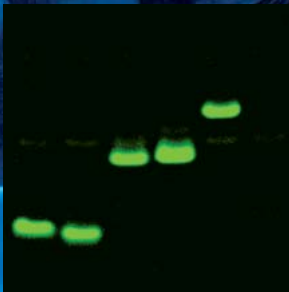


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Featured papers:

Specific, fluorescent detection
in *in vitro* expression
page 7

High-fidelity polymerase in a
ready-to-use SuperMix format
page 18

Identifying new transcription
start and polyadenylation sites
page 30



About The Cover:
 Photograph of moon jellyfish swimming in the ocean.

Data inset reflects specific, fluorescent detection of expressed protein using Lumio™ Technology. See page 7 for more information.

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Generation of linear templates using TOPO® Tools technology and subsequent expression *in vitro*

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Introduction

The use of *in vitro* expression systems has become increasingly popular as the first step in screening collections of open reading frames and cDNA libraries. However, the deterrent has traditionally been the time involved in subcloning the DNA into suitable circular expression constructs with the required regulatory elements. As an alternative, many researchers are turning to polymerase chain reaction (PCR) to generate linear templates for use with *in vitro* expression systems, but are still faced with time-consuming steps to add expression elements and are inevitably let down by low yields and poor biological activity.

The Expressway™ Linear Expression System is a prokaryotic *in vitro* expression system that has been designed specifically for use with linear DNA templates, generating higher yields and greater biological activity through enhanced template and transcript stability. This new system has a modified *E. coli* extract that has been developed to exhibit low exonuclease activity and contains known stabilizers of RNA transcripts. Researchers can now generate significant yields of active enzyme *in vitro* starting from linear DNA templates.

The Expressway™ Linear System also comes with an optional TOPO® Tools module that utilizes TOPO® Tools technology in combination with PCR to generate suitable linear templates for *in vitro* expression in only two PCR reactions. In the first reaction, a TOPO® adapter sequence (a specific 11 base pair-sequence containing a topoisomerase I recognition site) is added to the ends of

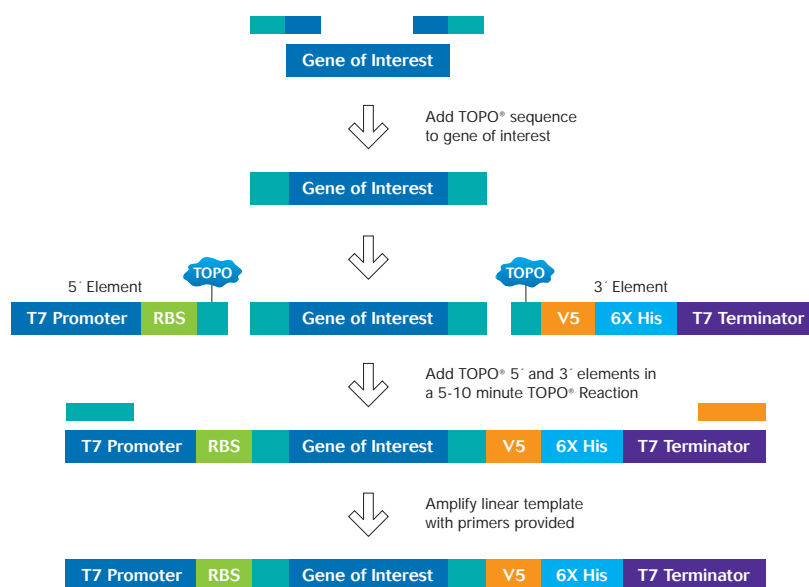
the gene of interest, allowing subsequent directional TOPO® joining of the PCR product to the TOPO® Tools 5' and 3' elements. The 5' element contains a T7 promoter, a prokaryotic ribosomal binding site (Shine-Dalgarno sequence) and start codon, and the 3' element contains a V5 epitope, a 6xHis tag, and a T7 terminator. Both elements are adapted with topoisomerase I and facilitate rapid ligation of the PCR product to each element, generating the linear DNA template in a short 5-10 minute reaction. This template is then amplified in a second PCR reaction using the primers provided, and may be used directly in the Expressway™ Linear System (Figure 1).

Materials and Methods

Preparation of Linear Templates. Linear

DNA templates for four different proteins (Green Fluorescent Protein (GFP), Chloramphenicol Acetyl Transferase (CAT), β -Glucuronidase (GUS), and β -Galactosidase (β -gal)) were generated using both the TOPO® Tools module and by amplifying directly from expression vectors already containing the appropriate regulatory sequences, as outlined in the Expressway™ Linear instruction manual. First, specific primers were designed for each gene of interest. For the TOPO® Tools templates, primer sets contained 18-20 nucleotides specific to the gene of interest and a 5' TOPO® adapter region of 11 nucleotides. The gene of interest was amplified in a 100- μ l reaction mixture containing 1X High Fidelity PCR buffer, 200 mM dNTPs, 2 mM MgSO₄, 200 ng each primer, 20 ng DNA template, and

Figure 1 – Generation of linear templates using the TOPO® Tools module



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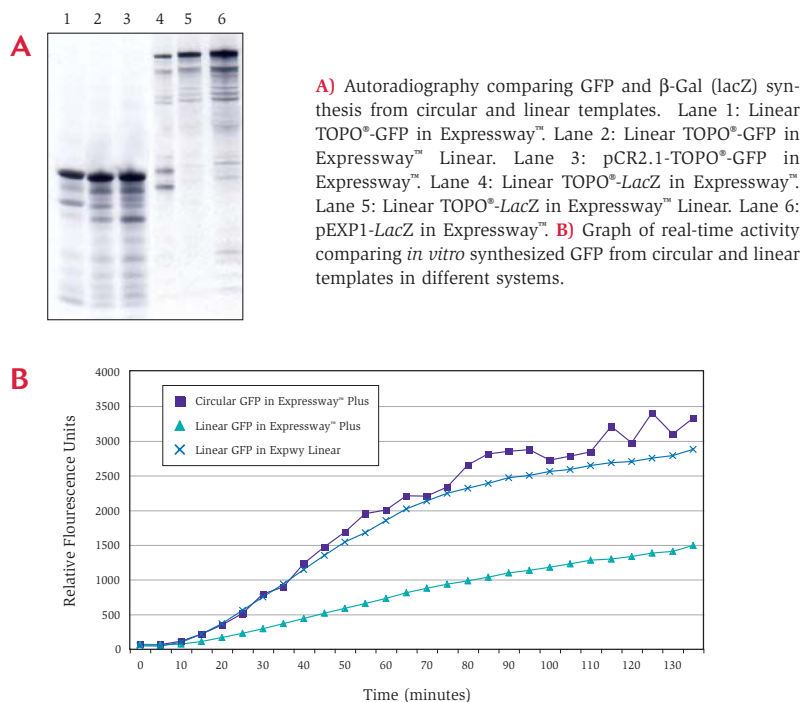
5 U Platinum® *Taq* DNA Polymerase High Fidelity. The reactions were performed using the following conditions: 4 minutes at 94°C; 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 68°C; 30 cycles. The elongation time was adjusted according to the length of the amplified segment (1 minute per kb).

The PCR products were purified using the S.N.A.P.™ MiniPrep Kit (Invitrogen) and quantitated by UV absorbance at 260 nm. Next, 20 ng of each TOPO® charged 5' and 3' element was incubated for 5 minutes at room temperature with 200 ng of the purified PCR product in a 5- μ l TOPO® reaction containing 50 mM Tris-HCl pH 7.5. The entire 5- μ l TOPO® reaction was then used in a second round of PCR to amplify the linear DNA template. The reaction conditions were the same as for the first round except the entire TOPO® ligation mixture was used instead of 20 ng of template DNA, and a forward primer for the 5' element and reverse primer for the 3' element were used as the primer set.

For linear template generation without using the TOPO® Tools module, primers consisted of 20-25 nucleotides flanking the T7 promoter and T7 terminator regions of the selected expression vector. PCR reactions were performed under the same conditions as outlined above. In general, the PCR reactions were used directly, however, in some cases, the linear templates were purified for long-term storage using the S.N.A.P.™ MiniPrep Kit (Invitrogen).

In Vitro Expression of Linear Templates. For standard Expressway™ Linear protein synthesis reactions, 20 μ l 2.5X IVPS Plus *E. coli* Reaction Buffer, 20 μ l of IVPS

Figure 2 – Yield and activity from linear templates is comparable to circular templates



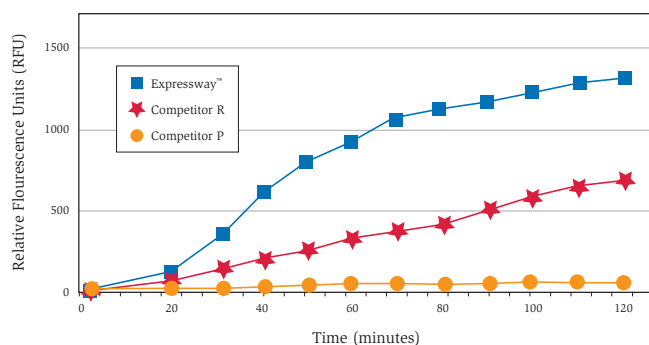
Linear *E. coli* extract, 1 μ l T7 Enzyme Mix-Linear, and 1 μ l of 75 mM methionine were pre-mixed on ice. Either 0.5-2 μ g of purified linear PCR template or 2-5 μ l of unpurified PCR product were added and the final volume of the reaction was brought up to 50 μ l with water. Reactions were incubated at 37°C for 2 hours in a Thermomixer or placed in 96-well plates in the fluorometer. After incubation, 5 μ l of RNase A (1 mg/ml) was added and reactions were incubated 15 more minutes. Competitor translation reactions were performed according to the manufacturers' instructions except a final concentration of 1.5 mM methionine was added.

Radiolabeled Reactions. Expressway™ Linear reactions were performed in the presence of 15 μ Ci [³⁵S] methionine (3,000 Ci/mmol, 15 μ Ci/ μ l). Total counts were determined by spotting 5 μ l of the reaction on individual glass filters and counting directly. Precipitable counts were determined by placing 5 μ l of RNase A-treated protein synthesis reactions into glass tubes, adding 10% TCA solution and incubating the tubes at +4°C for 20 minutes. After incubation, the precipitated proteins were passed over glass filters (grade 34 glass fiber) in a filtering apparatus, washed with 5% TCA, rinsed with 100% ethanol, and counted in the scintillation counter. Yield

continued on page 5

continued from page 4

Figure 3 – Competitive audit of linear *in vitro* transcription/translation



Real-time activity of *in vitro* synthesized GFP in three Linear IVTT systems. Real-time GFP activity was measured from 50- μ l protein synthesis reactions in 96-well plates at 37°C using a Molecular Devices Spectra Max GeminiXS Fluorometer (excitation 395 nm; emission 504 nm).

was determined as described in the Expressway™ Linear manual.

SDS PAGE. Gel samples were prepared by precipitating 5 μ l of the 50- μ l reactions in 20 μ l of 100% acetone and incubating at +4°C for >20 minutes. After centrifugation, the supernatant was removed and the pellet was resuspended in 20 μ l of 1X LDS sample buffer with reducing agent. The samples were heated for 10 minutes at 70°C, and run on 4-12% NuPAGE® Bis-Tris gels. Gels were stained with Coomassie® brilliant blue, dried, and exposed to MR film.

GFP and β -galactosidase Activity Assays. Real-time GFP activity was measured from 50- μ l protein synthesis reactions in 96-well plates at 37°C using a Molecular Devices Spectra Max GeminiXS Fluorometer (excitation 395 nm; emission 504 nm).

Fluorescence readings were taken at 10-minute intervals over a two-hour incubation period. Beta-galactosidase activity was measured from 1 μ l of 50 μ l protein synthesis reactions using Clontech's Luminescent β -galactosidase Reporter System 3 kit according to the manufacturer's instructions.

Results and Discussion

Linear Template DNA. Linear templates generated from direct amplification of plasmid DNA were compared to linear templates generated using the TOPO® Tools module. Both sets of templates contained identical sequence tags and differed only in the linker region between the gene of interest and expression elements (plasmid DNA constructs contained *attR* sites and TOPO® Tools templates contained the TOPO® recognition sequence). Linear template DNA

generated by both methods performed similarly, indicating that the presence of the TOPO® recognition sequence does not affect final protein yield or activity (data not shown). Use of the TOPO® Tools module allows convenient addition of required prokaryotic expression elements to any gene.

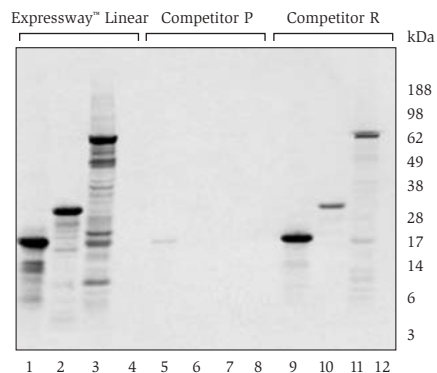
The linear template DNA does not need to be purified to perform in the Expressway™ Linear System, producing faster results. However, long-term storage of PCR reactions is not recommended, and PCR products should be purified to prevent degradation. The system can tolerate a wide concentration range of template DNA from 0.5-2 μ g and up to 8 μ l PCR reaction, making lengthy gel-based quantitation unnecessary.

Expression from Linear DNA Templates can Equal that of Circular Templates. Expression levels from linear templates have traditionally been lower when used with *in vitro* transcription/translation (IVTT) systems because these have been optimized for use with circular vectors. Expressway™ Linear is the first IVTT system to generate significant levels of synthesized product and activity from linear DNA templates. This system can generate up to 600 μ g/ml of active protein from linear templates. Synthesis and activity of GFP and β -galactosidase were compared from circular and linear templates in both the Expressway™ Plus and Expressway™ Linear Systems. Yields of full-length protein, as determined from [³⁵S] Methionine incorporation, TCA precipitation and autoradiography, were comparable (Figure 2A, page 4). The activity of GFP made from linear DNA in

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continued from page 5

Figure 4 – Competitive audit of linear *in vitro* transcription/translation



Expression of 500 ng purified linear TOPO®-GFP, CAT or GUS template DNA was compared in three commercially available expression kits by autoradiography. Approximately 1 μ l of each reaction was analyzed. Lanes 1,5,9: GFP. Lanes 2,6,10: CAT. Lanes 3,7,11: GUS. Lanes 4,8,12: No DNA control.

Expressway™ Linear was similar as well, indicating that the specific activity of the synthesized product, whether from circular template in Expressway™ Plus or linear template in Expressway™ Linear, is not affected (Figure 2B, page 4).

Competitive Audit of Linear IVTT Systems. The Expressway™ Linear System was compared to two competitor products currently available on the market. One vendor has a separate system for linear templates and the other offers a single system for both lin-

ear and circular templates. All three were evaluated for expression levels of CAT, GFP, and Gus and enzymatic activity of GFP. Expressway™ Linear clearly outperformed its competitors in both yield and activity (Figures 3, page 5, and 4).

Conclusions

Expressway™ Linear is the first true linear IVTT system to generate the quantities of soluble, active protein that the modern researcher demands. Yields can equal that of traditional systems while offering the convenience of synthesis from PCR-generated templates. All the required elements for transcription and translation can be added onto any gene adapted for use with TOPO® technology in a simple 5-minute incubation. Expressway™ Linear will soon become the standard first step for any researcher wishing to evaluate prokaryotic expression from their library collections.

Rapid detection of *in vitro* expressed proteins using Lumio™ Technology

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Introduction

In *in vitro* protein synthesis systems are known to be a quick and efficient means of expressing proteins while circumventing problems that can arise from *in vivo* expression of genes (1,2). Invitrogen's Expressway™ Plus Expression System is a straightforward and easy method to obtain high yields of functionally active protein (3). We now report an improved Expressway™ Plus System for the rapid detection of protein products using Lumio™ Technology. Lumio™ Technology is based on FLAsH, a biarsenical derivative of fluorescein that binds to an engineered tetracysteine sequence (Figure 1) (4). The tetracysteine sequence consists of Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa is any non-cysteine amino acid. Typically Xaa-Xaa is chosen to be Pro-Gly due to its reported pico-molar dissociation constants with FLAsH (5).

Lumio™ Technology coupled with a modified Expressway™ Plus System results in the rapid and easy detection of *in vitro* expressed proteins. The key elements of this kit are a modified cell-free extract (derived from an *E. coli* slyD mutant strain), pEXP3-DEST vector, and Lumio™ Green Detection Reagent. The versatility of the Lumio™ Technology allows co-translational monitoring of protein production during the Expressway™ Plus *in vitro* synthesis reaction. The Lumio™ Reagent has the unique characteristic of undergoing a marked transition from a virtually non-fluorescent state to a highly fluorescent state upon binding to a tetracysteine sequence. By taking advantage of this novel characteristic, real-time analysis of protein accumulation can be observed. Using a standard fluorescence

plate reader, the real-time incorporation of the Lumio™ sequence into newly synthesized proteins can be monitored directly in the *in vitro* reaction mixture. Due to the site-specific binding of the Lumio™ Green Detection Reagent, quantitative analysis of protein expression levels is possible.

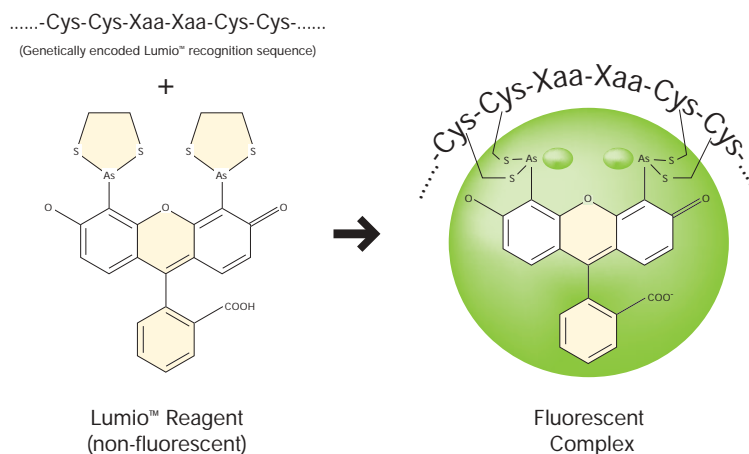
Alternatively, Lumio™ Green Detection Reagent can be directly added to protein samples before electrophoresis. This permits the visualization of protein products immediately after electrophoresis with a standard UV light box or a laser gel scanner, without the need for radioactivity. The robust, covalent attachment of the Lumio™ Reagent to the tetracysteine sequence eliminates any requirements for protein gel manipulation, such as the need to fix, stain, destain, or dry. In addition,

all safety, waste disposal, and regulatory issues associated with the use of radiolabeled amino acids are abolished. The Lumio™ Technology provides tremendous advantages for the detection and analysis of *in vitro* protein synthesis products and is likely to revolutionize the way protein expression is evaluated in cell-free systems.

Materials and Methods

Construction of pEXP3-DEST Expression Clones. The pEXP1 plasmid was used to create the N-terminal pEXP3-DEST vector and TEV protease cleavage site. Ultimate™ Human ORF kinase entry clones, CAT, GFP, and GUS were recombined with the pEXP3-DEST vector in single tubes by combining the entry clone, destination vector, and LR Clonase™ enzyme mix. After incu-

Figure 1 – Schematic diagram of Lumio™ Reagent binding to a tetracysteine sequence



In the unbound state, Lumio™ Reagent is essentially non-fluorescent, however, upon binding to a tetracysteine motif, Lumio™ Reagent becomes highly fluorescent. When bound to a tetracysteine-tagged peptide or protein, Lumio™ Reagent has peak absorption at 505 nm and maximum green-colored fluorescence emission at 528 nm.

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bating for one hour, the mixture was transformed into chemically competent TOP10 *E. coli* cells, plated, and colonies picked for amplification and DNA isolation.

Protein Synthesis. For standard Expressway™ Plus protein synthesis reactions, 4 µl DNase/RNase-free water, 20 µl 2.5X IVPS Plus *E. coli* Reaction Buffer, 1 µl T7 RNA polymerase, and 20 µl IVPS Plus *E. coli* extract were pre-mixed in

2-ml tubes on ice. One microgram of DNA templates was added and the final volume of the reaction brought to 50 µl with water and mixed thoroughly. Reactions were incubated at 37°C for 2 hours in a thermomixer or placed in 96-well plates in the fluorometer. Adenylate kinase 1 was produced *in vivo*.

In-gel Detection. Gel samples were prepared by precipitating 5 µl of the 50-µl

reactions into 20 µl 100% acetone and incubated at +4°C for >20 minutes. Reactions were centrifuged at maximum speed in a microcentrifuge for 5 minutes, acetone was aspirated, and the pellets were resuspended in 50 µl of 1X Lumio™ Green Detection Reagent. One microliter of this material was loaded onto 4-12% NuPAGE® gradient gels. Gels were visualized using a UV light box or Typhoon 8600 Variable mode Imager. For total protein profiling, gels were post-stained with Coomassie® brilliant blue.

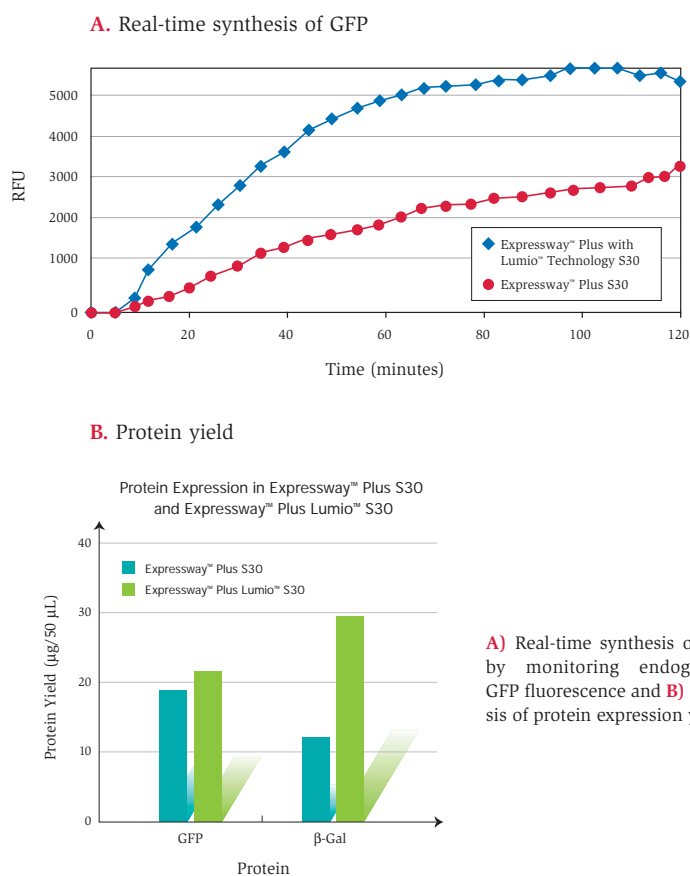
Real-time Detection of Protein Expression.

Real-time incorporation of the Lumio™ sequence was measured directly from 50-µl *in vitro* protein synthesis reactions with 20 µM Lumio™ Green Detection Reagent in a 96-well plate at 37°C using a Molecular Devices Spectra Max GeminiXS plate reader. The excitation wavelength was set at 500 nm, while emission was monitored at 535 nm. Readings were collected at 10-minute intervals over a 2-hour incubation period. Real-time monitoring of GFP production was performed in a similar manner without the addition of Lumio™ Green Detection Reagent.

Results and Discussion

Expressway™ Plus Expression System with Lumio™ Technology. The Expressway™ Plus system was re-engineered to maximize Lumio™ labeling technology through the construction of an *E. coli* slyD mutant strain. Construction of this mutant strain reduces non-specific binding of the Lumio™ Detection Reagent to endogenous SlyD protein, providing an optimal background for detection of tetracycline-tagged proteins. In addition,

Figure 2 – Comparison of extracts from *E. coli* strains Expressway™ Plus S30 and Expressway™ Plus with Lumio™ Technology S30



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it has been reported that SlyD is the major contaminant in His-tag protein purification (6). This makes the Expressway™ Plus Lumio™ cell-free extract ideal for the downstream purification of His-tagged proteins.

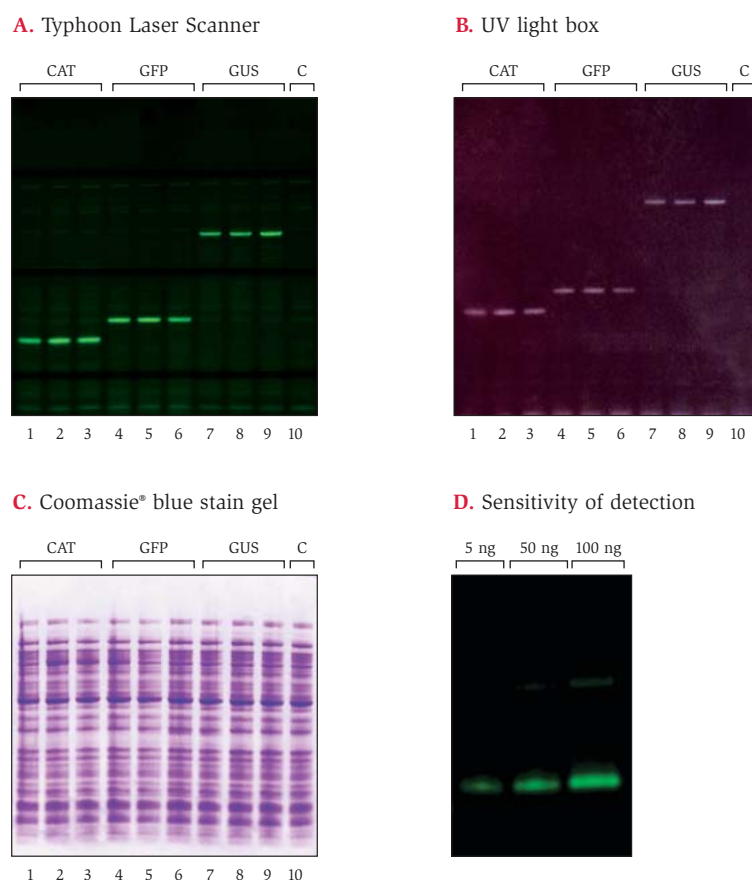
Functional Evaluation of S30 Extract from the *E. coli* SlyD Mutant Strain. To evaluate the performance of the translational extract from the *E. coli* slyD mutant strain, a comparison to Expressway™ Plus S30 extract was performed. The translation, folding, and chromophore maturation rate of Green Fluorescent Protein (GFP) was measured in S30 from the *E. coli* slyD mutant strain and compared to the current *E. coli* S30 extract. Based on the fluorescence of GFP, Figure 2A (page 8) reveals that the *E. coli* slyD mutant strain yields about two-fold higher functional GFP than the current S30 extract. Protein yields of GFP and β -galactosidase were also examined in S30 from the *E. coli* slyD mutant strain and current S30 extracts. In both cases higher protein expression levels were achieved in the S30 extract from the *E. coli* slyD mutant strain (Figure 2B, page 8).

In-gel detection. To test the ability of the Lumio™ Reagent to highlight proteins synthesized with the Expressway™ Plus system, tetracysteine-tagged chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), and glucuronidase (GUS) were expressed *in vitro*. The expressed proteins were then labeled with the Lumio™ Green Detection Reagent, separated by electrophoresis, and imaged with both a Typhoon laser scanner and a standard UV light box

(Figures 3A and 3B). In dramatic fashion, the tetracysteine-tagged proteins stand out against a low background signal in both images. Comparing the results of the fluorescent images to the total protein profile (Figure 3C) illus-

trates the sensitivity and ease of detection using the Lumio™ Reagent with the Lumio™ sequence. Figure 3D demonstrates that as little as 5 ng of purified Adenylate kinase 1 can be easily detected in-gel using the Lumio™ Detection

Figure 3 – In-gel detection of proteins synthesized using Expressway™ Plus Expression System with Lumio™ Technology and Lumio™ Green Detection Reagent



Gel imaged with **A)** Typhoon 8600 Variable mode Imager, **B)** standard UV light box, and **C)** white-light for total protein profile with Coomassie® blue stain. Lanes 1-3: CAT; Lanes 4-6: GFP; Lanes 7-9: GUS; Lane 10: no DNA control reaction. **D)** Analysis of the sensitivity of Lumio™ Green Detection Reagent using purified Adenylate Kinase 1. CAT, GFP, and GUS were expressed from pEXP3-DEST plasmid.

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Reagent. In contrast to modified amino acid methods, the consistent 1:1 stoichiometry of the Lumio™ Reagent binding to tetracysteine sequences results in uniform labeling of proteins and, perhaps, allows quantitative assessment of protein expression levels independent of radioactivity.

Real-time Incorporation of the Lumio™ Reagent. Monitoring real-time protein production was performed directly in cell-

free extract reactions with Lumio™ technology. *In vitro* transcription/translation reactions were set up as usual (see materials and methods) and then Lumio™ Green Detection Reagent was added to the reactions prior to 37°C incubation. As the reaction proceeded, an increase in fluorescence was monitored with a standard fluorometer (Figure 4A). Approximately 2-3 fold increase in fluorescence of the Lumio™ sequence is seen compared to a vector DNA control. In-gel detection of

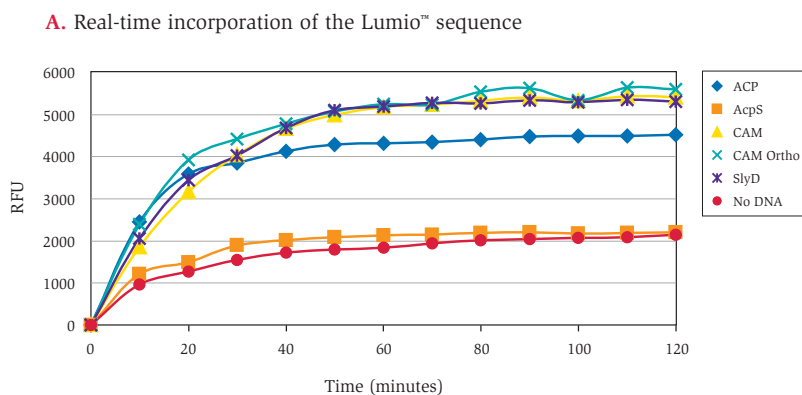
the co-translationally monitored proteins is shown for comparison in Figure 4B.

Screening Ultimate™ Human ORF Clones. The easy detection of proteins expressed using the Expressway™ Plus System with Lumio™ Technology was demonstrated using Gateway® Technology and the Ultimate™ Human ORF clone collection. Human ORF clones were recombined into pEXP3-DEST using Gateway® Technology, expressed in Expressway™ Plus Lumio™, detected in-gel, and monitored in real-time with the Lumio™ Detection Reagent (Figure 5, page 11). This result demonstrates that the Lumio™ Detection Reagent is a rapid and efficient method for detecting gene expression and eliminates the need for antibody production and radioactivity.

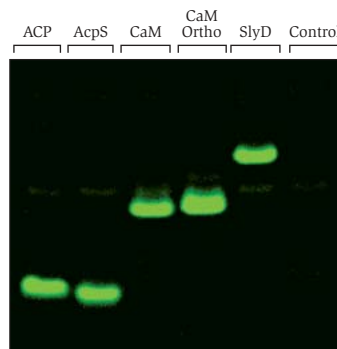
Conclusions

This newly designed Expressway™ Plus Expression System with Lumio™ Technology has tremendous advantages in the detection and analysis of *in vitro* expressed proteins. The striking transition to a highly fluorescent species upon tetracysteine binding combined with minimal background labeling of endogenous proteins makes the Expressway™ Plus System with Lumio™ Technology a powerful technique for detecting *in vitro* expressed proteins while still maintaining protein activity. Although Expressway™ Plus System with Lumio™ Technology is uniquely formulated to be ideally suited with the Lumio™ Green Detection Reagent, the original Expressway™ Plus System is also compatible with Lumio™ Technology. We have shown that the Expressway™ Plus System with Lumio™ Technology is

Figure 4 – In-gel detection and real-time incorporation of the Lumio™ sequence into *in vitro* synthesized proteins



B. In-gel detection



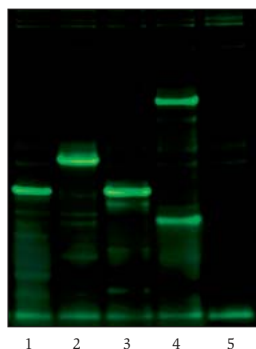
A) Real-time incorporation of Lumio™ to *in vitro* synthesized proteins. These proteins were expressed from the plasmid: pRSETb. **B)** In-gel detection of expressed proteins; ACP (Acyl carrier protein with C-terminal CCPGCC), AcpS (Acyl carrier protein S protein with C-terminal CCGGKNGGGCGC), CaM (Calmodulin with N-terminal CCEQCC), CaM Ortho (Calmodulin with N-terminal CCEQCC and C-terminal CGPCCGPC), and SlyD (full-length SlyD with naturally occurring C-terminal CCGGKNGGGCGC).

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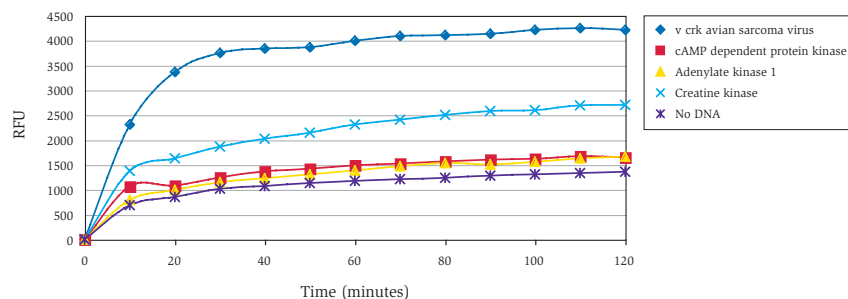
Figure 5 – Expression of Human ORFs in Expressway™ Plus System with Lumio™ Technology using pEXP3-DEST Vector

A. In-gel detection



A) In-gel detection of expressed proteins. Lane 1: v-crk avian sarcoma virus, 2: cAMP-dependent protein kinase, 3: adenylate kinase, 4: creatine kinase 5: no DNA control. **B)** Real-time incorporation of Lumio™ Green Detection Reagent into *in vitro* synthesized proteins.

B. Real-time incorporation of the Lumio™ sequence in Human ORFs



amendable for detecting proteins both in gels and in real-time by monitoring Lumio™ fluorescence. Finally, the unique site-specific binding of a single Lumio™ Reagent molecule to a tetracysteine sequence opens up the possibility for quantitative analysis of protein expression levels without the need for radioactivity.

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Enhanced TEV Protease extends enzyme stability for long-term activity

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Introduction

Additional sequences are commonly added to recombinant proteins to facilitate purification, improve detection, enhance solubility, or specify cellular localization. Frequently, it is unnecessary or undesirable to have a fusion moiety for subsequent applications. A protease site is usually included between the fusion moiety and the protein of interest so that the fusion moiety can be removed following purification. Proteases used for this cleavage, such as Thrombin and Factor Xa, typically have low specificity and are relatively inefficient.

TEV protease is an extremely efficient and highly specific protease useful for the removal of solubility and affinity tags from fusion proteins. TEV recognizes a seven amino acid sequence and cleaves it with high specificity. The consensus TEV protease recognition sequence is Glu-Asn-Leu-Tyr-Phe-Gln-Gly and is cleaved between Gln↓Gly (1-4,8,9). TEV protease is highly active over a broad range of temperatures (+4°C to 30°C) and pH (pH 6.0 to pH 8.5) (6). The polyhistidine tag at the N-terminus of TEV is useful for the removal of the protease after the cleavage reaction is complete.

Although TEV is highly specific for a seven amino acid recognition sequence, a drawback to the wild-type sequence of TEV protease is that it contains a cryptic auto-proteolysis site (5,7). The truncated version of TEV has a significantly reduced activity (5,7). An enhanced version of TEV protease (AcTEV™ Protease) was engineered to have significantly enhanced stability (resistance to self cleavage) without any change in perform-

ance. This results in long-term activity, a unique feature of AcTEV™ Protease.

Materials and Methods

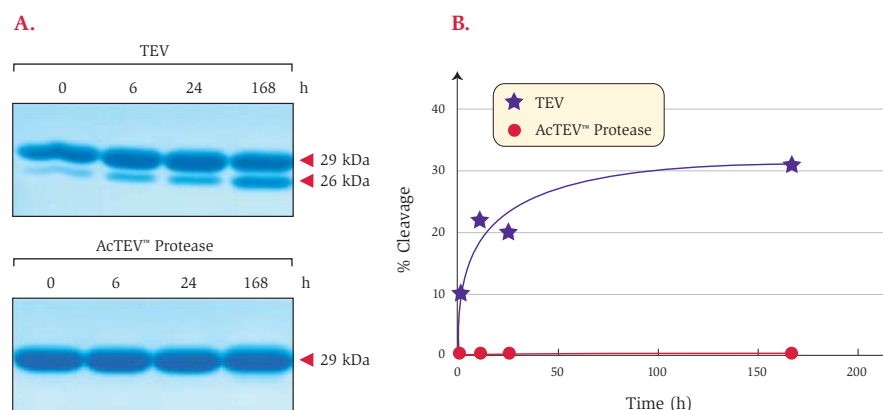
Unit Definition and Control Substrate. One unit of TEV protease cleaves $\geq 85\%$ of 3 μg of Control Substrate in 1 h at 30°C. The Control Substrate is a 30 kDa fusion protein that generates a 26 kDa GST fragment and 4 kDa peptide upon cleavage by TEV protease.

Stability Assay. Two hundred-fifty units of AcTEV™ Protease (Invitrogen, Cat. no. 12575-015) or TEV Protease were incubated in 1X TEV reaction buffer supplemented with 1 mM DTT at +4°C in a reaction volume of 100 μl . Aliquots (15 μl) were removed at the indicated times and inactivated with an equal vol-

ume of 2X SDS-PAGE loading dye. The inactivated aliquots were stored at -20°C until a fraction of each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel, Invitrogen, Cat. no. EC60255BOX) and SimplyBlue™ SafeStain (Invitrogen, Cat. no. LC6060). The gels bands were quantified using the AlphaImager™ 2200 gel documentation system and AlphaEaseFC™ software. The stability analysis was also repeated at a 30°C incubation temperature.

Time Course of Cleavage. The Control Substrate (24 μg) was incubated with 4 units of AcTEV™ or TEV Protease in 1X TEV reaction buffer supplemented with 1 mM DTT at 30°C in a reaction volume of 160 μl . Aliquots (20 μl) were removed at the indicated times and inactivated

Figure 1 – AcTEV™ and TEV Protease stability at +4°C



250 units of either TEV or AcTEV™ Protease was incubated in 1X TEV reaction buffer supplemented with 1 mM DTT at +4°C in a reaction volume of 100 μl . Twenty microliters aliquots were removed at t = 0, 6 h, 24 h, and 168 h (1 week) and inactivated with an equal volume of 2X SDS-PAGE loading dye. The inactivated aliquots were stored at -20°C until a fraction of each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain (Panel A). The amount of self-cleavage product was quantified and plotted as a function of time (Panel B).

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with an equal volume of 2X SDS-PAGE loading dye. A fraction of each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain. The gels bands were quantified as described above.

Unit Titration. The Control Substrate (10 µg) was incubated with 0-6 units of AcTEV™ or TEV Protease in 1X TEV reaction buffer supplemented with 1 mM DTT for 1 hour at 30°C in a reaction volume of 75 µl. The reactions were inactivated by addition of 75 µl of 2X SDS-PAGE loading dye. A fraction of each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain. The gels bands were quantified as described above.

Temperature Dependence of Cleavage

Reaction. The Control Substrate (18 µg) was incubated with 6 units of either AcTEV™ or TEV Protease in 1X TEV reaction buffer supplemented with 1 mM DTT at the indicated temperatures in a reaction volume of 120 µl. Aliquots (20 µl) were removed at the indicated times and quenched with an equal volume of 2X SDS-PAGE loading dye. A fraction of the each sample was analyzed on SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain. The gels bands were quantified as described above.

pH Dependence of Cleavage Reaction. The Control Substrate (18 µg) was incubated with 6 units of either AcTEV™ or TEV Protease in 1X TEV reaction buffer (50 mM Tris-HCl (pH 6, 7.5, 8 or 8.5), 0.5 mM EDTA) supplemented with 1mM DTT in a reaction volume of 120 µl. Aliquots (20 µl)

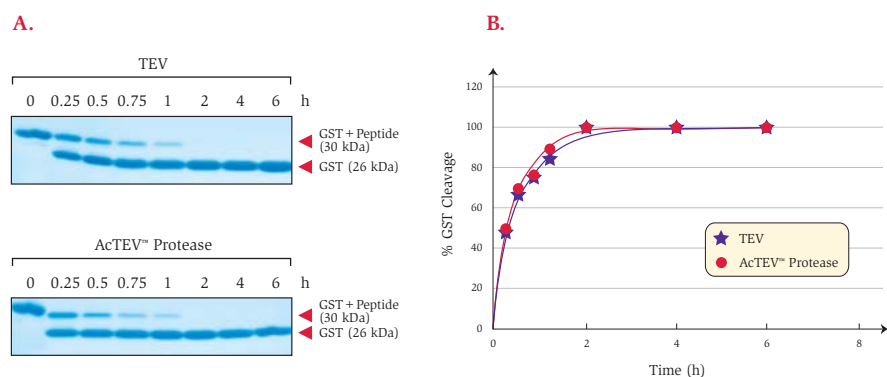
were removed at the indicated times and inactivated with an equal volume of 2X SDS-PAGE loading dye. A fraction of each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain. The gels bands were quantified as described above.

Result and Discussion

Stability Assay. We compared the stability of AcTEV™ and TEV Protease by incubating each enzyme in 1X TEV reaction buffer supplemented with 1 mM DTT at +4°C for a period of 1 week (Figures 1A and 1B, page 12). We observed significant self-cleavage of TEV (30% cleaved) during 1 week of incubation at +4°C. Typically, 30% cleavage of TEV protease was observed at the end of 1 week, but we have detected as much as 40% cleavage. In contrast to TEV, however, AcTEV™ Protease did not exhibit any self-cleavage over the same period of time. Similar results were observed when repeated at 30°C (data not shown). Therefore, AcTEV™ Protease is considerably more resistant to self-cleavage than traditional TEV.

Time Course of Cleavage. We examined the activity of TEV and AcTEV™ Protease by following the digestion of the Control Substrate over time (Figures 2A and 2B). In this experiment, TEV was able to cleave 50% of the Control Substrate in 0.25 hours and >85% of the substrate within 1 hour at 30°C. Similar results were observed for AcTEV™ Protease. At each time point, AcTEV™ Protease was able to cleave the equivalent amount of Control Substrate as TEV (Figure 2A). Thus, the enhanced stability of AcTEV™ Protease does not affect its enzymatic activity.

Figure 2 – AcTEV™ and TEV Protease time course of Control Substrate cleavage



24 µg of Control Substrate was incubated with 4 units of AcTEV™ or TEV Protease in 1X TEV reaction buffer supplemented with 1 mM DTT at 30°C in a reaction volume of 160 µl. Twenty-microliter aliquots were removed at t=0, 0.25 h, 0.5 h, 0.75 h, 1 h, 2 h, 4 h, and 6 h and inactivated with an equal volume of 2X SDS-PAGE loading dye. A fraction of each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain (Panel A). The amount of Control Substrate cleaved was quantified and plotted as a function of time (Panel B).

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Unit Titration. The amount of protein cleavage can be increased by prolonging the incubation time with TEV protease (Figure 2, page 13) or by adding additional TEV units for the same period of time. In Figure 3, we analyzed the amount of Control Substrate cleaved per unit of AcTEV™ or TEV Protease in 1 hour at 30°C.

Only 0.25 units of TEV was required to cleave 50% of the substrate and 2 units required to cleave 100% of the substrate. AcTEV™ Protease was able to cleave a similar amount of Control Substrate as TEV at each unit concentration. These results further confirm that the activity of AcTEV™ Protease is essentially the same as TEV.

Temperature and pH Dependence of Cleavage Reaction. A useful feature of TEV is that the enzyme is very active over a broad temperature range. This is illustrated in Figure 4, where TEV was capable of proficiently cleaving > 85% of the Control Substrate between +4°C and 30°C within a relatively short period of time (approximately 3 hours). AcTEV™ Protease also appears to be active over a broad temperature range. AcTEV™ Protease reactions at each tested temperature produced a similar amount of cleavage product as compared to TEV. The performance of AcTEV™ was also similar to that of TEV from pH 6.0-8.5 (data not shown). Overall, the temperature and pH profile of AcTEV™ Protease parallels that of TEV.

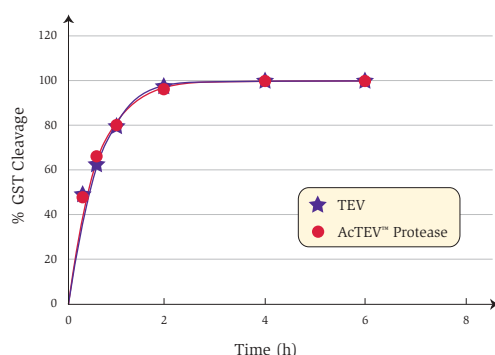
Conclusions

Our results demonstrate that AcTEV™ Protease has significantly increased stability compared to TEV protease. Although AcTEV™ Protease was enhanced for prolonged stability, there was no reduction in the enzymatic activity or specificity. In conclusion, AcTEV™ Protease is a very stable, efficient, and highly specific protease that is recommended for the removal of solubility and affinity tags from fusion proteins.

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Figure 3 – AcTEV™ and TEV Protease unit titration



10 µg of Control Substrate was incubated with 0, 0.25, 0.5, 1, 2, 4, and 6 units of AcTEV™ or TEV Protease in 1X TEV reaction buffer supplemented with 1 mM DTT for 1 hour at 30°C in a reaction volume of 75 µl. The reactions were inactivated by addition of 75 µl of 2X SDS-PAGE loading dye. A fraction of each sample was analyzed on SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain. The amount of Control Substrate cleaved was quantified and plotted as a function of TEV units.

Figure 4 – Cleavage of Control Substrate at various temperatures

A.

% GST Substrate Cleaved by TEV at Various Temperatures				
Time	4°C	16°C	21°C	30°C
0.25 h	27	58	56	71
0.5 h	37	70	69	82
1 h	65	91	93	100
2 h	81	95	100	100
3 h	91	100	100	100

B.

% GST Substrate Cleaved by AcTEV™ Protease at Various Temperatures				
Time	4°C	16°C	21°C	30°C
0.25 h	26	40	58	67
0.5 h	36	62	72	85
1 h	58	85	99	100
2 h	77	100	100	100
3 h	88	100	100	100

18 µg of Control Substrate was incubated with 6 units of either TEV (Panel A) or AcTEV™ Protease (Panel B) in 1X TEV reaction buffer supplemented with 1mM DTT at +4°C, 16°C, 21°C, 30°C in a reaction volume of 120 µl. 20 µl aliquots were removed at t = 0, 0.25 h, 0.5 h, 1 h, 2 h, and 3 h and inactivated with an equal volume of 2X SDS-PAGE loading dye. A fraction of the each sample was analyzed by SDS-PAGE (4-20% Novex® Tris-Glycine Gel) and SimplyBlue™ SafeStain. The amount of Control Substrate cleaved was quantified and the amount of Control Substrate cleaved at each time point at each temperature is reported in the tables above.

Increased specific amplification and flexibility of AccuPrime™ *Taq* DNA Polymerase High Fidelity in PCR

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Introduction

Polymerase Chain Reaction (PCR) is one of the most utilized techniques for the analysis of DNA and RNA (1). To accurately study particular stretches of genomic DNA, such as open reading frames, sequence specificity must be maintained. PCR is often prone to mistakes, resulting from non-specific priming during PCR cycling. Optimization of conditions, though time consuming, is frequently required for success.

Platinum® *Taq* DNA Polymerase High Fidelity is a hot-start enzyme mixture composed of recombinant *Taq* DNA polymerase, a proofreading polymerase, and *Taq* antibody (2). This enzyme blend increases PCR specificity and fidelity (six times over *Taq* polymerase). In addition, it can amplify a wide range of target sizes. Targets up to 12-20 kb can be amplified with minimal optimization. AccuPrime™ protein, a replication accessory protein, has been successfully utilized in AccuPrime™ *Taq* (3) and AccuPrime™ *Pfx* (4) to improve the specificity, yield, and sensitivity of PCR.

AccuPrime™ *Taq* DNA Polymerase High Fidelity is a next-generation PCR enzyme designed to further improve PCR specificity and yields. In this paper, we describe the AccuPrime™ protein and its introduction to existing hot-start Platinum® *Taq* DNA Polymerase High Fidelity. AccuPrime™ *Taq* DNA Polymerase High Fidelity can amplify DNA sequences with higher rates of success over a broader range of target sizes. Two AccuPrime™ PCR buffers are available for amplifying specific types of templates. Buffer I is optimized for plas-

mids, λ DNA, and cDNA, while Buffer II is optimized for use with genomic DNA.

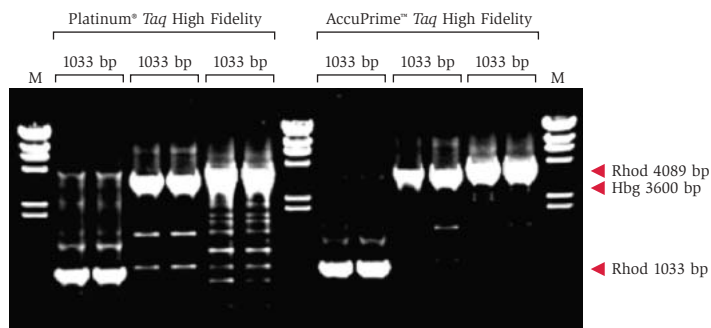
Materials and Methods

PCR. All reagents and enzymes were manufactured by the Invitrogen Corporation unless noted otherwise. Amplification reactions were prepared in 50- μ l volumes containing 0.2 μ M primers, 200 μ M of each dNTP, 1X PCR buffer, and 2 mM MgSO₄ using either 1 unit of Platinum® *Taq* DNA Polymerase High Fidelity or AccuPrime™ *Taq* DNA Polymerase High Fidelity. Templates varied from 20 ng to 100 ng, depending on the specific targets. Thermocycling was conducted using either the GeneAmp® PCR System 9600 or 2400 (Applied Biosystems, Inc.). Cycling conditions were a 2 min 94°C pre-incubation followed by 35 cycles of 94°C, 15 s; 52-62°C, 30 s; and 68°C, for 1 min/kb. Twenty percent of each amplification

reaction was analyzed by electrophoresis on a 0.5 X TBE 0.8% agarose gel containing 0.5 mg/ml ethidium bromide.

Competitive Audit. AccuPrime™ *Taq* DNA Polymerase High Fidelity was compared to other blended high-fidelity polymerases including Herculase™ Enhanced (Stratagene), Expand™ Long Template (Roche), Advantage™ 2 (Clontech), GeneAmp® High Fidelity (ABI), ProofStart™ (Qiagen), AccuTaq™ LA (Sigma), *Ex Taq*™ DNA Polymerase - hot start version (Takara) and KOD XL (Novagen) using five primer sets with amplicon sizes ranging from 2 kb to 15.1 kb (*c-Myc* 2.0 kb, Hbg 4.1kb, Hbg 7.5 kb, Hbg 12.3 kb, and Fac 15.1 kb). K562 human genomic DNA starting material (20 to 200 ng) was used in addition to 1 to 2.6 units of enzyme, based on each manufacturers' recommendation. For competitor polymerases, the cycling conditions followed manu-

Figure 1 – Increased flexibility in PCR conditions with AccuPrime™ *Taq* DNA Polymerase High Fidelity



Using 20 ng of human genomic DNA as a template, PCR reactions were performed as described in Methods under unoptimized conditions. The arrows indicated the specific amplified products. M: λ DNA/*Hind* III Fragments.

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facturer recommendations. Reactions were analyzed by electrophoresis as described above.

Results and Discussion

Increased Specificity and PCR Flexibility. To investigate PCR performance of AccuPrime™ *Taq* DNA Polymerase High Fidelity, we conducted PCR studies using more than 220 different primer sets.

AccuPrime™ *Taq* DNA Polymerase High Fidelity showed improvement in yield, sensitivity, and/or specificity over Platinum® *Taq* High Fidelity in 75% of the amplification reactions, in which Platinum® *Taq* High Fidelity did not perform well (Figure 1, page 15). We were able to produce a specific DNA band for these three targets using Platinum® *Taq* DNA Polymerase High Fidelity, but only after extensive optimiza-

tion of annealing temperatures and magnesium concentrations (data not shown). With AccuPrime™ *Taq* DNA Polymerase High Fidelity, specific products were obtained without individual optimization of the PCR conditions.

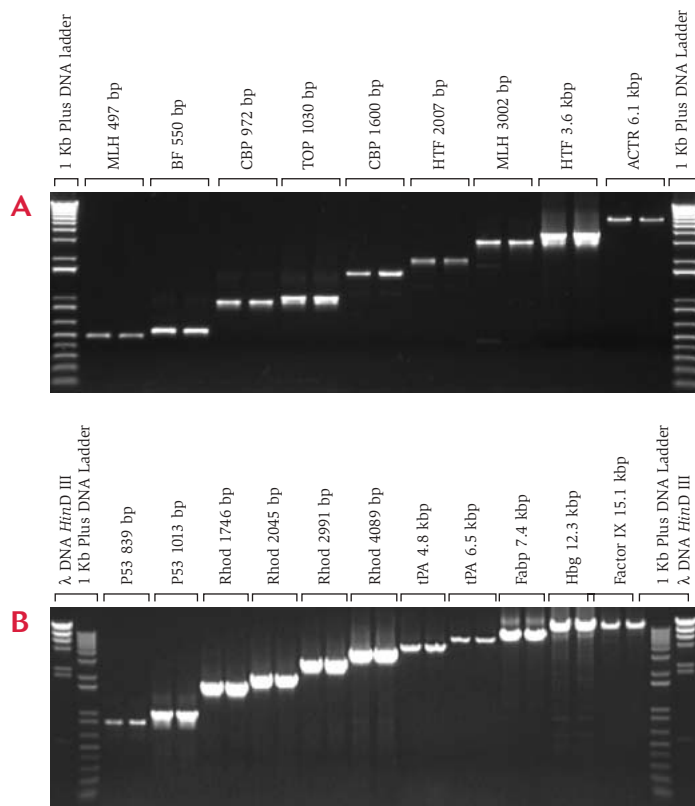
PCR performance of AccuPrime™ *Taq* DNA Polymerase High Fidelity. Two 10X AccuPrime™ PCR buffers are provided with AccuPrime™ *Taq* DNA Polymerase High Fidelity. Buffer I is optimized for plasmids, λ DNA, and cDNA, while Buffer II is optimized for genomic DNA. Figures 2A and 2B demonstrate the performance with cDNA and genomic DNA from 1 kb to 15 kb. All the PCR reactions produced specific PCR products without optimization of the PCR conditions.

Competitive Audit of AccuPrime™ *Taq* DNA Polymerase High Fidelity. The performance of AccuPrime™ *Taq* DNA Polymerase High Fidelity was directly compared against eight other commercially available blended polymerases using five primer sets with amplicons ranging from 2 kb to 15.1 kb (*c-Myc* 2.0 kb, *Hbg* 4.1kb, *Hbg* 7.5 kb, *Hbg* 12.3 kb, and *Fac* 15.1 kb). AccuPrime™ *Taq* DNA Polymerase High Fidelity clearly showed superior performance above all others in specificity and yield of the PCR products (Figures 3A and B, page 17).

Conclusion

We report the successful development of AccuPrime™ *Taq* DNA Polymerase High Fidelity. The Platinum® antibody complexes with *Taq* DNA polymerase and inhibits activity at ambient temperatures allowing room temperature reaction assembly of PCR. The thermostable

Figure 2 – Amplification performance of AccuPrime™ High Fidelity Buffer I and Buffer II

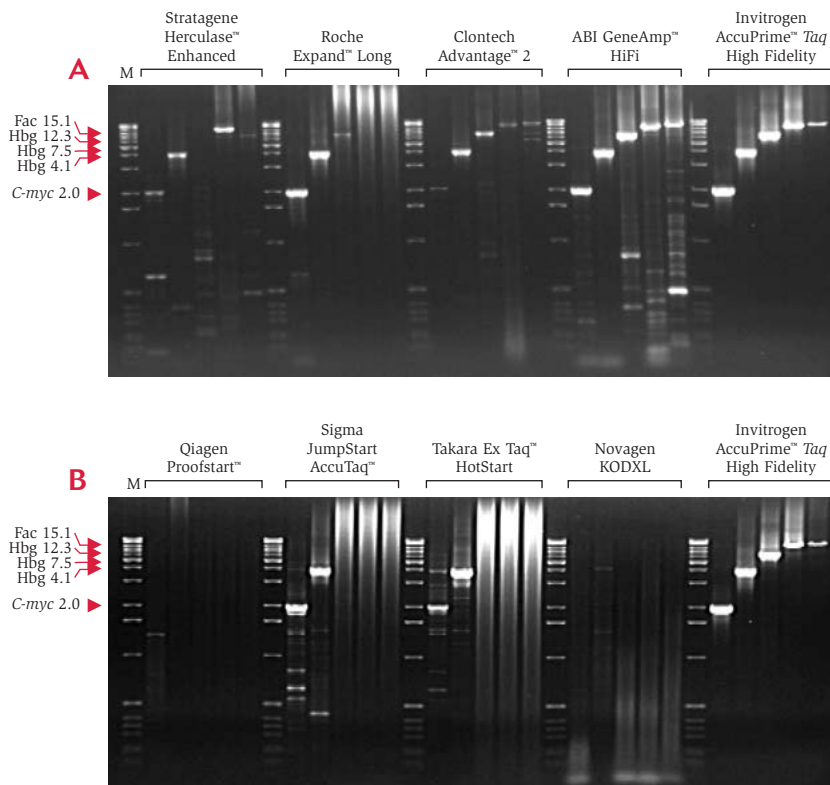


A) cDNA targets from 1.6 kb to 6.1 kb were amplified in duplicate with AccuPrime™ High Fidelity Buffer I. **B)** Genomic DNA targets ranging from 1013 bp to 15.1 kb were amplified in duplicate with AccuPrime™ High Fidelity Buffer II, all showing specific PCR products with very little, if any, non-specific product.

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Figure 3 – Competitive Audit of AccuPrime™ Taq DNA Polymerase High Fidelity



The performance of AccuPrime™ Taq DNA Polymerase High Fidelity was compared to eight blended high-fidelity polymerases. Each enzyme was used to amplify targets ranging from 2 kb to 15.1 kb using 20 to 200 ng of K562 human genomic DNA, using recommended amount of enzyme by respective manufacturer. The arrows in each picture indicate the specific amplified products. M: 1 Kb DNA Extension Ladder

AccuPrime™ protein enhances specific primer-template hybridization during every cycle of PCR. AccuPrime™ Taq DNA Polymerase High Fidelity dramatically improves specificity and provides a robust PCR for sub-optimal primer sets. It will amplify a broad range of target sizes, from 100 bp to 20 kb.

Acknowledgements

We thank Louis Leong, Adam Harris, and Jill Winer for critical reading of the manuscript and for helpful comments and suggestions.

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AccuPrime™ *Pfx* SuperMix: The robust high-fidelity PCR platform

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Introduction

Invitrogen's AccuPrime™ technology has proven to be a powerful method to achieve the high specificity, high sensitivity, and increased robustness needed for today's PCR applications. AccuPrime™ technology builds on the hot-start technology of the Platinum® family of enzymes to incorporate the AccuPrime™ protein as a specificity factor. In turn, this enhanced specificity renders AccuPrime™ polymerases with high sensitivity and robustness throughout all PCR cycles. The minimal optimization needed for PCR with AccuPrime™ *Pfx* DNA polymerase (1), unlike other high-fidelity proofreading polymerases, lends itself well to an all-inclusive SuperMix formulation.

The need for quick, effortless high-fidelity PCR was the hallmark under which AccuPrime™ *Pfx* SuperMix was developed. AccuPrime™ *Pfx* SuperMix incorporates all components needed for PCR reactions in a single solution. The AccuPrime™ *Pfx* SuperMix is further optimized to reduce assembly time and allow speedy results without sacrificing any of the high specificity and robustness achieved with the original AccuPrime™ *Pfx* DNA polymerase. Because no additional components beyond primers and template are required, it provides less opportunity for error or carryover contamination associated with pipetting. The ease and simplicity with which AccuPrime™ *Pfx* SuperMix can be used presents those needing the high specificity and fidelity shown with other *Pfx* DNA polymerases (2) a quick and useful approach to obtain similar and even improved results in less time. AccuPrime™ *Pfx* SuperMix retains all the vigor of

AccuPrime™ *Pfx* DNA polymerase and shows reproducible synthesis of specific products up to 15 kb.

In this article, we report the advantages and the performance of the new AccuPrime™ *Pfx* SuperMix and its utility with various templates, including genomic, cDNA, plasmid, and bacteriophage λ DNA. With its enhanced reproducibility and the high sensitivity and specificity associated with AccuPrime™ technology, AccuPrime™ *Pfx* SuperMix is ideal for a variety of PCR/RT-PCR applications, including high-throughput PCR, gene cloning, mutagenesis, and sequencing.

Materials and Methods

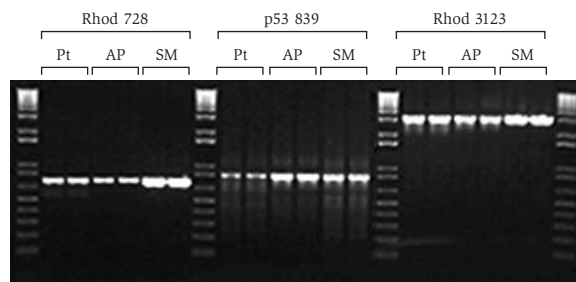
Standard PCR Reaction. PCR reactions were prepared in 25- μ l reaction volumes, unless indicated otherwise. The typical 25- μ l reaction contained 22.5 μ l of 1.1X AccuPrime™ *Pfx* SuperMix, 0.3 μ M of each primer, and template varying in concen-

tration from 100 pg (for plasmids and cDNA) to 100-200 ng (genomic DNA).

The PCR reactions were run following a standard protocol. Thermocycling was conducted using either the ABI GeneAmp® PCR System 9600 or the ABI GeneAmp® PCR System 2400. The standard PCR program consisted of one cycle of 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at a temperature between 55°C–65°C for 30 sec, and elongation at 68°C for a time equal to 1 min/kb. The reactions were then held at +4°C until further processing.

Following the completion of thermocycling, amplification products were mixed with 2.5 μ l of 10X BlueJuice™ and aliquots (5 μ l of total reaction volume per each lane) were analyzed by electrophoresis through an 0.8% agarose gel in 0.5X TBE containing ethidium bromide at a concentration of 0.5 μ g/ml.

Figure 1 – Comparison of AccuPrime™ *Pfx* SuperMix to Platinum® *Pfx* and AccuPrime™ *Pfx* DNA Polymerases



AccuPrime™ *Pfx* SuperMix show increased product yield compared to Platinum® *Pfx* and AccuPrime™ *Pfx*. Lanes M: 1 Kb Plus DNA Ladder; Pt: Platinum® *Pfx* DNA Polymerase; AP: AccuPrime™ *Pfx* DNA Polymerase; and SM: AccuPrime™ *Pfx* SuperMix. Standard PCR procedure was used (Materials & Methods) with 100 ng K562 DNA template and annealing temperature of 62°C for Rhod 728; 63°C for p53 839, and Rhod 3123.

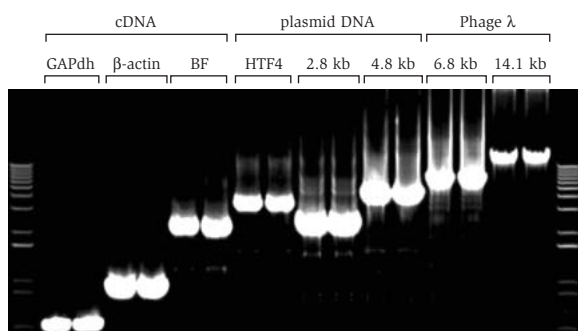
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Specificity and yield were compared among different samples on the same gel. *Freeze-thaw Endurance Testing.* The endurance of AccuPrime™ Pfx SuperMix

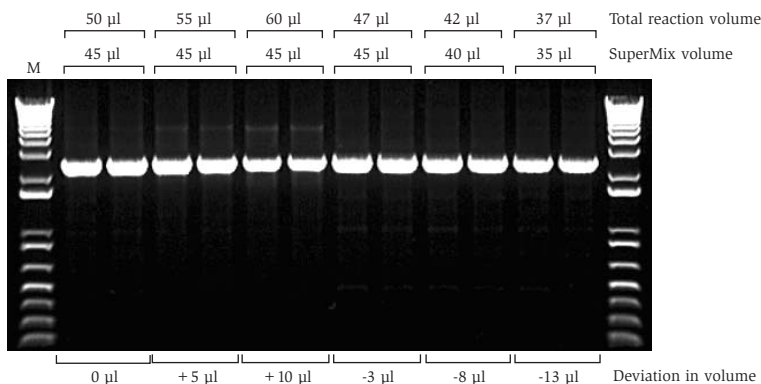
was tested by multiple freezing and thawing cycles. AccuPrime™ Pfx SuperMix was repetitively frozen at -20°C and thawed on ice. Aliquots were removed at designated intervals and left at -20°C until analyzed. Following completion of all freeze-thaw cycles, PCR reactions were performed using primer Hbg 3.6 for amplification of the template DNA.

Figure 2 – AccuPrime™ Pfx SuperMix on cDNA, plasmid, and bacteriophage λ templates



Standard reaction conditions were used with cDNA template from an RT reaction using 1 µg total RNA. Primer sets used were GAPdh 532 bp; β-actin 1026 bp; BF 2.4 kb; and HTF4 3.6 kb. Plasmid targets were the tepla plasmid vector backbone at 2.8 kb or with inserts for total sizes of 4.8 kb and 6.8 kb. Bacteriophage λ DNA targets of 12.7 and 14.1 kb were used with roughly 10 ng of λ DNA template. Annealing temperature was 60°C for all targets.

Figure 3 – AccuPrime™ Pfx SuperMix was tested for its tolerance of changes in reaction volume



Reactions were otherwise performed using standard conditions, 100 ng K562 genomic DNA, and 0.3 mM of the primer set. Even under the sub-optimal reaction volumes tested, AccuPrime™ Pfx SuperMix was able to produce specific product in high yields.

Competitive Audit of AccuPrime™ Pfx DNA Polymerase. Performance of AccuPrime™ Pfx SuperMix was compared with competitive high-fidelity PCR enzymes, including PfuTurbo® DNA Polymerase (Stratagene), PfuUltra™ DNA Polymerase (Stratagene), and Tgo DNA Polymerase (Roche). Each enzyme was used to amplify targets ranging from 773 bp to 6215 bp using 100 to 200 ng of human genomic DNA (K562, genotyping grade). PCR reactions were performed following manufacturers' recommendations. Annealing temperature for each primer set was identical for all the polymerases tested, 60°C for ATRN 6215 bp; 62°C for Rhod 773 bp, Rhod 1138 bp and Hbg 3.6 kb; and 63°C for p53 2424 bp.

Results and Discussion

Performance of AccuPrime™ Pfx SuperMix Formula. The AccuPrime™ Pfx SuperMix formulation was enhanced to achieve higher yields and minimize the need for optimization without sacrificing the high specificity and robustness of the original AccuPrime™ Pfx products. In the performance comparison, AccuPrime™ Pfx SuperMix performed equal or better than Platinum® Pfx in 90% of the cases. Primers sets with amplicons ranging from 500 bp to greater than 10 kb were tested.

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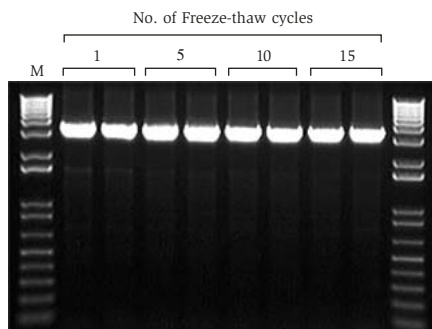
Figure 1 (page 18) shows comparisons of AccuPrime™ Pfx SuperMix to Platinum® Pfx and AccuPrime™ Pfx controls using genomic DNA template, while the performance of AccuPrime™ Pfx SuperMix with cDNA, plasmid, and bacteriophage λ DNA templates of varying sizes is demonstrated in Figure 2 (page 19).

AccuPrime™ Pfx SuperMix was tested for its tolerance of reaction volume variations, which may be required to accommodate underconcentrated templates and primers. Reactions were performed using otherwise standard conditions with 100 ng K562 template and 0.3 mM of the primer set. AccuPrime™ Pfx SuperMix showed little decrease in product yield on both sides of its normal concentration range. These results demonstrate the versatility of AccuPrime™ Pfx SuperMix under sub-optimal reaction conditions (Figure 3, page 19).

Freeze-thaw Endurance Testing. A concern of many SuperMix users is the possibility of deterioration of SuperMix components due to successive freezing and thawing throughout the product's lifetime. AccuPrime™ Pfx SuperMix was subjected to freeze-thaw endurance testing to assess stability during multiple freeze-thaw cycles. Figure 4 shows a gradual decrease in yield as the freeze-thaw cycles increased. However, AccuPrime™ Pfx SuperMix was able to continue to produce specific product even after more than 15 cycles; multiple freezing and thawing did not pose a significant detriment to its activity.

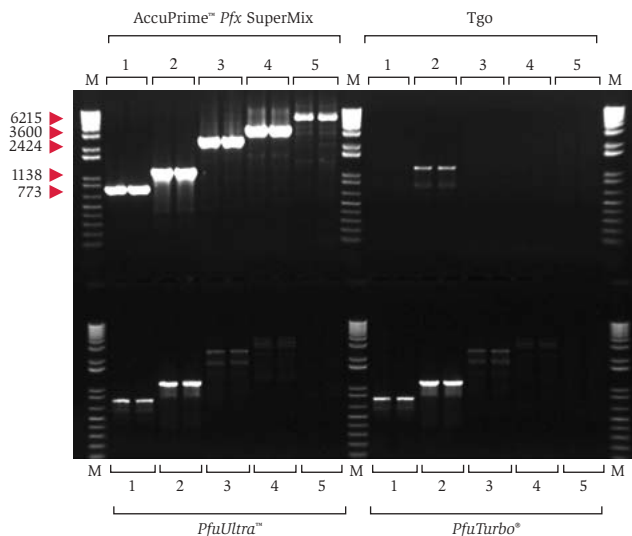
Competitive Audit of AccuPrime™ Pfx DNA polymerase. AccuPrime™ Pfx SuperMix

Figure 4 – Freeze-thaw endurance testing of AccuPrime™ Pfx SuperMix



The AccuPrime™ Pfx SuperMix was alternately frozen at -20°C then thawed on ice repetitively. Freeze-thaw intervals tested were 1, 5, 10 and 15 cycles of freezing and thawing. PCR reactions were performed following standard procedures using primer Hbg 3.6 kb as a target. A gradual decrease in yield as the freeze-thaw cycles increased is observed.

Figure 5 – Competitive audit of AccuPrime™ Pfx SuperMix against PfuUltra™, PfuTurbo®, and Tgo polymerases



Each DNA polymerase was used to amplify targets ranging from 773 bp to 6215 bp using 100 ng (or 200 ng for 6215 bp target) of human K562 genomic DNA template. Lane 1: Rhod 773 bp; Lane 2: Rhod 1138 bp; Lane 3: p53 2424 bp; Lane 4: Hbg 3.6 bp; and Lane 5: ATRN 6215 bp. AccuPrime™ Pfx SuperMix was successful in achieving highly specific and robust product for each target. Competitor enzymes were unable to produce longer target sizes and did not show yields comparable to AccuPrime™ Pfx SuperMix. Tgo DNA Polymerase only was able to produce one of the five targets tested. AccuPrime™ Pfx SuperMix was superior to all competitor enzymes tested.

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was directly compared to other proof-reading polymerases; *PfuUltra*[™] DNA Polymerase, *PfuTurbo*[®] DNA Polymerase, and Tgo DNA Polymerase. Five primer sets were used with amplicons ranging in size from 773 bp to 6215 bp (Rhod 773 bp, Rhod 1138 bp, p53 2424 bp, Hbg 3.6 bp, ATRN 6215 bp). In Figure 5 (page 20), AccuPrime[™] *Pfx* SuperMix showed superior performance over all other competitive enzymes tested. AccuPrime[™] *Pfx* SuperMix showed high specificity as well as superior yield.

Conclusion

The AccuPrime[™] *Pfx* SuperMix allows easy, high-fidelity PCR by combining all necessary components for PCR application into a single solution without compromising performance or the convenient hot-start capability. We show

that AccuPrime[™] *Pfx* SuperMix is as effective as AccuPrime[™] *Pfx* in producing specific and robust PCR products. The performance of AccuPrime[™] *Pfx* SuperMix shows 90% of cases being equal to or better than Platinum[®] *Pfx*. Comparison to competitors' high-fidelity enzymes shows AccuPrime[™] *Pfx* SuperMix as far superior in yield and specificity over a wide range of targets. The SuperMix formulation has been improved to minimize the need for optimization of reaction conditions. AccuPrime[™] *Pfx* SuperMix has proven itself to be stable and robust by its ability to withstand multiple cycles of freezing and thawing, and tolerance to volume alterations, thereby demonstrating its versatility.

By incorporating the general components necessary for PCR into a single

solution, we have provided a quick and useful tool for obtaining the high specificity and yield available from current AccuPrime[™] products with an improved SuperMix formula. The AccuPrime[™] *Pfx* SuperMix requires less time for set-up and optimization of PCR reactions and reduces the risk of error associated with pipetting. AccuPrime[™] *Pfx* SuperMix is a convenient and reliable way to achieve high-fidelity PCR ideal for variety of PCR applications, such as high-throughput PCR, gene cloning, mutagenesis, and sequencing.

Acknowledgments

We thank Jill Winer and Lisa Filippone for their critical reading of the manuscript and helpful suggestions.

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A look at the SuperScript™ III First-Strand Synthesis System for RT-PCR

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Introduction

RT-PCR is an effective and widely used tool in applications such as RNA detection, gene quantitation, and cloning of full-length genes. SuperScript™ II Reverse Transcriptase (RT), a version of M-MLV RT with reduced RNase H activity, has been shown to provide higher cDNA yields and longer target capacity than its parent enzyme (1,2). A new generation RT, SuperScript™ III RT, was engineered and the purification and biochemical characterization of the enzyme recently described (3). This enzyme has increased thermal stability over SuperScript™ II RT, a half-life of 220 minutes at 50°C, and can be used to synthesize cDNA at temperatures up to 55°C. Higher RT reaction temperatures can potentially alleviate RT pausing during cDNA synthesis by helping to melt RNA secondary structures (4,5,6), providing increased specificity, higher yields of cDNA, and more full-length product.

The SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Cat. no. 18080-051) incorporates the SuperScript™ III enzyme into a convenient package that contains all the necessary components for successful cDNA synthesis. The system was optimized and the protocol streamlined to provide a highly sensitive, specific, and robust RT-PCR system for a variety of applications. A wide range of RNA targets from 100 bp to >12 kb can be detected with this system, using 1 pg to 5 µg of total RNA. This report describes the application and performance evaluation of the SuperScript™ III First-Strand Synthesis System for RT-PCR.

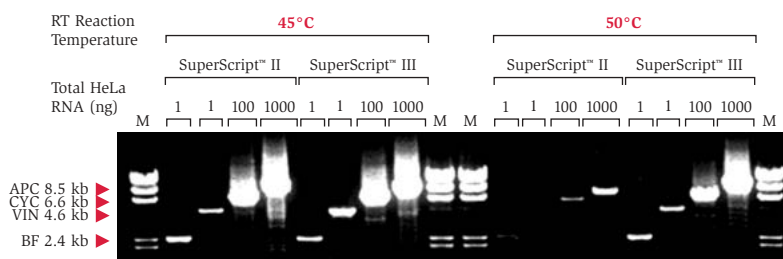
Materials and Methods

cDNA Synthesis. Total RNA was isolated from HeLa cells using TRIzol® Reagent (7). Mixtures containing 1 pg to 1 µg of total HeLa RNA, 1 µl of 10 mM dNTPs, and either 2.5 µM of oligo(dT)₂₀ or 2 µM of gene-specific primers (as indicated) in a 10-µl reaction volume were assembled on ice, followed by heating at 65°C for 5 min and cooling on ice. RT reactions (20 µl final reaction volume) were performed by adding 1X RT Buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 5 mM MgCl₂, 10 mM DTT, 40 units of RNaseOUT™ Recombinant Ribonuclease Inhibitor and 200 units of SuperScript™ III RT to the above mixture on ice. The reactions were incubated in a thermocycler for 50 min. at various temperatures ranging from 42 to 55°C. The reactions were placed on ice, followed by treatment with 2 units of *E. coli* RNase H at 37°C for 20 min. Following completion of cDNA synthesis and RNase H digestion, the cDNAs were placed on ice or stored at -20°C for immediate or future use in PCR amplification.

Gene-specific primers used in RT reactions are listed in Table 1 (page 23).

PCR Amplification of cDNA. For the evaluation of SuperScript™ III performance in RT-PCR, standard 50-µl PCR reactions were performed. For β-actin and GAPDH primer sets, PCR reactions consisted of 0.2 µM primers, 200 µM each dNTP, 1X PCR Buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM MgCl₂, 2 µl of the cDNA reaction, and 2 units of Platinum® Taq DNA Polymerase. For all other primer sets, PCR reactions consisted of 0.2 µM primers, 200 µM each dNTP, 1X High Fidelity PCR Buffer (60 mM Tris-SO₄ [pH 8.9]), 18 mM (NH₄)₂SO₄, 2 mM MgSO₄, 2 µl of the cDNA reaction, and 1 unit of Platinum® Taq DNA Polymerase High Fidelity. After incubation at 94°C for 2 min., amplification was 35 to 40 cycles at 94°C for 15 s, 55-60°C for 30 s, and 68°C (Platinum® Taq DNA Polymerase High Fidelity) or 72°C (Platinum® Taq DNA Polymerase) for 1 min/kb. Following completion of thermal cycling,

Figure 1 – RT reactions at 45°C and 50°C with SuperScript™ II and SuperScript™ III Reverse Transcriptases



RT reactions containing 1 to 1000 ng of total HeLa RNA were performed at 45 or 50°C for 50 min, using 200 units of each enzyme, followed by PCR with indicated primer sets.

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PCR amplification products were mixed with 10% (v/v) of 10X BlueJuice® Gel Loading Buffer. Twenty percent (11 µl) of the PCR reactions were analyzed on agarose gels containing 0.4 µg/ml EtBr. The DNA size marker (M) was λ DNA/*Hind* III Fragments (Figure 1,

page 22), or the 1 Kb Plus DNA Ladder (Figures 2-5). The primers used in PCR are listed in Table 1.

Results and Discussion

RT Temperature Range. SuperScript™ III RT is a mutant derivative of M-MLV RT with

reduced RNase H activity and increased thermal stability over its predecessor, SuperScript™ II RT. Increased thermal stability allows for cDNA synthesis at higher temperatures, up to 55°C. A temperature profile comparison was made between SuperScript™ II and SuperScript™ III RTs (Figure 1, page 22). RT reactions were performed with 200 units of each enzyme, using oligo(dT)₂₀ for priming, for 50 minutes at 45°C or 50°C. Resulting cDNAs were amplified using Platinum® *Taq* DNA Polymerase High Fidelity in standard PCR reactions, as described in Materials and Methods. Both enzymes performed similarly at 45°C, detecting as little as 1 ng of total HeLa RNA with BF 2.4 kb and VIN 4.6 kb primer sets. At 50°C, however, the RT-PCR product yield obtained with SuperScript™ III RT was significantly higher than that of SuperScript™ II RT for four different gene targets. Thus, SuperScript™ III RT provides a temperature advantage over SuperScript™ II, offering a tool for making cDNA from difficult RNA targets, such as templates with complex secondary structure or high GC content.

Next, the performance of the SuperScript™ III First-Strand Synthesis System for RT-PCR was evaluated at temperatures ranging from 42°C to 55°C, using several different gene targets (Figure 2, page 24). RT reactions were performed using varying amounts of total HeLa or rat brain RNA as described in the figure legend. The performance of SuperScript™ III RT was similar for all targets over the entire temperature range, providing a robust yield of product from 1.6 to 12.3 kb in length. These data indicate the versatility of the SuperScript™ III First-Strand Synthesis System in RT-PCR applications.

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Table 1 – List of primers used with the SuperScript™ III First-Strand Synthesis System for RT-PCR. Gene-specific primers used for cDNA synthesis are indicated with an asterisk.

β-actin 353 bp (human beta actin)	Sense primer: Antisense primer:	5' GCT CGT CGT CGA CAA CGG CTC 5' CAA ACA TGA TCT GGG TCA TCT TCT C
GAPDH 555 bp (human glyceraldehyde-3-phosphate dehydrogenase)	Sense primer: Antisense primer:	5' GTG AAG GTC GGA GTC AAC GGA TTT 5' CAC AGT CTT CTG GGT GGC AGT GAT
PP2A 1093 bp (human phosphatase 2A)	Sense primer: Antisense primer:	5' GTTCGATGTCAGTTACTGTCT 5' CACATCTTATTATCTGCAGTCTCTCAGAG
CBP 1.6 kb (human cap binding protein)	Sense primer: * Antisense primer:	5' ATGGCGATCGTCGAACCGGA 5' CACTGTCTTAATATGAATGGGACCTACTGAG
BF 2.4 kb (human B-factor properdin)	Sense primer: Antisense primer:	5' GAGCCAAGCAGACAAGCAAAGCAAGC 5' TGTTTTAAT TCAATCCCACGCCCTGT
HTF4 3.6 kb (human transcription factor 4)	Sense primer: * Antisense primer:	5' GCC GAA GAT GAA TCC CCA GCA ACA A 5' TCC TTT CCT CTG TGT GCT GAT TGC CA
VIN 4.6 kb (human vinculin)	Sense primer: Antisense primer:	5' GAGGAGGGCGAGGTGGACGGC 5' GAACTAACACACAGCGATGGGTGGGAA
CYC 6.6 kb (human cyclophilin-related protein)	Sense primer: Antisense primer:	5' GTTAGCGGCGTTGGGTTTGGC 5' AGGCTGGTTGAAGCTGGAGGGGA
Pole 6.8 kb (human DNA polymerase ε)	Sense primer: Antisense primer:	5' CGCCAAATTTCTCCCTGAA 5' CCGTAGTGCTGGGCAATGTTT
APC 8.5 kb (human adenomatous polyposis coli)	Sense primer: Antisense primer:	5' GCTGCAGCTTCATATGATCAGTTGTTA 5' AATGGCGCTTAGGACTTTGG
APC 8.9 kb (human adenomatous polyposis coli)	Sense primer: * Antisense primer:	5' GCTGCAGCTTCATATGATCAGTTGTTA 5' ATACCAATTTTCCCTGATGTAAGTTAGTCA
FIB 9.4 kb (human fibrillin)	Sense primer: Antisense primer:	5' TGGAGGCTGGGAACGTGAAGGAAA 5' ACAGGAATGACCGAGGTAATCTTGGC
Dynein 12.3 kb (rat dynein)	Sense primer: Antisense primer:	5' GCGGCGCTGGAGGAGAA 5' AGGTGGCGGCTCAAACACAAAG

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RT-PCR with Gene-specific Primers. Using gene-specific primers, rather than oligo(dT) primers, for RT reactions can sometimes lead to problems with non-specific priming and lower cDNA yield. The performance of the SuperScript™ III First-Strand Synthesis System for RT-PCR was tested in the presence of three sets of gene-specific primers, with targets ranging in size from 1.6 to 8.9 kb (Figure 3). For the smaller targets, CBP 1.6 kb and HTF4 3.6 kb, a similar cDNA yield was obtained when RT reactions were performed at 42°C, 46°C, 50°C, or 55°C. In contrast, a larger yield of the APC 8.9 kb product was observed with increasing temperatures. These data show that gene-specific primers can be used with the SuperScript™ III First-Strand Synthesis System to yield highly specific RT-PCR products at a variety of temperatures. In addition, for certain gene targets, higher cDNA yields can be obtained with increasing RT temperatures, indicating the high-temperature advantage of SuperScript™ III RT.

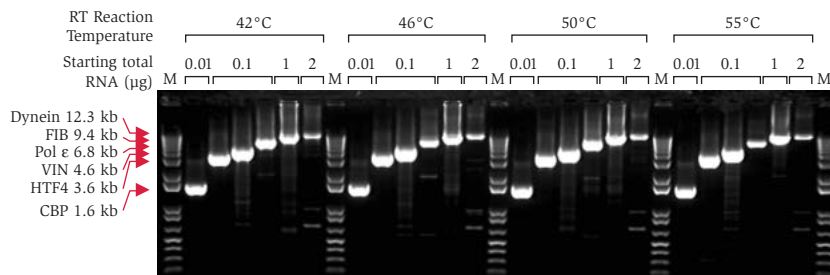
Sensitivity of the SuperScript™ III First-Strand Synthesis System. The ability to detect RT-PCR products from low amounts of starting material is of great importance when the available RNA is either limiting or very precious. One of the goals for developing the SuperScript™ III First-Strand Synthesis System was to generate a highly sensitive tool for RT-PCR. Using 1-100 pg of total HeLa RNA, RT reactions were performed with oligo(dT)₂₀ and 200 units of SuperScript™ III RT at 50°C for 50 min, followed by PCR amplification with primer sets to β-actin and GAPDH housekeeping genes (Figure 4, page 25). As little as 1 pg of starting total HeLa

RNA, or about 50-100 copies of β-actin and GAPDH, can reproducibly be detected, indicating the sensitivity as well as robustness of the system.

Comparing the SuperScript™ III First-Strand Synthesis System to Other First-strand Systems. In recent years, sev-

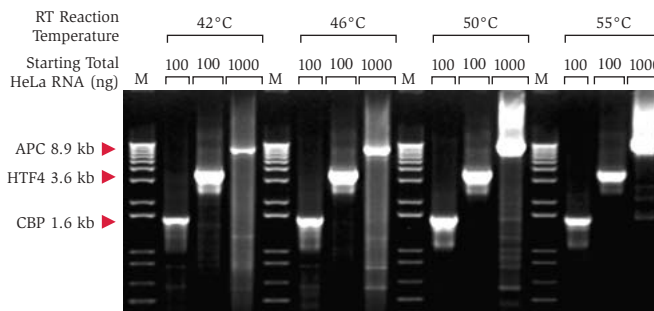
eral companies have launched products for RT-PCR applications. Four competing RT-PCR systems were tested in order to compare their performance to that of the SuperScript™ III First-Strand Synthesis System (Figure 5, page 25). RT reactions containing 1 or 100 ng of total HeLa RNA were performed according to each manu-

Figure 2 – Performance of the SuperScript™ III First-Strand Synthesis System for RT-PCR with several targets at various RT temperatures



RT reactions containing 10 ng to 1 μg of total HeLa RNA or 2 μg of total rat brain RNA (Dynein 12.3 kb target) were performed at 42, 46, 50 and 55°C for 50 min with 200 units of SuperScript™ III RT, followed by PCR with indicated primer sets.

Figure 3 – SuperScript™ III RT-PCR with gene-specific primers



RT reactions containing 100 or 1000 ng of total HeLa RNA and gene-specific reverse primers were performed at 42, 46, 50 and 55°C for 50 min with 200 units of SuperScript™ III RT. PCR amplification was performed using the indicated primer sets.

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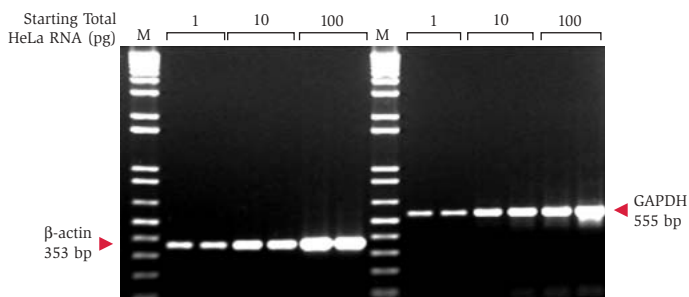
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facturer's directions. SuperScript™ III RT reactions were performed at 50°C for 50 min., using oligo(dT)₂₀ for priming, as described in Materials and Methods. Ten percent (2-5 µl) of each cDNA reaction was subsequently amplified by PCR with

Platinum® Taq DNA Polymerase High Fidelity. All five systems were able to detect down to 1 ng of a small (PP2A 1093 bp) gene target. However, whereas the SuperScript™ III First-Strand Synthesis System showed a consistently high prod-

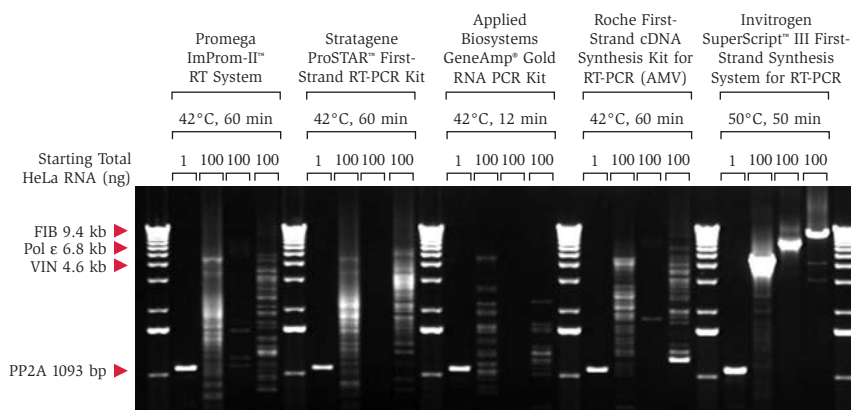
uct yield for three additional gene targets up to 9.4 kb in length, the other systems showed very little to no detectable product, as well as a considerable amount of non-specific products. Thus, the SuperScript™ III First-Strand Synthesis System outperforms these competitors in sensitivity, specificity, product yield, and target length.

Figure 4 – Sensitivity of SuperScript™ III RT



RT reactions containing 1 to 100 pg of total HeLa RNA and 200 units of SuperScript™ III RT were performed at 50°C for 50 min, followed by PCR with β-actin 353 and GAPDH 555 bp primer sets.

Figure 5 – Performance comparison of various RT-PCR systems



RT reactions containing 1 or 100 ng of total HeLa RNA were performed with each RT using the reagents and conditions specified in the manufacturer's protocol. Ten percent of each resulting cDNA was added to PCR reactions containing the indicated primer sets.

Conclusions

The SuperScript™ III First-Strand Synthesis System for RT-PCR was developed to provide a sensitive, robust, and reliable tool for use in RT-PCR applications. cDNA synthesis can be performed at temperatures up to 55°C, increasing the specificity, cDNA yield, and target length capacity over other RT-PCR systems. The system is able to detect as little as 50 to 100 gene copies from 1 pg of starting total HeLa RNA and to produce high yields of cDNA from targets up to 12.3 kb in size. The system contains all necessary components to perform first-strand cDNA synthesis, making it an ideal and convenient choice for a variety of RT-PCR applications.

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Sensitive one-step, one-tube RT-PCR with the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase

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Abstract

The SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase was developed to offer a convenient tool for a variety of RT-PCR applications, including multiplex RT-PCR. SuperScript™ III RT is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The advanced capabilities of SuperScript™ III were combined with the automatic “hot-start” feature of Platinum® *Taq* DNA Polymerase to provide a sensitive, robust, and reliable one-step, one-tube RT-PCR system. SuperScript™ III RT can synthesize cDNA at a temperature range of 45-60°C, resulting in increased specificity, higher yields of cDNA, and more full-length product than other RTs. A wide range of RNA targets from 200 bases to 4.5 kb can be detected with this system, using 0.01 pg to 1 µg of total RNA. The system contains all the components needed for first-strand cDNA synthesis and subsequent PCR amplification.

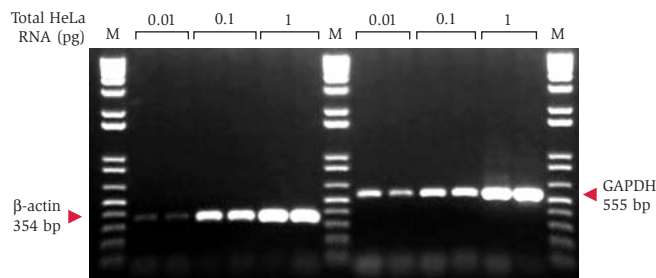
Introduction

Q In recent years, one-step RT-PCR has gained a strong position as a tool for high-throughput RT-PCR applications, including RNA detection and gene quantitation. Several companies offer one-step RT-PCR systems, however, only a few claim a detection capability of less than 1 pg of starting total HeLa RNA. The original SuperScript™ One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase has proven to be a reliable method for one-step RT-PCR. The system uses SuperScript™ II Reverse Transcriptase (RT), an RNase H mutant version of M-MLV RT, for higher cDNA yields and improved target length capacity (1,2). Combined with the automatic “hot-start” feature of Platinum® *Taq* DNA Polymerase, this system provides a convenient tool for one-step RT-PCR. A new generation RT, SuperScript™ III RT, was recently engineered and biochemically characterized (3). SuperScript™ III has a half-life of 220 minutes at 50°C and

increased thermal stability over its predecessor, and can therefore be used to synthesize cDNA at higher temperatures (55-60°C) than most other RTs. Since RTs have a propensity to pause during cDNA synthesis, higher temperatures may help increase the processivity of the enzyme through difficult RNA templates, providing increased specificity, higher yields of cDNA, and more full-length product (4,5,6).

The SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase was developed to incorporate SuperScript™ III RT into a convenient one-step, one-tube RT-PCR system. The system was optimized to provide a highly sensitive, specific, and robust RT-PCR system for a variety of applications. RNA targets up to 4.5 kb in length can be detected with this system, and as little as 0.01 pg of total RNA can be used for the detection of housekeeping genes. For increased specificity, cDNA synthesis can be performed at up to 60°C with the SuperScript™ III One-Step RT-PCR System.

Figure 1 – Sensitivity of the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq*



RT reactions containing 0.01 to 1 pg of total HeLa RNA and the β-actin 354 or GAPDH 555 bp primer set were performed for 30 min at 55°C, followed by 40 PCR cycles at 1 kb/min.

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This report describes the performance evaluation of the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq*.

Materials and Methods

RT-PCR. Total HeLa RNA was isolated from HeLa cells using TRIzol® Reagent (7). RT-PCR reactions (50 µl) containing 0.01 pg to 100 ng of total HeLa RNA, 10 µM of gene-specific sense and anti-sense primers, 2 µl of SuperScript™ III RT/Platinum® *Taq* Mix, and 25 µl of 2X Reaction Mix were assembled on ice. Multiplex RT-PCR reactions contained 10 µM of each primer set and 10 or 100 ng of total HeLa RNA. The SuperScript™ III RT/Platinum® *Taq* Mix contains a proprietary mixture of each enzyme for optimal cDNA synthesis and PCR amplification.

Table 1 – List of gene targets studied with the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq*

Human elongation factor 1A	EF1A 150 bp
Human beta actin	β-actin 301 bp β-actin 354 bp β-actin 406 bp
Human glyceraldehyde-3-phosphate dehydrogenase	GAPDH 555 bp GAPDH 1181 bp
Human cap binding protein	CBP 972 bp
Human guanine nucleotide exchange factor	GENE 1492 bp
Human BRCA1-binding helicase-like protein	BACH 2000 bp
Human DNA polymerase ε	Pol ε 2012 bp
Human B-factor properdin	BF 2441 bp
Human nuclear receptor coactivator	ACTR 491 bp ACTR 3009 bp
Human polymerase (RNA) II (DNA directed) polypeptide A	POLY 3508 bp
Human vinculin	VIN 347 bp VIN 4497 bp

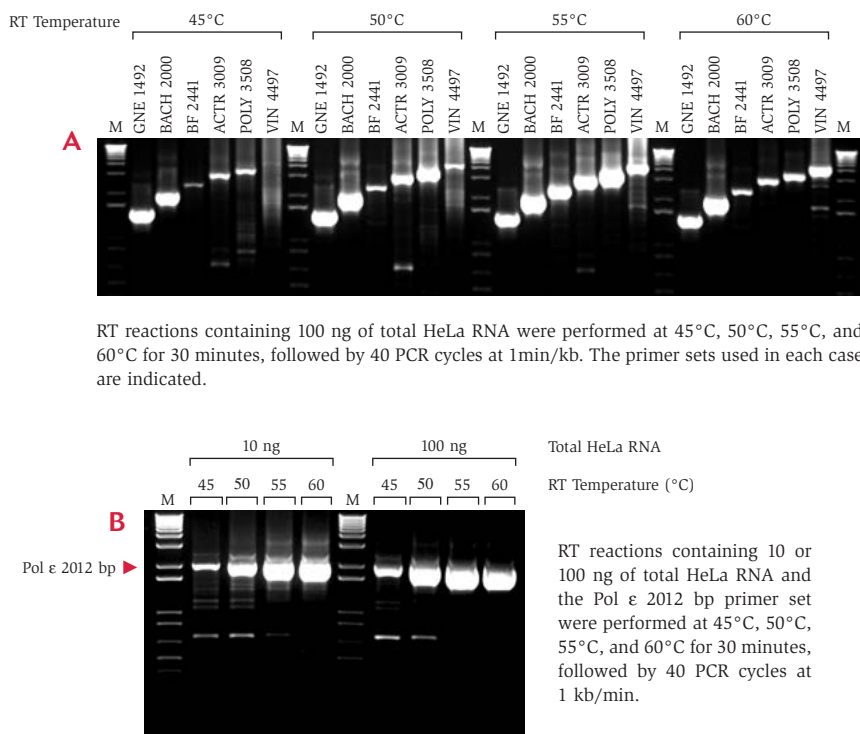
The 2X Reaction Mix is a proprietary buffer containing 0.4 mM of each dNTP and 3.2 mM magnesium sulfate (MgSO₄). The gene targets studied by RT-PCR are listed in Table 1.

The cDNA synthesis reaction and PCR amplification were performed in a thermal cycler in a single tube, using consecutive cycling steps as follows. After incubation at 45-60°C for 30 min (cDNA synthesis), the reactions were heated to 94°C for 2 min (pre-denaturation), followed by 40 cycles of amplification at 94°C for 15 s, 55-66°C for 30 s, and 68°C for 1 min/kb. PCR annealing tempera-

tures were empirically determined for each primer set. RT-PCR reactions were performed in duplicates.

RT-PCR amplification products were mixed with 10% (v/v) of 10X BlueJuice™ Gel Loading Buffer. Twenty percent (11 µl) of the RT-PCR reactions were analyzed by electrophoresis on 1% (w/v) agarose gels in 0.5X TBE with 0.4 µg/ml ethidium bromide. Multiplex RT-PCR products were separated on a 2% agarose gel next to 25 bp, 50 bp, and 100 bp DNA Ladders. The 1 Kb Plus DNA Ladder was used as a DNA size marker in all other experiments.

Figure 2 – RT temperature profile of the SuperScript™ III One-Step RT-PCR System



RT reactions containing 100 ng of total HeLa RNA were performed at 45°C, 50°C, 55°C, and 60°C for 30 minutes, followed by 40 PCR cycles at 1min/kb. The primer sets used in each case are indicated.

Total HeLa RNA
RT Temperature (°C)

RT reactions containing 10 or 100 ng of total HeLa RNA and the Pol ε 2012 bp primer set were performed at 45°C, 50°C, 55°C, and 60°C for 30 minutes, followed by 40 PCR cycles at 1 kb/min.

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Results and Discussion

RT-PCR conditions were optimized for SuperScript™ III RT and Platinum® *Taq* DNA Polymerase concentrations, as well as for the components of the 2X Reaction Mix, including optimization of the MgSO₄ concentration (data not shown). Using the optimized reaction conditions, a series of experiments was conducted to test the performance of the SuperScript™ III One-Step RT-PCR System in RT-PCR.

Sensitivity. The sensitivity of the SuperScript™ III One-Step RT-PCR System was evaluated in RT-PCR reactions, using picogram quantities of starting total HeLa RNA and primers to β -actin and GAPDH housekeeping genes (Figure 1, page 26). The system was able to reproducibly detect as little as 0.01 pg of RNA, indicating a 10-fold improvement in sensitivity over the existing SuperScript™ One-Step RT-PCR System with Platinum® *Taq* (data not shown).

Temperature Range. Primers to several gene

targets were used to test the performance of the SuperScript™ III One-Step RT-PCR System under different RT temperature conditions (Figure 2A, page 27). RT reactions were performed at 45°C, 50°C, 55°C, and 60°C, using 10 to 100 ng of total HeLa RNA. For the two smallest targets (GNE 1492 and BACH 2000 bp), similar product yields were observed throughout the temperature range. As target length increased, an improvement in sensitivity (increased product yield) and/or specificity was generally observed with increasing RT temperatures. This high-temperature advantage was true in particular for targets such as the Pol ϵ 2012-bp target, for which both a higher yield as well as specificity were observed at 55°C and 60°C (Figure 2b, page 27). The SuperScript™ III One-Step RT-PCR System can thus be used at a wide temperature range from 45°C to 60°C for overall improved RT-PCR performance.

Target Size Range. Gene targets ranging in size from 406 (β -actin) to 4497 bp (VIN) were used in RT-PCR reactions with the

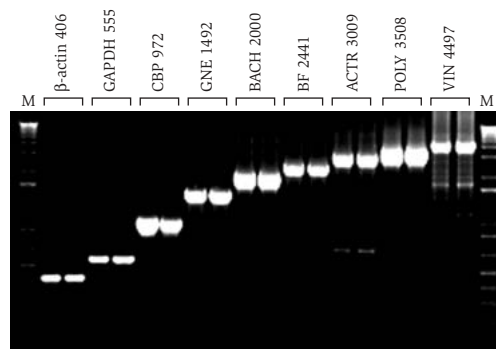
SuperScript™ III One-Step RT-PCR System (Figure 3). Results showed robust product yields for all nine targets tested, including the VIN 4497 bp target, representing a 1-kb improvement in target length capacity over the existing SuperScript™ One-Step RT-PCR System. Thus, the SuperScript™ III One-Step RT-PCR System provides a robust method for amplifying genes up to 4.5 kb in length.

Multiplex RT-PCR. The ability to use several different primer sets simultaneously to amplify genes from the same RNA source (multiplex RT-PCR) is an important consideration when the starting material is precious or limiting. The multiplex RT-PCR capability of the SuperScript™ III One-Step RT-PCR System was tested by using two to five sets of primers to detect different gene products from 10 or 100 ng of total HeLa RNA (Figure 4, page 29). Results showed robust and specific amplification of up to five different gene targets in the 150 bp to 1 kb range, indicating that the system is suitable for multiplex RT-PCR applications.

Conclusions

The SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* was developed to provide a sensitive, robust, and reliable tool for use in one-tube, one-step RT-PCR. cDNA synthesis can be performed at temperatures up to 60°C, increasing the specificity, cDNA yield, and target length capacity over other one-step RT-PCR systems. The system is able to detect as little as 0.01 pg of starting total HeLa RNA and to produce high yields of cDNA from targets up to 4.5 kb in size. This system contains all neces-

Figure 3 – Target size range of the SuperScript™ III One-Step RT-PCR System

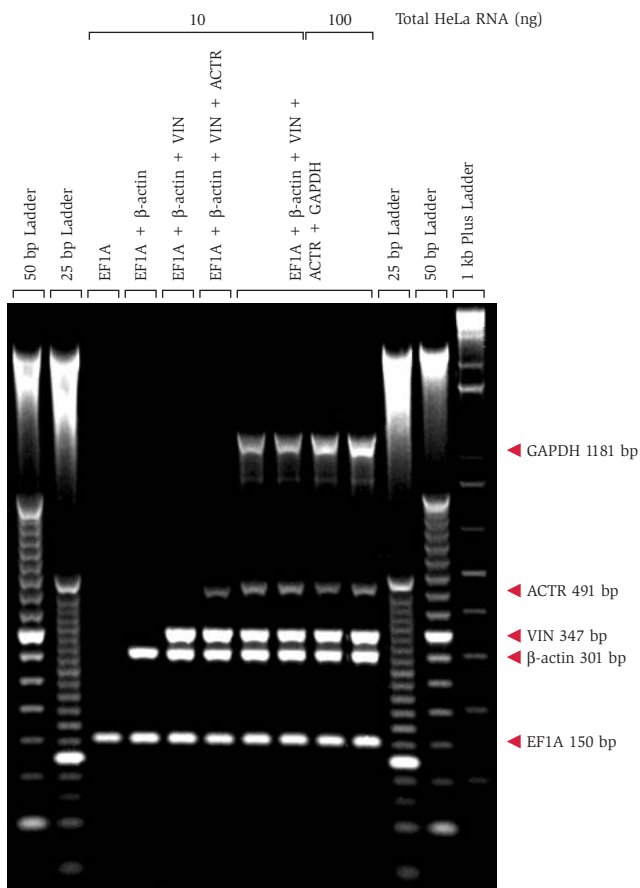


RT reactions were performed at 55°C for 30 minutes, followed by 40 PCR cycles at 1min/kb. Reactions contained 1 pg (β -actin and GAPDH primer sets), or 100 ng (all others) of total HeLa RNA.

continued on page 29

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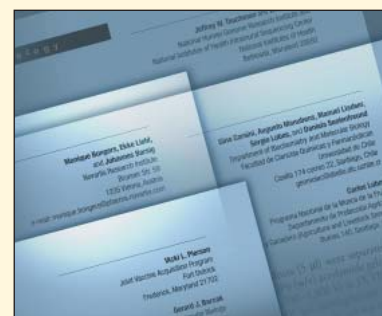
Figure 4 – Multiplex RT-PCR with the SuperScript™ III One-Step RT-PCR system



RT reactions were performed at 55°C for 30 minutes, followed by 40 PCR cycles at 1 min/kb. Reactions contained 10 or 100 ng of total HeLa RNA and one to five different primer sets, as indicated.

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sary components to perform first-strand cDNA synthesis and subsequent PCR amplification, making it an ideal and convenient choice for a variety of RT-PCR applications, including multiplex RT-PCR.

Acknowledgments

We thank Ginger Lucero and Qingli Mi for their assistance with experiments during the development of this product.

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Identification of heterogeneous transcription start and polyadenylation sites of the *APC* gene using the GeneRacer™ RACE ready cDNA

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Abstract

Full-length 5' and 3' cDNA ends of an adenomatous polyposis coli (*APC*) gene were obtained and analyzed using GeneRacer™ RACE ready cDNA from various human tissues. Two new alternative polyadenylation regions, 1935 bp and 2113 bp downstream from the TAA stop codon, were identified after sequencing the *APC* 3' RACE products. Sequence analysis of the *APC* 5' RACE clones identified four alternatively spliced transcripts of the untranslated first exon of the *APC* gene that were previously reported. Transcription start regions for each of the four transcript types A, B1, B2, and B3 were identified and a new transcription start region was found for transcript type B2.

Introduction

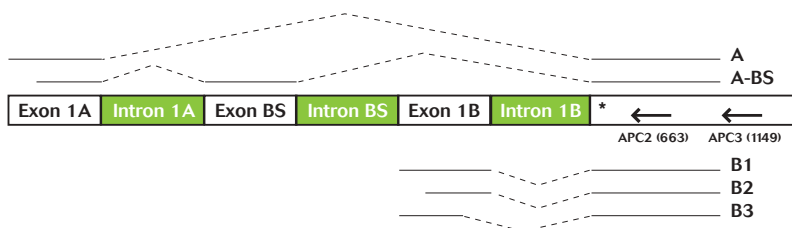
GeneRacer™ RACE ready cDNA uses an advanced RACE technology to provide an efficient method for accurate determination of complete full-length sequences of cDNA ends. The GeneRacer™ technology is based on the RNA ligase-mediated or oligo-capping RACE methods and its advancement was demonstrated by selective amplification of only full-length cDNA ends during RACE PCR (1-4). To demonstrate the sensitivity and effectiveness of the GeneRacer™ RACE ready cDNA, RACE reactions were performed on cDNA from various human tissues to amplify full-length cDNA ends of the 10.4 kb *APC* gene.

Transcription initiation sites of the adenomatous polyposis coli (*APC*) gene were studied previously using a conventional RACE technique (5). Initiation was found to occur at three sites in two distinct non-translating exons, termed 1A and 1B, at the 5' end of the *APC* gene (Figure 1) (5). At least five different tran-

script types generated by alternative splicing of the exons 1A and 1B were identified. There was one type from exon 1A (type A) and three types from exon 1B (type B1, B2, and B3) (Figure 1) (5). When studied in various tissues, most tissues were found to express all four alternative splicing forms with a variable proportion of each transcript among various tissues (5). An additional transcript type A-BS was also identified in the brain (Figure 1) (5).

To our knowledge, there are no reports on the polyadenylation sites of the *APC* gene. The specificity of polyadenylation sites in eukaryotic mRNA is defined by the presence of signal sequence, the hexanucleotide AAUAAA, 10-30 bases upstream of the cleavage/polyadenylation site and GU-rich motifs located 20-40 bases downstream of the cleavage site (6). The heterogeneity in mRNA polyadenylation sites is common and can be due to the presence of alternative polyadenylation signals in the 3' UTR as well as heteroge-

Figure 1 – Multiple forms of the *APC* gene first untranslated exon (adapted from Horri, A. *et al.*, Hum Mol Gen. 2, No 3, 283-287, 1993)

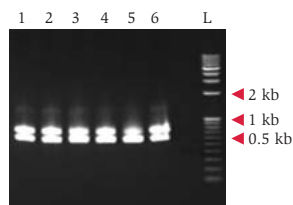


Alternative splicing in 5 transcript types (A, A-BS, B1, B2 and B3) is shown with dashed lines.
*-ATG start codon coinciding with the 39th base of *APC* mRNA (GenBank NM_000038). The positions of two gene specific primers used in nested 5' RACE PCR are also shown relative to NM_000038 sequence.

continued on page 31

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Figure 2 – Nested 3' RACE PCR amplification of the APC gene using GeneRacer™ RACE ready cDNA from six human various tissues



cDNA synthesis and PCR conditions are described in Materials and Methods. Lane L: mixture of 1 kb and 100 bp DNA ladders.

Lane	Tissue
1	HeLa cells
2	Breast
3	Uterus
4	Liver
5	Skin
6	Stomach

neous cleavage downstream from a single polyadenylation signal (7). The advanced RACE method of the GeneRacer™ RACE ready cDNA provided us an opportunity to study full-length cDNA ends and to identify transcription start and polyadenylation sites of the APC gene.

Materials and Methods

mRNA. HeLa mRNA was isolated from HeLa cell pellets (Cellexbio) using the FastTrack® 2.0 mRNA Isolation Kit (Invitrogen). Human normal tissues were processed to isolate total RNA using TRIzol® reagent (Invitrogen) followed by mRNA extraction using the FastTrack® 2.0 Kit.

GeneRacer™ RACE ready cDNA. GeneRacer™ RACE ready cDNA was prepared as described previously (8).

RACE PCR. RACE PCR reactions were performed using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) (2.5 U per 50- μ l reaction) and the nested PCR protocol with the first touchdown PCR and the second nested PCR. The GeneRacer™ PCR primers are described elsewhere (1,4). The positions of gene specific primers (GSPs) can be found in Figures 1 and 3. The touchdown PCR reactions were performed using 1 μ l of GeneRacer™ RACE ready cDNA, 30 pmol of GeneRacer™ 5' 1 primer or GeneRacer™ 3' primer, and 10 pmol of GSP APC3 (CCAACAATACAGAGTCTTTGTCATTGCCA) or APC4 (CCATGCGTTGGCAC-TTATCTATTCCCTGA) for 5' or 3' RACE respectively. The touchdown PCR program was as follows: 94°C for 2 min, 5 cycles of 94°C for 30 sec and 72°C for 2 min, 5 cycles of 94°C for 30 sec and 70°C for 2 min, 25 cycles of 94°C for 30 sec, 65°C for 30 sec and 68°C for

2 min followed by 68°C for 10 min. Nested PCR reactions were performed using 1 μ l of the touchdown PCR product and 10 pmol of either the GeneRacer™ 5' nested primer and the nested GSP APC2 (CCTGGCAGGTACCTAGTTGTCTTCCA) or the GeneRacer™ 3' nested primer and the nested GSP APC5 (GGGCAAGATCTCAGCAGTGAAGTATA) for 5' or 3' RACE respectively. The nested PCR program was as follows: 94°C for 2 min, 25 cycles of 94°C for 30 sec, 65°C for 30 sec and 68°C for 2 min followed by 68°C for 10 min. The 5' RACE reactions were repeated 4-6 times for each tissue.

Cloning and Sequencing RACE PCR Products. APC gene RACE products obtained from various tissues were cloned and sequenced. Following electrophoresis on a 1.2% E-Gel® pre-cast agarose gel (Invitrogen), the desired PCR bands were excised with a razor blade. Gel slices

Figure 3 – Genomic sequence (GenBank accession no. AC008575) of the 3' UTR of the APC gene showing polyadenylation cleavage sites (V) determined by the 3' RACE of the GeneRacer™ RACE ready cDNA from human breast tissue

```

133501 GTTTAAaagagaggaagaatgaaactaagaaaattctat.....
          APC4 → 134566          APC5 → 134903
135301 aatacactactctggtg ccttgaataatcacatcaagtagtaattatcacccttacct
135361 gtgtttataactccaggaatgagaatgatttttttaaagctaaaatgccagta aata
135421 aa agtgcctatgacttga1gcta5vagatattga1ctccaatgcctgtactgtctactgac
135481 cactttgtaaacacttcaattactatcttggaaatgattgaccttaaatTTTTGCCAA
135541 atgttatctgaaattgtctatgaaatcacatctactctgtgttttcccaggcttc cata
135601 aa caatggagatacatgca3vtaggtca1tactggttcctttcattttttgattttctat
135661 ttctaattttctgaattactgcatgccagttgtgcaaacactcaagtaactctctatgg

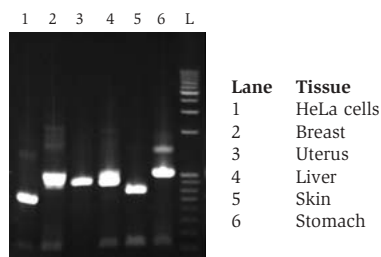
```

Upstream polyadenylation signals (A/CAUAAA) are underlined and the downstream putative U/GU-rich regions are shown in bold. The coding sequence is shown in upper case letters with the TAA stop codon in bold. Lower case letters in italics show the available mRNA sequence (GenBank accession no. NM_000038). The relative positions of two gene specific primers used in nested 3' RACE PCR are also shown. The number of sequenced 3' RACE clones found with a particular cleavage site is shown above the sequence.

continued on page 32

continued from page 31

Figure 4 – Nested 5' RACE PCR amplification of the *APC* gene using GeneRacer™ RACE ready cDNA from six human tissues



cDNA synthesis and PCR conditions are described in Materials and Methods. Lane L: mixture of 1kb and 100 bp DNA ladders.

were put into a S.N.A.P.™ purification columns (Invitrogen) fit into 1.7-ml sterile tubes and centrifuged at 14,000 rpm for 1 min. Gel and liquid matter were separated on the column and liquid containing the PCR product was collected in the tube. Four microliters of the purified product was cloned into the pCR®4-TOPO® or pCR®-XL-TOPO® vectors (Invitrogen) and transformed into chemically competent TOP10 cells (Invitrogen). Eight colonies from each transformation were picked and grown overnight. Plasmid DNA was prepared using the S.N.A.P.™ MiniPrep kit (Invitrogen). Inserts were sequenced using M13 reverse and M13 forward primers. Sequences were analyzed by BLAST search and GenBank sequence comparison.

Results and Discussion

APC Gene 3' RACE. GeneRacer™ RACE ready cDNA from six tissues was used to obtain 3' RACE products for the 10.4 kb *APC* gene. 3' RACE products were obtained with nested RACE PCR for all six

Figure 5 – DNA sequence of exons (underlined) and surrounding introns of untranslated exons 1A and 1B of the *APC* gene showing a distribution of transcription start sites (in bold) identified by the 5' RACE PCR of the GeneRacer™ RACE ready cDNA from various tissues (H-HeLa cells, B-breast, U-uterus, L-liver, Sk-skin, S-stomach)

A. Exon 1A, transcript type A. Total 5' RACE clones: 15.

```

361 aaatccgctg atgccaccag cgctccccga ttggctgggt gtgggcgcac gtgaccgaca
      1 2          4          2 6
421 tgtggtgtgta ttggtgcaggc ccgccagggt gtcactGgag acagaatgga ggtgctgccc
      H H          H          L L
481 gactcggaaa tgggtaggt gctggagcca ccatggccag gttctgtgtg ggggaggggg

```

B. Exon 1B, transcript type B1. Total 5' RACE clones: 16.

```

181 catgcgcatt gtagtcttcc cacctccc c Ac aagatggcgg agggcaagta gcaagggggc
      U          S U          L L1 L
      H1
241 ggggtgtggc cgccggaagc ctgaccgctg ctgggggggg acctgcccgc tcaggcccgc

301 gagctcggga ccgaggttgg ctcgatgctg ttcccaggta ctgttgttgg ctgttgggtga
361 ggaagtgtaa gcacgcagtt gccttctcgg gcctcggcgc ccctatgta cgctccctg
421 ggctcgggtc ccgtcgcgcc ttgcccgcct tctgtaccac cctcagttct cgggtcctgg
481 agcaccggcg ccagcaggag ctgcgtccgg caggagacga agagcccggg cggcgctcgt
541 acttctggcc actgggcgag cgtctggcag gtgagtggag ctgcag

```

C. Exon 1B, transcript type B2. Total 5' RACE clones: 25.

```

181 catgcgcatt gtagtcttcc cacctccc aagatggcgg agggcaagta gcaagggggc
      1 1          1 2 6 2 1 4
241 ggggtgtggc cgccggaagc ctagccgctg ctgggggggg ac ct gcgggc tcaggcccgc
      B L          L B U5 U B L3
      B1          B1
      5 1 1
301 gagctcggga ccgaggttgg ctcgatgctg ttcccaggta ctgttgttgg ctgttgggtga
      L4 H H
      Sk1
361 ggaagtgtaa gcacgcagtt gccttctcgg gcctcggcgc ccctatgta cgctccctg
421 ggctcgggtc ccgtcgcgcc ttgcccgcct tctgtaccac cctcagttct cgggtcctgg
481 agcaccggcg ccagcaggag ctgcgtccgg caggagacga agagcccggg cggcgctcgt
541 acttctggcc actgggcgag cgtctggcag gtgagtggag ctgcag

```

D. Exon 1B, transcript type B3. Total 5' RACE clones: 6

```

181 catgcgcatt gtagtcttcc cacctcccAc aagatggcgg agggcaagta gcaagggggc
      L Sk          L
      1
241 ggggtgtggc cgccggaagc ctgaccgctg ctgggggggg acctgcccgc tcaggcccgc
      H
301 gagctcggga ccgaggttgg ctcgatgctg ttcccaggta ctgttgttgg ctgttgggtga

```

The number of sequenced 5' RACE clones found with a particular transcription start site is shown above the sequence. Exons and major initiation sites (upper case) are according to GenBank accession numbers D13980 and D13981 and Ref. 5. A. Exon 1A: GenBank accession number D13980, transcript type A, exon positions 436-494, major initiation site position 457. B. Exon 1B: GenBank accession number D1398, transcript type B1, exon positions 207-570, major initiation site position 209. C. Exon 1B: GeneBank accession number D1398, transcript type B2, exon positions 259-570, major initiation site position 259. D. Exon 1B: GeneBank accession number D1398, transcript type B3, exon positions 207-337, major initiation site position 209.

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tissues (HeLa cells, breast, uterus, liver, skin, and stomach) (Figure 2, page 31). The 3' RACE products appeared as a double PCR band of about 600 bp and 780 bp each for all six tissues. Sequencing of those two bands from breast tissue revealed two distinct heterogeneous populations of transcripts. Both populations had mRNA sequences beyond the last base in the GenBank mRNA sequence of the *APC* gene and in every case the extra sequence matched the available genomic sequence (Figure 3, page 31). The two populations varied in the amount of extra bases and contained either around 123 or 301 additional bases compared to the GenBank mRNA sequence (Figure 3).

Our results indicate the presence of two distinct polyadenylation sites at the 3' UTR of the *APC* gene flanked by specific signal sequences (Figure 3). The two sites are around 1935 bp and 2113 bp downstream from the TAA stop codon (Figure 3). Both sites appear to also have heterogeneous polyadenylation cleavage sites spanning at least 14 and 9 bases, respectively (Figure 3). The two polyadenylation sites appear to be common to all the tissues studied (Figure 2).

APC Gene 5' RACE. *APC* gene 5' RACE products were obtained from six tissues (Figure 4, page 32). Sequencing of the 5' RACE products revealed a highly heterogeneous population of the 5' ends. GenBank search confirmed that all of the *APC* 5' RACE products matched various sequences representing multiple forms of the first non-translating exons of the *APC* gene reported previously (Figure 5) (5). Transcript types A, B1, B2, and B3 were found among various tissues (Figure 5,

Table 1 – Distribution of various transcripts of the *APC* first untranslated exon among six human tissues as determined by 5' RACE of GeneRacer™ RACE ready cDNA¹

Transcript type	5' RACE clones in various tissues					
	HeLa cells	Liver	Uterus	Breast	Skin	Stomach
1A	7	8	0	0	0	0
1B1	1	8	2	0	0	5
1B2	2	9	7	6	1	0
1B3	1	2	0	0	3	0
Total <i>APC</i> 5' RACE clones sequenced	11	27	9	6	4	5

¹ See Figure 5 for definition of transcript types and references.

page 32). From the six tissues analyzed, a total of 62 *APC* 5' RACE PCR clones were identified including 15 clones with transcript type A, 16 clones with transcript type B1 and 6 clones with transcript type B3 (Figure 5). All clones with transcript types A and B1 and five out of six clones with transcript type B3 started at, or upstream from, the predicted major initiation sites reported previously (Figure 5). The remaining 25 clones represented exon 1B, however, no obvious transcript type was observed (Figure 5). The *APC* sequence in these 25 clones started at various positions within exon 1B, but downstream from the predicted major initiation site for transcript type B2 reported previously (Figure 5) (5). While it is possible that some of these clones represent less than full-length transcripts of type B1 or B2, it is more likely that they represent type B2 transcripts with a new initiation region around positions 283-302 of exon 1B (Figure 5). Our data does not support the predicted previously major initiation sites for other transcript types as well. The majority of the clones often initiated at alternative upstream positions within

transcripts A, B1, and B3 (Figure 5).

Contrary to the GeneRacer™ method, the RACE technique used in the previous study was based on poly(A) tailing of cDNA which does not have an advantage of selecting for full-length 5' capped transcripts (5). It is possible, therefore, that the major initiation sites were misidentified in this study, especially for transcripts type B2 for which only 3 out of 9 sequenced clones started from the predicted major initiation site at position 259 (5). The GeneRacer™ method, on the other hand, provides a full-length selection for the 5' capped mRNA, ensuring full-length 5' RACE PCR results. It is, therefore, expected to produce more accurate determination of transcription start sites.

The wide distribution of the initiation sites suggests that there might not be a single major initiation site for each individual transcript type of the *APC* gene as was suggested previously (Figure 5). The heterogeneity of transcription initiation and polyadenylation sites is observed regularly when studied by full-length RACE PCR (our unpublished data and S. Sugano, personal communication, 6,7). In the case of

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some genes it becomes more appropriate to talk about the transcription initiation and polyadenylation regions rather than sites. It certainly is the case for the *APC* gene as shown here. The *APC* transcripts type A, B1, B2, and B3 had initiation regions spanning 30, 24, 73, and 24 bases, respectively (Figure 5). Two identified polyadenylation sites spanned 9- and 14-base regions respectively (Figure 3).

The distribution of various transcript types among the tissues studied appears to be tissue specific (Table 1, page 33). Although the number of clones sequenced is not large enough to make definite conclusions and the differences in the efficiency of RACE PCR of various transcripts could have affected the results, tissues with the most number of clones

sequenced (HeLa cells and liver) had all four transcripts (Table 1). This agrees with the previous study that found all tissues (including stomach and liver), except lung, expressing all four transcripts with the proportion of each transcript differing among various tissues (5).

Conclusion

Using the GeneRacer™ RACE ready cDNA from various human normal tissues, full-length cDNA ends were obtained for a 10.4 kb *APC* gene. Transcription start regions for four alternatively spliced transcript types of the first untranslated exon and the heterogeneous cleavage sites within two alternative polyadenylation regions of the *APC* gene were identified. We demonstrated how the GeneRacer™

RACE ready cDNA can be used to study the heterogeneous transcription start and polyadenylation sites, alternative splicing and gene expression in various tissues.

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Acknowledgements

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Molecular weight estimation of proteins by gel electrophoresis revisited

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Introduction

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the most popular method for protein separation, has been used for separation and characterization of protein and peptides for well over 30 years. The popularity of the method comes from its efficiency, sensitivity, reproducibility, and with the availability of pre-cast gels, its simplicity.

Shapiro *et al.* (1) showed that in the presence of the anionic detergent SDS, migration of proteins is dependent on the MW of the proteins and molecular weight (MW) estimates can be made by comparing protein and peptide migration with standards. After Weber and Osborn (2) tested the reliability of the technique on 40 proteins ranging in MW from 12 to 220 kDa, it became a generally accepted method for MW estimation of polypeptides.

In SDS-PAGE, proteins are denatured prior to separation, by heating in the presence of a thiol reagent and excess SDS. Surfactant-induced unfolding takes place and large amounts of SDS bind strongly to the protein. A weight ratio of 1.4 g SDS to 1 g protein is often quoted for the SDS-protein complex (3). It is assumed that in the presence of this high ratio of SDS to protein, the intrinsic charge of the protein becomes insignificant. The result is that polypeptide chains end up with identical charge densities and conformation and, therefore, should migrate in the gel merely according to their size.

MW estimations based on this technique make the following assumptions: SDS binds to all peptides and proteins at

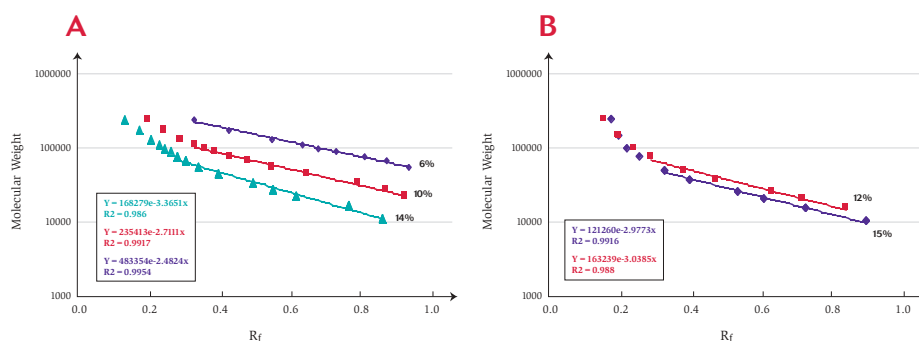
the same weight ratio, all SDS complexes have the same conformation (*i.e.*, all charge and shape variables have been removed by SDS), and hydrodynamic mobilities of the proteins of interest and the protein standards are similar. These assumptions may not always be valid. We revisited these methods using commercially available protein sets provided as standards for MW estimation. We employed mass spectrometry to accurately determine the mass of the proteins used in the standard sets, and we compared these masses to the “apparent molecular weight” of these proteins in gel. We then evaluated their behavior in standard methods for MW estimation using gels. We show that the use of standard methods can provide good estimates of MW and we note some of the limitations for accuracy in the estimations along with certain approaches that can be applied to reduce these limitations.

Methods

The gels used in this study were:

Invitrogen NuPAGE® Novex® Bis-Tris, Novex® Tris-Glycine (TG), Novex® Tris-Acetate (TA), and BioRad Ready Gel® Tris-HCl gels along with the unstained protein standards: MagicMark™, MagicMark™ XP, Mark12™, BenchMark™, and HiMark™ Unstained High Molecular Weight Protein Standard from Invitrogen (Carlsbad, CA); and Broad Range and Precision Plus Protein™ Standards from BioRad (Hercules, CA). Protein samples were purchased from CalBiochem (San Diego, CA) and Sigma (St. Louis, MO). The NuPAGE® Bis-Tris, TG, and TA gels were run on the XCell Surelock™ Mini-Cell apparatus (Invitrogen), using the corresponding running buffers. Ready Gel® Tris-HCl gels were run on the Mini-PROTEAN III Electrophoresis System (BioRad). Standards and reduced samples (DTT was used as the reducing reagent) were run under denaturing conditions for 35 minutes at 200 V (NuPAGE®), 90 minutes at 125 V (TG), 60 minutes at 150 V (TA), and 35 minutes at 200 V (Tris-HCl).

Figure 1 – Molecular weight vs. relative mobility over a limited range



Plots of MW as a function of R_f (relative mobility) for: (A) unstained BenchMark™ Standard on Novex® Tris-Glycine gel, 6%, 10% and 14% (B) Precision Plus Protein™ Standard on Ready Gel® 12% and 15% Tris-HCl gels.

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Unstained standards and proteins were stained with SimplyBlue™ SafeStain (Invitrogen). R_f measurements were made according to the equation:

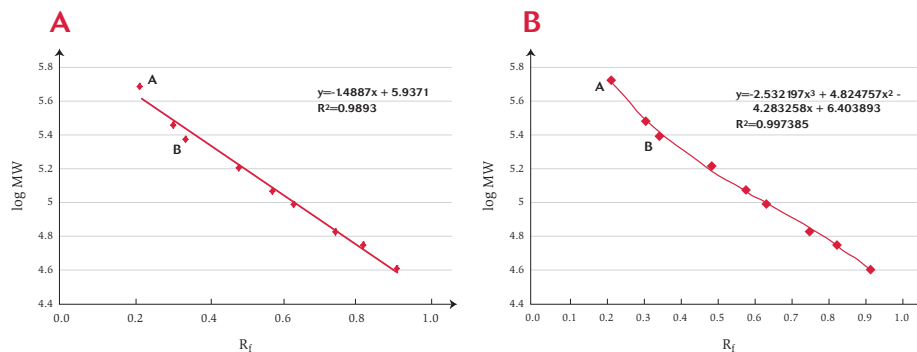
$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

in which the dye used was that of the sample buffer. Measurements were made using the AlphaImager™ 3.0 package (Alpha Innotech, San Leandro, CA).

MWs of the standards were measured by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Mass spectra were obtained on the VOYAGER-DE/VOYAGER-DE-STR systems (ABI, Foster City, CA). For proteins below 10 kDa, α -cyano-4-hydroxycinnamic acid (CHCA) dissolved in a 1:1 v/v acetonitrile and 0.1% trifluoroacetic acid (TFA) was used as the matrix. Protein standards with masses above 10 kDa were analyzed using sinapinic acid (SA) dissolved in the same solvent. All peptides/proteins were calibrated internally or externally using an internally prepared calibration set, BSA, myoglobin, and chymotrypsinogen.

We accurately measured the molecular weight of the unstained standard set of markers, MagicMark™, BenchMark™, and Mark12™. The results of this comparison are presented in Table 1 (page 38). It can be seen that the assigned masses are very close to the measured mass by MALDI, confirming the accuracy of calibration curves based on the standards. The largest deviation is seen for myosin; mass spectrometry data showed a MW of 224 kDa, which is significantly different from the MW value of 200-205 kDa generally referenced by various vendors and literature.

Figure 2 – Log molecular weight vs. relative mobility of large proteins



For the log MW vs. R_f plot of higher molecular weight proteins, (a) a linear fit, despite the relatively high correlation coefficient (0.9893), can produce large errors in the high MW region (A and B) while (b) a polynomial trend fits the data well and ensures higher accuracy. Data shown is the HiMark™ Unstained High Molecular Weight Protein Standard on a Novex® 3-8% TA gel.

Results and Discussion

The assumptions that are made in using SDS-PAGE to estimate MW are an oversimplification, nevertheless, the linearity holds over a useful range of protein MWs and gel concentrations, %T (%T = (grams of acrylamide + grams of bis-acrylamide) per 100 ml of solution). Figure 1A (page 34) shows MW vs. R_f plot for BenchMark™ proteins run on 6%, 10%, and 14% TG gels. A closer look at these plots shows that the plot is sigmoidal and an assumption of linearity is valid only over a finite but useful range. The non-linearity is even more pronounced when high MW proteins are analyzed (Figure 2). The extent of the linear relationship and useful molecular weight range depends on the gel concentration and gel system (4). Note that on a 6% gel, proteins with molecular weights below 50 kDa were not included on the plot, since they ran with the dye front and were not separated. On the 14% gel, the

higher molecular weight proteins were compressed. Hence, the 6% gel is appropriate for MW estimations of > 50 kDa (correlation coefficient, $R^2 = 0.99$) while the 14% gel can be used up to 70 kDa ($R^2 = 0.99$). A linear regression of the 20-100 kDa region of the 10% gel gives a R^2 of 0.99, and is therefore useful for this range. The same trend is observed on all uniform gels regardless of the vendor and the markers used. Figure 1B shows the Precision Plus Protein™ Standard run on 12% and 15% Ready Gel® Tris-HCl gels. The linearity of the calibration curve holds up to 50 kDa on the 15% gel and to 75 kDa on the 12% gel. The correlation coefficient for both linear fits is 0.99. Estimates of apparent MW beyond these ranges should be approached with caution.

The non-linearity of the log MW vs. R_f is most pronounced in the high MW range. Attempts to use a linear fit in this range will result in large errors. Figure 2 shows a

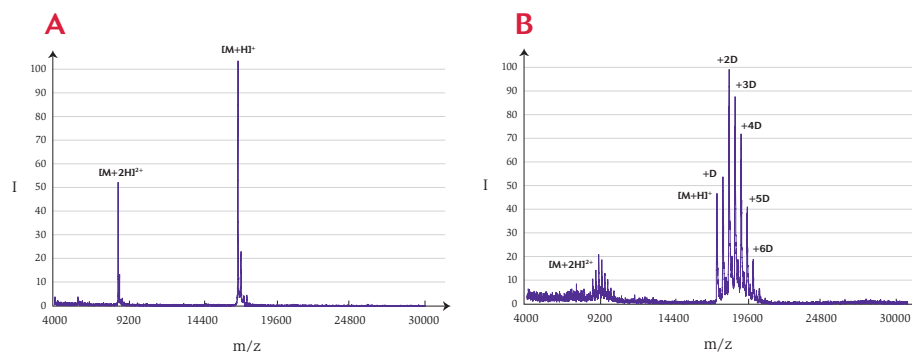
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set of high MW standards, HiMark™ Unstained High Molecular Weight Protein Standard (40 kDa-500 kDa) run on a 3-8% TA gel. Note that if a linear fit is used for the estimation of proteins of interest around the A and B region (Figure 2A), large errors in the apparent MW are inevitable. If MW estimations across the whole range of the gel are required, the use of a non-linear fit (most often a polynomial fit) is recommended (Figure 2B). To make these calculations simple, we have provided pre-made Excel spreadsheets, which automatically perform the necessary calculations, based on pre-selected non-linear equations for the particular gel type. Once the R_f values for the standards and the proteins of interest are entered into the spreadsheet, the program will return, depending on the type of gel used, the estimated MWs of the proteins. The Excel spreadsheets are available for free download from the Invitrogen Website at <http://www.invitrogen.com>.

MWs of a number of proteins/glycoprotein based on calibrations with unstained standards on NuPAGE® and TG gels are presented in Table 2 (page 39). Using the linear range of the standard curves, it can be seen that only measurements of the glycoprotein deviate more than 10% from the theoretical and 33% deviate <5%. Note that a more accurate MW assignment for the glycoprotein was obtained on NuPAGE® gels compared to TG and Tris-HCl gels. It is important to remember that the accuracy of the MW estimation depends greatly on the similarity between the hydrodynamic properties of the protein of interest and the proteins in the standard set. That is, it depends on how well the assumptions

Figure 3 – MALDI mass spectrum of myoglobin compared to a myoglobin-dye conjugate



MALDI mass spectrum of (a) myoglobin, M, compared to that of the (b) myoglobin-dye conjugate. The peaks in the protein-dye complex correlate with the attachment of up to 6 dyes (D) to the protein.

that support the technique apply to both standards and the protein of interest. If the assumptions do not apply equally well to the protein of interest and the standards, even if the calibration curve is linear over the mass range of interest, MW estimations will not be accurate. This may not be obvious unless the Ferguson plots ($\log R_f$ vs. %T) are graphed for both the standards and the protein of interest. If Y_0 , relative free mobility, which is the mobility of the protein extrapolated to %T=0, *i.e.*, in solution with no gel, is different for the protein of interest and the standards, MW measurements may not be reliable. Consult references 3 and 5 for a thorough review on this subject.

It is also important to keep in mind that for more accurate MW estimations, non-stained standards should be used. In general, pre-stained standards are not recommended for MW estimations and are usually used to monitor the electrophoretic run or the transfer of proteins

during western blotting. This is because pre-stained standards are usually a mixture of different protein-dye conjugates. Mass spectrometry data show a wide mass range of protein-dye conjugates in these mixtures with an average mass that is much higher than the MW of the protein. For example, mass spectra of aprotinin, lysozyme, myoglobin, and glutamic dehydrogenase protein-dye complexes show *average* MWs of 8073, 16121, 18188, 79217 Da vs. the measured undyed protein MWs of 6511, 14306, 16951, and 55658 Da, respectively.

Figure 3 (page 37) shows MALDI mass spectra of myoglobin and myoglobin dyed with 5 or 6-carboxy tetramethylrhodamine (TAMRA). Note that in the dyed myoglobin, a cluster of peaks from 16,950 Da to 19,430 Da, which is consistent with the undyed myoglobin and mass variants with 1-6 dyes attached, is observed. The difference in mass between the peaks corresponds to the expected mass increase of 413 Da on the

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Table 1 – Comparison of molecular weights determined by mass spectrometry with the assigned mass of three unstained MW standards: MagicMark™ XP, BenchMark™, and Mark12™. % deviations from the mass spec determined mass, are provided in the parentheses

MagicMark™:	"Assigned Mass" in Standard Set	Mass Spec. MW
	220,000 (+ 0.0)	219650
	120000 (-0.7)	120832
	100000 (-2.7)	102082
	80000 (-0.6)	80514
	60000 (-0.3)	60170
	50000 (-0.4)	50176
	40000 (-0.1)	40035
	30000 (0.3)	29902
	20000 (0.1)	19974
BenchMark™:	"Assigned Mass" in Standard Set	Mass Spec. MW
	220000 (-0.4)	220822
	160000 (0.0)	159971
	120000 (0.3)	119607
	100000 (0.5)	99467
	90000 (0.2)	89815
	80000 (0.2)	79827
	70000 (0.9)	69380
	60000 (0.4)	59748
	50000 (0.4)	49825
	40000 (1.6)	39364
	30000 (0.7)	29788
	25000 (0.2)	24938
	20000 (0.5)	19895
	15000 (0.7)	14899
	10000 (-1.7)	10170
Mark12™:	"Assigned Mass" in Standard Set	Mass Spec. MW
	200000 (-11)	224651
	116300 (0.2)	116080
	97400 (-0.2)	97623
	66300 (-0.2)	66454
	55400 (-0.5)	55658
	36500 (0.1)	36472
	31000 (6.9)	28994
	21500 (7.6)	19973
	14400 (0.7)	14303
	6000 (-7.8)	6511
	3500 (1.1)	3463
	2500 (6.9)	2338

formation of a dye-protein amide bond. It is important to realize that since the number of amine groups in proteins and their surface positions differ greatly, the efficiency of the reaction will vary from protein to protein. Moreover, the ratio of SDS to unit mass of protein may vary among dyes used. Some dye moieties remain charged in solution, which will cause protein migration to change in gels of different operating pH. Due to the above reasons, unstained standards are preferred for molecular weight estimation.

Proteins with unusual amino acid compositions or post-translational modifications can behave anomalously in MW estimates using gels. Proteins such as pepsin, which do not bind significant amounts of SDS, or those with molecular weights below 15,000 Da, are good examples of anomalous proteins. Several factors can contribute to the behavior of small proteins. The inherent charge of the polypeptide can contribute to the overall charge due to its small size or the shape and conformation of the polypeptide (5). Systems specifically for smaller proteins have been developed (7). Moreover, we assume that the large charges associated with the binding of SDS, overwhelm the intrinsic charges of proteins. In the case of proteins such as histones that carry a high net positive charge, the intrinsic charge of the protein contributes to the overall charge of the protein even in the presence of large amounts of SDS (5). These basic proteins migrate slower in the gel and their MWs are usually overestimated. Certain modifications are made in the gel systems for histones (5). Also, unreduced proteins can bind lower amounts of SDS, e.g., unreduced BSA

binds only 0.9 g SDS per gram of protein, emphasizing the fact that reduction of the protein samples plays an important role in more accurate estimations of molecular weights (8).

Glycoproteins are well known to behave anomalously even in the presence of SDS (3,5) and the MW inferred from R_f measurements are often higher than their real MWs, i.e., the weight of protein sequence and the glycan structure. This is probably related to the fact that SDS only binds, in the manner that we expect, to the amino acid portion, leaving the mobility of the molecule lower than expected. The presence of a prosthetic group in proteins in general seems to cause anomalous behavior; glycoproteins, lipoproteins, and maleylated proteins all behave anomalously in gels (3). Other post-translational modifications, such as phosphorylation can also contribute to the errors associated with MW estimations. Early on in the investigation of the precision of SDS-PAGE, Frank and Rodbard investigated the membrane protein rhodopsin (9). Molecular weights obtained for rhodopsin gave errors as high as 36%. Results in Table 2 (page 39) show that relative errors can be as high as 46% for fetuin, which is a highly glycosylated protein. NuPAGE® gels provided better MW estimations than TG gels.

There are a number of theories for explaining the structure of protein-SDS complexes, among which the "necklace" model by Shirahama, *et al.* (10), seems to be supported best by experimental evidence (11,12). In this model, where SDS micelle aggregates form along the protein like pearls on a string, it is easy to visualize that the ratio of SDS/protein will

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Table 2 – Molecular weight estimation for eight different proteins and glycoproteins, some commonly used as MW standards, obtained from calibration curves using different calibrator sets on NuPAGE® Bis-Tris (10%) and Novex® Tris-Glycine (14%). Relative errors from the expected MW are given in the parenthesis

NuPAGE® Bis-Tris	MW*	MagicMark™	
Bovine Serum Albumin	66431	68128 (2.6)	
Chymotrypsinogen A	25656	25594 (-0.2)	
β-Lactoglobulin B	18277	17224 (-5.8)	
Myoglobin	16951	15687 (-7.5)	
Cytochrome c	12360	11591 (-6.2)	
Fetuin (glycoprotein)	45548	58823 (29.1)	
Novex® Tris-Glycine	MW	MagicMark™	Precision Plus Protein™
Bovine Serum Albumin	66431	65306 (-1.7)	64907 (-2.3)
Chymotrypsinogen A	25656	27039 (5.4)	27396 (6.8)
β-Lactoglobulin B	18277	17399 (-4.8)	17290 (-5.4)
Myoglobin	16951	16175 (-4.6)	15598 (-8.0)
Cytochrome c	12360	13043 (5.5)	13152 (6.4)
Fetuin (glycoprotein)	45548	66609 (46.2)	663871 (40.2)

* MW corresponds to the theoretical mass of the protein sequence except for the glycoprotein, fetuin, for which the MW determined by mass spectrometry was used.

depend on the hydrophobic and hydrophilic regions of the protein and also on the charges of these domains. Electrostatic repulsion/attraction between SDS and negative/positive regions of the proteins will affect the mobility of the protein (12). Since the pH of the buffer can affect the charges, we believe that this can contribute to the different mobilities observed on different gels with different running pHs. For this reason, it is important to use the “assigned” mass of the calibrant for each gel system in each calibration curve, especially in the case of dyed proteins. Nevertheless, discrepancies between different gel systems can still be seen.

Conclusions

Gel electrophoresis can be used to esti-

mate protein MW with deviations of 1-10% under optimal conditions but errors of up to 20% can be expected. Nevertheless, this degree of accuracy is adequate for many applications. It is important to select the appropriate gel for the range of interest and to stay within the linear range of the standard curves. If one needs to use the entire range of the gel, the use of non-linear fits such as the polynomial fit, especially in the high MW regions is highly recommended. Today, a more accurate mass can be determined by mass spectrometry techniques such as MALDI and Electrospray Ionization (ESI), which can provide mass accuracies on the parts per million (ppm) levels. But mass spectrometers are expensive and are not available in every lab, samples need to be extensively purified, and these

methods are usually intolerant of salts/buffers/detergents commonly used in biological labs. Also, mass spectrometry analysis of large proteins exceeding 150 kDa is still a challenging task in part due to the lack of mass spectrometry compatible solvents for larger proteins, the large quantities required for their analysis, and in general problems associated with their desorption/ionization and detection. Gel electrophoresis still remains a robust, reliable, and high-throughput method for the MW estimation of these larger proteins. In the end, a thorough understanding of gel electrophoresis and the assumptions on which MW estimations are based can provide the means to more accurate “apparent” MW measurements.

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