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Introduction

The E.Z.N.A.™ family of products is an innovative system that radically simplifies the 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 Kits combine the power of HiBind® technology with the time-tested consistency of alkaline-SDS lysis of bacterial cells to deliver high quality DNA, low in endotoxins 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 copurification 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 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.

The purified plasmid DNA obtained with Omega Bio-Tek's E.Z.N.A.™ Endo-Free Plasmid System is suitable for automated fluorescent DNA sequencing, restriction endonuclease digestion, transfection of mammalian cells, and other manipulations.

Storage and Stability

A new column Equilibration Buffer is introduced into the End-Free Plasmid kits to improve the DNA yield and kit performance.

Storage and Stability

All of the E.Z.N.A.™ Endo-Free Plasmid Kits components are guaranteed for at least 12 months from the date of purchase when stored as follows: Solution I (once RNase A is added) and ETR Solution at 4°C, all other material at 22-25 °C. Store Solution II tightly capped when not in use

E.Z.N.A.™ Endo-free Plasmid Mini Kit I

Product No.	D6948-00	D6948-01	D6948-02
HiBind® DNA Mini Columns (I)	5	50	200
2 ml Collection Tubes	5	50	200
Solution I	5 ml	20 ml	60 ml
Solution II	5 ml	20 ml	60 ml
Buffer N3	2.5 ml	10 ml	30 ml
Equilibration Buffer	1.5 ml	7 ml	25 ml
ETR Solution	1 ml	5 ml	18 ml
Buffer HB	5 ml	30 ml	120 ml
DNA Wash Buffer	1.5 ml	15 ml	3 x 25 ml
RNase A	Pr e-added	100 µl	300 µl
Endotoxin-Free Elution Buffer	1.2 ml	10 ml	30 ml
Instruction Booklet	1	1	1

E.Z.N.A.™ Endo-free Plasmid Mini Kit II

Product No.	D6950-00	D6950-01	D6950-02
HiBind® DNA Mini Columns (II)	5	50	200
2 ml Collection Tubes	5	50	200
Solution I	5 ml	30 ml	120 ml
Solution II	5 ml	30 ml	120 ml
Buffer N3	2.5 ml	15 ml	60 ml
Equilibration Buffer	1.5 ml	7 ml	25 ml
ETR Solution	1.2 ml	10 ml	40 ml
Buffer HB	5 ml	30 ml	120 ml
DNA Wash Buffer	1.5 ml	15 ml	3 x 25 ml
RNase A	pre-added	100 µl	400 µl
Endotoxin-Free Elution Buffer	1.5 ml	15 ml	40 ml
Instruction Booklet	1	1	1

E.Z.N.A.™ Endo-free Plasmid Midi Kit

Product No.	D6915-00	D6915-01	D6915-03	D6915-04
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	7 ml	30 ml	80 ml	270 ml
Solution II	7 ml	30 ml	80 ml	270 ml
Buffer N3	4 ml	20 ml	40 ml	140 ml
Equilibration Buffer	3 ml	25 ml	30 ml	110ml
ETR Solution	4ml	15 ml	35 ml	150 ml
Buffer HB	8 ml	35 ml	85 ml	330ml
DNA Wash Buffer	4 ml	15 ml	40 ml	200 ml
RNase A	50 ul	100 µl	400 µl	1.2 ml
Endotoxin-Free Elution Buffer	4 ml	20 ml	60 ml	200 ml
Instruction Booklet	1	1	1	1

E.Z.N.A.™ Endo-Free Plasmid Maxi Kit

Product No.	D6926-00	D6926-01	D6926-03	D6926-04
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	25 ml	70 ml	270 ml	1050 ml
Solution II	25 ml	70 ml	270 ml	1050 ml
Buffer N3	15 ml	35 ml	135 ml	530 ml
Equilibration Buffer	7 ml	20 ml	80 ml	320 ml
ETR Solution	15 ml	35 ml	140 ml	550 ml
Buffer HB	25 ml	70 ml	270 ml	1050 ml
DNA Wash Buffer	15 ml	30 ml	150 ml	3 x 200 ml
RNase A	100 ul	300 µl	1.2 ml	5 ml
Endotoxin-Free Elution Buffer	15 ml	40 ml	90 ml	2 x 160 ml

Product No.	D6926-00	D6926-01	D6926-03	D6926-04
HiBind® DNA Maxi Columns	2	6	25	100
50 ml Collection Tubes	2	6	25	100
Lysate Clearance Filter Syringe	2	6	25	100
Instruction Booklet	1	1	1	1

* Endo-free Elution Buffer = 10mM Tris-HCl, pH 8.5
* The Equilibration Buffer contains Sodium Hydroxide. Take appropriate laboratory safety measures and wear gloves when handling.

Before Starting

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

Equipments and Reagents Supplied by User:

All Protocols	Mini Kits	Midi Kit	Maxi Kit
Bacteria growing and harvesting equipment. ■ Inoculating loop ■ culture tubes and flasks ■ 37°C Shaking Incubator ■ LB agar plates (with antibiotic) ■ LB medium (with antibiotic)	■ 1.5 ml centrifuge tubes	■ 15ml or 50 ml centrifuge tubes	■ 50ml centrifuge tubes
■ swinging bucket rotor capable of 3,000-5,000 x g	■ Vacuum manifold and Vacuum Source (for vacuum protocols)		
■ 96-100% Ethanol			
■ 42°C Water Bath			
■ Ice			

- **Add vial of RNase A to the bottle of Solution I provided and store at 4 °C.** RNase A has been pre-added to Solution I in Trial Kits (D6948/D6950-00).

- **Pre-chill Buffer N3 at 4°C**
- **Pre-chill ETR Solution at 4 °C**

NOTE: When purifying large plasmids, **pre-warm elution buffer to 65**°C to help increase yields.

NOTE: All centrifugation steps are to be carried out at room temperature

Dilute DNA Wash Buffer with absolute ethanol

E.Z.N.A.™ Endo-Free Plasmid Mini Kit I	
D6948-00 D6948-01 D6948-02	Add 6 ml of absolute ethanol to bottle Add 60 ml of absolute ethanol to bottle Add 100 ml of absolute ethanol per bottle
E.Z.N.A.™ Endo-Free Plasmid Mini Kit II	
D6950-00 D6950-01 D6950-02	Add 6 ml of absolute ethanol to bottle Add 60 ml of absolute ethanol to bottle Add 100 ml of absolute ethanol per bottle
E.Z.N.A.™ Endo-Free Plasmid Midi Kit	
D6915-00 D6915-01 D6915-03 D6915-04	Add 16 ml of absolute ethanol to bottle Add 60 ml of absolute ethanol to bottle Add 160 ml of absolute ethanol to bottle Add 800 ml of absolute ethanol to bottle
E.Z.N.A.™ Endo-Free Plasmid Maxi Kit	
D6926-00,D6926-01 D6926-03 D6926-04	Add 60ml , 120 ml of absolute ethanol to bottle Add 600ml of absolute ethanol to bottle Add 800 ml of absolute ethanol per bottle
Diluted DNA Wash Buffer must be stored at Room Temperature	

Growth and Culture of Bacteria

Bacterial Strain Selection

It is strongly recommended that an endA negative strain of E.coli be used for routine plasmid isolation. Examples of such strains include DH5α® , DH 1, and C600. These host strains yield high quality DNA with 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 Buffer 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, and then incubated for 12-16 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.™ Endo-Free 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® spin column.
NOTE: As culture ages DNA yield may begin to decrease due to cell death and lysis within the culture.

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.

E.Z.N.A.™ Endo-Free Plasmid Mini Kit I Spin Protocol

For the isolation of ***8-40µg of high-copy*** plasmid DNA from a 1-5ml culture using the E.Z.N.A.™ spin-column format.

For ***low-copy-number plasmids***, larger volumes ***(5-10ml)*** of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol" ***(yields = 1-10µg)***.

❑ **Preparation of Bacteria**

1. **Obtain a single isolated colony from a fresh LB/ampicillin (50 µg/ml) agar plate and inoculate a starter culture of 1-5 ml LB/ampicillin medium placed in a 10-20 ml culture tube with E.coli carrying desired plasmid and grow at 37°C with agitation for 12-16 h.** It is strongly recommended that an endA negative strain of E.coli be used for routine plasmid isolation.

NOTE: An OD600 reading between 2.0 and 3.0 is an indication that bacterial cells are at an optimal density for Harvesting, and plasmid DNA isolation.

■ **Harvesting of Bacterial Cells**

IMPORTANT: DO NOT EXCEED MAXIMUM CULTURE VOLUMES!
High-copy-number maximum culture volume = ***5 ml***

2. **Harvest the bacterial cells by centrifugation at 10,000 x g for 1 min at room temperature**.

■ **Alkaline-SDS Lysis of Bacterial Cells**

3. **Decant or aspirate medium**. 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 250 µl Solution I/RNase A to the bacterial pellet. Resuspend cells completely by vortexing or pipetting. Transfer the resuspended cells to a sterile 1.5 ml microcentrifuge tube.**

   NOTE: Complete resuspension of cell pellet is vital for obtaining good yields. **DO NOT VORTEX FOLLOWING STEP 4 , DOING SO WILL SHEAR CHROMOSOMAL DNA.**

5. **Add 250 µl Solution II and mix GENTLY but thoroughly by inverting and rotating the tube 4-6 times to obtain a cleared lysate**. A 1-2 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.)

6. **Add 125 µl ice-cold Buffer N3 and mix GENTLY but thoroughly by inverting the tube several times until a flocculent white precipitate forms**. **Centrifuge at maxi speed (≥13,000 x g) for 10 minutes at room temperature.**

■ **Endotoxin Removal using ETR Solution**

7. **CAREFULLY transfer the cleared supernatant to a sterile1.5 ml microcentrifuge tube. Add 0.1 volume of ice cold ETR Solution to the cleared lysate. Mix by inverting tube 7-10 times and incubate on ice for 10 minutes. Invert the tube several times during the incubation.**

8. **Incubate the lysate at 42°C for 5 minutes.** The lysate should appear turbid again. **Centrifuge at maxi speed (≥13,000 x g) for 3 minutes at room temperature (22-25°C).** The ETR Reagent should form a blue layer at bottom of the tube.

9. **Transfer the top aqueous phase into a new 1.5 ml microtube and add 0.5**

volume of absolute ethanol (room temperature, 96-100%). **GENTLY mix by inverting tube 6-7 times. Incubate at room temperature for 1-2 minutes.**

- **Plasmid DNA Purification with the HiBind® DNA Mini Column I**

10. **Equilibrate the HiBind® DNA column: Add 100µl Equilibration Buffer inro the column. Incubate at room temperature for 5 minutes. Spin at maximum speed (>13,000 x g) for 1 minute.**

11. **Apply 700 µl of mixture from step 9 to the HiBind® DNA Mini column I (blue) pre-inserted in a 2 ml collection tube, centrifuge at 10,000 x g for 1 minute at room temperature.**

12. **Discard the flow-through and load the remaining of the mixture from step 9 into the column and centrifuge as above. Discard the flow-through and re-use the collection tube.**

13. **Apply 500 µl Buffer HB to the column and centrifuge as above.**

    NOTE: This step ensures that residual protein contamination is removed and must be included for downstream application requiring high quality DNA.

14. **Discard the flow-through liquid and wash the column by adding 700 µl DNA Wash Buffer diluted with ethanol. Centrifuge as above and discard flow-through.**

    NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions. If refrigerated, DNA Wash Buffer must be brought to room temperature before use.

15. **REPEAT** wash step 14 with another 700 µl DNA Wash Buffer.

16. **Discard the flow-through liquid. Centrifuge the empty column at maxi speed (≥13,000 x g) for 2 min to dry the column matrix.**
    NOTE: Do not skip this step-it is critical for removing ethanol from the column.

- **Elution of Purified Plasmid DNA**

17. **Place column into a new clean 1.5 ml micro-centrifuge tube. Add 30-50 µl (depending on desired concentration of final product) of Endotoxin-Free Elution Buffer directly onto the column matrix and let it sit at room temperature for 1-2 minutes. Centrifuge at ≥13,000 x g for 1 min to elute DNA.** This represents approximately 70-85% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

Endo-Free Plasmid Mini Kit I Centrifuge/Vacuum Protocol

For the isolation of ***8-40µg of high-copy*** plasmid DNA from a 1-5ml culture using the

E.Z.N.A.™ Vacuum format.

For ***low-copy-number plasmids***, larger volumes ***(5-10ml)*** of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol" ***(yields = 1-5µg)***.

- **Preparation of Bacteria**

1. **Obtain a single isolated colony from a fresh LB/ampicillin (50 µg/ml) agar plate and inoculate a starter culture of 1-5 ml LB/ampicillin medium placed in a 10-20 ml culture tube with E.coli carrying desired plasmid and grow at 37°C with agitation for 12-16 h.** It is strongly recommended that an endA negative strain of E.coli be used for routine plasmid isolation.

   NOTE: An OD600 reading between 2.0 and 3.0 is an indication that bacterial cells are at an optimal density for Harvesting, and plasmid DNA isolation.

- **Harvesting of Bacterial Cells**

   IMPORTANT: DO NOT EXCEED MAXIMUM CULTURE VOLUMES!
   High-copy-number maximum culture volume = ***5 ml***

2. **Harvest the bacterial cells by centrifugation at 10,000 x g for 1 min at RT.**

- **Alkaline-SDS Lysis of Bacterial Cells**

3. **Decant or aspirate medium.** 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. **To the bacterial pellet add 250 µl Solution I/RNase A. Resuspend cells completely by vortexing or pipetting up and down**. Complete resuspension of cell pellet is vital for obtaining good yields.

   DO NOT VORTEX FOLLOWING STEP 4 , DOING SO WILL SHEAR CHROMOSOMAL DNA!

5. **Transfer the sample to a 1.5 ml centrifuge tube. Add 250 µl Solution II and mix gently but throughly by inverting and rotating the tube 4-6 times to obtain a cleared lysate**. A 1-2 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.)***

6. **Add 125 µl ice-cold Buffer N3 and mix gently but throughly by inverting tube several times until a flocculent white precipitate forms. Centrifuge at maxi speed (≥13,000 x g) for 10 minutes at room temperature.**

- **Endotoxin Removal using ETR Solution**

7. CAREFULLY transfer the cleared supernatant to a clean 1.5ml centrifuge tube. Add 0.1 volume of ice cold ETR Solution to the cleared lysate. Mix by inverting tube 7-10 times and incubate on ice for 10 minutes. Invert the tube several times during the incubation.
NOTE: After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

8. **Incubate the lysate at 42°C for 5 minutes.** The lysate should appear turbid again. **Centrifuge at maximum speed (>13,000 x g) for 3 minutes at 25°C.** The ETR Solution will form a blue layer at bottom of tube.

9. **Transfer the top aqueous phase into a new 1.5 ml tube and add 0.5 volume of absolute ethanol (room temperature, 96-100%). Gently mix by inverting tube 6-7 times and incubate at room temperature for 1-2 minutes.**

- **Plasmid DNA Purification using a Vacuum Manifold**

10. **Prepare the vacuum manifold by following the manufacturer's instructions. Insert the HiBind® DNA column into a leur connector on the manifold.**

11. **Prepare the HiBind® DNA Mini column: Add 100µl Equilibration Buffer into the column. Wait 5 minutes at room temperature. Turn on the vacuum to draw the liquid through the membrane. Add 700µl water into the column and continue the vacuum until all the liquid through the membrane.**

12. Apply the sample from step 9 into the HiBind® DNA Mini column by pipetting. Switch on the vacuum source to draw the entire mixture through the HiBind® DNA Mini columns, and then switch off the vacuum source.

13. **Wash the HiBind® DNA Mini column by adding 500 µl Buffer HB. Switch on the vacuum source to draw the solution through the HiBind® DNA Mini columns, and then switch off the vacuum source.**

14. **Wash the HiBind® DNA Mini column by adding 700 µl DNA Wash Buffer diluted with absolute ethanol. Switch on the vacuum source to draw the solution through the HiBind® DNA Mini columns, and then switch off the vacuum source.**
NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions. If refrigerated. DNA Wash Buffer must be brought to room temperature before use.

15. **Wash the HiBind® DNA Mini column by adding another 700 µl DNA Wash Buffer diluted with absolute ethanol. Switch on the vacuum source to draw the solution through the HiBind® DNA Mini columns, and then switch off the vacuum source.**

16. **Transfer the column to a 2 ml collection tube (Supplied). Centrifuge the empty column at maxi speed (≥13,000 x g) for 2 min to dry the column matrix**.
Do not skip this step-it is critical for removing ethanol from the column.

- **Elution of Purified Plasmid DNA**

17. **Place the HiBind® DNA Mini column in a clean 1.5 ml microcentrifuge tube (not supplied). Add 30-50 µl (depending on desired concentration of final product) Endotoxin-Free Elution Buffer directly onto the column matrix and let it sit at room temperature for 2 minutes. Centrifuge at ≥13,000 x g for 1 min to elute DNA.** This represents approximately 70-85% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

E.Z.N.A.™ Endo-Free Plasmid Mini Kit II Spin Protocol

For the isolation of ***40-70 µg of high-copy*** plasmid DNA from a ***10-15 ml*** culture using the E.Z.N.A.™ spin-column format.

For ***low-copy-number plasmids***, larger volumes ***(15-25ml)*** of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol" ***(yields = 2-20µg)***.

- **Harvesting of Bacterial Cells**

IMPORTANT: DO NOT EXCEED MAXIMUM CULTURE VOLUMES!
High-copy-number maximum culture volume = ***15 ml***

1. **Harvest the bacterial cells: Pellet 10-15ml overnight culture by centrifugation. Transfer appropriate volume of culture to a 15 ml centrifuge tube and centrifuge at 5,000 x g for 10 minutes.**

- **Alkaline-SDS Lysis of Bacterial Cells**

2. **Decant or aspirate medium**. 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 500 µl Solution I/RNase A. Resuspend cells completely by vortexing or pipetting up and down**. Complete resuspension of cell pellet is vital for obtaining good yields.

3. **Transfer the sample to a 1.5 ml centrifuge tube**. **Add 500 µl Solution II and mix gently but throughly by inverting and rotating the tube 7-10 times to obtain a cleared lysate.** This may require a 2-3 min incubation at room temperature with occasional mixing. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. (Store Solution II tightly capped when not in use.)

4. **Add 250 µl ice-cold Buffer N3 and mix gently but throughly by inverting tube several times until a flocculent white precipitate forms. Centrifuge at maxi speed (≥13,000 x g) for 10 minutes at room temperature.**

   NOTE: The Buffers must be mixed throughly. If the mixture appears still viscous, brownish and conglobated, more mixing is required to completely neutralize the solution. Complete neutralization of the solution is vital of obtaining good yields.

■ **Endotoxin Removal using ETR Solution**

5. **CAREFULLY transfer the cleared supernatant to a clean 1.5 ml centrifuge tube. Add 0.1 volume of ice cold ETR Solution to the cleared lysate. Mix by inverting tube 7-10 times and incubate on ice for 10 minutes. Invert the tubeseveral times during the incubation.**

6. **Incubate the lysate at 42°C for 5 minutes.** The lysate should appear turbid again. **Centrifuge at maxi speed (≥13,000 x g) for 3 minutes at room temperature (22-25°C).** The ETR Reagent should form a blue layer at bottom of the tube.

7. **Transfer the top aqueous phase (cleared lysate) into a new 2 ml microtube and add 0.5 volume of absolute ethanol (room temperature, 96-100%). Gently mix by inverting tube 6-7 times and incubate at room temperature for1-2 minutes.**

■ **Plasmid DNA Purification with the HiBind® DNA Mini Column II**

8. **Equilibrate the HiBind® DNA column: Add 100µl Equilibration Buffer inro the column. Incubate at room temperature for 5 minutes. Spin at maximum speed (>13,000 x g) for 1 minute.**

9. **Apply 750 µl of mixture from step 7 to the HiBind® Mini column II (purple) pre-inserted in a 2 ml collection tube, centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow-through and re-use the collection tube for next step.**

10. **REPEAT** step 9 by loading the remaining of the mixture into the same column until all of the mixture has passed through the column.

11. **Wash column with 500 µl Buffer HB and centrifuge as above**. This step ensures that residual protein contamination is removed and must be included for downstream application requiring high quality DNA.

12. **Discard the flow-through liquid and wash the column by adding 700 µl DNA Wash Buffer diluted with ethanol. Centrifuge as above and discard flow-through.**

    NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions. If refrigerated. DNA Wash Buffer must be brought to room temperature before use.

13. **REPEAT** wash step 12 with another 700 µl DNA Wash Buffer diluted with ethanol.

14. **Discard the flow-through liquid. Centrifuge the empty column at maxi speed (≥13,000 x g) for 2 min to dry the column matrix**. *Do not skip this step-it is critical for removing ethanol from the column.*

■ **Elution of Purified Plasmid DNA**

15. **Place the column into a new clean 1.5 ml micro-centrifuge tube. Add 60-100 µl (depending on desired concentration of final product) Endotoxin-Free Elution Buffer directly onto the column matrix and let it sit at room temperature for 2 minutes. Centrifuge at ≥13,000 x g for 1 min to elute DNA.** This represents approximately 65-85% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

E.Z.N.A.™ Endo-Free Plasmid Midi Kit Spin Protocol

For the isolation of ***100-200 µg of high-copy*** plasmid DNA from a ***20-50ml*** culture using the E.Z.N.A.™ spin-column format.

For ***low-copy-number plasmids***, larger volumes ***(50-100ml)*** of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol"***.***

■ **Harvesting of Bacterial Cells**

1. **Pellet 20-50 ml of overnight culture by centrifugation.** Optimal volume to use depends on the culture density and plasmid copy number (see instruction in the notes below). **Transfer appropriate volume of culture to a 50 ml centrifuge tube and centrifuge at 3,000-5,000 x g for 10 minutes**.

   NOTE: ***The optimal cell mass (OD600 x ml of culture) for HiBind® Midi column is around 80-100.*** For example, if the OD600 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 unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing which may result in shearing of genomic DNA and contaminate the plasmid DNA. For low copy number plasmids, use twice as much cell mass (160-200) and double the volume of each reagent and refer to the "Low Copy-Number Plasmids" Protocol (page27).

■ **Alkaline-SDS Lysis of Bacterial Cells**

2. **Decant or aspirate medium**. 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 2.5 ml Solution I/RNase A. Resuspend cells completely by vortexing or pipetting up and down**. ***Complete resuspension of cell pellet is vital for obtaining good yields.***

3. **Add 2.5 ml Solution II and mix gently but throughly by inverting and rotating the tube 7-10 times to obtain a cleared lysate**. This may require a 2-3 min incubation at room temperature with occasional mixing.

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

4. **Prepare a Lysate Clearance Filter Syringe by removing the plunger and place the barrel in a tube rack to keep the syringe upright.**

5. **Add 1.25 ml ice-cold Buffer N3 and gently mix 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 solution must be mixed throughly. If the mixture appears still viscous, brownish and conglobated, more mixing is required to completely neutralize the solution. Complete neutralization of the solution is vital of obtaining good yields.

■ **Obtaining a Clear Lysate using a Lysate Clearance Syringe**

6. **Immediately pour the lysate into the barrel of the Lysate Clearance Filter Syringe. Allow the cell lysate to sit for 2 minutes**. The white precipitate should float to the top. **Use a new 15ml or 50 ml tube to collect the cell lysate. Insert the plunger back into the barrel of the syringe**.

7. **Hold the Lysate Clearance filter syringe barrel over the 15ml or 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 a Lysate Clearance Filter Syringe. A tightly packed cell debris

pellet indicates efficient lysis.

■ **Endotoxin Removal using ETR Solution**

8. **Add 0.1 volume of ice cold ETR Solution (blue) to the filtered lysate, mix by inverting the tube 7-10 times and incubate on ice for 10-20 minutes. Invert the tube several times during the incubation.** After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

9. **Incubate the lysate at 42°C for 5 minutes. The lysate should appear turbid again. Centrifuge at 3,000-5,000 x g for 5 minutes at 25°C (let the centrifugation slow down)**. The ETR Solution will form a blue layer at the bottom of tube.

10. **OPTIONAL:** Transfer the top aqueous phase into a new 15 ml or 50 ml tube and **repeat steps 8 and 9 to further remove endotoxins.**

11. **CAREFULLY transfer the top aqueous phase into a new 15 or 50 ml tube. Add 0.5 volume of absolute ethanol (room temperature,96-100%). Gently mix by inverting the tube 6-8 times. Incubate at room temperature for 2 min.**

■ **Plasmid DNA Purification with the HiBind® DNA Midi Column**
NOTE: Steps 12-18 should be performed in swinging-bucket rotor for maximal yields. All centrifugation steps should be carried out at room temperature.

12. **Equilibrate the HiBind® DNA column: Place a HiBind® DNA Midi Column into a 15 ml collection tube (supplied). Add 1 ml Equilibration Buffer into the column. Incubate at room temperature for 5 minutes. Spin at maximum speed (>3,000 x g) for 3 minute.**

13. **Add 3.5 ml of the mixture from step 11 into the HiBind® DNA Midi column, centrifuge at 3,000-5,000 x g for 3-5 minutes. Discard the flow-through and re-use the collection tube.**

13. **REPEAT step 13 by loading the remaining of the mixture into the same column until all of the mixture has been passed through the column**.

14. **Add 3.0 ml Buffer HB to the DNA Midi column and centrifuge as above. Discard the flow-through and re-use the collection tube.**

15. **Add 3.5 ml DNA Wash Buffer diluted with absolute ethanol to the DNA Midi column and centrifuge as above. Discard the flow-through and re-use the collection tube.**

    NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions. If refrigerated, DNA Wash Buffer must be brought to room temperature before use.

16. **Add another 3.5 ml DNA Wash Buffer to the DNA Midi column and centrifuge**

as above. Discard the flow-through and re-use the collection tube.

17. **Centrifuge the empty column at maxi speed (no more than 8,000 x g) for 10-15 min to dry the column matrix**. ***Do not skip this step - it is critical for removing ethanol from the column.***

■ **Elution of Purified Plasmid DNA**

18. **Place column into a clean 15 ml centrifuge tube. Add 0.5-1.0 ml (depending on desired final concentration) Endotoxin-Free Elution Buffer onto the column matrix and let it sit at room temperature for 2-3 minutes. Centrifuge at maxi speed (no more than 8000 x g) for 5 min to elute DNA.** This represents approximately 60-80% 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 water to 65 °C and allowing the column to soak 2 min at room temperature before elution may significantly increase yields.***

E.Z.N.A.™ Endo-Free Plasmid Midi Kit Centrifuge/Vacuum Protocol

For the isolation of ***100-200 µg of high-copy*** plasmid DNA from a 20-50ml culture using a vacuum manifold format.

For ***low-copy-number plasmids***, larger volumes **(50-100ml)** of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol" (page27).

■ **Harvesting of Bacterial Cells**

1. **Pellet 20-50 ml of overnight culture by centrifugation**. Optimal volume to use depends on the culture density (see instruction in the notes below). Transfer appropriate volume of culture to a 50 ml centrifuge tube and centrifuge at 3,000-5,000 x g for 10 minutes.

   NOTE: The optimal cell mass (OD600 x ml of culture) for HiBind® Midi column is around 80-100. For example, if the OD600 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 unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing which may result in shearing of genomic DNA and contaminate the plasmid DNA. For low copy number of plasmid, see "Low Copy-Number Plasmids Protocol".

■ **Alkaline-SDS Lysis of Bacterial Cells**

2. **Decant or aspirate medium**. 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 2.5 ml Solution I/RNase A. Resuspend cells completely by vortexing or pipetting up and down.** ***Complete resuspension of cell pellet is vital for obtaining good yields .***

3. **Add 2.5 ml Solution II and mix gently but throughly by inverting and rotating the tube 7-10 times to obtain a cleared lysate**. This may require a 2-3 min incubation at room temperature with occasional mixing.

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

4. **Prepare a Lysate Clearance Filter Syringe by placing the barrel in a tube rack to keep the syringe upright.**

5. **Add 1.25 ml ice-cold Buffer N3 and gently mix 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 solution must be mixed throughly. If the mixture appears still viscous, brownish and conglobated, more mixing is required to completely neutralize the solution. Complete neutralization of the solution is vital of obtaining good yields.

■ **Obtaining a Clear Lysate using a Lysate Clearance Syringe**

6. **Immediately pour the lysate into the barrel of the Lysate Clearance Filter Syringe. Allow the cell lysate to sit for 2 minutes**. The white precipitate should float to the top. **Use a new 15ml or 50 ml tube to collect the cell lysate. Insert the plunger back into the barrel of the syringe.**

7. **Hold the Lysate Clearance filter syringe barrel over the 15 or 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 Clearance Filter Syringe in step 6-7.

■ **Endotoxin Removal using ETR Solution**

8. **Add 0.1 volume of ice cold ETR Solution (blue) to the filtered lysate, mix by inverting the tube 7-10 times and incubate on ice for 10-20 minutes. Invert the tube several times during the incubation**. After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

9. **Incubate the lysate at 42°C for 5 minutes**. The lysate should appear turbid again. **Centrifuge at 3,000-5,000 x g for 5 minutes at 25°C (let the centrifugation slow down).** The ETR Solution will form a blue layer at the bottom of tube.

10. **OPTIONAL**: Transfer the top aqueous phase into a new 15 or 50 ml tube and **REPEAT** steps 8-9 to further remove endotoxins.

11. **Carefully transfer the top aqueous phase into a new 15 or 50 ml tube. Add 0.5 volume of absolute ethanol (room temperature,96-100%). Gently mix by inverting the tube 5-6 times. Incubate at room temperature for 2 min**.

- **Plasmid DNA Purification using a Vacuum Manifold**

12. Prepare the vacuum manifold by following the manufacturer's instructions. **Insert the HiBind® Midi DNA Column into a leur connector on the manifold.**

13. **Prepare the HiBind® DNA Mini column: Add 1 ml Equilibration Buffer into the column. Wait 5 minutes at room temperature. Turn on the vacuum to draw the liquid through the membrane. Add 3.5 ml water into the column and continue the vacuum until all the liquid through the membrane.**

14. **Apply 3.5 ml of the sample from step 11 to the HiBind® Midi column by pipetting. Switch on the vacuum source to draw the solution through the HiBind® DNA Midi columns, and then switch off the vacuum source**.

15. **REPEAT** step 12 until all the remaining of the sample pass through the column.

16. **Apply 3.0 ml Buffer HB to the column by pipetting. Switch on the vacuum source to draw the solution through the columns, and then switch off the vacuum source.**

17. **Apply 3.5 ml DNA Wash Buffer diluted with absolute ethanol to the by pipetting. Switch on the vacuum source to draw the solution through the column, and then switch off the vacuum source.**

    NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions.

18. **Apply another 3.5 ml DNA Wash Buffer to the column by pipetting. Switch on the vacuum source to draw the solution through the column. After the solution has moved through the column, continue the vacuum for another 5-10 minutes. and then switch off the vacuum source.**

19. **Place the column into a 15 ml centrifuge tube. Centrifuge the empty column at maxi speed (no more than 8,000 x g) for 10-15 min to dry the column matrix. *Do not skip this step - it is critical for removing ethanol from the column.***

- **Elution of Purified Plasmid DNA**

20 **Place column into a clean 15 ml centrifuge tube. Add 0.5-1 ml (depending on desired final concentration) Endotoxin-Free Elution Buffer onto the column matrix and let it sit at room temperature for 2-3 minutes. Centrifuge at maxi speed (no more than 8000 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.

E.Z.N.A.™ Endo-Free Plasmid Maxi Kit Spin Protocol

For the isolation of ***0.6-1.2mg of high-copy*** plasmid DNA from a 50-200ml culture using a spin column format.
For ***low-copy-number plasmids***, larger volumes (200-400ml) of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol"***.***

- **Harvesting of Bacterial Cells**

1. **Pellet 50-200 ml of overnight culture by centrifugation**. Optimal volume to use depends on the culture density and plasmid copy number (see instruction in the notes below). **Transfer appropriate volume of culture to a centrifuge tube and centrifuge at 3,000-5,000 x g for 10 minutes**.

   NOTE: The optimal cell mass (OD600 x ml of culture) for HiBind® Maxi column is around 300-400. For example, if the OD600 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 unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing which may result in shearing of genomic DNA and contaminate the plasmid DNA. For low copy number of plasmids, see "Low Copy-Number Plasmids Protocol" (page 27).

■ **Alkaline-SDS Lysis of Bacterial Cells**

2. **Decant or aspirate medium**. 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 cell pellet is vital for obtaining good yields.

3. **Add 10 ml Solution II and mix gently but throughly by inverting and rotating the tube 7-10 times to obtain a cleared lysate**. This may require a 2-3 min incubation at room temperature with occasional mixing. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. ***(Store Solution II tightly capped when not in use.)***

4. **Prepare a Lysate Clearance Filter Syringe by removing the plunger and place the barrel in a tube rack to keep the syringe upright.**

5. **Add 5 ml ice-cold Buffer N3 and gently mix 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 solution must be mixed throughly. If the mixture appears still viscous, brownish and conglobated, more mixing is required to completely neutralize the solution. Complete neutralization of the solution is vital of obtaining good yields.

■ **Obtaining a Clear Lysate using a Lysate Clearance Syringe**

6. **Immediately pour the lysate into the barrel of the Lysate Clearance Filter Syringe. Allow the cell lysate to sit for 2 minutes.** The white precipitate should float to the top. **Use a new 50 ml tube to collect the cell lysate. Insert the plunger back into the barrel of the syringe.**

7. **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 Clearance Filter Syringe in steps 6-7.

■ **Endotoxin Removal using ETR Solution**

8. **Add 0.1 volume of ice cold ETR Solution (blue) to the filtered lysate, mix by inverting the tube 7-10 times and incubate on ice for 10-20 minutes. Invert the tube several times during the incubation**. After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

9. **Incubate the lysate at 42°C for 5 minutes**. The lysate should appear turbid again. **Centrifuge at 3,000-5,000 x g for 5 minutes at 25°C** (let the centrifugation slow down). The ETR Solution will form a blue layer at the bottom of tube.

10. **Optional:** Transfer the top aqueous phase into a new 50 ml tube. **REPEAT** steps 8 and 9 to further remove endotoxins.

11. **Carefully transfer the top aqueous phase into a new 50 ml tube. Add 0.5 volume of absolute ethanol (room temperature,96-100%). Gently mix by inverting the tube 7-8 times. Incubate at room temperature for 2 min.**

■ **Plasmid DNA Purification with the HiBind® DNA Maxi Column**

NOTE: Steps 12-18 should be performed in swinging-bucket rotor for maximal plasmid DNA yields. All of centrifugation steps should be carried out at room temperature.

12. **Equilibrate the HiBind® DNA column: Place a HiBind® DNA maxi Column into a 50 ml collection tube (supplied). Add 3 ml Equilibration Buffer into the column. Incubate at room temperature for 5 minutes. Spin at maximum speed (>3,000 x g) for 3 minute.**

13. **Add 20 ml of the mixture from step 11 into the HiBind® DNA Maxi column, centrifuge at 3,000-5,000 x g for 3-5 minutes. Discard the flow-through and re-use the collection tube.**

14. **REPEAT** step 13 by loading the remaining of the mixture into the same column until all of the mixture has been passed through the column.

15. **Add 10 ml Buffer HB to the DNA Maxi column and centrifuge as above. Discard the flow-through and re-use the collection tube.**

16. **Add 15 ml DNA Wash Buffer diluted with absolute ethanol to the column and centrifuge as above. Discard the flow-through and re-use the collection tube.**

    NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions. If refrigerated, DNA Wash Buffer must be brought to room

temperature before use

17. **Add another 10 ml DNA Wash Buffer diluted with ethanol to the column and centrifuge as above. Discard the flow-through and re-use the collection tube.**

18. **Centrifuge the empty column at maxi speed (no more than 6,000 x g) for 10-15 min to dry the column matrix**. *Do not skip this step - it is critical for removing ethanol from the column.*

- **Elution of Purified Plasmid DNA**

19. **Place column into a clean 50 ml centrifuge tube. Add 1.5-3.0 ml (depending on desired final concentration) Endotoxin-Free Elution Buffer onto the column matrix and let it sit at room temperature for 2-3 minutes. Centrifuge at maxi speed (no more than 6,000 x g) for 5 min to elute DNA.** 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.

E.Z.N.A.™ Endo-Free Maxi Kit Centrifuge/Vacuum Protocol

For the isolation of ***0.6-1.2mg of high-copy*** plasmid DNA from a 50-200ml culture using a vacuum format.

For ***low-copy-number plasmids***, larger volumes (200-400ml) of bacterial culture must be processed, and buffer volumes will double (to ensure optimal DNA recovery). Proceed to the "Low-copy-number plasmids Protocol" (page 27).

- **Harvesting of Bacterial Cells**

1. **Pellet 50-200 ml of overnight culture by centrifugation**. Optimal volume to use depends on the culture density (see instruction in the notes below) and plasmid copy number. **Transfer appropriate volume of culture to a centrifuge tube and centrifuge at 3,000-5,000 x g for 10 minutes.**

   NOTE: The optimal cell mass (OD600 x ml of culture) for HiBind® Maxi column is around 300-400. For example, if the OD600 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 unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing which may result in shearing of genomic DNA and contaminate the plasmid DNA. For low copy number of plasmid, use twice as much

cell mass (600-800) and double the volume of each reagent see the "Low Copy-Number Plasmids Protocol" (page 27).

- **Alkaline-SDS Lysis of Bacterial Cells**

2. **Decant or aspirate medium**. 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 cell pellet is vital for obtaining good yields.

3. **Add 10 ml Solution II and mix gently but throughly by inverting and rotating tube 7-10 times to obtain a cleared lysate**. This may require a 2-3 min incubation at room temperature with occasional mixing.

4. **Prepare a Lysate Clearance Filter Syringe by removing the plunger and place the barrel in a tube rack to keep the syringe upright**.

5. **Add 5 ml ice-cold Buffer N3 and gently mix 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 solution must be mixed throughly. If the mixture appears still viscous, brownish and conglobated, more mixing is required to completely neutralize the solution. Complete neutralization of the solution is vital of obtaining good yields.

- **Obtaining a Clear Lysate using a Lysate Clearance Syringe**

6. **Immediately pour the lysate into the barrel of the Lysate Clearance Filter Syringe. Allow the cell lysate to sit for 2 minutes.** The white precipitate should float to the top. **Use a new 50 ml tube to collect the cell lysate. Insert the plunger back into the barrel of the syringe**.

7. **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 Clearance Filter Syringe in step 6-7.

■ **Endotoxin Removal using ETR Solution**

8. **Add 0.1 volume of ice cold ETR Solution (blue) to the filtered lysate, mix by inverting the tube 7-10 times and incubate on ice for 10-20 minutes. Invert the tube several times during the incubation.** After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

9. **Incubate the lysate at 42°C for 5 minutes**. The lysate should appear turbid again. **Centrifuge at 3,000-5,000 x g for 5 minutes at 25°C (let the centrifugation slow down)**. The ETR Solution will form a blue layer at the bottom of tube.

10. **Optional:** Carefully transfer the top aqueous phase into a new 50 ml tube. **REPEAT** step 8-9 to further remove endotoxins.

11. **Carefully transfer the top aqueous phase into a new 50 ml tube and add 0.5 volume of absolute ethanol (room temperature,96-100%). Gently mix by inverting the tube 7-8 times and incubate at room temperature for 2 min.**

■ **Plasmid DNA Purification with a Vacuum Manifold**

12. Prepare the vacuum manifold by following the manufacturer's instructions. Insert the HiBind® DNA Maxi column into a leur connector on the manifold.

13. **Prepare the HiBind® DNA Mini column: Add 3 ml Equilibration Buffer into the column. Wait 5 minutes at room temperature. Turn on the vacuum to draw the liquid through the membrane. Add 20 ml water into the column and continue the vacuum until all the liquid through the membrane.**

14. **Apply 20 ml of the sample from step 11 to the HiBind® maxi column by pipetting. Switch on the vacuum source to draw the solution through the HiBind® DNA Maxi columns, and then switch off the vacuum source.**

15. **REPEAT** step 14 until all the remaining of the lysate pass through the column.

16. **Apply 10 ml HB Buffer to the column by pipetting. Switch on the vacuum source to draw the solution through the columns, and then switch off the vacuum source.**

17. **Apply 15 ml DNA Wash Buffer diluted with absolute ethanol to the column by pipetting. Switch on the vacuum source to draw the solution through the columns, and then switch off the vacuum source**.

    NOTE: DNA Wash Buffer must be diluted with absolute ethanol before use. See label for directions.

18. **Apply another 10 ml DNA Wash Buffer to the column by pipetting. Switch on the vacuum source to draw the solution through the column. After the solution has moved through the column, continue the vacuum for another 5 minutes. and then switch off the vacuum source**.

19. **Place the column into a 50 ml centrifuge tube. Centrifuge the empty column at maximum speed (no more than 6,000 x g) for 10-15 min to dry the column matrix**. Do not skip this step - it is critical for removing ethanol from the column.

■ **Elution of Purified Plasmid DNA**

20. **Place column into a clean 50 ml centrifuge tube. Add 1.5- 3.0 ml (depending on desired final concentration) Endotoxin-Free Elution Buffer onto the column matrix and let it sit at room temperature for 2-3 minutes. Centrifuge at maximum speed (no more than 6000 x g) for 5 min to elute DNA**. 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. ***Preheating the water to 65 °C and allowing the column to soak 2 min at room temperature before elution may significantly increase yields.***

DNA Precipitation

The plasmid DNA eluted from column normally performs well in most downstream applications include PCR, restriction enzyme digestion, lipid mediated transfection and transformation. The eluted plasmid DNA concentration varies based on the copy number, host strain and growing conditions. In some cases, residual ethanol may also present in the eluted plasmid. To adjust the concentration of the DNA after the elution or remove the residue of ethanol, one can perform ethanol precipitation as following:

1. **Carefully transfer the eluted plasmid to a clean tube suitable for precipitation.**
2. **Add 1/10 volume of 3M NaAC and 0.7 volume of isopropanol (room temperature).**
3. **Vortex to mix and centrifuge at >15,000 x g for 20 min at 4°C. Carefully decant the supernatant.**
4. **Wash DNA pellet once with 1-2 ml 70% ethanol and centrifuge at > 15,000 x g for 10 min at 4°C.**
5. **Carefully decant the supernatant without disturbing the pellet and air dry the pellet for 5-10 min.**
6. **Finally resuspend DNA pellet in 200-500µl (depending on desired concentration of final product) Endotoxin-Free Elution Buffer.**

Purification of Low-Copy-Number Plasmid and Cosmid DNA

Low copy number plasmids generally give 0.1-1 µg 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) bacteria, use following modified protocol.

1. Starting bacterial volume: **Use twice the volume of culture used in the high copy number plasmid protocols.** Use up to ***5-10 ml*** culture for E.Z.N.A™ Endo-Free Plasmid Mini Kit I (D6948), ***15-25 ml*** culture for E.Z.N.A™ Endo-Free Plasmid Mini Kit II (D6950), ***50-100 ml*** for E.Z.N.A™ Endo-Free Plasmid Midi Kit (D6915) and ***200-400 ml*** for E.Z.N.A™ Endo-Free Plasmid Maxi Kit (D6926).

2. **Pellet the bacterial cells by centrifugation.**

3. **Carry out resuspension, lysis, neutralization and endotoxin removal steps by doubling the volumes of Solution I, Solution II, Buffer N3, ETR Solution and absolute ethanol.**

4. **Continue with each standard protocol for wash, drying and elution steps.** There is no need to increase the volume of Buffer HB, DNA Wash Buffer and Elution Buffer.When plasmid or cosmids are > 10kb, preheat the Endo-free Elution Buffer to 70°C prior to eluting DNA from HiBind® membrane.

Further Purification of Endotoxin-Free Plasmid DNA Obtained by Other Methods.

Plasmid DNA isolated by other methods can be further purified using any of the E.Z.N.A.™ Endo-free Plasmid protocols in this hand book.

1. **Bring up the volumes of plasmid** by **100 µl** (Kit I), **100 µl** (Kit II), **600 µl** (Midi Kit) and **2 ml** (maxi kit) with water. **Add 0.1 volume of ice cold ETR Solution (blue) and mix by inverting the tube 7-10 times and incubate on ice for 10-20 minutes. Invert the tube several times during the incubation**. After addition of ETR Solution, the lysate should appear turbid, but it should become clear after incubation on ice.

2. **Incubate the lysate at 42°C for 5 minutes**. The lysate should appear turbid again. **Centrifuge at 10,000 x g for 5 minutes at 25°C to remove ETR Solution**. The ETR Solution will form a blue layer at the bottom of tube.

3. **OPTIONAL: Transfer the top aqueous phase (cleared lysate) into a new tube and REPEAT steps 1-2 to further remove endotoxins**.

4. **Transfer the cleared supernatant to a new tube and add a 5 volume amount of HB Buffer and mix throughly by vortexing briefly.**

5. **Apply the sample to the HiBind® DNA column. Draw the sample through the membrane by centrifugation or vacuum and proceed to the appropriate protocol beginning with the Wash Step (with DNA Wash Buffer).**

Guidelines for Vac-8 manifold

Vac-8 manifold facilitate DNA preparations by providing a convenient modular vacuum manifold for use with HiBind® spin columns. The following recommendations should be followed when handling a Vac-8 manifold:

1. Keep the Vac-8 manifold in a clean and dry condition. To clean, simply rinse all components with water and dry with absorbent paper. Do not use abrasives or solvents to clean the manifold.

2. The components of Vac-8 manifold are not resistant to ethanol, methanol, or other organic solvents. Do not bring solvent into contact with the vacuum manifold. Rinse the manifold with water throughly if solvents are spilled on the unit.

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:

DNA concentration = Absorbance$_{260}$ × 50 × (Dilution Factor) µg/ml

A value 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 depend on a number of factors including plasmid copy number, size of insert, host strain, culture volume, culture medium and binding capacity of the kits. In these factors, The copy number of vector, culture volume and binding capacity of the kits are most important. Copy number of plasmid is 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 of 50 ml overnight cultures (LB medium) with E.Z.N.A.™ Fastfilter Endo-Free Plasmid Midi Kit are indicated in the table.

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	37540	5-10 µg
pSC101 and its derivatives	pSC101	~5	5 µg

Troubleshooting Guide

Problem	Cause	Possible Solution
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.
		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 16 hr at 37°C. Storage of cultures for extended periods prior to plasmid isolation is detrimental.
	Low elution efficiency	The pH of Elution Buffer or water must be 8.5 and increase the volume of Elution Buffer.
	Low copy-number plasmid used	Such plasmids may yield as little as 0.1µg of DNA from a 1ml overnight culture. Double culture volume and follow suggested modifications with low copy number plasmid protocol on page 27.
	Binding columns were spun in a fixed angle rotor or with insufficient g-force	For midi and maxi kit, binding columns must be spun in a swinging bucket rotor at 3,000-5,000 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 OD600. 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.	Prepare DNA Wash Buffer Concentrate according to instructions on page 6.
High molecular weight DNA contamination of product	Over mixing of lysate upon addition of Solution II.	Do not vortex or mix aggressively after adding Solution II.
	Culture overgrown	Overgrown culture contains lysed cells and degraded DNA. Do not grow cell for longer than 16 hours.
RNA visible on agarose gel	RNase A not added to Solution I.	Check that RNase A provided with the kit has been used. If Solution I is more than 6 months old, add more RNase A.
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 or precipitate plasmid as page 27.
Absorbance of purified DNA does not accurately reflect quantity of the plasmid (A260/A280 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 absorbency. 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)
	Purification is incomplete due to column overloading.	Reduce the initial volume of culture.

Ordering Information

Product	Applications	Cat. No.
Plasmid Miniprep Kit I	Isolation of up to 30 µg Plasmid DNA	D6942-01/02 D6943-01/02
Plasmid Miniprep Kit II	Isolation of up to 70 µg Plasmid DNA	D6945-01/02
Plasmid Midiprep Kit	Isolation of up to 250 µg Plasmid DNA	D6904-03/04
Plasmid Maxiprep Kit	Isolation of up to 1.5 mg Plasmid DNA	D6922-01/02
Fastfilter® Plasmid Midiprep Kit	Isolation of up to 250 µg Plasmid DNA, featuring filter syringes for lysate clearance	D6905-03/04
Fastfilter® Plasmid Maxiprep Kit	Isolation of up to 1.5 mg Plasmid DNA, featuring filter syringes for lysate clearance	D6924-01/03/04
HP Plasmid Miniprep Kit I	Isolation of up to 30 µg of High Purity Plasmid DNA	D7042-01/02 D7043-01-02
HP Plasmid Miniprep Kit II	Isolation of up to 70 µg of High Purity Plasmid DNA	D7045-01-02
HP Plasmid Midiprep Kit	Isolation of up to 200 µg of High Purity Plasmid DNA	D7004-01/02
HP Plasmid Maxiprep Kit	Isolation of up to 1.5 mg of High Purity Plasmid DNA	D7022-01/02
E-Z 96® Fastfilter® Plasmid Isolation Kit	Isolation of Plasmid DNA using a 96-well format	D1097-01/02
M13 Isolation Kit	Isolation of up to 15µg of single stranded phage DNA	D6900-01/02
E-Z 96® M13 Isolation Kit	Isolation of up to 15µg of single stranded phage DNA using a 96-well format	D1900-01
E-Z 96® Mag-Bind® Plasmid Isolation Kit	Isolation of Plasmid DNA using a 96-well format and paramagnetic beads	M1256-01/02
E-Z 96® Mag-Bind® Endo-Free® Plasmid Isolation Kit	Isolation of Endotoxin free Plasmid DNA using a 96-well format and paramagnetic beads	M1258-01/02
Mag-Bind® Plasmid Maxiprep Kit	Isolation of up to 1.5 mg Plasmid DNA using magnetic beads technology	M1257-01/02
Mag-Bind® Plasmid Megaprep Kit	Isolation of up to 5 mg Plasmid DNA using magnetic beads technology	M1259-01/02
BAC/PAC DNA Isolation Kit	Effective purification of BAC or PAC DNA	D2156-01/02
E-Z 96® BAC/PAC DNA Kit	Parallel purification of BAC or PAC DNA using a 96-well format	D1056-01/02
Mag-Bind® Endo-Free® Plasmid Mega Kit	Isolation of up to 5 mg of Endotoxin free plasmid DNA using magnetic beads	M6262-02

Product	Size	Cat. No.
Solution I	250 ml	PS001
Solution II	250 ml	PS002
ETR Solution	100 ml	PS012
Buffer N3	250 ml	PS017
Endo-Toxin Free Elution Buffer	100 ml	PS016
DNA Wash Buffer Concentrate	100 ml/500 ml	PS010/PS011
Buffer HB	250 ml	PS009
RNase A	400µl / 5ml	AC117/AC118
2ml capless collection tubes	500/BAG	SS1-1370-00
1.5ml DNase/RNase Free Centrifuge Tubes	500/BAG	SS1-1210-00

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