

Protein Expression Application Note No. 2

Optimization of a DNA Polymerase Expressed in Insect Cells

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1 Introduction

The polymerase chain reaction (PCR) is a technique widely used in molecular biology, microbiology, genetics and diagnostics. The name, polymerase chain reaction, comes from the Taq DNA polymerase used to amplify (replicate many times) a piece of DNA using *in vitro* enzymatic replication. DNA polymerases are enzymes that catalyze the polymerization of deoxyribonucleotides into a DNA strand. They are best-known for their role in DNA replication, in which the polymerase “reads” an intact DNA strand as a template and uses it to synthesize the new strand. With PCR it is possible to amplify a very small number of pieces of DNA, over many cycles, generating millions of copies of the original DNA molecule. PCR has been extensively modified to perform a wide array of genetic manipulations and diagnostic tests.

DNA polymerases have a highly conserved structure, which means that their overall catalytic subunits vary very little from species to species. Based on sequence homology, DNA polymerases can be further subdivided into seven different families: A, B, C, D, X, Y, and reverse transcriptases.¹

The Baculovirus Expression Vector System (BEVS) is a convenient and versatile eukaryotic system for heterologous gene expression and large-scale protein expression (yields of up to 100 mg of protein per 10⁹ cells).²

Baculovirus expression provides correct folding of recombinant protein, as well as disulfide bond formation, oligomerization, and other important post-translational modifications. Consequently, the overexpressed protein exhibits the proper biological activity and function.

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The Baculovirus Expression Vector System is based on the introduction of a foreign gene into a nonessential region of the viral genome via homologous recombination with a transfer vector containing the cloned gene (accommodates genes up to 15 kb); this event occurs in the cotransfected insect cells. The production of foreign protein is then achieved by infection of additional insect cell cultures with the resultant recombinant virus.

The InsectDirect™ System (Novagen) is an enhancement

of the BEVS, providing a rapid, high throughput and virus-free method for heterologous protein expression in insect cells.³

The aim of the current study was to optimize a DNA polymerase for one-step RT-PCR that shows optimal performance in the combined RT and PCR step, and to improve methods for introducing mutations based on structure and sequence comparisons with known polymerases.

2 Methods

1. Cloning and Preparation of the DNA Polymerase Mutants

1.1 Site-directed mutagenesis by overlap extension PCR

The overlap extension polymerase chain reaction is a variant of PCR for inserting mutations at specific points in a sequence.⁴

To create the DNA polymerase mutants in two separate PCR reactions, two overlapping subfragments of the target sequence were amplified using a high fidelity DNA polymerase (0.5 units). Each reaction contained one flanking primer and one internal primer containing the desired mutation (0.4 μM final primer concentration). The template was the eukaryotic baculovirus expression vector pFBDM (10 pg to 200 ng), carrying the wild type DNA polymerase sequence. Generated subfragments were analyzed, using gel electrophoresis, purified with the High Pure PCR Product Purification Kit (Roche), and used as templates in a second round of PCR for the synthesis of the DNA polymerase mutants. After amplification, the end product was again analyzed by gel electrophoresis and purified with the High Pure PCR Purification Kit (Roche, see Figure 1). Purification was performed as described in the package insert.

1.2 RE digest

1 μg DNA was incubated with 1 to 2 units of the appropriate restriction enzyme (Roche). Incubation was carried out using the matched SuRE/Cut buffer (Roche) and temperature, according to the manufacturer's protocol, for at least one hour.

1.3 Dephosphorylation

To prevent re-ligation of the digested vector, the 5'-phosphate groups were removed by addition of 1 unit of rAPid Alkaline Phosphatase from the Rapid DNA Dephos & Ligation Kit (Roche) according to the manufacturer's instruction; this was added directly to the restriction digest after one hour. This mixture was incubated for 10 min. at +37°C, followed by 2 min at 75°C to inactivate the rAPid Alkaline Phosphatase.

1.4 Ligation of DNA-fragments

50 ng of vector DNA was ligated with 150 ng DNA insert for 5 minutes at room temperature using the Rapid DNA Dephos & Ligation Kit (Roche). 2 μl of the ligation reaction was directly used for the transformation into competent *E. coli* XL1-blue cells.

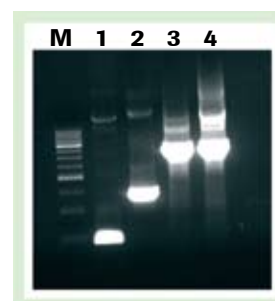


Figure 1: Site-directed mutagenesis by overlap extension PCR.

Amplification of two different DNA polymerase mutant subfragments (lanes 3 and 4), a short subfragment (lane 1), and eGFP (lane 2) to measure transfection efficiency. All fragments were cloned in the vector pLEX-1 (Novagen) for expression in the InsectDirect™ System (Novagen). After PCR

amplification, the fragments shown here were cut out of the agarose and DNA was purified using the High Pure PCR Product Purification Kit, followed by restriction enzyme digest with the appropriate SuRE/Cut buffers, and ligation with the Rapid DNA Dephos & Ligation Kit.

1.5 Plasmid preparation

The eukaryotic baculovirus expression vectors pFBDM (containing the mutated DNA polymerase genes) were purified from *E. coli* XL1-blue cells using the Genopure Plasmid Midi and Maxi Kit (Roche). One vial with 1 ml LB Medium was inoculated with a bacteria colony containing the respective plasmids and incubated at +37°C with vigorous shaking for eight hours. This preculture was then added to 150 ml LB-Medium (100 µg/ml) and incubated overnight with vigorous shaking at +37°C. The DNA preparation was performed according to the Genopure Plasmid Maxi Kit manual. Yields ranged from 630 to 1480 ng/µl.

The expression vector pIEX-1 for the InsectDirect™ System (containing the mutated DNA polymerase gene) was purified from *E. coli* XL1-blue cells using the Endofree Plasmid Maxi Kit (Qiagen). This removes endotoxins, which can disrupt an effective transfection of insect cells. One vial with 1 ml LB Medium was inoculated with a bacteria colony containing the respective plasmids and grown at +37°C with vigorous shaking overnight. This preculture was then added to 150 ml LB-Medium (100 µg/ml) and incubated overnight with vigorous shaking at +37°C. The DNA preparation differs from a normal purification, with a separate step for the removal of endotoxins, performed according to the manufacturer's protocol.

2. Expression of the recombinant Proteins in Insect Cells

2.1 Baculovirus Expression Vector System

Sf9 cells (Invitrogen) were grown in serum-free SF-900 II SFM Medium (Gibco) in suspension at +27°C using soft shaking under 5% CO₂. One hour prior to transfection, cells were seeded in a 6-well cell culture plate at a density of 1 x 10⁶ cells/well. In the meantime 10 µg recombinant pFBDM vector carrying the mutated DNA polymerase gene were mixed with FuGENE® HD Transfection Reagent at a molar ratio of 1.5:1, and incubated for 30 minutes at room temperature. This transfection-DNA-complex was added drop wise to the cells. After an incubation period of five days at +27°C, supernatants from each well were combined in one vial. About 2.5 ml of this P1 generation was used for the infection of a 50 ml suspension culture

(P2) of insect cells with a cell density of 1 x 10⁶ cells/ml. This culture was incubated for five days at +27°C with soft shaking. 25 ml of the supernatant was used to infect a 200 ml Sf9-culture (1 x 10⁶ cells/ml; P3). After the incubation period of three days at +27°C with soft shaking, cells were harvested to extract total protein. The supernatant was stored at +4°C.

2.2 InsectDirect™ System

For the expression of recombinant DNA polymerase mutants with the InsectDirect™ System (Novagen), DNA concentrations in a range of 2 to 4 µg/µl were used. Transfection was performed in 6-well cell culture plates and in 50 ml suspension cultures, each with a cell density of 1 x 10⁶ cells/well or ml. In addition to the FuGENE® HD Transfection Reagent, a transfection reagent X from another supplier was used for the formation of the transfection-DNA-complex in molar ratios from 0.5:1 to 7:1. DNA was diluted with ultrapure water, to a final concentration of 0.02 µg/µl and 0.04 µg/µl. Afterwards the transfection reagent was either added drop wise directly to the DNA dilution (FuGENE® HD Transfection Reagent) or first diluted separately (transfection reagent X) in 1 ml serum-free SF-900 II SFM medium, and then added to DNA dilution. These transfection mixtures were incubated at room temperature for 15 to 60 minutes until the transfection-DNA-complexes were added drop wise to the cells. Both culture types (adherent and suspension) were incubated for 48 to 72 h at +27°C, before the cells were harvested by chemical lysis (adherent cell culture) or centrifugation (suspension culture).

2.3 Cell lysis

Total protein of the BEVS was extracted by sonification. The cell pellet was suspended in buffer containing 1x cOmplete, EDTA-free Protease Inhibitor Cocktail Tablet (Roche), 1 mM PMSF (Roche) and 1 mM β-Mercaptoethanol, and sonificated two times for 60 seconds with an amplitude of 50%. After sedimentation of the cell debris by centrifugation (15,000 x g, 50 min, +4°C), the supernatant was used for the purification by Ni-NTA columns.

Cells of the adherent cell culture of the InsectDirect™ System were washed once with PBS, and then chemically solubilized using 200 µl M-Per mammalian Protein Extraction Reagent (Pierce) per well, according to the manufacturer's protocol.

3. Verification of Protein Expression after Transfection

Epitope tagging

20 μ l of the cell lysates were pipetted into SDS-PAGE (XCell SureLock Mini-Cell chambers (Invitrogen) to determine the quality of the protein expression. For the analysis of the specificity of the protein purification, SDS gels were transferred onto a nitrocellulose membrane for use with the XCell 2 Blot Module (Invitrogen). After the membrane was blocked and washed with 1x TBST, it was incubated with a 1:10,000 antibody dilution of a monoclonal mouse Anti-His₆-Peroxidase (Roche) for one hour at room temperature. After three washes, the membrane was incubated with a 1:4,500 dilution of a secondary goat anti-mouse IgG antibody (Chemicon) for another hour at room temperature. After washing for three times, bound antibody was detected using the Lumi-Light^{PLUS} Western Blotting Substrate (Roche), and subsequent exposure to photographic film, as described in the Lumi-Light^{PLUS} Western Blotting manual.

4. Purification of the recombinant Proteins

Ni-NTA columns

One way to purify recombinant proteins is the use of Ni-NTA affinity chromatography. A short sequence of six histidines was added to the gene of interest in the expression vector. Through the formation of a complex between these histidines and the Ni-NTA matrix on the purification column, recombinant proteins were isolated and eluted using the stronger complexing reagent imidazol. Supernatants of the BEVS (P3) and the InsectDirectTM System were added to the 50% Ni-NTA column (Qiagen), washed twice with each 20 ml of 50 mM imidazol buffer, and eluted with 5 ml 400 mM imidazol buffer. Isolated DNA polymerases were concentrated using a heparin-sepharose column (Amersham) and transferred to storage buffer by dialysis.

5. Functional Characterization of the recombinant DNA Polymerase Mutants

5.1 One-step RT-PCR on thermal cycler

The improved properties of the DNA polymerase mutants generated in the BEVS were tested using an one-step RT-PCR approach. Based on human liver or skeleton RNA (5 ng/ μ l) as template, a 350 bp and 587 bp fragment of β -actin was transcribed and amplified using the DNA polymerase mutants. A reaction buffer was prepared containing 3.4 mM MgCl₂ (Roche), 0.6 mM deoxynucleotides (Roche), 2.6 U/ μ l Protector RNase Inhibitor (Roche), 1 U/ μ l reverse transcriptase, and 1.25 U/ μ l of each DNA polymerase mutant. Approximately 25 ng RNA was used as template per reaction. The reaction was set up at +45°C for 30 min for the reverse transcription. The resulting cDNA was directly amplified in the same tube in the following PCR step. DNA end products were analyzed using agarose gel electrophoresis.

5.2 One-step qRT-PCR on real-time instrument

DNA polymerase mutants were also tested in a quantitative one-step RT-PCR using an Applied Biosystems 7500 Real Time PCR System (ABI) in combination with a primer for the amplification of a 86 bp fragment of the β -2 microglobulin gene (as reference gene). To test the DNA polymerase mutants, we optimized the reaction mix by testing different amounts of the individual components.

3 Results

1. Expression and Purification of the DNA Polymerase Mutants in the Baculovirus Expression Vector System

Mutants of a DNA polymerase were expressed in the BEVS to produce an optimized DNA polymerase for one-step RT-PCR that shows improved features. Expression plasmids carrying the genes of the DNA polymerase mutants were prepared using the Genopure Plasmid Maxi Kit (Roche) and transfected into insect cells using FuGENE® HD Transfection Reagent (Roche).

The DNA polymerase mutants were purified using Ni-NTA columns and analyzed by SDS-PAGE (see Figure 2). Yields range from 380 to 740 µg/ml purified enzyme.

The amount of purified DNA polymerase protein was estimated by SDS-PAGE and BCA Protein Assay Kit (Pierce), and adjusted to 20 U/µl using a reference of known concentration.

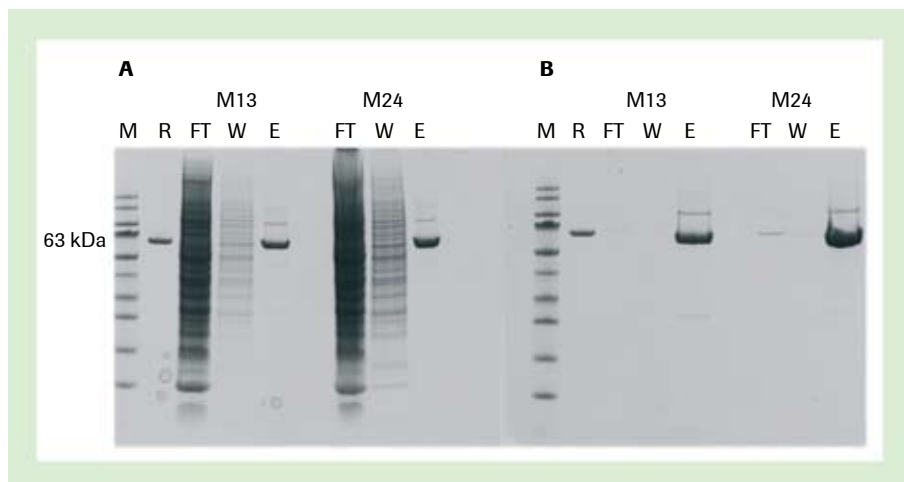


Figure 2: Purification of the DNA polymerase mutants.

Insect cells transfected with the expression plasmid were harvested and purified with Ni-NTA for the isolation of the DNA polymerase mutants. Wild type DNA polymerase was used as control (R).

A. Ni-NTA column; **B.** Heparin-Sepharose column for concentrating the amount of purified enzymes.

Abbreviations are: **FT** for flow through, **W** for wash, and **E** for eluates. Protein yields range from 380 to 740 µg/ml.

2. Test of the InsectDirect™ System

The InsectDirect™ System is an enhancement of the BEVS. We used it to test the expression of the DNA polymerase mutants. First, we needed to demonstrate that genes such as eGFP can be delivered into insect cells using the FuGENE® HD Transfection Reagent, and that a high proportion of cells is transfected and that the cells remain viable.

The expression plasmid carrying eGFP (pIEX-1_eGFP) was prepared using the Genopure Plasmid Maxi Kit (Roche), and transfected into insect cells using the FuGENE® HD Transfection Reagent (Roche) at a molar ratio of 2.5:1, and transfection reagent X at a molar ratio of 7:1.

To estimate transfection efficiency and cytotoxicity, cells were analyzed using fluorescence microscopy. Representative images were taken, and the transfected cell number was determined. Using FuGENE® HD Transfection Reagent, 30% of the cells were eGFP positive 24 h after transfection, whereas cells transfected with reagent X show a 20% transfection efficiency, and an increased cytotoxicity of up to 60% (see Figure 3).

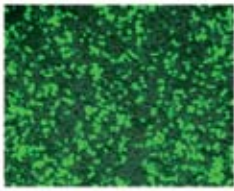
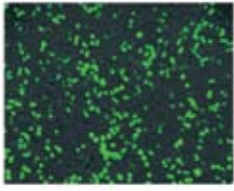
Transfection reagent		Transfection efficiency	Cytotoxicity
FuGENE® HD 2.5:1		30%	15%
Transfection reagent X 7:1		20%	60%

Figure 3: Estimation of transfection efficiency.

Insect cells were transfected with the expression plasmid pIEX-1_eGFP using either the FuGENE® HD Transfection Reagent (Roche) or transfection reagent X (from a different supplier). Transfection with FuGENE® HD Transfection Reagent yielded 30% eGFP transfected cells with low cytotoxic effects, whereas transfection with reagent X resulted in up to 60% cytotoxicity with 20% transfection efficiency.

To optimize transfection efficiency, different FuGENE® HD Transfection Reagent:DNA-complexes with molar ratios from 0.5:1 to 3.5:1 and 0.02 or 0.04 µg/µl DNA dilutions were tested (see Figure 4). The 1.5:1 transfection-DNA-complex with the 0.04 µg/µl DNA dilution showed a 85% transfection efficiency 72 h after transfection.

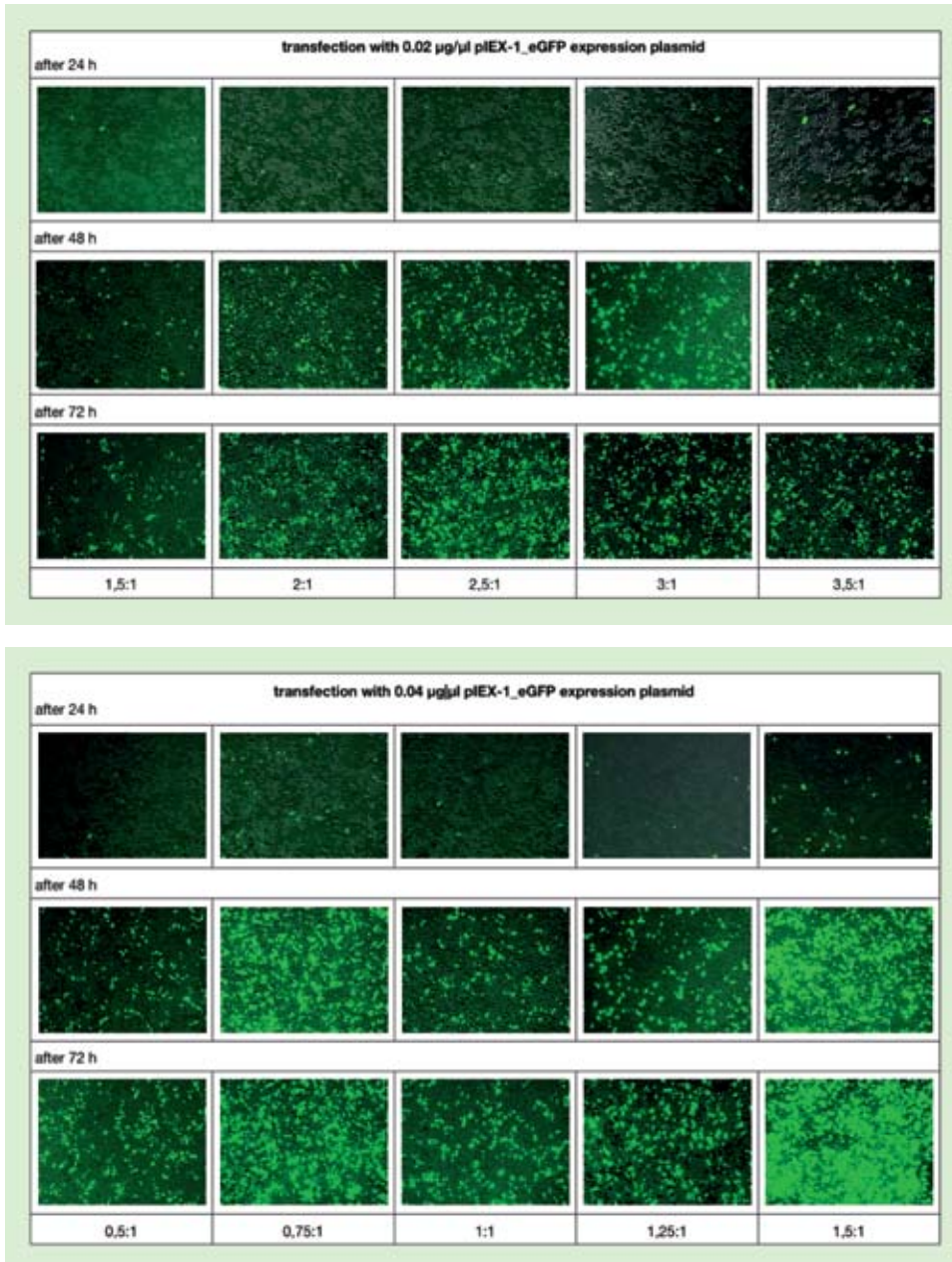


Figure 4: Optimization of transfection efficiency of FuGENE® HD Transfection Reagent in insect cells.

Insect cells were transfected with the expression plasmid pLEX-1_eGFP using either 0.02 or 0.04 µg/µl DNA in molar ratios from 0.5:1 to 3.5:1 (FuGENE® HD Transfection Reagent:µg DNA). The expression plasmid was generated using the Genopure Plasmid Maxi Kit (Roche). Successful transfection was determined after 24, 48 and 72 h. Approximately 85% of the cells transfected with 0.04 µg/µl DNA in a molar ratio of 1.5:1 were eGFP positive 72 h after transfection.

The same approach was used to examine the expression of a DNA polymerase mutant as was used for the eGFP reporter gene assay. Expression and transfection were estimated using western blot analysis (see Figure 5). With the exception of nonspecific endogenous protein bands, no expression of the DNA polymerase mutant could be detected. Western blot analysis showed that the Anti-His₆-Peroxidase antibody (Roche) produced less unspecific background than the Penta anti-his antibody (Qiagen).

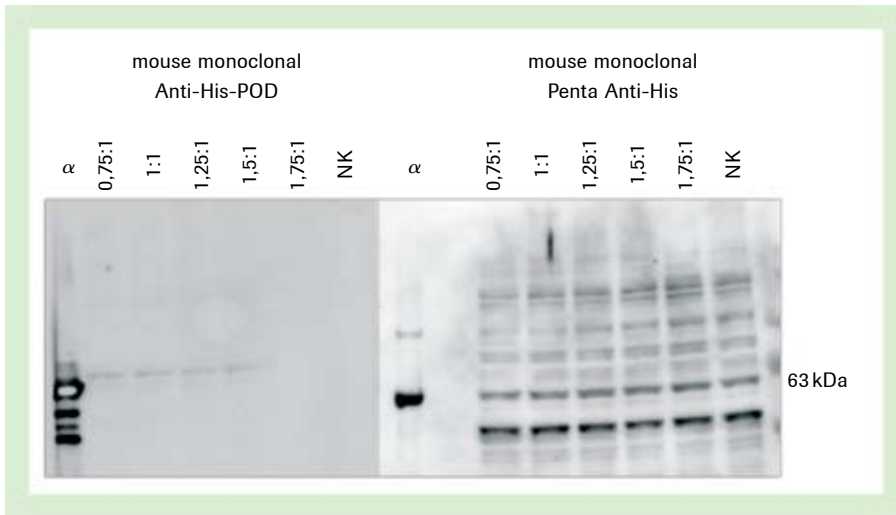


Figure 5: Comparison of two different antibodies against the His₆ tag of proteins.

Two 6-well plates with 1×10^6 insect cells / well were transfected with $0.04 \mu\text{g}/\mu\text{l}$ expression plasmid carrying a mutated DNA polymerase gene using FuGENE[®] HD Transfection Reagent in molar ratios from 0.75:1 (FuGENE[®] HD:DNA). Cells were harvested after 72 h, and $20 \mu\text{l}$ supernatant were used for western blot analysis using either a 1:10,000 dilution of the mouse monoclonal Anti-His₆-Peroxidase antibody (Roche) or a 1:3,500 dilution of the mouse monoclonal Penta Anti-His antibody (Qiagen). Protein expression was detected using the Lumi-Light^{PLUS} Western Blotting Substrate (Roche).

3. Functional characterization of the DNA polymerase Mutants

To test the performance of DNA polymerase mutants successfully expressed in the Baculovirus Expression Vector System, two different one-step RT-PCR were performed.

Overall performance of the enzyme mutants was examined using semiquantitative one-step RT-PCR on a thermal cycler. Using human liver and skeleton RNA, a 350 and 587 bp fragment of the β -actin gene was transcribed and amplified. The amount of DNA end product was analyzed using gel electrophoresis. Figure 6 shows the functionality and ability of the DNA polymerase mutants to synthesize both 350 and 587 bp DNA fragments.

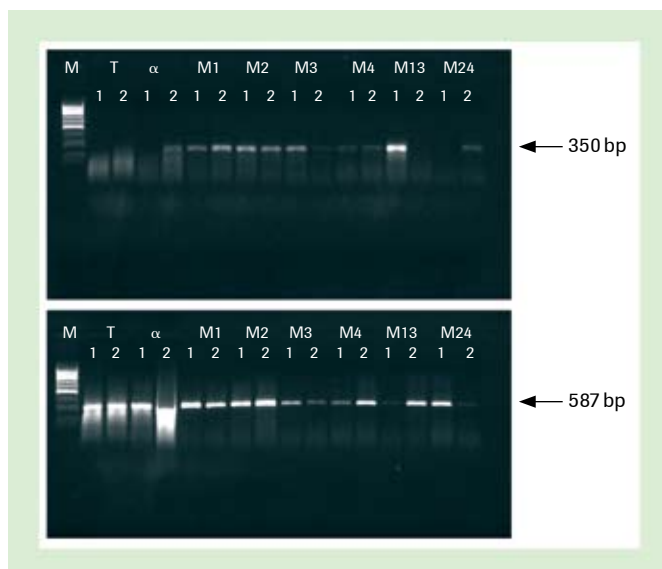


Figure 6: Performance of expressed DNA polymerase mutants in the one-step RT-PCR test system.

Mutants of a DNA polymerase were generated using the Baculovirus Expression Vector System and purified using Ni-NTA. With 25 ng human liver RNA, all tested enzymes were able to amplify both the 350 and 587 bp DNA fragment of β -actin.

Quantitative analysis of resulting DNA was examined using one-step qRT-PCR using an Applied Biosystem 7500 Real Time PCR System (ABI) with a primer for the amplification of a 86 bp fragment of the β -2 microglobulin gene (see Figure 7). All DNA polymerase mutants were found to be more active than the wild type, and able to synthesize up to 48.5 times more DNA than the native DNA polymerase.

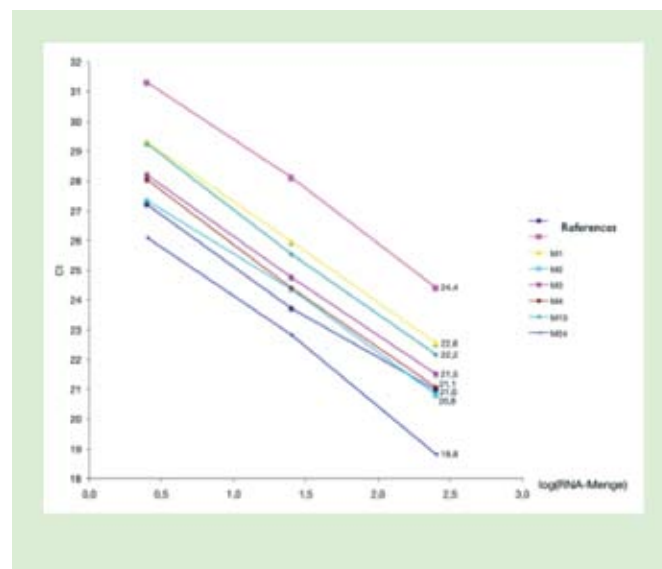


Figure 7: Performance of expressed DNA polymerase mutants in the one-step qRT-PCR test system.

Mutants of DNA polymerase were generated using the Baculovirus Expression Vector System and purified using Ni-NTA. With 2.5, 25 and 250 ng human liver RNA, all enzymes were able to synthesize the 86 bp DNA fragment of the β -2 microglobulin gene. All mutated enzymes were also more active than the wild type polymerase.

4 Conclusion

The aim of the present study was to design an easy-to-carry-out workflow for the generation of an optimized DNA polymerase with improved properties, using site-directed mutagenesis. We evaluated two different insect expression systems. The newly developed InsectDirect™ System was tested to quickly generate milligram quantities of recombinant proteins. Our eGFP reporter gene assay showed that the FuGENE® HD Transfection Reagent was able to transfect 85% insect cells without inducing cytotoxic effects. Although the cells were successfully transfected, further optimization would be necessary to express the DNA polymerase mutants.

Using the Baculovirus Expression Vector System and FuGENE® HD Transfection Reagent, our findings show that we were able to design and express milligrams of nearly pure and highly active enzymes that synthesize up to 48.5 times more DNA than the wild type. The improved performance of these mutant DNA polymerases was evaluated using both semiquantitative one-step RT-PCR and one-step qRT-PCR.

References

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Product	Cat. No.	Pack Size
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	11 732 676 001	1 kit (up to 250 purifications)
Restriction Enzymes	For all restriction enzymes please visit our special interest site at www.restriction-enzymes.com	
Rapid DNA Dephos & Ligation Kit	04 898 117 001	40 reactions
	04 898 125 001	160 reactions
rAPid Alkaline Phosphatase	04 898 133 001	1,000 reactions
	04 898 141 001	5,000 reactions
Genopure Plasmid Midi Kit	03 143 414 001	1 kit (for up to 20 isolations)
Genopure Plasmid Maxi Kit	03 143 422 001	1 kit (for up to 10 isolations)
FuGENE® HD Transfection Reagent	04 883 560 001	1 trial pack
	04 709 691 001	0.4 ml
	04 709 705 001	1.0 ml
	04 709 713 001	5 x 1 ml
	05 061 369 001	10 ml
PMSF	10 837 091 001	10 g
	11 359 061 001	25 g
cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets supplied in EASYpacks	04 693 132 001	20 tablets
Lumi-Light^{PLUS} Western Blotting Substrate	12 015 196 001	100 ml
Anti-His₆-Peroxidase	04 905 270 001	80 U
Magnesium Chloride Solution	11 699 113 001	3 x 1 ml
Protector RNase Inhibitor	03 335 399 001	2000 U
	03 335 402 001	10 000 U (5 x 2,000 U)
PCR Nucleotide Mix	11 581 295 001	200 µl (for 200 reactions)
	11 814 362 001	2,000 µl (for 2,000 reactions)

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