RTS™ pIVEX *E. coli* His-tag 2nd Generation Vector Set Manual

For high-level expression of His_6-tagged proteins in the cell-free

RTS *E. coli* system

RTS pIVEX *E. coli* His-tag 2nd Generation 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**
Protocol 1: Cloning into pIVEX vectors 6
Protocol 2: Cloning procedure 9
Protocol 3: Detection of expressed His_6-tagged proteins 9
Supporting information **14**
Additional information for restriction enzyme-mediated cloning 14
Vector maps 17
Typical results 17
References 19
Troubleshooting guide 20
Ordering information **24**

Product specifications

Product description

The RTS pIVEX *E. coli* His-tag 2nd Generation Vector Set is designed for high-level expression of His_6-tagged proteins in the cell-free rapid translation system (RTS) *E. coli* system. Cloning into pIVEX His_6-tag 2nd Generation Vectors allows optimal protein expression in all RTS *E. coli* systems. The Vectors contain all regulatory elements necessary for *in vitro* expression based on a combination of T7 RNA polymerase and prokaryotic cell lysates. The introduction of either an N- or a C-terminal His_6-tag provides a rapid method to detect and purify proteins of interest.

Product limitations

The RTS pIVEX *E. coli* His-tag 2nd Generation 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

RTS pIVEX *E. coli* His-tag 2nd Generation Vector Set	Vial contents and use
Ordering number	BR1400701
pIVEX2.3d Vector DNA; 2nd Gen. Vec. Set (vial 1)	→ 10 µg (20 µl) plasmid DNA → Cloning vector with C-terminal His_6-tag → Contains a multiple cloning site (MCS)
pIVEX2.4d Vector DNA; 2nd Gen. Vec. Set (vial 2)	→ 10 µg (20 µl) plasmid DNA → Cloning vector with cleavable N-terminal His_6-tag → Contains a multiple cloning site (MCS)

Shipping and storage conditions

The RTS pIVEX *E. coli* His-tag 2nd Generation Vector Set is shipped on dry ice. Vectors are stable for 1 week at 2–8°C and at −15 to −25°C 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

Protocol 1: Cloning into pIVEX vectors

Vector description

- pIVEX is the abbreviation for ***I****n* ***V****itro* **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
- The letter 'd' indicates a new generation of vector

Use and location of His_6-tag

Two different vectors are contained in the set. The general architecture is shown in Figure 1. Both vectors contain a sequence encoding a stretch of six consecutive histidines. This hexa-histidine (His_6-) tag allows easy detection (see Protocol 3, page 13) and purification of the expressed protein.

- Use pIVEX2.3d for fusing the gene to a C-terminal His6-tag.
- Use pIVEX2.4d for fusing the gene to an N-terminal His6-tag.

For native expression without tag use pIVEX2.3d and incorporate a stop codon (TAA) at the end of the gene (see 'Supporting information', page 15).

For detailed vector maps, see 'Vector maps', page 17. The complete vector sequences can be viewed and downloaded at www.biotechrabbit.com.

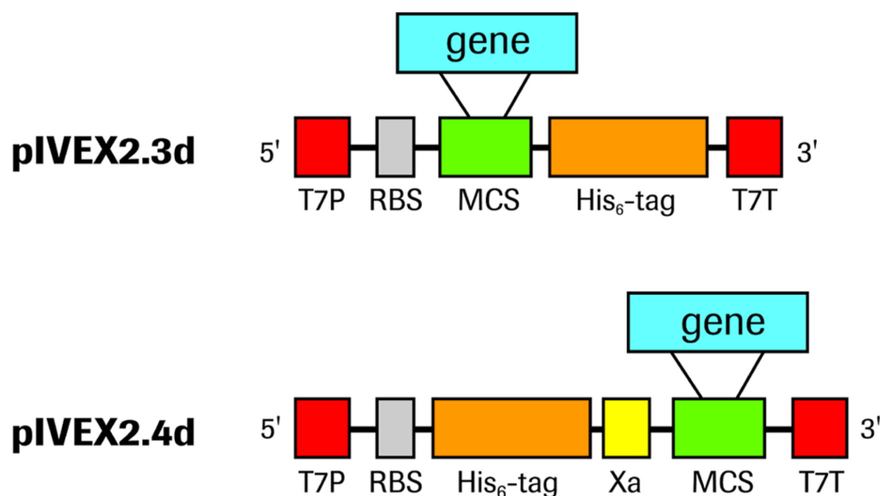


Figure 1. Functional elements of pIVEX vectors.

T7P*: T7 promoter;* ***RBS****: Ribosome binding site;* ***His$_6$-tag****: Tag sequence at C- or N-terminal position;* ***Xa****: Factor Xa restriction protease cleavage site;* ***MCS****: M Multiple cloning site for the insertion of the target gene.;* ***T7T****: T7 terminator.*

Selecting the cloning strategy

In general, the *Nco* I/*Sma* I restriction site combination is recommended for cloning into pIVEX vectors, since this approach provides optimal flexibility to switch into all available pIVEX 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 14.

Table 1. Cloning strategies

Issue	Suggestion solution
The target gene has no internal *Nco* I and *Sma* I sites	Use *Nco* I and *Sma* 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 14.
The target gene has an internal *Sma* 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. *Eco* RV, *Ssp* I, *Sca* I). Cut pIVEX 1.3 WG or pIVEX 1.4 WG with *Nco* I and *Sma* I.
Blunt end cloning at the 3' end should be avoided	Use *Xma* I, if your gene does not contain an internal *Xma* I site. *Xma* I recognizes the same sequence as *Sma* I but leaves a cohesive (sticky) end. Alternatively, *Pin* AI, *Sgr* AI, *Bse* AI, or *Ngo* MIV can be used to generate compatible, cohesive (sticky) ends.
The target gene has an internal *Nco* I site	Use an *Rca* I or *Bsp* LU11I site in the forward primer, if no *Rca* I or *Bsp* LU11I site is present in the target gene. These enzymes generate cohesive (sticky) ends compatible with *Nco* I. Cut pIVEX2.3d or pIVEX2.4d with *Nco* I and *Sma* I.
The target gene has internal *Nco* I, *Rca* I, and *Bsp* LU11I sites	Introduce an *Nde* I sequence into the forward primer. Use the *Nde* I site in pIVEX2.3d or pIVEX2.4d.
The target gene has internal *Nco* I, *Rca* I, *Bsp* LU11 I , and *Nde* I sites	Check for any of the additional restriction sites present in the MCS of pIVEX2.3d or pIVEX2.4d. Include one of these sites into the forward primer. OR eliminate the restriction site by mutation (e.g. conservative codon exchange [1, 2]). OR prepare a cloning fragment by limited digestion if the restriction site of your choice is present in the gene [1, 2].

Improved success rate

RTS pIVEX vectors have been specifically optimized for use in the RTS 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. Therefore, biotechrabbit recommends cloning the target gene into more than one expression vector.

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 efficient restriction enzyme cleavage (for examples see ‘Supporting information’, page 14)
- 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 15) and use pIVEX2.3d for cloning
- Design forward and reverse primers with comparable (±2°C) melting temperature (for calculation of melting temperatures see ‘Supporting information’, page 14)
- 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. 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. GenUP™ Gel Extraction Kit, cat. no. BR0700401, biotechrabbit).

Table 2. Example digestions

Restriction enzyme	Procedure
Nco I and *Sma* I (or *Xma* I)	➜ Digest 2 µg (4 µl) of DNA using 20 units of *Sma* I in 20 µl *Sma* I buffer at 25°C (or 20 units of *Xma* I in *Xma* I buffer at 37°C) for 1 h ➜ Check an aliquot to ensure the plasmid is linearized ➜ Add 20 units *Nco* I and digest for 1 h at 37°C
Nde I and *Sma* I (or *Xma* I)	➜ Digest 2 µg (4 µl) of DNA using 20 units of *Sma* I in 20 µl *Sma* I buffer at 25°C (or 20 units of *Xma* I in *Xma* I buffer at 37°C) for 1 h ➜ Check an aliquot to ensure the plasmid is linearized ➜ Add 20 units *Nde* I and digest for 1 h at 37°C ➜ See 'Supporting information', page 15 for hints on *Nde* I digests
Not I and *Sma* I (or *Xma* I)	➜ Digest 2 µg (4 µl) of DNA using 20 units of *Sma* I in 20 µl of *Sma* I buffer at 25°C (or 20 units of *Xma* I in *Xma* I buffer at 37°C) for 1 h ➜ Check an aliquot to ensure the plasmid is linearized ➜ Add 40 units of *Not* I in 40 µl *Not* I buffer and digest for 1 h at 37°C. See 'Supporting information', page 15 for hints on *Not* 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 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 alkaline phosphatase by heating to 65°C for 15 min.

Generation of PCR fragments

- ➜ Design PCR primers according to Protocol 2, page 9.
- ➜ 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 15).
- → 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).

Subcloning of PCR fragments using PCR vectors

Restriction enzymes often do not cut efficiently if the restriction site is located at the very end of a fragment. The completeness of the digest is difficult to analyze due to the small difference in size. Subcloning of PCR fragments using PCR cloning vectors can avoid this uncertainty. For instructions on this procedure, see 'Supporting information', page 16).

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 17). For cloning into pIVEX2.3d, check whether the start codon ATG and the tag sequence are in the correct reading frame. For cloning into pIVEX2.4d, 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 vector (using e.g. the T4 DNA Ligase Rapid, cat. no. BR1100301, biotechrabbit). For ligation of DNA fragments digested with *Nde* I see 'Supporting information, page 15.

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 RTS reaction, approximately 0.5 µg plasmid is required. For a single 1 ml reaction 10–15 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 Kit (cat. no. BR0700201, biotechrabbit) and GenUP™ Plasmid Plus Kit (cat. no. BR0701201, 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 (OD260/280 ≤1.7), a phenol treatment to remove proteins (e.g. traces of RNase) may enhance expression.

Analysis of the new expression vector

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.

5'- primer: 5’- TAATACGACTCACTATAGGG -3'

3'- primer: 5’- GCTAGTTATTGCTCAGCGG -3'

Protocol 3: Detection of expressed His_6-tagged proteins

His_6-tagged proteins can be detected easily after SDS-PAGE by western blotting using an anti-His_6 antibody. For methods and basic procedures, refer to the literature [e.g. reference 2].

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-His_6-Peroxidase (e.g. 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-His_6-Peroxidase solution (final concentration 12.5 mU/ml Anti-His_6-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/cm^2).
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:

Met Stop

5'-**ATG**CTAGCAAACTTACCTAAGGGTNNNNNNTTGTTCCCGTTCAAAATATTG**TAA**-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 X**CC ATG G**TA GCA AAC TTA CCT AAG GGT-3'

  $T_m = 12 \times 2°C + 8 \times 4°C = 56°C$

  (for melting point calculation see formula below)

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'

  $T_m = 14 \times 2°C + 7 \times 4°C = 56°C$

Formula for melting point (Tm) calculation

T_m = (number of A+T) $\times$ 2°C + (number of G+C) $\times$ 4°C

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

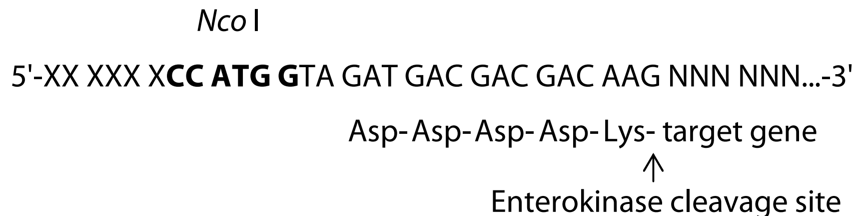
Expression of proteins with a minimized number of additional amino acids at the N-terminus

If a protein is to be expressed that has only few additional amino acids at the N-terminus, use one of the following two strategies:

- Cloning into the *Ksp* I site of pIVEX2.4d will result in one additional glycine at the N-terminus.

Note: When designing the forward primer, one ambiguous base has to be inserted between the *Ksp* I site and the target gene to maintain the right reading frame.

- For a protein without any additional amino acids biotechrabbit recommends the insertion of a protease cleavage site directly upstream of the target gene sequence into the forward primer, e.g. an enterokinase cleavage site:



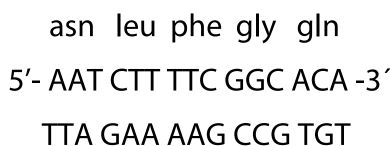
Cloning using restriction enzymes *Nde* I and *Not* I

- *Nde* I is sensitive to impurities in DNA preparations. To avoid cleavage at lower rates, make sure that the DNA preparations are highly pure (DNA purified by 'quick-and-dirty' miniprep procedures is often not sufficiently pure). If necessary, increase *Nde* I concentrations used for restriction digest.
- DNA digested with *Nde* I is more difficult to ligate using T4 DNA ligase. The ligation efficiency can be increased by adding 15% polyethylenglycol (PEG).
- *Not* I inefficiently cuts supercoiled plasmids. Linearize the DNA with the other enzyme or increase *Not* I up to 5- fold for complete digestion.

Example for cloning and expression of a gene without any tag

- Use pIVEX2.3d 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:



For this gene order the following reverse primer:

Subcloning of PCR fragments using PCR cloning vectors

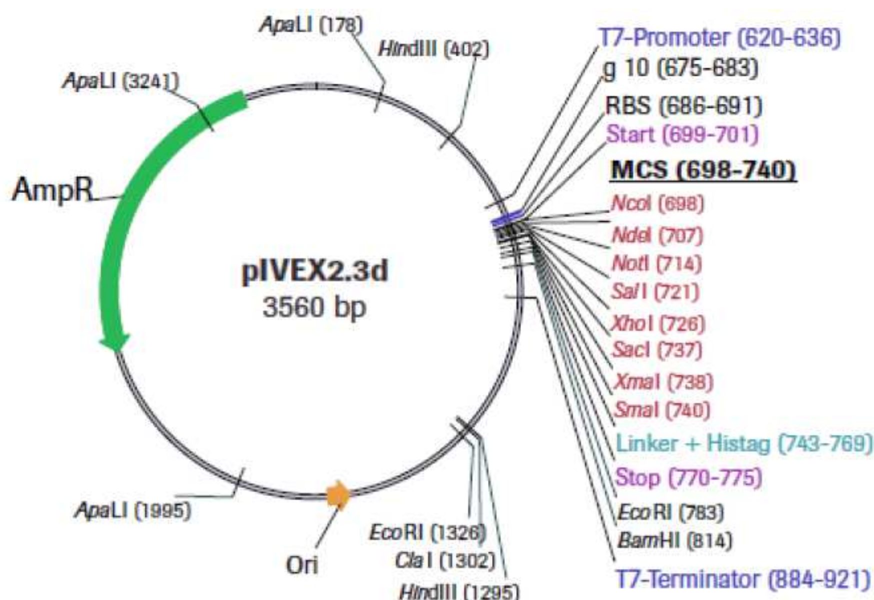
A disadvantage of direct cloning may, in some cases, be the inefficient cutting of restriction sites located at the very end of a fragment. As the restriction digest creates only a small difference in the fragment size, incomplete digestion will not be easily visible on agarose gels. Subcloning in PCR cloning vectors may avoid this problem.

Cloning issue	Suggestion
Subclone in blunt end cloning vectors	➔ Perform the PCR with thermostable DNA polymerase (with with 3'–5' exonuclease activity, e.g. Exact Polymerase (5 PRIME) to create PCR fragments with blunt ends ➔ Ligate into a blunt end cut cloning vector (e.g. using a PCR Cloning Kit) ➔ Cut out the template gene from the subcloning vector and clone into the pIVEX vector cut with compatible restriction enzymes
Subclone in T-overhang cloning vectors	➔ Perform the PCR with *Taq* DNA Polymerase (e.g. Hot Start *Taq* DNA Polymerase, biotechrabbit) to create PCR fragments with single deoxyadenosine residue overhangs at the 3' ends ➔ Ligate into a linearized cloning vector with a T-overhang and continue as described above

Vector maps

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

pIVEX2.3d




```
                                   T7-Promoter
601  GATCTCGATC CCGCGAAATT AATACGACTC ACTATAGGGA GACCACAACG
     CTAGAGCTAG GGCGCTTTAA TTATGCTGAG TGATATCCCT CTGGTGTTGC
                               g10 ε          RBS          NcoI
651  GTTTCCCTCT AGAAATAATT TTGTTTAACT TTAAGAAGGA GATATACCAT
     CAAAGGGAGA TCTTTATTAA AACAAATTGA AATTCTTCCT CTATATGGTA
                                                          Me
                                                XmaI
        NdeI       NotI      SalI   XhoI     SacI  SmaI    Linker
701  GGCACATATG AGCGGCCGCG TCGACTCGAG CGAGCTCCCG GGGGGGGTTC
     CCGTGTATAC TCGCCGGCGC AGCTGAGCTC GCTCGAGGGC CCCCCCCAAG
     tAlaHisMet SerGlyArgV alAspSerSe rGlu            GlyGlySe

      Histag                                EcoRI
751  TCATCATCAT CATCATCATT AATAAAAGGG CGAATTCCAG CACACTGGCG
     AGTAGTAGTA GTAGTAGTAA TTATTTTCCC GCTTAAGGTC GTGTGACCGC
     rHisHisHis HisHisHis* *****
```

Figure 2. pIVEX2.3d.

pIVEX2.4d

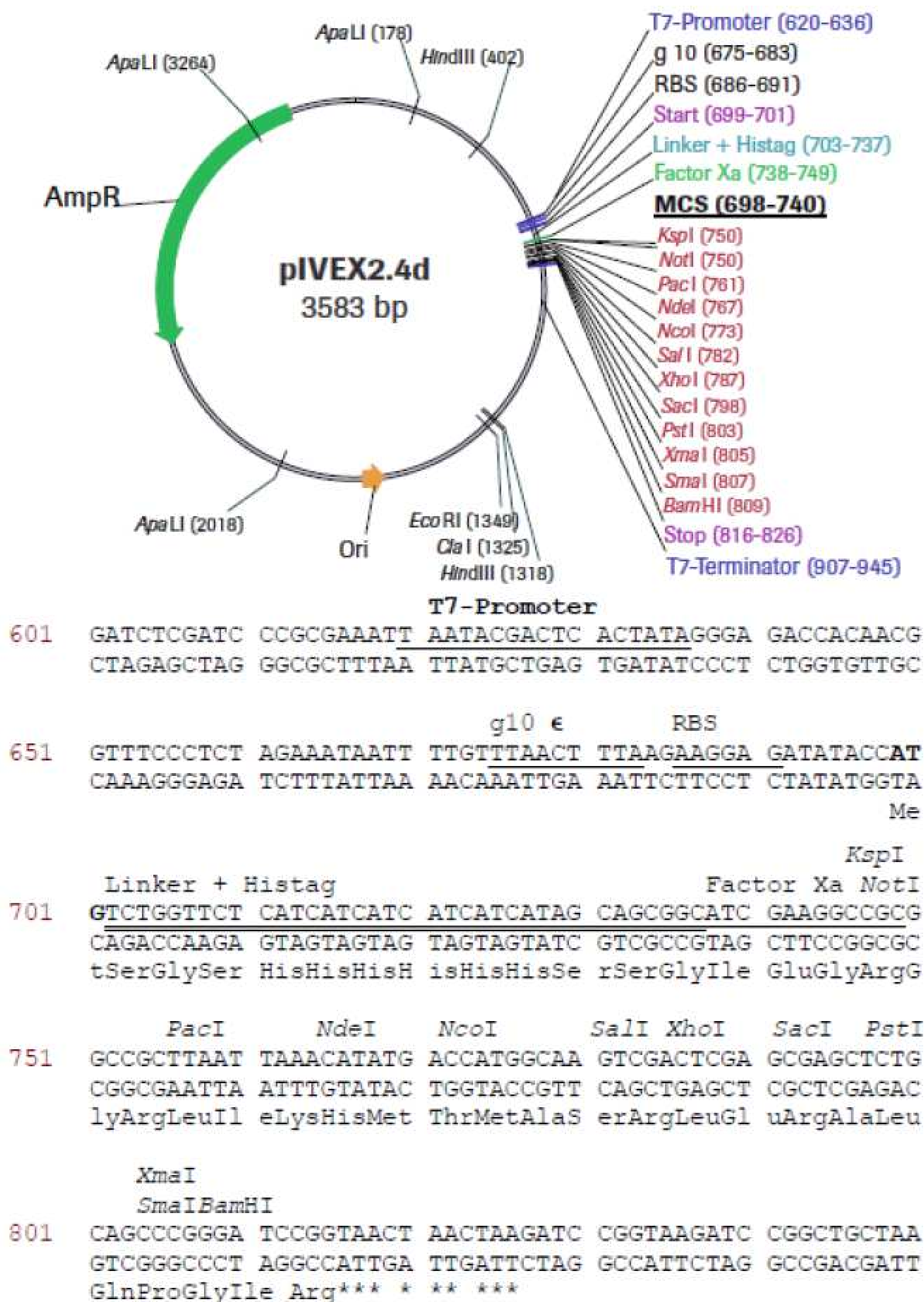


Figure 3. pIVEX2.4d.

Typical results

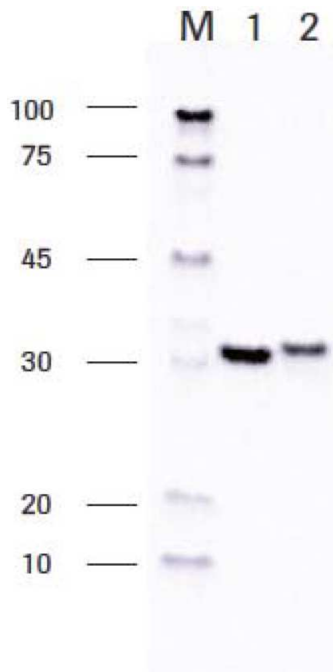


Figure 4. Expression of His-tagged green fluorescent protein (GFP; lane 1) and GFP mutant (lane 2) proteins using the RTS 500 E. coli HY Kit. The western blot was incubated with Anti-His$_6$-POD conjugate (Roche) according to the manufacturer's instructions, see Protocol 3, page 13. M: Multi-tag marker.

References

1. Sambrook J. et al., (1989). "Molecular Cloning - A Laboratory Manual" Second Edition, Cold Spring Harbor Laboratory Press, New York.
2. 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	Make sure that the right restriction buffer and reaction conditions were chosen. Note: *Sma* I is optimally active at 25°C. ➜ For restriction digest with *Nde* I and *Not* I, see 'Cloning using restriction enzymes *Nde* I and *Not* I', page 15. ➜ 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 page 16).

Possible cause	Unsuccessful ligation
Resolving	Check activity of T4 DNA ligase by performing a control ligation reaction.
Resolving	Use fresh ligase. Store the ligation buffer aliquotted at −15 to −25°C, as freezing and thawing results in degradation of ATP.
Resolving	Vary the ratio of vector DNA to insert DNA. 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). When vector and insert DNA differ in length, try other molar ratios (e.g. 1:1, 1:2).
Resolving	Use restriction enzymes providing sticky ends at both ends of the gene fragment to be cloned (e.g. use *Xma* I instead of *Sma* I). Note: For ligation of DNA fragments digested with *Nde* I, see 'Cloning using restriction enzymes *Nde* I and *Not* I', page 15.

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 *E. coli* HY Kit	24 reactions	BR1400101
RTS 100 *E. coli* HY Kit	96 reactions	BR1400102
RTS 500 ProteoMaster *E. coli* HY Kit	5 reactions	BR1400201
RTS 9000 *E. coli* HY Kit	1 reaction	BR1400301
RTS 100 *E. coli* Disulfide Kit	24 reactions	BR1400401
RTS 500 *E. coli* Disulfide Kit	5 reactions	BR1400501
RTS 100 *E. coli* Fab Kit	10 reactions	BR1400601
RTS 100 *E. coli* Fab Kit	96 reactions	BR1400602
RTS pIVEX *E. coli* His-tag, 2nd Gen. Vector Set	2 vectors, 10 µg each	BR1400701
RTS Wheat Germ LinTempGenSet, His6-tag	96 reactions	BR1401201
RTS pIVEX Wheat Germ His6-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