Development of Standardized Pathogen Detection Assays Using CleanAmp™ Master Mixes

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Abstract

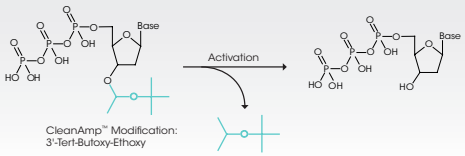
Acute gastroenteritis (AGE) affects over 179 million people and causes over 600,000 hospitalizations and 5000 deaths in the U.S. annually (CDC). Infectious AGE can be caused by a wide variety of different pathogens including bacteria, viruses and parasites. Rapid, definitive and economical identification of the causative AGE agent could inform medical decisions while reducing the inappropriate use of antibiotics. Standardized molecular methods and multiplexing will make pathogen identification more efficient and economical, with a huge potential global impact.

The molecular diagnostics field demands assays with more speed, specificity, and sensitivity. PCR is an established and reliable technology that meets these criteria. Although there are a myriad of published protocols using PCR for detection of specific diseases there is still a lack of established and standardized assays that cover the detection of certain infections.

CleanAmp™ dNTPs are a universal Hot Start technology that can be applied to many different types of PCR assays including Real-Time PCR, Multiplex PCR and RT-PCR. CleanAmp™ dNTPs are 2' deoxynucleotide triphosphates with a 3' thermolabile protecting group that prevents incorporation of the dNTPs at room temperature. As temperature is increased the protecting group begins to fall off creating a standard dNTP that can be naturally incorporated into the growing cDNA or PCR strand. CleanAmp™ dNTPs do not have a specific activation temperature but their kinetics are determined by a combination of heat, time and pH that can be optimized for each assay type. CleanAmp™ dNTPs have proved to increase amplification specificity and yield in a variety of assays.

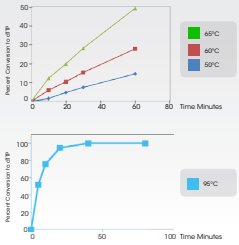
We will demonstrate the use of CleanAmp™ dNTPs in robust Multiplex Real-Time PCR and RT-PCR assays that are designed for screening and detection of pathogenic bacteria and viruses in patient stool samples. Primer sets were designed to consensus regions of several strains of each pathogen to maximize detection. Assays were optimized using positive control bacterial isolates and were then tested on DNA and RNA isolated from patient stool samples that may contain inhibitors. Using commercially available CleanAmp™ Master Mixes (CleanAmp™ 2x Multiplex Master Mix and CleanAmp™ 2x RT-PCR Master Mix) we aim to establish and standardize several assay panels for detection of the pathogens causing infectious diarrhea that will make diagnosis and treatment faster and more reliable

Figure 1: CleanAmp™ Hot Start dNTP Structure & Activation



Activation occurs with a temperature increase or pH decrease. CleanAmp™ dNTPs deprotect with a $T_{1/2}$ of 5 min at 95°C in PCR buffer.

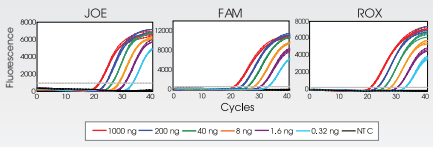
Figure 2: CleanAmp™ Deprotection Kinetics Allow for Application to a Wide Variety of Assays

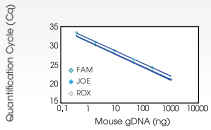


$T_{1/2}$ of 5 min @ 95°C in 1X PCR buffer

Applications for CleanAmp™
- PCR/qPCR
- RT-PCR/qRT-PCR
- Multiplex PCR
- Fast PCR
- GC-Rich PCR
- UNG Mediated Carry over assays

Figure 3: Efficient Fast Multiplex PCR with CleanAmp™ dNTPs





	Efficiency	R^2
FAM - 185 bp target	1.02	0.998
JOE- 125 bp target	1.02	0.998
ROX- 214 bp target	0.98	0.999

Figure 4: One-Step RT-PCR Specificity is Improved by Hot Start Activation of Both the RT and PCR Steps

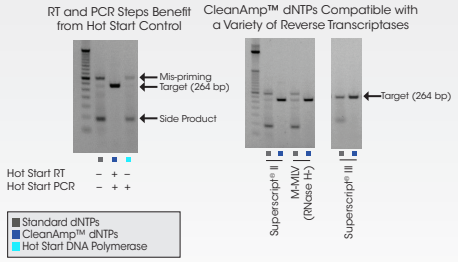
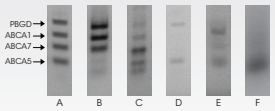


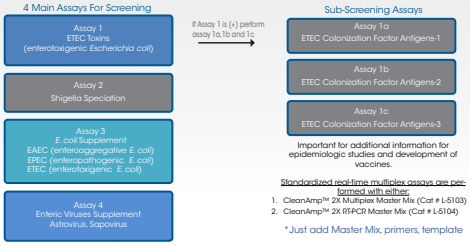
Figure 5: CleanAmp™ Enables Increased Accuracy and Consistent Results with Multiplexed One-Step RT-PCR



A CleanAmp™ One-Step RT-PCR Master Mix
B Path-ID™ Multiplex One-Step RT-PCR Kit (Life Technologies)
C Verso 1-Step RT-PCR Kit with Thermo-Start *Taq* (Thermo Scientific)
D Superscript® III One-Step RT-PCR System with Platinum *Taq* (Life Technologies)
E iTaq® Universal Probes One-Step Kit (Bio-rad)
F OneTaq® RT-PCR Kit (New England Biolabs)

Figure 6: Standardized Real-Time Multiplex Assay Panels for Detection of Pathogenic Bacteria and Viruses

Development of standardized real-time multiplex assays:
- Primer sets and hydrolysis probes were designed and tested for yield and specificity for individual target genes
- Optimal primers were combined for each assay and screened against well established bacterial reference strains positive for specific pathogens
- Clinical stool samples (previously validated) were screened for verification of positive pathogen detection
- All assays designed to include primers to detect an endogenous internal control (16S rRNA)



Important for additional information for epidemiologic studies and development of vaccines.

Standardized real-time multiplex assays are performed with either:
1. CleanAmp™ 2X Multiplex Master Mix (Cat # L-5103)
2. CleanAmp™ 2X RT-PCR Master Mix (Cat # L-5104)

* Just add Master Mix, primers, template

Figure 7: Real-Time Multiplex PCR Detection of Enterotoxigenic *E. coli*

Assay 1- ETEC Toxins		Sample Type: Bacterial Control Lysates	Stool Samples					NTC
Target Enterotoxin	Target Gene	ATCC 35401	USN50070	USN50113	USN50120	USN50123	USN50132	
STp (porcine heat stable enterotoxin)	estA1	+	+	+		+	+	
STh (human heat stable enterotoxin)	estA2-4	+			+		+	
LT (heat labile enterotoxin)	eltB1	+		+	+		+	
rrsA (16S rRNA)	rrsA	+	+	+	+	+	+	

+ Pathogen Detected.
Note: Multiple pathogens may contribute to infection and result in more than one positive per sample.

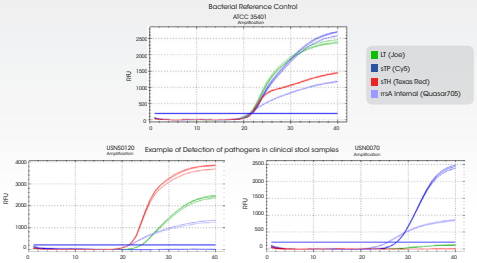


Figure 8: Real-Time Multiplex PCR Detection of Various Types of Pathogenic *E. coli*

Assay 3 - E. Coli Supplement		Sample Type & Number: Bacterial Control Lysates						Stool Samples						NTC
Type of E. coli	Target Gene	ATCC 43890	ATCC 43889	ATCC 43887	ATCC 35401	BAA 2326	ATCC 29552	70326	20379	10253	20444	20381	USNS 0129	
EPEC	bfpA			+										
EAEC	pCVD					+	+		+			+		
EPEC	eaeA	+	+	+				+		+	+		+	
ETEC (STh)	estA2-4				+								+	
rrsA (16S rRNA)	rrsA	+	+	+	+	+	+	+	+	+	+	+	+	

+ Pathogen Detected.
Note: Multiple pathogens may contribute to infection and result in more than one positive per sample.

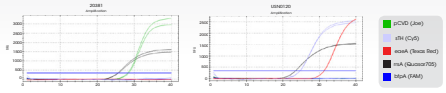


Figure 9: Pushing the Limit of Detection of *E. coli* Supplement Assay

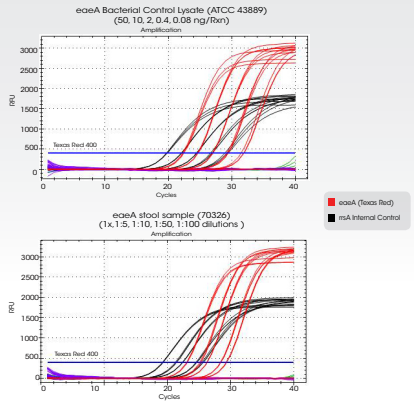
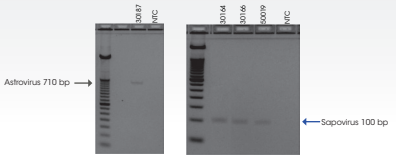


Figure 10: RT-PCR Detection of Enteric Viruses in Patient Stool Samples

Assay 4 – Enteric Viruses Supplement		Stool Sample Type and Number: Astrovirus	NTC	Sapovirus			NTC
Target Virus	Target Gene	30187		30164	30166	30019	
Astrovirus	Capsid	+					
Sapovirus	Capsid			+	+	+	

+ Pathogen Detected.



Conclusion

- CleanAmp™ deprotection kinetics allow for application to a wide variety of biological assays.
- CleanAmp™ 2X Multiplex Master Mix allows for efficient amplification of multiple targets simultaneously.
- CleanAmp™ 2X RT-PCR Master Mix improves specificity by Hot Start control at both the RT and PCR steps.
- CleanAmp™ 2X RT-PCR Master Mix efficiently amplifies multiple targets at once.
- ETEC Toxins Assay and *E. coli* Supplement Assay successfully identify positive pathogens using CleanAmp™ 2X Multiplex Master Mix.
- *E. coli* Supplement Assay has a low limit of detection with CleanAmp™ 2X Multiplex Master Mix.
- CleanAmp™ 2X RT-PCR Master Mix identifies positive pathogens by single-plex reactions for Enteric Viruses.

Acknowledgments

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Reaction conditions can be found at www.trilinkbiotech.com/posters

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