

Focus[®]

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for real-time PCR
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demonstrates higher
fidelity and sensitivity
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Rapid transfer of genes
from one vector to another
page 25





About The Cover:
Time-exposed shot of a molecule model of an antibody that attacks bacteria in mice.

Focus®

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PCR/RT-PCR

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LUX™ Primers: A versatile detection platform for real-time PCR

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Abstract

Real-time PCR with LUX™ Primers reports generation of amplicons at every PCR cycle by using single-labeled fluorogenic primers. The amount of initial template in the reaction can be accurately determined because it is directly proportional to the time point when a certain measurable amount of product is accumulated. The LUX™ (Light Upon eXtension) principle depends upon a single fluorophore being placed close to the 3' end in a specific sequence context that provides self-quenching. Using synthetic oligonucleotides, we have shown that de-quenching occurs upon extension of the primers during PCR and formation of the double-stranded PCR product. Here we show data using LUX™ Primers on different instrument platforms, such as the ABI Prism® 7700, the Roche LightCycler®, and the Corbett Rotor-Gene™ and provide conditions for successful multiplexing using FAM- and JOE-labeled LUX™ Primers.

Quantitative, fluorogenic, real-time PCR (qPCR) and RT-PCR (qRT-PCR) are used to determine the number of DNA copies and RNA transcripts from various biological samples like tissues and cell extracts (1,2). The qPCR methods employed usually involve the use of fluorescence techniques to detect the amplified DNA product. PCR amplification is directly linked to the generation and detection of signal, hence the term “real-time”. The reaction occurs in a homogeneous solution and no post-PCR procedures are required. The appearance of measurable product directly correlates with the amount of starting material and quantification can be performed from the fluorescence measured at each cycle, correlating it to a standard curve or a reference gene.

Several detection techniques are used in qPCR. Certain double-stranded DNA binding dyes, such as SYBR® Green I, fluoresce more brightly when bound to DNA and have been used for detection (3). These dyes, however, cannot be used in multiplex qPCR, where several target sequences are amplified in the same tube. Other detection methods incorporate the use of an oligonucleotide probe labeled with a fluorophore and a quencher (4,5). These probe-based techniques, while specific, have complex kinetics of hybridization and amplification and are relatively expensive to produce.

Invitrogen has developed and made available a real-time qPCR detection system that utilizes a fluorogenic primer labeled with a single fluorophore (6,7). The fluorogenic primer is self-quenched and its fluorescence increases upon its

incorporation into product during PCR. The design is based on the effects of the primary and secondary structure of oligonucleotides on the emission properties of attached fluorescent dyes (6). The design factors are used in a proprietary primer design software, called LUX™ Designer, that is used to design and order fluorogenic LUX™ Primers and unlabeled counterparts against the gene or sequence target of interest. The software can be accessed in three ways, as a desktop format that can be downloaded and installed on your computer, as a web-based primer design and ordering tool at <http://www.invitrogen.com/lux>, or by request from Invitrogen.

Material and Methods

Plasmids, template, and primers. Plasmids used as templates in qPCR were generated from first-strand cDNA by PCR with gene-specific primers and cloned into the pCR®4-TOPO® vector using a TOPO TA Cloning® Kit for sequencing (Invitrogen, Cat. no. K4595-01). The concentration of plasmid preparations was determined by OD₂₆₀ measurements. In order to convert concentrations to copy numbers, we used 9.1×10^{11} copies for one microgram of plasmid, divided by the size (plus insert) in kb.

RNA was isolated from human HeLa cells and mouse NIH 3T3 cells using the Micro-to-Midi Total RNA Purification System (Invitrogen, Cat. no. 12183-018) as described in the manual. RNA preparations were treated with DNase I (Invitrogen, Cat. no. 18068-015) to digest genomic DNA contamination using the protocol described in the DNase I manual.

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First-strand cDNAs were synthesized from total RNA by reverse transcription (20- μ l reaction volume) using the SuperScript™ II First-Strand Synthesis System (Invitrogen, Cat. no. 11904-018) primed with oligo-(dT)₁₂₋₁₈ and random hexamers as described in the manual.

Real-time PCR Protocols

ABI Prism® 7700 (Applied Biosystems). Real-time PCR on the ABI Prism® 7700 instrument was performed in 50- μ l assays with Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Cat. no. 11730-025) with the following assay composition:

- 25 μ l of SuperMix-UDG
- 1 μ l of ROX Reference Dye (Invitrogen, Cat. no. 12223-012)
- 1 μ l of LUX™ Primer FAM- or JOE-labeled, 10 μ m (200 nM final)

- 1 μ l of unlabeled primer, 10 μ m (200 nM final)
- 10 μ l of template
- 12 μ l of sterile distilled water (Invitrogen, Cat. no. 15230-162)

PCR program:

- UDG treatment: 2 min. at 50°C
- Denaturation: 2 min. at 95°C
- Cycling (45 cycles): 15 sec. at 95°C, 30 sec. at 55°C, 30 sec. at 72°C

For melting curve analysis, the reactions are cycled at 40°C for 1 minute and then ramped to 95°C over a period of 19 minutes followed by incubation at 25°C for 2 minutes.

LightCycler® (Roche). Real-time PCR experiments were performed in 20- μ l assays with Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Cat. no. 11730-025) with the following assay composition:

- 10 μ l of SuperMix-UDG
- 1 μ l of LUX™ Primer FAM-labeled, 10 μ m (500 nM final)
- 1 μ l of unlabeled primer, 10 μ m (500 nM final)
- 1 μ l of bovine serum albumin 5 mg/ml (final concentration of 250 ng/ μ l)
- 0.6 units of Platinum® *Taq* DNA polymerase (Invitrogen, Cat. no. 10966-018) (to bring the total to 1.2 units)
- 2 μ l of the template sample
- Sterile distilled water (Invitrogen, Cat. no. 15230-162) to 20 μ l

PCR program:

- Fluorescence: F1
- Program choice: amplification. Analysis mode: Quantification
- UDG treatment: 2 min. at 50°C
- Denaturation: 2 min. at 95°C
- Cycling (50 cycles): 5 sec. at 94°C, 10 sec. at 55°C single acquire, 10 sec. at 72°C

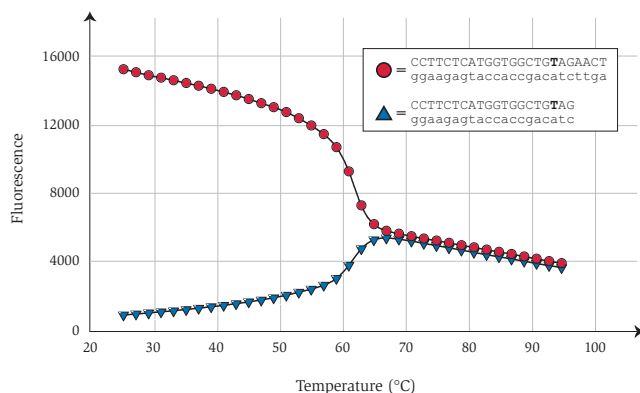
Melting curve analysis:

Program choice: melting curve. Analysis mode: Melting Curves

- 0 sec. at 95°C
- 15 sec. at 55°C
- 0 sec. at 95°C temperature, increase 0.1°C/sec with continuous acquisition
- 0 sec. at 40°C

Rotor-Gene™ RG-3000 (Corbett Research). Real-time PCR on the Corbett Rotor-Gene™ instrument was performed with Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Cat. no. 11730-025). For the 36-tube rotor, 25- μ l assays were used and for the 72-tube rotor, 20- μ l assays were used. The following protocol describes the 20- μ l assays for the 72-position rotor:

Figure 1 – The LUX™ principle: Melting curves showing de-quenching upon extension



The fluorescent intensity is modulated due to the proximity of the fluorophore to the 3' end of the oligonucleotide. The shorter sequence (blue) represents the equivalent to a LUX™ Primer and exhibits low fluorescence due to self-quenching. When this sequence is extended (red), as occurs during PCR, the quenching is released and the fluorescent signal increases several fold.

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- 10 μ l of SuperMix-UDG
- 1.6 μ l of LUX™ Primer 2.5 μ M (200 nM final)
- 1.6 μ l of unlabeled primer 2.5 μ M (200 nM final)
- 1 μ l of template
- 5.8 μ l of sterile distilled water (Invitrogen, Cat. no. 15230-162)

PCR program:

- UDG treatment: 2 min. at 50°C
- Denaturation: 2 min. at 95°C
- Cycling (50 cycles): 5 sec. at 95°C, 10 sec. at 60°C, 15 sec. at 72°C (data acquisition step)

For melting curve analysis, reactions were incubated from 45°C to 95°C in 1 degree steps with a ramp of 5 sec. at each step.

Multiplex PCR. Multiplex experiments were carried out with FAM-labeled LUX™ Primers targeted towards the human SDHA gene (Succinate dehydrogenase complex, subunit A, Acc. no. NM_004168) and JOE-labeled LUX™ Primers targeted towards mouse GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, Acc. no. NM_008084). Real-time multiplex PCR was performed in 50- μ l assays on the ABI Prism® 7700 instrument with Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Cat. no. 11730-025) with the following assay composition:

- 25 μ l of SuperMix-UDG
- 1 μ l of ROX Reference Dye (Invitrogen, Cat. no. 12223-012)
- 1 μ l of LUX™ SDHA primer, FAM-labeled, 10 μ M (200 nM final)
- 1 μ l of unlabeled SDHA primer, 10 μ M (200 nM final)
- 0.5 μ l of LUX™ mouse GAPDH, JOE-

- labeled, 10 μ M (100 nM final)
- 0.5 μ l of unlabeled GAPDH primer, 10 μ M (100 nM final)
- 10 μ l of template
- Sterile distilled water (Invitrogen, Cat. no. 15230-162) to 50 μ l

PCR program:

- UDG treatment: 2 min. at 50°C
- Denaturation: 2 min. at 95°C

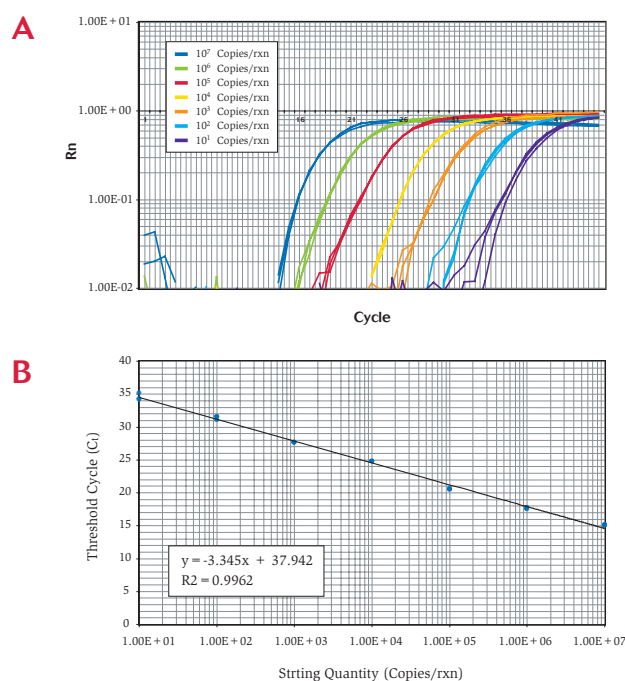
- Cycling: 15 sec. at 95°C, 30 sec. at 60°C 50 cycles

For melting curve analysis, the reactions are cycled at 40°C for 1 minute and then ramped to 95°C over a period of 19 minutes followed by incubation at 25°C for 2 minutes.

Results and Discussion

The LUX™ Principle. Real-time PCR/RT-

Figure 2 – Amplification plot for real-time PCR with human β -actin LUX™ Primers on the ABI Prism® 7700



Panel A: Real-time PCR with LUX™ Primers on an ABI Prism® 7700. Serial dilutions of a β -actin cDNA clone were prepared and PCR was performed using 200 nM FAM-labeled LUX™ Primer, 200 nM unlabeled primer, Platinum® Quantitative PCR SuperMix-UDG, and ROX Reference Dye. Reactions (3 replicates per dilution) were incubated as described in Materials and Methods.

Panel B: Standard curve showing the initial copy number of template versus the cycle threshold (Ct).

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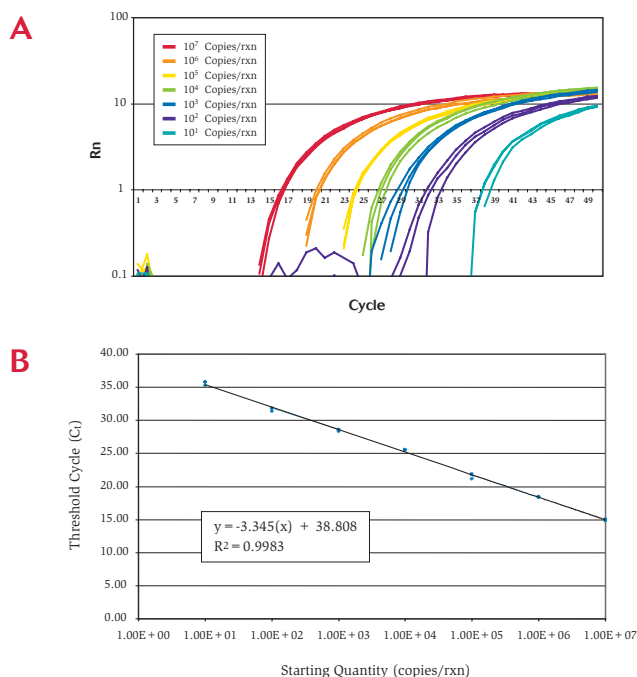
PCR based on fluorescent detection has become the technology of choice for accurate, reproducible quantification of DNA and RNA. LUX™ Fluorogenic Primers present a high-performing, yet cost-effective, detection platform for real-time PCR. LUX™ Primers are oligonucleotides labeled with a single fluorophore and are custom-synthesized for a specific gene target of interest. Typically 20-30 bases in length,

they are designed with a fluorophore close to the 3' end and 5' extension that confers a hairpin structure which contributes to the quenching of the dye. LUX™ Primers are currently offered with either a FAM label (6-carboxy-fluorescein) or a JOE label (6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein). Based on initial observations of quenching effects upon hybridization of labeled

oligodeoxynucleotides, a systematic study of the factors that can influence the fluorescence intensity of dyes linked to DNA was undertaken by Invitrogen (6,7). We have examined the importance of primary sequence for the fluorescence of internally labeled oligonucleotides as well as oligonucleotides labeled at the 5' and 3' terminus. In addition, the significance of secondary structure on fluorescence has been investigated through the use of single-stranded and double-stranded oligonucleotides. The picture that emerges from this work shows that actual fluorescence depends on secondary structure, local sequence context, and the proximity of the 3' or the 5' end of the oligonucleotide. Figure 1 (page 4) shows the melting curve analysis of two very similar oligonucleotides and highlights the dramatic changes in fluorescence upon extension of the sequence.

Use Of LUX™ Primers On Different Instrument Platforms. A variety of instrument platforms are available to conduct real-time PCR experiments. The three main categories of instruments that can be distinguished are (i) machines that use plastic tubes in fixed 96- or 384-well blocks, (ii) machines that use plastic tubes in a spinning rotor, and (iii) machines that use glass capillaries in a spinning rotor. Here we have chosen the ABI Prism® 7700, the Corbett Rotor-Gene™, and the Roche LightCycler® to represent each class. As fluorescent dye we have selected FAM labels that can be detected efficiently by all qPCR instruments currently on the market and the JOE label, that is detected efficiently by a large majority of instruments with the excep-

Figure 3 – Amplification plot for real-time PCR with human β -actin LUX™ Primers on the Roche LightCycler®



Panel A: Real-time PCR with LUX™ Primers on a Roche LightCycler®. Serial dilutions of a β -actin cDNA clone were prepared and PCR was performed using 500 nM FAM-labeled LUX™ Primer, 500 nM unlabeled primer, Platinum® Quantitative PCR SuperMix-UDG with added BSA (250 ng/ μ l) and Platinum® Taq DNA Polymerase (1.2 units final). The use of ROX Reference Dye is not necessary on this instrument. Reactions (3 replicates per dilution) were incubated as described in materials and Methods.

Panel B: Standard curve showing the initial copy number of template versus the cycle threshold (Ct).

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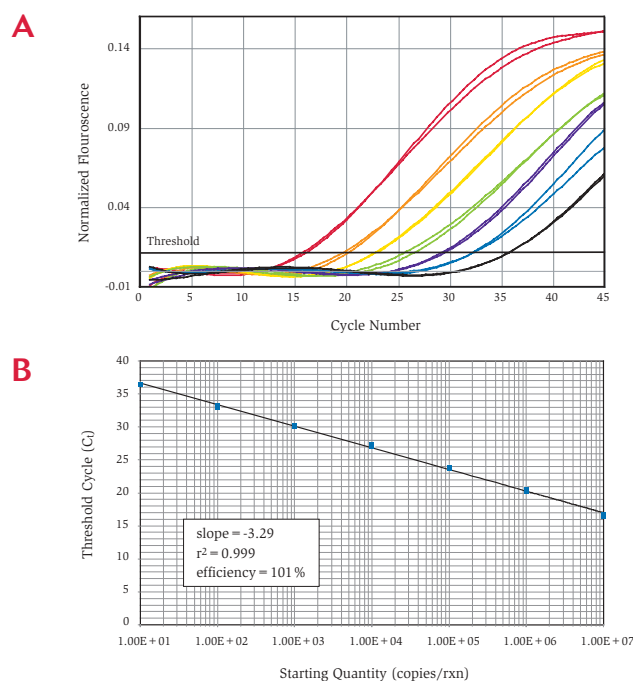
tion of the LightCycler®. Real-time PCR experiments with FAM-labeled LUX™ Primers with the three instrument platforms are shown in Figures 2 (page 5), 3 (page 6), and 4 (page 7).

The composition of the assays, volumes, and PCR programs are described in Materials and Methods. The assay volumes used here vary from instrument to instrument but 20 µl for glass capillaries and 20 to 50 µl for plastic tubes can be used as general guidelines. The number of PCR cycles used depends upon the copy number of the gene target in question and again 40 to 50 cycles should be used as a general guideline. Invitrogen™ Quantitative PCR SuperMix-UDG was used for all the assays described here. It provides UDG-carryover prevention and antibody-based *Taq* hot-start capability in an efficient and highly sensitive 2X ready-to-use reaction mix. ROX reference dye is provided in a separate tube at no charge and was used on the ABI Prism® 7700 but was omitted for assays on the Rotor-Gene™ and the LightCycler®. It is important to note that the LightCycler® requires the use of glass capillaries. This necessitated the addition of two components to the SuperMix-UDG; bovine serum albumin (BSA) to a final concentration of 250 ng/µl and the doubling of the Platinum® *Taq* DNA polymerase from 0.6 to 1.2 units for a 20-µl assay. Several commercial suppliers of BSA were tested (Sigma, NEB, Idaho Technology) and found to be equally effective in stimulating the PCR. LUX™ Primers performed well on all instrument platforms tested and the standard curves were of high quality in terms of sensitivity (low cycle thresholds), PCR efficiency (slopes close to -3.32 which

indicates 100% PCR efficiency) and correlation coefficients (close to 1.00, indicating good reproducibility and accuracy). In comparing the different instruments, the three platforms performed equally well in terms of specificity and sensitivity, although the choice of fluorescent dyes is more limited on the LightCycler®. Melting curve analysis was performed for the three

instrument platforms (data not shown) and only showed one peak in each template sample that corresponded to the amplicon targeted by the LUX™ Primer pair. Based on the open rotor design and the resulting rapid heat exchange, the Rotor-Gene™ and the LightCycler® afforded faster run times compared to the fixed heat block design of the ABI Prism® 7700.

Figure 4 – Amplification plot for real-time PCR with human 18S rRNA LUX™ Primers on the Corbett Rotor-Gene™



Panel A: Real-time PCR with LUX™ Primers on a Corbett Rotor-Gene™. Serial dilutions of a 18S rRNA cDNA from 300 ng to 0.3 pg were prepared and PCR was performed using 200 nM JOE-labeled LUX™ Primer, 200 nM unlabeled primer, and Platinum® Quantitative PCR SuperMix-UDG. The use of ROX reference Dye is not necessary on this instrument. Reactions (3 replicates per dilution) were incubated as described in Materials and Methods. Panel B: Standard curve showing the initial copy number of template versus the cycle threshold (Ct).

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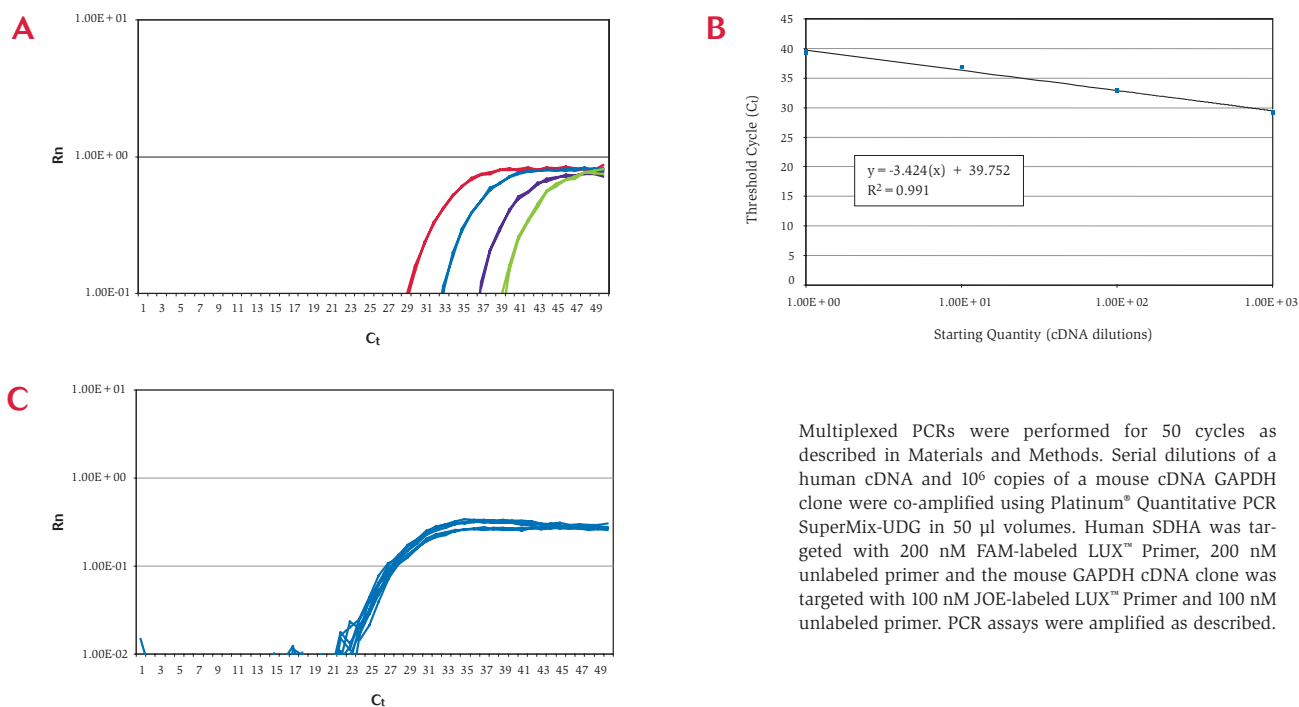
LUX™ Primers In Multiplex Format. In multiplex real-time PCR, more than one set of gene-specific primers are used to amplify separate genes from the template DNA in a single tube. Because more than one PCR product has to be accurately quantified in the same tube, different fluorescent labels are attached to the different fluorogenic primers. LUX™ Primers have been tested in multiplex reactions using FAM and JOE labels. Figure 5 shows the typical results of a multiplex PCR with LUX™ Primers using a simulated gene of interest (human SDHA) at different concentrations and

the housekeeping gene (mouse GAPDH) at a fixed concentration.

In comparison to a standard PCR, multiplex PCR is more demanding and may require the optimization of several parameters. In the example shown in Figure 5, we have decreased the primer concentration for the internal control gene GAPDH from 200 nM to 100 nM. Additional steps to optimize the multiplex reaction can include increasing the MgCl₂ in the reaction from 3 mM to 6 mM and increasing the amount of *Taq* polymerase enzyme. Platinum® *Taq* DNA polymerase stand-alone enzyme (Invitrogen, Cat. no.

10966-018) can be used for this purpose. The data show that multiplex experiments with LUX™ Primers are easy to optimize even on different instrument platforms. Multiplex PCR has also been performed on the Rotor-Gene™ (Corbett Research) using the same protocol (data not shown). Although LUX™ primers are currently available with two different labels (FAM and JOE), we have already shown that the LUX™ principle is compatible with a number of other dyes, including HEX and TET, allowing for expansion of the available dye spectrum in the future.

Figure 5 – Amplification plot for real-time multiplex PCR with human SDHA LUX™ Primers



Multiplexed PCRs were performed for 50 cycles as described in Materials and Methods. Serial dilutions of a human cDNA and 10⁶ copies of a mouse cDNA GAPDH clone were co-amplified using Platinum® Quantitative PCR SuperMix-UDG in 50 µl volumes. Human SDHA was targeted with 200 nM FAM-labeled LUX™ Primer, 200 nM unlabeled primer and the mouse GAPDH cDNA clone was targeted with 100 nM JOE-labeled LUX™ Primer and 100 nM unlabeled primer. PCR assays were amplified as described.

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AccuPrime™ *Pfx*: High-fidelity DNA polymerase with a zing

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Carlsbad, CA 92008

Though PCR is a powerful tool in nucleic acid research, the most widely used DNA polymerase, *Taq* DNA polymerase, has been plagued with a high error rate at about 2 to 5 x 10⁻⁵ (1, 2). This error frequency predicts that one base in every 20,000 nucleotides incorporated would be erroneous, or one or more 1 Kb-long amplicons would contain an error after only 15 PCR cycles, based on a theoretical doubling time of templates in PCR.

There have been two different approaches to achieve PCR amplification with a higher fidelity: 1) use of a proofreading DNA polymerase; and 2) blend *Taq* DNA polymerase with a proofreading enzyme. Using the proofreading enzymes by themselves in PCR can be arduous at times due to the very nature of proofreading activity. The proofreading activity of a polymerase comes from its intrinsic 3'→5' exonuclease activity that can occasionally chew off free 3' ends of primers or amplicons, making the enzymes difficult to use without extensive optimization of PCR parameters. The blended polymerases, as the second group is known, were devised because of the arduous nature of the proofreading enzymes at the expense of higher fidelity. Alternatively, a third factor could be introduced to improve the user-friendliness of the proofreading enzymes without compromising performance. By introducing thermostable accessory factors to polymerases, PCR enzymes are more efficient, without sacrificing the high fidelity.

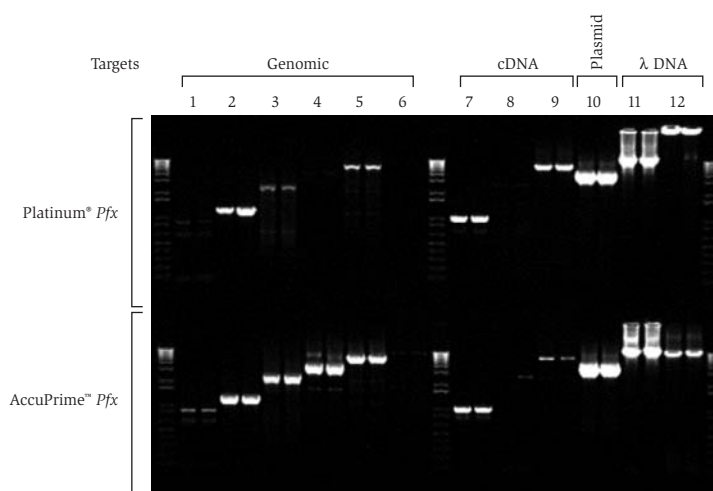
Invitrogen introduced the first member of a new line of next-generation PCR enzymes in 2001, AccuPrime™ *Taq* DNA

polymerase (2,3). This new technology uses a replication associated factor, AccuPrime™ Protein, to police primer annealing and guide the polymerase to primed sites for higher specificity and efficiency. AccuPrime™ technology improves PCR performance by promoting specific primer-template hybridization before, as well as during, every cycle of PCR. The same technology has been updated and successfully applied to a proofreading enzyme to create the most robust high-fidelity enzyme, AccuPrime™ *Pfx* DNA polymerase. In this case, AccuPrime™ technology has been updated to include a second, complementary protein, AccuPrime™ Protein II, that

enhances the robustness of the enzyme. The blending of AccuPrime™ proteins is unique to AccuPrime™ *Pfx* DNA polymerase, making a robust high-fidelity PCR enzyme.

In this manuscript, we describe the enhanced performance of this next-generation, high-fidelity PCR enzyme with the updated AccuPrime™ technology for PCR applications that require high fidelity, high sensitivity, and robustness. AccuPrime™ *Pfx* DNA polymerase shows improvement in yield, sensitivity, and/or specificity over other high-fidelity enzymes in about 50% of the cases with various target templates, such as, genomic DNA, plasmid, bacteriophage λ DNA,

Figure 1 – Performance comparison of AccuPrime™ *Pfx* DNA polymerase with Platinum® *Pfx* DNA polymerase using various templates



The template DNA used were human genomic DNA (K562, genotyping grade) ranging from 874 bp to 8.9 kb at 100 or 200 ng per reaction: 1) Rhod 874 bp; 2) Rhod 1237 bp; 3) p53 2380 bp; 4) Hbg 3.6 kb; 5) Rhod 6.8 kb; and 6) Fabp 8.9 kb, cDNA templates: 7) β actin 1 kb; 8) BF 2.4 kb; and 9) ATCR 6.1 kb, plasmid DNA; 10) pMOL21 4.1 kb, and λ DNA templates: 11) 9.1 kb; and 12) 9.8 kb. The gel shows a consistent performance enhancement in specificity, sensitivity, and robustness of AccuPrime™ *Pfx* DNA polymerase over Platinum® *Pfx* DNA polymerase.

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or cDNA. With its enhanced sensitivity, specificity, and reproducibility, AccuPrime™ *Pfx* DNA polymerase is ideal for a variety of PCR/RT-PCR applications, while its robustness minimizes the need for optimization. AccuPrime™ *Pfx* DNA polymerase maintains the high fidelity of Platinum® *Pfx* DNA polymerase and complements AccuPrime™ *Taq* DNA polymerase in generating a premier, next-generation PCR enzyme family.

Materials and Methods

PCR. Unless otherwise indicated, all PCR reactions were run following a standard protocol (below). PCR reactions were prepared in 50-µl reaction volumes containing 1X *Pfx* Amplification buffer with 1 mM MgSO₄ and 0.3 µM of each primer. The concentration of each of the four deoxynucleoside triphosphates (dNTPs) was 0.3 mM. The AccuPrime™ *Pfx* reaction mix contained AccuPrime™ Protein I and AccuPrime™ Protein II and is optimized for most PCR applications. Template concentration varied from 20 ng to 200 ng depending on the application. One unit of AccuPrime™ *Pfx* DNA polymerase (Invitrogen, Cat. no. 12344-024) was used in a typical 50-µl reaction. Thermocycling was conducted using either the GeneAmp® PCR System 9600 (Applied Biosystems, Inc.), GeneAmp® PCR System 2400 (Applied Biosystems, Inc.), or Peltier Thermal Cycler (PTC) 200 (MJR).

Standard PCR program

Pre-incubation
 95°C 5 minutes
 35 cycles of
 95°C 15 seconds
 55°C–65°C 30 seconds (5 degrees

below T_m)
 68°C 1 min/kb
 Hold at +4°C

Following the completion of thermocycling, PCR amplification products were mixed with 5 µl of 10X BlueJuice™ Gel Loading Buffer (Invitrogen) and aliquots (20% of total reaction volume, or 10 µl, per lane) were analyzed by electrophoresis on a 0.8%-1.5% agarose gel with an ethidium bromide concentration of 0.4 µg/ml pre-mixed in 0.5X TBE. The resulting gels were analyzed visually for specificity and yield among different samples.

Competitive Audit. The performance of AccuPrime™ *Pfx* DNA polymerase was compared with other high-fidelity PCR enzymes, such as *PfuTurbo*® DNA Polymerase (Stratagene), *PfuUltra*™ DNA Polymerase (Stratagene), Tgo DNA Polymerase (Roche), and KOD Hot Start DNA Polymerase (Novagen). Each enzyme was used to amplify targets ranging from 822 bp to 6816 bp using 100 to 200 ng of human genomic DNA (K562, genotyping grade). The following primer sets were used: *c-myc* 822 bp; p53 2380 bp; Human β globin (Hbg) 3.6 kb; Rhod 6173 bp; and Rhod 6816 bp. PCR reactions were performed following manufacturers' recommended conditions.

The annealing temperature for each primer set was identical for all polymerases tested: 65°C for *c-myc* 822 bp and p53 2380; 62°C for Hbg 3.6 kb; and 64°C for Rhod 6173 bp and Rhod 6816 bp. PCR amplification products were processed and analyzed as above.

Results and Discussion

Performance Enhancement of AccuPrime™ Pfx DNA Polymerase. Performance of AccuPrime™ *Pfx* DNA polymerase was assayed using genomic DNA, plasmid, phage λ DNA, and cDNA (Figure 1, page 10). Performance criteria were the amount of specific PCR product generated as measured by the band intensity (yield), the ratio between specific and non-specific bands (specificity), the minimum amount of template needed to generate the specific band (sensitivity), and consistency between duplicate reactions (reproducibility or robustness). AccuPrime™ *Pfx* was compared with Platinum® *Pfx* DNA polymerase using 89 primer sets with amplicons ranging from 500 bp to 13 Kb. The new AccuPrime™ *Pfx* out-performed Platinum® *Pfx* DNA polymerase in 47% of the cases (Table 1).

When the data was more closely scrutinized, however, it was found that PCR of amplicons less than 3 Kb showed a higher rate of improvement over

Table 1 – Statistical analysis of the performance of AccuPrime™ *Pfx* DNA polymerase compared to that of Platinum® *Pfx* DNA polymerase

Total Primers tested	Marked improvement	Slight improvement	Already good	No improvement	Inhibited
89	28 (31%)	14 (16%)	20 (22%)	25 (28%)	2 (2%)

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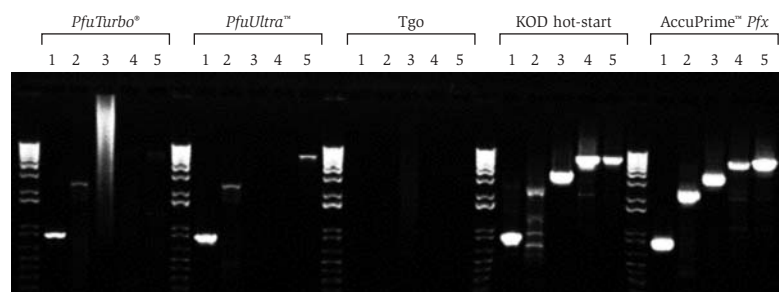
Platinum® *Pfx* DNA polymerase. Considering Platinum® *Pfx* already outperforms most other commercially available proofreading PCR enzymes, the higher performance of AccuPrime™ *Pfx* DNA polymerase over Platinum® *Pfx* implies the superiority of the new enzyme. Since most functional genes are less than 3 Kb (coding about 120 kDa polypeptides), AccuPrime™ *Pfx* DNA polymerase with its high fidelity would be ideal in amplifying functional genes for cloning or other proteomic applications.

Most significantly, AccuPrime™ *Pfx* DNA polymerase maintained the high fidelity of Platinum® *Pfx* DNA polymerase, which is about 26-times higher than that of *Taq* and two times that of *Pfu* DNA polymerase (1).

PCR Optimization for AccuPrime™ Pfx DNA Polymerase. Though the performance of AccuPrime™ *Pfx* DNA polymerase is robust and unsurpassed, there is no magic formula for PCR that works in all reactions. The outcome of PCR, in general, depends very much upon specific primer sets: 1) the annealing temperature of the PCR cycles should be adjusted according to the primers; and 2) the composition of an amplicon, as defined by the primers used, could affect the outcome of a PCR reaction. There are several helpful hints and pointers for primer design available online or in publications (4). In addition, there are a few software programs for PCR primer design as well, such as Vector NTI™ from InforMax®.

PCR optimization should begin with the primer annealing temperature, as described above, once the primers are prepared. Annealing temperature should

Figure 2 – Competitive audit of AccuPrime™ *Pfx* DNA polymerase



The performance of AccuPrime™ *Pfx* DNA Polymerase was compared to that of *PfuTurbo*® and *PfuUltra*™ DNA Polymerases (Stratagene), *Tgo* DNA Polymerase (Roche), and KOD Hot Start DNA Polymerase (Novagen). Each enzyme was used to amplify targets ranging from 822 bp to 6816 bp using 100 to 200 ng of human genomic DNA (K562, genotyping grade). The gel shows clear and consistent performance of AccuPrime™ *Pfx* DNA polymerase over all other products.

Lane 1: *c-myc* 822 bp
Lane 2: p53 2380 bp
Lane 3: Hbg 3.6 kb
Lane 4: Rhod 6173 bp
Lane 5: Rhod 6816 bp

be set at 5 to 10°C below the melting temperature (T_m) of the primers used. A higher annealing temperature may help in achieving a higher specificity. An increase in the amount of the template DNA could increase the yield. Another optimization option for AccuPrime™ *Pfx* DNA polymerase is to add KCl up to a final concentration of 50 mM. Several primer sets tested with KCl demonstrated that titration of KCl for PCR optimization is a viable option (data not shown).

Competitive Audit of AccuPrime™ Pfx DNA Polymerase. Performance of AccuPrime™ *Pfx* DNA polymerase was directly compared with other major high-fidelity polymerases, such as, *PfuTurbo*® and *PfuUltra*™ DNA Polymerases (Stratagene),

Tgo DNA Polymerase (Roche), and KOD Hot Start DNA Polymerase (Novagen), using five primer sets with amplicons ranging from 822 to 6,816 bp (*c-myc* 822 bp; p53 2380 bp; Hbg 3.6 kb; Rhod 6173 bp; and Rhod 6816 bp). AccuPrime™ *Pfx* DNA polymerase showed superior performance over all others tested in yield, specificity, robustness, and consistency (Figure 2).

Conclusion

We report here the development of another member of the next-generation AccuPrime™ PCR enzyme family. AccuPrime™ technology was introduced last year with the launch of AccuPrime™ *Taq* DNA polymerase and its companion, AccuPrime™ *Taq* PCR SuperMix. These

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systems incorporate a thermostable AccuPrime™ protein into the Platinum® technology that already enhances the performance of *Taq* DNA polymerase in PCR beyond that of other hot-start *Taq* DNA polymerases (2,3). The enhancement is shown in all aspects of PCR performance of the enzyme such as the specificity, sensitivity, and robustness. Such enhancement makes the AccuPrime™ *Taq* DNA polymerase system suitable for many areas of PCR application, including, high-throughput PCR, multiplex PCR, and PCR miniaturization.

Here we have applied an updated AccuPrime™ technology to the most arduous PCR application of all, amplification with a high-fidelity DNA polymerase. The

high-fidelity nature of an enzyme comes from the DNA polymerase having a proof-reading, or 3'→5' exonuclease, activity in addition to the polymerase activity. These counteracting activities of the polymerase make it useful, but difficult to harness, in PCR applications. In the updated AccuPrime™ technology, the two AccuPrime™ proteins complement each other in their functions to harness the high fidelity of *Pfx* DNA polymerase to create the most robust high-fidelity enzyme. AccuPrime™ *Pfx* DNA polymerase, with higher specificity and sensitivity than all other high-fidelity enzymes in the current market, makes a natural extension of the next-generation AccuPrime™ PCR enzyme family.

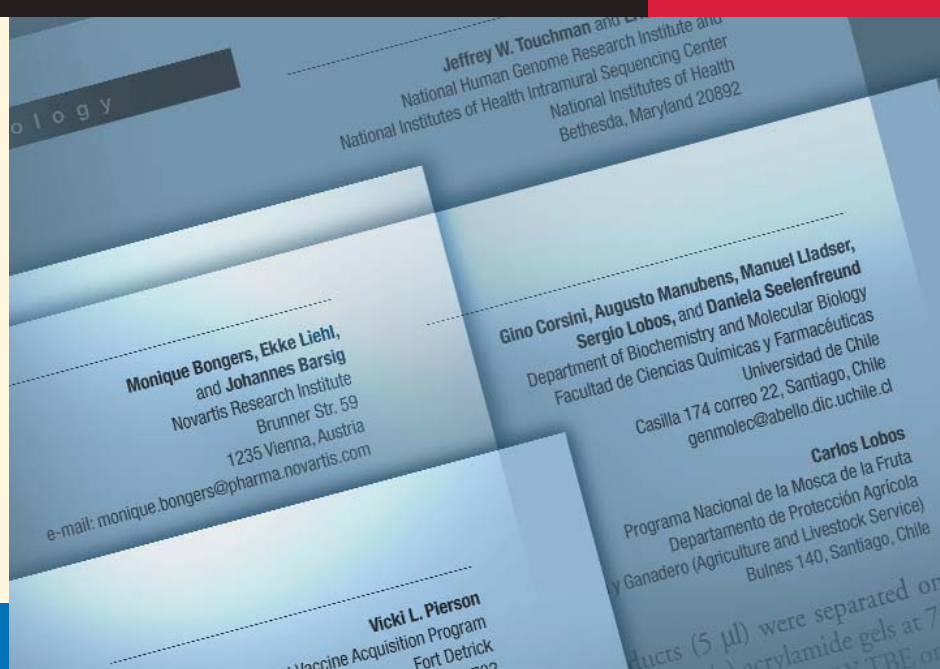
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Platinum® PCR SuperMix 96: A stable, versatile, and time-saving approach to high-throughput PCR amplification

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PCR amplification can be a time-consuming and laborious process. However, using a pre-assembled reaction mix that has been aliquoted into wells of a 96-well plate can save time and money while reducing experimental variability. We have developed Platinum® PCR SuperMix 96, a modified formulation of Platinum® PCR SuperMix, which contains recombinant *Taq* DNA polymerase, Platinum® *Taq* antibodies, dNTPs, magnesium, and buffer salts in a single mixture aliquoted into 96-well plates. The anti-*Taq* DNA polymerase antibody inhibits polymerase activity at low temperatures, providing an automatic “hot start” for PCR (1,2). Hot-start activation increases specificity and yield while also allowing reaction assembly at room temperature.

This report demonstrates the performance and advantages of this new PCR-ready, pre-aliquoted configuration. A key feature of Platinum® PCR SuperMix 96 is convenience as all components are combined and pre-aliquoted except for template and primers. Fewer pipetting steps are required which lowers the risk of contamination and shortens preparation time. The convenience of this configuration, available in plates compatible with robotics as well as plates in a breakable format, improves the efficiency and reliability of high-throughput and multiple-sample PCR.

Materials and Methods

Standard PCR Reactions. Unless otherwise indicated, all PCR reactions were carried out following a standard protocol. PCR reactions were prepared in 25- μ l reaction volumes containing 1X PCR

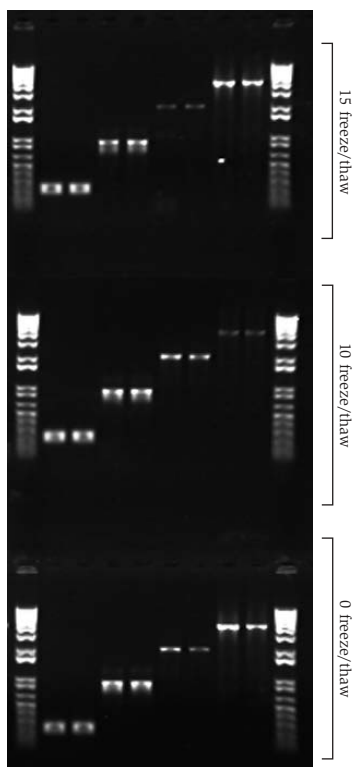
buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, and 0.2 μ M of each primer. The concentration of each of the four deoxynucleoside triphosphates (dNTPs) was 0.2 mM. Genomic DNA template concentration varied from 5 ng to 400 ng, depending on the application. Thermal cycling was conducted using MJ Research thermocyclers that accommodate the Platinum® PCR SuperMix 96 96-well skirted plate (Invitrogen, Cat. no.

11306-065) as well as the 96-well perforated plate (Invitrogen, Cat. no. 11306-073).

The standard PCR program cycled as follows, 94°C for 2 min. followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds (\sim 5 degrees below T_m), and 72°C for one min/kb. Following the 35 cycles, the temperature was held at +4°C until further processing. Following the completion of thermal cycling, PCR amplification products were mixed with 10% (v/v) 10X BlueJuice® Gel Loading Buffer. Twenty percent of the total reaction volume was loaded per lane of an 0.8% agarose gel. Samples were electrophoresed in a 0.5X TBE buffer containing ethidium bromide (0.5 μ g/ml). The resulting PCR products were visually inspected for specificity and yield.

Freeze/thaw Stability Assay. Stability of enzymes is often adversely affected by freezing followed by thawing. Freeze/thaw stability assays were performed to investigate the sensitivity of Platinum® PCR SuperMix 96 to freeze/thaw cycles. Pre-aliquoted plates were thawed at ambient temperature and subsequently placed in a -20°C freezer until the solutions were frozen, a period of time ranging from 20 min. to overnight. Following each freezing step, the plates were removed and allowed to warm to ambient temperature. This cycle of freezing and thawing continued for up to 15 cycles. Following the completion of all freeze/thaw cycles, PCR reactions were prepared by combining primer/template cocktail mixtures. These mixtures consisted of 5 ng (200 ng for primers sets over 3.6 kb) genomic

Figure 1 – Platinum® PCR SuperMix 96 is functional through 15 freeze-thaw cycles



Targets tested were 264, 913, and 2021 bp from 5 ng genomic DNA or 4100 bp from 200 ng genomic DNA as template.

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DNA isolated from K562 cells and 0.2 μ M of the indicated primer set. PCR reactions were then pre-incubated at 94°C for 2 min. The reactions were further processed with 35 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C at 1 min/kb. The PCR products were analyzed by electrophoresis with 0.5X TBE and 0.8% agarose containing 0.5 μ g/ml ethidium bromide.

Effect of Additional Volume in PCR. Additional primer/template volume assays were conducted to determine how much additional volume could be added to each well while still maintaining quality PCR products. PCR reactions had been optimized (data not shown) to a final volume of 25 μ l by adding 2.5 μ l of a template/primers/water mix to the pre-aliquoted 22.5 μ l of SuperMix. DNA, isolated from K562 cells, was used as template (5-400 ng dependent on product size) in conjunction with 5 pmol of each primer. In all reactions, the template and primer mass amounts were held constant while the total volume of the reaction was adjusted with water. The combined primer, template, and water mixture was added to each well pre-filled with 22.5 μ l of Platinum® PCR SuperMix 96 in the amounts of 1, 2.5 (optimum), 5, 10, 15, or 20 μ l. Primer sets used for this assay included Pr.i.1 264, p53 913, Rhod 4.1 kb. Thermal cycling proceeded with a pre-incubation step of 94°C for 2 min., and the reactions were further processed by 35 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 1min/kb. The PCR

Table 1 – List of primers used with the Platinum® PCR SuperMix 96 system

Pr1.3 264 bp	Sense primer: 5'- GCATCTGGGGCCTGGGATTTAG Antisense primer: 5'- TACAAGGCAGGCATCATGACTCACG
Caaf1 300bp	Sense primer: 5'- GCCCCCACCCAGGTGGTTTCTA Antisense primer: 5'- ATGCCTTCATCTGGCTCAGTGAA
Rhodopsin gene 462 bp	Sense primer: 5'- GCC CTA ACT TCT ACG TGC CCT TCT Antisense primer: 5'- CAT TGA CAG GAC AGG AGA AGG GA
Rhodopsin gene 1282 bp	Sense primer: 5'- GGG CCC ACA GGA TGC AAT TT Antisense primer: 5'- TGC TCA CCA CCC CAT GAA GTT T
Rhodopsin gene 2021 bp	Sense primer: 5'- CTT CCC CCA TTC CCT GGA ATT T Antisense primer: 5'- CCC TGG TGG GTG AAG ATG TAG AA
Rhodopsin gene 2579 bp	Sense primer: 5'- CAA AGC CCT CAT ATA TTC AGT CAA CA Antisense primer: 5'- AGG CTT CCA GCG CAC GTC ATT
Rhodopsin gene 4100 bp	Sense primer: 5'- CCC ATC AAC TTC CTC ACG CTC TA Antisense primer: 5'- TGG GCT TCC AGA ACT AGC TGA CT
p53 gene 913 bp	Sense primer: 5'- CCT TGG CTT TTG AAA ATA AGC TCC TGA Antisense primer: 5'- TGT CAA CCA CCC TTA ACC CCT CC
p53 gene 1587 bp	Sense primer: 5'- GCT GCC GTG TTC CAG TTG CTT TAT C Antisense primer: 5'- GCA GCT CGT GGT GAG GCT CCC
pBR 1989 bp (plasmid)	Sense primer: 5'- GCA GTC AGG CAC CGT GTA Antisense primer: 5'- CTC ATC AGC GTG GTC GTG
pMOL21 4100 (plasmid)	Sense primer: 5'- biotin-AAA AAC GCG TCA CCA GTC ACA GAA AAG CAT CTT AC Antisense primer: 5'- AAA ACG CGT CAA CCA AGT CAT TCT GAG AAT AGT
bact 353 (cDNA)	Sense primer: 5'- GCT CGT CGT CGA CAA CGG CTC Antisense primer: 5'- CAA ACA TGA TCT GGG TCA TCT TCT C
GAPDH 532 (cDNA)	Sense primer: 5'- GTG AAG GTC GGA GTC AAC GGA TTT Antisense primer: 5'- CAC AGT CTT CTG GGT GGC ACT GAT
bact 1026 (cDNA)	Sense primer: 5'- ATG GCC ACG GCT TCC AGC TCC Antisense primer: 5'- ATT CAA CTG GTC TCA AGT CAG TGT A
CBP 1.6 (cDNA)	Sense primer: 5'- ATGGCGATCGTCGAACCGGA Antisense primer: 5'- CACTGTCTTAATATGAATGGGACCTACTGAG
THRC 2.7 (cDNA)	Sense primer: 5'- GCC CGC AAG CCC AAG CTC TAT CT Antisense primer: 5'- ACA GAG CCA TGG TCC ATC CGA GAG
HTF4 3.6 (cDNA)	Sense primer: 5'- GCC GAA GAT GAA TCC CCA GCA ACA A Antisense primer: 5'- TCC TTT CCT CTG TGT GCT GAT TGC CA

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products were visualized by electrophoresis using 0.5X TBE and 0.8% agarose containing 0.5 µg/ml ethidium bromide.

SuperMix PCR Applications—Template Variation. To demonstrate product reproducibility, Platinum® PCR SuperMix 96 was used to amplify targets of varying lengths in duplicate using either genomic DNA or cDNA. Plasmid DNA was also amplified by colony PCR. The primer sets used for genomic DNA analysis were Caaf1 300, Rhod 462, Rhod 1242, Rhod 1302, p53 1587, Rhod 1705, Rhod 2579, and Rhod 4100 (Table 1, page 15). PCR cycling was conducted using the standard protocol mentioned previously with slight variations in annealing and extension conditions as indicated. All reactions con-

tained 100 ng of K562 genomic DNA with the exception of Rhod 4100 where 200 ng was used. To confirm that Platinum® PCR SuperMix 96 was not limited to amplification of only purified genomic targets or cDNA, experiments were conducted in which amplified targets were isolated from plasmid DNA present in a transformed bacterial colony. The plasmids were transformed into DH5α™ *E. coli*, plated onto LB-ampicillin, and grown overnight. A colony was picked with a sterile pipette tip and dipped directly into the PCR reaction with no other treatment followed directly by PCR as previously described.

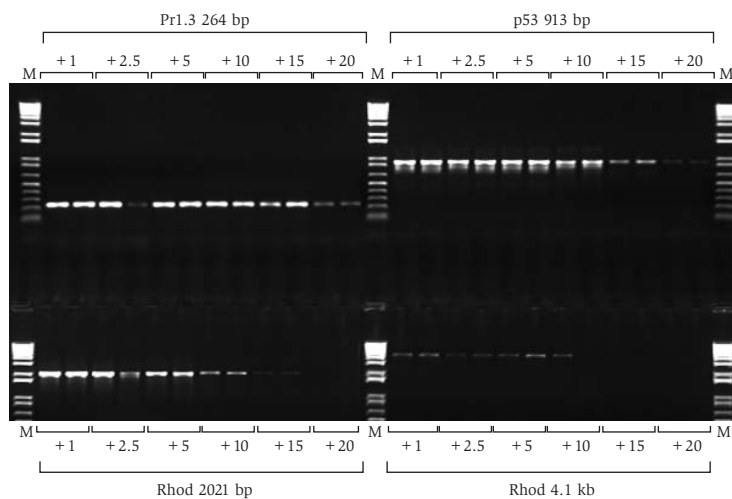
We isolated total HeLa RNA using the TRIzol® method (3) for cDNA comparison. Starting with total HeLa RNA (1 µg), cDNA

was prepared using SuperScript™ II Reverse Transcriptase (Invitrogen). From a 20-µl reverse transcription reaction, 2 µl was used in PCR. The cDNA primer sets used were β-act 353, GAPDH 532, β-act 1026, 1600, 2700, and HTF4 3.6. The PCR reaction final volume was 25 µl in the 96-well skirted plate. The PCR protocol was 94°C for 2 min; 35 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 1min/kb.

Results and Discussion

Stability of Enzyme in SuperMix Formulation. Platinum® PCR SuperMix 96 is a slight modification of the Platinum® PCR SuperMix pre-aliquoted into 96-well plates. We focused on ensuring reproducibility and maintaining the sensitivity of detection of the product. A concern for many SuperMix users may be the possibility of rapid enzyme activity deterioration following several freeze/thaw cycles. To address the possibility that freezing and thawing the SuperMix could negatively affect the reaction mix, we assessed the performance of Platinum® PCR SuperMix 96 following multiple freeze-thaw cycles. The supermix used to aliquot the 96-well plate of Platinum® PCR SuperMix 96 was pre-aliquoted into PCR reaction plates and subsequently placed at -20°C until frozen, a period of 30 minutes to overnight. For each cycle, the partially filled plate containing SuperMix was thawed to ambient temperature (30 min.) and additional wells were filled after the plate was warmed and subsequently returned to -20°C. After subjecting the 96-well pre-filled plate to multiple freeze/thaw cycles, PCR reactions were

Figure 2 – Additional volumes that can be added to the reaction



A range of volumes of a primer and template mixture were added to 22.5 µl of SuperMix. PCR products were seen with up to 20 µl of additional material added to the SuperMix for some primers. The absolute limit was 10 µl for Rhod 4.1.

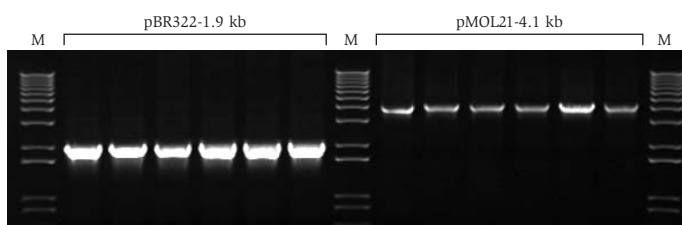
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prepared in a standard format and resulting products analyzed on an agarose gel. Platinum® PCR SuperMix 96 remained consistently stable after ten freeze-thaw cycles. This study was repeated with additional freeze/thaw cycles. Platinum® PCR SuperMix 96 maintains its stability even after 15 freeze/thaw cycles (Figure 1, page 14). Platinum® PCR SuperMix 96 is extremely stable and is suitable for use in the event that the plates or portions of the plate that had been separated at the perforations have been exposed to multiple freeze/thaw cycles.

Effect of Additional Volume in PCR. Often times a user may require the addition of a larger volume, for example when the template is not concentrated prior to its use. Alternatively, primers for the template may be too dilute for those suggested by the manufacturer. To address the volume limits or capacity of the reaction, we tested various volume sizes other than those recommended with the Platinum® PCR SuperMix 96 plates. Our interest was to determine a range of template and primer

Figure 4 – Amplification of plasmid DNA sequences detected from a bacterial colony transformed with plasmid DNA



PCR products are successfully amplified with Platinum® PCR SuperMix 96.

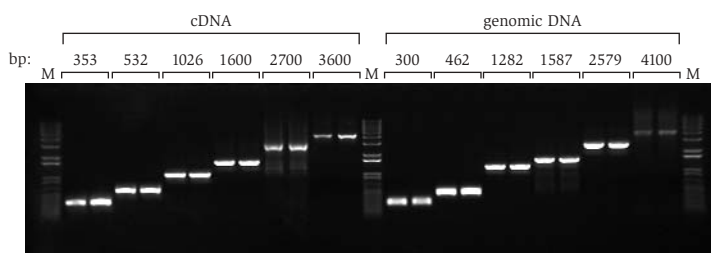
volumes that could be added to each well of the plate without negatively affecting the reaction. The amplification of four different sized targets was tested in standard reactions, where the standard addition of 2.5 µl of template and primers was compared to the addition of 1 to 20 µl template and primer while maintaining mass amounts in the reaction. In general, the amount of PCR product produced remained constant with the addition of up to 10 µl of template/primer mix, but fell when higher volumes were added

when attempting to amplify longer templates (Figure 2). We recommend additional volumes of up to 10 µl as acceptable.

Varied Template Amplification. To confirm that the 96-well format was useful in amplifying DNA targets other than genomic DNA, cDNA was amplified and evaluated after electrophoresis and compared to genomic DNA amplification (Figure 3). Products from cDNA were successfully amplified from 353 bp to 3.6 kb.

To further demonstrate the versatility of Platinum® PCR SuperMix 96, colony PCR was performed using two plasmids, pBR322 and pMOL21. A 1.9 kb product was amplified from pBR322 and a 4.1 kb product was amplified from pMOL21, both directly from transformed bacterial colonies. These results show robust amplification from five colony picks from transformed bacteria plated onto LB agar containing ampicillin. Platinum® PCR SuperMix 96 correctly amplified DNA products from bacteria transformed with either pMOL21 (4.1 kb product) or pBR322 (1.9 kb product) (Figure 4).

Figure 3 – Amplification of cDNA and genomic DNA targets using Platinum® PCR SuperMix 96



All targets were successfully amplified from a range of cDNA templates and a range of genomic DNA targets.

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Conclusions

We report the development and successful testing of the Platinum® PCR SuperMix 96 including freeze/thaw stability, effects of additional volume, and effectiveness using various templates. This 96-well format has all the benefits of Platinum® PCR SuperMix and the additional conveniences of a pre-aliquoted 96-well plate that is PCR-ready. The convenience is furthered by the fact that the user does not have to utilize the entire plate each time. An option is to use the non-skirted, perforated plate so that the unused portion can be placed at

+4°C or -20°C until the next use. We have demonstrated that Platinum® PCR SuperMix 96 has excellent freeze/thaw stability, in part, for this purpose. Alternatively, the skirted 96-well plate is ideal for robotics and high-throughput applications. Platinum® PCR SuperMix 96 can amplify targets up to 5 kb. The ability to assemble reactions at room temperature with ease and reliability make Platinum® PCR SuperMix 96 an ideal product for high-throughput PCR amplification as well as for benchtop applications and a strong addition to the Platinum® PCR product line.

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Thermal stability and cDNA synthesis capability of SuperScript™ III reverse transcriptase

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Abstract

SuperScript™ III reverse transcriptase (RT) is a genetically engineered mutant of Moloney murine leukemia virus (M-MLV) RNase H RT (also known as SuperScript™ II) that exhibits enhanced thermal stability for use at temperatures from 42°C up to 55°C. The RT half-life of SuperScript™ III is 220 minutes at 50°C compared to 6.1 minutes for SuperScript™ II. SuperScript™ III was able to synthesize a 9.5-kb full-length cDNA at 55°C. This enhanced thermal stability was achieved by increasing the intrinsic thermal stability of M-MLV RT, without diminishing the DNA polymerase catalytic activity of the enzyme. Processivity, fidelity, and catalytic constants remained relatively unchanged. SuperScript™ III RT exhibits significantly decreased non-template directed nucleotide addition, or TdT activity, for a more accurate transcript. As with SuperScript™ II, the RNase H mutation in SuperScript™ III results in higher yields and longer cDNA transcripts than RTs containing active RNase H. SuperScript™ III provides a tool for higher cDNA yield and synthesis of difficult RNA targets such as high GC and secondary structured templates.

M-MLV reverse transcriptase (RT) is widely used for the synthesis and cloning of cDNA. In addition to polymerase activity, retroviral RT possesses RNase H activity that degrades the RNA strand in an RNA:DNA hybrid (1). The presence of this degradative activity is responsible in part for the limitation on efficient synthesis of long cDNA (2,3). SuperScript™ II is an RNase H⁻ form of M-MLV RT, the RNase H domain of which was mutated to eliminate RNase H activity while maintaining complete mRNA-directed DNA polymerase activity (4,5), improving the efficiency of cDNA synthesis (4).

A second significant drawback to copying mRNA is the tendency of RTs to pause during cDNA synthesis, resulting in the generation of truncated products (6,7). This pausing is due in part to the secondary structure of RNA (6,8). Performing cDNA synthesis at reaction temperatures that melt the secondary structure of mRNA helps to alleviate this problem (9). In addition, the oligo(dT)_n primer often used to initiate cDNA synthesis tends to prime at internal stretches of A residues in mRNA at lower temperatures, resulting in the synthesis of 3'-end truncated cDNA products. M-MLV RT does not efficiently synthesize cDNA from mRNA above 43°C (10). SuperScript™ II can be used at temperatures up to 48°C because in the absence of RNase H activity, the mRNA template-DNA product complex is maintained during cDNA synthesis in a structural form that protects RT from thermal inactivation.

In an effort to raise the temperature at

which SuperScript™ II RT can be used to synthesize cDNA, we have randomly mutagenized the SuperScript™ II RT gene and screened for thermal stable mutants. A thermal stable mutant named SuperScript™ III (Invitrogen, Cat. no. 18080-044) was identified. Here we characterize the mutant and show that SuperScript™ III has increased RT thermal activity without altering catalytic activity.

Experimental Procedures

Half-life Determination. Mixtures (20 µl) containing 50 mM Tris-HCl (pH 8.4), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.05% (v/v) NP-40, and 0.2-0.5 unit/µl RT were incubated for various times in 0.5-ml tubes in a thermocycler at 50°C or 55°C. Incubation was stopped by placing the tubes in ice. An aliquot (5 µl) was assayed for residual activity with 5 mM poly(A)/3 mM oligo(dT)₂₅. The unit assay reaction contained 1X First-Strand Buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), 10 mM DTT, 3 mM MgCl₂ (total to 6 mM), 0.5 mM dTTP, [methyl-³H]dTTP (20 µCi/ml), and 5 µl of the heated RT aliquot. One unit of RT DNA polymerase activity is the amount of RT that incorporates one nmol of deoxynucleoside triphosphate into acid-insoluble product at 37°C in 10 min. The half-life is the time needed for the RT units activity to be reduced by 50%.

Specific Activity Assay. RT DNA polymerase unit activity was assayed with (rA)₆₃₀•p(dT)₁₂₋₁₈ (12). One unit of DNA polymerase activity is the amount of RT that incorporates one nmole of deoxynu-

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cleoside triphosphate into acid insoluble product at 37°C in 10 min.

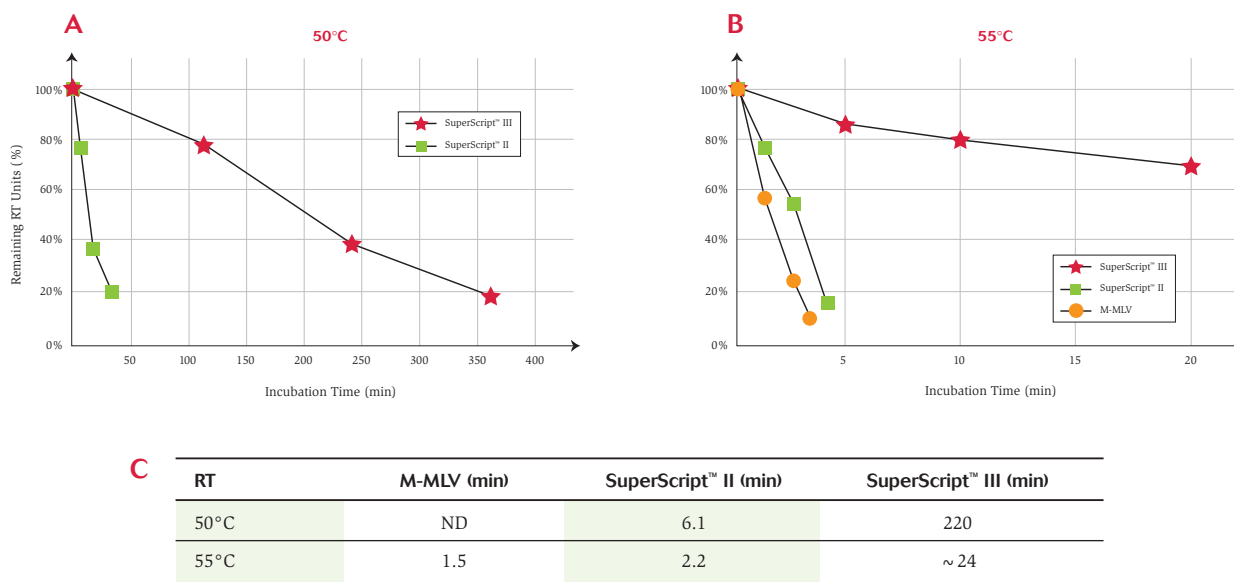
Processivity Assay. RT processivity was assayed on CAT cRNA annealed to a 22-mer DNA primer (5'-GCCTTGCGTA-TA-ATATTTGCC-3') [³²P]-labeled at the 5'-end with T4 polynucleotide kinase (template:primer, 2:1). The assay mixture (15 µl) containing 1.6 pmol template-primer, 250 µM dNTPs, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT was pre-heated for 5 minutes at 45°C. SuperScript™ II RT

(Invitrogen, Cat. no. 18064-022) and SuperScript™ III (Invitrogen, Cat. no. 18080-044) enzymes were serially diluted and 5 µl of each dilution added to the reaction mixtures, resulting in ratios from 1:10 to 1:20,000 enzyme:template-primer. The reaction was stopped after 3 minutes by addition of 5 µl of 50 mM EDTA, denatured by incubating at 85°C for 2 minutes and analyzed by electrophoresis on urea 6% polyacrylamide gels. Processivity was determined to be the average of extended bases at the dilutions where a single round of primer

extension predominated.

Steady-state Kinetic Measurements. The steady-state kinetic parameter, $K_m(dTTP)$, and catalytic rate constant, K_{cat} , were determined as described (15), using $(A)_n:(dT)_{30}$. A range of five [³²P]dTTP concentrations, which bracketed the $K_m(dTTP)$ value, were used for each determination of the kinetic parameters. The concentration of the template-primer and enzyme in the reaction were 2 µM (in primer termini) and 4 µM, respectively. Reaction mixtures (50 µl) also contained

Figure 1 – Half-lives of M-MLV, SuperScript™ II, and SuperScript™ III RTs at 50°C and 55°C



The half-lives were determined at 50°C (A) and 55°C (B) in the presence of 0.05% NP-40 using the reaction conditions described in Methods. (C) Summary of half-life data for the RTs at each temperature. The half-life is the time needed for the RT units activity to be reduced by 50%.

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50 mM Tris-HCl, pH 8.4, 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT and were incubated at 37°C.

Measurement of KD by Filter Binding. A nitrocellulose filter-binding assay (13,14) was used to determine the nucleic acid binding constants (K_D) of RTs for CAT cRNA•(dT)₂₀. Dephosphorylated CAT cRNA was labeled at the 5' end with [α -³²P]ATP and T4 polynucleotide kinase. Oligo(dT)₂₀ was annealed to the poly(A)-tailed CAT cRNA in a buffer containing 10 mM Tris-HCl, pH 7.5, and 80 mM KCl at 65°C for 5 minutes followed by chilling on ice. Reaction mixtures (100 μ l) containing binding buffer (50 mM Tris-HCl, pH 8.4, 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT), 0.05 nM ³²P-labeled CAT cRNA, 1 nM (dT)₂₀, and 1 to 50 nM RT were incubated at 23°C for 5 minutes. After incubation, the mixture was filtered through a nitrocellulose filter (Millipore, HA 0.45 mm) soaked in binding buffer, which was then washed once with binding buffer. The K_D is equal to that enzyme concentration at which 50% of the labeled CAT cRNA is bound. For this method of analysis to be valid, the CAT cRNA concentration in the reaction must be substantially below K_D , so that the total enzyme concentration approximates the concentration of free unbound enzyme.

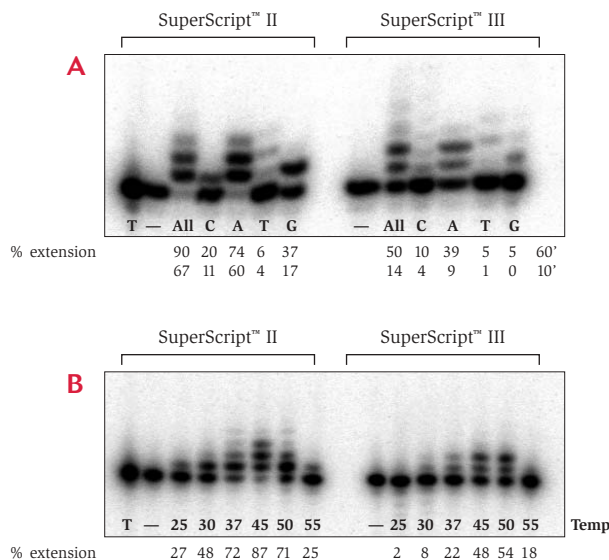
TdT Activity. The template-primer was prepared by annealing a 47-mer template (either RNA or DNA) 5'-GAGTTACAGTGT TTTTGTCCAGTCTGTAGCAGTGTGTGAATGGAAG-3' to an 18-mer DNA primer (5'-GAACAAAA-

CACTGTAAGTC-3') [³²P]-labeled at the 5'-end with T4 polynucleotide kinase (template:primer, 3:1). The assay mixture (10 μ l) contained 5 nM template-primer, 200 units each of either SuperScript™ II or SuperScript™ III, all 4 dNTPs, none, or dCTP, dATP, dTTP, dGTP (250 μ M each alone, as shown in the figure legends), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT. Reactions were incubated at various temperatures for 10 or 60 min. and terminated by the addition of 5 μ l of

40 mM EDTA, 99% formamide. Reaction products were denatured by incubating at 85°C for 2 minutes and analyzed by electrophoresis on urea 6% polyacrylamide gels. Percent extension of the primer was determined by band quantitation.

Full-length cDNA Profiling. cDNA synthesis from cRNA by SuperScript™ III was carried out in reaction mixtures (20 μ l) containing: 1X First-Strand Buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), 10 mM dithiothreitol (DTT),

Figure 2 – Extension of DNA primer on RNA template forming a blunt end



(A) Reaction performed at 45°C for 60 minutes. 200 units of enzyme used, 250 μ M each dNTP. T is Template-Primer only. Lanes marked (-) have T-P and enzyme but no dNTPs. Lanes marked (All) have all 4 dNTPs (250 μ M). Percent of primer extended also shown for reactions after 10-minute incubation. (B) Extension for 60 minutes at various temperatures of a labeled DNA primer on RNA template forming a blunt end. T is template only. Lanes marked (-) is T-P plus enzyme without dNTPs. Since SuperScript™ III is more thermostable, its TdT activity appears greater at 50°C than SuperScript™ II.

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500 μ M each of dATP, dTTP, dGTP, and dCTP, [α - 32 P]-dCTP (150-300 cpm/pmole), 40 units RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Cat. no. 10777-019), different amounts of RNA, and 0.5 μ g oligo(dT)₂₅. The mixes were pre-warmed at various temperatures for 2 minutes. After the addition of 200 or 400 units of SuperScript™ III RT, the mixtures were incubated at the same temperature for another 50 minutes in individual tubes. The reactions were stopped by the addition of 10 μ l of 0.5 M EDTA. An aliquot (5 μ l) of each reaction mixture was precipitated with 10% TCA to determine yield of cDNA synthesized and the remaining cDNA product was size fractionated on an alkaline 1.2% agarose gel (16). RTs from other vendors were assayed according to their own manual's suggestions.

Results

Thermal Stability of Purified SuperScript™ III RT. In order to characterize their intrinsic

Table 1 – Specific activity, catalytic constants, and nucleic acid dissociation constant of SuperScript™ II and SuperScript™ III RTs

RT	Specific Activity (U/mg)	K_m (mM)	K_{cat} (sec ⁻¹)	K_{cat}/K_m	K_D (nM)
SuperScript™ II	350,000	390 \pm 98	45 \pm 18	0.115	8 \pm 0.5
SuperScript™ III	350,000	274 \pm 92	46 \pm 11	0.17	11 \pm 1.0

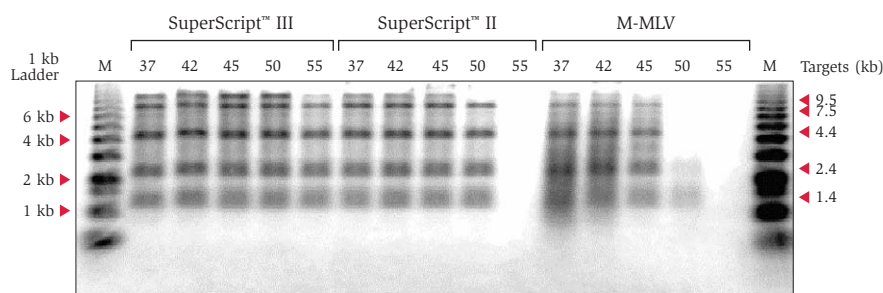
Mean \pm standard deviation of two or three determinations.

thermal stability, the half-lives of M-MLV, SuperScript™ II, and SuperScript™ III RTs were measured at elevated temperatures. The RT enzymes were first diluted to 0.2-0.5 units/ μ l in 1X First-Strand buffer in the presence of 0.05% NP-40. If diluted in the absence of detergent, the RT activity of all three is reduced to more than 90% without incubation (data not shown). Figure 1C (page 20) shows the half-lives of M-MLV, SuperScript™ II, and SuperScript™ III RTs. The half-life of SuperScript™ III at 50°C was 220 minutes, about 35-fold longer than that of SuperScript™ II. The half-life of

SuperScript™ III at 55°C was extrapolated to be approximately 24 minutes, about 10-fold longer than that of SuperScript™ II.

Enzymatic Characterization of SuperScript™ III RT. A number of catalytic properties of SuperScript™ III RT were compared to ascertain if mutations in the RT altered its DNA synthetic activity. The catalytic efficiency (K_{cat}/K_m) of SuperScript™ II and SuperScript™ III were within two-fold of each other and their K_m s for nucleotide substrate were similar (Table 1). The specific activity of both enzymes was similar at 350,000 U/mg. The processivity of the two enzymes were found to be similar at around 60 bases (data not shown). Fidelity was determined using the *rpsL* and *lacZ α* fidelity assays (17,18). The error rates using the *rpsL* assay were 1:32,000 for SuperScript™ III RT and 1:29,000 for SuperScript™ II. Using the *lacZ α* assay, the error rates were 1:22,000 for SuperScript™ III and 1:27,000 for SuperScript™ II. These results indicate that the mutations that substantially increased the thermal stability of SuperScript™ III did not appreciably affect its DNA polymerase catalytic capability. In the presence of a template-primer, the half-life of M-MLV H⁻ RT at 50°C is increased by a factor of 3 to 4 relative to the half-life in the absence of nucleic

Figure 3 – Full-length cDNA profiling using poly (A)-tailed RNA ladder



An autoradiograph is shown of 32 P-labeled cDNA synthesized from a mixture of 0.25 μ g each of 1.35 kb, 2.4 kb, 4.4 kb, 7.5 kb, and 9.5 kb and 0.5 μ g of oligo(dT)₂₅ with 200 units of each RT at various temperatures. Lane M is 32 P-labeled 1 kb DNA ladder.

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acids. This is the result of the enzyme being more resistant to heat inactivation when bound to template-primer than when unbound in solution. Mutations that impact the affinity of RT for template-primer will affect the apparent thermal stability of RT when it is engaged in cDNA synthesis. Therefore the affinities (K_D) of the SuperScript™ II and III RTs for CAT

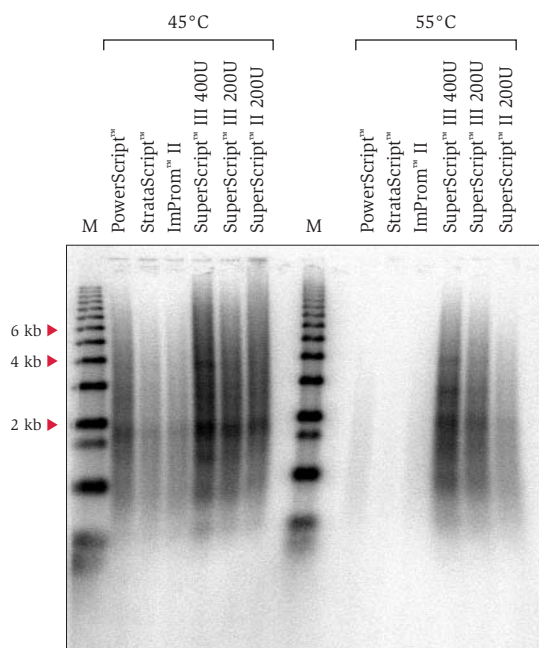
cRNA•(dT)₂₀ were determined (Table 1). The K_D of SuperScript™ III was increased somewhat from 8 nM to 11 nM, indicating that nucleic acid binding affinity was reduced slightly by the mutation. We conclude that when SuperScript™ III is engaged in cDNA synthesis, any increase in apparent thermal stability observed relative to SuperScript™ II is not due to

increased protection by virtue of binding more tightly to template-primer.

TdT Activity. TdT activity, or non-template directed nucleotide addition to the 3' primer end, is a well known property of many polymerases, including RTs (11). Using a DNA template, SuperScript™ II adds 1-3 additional nucleotides to the end of the transcript. SuperScript™ III RT has a greatly reduced TdT activity and, on a DNA template, will not add any detectable nucleotides (data not shown). On an RNA template, SuperScript™ II adds 1-2 bases with 69% of the primer extended after 10 minutes and 90% extended after an hour. SuperScript™ III RT exhibits significantly reduced TdT activity on an RNA template, with about 14% of the the primer extended after 10 minutes and 50% extended after 1 hour at 45°C (Figure 1A, page 20). The TdT activity is biased for both SuperScript™ II and III RT with the addition of dATP being strongly favored followed by dGTP, dCTP, and dTTP. TdT activity is temperature- and time-dependent (11). The optimal temperature for TdT activity for SuperScript™ III RT is 45°C to 50°C while for SuperScript™ II it is 45°C (Figure 1B, page 20). Reducing the extension time can further reduce TdT activity for both enzymes.

Full-length cDNA Profiling of RT. A practical method for judging the useful higher temperature of the DNA polymerase activity of RT is an assessment of the effect of increasing reaction temperature on the amounts of full-length cDNA products synthesized by RT from a mixture of

Figure 4 – Competitive audit for cDNA synthesis from total RNA



The cDNA synthesis reactions were performed in 20 μ l with 20 μ g of HeLa total RNA and 0.5 μ g of oligo(dT)₂₅. If provided, buffers and other components used were from their own kits plus 0.1 μ l of α -³²P-dCTP (10 mCi/ml). All the reactions were "hot start", 1 μ l of RT was added and incubated at 45°C or 55°C for 50 min. 400 or 200 units of SuperScript™ III RT were used for comparison. The reactions were stopped by the addition of 10 μ l of 0.5 M EDTA. Total cDNA synthesis was obtained by TCA-precipitation of 5 μ l of each mixture. After ethanol precipitation of the remainder of each mixture, the products were loaded onto a 1.2% agarose gel. The dried gel was exposed on the film for 1 hour.

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cRNAs of various lengths. The labeled full-length cDNA products can be separated and quantified on an alkaline agarose gel. As the reaction temperature is increased, full-length cDNA products disappear starting with those derived from longer cRNAs until a temperature is reached where no discernible full-length product of any length is synthesized. The results of such a gel analysis carried out at 45°C, 50°C, and 55°C are shown in Figure 2 (page 21) with the 0.24-9.5 Kb RNA Ladder (Invitrogen, Cat. no. 15620-016). SuperScript™ III RT shows great advantages at 50°C and 55°C for full-length 9.5 kb. SuperScript™ III can synthesize a 9.5 kb full-length band at both 50°C and 55°C while SuperScript™ II can synthesize up to 7.5 kb products at 50°C and MMLV produces only a faint 9.5-kb band at 45°C.

cDNA Synthesis From Total RNA. A competitive audit for cDNA synthesis from 20 µg HeLa total RNA is shown in Figure 3. It demonstrates the size distribution of cDNA synthesized from total RNA. The cDNA sizes synthesized by SuperScript™ III RT at 45°C and 55°C were from 0.5-10 kb.

Conclusion

By screening randomly mutated SuperScript™ II clones, we identified the thermal stable mutant SuperScript™ III. SuperScript™ III RT can synthesize full-length cDNA of at least 9.5 kb at temperatures up to 55°C. SuperScript™ III RT uses the same buffer conditions as SuperScript™ II and has very similar catalytic properties. Additionally,

SuperScript™ III RT has reduced non-tem-plate directed nucleotide extension activity. SuperScript™ III RT outperforms competitors' RTs with a strong advantage in producing cDNA at temperatures above 45°C. Use of SuperScript™ III RT at high temperatures can help overcome problems synthesizing difficult RNA targets with pauses at RNA secondary structures or high GC content, as well as reduce internal priming by oligo (dT)_n.

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Methods

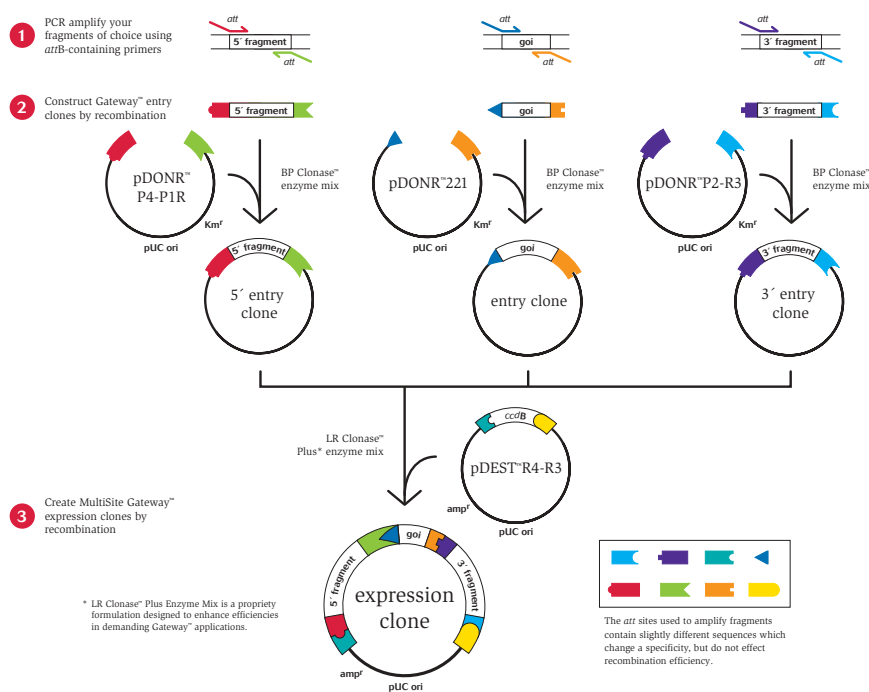
Construction of Gateway® Expression Clones. The MultiSite Gateway® Three-

Fragment Vector Construction Kit (Invitrogen, Cat. no. 12537-023) allows generation of a Gateway® expression clone containing specific 5' and 3' elements along with the gene of interest. This technology was used to create expression clones containing the GS2 (MAP kinase activated protein kinase) or GS5 (homologue of CSBP/p38 MAP kinase) genes. GS2 and GS5 were PCR amplified using *attB*-flanked gene-specific PCR primers. Each of the resulting PCR products was recombined into a Gateway® donor vector, pDONR™221, by a BP recombination reaction (page 27). A 5' entry element containing the CMV promoter and a 3' entry element encoding a

V5-His epitope tag were also amplified with primers including modified *attB* sites and the resulting PCR products recombined into the pDONR™P4-P1R and pDONR™P2-P3 vectors, respectively. The GS2 and GS5 entry clones were then recombined with both MultiSite Gateway® entry elements into the pDEST™R4-R3 vector via an LR reaction to generate expression clones (Figure 1).

In order to compare expression levels from clones generated using Gateway® Technology to that of clones generated without Gateway® Technology, GS2 or GS5 ORFs in the pcDNA™3.1/D-TOPO® vector were obtained from the expression-ready GeneStorm™ Clone Collection.

Figure 1 – How the MultiSite Gateway® Vector Construction Kit works



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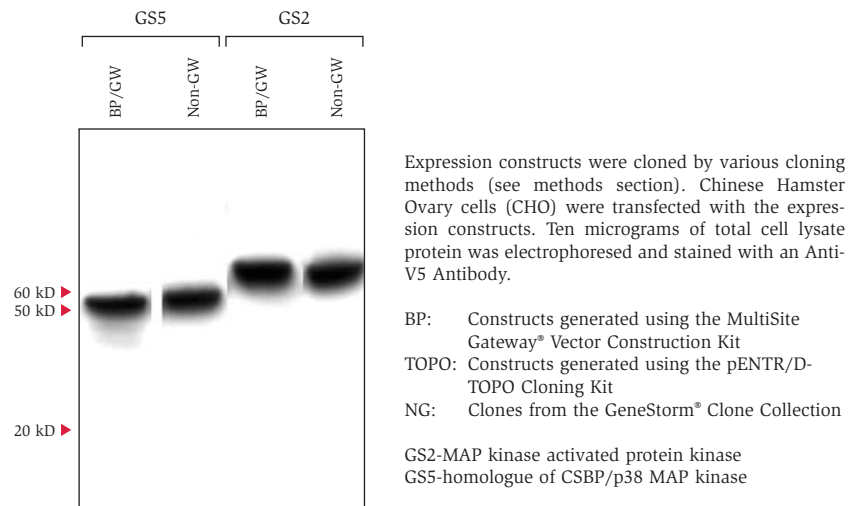
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These clones do not have the additional *att* sequence introduced via Gateway® or MultiSite Gateway® recombination (Figure 2).

Transfection. CHO cells were seeded at 100,000 cells/well in a 24-well plate one day before transfection. For each well of cells, 0.8 µg DNA was diluted into 50 µl of OptiMEM® media. Two microliters of Lipofectamine™ 2000 Reagent was diluted into 50 µl of OptiMEM® media. The diluted DNA and lipid mixtures were combined and incubated for 20 minutes at room temperature. The DNA-lipid complex was then added directly to the CHO cells, mixed gently, and incubated at 37°C in a CO₂ incubator for 48 hours.

Western Analysis. Growth medium was removed from the transfected cells. Cells were then lysed in 100 µl of lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0). Ten micrograms of the total pro-

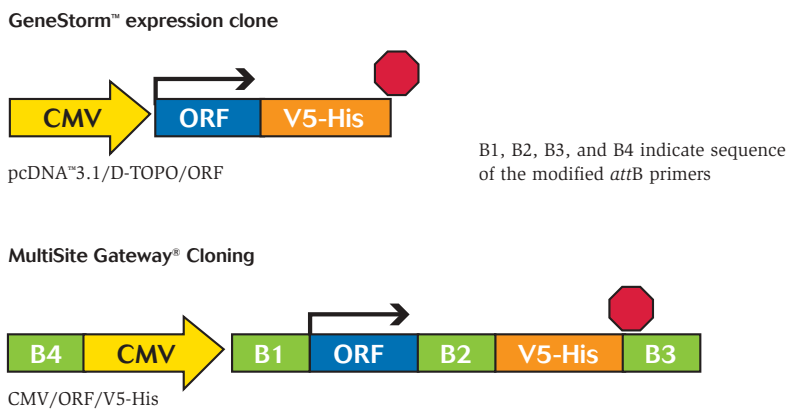
Figure 3 – Western analysis of GS2 and GS5 expression



tein was electrophoresed on a NuPAGE® 10% Bis-Tris gel. Proteins were transferred onto a PVDF membrane, using NuPAGE® transfer buffer, for 1 hour at 25

constant volts. The proteins were detected using an Anti-V5 Antibody (1:5000 dilution) and the WesternBreeze® Western Detection reagents and protocol.

Figure 2 – The composition of each expression clone



Results and Discussion

The expression of GS2 and GS5 was determined by western analysis using the Anti-V5 Antibody. As shown in Figure 3, expression results were comparable for all constructs, regardless of the cloning method used. The expression constructs generated via Gateway® recombination contain an additional peptide sequence representing an *attB2* site between the ORF and C-terminal tag. This peptide sequence did not affect the expression level or detection of the expressed protein. Furthermore, transcription was not affected by the presence of *attB1* between the CMV promoter and ORF.

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Conclusion

In conclusion, *attB* sequences were found to not interfere with transcription or translation of the expression constructs. Additionally, the entry clones generated in the MultiSite Gateway® Three-Fragment Vector Construction Kit did not affect GS2 or GS5 expression. Although not specifically shown in this study, with MultiSite Gateway® Technology, other 5' or 3' entry clones can be created for expression and detection with a variety of promoters, tags, or selection markers.

Traditional cloning procedures allow a gene to be cloned into a single vector designed for a specific experiment. For further analysis, the gene needs to be recloned and resequenced in each additional vector. The Gateway® Technology enables rapid transfer of genes from one vector to another, while maintaining the orientation and reading frame necessary for transcription and translation. The genes are transferred between vectors via recombination between various λ phage attachment (*att*) sequences. The *att* sites

do not interfere with transcription or expression. Once a Gateway® entry clone is created, the ORF can be seamlessly transferred into other expression or functional vectors to meet subsequent research needs.

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What are BP and LR reactions?

Gateway® Technology uses four recombination sites—*attB*, *attP*, *attL*, and *attR*—from phage lambda to transfer genes from one vector into another. *attB* sites always recombine with *attP* sites. This is known as a BP reaction. It is the basis for the reaction between the Gateway® entry vector and the PCR products containing *attB* sites. The *attB* x *attP*

recombination reaction yields *attL* and *attR* sites on the resulting molecules. *attL* sites always recombine with *attR* sites. This is known as an LR reaction. It is the basis for the entry clone x destination vector reaction. The *attL* x *attR* recombination reaction yields *attB* and *attP* sites on the resulting molecules. Note that cloning is directional.

The Gateway® Technology

BP Reaction



LR Reaction



In the BP reaction, the gene is transferred into an entry clone. This entry clone can be used as a reactor in the LR reaction to create the final expression clone.

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