

Axyprep-96 Plasmid Kit

For high-throughput plasmid purification in a 96-well format

Kit contents, storage and stability

Cat. No.	AP-96-P-4	AP-96-P-24
Kit size	4×96 preps	24×96 preps
96-well 2.2 ml growblock	4	24
96-well 1.6 ml growblock	2	4
96-well Filtration plate	4	24
96-well DNA plate	4	24
96-well V-bottom sample plate	6	28
BF-400 breathable film	4	24
Adhesive film	10	60
Silicone mat	8	48
RNase A	300 µl	2×900 µl
Buffer S1	135 ml	2×420 ml
Buffer S2	135 ml	2×420 ml
Buffer S3	200 ml	3×400 ml
Buffer W1	230 ml	3×460 ml
Buffer W2 concentrate	2×72 ml	-
10×Buffer W2 concentrate	-	180 ml
Buffer W2 bottle (empty)	-	1
Eluent	50 ml	250 ml
Protocol Manual	1	1

Except for Buffer S1 (after addition of RNase A), all buffers are stable for a period of at least 12 months when stored under ambient conditions. Please avoid exposure to direct sunlight and extremes in temperature. To preserve RNase activity, 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: Resuspension buffer. Store at 4°C after addition of RNase A.

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

Buffer S3: Neutralization buffer. Store at room temperature.

Buffer W1: Wash buffer. Store at room temperature.

Buffer W2 concentrate: Desalting buffer. Add the amount of ethanol specified and store at room temperature. Either 100% or 95% denatured ethanol can be used.

10×Buffer W2 concentrate (only in 24×96 kit): Before use of the kit, add 15 ml of 10× Buffer W2, 135 ml of deionized water, 350 ml of ethanol to the empty 500 ml bottle supplied with the kit. Mix well and store at room temperature. Either 100% or 95% denatured ethanol can be used.

Eluent: 2.5mM Tris-HCl, pH 8.5. Store at room temperature.

Introduction

The AxyPrep-96 Plasmid Purification Kit is suitable for the isolation of up to 20 µg of plasmid or cosmid DNA (per well) from up to 1.3 ml of bacterial culture. This method is based on a modified alkaline lysis of the bacteria in combination with the selective binding of the plasmid DNA to a special DNA plate. The purified plasmid or cosmid DNA is free from protein, genomic DNA and RNA and is suitable for a variety of applications, such as automated fluorescent sequencing, in vitro transcription, restriction analysis, PCR, eukaryotic cell transfection, etc. The AxyPrep-96 Plasmid Kit is ideal for use on automated sample preparation systems as a method for the high-throughput purification of plasmid intended for sequence analysis on platforms such as the ABI PRISM® 3700 or 3730 DNA Analyzers. This kit is not recommended for the purification of large recombinant constructs, such as BAC and P1. Rather, for the purification of BACs and P1 in a 96-well format, we recommend the AxyPrep Easy-96 Plasmid Purification Kit (catalog #AP-E96-P-4 or -24).

Comments about the various protocol steps

Carefully read through the entire protocol, including the various “Notes” before beginning the procedure.

1. Bacterial Culture

This kit is designed for the isolation of high-copy plasmid or cosmid from 1.3 ml bacterial cultures, grown in rich media in a 96-well format. For the isolation of low-copy plasmids or cosmids containing pMB1 or ColE1 origins or replication, the culture should be supplemented with chloramphenicol according to standard protocols to increase the titer of plasmid. 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-Bertain broth+2% glycerol) or 2×YT containing the appropriate antibiotic. We recommend LBG for routine bacterial growth. TB is not recommended for bacterial growth and plasmid propagation. 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 96-well 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 2.2 ml growblock(s) to a benchtop centrifuge with a swinging bucket rotor and plate carriers and centrifuging at 4°C for 5 minutes at 1,500×g. At this g-force the 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. The lysis procedure should not extend 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 neutralized by a high-salt buffer (S3) which also establishes the proper conditions for binding the plasmid to the AxyPrep DNA plate. During neutralization, the bacterial chromosomal DNA and cellular debris form a complex with the SDS, which is then precipitated out of solution by the high salt present in Buffer S3.

4. Removal of impurities by filtration and selective binding of DNA

The insoluble debris complex, consisting of bacterial genomic DNA, bacterial cell wall debris and SDS/salt is removed by filtration through the 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.

5. Washing, desalting and elution

The membrane within the DNA plate is then washed consecutively with Buffer W1 and Buffer W2 to remove residual debris and to desalt the plasmid. After thoroughly removing all residual wash solution, the purified plasmid is eluted into a small volume of Tris solution and collected in a V-bottom sample plate. Large recombinant constructs, such as BAC and P1 often exhibit diminished elution efficiency and this kit is, therefore, not recommended for their purification.

Caution

Buffer S2 contains NaOH, a caustic reagent. Buffer S3 and Buffer W1 contain chemical irritants. When working with the buffers, 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

- Heated water bath
- 8- or 12-channel pipette
- Disposable buffer trays
- Vortex
- AxyVac vacuum manifold (Catalog #AP-VAC) or comparable model
- Vacuum source capable of -25-30 inches Hg
- Vacuum regulator
- Centrifuge with a swinging bucket rotor and plate carriers
- 100% or 95% (denatured) ethanol

Preparation before experiment

- 1) Supplement rich media with appropriate antibiotics.
- 2) Add one tube of RNase A into one bottle of Buffer S1, as described on the bottle label, and mix well. Use a volume of Buffer S1 to dissolve any precipitate and transfer it from the RNase A tube into the Buffer S1 bottle. Make a notation, including the date of RNase A addition to the Buffer S1 label.
- 3) Before the use of the kit, add the amount of ethanol specified on the bottle label into the Buffer W2 concentrate. Mix well. Either 100% or 95% (denatured) ethanol can be used.
- 4) Preparation of Buffer W2 (only in 24×96 kit). Add the following to the empty "Buffer W2 bottle" bottle provided in the kit:
 - 15 ml of 10× Buffer W2 concentrate
 - 135 ml of distilled water
 - 350 ml of ethanol (100% or 95%)

- 5) Before each use, check Buffer S2 for precipitation. If precipitation occurs, warm in a 37°C water bath, accompanied by gentle swirling until the precipitate returns to solution. Equilibrate to room temperature before use.
- 6) Source some lint-free absorbent toweling.

Protocol

Bacterial culture and collection

1. Fill each well of the 96-well 2.2 ml growblock with 1.3 ml of rich medium containing the appropriate antibiotic. Inoculate each well with a single bacterial colony and attach a piece of the BF-400 breathable film to the top of the 2.2 ml growblock. Incubate the cultures for 20-24 hours at 37°C with shaking at 250-300 rpm.
2. Centrifuge at 1,500×g for 5 minutes to pellet the bacterial cells. Discard the supernatant by inverting the 96-well growblock quickly. Invert the 2.2 ml growblock on a paper towel for 2 minutes to drain off the residual medium.

Cells lysis and neutralization

3. Add 0.3 ml of Buffer S1 containing RNase A to each well, and carefully seal the top of the 2.2 ml growblock with a piece of adhesive film. Resuspend the bacterial cells by vortexing. The bacterial suspension in each well should be completely homogenous with no visible aggregates.

Note: Make sure that RNase A has been added to Buffer S1.

Note: Incomplete bacterial resuspension will result in diminished lysis efficiency, plasmid yield and purity.

4. Remove the adhesive film and discard. Add 0.3 ml of Buffer S2 to each well and seal the top of the 2.2 ml growblock with a piece of silicon mat. Mix gently but thoroughly by inverting the 2.2 ml growblock 6-8×. This step should be completed within 5 minutes.

Note: It is important to mix gently when inverting the 2.2 ml growblock. Vigorous agitation will shear the genomic DNA and result in contamination of the plasmid sample with genomic DNA fragments. If necessary, continue inverting the block until the solution becomes viscous and clear.

Note: After Buffer S2 is used, close the bottle immediately to avoid neutralization of the NaOH in Buffer S2 by ambient CO₂.

Note: Buffer S3 must be added within 5 minutes.

Note: The bacterial suspension will become viscous after lysis. To avoid cross-contamination between wells, centrifuge briefly at 2,500×g before proceeding with the next step. Allow the centrifuge to reach 2,500×g, then stop.

5. Discard the silicone mat. Add 0.45 ml of Buffer S3 to each well and reseal the 2.2 ml growblock with a new silicone mat. Mix gently but thoroughly by inverting the block 6-8× or until a uniform precipitate is formed within each well. Incubate at room temperature for 5 minutes. Remove the silicone mat and discard.

Note: Do not shake or vigorously agitate the neutralized lysate.

Lysate filtration, plasmid binding and desalting

The AxyVac Vacuum Manifold (catalog #AP-VAC) is required for this procedure. Other vacuum manifolds which can accommodate 96-well plates may also be suitable. We also recommend the use of a vacuum regulator. The vacuum source must be capable of -25-30 inches Hg. This is equivalent to approximately -850-1,000 mbar and -12-15 psi.

6. Connect the vacuum manifold to the vacuum source. Remove the manifold top and place a 96-well V-bottom sample plate and an AxyPrep DNA plate into the manifold base. Replace the manifold top and place an AxyPrep Filtration plate onto the manifold top. Be sure that filtration plate drip directors are aligned with the wells of the DNA plate underneath (Please refer to Figure 1).

Note: It is advisable to connect a trap between the vacuum manifold and the vacuum source to avoid contamination and damage to the vacuum pump or source.

7. Using a pipetter, transfer as much lysate as is possible from Step 5 to the corresponding wells of the Filtration plate. Turn on the vacuum source and adjust it to -25-30 inches Hg. Continue to draw vacuum through the plates until no liquid remains in any of the wells of the Filtration plate. Turn off the vacuum source and release the vacuum from inside of the vacuum manifold using the vacuum regulator. Discard the Filtration plate. Remove the DNA plate from the manifold and temporarily set it aside on a piece of absorbent toweling or plastic wrap.

Note: Be careful when handling the DNA plate. It will contain a substantial volume of clarified lysate.

Note: Wash and save V-bottom sample plate aside for next use.

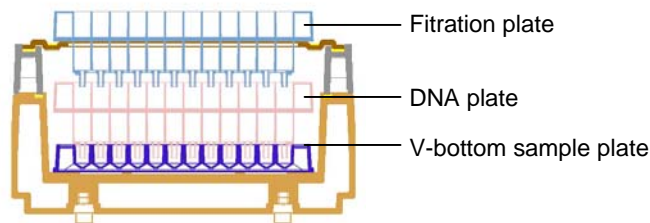


Figure1: Filtration and binding set

8. Place the waste collection tray into the manifold base. Replace the manifold top. Place the DNA plate onto the manifold. Turn on the vacuum and adjust to -25-30 inches Hg. Continue to draw the vacuum until no liquid remains in any of the DNA plate wells. Leave the vacuum on.
9. **Optional step:** Add 0.5 ml of Buffer W1 to each well of the DNA plate. Continue to draw the vacuum until no Buffer W1 is present in any of the wells.

Note: The use of Buffer W1 is only necessary to remove traces of endonuclease when endA+ bacterial strains have been used for plasmid propagation. These include the JM series and HB101. Host strains, such as XL-1 Blue and DH5 α do not require a Buffer W1 wash.

10. Add 0.9 ml of Buffer W2 to each well. Apply vacuum until no Buffer W2 remains in any of the plate wells. Repeat the Buffer W2 wash.

Note: Make sure that ethanol has been added to the Buffer W2 concentrate.

11. Apply maximum vacuum (up to -30 inches Hg) for 10 minutes to remove residual ethanol.

Note: Residual ethanol from Buffer W2 may inhibit subsequent enzymatic reactions and must be removed completely.

12. Turn off the vacuum source. Remove the DNA plate from the vacuum manifold and vigorously tap the plate approximately 6-8 \times on several layers of absorbent toweling. Be careful not to damage the drip directors on the underside of the plate.

Note: We recommend the use of “lint-free” absorbent toweling to avoid the release of tiny fibers which could contaminate the plasmid and interfere with subsequent capillary electrophoresis.

Note: Alternatively, the DNA plate can be centrifuged to remove residual Buffer W2. Please follow the following procedure: Turn off the vacuum. Place the DNA plate onto a 1.6 ml growblock. Transfer to a centrifuge with a swinging bucket rotor and plate carriers. Centrifuge at $\geq 3,000 \times g$ for 5 minutes.

Note: The kit contains a limited number of 1.6 ml growblocks. Save and use in subsequent preparations.

Elution

13. Remove the manifold top and place a 1.6 ml growblock into the manifold base. Place a V-bottom sample plate onto the 1.6 ml growblock. Reposition the manifold top and place the DNA plate on the manifold (Please refer to Figure 2).

Note: Be sure that the drip directors of the 96-well DNA plate are aligned directly over the wells of the V-bottom sample plate.

14. Pipette 80-100 μ l of Eluent or deionized water to the center of each well, and let it stand at room temperature for 1 minute. Turn the vacuum on and gradually increase the negative pressure to -15-30 inches Hg. Allow the vacuum to continue for 5 minutes.

Note: Pre-warming the Eluent to 65°C will generally improve elution efficiency.

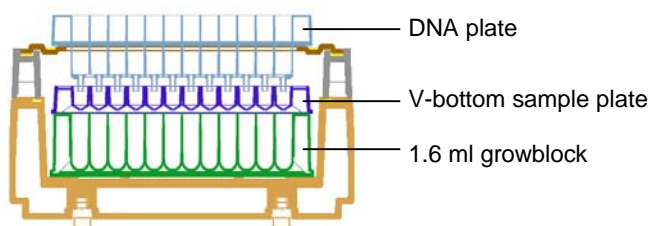


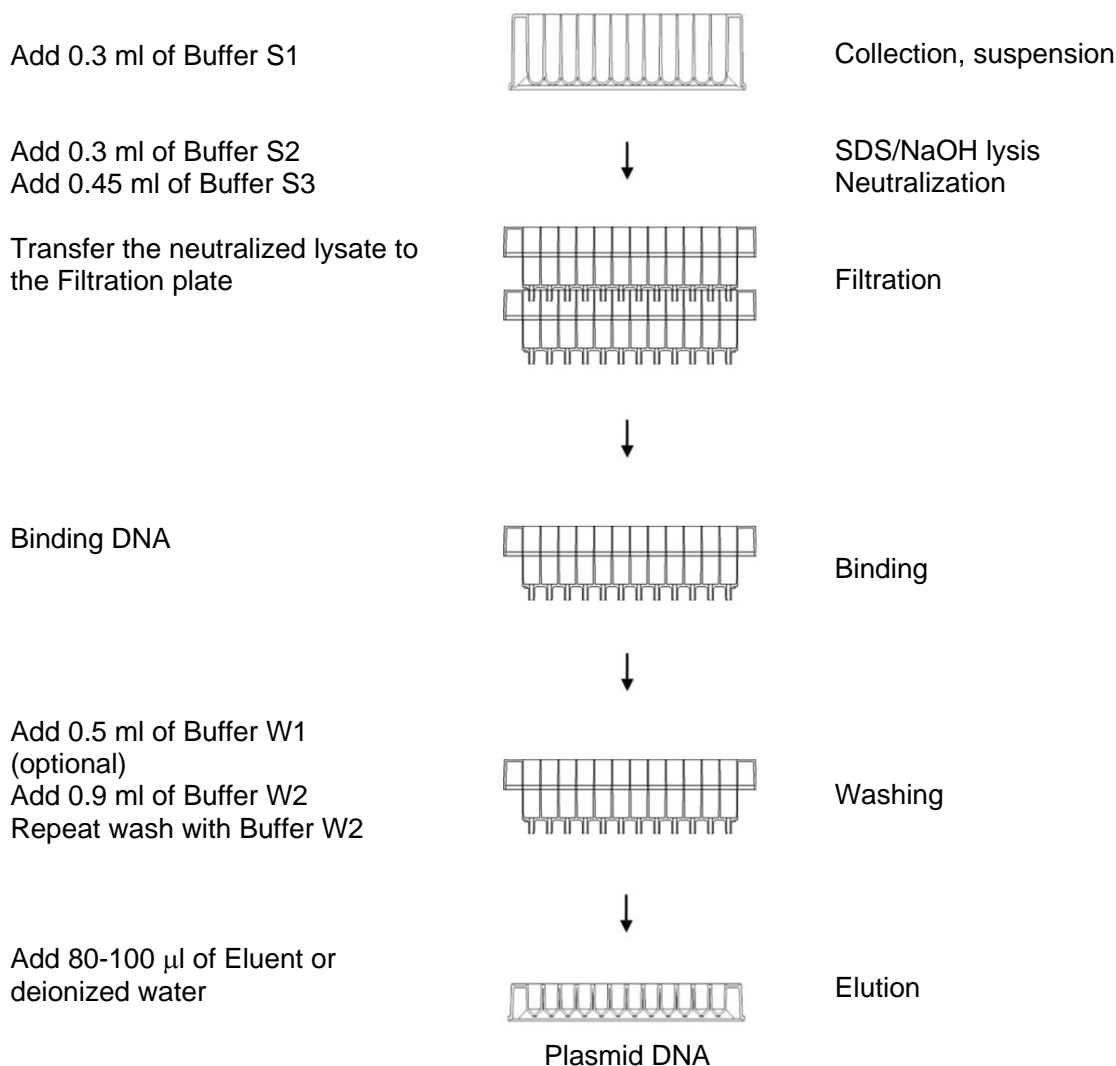
Figure2: Elution of plasmid using vacuum manifold

15. Turn the vacuum off and use the vacuum regulator to gradually release the vacuum from the manifold. Disassemble the manifold and remove the V-bottom sample plate containing the purified plasmid samples.

Centrifuge-mediated elution (alternative)

Place the DNA plate on a V-bottom sample plate. Pipette 80 μ l of Eluent or deionized water to the center of each well, and let it stand at room temperature for 1 minute. Centrifuge at $\geq 3,000 \times g$ for 5 minutes.

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 transformation 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. If a precipitate is present, warm Buffer S2 to 37°C accompanied by gentle agitation.

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.

Premature elution of plasmid during Buffer W2 wash step

Check to make sure that 95-100% ethanol has been added to the Buffer W2 concentrate, and in the correct amount. If unsure, replace with new stock of Buffer W2. Do not use 70% ethanol (common to many labs) to dilute Buffer W2 concentrate. 95% denature ethanol is 95% ethanol, plus 5% combined isopropanol and methanol and is completely satisfactory for use. 70-80% ethanol can be substituted for Buffer W2 temporarily.

Failure of plasmid to elute

Occasionally, excessive drying of the membrane and bound plasmid may result in diminished elution efficiency. Try decreasing the amount of time that the plate membranes are dried under vacuum prior to elution. Warm the eluent (or deionized water) to 65°C and allow the column or plate to sit for 5 minutes after the addition of the eluent before centrifugation.

2. 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 following 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

3. 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 end A+ 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 S3 step)

4. Multiple 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 S3.

5. 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 S3 may also result in the carryover of debris onto the plate.

- 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 S3 step)

6. 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 S3
- Incomplete lysis of bacteria (Buffer S2 step)
- Incomplete neutralization of bacterial lysate (Buffer S3 step)
- Excessively long exposure to Buffer S2 (too long before addition of Buffer S3)

7. RNA contamination

While limited amounts of residual bacterial RNA are generally not problematic in many applications, the presence of RNA may be viewed as an indication that certain aspects of the procedure have been compromised (albeit to a limited extent). The most likely reasons for the incomplete removal of bacterial RNA are:

- Failure to add RNase A to Buffer S1
- Buffer S1 dated or improperly stored (RNase activity compromised)
- Processing too much bacterial culture (overwhelming the RNase A)

- Incomplete resuspension and mixing during Buffer S1 and Buffer S2 steps

8. 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 S3K
- Nuclease contamination, plasmid degradation
- Deletions

9. 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 reduced 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 highly purified DNA is used.

Ensure 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%.