APPENDIXES
Appendix 1A

Preparation of an Agarose Gel for Gel Electrophoresis

Solutions Needed:

- DNA samples
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL DNA ladder with loading dye (Invitrogen)
- 6x Loading dye containing xylene cyanol and/or bromophenol blue dyes

Materials Needed:

- Pipettes (P-20)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- 250 mL Erlenmeyer flask
- 25 mL Erlenmeyer flask
- Saran wrap
- Scale
- Microwave
- 55°C water bath
- Hot hand protector
- Gel cast
- Gel comb
- Round bubble level
- Gel box
- Cables
- Electrophoresis power supply
- Plastic container for carrying the gel
- Gel document system (Bio-Rad)

PROCEDURE

*Note: SYBR Safe gel stain is unstable in UV or bright room light. If possible, keep the gel in the **dark** by either turning off the lights, covering the gel with a cardboard box or aluminum foil, or run the gel inside of a drawer. Realistically, hours of constant UV or bright room light exposure are required to cause any significant loss of signal.*

1. For a **1% agarose gel**, weigh out **1 gram** of agarose (powder) into a weigh boat on the scale in room 4128A2. *Note: The **percentage** of agarose in the gel reflects the amount of agarose (in **grams**) in **100 mL** of 1x TAE buffer.*
   Example: If you want to make a 0.7% agarose gel (0.7 g/100 mL, w/v), weigh out 0.7 g of agarose for 100 mL of 1x TAE buffer.
2. Carefully, pour the agarose into a 250 mL Erlenmeyer flask.
3. Measure **100 mL** of **1x TAE** buffer using a graduated cylinder, and add it to the flask in step 2.
4. Cover the flask with an inverted 25-mL Erlenmeyer flask or saran wrap. Swirl the solution to break up any lumps of agarose. *Note: The inverted flask will collect condensation from the steam produced during microwaving in step 6 below.*
5. Microwave the solution for **1-2 minute** or until the agarose granules have completely melted and the solution looks **clear**. Gently **swirl** the solution every 15 sec during microwaving to help melt the agarose evenly. *Caution: The solution gets **very hot**. Use a hot hand protector. Note: Constantly watch over the solution because if it starts boiling, it might overflow.*
6. Cool down the agarose solution for **10 min** in a **55°C water bath.**
7. While the agarose solution is cooling, prepare the gel cast with the appropriate comb. Use the round bubble level to make the gel cast level.
   *Note: **The comb depends on the number of samples to be loaded on the gel**. For example, if there are ≤ 18 samples, then use a 20-tooth comb; but, if there are ≥ 19 samples, then use a 30-tooth comb. Note: Remember that **two wells** will be for loading **DNA ladder** (on left and right sides of loaded samples).*
8. After the agarose solution has cooled down, add 10 μL of **10,000x SYBR Safe DNA gel stain** to 100 mL of agarose solution and **swirl** the flask **GENTLY** to mix. *Note: Do NOT swirl vigorously to avoid generating bubbles.*
9. Pour the **agarose/gel stain** solution into the gel cast with the appropriate gel comb. Wait **30 min** for the agarose solution to solidify. *Note: IMMEDIATELY after pouring the agarose solution, inspect the agarose solution's surface for the present of bubbles. If there are bubbles floating on the surface of the gel solution, use a pipette*

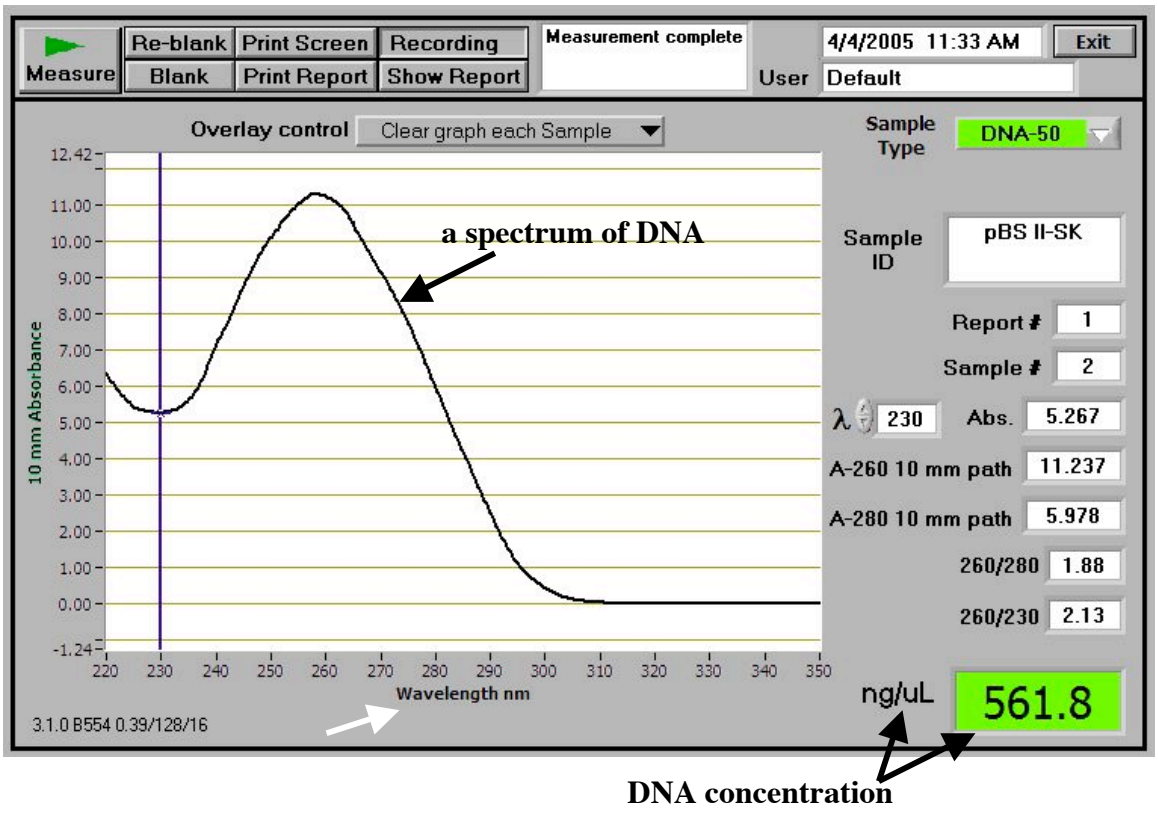
tip to pop them or move them to the sides of the gel before the gel has completely solidified.

10. Pour ~600 mL of **1x TAE** buffer into the gel box.
11. After the agarose has solidified into a gel, take out the comb by **gently** pulling it side to side and out of the gel. Put the gel in its cast into the gel box containing the 1x TAE running buffer.
12. Add **6x loading dye** to your samples. *Note: Do not use a loading dye that will travel to the same place as your DNA on the gel because the dye will obscure the DNA band. Xylene cyanol runs at ~3-4 kb, and bromophenol blue runs at ~400 bp on a 1% agarose gel.*
13. Load **10 µL** of **50 ng/µL DNA ladder with loading dye**.
14. Load samples and record the identity of the sample in each lane.
15. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (red) side of the gel box. Pipet up and down to mix. *Note: Add 5 µL of 10,000x SYBR Safe DNA gel stain for a small (50 mL) gel.*
16. Put the lid on the gel box and connect the electrodes to the power supply (**RED** to **RED** and **BLACK** to **BLACK**).
17. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) has migrated one-half or two-thirds of the gel length.
18. After 1-2 hours of running the gel, turn **off** the power supply.
19. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 4128A2. ***Caution:*** *It is a* ***MUST*** *to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking.*
20. Take a picture of the gel using the Bio-Rad Gel Document System. Label the picture using the text program of the Gel Document System. (*Your TA will show you how.*) *Alternatively: Print out the picture. Tape it to a piece of paper by putting a piece of white tape at a position immediately above the wells. Label the wells with the sample names.*
21. Print out the picture. Store the labeled picture in your lab notebook.

Appendix 1B

What is a **spectrophotometer**?

It is an **instrument** that **measures** the **amount** of **molecules absorbing a given wavelength of energy**. In this exercise, we measure the amount of DNA molecules in a given volume in the **ultraviolet wavelengths** of **200 – 280 nm** (**nm** stands for **nanometer**, which is **1 billionth** of a **meter**). The bases of DNA absorb at a wavelength of 254 nm. The absorbance of DNA molecules over the wavelength range of 220 - 350 nm is represented as a **spectrum** with a **peak** at **near 260 nm** as illustrated by the **NanoDrop spectrophotometer readout** below:



OPERATION OF NANODROP SPECTROPHOTOMETER ND-1000

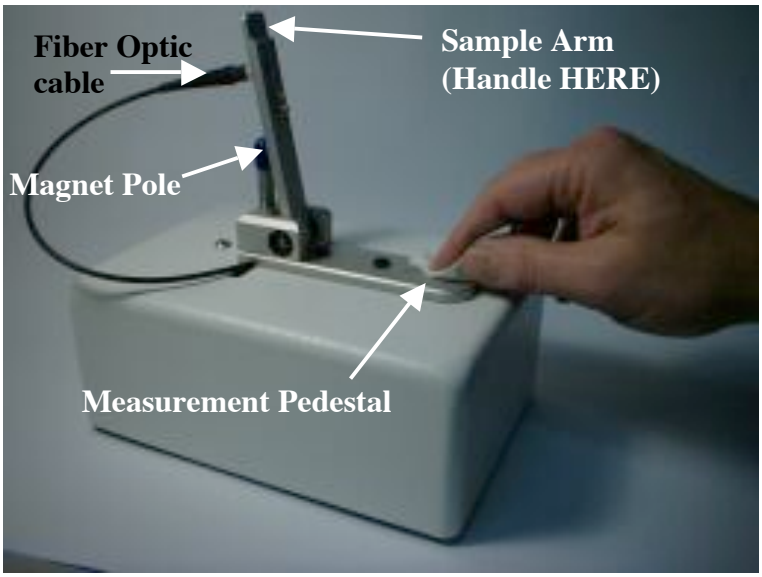
Note: The NanoDrop is powered by the laptop via the USB port.

1. **Open** the NanoDrop program by clicking the "**ND-1000 v3.7.1**" icon on the computer desktop. Wait for a few seconds for the program to open. You see the NanoDrop 1000 3.7.1 menu panel with **user field** set to **default.**
2. To read the concentration of DNA or RNA solutions, **click** on the "**Nucleic Acid**" button on the top left column.

   Note: You see the following message: "Ensure sample pedestals are clean and then load a water sample. After loading water sample, click OK to initialize instrument."
3. **Clean** the **measurement pedestals** as following:
   a) **Raise** the **sample arm** by holding its end.

   Caution: ***NEVER*** *hold the fiber optic cable when lifting and lowering the sample arm because the cord is fragile, and it is very expensive to replace it.*

   b) **Blot BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes slightly wetted with distilled water.



c) **Pipet 1.5 - 2 µL** of water onto the **measurement pedestal**.

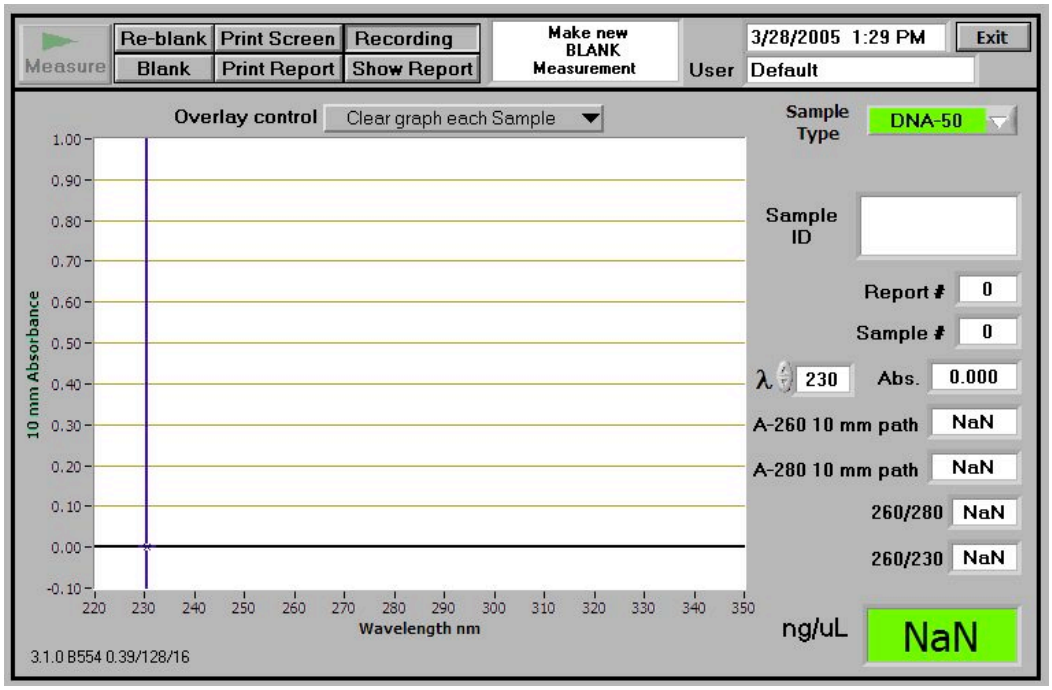
Note: Even though NanoDrop Inc. claims that the NanoDrop can read as low as 1 µL, the concentration reading is NOT consistent at this volume. Therefore, the ***minimal*** *volume for the concentration reading is* ***1.5 µL.***

d) **Slowly lower** the sample arm to its horizontal position.

Caution: *NEVER let the arm fall freely.*

e) **Click** the **OK** button.

Note: Now the NanoDrop is ***INITIALIZED.*** *You will see the* ***dialog panel*** *as shown below.*



f) Raise the sample arm to the vertical position.

g) **Wipe off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.

4. **Change SAMPLE TYPE** (if necessary) from **DNA-50** (default, for DNA) to **RNA-40** (for RNA) or **ssDNA-33** (for oligonucleotides), depending on your sample.
5. (Optional) **Change** the **OVERLAY CONTROL** field from the default setting of "CLEAR GRAPH EACH SAMPLE" to "CLEAR GRAPH ON NEW REPORT" or "ACCUMULATE UNTIL CLEAR" or "CLEAR GRAPH NOW."
6. Make a **NEW BLANK measurement.**
   a) Pipet 1.5 - 2.0 µL of either double-distilled water or TE (depending on whether your sample solution is in double-distilled water or TE) on the measurement pedestal.
   b) Lower the sample arm to the horizontal position.
   c) **Click** the **BLANK** button. *The blank was made*.
   d) After the reading is done, raise the sample arm to the vertical position.
   e) **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.
7. **Confirm** that the blank was made.
   a) Pipet 1.5 - 2.0 µL of either double-distilled water or TE (depending on whether your sample solution is in double-distilled water or TE) on the measurement pedestal.
   b) Lower the sample arm to the horizontal position.
   c) In the **SAMPLE ID** field, **type** the **identity** of the solution that you are measuring (either TE or ddH_2O).
   d) **Click** the **MEASURE** button.

      Note: After the reading is done, a ***concentration*** *(in* ***ng/µL****) and a* ***spectrum*** *of the absorbance, along with other information, are shown. The reading should be* ***less than 1 ng/µL****. If it is not, make a new blank measurement by repeating step 6.*
   e) Raise the sample arm to the vertical position.
   f) **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.

8. Measure the **samples**.
   a) In the **SAMPLE ID** field, **type** the **identity** of the sample solution.
   b) Pipet 1.5 - 2.0 μL of SAMPLE on the measurement pedestal.
   c) Lower the sample arm to the horizontal position.
   d) **Click** the **MEASURE** button to determine concentration of your sample.
   e) After the reading is done, a **sample concentration** (in **ng/μL**) and a **spectrum** of the sample, along with other information, are shown. You can either:
      i. **Save** the **window** of measured sample.
         1. Click on FILE.
         2. Choose SAVE WINDOW.
         3. Select an existing folder or create a new folder (give a name for the new folder).
         4. Type in a file name in the FILE NAME field.
         5. Click the SAVE button to save the file.
      ii. **Or**, **print** the **window** by **clicking** the **PRINT SCREEN** button.
      Note: To print the spectrum of the current sample, you MUST print it before reading the next sample. Otherwise, you need to repeat reading the sample.
   f) Raise the sample arm to the vertical position.
   g) **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.
9. **Repeat** step 8 for other samples.
10. After reading the **last sample**, **click** the **PRINT REPORT** button to print the concentrations of all samples.
11. If you are done with the NanoDrop, **click** the **EXIT** buttons.
12. **Blot BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes slightly wetted with distilled water.

Appendix 1C
100 bp DNA Ladder

Invitrogen™
life technologies

100 bp DNA Ladder

Cat. No. 15628-019 **Size: 50 µg**
Concentration: 1 µg/µl **Store at -20°C.**

Description:

The 100 bp DNA Ladder consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp. The 600 bp band is approximately 2 to 3 times brighter than the other ladder bands to provide internal orientation. This ladder is not designed for quantitation.

Storage Buffer:

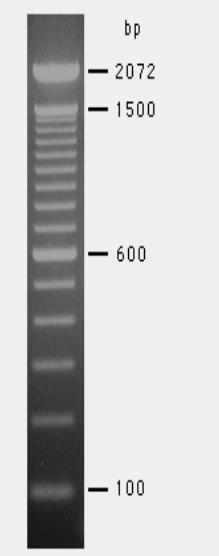
10 mM Tris-HC1 (pH 7.5)
1 mM EDTA

Recommended Procedure:

A final concentration of 20 mM NaCl is recommended for gel electrophoresis. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that the bands between 100 to 1500 bp are distinguishable. The 600 bp band must be more intense than any other band except the band at 2072 bp.



Note:

During 2% agarose gel electrophoresis with tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates near the 100-bp fragment. The 100-bp band migrates behind the bromophenol blue marker on 6% polyacrylamide gels with tris-borate (pH 8.0) as the running buffer.

Part of the 600-bp band may migrate anomalously slowly in polyacrylamide gels (1,2,3). This band may appear as an extra band near or on top of the 700-bp band.

References:

1. Hsieh, C., et al. (1991) *Mol. Gen. Genet. 225*, 25.
2. Stellwagen, N.C. (1983) *Biochemistry 22*, 6186.
3. Jordan, H. and Hartley, J. (1997) *Focus® 19*, 9.

100 bp DNA Ladder
0.5 µg/lane
2% agarose gel stained with ethidium bromide.

Cat. No. 15628-019

Appendix 1D
1 Kb Plus DNA Ladder

Invitrogen
life technologies

1 Kb Plus DNA Ladder

Cat. No. 10787-018 **Size: 250 µg**
Conc.: 1 µg/µl **Store at -20°C**.

Description:

The 1 Kb Plus DNA Ladder is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000 bp to 12,000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band contains approximately 8% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ^{32}P input; (ii) Labeling the 5′ ends with T4 polynucleotide kinase; (iii) Filling in the 3′ recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:

10 mM Tris-HCl (pH 7.5)
1 mM EDTA
50 mM NaCl

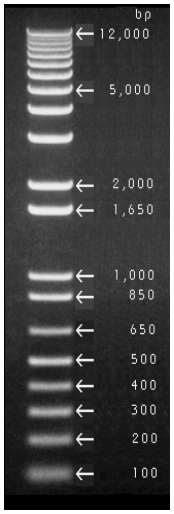
Recommended Procedure:

Invitrogen recommends the use of 10X BlueJuice™ Gel Loading Buffer (Cat. No. 10816-015) at a concentration of 2X for electrophoresis of DNA standards on agarose gels. Alternately, the DNA standard can be diluted such that the final concentration of NaCl is 20 mM. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that all bands in the ladder are distinguishable and are of approximate equal intensity by ethidium bromide staining.

Doc. Rev.: 021802



1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

5′-GATCC ——— G
G ——— CCTAG-5′
$(996\ bp)_n$

Notes:

During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

Cat. No. 10787-018

Appendix 1E
Ex Taq DNA Polymerase Manual

10XPCR Buffer,dNTP Mixture for PCR

TaKaRa Ex Taq™

Code No. RR001A
Size: 250 units

Shipping at -20˚C
Stored at -20˚C

Supplied Reagents : 10X *Ex Taq*™ Buffer
dNTP Mixture

Lot No.
Conc. : units/μl
Volume : μl
Expiry Date :

Storage Buffer:

20 mM	Tris-HCl (pH8.0)
100 mM	KCl
0.1 mM	EDTA
1 mM	DTT
0.5%	Tween®20
0.5%	Nonidet P-40®
50%	Glycerol

Unit definition: One unit is the amount of the enzyme that will incorporate10 nmol of dNTP into acid-insoluble products in 30 minutes at 74˚C with activated salmon sperm DNA as the template-primer.

Reaction mixture for unit definition:

25 mM	TAPS (pH 9.3 at 25˚C)
50 mM	KCl
2 mM	$MgCl_2$
1 mM	2-mercaptoethanol
200 μM	each dATP,dGTP,dTTP
100 μM	[α-^{32}P]-dCTP
0.25 mg/ml	activated salmon sperm DNA

Purity: Nicking activity, endonuclease and exonuclease activity were not detected after the incubation of 0.6 μg of supercoiled pBR322 DNA, 0.6 μg of λ DNA or 0.6 μg of λ-*Hin*d III digest with 10 units of this enzyme for 1 hour at 74˚C.

Applications:
For DNA amplification by Polymerase Chain Reaction (PCR).

PCR products : As most PCR products amplified with *TaKaRa Ex Taq* ™ have one A added at 3'-termini, the obtained PCR product can be directly used for cloning into T-vector. Also it is possible to clone the product in blunt-end vectors after blunting and phosphorylation of the end.

PCR test : Good performance of DNA amplification by Polymerase Chain Reaction (PCR) was confirmed by using λ DNA as the template (amplified fragment : 20 kbp).
Good performance of DNA amplification of β-globin gene by PCR was also confirmed by using human genomic DNA as the template (amplified fragment : 17.5 kbp).

PCR condition (an example)
When amplifying 1 kbp DNA fragment

98°C	10 sec	┐		98 °C	10 sec	┐	
55°C	30 sec	┘ 30 cycles	or	68 °C	1 min	┘	30 cycles
72°C	1 min	┘					

Note:Denaturation condition varies depending on an used thermal cycler and tube. It is recommended for 10-30 sec. at 94°C, or 1-10 sec. at 98°C.

General reaction mixture for PCR (total 50 μl)

TaKaRa Ex Taq ™ (5 units/μl)	0.25 μl
10X *Ex Taq* Buffer	5 μl
dNTP Mixture (2.5 mM each)	4 μl
Template	< 500 ng
Primer 1	0.2 ~ 1.0 μM (final conc.)
Primer 2	0.2 ~ 1.0 μM (final conc.)
Sterilized distilled water	up to 50 μl

Supplied 10X *Ex Taq* Buffer
Supplied Size : 1 ml/vial
Mg^{2+} concentration (10X): 20 mM
Storage : –20˚C

Supplied dNTP Mixture
Mixture of dNTP, ready for use in Polymerase Chain Reaction (PCR) without dilution.

Supplied Size	: 800 μl/vial
Concentration	: 2.5 mM of each dNTP
pH	: pH 7 ~ 9
Form	: Solved in water (sodium salts)
Purity	: ≥ 98% for each dNTP
Storage	: –20˚C

< **Cool Start Method** >
"Cool Start Method' , enables to minimize the amplification of non-specific band in PCR and achieves more accurate amplification. This is a simpler method without need for special enzyme nor additional reagents.⁺ Higher reaction specificity can be achieved by combining Hot Start PCR techniques with Taq Antibody (Code.9002A) and Cool Start method.
Protocol of Cool Start Method
1) Keep all reagents on ice until use.
2) Prepare the reaction mixture on ice.*,**
*The adding order of reagents dose not influence on results.
**The result will not be affected even when the mixture is left on ice 30 min. before thermal cycling.
3) Set a thermal cycler ready to start with the designated program.***
***No need to change PCR conditions especially for Cool Start.
4) Set the tubes in a thermal cycler and start thermal cycling immediately.
⁺JAPAN Patent 2576741 for Cool Start Method is owned by SHIMADZU CORPORATION

Note
For research use only. Not for use in diagnostic or therapeutic procedures.

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V2005.06

March 2008

QIAquick® Spin Handbook

QIAquick PCR Purification Kit
For purification of PCR products, 100 bp to 10 kb

QIAquick Nucleotide Removal Kit
For oligonucleotide (17-40mers) and DNA
(40 bp to 10 kb) cleanup from enzymatic reactions

QIAquick Gel Extraction Kit
For gel extraction or cleanup of DNA
(70 bp to 10 kb) from enzymatic reactions

QIAGEN®

Sample & Assay Technologies

Kit Contents

QIAquick PCR Purification Kits Catalog no.	(50) 28104	(250) 28106
QIAquick Spin Columns	50	250
Buffer PB*	30 ml	150 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
pH Indicator I	800 µl	800 µl
Collection Tubes (2 ml)	50	250
Loading Dye	110 µl	550 µl
Handbook	1	1

QIAquick Nucleotide Removal Kits Catalog no.	(50) 28304	(250) 28306
QIAquick Spin Columns	50	250
Buffer PN*	30 ml	140 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
Collection Tubes (2 ml)	100	500
Loading Dye	110 µl	550 µl
Handbook	1	1

QIAquick Gel Extraction Kits Catalog no.	(50) 28704	(250) 28706
QIAquick Spin Columns	50	250
Buffer QG*	2 x 50 ml	2 x 250 ml
Buffer PE (concentrate)	2 x 10 ml	2 x 50 ml
Buffer EB	15 ml	2 x 15 ml
Collection Tubes (2 ml)	50	250
Loading Dye	110 µl	550 µl
Handbook	1	1

* Buffers PB, PN, and QG contain chaotropic salts which are irritants. Take appropriate laboratory safety measures and wear gloves when handling.

Storage

QIAquick Spin Kits should be stored dry at room temperature (15–25°C). Under these conditions, QIAquick Spin Kits can be stored for up to 12 months without showing any reduction in performance and quality. Check buffers for precipitate before use and redissolve at 37°C if necessary. The entire kit can be stored at 2–8°C, but in this case the buffers should be redissolved before use. Make sure that all buffers and spin colums are at room temperature when used.

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 each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer PB contains guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

In case liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the QIAquick system.

Buffer PB

Contains guanidine hydrochloride and isopropanol: harmful, irritant, flammable. Risk and safety phrases*: R10-22-36/38. S23-26-36/37/39-46

Buffer PN

Contains sodium perchlorate and isopropanol: harmful, highly flammable. Risk and safety phrases*: R11-22. S13-16-23-26-36-46

Buffer QG

Contains guanidine thiocyanate: harmful. Risk and safety phrases*: R20/21/22-32. S13-26-36-46

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany
Tel: +49-6131-19240

* R10: Flammable. R11: Highly Flammable. R22: Harmful if swallowed. R20/21/22: Harmful by inhalation, in contact with skin and if swallowed. R32: Contact with acids liberates very toxic gas. R36/38: Irritating to eyes and skin. S13: Keep away from food, drink and animal feedingstuffs. S16: Explosive when mixed with oxidizing substances. S23: Do not breathe vapour/spray. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S36: Wear suitable protective clothing. S36/37/39: Wear suitable protective clothing, gloves and eye/face protection. S46: If swallowed, seek medical advice immediately and show the container or label.

Product Specifications

	QIAquick PCR Purification Kit	QIAquick Nucleotide Removal Kit	QIAquick Gel Extraction Kit
Maximum binding capacity	10 µg	10 µg	10 µg
Maximum weight of gel slice	—	—	400 mg
Minimum elution volume	30 µl	30 µl	30 µl
Capacity of column reservoir	800 µl	800 µl	800 µl
Typical recoveries			
Recovery of DNA	90–95% (100 bp – 10 kb)	80–95% (40 bp – 10 kb)	70–80% (70 bp – 10 kb)
Recovery of oligonucleotides (17–40mers)	0	60–80%	10–20%
Recovered			
Oligonucleotides	—	17–40mers	—
dsDNA	100 bp – 10 kb	40 bp – 10 kb	70 bp – 10 kb
Removed			
<10mers	**YES**	**YES**	**YES**
17–40mers	**YES**	no	no

Introduction

The QIAquick system, designed for rapid DNA cleanup, includes:

- **QIAquick PCR Purification Kits** for direct purification of double- or single-stranded PCR products (100 bp – 10 kb) from amplification reactions and DNA cleanup from other enzymatic reactions.
- **QIAquick Nucleotide Removal Kits** for general cleanup of oligonucleotides and DNA up to 10 kb from enzymatic reactions (e.g., labeling, dephosphorylation, restriction, and tailing).
- **QIAquick Gel Extraction Kits** for extraction of DNA fragments (70 bp – 10 kb) from standard, or low-melt agarose gels in TAE (Tris·acetate/EDTA) or TBE (Tris·borate/EDTA) buffer and DNA cleanup from enzymatic reactions.

QIAquick PCR Kits are also available in multiwell format for preparation of 8 to 96 samples (see page 37 for ordering information).

Enzymatic reaction cleanup using QIAquick Kits

The QIAquick system is suitable for fast cleanup of up to 10 µg of DNA fragments from enzymatic reactions and agarose gels (Table 1). Enzyme contamination of DNA samples can interfere with subsequent downstream applications. QIAquick Spin Kits can be used for highly efficient removal of a broad spectrum of enzymes widely used in molecular biology. In addition, QIAGEN offers the MinElute® Reaction Cleanup Kit, which is specially designed for fast and easy DNA cleanup from all enzymatic reactions. Using proven microspin technology, the MinElute Reaction Cleanup Kit delivers highly concentrated purified DNA by using an elution volume of only 10 µl (see ordering information, page 37).

Table 1. QIAquick DNA Cleanup Guide

	From solutions			From gels
	QIAquick PCR Purification Kit	QIAquick Nucleotide Removal Kit	QIAquick Gel Extraction Kit	QIAquick Gel Extraction Kit
Alkaline phosphatase	**YES**	**YES**	**YES**	**YES**
cDNA synthesis	**YES**	no	no	**YES**
DNase, nuclease digestion	**YES**	**YES**	**YES**	**YES**
Kinase:				
DNA fragments	**YES**	**YES**	**YES**	**YES**
Oligonucleotides	no	**YES**	no	no
Ligation	**YES**	**YES**	**YES**	**YES**
Nick translation	**YES**	**YES**	**YES**	**YES**
PCR	**YES**	no	no	**YES**
Random priming	**YES**	**YES**	**YES**	**YES**
Restriction digestion	**YES**	**YES**	**YES**	**YES**
Tailing:				
DNA fragments	**YES**	**YES**	**YES**	**YES**
Oligonucleotides	no	**YES**	no	no

QIAquick Kits provide high yields of pure nucleic acids, for direct use in applications such as:

- Fluorescent and radioactive sequencing
- Restriction
- Labeling
- Hybridization
- Ligation and transformation
- Amplification
- In vitro transcription
- Microinjection

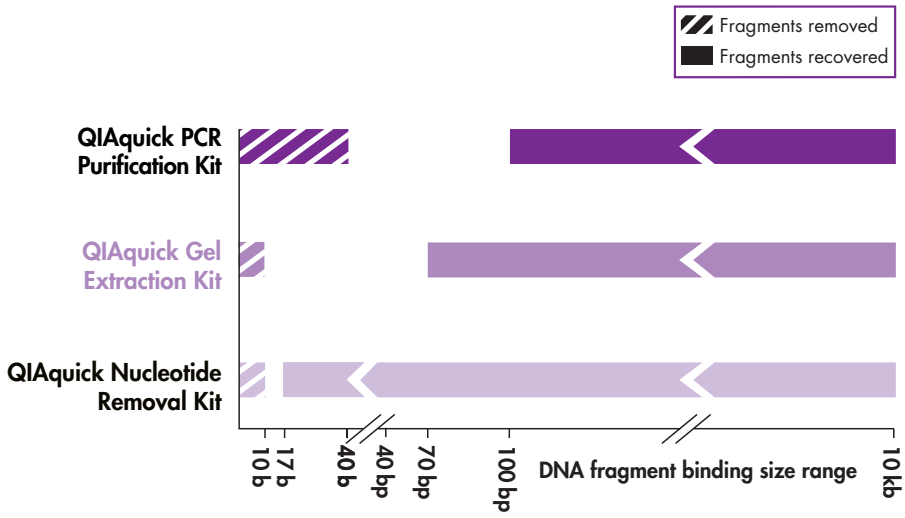


Figure 1. DNA fragment binding-size range. Recoveries of DNA fragments in the size range between "removed" and "recovered" are not defined.

Automated DNA cleanup

The QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit can be fully automated on the QIAcube. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., bind, wash, and elute) enabling purification of high-quality DNA.

The QIAcube is preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/MyQIAcube .

A detailed protocol for using QIAquick spin columns on the QIAcube is provided with the QIAcube.

Note: It is not necessary to add pH indicator I to Buffer PB when using the QIAcube.

The QIAquick Principle

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water (see page 17). QIAquick spin columns offer 3 handling options — as an alternative to processing the spin columns in a microcentrifuge, they can now also be used on any commercial vacuum manifold with luer connectors (e.g., QIAvac 6S or QIAvac 24 Plus with QIAvac Luer Adapters) or automated on the QIAcube.

Adsorption to QIAquick membrane — salt and pH dependence

The QIAquick silica membrane is uniquely adapted to purify DNA from both aqueous solutions and agarose gels, and up to 10 µg DNA can bind to each QIAquick column. The binding buffers in QIAquick Spin Kits provide the correct salt concentration and pH for adsorption of DNA to the QIAquick membrane. The adsorption of nucleic acids to silica surfaces occurs only in the presence of a high concentration of chaotropic salts (1), which modify the structure of water (2).

Adsorption of DNA to silica also depends on pH. Adsorption is typically 95% if the pH is ≤7.5, and is reduced drastically at higher pH (Figure 1). If the loading mixture pH is >7.5, the optimal pH for DNA binding can be obtained by adding a small volume of 3 M sodium acetate, pH 5.0.

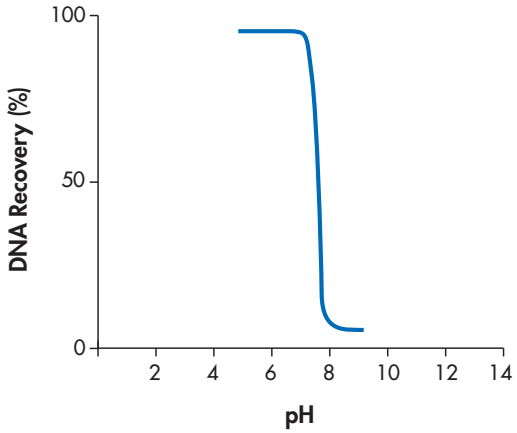


Figure 2. pH dependence of DNA adsorption to QIAquick membranes. 1 µg of a 2.9 kb DNA fragment was adsorbed at different pHs and eluted with Buffer EB (10 mM Tris·Cl, pH 8.5). The graph shows the percentage of DNA recovery, reflecting the relative adsorption efficiency, versus pH of adsorption.

Optimized binding buffers for every DNA cleanup task

All QIAquick Spin Kits contain identical QIAquick spin columns but different binding buffers optimized for each specific application:

- Buffer PB in the QIAquick PCR Purification Kit allows the efficient binding of single- or double-stranded PCR products as small as 100 bp and the quantitative (99.5%) removal of primers up to 40 nucleotides. This kit can therefore be used to remove oligo-dT primers after cDNA synthesis or to remove unwanted linkers in cloning experiments.
- Buffer PN in the QIAquick Nucleotide Removal Kit promotes the adsorption of both oligonucleotides ≥17 bases and DNA fragments up to 10 kb to the membrane.
- Buffer QG in the QIAquick Gel Extraction Kit solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane.

All of these buffers are available separately (see ordering information, page 37).

pH indicator

Binding buffer PB and binding and solubilization buffer QG are specially optimized for use with the QIAquick silica membrane. Buffer QG contains an integrated pH indicator, while an optional pH indicator can be added to Buffer PB allowing easy determination of the optimal pH for DNA binding. DNA adsorption requires a pH ≤7.5, and the pH indicator in the buffers will appear yellow in this range. If the pH is >7.5, which can occur if during agarose gel electrophoresis, the electrophoresis buffer had been used repeatedly or incorrectly prepared, or if the buffer used in an enzymatic reaction is strongly basic and has a high buffering capacity, the binding mixture turns orange or violet (Figure 2). This means that the pH of the sample exceeds the buffering capacity of Buffer PB or QG and DNA adsorption will be inefficient. In these cases, the pH of the binding mixture can easily be corrected by addition of a small volume of 3 M sodium acetate*, pH 5.0, before proceeding with the protocol. In addition, in the QIAquick Gel

Figure 3. Indicator enables easy checking of the optimal pH. Indicator dye in solubilization and binding Buffers QG and PB identifies optimal pH for DNA binding.

* 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) available from the product supplier.

Extraction Kit procedure, the color of the binding mixture allows easy visualization of any unsolubilized agarose, ensuring complete solubilization and maximum yields. The indicator dye does not interfere with DNA binding and is completely removed during the cleanup procedure. Buffers PB and QG do not contain sodium iodide (NaI). Residual NaI may be difficult to remove from DNA samples, and reduces the efficiency of subsequent enzymatic reactions such as blunt-end ligation.

Washing

During the DNA adsorption step, unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, and detergents (e.g., DMSO, Tween® 20) do not bind to the silica membrane but flow through the column. Salts are quantitatively washed away by the ethanol-containing Buffer PE. Any residual Buffer PE, which may interfere with subsequent enzymatic reactions, is removed by an additional centrifugation step.

Elution in low-salt solutions

Elution efficiency is strongly dependent on the salt concentration and pH of the elution buffer. Contrary to adsorption, elution is most efficient under basic conditions and low salt concentrations. DNA is eluted with 50 or 30 µl of the provided Buffer EB (10 mM Tris·Cl, pH 8.5), or water. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water to elute, make sure that the pH is within this range. In addition, DNA must be stored at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent. Elution with TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) is possible, but not recommended because EDTA may inhibit subsequent enzymatic reactions.

DNA yield and concentration

DNA yield depends on the following three factors: the volume of elution buffer, how the buffer is applied to the column, and the incubation time of the buffer on the column. 100–200 µl of elution buffer completely covers the QIAquick membrane, ensuring maximum yield, even when not applied directly to the center of the membrane. Elution with ≤50 µl requires the buffer to be added directly to the center of the membrane, and if elution is done with the minimum recommended volume of 30 µl, an additional 1 minute incubation is required for optimal yield. DNA will be up to 1.7 times more concentrated if the QIAquick column is incubated for 1 minute with 30 µl of elution buffer, than if it is eluted in 50 µl without incubation (Figure 4, page 14).

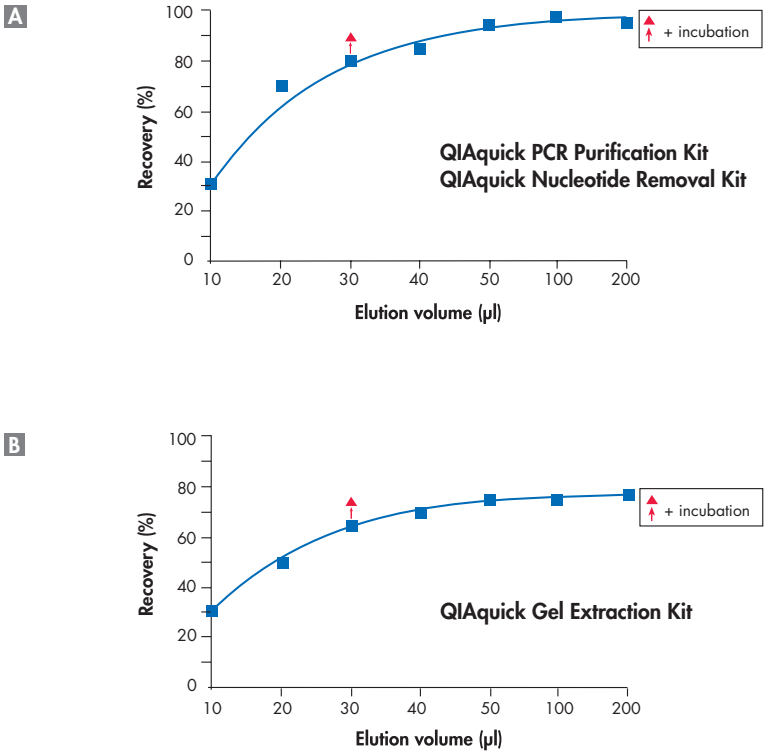


Figure 4. Highly concentrated DNA. Effect of elution buffer volume on DNA yield for A the QIAquick PCR Purification and QIAquick Nucleotide Removal Kit; B the QIAquick Gel Extraction Kit. 5 µg of a 2.9 kb DNA fragment were purified and eluted with the indicated volumes of Buffer EB. 30 µl plus 1 minute incubation on the QIAquick column gives DNA yields similar to 50 µl without incubation, but at a concentration 1.7 times greater.

Loading dye

Loading dye is provided for analysis of purified DNA samples using electrophoresis. It contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type. Loading dye is supplied as a 5x concentrate; thus 1 volume of loading dye should be added to 5 volumes of purified DNA.

Table 2. Migration Distance of Gel Tracking Dyes

%TAE (TBE) agarose gel	Xylene cyanol (light blue)	Bromophenol blue (dark blue)	Orange G (orange)
0.8	5000 bp (3000 bp)	800 bp (400 bp)	150 bp (<100 bp)
1.0	3000 bp (2000 bp)	400 bp (250 bp)	<100 bp (<100 bp)
1.5	1800 bp (1100 bp)	250 bp (100 bp)	<100 bp (<100 bp)
2.0	1000 bp (600 bp)	200 bp (<100 bp)	<100 bp (<100 bp)
2.5	700 bp (400 bp)	100 bp (<50 bp)	<50 bp (<50 bp)

Agarose gel analysis of yield

Yields of DNA following cleanup can be determined by agarose gel analysis. Table 3 shows the total yield obtained following extraction of 1 µg or 0.5 µg starting DNA from an agarose gel with a recovery of 80% or 60% using the QIAquick Gel Extraction Kit. The corresponding amount of DNA in a 1 µl aliquot from 50 µl eluate is indicated. Quantities of DNA fragment corresponding to these 1 µl aliquots are shown on the agarose gel in Figure 4.

Table 3. Amount of DNA in 1 µl aliquots of a 50 µl eluate following QIAquick purification

Starting DNA	Recovery	Total yield (50 µl eluate)	Amount of DNA in 1 µl
1 µg	80%	0.8 µg	16 ng
	60%	0.6 µg	12 ng
0.5 µg	80%	0.4 µg	8 ng
	60%	0.3 µg	6 ng

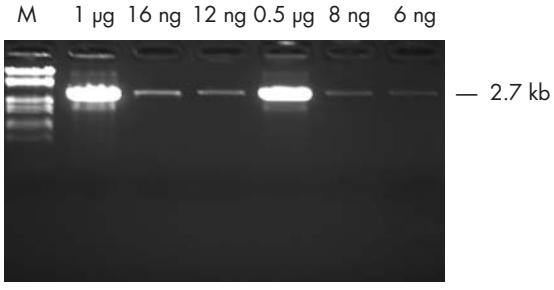


Figure 5. High DNA recovery. Quantities of purified 2.7 kb DNA fragment corresponding to 1/50 of the DNA obtained following purification from 1 µg or 0.5 µg starting DNA with a recovery of 80% or 60% (see Table 1). Samples were run on a 1% TAE agarose gel. **M**: lambda–EcoRI-HindIII markers.

Quantification of DNA fragments

DNA fragments can be quantified by running a sample alongside standards containing known quantities of the same-sized DNA fragment. The amount of sample DNA loaded can be estimated by visual comparison of the band intensity with that of the standards (Figure 5).

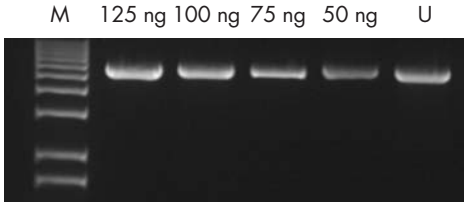
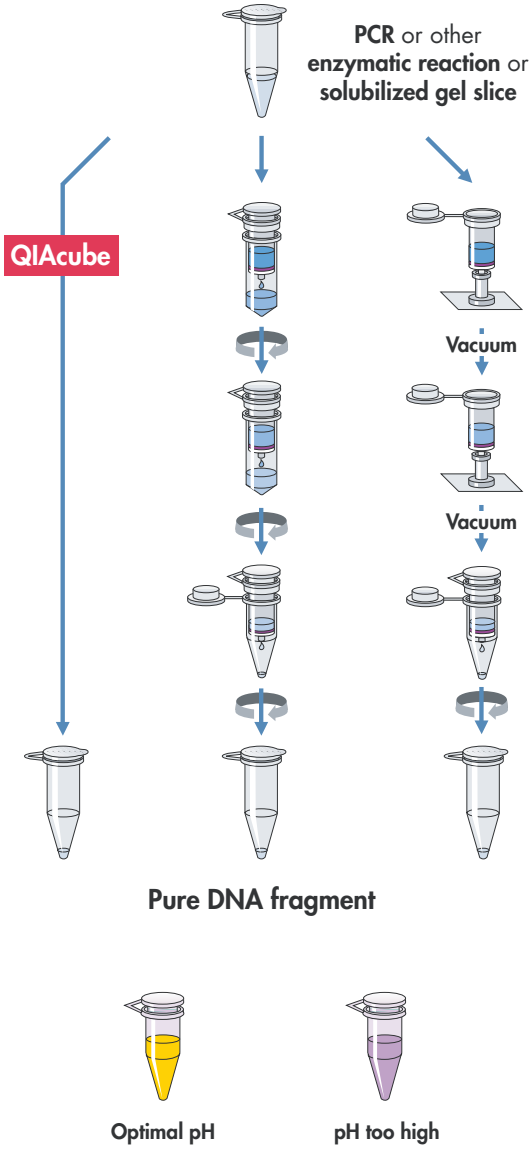


Figure 6. Agarose gel analysis. An unknown amount of a 5.5 kb DNA fragment (**U**) was run alongside known quantities (as indicated in ng) of the same DNA fragment. The unknown sample contained 75–100 ng DNA, as estimated by visual comparison with the standards. **M**: 1 kb DNA ladder.

Applications using QIAquick purified DNA

DNA purified with QIAquick is suitable for any subsequent application, such as restriction, labeling, hybridization, PCR, ligation and transformation, radioactive and fluorescent sequencing, in vitro transcription, or microinjection.

The QIAquick Procedure



Equipment and Reagents to Be Supplied by User

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) available from the product supplier.

For all protocols

- Ethanol (96–100%)*
- Microcentrifuge
- 1.5 or 2 ml microcentrifuge tubes
- 3 M sodium acetate, pH 5.0, may be necessary for PCR purification and gel extraction protocols.
- Optional: Distilled water or TE buffer (10 mM Tris·Cl. 1 mM EDTA, pH 8) for elution of DNA.

Vacuum protocols

- Vacuum manifold (e.g., QIAvac 24 Plus or QIAvac 6S)
- Vacuum pump (e.g., QIAGEN Vacuum Pump, see ordering information).

Gel extraction protocols

- Isopropanol (100%)
- Heating block or water bath set at 50°C

* Do not use denaturated alcohol, which contains other substances such as methanol or methylethylketone.

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx . The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and Suggestions
Low or no recovery		
a)	Buffer PE did not contain ethanol	Ethanol must be added to Buffer PE (concentrate) before use. Repeat procedure with correctly prepared Buffer PE.
b)	Inappropriate elution buffer	DNA will only be eluted efficiently in the presence of low-salt buffer (e.g., Buffer EB: 10 mM Tris·Cl, pH 8.5) or water. See "Elution in low-salt solutions", page 13.
c)	Elution buffer incorrectly dispensed	Add elution buffer to the center of the QIAquick membrane to ensure that the buffer completely covers the membrane. This is particularly important when using small elution volumes (30 µl).
Gel		
d)	Gel slice incompletely solubilized	After addition of Buffer QG to the gel slice, mix by vortexing the tube every 2–3 min during the 50°C incubation. DNA will remain in any undissolved agarose.
e)	pH of electrophoresis buffer too high (binding mixture turns orange or violet)	The electrophoresis buffer has been repeatedly used or incorrectly prepared, resulting in a sample pH that exceeds the buffering capacity of Buffer QG and leads to inefficient DNA binding. Add 10 µl of 3 M sodium acetate, pH 5.0, to the sample and mix. The color of the mixture will turn yellow indicating the correct pH for DNA binding. Even for binding mixtures with only small color changes (slight orange color), add the 10 µl sodium acetate.
f)	Gel slice was too large (>400 mg)	70–80% recovery can only be obtained from ≤400 mg gel slice per QIAquick column. For gel slices >400 mg, use multiple QIAquick columns.

Gel: refers to QIAquick Gel Extraction Kits only.

PCR: refers to QIAquick PCR Purification Kits only.

Other notes refer to all kits.

		Comments and Suggestions
PCR		
g)	Insufficient/no PCR product	Estimate DNA recovery by running 10% of PCR product before and after purification on an agarose gel.
PCR/Gel		
h)	Cloudy and gelatinous appearance of sample mixture after addition of isopropanol	This may be due to salt precipitation, and will disappear upon mixing the sample. Alternatively, the gel slice may not be completely solubilized. In this case, apply the mixture to the QIAquick column, centrifuge, and then add 0.5 ml Buffer QG to the column. Let stand for 1 min at room temperature, and then centrifuge and continue with the procedure. This additional wash will solubilize remaining agarose.
i)	Binding mixture turns orange or violet	The pH in the sample exceeds the buffer capacity of Buffer QG or PB respectively. Add 20 µl of 3 M sodium acetate, pH 5.0, to the sample and mix. The color of the mixture will turn yellow indicating the correct pH for DNA binding. Even for samples with slight color changes (orange color), add 10 µl sodium acetate.
DNA does not perform well (e.g., in ligation reactions)		
a)	Salt concentration in eluate too high	Modify the wash step by incubating the column for 5 min at room temperature after adding 750 µl of Buffer PE, then centrifuge.
b)	Eluate contains residual ethanol	Ensure that the wash flow-through is drained from the collection tube and that the QIAquick column is then centrifuged at 17,900 x *g* (13,000 rpm) for an additional 1 min.
Gel		
c)	Eluate contaminated with agarose	The gel slice is incompletely solubilized or weighs >400 mg. Repeat procedure, including the optional Buffer QG column-wash step.
PCR		
d)	Eluate contains primer-dimers	Primer-dimers formed are >20 bp and are not completely removed. After the binding step, wash the QIAquick column with 750 µl of a 35% guanidine hydrochloride aqueous solution (35 g in 100 ml). Continue with the Buffer PE wash step and the elution step as in the protocol.

		Comments and Suggestions
e)	Eluate contains denatured ssDNA, which appears as smaller smeared band on an analytical gel	Use the eluted DNA to prepare the subsequent enzymatic reaction but omit the enzyme. To reanneal the ssDNA, incubate the reaction mixture at 95°C for 2 min, and allow the tube to cool slowly to room temperature. Add the enzyme and proceed as usual. Alternatively, the DNA can be eluted in 10 mM Tris buffer containing 10 mM NaCl. The salt and buffering agent promote the renaturation of DNA strands. However the salt concentration of the eluate must then be considered for subsequent applications.

References

1. Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA ***76***, 615.
2. Hamaguchi, K. and Geiduschek, E.P. (1962) The effect of electrolytes on the stability of deoxyribonucleate helix. J. Am. Chem. Soc. ***84***, 1329.

Appendix 1G
Qiagen RNeasy Plant Mini Kit Handbook

Fourth Edition September 2010

RNeasy® Mini Handbook

RNeasy Mini Kit

For purification of total RNA from animal cells, animal tissues, bacteria, and yeast, and for RNA cleanup

RNeasy Protect Mini Kit

For immediate stabilization of RNA in harvested animal tissues and subsequent total RNA purification

RNeasy Plant Mini Kit

For purification of total RNA from plants and filamentous fungi

WWW.QIAGEN.COM

RNeasy Plant Mini Kit	(20)	(50)
Catalog no.	74903	74904
Number of preps	20	50
RNeasy Mini Spin Columns (pink)	20	50
QIAshredder Spin Columns (lilac)	20	50
Collection Tubes (1.5 ml)	20	50
Collection Tubes (2 ml)*	20	50
Buffer RLT*†	18 ml	45 ml
Buffer RLC†	18 ml	45 ml
Buffer RW1†	18 ml	45 ml
Buffer RPE‡ (concentrate)	5 ml	11 ml
RNase-Free Water	10 ml	10 ml
Handbook	1	1

* Also available separately. See page 74 for ordering information.

† Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 8 for safety information.

‡ Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Storage

The RNeasy Mini Kit, RNeasy Protect Mini Kit (including RNA*later* RNA Stabilization Reagent), and RNeasy Plant Mini Kit should be stored dry at room temperature (15–25°C) and are stable for at least 9 months under these conditions.

Storage of RNA*later* Reagent at lower temperatures may cause precipitation. Before use, redissolve the precipitate by heating to 37°C with agitation.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RNeasy Mini Kit, RNeasy Protect Mini Kit, and RNeasy Plant Mini Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

The RNeasy Mini Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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 each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer RLT contains guanidine thiocyanate, Buffer RLC contains guanidine hydrochloride, and Buffer RW1 contains a small amount of guanidine thiocyanate. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is split, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the RNeasy Mini Kit, RNeasy Protect Mini Kit, and/or RNeasy Plant Mini Kit.

Buffer RLT

Contains guanidine thiocyanate: harmful. Risk and safety phrases:* R20/21/22-32, S13-26-36-46

Buffer RLC

Contains guanidine hydrochloride: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-26-36-46

Buffer RW1

Contains ethanol: flammable. Risk phrase:* R10

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R22: Harmful if swallowed; R32: Contact with acids liberates very toxic gas; R36/38: Irritating to eyes and skin; S13: Keep away from food, drink and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S46: If swallowed, seek medical advice immediately and show the container or label.

Introduction

The *RNeasy Mini Handbook* provides protocols for use with the following kits:

- **RNeasy Mini Kit** — for purification of total RNA from animal cells, animal tissues, and yeast, and for cleanup of RNA from crude RNA preps and enzymatic reactions (e.g., DNase digestion, proteinase digestion, RNA ligation, and labeling reaction)
- **RNeasy Protect Mini Kit** — for immediate stabilization of RNA in harvested animal tissues and subsequent purification of total RNA
- **RNeasy Plant Mini Kit** — for purification of total RNA from plant cells and tissues and filamentous fungi

The RNeasy Mini Kit can also be used to purify total RNA from bacteria. In this case, we strongly recommend using the kit in combination with RNAprotect® Bacteria Reagent (available separately), which provides in vivo stabilization of RNA in bacteria to ensure reliable gene expression analysis. Various protocols for stabilizing and purifying RNA from different bacteria species are included in the *RNAprotect Bacteria Reagent Handbook*. The RNeasy Mini Kit and RNAprotect Bacteria Reagent can also be purchased together as the RNeasy Protect Bacteria Mini Kit. For ordering information, see pages 75–76. It is also possible to use the RNeasy Mini Kit to purify cytoplasmic RNA from animal cells. The protocol can be downloaded at www.qiagen.com/literature/protocols/RNeasyMini.aspx .

The RNeasy Kits are designed to purify RNA from small amounts of starting material. They provide a fast and simple method for preparing up to 100 µg total RNA per sample. The purified RNA is ready for use in downstream applications such as:

- RT-PCR and real-time RT-PCR
- Differential display
- cDNA synthesis
- Northern, dot, and slot blot analyses
- Primer extension
- Poly A^+ RNA selection
- RNase/S1 nuclease protection
- Microarrays

The RNeasy Kits allow the parallel processing of multiple samples in less than 30 minutes. Time-consuming and tedious methods, such as CsCl step-gradient ultracentrifugation and alcohol precipitation, or methods involving the use of toxic substances, such as phenol and/or chloroform, are replaced by the RNeasy procedure.

Principle and procedure

RNA purification using RNeasy technology

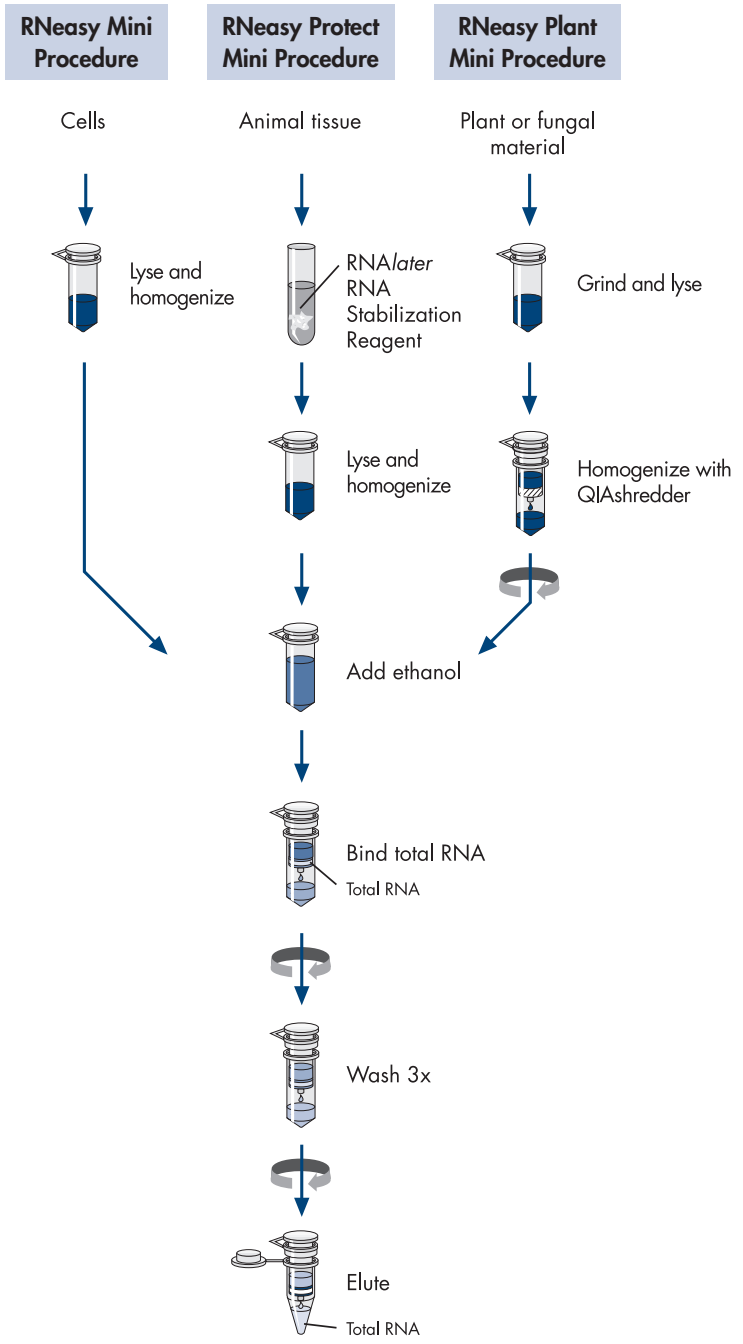
The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl water.

With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently. Protocols for purification of small RNA using RNeasy Kits are available at www.qiagen.com/goto/microRNAprotocols .

In this handbook, different protocols are provided for different starting materials. The protocols differ primarily in the lysis and homogenization of the sample and in the adjustment of the conditions for binding RNA to the RNeasy membrane. Once the sample is bound to the membrane, the protocols are similar (see flowchart, next page).

RNA stabilization using RNA*later* technology

RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analyses, such as microarray analyses, quantitative RT-PCR, such as TaqMan® and LightCycler® technology, and other nucleic acid-based technologies.


RNeasy Mini Procedure
RNeasy Protect Mini Procedure
RNeasy Plant Mini Procedure
Cells
Animal tissue
Plant or fungal material
Lyse and homogenize
RNA*later* RNA Stabilization Reagent
Grind and lyse
Lyse and homogenize
Homogenize with QIAshredder
Add ethanol
Bind total RNA
Total RNA
Wash 3x
Elute
Total RNA

The RNeasy Protect Mini Kit is supplied with RNA*later* RNA Stabilization Reagent, which represents a novel technology for the immediate preservation of the gene expression pattern in animal tissues, enabling reliable gene expression analysis. After harvesting, tissues are immediately submerged in RNA*later* RNA Stabilization Reagent, which rapidly permeates the tissues to stabilize and protect cellular RNA in situ. The reagent preserves RNA for up to 1 day at 37°C, 7 days at 15–25°C, or 4 weeks at 2–8°C, allowing transportation, storage, and shipping of samples without ice or dry ice. Alternatively, the samples can be archived at –20°C or –80°C. During storage or transport in RNA*later* RNA Stabilization Reagent, even at elevated temperatures (e.g., room temperature or 37°C), the cellular RNA remains intact and undegraded. RNA*later* technology allows large numbers of samples to be easily processed and replaces inconvenient, dangerous, and equipment-intensive methods, such as snap-freezing of samples in liquid nitrogen, storage at –80°C, cutting and weighing on dry ice, or immediate processing of harvested samples.

Note: RNA*later* RNA Stabilization Reagent is not for stabilization of RNA in animal cells, whole blood, plasma, or serum.

Description of protocols

Purification of Total RNA from Animal Cells Using Spin Technology

Up to 1 x 10^7 cells, depending on the cell line, are disrupted in Buffer RLT and homogenized. An overview of disruption and homogenization methods is given on pages 20–23. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. All bind, wash, and elution steps are performed by centrifugation in a microcentrifuge.

Purification of Total RNA from Animal Cells Using Vacuum/Spin Technology

Up to 1 x 10^6 cells, depending on the cell line, are disrupted in Buffer RLT and homogenized. An overview of disruption and homogenization methods is given on pages 20–23. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. The bind and wash steps are performed on a QIAvac 24, QIAvac 24 Plus, or QIAvac 6S vacuum manifold, and the final elution step is performed by centrifugation in a microcentrifuge.

Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi

Up to 100 mg of sample is first ground in liquid nitrogen and then lysed under highly denaturing conditions. The RNeasy Plant Mini Kit provides a choice of lysis buffers: Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively. The higher cell disruption and denaturing properties of Buffer RLT frequently make it the buffer of choice. However, some tissues, such as milky endosperm of maize or mycelia of filamentous fungi, solidify in Buffer RLT, making the extraction of RNA impossible. In these cases, Buffer RLC should be used instead. After lysis with either buffer, samples are centrifuged through a QIAshredder homogenizer. This simultaneously removes insoluble material and reduces the viscosity of the lysates by disrupting gelatinous material often formed in plant and fungal lysates. Ethanol is added to the cleared lysate, creating conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

RNA Cleanup

This protocol can be used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labeling) or to desalt RNA samples (up to 100 µg RNA). Buffer RLT and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For all protocols

- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- 96–100% ethanol*
- Disposable gloves
- Equipment for sample disruption and homogenization (see pages 20–23). Depending on the method chosen, one or more of the following are required:
  - Trypsin and PBS
  - QIAshredder homogenizer (see ordering information, page 76)
  - Blunt needle and syringe
  - Mortar and pestle
  - TissueLyser (see ordering information, page 76)
  - Rotor–stator homogenizer

For RNA purification from animal cells

- 70% ethanol*

For RNA purification from animal cells using vacuum technology

- QIAvac 24 (no longer available); QIAvac 24 Plus (cat. no. 19413); QIAvac 6S (cat. no. 19503) with the QIAvac Luer Adapter Set (cat. no. 19541); or other vacuum manifold with luer connectors and capable of dealing with vacuum pressures of –800 to –900 mbar
- QIAGEN Vacuum Pump (see page 75 for ordering information); or other vacuum pump capable of generating a vacuum pressure of –800 to –900 mbar and with a capacity of 18–20 liter/min

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Note: Use of insufficient vacuum pressure may reduce RNA yield and purity. The RNeasy procedure requires higher vacuum pressures compared with other QIAGEN procedures. Most water pumps or house vacuums do not provide sufficient vacuum pressure.

- Optional: Vacuum Regulator (cat. no. 19530) to measure the pressure difference between the inside and outside of a vacuum system

  A vacuum pressure of –800 to –900 mbar should develop when RNeasy Mini spin columns are used on the vacuum manifold. Vacuum pressures exceeding –900 mbar should be avoided. The vacuum pressure is the pressure difference between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 mbar or 760 mm Hg) and can be regulated and measured using a pressure gauge or vacuum regulator. Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere.

- Optional: VacConnectors (cat. no. 19407)

  These disposable connectors fit between the RNeasy Mini spin columns and the luer extensions on the QIAvac 24 or QIAvac 24 Plus or the QIAvac Luer Adapters on the QIAvac 6S. They prevent direct contact between the RNeasy Mini spin columns and luer connectors during RNA purification, avoiding any cross-contamination between samples. VacConnectors are discarded after single use.

For RNA purification from animal tissues

- 70% ethanol*
- Optional: Dithiothreitol (DTT)

For RNA purification from yeast using enzymatic lysis

- 70% ethanol*
- Buffer for enzymatic lysis

  In most cases, Buffer Y1 (containing sorbitol, EDTA, β-ME, and lyticase or zymolase) can be used. See the protocol on page 45 for details on preparing Buffer Y1.

For RNA purification from yeast using mechanical disruption

- 70% ethanol*
- Glass beads, 0.45–0.55 mm diameter

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

- Concentrated nitric acid, deionized water, and baking oven
- TissueLyser or other bead-mill homogenizer

For RNA purification from plants and fungi

- Liquid nitrogen
- Mortar and pestle (alternatively, TissueLyser or other bead-mill homogenizer)

Suppliers of equipment for disruption and homogenization*

Rotor–stator homogenizers can be purchased from:

- BioSpec Products, Inc. (www.biospec.com): Tissue-Tearor™ homogenizer
- Charles Ross & Son Company (www.mixers.com)
- IKA (www.ika.de): ULTRA-TURRAX® dispersers
- KINEMATICA AG (www.kinematica.ch) or Brinkmann Instruments, Inc. (www.brinkmann.com): POLYTRON® laboratory dispersing devices
- Omni International, Inc. (www.omni-inc.com)
- Silverson (www.silverson.com)
- VirTis (www.virtis.com)

Bead-mill homogenizers and stainless steel and tungsten carbide beads can be purchased from:

- QIAGEN (TissueLyser system, see page 76 for ordering information)

Glass, stainless steel, and tungsten carbide beads can be purchased from:

- Retsch (www.retsch.de)

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

Important Notes

Determining the amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The maximum amount that can be used is determined by:

- The type of sample and its RNA content
- The volume of Buffer RLT required for efficient lysis
- The RNA binding capacity of the RNeasy spin column

When processing samples containing high amounts of RNA, less than the maximum amount of starting material shown in Table 1 should be used, so that the RNA binding capacity of the RNeasy spin column is not exceeded.

When processing samples containing average or low amounts of RNA, the maximum amount of starting material shown in Table 1 can be used. However, even though the RNA binding capacity of the RNeasy spin column is not reached, the maximum amount of starting material must not be exceeded. Otherwise, lysis will be incomplete and cellular debris may interfere with the binding of RNA to the RNeasy spin column membrane, resulting in lower RNA yield and purity.

More information on using the correct amount of starting material is given in each protocol. Table 2 shows expected RNA yields from various sources.

Table 1. RNeasy Mini Spin Column Specifications

Maximum binding capacity	100 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >200 nucleotides
Minimum elution volume	30 µl
Maximum amount of starting material	
■ Animal cells	1×10^7*
■ Animal tissues	30 mg*
■ Yeast	5×10^7*
■ Plant tissues	100 mg
■ Filamentous fungi	100 mg

* For larger sample sizes, RNeasy Kits and RNeasy Protect Kits are available in midi and maxi formats. For details, visit www.qiagen.com/RNA .

Note: If the binding capacity of the RNeasy spin column is exceeded, RNA yields will not be consistent and may be reduced. If lysis of the starting material is incomplete, RNA yields will be lower than expected, even if the binding capacity of the RNeasy spin column is not exceeded.

Table 2. Typical Yields of Total RNA with RNeasy Mini Spin Columns

Source	Yield of total RNA* (µg)
Cell cultures (1 x 10^6 cells)	
NIH/3T3	10
HeLa	15
COS-7	35
LMH	12
Huh	15
Mouse/rat tissues (10 mg)	
Embryo (13 day)	25
Kidney	20–30
Liver	40–60
Spleen	30–40
Thymus	40–50
Lung	10–20
Yeast (1 x 10^7 cells)	
S. cerevisiae	25
Plants (100 mg leaves)	
Arabidopsis	35
Maize	25
Tomato	65
Tobacco	60

* Amounts can vary due to factors such as species, developmental stage, and growth conditions. Since the RNeasy procedure enriches for mRNA and other RNA species >200 nucleotides, the total RNA yield does not include 5S rRNA, tRNA, and other low-molecular-weight RNAs, which make up 15–20% of total cellular RNA.

Handling and storing starting material

RNA in animal and plant tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent (animal tissues only), flash-frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that tissue samples are immediately frozen in liquid nitrogen and stored at –70°C, or immediately immersed in RNA*later* RNA Stabilization Reagent.

The procedures for tissue harvesting and RNA protection should be carried out as quickly as possible. Frozen tissue samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT (lysis buffer), samples can be stored at –70°C for months.

Animal and yeast cells can be pelleted and then stored at –70°C until required for RNA purification. However, if performing RNA purification from yeast cells with enzymatic lysis, only freshly harvested samples can be used.

Disrupting and homogenizing starting material

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA purification procedures. Disruption and homogenization are 2 distinct steps:

- **Disruption**: Complete disruption of cell walls and plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced RNA yields.
- **Homogenization**: Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy spin column membrane and therefore significantly reduced RNA yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 3 (page 21) gives an overview of different disruption and homogenization methods, and is followed by a detailed description of each method. This information can be used as a guide to choose the appropriate methods for your starting material.

Note: After storage in RNA*later* RNA Stabilization Reagent, tissues become slightly harder than fresh or thawed tissues. Disruption and homogenization of these tissues, however, is usually not a problem.

Table 3. Disruption and Homogenization Methods

Sample	Disruption method	Homogenization method	Comments
Animal cells	Addition of lysis buffer	Rotor–stator homogenizer or QIAshredder homogenizer* or syringe and needle	If processing ≤1 x 10^5 cells, lysate can be homogenized by vortexing
Animal tissues	TissueLyser	TissueLyser	The TissueLyser gives results comparable to using a rotor–stator homogenizer
	Rotor–stator homogenizer	Rotor–stator homogenizer	Simultaneously disrupts and homogenizes
	Mortar and pestle	QIAshredder homogenizer* or syringe and needle	Rotor–stator homogenizer usually gives higher yields than mortar and pestle
Yeast	Enzymatic digestion of cell wall followed by lysis of spheroplasts	Vortexing	
	TissueLyser with glass beads	TissueLyser with glass beads	TissueLyser simultaneously disrupts and homogenizes; cannot be replaced by vortexing
Plants and filamentous fungi	Mortar and pestle	QIAshredder homogenizer*	Mortar and pestle cannot be replaced by rotor–stator homogenizer

* QIAshredder homogenizers are supplied in the RNeasy Plant Mini Kit and can be purchased separately for use with the RNeasy Mini Kit or RNeasy Protect Mini Kit. See page 76 for ordering information.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the animal or plant tissue immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen–cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the homogenization according to one of the 2 methods below.

Note: Grinding the sample using a mortar and pestle will disrupt the sample, but will not homogenize it. Homogenization must be performed afterwards.

Homogenization using QIAshredder homogenizers

Using QIAshredder homogenizers is a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples. Up to 700 µl of lysate is loaded onto a QIAshredder spin column placed in a 2 ml collection tube, and spun for 2 minutes at maximum speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column. QIAshredder spin columns are supplied in the RNeasy Plant Mini Kit and can be purchased separately for use with the RNeasy Mini Kit and RNeasy Protect Mini Kit. See page 76 for ordering information.

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. Lysate is passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize loss.

Eliminating genomic DNA contamination

Generally, DNase digestion is not required with RNeasy Kits since RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). In these cases, residual DNA can be removed by optional on-column DNase digestion using the RNase-Free DNase Set (see Appendix D, page 69). The DNase is efficiently removed in subsequent wash steps. Alternatively, residual DNA can be removed by a DNase digestion after RNA purification (see Appendix E, page 71). The DNase digestion can then be cleaned up, if desired, using "Protocol: RNA Cleanup" (page 56).

The RNeasy Plus Mini Kit, which is designed for RNA purification from animal cells and tissues, integrates unique gDNA Eliminator spin columns with RNeasy technology. Genomic DNA is effectively removed in a single, rapid centrifugation step, avoiding the need for DNase digestion. See page 76 for ordering information.

If the purified RNA will be used in real-time, two-step RT-PCR, we recommend using the QuantiTect® Reverse Transcription Kit. The kit provides a fast and convenient procedure, enabling cDNA synthesis and genomic DNA removal in only 20 minutes. For ordering information, see page 78.

Protocol: Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi

This protocol requires the RNeasy Plant Mini Kit.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 100 mg plant material or 1 x 10^7 cells can generally be processed. For most plant materials, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Average RNA yields from various plant materials are given in Table 2 (page 19).

If there is no information about the nature of your starting material, we recommend starting with no more than 50 mg plant material or 3–4 x 10^6 cells. Depending on RNA yield and purity, it may be possible to use up to 100 mg plant material or up to 1 x 10^7 cells in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

Counting cells or weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5 cm diameter leaf disc weighs 25–75 mg.

Important points before starting

- If using the RNeasy Plant Mini Kit for the first time, read "Important Notes" (page 18).
- If working with RNA for the first time, read Appendix A (page 63).
- Fresh or frozen tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.
- The RNeasy Plant Mini Kit provides a choice of lysis buffers: Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively. In most cases, Buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of guanidine thiocyanate. However, depending on the amount and type of secondary metabolites in some tissues (such as milky endosperm of maize or mycelia of filamentous fungi), guanidine thiocyanate can cause solidification of the sample, making extraction of RNA impossible. In these cases, Buffer RLC should be used.

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT, Buffer RLC, and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 8 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT or Buffer RLC before use. Add 10 µl β-ME per 1 ml Buffer RLT or Buffer RLC. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT or Buffer RLC containing β-ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69).

Procedure

1. **Determine the amount of plant material. Do not use more than 100 mg.**

   Weighing tissue is the most accurate way to determine the amount.

2. **Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 3.**

   RNA in plant tissues is not protected until the tissues are flash-frozen in liquid nitrogen. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

3. **Add 450 µl Buffer RLT or Buffer RLC (see "Important points before starting") to a maximum of 100 mg tissue powder. Vortex vigorously.**

   A short 1–3 min incubation at 56°C may help to disrupt the tissue. However, do not incubate samples with a high starch content at elevated temperatures, otherwise swelling of the sample will occur.

Note: Ensure that β-ME is added to Buffer RLT or Buffer RLC before use (see "Things to do before starting").

4. **Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.**

   It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.

5. **Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.**

   Note: The volume of lysate may be less than 450 µl due to loss during homogenization.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. **Transfer the sample (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   Reuse the collection tube in step 7.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.*

   Optional: If performing optional on-column DNase digestion (see "Eliminating genomic DNA contamination", page 23), follow steps D1–D4 (page 69) after performing this step.

7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.***

   Reuse the collection tube in step 8.

* Flow-through contains Buffer RLT, Buffer RLC, or Buffer RW1 and is therefore not compatible with bleach. See page 8 for safety information.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

Skip this step if performing optional on-column DNase digestion (page 69).

8. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Reuse the collection tube in step 9.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.**

   The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

    If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

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 protocols in this handbook or molecular biology applications (see back cover for contact information).

		Comments and suggestions
Clogged RNeasy spin column		
a)	Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (pages 20–23) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see protocols) and/or increase the volume of lysis buffer and the homogenization time. If working with tissues rich in proteins, we recommend using the RNeasy Fibrous Tissue Mini Kit (see page 76 for ordering information).
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material. It is essential to use the correct amount of starting material (see protocols).
c)	Centrifugation before adding ethanol not performed (protocols for tissues and mechanical disruption of yeast)	Centrifuge the lysate before adding ethanol, and use only this supernatant in subsequent steps (see protocols). Pellets contain cell debris that can clog the RNeasy spin column.
d)	Centrifugation temperature too low	The centrifugation temperature should be 20–25°C. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause formation of precipitates that can clog the RNeasy spin column. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring it to the RNeasy spin column.

		Comments and suggestions
Low RNA yield		
a)	Insufficient disruption and homogenization	See "Disrupting and homogenizing starting material" (pages 20–23) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see protocols) and/or increase the volume of lysis buffer and the homogenization time. If working with tissues rich in proteins, we recommend using the RNeasy Fibrous Tissue Mini Kit (see page 76 for ordering information).
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material. It is essential to use the correct amount of starting material (see protocols).
c)	RNA still bound to RNeasy spin column membrane	Repeat RNA elution, but incubate the RNeasy spin column on the benchtop for 10 min with RNase-free water before centrifuging.
d)	Ethanol carryover	During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C to dry the RNeasy spin column membrane. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. To eliminate any chance of possible ethanol carryover, place the RNeasy spin column in a new 2 ml collection tube and perform the optional 1-min centrifugation step as described in the protocols.
e)	Incomplete removal of cell-culture medium (cell samples)	When processing cultured cells, ensure complete removal of the cell-culture medium after harvesting cells (see protocols).

	Comments and suggestions
Low A_{260}/A_{280} value	
Water used to dilute RNA for A_{260}/A_{280} measurement	Use 10 mM Tris·Cl, pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 65).
RNA degraded	
a) Harvested animal tissue not immediately stabilized	Submerge the tissue in the appropriate volume of RNA*later* RNA Stabilization Reagent immediately after harvesting.
b) Too much animal tissue for proper stabilization	Reduce the amount of tissue or increase the amount of RNA*later* RNA Stabilization Reagent used for stabilization (see protocol on page 36).
c) Animal tissue too thick for stabilization	Cut large samples into slices less than 0.5 cm thick for stabilization in RNA*later* RNA Stabilization Reagent.
d) Frozen animal tissue used for stabilization	Use only fresh, unfrozen tissue for stabilization in RNA*later* RNA Stabilization Reagent.
e) Storage duration in RNA*later* RNA Stabilization Reagent exceeded	RNA*later* stabilized tissue can be stored for up to 1 day at 37°C, up to 7 days at 15–25°C, or up to 4 weeks at 2–8°C, and can be archived at –20°C or –80°C.
f) Inappropriate handling of starting material	Ensure that tissue samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent. For frozen cell pellets or frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the RNeasy procedure quickly, especially the first few steps. See Appendix A (page 63), "Handling and storing starting material" (page 20), and the RNA*later* protocol (page 36).

		Comments and suggestions
g)	RNase contamination	Although all RNeasy buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the RNeasy procedure or later handling. See Appendix A (page 63) for general remarks on handling RNA. Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
DNA contamination in downstream experiments		
a)	Optimal procedure not used (cell samples)	For animal cells, we recommend purifying cytoplasmic RNA for applications where the absence of DNA contamination is critical, since intact nuclei are removed at the start of the procedure. The protocol can be downloaded at www.qiagen.com/literature/protocols/RNeasyMini.aspx .
b)	No incubation with Buffer RW1	In subsequent preparations, incubate the RNeasy spin column for 5 min at room temperature (15–25°C) after addition of Buffer RW1 and before centrifuging.
c)	No DNase treatment	Perform optional on-column DNase digestion using the RNase-Free DNase Set (see Appendix D, page 69) at the point indicated in the individual protocols. Alternatively, after the RNeasy procedure, DNase digest the RNA eluate. After inactivating the DNase by heat treatment, the RNA can be either used directly in the downstream application without further treatment, or repurified using the RNA cleanup protocol (page 56).

		Comments and suggestions
RNA does not perform well in downstream experiments		
a)	Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C. When reusing collection tubes between washing steps, remove residual flow-through from the rim by blotting on clean paper towels.
b)	Ethanol carryover	During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C to dry the RNeasy spin column membrane. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. To eliminate any chance of possible ethanol carryover, place the RNeasy spin column in a new 2 ml collection tube and perform the optional 1-min centrifugation step as described in the protocols.

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 64). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS),* thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

† Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.

Appendix B: Storage, Quantification, and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260}=1 → 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 66), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 64). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)

A_{260} = 0.2

Concentration of RNA sample = 44 µg/ml x A_{260} x dilution factor
= 44 µg/ml x 0.2 x 50
= 440 µg/ml

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Total amount	= concentration x volume in milliliters
	= 440 µg/ml x 0.1 ml
	= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA", page 65).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While RNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample.

For analysis of very low abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in real-time RT-PCR applications, such as with ABI PRISM® and LightCycler instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Assays from QIAGEN are designed for real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible. For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination (see ordering information, page 78).

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

For other sensitive applications, DNase digestion of the purified RNA with RNase-free DNase is recommended. A protocol for optional on-column DNase digestion using the RNase-Free DNase Set is provided in Appendix D (page 69). The DNase is efficiently washed away in subsequent wash steps. Alternatively, after the RNeasy procedure, the RNA eluate can be treated with DNase. The RNA can then be repurified according to the RNA cleanup protocol (page 56), or after heat inactivation of the DNase, the RNA can be used directly in downstream applications.

The protocol for purification of cytoplasmic RNA from animal cells (available at www.qiagen.com/literature/protocols/RNeasyMini.aspx) is particularly advantageous in applications where the absence of DNA contamination is critical, since intact nuclei are removed. Using this protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and RNeasy technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, even further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). Using the cytoplasmic RNA protocol with optional DNase digestion results in undetectable levels of DNA, even in sensitive quantitative RT-PCR analyses.

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using an Agilent 2100 bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S RNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

RNeasy® Plant Mini Kit

The RNeasy Plant Mini Kit (cat. nos. 74903 and 74904) can be stored at room temperature (15–25°C) for at least 9 months.

For more information, additional and more detailed protocols, and safety information, please refer to the *RNeasy Mini Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- The RNeasy Plant Mini Kit provides a choice of lysis buffers. Buffer RLT is the lysis buffer of choice but Buffer RLT can cause solidification of some samples, depending on the amount and type of secondary metabolites in the tissue. In these cases, Buffer RLC should be used.
- Add either 10 µl β-mercaptoethanol (β-ME), or 20 µl 2 M dithiothreitol (DTT)*, to 1 ml Buffer RLT or Buffer RLC before use. Buffers with DTT or β-ME can be stored at room temperature for up to 1 month.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

* This option not included for plant tissue in handbook; handbook to be updated.

1. Disrupt a maximum of 100 mg plant material according to step 1a or 1b.

1a. Disruption with mortar and pestle
Immediately place tissue in liquid nitrogen. Grind thoroughly. Decant tissue powder and liquid nitrogen into RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 2.

1b. Disruption using the TissueLyser II, TissueLyser LT, or TissueRuptor®
For detailed information on disruption of plant tissues for purification of RNA, see *TissueLyser Handbook*, *TissueLyser LT Handbook,* or *TissueRuptor Handbook*. (The *RNeasy Mini Handbook* will be updated with this option.)

January 2011

Sample & Assay Technologies

2. Add 450 µl Buffer RLT or Buffer RLC to a maximum of 100 mg tissue powder. Vortex vigorously.
3. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at full speed. Transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet.
4. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 5.
5. Transfer the sample (usually 650 µl), with any precipitate, to an RNeasy Mini spin column (pink) in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.
6. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*.

   Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to dry the membrane.
9. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x *g* to elute the RNA.
10. If the expected RNA yield is >30 µg, repeat step 9 using another 30–50 µl of RNase-free water. Alternatively, use the eluate from step 9 (if high RNA concentration is required). Reuse the collection tube from step 9.

Appendix 1H
Turbo DNA-free kit

TURBO DNA-*free*™ Kit

REVISED 4/2009
Inactivation time extended in step 4 on page 7.

TURBO DNase Treatment and Removal Reagents
Part Number AM1907

Applied Biosystems

A. Product Description

Ambion® TURBO DNA-*free*™ DNase Treatment and Removal Reagents are designed to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the sample. The included TURBO DNase (patent pending) is an engineered version of wild type DNase I with 350% greater catalytic efficiency. TURBO DNase has a markedly higher affinity for DNA than conventional DNase I, and is thus more effective in removing trace quantities of DNA contamination. In addition, TURBO DNase maintains up to 50X greater activity than DNase I in solutions containing physiological salt concentrations. The TURBO DNase provided in the kit is overexpressed in an animal-free system, and is then extensively purified in a bovine-free process and tested. It is guaranteed to lack any contaminating RNase activity. The kit also includes an optimized DNase reaction buffer that contains a small molecule enhancer that extends the activity of the TURBO DNase enzyme by 100-fold or more. Using TURBO DNA-*free*, contaminating DNA is digested to levels below the limit of detection by routine PCR (Figure 1). The DNase is then removed rapidly and easily using a novel method which does not require phenol/chloroform extraction, alcohol precipitation, heating, or the addition of EDTA (see Table 1). TURBO DNA-*free* treated RNA is suitable for endpoint or real-time RT-PCR, microarray analysis, RPAs, Northerns, and all other RNA analysis methods.

Ambion®

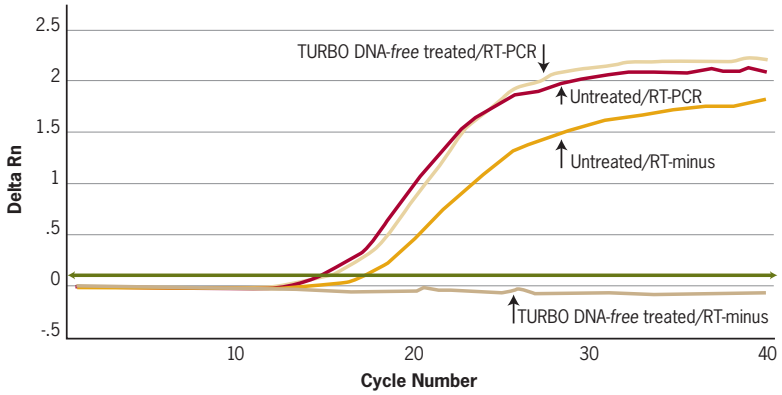


Figure 1. TURBO DNA-*free*™ Reduces Genomic DNA Contamination by Greater than 5 Million Fold.

Equal amounts of mouse spleen total RNA (purified using Ambion's RNAqueous® Kit) were either treated with 7.8 U of TURBO DNase in a 130 µL reaction for 20 min at 37°C, or were left untreated. The digestions were stopped by adding 22 µL DNase Inactivation Reagent. 5 µL (1 µg RNA) was amplified in a one step 25 µL RT-PCR using a TaqMan® primer probe set for mouse GAPDH. Treated and untreated samples were reverse transcribed with Ambion's MessageSensor™ RT Kit. RT-minus samples were subjected to PCR to control for DNA contamination. Results are shown using a linear scale so that the amplification plot for the TURBO DNase-treated, RT-minus sample is visible.

The fold-removal (5.4×10^6 fold) of genomic DNA was calculated as follows: The C_t value from the untreated RNA in the RT-minus reaction is the level of gDNA contamination. The fold-removal was determined by subtracting the RT-minus reaction C_t value for the treated RNA sample, 39.5 (the other duplicate's signal was undetectable) from the C_t value of the untreated sample, 17.13, and raising the 17.13 as the exponent with a base of 2.

Table 1. Treatment of RNA with TURBO DNA-*free*™ Maintains Target Sensitivity in Real-time RT-PCR

	Ct for β-actin (duplicates)	
RNA treatment	100 pg RNA	1 pg RNA
none	24.78 / 24.67	31.83 / 31.53
TURBO DNA-*free* treated	24.50 / 24.62	30.89 /30.88

	Ct for CDC-2 (duplicates)	
none	28.88 / 28.24	34.41 / 35.50
TURBO DNA-*free* treated	27.71 / 28.10	34.04 / 33.99

Total RNA from HeLa S3 cells was treated with the TURBO DNA-*free*™ Kit following the standard protocol. 5 µL of the treated RNA was then reverse transcribed using Ambion's MessageSensor RT Kit, and the resulting cDNA was amplified by real-time RT-PCR using primer and probe sets for either human β-actin or CDC-2 with TaqMan® detection.

In addition to removing the DNase enzyme, DNase Inactivation Reagent also removes divalent cations, such as magnesium and calcium, which can catalyze RNA degradation when RNA is heated with the sample (Figure 2).

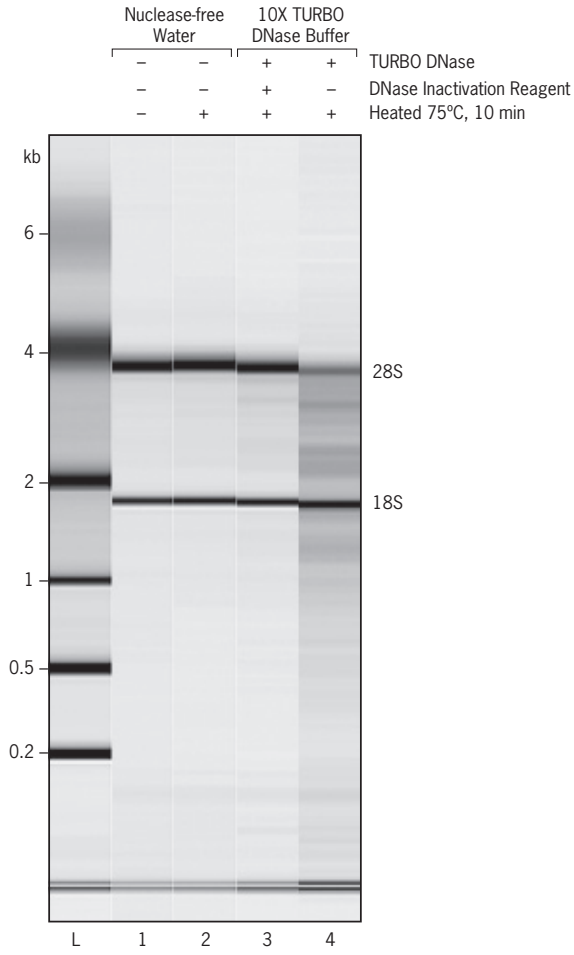


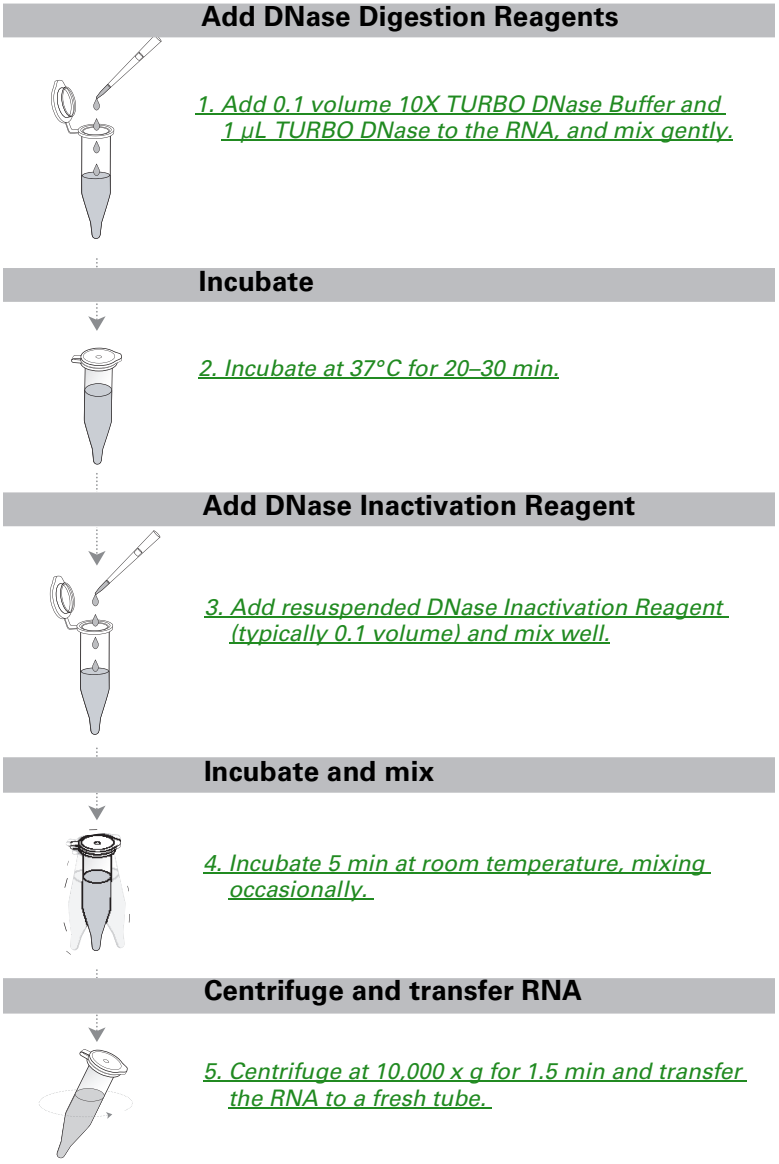
Figure 2. Removal of divalent cations by DNase Inactivation Reagent

HeLa-S3 total RNA (100 ng), in 50 µL 1X TURBO DNase Buffer or in nuclease-free water, was treated with components from the TURBO DNA-*free*™ kit as indicated. Samples were heated for 10 min at 75°C (Lanes 2, 3, & 5), or 3 min at 90°C (Lane 4), to determine if divalent cations from the TURBO DNase Buffer remained in solution, and degraded the RNA. 1 µL of each sample was analyzed on an RNA LabChip® using the Agilent 2100 bioanalyzer. Note that RNA was degraded in the sample that contained TURBO DNase Buffer, but was not treated with the DNase Inactivation Reagent (Lane 5); this degradation is due to the presence of divalent ions that induce heat-mediated RNA cleavage.

B. Procedure Overview

For the detailed procedure, see section E on page 5.

Figure 3. TURBO DNA-*free*™ Procedure Overview



C. How Much RNA Can Be Treated with TURBO DNA-*free*™?

This protocol is designed to remove trace to moderate amounts of contaminating DNA (up to 50 µg DNA/mL RNA) from purified RNA to a level that is mathematically insignificant by RT-PCR. No RNA isolation method can extract RNA that is completely free from DNA contamination; in fact, RNA isolated from some tissues, such as spleen, kidney, or thymus, often contain relatively high levels of DNA. Other potential sources of DNA contamination include carryover of the interface during organic extractions, and overloaded glass-fiber filters during RNA purification.

D. TURBO DNA-*free* Components and Storage

Reagents are provided for 50 TURBO DNA-*free* treatments (up to 100 µL each).

Amount	Component	Storage
120 µL	TURBO DNase (2 Units/µL)	–20°C
600 µL	10X TURBO DNase Buffer	–20°C
600 µL	DNase Inactivation Reagent	–20°C
1.75 mL	Nuclease-free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C or room temp

Store the TURBO DNA-*free* Kit at –20°C in a non-frost-free freezer for long-term storage. For convenience, the 10X TURBO DNase Buffer and the DNase Inactivation Reagent can be stored at 4°C for up to 1 week.

E. TURBO DNA-*free* Procedure

Procedure Notes

- We recommend conducting reactions in 0.5 mL tubes to facilitate removal of the supernatant after treatment with the DNase Inactivation Reagent.

- TURBO DNA-*free* reactions can be conducted in 96-well plates. We recommend using V-bottom plates because their shape makes it easier to remove the RNA from the pelleted DNase Inactivation Reagent at the end of the procedure.
- The recommended reaction size is 10–100 µL. A typical reaction is 50 µL.

1. Add 0.1 volume 10X TURBO DNase Buffer and 1 µL TURBO DNase to the RNA, and mix gently.

There are separate DNase digestion conditions depending on the amount of contaminating DNA and the nucleic acid concentration of the sample.

- ***Routine DNase treatment:*** ≤200 µg nucleic acid per mL
- ***Rigorous DNase treatment:*** >200 µg nucleic acid per mL or RNA that is severely contaminated with DNA (i.e. >2 µg DNA/50 µL)

Routine DNase treatment: Use 1 µL TURBO DNase (2 U) for up to 10 µg of RNA in a 50 µL reaction. These reaction conditions will remove up to 2 µg of genomic DNA from total RNA in a 50 µL reaction volume.

Rigorous DNase treatment: If the RNA contains more than 200 µg of nucleic acid per mL, dilute the sample to 10 µg nucleic acid/50 µL before adding the TURBO DNase Buffer and TURBO DNase. It is also helpful to add only half of the TURBO DNase to the reaction initially, incubate for 30 min, then add the remainder of the enzyme and incubate for another 30 min.

If the sample cannot be diluted, simply increase the amount of TURBO DNase to 2–3 µL (4–6 U). It may be possible to successfully remove contaminating DNA from samples containing up to 500 µg/mL nucleic acid in a 10–100 µL TURBO DNA-*free* reaction. However, the efficacy of treating highly concentrated nucleic acid samples depends on the absolute level of DNA contamination, and residual DNA may or may not be detectable by PCR after 35–40 cycles.

2. Incubate at 37°C for 20–30 min.

If only half of the TURBO DNase was added in step 1, incubate for 30 min, then add the rest of the TURBO DNase and incubate for 30 min more.

3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume) and mix well.

Always resuspend the DNase Inactivation Reagent by flicking or vortexing the tube before dispensing it.

- For ***routine DNase treatment*** use 2 µL or 0.1 volume DNase Inactivation Reagent, whichever is greater. For example, if the RNA volume is 50 µL, and 1 µL of TURBO DNase was used in step 1, add 5 µL of DNase Inactivation Reagent.
- For ***rigorous DNase treatments***, where 2–3 µL of TURBO DNase was used, add 0.2 volumes of DNase Inactivation Reagent.

 **IMPORTANT**

Always use at least 2 µL of DNase Inactivation Reagent, even if it is more than 0.1 volume.

 **NOTE**

The DNase Inactivation Reagent may become difficult to pipette after multiple uses due to depletion of fluid from the interstitial spaces. If this happens, add a volume of Nuclease-free Water (supplied with the kit) equal to approximately 20–25% of the bed volume of the remaining DNase Inactivation Reagent, and vortex thoroughly to recreate a pipettable slurry.

4. Incubate 5 min at room temperature, mixing occasionally.

Flick the tube 2–3 times during the incubation period to redisperse the DNase Inactivation Reagent.

 **NOTE**

If room temperature cools below 22–26 °C, move the tubes to a heat block or oven to control the temperature. Cold environments can reduce the inactivation of the TURBO DNase, leaving residual DNase in the RNA sample.

5. Centrifuge at 10,000 x g for 1.5 min and transfer the RNA to a fresh tube.

- For 96-well plates, centrifuge at 2000 x g for 5 min.

This centrifugation step pellets the DNase Inactivation Reagent. After centrifuging, carefully transfer the supernatant, which contains the RNA, into a fresh tube. Avoid introducing the DNase Inactivation Reagent into solutions that may be used for downstream enzymatic reactions, because it can sequester divalent cations and change the buffer conditions.

F. Troubleshooting

1. No RT-PCR product is detectable from RNA treated with TURBO DNA-*free*

DNase Inactivation Reagent could inhibit RT-PCR.
In step .E.5 on page 8, remove the RNA solution from the pelleted DNase Inactivation Reagent carefully to avoid transferring it to the tube of RNA. You may have to leave a small amount of RNA behind to accomplish this. If you accidentally touch the pellet while removing the RNA, recentrifuge to pack the DNase Inactivation Reagent.

TURBO DNA-*free* treated RNA should comprise only ~20% of an RT-PCR reaction volume.
For RT-PCR, we recommend that TURBO DNA-*free* treated RNA makes up ~20%, and no more than 40%, of the final RT-PCR volume. Otherwise, components from the TURBO DNase Buffer and the DNase Inactivation Reagent could interfere with the reaction. If necessary, RT-PCR volumes can be increased to 50 µL or more to accommodate your RNA without exceeding the 20–40% limit.

RNA used in RT-PCR should be treated only once with TURBO DNA-*free*.
The salt in TURBO DNA-*free* reactions is carefully balanced for optimal TURBO DNase activity. Subjecting RNA to a second TURBO DNA-*free* treatment will introduce additional salts that could interfere with downstream enzymatic reactions. If a second DNase treatment is required, please refer to the "TURBO DNA-*free* 2nd Digest Protocol" available online at:

www.ambion.com/techlib/append/supp/digest.html

2. RNA is degraded upon heating to >60°C

RNA samples that contain divalent cations, such as magnesium or calcium, will degrade when heated to temperatures above 60°C. To ensure that divalent cations are removed during step .E.4 on page 7, redisperse the DNase Inactivation Reagent by mixing the reaction 2–3 times during the incubation period.

3. The RNA absorbance spectrum has an unusual profile after treatment with TURBO DNA-*free*.

If the concentration of RNA in the sample is less than about 50 ng/µL, you may notice significant absorbance at ~230 nm. A_{260}/A_{280} ratios may also be slightly lower than normal when the RNA concentration is ≤25 ng/µL. These differences in the absorbance profile are caused by the enhancer in the TURBO DNase Buffer. Exhaustive comparisons at Ambion with both treated and untreated RNA samples indicate that the enhancer has no affect on accurate RNA quantification unless the RNA concentration is below 10 ng/µL. For more information, please see:

www.ambion.com/catalog/supp/absorbance.html

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.

Appendix 1I
iScript cDNA Synthesis

BIO-RAD

iScript™cDNA Synthesis Kit

25 x 20 µl reactions	170-8890
100 x 20 µl reactions	170-8891

For Research purposes only
Store at -20 °C (not frost-free)

iScript cDNA Synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. This kit includes just three tubes - comprehensive of the reagents required for successful RT-PCR.

The iScript reverse transcriptase is RNAse H+, resulting in greater sensitivity than RNAse H- enzymes. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided pre-blended with RNAse inhibitor. The unique blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length.

iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

Storage and Stability

Store the iScript™ cDNA Synthesis Kit at -20 °C in a constant temperature freezer. When stored under these conditions the kit components are stable for a minimum of one year after ship date. Nuclease-free water can be stored at room temperature.

Kit Contents

Reagent	Volume
25 reaction kit	
5x iScript Reaction Mix	100µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	25µl
100 reaction kit	
5x iScript Reaction Mix	400µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	100µl

Reaction Set Up

Component	Volume per reaction
5x iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	xµl
RNA template (100fg to 1µg Total RNA)*	xµl
Total Volume	20 µl

Reaction Protocol

Incubate complete reaction mix:
- 5 minutes at 25ºC
- 30 minutes at 42ºC
- 5 minutes at 85ºC
- Hold at 4ºC (optional)

Reagents and Materials Not Supplied

Reagents for PCR or real-time PCR
- Such as:
- iTaq™ DNA polymerase, 170-8870
- iQ™ Supermix, 170-8860 or
- iQ™ SYBR® Green Supermix, 170-8880

Pipette tips, aerosol barrier tips
- Such as:
- the Xcluda® Style B, 211-2006

Nuclease-free tubes
- Such as:
- 0.2ml Thin-Wall Tubes, 223-9473 or
- plates, 223-9441

RNA purification kit
- Such as the:
- Aurum™ Total RNA Mini Kit, 732-6820 or
- Aurum Total RNA Kit, 2 x 96 well, 732-6800

Recommendations for optimal results using the iScript cDNA Synthesis Kit:

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2µl.

*When using larger amounts of input RNA (>1µg) the reaction should be scaled up e.g. 40µl reaction for 2µg, 100µl reaction for 5µg to ensure optimum synthesis efficiency.

Appendix 1J
iProof High-Fidelity DNA Polymerase

BIO-RAD

iProof™ High-Fidelity PCR Kit

2 units/µl, 25 µl	50U	172-5330
2 units/µl, 100 µl	200U	172-5331

For research purposes only
Store at -20°C

iProof is a high-fidelity DNA polymerase that offers extreme performance for all PCR applications. Incorporating an exciting new and patented technology, iProof DNA polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity enhancing domain. This allows for the generation of long templates with an accuracy and speed previously unattainable with a single enzyme. The extreme fidelity of iProof makes it a superior choice for cloning. The error rate of iProof polymerase is determined to be 4.4×10^{-7} in iProof HF buffer, which is approximately 50-fold lower than that of *Thermus aquaticus*, and 6-fold lower than that of *Pyrococcus furiosus*.

The iProof™ High Fidelity PCR Kit includes lambda DNA control template and primers for 1.3 kb and 10 kb positive control amplicons. Sufficient template is included for performing 20 x 50 µl or 50 x 20 µl reactions.

Storage and Stability

Store the iProof™ High-Fidelity PCR Kit at -20°C in a constant temperature freezer. When stored under these conditions, the polymerase is stable for one year after the ship date.

Kit Contents

Reagent	50U	200U	Description
iProof Polymerase	25 µl	100 µl	iProof™ High Fidelity DNA Polymerase, 2 units/µl
iProof HF Buffer	1.5 ml	3 x 1.5 ml	5X HF Buffer, 7.5 mM $MgCl_2$
iProof GC Buffer	1.5 ml	3 x 1.5 ml	5X GC Buffer, 7.5 mM $MgCl_2$
dNTP mix	100 µl	100 µl	dNTP solution, 10 mM each
$MgCl_2$	1.5 ml	1.5 ml	50 mM $MgCl_2$ solution
Control 1template	40 µl	40 µl	Control 1template, 0.5 ng/µl
1.3 kb primers	50 µl	50 µl	4 µM each
10 kb primers	50 µl	50 µl	4 µM each
DNA Standard	200 µl	400 µl	DNA size standard
DMSO	500 µl	500 µl	100% DMSO solution

iProof DNA polymerase is unlike other enzymes. Please read the *QuickGuide* to modify your protocol for optimal results.

QuickGuide (See *Notes About Cycling Conditions* for details)

- Use 98°C for denaturation.
- Anneal at T_m +3°C (>20nt oligo).
- Use 15–30 sec/kb for extension times. Do not exceed 1 min/kb.
- Use iProof at 0.5–1.0 U per 50 µl reaction. Do not exceed 2 U/50 µl.
- Use 200 µM dNTPs. Do not use dUTP.
- iProof produces blunt end DNA products.

Notes About Cycling Conditions

1. **Denaturation**
   Template denaturation should be performed at 98°C. Due to the high thermostability of iProof, denaturation temperatures greater than 98°C can be used. A 30 s initial denaturation time is recommended, but this can be extended to 3 min for difficult DNA templates. Subsequent denaturation should be performed for 5–10 s at 98°C.
2. **Annealing**
   When using iProof, a general rule is to anneal primers (>20 nt) for 10–30 s at +3°C above the primer with the lowest T_m. Primer T_m should be calculated using the nearest-neighbor method as results can vary significantly depending on the method used. For primers $\leq$ 20 nt, use an annealing temperature equal to the primer with the lowest T_m.
3. **Extension**
   Template extension should be performed at 72°C and extension time depends on amplicon length and complexity. For low complexity DNA (e.g. plasmid, lambda, or BAC DNA) use 15 s per kb. For high complexity DNA (e.g. genomic DNA) use 30 s per kb. **Do not exceed 1 min per kb for amplicons that are >5 kb.**

Related Amplification Products From Bio-Rad Laboratories

Reagents for PCR or Real-Time PCR

iProof™ High-Fidelity DNA Polymerase	172-5301
iProof HF Master Mix	172-5310
iProof GC Master Mix	172-5320
iTaq™ DNA Polymerase	170-8870
iTaq Supermix With ROX	170-8854
iTaq SYBR Green Supermix With ROX	170-8850
iQ™ Supermix	170-8860
iQ SYBR Green Supermix	170-8880
iScript™ cDNA Synthesis Kit	170-8890
iScript Select cDNA Synthesis Kit	170-8896
iScript One-Step RT-PCR Kit with SYBR Green	170-8892
iScript One-Step RT-PCR Kit for Probes	170-8894

For ordering information on larger pack sizes, or to learn more about Bio-Rad amplification reagents and instruments, visit www.bio-rad.com/amplification/

Bio-Rad Laboratories
2000 Alfred Nobel Drive, Hercules, CA 94547
510-741-1000

10002300 Rev B

Reaction Setup

Important Note – Please Read Before Starting

Spin all tubes before opening to improve recovery. Reactions should be set up on ice. Pipet all components in the order given below. Always add iProof DNA Polymerase last to the reaction as primer degradation may occur in the absence of dNTPs. It is recommended that you prepare a master mix for the appropriate number of samples to be amplified.

Typical Reaction Setup

Component	Volume for 50 µl reaction	Volume for 20 µl reaction	Final Conc.
5X iProof HF Buffer*	10 µl	4 µl	1X
dNTP mix	1 µl	0.4 µl	200 µM each
Primer 1**	x µl	x µl	0.5 µM
Primer 2**	x µl	x µl	0.5 µM
DNA template	x µl	x µl	
Sterile H_20	x µl	x µl	
iProof DNA Polymerase	0.5 µl	0.2 µl***	0.02 U/µl
Total Volume	50 µl	20 µl	

* For difficult or GC-rich templates, 5X iProof GC Buffer can be used.
** Recommended final primer concentration is 0.5 µM; can range between 0.2–1.0 µM.
*** Enzyme should be diluted to avoid pipeting errors.

Control Template Reaction Setup

Component	Volume for 50 µl reaction	Volume for 20 µl reaction	Final Conc.
5X iProof HF Buffer	10 µl	4 µl	1X
dNTP mix	1 µl	0.4 µl	200 µM each
Primers *	2.5 µl	1 µl	0.2 µM
Control DNA Template	2 µl	0.8 µl	
Sterile H_20	34 µl	13.6 µl	
iProof DNA Polymerase	0.5 µl	0.2 µl**	0.02 U/µl
Total Volume	50 µl	20 µl	

* Either 1.3 kb or 10 kb primers
** Enzyme should be diluted to avoid pipetting errors.

Notes About Reaction Components

1. **iProof DNA Polymerase**
   The optimal amount of enzyme depends on the amount of template and the length of the PCR product. Usually 1 unit of iProof DNA polymerase per 50 µl reaction will give good results, but optimal amounts could range from 0.5–2 units per 50 µl reaction depending on amplicon length and difficulty. **Do not exceed 2 U/50 µl (0.04 U/µl), especially for amplicons that are > 5kb**.
2. **Buffers**
   Two buffers are provided: 5x iProof HF buffer and 5x iProof GC buffer. The error rate of iProof polymerase in HF buffer (4.4×10^{-7}) is lower than that in GC buffer (9.5×10^{-7}). Therefore, the HF buffer should be used as the default buffer for high fidelity amplification. However, the GC buffer can improve iProof performance on certain difficult or long templates, i.e. GC rich templates or those with complex secondary structures. Only use GC buffer when amplification with HF buffer does not provide satisfactory results.

3. **Mg^{2+} and dNTP**
   Mg^{2+} concentration is critical since iProof is a Mg^{2+}-dependent enzyme. Excessive Mg^{2+} stabilizes dsDNA, preventing complete denaturation, and can also promote inaccurate priming. Conversely, insufficient amounts of Mg^{2+} can lead to low product yield. The optimal Mg^{2+} concentration also depends on dNTP concentration, the specific DNA template and the sample buffer composition. The optimal Mg^{2+} concentration is 0.5 to 1 mM over the total dNTP concentration for standard PCR. For optimization, increase or decrease Mg^{2+} concentration in 0.2 mM increments.

   Only high quality dNTPs should be used. Use of dUTP or other dUTP-derivatives or analogs is not recommended. Due to the increased processivity of iProof, there is no advantage to increasing dNTP amounts. For optimal results, use 200 mM dNTPs.

4. **DNA Template**
   General guidelines are 1 pg–10 ng of DNA template in a 50 µl reaction for low complexity DNA (e.g. plasmid, lambda, or BAC DNA). For high complexity DNA (e.g. genomic DNA), 50–500 ng of template DNA should be used in a 50 µl reaction.

5. **PCR Additives**
   The recommended reaction conditions for GC-rich templates include the addition of 3% DMSO which aids in template denaturation. Further optimization of DMSO should be made in 2% increments. In some cases, DMSO may be used to help relax supercoiled plasmid DNA. High DMSO concentrations (10%) will require lowering the annealing temperature by 5.5–6.0°C. Other PCR additives such as formamide, glycerol, and betaine are also compatible with iProof.

Cycling Conditions

Important Note – Please Read

Due to the novel nature of iProof DNA polymerase, optimal reaction conditions may differ from standard PCR protocols. iProof works better at elevated denaturation and annealing temperatures due to higher salt concentration in the reaction buffer.

Typical Thermal Cycling Protocol

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	5–10 s	
Annealing	45–72°C	10–30 s	25–35
Extension	72°C	15–30 s / kb	
Final Extension	72°C	5–10 min	1

Control Template (1.3 kb) Cycling Protocol (2-step)

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	1 min	1
Denaturation	98°C	5 s	
Annealing/Extension	72°C	20 s	25–35
Final Extension	72°C	10 min	1

Control Template (10 kb) Cycling Protocol (3-step)*

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	1 min	1
Denaturation	98°C	5 s	
Annealing	60°C	15 s	25–35
Extension	72°C	2 min 30 sec	
Final Extension	72°C	10 min	1

* Both control template reactions can be run using the 10 kb cycling protocol.

Appendix 1K
pENTR/D-TOPO Cloning Instruction Manual

pENTR™ Directional TOPO® Cloning Kits

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an entry vector for the Gateway® System

Catalog nos. K2400-20, K2420-20, K2525-20, K2535-20, K2435-20, and K2635-20

Version G
6 April 2006
25-0434

User Manual

Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Design PCR Primers</td><td>• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer.
• Design the primers such that your gene of interest will be optimally expressed and fused in frame with the TEV recognition site (in pENTR™/TEV/D-TOPO® only) or any N- or C-terminal tags, if desired (after recombination with the Gateway® destination vector).</td></tr>
<tr><td>Amplify Your Gene of Interest</td><td>1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product.
2. Use agarose gel electrophoresis to check the integrity and determine the yield of your PCR product.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td>1. Set up the following TOPO® Cloning reaction. For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
<table>
<tr><th>Reagent</th><th>Chemical Transformation</th><th>Electroporation</th></tr>
<tr><td>Fresh PCR product</td><td>0.5 to 4 µl</td><td>0.5 to 4 µl</td></tr>
<tr><td>Salt solution</td><td>1 µl</td><td>--</td></tr>
<tr><td>Dilute salt solution (1:4)</td><td>--</td><td>1 µl</td></tr>
<tr><td>Water</td><td>to a final volume of 5 µl</td><td>to a final volume of 5 µl</td></tr>
<tr><td>TOPO® vector</td><td>1 µl</td><td>1 µl</td></tr>
<tr><td>Total volume</td><td>6 µl</td><td>6 µl</td></tr>
</table>
2. Mix gently and incubate for 5 minutes at room temperature.
3. Place on ice and proceed to transform One Shot® chemically competent E. coli, below.</td></tr>
<tr><td>Transform One Shot® Chemically Competent E. coli</td><td>1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® chemically competent E. coli cells and mix gently.
2. Incubate on ice for 5 to 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.
4. Add 250 µl of room temperature S.O.C. Medium.
5. Incubate at 37°C for 1 hour with shaking.
6. Spread 50-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.</td></tr>
</table>

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 23-25 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Size	Catalog no.
pENTR™/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2400-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2435-20
pENTR™/SD/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2420-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2635-20
pENTR™/TEV/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2525-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2535-20

Shipping/Storage

Each pENTR™ Directional TOPO® Cloning Kit is shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pENTR™ TOPO® Reagents	-20°C
2	One Shot® Chemically Competent *E. coli*	-80°C

continued on next page

Kit Contents and Storage, continued

pENTR™ TOPO® Reagents

The following reagents are supplied with each pENTR™ TOPO® vector (Box 1). **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pENTR™ TOPO® vector, TOPO®-adapted (pENTR™/D-TOPO® **or** pENTR™/SD/D-TOPO® **or** pENTR™/TEV/D-TOPO®)	15-20 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Water	---	1 ml
M13 Forward (-20) Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
M13 Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl

Sequences of the Primers

The table below provides the sequences of the M13 Forward (-20) and M13 Reverse sequencing primers.

Primer	Sequence	pMoles Supplied
M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´	407
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385

continued on next page

Kit Contents and Storage, continued

One Shot® Reagents

The following reagents are included with the One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store Box 2 at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 or Mach1™-T1^R cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of *E. coli* Strains

TOP10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Mach1™-T1^R: F$^-$ Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(r_k^-, m_k^+) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

Information for Non-U.S. Customers Using Mach1™-T1^R Cells

The parental strain of Mach1™-T1^R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S.A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Accessory Products

Introduction

The products listed in this section may be used with the pENTR™ Directional TOPO® Cloning Kits. For more information, refer to www.invitrogen.com or call Technical Service (see page 35).

Additional Products

Many of the reagents supplied in the pENTR™ Directional TOPO® Cloning Kits and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® TOP10 **Chemically Competent** *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 **Electrocompetent** *E. coli*	10 reactions	C4040-50
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03
M13 Forward (-20) Primer	2 µg (407 pmoles)	N520-02
M13 Reverse Primer	2 µg (385 pmoles)	N530-02
Kanamycin Sulfate	1 g	11815-016
LB Broth	500 ml	10855-021
LB Agar	500 g	22700-025
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
Gateway® LR Clonase™ Plus Enzyme Mix	20 reactions	12538-013
MultiSite Gateway® Three-Fragment Vector Construction Kit	1 kit	12537-023
AcTEV™ Protease	1,000 units	12575-015
	10,000 units	12575-023

x

Introduction

Overview

Introduction

The pENTR™ Directional TOPO® Cloning Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for entry into the Gateway® System or the MultiSite Gateway® System available from Invitrogen. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

A choice of pENTR™ Directional TOPO® vectors is available for optimal expression of your PCR product after recombination with the Gateway® destination vector of interest (see table below).

Vector	Benefit
pENTR™/D-TOPO®	For efficient expression of your gene of interest after recombination with a Gateway® destination vector
pENTR™/SD/D-TOPO®	Contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) for optimal expression of native protein after recombination with a prokaryotic Gateway® destination vector **Note:** Also suitable for efficient expression of your gene of interest in other hosts after recombination with a Gateway® destination vector (*e.g.* mammalian, insect, yeast)
pENTR™/TEV/D-TOPO®	Contains a Tobacco Etch Virus (TEV) recognition site for efficient TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein after recombination and expression from a Gateway® destination vector

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway® Technology, simply:

1. TOPO® Clone your blunt-end PCR product into one of the pENTR™ TOPO® vectors to generate an entry clone.
2. Generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway® destination vector of choice.
3. Introduce your expression construct into the appropriate host (*e.g.* bacterial, mammalian, yeast, insect) and express your recombinant protein.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 35).

continued on next page

Overview, continued

MultiSite Gateway® Technology

The MultiSite Gateway® Technology uses modifications of the site-specific recombination reactions of the Gateway® Technology (see the previous page) to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation. The MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023) facilitates simultaneous cloning of DNA fragments in three entry vectors to create your own expression clone. For more information about the MultiSite Gateway® Technology and the MultiSite Gateway® Three-Fragment Vector Construction Kit, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual which is available for downloading from our Web site or by contacting Technical Service.

Features of the pENTR™ TOPO® Vectors

The pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for entry into the Gateway® System. Features of the vectors include:

- *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway® destination vector
- T7 gene 10 translation enhancer and ribosome binding site for efficient translation of the PCR product in prokaryotes **(pENTR™/SD/D-TOPO® only)**
- TEV recognition site for TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein **(pENTR™/TEV/D-TOPO® only)**
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

How Directional TOPO® Cloning Works

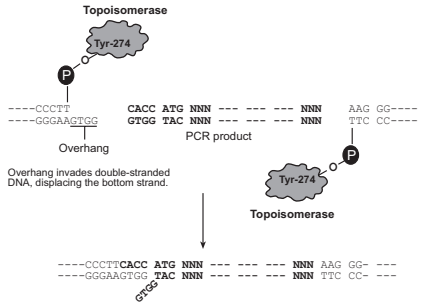
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT; see **Note** below) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



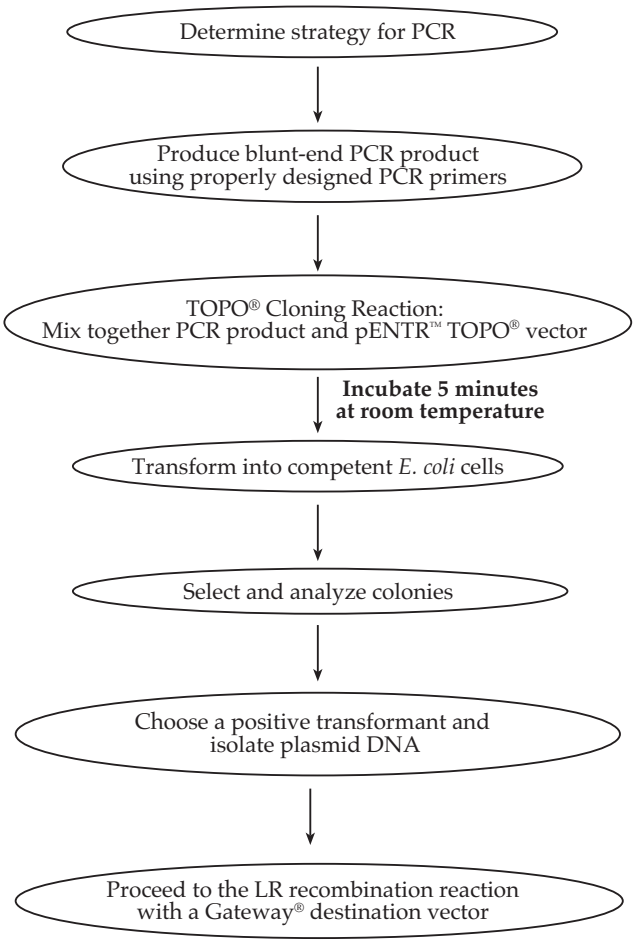
Note

The 5′ TOPO® recognition site in pENTR™/TEV/D-TOPO® is encoded by the sequence TCCTT rather than CCCTT. This is because the 5′ TOPO® recognition site directly follows the TEV recognition site, and studies have shown that TEV protease does not cleave efficiently if the first amino acid following the TEV recognition sequence is proline (Kapust *et al.*, 2002) as would be the case if the 5′ TOPO® recognition site was encoded by **CCC**TT. By changing the sequence of the 5′ TOPO® recognition site to **TCC**TT, the first amino acid following the TEV recognition site is now serine. **This change does not affect TOPO® Cloning efficiency and allows efficient TEV cleavage.**

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and clone your blunt-end PCR product.



4

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pENTR™ TOPO® vector you are using, consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal tag after recombination of your entry clone with a Gateway® destination vector

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pENTR™ TOPO® vector.
- If you plan to express your PCR product in mammalian cells as a native or C-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest should include a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)**NNATG**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** on the next page).
- If you plan to express your PCR product in mammalian cells as an N-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest does not need to contain a Kozak translation initiation sequence. A Kozak sequence is provided by the appropriate destination vector. **Note:** In this case, internal initiation may occur if your PCR product contains an endogenous Kozak sequence.
- If you plan to express your PCR product in **prokaryotic** cells without an N-terminal fusion tag (following recombination of the entry clone with a Gateway® destination vector), you should TOPO® Clone your PCR product into pENTR™/SD/D-TOPO®. pENTR™/SD/D-TOPO® contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) to enable efficient translation of the PCR product in *E. coli*. **To ensure optimal spacing for proper translation, design your forward PCR primer so that the ATG initiation codon of your PCR product directly follows the CACC necessary for directional cloning** (see **Example** on the next page).

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Designing PCR Primers, continued

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then:

- The ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.
- The ATG initiation codon is properly spaced from the RBS (in pENTR™/SD/D-TOPO® only), allowing proper translation of the PCR product in prokaryotic cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 on the next page).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you **do not** wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

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Designing PCR Primers, continued

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector). The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

…**GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′**

- To fuse the ORF in frame with a C-terminal tag (supplied by the destination vector after recombination), remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.
- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that the pENTR™ TOPO® vectors accept blunt-end PCR products.
- Do not add 5´ phosphates to your primers for PCR. This will prevent ligation into the pENTR™ TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

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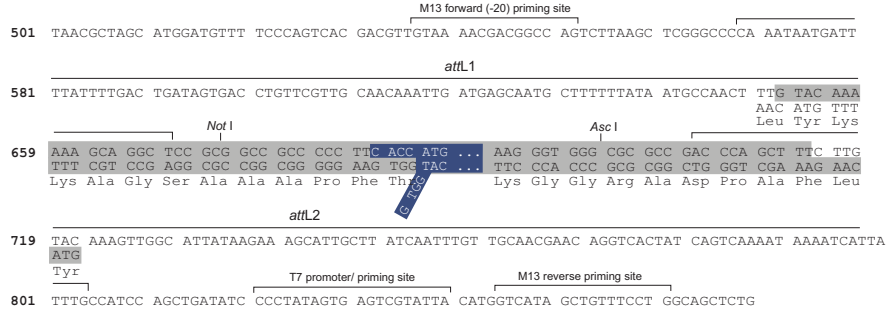
Designing PCR Primers, continued

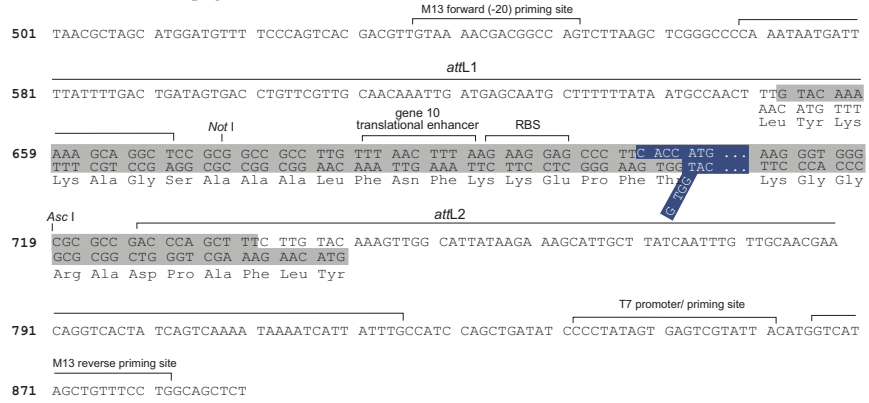
TOPO® Cloning Site for pENTR™/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/D-TOPO®, see pages 28-29.



TOPO® Cloning Site for pENTR™/SD/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/SD/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/SD/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/SD/D-TOPO®, see pages 30-31.



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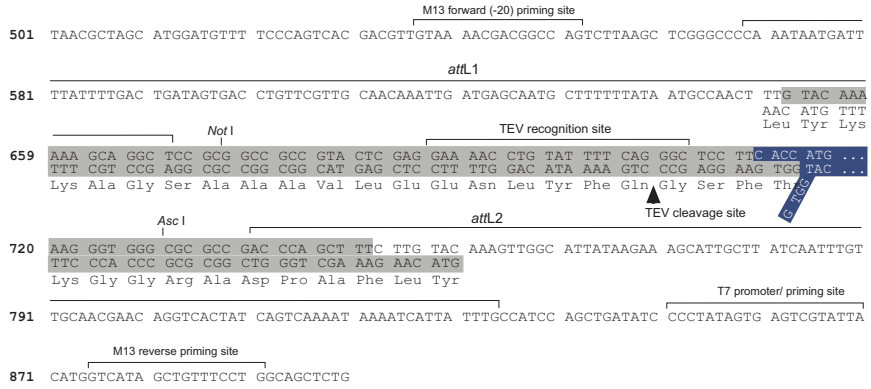
Designing PCR Primers, continued

TOPO® Cloning Site for pENTR™/TEV/ D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/TEV/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/TEV/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/TEV/D-TOPO®, see pages 32-33.

Note: The sequence of the 5′ TOPO® recognition site has been changed from **C**CCTT to **T**CCTT, resulting in an amino acid substitution of serine for proline**. This amino acid change increases the efficiency of TEV protease cleavage (Kapust *et al.*, 2002), but does not affect the efficiency of TOPO® Cloning.**



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

You will need the following reagents and equipment for PCR. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers to produce the PCR product

Producing Blunt-End PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at –20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 26-27).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Setting Up the TOPO® Cloning Reaction

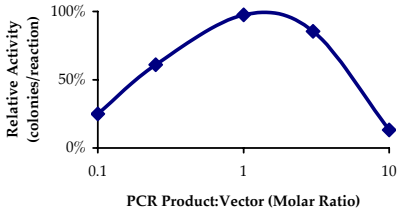
Introduction

Once you have produced the desired blunt-end PCR product, you are ready to TOPO® Clone it into the pENTR™ TOPO® vector and transform the recombinant vector into One Shot® competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent *E. coli*** (pages 13-14) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 23-25 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For pENTR™ TOPO® vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Setting Up the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page x for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Competent *E. coli***, next page.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pENTR™ TOPO® construct into competent *E. coli*. One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Needed

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment:

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* (supplied with the kit, Box 2)
- S. O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (to verify transformation efficiency, if desired, Box 2)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 50 µg/ml kanamycin (two for each transformation)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of One Shot® competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw **on ice** one vial of One Shot® cells from Box 2 for each transformation.

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Transforming One Shot® Competent *E. coli*, continued

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1^R chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature S.O.C. Medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the kanamycin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

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Transforming One Shot® Competent *E. coli*, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5-10 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml kanamycin.

   Note: If you transformed One Shot® Mach1™-T1^R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for only 4 hours in pre-warmed LB medium containing 50 µg/ml kanamycin before isolating plasmid DNA. For optimal results, inoculate as much of a single colony as possible.

2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

3. Analyze the plasmids by restriction analysis or PCR (see below) to confirm the presence and correct orientation of the insert.

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a combination of the M13 Forward (-20) primer or the M13 Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template.

Materials Needed:

- PCR Super Mix High Fidelity (Invitrogen, Catalog no. 10790-020)
- Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5-10 colonies and resuspend them individually in 50 µl of the PCR SuperMix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation. Use the M13 Forward (-20) and M13 Reverse included to help you sequence your insert (see the diagrams on pages 8-9 for the location of the priming sites in each pENTR™ TOPO® vector). For the complete sequence of each pENTR™ TOPO® vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 35).

Note: The M13 Forward (-20) and M13 Reverse primers are available separately from Invitrogen (see page x for ordering information).

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Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 23-25 or refer to the **Troubleshooting** section, page 21 for tips to help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Guidelines to Perform the LR Recombination Reaction

Introduction

Once you have obtained your entry clone, you may:

- Perform an LR recombination reaction using Gateway® LR Clonase™ II enzyme mix (see page x for ordering information) to transfer your gene of interest from the entry construct into any Gateway® destination vector of choice to generate an expression clone.
- Perform a MultiSite Gateway® LR recombination reaction with 5′ and 3′ entry clones, the appropriate MultiSite Gateway® destination vector, and LR Clonase™ Plus enzyme mix (see page x for ordering information) to generate an expression clone.

General guidelines are provided below.

Important

For most applications, we recommend performing the LR recombination reaction or the MultiSite Gateway® LR recombination reaction using a:

- Supercoiled entry clone(s) **and**
- Supercoiled destination vector

To catalyze the LR recombination reaction, we recommend using Gateway® LR Clonase™ II Enzyme Mix (see page x for ordering information). The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied by Invitrogen as separate components in LR Clonase™ enzyme mix (Catalog no. 11791-019) into an optimized single tube format to allow easier set-up of the LR recombination reaction. Follow the instructions included with the product to perform the LR recombination reaction.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired.

Destination Vectors

A large selection of Gateway® destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available, see our Web site (www.invitrogen.com) or call Technical Service (see page 35). Manuals supporting all of the destination vectors are available for downloading from our Web site or by contacting Technical Service.

***E. coli* Host**

Once you have performed the LR recombination reaction or the MultiSite Gateway® LR recombination reaction, you will transform the reaction mixture into competent *E. coli* and select for expression clones. You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α™, or equivalent for transformation. Do not transform the Gateway® or MultiSite Gateway® LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

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Guidelines to Perform the LR Recombination Reaction

Performing the LR Recombination Reaction

To perform the Gateway® LR recombination reaction, you will need:

- Purified plasmid DNA of the entry clone containing your gene of interest
- A destination vector of choice
- LR Clonase™ II enzyme mix (see page x for ordering information)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate chemically competent *E. coli* host and growth media for expression
- Appropriate selective plates

For instructions to perform the LR recombination reaction, refer to the Gateway® Technology with Clonase™ II manual or to the manual for the destination vector you are using.

Performing the MultiSite Gateway® LR Recombination Reaction

Before you can perform the MultiSite Gateway® LR recombination reaction, you will first need to generate 5′ and 3′ entry clones using Invitrogen's MultiSite Gateway® Three-Fragment Vector Construction Kit (Catalog no. 12537-023). Once you have generated the 5′ and 3′ entry clones, you will use the 5′ and 3′ entry clones, the entry clone containing your gene of interest, and the other reagents supplied in the MultiSite Gateway® Three-Fragment Vector Construction Kit (including LR Clonase™ Plus enzyme mix and the pDEST™R4-R3 destination vector) in a MultiSite Gateway® LR recombination reaction to generate an expression clone.

For instructions to generate 5′ and 3′ entry clones and to perform the MultiSite Gateway® LR recombination reaction, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 23-25) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	• Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector. • Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify your PCR product.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies, continued	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and smaller PCR products. • Optimize your PCR. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	Make sure that the forward and reverse PCR primers are designed correctly.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the proofreading polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Component	Amount
Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile water	41.5 µl
Proofreading polymerase (1-2.5 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. Make sure that you see a single, discrete 750 bp band.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pENTR™ TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pENTR™/D-TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® competent cells using the protocol on page 14.
4. Spread 50-200 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

continued on next page

Performing the Control Reactions, continued

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pENTR™/D-TOPO®	*Not* I	Correct orientation: 127, 3203 Reverse orientation: 646, 2684 Empty vector: 2580
pENTR™/SD/D-TOPO®	*Not* I	Correct orientation: 148, 3203 Reverse orientation: 667, 2684 Empty vector: 2601
pENTR™/TEV/D-TOPO®	*Eco*R V/*Pst* I	Correct orientation: 757, 2602 Reverse orientation: 250, 3109 Empty vector: 2610

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation.

Relatively few colonies should be produced in the vector-only reaction.

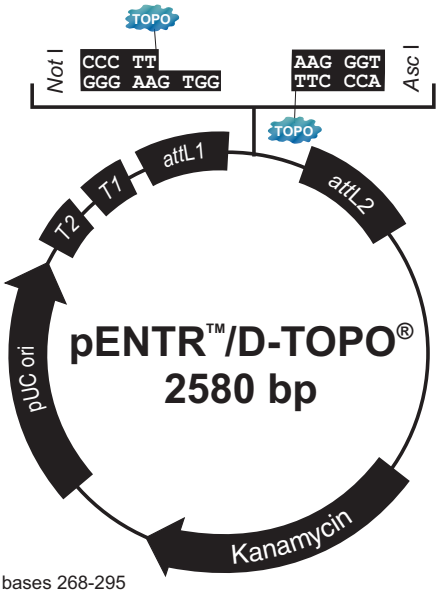
Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® competent cells with 10 pg of pUC19 using the protocol on page 14. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^9 cfu/µg DNA.

Map and Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® Map

The figure below shows the features of pENTR™/D-TOPO® vector. **The complete sequence of pENTR™/D-TOPO® is available for downloading from www.invitrogen.com)or by contacting Technical Service (see page 35).**



Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

continued on next page

Map and Features of pENTR™/D-TOPO®, continued

Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® (2580 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (-20) priming site	Allows sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Appendix 1L
QIAprep Spin Miniprep Handbook

Second Edition December 2006

QIAprep® Miniprep Handbook

For purification of molecular biology grade DNA

Plasmid
Large plasmids (>10 kb)
Low-copy plasmids and cosmids
Plasmid DNA prepared by other methods

QIAGEN

WWW.QIAGEN.COM

Kit Contents

QIAprep Spin Miniprep Kit	(50)	(250)
Catalog no.	**27104**	**27106**
QIAprep Spin Columns	50	250
Buffer P1	20 ml	73 ml
Buffer P2	20 ml	73 ml
Buffer N3*	30 ml	140 ml
Buffer PB*	30 ml	150 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
LyseBlue	20 µl	73 µl
RNase A†	200 µl	730 µl
Collection Tubes (2 ml)	50	250
Handbook	1	1

* Buffers N3 and PB contain chaotropic salts which are irritants and not compatible with disinfecting agents containing bleach. Take appropriate laboratory safety measures and wear gloves when handling. See page 9 for further information.

† Provided as a 10 mg/ml solution.

Storage

QIAprep Miniprep Kits should be stored dry at room temperature (15–25°C). Kits can be stored for up to 12 months without showing any reduction in performance and quality. For longer storage these kits can be kept at 2–8°C. If any precipitate forms in the buffers after storage at 2–8°C it should be redissolved by warming the buffers to 37°C before use.

After addition of RNase A and optional LyseBlue reagent, Buffer P1 is stable for 6 months when stored at 2–8°C. RNase A stock solution can be stored for two years at room temperature.

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of QIAprep Miniprep Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

QIAprep Miniprep 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.

The QIAcube, BioRobot 3000, BioRobot 8000 and BioRobot Universal System workstations are intended for research applications. No claim or representation is intended for their use to provide information for the diagnosis, prevention, or treatment of a disease.

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 back 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 each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffers N3 and PB contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to QIAprep Miniprep Kits.

Buffer N3

Contains guanidine hydrochloride, acetic acid: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-23-26-36/37/39-46.

Buffer P2

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

Buffer PB

Contains guanidine hydrochloride, isopropanol: harmful, flammable, irritant. Risk and safety phrases:* R10-22-36/38, S13-23-26-36/37/39-46

RNase A

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

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R22: Harmful if swallowed; 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 protecting clothing and gloves; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S46: If swallowed seek medical advice immediately and show the container or label.

Introduction

The QIAprep Miniprep system provides a fast, simple, and cost-effective plasmid miniprep method for routine molecular biology laboratory applications. QIAprep Miniprep Kits use silica membrane technology to eliminate the cumbersome steps associated with loose resins or slurries. Plasmid DNA purified with QIAprep Miniprep Kits is immediately ready for use. Phenol extraction and ethanol precipitation are not required, and high-quality plasmid DNA is eluted in a small volume of Tris buffer (included in each kit) or water. The QIAprep system consists of four products with different handling options to suit every throughput need.

Low throughput

The **QIAprep Spin Miniprep Kit** is designed for quick and convenient processing of 1–24 samples simultaneously in less than 30 minutes. QIAprep spin columns can be used in a microcentrifuge or on any vacuum manifold with luer connectors (e.g., QIAvac 24 Plus, or QIAvac 6S with QIAvac Luer Adapters).

The **QIAprep Spin Miniprep Kit** can be fully automated on the **QIAcube**. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute) enabling you to continue using the QIAprep Spin Miniprep Kit for purification of high-quality plasmid DNA.

The QIAcube is preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/MyQIAcube .

Medium throughput

For medium throughput requirements the **QIAprep 8 Miniprep Kit and QIAprep 8 Turbo Miniprep Kit** utilize 8-well strips on QIAvac 6S allowing up to 48 minipreps to be performed simultaneously in approximately 40 and 30 minutes respectively. In addition, the **QIAprep 8 Turbo BioRobot® Kit** enables automated purification of up to 48 minipreps in 50 minutes on BioRobot systems.

High throughput

The **QIAprep 96 Turbo Miniprep Kit** enables up to 96 minipreps to be performed simultaneously in less than 45 minutes on the QIAvac 96. For automated high-throughput plasmid purification the **QIAprep 96 Turbo BioRobot Kit** enables up to 96 minipreps to be processed in 70 minutes.

Applications using QIAprep purified DNA

Plasmid DNA prepared using the QIAprep system is suitable for a variety of routine applications including:

- Restriction enzyme digestion
- Library screening
- In vitro translation
- Sequencing
- Ligation and transformation
- Transfection of robust cells

Principle

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (1). The unique silica membrane used in QIAprep Miniprep Kits completely replaces glass or silica slurries for plasmid minipreps.

The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrane
- Washing and elution of plasmid DNA

All steps are performed without the use of phenol, chloroform, CsCl, ethidium bromide, and without alcohol precipitation.

Preparation and clearing of bacterial lysate

The QIAprep miniprep procedure uses the modified alkaline lysis method of Birnboim and Doly (2). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. After lysate clearing, the sample is ready for purification on the QIAprep silica membrane. For more details on growth of bacterial cultures and alkaline lysis, please refer to Appendix A on pages 39–42. In the QIAprep Spin and QIAprep 8 miniprep procedures, lysates are cleared by centrifugation, while the QIAprep 8 and 96 Turbo Miniprep kits provide TurboFilter strips or plates for lysate clearing by filtration.

LyseBlue reagent*

Use of LyseBlue is optional and is not required to successfully perform plasmid preparations. See “Using LyseBlue reagent” on page 14 for more information.

* LyseBlue reagent is only supplied with QIAprep Spin Miniprep Kits since multiwell or automated formats do not allow visual control of individual samples.

LyseBlue is a color indicator which provides visual identification of optimum buffer mixing. This prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. This makes LyseBlue ideal for use by researchers who have not had much experience with plasmid preparations as well as experienced scientists who want to be assured of maximum product yield.

DNA adsorption to the QIAprep membrane

QIAprep columns, strips, and plates use a silica membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. The optimized buffers in the lysis procedure, combined with the unique silica membrane, ensure that only DNA will be adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane but are found in the flow-through.

Washing and elution of plasmid DNA

Endonucleases are efficiently removed by a brief wash step with Buffer PB. This step is essential when working with *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, to ensure that plasmid DNA is not degraded. The Buffer PB wash step is also necessary when purifying low-copy plasmids, where large culture volumes are used.

Salts are efficiently removed by a brief wash step with Buffer PE. High-quality plasmid DNA is then eluted from the QIAprep column with 50–100 µl of Buffer EB or water. The purified DNA is ready for immediate use in a range of applications — no need to precipitate, concentrate, or desalt.

Note: Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range. Store DNA at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent.

DNA yield

Plasmid yield with the QIAprep miniprep system varies depending on plasmid copy number per cell (see page 39), the individual insert in a plasmid, factors that affect growth of the bacterial culture (see pages 39–42), the elution volume (Figure 1), and the elution incubation time (Figure 2). A 1.5 ml overnight culture can yield from 5 to 15 µg of plasmid DNA (Table 1, page 14). To obtain the optimum combination of DNA quality, yield, and concentration, we recommend using Luria-Bertani (LB) medium for growth of cultures (for composition see page 41), eluting plasmid DNA in a volume of 50 µl, and performing a short incubation after addition of the elution buffer.

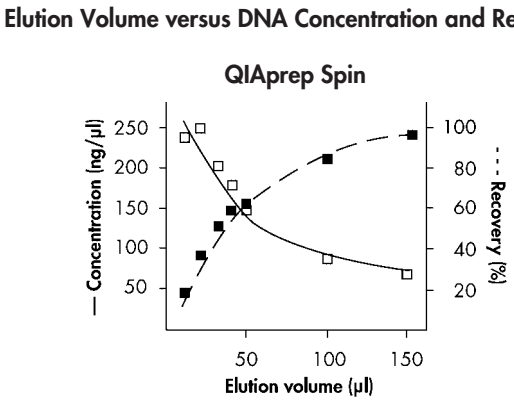


Figure 1 10 µg pUC18 DNA was purified using the QIAprep Spin protocol and eluted with the indicated volumes of Buffer EB. The standard protocol uses 50 µl Buffer EB for elution, since this combines high yield with high concentration. However the yield can be increased by increasing the elution volume.

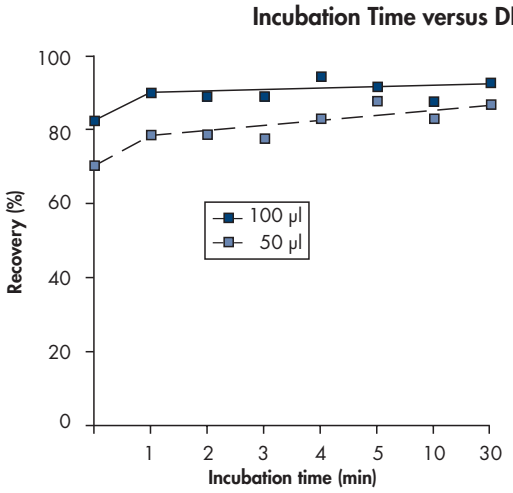


Figure 2 10 µg pBluescript DNA was purified using the QIAprep Spin Miniprep protocol and eluted after the indicated incubation times with either 50 µl or 100 µl Buffer EB. The graph shows that an incubation time of 1 minute and doubling the elution buffer volume increases yield.

Table 1. Effect of Different Compositions of Growth Medium LB on DNA Yield

Culture media	Yield
LB (containing 10 g/liter NaCl)	11.5 µg
LB (containing 5 g/liter NaCl)	9.5 µg

QIAprep Spin Miniprep Kit was used to purify DNA from 1.5 ml LB overnight cultures of XL1-Blue containing pBluescript®. Elution was performed according to the standard protocol (50 µl Buffer EB and 1 min incubation). Use of the recommended LB composition (with 10 g/liter NaCl, also see Appendix A, p. 43) provides optimal plasmid yield.

Using LyseBlue reagent

Using a simple visual identification system, LyseBlue reagent prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, cell debris, and genomic DNA.

LyseBlue can be added to the resuspension buffer (Buffer P1) bottle before use. Alternatively, smaller amounts of LyseBlue can be added to aliquots of Buffer P1, enabling single plasmid preparations incorporating visual lysis control to be performed.

LyseBlue reagent should be added to Buffer P1 at a ratio of 1:1000 to achieve the required working concentration (e.g., 10 µl LyseBlue into 10 ml Buffer P1). Make sufficient LyseBlue/Buffer P1 working solution for the number of plasmid preps being performed.

LyseBlue precipitates after addition into Buffer P1. This precipitate will completely dissolve after addition of Buffer P2. Shake Buffer P1 before use to resuspend LyseBlue particles.

The plasmid preparation procedure is performed as usual. After addition of Buffer P2 to Buffer P1, the color of the suspension changes to blue. Mixing should result in a homogeneously colored suspension. If the suspension contains localized regions of colorless solution or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

Upon addition of neutralization buffer (Buffer N3), LyseBlue turns colorless. The presence of a homogeneous solution with no traces of blue indicates that SDS from the lysis buffer has been effectively precipitated.

Important Notes

Please read the following notes before starting any of the QIAprep procedures.

Growth of bacterial cultures in tubes or flasks

1. **Pick a single colony from a freshly streaked selective plate and inoculate a culture of 1–5 ml LB medium containing the appropriate selective antibiotic. Incubate for 12–16 h at 37°C with vigorous shaking.**

   Growth for more than 16 h is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 x *g*) in a conventional, table-top microcentrifuge for 3 min at room temperature (15–25°C).**

   The bacterial cells can also be harvested in 15 ml centrifuge tubes at 5400 x *g* for 10 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

Buffer notes

- Add the provided RNase A solution to Buffer P1, mix, and store at 2–8°C.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Check Buffers P2 and N3 before use for salt precipitation. Redissolve any precipitate by warming to 37°C. Do not shake Buffer P2 vigorously.
- Close the bottle containing Buffer P2 immediately after use to avoid acidification of Buffer P2 from CO_2 in the air.
- Buffers P2, N3, and PB contain irritants. Wear gloves when handling these buffers.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (spin down briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

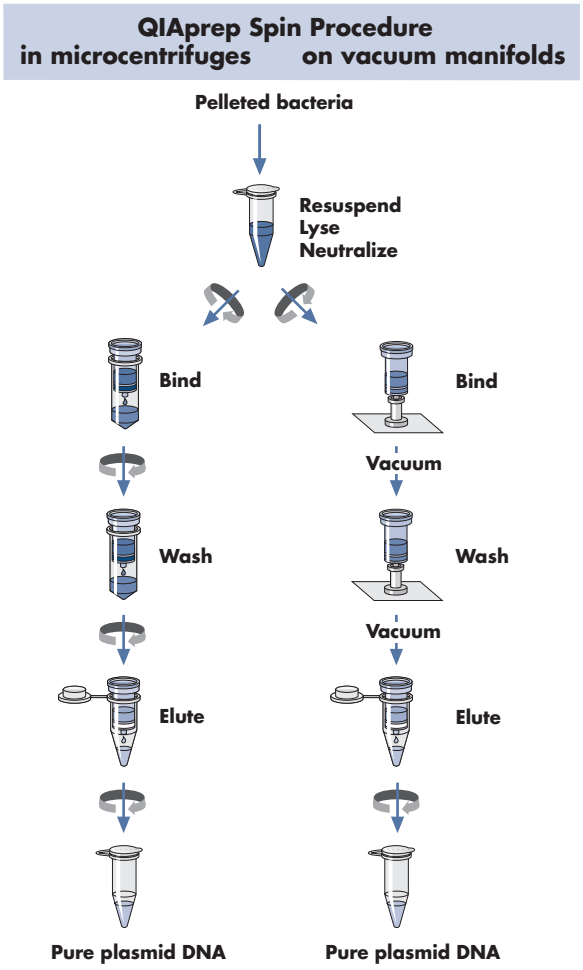
Centrifugation notes

- All centrifugation steps are carried out at 13,000 rpm (~17,900 x *g*) in a conventional, table-top microcentrifuge.

Elution notes

- Ensure that the elution buffer is dispensed directly onto the center of the QIAprep membrane for optimal elution of DNA. Average eluate volume is 48 µl from an elution-buffer volume of 50 µl (QIAprep spin procedures), and 60 µl from an elution-buffer volume of 100 µl (QIAprep multiwell procedures).
- For increased DNA yield, use a higher elution-buffer volume. For increased DNA concentration, use a lower elution-buffer volume (see "DNA yield", pages 13–14).
- If water is used for elution, make sure that its pH is between 7.0 and 8.5. Elution efficiency is dependent on pH and the maximum elution efficiency is achieved within this range. A pH <7.0 can decrease yield.

  Note: Store DNA at –20°C when eluted with water, as DNA may degrade in the absence of a buffering agent.
- DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.


QIAprep Spin Procedure
in microcentrifuges
on vacuum manifolds
Pelleted bacteria
Resuspend
Lyse
Neutralize
Bind
Bind
Vacuum
Wash
Wash
Vacuum
Elute
Elute
Pure plasmid DNA
Pure plasmid DNA

Protocol: Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 44.

Please read "Important Notes" on pages 15–21 before starting.

Note: All protocol steps should be carried out at room temperature.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.
8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
10. **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

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 back cover for contact information).

		Comments and suggestions
Low or no yield		
General		Low yields may be caused by a number of factors. To find the source of the problem, analyze fractions saved from each step in the procedure on an agarose gel (e.g., Figure 6, page 43). A small amount of the cleared lysate and the entire flow-through can be precipitated by adding 0.7 volumes isopropanol and centrifuging at maximum speed (13,000 rpm or ~17,000 x *g*) for 30 minutes. The entire wash flow-through can be precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.0, and 0.7 volumes of isopropanol.
No DNA in the cleared lysate before loading		
a)	Plasmid did not propagate	Read "Growth of bacterial cultures" (pages 39–41) and check that the conditions for optimal growth were met.
b)	Lysate prepared incorrectly	Check storage conditions and age of buffers.
c)	Buffer P2 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.
DNA is found in the flow-through of cleared lysate		
a)	QIAprep membrane overloaded	If rich culture media, such as TB or 2x YT are used, culture volumes must be reduced. It may be necessary to adjust LB culture volume if the plasmid and host strain show extremely high copy number or growth rates. See "Culture media" on page 41.

		Comments and suggestions
b)	RNase A digestion omitted	Ensure that RNase A is added to Buffer P1 before use.
c)	RNase A digestion insufficient	Reduce culture volume if necessary. If Buffer P1 containing RNase A is more than 6 months old, add additional RNase A.

DNA is found in the wash flow-through

Ethanol omitted from wash buffer		Repeat procedure with correctly prepared wash buffer (Buffer PE).

Little or no DNA in eluate

a)	Elution buffer incorrect	DNA is eluted only in the presence of low-salt buffer (e.g., Buffer EB [10 mM Tris·Cl, pH 8.5] or water). Elution efficiency is dependent on pH. The maximum efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range.
b)	Elution buffer incorrectly dispensed onto membrane	Add elution buffer to the center of the QIAprep membrane to ensure that the buffer completely covers the surface of the membrane for maximum elution efficiency.

Low DNA quality

DNA does not perform well in downstream applications

a)	Eluate salt concentration too high	For the QIAprep spin column, modify the wash step by incubating the column for 5 minutes at room temperature after adding 0.75 ml of Buffer PE and then centrifuging. For QIAprep 8 preparations and QIAprep 8 and 96 Turbo preparations, ensure that two wash steps are carried out prior to elution.
b)	Nuclease contamination	When using *endA*$^+$ host strains such as HB101 and its derivatives, the JM series, or any wild-type strain, ensure that the wash step with Buffer PB is performed.
c)	Eluate contains residual ethanol	Ensure that step 9 in the QIAprep Spin Miniprep protocol and steps 9 and 10 in the QIAprep 8 Miniprep, QIAprep 8 Turbo Miniprep, or QIAprep 96 Turbo Miniprep protocols are performed.

		Comments and suggestions
RNA in the eluate		
a)	RNase A digestion omitted	Ensure that RNase A is added to Buffer P1 before use.
b)	RNase A digestion insufficient	Reduce culture volume if necessary. If Buffer P1 containing RNase A is more than 6 months old, add additional RNase A.
Genomic DNA in the eluate		
a)	Buffer P2 added incorrectly	The lysate must be handled gently after addition of Buffer P2 to prevent shearing. Reduce culture volume if lysate is too viscous for gentle mixing.
b)	Buffer N3 added incorrectly	Upon addition of Buffer N3 in step 3, mix immediately but gently.
c)	Lysis too long	Lysis in step 2 must not exceed 5 minutes.
d)	Culture overgrown	Overgrown cultures contain lysed cells and degraded DNA. Do not grow cultures for longer than 12–16 hours.

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 (3,4). The yield and quality of 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 5), depending on their origin of replication (e.g., pMB1, ColE1, or pSC101) 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 5. Origins of replication and copy numbers of various plasmids (3).

DNA construct	Origin of replication	Copy number	Classification
Plasmids			
pUC vectors	pMB1*	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

* 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, DH5α, 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 (4). 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 and DH5α are highly recommended for reproducible and reliable results.

Inoculation

Bacterial cultures for plasmid preparation should always be grown from a single colony picked from a freshly streaked selective plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures may lead to uneven plasmid yield or loss of the plasmid. Inoculation from plates that have been stored for a long time may also lead to loss or mutation of the plasmid.

The desired clone should be streaked from a glycerol stock onto a freshly prepared agar plate containing the appropriate selective agent so that single colonies can be isolated. This procedure should then be repeated to ensure that a single colony of an antibiotic-resistant clone can be picked. A single colony should be inoculated into 1–5 ml of media containing the appropriate selective agent, and grown with vigorous shaking for 12–16 hours. Growth for more than 16 hours is not recommended since cells begin to lyse and plasmid yields may be reduced.

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 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 6 are based on these considerations.

Table 6. Concentrations of Commonly Used Antibiotics

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

Culture media

Luria-Bertani (LB) broth is the recommended culture medium for use with QIAprep Kits, since richer broths such as TB (Terrific Broth) or 2x YT 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 broth. If these media are used, recommended culture volumes must be reduced to match the capacity of the QIAprep membrane. If excess culture volume is used, alkaline lysis will be inefficient, the QIAprep membrane will be overloaded, 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. In such cases, doubling the volumes of Buffers P1, P2, and N3 may be beneficial. 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.

Table 7. Recommended composition of Luria Bertani medium

Contents	Per liter
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Preparation of cell lysates

Bacteria are lysed under alkaline conditions. After harvesting and resuspension, the bacterial cells are lysed in NaOH/SDS (Buffer P2) in the presence of RNase A (2, 5). 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 N3. 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 not be separated on QIAprep membrane and will elute under the same low-salt conditions. Mixing during the lysis procedure must therefore be carried out by slow, gentle inversion of the tube.

Appendix B: Agarose Gel Analysis of Plasmid DNA

The QIAprep Miniprep procedure can be analyzed using agarose gel electrophoresis as shown in Figure 6. Samples can be taken from the cleared lysate and its flow-through, precipitated with isopropanol and resuspended in a minimal volume of TE buffer. In Figure 6 the cleared lysate shows closed circular plasmid DNA and degraded RNase A-resistant RNA. The flow-through contains only degraded RNA and no plasmid DNA is present. The eluted pure plasmid DNA shows no contamination with other nucleic acids.

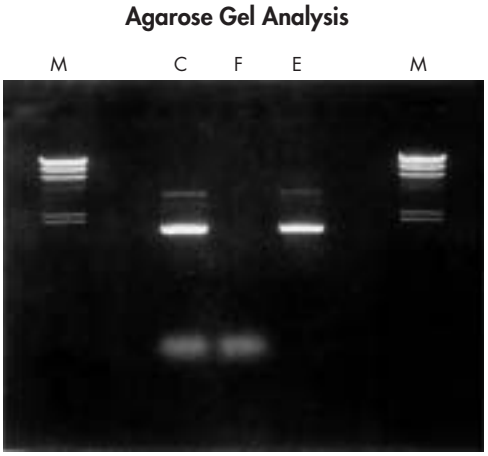


Figure 6 Agarose gel analysis of the QIAprep Miniprep procedure. **C**: cleared lysate; **F**: flow-through; **E**: eluted plasmid; **M**: markers.

Appendix C: Special Applications

Purification of low-copy plasmids and cosmids

All QIAprep miniprep protocols in this handbook can be used for preparation of low-copy-number plasmid or cosmids from 1–10 ml overnight *E. coli* cultures grown in LB medium.

Only two slight modifications to the protocols are required:

- The wash step with Buffer PB is required for all strains.
- When plasmid or cosmids are >10 kb, pre-heat Buffer EB (or water) to 70°C prior to eluting DNA from the QIAprep membrane. A 10 ml overnight LB culture typically yields 5–10 µg DNA.

Note: When using 10 ml culture volume, it is recommended to double the volumes of Buffers P1, P2, and N3 used.

Purification of very large plasmids (>50 kb)

Plasmids >50 kb elute less efficiently from silica than smaller plasmids, but do elute efficiently from QIAGEN anion-exchange resin. QIAGEN provides the anion-exchange-based QIAGEN Large-Construct Kit for efficient large-scale purification of ultrapure genomic DNA-free BAC, PAC, P1, or cosmid DNA. For high-throughput, small-scale purification of BACs, PACs, and P1s, an optimized alkaline lysis protocol in R.E.A.L.® Prep 96 Kits yields DNA suitable for sequencing and screening. Call QIAGEN Technical Services or your local distributor for more information on these kits, or see ordering information on page 47.

Purification of plasmid DNA prepared by other methods

Plasmid DNA isolated by other methods can be further purified using QIAprep modules and any of the QIAprep protocols in this handbook.

1. Add 5 volumes of Buffer PB to 1 volume of the DNA solution and mix (e.g., add 500 µl Buffer PB to 100 µl of DNA sample).
2. Apply the samples to QIAprep spin columns or to the wells of a QIAprep 8 strip or 96-well plate. Draw the samples through the QIAprep membrane by centrifugation or vacuum, and continue the appropriate protocol at the Buffer PE wash step. The optional wash step with Buffer PB is not necessary.

References

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Bench Protocol: QIAprep Spin Miniprep Kit Using a Microcentrifuge

This protocol is designed for the purification of up to 20 µg high-copy plasmid DNA from 1–5 ml overnight *E. coli* culture in LB medium. New users and users wanting to purify low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods should refer to the detailed protocols provided in the *QIAprep Miniprep Handbook*, 2nd ed.

Things to do before starting

- Add RNase A solution to Buffer P1.
- Optional: Add LyseBlue reagent to Buffer P1.
- Add ethanol (96–100%) to Buffer PE.
- Check Buffers P2 and N3 for salt precipitation and redissolve at 37°C if necessary.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**
2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   If using LyseBlue reagent, solution turns blue.
3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   If using LyseBlue reagent, solution turns colorless.
4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**
5. **Apply the supernatant (from step 4) to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is only required when using *endA*$^+$ or other bacteria strains with high nuclease activity or carbohydrate content (see *QIAprep Miniprep Handbook* for more details)
8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**
10. **To elute DNA, place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 50 µl Buffer EB or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**