User Guide

Catalog Nos.
PLED35

GenElute™ Endotoxin-free Plasmid Midiprep Kit

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Ordering Information

Cat. No.	Product Description	Pkg Size
PLED35	GenElute™ Endotoxin-free Plasmid Midiprep Kit	35 preps

Related Products

Related Products	Cat. No.	Related Products	Cat. No.
Water, Molecular Biology Reagent	**W4502**	Ethidium bromide, aqueous, 10 mg/mL	**E1510**
LB Broth, EZMix™	**L7658**	β-Galactosidase Reporter Gene Activity Dectection Kit	**GALA**
LB Agar, EZMix™	**L7533**	β-Galactosidase Fluorescence Activity Detection Kit	**GALF**
Terrific Broth, EZMix™	**T9179**	Chloramphenicol Acetyltransferase Reporter Gene Activity Detection Kit	**CATA**
Precast Agarose Gels, 1.0%, 8 well	**P5472**	Escort III Transfection Reagent	**L3037**
TAE Buffer (10×)	**T9650**	Excort IV Transfection Reagent	**L3287**
TBE Buffer (10×)	**T4415**	Gel Loading Solution	**G2526**
DirectLoad™ Wide Range DNA Marker	**D7058**	GenElute™ Endotoxin-free Plasmid Maxiprep Kit	**PLEX15**

GenElute Endotoxin-free Plasmid Midiprep Kit

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Product Description

Endotoxins (also known as lipopolysaccharides or LPS) are often co-purified with plasmid DNA and significantly reduce transfection efficiencies in endotoxin-sensitive cell lines. The GenElute Endotoxin-free Plasmid Midiprep Kit offers a simple, rapid, cost-effective method for isolating up to 250 µg of endotoxin-free plasmid DNA (≤ 0.1 EU/µg DNA) for use in cell transfection.

Overnight recombinant *E. coli* culture is harvested by centrifugation and subjected to a modified alkaline-SDS lysis procedure. Endotoxins are removed from the cleared lysate by simple extraction and phase separation. Plasmid DNA is further purified by absorption onto silica in the presence of high salt. After a spin-wash step, the bound plasmid DNA is eluted in endotoxin-free water.

The recovered plasmid DNA is predominately in its supercoiled form. There is no visual evidence of genomic DNA or RNA contamination by agarose gel electrophoresis. The DNA is ready for immediate use in downstream applications such as transfection, restriction endonuclease digestion, cloning, sequencing, and PCR amplification.

Reagents Provided	Catalog No.	PLED35 35 Preps
Resuspension Solution	**R1149**	100 mL
RNase A Solution	**R6148**	0.6 mL
Lysis Solution	**L1912**	100 mL
Neutralization Solution	**N2409**	60 mL
Endotoxin Removal Solution	**E4274**	45 mL
DNA Binding Solution	**B1555**	60 mL
Column Preparation Solution	**C2112**	225 mL
Optional Wash Solution	**W4011**	80 ml
Wash Solution Concentrate	**W3886**	25 mL
Endotoxin Free Water	**2107**	100 mL
GenElute Midiprep Binding Columns in tubes	**G6540**	35 each
Collection Tubes, 15 mL	**C4228**	35 each

Reagents and Equipment Required But Not Provided

- Ethanol (95–100%), Catalog Nos. **E7148**, **E7023**, or **459836**
- Centrifuge and tubes capable of 15,000 × *g*
- Centrifuge and swinging bucket rotor capable of 3000–5000 × *g* with adapters for Corning® or equivalent 15 mL conical tubes
- Centrifuge tubes, Oak Ridge, Catalog No. **T2918**
- Centrifuge tubes, Corning or equivalent 15 mL conical, Catalog Nos. **C3048**, **C8046**, or **C8171**
- 37 °C water bath

Precautions and Disclaimer

The GenElute Endotoxin-free Plasmid Midiprep Kit is for laboratory use only, not for drug, household, or other uses. The Column Preparation Solution is an irritant; the DNA Binding Solution and the Optional Wash Solution contain guanidine, which is harmful. Wear gloves, safety glasses, and suitable protective clothing when handling these solutions or any reagent provided in the kit. See the Material Safety Data Sheet (MSDS).

Storage and Stability

Store the kit at room temperature. If any kit reagent forms a precipitate, warm at 65 °C until dissolved and allow to cool to room temperature before use.

Preparation Instructions

Before beginning the procedure, prepare the following:

1.	**Thoroughly mix reagents**	Examine reagents for precipitation. If any kit reagent forms a precipitate upon storage, warm at 55–65 °C until the precipitate dissolves. Allow the reagent to cool to room temperature before use.
2.	**Resuspension Solution**	Spin the tube of RNase A Solution briefly. Add 500 µL of the RNase A Solution to the Resuspension Solution. Mix thoroughly prior to initial use.
3.	**Wash Solution**	Add 100 mL of 95–100% ethanol to the Wash Solution Concentrate prior to initial use. Tightly cap the bottle after each use to prevent the evaporation of ethanol.
4.	**Endotoxin Removal Solution**	Mix briefly and incubate the bottle on ice for >10 minutes before use.
5.	**Heat water bath**	Heat water bath to 37 °C.

Procedure

Note: All centrifugation speeds are given in units of *g*. Please refer to Table 1 for information on converting *g*-force to rpm. If centrifuges/rotors for the required *g*-forces are not available, use the maximum *g*-force possible and increase the spin time proportionally. Spin until all liquid passes through the column. A swinging bucket rotor is necessary for steps 5–11.

Table 1. Conversion of Centrifugal Force (in units of *g*) to RPM for Common Rotors

Centrifuge	Rotor	Type*	Radius (cm)	RPM at 3,000 x *g*	RPM at 5,000 x *g*	RPM at 15,000 x *g*
Beckman						
Allegra 6	GH-3.8	SB	20.4	3,631	4,688	N/A
Allegra 21(R)	S4180	SB	16.1	4,081	5,268	N/A
Allegra 64	F0485	FA	9.0	N/A**	N/A	12,211
	F0685	FA	9.7	N/A	N/A	11,764
TJ-25	TS-5.1-500	SB	19.0	3,756	4,849	N/A
	TA-10-250	FA	13.7	N/A	N/A	9,901
Rotors for older Beckman centrifuges	JA-10	FA	15.8	N/A	N/A	9,215
	JA-14	FA	13.7	N/A	N/A	9,896
	JA-20	FA	10.8	N/A	N/A	11,146
	JS-13	FA	14.0	N/A	N/A	9,790
IEC	215	SB	13.0	4,537	5,857	N/A
MP4(R)	224	SB	35.9	2,733	3,528	N/A
PR-7000M	966	SB	24.5	3,310	4,274	N/A
B22M	877	FA	12.6	N/A	N/A	10,318
Sorvall	HB-4	SB	14.7	4,277	5,522	N/A
	HB-6	SB	14.6	4,284	5,531	N/A
	HS-4	SB	17.2	3,948	5,097	N/A
	SH-80	SB	10.1	5,142	6,639	N/A
	GSA	FA	14.5	N/A	N/A	9,604
	SA-300	FA	9.7	N/A	N/A	11,784
	SA-600	FA	12.9	N/A	N/A	10,179
	SE-12	FA	9.3	N/A	N/A	11,997
	SL-50T	FA	10.7	N/A	N/A	11,203
	SS-34	FA	10.7	N/A	N/A	11,203

*SB = swinging bucket; FA = fixed angle
**N/A = not appropriate for application

The correct rpm for unlisted rotors can be calculated using the formula:

$$RPM = \sqrt{RCF / 1.118 \times 10^{-5} r}$$

where RCF = required gravitational acceleration (relative centrifugal force) in units of *g*;
r = radius of the rotor in cm;
RPM = the number of revolutions per minute required to achieve the necessary *g*-force

All steps are carried out at room temperature unless otherwise noted.

1. Harvest Cells

Pellet 5–40 mL of overnight recombinant *E. coli* culture by centrifugation. Optimal volume of culture to use depends upon the culture density. For best yields, follow the instructions in the note below. Transfer the appropriate volume of the recombinant *E. coli* culture to an Oak Ridge-style tube (capable of ≥15,000 × *g*), and centrifuge at 3000–5000 × *g* for 10 minutes. Remove and discard all of the medium supernatant.

Note: For best results, start with a single colony from a freshly streaked plate. Grow in Luria broth (LB) containing the appropriate antibiotic at 37 °C with vigorous shaking (250–300 RPM) overnight. Measure the absorbance of the overnight culture at 600 nm. Use a total cell mass of approximately 80, where cell mass equals OD_{600} × mL of culture. To calculate the volume of culture to use, take the desired cell mass (80) and divide by the absorbance of the overnight culture at 600 nm. For example, with a very dense culture of recombinant *E. coli* grown to an OD_{600} of about 4.0, use only 20 mL of the culture. With a less dense culture, where OD_{600} is about 2.0, use 40 mL. For low copy plasmids, use twice as much culture (total cell mass of 160). Do not exceed a total cell mass of 100 (with high copy plasmids) or 200 (with low copy plasmids). Higher cell mass can cause a reduction in yield. For cultures grown in rich media, less volume may be necessary. Please contact Sigma Technical Service if you require further assistance.

2. Resuspend Cells

Prior to first time use, be sure to add the appropriate volume of the RNase A Solution to the Resuspension Solution. Completely resuspend the bacterial pellet with 1.2 mL of Resuspension Solution by pipetting up and down. Make sure that the cells are completely resuspended until homogenous. Incomplete resuspension will result in poor recovery.

3. Lyse Cells

Lyse the resuspended cells by adding 1.2 mL of Lysis Solution. Immediately mix the contents by gentle inversion (6-8 times) until the mixture becomes clear and viscous. **Do not vortex.** Harsh mixing will shear genomic DNA, resulting in chromosomal DNA contamination in the final recovered plasmid DNA. **Do not allow the lysis reaction to exceed 5 minutes.** Prolonged alkaline lysis may permanently denature supercoiled plasmid DNA, which may render it unsuitable formost downstream applications.

4. Neutralize

Precipitate the debris by adding 0.8 mL of Neutralization Solution to the lysate. Immediately mix the contents thoroughly by gentle inversion.
Do not vortex. Pellet the cell debris by centrifuging at ≥15,000 × *g* for 15 minutes at 2-8 °C. Transfer the cleared lysate into a Corningor equivalent 15 mL conical tube. If the supernatant contains a large amount of floating cell debris after centrifugation, re-centrifuge the supernatant before proceeding to step 5.

5. Remove Endotoxin

a. Add 300 µL of the Endotoxin Removal Solution to the lysate. Mix thoroughly by inversion for 1 minute. Chill the tube on ice for ≥ 5 minutes. Mix 1–2 times during the ice incubation. The solution should be light blue and clear.

b. Warm the tube in a 37 °C water bath for 5 minutes. The solution will turn cloudy. To separate the phases, centrifuge the tube at 3000–5000 × *g* in a swinging bucket rotor for 5 minutes at room temperature. The clear upper phase contains plasmid DNA. The blue lower phase contains endotoxins.

c. Carefully transfer the clear upper phase into a fresh 15 mL conical tube. Discard the blue lower phase.

d. Repeat steps a, b & c, then continue to step 6.

Note: Take care not to introduce endotoxins in subsequent steps. Use only new, unhandled plasticware; it is considered to be endotoxin free.

6. Add DNA Binding Solution

Add 0.8 mL of the DNA Binding Solution to the endotoxin-free lysate. Mix the contents thoroughly by inversion or by vortexing.

7. DNA Binding Column Preparation

Insert a GenElute Midiprep Binding Column to a collection tube. Add 3 mL of Column Preparation Solution to each column and centrifuge in a swinging bucket rotor at 3000–5000 × *g* for 1–2 minutes. Discard the flow-through liquid.

Note: The Column Preparation Solution maximizes binding of DNA to the filter resulting in more consistent yields.

8. Bind DNA

Load the lysate from step 6 into a prepared GenElute Midiprep Binding Column seated in a collection tube. Centrifuge in a swinging bucket rotor at 3000–5000 × *g* for 1–2 minutes. Discard the flow-through liquid.

9. Optional Wash

Add 2.0 mL of the Optional Wash Solution to the column. Centrifuge in a swinging bucket rotor at 3000–5000 × *g* for 2 minutes. Discard the flow-through liquid.

Note: When working with bacterial strains containing the wild-type EndA$^+$ gene, such as HB101, JM101, and the NM and PR series, the Optional Wash step is necessary to avoid nuclease contamination in the final plasmid preparation. The Optional Wash also removes any residual endotoxins that may have been carried over.

10. Wash Column

Prior to first time use, be sure to add ethanol to the Wash Solution Concentrate. Add 3 mL of diluted Wash Solution to the column. Centrifuge in a swinging bucket rotor at 3000–5000 × *g* for 5 minutes. Make sure that the Wash Solution is completely removed from the column before proceeding to step 11.

11. Elute DNA

Transfer the column to a fresh collection tube. Add 1 mL of Endotoxin Free Water to the column. Centrifuge in a swinging bucket rotor at 3000–5000 × *g* for 3–5 minutes. The DNA is now present in the flow-through eluate, and is ready for immediate use or storage at –20 °C.

Note: Less than 0.8 mL of the eluate may be recovered after centrifugation, particularly if the maximum amount of starting culture was used. However, the plasmid DNA yield will not be compromised. For a more concentrated DNA preparation, the elution volume may be reduced to a minimum of 500 µL. For optimal recovery in 500 µl, preheat the elution solution to 65 °C and add directly to the binding filter. Allow the preheated elution solution to soak into the binding filter for 10 minutes before centrifugation. Incubating with the preheated elution solution will improve recovery, but the total plasmid DNA yield is likely to be less than with elution using the full 1 mL.

Results

Recovery and purity of the plasmid DNA may be determined by spectrophotometric analysis. The ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}) should be 1.7 to 1.9. The size and quality of DNA may be determined by agarose gel electrophoresis or pulsed field electrophoresis.

Troubleshooting Guide

Poor or low plasmid DNA recovery.	**Cause** — Binding columns were spun in a fixed angle rotor or with insufficient *g*-force. **Solution** — Binding columns must be spun in a swinging bucket rotor at 3000–5000 × *g* in steps 7–11 for liquids to pass through efficiently. Actual spin speed in RPM will depend upon rotor size (see note at beginning of the Procedure Section).
	Cause — Wash Solution is too concentrated. **Solution** — Confirm that the Wash Solution concentrate was diluted with the specified volume of ethanol. Keep the bottle tightly capped between uses to prevent evaporation.
	Cause — Culture is too old. **Solution** — Streak a fresh plate from a freezer stock, pick a single colony, and prepare a new culture.
	Cause — Too many or too few cells were used. **Solution** — Confirm cell density by measuring OD_{600}. To calculate the volume of culture to use, take the desired cell mass (80 for high copy or 160 for low copy plasmids) and divide by the absorbance of the overnight culture at 600 nm.
	Cause — Plasmid replication is poor. **Solution** — Confirm that the cells were grown in the appropriate medium under optimized conditions.
	Cause — Antibiotic activity is insufficient. **Solution** — Use fresh antibiotic solutions for growth of overnight cultures. Most antibiotic solutions are light sensitive and degrade during long term storage at 2–8 °C.
	Cause — Alkaline lysis is prolonged. **Solution** — Reduce the lysis time (step 3) to 3 minutes or until the suspended cells form a clear, viscous solution.
	Cause — Precipitation of cell debris is incomplete. **Solution** — Reduce the initial volume of cell culture.
	Cause — Lysis is incomplete. **Solution** — Reduce the initial volume of culture or increase the lysis time (step 3) while monitoring the lysis visually. Do not exceed a cell mass of 100 (cell mass = OD_{600} × mL of culture). For best results, use a total cell mass of 80 for high copy or 160 for low copy plasmids.

Absorbance of purified DNA does not accurately reflect the quantity of plasmid (A_{260}/A_{280} ratio is high or low).	**Cause** — Wash Solution is diluted with ethanol containing impurities. **Solution** — Check the absorbance of ethanol between 250 and 300 nm. Do not use ethanol with high absorbance. Traces of impurities may remain on the binding column after washing. Impurities may show up in the eluate and may contribute to the absorbance in the final product.
	Cause — Plasmid DNA is contaminated with RNA; RNase A treatment is insufficient. **Solution** — Confirm that the RNase A Solution was added to the Resuspension Solution prior to first use. The RNase A Solution may degrade due to high temperatures (>65 °C) or prolonged storage (>6 months at room temperature).
	Cause — Plasmid DNA is contaminated with chromosomal DNA. **Solution** — Do not use cultures that have grown for more than 24 hours or are in the cell death phase. Do not vortex or vigorously shake the cells during the lysis reaction (step 3) or neutralization procedure (step 4).
	Cause — Background reading is high due to silica fines. **Solution** — Spin the DNA sample at maximum speed for 1 minute; use the supernatant to repeat absorbance readings.
	Cause — Purification is incomplete due to column overloading. **Solution** — Reduce the initial volume of culture. Do not exceed 100 OD_{600} units of total cell mass (cell mass = OD_{600} × mL of culture). For best results, use a cell mass of 80.
Residual level of endotoxin is >0.1 EU/µg DNA.	**Cause** — Culture is overgrown or too much culture used. **Solution** — Grow culture 12–16 hours with vigorous shaking. Do not exceed the recommended maximum culture volume or cell mass.
	Cause — Endotoxin-enriched lower (blue) phase is carried over. **Solution** — Avoid pipetting any part of the blue lower phase when transferring the clear upper phase in step 5. Perform the Optional Wash step to remove endotoxins that may have been carried over.
Endotoxin Removal Solution is in two phases.	**Cause** — Storage temperature is higher than 25 °C. **Solution** — Mix the solution briefly and incubate on ice for >10 minutes before use. Solution will be clear, blue, and homogeneous (in one phase).

Additional band is migrating ahead of the supercoiled plasmid during electrophoresis.	**Cause** — A portion of the plasmid DNA is permanently denatured. **Solution** — Do not allow the lysis reaction (step 3) to exceed 5 minutes. Note that the nicked (covalently open) double-stranded plasmid DNA runs slower than the supercoiled DNA during electrophoresis.
Poor performance in downstream enzymatic applications.	**Cause** — Purification is incomplete. **Solution** — Salts in one or more of the solutions may have precipitated. Heat the solution at 65 °C until dissolved. Cool to room temperature prior to use.
	Cause — DNA concentration is too low. **Solution** — Precipitate the DNA with ethanol, then resuspend the DNA in a smaller volume of endotoxin-free water. **Or** Elute silica-bound DNA with less endotoxin-free water. Note that using less water may reduce the overall recovery.
	Cause — DNA was prepared from EndA$^+$ strains. **Solution** — The Optional Wash Step (step 9) must be included when recovering DNA from EndA$^+$ strains.
	Cause — The final DNA eluate contains too much salt. **Solution** — Precipitate the DNA using ethanol. Dry the pellet. Redissolve in endotoxin-free water.
	Cause — The column contains residual ethanol from diluted Wash Solution. **Solution** — Re-centrifuge the column for 1 minute after the washing (step 10) to remove any residual Wash Solution.

References

1. Birnboim, H.C.; Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA *Nucleic Acids Res.* **1979**, *7*, 1513–1522.
2. Vogelstein, B.; Gillespie, D. Preparative and analytical purification of DNA from agarose *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 615–619.

Experienced User Protocol

1 Prepare Cleared Lysate

❒ Pellet and resuspend cells, alkaline lyse, and neutralize.

2 Remove Endotoxin

❒ Phase separation and spin twice.

3 Bind DNA

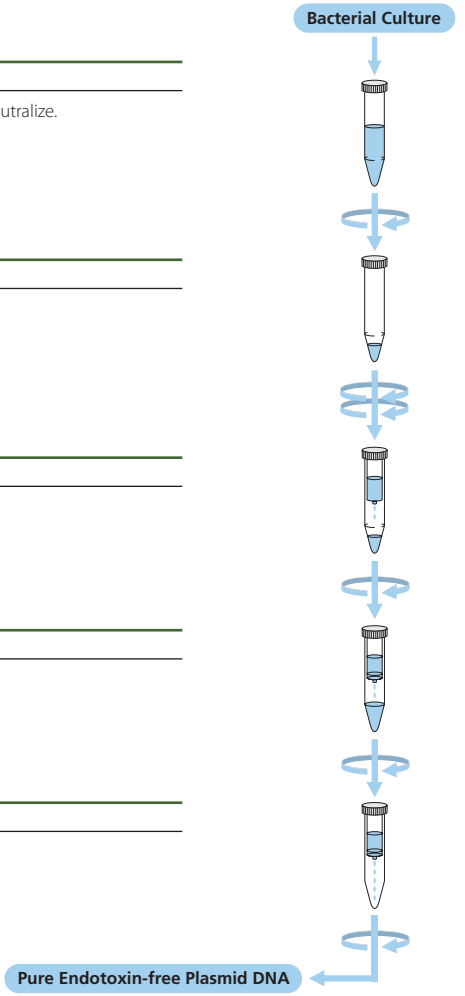
❒ Bind and spin.

4 Wash Column

❒ Wash and spin.

5 Elute DNA

❒ Elute and spin.



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