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# A New Direct Detection System for Antibiotic Resistant Bacteria

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/59345>

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## 1. Introduction

A new system based on the phenotypic expression of antibiotic resistance has been developed. The system, the Defined Substrate Utilization<sup>®</sup> (DSU<sup>®</sup>) process, enhances the metabolism of the target microbe while providing inadequate nutrients for the non-target microbes. Accordingly, the target microbe multiplies to a much greater extent than the non-target microbes and this differential allows for the specific detection of the target microbe. The non-target microbes multiply insufficiently to be noticed. Incorporated in the DSU<sup>®</sup> screening tools are substrates, that when metabolized, produces a sensible signal such as color or luminescence. In addition, specific inhibitors to the non-target microbes are included in the formulas. This format allows a more sensitive screen than parallel agar-based methods. In terms of time to positive, the liquid (once the powder is hydrated with sterile water), coupled with the optimization of nutrients generates results in the same time frame as genetic amplification tests. The mixing of the ingredients in powder form permits a much less expensive individual screen than either conventional agar-based or genetic amplification methods. In addition, the format permits utilization of the DSU<sup>®</sup> tools both in the remote field environment or the clinical microbiology laboratory.

There are two basic protocols utilized for the epidemiological screening of microbes. The first is the conventional agar-based culture technique. Here a specimen is plated on one or more selective agars and incubated 24 to 48 hours. A trained medical technologist examines the colonies and those compatible with the target microbe are subjected to specific biochemical and/or immunochemical testing, and antibiotic susceptibility procedures. These may require from 24 to 72 hours incubation [1]. In an attempt to facilitate the culture protocol, chromogenic substrate media have been introduced for some, but not all, bacteria of epidemiological interest. Chromogenic substrate agars have a short shelf life, must be stored cold and often in

the dark, and the colors differentiating the positive and negative colonies may not always be distinct, necessitating confirmatory testing [2]. They are generally held 48 hours before calling a negative, although 48 hours can result in false-positives [3, 4].

The second technique utilizes genetic amplification (GA) to detect the target microbe. A number of companies make different types of GA. However, in common to them all are: each requires an expensive instrument, utilizes reagents which are expensive, requires highly skilled labor in a centralized laboratory, and does not recover living bacteria [5]. In addition, each is subject to false-positives and false-negatives because of unexpected mutations [6, 7].

A new and novel, one-step, direct epidemiology screening method based on classical phenotypic parameters was developed as an alternative to conventional agar-based methods for a variety of antibiotic resistant bacteria. For example, for *Staphylococcus aureus* (*S. aureus*), the key enzyme is coagulase and specificity is achieved equal to that of conventional identification schemes [8]. The method of Defined Substrate Utilization® (DSU®) optimizes specific, selective, and differential biochemicals in powder form. Hence, it overcomes the observation by Selepek and Witebsky that the lot-to-lot variation of commercial plasma was too variable to be used directly from patient specimens [9]. For each target microbe, an optimum background of nutrients and inhibitors was generated tailored to the biochemical physiology of that bacterium. Accordingly, the target microbes are preferentially fed while the non-target microbes are in a nutrient deficit.

The powder format allows optimization of ingredients much more precisely than an agar gel. Moreover, the overall sensitivity of the DSU® method is enhanced since liquid culture (once the powder is hydrated with sterile water) detects a lower number of colony forming units (CFU) than on the surface of agar [10, 11]. The DSU® tools are generally positive from a colony within 4 to 5 hours and from a human or animal sample in 6-9 hours on average. The bacteria remain viable in the screens and thus provide the opportunity to further evaluate the target microbe.

In addition to be able to be done manually by visual observation, an inexpensive instrument was developed to read and analyze results of all the screens (Pilots Point Monitor 60®, Pilots Point LLC, Sarasota, FL). One may add any combination of any of the DSU® screening tools into the instrument and results are generated by an algorithm controlling the analysis. Results can be sent to cell phones, via Wi-Fi, or in any format to any location. For example, the instruments may be stationed at various clinics.

This paper shall describe in detail four of the epidemiological screens: methicillin-resistant *S. aureus* (MRSA), all *S. aureus* including MRSA and methicillin-susceptible *S. aureus* (MSSA), vancomycin-resistant enterococci (VRE), and carbapenemase-resistant Enterobacteriaceae (CRE). Screens for vancomycin-intermediate resistant *S. aureus* (VISA), and ciprofloxacin-resistant (cipR) bacteria will be described briefly as subsets of the above. In addition, the principles and function of the instrument will be presented.

While this paper presents six of the DSU® epidemiology screens, the format and phenotypic expression in the presence of defined nutrients, allows for the rapid development of others.

## 2. The Defined Substrate Utilization<sup>®</sup> Method

### 2.1. Screening Method for *S. aureus*: Both Methicillin-susceptible (MSSA) and Methicillin-resistant (MRSA)

#### 2.1.1. *EPI-M<sup>®</sup>* and *aureusAlert<sup>®</sup>* — Quantitative analysis with pure and anterior nares cultures

[*EPI-M<sup>®</sup>* is the DSU<sup>®</sup> epidemiology screen for MRSA, *aureusAlert<sup>®</sup>* is the DSU<sup>®</sup> epidemiology screen for all *S. aureus* – both MSSA and MRSA (Pilots Point LLC, Sarasota, FL)]

##### 2.1.1.1. *Pure cultures*

*S. aureus* ATCC 43300 (MRSA), *S. aureus* ATCC 34591 (MRSA), *S. aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 29213 (MSSA), *S. epidermidis* ATCC 12228, the MRSA USA 600 (6 isolates), MRSA USA 300 (2 isolates), and MRSA USA 100 (2 isolates), plus 5 clinical patient laboratory isolates of MRSA and 5 of MSSA were utilized.

A suspension of each of the bacterial isolates was made in normal physiological saline to a 0.5 McFarland standard. From this suspension, 0.1 mL was transferred to 9.9 mL of sterile normal saline and vortexed well. Using a quantitative pipette (Rainin, Rainin Instrument LLC, Oakland, CA) 1 and 10 microliters of the suspension was transferred to *aureusAlert<sup>®</sup>* and *EPI-M<sup>®</sup>* and a dilution series generated. Colony counts were made from the 0.5 McFarland standard and the 9.9 mL normal saline [8].

##### 2.1.1.2. *Human surveillance study*

Nasal swabs (Culturette<sup>™</sup> II, Becton, Dickinson and Company, Cockeysville, MD) were obtained from people entering or leaving a building at the following locations: Yale-New Haven Hospital (New Haven, CT, USA); Walter E. Washington Convention Center (Washington, D.C., DC, USA), George Washington University Hospital (Washington, D.C., DC, USA) and Tampa General Hospital (Tampa, FL, USA). Two swabs were obtained from the same nostril.

##### 2.1.1.3. *Conventional procedure*

*S. aureus*: Volunteer nasal swab specimens were aseptically transferred to Trypticase Soy Broth (TSB) containing 6.5% NaCl for 24 hours enrichment at 33°C-35°C. The enrichment samples were subcultured on to Tryptic Soy Sheep Blood Agar (TSBA) plates, and the plates were incubated aerobically for 24 hours at 35°C. Suspicious colonies of *S. aureus* were identified by using standard laboratory methods including catalase, tube or slide coagulase, Gram staining, and mannitol salt agar. Confirmed *S. aureus* colonies were tested for methicillin resistance using the CLSI recommended reference method (e.g., 30 mcg cefoxitin disk) [12]. In addition, an assay for PBP-2 (Remel, Lenexa, KS) was performed.

MRSA: Cultures were inoculated into TSB with 6.5% NaCl and incubated for 18-24 hours. A subculture was made to blood agar (BA) plates and a Staphaurex<sup>®</sup> (Remel, Lenexa, KS) test

performed. In addition, an assay for PBP-2 (Remel, Lenexa, KS) was performed. Antibiotic susceptibility test (AST) was performed by both an agar dilution method (Mueller-Hinton agar with 4 mcg/mL oxacillin, Becton, Dickinson and Company, Cockeysville, MD) and also by the Sensititre™ (Trek Diagnostic Systems, Oakwood Village, OH) microdilution method.

#### 2.1.1.4. Defined Substrate Utilization® screens

All ingredients of the Defined Substrate Utilization® screens are optimized in powder form in a tube and are performed in the same way. Each tube is labelled with the name of the screen, and a specific amount of water is transferred to the tube to hydrate the powder. The sample is added to the tube and incubated at 35°C. A positive result, which can occur any time after the initiation of incubation, is seen as follows:

*Interpretation:* aureusAlert® – a clot or coalescence forms in the liquid; no observable clot or coalescence is a negative result. EPI-M® – the liquid changes color from straw-colored to amethyst with an increase in coalescence; no change from straw-colored is a negative result. Tubes are held a maximum of 24 hours before calling them negative.

Control *S. aureus* standards ATCC 25923 (MSSA) and ATCC 43300 (MRSA) plus 5 MSSA and 5 MRSA isolates from patients were tested. MSSA and MRSA isolates were diluted from 7 log10 to 1 log10 and incubated at 35° and 23°C.

Based on quantitative analysis of pure cultures, the Defined Substrate Utilization® screens were able to detect as low as 20 CFU (18 hours of incubation) with both MSSA and MRSA (Table 1; Table 3). Tables 2A and 2B, and Tables 4A and 4B, present the results of the nasal screening of normal subject volunteers. For both the detection of *S. aureus* and MRSA there was no difference between the Defined Substrate Utilization® screens and conventional methods. aureusAlert® and EPI-M® showed a specificity of 100%.

#### 2.1.2. aureusAlert® for all *S. aureus* (MSSA & MRSA)



**Figure 1.** *S. aureus* Negative – Liquid



Figure 2. S. aureus Positive – Clot

MSSA ATCC 25923 (CFU/mL)	Detection Time at 35°C
7 log10	2.0 h
6 log10	3.0 h
5 log10	4.0 h
4 log10	7.0 h
3 log10	10.0 h
2 log10	15.0 h

Table 1. Detection time of Quality Control Clone ATCC 25923 in aureusAlert®

	Conventional Positive	Conventional Negative	Total
aureusAlert® Positive	450	20	470
aureusAlert® Negative	10	1787	1797
Total	460	1807	2267
(A)			
Site	aureusAlert® (Positive / Negative)	Conventional (Positive / Negative)	
Tampa General Hospital	69 / 322	72 / 322	
Yale-New Haven Hospital	63 / 289	58 / 289	
(B)			

Table 2. A&B. Detection comparison of S. aureus nasal swabs in aureusAlert® and conventional methods



2.1.3. EPI-M® (MRSA)

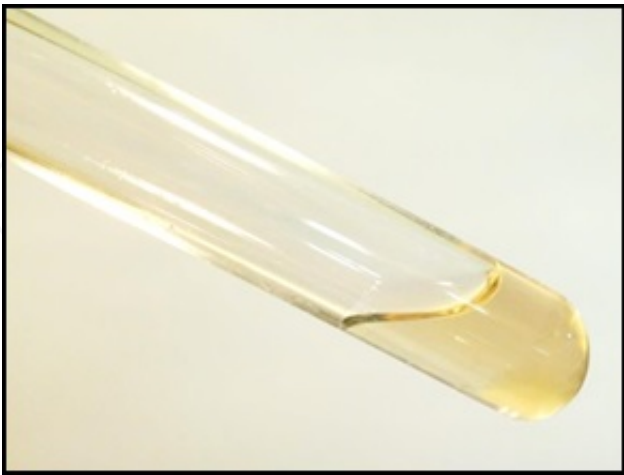


Figure 3. MRSA Negative – Straw-colored



Figure 4. MRSA Positive – Amethyst

MRSA ATCC 43300 (CFU/mL)	Detection Time at 35°C
7 log10	2.0 h
6 log10	3.0 h
5 log10	4.0 h
4 log10	6.5 h
3 log10	14.0 h
2 log10	18.0 h

Table 3. Detection time of Quality Control Clone ATCC 43300 in EPI-M®

	Conventional Positive	Conventional Negative	Total
EPI-M® Positive	49	2	51
EPI-M® Negative	1	480	481
Total	50	482	532
(A)			
Site	EPI-M® (Positive / Negative)	Conventional (Positive / Negative)	
Tampa General Hospital	33 / 317	32 / 317	
Yale-New Haven Hospital	19 / 217	18 / 217	
(B)			

**Table 4. A & B.** Detection comparison of MRSA nasal swabs in EPI-M® and conventional methods

Described here is a method to detect *S. aureus*, both MSSA and MRSA, directly from a human, animal, or environmental sample. The key innovation was to optimize all ingredients and generate a stable, ready-to-use powder. The system's central ingredient is an enhanced coagulase substrate. The enhanced coagulase reaction overcame the observation by Selepak and Witebsky that rabbit plasma variability precluded the ability to directly detect *S. aureus* directly from samples. In one form, aureusAlert®, all *S. aureus* are detected. Using the aureusAlert® as a base, the addition of a *mecA* inducer specifically distinguishes MRSA (EPI-M®). By employing the definitive tests in the right milieu, as low as 20 CFU of MSSA and MRSA could be detected.

This study demonstrated that the sensitivity was equivalent to conventional methods by direct comparison and molecular methods from literature references. The time to detection was in the clinically useful range for the epidemiological screening of anterior nares (Table 1; Table 3). Because coagulase was chosen as the core detection system, a specificity of 49/49 for EPI-M® was seen from sampled subjects. Likewise, aureusAlert® being an enhanced coagulase test also showed complete agreement with conventional identifications.

The bacteria remain viable in the aureusAlert® and EPI-M® epidemiology screening tools. The tubes can be transported, as is, from the field for further analysis (e.g., antibiotic susceptibility testing, molecular fingerprinting). The powder format offers great flexibility of use. It has the ability to gather information from a broad spectrum of sampling: in the field, in satellite facilities, in clinics, and in large volume central laboratories. It requires no skilled labor. Because it is a stable powder, it can be inexpensively transported. Its cost is 20% of genetic amplification and 75% of conventional processing surveillance samples.

#### 2.1.4. Additional *S. aureus* screen: EPI-VISA®

The screen for vancomycin-intermediate resistant *S. aureus* (VISA) utilizes the EPI-M® base with the substitution of 3 mcg/mL (3 mg/L) of vancomycin for cefoxitin. The colors produced are the same as the EPI-M®.



## 2.2. Screening Method for Vancomycin-resistant Enterococci (VRE)

Both asymptomatically colonized and infected patients serve as reservoirs for transmission of VRE, as well as contaminated surfaces and patient care equipment. European Union and United States public health agencies have developed recommendations for preventing the transmission of vancomycin resistance within and among hospitals and nursing homes. Current surveillance methods require a number of sequential steps and needs a special series of culture media and identification tests. A highly skilled and trained medical technologist is needed to perform the analysis. Molecular tests are expensive and require special equipment.

A new, simple, and one-step screening method for the detection of VRE (as defined as a minimum inhibitory concentration (MIC) to vancomycin of 6 mcg/mL) directly from rectal/perirectal specimens was compared to the Food and Drug Administration (FDA) reference or predicate method. The screening tool (EPI-V<sup>®</sup>, Pilots Point LLC, Sarasota, FL) contains all the required ingredients in a stable powder form, in a standard test tube. The presence of VRE in a rectal swab is denoted by the production of a two sequential biochemical reactions inside the test tube: substrate hydrolysis representing bile-esculin followed by pyrrolidonyl arylamidase (PYR) substrate hydrolysis. Bile-esculin plus PYR is the generally accepted identification of the genus *Enterococcus* [13].

### 2.2.1. Standard culture method

The FDA predicate VRE culture method is Bile Esculin Azide agar with 6 mcg/mL vancomycin (BEAV) (Remel, Lenexa, KS) [14]. Specimens consisted of 400 sequential human rectal/perirectal surveillance swabs obtained as part of the ongoing surveillance program. The collection device was a Culturette<sup>™</sup> II (Becton Dickinson and Company, Cockeysville, MD). There was no prior notification of individuals collecting the specimens. The Culturette<sup>™</sup> II swab was twirled vigorously in 1 mL of pH 7.0, 50 mM HEPES (Sigma-Aldrich, St. Louis, MO) buffer so that each of the two methods would be challenged with the same inoculum. From the HEPES buffer, 0.1 mL of the extracted patient specimen was plated on the surface of the BEAV agar plate. After incubation for 24 hours at 35°C in ambient air, colonies that were brown-black were gram-stained. Colonies showing gram-positive cocci in chains were then tested by the PYR reaction (Becton-Dickinson, Cockeysville, MD). The MIC to vancomycin from PYR positive colonies was performed by the Etest<sup>®</sup> method (bioMérieux, Durham, NC).

### 2.2.2. EPI-V<sup>®</sup>

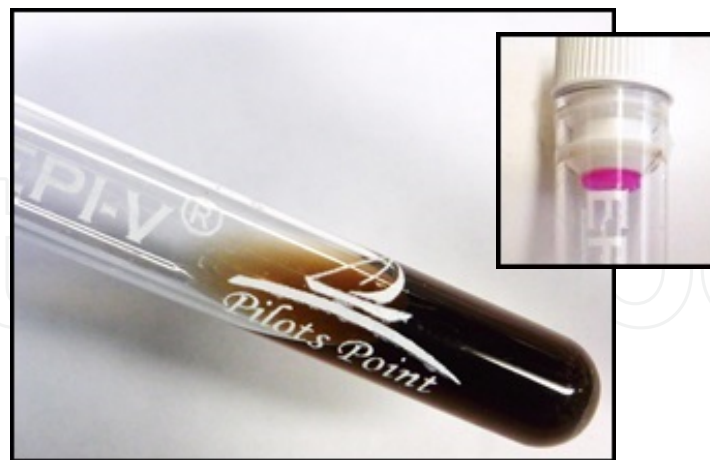
The EPI-V<sup>®</sup> screening tool is in a powder format, in a flange-capped test tube, and stored at room temperature. To use, 3 mL of sterile water was added to dissolve the powder. To this solution, 0.1 mL of the extracted patient's specimen was added. EPI-V<sup>®</sup> test tubes were incubated for a maximum of 24 hours at 35°C in ambient air to call a specimen negative. However, color development at any time, denoted a positive result. Test tubes demonstrating a brown-black color (bile-esculin positive) were tilted to coat the disk present in the EPI-V<sup>®</sup> cap. This cap contains an enhanced reagent for the detection of PYR hydrolysis [15]. The

development of a bright fuchsia color within 15 seconds is a positive reaction for PYR. Positive bile-esculin and PYR results demonstrated the presence of VRE.

In order to determine if a false-positive or false-negative reaction had occurred from the EPI-V<sup>®</sup>, approximately 0.1 mL was subcultured from all tests tubes to the surface of a Bile Esculin Azide agar plate containing 6 mcg/mL vancomycin. Plates were incubated at 35°C for 24 hours in ambient air. All colonies compatible with *Enterococcus* were then identified to species. If an *Enterococcus* species was isolated, an Etest<sup>®</sup> MIC to vancomycin was performed. All isolates were identified to species using the Vitek 2 system (bioMérieux, Durham, NC).



**Figure 5.** VRE Negative – Straw-colored with white disk



**Figure 6.** VRE Positive – Dark brown/black with bright fuchsia disk

The EPI-V<sup>®</sup> tool, from a diluted specimen, demonstrated a sensitivity of 107% and a specificity of 98.4%. Table 5 presents the comparison of the EPI-V<sup>®</sup> results versus the Bile Esculin Azide (BEAV) culture method. Table 6 presents the time course of the 120 positive analyses by the EPI-V<sup>®</sup> tool.

	BEAV Positive	BEAV Negative
EPI-V® Positive	111	9
EPI-V® Negative	0	280

**Table 5.** Comparison of EPI-V® and Bile Esculin Azide Media for the recovery of VRE from stool samples

Time	Number of Specimens Positive	Cumulative
5 h	12	12
6 h	6	18
7 h	5	23
8 h	11	34
9 h	3	37
10 h	7	44
16 h	73	107
18 h	8	115
20 h	5	120

**Table 6.** EPI-V® Time to Positive from 120 positive stool samples

The EPI-V® screening tool consists of both a substrate in the liquid (a bile-esculin substrate) and a detection disk for a second biochemical reaction (PYR).

The EPI-V® tool showed excellent sensitivity and specificity relative to the Bile Esculin Azide FDA referenced method and required only a maximum of 20 hours. In addition, the EPI-V® tool offered several significant advantages over the FDA referenced method. These included: no skilled technologist time required, simple quality control, highly conserved incubator and refrigerator space, and approximately 1/4-1/3 the cost of agar-based methods. The simplicity and ease of use of the EPI-V® epidemiology screen allows for more efficient and timely processing of large numbers of patient surveillance specimens for VRE. This new screen offers the clinical microbiologist, hospital epidemiologist, or infection control practitioner, flexibility as to the timing of collection and number of rectal or perirectal patient surveillance cultures.

**2.3. Screening Methods for Antibiotic Resistant Enterobacteriaceae**

Carbapenems are a class of antibiotics made by modifying penicillin. This chemical modification keeps part of the penicillin molecule, called the beta-lactam structure, and makes the rest artificial. Therefore, carbapenems are referred to, with other classes of penicillin modified antibiotics, as semi-synthetic penicillins. The modifications were made because bacteria rapidly became resistant to penicillin. Chemists added bits and pieces of chemical structures to penicillin to avoid this resistance. The bacteria figured out how to make enzymes to chemically destroy these new modifications. After 70+ years of this war between chemists and bacteria, the bacteria have topped the chemists by making a class of enzymes called carbapenemases [16].

The original carbapenem was first used in the U.S. in 1976. Since then better forms of this class have been made chemically. Currently, the most common carbapenem antibiotic used is meropenem. In the fight between chemists and bacteria, meropenem is the last antibiotic standing. If a bacterium is resistant to meropenem, there are no good alternatives [16].

### 2.3.1. What is CRE?

The clinical microbiology laboratory, where infections are diagnosed, divides bacteria into various shapes (spheres are cocci, cylinders are rods) and armor (gram-positive or gram-negative). A particular class of antibiotic generally has activity against either gram-negative or gram-positive bacteria, but not both. Carbapenems were developed to treat the gram-negative bacteria found in hospitals. Gram-negative bacteria in hospitals are associated with a very large morbidity and mortality, plus tremendous cost.

The gram-negative bacteria that have become resistant to carbapenems are known as CRE, or carbapenem-resistant enterics. Until a short time ago carbapenem resistance was only in one type of enteric, called *Klebsiella pneumoniae* (K. pneumoniae); therefore resistant bacteria were called *K. pneumoniae carbapenemase* (KPC). Unfortunately resistance has spread to bacteria related to *Klebsiella* and we now use the term CRE. These enteric bacteria normally live in the intestines of humans [16].

### 2.3.2. What is the CRE problem?

CRE is primarily a nosocomial associated problem. The likely reasons include the following: the use of large amounts of various antibiotics that select for the CRE bacteria; the transport from patient to patient of CRE on the hands of health care workers, including doctors and nurses; an environment such as a hospital room in which the bacteria can be deposited to infect new residents. For example, the CRE bacteria can remain alive in hospital rooms for a very long time period. In one study, after 30 days significant numbers of CRE could be found in a hospital room, and even after 100 days CRE were recovered. Death rates of up to 50% can be seen in patients with CRE isolated from the blood. The CDC has identified CRE as one of the prime problems for hospitalized patients [16].

### 2.3.3. Impediments to the control of CRE

*Lack of funding:* There is no billing code for CRE under Medicare and Medicaid. Therefore, reimbursement for the actual laboratory testing is not available. While there would be a huge financial benefit to the prevention of infections (in addition to the obvious benefit to the patient) hospitals do not balance costs and benefits between departments.

*Lack of a systems approach:* Many hospitals are now actually networks of hospitals. It has been difficult to achieve uniformity of action across the networks.

*Lack of a diagnostic test:* The IDSA says “New, rapid accurate diagnostic tests are sorely needed. Unfortunately, there is little impetus for companies...”

*Need for highly skilled labor:* Current methods require several days and utilize highly skilled and costly labor [16].

#### 2.3.4. Methods for the detection of Carbapenemase-producing Enterobacteriaceae

##### 2.3.4.1. Centers for Disease Control Method

The CDC method utilizes a series of sequential steps and can require up to 4 days to determine either positive or negative [17].

*Procedure:*

##### Step 1. Day One

1. Aseptically, place one 10- $\mu$ g ertapenem or meropenem disc in 5 mL trypticase soy broth (TSB).
2. Immediately inoculate the broth with the rectal culture swab.
3. Incubate overnight at  $35 \pm 2^\circ\text{C}$ , ambient air.

##### Step 2. Day Two

1. Vortex and subculture 100  $\mu$ l of the incubated broth culture onto a MacConkey agar plate.
2. Streak for isolation.
3. Incubate overnight at  $35 \pm 2^\circ\text{C}$ , ambient air.

##### Step 3. Day Three

1. Examine the MacConkey agar for lactose-fermenting (pink-red) colonies.
2. More than one colony morphology may represent different species of Enterobacteriaceae.

NOTE: Carbapenemases are known to exist in several different species of gram-negative bacilli including species of Enterobacteriaceae and *Pseudomonas aeruginosa* (*P. aeruginosa*). However, carbapenemases are more common in lactose-fermenting species of Enterobacteriaceae (e.g. *K. pneumoniae* and *E. coli*) than in non-lactose fermenting Enterobacteriaceae (e.g. *Serratia marcescens* and some *Enterobacter* spp.) and *P. aeruginosa*. In this procedure, it is suggested that laboratories focus their efforts on detection of resistant lactose-fermenting bacteria to reduce workload. Healthcare facilities that have identified clinical infections with carbapenemase-producing non-lactose fermenting gram-negative species should consider altering this procedure to include characterization of colonies with a morphology that is consistent with those species.

It may be necessary to subculture representative colonies of each morphology type to a non-selective media for isolation and/or for susceptibility testing. Screen representative isolated colonies using a phenotypic test for carbapenemase production, such as the Modified Hodge Test (MHT) or test for carbapenem susceptibility using a standardized method and follow the CLSI guidelines for identification of carbapenemase-producing Enterobacteriaceae [12].



#### Step 4. Day Four

1. For CRE and/or MHT-positive isolates, perform species-level identification.

##### 2.3.4.2. Hydrolysis of Carbapenem Methods

###### *Rapid Detection of Carbapenemase-producing Enterobacteriaceae*

This method takes advantages of the observation that the hydrolysis of imipenem results in an acid molecule. Therefore, the authors incorporate a pH indicator that changes from red to yellow when acid is produced in sufficient amounts. One advantage of the method is that by using a pH change, a broad spectrum of carbapenemases can be detected. A significant disadvantage is that imipenem is not stable and the whole formula must be made fresh each time it is used. This aspect seriously inhibits the method's use [18].

###### *Detection of Carbapenemase Producers in Enterobacteriaceae by Use of a Novel Screening Medium*

Different concentrations of several carbapenem molecules were tested, and finally, ertapenem was added to Drigalski agar medium at a concentration of 0.25 mg/mL. ZnSO<sub>4</sub> (70 mg/mL) was added to improve expression of metallo-beta-lactamases (MBLs) by MBL producers. Cloxacillin (250 mg/mL), which is a cephalosporinase (AmpC-type beta-lactamase) inhibitor, was used to prevent growth of isolates expressing high levels of cephalosporinases, such as *Enterobacter cloacae* (E. cloacae), *Enterobacter aerogenes* (E. aerogenes), *Morganella morganii* (M. morganii), and *Serratia marcescens* (S. marcescens). These isolates are clinically significant sources of carbapenem resistance associated with an outer membrane permeability defect [19].

#### 2.4. Screening Method for Carbapenem-resistant Enterobacteriaceae (CRE)

EPI-CRE<sup>®</sup> is a selective and differential medium containing 2 mcg/mL of meropenem, intended for use in the qualitative detection of gastrointestinal colonization with carbapenem-resistant Enterobacteriaceae (CRE) to provide epidemiological data. It is based on classical biochemicals in an optimized form. EPI-CRE<sup>®</sup> is performed with rectal swabs and fecal specimens from patients to screen for CRE colonization. The presence of CRE is established when the color of the medium changes from red to yellow. In addition, a violet precipitate may also be observed.

Specimens should be collected, handled and disposed of in compliance with accepted guidelines and in accordance with all regulations. Cotton swabs should be avoided with preference to synthetic fibers such as Dacron or rayon. A generally available reference is the Manual of Clinical Microbiology [8]. EPI-CRE<sup>®</sup> is performed with rectal swabs and fecal specimens which should be collected and handled following recommended guidelines [13].

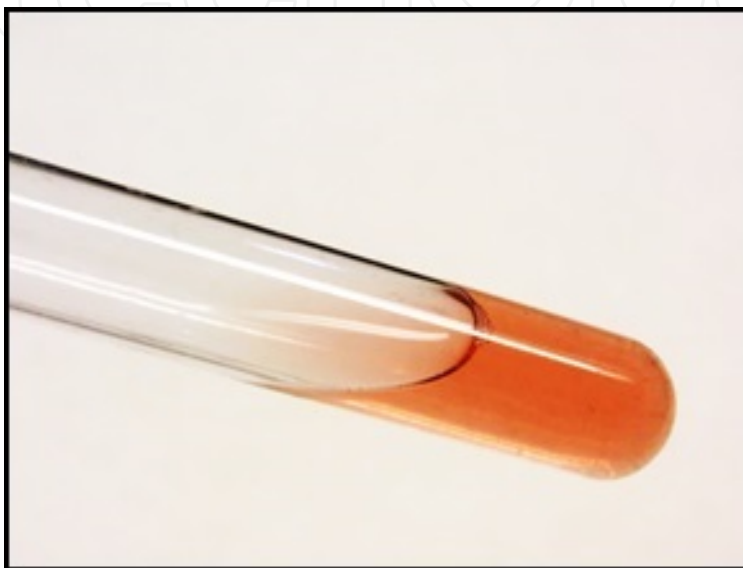
##### 2.4.1. EPI-CRE<sup>®</sup>

###### 2.4.1.1. Procedure

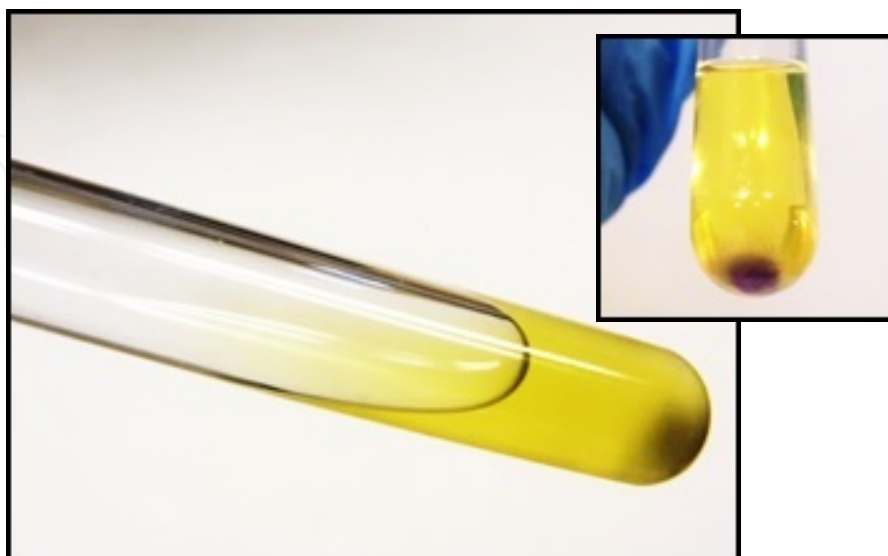
1. Inoculate the sample into the medium. If a swab is used, twirl the swab in the medium for 10 seconds and express excess fluid from the swab before removing and discarding into disinfectant. Do not use a cotton swab.



2. Incubate the tube(s) at 34-36°C.
3. The tube(s) may be examined any time up to 24 hours for a color change from red to yellow. If the medium has not become yellow in 24 hours, it is negative.
4. At any time after the start of incubation, if a yellow color is observed, EPI-CRE® is positive. In addition, a violet precipitate may be observed.



**Figure 7.** CRE Negative – Red



**Figure 8.** CRE Positive – Yellow with violet precipitate

#### 2.4.1.2. Note on the biology of EPI-CRE®

EPI-CRE® detects ONLY living bacteria. It is 100% specific (based on Bergy's Manual and the Manual of Clinical Microbiology).

*PCR positive and EPI-CRE®negative:* Genetic amplification is not to be used as a "test of cure". The reason is that PCR and other genetic amplification methods will detect dead bacteria. Therefore, if a patient is treated intra-nasally or systemically with an effective antibiotic, the bacteria may be killed but their genetic material still is present. EPI-CRE® only detects living bacteria so this situation is not a limitation with this method.

### 2.5. Screening Method for Ciprofloxacin-resistant (cipR) Bacteria

EPI-FLOX® is a selective medium that allows the differentiation of ciprofloxacin-resistant from ciprofloxacin-susceptible bacteria. EPI-FLOX® contains 2 mcg/mL of ciprofloxacin, intended for use in the qualitative detection of ciprofloxacin-resistant (cipR) bacteria to provide epidemiological data. It is based on classical biochemicals in an optimized form. EPI-FLOX® is performed with urethral swabs and specimens, or rectal swabs and fecal specimens from patients to screen for cipR bacterial colonization. The presence of cipR bacteria is established when the color of the medium changes from red to yellow. In addition, a violet precipitate may also be observed.

The base formula of the EPI-FLOX® is the same as that of EPI-CRE® with the substitution of ciprofloxacin for meropenem. Therefore the procedure for use and the color changes are the same.

#### 2.5.1. Note on the utility of the antibiotic resistant Enterobacteriaceae format

Because the base formula is designed to select for lactose positive Enterobacteriaceae, changes in the antibiotic can easily produce tailored screens. For example, by decreasing the concentration of meropenem to 0.25 mcg/mL, an epidemiological screen for OXA-48 can be produced.

## 3. Instrument: Pilots Point Monitor 60® (PPM 60®)

As described above, each of the DSU® epidemiology screens can be done manually. However, there are advantages to be able to place the screens in an instrument. Among these are:

*Standardization:* the instrument will read and interpret the screens in a completely uniform manner.

*Positives reported when detected:* the instrument monitors the color change of each screen every 15 minutes. Once the algorithm determines that a significant change has occurred compared to a control the results are reported at that time. Therefore there is continuous monitoring and reporting. Another benefit is that response time will be shortened.

*Satellite screening:* Because results are available from the instrument via Wi-Fi, internet, and intranet, screening at various locations can be controlled from a central location.

*Epidemiological pattern analysis:* Either from a central location or from satellite laboratories, data can be analyzed to determine if any increase of any of the epidemiological threats from any location is occurring.

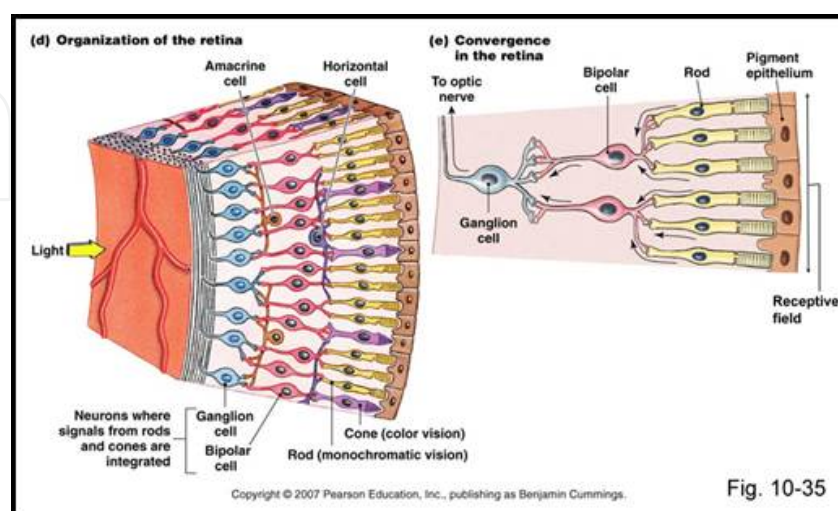
*Inexpensive cost:* the instrument is designed to minimize cost. As shall be discussed below the instrument's function is to produce light and measure a change. Data are transferred to a computer – either a personal computer (PC) or an information technology (IT) system – for analysis. This format lowers the cost of the instrument.

*Single instrument for all screens:* Because of the light source chosen (see below) all of the screens, regardless of color, can be performed in one instrument. Moreover, wavelengths from 360nm through the entire visible light can be accurately measured, which allowed both fluorescent (366nm) and visible light measurements. The ability to use both ultraviolet and visible wavelengths permits the development of two separate biochemical identification systems. The separation of two biochemicals can allow for the detection of two separate target microbes (if each is specific for each microbe) of an enhanced specificity for a single target microbe (if the two together provide enhance specificity over one alone).

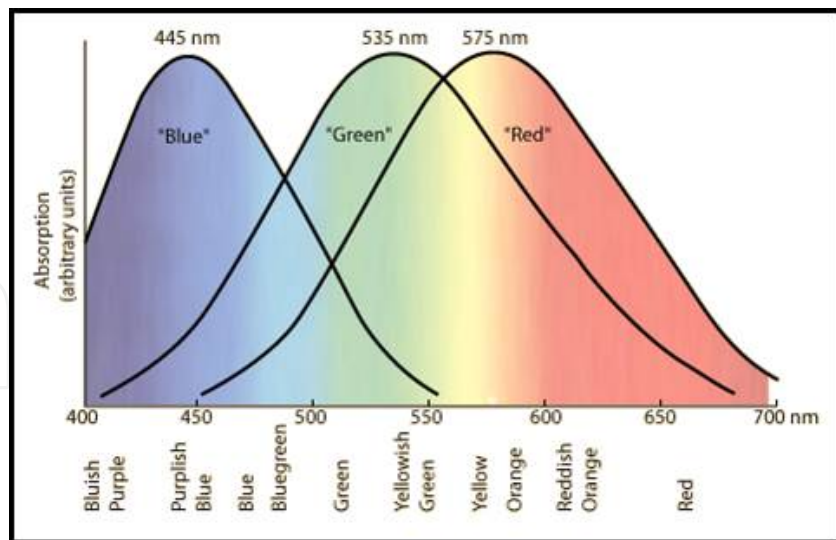
### 3.1. Principle of the instrument

The choice of the light source is critical to the generation of a functioning inexpensive instrument. Instead of using laser, fluorescence polarization, light scattering, reflectance spectrophotometry that are expensive and functionally limited, an inexpensive instrument that uses a white light generator that passes through the sample was generated. Absorption of one or more of the colors allows other to pass through. In addition an overall measure of turbidity, called luminosity, is also measured. For EPI-M® the coagulase coalescence change enhances the process.

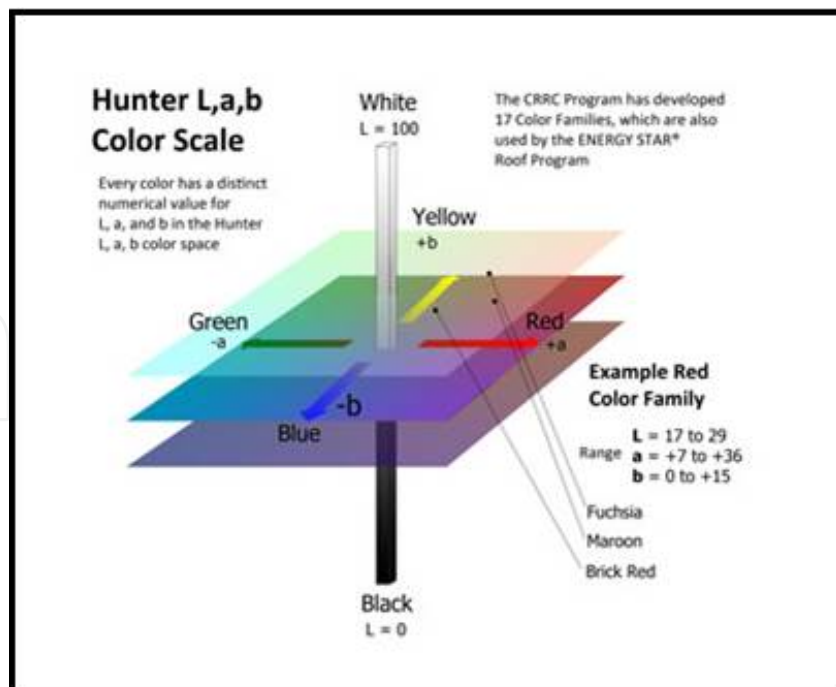
The principal of the instrument is based on the way the human eye sees color.



There are three primary colors that the cones see, which translate into the full range of colors.



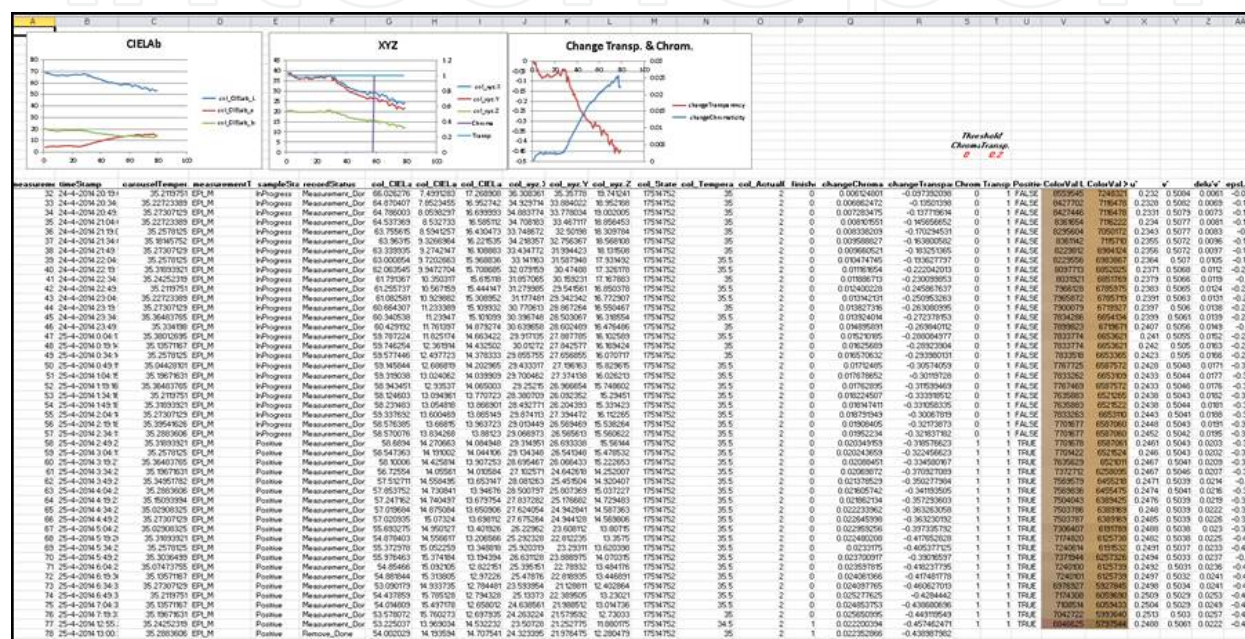
In the analysis program the light coming out of the sample is broken down as shown below. The first measure, which is called the “L” value, is essentially luminosity. It determines a scale from black to white. The second measure is the “a” value, which measures a change from green to red. The third measure is the “b” value, which measures a change from blue (and effectively includes as shown below yellow). Any particular reading can be determined exactly on the graph below by knowing the “L”, “a”, and “b” values. The sensitivity of the analysis program is enhanced because it can relate the changes of any combination of “L”, “a”, and “b” values.



An example of a typical analysis is shown below for an EPI-M<sup>®</sup> MRSA analysis with standard ATCC MRSA 43300. The tube is scanned every 15 minutes and “L”, “a”, and “b” values



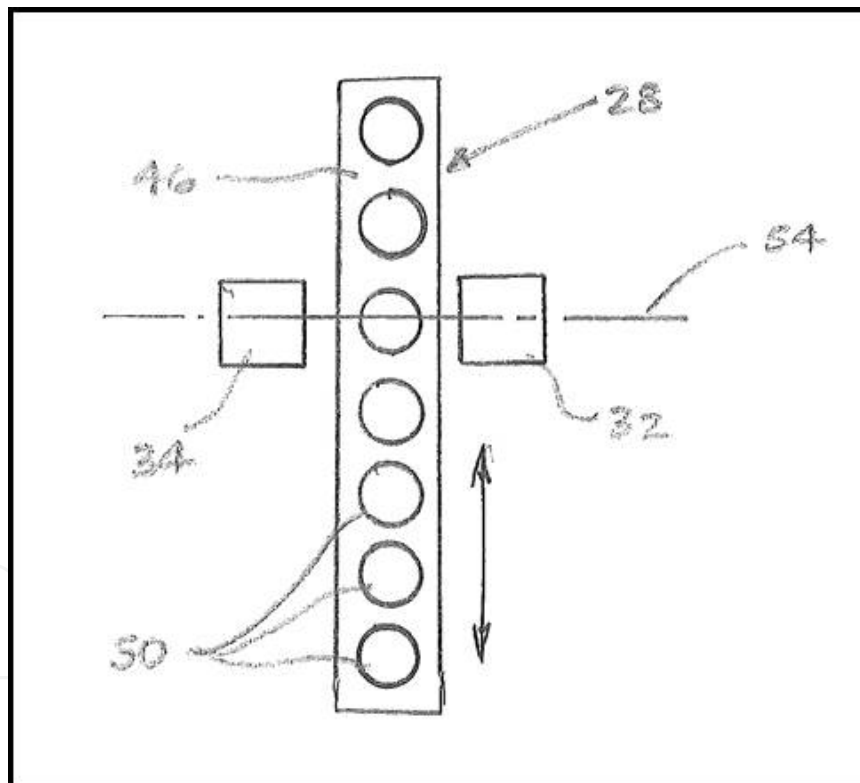
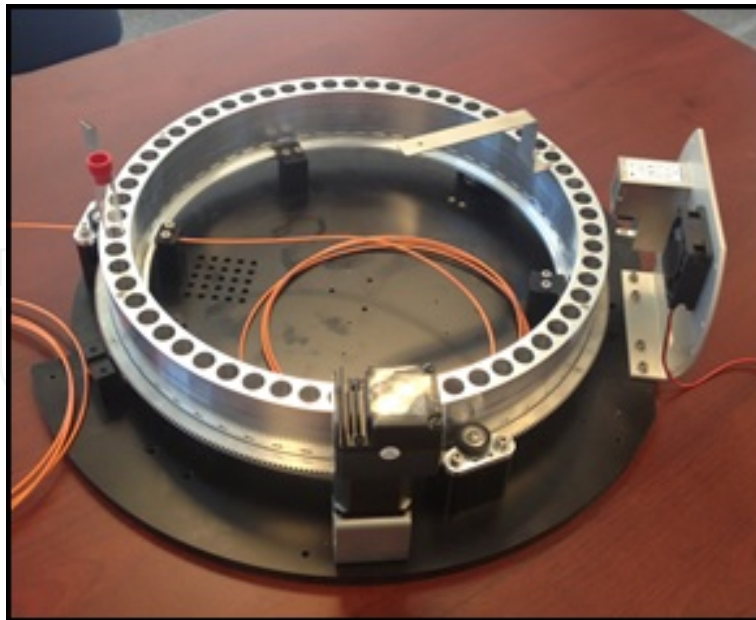
generated (see the spreadsheet below for the individual values). The analysis program then translates the values into graphical form, which are shown above the spreadsheet. The analysis program then determines by a combination of “L”, “a”, or “b”, or any combination thereof, if there is a significant change compared to a control. In this analysis 3 log<sub>10</sub> of ATCC 43300 was detected in 14.5 hours.



### 3.2. The instrument

The inside of the Pilots Point Monitor 60® is shown below. It is designed to accept the largest number of screens with the smallest footprint. It is approximately 0.8 meters in diameter. In the current configuration, 6 instruments can be connected to one PC. In practice the instrument cannot be opened. The tube (as shown in the opened instrument below) in practice is added to the instrument by sliding the blue plastic bar below the PPM 60® logo so as to expose only one slot. The instrument via a barcode on the test tube recognizes the name of the screen and all the demographics of the patient. The carousel moves past the light generator with each shift of position every 15 minutes. There is an incubator in the instrument that can be adjusted +/-0.2°C.

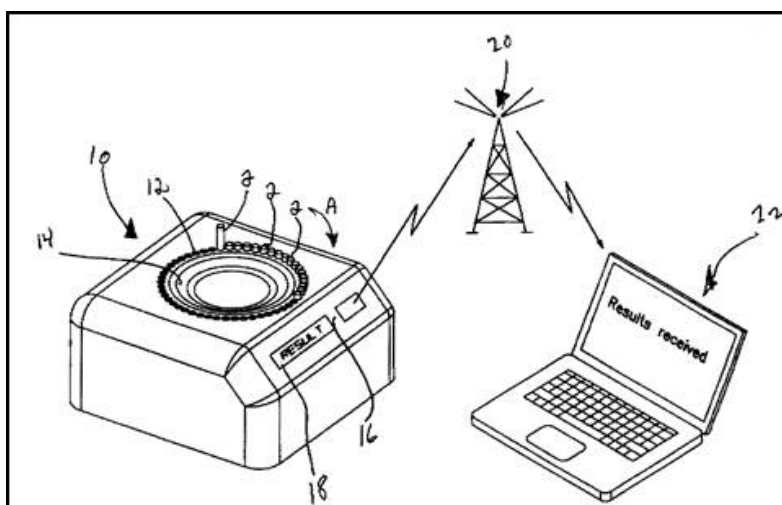
Below is a diagram of the working aspects of the instrument: 46 is the carousel block, 28 is the wall of the carousel. 50 are the holes in the carousel into which the tubes are place, 34 is the white light source, and 32 is the detector which reads the amount and quality of light (“L”, “a”, and “b”) that is represented by 54.



Below is the instrument as it would be used.

As the scanning of the tubes progresses, the information is sent to a PC or to a central IT station. This flexibility allows both large and small laboratories, epidemiologists in the field, researchers studying the prevalence of antibiotic resistant bacteria in animals, people, and even surfaces to use one system.





### 3.3. Enhanced sensitivity of the Defined Substrate Utilization® tools

#### 3.3.1. Agar compared to liquid

Compared to agar-based microbial detection systems, liquid with the same formulas are always more sensitive, meaning liquid has the ability to detect a smaller number of bacteria than agar and in shorter period of time [10]. The reasons for this difference are based first on the fact that on the surface of agar, bacteria must make colonies to be observed. A visible colony requires a minimum number of bacteria. The surface of agar has a surface tension that inhibits the spreading of the bacterial colony. By comparison in liquid, the bacteria are free to divide in three dimensions.

In addition, the nutrients from the agar must enter the colony from only one direction – from the agar vertically into the colony. Likewise the waste products, which are inhibitory to the growth of the microbial colony, diffuse in close proximity to the developing colony thus

inhibiting the colony's lateral development. The production of a colony can be thought to reach development when the nutrients below the colony (all that is available to it) combined with the production of waste products cause the bacteria to stop multiplying and expanding. By contrast, a bacterium in liquid suffers no such inhibitions. Nutrients, including the metabolizable substrate, are available from all directions. Therefore, there is no inhibition for the development of the bacteria. Likewise any waste products diffuse away from the developing bacteria nidus into the liquid, away from the nidus.

### 3.3.2. Optical parameters

Microbiological optical systems have over the years moved to a microtiter format. The microtiter structures are either the classical 96 well microtiter tray, or a variation, or a microtiter format in a vertical series of cupules, as exemplified by the Vitek 2 system (bioMérieux, Durham, NC). The decrease in the light path has significant negative effects on both sensitivity and the time to detection. The reason for this is that the optics of light absorbance is governed by the Beer-Lambert law. As shall be discussed below, the intensity of light is logarithmically related to the length of the light path. This means that a decrease in the light path results in a log decrease in the amount of measurable light that reaches the detector. This relationship was presented as the linear relationship between absorbance and concentration of an absorbing species.

The general Beer-Lambert law is usually written as:

$$A = a(\lambda) \cdot b \cdot c$$

where  $A$  is the measured absorbance,  $a(\lambda)$  is a wavelength-dependent absorptivity coefficient,  $b$  is the path length, and  $c$  is the analyte concentration.

When working in concentration units of molarity, the Beer-Lambert law is written as:

$$A = \epsilon \cdot b \cdot c$$

where  $\epsilon$  is the wavelength-dependent molar absorptivity coefficient with units of  $M^{-1} \text{ cm}^{-1}$

Regarding instruments, measurements are usually made in terms of transmittance ( $T$ ), which is defined as:

$$T = I / I_0$$

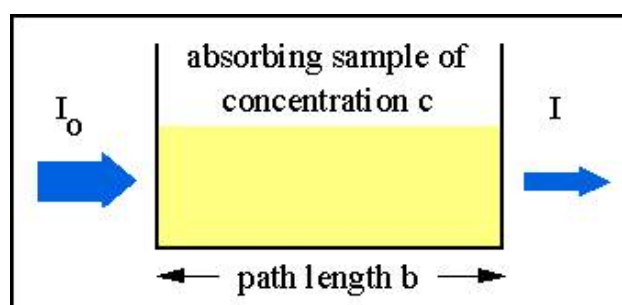
where  $I$  is the light intensity after it passes through the sample and  $I_0$  is the initial light intensity.

The relation between  $A$  and  $T$  is:

$$A = -\log T = -\log (I / I_0)$$

and this formula is the one that is generally associated with the Beer-Lambert law.

The PPM 60<sup>®</sup> instrument uses traditional 1 centimeter (cm) light path test tubes. By comparison, the light path of the well formatted microtiter trays is 0.1 cm and the light path of the Vitek 2 system even less. Therefore, the differences in the light paths inherently make the PPM 60<sup>®</sup> instrument on the order of 100 times more sensitive. In terms of number of bacteria per mL, 6



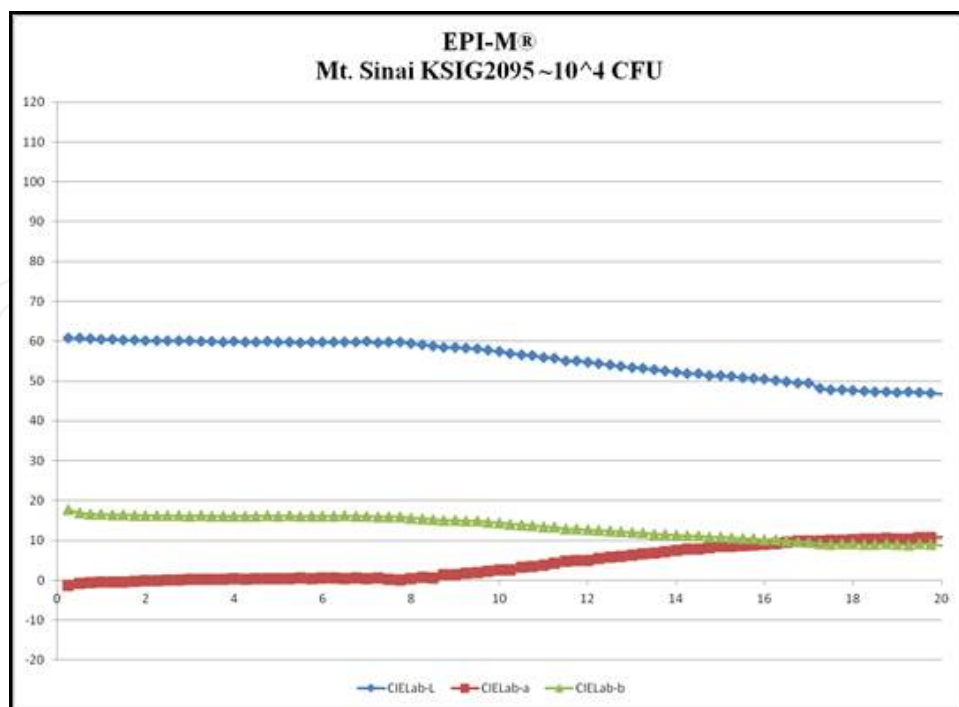
$\log_{10}/\text{mL}$  of bacteria is the lowest limit of detection in a 0.1 cm light path (based on turbidity, without a chemical signal, such as the release of a chromophore from a hydrolysable substrate or change in a pH indicator) and in a 1 cm pathway, the limit increases to 8  $\log_{10}/\text{mL}$ .

Practically, because bacteria in log phase have a generation time of 15-20 minutes, the time to detection in the PPM 60<sup>®</sup> instrument is much less than other methods, and significantly less than the observation of colonies on agar.

### 3.3.3. Example of data produced by the PPM 60<sup>®</sup> and analysis of a positive

Below are the results from the PPM 60<sup>®</sup> from a patient isolate of MRSA from the Mt. Sinai Hospital, New York City, NY, USA (courtesy of Dr. Camille Hamula). The initial inoculum was less than that found in the nose at  $6.0 \times 10^4$  CFU.

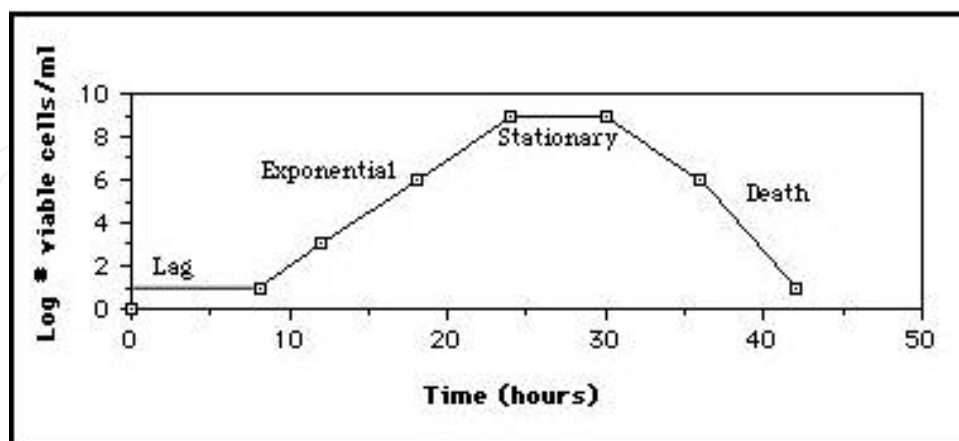
A	B	C	D	E	F	G	H	I	J	K
Time (min)	Hours	Temp (°C)	Gen	Averaging Cycles X	Y	Z	CELab-L	CELab-S	CELab-b	
900016	0.250004444	33.5	2	4	27.73106	28.087204	15.530171	50.858513	-1.246363	17.746902
9000047	0.500013056	34	2	4	27.722898	28.972702	15.063016	60.75753	-0.943823	16.900003
2700063	0.750015	34	2	4	27.541946	28.740337	15.932626	60.955773	-0.676334	16.633547
3600070	1.000021667	34	2	4	27.447348	28.600136	15.788754	60.427094	-0.516355	16.525212
4500094	1.250026711	34	2	4	27.467205	28.597708	15.812591	60.424307	-0.427723	16.467090
5400110	1.500039556	34	2	4	27.383285	28.425196	15.750627	60.270943	-0.359654	16.34885
6300141	1.750039167	34	2	4	27.40054	28.463761	15.784233	60.310791	-0.26732	16.335701
7200156	2.000043333	34	2	4	27.25528	28.254051	15.691315	60.18396	-0.18891	16.225374
8100172	2.250047778	34	2	4	27.291881	28.30476	15.73616	60.163078	-0.041455	16.198135
9000203	2.500056389	33.5	2	4	27.245409	28.259773	15.70869	60.122704	-0.072964	16.189547
9900219	2.750060833	33.5	2	4	27.207032	28.200922	15.675765	60.063824	0.006385	16.184818
10800235	3.000065278	33.5	2	4	27.227167	28.195044	15.677691	60.064545	0.164509	16.170001
11700266	3.250073889	33.5	2	4	27.100008	28.064308	15.586357	59.946795	0.160625	16.191685
12600297	3.5000825	33.5	2	4	27.25977	28.163034	15.674461	60.025744	0.243694	16.125244
13500328	3.750091111	33.5	2	4	26.94194	27.887069	15.519559	59.781036	0.236481	16.070240
14400344	4.000095556	33.5	2	4	27.11662	28.033354	15.588481	59.918861	0.327595	16.110781
15300360	4.2501	33.5	2	4	27.059534	27.976362	15.561252	59.864575	0.301391	16.10359
16200375	4.500104167	33.5	2	4	27.053071	27.937504	15.530626	59.832237	0.464196	16.13104
17100402	4.750117222	33.5	2	4	27.09558	28.000975	15.590474	59.889616	0.372976	16.180231
18000430	5.000121667	33	2	4	27.030632	27.978943	15.517954	59.812721	0.459852	16.138886
18900469	5.250130278	33.5	2	4	27.04677	27.944995	15.519638	59.845375	0.430954	16.190736
19800485	5.500134722	33.5	2	4	26.802299	27.695962	15.399378	59.756637	0.552325	16.024839
20700516	5.750143333	33	2	4	27.000376	27.982088	15.433487	59.782059	0.468761	16.136026
21600547	6.000151944	33	2	4	27.049575	27.916847	15.507915	59.810538	0.585521	16.154768
22500594	6.250165	33	2	4	27.005371	27.864738	15.497915	59.766342	0.596678	16.088022
23400641	6.500178556	33	2	4	27.087038	27.964676	15.440591	59.874886	0.480245	16.100134
24300703	6.750195278	33	2	4	27.003933	27.846949	15.489616	59.750214	0.618024	16.100525
25200735	7.000204167	33	2	4	27.102969	27.987844	15.566097	59.877747	0.470072	16.122389
26100766	7.250212778	33	2	4	26.772223	27.638933	15.429783	59.561027	0.497103	16.196789
27000791	7.500216944	33	2	4	26.97967	27.926277	15.582195	59.822075	0.196487	16.198872
27900828	7.75023	33	2	4	26.903717	27.953058	15.628884	59.846306	0.124574	16.197805
28800844	8.000234444	33	2	4	26.62483	27.514111	15.495367	59.447205	0.388414	16.152704
29700860	8.250238889	33	2	4	26.399705	27.186823	15.319544	59.146858	0.758648	16.173533
30600891	8.5002475	33	2	4	26.038628	26.860233	15.179551	58.844734	0.577596	16.101239
31500922	8.750256711	33	2	4	25.894731	26.421257	15.000735	58.430359	1.501441	16.030087
32400953	9.000264722	33	2	4	25.836641	26.433092	14.983352	58.445977	1.45939	16.103230
33300969	9.250269167	33	2	4	25.66745	26.177252	14.888306	58.204918	1.76018	14.902472
34200985	9.500273611	33	2	4	25.592785	26.078732	14.849304	58.111122	1.885576	14.787877
35100916	9.750282222	32.5	2	4	25.259933	25.854036	14.671161	57.707161	2.227129	14.61519
36001031	10.00028639	32.5	2	4	25.062403	25.379784	14.569389	57.442642	2.510052	14.448023
36901047	10.25029083	32.5	2	4	24.624963	24.900784	14.388033	56.985466	2.640456	14.140391
37801063	10.50029528	33	2	4	24.389302	24.956289	14.214195	56.640446	3.302047	13.962626
38701094	10.75030389	32.5	2	4	24.252323	24.330191	14.138654	56.41943	3.491094	13.775659
39601110	11.00030833	32.5	2	4	23.897129	23.893232	13.968018	55.980736	3.80826	13.471185
40501156	11.25032111	32.5	2	4	23.776279	23.676466	13.885352	55.762398	4.218055	13.368472
41401172	11.50032556	32.5	2	4	23.152481	22.910329	13.549113	54.989143	4.785886	12.887284
42301203	11.75033417	32.5	2	4	23.230804	22.970663	13.584412	55.042707	4.907459	12.875056
43201235	12.00034306	32.5	2	4	22.96487	22.684134	13.470819	54.745483	4.994244	12.670029
44101266	12.25035167	32.5	2	4	22.751898	22.369802	13.309974	54.419723	5.448133	12.540829



There was a significant change beginning at 9 hours and the positive was called at 11.25 hours.

### 3.3.4. Quantification of CFU/mL by the PPM 60® instrument

The time to a positive is proportional to the number of bacteria in the sample. Bacteria go through four stages of growth in liquid (but not agar): lag phase, log phase, stationery phase, and decline phase.

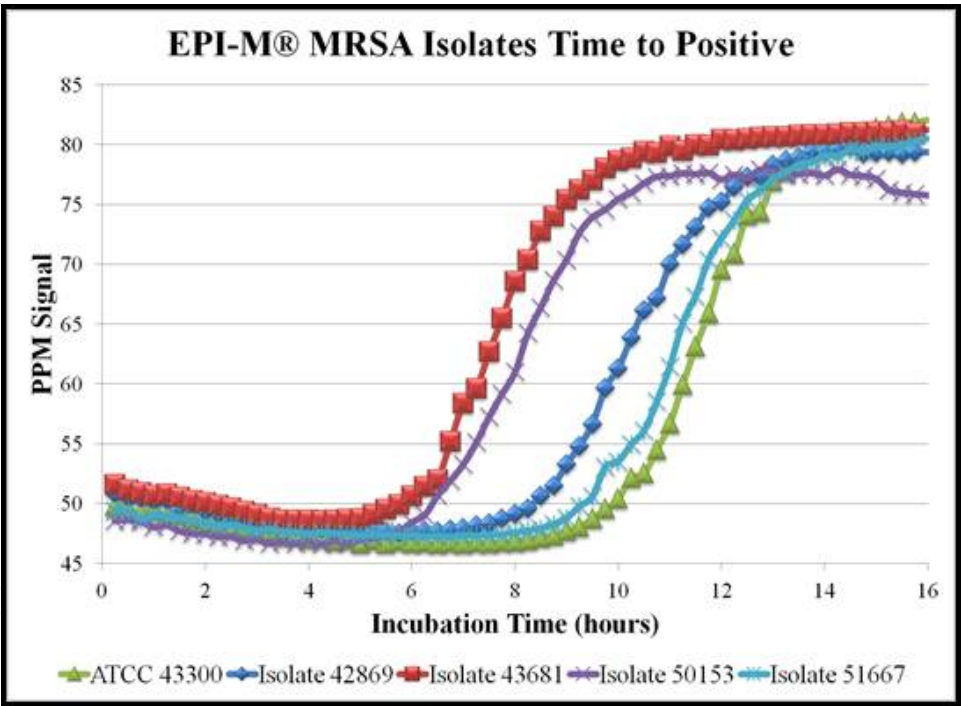


It is well known that the greater the number of bacteria in a sample, the shorter the lag phase. Lag phase is the time that the bacteria acclimate to a new environment. When bacteria enter a new environment, there is a heterogeneous population in terms of physiological health. Each of the cells acclimates to the new environment and leave lag phase and enter log phase at

different times. By analogy it is similar to a kettle of popcorn kernels; one adds the kernels and all are in the same oil at the same temperature. The “healthier” ones pop first, the number of pops increase, and then there are a very large number of pops. Then there is a declining number of pops. Likewise with bacteria, some leave lag phase and enter log phase before others. The greater the number of bacteria, the shorter is the overall time the population goes from lag phase to log phase. In the PPM 60® instrument as discussed above, once the bacteria enter log phase they are dividing every 15-20 minutes. Therefore, the more bacteria in a population that leaves lag phase and enters log phase, the shorter the time to a positive since each bacteria multiplying produces a signal.

Below are examples of the ability of the PPM 60® to provide quantitative information with a variety of target bacteria.

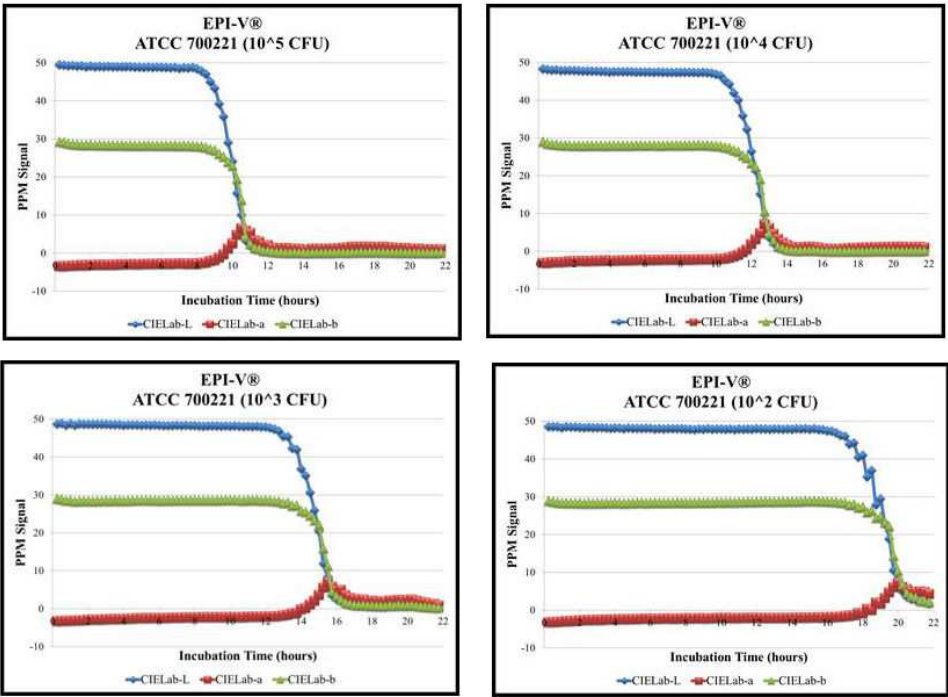
3.3.4.1. MRSA



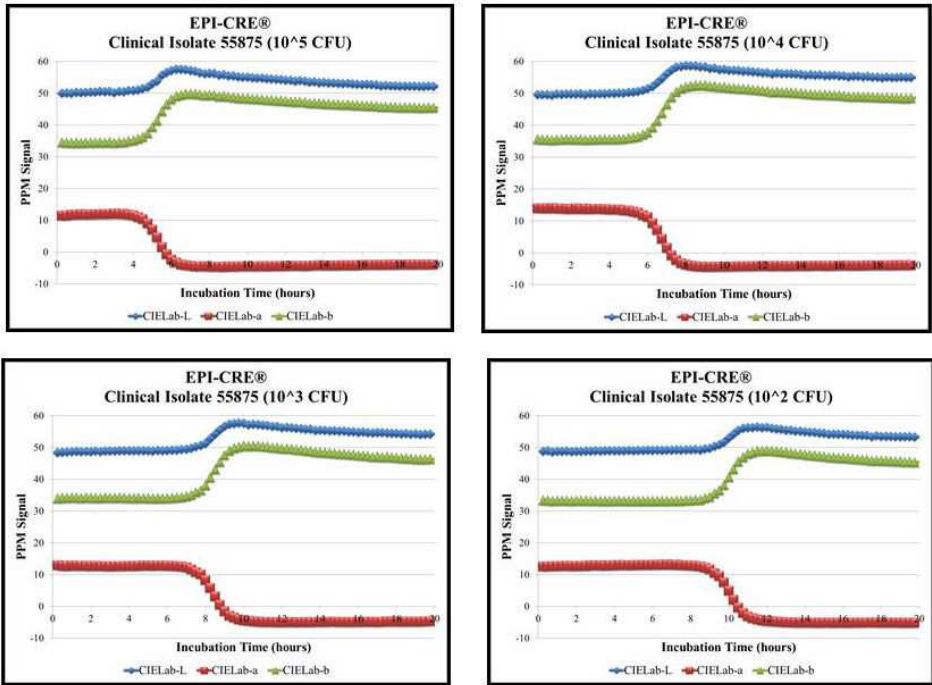
Isolate	Positive Result	Total CFU
Clinical Isolate 50153	6.25 hrs	24,000
Clinical Isolate 43681	6.0 hrs	14,800
Clinical Isolate 42869	8.5 hrs	10,400
Clinical Isolate 51667	9.0 hrs	4,800
ATCC 43300	9.75 hrs	4,000



3.3.4.2. VRE



3.3.4.3. CRE





## Acknowledgements

Lize-Mari Russo is thanked for excellent technical assistance with this project. SCE is the developer and patent holder of the DSU<sup>®</sup> epidemiology screens including EPI-M<sup>®</sup>, aureusAlert<sup>®</sup>, EPI-VISA<sup>®</sup>, EPI-V<sup>®</sup>, EPI-CRE<sup>®</sup> and EPI-FLOX<sup>®</sup>.

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