qPCR amplification of human gastrointestinal samples

Abstract

DNA extraction is an important step prior to most qPCR experiments, however complex samples such as those collected from the gastrointestinal tract (GI) tend to have a high concentration of PCR inhibitors present, even after DNA extraction. Here we demonstrate the utility of the PrimeTime™ One-Step 4X Broad-Range Master Mix and direct amplification enhancer in overcoming this common challenge as well as improving fluorescence and providing earlier detection of key microbial targets in GI samples. The results presented below clearly show that the PrimeTime One-Step 4X Broad-Range Master Mix can reduce the impact of PCR inhibitors that were carried over in GI samples, post-extraction, relative to another commercial master mix tested in this study.

Introduction

Gastrointestinal (GI) infections can be caused by bacteria, fungus, and viruses in GI system. There are multiple approaches researchers use to identify the microbes linked to GI infections. Traditionally, microbial identification has occurred through culturing of a presumptive positive sample. However, sample culturing approaches require a considerable amount of time and can miss polymicrobial samples. The advancement of the genomic age has introduced numerous improvements to alternative identification approaches of microbial pathogens including the addition of qPCR and next-generation sequencing (NGS). While NGS provides a wealth of information, the turnaround time associated with sequencing can be days to weeks, costs considerably more than qPCR-based tests, and requires potentially time consuming bioinformatic analyses.

Relative to NGS and culture-based approaches, qPCR is advantageous when it comes to costs and turnaround time. In addition, qPCR allows for increased accuracy in discriminating between pathogens and availability to query multiple targets in a single reaction through multiplexing assays. One major challenge encountered when performing qPCR is PCR inhibitors. Reaction inhibitors are common within GI samples and even though extraction protocols have been proven to reduce the presence of inhibitors, they do not eliminate them from the final sample. This can result in lack of amplification of key targets and lead to incorrect conclusions when analyzing samples.

The PrimeTime One-Step 4x Broad-Range Master Mix has allowed researchers to overcome this important challenge by reducing the impact of PCR inhibitors in qPCR reactions. This Master Mix includes a direct amplification enhancer solution that helps to neutralize any remaining inhibitors carried over through the extraction process.

Figure 1. Basic workflow of GI associated testing from rectal swabs. Utilizing the PrimeTime One-Step Broad-Range Master Mix promotes more robust amplification resulting in greater PCR confidence.

Using a standard qPCR workflow (Figure 1), we illustrate the combined utility of the PrimeTime One-Step 4X Broad-Range Master Mix and direct amplification enhancer in mitigating the impacts of PCR inhibitors present in DNA extracted from GI samples. Relative to another commercially available master mix, the PrimeTime One-Step 4X Broad-Range Master Mix provided amplification of all microbial targets, earlier amplification, and higher endpoint fluorescence per target.

Methods

Synthetic DNA target controls

Sixteen common pathogenic GI microbes were selected (Table 1) and gBlocks™ Gene Fragments were designed to mimic the target amplicon with a five base spacer added to the 3′ and 5′ ends. A gBlocks Gene Fragment was also designed for human RNase P. All gBlocks Gene Fragments were diluted to a final concentration of 1 x 10^3 copies per reaction.

Table 1. GI pathogens targeted.

	Target
Group #1	*Clostridium difficile*
	Human adenovirus
	Salmonella spp.
	Shigella spp.
Group #2	Enteroinvasive *E. coli*
	Norovirus G1
	Norovirus G2
	Human rotavirus
Group #3	*Campylobacter* spp.
	Enterohemmoragic verotoxin *E. coli*
	Human astrovirus
	Human sapovirus
Group #4	*Cryptosporidium* spp.
	Entameoba histolytica
	Giardi lamblia
	Yersinia enterocolitica

Seven replicates of each group (Table 1) were run with either the PrimeTime One-Step 4X Broad-Range Master Mix or another commercially available master mix on a Thermo Fisher QuantStudio™ 7 Flex. The final primer concentrations were 200 nM (IDT) while the final probe concentrations were 100 nM (IDT). Cycling conditions were 25°C for 2 min, 53°C for 10 min, 85°C for 10 min, 95°C for 2 min followed by 46 cycles at 95° C for 3 sec then 60°C for 32 sec.

DNA extraction and amplification of GI samples

Human GI samples were collected and stored in Cary-Blair media before DNA extraction. Extractions were completed using the Roche MagNA Pure 96 instrument. qPCR experiments were run using extracted DNA (5 µL per reaction), primers/probes targeting 16 GI pathogens (Table 1) and the human *RNase P* gene as well as either the PrimeTime One-Step 4X Broad-Range Master Mix or another commercially available master mix. Reactions with the PrimeTime One-Step 4X Broad-Range Master Mix also contained 2 µL of the direct amplification enhancer.

A single replicate reaction for each master mix was run on a Thermo Fisher QuantStudio™ 7 Flex. The final primer concentrations were 200 nM (IDT) while the final probe concentrations were 100 nM (IDT). Cycling conditions were 25°C for 2 min, 53°C for 10 min, 85°C for 10 min, 95°C for 2 min followed by 46 cycles at 95° C for 3 sec then 60°C for 32 sec.

Results and discussion

qPCR amplification of synthetic controls

To establish a baseline for amplification performance, synthetic controls designed to mimic 16 different GI-associated pathogens (Table 1) were amplified with either the PrimeTime One-Step 4X Broad-Range Master Mix (Figure 2A) or a another commercially available master mix (Figure 2B). The samples amplified at a similar cycle threshold (C_t) value (data not shown) however, controls amplified with the PrimeTime One-Step 4X Broad-Range Master Mix showed greater signal amplitude when compared to the commercially available master mix (Figure 2A compared to Figure 2B).

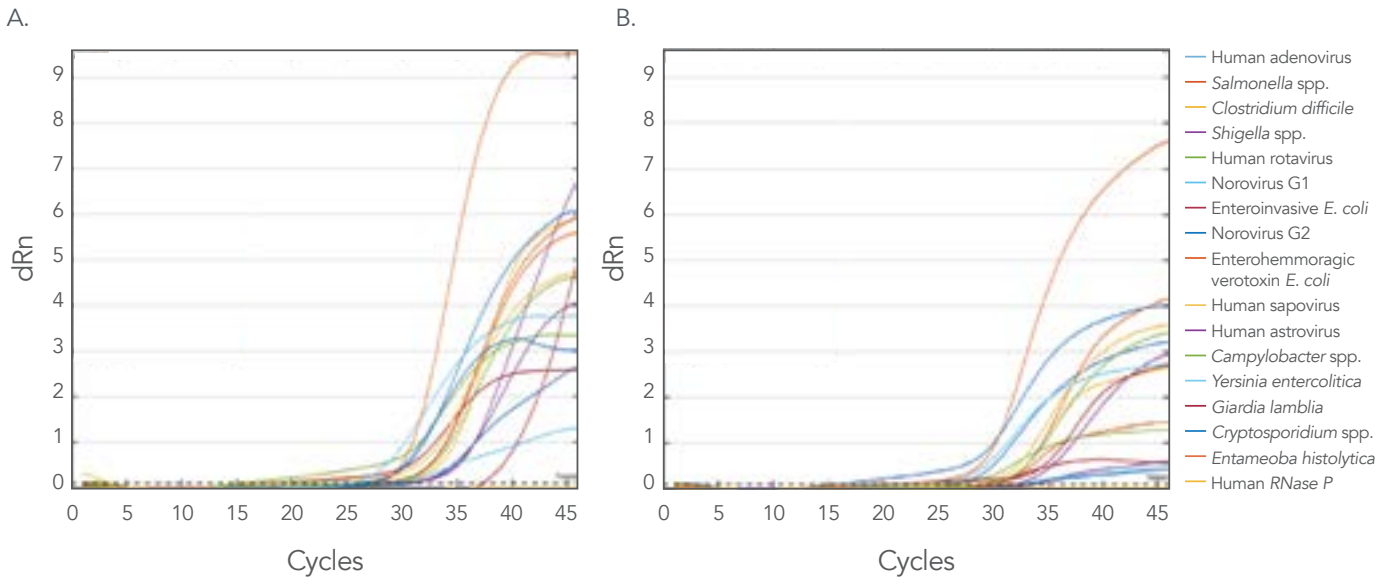

Figure 2. Amplification data for synthetic controls against different GI pathogens. gBlocks Gene Fragments were designed against the target amplicon and diluted to 1 x 10^3 copies per reaction. (**A**) Amplification of controls for GI-associated pathogens using the PrimeTime One-Step 4X Broad-Range Master Mix. (**B**) Amplification of controls for GI-associated pathogens using a commercially available Master Mix (n = 5). The figures are representative of one reaction.

qPCR amplification of GI samples

qPCR based GI testing workflows require sample collection by nucleic acid extraction to ensure clean DNA and removal of qPCR inhibitors. While extraction does reduce the presence of reaction inhibitors, it does not completely remove them, especially in inhibitor rich samples like those collected from the GI tract –i.e., stool and rectal swabs.

To determine the efficacy of the PrimeTime One-Step 4X Broad-Range Master Mix in overcoming PCR inhibitors in DNA extracted from GI samples, microbes previously associated with GI infections were targeted in GI samples and compared the results with another commercially available master mix.

While both master mixes worked well on the synthetic controls (Figure 2), only the PrimeTime One-Step 4X Broad-Range Master Mix in combination with the direct amplification enhancer amplified all 16 targets (Figure 3A). The other commercially available master mix only amplified 11 out of 16 microbial targets assayed in this experiment (Figure 3B). These differences suggest the presence of PCR inhibitors that were carried over from the DNA extraction and show the utility of the PrimeTime One-Step 4X Broad-Range Master Mix and direct amplification enhancer when preforming qPCR on inhibitor rich samples.

In addition to amplifying all the targets in the presence of PCR inhibitors, the PrimeTime One-Step 4X Broad-Range Master Mix also consistently generated higher fluorescence (Figure 3A,B) and earlier amplification (Figure 3C) than the other commercially available master mix.

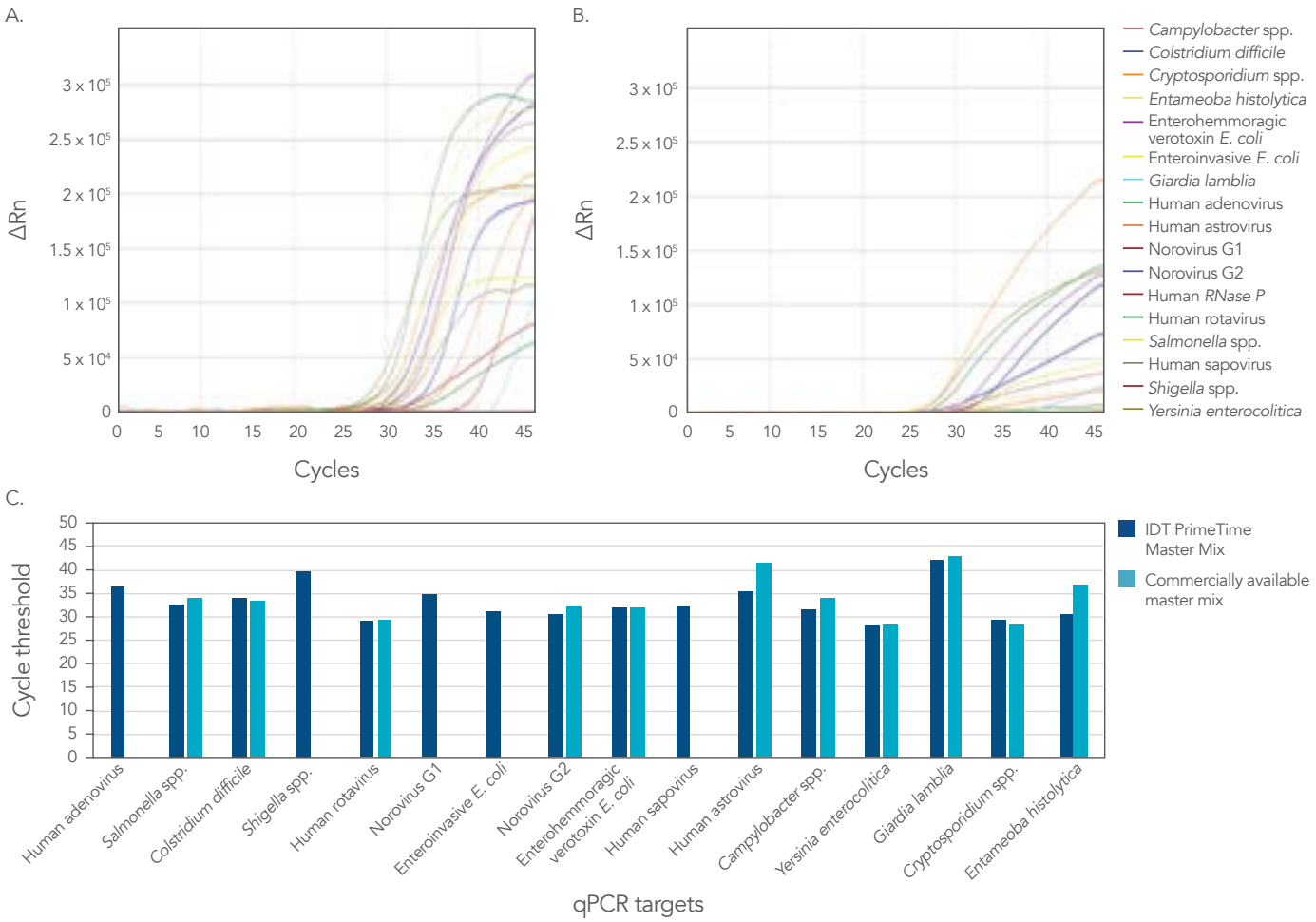

Figure 3. Amplification data for GI-associated targets. Pooled biological samples previously determined to be positive for *Campylobacter* spp., Enterohemmoragic verotoxin *E. coli*, human astrovirus, human rotavirus, *Yersinia enterocolitica*, *Colstridium difficile*, enteroinvasive *E. coli*, group 1 norovirus, *Salmonella* spp., *Cryptosporidium* spp., *Giardia lamblia*, group 2 norovirus, human sapovirus, *Entameoba histolytica*, human adenovirus, *Shigella* spp., and human *RNase P* amplified with the (**A**) PrimeTime One-Step 4X Broad-Range Master Mix with 2 µL per reaction of the direct amplification enhancer and (**B**) another commercially available master mix. (**C**) Amplification cycle threshold (C_t) values for GI-associated targets. Targets not showing amplification were set to C_t = 45, (n = 1).

Conclusions

Here we show that the use of the PrimeTime One-Step 4X Broad-Range Master Mix is an effective solution for qPCR amplification of DNA extracted from GI samples containing PCR inhibitors when targeting pathogens associated with GI infections.

When compared to another commercially available master mix the use of both the direct amplification enhancer and the PrimeTime One-Step 4X Broad-Range Master Mix resulted in:

- Amplification of all key microbial targets
- Earlier amplification of targets
- Higher endpoint fluorescence

All of these benefits translate to greater confidence when identifying GI pathogens in inhibitor-rich samples.

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