PCR protocol for generating optimized templates for Pierce Human *In Vitro* Expression Kits

TR0072.1

Introduction

The Thermo Scientific Pierce Human In Vitro Protein Expression System enables protein expression from DNA or mRNA templates in a cell-free extract containing essential components of the human translational machinery. These extracts sustain the synthesis of target proteins from DNA templates for up to 6 hours without the need to remove inhibitory byproducts. Current *in vitro* expression systems require that the gene be cloned into an optimized expression vector. Our human expression system includes the pT7CFE1-CHis expression vector for optimal protein production by the traditional cloning route.

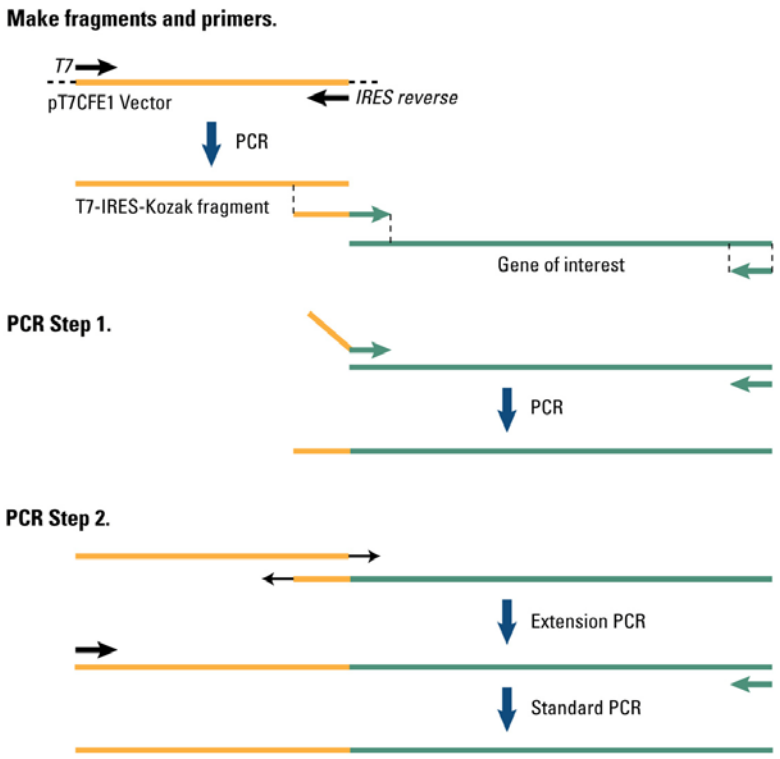
In addition, our system also is compatible with a PCR-based method to generate a linear DNA template containing the upstream and downstream elements required for optimal expression. This method is effective using any plasmid or open reading frame. The upstream elements added to a gene by this PCR method include a T7 promoter, an internal ribosome entry site (IRES) and a Kozak sequence. To improve mRNA stability, a poly(A) sequence (21nt) also can be added to the 3´ end of the gene. The PCR product can be either purified or added directly to the transcription mix for mRNA synthesis. This mRNA template is then added to the translation mix that contains all the machinery for protein expression.

PCR strategy for generating templates for *in vitro* translation.

Make fragments and primers: First, the necessary fragments and PCR primers are prepared or obtained. T7 and IRES primers can be used to amplify a 600bp T7-IRES-Kozak fragment from the pT7CFE1 vector (Part No. 88860). Also, gene-specific “overlap” primers are designed and synthesized.

PCR Step 1: Next, the overlap primers are used to amplify the gene with leading Kozak sequence and terminal polyA sequence.

PCR Step 2: T7-IRES-Kozak fragment is used to anneal and fully extend the previously amplified gene sequence. The T7 primer and anti-sense overlap primer are then added for 30 PCR cycles to amplify the full-length DNA template.



Pierce Biotechnology
3747 N. Meridian Road
PO Box 117
Rockford, IL 61105 USA
(815) 968-0747
(815) 968-7316 fax
www.thermoscientific.com/pierce

Generate linearized T7-IRES-Kozak fragment from pT7CFE1-CHis

A. Materials Required

- pT7CFE1-CHis (Part No. 88860)
- Upstream T7 primer: 5´- AACGACGGCCAGTGAATTGTAATA
- IRES Reverse primer: 5´- ATGGGTGGTGGCCATATTATCATC
- PCR Mix: complete, high-fidelity PCR mix (e.g., Invitrogen* Platinum PCR SuperMix High Fidelity)

B. PCR Protocol

1. Prepare 50µL PCR mixture:

Component	Stock Concentration	Volume per 50µL reaction
pT7CFE1-CHis	0.5µg/µL	1µL
Upstream T7 primer	100µM	1µL
IRES Reverse primer	100µM	1µL
PCR Mix	1.1X	47µL

2. PCR amplify gene of interest with target-specific sense and antisense primers described above.

Cycle Step	Temp	Time	No. of cycles
Initial denature	95°C	1 min	1
Denaturation	95°C	30 sec	30
Annealing	45 to 68°C (increase 0.8°C per cycle)	30 sec	
Extension	68°C	1 min/1000bps	
Final extension	68°C	5 min	1

3. Gel purify the resulting PCR fragment using gel extraction kit (e.g., Qiagen* QIAquick* Gel Extraction Kit)

Generate complete DNA template using two-step PCR protocol (4 to 6 hours)

A. Materials Required:

- DNA template for gene of interest
- Target-specific sense and antisense PCR primers (100µM stocks) (see next section)
- T7-IRES-Kozak fragment: purified 600bp PCR product generated above (approx. 200ng/µL)
- Upstream T7 primer: 5´- AACGACGGCCAGTGAATTGTAATA (100µM stock)
- Gel extraction kit (e.g., Qiagen* QIAquick* Gel Extraction Kit)
- PCR Mix (e.g., Invitrogen* Platinum PCR SuperMix High Fidelity)

B. PCR Step 1: Amplify of gene of interest with required overlapping sequences:

1. Design and obtain a sense primer with a 24nt sequence that overlaps with the T7-IRES-Kozak fragment and an 18nt sequence (gray Xs) that overlaps with the 5' region of your gene of interest (including the start codon sequence ATG):

2. Design and obtain an antisense primer for your gene of interest (gray Ys) that adds a 21nt poly T sequence (resulting in an mRNA with a poly A tail). If desired, include the antisense sequence for an affinity tag, such as HA or His, between the end of the gene and the poly A tail:

3. PCR amplify gene of interest with target-specific sense and antisense primers described above.
4. Gel purify the resulting PCR fragment using a gel extraction kit.

C. PCR Step 2: Anneal T7-IRES-Kozak fragment and amplify complete DNA template.

1. Prepare initial 48µL PCR mixture:

Component	Volume
T7-IRES-Kozak fragment (600bp)	2µL
Gene of interest with overlaps (from PCR Step 1).	2µL
PCR Mix	44µL

2. Perform PCR for 10 cycles:

Cycle Step	Temperature	Time	No. of cycles
Initial denature	95°C	1 min	1
Denaturation	95°C	30 sec	10
Annealing	45 to 68°C (increase 0.8°C per cycle)	30 sec	
Extension	68°C	1 min/1000bps	
Final extension	68°C	5 min	1

3. Add Upstream T7 primer and antisense gene primer:

Component	Stock Concentration	Volume
Upstream T7 primer	100µM	1µL
Antisense primer for gene of interest	100µM	1µL

4. Continue PCR for 30 additional cycles:

Cycle Step	Temperature	Time	No. of cycles
Initial denature	95°C	1 min	1
Denaturation	95°C	30 sec	30
Annealing	45 to 68°C (increase 0.8°C per cycle)	30 sec	
Extension	68°C	1 min/1000bps	
Final extension	68°C	5 min	1

5. Proceed directly to the Human *In Vitro* Protein Expression Kit procedure or store the PCR product overnight at -20°C for use the next day.

   Note: The resulting solution contains sufficient full-length DNA template for multiple transcription and translation reactions with one of the Thermo Scientific Pierce Human *In Vitro* Protein Expression Kits. Add 1µL (between 250-500ng) of final PCR product to each 25µL transcription reaction.

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