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Product Information

PhasePrep™ BAC DNA Kit

Catalog Number **NA0100**

TECHNICAL BULLETIN

Product Description

The PhasePrep BAC DNA Kit offers a scalable and cost-effective method for isolating large-molecular weight plasmids such as Bacterial Artificial Chromosomes (BAC) from recombinant *E. coli* cultures. The same kit can be used for preparations of four different sizes; sufficient reagents are provided for 300 micro, 180 mini, 30 midi, or 15 maxi preparations. Up to 2, 12, 50, or 100 µg of BAC DNA can be recovered from 5, 40, 250, or 500 ml of overnight recombinant *E. coli* culture, respectively. The purified BAC DNA contains very low levels of endotoxin (≤10 EU/µg DNA).

Recombinant *E. coli* culture is harvested by centrifugation and subjected to a modified alkaline-SDS lysis procedure. Nucleic acid is precipitated from the cleared lysate; residual RNA is removed by a short digestion at elevated temperature with an RNase cocktail. Endotoxins and other impurities are removed by simple temperature-mediated extraction and phase separation. Finally the BAC DNA is selectively precipitated from solution. The recovered BAC DNA is predominantly in its super-coiled form, free of RNA contamination, and ready for immediate use in downstream applications, such as sequencing, restriction digestion, cloning, and PCR†.

Reagents Provided (Sufficient for 300 micro, 180 mini, 30 midi, or 15 maxi preparations)	**Catalog Number**	**Quantity**
Resuspension Solution	R1149	2 x 200 ml
RNase A Solution (20 mg/ml)	R6148	2 x 1.5 ml
Lysis Solution	L1912	2 x 200 ml
Neutralization Solution	N4659	2 x 200 ml
Elution Solution	E5650	200 ml
RNase Cocktail	R2151	250 µl
Endotoxin Removal Solution	E4274	60 ml
Sodium Acetate Buffer Solution, 3 M, pH 7.0	S2404	25 ml
DNA Precipitation Solution	D4564	200 ml

Storage

Store the kit at room temperature. Store the RNase Cocktail at –20 °C after opening the vial.

Equipment and reagents required, but not provided

- Ethanol, 190 proof, Catalog Number E7148; 200 proof, Catalog Number E7023; or anhydrous, Catalog Number 459836
- 2- Propanol, Catalog Number I9516
- Centrifuge, capable of 15,000 x *g*, at 2-8 °C
- Centrifuge tubes: 15 ml conical, Catalog Number C3048; 50 ml Oak Ridge, Catalog Number T2918
- Centrifuge bottles (250 ml and 500 ml), Catalog Numbers B1908 and B2033
- Microcentrifuge
- Microcentrifuge tubes (1.5 ml and 2.0 ml), Catalog Numbers T9661 and T2795
- 37 °C and 60 °C water baths

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.
Wear gloves, safety glasses, and suitable protective clothing when handling any reagent provided in the kit.

Preparation Instructions

- **RNase A/Resuspension Solution:** Briefly centrifuge the tube containing the RNase A Solution. Add the RNase A Solution to the Resuspension Solution (one vial of RNase A Solution per bottle of Resuspension Solution), and mix thoroughly before use.
  Store the RNase A/Resuspension Solution at 2-8 °C.
- **Lysis Solution:** If the SDS in the Lysis Solution precipitates during storage, heat the solution at 37 °C until the SDS goes back into solution. Mix well and cool to room temperature before use.
- **Neutralization Solution:** Chill on ice before use.
- **Endotoxin Removal Solution:** Mix briefly and chill on ice before use.

Procedures

Micro Scale Preparation

1. **Harvest cells.** Pellet 3-5 ml of an overnight culture at 3,000-5,000 x *g* for 5-10 minutes. For best results, use a cell mass of 18 (cell mass = OD_{600} x ml of culture; volume to use is equal to 18 divided by the absorbance of the culture at 600 nm). Remove the entire medium supernatant.

Note: The PhasePrep BAC DNA Kit is optimized for use with cultures grown in standard LB medium. For best results, inoculate the culture with a colony from a freshly streaked plate; grow the culture to OD_{600} = 3-4. **Do not exceed a cell mass of 20.**

2. **Resuspend cells.**
   Add 250 µl of RNase A/Resuspension Solution to the bacterial cell pellet. Completely resuspend the bacterial pellet by pipetting up and down. Transfer the resuspended cells into a 1.5 ml microcentrifuge tube.

3. **Cell Lysis** Add 250 µl of Lysis Solution to the resuspended cells. Mix immediately by gently inverting the tube 4-6 times and incubate at room temperature for 5 minutes. **Do not vortex. Do not allow the lysis reaction to exceed 5 minutes.** Harsh mixing or prolonged lysis will shear the genomic DNA, resulting in genomic DNA contamination in the final preparation.

4. **Neutralization** Add 250 µl of chilled Neutralization Solution to the lysate. Mix immediately and thoroughly by gently inverting the tube 6-8 times. **Do not vortex or shake**, as this will result in shearing the DNA. Incubate the tube on ice for 5 minutes. Centrifuge at maximum speed (~16,000 x *g*) for 5 minutes at 2-8 °C to pellet the cell debris. Carefully transfer the cleared lysate (supernatant) with a pipette tip to a clean microcentrifuge tube.

Notes:
a. To avoid shearing the BAC DNA, use large orifice pipette tips.
b. To avoid taking floating particulates while transferring the supernatant, position the tip below the surface of the supernatant before aspirating.

5. **Nucleic acid precipitation.** Add 450 µl of 2-propanol at room temperature to the cleared lysate. Mix thoroughly by gentle inversion and centrifuge at maximum speed for 20 minutes at 2-8 °C. Remove the supernatant and wash the pellet with 100 µl of 70% ethanol at room temperature. Centrifuge at maximum speed for 5 minutes at 2-8 °C, remove the supernatant by pipetting, and air dry the pellet briefly (5 minutes).

6. **Residual RNA digestion** Add 500 µl of Elution Solution to resuspend the pellet. Dilute the RNase Cocktail 10-fold by mixing 1 µl of the cocktail with 9 µl of Elution Solution. Add 1 µl of the diluted cocktail to the tube, mix briefly, and incubate in a 60 °C water bath for 5 minutes. The diluted RNase cocktail is stable for at least 3 months at 2-8 °C.

7. **Adjust salt concentration**. After digestion, centrifuge the tube briefly (1 minute) to collect the liquid. Transfer the solution to a 1.5 ml microcentrifuge tube. Add 40 µl of Sodium Acetate Buffer Solution to the tube and mix briefly.

   Note: Large orifice tips are recommended for pipetting the viscous Endotoxin Removal Solution in the next step.

8. **Removal of endotoxins and other impurities.**
   a. Add 100 µl of Endotoxin Removal Solution and mix thoroughly by inversion. Chill the tube on ice for ≥ 5 minutes. Mix 1-2 times during the incubation. The solution will become light blue and clear.
   b. Warm the tube in a 37 °C water bath for 5 minutes. The solution will become cloudy. Centrifuge at maximum speed for 3 minutes at room temperature to separate the phases. The clear upper phase contains the BAC DNA. The blue lower phase contains endotoxins and other impurities.
   c. Carefully transfer the clear upper phase into a new 1.5 ml microcentrifuge tube. Discard the blue lower phase.
   d. **Optional**: Repeat steps a, b, and c, then continue to step 9.

9. **DNA Precipitation** Add 540 µl of room temperature DNA Precipitation Solution. Mix thoroughly by gentle inversion and centrifuge at maximum speed for 20 minutes at 2-8 °C. Remove the supernatant and wash the pellet with 150 µl of room temperature 70% ethanol. Centrifuge again for 5 minutes and pipette off the supernatant. Repeat the wash with 50 µl of room temperature 70% ethanol. Remove all of the supernatant and air-dry the pellet briefly (5 minutes). Dissolve the DNA in 20-25 µl of sterile deionized water (for sequencing) or Elution Solution. Rinse DNA from the side of the tube with gentle tapping. Use approximately one third of the preparation for a single sequencing reaction or restriction digestion.

Note: DNA aggregates may not be visible during the precipitation. After centrifugation the pellet may be loosely attached to the side of the centrifuge tube. Mark the tube for orientation relative to the centrifugal force prior to centrifugation. Carefully pipette off the supernatant from the side opposite the pellet to avoid the risk of aspirating the pellet. Dissolve the DNA overnight in a refrigerator before quantitation.

Mini Scale Preparation

1. **Harvest cells.** Transfer 30-40 ml of an overnight culture to an Oak Ridge style centrifuge tube (capable of ≥15,000 x *g*) and centrifuge at 4,000-5,000 x *g* for 10 minutes. For best results, use a cell mass of 140 (see description of cell mass in the Micro Scale Preparation procedure). Remove all of the medium supernatant.

Note: This kit is optimized for use with cultures grown in standard LB medium. For best results, inoculate the culture with a colony from a freshly streaked plate. Grow the culture to OD_{600} = 3-4. **Do not exceed a cell mass of 160.**

2. **Resuspend cells.**
   Add 2 ml of RNase A/Resuspension Solution to the bacterial cell pellet. Completely resuspend the bacterial pellet by pipetting up and down.

3. **Cell Lysis** Add 2 ml of Lysis Solution to the resuspended cells. Mix immediately by gently inverting the tube 4-6 times and incubate at room temperature for 5 minutes. **Do not vortex or shake. Do not allow the lysis reaction to exceed 5 minutes.** Harsh mixing or prolonged lysis will shear the genomic DNA, resulting in genomic DNA contamination in the final preparation.

4. **Neutralization** Add 2 ml of chilled Neutralization Solution to the lysate. Mix immediately and thoroughly by gently inverting the tube 6-8 times. **Do not vortex or shake**, as this will result in shearing the DNA. Incubate the tube on ice for 5-10 minutes. Mix again during the ice incubation by gently inverting the tube 2-3 times. Centrifuge at 15,000 x *g* for 20 minutes at 2-8 °C to pellet the cell debris. Carefully transfer the cleared lysate (supernatant) with a pipette to a clean centrifuge tube.

Notes
a. To avoid shearing the BAC DNA, use large orifice pipette tips.
b. To avoid taking floating particulates while transferring the supernatant, position the pipette below the surface of the supernatant before aspirating.

5. **Nucleic acid precipitation** Add 3.6 ml of room temperature 2-propanol to the cleared lysate. Mix thoroughly by gentle inversion and centrifuge at 15,000 x *g* for 20 minutes at 2-8 °C (mark the tube for orientation relative to the centrifugal force before centrifugation). Pour off the supernatant and wash the pellet with 2 ml of room temperature 70% ethanol. Centrifuge at 15,000 x *g* for 5 minutes at 2-8 °C and pipette off the supernatant. Use a pipette tip to remove the last drop of liquid and air-dry the pellet briefly (5 minutes).

6. **Residual RNA digestion** Add 650 µl of Elution Solution to resuspend the pellet. Rinse the pellet off the side of the tube with gentle swirling and dissolve the DNA for 5 minutes. Add 1 µl of the undiluted RNase Cocktail to the tube, mix briefly, and incubate in a 60 °C water bath for 10 minutes. Rinse the side of the tube 1-2 times with gentle swirling during digestion to dissolve any residual DNA pellet.

7. **Adjust salt concentration**. After digestion, centrifuge the tube briefly (1 minute) to collect the liquid. Transfer the solution to a 1.5 ml microcentrifuge tube. Add 50 µl of Sodium Acetate Buffer Solution and mix briefly.

   Note: The DNA solution may contain a small amount of insoluble particulates which will be removed during the endotoxin removal steps. Large orifice tips are recommended for pipetting the viscous Endotoxin Removal Solution in the next step.

8. **Removal of endotoxins and other impurities.**
   a. Add 120 µl of Endotoxin Removal Solution and mix thoroughly by inversion for 30 seconds. Chill the tube on ice for 5 minutes. Mix 1-2 times during the incubation on ice. The solution will become light blue and clear.
   b. Warm the tube in a 37 °C water bath for 5 minutes. The solution will become cloudy. Centrifuge at maximum speed (~16,000 x *g*) for 3 minutes at room temperature to separate the phases. The clear upper phase contains the BAC DNA. The blue lower phase contains endotoxins and other impurities.
   c. Carefully transfer the clear upper phase into a new 1.5 ml microcentrifuge tube. Discard the blue lower phase.
   d. **Optional**: Repeat steps a, b, and c, then continue to step 9.

9. **DNA precipitation** Transfer the clear upper phase into a 2 ml microcentrifuge tube. Add 700 µl of room temperature DNA Precipitation Solution. Mix thoroughly by gentle inversion and centrifuge at maximum speed for 20 minutes at 2-8 °C. Remove the supernatant and wash the pellet with 500 µl of room temperature 70% ethanol. Centrifuge again for 5 minutes and remove the supernatant. Repeat the wash with 100 µl of 70% ethanol. Remove all of the supernatant and air-dry the pellet briefly (5 minutes). Dissolve the DNA in Elution Solution or sterile deionized water (for sequencing). Rinse the DNA off the side of the tube with gentle tapping.

   Note: DNA aggregates may not be visible during the precipitation. After centrifugation, the pellet may be loosely attached to the side of the centrifuge tube. Mark the tube for orientation relative to the centrifugal force before centrifugation. Carefully pipette off the supernatant from the side opposite the pellet to avoid the risk of aspirating the pellet. Dissolve the DNA overnight in a refrigerator before quantitation.

Midi and Maxi Scale Preparations

1. **Harvest cells.** Transfer 150-250 ml (midi) or 300-500 ml (maxi) of an overnight culture to a centrifuge bottle; centrifuge at 4,000-5,000 x *g* for 10 minutes. For best results, use a cell mass of 750 for midi or 1500 for maxi (see description of cell mass in the Micro Scale Preparation procedure). Remove all of the medium supernatant.

   Note: The PhasePrep BAC DNA Kit is optimized for use with cultures grown in standard LB medium. For best results, inoculate the culture with a colony from a freshly streaked plate, and grow the culture to OD_{600} = 3-4. **Do not exceed a cell mass of 1000 for a midi prep or 1750 for a maxi prep.**

2. **Resuspend cells.** Add 12 ml (midi) or 24 ml (maxi) of RNase A/Resuspension Solution to the bacterial cell pellet. Completely resuspend the bacterial pellet by shaking on a shaker or pipetting up and down.

3. **Cell Lysis** Add 12 ml (midi) or 24 ml (maxi) of Lysis Solution to the resuspended cells. Mix immediately by gently inverting the bottle 4-6 times and incubate at room temperature for 5 minutes. **Do not vortex or shake. Do not allow the lysis reaction to exceed 5 minutes.** Harsh mixing or prolonged lysis will shear the genomic DNA, resulting in genomic DNA contamination in the final preparation.

4. **Neutralization** Add 12 ml (midi) or 24 ml (maxi)) of chilled Neutralization Solution to the lysate. Mix immediately and thoroughly by gently inverting the bottle 8-10 times. **Do not vortex or shake**, as this will result in shearing the DNA. Incubate the bottle on ice for 10 minutes. Mix again during ice incubation by gently inverting the bottle 4-6 times. Transfer the lysate to a 250 ml centrifuge bottle (if the lysate is processed in a 500 ml bottle) and centrifuge at 15,000 x *g* for 30 minutes at 2-8 °C to pellet the cell debris. Carefully transfer the cleared lysate (supernatant) with a pipette to a 250-ml centrifuge bottle.

Notes

a. To avoid shearing the BAC DNA, use large orifice pipette tips
b. To avoid taking floating particulates while transferring the supernatant, position the pipette below the surface of the supernatant before aspirating. If a large amount of cellular debris is carried over, re-centrifuge the supernatant for 10 minutes to remove the cellular debris. Alternatively, pass the cleared lysate through a fast flow filter paper prewetted with distilled water.

5. **Nucleic acid precipitation.** Add 22 ml (midi) or 44 ml (maxi) of room temperature isopropanol to the cleared lysate. Mix thoroughly by gentle inversion and centrifuge at 15,000 x *g* for 20 minutes at 4 °C (mark the bottle for orientation relative to the centrifugal force before centrifugation). Pour off the supernatant and wash the pellet with 10 ml of room temperature 70% ethanol. Centrifuge at 15,000 x *g* for 5 minutes at 4 °C and pipette off the supernatant. Use a pipette tip to remove the last drop of liquid and air-dry the pellet briefly (5 minutes).

6. **Residual RNA digestion.** Add 5 ml (midi) or 10 ml (maxi) of Elution Solution to resuspend the pellet. Rinse the pellet off the side of the bottle with gentle swirling and dissolve the DNA for 5 minutes. Add 5 µl (midi) or 10 µl (maxi) of the undiluted RNase Cocktail to the bottle, mix briefly, and incubate in a 60 °C water bath for 15 minutes. Rinse the side of the bottle 1-2 times with gentle swirling during the digestion to dissolve any residual DNA pellet.

7. **Adjust salt concentration**. After digestion, centrifuge the bottle briefly (1 minute) to collect the liquid. Transfer the solution to a 15 ml conical tube. Add 400 µl (midi) or 800 µl (maxi) of Sodium Acetate Buffer Solution and mix briefly.

Note: The DNA solution may contain a small mount of insoluble particulates, which will be removed during the endotoxin removal steps.. Large orifice tips are also recommended for pipetting the viscous Endotoxin Removal Solution in the next step.

8. **Removal of endotoxins and other impurities.**

a. Add 0.9 ml (midi) or 1.8 ml (maxi) of Endotoxin Removal Solution and mix thoroughly by inversion for 30 seconds. Chill the tube on ice for ≥ 10 minutes. Mix 1-2 times during the incubation on ice. The solution will become light blue and clear.
b. Warm the tube in a 37 °C water bath for 5 minutes. The solution will become cloudy. Centrifuge the tube at 3,000-4,000 x *g* for 5 minutes at room temperature to separate the phases (using a swinging bucket rotor). The clear upper phase contains the BAC DNA. The blue lower phase contains endotoxins and other impurities.
c. Carefully transfer the clear upper phase into a new tube. Discard the blue lower phase.
d. **Optional**: Repeat steps a, b, and c, then continue to step 9.

9. **DNA precipitation.** Transfer the clear upper phase into a 50-ml centrifuge tube. Add 5.4 ml (midi) or 10.8 ml (maxi) of room temperature DNA Precipitation Solution. Mix thoroughly by gentle inversion and centrifuge at 15,000 x *g* for 30 minutes at 4 °C. Remove the supernatant and wash the pellet with 5 ml (midi) or 10 ml (maxi) of room temperature 70% ethanol. Centrifuge again for 5 minutes and remove the supernatant. Repeat the wash with 2 ml of 70% ethanol. Remove all of the supernatant and air-dry the pellet for 5-10 minutes. Dissolve the DNA in Elution Solution or sterile deionized water (for sequencing). Rinse the DNA off the side of the tube with gentle swirling.

   Note: DNA aggregates may not be visible during the precipitation. After centrifugation in a fixed angle rotor, the pellet may form a thin film along the centrifugal side of the tube and may be difficult to see. Mark the tube for orientation relative to the centrifugal force before centrifugation. Pipette off the supernatant from the opposite side of the pellet. Do not over-dry the pellet. Make sure to rinse any DNA off the side of the tube with gentle swirling while dissolving the DNA. Dissolve the DNA overnight in a refrigerator (tilt the tube to the side where the pellet is expected) before quantitation.

Results

Recovery and purity of BAC DNA preparations may be determined by spectrophotometric analysis. The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) should be 1.7-1.9. The size and quality of DNA may be determined by restriction digestion and agarose gel electrophoresis or pulsed-field gel electrophoresis.

References

1. Birnboim, H. C., and Doly, J., A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*., **7**, 1513-1522 (1979).
2. Birnboim, H. C., A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.*, **100**, 243-255 (1983).
3. Cotton, M. *et al*., Lipopolysaccharide is a frequent contaminant of plasmid DNA preparation and can be toxic to primary human cells in the presence of adenovirus. *Gene Therapy*, **1**, 239-246 (1994).

Related Products

- Water, Molecular Biology Reagent, Catalog Number W4502
- LB Broth, EZMix™ , Catalog Number L7658
- LB Agar, EZMix™, Catalog Number L7533
- Agarose for pulsed field electrophoresis, Catalog Number A2929
- TAE Buffer (10X), Catalog Number T9650
- TBE Buffer (10X), Catalog Number T4415
- Gel Loading Solution, Catalog Number G2526
- Lambda DNA *Hin*d III Digest, 125-23,130 bp, Catalog Number D9780
- PulseMarker™ DNA marker, 0.1-200 kb, Catalog Number D2291
- Ethidium bromide, aqueous, 10 mg/ml, Catalog Number E1510

Troubleshooting Guide

Problem	Cause	Solution
Low DNA yield	Number of cells is insufficient	Inoculate the culture with a colony from a freshly streaked plate. For midi and maxi preps, grow a starter culture for 6-8 hr and inoculate an overnight culture with the starter culture in a 500 to 1000-fold dilution. Confirm that the cells were grown under optimal conditions (37 °C, 280-300 rpm) in a vessel at least four times larger than the volume of the medium. Grow the overnight cultures to OD_{600} = 3-4.
	Culture is too old	Streak a new plate from stock.
	Antibiotic activity is insufficient	Confirm that the medium contains the appropriate selective antibiotic. Use a sufficient amount of fresh antibiotic solution for growth of overnight cultures (for chloramphenicol, use 20 µg/ml for an overnight culture). Most antibiotic solutions are light sensitive and degrade during long term storage at 2-8 °C.
	Lysis reaction is incomplete	Reduce the cell density or the culture volume, or increase volumes of Rnase A/Resuspension Solution, Lysis Solution, and Neutralization Solution proportionally. Keep the Lysis Solution bottle tightly capped between uses to prevent acidification. Check Lysis Solution for SDS precipitation; redissolve SDS by warming at 37 °C and allow to cool to room temperature before use
	Neutralization Solution and Lysate were not well mixed	After adding the pre-chilled Neutralization Solution, mix immediately but gently, by inversion until a fluffy egg-white material forms. Mix again during the incubation on ice.
	DNA pellet was lost or not rinsed off from the side of the tube	Mark the tube for orientation before centrifuging to help locate the pellet. Pipette off the supernatant from the opposite side of the pellet; avoid disrupting the pellet. Rinse the DNA off the side of the tube by gentle tapping or swirling when re-dissolving the DNA pellet.
Sheared BAC DNA	Alkaline lysis was too long	Do not allow the lysis reaction to exceed 5 minutes.
	Excessive mixing caused shearing	Mix gently by inversion during lysis and neutralization. Do not shake or vortex.
	Shearing occurred during reconstitution	Reconstitute the DNA gently. Avoid vigorous pipetting. Use large orifice pipette tips for handling the BAC DNA solution.
Poor performance in downstream applications	The final BAC DNA eluate contains too much salt or protein	Perform DNA precipitation with room temperature 2-propanol in Step 5 and room temperature DNA Precipitation Solution in Step 9. Do not perform the precipitation at –20 °C. Wash the pellet twice in the final step with room temperature 70% ethanol and remove all of the liquid with pipette tips.
	EDTA is interfering by chelating cofactors	Precipitate the desired amount of BAC DNA and reconstitute in sterile deionized water for sequencing or other enzymatic reactions.
Absorbance of the final product does not match the actual quantity of BAC DNA	BAC DNA is contaminated with RNA	Confirm that the RNase A Solution was added to the Resuspension Solution prior to first time use. Store RNase A/Resuspension Solution at 2-8 °C. RNase A may degrade due to high temperatures (> 65 °C) or prolonged storage at room temperature. Keep the RNase Cocktail at –20 °C for long term storage.
	BAC DNA is contaminated with chromosomal DNA	Do not vortex or shake during lysis or neutralization. Do not allow the lysis reaction to exceed 5 minutes. Do not use cultures that have grown for over 24 hours or are in the cell death phase. Do not freeze and thaw bacterial cell pellets.
	DNA is not completely redissolved	Be sure that the DNA is completely redissolved before quantitation.