

pTUNE Inducible Vector

Application Guide

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Package contents and Storage Conditions:

The following components are included:

- One (1) vial containing pTUNE vector as 10 ug lyophilized plasmid DNA
- DNA sequencing primers (100 pmol) for pTUNE Inducible vector; dried onto the bottom of tubes
 - pTune-F Forward (5' TAGAGTCGACCTGCAGCCGG 3')
 - pTune-R Reverse (5' TCGCTGATTTGTGTAGGGGA 3')
- A copy of application guide

Store the DNA at room temperature. Once DNA is re-suspended in water or TE, store at -20°C .

The cDNA clone is shipped at room temperature, but should be kept at -20°C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related products:

TrueORF cDNA clones	http://www.origene.com/orf
TrueClone cDNA clones	http://www.origene.com/cdna
HuSH™ shRNA Plasmids	http://www.origene.com/shRNA
Validated Antibodies	http://www.origene.com/antibody
Functional Proteins	http://www.origene.com/protein
Transfection Reagents	http://www.origene.com/cdna/transfection.msp

Introduction

pTUNE Inducible Gene Expression System is an engineered, tunable genetic switch that tightly regulates gene expression in mammalian cells. This switch couples repressor proteins and an RNAi target design to effectively turn any gene off described by Tara Deans et al. (Cell 130, 2007). Unlike other inducible vectors using either tetracycline (tet) repressor or small interfering RNAs and having a certain degree of leakiness, pTUNE Inducible vector combines repression at a lactose operon (*lac*) with a tet-controlled promoter using RNA interference as a means to achieve tight inhibition.

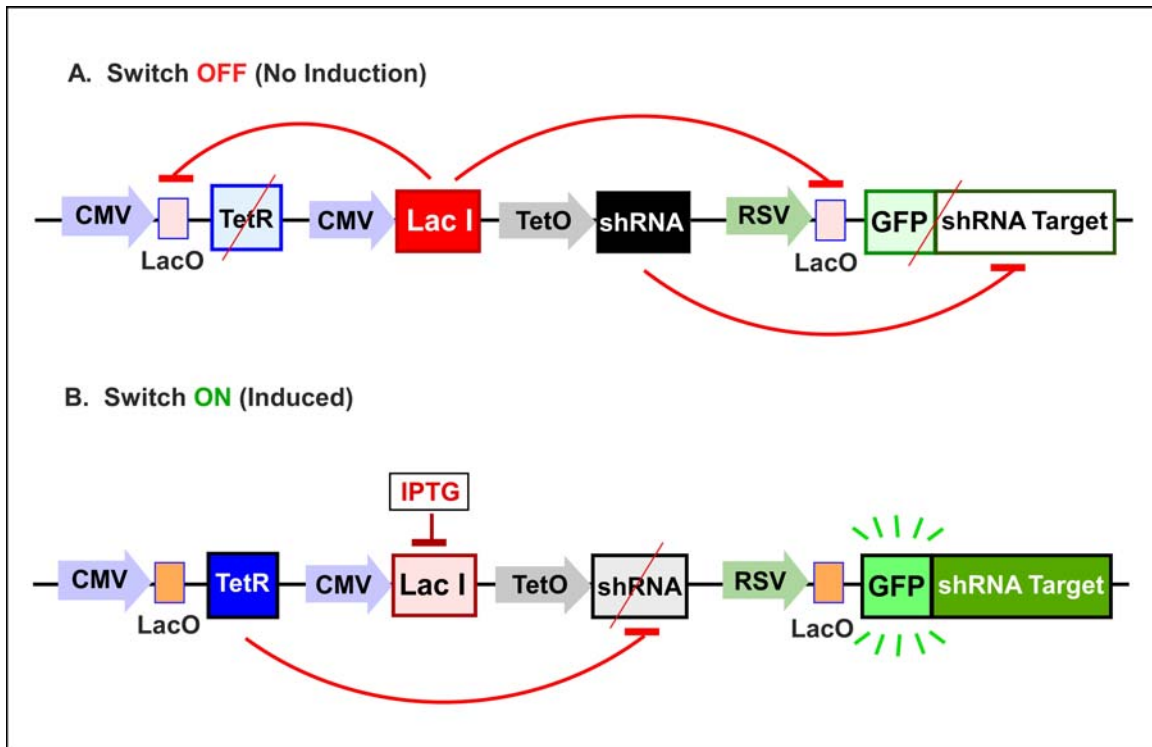


Figure 1. Schematic Diagrams of pTUNE Inducible vector.

(A) In the switch off state, repressor proteins (LacI) are expressed and work on the lac operator sites downstream of the RSV and CMV promoters. This results in repression of gene expression (shown here as GFP). The LacI repressor proteins also work on the lac operator sites within the tetR repressor module, which results in repression of TetR expression. As the result of repression of TetR, shRNA is transcribed by the U6 promoter and complementarily works on the target sequence located in the 3' end UTR of the gene as well as prevents any residual gene expression. **(B)** In the switch on state, isopropyl-β-thiogalactopyranoside (IPTG) binds to the LacI proteins, resulting in a conformational change in the repressor proteins. The addition of IPTG causes the release of repressor proteins from the lac operator sites, which allows for the transcription of the gene of interest and tetR. In the meantime, the Tet repressor proteins bind to the tet operator site located in the U6 promoter of the RNAi, shutting down the transcription of the inhibitory shRNA.

The advantages of this inducible Gene Expression System are:

- Tightly regulate gene expression.
- The gene expression can be fine-tuned with different IPTG concentrations.
- The switch is reversible.
- It can be used with any gene of interest.
- Express tagged protein

- Compatible with OriGene's 37000 (and growing daily) TrueORF cDNA clones. The gene of interest can be easily shuttled from any of the TrueORF clones.

Figure 2. Inducible expression of tGFP in the pTUNE Inducible vector. HEK293 cells transiently transfected with 100 ng of pTUNE-tGFP plasmid expressing tGFP in a 96 well culture plate. (A) In the absence of the IPTG for 72 hours. (B) In the presence of the 25 μ M IPTG for 72 hours.

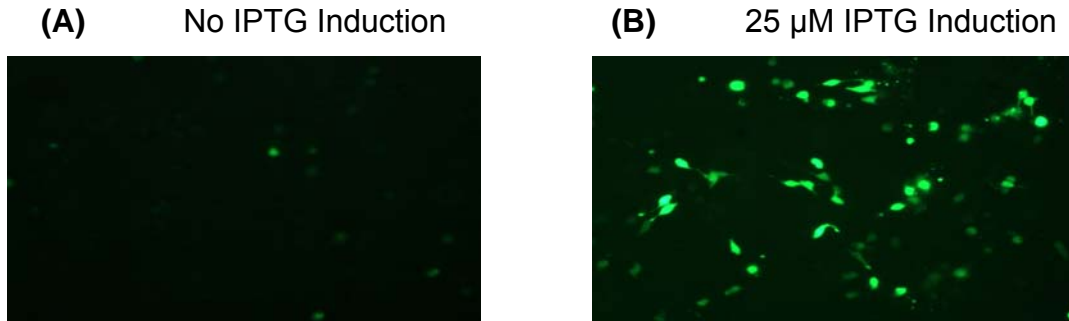
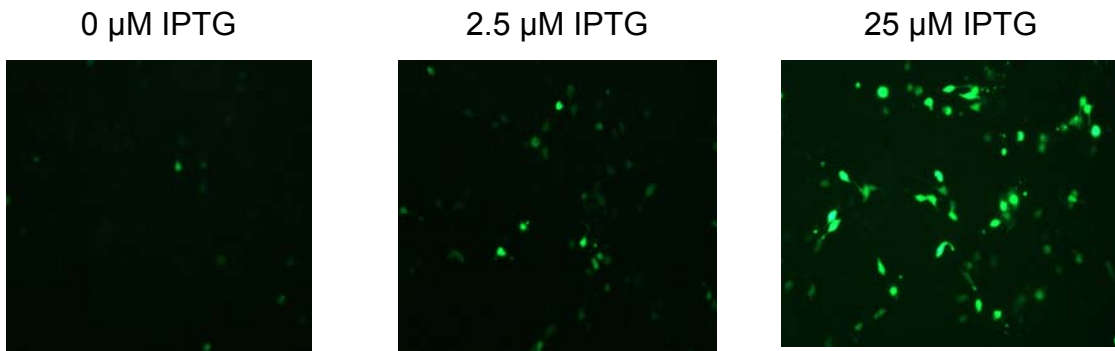
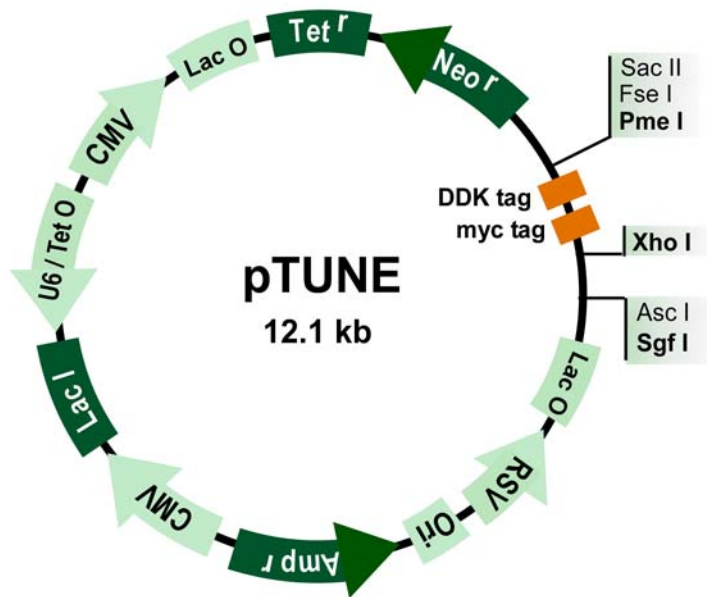


Figure 3. Gene Expression in pTUNE inducible vector can be controlled in a tunable way. pTUNE-tGFP was transiently transfected in HEK293 cells and incubated in the presence of IPTG of various concentrations.



pTUNE Inducible Vector Information

Figure 4. Schematic of the pTUNE Inducible Vector and its Multiple Cloning Sites.



pTune_F *Kpn I* RBS Kozac Consensus *Sgf I* *Asc I*
 CCTCTAGAGTCGACCTGCAGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGCGATCGCCGGCGCG

Rsr II * *Mlu I* * *Xho I* Myc Tag
 CCAGATCTCAAGCTTAACTAGCTAGCGGACCG ACG CGT ACG CGG CCG CTC GAG CAG AAA CTC ATC TCA
T R T R P L E Q K L I S

GAA GAG GAT CTG GCA GCA AAT GAT ATC CTG GAT TAC AAG GAT GAC GAC GAT AAG DDK Tag *Pme I*
E E D L A A N D I L D Y K D D D D K V Stop

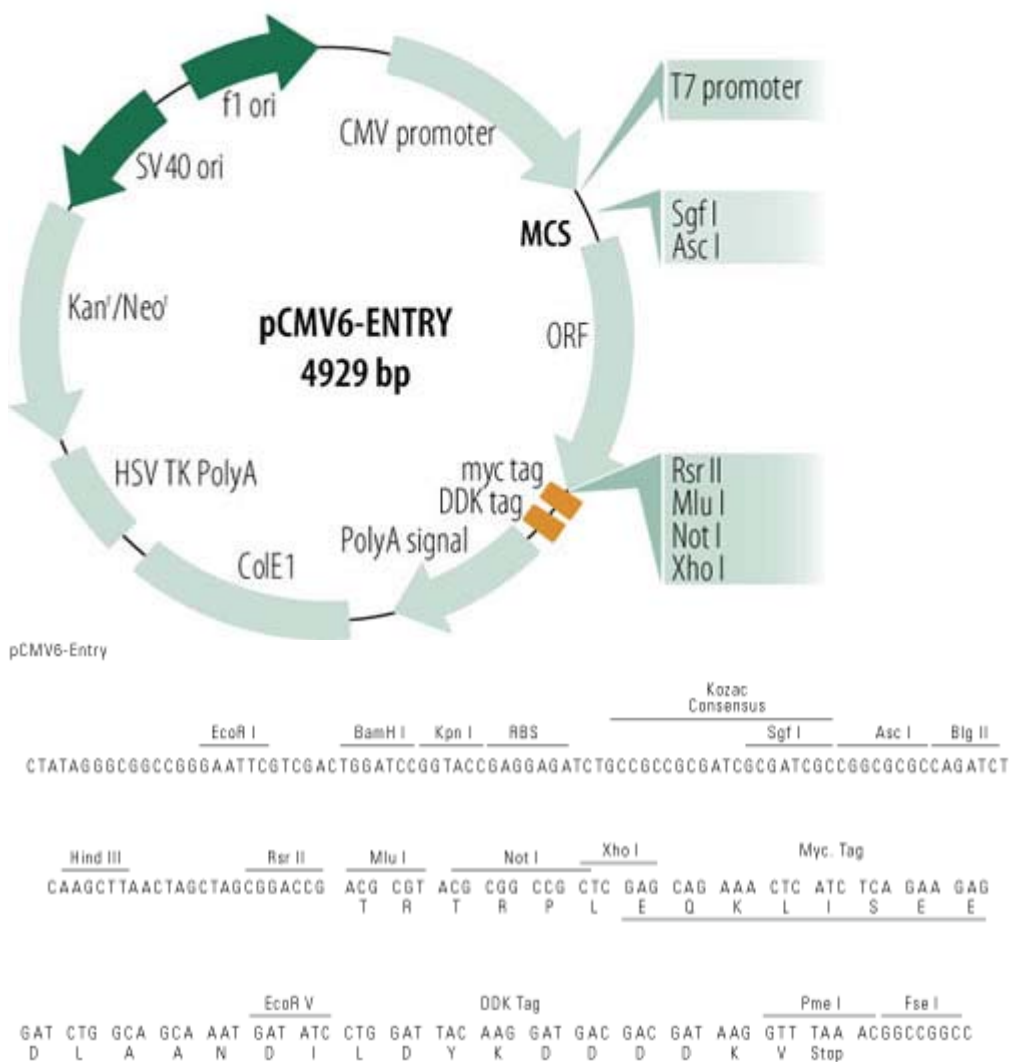
Fse I *Sac II* pTune_R
ACGGCCGGCCGGTCATAGCTGTTTCCTGAACAGATCCCCTACACAAATCAGCGATTTT

* Do not use it as the cloning site.

TrueORF Entry Vector Information

Figure 5. Schematic of the pCMV6-ENTRY Vector and its Multiple Cloning Sites.

pTUNE Inducible vector enables expression of the gene of interest as a C-terminal Myc and Flag (DDK) tagged protein, which facilitates multiple downstream applications that utilize an anti-tag antibody, such as protein detection, protein purification, subcellular localization, etc. All ORF (Open Reading Frame) inserts are cloned into an OriGene pCMV6-Entry vector and therefore can be easily shuttled by a simple 'cut-and-paste' mechanism into pTUNE Inducible vector.



Protocols

There are several ways you can clone the gene of interest into OriGene pTUNE Inducible vector.

- Shuttle the gene of interest from OriGene TrueORF cDNA clone into pTUNE Inducible vector. Now, there are 37,000 TrueORF cDNA clones in OriGene TrueORF collection database. These clones can be purchased and shuttled into pTUNE vector via a simple restriction-based cut and ligation. (PrecisionShuttle System). OriGene also provides the shuttling service.
- Utilize OriGene's TrueORF cloning service for our customer. If you can't find the gene of interest, please submit your request to techsupport (techsupport@origene.com), and then OriGene scientists will clone your gene of interest into your designated vector.
- If you choose to do it yourself, the following is detailed procedures. (Please note that this is only recommended if Sgf I and Xho I site are absent from your target ORF. Otherwise, it would be much easier to purchase the corresponding TrueORF in the pCMV6-Entry vector and then shuttle the insert to pTUNE vector.)

Cloning strategy:

	Restriction Site Combination	Destination Vector
No internal Xho I in ORF	Sgf I (Asc I)/Xho I	pTUNE inducible vector
There is (are) internal Xho I site(s) in ORF	Sgf I (Asc I)/Mlu I (Rsr II, Not I)	PCMV6-ENTRY vector
Shuttle from pCMV6-ENTRY to pTUNE vectors	Sgf I (Asc I)/Sac II (Fse I, Pme I)	pTUNE inducible vector

Directly clone ORF into pTUNE Inducible vector

1. Primer Design and PCR Amplification of ORF

The open reading frame (ORF) of the clone must be PCR amplified in order to append cloning sites to the 5' and 3' ends of the sequence.

Forward primer with Sgf I

5' GAGGCGATCGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

(Ns represent the sequence of the ORF beginning with the start codon, ATG)

Reverse primer with Xho I

5' GCGCTCGAGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

(Ns represent the last 8 codons of the ORF in reverse complement orientation. These do not include the stop codon that must be removed to generate a fusion protein for C-terminally tagged vectors,

We recommend using a full-length cDNA plasmid as the template for ORF cloning. The PCR error is rather high 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 improve the PCR.

The recommended PCR polymerase and buffer are available from New England Biolabs (Phusion™ High-Fidelity PCR Kit, F-553S).

PCR reaction setup:

Component	Volume (µl)
5X PCR Buffer	4.0
dNTPs (2.5 mM each)	1.6 (0.4ul each)
Phusion Polymerase (2U/µl)	0.2
Nuclease Free Water	11.0
Forward Primer (10 µM)	0.6
Reverse Primer (10 µM)	0.6
cDNA Template	2.0 (50-100 ng Plasmid)
Total Volume	20.0

PCR cycling conditions:

Temperature/Time	Cycles
95 °C, 1 min	1
95 °C, 10 sec, 62 °C 20 sec, 72 °C 4 min	2
95 °C, 10 sec, 60 °C 20 sec, 72 °C 4 min	2
95 °C, 10 sec, 58 °C 20 sec, 72 °C 4 min	2
95 °C, 10 sec, 56 °C 20 sec, 72 °C 4 min	15
72 °C 10 min	1 min per kb
4 °C Hold	

2. Prepare the insert

Verify that the size of the amplification product is correct by agarose gel electrophoresis, and purify the remainder of the reaction using a purification column or similar method. Elute the

DNA from the purification column in 26 μ l of 10 mM Tris buffer. Set up a digestion reaction as described below, substituting other restriction enzymes as appropriate.

Component	Volume (μ l)
10X Restriction Buffer	3.0
Sgf I (10U/ μ l)	0.6
Xho I (10U/ μ l)	0.6
Purified PCR Product	26.0
Total Volume	~ 30.0

Mix well, and incubate at 37 °C for 1 hour.

Purify the digestion reaction using a purification column and elute in 18 μ l of 10 mM Tris buffer.

3. Prepare the vector:

Digest pTUNE Inducible vector with the Sgf I and Xho I

Component	Volume (μ l)
10X Restriction Buffer	3.0
Sgf I (10U/ μ l)	0.8
Xho I (10U/ μ l)	0.8
PTUNE Inducible vector	10.0
Nuclease Free Water	15.4
Total Volume	30.0

Incubate at 37 °C for 1.5 hr, then add 0.5 μ l alkaline phosphatase, and continue the incubation at 37°C for another 30 min. A two hour digestion is recommended to ensure that the vector is completely digested. Dephosphorylation of the digested vector is essential to eliminate self-ligation.

Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolating the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 μ l of 10 mM Tris buffer.

4. Set up a ligation reaction with the purified vector and insert fragments:

Component	Volume (μ l)
10X T4 DNA Ligase Buffer	1.0
Nuclease Free Water	3.5
T4 DNA Ligase (4U/ μ l)	0.5
Digested pTUNE Inducible Vector Fragment from Step 3	2.0

Digested PCR Product from Step 2	3.0
Total Volume	10.0

Incubate the ligation reaction at 12 °C overnight.

5. Transform 1 µl of the ligation mixture using 20 µl high efficiency competent E. coli cells (ideally 1×10^8 CFU/ug). Following transformation, resuspend cells in 200 uL LB.

6. Plate the entire transformation reaction on a standard LB-agar plate containing 100 µg/ml Ampicillin. Incubate at 37 °C overnight.

7. Pick at least 4-8 independent colonies from each ligation. Confirm the insert by restriction digestion and/or vector primer sequencing (include in the shipment).

Transfer of ORF from pCMV6-Entry Vector to pTUNE Inducible Vector

The ORF of a C-terminally tagged protein is followed by a double tag (Myc and Flag) including a short spacer (5-6 amino acid residues). The MCS of pCMV6-Entry and pTUNE vectors are similar except some cloning sites in entry vector, such as Mlu I, Rsr II and Not I, are not unique restriction sites in pTUNE vector. Depending on the gene of interest, Sgf I or Asc I at 5' end of ORF and Pme I or Fse I or Sac II at 3' end ORF can be used to transfer entire ORF containing the Myc and Flag tags between pCMV6-Entry and pTUNE vectors. The ORF transfer protocol from pCMV6-Entry vector to pTUNE vector is detailed below.

1. Digest the TrueORF entry clone:

Component	Volume (µl)
10X Restriction Buffer	2.0
Sgf I or Asc I (10U/µl)	0.6
Sac II or Fse I or Pme I (10U/µl)	0.6
ORF cDNA Clone in pCMV6-Entry Vector (100-200ng)	3.0
Nuclease Free Water	13.8
Total volume	20.0

Incubate at 37 °C for 1 hour.

2. Digest the pTUNE Inducible vector:

Component	Volume (μl)
10X Restriction Buffer	2.0
Sgf I or Asc I (10U/μl)	0.6
Sac II or Fse I or Pme I (10U/μl)	0.6
pTUNE Inducible Vector (200ng)	3.0
Nuclease Free Water	13.8
Total Volume	20.0

Incubate at 37 °C for 1 hour. Add 0.4 μl calf intestine phosphatase to the digestion, and continue to incubate at 37 °C 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:

Component	Volume (μl)
10X T4 DNA Ligase Buffer	1.0
Nuclease Free Water	3.25
T4 DNA Ligase (4U/μl)	0.75
Digested ORF Insert in pCMV6-Entry Vector from Step 1	2.0
Digested pTUNE Vector fragment from Step 2	3.0
Total volume	10.0

Incubate the ligation reaction at 12 °C overnight.

5. Transform the ligation reaction into high-efficiency, competent *E. coli* cells ($\geq 1 \times 10^8$ CFU/μg DNA) following the appropriate transformation protocol. Plate the transformants on LB-agar plates supplemented with 100 μg/ml ampicillin.

6. Pick at least six colonies for subsequent DNA purification and screening. Amplify and purify the selected clone(s) by growing overnight in liquid LB-amp media, then isolating the DNA using standard plasmid purification procedures.

7. Confirm the insert by restriction digestion and/or vector primer sequencing using the provided pTune-F for 5' end sequencing and pTune-R for 3' end sequencing.

Protocol for sequencing Primer Re-suspension and DNA Sequencing

Carefully open the tube, and add 10uL of sterile, deionized H2O to the bottom to obtain a 10uM stock. Alternatively, a low TE solution (10mM Tris (8.0), 0.1mM EDTA) is advisable for long-term storage at -20°C.

Close the tube and let sit for at least 10 minutes at room temperature (or overnight at 4°C). After vortexing for 10 seconds, quick spin the tube to bring the contents to the bottom of the tube. The primer stock (10uM) is now ready to be added to a DNA sequencing reaction (1ul=10pmol).

Induced expression

1. Prepare IPTG

Prepare 1M stock of IPTG and dilute it with sterilized water.

2. IPTG Induction

For transient transfections, the cells were induced with desired amount (2.5uM -1mM) of IPTG 1-2 hours after the transfection was done.

For stable transfections, linearize 5ug plasmid DNA with AhdI before transfections. The medium was changed daily containing fresh IPTG at the desired dilution.

Troubleshooting

For questions not addressed here, please contact OriGene's Technical Support professionals. You may dial 888-267-4436 from any US location, or 301-340-3188 outside the US. E-mail inquiries to techsupport@origene.com are also invited.

No colonies or low number of colonies from transformation

Cause	Remedy
The competent cells used in the transformation were not as efficient as necessary.	Obtain a fresh batch of competent cells and ensure that the efficiency is $\geq 1 \times 10^8$ CFU/ μ g DNA by performing a separate transformation reaction with a transformation-qualified control (usually a fixed amount of supercoiled plasmid such as pUC19). In some extreme cases, especially for larger inserts (>5 kb), higher efficiency cells or electroporation may be required. Should a gene prove to be toxic to the cells, transforming into strains that reduce the copy number can increase the odds of

	obtaining colonies (i.e. ABLE-C or ABLE-K strains; Stratagene, La Jolla CA).
Too little DNA was used in the transformation reaction.	Add more DNA (but not more than 10% of the volume of competent cells used).
The ligation of the ORF donor DNA into the recipient plasmid was not successful.	The ligase enzyme may not work properly. Repeat the reaction with fresh ligase and ligation buffer (which contains the temperature-sensitive component, ATP) or perform troubleshooting as recommended by the manufacturer of the ligase.
The wrong antibiotic selection plate was used.	Make sure to use an LB-agar plate containing the correct antibiotics (e.g. ampicillin for pTUNE Inducible vector and kanamycin for entry vector).

Too high self-ligation background (no insert) from destination vector

Cause	Remedy
The pTUNE Inducible vector was not completely digested.	Allow the digestion reaction to continue for 1-2 hours at 37°C.
The dephosphorylation of the pTUNE Inducible vector was not complete, and the vector religated with its own fragment.	Increase the concentration of CIP and/or the length of the dephosphorylation incubation as recommended by the ligase manufacturer.

Frequently Asked Questions

Why should I use OriGene's TrueORF clones?

All TrueORF Clones are derived from OriGene's unique TrueClone Collection, and were isolated from high quality cDNA libraries made from a variety of tissues. TrueORF Clones allow customers to directly apply these expression- ready, tagged ORF clones to experiments designed for protein expression, purification, protein-protein interaction and stable clone selection.

TrueORF clones also serve as the entry clone for this pTUNE Inducible system and allow easy construction of this inducible, tagged ORFs. This saves valuable time by eliminating the need for subcloning, verification and amplification. All TrueORF and pTUNE Inducible vectors share the similar multiple cloning sites (MCS). Customers can easily shuffle the cDNA of a TrueORF clone to pTUNE Inducible vector.

What restriction enzymes should I use if Sgf I or Mlu I sites are present in my ORF?

While 96% of all human and mouse ORFs can use the Sgf I - Mlu I combination, some ORFs do contain internal Mlu I site(s). Most of those ORFs with an internal Mlu I site can be transferred using another rare cutter (Rsr II), whose restriction site is upstream of Mlu I, or Not I, whose site is immediately downstream of Mlu I. Using one of the four different subcloning combinations, any ORF can be cloned into pCMV6 Entry vector, and then shuttled to pTUNE Inducible vector using Sac II or Pme I or Fse I.

Can I directly clone ORF into pTUNE Inducible vector?

If no internal Xho I site(s) in ORF, you can use Sgf I/Xho I combination to clone the ORF into pTUNE Inducible vector.

Has OriGene fully sequenced all TrueORF clones?

Not always. When transferring the cDNA into the TrueORF Entry Vector, OriGene always uses fully sequenced plasmids as templates and Phusion High-Fidelity DNA Polymerase (New England Biolabs), which has a mutation rate less than 4×10^{-7} . This ensures the highest fidelity of every TrueORF clone. After cloning into the entry vector, each of OriGene's TrueORF clones was sequenced at both the 5' and 3' ends, and the resulting sequence was matched to the corresponding reference sequence. For many ORFs 1 Kb or less in length, the 5' and 3' sequencing reads have covered the full ORF. For longer cDNAs, the ORF was not fully covered by sequencing reads.

Do TrueORF clones exactly match the reference gene sequence?

All TrueORF clones are guaranteed to match the ORF of the corresponding gene sequences as published on OriGene's website. However, some clones may contain nucleotide changes compared to the published reference sequences. This is due to SNPs (single nucleotide polymorphisms) reflecting the unique differences from genes expressed in different tissues and different individuals. Published references may represent a different SNP than the OriGene transcript. Should a specific SNP be required, this can be contracted from OriGene at an additional charge.

Can I transfer large ORFs using this system?

It has been reported that ORFs larger than 4 Kb are unstable in recombination-based Systems. For OriGene inducible system, several ORFs ranging from 0.7 to 10 kb have been cloned into this inducible vector. The ORF larger than 10 kb has not been tested.

What sites should I use to transfer a TrueORF clone into the Gateway system?

There are multiple sites in pCMV6-Entry than can be used to move the insert of a

TrueORF clone into any of Gateway's entry vectors (pENTR-1A, -2B, -3C, -4 and -11). These sites are EcoR I, Sal I, BamH I and Kpn I at the 5' end, and Not I at the 3' end.

What does your disclaimer mean?

OriGene's disclaimer for the TrueORF clones reads as follows: "Our molecular clone sequence data has been matched to the accession number below as a point of reference. Note that the complete sequence of our molecular clones may differ from the sequence published for this corresponding accession number, e.g., by representing an alternative RNA splicing form or single nucleotide polymorphism (SNP)." The NCBI RefSeq mRNA sequences are continuously being revised, as some may have been derived from aberrantly spliced transcripts or generated by incorrect prediction of intron-exon junctions in silico. These sequences are therefore used only as a "reference" and not as a "standard". OriGene's clones are isolated from full-length cDNA libraries and may differ from the reference sequence for this reason.

What is the TrueORF Guarantee?

OriGene warrants that the product will meet specifications listed. At OriGene's discretion, free replacement of any non-conforming product will be made if OriGene is notified within 30 days of product receipt. If you experience any difficulty with any OriGene product, please contact our Technical Support Staff at 888-267-4436, or 301-340-3188 outside the US.

References

Deans, T.L. *et al.* A tunable genetic switch based on RNAi and repressor proteins for regulating gene expression in mammalian cells. ***Cell* 130, 363–372 (2007)**

Rusk, N. Switching genes off—all the way, ***NATURE METHODS* 4 (9), 684-685 (2007)**