Contents

Introduction………………………………………………………… 2

Important Notes……………………………………………………… 2

Storage and Stability………………………………………………... 3

Before Starting……………………………………………………….. 4

Kit Contents………………………………………………...……… 5

Safety Information…………………………………………………. 5

EZgene™ EndoFree Plasmid Midiprep ezFilter Spin Protocol…….. 6

A: Removal of Endotoxin during Plasmid Purification ....………… 6

B: Removal of Endotoxin after Plasmid Purification ....………… 8

Purification of Low-Copy-Number Plasmid and Cosmid………….. 9

无内毒素质粒中提过滤法 I 型试剂盒简明步骤………………...…. 10

Trouble Shooting Guide...……... 12

Introduction

Key to the kit is our proprietary DNA binding systems that allow the high efficient binding of DNA to our ezBind™ matrix while proteins and other impurities are removed by wash buffer. Nucleic acids are easily eluted with sterile water or Elution Buffer.

Unlike other kits in the markets, our patented plasmid purification kit has no chaotropic salts in the buffer. The purified DNA is guanidine/anion exchange resin residues free.

Plasmid isolated with traditional protocol normally contains high level of endotoxins (Lipopolysaccharides or LPS). For transfection of endotoxin sensitive cell lines or microinjection, the endotoxins should be removed before the applications. The EZgene™ endofree system uses a specially formulated buffer that extracts the endotoxin from the plasmid DNA. Two rounds of extraction will reduce the endotoxin level to 0.1 EU (Endotoxin) per µg of plasmid DNA. The endofree plasmid miniprep kit provides an efficient endotoxin removal step into the traditional purification procedure to produce transfection grade plasmid DNA.

This kit is designed for fast and efficient purification of plasmid DNA from 15 to 50 mL of *E. coli* culture. The midi column has a DNA binding capacity of 250 µg.

The purified endofree DNA is ready for downstream applications such as transfection of endotoxin-sensitive cell lines, primary cultured cells or microinjection.

Important Notes

Plasmid Copy Numbers: The yield of plasmid DNA depends on the origin of the replication and the size of the plasmid. The protocols are optimized for high copy number plasmid purification. For low copy number plasmids, both the culture volume and the buffer volume need to be scaled up 2 times. Please contact our customer service for further information and reference Table 1 for the commonly used plasmids,

Table 1 Commonly used plasmid.

Plasmid	Origin	Copy Numbers	Expected Yield (µg per 50 mL)
pSC101	pSC101	5	5
pACYC	P15A	10-12	5-10
pSuperCos	pMB1	10-20	10-20
pBR322	pMB1	15-20	10-20
pGEM®	Muted pMB1	300-400	100-150
pBluescript®	ColE1	300-500	100-200
pUC	Muted pMB1	500-700	150-250

Host Strains: The strains used for propagating plasmid have significant influence on yield. Host strains such as Top 10 and DH5 yield high-quality plasmid DNA. *endA*+ strains such as JM101, JM110, HB101, TG1 and their derivatives, normally have low plasmid yield due to either endogenous endonucleases or high carbohydrates released during lysis. We recommend transform plasmid to an *endA*- strain if the yield is not satisfactory. For purifying plasmid DNA from *endA*+ strains (Table 2), we recommend use product PD1712.

Table 2 *endA* strains of *E. Coli*.

EndA*- Strains of *E. Coli							
DH5α	DH1	DH21	JM106	JM109	SK2267	SRB	XLO
TOP10	DH10B	JM103	JM107	SK1590	MM294	Stbl2™	XL1-Blue
BJ5182	DH20	JM105	JM108	SK1592	Select96™	Stbl4™	XL10-Gold
EndA*+ Strains of *E. Coli							
C600	JM110	RR1	ABLE® C	CJ236	KW251	P2392	BL21(DE3)
HB101	TG1	TB1	ABLE® K	DH12S ™	LE392	PR700	BL21(DE3) pLysS
JM101	JM83	TKB1	HMS174	ES1301	M1061	Q358	BMH 71-18
All NM strains				All Y strains			

Optimal Cell Mass (OD_{600} x mL of Culture): This procedure is designed for isolating plasmid grown in standard LB medium (Luria Bertani) for 12 -16 hours to a density of OD_{600} 2.0 to 3.0. If rich mediums such as TB or 2xYT are used, make sure the cell density doesn't exceed 3.0 (OD_{600}). A high ratio of biomass over lysis buffers result in low DNA yield and purity. The midi column has an optimal biomass of 100-150. For example, if the OD_{600} is 3.0, the optimal culture volume should be 25-50 mL.

Culture Volume: Use a flask or tube 4 times bigger in volume than the culture medium to secure optimal condition for bacteria growth. Don't exceed the maximum culture volume suggested in the protocol. Incomplete lysis due to over amount of bacterial culture results in lower yield and less purity.

Storage and Stability

Buffer A1 should be stored at 4 ℃ once RNase A is added. All other materials can be stored at room temperature (22-25 ℃). The Guaranteed shelf life is 12 months from the date of purchase.

Before Starting

Alternative endotoxin removal procedures are provided. Protocol A removes endotoxin during the purification of plasmid DNA and Protocol B removes endotoxin after the purification of plasmid DNA.

Prepare all components and get all necessary materials ready by examining this instruction booklet and become familiar with each step.

Important Notes

- RNase A: It is stable for half a year under room temperature. Spin down the RNase A vial briefly. Add the RNase A solution to Buffer A1 and mix well before use. Store at 4 ℃.
- **Buffer B1 precipitates below room temperature. It is critical to warm up the buffer at 50 ℃ to dissolve the precipitates before use.**
- Keep the cap tightly closed for Buffer B1 after use.
- Make sure the availability of centrifuge, especially, after mixing the lysate with ethanol, the sample needs to be processed immediately by centrifugation.
- Centrifuge speed at 5,000x g is recommended. In case the collection tube doesn't fit in high speed centrifuge rotor, use benchtop centrifuge and spin at 2,500 x g with double centrifugation time.
- ***Carry out all centrifugations at room temperature.***

Materials supplied by users

- 70% ethanol and 100% ethanol.
- High speed centrifuge and 30 mL high speed centrifuge tubes.
- 15 mL and 50 mL conical tubes.

Kit Contents

Catalog #	PD1416-00	PD1416-01	PD1416-02
Preps	2	10	25
EzBind™ Columns	2	10	25
Filter syringe (25 mL)	2	10	25
Buffer A1	6 mL	30 mL	70 mL
Buffer B1	6 mL	30 mL	70 mL
Buffer N3	8 mL	40 mL	100 mL
Buffer KB	7 mL	35 mL	85 mL
EndoClean Buffer	2 mL	10 mL	25 mL
RNase A (20 mg/mL)	0.6 mg (30 µL)	3 mg (150 µL)	7 mg (350 µL)
Endofree Elution Buffer	3 mL	15 mL	50 mL
User Manual	1	1	1

Safety Information

- Buffer N3 contains acidic acid, wear gloves and protective eyewear when handling.
- Buffer N3 and KB contains chaotropic salts, which may form reactive compounds when combines with bleach. Do not add bleach or acidic solutions directly to the preparation waste.

EZgene™ EndoFree Plasmid Midiprep Spin Protocol

A. Removal of Endotoxin *during* Plasmid Purification

This protocol is designed for removing the endotoxin during the plasmid purification.

1. Inoculate **15-50 mL** LB containing appropriate antibiotic with 50 µL fresh starter culture. Incubate at 37 ℃ for 14-16 hours with vigorous shaking.

   Note: The best way to prepare a starter culture: Inoculate a single colony from a freshly grown selective plate into 1 mL LB medium containing the appropriate antibiotic and grow at 37 ℃ for 6-8 hours with vigorous shaking (~250 rpm).
   Note: Do not use a starter culture that has been stored at 4 ℃.
   Note: Do not grow starter culture directly from glycerol stock.
   Note: Do not use more than 50 mL culture or cell mass greater than 150.

2. Harvest the bacterial by centrifugation at 5,000 x g for 10 minutes at room temperature. Pour off the supernatant and blot the inverted tube on paper towels to remove residual medium.

3. Add **2.5 mL Buffer A1** (Add RNase A to **Buffer A1** before use) and completely resuspend bacterial pellet by vortexing or pipetting **(Complete resuspension is critical for optimal yields).**

4. Add **2.5 mL Buffer B1,** mix gently but thoroughly by inverting 5 times and incubate for 5 minutes to obtain a slightly clear lysate.

   Note: Do not incubate longer than 5 minutes. Over-incubating causes genomic DNA contamination and plasmid damage.

5. Add **ice cold 600 µL Buffer N3**, mix immediately by inverting 5 times and sharp hand shaking for 5 times.

   Note: It is critical to mix the solution well. If the mixture still appears conglobated, brownish or viscous, more mix is required to completely neutralize the solution.

6. **Optional 1:** Transfer the lysate to a high speed centrifuge tube and centrifuge at 14,000 x g for 10 minutes at room temperature. Carefully transfer the clear supernatant into a 15 mL tube (avoid the floating precipitates).

   Note: If the rotor is cold, incubate the lysate at room temperature for 10 minutes and then perform centrifugation as described.

   Optional 2: Pour the lysate directly into the barrel of the filter syringe. Insert the syringe to a clean 15 mL tube (not supplied) set in a rack. Allow the cell lysate to sit for 10 minutes. The white precipitates should float to the top. Hold the filter syringe barrel over the 15 mL tube and gently insert the plunger to expel the cleared lysate to the tube, stop when feel major resistance, some of

the lysate may remain in the flocculent precipitate, **do not force the residual lysate through the filter.**

7. Transfer the clear lysate to a new high-speed centrifuge tube and add **0.1 volume** of **EndoClean Buffer**, vortex for 10s and incubate on ice for 10 minutes. Mix the sample several times without leaving ice.

   Note: Use a serological pipet or a tip cut with a clean razor in the end to transfer the EndoClean Buffer.
   Note: At room temperature (> 23 ℃), the sample becomes turbid after adding **EndoClean Buffer.** The solution becomes clear after incubating on ice.

8. Centrifuge at 13,000 x g for 10 min (Alternatively, the sample can be processed in a 15 mL conical tube and centrifuge at 2,500 x g for 15 min) at room temperature (the temperature must be greater than 23 ℃). The solution should separate into 2 phases, the upper clear phase contains DNA and the lower organic phase contains endotoxin. The two phases will not separate if the temperature is less than 23 ℃.

   Note: If phase partitioning is not observed after centrifugation：
   - Incubate the solution at 65 ℃ for 5 minutes. The solution becomes turbid again. And then repeat step 8.
   - Or add **200 µL Chloroform** (37 ℃), vortex to mix well, repeat step 8.

   Note: Up to 99% of the endotoxin can be removed by extracting with the EndoClean buffer once. Another extraction is necessary if less than 0.1 EU (Endotoxin)/ µg of DNA is desired by repeating step 7-8.

9. Carefully transfer the clear supernatant into a 15 mL conical tube (avoid the interface precipitates). Add **3 mL Buffer N3** and **3 mL 100% ethanol.** Mix immediately by sharp hand shaking for 5 times. The mixture of ethanol/lysate needs to be transfer to the DNA column immediately.

10. Immediately transfer **6 mL** the **lysate/ethonal mix** into a DNA column with a 15 mL collection tube. Centrifuge at > 2,500 x g for 1 min at room temperature. Remove the column from the tube and discard the flow-through liquid. Reinsert the column to the collection tube. Repeat step 10 till all the lysate/ethonal mix has been passed through the column.

11. **Optional:** Add **3.0 mL Buffer KB** into the spin column, centrifuge at > 2,500 x g for 1 minute. Remove the spin column from the tube and discard the flow-through. Put the column back to the collection tube.

    Note: Buffer KB is recommended for *endA+* strains such as HB101, JM101, TG1 or their derived strains. It is not necessary for isolating DNA from *endA-* strains such as Top 10 and DH5a. Please reference Table 2 on page 3.

12. Add **5 mL 70% ethanol** into the column, centrifuge at > 2,500 x g for 1 min. Remove the column from the tube and discard the flow through. Reinsert the column into the collection tube. Repeat step "12".

13. Centrifuge the column, **with the lid open**, at > 2,500 x g for 10 minutes. This step removes residual ethanol for optimal elution in next step.

    Note: Residual ethanol can be removed more efficiently with the column lid open. High centrifuge speed (5,000 x g) is suggested to remove the ethanol. It is critical to remove residual ethanol completely.

14. Carefully transfer the spin column into a clean 15 mL tube and add **0.5 mL Endofree Elution Buffer** to the center of the column and incubate for 1 minute at room temperature. Elute the DNA by centrifugation at > 2,500 x g for 5 minutes. If higher yield is desired, reload the eluate in the 15 mL tube to the column, incubate for 1 minute and centrifuge again.

    Note: Two elutions give rise to maximum DNA yield. Use less **Endofree Elution Buffer** if high concentration is desired.
    Note: The DNA is ready for downstream applications such as cloning/subcloning, RFLP, Library screening, *in vitro* translation, sequencing, transfection, and microinjection.

15. The DNA concentration can be calculated as follows,

    DNA concentration (μg/mL) = OD_{260nm} x 50 x dilution factor.

B. Removal of Endotoxin *after* Plasmid Purification

This protocol is designed for removing the endotoxin after the plasmid is purified.

1. Follow the protocol from Step 1 to 6 on page 6-7.

2. Transfer the lysate to a clean 15 mL conical tube and add **3 mL** of **Buffer N3** and **3 mL** of **100% ethanol**, mix well and go to step 10-15 on page 9-10.

3. After the plasmid is purified, add **0.1 volume** of **EndoClean Buffer** to the plasmid sample in a 2 mL centrifuge tube (For example, add **0.1 mL EndoClean Buffer** to **1 mL plasmid sample**). The solution becomes turbid after adding EndoClean Buffer.

4. Vortex the tube for 5s and put on ice for about 10 minutes Mix the sample several times without leaving ice. The solution becomes clean after incubating on ice.

5. Centrifuge at 12,000 x g at **room temperature** for 10 minutes **(the temperature must be greater than 23 ℃ for phase partitioning).**

   Note: If phase partitioning is not observed after centrifugation,
   - Incubate the solution at 65 ℃ for 5 minutes., and repeat step 5.
   - Or add **200 µL Chloroform** (37 ℃), votex for 10s, and repeat step 5.

6. Carefully transfer the upper clear layer solution to a 2 mL tube.

7. Precipitate plasmid DNA with **0.1 volume** of **3 M KAc (pH 5.2)** and **0.7 volume** of **Isopropanol**. Centrifuge at 12,000 x g for 10 minutes. Carefully decant.

8. Add **1 mL 70% ethanol** and centrifuge at 12,000 x g for 5 minutes. Carefully decant and air-dry the DNA for 30 minutes in a hood.

9. Resuspend the DNA with **Endofree Elution Buffer**.

   Note: The DNA is ready for downstream applications such as cloning/subcloning, RFLP, Library screening, *in vitro* translation, sequencing, transfection, and microinjection.

   DNA concentration (µg/mL) = $OD_{260\ nm}$ x 50 x dilution factor.

Purification of Low-Copy-Number Plasmid/Cosmid

The yield of low copy number plasmid is normally around 0.1 – 1 µg / mL of overnight culture. For isolating low copy number or medium copy number plasmid DNA, use the following guideline:

1. Culture volume: Use **2 x volumes** of the **high copy number** culture. Use up to 100 mL for midipreps.

2. Use **2 x volumes** of the **Buffer A1**, **Buffer B1**, **Buffer N3** and **100% ethanol**. Additional buffers can be purchased from Biomiga.

3. Use **same volumes** of **Wash Buffer (70% ethanol)** and **Endofree Elution Buffer**..

无内毒素质粒中提过滤法 I 型简明步骤（PD1416）

（详细内容请参考说明书英文部分）

I. 实验前准备

RNase A:室温下可稳定保存半年，使用前将提供的所有RNase A瞬时离心后加入Buffer A1, 使用后将Buffer A1/RNase A置于4°C保存。

Buffer B1: 在低于室温时会沉淀，请于50°C左右水浴加热至沉淀完全溶解，溶液澄清，使用后保证Buffer B1瓶盖旋紧。

准备70%和100%的乙醇。在室温下（22-25°C）进行所有离心操作。

II. 注意事项

质粒拷贝数：纯化中低拷贝的质粒时，使用2倍的菌液体积，2倍的Buffer A1,B1,N3,100%乙醇，相同体积的70% 乙醇和Endofree Elution Buffer..

转化菌: 若为-70°C甘油冻存的菌，请先涂布平板培养后，再重新挑选新的单个菌落进行培养。切勿直接取冻存在4°C的菌进行培养。

柱结合能力：250 µg。

对富含内源核酸酶的宿主菌（endA＋）如HB101, JM101, TG1等，需去核酸酶，请使用产品PD1712。

III. 操作步骤 （在提取质粒过程中去除内毒素）

1. 取50 µL新鲜的菌液接种到**15-50 mL** (勿超过 50 mL)的LB培养基（含适量抗生素），37°C震荡培养14-16小时。室温下5,000 x g离心10分钟，收集菌体，并尽可能的吸去上清。

   注：残留的液体培养基容易导致菌液裂解不充分，离心后沉淀较松，不能有效吸取上清。

   注：本说明书中的操作程序适用于标准 LB （Luria Bertani）培养基培养 12-16 小时后，OD_{600}（细菌密度）在 2.0-3.0 之间的菌液。若采用的是富集培养基，例如 TB 或 2×YT，请注意保证 OD_{600} 不超过 3.0。

2. 加入**2.5 mL Buffer A1**（确保已加入RNase A），用移液器或涡流震荡确保细菌沉淀重新悬浮。

   注：不完全悬浮易导致菌体裂解不完全，从而使产量降低。

3. 加入 **2.5 mL Buffer B1**, 轻轻地反转5-10 次以混合均匀，然后静置2-5分钟至溶液粘稠而澄清。

   注：切勿剧烈振荡。静置时间不应超过5分钟，时间过长会导致基因组DNA污染或质粒受到破坏。若溶液未清亮澄清，则表明菌体裂解不充分，应加大Buffer B1的用量或减少菌体量。

4. 加入**600 µL Buffer N3**, 立即反转5次，用手用力摇晃3-5次充分混匀，此时出现白色絮状沉淀。
5. **方法一：**将离心管转至高速离心机，在室温下14,000 x g 离心10分钟（若上清中有白色沉淀，可再次离心）小心吸取离心后的上清液至15 mL管中*（避免吸起沉淀）*。**注：**低温下RNase不工作，易有RNA污染。如果离心机转子较冷，将离心管在室温下温育10分钟后再离心。

   方法二：将裂解液转移至过滤器中，放在一个15 mL的试管上静置10分钟。管中的白色絮状沉淀浮上来，对准15 mL的管向下压，使裂解液尽可能多的通过，有些裂解液可能会残留在沉淀中。**注：**静置10 分钟时RNase A将工作，排除RNA污染。
6. 定量吸取离心后的上清液至新的15 mL管中（避免吸起沉淀），加入**0.1 倍体积**的**EndoClean Buffer**，混匀后冰浴10分钟，其间不时摇匀(若**EndoClean Buffer**粘稠难吸，可将枪头剪掉头后再吸取)。

   注：加入**EndoClean Buffer**后溶液变红并混浊，冰浴后变清亮。
7. 室温下(冰浴取出后务必使溶液温度恢复到23°C以上，否则溶液不分层)13,000 x g离心10分钟（也可在2,500 x g离心15 min)。此时溶液分为两层，上层水相含有质粒，下层红色有机相含有内毒素。
   注：若分层不明显，则是因为温度不够，可65°C温浴5分钟，再在13,000 x g离心10分钟。或者加入经温育后的**200 µL Chloroform** (>37°C)，混匀后再重复步骤7。**注：**此时可去除99%的无内毒，再重复第6-7步可使内毒素含量低于0.1 EU (Endotoxin)/ µg DNA.
8. 将上层水相转移至一个新的15 mL管中，加入**3 mL** 的**Buffer N3**及**3 mL**的**100% ethonal**，用手用力甩5次以混匀，该混合溶液需要需马上进行过柱吸附操作。
9. 立即转移**6.0 mL**裂解液至带收集管的DNA柱中，室温下> 2,500 x g 离心1分钟，倒掉收集管中的废液，将离心柱重新放回到收集管中。重复此步直至所有的溶液通过DNA柱。
10. **可选:** 向DNA柱中加入 **3.0 mL Buffer KB**, 室温下> 2,500 x g 离心1分钟，倒掉收集管中的废液，将离心柱重新放回到收集管中。
    注：此步对富含内源核酸酶的宿主菌（*endA*＋）来说是必须的，如HB101, JM101, TG1等；对*endA*-来说可省略，如Top 10和DH5a等，请参照说明书第3页表2.
11. 向离心柱中加入**5 mL 70% 乙醇**，室温下> 2,500 x g离心1分钟，倒掉收集管中的废液，将离心柱重新放回到收集管中。重复步骤“**11**”。
12. 将离心柱放回高速离心机中，室温下> 2,500 x g开盖离心10分钟，以彻底去除残留的乙醇。

注：此步骤中开盖离心将会更有效的去除残留的乙醇，较高的转速有利于有效的去除乙醇。乙醇是否去除干净将会影响最后的洗脱效率。

13. 将离心柱转至一个新的15 mL离心管中，向DNA柱膜的正中加入**0.5 mL**的**Endofree Elution Buffer**，室温放置1分钟，> 2,500 x g 离心5分钟，以洗脱质粒DNA。若想提高得率，可用洗脱得到的**0.5 mL**的**Endofree Elution Buffer**。再洗脱一次，收集到新的离心管中。
注：提取到的质粒DNA若用于转染内毒素敏感性细胞株，原代细胞及用于微注射，建议去除内毒素。

Trouble Shooting Guide

Problems	Possible Reasons	Suggested Improvements
Low Yield	Poor Cell lysis.	• Resuspend pellet thoroughly by votexing and pipetting prior adding Buffer B1. • Make fresh Buffer B1 if the cap had not been closed tightly. (Buffer B1: 0.2N NaOH and 1%SDS).
Low Yield	Bacterial culture overgrown or not fresh.	Grow bacterial 12-16 hours. Spin down cultures and store the pellet at -20 ℃. if the culture is not purified the same day. Do not store culture at 4 ℃ over night.
Low Yield	Low copy-number plasmid.	Increase culture volume and increase the volume of Buffer A1, B1, N3, and 100% ethanol according to instructions on page 9.
No DNA	Plasmid lost in Host *E.coli*	Prepare fresh culture.
Genomic DNA contamination	Over-time incubation after adding Buffer B1.	Do not vortex or mix aggressively after adding buffer B1. Do not incubate more than 5 minutes after adding Buffer B1.
RNA contamination	RNase A not added to Buffer A1.	Add RNase A to Buffer A1.
Plasmid DNA floats out of wells while running in agarose gel, DNA doesn't freeze or smell of ethanol	Ethanol traces not completely removed from column.	Make sure that no ethanol residual remaining in the silicon membrane before eluting the plasmid DNA. Re-centrifuge or vacuum again if necessary.

***FOR RESEARCH USE ONLY.**