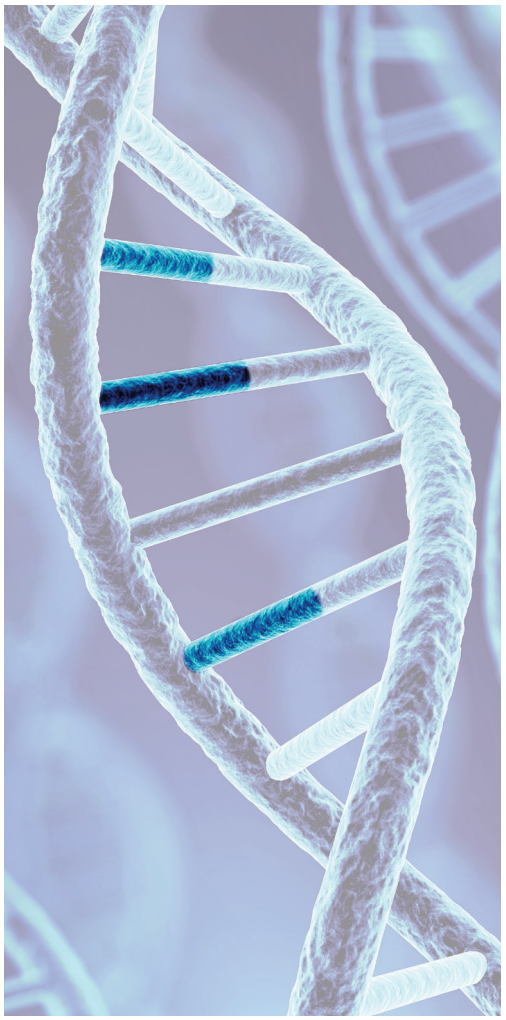
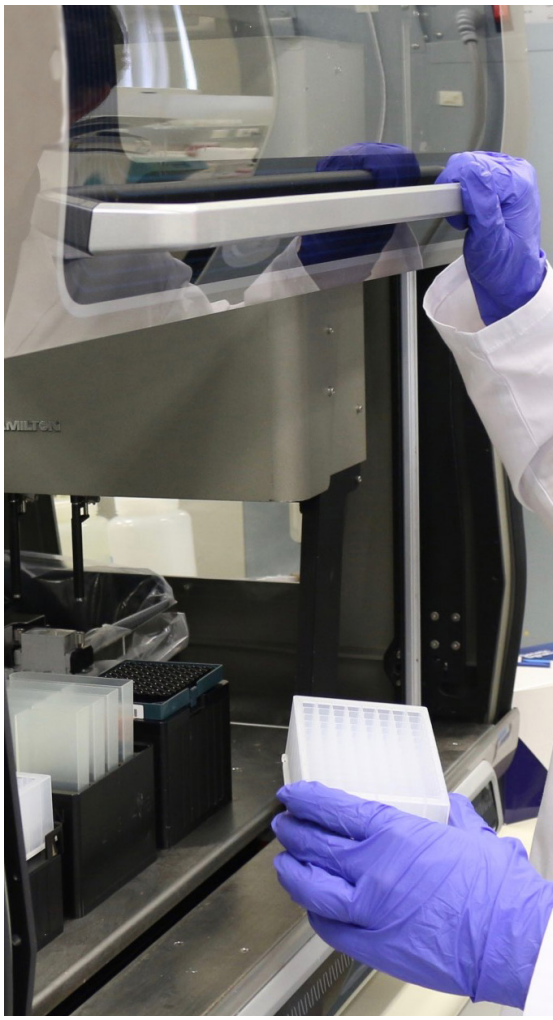
A GENETIC SIGNATURES WHITE PAPER

Advances in Gastrointestinal Protozoa Testing:

Molecular Ova and Parasite Investigations







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Abstract & Key Information

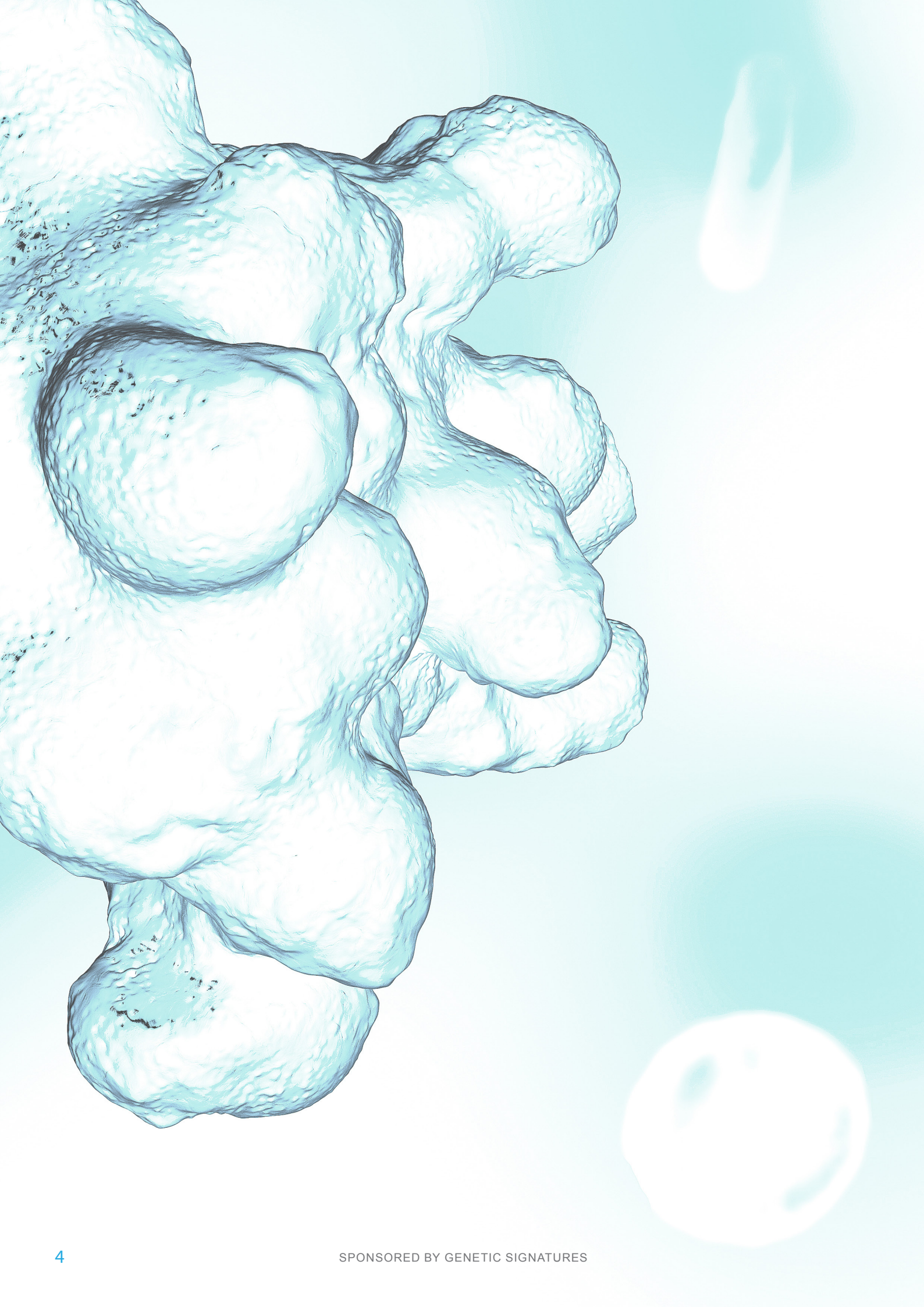
In the United States (US), gastrointestinal (GI) protozoan parasites are a significant healthcare burden, with more than 350 million cases of acute GI infections annually and are the leading cause of mortality of children under five years old.[1,2] Diagnosis that distinguishes between GI pathogens using traditional approaches remains a challenge. Specifically, the screening method for Ova & Parasites (O&P) has not seen advancement in diagnostic detention for decades. It is recognized within the parasitology field that traditional O&P tests are time consuming, have variable sensitivity, and are burdened by skilled labor shortage and costs.[3]

This paper highlights the challenges of current O&P testing, and introduces a new paradigm of a molecular Polymerase Chain Reaction (PCR)-based O&P screening method which would identify the significant majority of gastroenteritis-causing parasites, either as a standalone test, or to complement traditional O&P. The molecular O&P test is Genetic Signatures' *EasyScreen*™ Enteric Protozoan Detection Kit, which uses a novel 3base™ technology and screens for eight clinically relevant GI protozoa.

Market research assessed in this paper indicates the approximate tripling of GI protozoa outbreaks over the last decade in the US, and reveals the complexity of current O&P testing guidelines that stifle fast and effective patient management. In contrast to traditional O&P, US clinical trials and user experience demonstrate that molecular O&P can increase positive detection rate of GI protozoa and coinfection. In addition, patient results can be returned within one day compared to two to four days, or even longer. Molecular O&P therefore demonstrates benefits to the diagnostic workflow, healthcare system, and importantly patient management.

- GI protozoa diagnosis currently relies heavily on O&P examination, which has variable sensitivity and specificity, is labor intensive, time consuming, and requires highly skilled laboratory scientists.
- There is a growing willingness of laboratories to adopt PCR technologies for routine diagnostic procedures, due to the increased sensitivity and economic benefits.
- Genetic Signatures is a specialist molecular diagnostics company offering patented 3base™ multiplex PCR solutions that provide distinct advantages to molecular diagnostic testing compared to other commercial PCR alternatives.
- Genetic Signatures is expanding their US product offering to include the *EasyScreen*™ Enteric Protozoan Detection Kit. Food and Drug Administration (FDA) trials are in progress at ARUP Laboratories (Utah), Indiana University and TriCore Laboratories (New Mexico).
- There is significant clinical evidence that products within our *EasyScreen*™ Enteric Detection Kit range can support a molecular O&P approach for the syndromic testing of GI protozoa, yielding higher specificity and sensitivity than traditional O&P, improving turnaround times and maximizing workflow efficiency.[7,17]

Genetic Signatures' *EasyScreen*™ Enteric Protozoan Detection Kit is currently for Investigational Use Only in the US. Contact us for more information on regulatory status of our products in your region. When using our products always read the label and follow the directions for use.



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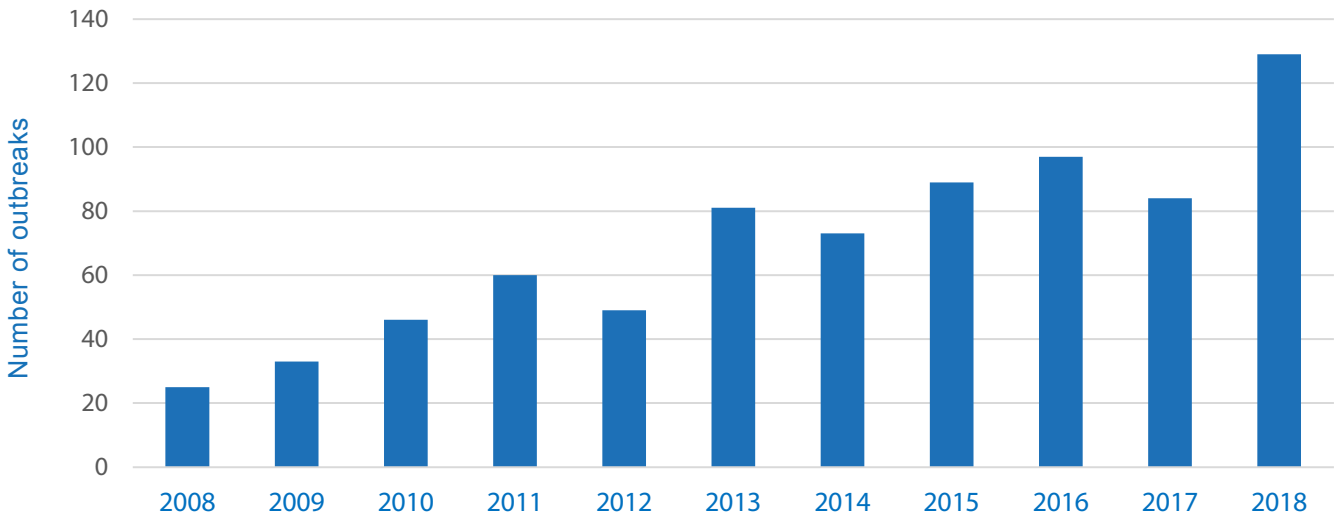
Burden of GI Protozoan Parasites in the United States

In the US, GI protozoan parasites are a significant healthcare burden, infecting millions of people and negatively impacting the healthcare system and patients.[1,2] The most prevalent GI protozoa in the US include members of the *Giardia* and *Cryptosporidium* genera, which cause regular waterborne outbreaks in the summer months.[4] Other notable protozoan parasites which are endemic to developing countries include *Cyclospora cayetanensis* which causes outbreaks linked to fresh produce, and dysentery-causing *Entamoeba histolytica* which can be identified in returning travelers.[5,6] Despite incidence and mortality from these types of infections being lower in developed countries, it is crucial to accurately diagnose all infections, as these can cause debilitating symptoms and complications in patients, and present a greater economic burden in developed countries due to higher medical and treatment costs.[7]

The graph below from the Center for Disease Control & Prevention (CDC) demonstrates the increasing trend in GI protozoan outbreaks identified in the US between 2008 and 2018. The data for each year include total outbreaks diagnosed as *Giardia, Cryptosporidium, Cyclospora, Blastocystis* and *Entamoeba*, and supports the increasing need to address infection control for these pathogens due to the likely increase in future outbreaks.

Figure 1. GI Protozoan Outbreaks in the US, Center for Disease Control & Prevention (CDC)

Sourced from CDC National Outbreak Reporting System (NORS) Dashboard on 18/10/21



An outbreak is defined as the occurrence of two or more cases of similar illness epidemiology linked to a common exposure. NORS data are collected from voluntary reporting only. Thus, outbreaks and illness will be underrepresented. Data includes voluntary reported outbreaks diagnosed as *Entamoeba*, *Cryptosporidium*, *Cyclospora*, *Blastocystis* and *Giardia*.



Dr Bobbi Pritt, MD
Director, Clinical Parasitology, Mayo Clinic

Dr Pritt is director of the Clinical Parasitology Laboratory in Mayo Clinic's Department of Laboratory Medicine and Pathology, coordinating the development of real-time PCR assays, predominantly for enteric and vector-borne pathogens. She received her medical degree from Larner College of Medicine at The University of Vermont.

Dr Pritt is active in research and education, authors expert publications in high-impact journals, and partners with patient-facing providers to improve the delivery of personalized patient care.

“

Cryptosporidium and *Giardia* are the two primary parasites that all of the labs in the United States see, and we do a number of different types of tests for them. They cause a significant burden on our population in terms of work loss and economic productivity. The symptoms of *Cryptosporidium* and *Giardia* infection can last for weeks.

We also see cases of *Cyclospora* infection every year now, usually linked to produce from Latin America. If we see other parasites, it’s usually in someone who’s traveled.

These infections can be potentially serious and even life-threatening in immunocompromised patients. Any of them could be missed on routine O&P exams if the proper stains or studies aren’t performed.

They all require some extent of special testing for maximum sensitivity.

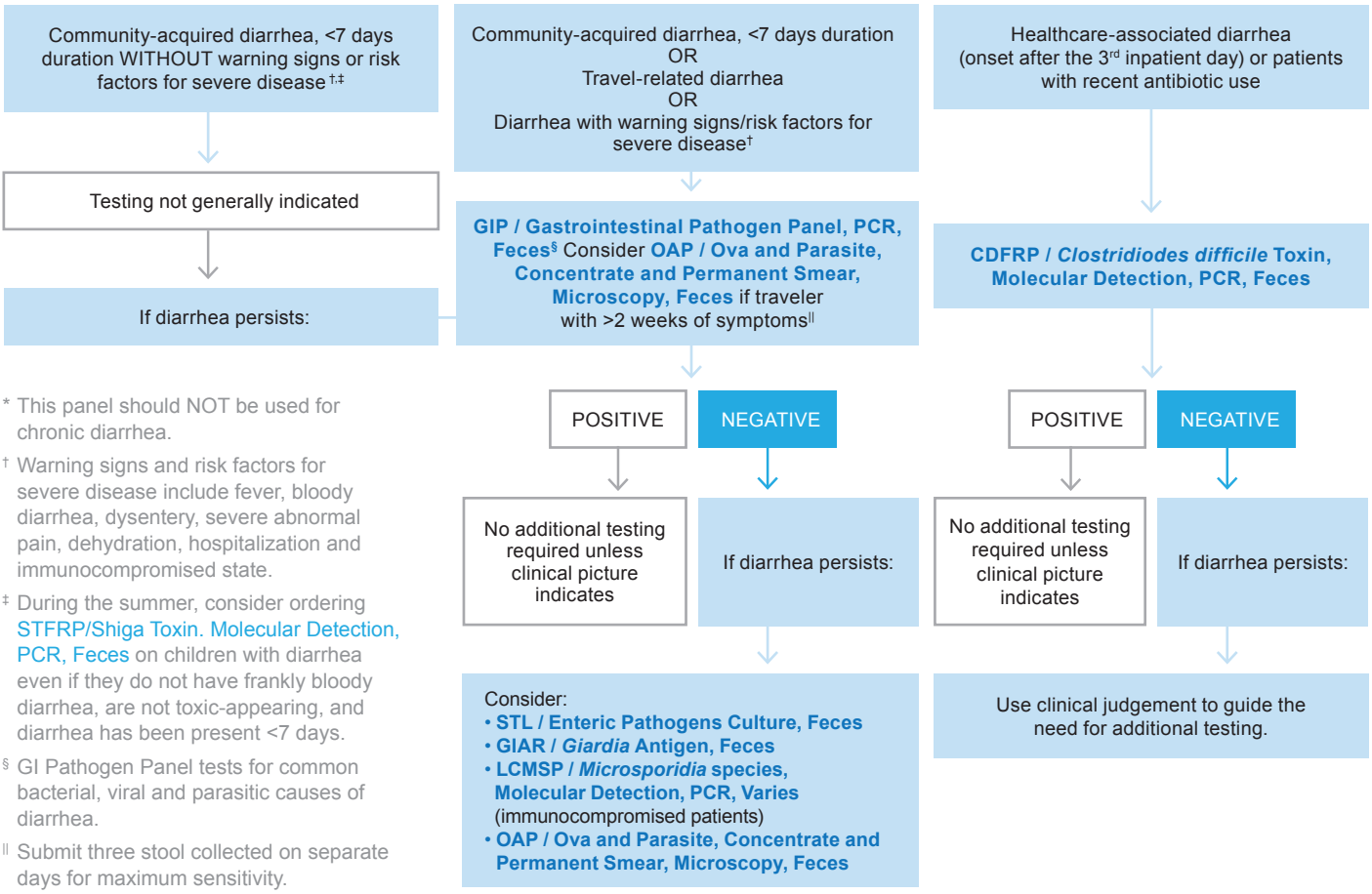
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In the US, the laboratory diagnosis of GI infections has traditionally relied on a combination of techniques including O&P (microscopy and culture), antigen detection, and individual real-time PCR assays.[3] Although these methods have demonstrated good performance, they are labor intensive, time consuming, and rely on ordering physicians to request the appropriate tests. Sensitivities as low as 54.8% and 66.4% by microscopy have been reported for *Cryptosporidium* and *Giardia* respectively.[8] Whilst rapid fecal antigen testing has been approved by the FDA for *Giardia, Cryptosporidium spp.* and *E. histolytica*, none have been commercially developed to date for the identification of *Cyclospora* and *Dientamoeba*.[8]

Figure 2, sourced from the Mayo Clinic, demonstrates the complexity with traditional guidelines, posing challenges for physicians when distinguishing between pathogens that can cause similar symptoms. Therefore, there is a need for a syndromic approach to testing that can broadly and specifically screen for a number of pathogens at once. Faster diagnoses achieved with molecular O&P will ultimately enable physicians to prescribe the best treatment for patients more quickly. For the majority of cases, they can be treated with fast-acting antiparasitic medication.

Figure 2. Laboratory Testing for Infectious Causes of Diarrhea*

Sourced from the Mayo Foundation for Medical Education and Research (MFMER) on 18/10/21



* This panel should NOT be used for chronic diarrhea.

† Warning signs and risk factors for severe disease include fever, bloody diarrhea, dysentery, severe abnormal pain, dehydration, hospitalization and immunocompromised state.

‡ During the summer, consider ordering STFRP/Shiga Toxin. Molecular Detection, PCR, Feces on children with diarrhea even if they do not have frankly bloody diarrhea, are not toxic-appearing, and diarrhea has been present <7 days.

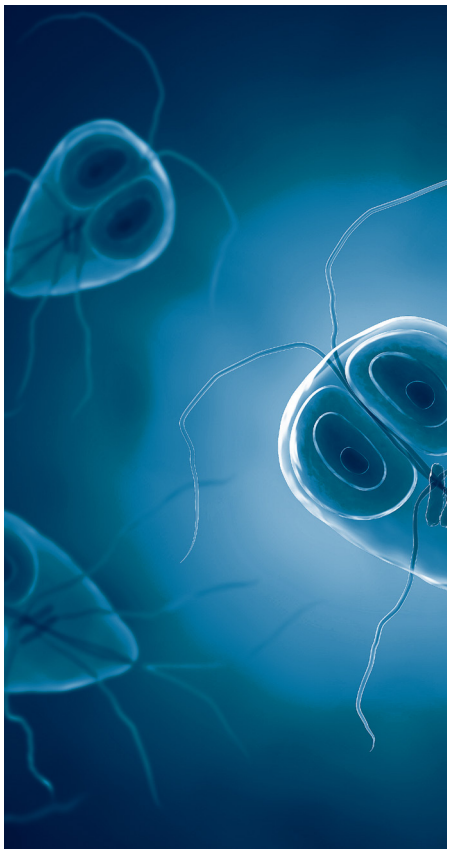
§ GI Pathogen Panel tests for common bacterial, viral and parasitic causes of diarrhea.

‖ Submit three stool collected on separate days for maximum sensitivity.

Note: in outbreak scenarios with a known organism, consider ordering a specific test for that organism. (CYCL/*Cyclospora* Stain, Feces; CRYPS / *Cryptosporidium* Antigen, Feces; GIAR / *Giardia* Antigen, Feces; bacterial stool culture).

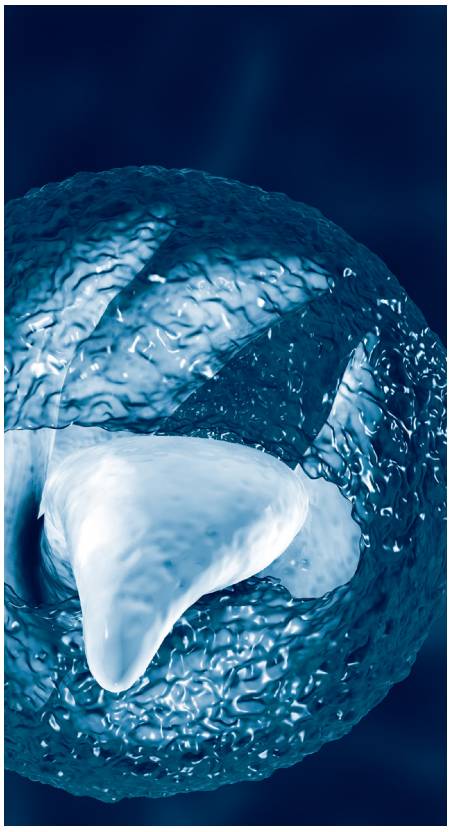
GI Protozoa Epidemiology and Public Health Impact in the US

Giardia



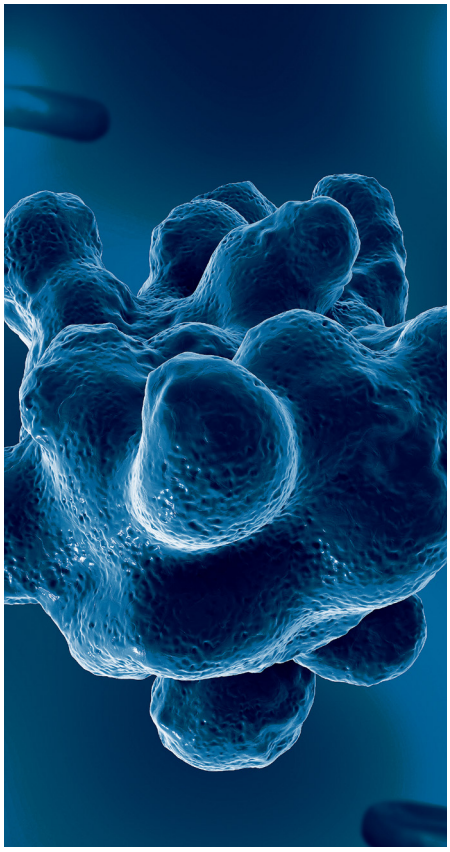
General information	*Giardia duodenalis* (also known as *Giardia lamblia* and *Giardia intestinalis*) is one of the most common GI parasites in humans. It is protected by an outer shell that allows it to survive outside the body for long periods of time and makes it tolerant to chlorine disinfection. *G. duodenalis* is the causative agent of giardiasis.
Transmission	Water is the most common mode of transmission between humans. It is a common cause of waterborne outbreaks.[9]
Symptoms	Giardiasis symptoms include acute, watery diarrhea. Chronic infections can result in weight loss and malabsorption, and can cause long-term complications, including irritable bowel syndrome and chronic fatigue.
Number of infections and deaths	*G. duodenalis* infects >280 million people annually.[10] Giardiasis is underreported at 20,000 cases in the US each year. It is estimated that the real figure is 1–2 million cases annually in the US alone.[9,11] Rarely do *Giardia* infections cause death in the US, however, 4,600 people are hospitalized in the US with giardiasis each year. Hospitalized cases are primarily children under five years of age.[12]

Cryptosporidium



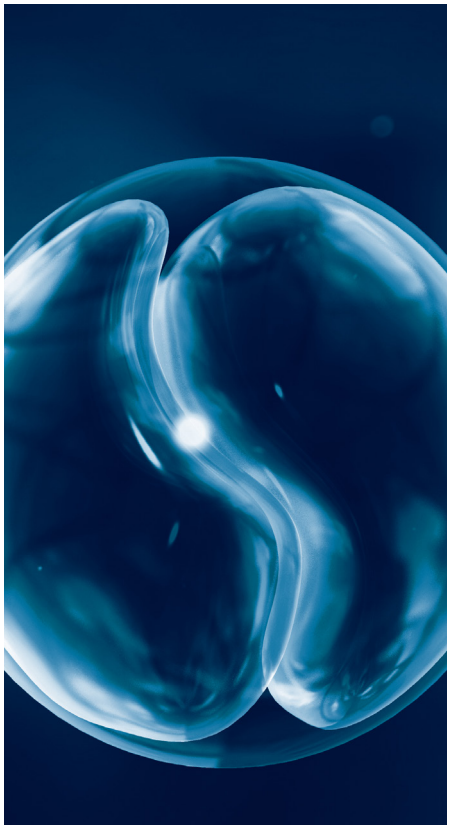
General information	At present, there are 26 different species of *Cryptosporidium*. The species most frequently identified in humans is *Cryptosporidium hominis*. It causes cryptosporidiosis.
Transmission	During the summer months, the number of diagnoses increases due to outbreaks associated with the use of recreational waters. The consumption of raw or undercooked foods or unpasteurized drinks has been linked to foodborne outbreaks.[11]
Symptoms	It is a major cause of moderate to severe diarrhea worldwide. Symptoms include abdominal pain, fever, vomiting, malabsorption, and self-limiting diarrhea.
Number of infections and deaths	There are >2.9 million cases of cryptosporidiosis in children aged <24 months in sub-Saharan Africa alone annually.[10] In the US, an estimated 748,000 cryptosporidiosis cases occur each year.[4]

E. histolytica



General information	*E. histolytica* is the causative agent of amebiasis. It is one of the few *Entamoeba* which infect humans.[6]
Transmission	Transmission occurs via the fecal–oral route, mainly via ingestion of food and water contaminated with amebic cysts.
Symptoms	Clinical symptoms of amebiasis range widely from asymptomatic to severe symptoms, including dysentery (bloody diarrhea) and extraintestinal abscesses.[10,13]
Number of infections and deaths	Amebiasis results in 100,000 human deaths annually on a global scale.[10] In the US, incidence of amebiasis is low and mostly seen in returning travelers or immigrants from developing countries. Severe cases can be associated with high case fatality. In the US, amebiasis-related deaths still occur occasionally, at around five deaths per year.[6]

Cyclospora



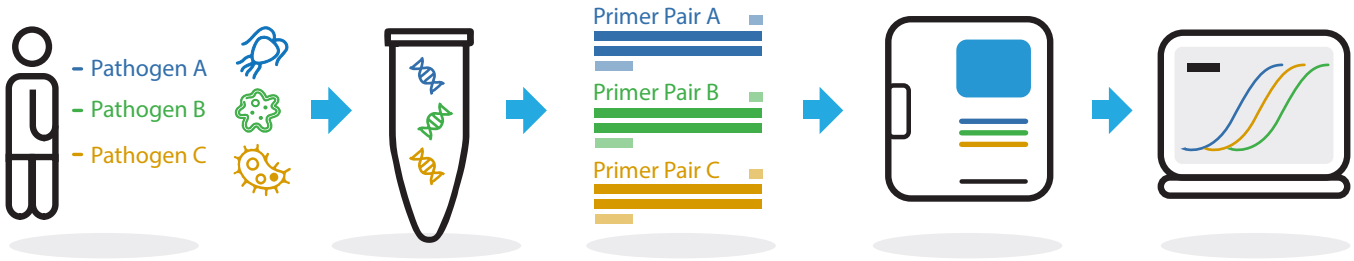
General information	*Cyclospora cayetanensis* causes cyclosporiasis. It is endemic in developing countries and associated with sporadic outbreaks in developed countries.[10]
Transmission	People can become infected with *Cyclospora* by consuming food or water contaminated with feces that contain the parasite.
Symptoms	Symptoms of cyclosporiasis include watery diarrhea, loss of appetite, weight loss and cramping.
Number of infections and deaths	People traveling in countries where *Cyclospora* is endemic may be at increased risk of infection. There are regular cyclosporiasis outbreaks in the US, mostly linked to infected food or drink. Recent outbreaks were linked to fresh basil, vegetable trays and bagged salad. The number of people infected with *Cyclospora* varies each year, depending on the number and extent of these outbreaks.[14]

3

Molecular O&P as a Screening Method for GI Protozoa

The field of molecular diagnostics in the clinical environment has gained momentum throughout the past decade, but has been further embraced since the SARS-CoV-2 pandemic. Molecular diagnostics involves the identification of a target organism by detecting the presence of the organisms' genetic code in the sample. PCR is generally used for the detection of infectious pathogens and is recognized as the gold-standard method due to its superior sensitivity and specificity over other methods. More so, the versatility of multiplex PCR enables multiple pathogens to be screened simultaneously, as outlined in Figure 3.

Figure 3. Overview of Multiplex PCR



Patient samples are collected which may contain more than one pathogen

Nucleic acids from both the patient and pathogens are extracted

PCR components include primers that bind specifically to pathogen target sequences of interest

A thermocycler enables amplification of target sequences, triggering fluorescent reading

Analysis shows positive or negative results

Multiplex PCR is achieved by obtaining patient samples such as saliva, blood or stool. The sample will contain nucleic acids from both the patient and any pathogens present, which undergo an extraction process in either a test-tube or multi-well PCR plate. In each test-tube or well, a mix of PCR components are combined with the nucleic acids, which include primer and probe molecules, specifically designed by the assay manufacturer. The primers enable the amplification of a single DNA sequence into millions of copies, through the repeated heating and cooling processes of thermocycling. Fluorescent probes, also specific to each target sequence, allow the amount of DNA present to be measured as it is amplified, and generates an amplification curve to distinguish between a positive or negative result.



Dr Marc R. Couturier, PhD
Medical Director, ARUP Laboratories

Dr Couturier is the Medical Director of Parasitology and Fecal Testing, Infectious Disease Rapid Testing, and Associate Director of Bacteriology at ARUP Laboratories. He is also a professor of pathology at the University of Utah. Dr Couturier received his PhD in medical microbiology and immunology with specialty in bacteriology from the University of Alberta in Edmonton, Alberta. He served as a Research Associate/Post-Doctoral Fellow at the Alberta Provincial Laboratory for Public Health, and completed a medical microbiology fellowship (ABMM) at the University of Utah.

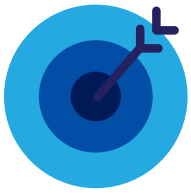
Through his career, he has developed molecular diagnostic methods for the detection of enteric pathogens and continues to pursue research in artificial intelligence for improved detection of parasites.

“For parasites, we were doing everything manually, everything with the same methods, everything done by British Naval doctors in the 1800s. To think that we hadn’t come very far in 200 years and as a community bemoaning how insensitive microscopy is and all the flaws with it and all the challenges, it just didn’t make sense that we wouldn’t try molecular. At ARUP, we embrace new technologies and try to modernize our tests and make them better. Once ordering physicians have used molecular for parasite screening, they become users. They are not going to go back to O&P. Unless it’s a scenario where they think the patient has schistosoma or a hookworm or something that isn’t targeted, then they would supplement with an O&P.”

Advantages of Molecular O&P for GI Protozoa

Dr Couturier explains that "we should be able to cut out 95% of O&Ps" if the resources and recommendations were in place for all ordering clinicians to request molecular testing. He adds that a more short-term result would be to "eliminate 30–40%" of traditional O&Ps.

> "Molecular panels would be used as a screen and only negative specimens with high suspicions would go for full O&P."



Higher sensitivity and specificity

A number of studies have compared assays in our *EasyScreen*™ Enteric Detection Kit range to traditional O&P examinations.[7,17,18] In a study with 358 samples, the *EasyScreen*™ Kit yielded 92–100% sensitivity (compared to 55–77% for traditional O&P) and 100% specificity (compared to 95–100% for traditional O&P).[17]



Ability to combine multiple targets in one multiplex assay

The *EasyScreen*™ Enteric Protozoan Detection Kit includes eight targets and enables screening of up to 95% of samples that would undergo traditional O&Ps for parasite investigations. This can allow rapid changes to the care pathway and allow prescribing physicians to shift from broad empiric therapy to targeted therapy.



Workflow automation

Molecular screening processes do not require the involvement of as many laboratory employees and can therefore solve skill-shortage challenges. *EasyScreen*™ allows for automation of the GI pathogen screening progress.

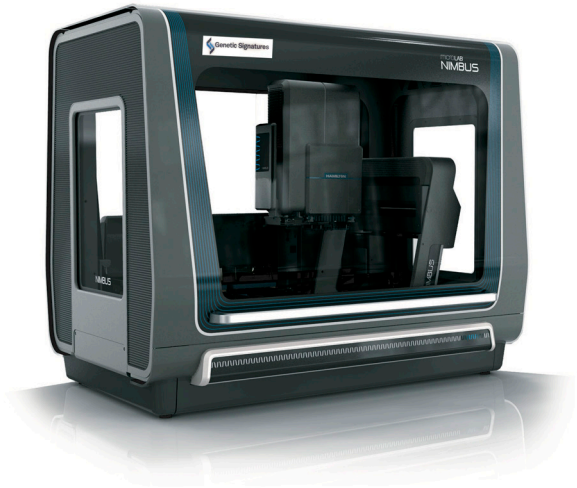


Quicker turnaround time

The *EasyScreen*™ Enteric Protozoan Detection Kit yields results within approximately 4.5 hours (Figure 4) compared to traditional O&P which can take up to four days or longer (Figure 5).

Genetic Signatures' *EasyScreen*™ Enteric Protozoan Detection Kit is currently for Investigational Use Only in the US. Contact us for more information on regulatory status of our products in your region. When using our products always read the label and follow the directions for use.

The GS1 Automated System

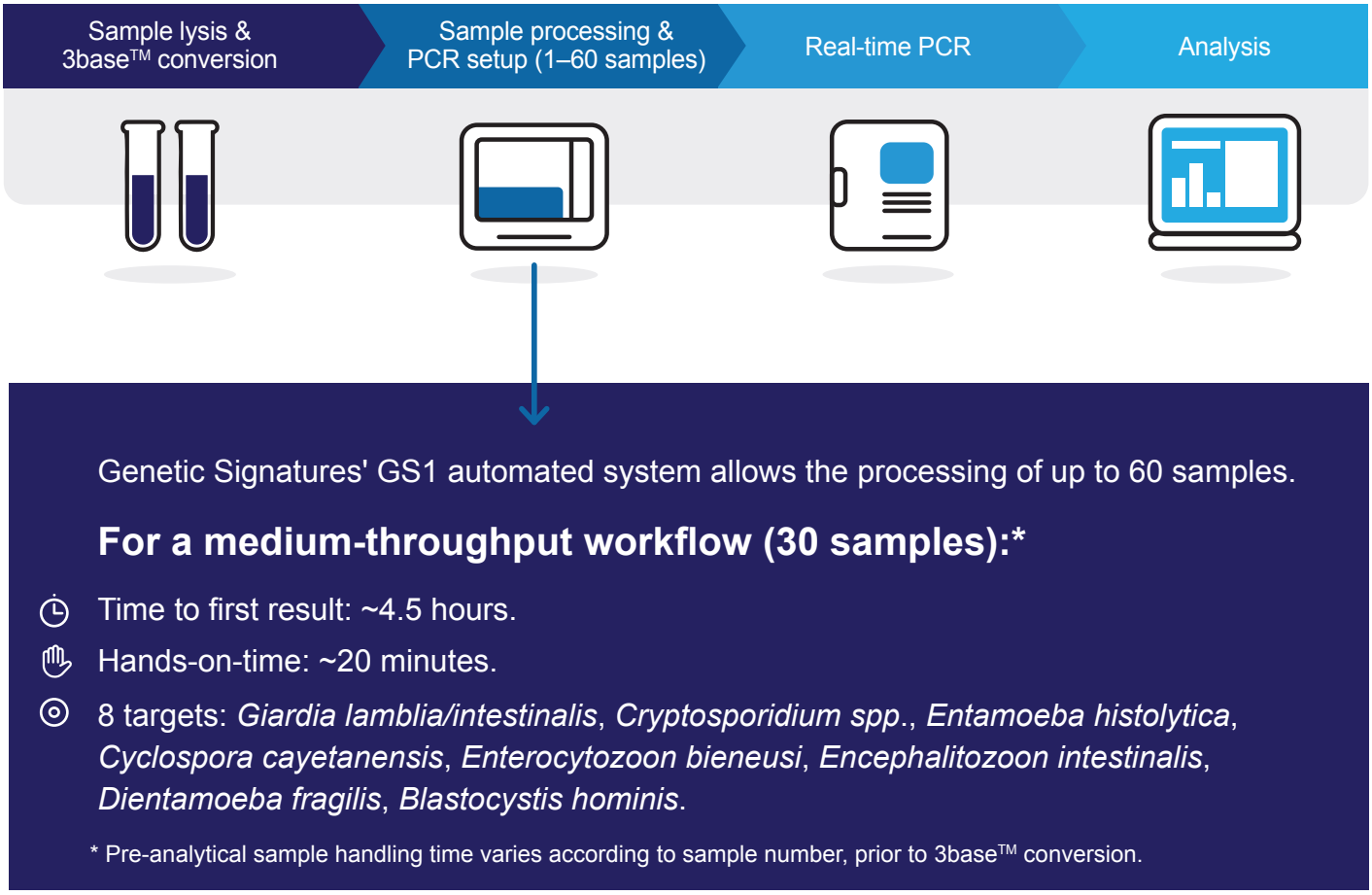


The *EasyScreen*™ Enteric Protozoan Detection Kit is compatible with Genetic Signatures' automated instrument, the GS1, providing a workflow solution that combines both nucleic acid extraction and PCR setup on a single system.

Advantages of the GS1:

- Performs sample extraction and PCR setup.
- Fast sample processing of up to 60 samples.
- 96-well PCR setup on one system.
- Significant walk-away-time and improved staff productivity.
- Wizard-driven interface enables on-board sample and reagent traceability, and supports minimal wastage.
- Automated data analysis with integration to Laboratory Information System (LIS).
- Small footprint allows improved use of space.

Figure 4. Workflow on the GS1 using the *EasyScreen*™ Enteric Protozoan Detection Kit



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4

Healthcare Benefits with Molecular O&P versus Traditional Methods

Microscopic O&P examination is the traditional method for detecting protozoan GI infection in many developed countries, including the US.

Currently, testing of fecal specimen requires ordering physicians to consider which specific pathogens might be associated with individual cases of diarrhea and then choose a testing scheme that ensures all the appropriate pathogens are targeted. Unsurprisingly, this approach often fails to yield positive results, due to the diverse array of pathogens associated with diarrhea and the diversity of testing methods. The prevalence and clinical impact of enteric parasites are underestimated due to a lack of adequate detection.

Additionally, the cause of infectious diarrhea is not determined in up to 80% of cases, which can result in the inappropriate use of antibiotics.[10] The appropriate use of antibiotics is a major emphasis in the US and better tools to assist antibiotic stewardship are greatly needed.

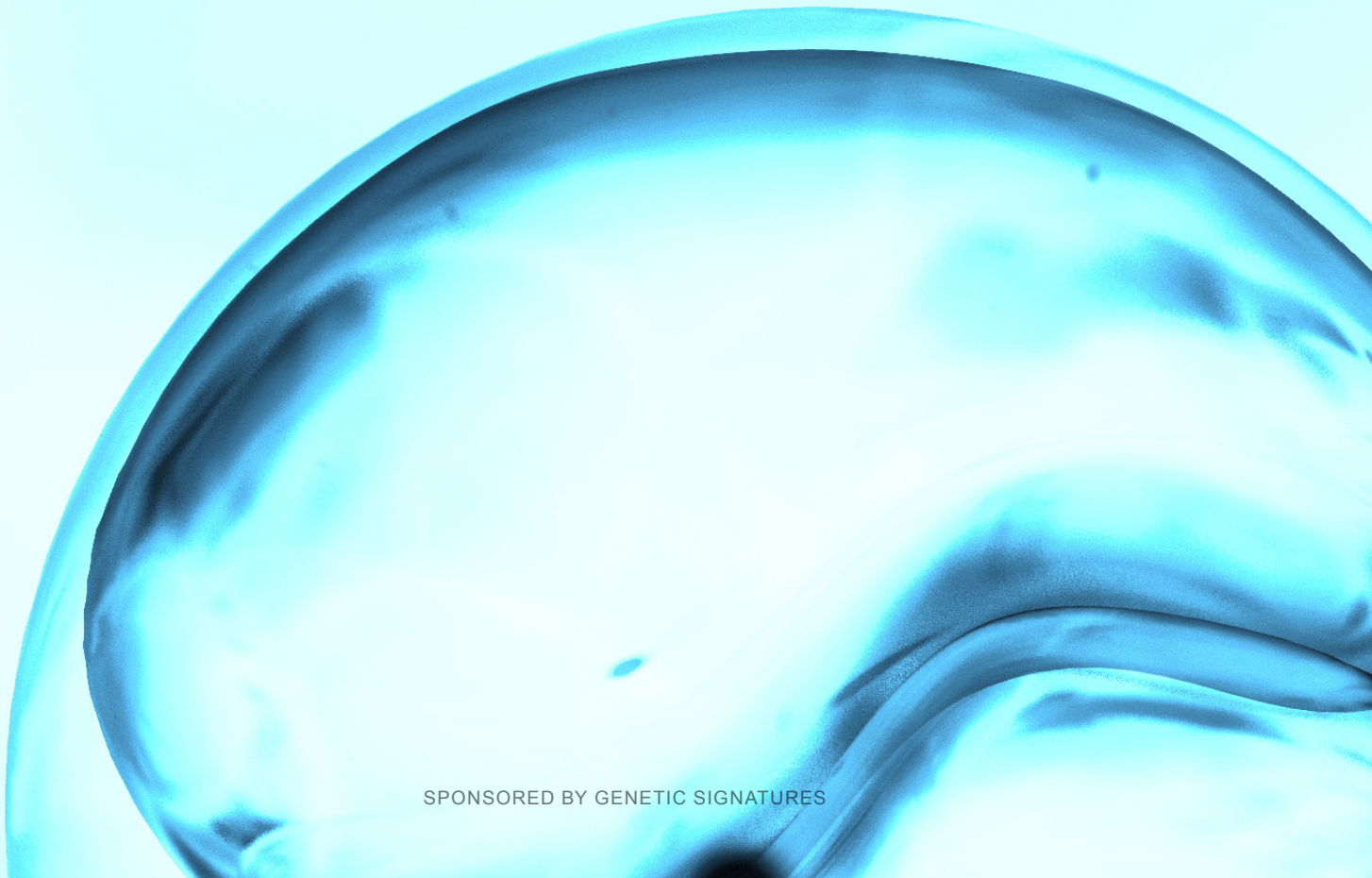
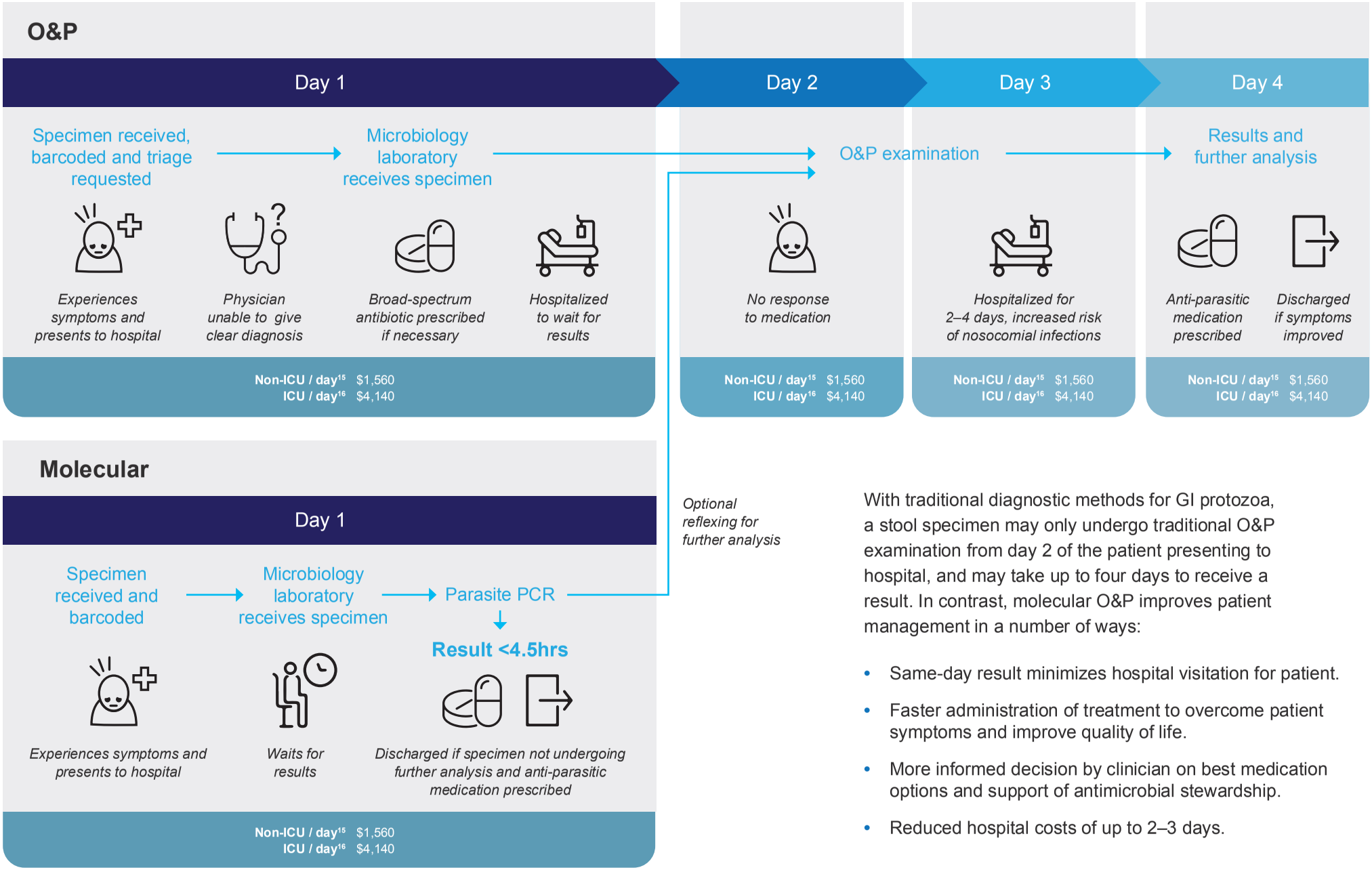


Figure 5. Improved Patient Journey with Molecular O&P



With traditional diagnostic methods for GI protozoa, a stool specimen may only undergo traditional O&P examination from day 2 of the patient presenting to hospital, and may take up to four days to receive a result. In contrast, molecular O&P improves patient management in a number of ways:

- Same-day result minimizes hospital visitation for patient.
- Faster administration of treatment to overcome patient symptoms and improve quality of life.
- More informed decision by clinician on best medication options and support of antimicrobial stewardship.
- Reduced hospital costs of up to 2–3 days.



Dr Glen Hansen, PhD
Medical Director, Microbiology & Molecular Diagnostics Laboratory, Hennepin County Medical Center

Dr Hansen is a microbiologist specializing in both medical microbiology and molecular diagnostics. He graduated with honors from The University of Saskatchewan, earning dual bachelor's degrees in Microbiology and Immunology, and completing a PhD in Clinical Microbiology. His post-doctoral education includes a Fellowship in Molecular Diagnostics, and in Medical Microbiology and Public Health Laboratory Microbiology. Dr Hansen is also a Fellow of the Canadian College of Microbiologists, and Diplomat of the American Board of Medical Microbiology.

Molecular testing methods offer significant value to busy laboratories, as they have an impact on workflow and efficiency.

"The greatest resource for laboratories is their workforce and when that workforce is in limited supply, molecular technology becomes a very attractive value proposition. To use that limited resource in areas that really impact patient care and using molecular power specifically to screen negative cases is a much more effective way of screening than using our limited resources to look at negative smears.

Molecular is going to impact patient management in three ways. Your results will come back quicker, they will potentially be more accurate and they will also help redirect the evolving differential diagnosis by providing both positive and negative results with accuracy and efficiency."

5

Clinical Evidence

There is an urgent need for testing that can broadly and specifically screen for a number of pathogens at once in the US, as GI protozoan parasites are a significant burden to the healthcare system.

Genetic Signatures is addressing the need for molecular O&P testing in the US with their GI protozoan testing solution, the *EasyScreen*™ Enteric Protozoan Detection Kit. FDA trials are in progress at important North American laboratories, including ARUP Laboratories (Utah), Indiana University, and TriCore Laboratories (New Mexico).

The *EasyScreen*™ Enteric Protozoan Detection Kit detects the pathogens listed below. The selected targets meet market demands for a broad and specific testing solution for the most clinically relevant GI protozoa in the US.

- *Giardia lamblia/intestinalis*
- *Cryptosporidium spp.*
- *Entamoeba histolytica*
- *Cyclospora cayetanensis*
- *Enterocytozoon bieneusi*
- *Encephalitozoon intestinalis*
- *Dientamoeba fragilis*
- *Blastocystis hominis*

To use the *EasyScreen*™ Enteric Protozoan Detection Kit, a portion of fixed or fresh stool specimen should undergo direct nucleic acid extraction using the *EasyScreen*™ Sample Processing Kit and GS1 automated extraction system (Genetic Signatures, US). Then, the *EasyScreen*™ PCR Detection Kit can be used to perform PCR on various thermocyclers.[17]

The team at ARUP Laboratories had the opportunity to trial the *EasyScreen*™ Enteric Protozoa Detection Kit and have shared their experience.

> We had everything possible to be able to support a clinical study with Genetic Signatures. It seemed like it would be something we'd love to get our hands on to see. And it was a win–win because they needed a clinical trial site that could do all this. And it gives us an early hands-on experience with the technology.

Dr Marc Couturier, PhD
Medical Director,
ARUP Laboratories

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Lexi Bracken
Research Scientist, ARUP Laboratories

Ms Bracken received a Medical Laboratory Scientist degree from Brigham Young University, after which she worked as a generalist at Central Valley Medical Center. She later completed her Master of Public Health at the University of Utah whilst working at ARUP Laboratories as a Medical Laboratory Scientist specializing in parasitology. Just prior to the COVID-19 pandemic, Ms Bracken was promoted to Research Coordinator which oversees the clinical trials related to infectious disease medical diagnostics.

“

One of the things I really liked about the *EasyScreen*™ method was the range of targets that it can detect. I've seen a lot of other molecular testing that has a narrower range of targets. So the fact that this one has eight was wonderful to me.

It was very easy to use as well. The computer setup has a very user-friendly format. And it's very thorough in telling you what to do as the operator so that if you don't have a lot of molecular background, which applied to me, you can still feel confident in what you are doing.

Some of our preliminary results have picked up possible *E. histolytica* a few times, which is something of a legend in the clinical lab.

”

Clinical Study Results

An earlier assay within our *EasyScreen*™ Enteric Detection Kit range has previously undergone clinical trials in Australia and Europe. This kit has TGA and CE-IVD registration, and includes five targets: *D. fragilis*, *Cryptosporidium spp.*, *B. hominis*, *E. histolytica* and *G. intestinalis*. Results of these trials are summarized below.



Australia

Researchers in Sydney screened 358 samples using *EasyScreen*™ and traditional microscopy.

- The *EasyScreen*™ Enteric Protozoan Detection Kit yielded 92–100% sensitivity, 100% specificity and detected all commonly found genotypes and subtypes of clinically-important human protozoan parasites.
- Microscopy yielded 55–75% sensitivity and returned a number of false-positives.[17]

Another study in Australia compared the *EasyScreen*™ Enteric Protozoan Detection Kit to conventional microscopy. A total of 81 samples previously tested for the presence of protozoa were screened.

- *EasyScreen*™ yielded 10 additional positive screens which had come back negative through microscopy.
- Co-infection occurred in 28.4% of samples tested using the *EasyScreen*™ method, but only in 8.6% of samples when assayed using conventional microscopy.[18]



Italy

A total of 632 fecal samples were submitted for routine screening **at two centers in Italy.** Molecular screening with *EasyScreen*™ was compared to conventional methods, including microscopy and immunoassays.

- Of 32 samples testing positive through microscopy and immunoassays, 31 were detected as concordantly positive using *EasyScreen*™.
- A total of 91 of 632 samples only tested positive with molecular testing, therefore increasing the positive detection rate by 275%.
- The *EasyScreen*™ assay detected 14 co-infections compared to three co-infections identified by conventional methods.
- *EasyScreen*™ provided a rapid and sensitive simultaneous identification of the most important diarrhea-causing protozoa that infect humans.[7]

TGA: Therapeutic Goods Administration (Australia)
CE-IVD: Conformité Européenne' (European Conformity)

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Genetic Signatures

Genetic Signatures uses its proprietary 3base™ technology to improve patient care and outcomes by developing innovative diagnostic technologies that simplify molecular pathology.

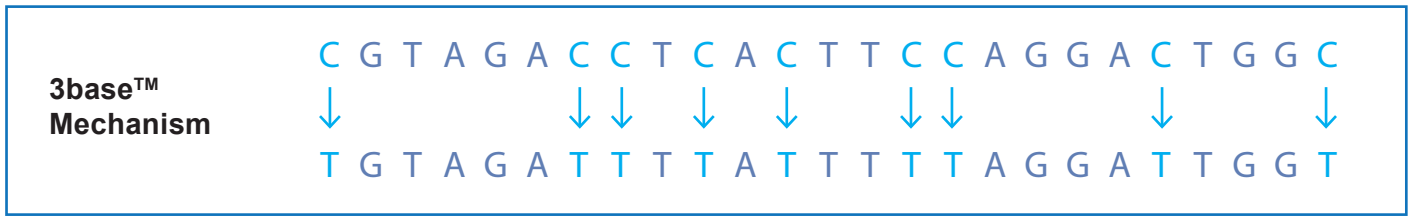


Genetic Signatures is a specialist molecular diagnostics company and has been providing advanced syndromic molecular testing solutions for infectious diseases for over 15 years. Genetic Signatures' proprietary 3base™ real-time PCR multiplex technology was pioneered by Dr Geoffrey Grigg, an acclaimed molecular biologist who conceived 'bisulphite conversion' as a method to detect methylated cytosines.[19] The potential of Dr Grigg's groundbreaking research for screening infectious pathogens was quickly recognized, with the commercialization and ongoing advancement of this technology, evolving into the Genetic Signatures *EasyScreen*™ Detection Kits sold today. These assays can detect groups of organisms across a broad range of infectious diseases, up to 20 at a time from one patient, significantly improving patient diagnosis and subsequent treatment solutions.[20]

Whilst all *EasyScreen*™ test kits are platform agnostic, Genetic Signatures offers a complete solution, including kits, automated instrumentation, service, and support. This allows hospitals and pathology laboratories to improve their operational efficiency and diagnostic workflow, ultimately having a positive impact on patient management and outcomes.

Genetic Signatures' *EasyScreen*™ products are accessible in over 30 countries, with direct representation in the US, United Kingdom, Germany, Australia and New Zealand, and distribution partners in Canada, Africa and other European nations.

Figure 6. Overview of the 3base™ Mechanism



With Genetic Signatures' 3base™ reagent, patient samples undergo '3base™ conversion' resulting in the change from cytosine (C) bases to thymine (T) bases prior to detection with PCR.

3base™ Technology

Methylation is a naturally occurring biological process in every organism that controls functions such as gene expression, cell differentiation and growth.[21] Methylation involves a chemical change to the cytosine (C) nucleotide base in DNA. Although this process can be normal, irregular patterns of cytosine methylation are well known to contribute to disease processes such as cancer due to negative mutational events.[22]

Methylation can be detected and quantified by a method of 'bisulphite conversion', a process where unmethylated cytosine bases are converted to a thymine (T) base but methylated cytosine (C) bases remain unchanged. Dr Grigg conceived the in vitro process of bisulphite conversion[17] and Genetic Signatures staff noticed that treating microbial DNA or RNA with sodium bisulphite creates a genome with three bases, rather than the naturally occurring four-base genome (Figure 6).[19] This discovery led to the foundation of Genetic Signatures' patented 3base™ technology.

3base™ works by converting C bases to T bases. This has many advantages for multiplex real-time PCR, including improving sequence similarity for more efficient detection of subtypes or possible variants present in a sample. Figure 7 shows the increase in sequence similarity between sequences of 10 different viral isolates from 75% ('before') to 95% ('after') via 3base™ conversion.

Figure 7. Improved Sequence Similarity with 3base™

Sequence	Before	After
Seq 1	G A T G G C G A T A T G G T T G A C A C	G A T G G T G A T A T G G T T G A T A T
Seq 2	G A T G G T G A C A T G G T A G A T A C	G A T G G T G A T A T G G T A G A T A T
Seq 3	G A T G G T G A T A T G G T G G A C A C	G A T G G T G A T A T G G T G G A T A T
Seq 4	G A T G G T G A T A T G G T A G A T A T	G A T G G T G A T A T G G T A G A T A T
Seq 5	G A T G G T G A T A T G G T G G A C A C	G A T G G T G A T A T G G T G G A T A T
Seq 6	G A T G G C G A C A T G G T T G A T A T	G A T G G T G A T A T G G T T G A T A T
Seq 7	G A T G G T G A T A T G G T G G A C A C	G A T G G T G A T A T G G T G G A T A T
Seq 8	G A T G G T G A C A T G G T A G A T A C	G A T G G T G A T A T G G T A G A T A T
Seq 9	G A T G G T G A T A T G G T A G A T A C	G A T G G T G A T A T G G T A G A T A T
Seq 10	G A T G G T G A T A T G G T G G A T A C	G A T G G T G A T A T G G T G G A T A T

Consensus

Sequence	G A T G G Y G A Y A T G G T D G A Y A Y	G A T G G T G A T A T G G T D G A T A T
	• 75% sequence similarity over 20 bases • 48 possible primer combinations	• 95% sequence similarity over 20 bases • 3 possible primer combinations

Figure 8 shows an example of conventional and 3base™ DNA sequences for two primers and two probes. The primers and probes used in 3base™ assays have a more similar melting temperature (Tm) which improves the efficiency of multiplex real-time PCR.

Figure 8. Uniform Melting Temperatures with 3base™

Conventional Sequence		Tm
Primer 1	G T A C A C A C C G C C C G T C G C T C C T A C C	77°C
Primer 2	G A A G G A G A A A G T C G T A A C A A G	56°C
Probe 1	T G A A T A A A G A G G T G A A A T T C T A G G	59°C
Probe 2	G A A G G G C C G C G A G C C C C C G C G C	87°C

3base™ Sequence		Tm
Primer 1	G T A T A T A T T G T T T G T T G T T T T T A T T	52°C
Primer 2	G A A G G A G A A A G T T G T A A T A A A G	50°C
Probe 1	T G A A T A A A G A G G T G A A A T T T T A G G	59°C
Probe 2	G A A G G G T T G T G A G T T T T T G T G T	62°C

Advantages of 3base™ for Infectious Disease Testing

- **More efficient multiplex assays:** Fewer primers, less competition and improved sensitivity and specificity
- **Detection of subtypes:** Due to increased sequence similarity, fewer primers can be used for the detection of known subtypes or gene families, providing screening capabilities for a wide number of clinically relevant targets
- **Immunity to genetic variation:** 3base™ conversion of C to T also provides 'immunity' to potential genetic variation that may arise at random in organisms and was demonstrated with Genetic Signatures' SARS-CoV-2 assay where 3base™ enabled immunity to both C>T and T>C mutations, with C>T being the most frequently occurring spontaneous mutation in nucleic acids[23,24]
- **Reduced G-C content:** Conversion of cytosine bases to thymine bases within target sequences contributes to improved PCR efficiency for organisms with high G-C content such as *giardia*, where inefficiencies with amplification have been reported using 4 base assays[25]
- **Uniform sample processing conditions:** 3base™ provides a universal extraction process regardless of sample type, and is compatible with both DNA and RNA from bacteria, protozoa, viruses and fungi
- **Reduced contamination:** 3base™ conversion of samples ensures 3base™ assays are not affected by environmental contamination by native 4 base sequences
- **Open platform:** Compatible with most automated sample preparation systems and PCR instrumentation



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Dr Stark holds a doctorate in Philosophy, Molecular and Clinical Parasitology from the University of Technology Sydney. Dr Stark is the Laboratory Manager for Microbiology, Molecular and Precision Medicine at St Vincent's Hospital Sydney, which oversees SARS-CoV-2 testing and the syndromic testing of respiratory, STI and gastroenteritis samples. He has a passion for tropical medicine involving parasitology and has been published in over a hundred leading scientific journals. He is a leading expert in parasitology, but his interests are with raising awareness of less well-known parasites that can be pathogenic to humans and are often underdiagnosed in many geographies around the world. These included *Leishmania*, *Microsporidia* and *Dientamoeba fragilis*.

"

Since using Genetic Signatures' molecular panel for GI infections, I am able to rationalize and streamline our workflow. That allows for significant staff savings and cost savings.

We routinely screen all of our patients for protozoan parasites, and we've picked up additional infections that would have been missed by not using molecular screening methods. I think there is a definite benefit to the patient. The only reason we would do microscopy now is if it's a specific request for something that is not on the molecular panel.

We have had positive feedback from the clinicians and the requesting practitioners. We have been providing them with quicker, more comprehensive and more sensitive results. The assay is very specific, so there are not going to be any false positives.

"

7

FAQ

GI protozoan parasite-related illnesses are lower in the US than in developing countries. Why invest extra money?

Although GI parasite infections are lower in prevalence compared to developing countries, traditional O&P testing takes far too long. Improvements in testing regimes can deliver more value to healthcare and hospital systems, Money invested in molecular O&P ultimately allows cost savings in hospital stays (as shown in Figure 5), and maximizes staff and laboratory efficiency.

Would 3base™ technology be less specific and less effective due to increased sequence similarity?

Since 3base™ involves the conversion of cytosine to thymine in the nucleic acids of a pathogen, the resulting strands of DNA (or RNA) no longer have their normal 'complementary' pairing, with each strand now containing its own genetic sequence.

3base™ sequences still allow the same specificity as a traditional four-base genome; all that is required is a slight increase in primer and probe length to obtain a unique sequence.

In addition, the increased sequence similarity across sub-species and variants can be advantageous for species identification, where a single pair of 3base™ primers can amplify multiple closely-related sub-species or variants. The 3base™ designs can provide increased immunity to genetic mutations/ viral variation, thus preserving the specificity, and minimising the need to constantly redesign assays. Genetic Signatures' assays have performed with equal sensitivity and specificity in external quality assurance programs compared to other traditional four-base molecular assays.

Is it expensive to set up a molecular O&P screening solution in my laboratory?

Genetic Signatures offers flexible options to adopt a molecular O&P screening solution. The company aims to enable laboratories to perform testing in house that would ordinarily send specimens away, improving cost-effectiveness, turnaround time and positively impacting patient management. The molecular O&P testing solution can often change the economics of testing from a being a cost burden to becoming income generating if implemented correctly.

Do I need to invest in specific processing kits and equipment to be able to use the Genetic Signatures solution?

Yes. However, Genetic Signatures offers flexible options to ensure customers of various needs and budget can access this technology and workflow. Customers are provided with a solution that offers plenty of flexibility and capacity for future growth needs.

How much would one *EasyScreen*™ Enteric Protozoan Detection Kit cost my laboratory?

Depending on the number of samples processed per day, the acquisition costs are flexible and dependent on the customer's situation. Each customer has specific diagnostic requirements, therefore, requiring a tailored solution to meet their needs. Compared to laboratories using traditional O&P testing, laboratories using molecular O&P solutions should be in a better economical position due to efficiency savings and the existing funding models.

My staff have been doing traditional O&P testing for years. What type of training do laboratory staff need to undergo to use this type of technology?

Genetic Signatures has a team of experienced technical personnel that specializes in this area. We provide all the training and ongoing support for the customer. 3base™ technology makes this a simple process. Customers who use the Genetic Signatures workflow praise the ease of use and simplicity of results compared to traditional O&P methods, and the subjectivity that comes with it.

Notes

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