

Wizard[®] Plus SV Minipreps DNA Purification System: The Next Generation in Miniprep Purification



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Promega's new Wizard[®] Plus SV Minipreps DNA Purification System provides highly purified plasmid DNA and offers the flexibility of using either a spin or vacuum manifold format. The system incorporates a novel alkaline protease digestion step allowing the use of endA positive bacterial strains expressing endonuclease I. In this article, we demonstrate that plasmid DNA isolated using this system is optimal for demanding applications such as automated fluorescent sequencing and coupled in vitro transcription and translation systems.

Introduction

Purification of plasmid DNA is an essential technique for molecular biologists. The 1970s witnessed the development of cornerstone molecular biology techniques such as restriction enzyme digests and cloning of plasmid DNA. Subsequent developments included rapid, small-scale ("miniprep") plasmid DNA purification techniques and the improvement of cloning plasmids by incorporation of transcription promoters, translation initiators and multiple cloning sites into plasmid vectors. In the 1990s, purified miniprep plasmid DNA is commonly used in demanding applications such as fluorescent sequencing and coupled transcription/translation reactions (e.g., TNT[®] Lysate Systems). As the complexity of downstream applications of purified miniprep plasmid DNA has increased, so has the need for high-quality plasmid DNA preparations.

Promega recently introduced a new member of our DNA Purification Systems product line: the Wizard[®] Plus SV Minipreps DNA Purification System. This system uses a silica-based membrane instead of a silica gel resin. This membrane-based system provides high integrity and high purity plasmid DNA preparations with a choice of purification formats: spin (microcentrifuge) or vacuum, or simply, "SV". Plasmid DNA purified with the Wizard[®] Plus SV DNA Purification System supports traditional downstream molecular biology applications, as well as automated fluorescent DNA sequencing and coupled transcription/translation. The Wizard[®] Plus SV Minipreps DNA Purification System is an inexpensive and rapid miniprep plasmid DNA purification system optimized for many demanding applications in molecular biology.

Purification format

The Wizard[®] Plus SV Minipreps DNA Purification System is designed for both spin (microcentrifuge) and vacuum formats for plasmid DNA purification ([Figure 1](#)). In the spin format, the Wizard[®] Plus SV Minipreps DNA Purification System can be used to process 20 samples in 45-55 minutes. The design of the Wizard[®] Plus SV Minipreps Columns allows them to be placed directly in a microcentrifuge tube and spun at 14,000 x g (i.e., top speed for most microcentrifuges). In the vacuum format, the Wizard[®] Plus SV Minipreps DNA Purification System can be used to process 20 samples in 35-45 minutes. To use the vacuum format, simply attach the Vacuum Adapter (available with some of the Wizard[®] Plus SV Minipreps DNA Purification Systems) to the Miniprep Column. The Miniprep Column is then compatible with Luer-Lok[®] vacuum systems such as the Vac-Man[®] Laboratory Vacuum Manifold (Cat.# A7231) and the Vac-Man[®] Jr. Vacuum Manifold (Cat.# A7660). Both formats yield high quality plasmid DNA minipreps. The spin format is more efficient when processing a small number of samples (<16), while the vacuum format is recommended for processing a larger number of samples.

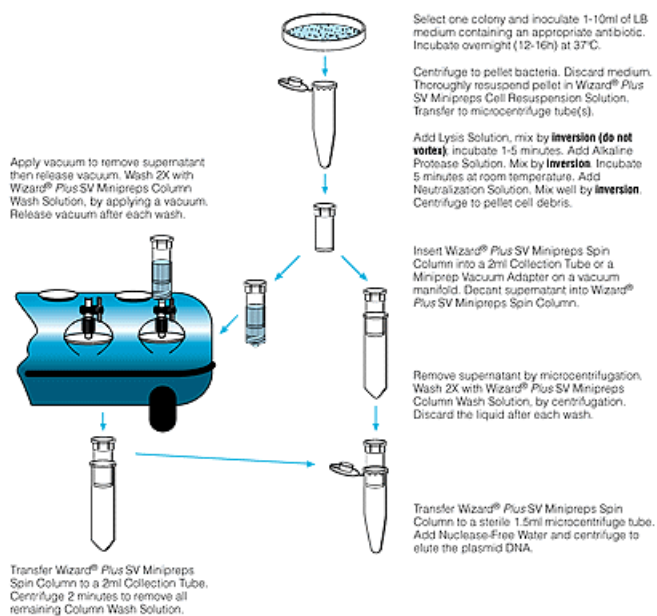


Figure 1. Flow diagram of plasmid DNA isolation and purification using the Wizard® Plus SV Minipreps DNA Purification System.

Plasmid DNA yield

Plasmid DNA yield can be affected by a number of factors, including plasmid copy number, insert size, the volume of culture processed, culture media and the overall binding capacity of the purification system. Of these factors, plasmid copy number, culture volume and binding capacity have the greatest influence on plasmid yield.

Copy number is determined primarily by the region of DNA surrounding and including the origin of replication in the plasmid. This area, known as the replicon, controls replication of plasmid DNA by bacterial enzyme complexes. In many cases, the exact copy number of a particular construct will be unknown. However, most plasmids are derived from a small number of commonly used parent constructs. Plasmid yield may vary more than 100-fold depending on the plasmid construct.

To compensate for low copy number plasmids, it is often necessary to process increased volumes of bacterial culture to obtain the required yield of plasmid DNA. This can be problematic if the amount of culture processed overloads the purification system, resulting in an increased level of contaminants in the DNA.

The Wizard® Plus SV Minipreps DNA Purification System is designed to obtain optimal yields of both high and low copy number plasmids. **Figure 2** illustrates the average yield of plasmid DNA obtained from cultures of DH5alpha® cells transformed with either pGEM®*-3Zf(+) Vector (high copy number plasmid) or pALTER®-1 *amp^r* Vector (low copy number plasmid). The average yield of pGEM®*-3Zf(+) Vector was 4.8µg (±0.5µg) from a 1.5ml culture and 2.6µg (±0.3µg) of pALTER®-1 *amp^r* Vector from a 10ml culture. When normalized for the volume of culture processed, the high copy number plasmid pGEM®*-3Zf(+) Vector (3.2µg DNA/ml of culture) yields approximately 12-fold more DNA than the low copy number plasmid pALTER®-1 *amp^r* Vector (0.26µg DNA/ml of culture).

*U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

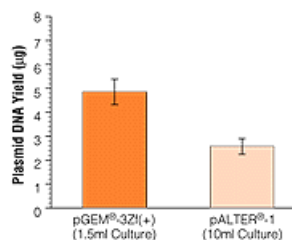


Figure 2. Plasmid DNA yield from high and low copy plasmids using the Wizard® Plus SV Minipreps DNA Purification System. *E. coli* DH5alpha® cells were transformed with either the pGEM®-3Zf(+) Vector (high copy number plasmid) or the pALTER®-1 *amp*^r Vector (low copy number plasmid) and grown in LB medium containing 50µg/ml of ampicillin (16 hours at 37°C, 200rpm). Plasmid DNA was isolated in sets of 42 on three consecutive days (126 total samples) from 1.5ml (pGEM®-3Zf(+) Vector) and 10ml (pALTER®-1 *amp*^r Vector) cultures using the Wizard® Plus SV DNA Purification System as described (7). Aliquots of each plasmid DNA preparation were electrophoresed on a 1% agarose gel along with a titration series of plasmid DNA of known concentration. The gel was stained with iodopropyl thiazole orange (a fluorescent dye that is approximately 10-fold more sensitive than ethidium bromide) and the fluorescence intensity of the samples was compared to the plasmid standards using a Molecular Dynamics FluorImager™. Each bar represents the average of 126 samples.

Figure 3 shows the effect of culture volume on the yield of pGEM®-3Zf(+) Vector. The yield of plasmid was essentially linear over the range of culture volumes tested in this experiment, with a maximum yield of 13.3µg (±0.3µg) obtained from 5ml cultures using the Wizard® Plus SV Minipreps DNA Purification System. We do not recommend using >5ml of culture transformed with a **high copy number plasmid**. Higher culture volumes saturate the purification matrix, reducing plasmid DNA yields and the quality of the preparation.

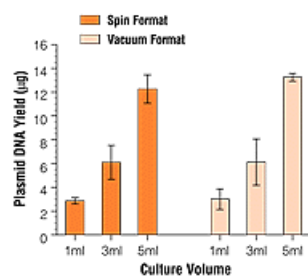


Figure 3. Plasmid DNA yield versus culture volume using both the spin and vacuum formats. pGEM®-3Zf(+) Vector DNA was propagated in 1ml, 3ml and 5ml cultures of *E. coli* DH5alpha® as described in Figure 2. Plasmid DNA was isolated from 10 samples of each culture volume using the Wizard® Plus SV Minipreps DNA Purification System spin and vacuum formats (7). Plasmid DNA yield was estimated by agarose gel electrophoresis as described in Figure 2. Each bar represents the average yield from 10 samples.

Effect of alkaline protease on plasmid DNA stability

We have previously reported plasmid DNA instability associated with DNA isolated from *E. coli* strains containing the wild-type gene for endonuclease I (8). These studies were continued during the development of the Wizard® Plus SV Minipreps DNA Purification System and have resulted in a novel method to inactivate endonuclease activity**.

**Patent pending.

Proteases have been used to enzymatically degrade proteins in nucleic acid isolation procedures for many years. Use of Proteinase K for DNA purification was first reported by Gross-Bellard *et al.* (9). Proteinase K is optimally active in the neutral pH range (pH 7-8) but is inactive at pH 10.5 and above, the typical pH of an alkaline lysate. An acid protease has also been reported to be useful in reducing protein levels in the DNA isolation process (10), but it has been noted that the low pH can result in depurination and chain breakage in the DNA. In an effort to identify a protease that would work well in an alkaline lysis procedure, we investigated the alkaline proteases (proteases active at pH 10 and above) that have been used in the detergent industry to remove protein based stains (11,12). Alkaline protease, originally identified as subtilisin Carlsberg, is isolated from the bacterium *Bacillus licheniformis* (13). During development of the Wizard® Plus SV Minipreps DNA Purification System, we found that the addition of alkaline protease during the lysis step in preparation of the cell lysate inactivates endonuclease I and reduces the overall level of protein contamination in the final plasmid DNA sample. This protease is particularly suitable for use during alkaline lysis steps because it is very efficient at protein digestion in detergents and alkaline pH.

To examine the effect of alkaline protease on plasmid stability, increasing amounts of alkaline protease was added to the cell lysate and the isolated DNA was incubated for 16 hours in the presence or absence of MULTI-CORE™ Restriction Enzyme Buffer (25mM Tris acetate (pH 7.8), 100mM potassium acetate, 10mM magnesium acetate and 1mM DTT). MULTI-CORE™ Buffer is specifically formulated to support the activity of a wide range of restriction enzymes and should provide a very favorable environment for activation of any contaminating nucleases. DNA degradation was monitored by agarose gel electrophoresis (Figure 4). In these experiments, pALTER®-1 *amp*^r Vector was isolated from 10ml overnight cultures of *E. coli* LE392 (*endA* positive) grown in 2X YT medium supplemented with glucose. This combination of bacterial host strain and culture media results in very high expression levels of

endonuclease. In the absence of alkaline protease treatment, plasmid DNA samples were completely degraded whether or not they were incubated for 16 hours in MULTI-CORE™ Buffer. When the lysate was treated with 125µg of alkaline protease, plasmid stability in MULTI-CORE™ Buffer was significantly improved. Although each of the samples appeared to show an increased amount of nicked plasmid during the 16 hour incubation, they were not completely degraded. Upon increasing the alkaline protease concentration in the cell lysis step to 250µg (as recommended in the Technical Bulletin; 7), nicking was still observed but there was no detectable degradation of the plasmid DNA. The use of higher levels of alkaline protease did not significantly affect plasmid stability, and is not recommended because it leads to increased RNA contamination due to proteolytic inactivation of RNase A supplied in the Cell Resuspension Solution (data not shown).

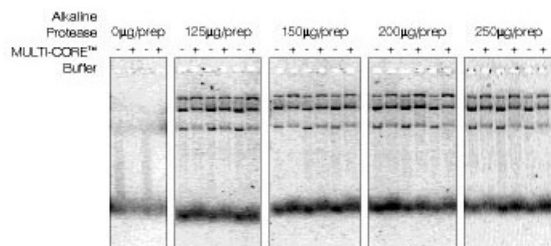


Figure 4. Effects of alkaline protease on plasmid DNA stability. *E. coli* LE392 cells were transformed with the pALTER®-1 *amp^r* Vector and grown in 10ml cultures (2X YT medium containing 1% glucose and 50µg/ml of ampicillin for 16 hours at 37°C, 200rpm). DNA was isolated using the Wizard® Plus SV Minipreps DNA Purification System spin protocol as described (7), except increasing amounts of alkaline protease (0-250µg) were added to each cell lysate and the lysate was incubated at room temperature for 5 minutes. Multiple plasmid samples were isolated for each of the conditions tested. The isolated plasmid DNAs (0.5µg) were subjected to agarose gel electrophoresis after a 16 hour incubation at 37°C in the presence or absence of 1X MULTI-CORE™ Buffer. The iodopropyl thiazole orange stained gel was imaged on a Molecular Dynamics FluorImager™.

The carryover of alkaline protease into the purified plasmid preparation was thoroughly evaluated using a sensitive fluorescent synthetic protease substrate. Table 1 shows the carryover of alkaline protease in pGEM®-3Zf(+) and pALTER®-1 *amp^r* Vector preparations purified from 1.5ml and 10ml cultures, respectively, using the Wizard® Plus SV Minipreps DNA Purification System.

Plasmid Vector	Protocol	Culture Volume	Average Alkaline Protease/Prep	Range of Alkaline Protease/Prep
pGEM®-3Zf(+)	Spin	1.5ml	1.65ng (±1.92)	0-3.5ng
pGEM®-3Zf(+)	Vacuum	1.5ml	0ng	0ng
pALTER®-1 <i>amp^r</i>	Spin	10ml	6.1ng (±0.93)	5-7.2ng
pALTER®-1 <i>amp^r</i>	Vacuum	10ml	5.35ng (±0.7)	5-6.4ng

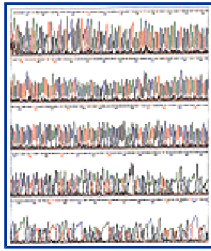
These assays were performed under optimum conditions for protease activity. When assays were performed at neutral pH without added CaCl₂, no activity could be detected.

Use of Wizard® Plus SV Minipreps purified plasmid DNA in downstream applications

Fluorescent sequencing

The quality of automated fluorescent DNA sequencing data is dependent upon a number of factors, including the bacterial host strain in which the plasmid is propagated, and the purity and the quantity of the sequencing template (14). For example, plasmid DNA isolated from bacterial strains producing endonuclease I is frequently degraded and yields poor quality sequence data (8,15). Contaminating salts and chelating agents can result in reduced signal strength and shortened read length (16), as does insufficient template DNA. Excessive amounts of template DNA result in band broadening and truncated data (14).

An important goal during the development of the Wizard® Plus SV Minipreps DNA Purification System was optimization of the system to facilitate automated fluorescent DNA sequencing applications. High copy number plasmid DNA (e.g., pGEM®-3Zf(+)) Vector) isolated from 1.5ml bacterial cultures routinely yields high quality fluorescent sequencing data (Figure 5). Successful sequencing is dependent upon accurate quantitation of the purified plasmid DNA. Because optical density frequently yields erroneously high DNA concentration estimates, we routinely estimate the plasmid DNA concentration by agarose gel electrophoresis. Prior to sequencing, the fluorescence intensity of the purified plasmid DNA is compared to the intensity observed with a dilution series of plasmid DNA of known concentration. If the plasmid DNA solution is too dilute to be used directly for sequencing, it can be concentrated by vacuum drying in a SpeedVac® apparatus or by ethanol precipitation, and then resuspended in the appropriate volume of sterile, deionized water.

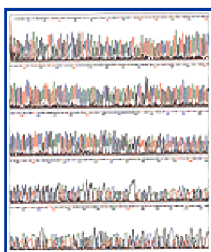


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Figure 5. ABI377 trace from Wizard[®] Plus SV Minipreps DNA Purification System purified, high copy number plasmid DNA sequenced with fluorescent dye primer chemistry. The pGEM[®]-3Zf(+) Vector was isolated from a 1.5ml culture of transformed *E. coli* DH5alpha[®], grown in LB medium containing 50µg/ml of ampicillin (16 hours at 37°C, 200rpm), using the Wizard[®] Plus SV Minipreps DNA Purification System as described (7). Aliquots of the purified plasmid DNA were separated on an agarose gel and the fluorescence intensity of the purified DNA compared to that observed from known amounts of plasmid DNA. Aliquots containing 1µg of plasmid DNA were then dried in a SpeedVac[®] and resuspended in 6µl of water. Fluorescent -21M13 dye primer sequencing reactions were performed using a Perkin Elmer/Applied Biosystems ABI PRISM[™] Dye Primer Cycle Sequencing Ready Reaction Kit with AmpliTaq[®] DNA Polymerase, FS as recommended by the manufacturer (14). The trace is from an ABI377 fluorescent sequencing instrument. Data were collected for 3.0 hours.

Low copy number plasmids (e.g., pBR322-based plasmid DNA) present special problems for fluorescent DNA sequencing. The low amount of plasmid DNA recovered necessitates processing of larger culture volumes, typically 10ml. Consequently, the silica purification matrix is burdened with higher levels of chromosomal DNA, protein, RNA and other culture components that can compete for available binding sites on the matrix. Although wash buffers eliminate most of the bound contaminants, some of the material remains bound to the matrix. Subsequent elution of the plasmid DNA from the matrix also releases some of these contaminants. The resulting plasmid DNA solution thus contains a higher proportion of chromosomal DNA, protein and RNA than observed with a high copy number plasmid isolated from a low culture volume (1.5ml). It should be noted that this phenomenon is observed with all silica-based purification systems.

A few added steps will significantly improve the performance of low copy number plasmid DNA in fluorescent DNA sequencing. First, it is absolutely critical to use an agarose gel-based method as described above for accurate DNA quantitation. Optical density measurements of silica-purified, low copy number plasmid DNA are especially prone to overestimation. Second, the plasmid DNA should be concentrated by ethanol precipitation to yield the requisite 100-200ng/µl recommended for fluorescent DNA sequencing. Vacuum drying low copy number plasmid preparations, as described above for high copy number plasmid preps, results in inconsistent DNA sequence quality. Low copy number plasmid DNA (e.g., pALTER[®]-1 *amp^r*) isolated from 10ml bacterial cultures routinely yields high quality fluorescent sequencing data (Figure 6), if these two precautions (accurate quantitation and ethanol precipitation) are observed.



(Click on image for detailed view; 221K GIF file)

Figure 6. ABI377 trace from Wizard[®] Plus SV Minipreps DNA Purification System purified low copy number plasmid DNA sequenced with fluorescent dye primer chemistry. The pALTER[®]-1 *amp^r* Vector was isolated from a 10ml culture of transformed *E. coli* DH5alpha[®], grown in LB medium containing 50µg/ml of ampicillin (16 hours at 37°C, 200rpm), using the Wizard[®] Plus SV Minipreps DNA Purification System as described (7). The DNA was quantitated as described in Figure 5, and aliquots containing 1µg of plasmid DNA were ethanol precipitated (7) and resuspended in 6µl of water. Fluorescent 21M13 dye primer sequencing reactions were performed using a Perkin Elmer/Applied Biosystems ABI PRISM[™] Dye Primer Cycle Sequencing Ready Reaction Kit with AmpliTaq[®] DNA Polymerase, FS as recommended by the manufacturer (14). The trace is from an ABI377 fluorescent sequencing instrument. Data were collected for 3.0 hours.

The overall consistency from prep to prep for both high and low copy plasmids has also been evaluated. In these experiments, a total of 18 plasmid DNA samples were isolated in sets of six on three consecutive days. These data are displayed in Figure 7. Of the 36 plasmids isolated, only three samples had a read accuracy of less than 98% over a range of 500 bases.

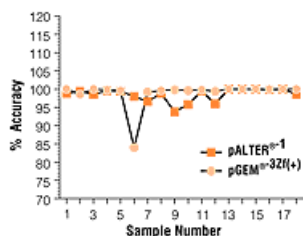


Figure 7. Automated fluorescent DNA sequencing. pGEM[®]-3Zf(+) and pALTER[®]-1 *amp*^r Vector DNAs were purified, quantitated and sequenced as described in Figures 5 and 6, respectively. The graph indicates the accuracy of the sequence data observed with 18 preparations of each plasmid DNA (six samples of each plasmid per day for three days). Ambiguities (N's) and miscalls throughout the 500 base read are counted as errors.

Each lot of the Wizard[®] Plus SV Minipreps DNA Purification System is tested for its ability to isolate plasmid DNA that can be used in fluorescent DNA sequencing applications. The minimum specification is $\geq 98\%$ accuracy over a 500 base range, although $>99\%$ accuracy is typically observed.

Restriction enzyme digestion

Restriction enzyme digestion is a common application for the manipulation of plasmid DNA. The digestion efficiency of both high and low copy number plasmids isolated using the Wizard[®] Plus SV Minipreps DNA Purification System were examined in detail. Low copy number plasmids generally require more restriction endonuclease for complete digestion. The pALTER[®]-1 *amp*^r Vector, for example, typically requires 5-10 units of enzyme per microgram of plasmid DNA (Figure 8) compared to 1-3 units of enzyme with high copy number plasmids (data not shown). Based on this observation, we recommend that restriction enzyme digests of low copy number plasmids include at least 5 units of enzyme per microgram of DNA.

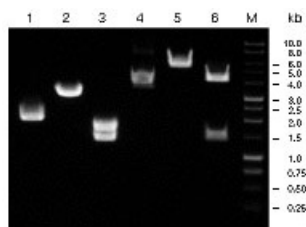


Figure 8. Restriction enzyme digestion of high and low copy number plasmids. The plasmid vectors pGEM[®]-3Zf(+) and pALTER[®]-1 *amp*^r (0.9 μ g), isolated from *E. coli* DH5alpha[®] using the Wizard[®] Plus SV Minipreps DNA Purification System (7), were digested with 10 units of *Sph* I or 10 units of *Sph* I and *Sca* I for 1 hour at 37°C. The digested samples were resolved on a 1% agarose gel, stained with ethidium bromide. Lanes: Lane 1, uncut pGEM[®]-3Zf(+) Vector; Lane 2, *Sph* I cut pGEM[®]-3Zf(+) Vector; Lane 3, *Sph* I and *Sca* I cut pGEM[®]-3Zf(+) Vector; Lane 4, uncut pALTER[®]-1 *amp*^r Vector; Lane 5, *Sph* I cut pALTER[®]-1 *amp*^r Vector; Lane 6, *Sph* I and *Sca* I cut pALTER[®]-1 *amp*^r Vector; Lane M, 1kb Ladder Marker DNA (Cat.# G5711).

In vitro transcription and translation

We also tested plasmid DNA isolated using the Wizard[®] Plus SV Minipreps DNA Purification System for its efficiency as a template for *in vitro* coupled transcription/translation in rabbit reticulocyte lysate, another application where it is essential to use pure plasmid DNA. The T7 Luciferase Control Plasmid, provided with the TNT[®] T7 Quick Coupled Transcription/Translation System, was transformed into *E. coli* DH5alpha[®]. Plasmid DNA was isolated from 5ml cultures using the Wizard[®] Plus SV Minipreps DNA Purification System. The efficiency of the purified plasmid DNA as a template for transcription/translation *in vitro* was analyzed by three methods. In the first assay, total protein synthesis was quantitated by measuring the amount of [³⁵S]methionine incorporated into trichloroacetic acid (TCA) precipitable material. Secondly, the efficiency of [³⁵S]methionine incorporation into firefly luciferase was determined by resolving the reaction products on a denaturing polyacrylamide gel and quantitating the luciferase band using a PhosphorImager[™]. Finally, the amount of active luciferase synthesized in the reaction was quantitated using the Promega Luciferase Assay System. In each case, Luciferase T7 Control Plasmid DNA taken from Promega inventory was used as a control. The results of these experiments, presented as a percentage of the value obtained using the control plasmid preparation, are summarized in Figure 9.

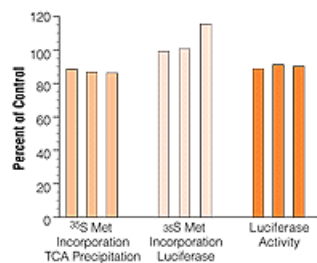


Figure 9. Coupled *in vitro* transcription/translation reactions in the TNT[®] T7 Quick Coupled Transcription/ Translation System. Luciferase T7 Control DNA, either isolated from 5ml cultures of transformed *E. coli* DH5alpha[®] using the Wizard[®] Plus SV Minipreps DNA Purification System (7) or taken from Promega inventory (Cat.# L4824), was used as a template for *in vitro* transcription/translation in Promega's TNT[®] T7 Quick Coupled Transcription/ Translation System (Cat. #L1170) as described (15). The concentration of DNA used was 1µg/50µl reaction and all reactions were performed in triplicate. Total protein synthesis was analyzed by precipitating the [³⁵S]methionine-labeled proteins with TCA, collecting the precipitated proteins on Whatman[®] CF/A filters and quantitating the [³⁵S]methionine by liquid scintillation counting. Synthesis of the firefly luciferase protein was assayed by resolving the reaction products by SDS-PAGE (4-20% polyacrylamide) and quantitating the luciferase band using a PhosphorImager[™]. Active luciferase enzyme was quantitated using the Luciferase Assay System (Cat.# E1500) as described (17). The data obtained using the Wizard[®] Plus SV Minipreps isolated plasmid DNA as the template is presented as a percentage of the values obtained using the control (i.e., inventory) Luciferase T7 Control DNA.

The results indicate Wizard[®] Plus SV Minipreps DNA Purification System-purified plasmid DNA is transcribed and translated at 80-95% efficiency, compared with the Luciferase T7 Control Plasmid DNA. Based on the results of this analysis, we recommend the use of the Wizard[®] Plus SV DNA Purification System as a fast and simple method to prepare DNA for *in vitro* transcription/translation reactions.

Summary

The combination of spin and vacuum formats offered by the Wizard[®] Plus SV Minipreps DNA Purification System provides a significant improvement in sample handling over previous silica gel-based Wizard[®] DNA Purification Systems. In addition, the system incorporates a novel alkaline protease digestion step that significantly decreases the level of protein carryover and allows for plasmid propagation in bacterial strains expressing endonuclease I (*endA* positive). Plasmid DNA isolated with the Wizard[®] Plus SV Minipreps DNA Purification System has been extensively tested for purity and performance in most routine molecular biology applications. The quality of the DNA will provide excellent results in automated fluorescent sequencing systems and is recommended for use with coupled *in vitro* transcription/translation systems.

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Ordering Information

Product	Size	Cat.#
Wizard [®] Plus SV Minipreps DNA Purification System	50 preps	A1330
	250 preps	A1460
Wizard [®] Plus SV Minipreps DNA Purification System	50 preps	A1340
	250 preps	A1470

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