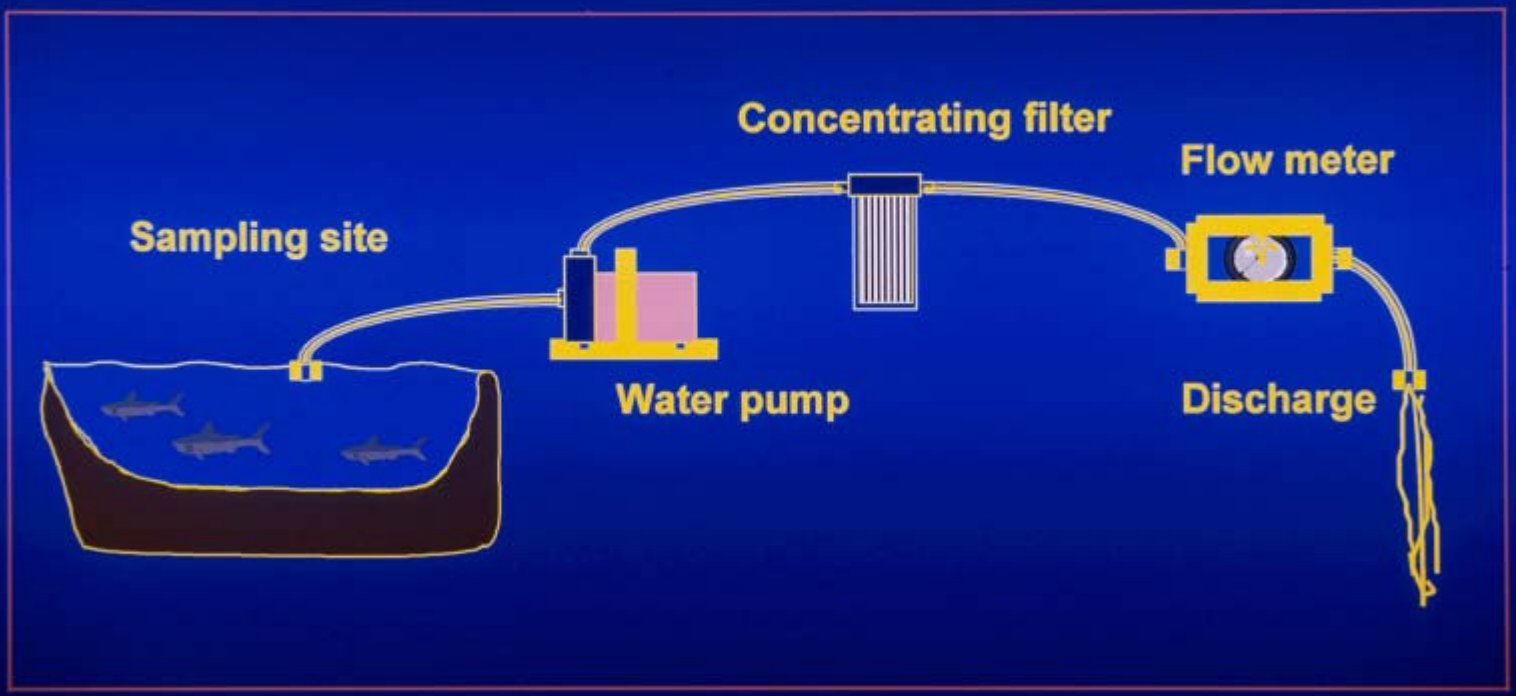
↓

Quantification

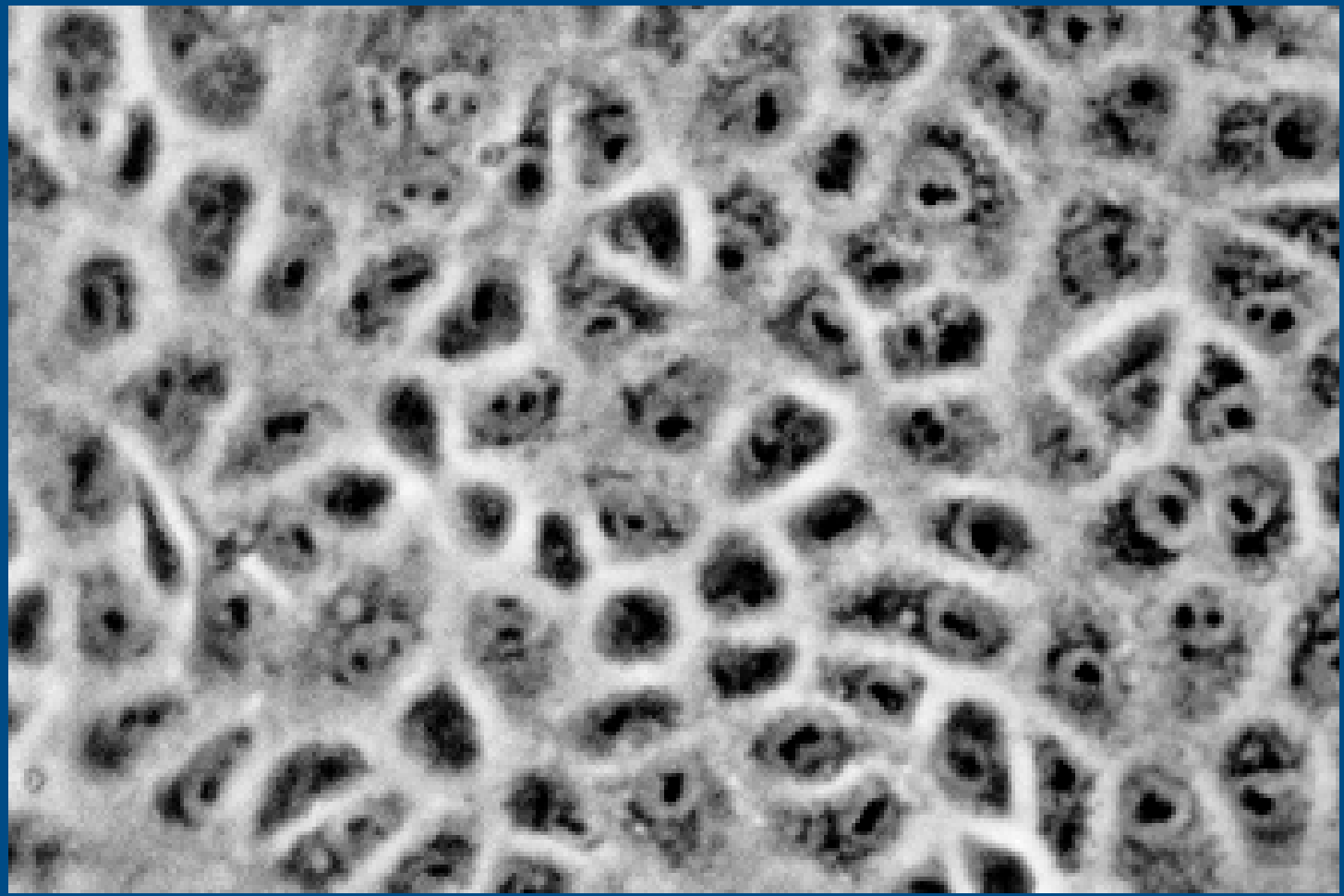
↓

Isolation and/ or Characterization

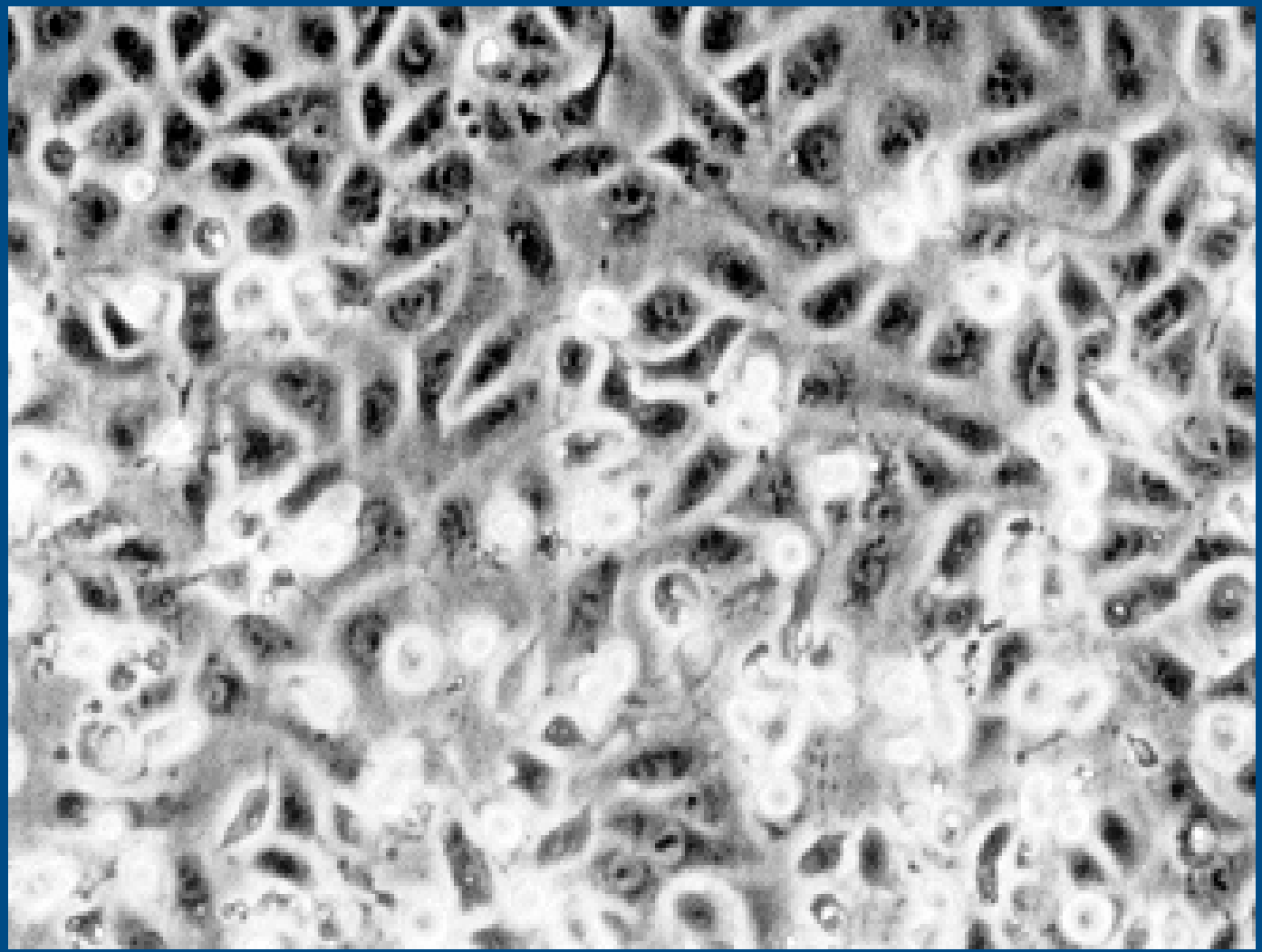
Concentration of viruses from water

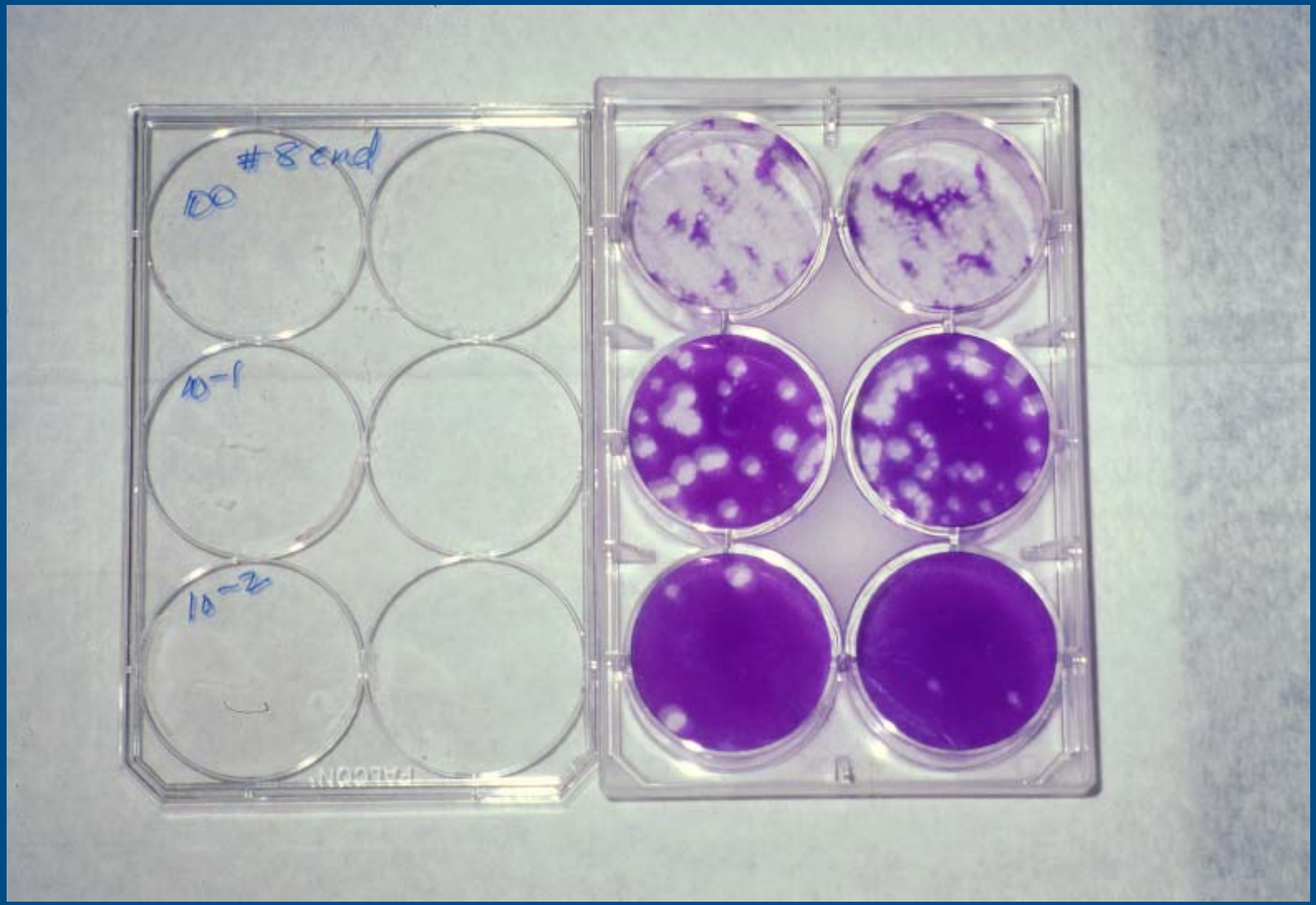


Noninfected monkey kidney cells



Monkey cells infected with poliovirus




#8 end
10-1
10-2

Principles of Protozoan Detection in the Environment

- Filtration of large volumes of water
- Concentration
- Purification
- Method of detection coupled with an ability to quantify the desired microorganisms

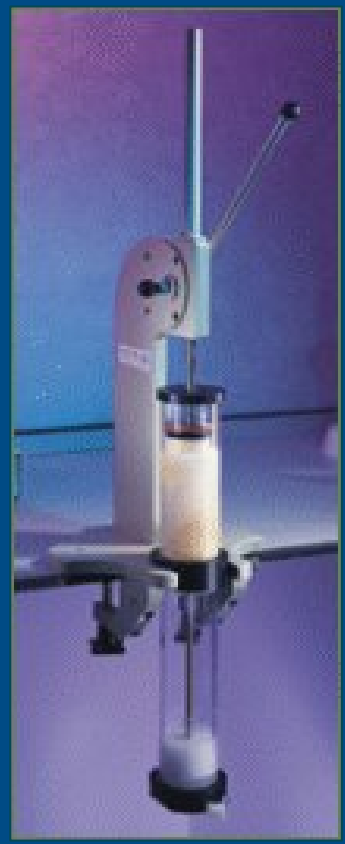
Parasite Concentration

- Pall Gelman Envirocheck Sampling Capsule
- Used for US EPA method 1622
  - Protozoa detection: *Cryptosporidium* and *Giardia*

Envirocheck Sampling Capsule

Methodology (filtration/concentration with Filta-Max)

- After a volume of water is passed through a filter cartridge, the eluted solution is then concentrated



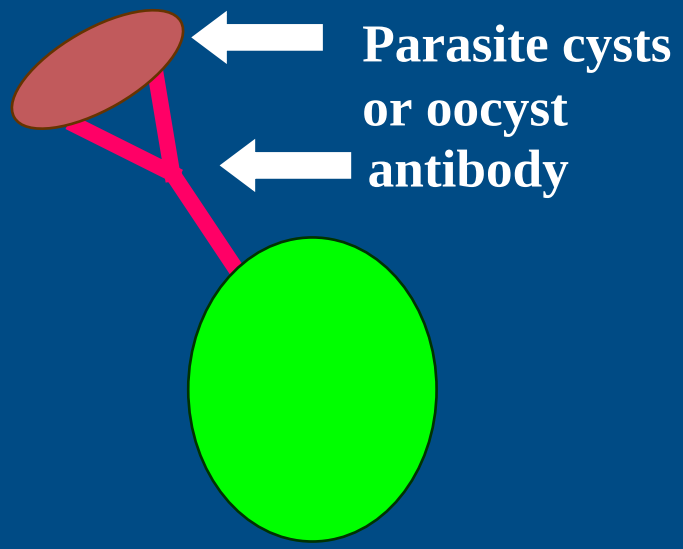
Filta-Max™

Methodology

- The concentrate is then purified
- This is accomplished by immunomagnetic separa (IMS)

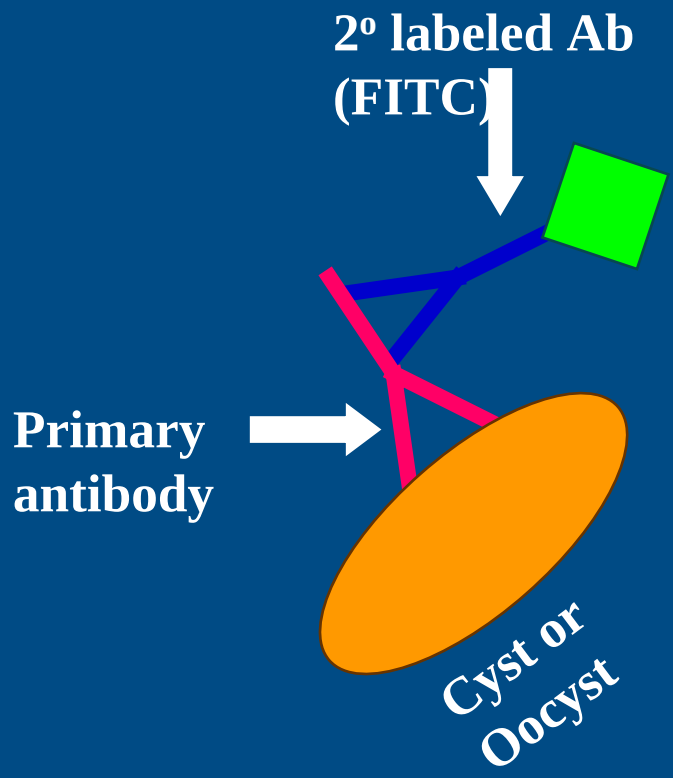
Puri-Max™

Purification by IMS



Detection by IF

- Detection method is based on an indirect immunofluorescent antibody (IFA) stain



Examination

Read slide under UV epifluorescent microscope

Presumptive

Cryptosporidium
4-6 mm

Giardia
8-12 mm

Confirm

using Differential Interference Contrast

see up to four sporozoites

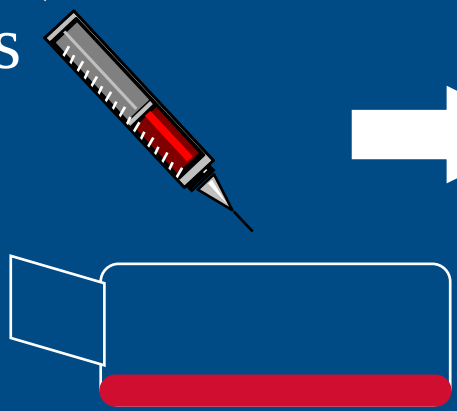
see nuclei, axoneme & median bodies

Cell Culture-*Cryptosporidium*

Take purified sample expose to 10% bleach for 10 min to inactivate viruses and bacteria, algae, fungi

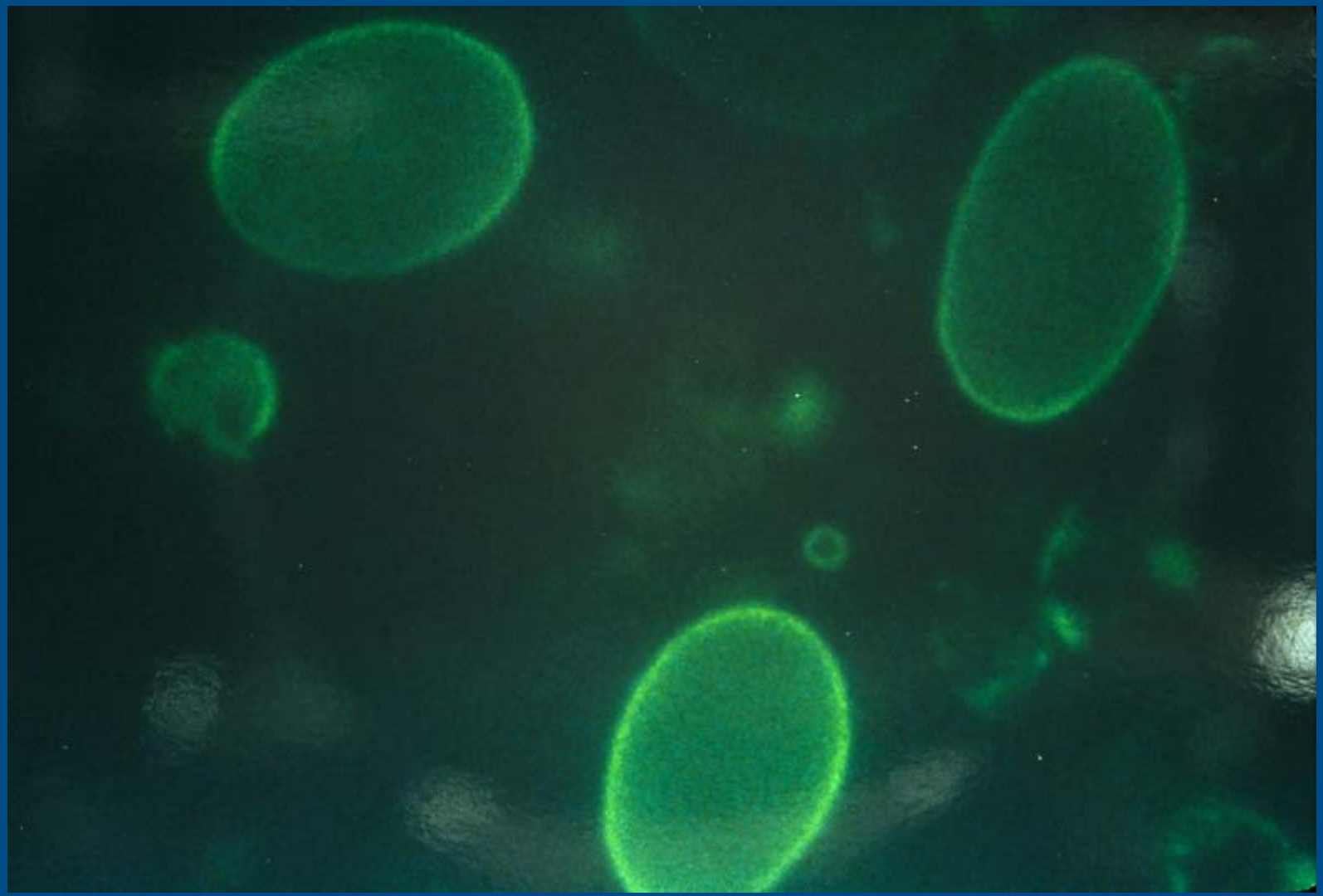
Wash and perform excystation

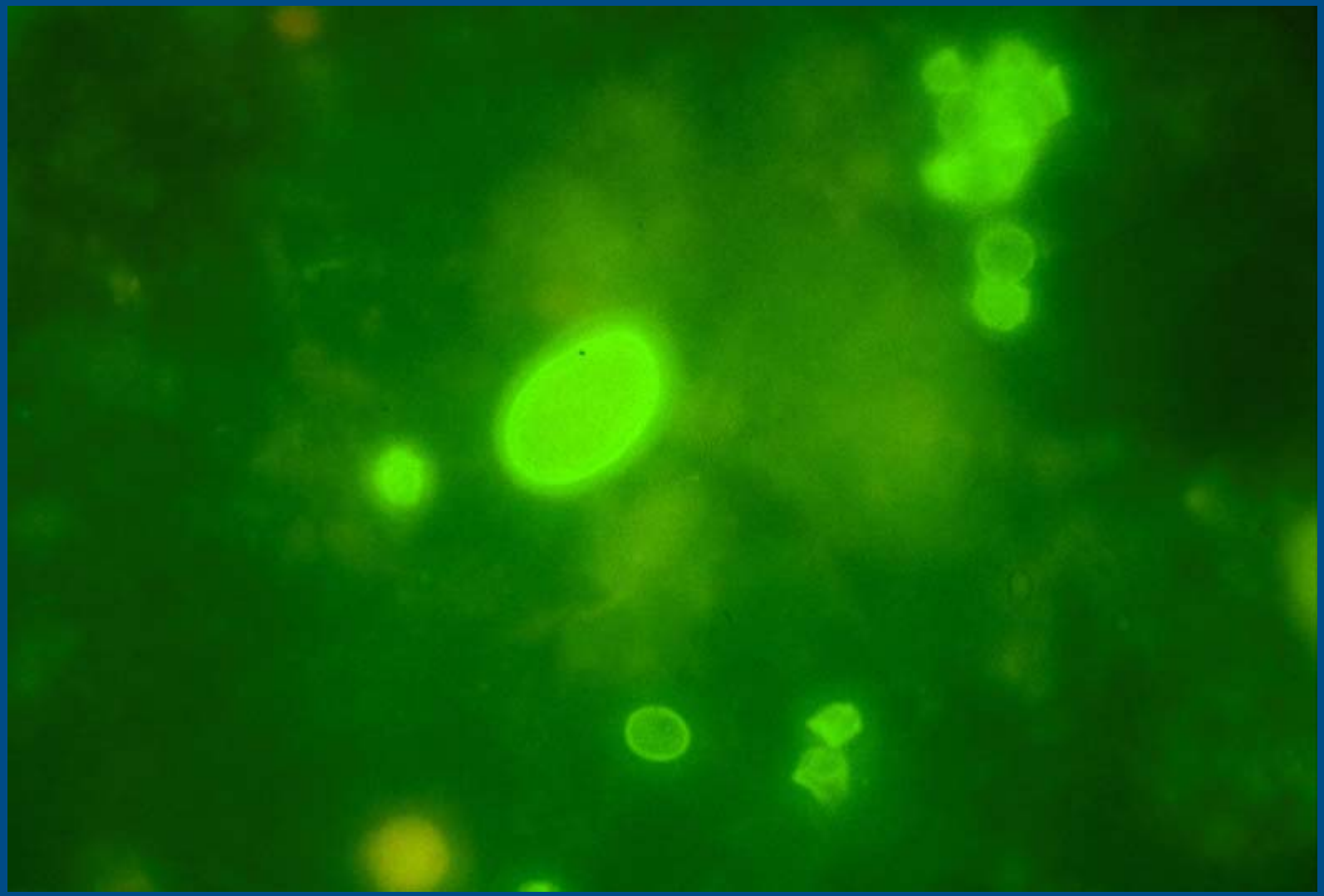
Inoculate sample onto HCT-8 cells



Examine cells for evidence of infection and growth of *Cryptosporidium* life stages

- microscope (bright field/IFA)
- ELISA
- PCR





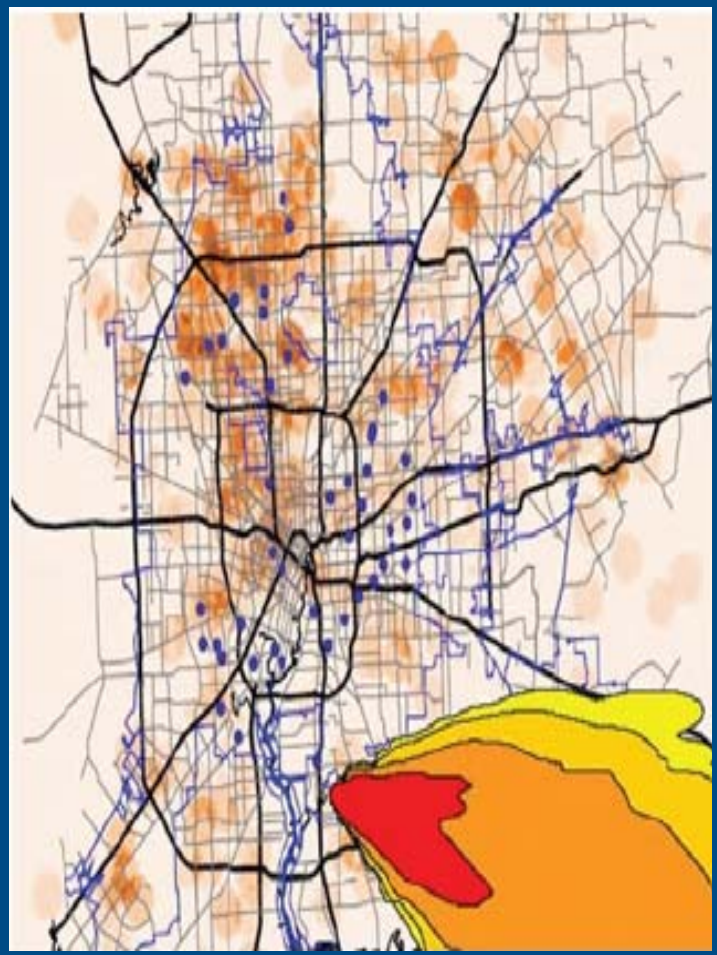
Aerosol Sampling

- Impingers
- Impact- Anderson Aerosol
- Filters
- High volume fluid samplers
- Electrostatic participators
- Settling plates

Aerosol Sampling

- Efficacy affected by
  - Collection media
  - Relative humidity
  - Desiccation

BioWatch Program



Background

- Rising concerns
  - Terrorists could aerosolize biological agents in the air
  - Potentially causing thousands of causalities
  - Without early detection, the magnitude of the problem might only be revealed as people arrived at hospitals with symptoms
- Early-warning system
  - Department of Homeland Security
  - Tested at the winter Olympic, Salt Lake City in 2002
  - BioWatcat Program in 2003

Agencies & Responsibilities

- Department of Homeland Security (DHS)
- Environmental Protection Agency (EPA)
- Center for Disease Control and Prevention (CDC)
- Department of Energy (DOE)
- Department of Defense (DOD)

DHS
Funding,
Oversight,
Coordination

DOE and DOD
Site selection,
Sampler unit,
Tech support

EPA
Field coordination,
Site maintenance,
Sampling operation

CDC
Sample analysis &
data management,
Consequence management
strategy

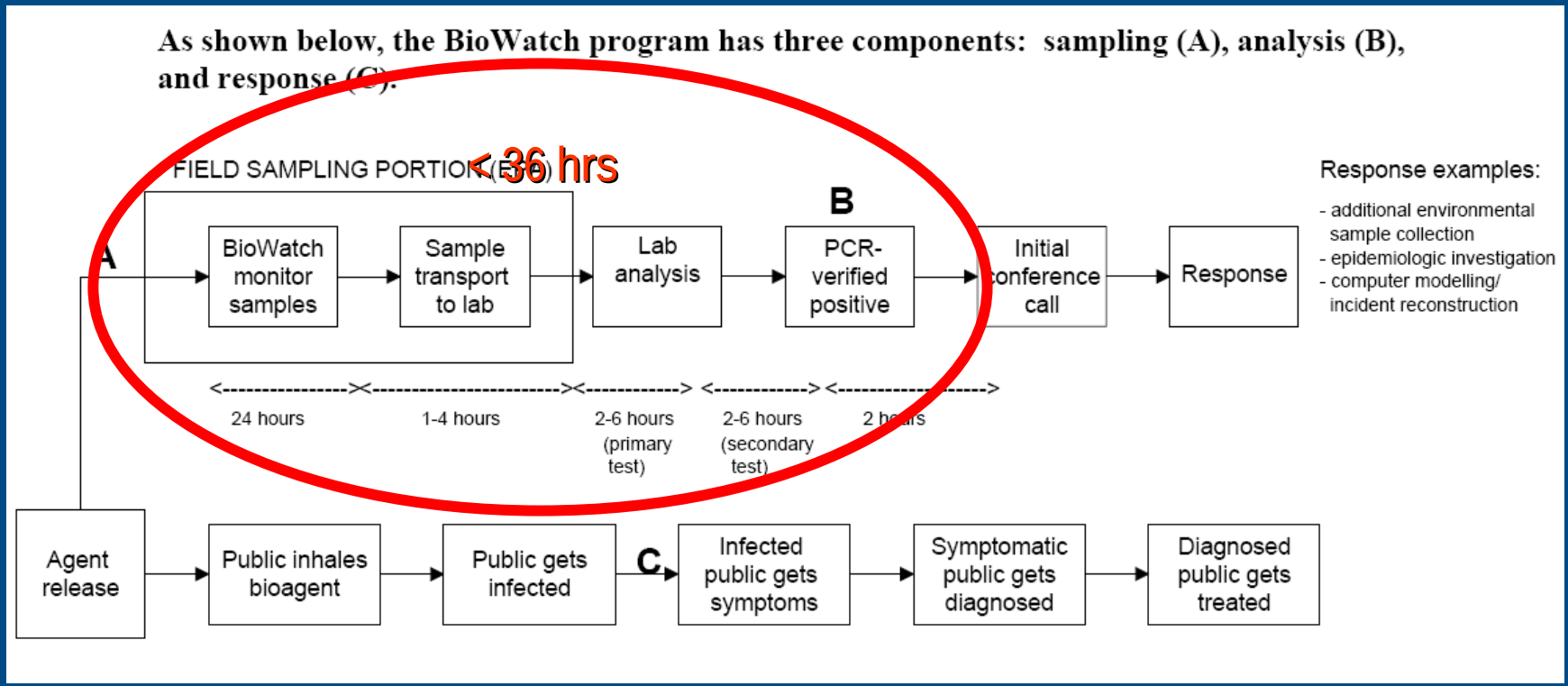
Sampling

- Outdoor
  - Pre-existing EPA air quality station
  - Manually collect samples 24-hr intervals, 7 days/week, 365 days/year
    - Potable sampling unit (PSU)
    - Dry filter unit (DFU)
    - Distributed sampling unit
  - Deliver samples daily to CDC-operated facilities
- Indoor (enhancing & developing the protocols)
  - Suggested methods
    - HEPA socks?
    - Swabs for areas where water pools?
    - Swipes for surfaces, e.g. computer screens, air intake vents?

Analysis & Responses

- CDC-operated BioWatch facility
  - Federal, State, or local public health laboratories
  - Primary analysis
    - Polymerase chain reaction (PCR)
    - If a biological agent is detected, perform 2ndary test
  - Secondary analysis
    - PCR-verified positive
      - existence of a biological material in the air due to either an intentional or a natural
- Notification by local level with health authorities
- Low enforcement by Federal Bureau Investigation (FBI)

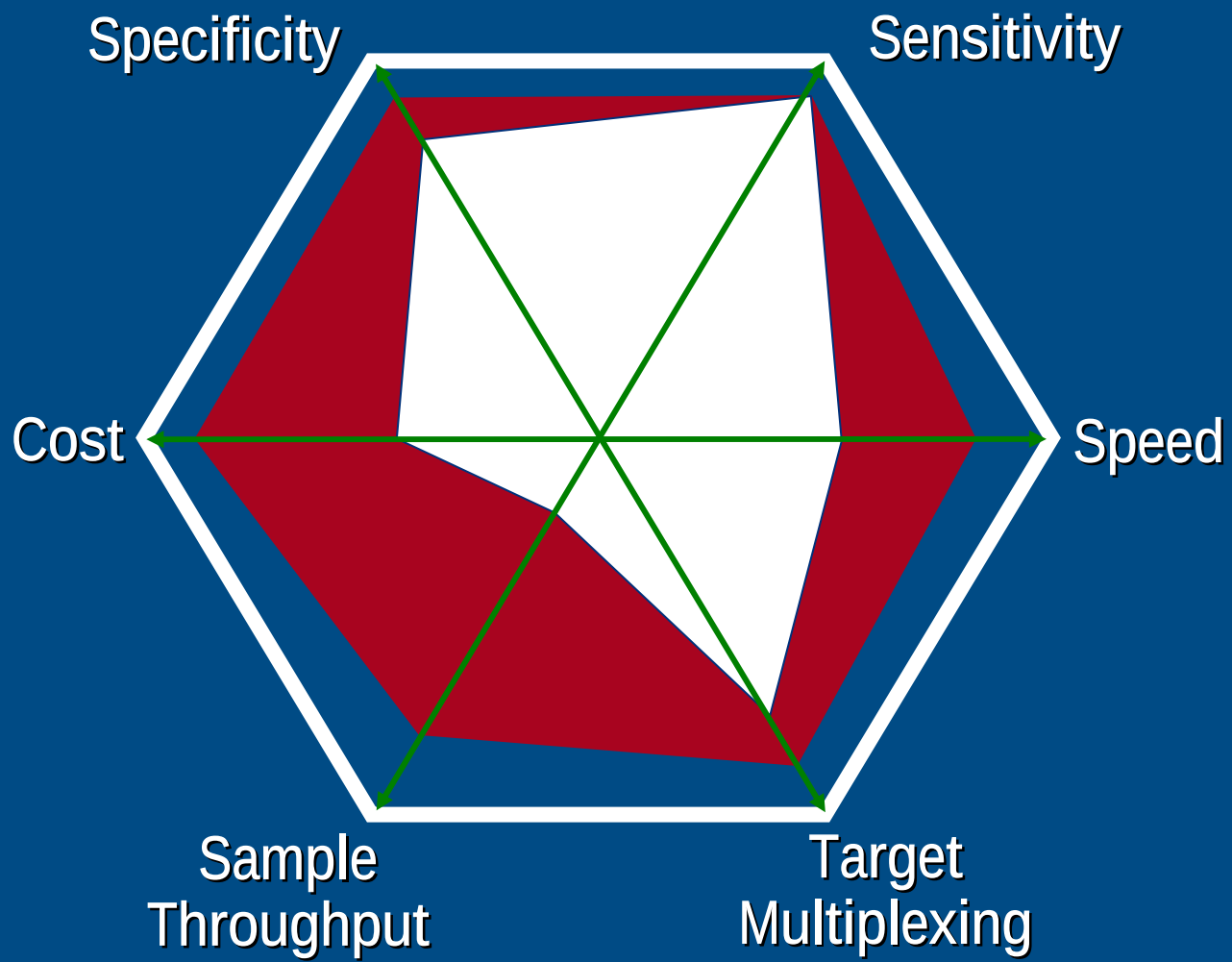
BioWatch Program Model



Evaluations of the Program

- EPA concerns
  - Quality assurance and quality control (QAQC)
  - Protocols and response systems

Detection Goals



Three Dimensions of Detection

Viruses

Rotavirus
Hepatitis A
Norwalk virus

Protozoa

Cryptosporidium
Giardia

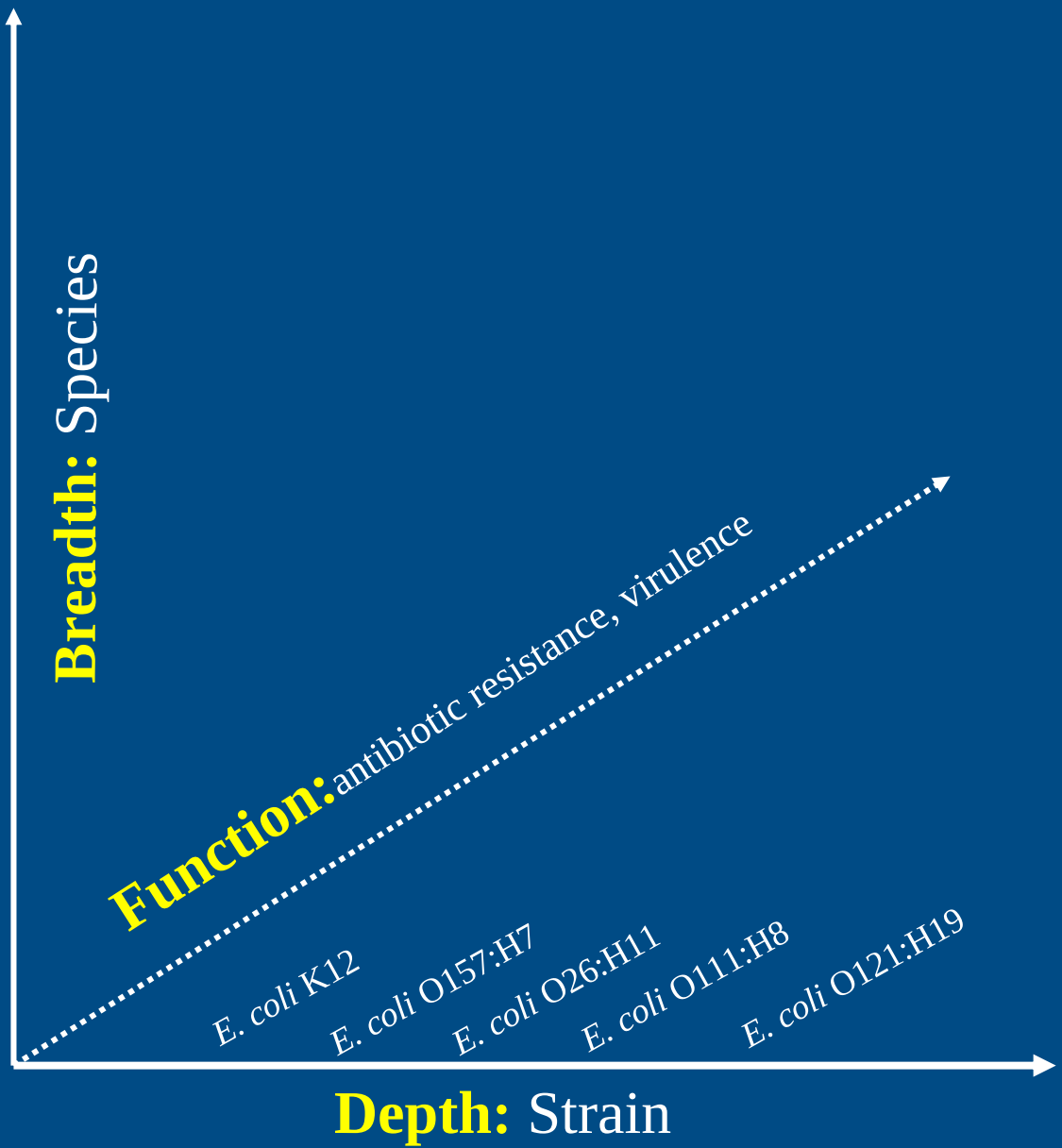
EPA: Indicator organisms

E. coli
Fecal enterococci
Bacteroides spp.
Bifidobacteirum spp.
Clostridium spp.
Eubacterium spp.
Background Organism (List of phgylogentic groups)

CDC Category B- Water

Yersinia enterocolitica
Campylobacter
Listeria monocytogenes
Salmonella (Newport and DT104)
Pathogenic vibrios

Shigella
Diarrheagenic *E. coli*



Polymerase Chain Reaction (PCR)

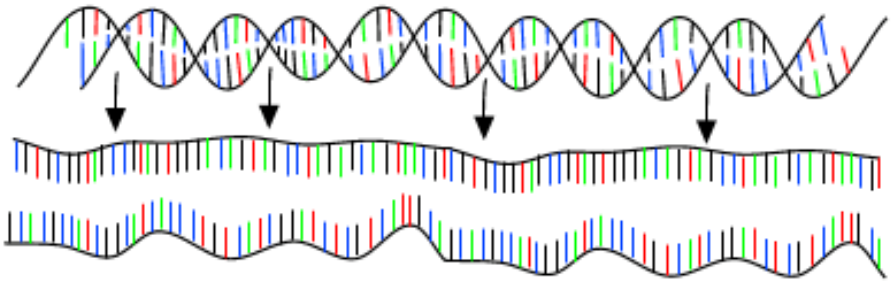
- Developed in 1985 by Kary Mullis
- Dr. Mullis received the Nobel Prize in Chemistry in 1993
- PCR is considered as one of the most important discoveries in molecular biology

What is PCR?

- Enzymatic reaction that makes many copies of DNA from single molecule
- 2^n copies of DNA from single molecule where n = No. of cycles
- So, 35 cycles of PCR would yield 2^{35} copies of DNA

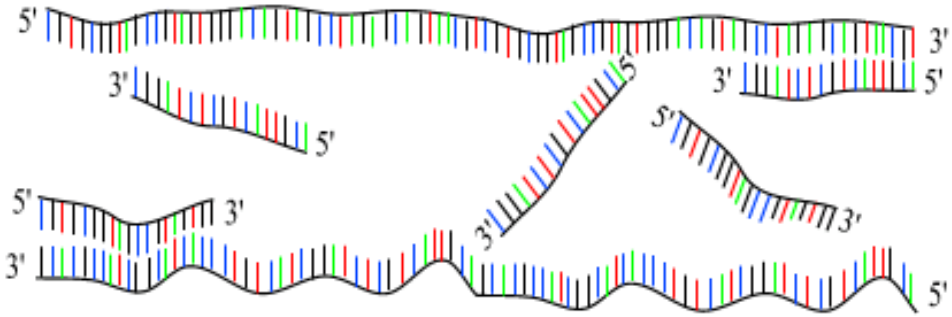
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

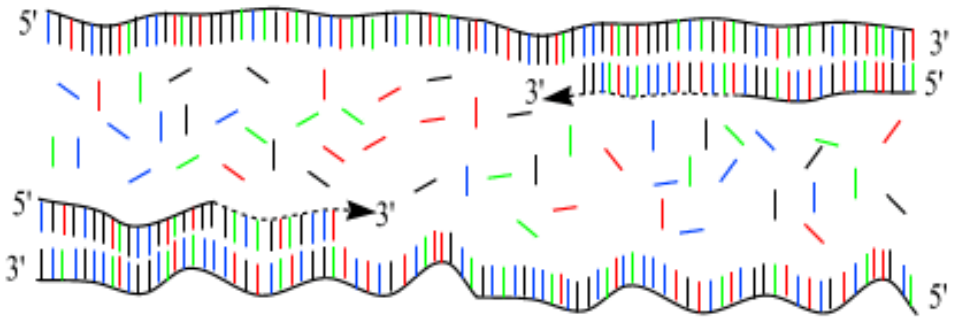
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

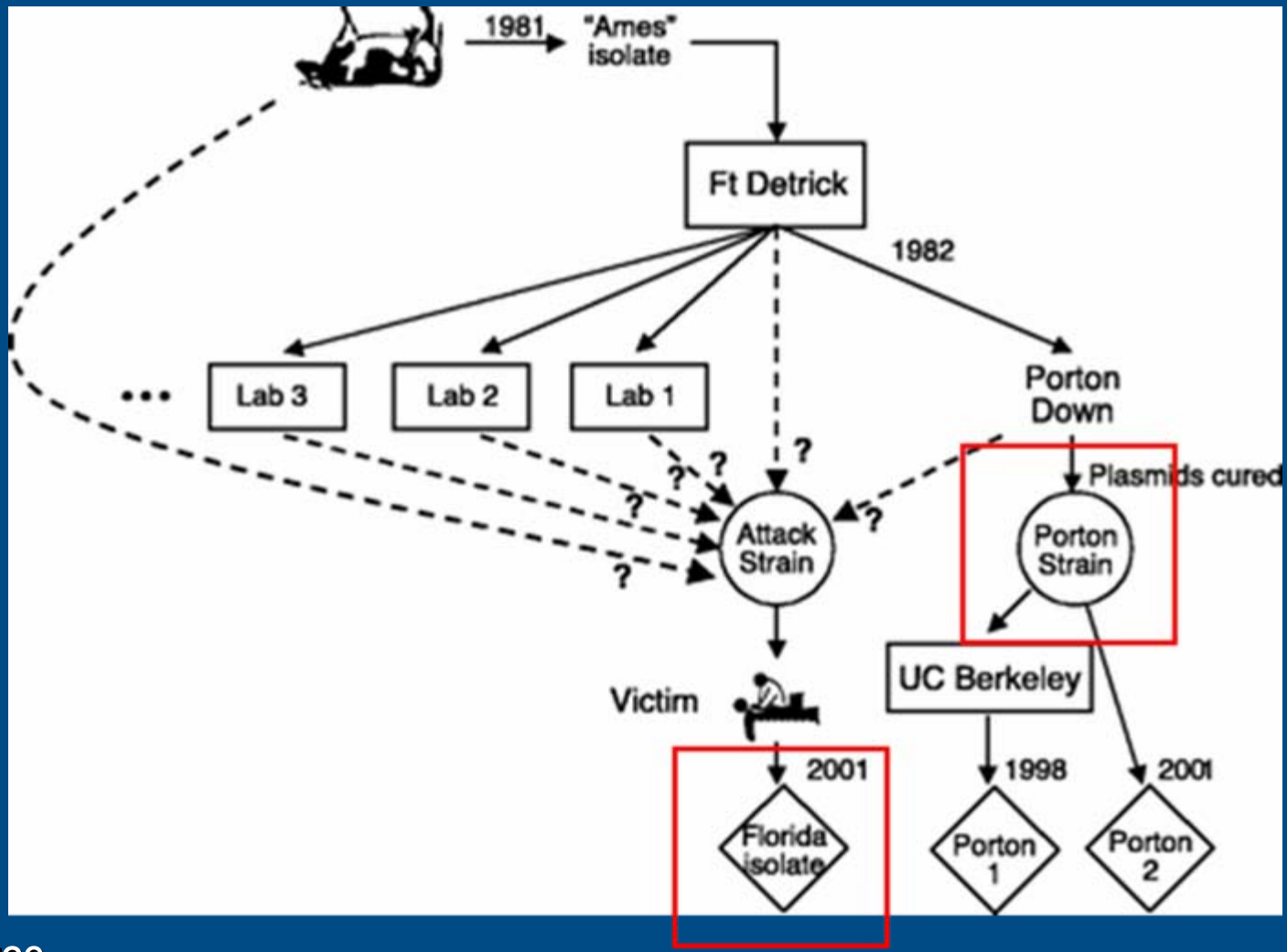
2 minutes 72 °C

only dNTP's

(Andy Vierstraete 1999)

Applications

- Allows for amplification and identification of specific gene sequences from specific organisms (i.e. bacteria, parasites, viruses, etc.) that would otherwise be undetectable
- Provides information for HAZ ID
- Provides information for sources (HAZ)
- Used for Forensic studies (Bioterrorism)
- Used for molecular epidemiology (outbreak investigation)



Source

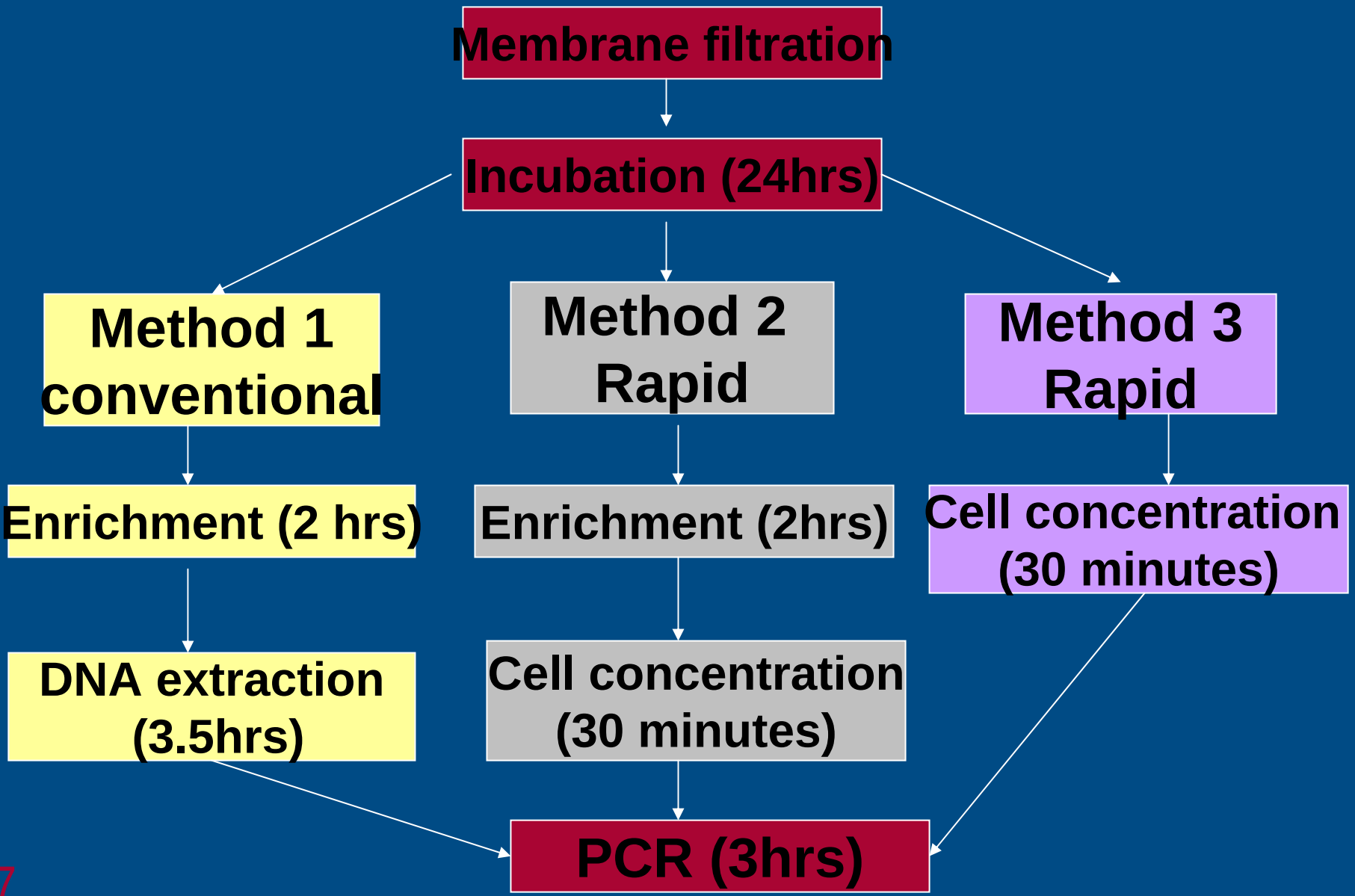
Timothy D. Read *et al*. Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. *Science* 296:2028-2033 (2002).

Esp marker

- Host specific genetic marker

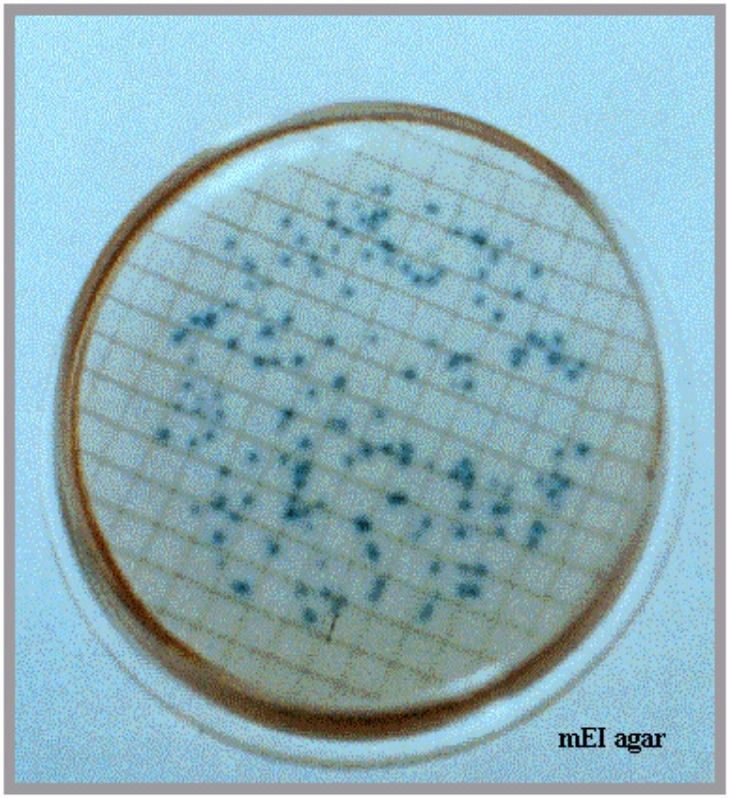
  Differentiate Human/non human pollution
- Present in Indicator bacteria (Enterococci)
- Traditional MF method and PCR used to detect the gene
- A culture dependent library independent method

Methods


Membrane filtration
Incubation (24hrs)
Method 1 conventional
Method 2 Rapid
Method 3 Rapid
Enrichment (2 hrs)
Enrichment (2hrs)
Cell concentration (30 minutes)
DNA extraction (3.5hrs)
Cell concentration (30 minutes)
PCR (3hrs)

Membrane Filtration (MF)

Enterococci (After Incubation)



Colonies that have a blue halo are counted as enterococci.

Enrichment

Polymerase Chain Reaction (PCR)

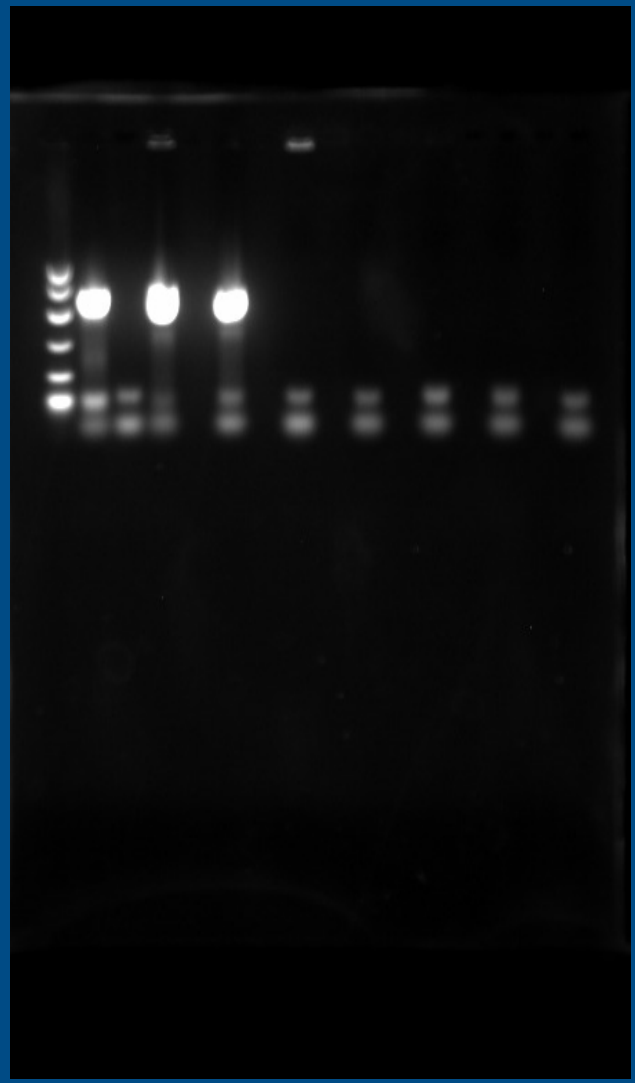
- Enzymatic reaction that makes many copies of DNA from single molecule

- Forward primer, which is specific for the *E. faecium esp* gene is: (5'-TAT GAA AGC AAC AGC ACA AGT T-3')
- Conserved reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3')

Scott *et al.,* 2005. Env. Sci. Technol., 39: 283-287

Gel loading

Esp detection after PCR



qReal Time PCR: What can it do for my project?

Real time PCR : in situ detection

Detect PCR product fluorescently in each well of PCR plate.

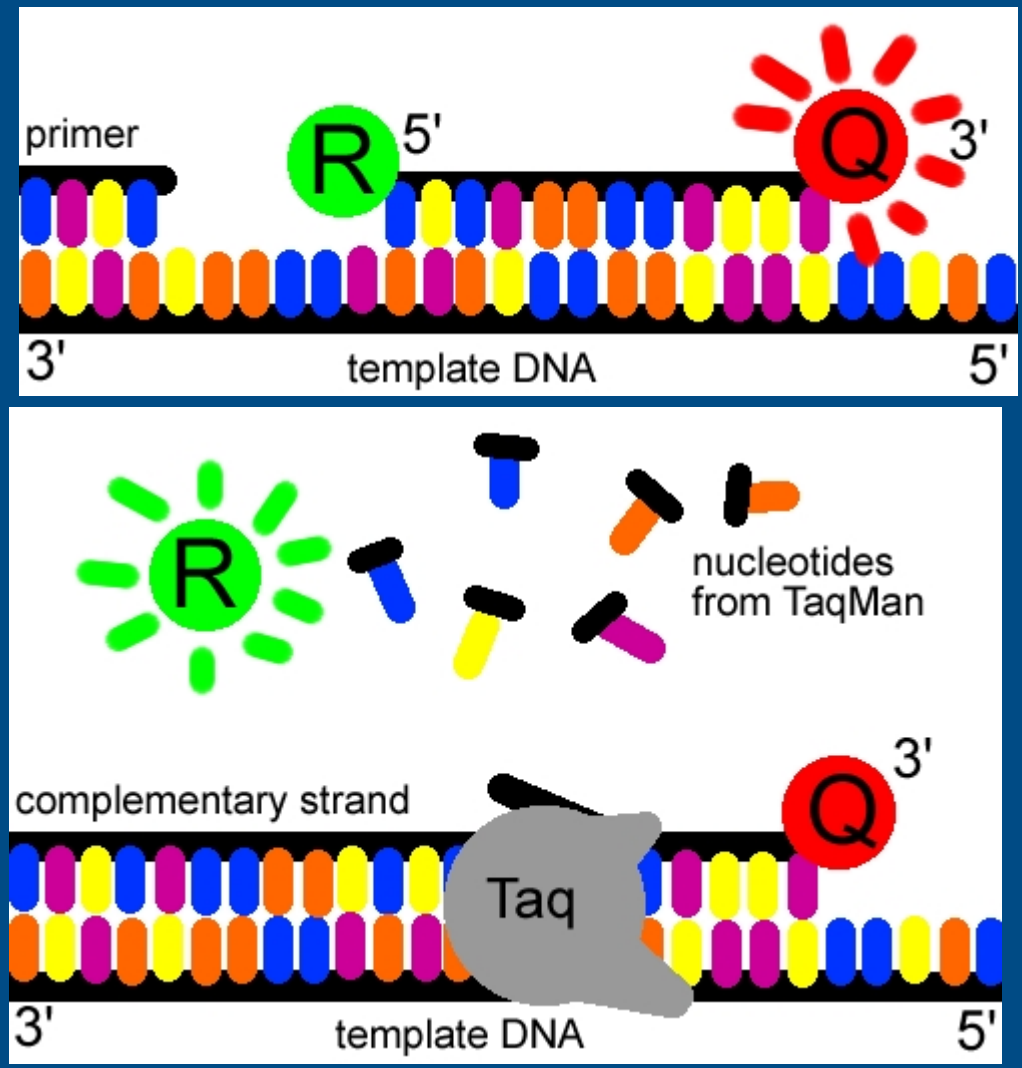
Fast PCR screening without gels.

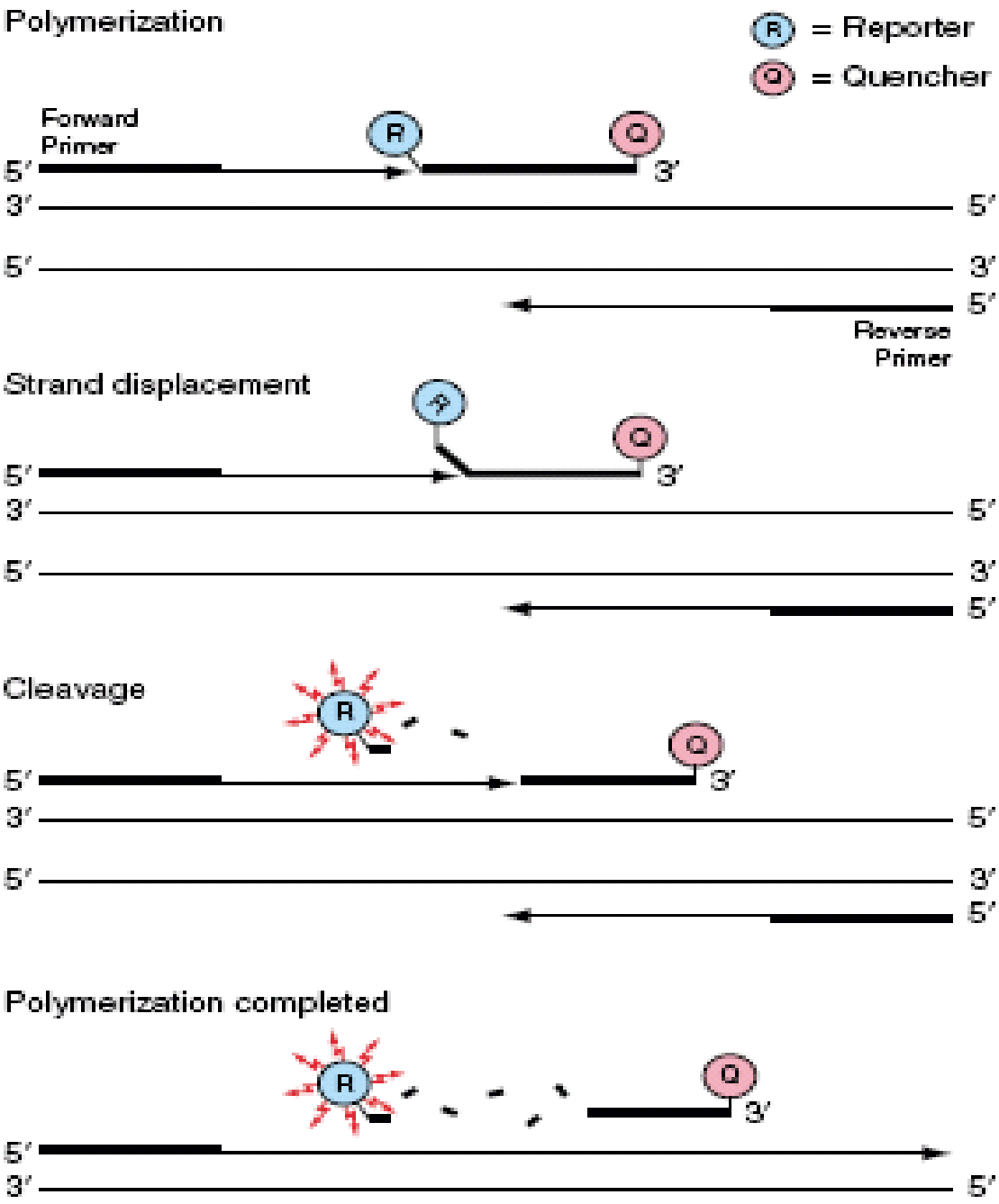
Quantify amount of PCR product at each cycle.

Detect presence or quantify fraction of sample made up by particular species using species specific primers.

Use threshold detection for relative abundance.

REAL TIME PCR using Taqman probe method




Polymerization
R = Reporter
Q = Quencher
Forward Primer
R
Q
5′
3′
3′
5′
5′
3′
5′
Reverse Primer
Strand displacement
R
Q
5′
3′
3′
5′
5′
3′
5′
Cleavage
R
Q
5′
3′
3′
5′
5′
3′
5′
Polymerization completed
R
Q
5′
3′
3′
5′

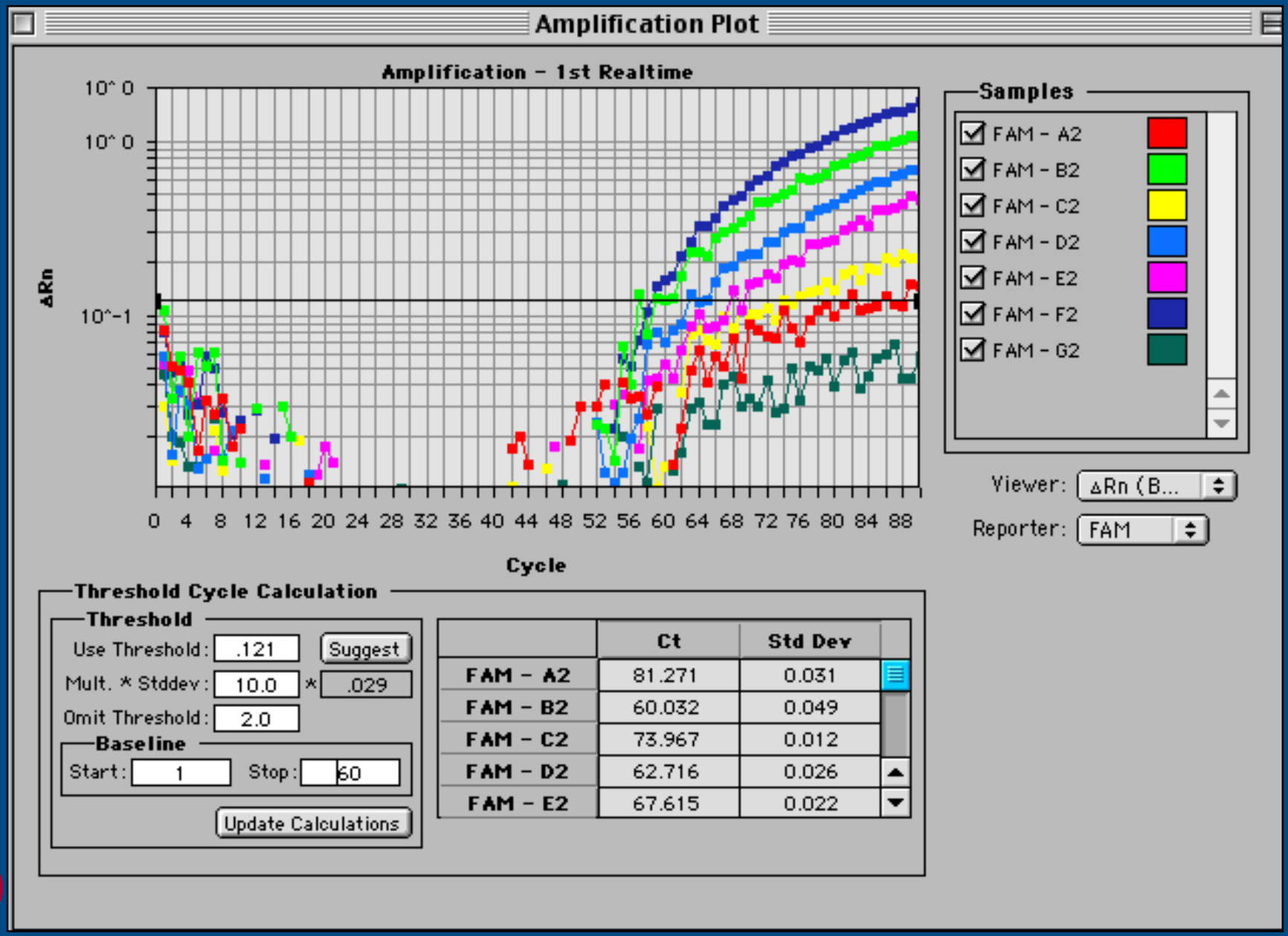
Well-designed TaqMan probes require very little optimization.

In addition, they can be used for multiplex assays by designing each probe with a spectrally unique fluor/quench pair.

However, TaqMan probes can be expensive to synthesize, with a separate probe needed for each DNA target being analyzed.

Each probe is ~$ 400.00

Real-time QPCR using molecular beacon



Factors Important in Developing Detection Tools

Factors to Consider in Developing Parallel Detection Tools for Microorganisms

TARGET SELECTION

- Bacteria
  - *Bacillus anthracis*
  - *E. coli* O157:H7
  - Etc.
- Protozoa
  - *Cryptosporidium*
  - *Giardia*
  - Etc.
- Viruses
  - Norwalk virus
  - SARS
  - Etc.

PROBE DESIGN

- Gene Target
  - Small subunit rRNA
  - Functional genes
  - Intragenic spacer region
  - Virulence factors
- Specificity
  - Probe size
  - Perfect match/mismatch strategy
- Resolution
  - Genus & species level
  - Strain fingerprinting

MICROARRAY PLATFORM

- Glass slide arrays
  - In-house printed arrays
  - Commercial e.g., Agilent
- In situ synthesized arrays
  - e.g., Affymetrix
  - NimbleGen
  - Xeotron
- Gel-pad arrays
- Diagnostic arrays
  - e.g., Motorola's eSensor
  - Lab-on-a-chip systems

SAMPLE PROCESSING

- Matrices
  - Air, water, food
  - Body fluid, tissue, soil
  - Others
- Sample Concentration
  - Micro- & Ultrafiltration
  - Centrifugation, others
- Target Enrichment & Amplification
  - Bead-based separation
  - Polymerase chain reaction
  - Biological amplification on media

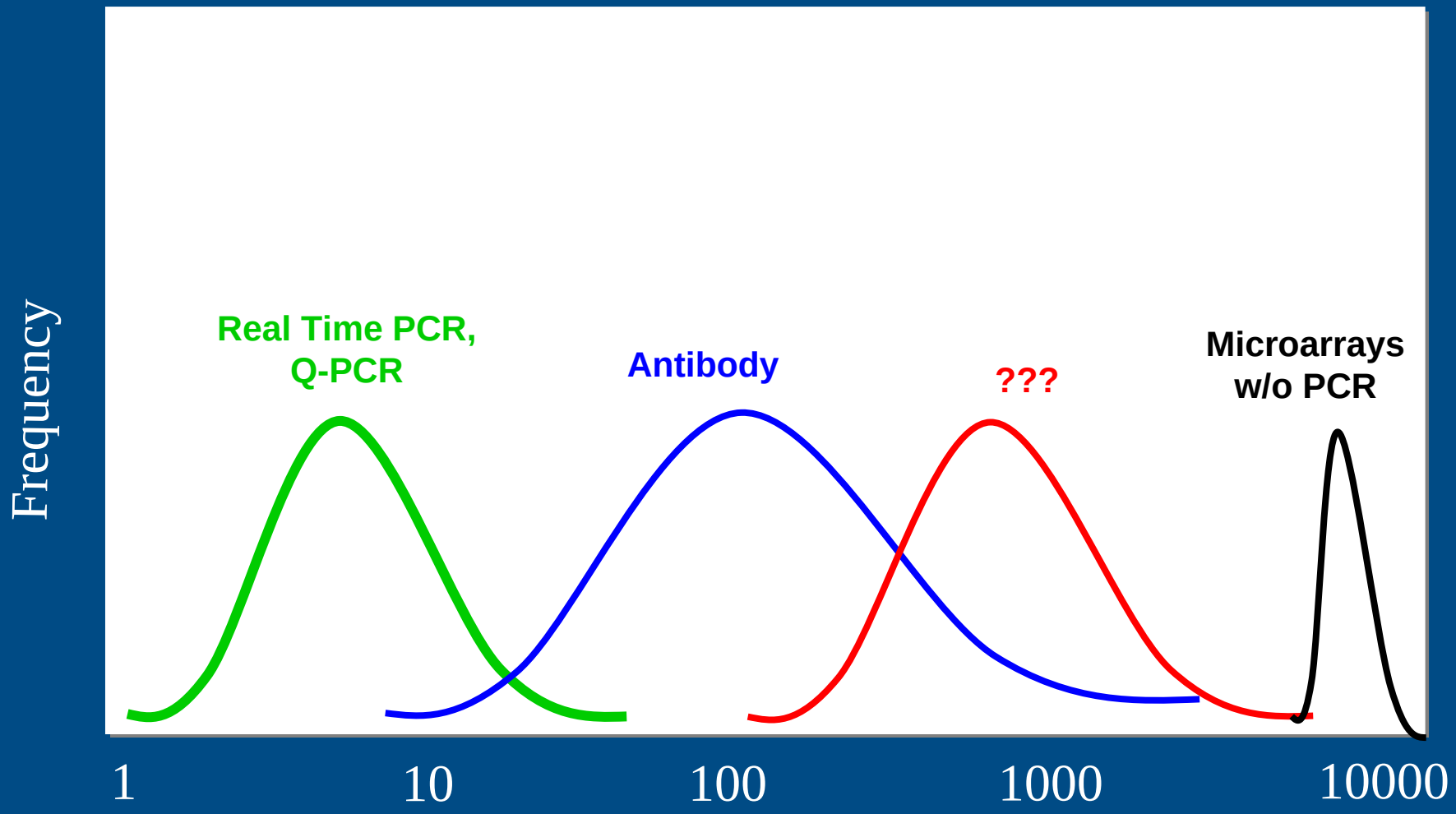
VALIDATION & DATA ANALYSIS

- Detection
  - Identification
  - Reliability
  - False positives
  - False negatives
  - Detection limit
  - Signal amplification
- Quantification
  - Dissociation curves
  - Standard mixtures
  - Chip-based PCR

USABILITY

- Upgradability
  - Database growth
- User-friendliness
  - Simplicity
  - Speed
  - Portability
  - Robustness
- Competitiveness
  - Cost
  - Niche
  - Competing technologies

Limit of Detection



Comparison of 6 PCR-based and 1 Antibody-based Assay

	PCR Chip Hand-Held	Arrow-Tech Bio-Seeq (HANAA)	Idaho RAZOR	PCR-Chip Bench top	Idaho R.A.P.I.D.	BioTrove NTCycler	BioVeris M1M analyzer
Base Unit	$1,000	$31,000	$35,000	$8,000	$55,000	$100,000	$70,000
Consumable Price (U)	$10	$38	$18	$100		$400	$1,000
Time	30 min	20-30 min	30 min	30 min	30 min	3.5 hr	90 min
# of Samples	14	6	12	56	10	48	96
# of Targets	50	1-4	12	50	10	18-56	1
Sample volume	10-500 nl		5-20 µl	10-500 nl	5-20 µl	33 nl	100 µl
Quantitation	Yes	No	No	No	Yes	Yes	No
Sensitivity	5 copies	5 copies	5 copies	5 copies	5 copies	5 copies	1000 cells
Portability (Weight)	< 1 lb	6.5 lb	9 lb	30 lb	50 lb	100 lb	100 lb
	Goal*						







Thank You