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Contents

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High Multiplex Molecular Diagnostics

Shifting the Diagnostics Paradigm

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Historically, the Polymerase Chain Reaction (PCR) has been quintessential to the development and growth of molecular biology and molecular diagnostics. PCR is a robust universal chemistry that enables detection of a wide range of low-level targets through the amplification of nucleic acid oligonucleotides. The chemistry has been essential for the success of the Human Genome Project; gene expression analysis that identified novel mutations and polymorphisms in diseases such as cancer; cloning of gene sequences used as therapeutic proteins; and the sensitive detection of pathogens through molecular In Vitro Diagnostics (1).

Molecular diagnostics are uniquely suited for highly selective and sensitive diagnosis of disease. Unfortunately, due to high costs and questionable performance, molecular diagnostics have been primarily restricted to confirmatory testing in response to standard clinical diagnosis in the clinic, limiting a potent resource for precision screening and early therapeutic intervention. By increasing quality and decreasing cost while taking advantage in the explosion of genomic-based information, a paradigm shift can occur in which molecular diagnostics are routinely used in systematic screening as well as syndrome-based diagnosis. The challenge – and the next step – for the molecular diagnostics industry is to develop robust multiplexed PCR-based diagnostics, with the goal of affordable identification of multiple pathogens, resistance mutations carried by pathogens, cancer-associated mutant genes, or host-related gene expressions associated with chronic diseases.

PCR is well positioned for multiplexing, in which the simultaneous amplification of multiple target nucleic acids enables detection of often low-level infectious agents, target gene mutations and gene expression events, even before phenotypic or pathological manifestations are evident. Multiplex diagnostic assays have already demonstrated their utility in clinical diagnostics as shown during the 2009 H1N1 influenza pandemic, their use of BCR-Abl genotyping for adult leukemia, and real-time tests to detect the causative pathogens of sepsis (2-4). Unfortunately, multiplex real-time PCR is also limited because of the complexity of multiplex assay design and instrumentation limitations. Proper design and development of multiplex molecular assays will be critical in the development of healthcare processes that are highly focused on the patient.

In nucleic acid-based testing, value depends on accurately determining low-level pathogens or mutations in samples that are limited in amount or difficult to obtain multiple times. Consequently, development of assays that can detect less than 100 copies per target is an extremely difficult but desirable goal. When applied to high multiplex reactions (Table 1), that challenge grows substantially, especially in relation to the design, development and validation of the diagnostic test.

Table 1. Benefits of High Multiplex Reactions

Feature	Multiplex Benefit
Multiple targets per reaction	Increases number of analytes per reaction
	Simplifies assay assembly
	Standardizes internal control across multiple analytes
	Convenient for screening
Cost Savings	Fewer reactions, reduced cost of reagents
Sample Conservation	Reduced sample requirements and conserves precious samples
Reliability	Minimizes number of points for pipetting errors for the same number of singleplex reactions

In recent years, the industry has made strides to address the growing challenge of PCR multiplexing, mainly through advances in instrumentation and detection platforms. However, very little in the area of PCR chemistry has been done to increase the ease of multiplexing.

Challenges

Real-time PCR currently is considered a gold standard for clinical molecular diagnostics because of its versatility, speed and ease of use. But those benefits come at a price. Oligonucleotide chemistry and design still present issues for both reaction kinetics and target specificity. In particular, target specificity is a major concern because of the false positives associated with cross-reacting targets. Furthermore, competition between real target and cross-reacting species, as well as potential primer-primer interactions, exhausts reaction components and reduces the assay's robustness, specificity and sensitivity (1).

In a reaction, temperature settings, target denaturation and reaction components all can be optimized for the single target. But in a multiplex reaction, these issues are compounded. Therefore, multiple primer pairs must be designed with high specificity for individual targets, while functioning under generic assay conditions. Table 2 outlines several requirements that must be addressed to successfully validate multiplex reactions. For a multiplex test at best one designs and optimizes to an average. As the complexity of the multiplexing increases the compromises in the design and optimal conditions continues and in most cases imparts dramatic changes in the performance of the assay.

Despite these challenges, instrumentation currently is viewed as the major limiting factor in real-time PCR multiplexing. Existing instrumentation typically is limited to four to five fluorescent channels with the common understanding of "one channel, one target". With that limitation, a maximum number of four to five primer pairs must be carefully designed to match annealing temperature and oligonucleotide size while minimizing secondary structures, complementary 3' ends, and cross-reactivity to non-specific targets. Likewise fluorescently-labeled probes must be designed to minimize primer-probe interactions, while maximizing probe-target interactions, quencher function and/or fluorescent probe interactions that result in the real-time readout.

Additionally, overall variability in the levels of the targets can result in preferential amplification of one target over another, as well as PCR drift (i.e., stochastic variation caused by low template concentration). Variability in the physicochemical characteristics of the amplified sequences, GC content, flanking regions and secondary structures also may add to the imbalance of the reaction and the subsequent outcomes.

Although predictive molecular modeling, bioinformatics and instrument performance have greatly enhanced the overall success of multiplex real-time PCR, the significance of the optimized reaction conditions cannot be overstated. Innovations in the chemistry, primer design and reaction conditions to circumvent the limiting issues are keys to leapfrogging incremental advances to take multiplexing PCR capabilities to the next level.

Table 2. Limitations of Current Approaches to Multiplexing

Assay Requirement	Potential Issues
Sample	Quality and quantity of sample – inhibitor carry over due to increased sample to increase sensitivity
Reaction Conditions	Mg^{2+} concentrations – optimal for both single and multiple analyte detection without change in performance
	Additional polymerase and NTPs required – increased cost
Oligonucleotide Design	Specificity to diagnostic target site, especially challenging for single nucleotide polymorphism (SNP) or mutational analysis
	Off-target cross-reactivity and non-specific amplification due to spurious primer interactions, and primer-primer/primer-probe interactions, leading to false positives
	Secondary structures, leading to both false negatives and false positives
Assay Performance	Decreased Sensitivity – interaction of oligonucleotides (primers and probes)
	Decrease Sensitivity for mixed analyte detection due to high concentrations of one analyte affect the sensitivity for other analytes
	Decreased Specificity through oligonucleotide (primers and probes) with off-target interactions

Addressing the Challenges with Innovation: DPO™ and TOCE

Two major advancements in the area of multiplex PCR, Dual-Priming Oligonucleotides (DPO™) and Tagging Oligonucleotide Cleavage and Extension (TOCE), enable multiplex assays on existing real time platforms that are currently found in most clinical laboratories. These new and emerging technologies are redefining high multiplexing by enabling "one channel, many targets".

DPO technology grew out of a need to enhance specificity and minimize the non-specific amplification common in multiplex PCR. DPO primers are structurally different from conventional primers and comprise two distinct annealing regions separated by a unique polydeoxyinosine linker (Figure 1). The structure formed by the Poly-dI linker controls the two-step primer binding event required for polymerase to extend a DPO primer. With a DPO primer, initial binding is through the 5'-target Stabilizing portion (Stabilizer) of the primer. The Stabilizer acts as any other primer because of its inherent thermodynamic properties. However, primer extension is controlled through the more specific annealing of the shorter 3'-Target Determinant portion of the primer (5).

This patented technology enhances target specificity, and is well-suited for multiplexing. By practically eliminating productive primer-primer interactions and non-specific binding and elongation of the primer, DPO technology optimizes the reaction conditions necessary for higher-order multiplex capability. In addition, the enhanced specificity exhibited by DPO primers extends the annealing temperature range, thus increasing the robustness of multiplex PCR reactions.

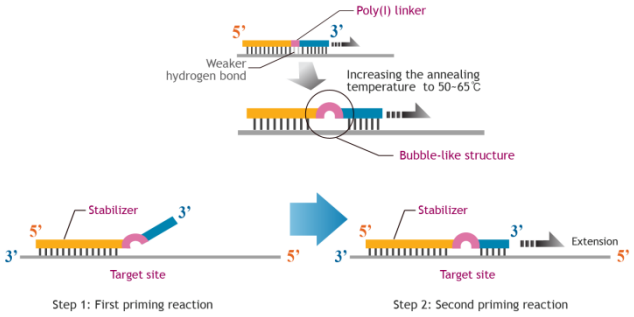

Figure 1. Dual Priming Oligonucleotide (DPO™) Technology

While DPO technology focuses on increasing the specificity, sensitivity and robustness of PCR, TOCE focuses on the readout of the real-time reaction, fostering dramatic improvements in sensitivity and specificity. TOCE overcomes the current limitations and fully exploit the potential of real-time PCR in high multiplex analysis, through signal generation and melting temperature analysis using an artificial template system instead of the current target-based probes that rely on variable and shifting target templates (6).

As illustrated in Figure 2, TOCE is a very elegant chemistry solution. TOCE introduces two novel components, the Pitcher and Catcher, to accomplish a unique signal generation in real time. In a TOCE reaction, the 5' nuclease activity specifically cleaves a target-bound Pitcher in such a manner that a designed Tagging portion is released. The released Tagging portion hybridizes to the Capturing portion of the Catcher. The Formation of the Duplex Catcher through Tagging portion extension and physical distancing of the quencher from the fluorescent moiety results in the fluorescent signal. What becomes apparent is the control over the melting temperature properties of the Catcher: by designing unique Catchers, the resulting Duplex Catcher will have a predictable and unique Tm profile. As a result, multiple Catchers with unique Tm profiles can be detected by Catcher Melting Temperature Analysis (CMTA), in the same reaction and in the same color channel. In a standard four-channel real-time thermocycler, that will amount to as many as 28

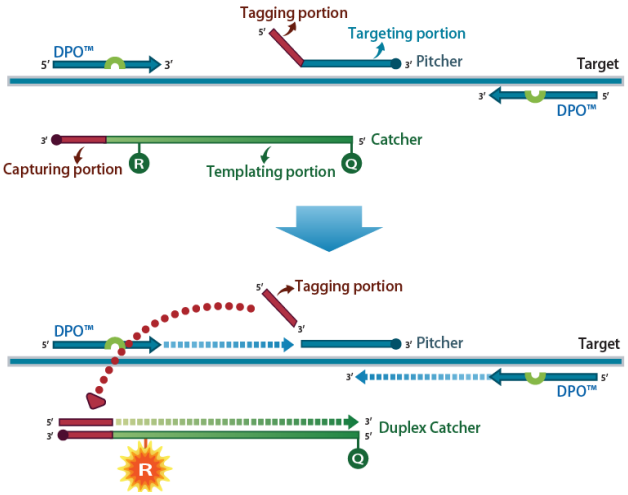

Figure 2. Tagging Oligonucleotide Cleavage and Extension (TOCE) Technology

different targets visualized in one reaction.

With the incorporation of DPO and TOCE chemistries into real-time PCR assay design and development, the industry will be able to overcome the design constraints inherent in current multiplex capabilities. TOCE is a major advancement in high-multiplex real-time PCR, allowing assay design that works with, and greatly expands the capabilities of, conventional real-time platforms. The control and ease of design of the Tagging portion and the Catcher, independent of the target sequences, facilitates flexibility in assay development and optimization. Therefore, the specificity and ease of use of TOCE will enable multiplex assay development across a wide range of applications and platforms, including quantitative real-time PCR and highly selective mutational analysis.

DPO and TOCE multiplex PCR assays combine the cost-effectiveness of identifying multiple analytes in a single reaction with the ease of development, increased specificity, enhanced sensitivity and high level of reproducibility necessary to validate assays for *in vitro* diagnostics (Table 3). These unique and emerging technologies, DPO and TOCE, are redefining multiplexing by fully exploiting the number of analytes that can be detected in a single reaction, using current, real-time instrumentation.

In the following papers, the power of TOCE to generate real-time results in multiplex assays with high specificity and sensitivity is presented. A particularly exciting development is multiplex quantitative assessment afforded by cyclic-Catcher Melting Temperature Analysis (cyclic-CMTA). This innovative application of TOCE combines the sensitivity of real-time PCR with the discriminating capabilities of each Catcher, resulting in quantitative analysis of multiple targets in the same channel. The combination of DPO and TOCE provides the capability to advance high multiplex quantitative real-time PCR into routine laboratory testing for a variety of applications, moving multiplex analysis forward from the "exception" to the "exceptional".

Table 3. Benefits of Seegene Technologies

Differentiator	Benefit
Flexible and Agile	**Rapid primer design** and assay development; minimal redesign required **Ease of development** due to robustness of the chemistry **Sensitivity** is not sacrificed for specificity **Built-in** quality check due to dual priming events being required for each primer
Multiplexing Capabilities	**Low spurious cross-reactivity** ensures that most acquired targets can be tested **Minimal to no oligonucleotide interactions** (primer dimers, complex secondary structures) simplify primer design and increases multiplexing capabilities
Analytical and Clinical Performance	**High Specificity** ensures a low-to-no False Negative Rate, increasing confidence in clinical determination **High Sensitivity** enables lower detection limits and ensures low-to-no False Positive Rate

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TOCE: Innovative Technology for High Multiplex Real-time PCR

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ABSTRACT

Real-time PCR offers many advantages, but equally many hurdles, to the development of multiplex diagnostic tools. To overcome the current limitations and fully exploit the potential of real-time PCR in high multiplex analysis, a new concept in high multiplex real-time PCR technology, Tagging Oligonucleotide Cleavage and Extension (TOCE) is proposed. TOCE enables the detection of multiple targets in a single fluorescence channel through melting temperature analysis (MTA) of an artificial template, the Catcher. Our data show that signal generation from the Catcher is proportional to the amplification of the target, but independent of the target sequence. Catchers with specific T_m profiles were designed, resulting in generation of Catcher Melting Temperature Analyses (CMTA) that were consistent and predictable. Furthermore, we show that detection of multiple virus samples was as specific and sensitive as detection of each virus separately. TOCE represents a new paradigm for real-time PCR assay development, greatly expanding the capabilities of conventional and widely-used real-time PCR platforms, as well as new instrumentation.

INTRODUCTION

The major advantage of real-time PCR is the development of homogeneous reactions, the amplification and detection of an analyte in real time (1, 2). As a result, homogeneous real-time PCR has rapidly become the key tool for molecular assay development, expanding into applications as diverse as *in vitro* diagnostics (IVDs), food safety testing and pharmacogenomics (3, 4). The sophisticated engineering of real-time PCR instrumentation, with 4-5 fluorescence detection channels, has expanded the capacity to detect multiple analytes in a homogeneous assay, increasing the information gathered per reaction with less sample input while enhancing overall assay reliability through addition of internal controls and standards (5, 6).

Despite the advances in multiplex real-time PCR, there are several drawbacks that have limited the use of existing instrumentation platforms. With the current "one channel, one target" PCR chemistry, conventional real-time thermal cyclers can only detect and discriminate five or fewer analytes, a major obstacle for the high multiplicity (7). Although melting analyses using fluorescent hybridization probes have been proposed as a solution for detection of multiple targets in a single channel (8-10), the number of detectable analytes is still restricted by limited choices with the target sequences (11, 12). Normal sequence variation and genetic drift will make it difficult to define target-based probe binding regions that result in consistent and reliable multiple T_m profiles (13-15).

To overcome the current limitations and fully exploit the potential of real-time PCR in high multiplex analysis, a new approach to high multiplex real-time PCR technology, Tagging Oligonucleotide Cleavage and Extension (TOCE) has been developed. TOCE is a new concept of melting temperature analysis using artificial template-based T_m instead of the current probe-based T_m. In the following analysis, the ability of TOCE technology to consistently produce the predicted melting temperature profile in both singleplex and multiplex assays, as well as to distinguish multiple targets in a single channel in a homogeneous real-time PCR reaction, was demonstrated.

RESULTS

TOCE is a novel approach to real-time PCR

As shown in Figure 1, two novel components, the "Pitcher" and the "Catcher", play a critical role in signal generation in the TOCE assay. The Pitcher is a single-stranded oligonucleotide composed of a Tagging portion adjacent to a Targeting portion that can specifically hybridizes to the target region of interest. The Tagging portion of the Pitcher is a unique sequence and does not hybridize to any target sequences. The Catcher is a dual-labeled, single-stranded artificial template composed of a Capturing portion (complementary to the Tagging portion) adjacent to a Templating portion (Figure 1).

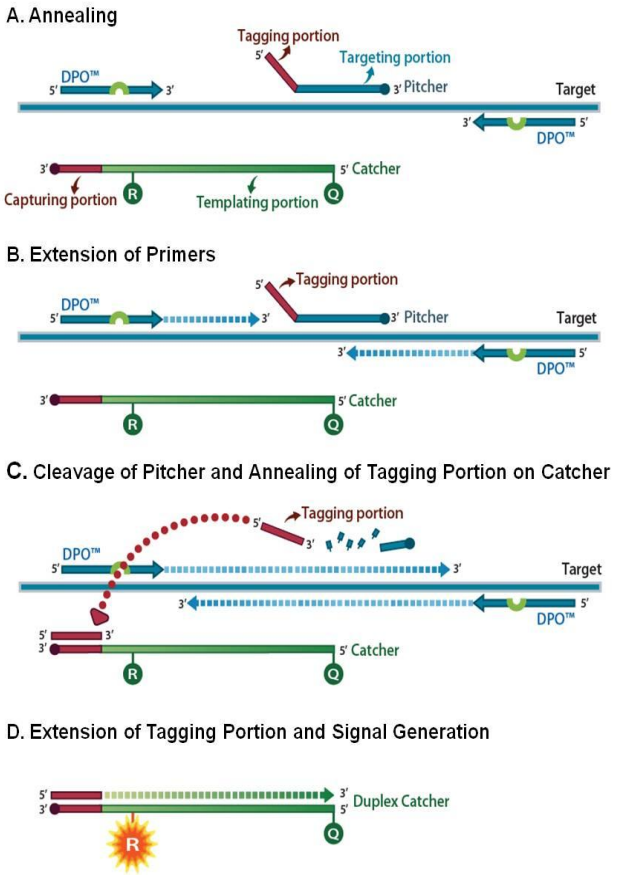


Figure 1. Schematic Diagram of Tagging Oligonucleotide Cleavage and Extension (TOCE) Assay. (A) TOCE assay is initiated with hybridization of upstream and downstream primers (DPO™) and Pitcher to the selected target sequence. **(B)** *Taq* polymerase-mediated extension of primers. **(C)** *Taq* polymerase having a 5' nuclease activity encounters the target-bound Pitcher, and cleaves the Pitcher, releasing the Tagging portion. The sequence of released Tagging portion is complementary to Capturing portion of the Catcher. **(D)** As the Tagging portion is fully extended on the Catcher to create the Duplex Catcher, quenching is diminished and the fluorescent signal can be detected.

As the PCR reaction initiates, the DPO™ primers (16) and the Pitcher hybridize to the specific sequence of the target region. During each PCR cycle, extension of upstream primer by *Taq* polymerase having 5' nuclease activity results in the cleavage of the target-bound Pitcher and release of the Tagging portion. The Tagging portion hybridizes to the Capturing portion of the Catcher. Extension of the Tagging portion to create the Duplex Catcher causes the physical distancing of the fluorescent reporter from the quencher, generating a signal (Figure 1). Therefore, generation of the signal in the TOCE assay is proportional to the amplification of the target, but independent of the target sequence. Because the sequence of the Catcher is defined during assay design, the resulting Duplex Catcher will yield a distinct and predictable melting profile in melting temperature analysis, referred to as Catcher Melting Temperature Analysis (CMTA).

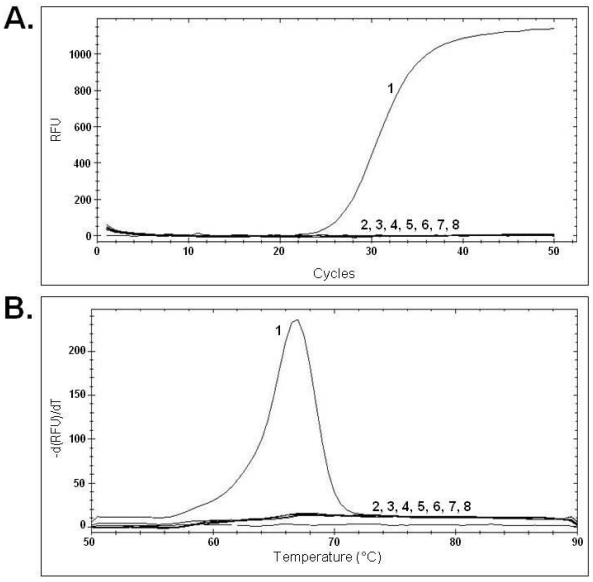
Signal generation is directly dependent on Pitcher cleavage and Duplex Catcher formation

The diagram of the TOCE assay would indicate that signal generation is directly dependent on the extension of Tagging portion on the Catcher, and not template amplification. To demonstrate the principle of TOCE assay, individual components of the reaction were systematically omitted or substituted. Results show that a fluorescent signal was generated only when all the components of the assay were present (Figure 2A; curve 1). CMTA yielded a single peak at a T_m of 67°C, consistent with the predicted melting temperature for the Duplex Catcher (Figure 2B; curve 1). As expected, 210-bp amplicon was generated (Figure 2C; lane 1). No fluorescent signal was produced in the absence of target amplification (Figure 2A and B, curve 2-4, and Figure 2C, lane 2-4). Strikingly, no signal was generated in the absence of Pitcher, nor in the presence of Pitcher without a Tagging portion (Figure 2A and B, curve 5 and 6), even though the predicted target amplicon was clearly visualized on agarose gel electrophoresis (Figure 2C, lane 5 and 6). Furthermore, no signal was generated in the absence of Catcher, or in the presence of a randomly-designed Catcher (Figure 2A, curve 7 and 8), despite synthesis of the amplicon (Figure 2C, lane 7 and 8).

Figure 2D summarizes the data, showing that the fluorescent signal generated in the TOCE reaction is directly dependent on cleavage of the Pitcher and Tagging portion-Catcher extension.

T_m profile is controlled by the engineered sequence of the Catcher

Because the observed T_m is equal to the predicted T_m of the Duplex Catcher, it should be possible to generate different CMTA profiles by simply modifying or lengthening the Catcher sequence (as illustrated in Figure 3A). To demonstrate this hypothesis, five different Catchers were designed, with T_m ranging from 61.5°C to 81.0°C respectively. Each Catcher contained the same Capturing portion complementary to the Tagging portion of a single Pitcher. Each Catcher was tested separately in an identical TOCE reaction with the same target template, DPO primer pair and Pitcher. As shown in Figure 3B, the same TOCE reaction yielded five different T_m profiles corresponding to the designed T_m of the input Catcher.



D.

No.	Template	Primers	Pitcher	Catcher	Results	
					C_t	Tm (°C)
1	+	+	+	+	27.27	67.0
2	-	+	+	+	N.D.	N.D.
3	Non-target	+	+	+	N.D.	N.D.
4	+	-	+	+	N.D.	N.D.
5	+	+	-	+	N.D.	N.D.
6	+	+	No Tagging	+	N.D.	N.D.
7	+	+	+	-	N.D.	N.D.
8	+	+	+	+**	N.D.	N.D.

**Catcher with non-complementary Capturing portion to Tagging portion
N.D. , Not Detected

Figure 2. Signal generation in TOCE assay is directly dependent on Pitcher cleavage and formation of the Duplex Catcher. A series of TOCE assays were assembled for detection of a *Neisseria gonorrhea* (NG) genomic sequence: (1) NG genomic DNA (gDNA) template, DPO primer set, Pitcher and Catcher; (2) without gDNA; (3) with non-target template (*Chlamydia trachomatis* gDNA) (4) without DPO primer set; (5) without Pitcher; (6) with Pitcher without the Tagging portion; (7) without Catcher; or (8) with Catcher non-complementary to the Pitcher. The results of the TOCE assay are shown as **(A)** Amplification curve analysis, **(B)** CMTA and **(C)** agarose gel electrophoresis to detect the 210-bp target amplicon. **(D)** Results for experiment are summarized in tabular form.

TOCE assay enables simultaneous detection of multiple targets in the same channel

A predictable CMTA for each Catcher introduces the possibility that multiple Catchers with controlled T_m profiles can be distinguished in the same channel of a multiplex TOCE assay. To evaluate this possibility, cDNAs generated from three different respiratory viruses, Respiratory Syncytial Virus A

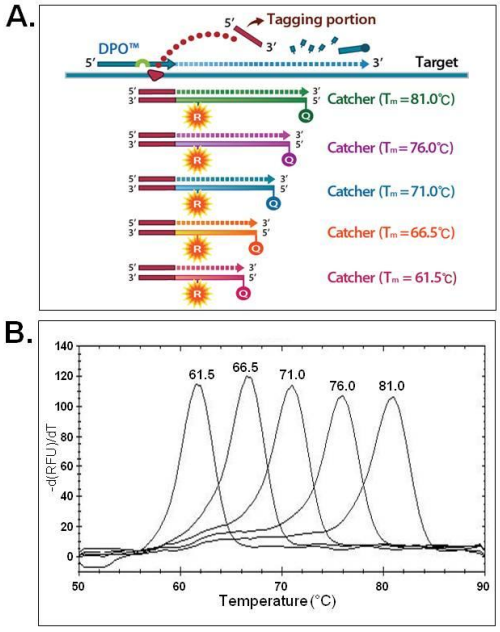


Figure 3. Controlled T_m profiles through designed sequence variations of the Catcher. **(A)** Illustration of the output T_m of the resulting Duplex Catcher by the controlled design of the Catcher sequence. **(B)** CMTA for 5 separate TOCE reactions. Five different Catchers with varying T_m ranging from 61.5°C to 81.0°C were designed by changing the sequence composition of the Templating portion only. The Capturing portion is the same for all Catchers. Each TOCE reaction contained one Catcher, as well as the NG gDNA template, DPO primer set and Pitcher.

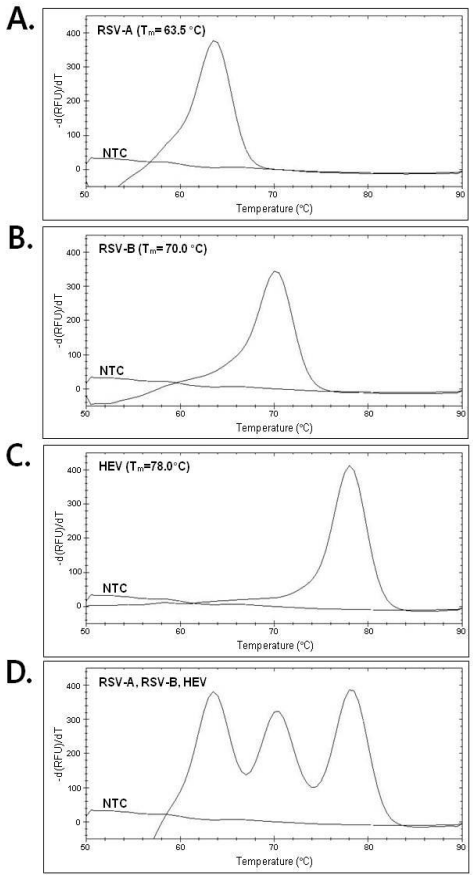


Figure 4. Multiplex assay for the detection of three different viruses in single channel. TOCE assays were performed with all components (primer sets, Pitchers and Catchers) necessary to simultaneously detect target template derived from three different viral genomes. **(A-C)** CMTA using a single target template derived from (A) Respiratory Syncytial Virus A [RSV-A]; (B) Respiratory Syncytial Virus B [RSV-B]; or (C) Human Enterovirus [HEV]. **(D)** CMTA for multiplex reaction using target template derived from all three viral genomes. NTC, No Template Control.

(RSV-A), Respiratory Syncytial Virus B (RSV-B), and Human Enterovirus (HEV), were used as templates. A master mix containing DPO primer pairs, Pitchers and Catchers required to detect all three viral target sequences was assembled. Multiplex real-time PCR was performed in the presence of a single viral cDNA (Figure 4A-C) or all three viral cDNAs (Figure 4D).

CMTA for each assay with a single cDNA as a template showed a single T_m profile corresponding to the designated Catcher: T_m of 63.5°C for RSV-A, T_m of 70.0°C for RSV-B, and T_m of 78.0°C HEV. When all three viral cDNAs were present in the multiplex TOCE assay, a three-peak T_m profile was observed that matched the predicted melting temperature for each Catcher (Figure 4). Therefore, the CMTA for multiple targets in the TOCE assay reflects the signal generated from each Catcher in the reaction. Furthermore, the signal intensity for the multi-target reaction was comparable to that of any single target reaction, suggesting that TOCE retains full sensitivity in the presence of multiple analytes.

DISCUSSION

This study introduces TOCE, an innovative technology that surpasses the current capabilities of real-time PCR. Currently, homogeneous T_m analysis is based on detection of dsDNA-specific intercalating dye(s) binding to target amplicon, or fluorescence from target-based probes (9, 10). All these techniques depend on direct interaction with target DNA and, as such, are hampered by ambiguity resulting from variable target sequences. This is particularly problematic in multiplex target-specific MTA, in which the "predicted" T_m of an amplicon or target-based probe may actually vary by several degrees in the melting profile (13-15).

TOCE bypasses the need to directly measure the target, employing an artificial template of defined and predictable T_m, the Catcher, to detect the presence of target. While signal generated in the TOCE reaction is proportional to the amplification of the target (Figure 2), the T_m of any given Catcher is controlled and invariant. That controlled T_m results in the discrete and predictable melting profiles shown in Figures 3 and 4. This is the first time that such an innovative strategy (i.e. the use of an artificial template to generate a multiplex MTA) has been successfully used to analyze multiple targets in one channel. As a result, multiple Catchers with unique Tm profiles can be detected by CMTA in the same reaction and in the same color channel, significantly increasing the number of targets that can be simultaneously detected in the conventional 4-channel real-time thermocycler.

The Catcher exchange experiment (Figure 3) demonstrates the design versatility of the Pitcher-Catcher detection system. Without changing any other component in the assay, the same target could be detected at five different T_m, simply by changing the sequence composition and/or length of the Templating portion of the Catcher. This controllable T_m property is a key to the ease of design of a TOCE assay. Catcher combinations are flexible, simplifying assay optimization through selection of Tagging portion and Catcher combinations to give

the desired T_m profile. Moreover, these combinations are interchangeable, so that Tagging portions or Catchers can easily be selected to minimize non-specific interactions.

The effectiveness of the TOCE optimization in multiplex assay design was demonstrated in the viral detection experiment (Figure 4), in which three different viruses were simultaneously detected by CMTA in a single channel. Furthermore, the detection sensitivity of all three viruses in the multiplex TOCE reaction was comparable to the sensitivity of each virus in a singleplex assay, confirming the robustness of TOCE. In a related experiment *(data not shown)*, the dynamic range and sensitivity of TOCE assay was shown to be comparable to a standard hydrolysis probe reaction, with a limit of detection of ~1 copy/reaction. Therefore, TOCE retains all the detection capabilities of standard target-based real-time PCR, but with the added advantage of detection of multiple targets in the same channel.

Dual Priming Oligonucleotide (DPO) primers are another component required for optimization of the TOCE assay. DPO primers function normally in PCR but increase specificity of target amplification while minimizing non-target interactions and primer-based artifacts (17). This is particularly important in high-multiplex TOCE assay, in order to prevent spurious oligonucleotide interactions that could generate non-specific signaling from the Catcher, or interfere with Tagging portion and Catcher interaction. Ultimately, the use of DPO primers, in combination with the flexibility of TOCE technology, contributes to exceptional ease of use in design and development of TOCE assays. This further distinguishes TOCE from other technologies that require highly sophisticated bioinformatics and extensive depth of target information to deliver specificity, but for a limited number of targets.

TOCE is a major advancement in high-multiplex real-time PCR capabilities, offering assay design that greatly expands the capabilities of conventional real-time instrumentation. In addition, TOCE should provide assay development solutions for next generation instrumentation platforms. TOCE will enable multiplex assay development across a wide range of applications, including quantitative real-time PCR and highly selective mutational analysis, providing a powerful new tool for high-multiplex molecular assay development in real time.

MATERIALS AND METHODS

TOCE assay

The TOCE reaction was conducted in the final volume of 20 μL containing 100 pg of *Neisseria gonorrhoeae* (NG) gDNA, 10 pmole of each primer, 5 pmole of Pitcher, 1 pmole of Catcher and 10 μL of 2X Master Mix containing 2.5 mM $MgCl_2$, 200 μM of dNTPs and 1.6 units of *Taq* DNA polymerase. In case of non-target template, *Chlamydia trachomatis* (CT) gDNA was used. The reaction mixture was denatured for 15 min at 95℃ and subjected to 50 cycles of 30 sec at 95℃, 60 sec at 55℃, 30 sec at 72℃ in the real-time thermocycler (CFX96, Bio-Rad). Fluorescent signal was observed at 55℃ during each cycle (Figure 2A). After the reaction, CMTA was performed by cooling the reaction mixture to 50℃, holding at 50℃ for 30 sec, and heating slowly at 50℃ to 90℃. The fluorescence was measured continuously during the temperature rise to monitor dissociation of Duplex Catcher.

Controlled T_m profile

Five unique Catchers with Capturing portion complementary to the Tagging portion of the Pitcher but with distinct T_m profiles were designed. The TOCE reaction was conducted in the final volume of 20 μL containing 100 pg of NG gDNA, 10 pmole of each primer, 5 pmole of Pitcher, 0.5 pmole of Catcher and 10 μL of 2X Master Mix containing 2.5 mM $MgCl_2$, 200 μM of dNTPs and 1.6 units of *Taq* DNA polymerase. The reaction mixture was denatured for 15 min at 95℃ and subjected to 40 cycles of 30 sec at 95℃, 60 sec at 60℃, 30 sec at 72℃ in the real-time thermocycler (CFX96). CMTA was performed as described above.

Multiple target detection in a single channel

cDNAs from RSV-A (ATCC VR-26), RSV-B (ATCC VR-955), and HEV (ATCC VR-784) were generated by a random hexamer reverse transcriptase reaction. The TOCE reaction was conducted in the final volume of 20 μL containing 2 μL of each cDNA, 10 pmole of each primer, each 5 pmole of Pitcher, each 1 pmole of Catcher and 10 μL of 2X Master Mix containing 2.5 mM $MgCl_2$, 200 μM of dNTPs and 1.6 units of *Taq* DNA polymerase in the real-time thermocycler (CFX96). The reaction mixture was denatured for 15 min at 95℃ and subjected to 50 cycles of 30 sec at 95℃, 60 sec at 60℃, 30 sec at 72℃. CMTA was performed as described above.

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cyclic-CMTA: An Innovative Concept in Multiplex Quantification

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ABSTRACT

In this study, we first introduce cyclic-Catcher Melting Temperature Analysis (cyclic-CMTA), an innovative technology to overcome the limitations inherent in endpoint melting temperature analysis. The key feature, repeating melting temperature analysis at discrete cycles during the TOCE reaction, was used to monitor the appearance of the melting profile, which is correlated with the amount of targets. cyclic-CMTA does not affect the quality of the melting profile for either single or multiple targets. cyclic-CMTA exhibited a wide dynamic range for qualification of multiple targets in the same channel. Furthermore, the technique was successfully demonstrated in a viral multi-target sample, where the concentration of three different viruses was unbalanced. Therefore, combining TOCE and cyclic-CMTA provides an innovative and powerful tool for reliable, multiplex quantification in real time that will be readily adaptable to a variety of diagnostic scenarios.

INTRODUCTION

The melting temperature analysis (MTA) in real-time PCR is typically done at the end of PCR to confirm the correct amplicon product (1). It also provides the multiplex detection capability using the target-specific melting temperatures in a single channel, thereby increasing the number of analysts in a single tube (2-6).

Despite the advantage of MTA, it is still challenging to quantify targets because the values are obtained after an amplification step is completed, so-called endpoint MTA. Since the melting peaks are measured at the plateau phase where PCR amplicons are saturated, it is difficult to discriminate the actual quantity of any one target. It can also give inconsistent results because the amplification efficiency is more likely to decrease during later cycles as unknown factors are accumulated, which causes sample-to-sample variation, especially for low level of target (7, 8). Due to the limitations of the current MTA, quantitative melting temperature techniques have never been undertaken, despite high demand in many fields of investigation such as basic research, clinical testing, environmental monitoring and bioterrorism surveillance.

Recently, our laboratory developed a unique multiplex real-time PCR technology, Tagging Oligonucleotide Cleavage and Extension (TOCE) using the Catcher Melting Temperature Analysis (CMTA) (9). Herein, we first report an innovative application of TOCE with repeated melting temperature analysis, named cyclic-CMTA. As the name implies, melting steps are incorporated at different cycles as the amplification progresses. As illustrated in Figure 1, CMTA can be monitored at pre-selected cycles (referred to as CMTA points); the CMTA point where the melting peak first appears represents an initial concentration of a target. For example, the melting peak of a high titer target appears at the first CMTA point, while that of an intermediate titer target appears at the second CMTA point and finally, the melting peak for a low titer target appears at the last CMTA point.

In this paper, we demonstrate the power of cyclic-CMTA to quantify multiple targets in TOCE assay.

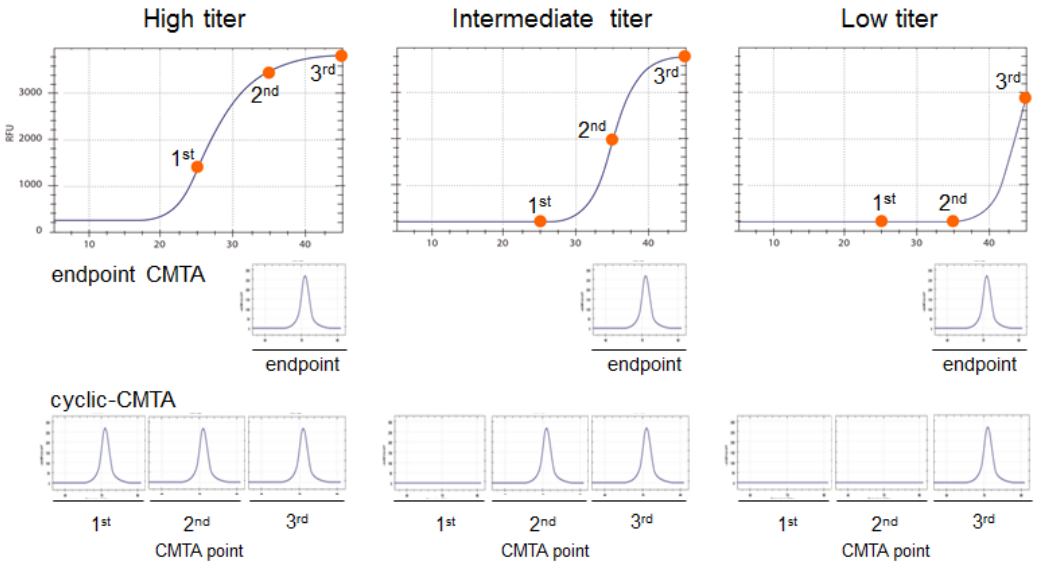


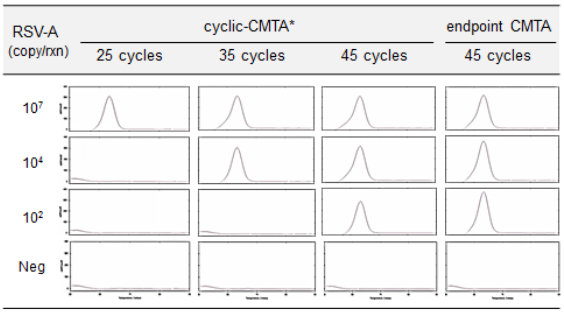
Figure 1. Process of cyclic-CMTA for quantification of target in TOCE assay. In the top panels, CMTA points, preselected cycles in amplification process when melting temperature analysis is perform, are indicated (●). The middle panels show the endpoint CMTA for each titer of the target. The bottom panels show the appearance of the melting peak for the three different titer of the target. The melting peak appears at the first CMTA point for the High titer, at the second CMTA point for the Intermediate titer, and at the third CMTA point for the Low titer.

RESULTS

Impact of cyclic-CMTA on assay performance and quantification

In order to assess i) whether the results of the melting peak profile are affected and ii) whether input concentration of a target can be discriminated when CMTA points are incorporated more than once, cyclic-CMTA was performed with three different dilutions of RSV-A ranging from 10^7 to 10^2 copies. CMTA was repeated three times after cycles 25, 35 and 45, and compared to endpoint CMTA. Qualitatively, the melting profiles at the last cyclic-CMTA point were nearly identical to the melting profiles obtained at the endpoint CMTA, with superimposable melting peaks regardless of the amount of RSV-A (Figure 2). These results show that cyclic-CMTA does not affect the performance of TOCE assay. Furthermore, the quantitative capability of cyclic-CMTA showed that the melting profiles for 10^7 and 10^4 copies of RSV-A appeared at the first (cycle 25) and the second CMTA point (cycle 35), respectively, while the melting profile of 10^2 copies appeared only at the last CMTA point (cycle 45) (Figure 2). Taken these results together, this indicates that the appearance of the melting peak quantitatively correlated to the copy number of a target.

A.



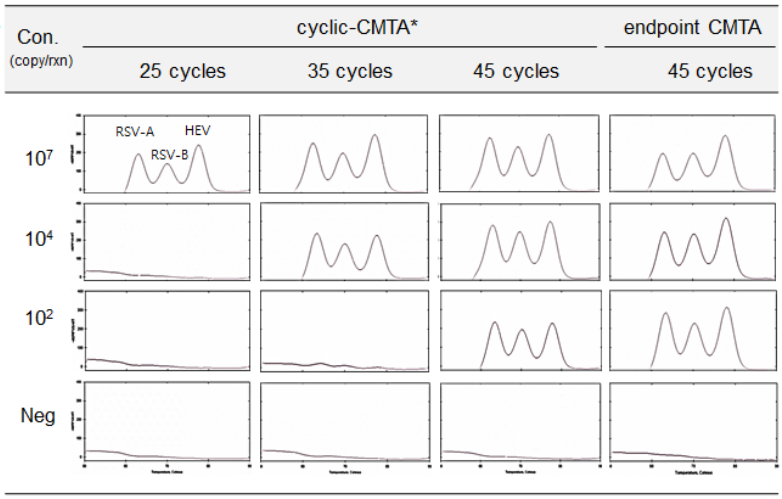
B.

RSV-A (copy/rxn)	cyclic-CMTA*			endpoint CMTA
	25 cycles	35 cycles	45 cycles	45 cycles
10^7	+	+	+	+
10^4	ND	+	+	+
10^2	ND	ND	+	+
Neg	ND	ND	ND	ND

* Cycles 25, 35 and 45 are 1st, 2nd and last CMTA point, respectively.

Figure 2. Comparison of cyclic-CMTA to endpoint CMTA for a RSV-A quantification and the correlation of concentration to the appearance of the melting peak at various CMTA points. A. A series of TOCE assays were assembled for detection of serially diluted RSV-A. cyclic-CMTA was performed at pre-selected CMTA points (cycles 25, 35 and 45), and at the end of the PCR amplification program (endpoint CMTA). No melting peak was observed for the negative control (Neg) **B**. Table summarizing the results of cyclic-CMTA. + : detected; ND : not detected; Neg : negative control.

A.



B.

Con. (copy/rxn)	cyclic-CMTA*									endpoint CMTA		
	25 cycles			35 cycles			45 cycles			45 cycles		
	RSV-A	RSV-B	HEV	RSV-A	RSV-B	HEV	RSV-A	RSV-B	HEV	RSV-A	RSV-B	HEV
10^7	+	+	+	+	+	+	+	+	+	+	+	+
10^4	ND	ND	ND	+	+	+	+	+	+	+	+	+
10^2	ND	ND	ND	ND	ND	ND	+	+	+	+	+	+
Neg	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

* Cycles 25, 35 and 45 are 1st, 2nd and last CMTA point, respectively.

Figure 3. Quantification of multiple targets in a single channel by cyclic-CMTA. A. A series of TOCE assays were assembled in which three different viral templates (RSV-A, RSV-B and HEV) were added in equal concentration, as indicated. cyclic-CMTA was performed at pre-selected CMTA points (cycles 25, 35 and 45), and at the end of the PCR amplification program (endpoint CMTA). Three different peaks were observed in the same channel corresponding to the Catcher Tm for RSV-A (63.5℃), RSV-B (70.0℃) and HEV (78.0℃). No melting peak was observed for the negative control (Neg). **B.** Table summarizing the result of cyclic-CMTA. + : detected; ND: not detected; Neg : negative control.

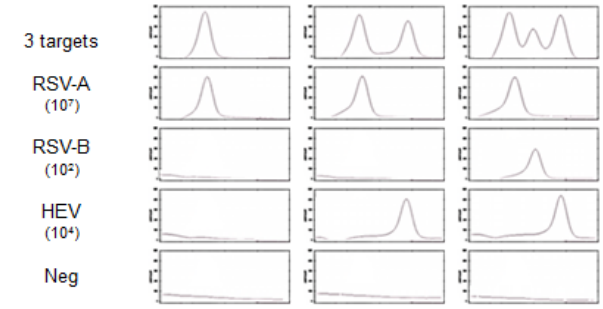
Utilization of cyclic-CMTA for multiple quantification

To examine whether cyclic-CMTA enables quantification of multiple targets in the same channel, combinations of RSV-A, RSV-B and HEV at same concentration of $10^{7,}$ 10^4 and 10^2 copies per reaction were used. Similar to the results of single target cyclic-CMTA, additional CMTA did not affect the performance of a multiple target assay when compared to endpoint CMTA. Also, the points at which the melting peak appeared were dependent on the input copies of three different targets (Figure 3). The CMTA point at which the melting peaks appeared corresponded to the initial viral load, with viral titer of 10^7 appearing at the first CMTA point (cycle 25), 10^4 at the second CMTA point (cycle 35) and 10^2 at the last CMTA point (cycle 45) (Figure 3). Moreover, neither increase in nonspecific signal nor decrease in sensitivity was observed at any concentration. These results indicate that cyclic-CMTA can be used with TOCE assay to quantify multiple targets reliably and consistently.

The sensitivity of multiple target detection in cyclic-CMTA

Using current multiplex formats, preferential amplification of a high concentration target is a common phenomenon, which makes detection of a low concentration target problematic (10). To examine if cyclic-CMTA can quantify all targets in a multiplex assay in which one target concentration is much greater than the others, a mixture of varying amount of RSV-A, RSV-B and HEV was used. The results show that the melting profiles of the mixture were consistent with that of individual target (Figure 4). RSV-A with the highest titer (10^7 copies) in this mixture appeared at the first CMTA point, which was consistent with the melting profile of 10^7 copies of

A.



B.

Target (copy/rxn)	cyclic-CMTA*								
	25 cycles			35 cycles			45 cycles		
	RSV-A	RSV-B	HEV	RSV-A	RSV-B	HEV	RSV-A	RSV-B	HEV
RSV-A (10^7) RSV-B (10^2) HEV (10^4)	+	ND	ND	+	ND	+	+	+	+
RSV-A (10^7)	+	ND	ND	+	ND	ND	+	ND	ND
RSV-B (10^2)	ND	ND	ND	ND	ND	ND	ND	+	ND
HEV (10^4)	ND	ND	ND	ND	ND	+	ND	ND	+
Neg	ND	ND	ND	ND	ND	ND	ND	ND	ND

* Cycles 25, 35 and 45 are 1st, 2nd and last CMTA point, respectively.

Figure 4. Quantification of multiple targets with varying amounts of three different viral templates in a single channel by cyclic-CMTA. A. A series of TOCE assays were assembled with unequal amounts of three different viral templates (RSV-A, RSV-B and HEV) either as a mixture (3 targets) or individually, as indicated. Three different melting peaks corresponding to the Catcher Tm for RSV-A (63.5℃), RSV-B (70.0℃) and HEV (78.0℃) were observed in the same channel. The appearance of each melting peak at various CMTA points corresponds to the concentration of each viral template. No melting peak was observed for the negative control (Neg). **B.** Table summarizing the result of cyclic-CMTA. + : detected; ND: not detected; Neg : negative control.

RSV-A alone. Similarly, HEV (10^4 copies) and RSV-B (10^2 copies) appeared at the second and last CMTA point, respectively, the same CMTA points at which they were measured individually (Figure 4). These results demonstrate that the dominant target does not interfere with detection of other targets, even across 5 orders of magnitude, and thus can be utilized to quantify multiple targets simultaneously.

DISCUSSION

In this report, cyclic-CMTA, a novel and innovative application of TOCE technology, has been shown to be an effective quantitation tool for multiplex real-time PCR. Using cyclic-CMTA, we demonstrated for the first time that repeated melting temperature analyses can be used not only to detect a target, but to quantify it. Furthermore, cyclic-CMTA can be used to quantify multiple targets in the same channel.

cyclic-CMTA has the capability to detect melting peaks in complete concordance with that of endpoint CMTA, which eliminates the concern that cyclic-CMTA may induce a decrease of performance (i.e. quality of melting profile).

Recent reports have touted the multiplex quantification in real-time PCR; however, their results are still restricted to "one channel, one target" based on Threshold Cycle (C_t) for each target (11-16). Therefore, cyclic-CMTA is the first successful demonstration of multiplex quantification in a single channel.

As such, this technology surpasses standard endpoint MTA technique in multi-target detection. While both techniques provide confirmation of target amplification, only cyclic-CMTA enables quantification of all targets in the assay, thus greatly expanding the capabilities of current real-time PCR instrumentation.

A major challenge for current real-time PCR is discrimination of multiple targets simultaneously when one target is dominant. However, cyclic-CMTA was able to identify and quantify every target in the sample accurately, even when one target is present in significantly higher concentration (Figure 4). This experiment more precisely reflects the "real world" experience of various laboratories. Given the limitations of quantitative capability in multiplex real-time PCR, cyclic-CMTA will be powerful tool to address the knowledge gap in certain diseases, due to the lack of quantitative ability in current *in vitro* diagnostic assay (i.e. HPV and respiratory viruses) (17-19), toxicology screening and detection of multiple food-borne pathogens (20, 21).

Beyond current routine diagnostic applications, this new technique will become valuable in research aimed at gaining insight into quantitative relationship of multiple agents on pathogenicity, disease progression and comorbidity in order to optimize patient care. The same can be said of understanding the impact of multiple mutations in cancer progression or genetic determinants of multi-drug resistance. cyclic-CMTA will become a game-changing strategy in formulating and answering these pivotal clinical challenges.

MATERIALS AND METHODS

Sample Preparation

Genomic sequences from three different viruses, RSV-A, RSV-B and HEV, were amplified and cloned into a plasmid to be used as templates in TOCE assays. Serial dilutions of each stock of purified plasmid template were added to TOCE assays as described in the figure legends.

cyclic-CMTA

TOCE reactions were conducted in the final volume of 20 μL containing diluted single or mixed plasmid templates, target-specific components (10 pmole of each DPO primer, 5 pmole of Pitcher, 1 pmole of Catcher) and 10 μL of 2X Master Mix containing 2.5 mM $MgCl_2$, 200 μM of dNTPs and 1.6 units of *Taq* DNA polymerase. As a negative control, distilled water was used instead of the template.

Reaction mixtures were denatured for 15 min at 95℃ and subjected to 45 cycles of 30 sec at 95℃, 60 sec at 60℃, 30 sec at 72℃ in the real-time thermocycler (CFX96, Bio-Rad). CMTA was performed by cooling the reaction mixture to 50℃, holding at 50℃ for 30 sec, and heating from 50℃ to 90℃. The fluorescence was measured continuously during the temperature rise. The melting peaks were derived from the initial fluorescence (F) versus temperature (T) curves by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T).

cyclic-CMTA was performed after PCR Cycle 25, 35 and 45, respectively. Endpoint CMTA was performed after 45 cycles of the reaction.

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