invitrogen™

HTP TOPO TA Cloning® Kit Dual Promoter

Five-minute cloning of *Taq* polymerase-amplified PCR products for high-throughput cloning

Catalog nos. K4600-05, K4600-480, K4600-500

Version G
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25-0365

User Manual

Table of Contents

Kit Contents

Shipping and Storage

The HTP TOPO TA Cloning® Dual Promoter Kits are shipped on dry ice. Each kit contains a box with TOPO TA Cloning® reagents and one with TOP10 Chemically Competent E. coli in bulk or in a 96-well format (MultiShot™ or MultiShot™ StripWell TOP10 Chemically Competent E. coli). Store TOPO TA Cloning® reagents at -20°C and store the cells at -80°C.

Types of Kits

The table below lists the catalog number, type of competent E. coli, and number of reactions in the kits available.

Catalog no.	Competent Cells	Reactions
K4600-500	5 x 5 ml; 1 x 50 µl	500
K4600-480	Five 96-well plates, 15 µl/well 1 test plate with 12 wells of cells only	480
K4600-05	Five 96-well plates, 50 µl/well	480

HTP TOPO TA Cloning® Reagents

HTP TOPO TA Cloning® reagents are listed below. **Please note that the user must supply *Taq* polymerase.** Store at -20°C.

Item	Concentration	Amount
pCR®II-TOPO® vector	10 ng/ l plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 g/ml BSA 30 µM phenol red	500 µl
dNTP Mix	12.5 mM each dATP, dCTP, dGTP, dTTP in water, pH 8	300 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	500 µl
Sterile Water	--	3 x 1 ml
M13 Forward (-20) Primer	0.1 µg/µl in TE Buffer, pH 8	2 x 20 µl (407 pmol each tube)
M13 Reverse Primer	0.1 µg/µl in TE Buffer, pH 8	2 x 20 µl (385 pmol each tube)

continued on next page

Kit Contents, Continued

Primer Sequence

The table below lists the sequence of the sequencing primers included in the kit.

Primer	Sequence
M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´

Ordering Information

Additional primer may be ordered if needed.

Primer	Quantity	Catalog no.
M13 Forward (-20)	2 µg (407 pmol)	N520-02
M13 Reverse	2 µg (385 pmol)	N530-02

TOP10 Chemically Competent *E. coli*

TOP10 Chemically Competent *E. coli* is supplied in one of two formats (bulk or MultiShot™) depending on the catalog number (see page iv). Reagents supplied with each format are described below and on the following page.

Bulk TOP10

Catalog no. K4600-500 supplies the following reagents.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	5 x 15 ml
TOP10 cells	--	5 x 5 ml
	--	1 x 50 µl (control reaction)
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

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MultiShot™ TOP10

Catalog no. K4600-480 supplies the following reagents.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	5 x 15 ml
TOP10 cells	--	5 x 96-well plates (15 µl per well)
	--	Test plate with 12 wells of cells (1 row)
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

MultiShot™ StripWell TOP10

Catalog no. K4575-05 supplies the following reagents.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	10 x 15 ml
TOP10 cells	--	5 x 96-well plates (50 µl per well)
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

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

Overview

Introduction

The HTP TOPO TA Cloning® Dual Promoter Kits are specifically designed to simplify high-throughput (HTP) applications. Sufficient pCR®II-TOPO® is provided in bulk to perform 500 TOPO® Cloning reactions. Chemically competent TOP10 *E. coli* are provided in a choice of three formats:

- Cells are provided in bulk aliquots of 5 ml to allow simple transfer of the cells from a sterile trough into a 96-well plate containing the TOPO® Cloning reaction.
- Cells are provided pre-aliquoted in 96-well plates (MultiShot™ format) to allow addition of the TOPO® Cloning reaction to the cells.
- Cells are provided pre-aliquoted in 96-stripwell plates (MultiShot™ StripWell format) to allow addition of the TOPO® Cloning reaction to only those cells needed for your particular application.

Experimental Outline

Most users will already have a process for their own high-throughput application. The table below provides a basic outline of the experimental protocol for reference and to evaluate your own protocol.

Step	Action	Page
1	Produce PCR products using your own protocol. Guidelines are provided for your convenience.	2
2	Set up TOPO® Cloning reactions for use with— Bulk cells MultiShot™ cells MultiShot™ StripWell	 3 5 7
3	Transform the TOPO® Cloning reaction into— Bulk cells MultiShot™ cells MultiShot™ StripWell cells	 3 5 7
4	Analyze plasmid DNA for the correct clone.	10
5	Sequence plasmid DNA using your method of choice.	10

Methods

Producing PCR Products

Introduction

The HTP TOPO TA Cloning® Dual Promoter Kits are specifically designed for high-throughput applications. While most users will already have a procedure and reagents for producing PCR products, we include some reminders for your convenience.

Synthesizing Primers

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®II-TOPO®.

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase (i.e. Expand™ or eLONGase™), *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

Multiple PCR Bands

If you obtain multiple bands from your PCR, there are several options to consider, depending on your application. Please note that primer-dimers are preferentially cloned because of their small size. You may--

- Proceed directly to TOPO® Cloning (page 3, 5, or 7).
- Gel-purify your fragment before TOPO® Cloning. A variety of methods and kits are available. We recommend the S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25).
- Optimize your PCR to eliminate multiple bands and smearing. The PCR Optimizer™ Kit (Catalog no. K1220-01) contains additional reagents to help you optimize your PCR.

The Next Step

After producing your PCR products, you are ready to TOPO® Clone and transform *E. coli*. Three sample protocols are provided to help you develop a protocol specifically for your needs. These protocols were created in response to specific customer needs. To TOPO® Clone and transform using:

- Bulk chemically competent *E. coli*, see page 3
- MultiShot™ chemically competent *E. coli*, see page 5
- MultiShot™ StripWell chemically competent *E. coli*, see page 7

High-Throughput TOPO® Cloning and Transformation with Bulk Cells

Description

In this protocol, the TOPO® Cloning reaction is set up in a 96-well U bottom, polystyrene plate (Costar, Catalog no. 3366, 330 µl/well) and the competent cells are placed in a trough for dispensing.

Before Starting

- Chill a 96-well metal heating block (VWR, Catalog no. 13259-260) on ice until the block is cold.
- Bring a vial of SOC to room temperature.
- Pre-heat a heat block or thermocycler containing a 96-well metal block to 42°C. **Note**: You can also use a water bath, but be careful not to contaminate the cells.
- Thaw 1 tube (5 ml) of TOP10 chemically competent *E. coli* on ice (30-40 minutes).
- Warm agar plates containing 50-100 µg/ml ampicillin or 50 µg/ml kanamycin to 37°C.
- If you wish to test the transformation efficiency of the cells with pUC19, dilute a portion of the 10 pg/µl stock solution to 2.5 pg/µl and store on ice.

Controls

A 50 µl aliquot of competent cells is provided to perform a test TOPO® Cloning and transformation reaction. You can also include the pUC19 plasmid as an internal control (see **Procedure** below).

Procedure

1. Set up the 6 µl TOPO® Cloning reaction in each well as follows. If you include pUC19 as a control, leave 2-3 wells empty.

PCR product	1 µl
Salt Solution	1 µl
Sterile Water	3 µl
pCR®II-TOPO®	1 µl
Final Volume	6 µl

2. Incubate 5-10 minutes at room temperature.
3. Place the 96-well plate on the cooling block for 5 minutes.
4. If you are including pUC19, add 2 µl (5 pg) of the diluted plasmid to 2-3 empty wells.
5. Pour thawed TOP10 *E. coli* into a sterile trough and immediately dispense 45 µl/well. Gently pipet up and down 1-2 times to mix.
6. Cover the plate with Parafilm® and incubate it on the chilled block for 20 minutes. Proceed to the next page.

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High-Throughput TOPO® Cloning and Transformation with Bulk Cells, Continued

Procedure, continued

7. Transfer the plate to either the pre-warmed heat block or the thermocycler and heat-shock the cells at 42°C for 30 seconds.
8. Transfer the plate back to the cooling block and press down to ensure the plate is in complete contact with the cooling block. Incubate for 1 minute.
9. Remove the Parafilm® and add 150 µl/well of SOC.
10. Re-cover the plate and incubate the plate at 37°C for 1 hour. **Note**: Gentle shaking (125 RPM) is optional.
11. Plate 50 µl from each well onto selective plates. Use LB plates containing 100 µg/ml ampicillin for selection for the pUC19 controls. Incubate overnight at 37°C.
12. The next day, select 5-10 colonies and process as desired.

Plating Volumes and Expected Results

The table below describes the type of DNA, amount transformed into chemically competent cells, the volume plated, and the number of colonies.

Note: We use pUC19 to qualify the kit. Transformation efficiency should be $> 1 \times 10^8$ cfu/µg and yield 100-300 colonies per plate.

DNA	Type	Amount Transformed	Volume Plated	Number of Colonies
pUC19	Supercoiled	5 pg vector	50 µl	100-300
pCR®II-TOPO® plus PCR-amplified 750 bp insert	TOPO TA Cloning®	10 ng vector + 20 ng insert	50 µl	150-300

Too Many Colonies

If you obtain too many colonies, reduce the amount plated and/or dilute the transformation with additional SOC.

High-Throughput TOPO® Cloning and Transformation with MultiShot™ Cells

Description

In this protocol, the TOPO® Cloning reaction is set up in a 96-well plate and 2 µl are transferred to each well of a MultiShot™ 96-well plate containing 15 µl of chemically competent cells per well.

Before Starting

- Chill two 96-well metal heating blocks (VWR, Catalog no. 13259-260) on ice until the blocks are cold.
- Bring a vial of SOC to room temperature.
- If you are planning to use pUC19 as a control for transformation, dilute a portion of the 10 pg/µl stock solution to 2.5 pg/µl and store on ice.
- Warm agar plates containing 50-100 g/ml ampicillin or 50 g/ml kanamycin to 37°C.
- Pre-heat a heat block or thermocycler containing a 96-well metal block to 42°C. **Note**: You can also use a water bath, but be careful not to contaminate the cells.
- If you are using a thermocycler, program the machine to hold the temperature at 42°C.

Controls

A test plate containing 1 row (12 wells) of cells is included to perform test TOPO® Cloning reactions and transformations. In addition, you can include the pUC19 plasmid as an internal control (see **Procedure** below).

Procedure

1. In a 96-well plate, set up the 6 µl TOPO® Cloning reaction in each well as follows.

PCR product	1 µl
Salt Solution	1 µl
Sterile Water	3 µl
pCR®II-TOPO®	1 µl
Final Volume	6 µl

2. Incubate 5-10 minutes at room temperature.
3. Place the 96-well plate on one of the cooling blocks for 5 minutes.
4. Remove a MultiShot™ plate from the freezer and place it in the second cooling block. Cells should thaw within 30 seconds.
5. Carefully remove the aluminum foil seal.
6. Use a multi-channel pipet to add 2 µl of each TOPO® Cloning reaction (~3.3 ng) to each well of the MultiShot™ plate. Keep the volume around 2 µl for uniform results. To use the pUC19 control, add 2 µl (5 pg) of the 2.5 pg/µl solution. Proceed to the next page.

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Procedure, continued

7. Cover the cells with the supplied plastic lid and incubate the cells and DNA in the chilled block for 20 minutes.
8. Transfer the cell plate to either the pre-warmed heat block or thermocycler and heat-shock for 30 seconds at 42°C.
9. Transfer the cell plate back to a cooling block, press the plate into the block, and allow the plate to cool for 1 minute.
10. Remove the plastic lid and add 90 µl SOC to each well.
11. Cover the plate with the lid and incubate the plate at 37°C for 1 hour. **Note**: Gentle shaking (125 RPM) is optional.
12. Plate 100 µl from each well on selective plates. Plate the pUC19 control reactions on LB plates containing 100 µg/ml ampicillin. Incubate overnight at 37°C. See below for the expected number of colonies.

Plating Volumes and Expected Results

The table below describes the type of DNA, amount transformed into MultiShot™ chemically competent cells, the volume plated, and the number of colonies.

Note: We use pUC19 to qualify the kit. Transformation efficiency should be > 1 x 10^8 cfu/µg and yield 25-100 colonies per plate.

DNA	Type	Amount Transformed	Volume Plated	Number of Colonies
pUC19	Supercoiled	5 pg vector	10 µl	50-200
pCR®II-TOPO® plus PCR-amplified 750 bp insert	TOPO TA Cloning®	3.3 ng vector + insert	100 µl	25-100

Too Many Colonies

If you obtain too many colonies, you can reduce the amount plated or dilute the TOPO® Cloning reactions with sterile water or TE buffer prior to adding the reaction to the cells.

High-Throughput TOPO® Cloning and Transformation with MultiShot™ StripWell Cells

Description

In this protocol, the TOPO® Cloning reaction is set up in a 96-well plate and 2 µl are transferred to each well of a MultiShot™ StripWell 96-well plate containing 50 µl of chemically competent cells per well.

Before Starting

- Chill a 96-well metal heating block (VWR, Catalog no. 13259-260) on ice until the block is cold.
- Prepare a container of ice large enough to chill the number of wells you will be using.
- Bring the vial of SOC to room temperature.
- If you are planning to use pUC19 as a control for transformation, dilute a portion of the 10 pg/µl stock solution to 2.5 pg/µl and store on ice.
- Warm agar plates containing 50-100 µg/ml ampicillin or 50 µg/ml kanamycin to 37°C.
- Pre-heat a water bath to 42°C.

Procedure

1. In a 96-well plate, set up the 6 µl TOPO® Cloning reaction in the desired number of wells as follows.

PCR product	1 µl
Salt Solution	1 µl
Sterile Water	3 µl
pCR®II-TOPO®	1 µl
Final Volume	6 µl

2. Incubate 5-10 minutes at room temperature.
3. Place the 96-well plate on the cooling block for 5 minutes.
4. Remove a MultiShot™ StripWell plate from the freezer and remove the number of wells you need. Return any unused wells to the freezer. Place the wells in the container of ice. Cells should thaw within 1 minute.
5. Carefully remove the strip of caps from each set of 8 wells and keep them for further use.
6. Use a multi-channel pipet to add 2 µl of each TOPO® Cloning reaction (~3.3 ng) to the wells. Keep the volume around 2 µl for uniform results. To use pUC19 as an internal control, add 2 µl (5 pg) of the 2.5 pg/µl solution to 2-3 separate wells.

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High-Throughput TOPO® Cloning and Transformation with MultiShot™ StripWell Cells, continued

Procedure, continued

7. Cover the cells with the caps and incubate the cells and DNA on ice for 30 minutes.
8. Transfer the wells to the water bath and heat-shock for 30 seconds at 42°C. **Note:** Be careful not to contaminate the cells.
9. Transfer the wells back to the ice and allow the wells to cool for 1 minute.
10. Remove the caps and add 250 µl SOC to each well. **Re-cap the wells tightly.**
11. Incubate the wells at 37°C for 1 hour with shaking (225 RPM). We turn the wells on their side to increase aeration and secure them to the shaker.
12. Plate 25 µl from each well on selective plates. Plate the pUC19 control reactions on LB plates containing 100 µg/ml ampicillin. Incubate overnight at 37°C. See below for the expected number of colonies.

Plating Volumes and Expected Results

The table below describes the type of DNA, amount transformed into MultiShot™ StripWell chemically competent cells, the volume plated, and the number of colonies.

Note: We use pUC19 to qualify the kit. Transformation efficiency should be $> 1 \times 10^8$ cfu/µg and yield 100-300 colonies per plate.

DNA	Type	Amount Transformed	Volume Plated	Number of Colonies
pUC19	Supercoiled	5 pg vector	10 µl	100-300
pCR®II-TOPO® plus PCR-amplified 750 bp insert	TOPO TA Cloning®	3.3 ng vector + insert	25 µl	100-300

Too Many Colonies

If you obtain too many colonies, you can reduce the amount plated or dilute the TOPO® Cloning reactions with sterile water or TE buffer prior to adding the reaction to the cells.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.

- After combining the TOPO® Cloning reaction with chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Analyzing and Sequencing Clones

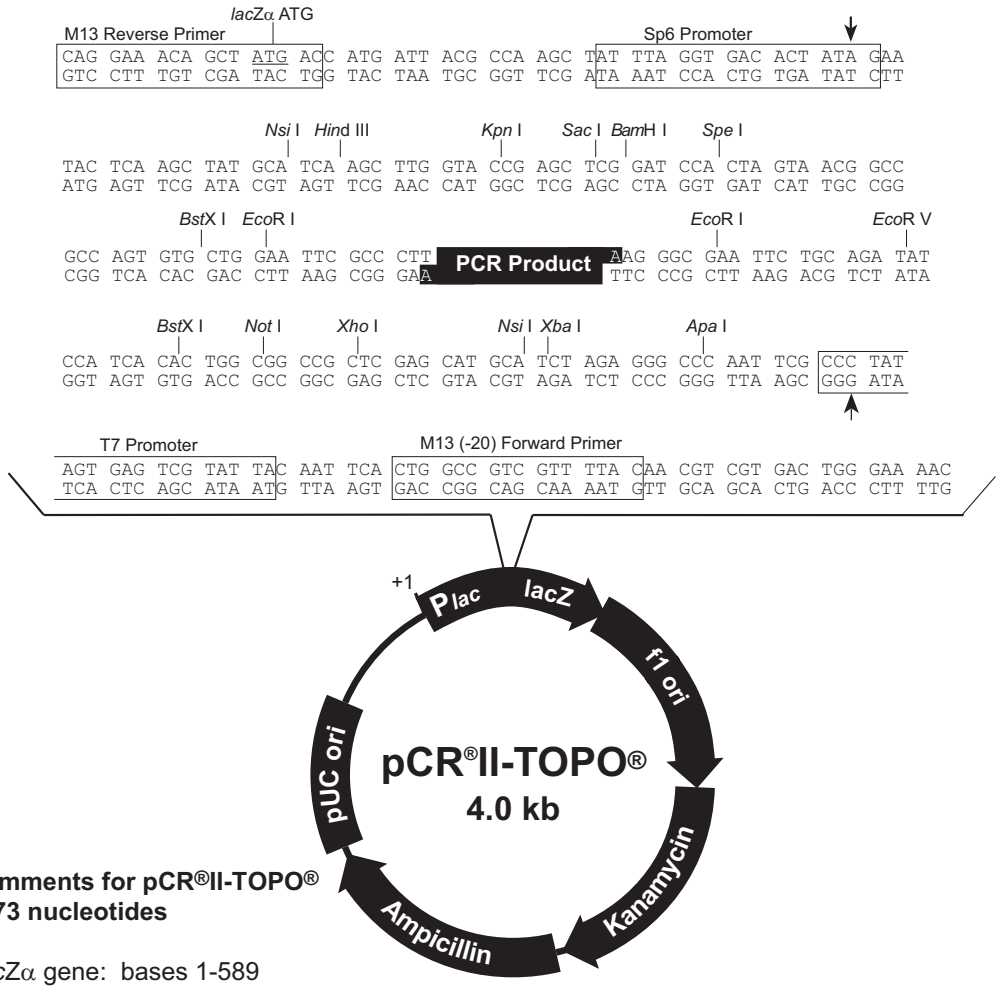
Analysis	Use your own HTP method to analyze clones for the desired insert.
Sequencing	Once you have identified the correct clone, you are ready to sequence your insert. Two primers (M13 Forward (-20) and M13 Reverse) have been included to help you sequence your insert. If you need more primer, please see page v for ordering information. Please refer to the map on page 11 for the sequence surrounding the TOPO® Cloning site. For the full sequence of the vector, please visit our Web site or contact Technical Service (page 12).

Appendix

Map of pCR®II-TOPO®

pCR®II-TOPO® Map

The map below shows the features of pCR®II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (page 12).**



Comments for pCR®II-TOPO®
3973 nucleotides

*LacZ*α gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

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Limited Warranty

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Product Qualification

Introduction

Invitrogen qualifies the HTP TOPO TA Cloning® Dual Promoter Kits as described below.

Restriction Digest

The parental supercoiled pCR®II vector is qualified by restriction digest prior to linearization and adaptation with topoisomerase. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pCR®II
*Hin*d III (linearizes)	3971 bp
Xba I (linearizes)	3971 bp
Nsi I (linearizes)	112, 3859 bp
Pst I	1190, 2781 bp
*Eco*R I and *Afl* II	16, 450, 716, 2789 bp

TOPO® Cloning Efficiency

Once the pCR®II-TOPO® vector has been adapted with topoisomerase I, it is lot-qualified using the method described in this kit. A 750 bp control PCR product is TOPO® Cloned into the vector and subsequently transformed into the TOP10 competent *E. coli* included with each kit.

Each lot of vector should yield greater than 95 (+/- 4%) cloning efficiency.

Primers

The primers included in this kit have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

TOP10 Competent *E. coli*

Bulk cells, MultiShot™, and MultiShot™ StripWell 96-well plates are functionally qualified with the kit. The three formats are tested with the TOPO® Cloning reaction and the pUC19 plasmid using the protocols in the manual. Expected results are summarized below.

Vector	Bulk Cells	MultiShot™	MultiShot™ StripWell
TOPO® Cloning reaction with pCR®II-TOPO® (30 ng transformed)	150-300 colonies (50 µl plated)	50-200 colonies per plate (100 µl plated)	100-300 colonies per plate (25 µl plated)
pUC19 plasmid only (5 pg transformed)	1 x 10^8 cfu/µg DNA 100-300 colonies per plate	1 x 10^8 cfu/µg DNA 100-300 colonies per plate	1 x 10^8 cfu/µg DNA 100-300 colonies per plate

Purchaser Notification

Limited Use Label License No: 5

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