



RTS™ pIVEX Wheat Germ His₆-tag Vector Set Manual

For high-level expression of His₆-tagged proteins in the cell-free RTS
Wheat Germ CECF system

RTS pIVEX Wheat Germ His₆-tag Vector Set, April, 2015

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The continuous-exchange cell-free (CECF) technology applied in the RTS 100 Wheat Germ, RTS 500 Wheat Germ, RTS 100 Disulfide, RTS 500 Disulfide, RTS 500 E. coli and RTS 9000 E. coli products is based on patented technology (U.S. Patent 5,478,730). The purchase price of this product includes practicing a cell-free expression achieving continuous production of a polypeptide in the presence of a semi-permeable barrier and related processes described in said patents.

Contents

Product specifications	4
Product description	4
Product limitations	4
Materials supplied	4
Shipping and storage conditions	4
Safety information	5
Quality assurance	5
Product warranty	5
Protocols	6
Product principle	6
Protocol 1: Cloning into RTS pIVEX Wheat Germ vectors via restriction sites	8
Protocol 2: Cloning procedure	11
Protocol 3: In-Fusion cloning into pIVEX WG vectors	15
Protocol 4: Detection of expressed His ₆ -tagged proteins	16
Supporting information	17
Additional information for restriction enzyme-mediated cloning	17
Vector maps	20
Typical results	22
References	22
Troubleshooting guide	23
Ordering information	27

Product specifications

RTS pIVEX Wheat Germ vectors are designed for high-level expression of His₆-tagged proteins in the cell-free RTS Wheat Germ Continuous Exchange Cell-Free (CECF) system.

Product description

The RTS pIVEX Wheat Germ His₆-tag Vector Set provides all regulatory elements necessary for *in vitro* protein expression based on a combination of T7 RNA polymerase and wheat embryo lysates.

Product limitations

The RTS pIVEX Wheat Germ His₆-tag Vector Set is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials described in this text.

Materials supplied

Vial	Contents and use
Ordering number	BR1401301
pIVEX 1.3 WG;	→ 10 µg (20 µl) plasmid DNA
His ₆ -tag Vector Set (vial 1)	→ Cloning vector with C-terminal His ₆ -tag → Contains a multiple cloning site (MCS)
pIVEX 1.4 WG;	→ 10 µg (20 µl) plasmid DNA
His ₆ -tag Vector Set (vial 2)	→ Cloning vector with cleavable N-terminal His ₆ -tag → Contains a multiple cloning site (MCS)

Shipping and storage conditions

The RTS pIVEX Wheat Germ His₆-tag Vector Set is shipped on dry ice. Vectors are stable at -20°C in the dark until the expiration date printed on the label. Repeated freezing and thawing decreases the amount of supercoiled plasmid.

Safety information

All due care and attention should be exercised in the handling of this product. We recommend all users of biotechrabbit products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines. Specifically, always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

Neither of the vials contains hazardous substances in reportable quantities. The usual precautions taken when handling chemicals should be observed. Used reagents can be disposed of in waste water in accordance with local regulations. In case of eye contact, flush eyes with water. In case of skin contact, wash off with water. In case of ingestion, seek medical advice.

Quality assurance

biotechrabbit products are manufactured using quality chemicals and materials that meet our high standards. All product components are subjected to rigorous quality assurance testing process:

- **Component testing:** each component is tested to ensure the composition and quality meet stated specifications.
- **Performance testing:** each product is tested to ensure it meets the stated performance specification.

Additional quality information is available from www.biotechrabbit.com. Certificate of analysis sheets for biotechrabbit products can be obtained on request.

Product warranty

biotechrabbit is committed to providing products that improve the speed, ease-of-use and quality of enabling technologies.

biotechrabbit guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use.

This warranty is in place of any other warranty or guarantee, expressed or implied, instituted by law or otherwise. biotechrabbit provides no other warranties of any kind, expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall biotechrabbit be responsible for any direct, indirect, consequential or incidental damages or loss arising from the use, misuse, results of use or inability to use its products, even if the possibility of such loss, damage or expense was known by biotechrabbit.

Protocols

Product principle

Introduction

Cloning into rapid translation system (RTS) pIVEX Wheat Germ His₆-tag vectors allows optimal protein expression in all RTS 100 and RTS 500 Wheat Germ CECF Kits (see Ordering Information). The introduction of either an N- or C-terminal His₆-tag provides a rapid method to detect and purify proteins of interest.

Before designing a strategy for cloning into RTS pIVEX Wheat Germ vectors, consider this alternative for RTS template generation.

At the start of most expression projects, a gene-specific PCR performed on the cDNA encoding the protein of interest will be required (e.g. to add restriction sites needed for subsequent cloning). Since cell-free expression offers the possibility of expressing proteins directly from PCR fragments, an interesting concept at this point is to first screen for expression by directly using one (or a whole set) of these PCR fragments (linear expression templates) in RTS 100 small-scale reactions (e.g. RTS 100 Wheat Germ CECF Kit, biotechrabbit). Regulatory regions necessary for transcription and translation control can easily be added to the cDNA of interest by overlap-extension PCRs carried out using the RTS Wheat Germ LinTempGenSet (biotechrabbit), with the products of the first gene-specific PCR as template (Figure 1). Cloning into a pIVEX vector (e.g. to produce a template for expression scale-up, or to ensure a well characterized (sequenced) and stabilized template is used in subsequent experiments) can be done at a later step in the expression project, for example, using standard cloning via restriction sites (see Protocols).

A time-saving and more convenient alternative to standard cloning, especially for the simultaneous processing of multiple cDNAs in parallel, is based on the In-Fusion™ PCR Cloning technology (Clontech).

Description of procedure

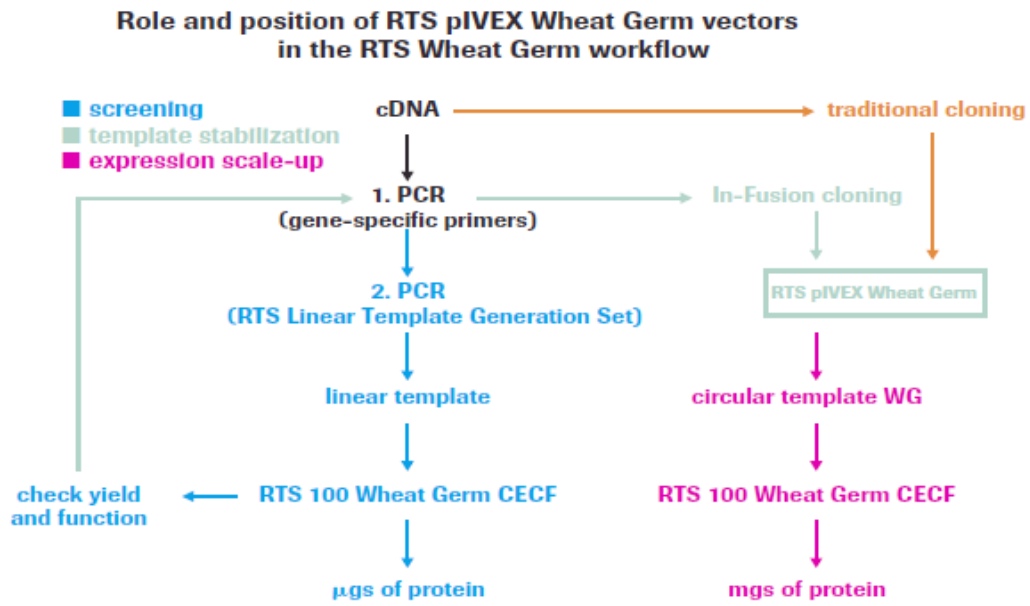


Figure 1. Role of pIVEX Wheat Germ vectors (boxed) in the RTS Wheat Germ system.

Protocol 1: Cloning into RTS pIVEX Wheat Germ vectors via restriction sites

Vector description

- pIVEX is the abbreviation for *In Vitro* **EX**pression
- The first number indicates the basic vector family
- The second number indicates the type and position of the tag
 - Even numbers indicate tags fused to the N-terminus
 - Odd numbers indicate tags fused to the C-terminus

Use and location of His₆-tag

Two different vectors are contained in the set. The general architecture is shown in Figure 2. Both vectors contain a sequence encoding a stretch of six consecutive histidines. This hexa-histidine (His₆-) tag allows easy detection (see Protocol 4, page 16) and purification of the expressed protein.

- Use pIVEX 1.3 WG for fusing the gene to a C-terminal His₆-tag.
- Use pIVEX 1.4 WG for fusing the gene to an N-terminal His₆-tag.

For native expression without tag use pIVEX 1.3 WG and incorporate a stop codon (TAA) at the end of the gene (see 'Vector maps', page 20).

For detailed vector maps, see 'Vector maps', page 20. The complete vector sequences can be viewed and downloaded at www.biotechrabbit.com.

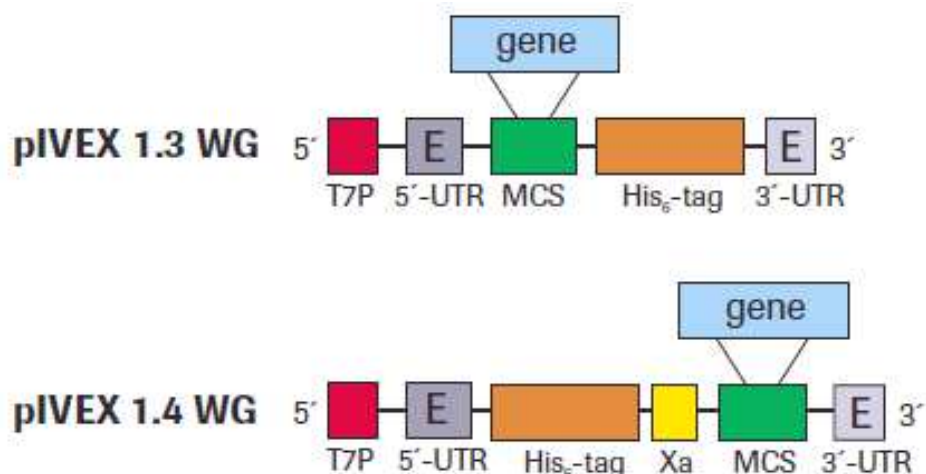


Figure 2. Functional elements of pIVEX Wheat Germ vectors.

T7P: T7 promoter; **UTR:** Untranslated regulatory regions (UTRs) containing optimized translation enhancer (E) elements; **His₆-tag:** Tag sequence at C- or N-terminal position; **Xa:** Factor Xa restriction protease cleavage site; **MCS:** Multiple cloning site in three different reading frames for the insertion of the target gene.

The 5' and 3' UTR regions were designed and optimized specifically for the RTS Wheat Germ system [1]. They correspond to a repetition of a sequence complementary to the 18 S ribosomal RNA (5' UTR) and a sequence derived from TMV (3' UTR), respectively.

Selecting the cloning strategy

In general, the *Nco* I/*Sma* I restriction site combination is recommended for cloning into pIVEX WG vectors, since this approach provides optimal flexibility to switch into all available pIVEX vectors (and also for switching between RTS pIVEX Wheat Germ and RTS pIVEX *E. coli* vectors) and normally results in good expression efficiencies. Once a PCR fragment containing the appropriate restriction sites is prepared, cloning into different pIVEX vectors can be done easily in parallel or successively.

To minimize problems, biotechrabbit recommends selecting the cloning strategy strictly according to Table 1. For cloning strategies allowing the minimization of additional amino acids added to the N-terminus of an expressed protein, see 'Supporting information', page 18.

Table 1. Cloning strategies

Issue	Suggestion solution
The target gene has no internal <i>Nco</i> I and <i>Sma</i> I sites	Use <i>Nco</i> I and <i>Sma</i> I sites for cloning. Note: The second amino acid will be changed in most cases. Design primers according to the example given in 'Supporting information', page 17.
The target gene has an internal <i>Sma</i> I site (generates blunt ends)	Use an alternative blunt end restriction site in the reverse primer that does not cut inside the target gene (e.g. <i>Eco</i> RV, <i>Ssp</i> I, <i>Sca</i> I). Cut pIVEX 1.3 WG or pIVEX 1.4 WG with <i>Nco</i> I and <i>Sma</i> I.
Blunt end cloning at the 3' end should be avoided	Use <i>Xma</i> I, if your gene does not contain an internal <i>Xma</i> I site. <i>Xma</i> I recognizes the same sequence as <i>Sma</i> I but leaves a cohesive (sticky) end. Alternatively, <i>Pin</i> AI, <i>Sgr</i> AI, <i>Bse</i> AI, or <i>Ngo</i> MIV can be used to generate compatible, cohesive (sticky) ends.
The target gene has an internal <i>Nco</i> I site	Use an <i>Rca</i> I or <i>Bsp</i> LU11I site in the forward primer, if no <i>Rca</i> I or <i>Bsp</i> LU11I site is present in the target gene. These enzymes generate cohesive (sticky) ends compatible with <i>Nco</i> I. Cut pIVEX 1.3 WG or pIVEX 1.4 WG with <i>Nco</i> I and <i>Sma</i> I.
The target gene has internal <i>Nco</i> I, <i>Rca</i> I, and <i>Bsp</i> LU11I sites	Introduce an <i>Nde</i> I sequence into the forward primer. Use the <i>Nde</i> I site in pIVEX 1.3 WG or pIVEX 1.4 WG.
The target gene has internal <i>Nco</i> I, <i>Rca</i> I, <i>Bsp</i> LU11 I, and <i>Nde</i> I sites	Check for any of the additional restriction sites present in the MCS of pIVEX 1.3 WG or pIVEX 1.4 WG. Include one of these sites into the forward primer. <u>OR</u> eliminate the restriction site by mutation (e.g. conservative codon exchange [2, 3]). <u>OR</u> prepare a cloning fragment by limited digestion if the restriction site of your choice is present in the gene [2, 3].

Improved success rate

RTS pIVEX WG vectors have been specifically optimized for use in the RTS Wheat Germ cell-free protein expression system. However, any DNA inserted into these vectors results in a unique constellation. Interactions (base pairing on mRNA level) between the coding sequence of the target gene and the 5' untranslated region containing regulatory elements from the vector cannot be easily predicted and may impede or improve the translation process. N-terminal extensions may be helpful in overcoming problems with expression yield in such cases.

Protocol 2: Cloning procedure

Primer design for PCR cloning

- Use forward and reverse primers (about 20 bases) complementary to the gene, the restriction sites of choice (in same frame as on vector), and 5–6 additional base pairs to allow restriction enzyme cleavage (for examples see 'Supporting information', page 17)
- For efficient digestion with *Nde* I or *Not* I, the number of additional base pairs must be higher. For *Nde* I cleavage, add 8 additional base pairs. For *Not* I cleavage, add 10 additional base pairs
- To express a gene without a tag, insert a stop codon at the end of the gene (see 'Supporting information', page 19) and use pIVEX 1.3 WG for cloning
- Design forward and reverse primers with comparable ($\pm 2^\circ\text{C}$) melting temperature (for calculation of melting temperatures see 'Supporting information', page 17)
- Try to minimize secondary structure and dimer formation by means of primer design
- High-quality primers (HPLC-purified) are recommended

Digestion of pIVEX vectors for cloning

Procedure

1. Briefly centrifuge the vials containing the pIVEX vectors.
2. Digest the selected pIVEX vector(s) using the appropriate restriction enzymes and buffers. Restriction enzymes and buffers are available from Roche. See Table 2 for sample digestions.
3. Run an agarose gel to control the reaction and to separate the linearized vector from undigested vector and smaller fragments.
4. Isolate and purify the fragment with the correct size from the gel (e.g. using the GenUP™ Gel Extraction Kit, cat. no. BR0700401, biotechrabbit).

Table 2. Example digestions

Restriction enzyme	Procedure
<i>Nco</i> I and <i>Sma</i> I (or <i>Xma</i> I)	<ul style="list-style-type: none"> → Digest 2 µg (4 µl) of DNA using 20 units of <i>Sma</i> I in 20 µl <i>Sma</i> I buffer at 25°C (or 20 units of <i>Xma</i> I in <i>Xma</i> I buffer at 37°C) for 1 h → Check an aliquot to ensure the plasmid is linearized → Add 20 units <i>Nco</i> I and digest for 1 h at 37°C
<i>Nde</i> I and <i>Sma</i> I (or <i>Xma</i> I)	<ul style="list-style-type: none"> → Digest 2 µg (4 µl) of DNA using 20 units of <i>Sma</i> I in 20 µl <i>Sma</i> I buffer at 25°C (or 20 units of <i>Xma</i> I in <i>Xma</i> I buffer at 37°C) for 1 h → Check an aliquot to ensure the plasmid is linearized → Add 20 units <i>Nde</i> I and digest for 1 h at 37°C → See 'Supporting information', page 18 for hints on <i>Nde</i> I digests
<i>Not</i> I and <i>Sma</i> I (or <i>Xma</i> I)	<ul style="list-style-type: none"> → Digest 2 µg (4 µl) of DNA using 20 units of <i>Sma</i> I in 20 µl of <i>Sma</i> I buffer at 25°C (or 20 units of <i>Xma</i> I in <i>Xma</i> I buffer at 37°C) for 1 h → Check an aliquot to ensure the plasmid is linearized → Add 40 units of <i>Not</i> I in 40 µl <i>Not</i> I buffer and digest for 1 h at 37°C. See 'Supporting information', page 18 for hints on <i>Not</i> I digests.

Phosphatase treatment of digested vectors

This step is optional in the case of cohesive end cloning but necessary for ligation of blunt ended inserts.

Treat 300 ng of digested pIVEX WG vector with 3 units of shrimp alkaline phosphatase in a total volume of 10 µl in 1× phosphatase buffer for 90 min at 37°C.

Inactivate the shrimp phosphatase by heating to 65°C for 15 min.

Generation of PCR fragments

- Design PCR primers according to Protocol 2, page 11.
- Optimal PCR reaction conditions depend on the template/ primer pairs and have to be calculated accordingly
- Avoid nonspecific products and misincorporation, by keeping cycle numbers as low as possible (<25). Use a thermostable DNA polymerase that includes a hot-start function (e.g. Hot Start *Taq* DNA Polymerase, cat. no. BR0200101, biotechrabbit)
- Cut the end of the PCR product using the restriction sites introduced with the primers
- Note: The cutting efficiency of many restriction enzymes is reduced if their recognition sites are located less than 6 base pairs (8 base pairs for *Nde* I and 10 base pairs for *Not* I) from the 5' end. Therefore, restriction digests require higher enzyme concentrations and longer incubation times (see 'Supporting information', page 18).
- Run the digested PCR product on an agarose gel. Excise the fragment from the gel and purify it (e.g. GenUP™ Gel Extraction Kit, cat. no. BR0700401, biotechrabbit).

Excision of restriction fragments from existing vectors

Under certain conditions the target gene can be excised from an existing vector construct. This strategy can be applied if the gene is already flanked by restriction sites contained in the MCS of both pIVEX vectors (see 'Vector maps', page 20). For cloning into pIVEX 1.3 WG, check whether the start codon AUG and the tag sequence are in the correct reading frame. For cloning into pIVEX 1.4 WG, ensure that the first triplet of the gene of interest and the stop codon behind the *Bam* HI site are in the correct reading frame.

Vector ligation, transformation, and purification

Ligation

Ligate the purified DNA fragment into the linearized pIVEX WG vector. For ligation of DNA fragments digested with *Nde* I see 'Supporting information, page 18.

Transformation

Transform a suitable *E. coli* strain (e.g. JM109 or XL1 blue) to amplify the expression plasmid.

Amplification of the plasmid in *E. coli*

Prepare an appropriate amount of plasmid for the subsequent transcription/translation reactions. For a single 50 µl reaction, approximately 2 µg plasmid is required. For a single 1 ml reaction 40–60 µg plasmid is required. Preparation of a sufficient amount of plasmid for multiple reactions, including characterization by sequencing, (see below) is recommended. The GenUP™ Plasmid (cat. no. BR0700201) and GenUP™ Plasmid Plus (cat. no. BR0701201) Kits (biotechrabbit) are recommended.

Purity of the plasmid preparation

The purity of plasmids obtained from commercially available DNA preparation kits is sufficient for the use as template in the RTS. When DNA purity is insufficient (OD_{260/280} ≤ 1.7), a phenol treatment to remove proteins (e.g. traces of RNase) may enhance expression.

Restriction mapping

Successful cloning should be verified by restriction mapping of the construct and subsequent analysis on an agarose gel. biotechrabbit recommends using a restriction enzyme with a single cleavage site in the vector (such as *Cla* I or *Bam* HI) together with another enzyme that has one or two cleavage sites within the target gene.

Sequencing

The generated expression vectors should be sequenced to verify the correctness of the PCR-generated DNA fragments and correct cloning. Use a 5' primer complementary to the T7 promoter and a 3' primer complementary to the 3' untranslated region of mRNA downstream of termination codon.

Protocol 3: In-Fusion cloning into pIVEX WG vectors

The use of the In-Fusion PCR Cloning technology (offered by Clontech) greatly facilitates cloning into RTS pIVEX vectors. The enzyme mix contained in that system non-specifically recognizes the presence of identical sequences at the ends of (i) a PCR fragment and (ii) a linearized plasmid vector and then links both together in a 5–10-minute reaction. Restriction digestions of the inserts for cloning and DNA purifications from agarose gels can be avoided, while stocks of linearized and purified vector can be stored at –20°C for 2–3 months.

To prepare a cDNA of interest for cloning into RTS pIVEX Wheat Germ vectors via In-Fusion, the following primers should be used for PCR amplification:

To create an expression vector with	Gene-specific sense primer	Gene-specific antisense primer
C-terminal His₆-tag	5'-CCACAACAGCTTGTCGAACC +ATG +15–20 nt specific for the gene of interest (beginning with ATG)	5'-TGATGATGAGAACCCCCCCC +15–20 nt specific for the gene of interest (ensure no stop codon from the gene-specific sequence inhibits tag expression)
N-terminal His₆-tag	5'-CGCTTAATTAACATATGACC +15–20 nt specific for the gene of interest (no ATG necessary)	5'-TTAGTTAGTTACCGGATCCC +TTA +15–20 nt specific for the gene of interest (optional: Include TTA stop codon to avoid 3 additional amino acids)

The vector should be digested according to the following protocol.

Procedure

1. Digest 2 µg (4 µl) of DNA with 20 units of *Sma* I in 20 µl of *Sma* I buffer at 25°C for 1 hour.
2. Check an aliquot to ensure that the plasmid is completely linearized.
3. Add 20 units of *Nco* I and digest for 1 hour at 37°C.
4. Run the pIVEX WG plasmid on a 0.8% agarose gel to separate it thoroughly from the small excised fragment.
5. Elute the linearized plasmid band.

Protocol 4: Detection of expressed His₆-tagged proteins

His₆-tagged proteins can be detected easily after SDS-PAGE by western blotting using an anti-His₆ antibody. For methods and basic procedures, refer to the literature [e.g. reference 3].

Procedure

1. Dilute the Western Blocking Reagent 1:10 in TBST (50 mM Tris·HCl, 150 mM NaCl, 0.1% (v/v) Tween[®] 20, pH 7.5) and incubate the blot in 20 ml of this blocking buffer for 90 min at 15–25°C (or at 2–8°C overnight).
2. Wash three times with TBST for 5 minutes each time.
3. Dissolve Anti-His₆-Peroxidase (Roche) at a concentration of 50 U/ml in water.
4. Incubate the blot in 50 ml blocking buffer with 12.5 µl of the Anti-His₆-Peroxidase solution (final concentration 12.5 mU/ml Anti-His₆-Peroxidase) for 60 min at 15–25°C with gentle agitation.
5. Wash four times with TBST for 5 minutes each time.
6. Incubate the blot for 5 minutes in a quantity of Lumi-Light^{PLUS} substrate solution sufficient to cover the membrane (0.1 ml/cm²).
7. Expose on an appropriate imager or X-ray film for 1 minute. Adjust the exposure time between 10 s and 20 min according to the result of the first film.

Supporting information

Additional information for restriction enzyme-mediated cloning

Designing a *Nco* I/*Sma* I primer pair

Example target gene sequence:

MetStop

5'-**ATG**CTAGCAAACCTTACCTAAGGGTNNNNNNTTGTCCCGTTCAAATATT**GTAA**-3'

3'-TACGATCGTTTGAATGGATTCCCANNNNNNAACAAGGGCAAGTTTTATAACATT-5'

For cloning a gene into a pIVEX vector use:

→ A forward primer with *Nco* I site (bold letters):

5'-XX XXX **XCC ATG GTA** GCA AAC TTA...
 ...CCT AAG GGT-3'

$$T_m = 12 \times 2^\circ\text{C} + 8 \times 4^\circ\text{C} = 56^\circ\text{C}$$

Note: The second amino acid will be mutated in this example. This is true for all cases (ca. 75%) where the target sequence has A or C or T (not G) after the ATG start codon and a G is required in the primer sequence to introduce the *Nco* I site. If the possibility to recut the inserted DNA with *Nco* I after cloning is not important for you, you can use e.g. *Rca* I or *Bsp* LU11 I that generate ends compatible with *Nco* I, but have an A and a T in the sixth position of the recognition sequence, respectively.

→ A reverse primer with *Sma* I site (bold letters):

5'-XXX XXX **CCC GGG** CAA TAT TTT GAA CGG...
 ...GAA CAA-3'

$$T_m = 14 \times 2^\circ\text{C} + 7 \times 4^\circ\text{C} = 56^\circ\text{C}$$

Formula for melting point (T_m) calculation

$$T_m = (\text{number of A+T}) \times 2^\circ\text{C} + (\text{number of G+C}) \times 4^\circ\text{C}$$

Optimal annealing temperatures for PCR are 5–10°C lower than the T_m values of the primers.

Example for cloning and expression of a gene without any tag

- Use pIVEX 1.3 WG and add a TAA stop codon between the last amino acid and the *Sma* I site.
- Add an AT-rich stretch of 6 bases 5' of the *Sma* I site to allow a more efficient restriction cleavage (complementarity of this short sequence to the rest of the primer should be avoided).

Example: Target gene 3'-terminal sequence:

Asn Leu Phe Gly Gln
5'- AAT CTT TTC GGC ACA -3'
TTA GAA AAG CCG TGT

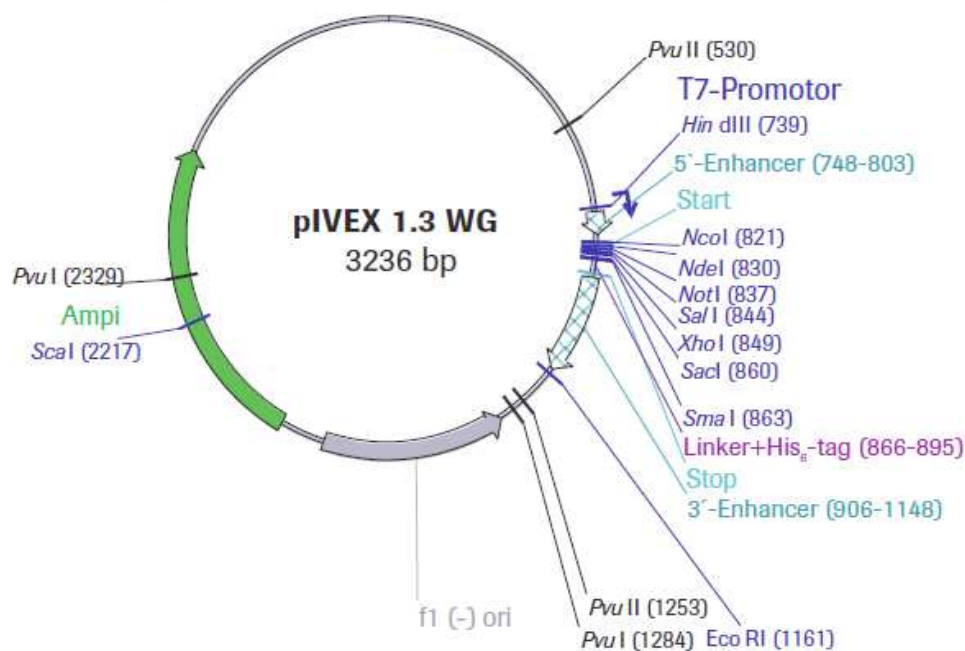
For this gene order the following reverse primer:

Sma I
5'-XXX XXX **CCC GGG** TTA TGT GCC GAA AAG ATT-3'

Vector maps

The complete vector sequences can be viewed and downloaded from www.biotechrabbit.com.

pIVEX 1.3 WG



	T7-Promotor		HindIII	
701	TTACGCCAAG	CTCATTAATA	CGACTCACTA	TAGGCCTAAG
	AATGCGGTTT	GAGTAATTAT	GCTGAGTGAT	ATCCGGATTG
				GAATGTTTAT

	5'-Enhancer			
751	CTCCCCACA	ACAGCTTACA	ATACTCCCCC	ACACAGCTTA
	GAGGGGGTGT	TGTCGAATGT	TATGAGGGGG	TGTGTCTGAAT
				GTTTATGAGG

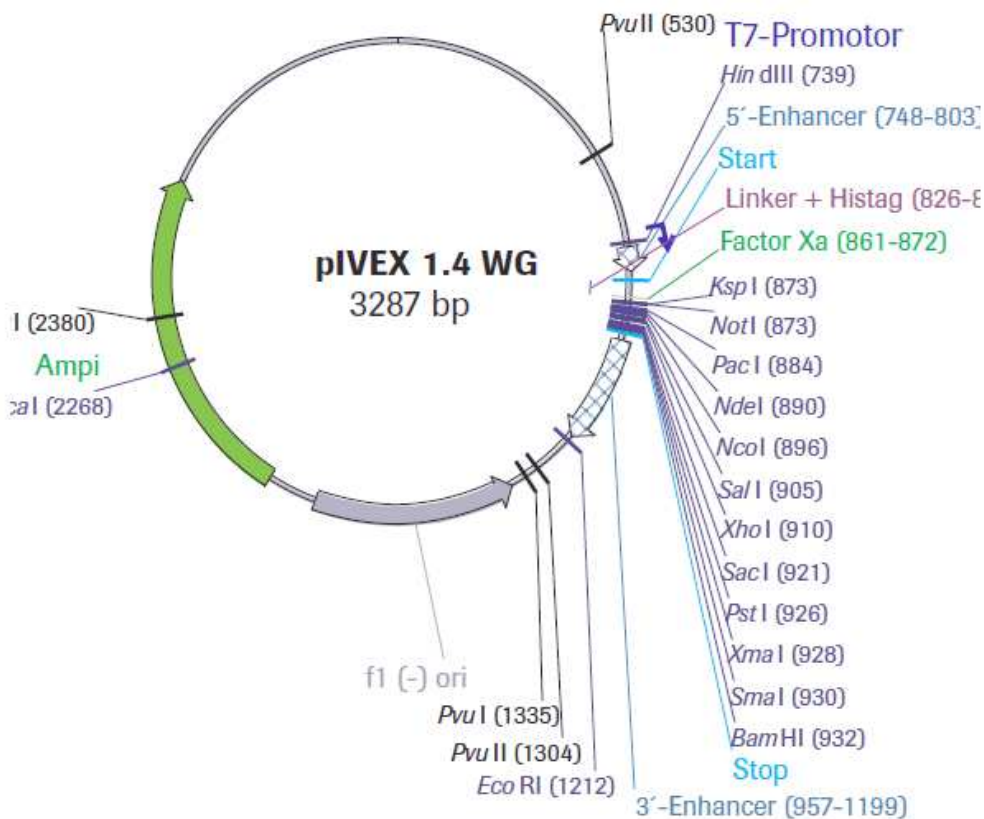
	Start			
		NcoI	NdeI	NotI
				SalI
801	CCCACAACAG	CTTGTCGAAC	CATGGCACAT	ATGAGCGGCC
	GGGTGTTGTC	GAACAGCTTG	GTACCGTGTA	TACTCGCCGG
				CGCAGCTGAG

	XhoI	SacI	SmaI	Linker+His₆-tag		Stop
851	GAGCGAGCTC	CCGGGGGGGG	TTCTCATCAT	CATCATCATC	ATTAATAAGG	
	CTCGCTCGAG	GGCCCCCCCC	AAGAGTAGTA	GTAGTAGTAG	TAATTATTCC	

	3'-Enhancer				
901	TACCCAGCTC	TTCTGGTTTG	GTTTGGACCT	CTGGTCCTGC	AACTTGAGGT
	ATGGGTCGAG	AAGACCAAAC	CAAACCTGGA	GACCAGGACG	TTGAACTCCA

Figure 3. pIVEX 1.3 WG.

pIVEX 1.4 WG



T7-Promotor

701 TTACGCCAAG CTCATTAATA CGACTCACTA TAGGCCTAAG CTTACAAATA
 AATGCGGTTC GAGTAATTAT GCTGAGTGAT ATCCGGATTC GAATGTTTAT

5'-Enhancer

751 CTCCCCCACA ACAGCTTACA ATACTCCCCC ACACAGCTTA CAAATACTCC
 GAGGGGGTGT TGTGGAATGT TATGAGGGGG TGTGTGGAAT GTTTATGAGG

Start

Linker + His₆-tag

801 CCCACAACAG CTTGTGGAAC CATGTCTGGT TCTCATCATC ATCATCATCA
 GGGTGTGTC GAACAGCTTG GTACAGACCA AGAGTAGTAG TAGTAGTAGT

Factor Xa Ksp I Not I

Nde I Nco I

851 TAGCAGCGGC ATCGAAGGCC GCGGCCGCTT AATTAAACAT ATGACCATGG
 ATCGTCGCCG TAGCTTCCGG CGCCGGCGAA TTAATTTGTA TACTGGTACC

Xma I

Sa I Xho I Sac I Pst I Sma I Bam HI Stop

901 CAAGTCGACT CGAGCGAGCT CTGCAGCCCG GGATCCGGTA A
 GTTCAGCTGA GCTCGCTCGA GACGTCGGGC CCTAGGCCAT T

Figure 4. pIVEX 1.4 WG.

Typical results

Different ORFs were cloned in pIVEX 1.3 WG (C-terminal tag) and pIVEX 1.4 WG (N-terminal tag) vectors. Resulting vectors were expressed using the RTS 100 Wheat Germ CECF Kit.

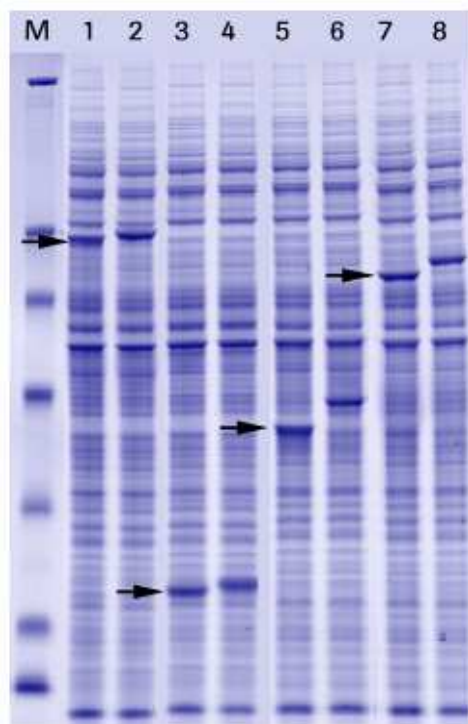


Figure 5. Expression of different His₆-tagged proteins in RTS 100 WG CECF.

Expression reactions were analyzed by SDS-PAGE and gel was stained with Coomassie® Brilliant Blue.

M: Marker; **Lanes 1 and 2:** Glucuronidase (GUS); **Lanes 3 and 4:** Survivin; **Lanes 5 and 6:** Human kinase; **Lanes 7 and 8:** Receptor protein. **Lanes 1, 3, 5, and 7:** C-terminal tag; **Lanes 2, 4, 6, and 8:** N-terminal tag.

References

1. Akbergenov, R.Z. et al. (2003). Complementary interaction between the central domain of 18S rRNA and the 5' untranslated region of mRNA enhances translation efficiency in plants. In: "Cell-free protein expression", Swartz, J.R. Ed., Springer, pp. 199–208.
2. Sambrook J. et al., (1989). "Molecular Cloning - A Laboratory Manual" Second Edition, Cold Spring Harbor Laboratory Press, New York.
3. Ausubel, U.K. et al., (1993). "Current Protocols In Molecular Biology" John Wiley & Sons Inc., New York.

Troubleshooting guide

The following troubleshooting recommendations are designed to address unexpected or undesired results. To ensure optimal use, follow the guidelines and recommendations in the manual.

Observation	No PCR product
Possible cause	Secondary structures of the primers
Resolving	Try to minimize secondary structure and dimer formation when designing primers.
Resolving	Raise the primer concentration in the PCR reaction or use longer primers without G or C nucleotides at the 3'-end if a G+C content of 60% is not feasible.
Possible cause	Inadequate annealing temperature
Resolving	Check whether the right annealing temperature was used for the PCR reaction (5–10°C lower than T_m).
Resolving	Adapt the annealing temperature to the primer with the lowest melting temperature.
Possible cause	Concentration of Mg ²⁺ too low
Resolving	Determine the optimal MgCl ₂ concentration specifically for each template/primer pair by preparing a reaction series containing 0.5–4.5 mM MgCl ₂ .
Resolving	Optimize the concentration of template DNA in the PCR reaction.

Observation	Non-specific amplification
Possible cause	Low primer specificity
Resolving	Ensure the primers specifically flank the 5'- and 3'- ends of the gene and are not complementary to other sequence regions of the template DNA. Increase primer length, if necessary.
Resolving	Use hot start techniques.
Possible cause	Concentration of Mg ²⁺ too high
Resolving	Avoid excess of free magnesium leading to unspecific amplification.
Resolving	Determine the optimal concentration by preparing a reaction series containing 0.5–4.5 mM MgCl ₂ .
Resolving	Raise the annealing temperature, if necessary.

Observation	No or only a few colonies after transformation
Possible cause	Inappropriate selection medium
Resolving	Ensure that plates contain 50 µg/ml ampicillin or carbenicillin and no other antibiotics.
Possible cause	Inactive competent cells
Resolving	Avoid frequent freezing and thawing of competent cells.
Resolving	Perform a test transformation with 10 pg supercoiled control plasmid.
Possible cause	Excess of ligation reaction during transformation
Resolving	Limit the volume of the ligation reaction to less than 20% of the whole transformation reaction volume to avoid inhibitory effects due to ligation buffers.

Possible cause	Unsuccessful restriction digest of the PCR product
Resolving	<p>Make sure that the right restriction buffer and reaction conditions were chosen.</p> <p>Note: <i>Sma</i> I is optimally active at 25°C.</p> <ul style="list-style-type: none"> → For restriction digest with <i>Nde</i> I and <i>Not</i> I, see 'Cloning using restriction enzymes <i>Nde</i> I and <i>Not</i> I', page 18. → Increase incubation time. → Subclone the PCR product into a PCR cloning vector if direct cloning after digestion of the PCR product is not successful (see Protocol 3).
Possible cause	Unsuccessful ligation
Resolving	Check activity of T4 DNA ligase by performing a control ligation reaction.
Resolving	<p>Use fresh ligase.</p> <p>Store the ligation buffer aliquoted at –15 to –25°C, as freezing and thawing results in degradation of ATP.</p>
Resolving	<p>Vary the ratio of vector DNA to insert DNA.</p> <p>Adjust the molar ratio of vector DNA to insert DNA to 1:3 (e.g. for a vector/insert size ratio of 3:1 use 50 ng linearized dephosphorylated vector and 50 ng insert).</p> <p>When vector and insert DNA differ in length, try other molar ratios (e.g. 1:1, 1:2).</p>
Resolving	<p>Use restriction enzymes providing sticky ends at both ends of the gene fragment to be cloned (e.g. use <i>Xma</i> I instead of <i>Sma</i> I).</p> <p>Note: For ligation of DNA fragments digested with <i>Nde</i> I, see 'Cloning using restriction enzymes <i>Nde</i> I and <i>Not</i> I', page 18.</p>
Possible cause	Alkaline phosphatase not inactivated after dephosphorylation of the vector
Resolving	Inactivate the alkaline phosphatase (note: shrimp alkaline phosphatase can be inactivated simply by heat treatment whereas complete inactivation of calf intestine phosphatase requires additional treatments [e.g. phenolization]).

Observation	High background of non-recombinants after transformation
Possible cause	Inappropriate medium
Resolving	Ensure that the selection medium contains the correct, active antibiotic by performing a mock transformation reaction without DNA. No colonies should be obtained.
Possible cause	Incomplete digestion of vector or insert
Resolving	Purify the vector or insert after the first digestion step using the GenUP™ PCR Cleanup Kit (cat. no. BR0700301, biotechrabbit). Perform the second digestion step of the vector or insert in the optimal buffer.
Possible cause	Unsuccessful dephosphorylation of the vector
Resolving	Perform a religation control reaction without insert where only few colonies should be obtained.
Resolving	Use fresh (shrimp) alkaline phosphatase.
Resolving	Increase the incubation time.
Possible cause	Excess of linearized, phosphorylated vector
Resolving	Depending on background strongly reduce the amount of linearized vector in the ligation reaction 2–5-fold. Note: If the vector:insert ratio is too high, religation is favored.

Ordering information

Product	Size	Order no.
RTS Linear Template Kit Plus	20 reactions	BR1402401
RTS pIX3.0 Vector	1 vector, 25 µg	BR1402701
RTS 100 <i>E. coli</i> HY Kit	24 reactions	BR1400101
RTS 100 <i>E. coli</i> HY Kit	96 reactions	BR1400102
RTS 500 ProteoMaster <i>E. coli</i> HY Kit	5 reactions	BR1400201
RTS 9000 <i>E. coli</i> HY Kit	1 reaction	BR1400301
RTS 100 <i>E. coli</i> Disulfide Kit	24 reactions	BR1400401
RTS 500 <i>E. coli</i> Disulfide Kit	5 reactions	BR1400501
RTS 100 <i>E. coli</i> Fab Kit	10 reactions	BR1400601
RTS 100 <i>E. coli</i> Fab Kit	96 reactions	BR1400602
RTS pIVEX <i>E. coli</i> His-tag, 2nd Gen. Vector Set	2 vectors, 10 µg each	BR1400701
RTS Wheat Germ LinTempGenSet, His ₆ -tag	96 reactions	BR1401201
RTS pIVEX Wheat Germ His ₆ -tag Vector Set	2 vectors, 10 µg each	BR1401301
RTS 100 Wheat Germ CECF Kit	24 reactions	BR1401001
RTS 500 Wheat Germ CECF Kit	5 reactions	BR1401101
RTS 500 Adapter	1 adapter	BR1401901
RTS GroE Supplement	For five RTS 500 reactions	BR1401701
RTS DnaK Supplement	For five RTS 500 reactions	BR1401601
RTS Amino Acid Sampler	1 set	BR1401801
RTS 100 Insect Membrane Kit	5 reactions	BR1401501
RTS 100 Insect Membrane Kit	20 reactions	BR1401502
RTS 100 Insect Disulfide Kit	10 reactions	BR1401401
RTS 100 Insect Disulfide Kit	96 reactions	BR1401402
RTS Linear Template Fab Kit	96 reactions	BR1402201
RTS pIX4.0 Insect Vector	1 vector, 25 µg	BR1400901

