

E.Z.N.A.® Endo-Free Plasmid Mega Kit

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Introduction

The E.Z.N.A.® family of products is an innovative system that radically simplifies extraction and purification of nucleic acids from a variety of sources. Key to the system is the Omega Bio-tek's proprietary HiBind matrix that avidly, but reversibly, binds DNA or RNA under certain optimal conditions allowing proteins and other contaminants to be removed. Nucleic acids are easily eluted with deionized water or low salt buffer.

The E.Z.N.A.™ Endo Free Plasmid Mega Kit combines time-tested consistency of alkaline SDS lysis of bacterial cells with Omega Bio-tek's innovative high efficiency DNA binding technology to recovery large scale high quality plasmid DNA. This new method facilitates the binding, washing, and elution steps, enabling multiple samples to be simultaneously processed. This kit uses a Syringe-Format System is designed to replace the centrifugation step following alkaline lysis of bacterial cells, the Lysate Clearance Filter Syringes completely remove SDS precipitates and clear bacterial lysates in a fraction of the time required for centrifugation.

Yields vary according to plasmid copy number, E.coli strain, and conditions of growth, but 250-500 mL of overnight culture in LB medium typically produces 1-2.5 mg high-copy plasmid DNA. Up to 1 liter overnight culture may be processed when working with low-copy number plasmids. The product is suitable for automated fluorescent DNA sequencing, restriction endonuclease digestion, transfection of mammalian cells, and other manipulations.

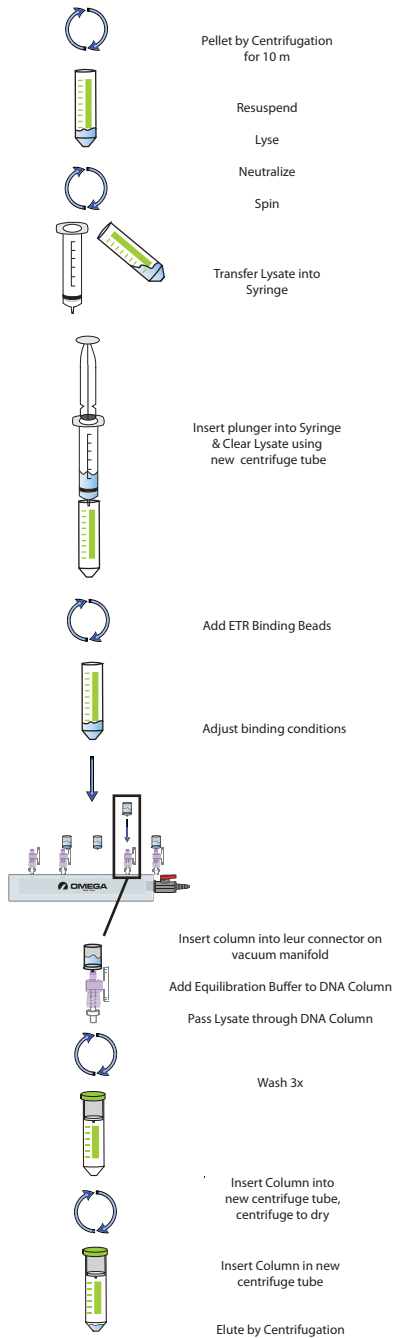
New In this Edition

- The latest edition of this manual has been redesigned to enhance readability and protocol quality.

Vacuum Manifold Required

- Since large volume of buffers are used in this protocol, it is strongly recommended to use a vacuum manifold to significantly reduce the hands on time. For information on vacuum manifold settings and user instructions, please see page 6.

Illustrated Protocol



Kit Contents

Product Number	D6228-00	D6228-01	D6228-02
Purification	2 Preps	5 Preps	20 Preps
HiBindDNA Mega Columns	2	5	20
50 mL Collection tubes	2	5	20
Lysate Clearance Syringe	2	5	20
Solution I	50 mL	110 mL	450 mL
Solution II	50 mL	110 mL	450 mL
Neutralization Buffer	50 mL	110 mL	450 mL
PFC Binding Buffer	50 mL	110 mL	450 mL
Buffer EWR	35 mL	80 mL	320 mL
ETR Binding Beads	12 mL	30 mL	55 mL
DNA Wash Buffer	25 mL	50 mL	200 mL
Endotoxin-Free Water	25 mL	60 mL	140 mL
Rnase A	450 μ L	15 mL	40 mL
Instruction Booklet	1	1	1

Storage and Stability

All E.Z.N.A.® Endo Free Mega Plasmid Isolation Kit components are guaranteed for at least 12 months from the date of purchase when stored as follows: Solution I/RNaseA at 2-8°C after being combined, see page 5. Store ETR binding Beads at 2-8°C. All other material at 22-25°C. Solution II and PFC Binding Buffer may form precipitate under lower temperature during shipping or storage, incubate at 50°C to dissolve the precipitate.

Preparing Reagents

- Add vial of RNase A to the bottle of Solution I provided and store at 2-8°C.
- Dilute DNA Wash Buffer with absolute ethanol (96-100%) as follows:

Kit	Ethanol To Be Added
D6228-00	Add 100 mL ~96-100% absolute ethanol
D6228-01	Add 200 mL ~96-100% absolute ethanol
D6228-02	Add 800 mL ~96-100% absolute ethanol

Store diluted DNA Wash Buffer at room temperature.

- **Prepare ETR Binding Beads as Follows:**

1. Aliquote the amount of ETR Binding Beads. Prepare ETR Binding beads fresh within 4 hours of processing. Each Prep will require 5 mL of Beads
2. Centrifuge at 3,000 x g for 3 minutes and remove the supernatant
3. Add 2 Volumes of Water(not provided) to the ETR Binding Beads
4. Mix by Vortexing until the beads are resuspended
5. Centrifuge at 3,000 x g for 3 minutes and remove the supernatant
6. Add 2 Volumes of Water (not provided) to the ETR Binding Beads
7. Mix by Vortexing until the beads are resuspended
8. Centrifuge at 3,000 x g for 3 minutes and remove the supernatant
9. Add 1 Volume of Water (not provided) and vortex to fully resuspend the beads.

Recommended Settings

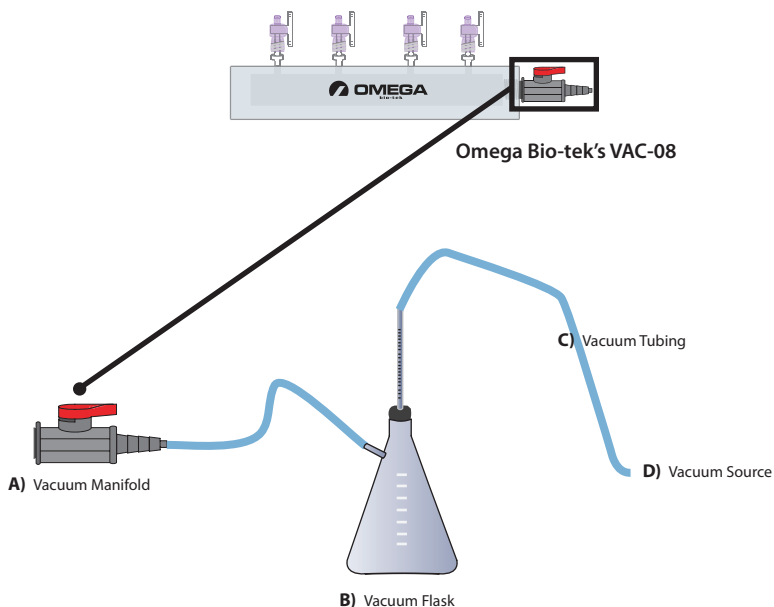
The Vacuum/Spin Protocol is the recommended protocol as it decreases hands on and procedure time. The following is required for use with the Vacuum /Spin Protocol:

- A) Vacuum Manifold (We recommend Omega Bio-tek VAC-08)
Other Compatible Vacuum Manifolds: Qiagen QIAvac24, Sigma Aldrich VM20, Promega Vacman®, or any manifold with standard Luer connectors
- 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 (mm Hg)	0.75
Kilopascals (kPa)	0.1
Inches of mercury (inch Hg)	0.0295
Torrs (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per Square Inch (psi)	0.0145

Illustrated Vacuum Setup:



Recommended Settings Continued

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 the E.Z.N.A.[®] Plasmid Isolation 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 activities 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, II, and N3, 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. It should then be incubated for 12-16 hours at 37°C with vigorous shaking (~300rpm; 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.[®] Plasmid 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 Continued

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 Mega - Vacuum/Spin Protocol

E.Z.N.A. Endo-Free Plasmid Mega Vacuum Protocol

This Protocol is designed to isolate 2.5 mg of high Copy-Number plasmids or 200 -500µg of low Copy-Number Plasmids from 500 mL overnight cultures using the E.Z.N.A.® Endo-Free Plasmid Mega Kit.

User Supplied Equipment:

- Absolute ethanol (96-100%)
- Centrifuge with swinging bucket rotor capable of 5,000 x g with adapter for 50 ml centrifuge tube and 125 mL Centrifuge bottles
- Vacuum pump capable of generating -200 to -600 mbar
- Vacuum manifold with standard leur connector
- 50 ml Centrifuge tube
- 125 or 250 mL Centrifuge Bottle
- Centrifugation tube (i.e Nalgene 3120)
- Vacuum Manifold (Cat No. Vac-08)
- Ice

Things to do before starting:

- Pre chill Neutralization Buffer on Ice
 - Prepare ETR Binding Beads and DNA Wash Buffer and Solution 1 according to directions on page 5
 -
1. Culture volume: Inoculate 500 mL LB/ampicillin (50 µg/mL) medium placed in a 2-5 liter culture flask with E. Coli carrying desired plasmid and grow at 37°C with agitation for 12-16 hours. For best results, use overnight culture as the inoculum. It is strongly recommended that an end A negative strain of E.coli be used for routine plasmid isolation. Examples of such strains include DH5α® and JM109®.

Note: Optimal growth conditions of bacteria is vital of obtaining maximal plasmid DNA yields. The best conditions are achieved by picking a single isolated colony from a freshly transformed or freshly grown plate to inoculate a 2-5mL starter culture containing the appropriate antibiotic. Incubate for ~8 hours at 37°C with vigorous shaking (~300rpm). Then used to inoculate appropriate volume of Pre-warmed liquid growth medium with antibiotic. Grow at 37°C for 12-16 hr with vigorous shaking(~300rpm). Using a flask or vessel with a volume of a least 3-4 times the volume of the culture and diluting the starter culture 1/500 to 1/1000 into the growth medium.

Following overnight bacterial growth, an OD₆₀₀ of 1.5~2.0 indicates a well-grown culture. For the best result determination of OD₆₀₀ for each culture is recommended. it is important to dilute the bacterial culture (10 to 20 fold) to enable photometric measurement in the linear range between 0.1 and 0.5 OD₆₀₀. We recommend a bacterial density of between 2.0 and 3.0 at OD₆₀₀. When using nutrient-rich media,

care should be taken ensure that the cell density does not exceed an OD₆₀₀ of 3.0. If using a frozen glycerol stock as inoculum, streak it onto an agar plate containing

Endo-Free Plasmid Mega - Vacuum/Spin Protocol

the appropriate antibiotic for single colony isolation. Then pick a single colony and inoculate the 2-5mL starter culture as described above.

2. Pellet up to bacteria in appropriate vessels by centrifugation at $5,000 \times g$ for 10 min at room temperature.
3. Decant or aspirate medium and discard. To ensure that all traces of the medium are removed, use a clean paper towel to blot excess liquid from the wall of the vessel.
4. Add 20 mL of Solution I/RNase A. Resuspend cells completely by vortexing or pipetting up and down.

Note: Complete resuspension of cell pellet is vital for obtaining good yields.

5. Add 20 mL of Solution II, gently mix by inverting and rotating the tube 7 times to obtain a cleared lysate. Incubate 3 minutes at room temperature.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Prolonged incubation may lead to nicking of plasmid DNA. (Store Solution II tightly capped when not in use.)

6. Add 20 mL of Ice-cold Neutralization Buffer, cover, and gently mix by inverting tube several times until a flocculent white precipitate forms.

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

7. Centrifuge at $4,000 \times g$ for 5 minutes. Lysate will not completely clear until after step 8.

Note: Increasing centrifugation speed is helpful to completely remove the precipitated bacterial cell material. After centrifugation, a tightly packed cell debris pellet indicates efficient lysis.

8. Finish clearing the lysate: Pour the semi cleared lysate into the barrel of the Lysate Clearance Filter Syringe. Allow the cell lysate to sit for 2 minutes.

9. Hold the Lysate Clearance filter syringe barrel over the 125 or 250 mL centrifuge vessel and gently insert the plunger to expel the cleared lysate to the tube. Some of the lysate may remain in the flocculent precipitate, do not force this residual lysate through the filter.

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

Endo-Free Plasmid Mega - Vacuum/Spin Protocol

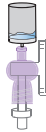
10. Add 5.0 ml of ETR Binding Beads. Mix thoroughly by vortexing for 10-20seconds, and incubate for 5 minutes at room temperature.

Important: ETR Binding Beads must be prepared according to preparing reagents section on page 5 before use.

11. Centrifuge at 3,000 x g for 10 minutes.
12. Carefully remove the cleared supernatant to a new 125/250 mL vessel avoid transferring the ETR Binding Bead Pellet

Note: Pelleted ETR Binding Beads contains high levels of Endo-toxins and should not be transferred to the new vessel.

13. Measure the volume of the supernatant, add 1/3 volume of the PFC Binding Buffer. Mix thoroughly by vortexing.
14. Insert a HiBind DNA Mega column to the vacuum manifold.
15. Pour the lysate from step 13 in to the HiBind DNA Mega column. Switch on the vacuum source to draw the solution through the HiBind DNA Mega column. Continue applying lysate from step 10 to the HiBind DNA Mega column. Do not allow the HiBind DNA Mega column to become empty until you have applied all of the lysate from step 10.



By not allowing the HiBind DNA Mega column to empty until the end of step 12, clogging or foaming of the HiBind DNA Mega column will be reduced.

16. Add 15 ml of Buffer EWR to the HiBind DNA Mega column and apply the vacuum to draw all the liquid through the column.
17. To wash the DNA, add 20 ml of DNA Wash Buffer to the HiBind DNA Mega column and apply the vacuum to draw all the liquid through the column.
18. Add an additional 20 ml of DNA Wash Buffer to the HiBind DNA Mega column and apply the vacuum to draw all the liquid through the column.
19. Transfer the HiBind DNA Mega column into a 50 ml centrifuge tube (supplied). Centrifuge at 5,000 x g for 10 minutes to dry the membrane.
20. Place the HiBind DNA mega column into a new 50 ml centrifuge tube (not supplied). Add 2-3 ml of Endotoxin Free Water. Incubate at room temperature for 5 minutes.

Endo-Free Plasmid Mega - Vacuum/Spin Protocol

21. Centrifuge at 5,000 x g for 5 minutes to elute the DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

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 (800-832-8896).

Possible Problems and Suggestions

Low DNA Yields	
Poor cell lysis	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 bacterial cells. Solution II if not tightly closed, may need to be replaced. Prepare as follows: 0.2 N NaOH, 1% SDS.
Bacterial Clone is overgrown or not fresh.	Do not incubate cultures for more than 24 hr at 37°C. Storage of cultures for extended periods prior to plasmid isolation is detrimental.
Low elution efficiency	If using Endotoxin-free water for elution, adjust the pH of the water to pH 8.0
Low copy-number plasmid used	Such plasmids may yield as little as 0.1 µg of DNA from a 1 ml overnight culture. Double culture volume and follow suggested modifications with low copy number plasmid protocol.
Binding columns were spun in a fixed angle rotor or with insufficient g-force	For midi and Midi kit, binding columns must be spun in a swinging bucket rotor at 4,000 x g for liquids to pass through efficiently.
Alkaline lysis is prolonged	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	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.
DNA Wash Buffer not diluted with ethanol.	Prepare DNA Wash Buffer Concentrate according to instructions on page 5.

Troubleshooting Guide

High molecular weight DNA contamination of product.	
Over mixing of cell lysate upon addition of Solution II.	Do not vortex or mix aggressively after adding Solution II.
Culture overgrown	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 completely been removed from column following wash steps.	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 for drying
Absorbance of purified DNA does not accurately reflect quantity of the plasmid (A_{260}/A_{280} ratio is high or low).	
DNA Wash Buffer is diluted with ethanol containing impurities.	Check the absorbance of the ethanol between 250nm and 300nm. Do not use ethanol with high absorbance. Traces of impurities may remain on the binding column after washing and 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 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 silica fines.	Spin the DNA sample at maximum speed for 1 minutes; use the supernatant to repeat the absorbance readings.
Purification is incomplete due to column overloading.	Reduce the initial volume of culture.
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 or Neutralization Buffer step.

Troubleshooting Guide

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 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)

Buffer (Size)	Part Number
Solution I (250 mL)	PS001
Solution II (250 mL)	PS002
Neutralization Buffer (250mL)	PS004
DNA Wash Buffer Concentrate (100 mL)	PS010
RNase A (5mL)	RNA-03

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