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Introduction

The E.Z.N.A.® family of products is an innovative system that radically simplifies extraction and purification of nucleic acids from a variety of sources. Key to the system is 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 96® Fastfilter BAC/PAC DNA Isolation Kit is designed for rapid high-throughput purification of BACs, PACs, and P1s from small volume of *E. Coli.* bacterial cultures grown and processed in a 96-well plate format. Process may be performed by using either a centrifugation or full vacuum protocol. One 96-well plate can be processed manually within 60 minutes and two plate in less than 80 minutes. 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.

The E-Z 96® Fastfilter BAC/PAC DNA Isolation procedure is based on modified alkaline lysis procedure in which the bacterial cells are lysed in the presence of RNase A, After the naturalization state, the cell lysates are cleared by filtration using E-Z 96 Lysate Clearance Plate. The liberated BAC, PAC, P1, or plasmid is trapped on to the membrane in the E-Z 96® DNA Plate with a propriety technology. After two quick wash step, the purified BAC, PAC or plasmid DNA can be eluted with low Elution Buffer or water. The eluted BAC, PAC or plasmid can be directly used in downstream applications.

Storage and Stability

All E-Z 96® Fastfilter BAC/PAC DNA Kit components are guaranteed for at least 12 months from the date of purchase when stored as follows: The mixture of T1/Rnase A at 4oC; all other material at 22-25oC. Store Solution II tightly capped.

Kit Contents

Product Number	D1055-00	D1055-01	D1055-02
Purification times	1 x 96	4 x 96	20 x 96 Preps
E-Z 96® DNA Plate	1	4	20
Deep Well Collection Plate (2.0 mL)	1	4	20
Collection Plate (500µl)	1	4	20
Sealing film	3	12	60
E-Z 96® Lysate Clearance Plate	1	4	20
Buffer T1	45mL	180 mL	900mL
Buffer T2	45 mL	180 mL	900 mL
Buffer T3	45 mL	180 mL	900 mL
BAC Binding Buffer	15 mL	50 mL	250 mL
SPM Wash Buffer	60 mL	300 mL	2 x 300 mL
Elution Buffer	30 mL	120 mL	500 mL
RNase A Concentrate	100 µL	600 µL	2.4 mL
Instruction Booklet	1	1	1

* The 2mL 96-well collection plates are reusable. See page 7 for detailed instructions.

Before Starting

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

- Add vial of RNase A to bottle of T1 and Store at 4°C.
- **BAC Binding Buffer** has to be diluted with isopropanol (96%-100%) as follows:

  D1055-00 Add 45 ml isopropanol (96-100%) to the bottle

  D1055-01 Add 150 ml isopropanol (96-100%) to the bottle

  D1055-02 Add 750 ml isopropanol (96-100%) to the bottle

- **SPM Wash Buffer** has to be diluted with absolute ethanol (~96-100%) as follows.

  D1055-00 Add 140 mL ~96%-100% ethanol

  D1055-01 Add 700 mL ~96%-100% ethanol

  D1055-02 Add 700 mL ~96%-100% ethanol per bottle

Store the diluted SPM Wash Buffer and BAC Binding at room temperature !

Vacuum Manifold Protocol

Materials Supplied By User

- Centrifuge with swinging-bucket rotor at room temperature capable of 4000 x g
- Adapter for 96-well collection plate
- 2.2 ml 96 deep well plate for culture bacteria
- Vacuum pump or vacuum aspirator capable of achieving a vacuum of 20-24 inches Hg
- Standard vacuum manifold (i.e: Omega Product #VAC-03)
- Vacuum oven or incubator preset to 70°C
- Isopropanol (96-100%)
- Absolute ethanol (96-100%)

1. Culture Volume: **Innoculate 1.0-1.2 mL LB or YT medium with proper antibiotic(s) placed in a 96-well 2 mL culture block with *E.coli* carrying desired BAC, PAC or P1 and grow at 37°C with agitation (180-300 rpm) for 20-24 h**.

2. Seal the plate with sealing film and pellet bacteria by **centrifugation at 2,000 x g for 10 minutes** in a swinging-bucket rotor at room temperature.

3. **Remove the sealing film and discard supernatant into a waste container. Dry the plate by tapping the inverted block firmly a paper towel to remove excess media. Add 200 µl T1/Rnase A to the bacterial pellet in each well of the plate**. Resuspend cells completely by vortexing and/or pipetting. No cell clumps should be visible after resuspension of the pellet. Complete resuspension of cell pellet is vital for obtaining good DNA yields.

4. **Add 200 µl T2 into each well and mix sample throughly by gently shaking and rotating the plate for 1 minute to obtain a cleared lysate.** A 2-3 min incubation at room temperature may be necessary. The solution should become viscous and slightly clear. Avoid vigorous mixing as doing so will shear chromosomal DNA and cause lower purity on purified BAC, PAC, P1 DNA. (Store T2 tightly capped when not in use.)

5. **Add 200 µl T3 to each well.** Dry the top of the plate with a paper tower. Seal the plate with another sealing film and mix by gently inverting the plate for 5-6 times until a flocculent white precipitate forms.

6. **Assemble the vacuum manifold**: 1). Place E-Z 96® Lysate Clearance Plate in the top plate of manifold; 2). Place one Deep Well Collection plate (supplied) inside the manifold base; 3). Place the E-Z 96® DNA Plate into the plate holder. 4). Place the top plate of manifold over the base, the Deep Collection Plate now should be positioned under the E-Z 96® Lysate Clearance Plate. Seal the unused wells of E-Z 96® Lysate Clearance Plate with sealing film.

7. **Carefully peel off the sealing film and transfer the lysates from the culture plate into the wells of E-Z 96® Lysate Clearance Plate.** Allow the lysate to stand for 5 minutes. The white precipitate should float to the top.

8. **Turn on the vacuum pump and apply the vacuum by switching vacuum valve to the opening position. It may be necessary to apply light pressure to the top of**

the plate by pressing down to engage the vacuum. Vacuum until all the liquid passes through the membrane of E-Z 96 Lysate Clearance Plate.

9. **Turn off the vacuum by slowly turn the vacuum valve to the close position. Allow the vacuum manifold pressure to equalize to ambient pressure.**

   Note: If some wells of the Lysate Clearance Plate clog, cover plate with a sealing film while the vacuum is been drawn. Cover the plate with sealing film will increase the vacuum pressure. Some appearance of the white flocculent material in wells is normal and should not mistaken for clogging. No more than 5 minutes should be spent in trying to get clogged wells to clear through the Lysate Clearance Plate.

10. **Discard the E-Z 96® Lysate Clearance Plate. Remove the Deep Well Collection Plate which contains cleared lysate from the vacuum manifold. Add 200µl of BAC Binding Buffer to each well of the cleared lysate. Seal the Deep Well Collection plate with sealing film. Mix the sample throughly by pipetting or inverting the plate 4 times**.

11. **Place a waste collection tray inside the vacuum manifold and place top plate over the base. Making sure that the E-Z 96® DNA Plate is seated securely.**

12. **Transfer entire sample from the Deep well plate to the E-Z 96 DNA plate.**

13. **Turn on the vacuum pump and apply the vacuum by switching vacuum valve to the opening position. It may be necessary to apply light pressure to the top of the plate by pressing down to engage the vacuum. Vacuum until all the liquid passes through the membrane of E-Z 96 DNA Plate.**

14. **Turn off the vacuum, wash the wells by adding 750µl SPM Wash Buffer diluted with absolute ethanol to each well of the E-Z 96 DNA® Plate. Apply the vacuum until all liquid passes through.**

15. **Turn off the vacuum, wash the wells by adding 750µl SPM Wash Buffer diluted with absolute ethanol to each well of the E-Z 96 DNA® Plate. Apply the vacuum until all liquid passes through.**

16. **After the all liquid pass through the membrane, dry the membrane by applying maximum vacuum for another 15-12 minutes**.

17. **Remove the E-Z 96® DNA Plate from the vacuum manifold, then vigorously tap the plate on a stack of absorbent paper towels until no drops come out. Remove any residual moisture from the tip ends of the DNA plate with clean absorbent paper towels.**

18. **Place the E-Z 96® DNA Plate back to the vacuum manifold and apply the maximum vacuum for another 5 minutes. This step will ensure to evaporate any remaining ethanol from membrane.**

19. **Elution with provided microplate:** Assemble the vacuum manifold by place a new 500µl collection plate (provided) inside the base of manifold. If Omega manifold (Vac-03) is used in this procedure, a used E-Z 96® DNA Plate or a 800 µl plate should be placed under the 500 µl collection plate as a support to give the collection plate a proper position.

20. **Add 75 µl Elution Buffer (10mM Tris-HCl, pH 8.5) or sterile water** to the center of well of the E-Z 96® DNA Plate, let stand for 5 minutes. Apply maximum vacuum for5-10 minutes to elute DNA from the plate. Turn off the vacuum and ventilate the manifold slowly.

21. Remove the collection plate containing eluted DNA and seal with film. Store sample at -20°C.

    Note: When increased yield and concentration is required, use the optional elution method on page 10.

Centrifugation Protocol

Materials Supplied By User

- Centrifuge with swinging-bucket rotor at room temperature capable of 3000 x g (such as Eppendorf 5810 with MTP rotor.)
- Adapter for 96-well collection plate
- Deep well culture plates for bacterial culture
- Absolute (96%-100%) ethanol
- Vacuum oven or incubator preset to 70°C

1. **Culture Volume: Innoculate 1.0-1.2 mL LB or YT medium with proper antibiotic(s) placed in a 96-well 2 mL culture block with *E.coli* carrying desired BAC, PAC, P1 and grow at 37°C with agitation (180-300 rpm) for 20-24 h.**

2. **Seal the plate with sealing film and pellet bacteria by centrifugation at 2,000 x g for 5 minutes in a swinging-bucket rotor at room temperature**

3. **Remove the sealing film and discard supernatant into a waste container. Dry the plate by tapping the inverted block firmly a paper towel to remove excess media. Add 200 µl T1/Rnase A to the bacterial pellet in each well of the plate. Resuspend cells completely by vortexing and/or pipetting. No cell clumps should be visible after resuspension of the pellet. Complete resuspension of cell pellet is vital for obtaining good DNA yields.**

4. **Add 200 µl T2 into each well and mix throughly by gently shaking and rotating the plate for 1 minute to obtain a cleared lysate.** A 2-3 min incubation at room temperature may be necessary. The solution should become viscous and slightly clear. Avoid vigorous mixing as doing so will shear chromosomal DNA and cause lower purity on purified BAC, PAC, P1 DNA. (Store T2 tightly capped when not in use.)

5. **Remove the sealing film and add 200 µl T3 to each well. Dry the top of the plate with a paper tower. Seal the plate with another sealing film and mix by gently inverting the plate for 5-6 times until a flocculent white precipitate forms**.

6. **Place a new E-Z 96® Lysate Clearance Plate on top of a clean deep well plate (supplied).**

7. **Transfer the lysate into E-Z 96® Lysate Clearance Plate. Allow the cell lysate to sit for 2-3 minutes. A white precipitate should float to the top.**

8. **Place the Clearance/Collection plates in centrifuge's swing-bucket rotor with adapter for deep well plate. Centrifuge at 3000 x g for 5 minutes.**

9. **Discard the E-Z 96® Lysate Clearance Plate.**

10. **Add 200µl of BAC Binding Buffer to each well of the cleared lysate. Seal the Deep Well Collection plate with sealing film. Mix the sample throughly by pipetting or inverting the plate 4-5 times.**

11. **Place a new E-Z 96® DNA Plate on top of a 96-well deep well plate (e.g., 2 mL deep well plate supplied with kit) and transfer the cleared cell lysate into E-Z 96® DNA Plate**.

12. **Centrifuge at 3000 x g for 5 minutes.**

13. **Discard the flow-through liquid and re-use the deep well collection plate for next step**.

14. **Add 900µl SPM Wash Buffer to each well. Centrifuge at 3000 x g for 2 minutes. Discard the flow-through and re-use the collection plate for next step.**

15. **Place the E-Z 96® DNA Plate back on top of the deep well collection plate, centrifuge at 3000 x g for 5 minutes.**

16. **Remove the E-Z 96® DNA Plate, tap the plate on a stack of absorbent paper until no drops come out, clean the nozzles of the DNA Plate with clean absorbent paper.**

17. **Elution: Place the E-Z 96® DNA Plate on top of a clean 500µl collection plate (supplied) .**

18. **Add 75 µl Elution Buffer (10mM Tris-HCl, pH 8.5) or sterile water to each well of the E-Z 96® DNA Plate**. Let stand for 5 minutes.

19. **Centrifuge the E-Z 96 DNA plate/Collection Plate at 3000 x g for 10 minutes.**

20. **Centrifuge the E-Z 96 DNA plate/Collection Plate at 3000 x g for 5 minutes.**

21. **Remove the collection plate containing eluted DNA and seal with film. Store sample at -20°C**

Isolating BAC DNA From 10 mL Culture Protocol

Materials Supplied by User:

- Centrifuge Capable of 45000 x g with adapter for deep well plates
- Incubator set to 37°C
- The following protocol is developed by using centrifugation, however, a vacuum manifold can be used after step 9.
- Isopropanol
- Absolute Ethanol

1. Grow the bacterial cells overnight at 37C in 15 ml 2 x YT in a appropriate vessel

2 Transfer 2 ml of the culture into a 2.2 ml 96 deep well plate. Pellet cells by centrifuging for 15 minutes at 2500 x g in a table top centrifuge. Pour off the

supernatant and repeat 4 more times to process total of 10 ml culture.

3. Add 400µl Cell Suspension Solution (Buffer T1) to each plate well. Resuspend the bacterial pellet by pipetting and shaking.

4. Add 400µl Cell Lysis Buffer (Buffer T2) to each to each well and mix by shaking. Complete lysis is critical for yield and quality. Perform a trial run to check the cell lysis. A 3-5 minutes incubation may needed, do not incubate more than 5 minutes.

5. Add 400µl of Neutralization Buffer (Buffer T3) to each well. Seal the plate with a sealing film and vortex at maximum speed for 1 minute.

6. Centrifuge at 4500 x g for 10 minutes.

7. Place a new E-Z 96 Lysate clearance plate on top of a new 2.2 ml deep-well plate. Transfer the supernatant into a new E-Z 96 Lysate Clearance Plate. Note: After the centrifugation, visible precipitates may be still in the supernatant, those particles will be cleared after the lysate clearance plate.

8. Centrifuge at 3000 x g for 5 minutes at room temperature.

9. Discard the Lysate Clearance Plate, add 400 µl of BAC Binding Buffer into each well of the deep-well plate contains the cleared cell lysate. Mix the sample throughly by pipetting 10-20 times or inverting the plate 3-5 time. Note: It is critical to mix the BAC Binding Buffer with cell lysate throughly to obtain optimized yield.

10. Transfer the Cell Lysate into a new E-Z 96 DNA Plate pre-sit on top of a 2.2 ml deep well pate. (A used 2.2 ml plate from previous step can be used at this step)

11. Centrifuge at 2500 x g for 5 minutes

12. Add 800 µl DNA Wash Buffer into each well and centrifuge at 2500 x g for 5 minutes. Discard the flow-through and reused the deep-well plate.

13. Wash the plate with another 800 µl DNA Wash Buffer. Discard the flow-through and reused the deep-well plate.

14. Centrifuge the empty plate for 1o minutes at maximum speed (4000 x g) at room temperature to dry the membrane of E-Z 96 DNA plate.

15. Add 150-200 µl Elution Buffer to each well, incubate at 37°C for 10 minutes.

16. Place the E-Z 96 DNA plate onto a new 96-well collection plate, centrifuge at maxim speed (4000 x g) for 5 minutes to elute DNA.

Alternative Elution Method

The following optional elution method can be used when higher yield and concentration are required. A additional drying step is required following elution to reduce the final volume of elute and concentrate the DNA. Start this procedure after the drying step (step 18 of the standard protocols).

1. **Add 100µl of the Elution Buffer to each well of the E-Z 96 DNA plate. Ensure that the Elution Buffer was applied on the center of the membrane. Do not apply the Elution Buffer on the side of the well.**

2. **Incubate for 5 minutes.**

3. **Turn on the vacuum pump and apply the vacuum by switching vacuum valve to the opening position. It may be necessary to apply light pressure to the top of the plate by pressing down to engage the vacuum. Vacuum until all the liquid passes through the membrane of E-Z 96 DNA Plate.**

4. **Turn off the vacuum by slowly turn the vacuum valve to the close position. Allow the vacuum manifold pressure to equalize to ambient pressure.**

5. **Add a second 100 µl Elution Buffer (10mM Tris-HCl, pH 8.5) or sterile water to each the center of well of the E-Z 96® DNA Plate, let stand for 5 minutes. Apply maximum vacuum for 5-10 minutes to elute DNA from the plate. Turn off the vacuum and ventilate the manifold slowly. Allow the vacuum manifold pressure to equalize to ambient pressure.**

6. **Dry the eluted DNA sample in the Collection plate for 2-3 hours at 60°C in a vacuum chamber. If the vacuum chamber is not accessible, the sample can be dried down in a incubate at 37°C for overnight.**

Cleaning of 2mL 96-Well Collection Plates

The 2mL 96-well collection plates are reusable. To avoid cross-contamination, rinse the plates throughly with tap water after each user. Rinse with 0.5M HCl for 5minutes, then rinse throughly with distilled water. 2mL 96-well collection plates can also be autoclaved after washing.

Troubleshooting Guide

Please use this guide to solve any problems that may arise. We hope that it will aid in

clearing up any questions for you. If for any reason you need further assistance, please contact our technical support staff (see contact information on page 16).

Possible Causes and Suggestions

Low DNA yields	
■ Poor cell lysis or cell growth Condition	Only use LB or YT medium to achieve the maximum yield with the growth condition outline in the protocol. If other media are used, the growth time may have to be adjusted
	Cells may not have been dispersed adequately prior to the addition of T2. Make sure to vortex cell suspension to completely disperse.
■ Bacterial clone is overgrown or not fresh.	Increase incubation time with T2 to obtain a clear lysate. T2 if not tightly closed, may need to be replaced. Prepare as follows: 0.2 N NaOH, 1% SDS. Do not incubate cultures for more than 26 hrs at 37° C. Storage of cultures for extended periods prior to DNA isolation is detrimental.
■ Low elution efficiency	The pH of Elution Buffer or water must be ≥8.0
■ Poor BAC PAC DNA replication during host cell growth	Some BAC or PAC clones do not replicate efficiently within their host cells. Even with lower yield, it is still very possible to obtain good sequencing reading.

■ Elution Buffer is added improperly to the membrane	Apply the Elution Buffer to the center of the membrane. Do not apply Elution Buffer to the side of wells which will cause poor elution.
No DNA Eluted	
■ SPM Wash Buffer is not diluted with 96-100% ETOH.	Prepare SPM Wash Buffer according to instructions on page 4.
High molecular weight DNA contamination of product.	
■ Over mixing of cell lysate upon addition of T2.	Do not vortex or mix aggressively after adding T2.
RNA visible on agarose gel	
■ RNase A not added to T1.	Check that RNase A provided with the kit has been used. If T1 is more than 6 months old, add more RNase A.
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
Poor DNA sequencing	

Poor cell growth condition	Proper cultivation of cell clone is essential to obtain optimal good quality of template for DNA sequencing. Use proper inoculation and growth condition to grow the bacteria clone.
Poor sequencing primer design	Re-design the sequencing primers
Ethanol contamination in the eluted DNA sample	Completely dry the E-Z 96 DNA plate before the elution. If the Eluted DNA contains ethanol residue from the SPM Wash Buffer, it could interfere the cycle sequencing reactions, resulting in short sequencing read length. Longer drying time (by either centrifugation and vacuum) may be necessary. Remove any liquid residue from drips and inside of each wells.