# APPENDIXES

## Appendix 1A

### Preparation of a 1% Agarose Gel for Gel Electrophoresis

2. For a **1% agarose gel**, weigh out **1 gram** of agarose (powder) on a weighing scale.
   *Note: **percentage** of the gel reflects the **amount of agarose** in **gram** in **100 mL** of 1X TAE buffer, depending on the final percentage of agarose in the gel.*
   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 agarose solution
3. Carefully, put the agarose in a 250-mL Erlenmeyer flask.
4. Measure out 100 mL of 1X TAE buffer using a plastic or glass graduated cylinder.
5. Add 100 mL of 1X TAE buffer into the flask in step 2.
6. Cover the flask with a piece of plastic wrap. Poke 3-4 holes on the plastic wrap using a pointed end of a pencil or pen (*Note: the holes allow the steam to escape during microwaving in step 6 below*). Swirl the solution to break up any lumps of agarose granules.
7. Microwave the solution for about 2 minutes or until the agarose granules have completely melted.
   - *Be careful with the flask. The solution gets very hot.*
   - *Constantly watch over the solution because when it starts boiling, it might overflow.*
   - *Swirl gently the solution several times while microwaving to help melt agarose evenly.*
   - Once the agarose has melted completely, the solution is clear.
8. Cool down the agarose solution for at least 30 min in a 55ºC water bath.
9. While the agarose solution is cooling, prepare the gel cast with the appropriate comb.
   - ***The comb depends on the number of samples to be loaded on the gel.*** *For example, if there are less than 18 samples, then use a 20-tooth comb; but, if there are 21 samples, then use a 30-tooth comb.*

- *Remember to add two more wells to the number of wells needed for the samples. These two wells will be for loading 1kb DNA ladder in the first and the last wells (or left and right sides of loaded samples).*

*10.* After the agarose solution has been cooled down, add 5 μL of Ethidium Bromide (EtBr) into the solution and swirl the flask GENTLY to mix. *<u>Note:</u> Do NOT swirl vigorously to generate many bubbles.*

11. Pour the agarose/EtBr solution into the gel cast. Wait for 30 min for the agarose solution to solidify.

    *<u>Note:</u> IMMEDIATELY after pouring the agarose solution, inspect the agarose solution's surface for the present of bubbles. If there are many bubbles floating on the surface of the gel solution, use a pipette tip to pop them before the gel is completely solidified.*

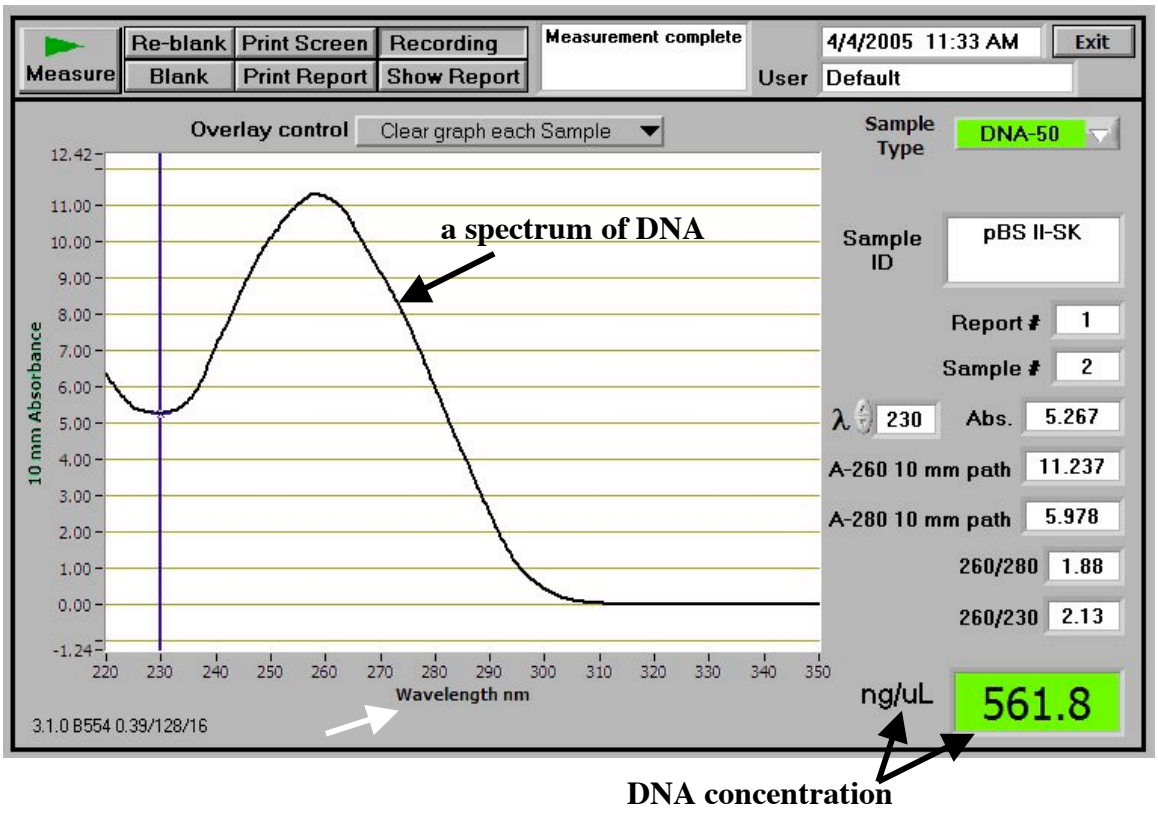
12. Pour ~600 mL of 1X TAE buffer into the gel box.

After the agarose has solidified into a gel, take out the comb gently by pulling it straight up out of the gel and put the gel in the gel box containing the 1XTAE running buffer.

## Appendix 1B

What is a **spectrophotometer**?

It is an **instrument** that **measures** the **amount** of **molecules absorbing at 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 have the absorbance at the 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 computer via the USB port.

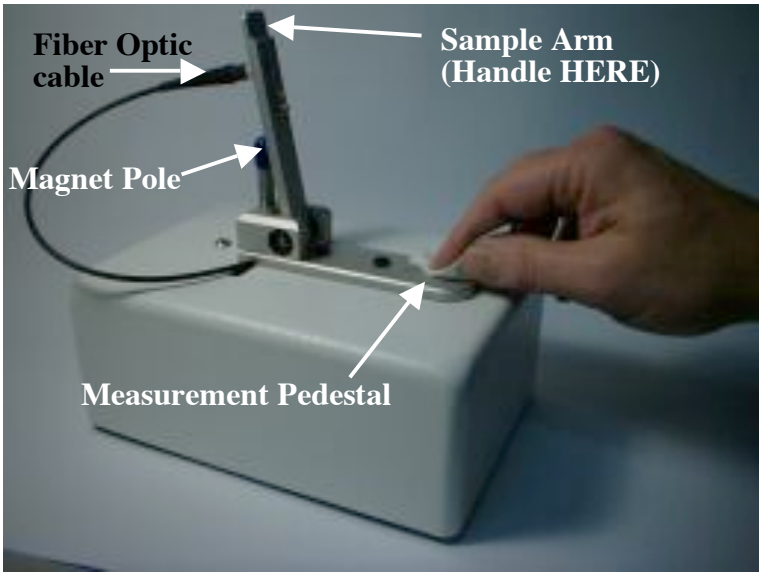
1. **Turn ON** the **Computer (laptop)** that connects to the Nanodrop.
2. **Turn ON** the **Nanodrop** by clicking an "**ND-1000 v3.1.0**" icon on the computer desktop. Wait for a few seconds for the Nanodrop to be up. You see the Nanodrop 3.1.0 Diagnose panel with **User field** as **Default.**
3. **Click** on "**Nucleic Acid**" button on the top left column for reading concentration of DNA and RNA solutions.
   - ❖ You see a following message:

   **"Ensure Sample Pedestals are clean and then load a water sample. After loading water sample, click OK to initialize instrument"**
4. a. **Clean** the **sample pedestals as followings:**

   i. **Raise** the **Sample Arm up** by holding at its end as shown on the picture below.

   **Caution: NEVER** hold the Optical Cord when lifting and lowering the Sample Arm because the cord is fragile; and it is very expensive to replace it.

   ii. **Wipe both** the **Measurement Pedestal** and the **Sample Arm** with a piece of Kimwipes slightly wetted with distilled water.



b. **Pipet 1.5 - 2 µL** of water on the **Measurement pedestal**.

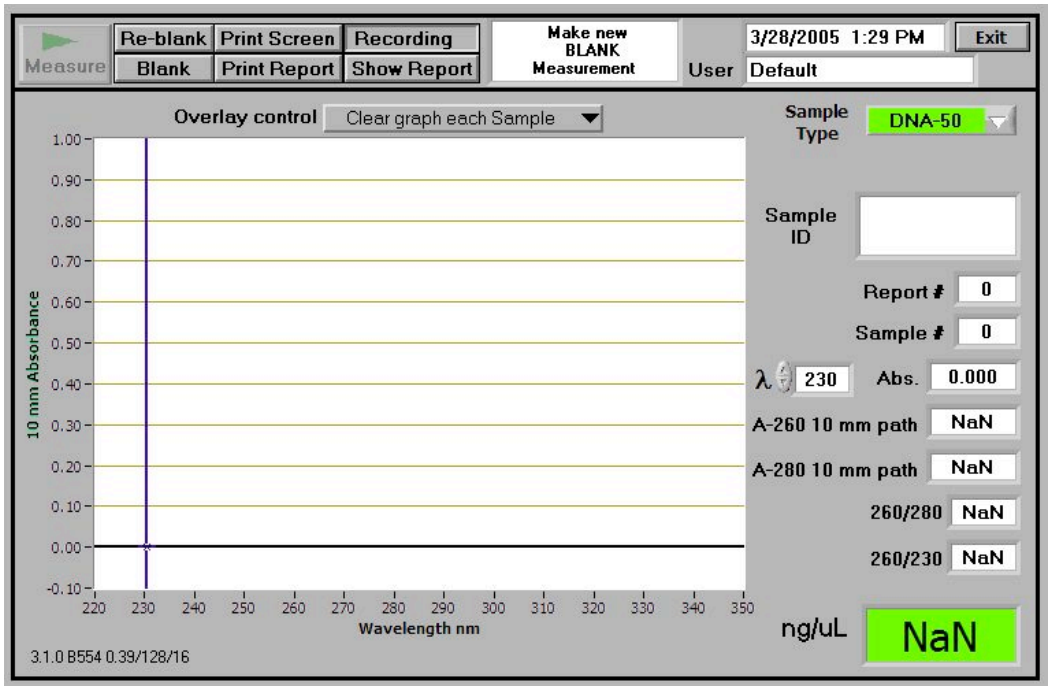
Note: Even though the Nanodrop Inc. claims that the Nanodrop can read as low as **1 µL**, the concentration reading is NOT consistent at this volume. Therefore, the **minimal** volume for the concentration reading is **1.5 µ□**

c. **Slowly Lower** the SAMPLE Arm to its horizontal position.

Caution: NEVER let the arm fall freely.

d. **Click** the **OK** button. The Nanodrop is **INITIALIZED.**

You see a **Dialog panel** as shown below



**What do you need to do, NEXT?**

a. **Change SAMPLE TYPE** (if necessary) from **DNA-50** (by Default) to **RNA-40** or **Other** (for Oligonucleotides), depending on your sample.

b. **Type in** the **SAMPLE ID** field the Information of your sample.

c. **Make** a **NEW BLANK measurement**

d. (Option) **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"

5. **Wipe off** the **liquid** on **BOTH** the **ARM** and **Measurement pedestals** with a piece of Kimwipes.
6. **Make** a **Blank measurement** by pipetting 1.5 - 2.0 □L of either double-distilled water or TE (depending on whether your sample solution is in double-distilled water or TE) on the Measurement Pedestal. Then lower the Sample Arm to its horizontal position.
7. **Click** the **BLANK** button. The blank was made.
8. After the reading is done, **bring** the Sample Arm **up** to the vertical position and **Wipe off** the **liquid** on **BOTH** the **ARM** and **Measurement pedestals** with a piece of Kimwipes.
9. **Pipet** 1.5 - 2.0 □L of SAMPLE on the Measurement Pedestal and lower the Sample Arm.
10. **Type in** the **SAMPLE ID** field **Information** of a sample solution.
11. **Click** the **MEASURE** button to determine concentration of your sample.
12. After the reading is done, a sample concentration (in **ng/□L**) and a spectrum of the sample along other information are shown. You can either
    a. **Save** the **window** of measured sample by
    clicking on FILE → choose SAVE WINDOW → Select an existing folder or Create a NEW folder (give a name for the NEW folder) → Type in a Name file in the FILE NAME field → Click the SAVE button to save the file **or**
    b. **Print** the **window** by **clicking** the **PRINT SCREEN** button.

Note: To print the current spectrum of the sample, you MUST print it before reading the next sample. Otherwise, you need to repeat reading the sample.

13. **Repeat** steps 8-12 for other samples.
14. After reading the **last sample**, **click** the **PRINT BATCH** button to print concentrations of all read samples.
15. If done with the Nanodrop, **click** the **EXIT** buttons.
16. **Clean** the **Measurement Pedestal** and the **Sample Arm** with a piece of Kimwipes slightly wetted with distilled water.

## Appendix 1C

## 1-kb DNA Ladder (Taken from Invitrogen website)

Invitrogen
life technologies

# 1 Kb DNA Ladder

**Cat. No. 15615-016** **Size: 250 µg**
**Conc.: 1.0 µg/µl** **Store at -20°C.**

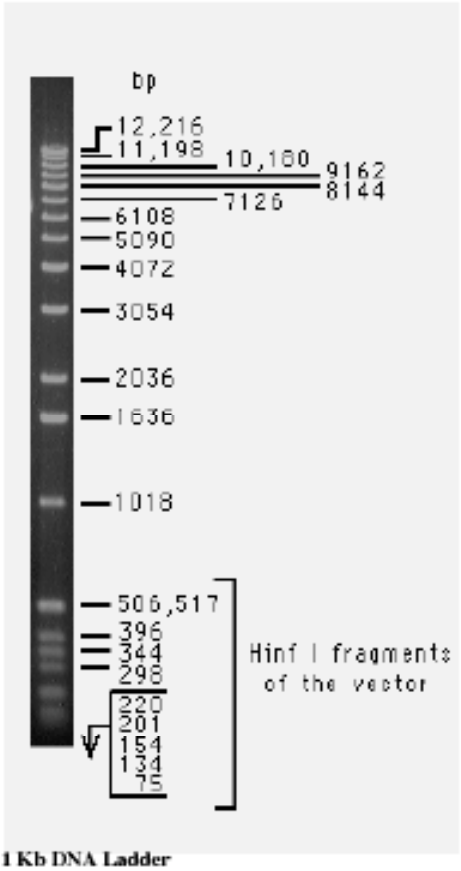
Description:
The 1 Kb DNA Ladder (U.S. Patent No. 4,403,036) is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. The bands of the ladder each contain from 1 to 12 repeats of a 1018-bp DNA fragment. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1636 bp. The 1636-bp band contains 10% 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)
50 mM NaCl
0.1 mM EDTA

Recommended Procedure:
Invitrogen recommends the use of 10X BlueJuice® Gel Loading Buffer (10816-015) at a concentration of 2X [for electrophoresis of this ladder on agarose gels]. Alternatively, the DNA ladder can be diluted in a buffer such that the final concentration of NaCl is 20 mM. Apply approximately 0.1 µg of standard per mm lane width. DO NOT HEAT!

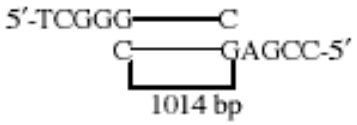
Quality Control:
Agarose gel analysis shows that all bands larger than 500 bp are distinguishable.

Doc. Rev.: 011602




**1 Kb DNA Ladder**
**0.5 µg/lane**
**0.9% agarose gel**
**stained with ethidium bromide**

Structure of Fragment (1):




Notes:

During 1.2% agarose gel electrophoresis with Tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates together with the 506/517 bp doublet band.

The 1636 bp band and all bands less than 1000 bp are generated from pBR322.

If the ionic strength of the sample is too low, blurring of the bands can occur.

Cat. No. 15615-016

## Appendix 1D

## iProof High-Fidelity DNA Polymerase (Taken from Bio-Rad Website)

BIO-RAD

# iProof™ High-Fidelity DNA Polymerase

| | | |
|---|---|---|
| 2 units/µl, 10 µl | 20U | 172-5300 |
| 2 units/µl, 50 µl | 100U | 172-5301 |
| 2 units/µl, 250 µl | 500U | 172-5302 |

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 x $10^{-7}$ in iProof HF buffer, which is approximately 50-fold lower than that of *Thermus aquaticus*, and 6-fold lower than that of *Pyrococcus furiosus*.

### Storage and Stability

Store iProof™ High-Fidelity DNA Polymerase 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 | 20U | 100U | 500U | Description |
|---|---|---|---|---|
| iProof Polymerase | 10 µl | 50 µl | 250 µl | iProof™ High Fidelity DNA Polymerase, 2 units/µl |
| iProof HF Buffer | 1.5 ml | 1.5 ml | 4 x 1.5 ml | 5X HF Buffer, 7.5 mM $MgCl_2$ |
| iProof GC Buffer | 1.5 ml | 1.5 ml | 4 x 1.5 ml | 5X GC Buffer, 7.5 mM $MgCl_2$ |
| $MgCl_2$ | 1.5 ml | 1.5 ml | 2 x 1.5 ml | 50 mM $MgCl_2$ solution |
| DMSO | 500 µl | 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.

10002298 Rev A

## 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.

| Component | Volume for 50 µl reaction | Volume for 20 µl reaction | Final Conc. |
|---|---|---|---|
| 5X iProof HF Buffer* | 10 µl | 4 µl | 1X |
| 10 mM 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.

### 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 ($4.4 \times 10^{-7}$) is lower than that in GC buffer ($9.5 \times 10^{-7}$). 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 µM 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 |

## 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.**

## Component Specifications

**Storage buffer**
20 mM Tris-HCl (pH 7.4 at 25°C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.5% Tween 20, 0.5% Nonidet P 40, 200 µg/ml BSA and 50% Glycerol

**Unit Definition**
One unit is defined as the amount of enzyme required to incorporate 10 nmoles of dNTPs into acid-insoluble form at 74°C in 30 minutes under the stated assay conditions.

**Enzyme Stability**
Each lot of DNA polymerase is tested for stability under normal storage conditions (-20°C). Enzyme stability is monitored at regular intervals for a two year period after the original assay date.

**Appendix 1E**

**pENTR/D-TOPO Cloning Instruction Manual (Taken from Invitrogen Website)**


# 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*

A Limited Use Label License covers this product (see Purchaser Notification). By use of this product, you accept the terms and conditions of the Limited Use Label License.

User Manual

## 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™-T1R 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™-T1R 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™-T1R 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

**One Shot® Reagents**

The following reagents are included with the One Shot® TOP10 or Mach1™-T1R 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<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose | 6 ml |
| TOP10 or Mach1™-T1R 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*lacZ*ΔM15 Δ*lacX74 rec*A1 *ara*D139 Δ(*ara-leu*)7697 *galU galK rps*L (Str$^R$) *end*A1 *nup*G

Mach1™-T1R: F- Φ80*lacZ*ΔM15 Δ*lacX74 hsdR*($n_k^-$, $m_k^+$) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

**Information for Non-U.S. Customers Using Mach1™-T1R Cells**

The parental strain of Mach1™-T1R *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.

## 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:<br>50% glycerol<br>50 mM Tris-HCl, pH 7.4 (at 25°C)<br>1 mM EDTA<br>2 mM DTT<br>0.1% Triton X-100<br>100 µg/ml BSA<br>30 µM bromophenol blue | 20 µl |
| dNTP Mix | 12.5 mM dATP<br>12.5 mM dCTP<br>12.5 mM dGTP<br>12.5 mM dTTP<br>in water, pH 8 | 10 µl |
| Salt Solution | 1.2 M NaCl<br>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*

# 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<br>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

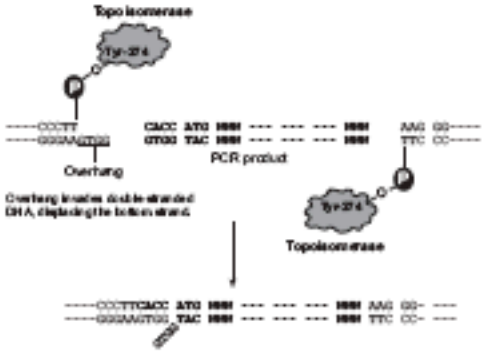
## How Topoisomerase I Works

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%.



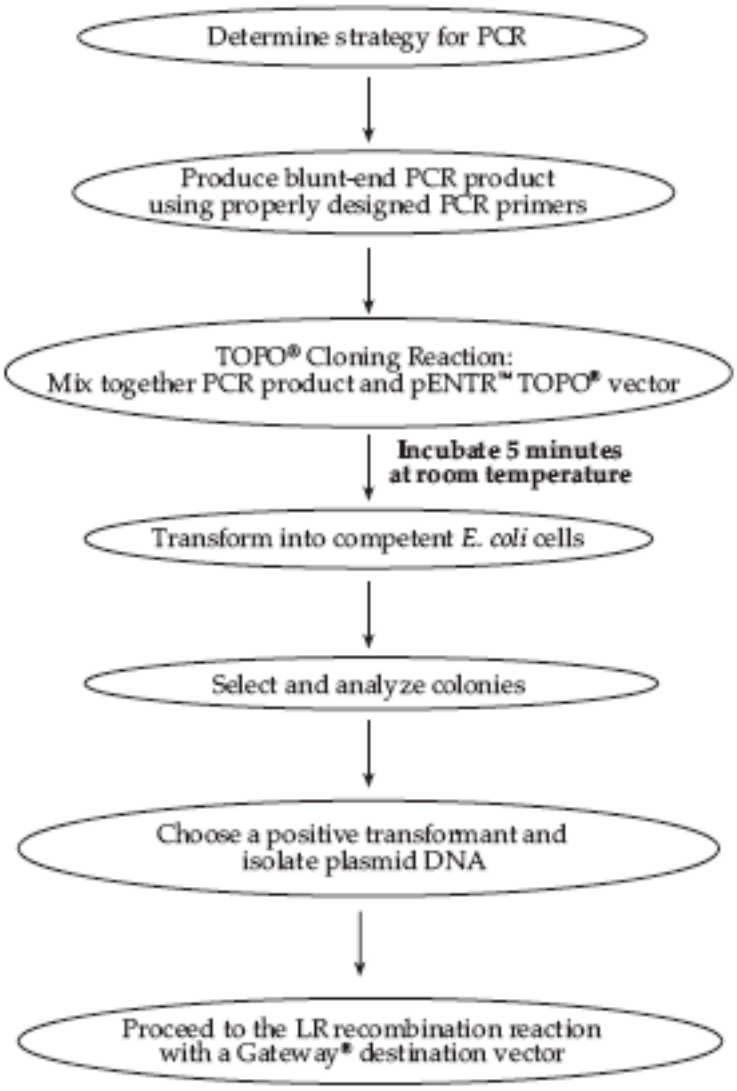

**Note**

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 CCCTT. By changing the sequence of the 5' TOPO® recognition site to TCCTT, 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.



# Methods

## 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)NNA**T**GG. 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).

*continued on next page*

## 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).

*continued on next page*

**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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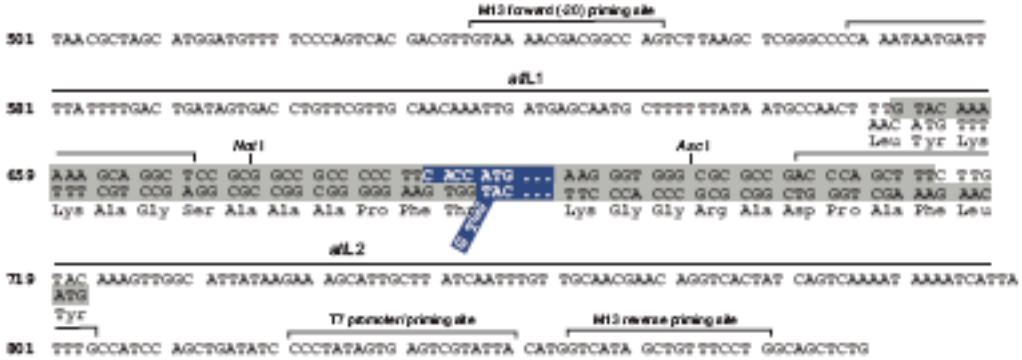
# Designing PCR Primers, continued

## 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.


*continued on next page*

# 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 –20°C 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

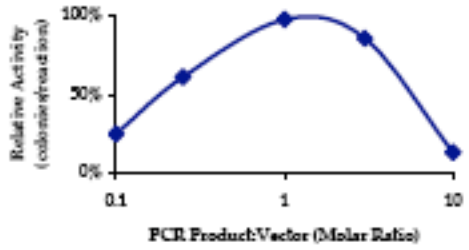
## Introduction

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® 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® 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® 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.

# 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.<br>• 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.<br>• 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.<br>• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes.<br>• 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.<br>• Gel-purify your PCR product. |

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## 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.<br>• 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.<br>• Optimize your PCR.<br>• 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.<br>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*

## 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*

## 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<br>Reverse orientation: 646, 2684<br>Empty vector: 2580 |
| pENTR™/SD/D-TOPO® | *Not* I | Correct orientation: 148, 3203<br>Reverse orientation: 667, 2684<br>Empty vector: 2601 |
| pENTR™/TEV/D-TOPO® | *EcoR* V/*Pst* I | Correct orientation: 757, 2602<br>Reverse orientation: 250, 3109<br>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.

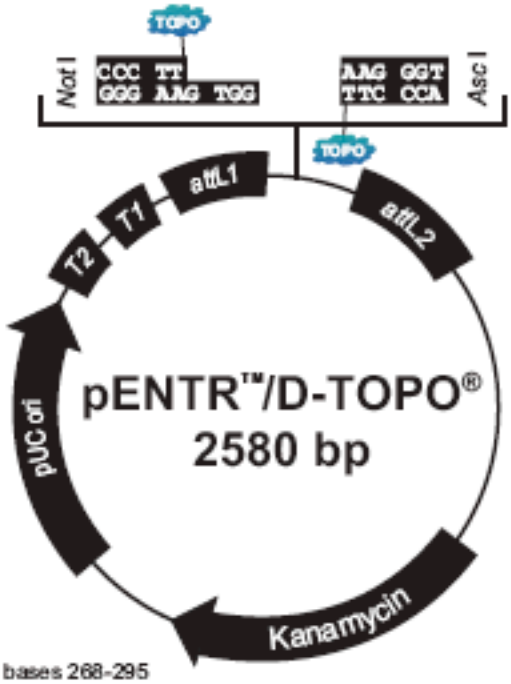
## Transformation Control

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
*attL1*: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*attL2*: 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

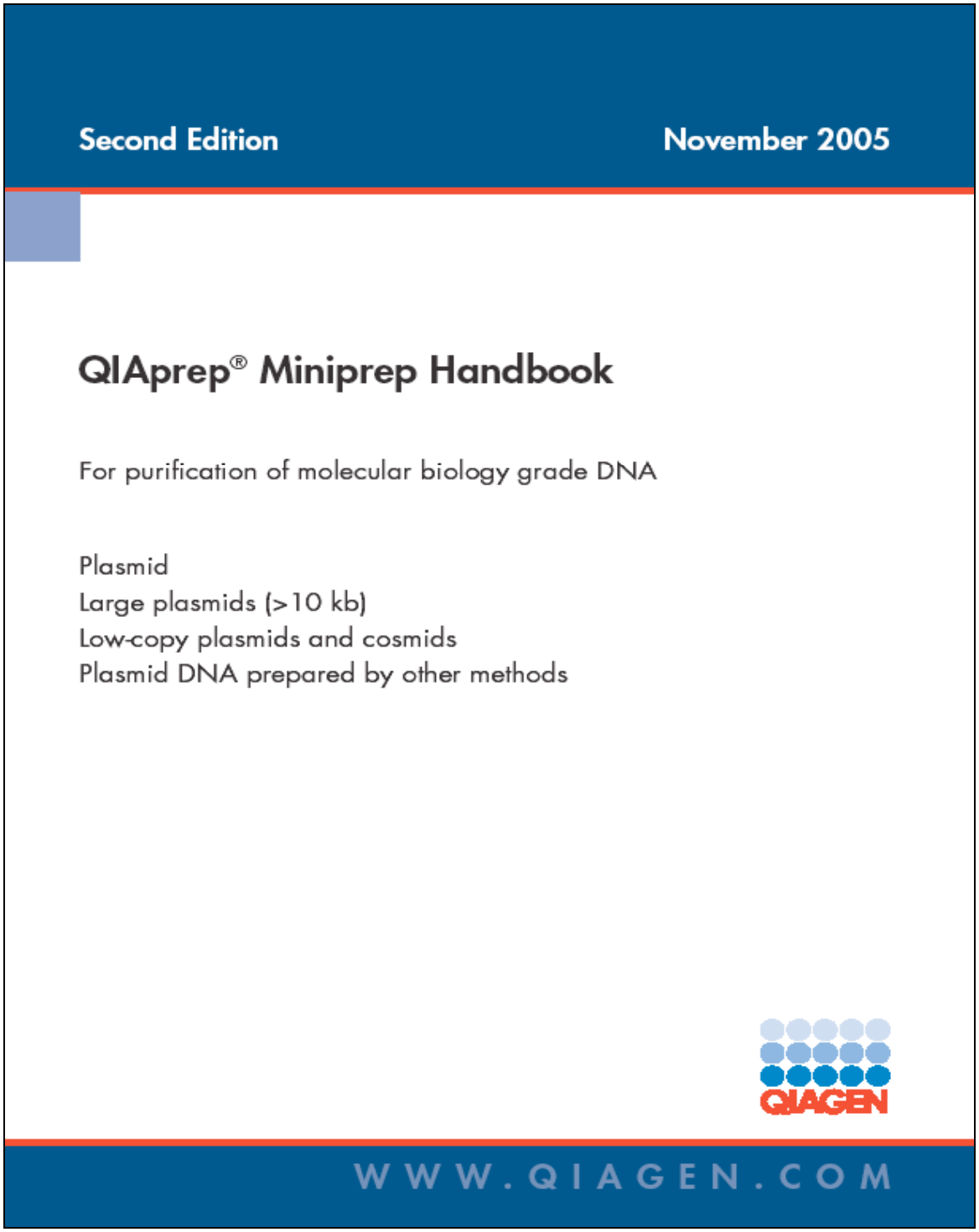
**Features of pENTR™/D-TOPO®**

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. |
| *attL1* and *attL2* 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 1F**

**QIAprep Miniprep Handbook (Taken from Qiagen Website)**



## Kit Contents

| QIAprep Spin Miniprep Kit | (50) | (250) |
|---|---|---|
| **Catalog no.** | **27104** | **27106** |
| QIAprep Spin Columns | 50 | 250 |
| Buffer P1 | 20 ml | 70 ml |
| Buffer P2 | 20 ml | 70 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 ml | 70 ml |
| RNase A† | 200 µl | 700 µl |
| Collection Tubes (2 ml) | 50 | 250 |
| Handbook | 1 | 1 |

## 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.

## 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).

### 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.

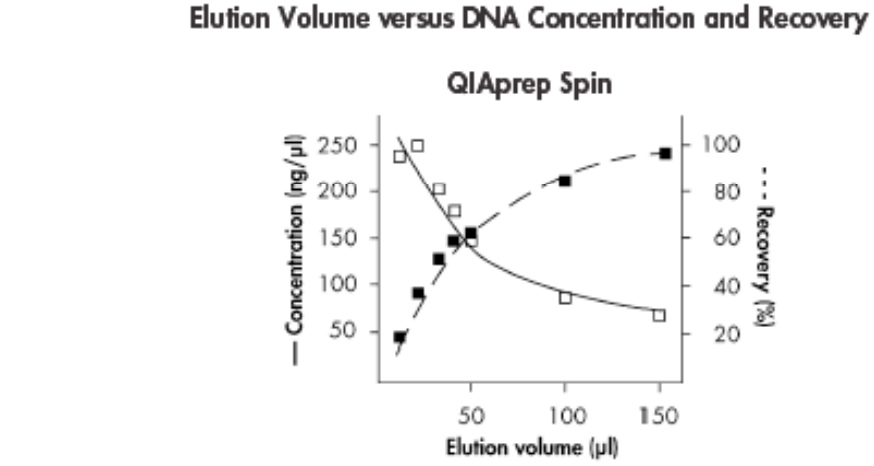



**Figure 1** 10 µg pUC18 DNA was purified using the QIAprep Spin protocol and eluted with the indicated volumes of Buffer EB. The standard protocol uses 50 µl Buffer EB for elution, since this combines high yield with high concentration. However the yield can be increased by increasing the elution volume.

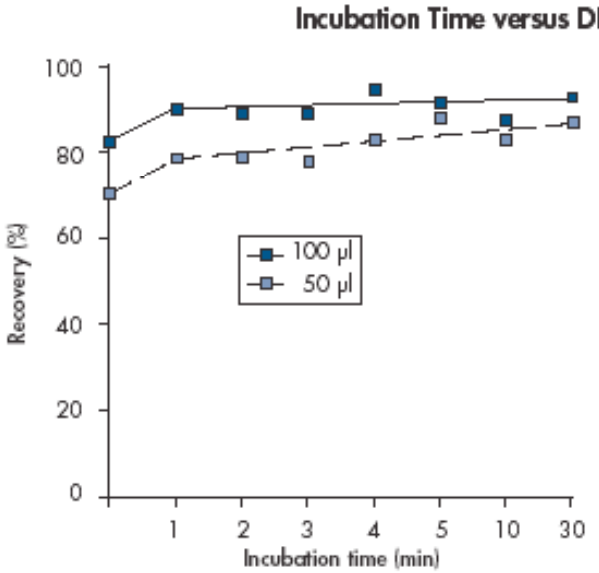



**Figure 2** 10 µg pBluescript DNA was purified using the QIAprep Spin Miniprep protocol and eluted after the indicated incubation times with either 50 µl or 100 µl Buffer EB. The graph shows that an incubation time of 1 minute and doubling the elution buffer volume increases yield.

**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.

**Cell Cultivation in a 96-Well Block for QIAprep Turbo 96**

1. **Fill each well of a 96-well flat-bottom block with 1.3 ml of growth medium containing the appropriate selective agent. Inoculate each well from a single bacterial colony. Incubate the cultures for 20–24 h at 37°C with vigorous shaking.**

   The wells in the block may be protected against spill-over by covering the block with a plastic lid or adhesive tape. AirPore microporous tape sheets promote gas exchange during culturing (see ordering information, page 49). If non-porous tape is used, pierce 2–3 holes in the tape with a needle above each well for aeration.

2. **Harvest the bacterial cells in the block by centrifugation for 5 min at 2100 x *g* in a centrifuge with a rotor for microtiter plates (e.g., QIAGEN Centrifuge 4K15C, or Heraeus Minifuge® GL), preferably at 4–10°C. The block should be covered with adhesive tape during centrifugation. Remove media by inverting the block.**

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

   **WARNING:** Ensure that the buckets on the rotor have sufficient clearance to accommodate the 2 ml flat-bottom blocks before starting the centrifuge.

**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.

**Vacuum notes**

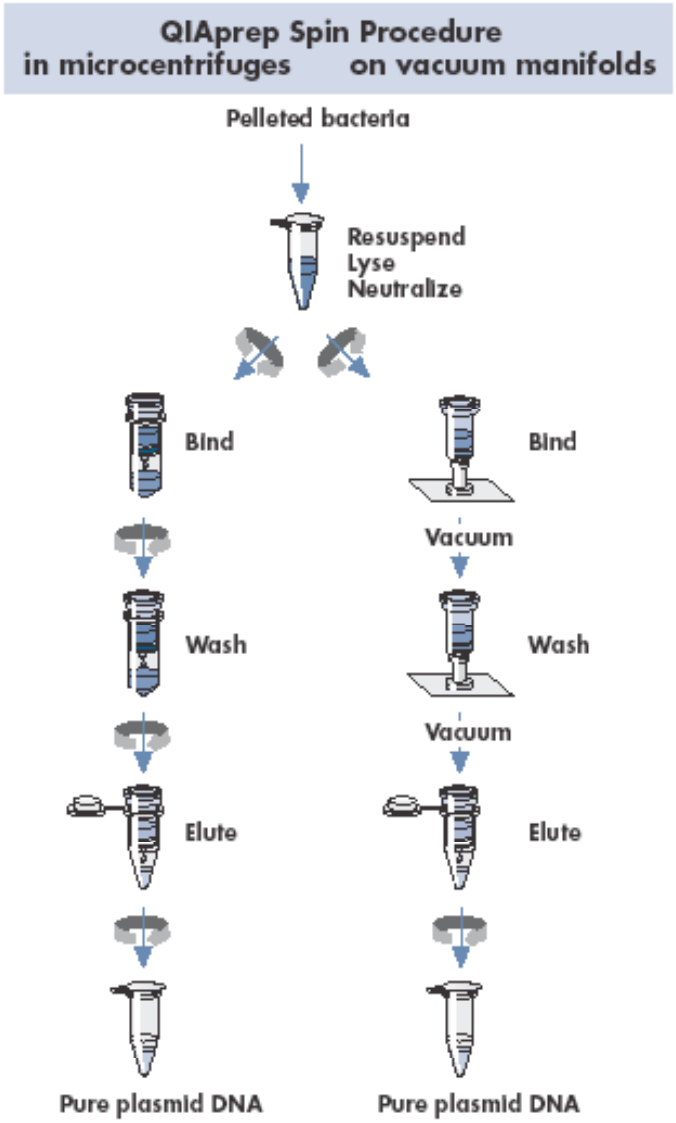
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during manipulations.
- Wear safety glasses when working near a manifold under pressure.
- For safety reasons, do not use 96-well plates that have been damaged in any way.
- For QIAprep 8, QIAprep 8 Turbo, and QIAprep 96 Turbo miniprep procedures, the negative pressure (vacuum) should be regulated before beginning the procedure by applying the vacuum to the appropriate number of **empty** QIAprep modules (indicated in Table 2) on the QIAvac manifold.

  The vacuum pressure is the pressure differential between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 millibar or 760 mm Hg) and can be measured using a vacuum regulator (see ordering information, page 48). Vacuum recommendations are given in negative units (Table 2) to indicate the required reduction in pressure with respect to the atmosphere. Table 3 provides pressure conversions to other units.
- Use of a vacuum pressure lower than recommended may reduce DNA yield and purity.

**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. |

| | | |
|---|---|---|
| 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.

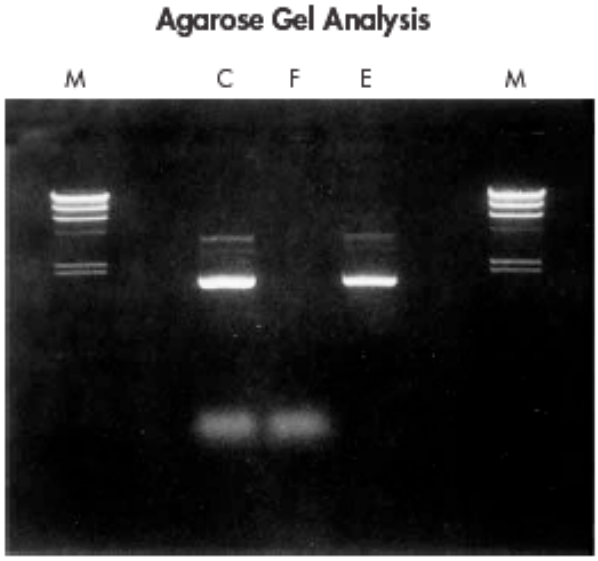



**Figure 6** Agarose gel analysis of the QIAprep Miniprep procedure. **C**: cleared lysate; **F**: flow-through; **E**: eluted plasmid; **M**: markers.

# 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.

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