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Original Article

Gene disruption/knock-in analysis of *mONT3:* vector construction by employing both *in vivo* and *in vitro* recombinations

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ABSTRACT We report the isolation, spatial/temporal expression and gene disruption phenotype of the mouse *ONT3 (mONT3)* gene, which encodes a novel secreted signaling protein belonging to the Olfactomedin/Noelin/Tiarin family. During early embryogenesis, *mONT3* is detected in the proximal region of the allantois on embryonic day (E) 7.25, in the lateral plate mesoderm on E 8.0 and in the CNS and heart on E 8.5. The homozygous mutant is born normal and fertile. For the expression pattern and loss-of-function analyses, we have successfully generated the LacZ-knock-in targeting vector directly from BACs carrying mouse genomic fragments by combining *in vivo* and *in vitro* recombination techniques. This approach enables rapid and reproducible construction of the fully functional vectors within two weeks without the use of restriction enzyme digestion and ligation, or the use of PCR-amplification of large genomic fragments. In addition, this method is applicable to rapid generation of transgenic vectors, demonstrating its versatility in reverse genetic studies.

KEY WORDS: *ONT, EG construction, gene expression, knock-in, recombination*

Introduction

Tissue interactions mediated by secreted signals play major roles in vertebrate embryogenesis. Recently, biochemical and molecular embryological studies have identified a novel secreted protein family, which includes bullfrog Olfactomedin, chicken Noelin and *Xenopus* Tiarin (Yokoe and Anholt, 1990; Barembaum *et al.*, 2000; Tsuda *et al.*, 2002). *Noelin* is expressed in the neural crest of early chick embryos and controls the generations of migratory neural crest cells (Barembaum *et al.*, 2000). *Tiarin* is expressed in the non-neural ectoderm flanking the *Xenopus* neural plate and exhibits potent dorsalizing activities on developing central nervous system (CNS) tissues (Tsuda *et al.*, 2002).

To further understand the roles of the Olfactomedin/Noelin/Tiarin (ONT) family factors, we have searched for other members of the ONT family expressed in chick and mouse embryos. Chick *ONT1* (*cONT1*) has been isolated by the degenerative PCR approach and is expressed in various tissues of the early chick embryo (M.S., M.I., M.K., Y.S., manuscript in preparation). Using the sequence information of *cONT1*, we have identified another *ONT* -related gene expressed in the mouse embryo, named mouse *ONT3* (*mONT3*). In this study, we report the isolation and characterization of *mONT3* by using homologous recombination techniques.

Additionally, in the course of these analyses, we have made a major modification in the construction method for targeting plasmids containing large genomic fragments and improved the speed, reproducibility and versatility of the procedure. We provide the data and the protocol for this approach, which combines *in vivo* and *in vitro* DNA recombinations.

Results

Mouse *ONT3* (GenBank: NM_133859; also as olfactomedin-like 3/ HNOEL-iso/ 2810002E22Rik) encodes a novel secreted molecule that belongs to the ONT family. In a database search, we identified two new members of the family: *mONT2* (Genbank: NM_172907; also as olfactomedin-like 1) and *mONT3*. A phylogenetic analysis reveals that *cONT1*, *mONT2* and *mONT3* fall into a new subfamily (Fig. 1A); *mONT3* is more closely related to

Abbreviations used in this paper: CNS, central nervous system; BAC, bacterial artificial chromosome; ONT, olfactomedin/noelin/Tiarin; LacZ, β-Galactosidase; HH, Hamburger and Hamilton stage.

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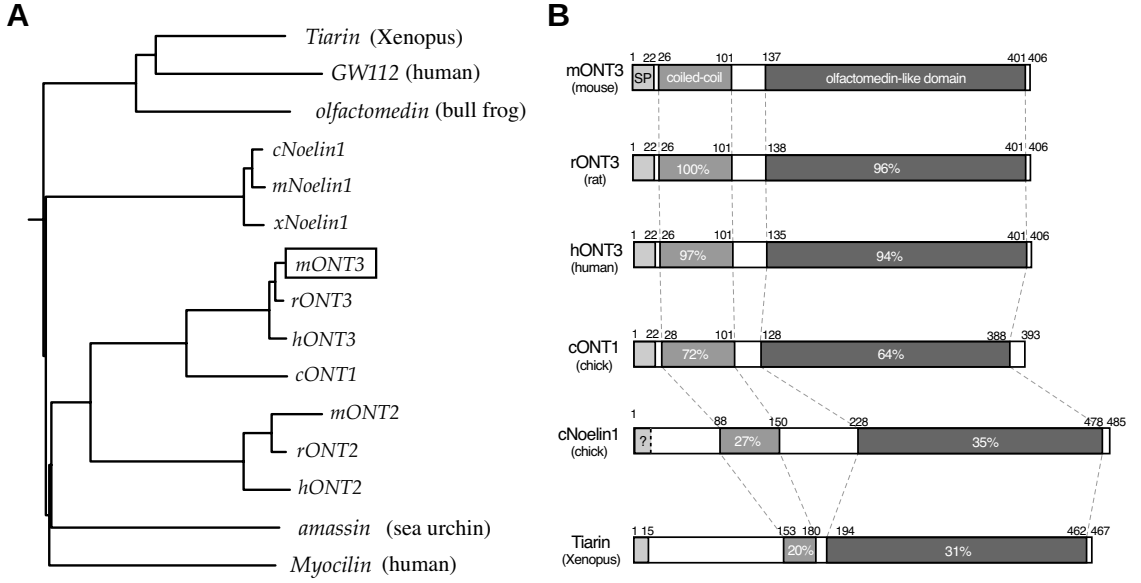
cONT1. The deduced protein of mONT3 consists of 406 amino acid residues with a putative signal peptide at the N-terminus, a coiled-coil domain in the middle and an olfactomedin-like domain at the C-terminus (Fig. 1B). These domains are highly conserved in the rat and human counterparts (Fig. 1B, C). According to the genomic database, the *mONT3* gene is located on mouse chromosome 3F2.2 and contains three exons spanning 2549 bp (Fig. 2C).

To understand the *in vivo* expression pattern and requirement of *mONT3*, we generated LacZ-knock-in mice by gene targeting at the *mONT3* locus. To maximize speed and simplicity and to minimize the risk of mutation, we have tested a strategy of generating gene-targeting vectors by combining *in vivo* recombination with *in vitro* recombination. For the sake of versatility, we use commercial kits for these recombinations: the Red/ET cloning technology (Gene Bridges GmbH) for '*in vivo*' subcloning of 5' and 3' genomic fragments from BACs (Fig. 2A) and the Multisite GATEWAY cloning system for '*in vitro*' assembling of genomic fragments and a universal marker cassette into a destination vector (Fig. 2B). In this report, the combination strategy for vector construction using the Red/ET and GATEWAY systems is hereafter referred to as EG construction (ET and GATEWAY).

To start the vector construction, we first prepared the linear Entry clone vector backbones by using PCR for the 5' and 3' fragments (an Entry clone vector is a carrier plasmid of the insert for *in vitro* recombination). We designed PCR primers that carried



C

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mONT3aa     1 MGPSAPLLLLFFLSWTGPLQGQQHHLVEYMERRLAALEERLAQCQDQSSRHAAELRDFKN  60
hONT3aa     1 MGPSTPLLILFLLSWSGPLQGQQHHLVEYMERRLAALEERLAQCQDQSSRHAAELRDFKN  60

mONT3aa    61 KMLPLLEVAEKERETLRTEADSISGRVDRLEREVDYLETQNPALPCVELDEKVTGGPGAK 120
hONT3aa    61 KMLPLLEVAEKEREALRTEADTISGRVDRLEREVDYLETQNPALPCVEFDEKVTGGPGTK 120

mONT3aa   121 GKGRRNEKYDMVTDCSYTVAQVRSMKILKRFGGSAGLWTKDPLGPAEKIYVLDGTQNDTA 180
hONT3aa   121 GKGRRNEKYDMVTDCGYTISQVRSMKILKRFGGPAGLWTKDPLGQTEKIYVLDGTQNDTA 180

mONT3aa   181 FVFPRLRDFTLAMAARKASRIRVPFPWVGTGQLVYGGFLYYARRPPGGPGGGGELENTLQ 240
hONT3aa   181 FVFPRLRDFTLAMAARKASRVRVPFPWVGTGQLVYGGFLYFARRPPGRPGGGGEMENTLQ 240

mONT3aa   241 LIKFHLANRTVVDSSVFPAESLIPPYGLTADTYIDLAADEEGLWAVYATRDDDRHLCLAK 300
hONT3aa   241 LIKFHLANRTVVDSSVFPAEGLIPPYGLTADTYIDLAADEEGLWAVYATREDDRHLCLAK 300

mONT3aa   301 LDPQTLDTEQQWDTPCPRENAEAAFVICGTLYVVYNTRPASRARIQCSFDASGTLAPERA 360
hONT3aa   301 LDPQTLDTEQQWDTPCPRENAEAAFVICGTLYVVYNTRPASRARIQCSFDASGTLTPERA 360

mONT3aa   361 ALSYFPRRYGAHASLRYNPRERQLYAWDDGYQIVYKLEMKKKEEEV 406
hONT3aa   361 ALPYFPRRYGAHASLRYNPRERQLYAWDDGYQIVYKLEMRKKEEEV 406
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Fig. 1. Characterization of mouse ONT3. (A) *A phylogenetic tree generated by ClustalW. The N-J tree with branch length program was selected. Source for sequences was as follows:* amassin *(accession number NM_214633),* GW112 *(accession number NM_006418),* Myocilin *(accession number NM_000261),* cNoelin1 *(accession number NM_204767),* mNoelin1 *(accession number NM_019498) and* xNoelin1 *(accession number AY071869),* olfactomedin *(accession number L13595),* hONT2 *(accession number NM_198474),* mONT2 *(accession number NM_172907),* rONT2 *(accession number NM_001013192),* hONT3 *(accession number NM_020190),* mONT3 *(accession number NM_133859),* rONT3 *(accession number XM_227535),* Tiarin *(accession number AB075925).* **(B)** *Structures of mouse protein sequences and comparison of other olfactomedin family member proteins.* **(C)** *Comparison of mouse and human ONT3 proteins. The protein sequences were aligned by ClustalW (http://clustalw.genome.jp/) and shaded by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).*

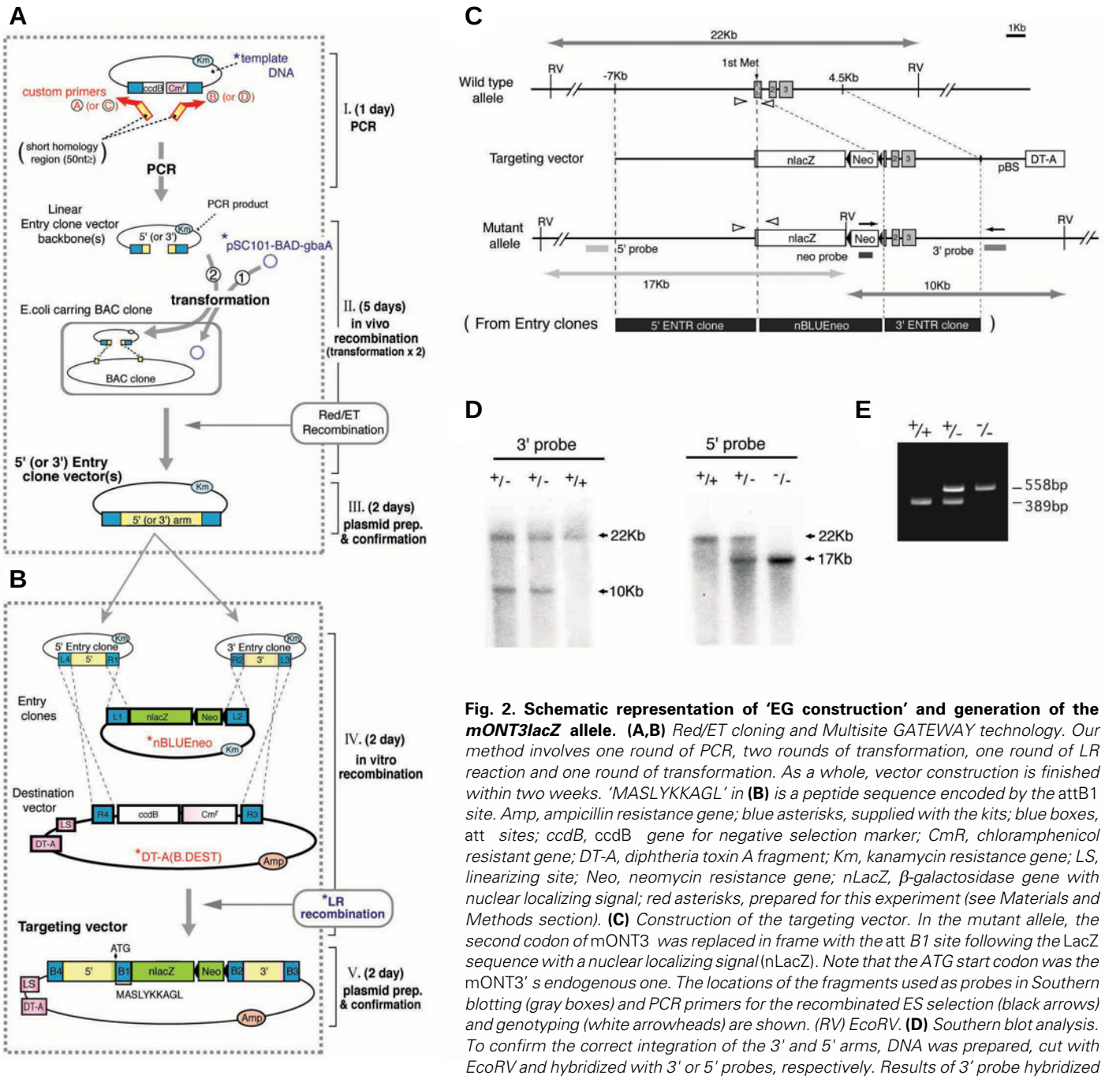

Fig. 2. Schematic representation of 'EG construction' and generation of the *mONT3lacZ* allele. (A,B) *Red/ET cloning and Multisite GATEWAY technology. Our method involves one round of PCR, two rounds of transformation, one round of LR reaction and one round of transformation. As a whole, vector construction is finished within two weeks. 'MASLYKKAGL' in* **(B)** *is a peptide sequence encoded by the* attB1 *site. Amp, ampicillin resistance gene; blue asterisks, supplied with the kits; blue boxes,* att *sites; ccdB,* ccdB *gene for negative selection marker; CmR, chloramphenicol resistant gene; DT-A, diphtheria toxin A fragment; Km, kanamycin resistance gene; LS, linearizing site; Neo, neomycin resistance gene; nLacZ, β-galactosidase gene with nuclear localizing signal; red asterisks, prepared for this experiment (see Materials and Methods section).* **(C)** *Construction of the targeting vector. In the mutant allele, the second codon of* mONT3 *was replaced in frame with the* att *B1 site following the* LacZ *sequence with a nuclear localizing signal* (nLacZ). *Note that the ATG start codon was the* mONT3' *s endogenous one. The locations of the fragments used as probes in Southern blotting (gray boxes) and PCR primers for the recombinated ES selection (black arrows) and genotyping (white arrowheads) are shown. (RV) EcoRV.* **(D)** *Southern blot analysis. To confirm the correct integration of the 3' and 5' arms, DNA was prepared, cut with EcoRV and hybridized with 3' or 5' probes, respectively. Results of 3' probe hybridized with the DNA from recombinant ES cells and 5' probe hybridized with the DNA from mice are presented.* **(E)** *PCR genotyping of each allele. Genomic DNA was prepared from the tails of wild-type, heterozygous and homozygous mice. The DNA was amplified by PCR with allele-specific primers to detect the wild-type allele (389 bp; mONT3-5'A and mONT3-3'A) and the mutant allele (558 bp; mONT3-5'A and mONT3-LA).*

appropriate *att* sites for Multisite GATEWAY recombination and short genomic sequences for Red/ET-mediated homologous recombination at corresponding ends (Fig. 2A, step I). In parallel, *E. coli* cells with a BAC clone containing the *mONT3* gene (purchased from BACPAC Resources Center) were transformed with pSC101-BAD-gbaA that encoded proteins necessary for Red/ET recombination (Fig. 2A, step II). The Entry clone vectors for the 5' and 3' genomic arms were generated *in vivo* by transfecting *E. coli* with linear 5' and 3' Entry vector backbones, respectively (Fig. 2A, steps II and III). Next, we performed Multisite GATEWAY cloning. The Entry clone vectors for the 5' and 3' arms were used for an LR recombination reaction with nBLUEneo as a marker cassette and DT-A(B.DEST) as a Destination vector (Fig. 2B; see Methods for the preparation of nBLUEneo and DT-A(B.DEST)). In this series of experiments, the correct recombination via the *in vivo* Red/ET reaction was observed in 3/16 clones for the 5' Entry

clone and in 4/16 for the 3' Entry clone. For the *in vitro* LR reaction, expected tetrameric ligation occurred in 2/16 clones (Table 1).

We next applied the targeting vector to homologous recombination in the ES cells. We observed proper homologous recombination in 23/96 revertant ES cells (Fig. 2D and data not shown) and generated chimera mice with the knock-in ES cells. Genotyping of the knock-in mice was done by Southern blot and/or PCR (Fig. 2D, E and data not shown). To analyze the expression pattern of *mONT3*, heterozygous embryos were stained with X-gal solution. LacZ signals were first detected on E7.25 in the proximal region of the allantois (Fig. 3A, F) and expanded to the presumptive lateral plate mesoderm by E8.0 (Fig. 3B, G). The staining was also observed in the midbrain-hindbrain boundary, rhombomere 3 and 5 of the hindbrain, the neural tube and the heart on E8.5 (Fig. 3C, H) and in the otic vesicles on E9.5 (Fig. 3D, I). On E10.5, we observed LacZ expression in the lens (Fig. 3E, J, K), neural tube, roof plate of the neural tube posterior to the forelimb level (Fig. 3E, J, L), ventral portion of the diencephalon and midbrain, branchial arches and mesenchymal tissues dorsal to the telencephalon (Fig. 3E, J). These expression patterns were reproduced in the knock-in mice from at least two independent ES cell lines. Whole-mount *in situ* hybridization revealed consistent expression patterns (data not shown).

Next, we intercrossed heterozygotes to evaluate homozygous phenotypes. Genotyping analysis showed that litters of all three genotypes were born according to the Mendelian ratio (Table 2). Homozygotes were indistinguishable from wild-type and heterozygous mice in external appearance, growth and behavior. Both male and female were fertile and produced a normal litter size (data not shown). These results demonstrate that *mONT3* (zygotic and maternal) is dispensable for normal embryogenesis.

As shown above, the EG construction approach works satisfactorily for the construction of gene targeting vectors from BACs. This prompted us to ask whether this technique is applicable to the generation of transgenic vectors. Until recently, the bottleneck for *in vivo* analyses of region-specific enhancers has been the production of transgenic mice and expansion of the colonies. The recent invention of the *in ovo* chick electroporation method has provided an alternative approach that remarkably accelerates the finding of the regulatory regions working in the embryo (Uchikawa *et al.*, 2003). In this approach, the rate-limiting step is shifted to the construction step of transgenic vectors. To test the compatibility of our strategy, we generated a transgenic vector that drives the expression of a fluorescence protein (in this case, Venus GFP (Nagai *et al.*, 2002)) widely in the developing CNS. A recent report has demonstrated that *mSox3* enhancers are clustered in a relatively small genomic region adjacent to the coding region (Brunelli *et al.*, 2003). This situation is in contrast to that of chick *Sox2* enhancers, which are dispersed in long genomic areas (spanning over 50 Kb; Uchikawa *et al.*, 2003). We used 3.3 Kb of 5' and 3.9 Kb of 3' fragments containing sufficient neural-specific enhancers according to the previous report (Fig. 4A). In the subcloning of these fragments by the *in vivo* Red/ET reaction,

TABLE 1

EFFICIENCY OF RECOMBINATION REACTIONS

	Red/ET recombination		GATEWAY reaction
	arm (length)	positive/total	positive/total
mONT3	5' (7 Kb)	3/16	2/16
	3' (4.5 Kb)	4/16	
mSox3	5' (3.3 Kb)	8/15	7/8
	3' (3.9 Kb)	1/3	
mFoxd3	5' (8 Kb)	1/94	5/5
	3' (5 Kb)	1/16	

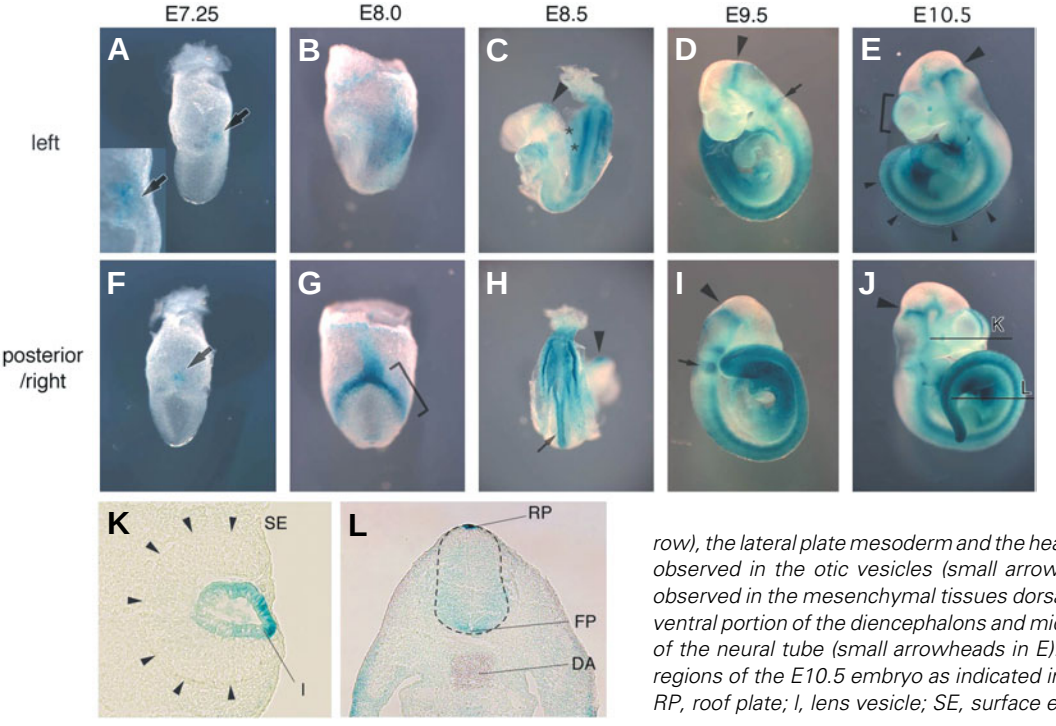

Fig. 3. X-gal staining of *mONT3lacZ/+* mice. *Left side view (A-E), right side view (I,J) and posterior view (F-H) of each stage indicated at the top. Anterior is to the left (A-C).* **(A,F)** *At E7.25, lacZ staining was observed in the proximal region of the allantois (arrows).* **(B,G)** *At E8.0, the staining was observed in the allantois and the presumptive lateral plate mesoderm (bracket).* **(C,H)** *At E8.5, the staining was observed in the midbrain-hindbrain boundary (arrowheads), rhombomere 3 and 5 (asterisks), the neural tube (small gray arrow), the lateral plate mesoderm and the heart.* **(D, I)** *At E9.5, the staining was also observed in the otic vesicles (small arrows).* **(E,J)** *At E10.5, the staining was observed in the mesenchymal tissues dorsa to the telencephalon (bracket), lens, ventral portion of the diencephalons and midbrain, branchial arches and roof plate of the neural tube (small arrowheads in E).* **(K,L)** *Sections of the eye and trunk regions of the E10.5 embryo as indicated in (J). DA, dorsal aorta; FP, floor plate; RP, roof plate; l, lens vesicle; SE, surface ectoderm.*

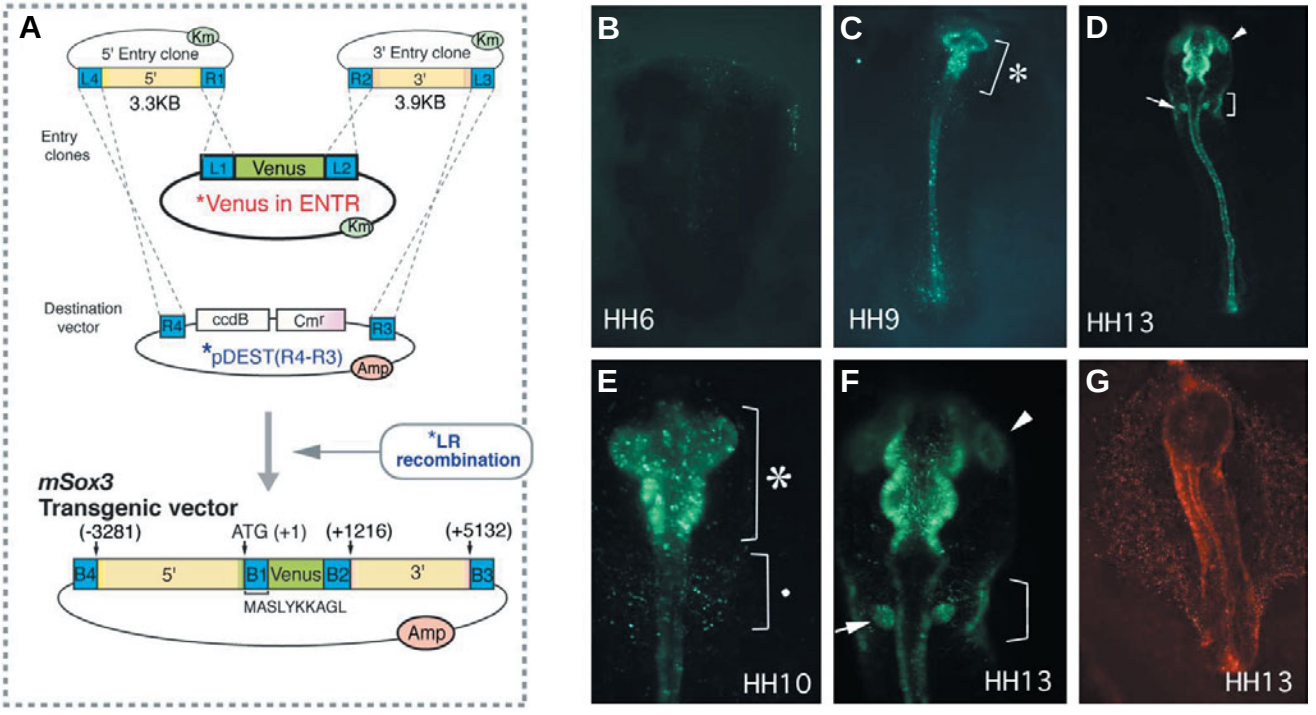

Fig. 4. Construction of *mSox3* transgene and electroporation of chick embryos. (A) *Schematic representation of* mSox3 *transgenic construct, which contains a 3.3 Kb fragment upstream and a 3.9 Kb fragment downstream of the* mSox3 *gene.* **(B-G)** *Electroporated embryos at different stages. Venus fluorescence was observed in the fore- and midbrain (asterisk), the neural crest (dot) and the spinal cord at stages HH9-10* **(C,E)** *and eyes (arrowhead), otic vesicles (arrow), branchial arches (bracket), the fore- and midbrain and the spinal cord at stage HH13* **(D,F)**. *The electroporated area was confirmed by DsRed2 fluorescence driven under the control of the CMV promoter (Clontech)* **(G)**.

correct recombination was observed in 8/15 for the 5' Entry clone and 1/3 for the 3' Entry clone. The *in vitro* LR recombination step was successful in 7/8 (Table 1).

The resulting transgenic vector was tested by using the *in ovo* electroporation technique. We introduced the vector DNA into the epiblast of HH4 chick embryos and cultured them in the modified EC cultures (Chapman *et al.*, 2001). We did not observe any strong fluorescence until HH6 (Fig. 4B), while we observed GFP expression driven by the control CMV promoter as early as HH 5 (data not shown). At HH9, we observed strong fluorescence restricted to the neural tube (signals at the hindbrain region were relatively weak; Fig. 4C, E). We also saw GFP expression in migrating neural crest cells in the hindbrain region (Fig. 4E). At HH13, we observed GFP signals in the optic and otic vesicles, branchial arches, forebrain, midbrain and spinal cord (Fig. 4D, F). These expression patterns are largely consistent with LacZ expression in the transgenic mouse and *Xenopus* embryos (Brunelli *et al.*, 2003) as well as *Sox3* mRNA distribution in the chick (Abu-Elmagd *et al.*, 2001). These results suggest that the regulatory system underlying *Sox3* expression is conserved between chick and mice and showed that this *Sox3* transgenic vector is useful for driving the expression of a gene of interest in a large region of the neural tissues after HH9, since a one-step LR reaction can easily replace the *Venus GFP* portion with any other cDNA.

TABLE 2

***MONT3* GENOTYPES OF THE F2 PROGENY FROM F1 HETEROZYGOTE INTERCROSS**

	No.(%) of F2 mice per genotype		
Genotype	**+/+**	**+/-**	**-/-**
male	17(22.7)	38(50.7)	20(26.7)
female	13(25.5)	24(47.1)	14(27.8)
total	30(23.8)	62(49.2)	34(27.0)

Discussion

In this study, we have performed the expression pattern and gene disruption analyses of *mONT3*. The ONT family is a relatively new group of secreted signals that seem to play regulatory roles in vertebrate embryogenesis (Barembaum *et al.*, 2000; Tsuda *et al.*, 2002). However, only gain-of-function studies on the family members have been so far reported while knowledge on the *in vivo* roles of endogenous ONT factors is still limited. As shown in figure 3, intriguing expression patterns of *mONT3* are observed in mouse embryos. Our preliminary gain-of-function study has shown that *mONT3* exhibits a dorsalizing effect, as shown for *xTiarin*, when overexpressed in *Xenopus* embryos. While overexpression of *mONT3* at a low dose (500 pg mRNA per cell: animal blastomere injection at the eight cell stage) does not induce expansion of the dorsal CNS markers *Zic-r1* (n=12) and *Pax3* (n=15), a high dose injection of *mONT3* (2000pg) induces moderate expansion of the expression domains of these dorsal marker genes (40.9%, n=22 for Zic-r1; 31.6%, n=19 for Pax3; Fig. 5), suggesting the *mONT3* has a weak dorsalizing activity in the context of *Xenopus* ectodermal patterning. In contrast, homozygous *mONT3* mutants are viable, apparently normal (including

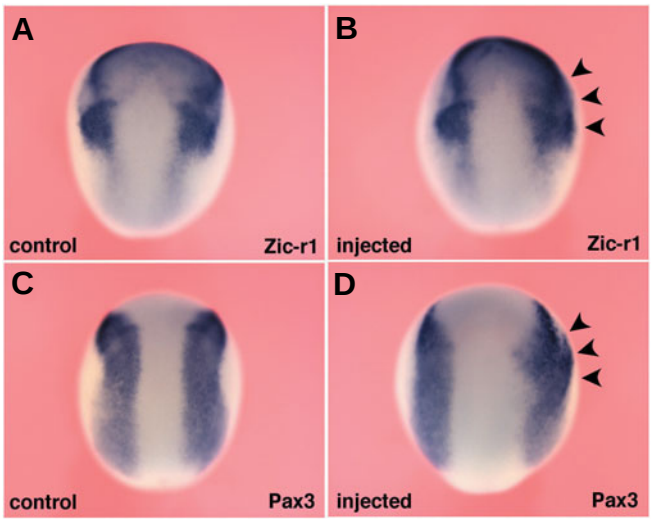

Fig. 5. Microinjection of *mONT3* mRNA at a high dose induces moderate expansion of dorsal neural markers. *Control* **(A,C)** *or* mONT3 **(B,D)** *mRNA injected embryo. When* mONT3 *mRNA was injected at a higher dose (2000 pg per cell), weak expansion of the dorsal CNS markers (Zic-r1 and Pax3) was observed at the neurula stage.*

development and basic functions of the brain) and fertile. These observations suggest the possibility that the *mONT3* gene function is dispensable and possibly compensated by other family genes. In future, the generation and detailed analyses of individual and compound KO mice of other ONT family members are required for understanding the exact *in vivo* roles of this gene family during embryogenesis.

In addition, we have explored a rapid construction method for the generation of the target vector. The recent introduction of the total genomic sequence database has highlighted the promise for high-throughput analysis in functional genomics to elucidate the *in vivo* functions of gene networks. Although transgenic and gene targeting analyses are key approaches in the study of large-scale reverse genetics, they require elaborate processes of isolating and subcloning large genomic fragments. The large collection of mouse genomic BAC clones is an attractive source for genomic fragment isolation. However, the handling of BACs is technically demanding and the conventional digestion/subcloning processes are time-consuming and often problematic.

This study has shown the usefulness of the 'EG construction' approach for generating transgenic and targeting constructs. This strategy takes advantage of the two recombination systems. First, by using the Red α/β-based *in vivo* recombination, one can isolate genomic fragments from the large collection of BAC libraries without the handling of BAC DNAs or the use of PCR, which may cause mutations. Second, by using the *in vitro* recombination, one can perform simultaneous directional subcloning of three DNA fragments (containing 5', 3' and marker insert fragments) into a targeting vector in a seamless manner. Furthermore, the same vectors containing the 5' and 3' genomic fragments can be used for generating constructs for KO, knock-in, conditional KO, transgenic and promoter analysis studies by utilizing ready-made universal marker cassettes (e.g. drug selection and GFP markers). By combining two types of recombination techniques, this strategy provides satisfactory speed, efficiency and reproducibility, as demonstrated in this study. In addition to the *mONT3* and *Sox3* constructs, we have applied the EG construction to the generation of a GFP transgenic vector carrying the 8 Kb 5' fragment and 5 Kb 3' fragment of the *Foxd3* gene. In this case, 1/94 for the 5' Entry clone, 1/16 for the 3' Entry clone and 5/5 of the transgenic vector underwent correct recombination (Table 1). Taken together, at all recombination steps involving 3-8 Kb genomic fragments (except for the Red/ET step of the long 5' *Foxd3* fragment), correct plasmids were obtained by DNA preparation with less than 24 colony pick-ups. This means that, in most cases, the desired construction can be obtained within a single trial of the standard mini-prep procedure using a typical bench-top centrifuge (18-24 slots). Similar efficiencies were observed in the construction of three other targeting vectors (MI, MK and YS, unpublished observations). In summary, we propose EG construction as a versatile and practical approach for constructing targeting and transgenic vectors. A detailed step-by-step protocol for practical use of the EG construction is provided in the Appendix to this paper.

Materials and Methods

Construction of the 5', 3' and reporter Entry clone plasmids

Red/ET cloning was performed according to the manufacturer's instructions (Gene Bridges GmbH, Germany) and the procedures described previously (Testa *et al.*, 2003) with some modifications. To combine with the Multisite GATEWAY technology (Invitrogen Corp., Carlsbad, CA USA), we first created linear 5' and 3' Entry vector backbones that have appropriate *att* sites and short sequences of homologous recombination regions (blue and checked boxes in Fig. 2A) by using PCR. Each PCR primer (A, B, C and D in Fig. 2A) was designed to include 50 to 54 nucleotides of homologous regions in each genomic sequence, followed by 27 or 28 nucleotides of GATEWAY vector sequences at the 3' end (underlined). The templates and primers were:

< mSox3-5' Entry vector >
Template: pDONR-P4P1R (linearized with BamH1)
Sox3primerA: 5'-tgcccgccccccatgccccaccccgctccaaggagcgtt aagactccctcaacttttctatacaaagttggcattat-3'
Sox3primerB: 5'-gagcgccgtctcctcccgccacgctggcgcacctccttcccg ccccggcgatggcaagtttgtacaaaaaagttgaacgag-3'
< mSox3-3' Entry vector >
Template: pDONR-P2RP3 (linearized with BamH1)
Sox3primerC: 5'-tacggccaaactttcggtcctcctcatcttcggtacaagg caacggtcccccacttttgtacaagaaagttgaacgag-3'
Sox3primerD: 5'-tgcctaacagtcttggattgcaaggagcaggggccaccaggg tcccccaacaacttttattatacaaagttggcattat-3'
< mFoxd3-5' Entry vector >
Template: pDONR-P4P1R (linearized with BamH1)
Foxd3(A): 5'-gtactctgtgaaatgcctaccggacttctgcaatgccagggg catttagtcaacttttctatacaaagttggcattat-3'
Foxd3(B): 5'-ctcgctctctgctcgctcctggccgccgccaccaaccccgcgga gggatggcaagtttgtacaaaaaagttgaacgag-3'
< mFoxd3-3' Entry vector >
Template: pDONR-P2RP3 (linearized with BamH1)
Foxd3(C): 5'-gagcaggctgctgccagaggccgccgtccggcccggcc actagctcatcaccacctttgtacaagaaagttgaacgag-3'
Foxd3(D): 5'-agcagacttgggccttgggaccttccttccaccccgggtgcc ttcccttacaacttttattatacaaagttggcattat-3'
< mONT3-5' Entry vector >
Template: pDONR-P4P1R (linearized with BamH1)
A: 5'-ttagaaccagctgatttaagtaagtggctttctctgcc

ttgcctcaggtacaactttctatacaaagttggcattat-3'
B: 5'-gaaagaggccgaatgcacacactcctctggccccactt
aaggctgccatggcaagtttgtacaaaaaagttgaacgag-3'
< mONT3-3' Entry vector >
Template: pDONR-P2RP3 (linearized with BamH1)
C: 5'-caagtccctcacctctaaggcagctagtcggcgttcc
atgtactccacaaccactttgtacaagaaagttgaacgag-3'
D: 5'-aacactcctcctttttgtctgtctttcttctctccacagg
cagagctgctccaacttttattatacaaagttggcattat-3'

BAC-containing *E. coli* was identified by the alignment of BAC end sequences in the NCBI database and purchased from BACPAC Resources Center (Children's Hospital of Oakland Research Institute, URL http://bacpac.chori.org/). They were transformed with pSC101-BAD-gbaA according to the standard procedures. Cells were prepared for electroporation after inducing expression of recombination proteins. Linear 5' and 3' Entry vector generated formerly was transfected individually into the *E. coli* carrying the BAC clone. Entry clones containing *att*-flanked genomic fragments were generated by Red/ET recombination. Proper recombination was confirmed by restriction enzyme mapping.

For a transgenic construct, a DNA fragment containing the *Venus* gene flanked with *att* sites was amplified by PCR from Venus/pCS2 (Nagai *et al*., 2002).
[primers att-Venus-:
ggggacaagtttgtacaaaaaagcaggcttcatggtgagcaagggcgaggagctgttcacc
att-Venus-R:
ggggaccactttgtacaagaaagctgggtttacttgtacagctcgtccatgccgagagt]
and subcloned into a pDONR 221 vector (named Venus-in-ENTR) by using BP clonase (Invitrogen). For a targeting vector, the NLS-LacZ-pA cassette from BAT-gal (Maretto *et al*., 2003) was subcloned into a *loxP*-flanked PGK-neo vector using the conventional method and the NLS-LacZ-pA-loxP-PGK-neo-loxP cassette was subcloned into pDONR221 by Red/ET-cloning (named nBLUEneo in ENTR).

Construction of DT-A (B.DEST)

For targeting vector construction, we made a Destination vector, pDT-A(B.DEST), which contains a DT-A cassette and a linearizing site. First, *att*-flanked *ccdB* and chloramphenicol resistance gene cassette 3' to the linearizing site was amplified from the pDEST(R4-R3) vector (Invitrogen) using the following PCR primers:
[Forward: ataagaatgggcccggcgcgccttaattaaggccggccgttt
aaacgcggccgcatttaaatcaggaaacagctatgac
and Reverse: ccgctcgaggtaaaacgacggccag] and cut with PspOMI and XhoI. Second, pMCDT-A(A+T/pau), which contains diphtheria toxin A fragment gene with MC1 promoter for the negative selection of homologous recombinant (Yagi *et al*., 1993b), was cut with NotI and XhoI and ligated with the PCR fragment. The linearizing site contains sequences recognized with restriction enzymes of AscI, PacI, FseI, PmeI, NotI and SwaI (underlined).

Generation of the transgenic construct and the targeting vector

To make three DNA fragments subcloned directionally into one Destination vector, we used the Multisite GATEWAY Three-fragment Vector Construction Kit (Invitrogen). Two Entry clone plasmids carrying 5' and 3' genomic fragments were prepared from each recombinant *E. coli* using QIAprep Spin Miniprep Kit (QIAGEN). Following the manufacture's protocol, we used these Entry clone plasmids for LR plus recombination reactions with the Venus in ENTR and a pDEST(R4-R3) for the transgenic construct and with the nBLUEneo and the DT-A(B.DEST) for the targeting vector.

Establishment of mONT3 *targeted mice and X-gal staining analysis*

The targeting vector was transfected to TT2 embryonic stem cells (Yagi *et al*., 1993a) and homologous recombinant ES cells were primary screened by PCR with
neoC2 primer: (5'-GCCTGCTTGCCGAATATCATGGTGGAAAAT-3') and Select5 primer (5'-ATTAAGTGGCGCGGACAAGCACTTC-3') and confirmed by Southern blot analysis. After electroporation to TT2 ES cells, 23 of 96 clones were PCR positive. We selected 13 clones for Southern blot analysis and confirmed that all of them had correct recombination. Recombinant ES cells were injected into an eight-cell embryo of CD-1 mice. We obtained germline chimeras from three independent recombinants and observed no differences in the phenotypes among these lines.

Embryos were stained with X-gal as described in Kimura *et al*. (Kimura *et al*., 1997).

PCR-genotyping of mice

Genomic DNA was isolated from tails or yolk sacs. They were digested overnight at 55ºC in 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.2% SDS and 200 mM NaCl in the presence of 100 μg/ml of Proteinase K. Samples were then boiled for 15 minutes, diluted 5 times with sterile water, spun at 13000 rpm for 15 minutes to pellet the debris. 1 μl of the supernatant was subjected to PCR. Primers were designed against the sequence 5' and 3' to the ATG start codon of *mONT3* (mONT3-5'A and mONT3-3'A, respectively) and against the sequence of β-*galactosidase* (mONT3-LA). The primers were:

mONT3-5'A:	AAAGAGGCCGAATGCACACACTCCT
mONT3-3'A:	GACTCTCATTTCCCCTGCCCCCTTA
mONT3-LA:	GCACCACAGATGAAACGCCGAGTTA

Electroporation to chick embryos

Chick electroporation was performed as described previously (Uchikawa *et al*., 2003). After vector DNA was introduced by electroporation, the embryos were cultured on an agar albumen plate according to the EC method (Chapman *et al*., 2001) with some modification. We placed the embryos with the dorsal side up, overlaid with a drop of liquid albumen and incubated them at 37ºC.

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We are grateful to R. Ladher and T. Sakamoto for advice on chick tissue culture, to K. Furushima and A. Miyawaki for plamids and to S.C. Suzuki for discussion on recombination techniques. We also thank to H. Ikeda for critical reading of the manuscript. This work was supported by grants-in-aid (YS) from MEXT, the Kobe Cluster Project and the Leading Project.

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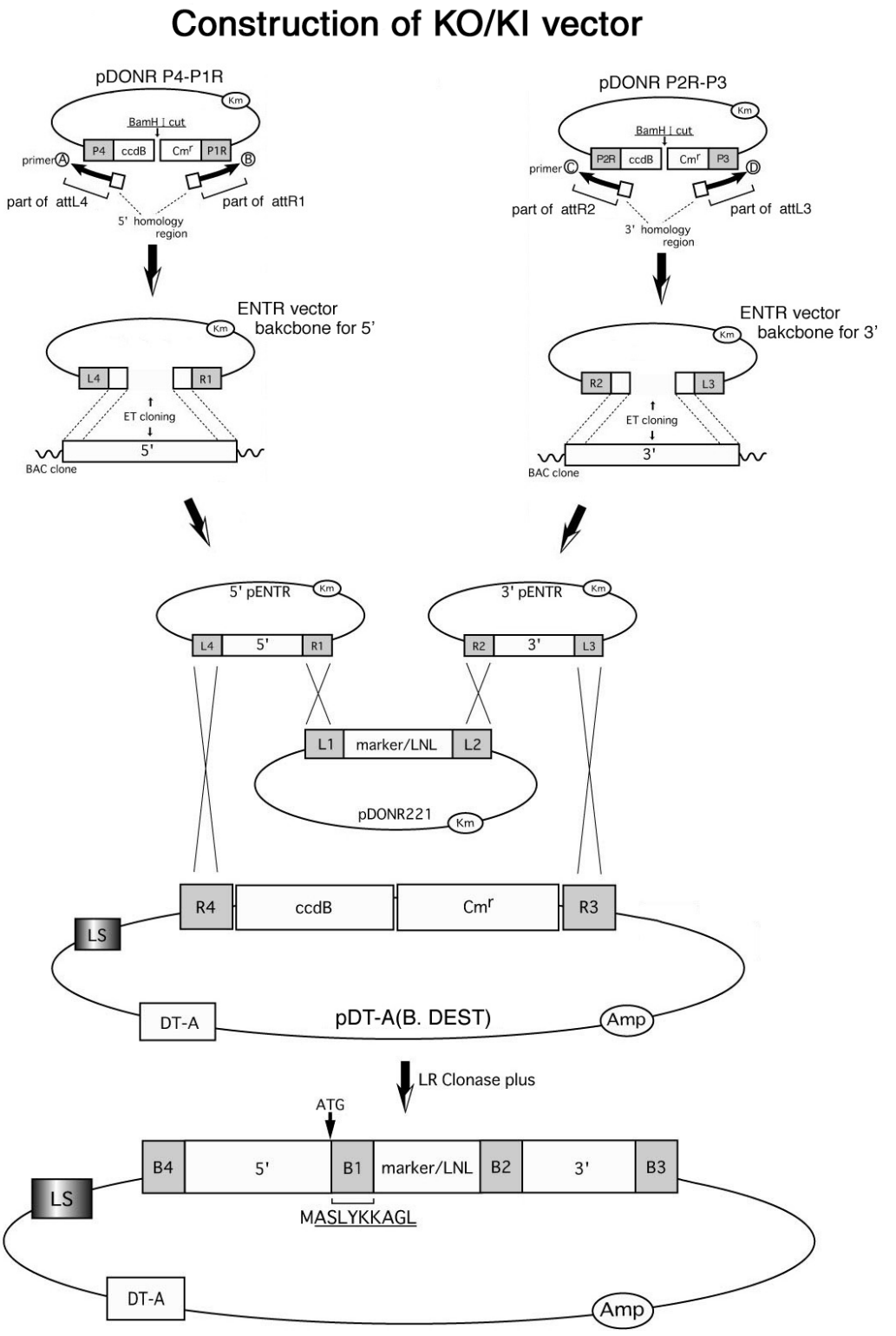
APPENDIX

The EG Construction Protocol

A construction manual of KO/KI Vectors by *in vivo* and *in vitro* recombinations

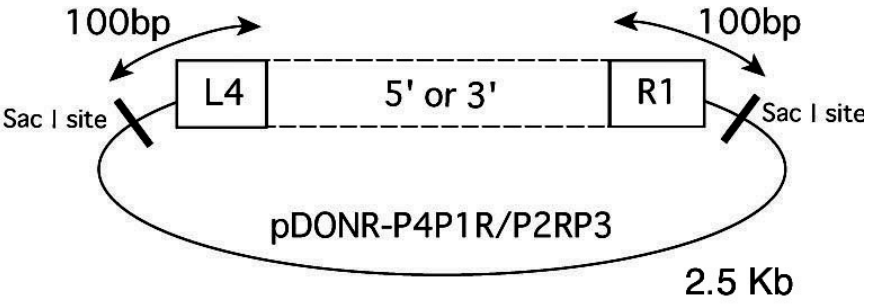
Contents

1. Overview

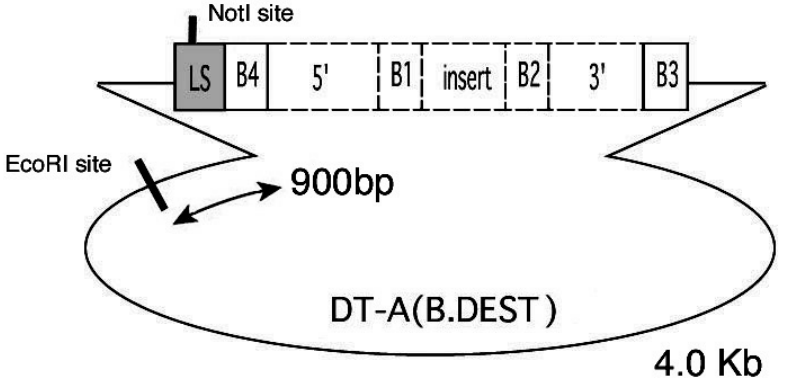

Construction of KO/KI vector
pDONR P4-P1R
Km
BamH I cut
P4
ccdB
Cmr
P1R
primer A
B
part of attL4
part of attR1
5' homology region
pDONR P2R-P3
BamH I cut
P2R
ccdB
Cmr
P3
primer C
D
part of attR2
part of attL3
3' homology region
ENTR vector bakcbone for 5'
L4
R1
ET cloning
5'
BAC clone
ENTR vector bakcbone for 3'
R2
L3
ET cloning
3'
BAC clone
5' pENTR
L4
5'
R1
3' pENTR
R2
3'
L3
L1
marker/LNL
L2
pDONR221
R4
ccdB
Cmr
R3
LS
DT-A
pDT-A(B. DEST)
Amp
LR Clonase plus
ATG
B4
5'
B1
marker/LNL
B2
3'
B3
LS
MASLYKKAGL
DT-A
Amp

2. Maps and Enzyme Sites

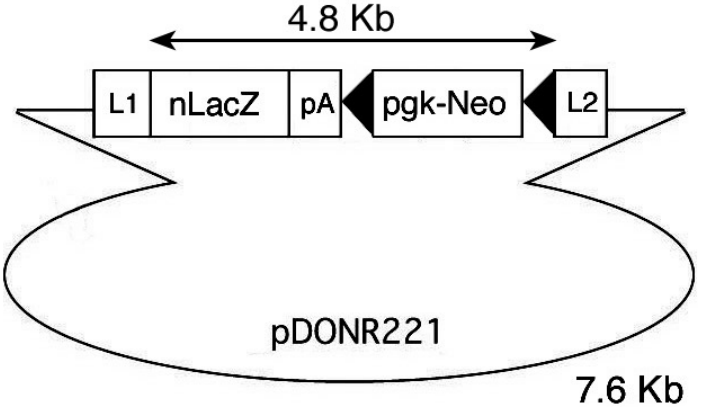
pDONR-P4P1R/P2RP3(for 5' and 3')



DT-A(B.DEST)



nBLUEneo in ENTR



3. Materials

3.1. BAC clones

The clone with the name starting with RP23xxx can be purchased from the BACPAC Resource Center (e-mail address: bacpacorders@chori.org, URL: http://bacpac.chori.org/). We usually order two clones for one gene, just in case.

3.2. Primers

Oligonucleotides should be purchased from a reliable provider. We recommend purifying the primers for Red/ET cloning with HPLC because it enriches full-length oligonucleotides. Primers for other purposes do not usually require special purification.

3.3. Plasmids

1. Prior to use, pDONR-P4-P1R and pDONR-P2R-P3 (provided with the 'Multisite GATEWAY Three-Fragment Vector Construction Kit', Invitrogen, #12537-023) should be linearized with BamHI, purified with a QIAperp PCR Purification Kit and its concentration adjusted to about 15 ng/μl.
2. pSC101-BAD-gbaA(Tet^r) (Gene Bridges GmbH)
3. DT-A(B.DEST), containing a negative selection marker and linearizing sites (constructed in the Sasai Lab.)
4. nBLUEneo in ENTR, containing a lacZ reporter and a positive selection marker (constructed in the Sasai Lab.)

3.4. Equipment

Standard laboratory equipments are used including: PCR machine, spectrophotometer, high-speed refrigerated microcentrifuge, electroporator (Genepulser II with Pulse Controller Plus; Bio-Rad) and cuvettes with 0.1 cm gaps; an incubator and a shaking incubator set at 30ºC and an incubator and a shaking incubator set at 37ºC.

3.5. Reagents and media

1. For PCR amplification, DNA polymerase with proof-reading activity should be used. We routinely use KOD plus DNA polymerase (TOYOBO, # KOD-201) with KOD Dash Buffer (TOYOBO, #LDP-101) with 10% DMSO for amplifying ENTR vector backbones. For other purposes, we use KOD Dash polymerase.
2. QIAquick PCR Purification Kit (Qiagen) or equivalent
3. Restriction enzymes and buffers. DpnI restriction enzyme (New England Biolabs) is used to digest the template DNA.
4. QIAquick Gel Extraction Kit (Qiagen) or equivalent
5. LB plates with or without antibiotics (ampicillin 100 μg/ml, chloramphenicol 20 μg/ml, tetracycline 3 μg/ml, chloramphenicol 20 μg/ml plus tetracycline 3μg/ml and kanamycin 20 μg/ml).

3.6. Antibiotics

Chloramphenicol (100 mg/ml); Chloramphenicol 100 mg + ethanol 1 ml -> mixed and stored at -20ºC

Tetracycline (5 mg/ml); Tetracycline 5 mg + EtOH 0.5 ml + DMSO 0.5 ml -> mixed and stored at -20ºC

Kanamycin (20 mg/ml); Kanamycin 20 mg + DW (distilled water)1ml -> mixed, filterated (ø 0.2 μm) and stored at -20ºC

Ampicillin (100 mg/ml); Ampicillin 100mg + DW 1 ml -> mixed, filterated (ø 0.2 μm) and stored at -20ºC

3.7. QIAprep Spin Miniprep Kit (QIAGEN) or equivalent

3.8. 10% glycerol/DW

Glycerol 5 ml + DW 45 ml -> mixed, filterated (ø 0.2 μm) and stored at 4ºC or RT

3.9. Liquid nitrogen

3.10. 20% L-arabinose

L-arabinose 5 g + DW 25 ml in a conical tube -> mixed, filterated (ø 0.2 μm) and stored at 4ºC or RT

3.11. LR Clonase Plus Enzyme Mix,

5 x LR Clonase Plus reaction buffer and Proteinase K (provided with 'Multisite GATEWAY Three-Fragment Vector Construction Kit,' Invitrogen, #12537-023).

3. 12. TOP10 electrocompetent cells (Invitrogen).

4. Experimental Procedures

4.1. Primer design

(Note that this protocol is for replacing the gene with the marker gene casette at the 1st ATG to the marker gene.)

1) Prepare the sequence data of the gene of interest.
2) To find the position of the 1st exon on a chromosome and exon-intron structure of the gene, submit the sequence data to NCBI mouse genomic BLAST (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html).
3) Click 'Genome View' and find the position of the gene. To display the 'Map View,' click the chromosome number.
4) Click 'Maps & Option.' Add 'BES_Clone' to 'Maps displayed' and move it down. Click 'apply' to display the names of the BAC clones that contain the gene.
5) Select BAC clones that contain a 20 Kb region around the 1st ATG. The clone with the name starting with RP23xxx can be purchased from the BACPAC Resource Center (e-mail address: bacpacorders@chori.org). We usually order two clones for one gene. For more information, visit the URL: http://bacpac.chori.org/.
6) Click 'Download/View Sequence/Evidence' to get the genomic sequence. We usually extract the 20 Kb sequences that contain the regions 10 Kbp above and 10 Kbp below the 1st ATG (total 20 Kbp; place the A of the 1st ATG on the 1001st of the sequence data). (In some cases, the sequence data contain 'NNN…' sequences. If the ambiguous regions participate in an important region for primer design, determine the sequence manually.)
7) Primers (we recommend to use HPLC purified primers.):

for 5' arm:	A (reverse)	50 nucleotides (nts) of the complementary sequence that is around 8 Kb above the 1st ATG [for homology arm] +28 nts (caacttttctatacaaagttggcattat) [for attL4]
	B (forward)	47 nts just above the 1st ATG [for homology arm] +ATGG (this 'ATG' represents the endogenous one) +27 nts (caagtttgtacaaaaaagttgaacgag)[for attR1]
for 3' arm:	C(reverse)	50 nts containing the complementary sequence of the 3' end of the 1st exon [for homology arm] +27 nts (ccactttgtacaagaaagttgaacgag) (for attR2)
	D(forward)	50 nts around the sequence 4 Kb below the 3' end of the 1st exon [for homology arm] +28nts (caactttattatacaaagttggcattat) (for attL3)

	primer set	template	band size
pENTR5'	A B	pDONR-P4P1R (linearized)	2.8 kb
pENTR3'	C D	pDONR-P2RP3 (linearized)	2.8 kb

4.2. Preparation of ENTR vector backbones

8) Mix the following solutions in a PCR tube on ice and perform PCR according to the following program. We usually prepare 8 tubes (total 160 μl) to get ≥ 10 μg of each PCR product. Templates are linearized with BamHI and purified with QIAquick PCR Purification Kit before use.

Template DNA (about 15 ng/μl):	1 μl
10 x buffer for KOD Dash:	2 μl
Primers (10 μM):	1 μl each
2 mM dNTPs:	2 μl
KOD plus polymerase:	0.4 μl
DMSO:	1 μl
DW:	11.6 μl / 20 μl

PCR condition

94ºC hold (Easy hot start; make the machine ready to start, then insert the tubes and immediately start the reaction)

94ºC for 5 min
{94ºC for 30 sec / 50ºC for 2 sec / 74ºC for 4 min} x 25
74ºC for 5 min
4ºC ∞

9) Apply 1 μl of the PCR product on agarose gel and check the amount of the PCR product. (Usually there is ≥ 100 ng within one band. Using KOD plus enzyme with KOD Dash buffer and ASTEC PC801 thermal cycler, we can reproducibly amplify the Entry vector backbone. If you observe only a weak band, run ten additional cycles. If the PCR product consists of multiple bands, increase the annealing temperature to 60ºC, change the PCR enzyme, or try touchdown PCR (see STEP 14).)

10) Purify the products by QIAquick PCR Purification Kit. First, add 0.2 μl of 5 M CH_3COONa pH5.2 to 20 μl PCR product to decrease the pH below 7.5 and proceed according to the manufactures protocol. Collect eight tubes of PCR product in one tube. To elute DNA, add 90 μl of Buffer EB to the center of the membrane. The total amount of purified DNA should be about 10 μg in 90 μl of Buffer EB.

11) Add 5 μl of DpnI (NEB) and 10 μl of 10 x NEBuffer4 and incubate at 37ºC for ≥1 hour to digest the template DNA.

12) Apply the DNA solution on agarose gel, excise the DNA fragment from the gel, purify by using the QIAquick Gel Extraction Kit and elute with 50 μl of Buffer EB.

13) Check the OD_{260}. If all procedures are successful, the final concentration should be around 150 ng/μl. If the concentration is below 75 ng/μl, precipitate with ethanol and concentrate again, or perform PCR again. If the concentration is about 100 ng/μl, use 3 μl of the eluted solution at STEP 44.

4.3. Confirmation of BAC clones

(Note that the following procedure is for the BAC clones derived from the RPCI-23 library. If you use another library, the antibiotics should be changed accordingly.)

14) The BAC clones derived from the RPCI-23 library are purchased from the BACPAC Resource Center and sent as LB stab stocks. When they arrive, make glycerol stocks and ensure that the clones include the gene of interest by PCR using gene-specific primers.

BAC clone preparation

We routinely use a QIAprep Spin Miniprep Kit. The concentrations of the eluted BAC DNA solutions are low, but sufficient for use as PCR templates. Prepare cultured E. coli at STEP 17. At the elution step, apply 100 μl of pre-warmed (65ºC) Buffer EB to the filter, leave for 1 min at RT and spin at 13,000 rpm for 1 min. If you apply 10 μl of the eluted solution on agarose gel, you can usually observe a faint band (about 10 ng)).

Clone check by PCR

i) Design the PCR primers around the 1st ATG. (Later, the 5' primer is also used as a sequence primer to check the correct recombination of the final construct.

We routinely use the 'Primer3 program'

(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The parameter settings:

Mispriming Library: RODENT AND SIMPLE
Product Size Range: 300 - 600
Primer Size: Min: 20 Opt: 25 Max: 30
Product Tm: Min: 60 Opt: 65 Max: 70
Primer GC%: Min 50 Max: 80
Other parameters: default

ii) Mix on ice

- Template DNA: (BAC mini prep) 1 μl,
  (B6 genome) 1 μl (as a positive control)
- 10 x KOD Dash buffer: 2 μl
- Primers (10 μM): 1 μl each
- 2 mM dNTPs: 2 μl
- KOD Dash polymerase: 0.2 μl
- DMSO: 1 μl
- DW: 10.8 μl / 20μl

iii) PCR conditions

94ºC hold (Easy hot start)
94ºC for 5 min
{94ºC for 30 sec / * XºC for 2 sec / 74ºC for 30 sec} x 35
74ºC for 5 min
4ºC for ∞

iv) Apply 5 μl of PCR product on agarose gel and check the correct amplification.

*We routinely perform touchdown PCR. During the initial ten cycles, the annealing temperature is decreased from 62ºC to 57ºC. Then, we perform PCR for 25 cycles with an annealing temperature of 57ºC.

4.4. Preparation of electrocompetent cells (for 5 tubes)

[Reagents]

LB medium

10 ml x the number of BAC clones

10% Glycerol/DW

LB plate with 20 μg/ml of chloramphenicol (x the number of BAC clones)

We routinely prepare antibiotic-free LB plates (25 ml of LB in a 9-cm Petri dish) and spread 5 μl of chloramphenicol (100 mg/ml) diluted with 45 μl of DW and dry briefly just before use (final concentration should be about 20 μg/ml).

Chloramphenicol (100 mg/ml)

Day –1

15) Streak the E. coli containing BAC clone on an LB plate (+Cm: final 20 μg/ml (1/5000 dilution)) and incubate at 37ºC overnight.

Day 0

16) Pick a single colony and inoculate it into 5 ml of LB medium (+Cm: final 20 μg/ml). Incubate at 37ºC overnight.

Day 1

17) Make glycerol stocks. (Mix 100 μl of the culture and 100 μl of 60% glycerol/DW.) Transfer 3 ml of the culture for the mini-prep (STEP 14).

18) Transfer 100 μl of the culture into 10 ml (+Cm: 20 μg/ml) of LB medium and grow at 37ºC until the cells are at OD_{600} = 0.3 ~ 0.4 (this usually takes about 3 hours; to be sure, check OD_{600} at 60 min and 120 min of culture). During this culture period, prepare 10% of glycerol/DW, 1.5 ml eppendorf tube and a tube stand and keep them on ice for the following procedures.

19) Transfer 1.4 ml of the culture into the eppendorf tube on ice.

20) Spin at 11,000 rpm (11,000 g) for 30 sec at 4ºC. Remove as much supernatant as possible by using a yellow tip.

21) Resuspend the cells in 1 ml of 10% glycerol/DW on ice.

22) Spin at 11,000 rpm for 30 sec at 4ºC. Remove as much supernatant as possible by using a yellow tip.

23) Resuspend the cells in 1 ml of 10% glycerol/DW on ice.

24) Spin at 11,000 rpm for 30 sec at 4ºC. Remove as much supernatant as possible by using a yellow tip.

25) Adjust the final volume to 40 μl with 10% glycerol/DW. Use them for electroporation immediately or freeze them in liquid N_2 and store at -80ºC until use.

4.5. Transformation of pSC101-BAD-gbaA(Tet^r) to BAC containing E. coli

[Reagents]

pSC101-BAD-gbaA (9.7 kb, 60 ng/μl) (Genebridges)

Cuvettes for electroporation (0.1 cm electrode gap) -> on ice

Bio-Rad gene pulser

LB plate (+Cm(20 μg/ml) and Tet(3 μg/ml)) x the number of BAC clones

(We routinely stock antibiotic-free LB plates, spread them with 5 μl of Chloramphenicol and 15 μl of tetracycline diluted with 30 μl of DW and dry briefly just before use.)

Tetracycline (5 mg/ml)

Chloramphenicol (100 mg/ml)

Day 1

26) Thaw competent cells on ice, add 30 ng of pSC101-BAD-gbaA (= 60 ng/μl x 0.5 μl) and transfer to the ice-cold cuvettes.

27) Electroporate the cells at 2.3 kV, 25 μF capacitance and 200 Ω resistance (time const. should be about 5 msec). Place the sample on ice immediately after electroporation.

28) Add 450 μl of LB medium (antibiotic-free), transfer to 1.5 ml eppendorf tube and culture at 30ºC for 70 min. (During this period, we usually spread 15 μl of tetracycline and 5 μl of chloramphenicol on antibiotic-free LB plates and dry them briefly at 30ºC.)

29) Spread 100 μl of the culture on an LB plate (Cm+ and Tet+) and incubate at 30ºC for ≥ 18 hours.

Day 2

30) Colony count (Usually you can observe hundreds of colonies.)

31) Prepare 5 ml of LB medium (Cm+ and Tet+) (Add 3 μl of tet and 1 μl of Cm to 5 ml of LB medium). Pick 4 colonies and incubate at 30ºC for 18 hours.

Day 3

32) Make the glycerol stock for each clone.

33) Transfer 3 ml of the culture and perform a mini-prep to check the correct transformation. We usually store the rest of the cells at RT during the mini-prep for confirmation, or start STEP 35 prior to the confirmation step. For the mini-prep, we usually use QIAprep Spin Miniprep Kit. Elute with 50 μl of Buffer EB, apply 10 μl of eluted DNA on agarose gel and observe a faint band of pSC101-BAD-gbaA (9.3 Kb in size).

34) Choose the clone that produces pSC101-BAD-gbaA most and make it electrocompetent as follows.

4.6. Preparation of electrocompetent cells that obtain BAC clone and pSC101-BAD-gbaA

(for 5 tubes)

[Reagents]

LB medium
10 ml x number of clones
Tetracycline (5 mg/ml)
Chloramphenicol (100 mg/ml)
10% Glycerol
L-arabinose (20%)

35) Transfer 100 μl of E. coli into 10 ml of LB medium (+ Tet: 6 μl and Cm: 2 μl) and grow at 30ºC until the cells reach OD_{600} = ~ 0.2 (about 3 hours; to be sure, also check OD_{600} at 60 min and 120 min of culture).

36) When the cells reach OD_{600} = ~ 0.2, add 75 μl of L-arabinose (20%) to 7.5 ~ 10 ml of culture (final 0.1 ~ 0.2%) to induce the recombination proteins and grow at 37ºC until the cells reach OD_{600} = 0.35 ~ 0.4 (about 45 minutes; to be sure, also check OD_{600} at 30 min).

Put the eppendorf tube, tube stand and 10% glycerol/DW on ice.

37) Transfer 1.4 ml of the culture in each eppendorf tube (usually 5 tubes for one culture).

38) Spin at 11,000 rpm for 30 sec at 4ºC. Remove as much supernatant as possible by using a yellow tip.

39) Resuspend the cells in 1 ml of 10% glycerol/DW on ice.

40) Spin at 11,000 rpm for 30 sec at 4ºC. Remove as much supernatant as possible by using a yellow tip.

41) Resuspend the cells in 1 ml of 10% glycerol/DW on ice.

42) Spin at 11,000 rpm for 30 sec at 4ºC. Remove as much supernatant as possible by using a yellow tip.

43) Adjust the final volume to 40 μl with 10% glycerol/DW. Use them for electroporation immediately or freeze them in liquid N_2 and store at -80ºC until use.

4.7. Electroporation for ET cloning

[Reagents]

ENTR vector backbones (prepared at STEP 13)
Electrocompetent cells (prepared at STEP 34)
Cuvette for electroporation (0.1 cm electrode gap) -> on ice
Bio-Rad gene pulser
LB plate (+Kan: 20μg/ml)
(We usually stock antibiotic-free LB plates and spread them with 25 μl of Kanamycin diluted with 25 μl of DW and dry briefly just before use.)
Kanamycin (20 mg/ml)

Day 1

44) Prepare 0.3 μg of each ENTR vector backbone generated at STEP13.

45) Thaw competent cells on ice, add 2 μl (300 ng) of ENTR vector backbones and transfer to the ice-cold cuvette.

46) Electroporate the cells at 2.3 kV, 25 μF with the Pulse controller set to 200 Ω (time const. should be about 5 msec). Place the sample on ice immediately after electroporation.

47) Add 1 ml of antibiotic-free LB medium, transfer to 15 ml conical tube and grow at 37ºC for 70 min. (During this culture time, spread 25 μl of Kanamycin diluted with 25 μl of DW on antibiotic-free LB plates and dry them briefly.)

48) Spread 100 μl of the culture on an LB plate (+Kan), spin the rest at 11,000 rpm for 30 sec, resuspend in 100 μl and plate that on another LB plate (+Kan).

49) Incubate at 37ºC overnight.

Day 2

50) Colony count (Usually you can observe hundreds of colonies.)

51) Pick the colonies and inoculate into the LB medium (Kan: 1/1000). (We usually pick 24 colonies for each ENTR vector.)

Day 3

52) Mini-prep by a QIAquick Spin Miniprep Kit

53) Check the integrity of plasmid by using enzyme digestion. (We usually use SacI because it cuts both ends of the ENTR vector backbone, as shown in Section 2. *Maps and Enzyme Sites)*

54) Check the sequence using the M13 forward (-20) and M13 reverse primers. If you cannot obtain correctly recombined plasmids, we recommend increasing the number of pick-ups up to 192. When performing the min-prep, mix eight cultures together (0.5 ml of each culture -> total 4 ml), then perform the mini-prep. Elute with 100 μl of Buffer EB and apply 8 μl of the eluted DNA without enzyme digestion. Because the molecular weight of each ENTR clone is usually higher, you can distinguish the correct plasmids from the false ones. If you find the correct plasmids, perform the mini-prep from the individual cultures.

4.8. Multisite GATEWAY

<Reagents>

LR Clonase Plus Enzyme Mix (Invitrogen, #12538-013, store at -80ºC)
(Containing 5 x LR Plus reaction buffer, LR Clonase Plus enzyme mix, ProteinaseK)
nBLUEneo in ENTR (50 μg/μl = 10 fmol/μl)
pDT-A(B.DEST) (60 ng/μl)
Entry clone vectors
LB(+Amp) plate

<Procedure>

55) Mix at RT[1]

- Entry clones (supercoiled, 20-25 fmols each)
  - 5'-ENTR clone vector
  - 3'-ENTR clone vector
  - nBLUEneo in ENTR (50 μg/μl): 2 μl
- pDT-A(B.DEST) (60 ng/μl): 1 μl
- 5 x LR Plus reaction buffer: 4 μl
- DW: to 16 μl

56) Thaw the LR Clonase Plus enzyme mix on ice and vortex it briefly twice.

57) Add 4 μl of LR Clonase Plus enzyme mix to each of the samples above

and mix by vortexing briefly twice.

58) Incubate at 25ºC for 16 hours.

59) Add 2 μl of Proteinase K and incubate at 37ºC for 10 min.

Desalt (by QIAGEN PCR purification kit)

60) Add 30 μl of DW (up to 50 μl).

61) Add 250 μl of Buffer PB, apply to the spin column and spin at 13,000 rpm x 1 min. Discard the flow-throw.

62) Add 750 μl of Buffer PE, spin at 13,000 rpm for 1 min and discard the flow-throw.

63) Spin once again at 13,000 rpm for 1 min.

64) Apply 30 μl of DW to the center of the column and leave at RT for ≥ 30 min.

65) Spin at 13,000 rpm for 1 min.

Electroporation

66) Thaw the TOP10 Electrocompetent cells on ice.

67) Add 2 μl of desalted DNA solution and transfer to the ice-cold cuvette.

68) Electroporate the cells (settings are 2.5 kV, 25 μF, 100 Ω) (time const. Should be about 2 msec), then immediately place on ice.

69) Add 950 μl of antibiotic-free LB medium, transfer to 1.5 ml tube and grow at 37ºC for 1 hour.

70) Spread 100 μl of culture on an LB plate (+Amp) and incubate at 37ºC overnight.

71) Pick colonies (usually 8 colonies for the first trial) -> mini-prep and check sequence.