**Experimental Protocol**

**Reconstitution & Amplification**

The vector is provided as 10 ug lyophilized plasmid DNA. Reconstitute the DNA in 100 µL dH2O (final concentration 100 ng/µL). Transform 1 ul of the DNA using 20 ul of high efficiency competent E. coli cells (ideally 1x108 CFU/ug). Following transformation, resuspend cells in 200 uL LB media. Plate the entire transformation reaction on a standard LB-agar plate containing 25ug/ml kanamycin for PS100124 vector and 100ug/ml Ampicillin for PS100125 vector. Incubate at 37oC overnight. Pick at least 4-8 independent colonies to do mini-prep from each transformation. Confirm the insert by restriction digestion and/or vector primer sequencing.

\*OriGene offers specific sequencing primers for PS100124 and PS100125 (CAT# PRIMER, including VP1.5 and XL39 primers).

**Cloning of OriGene ORF into the Tet-ON inducible Vectors (PS100124 and PS100125)**

OriGene offers ORF clones for every human, mouse and rat genes in the pCMV6-Entry (CAT# PS100001) vector, which has compatible cloning sites with all-in-one Tet-ON inducible vectors (CAT# PS100124 or PS100125). To transfer the insert gene from OriGene’s ORF clone (donor) into the new inducible vector (recipient), choose the appropriate cloning sites and follow the general cut-and-paste subcloning protocols as below.

1. Digest the donor ORF clone:

|  |  |
| --- | --- |
| Components | Volume |
| 10X restriction buffer | 2 µl |
| Sgf I (10 U/μl) | 0.6 µl |
| Mlu I (10 U/μl) | 0.6 µl |
| nuclease-free water | 13.8 µl |
| Donor ORF clone (200 ng) | 3 µ |
| Total volume | **20 µl** |

Incubate at 37o C for 3 hrs.

1. Digest the inducible Tet-ON destination vector:

|  |  |
| --- | --- |
| Components | Volume |
| 10X restriction buffer | 2 µl |
| Sgf I (10 U/μl) | 0.6 µl |
| Mlu I (10 U/μl) | 0.6 µl |
| nuclease-free water | 13.8 µl |
| Tet-ON destination Vector (200 ng) | 3 µ |
| Total volume | **20 µl** |

\* For the 4% of the clones that have internal Sgf I or Mlu I sties, please use the appropriate combination of restriction sites as recommended by OriGene.

Incubate at 37oC for 3 hrs. Add 0.5 μl antarctic phosphatase (units used according to the manufacturer’s protocol) to the digestion, and continue to incubate at 37oC for an additional 30 minutes.

3. Purify the digestion using a commercial PCR purification column and elute in 20 ul 10 mM Tris.

4. Set up a ligation reaction:

|  |  |
| --- | --- |
| Components | Volume |
| 10X T4 DNA ligation buffer | 1 µl |
| T4 DNA Ligase (4U/ µl) | 0.75 µl |
| Nuclease-free water | 3.25 µl |
| Digested DNA from Step 1 (ORF clone) | 2 µl |
| Digested DNA from Step 2 (Tet-ON vector) | 3 µl |
| Total Volume | **10 µl** |

 5. Incubate the ligation reaction at room temperature for 1 hour.

6. Transform the ligation reaction into high-efficiency, competent E. coli cells (≥ 1×108 CFU/µg DNA) following the appropriate transformation protocol. Plate the transformants on LB-agar plates supplemented with either 100 µg/ml ampicillin for PS100125 or 25 μg/ml kanamycin for PS100124.

7. Pick at least four colonies for subsequent DNA purification and screening. Amplify and purify the selected clone(s) by growing overnight in liquid LB containing the corresponding antibiotics (ampicillin or Kanamycin), then isolating the DNA using standard plasmid purification procedures.

8. Confirm the insert by restriction digestion and/or vector primer sequencing using the provided V1.5 for 5’ end sequencing and XL39 for 3’ end sequencing.

**Cloning of ORFs into Inducible Tet-ON vector (PS100124 and PS100125) using PCR Amplification**

The ORF sequence can be PCR amplified and cloned into the inducible Tet-On vectors (PS100124 and PS100125). In order to append cloning sites to the 5’ and 3’ ends of the ORF sequence, you need to add the target sequences of the selected restriction enzymes to the forward and reverse PCR primers; examples are shown below.

Forward primer with Sgf I

5’ GAGGCGATCGCCNNNNNNNNNNNNNNNNNNNNNNN 3’

Ns represent the sequence of the ORF beginning with the start codon, ATG. It is important to add the additional “C” base after the Sgf I site to maintain appropriate reading frames with N-terminal tags in some destination vectors.

Reverse primer with Mlu I

5’ GCGACGCGTNNNNNNNNNNNNNNNNNNNNNNNN 3’

Ns represent the reverse complement of the ORF sequence starting with the stop codon for N-terminally tagged or untagged destination vectors. This ensures that the expressed fusion protein will end at the native C-terminal end of the ORF. For C-terminally tagged vectors, the reverse complement of the ORF sequence should start with the second-to-last codon, as the stop codon must be removed to generate a fusion protein.

If the recognition sites for Sgf I or Mlu I are present internally in the ORF, another rare cutter such as Asc I, Rsr II or Not I can be used in the cloning strategy. In these cases, the sequences of these alternate restriction sites should be used in place of Sgf I and/or Mlu I (examples below). This same primer design strategy described above should be used for the design of other primers. The Ns in the forward primer represent the sequence of the ORF beginning with the start codon, ATG. The Ns in the reverse primers represent the reverse complement of the ORF sequence starting with the stop codon for N- terminally tagged or untagged destination vectors, or starting with the second- to-last codon for C-terminally tagged vectors.

Forward primer with Asc I:

5’ GCCGGCGCGCCANNNNNNNNNNNNNNNNNNNNNNN 3’

An extra nucleotide after Asc I is important to maintain reading frames with N- terminal tags in some destination vectors.

Reverse primer with Rsr II:

5’ GCGTCGGTCCGCTNNNNNNNNNNNNNNNNNNNNNNNN 3’

Extra nucleotides after Rsr II are important to maintain appropriate reading frames with C-terminal tags in some destination vectors.

Reverse primer with Not I:

5’ GCGACGCGGCCGCGTACGCGTNNNNNNNNNNNNNNNNNNNNNNNN 3’

Mlu I is also added for downstream subcloning.

We recommend using a full-length cDNA clone as the template for ORF cloning. The success rate is low when a cDNA pool is used as the template for a PCR cloning reaction. When the GC content of an ORF (or a region of the ORF longer than 100 bp) is above 75%, a special PCR buffer with DMSO or other additive should be used to increase the success rate. The recommended PCR polymerase and buffer are available from New England Biolabs (Phusion™High-Fidelity PCR Kit, F-553S).

**PCR reaction setup:**

|  |  |
| --- | --- |
| Component | Volume |
| 5X PCR buffer | 4 µl |
| dNTPs (2.5 mM each) | 1.6 µl |
| Phusion polymerase (2U/ µl) | 0.2 µl |
| Nuclease free water | 11 µl |
| Forward Primer (10 µM) | 0.6 µl |
| Reverse Primer (10 µM) | 0.6 µl |
| cDNA template | 2 µl (50-100ng plasmid) |
| Total Volume | **20 µl** |

All of the components should be kept on ice. When setting up multiple reactions, a master mix can be prepared without cDNA template or primers. After aliquoting the master mix, the cDNA template and primers can be added individually to each tube.

**PCR cycling conditions**:

The optimum Tm for annealing should be 55-60°C. The extension time depends upon the length of the ORF. The following program is generally used for ORFs from 500 bp-4000 bp.

1 cycle of 95 ͦ C 1 min

2 cycles of 95 ͦ C 10 sec

 62 ͦ C 20 sec

 72 ͦ C 4 min

2 cycles of 95 ͦ C 10 sec

 60 ͦ C 20 sec

 72 ͦ C 4 min

2 cycles of 95 ͦ C 10 sec

 58 ͦ C 20 sec

 72 ͦ C 4 min

15 cycles of 95 ͦ C 10 sec

 56 ͦ C 20 sec

 72 ͦ C 4 min

Final extension 72oC 10 min 4 ͦ C hold

 **Induction and expression testing**

Tet-OFF system can be induced by both tetracycline and doxycycline, but the Tet-ON system only responds to the doxycycline (Dox), not tetracycline (Gossen & Bujard, 1995). Also doxycycline is more stable in the culture medium (48 hours versus 24 hours for tetracycline) and appears to be more potent than tetracycline. In vitro 0.01–1.0 µg/ml of Dox has been shown to induce Tet-ON expression within 48 hours. Titration of different Dox concentration, as well as determination of the optimum incubation period to achieve maximal expression, is recommended for each individual target gene.

Here, we describe a protocol to test our Tet-On systems (CAT# PS100124 and PS100125). Our test was conducted on HEK 293 cells, however, when using different cell lines, we recommend using this protocol as a reference while taking into consideration cell culture requirements, transfection method, etc., that are appropriate to the host cell line you will be experimenting on.

1. 24 h prior to transfection, 3x104 HEK293 cells/well were seeded on to a 96 well plate in growth media consisting of DMEM supplemented with antibiotics and 10% FBS.

 2. On the following day, cells were transfected with 0.2 ug of Tet-ON vector (Cat#PS100125) per well using Turbofectin transfection reagent (TF81001, OTI) at 1:3 ratio (DNA:Turbofectin reagent).

3. 24 h post transfection 4 different dosages of Dox (NC0424034, Fisher Scientific) was tested by adding 0.01, 0.1, 1.0, and 10 (ug/ml) in duplicate wells for each dosage respectively. Doxuntreated wells served as control.

4. Post treatment with Dox, Tet-On induced GFP fluorescence in cells was scanned with a fluorescence plate reader on three consecutive days (in case of CAT# PS100124, perform IHC on the transfected cells with Anti-DDK antibody and assay for myc-DDK tag expression). Raw florescence readouts expressed in arbitrary units (a.u.) for each dosage of Dox treatment was compared to the control group (Dox-untreated) to determine the induction efficiency of our TetON system (CAT# PS100125).