

Molecular Diagnostics

Developing bacteriophage
amplification for bacterial
identification

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Bacteriophage amplification for bacterial identification

New bacteriophage technologies are being developed to create rapid tests for bacterial infections. BY DREW SMITH

Anyone who has taken an introductory biology course is familiar with the role that bacteriophage (viruses that infect bacteria) have played in the development of modern biology. As the simplest of life forms, bacteriophage are especially amenable to study and manipulation. Many of the most profound insights of twentieth-century biology (e.g., establishment of DNA as the genetic material, the nature of genes and the genetic code, the workings of genetic switches) were teased out by clever yet low-tech experiments made possible by phage biology.

The essence of this biology is that a few phage will quickly become billions if they are provided with a host and growth medium, and nothing is done to poison either host or phage. This property of massive spontaneous amplification makes phage growth a good surrogate marker for following biological processes.

Phage have continued to be a source of useful enzymes for biotechnology and provide a substrate for bioengineering and nanotechnology development. But their usefulness to science and medicine diminished during the course of the last century as the focus of biology shifted to higher levels of complexity, and medicine in wealthy countries focused on treat-



ing diseases of affluence. Like phage themselves, phage technology development depends on bacteria. When it was decided that bacterial diseases could be ignored, bacteriophage also started to be ignored.

It is no secret that bacterial infectious diseases are making a comeback and that antibiotic resistance is spreading fast while the pace of new antibiotic development remains slow. As the bacterial threat increases, so does the need for better tools to identify and classify bacteria such that appropriate therapies can be administered.¹ Phage-based bacterial diagnostics now being developed offer the promise of providing accurate, actionable information

to doctors while retaining the low costs and simplicity always associated with phage technology.

Administering appropriate antibiotic therapies as soon as possible has always been the key to successful treatment of serious bacterial infections.² Fifty years ago, this was easy. Today, a patient's chances of receiving appropriate antibiotics are "no better than a coin flip," as a recent U.S. study showed.³ The reasons for this are not hard to understand: nearly all strains of clinically important bacteria are resistant to at least one antibiotic.

Empiric therapy, also known as guessing, will regularly result in the wrong choices of antibiotics. Selecting appropriate therapies requires antibiotic susceptibility testing. However, current methods for identifying bacteria and determining antibiotic susceptibility are too slow (usually three days to results), or too expensive for constrained lab budgets, or too complicated, especially for community hospitals with limited expertise. As a result, susceptibility testing is often not performed, or is ignored because the patient has gotten better (or died) by the time results are available.

Molecular diagnostic technologies, principally polymerase chain reaction (PCR) and isothermal amplification technologies, have advanced considerably in the last decade and are becoming faster, cheaper, and less

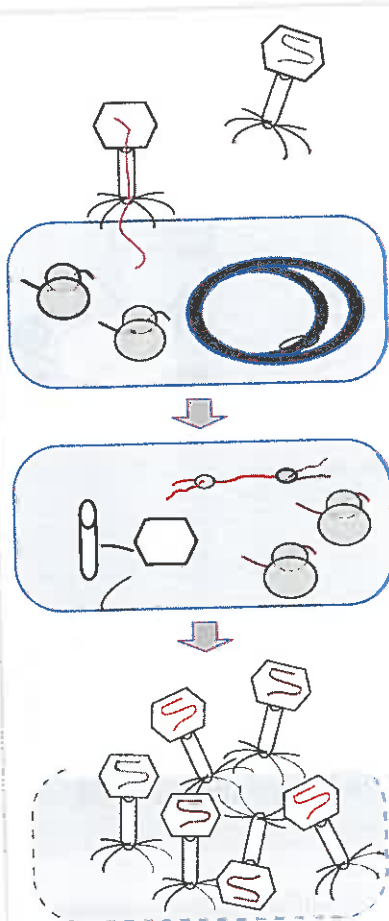


Figure 1. Phage life cycle. Top: Bacteriophage scan the bacterial surface. If correct receptors are found, phage DNA (or RNA) is injected. Middle: The host chromosome is destroyed, ribosomes are reprogrammed with phage mRNA, phage DNA replication commences, and phage proteins begin to assemble. Bottom: The host cell wall is degraded and mature phage particles are released.

complicated. There is every reason to believe that these trends will continue. Molecular technologies are, or should be, the gold standard for species identification. After all, it is a gene sequence that best defines a microbial species. Well executed molecular assay technologies will not likely be surpassed for their speed and accuracy for species identification.

However, speciation is only half, and arguably the less important half, of the diagnostic information needed

for treating bacterial infections. An effective treatment depends critically on identifying the antibiotics to which an infectious agent is susceptible. While an effective treatment is possible without species identification, speciation is not sufficient to inform an effective treatment.

A look at phage biology suggests why phage are especially well suited to assay bacterial antibiotic susceptibility and resistance (see Figure 1). Phage are parasites, and they depend completely on their bacterial host for growth and amplification. Any agent that kills the host or shuts down its metabolism (i.e., an antibiotic) must necessarily prevent phage growth. The key point to remember is that this effect is pathway- and gene-independent. Phage technology thus provides the basis for a method of determining antibiotic susceptibility that is both universal and agnostic, and no underlying mechanism of resistance needs to be assumed.

In principle then, phage technology

can provide a platform for the development of rapid, simple, and accurate bacterial diagnostics for a range of infectious diseases. Historically, there have been two approaches to using phage as diagnostic reagents: the phage are labeled or are engineered to express a label such as luciferase, or phage amplification itself is detected.⁴ The first strategy has the advantage of high analytical sensitivity, resulting in assays capable of single-cell detection. However, by reporting only an initial binding or gene expression event, some specificity is lost. In addition, the labeling steps result in increased complexity of manufacturing. As a result of these factors, no phage labeling schemes have yet been implemented in a commercially viable assay.

Detection of phage amplification as a surrogate marker for bacterial viability has advanced further and will be described in more detail. Two phage-based assays have reached commercial development: the FastPlaque assay



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by BioTec Ltd. for tuberculosis (TB) detection, and the MRSA/MSSA Blood Culture Test by MicroPhage Inc. for detecting and classifying bloodstream infections.

Tuberculosis Diagnostics

Identifying *Mycobacterium tuberculosis* infections is among the most challenging problems in bacterial diagnostics. The sample type (sputum) is notoriously difficult to work with, the number of bacteria in a positive sample can be small, and with a doubling time of five days or more, the organism grows painfully slowly. Clinics in affluent countries can cope with these challenges since reliable PCR methods have been developed, case loads are small, and multiple drug-resistant strains are still relatively rare.

The problem is that diagnostic resources are scarcest where the needs are greatest. PCR methods that are largely restricted to university hospitals in affluent countries are of no use to the rest of the world. Not surprisingly, developing TB diagnostics usable in resource-poor clinics has been identified by the Gates Foundation as a top priority for its Grand Global Challenges program.

The Fast-Plaque assay by BioTec is a solid step toward the goal of developing simple and usable TB diagnostics, and illustrates both the strengths and weaknesses of phage-based diagnostics. The weaknesses arise from the fact that phage must find the bacteria, and that both phage and bacteria are large. Large, that is, for diagnostic reagents. Their diffusion constants are 2–3 orders of magnitude slower than those of macromolecules such as antibodies or PCR primers.

A useful number to keep in mind for thinking about the timing of phage binding is 10^{-9} ml/min. This obtuse-sounding rate constant makes sense once it is multiplied by the concentration of bacteria in a test sample: at 10^7 bacteria/ml, about 1% of the bacteria will be infected per minute. A problem with TB, and some other sample types, is that there may be no more

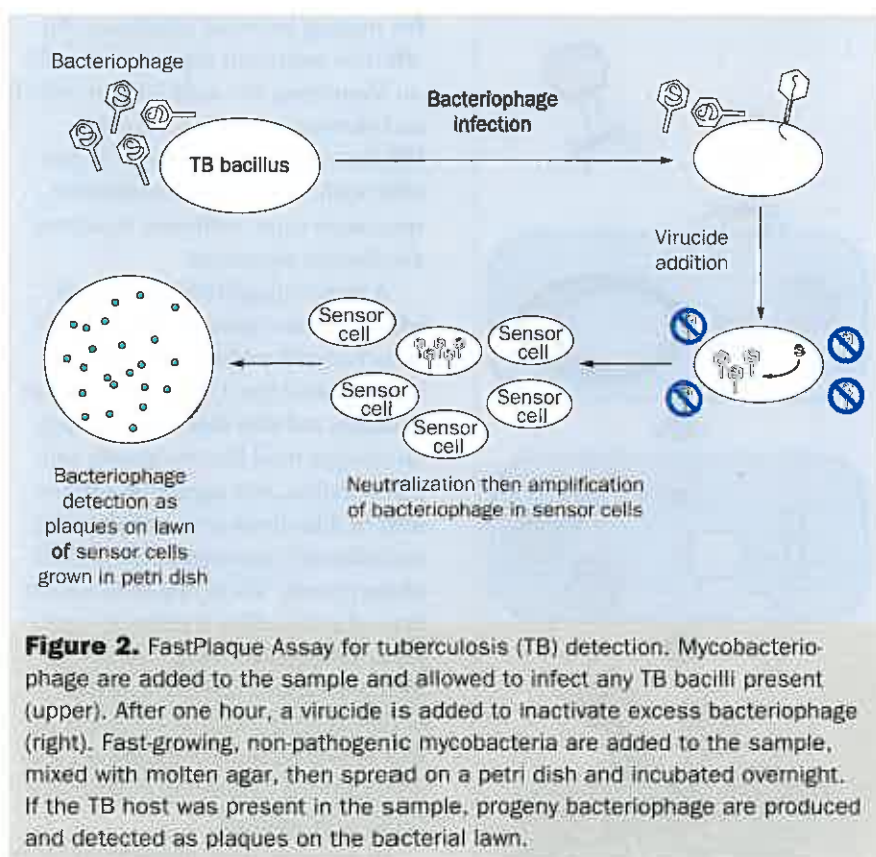


Figure 2. FastPlaque Assay for tuberculosis (TB) detection. Mycobacteriophage are added to the sample and allowed to infect any TB bacilli present (upper). After one hour, a virucide is added to inactivate excess bacteriophage (right). Fast-growing, non-pathogenic mycobacteria are added to the sample, mixed with molten agar, then spread on a petri dish and incubated overnight. If the TB host was present in the sample, progeny bacteriophage are produced and detected as plaques on the bacterial lawn.

than 10^2 bacteria per ml in a sample. Getting phage to bind even one cell will take roughly 10^5 min. if the phage are at a similar concentration.

A solution to the rate problem is to drive the binding kinetics with high phage concentrations. For example, phage at 10^8 /ml will infect all the cells in a sample in a few minutes. But this approach creates a background problem, as 100 cells producing 100 progeny phage each (10^4 total) will be a small fraction of the input phage level. The background problem can be solved by killing or removing the input phage, either by filtering or adding a virucide that does not kill internalized phage.⁵

The Fast-Plaque assay employs the latter method, treating the phage-exposed sample with a virucide that is removed before progeny phage are produced (see Figure 2). Only a few hundred phage (a few femtograms in mass) are produced by the initial phage reaction, so an amplification step is required to permit detection by the operator. The assay employs the

phage's self-amplifying properties to accomplish this step: a fast-growing *Mycobacterium* species is added to the sample then spread in soft agar on a plate for a plaque assay.

The plaque assay is the lowest tech example of a single-particle detection assay. As host bacteria grow and infiltrate the soft agar, they will encounter and be infected by single phage particles. Each infected cell will release hundreds of new phage particles that will in turn infect and lyse hundreds of new bacteria. After an overnight incubation period, the result is an approximately 2-mm zone of clearing (a plaque) in a lawn of bacteria that is easily detected by visual inspection.

The Fast-Plaque assay thus requires no instrument more sophisticated than an autoclave and an incubator to attain detection of TB in about 48 hours. True to the promise of phage technology in delivering useful antibiotic susceptibility information, the basic ID test can be extended with a parallel assay (FastPlaque Response) that detects

rifampin resistance and susceptibility.

Test execution requires several steps, each of which is fairly simple: add decontaminating solution, centrifuge to concentrate and remove supernatant, add bacteriophage and incubate, add virucide, add sensor cells and media, mix with molten agar and plate, incubate overnight, and count plaques.

The performance of the FastPlaque assay in the field has been mixed. Well-equipped laboratories have reported sensitivity and specificity values of 95% or better.⁶ However, these sensitivity values are somewhat misleading as typically only 70-80% of samples are interpretable. The leading cause of test failure is due to overgrowth or inhibition by competing bacteria present in the sample.

The FastPlaque product was reformulated to include an antibiotic cocktail that suppresses the growth of non-*Mycobacterium* species, resulting in an increase of interpretable samples from 70% to 88%.⁷

Reported performance in resource-constrained laboratories has been highly variable. Although specificity is generally high (83-100%), sensitivity can be poor, ranging from 88% all the way down to 21%.⁸ Lack of sensitivity is most plausibly explained by low bacterial loads. When results from untreated patients only are considered, reported sensitivities are 72% or higher. Loss of viable target bacteria during storage and transport and contamination are also factors likely to contribute to decreased sensitivity.

The overall opinion seems to be that the FastPlaque assay is at best comparable to smear microscopy.⁹ Although it is more specific than microscopy, it is also more complex in its present form. However, the expansion of the FastPlaque assay to include rifampin susceptibility testing provides a qualitative advantage over smear microscopy, which cannot

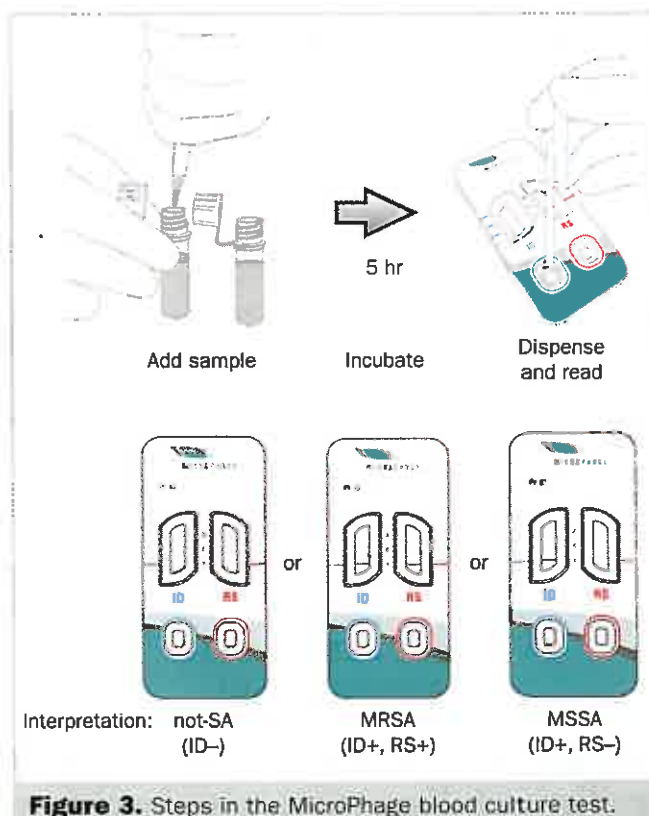


Figure 3. Steps in the MicroPhage blood culture test.

assess antibiotic susceptibility. With the rapid spread of multidrug-resistant strains in many countries, this advantage is significant as it enables good antibiotic stewardship practices without depriving patients of the best available treatments.

Timely *S. aureus* Testing

MicroPhage Inc. (Longmont, CO) is nearing commercial launch of its first product, a test that identifies *S. aureus* in positive blood cultures and classifies them as methicillin-susceptible or -resistant. The MicroPhage platform is simpler than the FastPlaque assay and employs a rapid-test immunoassay technology for its readout: mix liquid reagents (broth plus phage) with dry reagents (antibiotics), add clinical sample and incubate, and add test sample to detector and read (see Figure 3).

Because *Staph* bacteria grow much faster than *Mycobacteria* (doubling time is one half-hour versus five days), the phage amplification reaction takes place in a single step incubation of five hours. Because of the rapid-test

readout, the results of the amplification reaction are available in 20 minutes rather than after an overnight incubation, and are not susceptible to invalidation by contamination. The advantage of this approach is to convert microbiology tests, which usually require a high degree of knowledge and experience, into simple immunoassays that all lab personnel can reliably perform.

The *Staph* bacteremia assay is a key test for the specificity of phage amplification technology. Bacteriophage occupy a number of ecological niches. Generalist phage infect a wide range of bacterial species but amplify with modest success on any particular species. Specialist phage are very good at infecting one or a few species.

Obviously a bacterial identification test should make use of the latter category. But the level of specificity attainable with bacteriophage in a clinical assay has been an unanswered question. The bacteremia application is a particularly challenging application in this regard. Closely-related non-pathogenic *Staphylococcus* species are three times as prevalent in positive blood cultures as is *S. aureus*.¹⁰

A multicenter clinical trial of the MicroPhage MRSA/MSSA Blood Culture Test was completed in January 2010 and is undergoing FDA review. Results indicate that bacteriophage technology can indeed deliver high diagnostic specificity (see Table I). Furthermore, the phage test is able to classify *S. aureus* strains as methicillin-susceptible or -resistant with an accuracy that approaches reference methods.

Because of the rapid time to results, the test has the potential to improve the detection of *S. aureus* bloodstream infections, which are highly lethal (greater than 20% mortality rate) and commonly under-treated or mistreated with respect to

appropriate antibiotic therapies.¹¹ *S. aureus* infections cause a number of other significant healthcare problems. MicroPhage is planning to expand its *S. aureus* tests to include wounds and surgical-site infections, pneumonia, and a screening test for nasal carriage.

Regulatory Regime and Barriers to Adoption

Bacteriophage technology already has some precedents at FDA as a Class I IVD, with plaque assays registered from CDC and the U.S. Army for a *Staphylococcus sp.* phage typing scheme and a *B. anthracis* (Anthrax) test using gamma phage, respectively. MicroPhage's application for use of phage amplification in a Class II IVD will likely benefit other companies in the same space since they will be able to use it as a predicate device for their submissions to FDA.

There are few barriers to adopting phage technology, particularly bacteriophage amplification, as the

performance of the first test on this platform has shown equivalence to other standard methods. Clinical microbiologists, the primary end users, are familiar with bacteriophage and understand that these are bio-safety Level I organisms and do not pose any undue risk to test users.

There is no established reimbursement that is currently assigned to bacteriophage technology. MicroPhage plans to pursue a standard approach to demonstrate test performance and economic return compared to molecular methods in order to establish a new CPT code. In the interim, MicroPhage also plans to use a miscellaneous code while petitioning for a new code and pricing assignment.

Future Developments

The growth and development of phage diagnostic technologies can be surmised by an assessment of their strengths and weaknesses versus competing technologies. Most technology

Parameter	Results
<i>S. aureus</i> sensitivity	93%
<i>S. aureus</i> specificity	98%
MRSA overcalls	0.3%
MSSA overcalls	0.6%
MRSA, MSSA PPV	94%
MRSA, MSSA NPV	99%

Table I. Performance of the MicroPhage Blood Culture Test in Multicenter Trials. Total samples tested = 1116, total *S. aureus* samples = 366. Sample: 10 µL positive blood culture. Reference methods: Tube coagulase and Staphaurex for bacterial ID; cefoxitin disk diffusion for antibiotic susceptibility.

developments in bacterial diagnostics are based on nucleic acid test (NAT) technologies, especially PCR and isothermal amplification methods. Table II summarizes the key differentiators between bacteriophage amplification

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Property	NAT	BAT
Bacterial ID	Yes	Yes
Antibiotic susceptibility	No	Yes
Complexity	Low to High	Low
Capital costs	> \$30 K	None
Per-test costs	\$30-50	\$12-30

Table II. Comparison of nucleic acid and bacteriophage amplification technologies.

technology and NATs with respect to bacterial diagnostics.

Complexity has been a constraining factor in developing NATs. Inhibitors of DNA polymerases are found in a variety of specimen types, necessitating extensive sample processing steps, resulting in a tradeoff between cost and complexity. Automated sample preparation instruments reduce complexity and hands-on time but result in significant capital costs. By contrast, bacteriophage have evolved to work in an extracellular biological milieu and are generally insensitive to sample interference. For example, the FastPlaque test requires only decontamination and concentration steps for sputum samples, and the MRSA/MSSA Blood Culture Test requires no sample preparation.

While NATs are very good at identifying bacterial species, they have had limited success in accurately determining resistance and susceptibility. The limiting factors appear to be not the technology per se, but the underlying biology. NAT methods have been successful in determining methicillin resistance in *S. aureus*, but MRSA may well be the low-hanging fruit for NATs. The vast majority of resistant strains harbor a specific, well-conserved gene cassette (*scfMec*) that integrates into a unique site in the *S. aureus* chromosome. The resistance factor is physically linked to chromosomal sequences that identify *S. aureus*, which is unusual. Most resistance factors are found on plasmids that shuttle promiscuously between species, picking up numerous rearrangements and point mutations along the way. These rearrangements confound efforts to design universal primer sets. Furthermore, resistance

plasmids may be found in nonpathogenic organisms, creating the potential for false resistance calls in mixed infections.

The consequence of this biology is that NAT diagnostics focus primarily on species identification. While species identification is valuable in diagnosing viral infections, it is less valuable for bacterial infections, which additionally require antibiotic susceptibility information to guide therapy.

The gold standard for susceptibility testing will likely continue to be based on culture methods. Bacteriophage amplification is a method of accelerating culture results by using a surrogate marker. The ability of phage technology to create new antibiotic susceptibility tests is illustrated by a proof-of-concept experiment at MicroPhage. A panel of *S. aureus* clinical isolates resistant to one or more of a set of eight antibiotics was identified and tested for susceptibility determination using phage technology (see Table III). The antibiotics tested include all major categories, including inhibitors of DNA synthesis, protein synthesis, and cell wall synthesis. The phage test could accurately call susceptibility and resistance in all antibiotic categories. Although preliminary, this experiment offers support for the proposition that phage technology provides a general platform for developing

bacterial diagnostics.

As bacteriophage technology is a biological method, it bears comparison with commercialized culture methods, principally the chromogenic media such as ChromAgar and MRSA Select, and automated ID/AST systems such as Vitek and Phoenix. Like the walkaway ID/AST systems, phage tests (assuming acceptable performance) can determine antibiotic susceptibility and guide therapy. The advantage of phage technology in this respect is that direct sample testing is possible, allowing reports to be generated in hours, rather than 2-3 days with the walkaways. For critical indications such as bloodstream infections and pneumonias, this time difference could have significant clinical value.

Both the phage tests and chromogenic media require only an incubator, and both currently report only a single species. They differ from automated systems in having very low capital costs, making them more accessible to a range of institutions. But they do not provide a comprehensive report on species and antibiotic susceptibilities. The chromogenic media are currently labeled only for presumptive identification of bacterial species and resistant strains, usually in 18-24 hours, and optimally with a pre-enrichment step.

Because all bacteria have bacteriophage, phage technology can be expanded to cover the entire gallery of pathogenic bacteria and, most

Antibiotic	R	S	Accuracy	Target
Cefazolin	6	6	75%	Cell wall
Cefoxitin	126	128	95%	Cell wall
Clindamycin	5	11	100%	Ribosome
Daptomycin	2	19	100%	Cell wall
Levofloxacin	7	5	100%	DNA gyrase
Meropenem	7	17	100%	Cell wall
Piperacillin + Tazobactam	4	12	100%	Cell wall
Tetracycline	2	10	100%	Ribosome
Tigecycline	0	24	100%	Ribosome
Trimethoprim + Sulfamethoxazole	3	9	100%	Thymidine synthesis

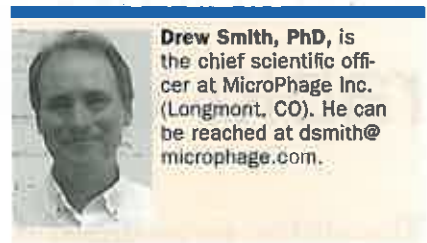
Table III. Antibiotic susceptibility testing in *S. aureus*. R = number of resistant strains, S = number of sensitive strains tested. All strains tested in blood culture.

crucially, provide timely determination of antibiotic susceptibility. As the technology matures, tests will become more streamlined and may be combined into panels with expanded menus of target bacteria and antibiotics. There are no significant barriers to automating the test process so some degree of instrumentation is likely to be implemented, particularly with respect to calling, recording, and communicating test results to clinical information systems. Another potential area for progress will be the development of near-patient tests at clinics, urgent-care centers, and long-term care facilities. Since the tests require no instrumentation other than an incubator, streamlined kits with a small heat block could be run by minimally trained personnel.

Bacteriophage have long been a friend to science, facilitating numerous discoveries on the workings of cells. Phage technology is now ready to become a friend to clinics, enabling faster, better, and less expensive diagnosis of serious bacterial infections.

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