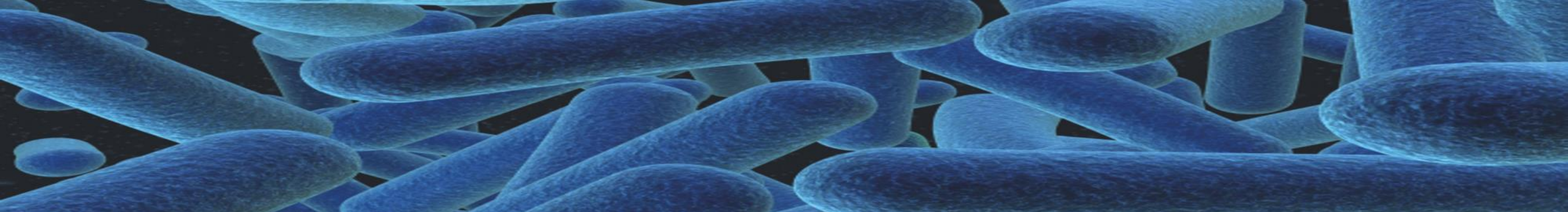
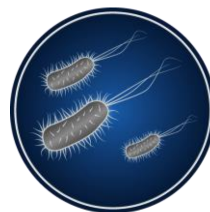


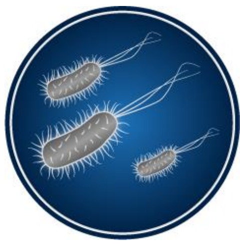
Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods

Cellular Component-based Technologies

Michael J. Miller, Ph.D.

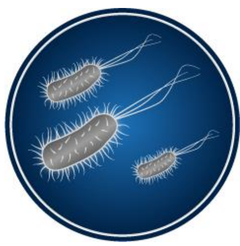


MICROBIOLOGY
CONSULTANTS, LLC



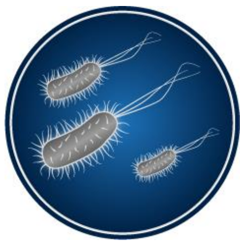
Cellular Component-based Technologies

- Rely on the analysis of cellular components or the use of probes that are specific for microbial target sites of interest
  - ATP
  - Fatty acids in cell membranes
  - Surface macromolecules
  - Bacterial endotoxin
  - Proteins
  - Nucleic acids



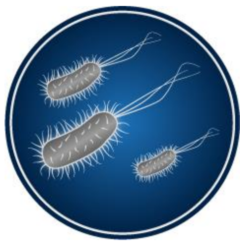
Scientific Principles

- ATP Bioluminescence
- Endotoxin Detection
- Fatty Acid Profiling
- Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Mass Spectrometry
- Surface Enhanced Laser Desorption Ionization Time of Flight (SELDI-TOF) Mass Spectrometry
- Fourier Transform-Infrared (FT-IR) Spectrometry



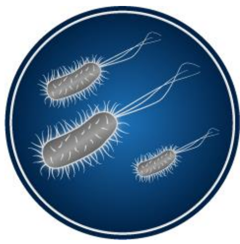
ATP Bioluminescence

- Bioluminescence is the generation of light by a biological process
- 1947: William McElroy discovered the mechanism by which bioluminescence occurs
- Observed in the tails of the American firefly *Photinus pyralis*
- Specific enzyme reaction catalyzing the consumption of ATP (Adenosine Triphosphate)



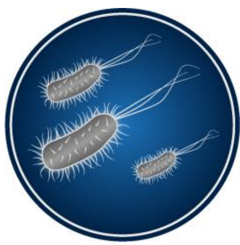
ATP Bioluminescence

- In the presence of the substrate luciferin, the enzyme luciferase will use the energy from ATP to oxidize luciferin and produce photons (hv; light at a wavelength of 562nm)



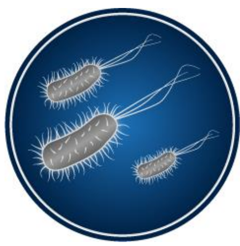
ATP Bioluminescence

- Because all living cells store energy in the form of ATP, it can be used as a measure of organism viability
- Capture microorganisms, release ATP from within the cells, and measure the amount of bioluminescence generated
- Instruments utilize a luminometer equipped with a photomultiplier tube to detect the photons



ATP Bioluminescence

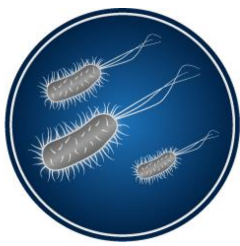
- The concentration of ATP required for measurement is about 200 attomoles, which is equivalent to one yeast or mold cell or approximately 100 bacterial cells, depending on their metabolic state.
  - May require up to 1000 bacterial cells
- When low numbers of cells are expected, an enrichment step in media is required to allow the cells to multiply and produce a sufficient level ATP for detection



Pall Pallchek

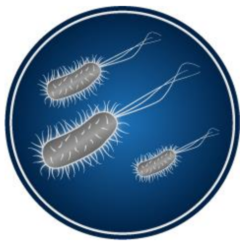
- **No longer sold by Pall Corporation**
- Enrich sample (e.g., 18 h) on media to allow growth of bacteria
- Add luciferin and luciferase
- Results were provided as relative light units (RLU)
- It is worthwhile presenting a case study on this technology as this can be used for similar systems

Rapid Microbiological Methods. © Michael J. Miller, Ph.D. 2018.



Case Study

- GSK received FDA approval to use the Pallchek system for the early release of a non-sterile prescription nasal spray product (up to four days earlier than conventional methods)
- They were the first pharmaceutical company to obtain an approval under the FDA PAT initiative
- The firm used a comparability protocol and implemented the technology under a CBE-0
- Filtered the product, enriched overnight and tested the filter
- They used a 2-tiered approach to product release



Case Study

Prepare samples

Incubate

Measure ATP levels

ATP Positive (RLU > set limit)

ATP Negative (RLU < set limit)

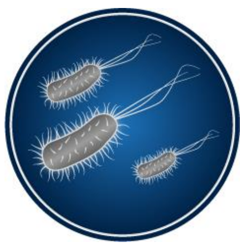
Enumerate & Identify (2-4 Days)

Specification met

PASS

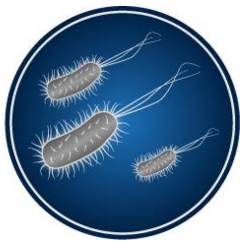
FAIL

RELEASE



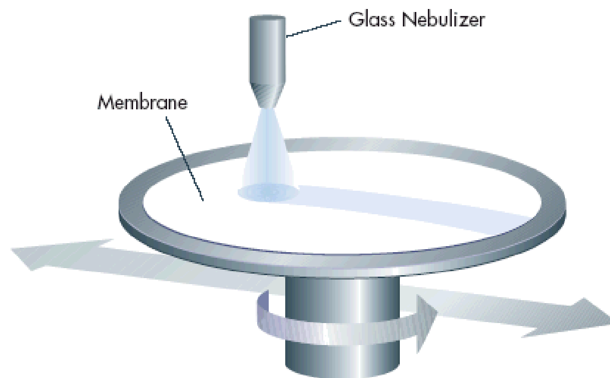
Millipore Milliflex Rapid Microbiology Detection System

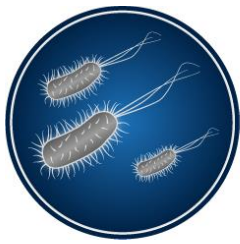
- Utilizes a filter membrane to capture individual cells, allow them to grow into micro-colonies and provide an actual cell count
- Pass sample through 0.45 micron PVDF membrane
- Can rinse filter to reduce bioluminescence inhibition or interference



Millipore Milliflex Rapid Microbiology Detection System

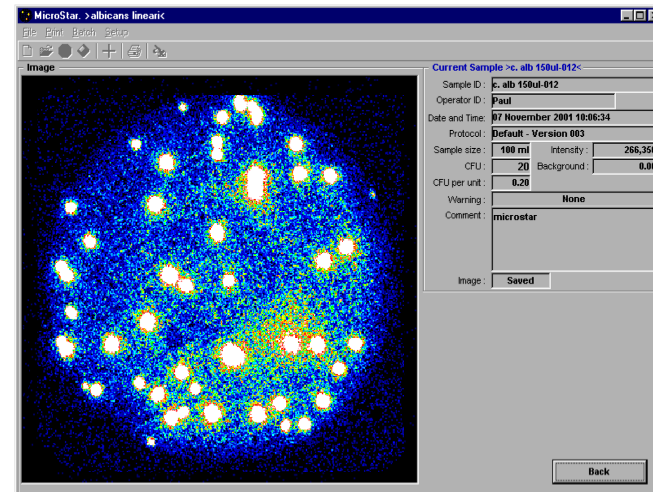
- For bacterial detection, incubate on appropriate medium to form micro-colonies (e.g., 18 hrs)
  - Growth is not required for yeast or vegetative mold
- The filter is then placed into the AutoSpray station, where ATP releasing agent and bioluminescence reagents are applied

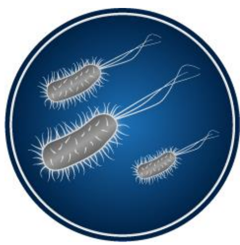




Millipore Milliflex Rapid Microbiology Detection System

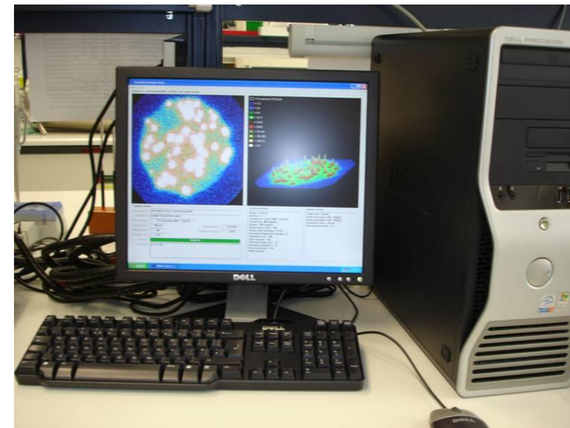
- The filter is then transferred to the Detection Tower
  - The detection tower intensifies bioluminescence from each cell (or micro-colony) thousands of times
  - CCD camera sees signal, image processor provides cell count
  - Each image theoretically arises from a single cell
  - May be non-destructive (continue to grow micro-colonies)

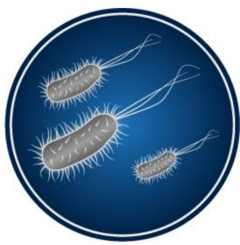




Case Study

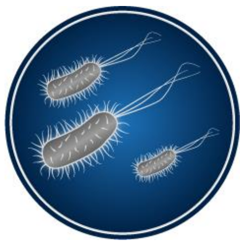
- In 2010, Novartis received FDA, EMA and MHRA approval to use the Milliflex Rapid system as an alternative to the compendial sterility test
- Reduces the 14 day sterility test to 5 days
- Same filtration volumes and rinse fluids as compendial test, then uses agar media for growth of micro-colonies





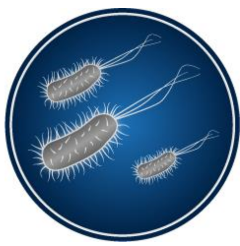
Case Study

- They conducted extensive testing to determine the most optimal growth medium and incubation parameters where micro-colonies can develop
  - Modified Schaedler blood agar (Rapid Sterility Test Medium)
  - Aerobically at 20-25° C, aerobically at 30-35° C and anaerobically at 30-35° C in an anaerobic jar
  - Stressed organisms (heat, UV, exposure to preserved product)
- Incubation time has been validated at 5 days
  - The time required to detect a "worst-case, slow growing microorganism" (*Propionibacterium acnes*), plus additional time to account for variability and testing in the laboratory



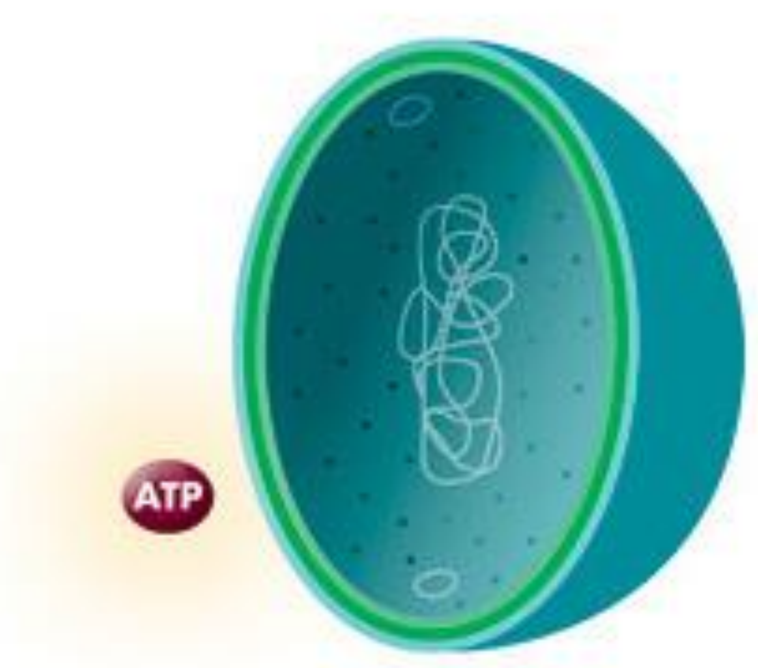
Celsis/Charles River Labs Advance II System

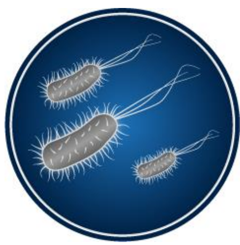
- Same ATP detection principle as previously discussed
- Celsis RapiScreen reagents contain luciferin and luciferase
- Measures Relative Light Units (RLU)



Celsis/Charles River Labs Advance System

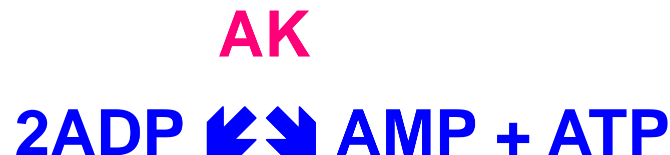
- ATP bioluminescence is limited by the fact that an organism can contain only a finite amount of ATP
- An average bacterial cell contains 1 attomole of ATP
- Celsis developed a method to amplify the amount of ATP generated in the cell

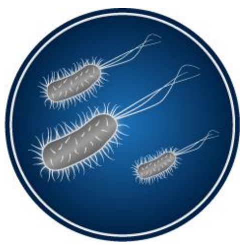




Celsis/Charles River Labs Advance System

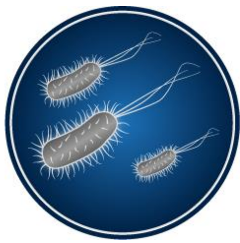
- All living organisms also contain adenylate kinase (AK), another vital part of energy metabolism
- Because AK is an enzyme, rather than a metabolite, it is possible to use AK to generate almost unlimited amounts of its products
- AK catalyzes the linear amplification of ADP to high levels of ATP:





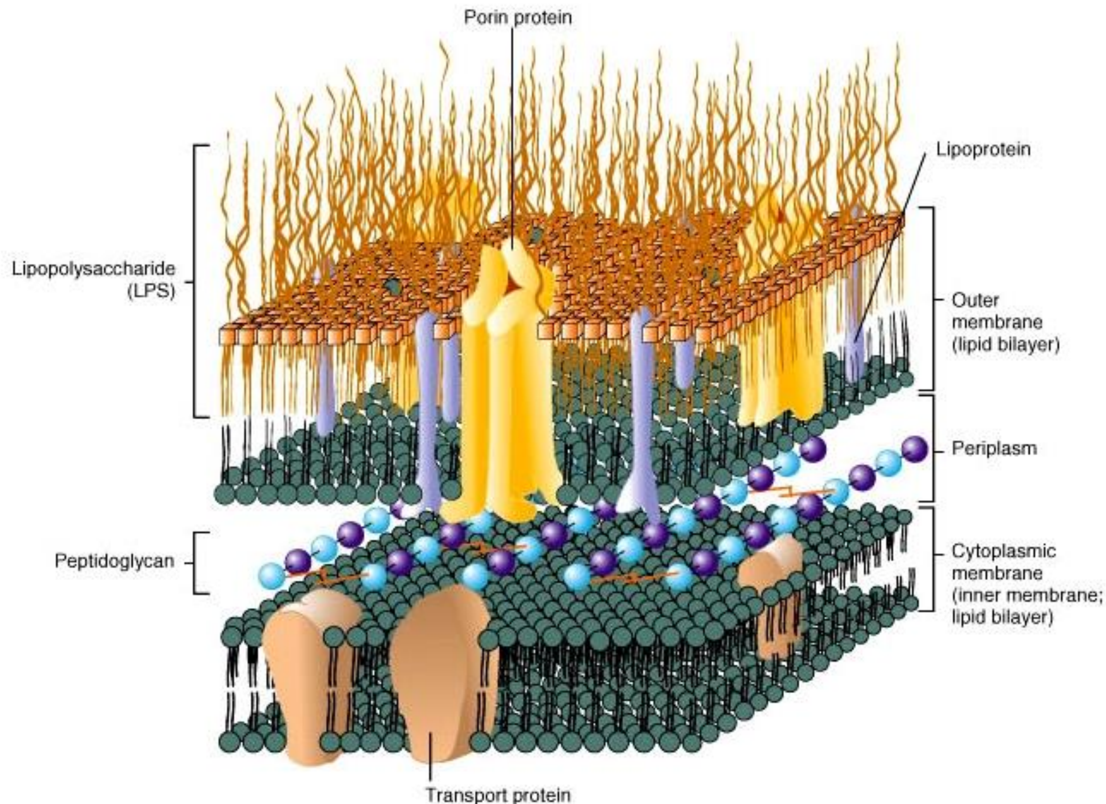
Celsis/Charles River Labs Advance System

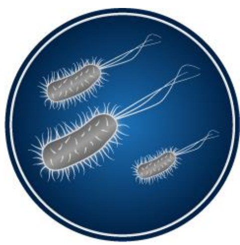
- Extract AK and ATP from the cells
- Add Celsis AMPiScreen reagent, which contains ADP
- Add luciferin and luciferase
- The resulting reaction produces a 1000-fold increase in ATP
- This procedure is being used to detect growth during a sterility test after a 7-day incubation period in media



Rapid Detection of Endotoxin

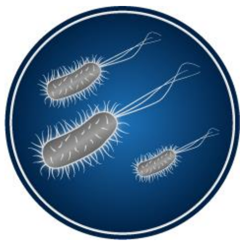
- Lipopolysaccharide from Gram-negative bacteria





Rapid Detection of Endotoxin

- The most widely used methods employ Limulus Amebocyte Lysate (LAL), which is isolated from the blood of the horseshoe crab *(Limulus polyphemus)*



Rapid Detection of Endotoxin

Endotoxin

Glucan

Factor C → Activated Factor C

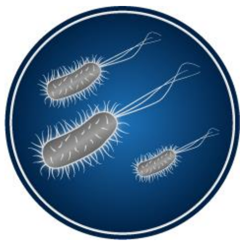
Activated Factor G ← Factor G

Factor B → Activated Factor B

Proclotting Enzyme → Clotting Enzyme

Coagulogen → Coagulin (gel-clot)

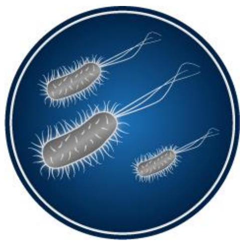
Chromogenic Substrate → Yellow Color



Charles River Labs EndoSafe PTS

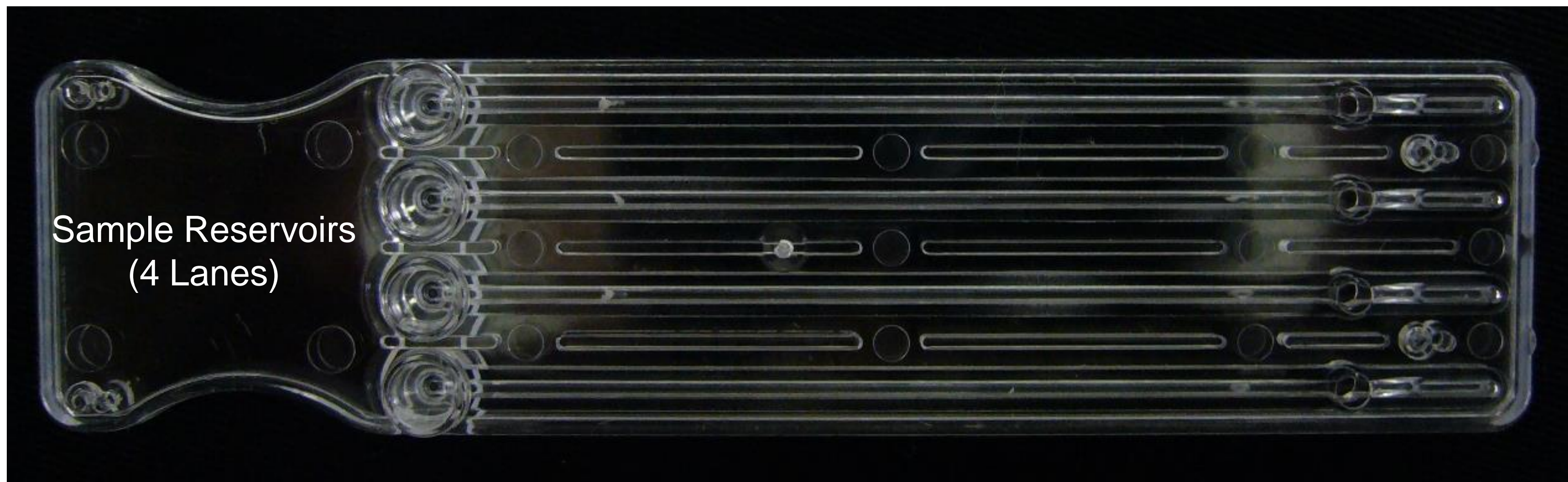
- Endotoxin testing using LAL
- Rapid (15-20 minutes), point-of-use using a disposable cartridge and hand-held, touch screen incubating spectrophotometer
- Quantitative kinetic chromogenic method by measuring color intensity directly related to the endotoxin concentration in a sample

Rapid Microbiological Methods. © Michael J. Miller, Ph.D. 2018.



Charles River Labs EndoSafe PTS

- Each cartridge contains LAL reagent, chromogenic substrate and control standard endotoxin (CSE; mid-point within cartridge range)

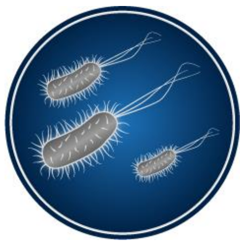


Endotoxin Spikes (Lanes 3-4)

LAL Reagent

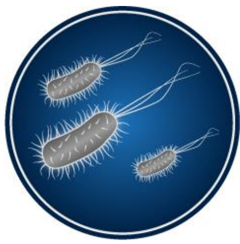
Chromogenic Substrate

Optical Wells



Charles River Labs EndoSafe PTS

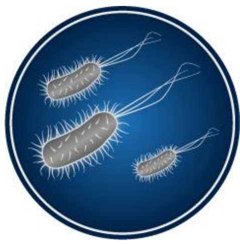
- Add 25 µL of sample into each of the four channels of the cartridge
- The reader draws and mixes the sample with the LAL reagent in two Sample Channels, then with the LAL reagent and positive product control in the Spike Channels
- Sample is incubated and combined with chromogenic substrate
- After mixing, the optical density of the wells are measured and analyzed against an internally-archived standard curve



Charles River Labs EndoSafe PTS

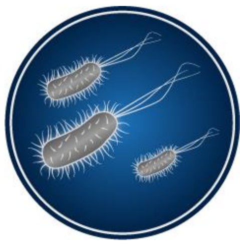
- Test results appear on the screen
- Sensitivity includes 0.01-1.0 and 0.05-5.0 EU per mL
- FDA-approved as an alternative to traditional LAL testing methods for final product release

Version:	: Experimental 0.9
Date / Time:	8/25/15 2:57 PM
Device:	w2515008075
Operator ID:	
Cartridge:	Endotoxin
Temperature:	Start: 37.0C End: 37.0C
Method:	KX-122
Cartridge Lot#:	5146152
Cartridge Cal Code:	511532565565
Range:	5-0.05
Range Time:	Sec: 115-725
Onset Time(s):	> 725 266 > 725 252
Dilution:	1
Sample Lot:	99732359
Sample ID:	LRW
Sample Reaction Time CV:	0.0% Pass
Spike Value:	0.606 EU/mL
Spike Reaction Time CV:	3.8% Pass
Spike Recovery:	81% Pass
Test Suitability:	Pass
Sample Value:	<0.050 EU/mL



Hyglos EndoLISA

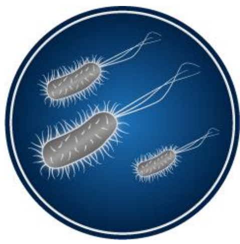
- Uses a phage binding protein for endotoxin detection
- Correlates with kinetic, chromogenic LAL test
- 0.05 - 500 EU/mL sensitivity
- Time to result is ~ 3 hours
- Uses recombinant Factor C



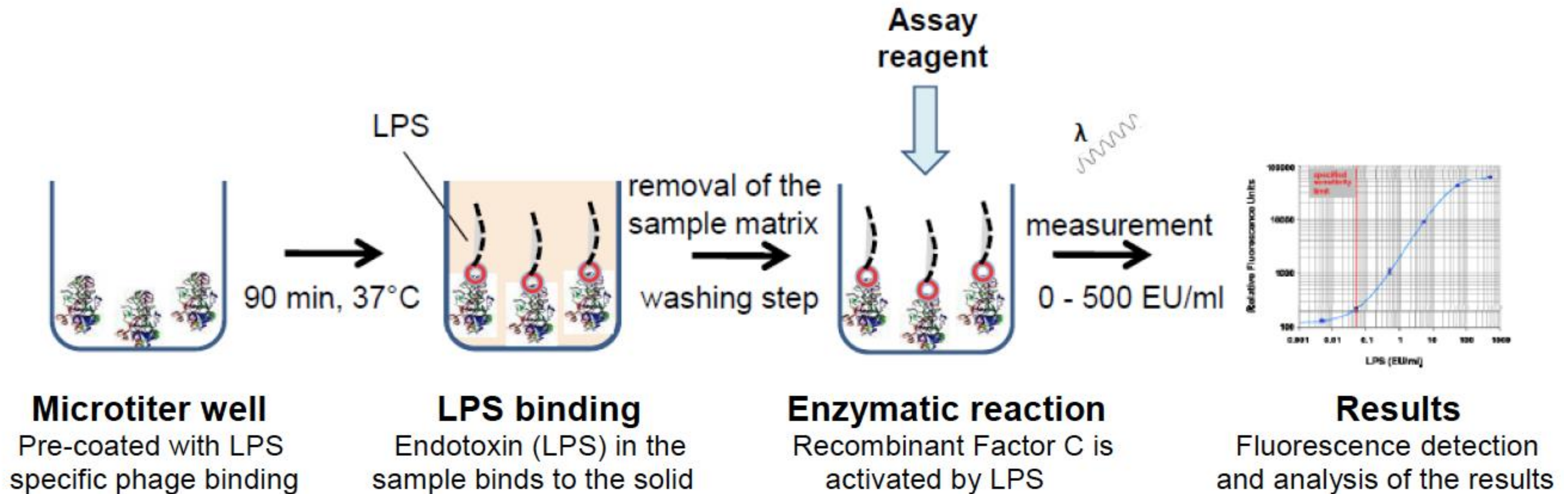
Hyglos EndoLISA

- Microplate is pre-coated with a phage-derived receptor protein that has a high affinity and specificity for the conserved core region of LPS
- 100 µl of the sample is added to a microplate well
- After LPS binding, the adhered sample matrix is washed to remove potential interfering substances
  - Salts
  - Buffers with extreme pH conditions (pH 4-9)
  - Detergents
  - Antibiotics
- LPS is detected using recombinant Factor C and a fluorescent substrate

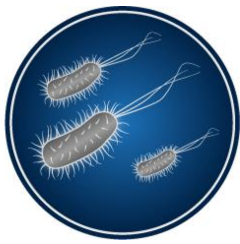
Rapid Microbiological Methods. © Michael J. Miller, Ph.D. 2018.



Hyglos EndoLISA

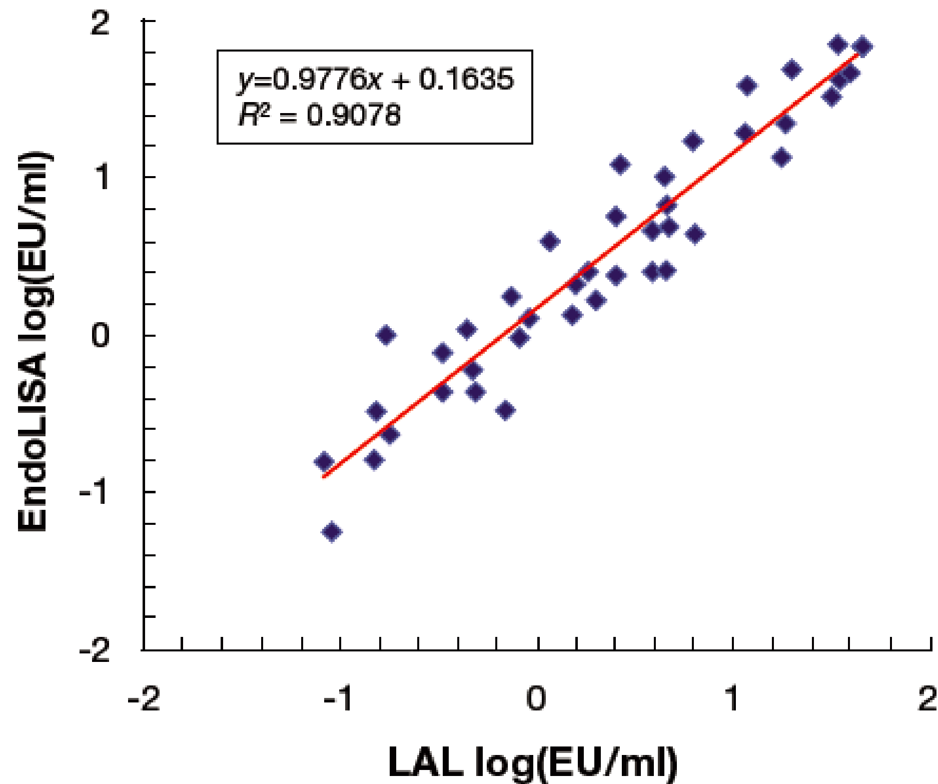


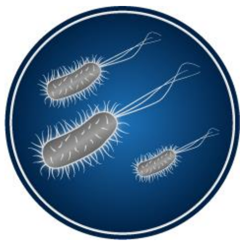
- 90 min binding step in microplate
- Wash 3X
- Add assay reagent, incubate at 37° C for 90 min, detect LPS in a fluorescence reader



Hyglos EndoLISA

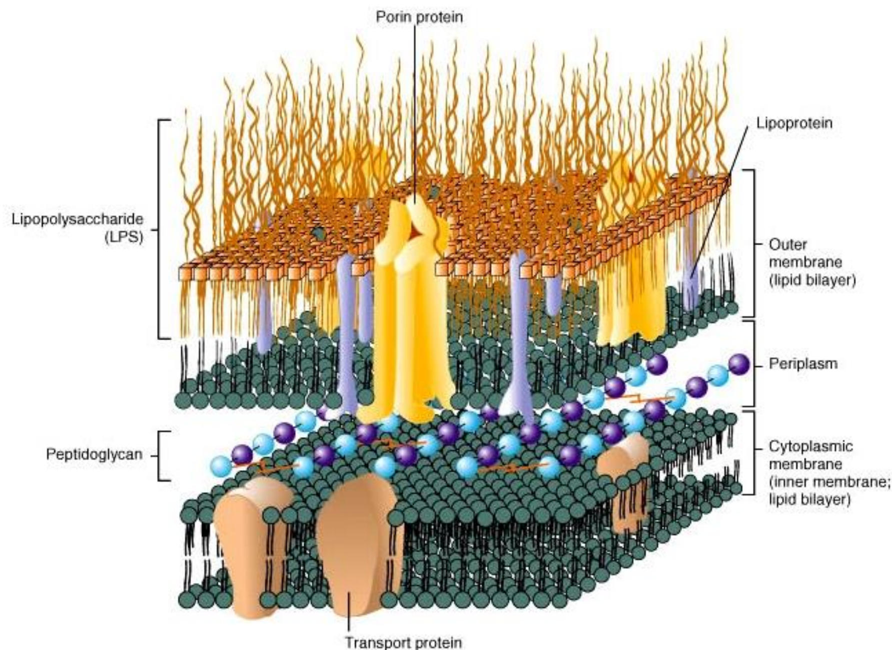
- Recombinant Factor C correlates with LAL assay using *E. Coli, Salmonella* and *Pseudomonas*

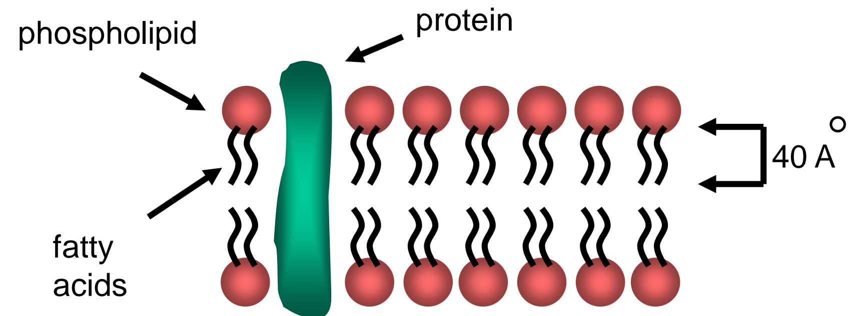


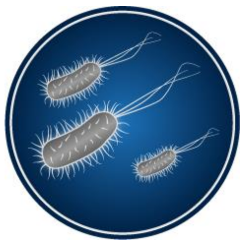


Fatty Acid Analysis

- The cellular membrane contains lipid biopolymers
- Fatty acid profiles provide a fingerprint for microorganism identification

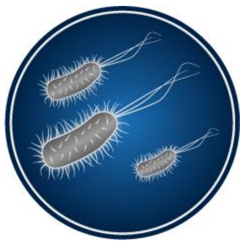






Sherlock MIDI System

- Fatty acids from microorganisms are extracted and used to provide a gas chromatographic profile that can be compared with an internal database
- A loopful of organisms from an isolated colony are lysed to release fatty acids from cellular lipids; fatty acids are processed and placed in a gas chromatography instrument



Sherlock MIDI System

E-FAME™ Rapid Bacterial ID Method

Derivatize

Extract

E1

E2

E3

Pure Culture

Transfer

~5 mg

Heat – 100°C

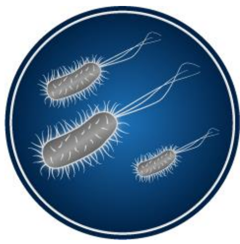
7 min

Top Layer

70 µL

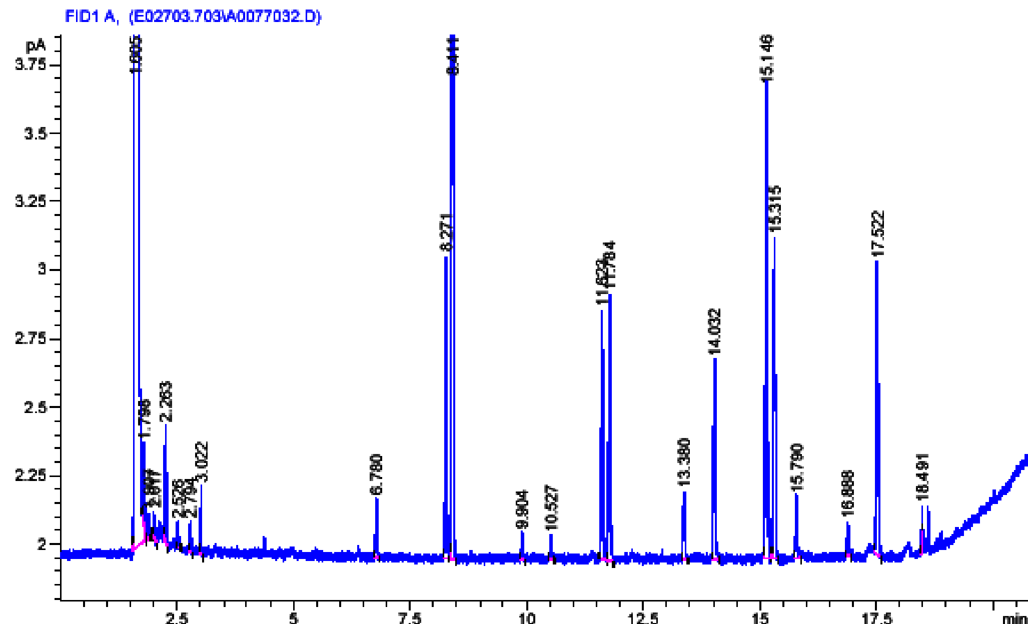
Analysis & Identification

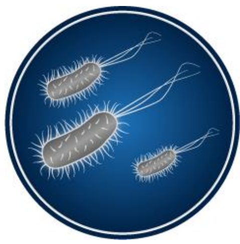
< 20 Minutes



Sherlock MIDI System

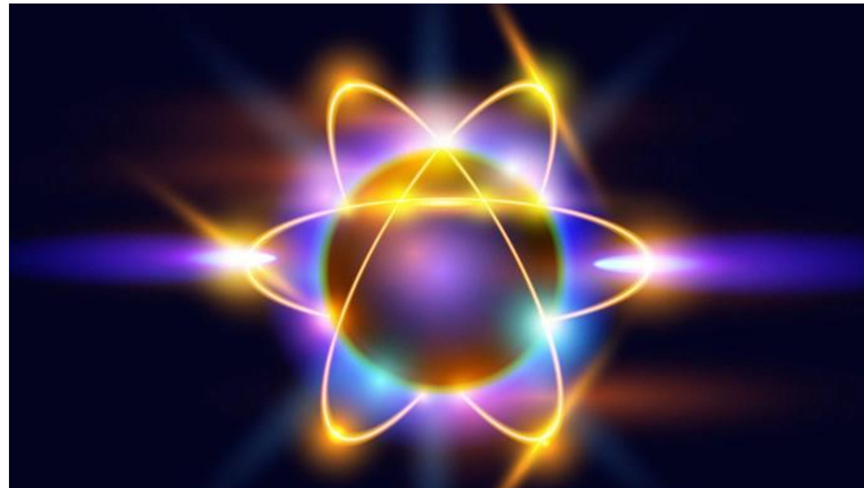
- Each peak corresponds to a specific fatty acid
- Compare fatty acid profile to an internal database to identify more than 1200 bacteria and 200 fungi (yeast and mold)

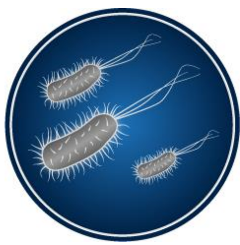




MALDI TOF Mass Spectrometry

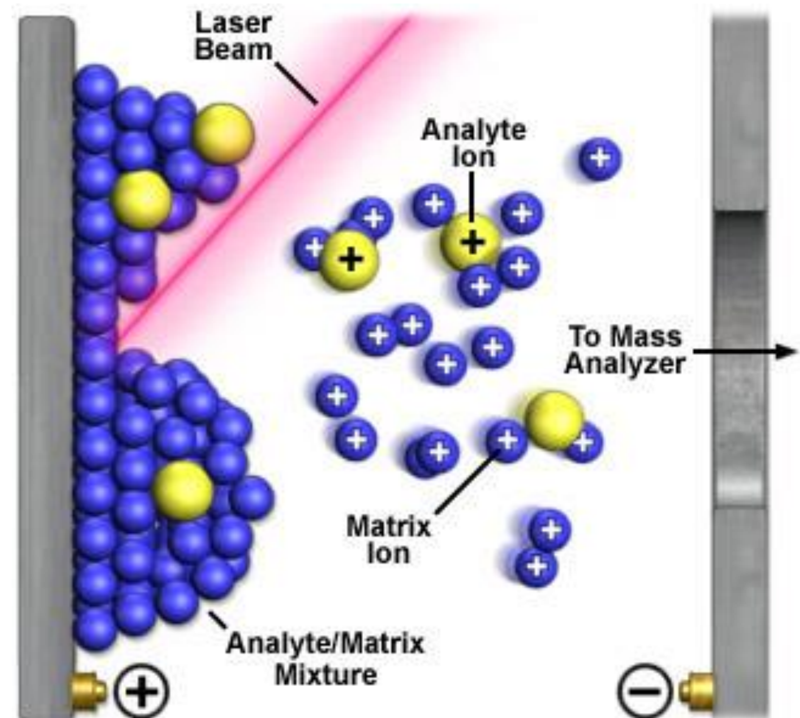
- Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry
- Accurate molecular weight measurement (and characterization) of biomolecules, including proteins, peptides, polysaccharides and nucleic acids

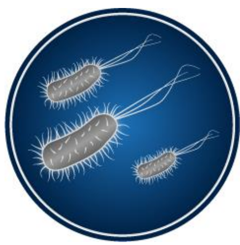




MALDI TOF Mass Spectrometry

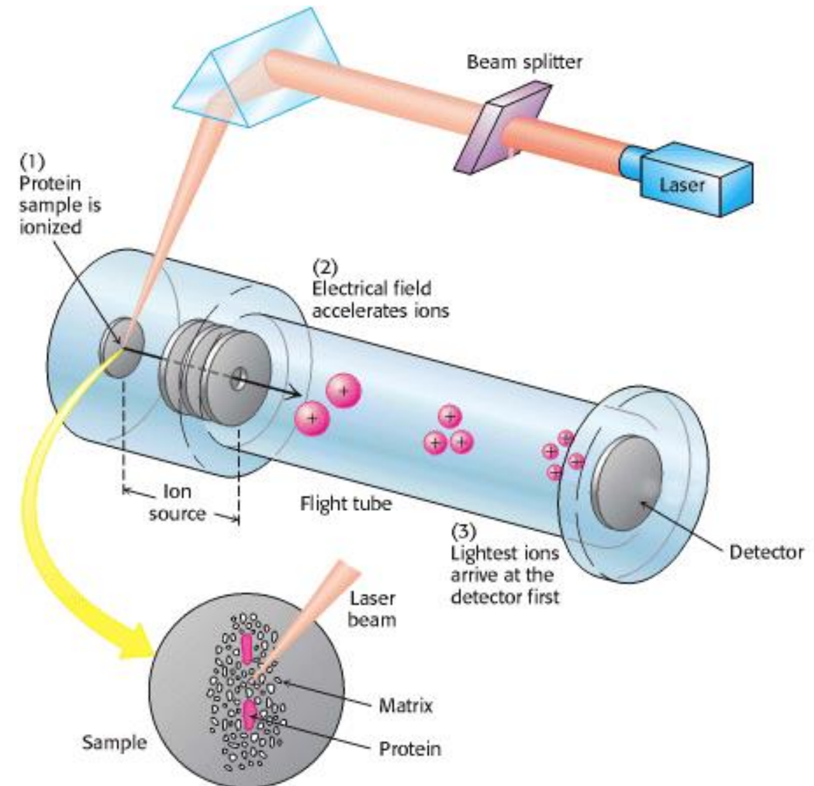
- A biomolecule is combined with a UV-absorbing matrix and ionized by a laser
- The laser energy is absorbed by the matrix, preventing unwanted fragmentation of the biomolecule

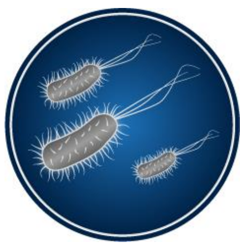




MALDI TOF Mass Spectrometry

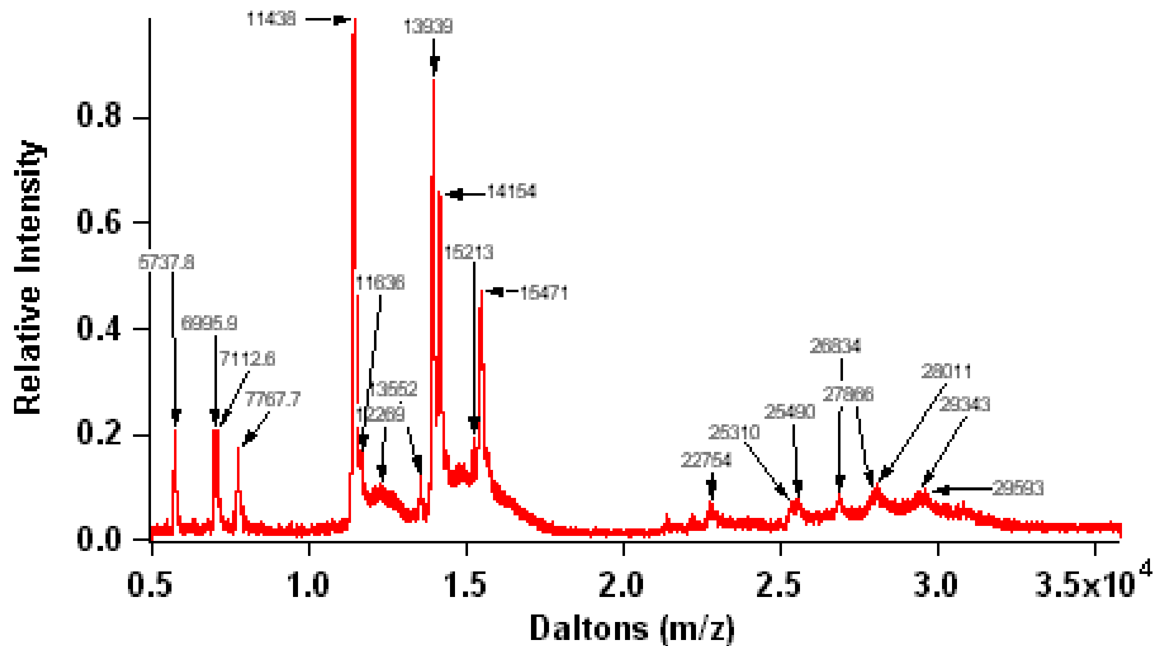
- The ionized particles are then accelerated in an electric field and enter the flight tube
- Different molecules are separated according to their mass to charge ratio and reach the detector at different times

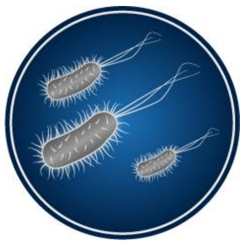




MALDI TOF Mass Spectrometry

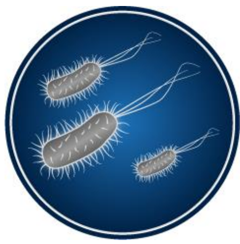
- A mass spectrum is generated within seconds
- Mass spectra can be used for microbial identification





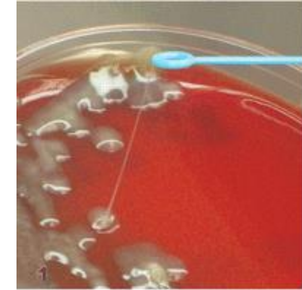
Bruker microflex and MALDI BioTyper Software

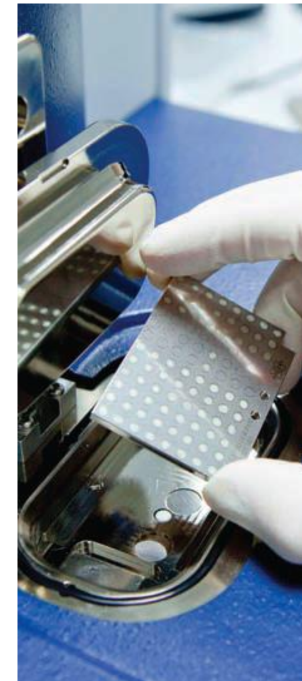
- No need for pre-screening (Gram staining)
- Applicable for bacteria, yeast, mold and Mycobacteria
- Detects proteins and peptides
- Need minimum of 10^5 cells for accurate measurement

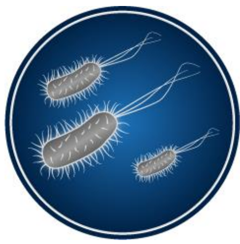


Bruker microflex and MALDI BioTyper Software

- Intact cells from a pure culture are added to a stainless steel target plate and allowed to co-crystallize with the UV-absorbing matrix
- After drying, the target plate is placed into the mass spectrometer, and exposed to a laser
- Ionized proteins and peptides are arranged in a spectrum with increasing mass (between 2-20 kDa)
- Mass spectra are compared with an internal database
- >4,600 spectra; >2,000 species



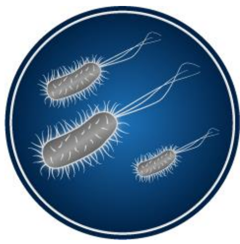




Bruker microflex and MALDI BioTyper Software

- Spectrum of *E. coli* (mass of ribosomal proteins highlighted)

Intens. [a.u.]
4364.06
5380.64
6254.64
6315.49
5096.01
6410.90
7157.65
7273.87
7870.62
8368.99
0 1000 2000 3000 4000 5000
4000 4500 5000 5500 6000 6500 7000 7500 8000
m/z



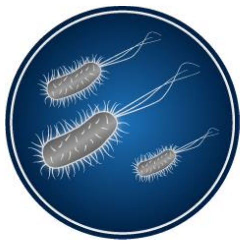
Bruker microflex and MALDI BioTyper Software

Aspergillus fumigatus

Bacillus subtilis

Candida albicans ATCC 10231

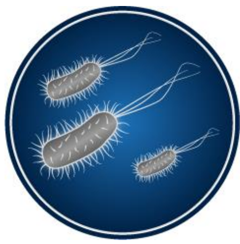
Escherichia coli DH5alpha



Bruker microflex and MALDI BioTyper Software

- *Pseudomonas oleovorans* grown on different media

YPM Medum
Plate count agar
Corynebacterium agar
Gym Streptomyces Medium
Rhizobium Medium
1:10 Dilution MRS medium
YPD Medium
4000 5000 6000 7000 8000 9000 10000 11000
m/z



bioMérieux Vitek MS

- MALDI-TOF mass spec
- >25,000 spectra for clinically-relevant microorganisms