

Product Information

GenElute™ Endotoxin-free Plasmid Maxiprep Kit

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TECHNICAL BULLETIN

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 Maxiprep Kit offers a simple, rapid, cost-effective method for isolating up to 1.2 mg of endotoxin-free plasmid DNA (≤ 0.1 EU/ μ g DNA) for use in cell transfection. Note that the actual yield and optimal volume of culture to use depend on the plasmid and the culture medium (see Procedure, step 1).

An 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 with simple extraction and phase separation steps. The 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.

Precautions and Disclaimer

The GenElute Endotoxin-free Plasmid Maxiprep 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 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).

Reagents Provided (Sufficient for 15 preparations)	Amount
Resuspension Solution, R 1149	100 ml
RNase A Solution (20 mg/ml), R 6148	0.6 ml
Lysis Solution, L 1912	100 ml
Neutralization Solution, N 2409	60 ml
Endotoxin Removal Solution, E 4274	45 ml
DNA Binding Solution, B 1555	60 ml
Column Preparation Solution, C 2112	225 ml
Optional Wash Solution, W 4011	135 ml
Wash Solution Concentrate, W 3886	50 ml
Endotoxin Free Water, 210-7	100 ml
GenElute Maxiprep Binding Columns in tubes, G 6665	15 each
Collection Tubes, 50 ml, C 4353	15 each

Equipment and Reagents Required But Not Provided

- Ethanol (95-100%), Product Codes E 7148, E 7023, and 45,983-6
- Centrifuge capable of 15,000 x g
- Centrifuge tubes, Oak Ridge, Product Code T 2918
- Centrifuge and swinging bucket rotor capable of 3,000-5,000 x g with adapters for Corning[®] or equivalent 15 ml conical tubes
- Centrifuge bottles, Product Code Z35,373-6
- Centrifuge tubes: 15 ml conical, Product Code C 3048; and 50 ml conical, Product Code C 4353
- 37 °C water bath

Preparation Instructions

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 200 ml of 95-100% ethanol to 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 to 37 °C.**

Storage

Store the kit at room temperature. If any kit reagent forms a precipitate upon storage, see Preparation Instructions.

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						
MP4(R)	215	SB	13.0	4,537	5,857	N/A
	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

See previous table for spin speeds in rpm for selected common centrifuges and rotors. 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

1. **Harvest cells.** Pellet 25-200 ml of an overnight recombinant *E. coli* culture by centrifugation. The 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 a centrifuge bottle and pellet the cells at 3,000-5,000 x g for 5-10 minutes. Remove and discard all of the medium supernatant.

Note: For best results, start with a single colony from a freshly streaked plate. Grow overnight in Luria broth (LB) containing the appropriate antibiotic at 37 °C with vigorous shaking (250-300 RPM). Measure the absorbance of the overnight culture at 600 nm. Use a total cell mass of approximately 300, where cell mass equals $OD_{600} \times \text{ml of culture}$. To calculate the volume of culture to use, take the desired cell mass (300) 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 75 ml of the culture. With a less dense culture, where OD_{600} is about 2.0, use 150 ml. For low copy plasmids, use a total cell mass of 500. A 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 amount of RNase A Solution to the Resuspension Solution. Completely resuspend the bacterial pellet with 4.8 ml of Resuspension Solution by pipetting up and down. Incomplete resuspension will result in poor recovery. Transfer the resuspended pellet into a centrifuge tube (Oak Ridge style or equivalent) capable of reaching $\geq 15,000 \times g$.
3. **Lyse cells.** Lyse the resuspended cells by adding 4.8 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 the 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 that may render it unsuitable for most downstream applications.
4. **Neutralize.** Precipitate the cell debris by adding 3.2 ml of the Neutralization Solution to the lysate. Immediately mix the contents thoroughly by gentle inversion. **Do not vortex.** Pellet the cell debris by centrifuging at $\geq 15,000 \times g$ for 15 minutes at 2-8 °C. If the supernatant contains a large amount of floating cell debris after centrifugation, re-centrifuge the supernatant before proceeding to step 5. Transfer the cleared lysate into a Corning or equivalent 15 ml conical tube.
5. **Remove endotoxin**
 - a. Add 1.2 ml of **ice cold** Endotoxin Removal Solution to the lysate. Mix thoroughly by inversion for 1 minute. Chill the tube on ice for ≥ 10 minutes. Mix 2-3 times during the ice incubation. The solution should be light blue and clear.
 - b. Warm the tube in a 37 °C water bath for 10 minutes. The solution will turn cloudy. To separate the phases, centrifuge the tube at 3,000-5,000 x 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 and b; then continue to step 6.

Note: To avoid introducing endotoxins in subsequent steps, use only new, unhandled plastic-ware, which is considered to be endotoxin-free.

6. **Add DNA Bind Solution.** Carefully transfer the clear upper phase into a fresh 50 ml tube. Add 3.2 ml of DNA Binding Solution to the endotoxin-free lysate. Mix the contents thoroughly by inversion or vortexing.
7. **DNA Binding Column Preparation.** Insert a GenElute Maxiprep Binding Column to a collection tube. Add 12 ml of Column Preparation Solution to each column and centrifuge in a swinging bucket rotor at 3,000-5,000 x *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 into a prepared GenElute Maxiprep Binding Column seated in a collection tube. Centrifuge in a swinging bucket rotor at 3,000-5,000 x *g* for 1-2 minutes. Discard the flow-through liquid.
9. **Optional Wash.** Add 8.0 ml of Optional Wash Solution to the column. Centrifuge in a swinging bucket rotor at 3,000-5,000 x *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. 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 15 ml of the diluted Wash Solution to the column. Centrifuge in a swinging bucket rotor at 3,000-5,000 x *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 3-5 ml of Endotoxin Free Water to the column. Centrifuge in a swinging bucket rotor at 3000-5000 x *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: If a more concentrated plasmid DNA preparation is required, the elution volume may be reduced to a minimum of 1 ml. For optimal recovery in 1 ml, preheat the Endotoxin Free Water to 65 °C and add directly to the binding column. Allow the preheated Endotoxin Free Water to soak into the binding column for 10 minutes before centrifugation. Incubating with preheated Endotoxin Free Water will improve recovery, but the total plasmid DNA yield will likely be less than with elution in the full 5 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 the DNA may be determined by agarose gel electrophoresis or pulsed field electrophoresis.

Troubleshooting Guide

Problem	Cause	Solution
Poor or low Plasmid DNA recovery	Binding columns were spun in a fixed angle rotor, or with insufficient <i>g</i> -force.	Binding columns must be spun in a swinging bucket rotor at 3,000-5,000 x <i>g</i> in steps 7-11 for liquids to pass through efficiently. Actual spin speed in RPM will depend on rotor size (see note at beginning of the Procedure section).
	Wash Solution is too concentrated.	Confirm that the Wash Solution Concentrate was diluted with the specified volume of ethanol. Keep the bottle tightly capped between uses to prevent evaporation.
	Culture is too old.	Streak a fresh plate from a freezer stock, pick a single colony, and prepare a new culture.
	Too many or too few cells were used.	Confirm the cell density by measuring OD ₆₀₀ . To calculate the volume of culture to use, take the desired cell mass (300 for high copy plasmids or 500 for low copy plasmids) and divide by the absorbance of the overnight culture at 600 nm.
	Plasmid replication is poor.	Confirm that the cells were grown in an appropriate medium under optimized conditions.
	Antibiotic activity is insufficient.	Use a fresh antibiotic solution for growth of overnight cultures. Most antibiotic solutions are light sensitive and degrade during long term storage at 2-8 °C.
	Alkaline lysis is prolonged.	Reduce the lysis time (step 3) to 3 minutes or until the suspended cells form a clear viscous solution.
	Precipitation of cell debris is incomplete.	Reduce the initial volume of cell culture.
	Lysis is incomplete.	Reduce the initial volume of cell culture or increase the lysis time (step 3) while monitoring the lysis visually. For best results, use a total cell mass of 300 for high copy plasmids or 500 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).	Wash Solution is diluted with ethanol containing impurities.	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.
	Plasmid DNA is contaminated with RNA; RNase A treatment is insufficient.	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).
	Plasmid DNA is contaminated with chromosomal DNA.	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).
	Background reading is high due to silica fines.	Spin the DNA sample at maximum speed for 1 minute; use the supernatant to repeat the absorbance readings.
	Purification is incomplete due to column overloading.	Reduce the initial volume of cell culture.
The Endotoxin Removal Solution is in two phases.	Storage temperature is higher than 25 °C.	Mix the solution briefly and incubate on ice for >10 minutes before use. The solution will be clear, blue, and homogeneous (in one phase).
Residual level of endotoxin is >0.1 EU/μg DNA.	Culture is overgrown or too much culture is used.	Grow culture 12-16 hr. with vigorous shaking. Do not exceed the recommended maximum culture volume or cell mass.
	Endotoxin-enriched lower (blue) phase is carried over.	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.

Troubleshooting Guide (continued)

Problem	Cause	Solution
Additional band is migrating ahead of the supercoiled plasmid during electrophoresis.	A portion of the plasmid DNA is permanently denatured.	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	Purification is incomplete.	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.
	DNA concentration is too low.	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.
	DNA was prepared from EndA ⁺ strains.	The Optional Wash (step 9) must be included when recovering DNA from EndA ⁺ strains.
	The final plasmid DNA eluate contains too much salt.	Precipitate the DNA using ethanol. Dry the pellet. Redissolve in endotoxin-free water.
	The column contains residual ethanol from diluted Wash Solution.	Re-centrifuge the column for 1 minute after washing (step 10) to remove any residual Wash Solution.

References

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

Related Products	Product Code	Related Products	Product Code
Water, Molecular Biology Reagent	W 4502	Ethidium bromide, aqueous, 10 mg/ml	E 1510
LB Broth, EZMix™	L 7658	β-Galactosidase Reporter Gene Activity Detection Kit	GAL-A
LB Agar, EZMix™	L 7533	β-Galactosidase Fluorescence Activity Detection Kit	GAL-F
Terrific Broth, EZMix™	T 9179	Chloramphenicol Acetyltransferase Reporter Gene Activity Detection Kit	CAT-A
Precast Agarose Gels, 1.0%, 8 well	P 5472	Luciferase Assay Kit	LUC-1
TAE Buffer (10x)	T 9650	Escort III Transfection Kit	L 3037
TBE Buffer (10x)	T 4415	Excort IV Transfection Kit	L 3287
Gel Loading Solution	G 2526		
DirectLoad™ Wide Range DNA Marker	D 7058		
GenElute™ Endotoxin-free Plasmid Midiprep Kit	PLED-35		

† The PCR process is covered by patents owned by Hoffman-LaRoche, Inc. Corning is a registered trademark of the Corning Corp.

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