Rapid Translation System
RTS pIVEX His-tag Vector Set

Cat. No. 3 253 538

Version 1, May 2001

Store at –15 to –25°C

1. Preface

Kit contents

Vial	Label	Contents and use
1	pIVEX 2.3	• 10 µg (20 µl) plasmid • cloning vector with C-terminal His-Tag • for cloning using *Nco* I and *Sma* I
2	pIVEX 2.3-MCS	• 10 µg (20 µl) plasmid • cloning vector with C-terminal His-Tag • contains a multiple cloning site (MCS)
3	pIVEX 2.4a	• 10 µg (20 µl) plasmid • cloning vector with N-terminal His-Tag • contains a multiple cloning site • for reading frame **a**
4	pIVEX 2.4b Nde	• 10 µg (20 µl) plasmid • cloning vector with N-terminal His-Tag • contains a multiple cloning site (MCS) and an • additional *Nde* I site • for reading frame **b**
5	pIVEX 2.4c	• 10 µg (20 µl) plasmid • cloning vector with N-terminal His-Tag • contains a multiple cloning site (MCS) • for reading frame **c**

Safety Information

All bottles contain no 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.

2. Introduction

Roche's Rapid Translation System RTS pIVEX His-tag Vectors are designed for high-level expression of His-tagged proteins in the cell free RTS *E.coli* system. The vectors contain all regulatory elements necessary for *in vitro* expression with T7 RNA polymerase in the prokaryotic system. The introduction of either a N-, or a C-terminal His-tag provides a rapid method to purify proteins of interest. Cloning into pIVEX His-tag Vectors allows optimal protein expression in all RTS *E.coli* systems (see Related products in chapter 4.7).

3. Cloning into pIVEX vectors

Literature

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

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

3.1 Vector description

Cloning 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 kind and position of the tag
  - Even numbers mean tags, fused to the N-terminus
  - Odd numbers mean tags, fused to the C-terminus
- Letters **a, b, c** indicate different reading frames.

Functional elements of the cloning vectors

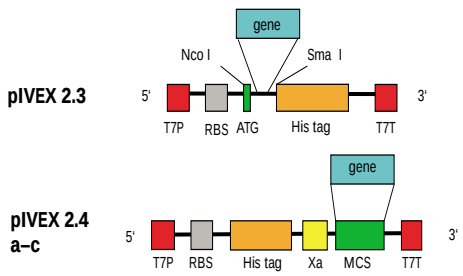


Fig. 1: Cloning pIVEX cloning vectors.
Abbreviations
T7 P = T7 Promotor, RBS = Ribosomal binding site, ATG = Start codon, *Nco* I, *Sma* I = Restriction enzyme recognition sites for cloning of the target gene, C-Tag, N-Tag = C- or N-terminal tag position, Xa = Factor Xa restriction protease cleavage site, MCS = Multiple cloning site in three different reading, frames for the insertion of the target gene, T7 T = T7 Terminator

Cloning vectors supplied in the set

Five different cloning vectors are supplied in the set. All vectors contain the hexa-Histidine tag. The general architecture is shown in Fig. 1.

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

3.2 Guidelines for vector selection

Vectors available

Five pIVEX cloning vectors are provided with this set. 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**.

Use and location of the tag

Each pIVEX vector contains a hexa-His fusion tag to allow easy detection and purification of the expressed protein. (For purification and detection protocols please refer to our web site www.proteinexpression.com).

- Use pIVEX 2.3 or pIVEX 2.3-MCS for fusing the gene with a C-terminal His-tag.
- Use pIVEX 2.4(a-c) for fusing the gene with a N-terminal His-tag.

For expression without tag use pIVEX 2.3 or pIVEX 2.3-MCS and incorporate the stop codon (TAA) at the end of the gene (see chapter 4.3).

Selecting the cloning strategy

The pIVEX vectors provide high flexibility regarding the cloning strategy. To minimize problems, we recommend selecting the cloning strategy strictly according to the following decision matrix.

IF...	THEN...
The target gene does not contain internal *Nco* I and *Sma* I or *Xma* I sites	• Use *Nco* I and *Sma* I (or *Xma* I) sites for cloning. • Use pIVEX 2.3 or pIVEX 2.4b *Nde* cut with *Nco* I and *Sma* I, or *Xma* I ***Note***: *Xma* I recognizes the same sequence as *Sma* I but leaves a cohesive (sticky) end. Cloning of fragments containing sticky ends is generally easier than cloning of blunt ended inserts.
The target gene has an internal *Sma* I site (generates blunt ends)	• Check the gene to be free of any other blunt end restriction enzyme (e.g. *Eco* RV, *Ssp* I, *Sca* I). • Use an alternative blunt end restriction site in the reverse primer. • Use pIVEX 2.3 or pIVEX 2.4b *Nde* cut with *Nco* I and *Sma* I.
You want to avoid blunt end cloning at the 3'end	Check whether the gene is free of *Xma* I sites. If you find *Xma* I sites you can use *Pin* AI, *Sgr* AI, *Bse* AI, or *Ngo* MIV. These enzymes generate compatible, cohesive (sticky) ends.
The target gene has an internal *Nco* I site	• Check if no *Rca* I or *Bsp* LU11 I site is present in the target gene. If not, use a *Rca* I site (*Bsp* HI = isoschizomer) or *Bsp* LU11I in the forward primer. An *Rca* I or *Bsp* LU11I digested fragment can also be ligated into the *Nco* I site. • Use pIVEX 2.3 or pIVEX 2.4 b Nde cut with *Nco* I and *Sma* I.
No *Nde* I site is present internally in the target gene:	• Use *Nde* I sequence in the forward primer. • Use *Nde* I site in pIVEX 2.3-MCS or pIVEX 2.4b Nde.
The target gene has internal *Nco* I, *Rca* I, *Bsp* LU11I and *Nde* I sites	• Check for any of the additional restriction sites present in pIVEX 2.3 MCS or pIVEX 2.4 (a-c). • Include one of these sites into the forward primer. ***Note*: The rare cutting restriction enzyme *Not* I with an 8 base pair recognition sequence is recommended if the gene is to be cloned into vectors with N- and C-terminal tag position.** • Use pIVEX 2.3-MCS or pIVEX 2.4b cut with *Not* I and *Sma* I. This will introduce additional amino acids. or • Prepare a cloning fragment by limited digestion if desired restriction site is present in the gene (refer to the literature given at the top of section 3.2). • Eliminate the restriction site by mutation (e.g. conservative codon exchange, refer to the literature given at the top of section 3.2).

Improving success rate

The pIVEX vectors are especially optimized for use in RTS 500 *in vitro* protein expression. Every DNA, inserted into the expression vector, results in an unique constellation. Interactions (base pairing on mRNA level) between the coding sequence of the target gene and the 5'- untranslated region that contain regulatory elements from the vector can hardly be predicted. Therefore, we recommend to clone the gene in more than one expression vector.

- Once the PCR fragment is prepared, cloning into different pIVEX vectors can easily be done in parallel.
- In particular, N-terminal extensions have proven to exhibit mostly positive impact on expression yields.

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 basepairs to allow proper restriction enzyme cleavage (for examples see appendix).
  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 4.3).
- Design forward and reverse primers to have a comparable (± 2°C) melting temperature (for calculation of melting temperatures see appendix 4.3).
- Exclude the possibility of secondary structure formation within the primers.
- Exclude complementary regions in the 3' ends of the primer pair.
- Use high quality primers that are purified on HPLC or acrylamide gels.

3.3.2 Restriction digest of the pIVEX vectors

Stability of pIVEX vectors

- Briefly centrifuge down the contents of the vial with the 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 decrease the amount of supercoiled plasmid.

Digestion of pIVEX vectors for cloning

- 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.
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. ***Note*:** The cutting efficiency of many restriction enzymes is reduced, when their recognition sites are less than 6 base pairs from the terminus. Therefore the use of adjacent restriction sites requires higher enzyme concentrations and longer incubation times.

Phosphatase treatment of the digested pIVEX vectors

This optional step is necessary for ligation of blunt ended inserts

- Treat 300 ng of digested pIVEX vector with 3 units of alkaline phosphatase (shrimp) in a total volume of 10 µl in 1× phosphatase buffer for 90 minutes at 37°C.
- Inactivate the phosphatase (shrimp) 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 must be calculated accordingly.
  - To avoid nonspecific products and misincorporation, run a minimal number of cycles.
  - To reduce the error rate use high fidelity PCR systems (e.g. Expand High Fidelity PCR-Sytem), 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, when their recognition sites are located less than 6 base pairs (for *Nde* I 8 basepairs and for *Not* I 10 basepairs) from the terminus. Therefore, restriction digests require higher enzyme concentrations and longer incubation times.
- 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).

Subcloning of PCR fragments using PCR cloning vectors

Restriction enzymes do often 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 circumvent this step of uncertainty. An instruction for this strategy is given in the appendix.

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 the correct restriction sites (see chapter 4.2 for vector maps).

- Use the restriction sites *Nco* I and *Sma* I (or *Xma* I) if you want to clone into pIVEX 2.3.
- Use two of the restriction sites between *Nde* I and *Sma* I (or *Xma* I) if you want to clone into pIVEX 2.3-MCS.
- Use two of the restriction sites between Ksp I and *Bam* HI if you want to clone into pIVEX 2.4(a-c).
- Check whether the start codon AUG and the stop codon (behind the *Bam* H I site) are in the correct reading frame.

3.3.4 Vector ligation, transformation and purification

Ligation

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

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 is 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 Rapid Translation System. When DNA purity is insufficient ($OD_{260/280} \leq 1.7$), a phenol treatment to remove 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 *Xba* I or *Bam* HI) together with another enzyme that has one or two cleavage site(s) within the target gene.

Sequencing

The ultimate proof for the fidelity of PCR and cloning is provided by sequencing. For sequencing use a 5' primer coding for the T7 promotor and a 3' primer, complementary to the T7 terminator.

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

3.4 Use of other vectors than pIVEX

General recommendations

The pIVEX vector family has been developed and optimized for use in the Rapid Translation System. Therefore, we strongly recommend cloning target genes into a pIVEX vector prior to *in vitro* expression.

Required vector elements

Any vector to be used in combination with the Rapid Translation System must include the following elements and structural features:

- circular closed form
- target gene under control of T7 promotor located behind a RBS (ribosomal binding site) sequence
- distance between T7 promotor and start ATG should not exceed 100 base pairs
- distance between the RBS sequence and start ATG should not exceed 5-8 base pairs
- T7 terminator sequence at the 3' end of the gene

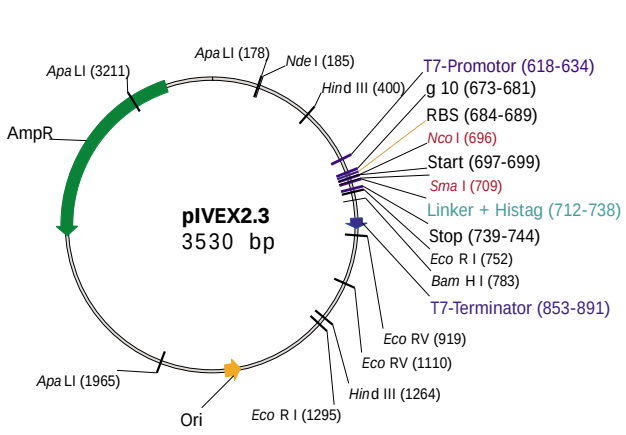
4. Appendix

4.1 Problems with the cloning procedure

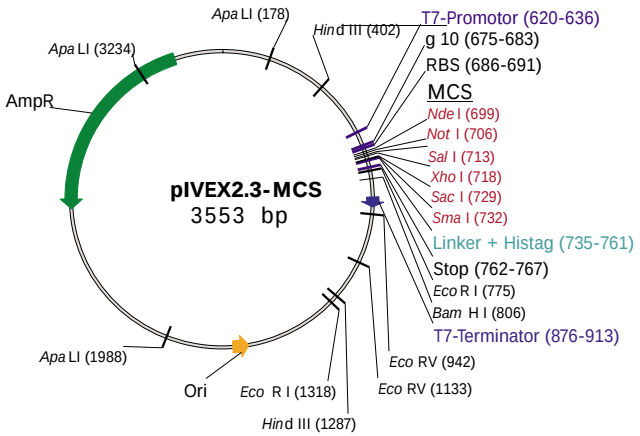
Observation	Potential Reason	Recommendation
No PCR product	Secondary structures of the primers	• Avoid complementary primer regions to exclude dimer formation by appropriate primer design. • Design primers that contain less than 60% G+C to avoid secondary structure formation. • Raise the primer concentration in the PCR reaction or use longer primers without G or C nucleotides at the 3'-end if a G+C content $<$60% is not feasible.
	Inadequate annealing temperature	• Check whether the right annealing temperature was used for the PCR reaction. • 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. • 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	• Use fresh competent cells. • Avoid frequent freezing and thawing of competent cells. • Perform a test transformation with supercoiled control plasmid (e.g. 10 pg of pUC18 plasmid) to verify the competence of your cells.
	Excess of ligation products during transformation	Limit the volume of the ligation products to less than 20% of the whole transformation reaction volume to avoid an inhibitory effect by an excess of DNA.
	Unsuccessful restriction digest of the PCR product	• Make sure that the right restriction buffer and reaction conditions were chosen. ***Note:*** 1. *Sma* I is optimally active at 25°C. 2. For restriction digest with *Nde* I and *Not* I, see appendix (section 5.3). • Increase incubation time. • Subclone the PCR product into a PCR cloning vector, if direct digestion of the PCR product is not successful (see section 5.3). ***Note***: A successful restriction digest of PCR products is not obviously visible in agarose gels.
No or only few colonies after transformation, continued	Unsuccessful ligation	• Check activity of T4 DNA ligase by performing a control ligation reaction. • Use fresh ligase • Use fresh ligation buffer • 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 insert / vector size ratio of 1:3). – When vector and insert DNA differ in length, try other molar ratios (1+1, 1+2). • Make sure that the digested vector ends are compatible with the ends of your insert. • Use restriction enzymes providing sticky ends at both ends of the gene fragment to be cloned use *Xma* I instead of *Sma* I. ***Note:*** For ligation of DNA fragments digested with *Nde* I, see appendix (section 5.3).
	Alkaline phosphatase not inactivated after vector dephosphorylation	Inactivate the alkaline phosphatase (shrimp alkaline phosphatase is inactivated by heat treatment).
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.
	Unsuccessful dephosphorylation of the vector	• Perform a negative control ligation 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 in two- to fivefold dilutions. ***Note*:** If the ratio vector/insert ratio is too high, religation is favored.

4.2 Vector maps

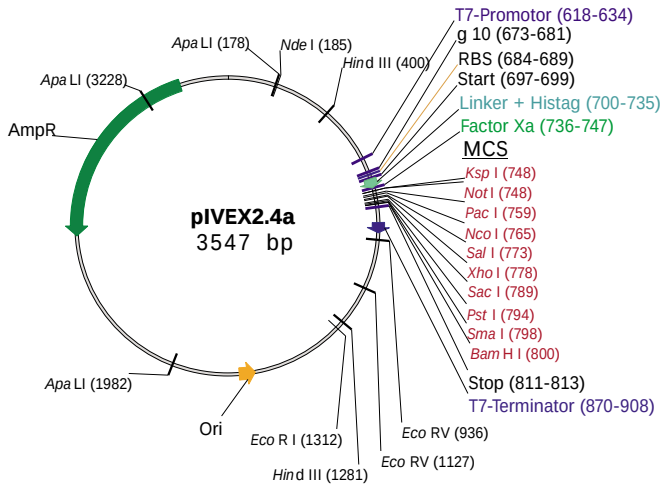
pIVEX 2.3 vector



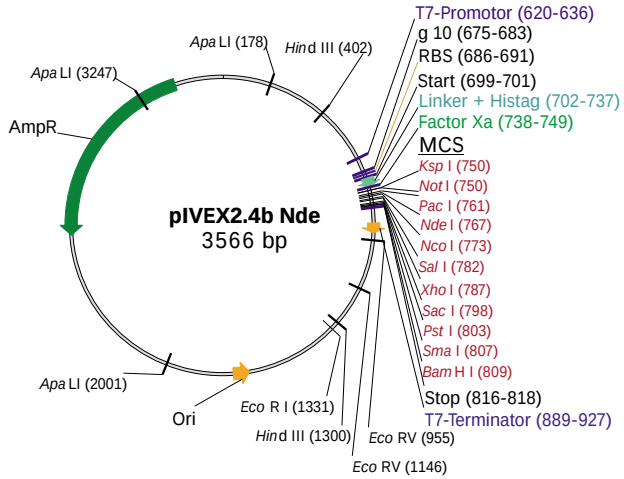
pIVEX2.3-MCS vector



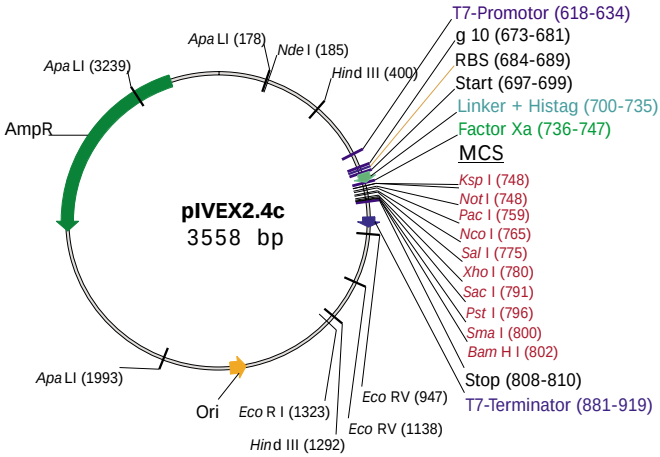
pIVEX2.4a vector



pIVEX2.4b vector



pIVEX2.4c vector



4.3 Additional information for cloning

Formula for melting point (T_m) calculation

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

Example for designing a Nco I/Sma I primer pair

Target gene sequence (example):

```
   Met                                   Stop
5′-ATGCTAGCAAACTTACCTAAGGGT.....
     ....AATTTGTTCCCGTTCAAAATATTGTAA -3′
3′-TACGATCGTTTGAATGGATTCCCA.....
     ....TTAAACAAGGGCAAGTTTTATAACATT -5′
```

For cloning a gene into a vector with a C-terminal tag-sequence use a forward primer with *Nco* I site (bold letters):

5´-XXXXXX***CCATGG***TAGCAAACTTACCTAAGGGT-3′

T_m= 12 × 2°C + 8 × 4°C = 56°C

and a reverse primer with *Sma* I site (bold letters):

5′-XXXXXX***CCCGGG***CAATATTTTGAACGGGAACAA-3′

T_m = 14 × 2°C + 7 × 4°C = 56°C

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. 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 first enzyme or use up to 5-fold more *Not* I for complete digestion.

continued on next page

4.3 Additional information for cloning, continued

Example for cloning a gene without expressing a tag

Target gene 3'- terminal sequence (example):

```
     asn leu phe gly gln
5′- AAT CTT TTC GGC ACA -3′
3′- TTA GAA AAG CCG TGT -5′
```

- Add a stop codon TAA (**ATT** in reverse orientation) between the last amino acid and the *Sma* I site.
- Add 6 bases *XXXXXX* after the *Sma* I site with any AT rich sequence and no complementarity to the reverse primer to allow a more efficient restriction cleavage

For this gene order the following reverse primer:

Reverse primer in 3'- 5' orientation

```
                                SmaI
3′- TTA GAA AAG CCG TGT ATT GGGCCC XXXXXX -5′
```

Reverse primer in 5'- 3' orientation

```
              SmaI
5′- XXXXXX CCC GGG TTA TGT GCC GAA AAG ATT -3′
```

Subcloning of PCR fragments using PCR cloning vectors

A disadvantage of direct cloning is 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 Tgo DNA polymerase 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). • Transform as usually and make minipreps from 2-6 transformants. • 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 Polymerase to create PCR fragments with single deoxyadenosine residue overhangs at the 3´ends. • Then ligate into a linearized cloning vector vectors with a T-overhang. • Transform as usually and make minipreps from 2-6 transformants. • Cut out the template gene from the subcloning vector and clone into the pIVEX vector cut with compatible restriction enzymes.

4.4 How to contact Roche Molecular Biochemicals

Three ways to contact us

To contact Roche Molecular Biochemicals for technical assistance, please choose one of the following:

IF you are using...	THEN...
the Internet	Access our web site at: http://www.proteinexpression.com or http://biochem.roche.com
E-mail	Please refer to the address that corresponds to your particular location, printed on the last page of this instruction manual.
the telephone	Please refer to the telephone number that corresponds to your particular location, printed on the last page of this instruction manual.

4.5 Related products

Product	Pack size	Cat. No.
Rapid Translation System RTS 100 *E.coli* HY Kit	24 reactions (50 µl each)	3186 148
Rapid Translation System RTS 500 Instrument	1 instrument	3064 859
Rapid Translation System RTS 500 *E.coli* Circular Template Kit	5 reactions (1 ml each)	3018 008
Rapid Translation System RTS 500 *E.coli* HY Kit	2 reactions (1 ml each) 5 reactions (1 ml each)	3246 817 3246 949
Tgo DNA Polymerase	50 reactions	3186 172
Expand High Fidelity PCR- System	10 × 250 units	1759 078
PCR Cloning Kit (blunt end)	1 kit	1939 645
Agarose MP	100 g	1388 983
Agarose Gel DNA Extraction Kit	1 kit	1696 505
Rapid DNA Ligation Kit	1 kit	1635 379
Phosphatase, alkaline, shrimp	1000 units	1758 250
Restriction Enzymes (for a complete listing of all restriction enzymes and pack sizes, refer to the Roche Molecular Biochemicals Catalog)		
Bam HI	1000 units	220 612
Bse AI	200 units	1417 169
Bsp LU11I	200 units	1693 743
Eco RV	2000 units	667 145
Nco I	200 units	835 315
Nde I	200 units	1040 219
Not I	200 units	1014 706
Pin AI (*Age* I)	200 units	1464 841
Rca I (= *Bsp* HI)	200 units	1467 123
Sca I	500 units	775 258
Sgr AI	200 units	1277 014
Sma I	1000 units	220 566
Ssp I	200 units	972 967
Xba I	1000 units	674 257
Xma CI (=*Xma* I)	200 units	1743 392

E-mail Address	Country	E-mail Address	Country
argentina.biochem@roche.com	Argentina	Raitis@invitros.lv	Latvia
biochem.au@roche.com	Australia	Sakkijha@rdleb.com	Lebanon
Gerhard.Muehlbauer@roche.com	Austria	Gintaras@eksma.lt	Lithuania
biochem.be@roche.com	Belgium	diagnostics@prophac.lu	Luxembourg
Valent@mbox.cit.bg	Bulgaria	Vccl@vol.net.mt	Malta
africhem@camnet.cm	Cameroon	Aiouche.echo@dounia.net.ma	Morocco
biochem.ca@roche.com	Canada	biocheminfo.nl@roche.com	Netherlands
biochem.cn@roche.com	China	biochem.nz@roche.com	New Zealand
Info@medisell.com.cy	Cyprus	bofungwu@linkserve.com.ng	Nigeria
Bm-comp@bm-comp.cz	Czech Republic	biochem.se@roche.com	Norway
dk.biochem@roche.com	Denmark	biochem.pt@roche.com	Portugal
ou.melestrum@neti.ee	Estonia	Topdiag@fx.ro	Romania
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Bm_roche@hotmail.com	India	biochem.se@roche.com	Sweden
h.hajian@tebtech.com	Iran	BiochemInfo.CH@roche.com	Switzerland
tubanegin@istn.irost.com	Iran	Jean-Marie.kindbeiter@roche.com	Tunisia
Dyn@netvision.net.il	Israel	bmuae@emirates.net.ae	United Arab Emirates
it.biochem@roche.com	Italy	uk.biochem@roche.com	United Kingdom
biochemicals@rdj.co.jp	Japan	biochemts.us@roche.com	USA
pharmakp@net2000ke.com	Kenya	Mvalentiner@telcel.net.ve	Venezuela
Bmskorea@chollian.net	Korea	dusica@eunet.yu	Yugoslavia
react@ncc.moc.kw	Kuwait	biochemts.row@roche.com	All other countries

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Argentina 541 954 5555; **Australia** (02) 9899 7999; **Austria** (01) 277 87; **Belgium** (02) 247 4930; **Brazil** +55 (11) 3666 3565; **Bulgaria** +35929625408; **Cameroon** 237-370269; **Canada** (450) 086 7050; (800) 361 2070; **Chile** 00 56 (2) 22 33 737 (central) 00 56 (2) 22 32 099 (Exec); **China** 86 21 6427 5586; **Colombia** 0057-1-3412797; **Cyprus** +357-2-311362; **Czech Republic** (0324) 45 54, 58 71-2; **Denmark** +45 363 999 58; **Egypt** 20-2-3619047; **Estonia** 372-7-447600; **Ethiopia** 251-1-552799; **Finland** +358 9 525 333 66; **France** 04 76 76 30 87; **Germany** (0621) 759 8568; **Greece** +3 01 61 66 100; **Hong Kong** (852) 2485 7596; **India** +91-22-8379906; **Indonesia** 62 (021) 252 3820 ext. 755; **Iran** +98-21-8072374 / +98-21-8797027; **Israel** 972-6- 6380569; **Italy** 039 247 4109-4181; **Japan** 03 3432 3155; **Kenya** +254-2-750112; **Korea** 82-2-3471-6500; **Kuwait** +965-4837859; **Latvia** 371-787828309; **Lebanon** Fax: 00961-1-399667; **Lithuania** 370-2-729715; **Luxembourg** +352-496098; **Malta** Fax: +356-341087; **Morocco** Fax: +212-2-944040; **Malaysia** 60 (03) 755 5039; **Mexico** (5) 227 8967; **Netherlands** (036) 539 4911; **New Zealand** (09) 276 4157; **Nigeria** +234-1-521767; **Norway** (47) 23 373300; **Philippines** (632) 810 7246; **Poland** +48 (22) 22 66 84 305; **Portugal** (01) 4171717; **Republic of Ireland** 1 800 40 90 41; **Romania** +40-1-2123763; **Russia** (49) 621 759 8636 Fax: (49) 621 759 8611; **Saudia Arabia** +966-1-4010364; **Singapore** 0065 272 9200; **Slovenia** +386 61 1363528; **South Africa** (011) 886 2400; **South Korea** 02 569 6902; **Spain** (93) 201 4411; **Sweden** (08) 404 8800; **Switzerland** +41 (41) 799 6161; **Taiwan** (02) 736 7125; **Thailand** 66 (2) 274 07 08 (12 line); **Turkey** 0090 212 216 32 80; **United Arab Emirates** +971-4-694351; **United Kingdom** (0800) 521578; **USA** (800) 428 5433. **Venezuela** Fax: +0058-4810697; **Yugoslavia** +381 11 137163.

Roche Diagnostics GmbH
Roche Molecular Biochemicals
Sandhofer Strasse 116
D-68305 Mannheim
Germany