*micro*R.E.A.L.™ Prep 384 Plasmid Handbook

For

Very high-throughput rapid extraction alkaline lysis minipreps of plasmid DNA

June 2001
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Kit Contents

	***micro*R.E.A.L. Prep 384 Plasmid Kit (20)**
	Catalog No. 26363
No. of preparations	7680
384-Well Filter Plates	20
384-Well Collection Plates	20
Buffer R1	2 x 150 ml
Buffer R2	2 x 150 ml
Buffer R3	2 x 150 ml
RNase A*	2 x 15 mg
Tape Pads	14 (25 sheets per pad)

** Provided as a 100 mg/ml solution.*

For cultivation of bacterial cultures in a 96-well microplate format, we recommend 96-Well Microplates MC. These plates are available separately (cat. no. 19586). For cultivation of bacterial cultures in 384-well format we recommend UniPlates® 400 (available from Polyfiltronics Inc., www.polyfiltronics.com).

Storage Conditions

*micro*R.E.A.L.™ Prep 384 Plasmid Kits should be stored at room temperature (15–25°C). Kits can be stored for up to 12 months without showing any reduction in performance or quality.

Note: After addition of RNase A, Buffer R1 is stable for 6 months when stored at 2–8°C.

Quality Control

As part of the stringent QIAGEN quality assurance program, the performance of *micro*R.E.A.L. Prep 384 Plasmid Kits is monitored routinely on a lot-to-lot basis. All kit components are tested separately to ensure highest performance and reliability.

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 *micro*R.E.A.L. Prep 384 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 local distributors (see inside front cover).

Product Use Limitations

*micro*R.E.A.L. Prep 384 Kits are developed, designed, and sold for research purposes only. They are 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.

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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).

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 convenient and compact PDF format at **www.qiagen.com/ts/msds.asp** where you can find, view, and print the MSDS for this kit and its components.

The following risk and safety phrases apply to components of the *micro*R.E.A.L. Prep 384 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

** 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 clothing and gloves; S46: If swallowed, seek medical advice immediately and show this container or label.*

Introduction

The *micro*R.E.A.L. Prep 384 procedure is designed for rapid very high-throughput purification of plasmids from small-volume bacterial cultures. It is based on a modified alkaline lysis procedure, which has been adapted for 384-well processing.

Cultivation and lysis can be carried out either in four 96-Well Cultivation Plates (cat. no. 19586) or in 384-well UniPlate® 400 blocks (available from Polyfiltronics Inc., www.polyfiltronics.com). When using 96-well plates, lysates from all four plates are transferred to a 384-well filter plate. For 384-well blocks, the format remains the same. Protocols are provided in this handbook for both 96-well and 384-well methods.

The *micro*R.E.A.L. Prep 384 Principle

The *micro*R.E.A.L. Prep 384 System provides a fast, simple, and cost-effective method for high-throughput purification of plasmid DNA for use in routine molecular biology applications.

Alkaline lysis of bacteria

After clearing of the lysates, plasmid DNA is purified and concentrated by an isopropanol precipitation. After a single wash step, the precipitated plasmid DNA is resuspended in low-salt buffer and is ready for direct use in downstream applications.

Applications and yields

The yield and quality of DNA obtained with the *micro*R.E.A.L. Prep 384 procedure depends on the cultivation conditions and the host–vector system. Yields of 1–1.5 µg plasmid DNA are typically obtained if the recommended cultivation conditions in the protocol are followed.

The plasmid DNA is suitable for automated sequencing applications, in particular those using cycle sequencing chemistries based on improved thermostable DNA polymerases (AmpliTaq® DNA Polymerase FS or ThermoSequenase™ II). For fluorescent sequencing reactions, we strongly recommend BigDye™ Terminator chemistry (PE Biosystems) or DYEnamic™ ET dye-terminator chemistry (Amersham Pharmacia Biotech).

Streamlining the Procedure Using Automated Devices

The procedure can be streamlined using automated liquid-handling devices. We recommend the following systems for individual steps of the procedure:

- Lysis: 96- and 384-channel pipetting systems
- Transfer of lysates: 96-needle microdispenser capable of changing from 96-well format for aspirating to 384-well format for dispensing
- Precipitation and wash steps: 384-channel pipetting system

Recommendations

Lysis and wash steps can be easily carried out by the use of multi-channel dispensing devices. The most suitable systems are the Multidrop® 384 Microplate Dispenser from Labsystems (www.finnpipette.com) or the QFill2 System from Genetix Ltd. (www.genetix.co.uk). Both systems are able to handle 96-well and 384-well formats.

A suitable device for the transfer of lysates is the Hydra® 96 Microdispenser from Robbins Scientific, together with their 384-well Plate Positioner accessory. Note that when using this accessory, a frame or a rack is required to ensure that the 384-well plates fit into the positioner. Other suitable automated systems (e.g., Zymark or TomTek systems) can also be used for streamlining the *micro*R.E.A.L. Prep 384 procedure.

Limitations of the use of these devices in the individual steps of the protocol are given in Table 1.

Table 1. Suitability of various devices for automation of the microR.E.A.L. Prep 384 procedure

	Dispensing lysis buffer		Transfer of lysates		Dispensing buffer for precipitation	Dispensing resuspension buffers
Format	96-well	384-well	96-well	384-well	96- and 384-well	96- and 384-well
Device						
Multidrop 384	+	–	–	–	+*	+*
QFill2	+	+	–	–	+*	+*
Hydra™ 96	+	–	+	–	+	+
Hydra™PP (Automated Plate Positioner)	+	+	+	+	+	+

*+ : suitable; – : not suitable; * modification may be necessary*

Due to the relatively high dispensing speed of most robotic systems, there is no need to mix lysates by vortexing when cultivating in a 96-well format. This is an advantage over cultivation in a 384-well format as thorough mixing of lysates by shaking or vortexing can be problematic.

The following are general recommendations for lysis in a 96-well format:

- After dispensing Buffer R1, optimal resuspension is obtained if the plates are attached to a vortex shaker or a similar device with a horizontal platform. If this is not available, plates may also be vortexed manually.
- Check if the automatic device for buffer delivery is capable of high-speed dispensing or already has a high dispensing speed built in. If this is the case, time for the mixing of buffers can be saved.

For the Hydra™ 96 Microdispenser using 96-well plates for cultivation and lysis, start out with the settings as recommended in Table 2.

Table 2. Recommended settings for the Hydra 96 Microdispenser using the 96-well cultivation protocol

Program File	1	2	3	4
Action	Lysis	Transfer	Precipitation	Wash
	Dispensing lysis buffers	Aspirating lysates in 96-well format; transfer into 384-well plate	Dispensing isopropanol into the 384-well collection plate	Dispensing wash buffer into 384-well plate
Parameters				
DV	30	90	65	100
DH	3980	890	1070	1070
FV	30	95	140	200
FH	4030	3990	3900	3900
EH	3600	3600	3600	3600
WV	150	150	150	150
WH	3600	3600	3800	3600
Number of washes	1	2 or 3	3	3
Plates	Solution tray filled with buffer	Cultivation plate filled with lysates	Solution tray filled with isopropanol	Solution tray filled with 70% ethanol
	96-well cultivation plate	384-well filter plate	384-well collection plate	384-well collection plate
	Solution tray filled with water	Solution tray filled with water	Solution tray filled with water	Solution tray filled with water

Hydra 96 Microdispenser Procedures

Program file 1: Lysis

Fill syringes with buffer from the solution tray and dispense buffer into cultivation plate. Wash needles once with water. Repeat for each plate. After dispensing, fill solution tray with water and perform 3 wash cycles.

Program file 2: Transfer

Place 96-well plate containing lysates into the device and aspirate lysates; place 384-well filter plate on top of a standard 384-well microplate into a 384-well plate positioner and dispense the lysates. After dispensing, fill the tray with water and perform 2 wash cycles. Repeat for remaining 3 plates, each time adjusting the thumbwheel of the plate positioner to the next position. After dispensing, fill solution tray with water and perform 3 wash cycles.

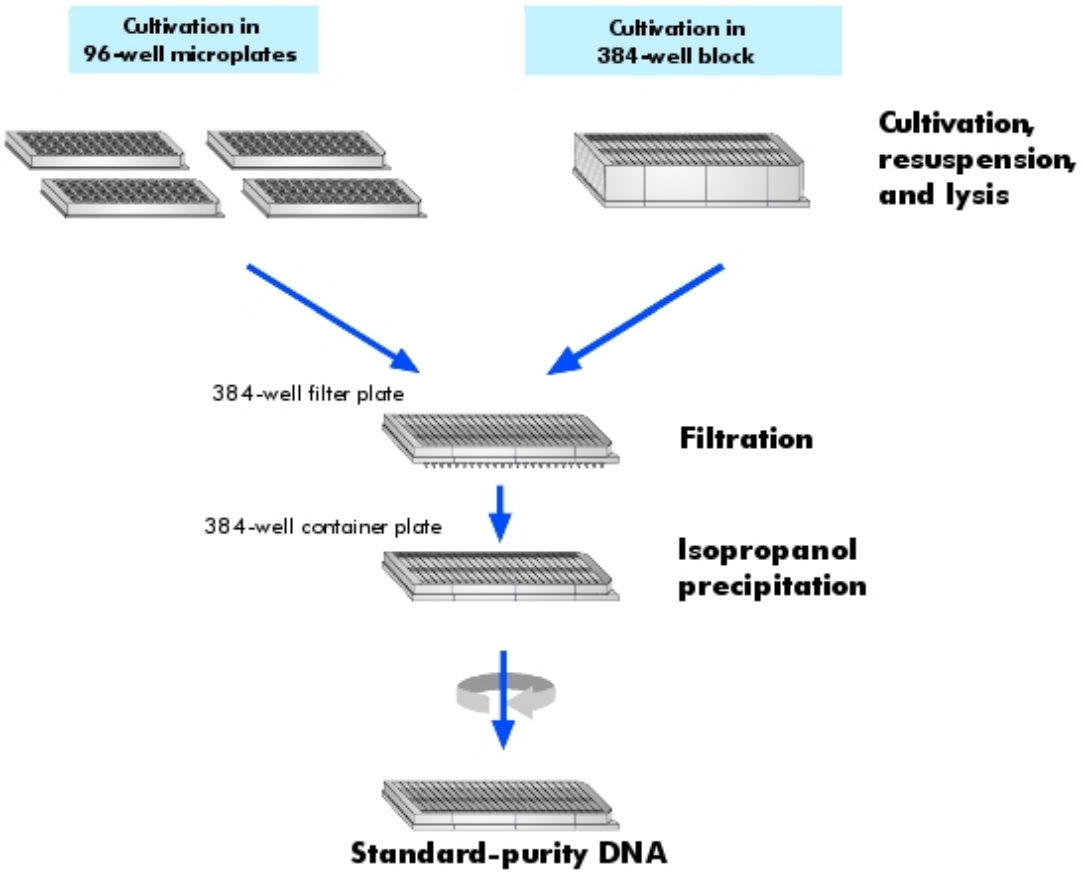
Program file 3: Precipitation

Place solution tray filled with isopropanol into the device and fill syringes; place 384-well collection plate into 384-well plate positioner and dispense isopropanol in two strokes. After each plate, adjust the thumbwheel of the plate positioner to the next position. Repeat dispensing for the remaining 2 positions without wash cycle. After dispensing, fill solution tray with water and perform 3 wash cycles.

Program file 4: Wash

Place solution tray filled with 70% ethanol into the device and fill syringes; place 384-well collection plate into 384-well plate positioner and dispense ethanol in two strokes. After each, adjust the thumbwheel of the plate positioner to the next position. Repeat dispensing for the remaining two positions without the wash cycle. After dispensing, fill solution tray with water and perform 3 wash cycles.

The *micro*R.E.A.L. Prep 384 Plasmid Kit Procedure



*micro*R.E.A.L. Prep 384 Plasmid Kit Protocol

This protocol is designed for very high-throughput purification of high copy number plasmids in a 384-well format.

Important notes before starting

General notes

- Bacterial cultures for plasmid preparation with the *micro*R.E.A.L. Prep 384 procedure should be grown in 2x YT media. When using the 384-well blocks for cultivation, Magnificent Broth (MacConnell Research Corp.; www.macconnell.com) can also be used, see page 24 for limitations. Use 350 µl media per well when cultivating bacteria in 96-well microplates, or 300 µl for cultivation in 384-well blocks.
- The density of the bacterial culture should be 3 x 10^8 – 2 x 10^9 cells/ml. If the cultures have higher densities, wells of the filter plate may be become clogged, leading to significantly lower yields.
- The *micro*R.E.A.L. Prep 384 procedure works optimally when the same vector–host strain combination is used for all the samples in a block or set of blocks that are prepared together. Cell cultivation conditions should be individually optimized for different vector–host strain combinations.
- If cultivation is carried out in a 96-well format, 4 plates of bacterial cultures are required for DNA purification in a 384-well format.
- DNA concentration should be determined for a small number of random samples using agarose gel electrophoresis. The concentration of plasmid DNA is generally too low to allow photometric quantitation at 260 nm using a 96-well plate OD reading system.

Notes on buffers

- Prior to preparation, add one vial of the RNase A solution provided to one bottle of Buffer R1. This solution is stable for 6 months when stored at 2–8°C.

Equipment to be supplied by the user

- Centrifuge capable of attaining 5796 x *g* (6000 rpm) with a swinging-bucket rotor for 2 or more 96-well microplates and sufficient clearance for 384-well blocks. We recommend the QIAGEN 96-Well-Plate Centrifugation System.
- AirPore™ Tape.

Procedure

A. 96-well procedure

For growth of bacterial cultures, we recommend the use of 96-Well Microplates MC (cat. no. 19586) which have a maximum volume of 0.5 ml.

Growing bacterial cultures in 96-well microplates

1. **Fill each well of four 96-well cultivation plates with 350 µl of 2x YT medium containing an appropriate selective antibiotic. Inoculate each well from a single bacterial colony or a glycerol stock using an automated colony picker or sterile toothpicks. Incubate the cultures for 18 h at 37°C with shaking at 180 rpm.**

   Plates should be covered with adhesive tape to prevent evaporation and cross-contamination. Aeration can be achieved by piercing 2 holes above each well with a standard injection needle. Do not use AirPore Tape as the evaporation rate is too high with this tape.

   Tip: An old 8-well strip can be easily adapted to make an 8-channel piercing device by inserting needles into each well. Take care to prevent the needles touching the medium as this will cause cross-contamination. To avoid cross-contamination we recommend piercing the tape pads on an empty plate and then sealing the inoculated culture plate with the pre-pierced tape pad sheets.

2. **Harvest the bacterial cells in the plates by centrifugation for 5 min at 2500 x *g* in a centrifuge with a rotor for 96-well microplates. Remove medium by inverting the plates.**

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

   Note: Two microplates can be centrifuged on top of each other in one rotor bucket. For safety, a rubber pad should be placed between the two plates.

   WARNING: Ensure that the buckets on the rotor have sufficient clearance to accommodate the microplates before starting the centrifuge.

Preparing bacterial lysates in 96-well microplates

3. **Resuspend each bacterial pellet in 30 µl Buffer R1. Use an 8-channel pipet or an automated device for buffer delivery. Cover the plates with adhesive tape and mix by vortexing.**

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

4. **Add 30 µl Buffer R2 to each well, seal the plates with a new sheet of tape, mix gently by vortexing, and incubate at room temperature for 5 min.**

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

   If a high pipetting speed is used, mixing the buffers is not necessary, otherwise vortex carefully at the lowest speed setting to avoid shearing the bacterial genomic DNA.

   Do not incubate the lysates for more than 5 min. Longer 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 by CO_2 in the air.

5. **Add 30 µl Buffer R3 to each well, seal the plates with a new sheet of tape, and mix gently by vortexing.**

   Centrifuge the plates for 5 min at 2500 x *g*.

   Note: Two 96-well microplates can be centrifuged on top of each other in one rotor bucket.

   WARNING: Ensure that the buckets on the rotor have sufficient clearance to accommodate the microplates before starting the centrifuge.

B. 384-well procedure

Growing bacterial cultures in 384-well blocks

For this cultivation procedure, we recommend the use of an Elevator Model Levitation Stirrer (VP 707A) or a Carousel Model Levitation Stirrer (VP 707B) from V&P Scientific, Inc. (www.vp-scientific.com). The use of these devices is a reliable way to solve the problems of aeration of cultures grown in 384-well UniPlate 400 blocks (available from Polyfiltronics Inc., www.polyfiltronics.com). In these systems aeration is facilitated by the movement of a magnetic ball which is placed in each well.

1. **Prior to dispensing media, add a stirring ball available from V&P Scientific Inc., to each well. Fill each well with 300 µl 2x YT medium or Magnificent Broth containing the appropriate selective antibiotic. Inoculate each well from a single bacterial colony or a glycerol stock using a colony picker or a 96-pin tool. Incubate the cultures for 20 h at 37°C.**

   The blocks should be sealed with an AirPore Tape Sheet to enable good aeration. When using the Elevator Model Levitation Stirrer, the controlling wheel should be set to the scale value ‘50’.

2. **Harvest the bacterial cells in the blocks by centrifugation for 5 min at 2500 x *g* in a centrifuge with a rotor for 96-well microplates.**

   Do not remove the balls, because they are needed to aid the resuspension and the mixing of the lysis buffer in the wells of the 384-well block. Remove medium using a suitable automated pipetting device.

Preparing bacterial lysates in 384-well blocks

3. **Resuspend each bacterial pellet in 30 µl Buffer R1. Use an 8-channel pipet or an automated liquid-handling device. Tape the block with an adhesive tape sheet and resuspend the cells by placing the blocks for 2 min in the V&P Scientific Elevator Model Levitation Stirrer. The controlling wheel should be set to the scale value '50'.**

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

4. **Add 30 µl Buffer R2 to each well, seal the blocks with a new sheet of tape, and mix by placing the blocks in the V&P Scientific Elevator Model Levitation Stirrer. Allow the block to perform 5 up-and-down strokes.**

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

   At the end of the incubation, the lysate should appear viscous and free of bacterial cell clumps.

5. **Add 30 µl Buffer R3 to each well, seal the block with a new sheet of tape, and mix by placing the block in the V&P Scientific Elevator Model Levitation Stirrer. Allow the block to perform 5 up-and-down strokes.**

6. **Centrifuge the block for 5 min at 2500 x *g*.**

Transfer and filtration of lysates in the 384-well plate

1. **Transfer the lysates (total volume 90 µl) into the wells of the 384-well filter plate using an 8-channel pipet or an automated liquid-handling device.**

   Ensure that the numbering and orientation of the 96-well plates are in accordance with the 384-well filter plate. When transferring samples manually, make sure that samples from each 96-well plate are transferred to the appropriate positions in the 384-well plate.

   Tip: Using wide-bore pipet tips may make manual transfer easier.

2. **Place the 384-well filter plate on top of a 384-well collection plate and centrifuge together for 10 min at 2500 x *g*.**

   If lysate remains in any of the wells, repeat this centrifugation step.

Desalting and concentrating DNA by isopropanol precipitation

3. **Add 65 µl isopropanol to each well of the 384-well collection plate, carefully seal the plate with a tape sheet, and mix the samples by inverting the plate 10 times.**

4. **Centrifuge the plates at 5796 x *g* (6000 rpm) for 30 min at room temperature to pellet the DNA. Remove the supernatants by quickly inverting the plate over a waste container, then tapping it firmly, upside down, onto a paper towel.**

   With certain centrifuges, centrifugation may cause the outer rim of the 384-well collection plate to crack. This can be avoided by placing a small stack of tape pads, or a piece of plastic or cardboard (which fits within the rim of the plate) under the 384-well collection plate, to avoid direct contact between the rim of the plate and the centrifuge bucket.

   Mark the orientation of the plate before centrifugation so that it can be spun in the same orientation during 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 is recommended. Alternatively, the blocks can be spun at a lower *g* force (2500–5796 x *g*), but the centrifugation time may need to be extended (up to 60 min).

   Tip: Depending on the height of the centrifuge bucket, two plates can be centrifuged on top of each other.

5. **Remove the supernatants by quickly inverting and firmly tapping the plate upside down onto a stack of paper towels.**

6. **Wash each DNA pellet with 100 µl 70% ethanol. Centrifuge the plates (in the same orientation as before) at 5796 x *g* (6000 rpm) for 10 min. Remove the wash solutions by inverting the plate, and tapping it firmly, upside down, onto a stack of paper towels. 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.

7. **Redissolve the DNA pellets in 30 µl 10 mM Tris·Cl, pH 8.5, seal the 384-well plate with a sheet of adhesive tape, mix by vortexing, and centrifuge briefly to collect the droplets.**

   The plasmid DNA samples can be used directly in downstream applications.

Troubleshooting Guide

	Comments and suggestions
Poor growth of cultures	Determine the optimal cultivation time for the particular vector–host strain combination and adjust if necessary. See page 28 "Appendix C: Optimization of Culture Conditions".
Clogged wells of 384-well plate	Clogging may occur if the bacterial density of the culture is too high. Adjust culture conditions for the vector–host strain combination by decreasing the cultivation time or the culture volume, or use of an alternative growth medium. See page 28 "Appendix C: Optimization of Culture Conditions".
RNA contamination in the eluate	Ensure that RNase A is added to Buffer R1. A low level of RNA contamination will usually not affect the quality of sequencing data.
Genomic DNA in the eluate	The lysate must be handled gently after addition of Buffer R2 to prevent shearing of DNA. Incubation in Buffer R2 should not exceed 5 minutes. A low level of genomic DNA contamination will usually not affect the quality of sequencing data.
Low signal intensity in sequencing applications	Increase the number of sequencing reaction cycles to 40. Use 3–4 µl BigDye Terminator Ready Reaction Premix for sequencing reactions.

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 (1, 2). 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.

Vector systems

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 are generally present in lower copy numbers.

For plasmid purifications using the *micro*R.E.A.L. Prep 384, we strongly recommend the use of pUC vectors or pUC derivatives carrying a ColE1 origin of replication leading to 500–700 copies per cell.

Table 3. Origins of replication and copy numbers of various plasmids (1)

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

** 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 purified DNA. Host strains such as DH1, DH10B™, DH5α™, and C600 give high-quality DNA. The slower growing strain XL1-Blue also yields DNA of a 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. 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. DH10B and DH5α 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. We recommend bacterial cultures be prepared from a single colony, but glycerol stocks maybe effectively used if due care is taken. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures is 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 be stored at 4°C and should not be more than one week old. 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 according to self-optimized growth conditions, dependent on the medium, host–vector combination, and cultivation format (96- or 384-well). 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 that do not receive plasmids will replicate much faster than plasmid-containing cells and will 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 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 are based on these considerations.

Table 4. Concentrations of commonly used antibiotics

Antibiotic	Stock solutions Concentration	Storage	Working concentration (dilution)
Ampicillin (sodium salt)	50 mg/ml in H2O	–20°C	100 µg/ml (1/5000)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml (1/200)
Kanamycin	10 mg/ml in H2O	–20°C	50 µg/ml (1/200)
Streptomycin	10 mg/ml in H2O	–20°C	50 µg/ml (1/200)
Tetracycline HCl	5 mg/ml in ethanol	–20°C	50 µg/ml (1/100)

Culture media

2x YT broth is the recommended culture medium for the *micro*R.E.A.L. Prep 384 procedure. This medium allows high cell densities to be achieved leading to high yields of plasmid DNA. 2x YT medium should contain 16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, and be adjusted to pH 7.0.

Richer broths such as Terrific Broth (TB), and Magnificent Broth lead to extremely high cell densities which can overload the purification system if the culture conditions are not optimized for these media. The membrane of the 384-well filter plate can become overloaded and clogged, and the performance of the system will be unsatisfactory. In addition, the alkaline lysis will be inefficient. If these media are used for plasmid purification, the recommended culture volumes must be adapted, or the cultivation time reduced to match the capacity of the 384-well filter plate. A general guide is given in "Appendix C: Optimization of Culture Conditions" (page 28).

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. 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. This denatured form 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 SDS 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 co-purified. 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 *micro*R.E.A.L. Prep 384 procedure can be analyzed by agarose gel electrophoresis as shown in Figure 1.

Reproducible Plasmid Purification

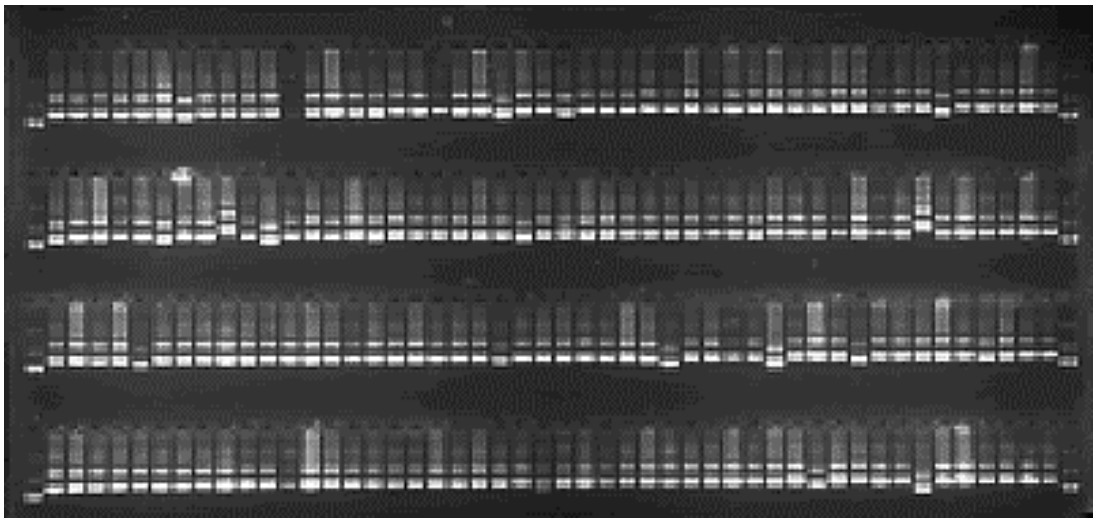


Figure 1. *Agarose gel analysis of shotgun clones purified using the* micro*R.E.A.L. Prep 384 procedure. 20% (6 µl from 30 µl) of the yielded plasmid DNA was loaded per lane.*
Outer lanes: *100 ng marker plasmid.*

Appendix B: Sequencing Recommendations for Capillary Sequencers.

Sequencing reactions using plasmid DNA prepared with the *micro*R.E.A.L. Prep 384 Plasmid Kit have so far only been tested with ABI PRISM® Sequencers (PE Biosystems). Below are sequencing reaction recommendations for fluorescent DNA sequencing with ABI PRISM Sequencers.

Sequencing setup

Device	**ABI PRISM Sequencer**
DNA template	6 µl
Primer	8 pmol
Premix	2 µl BigDye Terminator Ready Reaction Premix
5x *Taq* DNA Polymerase Buffer	1 µl
Final reaction volume	10 µl
Cycling conditions	
Denaturation	95°C; 30 s
Annealing	50°C; 10 s
Elongation	60°C; 4 min
Number of cycles	30

Dye-terminator removal

For the removal of unincorporated dye terminators, we strongly recommend use of the DyeEx™ 96 Kit. Detailed protocols for the purification of sequencing reactions can be found in the handbook supplied with the DyeEx 96 Kit. For the ABI PRISM 377 Sequencer, resuspend the dry reaction pellets in 1.5 µl loading buffer and load 1 µl. For the ABI PRISM 3700 Sequencer, directly load 2.5 µl of the flow-through of the DyeEx 96 purification procedure.

Sequencing results

Analysis of sequencing quality using Phred analysis showed a consistently high performance (Figure 2).

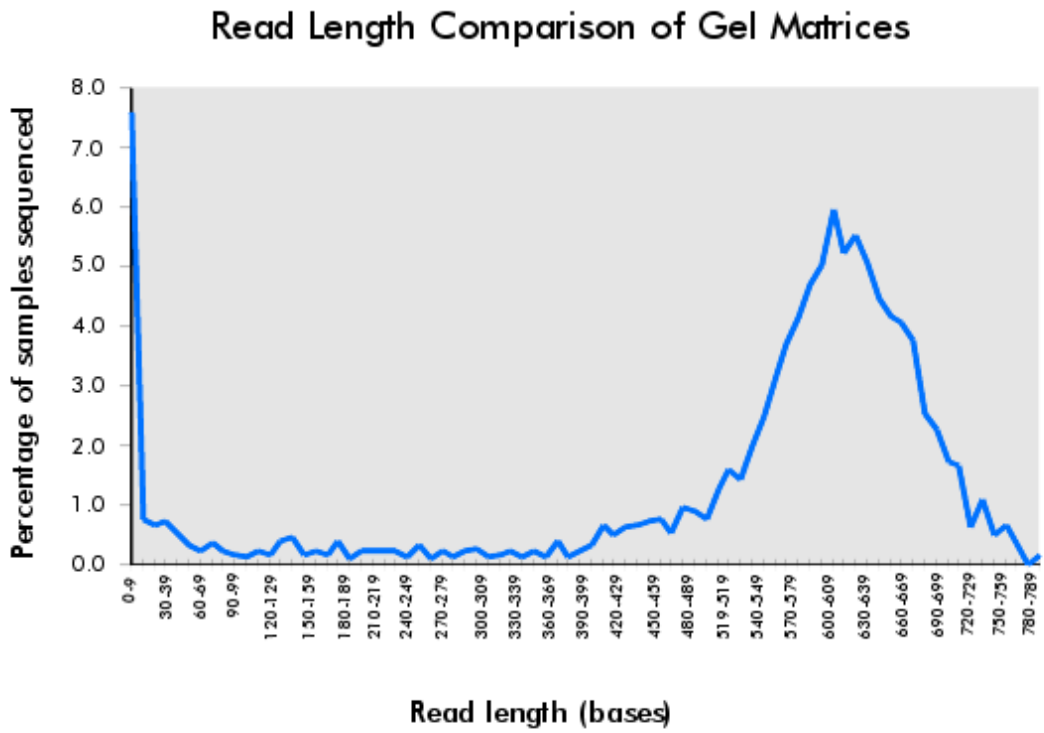


Figure 2. *Phred analysis (Q>20) of 2323 sequencing reactions using templates from various bacterial shotgun clone libraries prepared using the* micro*R.E.A.L. Prep 384 Kit.*

Appendix C: Optimization of Culture Conditions

DNA yield can vary depending on factors such as the host–vector system, the medium used, the cultivation conditions, and the cultivation format. Guidelines for optimization of growth conditions are given below.

Cultivation in 96-Well Microplates MC

Follow the cultivation procedure as described in the protocol.

If the yield is too low:

- Ensure the vector has a high copy number. Perform control experiments of vector only (without insert) to assess yield.
- Increase the cultivation time. Cultivate cells for 20 h, 22 h, and 24 h and assess yields to determine optimal cultivation time.

If the filter plates are clogged:

- Decrease cultivation time to 16 h
- Decrease volume of medium used to 300 µl or 250 µl (optional)
- Check inoculum size

If medium other than 2x YT is used for cultivation:

Determine, by agarose gel analysis, which cultivation conditions lead to the highest plasmid yield without clogging the filter plates. The conditions we recommend testing are as follows:

- Prepare three 96-well plates, varying the volume of medium in the wells of each plate (250 µl, 300 µl, and 350 µl).
- Using 24 individual clones, inoculate each plate with 1 µl of each of the pre-cultures. Repeat inoculation three times per plate. Try to inoculate the plates uniformly.
- Incubate plates at 37°C with shaking.
- After 16 h, aspirate one sample of each clone from each plate. Dispense the cultures into a new 96-Well Microplate MC and store at 4°C.
- Continue the incubation and repeat aspiration and culture transfer at 18, 20, and 22 h, storing the culture aliquots at 4°C until all samples have been collected.
- Prepare the clones according to the *micro*R.E.A.L. Prep 96-well procedure and compare yields on an agarose gel.

Cultivation in a 384-well format

The cultivation of clones in 384-well format has to be adapted for all libraries. When performing this procedure, please remember that cultivation is not the only step which requires optimization. The lysis procedure must also be adapted for 384-well blocks. In addition, special equipment is required. We recommend the use of a Levitation Stirrer (VP 707A) from V&P Scientific Inc., (www.vp-scientific.com). An ultra-high-throughput capacity can be achieved by use of the Carousel Levitation Stirrer (VP 707B) situated in a 37°C warm room. The limiting factor for any cultivation using 384-well blocks is aeration. For optimal aeration using the V&P Scientific equipment, control experiments should be performed where the following parameters are considered.

- Culture volume should be kept constant at 300 µl
- Cultivation times of 18, 20, 22, and 24 h should be tested
- Vary the settings of the Carousel Levitation Stirrer, e.g., speed and lifting height

References

1. Sambrook, J. et al., eds (1989) Molecular cloning: a laboratory manual. 2nd ed., Cold Spring Harbor, N.Y.: Laboratory Press.

2. Ausubel, F. M. et al., eds (1991) Current protocols in molecular biology. New York: Wiley Interscience.

Ordering Information

Product	Contents	Cat. No.
*micro*R.E.A.L Prep 384 Plasmid Kit (20)	For 20 x 384 rapid extraction alkaline lysis minipreps	26363
Accessories		
96-Well Microplates MC (40)	40 x 96-well microplates for bacterial cultivation	19586
Other related products		
R.E.A.L Prep 96 Plasmid Kit (4)*	For 4 x 96 rapid extraction alkaline lysis minipreps	26171
R.E.A.L Prep 96 Plasmid Kit (24)*	For 24 x 96 rapid extraction alkaline lysis minipreps	26173
R.E.A.L Prep 96 BioRobot Kit (4) †	For 4 x 96 automated rapid extraction alkaline lysis Minipreps	961141
QIAprep 96 Turbo Miniprep Kit (4)*†	For 4 x 96 high-purity plasmid Minipreps	27191
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks during bacterial cultivation	19571
DyeEx 96 Kit (4) †	4 DyeEx 96 Plates, waste collection plate (48-well)	63181

** Requires the use of QIAvac 96*

† Other kit sizes available; please inquire

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