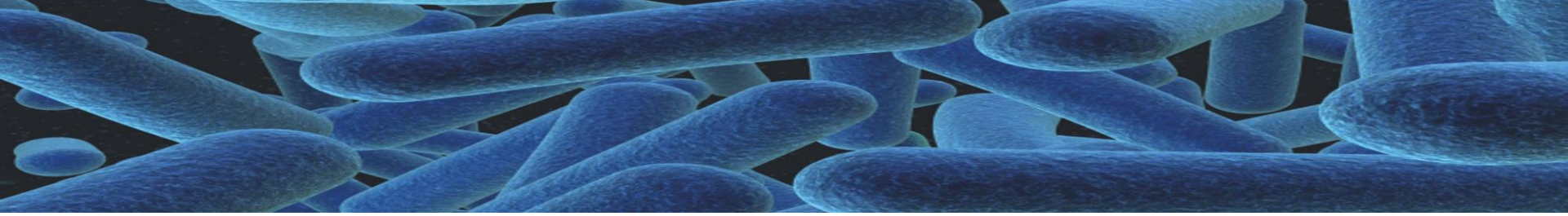


Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods

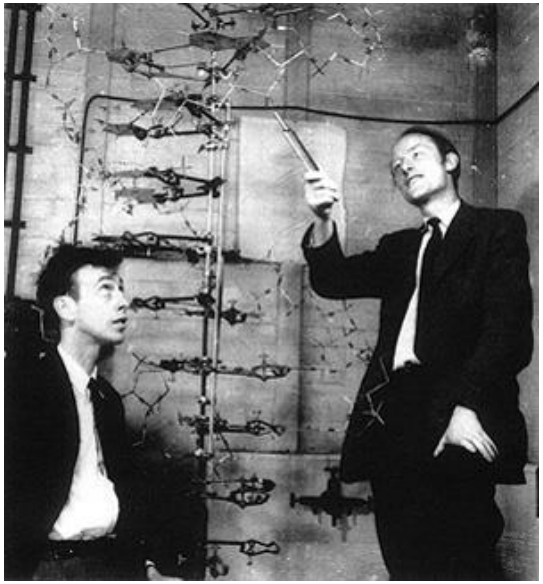
Genetic-based Technologies

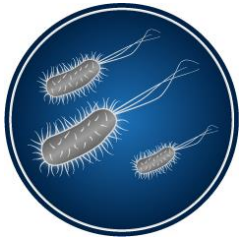
Michael J. Miller, Ph.D.

MICROBIOLOGY CONSULTANTS, LLC

Genetic-based Technologies

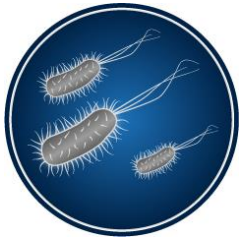
- Are now recognized as providing superior results when compared with other classical microbiology methods
- Detection of specific microorganisms
- Microbial identification
- Strain differentiation
- Estimation of viable cell count





Genetic-based Technologies

- Regulatory agencies and the compendia have acknowledged the strength in these technologies
  - The FDA aseptic processing guidance recommends the use of rapid genotypic methods for microbial ID and investigations into failures (e.g., sterility test; media fill contamination)
  - Ph. Eur. and JP recommend PCR for mycoplasma
  - JP has a chapter on using DNA sequencing for microbial ID
  - The Australian Therapeutic Goods Administration (TGA) requires that a sensitive method of microbial identification, such as molecular typing techniques using RNA/DNA homology, is utilized when the identification of an isolate is used to invalidate a sterility test

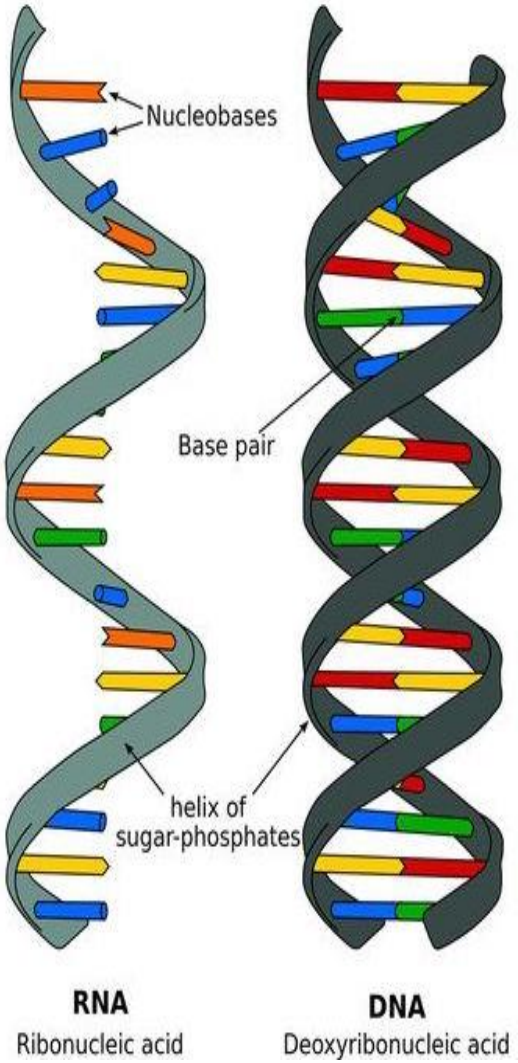


Scientific Principles

- Amplification and detection of target DNA or RNA sequences using polymerase chain reaction (PCR)
  - Quantitative (q) PCR (quantitate the PCR amplicons)
  - Reverse transcriptase (RT) PCR
  - Transcription Mediated Amplification (TMA) and Nucleic Acid Sequence Based Amplification (NASBA)
- Fragmentation of DNA sequences that encode for the rRNA operon (Ribotyping)
- Gene sequencing
- Applications include the detection of specific organisms in a sample, the identification of a microbial isolate, and an estimation of viable cell count

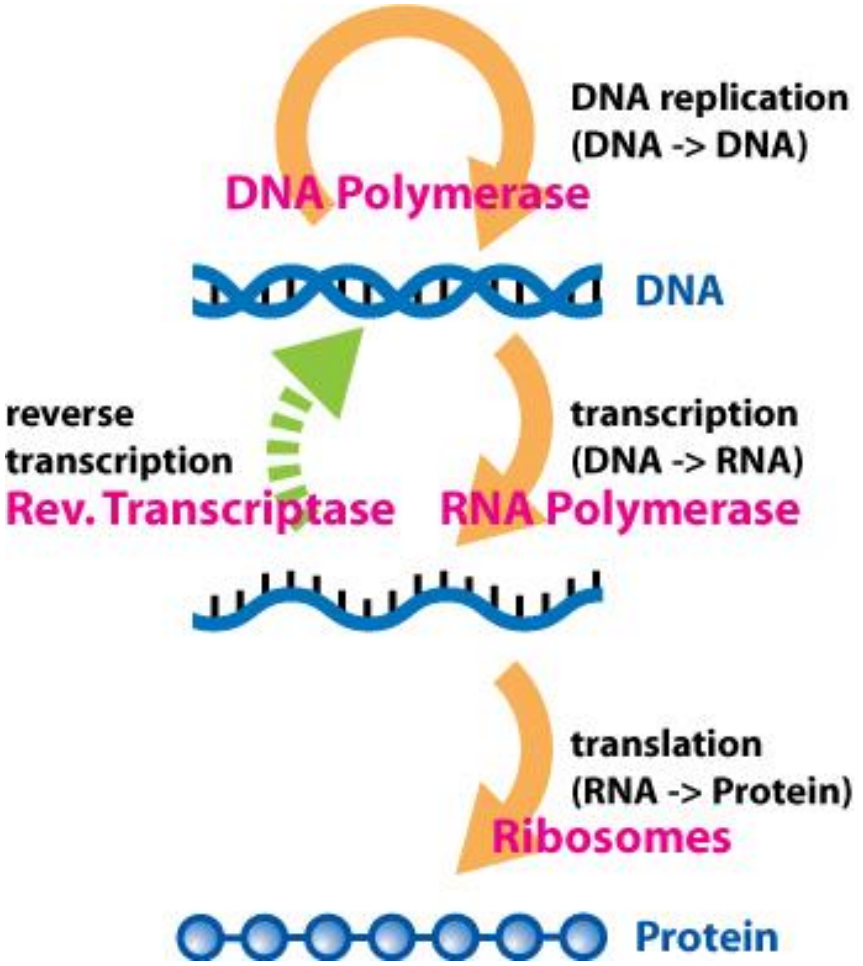
Review: DNA and RNA

	Deoxyribonucleic acid **DNA**	Ribonucleic acid **RNA**
Function	Genetic information storage	Converts DNA genetic information into proteins mRNA, tRNA and rRNA
Configuration	Double Stranded	Single Stranded
# Copies	1 - few	1000's of rRNA
Sugar	Deoxyribose	Ribose
Bases	Adenine, Guanine, Cytosine, Thymine	Adenine, Guanine, Cytosine, Uracil



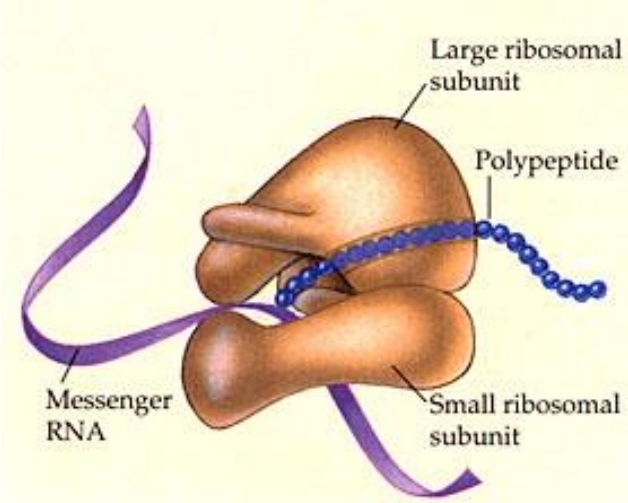
Review: DNA and RNA

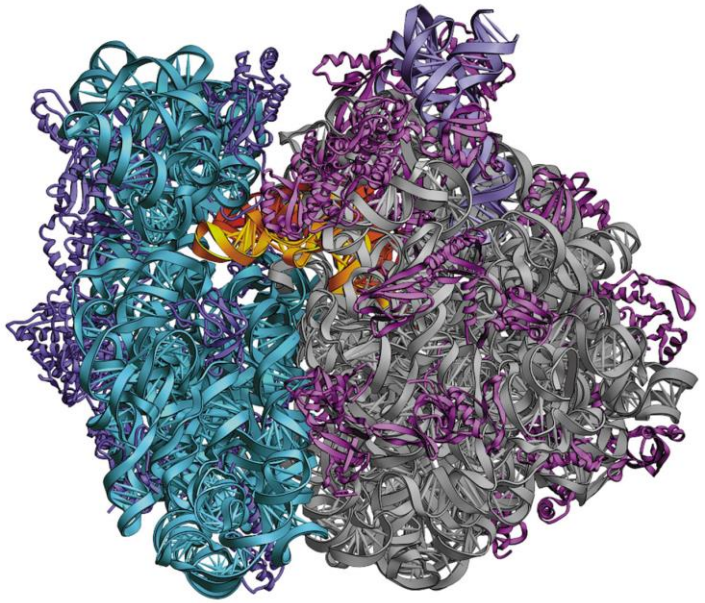
- DNA can replicate to form new DNA during cell division
- RNA is transcribed from DNA
- RNA is translated into proteins within ribosomes



Review: Ribsomes

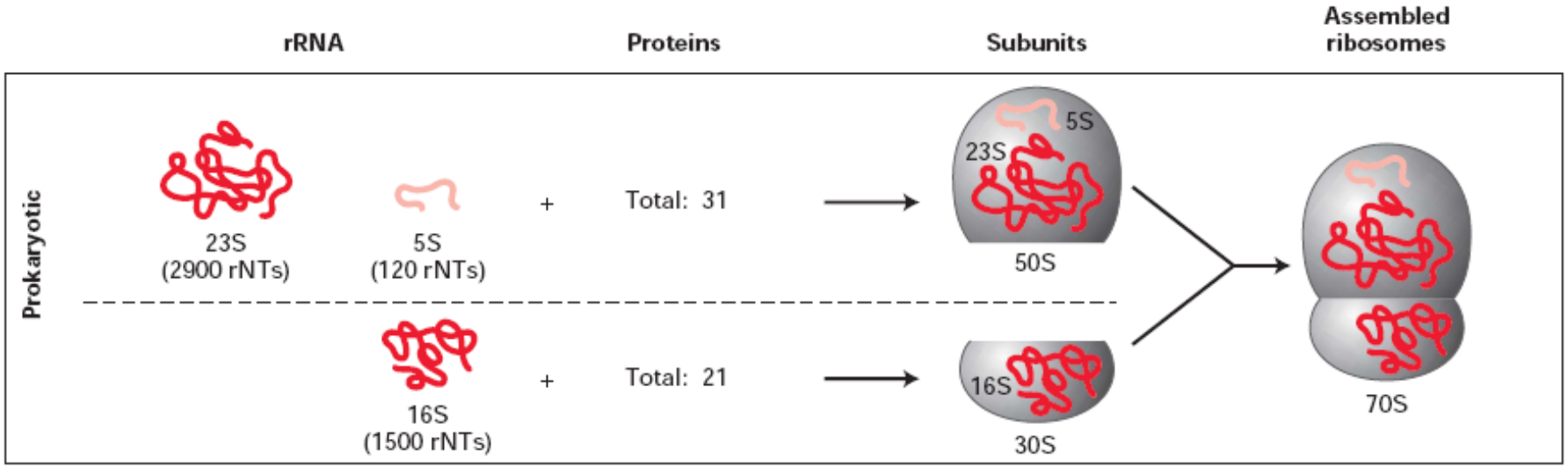
- Ribosome structure and ribosomal RNA (rRNA)
  - DNA sequences encode for rRNA





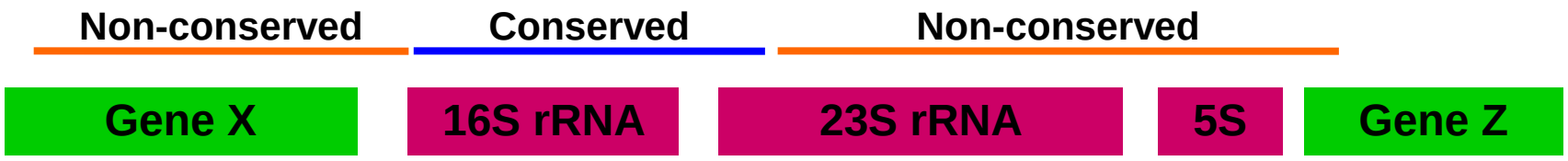
Review: Ribsomes

- Ribosome subunits and rRNA



Review: rRNA

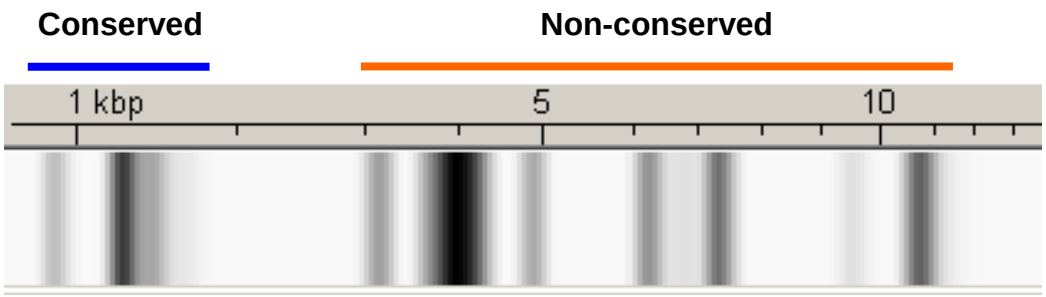
- rRNA fragments are highly conserved at the genus and species level (16S sequence)
  - Correct RNA structure is critical for ribosome function
  - Bergey's Manual of Systemic Bacteriology now uses 16S rDNA sequences as the standard for taxonomic classification of bacteria
- Non-conserved fragments (spacer and flanking regions of the 16S sequence) can be used to differentiate strains within a particular species



rRNA operon

16S rRNA Ribotyping

- Bacterial DNA is extracted from a cell and cut into fragments
- The fragments are hybridized with a DNA probe (derived from 16S rRNA operon and spacer/flanking regions)
- The probe has a detection marker (e.g., luminescent)
- The fragments can be viewed as banding patterns and compared with an identification database

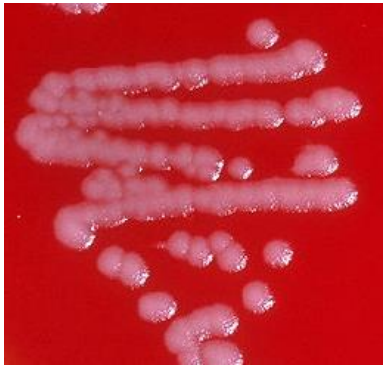


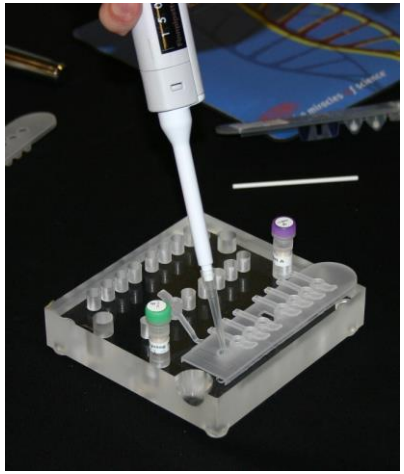
Hygiena RiboPrinter

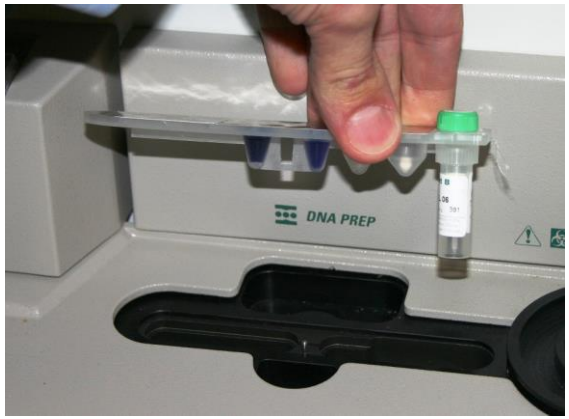
- Automated ribotyping system for microbial identification and strain tracking (original developed by Dupont Qualicon)

Hygiena RiboPrinter

- Dilute pure colony sample in buffer
- Pre-process samples: heat inactivation/add lysing agent
- DNA is extracted and cut into fragments by a restriction enzyme (e.g., EcoRI, PvuII)

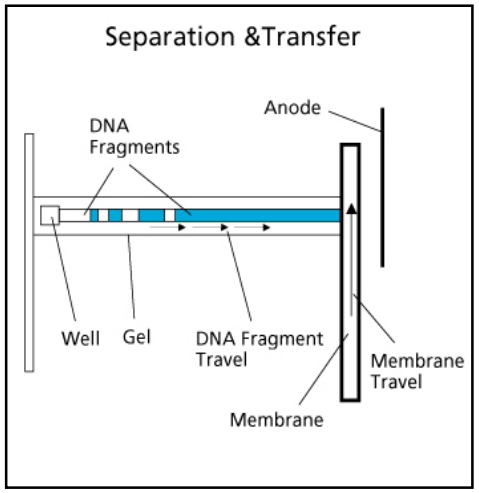


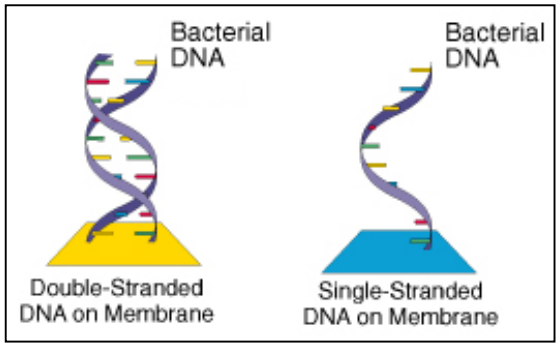




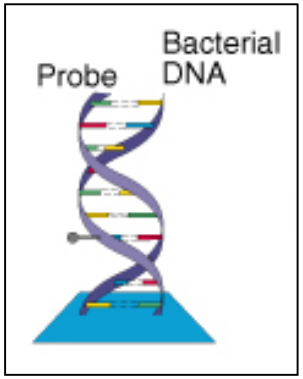
Hygiena RiboPrinter

- Fragments are separated according to size by gel electrophoresis, transferred and immobilized on a moving nylon membrane (automated Southern blot)
- The immobilized double-stranded DNA is denatured to single-stranded DNA

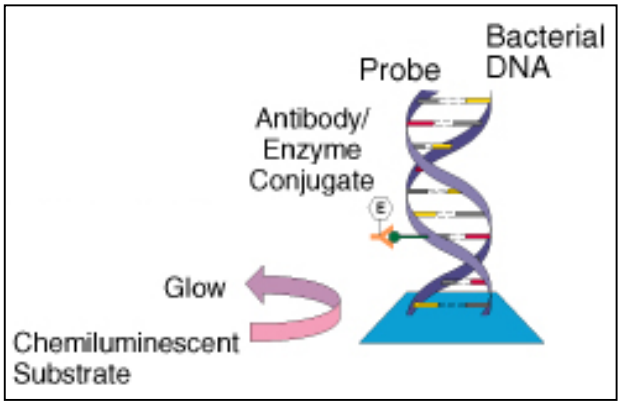




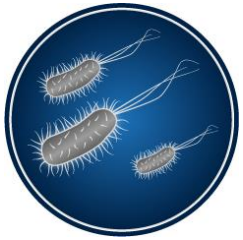
Hygiena RiboPrinter



The membrane is hybridized with the DNA probe (derived from *E. coli* rRNA operon; contains sequences encoding for 16S rRNA, 23S rRNA, 5S rRNA, and spacer region including Glu-tRNA)



An antibody-enzyme conjugate is bound to the probe and a chemiluminescent agent is added

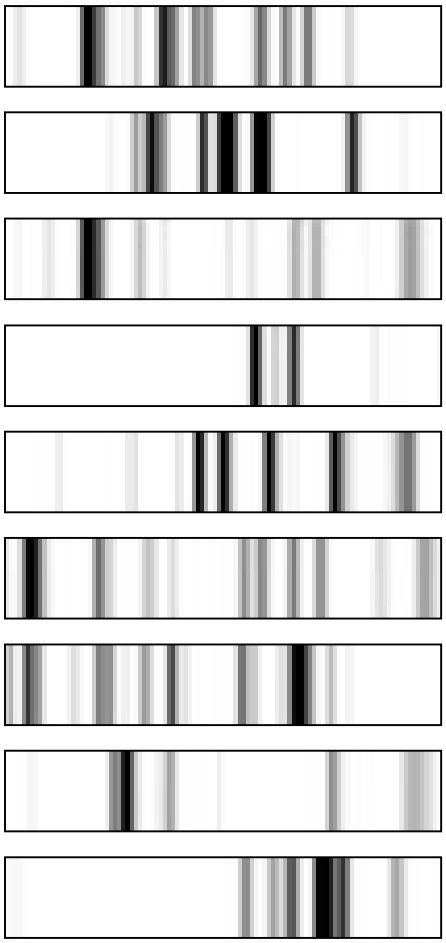


Hygiena RiboPrinter

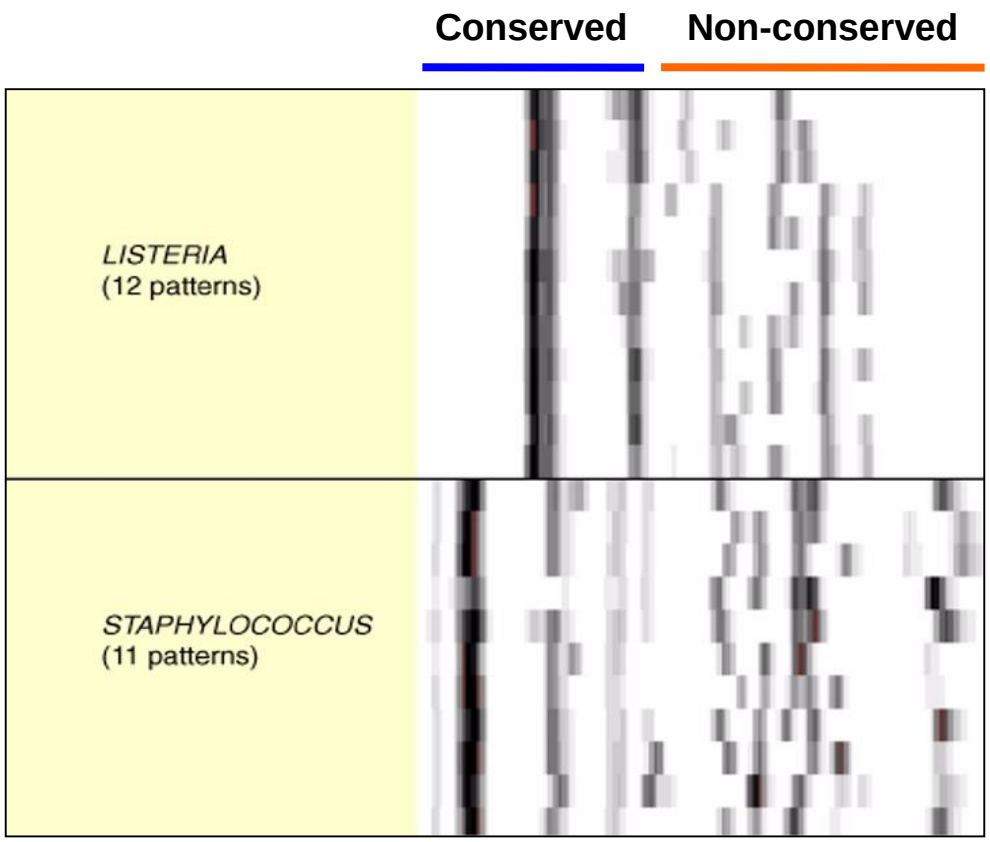
- Light emitted by the fragments are captured by a digitizing camera and stored as image data in the computer
- The software creates the RiboPrint pattern, which is then compared with patterns stored in the system database and the bacterium is identified
- System is fully automated; up to 32 ID's per 24 hours

Hygiena RiboPrinter

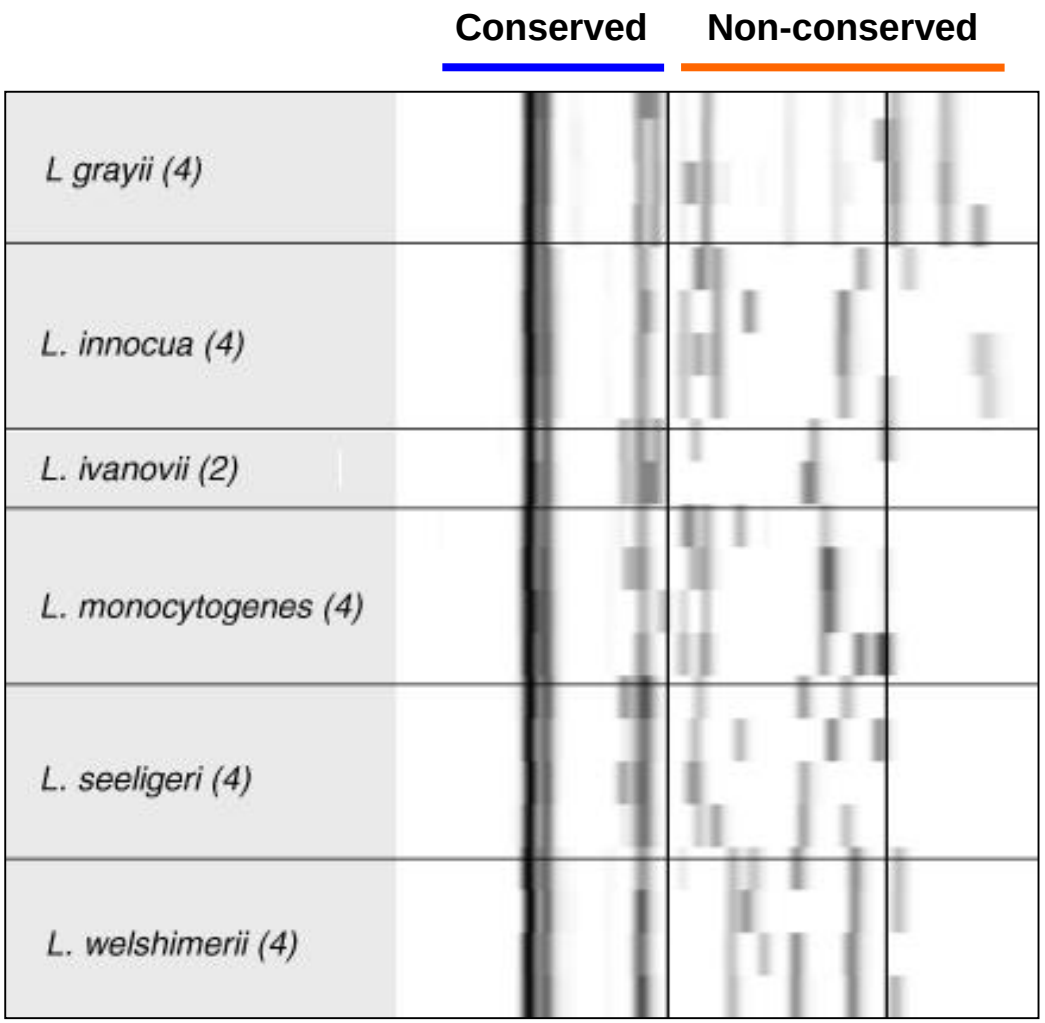
- Current database contains more than 6900 patterns
- 219 bacterial genera
- 1440 species



Hygiena RiboPrinter



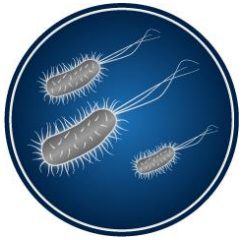
Hygiena RiboPrinter



Case Study

- Sterility test positive investigation, samples sent to lab

Vitek Identification	**RiboPrinter Pattern**
S. epidermidis (finished product)	
S. epidermidis (raw material)	
S. epidermidis (personnel gloves)	

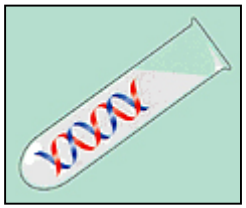


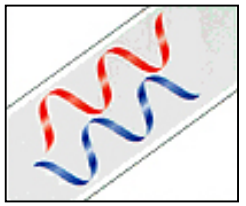
Review: PCR

- Polymerase Chain Reaction (PCR)
- Small fragments of DNA (primers) are used to find a specific sequence (target) on a sample of DNA
- A heat stable enzyme (e.g., *Taq* DNA polymerase) makes millions of copies of the target sequence, which can be easily detected
- We will explore different types of PCR processes
  - Real-time, quantitative PCR
  - Reverse transcriptase PCR
  - Rep-PCR: typing of non-coding repetitive DNA sequences

Review: PCR

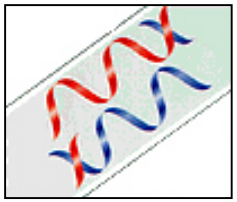
DNA is extracted and heated to separate the double strands





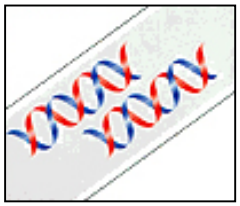
Heat to 90° C

DNA primers (short, synthetic sequences) are added, which bind to unique target sequences on the template DNA, if they are present



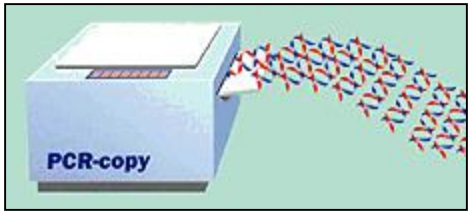
Lower heat to 55° C

Heat-stable DNA polymerase and nucleotide bases (A,T,G,C) are added. The primer is elongated, producing two new complete copies of the template DNA strands



Raise heat to 70° C

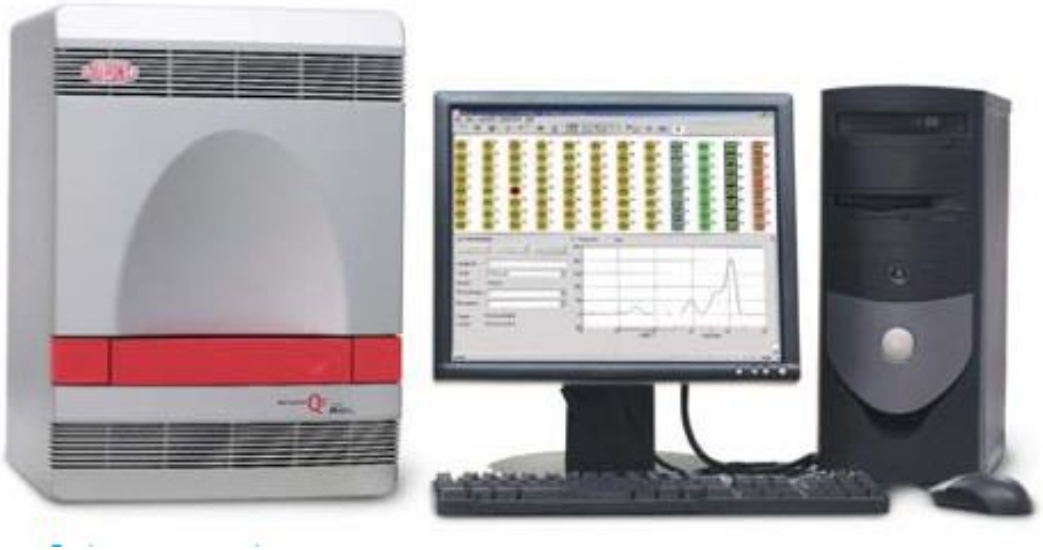
The process is repeated, resulting in millions of copies of the target DNA

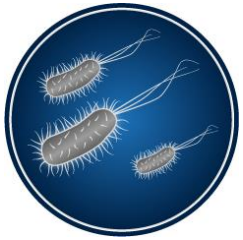


PCR Video

Hygiena BAX System Q7

- Automated real-time, quantitative PCR and probe-based detection



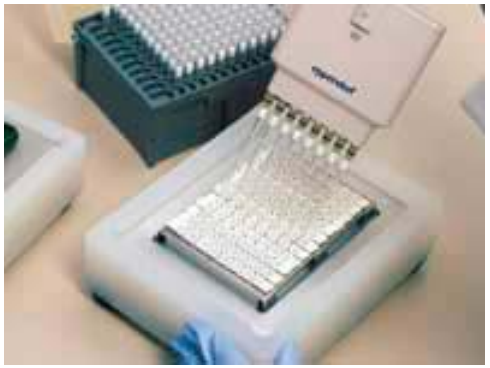


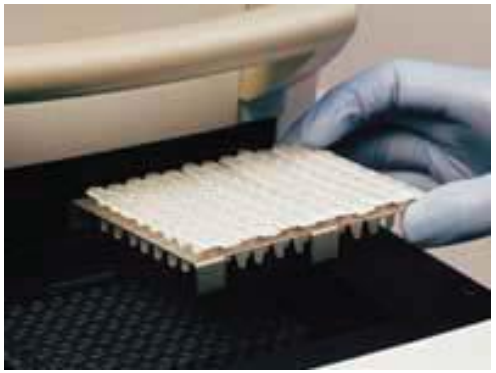
Hygiena BAX System Q7

- DNA amplification is measured as it occurs, allowing the detection of sequence-specific PCR copies as they accumulate in "real time"
- The test sample is enriched in media to provide enough DNA for analysis and to eliminate false positives from dead cells or residual DNA
- Two types of assays:
  - Intercalating dye (SYBR Green) chemistry
  - Taqman probe-based chemistry that allows more than one detection event

Hygiena BAX System Q7

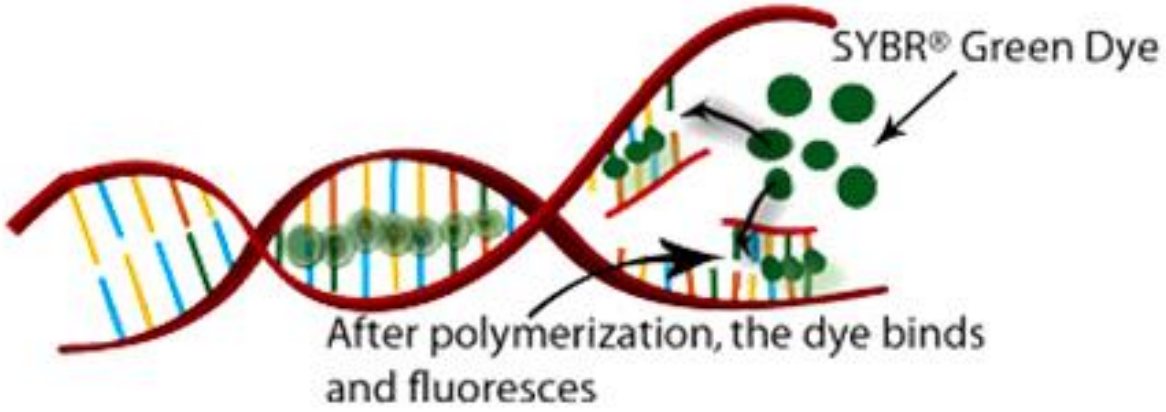
- Enriched samples are heated in a lysis reagent solution to rupture the bacterial cell wall and release DNA
- PCR reaction tubes contain tablets that are hydrated with the test samples
- The tablets contain intercalating dye or probes, along with all the reagents, enzymes and primers needed for the PCR reaction
- The primers match a unique genetic region of the target's DNA



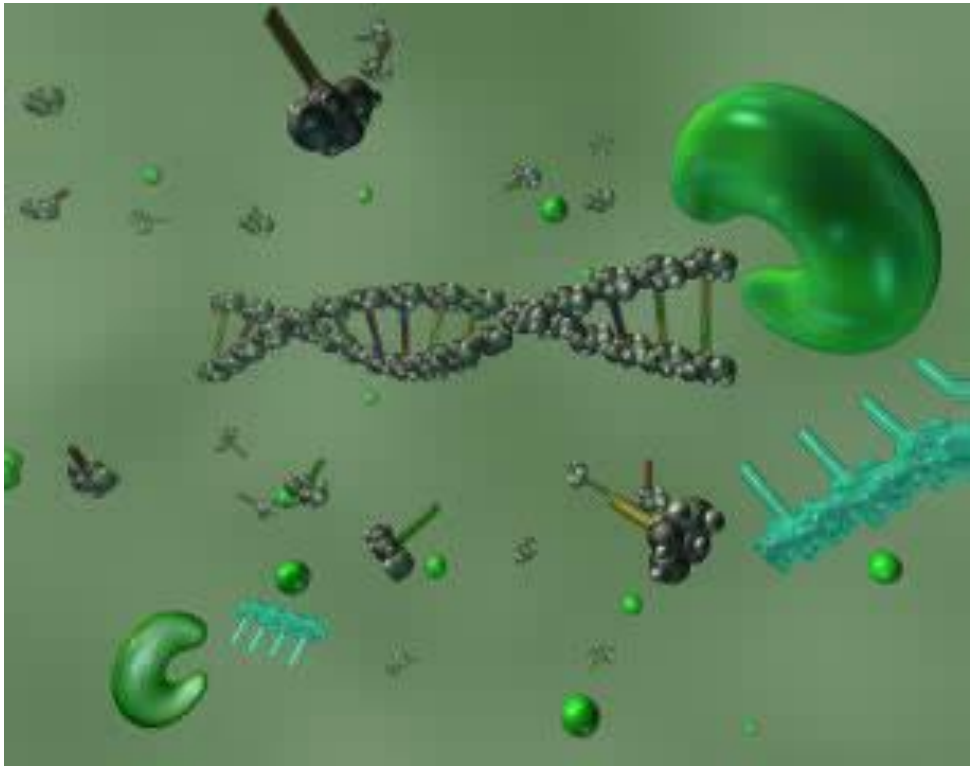


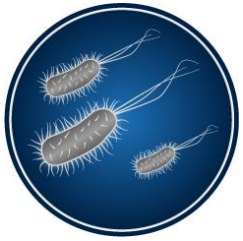
Intercalating Dye SYBR Green Assay

- During PCR, SYBR Green dye binds to a newly synthesized double-stranded DNA
- The bound dye emits a fluorescent signal when excited by light
- Each PCR cycle will result in an increase in fluorescence
- Plot the fluorescence in real time. No DNA target = no fluorescence



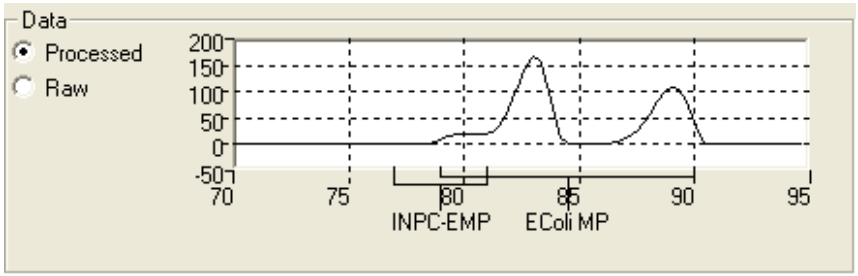
Intercalating Dye
SYBR Green Assay

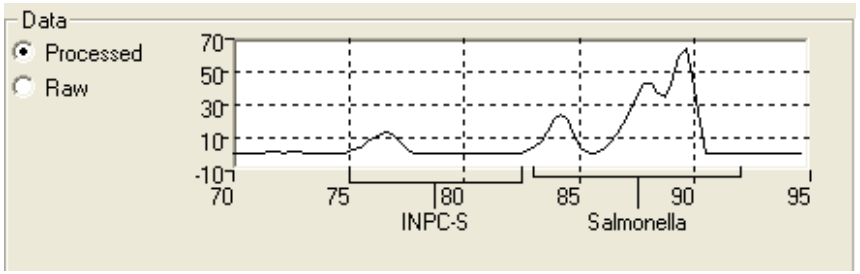




Intercalating Dye SYBR Green Assay

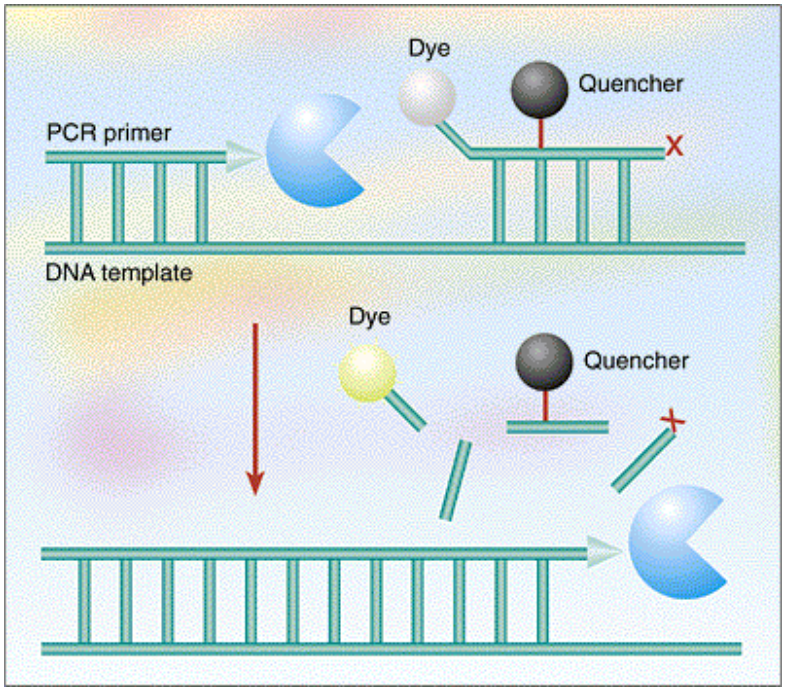
- After PCR detection, we will generate a melting curve
- Excite the dye to produce a fluorescence baseline signal
- The temperature is slowly raised to denature the DNA, releasing the dye and lowering the fluorescent signal
- Change in fluorescence is plotted against temperature
- Each target organism generates its own melting curve



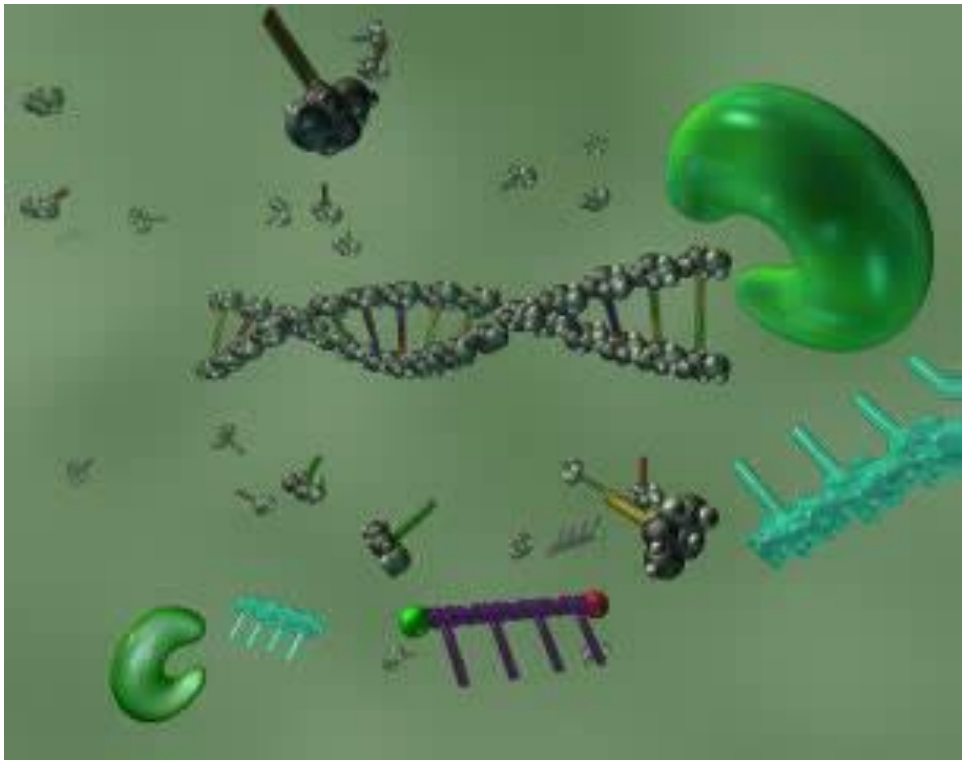


Taqman Probe-based Assay

- During PCR, at the same time primers bind to a target sequence, a Taqman probe binds to another region of the target sequence
- The probe contains a fluorescent reporter dye at one end and a quencher dye at the other end. There is no fluorescence.
- As Taq polymerase extends the primer, the probe is cleaved, releasing the reporter dye and increasing the fluorescence signal
- Each PCR cycle increases the level of fluorescence

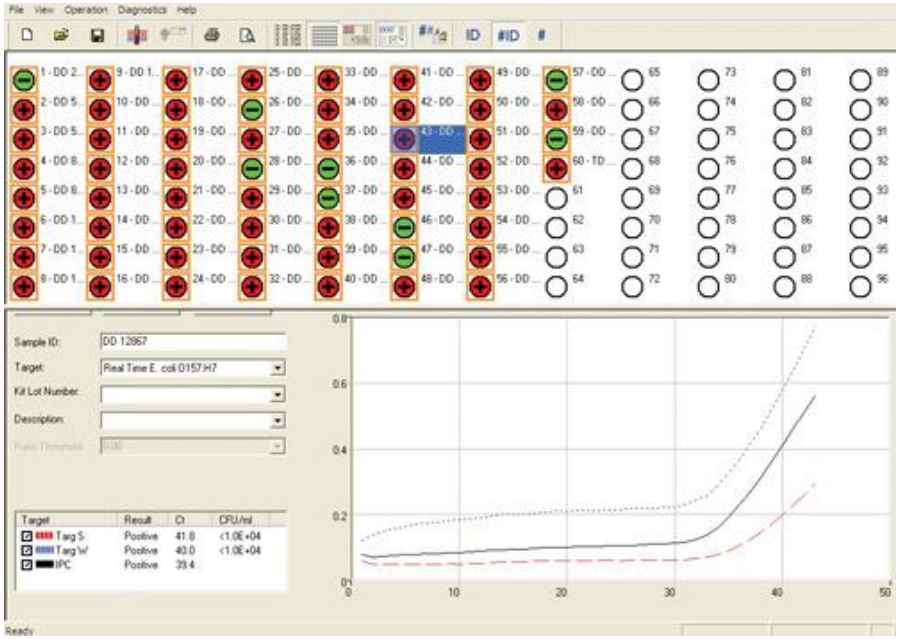


Taqman Probe-based Assay



Hygiena BAX System Q7

- Continuous wavelength detection allows the use of multiple dyes (and probes) in a single reaction and in real time
  - *Salmonella*, *S. aureus, Vibrio, Shigell*a, *Listeria monocytogenes, Camplyobacter jejuni/coli*, *E. coli* O157:H7, Cronobacter (*Enterobacter sakazakii*), yeast and mold

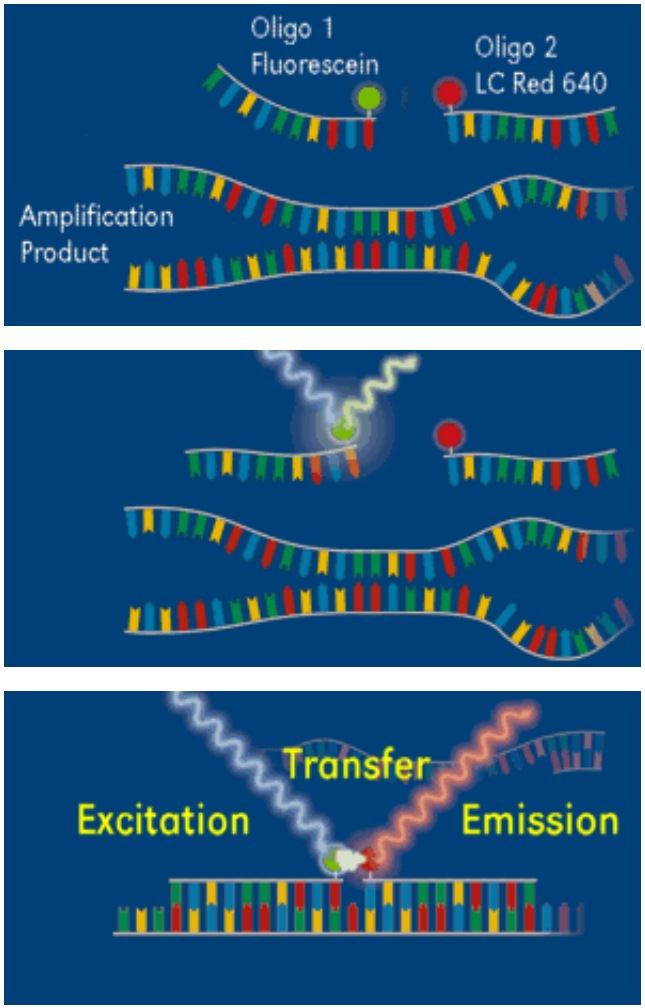


Biotecon Diagnostics microproof Hygiene Screening System

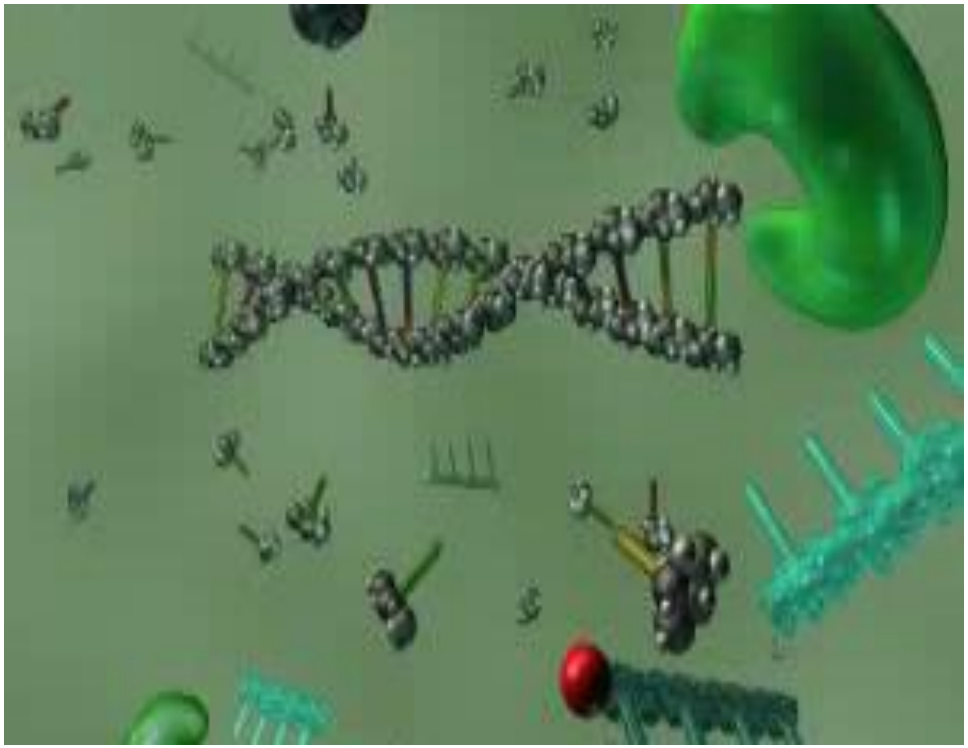
- PCR-based technology for the detection of microorganisms belonging to the genera *Corynebacterium, Staphylococcus, Micrococcus*
- PCR amplification in the Roche LightCycler
- Simultaneous detection within 90 minutes
- Start with an isolated colony

Biotecon Diagnostics Hygiene Screening System

- FRET probes to detect amplification
- Fluorescence resonance energy transfer (FRET) occurs between excited states of two dyes
- The donor dye is excited by a light source
- When the dyes are close to each other, excitation is transferred from the donor dye to the acceptor dye
- The acceptor dye emits light of a different wavelength; this is detected

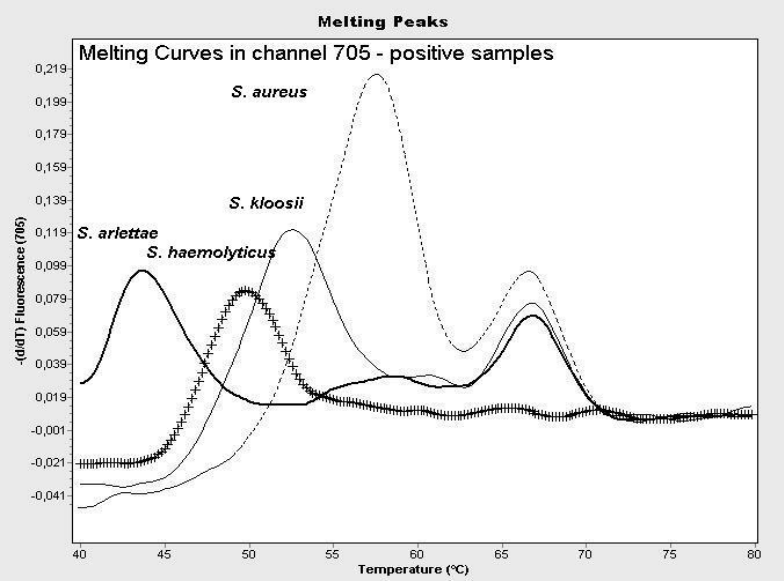


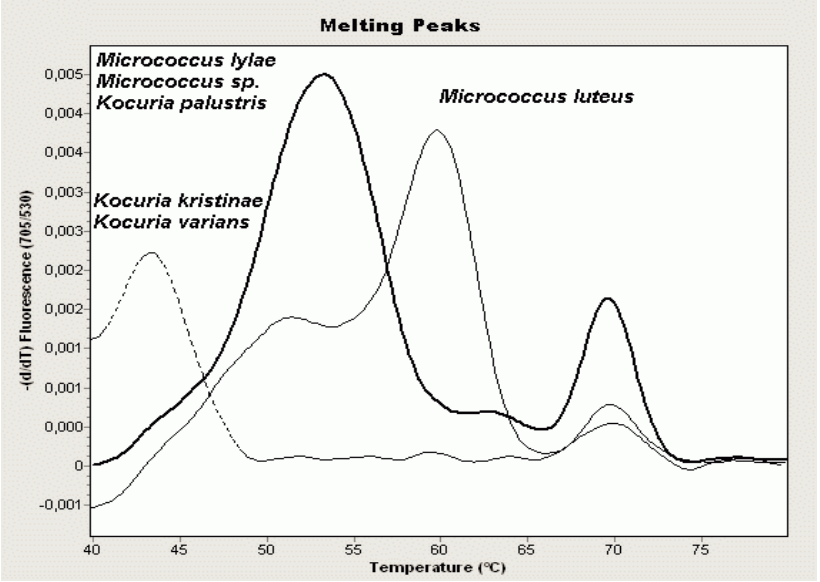
FRET Probe



Biotecon Diagnostics Hygiene Screening System

- Melting curves and melting peaks are used to differentiate and identify some organisms to the species level





Pall GeneDisc

- Quantitative (q)PCR to simultaneously detect multiple organisms in the same sample
- Different primers and probes/dyes for each DNA sequence is in the outside edge of the disc
- Taqman probe technology

Pall GeneDisc

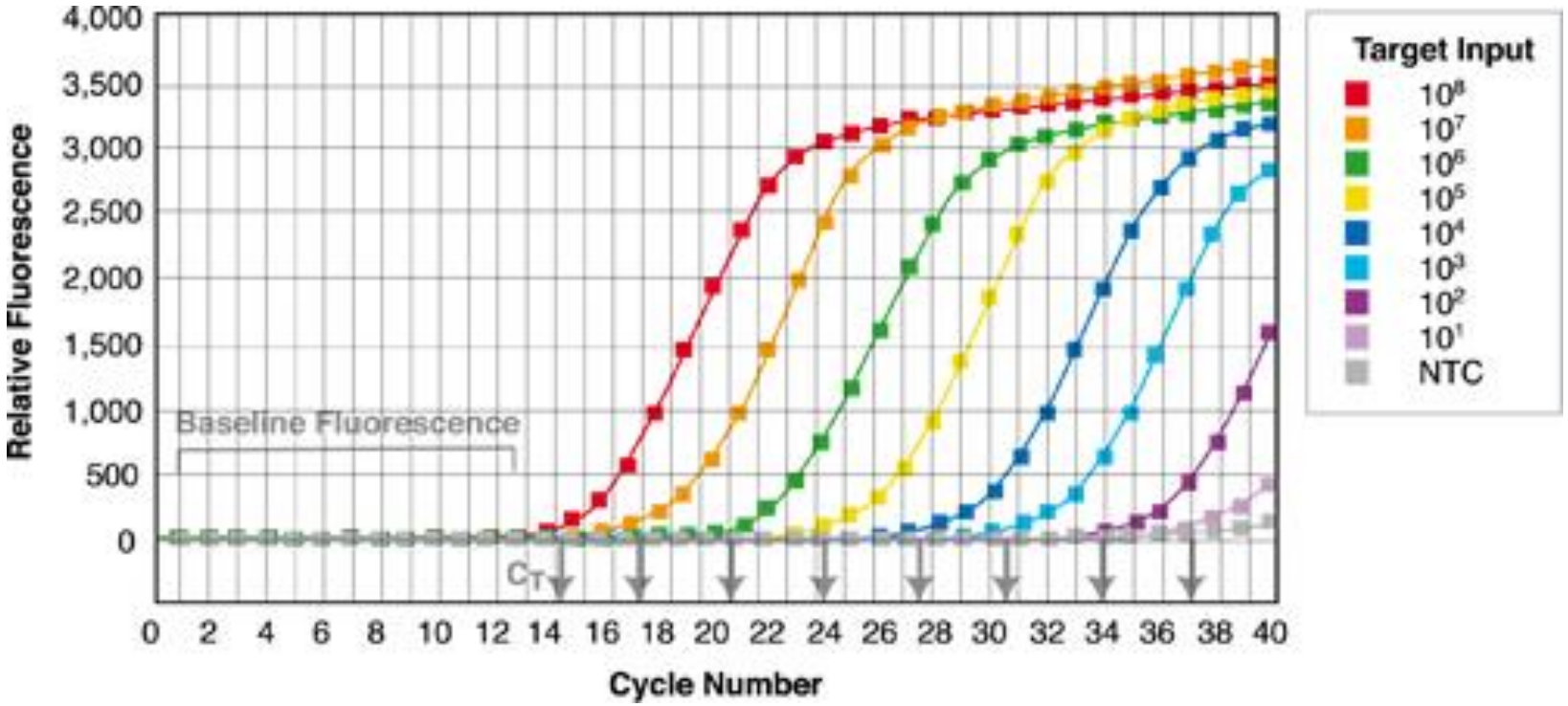
- Samples are filtered, media is added to the filter cartridge and the membrane is incubated for 6-16 hours
- The membrane is removed and placed in a lysis tube. Organisms on the membrane are lysed (sonicated for 8 min) and heated (100˚ C for 19 min) to release the DNA

Pall GeneDisc

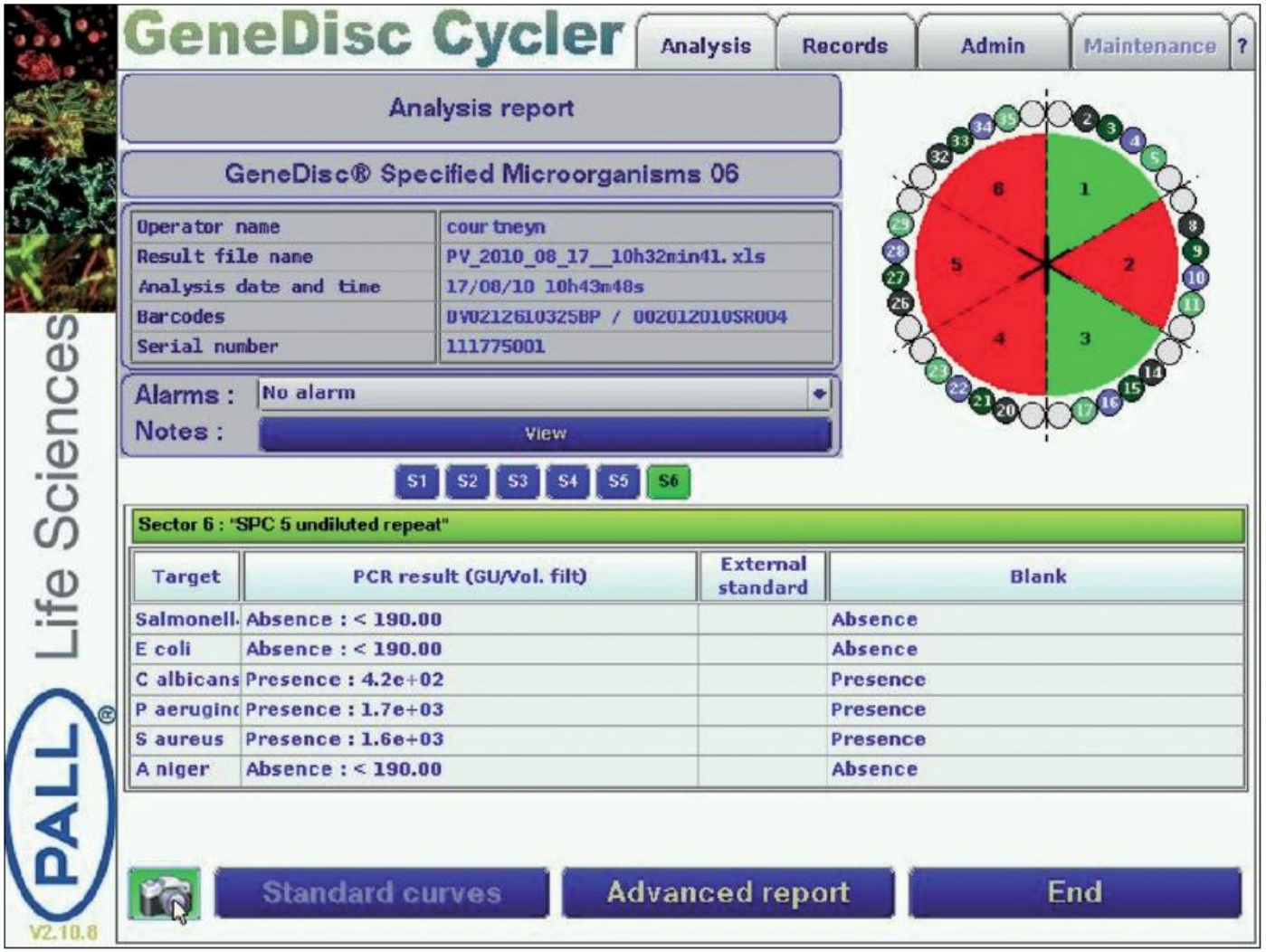
- The purified DNA and a Master Mix (polymerase and deoxynucleotides) are added to the upper hub of a GeneDisc plate
- The plate is inserted into the instrument, and the disk rotates through 4 different heating and cooling sections during the PCR amplification and detection process

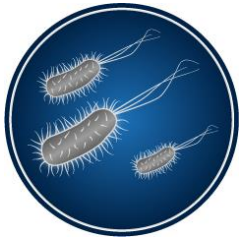
Pall GeneDisc

- Fluorescent signals increase during amplification



Pall GeneDisc Software Display



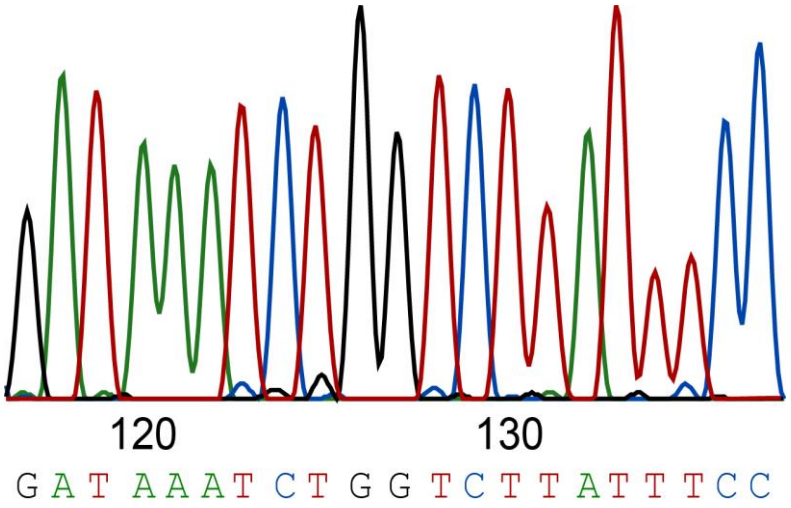


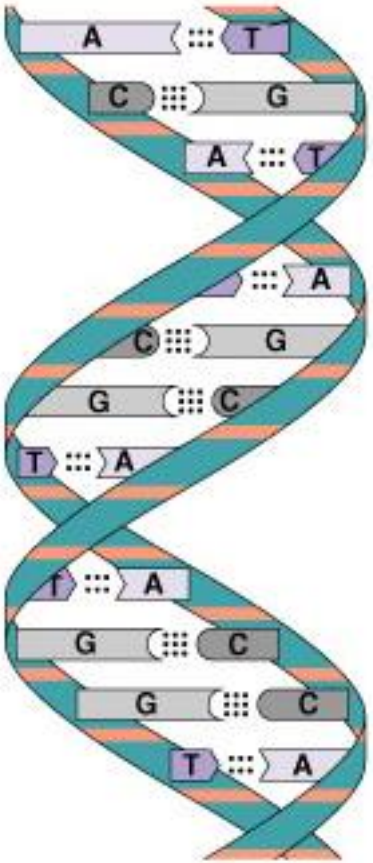
Pall GeneDisc

- Up to 6, 9 or 12 test samples may be assayed per disc
  - Depends on plate/application; includes positive and negative controls
  - 8 modular system units have the capability of testing up to 96 samples per run (~1 hour)
- Plate for Compendial Specified Microorganisms:
  - *Escherichia coli, Salmonella spp., Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Aspergillus brasiliensis (A. niger)*
- Food Testing
  - STEC, non-STEC and *E. coli* O157*, Salmonella, Listeria*
- Environmental
  - *Legionella, Pseudomonas, Enteroccocus, Cyanobacteria, E. coli*

Gene Sequencing

- The process of recording the exact sequence of nucleotides in a section of an organism's DNA
- DNA sequences can be used for microbial identification





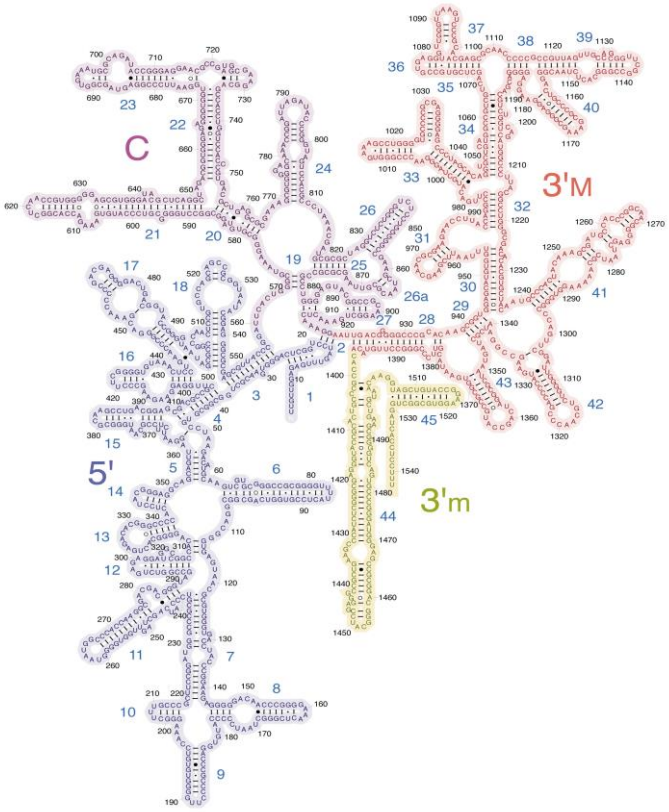
Gene Sequencing

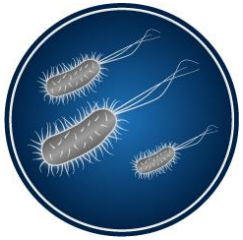
- Methods have simplified over the years!

Thermo Fisher MicroSEQ

Thermo Fisher MicroSEQ

- Sequencing the first 500 base pairs of 16S rRNA gene for bacteria and the D2 region of large-subunit rRNA gene for fungi
  - Universal among bacteria and fungi
  - Functionally conserved
  - Provides a high level of discrimination
  - Stable, not influenced by environmental conditions
  - Can sequence the entire 16S gene for greater bacterial differentiation (1500 bps)



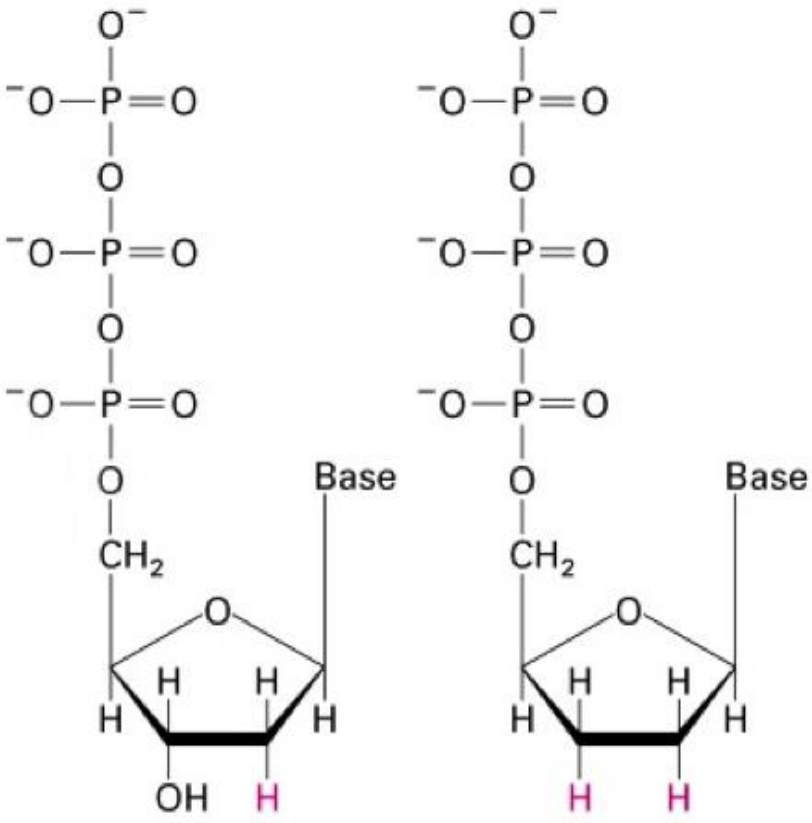


Thermo Fisher MicroSEQ

- Start with a loopful of cells from a plate or pure culture
- Extract DNA
- For bacteria, add primer for 16S rDNA and amplify DNA by PCR in a thermal cycler
- Perform cycle sequencing
  - Primers direct the sequencing of the forward or reverse reaction for each of the DNA strands
- Use a mixture of standard deoxyribonucleotides (dA, dT, dC, dG) and dideoxyribonucleotides (ddNTP)

Thermo Fisher MicroSEQ

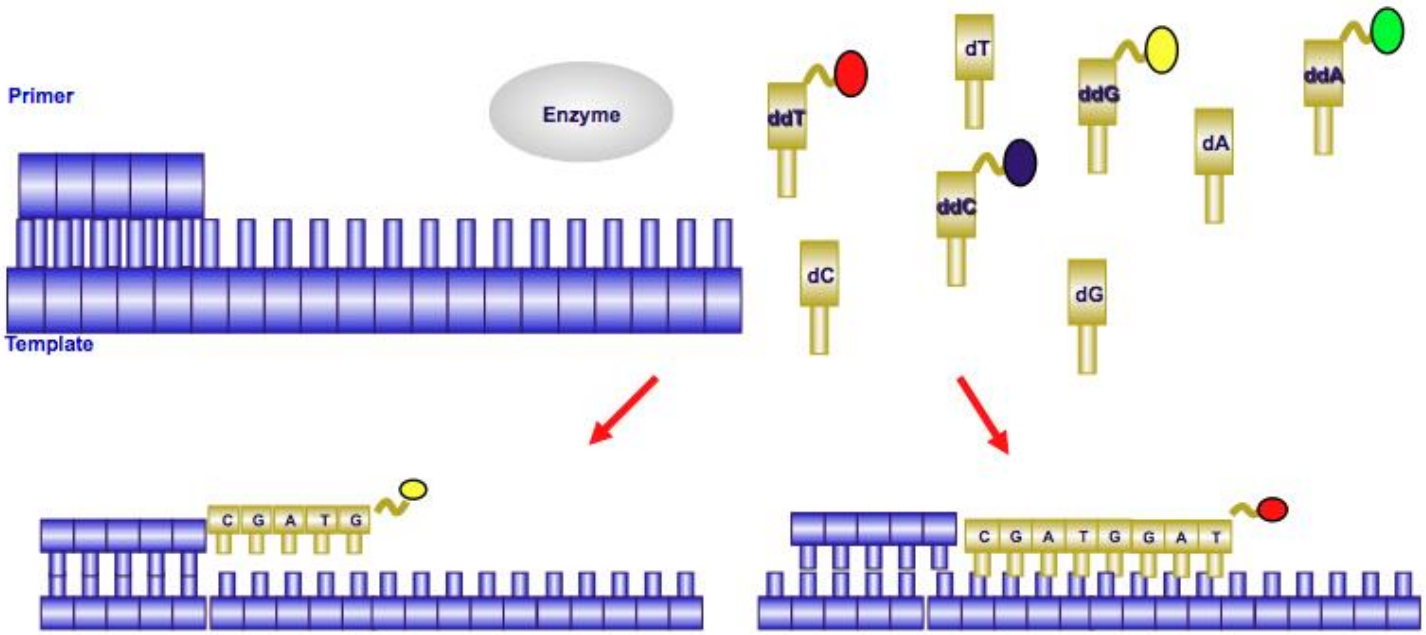
- ddNTPs lack a 3'-hydroxyl (-OH) group on their deoxyribose sugar
- Once a ddNTP is incorporated during sequencing, DNA elongation is terminated



dNTPs ddNTPs

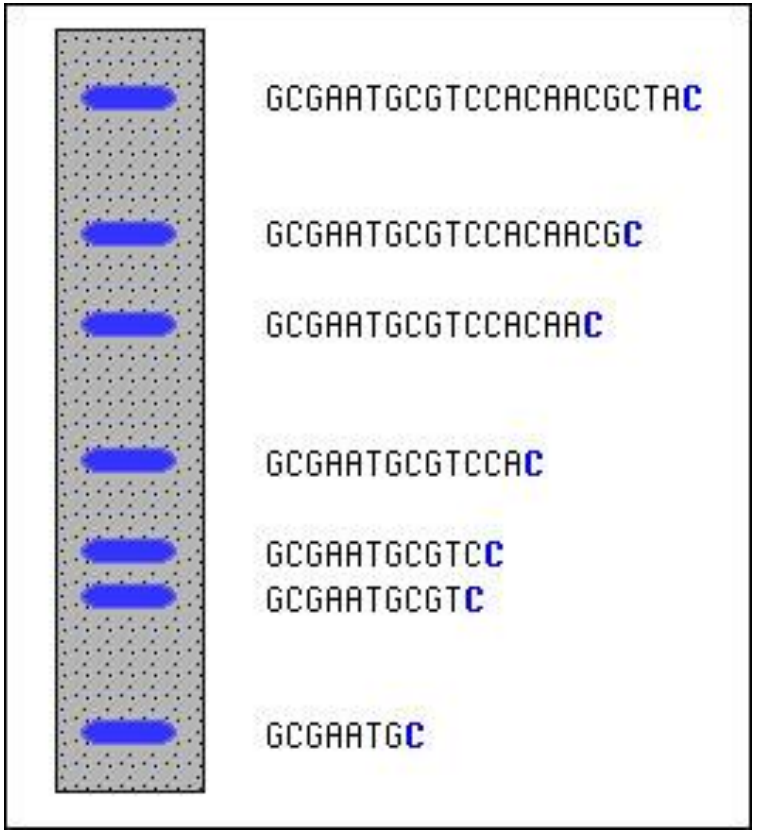
Thermo Fisher MicroSEQ

- Each ddNTP is also labeled with a different fluorescent dye
- The terminating DNA strand extensions result in DNA fragments of varying length and ending in a different dye



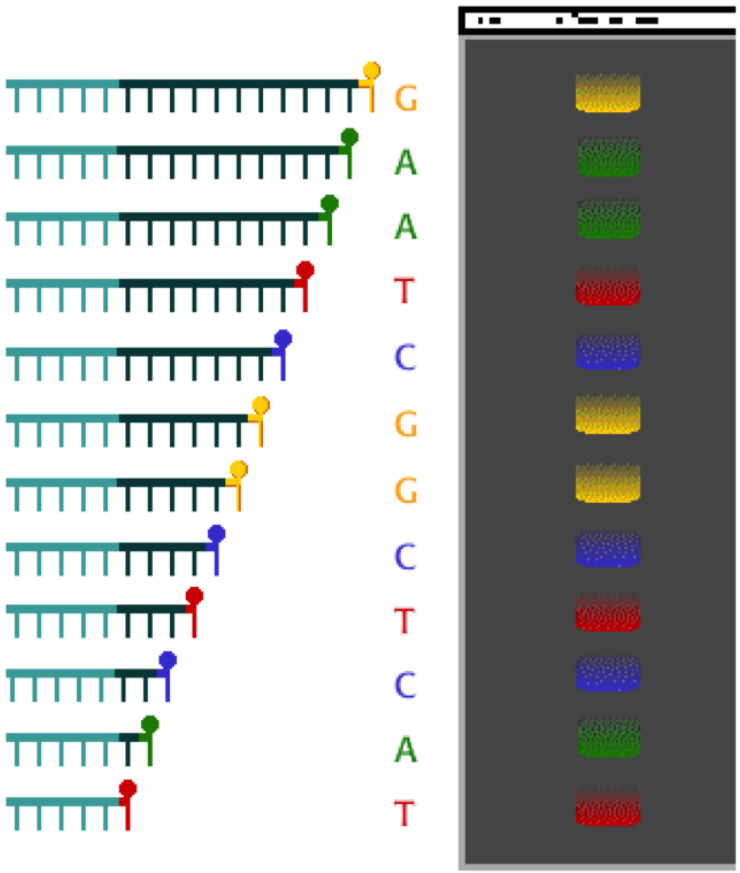
Thermo Fisher MicroSEQ

- The following illustrates different lengths of DNA all ending in a cytosine dideoxyribonucleotide
- Now we have to do the same with the other three nucleotides



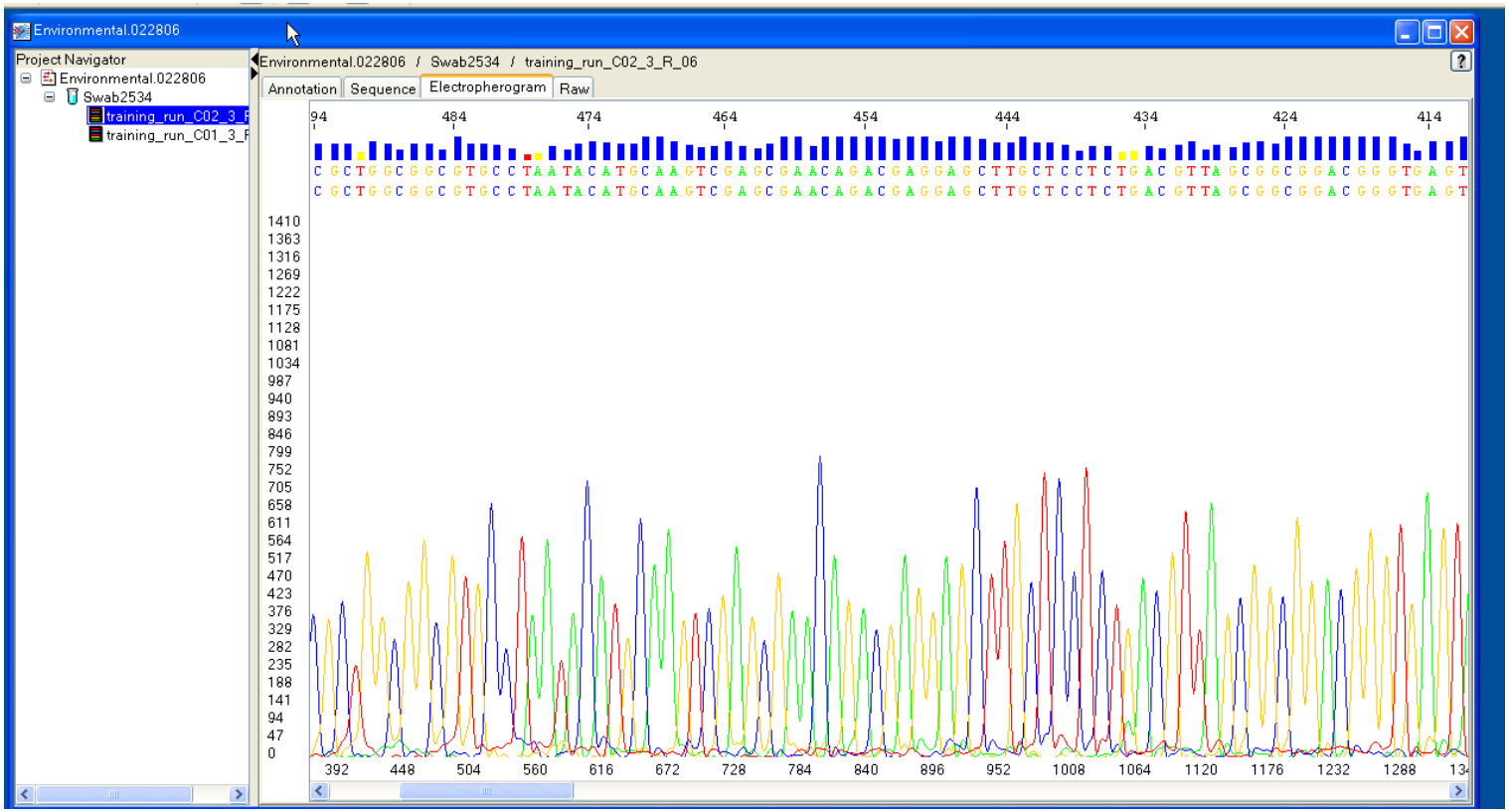
Thermo Fisher MicroSEQ

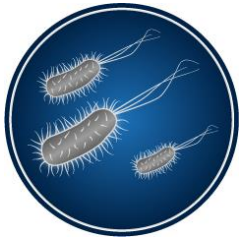
- A genetic analyzer separates all DNA fragments using capillary electrophoresis and a laser detects the last nucleotide in each fragment via fluorescence of the dye at the end
- Fragments are arranged by size and this represents a DNA sequence in the correct order



Thermo Fisher MicroSEQ

- The sequence is compared with a database or library and the best match for a microbial ID is provided



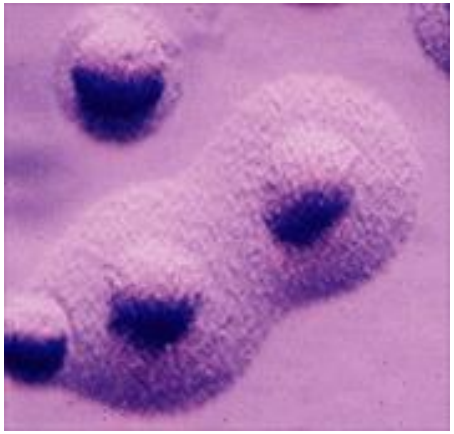


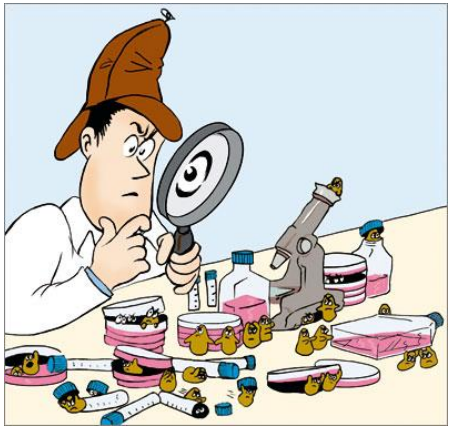
Thermo Fisher MicroSEQ

- 80 IDs/day
- Higher throughput if using greater capacity capillary analyzers
- Database
  - > 1800 bacteria
  - > 90 Mycoplasma
  - > 1100 yeast and mold

Mycoplasma Detection RMMs

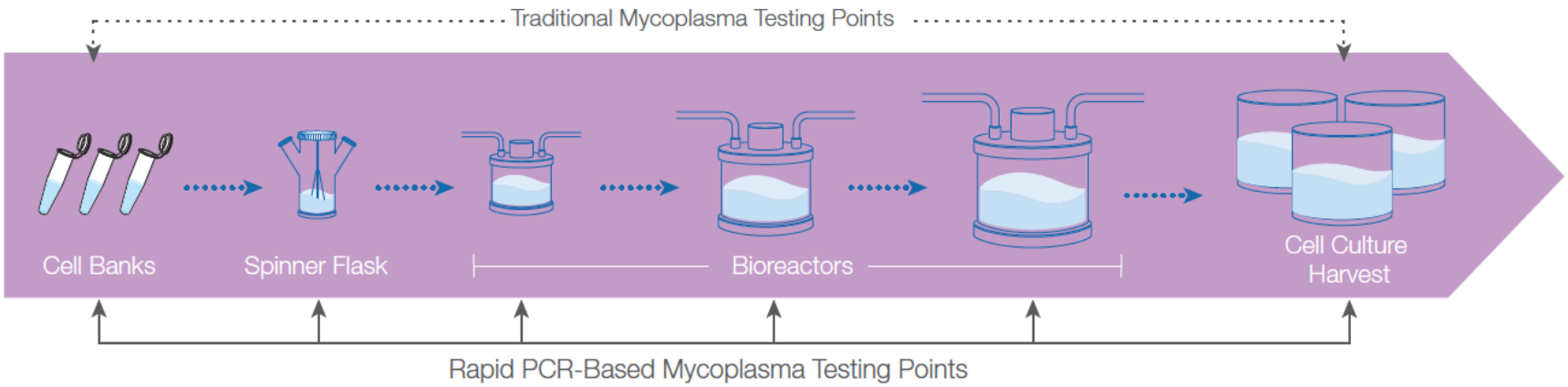
- The current Mycoplasma test is a 28-day culture test
- In 2007, the Ph. Eur. accepted nucleic acid tests (real-time PCR) as an alternative to traditional Mycoplasma detection (after validation)
- JP has a similar chapter
- RMMs utilizing nucleic-acid amplification techniques can now detect Mycoplasma within hours





Mycoplasma Detection RMMs

- Rapid PCR-based testing for Mycoplasma can now be conducted throughout the cell culture manufacturing process, from inoculation through harvest
- Thermo Fisher MycoSEQ, EMD Millipore MilliPROBE, Greiner Bio-One CytoCheck, Roche MycoTool

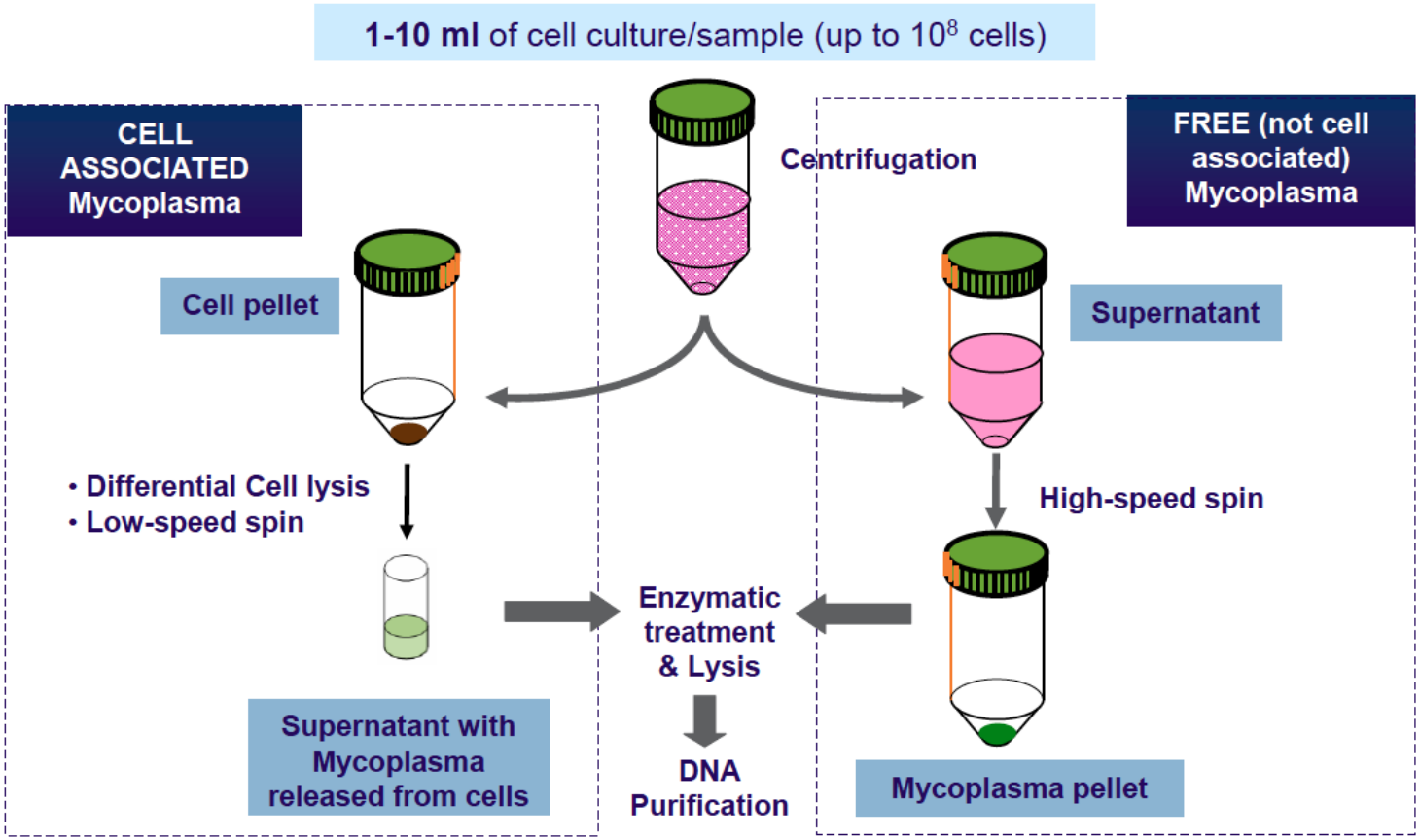


Thermo Fisher MycoSEQ

- Detection of greater than 90 Mycoplasma species, while excluding species closely related at the genetic level
- Limit of detection has been demonstrated as low as 1 CFU or copy equivalent per ml of test sample as compared with culture method
  - Amgen required a 7-day enrichment in liquid medium to eliminate interference from their commercial protein product
- SYBR Green probe
- Time-to-results <5 hr

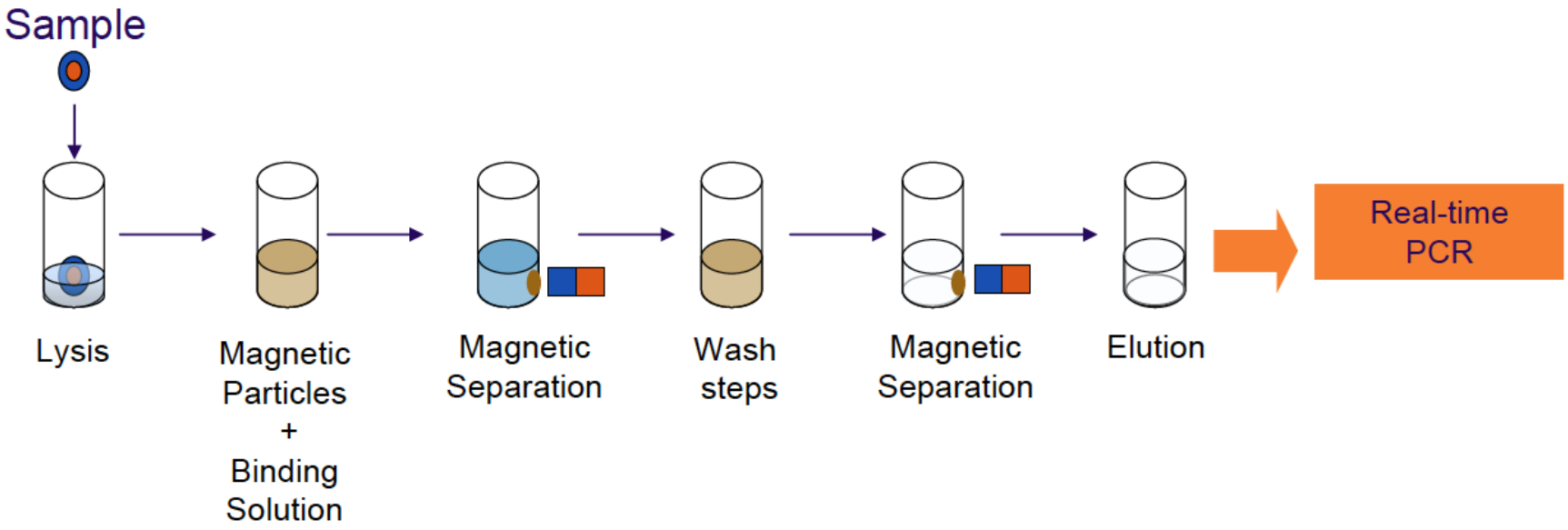
Thermo Fisher MycoSEQ

- Sample preparation



Thermo Fisher MycoSEQ

- DNA purification

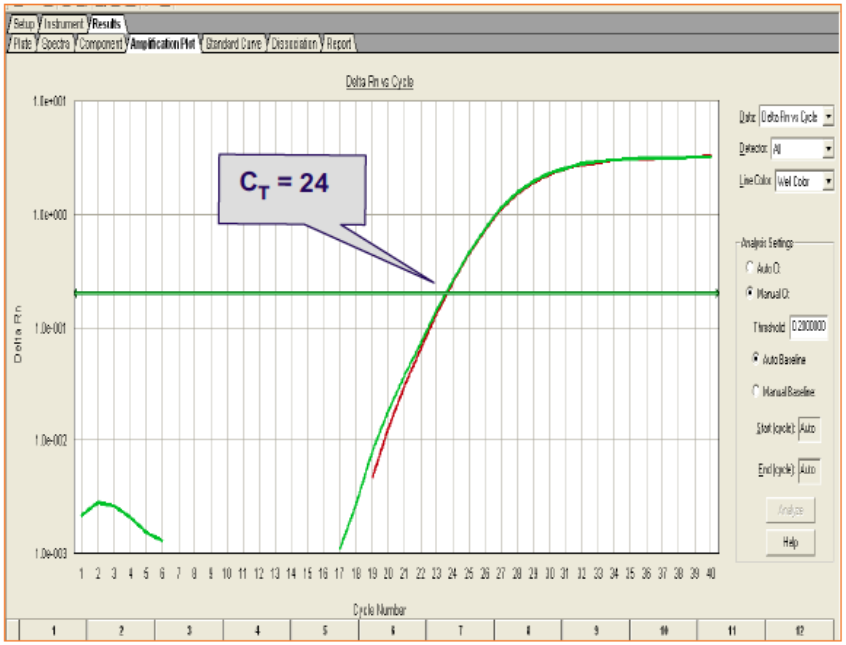


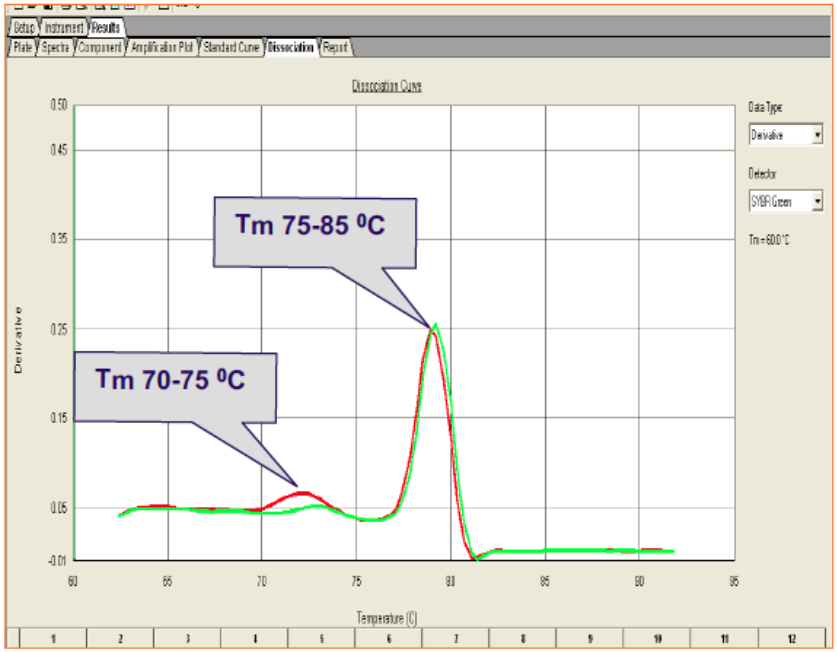
Thermo Fisher MycoSEQ

- AutoMate Express™ Nucleic Acid Extraction System
- Up to 13 samples per run
- Purification time ≤ 1 hr

Thermo Fisher MycoSEQ

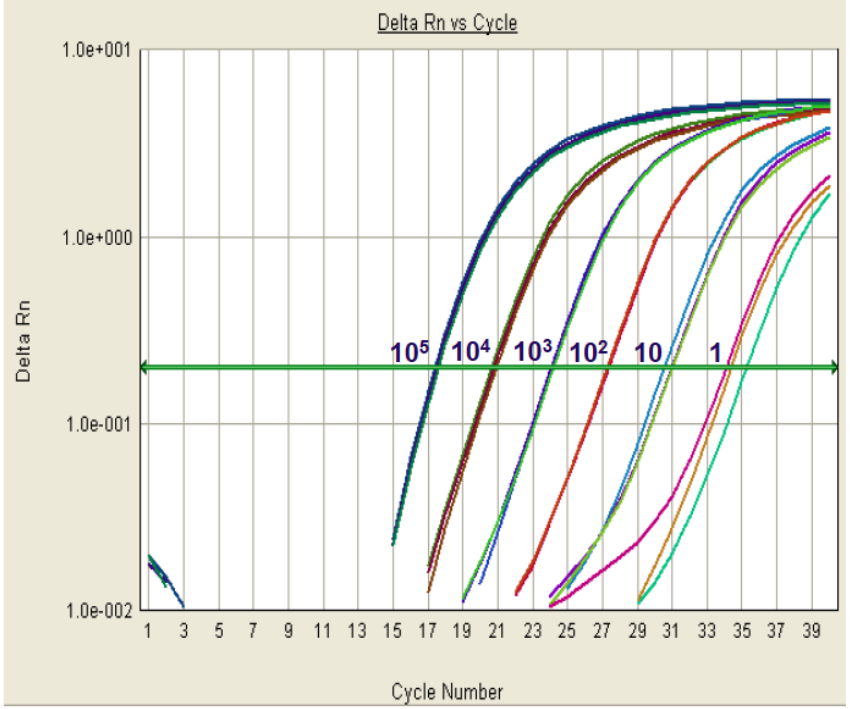
- Detection via real-time PCR and DNA melting curve analysis

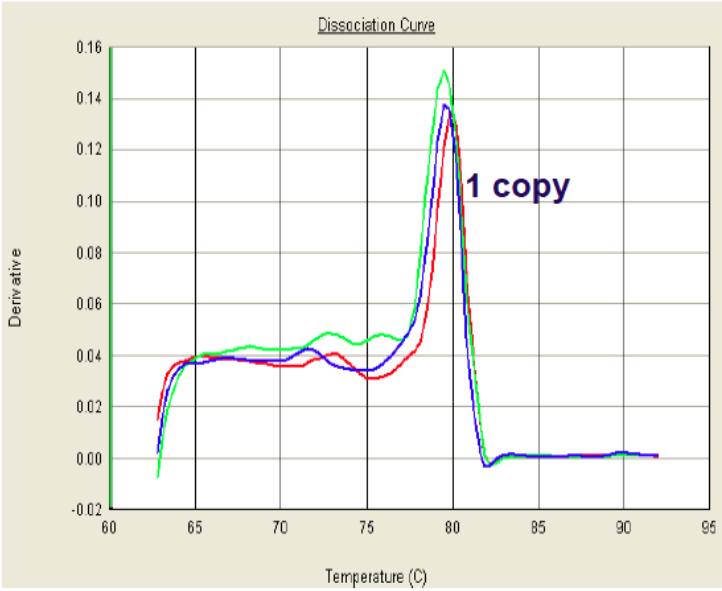




Thermo Fisher MycoSEQ

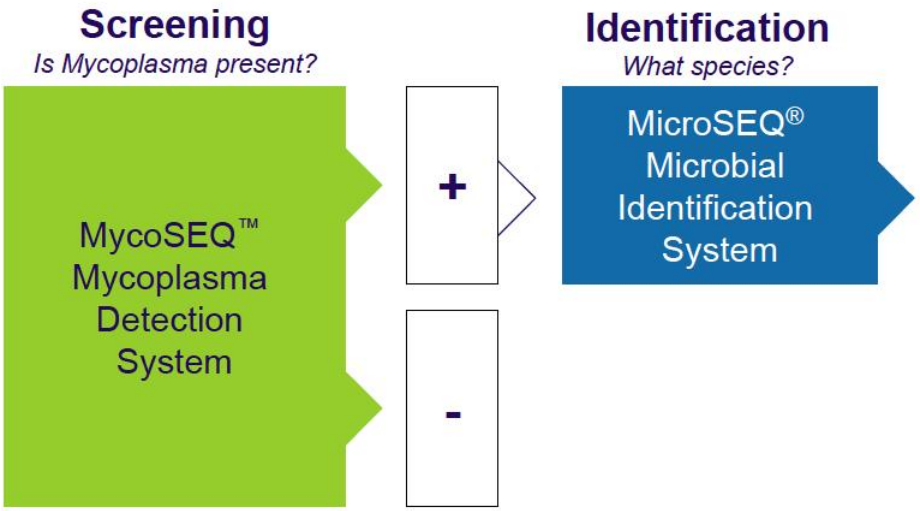
- Assay Sensitivity (example *Mycoplasma arginini*)



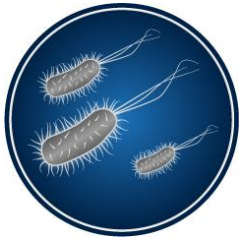


Thermo Fisher MycoSEQ

- If a positive result is obtained, can use MicroSEQ to ID



MICROSEQ® ID 2.1 500BP LIBRARY	
Mycoplasma	*alkalescens*
Mycoplasma	*arginini*
Mycoplasma	*bovigenitalium*
Mycoplasma	*bovirhinis*
Mycoplasma	*bovis*
Mycoplasma	*bovis*
Mycoplasma	*californicum*
Mycoplasma	*canadense*
Mycoplasma	*cynos*
Mycoplasma	*dispar*
Mycoplasma	*equigenitalium*
Mycoplasma	*felis*
Mycoplasma	*fermentans*
Mycoplasma	*gallisepticum*
Mycoplasma	*gateae*
Mycoplasma	*genitalium*
Mycoplasma	*hominis*
Mycoplasma	*hyopneumoniae*
Mycoplasma	*hyorhinis*
Mycoplasma	*iowae*
Mycoplasma	*meleagridis*
Mycoplasma	*orale*
Mycoplasma	*ovipneumoniae*
Mycoplasma	*penetrans*
Mycoplasma	*pirum*
Mycoplasma	*pneumoniae*
Mycoplasma	*pulmonis*
Mycoplasma	*putrefaciens*
Mycoplasma	*salivarium*
Mycoplasma	*synoviae*

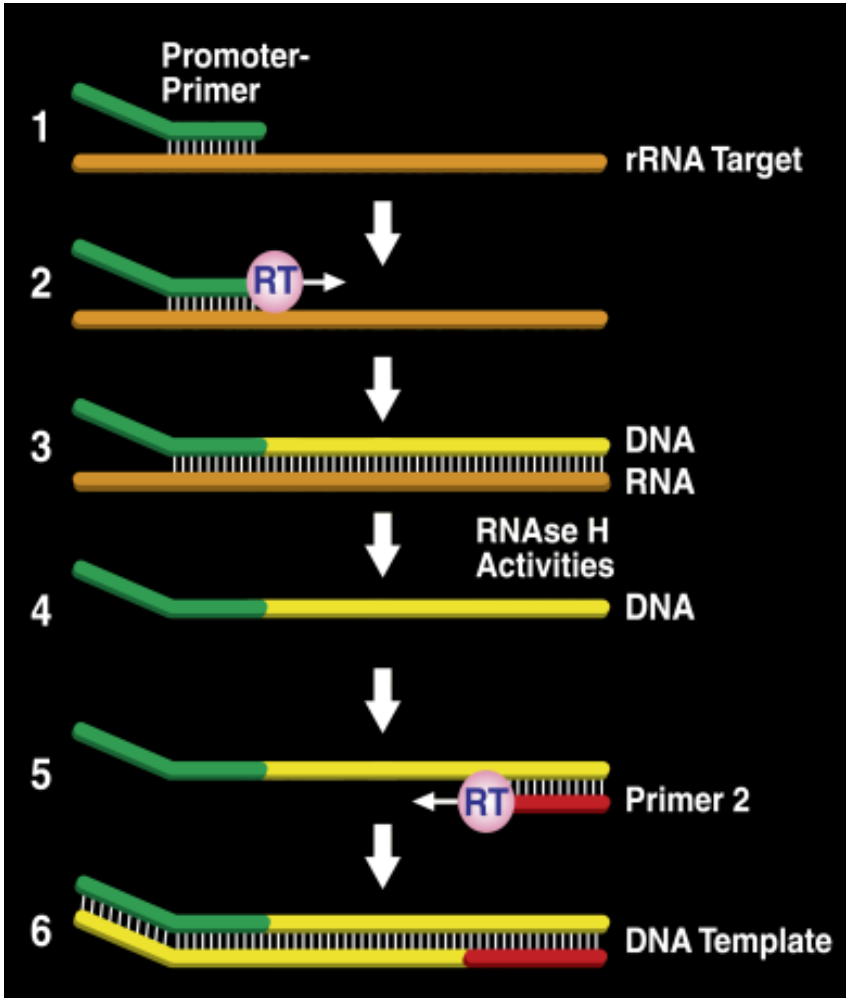


Transcription-Mediated Amplification

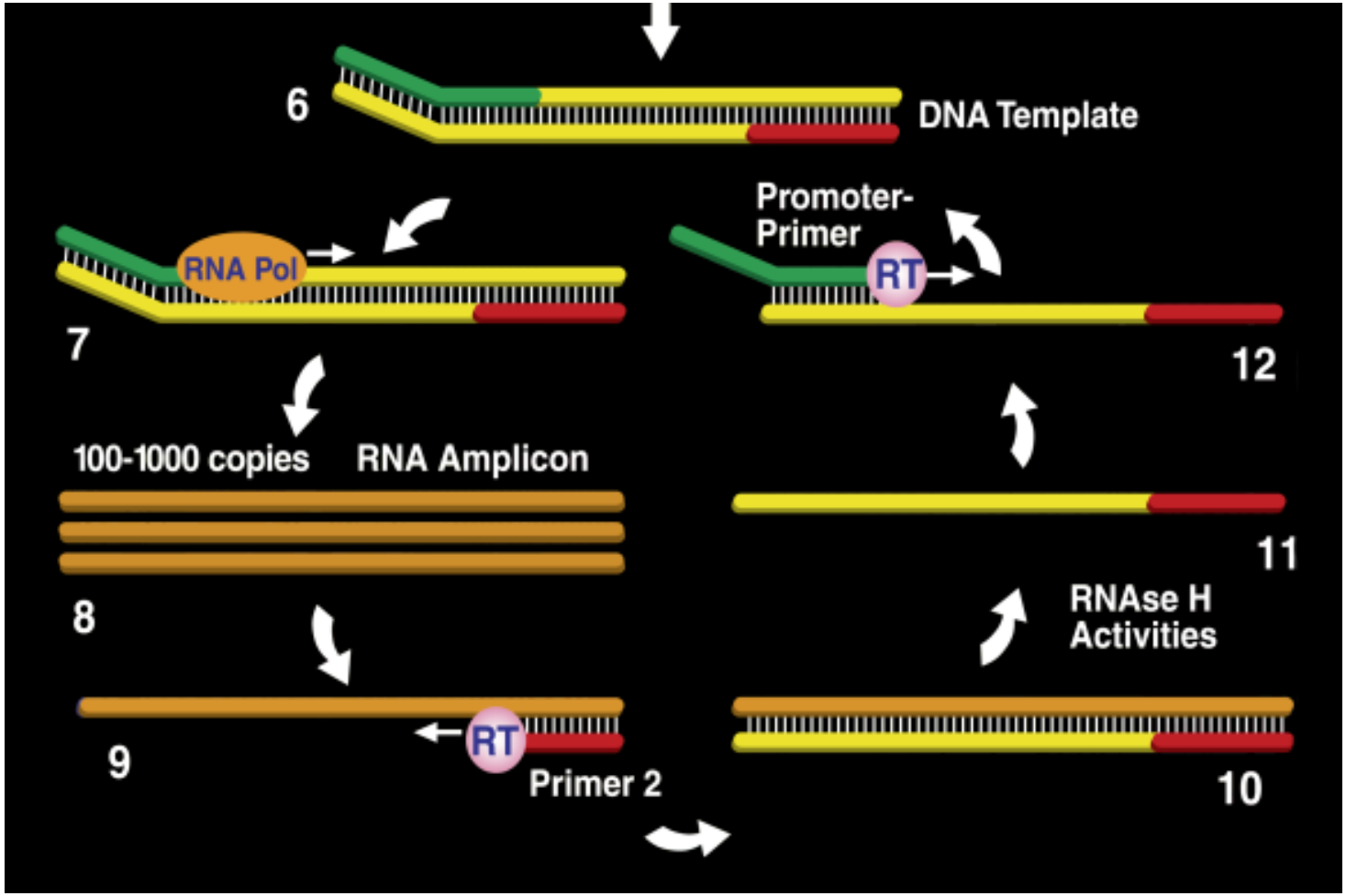
- Real-Time Transcription Mediated Amplification (TMA) is now being used for Mycoplasma testing
- Targets ribosomal RNA
- RNA is a better marker of cellular viability
- RNA is not as stable outside of the cell as DNA
- Less risk of detecting DNA from non-viable cells or contamination from residual DNA in the sample and/or the work environment

Transcription-Mediated Amplification

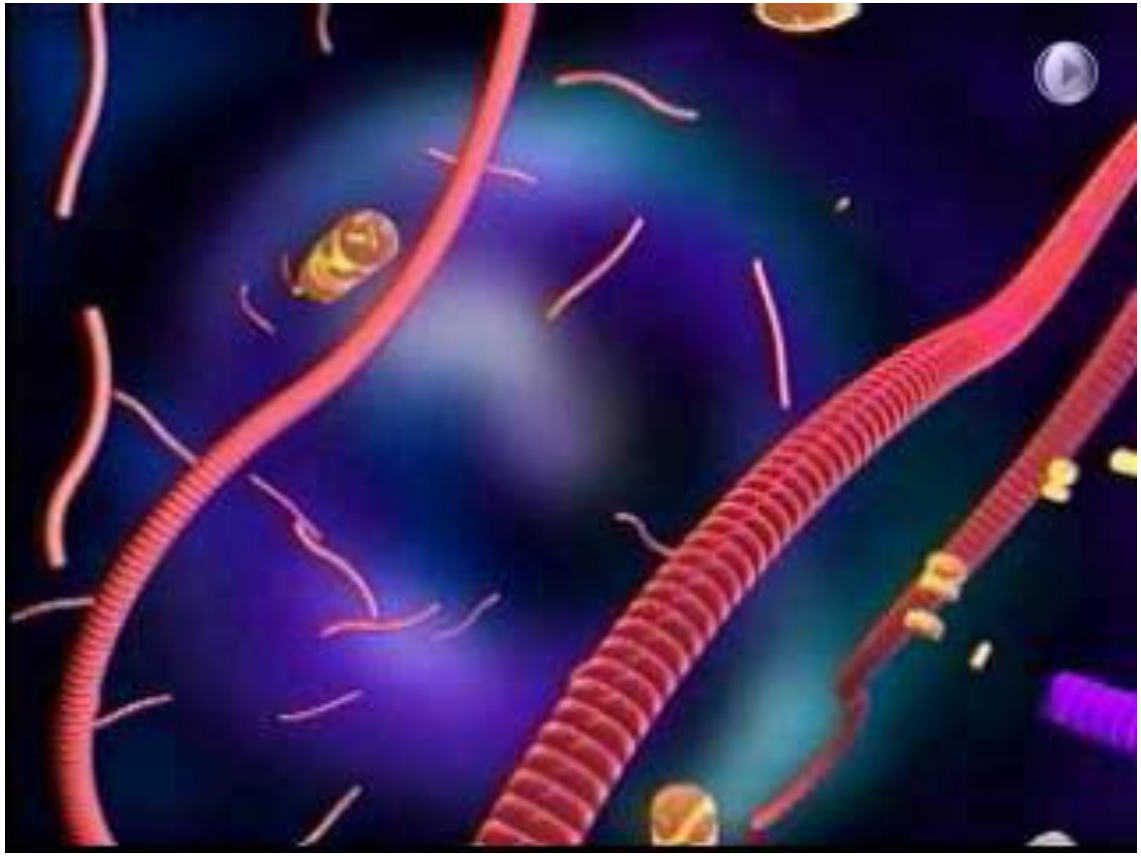
- Primer binds to RNA sequence
- Reverse transcriptase (RT) synthesizes complimentary DNA
- RT mimics RNAse H activity and removes the single-strand RNA
- Second primer binds to remaining cDNA strand
- RT synthesizes second cDNA strand
- RNA polymerase makes millions of copies of RNA and the process is repeated (next slide)

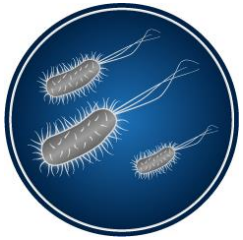


Transcription-Mediated Amplification



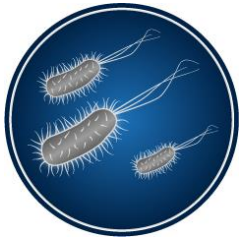
Transcription-Mediated Amplification





TMA Advantages Over PCR

- Easy to use; single tube format with no washing steps
- Isothermic reaction (e.g., 42°C); no need for a thermocycler
- Enzymes are less sensitive to interference
- Less risk of false positives from other organisms, nonviable cells or residual DNA
- Targets 1000's of RNA copies per cell vs. 1 DNA target
- Produces 100-1000 RNA copies per cycle compared with two DNA copies per cycle (10 billion fold increase of copies within 15-30 min)

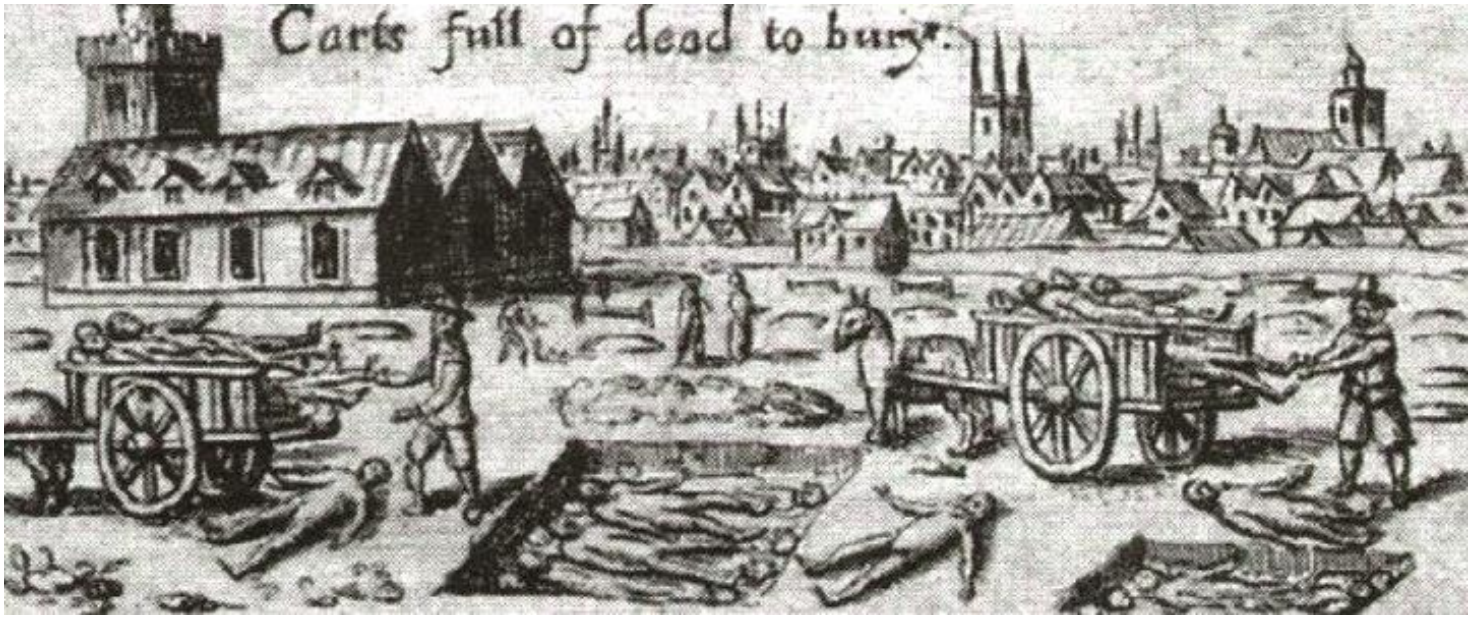


TMA vs. NASBA

- TMA (transcription-mediated amplification)
  - Uses reverse transcriptase (RT) with RNAse *activity* and RNA polymerase
- NASBA (nucleic acid sequence-based amplification)
  - Uses reverse transcriptase (RT), RNA polymerase, and RNase H added to the reaction mixture

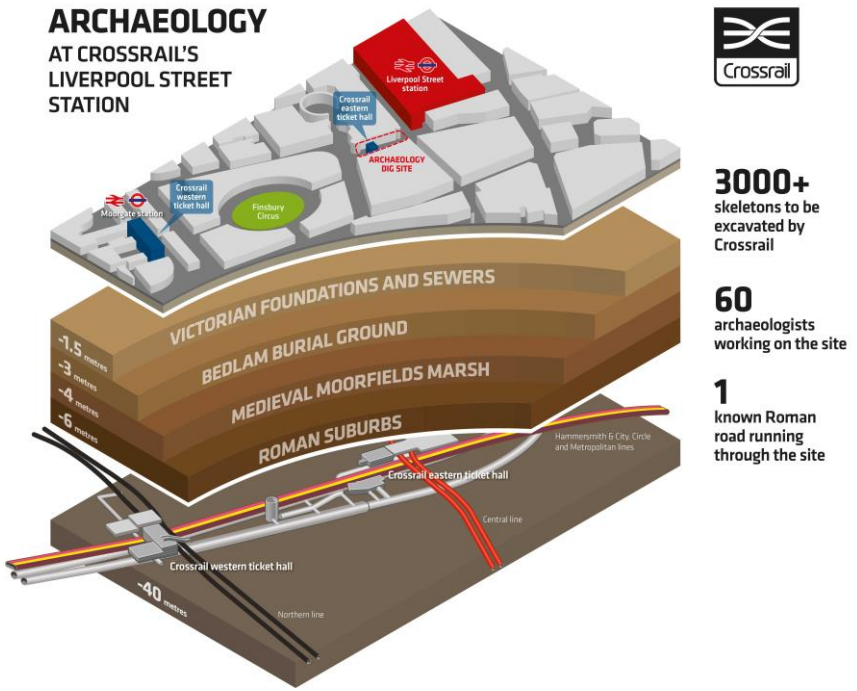
Final Note

- 1665-1666: The Great Plague of London (100,000 dead)
- We always assumed this was caused by *Yersinia pestis*



Final Note

- 2016: London Underground extension; human remains were excavated from a supposed “plague pit”
- Confirmed *Y. pestis* using nucleic acid amplification



The End of Genetic-based RMMs