An Assessment of the Technological Issues and Options for Point-Of-Care Diagnostic Tests in Resource-Limited Settings

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Introduction

The enormous strides that have been made in the scientific disciplines of molecular biology, genomics, chemistry, materials science, physics and engineering have been combined in the last 20 years to deliver a breathtaking array of technologies for the diagnosis of disease. Sensitive and specific diagnostic tests already exist for most infectious diseases. Unfortunately, this arsenal of tools is accessible only to the relatively wealthy in the developed world. A large portion of the earth's population has no access to these diagnostic tests that, if available, would assist local health care workers to better diagnose, manage, and ultimately overcome infectious diseases such as tuberculosis, malaria, and AIDS that continue to devastate these populations and remain some of humanity's greatest health burdens.

Diagnostic tests are a crucial component of strategies to combat many infectious diseases, not just at the level of the individual patient, but also for disease surveillance, which is the foundation of successful disease control and elimination programs. Many individuals at risk for these diseases have significant geographic and economic barriers to reaching even the most basic of health care settings, and often have a limited ability to return to the health care setting in a meaningful time frame for follow up. Such peripheral health care settings may have very limited medical supplies and staff with very minimal laboratory training. In order to be effectively implemented in a peripheral health care setting, or in any setting that might be limited by resources, a diagnostic test would ideally require no equipment, electrical power, sterile conditions, or extensive training. In places such as Sub-Saharan Africa, where temperatures can exceed 120°F in the laboratory itself, stability of the test and performance in extreme conditions become of paramount importance. In order to deliver the care required during a patient's visit, a diagnostic test should be rapid, so that treatment can be administered before the patient leaves. Most importantly, the tests must be affordable in context of the local health care setting.

Despite the vast array of diagnostic tests for infectious diseases available in the developed world, very few tests exist with the necessary performance characteristics and with a cost that is low enough to be routinely employed in resource-limited settings. Therefore, tests designed with the developing world in mind are urgently needed.

The good news is that the diagnostics research and industrial communities appear to be on the verge of overcoming many of the technological barriers to delivering these new tests. Demand in recent years for point-of-care testing devices for the developed world, and for "field-usable" tests for biosecurity applications, have led to incremental improvements to more traditional diagnostic platforms, as well as to truly innovative technologies and products. As this technological explosion and synergy between scientific disciplines continues to gain momentum, we can expect to see significant progress in the number of products on the market that are practical for resource-limited settings.

In order to enhance the likelihood of success, it is very important for diagnostic test developers to understand the challenges that exist for implementing disease diagnostics for the developing world. This paper reviews these challenges, the current state of diagnostic technologies that could be suitable, and areas where further improvements are needed. Finally, selected technologies under development are reviewed that may some day radically alter the landscape of diagnostic testing in resource-limited settings.

Section 1. Specimen Collection and Processing

Obtaining and testing clinical specimens in resource-limited environments presents a unique set of challenges. Ideally the specimen can be obtained and tested in the same location, and the test carried out within the timeframes that are practical for the patient-healthcare encounter. The most successful specimen collection and processing approaches will not require special equipment or supplies, sterile conditions, electrical power, extensive training, or generate biohazardous waste, and will be integrated into the assay test device.

In resource-limited environments, the practicality of collecting and processing a particular specimen type is a more important factor in the successful adoption of a particular test than it is in resource-rich sites. Factors such as the quantity of the analyte in different specimen types and the sensitivity that is required for a useful diagnostic test for a particular infectious agent must be balanced against the ease of collecting a particular specimen type, and the sensitivity that can be obtained by an assay format with a particular specimen type. For instance, if HIV virions are present in sufficient quantities to be adequately detected by a one-step immunodiagnostic method in a sample type that is easy to obtain, such as oral fluid, then this is usually preferable to another sample type or assay format that presents greater challenges in sample processing (such as a nucleic acid test that requires nucleic acids to be purified from whole blood). The following discussion reviews the specific challenges surrounding the collection and processing of blood, oral fluid, urine, respiratory, and stool specimens.

1A. Blood

Blood is the most commonly used specimen in human diagnostic tests.[1,2,3] In many diseases caused by infectious organisms such as HIV or HBV,[4] blood contains the analytes that are used to diagnose the disease. In resource-rich environments, blood is often obtained by healthcare workers via phlebotomy (venous puncture) and collected in vacuum tubes. Because of the wide variety of components of blood and the potential of many of these components to interfere with diagnostic tests, it is often necessary to then separate the blood via centrifugation into its cellular (solid) and either plasma (liquid) or serum (clotting-factor depleted liquid) components, after which only one component is tested in the assay. However, in resource limited environments, this sample collection and processing strategy is not

necessarily practical due to the requirements for training, supplies such a syringes and vacuum tubes, biohazardous waste disposal, equipment, and power for centrifugation.

Testing of a particular fraction of blood can often allow higher sensitivities and specificities to be achieved. For example, state-of-the-art immunoassays to detect hepatitis B surface antigen performed using plasma and sophisticated instruments have sensitivities that exceed 0.1 ng/ml. Rapid devices that measure the presence of this antigen in whole blood are commercially available,[5] however sensitivity (typically limited to 1.0 ng/ml) has been sacrificed in favor of speed and the ability to use whole blood as a sample.[6]

The most practical approach currently available for the collection of blood in resource-limited environments involves pricking a finger (or heel) with a lancet and capturing blood drops on a solid support, such as filter paper or a sample collection pad or in a microcapillary. Capillary collection devices can be subsequently centrifuged in a microhematocrit system to prepare plasma and whole cell fractions.[7] Newer paper or membrane materials for rapid point-of-care (POC) devices have been designed to allow some level of separation, where certain components remain bound to the material, and other can be eluted off or wicked out of the material. Manufacturers of synthetic membranes have developed specialized glass fiber, polyester, nitrocellulose, or cellulose products that are used in rapid POC devices that are designed to accept a sample of 30 to 100 µl (one to two drops) of blood from the fingertip.[8] The drops of blood are placed on a sample separation material ("sample pad") at one end of a lateral flow device (see assay format section). Pads of varying thickness can be used to control the blood wicking rate as well as the maximum working volume. Blood cells are trapped in the pad, with minimal lysis of the erythrocytes, and within 20 to 45 seconds, the plasma in the sample is wicked through the pad on to the chromatographic membrane of the device. Recent advances in membrane production have allowed the production of a single membrane material that functions as both the sample pad for separation of the blood and the chromatographic membrane.[9]

Cells can be removed from blood (or other specimens) with other technologies such as track etched membranes (TEMs), which are extremely thin yet have very consistent and specific pore sizes.[9] Thus cells of particular sizes can either pass through or be retained on the membrane. For instance, microparticles coated with specific antibodies can be added to a sample that bind to particular cell types, creating large particle-cell complexes that are retained by TEMs, while other cell types and plasma components pass through the membrane. Although devices using this approach have yet to be commercialized, it is conceivable that such an approach could be used in a device to collect specific cells for analysis.

Other approaches are under development that might someday be appropriate for processing clinical samples in resource-limited settings, including the use of microbeads coated with specific antibodies,[10] self-contained microfluidic cartridges, or microelectrical mechanical systems (MEMs), though these devices have not yet achieved the cost or simplicity requirements for resource limited sites.

1B. Oral Fluid

Oral fluid is probably the easiest specimen to collect and is typically collected via swab or similar collection device.[11] The specimen is then either dried or placed into a stabilization media[12] for storage until an assay is conducted. Oral fluid specimens contain both liquid (mostly saliva) and cells that have been exfoliated from the oral epithelium. In resource-rich environments, the cells might be removed from the specimen via centrifugation or filtering[12] though newer assay platforms have been designed to use non-fractionated oral fluids and still provide excellent concordance with results obtained in assays that use blood. DNA for genotyping can also be readily prepared from oral cheek (buccal) swabs or from the cellular components in saliva.[13]

Crevicular fluid, usually collected with a sterile paper point in the space between the teeth and gum, represents a different type of oral specimen that is comprised of serum, connective tissue and epithelial, and in the case of disease inflammatory components. In the dental literature this specimen has been extensively cited as a source for new biomarkers associate with periodontal and other oral disorders.[14]

Compared to blood, oral fluids specimens have significant advantages for resource limited environments. Oral fluid is easy and painless to collect, contains lower concentrations of components that interfere with the performance of immunoassays or nucleic acid amplification methods, it is easier to prepare for analyses, and it is less biohazardous (infectious agents are often present at lower levels than in blood).[15] The disadvantages with oral fluid are that it is more difficult to volumetrically standardize the quantity of a biomarker, due to inter-individual differences in fluid constituents, and that oral fluids may have lower concentrations of particular analytes, depending on the infectious agent to be detected.

1C. Urine

Urine is usually a sterile, cell-free specimen that can be easily self-collected using a variety of collection devices[16] and has been the specimen of choice for particular biomarkers. Antibodies, DNA, carbohydrates, and the antigens of some infectious agents are present in urine. For example, Hilton et al.[17] demonstrated that urine-based detection of antibodies for HIV in high-prevalence inner city communities can be effective in identifying infected individuals. Recently it has been demonstrated that urine can be used in a 15 minute immunochromatographic assay designed to detect *S. pneumoniae* C polysaccharide in a high percentage of individuals infected with pneumococcal bacteremia.[18] Botezatu et al. have shown that large fragments of DNA can cross the kidney barrier and can be found in urine,[19]

which can be collected and used in a wide variety of genotyping and DNA analysis applications.[20] Despite these advantages, urine has several limitations. There is significant inter-individual variability in the specimen volume, pH, salt, urea, and other constituents, and therefore the assay must be sufficiently robust to perform with diverse specimens. In addition, some analytes are present at lower levels than in other specimen types, and for some assays it might be necessary to concentrate the analyte before analysis. Finally, it is normally devoid of cells and hence is not useful for the analysis of cellular components.

1D. Respiratory Specimens

Expectorated sputum is the most commonly obtained specimen for analyses of lower respiratory infections.[21] Although it is relatively easily obtained from symptomatic patients, a quality specimen requires following strict collection protocols. Sputum may be induced using chemical techniques involving inhalation of an aerosolized 3% saline solution. A good quality specimen contains mucin and is highly viscous by nature. Standard microbiological procedures require that sputum be homogenized using chemical and physical methods such as the addition of equal volumes of dithiothreitol followed by vigorous vortexing for 20 to 30 seconds.[21] Although used in the routine clinical microbiology laboratory, these procedures for specimen collection are difficult in resource-limited settings. Because of the challenges in properly collecting a specimen and the need for extensive specimen processing, sputum will be a more difficult sample type to work with in resource-limited settings. Several newer approaches might make the collection and use of sputum specimens easier in the future, including techniques that have been used in patients with cystic fibrosis to improve the mobilization of sputum. Mechanical methods which cause the chest to vibrate at particular frequency have been tried, but the results of clinical studies have been inconclusive. Various chemical agents have also been tried to reduce the viscosity of the sputum which allow for easier expectoration. DNase has been used in this manner and Inspire Pharmaceutical has a drug in clinical evaluation (denufosol tetrasodium) which increases mucociliary clearance.[22] Further work needs to be done to determine if such approaches could be applied to the collection of sputum samples in resource limited settings. In addition to the above considerations, sputum is even further difficult to collect from children and patients infected with HIV. The challenges of specimen collection will need to be overcome to deliver tests for the organisms involved in acute lower respiratory infections, especially in children and HIV infected patients. Sputum collection is an area where an innovative practical solution is still needed.

Other specimens obtained for respiratory analysis include nasal washes, nasopharyngeal swabs, throat swabs, tracheal aspirates, and bronchoalveolar lavage.[21] These specimen types provide varying degrees of difficulty in collection, with swabs being the easiest. Swab specimens obtained from the upper respiratory tract have been successfully used to detect infectious agent antigens in a variety of point-of-

care tests. These sample types are usually placed into a diluent containing detergents and mucolytic agents and then transferred to the testing device. Compared to sputum, these upper respiratory tract specimens are easier to collect and require minimal processing.

1E. Stool

Stool is collected routinely for a variety of diagnostic analyses ranging from occult blood for colorectal cancer screening to parasitology. Bacteria, intestinal cells, and cell debris are all found in this specimen type. For parasitological analyses, fresh specimens have traditionally been examined by microscopy.[23] Due to the mode of collection and the presence of a broad number of generally uncontrollable substances (e.g. pigments, enzymes, inhibitors, and nucleases) stool specimens will provide significant challenges for resource-limited environments. Nevertheless, it may be a useful specimen type for detecting infectious agents of the gastrointestinal tract. For example, stool antigen testing has been used for the non-invasive detection of *H. pylori* that is indicative of active infection.[24] Specimen preparation involves emulsification, vortexing, and dilution prior to analysis. Recently a rapid POC test for *H. pylori* has been developed that uses stool as the specimen type.[25] Specimen processing vials containing diluent and snap off caps simplify the specimen preparation. Additional studies have documented the value of antigen testing for a range of parasites (e.g. *Giardia, Cryptosporidium,* and *Schistosomiasis*) in stool using standard ELISA and lateral flow assays.[26] Thus, technologies and approaches have been developed and are available for preparation of stool specimens for use in POC settings.

1F. Stabilization of Clinical Samples

There have been advances in the stabilization of biological materials through the addition of agents that promote "glass" formation or vitrification. Compounds such as trehalose and phosphate promote vitrification.[27] Cells stored in glass form are often nearly as stable as deep frozen materials. One potential limitation is that some procedures require vacuum drying in addition to trehalose addition, a clearly impractical process for resource-limited settings. Whether this method could be employed to stabilize blood or other samples in resource limited sites remains to be tested.

1G. Summary of Specimen Collection and Preparation

Each specimen type provides a unique set of challenges in their collection and preparation. These features are summarized and ranked in Table 1. It is important to note that even though a specimen is relatively easy to collect and prepare, it may not be appropriate because its clinical utility in the context of disease diagnosis and patient management has not been established. Furthermore, once a specimen is prepared, it may require further processing prior to analyses. For example, leukocytes prepared from whole blood, or a sputum specimen that has been rendered homogeneous, will require further processing

depending upon the biomarker being analyzed. These considerations will be further discussed in the section that follows.

1H. The Impact of Biomarker Type on Specimen Processing

The manner in which a sample must be collected and processed depends upon the type of biomarker to be detected. Protein biomarkers, either antibodies or infectious agent antigens, generally require the least amount of preparation or purification, and they are generally stable in a variety of sample types. Of note however, proteins are susceptible to proteolytic degradation and conformational changes which could profoundly affect the ability of a binder such as a monoclonal antibody to recognize and bind the protein biomarker. Protein biomarkers that might present the greatest challenge in resource limited sites are those that are used to enumerate cell types, because of the requirements to stabilize the cells. Studies have shown that CD4 cell enumeration can be performed by plate-based ELISA assay methods with favorable concordance with flow cytometry.[28] Adoption of this technique to a field-usable immunoassay device would require lysis and analysis of whole blood specimens. An alternative approach for CD4 cell enumeration involves the trapping and analysis of cells on a disposal membrane chip. LabNow has developed and integrated this approach into a small instrument designed for point of care settings.[29]

DNA is generally stable during purification and storage,[8] and is relatively stable in whole blood for extended periods of time.[30] Even relatively crude methods of storing samples, such as spotting them on to filter paper or membrane membranes such as Whatman FTA cards,[9] can be used in resource-limited environments for preserving the DNA in a specimen. The FTA cards have been used to store DNA samples at room temperature for up to twelve years. To date, one of the main challenge for DNA diagnostics has been the requirement for purification of the DNA away from the other protein components in a sample before it can be analyzed. Though a large number of DNA purification methods exist, few are practical for use in resource limited settings, due to their requirements for laboratory settings, special equipment, extensive training, and cost. However, there are recent reports of filter papers that bind DNA from directly-spotted crude samples, and the DNA can then be eluted from the filter paper, after a brief wash, and used directly in analyses such as PCR.[31] Simple approaches such as this for DNA purification will facilitate the use of DNA-based diagnostics in resource-limited settings.

The difficulties in purifying DNA are even greater for particular infectious agents such as *M. tuberculosis*. These mycobacteria are protected by exceptionally strong cell walls containing mycolyl ester cross linked lipids and strong chemical methods are required to release the DNA for analysis, which typically involves physical crushing of the cells. This process can unduly expose the person conducting the test to biohazardous material. In such cases, a field-usable, inexpensive method to purify DNA, or analytical assays for DNA that does not require extensive purification would greatly enable the use of DNA diagnostics.

A particularly challenging biomarker type is RNA, primarily due to its instability. RNA is highly susceptible to attack by a variety of enzymes termed ribonucleases. Exposure to ribonucleases can begin as soon as a specimen is collected, because many individuals produce ribonucleases on their fingers. If left to stand for extended periods, the RNA in clinical specimens becomes degraded, and this can be more of a problem with some RNA species than others.[32] Special collection systems are generally required to preserve RNA, and a number of methods that inhibit proteases and ribonucleases have been developed for resource rich environments.[9,33] One approach that is amenable to resource-limited sites are synthetic membrane cards that are used to collect several drops of blood or other sample type, and whose material disrupts cells, denatures enzymes, and therefore stabilizes RNA.[9] Like methods for analyzing DNA, the methods that are currently available to analyze RNA require that the RNA be purified away from other cellular components, and therefore there is a significant need for field-usable, inexpensive methods to stabilize and purify RNA for analysis, or for analytical methods that do not require extensive purification.

Table 1. Relative ranking of specimen collection and processing

Specimen Type	Collection method	Ease to collect	Equipment to Collect	Specimen Preparation	Cost
Whole Blood	Phlebotomy	4	3	1	2
	Finger/heel prick	2	1	1	1
Blood Serum/plasma	Phlebotomy	4	3	2 serum 4 plasma	2 serum 3 plasma
	Capillary	2	2	3	2
Blood Cells	Phlebotomy	4	3	4	4
	Capillary	2	2	4	3
Oral Fluids	Swab/pipette	1	1	1	1
Urine	Cup/tube	1	1	1	1
Upper Respiratory	Swab	1	1	1	1
Lower Respiratory	Expectorated or Induced Sputum	5	3	5	5
Stool	Cup/paddle	2	1	3	1

Table 1. Relative ranking of specimen collection and processing. A 5 point relative 5 point scale was used, with a ranking of "1" being the most applicable and a ranking of "5" being the least applicable. In the context of resource limited environments a ranking of 1 or 2 is considered acceptable, a 3 is considered marginally acceptable (with concerns), and a 4 or 5 is considered unacceptable. The continuum used for ranking was based upon the following considerations: Ease to collect: 1, simple, non invasive, no specialized skills, one minute or less time required; 5, complex, potentially invasive, special skills required, difficult specimen to obtain. Equipment to collect: 1, simple, few skills required, readily available; 5, complex, specialized skills required, special reagents potentially required. Specimen preparation: 1, specimen requires no processing, use as collected; 5, extensive processing required with chemical reagents that may need refrigeration, requires specialized electrical equipment. Cost of collection and processing: 1, <$0.10; 2, $0.10-$1; 3, $1-3; 4, 3-10; 5>$10; Note that cost of >$10 includes, equipment, devices, chemicals, reagents, and disposables

Section 2. Assay Components

An analyte is the item that is detected by a diagnostic test, and an analyte can be a component of an infectious agent, or a component of the host immune response (e.g. antibodies or cytokines) elicited to fight off an infectious agent. Binders are reagents that bind to the analyte. Binders are often attached to some form of solid support and/or tagged with a molecule that can generate a detectable signal (e.g. a fluorescent dye or an enzyme). In biological systems, a ligand is a molecule that binds to a receptor.

Table 2. Summary of Analytes and Binder Options

Biomarker Type	**Analyte**	**Binder Options**	**Examples**
Proteins	Proteins (non-antibody), either large or small	Antibodies, ligands, aptamers	Standard antigen assay, e.g. HIV p24 antigen
	Antibodies	Antigens, antibodies	An antibody response to a protein, e.g. anti-HIV antibodies
	Protein isoforms; post translational modifications	Antibodies, ligands, receptors, lectins	Evaluate differences in phosphorylation of a given protein
	Protein isoforms; genetic variants	Antibodies, ligands, receptors, aptamers	Differences in HIV envelope protein amino acid sequences
	Protein complexes	Antibodies	HBsAg; polymerization of subunits
Nucleic Acids	Infectious agent RNA genome	DNA & RNA Probes, Primers, and modified nucleic acid probes and primers	HIV RNA genome
	Infectious agent mRNA		HIV gene expression in infected cells
	Host mRNA		Expression of host genes
	Host genomic DNA		Host genotype; e.g. polymorphisms in disease susceptibility
	Infectious agent genomic DNA		Hepatitis B Virus genome
	Epigenetic changes	Antibodies, aptamers	Methylation patterns of genomic DNA
Polysaccharides	Cell membrane: host	Aptamers lectins, antibodies	Differences in glycoprotein or glycol lipid patterns
	Cell membrane or cell wall: Infectious agent	Antibodies, lectins	Differences in viral or bacterial sub-species

As shown in Table 2, a multitude of binder options are available. The binder used will impact the specificity and sensitivity of an assay. Though the goal is to maximize the sensitivity and specificity, it is often difficult to maximize both at the same time. Decisions regarding the binder are often made early in the assay design process in order to achieve an acceptable compromise between these performance specifications, as well as other practical concerns such as cost, stability, and manufacturing logistics. In general, for assays used to detect and/or measure the amount of infectious agents, nucleic acid-based detection is generally more specific and sensitive than methods that use an immunological-based detection, while immunological-based detection is generally faster, easier, and more robust.[34]

2A. Binders Used to Detect Proteins and Other Non-Nucleic Acid Molecules

The most widely used binders to detect or quantify proteins and other non-nucleic acid analytes are antibodies, and assays that use antibodies are generally termed immunoassays.

Antibodies which are polyclonal can be created by immunizing animals with the entire analyte (antigen), analyte component (peptide), or analyte clone (cDNA). The best immune responses, and hence broadest spectrum of antibodies, are obtained by choosing animals that are distantly related to the analyte's species,[35] hence rabbits, goats, and more recently chickens (see Table 3) have been the animals of choice.[36] The production of IgY in chickens has the potential to provide high-affinity antibodies, while also providing the advantages of persistent titer, a collection method which is non-invasive, scalable, and has a high yield, as well as a simpler and more economical isolation process than is required for the isolation of polyclonal antibodies from mammalian blood.[36,37]

A polyclonal antibody response to the antigen is a complex mixture of antibodies that recognizes different epitopes of the analyte with varying affinities. Affinity purification is used to remove antibodies that do not bind to the analyte, and the affinity purification conditions can be modulated to improve the overall assay specificity of the collection of antibodies. Affinity-purified polyclonal antibodies are the reagent of choice when broad recognition of many epitopes on the analyte and therefore strong polyvalent analyte binding is desired. However, recognition of many epitopes is often undesirable when the assay needs to be able to distinguish closely related analytes (e.g. measuring differences in protein phosphorylation patterns or typing closely related organisms).

Monoclonal antibody technology allows a single antibody clone to be produced that binds a specific epitope on the analyte. Closely related analytes are employed in the monoclonal antibody screening process to select for high affinity and specificity, and reduce cross reactivity. Monoclonal antibodies can be highly specific, as evidenced by their ability to distinguish closely related analytes such as human chorionic gonadotropin and human luteinizing hormone.[38,39,40] However, by using a monoclonal

antibody, polyvalent (i.e. multi-epitope) binding is not possible, which results in much lower affinities than with polyclonal antibody preparations (K_Ds 10^{-6} and 10^{-9}, respectively). Traditional monoclonal antibody production in mice has been hampered by the observation that many small compounds and peptides do not illicit a good immune response in mice. This limitation was addressed with the introduction of monoclonal antibody production in rabbits.[41] Furthermore, rabbits generate greater antibody diversity and affinity, therefore providing a greater likelihood of obtaining an antibody-producing clone that produces a very high-affinity monoclonal antibody (K_D 10^{-8} to 10^{-9}).[42] The production of monoclonal antibodies from chickens has been reported in several instances, but these methods require further development before widespread use would be possible.[43,44]

Assay performance can be hindered by background signal from a variety of sources related to the use of antibodies as binders, and several strategies have been employed to reduce this background. Assay specificity is often enhanced by employing antibodies that have been engineered or enzymatically treated to remove domains (e.g. Fc domain), which bind factors in human sera (e.g. Rheumatoid factor or IgM) and create undesirable background signal.[45] However, this strategy adds additional complexity to the assay manufacturing process, and can increase assay reagent costs.

When using antibody binders that have been produced in animals such as mouse, rabbit or goat, background signal can result when testing samples from patients who have been exposed to those animals, and therefore have naturally-occurring antibodies to those animal's antibodies in their serum. This kind of background can be avoided by "humanizing" monoclonal antibodies, which involves engineering amino acid changes into the cell line that produces the antibody, to give it a sequence that is more like that found in human antibodies, or via the use of specific blocking agents developed to reduce the kind of background.[46,47] Although humanization has not been leveraged in the development of immunoassays, it may be useful to minimize heterophile (e.g. human anti-mouse) antibody reactivity and enhance assay specificity. Another strategy that could be used to reduce this kind of background signal is the use of chicken IgY antibodies, which do not react with mammalian IgG or IgM, human anti-mouse IgG antibodies (HAMA), or rheumatoid factor, which often gives false positive results by interaction in immunoassays.

Antibodies and antibody-like reagents can be identified and improved upon using non-mammalian selection systems. The best example of this approach termed phage display, in which filamentous viruses known as phage that infect bacteria are used to select engineered antibodies.[48] Phage display facilitates the selection of antibodies with regard to their size, valency, affinity, and selectivity. Antibodies selected via phage display methods can be subsequently engineered to contain "human like" domains.[47] Both of these steps can be time-consuming and expensive as methods to identify suitable antibodies and anti-body-like reagents. Once a binding reagent has been identified and/or engineered using these methods, it

must still be produced in mammalian cell culture systems, which costs that are similar to that of monoclonal antibodies.[49]

Another binder class, apart from antibodies, that can be used for detecting glycosylation patterns (e.g. glycoproteins, glycolipids, etc.) are the lectins, which are proteins produced by certain plant and animal species that are highly specific binders for specific glycosylation.[50]

While antibodies and other proteins are used as binders in many existing immunoassays, they have several intrinsic properties that might limit their use as binders in assays to be performed in resource-limited sites. Protein binders in liquid state generally require refrigeration or freezing for shipping and storage, as well as sterile conditions to prevent their degradation by bacteria or molds. For these reasons, and the challenges associated with the consistent production of large quantities of antibodies, other approaches have been pursued to develop synthetic substances that could provide the sensitivity and specificity of antibodies, but that would have higher thermal and enzymatic stability, and reduced manufacturing costs.

One promising approach involves aptamer technology. Aptamers are synthetic DNA or RNA oligonucleotides that can adopt a vast spectrum of three dimensional shapes. It is the three dimensional shape of the aptamer that recognizes the molecular structure of the analyte, and aptamers can be selected to have a high affinity and sensitivity for an analyte. Aptamers hold promise as a binder class that will have equivalent or superior performance to antibodies in diagnostic immunoassays, and may provide advantages in ease of production and cost.[51,52]

Another strategy uses technologies that were developed for combinatorial chemistry to select and iteratively design polymer surfaces. They polymers are designed using rational design techniques, where structure-activity relationships for the interactions between surfaces and other molecules are predicted. Then, using combinatorial chemistry methods, a library of hundreds of novel polymer surfaces are synthesized that are expected to have superior characteristics for binding a particular analyte, and to minimize non-specific binding. These polymers are synthesized in an array format, screened in a high-throughput manner, and the performance of all the surfaces in the library is tested. Those polymers with the best properties are expanded upon and optimized. Successive rounds of optimization can be performed until a surface with the desired properties is identified. Optimized polymers can be layered on a range of standard substrates, such as microtiter plates, beads, or porous membranes.[53]

Molecular imprinting is another technology under development that allows the formation of recognition sites for specific analytes in synthetic polymer films, which could then be used as binders in biomarker assays.[54] These approaches for developing synthetic polymer films have the potential to develop binding substances that are much more stable than antibodies, as well as easier and therefore less costly to

manufacture. However, realization of this potential, especially with reference to biomarker assays to be performed at resource-limited sites, remains to be demonstrated.

Table 3: Summary of Binders Used to Detect Proteins

Class	Type	Comments	References
Antibodies	Polyclonal-mammalian	Broad spectrum of analyte recognition and generally provide strong binding affinities. Produced in rabbits, goats, and other mammalian species.	55
	Polyclonal-Avian	Broad spectrum of analyte recognition. High antigenicity for mammalian antigens.	36
	Monoclonal-mouse	Highly specific, lower binding affinity, K_D 10ee-6	38, 39
	Monoclonal-rabbit	Very specific and higher affinity compared to mouse monoclonals	42
	Monoclonal-"human"	Different approaches used to create "human like" monoclonal antibodies, K_D 10^{-8} to 10^{-9}	48, 47
Antibody-like molecules	Monoclonal-bacteriophage	Libraries of 10ee9 antibodies are available. Broad coverage of antigens and relatively high affinity binding	49
Antigens	Potentially bind many antibodies	Used to detect antibodies (host response) to an infectious agent	37
Lectins	Monoclonal-like		50
Aptamers	Monoclonal-like	Synthetic DNA or RNA oligonucleotides that recognize three dimensional structures	51, 52
Synthetic polymers	Chemical polymers, molecular imprints	Synthetic polymer provides specific recognition sites for analyte	53, 54

It is important to consider the assay application when choosing a protein binder. If a highly specific assay is desired, binders that provide a high level of selectivity, such as monoclonal antibodies or aptamers, will be required. A variety of additional factors must be considered including manufacturability, ease of assay formatting, cost, and availability. In Table 4, binder types were ranked relative to each other in the context of limited resource environments. From this analysis it is clear that a superior protein binder does not exist. Instead, each binder has its own attributes that can be exploited for specific applications. For example, mouse monoclonal antibodies are highly specific but generally they bind analytes with lower affinity. Higher affinity antibodies might be obtained using rabbit sources; however this needs to be balanced with cost ($5 to 7,000 mouse vs. $15 to 20,000 rabbit) as well as availability (many sources and no intellectual property (IP) barriers for making mouse monoclonal antibodies but only one source and IP barriers for making rabbit monoclonal antibodies). Costs for producing gram quantities (generally sufficient for millions of assays) are similar once the clone is obtained. If a highly specific antibody is not

required, then polyclonal antibodies should be considered since they generally provide strong multivalent binding, are easy to produce (in rabbits or goats), non-encumbered with IP, are generally more stable during assay formatting, chemical modification and storage. In resource limited environments reagent stability presents challenges, some of which can be reduced by the ability to provide reagents in a lyophilized form that can be reconstituted. Generally both mono-and polyclonal antibodies can be lyophilized; however, polyclonal antibodies generally are more suitable for such treatment. In general newer emerging technologies such as aptamers, molecular imprints, and antibody-like molecules provide a unique set of challenges. First, they have not been widely used in the development of diagnostic assays and hence technological challenges are not well documented. Second, the technology is not broadly available and furthermore the technology itself is often protected via IP which limits availability and hence increases costs. Taken together, due to their broad availability, multiple assay format capability and reasonable stability antibodies should be considered as the first choice in choosing a binder. If they can not provide the needed assay sensitivity, specificity, stability and format-ability other options are available and should be considered.

Table 4. Relative ranking of binders used to detect proteins and other non-nucleic acid molecules.

Class	Type	Affinity	Specific	Ease to make	Availability	Ease to format	Stability	Cost	Amenable to Chemistries
Antibodies	Polyclonal-mammalian	1	4	2	1	1	2	1	2
	Polyclonal-Avian	1	2	1	3	1	2	1	2
	Monoclonal-mouse	3	1	3	1	2	3	2	3
	Monoclonal-rabbit	2	1	4	3	2	3	3	2
	Monoclonal-"human"	2	1	4	4	2	3	4	3
Antibody-"like"	Monoclonal-bacteriophage	2	1	3	3	2	2	3	2
Aptamers	Monoclonal-like	2	2	1	3	2	1	3-4	1
Molecular Imprints	Monoclonal like	3	2	3	5	2	1	?	3

Table 4. Relative ranking of binders used to detect proteins and other non-nucleic acid molecules. A 5 point relative 5 point scale was used, with a ranking of "1" being the most applicable and a ranking of "5" being the least applicable. In the context of resource limited environments a ranking of 1 or 2 is considered acceptable, a 3 is considered marginally acceptable (with concerns), and a 4 or 5 is considered unacceptable. . The continuum used for ranking was based upon the following considerations: Affinity: 1, KD <10ee-11; 2, KD 10ee-9 to 10ee-11; 3,

KD 10ee-6 to 10ee-9. Specific: 1, low cross reactivity, only one epitope recognized; 2, mild cross reactivity; 4, broad cross reactivity, difficult to specifically detect closely related proteins. Ease to Make:1, generally <3 months to make, highest production capability; 2, generally 3-4 months to make, high production capability; 3, 4-6 months to make, routine techniques available; 4, 4-6 months to make, specialized techniques required; 5, not routinely made

Availability: 1, technology readily available, many sources, no IP; 3, limited sources, IP may be an issue; 4, Limited sources, limited capacity, IP is an issue; 5, Ease of making not clear or established

Stability: 1, generally stable in solution at a wide range of temperatures; 2, generally stable in solution if refrigerated or stable at range of temperatures when lyophilized; 3, Generally require refrigeration or freezing for stability. Cost to Make: 1, <$5,000; 2, $5,000-10,000; 3, $10,000-20,000; 4, > $20,000; ?, unknown. Cost is based upon the usual contract rate to create the binder. Once binders are created the costs to produce them are similar, usually costing <$0.05 for each assay in which they are used.

Amenable to chemistries: 1, can be highly modified and retains reactivity, 2, generally most attachment chemistries can be used; 3, Subject to issues with many chemistries.

2B. Binders Used to Detect Nucleic Acids

The binders that are used to detect nucleic acids are nucleic acids themselves and are often called probes or primers. Although probes and primers are very similar in structure (single stranded DNA or DNA/RNA constructs) they are used for different purposes. While probes are used to simply detect the presence of a nucleic acid sequence in an assay, primers are used to initiate the duplication (amplification) of specific adjacent nucleic acid sequences. Amplified nucleic acid sequences can either be detected as part of the amplification process, or detected in later steps of the assay that are not part of the amplification process. Assay formats, including those based upon amplification, are covered in Section 3. Different classes and types of probes and primers are summarized in Table 5. DNA and RNA probes bind to their complementary target sequences in a process called hybridization, and nucleic acids that are bound together in this way are termed hybrids. RNA-DNA hybrids are substantially more thermostable than DNA-DNA hybrids under certain conditions; therefore RNA probes offer advantages when the creation of strong hybrids is required.[56] Of important note, single stranded RNA probes are highly susceptible to chemical, thermal, and enzymatic degradation, even though RNA-DNA hybrids are generally stable. This negative feature should be carefully considered before choosing RNA probes as a nucleic acid binder.

The length of probe that will be used in an assay will determine many of the assay's performance characteristics, and therefore is an important design consideration. Longer probes generally form more stable hybrids. For instance, the presence of mismatched bases in a hybrid, which can result from the presence of genetic variants, does not impact the hybridization capability of longer probe sequences (>50 nucleotides). Generally one nucleotide mismatch can be tolerated every 25 to 50 nucleotides.[57] Another advantage is that long probes may allow the assay signal to be increased because they have more sites for attaching a label either directly (e.g. attaching a fluorescent dye) or indirectly (e.g. attaching a "linker" such as biotin), which can generally be attached every 10 to 25 nucleotides.[58] In contrast, when the assay

specificity requires the detection of only a short (15 to 50 nucleotides) sequence, a shorter probe must be used. This is often the case for assays that detect infectious agents, where it is often useful to distinguish closely related species, and therefore short target sequences must be chosen.[59]

The ability to chemically synthesize DNA and RNA oligonucleotide sequences (oligos) in large quantities has profoundly affected the design of assays that detect specific nucleic acids. Currently, single stranded oligos are the binder of choice to detect nucleic acids. Oligos ranging from 10 to 80 nucleotides in length can be routinely obtained from a plethora of commercial sources. If longer probes are required for an assay, then individually synthesized oligos can be chemically ligated to achieve the desired length.[60] Such ligation approaches do have practical limitations in yield and cost, as yield decreases and cost increases with the number of ligations. Although oligos shorter than 18 nucleotides can provide a highly specific hybridization event, their short length makes hybrid instability either an advantage to exploit or a concern. This is because the change in temperature at which a hybrid dissociates (the melting temperature, or Tm, defined as the temperature at which 50% of a population of hybrids has dissociated) at lengths below 18 nucleotides is much greater per nucleotide than above 18 nucleotides.[61,59] Short probes can therefore be designed to distinguish single nucleotide differences. In contrast, the ability of a single nucleotide variation to greatly impact the hybridization of a probe to its target sequence is a concern when designing assays for sequences that are highly susceptible to spontaneous mutations, as occurs in certain infectious organisms.

Furthermore, assays often detect multiple unique target sequences, and each probe-target hybrid has a unique melting temperature. Thus some hybrids may be stable and some may be unstable under a specific set of assay conditions. There are a number of approaches to address the challenge of designing multiple probes for multiple target sequences with similar melting temperatures, including modifications in assay design and formatting (Section 3), the addition of reagents that minimize melting temperature differences, as well as through the incorporation of nucleic acid analogs which create more thermally stable hybrids.

Incorporation of peptide nucleic acids (PNAs),[62] locked nucleic acids (LNAs),[63] 2'O-Methyl nucleic acids,[64] and other analogs[65] have been used to create short oligomers that have high thermal stability, are highly specific, and display resistance to degradation by nucleases that might be accidentally introduced into assays. Oligomers synthesized using iso-C/G analogs (isomers of cytosine and guanine) hybridize only to synthetic nucleic acid analogs that contain the complementary iso-C/G analog.[66]

Table 5. Summary of Binders Used to Detect Nucleic Acids

Class	Type	Comments	Reference
DNA Probe	Cloned double stranded DNA	Long sequences (0.5 – 10 kb) provide stable binding. Usually produced in prokaryotic systems	67
	Synthetic single stranded DNA	Short sequence provides specific but weaker binding	67
RNA probe	Cloned and transcribed single stranded RNA	Long sequences (0.5 kb or greater) provide highly stable binding. RNA probes are highly susceptible to degradation	67
	Synthetic single stranded RNA	Short sequence provides specific binding. RNA probes are highly susceptible to degradation	65
Primers	Synthetic single stranded DNA or DNA/RNA constructs	Usually 15-25 nucleotides in length are designed to flank nucleic acid sequence(s) to be amplified	68,69
Nucleic Acid analogs	Protein Nucleic Acids (PNAs)	DNA mimic with pseudo-peptide backbone. Bind both single and double stranded DNA (strand invasion)	62
	Locked Nucleic Acids (LNAs)	Nucleic acid analog containing 2'-O, 4'_c methylene bridge. Provide highly stable binding. Produced synthetically	63, 64, www.proligo.com
	Super A-Super T	Nucleic acid analogs that enhance A-T base pairing	65
	Isomers- IsoC/G	Synthetic analogs incorporated into synthetic DNA. Only bind to sequences containing the isomer compliment	66, www.eragen.com

Numerous nucleic-acid binder attributes need to be considered during the assay design process (see Table 6). The number of nucleic acid binders required, their length (number of nucleotides), the composition of the binders (DNA, RNA, or a combination), and any requirements for nucleic acid analogs that might provide hybridization specificity or robustness, will all affect the cost and the assay's performance.

The goal of the assay, and therefore the specificity of hybridization that is required to meet that goal, is of primary importance. The goal of the assay may be the qualitative detection of a nucleic acid (e.g. an organism like HIV), or the quantification of a nucleic acid species produced by that organism (e.g. quantification of HIV genomes, or "viral load"), in which case binders must be chosen that recognize only the sequence of the organism (e.g. HIV and not other retroviruses), yet still recognize all subspecies. The amount of genetic variation that occurs among subspecies varies from organism to organism. Short oligo probes (18 nucleotides or less) might be inappropriate for organisms that have a great deal of genetic variation from isolate to isolate, because single base changes can significantly change their melting temperature. Therefore in some cases, longer oligos (>30 nucleotides) will be more appropriate, as they will be less impacted by a single nucleotide mismatch. If the range of variation in the target sequence is

known, then a mixture of probes can be synthesized that contains all the possible variants, (a "degenerate" probe mix).

Another strategy that can overcome the challenges of detecting organisms with a high degree of subspecies variation is to design the assay to detect a number of distinct target sequences of the organism. This strategy can improve the assay's robustness, for even if one or more target sequences is not detected due to a high degree of variation in that particular isolate, there is a high likelihood that at least one target sequence will be detected. The use of hybrid-stabilizing analogs and reagents can help to minimize these issues; however these reagents add additional costs to the assay manufacturing. Thus the improved performance of assays that use such reagents must be balanced with the significant increases in cost, the existence of fewer suppliers for these reagents, and the risks associated with using newer reagents with which the diagnostic industry has less experience.

If the goal for the assay is to distinguish closely related nucleic acid sequences (e.g. genotyping to determine drug resistance), probes must be designed that will distinguish (i.e. bind differentially) to the nucleic acid sequences that must be distinguished. Probes that are longer than 50 nucleotides can form stable hybrids despite the presence of mismatches in hybridization, and are therefore not generally suitable for such discrimination. The thermal instability of short probes can be exploited to provide binders that hybridize only to a completely complimentary sequence.

If the desired assay sensitivity cannot be obtained by directly detecting a nucleic acid target using the preferred detection technology, then the assay sensitivity can be improved by making many copies of (amplifying) the nucleic acids to be detected. All amplification methods use one or more oligonucleotide primers that are either single stranded DNA or single stranded DNA-RNA chimeras. The primers must be chosen 1. To hybridize proximal to the sequence to be amplified, 2. Hybridize to and amplify only the sequence of interest, 3. Not be impacted by genetic variation in the target sequence, 4. Hybridize with a similar Tm of the other primers in the assay (if multiple target sequences are to be amplification ("multiplex detection). Oligomers ranging in length from 25 to 50 nucleotides generally fulfill these requirements. However, some assays are designed to detect specific genotypes or subspecies by selecting primers that will only hybridize to, and therefore amplify and detect, one genotype variant (allele) or subspecies' sequence. The primers for such assays are therefore shorter oligonucleotides, which require a perfect match to their complementary sequence to form a stable hybrid.

There are other considerations that are unique to the design of assays for use in resource-limited sites, where higher standards for stability, robustness, and ease-of-use must be met. Although RNA probes provide high affinity and specific hybridization, they are highly susceptible to degradation during reagent manufacturing (e.g. chemical modification for attachment of signaling systems), storage, and during the

assay procedure itself. This is a significant concern for their applicability to assays that are to be performed at resource-limited sites. Generally they need to be frozen during shipping and storage, thawed only once, and used in an aseptic environment. DNA primers are more resistant to degradation (can be stored dry at ambient temperatures) and are less expensive than RNA primers, which are significant advantages for assays that will be run in resource-limited sites. Primers that are constructs of DNA and RNA are expensive, more difficult to procure, and highly unstable due to the labile nature of single stranded RNA. However once such primers hybridize to their DNA target the RNA component is resistant to degradation by routinely encountered ribonucleases.

Table 6. Relative comparison of binders used to detect nucleic acids.

Class	Type	Affinity	Specific	Ease to make	Availability	Ease to format	Stability	Cost	Amenable to Chemistries
DNA Probe	Cloned	1	3	1	1	1	2	2	2
	Synthetic	3	1	1	1	1	2	1	1
RNA Probe	Cloned	1	3	2	3	3	4	3	4
	Synthetic	2	2	2	2	3	4	3	4
Primers	DNA	3	1	1	1	1	2	1	1
	DNA/RNA	3	1	3	3	2	4	3	4
Nucleic acid analogs	PNAs	2	1	2	3	1	1	4	1
	LNAs	2	1	2	3	1	1	4	1
	2'O-Methyl nucleic acids	2	1	2	3	1	1	4	1
	Isomers	2	1	2	3	2	2	3	1

Table 6. Relative comparison of Binders to detect nucleic acids. Binders were scored on a relative 5 point scale with a ranking of "1" being the most favorable and a ranking of "5" being the least favorable. In the context of resource limited environments a ranking of 1 or 2 is considered acceptable, a 3 is considered marginally acceptable (with concerns), and a 4or 5 is considered unacceptable. . The continuum used for ranking was based upon the following considerations: Affinity; 1, Hybrids generally stable up to 25oC > Tm; 2, hybrids generally stable up to 20oC> Tm; 3, hybrids generally stable up to 15oC> Tm. Specific; 1, can distinguish a single base mismatch; 2, can differentiate 2 closely related sequences; 3, can differentiate closely related sequences if long > 500 nucleotide unique sequences are present in the target

Ease to make; 1, routine; 2, some specialized approaches required; specific reagents and know-how require Availability; 1, common, many vendors, no IP; 2, fewer vendors, no IP; significant know-how required, possible IP. Ease to format: 1, many options; 2, specific knowledge required; 3, moderately difficult. Stability; 1, highly stable dried or in solution; resistant to nucleases; 2, usually stable dried or in solution, susceptible to nucleases; 3, significant care must be taken, very susceptible to degradation

Cost; 1, <$100; 2, $100-$300; 3, $300-$500; 4, generally >$500. Amenable to chemistries: 1, easily modified with no impact on performance; 2, most chemistries can be used; 4, most chemistries negatively impact performance or degrade the binder

Section 3. Assay Formats, Detection Methods, and Assay Interpretation

Diagnostic tests with sufficient sensitivities and specificities already exist for most infectious diseases; but, the vast majority of these tests are not appropriate for use in resource-limited sites.[70] Many tests require expensive, complex equipment, well-trained personnel, or are prohibitively expensive. The World Health Organization has suggested criteria for ideal diagnostic tests to be used in resource-limited settings and use the acronym ASSURED: Affordable by those at risk of infection, Sensitive, Specific, User-friendly (simple to perform with minimal training), Rapid (enabling treatment at first visit) and Robust (does not require refrigerated storage), Equipment-free, and Delivered to those who need it. Though it is challenging to meet all these criteria in one test, they are all important to the successful use of a test in resource-limited environments, and therefore it is worthwhile to strive to achieve them.

Even where rapid, inexpensive, easy to perform tests already exist, often their performance has not been adequately tested or optimized for use in resource-limited sites, and in cases where their performance has been measured, it may fall well below that of evaluation performed under the controlled settings of clinical trials and resource-rich sites.[70,71,72] Therefore further improvements are still needed for diagnostic tests for many infectious diseases in the areas of assay performance in field-use settings, or cost (or both) for many infectious disease tests that are appropriate for resource-limited sites.

Resource limited settings present unique challenges to most developers of diagnostic tests who are accustomed to having a place to plug their devices, and an uninterrupted source of AC power. To maximize the ability to utilize new diagnostics in remote locations, it should be presumed that there will be no constant source of centrally distributed electricity. The devices should be self-powered. The two alternative power sources that are usually considered are batteries and solar power. Batteries can provide substantial power. However they either need to be re-charged through a central power source or replaced, which requires a consistent and reliable channel for distribution and disposal of batteries at many sites. For these reasons, solar power is particularly attractive. It is not uncommon to see solar grids on the roof of remote clinics in Africa today. Solar powering of the devices themselves would be particularly attractive, but this presents a challenge to the developer of the tests to keep the power utilization low so that inexpensive solar batteries, such as are used to power inexpensive calculators, can be employed. A practical alternative might be the use of solar rechargeable standard batteries to power the required instruments.

Ease of interpretation is an important consideration in the development of diagnostic tests for resource-limited settings. Some of the most practical test formats for these settings have no instrumentation, and therefore use a visually detected output which is often generated by a colorimetric reaction. The operator of the test must then interpret this visual output. Potential disadvantages of visually detected outputs are

that they are generally less sensitive, qualitative or semi-quantitative at best, and are more prone to inter-operator variation in interpretation. These disadvantages can lead to reduced performance of a test in resource-limited settings, compared to a sophisticated laboratory environment. Tests with robustly configured positive and negative control signals, those that minimize the fraction of tests that produce boarder-line or ambiguous results, and those that require minimal training to achieve the correct interpretation, will have the least amount of variation due to interpretation and have better performance in resource-limited settings.

When a visually detected output cannot deliver the required sensitivity, an instrument could be used to detect a colorimetric or turbidity change, or an alternative detection mode, such as fluorescence or luminescence, and more recently, magnetic or electrical. The increase in sensitivity and quantification that is gained is accompanied by the need for an instrument. In resource-limited sites, this can add disadvantages such as additional cost, power requirements, complexity, and training requirements, and may affect the robustness of the testing method. Test output from instruments often uses language, which may not be the most easily interpretable for use in settings where that language is not spoken. Easily understood symbols are preferable than any one particular language. Instruments designed for laboratory use may use a screen or paper to output their results, and the effect of these output modes on interpretation should be considered. As tests that use non-visual detection move in to field use, these challenges for interpretation should be considered as well. Ideally an instrument would have software that facilitates the interpretation for the operator. Devices that are already on the market for biosecurity applications may provide examples of output and interpretation, such as a sound or simple color output, that are worth considering when optimizing the ease-of-interpretation of a test for resource-limited settings.

3A. Immunological Formats and Detection Methods

Immunologic detection formats have been the most widely used for the detection of infectious agents, toxins, or host antibodies to infectious agents, particularly in resource-limited environments. Many formats are available from commercial sources. The advantages of immunoassay technology relative to other analytical techniques include high analyte selectivity, reduced sample preparation, and adaptability to field use. The limitations of immunoassay technology can include interferences from certain constituents of samples, cross-reactivity to structural analogs of the analyte, poor suitability for some multi-analyte applications, low availability of reagents, and longer assay development time than some methods.[73]

The main categories of immunological methods are the agglutination format, membrane based formats (e.g. immunochromatographic cards, lateral flow devices, or flow through devices), solid phase devices (e.g. the free standing “dipstick” format) and array-based formats (e.g. microtiter-plate based ELISAs),

including virtual arrays (e.g. bead-based formats which use flow cytometry for detection). Each of these categories is discussed below.

There are also two major approaches for immunodiagnostics: detection of antigens with antibodies or detection of antibodies with antigens (serology). Antibodies or engineered binders can be used in an assay to detect antigen(s) from an infectious agent. Alternatively, antigen(s) from an infectious agent can be used as the binder in an assay, which serves to detect the presence of anti-infectious agent antibodies produced by the host patient (a serological assay). The information these alternate approaches provide are not always identical. The presence of the antigen usually indicates current infection, but not always (e.g. empty Dane particles of HBV surface antigen). In some diseases, such as filiarisis and leptospirosis, individuals can remain antigen positive for years after treatment and presumptive cure.[74,75] Antibodies against the infectious agent are not present during the acute phase of a first infection and won't appear until "seroconversion' many days to months later, if at all. Vaccinations against the agent of interest also make the use of antibody detection less useful.

All of the immunodiagnostics formats can use either of these approaches, and newer products combine the detection of both analyte types, such as "fourth generation" of the HIV diagnosis tests, which detects the presence of antibodies to HIV 1 and 2, as well as the p24 viral capsid protein, which is detectable earlier in the acute phase of infections before antibody production.[76]

The use of antigen as the binder in an assay may be easier, and in some cases cheaper to manufacture, than using an antibody as a binder. Newer capabilities to produce antigens from cloning and expression systems or occasionally from peptide synthesis have generally replaced the traditional use of sometimes crude preparations of antigen from infectious agents cultured in the laboratory. The ability to engineer the antigen in vitro may further improve the feasibility and cost of manufacturing particular antigens perhaps even improving the assay performance and product stability. For instance, the purposeful introduction of disulfide linkages in a non-antigenic portion of a viral protein could help to preserve its structure under harsh conditions. There are several challenges for serology assays in resource-limited settings. Serological tests detect antibodies from past as well as current infections, which may persist for many years or even the lifetime of an individual. Serological tests that specifically detect the presence of IgM antibodies rather than IgG antibodies can overcome this problem in some cases since IgM antibodies are usually indicative of a relatively recent infection.[77] Serological assays can also result in a high false positive rate in areas where individuals are permanently in contact with various infectious agents, may develop cross-reacting antibodies and/or may be infected with multiple infectious agents which occurs frequently.[78,79] Another potential disadvantage of serological tests is that they may frequently produce false negative results in immuno-compromised individuals, such as HIV positive individuals, premature babies, and diabetics, which is a growing problem in many parts of the under-resourced world.[80]

However, both serology and antigen detection approaches can be hampered by sensitivity in the early stages of an infection, before there is sufficient analyte to detect and therefore the utility of an immunodiagnostic format for a particular infectious agent depends on how important speed is in the diagnosis and management is with a particular infection.[75,81]

3B. Agglutination Formats

In agglutination immunoassay formats, binders are coated onto white or colored latex particles, gelatin beads, charcoal particles, dyes or colloidal particles. The specimen is mixed with a solution containing the coated particles, often directly on a microscope slide, then rotated or agitated to allow the binders to interact with the analyte if it is present. If the analyte is present, large multimeric complexes form and agglutinate and possibly precipitate from solution. The mixture is allowed to settle for a few minutes or more and the qualitative results are determined by observing whether a clear change in apparent particle size or a precipitate forms on the slide or card. Latex bead agglutination assays have been widely used to diagnose a wide variety of infections. The agglutination test for Group A *Streptococcal* antigen may be one of the easiest and most widely known in resource-rich environments, though there are others that have been more widely used in resource-limited environments such as the direct agglutination test (DAT) for visceral leishmaniasis[82] and the rapid plasma reagin (RPR) test for syphilis.[83] The tests that are most appropriate for field-use can either be performed without a rotator, be hand-rotated or shaken, or use a rotator with an alternative power source, such as solar power.[83] Advantages of the agglutination format are its generally low cost, speed, ease-of-use, generally long shelf lives for the reagents and minimal equipment requirements.[84] However, some agglutination tests require extended precipitation times, refrigeration of the reagents, or are difficult to interpret in samples with low quantities of analyte and in some cases there can be problems with specificity due to interfering substances in the sample.[85]

Aside from these logistic disadvantages, agglutination formats have generally been limited by sensitivity, especially for tests that detect the presence of antigen.[85] The sensitivity is dependent in part of the quantity of the analyte, which is often an antibody to an infectious agent, in a sample type that is practical to collect. Some newer tests use channeled plastic devices and/or automated readers which have significantly improved sensitivity, but these features add cost and complexity and have not yet been evaluated in field-use settings. Agglutination assays can also be implemented in a quantitative way, where turbidometric measurements determine the analyte concentration, but this requires instrumentation, and therefore has not generally been applied to field-use assays.

Recently ultrasound has been used to improve the sensitivity of agglutination assays in a format called ultrasound enhanced latex agglutination tests (USELAT) in which the mixture of sample and latex beads is sonicated for a brief period. The ultrasound generates standing waves within the liquid suspension which greatly increases the rate of particle collisions needed to drive the binding of the analyte and the

binder. This serves to improve both the speed and sensitivity of analyte detection in comparison with standard agglutination methods. After sonication, the assay mix is put on to a slide, stirred manually with a mixing stick and then examined using a microscope for agglutination. When tested on commercially available assays, improvements ranging from 10 fold to 10,000 fold in sensitivity were noted and reductions in the time of the assay from hours to minutes were obtained.[86,87] The requirement for a microscope and an ultrasonic device (which may ultimately cost several thousand dollars) could be a barrier in most resource-limited settings, and a robust ultrasound device would still need to be developed.

Because of the limitations of the agglutination formats, particularly in sensitivity, other immunodiagnostic formats such as the lateral flow format, have emerged as an alternative format for resource-limited environments.

3C. Membrane-Based Formats

Membrane-based assays have become a promising format for use in resource limited sites because they are simple to perform, rapid, stable under a wide range of shipping and storage conditions, require no equipment and are relatively inexpensive. A wide variety of tests to detect infectious agents have been developed for point-of-care testing and, in some cases, for use in resource limited environments as well. The most popular formats are the cassette lateral flow format where the immunochromatographic strip is encased in a plastic cassette and the dipstick format, which may be free-standing. In the lateral flow format, the base substrate is typically a nitrocellulose strip which has at its base a “sample pad” (area where the sample is applied), followed by an area where a conjugate of the binder and label is deposited (often a binder adsorbed to colloidal gold, colored latex, or carbon particles), and further up the immunochromatographic strip, a line of binder that can capture the conjugate of analyte and label and a separate line that serves as a control. When a liquid sample is applied to the sample pad, the sample migrates up the strip by capillary diffusion and moves through the conjugate pad allowing the interaction of any analyte in the sample with the binder-label-conjugate. These components then continue to wick through the membrane strip and migrate towards the line of capture binder where they become immobilized and can be visualized as a colored line. The current generation of products typically detects only one analyte and provides qualitative results. Newer products are emerging that allow the detection of multiple analytes and/or provide semi-quantitative results. In the semi-quantitative products, the lines of capture binder are designed to capture only a specific quantity of analyte and any excess is captured by subsequent capture lines creating a ladder of signal lines that can approximate the quantity of analyte in the sample.

Other implementations of this format have been used in resource-rich environments for several years that generate a signal (often fluorescence) that is detected by a small, portable analyzer that converts the signal from the test line into a digital signal.[88] Several small, portable devices for point-of-care use have been

released (Response Biomedical, and Inverness) that quantitatively detect the signal from lateral flow devices that use fluorescently dyed latex particles. These assays are run on whole blood, urine, or serum, depending on the assay. Though many of the lateral flow cassette devices are used for cardiovascular disease testing, tests are available for West Nile Virus and Group A *Streptococcal* antigen. It should be possible to develop other tests for infectious agents that use this format as well.[89] Under laboratory conditions, these platforms allow for the detection and quantification of analytes at sensitivities and dynamic ranges that are similar to the plate-based enzyme-linked immunosorbant assays (ELISAs), though their performance in resource-limited setting remains to be determined. The disposable immunochromatographic cassettes cost about $1, and the readers cost approximately $1000.[89]

The dipstick format is a solid-phase assay in which lines or dots of binder on a strip of material are immersed directly in the sample that has been mixed with other assay components. Because there is no separation in this assay format, it cannot reduce the complexity of the sample before it reaches the binder and therefore this format has been used primarily for less complex sample types such as urine.

There are many manufacturers of dipstick and lateral flow devices. These type of tests are either already in use, or hold promise for use in resource-limited settings, for the detection of *Leishmania*, Dengue virus, *Treponema pallidum* (syphilis), *Mycobacterium tuberculosis, Hepatitis B* virus and *Hepatitis C* virus, as well as at least 2 membrane-based products that have useful sensitivities and specificities for both *Plasmodium falciparum* and *P. vivax* (malaria parasites).[90,91,92] A large number of tests are sold for the detection antibodies to HIV.[93] The latest generation of products can be performed on minimally processed blood, oral fluid, or urine samples due to advances in the sample pad materials. Some have been evaluated in resource-limited settings,[92] though many have not been adequately assessed to determine if their performance will match that observed in resource-rich environments. Even though products with useful sensitivities and specificities in resource-limited setting already exist for a number of disease, it may still beneficial to improve these tests or develop additional capabilities, such as achieving higher sensitivity and specificity for a wider range of subspecies (e.g. the non-*falciparum* species of *Plasmodium*), producing quantitative rather than qualitative results in cases where this information is useful (e.g. a field-usable immunodiagnostic for viral load), as well as increasing the robustness and extending the shelf-life.

The price per assay for some of the membrane-based immunodiagnostics designed for field use ranges from 50 cents to $5,[92,94] which is still an order of magnitude too high to be practical in resource- limited environments. The costs of production are estimated to be around 25 to 50 cents, and are largely driven by the manufacturing costs and individual packaging. It is likely that further opportunities exist to reduce the manufacturing costs, perhaps through manufacturing in lower-cost environments.[6]

The general limitation for the membrane-based immunodiagnostics has been sensitivity. Recent improvements in the manufacture of the materials, protein-loading capacity, quality and consistency in colloidal gold conjugate manufacture, and technologies for printing of the test line of capture binder have all combined to improve the sensitivity achievable by this assay format. Sensitivities achievable are similar to plate-based ELISA formats, which for many analytes can be 1 ng/ml or less.[88] For many analytes, however, this is still not sensitive enough for the sample types that are practical to use in resource-limited sites.

Several approaches for enhancing the signal, and therefore improving the sensitivity, are emerging that hold promise to expand the number of infectious agents that are feasible to detect via membrane-based immunodiagnostics and sample types that are easily obtained and that require minimal processing. The first technique, which could be applicable to assays appropriate for resource-limited sites, is silver enhancement of the signal produced by the colloidal gold particles. A pad of silver salt is applied between the sample pad and the gold-binder conjugate pad. This technique provides greater sensitivity without the need for a specialized instrument to detect and interpret the assay result.[88]

The second technology uses paramagnetic particles instead of the traditional colloidal gold particles as a label. The paramagnetic particles are only magnetic when placed in a very strong magnetic field and do not aggregate until placed in the field. Quantum Design (San Diego) has developed antibody-coated paramagnetic particles that are designed to be used in lateral flow formats. In conventional lateral flow assays, the results are read as a colored line which is detected visually or by an optical instrument that measures reflectance, contrast, color change or fluorescence. Only the signal generated in the top 10 um of the membrane is detected using these methods although the membranes are a few hundred microns thick. Because the labeled analyte moves throughout the entire thickness of the membrane, not just on the surface, the vast majority of the label cannot be detected by conventional detection methods. The use of a magnetic label avoids this issue. The entire capture zone, throughout the thickness of the membrane, is measured, not just the surface, and the background is low because magnetic substances are not commonly found in biological samples. The magnetic particle label requires a small assay reader device that exposes the membrane to a group of small coils residing between two ferrite E-core magnets. The device detects the presence of the paramagnetic particles in specific locations on the membrane. The signal from these sensors is then amplified and processed to return a value that indicates the quantity of magnetic particles in the analytical region. The resolution of the assay reader device allows the multiplexing of several analyte regions along the analytical membrane that should allow one strip to detect a panel of analytes. The power requirements for the assay reader device are modest enough that is should be practicable to have a portable, handheld instrument capable of running on batteries, though a truly field-usable device, in terms of robustness and cost, remains to be developed. The dynamic range on prototype assay readers

is 4 orders of magnitude with signal to noise ratio of 3 to 1. Initial feasibility experiments allowed the detection of 10 fg/ml of *staphylococcus* enterotoxin B. Due to the sensitivity and potential for quantification for this format and the many sources of assay variation in a lateral flow format, rigorous manufacturing processes will be required for the binder-label-conjugate pad, the immunochromatic membrane (pore size consistency and membrane thickness), flow consistency and reagent deposition. In addition, the production versions of the cassettes will need to be much thinner in order to allow the detection head in the device to be sufficiently close to the membrane.[95] It is still unclear how these requirements will translate into the cost-per assay and if or when this approach would be feasible for resource-limited sites.

A third approach to improving the sensitivity of lateral flow devices involves the use of other labels such as luminescent europium III chelate nanoparticles. The use of this label improved the sensitivity of detection of *P. falciparum* in a lateral flow format (in a laboratory setting) to 10 parasites per µl, which is a 10 to 20 fold improvement in the detection limit compared to colloidal gold.[96] If these materials can be implemented in a field-usable format, the alternative labels could improve the sensitivity of membrane-based immunodiagnostics for other infectious agents where sensitivity has been the limitation.

3D. Array-Based Formats

Many platforms that are currently available are similar to or derived from classic sandwich or competitive assays based on the enzyme-linked immunosorbant assay (ELISA) design. The most common of these are the plate-based ELISA formats and the bead-based formats, such as the Luminex system. Few if any of these formats as currently employed are appropriate for field use since they require strictly controlled laboratory conditions (aseptic conditions, temperature control, refrigeration of reagents), technical personnel for performance and/or interpretation, and expensive, complicated equipment, and they will not be discussed further in this review. Other products that are currently available, such as the Bio-Detector (Smiths Detection) have been developed for the field-use immunodetection of pathogens for biosecurity applications, but they have not generally been developed for use with clinical specimens and the very high cost of both the instrument and each individual assay make this system impractical in resource-limited settings.

3E. Formats and Detection Methods for Nucleic Acid Based Diagnostic Tests

The usefulness of an immunoassay, compared to a nucleic acid assay, depends on a range of factors including whether sufficient analyte is present in sample types that are practical to obtain to provide useful clinical information, and whether detecting the presence of antibodies to or antigens of an infectious agent is sufficient for diagnosis of a particular infectious disease. It is a trade off between the performance needs and the clinical information provided by the analyte.

In resource-rich sites, the detection of an infectious organism is most reliably accomplished with nucleic acid methods. The shortcomings of antibody detection have been discussed above. Antigens from the organism can appear without the organism being present in its entirety. Outside of the host cell, the presence of the organism's genome is definitive for its presence. The quantification of the nucleic acid is the quantification of the organism. However, nucleic acid detection has its own limitations. For bacterial detection, it is not clear from the detection of the genomic DNA whether the organism is alive or dead, an issue that created a long delay in the approval of a *Mycobacterium tuberculosis* test by the FDA in the United States. Liver cell collection by biopsy and subsequent lysis can provide HBV DNA that is not associated with the virus, but is a dormant intracellular form (so called CCC DNA). Similarly, HIV proviral DNA is found in infected cells, but since it is an RNA virus, this is rarely an issue.

Generally, there is too low a concentration of nucleic acid to be detected with simple uni-labeled probes in direct hybridization formats. Therefore, one of two amplification techniques is employed: target amplification or signal amplification. Target amplification techniques, such as polymerase chain reaction (PCR) produce more of the target nucleic acid (RNA or DNA; for RNA, a reverse transcription step is used), and then use low-sensitivity detection schemes. In contrast, signal amplification is used to detect the nucleic acid at its physiological concentration by direct probe hybridization followed by the incorporation of a very large number of labels. The first such technique, the branched DNA or bDNA method, has been used to incorporate more than 18,000 labels onto each RNA molecule of the HIV genome. As a result, the method can be used to detect as few as 50 molecules of the HIV viral RNA in 1 ml of blood. There are advantages and disadvantages to both methods. Assay formats that use these techniques are addressed below.

Tests that are used to detect nucleic acids for most infectious agents already exist and many have the highest sensitivities and specificities that can be obtained with any format; however, the majority of the current generation of products require complex sample preparation, laboratory environments, highly trained personnel, and come at a price that is not practical for use in resource-limited sites. Recently, a number of formats and platforms have emerged that could be suitable for field use. The challenge remains to reduce the cost of these diagnostic tests to a cost that is practical for resource-limited settings (from dollars to cents per test).

3F. Field-Use Platforms That Amplify the Nucleic Acid Analyte

There are many chemistries for nucleic acid amplification that are used or could be used in diagnostic test formats, though by far the most commonly used is PCR. Scientists' familiarity with PCR has made it the first choice among diagnostic test manufacturers who are relatively unconstrained by assay complexity, speed, and cost and are primarily producing tests for use in resource-rich sites. Some test developers have attempted to adapt existing PCR-based tests to the requirements of relatively resource-limited settings.

However, because PCR assays require many cycles of precise temperatures changes, the complexity of the instruments is higher than for some other newer target amplification chemistries, and therefore it is worthwhile to consider other technologies in addition to PCR for resource limited sites.

PCR amplification chemistries that provide a quantitative amplification (so called Q-PCR) and couple the amplification and detection of the nucleic acid are most commonly used such as the TaqMan® (double-dye) chemistry. Though many Q-PCR thermocycler instruments are available, very few have been developed or adapted for field use. Several manufactures now market instruments and kits that significantly reduce the complexity of the analyses to a "low complexity test" in a clinical diagnostics laboratory in a resource-rich site, including the GeneXpert (Cepheid), the LightCycler (Roche) and the PathAlert kits (Invitrogen). However, these instruments are still sophisticated and were designed to be used in a laboratory setting.[97]

The GeneXpert system is widely used by the United States Postal Service, and while this is clearly a non-laboratory environment, it is not an extremely resource-limited setting. This platform is unique in its full automation and integration of sample purification and analysis in a self-contained, single-use microfluidic cartridge, its ease-of-use, speed and relative robustness. The cartridge incorporates a syringe drive, rotary drive, and a sonic horn that delivers ultrasonic energy to lyse the raw specimen thereby releasing nucleic acids from cells. The syringe drive and rotary drive move liquid between cartridge chambers to wash, purify, and concentrate the nucleic acids. The system has been validated for use with whole blood, oral fluid, urine, as well as a variety of environmental sample types. After purification, the concentrated nucleic acid is moved into the cartridge reaction chamber where it is amplified via PCR and the amplified nucleic acid is detected by the instrument's real time fluorimeter using any number of fluorescent labels or intercalating dyes. Cartridges are available to purify either DNA or RNA and can perform the reverse transcription steps required to amplify RNA targets. The instrument's rapid temperature-controlled fluorimeter has reduced power consumption requirements and produces results in less than 30 minutes. The cartridges contain lyophilized reagents and have an extended shelf-life at ambient temperatures. However, the instrument was designed to be run in a laboratory setting, not in resource-limited sites, and further modifications might be required to produce a prototype that is worth evaluating in resource limited sites. The most significant barrier for the use of this system in resource limited sites is the cost. Current list prices for the cartridges are $40 to $60 per assay.

Several other PCR analysis platforms, such as the RAPID System (Idaho Technologies) and the Bio-Seeq (Smiths Detection) can perform PCR analyses under field-use conditions; however these devices have been developed for biosecurity applications, rather than the ASSURED criteria. The RAPID uses the LightCycler technology, which is a high-speed thermocycler with a built-in fluorescence monitoring system that controls the temperature of the air around the assay chambers (rather than controlling a solid

material around the assay chambers). Though both these platforms are rugged, rapid, adapted to a variety of power sources, use reagents with long shelf lives over a fairly wide range of temperatures, and have somewhat simplified procedures, neither of them have on-board capacity to process or purify nucleic acids from clinical samples, and both have a relatively high cost per assay (tens of dollars). These platforms are therefore not well suited for use in resource-limited environments.

Given the challenges of implementing PCR-based assays that meet the ASSURED criteria and are affordable for resource limited settings, it is worthwhile asking what alternative approaches might circumvent some or all of the difficulties. There are at least three alternatives that are worth considering

- Approaches that do not require thermal cycling, and therefore a simpler instrument could be deployed
- Approaches that integrate and/or automate the purification of nucleic acids at a more reasonable price
- Approaches that do not require purified nucleic acids.

3G. Approaches That Use a Simpler Instrumentation

While PCR can provide a very high sensitivity, the instrumentation is complicated because of the temperature cycling required. However, there are a number of nucleic acid amplification chemistries that do not require thermal cycling, which might allow a simpler and more robust instrument to be used that would be better suited to resource-limited settings.

Two of these chemistries which are similar in certain ways are Transcription Mediated Amplification (TMA, GenProbe) and NASBA (BioMérieux).[98] Both chemistries use a series of enzymatic reactions to produce single stranded RNA amplification products (amplicons) without the need for temperature cycling. Another enzymatic chemistry for amplifying DNA called Strand Displacement Assay (SDA) (Becton Dickinson) utilizes a number of different enzymatic reactions including a restriction endonuclease and DNA polymerase. DNA polymerase is used to synthesize the target which contains the recognition site nicking by the restriction endonuclease. In the presence of DNA polymerase, the nicked DNA serves as a template for synthesis of the target. The reaction can be complete in one hour and does not require thermal cycling, though the original double stranded DNA target must be denatured at 95°C to initiate the reaction.[99] Yet another novel isothermal system for the amplification of DNA or RNA has recently been described by Kurn et al. This method, termed SPIA, utilizes a mixture of enzymes along with a chimeric DNA/RNA primer to achieve 10^{12} fold amplification in 15 minutes. Although SPIA has been commercialized for amplification of all mRNA in a specimen, it could be adapted for the amplification of specific DNA or RNA sequences.[100] All these amplification chemistries have the advantage that they do not require thermal cycling. However, as of yet, no manufacturer has developed a

instrument that would perform assays using these chemistries, and the sample purification steps required, that is appropriate for use in resource-limited settings.

Another amplification chemistry that does not require thermal cycling called Exponential Amplification Reaction (EXPAR) is being developed by Ionian Technologies. This technology is similar to the SDA technology in that a restriction endonuclease is used. However, the restriction endonuclease in this case is used to nick the double-stranded DNA template and allow release of a detection oligonucleotide. This oligonucleotide can be detected by a variety of methods. For efficient release of the oligonucleotide, the reaction must be run at 60°C. Because of the multiple enzymatic reactions required in this technology, purified nucleic acid samples are required.[101]

Ionion is also developing a small, ultra rapid, extremely simple, rugged, and portable instrument which will be suitable for field use. The device is designed to run assays that use the isothermal EXPAR chemistry. The prototype instrument currently use SYBR green detection of double stranded, amplified DNA, and the device can provide a qualitative or quantitative interpretation for single-plex assays. The assays could also be adapted to other detection methods. Advantages of this system are anticipated to be the speed of amplification and detection, (results are obtained in 5 to 10 minutes), low power usage (small external battery pack), the wide range of temperatures in which the instrument functions (110°F to 0°F), and sensitivity, as the current prototype devices can robustly detect 100 copies of a nucleotide sequence per sample which provides a sensitivity on par with PCR.[102] Currently, the device does not have the capabilities to purify nucleic acids from clinical samples. The hand-held device is anticipated to cost around $1,000, and reagent costs are expected to be a fraction of a dollar per assay.

These amplification chemistries provide similar sensitivities and dynamic ranges as PCR, and several are more rapid. The fact that they do not require thermal cycling may allow them to be used with simpler, more robust, and less costly instrumentation that requires less power. However, because they require multiple enzymes and/or a more complex set of oligomers, they may end up having a higher cost per assay. In addition, most of the work to date using these alternative chemistries has generally been done by a few researchers at specific companies, and the assay design and optimization strategies are less well-known in the general research community. All the chemistries that amplify nucleic acids, including PCR, can be inhibited by substances in clinical specimens, and therefore all these methods have the disadvantage that the nucleic acids need to be purified before the assay reaction can be performed.

3H. Approaches That Use a Simpler Detection and Interpretation Method

The Innogenetics LiPA platform provides a simple method for the detection of PCR products in a small disposable device that resembles a dipstick. The PCR is carried out in the presence of a biotinylated primer. The PCR product is then incubated in a small tray with a strip (dipstick) which contains

immobilized target-specific capture probes. The hybridized complex bound to the strip is detected visually by the addition of a horseradish peroxidase strepavidin conjugate with a colorimetric substrate, which results in a red colored line. The qualitative result is interpreted by the operator without the need for instrumentation. Total time for incubation and wash steps is about 90 minutes.[103] This technology is being used in a number products, including detection of infectious agents and genotyping viral strains.

An academic group has reported the development of a multi-analyte lateral-flow system for the detection of multiple strains of Dengue virus. The RNA genome of the virus was amplified via the NASBA chemistry, and the detection of specific serotypes of Dengue virus was accomplished using a single lateral flow device in 25 minutes. A reporter probe, which hybridizes to all Dengue serotypes, was coupled to dye-entrapping liposomes. Serotype-specific capture probes were immobilized through biotin-streptavidin interaction on the surface of a polyethersulfone membrane strip, each in a separate capture location. The amplified Dengue virus RNA was mixed with the set of probes, and allowed to migrate up the test strip. The amount of liposome that is captured in each location is quantified by a portable reflectometer. The detection limits of this prototype device were different for each strain of the virus (50 RNA molecules for serotype 2, 500 RNA molecules for serotypes 3 and 5, and 50,000 molecules for serotype 1). Though this prototype system required laboratory equipment and expertise, the authors anticipate that such a system could be implemented in a way that would be portable, inexpensive, and very easy to use.[104]

Another strategy that uses a lateral flow device for the detection and identification of Dengue Virus species is under development at Access Bio Inc. (Monmouth, NJ). In this system, the Dengue virus' RNA genome is purified from a sample, reverse-transcribed, and then amplified using a combination of a common primer (conjugated to biotin) and serotype-specific primers (each conjugated to a different hapten such as rhodamine red). If a particular serotype is present in the sample, an amplicon is produced that has both biotin and a specific hapten conjugated to it. The amplification products are then applied to a lateral flow device, and while wicking through the device, they pass through the dye pad, and bind to a conjugate of strepavidin and colloidal gold. These reaction complexes continue to wick through the device, and pass through the capture zones (one for each Dengue serotype) where antibodies to each of the specific haptens have been immobilized (e.g. anti-rhodamine red antibody). In this manner, the colloidal gold conjugate only generates a signal in a serotype-specific capture zone if a serotype-specific amplification product was produced from the sample. The authors report that detection via their lateral flow device can be completed in about 10 minutes, can be visually interpreted by an operator without extensive training, and that the detection limits were equivalent to those obtained with a fluorogenic real-time PCR instrument.[105]

3I. Approaches to Integrate or Automate the Nucleic Acid Preparation in an Inexpensive Way

The fact that most amplification chemistries require purified nucleic acids has been a barrier to the application of nucleic-acid based tests in resource-limited settings. Several integrated platforms for dealing with issue are being developed. The GeneXpert instrument and self-contained cartridges (Cepheid) have already been discussed. Another system that uses self- contained cartridges is in development by IQuum (Allston, MA). Their system is based on their proprietary lab-in-a-tube (Liat™) technology. The Liat™ tube is a self-contained, closed device that contains all the necessary purification and assay reagents in separate chambers (segments) of the tube, which are separated by seals. Pressure is applied to break the seals and allow reagents to mixed and moved. In one segment, magnetic beads are be used for the isolation and purification of nucleic acids. The system can be used for variety of sample types including whole blood, urine, plasma, and swabs. The beads are washed and the purified nucleic acids are eluted and moved into another segment of the tube for subsequent amplification and detection. To complete the amplification, the assay mixture is moved rapidly between two segments of the tube, which are kept at different temperatures. This rapid transfer of the assay mixture between two segments which are kept at two temperatures allows the entire 30 cycle PCR reaction to be completed in 12 minutes.[106] Detection is accomplished using fluorescent TaqMan® probes, and interpretation of the quantitative test result is reported on the screen of the instrument.

The company HandyLab (Ann Arbor, MI) is also developing a fully self-contained cartridge with sample purification capabilities. The small microfluidic fabricated device contains a "bubble" into which up to 1 ml of blood can be introduced. The material is lysed, nucleic acids are bound to a set of beads, washed, and eluted in a small volume for automatic dispensing into the chip for processing in a PCR assay. A unique micro-valve system permits metering of reagents and reaction mixtures. Localized heating and cooling on the processing system controls the rapid thermocycling.[107]

Another approach for dealing with sample preparation was developed by Xtrana. The technology was acquired by Applied Biosystems in 2004. Xtrana developed a simple solid phase sample extraction method. The solid phase material, called XtraBind, binds both single stranded DNA and RNA. DNA bound the XtraBind is quite stable and can be stored up to 18 months and RNA has stability of more than 8 weeks when bound to the membrane. The nucleic acid can be eluted from the membrane and applied to any amplification method. For specific capture of nucleic acid targets, target specific probes can be derivatized to the XtraBind. The test sample is mixed in a hybridization buffer and captured on the membrane allowing washing steps to remove inhibitors and contaminants.[108]

3J. Approaches That Do Not Require Purified Nucleic Acids

An example of a signal amplification technology which does not require any enzymatic amplification of the nucleic acid target is the bDNA chemistry (Bayer Diagnostics and Genospectra). This technology uses synthetic DNA multimers to provide multiple binding sites for a detection probe. The chemistry can be used on a variety of specimens without the need for purification of the nucleic acid, which may be a significant advantage under certain circumstances. Several different temperatures are used (up to 55°C), but thermal cycling is not required. Detection limits of 50 molecules are observed in routine use of the FDA approved HIV viral load assay (Bayer Diagnostics). For detection of viruses and intracellular mRNA, a plasma or cellular sample is placed in the reaction vessel, lysed and hybridized without any purification steps. Thousands of mRNA assays for human mRNAs have been developed and are commercially available.[109] A significant disadvantage to the method is that a long hybridization (18 hours) is required. A similar signal amplification method has been developed by Genisphere.[110]

An approach that does not require purified nucleic acids is the Invader amplification chemistry (Third Wave Technologies). This amplification process can be performed on crude cell lysates and relies on the enzymatic cleavage of specially designed oligonucleotides in the presence of the target nucleic acid (RNA or DNA). The cleaved oligonucleotides then hybridize in a secondary reaction to another set of oligonucleotides which have both a fluorescent label and a quencher label. Cleavage of this secondary oligonucleotide results in the release of the quencher in to solution, and the fluorescence of the part that remains hybridized can be detected. This amplification process is typically run at 60 to 65°C and takes up to two hours to generate a result.[111] These assays can be run using standard laboratory microplate readers, but the sensitivity of the assays is less than that generally achieved with target amplification assays.

Another platform that does not require nucleic acid purification is under development by Investigen, and has been named the smartDNA platform. In this platform, a protein nucleic acid (PNA) oligomer that is specific for an infectious agent and specific cyan dyes are added to a minimally processed specimen, and incubated for several minutes. The PNA oligomer binds invasively and specifically to its complementary nucleic acid (NA) in the genome of the infectious agent, if it is present.[112] The sample is then exposed to light for several more minutes. These specific cyan dyes undergo an irreversible, light-mediated reaction in the presence of PNA:NA hybrids, possibly due to the formation of a catalytic site by the PNA:NA hybrid. When this occurs, a visible color change can be observed, and the qualitative result is visually interpreted by the operator.[113] The steps in the assay are very easy to perform, very rapid (minutes), no equipment is required, and the reagents are stable indefinitely in the dark. Preliminary evidence suggests that useful sensitivities and specificities will be achieved for resource-limited settings.[114]

Nanosphere is developing a technology for the direct detection of nucleic acids and proteins. Their nucleic acid detection based on the use of oligonucleotide probes attached to gold nanoparticles.

Originally a simple "spot and read" colorimetric detection was evaluated. The color change is due to the aggregation of the gold particles which causes a visual change in the color from red or purple to blue. However, this simple direct method suffered from poor sensitivity (10 fmol target) .[115] To improve the sensitivity of detection, the size of the nanoparticles were increased. Also, detection was now based on the change in color of the scattered light from a waveguide. Detecting this change requires a color CMOS detector, instrumentation requirements unlikely adaptable to a field setting

A novel approach to malaria diagnosis, called spectral acquisition process detection (SAPD), is under development, which might be applicable to the detection, identification, and enumeration of other infectious agents as well. In this system, multi-wavelength ultraviolet/visible (UV-VIS) spectroscopy is used to generate transmission spectra over a broad range of wavelengths (190 – 1100 nm) and/or with the scattered light measured at one or many different angles. The spectrum that is generated is affected by the size and chemical composition of infectious agents that are present. The spectral pattern that identifies an infectious agent is based on light scattering theory, spectral deconvolution techniques, and on the approximation of the frequency-dependent optical properties of the basic constituents of living organisms. The assay performed for the detection of *Plasmodium* consisted of collecting 2 to 5 µl of whole blood, diluting it with normal saline and recording the spectrum using a Hewlett Packard 8453 spectrophotometer with an acceptance angle of less than 2 degrees. The results would be downloaded onto a laptop computer for interpretation. In pilot experiments, a sensitivity of one infected cell per 1 million red blood cells was achieved. The approach is unique in that it requires no reagents or expensive instrumentation, and the authors anticipate that this method will be a quantitative, rapid, sensitive, inexpensive, and simple to use diagnostic method that is appropriate for resource-limited environments.[116]

3K. Other Promising Technologies for the Future

A number of diverse technical approaches are being used in research settings to improve the sensitivity of detection or ease of use for assays. While these technologies are still in the research phase, advances in manufacture and instrumentation could make such technologies feasible for field use.

To improve the sensitivity of protein detection several companies have combined immunoassays with nucleic acid detection. Leucadia Technologies and Nanosphere have both looked at means to combine the sensitive detection of nucleic acids with antibody detection. Leucadia Technologies has developed a system (NADIA) in which they attach partially overlapping oligonucleotides to two different antibodies to the same analyte.[117] Binding of the antibodies to the analyte brings the oligonucleotides into proximity and the overlapping oligonucleotides can be amplified by any of the nucleic acid amplification technologies. Nanosphere uses two types of particle in their assay; one particle is a magnetic particle conjugated to a capture antibody and the second particle is a gold nanoparticle that contains both the capture antibody and multiple copies of an oligonucleotide. The binding of the target to the particles

allows for magnetic separation of the target containing the bound gold nanoparticle. The gold nanoparticle has multiple copies of the oligonucleotide which can be detected by a number of nucleic acid technologies.[118]

Members of a Cornell University research group headed by engineering professor Harold Craighead report they have used tiny oscillating cantilevers to detect masses as small as 6 attograms by noting the change an added mass produces in the frequency of vibration. The researchers manufactured a microcantilever out of silicon and silicon nitride. The cantilever can be put into motion by applying an electric field. The frequency of vibration can be measured by shining a laser and measuring the change in reflection of the light. The frequency of vibration is a function of the mass. Antibodies or other capture agents can be attached to the surface and binding of the target to the cantilever surface causes a chance in the vibration frequency.[119] This technology could be applied to the direct detection of DNA, proteins, or entire microbes where no labeling of a specific binding agent is required. Many other companies and laboratories are busy developing these cantilever processes and devices. Thus far, the sensitivity of the method has not met expectations, but in the long term, the prospect of developing very inexpensive microfabricated chips will continue to drive interest and investment.

Another interesting approach that is currently under development for the sensitive, direct detection of biomolecules is called rupture event scanning (Akubio, Cambridge, UK) and initial pilot studies have focused on detecting virus particles. Antibodies that are specific for virus particles are attached to the oscillating surface on a quartz crystal microbalance (QCM). After allowing the virions in a sample to bind to the antibodies, the virions are subsequently detached from the surface by increasing the oscillation of the crystal. The breaking of the bonds between the virus and the surface of the crystal creates a sound that can be detected by a microphone and converted into an electrical signal. Pilot studies demonstrated that the virus could be directly detected in serum without any (additional) sample preparation.[120] QCMs are already used in a number of electronic devices and are inexpensive; it is anticipated that the instrumentation for this technology could be produced very economically.

Carbon nanotubes may also be applied for the sensitive detection of biomolecules. Work at Nanomix (Emeryville, California) has shown that nanotubes can be derivatized with a polymer to allow the attachment of capture probes on the surface the nanotubes. The binding of the target to the capture probe changes the electrical properties of the nanotube device allowing for sensitive label free detection.[121] Challenges still exist for reliable and reproducible manufacture of nanotubes. However, if the manufacturing issues can be solved, nanotubes may provide a means for the simple instrumentation for the detection of biomolecules.

BioFlash™ system (Innovative Biosensors, College Park, MD) is based on the CANARY™ (Cellular Analysis and Notification of Antigen Risks and Yields) technology. BioFlash™ assays are very rapid, and have the potential to be very sensitive and specific, and easy-to-use. The system consists of engineered biosensors expressing membrane bound, pathogen-specific antibodies and a calcium sensitive bioluminescent molecule. Cross-linking of the antibodies by even small amounts of the specific pathogen leads to elevation of intracellular calcium and light emission. The amplified light output is detected using a luminometer. To work with complex samples, an additional filtration and centrifugation step are required. The complete assay from sample to result can be performed in less than 5 minutes.[122] The feasibility of a field-usable system for resource-limited settings remains to be explored.

Table 7. Commercial Platforms on the Market or In Development that are Appropriate for Resource Limited Sites

Product	**Status****	**Training**	**Instrument********	**Sample Purity**	**Sensitivity**#**	**Speed**	**Interpretation**	**Cost**+**	**Power**++**
Immunoassay									
Agglutination	many	low-mod	low-mod	crude	low	min - hr	easy - mod	low	none - low
Dipstick	many	low	none	crude	mod	min	easy - mod	low-mod	none
Dipstick + Device	several	low	mod	crude	mod-high	min	easy	mod	none-low
Lateral Flow	many	low	none	crude	mod	min	easy - mod	low-mod	none
Lateral Flow + Device	several	low	mod	crude	mod-high	min	easy	mod	none-low
Nucleic Acid									
GeneXpert (PCR)	several	low	high	crude	high	min	easy	high	mod
RAPID (PCR)	several	mod	high	purified	high	min	easy	high	mod
IQuum (PCR)	in dev	low*	high	crude	high	min	easy	-	?
Ionian (EXPAR)	In dev	low*	mod $$^{+++}$	purified	high	min	easy	-	mod

Table 7. *expected; ** for field or POC use; ****low simple inexpensive such as a battery operated rotator, mod small moderately complex instrument, high, expensive complex instrument; + cost is end user cost and is not cost of goods, additionally cost of instrument is spread over a number (e.g. 100-1,000 tests), low< $1, mod $1-$10, high >$10; + power can be battery based, low implies a small flashlight type of battery, mod implies a larger battery such as a car battery; +++ does not include sample prep; # sensitivity means the sensitivity of the system to detect an analyte, low is the least sensitive and high is the most sensitive, such as that obtained with PCR (10 - 50 molecules).

Table 8. Technologies in Development

Technology (Company)	Capability	Commercial Status	Instrumentation	Sample Purity	Sensitivity*	Speed**	Detection Options	Power Requirements***
HandyLab	nuc. acid. purification	development	complex	NA	NA	TBD	NA	moderate
Xtrana/ABI	nuc. acid purification	development	none	NA	NA	rapid	NA	minimal
PCR (Many)	nuc. acid amplification	varies	complex	purified	high	rapid	fluorescent/others	high
TMA (Gen-Probe)	nuc. acid amplification	mature /no POC	mod complex	purified	high	rapid	chemiluminescent	moderate
NASBA (bioMerieux)	nuc. acid amplification	mature/no POC	mod complex	purified	high	rapid	fluorescent	moderate
SDA (Beckton Dickenson)	nuc. acid amplification	mature/no POC	mod complex	purified	high	mod	fluorescent	moderate
bDNA (Bayer)	signal amplification	mature/no POC	complex	crude	high	slow	chemiluminescent	high
Invasive Cleavase (Third Wave)	signal amplification	mature/no POC	complex	crude	mod	mod	fluorescent	moderate
Gold Nanoparticles (Nanosphere)	signal amplification	development	complex	TBD	mod	TBD	colorimetric	TBD
NADIA (Leucadia)	signal amplification for immunoassay	development	complex	crude	TBD	mod	fluorescent/others	moderate
BioFlash (Innovative Biosensors)	signal amplification for immunoassay	development	complex	crude	TBD	rapid	luminescence	moderate
smartDNA (Investigen)	homogen. direct detect	development	mod complex	crude	mod	rapid	colorimetric	moderate
SAPD	label-free detection	development	mod complex	crude	high	rapid	spectral analysis	moderate
Micro-cantilever	label-free detection	development	complex	TBD	high	rapid	electrical	TBD
Acoustic detection (Akubio)	label-free detection	development	mod complex	TBD	high	rapid	electrical	TBD
Carbon nanotube (Nanomics)	label-free detection	development	mod complex	TBD	high	rapid	electrical	TBD

Table 8. TBD, to be determined; NA, information not available; *, sensitivity, high <50 molecules, moderate > 50 molecules; *rapid, <10-20 min, mod 20min-2 hrs, slow >2 hrs, ***power requirements are projected and could be less that stated here, minimal small battery, moderate larger battery, high, significant power source required for thermocycling or temperature maintenance of extended periods of time.

Discussion

The criteria for diagnostic tests that will be used in resource-limited settings are clearly different from those for tests in conventional diagnostic laboratory settings. Interestingly, the criteria most closely resemble the "self testing" methods such as home glucose monitors or pregnancy tests. An awareness of these different criteria, and the day to day challenges that are faced when performing diagnostic tests "in the field," will hopefully encourage academic and commercial diagnostics developers to produce more of these robust and inexpensive tests that are so urgently needed. Given all the improvements that have been made in the point-of-care formats, and all the innovative new approaches that combine the expertise of many diverse scientific disciplines, the diagnostic industry is surely on the verge of overcoming many of the technological barriers to producing products that are suitable for resource limited sites.

Meeting the ASSURED criteria for diagnostic tests will be challenging. To be affordable within local health care systems in remote locations, a test will need to cost relatively little. The sensitivity and specificity requirements will be determined by disease-specific criteria. To be user-friendly, the sample type must be easy to collect, and the test should be simple to perform and interpret with minimal training. To be rapid, the time to perform a test should be measured in minutes, not hours, in order to enable treatment of the patient during the visit. To be robust, the test should not require refrigeration for delivery or storage and should be stable under extreme conditions of temperature and humidity. To be equipment-free, a test should require no extra equipment for sample processing or to perform the assay. To be delivered to those who need it, the test needs to be portable and function under a wide range of conditions, without a sophisticated laboratory environment, and with practical power sources.

Resource-rich sites can often overcome the challenges of a test that is less than ideal for their setting, such as a test that takes a long time to perform, or a test that requires a specialist to obtain the sample type. However, almost by definition, resource-limited sites have no resources to overcome the criteria that are not met by a particular test, and therefore a deficiency in any of these criteria (except one) is an insurmountable barrier in these settings, no matter how well the test fulfills the other criteria. Therefore, until recently, the populations that live in resource-limited areas have had to live with diagnostic tests that are deficient in the one criterion that they can compromise on, but should not have to sacrifice, which is performance (sensitivity and specificity).

Favorable conditions in related diagnostic markets which have test criteria that are similar in certain ways to the ASSURED criteria, have helped drive innovation and the number of tests on the market today that are appropriate for resource-limited sites. The upswing in demand in resource-rich sites for point-of-care testing devices has helped in this evolution, as these tests often fit many of the ASSURED criteria. The demand for "field-usable" tests for biosecurity applications has driven improvements in other areas. Hopefully the

demand in these more lucrative markets will continue to drive the technological innovation and synergy between scientific disciplines and the arsenal of high-performing tests that are practical for resource limited settings will continue to expand.

The emerging technologies and approaches give the diagnostics community a good reason to take a step back and re-evaluate why certain approaches that are currently used in laboratory environments are unlikely to be adoptable in resource-limited settings. There often several test formats and sample types that could be used to detect, identify, or quantify a particular infectious organism. Instead of trying to force laboratory formats to work in resource-limited settings, a completely different approach could be taken that would be more successfully implemented in the field

Once an accurate assessment is made of the sensitivity and specificity requirements for a particular infectious agent in a particular population with the sample types that are practical and clinically meaningful to obtain, then the available approaches can be evaluated to determine the most practical and least costly way to achieve the essential performance criteria. Some of the most exciting diagnostics to emerge are those that appear to be a simple disposable device, but actually contain complex and innovative technologies. If these devices can be made robust and cost-effective, the panoply of diagnostics tests available today to only a select few will become accessible to the vast majority of the earth's population.

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