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CopyControl™ cDNA, Gene & PCR Cloning Kit with Chemically Competent TransforMax™ EPI300™ *E. coli*

Cat. No. CCPCR1CC

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1. Introduction

The CopyControl Cloning System,* based on technology developed by Dr. Waclaw Szybalski [1-3] at the University of Wisconsin-Madison, combines the clone stability afforded by single-copy cloning with the advantages of high yields of DNA obtained by "on-demand" induction of the clones to high-copy number. For example, CopyControl BAC (Bacterial Artificial Chromosome) clones can be induced to 10-20 copies per cell and CopyControl Fosmid and PCR clones can be induced from single copy to 10-200 copies per cell to improve DNA yields for sequencing, fingerprinting, subcloning, *in vitro* transcription, and other applications. CopyControl Cloning Kits for BAC cloning, fosmid library production, and cDNA, Gene & PCR cloning are available.

The CopyControl Cloning System Has Two Required Components

1. The CopyControl pCC1™ (Blunt Cloning-Ready) Vector contains both the *E. coli* F factor single-copy origin of replication and the high-copy *ori*V origin of replication.
2. Initiation of replication from *ori*V requires the *trf*A gene product that is supplied by the TransforMax™ EPI300 *E. coli* cells.* The cells have been engineered so that a mutant *trf*A gene is under tight control of an inducible promoter. In the absence of the induction agent (a simple sugar), *trf*A gene expression is repressed and the clones are maintained at single-copy number in the EPI300 cells. Addition of the CopyControl Fosmid AutoInduction Solution induces expression of the *trf*A gene resulting in amplification of the clone to high-copy number.

Features of the CopyControl pCC1 (Blunt Cloning-Ready) Vector (Fig. 1)**:**

- Chloramphenicol resistance as an antibiotic selectable marker.
- *E. coli* F factor-based partitioning and single-copy origin of replication.
- *ori*V high-copy origin of replication.
- Bacteriophage lambda *cos* site for lambda packaging or lambda-terminase cleavage.
- Bacteriophage P1 *lox*P site for Cre-recombinase cleavage.
- Bacteriophage T7 RNA polymerase promoter flanking the cloning site.
- Suitable for "blue/white" screening.

2. Kit Contents

Desc.	Concentration	Quantity
Reagents included in the kit are sufficient to clone 20 PCR products.		
CopyControl™ pCC1™ (Blunt Cloning-Ready) Vector (linearized at the unique *Eco*72 I site and dephosphorylated)	(75 ng/µl)	20 µl
PCR Precipitation Solution		1 ml
PCR 10X Cloning Buffer		50 µl
PCR End-Repair Enzyme Mix		25 µl
Fast-Link™ DNA Ligase	(50 U @ 2 U/µl)	25 µl
EpiBlue™ Solution		2.5 ml
EpiLyse™ Solution	10 ml	
Control PCR Product	(4 ng/µl, 1,360 bp)	20 µl
Supercoiled DNA Marker	(10 ng/µl, ~8.1 Kb)	200 µl
Sterile Water		500 µl
Chemically Competent TransforMax™ EPI300™ *E. coli* [F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) (Str^R) φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G *trf*A *dhfr*]		20 x 50 µl
CopyControl™ Fosmid AutoInduction Solution	(500X concentrate)	4 x 1 ml

Note: *EpiBlue and EpiLyse Solutions are supplied together in a poly bag. TransforMax EPI300 cells and 1 ml of the CopyControl Fosmid AutoInduction Solution are supplied together in their own box.*

Storage: Store the Electrocompetent TransforMax EPI300 *E. coli* at –70°C. Store the EpiBlue and EpiLyse Solutions at room temperature. Store the remainder of the kit components at –20°C.

Additional Required Reagents

In addition to the components supplied, the following reagents are required:

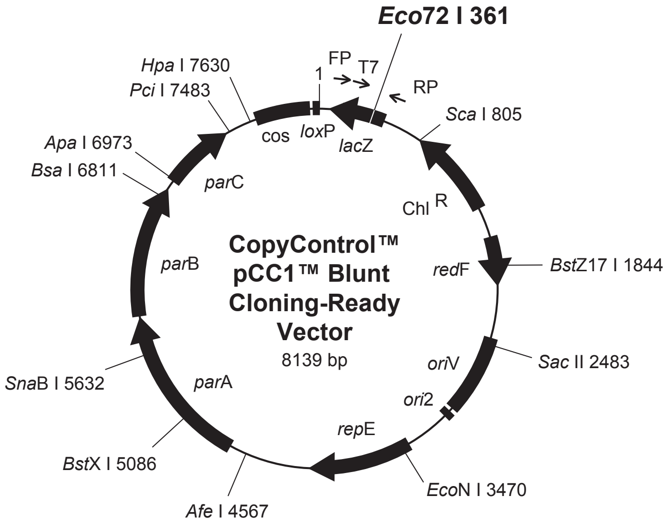
LB plates (1% Tryptone, 0.5% Yeast Extract, 1% NaCl, 15 g/L agar, [pH 7.0]) + 12.5 µg/ml chloramphenicol and (optional components) X-Gal (40 µg/ml) and IPTG (0.4 mM)

TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA)

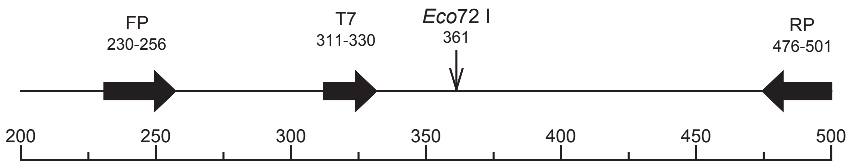
Supercoiled DNA Molecular Weight Markers (>8 Kb)

Quality Control

The CopyControl cDNA, Gene & PCR Cloning Kit with Chemically Competent TransforMax EPI300 *E. coli* cells is function-tested using the Control PCR Product provided. Greater than 1 x 10^6 white or light blue colonies are obtained per microgram of the Control PCR Product when using Chemically Competent TransforMax EPI300 cells with a transformation efficiency of >1 x 10^8. At least 70% of all white colonies must contain the correct size insert.



Note: Not all restriction enzymes that cut only once are indicated above.
See Appendix B for complete restriction information.
Primers are not drawn to scale.



FP = pCC1™/pEpiFOS™ Foward Sequencing Primer 5' GGATGTGCTGCAAGGCGATTAAGTTGG 3'
RP = pCC1™/pEpiFOS™ Reverse Sequencing Primer 5' CTCGTATGTTGTGTGGAATTGTGAGC 3'
T7 = T7 Promoter Primer 5' TAATACGACTCACTATAGGG 3'

Figure 1. **pCC1™ (Blunt Cloning-Ready) Vector Map.**

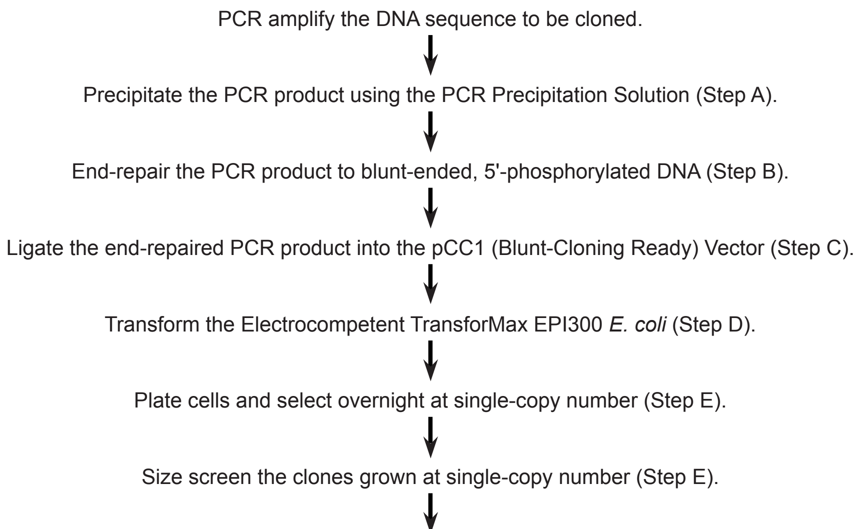
3. Overview of the CopyControl PCR Cloning Process

The CopyControl cDNA, Gene & PCR Cloning Kit can be used to clone 20 PCR products, RT-PCR products, restriction fragments, or cDNAs. The entire process, including cloning, selecting, and screening of the clones can be completed in less than 24 hours.

In the CopyControl PCR cloning process, clones are selected and grown at single-copy number to ensure the stability of the insert and viability of the clone. This is crucial if the PCR product may be unstable (e.g., contains repetitive sequences, high GC or AT content) or encodes a product that may be lethal or detrimental to the host cell. Then, clones are grown in culture and induced to high-copy number by adding of the CopyControl Fosmid AutoInduction Solution.

CopyControl Fosmid AutoInduction Solution, which replaces the standard CopyControl Induction Solution, was developed for induction of the copy number of CopyControl single-copy plasmid and fosmid` DNA to 200 and 50 copies respectively. The new protocol is simple and requires addition of the Autoinduction Solution to the culture medium prior to inoculation of the culture, bypassing hands-on subculturing steps. The autoinduction process can be performed in any culture volume based on your needs. Generally, 200 µl to 1 ml of culture will provide sufficient DNA for most applications. We provide a procedure for amplifying clones from 200-µl to 50-ml cultures on page 11.

An overview of the steps involved in using the CopyControl cDNA, Gene & PCR Cloning Kit is presented below.



4. CopyControl PCR Cloning Procedure

General Considerations

1. PCR products up to 15 kb, produced using any thermostable DNA polymerase(s), including Taq, proofreading polymerases, and enzyme blends, can be efficiently cloned without the need for gel or column purification. Although it is not necessary, if you wish to gel-purify the PCR product prior to cloning, it is extremely important to avoid damage of DNA due to exposure to UV light. Even exposure for short periods of time can decrease the efficiency of cloning by two orders of magnitude or more.
2. The CopyControl pCC1 (Blunt Cloning-Ready) Vector supplied in the kit has been linearized, dephosphorylated, extensively purified, and is ready to use in the ligation step.

A. Precipitation of the PCR Product

Kit component used in this step: **PCR Precipitation Solution.**

Use the procedure described in Steps A1-A5 to purify PCR products >500 bp. However, the recovery of PCR products less than 500 bp is not efficient, using this method. Therefore, we recommend that PCR products of <500 bp be ethanol-precipitated. Ethanol precipitation will not get rid of primer dimers, but these will not cause any problem in obtaining the desired PCR clones.

1. To each PCR reaction with PCR products >500 bp, add an equal volume of PCR Precipitation Solution. Vortex vigorously to mix. If the concentration of the PCR product is expected to be >10 ng/µl, proceed to centrifugation (Step A-2) immediately. If the concentration of the PCR product is expected to be ≤10 ng/µl, incubate the mixture on ice for 30 minutes before centrifugation to maximize recovery.
2. Centrifuge for 5 minutes at 12,500 rpm at 4°C.

   Important: *Centrifugation at 4°C is required to obtain maximum recovery of the PCR product.*
3. Carefully aspirate and discard all the supernatant without disrupting the DNA pellet. Do not pour out the supernatant. The DNA pellet will not be visible. Since it is important to get rid of all the supernatant, centrifuge again briefly if necessary.
4. Resuspend the pellet in 15 µl of TE buffer if the starting PCR product concentration is ≤ 25 ng/µl. Resuspend the pellet in 25 µl of TE buffer if the starting PCR product concentration is > 25 ng/µl.
5. Measure DNA concentration using a fluorometer, if available.

   Note: *A spectrophotometer may not provide an accurate DNA concentration, which is important for a successful ligation reaction to achieve the highest cloning efficiency.*

B. End-Repair to Generate Blunt-Ended and 5′-Phosphorylated PCR Product

Kit components used in this step: **PCR 10X Cloning Buffer, PCR End-Repair Enzyme Mix and Sterile Water.**

***Note:** In the reaction, 0.014 pmol of PCR product will provide the recommended vector to insert ratio of 1:1. One can calculate the amount (ng) of PCR product needed in each reaction as follows:*

$$\text{ng of PCR product} = \frac{\text{75 ng (amount of pCC1 vector in the ligation) x Length of PCR product (in bp)}}{\text{8,139 (size in bp of the CopyControl pCC1 vector)}}$$

1. Thaw and thoroughly mix all of the reagents. Set up the following reaction:

x	µl	sterile water
1	µl	PCR 10X Cloning Buffer
x	µl	PCR product (0.014 pmol)* from Part A
1	µl	PCR End-Repair Enzyme Mix
10	µl	Total reaction volume

2. Mix by vortexing or flicking the bottom of the tube. Centrifuge briefly to collect all solution in the bottom of the tube. Incubate the reaction at room temperature for 30 minutes.
3. Heat the reaction at 70°C for 10 minutes to inactivate the enzymes in the PCR End-Repair Enzyme Mix.

   ***Important:** Complete heat inactivation of the enzymes is crucial to avoid background clones caused by vector self-ligation.*
4. Cool the reaction on ice for 3-5 minutes. Proceed to Part C.

C. Ligation of End-Repaired PCR Product into CopyControl pCC1 (Blunt Cloning-Ready) Vector

Kit components used in this step: **Fast-Link DNA Ligase and CopyControl pCC1 (Blunt Cloning-Ready) Vector.**

1. To the 10 µl end-repair reaction from Step B, add 1 µl of the CopyControl pCC1 (Blunt Cloning-Ready) Vector and 1 µl of Fast-Link DNA Ligase.
2. Mix by vortexing or by flicking the bottom of the tube. Centrifuge the tube briefly to collect all of the solution in the bottom.
3. Incubate the reaction at room temperature for 1 hour.

   ***Note:** Longer incubation times, up to 2 hours, may increase the total number of clones. However, it is not necessary in most cases because more than enough clones with the desired PCR product will be generated under standard reaction conditions.*
4. Heat the reaction at 70°C for 10 minutes to inactivate the Fast-Link DNA Ligase.

   ***Important:** Complete heat-inactivation of the enzyme will ensure the highest cloning efficiency.*
5. Cool the reaction on ice for 3-5 minutes.

D. Transformation of the Chemically Competent TransforMax EPI300 *E. coli*

Kit components used in this step: **Chemically Competent TransforMax EPI300 *E. coli.***

Reagents supplied by the user: SOC or LB Medium.

1. Thaw the Chemically Competent TransforMax EPI300 *E. coli* on ice and keep them on ice until used.
2. Combine 1-5 µl of the Ligation Reaction from Step C with 50 µl of the competent cells in a 0.5-ml microcentrifuge tube. Mix gently.
3. Incubate on ice for 30 minutes.
4. Heat shock the mixture at 42°C for exactly 30 seconds.
5. Ice the mixture for 2 minutes.
6. Add 250 µl of SOC medium and transfer to a 1.5-ml microcentrifuge tube.
7. Shake gently at 37°C for 1 hour to allow the cells to recover and the antibiotic resistance gene to be expressed.

E. Selecting, Screening and Amplifying the CopyControl PCR Clones Grown at Single-Copy Number

Kit components used in this step: E**piLyse Solution, EpiBlue Solution, Supercoiled DNA Size Marker and CopyControl Fosmid AutoInduction Solution.**

Reagents supplied by the user: **LB Medium + chloramphenicol (12.5 µg/ml) and Supercoiled DNA Size Markers (>8 Kb).**

Blue/white screening of the clones is not necessary because of the very low number of blue (nonrecombinant) clones that will be obtained. However, if blue/white screening is desired, include X-Gal (40 µg/ml) and IPTG (0.4 mM) in the plates.

Plating and Selecting the CopyControl PCR Clones at Single-Copy Number.

1. Prepare and prewarm to 37°C three LB plates containing chloramphenicol (12.5 µg/ml) (and X-Gal and IPTG if desired). Prepare 100 µl each of a 1:10 and a 1:100 dilution of the transformed cell culture from Step D in SOC or LB medium. Spread 100 µl of undiluted cell culture, and the 1:10 and 1:100 dilutions, individually onto the plates.

   Note: *Although it is not required to plate all three concentrations of cells, doing so increase the chances of obtaining a sufficient number of evenly spread out colonies.*

2. Grow the plates overnight at 37°C. We recommend incubating the plates for at least 20 hours.

   Note: *If X-Gal and IPTG have been included in the plates for blue/white screening, some white colonies (recombinant clones) may develop a light blue center if grown to larger sizes or during extended storage at 4°C. This phenomenon is likely due to the production of a small amount of functional LacZ α-peptide that can result when cloning relatively small DNAs. Most of these light blue clones will contain the desired PCR product.*

Size-Screening the CopyControl PCR Clones Grown at Single-Copy Number.

3. Add 300 µl of LB medium + chloramphenicol (12.5 µg/ml) to each of ten 1.5-ml microcentrifuge tubes.
4. Randomly pick 10 colonies (white or light blue colonies if blue/white screening was done) with sterile pipette tips and transfer them individually into the 1.5-ml tubes.

   Note: *If the colony size is ≤1 mm diameter, pick the whole colony; if the size is >1 mm diameter, only pick a small amount of the cells, because too much cell material may interfere with the screening process.*
5. Incubate at 37°C for 30 minutes with vigorous shaking.

   Important: *Vigorous shaking is crucial! For example, tape the tubes horizontally to the shaker. Placing the tubes in a rack and taping the rack to the bottom of the shaker is not sufficient.*
6. Transfer 100 µl of the cell cultures into separate 1.5-ml microcentrifuge tubes and store them at 4°C. These aliquots will be used as inocula for the clone amplification process in Part E, Step 12. Do not add CopyControl Fosmid AutoInduction Solution to these tubes.
7. Centrifuge the remaining 200 µl of cell cultures for 1 minute at >10,000 x g.
8. Discard the supernatant. Resuspend the cell pellets in 20 µl (10-50 µl can be used) of the EpiBlue Solution. Vortex to completely resuspend the cells. It is critical to completely resuspend the cell pellets to maximize the amount of DNA released from the cells.
9. Add an equal volume of the EpiLyse Solution and vortex vigorously. Load 10-15 µl of the lysed cell solutions on a 1% agarose gel. If the lysed cell solutions are too viscous to load onto a gel, add more EpiLyse Solution until loading is manageable. It is important to make sure the lysed cell solution stays in the wells since the viscous solution may be easily pulled out of the wells when retracting the pipette tips. The remaining lysed cell solutions can be stored at room temperature for up to 1 week in the event additional gels are run.

   Note: *If a cleaner gel is desired (e.g., less RNA in the gel), the lysed cell solutions can be incubated at 70°C for 15 minutes before loading onto a gel.*
10. Load 10 µl of the Supercoiled DNA Size Marker and other supercoiled DNA markers, if available (not supplied in the kit), to lanes of the gel adjacent to the lysed cell extracts. Run the gel for 45-60 minutes. Stain with a 1:10,000 dilution of SYBR® Gold in gel-running buffer or water, or prolonged staining with ethidium bromide.
11. The Supercoiled DNA Size Marker is ~8.1 Kb, the same size as the CopyControl pCC1 Cloning Vector. Therefore, the clones containing the PCR product will migrate at a size equal to 8.1 kb + the size of the cloned PCR product. Once the desired clones are identified, be sure to prepare a glycerol stock of each for long-term storage.

Inducing the CopyControl PCR Clones, Grown at Single-Copy Number, to High-Copy Number.

The autoinduction process can be performed in any culture volume based on your needs. Generally, 200 µl to 1 ml of culture will provide sufficient DNA for most applications. In this step, we provide the procedure for amplifying the clones in 200-µl to 50-ml cultures.

12. Once the desired clones are identified, use the 100 µl of uninduced cell cultures stored at 4°C from Part E, Step 6, to start overnight cultures as needed. Alternatively, choose the desired clones directly from the overnight transformation plate.
13. Choose the preparation size and vessel to be used from the table below prior to inoculation of the culture. Supplement the appropriate amount of LB medium + 12.5 µg/ml chloramphenicol with the 500X CopyControl Fosmid AutoInduction Solution.

Volume of Starting Culture (LB + Chloramphenicol [12.5 µg/ml])	Volume of 500X CopyControl Fosmid AutoInduction Solution*	Size of Vessel to be Used
200 µl	0.4 µl	1-ml 96-well plate
1 ml	2 µl	2.2-ml deep-well 96-well plate
2 ml	4 µl	14-ml Falcon tube
5 ml	10 µl	14-ml Falcon tube
50 ml	100 µl	250-ml Erlenmeyer flask

**Mix thoroughly after thawing.*

14. Inoculate the medium with a small portion of the desired CopyControl clones from an overnight plate.
15. Incubate the culture overnight (16-20 hours) at 37°C with vigorous shaking.

    Important: *Vigorous shaking is crucial for proper aeration! Cultures incubated for longer or shorter periods of time may not properly induce.*

16. Collect the cells by centrifugation and purify the DNA. For 1-ml cultures, use the BACMAX96™ DNA Purification Kit (Epicentre); for 2-ml, 5-ml, and 50-ml cultures, use the FosmidMAX™ DNA Purification Kit (with consumable plastics) (Epicentre). Alternatively, standard laboratory methods for DNA purification can be used.

5. Appendix

Appendix A

Cloning the Control PCR Product

The 1,360-bp Control PCR Product is supplied to test the cloning efficiency of the kit.

1. End-repair the Control PCR Product to generate blunt-ended and 5′-phosphorylated DNA.

   Thaw and thoroughly mix all of the reagents. Set up the following reaction:

5	µl	sterile water
1	µl	PCR 10X Cloning Buffer
3	µl	Control PCR Product
1	µl	PCR End-Repair Enzyme Mix
10	µl	Total reaction volume

2. Mix by vortexing or flicking the bottom of the tube. Centrifuge briefly to collect all the solution in the bottom of the tube. Incubate the reaction at room temperature for 30 minutes.
3. Heat the reaction at 70°C for 10 minutes to inactivate the PCR End-Repair enzymes.
4. Cool the reaction on ice for 3-5 minutes.
5. Ligate the blunt-ended, 5′-phosphorylated Control PCR product into the CopyControl pCC1 (Blunt Cloning-Ready) Vector, transform the Electrocompetent TransforMax EPI300 *E. coli*, and plate as described in Parts C and D. Then, select and size screen the
   clones, and induce them to high-copy number according to the procedure in Part E. Greater than 70% of the white colonies will have the correct control PCR product, with a size of ~9.5 kb, including the vector.

Appendix B

pCC1 (Blunt Cloning-Ready) Vector and Sequencing Primers Data

pCC1/pEpiFOS-5 Sequencing Primers

Primers are available separately:

pCC1™/pEpiFOS™ Forward Sequencing Primer Cat. No. F5FP010
5′ GGATGTGCTGCAAGGCGATTAAGTTGG 3′1 nmol supplied in TE Buffer at 50 µM

pCC1™/pEpiFOS™ Reverse Sequencing Primer Cat. No. F5RP011
5′ CTCGTATGTTGTGTGGAATTGTGAGC 3′1 nmol supplied in TE Buffer at 50 µM

Note: *The pCC1/pEpiFOS Forward and Reverse Primers do not function well as IRD800-labeled sequencing primers. We recommend using the T7 and pCC1/pEpiFOS RP-2 Primers instead of the pCC1/pEpiFOS Forward and Reverse Primers respectively, for this purpose.*

pCC1™/pEpiFOS™ RP-2 Reverse Sequencing Primer

5′ TACGCCAAGCTATTTAGGTGAGA 3′

Orientation for pCC1 (Blunt Cloning-Ready) End-Sequencing

The following is the nucleotide sequence of pCC1 (Blunt Cloning-Ready) Vector (bases 230-501) from the pCC1/pEpi-FOS Forward Sequencing Primer (230-256) to the pCC1/pEpiFOS Reverse Sequencing Primer (501-476) encompassing the T7 RNA polymerase promoter (311-330) and the *Eco*72 I site (359-364).

```
230 GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT TTTCCCAGTC
280 ACGACGTTGT AAAACGACGG CCAGTGAATT GTAATACGAC TCACTATAGG
330 GCGAATTCGA GCTCGGTACC CGGGGATCCC AC - - Cloned Insert
      - - Cloned Insert - - - - - - -   GTGGGATC CTCTAGAGTC
380 GACCTGCAGG CATGCAAGCT TGAGTATTCT ATAGTCTCAC CTAAATAGCT
430 TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC
480 ACAATTCCAC ACAACATACG AG
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An electronic copy of the pCC1 (Blunt Cloning-Ready) Vector sequence can be downloaded at www.epibio.com/sequences.

Restriction Analysis of the pCC1 (Blunt Cloning-Ready) CopyControl Vector

Restriction Enzymes that cut pCC1 (Blunt Cloning-Ready) Vector one to three times:

Enzyme	Sites	Location
Acc65 I	2	344, 5207
Acl I	2	1133, 5599
Afe I	1	4566
Afl II	2	6608, 6848
Afl III	3	4973, 5147, 7482
Age I	3	3827, 5057, 5950
Ahd I	1	7486
Ale I	1	6543
Apa I	1	6972
ApaB I	3	96, 1946, 7646
ApaL I	1	87
BamH I	2	353, 365
Bbs I	3	5050, 5239, 6116
BciV I	1	2497
Bcl I	1	5798
Bgl I	3	651, 3171, 7620
Bgl II	2	3146, 5213
Blp I	1	4479
BmgB I	3	2624, 5037, 7797
Bmr I	3	268, 7018, 7147
Bpu10 I	3	1446, 3927, 5122
Bsa I	1	6810
BsaB I	2	7754, 7838
BsaH I	1	146
BseY I	3	2412, 5890, 6647
Bsm I	2	824, 1231
BsmB I	3	994, 1547, 3942
BspE I	2	1222, 5767
BspLU11 I	1	7482
BsrB I	3	476, 1660, 2282
BsrG I	1	3780
BssH II	2	5464, 6008
BssS I	3	5157, 6807, 7370
BstAP I	3	95, 1945, 7645
BstE II	1	7604
BstX I	1	5085
BstZ17 I	1	1844
Bts I	2	570, 5559
Dra III	2	1945, 7823
Eco47 III	1	4566
Eco72 I	**1**	**361**
EcoN I	1	3469
EcoO109 I	2	1728, 7858
EcoR I	1	332
EcoR V	2	4128, 4357
Fse I	1	2489
Fsp I	3	167, 3752, 7578
Hind III	1	395
Hpa I	1	7629
Kpn I	2	348, 5211
Mfe I	1	4987
Msc I	3	955, 2634, 5418
Nar I	1	146
Nco I	2	917, 7187
Nde I	2	94, 5005
Not I	2	2, 643
Nru I	2	1644, 7674
Nsp I	3	393, 1831, 7486
Pci I	1	7482
PflF I	1	5271
Pml I	1	361
PpuM I	2	1728, 7858
Psi I	2	2926, 3122
PspOM I	1	6968
Pst I	3	387, 4025, 5566
Pvu I	2	188, 5873
Sac II	1	2483
Sal I	3	377, 657, 7662
Sap I	2	4603, 5813
Sbf I	2	387, 4025
Sca I	1	805
SexA I	1	7600
Sfi I	1	651
Sfo I	1	147
SgrA I	3	2492, 5057, 6214
Sim I	2	5171, 7858
Sma I	3	350, 651, 3493
SnaB I	1	5631
Spe I	1	6722
Sph I	1	393
Srf I	1	651
Sse8647 I	1	1728
Stu I	1	3174
Tat I	3	77, 803, 3780
Tth111 I	1	5271
Xba I	2	371, 3192
Xcm I	1	2687
Xma I	3	348, 649, 3491

Restriction Enzymes that cut pCC1 (Blunt Cloning-Ready) Vector four or more times:

Acc I	BsmA I	Dsa I	HpyCH4 V	PspG I
Aci I	Bsp1286 I	Eae I	Mae II	Pvu II
Alu I	BspH I	Eag I	Mae III	Rsa I
Alw I	BspM I	Ear I	Mbo I	Sac I
AlwN I	Bsr I	Fau I	Mbo II	Sau3A I
Apo I	BsrD I	Fnu4H I	Mly I	Sau96 I
Ase I	BsrF I	Gdi II	Mnl I	ScrF I
Ava I	BssK I	Hae I	Mse I	SfaN I
Ava II	BstDS I	Hae II	Msl I	Sfc I
Ban I	BstF5 I	Hae III	Msp I	Sml I
Ban II	BstN I	Hha I	MspA1 I	Ssp I
Bfa I	BstU I	Hinc II	Mwo I	Sty I
BfuA I	BstY I	Hinf I	Nae I	Taq I
Bme1580 I	Btg I	HinP I	Nci I	Tfi I
BsaA I	Cac8 I	Hpa II	NgoM IV	Tse I
BsaJ I	CviJ I	Hph I	Nla III	Tsp45 I
BsaW I	Dde I	Hpy188 I	Nla IV	Tsp4C I
BsiE I	Dpn I	Hpy99 I	PflM I	Tsp509 I
BsiHKA I	Dra I	HpyCH4 III	Ple I	TspR I
Bsl I	Drd I	HpyCH4 IV	PshA I	Xmn I

Restriction Enzymes that do not cut pCC1 (Blunt Cloning-Ready) Vector:

Aat II	BfrB I	Cla I	PaeR7 I	Tli I
Asc I	BsiW I	Mlu I	Pme I	Xho I
AsiS I	BspD I	Nhe I	Rsr II	
Avr II	BstB I	Nsi I	SanD I	
BbvC I	Bsu36 I	Pac I	Swa I	

6. References

1. Hradecna, Z., Wild, J., and Szybalski, W. (1998) *Microbial. And Comp. Genomics* **3**, 58.
2. Wild, J., Hradecna, Z., and Szybalski, W. (2001) *Plasmid* **45**,142.
3. Wild, J. *et al*., (2002) *Genome Research* **12**, 1434.

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