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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 (OBI) 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 endotoxin-free Elution Buffer.

Endotoxins are lipopolysaccharides (LPS) found in the outer cell membrane of gram negative bacteria such as *E.Coli*. In mammalian systems, the endotoxins are pyrogenic; they can cause fever and endotoxin shock syndrome. Endotoxin contamination is one of the major concerns for gene therapy. Endotoxin contamination in plasmids can cause lower transfection efficiency for endotoxin sensitive cell lines. Since endotoxins are negatively charged molecules like DNA, both DNA and endotoxin molecules behave similarly on the surface of silica and anion-exchange chromatography columns, which are the most commonly used technologies for plasmid purification.

The E.Z.N.A.® Fastfilter Endo-free Plasmid Maxi Kit combines the power of HiBind® technology with the time-tested consistency of alkaline-SDS lysis of bacterial cells to deliver high quality DNA. Omega Bio-Tek's HiBind® Maxi columns facilitate the binding, washing, and elution steps, thus enabling multiple samples to be simultaneously processed. This kit also includes a special filter cartridge, which replaces the centrifugation step following alkaline lysis. Following cell lysis, endotoxins are removed from the cleared cell lysate with simple extraction-phase-separation steps. DNA is bound to the silica membrane and then contaminants are removed with a simple wash step. Yields vary according to plasmid copy number, *E.coli* strain, and conditions of growth, but up to 600-1200 µg of high copy number plasmid or 50-400 µg of low copy number plasmid can be purified from a 200 ml overnight culture. Up to 500 ml culture may be processed when working with low-copy number plasmid. The product is suitable for automated fluorescent DNA sequencing, restriction endonuclease digestion, transfection of mammalian cells, and other manipulations.

Storage and Stability

All E.Z.N.A.® Fastfilter Endo-Free Plasmid Maxi Kit components are guaranteed for at least 12 months from the date of purchase when stored as follows: Solution I (once RNase A is added) at 4°C, all other material at 22-25°C.

Kit Contents

E.Z.N.A.[®] Fastfilter Endo-Free Plasmid Maxi Kit

Product Number	D6926-01	D6926-03	D6926-04
Purifications	6	25	100
HiBind [®] DNA Maxi Columns	6	25	100
Lysate Clearance Filter Syringes	6	25	100
Solution I	70 ml	270 ml	1,050 ml
Solution II	70 ml	270 ml	1,050 ml
Buffer N3	70 ml	270 ml	1,050 ml
ETR Solution	20 ml	90 ml	320 ml
Buffer HB	70 ml	260 ml	1,050 ml
EPW Wash Buffer	40 ml	200 ml	3 × 200 ml
RNase A	250 µl	1 ml	3.25 ml
Endotoxin-free Elution Buffer	25 ml	100 ml	400 ml
Instruction Booklet	6	1	1

Before Starting

Briefly examine this booklet to become familiar with each step. Prepare all components and have the necessary materials ready before starting.

- Items Supplied By User:**
- Laboratory centrifuge equipped with **swinging-bucket** rotor capable of 2,000-5,000 × g.
 - Water bath preset at 42°C
 - Sterile 50 ml centrifuge tubes. (Falcon[®] tubes recommended.)
 - Absolute (96%-100%) ethanol
 - Endotoxin-free water

IMPORTANT	1. Add a vial RNase A to bottle of Solution I provided. Store at 4°C.
	2. EPW Wash Buffer Concentrate is to be diluted with absolute ethanol (96%-100%) as follows: D6926-01: Add 160 ml absolute ethanol D6926-03: Add 800 ml absolute ethanol per bottle D6926-02: Add 800 ml absolute ethanol per bottle
	3. Optional: Preheat Endotoxin-free Elution Buffer at 70°C.

Protocol 1: Endo-Free Plasmid Maxi Kit Spin Protocol

This Protocol is designed to isolate 600-1200 µg of high copy-number plasmids or 50-400 µg of low copy-number plasmids from 200 ml (500 ml for low copy-number) overnight cultures using the E.Z.N.A.[®] Fastfilter Endo-Free Plasmid Maxi Kit. For increasing yield of low copy-number plasmids, proceed to the Low Copy-Number Plasmids Protocol on page 9.

■ Growth of bacterial cultures

1. Inoculate 200 ml LB/ampicillin (50 µg/ml) medium placed in a 1-4 liter culture flask with *E.coli* carrying desired plasmid and grow at 37°C with agitation for 12-16 h. For best results use overnight culture as the inoculum. It is strongly recommended that an *endA* negative strain of *E.coli* be used for routine plasmid isolation. Examples of such strains include DH5α[®] and JM109[®].

Optimal growth conditions for bacteria are vital for obtaining maximal plasmid DNA yields. The best conditions are achieved by picking a single isolated colony from a freshly transformed plate or a fresh plate to inoculate a 2-5 ml starter culture containing the appropriate antibiotic. Incubate for ~8hr at 37°C with vigorous shaking (~300rpm). Then use to inoculate appropriate volume of pre-warmed liquid growth medium with antibiotic. Grow at 37°C for 12-16 hr with vigorous shaking (~300rpm). Use a flask or vessel with a volume of at least 3-4 times the volume of the culture, then dilute the starter culture 1/500 to 1/1000 into 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 the appropriate antibiotic for single colony isolation. Then pick a single colony and inoculate the 2-5ml starter culture as described above.

Growth of bacteria in rich media such as 2 x YT or TB is not recommended for use with this kit.

■ Lyse the bacterial cells with alkaline-SDS solution

2. Pellet up to 200 ml bacterial culture in appropriate vessels by centrifugation at 2,000-5,000 × 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. **To the bacterial pellet add 10 ml Solution I/RNase A.** Resuspend cells completely by vortexing or pipetting up and down.

Complete resuspension of the cell pellet is vital for obtaining good yield.

4. **Add 10 ml Solution II and mix gently but thoroughly by inverting and rotating the tube 7-10 times to obtain a cleared lysate.** A 5 min incubation at room temperature may be necessary.

Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. (Store Solution II tightly capped when not in use.)

5. Prepare a Lysate Clearance Filter Syringe by removing the plunger and placing the barrel in a tube rack to keep the syringe upright.
6. **Add 10 ml Buffer N3 and mix gently but thoroughly by inverting tube several times until a flocculent white precipitate forms.** This may require a 2-3 min incubation at room temperature with occasional mixing.

Note: The Buffers must be mixed thoroughly. If the mixture still appears to be viscous, brownish and conglutinated, more mixing is required to completely neutralize the solution. Complete neutralization of the solution is vital to obtain good yields. Using ice-cold Buffer N3 is helpful to precipitate more bacterial proteins.

■ **Clear the lysate with Lysate Clearance Filter Syringe**

7. Immediately pour the lysate into the barrel of the Lysate Clearance Filter Syringe. Allow the cell lysate to sit for 5 minutes. The white precipitate should float to the top. Remove the cap from outlet nozzle of the syringe, the cell lysate may start to pass through the filter, use a new 50 ml tube to collect the cell lysate. Insert the plunger back into the barrel of the syringe.
8. Hold the Lysate Clearance filter syringe barrel over the 50 ml tube and gently insert the plunger to expel the cleared lysate to the tube.

Note: Some of the lysate may remain in the flocculent precipitate, do not force this residual lysate through the filter.

Alternatively, the cell debris and KDS-precipitation can be removed by centrifugation at 15,000 x g for 10 min at 4° C, instead of using the syringe in steps 7-8. A tightly packed cell debris pellet indicates efficient lysis. Using this alternate clearance step may improve the yield because all of the sample can be collected, whereas some is left behind when using the syringe method.

■ **Remove endotoxins with ETR Solution**

9. **Add 0.1 volume of ETR Solution (blue) to the filtered lysate. Mix by inverting the tube 7-10 times and incubate on ice for 10 minutes.**

Note: After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

10. **Incubate the lysate at 42°C for 5 minutes. The lysate should appear turbid again. Centrifuge at 2,000-5,000 × g for 5 minutes at room temperature. The ETR Solution will form a blue layer at the bottom of the tube.**

If there is any ETR Solution suspended in the lysate, add 3 ml pre-heated water(42°C) and vortex to mix, then centrifuge at 5,000 × g for 5 minutes again.

11. **Carefully transfer the top aqueous phase (cleared lysate) to a new 50 ml tube. Add 0.5 volume of absolute ethanol (room temperature) and gently mix by inverting tube 5-6 times. Incubate at room temperature for 2 min.**

Note: If the total volume of cleared cell lysate is 30 ml, add 15 ml absolute ethanol. Avoid transferring any blue ETR Solution since it contains highly concentrated LPS.

■ **Purify plasmid DNA with HiBind® DNA maxi column**

Note: Step 12 to 18 should be performed in swinging-bucket rotor for maximal plasmid DNA yields. All of the centrifugation step should be carried out at room temperature.

12. Take a HiBind® DNA Maxi Column pre-inserted in a 50 ml collection tube (supplied) and add 20 ml of cleared lysate (from step 11) into the HiBind® DNA Maxi column. Centrifuge at 2,000-3,000 × g for 2 minutes. Discard the flow-through and re-use the collection tube.
13. Repeat step 12 until all remaining cleared cell lysate has been passed through the column. Discard the flow-through and re-use the collection tube.
14. **Add 10ml Buffer HB to the DNA maxi column.** Centrifuge at 2,000 × g for 2 minutes. Discard the flow-through and re-use the collection tube.
15. **Add 15 ml EPW Wash Buffer (diluted with absolute ethanol) to the DNA maxi column** and centrifuge as above. Discard the flow-through and re-use the collection tube.

Note: EPW Wash Buffer Concentrate must be diluted with absolute ethanol before use. See label for directions. EPW Wash Buffer must be at room

temperature when used.

16. **Add 10 ml EPW Wash Buffer to the DNA maxi column** and centrifuge as above. Discard the flow-through and re-use the collection tube.
17. **Centrifuge the empty HiBind® DNA maxi column at maximum speed (no more than 5,000 x g) for 10 min to dry the column matrix. Do not skip this step - it is critical for removing ethanol from the column.**

■ **Elute Plasmid DNA from the HiBind® DNA maxi column**

Optional: For maximal yield and high concentration of plasmid, see alternative protocol of elution on page 8. For fast elution, proceed step 18-19.

18. **Drying the column:** choose either of the methods below to further dry the column before eluting DNA.
 - A. Remove the column and place into a vacuum chamber at room temperature. Any device connected to a vacuum source may be used. Seal the chamber and apply vacuum for 15 min. Remove the column and proceed to step 19.
 - B. Bake the maxi column at 65° C in a vacuum oven or incubator for 10-15 minutes. Proceed to step 19.
19. Place the HiBind® DNA maxi column into a clean 50 ml centrifuge tube. Add 2-3 ml (depending on desired final concentration) Endotoxin-Free Elution Buffer (or endotoxin-free water) onto the center of membrane and centrifuge at maximum speed (no more than 5000 x g) for 5 min to elute DNA. This represents approximately 70-80% 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. Preheating the elution buffer or water to 70°C and allowing the column to soak 3 min at room temperature before elution may significantly increase yields.

Note: The plasmid DNA obtained using this protocol performs well in PCR, restriction digests, lipid mediated transfection and transformation. The expected concentration of plasmid varies between different copy number vector. However, the concentration of high copy-number plasmid is 150-600ug/ml. Some residual ethanol is present, but does not interfere with these downstream applications. One may get high concentration and absolutely remove ethanol with the alternative elution step as follows.

■ **Alternative protocol for eluting plasmid DNA from column**

1. **Place the HiBind® DNA Maxi column into a clean 50 ml centrifuge tube. Add 6 ml Endotoxin-Free Elution Buffer (or endotoxin-free water) directly onto the column matrix.** Allow column to sit 2 min at room temperature. Centrifuge at maximum speed (no more than 5,000 x g) for 5 min to elute DNA. Preheating the water to 70°C and allowing the column to soak 3 min at room temperature before elution may significantly increase yields.
2. **Carefully transfer eluted, purified plasmid DNA from the 50 ml centrifuge tube to a clean tube suitable for precipitation. Add 260 µl 5M NaCl and 4.4 ml isopropanol at room temperature.** Vortex to mix and centrifuge at >15,000 x g for 30 min at 4°C. Carefully decant the supernatant.
3. **Wash the DNA pellet once one with 2 ml 70% ethanol and centrifuge at >5,000 x g for 10 min.** Carefully decant the supernatant without disturbing the pellet.
4. Air-dry the pellet for 5-10 min.
5. Finally resuspend DNA pellet in 200-500 µl (depending on desired concentration of final product) Endotoxin free Elution Buffer or endotoxin-free water.

Protocol 2: Endo-free Plasmid Maxi Kit Vacuum Protocol

1. Prepare the cleared endotoxin-free cell lysate by following step 1-11 of the Spin Protocol on pages 4-6.
2. Transfer the cleared cell lysate to the HiBind® DNA Maxi column. Be careful not to overfill the column. Apply the vacuum to allow all of the sample to pass through the column.
3. Add 10 ml Buffer HB to the column and apply the vacuum to draw the liquid through the column.
4. Wash the sample: Add 15 ml SPW Wash buffer (pre-diluted with absolute ethanol) to the column and allow it pass through the column via the vacuum.
5. Wash the column again with 10 ml SPW Wash buffer by repeating step 4. Keep the vacuum on for another 10 minutes after the liquid pass through the column to dry the membrane (critical).
6. Proceed with either elution procedure on pages 7 or 8.

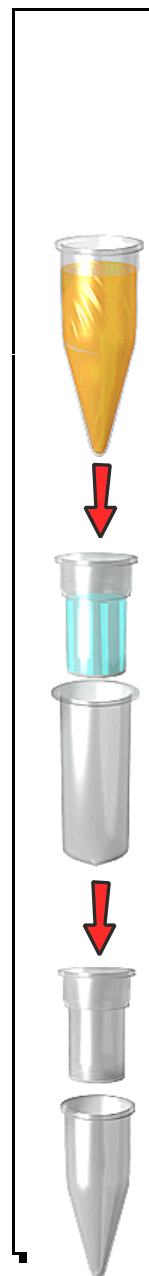
Protocol 3: Low Copy-Number Plasmids Protocol

Low copy plasmids generally give 0.1-1 µg plasmid DNA per ml overnight culture. For isolation of plasmid from low copy-number plasmids (0.1-1 µg/ml culture) or low-midi copy-number plasmids (1-2 µg/ml culture), the method can be modified to essentially increase the yield if necessary.

Start with 400-500ml bacterial culture. Centrifuge for 10 min at 3,500-5,000 x g in a centrifuge tube. Proceed from step 3 (page 4) and double the volumes of Solutions I, II, and N3. Continue as above using only one HiBind® DNA Maxi column per sample. There is no need to increase the volumes of Buffer HB and Wash Buffer used. Additional volumes of Solutions I, II, and N3 and ETR Solution can be purchase separately.

Note: This method is not recommended for high copy number plasmids because beyond 200 ml culture, the HiBind® DNA Maxi column quickly becomes saturated. In this situation we recommend processing of multiple samples from the same culture.

Protocol 4: Short Protocol For Experienced Users



1. Pellet cells from 200 ml (high copy) or 500 ml (low copy) overnight culture.
2. Resuspend cells in 10 ml (high copy) or 20 ml (low copy) Solution I/RNase A.
3. Add 10 ml (high copy) or 20 ml (low copy) Solution II. Mix gently but thoroughly by inverting 4-6 times to obtain cleared lysate. A brief incubation at RT may be required.
4. Add 10 ml (high copy) or 20 ml (low copy) N3 and mix well to form white precipitate.
5. Centrifuge at maximum (at least 10,000 x g) speed for 10 min at 4°C or Clear the lysate with Lysate Clearance Filter Syringe
6. Remove endotoxins with ETR Solution
7. Transfer cleared lysate to a HiBind™ DNA Maxi column placed in a 50 ml collection tube. Centrifuge at 2000-3000 x g for 2 min. Discard liquid. Repeat this step until the entire sample has been passed through.
NOTE: Steps 7 to 13 should be performed in a swinging-bucket rotor for maximal plasmid DNA yields. All centrifugation steps should be carried out at room temperature.
8. Wash column with 10 ml Buffer HB. Centrifuge as above. Discard liquid.
9. Using same collecting tube, wash column with 15 ml EPW Wash Buffer diluted with ethanol. Centrifuge as above.
10. Optional: Wash column a second time with 10ml EPW Wash Buffer. Centrifuge as above.
11. Centrifuge the empty column for 10 min at max speed to dry.
12. Elute plasmid with 3 ml Endotoxin Free Elution Buffer. And Centrifuge at max speed for 2 min.
13. Remove the column from collection tube and store the eluted DNA at -20°C.

Yield and Quality of DNA

Determine the absorbance of an appropriate dilution (10- to 20-fold) of the sample at 260 nm and then at 280 nm. The DNA concentration is calculated as follows:

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

The ratio of (Absorbance₂₆₀)/(Absorbance₂₈₀) is an indication of nucleic acid purity. A value greater than 1.8 indicates > 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 depend on a number of factors, including plasmid copy number, size of insert, host strain, culture volume, culture medium and binding capacity of the kit. Of these factors, the copy number of vector, culture volume and binding capacity of the kit are most important. Copy number of plasmids can vary from one copy to several hundred copies per cell, as dictated by their origin of replication. But very large plasmids are often maintained at very low copy number per cell. The expected yield from 200 ml overnight cultures (LB medium) are indicated in the table.

Plasmid	Replicon	Copy Number	expected Yield of 200ml culture
pUC vector	pMB1	500-700	700-1200 μg
pBR322 and its derivatives	pMB1	15-20	40-80 μg
PACYC and its derivatives	p15A	10-12	20-40 μg
pSC101 and its derivatives	pSC101	~5	10-20 μg
pBluescript	ColE14	300-500	600-900 μg
ColE14	ColE14	15-20	10-40 μg
pGEM	pMB1	300-700	800-1000 μg

Trouble Shooting Guide

Problem	Likely Cause	Suggestions
Low DNA yields	Poor cell lysis	Only use LB or YT medium containing ampicillin. Do not use more than 200 ml (high copy-number) or 500 ml (low copy-number). Cells may not be dispersed adequately prior to addition of Solution II. Vortex/ pipet cell suspension to completely disperse bacterial clumps. Increase incubation time with Solution II to obtain a clear lysate. Solution II, if not tightly closed, may need to be replaced. Prepare as follows: 0.2 N NaOH, 1% SDS.
	Bacterial culture overgrown or not fresh.	Do not incubate cultures for more than 16 hr at 37°C. Storage of cultures for extended periods prior to plasmid isolation is detrimental to yield and quality.
	Low copy-number plasmid used	Such plasmids may yield as little as 0.1 μg DNA from a 1 ml overnight culture. Increase culture volume to 500 ml.
No DNA eluted.	EPW Wash Buffer Concentrate not diluted with absolute ethanol.	Prepare EPW Wash Buffer Concentrate as instructed on the label.
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. Adequate mixing is obtained by simply inverting and rotating tube to cover walls with viscous lysate.
Optical densities do not agree with DNA yield on agarose gel.	Trace contaminants eluted from column increase A_{260} .	Make sure to wash column as instructed. Alternatively, rely on agarose gel/ethidium bromide electrophoresis for quantitation.
RNA visible on agarose gel.	RNase A not added to Solution I.	Add vial(s) of RNase to each bottle of Solution I.
Plasmid DNA floats out of well while loading agarose gel, does not freeze, or smells of ethanol.	Ethanol traces not completely removed from column following wash steps.	Centrifuge column at 3,000 x g for 10 min to dry the column. A swinging-bucket rotor is recommended for centrifugation. Use of the Alternative Protocol for Eluting Plasmid DNA from Column on page 8 will assure removal of ethanol.