- High Throughput
- High Quality

ALINE SequeMid™ Plasmid Prep Kit

Catalog Numbers: **S-8001-S, S-8001-M, S-8001-L**

Contents

Protocol Manual *Revision v1.1*

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Please refer to http://www.alinebiosciences.com Support section for updated protocols and MSDS when handling or shipping any chemical hazards. The information provided is subject to change without notice.

Introduction

ALINE SequeMid™ 96-well plasmid purification kit utilizes a proprietary magnetic bead based technology for high-throughput purification of plasmid DNA from *E.coli* cells. SequeMid™ can also be used for purification of fosmid and BAC vector-based constructs ranging from 5kb to 150kb. The purification process consists of steps of pelleting cells, resuspension, lysis, neutralization, centrifugation, DNA binding, ethanol washing and plasmid elution. The protocol is automation friendly for most commercially available platforms. Plasmid DNA purified with this system can be used in a variety of molecular biology applications, such as:

- DNA sequencing (including capillary sequencing and Next Gen Sequencing library construction)
- PCR amplification
- Restriction enzyme digestion
- Cloning

SequeMid™ yields approximately 500 ng – 2 µg of DNA per purification with high-copy plasmids. For low-copy plasmids and larger constructs, the average yield is about 300 ng - 1 µg. These yields vary depending on the cell line, cell culture conditions, vector type, and size of the construct.

Process Overview:

The ALINE Biosciences SequeMid™ procedure is performed in the following stages:

1 Pelleted *E.coli* cells are resuspended in R1 solution,
2 Addition of L2 solution to lyse the bacterial cultures.
3 Addition of N3 solution neutralizes the high pH and maintains DNA integrity.
4 Centrifugation to pellet cell debris.
5 Supernatant is transferred out from under the flocculent and moved into a fresh 96-well round bottom microtiter plate.
6 Addition of isopropanol and Bind4 solution bind plasmid DNA to the magnetic beads.
7 Beads are washed with 70% ethanol to remove salts and other contaminants.
8 Elution of plasmid.

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Product Specifications:

Product Number	Description	Number of Reactions	Storage Conditions
S-8001 S	ALINE SequeMid™- Small	50	See Kit Components (*Page 2*) section for the conditions of each component.
S-8001 M	ALINE SequeMid™ - Medium	384	
S-8001 L	ALINE SequeMid™- Large	3840	

Materials Supplied in the SequeMid™ Kit:

ALINE SequeMid™ reagents have a shelf life of 6 months if stored as instructed.

Kit Components:

- **SequeMid™ R1 Solution** - Resuspension and elution solution

  Store at 4°C. R1 solution contains RNase activity.
- **SequeMid™ L2 Solution** - Lysis solution
  Store at room temperature. (Or at 37°C to prevent precipitation in colder labs.)
  If white precipitate forms due to low temperature, then warm the solution and shake it to ensure precipitates are completely dissolved before use.

- **SequeMid™ N3 Solution -** Neutralization solution
  Store at room temperature.

- **SequeMid™ Bind4 Solution –** Plasmid bind solution
  Store at 4°C. DO NOT FREEZE.
  Shake well to resuspend magnetic particles before using. This solution should appear homogenous.

Materials Supplied by the User:

Consumables and Hardware:

For 96 well format: 96 well ring stand

• Source Plate: 96-well 2.2 mL deep well culture block (ABGene/Marsh # DW9622

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http://www.abgene.com/) for BACs

OR 96-well 1.1 mL culture block (ABGene/Marsh # DW9611 http://www.abgene.com/) for plasmids/fosmids (see individual protocols for details)

- Destination Plate:
  - 300 µL round bottom microtiter plate (96-well (300 µL well capacity) round bottom plate [Costar # 07-200-105; www.fishersci.com]
- Gas permeable seals
- Liquid handling robotics if applicable

Reagents:

- 2xYT bacterial growth media containing the appropriate antibiotic (TAE buffered to pH7)
  LB with 10% Glycerol growth media containing the appropriate antibiotic (TAE buffered to pH7)
- 100% Isopropanol
- Fresh 70% Ethanol (EtOH) **Note: Fresh 70% ethanol should be prepared and used for optimal results.**

Procedures – 96 well format

1. **Pipette 800 µL 2xYT bacterial growth media containing the appropriate antibiotic (see recommendations below) into each well of a 1.2 mL deep well culture block.**
   NOTE: High copy plasmids may be grown using only 600 µL of 2xYT. ALINE Biosciences recommends reduced antibiotic concentrations for overnight cultures as conventional concentrations can delay the propagation of cells. Final concentration in media or glycerol: Chloramphenicol 12.5 µg/mL, Ampicillin 50µg/mL, Kanamycin 35 µg/mL, Carbenicillin 50 µg/mL, Zeocin 25 µg/mL, Tetracycline 2.5 µg/mL.

   If using different media, antibiotic and/or growth parameters, increase or decrease your growth time to meet the following final cell density averages in the overnight culture:

   Plasmids in 800 µL: 1.32×10^{9} cells total

   Cosmids or Fosmids in 800 µL: 1.52×10^{9} cells total

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2. **Inoculate each well with a single *E. coli* bacterial colony.**

   NOTE: Overnight cultures can be inoculated directly from colony-containing agar lawns or from glycerol stock plates.

3. **Cover the plate with a gas permeable seal and shake at 300 rpm at 37°C. Grow plasmids for 17. 5 hours and fosmids/cosmids for 19 hours.**

   NOTE: Do not allow culture blocks to grow longer than recommended. If the plates grow too long, the cells may die and the SequeMid™ purification process will not yield optimum results.

4. **Pellet bacterial cells by centrifuging culture plates at 2500 x g for 10 minutes.**

   NOTE: The following settings for two common centrifuges and rotors amy be used:

   Beckman GH 3.8 rotor with MicroPlus carrier: 2500 x g = 3300 rpm

   Jouan P60 rotor with microplate carrier: 2500 x g = 2900 rpm

5. **Remove the seal after centrifugation and invert the block to decant the media away from the cell pellets. Blot the inverted block on a paper towel to remove excess media.**

   NOTE: Blot gently to avoid dislodging the cell pellet. At this point, pelleted blocks can be sealed and stored at -20°C or -80°C.

6. **Add 100 µL of R1 solution and thoroughly resuspend cell pellets by vortexing, shaking or pipette mixing.**
   NOTE: Pipette mixing and shaking are often used for automated processes. Pipette mix 20 times (if possible, mix 5 times at 4 different locations in the well) for an even resuspension. Aspirate and dispense 80 µL each time at a mixing speed of 150 µL/sec. Alternatively, shake 4 minutes at 600-1200 RPM on a shaker. Shaking speeds varies depending on the shaker. Vortexing on a high setting takes 2-3 minutes. The cell pellet should be completely resuspended so that there are no cell clumps and the mixture appears homogenous.

7. **Add 100 µL of L2 solution, mix gently and allow the samples to lyse for 5 minutes.**

   NOTE: Shake the plate for 5 minutes at 300-600 RPM. Alternatively, pipette mix gently 2 times then allow the samples to sit for 5 minutes. Avoid vigorous pipette mixing as large plasmids can be easily sheared. Do not lyse for longer than 10 minutes.

   If there is a white precipitate in the L2 solution prior to addition, warm the bottle in a 37°C water bath while shaking periodically until the precipitate dissolves prior to use. When

handling L2, wear gloves in case L2 may contact skin, as it is a basic solution.

8. **Add 100 µL of N3 solution, mix gently and allow the samples to neutralize for 10 minutes.**

   NOTE: Shake the samples for 10 minutes at 300-600 RPM to complete the neutralization. Alternatively, pipette mix very gently near the bottom of the plate, avoiding the flocculent at the top of the well. Wear gloves when handling N3. Addition of N3 neutralizes the solution and precipitates proteins and cellular debris, creating a white flocculent.

9. **Pellet cell debris by centrifuging culture plates at 2500 x g for 15 minutes.**

10. **Transfer 110 µL of the clear supernatant to a 300 µL round bottom microtiter plate.**

    NOTE: The supernatant should be free of flocculent for optimal results.

11. **Add 10 µL of Bind4 solution and 85 µL of 100% isopropanol. Pipette mix 10 times.**

    NOTE: Bind4 contains magnetic beads and should appear homogeneous after mixing. Shaking

    samples, instead of Pipette mixing, may result in incomplete mixing and reduced yield.

    The final concentration of isopropanol in the well should be 40%. If the initial transfer volume at step 10 is different from the specified due to robotic optimizations or limitations, the volume of isopropanol to be added can be determined using the following equation:

    Vol_Iso = 0.71 * (Vol_lysate + 10) Where:

    Vol_Iso = volume of isopropanol to be added per well

    Vol_lysate = volume of cleared lysate transferred

12. **Place the round bottom microtiter plate on a magnet and allow the beads to separate for 15 minutes.**

    NOTE: The supernatant should be clear and the beads should form a ring around the bottom of the plate. The supernatant may be slightly yellowish-brown, but should otherwise be translucent.

13. **With the plate on the magnet, aspirate the supernatant from the plate and discard.**

    NOTE: If the magnetic particles are disturbed during aspiration, more separation time may be required before removing the supernatant. The discarded solution should not

have magnetic beads.

14. **With the plate on the magnet, dispense 200 µL of fresh 70% ethanol into each well of the plate to wash the magnetic beads. Allow samples to incubate for 30 seconds, then remove and discard the ethanol wash solution. Repeat the wash twice, for a total of 3 washes.**

    NOTE: For best results make fresh 70% ethanol each day. If the concentration of the ethanol is less than 70%, some of the product may be washed away. Do not disturb the beads during the wash step as beads have the target DNA bound to them.

15. **Dry the plate at room temperature or 37°C for 10 minutes.**

    NOTE: After drying, the plates may be stored at room temperature for several days.

16. **Add 40 µL of R1 solution to each well of the plate, incubate for 5 minutes at 37°C and shake to elute samples.**

    NOTE: Vortex or shake the plate for 30 seconds after incubating the plates for 5 minutes at 37°C to fully elute the plasmid from the beads. For large DNA, such as BACs, it is helpful to let the plates sit for 5-10 minutes after vortexing to allow the large DNA extra time to dissociate from the beads. It is not necessary for the beads to go back into solution for complete elution to occur, however, it is extremely important the elution buffer completely covers the ring of beads for maximum recovery.

    R1 contains RNase and is recommended for elution. Other aqueous low-salt buffers may also be used for elution.

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Calculation of Cell Density:

ALINE Biosciences recommends quantitation of cell number based on light obsorption at 600nm on a spectrophotometer. Use a 1:20 dilution of the overnight culture to perform reading, as the concentration of cells in non-diluted culture is generally too high to obtain an accurate OD 600nm reading. Use clear media to blank the system.

The following protocol uses a 96 well half area (150 µΛ) flat bottom plate (Costar #3695, item #29444320 from http://www.vwrsp.com/).

1 Measure the total volume left in the well after overnight growth. This should be slightly less than the starting volume due to evaporation overnight. The final volume is necessary to determine the total number of cells in the well.

2 Fill the wells a half area flat bottom plate with 142.5 µL DiH2O.

3 Add 7.5 µL of 2xYT (or sterile growth media used for the overnight culture) to each well and pipette to mix.

4 Take an OD 600 reading of the plate or select blank. These readings are the blank/background.

5 Fill the wells of a second half area flat bottom plate with 142.5 µL DiH2O.

6 Add 7.5 µL of fresh overnight culture to each well and pipette to mix. Pipette mix the overnight culture prior to taking a sample and make sure no cells are clustered at the bottom of the well. Do not let the diluted sample plate sit too long once they are prepared, as the cells will begin to settle that will affect accurate reading.

7 Take an OD 600 reading of the plate. These readings are the raw ODs.

Use these values to calculate the total number of cells in the overnight culture. When evaluating 96 samples it is helpful to program an Excel spreadsheet to automatically calculate these values, given the Blank and Raw OD values:

Subtract the blank: ODRaw – ODBlank = ODAdjusted

Multiply by path length (1.1 for Costar half area plate with 150 µL total volume) **and dilution factor** (20 for 1:20 dilution): ODAdjusted x Path Length x Dilution Factor = OD of culture in half area plate

Multiply by 1 OD (5.0 x 108 cells/mL): OD x (5.0 x 108) cells/mL = Number of cells in 1 mL of overnight culture

Multiply by the volume (in milliliters) of the overnight culture: No. Cells in 1 mL x Total Overnight Culture Volume (mL) = Total Number Cells in the Overnight Culture

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Simplified:
(ODRaw – ODBlank) x Path Length x Dilution Factor x (5.0 x 108) x Final Overnight Culture Volume (mL)
(ODRaw – ODBlank) x 1.1 x 20 x (5.0 x 108) x Final Overnight Culture Volume (mL)
(ODRaw – ODBlank) x (1.1 x 1010) x Total Overnight Culture Volume (mL) = Total Number Cells in Overnight Culture

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