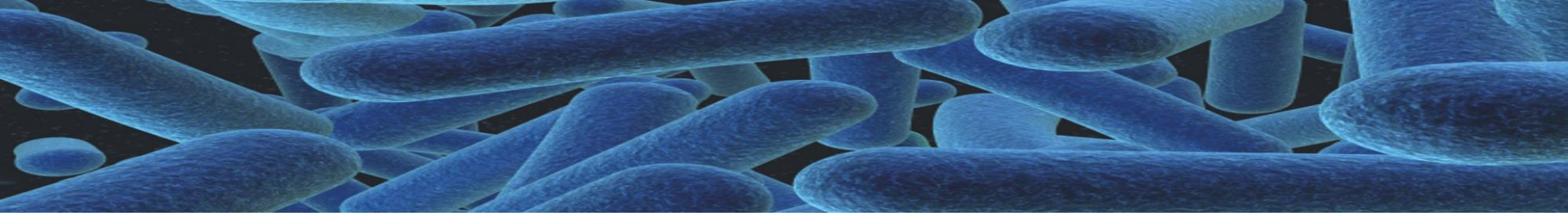


Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods

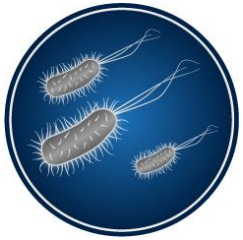
Growth-based Technologies

Michael J. Miller, Ph.D.

MICROBIOLOGY CONSULTANTS, LLC

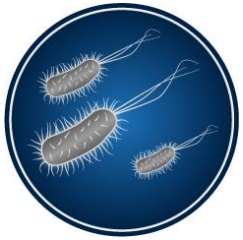
Growth-based Technologies

- Rapid detection, enumeration and identification of actively growing microorganisms
- Usually use conventional media (liquid or solid)
- Applications can include bioburden testing, environmental monitoring, sterility testing, and the identification of microorganisms



Scientific Principles

- Electrochemical Measurement
- Detection of Carbon Dioxide (CO_2)
- Utilization of Biochemical and Carbohydrate Substrates
- Digital Imaging and Auto-fluorescence of Micro-Colonies
- Fluorescent Staining and Laser Excitation of Micro-Colonies
- Use of Selective Media for the Detection of Specific Microorganisms
- Measurement of Change in Head Space Pressure
- Microcalorimetry

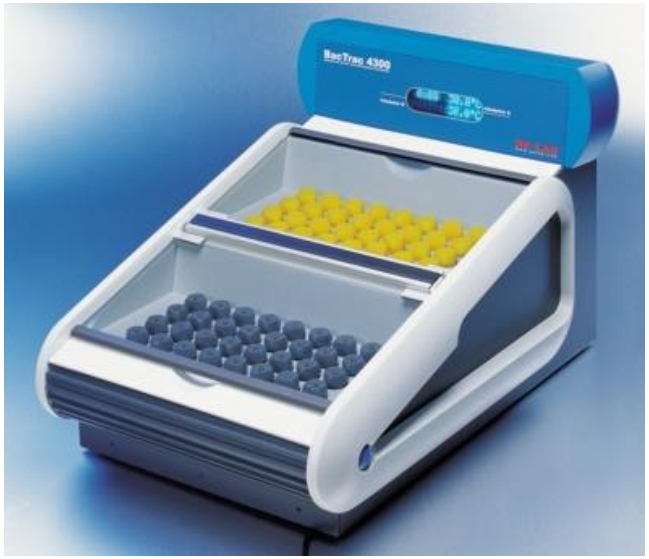


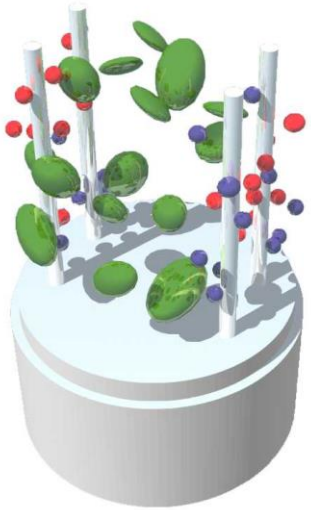
Impedance Microbiology

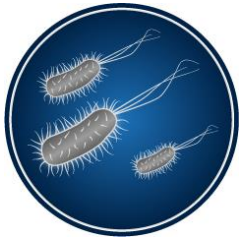
- Microbial growth results in the breakdown of larger, relatively uncharged molecules into smaller, highly charged molecules
  - Proteins into amino acids
  - Fats into fatty acids
  - Polysaccharides/sugars into lactic acid
- Growth is detected by monitoring the movement of ions between electrodes (conductance), or the storage of charge at the electrode surface (capacitance)

Sy-Lab BacTrac 4300
Microbiological Impedance Analyser

- Uses culture vials with 4 electrodes on the botom
- Test samples and liquid media are added to the well
- The holder is placed in an incubator and monitored







Sy-Lab BacTrac 4300 Microbiological Impedance Analyser

- If microbial growth occurs in the liquid media (~100,000 cfu for bacteria and ~10,000 cfu for yeast and mold), changes in impedance can be detected faster than observing turbidity in the media
- The system holds up to 64 samples and incubated in two temperature zones
- Different media can also detect specific organisms:
  - Enterobacteriaceae, coliforms, *E. coli, Pseudomonas aeruginosa*, entrerococci*, Salmonalla, Listeria*, coagulase positive Staphylococci, *Bacillus cereus*, clostridia, lactic acid bacteria, yeasts and mold

Detection of CO_2

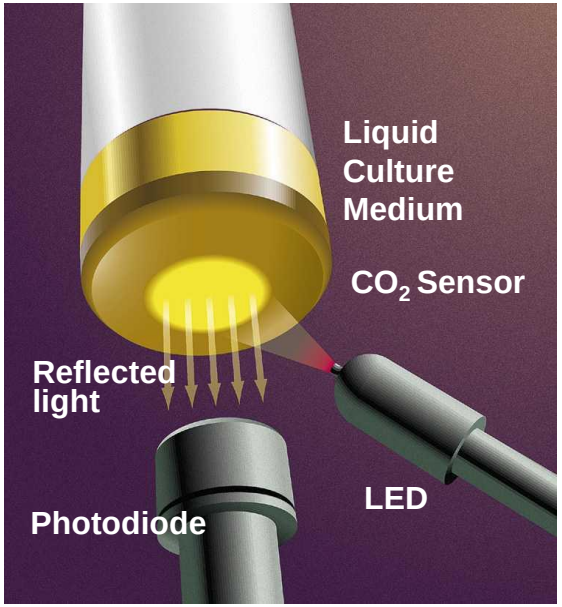
- Microorganisms, when grown in liquid culture, produce carbon dioxide (CO_2) and other metabolites
- In a closed container, the amount of CO_2 produced may be monitored

bioMérieux BacT/ALERT

- Historically used in hospital clinical labs; more recently targeting the pharmaceutical industry
- Samples are added to media bottles that have a liquid emulsion (silicone) sensor
- During microbial growth, CO_2 in the medium diffuses into the sensor

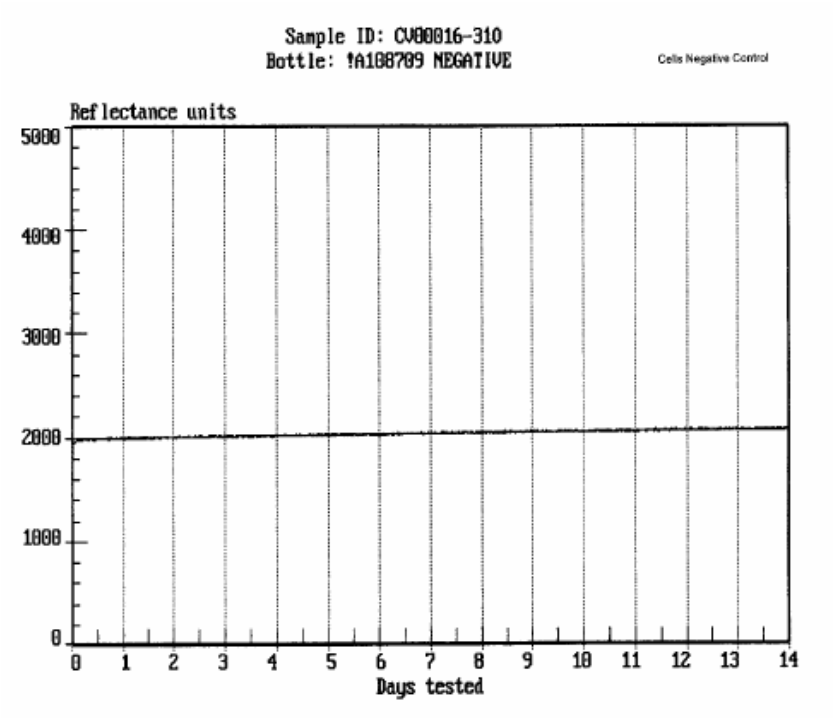
bioMérieux BacT/ALERT

- Hydrogen ions interact with the sensor resulting in a decrease in pH
- The liquid emulsion sensor changes to a yellow color
  - Sensitivity level to produce a color change is not known



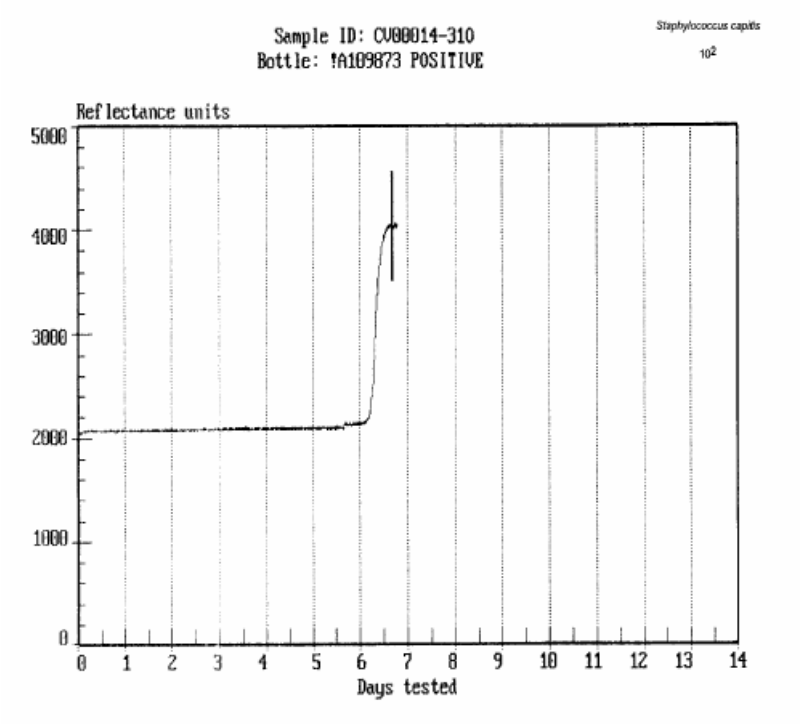
bioMérieux BacT/ALERT

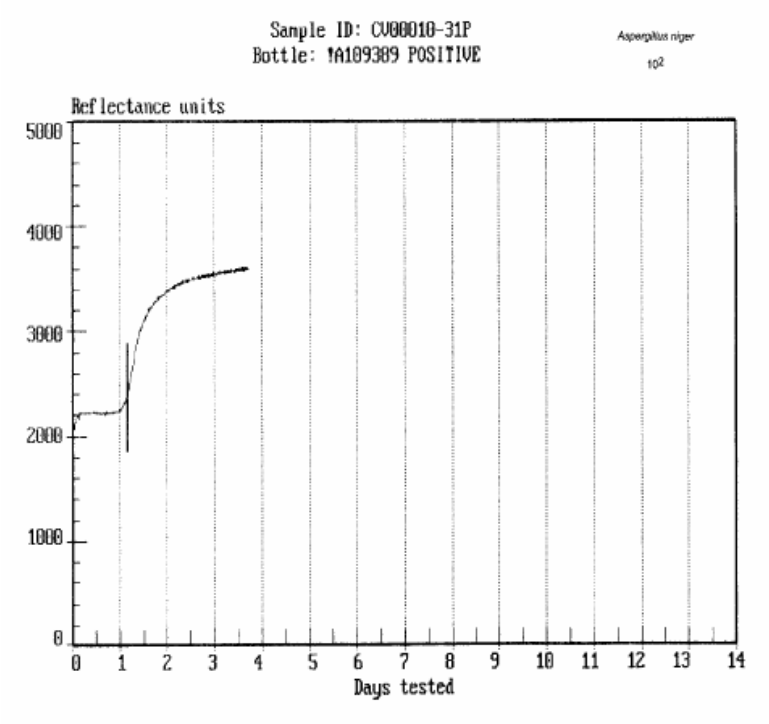
- The rate at which CO_2 is detected depends on the initial concentration of microorganisms
- Example of response with no microorganisms:

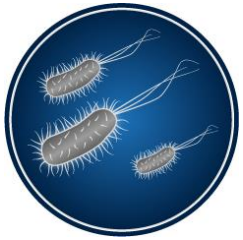


bioMérieux BacT/ALERT

- Example slow and fast growing microorganisms:





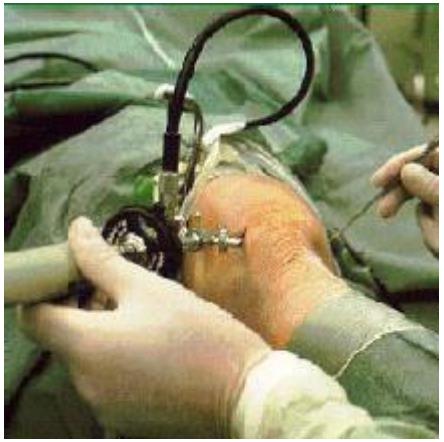


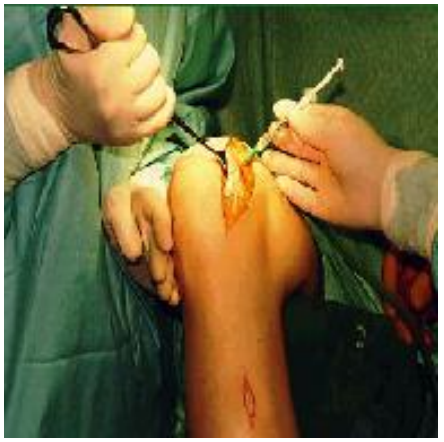
bioMérieux BacT/ALERT

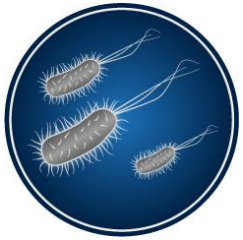
- Primarily used as a presence/absence test
- Offers dual incubation temperatures
- Readings every 10 minutes
- Currently FDA-approved as a rapid sterility test for cell culture products

Case Study

- Genzyme Biosurgery has received FDA approval to release Autologous Cultured Chondrocytes based on interim negative results at 3 days of a 14 day assay, as an alternate to the conventional Steritest system





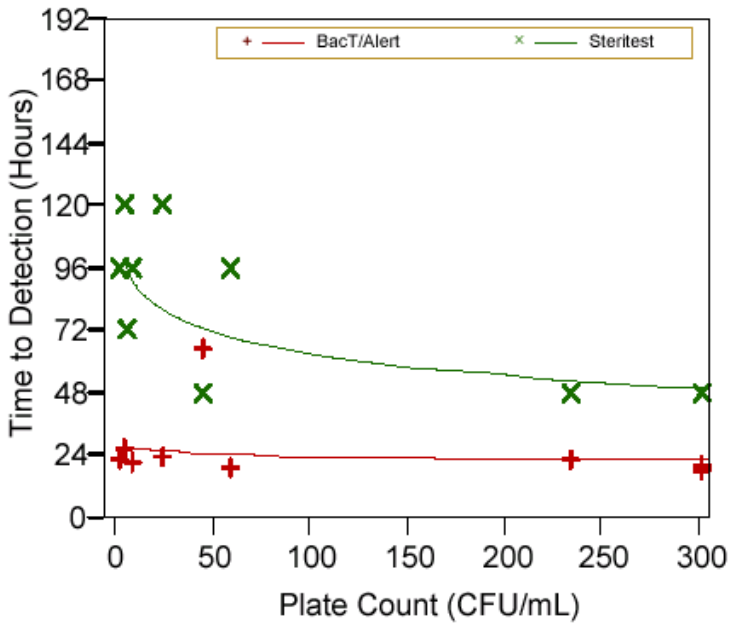


Case Study

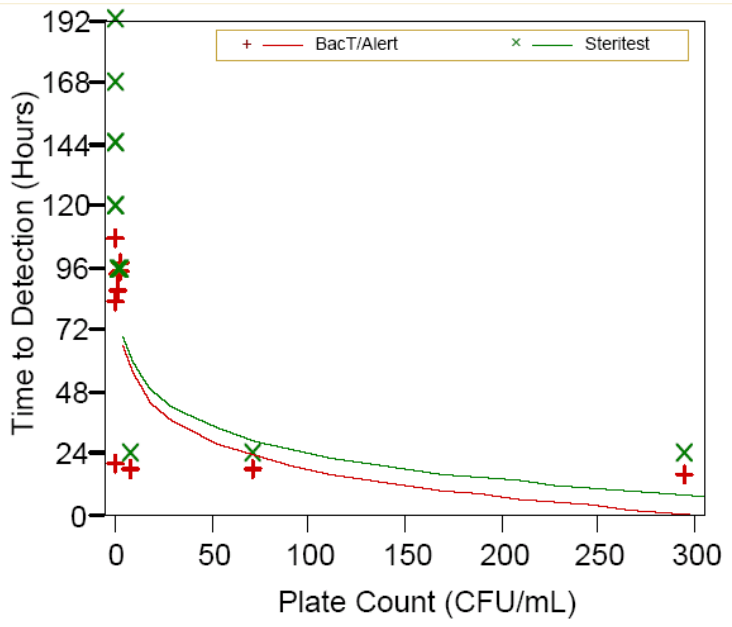
- Sterility test challenges:
- The cell therapy product has a shelf life of 3 days
- The firm cannot wait for the 14 day results to release the product for implantation
- The product may appear turbid when placed in sterility test media
- Each patient represents a unique testing lot with multiple tests per lot

Case Study

- Time to detection compared with Steritest system



Staphylococcus warneri



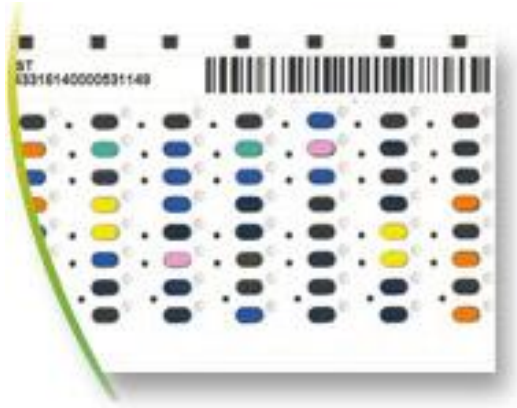
Propionibacterium acnes

Other CO_2 Detection Systems

- BD Diagnostic Systems BACTEC FX
  - Primarily used in clinical labs
  - Fluorometric sensor

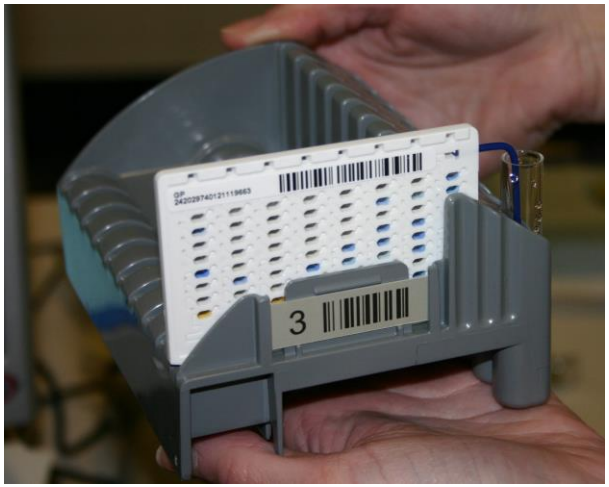
bioMérieux Vitek 2 Compact

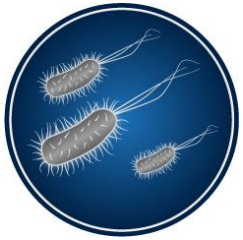
- Measures the ability of microorganisms to utilize a variety of biochemical and carbohydrate substrates dehydrated onto 64 well cards
  - Gram-negative bacilli
  - Gram-positive cocci & bacilli
  - Yeasts
  - Neisseria, *Haemophilus* and other fastidious Gram negative bacteria
  - Anaerobic bacteria and coryneform bacteria



bioMérieux Vitek 2

- Organisms are grown on media and isolated colonies are used to inoculate Vitek cards
  - A pure culture is required; usually from 24-hr growth
  - Gram stain to determine correct card to use
  - Inoculate tubes and adjust turbidity





bioMérieux Vitek 2

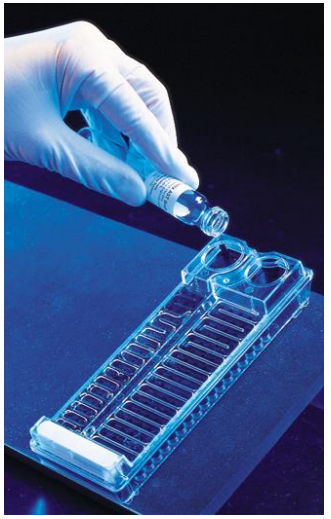
- Every 15 minutes, an optical system monitors changes in each well using different wavelengths in the visible spectrum
  - Turbidity (microbial growth) or colored products of substrate metabolism
- Time to result is 2-14 hours; reader can accommodate up to 60 cards
- The patterns of positive and negative responses in each biochemical and carbohydrate substrate well are compared to an internal database and if a match is found, a microbial identification is provided
- Database contains over 330 species

Becton Dickinson Phoenix

- Gram positive and Gram negative identifications
- Cards contain 45 biochemical substrates
  - Color change
  - Fluorescence changes
  - Also includes antibiotic resistance

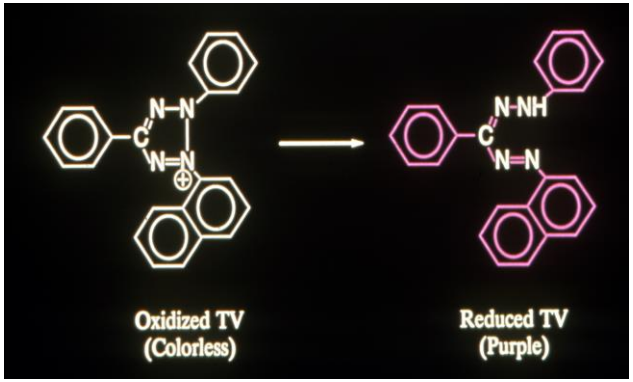
Becton Dickinson Phoenix

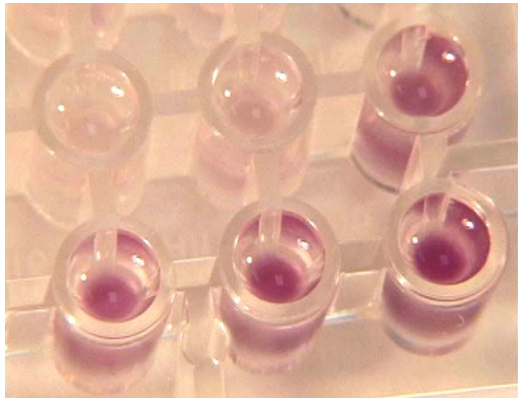
- Grow the organisms on media and isolate pure culture
- Gram stain to determine correct card to use (Gram + or -)
- Prepare suspension and pour broth into panel
- Cap and place panel in incubator
- Panels are read every 20 minutes
- Compare results with database
  - Over 225 bacterial entries



Biolog OmniLog

- Multiple carbon utilization tests using a 96-well microtiter plate
- Each well contains a specific carbon source and tetrazolium violet dye
  - Microorganisms reduce tetrazolium violet during growth, producing a purple colored well

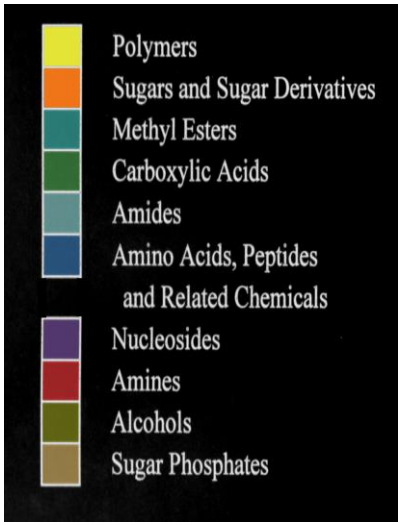




Biolog OmniLog

GN MicroPlate™

A1 water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D-galactosamine	A8 N-acetyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 i-erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentiobiose	B6 α-D-glucose	B7 m-inositol	B8 α-D-lactose	B9 lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 D-psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turanose	C10 xylitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 cis-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D-glucosaminic acid	D9 D-glucuronic acid	D10 α-hydroxybutyric acid	D11 β-hydroxybutyric acid	D12 γ-hydroxybutyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D,L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl-glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl-L-aspartic acid	F12 glycyl-L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenylalanine	G6 L-proline	G7 L-pyroglutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D,L-carnitine	G12 γ-amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3-butanediol	H9 glycerol	H10 D,L-α-glycerol phosphate	H11 glucose-1-phosphate	H12 glucose-6-phosphate

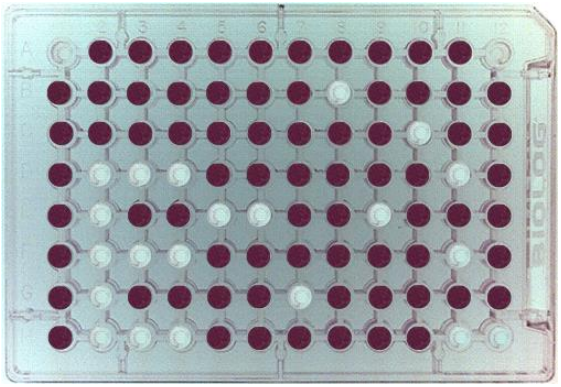


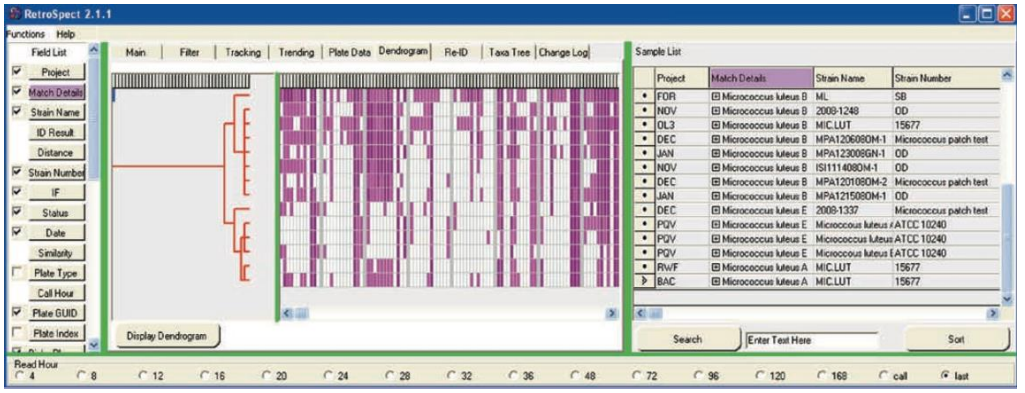
Biolog OmniLog

- Grow organisms on media and isolate pure culture
- Gram stain to determine which microplate to use
  - GEN III card for bacteria does not require Gram staining
- Prepare suspension, inoculate microplate and incubate
  - 4 to 24 hr for bacteria; 48 to 72 hr for yeast and mold

Biolog OmniLog

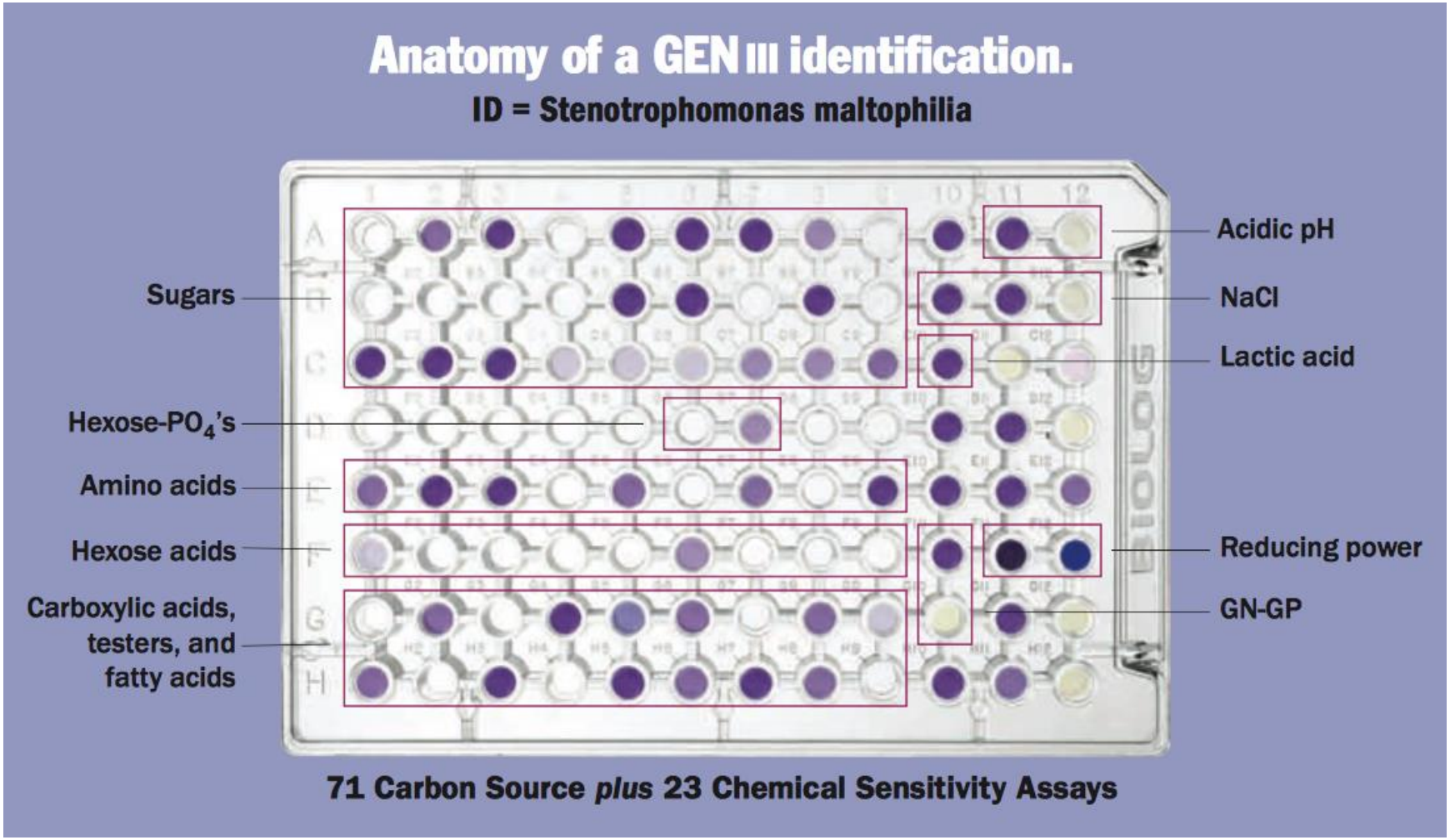
- If microbial growth occurs, the well turns purple





- The pattern of purple wells is compared with the reference library
- Database contains more than 2,500 organisms including aerobic and anaerobic bacteria, yeast and mold species
- Smaller/manual instruments are also available

Biolog OmniLog

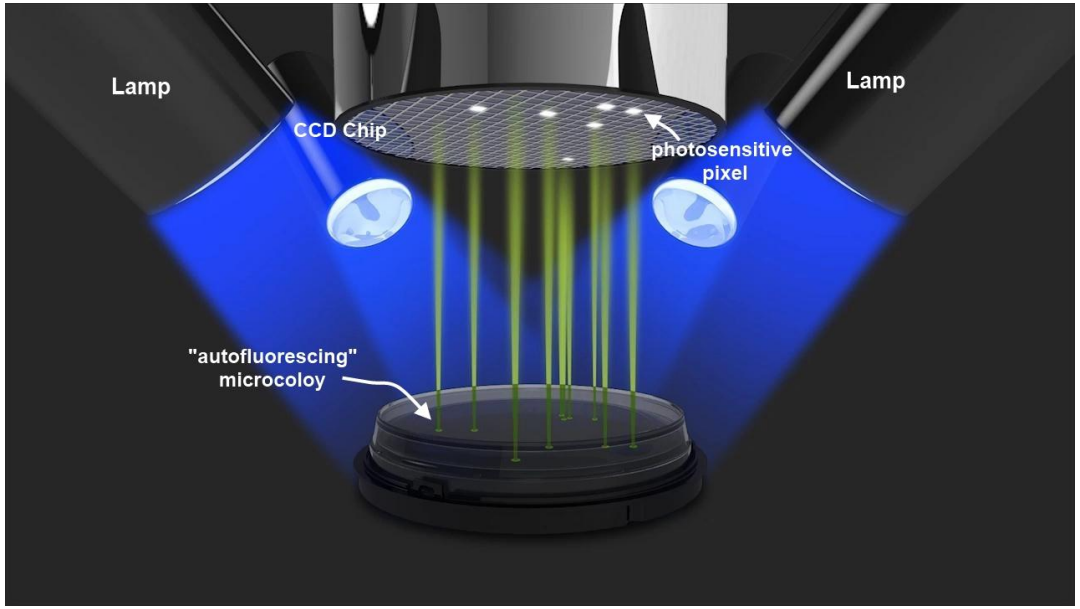


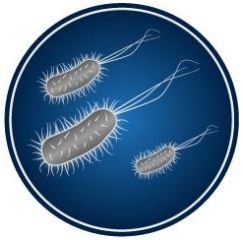
Rapid Micro Biosystems Growth Direct

- Digital imaging technology that enumerates micro-colonies in one-half the time to visualize colonies
- The sample is filtered and the filter is placed onto a flat agar medium cassette with an optically clear lid
- A light emitting diode (LED) excites micro-colonies to autofluoresce, which are enumerated by a CCD imaging system

Rapid Micro Biosystems Growth Direct

- Cells fluoresce in the yellow-green spectral region when illuminated with blue light due to oxidized flavins
  - Photosensitive pixels in the CCD camera chip detect auto-fluorescing micro-colonies





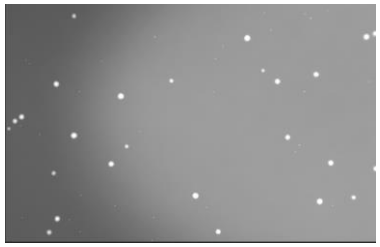
Rapid Micro Biosystems Growth Direct

- The system automatically incubates and analyzes each cassette over time
  - Particles that do not grow in size over time are ignored
- Non-destructive – can continue to incubate media to obtain colonies for microbial identification
- Considered an automated version of the existing compendial method
- Bioburden and environmental monitoring
- One or two temperatures
- Capacity: up to 350 plates

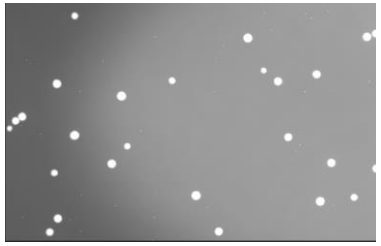
Rapid Micro Biosystems Growth Direct

0 hr

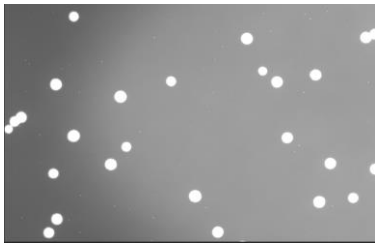
6 hr



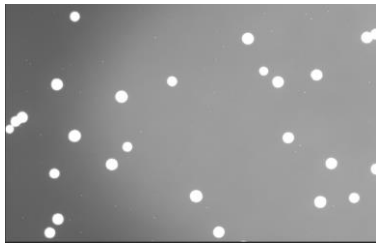
7 hr



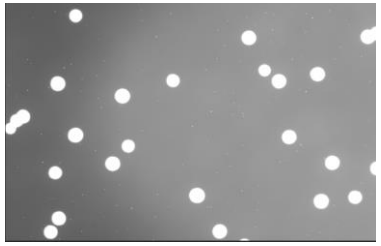
8 hr



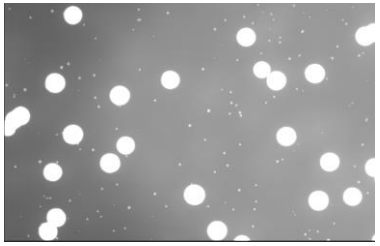
9 hr



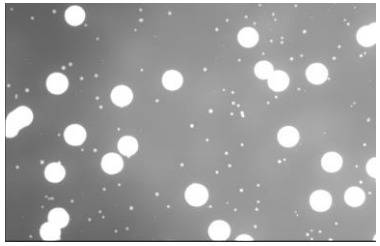
10 hr



11 hr



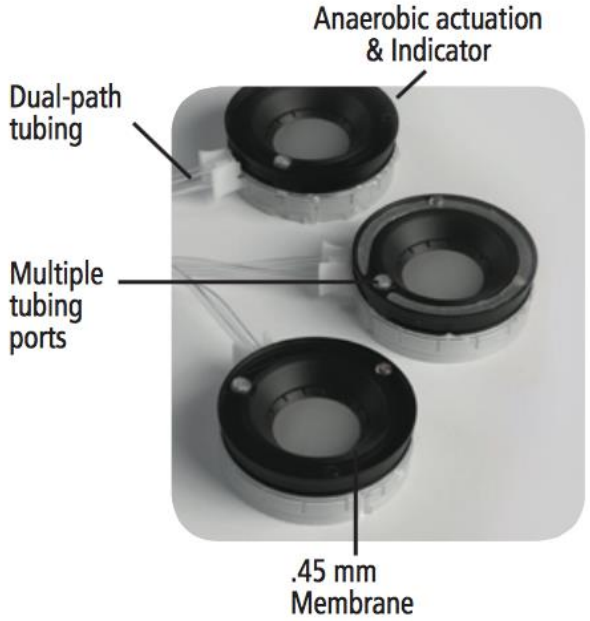
12 hr

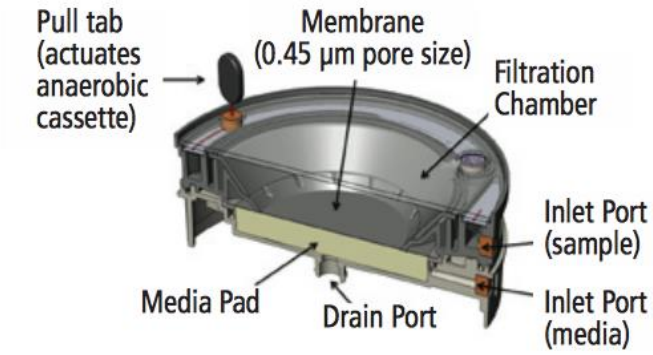


13 hr

Rapid Micro Biosystems Growth Direct

- IN DEVELOPMENT:
- Sterility testing (solid media cassette)
- Claims detection within hours; full test is 7 days (you must validate equivalence to the compendial test)
- Aerobic and anaerobic incubation; 2 temperatures
- Closed-loop sampling; 280 plate capacity



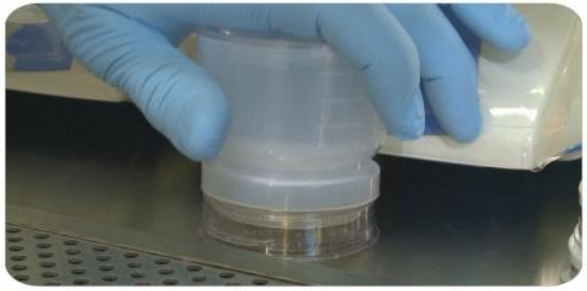
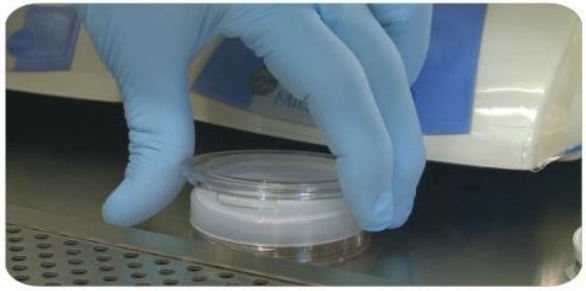
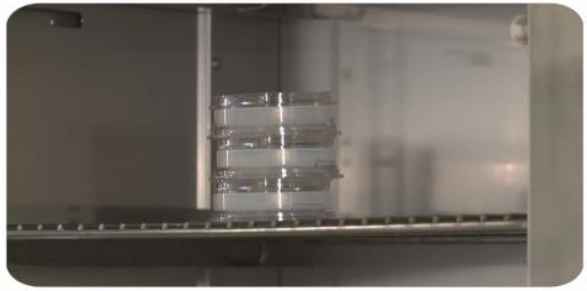


EMD Millipore Milliflex Quantum

- Fluorescent staining and laser excitation of micro-colonies on a membrane
- Applicable for all filterable samples, including water, in-process and finished product
- Non-destructive – can continue to incubate media to obtain colonies for microbial identification

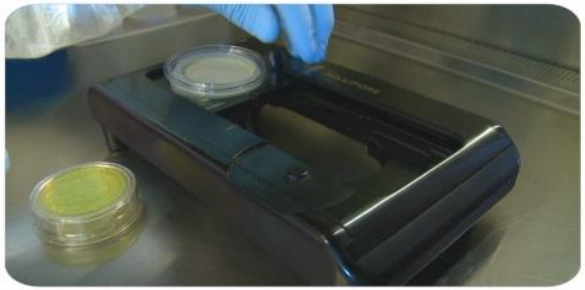
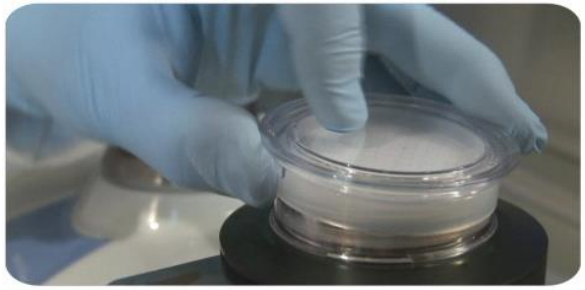
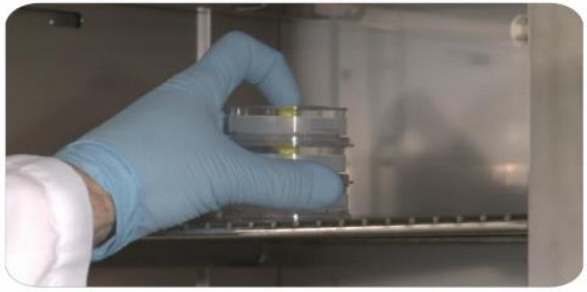
EMD Millipore Milliflex Quantum

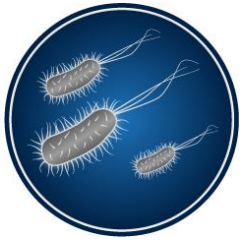
- Filter the sample, place the membrane onto an agar cassette and remove the funnel
- Incubate for an appropriate time period

EMD Millipore Milliflex Quantum

- Saturate the staining cassette with a non-fluorescent substrate, remove the agar cassette from the incubator, place the membrane onto the staining cassette and incubate for 30 minutes at 32.5˚ C

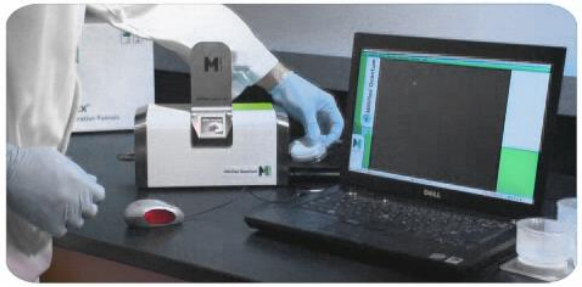


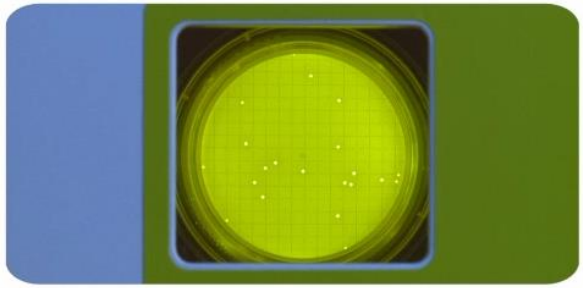
EMD Millipore Milliflex Quantum

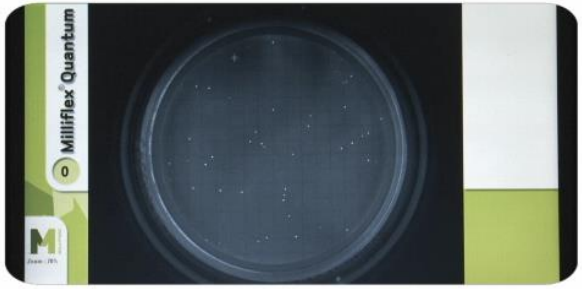
- Microorganisms retained on the membrane will take up the non-fluorescent substrate
- Within viable and culturable cells, the non-fluorescent substrate is enzymatically cleaved
- The cleaved substrate liberates free fluorochrome into the microorganism cytoplasm
- As fluorochrome accumulates inside the cells, the signal is naturally amplified

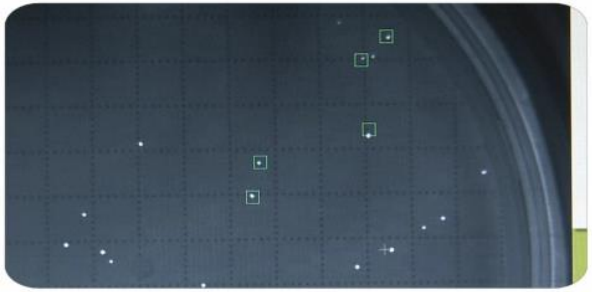
EMD Millipore Milliflex Quantum

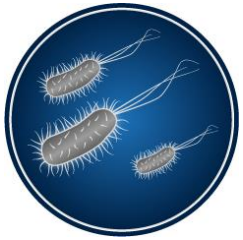
- Following incubation, the membrane is placed into the reader and exposed to the excitation wavelength of the dye
- Fluorescent micro-colonies can then be counted in the instrument window or on a computer via a camera











EMD Millipore Milliflex Quantum

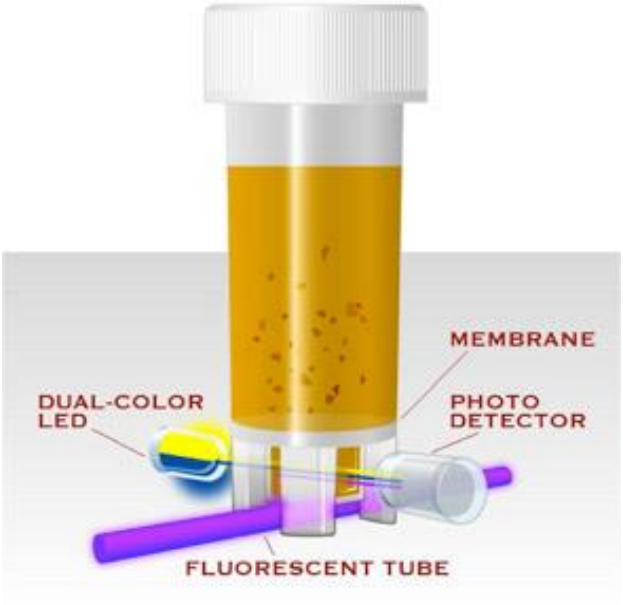
- Following staining and counting of micro-colonies, the membrane can be placed onto the agar cassette and re-incubated to allow larger colonies to form which can then be used for microbial identification (non-destructive)
- Instrument is marketed as the “EZ-Fluo” in Europe

BioLumix

- Detects target microorganisms by monitoring changes in color or fluorescence in selective media, and/or by monitoring the generation of CO_2

BioLumix

- Each vial contains a broth medium and/or other reagents specific for the target organism with unique dyes in which target microorganisms grow and are detected by changes in color or fluorescence
- These changes, expressed as light intensity units, are detected by an optical sensor

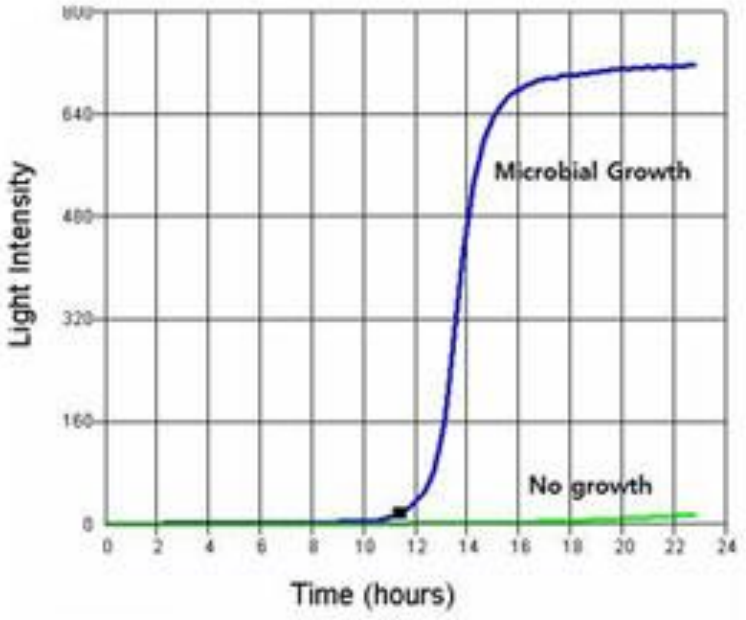


BioLumix

- Disposable two-zone vials contain an incubation zone (top of vial) for the sample and microorganism, and a reading zone (bottom of vial)
- The two-zones eliminates masking of the optical pathway by the product and by microbial turbidity

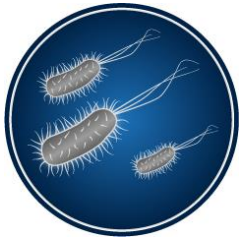
BioLumix

- One bacterial cell is usually detected within 8-18 hours, a single yeast cell is detected in 20-30 hours, and mold requires 35-48 hours
- The threshold for bacteria is 100,000 cells/ml and the threshold for yeast/mold is 10,000 cells/ml
- The time to detection depends on the initial concentration of organisms in the product sample



BioLumix

- Absence of specified microorganisms
- Tests include total aerobic count, yeast & mold, coliforms, *E. coli*, lactic acid bacteria, Enterobacteriaceae, *Salmonella, Pseudomonas,* and *Staphylococcus*



BioLumix

- The system can be used to screen for an estimation of organisms in a test sample that are above or below a certain quantitative specification (“dilute-to-spec”)
- Dilute the test sample to a level that represents the specification level (e.g., 1:100 dilution for a spec of not more than 100 cfu)
- No response: there is <100 cfu in the sample
- Positive response: ≥100 cfu in the sample
- May consider diluting to one-log lower than the spec to avoid variability at the specification level

BACTEST Speedy Breedy

- Portable respirometer
- Monitors pressure changes relating to gaseous exchanges within a closed culture vessel as a result of microbial respiration.

BACTEST Speedy Breedy

- 50 ml closed culture vessel
- Real time analysis of pressure changes in the vessel headspace
- Aerobes, facultative anaerobes, anaerobes, and microaerophilic bacteria; yeast
- Uses general or selective media
- 1 CFU sensitivity after growth

BACTEST Speedy Breedy

- Measures both positive and negative pressure such that monitoring can be performed on a range of microbial processes reacting to differing conditions