

R.E.A.L.[®] Prep 96 Handbook

For

Rapid Extraction Alkaline Lysis Minipreps
of plasmid, cosmid, or BAC DNA

February 2002



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Kit Contents

R.E.A.L.® Prep 96 Plasmid Kit	(4)	(24)
Catalog No.	26171	26173
No. of Preparations	384	2304
QIAfilter™ 96 Plates	4	24
Buffer R1	150 ml	2 x 500 ml
Buffer R2	150 ml	2 x 500 ml
Buffer R3	150 ml	2 x 500 ml
RNase A*	15 mg	2 x 50 mg
Tape Pads	2	7
Square-Well Blocks	8	48
Lids	4	24
Handbook	1	1

* Provided as a 100 mg/ml solution.

Storage Conditions

All kit components and buffers can be stored at room temperature (15–25°C) for up to 12 months.

After addition of RNase A, Buffer R1 should be stored at 2–8°C and is stable for 6 months. RNase A stock solution can be stored at room temperature for up to 2 years.

Quality Control

As part of the stringent QIAGEN® quality assurance program, the performance of R.E.A.L. Prep 96 Plasmid Kits is monitored routinely on a lot-to-lot basis. All kit components are tested separately to ensure highest performance and reliability.

Product Use Limitations

The R.E.A.L. Prep 96 Plasmid Kit is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside front cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the R.E.A.L. Prep 96 Plasmid Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or your local distributor (see inside front cover).

Safety Information

When working with chemicals always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in a convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

The following risk and safety phrases apply to the components of the R.E.A.L. Prep 96 Plasmid Kit.

Buffer R2

Contains sodium hydroxide: irritant. Risk and safety phrases: * R36/38, S13-26-36-46

Buffer R3

Contains acetic acid: irritant. Risk and safety phrases: * R36/38, S13-26-36-46

RNase A

Contains ribonuclease: sensitizer. Risk and safety phrases: * R42/43, S23-24-26-36/37

24-hour emergency information

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R36/38: Irritating to eyes and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink, and animal feedingstuffs; S23: Do not breathe spray; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S46: If swallowed, seek medical advice immediately and show this container or label.

Introduction

The R.E.A.L. Prep 96 Plasmid Kit is designed for rapid high-throughput purification of plasmids, BACs, PACs, and P1s from small-volume bacterial cultures. It is based on a modified alkaline lysis procedure which has been specially adapted for high-throughput DNA applications. The procedure has been developed and tested using a variety of high copy plasmids, low-copy cosmids, BACs, PACs, P1s, and *E. coli* strains (Table 1).

Two protocols are provided in this handbook: one for high-throughput preparation of high-copy plasmids and cosmids (page 14) and a special application protocol for high-throughput preparation of low-copy cosmids, BACs, PACs, and P1s (page 19). In addition, detailed background information and a useful troubleshooting guide, as well as hints for downstream applications with BAC DNA and for cultivation of BAC clones are provided (pages 23–38).

Table 1. Plasmid constructs and bacterial strains used with the R.E.A.L. Prep 96 procedure

Cosmids Plasmids	BACs, PACs, P1s		Bacterial strains	
	(45–50 kb)	(80–120 kb)	endA ⁻	endA ⁺
pUC18/19	Lorist	pBeloBAC	DH5 α TM	HB101
pBluescript [®]	Lawrist	pCYPAC-1	XL1-Blue	NM554
pGEM [®]	pWE15	pAD10sacBII	SOLR TM	
pTZ19R	pOU61		DH10B TM	

The R.E.A.L. Prep 96 Principle

The R.E.A.L. Prep 96 system provides a fast, simple, and cost-effective method for small-scale purification of plasmid DNA for use in routine molecular biology laboratory applications. The R.E.A.L. Prep 96 procedure is based on modified alkaline lysis of bacterial cells, followed by clearing of the lysates by filtration using the QIAfilter module, and further purification and concentration of DNA by isopropanol precipitation. The DNA obtained is resuspended in a small volume of Tris buffer and is ready for use. All steps are performed without the use of phenol, chloroform, CsCl, and ethidium bromide.

Alkaline lysis of bacteria

The R.E.A.L. Prep 96 procedure uses the modified alkaline lysis method of Birnboim and Doly (1). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt conditions in one step, ready for isopropanol precipitation. For more details on growth of bacterial cultures and alkaline lysis, refer to Appendix A on page 23.

Clearing bacterial lysates using the QIAfilter module

QIAfilter 96 modules eliminate the need for lysate centrifugation. Following alkaline lysis of bacterial cultures, the crude lysates are loaded directly onto the QIAfilter 96 plate. Denatured and precipitated cellular components and SDS are removed by filtration through the QIAfilter membrane. Particle-free filtrates flow directly into the wells of the 96-well block positioned underneath.

DNA purification and concentration by isopropanol precipitation

The cleared lysates are of an appropriate ionic strength for isopropanol precipitation. Therefore only isopropanol needs to be added for precipitation and purification of the plasmid DNA. The isopropanol precipitation step is carried out by centrifugation.

Applications

The amount and quality of DNA obtained with the R.E.A.L. Prep 96 procedure is suitable for automated sequencing applications, in particular those using cycle sequencing chemistries based on improved thermostable DNA polymerases (e.g., AmpliTaq® DNA Polymerase FS, ThermoSequenase™, and DYEnamic™ ET dye terminator) and applications using capillary electrophoresis sequencers (e.g., ABI PRISM® 3700 and MegaBACE™ -1000). R.E.A.L. Prep 96-purified DNA is also suitable for screening procedures such as restriction digestion and microarray-based analysis.

QIAGEN also offers alternative products for high-throughput plasmid purification. The QIAwell® 96 Ultra Plasmid Kit and the QIAprep® 96 Turbo Plasmid Kit provide a higher level of purity and should be used for sequencing projects requiring maximum read length and accuracy. For fully automated walkaway plasmid purification the MagAttract™ 96 Miniprep System, QIAprep 96 Turbo BioRobot Kit, and QIAwell 96 Ultra BioRobot Kits can all be used. In addition, for very high-throughput QIAGEN offers the *micro*R.E.A.L.™ Prep 384 Plasmid Kit, which uses rapid extraction alkaline lysis technology, and can process up to two 384-well plates in 135 minutes. Please call QIAGEN Technical Services or your local distributor (see inside front cover) for more information, or see Ordering Information on pages 40–42.

The QIAGEN Guide to Template Purification and DNA Sequencing (2nd Edition) provides a general overview of commonly used sequencing methods and technical information on sequencing optimization and troubleshooting. This comprehensive guide is available free on request or can be downloaded at www.qiagen.com.

Automation of the R.E.A.L. Prep 96 procedure on BioRobot® Systems

R.E.A.L. Prep 96 procedures for purification of plasmid, cosmid, BAC, PAC, and P1 DNA can be automated on QIAGEN BioRobot® Systems. A special kit format optimized for use with the BioRobot 3000 workstation is available (see Ordering Information, page 40). Purification protocols are provided in the BioRobot 3000 user manual.



BioRobot Systems are versatile workstations designed to automate high-throughput liquid handling and sample processing. Ready-to-run protocols are available for purification of nucleic acids, setup of reactions for sequencing, PCR, and RT-PCR and other liquid-handling applications. Chemistry, hardware, software, and technical support are all supplied by QIAGEN, providing a complete automation system for molecular biology and molecular diagnostic laboratories. For detailed specifications and ordering information, call QIAGEN*.

Recommendations for the R.E.A.L. Prep 96 Procedure

Vacuum recommendations

- The QIAvac 96 manifold operates with a house vacuum, vacuum pump, or water aspirator. The components of the QIAvac 96 manifold are shown in Figure 1 on page 11.
- The negative pressure (vacuum) should be assessed before beginning the procedure by applying the vacuum to an empty QIAfilter 96 plate on the QIAvac 96 manifold. Optimal vacuum is between -200 and -300 mbar (conversions to other units are provided in Table 2 on page 11).
- The vacuum pressure is the differential pressure between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 millibar or 760 mm Hg) and can be measured using a vacuum regulator (see Ordering Information, page 40). Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere.

* QIAGEN Robotic Systems are not available in all countries; please inquire.

- Excessive vacuum can cause sample spattering, while insufficient vacuum increases sample preparation time.
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during manipulations.
- Wear safety glasses when working near a manifold under pressure.
- For safety reasons, do not use 96-well plates that have been damaged in any way.

Table 2. Pressure conversions

To convert from millibars (mbar) to:	Multiply by:
Millimeters of mercury (mm Hg)	0.75
Kilopascals (kPa)	0.1
Inches of mercury (inch Hg)	0.0394
Torr (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per square inch (psi)	0.0145

The QIAvac 96 Manifold

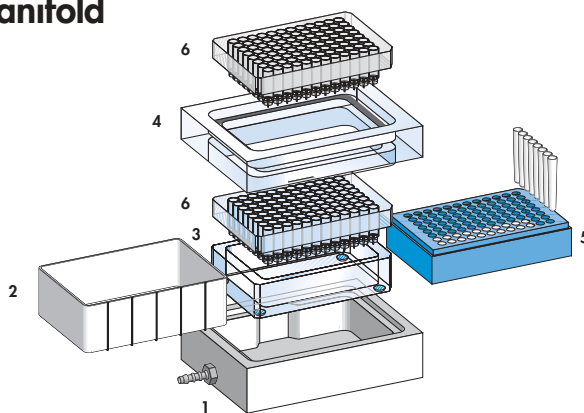


Figure 1. Components of the QIAvac 96 manifold.

1. QIAvac base, which holds a waste tray, a plate holder, or a microtube rack
2. Waste tray
3. Plate holder (shown with 96-well plate)
4. QIAvac 96 top plate with aperture for 96-well plate
5. Disposable microtube rack
6. 96-well plate*

*Not included with QIAvac 96. Included in the R.E.A.L. Prep 96 Plasmid Kit.

Multichannel pipet recommendations

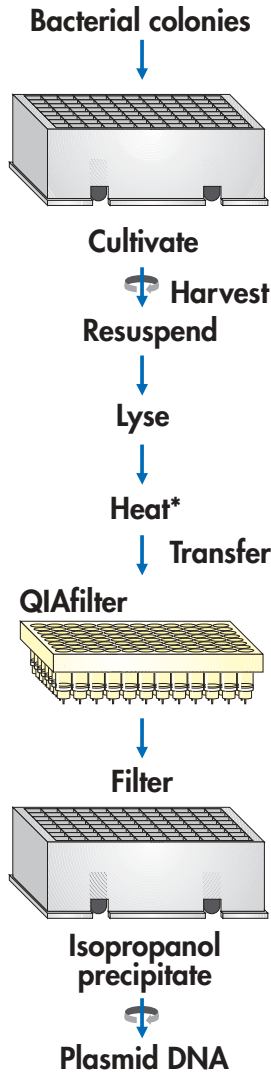
Many steps of the R.E.A.L. Prep 96 procedure require repeated pipetting, and a reservoir or multichannel pipet can greatly facilitate liquid handling. The Matrix Impact® cordless multichannel pipet or the Matrix Multi-8 Electrapette® can be purchased with an expandable tip-spacing system for direct liquid transfer from tubes to microtiter plates. For information on distributors, contact Matrix Technologies Corporation, 44, Stedman Street, Lowell, MA 01851 USA. www.matrixtechcorp.com.

Pipet tip recommendations

Some standard 1 ml pipet tips are not easily accommodated in the 96-well square-well blocks which are used in the R.E.A.L. Prep 96 protocol. When pipetting into square-well blocks, we recommend using pipet tips with 1.25 ml or 1.5 ml fill volume, such as:

- Matrix pipet tips (Cat No. 8051) for use with the Matrix pipet models mentioned above. These can be purchased from the distributors listed above.
- Finntip Multistepper pipet tips for use with single-channel pipets. These are available from: Labsystems Oy, Pultitie 8, P.O. Box 8, FIN-00881 Helsinki, Finland. www.labsystems.fi.

The R.E.A.L. Prep 96 Plasmid Procedure



* *Optional, depending on E. coli host strain (see step 6, page 16).*

R.E.A.L. Prep 96 Plasmid Protocol

This protocol is designed for high-throughput preparation of high-copy-number plasmids and cosmids from 1.3 ml bacterial cultures in 96-well format.

Important notes before starting

General notes

- Bacterial cultures for plasmid preparation with the R.E.A.L. Prep 96 procedure can be grown in Luria-Bertani (LB) or 2x YT media. Volumes can be up to 1.3 ml in the provided 96-well blocks. Use of enriched media (e.g., 2x YT) may result in higher plasmid DNA yield since such media may generate higher cell densities for certain vector/host strain combinations than LB medium, especially when using low-copy-number vectors.
- For the purification of cosmids, we strongly recommend using 2x YT media for cultivation.
- The R.E.A.L. Prep 96 procedure works optimally when the same vector/host strain combination is used for all of the samples in a block or set of blocks that are prepared together. Different vector/host strain combinations should be individually optimized for cell resuspension, lysis and filtration conditions, and volumes used for DNA resuspension.
- Growth of cultures in square-well blocks can vary depending on the growth medium, the bacterial strain, and the plasmid construct, giving rise to varied DNA concentrations in the final samples. When using the R.E.A.L. Prep 96 Plasmid Kit for the first time to prepare DNA for sequencing, we recommend preparing sequencing reactions using a range of different amounts of plasmid DNA in order to optimize the quantity of template DNA for the particular sequencing chemistry. Good starting ranges would be 0.6–1.2 µg total for fluorescent dye primer cycle sequencing and 0.3–0.5 µg total for fluorescent dye-terminator cycle sequencing.
- The DNA concentration of each sample is best determined using either a fluorimeter or agarose gel electrophoresis followed by ethidium bromide staining. For agarose gel quantitation, compare 1 µl aliquots of sample to known concentrations (50 ng, 100 ng, 150 ng) of a supercoiled plasmid DNA standard (e.g., the standards provided with each sequencing kit).

Notes on buffers

- Before using the kit for the first time, add the RNase A solution provided with the kit to Buffer R1. This solution is stable for six months when stored at 2–8°C.
- Buffer R2 should be kept at room temperature. Check before use for SDS precipitation, and if necessary redissolve SDS by warming. Close the Buffer R2 bottle immediately after use to avoid acidification of Buffer R2 from CO₂ in the air.

Equipment to be supplied by user

- The QIAvac 96 vacuum manifold is required for the production of cleared alkaline lysates using the QIAfilter 96 plate (see “Vacuum recommendations” on page 10).
- A centrifuge capable of attaining 2500 x g with a swinging-bucket rotor for 2 or more 96-well microplates and sufficient clearance for square-well blocks* is required (e.g., the QIAGEN 96-Well-Plate Centrifugation System, see page 41).
- For maximum convenience with minimum hands-on time, an 8-channel pipet with ≥ 1 ml fill and dispense volume is recommended.

Procedure

Growing 96-well bacterial cultures

1. **Fill each well of a square-well block* with 1.3 ml growth medium containing the appropriate selective antibiotic. Inoculate each well from a single bacterial colony. Incubate the cultures for 20–24 h at 37°C with shaking at 220 rpm.**

Aeration of the blocks can be achieved by using one of the loose-fitting plastic lids provided or AirPore™ Tape Sheets (see Ordering Information, page 41). Alternatively, adhesive tape with 2–3 holes pierced above each well can be used.

2. **Harvest the bacterial cells in the block by centrifugation for 5 min at 1500 x g in a centrifuge with a rotor for 96-well microplates. The block should be covered with adhesive tape during centrifugation. Remove medium by inverting the block.**

To remove the medium, peel off the tape and quickly invert the block over a waste container. Tap the inverted block firmly on a paper towel to remove any remaining droplets of medium.

WARNING: Ensure that the buckets on the rotor have sufficient clearance to accommodate 2.2 ml square-well blocks before starting the centrifuge.

Preparing bacterial alkaline lysates

3. **Resuspend each bacterial pellet in 0.3 ml Buffer R1. Use an 8-channel pipet with a large fill volume (≥ 1 ml per channel) for buffer delivery. Tape the block and mix by vortexing.**

Ensure that RNase A has been added to Buffer R1. The pelleted cells should be resuspended completely leaving no cell clumps.

* For automated plasmid purification using the R.E.A.L. Prep 96 procedure on BioRobot Systems, cell cultivation, harvesting, and lysis should be performed in 2 ml, 96-well flat-bottom blocks (included in BioRobot Kits).

- 4. Add 0.3 ml Buffer R2 to each well, seal the block with new tape, mix gently but thoroughly by inverting 10 times, and incubate at room temperature for 5 min.**

Buffer R2 should be checked before use for SDS precipitation caused by low storage temperatures. If necessary, redissolve SDS by warming. Do not vortex the lysates at this step, as this may cause shearing of the bacterial genomic DNA. Do not incubate for more than 5 min. Additional incubation may result in increased levels of open circular plasmid. At the end of the incubation, the lysate should appear viscous and free of bacterial cell clumps.

Close the Buffer R2 bottle immediately after use to avoid acidification of Buffer R2 from CO₂ in the air.

- 5. Add 0.3 ml Buffer R3 to each well, seal the block with new tape, and mix immediately by inverting 10 times.**

Gently inverting the taped block 10 times ensures uniform precipitation.

- 6. Optional: Place the block in a boiling water bath for 5 min.**

This heating step denatures and precipitates proteins and carbohydrates that are not removed by alkaline lysis. There should be sufficient water in the bath to float the 96-well block.

Note: The boiling step is essential for bacterial strains that have not been mutated for reduced endonuclease levels (endA⁺ strains). However, in many endA⁻ strains the boiling step can be eliminated without any risk of plasmid degradation. The elimination of this step is advantageous for very high-throughput minipreps.

WARNING: Wear protective gloves when removing blocks from the boiling water bath. The blocks are hot and can cause burns.

- 7. Optional: Cool the block to room temperature by incubating on ice for 10 min.**

This step is only necessary if the boiling step (step 6) has been performed. Cooling enhances precipitation of contaminants after boiling and is required to prevent excessive isopropanol volatilization in subsequent steps.

Clearing lysates with QIAfilter 96

- 8. Place a QIAfilter 96 plate (yellow) in the top plate of the QIAvac 96 manifold. Place a new square-well block into the base and reassemble the manifold.**

Ensure that the numbering and orientation of the plate and new square-well block are in accordance with the lysis block, and that the wells of the block are properly aligned with the outlet nozzles of the QIAfilter 96 plate.

9. Transfer the lysates to the wells of the QIAfilter 96 plate.

Use an 8-channel pipet adjusted to 1 ml fill volume. Most of the precipitated material will stick to the walls of the culture block. This material contains virtually no plasmid DNA and can be safely left behind. Occasionally, precipitate will clog the end of a pipet tip. Lightly tapping the tip on the bottom of the well of the culture block will break through the precipitate and allow the remaining material to be transferred. Any unused wells may be sealed with tape for later use.

10. Apply vacuum (–200 to –300 mbar) until the lysates are completely transferred to the square-well block in the QIAvac base.**Desalting and concentrating DNA by isopropanol precipitation****11. Take the square-well block containing the cleared lysates from the vacuum manifold. Add 0.7 volumes of room-temperature isopropanol to each well (0.63 ml for 0.9 ml of lysate), tape the block, and mix immediately by inverting 3 times.**

If the lysate is not properly cooled prior to this step, isopropanol volatilization will cause the tape to detach from the wells. If this occurs, increase the length of time the samples are held on ice prior to filtration (step 7).

When preparing multiple sets of 96 samples, add isopropanol to one block, tape, and mix by inversion before proceeding to the next block. This will minimize separation of the tape from the block before the samples are mixed.

12. Centrifuge the block at 2500 x g for 15 min at room temperature to pellet the plasmid DNA. Remove the supernatants by quickly inverting the block over a waste container, then tapping the block firmly, upside down, onto a paper towel.

Mark the orientation of the block before centrifugation so that it can be spun in the same orientation in the ethanol wash step. DNA pellets from isopropanol precipitations have a glassy appearance and may be difficult to see. Handle the block carefully to avoid dislodging the pellets.

13. Wash each DNA pellet with 0.5 ml of 70% ethanol. Centrifuge the block (in the same orientation as before) at 2500 x g for 2 min to reconcentrate the pellets. Remove the wash solutions by inverting the block, then tapping it firmly, upside down, onto a paper towel. Air dry the pellets for 15 min or dry under vacuum for 10 min.

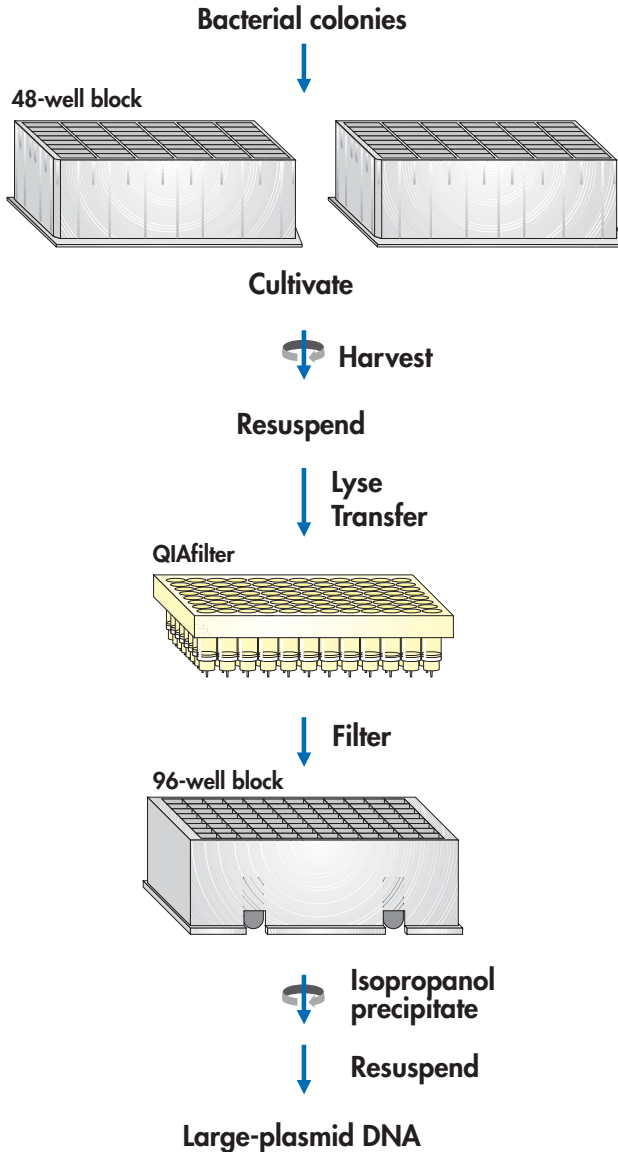
Ensure that no alcohol droplets are visible after air drying, but do not overdry the DNA pellets as this will make them difficult to dissolve. If this occurs, the DNA pellets may be heated at 50°C until they are completely redissolved.

14. Redissolve the DNA pellets in 50–250 µl 10 mM Tris-Cl, pH 8.5, or distilled water.

The optimal volume of buffer to use will depend on the copy number of the plasmid and the desired DNA concentration. Cosmids prepared with this protocol should be resuspended in a maximum volume of 50 µl.

Avoid repeated pipetting, which can shear the DNA.

The R.E.A.L. Prep 96 BAC Procedure



R.E.A.L. Prep 96 BAC Protocol

This protocol is a special application protocol designed for high-throughput preparation of 600–800 ng large plasmid DNA (P1s, PACs, BACs; 50–250 kb in size) from 2.5 ml bacterial cultures. It can be also used for the purification of cosmids. The protocol requires the use of 48-well blocks (purchased separately, see Ordering Information, page 41) which provide optimal conditions for cultivation of BAC clones. Alternatively, the bacterial cultures can also be grown in the 96-well square-well block provided.

Important notes before starting

- Please read also the “Important notes before starting” for the R.E.A.L. Prep 96 Plasmid Protocol on pages 14–15 before beginning the procedure. Additional information on cultivation of BAC clones is given in Appendix D on page 29.
- Yields of BAC DNA are most consistent when cultures are inoculated from fresh precultures grown in a 96-well block such as the square-well block provided. Precultures should be grown in 2x YT medium for 16 h at 37°C with shaking. Inoculating cultures for BAC preparations directly from single colonies, glycerol stocks, or after several passages of a culture may result in high differences in yield.
- For precultivation, fill each well of a standard 96-well microplate (U shape bottom; 300 µl maximum filling volume) with 150 µl of growth medium (LB or 2x YT) containing the appropriate selective antibiotic. Inoculate each well from a single bacterial colony or from glycerol stocks using a 96-pin device or an 8-channel pipet. Incubate the cultures for 12–16 h at 37°C with shaking at 180 rpm.

Aeration of the plate can be achieved by using adhesive tape with 1–2 holes pierced above each well.

The precultures can be used as glycerol stocks after inoculation of the main overnight culture by adding 100 µl sterile 60% glycerol. Seal the plate with a tape pad and store at –70°C.

- BAC, PAC, or P1 miniprep cultures should be grown in 48-well blocks for 16 hours at 37°C with shaking. Use 2.5 ml 2x YT per well. The antibiotic concentration in the medium should be 25 µg/ml for kanamycin or 12.5 µg/ml for chloramphenicol.
- To facilitate inoculation of the main cultures, use a 48-pin device or an 8-channel pipet for more reproducible inoculation. When using a multichannel pipet, inoculate using a dilution of approximately 1/1000 (2.5 µl into 2.5 ml).
- A centrifuge capable of attaining at least 2500 x g and preferably up to 6000 x g (e.g., the QIAGEN 96-Well-Plate Centrifugation System, see page 41), with a swinging-bucket rotor for two or more microplates and sufficient clearance for square-well blocks is required.

Procedure

Growing bacterial cultures

1. **Fill each well of a 48-well block with 2.5 ml of 2x YT containing the appropriate selective antibiotic. Using a 48-pin device or an 8-channel pipet, inoculate each well with clones from precultures grown in a 96-well block. Incubate the cultures for 16 h at 37°C with shaking at 175 rpm.**

48-well blocks must be purchased separately (see Ordering Information, page 41). As an alternative option, BAC cultures can also be grown in 96-well square-well blocks.

Aeration of the blocks can be achieved by using AirPore Tape Sheets (see Ordering Information, page 41). Alternatively, adhesive tape with 2–3 holes pierced above each well can be used.

Note: Yields of BAC DNA are most consistent when cultures are inoculated from precultures grown in a 96-well block such as the provided square-well block. Inoculating directly from single colonies may result in high differences in yield.

2. **Harvest the bacterial cells in the block by centrifugation for 10 min at 2500 x g in a centrifuge with a rotor for 96-well microplates. The block should be covered with adhesive tape during centrifugation. Remove medium by inverting the block.**

To remove the medium, peel off the tape and quickly invert the block over a waste container. Tap the inverted block firmly on a paper towel to remove any remaining droplets of medium.

WARNING: Ensure that the buckets on the rotor have sufficient clearance (4.5 cm) to accommodate the blocks before starting the centrifuge.

Preparing bacterial alkaline lysates

3. **Resuspend each bacterial pellet in 0.3 ml Buffer R1. Use an 8-channel pipet with a large fill volume (≥ 1 ml per channel) for buffer delivery. Tape the block and mix by vortexing.**

Ensure that RNase A has been added to Buffer R1. The pelleted cells should be resuspended completely, leaving no cell clumps.

4. **Add 0.3 ml Buffer R2 to each well, seal the block with new tape, mix gently but thoroughly by inverting 10 times, and incubate at room temperature for 5 min.**

Buffer R2 should be checked before use for SDS precipitation caused by low storage temperatures. If necessary, redissolve SDS by warming.

Do not vortex the lysates at this stage, as this will cause shearing of the bacterial genomic DNA. Do not incubate for more than 5 min. Additional incubation may result in increased levels of open circular plasmid. At the end of the incubation, the lysate should appear viscous and free of bacterial cell clumps.

Close the Buffer R2 bottle immediately after use to avoid acidification of Buffer R2 from CO₂ in the air.

- 5. Add 0.3 ml Buffer R3 to each well, seal the block with new tape, mix immediately by inverting 20 times, and incubate on ice for 10 min.**

Thorough mixing ensures uniform precipitation of potassium dodecylsulfate (KDS) and cellular debris.

Clearing lysates with QIAfilter 96

- 6. Place a QIAfilter 96 plate (yellow) in the top plate of the QIAvac 96 manifold. Place a new square-well block in the base and reassemble the manifold.**

Ensure that the numbering and orientation of the plate and new square-well block are in accordance with the lysis block, and that the wells of the block are properly aligned with the outlet nozzles of the QIAfilter 96 plate.

- 7. Transfer the lysates from step 5 to the wells of the QIAfilter 96 plate.**

Use a multichannel pipet with a sufficiently large fill volume (>1 ml per channel). Most of the precipitated material will stick to the walls of the culture block. This material contains virtually no BAC DNA and can be safely left behind. Occasionally, precipitate will clog the end of a pipet tip. Lightly tapping the tip on the bottom of the well of the culture block will break through the precipitate and allow the remaining material to be transferred.

Any unused wells may be sealed with tape for later use.

- 8. Apply vacuum (-200 to -300 mbar) until the lysates are completely transferred to the square-well block in the QIAvac base.**

Desalting and concentrating DNA by isopropanol precipitation

- 9. Take the square-well block containing the cleared lysates from the vacuum manifold. Add 0.7 volumes of room-temperature isopropanol to each well (0.63 ml for 0.9 ml of lysate), tape the block, and mix immediately by inverting 3 times.**

Precipitation may be enhanced by adding 2 μg glycogen (Sigma; working solution: 1 $\mu\text{g}/\mu\text{l}$).

When preparing multiple sets of 96 samples, add isopropanol to one block, tape, and mix by inversion before proceeding on to the next block. This will minimize separation of the tape from the block before the samples are mixed.

- 10. Centrifuge the block at 6000 x g for 30 min at room temperature to pellet the DNA. Remove the supernatants by quickly inverting the block over a waste container, then tapping it firmly, upside down, onto a paper towel.**

Mark the orientation of the block before centrifugation so that it can be spun in the same orientation in the ethanol wash step. DNA pellets from isopropanol precipitations have a glassy appearance and may be difficult to see. Handle the block carefully to avoid dislodging the pellets.

WARNING: The centrifugation conditions are close to the maximum specifications of most common rotors. Use of the QIAGEN 96-Well-Plate Centrifugation System 4K15C is recommended. Alternatively, the blocks can be spun at a lower *g* force (2500–6000 x *g*), but the centrifugation time may need to be extended (up to 60 min).

- 11. Wash each DNA pellet with 0.5 ml of 70% ethanol. Centrifuge the block (in the same orientation as before) at 6000 x g for 15 min to reconcentrate the pellets. Remove the wash solutions by inverting the block, then tapping it firmly, upside down, onto a paper towel. Air dry the pellets for 15 min or until no ethanol remains.**

Ensure that no alcohol droplets are visible after air drying, but do not overdry the DNA pellets as this will make them difficult to dissolve.

- 12. Redissolve the DNA pellets in 22 μ l 10 mM Tris-Cl, pH 8.5 by incubating overnight at room temperature.**

When the BAC DNA is to be used for sequencing, the tubes can be vortexed to accelerate resuspension. Although vortexing leads to shearing of the high-molecular-weight DNA, this does not affect sequencing applications. However, if the BAC DNA is to be used for mapping applications, vortexing and repeated pipetting should be avoided to prevent shearing of the DNA.

Appendix A: Background Information

Growth of bacterial cultures

Plasmids are generally prepared from bacterial cultures grown in the presence of a selective agent such as an antibiotic (2, 3). The yield and quality of prepared plasmid DNA may depend on factors such as plasmid copy number, host strain, inoculation, antibiotic, and type of culture medium.

Plasmid copy number

Plasmids vary widely in their copy number per cell (Table 3), depending on the origin of replication they contain (pMB1, ColE1, or pSC101 for example) which determines whether they are under relaxed or stringent control, and depending on the size of the plasmid and its associated insert. Some plasmids, such as the pUC series and derivatives, have mutations which allow them to reach very high copy numbers within the bacterial cell. Plasmids based on pBR322 and cosmids are generally present in lower copy numbers. Very large plasmids and cosmids are often maintained at very low copy numbers per cell.

Table 3. Origins of replication and copy numbers of various plasmids (2).

DNA construct	Origin of replication	Copy number	Classification
Plasmids			
pUC vectors	ColE1	500–700	high copy
pBluescript vectors	ColE1	300–500	high copy
pGEM vectors	pMB1 *	300–400	high copy
pTZ vectors	pMB1 *	>1000	high copy
pBR322 and derivatives	pMB1 *	15–20	low copy
pACYC and derivatives	p15A	10–12	low copy
pSC101 and derivatives	pSC101	~5	very low copy
Cosmids			
SuperCos	ColE1	10–20	low copy
pWE15	ColE1	10–20	low copy
BACs, PACs, P1s			
pBeloBAC	F Plasmid	1–2	very low copy
pCYPAC-1	P1	1–2	very low copy
pAD10sacBII	P1	1–2	very low copy

* The pMB1 origin of replication is closely related to that of ColE1 and falls in the same incompatibility group. The high copy number plasmids listed here contain mutated versions of this origin.

Host strains

Most *E. coli* strains can be used successfully to isolate plasmid DNA, although the strain used to propagate a plasmid has an effect on the quality of the purified DNA. Host strains such as DH1, DH10B, DH5 α , and C600 give high-quality DNA. The slower growing strain XL1-Blue also yields DNA of very high quality which works extremely well for sequencing. Strain HB101 and its derivatives, such as TG1 and the JM series, produce large amounts of carbohydrates which are released during lysis and can inhibit enzyme activities if not completely removed (3). In addition, these strains have high levels of endonuclease activity which can reduce DNA quality. The methylation and growth characteristics of the strain should also be taken into account when selecting a host strain. XL1-Blue, DH10B, and DH5 α are highly recommended for reproducible and reliable results.

Inoculation

Bacterial cultures for plasmid preparation should ideally be grown from a single colony picked from a freshly streaked selective plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures is also possible, but may lead in rare cases to uneven yields of plasmid DNA.

The desired clones should be streaked directly after transformation onto a freshly prepared agar plate containing the appropriate selective agent, so that single colonies can be isolated. The agar plate used for the inoculation should not be older than one week. The colonies should be inoculated into a multiwell block containing media supplemented with the appropriate selective agent and grown with vigorous shaking for 16–24 hours. Cultivating bacteria for more than 24 hours is not recommended since cells begin to lyse, potentially reducing plasmid yields.

Antibiotics

Antibiotic selection should be applied at all stages of growth. Many plasmids in use today do not contain the *par* locus which ensures that the plasmids segregate equally during cell division. Daughter cells which do not receive plasmids will replicate much faster than plasmid-containing cells in the absence of selective pressure, and can quickly take over the culture.

The stability of the selective agent should also be taken into account. Resistance to ampicillin, for example, is mediated by β -lactamase which is encoded by the plasmid-linked *bla* gene and which hydrolyzes ampicillin. Levels of ampicillin in the culture medium are thus continually depleted. This phenomenon is clearly demonstrated on ampicillin plates, where “satellite colonies” appear as the ampicillin is hydrolyzed in the vicinity of a growing colony. Ampicillin is also very sensitive to temperature, and when in solution should be stored frozen in single-use aliquots. The recommendations given in Table 4 on page 25 are based on these considerations.

Table 4. Concentrations of commonly used antibiotics

Antibiotic	Stock solutions		Working concentration (dilution)
	Concentration	Storage	
Ampicillin (sodium salt)	50 mg/ml in H ₂ O	-20°C	100 µg/ml (1/500)
Chloramphenicol	34 mg/ml in ethanol	-20°C	170 µg/ml (1/200)
Kanamycin	10 mg/ml in H ₂ O	-20°C	50 µg/ml (1/200)
Streptomycin	10 mg/ml in H ₂ O	-20°C	50 µg/ml (1/200)
Tetracycline HCl	5 mg/ml in ethanol	-20°C	50 µg/ml (1/100)

Culture media

High-copy plasmids

Luria-Bertani (LB) broth is the recommended culture medium for high-copy-number plasmid purification, whereas 2x YT is recommended for low-copy-number plasmid purification with the R.E.A.L. Prep 96 Plasmid Kit. Richer broths such as TB (Terrific Broth) lead to extremely high cell densities which can overload the purification system. It should be noted that cultures grown in TB may yield 2–5 times the number of cells compared to cultures grown in LB. If these media are used for high-copy-number plasmid purification, recommended culture volumes must be reduced or the cultivation time must be decreased to match the capacity of the QIAfilter membrane. If too high a culture volume is used, alkaline lysis will be inefficient, the QIAfilter membrane will be overloaded and will become clogged, and the performance of the system will be unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing, which may result in shearing of bacterial genomic DNA and contamination of the plasmid DNA. Care must also be taken if strains are used which grow unusually fast or to very high cell densities. It is best to calculate culture cell density and adjust the volume accordingly.

Please note that a number of slightly different LB culture broths, containing different concentrations of NaCl, are in common use. Although different LB broths produce similar cell densities after overnight culture, plasmid yields can vary significantly. We recommend growing cultures in LB medium containing 10 g NaCl, 10 g tryptone, and 5 g yeast extract per liter to obtain the highest plasmid yields with the R.E.A.L. Prep 96 System.

Low-copy plasmids

2x YT broth is the recommended culture medium for cultivating BACs, PACs, P1s and other low copy number plasmids for purification using the R.E.A.L. Prep 96 procedure. This medium allows higher cell densities to be achieved which permits higher yields of plasmid DNA. 2x YT medium should contain 16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, adjusted to pH 7.0.

Preparation of cell lysates

Bacteria are lysed under alkaline conditions. After harvesting and resuspension, the bacterial cells are lysed in NaOH/SDS (Buffer R2) in the presence of RNase A (1, 4). SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents while the alkaline conditions denature the chromosomal and plasmid DNAs, as well as proteins. The optimized lysis time allows maximum release of plasmid DNA without release of chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline conditions may cause the plasmid to become irreversibly denatured (2). This denatured form of the plasmid runs faster on agarose gels and is resistant to restriction enzyme digestion.

The lysate is neutralized and adjusted to high-salt binding conditions in one step by the addition of Buffer R3. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris, and KDS* to precipitate, while the smaller plasmid DNA renatures correctly and stays in solution. It is important that the solution is thoroughly and gently mixed to ensure complete precipitation.

To prevent contamination of plasmid DNA with chromosomal DNA, vigorous stirring and vortexing must be avoided during lysis. Separation of plasmid from chromosomal DNA is based on coprecipitation of the cell-wall-bound chromosomal DNA with insoluble complexes containing salt, detergent, and protein. Plasmid DNA remains in the clear supernatant. Vigorous treatment during the lysis procedure will shear the bacterial chromosome, leaving free chromosomal DNA fragments in the supernatant. Since chromosomal fragments are chemically indistinguishable from plasmid DNA under the conditions used, the two species will be copurified. Mixing during the lysis procedure must therefore be carried out by slow, gentle inversion of the block.

Agarose gel analysis of plasmid DNA

The quality and yield of plasmid DNA obtained with the R.E.A.L. Prep 96 procedure can be analyzed by agarose gel electrophoresis as shown in Figure 2.

Reproducible Plasmid Purification

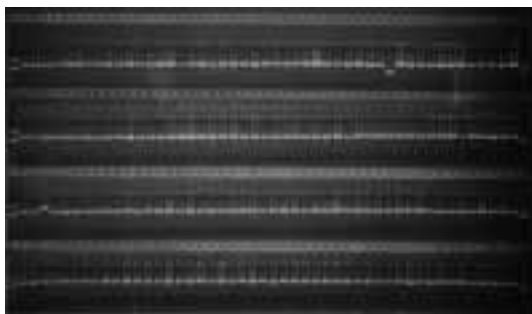


Figure 2. Agarose gel analysis of shotgun clones purified using the R.E.A.L. Prep 96 procedure. Approximately 200 ng DNA was loaded per lane onto a 1% TAE agarose gel. Outer lanes: markers.

* Potassium dodecyl sulfate.

Appendix B: Overview of Protocol Recommendations

Recommendation	Construct				
	High-copy plasmid	Low-copy plasmid	Cosmid	Cosmid (alternative)	BAC, PAC, P1
Blocks for cultivation	96-well	96-well	96-well	48-well	48-well
Volume of medium per well	1.3 ml	1.3 ml	1.3 ml	2.5 ml	2.5 ml (2 x 2.5 ml)*
Medium	LB	2x YT	2x YT	2x YT	2x YT
Cultivation time	20–24 h	20–24 h	20–24 h	16 h	16 h
Shaker settings	220 rpm	220 rpm	220 rpm	175 rpm	175 rpm
Clones	Single colonies	Single colonies	Single colonies; precultures from glycerol stocks	Single colonies; precultures from glycerol stocks	Precultures from glycerol stocks
Volume for resuspension of DNA	50–250 µl	50 µl	50 µl	50 µl	22 µl

* see Appendix F, page 34.

Appendix C: Recommendations for Capillary Sequencers

Shotgun plasmid clones purified using a R.E.A.L. Prep 96 Plasmid Kit were tested at QIAGEN for fluorescent capillary sequencing using the MegaBACE 1000 and the ABI PRISM 3700. Successful sequencing results were achieved using the following conditions.

Sequencing setup

Device	MegaBACE 1000	ABI PRISM 3700
Template	300 ng	300 ng
Primer	8 pmol	8 pmol
Premix	4 μ l DYEnamic™ ET Terminator Premix	2 μ l BigDye™ Terminator Ready Reaction Premix
10x Taq Polymerase Buffer	–	1 μ l
Final Reaction Volume	10 μ l	10 μ l

Cycling conditions

Device	MegaBACE 1000	ABI Prism 3700
Denaturation	95°C; 20 s	95°C; 30 s
Annealing	50°C; 15 s	50°C; 10 s
Elongation	60°C; 1 min	60°C; 4 min
Number of cycles	25	25 or 30

Dye-terminator removal

For the removal of the non-incorporated sequencing dyes, we strongly recommend use of the DyeEx™ 96 Kit. Detailed protocols for the purification of fluorescent sequencing reactions can be found in the handbook supplied with the DyeEx 96 Kit.

Sample loading

The best results at QIAGEN for both sequencers were obtained by using the “water loading” protocol.

Appendix D: Recommendations for Cultivation of BAC Clones

The most critical factor in BAC template preparation for end sequencing is proper cultivation of BAC clones. The following conditions are recommended for cultivation:

- Always inoculate cultures for BAC preparations from fresh precultures. We recommend using a multichannel pipet to obtain the most reproducible inoculation.
- For BAC clone preculture procedures please refer to the “Important notes before starting” section of the R.E.A.L. Prep 96 BAC Protocol on page 19.
- Do not inoculate cultures for BAC preparations directly from glycerol stocks or single colonies from agar plates, or from precultures which are more than one day old.
- Do not passage the preculture. After inoculating the culture for preparation, discard the preculture or prepare a glycerol stock.
- Avoid repeated thaw/freezing cycles of glycerol stocks. If stocks will be frequently needed, prepare and use replica plates.

After proper cultivation, up to three sequencing reactions or, alternatively, one control restriction digest and two sequencing reactions can be performed per BAC preparation. Yields should be checked by restriction digestion and subsequent electrophoresis on a 0.7% agarose gel (see Figure 3, page 31). Based on the results from this analysis, appropriate clones for one or two sequencing reactions can be chosen.

Appendix E: Downstream Applications with BAC

P1, PAC, or BAC DNA obtained with the R.E.A.L. Prep 96 BAC Protocol (page 19) can be used directly for restriction digestion, PCR, sequencing, and other applications. Typical yields are in the range 30–40 ng/μl, depending on the copy number and growth characteristics of the large construct.

Restriction digestion

Restriction digests using 7–10 μl purified DNA should be readily detectable on an ethidium bromide-stained agarose gel (0.6–0.7 %) after incubation for 1 h with 5–10 units of restriction endonuclease and the appropriate reaction buffer (see Figure 3 on page 31 for a typical result).

Restriction Analysis of BAC Clones

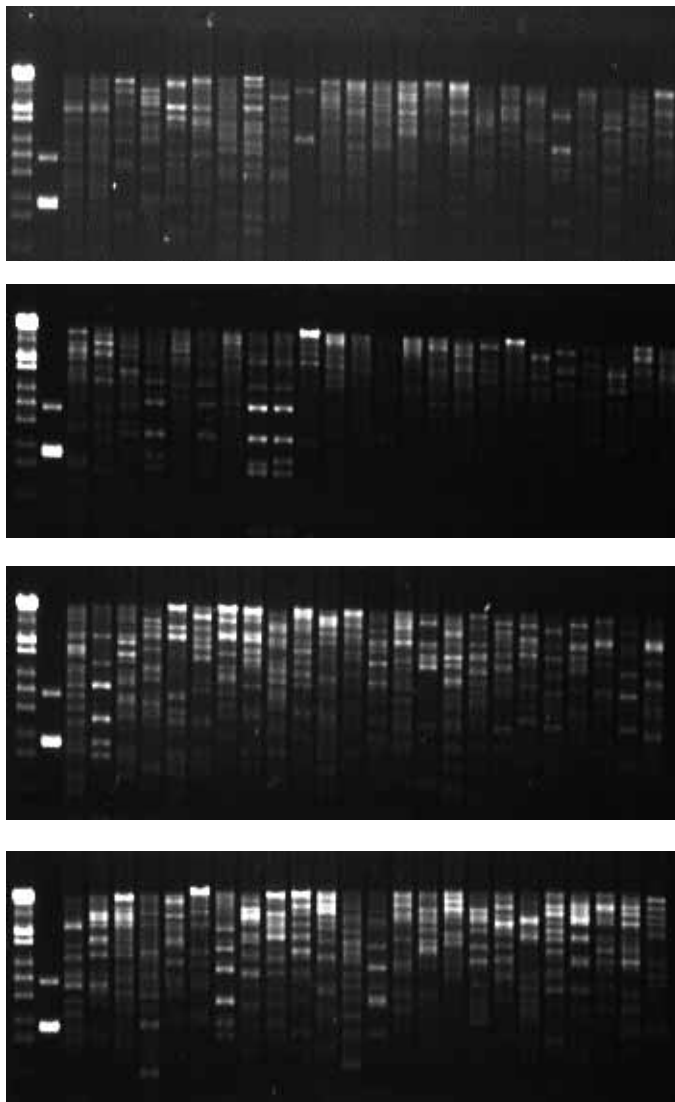


Figure 3. Typical agarose gels of control restriction digests of 96-well plate IGF-BAC clones from an *Arabidopsis thaliana* BAC library. For restriction digestion 7 μ l BAC DNA purified using the R.E.A.L. Prep 96 BAC Protocol was incubated for 1 h with 5 units of EcoRI and the appropriate reaction buffer. The completed reaction was loaded on an ethidium bromide-stained agarose gel (0.7 %). Markers, 1st lane of each gel: Lambda-Styl (~300 ng); 2nd lane of each gel: pGEM (100 ng).

Fluorescent DNA sequencing

BAC end sequencing is a very common method for mapping BAC libraries. This method only became feasible for small-scale BAC preparations with the introduction of sequencing dyes such as BigDye Terminator chemistry or ET-Primers. Below are recommended sequencing conditions for BAC end sequencing using a sequencer such as the ABI PRISM 377XL DNA Sequencer.

Sequencing reactions

For fluorescent DNA sequencing with BigDye Terminator chemistry, we recommend using: 7–10 μ l DNA, 3.2 pmol primer, and 8 μ l ready reaction premix in a total reaction volume of 20–22 μ l (depending on volume of DNA template used), with the following cycling conditions:

5 min denaturation at	95°C
30 cycles of:	95°C for 30 s
	50°C for 10 s
	60°C for 4 min

Or alternatively:

7–10 μ l DNA, 10–12 pmol primer, and 8 μ l ready reaction premix in a total reaction volume of 20–22 μ l (depending on volume of DNA template used), with the following cycling conditions:

2 min denaturation at	96°C
75 cycles of:	96°C for 10 s
	50°C for 5 s
	60°C for 4 min

For efficient removal of the non-incorporated sequencing dyes, we recommend use of the DyeEx 96 Kit, or the DyeEx Spin Kit. The purified and dried sequencing reactions should be resuspended in 1.0 μ l loading buffer, and the entire sequencing reaction loaded.

Gel and electrophoresis conditions

The following gel and electrophoresis conditions are recommended: 2x gel; 5% polyacrylamide (29:1); 100 B/h; 36 cm well-to-read gel in combination with a 48-lane comb. These conditions give the best results in terms of resolution, signal strength, reading length, and accuracy.

Software version: ABI PRISM 377XL Collection 2.0

Plates: Standard length 36 cm WTR, 0.2 mm spacers, and a 48-lane comb

Gel preparation (5% polyacrylamide, 1x TBE)

Mix 21 g urea and 8.4 ml of a 30% acrylamide solution (29:1 acrylamide:bisacrylamide ratio) in 20 ml H₂O. Dissolve the urea by stirring and warming (take care not to heat to above 50°C), and deionize the gel solution with Serva SERDOLIT® for 10 minutes. Pass 6 ml of 10x TBE buffer through a 0.2 µm filter, and then add the gel solution. Degas the mixture for 1 minute under vacuum. Add 350 µl ammonium persulfate (10%) and 15 µl TEMED for the polymerization (>3 h). After 1 hour place a wet paper towel at the bottom of the gel to avoid the occurrence of the “red rain” phenomenon.

Electrophoresis conditions

A 10 minute pre-run with the pre-run module PR36A-1200, followed by a 9 hour run with the run module 36(E)-1200 is recommended.

Modules:	PR36A-1200	Voltage	1000 V
		Current	35 mA
		Power	50 W constant
		Collection time	1 h
		Gel temperature	51°C
	36(E)-1200	Voltage	3000 V
		Current	50 mA
		Power	48 W constant
		Collection time	9 h
		Gel temperature	45°C

These recommended electrophoresis conditions normally result in high accuracy for read lengths of up to 700 bases. If only short reads of up to 500 bases are required, the electrophoresis should be performed under the same gel conditions, except that the time setting in the run module should be decreased to 6.5–7 hours.

Sequence troubleshooting

For sequencing of BAC clones, the gel and electrophoresis conditions given above are generally optimal. Other conditions such as 4 x runs or use of a 36- or 96-lane comb lead to decreased signal strength and resolution, resulting in smaller read lengths and lower accuracy. Typical sequence traces have signal strengths of between 50 and 120 for each dye. If the signal strength is below 40, use the Weak Signal Basecaller module for analysis, or increase the number of sequencing cycles to 45. The background should normally be low due to the good resolution of the gel. A high background in the electropherogram may reflect an excess of primer. For setting up sequencing reactions, start with a primer concentration of 0.8 pmol/µl as a working solution and optimize as required.

Appendix F: Modified R.E.A.L. Prep 96 BAC Procedure for Capillary Sequencing

Due to the different loading procedures of slab-gel and capillary sequencers, increased amounts of BAC DNA must be used for BAC end sequencing on capillary sequencers. The reason for this is that only a small amount of the reaction is injected into the capillary during electrokinetic injection, whereas on a slab-gel sequencer the entire reaction can be loaded onto the gel.

In order to enable BAC end sequencing on the ABI PRISM 3700 DNA Analyzer and to overcome yield problems for difficult BAC libraries, we have developed a modified BAC preparation procedure using the R.E.A.L. Prep 96 Kit. The major differences to the previously described protocol are; the doubling of the culture volume (e.g., for 96 clones use four 48-well growth blocks) and a second purification of the clones using the DyeEx 96 Kit.

Procedure

- Fill four 48-well growth blocks with medium and inoculate each clone into two wells, in order to double the volume of medium per clone.
- After cultivation and sedimentation of the cells, resuspend each cell pellet in 200 μ l Buffer R1.
- Combine the corresponding, resuspended clones in a well of a 48-well block.
- Follow the lysis and purification protocol as listed on pages 19–21, with following exceptions:
 - use 400 μ l Buffer R2 and Buffer R3.
 - Take 840 μ l isopropanol for the precipitation (since the 96-well block used for the precipitation is almost full, carefully tape the block before mixing).
- To further increase purity of the DNA, after resuspension in 10 mM Tris-Cl, pH 8.5, load the entire BAC preparation onto a DyeEx 96 plate, spin and collect the eluate.
- The BAC DNA can be directly used for downstream reactions. Sequencing reactions should be set up as recommended in Appendix E. For BAC end sequencing on the 3700 DNA Analyzer we recommend the use of the DyeEx 96 Kit for dye-terminator removal and the “water loading” protocol.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol(s) in this handbook or molecular biology applications (see inside front cover for contact information).

Comments and suggestions

Low or no yield

General

Low yields may be caused by a number of different factors. To find the source of the problem, check each suggestion below.

Low DNA yield (BAC DNA preps)

Use fresh glycerol cultures and avoid repeated freeze/thaw cycles of clones. 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 precultures for inoculation. The remainder of the precultures can be used to set up new glycerol stocks.

No DNA in eluates

a) Plasmid did not propagate

Read "Growth of bacterial cultures" section (pages 23–25) and check that the conditions for optimal growth were met.

b) Lysate prepared incorrectly

Check storage conditions and age of buffers and check buffer volumes added to the samples.

c) Buffer R2 precipitated

Redissolve by warming to 37°C.

d) Cell resuspension incomplete

Pelleted cells should be completely resuspended in Buffer P1. Do not add Buffer P2 until an even suspension is obtained.

Little DNA in eluates

Poor plasmid propagation

Check the age of the clones and the culture conditions. Try again with freshly prepared single colonies or a new clone library.

Low DNA quality

RNA in eluates

- a) RNase A digestion omitted
- b) RNase A digestion insufficient

Ensure that RNase A is added to Buffer R1 before use.

Reduce culture volume if necessary. If Buffer R1 containing RNase A is more than 6 months old, add fresh RNase A.

Genomic DNA in eluates

- a) Buffer R2 added incorrectly
- b) Buffer R3 added incorrectly
- c) Lysis too long
- d) Culture overgrown

The lysate must be handled gently after addition of Buffer R2 to prevent shearing. Reduce culture volume if lysate is too viscous for gentle mixing.

Upon addition of Buffer R3, mix the sample immediately, but gently.

Lysis in step 4 must not exceed 5 minutes.

Overgrown cultures contain lysed cells and degraded DNA. Do not grow cultures for longer than 16–24 hours.

DNA does not perform well

General

- a) Eluate salt concentration too high
- b) Nuclease contamination

Check that the ethanol wash was carried out properly. If the problem remains, perform a second ethanol wash step.

Do not perform isopropanol precipitation in a cold centrifuge, as this encourages salt precipitation.

When using endA⁺ host strains such as HB101 and its derivatives, the JM series, or any wild-type strain, perform the heat inactivation step (step 6) on page 16.

c) Eluate contains residual ethanol

Ensure that the precipitated DNA is dried correctly before resuspension.

Poor restriction digestion (BAC DNA)

Poor quality BAC DNA

Incorrect volumes of the lysis buffers added to the samples. Repeat with correct volumes of buffers.

Sequencing-related problems

General

a) 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.

b) Low signal

Increase the number of cycles to 45 or 75 for the sequencing reactions or increase the amount of template DNA used.

c) Short read length

Check the template concentration; ensure that the 70% ethanol wash step is performed correctly to avoid salt contamination; increase the number of cycles to 45 or 75 for the sequencing reactions; or increase the amount of DNA template used.

Fluorescent DNA sequencing

a) General

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 can be problematic, even if ultrapure quality DNA is used.

b) Read length too short

Check that the isopropanol precipitation and the ethanol wash were carried out properly to avoid problems of salt and ethanol contamination.

If using the ABI PRISM 3700 sequencer, ensure that dye terminators are removed from sequencing reactions using DyeEx technology (spin columns or 96-well plates; see Ordering Information on page 41). If sequencing reactions are not cleaned up prior to loading, electroinjection into capillaries is inefficient, and short sequence reads are obtained due to salt contamination. With the capillary sequencing recommendations on page 34, very long sequence reads, typically >600 bases, can be achieved with R.E.A.L. Prep 96-purified DNA.

References

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Ordering Information

Product	Contents	Cat. No.
R.E.A.L. Prep 96 Plasmid Kit (4)*	For 4 x 96 rapid extraction alkaline lysis minipreps: 4 QIAfilter 96 Plates, Square-Well Blocks, Tape Pads, Reagents, Buffers	26171
R.E.A.L. Prep 96 Plasmid Kit (24)*	For 24 x 96 rapid extraction alkaline lysis minipreps: 24 QIAfilter 96 Plates, Square-Well Blocks, Tape Pads, Reagents, Buffers	26173
R.E.A.L. Prep 96 BioRobot Kit (4)†	For 4 x 96 automated rapid extraction alkaline lysis minipreps: 4 QIAfilter 96 Plates, Flat-Bottom Blocks, Square-Well Blocks, Reagents,	961141
Related products		
micro.R.E.A.L. Prep 384 Plasmid Kit (20)	For 20 x 384 rapid extraction alkaline lysis minipreps: 20 x 384-Well Filter Plates, 20 x 384-Well Collection Plates, Reagents, Buffers, Tape Pads Buffers, Tape Pads	26363
Accessories		
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder, Rack of Collection Microtubes (1.2 ml)	19504
Vacuum Regulator	For use with QIAvac manifolds	19530
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570

* Requires use of QIAvac 96

† Larger kit sizes also available; please inquire.

Ordering Information

Product	Contents	Cat. No.
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks during bacterial cultivation: 50 sheets per pack	19571
Square-Well Blocks (24)	96-well blocks with 2.2 ml wells, 24 blocks per case	19573
48-Well Blocks (24)	48-well blocks with 5 ml wells, 24 blocks per case	19577
96-Well-Plate Centrifugation System		
Plate Rotor 2 x 96	Rotor for 2 QIAGEN 96 plates for use with QIAGEN Centrifuges*	81031
Centrifuge 4-15C†	Universal laboratory centrifuge with brushless motor	Inquire
Centrifuge 4K15C†	Universal refrigerated laboratory centrifuge with brushless motor	Inquire
DyeEx Kits for dye-terminator removal		
DyeEx Spin Kit (50)	50 DyeEx Spin Columns, Collection Tubes (2 ml)	63104
DyeEx 96 Kit (4)‡	4 DyeEx 96 Plates; 48-Well Collection Plates	63181

* The Plate Rotor 2 x 96 is available exclusively from QIAGEN. Under the current liability and warranty conditions, the rotor may only be used in Centrifuge 4-15C and 4K15C from QIAGEN, the freely programmable models of centrifuges 4-15, 4K15, 6-10, 6K10, 6-15, and 6K15 from Sigma Laborzentrifugen GmbH.

† Not available in all countries; please inquire.

‡ Larger kit sizes also available; please inquire.

Ordering Information

Product	Contents	Cat. No.
Other related products		
MagAttract 96 Miniprep Core Kit (24)	MagAttract Suspension (Miniprep) and Buffers for 24 x 96 minipreps	120030
QIAprep 96 TurboKit (24)*†	For 4 x 96 high-purity plasmid minipreps. 4 each: TurboFilter® 96 Plates, QIAprep 96 Plates, Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27191
QIAprep 96 Turbo BioRobot Kit (4)	For 4 x 96 high-purity plasmid minipreps, 4 each: TurboFilter 96 and QIAprep 96 Plates; Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml) and Caps, 96-Well Microplates RB and Lids, Tape Pads	962141
QIAwell 96 Ultra Plasmid Kit (4)*†	For 4 x 96 ultrapure plasmid minipreps, 4 each: QIAfilter 96, QIAwell 96, and QIAprep 96 Plates; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	16191
QIAwell 96 Ultra BioRobot Kit (4)	For 4 x 96 ultrapure plasmid minipreps, 4 each: QIAfilter 96, QIAwell 96, and QIAprep 96 Plates; Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml) and Caps, 96-Well Microplates RB and Lids, Tape Pads	960141

* Requires use of QIAvac 96

† Larger kit sizes also available; please inquire.

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