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1 Construction of pDEST_R, a GATEWAY vector for gene disruption in filamentous fungi

2 For page heading: pDEST_R, a fungal gene disruption vector

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1 **Abstract** We have constructed pDEST_R, a destination vector of Gateway system especially
2 for gene targeting and disruption in filamentous fungi. The vector was constructed by
3 removing multicloning site of pGEM-T easy vector, and inserting hygromycin
4 phosphotransferase gene construct from pCB1004, and a Gateway vector conversion cassette.
5 In order to construct a DNA for gene disruption, only an inverse-PCR amplification of the
6 restricted, target sequence is needed. After the amplification with a 5'CACC-tagged primer
7 and an ordinary primer, the DNA fragment will be inserted into pENTR/D-TOPO vector and
8 then transferred into pDEST_R through LR-recombination reaction. The resulting vector has
9 the disruption construct, after being digested with the restriction enzyme used for the
10 inverse-PCR. The effectiveness of this vector was assessed in *Neurospora crassa*. The use of
11 pDEST_R will therefore simplify the construction of a targeting vector, where multiple ligation
12 steps are usually needed.

13

14 Key words: GATEWAY, Gene targeting/disruption, *Neurospora*, *Magnaporthe*, Hygromycin
15 B resistance

16

17 Recently, the number of filamentous fungi whose genome sequence is revealed is increasing
18 [3]. However, there are many hypothetical genes, i. e. “gene-like” sequences which shows
19 similarity with other known genes of *Saccharomyces* or other organisms or whose functions
20 are not clear. In order to know the function of any interesting hypothetical protein encoding
21 genes, gene disruption is inevitable [3].

22 In the gene disruption analyses in fungi, gene disruption construct which carries
23 substitution of (or a part of) the gene of interest with a marker gene is usually used. Double
24 crossing-over event will substitute the targeted gene with the disruption construct, and disrupt
25 the gene of interest. In order to obtain the disruption construct, several methods are available.

1 One of those, PCR-aided construction [12, 13,14] is a rapid and efficient method but not
2 always effective especially when the recombination frequency of the host organism is low,
3 because PCR will limit the length of homologous DNA region and the supply of the DNA.
4 Transposon-arrayed gene knockouts (TAGKO) is very effective when the DNA of interest is
5 inserted in a cosmid vector with the transposon [2]. But there is a bias of the integration
6 frequency throughout the cosmid insert, so one must screen the appropriate transposon-tagged
7 clone prior to use for the gene knockout experiment [2]. Inserting a marker gene into the gene
8 of interest by ordinary recombinant DNA technique is the most popular and reliable method.
9 However, it usually needs several ligation steps and is actually time consuming, especially
10 when many knockout experiments are intended.

11 Ligation with DNA ligase has become the second choice as the method to insert
12 DNA fragments into vectors. TOPO cloning and Gateway system are examples of alternatives
13 for ligation. The topoisomerase I from *Vaccinia* virus covalently bound to the 3' ends of the
14 vector ligates the DNA ends of the vector and the insert, with higher efficiency than
15 bacteriophage T4 DNA ligase. Gateway technology is a DNA strand exchanging system based
16 on the well-characterized lambda phage site-specific recombination system. This technology
17 enables us to make multiple destination vectors for different purposes such as expression in *E.*
18 *coli* or *S. cerevisiae*, from a single entry vector with inserted gene of interest. This technology
19 was already introduced to fungal reporter vector [10], and thus can be facilitated in the system
20 of fungal gene knockout.

21 In this paper, we attempted to construct a new destination vector which is specified
22 to be used in fungal gene disruption. The vector will carry the inverse PCR-amplified
23 fragment of the gene of interest, and will be cut with appropriate restriction enzyme prior to
24 use.

25

1 **Materials and methods**

2 **Bacterial and fungal strains.** *Escherichia coli* JM109 and TOP10 (Invitrogen, Carlsbad, CA)
3 was used for general DNA manipulation. *E. coli* DB3.1 was purchased from Invitrogen and
4 used for propagation of plasmid which contains *ccdB* gene. *Neurospora crassa* 74-OR8-1a
5 (FGSC988) and *fr* (FCSC102) were obtained from Fungal Genetics Stock Center (FGSC).
6 These strains were maintained on Vogel's 2% glucose agar.

7 **Vectors and DNAs.** pGEM-T easy (Promega, Madison, WI) was self-ligated, and extracted
8 from *E. coli* JM 109 culture prior to use. Plasmid pCB1004 [1] was obtained from FGSC.
9 pENTR/D-TOPO cloning system and Gateway vector conversion kit was purchased from
10 Invitrogen Corp., Carlsbad, CA. Oligonucleotide primers were synthesized by Date Concept
11 Co. Ltd, Sapporo, Japan.

12 **DNA manipulation.** All plasmids were extracted using Quantum prep plasmid extraction kit
13 (BIO-RAD, Hercules, CA). Restriction enzymes were purchased from Takara Bio Ohtsu,
14 Japan. Blunting and kination reactions was performed with Takara BKL kit (Takara Bio,
15 Ohtsu, Japan). For the ligation, T4 DNA ligase (New England Biolabs, Inc. Beverly, MA) was
16 used. TOPO cloning of PCR fragment into pENTR/D-TOPO and LR clonase (Invitrogen,
17 Corp., Carlsbad, CA) reactions were done following manufacturer's instructions.

18 **PCR and sequencing.** For the amplification of pGEM-Hyg, KOD-plus (Toyobo, Osaka,
19 Japan) was used. Expand long template PCR system (Roche Diagnostics, Penzberg,
20 Germany) was used for inverse PCR. All sequencing reaction was done with BigDye
21 Terminator v1.1 cycle sequencing kit and analyzed by ABI PRISM 310 Genetic Analyser
22 (Applied Biosystems, Foster City, CA). The nucleotide sequence of pDESTR is available
23 from DDBJ/EMBL/Genbank database under accession no. AB218275.

24 **Transformation of *N. crassa*.** *N. crassa* was transformed using electroporation, according to
25 the method described by Ninomiya et al. [6], with some modifications. Forty µl of conidia

1 suspension (10^8 / ml) and DNA (10 μ g) were mixed and placed in an electroporation cuvette
2 with 0.2 cm gap (BIO-RAD, Hercules, CA). Electroporation was performed by a charging
3 voltage of 2.0 kV, a resistance of 800 ohms, and a capacitance of 25 μ F, using a Gene Pulser
4 apparatus (BIO-RAD, Hercules, CA). After the electroporation, cells were transferred into 1
5 ml of Vogel's 1.5% glucose liquid medium, and incubated for 2 hrs at 30 °C with gentle
6 shaking. 200 μ l of the culture was then inoculated onto a basal agar plate (Vogel's 2% sorbose,
7 with 0.05% glucose and 0.05% fructose) containing 500 μ g/ml hygromycin B.

8 **Mutant analysis.** Genomic DNA of transformant (2 μ g) was extracted with the method by
9 Sone et al. [8]. Southern hybridization was performed using Alkphos direct nucleic acid
10 labeling and detection system (Amersham Biosciences, Piscataway, NJ). Microscopic
11 observation was done with Olympus BX 50 microscope equipped with DP 50 digital camera
12 (Olympus, Tokyo Japan).

13

14 **Results and Discussion**

15 **Basic concept of vector designing.** Generally, vectors for gene disruption is constructed by
16 inserting a selective marker gene such as drug resistant genes into the center part of the gene
17 to be disrupted. Alternatively, this assembly can be achieved by ligating inverse PCR product
18 of a restriction fragment including the gene of interest into a vector with a selective marker,
19 and digestion with the same restriction enzyme prior to use for transformation (Fig. 1). This
20 alternative method for construction has some advantages comparing with the conventional
21 method of ligating a marker gene into cloned fragment: 1) Cloning of the flanking region is
22 not necessary, even if the information of the sequence of the region is unknown. Southern
23 analysis of the gene of interest is enough to select a suitable restriction enzyme for the
24 fragmentation of the flanking region around the gene, and a little sequence information of the
25 gene of the interest is enough to design a pair of inverse primers. 2) Only one ligation step is

1 necessary to construct a vector. For this method, however, some important features are
2 required for the vector: 1) The number of restriction sites should be limited. In the final step
3 prior to transformation of target organism, the vector must be linearized by the restriction
4 enzyme used during fragmentation for inverse-PCR. Restriction sites already present in the
5 vector will affect the availability of the number of candidate restriction enzymes to be used in
6 the inverse PCR step. 2) Efficient ligation of the inverse PCR fragment into the vector is ideal
7 for universal usage.

8 In order to achieve the necessary conditions above, a strategy of construction was
9 made (Fig. 2). For the basic plasmid structure, pGEM-T easy was used. This may be
10 substituted by other pUC-related vectors such as pUC, pBluescript and other pGEM vectors.
11 First, by digestion with *Pvu* II, MCS (multi-cloning sites) were removed. This effectively
12 reduced the number of restriction sites included in the plasmid. On the other hand, the *hph*
13 gene for hygromycin resistance was amplified from pCB1004, with primers HygF
14 (5'-CCGTGGAGGTAATAATTGA-3') and HygR (5'-CGAAGAACGTTTTCCAATGA-3').
15 The cassette was the modified version of that in pCSN43, with the reduced number of
16 restriction sites [1]. The amplified, blunted-kinated *hph* cassette was ligated into the blunt *Pvu*
17 II site in the pGEM-T easy/ *Pvu* II longer fragment to be named as pGEM-Hyg. A pair of
18 primers (DESTR1, 5'- GATCGGTGCGGGCCTCTTCG-3' and DESTR2, 5'-
19 GTTGCGCAGCCTGAATGGCG-3') was designed to amplify the almost full length of the
20 pGEM-Hyg, and the amplified DNA was blunted, kinated and ligated with the Gateway
21 cassette rfb in the Gateway vector conversion kit, to become pDESTR, the final product. The
22 vector was checked for the ability to transform *N. crassa* wild type strain and *Magnaporthe*
23 *grisea* Ina168 strain to hygromycin resistant transformants (data not shown).

24 For the cloning of the inverse PCR fragment, Gateway system is to be utilized, i.e.
25 inverse PCR fragment will be first ligated into entry vector, such as pENTR/D-TOPO or

1 pCR8/GW/TOPO. For pENTR/D-TOPO, inverse PCR should be done with proofreading
2 enzyme and a pair of primers, one of which with CACC attached at its 5'-end. For
3 pCR8/GW/TOPO, inverse PCR should be performed with non-proofreading enzyme with any
4 primer pair, but *EcoRI* restriction sites at both sides of the cloning site eliminate the
5 possibility to use *EcoRI* as the enzyme the inverse PCR. The constructed entry vector will be
6 used for LR-recombination reaction with pDEST, and the inverted fragment will be
7 exchanged with chloramphenicol resistant gene - *ccdB* cassette. Finally, disruption construct
8 will be obtained by digesting the resulting plasmid DNA with the restriction enzyme used in
9 the inverse PCR.

10 Restriction enzymes suitable for the inverse PCR-based gene disruption with
11 pDEST are listed in Table 1. Most of enzymes present in MCS of most cloning vectors are
12 included. This will help the users to select an appropriate restriction enzyme for the inverse
13 PCR, and is also economically reasonable. In the case one cannot find natural restriction sites
14 flanking the gene of interest, preliminary PCR with a pair of primers with a restriction site of
15 choice at 5' end of each primer can be applied to obtain the inverse PCR product of the gene
16 of interest.

17

18 **Disruption of *Frost* gene of *Neurospora crassa* using pDEST system.** As a model
19 experiment, *Frost* gene of *N. crassa* was disrupted using pDEST (Fig. 3A). DNA sequence
20 of *Frost* flanking region was searched from *Neurospora* genome project web site
21 (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html), using the
22 sequence previously reported for *Frost* gene [9]. *Xho* I-digested 3658 bp fragment is selected,
23 and a pair of primers, Frinv2 (5'-TCGCCAAACCTCGCGTACTC-3') and Frinv3CACC
24 (5'-CACCTGATTGCCAGCCTTCCG-3') was designed for inverse PCR amplification of
25 *Frost*-flanking region. Genomic DNA of *N. crassa* wild type 74-OR8-1a was digested with

1 *Xho* I at 0.1, 0.5, 1.0 ng DNA/μl and combined prior to inverse PCR. A clear amplification
2 was observed and amplicon was used for the cloning into pENTR/D-TOPO after the cleanup
3 with the Microspin S-400HR (Amersham Biosciences, Piscataway, NJ). The resulting plasmid,
4 pENTR-fr was used for the LR-recombination reaction, and a destination vector pDESTR-fr
5 was constructed. pDESTR-fr was digested with *Xho* I prior to the transformation of the *N.*
6 *crassa* wild type. Transformation of *N. crassa* was performed with electroporation. On the
7 hygromycin containing selection medium, hygromycin resistant colonies appeared. Almost
8 half of the colonies showed abnormal, hyperbranching morphology similar to the *frost* mutant
9 (Fig.4). Six colonies with *frost*-like morphology were picked up and their genomic DNAs
10 were extracted. Southern hybridization was performed to confirm that the expected
11 recombination caused the *frost*-like morphology (Fig. 3B). In all 6 transformants, it was
12 revealed that the expected recombination happened at the flanking region of *frost*, and
13 contained *fr::hph* structure.

14

15 **Conclusion.** The gene disruption system using pDESTR, a novel Gateway destination vector
16 reported in this paper gives an efficient way of gene disruption in filamentous fungi. The
17 selection marker of *hph* cassette from pCB1004 was already known to work effectively in
18 other filamentous fungi [5, 11], indicating that pDESTR system will work also in many other
19 fungal strains. A similar Gateway destination vector for gene disruption harboring
20 phosphinothricin resistance gene (*bar*) cassette from pBARKS1 [7] is under construction.
21 This will enable us to disrupt multiple genes. These novel vectors may help the functional
22 characterization of hypothetical genes of genome-sequenced fungi, by combination with
23 non-homologous end joining (NHEJ) – deficient strains [6] and efficient transformation
24 methods [5].

25

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3 Education, Culture, Sports, Science and Technology of Japan (16780029).

4

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1 **Figure Legends**

2 Fig.1 Schematic illustration of the inverse PCR-based gene disruption. (1) Selection of an
3 appropriate restriction enzyme (E), which cuts both upstream and downstream of the gene of
4 interest. Then digestion of genomic DNA or larger clone which includes the gene of the
5 interest such as cosmid clone, and self ligation by DNA ligase will be performed. A pair of
6 inverse primers will be designed at the central part of the restriction fragment. The position of
7 these primers will be the junction with the disruption DNA, thus primers should be designed
8 at the desired position for the disruption. (2) Inverse PCR will produce the DNA fragment
9 which connects the upstream and downstream DNA fragments in an opposite order, at the
10 central restriction site (E). (3) Ligation of the inverse PCR fragment into a cloning site (CS)
11 of the vector, which contains a selectable marker. (4) Digestion of the ligated plasmid with the
12 restriction enzyme used for the inverse PCR. This will produce the gene disruption construct.

13

14 Fig. 2 The construction of pDESTR. Small arrows indicate primers for PCR. See Results &
15 Discussion for details.

16

17 Fig. 3 Disruption of *N. crassa Frost* gene. (A) Maps of genomic DNA of wild type *Frost*
18 locus (WT) and the desired transformant with the disrupted *frost* locus (TF). Disruption
19 construct for the transformation was 7.7 kb *Xho* I fragment and is shown with thick line in TF
20 map. *Frost* gene is indicated with a black solid arrow. *hph*-Amp DNA from pDESTR was
21 indicated with white box. Position of primers for the inverse PCR was indicated with small
22 arrows. The probe for the Southern analysis was indicated with a dotted box. The length of
23 *Eco*R I-*Bam*H I fragments detected in the Southern analysis were indicated. B, *Bam*H I; E,
24 *Eco*R I; X, *Xho* I. (B) Southern analysis of the transformants. Genomic DNA (2 μ g) was
25 double-digested with *Eco*R I and *Bam*H I and probed with *Frost* gene fragment (left) and *hph*

1 cassette (right). WT: 74-OR8-1a, TF: Six transformants with *frost* phenotype. Sizes are shown
2 in kb.

3

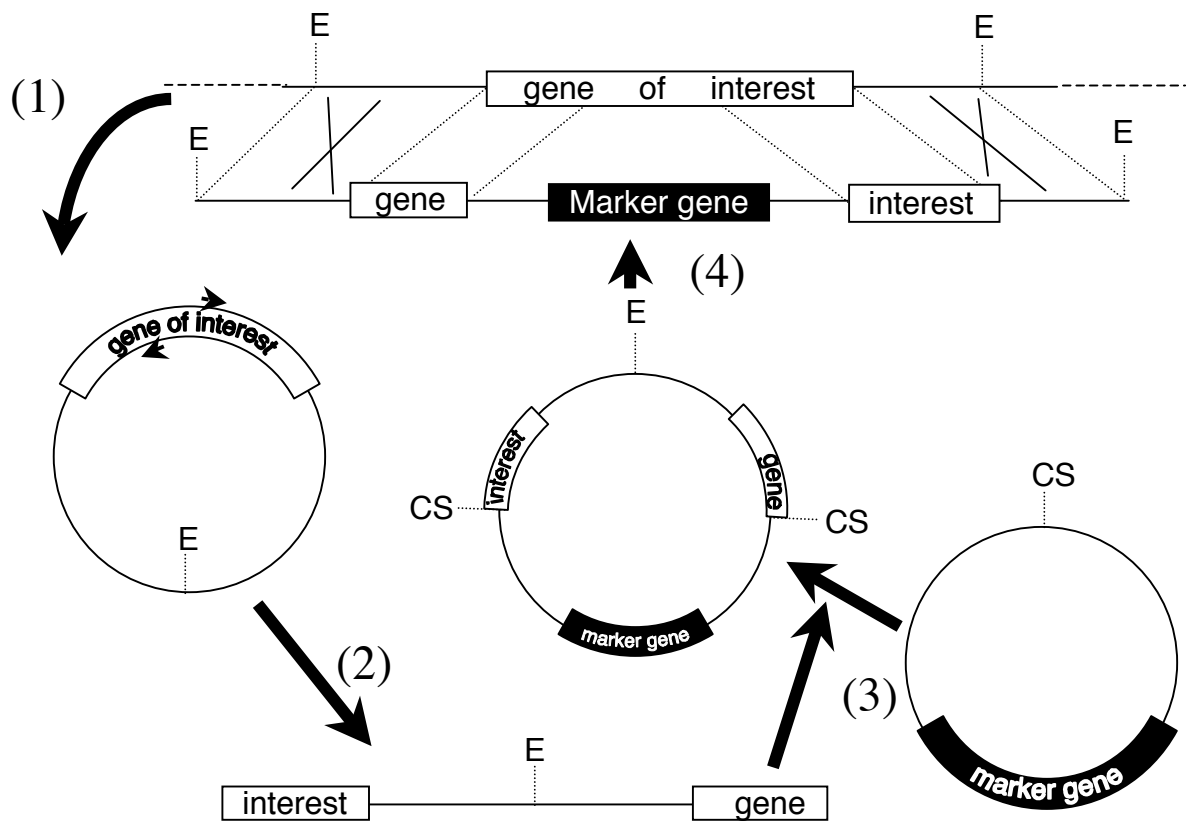
4 Fig 4 Morphology of *fr::hph* disruptant. Bar = 0.1mm.

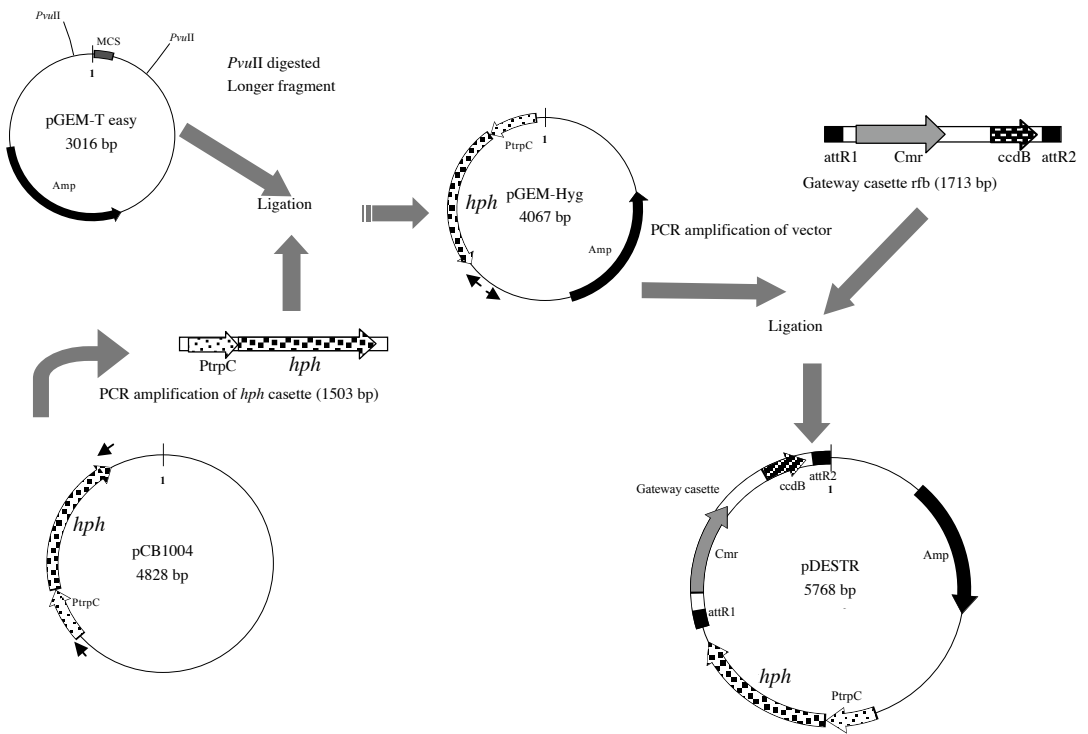
5

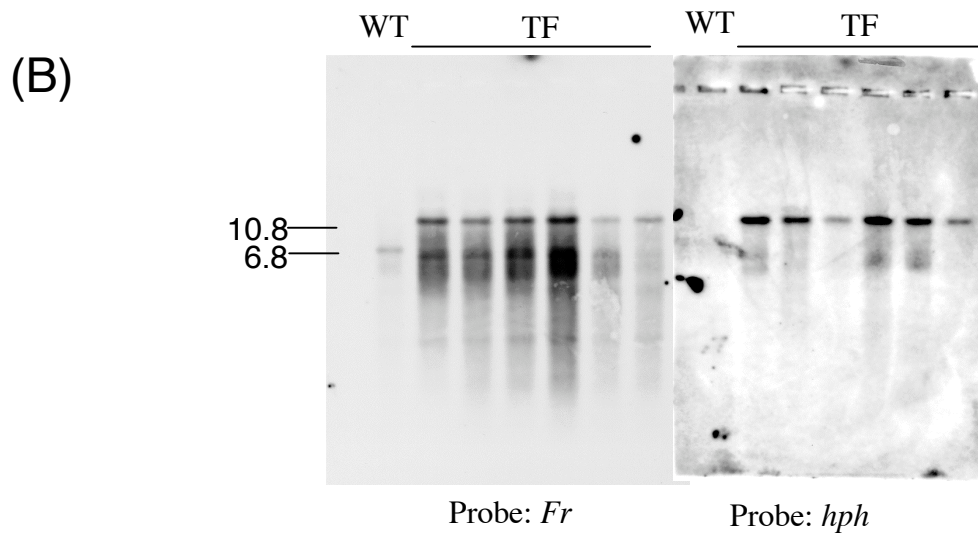
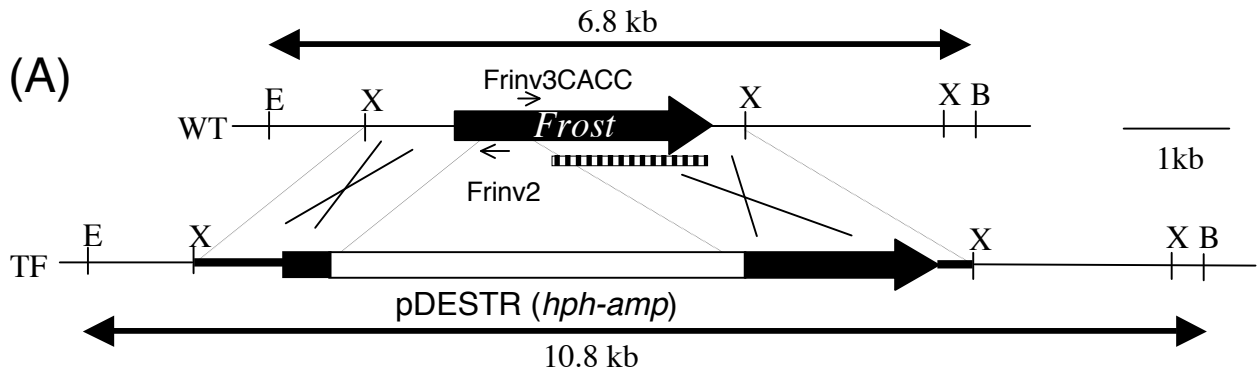
1 **Table.** List of restriction enzymes suitable for the inverse-PCR based gene disruption using pDESTR*

***Aat I/Eco147 I/Pce I/SseB I/Stu I, Aau I/Bsp1407 I/BsrG I/SspB I, Acc65 I/Asp718 I,*
Acv I/BbrP I/Eco72 I/PmaC I/Pml I, Afe I/Aor51H I/Eco47 III/Fun I,
Age I/AsiA I/BshT I/CspA I/PinA I, Ahl I/Bcu I/SpeI, Apa I, Asc I,
Asu II/Bpu14 I/BsiCI/Bsp119 I/BspT104 I/BstB I/Csp45 I/Lsp I/Nsp V/Sfu I, AsuNH I/Nhe I,
*Avr II/Bln I/BspA2 I/XmaJ I, Bal I/Mls I/Msc I/Msp20 I, **BamH I,***
Ban III/Bsa29 I/Bsc I/BseC I/BsiX I/Bsp106 I/BspD I/BspX I/Bsu15 I/BsuTU I/Cla I/Zho I,
Bbe I, BbvC I, Bcl I/BsiQ I/Fba I/Kso22 I, Bgl II, BmgB I/Btr I, BsiW I/Pfl23 II/PspL I/Sun I,
BseP I/BssH II/Pau I, Bsp68 I/Nru I, Bsp120 I/PspOM I, BssH I/PaeR7 I/Sfr274 I/Sla I/Tli I/Xho I,
BssNA I/BstZ17 I/Bst1107 I, BstSN I/Eco105 I/SnaB I, CciN I/Not I, Cfr9 I/PspA I/Vma I/XmaC I,
*Cfr42 I/Sac II, Eci36 II/EcoICR I, **EcoR I/Fun II, EcoR V/Eco32 I, EcoT22 I/Mph1103 I/Nsi I/Zsp2 I,***
*Ege I/Ehe I/Sfo I, Fse I, **Hind III, Kas I, Kpn I, Mfe I/Mun I, Mlu I, Mly113 I/Nar I, Mss I/Pme I, Pac I,***
Pst I, Pvu II, Psp124B I/Sac I/Sst I, Sal I, Sbf I/Sda I/Sse8387 I, Sma I, Smi I/Swa I, Srf I, Xba I

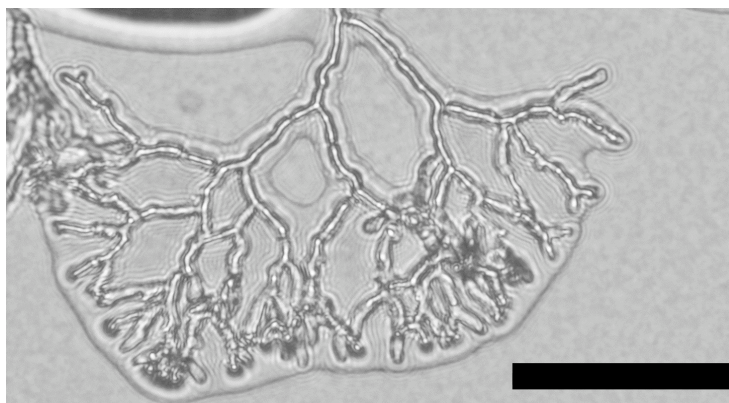
- 2 * Enzymes often present in multi cloning sites of cloning vectors are listed with **bold** letters. Enzymes with
3 ambiguous recognition sequence (for example, *Nci I* (CCSGG)) were not included in this list because such
4 restriction fragments were not always self-ligated. **Isoschizomers are shown with “/”.







fr (FGSC102)



fr::hph
disruptant

