RTS pIVEX His_6-tag, 2nd Generation Vector Set

Cat. No. 03 269 019 001

Version February 2008

Store at −15 to −25°C

1. Preface

Kit contents

Vial	Label	Contents and use
1	pIVEX2.3d	• 10 µg (20 µl) plasmid • cloning vector with C-terminal His_6-tag • contains a multiple cloning site (MCS)
2	pIVEX2.4d	• 10 µg (20 µl) plasmid • cloning vector with cleavable N-terminal His_6-tag • contains a multiple cloning site (MCS)

Safety Information

None of the bottles contain hazardous substances in reportable quantities. The usual precautions taken when handling chemicals should be observed. Used reagent can be disposed off 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.

Stability of pIVEX vectors

Vectors are stable for 1 week at 2–8°C and for 2 years at −15° to −25°C. Repeated freezing and thawing decreases the amount of supercoiled plasmid.

2. Introduction

Roche's RTS pIVEX His_6-tag Vectors are designed for high-level expression of His_6- tagged proteins in the cell free RTS *E. coli* system. The vectors contain all regulatory elements necessary for *in vitro* expression based on a combination of T7 RNA polymerase and procaryotic cell lysates. The introduction of either a N- or a C-terminal His_6-tag provides a rapid method to detect and purify proteins of interest. Cloning into pIVEX His_6-tag Vectors allows optimal protein expression in all RTS *E. coli* systems (see 4.6 Related products).

3. Cloning into pIVEX vectors

3.1 Vector description

Vector nomenclature

- 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 mean tags fused to the N-terminus
  - Odd numbers mean tags fused to the C-terminus
- Letter d indicates the new vector generation.

For additional vectors with alternative tags please refer to our current catalog or to our websites **http://biochem.roche.com** and http://www.proteinexpression.com.

Functional elements of pIVEX vectors

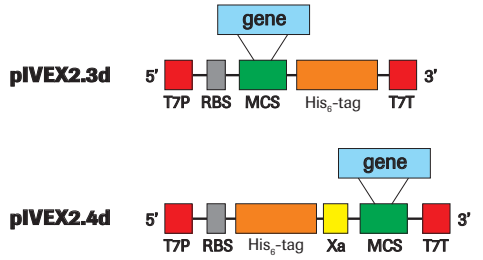


Fig. 1: Functional elements of pIVEX vectors.

Abbreviations
T7 P = T7 Promoter, RBS = Ribosomal binding site, Xa = Factor Xa restriction protease cleavage site, MCS = Multiple cloning site for the insertion of the target gene, T7 T = T7 Terminator

Use and location of the His_6-tag

Two different vectors are supplied within the set. Both vectors contain the hexa-histidine tag. The general architecture is shown in Fig. 1.
The hexa-histidine (His_6-)tag allows easy detection (see chapter 4.5) and purification of the expressed protein. (For purification protocols please refer to our web site www.proteinexpression.com).

- Use pIVEX2.3d for fusing the gene with a C-terminal His_6-tag.
- Use pIVEX2.4d for fusing the gene with a N- terminal His_6-tag.
- For native expression without tag use pIVEX2.3d and incorporate a stop codon (TAA) at the end of the gene (see chapter 4.1.4).

For detailed vector maps refer to chapter 4.3. The complete vector sequences can be viewed and downloaded from the Roche Molecular Biochemicals protein expression web site **www.proteinexpression.com.**

3.2 Selecting the cloning strategy

In general, it is recommended to use the *Nco* I/*Sma* I restriction site combination for cloning into pIVEX vectors, because this approach provides optimal flexibility to switch into all available pIVEX vectors and normally results in good expression efficiencies. Once the PCR fragment is prepared, cloning into different pIVEX vectors can easily be done in parallel or successively.

To minimize problems, we recommend to select the cloning strategy strictly according to the following decision matrix. For cloning strategies allowing to minimize the number of additional amino acids added to the N-terminus of an expressed protein, please refer to chapter 4.1.2.

IF...	THEN...
The target gene is free of 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 in chapter 4.1.1.

continued on next page

3.2 Selecting the cloning strategy, continued

IF...	THEN...
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 your target gene (e.g. *Eco* RV, *Ssp* I, *Sca* I). • Cut pIVEX2.3d or pIVEX2.4d with *Nco* I and *Sma* I.
You want to avoid blunt end cloning at the 3' end	• **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, you can use *Pin* AI, *Sgr* AI, *Bse* AI, or *Ngo* MIV which generate compatible, cohesive (sticky) ends.
The target gene has an internal *Nco* I site	• **Use a *Rca* I or *Bsp* LU11 I site** in the forward primer, if no *Rca* I or *Bsp* LU11 I 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* LU11 I sites	• **Introduce a *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, refer to the literature given at the end of chapter 4.1). or • Prepare a cloning fragment by limited digestion if desired restriction site is present in the gene (refer to the literature given at the end of chapter 4.1).

Improved success rates

The pIVEX vectors are especially optimized for use in RTS cell-free protein expression systems. However, any DNA inserted into the expression vector 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 can hardly be predicted and may impede or improve the translation process. In particular, N-terminal extensions have proven to exhibit mostly positive impact on expression yields. Therefore, we recommend to clone the target gene in more than one expression vector.

3.3 Cloning procedure

3.3.1 Primer design for PCR cloning

Rules for primer pair design

- Use forward and reverse primers consisting of about 20 bases complementary to the gene, the restriction sites of choice (in frame), and 5-6 additional base pairs to allow proper restriction enzyme cleavage (for examples see chapter 4.1.1).
- For efficient digestion with *Nde* I or *Not* I the number of additional basepairs must be higher. Include 8 additional basepairs in the primer to cut your PCR product with *Nde* I and 10 additional basepairs to cut it with *Not* I.
- To express a gene without a tag, insert a stop codon at the end of the gene (for an example see appendix 4.1.4) and use pIVEX2.3d for cloning.
- Design forward and reverse primers with comparable (±2°C) melting temperature (for calculation of melting temperatures see appendix 4.1.1).
- Try to minimize secondary structure and dimer formation by means of primer design.
- High quality primers (purified on HPLC or acrylamide gels) are recommended.

3.3.2 Restriction digest of the pIVEX vectors

Digestion of pIVEX vectors for cloning

Briefly centrifuge down the contents of the vial with the pIVEX vectors.

- Digest the selected pIVEX vector(s) using the appropriate restriction enzymes and buffers (for restriction enzymes and buffers please refer to our current catalog).
- Run an agarose gel to control the reaction and to separate the linearized vector from undigested vector and smaller fragments.
- Isolate and purify the fragment with the correct size from the gel (e.g. using the Agarose Gel DNA Extraction Kit).

Examples:

Digestion with..	Procedure
Nco I and *Sma* I (or *Xma* I)	• Digest 2 µg (4 µl) of DNA with 20 units of *Sma* I in 20 µl of 1× buffer A at 25°C (or 20 units of *Xma* I in buffer M at 37°C) for one hour. • Check an aliquot to be sure that the plasmid is linearized. • Add 20 units of *Nco* I and digest for another hour at 37°C.
Nde I and *Sma* I (or *Xma* I)	• Digest 2 µg (4 µl) of DNA with 20 units of *Sma* I in 20 µl of 1× buffer A at 25°C (or 20 units of *Xma* I in buffer M at 37°C) for one hour. • Check an aliquot to be sure that the plasmid is linearized. • Add 20 units of *Nde* I and digest for another hour at 37°C. • (see 4.1.3 for additional hints concerning *Nde* I digests)
Not I and *Sma* I (or *Xma* I)	• Digest 2 µg (4 µl) of DNA with 20 units of *Sma* I in 20 µl of 1× buffer A at 25°C (or 20 units of *Xma* I in buffer M at 37°C) for one hour. • Check an aliquot to be sure that the plasmid is linearized. • Add 40 units of *Not* I in 40 µl of 1× buffer H and digest for another hour at 37°C. • (see 4.1.3 for additional hints concerning *Not* I digests)

Phosphatase treatment of the digested pIVEX 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 phosphatase by heating to 65°C for 15 min.

3.3.3 Preparation of the inserts

Generation of PCR fragments

- Primer design
  Design PCR primers according to section 3.3.1.
- PCR conditions
  Optimal reaction conditions depend on the template/primer pairs and have to be calculated accordingly.
  - To avoid nonspecific products and misincorporation, try to keep cycle number as low as possible (<25).
  - To reduce the error rate use a high fidelity PCR system that includes a proofreading enzyme (e.g. Expand High Fidelity PCR-System), especially with templates longer than 2 kb.
- Restriction digest
  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 (for *Nde* I 8 basepairs and for *Not* I 10 basepairs) from the 5' end. Therefore, restriction digests require higher enzyme concentrations and longer incubation times (see 4.1.3 for additional hints concerning *Nde* I and *Not* I digests).
- Purification of the PCR fragment
  Run the digested PCR product on an agarose gel. Excise the fragment with the correct size from the gel and purify it (e.g using the Agarose Gel DNA Extraction Kit).

3.3.3 Preparation of the inserts, continued

Subcloning of PCR fragments using PCR cloning 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 circumvent this step of uncertainty. An instruction for this strategy is given in the appendix (4.1.5).

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 chapter 4.3 for vector maps). **In any case, for cloning into pIVEX2.3d check whether the start codon AUG and the tag sequence are in the correct reading frame. For cloning into pIVEX2.4d check whether the first triplet of your gene of interest and the stop codon behind the *Bam* HI site are in the correct reading frame.**

3.3.4 Vector ligation, transformation, and purification

Ligation

Ligate the purified DNA fragment into the linearized pIVEX vector (using e.g. the Rapid DNA Ligation Kit). For ligation of DNA fragments digested with *Nde* I see chapter 4.1.3.

Transformation

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

Amplification of the plasmid in *E. coli*

Prepare a suitable amount of plasmid for the subsequent transcription-translation reactions. For a single 50 µl reaction, approx. 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 3.3.5) is recommended. Geno Pure Plasmid Midi or Maxi Kits are best suited for this purpose (see 4.6 Related products).

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 Rapid Translation System. When DNA purity is insufficient ($OD_{260/280} \leqslant 1.7$), a phenol treatment to remove proteins (e.g. traces of RNase) may enhance expression.

3.3.5 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. We recommend using a restriction enzyme with a single cleavage site in the vector (like *Cla* I or *Bam* HI) together with another enzyme that has one or two cleavage site(s) 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 T7 terminator.

- 5'- primer: 5'- TAATACGACTCACTATAGGG -3'
- 3'- primer: 5'- GCTAGTTATTGCTCAGCGG -3'

4. Appendix

4.1 Additional information for cloning

4.1.1 Example for designing a *Nco* I/*Sma* I primer pair

Target gene sequence (example):

```
   Met
5'-ATGCTAGCAAACTTACCTAAGGGTNNN   Stop
         NNNTTGTTCCCGTTCAAAATATTGTAA-3'

3'-TACGATCGTTTGAATGGATTCCCANNN
         NNNAACAAGGGCAAGTTTTATAACATT-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'
```

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

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 you resign the possiblity to recut the inserted DNA with *Nco* I, 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.

- and 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 (T_m) calculation

T_m = (number of A+T) × 2°C + (number of G+C) × 4°C

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

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

If you want to express a protein with only few additional amino acids at the N-terminus, we recommend 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 we recommend to insert a protease cleavage site directly upstream of your target gene sequence into the forward primer, e.g. an enterokinase cleavage site:

```
          NcoI
5´-XX XXX XCC ATG...
 ...GTA GAT GAC GAC GAC AAG NNN NNN...-3'
       Asp-Asp-Asp-Asp-Lys-target gene
                        ↑
                enterokinase cleavage site
```

4.1.3 Special information for 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 your DNA preparations are highly pure (DNA purified by "quick-and-dirty" miniprep procedures is often *NOT* pure enough). If necessary, increase *Nde* I concentrations used for restriction digest.
- DNA digested with *Nde* I is more difficult to ligate with 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 use up to 5-fold more *Not* I for complete digestion.

4.1.4 Example for cloning and expression of a gene without <u>any</u> 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).

<u>Example:</u> 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:

```
          SmaI
5'-XXX XXX CCC GGG TTA TGT GCC GAA AAG ATT-3'
```

4.1.5 Subcloning of PCR fragments using PCR cloning vectors

A disadvantage of direct cloning may be the inefficient cutting of restriction sites located at the very end of a fragment in some cases. 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.

IF you want to...	THEN...
Subclone in blunt end cloning vectors	• Perform the PCR with thermostable DNA polymerase (with 3´-5´-Exonuclease activity, *e.g.*, Tgo or Pwo SuperYield) to create PCR fragments with blunt ends (the Expand High Fidelity PCR-System also creates a sufficient amount of blunt ended PCR fragments). • Then ligate into a blunt end cut cloning vector (*e.g.*, using the 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 Expand High Fidelity PCR-System or *Taq* DNA Polymerase to create PCR fragments with single deoxyadenosine residue overhangs at the 3´ends. • Then ligate into a linearized cloning vector with a T- overhang and continue as described above.

Literature

For information on basic cloning techniques, please refer to the following general references:

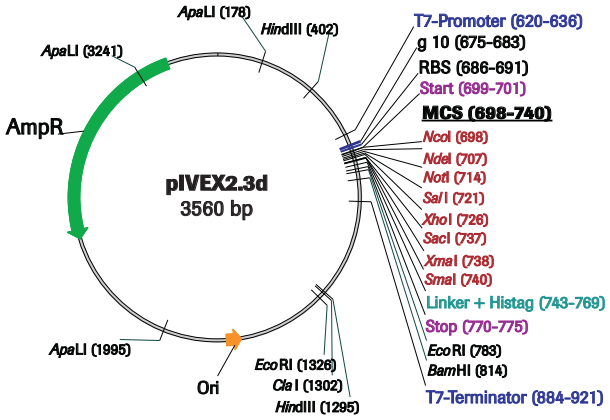
1 Sambrook et al. (1989) "Molecular Cloning - A Laboratory Manual" Second Edition, Cold Spring Harbor Laboratory Press, New York.
2 Ausubel, U. K. et al. (1993) "Current Protocols In Molecular Biology" John Wiley & Sons Inc., New York.

4.2 Trouble shooting guide

Observation	Potential Reason	Recommendation
No PCR product	Secondary structures of the primers	• Try to minimize secondary structure and dimer formation when designing primers. • 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.
	Inadequate annealing temperature	• Check whether the right annealing temperature was used for the PCR reaction (5 to 10°C lower than T_m). • Adapt the annealing temperature to the primer with the lowest melting temperature.
	Concentration of $MgCl_2$ too low	• Determine the optimal $MgCl_2$ concentration specifically for each template/primer pair by preparing a reaction series containing 0.5–4.5 mM $MgCl_2$. • Optimize the concentration of template DNA in the PCR reaction.
Nonspecific amplification	Low specificity of the primers	• Make sure that the primers specifically flank the 5'- and 3'- ends of your gene and are not complementary to other sequence regions of the template DNA. If necessary, increase primer length. • Use hot start techniques.
	Concentration of $MgCl_2$ too high	• Avoid excess of free magnesium leading to unspecific amplification. • Determine the optimal concentration by preparing a reaction series containing 0.5–4.5 mM $MgCl_2$. • Raise the annealing temperature if necessary.
No or only few colonies after transformation	Inappropriate selection medium	Make sure that your plates contain 50 µg/ml ampicillin or carbenicillin and no other antibiotics.
	Inactive competent cells	• Avoid frequent freezing and thawing of competent cells. • Perform a test transformation with 10 pg supercoiled control plasmid.
	Excess of ligation reaction during transformation	• 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.
	Unsuccessful restriction digest of the PCR product	• 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 appendix (section 4.1.3). • 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 section 4.1.5).
	Unsuccessful ligation	• Check activity of T4 DNA ligase by performing a control ligation reaction. • Use fresh ligase • Store the ligation buffer aliquoted at -20°C, as freezing and thawing results in degradation of ATP. • Vary the ratio of vector DNA to insert DNA: • Adjust the molar ratio of vector DNA to insert DNA to 1+3 (e.g. 50 ng linearized dephosphorylated vector and 50 ng insert (for a vector / insert size ratio of 3:1). • When vector and insert DNA differ in length, try other molar ratios (1+1, 1+2). • 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 appendix (section 4.1.3).
	Alkaline phosphatase not inactivated after vector dephosphorylation	Inactivate the alkaline phosphatase (please note: shrimp alkaline phosphatase can be inactivated simply by heat treatment whereas complete inactivation of calf intestine phosphatase requires additional treatments (e.g. phenolization).
High background of non-recombinants after transformation	Inappropriate medium	Make sure that your selection medium contains the correct, active antibiotic by performing a mock transformation reaction without DNA. No colonies should be obtained.
	Incomplete digestion of vector / insert	Purify the vector / insert after the first digestion step using the High Pure PCR Product Purification Kit. Perform the second digestion step of the vector / insert in the optimal buffer.
	Unsuccessful dephosphorylation of the vector	• Perform a religation control reaction without insert where only few colonies should be obtained. • Use fresh (shrimp) alkaline phosphatase. • Increase the incubation time.
	Excess of linearized, phosphorylated vector	• Depending on background strongly reduce the amount of linearized vector in the ligation reaction two- to fivefold. • ***Note:*** If the vector/insert ratio is too high, religation is favored.

4.3 Vector maps

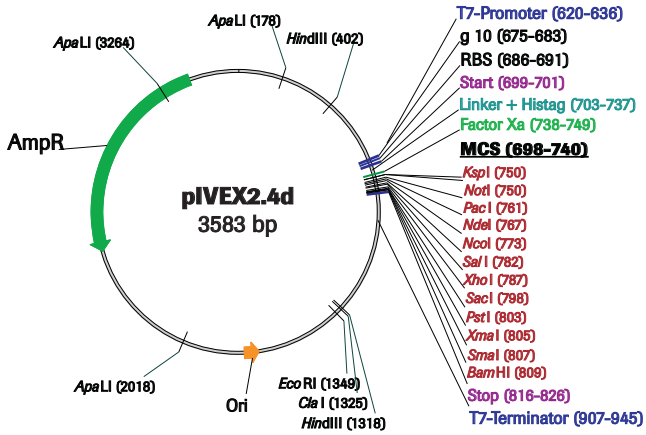
pIVEX2.3d vector




```
                                  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* *****
```

pIVEX2.4d vector




```
                                  T7-Promoter
601   GATCTCGATC CCGCGAAATT AATACGACTC ACTATAGGGA GACCACAACG
      CTAGAGCTAG GGCGCTTTAA TTATGCTGAG TGATATCCCT CTGGTGTTGC

                                  g10 ϵ       RBS
651   GTTTCCCTCT AGAAATAATT TTGTTTAACT TTAAGAAGGA GATATACCAT
      CAAAGGGAGA TCTTTATTAA AACAAATTGA AATTCTTCCT CTATATGGTA
                                                          Me
                                                       KspI
       Linker + Histag                          Factor Xa NotI
701   GTCTGGTTCT CATCATCATC ATCATCATAG CAGCGGCATC GAAGGCCGCG
      CAGACCAAGA GTAGTAGTAG TAGTAGTATC GTCGCCGTAG CTTCCGGCGC
      tSerGlySer HisHisHisH isHisHisSe rSerGlyIle GluGlyArgG

          PacI      NdeI    NcoI      SalI XhoI   SacI  PstI
751   GCCGCTTAAT TAAACATATG ACCATGGCAA GTCGACTCGA GCGAGCTCTG
      CGGCGAATTA ATTTGTATAC TGGTACCGTT CAGCTGAGCT CGCTCGAGAC
      lyArgLeuIl eLysHisMet ThrMetAlaS erArgLeuGl uArgAlaLeu

         XmaI
         SmaIBamHI
801   CAGCCCGGGA TCCGGTAACT AACTAAGATC CGGTAAGATC CGGCTGCTAA
      GTCGGGCCCT AGGCCATTGA TTGATTCTAG GCCATTCTAG GCCGACGATT
      GlnProGlyIle Arg*** * ** ***
```

4.4 Note to the purchaser

When using the Ni-NTA technology for the purification of polyhistidine-tagged proteins in research applications, it is recommended to purchase the purification resin from Qiagen for which they hold exclusive licenses from F. Hoffmann-La Roche under European Patent 0253303, US Patent 4,877,830 and corresponding patent rights.

When using the Ni-NTA technology and the purification resin from Qiagen for commercial purposes, a license is required in addition from F. Hoffmann-La Roche under the above mentioned patents.

4.5 Detection of expressed His_6-tagged proteins

The His_6-tagged proteins can be detected easily after SDS-PAGE and by Western blotting using an Anti-His_6 antibody. For methods in basic procedures refer to the literature (*e.g.*, Ausubel et al., cited in chapter 4.1.5). For Cat. No. of the products needed for detection, please refer to section 4.6.

Step	Action
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 room temperature (or at 4°C overnight).
2	Wash 3 × 5 min with TBST.
3	Dissolve Anti-His_6-Peroxidase 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 room temperature with gentle agitation.
5	Wash 4 × 5 min with TBST.
6	Incubate the blot for 5 min in a quantity of Lumi-Light ^{Plus} substrate solution sufficient to cover the membrane (0.1 ml/cm^2).
7	Expose on Lumi-Imager F1 Work Station or X-ray film for 1min. Adjust the exposure time between 10 s and 20 min according to the result of the first film.

Example:

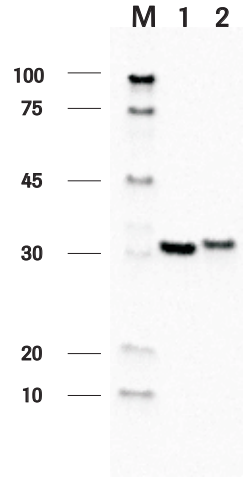


Fig. 2: Expression of His-tagged GFP (1) and GFP mutant (2) proteins in RTS 500 HY: Western blot was incubated with Roche´s Anti-His_6-POD conjugate as described. M = Multi-tag-Marker

4.6 Related products

Product	Pack Size	Cat. No.
Linear Template Generation by PCR		
RTS *E. coli* Linear Template Generation Set, His_6-tag	96 reactions	03 186 237 001
RTS *E. coli* Linear Template Generation Set, HA-tag	96 reactions	03 315 860 001
RTS *E. coli* Linear Template Generation Set, MBP fusion	96 reactions	03 358 828 001
Rapid Expression Screening and Optimization		
RTS 100 *E. coli* HY Kit [2]	24 reactions 96 reactions	03 186 148 001 03 186 156 001
Preparative-Scale Expression		
RTS 500 ProteoMaster *E. coli* HY Kit [2,3,4]	5 reactions	03 335 461 001
RTS 500 *E. coli* HY Kit [1,2,3,4]	2 reactions 5 reactions	03 246 817 001 03 246 949 001
RTS 9000 *E. coli* HY Kit [4,5,6]	1 reaction 3 reactions	03 290 395 001 03 290 468 001
AviTag Biotinylation Reagents		
RTS AviTag *E. coli* Biotinylation Kit, Plasmid	For 96 reactions (RTS 100) or 5 reactions (RTS 500)	03 514 919 001
RTS AviTag Biotinylation Kit	For 96 reactions (RTS 100) or 5 reactions (RTS 500)	03 514 935 001
Vectors		
RTS pIVEX HA-tag Vector Set	2 vectors, 10 µg each	03 268 993 001
RTS pIVEX MBP Fusion Vector [5]	1 vector, 10 µg	03 268 985 001
RTS pIVEX GST Fusion Vector [6]	1 vector, 10 µg	03 268 969 001
Other Reagents		
RTS 100 *E .coli* Disulfide Kit	1 Kit	04 349 741 001
RTS GroE Supplement	for five RTS 500 reactions	03 263 690 001
RTS Amino Acid Sampler	for five RTS 500 reactions	03 262 154 001
Expand™ High Fidelity PCR-System	10× 250 units	11 759 078 001
Pwo SuperYield	100 units	04 340 868 001
High Pure PCR Product Purification Kit	1 Kit	11 732 668 001
Anti-His_6-Peroxidase	50 U	11 965 085 001
Genopure Maxiprep Kits	1 kit (10 preps)	03 143 422 001
Restriction Enzymes For a complete listing of all restriction enzymes please visit our Special Interest Site at www.restriction-enzymes.com.		
Bam HI	1000 units	10 220 612 001
Bse AI	200 units	11 417 169 001
Eco RV	2000 units	10 667 145 001
Nco I	200 units	10 835 315 001
Nde I	200 units	11 040 219 001
Not I	200 units	11 014 706 001
Pin AI (*Age* I)	200 units	11 464 841 001
Rca I (= *Bsp* HI)	200 units	11 467 123 001
Sca I	500 units	10 775 258 001
Sgr AI	200 units	11 277 014 001
Sma I	1000 units	10 220 566 001
Ssp I	1000 units	10 972 975 001
Xba I	1000 units	10 674 257 001
Xma CI (=*Xma* I)	200 units	11 743 392 001

[1] For use with the RTS 500 Instrument

[2] For Research Use Only. Proteins expressed using the RTS, and data derived therefrom that would enable the expression of such proteins (collectively, "Expressed Proteins"), may be used only for the internal research of the purchaser of this system. Expressed Proteins may not be sold or transferred to any third party without the written consent of Roche Diagnostics."

[3] The purchase price of this product includes a limited, non-exclusive, non-transferable license under U.S. patents 6.168.931 and 6.337.191 and their foreign counterparts, exclusively licensed by a member of the Roche Group.

[4] The continuous-exchange cell-free (CECF) technology applied in the RTS 100, 500, and RTS 9000 products is exclusively licensed by a member of the Roche Group.

[5] The MBP expression component derived from pMAL™ (New England Biolabs) of Roche's Rapid Translation System (RTS) Reagents and Kits is licensed for use with Roche's Rapid Translation System only. For any commercial use of pMAL™ expression, licensing information may be obtained from New England Biolabs, Legal Department, 32 Tozer Road, Beverly, MA 01915, USA.

[6] The GST fusion protein expression component of Roche's Rapid Translation System (RTS) Reagents and Kits is covered by a non-exclusive license granted by Amersham Biosciences AB under European patent 0 293 249 and corresponding patents in other countries to Roche Diagnostics GmbH. Purchasing this product allows its use for scientific investigation and research only. The product shall not be used for any clinical or therapeutic use on humans or animals. Licenses for commercial use of products covered by the above mentioned patents must be negotiated directly with Chemicon International, Inc.

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