

E.Z.N.A.® Endo-Free Plasmid DNA Midi Kit
E.Z.N.A.® Endo-Free Plasmid DNA Maxi Kit

Table of Contents

Introduction.....2
Yield and Quality of DNA.....3
Illustrated Protocols.....4
Kit Contents.....5
Preparing Reagents / Storage and Stability.....6
Guidelines for Vacuum Manifold.....7
Endo-Free Plasmid DNA Midi Vacuum Protocol.....10
Endo-Free Plasmid DNA Midi Centrifugation Protocol.....15
Endo-Free Plasmid DNA Maxi Vacuum Protocol.....20
Endo-Free Plasmid DNA Maxi Centrifugation Protocol.....25
Modified Protocols.....30
Troubleshooting Guide.....33
Ordering.....35

Manual Revision: August 2013



Introduction

The E.Z.N.A.® Endo-Free Plasmid Midi Kit and the Endo-Free Plasmid Maxi Kit combine the power of HiBind® technology with Omega Bio-tek's innovative Endotoxin Removal Technology (ETR) to deliver high-quality plasmid DNA with low endotoxin levels for use in eukaryotic transfection, and *in vitro* experiments.

Endotoxins are lipopolysaccharides (LPS), found in the outer cell membrane of gram negative bacteria such as *E. coli*. One *E. coli* cell contains around 2 million LPS molecules, each having hydrophobic, hydrophilic, and charged regions. Bacteria release small quantities of endotoxins during growth, and large quantities at death. At the time of lysis during plasmid purification, endotoxins are shed into the lysate. The chemical and physical properties that endotoxin molecules possess lead to their co-purification with plasmid DNA by behaving similarly on the surface of silica and anion-exchange resins. The E.Z.N.A.® Endo-Free Plasmid System uses a specially formulated buffer that prevents endotoxin molecules from binding to the surface of the HiBind® matrix. In addition, the E.Z.N.A.® Endo-Free Plasmid Midi and Maxi Kits include specialized filter cartridges that replace the centrifugation step following alkaline lysis.

For the best interpretation of results it is crucial that the purified plasmid DNA be free of endotoxins. Endotoxin contamination lowers transfection efficiencies for endotoxin sensitive cell lines. For gene therapy, endotoxin contamination should be of major concern since endotoxins have the potential to cause fever, endotoxic shock syndrome, and interfere with *in vitro* transfection into immune cells.

New In this Edition:

- Equilibration Buffer is no longer included with this kit. An optional Column Equilibration Protocol has been added to the protocol for your convenience.
- Equilibration Buffer is replaced with 3M NaOH provided by the user.
- EHB Buffer has been replaced by EHBC Buffer. Isopropanol is required and supplied by the user.

Storage and Stability

All of the E.Z.N.A.® Endo-Free Plasmid DNA Midi and Maxi Kit components are guaranteed for at least 24 months from the date of purchase when stored as follows. After RNase A is added, Solution I should be stored at 2-8°C. All other components should be stored at room temperature. Store Solution II tightly capped when not in use. During shipment or storage in cool ambient conditions, precipitates may form in EHBC Buffer, Solution II, and ETR Binding Buffer. Dissolve such deposits by warming the solution at 37°C and gently shaking.

Yield and Quality of DNA

Determine the absorbance of an appropriate dilution (20 to 50 fold) of the sample at 260 nm and then at 280 nm. The DNA concentration is calculated as follows:

$$\text{DNA concentration} = \text{Absorbance 260} \times 50 \times (\text{Dilution Factor}) \mu\text{g/mL}$$

A ratio greater than 1.8 indicates greater than 90% nucleic acid. Alternatively, quantity (as well as quality) can sometimes best be determined by agarose gel/ethidium bromide electrophoresis by comparison to DNA samples of known concentrations. Typically, the majority of the DNA eluted is in monomeric supercoil form, though concatamers may also be present.

Plasmid Copy Number and Expected Yield

The yield and quality of the plasmid DNA obtained depends on a number of factors including plasmid copy number, size of insert, host strain, culture volume, culture medium, and binding capacity of the kits. Of these factors, the vector copy number, culture volume, and kit binding capacity are most important. Plasmid copy number ranges from one copy to several hundred copies per cell as dictated by their origin of replication. But very large plasmids often display a very low copy number per cell. The expected yield of 50 mL overnight cultures (LB medium) with the E.Z.N.A.® Endo-Free Plasmid DNA Midi or Maxi Kit are indicated in the following table.

Sample yields from 50 mL starting culture

Plasmid	Replicon	Copy Number	Expected Yield (50 mL culture)
pUC vectors	pMB1	500-700	150-250 µg
pBluescript® vectors	ColE14	300-500	100-180 µg
pGEM® vectors	pMB1	300-400	100-200 µg
pBR322 and its derivatives	pMB1	15-20	10-20 µg
ColE14	ColE14	15-20	10-20 µg
PACYC and its derivatives	p15A	37-40	5-10 µg
pSC101 and its derivatives	pSC101	~5	5 µg
pGEM	pMB1	300-700	100-200 µg

Centrifugation Protocol

Vacuum Protocol



Pellet by Centrifugation



Resuspend
Lyse
Neutralize



Transfer Lysate to Syringe



Insert Plunger into Syringe
and Clear Lysate
into 15 mL Collection Tube



Add ETR Binding Buffer



Pass Lysate through
HiBind® DNA Column



Wash 4X



Dry HiBind® matrix

Elute Endotoxin-free DNA



Pellet by Centrifugation



Resuspend
Lyse
Neutralize



Transfer Lysate to Syringe



Insert Plunger into Syringe
and Clear Lysate
into 15 mL Collection Tube

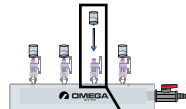


Prepare Vacuum Manifold

Add ETR Binding Buffer



Insert column into leuc connector
on vacuum manifold



Pass Lysate through
HiBind® DNA Column



Wash 4X



Dry HiBind® matrix

Elute Endotoxin-free DNA

Kit Contents

E.Z.N.A.® Endo-Free Plasmid DNA Midi Kit	D6915-00B	D6915-01B	D6915-03B	D6915-04B
Purifications	2	10	25	100
HiBind® DNA Midi Columns	2	10	25	100
15 mL Collection Tubes	2	10	25	100
Lysate Clearance Filter Syringe	2	10	25	100
Solution I	6 mL	30 mL	80 mL	270 mL
Solution II	6 mL	30 mL	80 mL	270 mL
N3 Buffer	3 mL	15 mL	40 mL	140 mL
ETR Binding Buffer	15 mL	60 mL	170 mL	3 x 215 mL
ETR Wash Buffer	6 mL	30 mL	60 mL	230 mL
EHBC Buffer	4 mL	21 mL	48 mL	2 x 100 mL
DNA Wash Buffer	4 mL	15 mL	40 mL	5 x 40 mL
RNase A	20 µL	100 µL	300 µL	1.2 mL
Endo-Free Elution Buffer	4 mL	20 mL	60 mL	200 mL
User Manual	✓	✓	✓	✓

E.Z.N.A.® Endo-Free Plasmid DNA Maxi Kit	D6926-00B	D6926-01B	D6926-03B	D6926-04B
Purifications	2	6	25	100
HiBind® DNA Maxi Columns	2	6	25	100
50 mL Collection Tubes	2	6	25	100
Lysate Clearance Filter Syringe	2	6	25	100
Solution I	30 mL	70 mL	270 mL	4 x 270 mL
Solution II	30 mL	70 mL	270 mL	4 x 270 mL
N3 Buffer	15 mL	35 mL	135 mL	2 x 270 mL
ETR Binding Buffer	55 mL	170 mL	3 x 215 mL	10 x 270 mL
ETR Wash Buffer	25 mL	70 mL	270 mL	4 x 270 mL
EHBC Buffer	14 mL	40 mL	165 mL	4 x 165 mL
DNA Wash Buffer	15 mL	40 mL	3 x 50 mL	12 x 50 mL
RNase A	120 µL	300 µL	1.2 mL	5 mL
Endo-Free Elution Buffer	10 mL	40 mL	90 mL	2 x 160 mL
User Manual	✓	✓	✓	✓

Preparing Reagents

1. Add vial of RNase A to the bottle of Solution I provided and store at 2-8°C.
2. Dilute DNA Wash Buffer with 100% ethanol as follows and store at room temperature.

Kit	100% Ethanol to be Added
D6915-00B	16 mL
D6915-01B	60 mL
D6915-03B	160 mL
D6915-04B	160 mL per bottle

Kit	100% Ethanol to be Added
D6926-00B	60 mL
D6926-01B	160 mL
D6926-03B	200 mL per bottle
D6926-04B	200 mL per bottle

3. Dilute EHBC Buffer with isopropanol as follows and store at room temperature.

Kit	Isopropanol to be Added
D6915-00B	2.6 mL
D6915-01B	14 mL
D6915-03B	32 mL
D6915-04B	65 mL per bottle

Kit	Isopropanol to be Added
D6926-00B	9.5 mL
D6926-01B	26 mL
D6926-03B	110 mL
D6926-04B	110 mL per bottle

4. Check Solution II, EHBC Buffer, and ETR Binding Buffer for precipitates before use. Redissolve any precipitates by warming to 37°C.

Guidelines for Vacuum Manifold

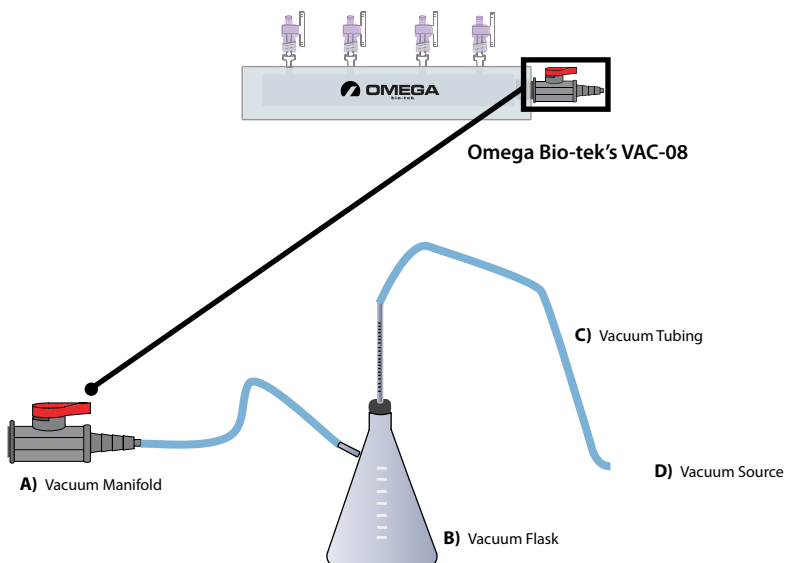
The following is required for use with the Vacuum/Spin Protocol:

- A) Vacuum Manifold (We recommend Omega Bio-tek's VAC-08)
Other Compatible Vacuum Manifolds: Qiagen QIAvac24, Sigma Aldrich VM20, Promega Vacman®, or manifold with standard Luer connector
- B) Vacuum Flask
- C) Vacuum Tubing
- D) Vacuum Source (review tables below for pressure settings)

Manifold	Recommended Pressure (mbar)
VAC-08	-200 to -600

Conversion from millibars:	Multiply by:
Millimeters of mercury (mmHg)	0.75
Kilopascals (kPa)	0.1
Inches of mercury (inch Hg)	0.0295
Torr (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per Square Inch (psi)	0.0145

Vacuum Setup:



Recommended Settings

Growth and Culture of Bacteria

Bacterial Strain Selection

It is strongly recommended that an end A negative strain of *E. coli* be used for routine plasmid isolation. Examples of such strains include DH5 α [™], DH1, and C600. These host strains yield high-quality DNA with E.Z.N.A.[®] Endo-Free Plasmid DNA Kit Protocols. XL1-Blue, although a slower growing strain is also recommended due to its yield of high-quality DNA.

Host strains derivatives from HB101 such as TG1 and the JM100 series release large amounts of carbohydrates during lysis, which may inhibit enzyme activity when not completely removed. Some strains may also lower DNA quality due to having high levels of endonuclease activity, and therefore are not recommended (i.e. JM101, JM110, HB101). One may reduce the amount of culture volume or double the volumes of Solution I, Solution II, and N3 Buffer, if problems are encountered with strains such as TG1 and Top10F.

Inoculation

Bacterial cultures for plasmid preparations should always be grown from a single colony picked from a freshly streaked plate. Subculturing directly from glycerol stock or liquid cultures may lead to uneven yields or plasmid loss. Optimal results are obtained by using one single isolated colony from a freshly transformed or freshly streaked plate to inoculate an appropriate volume of starter culture containing the appropriate antibiotic, and then incubated for 12-16 at 37°C with vigorous shaking (~300 rpm; shaking incubator).

Note: Aeration is very important. The culture volume should not exceed 1/4 the volume of the container.

Culture Media

The E.Z.N.A.[®] Endo-Free Plasmid DNA Kits are specially designed for use with cultures grown in Luria Bertani (LB) medium. Richer broths such as TB(Terrific Broth) or 2 x YT lead to high cell densities that can overload the purification system, and therefore are not recommended. If rich media has to be used, growth times have to be optimized, and the recommended culture volumes must be reduced to match the capacity of the HiBind[®] DNA Column.

Note: As culture ages, DNA yield may begin to decrease due to cell death and lysis within the culture.

Recommended Settings

Culture Volume and Cell Density

Do Not Exceed Maximum Recommended Culture Volumes

For optimal plasmid yields, the starting culture volume should be based on culture cell density. A bacterial density between 2.0 and 3.0 at OD₆₀₀ is recommended. When using nutrient-rich media, care should be taken to ensure that the cell density does not exceed an OD₆₀₀ of 3.0. Using a high-density culture outside of the recommended OD range may overload the purification system.

Endo-Free Plasmid DNA Midi Kit Vacuum Protocol

E.Z.N.A.® Endo-Free Plasmid DNA Midi Kit - Vacuum Protocol

All centrifugation steps should be performed with a swing bucket rotor for maximum plasmid DNA yields. All centrifugation steps should be carried out at room temperature. If high yields of low copy number plasmid DNA are desired, see “Low Copy Number Plasmids and Cosmids” protocol on Page 31.

Materials and Equipment to be Supplied by User:

- 100% Ethanol (Do not use denatured alcohol)
- Isopropanol
- Centrifuge with swing bucket rotor capable of at least 4,000 x g (See the “Troubleshooting” section on Page 34 if not available)
- Nuclease-free 15 mL and 50 mL centrifuge tubes
- Vacuum manifold
- Vortexer
- Ice bucket
- Optional: Water bath or incubator capable of 70°C
- Optional: 3M NaOH

Before starting:

- Chill N3 Buffer on ice
- Heat Elution Buffer to 70°C if Plasmid DNA is > 10 kb
- Prepare DNA Wash Buffer, EHBC Buffer, and Solution 1 according to “Preparing Reagents” section on Page 6

1. Transfer 20-50 mL overnight culture to a 50 mL centrifuge tube (not provided).

Note: The optimal volume to use depends on the culture density and plasmid copy number. The optimal cell mass ($OD_{600} \times \text{mL culture}$) for the HiBind® DNA Midi Column is 80-100. For example, if the OD_{600} of a culture is 4.0, the optimal culture volume should be 20-25 mL. If excess culture cell mass is used, alkaline lysis will be inefficient, the HiBind® membrane will be overloaded, and the performance of the system will be decreased. The increase in lysate viscosity will require vigorous mixing which may result in shearing of genomic DNA and contamination the plasmid DNA. For low copy number plasmids, see the “Low Copy Number Plasmids” protocol on Page 31.

2. Centrifuge at 4,000 x g for 10 minutes at room temperature.

Endo-Free Plasmid DNA Midi Kit Vacuum Protocol

3. Decant or aspirate and discard the culture media.

Note: To ensure that all traces of the medium are removed, use a clean paper towel to blot excess liquid from the wall of the tube.

4. Add 2.5 mL Solution I/RNase A. Vortex or pipet up and down to completely resuspend the cells.

Note: RNase A must be added to Solution I before use. Please see the instructions in the "Preparing Reagents" section on Page 6.

5. Add 2.5 mL Solution II. Invert and rotate the tube gently 8-10 times to obtain a cleared lysate. This may require a 2-3 minute incubation at room temperature with occasional mixing.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO₂ in the air.

6. Add 1.25 mL cold N3 Buffer. Gently invert 10 times or until a flocculent white precipitate forms. This may require a 2 minute incubation at room temperature with occasional mixing.

Note: The solution must be mixed thoroughly. This is vital for obtaining good yields. If the mixture still appears viscous, brownish, or conglobated, more mixing is required to completely neutralize the solution.

7. Prepare a Lysate Clearance Filter Syringe by removing the plunger. Place the barrel in a tube rack to keep upright. Make sure the end cap is attached to the syringe tip.
8. Immediately transfer the lysate from Step 6 into the barrel of the Lysate Clearance Filter Syringe.
9. Hold the Lysate Clearance Filter Syringe barrel over a clean 15 mL centrifuge tube (not provided) and remove the end cap from the syringe tip.

Endo-Free Plasmid DNA Midi Kit Vacuum Protocol

10. Gently insert the plunger into the barrel to expel the cleared lysate into the 15 mL centrifuge tube.

Note: Some of the lysate may remain in the flocculent precipitate. DO NOT force this residual lysate through the filter.

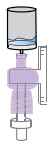
11. Measure the volume of cleared lysate.
12. Add 1 volume ETR Binding Buffer. Invert the tube gently 10 times.
13. Prepare the vacuum manifold by following the manufacturer's instructions.
14. Connect the HiBind® DNA Midi Column to the vacuum manifold. Refer to the Illustrated Vacuum Set Up on Page 7 for details.

Optional Protocol for Column Equilibration:

1. Add 1 mL 3M NaOH to the HiBind® DNA Midi Column.
2. Turn on the vacuum source to draw the buffer through the column.
3. Turn off the vacuum.

15. Transfer the cleared lysate from Step 13 to the HiBind® DNA Midi Column.
16. Turn on the vacuum source to draw the lysate through the column.
17. Continue adding lysate into until you have ~500 μ L lysate remaining in the column.

Note: Leaving the ~500 μ L lysate in the column prevents foaming in the following steps and allows for faster processing. See the illustration below.



Leaving 500 μ L solution in the column after Step 21 reduces foam which decreases total processing time and improves efficiency.

Endo-Free Plasmid DNA Midi Kit Vacuum Protocol

18. Turn off the vacuum.
19. Add 2 mL ETR Wash Buffer.
20. Turn on the vacuum source to draw the buffer through the column.
21. Turn off the vacuum.
22. Add 3 mL EHBC Buffer.

Note: EHBC Buffer must be diluted with isopropanol prior to use. Please see the instructions in the “Preparing Reagents” section on Page 6.
23. Turn on the vacuum source to draw the buffer through the column.
24. Turn off the vacuum.
25. Add 3.5 mL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please see the instructions in the “Preparing Reagents” section on Page 6.
26. Turn on the vacuum source to draw the buffer through the column.
27. Turn off the vacuum.
28. Repeat Steps 25-27 for a second DNA Wash Buffer wash step.
29. Transfer the HiBind® DNA Midi Column to a 15 mL Collection Tube (provided).

Endo-Free Plasmid DNA Midi Kit Vacuum Protocol

30. Centrifuge the empty HiBind® DNA Midi Column at 4,000 x *g* for 10 minutes to dry the column matrix.

Note: It is important to dry the HiBind® DNA Midi Column matrix before elution. Residual ethanol may interfere with downstream applications.

31. Transfer the HiBind® DNA Midi Column to a nuclease-free 15 mL centrifuge tube.
32. Add 0.5-1 mL Endo-Free Elution Buffer directly to the center of the column matrix.
33. Let it sit at room temperature for 3 minutes.
34. Centrifuge at 4,000 x *g* for 5 minutes.

Note: This represents approximately 65-80% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration. Alternatively, a second elution may be performed using the first eluate to maintain a high DNA concentration.

35. Store DNA at -20°C.

Endo-Free Plasmid DNA Midi Kit Centrifugation Protocol

E.Z.N.A.® Endo-Free Plasmid DNA Midi Kit - Centrifugation Protocol

The following protocol is designed for plasmid DNA isolation when a vacuum manifold is not available. All centrifugation steps should be performed with a swing bucket rotor for maximum plasmid DNA yields. All centrifugation steps should be carried out at room temperature. If high yields of low copy number plasmid DNA are desired, see “Low Copy Number Plasmids and Cosmids” protocol on Page 31.

Materials and Equipment to be Supplied by User:

- 100% Ethanol (Do not use denatured alcohol)
- Isopropanol
- Centrifuge with swing bucket rotor capable of at least 4,000 x g (See the “Troubleshooting” section on Page 34 if not available)
- Nuclease-free 15 mL and 50 mL centrifuge tubes
- Vortexer
- Ice bucket
- Optional: Water bath or incubator capable of 70°C
- Optional: 3M NaOH

Before starting:

- Chill N3 Buffer on ice
- Heat Elution Buffer to 70°C if Plasmid DNA is >10 kb
- Prepare DNA Wash Buffer, EHBC Buffer, and Solution 1 according to “Preparing Reagents” section on Page 6

1. Transfer 20-50 mL overnight culture to a 50 mL centrifuge tube (not provided).

Note: The optimal volume to use depends on the culture density and plasmid copy number. The optimal cell mass ($OD_{600} \times \text{mL culture}$) for the HiBind® DNA Midi Column is 80-100. For example, if the OD_{600} of a culture is 4.0, the optimal culture volume should be 20-25 mL. If excess culture cell mass is used, alkaline lysis will be inefficient, the HiBind® membrane will be overloaded, and the performance of the system will be decreased. The increase in lysate viscosity will require vigorous mixing which may result in shearing of genomic DNA and contamination the plasmid DNA. For low copy number plasmids, see the “Low Copy Number Plasmids” protocol on Page 31.

2. Centrifuge at 4,000 x g for 10 minutes at room temperature.

Endo-Free Plasmid DNA Midi Kit Centrifugation Protocol

3. Decant or aspirate and discard the culture media.

Note: To ensure that all traces of the medium are removed, use a clean paper towel to blot excess liquid from the wall of the tube.

4. Add 2.5 mL Solution I/RNase A. Vortex or pipet up and down to completely resuspend the cells.

Note: RNase A must be added to Solution I before use. Please see the instructions in the "Preparing Reagents" section on Page 6.

5. Add 2.5 mL Solution II. Invert and rotate the tube gently 8-10 times to obtain a cleared lysate. This may require a 2-3 minute incubation at room temperature with occasional mixing.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO₂ in the air.

6. Add 1.25 mL cold N3 Buffer. Gently invert 10 times or until a flocculent white precipitate forms. This may require a 2 minute incubation at room temperature with occasional mixing.

Note: The solution must be mixed thoroughly. This is vital for obtaining good yields. If the mixture still appears viscous, brownish, or conglobated, more mixing is required to completely neutralize the solution.

7. Prepare a Lysate Clearance Filter Syringe by removing the plunger. Place the barrel in a tube rack to keep upright. Make sure the end cap is attached to the syringe tip.

8. Immediately transfer the lysate from Step 6 into the barrel of the Lysate Clearance Filter Syringe.

9. Hold the Lysate Clearance Filter Syringe barrel over a clean 15 mL centrifuge tube (not provided) and remove the end cap from the syringe tip.

Endo-Free Plasmid DNA Midi Kit Centrifugation Protocol

10. Gently insert the plunger into the barrel to expel the cleared lysate into the 15 mL centrifuge tube.

Note: Some of the lysate may remain in the flocculent precipitate. DO NOT force this residual lysate through the filter.

11. Measure the volume of cleared lysate.
12. Add 1 volume ETR Binding Buffer. Invert the tube gently 10 times.

Note: Steps 13-32 should be performed in a swing bucket rotor for maximum plasmid DNA yield. All of centrifugation steps should be carried out at room temperature.

13. Insert a HiBind® DNA Midi Column into a 15 mL Collection Tube (provided).

Optional Protocol for Column Equilibration:

1. Add 1 mL 3M NaOH to the HiBind® DNA Midi Column.
2. Let sit at room temperature for 4 minutes.
3. Centrifuge at 4,000 x *g* for 3 minutes.
4. Discard the filtrate and reuse the collection tube.

14. Transfer 3.5 mL cleared supernatant from Step 12 to the HiBind® DNA Midi Column.
15. Centrifuge at 4,000 x *g* for 3 minutes.
16. Discard the filtrate and reuse the collection tube.
17. Repeat Steps 14-16 until all of the cleared supernatant has been transferred to the HiBind® DNA Midi Column.
18. Add 2 mL ETR Wash Buffer.

Endo-Free Plasmid DNA Midi Kit Centrifugation Protocol

19. Centrifuge at 4,000 x *g* for 3 minutes.

20. Discard the filtrate and reuse the collection tube.

21. Add 3 mL EHBC Buffer.

Note: EHBC Buffer must be diluted with isopropanol prior to use. Please see the instructions in the “Preparing Reagents” section on Page 6.

22. Centrifuge at 4,000 x *g* for 3 minutes.

23. Discard the filtrate and reuse the collection tube.

24. Add 3.5 mL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please see the instructions in the “Preparing Reagents” section on Page 6.

25. Centrifuge at 4,000 x *g* for 3 minutes.

26. Discard the filtrate and reuse the collection tube.

27. Repeat Steps 24-26 for a second DNA Wash Buffer wash step.

28. Centrifuge the empty HiBind® DNA Midi Column at 4,000 x *g* for 10 minutes to dry the column matrix.

Note: It is important to dry the HiBind® DNA Midi Column matrix before elution. Residual ethanol may interfere with downstream applications.

Endo-Free Plasmid DNA Midi Kit Centrifugation Protocol

29. Transfer the HiBind® DNA Midi Column to a nuclease-free 15 mL centrifuge tube.
30. Add 0.5-1 mL Endo-Free Elution Buffer directly to the center of the column matrix.
31. Let it sit at room temperature for 3 minutes.
32. Centrifuge at 4,000 x *g* for 5 minutes.

Note: This represents approximately 65-80% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration. Alternatively, a second elution may be performed using the first eluate to maintain a high DNA concentration.

33. Store DNA at -20°C.

Endo-Free Plasmid DNA Maxi Kit Vacuum Protocol

E.Z.N.A.® Endo-Free Plasmid DNA Maxi Kit - Vacuum Protocol

All centrifugation steps should be performed with a swing bucket rotor for maximum plasmid DNA yields. All centrifugation steps should be carried out at room temperature. If high yields of low copy number plasmid DNA are desired, see “Low Copy Number Plasmids and Cosmids” protocol on Page 31.

Materials and Equipment to be Supplied by User:

- 100% Ethanol (Do not use denatured alcohol)
- Isopropanol
- Centrifuge with swing bucket rotor capable of at least 4,000 x g (See the “Troubleshooting” section on Page 27 if not available)
- Vacuum manifold
- Vortexer
- Nuclease-free 50 mL centrifuge tubes
- Appropriate centrifuge bottle for Step 1
- Ice bucket
- Optional: Water bath or incubator capable of 70°C
- Optional: 3M NaOH

Before starting:

- Chill N3 Buffer on ice
- Heat Elution Buffer to 70°C if Plasmid DNA is >10 kb
- Prepare DNA Wash Buffer, EHBC Buffer, and Solution 1 according to “Preparing Reagents” section on Page 6

1. Transfer 50-200 mL overnight culture to an appropriate centrifuge bottle (not provided).

Note: The optimal volume to use depends on the culture density and plasmid copy number. The optimal cell mass ($OD_{600} \times \text{mL culture}$) for the HiBind® DNA Maxi Column is 300-400. For example, if the OD_{600} of a culture is 4.0, the optimal culture volume should be 75-100 mL. If excess culture cell mass is used, alkaline lysis will be inefficient, the HiBind® membrane will be overloaded, and the performance of the system will be decreased. The increase in lysate viscosity will require vigorous mixing which may result in shearing of genomic DNA and contamination the plasmid DNA. For low copy number plasmids, see the “Low Copy Number Plasmids” protocol on Page 31.

2. Centrifuge at 4,000 x g for 10 minutes at room temperature.

Endo-Free Plasmid DNA Maxi Kit Vacuum Protocol

3. Decant or aspirate and discard the culture media.

Note: To ensure that all traces of the medium are removed, use a clean paper towel to blot excess liquid from the wall of the bottle.

4. Add 10 mL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.

Note: RNase A must be added to Solution I before use. Please see the instructions in the "Preparing Reagents" section on Page 6.

5. Add 10 mL Solution II. Invert and rotate the tube gently 8-10 times to obtain a cleared lysate. This may require a 2-3 minute incubation at room temperature with occasional mixing.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO₂ in the air.

6. Add 5 mL cold N3 Buffer. Gently invert 10 times or until a flocculent white precipitate forms. This may require a 2 minute incubation at room temperature with occasional mixing.

Note: The solution must be mixed thoroughly. This is vital for obtaining good yields. If the mixture still appears viscous, brownish, or conglobated, more mixing is required to completely neutralize the solution.

7. Prepare a Lysate Clearance Filter Syringe by removing the plunger. Place the barrel in a tube rack to keep upright. Make sure the end cap is attached to the syringe tip.
8. Immediately transfer the lysate from Step 6 into the barrel of the Lysate Clearance Filter Syringe.
9. Hold the Lysate Clearance Filter Syringe barrel over a new 50 mL centrifuge tube (not provided) and remove the end cap from the syringe tip.

Endo-Free Plasmid DNA Maxi Kit Vacuum Protocol

10. Gently insert the plunger into the barrel to expel the cleared lysate into the 50 mL centrifuge tube.

Note: Some of the lysate may remain in the flocculent precipitate. DO NOT force this residual lysate through the filter.

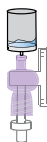
11. Measure the volume of cleared lysate.
12. Add 1 volume ETR Binding Buffer. Invert the tube gently 10 times.
13. Prepare the vacuum manifold by following the manufacturer's instructions.
14. Connect the HiBind® DNA Maxi Column to the vacuum manifold. Refer to the Illustrated Vacuum Set Up on Page 7 for details.

Optional Protocol for Column Equilibration:

1. Add 3 mL 3M NaOH to the HiBind® DNA Midi Column.
2. Let sit at room temperature for 5 minutes.
3. Turn on the vacuum source to draw the buffer through the column.
4. Turn off the vacuum.

15. Transfer the cleared lysate from Step 12 to the HiBind® DNA Maxi Column.
16. Turn on the vacuum source to draw the lysate through the column.
17. Continue adding lysate into until you have ~500 μ L lysate remaining in the column.

Note: Leaving the ~500 μ L lysate in the column prevents foaming in the following steps and allows for faster processing. See the illustration below.



Leaving 500 μ L solution in the column after Step 21 reduces foam which decreases total processing time and improves efficiency.

Endo-Free Plasmid DNA Maxi Kit Vacuum Protocol

18. Turn off the vacuum.
19. Add 10 mL ETR Wash Buffer.
20. Turn on the vacuum source to draw the buffer through the column.
21. Turn off the vacuum.
22. Add 10 mL EHBC Buffer.

Note: EHBC Buffer must be diluted with isopropanol prior to use. Please see the instructions in the "Preparing Reagents" section on Page 6.
23. Turn on the vacuum source to draw the buffer through the column.
24. Turn off the vacuum.
25. Add 15 mL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please see the instructions in the "Preparing Reagents" section on Page 6.
26. Turn on the vacuum source to draw the buffer through the column.
27. Turn off the vacuum.
28. Add 10 mL DNA Wash Buffer.
29. Turn on the vacuum source to draw the buffer through the column.
30. Turn off the vacuum.

Endo-Free Plasmid DNA Maxi Kit Vacuum Protocol

31. Transfer the HiBind® DNA Maxi Column to a 50 mL Collection Tube (provided).
32. Centrifuge the empty HiBind® DNA Maxi Column at 4,000 x *g* for 10 minutes to dry the column matrix.

Note: It is important to dry the HiBind® DNA Maxi Column matrix before elution. Residual ethanol may interfere with downstream applications.

33. Transfer the HiBind® DNA Maxi Column to a nuclease-free 50 mL centrifuge tube.
34. Add 1.5-3 mL Endo-Free Elution Buffer directly to the center of the column matrix.
35. Let it sit at room temperature for 5 minutes.
36. Centrifuge at 4,000 x *g* for 5 minutes.

Note: This represents approximately 65-80% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration. Alternatively, a second elution may be performed using the first eluate to maintain a high DNA concentration.

37. Store DNA at -20°C.

Endo-Free Plasmid DNA Maxi Kit Centrifugation Protocol

E.Z.N.A.® Endo-Free Plasmid DNA Maxi Kit - Centrifugation Protocol

The following protocol is designed for plasmid DNA isolation when a vacuum manifold is not available. All centrifugation steps should be performed with a swing bucket rotor for maximum plasmid DNA yields. All centrifugation steps should be carried out at room temperature. If high yields of low copy number plasmid DNA are desired, see “Low Copy Number Plasmids and Cosmids” protocol on Page 31.

Materials and Equipment to be Supplied by User:

- 100% Ethanol (Do not use denatured alcohol)
- Isopropanol
- Centrifuge with swing bucket rotor capable of at least 4,000 x g (See the “Troubleshooting” section on Page 27 if not available)
- Vortexer
- Nuclease-free 50 mL centrifuge tubes
- Appropriate centrifuge bottle for Step 1
- Ice bucket
- Optional: Water bath or incubator capable of 70°C
- Optional: 3M NaOH

Before starting:

- Chill N3 Buffer on ice
- Heat Elution Buffer to 70°C if Plasmid DNA is >10 kb
- Prepare DNA Wash Buffer, EHBC Buffer, and Solution 1 according to “Preparing Reagents” section on Page 6

1. Transfer 50-200 mL overnight culture to an appropriate centrifuge bottle.

Note: The optimal volume to use depends on the culture density and plasmid copy number. The optimal cell mass ($OD_{600} \times \text{mL culture}$) for the HiBind® DNA Maxi Column is 300-400. For example, if the OD_{600} of a culture is 4.0, the optimal culture volume should be 75-100 mL. If excess culture cell mass is used, alkaline lysis will be inefficient, the HiBind® membrane will be overloaded, and the performance of the system will be decreased. The increase in lysate viscosity will require vigorous mixing which may result in shearing of genomic DNA and contamination the plasmid DNA. For low copy number plasmids, see the “Low Copy Number Plasmids” protocol on Page 31.

2. Centrifuge at 4,000 x g for 10 minutes at room temperature.

Endo-Free Plasmid DNA Maxi Kit Centrifugation Protocol

3. Decant or aspirate and discard the culture media.

Note: To ensure that all traces of the medium are removed, use a clean paper towel to blot excess liquid from the wall of the bottle.

4. Add 10 mL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.

Note: RNase A must be added to Solution I before use. Please see the instructions in the "Preparing Reagents" section on Page 6.

5. Add 10 mL Solution II. Invert and rotate the tube gently 8-10 times to obtain a cleared lysate. This may require a 2-3 minute incubation at room temperature with occasional mixing.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO₂ in the air.

6. Add 5 mL cold N3 Buffer. Gently invert 10 times or until a flocculent white precipitate forms. This may require a 2 minute incubation at room temperature with occasional mixing.

Note: The solution must be mixed thoroughly. This is vital for obtaining good yields. If the mixture still appears viscous, brownish, or conglobated, more mixing is required to completely neutralize the solution.

7. Prepare a Lysate Clearance Filter Syringe by removing the plunger. Place the barrel in a tube rack to keep upright. Make sure the end cap is attached to the syringe tip.

8. Immediately transfer the lysate from Step 6 into the barrel of the Lysate Clearance Filter Syringe.

9. Hold the Lysate Clearance Filter Syringe barrel over a new 50 mL centrifuge tube (not provided) and remove the end cap from the syringe tip.

Endo-Free Plasmid DNA Maxi Kit Centrifugation Protocol

10. Gently insert the plunger into the barrel to expel the cleared lysate into the 50 mL centrifuge tube.

Note: Some of the lysate may remain in the flocculent precipitate. DO NOT force this residual lysate through the filter.

11. Measure the volume of cleared lysate.
12. Add 1 volume ETR Binding Buffer. Invert the tube gently 10 times.

Note: Steps 13-35 should be performed in a swing bucket rotor for maximum plasmid DNA yield. All of centrifugation steps should be carried out at room temperature.

13. Insert a HiBind® DNA Maxi Column into a 50 mL Collection Tube (provided).

Optional Protocol for Column Equilibration:

1. Add 3 mL 3M NaOH to the HiBind® DNA Maxi Column.
2. Let sit at room temperature for 4 minutes.
3. Centrifuge at 4,000 x *g* for 3 minutes.
4. Discard the filtrate and reuse the collection tube.

14. Transfer 20 mL cleared supernatant from Step 12 to the HiBind® DNA Maxi Column.
15. Centrifuge at 4,000 x *g* for 3 minutes.
16. Discard the filtrate and reuse the collection tube.
17. Repeat Steps 14-16 until all of the cleared supernatant has been transferred to the HiBind® DNA Maxi Column.

Endo-Free Plasmid DNA Maxi Kit Centrifugation Protocol

18. Add 10 mL ETR Wash Buffer.

19. Centrifuge at 4,000 x *g* for 3 minutes.

20. Discard the filtrate and reuse the collection tube.

21. Add 10 mL EHBC Buffer.

Note: EHBC Buffer must be diluted with isopropanol prior to use. Please see the instructions in the “Preparing Reagents” section on Page 6.

22. Centrifuge at 4,000 x *g* for 3 minutes.

23. Discard the filtrate and reuse the collection tube.

24. Add 15 mL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please see the instructions in the “Preparing Reagents” section on Page 6.

25. Centrifuge at 4,000 x *g* for 3 minutes.

26. Discard the filtrate and reuse the collection tube.

27. Add 10 mL DNA Wash Buffer.

28. Centrifuge at 4,000 x *g* for 3 minutes.

29. Discard the filtrate and reuse the collection tube.

Endo-Free Plasmid DNA Maxi Kit Centrifugation Protocol

30. Centrifuge the empty HiBind® DNA Maxi Column at 4,000 x *g* for 10 minutes to dry the column matrix.

Note: It is important to dry the HiBind® DNA Maxi Column matrix before elution. Residual ethanol may interfere with downstream applications.

31. Transfer the HiBind® DNA Maxi Column to a nuclease-free 50 mL centrifuge tube.
32. Add 1.5-3 mL Endo-Free Elution Buffer directly to the center of the column matrix.
33. Let it sit at room temperature for 5 minutes.
34. Centrifuge at 4,000 x *g* for 5 minutes.

Note: This represents approximately 65-80% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration. Alternatively, a second elution may be performed using the first eluate to maintain a high DNA concentration.

35. Store DNA at -20°C.

DNA Precipitation

The concentration of the eluted plasmid DNA varies with copy number, host strain, and growth conditions. In some cases, residual ethanol may also be present. To adjust the DNA concentration following plasmid DNA elution or for the removal of residual ethanol, perform the following isopropanol precipitation protocol.

1. Carefully transfer the eluted plasmid DNA to a clean tube suitable for precipitation. Add 1/10 volume 3M NaAC (pH 5.2) and 0.7 volume isopropanol (room temperature). Vortex to mix.
2. Centrifuge at $\geq 15,000 \times g$ for 20 minutes at 4°C.
3. Carefully decant the supernatant.
4. Add 1-2 mL 70% ethanol. Vortex to resuspend the pellet.
5. Centrifuge at $\geq 15,000 \times g$ for 10 minutes at 4°C.
6. Carefully decant the supernatant.
7. Air dry the pellet for 10 minutes.
8. Add 200-500 μL Endo-Free Elution Buffer.
9. Store DNA at -20°C.

Low Copy Number Plasmid and Cosmid DNA Purification

E.Z.N.A.[®] Endo-Free Plasmid DNA Midi/Maxi Kit Protocol - Low Copy Number Plasmid and Cosmid DNA Protocol

Low copy number plasmids generally give 0.1-1 µg DNA per mL overnight culture. For the isolation of plasmid DNA from low copy number plasmids (0.1-1 µg/mL culture) or low copy number plasmid (1-2 µg/mL culture) bacteria, use the following modified protocol.

Note: The E.Z.N.A.[®] Endo-Free Plasmid DNA Midi Kit and the E.Z.N.A.[®] Endo-Free Plasmid DNA Maxi Kit come with enough buffers to perform the standard protocols. Additional buffers are needed to perform the Low Copy Number Plasmid and Cosmid DNA Protocol. These buffers can be purchased separately. See Page 35 for ordering information.

1. Increase the volume of starting culture from that of high copy number plasmids. Use 50-100 mL bacterial culture for the E.Z.N.A.[®] Endo-Free Plasmid DNA Midi Kit and 200-400 mL bacterial culture for the E.Z.N.A.[®] Endo-Free Plasmid DNA Maxi Kit.
2. Pellet the bacterial cells by centrifugation.
3. Decant or aspirate and discard the culture media.
4. Perform Steps 4-12 in the standard protocols with double volumes of Solution I, Solution II, N3 Buffer, and ETR Binding Buffer.
5. Continue with Step 13 of the standard protocols by following the wash, drying, and elution steps. **There is no need to increase the volumes of EHBC Buffer, ETR Wash Buffer, DNA Wash Buffer, or Endo-Free Elution Buffer.**

Purification of Plasmid DNA prepared by other Methods

E.Z.N.A.® Endo-Free Plasmid DNA Midi/Maxi Kit Protocol - Plasmid DNA Purification

Plasmid DNA isolated using other methods can be further purified with the following protocol. The protocol includes the removal of endotoxins from the sample.

1. Bring the volume of plasmid DNA to 2 mL with sterile deionized water.
2. Add an equal volume ETR Binding Buffer. Vortex to mix thoroughly.
3. Transfer the sample to the HiBind® DNA Column. Draw the sample through the membrane by centrifugation or vacuum.
4. Continue the appropriate protocol beginning with the DNA Wash Buffer step and finishing with the elution step.

Troubleshooting Guide

Please use this guide to troubleshoot any problems that may arise. For further assistance, please contact the technical support staff, toll free, at 1-800-832-8896.

Possible Problems and Suggestions

Low DNA Yields	
Poor cell lysis	<ul style="list-style-type: none"> Reduce the initial volume of culture or increase the lysis time while monitoring the lysis visually. Cells may not have been dispersed adequately prior to the addition of Solution II. Make sure to vortex cell suspension to completely disperse. Solution II, if not tightly closed, may need to be replaced.
Bacterial culture is overgrown or not fresh	<ul style="list-style-type: none"> Do not incubate cultures for more than 16 hours at 37°C. Storage of cultures for extended periods prior to plasmid isolation is detrimental.
Low elution efficiency	<ul style="list-style-type: none"> If using endotoxin-free water for elution, the pH must be 8.0
Low copy number plasmid used	<ul style="list-style-type: none"> Such plasmids may yield as little as 0.1 µg plasmid DNA from a 1 mL overnight culture. Double culture volume and follow the Low Copy Number Plasmid and Cosmid DNA Protocol.
Columns were spun in a fixed angle rotor or with insufficient g-force	<ul style="list-style-type: none"> For the Midi and Maxi kits, the columns must be spun in a swing bucket rotor at 4,000 x g for liquids to pass through efficiently.
Alkaline lysis is prolonged	<ul style="list-style-type: none"> Reduce the lysis time (Solution II) to 3 minutes or until the suspended cells form a clear viscous solution.
Too many or too few cells were used	<ul style="list-style-type: none"> Confirm the cell density by measuring OD. To calculate the volume of culture to use, take the desired cell mass and divide by the absorbance of the overnight culture at 600 nm.
No DNA Eluted	
DNA Wash Buffer not diluted with ethanol	<ul style="list-style-type: none"> Prepare DNA Wash Buffer according to the instructions on Page 6.
EHBC Buffer not diluted with isopropanol	<ul style="list-style-type: none"> Prepare EHBC Buffer according to the instructions on Page 6.

Troubleshooting Guide

High molecular weight DNA contamination of product	
Over mixing of cell lysate upon addition of Solution II	<ul style="list-style-type: none"> Do not vortex or mix aggressively after adding Solution II.
Culture overgrown	<ul style="list-style-type: none"> Overgrown culture contain lysed cells and degraded DNA. Do not grow cell longer than 16 hours.
Plasmid DNA floats out of well while loading agarose gel	
Ethanol has not been removed completely from column following wash steps	<ul style="list-style-type: none"> Centrifuge column as instructed to dry the column before elution. Incubate columns for 10 minutes at 65°C to completely dry membrane after centrifugation step.
Absorbance of purified DNA does not accurately reflect quality of the plasmid (A_{260}/A_{280} ratio is too high or too low)	
DNA Wash Buffer is diluted with ethanol containing impurities	<ul style="list-style-type: none"> Check the absorbance of the ethanol between 250 nm and 300 nm. Do not use ethanol with high absorbance. Trace impurities may remain on the column after washing and can contribute to the absorbance.
Plasmid DNA is contaminated with RNA; RNase A treatment is insufficient.	<ul style="list-style-type: none"> Confirm that the RNase A was added to Solution I prior to first use. The RNase A Solution may degrade due to high temperatures (>65°C) or prolonged storage (>6 months at room temperature)
Background reading is high due to fine silica particulates	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Reduce the initial volume of culture.
Plasmid DNA is contaminated with chromosomal DNA	<ul style="list-style-type: none"> 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 or after adding N3 Buffer.
4,000 x g centrifuge not available	
4,000 x g centrifuge not available	<p>For centrifuges only capable of 2,000-4,000 x g, increase all centrifugation times by 2 minutes except for the drying of the column. Increase drying by 5 minutes. It may be necessary to incubate the empty column for drying step at 65°C for 10 minutes to completely dry the column.</p> <p><i>A Swing Bucket Centrifuge is Required.</i></p>

Ordering Information

The following components are available for purchase separately.
(Call Toll Free at 800-832-8896)

Product	Part Number
Solution I (250 mL)	PS001
Solution II (250 mL)	PS002
Endo-Free Elution Buffer (100 mL)	PS016

HiBind®, E.Z.N.A.®, and MicroElute® are registered trademarks of Omega Bio-tek, Inc.
Qiagen®, QIAvac® and Vacman® are all trademarks of their respective companies.
PCR is a patented process of Hoffman-La Roche. Use of the PCR process requires a license.

Notes: