epicentre®
an Illumina® company

EZ-Tn5™ pMOD™<R6Kγ*ori*/MCS> Transposon Construction Vectors

Cat. No. MOD1503

*,† See notes page 16.

Connect with Epicentre on our blog **(epicentral.blogspot.com)**,
Facebook **(facebook.com/EpicentreBio)**, and Twitter **(@EpicentreBio)**.

1. Introduction

The EZ-Tn5™ pMOD™<R6Kγ*ori*/MCS> Transposon Construction Vector* was developed for the preparation of custom EZ-Tn5 Transposons that can also be used for rescue cloning. The vector contains a multiple cloning site (MCS) and an *E. coli* conditional origin of replication (R6Kγ*ori*) flanked by the hyperactive 19-bp Mosaic Ends (ME) that are specifically recognized by EZ-Tn5 Transposase. Also included between the ME's are primer binding sites for bidirectional sequencing from any custom EZ-Tn5 Transposon. To prepare a transposon, clone any DNA sequence of interest into the MCS and then generate the transposon either by PCR amplification using the Forward and Reverse PCR Primers provided with the vector, or restriction enzyme digestion.

A custom EZ-Tn5 Transposon can be incubated with EZ-Tn5 Transposase in the absence of Mg^{2+} to form an EZ-Tn5 Transposome™ for random insertion into the genomic DNA of living cells.† The presence of an origin of replication enables you to propagate or rescue the region of genomic DNA into which the transposon has been inserted. A custom EZ-Tn5 Transposon can also be used for insertion into any target DNA *in vitro**. *In vitro* transposition of R6Kγ*ori* containing transposons can be used, for example, to rescue plasmids which ordinarily do not replicate in *E. coli* because they lack a recognizable origin of replication and/or a selectable marker.

The EZ-Tn5 pMOD-3<R6Kγ*ori*/MCS> Transposon Construction Vector contains a *colE*1 origin of replication outside the MCS (Fig. 4). This vector works well for constructing transposons in most cases. However, if the transposon is prepared by restriction enzyme digestion, there is a chance that the uncut pMOD vector will interfere with downstream applications. Replication from the R6Kγ origin is dependent on the *pir* gene product produced by TransforMax™ EC100D™ *pir*$^+$ and *pir*-116 *E. coli* cells which are available separately. Since most bacterial strains do not contain a *pir* gene, the uncut plasmid DNA that contaminates these transposon preparations cannot replicate and background problems are eliminated.

2. Product Specifications

Storage: Store at –20°C.

Quality Control:

The EZ-Tn5 pMOD<R6Kγ*ori*/MCS> Transposon Construction Vector is function tested for:

a) The presence of the MCS, and transposon-liberating, restriction sites;
b) PCR amplification of the vector with both pMOD Forward and Reverse PCR primers;
c) ME recognition by the EZ-Tn5 Transposase enzyme.

3. Kit Contents

Desc.	Concentration	Quantity
MOD1503		
pMOD™-3<R6Kγ*ori*/MCS> Vector	@ 1 µg/µl	20 µg
pMOD™<MCS> Forward PCR Primer	@ 50 µM	1 nmol
pMOD™<MCS> Reverse PCR Primer	@ 50 µM	1 nmol

4. Related Products

The following products are also available:

- TransforMax™ EC100D™ *pir*$^{+}$ Electrocompetent *E. coli*
- TransforMax™ EC100D™ *pir*-116 Electrocompetent *E. coli*
- EZ-Tn5™ pMOD™-2<MCS> Transposon Construction Vector
- APex™ Heat-Labile Alkaline Phosphatase
- Fast-Link™ DNA Ligation Kits
- T4 DNA Ligase
- Colony Fast-Screen™ Kits
- MasterPure™ Nucleic Acid Purification Kits
- End-It™ DNA End-Repair Kit
- EZ-Tn5™ Custom Transposome Construction Kits

5. Protocols

I. Cloning into an EZ-Tn5 Transposon Construction Vector

Creating a custom EZ-Tn5 Transposon requires that you clone your DNA fragment of interest into the MCS of the pMOD Vector. A map of the MCS and sequencing information are provided later in this document to assist in development of a successful cloning strategy.

Please consult a general molecular biology reference [e.g., Maniatis, T., *et al.*, (1982) *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.] for recommendations on restriction digests, dephosphorylation of vector and ligations. Epicentre offers the Fast-Link™ DNA Ligation and Screening Kit for efficient ligation and recombinant screening, APex™ Heat-Labile Alkaline Phosphatase for dephosphorylation of DNA and GELase™ Agarose Gel-Digesting Preparation for recovery of DNA from agarose.

Transform ligation mixtures into a competent bacterial strain and select on media containing 50-100 µg/ml ampicillin or other selective reagents dictated by the transposon insert. Use of a *rec*A^{-}, *end*A^{-} strain is preferable, for target stability and subsequent purification steps (e.g., Epicentre's TransforMax EC100™ Electrocompetent *E. coli**), but not absolutely necessary.

2. Isolation of a Custom EZ-Tn5 Transposon

A functional EZ-Tn5 Transposon can be isolated either by restriction enzyme digestion or PCR amplification.

A. Restriction enzyme digestion with *Pvu* II or *Psh*A I:

Note: *The cloned insert must not contain a recognition site(s) for the restriction enzyme chosen to liberate the EZ-Tn5 Transposon.*

1. Digest the recombinant pMOD<R6K*γori*/MCS> DNA with either *Pvu* II or *Psh*A I using conditions recommended by the enzyme supplier.
2. Heat-inactivate the enzyme (if applicable) by incubating at 70°C for 10 minutes.
3. To ensure that false positives are not generated from uncut, parental plasmid DNA, minimize this type of background by purifying the transposon following gel electrophoresis.

B. PCR Amplification:

Method 1 – Using the pMOD standard PCR Primers:

1. Amplify the transposon region using the pMOD<MCS> Forward and Reverse PCR Primers provided with the vector. A suggested cycling profile is outlined below.

   a. Initially, denature the template at 94°C for 2 minutes.

   b. Perform 30 cycles of:

   Denature at 94°C for 30 seconds.

   Anneal at 60°C for 45 seconds.

   Extend at 72°C for 1 minute for every kb of expected product.
2. We recommend PEG precipitation to remove small molecules (e.g., primers, nucleotides) that may interfere with transposition. Alternatively, a standard ethanol precipitation can be used.
   a. Dilute the PCR reaction to 500 µl with TE.
   b. Add 250 µl of 5 M NaCl and 250 µl of 30% PEG 8000/1.5 M NaCl.
   c. Mix well and incubate at 4°C for at least 30 minutes.
   d. Centrifuge at 4°C for 10 minutes at 10,000 x g. Discard the supernatant, centrifuge again for a few seconds, and discard any remaining supernatant.
   e. Dissolve the DNA in a suitable amount of TE.
3. Digest the PCR product with *Pvu*II or *Psh*A I restriction enzymes as described in Section 2-A.
4. Purify the PCR product using standard cleanup methods (spin column or gel purification).

Method 2 – Using the "ME Plus 9" PCR Primers (NOT Supplied):

1. Perform PCR using primers which incorporate the reverse complement of the Mosaic Element. We use the reverse complement of the ME plus 9 bases. It is VERY important that the ME Plus 9 Forward and Reverse Primers are 5'-phosphorylated for maximal transposition efficiency. If the primers are not phosphorylated you can easily add the 5-phosphate groups using T4 Polynucleotide Kinase.

Sequences of the two primers to use:

ME Plus 9 – 3′ primer

5′-CTGTCTCTTATACACATCTCAACCATCA-3′

ME Plus 9 – 5′ primer

5′-CTGTCTCTTATACACATCTCAACCCTGA-3′

Cycling protocol is:

a. Initially, denature the template at 94°C for 1 minute.
b. Perform 25-30 cycles of:
   Denature at 94°C for 30 seconds.
   Anneal at 55°C for 1 minute.
   Extend at 72°C for X minutes (one minute per kb PCR product)

Regarding buffer choice, each template will be different. You will want to use a high fidelity enzyme. Epicentre's FailSafe PCR System enzyme is an excellent choice as it will give you 3X the fidelity of Taq Polymerase but does not have the processivity issues of many pure proofreaders. We strongly recommend to use the FailSafe PCR Selection Kit, use all 12 of the FailSafe 2X PCR PreMixes, and find out the best one to use for each template. Alternatively, a pure proofreader, such as *Pfu* or Phusion® Polymerase, can be used.

After PCR, transposon cleanup may be performed using the methods in Part B, Step 2 or Step 4.

3. *In Vitro* Transposon Insertion Reaction

This reaction inserts an EZ-Tn5 Transposon into target DNA, *in vitro*. The target DNA should be free of contaminating chromosomal DNA which is a direct competitor of the target DNA for insertion. Reaction conditions given have been optimized to maximize transposition frequency while minimizing multiple insertion events. Be sure to calculate the moles of target DNA used in the reaction and add an equimolar amount of the EZ-Tn5 Transposon.

1. Prepare the transposon insertion reaction mixture by adding in the following order:

1	µl	EZ-Tn5 10X Reaction Buffer (see Note below)
0.2	µg	target DNA**(see below)
x	µl	molar equivalent EZ-Tn5 Transposon
x	µl	sterile water to a reaction volume of 9 µl
1	µl	EZ-Tn5 Transposase (Available in the EZ-Tn5 Custom Transposome Construction Kits)
10	µl	Total reaction volume

2. Incubate the reaction mixture for 2 hours at 37°C.
3. Stop the reaction by adding 1 µl EZ-Tn5 10X Stop Solution.
   Mix and heat for 10 minutes at 70°C.

4. Use 1 µl for electroporation into the appropriate bacterial strain and plate on selective media as dictated by the transposon insert. Use of a *rec*A⁻, *end*A⁻ strain is preferable but not absolutely necessary. R6Kγ*ori*-dependent replication requires the *pir* gene product produced by TransforMax EC100D *pir*⁺ and *pir*-116 *E. coli* cells (sold separately). Store unused reaction mixture at –20°C.

The actual number of EZ-Tn5 <R6Kγ*ori*/MCS>-based insertion clones obtained will vary depending on factors such as target DNA size and the transformation efficiency of the competent cells used to recover the transposon insertion clones.

** Calculation of µmol target DNA:

µmol target DNA = µg target DNA / [(# base pairs in target DNA) x 660]

For example: 0.2 µg of a 6,100-bp target clone
= 0.2 µg / [6,100 bp x 660] = 0.05 x10^{-6} µmol = 0.05 pmol

Note: *EZ-Tn5 10X Reaction Buffer (supplied with the EZ-Tn5 Transposase) is composed of 0.5 M Tris-acetate (pH 7.5), 1.5 M potassium acetate, 100 mM magnesium acetate, and 40 mM spermidine.*

4. Production of EZ-Tn5 Transposomes

Production of stable EZ-Tn5 Transposomes can only be accomplished in the absence of Mg^{2+}.

Do not use the EZ-Tn5 10X Reaction Buffer provided with the EZ-Tn5 Transposase to prepare EZ-Tn5 Transposomes.

1. Prepare the transposome reaction mixture by adding in the following order:‡

2	µl	EZ-Tn5 Transposon DNA (100 µg/ml in TE Buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA])
4	µl	EZ-Tn5 Transposase (Available in the EZ-Tn5 Custom Transposome Construction Kits)
2	µl	100% glycerol
8	µl	Total reaction volume

2. Mix by vortexing. Incubate for 30 minutes at room temperature.

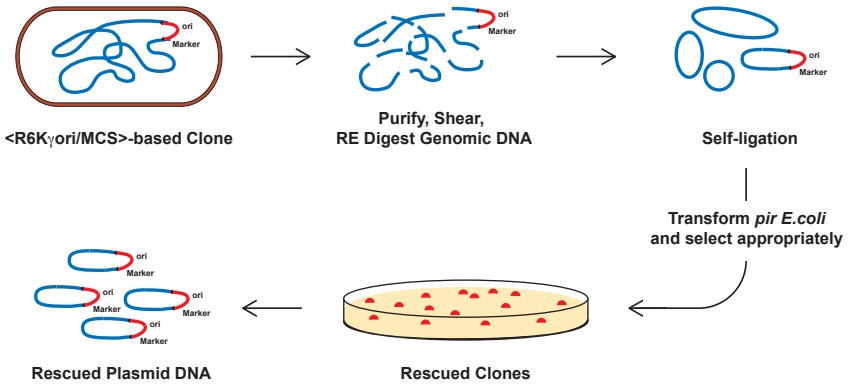


Figure 1. Rescue Cloning Overview.

3. Store the solution at –20°C.

   The solution will not freeze stored at –20°C and is stable for at least one year.

4. Electroporate electrocompetent cells using 1 µl of the EZ-Tn5 <R6Kγ*ori*/MCS>-based Tnp Transposome. Use cells of the highest transformation efficiency possible (at least >10^7 cfu/µg of DNA) to maximize the number of transposon insertion clones. Perform electroporation according to the equipment manufacturer's recommendations and plate on selective media as dictated by the transposon insert.

   ‡*The EZ-Tn5 Transposome production protocol can be scaled up or down as needed.*

5. Rescue Cloning of EZ-Tn5™ <R6Kγori/MCS>-based Transposed Genomic DNA

An overview of the process for rescue cloning of the EZ-Tn5 <R6Kγ*ori*/MCS>-based Transposon insertion site in genomic DNA is given below. The protocol is presented on the next page.

Figure 2. EZ-Tn5™ Transposon Insertion Site Junction.

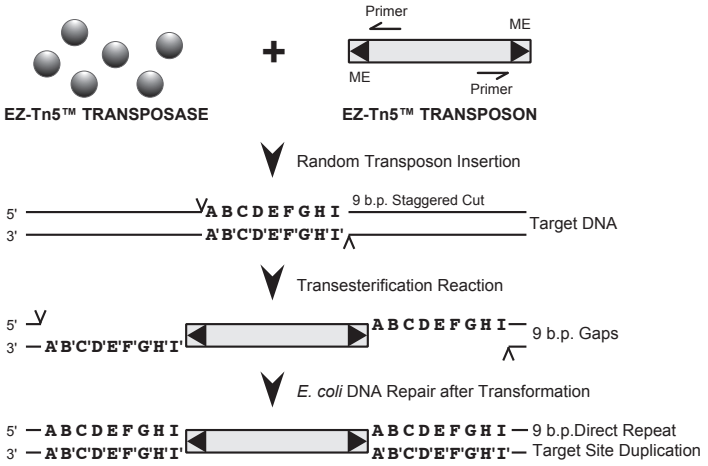


Figure 3. EZ-Tn5™ Transposase Insertion Site Duplication Process.

Protocol

1. **Preparation of Transposed Genomic DNA from Host Cells:** Prepare genomic DNA from chosen clones, for example, using the MasterPure™ DNA Purification Kit. Fragment 1 µg of the genomic DNA by random shearing or by restriction endonuclease digestion(s) (Choose restriction enzymes which do not cut within the transposon). If desired, size-select the fragmented genomic DNA (e.g., by low-melting point agarose gel electrophoresis). Genomic DNA that has been fragmented by random shearing or by digestion with two different restriction endonucleases must be end-repaired (made blunt-ended) and 5′ phosphorylated in order to be self-ligated. End-repair and 5′ phosphorylate the DNA as necessary (e.g., by using the End-It™ DNA End-Repair Kit).
2. **Ligation of Fragmented Genomic DNA:** Self-ligate 0.1-1 µg of DNA using 2 U of T4 DNA Ligase in 10-20 µl total volume for 1 hour at room temperature. The extent of ligation can be quickly monitored by running aliquots of the reaction before and after addition of the T4 DNA Ligase addition, on an agarose gel. Terminate the reaction and inactivate the T4 DNA Ligase by heating at 70°C for 10 minutes.
3. **Transformation and Selection of Rescue Clones:** Electroporate electrocompetent *pir E. coli* (*E. coli* expressing the Π protein, e.g. TransforMax EC100D *pir*$^+$ or TransforMax EC100D *pir*-116 Electrocompetent *E. coli*) using 1-2 µl of the ligation mix, and recover the electroporated cells as per the manufacturer's recommendations. Plate cells on selective media as dictated by the transposon insert.

6. DNA Sequencing of Transposon Insertion Clones

Information on the Forward and Reverse Sequencing Primers, is available separately (see Primer Information). Since these primers anneal to a region near the ends of the transposon, the first sequence data obtained from each sequencing reaction is that of transposon DNA. The 19-bp EZ-Tn5 Transposase recognition sequence (ME) found at the junction of the inserted transposon and the target DNA is a useful landmark for distinguishing transposon sequence from target sequence (see Fig. 2).

EZ-Tn5 Transposase-catalyzed transposon insertion results in the generation of a 9-bp target site sequence duplication where one copy immediately flanks each side of the inserted transposon. This is important to consider when assembling the nucleotide sequence of a recombinant clone insert. The process of transposon insertion site duplication is depicted in Fig. 3.

6. Primer Information

pMOD<MCS> Forward PCR Primer

5′ - ATTCAGGCTGCGCAACTGT - 3′

Storage: Store at –20°C.

Concentration: 1 nmol @ 50 µM ..... 20 µl

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

Length: 19 nucleotides

G+C content: 10

Molecular Weight: 5,786 daltons

Temperatures of Dissociation & Melting:

T_d: 66°C (nearest neighbor method)

T_m: 68°C (% G+C method)

T_m: 58°C ([2 (A+T) + 4 (G+C)] method)

T_m: 60°C ((81.5 + 16.6 (log [Na^+])) +
([41 (#G+C) - 500] / length) method)
where [Na^+] = 0.1 M

pMOD<MCS> Reverse PCR Primer

5′ - GTCAGTGAGCGAGGAAGCGGAAG - 3′

Storage: Store at –20°C.

Concentration: 1 nmol @ 50 µM ..... 20 µl

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

Length: 23 nucleotides

G+C content: 14

Molecular Weight: 7,206 daltons

Temperatures of Dissociation & Melting:

T_d: 74°C (nearest neighbor method)

T_m: 77°C (% G+C method)

T_m: 74°C ([2 (A+T) + 4 (G+C)] method)

T_m: 68°C ((81.5 + 16.6 (log [Na^+])) +
([41 (#G+C) - 500] / length) method)
where [Na^+] = 0.1 M

pMOD<MCS> Forward Sequencing Primer

5′ - GCCAACGACTACGCACTAGCCAAC - 3′

Storage: Store at –20°C.

Concentration: 1 nmol @ 50 µM ..... 20 µl

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

Length: 24 nucleotides

G+C content: 14

Molecular Weight: 7,328 daltons

Temperatures of Dissociation & Melting:

T_d: 74°C (nearest neighbor method)

T_m: 77°C (% G+C method)

T_m: 76°C ([2 (A+T) + 4 (G+C)] method)

T_m: 68°C ((81.5 + 16.6 (log $[Na^+]$)) +
([41 (#G+C) - 500] / length) method)
where $[Na^+]$ = 0.1 M

pMOD<MCS> Reverse Sequencing Primer

5′ - GAGCCAATATGCGAGAACACCCGAGAA - 3′

Storage: Store at –20°C.

Concentration: 1 nmol @ 50 µM ..... 20 µl

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

Length: 27 nucleotides

G+C content: 14

Molecular Weight: 8,294 daltons

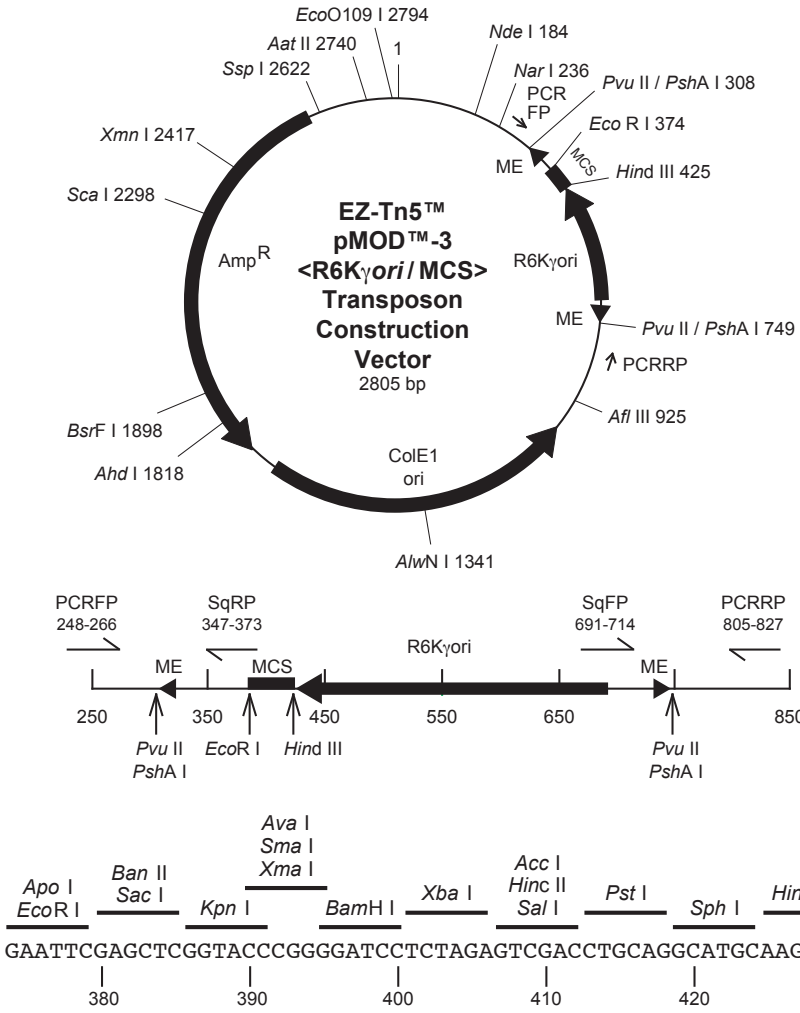
Temperatures of Dissociation & Melting:

T_d: 79°C (nearest neighbor method)

T_m: 78°C (% G+C method)

T_m: 82°C ([2 (A+T) + 4 (G+C)] method)

T_m: 68°C ((81.5 + 16.6 (log $[Na^+]$)) +
([41 (#G+C) - 500] / length) method)
where $[Na^+]$ = 0.1 M



SqFP = pMOD™<MCS> Foward Sequencing Primer 5' GCCAACGACTACGCACTAGCCAAC 3'
SqRP = pMOD™<MCS> Reverse Sequencing Primer 5' GAGCCAATATGCGAGAACACCCGAGAA 3'
PCRFP = pMOD™<MCS> Forward PCR Primer 5' ATTCAGGCTGCGCAACTGT 3'
PCRRP = pMOD™<MCS> Reverse PCR Primer 5' GTCAGTGAGCGAGGAAGCGGAAG 3'
ME = Mosaic End 5' AGATGTGTATAAGAGACAG 3'
MCS = Multiple Cloning Site

Figure 4. pMOD™-3<R6Kγ*ori*/MCS> Transposon Construction Vector.

The pMOD-3<R6Kγ*ori*/MCS> Transposon Construction Vector 2,805-bp sequence can be downloaded at http://www.epicentre.com/sequences.

Restriction Enzymes that cut the pMOD-3<R6Kγ*ori*/MCS> Transposon Construction Vector one to three times:

Enzyme	Sites	Location	Enzyme	Sites	Location
Aat II	1	2740	Drd I	2	97, 1033
Acc65 I	1	386	Eae I	2	764, 2206
Acc I	1	408	Ear I	3	296, 809, 2613
Acl I	2	2044, 2417	EcoO109 I	1	2794
Afl III	1	925	EcoR I	1	374
Ahd I	1	1818	Fsp I	2	258, 2040
AlwN I	1	1341	Gdi II	2	774, 2204
ApaB I	1	186	Hae I	3	940, 951, 1403
ApaL I	3	177, 1239, 2485	Hae II	3	239, 803, 1173
Apo I	2	342, 374	Hinc II	1	409
Ase I	2	504, 1990	Hind III	1	425
Ava I	2	349, 390	Hpy99 I	3	1030, 1824, 2087
Ava II	2	1956, 2178	Kpn I	1	390
BamH I	1	395	Msl I	3	2070, 2229, 2588
Ban I	3	235, 386, 1766	Nar I	1	236
Ban II	1	384	Nde I	1	184
BciV I	2	1128, 2655	Nsp I	3	41, 423, 929
BfuA I	1	420	Pci I	1	925
Bgl I	2	251, 1938	**PshA I**	**2**	**308, 749**
Bme1580 I	3	181, 1243, 2489	Psi I	1	460
Bmr I	1	1858	PspG I	3	951, 1072, 1085
Bsa I	1	1879	Pst I	1	417
BsaA I	3	537, 647, 669	Pvu I	2	279, 2188
BsaH I	3	236, 2355, 2737	**Pvu II**	**2**	**308, 749**
BsaJ I	3	390, 391, 1085	Sac I	1	384
BsaW I	3	1131, 1278, 2109	Sal I	1	407
BseY I	1	1229	Sap I	1	809
BsmB I	1	45	Sbf I	1	417
BspD I	1	337	Sca I	1	2298
BspH I	3	1645, 2653, 2758	Sfo I	1	237
BspLU11 I	1	925	Sim I	3	1117, 1600, 1886
BspM I	1	420	Sma I	1	392
Bsr I	3	1333, 1346, 2294	SnaB I	1	669
BsrB I	2	858, 2659	Sph I	1	423
BsrD I	2	1879, 2053	Ssp I	1	2622
BsrF I	1	1898	Tat I	2	167, 2296
BssS I	3	1098, 2482, 2789	Tfi I	2	760, 900
BstAP I	1	185	Tsp45 I	3	56, 2074, 2285
BstN I	3	953, 1074, 1087	Xba I	1	401
Bts I	2	2218, 2238	Xma I	1	390
Cla I	1	337	Xmn I	1	2417

Restriction Enzymes that cut the pMOD-3<R6Kγ*ori*/MCS> Transposon Construction Vector four or more times:

Aci I	BstU I	HinP I	Mnl I	ScrF I
Alu I	BstY I	Hpa II	Mse I	SfaN I
Alw I	Cac8 I	Hph I	Msp I	Sfc I
Bfa I	CviJ I	Hpy188 I	MspA1 I	Sml I
BsiE I	Dde I	HpyCH4 III	Mwo I	Taq I
BsiHKA I	Dpn I	HpyCH4 IV	Nci I	Tse I
Bsl I	Dra I	HpyCH4 V	Nla III	Tsp4C I
BsmA I	Fau I	Mae II	Nla IV	Tsp509 I
Bsp1286 I	Fnu4H I	Mae III	Ple I	TspR I
Bsr I	Hae III	Mbo I	Rsa I	
BssK I	Hha I	Mbo II	Sau3A I	
BstF5 I	Hinf I	Mly I	Sau96 I	

Restriction Enzymes that do not cut the pMOD-3<R6Kγ*ori*/MCS> Transposon Construction Vector:

Afe I	Bpu10 I	Dsa I	Nru I	SgrA I
Afl II	BsaB I	Eag I	Nsi I	Spe I
Age I	BsiW I	Eco47 III	Pac I	Srf I
Ale I	Bsm I	EcoN I	PaeR7 I	Sse8647 I
Apa I	BspE I	EcoR V	PflF I	Stu I
Asc I	BsrG I	Fse I	PflM I	Sty I
AsiS I	BssH II	Hpa I	Pme I	Swa I
Avr II	BstB I	Mfe I	Pml I	Tli I
Bbs I	BstDS I	Mlu I	PpuM I	Tth111 I
BbvC I	BstE II	Msc I	PspOM I	Xcm I
Bcl I	BstX I	Nae I	Rsr II	Xho I
BfrB I	BstZ17 I	Nco I	Sac II	
Bgl II	Bsu36 I	NgoM IV	SanD I	
Blp I	Btg I	Nhe I	SexA I	
BmgB I	Dra III	Not I	Sfi I	

7. Reference

1. York, D. *et al.*, (1998) *Nucl. Acids Res.* **26**, 1927.

Visit our technical blog: **epicentral.blogspot.com**