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Introduction

The E.Z.N.A.™ Endo-Free BAC/PAC DNA Isolation Kit is designed for rapid high-throughput purification of BACs, PACs, and P1s from small volume of bacterial cultures. It is based on a modified alkaline lysis procedure which has specially adapted for spin-column and high-throughput family of procedures. The procedure has been developed and tested using a variety of low copy cosmids, BACs, PACs, P1s , and *E. coli* strains. This kit can also be used for high copy plasmid isolation . Two protocols are provided in this handbook, one for preparation of low-copy cosmid, BACs, PACs, and P1s and another protocol for high-copy plasmids and cosmids.

The E.Z.N.A.™ Endo-Free BAC/PAC DNA Isolation Kit provided a fast, simple method for small scale purification of BCAs, PACs, P1s and Plasmids for use in routine molecular biology laboratory applications. E.Z.N.A.™ Endo-Free BAC/PAC DNA procedure is based on modified alkaline lysis of bacterial cells, followed by clearing of the lysates by filtration using spin filter column, and further purification and concentration of DNA by isopropanol precipitation. The DNA obtained is dissolved with small volume of TE buffer or water and is ready to use for most downstream applications.

Benefits

The E.Z.N.A.™ BAC/PAC DNA Kit means:

- Speed - BACs, PACs, P1s, and Plasmid DNA isolation in <60 min
- Reliability - optimized buffers guarantee pure DNA every time
- Safety - No organic extractions
- Quality - purified DNA suitable for most applications

Storage and Stability

All E.Z.N.A.™ Endo-Free BAC/PAC DNA Kit components are guaranteed for at least 12 months from the date of purchase when stored as follows: The mixture of Buffer T1/RNase A and ETR Solution at 4°C. All other material at 22-25°C.

Kit Contents

Product Number	D2157-00	D2157-01	D2157-02
Purification times	5 Preps	50 Preps	200 Preps
HiBind DNA Column	5	50	200
2 mL Collection Tubes	5	50	200
Buffer T1	5 mL	20 mL	60 mL
Buffer T2	5 mL	20 mL	60 mL
Buffer T3	5 mL	20 mL	60 mL
BAC Binding Buffer	1.5 mL	5 mL	15 mL
ETR Solution	1 mL	6 mL	20 mL
Endotoxin-Free Elution Buffer	2 mL	10 mL	20 mL
Linear Polyacrylamides	15 µL	120 µL	450µL
RNase A, Concentrate	50 µL	200 µL	600 µL
Instruction Booklet	1	1	1

Before Starting

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

Supplied By User:
Microcentrifuge capable of at least 13,000 x g.
Sterile 1.5 mL centrifuge tubes.
Sterile deionized water (or TE buffer)
96%-100% isopropanol
10-20 mL Culture tubes
70% ethanol

IMPORTANT

1. Add vial of RNase A to bottle of Buffer T1 and Store at 4°C.
2. Bacterial cultures for cosmids and BACs PACs and P1s, we strongly recommend using 2 x YT media for cultivation.
3. Prepare the BAC Binding Buffer as following:
   D2517-00: add 4.5 ml isopropanol before used.
   D2517-01: add 15 ml isopropanol before used.
   D2517-02: add 45 ml isopropanol before used.

Note: All steps must be carried out at room temperature.

1. **Isolate a single colony from a freshly streaked selective plate, and inoculate a starter culture of 2-5 ml LB or YT medium containing the appropriate selective antibiotic. Incubate for ~ 20-24 hr at 37˚C with vigorous shaking (~ 300 rpm)**. Use a flask with a volume at least 4 times the volume of the culture.

2. **Pellet 1.5-5 ml bacteria by centrifugation at 13,000 x g for 3 min at room temperature. Decant or aspirate medium and discard.**

3. **Resuspend the bacterial pellet by adding 200 µl of Buffer T1/RNase A solution, and vortexing.** Complete resuspension of cell pellet is vital for obtaining good yields.

4. **Add 200 µl of Buffer T2 and gently mix by inverting 5-10 times to obtain a clear lysate. Incubate at room temperature for 5 minutes.** Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. The lysate should appear viscous. Do not allow the lysis reaction to proceed more than 5 min. (Store Buffer T2 tightly capped when not in use).

5. **Add 200 µl of Chilled Buffer T3 and gently mix by inverting 15-20 times until a flocculent white precipitate forms. Incubate on ice for 5 minutes.** Do not vortex, as this will result in shearing.

6. **Centrifuge at ≥ 13,000 x g for 10 minutes at 4˚C.** Promptly proceed to the next step.

7. Transfer the lysate into a new 2 ml microcentrifuge tube. **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. Inverting the tube serval 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 10,000 × g for 3 minutes at 25°C. The ETR Solution will form a blue layer at the bottom of tube.

9. Carefully transfer the cleared supernatant to a new 1.5 ml tube (not supplied). Add 200 µl of BAC Binding Buffer diluted with isopropanol. Invert 3-5 times to mix throughly and incubate at room temperature for 5 min.

   Note: BAC Binding Buffer has to be diluted with isopropanol (96-100%) before use.

See the label on the bottle or page 3 for detail instructions.

10. Apply the sample to the HiBind® DNA column assembled in a 2 ml collection tube (provided).

11. Centrifuge at > 8,000 x g for 30 second at room temperature. Remove the HiBind® DNA column, discard the flow-through and re-use the collection tube for next step.

12. Place the HiBind® DNA column back into the collection tube and add 750µl of 80% ethanol. Centrifuge at > 8,000 x g for 30 seconds at room temperature. Discard the flow-through and re-use the collection tube.

13. Place the HiBind® DNA column back into the collection tube and centrifuge at maximum speed for 2 minutes to dry the column.

14. Place the HiBind® DNA column into a clean 1.5 ml centrifuge tube, apply 40 µl of Elution Buffer (10mM Tris-HCl, pH 8.5) or water onto the center of the membrane. Incubate 5 minutes at room temperature.

15. Centrifuge at maximum speed for 2 minutes to elute the DNA.

16. Store the eluted DNA at -20˚C.

Plasmid Isolation Protocol

Procedure

Before starting, we recommend you refer to page 3-4 of this booklet for important information on preparation of components and required materials.

1. **Inoculate 1.5-5 mL LB or 2 x YT medium containing appropriate selective antibiotic placed in a 10-20 mLculture flask 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. Examples of such strains include DH5α® and JM109®.

2. **Pellet bacteria by centrifugation at 10,000 x g for 1 min at room temperature.**

3. **Decant or aspirate medium and discard. To the bacterial pellet add 200 µL Buffer T1/RNase A. Resuspend cells completely** by vortexing. Complete resuspension of cell pellet is vital for obtaining good yields..

4. **Add 200 µL Buffer T2 and gently mix by inverting and rotating tube 5-10 times to obtain a cleared lysate.** Incubate at room temperature for 3-5 minutes. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. (Store Buffer T2 tightly capped when not in use.)

5. **Add 200 µLBuffer T3 and gently mix by inverting 15-20 times until a flocculent white precipitate forms.** Incubate on ice for 5 minutes.

6. **Optional: Place the tube on ice and incubate for 10 minutes.**

7. Centrifuge at ≥12,000 x g for 10 minutes at room temperature. **CAREFULLY aspirate and add the clear supernatant to a HiBind DNA Column assembled in a 2 mL centrifuge tube (provided).** Ensure that the pellet is not disturbed and that no cellular debris is carried over into the column. Centrifuge 1 min at 12,000 x g at room temperature to completely pass lysate through column.

8. **Discard HiBind DNA Column** and transfer the lysate into a new 2 ml microcentrifuge tube. **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. Inverting the tube serval times during the incubation.**

   Note: 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 12,000 × g for 3 minutes at 25°C. The ETR Solution will form a blue layer at the bottom of tube.**

10. **Carefully transfer the top aqueous phase into a new 1. 5 ml tube.** Add 2 µL of Linear Polyacrylamides (supplied) and 0.7 volume of room temperature isopropanol to the samples. Mix the sample by vortexing for 15 seconds.

11. **Centrifuge at ≥12,000 x g for 15 minutes at room temperature to pellet the DNA.** Carefully aspirate or decant the supernatant and discard, making sure not to dislodge the DNA pellet.

12. **Wash the DNA pellet with 500 µL of ice-cold 70% ethanol**. vortex at maxi speed for 15 seconds. Centrifuge the tube for 10 minutes to re-concentrate the DNA pellet. Discard the liquid and place inverted microfuge tube on a paper towel for 10-15 min to air dry the DNA pellet.

    Note: Ensure that no alcohol droplets are visible after air drying, but do not over dry the DNA pellet because this will make the pellet difficult to redissolve.

13. **Re-dissolve the DNA pellet in 30 µL Endotoxin-Free Elution Buffer by vortexing at maxi speed.**

14. **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

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 5 mL (with high copy plasmids or 10 mL with low copy plasmids) culture with the basic protocol. Cells may not be dispersed adequately prior to addition of Buffer T2 Vortex cell suspension to completely disperse. Increase incubation time with Buffer T2 to obtain a clear lysate. Buffer T 2 if not tightly closed, may need to be replaced. Prepare as follows: 0.2 N NaOH, 1% SDS.
	Bacterial clone is not fresh.	Use fresh glycerol cultures and avoid repeated freeze/thaw cycles of clones. Always make enough replica plates and use precultures for inoculation. The reminder of the precultures can be use to set up fresh glycerol stocks.
No DNA eluted.	Lysate prepared incorrectly.	Check the stock of buffers and age of the buffers. Make sure the correct volume of buffer added to the samples.
	Buffer T2 precipitated	Warm up the Buffer T2 to dissolve the precipitate.
	Cells are not resuspended completely.	Pelleted cells should be completely resuspended with Buffer T1. Do not add Buffer T2 until an even cell suspension are is obtained.
High molecular weight DNA contamination of product.	Over mixing of cell lysate upon addition of Buffer T2	Do not vortex or mix aggressively after adding Buffer T2. Adequate mixing is obtained by simply inverting and rotating tube to cover walls with viscous lysate. Reduce the culture volume if lysate is too viscous for gentle mixing.
	Culture overgrown	overgrown culture contains lysed cells and degraded DNA. Do not grow cell for longer than 16 hours
DNA degraded after the storage	high level of Endonuclease activity	Perform the heat inactivation step.
RNA visible on agarose gel.	RNase A not added to Buffer T2	Add 1 vial of RNase to each bottle of Buffer T2
DNA floats out of well while loading agarose gel	Ethanol not completely removed	Air dry the DNA pellet before redissolve the DNA .