Application note

RapiDxFire Thermostable Reverse Transcriptase

Introduction

A reverse transcriptase (RT) is a DNA polymerase enzyme that synthesises cDNA from an RNA template. RTs are widely used to study gene expression in cells or tissues, in next-generation sequencing (NGS) applications, and in conjunction with quantitative PCR (qPCR) or isothermal amplification methods to detect and identify RNAs that are of clinical or functional significance.

RT enzymes may differ in key characteristics that affect their performance in different applications. Their thermostability allows cDNA synthesis to be performed at higher temperatures (above 50 °C), enabling them to melt areas of secondary structure in the RNA target, which improves cDNA yield and subsequent detection sensitivity from difficult RNA targets. Thermostability also is an indicator of general enzyme stability for storage, automation applications, and lyophilisation. Processivity, which refers to the number of nucleotides incorporated into cDNA during a single enzyme binding event, can affect cDNA length, RT efficiency, and RT reaction time. Native RT enzymes also have RNase H endonuclease activity that will cleave the RNA from a DNA-RNA duplex and limit cDNA length. Some RT variants also have been engineered to reduce RNase H activity to allow longer cDNA products.

Commercially available RTs used in molecular biology are generally derived from Moloney

Lucigen®

BIOSEARCH™ TECHNOLOGIES
GENOMIC ANALYSIS BY LGC

Application note

RapiDxFire Thermostable Reverse Transcriptase

murine leukaemia virus (MMLV) or avian myeloblastosis virus (AMV), which have optimal activity at 37-42 °C. Cloned AMV RT and engineered variants of AMV RT and MMLV RT were developed to improve thermostability and other performance characteristics. Newer applications, and use of difficult sample types and targets, require RTs that are not only thermostable but also demonstrate high sensitivity and shorter reaction times. We have found that most commercially available engineered RTs have limited activity at, and tolerance to, higher temperatures, and suffer limited stability under ambient temperature conditions and in use with automated handling platforms.

RapiDxFire™ Thermostable Reverse Transcriptase is a unique, proprietary enzyme originally identified from a hot spring viral source. The RapiDxFire Thermostable RT retains its inherent stability under multiple storage and temperature conditions, and provides strong reverse transcriptase activity at high temperatures. This enzyme, which lacks RNase H and 3´→5´ exonuclease activity, efficiently synthesises short cDNA fragments (≤ 1 Kb). Like other RTs, RapiDxFire RT possesses DNA polymerase activity but lacks 5´→3´ exonuclease activity. RapiDxFire Thermostable Reverse Transcriptase performs fast and efficient first-strand cDNA synthesis using gene-specific primers. In this study, we compared RapiDxFire Thermostable Reverse Transcriptase thermostability, speed, and sensitivity to two leading thermostable RTs in the market.

Materials and Methods

Commercial samples

Commercial samples used included Human Skeletal Muscle Total RNA (Thermo Fisher Scientific Cat No. AM7982), RNA, MS2 (Roche Cat No. 10165948001), Zika virus ATCC® VR-1843™ (ATCC Cat No. VR-1843), poly (rC)-p(dG) 12-18 (Midland Certified Reagents, Cat No. P-4210), and poly (rA)-p(dT) 12-18 (Midland Certified Reagents, Cat No. P-4012).

Activity assay

Activity profiles were generated using the Invitrogen™ EnzChek™ Reverse Transcriptase Assay Kit (Thermo Fisher Scientific Cat No. E22064) utilising either poly(A) template or poly(C) template. The RNA-DNA heteroduplexes were then quantified utilising PicoGreen® fluorescence assay on a Tecan Infinite® M1000 Pro microplate reader.

cDNA synthesis

RapiDxFire Thermostable Reverse Transcriptase (Lucigen Cat No. 30250-2) was used according to the standard protocol. RTs from other suppliers were used according to their standard protocols unless otherwise stated. For all first-strand cDNA synthesis steps, gene-specific primers were utilised.

PCR

PCR was performed on samples in a second step following reverse transcription using EconoTaq DNA Polymerase (with Mg++) and its supplied buffer (Lucigen Cat No. 30031-3). Real-time detection of PCR products was performed using an intercalating dye (Dyomics Cat No. V13-01184) and a BioRad CFX C1000 Touch™ Thermal Cycler with maximum absorption/emission wavelengths of 481nm/526nm, respectively. The reverse transcription reaction contributed up to 10% of the total PCR assay volume.

Application note

RapiDxFire Thermostable Reverse Transcriptase

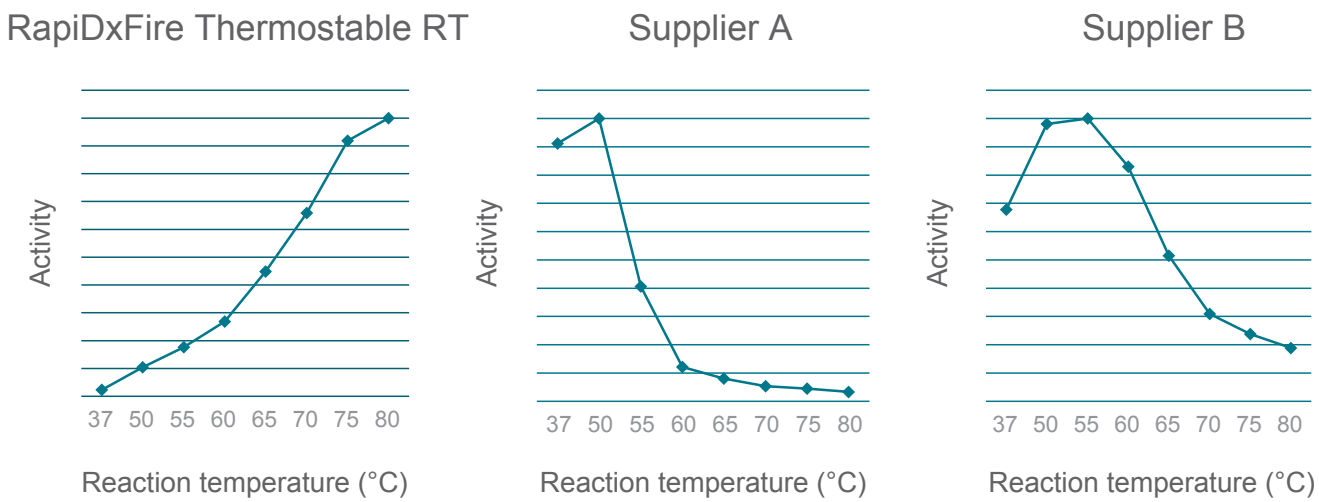

Figure 1. Reaction temperature profiles for thermostable reverse transcriptases. Triplicate reactions were set up on ice for each condition tested using each manufacturer's recommended buffer system, transferred to the indicated temperatures, and incubated for 40 minutes. RNA/cDNA RNA-DNA heteroduplexes were then detected utilising PicoGreen fluorescence assays on a Tecan Infinite M1000 Pro microplate reader.

Results

Reverse transcription activity thermal profile

Thermal activity profiles were determined for each enzyme by setting up triplicate reactions on ice for each condition tested using each manufacturer's recommended buffer system and then incubating the RT reactions with poly (rC)-p(dG) 12-18 template/primer for 40 minutes at eight different temperatures (37, 50, 55, 60, 65, 70, 75, and 80 °C). Following incubation, RNA/cDNA product was quantified by PicoGreen dye fluorescence as a measure of polymerisation activity. Each profile was represented as a percentage of each peak RFU (and not as a comparison against each other). RapiDxFire Thermostable RT exhibited increasing activity as the reaction temperature was increased up to 80 °C, the highest temperature tested, and retained ~60% activity after 10 minutes at 90 °C (data not shown). The reverse transcriptases from Supplier B and Supplier A had peak activities between 50-55 °C and 37-50 °C, respectively (Figure 1).

Time course and sensitivity

Time-course studies of cDNA synthesis were performed in a 2-step RT-qPCR process with different thermostable RTs. Zika cDNA synthesis was conducted for each enzyme in duplicate using target-specific primers in the recommended reaction buffer and incubation temperatures (RapiDxFire: 60 °C; Supplier A: 50 °C; and Supplier B: 55 °C) according to the suppliers' guidelines. After completion of synthesis and a 1-minute reverse transcription reaction (Figure 2A), qPCR was performed using one-tenth volume of each of the Zika cDNA samples using an intercalating dye. Additional samples were run through 1-, 2-, 5-, and 10-minute reverse transcription reactions for a time-course study prior to performing second-step qPCR. Encircled data points are derived from data represented in the qPCR curve from panel A (Figure 2).

Application note

RapiDxFire Thermostable Reverse Transcriptase

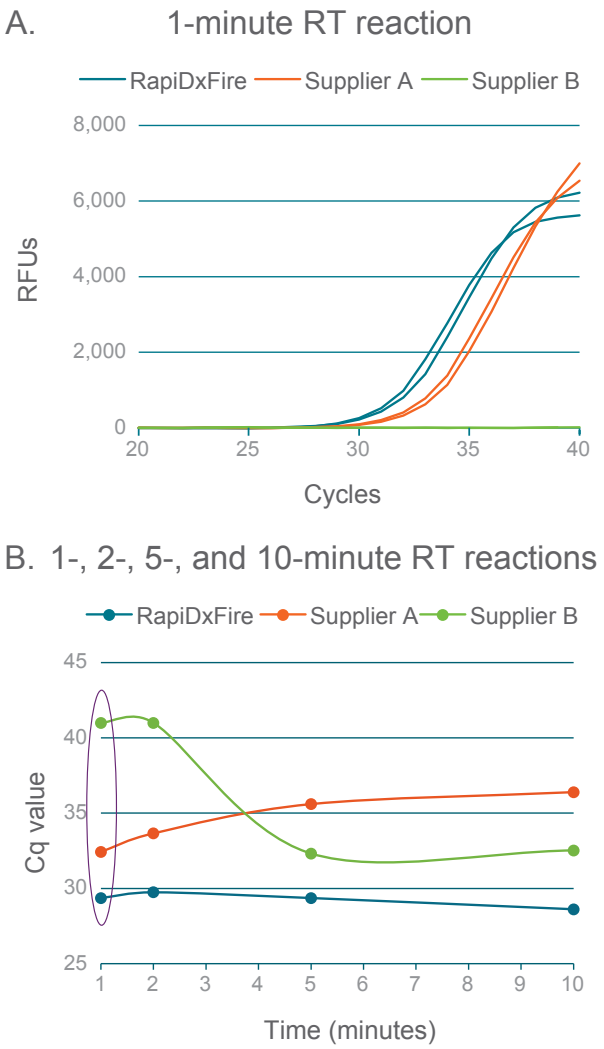

Figure 2. cDNA synthesis time course studies in a 2-step RT-qPCR process with different thermostable RTs. Zika cDNA synthesis for each enzyme performed in the recommended reaction buffer and incubation temperature recommended for RapiDxFire, Supplier A, and Supplier B. After synthesis and RT reaction, qPCR was performed using one-tenth volume of each of the cDNA samples using an intercalating dye in 1-minute (A) or (B) 1-, 2-, 5-, and 10-minute RT reactions. Data points derived from the qPCR curve in panel A are encircled in panel B.

Reaction temperature impact on sensitivity

Reaction temperature and sensitivity were evaluated using a two-step real-time RT-qPCR process. cDNA synthesis was conducted for each of the enzymes using standard conditions according to each supplier's guidelines. In

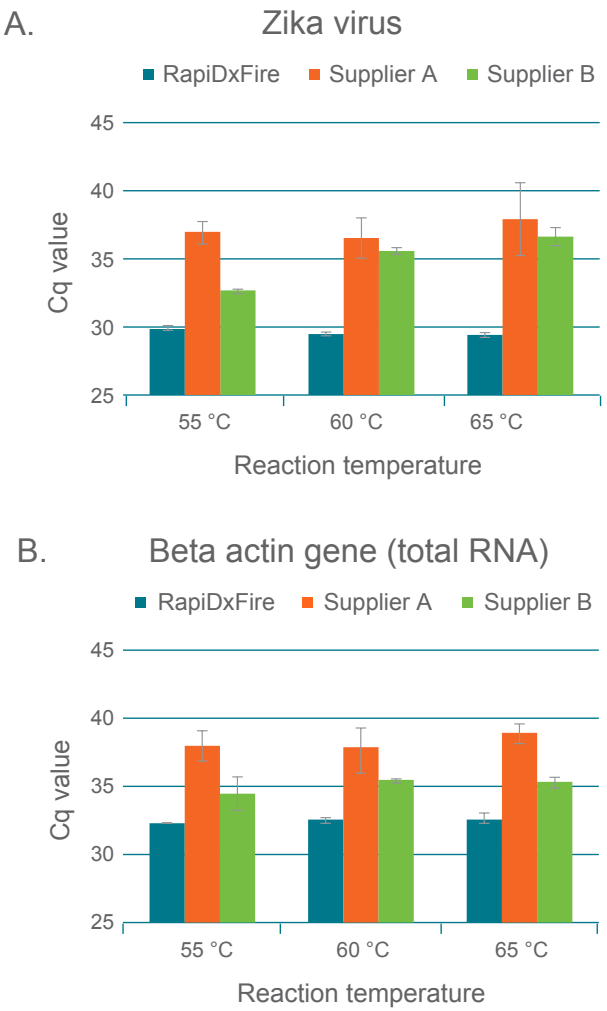

Figure 3. Reaction temperature sensitivity. Real-time qPCR detection after a two-step real-time RT-qPCR process that created cDNA from (A) Zika virus or (B) beta actin gene from total Human RNA using different RT reaction temperatures of 55, 60, and 65 °C for 10 minutes.

separate reactions each enzyme was used to create cDNA from Zika virus (Figure 3A) or using 0.5 ng of Human total RNA (Figure 3B) using RT reaction temperatures of 55, 60, and 65 °C for 10 minutes. After cDNA synthesis, real-time qPCR was run on each of the RT samples. RapiDxFire Thermostable RT provided consistent results across reaction temperatures with superior sensitivity.

Application note

RapiDxFire Thermostable Reverse Transcriptase

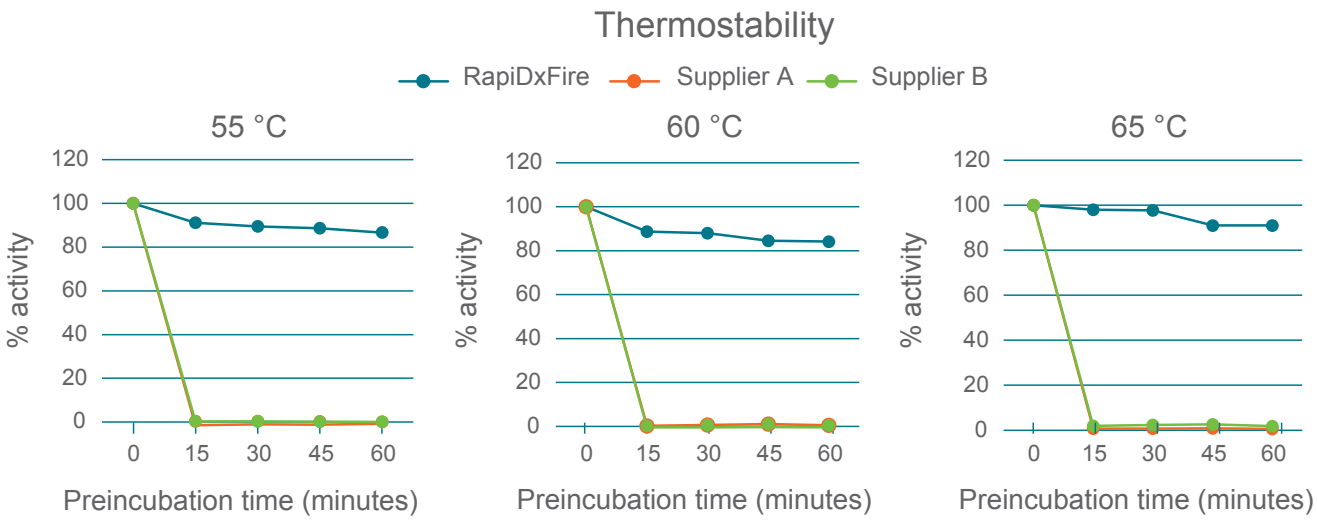

Figure 4. Thermostability studies of RTs at elevated temperatures. Reaction mixes containing each enzyme, but without dTTP and poly (rA)-p(dT) 12-18 template/primer were pre-incubated for 0 to 60 minutes at 55, 60, and 65 °C. After adding nucleotide and template/primer, 15-minute RT reactions at 55 °C were performed before quantifying the RNA/cDNA product by PicoGreen fluorescence. The polymerisation activity percentage (% Activity) was calculated based on the activity control of each enzyme measured without pre-incubation (0-minute control).

Thermotolerance

Thermotolerance of reverse transcriptases under reaction conditions was determined by pre-incubating reaction mixes containing each enzyme for 0, 15, 30, 45, and 60 minutes at 55, 60, and 65 °C. The reaction mix for each enzyme was prepared according to the standard buffer conditions in each supplier's instructions, but without dTTP and poly (rA)-p(dT) 12-18 template/primer, which were added after pre-incubation. RT reactions containing nucleotide and template/primer were then incubated for 15 minutes at 55 °C before quantifying the RNA/cDNA product by PicoGreen dye fluorescence to measure polymerisation activity. The activity percentage (% Activity) was calculated based on the activity control of each enzyme measured without pre-incubation (0-minute control). The RapiDxFire Thermostable RT was the only RT that remained stable under reaction conditions for extended periods of time at any of the temperatures tested (Figure 4).

Stability

Stability of reverse transcriptases were evaluated by storing enzyme stocks at ambient temperature and -20 °C in separate aliquots for

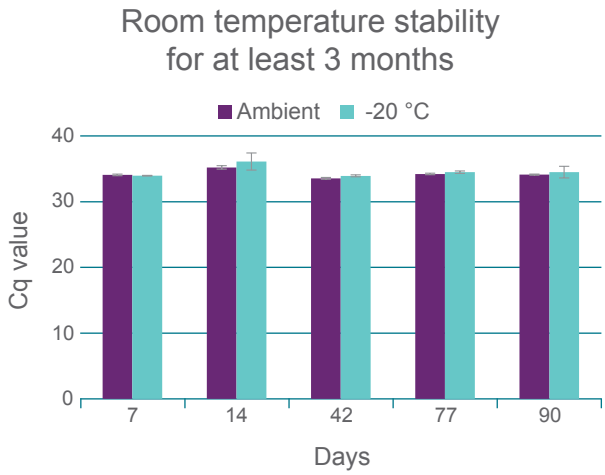

Figure 5. Long-term thermostability studies at ambient temperature. A three-month study was conducted by storing RapiDxFire Thermostable RT enzyme stock at ambient temperature and -20 °C in separate aliquots and evaluated by measuring first-strand cDNA synthesis at 60 °C for 5 minutes. Stability was measured as an increase in Cq value (or a decrease in cDNA synthesis) using real-time qPCR.

Application note

RapiDxFire Thermostable Reverse Transcriptase

7, 14, 42, 77, and 90 days. RapiDxFire Thermostable RT was evaluated at each time point by measuring first-strand cDNA synthesis of 10,000 copies of MS2 RNA. cDNA synthesis using a gene-specific primer for MS2 was carried out at reaction temperature of 60 °C for 5 minutes. cDNA was measured using real-time qPCR and two MS2 primers designed around a 520-base pair amplicon. Stability was evaluated directly based on the increase in Cq value or indirectly by the decrease in cDNA synthesis (Figure 5).

Conclusions

RapiDxFire Thermostable Reverse Transcriptase is a unique enzyme that provides superior thermostability when compared to other commercial reverse transcriptases. Its improved thermostability allows for higher reaction temperatures and flexible storage solutions, and when used at higher temperatures, RapiDxFire Thermostable Reverse Transcriptase outperforms other RTs.

Integrated tools.
Accelerated science.

@LGCBiosearch | lucigen.com

BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC