

Plasmid MiniPrep System

Catalog Number 100-100

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Plasmid Miniprep System (Rev. 3: 5/20/04)

I. Buffers

Only Buffer 1 requires storage at 4°C. All other buffers and kit components should be stored at room temperature. The shelf-life of Buffer 1 is approximately nine months when stored at 4°C. All other buffers have a minimum shelf-life of at least nine months when stored at room temperature.

Plasmid Buffer 1

- Proprietary composition
- Contains RNase A
- Nontoxic and noncaustic
- **Store at 4°C.**

Plasmid Buffer 2

- Proprietary composition
- Caustic: contains NaOH
- **Always wear eye protection and gloves when handling**

Plasmid Buffer 3

- Proprietary composition
- Acidic irritant: contains a mix of salts and acetic acid
- **Always wear eye protection and gloves when handling**

Plasmid Buffer 4

- 10 mM Tris-Cl, pH 7.5 (final)
- 80% ethanol (final)

IMPORTANT: The customer is required to add either 40 ml (Cat. No.100-050) or 80 ml (Cat. Nos.100-100 and 100-200) of 95-100% ethanol to the contents of the Buffer 4 bottle to generate the working wash buffer. Either denatured or nondenatured ethanol may be used.

Plasmid Buffer 5

- 12 mM Tris-Cl, pH 8.5
- Nontoxic and noncaustic

II. Introduction

The SuperPrepMini™ Miniprep Plasmid Kit.....A Plasmid Purification System Developed by Scientists who Sequence for a Living.

The SuperPrepMini™ Plasmid PurificationKit is designed specifically to purify plasmid and cosmid for fluorescent DNA sequencing. It will render exceptionally pure plasmid (or cosmid), free of RNA, genomic DNA and other cellular contaminants which could compromise cycle sequencing reactions and the incorporation of fluorescently labeled dye terminators and primers. When used according to the sequencing kit manufacturer's instructions, plasmid purified by the our method will routinely render maximum sequencing reads on the ABI Prism™ DNA Sequencers (Applied Biosystems, Foster City, CA). The following cycle sequencing kits have performed exceptionally well with SuperPrepMini™-purified templates:

- ABI DNA Sequencing Kit (Dye Terminator)
- ABI Prism™ BigDye Terminator Cycle Sequencing Kit ()
- Amersham Thermo Sequenase™ Dye Terminator Cycle Sequencing Kit
- Amersham Thermo Sequenase™ II Dye Terminator Cycle Sequencing Kit
- Amersham DYEnamic ET Terminator Cycle Sequencing Kit

As a result of the level of purity and mass amounts rendered, plasmid and cosmid produced with this system are also recommended for a number of other molecular biology applications, such as isotopic (manual) DNA sequencing, restriction digests, transformations, site-directed mutagenesis, etc. In other words, the SuperPrepMini™ System can be used whenever smaller amounts (up to 25 µg) of highly purified plasmid or cosmid are required. The SuperPrepMini™ spin columns can be used in either a standard benchtop microcentrifuge or in any vacuum manifold with luer fittings.

III. Protocol

This protocol is designed to produce up to approximately 25 µg of highly purified high copy number plasmid from overnight bacterial cultures grown to stationary phase in LB (Luria-Bertani) broth. This protocol is also recommended for medium- and lower copy number constructs, such as pET, pBR and cosmids. Richer broths such as SB (Super Broth), TB (Terrific Broth) or 2xYT can also be used but care must be taken not to overwhelm the system with an excessive number of cells. On those occasions when a richer broth must be used, we recommend a maximum bacterial culture volume of 2 ml.

The actual amount of plasmid (or cosmid) rendered is primarily contingent upon the copy number. High copy number plasmids, such as pGEM and pUC will generally produce approximately 2-5 µg/ml of LB culture. These same high copy number constructs will generate up to approximately 10-20 µg/ml when grown in richer broths, such as SB, TB or 2xYT. Medium copy number plasmids (e.g., pET and pBR322) and cosmids will generate approximately 0.2-1 µg/ml of LB culture and approximately 1-5 µg/ml of SB, TB or 2xYT. These are only general figures but are sufficiently accurate to allow one to plan the propagation and purification of their constructs accordingly.

Note: Always inoculate cultures with a single colony from a fresh plate and never grow for more than 16 hours. Also, do not store bacterial cultures for extended periods (particularly at room temperature) before initiating plasmid purification.

Very low- and single-copy plasmids

Very low copy number plasmids can also be purified using the SuperPrepMini™ procedure but the reagent volumes in the kit have not been configured for the routine purification of these constructs. These very low copy number constructs must typically be grown in large culture volumes in order to produce enough plasmid for sequencing and other applications which will quickly exhaust the kit reagents. Therefore, this kit is not recommended for their purification.

Steps

Read all steps carefully before proceeding.

1. Pellet the bacteria by centrifugation.

Bacterial cultures grown overnight to stationary phase. Select one of the following:

- up to 8 ml of high copy number construct in LB
- up to 8 ml of medium copy number construct in LB
- up to 2 ml of either high- or medium copy number construct in SB, TB or 2xYT

Any culture volumes greater than 1.5-2 ml can be pelleted in a variety of different centrifuge tubes, provided that a centrifugal force of approximately 3,000-5,000 x g can be applied. Typically, bacteria are pelleted by centrifugation for approximately 10 minutes at 5,000 x g, although considerably higher g-force can be applied over a much shorter period of time. For example, 1.5-2 ml of culture can be efficiently pelleted by centrifugation at top speed (approx. $\geq 12,000 \times g$) in a microcentrifuge for 2 minutes.

Note: *Alkaline lysis of the bacterial pellet (Step 3) is intended to occur in a 1.5-2 ml microcentrifuge tube (not included in kit). Therefore, either the bacterial pellet or the resuspended bacteria (Step 2) must be in a microcentrifuge tube prior to the addition of Buffer 2.*

IMPORTANT: DO NOT EXCEED THE RECOMMENDED CULTURE VOLUMES, ABOVE.

2. Resuspend the bacterial pellet in 250 μ l of Plasmid Buffer 1.

Add 250 μ l of Buffer 1 and either pipet or vortex the bacterial pellet until it is completely resuspended.

Note: *If the resuspended bacterial pellet is not in a 1.5-2 ml microcentrifuge tube, it must be transferred at this time. No transfer should occur after the addition of Buffer 2. The customer is expected to provide this tube. Do not use the 2 ml collection tubes supplied in the kit.*

3. Add 250 μ l of Plasmid Buffer 2.

Following the addition of Buffer 2, the contents should be mixed by gently inverting several times.

Caution: This step lyses the bacteria and releases both the plasmid and the bacterial chromosomal DNA into solution. The release of the bacterial DNA results in a noticeable increase in the viscosity of the solution. The bacterial DNA is very brittle and will fracture into smaller pieces if agitated excessively. Small pieces of bacterial chromosomal DNA will copurify with the plasmid if present. To avoid this, do not agitate the solution forcefully and **NEVER VORTEX.**

Note: *Following the addition of Buffer 2, neutralization with Buffer 3 (Step 4) should occur within 5 minutes.*

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4. Add 350 µl of Plasmid Buffer 3 and gently invert several times.

This step renders the bacterial chromosomal DNA and cell wall debris insoluble through a combination of rapid neutralization in conjunction with a high salt-detergent precipitation. The plasmid is left in solution. Following the addition of Buffer 3 and mixing, a white debris precipitate will immediately form.

Caution: Do not vortex.

Important: Do not agitate with excessive force but be sure that the contents are homogeneously mixed during inversion.

5. Centrifuge for 5 minutes at top speed ($\geq 12,000 \times g$) in a microcentrifuge.

This step will tightly pellet the debris against the bottom or wall of the tube allowing easy removal of the supernatant containing the plasmid.

Note: SuperPrepMini™ Plasmid Buffers 1-3 have been specifically formulated to produce firm, immobile genomic DNA pellets following precipitation and centrifugation. However, these reagents can be overwhelmed if too many bacteria are processed. If the white pellet appears gelatinous and diffuse following centrifugation, it is an indication that too many cells have been processed and an excessive amount of bacterial chromosomal DNA is present. This gelatinous mass can be removed by partially drawing it into the pipet tip and transferring it out of the tube. Following this, centrifuge again at top speed for 2 minutes. Generally, this will not affect the quality of the plasmid preparation. Reduce the culture volume by 20-25% in subsequent preparations.

6. Transfer (pipet or pour) the clarified supernatant into a SuperPrepMini™ spin column seated in a 2 ml collection tube (provided in kit).

Carefully remove the supernatant without disturbing the pellet. A small amount of precipitate transferred to the spin column is inconsequential, but plasmid quality will be optimized if this step is carried out with finesse.

Note: If the pellet is accidentally disturbed or drawn into the pipet, it is usually inconsequential. Simply expel the material back into the tube and centrifuge again at top speed for 2 minutes and continue.

Note: On occasion, there may be 50-100 µl of residual clarified lysate remaining. If purifying a high copy number plasmid, simply discard this residual volume. If purifying a lower copy number plasmid, this residual amount can be assimilated by repeating Steps 6-7.

Note: Do not load more than 750 µl at one time into the spin column.

7. Bind the plasmid to the spin column membrane with a low speed spin.

Centrifuge the spin column for **1 minute at 3,000-4,000 rpm (approx. 1,000 x g)**. The binding of the plasmid to the spin column membrane is dependent upon a diffusive interaction between the plasmid and the surface of the fibers comprising the membrane. This interaction is facilitated by moving the solution containing the plasmid through the membrane at a modest speed.

At the end of the spin, check to ensure that no solution remains in the spin column. Discard the solution in the collection tube but save the tube for reuse in the next step.

8. Wash the spin column with 750 µl of Plasmid Buffer 4.

Add 750 µl of Buffer 4 to the spin column and centrifuge for **1 minute at 3,000-4,000 rpm**. The removal of residual debris and salts which are loosely bound to the spin column or plasmid will be more efficient if this spin is carried out at a modest speed.

Note: Be sure that no buffer remains in the spin column before proceeding to the next step.

9. Empty the wash buffer from the collection tube and centrifuge the spin column for 1 minute at top speed ($\geq 12,000 \times g$).

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This high-speed spin is used to completely expel any residual wash buffer from the spin column.

10. Transfer the spin column to a clean 1.5 ml microcentrifuge tube (supplied by customer).

Check to ensure that no solution remains within the spin column. Remove the spin column from the collection tube and, if necessary, blot the luer end with a clean absorbent tissue, such as a kimwipe to remove any traces of wash buffer. Place the spin column into a new microcentrifuge tube from laboratory stock.

Note: Do not use one of the 2 ml kit collection tubes for this.

11. Elute the bound DNA with 60 µl of Plasmid Buffer 5.

Pipet 60 µl (standard eluent volume) onto the membrane at the base of the spin column. Be careful not to puncture the membrane with the pipet tip. **Allow 1 minute for detachment of the plasmid from the chromatographic membrane before centrifugation. Spin for 1 minute at top speed ($\geq 12,000 \times g$)** to completely expel all of the eluent and plasmid.

Note: TE or deionized water can also be used as eluents, provided that the pH is 8.0-8.5. However, the chemical stability of DNA is diminished when stored in pure (non-buffered) water for extended periods. Some investigators have reported the inhibition of enzymatic reactions, such as PCR, when even small amounts (0.5-1 mM) of EDTA are present. Speed-vac concentration of plasmids eluted in TE may concentrate the EDTA to a level which may interfere with subsequent enzymatic reactions.

12. Following elution and concentration (if any) accurately determine the quantity of purified plasmid DNA with a spectrophotometer.

A common problem encountered with fluorescence-based sequencing reactions is the use of too little plasmid template. Most dye terminator-type cycle sequencing reactions require 0.5-1 µg of plasmid template per reaction. It is also a good idea to run a small aliquot of the sample on an agarose gel. Gel analysis will also allow verification of the purity of the sample and provide a permanent pictorial record of the sample prep. This can be quite useful in the event that a problem is encountered.

III. Troubleshooting

It is not the objective of this section to address all of the possible problems which can occur when carrying out fluorescent sequencing. Certainly, many/most irregularities are best addressed by the manufacturers of the instruments and cycle sequencing kits. Rather, this information deals with common problems which can occur during the use of the SuperPrepMini™ Plasmid System and how these may result in suboptimal sequencing data.

It has been our experience that there is relatively little opportunity for problems to occur, either during plasmid purification or in the subsequent sequencing reactions, provided that the following conditions are observed:

- Inoculate the culture medium with a single colony from a fresh plate.
- Do not grow the culture for longer than 15 hours.
- Do not exceed the recommended culture volumes when performing the purification.
- Accurately quantitate the amount of plasmid before carrying out the sequencing reactions.
- Use high quality sequencing primers from a reputable supplier.
- Scrupulously follow the kit manufacturer's instructions for setting up the sequencing reactions.

1. Poor yield

The most likely causes are as follows:

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- A medium- or lower copy number construct is being purified from an excessively large culture volume and the RNase A activity of Buffer 1 has been compromised. In this scenario the much more abundant RNA is swamping the spin column membrane and excluding the plasmid from binding. This would be confirmed by the presence of a large amount of RNA when evaluating the preparation on a gel.
- Instead of adding 95-100% ethanol to the Buffer 4 concentrate, a lower concentration (e.g., 70%) was added instead. In this scenario, the plasmid is eluting prematurely in the wash buffer.
- The bacterial culture is heterogenous and contains a low titer of plasmid-bearing bacteria.
- The plasmid is simply present in a lower number of copies per cell than anticipated.

Remedial measures:

- Reduce the culture volume.
- Check to ensure that the RNase A has been added to Buffer 1.
- Replace the Buffer 1 if suspect.
- Restreak the bacterial culture and grow another culture from a single colony.
- Check efficacy of antibiotic used to select for plasmid-bearing bacteria.

2. Poor performance in fluorescent sequencing

There are many factors unrelated to the purity or quantity of the plasmid template which may be responsible. In general, "poor performance" means either low signal intensity, very short read length or garbled sequence. Running a control reaction with the same sequencing reagents but with a different plasmid is probably the best way to determine if the problem is related to the sequencing template, per se, or to some other factor. If the plasmid template is indicated, the most common causes are as follows:

- Low signal too little template added; possible plasmid degradation
- Short reads plasmid preparation contaminated with salt, ethanol or a bacterial metabolite which is compromising the DNA polymerase
- Garbled sequence genomic DNA contamination; possible presence of a second plasmid from a mixed culture; problem with primer design, etc.

Remedial measures:

- Run a control reaction with a different plasmid to determine the source of the problem.
- Accurately determine the amount of plasmid with a spectrophotometer and add *exactly* what the sequencing kit instructions specify. Run a small aliquot on a gel to verify the spectrophotometer reading and to check for possible degradation.
- Precipitate the plasmid with one-third volume 7.5 M ammonium acetate and two volumes of 95-100% ethanol. Wash 2x with ice-cold 70% ethanol to remove any residual salt.
- Run an aliquot of the plasmid preparation on a gel and inspect closely for either genomic DNA contamination or any other nucleic acid species. Gel-purify the plasmid if necessary.
- Carry out a restriction digest to verify the presence of only a single plasmid species.

3. RNA contamination

RNA contamination is visualized as a diffuse low molecular weight band or smear at the bottom of each lane on an agarose gel. However, residual RNA contamination is usually fairly innocuous in terms of interference with cycle sequencing reactions unless present in high concentration. The reason for this is that the different species of RNA present generally do not compete effectively with the sequencing primers for priming sites on the plasmid. The most common causes of residual RNA contamination are as follows:

- Processing excessive amounts of bacteria (the RNase A activity in Buffer 1 is overwhelmed).
- The RNase A activity in Buffer 1 has been compromised due to either age or improper storage.
- The RNase A was not added to Buffer 1.
- The bacterial pellet was incompletely resuspended in Buffer 1 prior to the addition of Buffer 2.

Remedial measures:

- Reduce the bacterial culture volume.
- Replace the Plasmid Buffer 1.
- Vortex or pipet until the cell pellet is completely resuspended. Visually inspect the cell suspension to ensure that complete resuspension has occurred.

4. Genomic DNA contamination

Residual bacterial genomic DNA is best visualized as a high molecular weight band in or near the well on an agarose gel. Unlike residual RNA, genomic DNA will interfere with the cycle sequencing reactions in a manner which is more or less proportional to the amount present. At lower concentrations, contaminating genomic DNA will probably only elevate the level of background on the electropherogram but still allow the sequence to be read. When present in higher concentrations, the sequence may be impossible to read. The most common causes of genomic DNA contamination in a plasmid preparation are as follows:

- Processing excessive amounts of bacteria.
- Incomplete (nonhomogenous) mixing of Plasmid Buffers 2 and 3.
- Excessive force when mixing in Buffers 2 and 3.

Remedial measures:

- Run a preparative agarose gel and gel-purify the plasmid to remove the contaminating genomic DNA.
- Repeat the purification and reduce the culture volume.
- Following the addition of Buffers 2 and 3 use gentle but thorough mixing to ensure that the contents are completely mixed without shearing the genomic DNA.

5. Plasmid looks smeary or “dirty” when run on a gel.

The most common cause is processing too many bacteria. Occasionally, the cell density of an overnight culture may be inordinately high, even when grown under recommended conditions. Alternatively, incomplete mixing of the first three reagents (particularly Buffers 2 and 3) may also compromise the removal of bacterial debris.

Remedial measures:

- Reduce the bacterial culture volume processed by 30-50% and repeat the purification.
- Be sure that the cells are completely resuspended in Buffer 1 and be sure that Buffers 2 and 3 are homogeneously mixed when added.
- Be sure to use LB, not SB, TB or other richer broth.
- Do not grow bacterial culture for an excessive period of time.
- Do not store bacteria for an excessive period of time before processing.

IV. SuperPrepMini™ Plasmid Kit sizes and contents

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50 purifications (Catalog No. 100-050)

- 50 SuperPrepMini™ spin columns
- 50 collection tubes
- Plasmid Buffer 1 (14 ml)
- Plasmid Buffer 2 (14 ml)
- Plasmid Buffer 3 (20 ml)
- Plasmid Buffer 4 (10 ml 5x)
- Plasmid Buffer 5 (10 ml)

100 purifications (Catalog No. 100-100)

- 100 SuperPrepMini™ spin columns
- 100 collection tubes
- Plasmid Buffer 1 (28 ml)
- Plasmid Buffer 2 (28 ml)
- Plasmid Buffer 3 (37 ml)
- Plasmid Buffer 4 (20 ml 5x)
- Plasmid Buffer 5 (10 ml)

200 purifications (Catalog No. 100-200)

- 2 x 100 SuperPrepMini™ spin columns
- 2 x 100 collection tubes
- Plasmid Buffer 1 (2 x 28 ml)
- Plasmid Buffer 2 (2 x 28 ml)
- Plasmid Buffer 3 (2 x 37 ml)
- Plasmid Buffer 4 (2 x 20 ml 5x)
- Plasmid Buffer 5 (2 x 10 ml)

V. Product Warranty

Ana-Gen Technologies (Ana-Gen) products are sold for research purposes only. Each production lot is subjected to appropriate quality assurance procedures and are warranted to perform as indicated when handled, stored and used according to the manufacturer's instructions. Please report any problems to Ana-Gen immediately by calling 404-223-5090. By using this product, the customer acknowledges and agrees that any liability which Ana-Gen incurs to the customer is at the discretion of Ana-Gen and is limited to either the replacement of the product or in certain cases, to a credit of the price paid to Ana-Gen for the product, including applicable shipping and handling costs. The customer further agrees that any liability assumed by, or any remedial action taken by Ana-Gen is contingent upon the customer providing full details of the nature of the problem and the circumstances of use to Ana-Gen, including return of the product to Ana-Gen for testing. Any remedial measures taken by Ana-Gen will occur only AFTER testing and verification of malperformance by Ana-Gen of the product in question. Ana-Gen will, at the customer's request, provide "custom" protocols which are intended to allow a kit or product to be employed in a particular set of unconventional circumstances, as determined by the customer. Ana-Gen makes no guarantee concerning the performance of its kits or products when used in custom protocols or any protocols which depart from the specific instructions provided in a kit. **Ana-Gen will not, under any circumstances provide the specific composition of solutions, reagents or any kit component which it regards and/or has designated as "proprietary".**