

AxyPrep Easy-96 Plasmid DNA Kit

For the purification of plasmid
from bacterial cultures in a 96-well format

Kit contents, storage and stability

Cat. No.	AP-E96-P-4	AP-E96-P-24
Kit size	4 × 96	24 × 96
96-well Filtration plate	4	24
96-well 2.2 ml growblock	8	48
Silicone mat	12	72
Adhesive film	20 sheets	120 sheets
BF-400 breathable film	5	25
RNase A	270 µl	2 × 820 µl
Buffer S1	135 ml	2 × 410 ml
Buffer S2	135 ml	2 × 410 ml
Buffer NP	135 ml	2 × 410 ml
Protocol manual	1	1

* The BAC protocol requires the use of a 48-well, 5 ml growblock (Axygen #P-5ML-48-C) and a 48-well silicone sealing mat (Axygen #AM-48-IMP) which are not provided in this kit and must be ordered separately. Please contact Axygen Scientific for more information.

Except for the RNase A (after addition to Buffer S1), all reagents are stable for a period of at least 12 months from the date of receipt when stored under ambient conditions. Please avoid exposure to direct sunlight or extremes in temperature. Buffer S2 contains SDS which may precipitate if exposed to cold temperatures. If this occurs, simply warm with a 37°C source and gently agitate to redissolve it. To preserve RNase activity during long-term storage, the RNase A is suspended in a solution containing a high concentration of ammonium sulfate. On occasion, a precipitate may form. If this occurs, the precipitate is easily dissolved in Buffer S1 and the RNase activity is unaffected.

RNase A: 50 mg/ml. Store at room temperature.

Buffer S1: Bacterial resuspension buffer. Store at 4°C after addition of RNase A.

Buffer S2: Bacterial lysis buffer. Store at room temperature.

Buffer NP: Neutralization buffer. Store at room temperature.

Introduction

The AxyPrep Easy-96 Plasmid DNA Kit employs a modified alkaline lysis method in combination with an optimized 96-well lysate Filtration plate and an alcohol precipitation step to purify plasmid and cosmid DNA from bacterial cultures. The kit is designed to process 1.3 ml aliquots of bacterial culture,

grown in a 96-well format. 1.3 ml of bacterial cultures (grown in rich media) containing high copy number constructs will generally yield up to 10-20 µg of plasmid, depending upon the vector. Plasmid or cosmid DNA prepared by the Easy-96 Plasmid DNA Miniprep Kit is suitable for automated fluorescent DNA sequencing on instruments such as the ABI PRISM® 3700 and 3730 DNA Analyzers. The purified plasmid or cosmid DNA can also be used in a variety of other applications, such as restriction analysis, PCR, probe synthesis, etc.

This kit is also recommended when a 96-well plate format is required for the purification of large recombinant constructs, such as BACs and P1.

Comments about the various protocol steps

Since no chromatographic medium is used to segregate the plasmid DNA, it is common to see some residual bacterial RNA copurified with the plasmid DNA. Degradation of the RNA by RNase A, following bacterial lysis, is intended to reduce the amount of RNA which co-precipitates with plasmid during the alcohol precipitation. However, the Easy-96 Plasmid Miniprep procedure includes an optional step in which the neutralized lysate is heated at 92°C for 8 minutes to further hydrolyze the RNA. The use of this heating step will substantially reduce the amount of bacterial RNA copurified with the plasmid.

1. Bacterial Culture

For the isolation of low-copy plasmids or cosmids containing pMB1 or ColE1 origins or replication, the culture should be supplemented with chloramphenicol to increase the titer of plasmid according to standard protocols. Please refer to the following table for details.

DNA construct	Origin of replication	Copy number	Classification
Plasmids			
pUC	ColE1	500-700	high copy
pBluescript	ColE1	300-500	high copy
pTZ	pMB1	>1,000	high copy
pBR322	pMB1	15-20	med-low copy
Cosmids			
SuperCos	ColE1	10-20	low copy
pWE15	ColE1	10-20	low copy

Bacterial cultures for plasmid isolation should always be grown from a single colony isolated from a freshly streaked plate. A single colony should be inoculated into rich media, such as LBG (Luria-Bertani broth + 2% glycerol) or 2×YT containing the appropriate antibiotic. We recommend LBG for routine bacterial growth. TB is not recommended. For bacterial growth, the kit includes 96-well 2.2 ml growblocks and BF-400 breathable film. The BF-400 breathable film is attached to the 2.2 ml growblock during growth to allow aeration of the bacterial cultures.

2. Collection of bacterial cells

The bacteria are harvested by transferring the 96-well growblock(s) to a benchtop centrifuge with a swinging bucket rotor and plate carriers and centrifuging for 5 minutes at 1,500×g. At this g-force, bacterial cells will be efficiently pelleted and can also be easily resuspended in Buffer S1.

3. Cells lysis and neutralization

After harvesting and resuspension, the bacterial cells are lysed by SDS under alkaline conditions. This alkaline lysis should not be extended beyond 5 minutes. Prolonged exposure to alkaline conditions may result in the formation of irreversibly denatured plasmid, which is refractory to many enzymatic reactions, such as restriction and sequencing. The alkaline lysate is then neutralized by an acidic buffer (NP). During neutralization, the bacterial chromosomal DNA and cellular debris form a complex with the SDS, which is then precipitated out of solution by a concentrated salt present in Buffer NP.

4. Removal of impurities by filtration

The insoluble debris complex, consisting of bacterial genomic DNA, bacterial cell wall debris and SDS/salt is removed by filtration through the 96-well Filtration plate. This plate contains specific porous materials which selectively remove this macroscopic debris complex while allowing the clarified filtrate containing the plasmid to pass through. The filtration medium will also remove tiny debris particles which could potentially interfere with capillary electrophoresis.

Caution

Buffer S2 contains chemical irritants. When working with this buffer, always wear suitable protective clothing such as safety glasses, laboratory coat and gloves. Be careful to avoid contact with eyes and skin. In the case of such contact, wash immediately with water. If necessary, seek medical assistance.

Equipment and consumables required

- 100% or 95% (denatured) ethanol
- 100% isopropanol (store at room temperature)
- 70% reagent-grade denatured ethanol (store at 4°C)
- 1 mM Tris-Cl, pH 8.5
- Shaker incubator
- Centrifuge capable of 3,000×g with swinging bucket rotor and plate carriers
- 92°C water bath
- AxyVac vacuum manifold (catalog #AP-VAC)
- Vacuum regulator
- Vacuum pump or source (-25-30 inches Hg required)
- 8- or 12-channel pipette
- Ice bath (for BAC preparation)

Preparation before experiment

- Before using the kit, add RNase A to Buffer S1. Mix well and store at 4°C. Use an aliquot of Buffer S1 to dissolve any precipitate in the RNase A tube and transfer it to the Buffer S1 bottle.
- Check Buffer S2 for precipitation before each use. If precipitation occurs, incubate at 37°C to dissolve the precipitate and equilibrate to room temperature. After use, the bottle should be closed immediately in order to avoid neutralization of the NaOH by ambient CO₂.

- Equilibrate Buffer NP to 4°C before use.
- Switch on a water bath to 92°C for the optional heating step (if elected).

Easy-96 Plasmid Protocol

1. Fill each well of the 2.2 ml growblock with 1.3 ml of rich medium containing the appropriate selective antibiotic. Inoculate each well with a single bacterial colony. Carefully apply a sheet of BF-400 breathable film. Incubate the cultures for 20-24 hours at 37°C with shaking at 260-300 rpm.

Note: If necessary, wipe droplets of media from the top of the 2.2 ml growblock before applying the BF-400 breathable film.

2. To harvest the bacteria, transfer the sealed plate to a centrifuge with a swinging bucket rotor and plate carriers. Centrifuge for 5 minutes at 1,500×g (ambient temperature).
3. Following centrifugation, remove the BF-400 breathable film and discard the supernatant by inverting the 2.2 ml growblock. Gently tap the inverted 2.2 ml growblock several times on a paper towel to dislodge the residual broth. Allow the 2.2 ml growblock to remain inverted on a paper towel for a few minutes to drain off residual medium.
4. Pipette 300 µl of Buffer S1 to each well. Seal the wells of the 2.2 ml growblock with a piece of clear Adhesive film provided in the kit. Resuspend the bacteria by vortexing.

Note: Please make sure that the RNase A has been added to Buffer S1.

Note: It may be necessary to dry the top of the 2.2 ml growblock with absorbent toweling before sealing with the Adhesive film.

Note: Vortex until the bacteria are completely resuspended and no clumps or aggregates are apparent.

5. Remove the Adhesive film from the top of the 2.2 ml growblock and pipette 300 µl of Buffer S2 into each well. Seal the 2.2 ml growblock with another sheet of Adhesive film and mix gently but thoroughly by inverting the 2.2 ml growblock 6-8×.

IMPORTANT: Buffer NP (Step 6 below) must be added within 5 minutes.

Note: Mix gently but thoroughly to avoid shearing the bacterial genomic DNA.

6. Remove the Adhesive film and discard. Pipette 300 µl of chilled (4°C) Buffer NP into each well. Seal the 2.2 ml growblock with a new Silicone mat (provided) and mix gently but thoroughly by inverting approximately 6-8×. Allow the 2.2 ml growblock to sit at room temperature for 5 minutes.

7. **Optional step:** Place the 2.2 ml growblock in a 92°C water bath for 8 minutes.

Note: This heating step hydrolyzes residual RNA and denatures and precipitates proteins that are not removed by alkaline lysis. This heating step is essential for bacterial strains that have not been mutated for reduced endonuclease levels (endA+ strains). In endA- strains the heating step can be eliminated. However, higher levels of RNA will be visible if this heating step is eliminated.

IMPORTANT: To avoid contamination with genomic DNA, the incubation temperature should not exceed 92°C.

IMPORTANT: Incubation time at 92°C should not exceed 15 minutes.

8. **Optional step:** Transfer the 2.2 ml growblock onto ice and incubate for 15 minutes.

Note: This step is only necessary if the heating step has been performed.

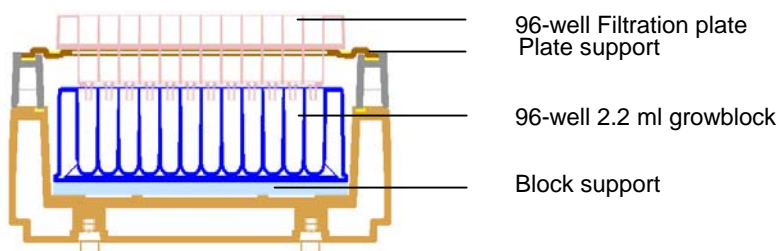
Either vacuum or centrifugation can be used to filter the lysate

Steps [9A-10A] describe the use of a vacuum manifold and Steps [9B-10B] describe the use of a centrifuge.

A. Vacuum manifold

A vacuum manifold, such as AxyVac (#AP-VAC) which can accommodate a 96-well plate is required for Steps 9A-10A. A negative pressure of $-25-30$ inches Hg is required. $-25-30$ inches Hg is equivalent to approximately $-850-1,000$ mbar and $-12-15$ psi.

9A. First place a block support and then a new 2.2 ml growblock into the base and reassemble the manifold. Place the Filtration plate on the top plate support of the vacuum manifold.



10A. Using a pipetter, transfer the neutralized lysates from the 2.2 ml growblock (Step 6 or Step 8) into the wells of the Filtration plate. Apply vacuum ($-25-30$ inches Hg) until no liquid remains in the Filtration plate wells.

Proceed to step 11 (below)

B. Centrifugation

A low-speed centrifuge with a swinging bucket rotor and plate carriers is required for this procedure.

9B. Place the Filtration plate onto a clean 2.2 ml growblock. Using a pipetter, transfer the neutralized bacterial lysates from the 2.2 ml growblock (Step 6 or Step 8) into the wells of the Filtration plate.

10B. Place the Filtration plate and the 2.2 ml growblock together into the plate carrier. Centrifuge at $2,000 \times g$ for 5 minutes at ambient temperature.

Note: If lysate is still present in the wells of the Filtration plate, increase to $2,500 \times g$ and spin for an additional 5 minutes.

Continue

11. Discard the Filtration plate. Add $650 \mu\text{l}$ of 100% isopropanol to the clarified lysates in the 2.2 ml growblock, and seal each well with a new Silicone mat. Immediately mix by inverting several times.

Note: To avoid possible cross-contamination, be sure that each well is individually sealed with the Silicone mat.

12. Centrifuge the 2.2 ml growblock at $3,000 \times g$ for 20 minutes at room temperature to precipitate the plasmid DNA. Discard the supernatant by inverting the block, and allow the 2.2 ml growblock to remain inverted on a paper towel for few minutes.

13. Desalt the plasmid DNA by adding 600 μ l of ice-cold 70% ethanol to each well, and seal each well with an Adhesive film. Centrifuge at 3,000 \times g for 3 minutes. Discard the supernatant and invert the 2.2 ml growblock on absorbent toweling to drain off residual solution. Dry the plasmid DNA for 15-20 minutes at room temperature or dry under vacuum for 10 minutes.

Note: It is not necessary to mix or invert the contents.

IMPORTANT: Make sure that no ethanol is left in DNA pellet after drying. Residual ethanol will inhibit many enzymatic reactions.

IMPORTANT: Do not over-dry the plasmid DNA. This will result in difficulty in resuspension.

14. Add 40-80 μ l of 1 mM Tris-HCl, pH 8.5 buffer to each well, and seal each well with an Adhesive film. Vortex to resuspend the plasmid DNA.

Note: If the plasmid pellet appears difficult to resuspend, seal the top of the plate and warm to 50-60°C, vortexing occasionally until the plasmid is completely resuspended. The resuspension solution can be preheated to 60°C in subsequent preparations.

Note: The volume of resuspension solution used will depend upon the final concentration of plasmid desired. For most high copy number constructs, resuspension in 50-60 μ l should provide a plasmid concentration of at least 100-200 ng/ μ l.

Note: Deionized water can be substituted for the 1 mM Tris-HCl (pH 8.5) but may be less effective in resuspending the plasmid DNA.

Easy-96 BAC Protocol

This protocol is designed to purify large recombinant DNA constructs (P1s, PACs, BACs; 50-250 kb in size) from 2.5 ml of bacterial culture. The BAC cultures are grown in 2 \times 48-well growblocks and then transferred to a single 96-well Filtration plate in Step 7A or 7B (below). The purification regimen should be designed such that two 48-well growblocks are used to propagate the BAC cultures. The cultures from the 48-well growblocks will then be consolidated into a single 96-well Filtration plate during the procedure. Bacteria should be grown in a rich medium in order to increase plasmid yield. LBG (Luria-Bertani broth + 2% glycerol) or 2 \times YT are recommended for use as the culture medium. Media containing high levels of phosphate are not suitable for use with this kit. TB is not recommended, for bacterial growth and plasmid propagation.

1. Fill each well of a 48-well growblocks (Axygen #P-5ML-48-C) with 2.5 ml of rich medium containing the appropriate selective antibiotic. Inoculate each well with clones from pre-cultures grown in a 96-well 2.2 ml growblock. Carefully apply a sheet of BF-400 Breathable Film to seal the wells. Incubate the cultures for 16 hr at 37°C with shaking at 175 rpm.

Note: The 48-well growblocks and 48-well Silicone mats are not included in this kit and must be purchased separately. Please contact your local Axygen distributor or contact Axygen Scientific directly at (510)494-8900 for ordering information.

Note: Yields of BAC DNA are most consistent when cultures are inoculated from precultures grown in a 96-well block such as the 96-well 2.2 ml growblock provided in this kit. Inoculating directly from single colonies may result in highly variable yields.

2. To harvest the bacteria, centrifuge at 2,500 \times g for 10 minutes (ambient temperature) in a swinging bucket rotor with plate carriers. After centrifugation, discard the supernatant by

inverting the 48-well growblock. Allow the 48-well growblock to remain upside down on absorbent toweling for few minutes to drain off residual medium.

3. Add 300 μ l of Buffer S1 to each well and seal the 48-well growblock with a piece of Adhesive film provided in the kit. Resuspend the bacteria by vortexing.

Note: Please make sure that the RNase A has been added to Buffer S1.

Note: It may be necessary to dry the top of the 48-well growblock with absorbent toweling before sealing with the Adhesive film.

Note: Vortex until the bacteria are completely resuspended and no aggregates are apparent.

4. Add 300 μ l of Buffer S2 to each well. Seal the 48-well growblock with a new 48-well Silicone mat and mix gently but thoroughly by inverting 6-8 \times .

Note: Buffer NP (Step 5 below) must be added within 5 minutes.

Note: Mix gently but thoroughly to avoid shearing the bacterial genomic DNA.

Note: The bacterial suspension will become viscous after lysis. To avoid cross-contamination between wells, centrifuge briefly at 3,000 rpm to collect any lysate from the Silicone mat. Allow centrifuge to reach 3,000 rpm, then stop.

5. Add 300 μ l of Buffer NP to each well. Seal the 48-well growblock with a new 48-well Silicone mat and mix gently but thoroughly by inverting approximately 6-8 \times . Incubate on ice for 5 minutes.

Either vacuum or centrifugation can be used to filter the lysate

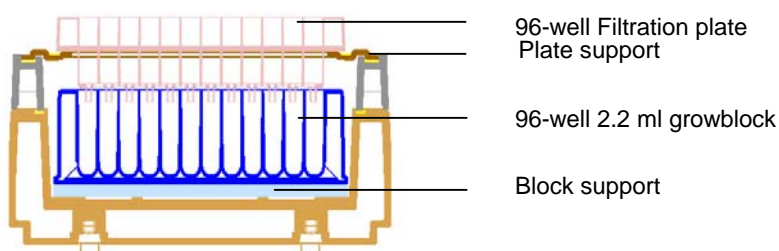
Steps [6A-7A] describe the use of a vacuum manifold and Steps [6B-7B] describe the use of a centrifuge.

The BAC-bearing bacterial cultures have been grown, lysed and neutralized in a 48-well growblock. The neutralized lysates will now be transferred to a 96-well Filtration plate. Each Filtration plate can be used to consolidate the cultures from two 48-well growblocks.

A. Vacuum manifold

A vacuum manifold, such as AxyVac (#AP-VAC) which can accommodate a 96-well plate is required for Steps 6A-7A. A negative pressure of -25-30 inches Hg is required. -25-30 inches Hg is equivalent to approximately -850-1,000 mbar and -12-15 psi.

- 6A. Place a block support and a new 2.2 ml growblock in turn into the base and reassemble the manifold. Place the 96-well Filtration plate on the top plate support of the vacuum manifold.



7A. Using a pipetter, transfer the lysates into the wells of the Filtration plate. Apply vacuum until no liquid remains in the Filtration plate wells.

Proceed to step 8 (below)

B. Centrifugation

A low-speed centrifuge with a swinging bucket rotor and plate carriers is required for this procedure.

6B. Place the Filtration plate onto a clean 2.2 ml growblock. Using a pipetter, transfer the neutralized bacterial lysate into the wells of the Filtration plate.

7B. Place the Filtration plate and the 2.2 ml growblock together into the plate carrier of a swinging bucket rotor. Centrifuge at $2,000 \times g$ for 5 minutes at ambient temperature.

Note: If lysate is still present in the wells of the Filtration plate, increase to $2,500 \times g$ and spin for an additional 5 minutes.

Continue

8. Discard the Filtration plate. Add $650 \mu\text{l}$ of 100% isopropanol to the clarified lysates in the 2.2 ml growblock, and seal each well with a new Silicone mat. Immediately mix by inverting $6-8 \times$.

Note: Be sure that each well is completely sealed with the Silicone mat.

9. Centrifuge 2.2 ml growblock at $3,000 \times g$ for 60 minutes at room temperature to pellet the BAC DNA. Discard the supernatant by inverting the block. Allow the block to remain inverted for several minutes on a piece of absorbent toweling.

IMPORTANT: Since BAC constructs are very low copy number, only a limited mass will be harvested from each culture, making precipitation and pelleting difficult. High g-force and long centrifugation times are essential in recovering BAC DNA. $6,000 \times g$ for 30 minutes can be substituted for the above conditions.

Note: On occasion, certain grades of paper toweling may contain fine fibers and particulates which can detach and contaminate the plate wells. These small fibers may interfere with subsequent capillary electrophoresis during sequence analysis. We recommend the use of lint-free absorbent toweling.

10. Desalt the BAC DNA by adding $600 \mu\text{l}$ of ice-cold 70% ethanol to each well, and seal each well with a Silicone mat. Centrifuge at $3,000 \times g$ for 10 minutes. Discard the supernatant and invert the block on absorbent toweling to drain off residual solution. Dry the plasmid DNA for 15-20 min at room temperature or dry under vacuum for 10-15 minutes.

Note: It is not necessary to mix or invert the contents.

IMPORTANT: Make sure that no ethanol is left in the DNA pellet after drying. Residual ethanol will inhibit many enzymatic reactions.

IMPORTANT: Do not over-dry the BAC DNA. This will result in difficulty with resuspension.

11. Add $25 \mu\text{l}$ of 1 mM Tris-HCl (pH 8.5) buffer to each well, and seal each well with a Silicone mat. Allow the BAC DNA to resuspend overnight under ambient conditions.

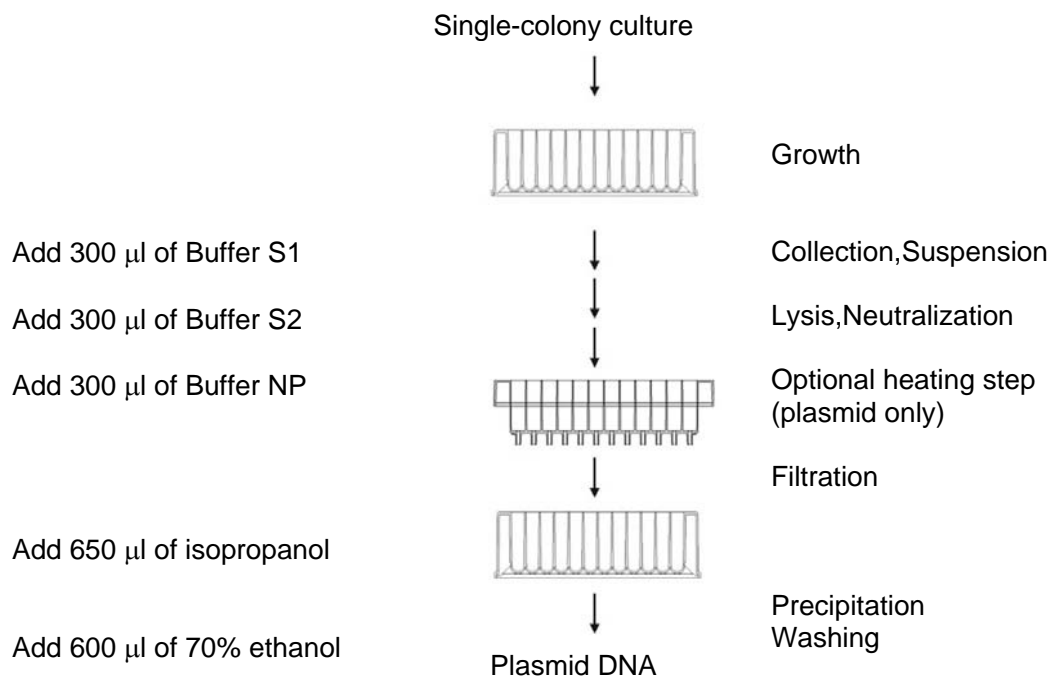
Note: If the BAC pellet appears difficult to resuspend, seal the top of the plate and warm to $50-60^\circ\text{C}$ until the BAC DNA is completely resuspended. Occasional gentle swirling or agitation may be required. The resuspension solution can be preheated to 60°C in subsequent preparations.

Note: If the BAC DNA is intended to be used immediately for sequencing, the plate can be vortexed to accelerate resuspension. Although the vortexing may induce some shearing of the BAC construct, this will

not effect its performance in sequencing. However, if the BAC DNA is intended for mapping applications, vortexing and repeated pipetting should be avoided to prevent shearing.

Note: Deionized water can be substituted for the 1 mM Tris-HCl (pH 8.5) but may be less effective in resuspending the BAC DNA.

Overview



Troubleshooting

1. Little or no plasmid DNA recovered

Plasmid did not propagate efficiently

Restreak fresh plates from glycerol stocks. Be sure that appropriate antibiotics are present and fresh. If using ampicillin, consider replacing with carbenicillin. If necessary, repeat transfection of bacteria with fresh plasmid. Try a different bacterial host strain.

Incomplete bacterial lysis

Generally attributable to processing too many bacteria or using outdated Buffer S2 in which the NaOH has been compromised through repeated exposure to ambient CO₂. Reduce the culture volume by 50% and repeat the purification to determine if this is the cause. Use fresh Buffer S2. Redissolve by warming to 37°C if precipitation occurs in Buffer S2.

Cell resuspension incomplete

After adding Buffer S1, use vigorous vortexing to ensure complete resuspension of the bacterial pellet. Visually inspect before proceeding with the addition of Buffer S2

Failure of plasmid to precipitate/pellet

Insufficient g-force or centrifugation time.

Be sure 100% isopropanol was used to precipitate plasmid.

2. Low BAC DNA yield

Use fresh glycerol cultures and avoid repeated freeze/thaw of frozen stocks. Depending on individual BAC clones and libraries, BAC glycerol cultures are sometimes very sensitive to freeze/thaw cycles. Always make enough replica plates and use pre-cultures for inoculation. The remainder of the pre-cultures can be used to set up new glycerol stocks.

3. Low DNA quality

Highly purified plasmid DNA will generally exhibit an $A_{260/280} = 1.7-1.9$. A reading <1.7 generally indicates protein contamination and a reading >1.9 generally indicates RNA contamination. While technically suboptimal in purity, plasmid preps outside the range of 1.7-1.9 will usually perform quite well in many applications. In the event that an inordinately low or high $A_{260/280}$ reading is accompanied by poor performance, the above guidelines should be used to determine the source cause of the impurity.

a. Low $A_{260/280}$

Plasmid preps with depressed spectrophotometric readings may also exhibit high background on agarose gels and poor performance in certain enzymatic reactions. This problem is usually attributable to the following:

- Processing too many bacteria
- Incomplete resuspension (Buffer S1)
- Incomplete lysis (Buffer S2)

b. High $A_{260/280}$

Plasmid preps with elevated $A_{260/280}$ readings may also exhibit RNA smears or bands on an agarose gel. Residual RNA contamination is usually attributable to the following:

- Failure to add RNase A to Buffer S1
- Buffer S1 too old or RNase A activity compromised
- Processing too many bacteria
- Incomplete resuspension
- Incomplete lysis

c. Plasmid band smeared on gel

A smeared plasmid band usually indicates enzymatic degradation of the plasmid within the bacterial host or during the purification process. This is usually attributable to:

- Use of an endA+ bacterial host
- Excessively long growth of bacterial culture
- Excessively long storage/handling of the harvested bacteria
- Improper storage of harvested bacteria
- Incomplete lysis of bacteria (Buffer S2 step)
- Incomplete neutralization of bacterial lysate (Buffer NP step)

d. BAC band smeared on gel

- See “Plasmid band smeared”, above
- Excessive agitation during Buffer S2 and Buffer NP steps

e. Multiple plasmid bands on gel

It is quite common to see multiple bands within a single lane when a plasmid sample is run on an agarose gel. These bands represent different forms of the plasmid. Usually, one of the bands is clearly predominant and this is the supercoiled form of the plasmid. Usually, there are 1-3 bands above the supercoil band, indicating plasmid species with slower electrophoretic mobility. These are usually the nicked and dimeric forms of the plasmid (or different combinations thereof). Occasionally, there may be a faint band which runs slightly ahead of the supercoil. This is referred to as the “irreversibly denatured” plasmid and is a byproduct of alkaline lysis. This form of the plasmid is refractory to many/most enzymatic reactions, including restriction and sequencing. The presence of the irreversibly denatured plasmid may become excessive if the plasmid is exposed to denaturing conditions (Buffer S2) for too long a period of time before the addition of Buffer NP.

f. High background on gel

The background material which stains weakly with ethidium bromide is usually a combination of bacterial debris and bacterial genomic DNA/RNA. Its presence may be attributable to bacterial death and lysis prior to purification or simply processing too many bacteria and overwhelming the ability of the protocol to segregate this debris from the plasmid. Alternatively, incomplete mixing of Buffers S2 and Buffer NP may also result in the carryover of debris.

- Excessively long growth of bacterial culture (cell death and lysis)
- Excessively long storage/handling of the harvested bacteria
- Improper storage of harvested bacteria
- Processing too much bacterial culture
- Incomplete lysis of bacteria (Buffer S2 step)
- Incomplete neutralization of bacterial lysate (Buffer NP step)

4. Genomic DNA contamination

- Excessively long growth of bacterial culture (cell death and lysis)
- Processing too much bacterial culture
- Excessive agitation after the addition of Buffer S2
- Excessive agitation after the addition of Buffer NP
- Incomplete lysis of bacteria (Buffer S2 step)
- Incomplete neutralization of bacterial lysate (Buffer NP step)
- Excessively long exposure to Buffer S2 (too long before addition of Buffer NP)

5. DNA does not perform well (general)

Failure of the plasmid to perform in enzymatic reactions is usually indicative of either the presence of an inhibitory contaminant (such as salt or ethanol) or modification of the plasmid. Occasionally, plasmids propagated through several generations may undergo deletions. This is fairly common

with cosmids. It may be necessary to confirm the sequence composition of the plasmid when no other causative factor is apparent.

- Contaminating salt present
- Contaminating ethanol present
- Excessively long exposure to denaturing conditions before neutralization with Buffer NP
- Nuclease contamination, plasmid degradation
- Deletions

6. Sequencing-related problems (fluorescent capillary)

Complete sequencing failure

Check the DNA yield, the sequencing reaction setup including the running conditions, and correct concentration. Try using less DNA in the sequencing reaction.

Low signal

Increase the number of cycles to 45-60 for the sequencing reactions or increase the amount of template DNA used. 400-500 ng of plasmid or cosmid should be optimal for most sequencing reactions.

Short read length

This may indicate the presence of a contaminant (usually salt) which is injurious to the DNA polymerase used in the sequencing reaction. Salt contamination will also interfere with electrokinetic uptake of labeled fragments into the capillaries during sequencing and this can result in shortened read lengths and/or diminished signal intensities. Alternatively, the amount of plasmid template may be insufficient. However, depending on the source and length of the insert DNA, it may be difficult to achieve the long sequence reads that are routinely obtained with standard short inserts or high copy number plasmids. Sequencing large template DNAs (cosmids and BACS) can also sometimes be problematic, even if ultrapure quality DNA is used.

Be sure that the 70% ethanol wash step is performed correctly to avoid salt contamination. Increase the number of cycles to 45-60 for the sequencing reactions. If necessary, use gel-filtration or ultrafiltration diagnostically to desalt a limited number of plasmid samples to verify salt contamination. Try increasing the amount of plasmid used in the sequencing reactions by 50%-100%.