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A High-fidelity Thermostable Reverse Transcriptase for RNA Seq Analysis of Transcriptomes.

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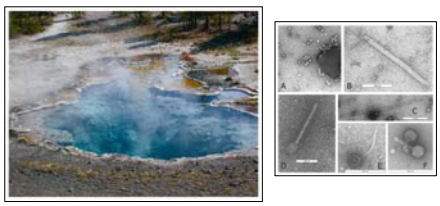
ABSTRACT

The future of research into human disease will require a better understanding of the regulation of gene expression. In addition, RNA viruses are common pathogens and virome sequencing is increasingly important in understanding a variety of viral diseases. Next generation sequencing platforms provide a powerful tool to study the human transcriptome and RNA virome; however, the value of this information depends directly on the accuracy of the libraries created for these analyses. Currently used library construction protocols are compromised by low fidelity enzymes for cDNA synthesis and amplification, and technical limitations in library construction. These limitations lead to frequent errors and rearrangements during cDNA synthesis, bias due to secondary structure and nucleotide composition, and the loss of information on the direction of transcription. This is due to complicated library construction methods and their reliance on retroviral RTs, particularly those of Moloney Murine Leukemia Virus (M-MLV) and Avian Myeloblastosis Virus (AMV), to synthesize cDNA. Deficiencies inherent to retroviral RTs include: 1) Low thermostability, 2) Low fidelity, 3) Frequent rearrangements of the products, 4) Secondary activities, 5) High bias for specific primers and templates, and 6) Interference with PCR enzymes. Novel thermostable DNA polymerases (Pols), discovered in viral metagenomes from natural thermal environments are being developed as improved reagents for detecting and analyzing nucleic acids. PyroScript Reverse Transcriptase (RT) is the first thermostable viral DNA polymerase and the first Pol from any source with RT activity for efficient single-enzyme RT-PCR. In addition, this enzyme has an inherently high fidelity PCR capability, significantly higher than Taq Pol. These attributes improve the detection and quantification of transcript RNAs and RNA viruses and should facilitate the more accurate, efficient construction of transcriptome and RNA virome libraries for sequence analysis.

Thermophilic Phage Metagenomics



Viral Metagenomics

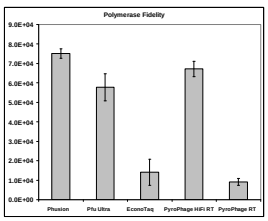
Thermophilic phage (electron micrographs, right) were isolated from the Hot Springs of Yellowstone National Park (left) and used to construct metagenomic libraries that were screened to identify novel DNA polymerases.

Applied Environmental Microbiology ***74****(13):4164-4174. 2008.*

High Fidelity PCR with PyroPhage RT

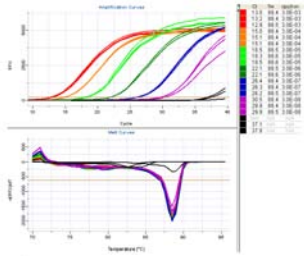
5'-3' exonuclease	-
3'-5' exonuclease	*Strong
Strand displacement	Strong
Extension from nicks	Strong
Thermostability (T½ @95°C)	10 min.
Km dNTPs	40 µM
Km DNA	5.3 nM
Processivity	47 nt
Fidelity	7 X 10^6
Template	DNA / RNA

*The 3'-5 exonuclease domain has been disabled in Pyrophage RT

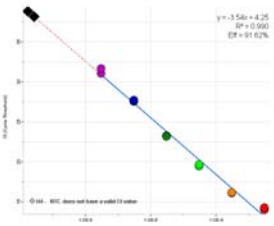


RT-PCR with PyroPhage RT

a.



b.



Linearity of RT-PCR

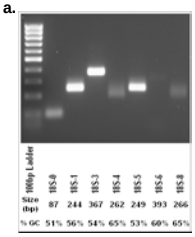
Triplicate single-enzyme RT-PCR amplifications of a 10^3 to 10^8-fold dilution series of the RNA target. Amplification was detected by EvaGreen fluorescence.

PyroScript RT-PCR 2X Master Mix, RNA Control and Control Primer Set were used for the analysis.

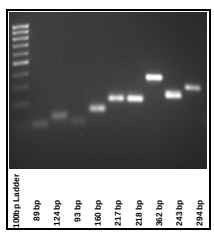
a. Real-time and post-reaction melt data. Two-step cycling conditions were used.

b. Linear regression of Cycle threshold (Ct) vs. $\log_{10}$ Dilution Factor.

a.



b.



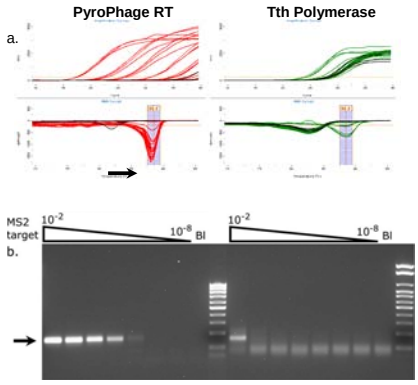
Single Enzyme RT-PCR

a.) Human 18S rRNA sequences were amplified from 100 pg total A549 cell line RNA. Seven primer sets targeting amplicons from 51 to 65% GC content and from 87 to 393 bp in length were tested.

b.) Viral RNA (Enterobacteriophage MS2, ATCC 15597-B1) was amplified by 40 cycles of RT-PCR without background. Products from 89 to 362 bp in length were amplified.

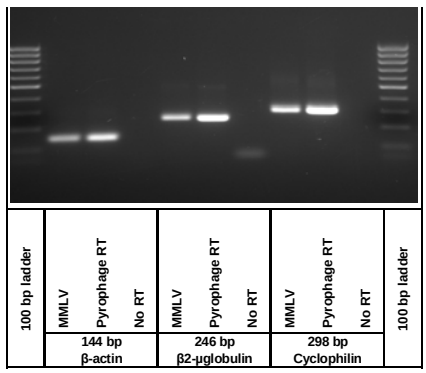
- One-step single-enzyme RT-PCR cycling conditions:
  15 sec @ 94˚C, (10s @ 94˚C, 30s @ 72˚C)*40

Comparison Data



PyroPhage RT vs. Tth Polymerase

Single-enzyme, one-step RT-PCR of a 160 bp amplicon using a 10^2 to 10^8-fold dilution series of MS2 RNA.
a. Real-time and post-reaction melt data
b. Corresponding Agarose gel data. Tth polymerase used with Mn^{2+} as directed (Epicentre).
Arrows show correct melt Tm (top) and amplicon (bottom).



PyroPhage RT vs. MMLV RT 2-step RT-PCR human mRNA

Total human Liver RNA (1 µg) was reverse transcribed by Moloney Murine Leukemia Virus or by PyroPhage RT, then PCR amplified using Lucigen EconoTaq® PLUS Master Mix.

Shown are targets of 144, 246 and 298 bp.

CONCLUSIONS

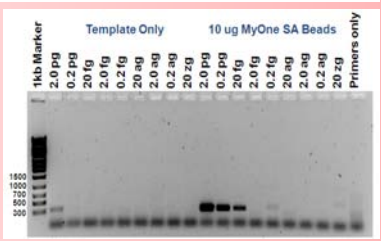
- **Thermostable PyroPhage RT directly detects and RT-PCR amplifies viral RNA and human transcripts.**
- **Effective for quantitative real-time and conventional RT-PCR analyses.**
- **Enhanced signal with magnetic beads.**
- **PyroPhage RT is effective in RT-LAMP.**
- **PyroScript RT-PCR 2X Master Mix is a robust and convenient RT-PCR solution.**

Enhanced PCR Amplification with SA Beads

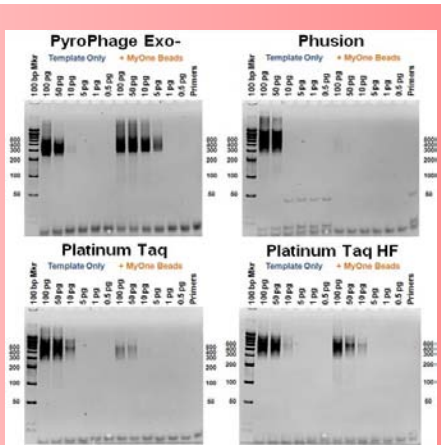
a.) PyroPhage Exo- shows enhanced activity in combination with streptavidin (SA) magnetic beads in a PCR reaction when compared to Phusion, Platinum Taq and Platinum Taq HF.

b.) The addition of 10 ug of MyOne SA Magnetic beads to a PCR reaction increased sensitivity by over 2 orders of magnitude.

b



a



Acknowledgements

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Sequencing of viral metagenomes was performed by JGI.

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