For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Rapid Translation System RTS 500

E.coli Circular Template Kit

In vitro protein synthesis system based on *E.coli* lysate

Cat. No. 3 018 008
For five 1 ml synthesis reactions

Store this kit at −15°C to −25°C

Instruction Manual

Version 2, September 2000

The Rapid Translation System website:
www.proteinexpression.com

Please refer to “Notice for the purchaser” on page 35.

Roche

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1. Preface

Kit contents

Bottle no./ cap color	Label	Contents and function
1 red	*E.coli* Lysate	• stabilized and lyophilized lysate from *E.coli* • contains components for transcription and translation • five bottles total, one bottle per reaction
2 green	Reaction Mix	• stabilized and lyophilized substrate mix to prepare 1 ml Reaction Solution • five bottles total, one bottle per reaction
3 blue	Feeding Mix	• stabilized and lyophilized substrate mix to prepare 10 ml Feeding Solution • five bottles total, one bottle per reaction
4 orange	Energy Mix	• energy regenerating system, solid • five bottles total, one bottle per reaction
5 white	Reconstitution Buffer	• ready-to-use buffer solution for the reconstitution of bottles 1, 2, 3, and 4 • two bottles containing 35 ml each
6 colorless	Control Vector GFP	• 50 µg lyophilized plasmid • GFP (green fluorescent protein) expression vector with C-terminal His-Tag for the control reaction
7 blue	pIVEX 2.3	• 10 µg lyophilized plasmid • cloning vector with C-terminal His-Tag • for cloning using *Nco* I and *Sma* I
8 blue	pIVEX 2.3-MCS	• 10 µg lyophilized plasmid • cloning vector with C-terminal His-Tag • contains a multiple cloning site (MCS)
9 blue	pIVEX 2.4a	• 10 µg lyophilized plasmid • cloning vector with N-terminal His-Tag • contains a multiple cloning site • for reading frame **a**
10 blue	pIVEX 2.4b Nde	• 10 µg lyophilized plasmid • cloning vector with N-terminal His-Tag • contains a multiple cloning site (MCS) and an • additional *Nde* I site • for reading frame **b**
11 blue	pIVEX 2.4c	• 10 µg lyophilized plasmid • cloning vector with N-terminal His-Tag • contains a multiple cloning site (MCS) • for reading frame **c**
12	Reaction Device	• disposable plastic device with 2 compartments, each containing 1 magnetic stirring bar. The two compartments are divided by a semi-permeable membrane. • five devices total • device fits into RTS 500 Instrument. The RTS 500 Instrument (stirring and temperature control unit) is available from Roche Molecular Biochemicals, Cat. No. 3 064 859.

Safety information

Bottles 2 and 3 contain < 25% dithiothreitol, < 20% EDTA. All other 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.

Laboratory requirements

To avoid contamination with RNases we recommend using RNase-free materials and wearing gloves. Heat-treated glassware is not required, since solutions can be reconstituted in the bottles provided. An UV lamp (360 nm) is needed for the detection of GFP (control reaction).

Additional equipment required

To perform protein expression with this kit, the following equipment is required:

- RTS 500 Instrument, Cat. No. 3 064 859 (Stirring and Temperature Control Unit)
- Pipettes 10-200 µl, 200-1000 µl, and 10 ml
- Pipette tips autoclaved at 121°C for 20 min

Additional reagents required

Besides the template vector coding for the protein of interest, no additional reagents are required. Use only deionized DNase- and RNase-free water for the reconstitution of the Control Vector GFP and the Cloning Vectors .

A GFP standard protein (e.g. r-GFP from Roche Molecular Biochemicals) is required for the accurate quantitation of GFP (control reaction) on SDS-polyacrylamide gels.

2. Introduction

2.1 Product overview

System components

The Rapid Translation System RTS 500, consists of:

- reagents for 5 coupled transcription/translation reactions,
- 5 disposable Reaction Devices,
- 4 different cloning vectors,
- 1 Control Vector GFP,
- The RTS 500 Instrument (stirring and temperature control unit, Cat. No. 3 064 859)

The RTS 500 *E.coli* Circular Template Kit contains all reagents and reaction devices to perform five 1 ml reactions. The kit also contains one control vector GFP and four different cloning vectors.

Reaction principle

Transcription and translation take place simultaneously in the 1 ml reaction compartment of the reaction device. Substrates and energy components essential for a sustained reaction are continuously supplied via a semipermeable membrane. At the same time, potentially inhibitory reaction by-products are diluted via diffusion through the same membrane into the 10 ml feeding compartment. Protein is expressed for up to 24 hours yielding up to 500 µg of functionally active protein.

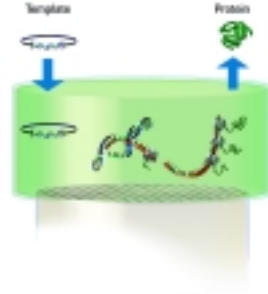

Fig 1: ***Schematic illustration of CECF protein expression.***
Transcription and translation take place simultaneously in the reaction compartment

Step	Description
1	The protein of interest is cloned into a template vector and added to the reaction compartment.
2a and 2b	In a coupled *in vitro* reaction DNA is first transcribed from the template vector into mRNA by T7 RNA polymerase (2a), followed by translation by the ribosomal machinery present in the *E.coli* lysate (2b).
3	Expressed protein accumulates in the reaction compartment and is harvested after a 4–24 hours run.

Application

The system consisting of the RTS 500 *E.coli* Circular Template Kit and the RTS 500 Instrument is designed for protein expression in the scale of 100 to 500 µg in a 1 ml reaction volume. Proteins in the molecular weight range from 10 to 120 kD have been successfully synthesized. For a current list of expressed proteins, please refer to our website at http://www.proteinexpression.com.

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Limitations of post-translational modifications

The RTS 500 *E.coli* Circular Template Kit is not able to introduce the following post-translational modifications:

- Glycosylation
- Phosphorylation
- Disulfide bond formation
- Signal sequence cleavage

Template DNA

The DNA of interest must be cloned into a pIVEX vector or another vector, designed for prokaryotic *in vitro* protein expression and containing a T7 promotor. The requirements are described in chapter 3.3.

Reaction time

Between 4 and 24 hours

Number of reactions

The kit provides reagents and plastic disposables for five 1 ml reactions. Reagents for each reaction are bottled separately.

Stability

This product is stable at −15°C to −25°C until the expiration date printed on the label.

Advantages

Feature	Advantage
• **Fast**	• Kit components reconstituted and mixed within minutes • Proteins expressed within 24 hours • RTS 500 Instrument (separately available) for controlled stirring and heating easy to set up and program
• **Cell-free**	• Expression of toxic proteins • Expression independent of codon usage due to excess of all tRNA species • No fermentation, safety regulations due to lack of recombinant organisms
• **Versatile**	• Easy adaptation of expression conditions by changing temperature, adding detergents, chaperones or protease inhibitors • Monitoring of the expression by taking samples throughout the run
• **Reliable**	• Kits tested to yield minimum 400 μg/ml GFP control protein • Reproducible results
• **Complete**	• Required components for the expression supplied with the kit

2.2 Background information

Coupled *in vitro* transcription/ translation

Instead of working with isolated mRNA it is more effective to synthesize the mRNA in an *in vitro* expression system (DeVries & Zubay (1967).

Upon addition of the DNA template and T7 RNA polymerase to the DNA-depleted *E.coli* lysate, transcription and translation are closely coupled in time and space: While the T7 RNA polymerase transcribes the template gene, the ribosomes provided by the *E. coli* lysate start to translate the 5'-end of the nascent mRNA.

Continuous Exchange Cell-Free protein synthesis (CECF)

During coupled transcription and translation energy components, nucleotides, and amino acids are consumed, whereas waste products like inorganic pyrophosphate, phosphate, nucleotide mono- and diphosphates, and other degradation products are formed.

The accumulation of inhibitory waste components and the depletion of substrates would quickly result in the stop of the reaction.

This is prevented by a continuous removal of waste and supply with substrate components through a semipermeable dialysis membrane, which separates the upper reaction compartment from the lower feeding compartment in the RTS 500 Reaction Device as shown in the figure.

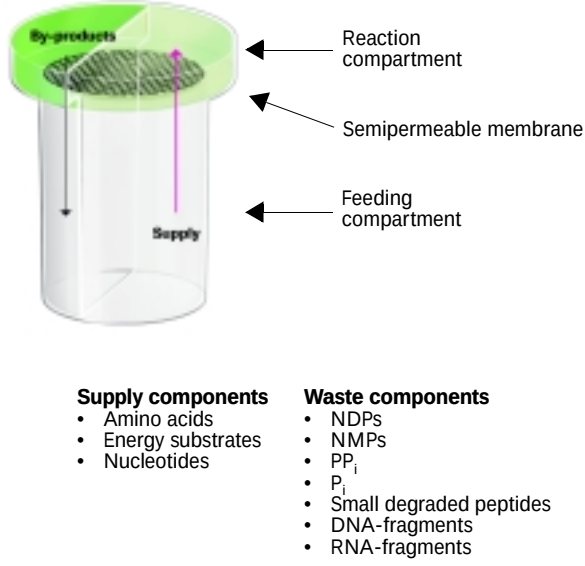

Fig 2: RTS Reaction Device. Principle of *Continuous Exchange Cell-Free* protein synthesis (CECF) as invented by Spirin (1988).

3. Procedures and required materials

3.1 Protein synthesis reaction

3.1.1 Before you begin

Kit reagents

- All required reagents are supplied with the kit.
- Do not combine reagents from different kit lots.
- Reconstitute only the bottles needed for the experiment.
- For reconstitution of bottles 1 to 4 use only Reconstitution Buffer supplied with the kit (bottle 5). For reconstitution of the plasmids (bottles 6 to 11) use sterile DNase- and RNase-free water.
- Reconstitution Buffer can be thawed at 25°C in a water bath.
- Store the reconstituted plasmids at −15°C to −25°C
- Reconstitute the lyophilized reagents directly prior to use.
- Keep reconstituted reagents and working solutions on ice before use.

DNA template

Prepare and purify as detailed in the cloning section (chapter 3.2).

Equipment

- Use the RTS 500 Instrument from Roche Molecular Biochemicals for optimal reaction performance and convenience (Cat. No. 3 064 859).
- Use only calibrated pipettes.
- Use RNase-free plastic and glassware.
- For precise quantification of the control reaction with GFP, apply the reaction products onto a SDS-polyacrylamid gel. A qualitative estimation can be made using an UV-lamp (360 nm).

3.1.2 Reconstitution of lyophilized components

Solution	Component	Reconstitution procedure	For use in
1	*E.coli* Lysate Bottle 1, red cap	Reconstitute the lyophilizate with 0.25 ml Reconstitution Buffer (bottle 5), mix carefully by rolling or gentle shaking. *DO NOT VORTEX*!	section 3.1.3 solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.8 ml Reconstitution Buffer (bottle 5), mix by rolling or shaking.	section 3.1.3 solution 8
3	Feeding Mix Bottle 3, blue cap	Reconstitute the lyophilizate with 10.5 ml Reconstitution Buffer (bottle 5), mix by rolling or shaking.	section 3.1.3 solution 7
4	Energy Mix Bottle 4, orange cap	Reconstitute the solid with 0.6 ml Reconstitution Buffer (bottle 5), mix by rolling or shaking.	section 3.1.3 solution 7 and 8
5	Reconstitution Buffer Bottle 5, white cap	• 2 × 35 ml ready-to-use solution • The solution is stable at 4°C but can also be stored at −20°C.	solution 1, 2, 3, 4
6	Control Vector GFP Vial 6, colorless cap	• Briefly centrifuge down the contents of the bottle • Reconstitute the lyophilizate with 50 µl sterile DNase- and RNase- free water • The solution is stable at −20°C.	section 3.1.5

Appearance of solutions

With the exception of the *E.coli* lysate all reconstituted lyophilizates should yield a clear solution. The *E.coli* lysate remains clowdy.

3.1.3 Preparation of working solutions

Solution	Component	Preparation of working solution	For use in
7	Feeding Solution	• Add 0.5 ml of the reconstituted Energy Mix (solution 4) to Feeding Mix (solution 3) to obtain the Feeding Solution • Mix by rolling or shaking.	section 3.1.4

8	Reaction Solution	• To *E.coli* lysate (solution 1) add
		  • 0.75 ml of the reconstituted Reaction Mix (solution 2),
		  • 50 µl of the reconstituted Energy Mix (solution 4)
		  • 5-15 µg of the DNA template (50 µl maximum volume)
		  • Mix carefully by rolling or gentle shaking
		DO NOT VORTEX!
			section 3.1.4

3.1.4 Running an experiment

Loading of the reaction compartment

The reaction compartment must be filled first.

- Open both lids of the 1 ml reaction compartment (large diameter).
- Fill with approx. 1 ml Reaction Solution (solution 8) through the circular opening using a 1 ml pipette. Let escape the air through the oval-formed opening by tipping the device slightly. It is not necessary to remove all air bubbles from the reaction compartment.
- Close the lids securely.

Loading of the feeding compartment

- Turn the reaction device upside-down.
- Open the two lids of the feeding compartment (small diameter).
- Fill with approx. 10 ml Feeding Solution (solution 7) through the circular opening. Let escape the air through the oval-formed opening by tipping the device slightly. **Air bubbles should be removed as completely as possible from the feeding compartment.**
- Close the lids securely and turn the reaction device (reaction compartment upright again).

Starting the run

Note: Make sure, that both stir bars are properly located at the bottom of their respective compartment. Otherwise **separate the stir bars by gently tapping the device on the table or with the help of another magnet**.

- Insert the loaded reaction device into the RTS 500 Instrument.
- Set instrument parameters according to the instrument manual.
- Start the run.
- Look through the illuminated window to be sure, that both stir bars are rotating.

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Points to consider

Parameter	Guideline
Stirrer speed	Stirring is essential to guarantee homogeneous distribution of components and to avoid membrane clogging. Stirrer speed should be set between 120 and 180 rpm.
Temperature	Optimum temperature for most proteins is 30°C. However, lower temperatures may be used for proteins that tend to aggregate.
Time	Protein synthesis continues for up to 24 hours. For unstable proteins the optimum yield of soluble active protein may be achieved at shorter reaction times.

End of run

- Stop the run.
- Remove the Reaction Device from the RTS 500 Instrument.
- Open both lids from the reaction compartment (large diameter).
- Remove Reaction Solution through the circular opening with a 1 ml pipette.
- Store the Reaction Solution at −20°C or at 0–4°C until purification or further processing.

3.1.5 GFP control reaction

Reaction procedure for GFP control protein

Step	Procedure
1	Reconstitute bottles 1, 2, 3, 4, and 6 according to chapter 3.1.2.
2	Prepare Feeding Solution according to chapter 3.1.2 and 3.1.3.
3	Prepare Reaction Solution according to chapter 3.1.2 and 3.1.3. Add 15 µg (15 µl) reconstituted Control Vector GFP (vial 6).
4	Fill the reaction chamber to **50%** with **0.5 ml** of Reaction Solution. For explanation see section "Oxidation of GFP" below this table.
5	Fill the feeding chamber with Feeding Solution.
6	Start the reaction by setting the following parameters: Temperature: 30°C Stirring speed: 120 rpm Time: 20 hours
7	After completing the run remove the Reaction Solution with a pipette through the round opening and store in a 2 ml vial for 24 hours at 4°C for maturation of GFP.
8	The fluorescence of GFP (excitation wavelength 395 nm, emisson at 504 nm) can be observed using an UV lamp (360 nm)
9	Apply 2–5 µl of the reaction onto SDS-polyacrylamide gels, and add 1 µl of a GFP standard (separately available) onto a separate lane.
10	Run the gel and stain with Coomassie Blue and quantify the synthesized GFP by comparison with the GFP standard. ***Note***: The GFP protein can also be detected on Western blots by using an anti-His antibody.

Oxidation of GFP

GFP needs molecular oxygen to post-translationally form the fluorophor (Coxon, A. & Bestor, T. H. (1995)). To enhance this conversion to the active form, air is provided during the reaction (and only for this control reaction) by filling the reaction chamber only to 50% with Reaction Solution. The yield of properly-folded fluorescent GFP is further increased by storing the Reaction Mix after the expression in a 2 ml vial for 24 hours at 4°C.

After 24 hours of storing at 4°C the maturation is almost completed.

3.1.6 Batch reaction

Introduction

Batch reactions using the Rapid Translation System RTS 500 *E.coli* Circular Template Kit can be used for fast, easy and economical selection of the optimal pIVEX vector, evaluation of the translation efficiency, and potential adaption of the RTS 500 reaction conditions.

The expression of the cloned pIVEX vectors yield His_6-tagged proteins that can easily be detected by Western Blotting using anti-His_6 antibody.

The components of the RTS 500 *E.coli* Circular Template Kit are sufficient for 20 batch reactions with a volume of 25 µl each, including a GFP control reaction (for control reaction see chapter 3.5.1).

Procedure for batch reaction

Step	Procedure
1	Prepare solutions 1, 2, and 4 by reconstituting bottles 1, 2 and 4 according to chapter 3.1.2.
2	Prepare solution 8 (Reaction Solution) according to chapter 3.1.3. without adding the DNA template.
3	Take 480 µl of the Reaction Solution and prepare maximally 20 aliquots (24 µl each) for setup of the batch reactions. The rest of the Reaction Solution is used for the GFP control reaction (see 3.1.5). The Reaction Solution is stable for approx. 2 weeks stored at -20°C. For longer storage -70 °C is recommended.
4	Add 1 µl of the DNA template (375 ng/µl) to one 24 µl aliquot.
5	Incubate 1 h at 30°C and store at -20°C if necessary.

The expressed protein is His-tagged and can be detected on Western Blot using anti-His-antibodies. Please refer to our website www.proteinexpression.com for a detailed protocol.

Notes

- It is possible to prepare larger aliquots of the Reaction Solution.
- Three freeze/thaw cycles can be performed without significant loss of activity.
- Reconstituted bottles 1, 2 and 4 can also be stored at -20°C for at least 2 weeks.

3.2 Cloning into pIVEX vectors

Literature

Customers with no experience in basic cloning methods may refer to one of the following books:

- 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.

3.2.1 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

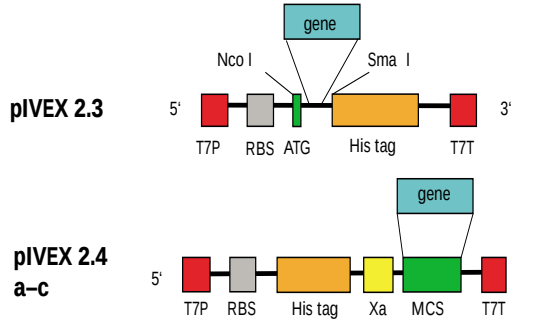

Fig. 3: Cloning vectors supplied with the kit

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

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Cloning vectors supplied with the kit

Five different cloning vectors are supplied with the kit. These vectors belong to vector family “2” containing the hexa-Histidine tag. The general architecture is shown in Fig. 3.

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

Cloning Vector	Description
pIVEX 2.3 Vial 7, blue cap	• Derivative of type 2 vector family • Contains C-terminal hexa-histidine tag • Cloning via *Nco* I and *Sma* I or *Xma* I
pIVEX 2.3-MCS Vial 8, blue cap	• Derivative of type 2 vector family • Contains C-terminal hexa-histidine tag • Cloning via *Nde* I or multiple cloning site
pIVEX 2.4a Vial 9, blue cap	• Derivative of type 2 vector family • Contains N-terminal hexa-histidine tag • Cloning via *Nco* I or multiple cloning site • Reading frame **a** starting at the *Sal* I restriction site
pIVEX 2.4b Nde Vial 10, blue cap	• Derivative of type 2 vector family • Contains N-terminal hexa-histidine tag • Cloning via *Nco* I, *Nde* I, or multiple cloning site • Reading frame **b** starting at the *Sal* I restriction site
pIVEX 2.4c Vial 11, blue cap	• Derivative of type 2 vector family • Contains N-terminal hexa-histidine tag • Cloning via *Nco* I or multiple cloning site • Reading frame **c** starting at the *Sal* I restriction site

3.2.2 Guidelines for vector selection

General recommendations

We recommend using only pIVEX cloning vectors in combination with the Rapid Translation System. Other vectors with similar architecture may also work, however with suboptimal results.

Vectors available

A set of five pIVEX cloning vectors are provided with this kit. 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 5.3).

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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...
If 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 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.
If 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).

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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 could 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 could be done easily in parallel.
- In particular, N-terminal extensions have proven to exhibit mostly positive impact on expression yields.

3.2.3 Cloning procedure

3.2.3.1 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 restriction 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 5.3).
- Design forward and reverse primers to have a comparable ($\pm$ 2°C) melting temperature (for calculation of melting temperatures see appendix 5.3).
- 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.

3.2.3.2 Restriction digest of the pIVEX vectors

Reconstitution of pIVEX vectors

- Briefly centrifuge down the contents of the vial with the pIVEX vectors.
- Dissolve the pIVEX vectors with 20 µl of sterile DNase- and RNase-free water.
- Reconstituted vectors are stable for one week at 0–4°C and for 1 year at −20°C.

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 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 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 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.

3.2.3.3 Preparation of the inserts

Generation of PCR fragments

- Primer design
  Design PCR primers according to section 3.2.3.1.
- 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 5.2 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.

3.2.3.4 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 at least 100 μg of plasmid to allow fine adjustment of the template concentration in the expression experiment.

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 unsufficient ($OD_{260/280} \leq 1.7$), a phenol treatment to remove traces of RNase may enhance expression.

3.2.3.5 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'

3.3 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 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. Typical results

Expression kinetics

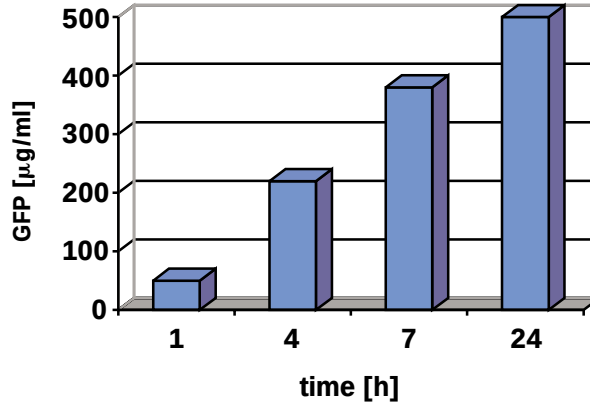

Fig. 4: Yields of Green Fluorescent Protein (GFP) at 30°C harvested at different time. Samples were taken at various time points from the reaction chamber.

Temperature dependency

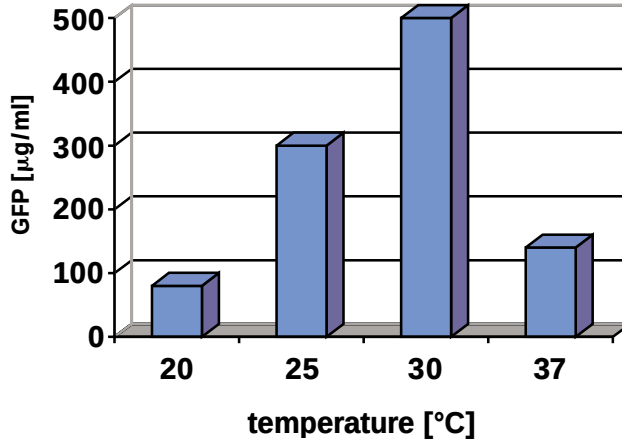

Fig. 5: Yield of Green Fluorescent Protein (GFP) at various temperatures (20, 25, 30 and 37°C) after 24 hours expression.

continued on next page

4. Typical results, continued

Stirring speed dependence

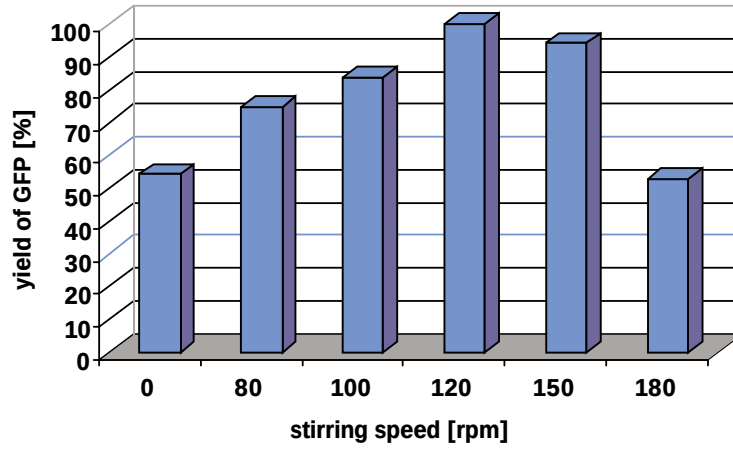

Fig. 6: Yield of Green Fluorescent Protein (GFP) at various stirring speed settings after 24 hours expression at 30°C.

SDS-PAGE of expressed GFP

Lane 1: Molecular weight markers
Lane 2: 2 µl aliquot of expressed GFP

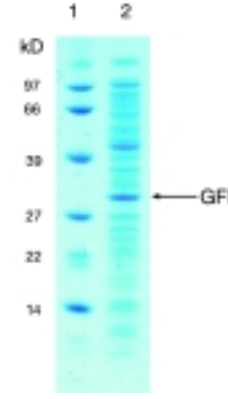

Fig. 7: *In vitro* expression of GFP after a 24 hour reaction. Samples were loaded onto a 12% SDS-PAGE gel and stained with Coomassie Blue.

5. Appendix

5.1 Trouble shooting

5.1.1 General problems

Observation	Potential Reason	Recommendation
No or weak stirring during a run	Stir bars stick to each other.	Separate stirring bars by gently tapping the device on the table or with the help of a magnet.
	Stirring speed is set to zero or too low.	Increase stirring speed.
White precipitate in the Feeding Solution after the run	Insoluble salts are formed.	Usually the expression is not affected by the precipitate.
No expression using the GFP control reaction	Kit expired	Order a new kit.
	Kit has not been stored at −20°C.	Order a new kit.
	Contamination with RNases	Repeat experiment and take care to work RNase-free at each step.
	Air bubbles in the large feeding chamber	Remove any air bubbles from the feeding chamber before starting a run.
	Wrong handling	Repeat the experiment exactly according to the working instructions.
	A kit component is inactive or degraded.	Contact Roche Molecular Biochemicals technical service. For addresses see the last page of this instruction manual.

continued on next page

5.1.2 Problems with the expression of the target protein, while the GFP control reaction works

Observation	Potential Reason	Recommendation
Sufficient protein expression, but low yield of active protein	Incorrect folding of the protein due to: • dependence on cofactors	• Add necessary cofactors.
	• necessity of disulfide bond formation	• Oxidize after the reaction to allow formation of disulfide bonds (see e.g. Ahmed et al (1975), Odorzinsky & Light (1979)
	• dependence on secondary modifications	• The *E.coli* lysate can not introduce post-translational modifications like glycosylation, phosphorylation, or signal sequence cleavage.
	• dependence on chaperones	• Add chaperones [see Rudolph (1997)].
Product appears in the pellet after centrifugation	Aggregation	• Add/adjust chaperones. • Change experimental conditions (time, temperature). • Add mild detergents (e.g. up to 0.1% Tween 20 (v/v) , or 0.1% Chaps (w/v) for membrane proteins).
Low expression yield	Expression time too short	• Extend expression
	The tag has a negative influence on the folding of the protein.	• Try expression in different pIVEX vectors.
Several product bands on SDS-PAGE or product smaller than expected	Proteolytic degradation	• Add protease inhibitors to the reaction. For example take 1 tablet of Complete mini EDTA-free for a 1 ml reaction. Use up to 10 mM EGTA.
	Internal initiation site	• Replace the corresponding methionine by point mutation.
	Premature termination of the translation	• Check the sequence of the target gene regarding reading frame and mutations which might yield a stop codon. • Search for strong secondary structures of the mRNA and eliminate them by conservative mutations.
No expression of the target gene, but normal expression of GFP (approx. 400 μg/ml)	Cloning error	• Check the sequence.
	Low purity of plasmid DNA	• Assure that the absorbance ratio 260 nm/280 nm is at least 1.7. • Perform a phenol extraction if purity is low. • Make a new plasmid purification.
	Contamination with RNases	• Repeat experiment and take care to work RNase-free at each step.
	No initiation of translation due to strong secondary structures of the mRNA	• Try expression in different pIVEX-vectors. • Try expression as N-terminally tagged fusion protein in pIVEX 2.4.
	Expressed protein interferes with the translation or transcription process.	• Express the gene of interest together with GFP. If GFP expression is inhibited the active protein can not be expressed with the kit.

continued on next page

5.1.3 Problems with the cloning procedure

Observation	Possible Cause	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.

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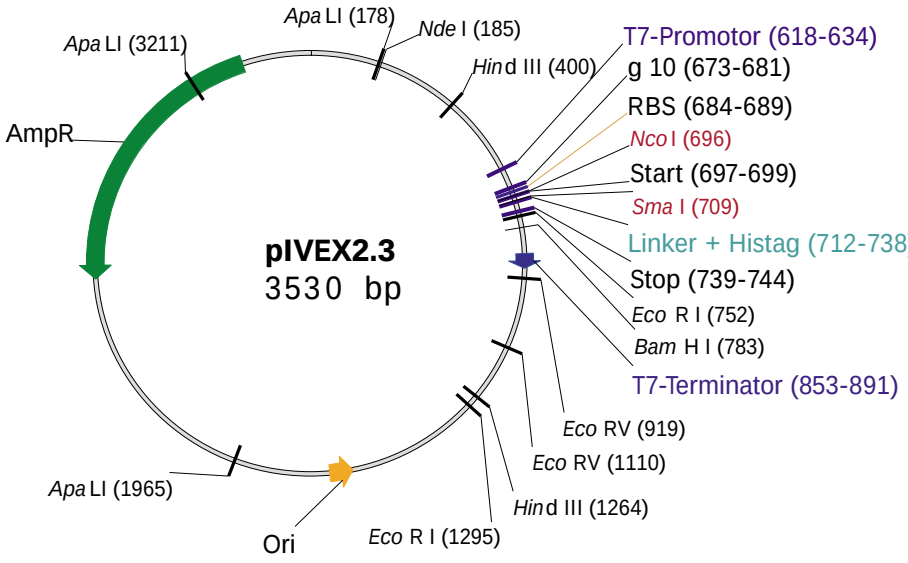
5.1.3 Problems with the cloning procedure, continued

Observation	Possible Cause	Recommendation
No or only few colonies after transformation, continued	Unsuccessful restriction digest of the PCR product	• Make sure that the right restriction buffer and reaction conditions were chosen. ***Notes:*** 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.
	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.

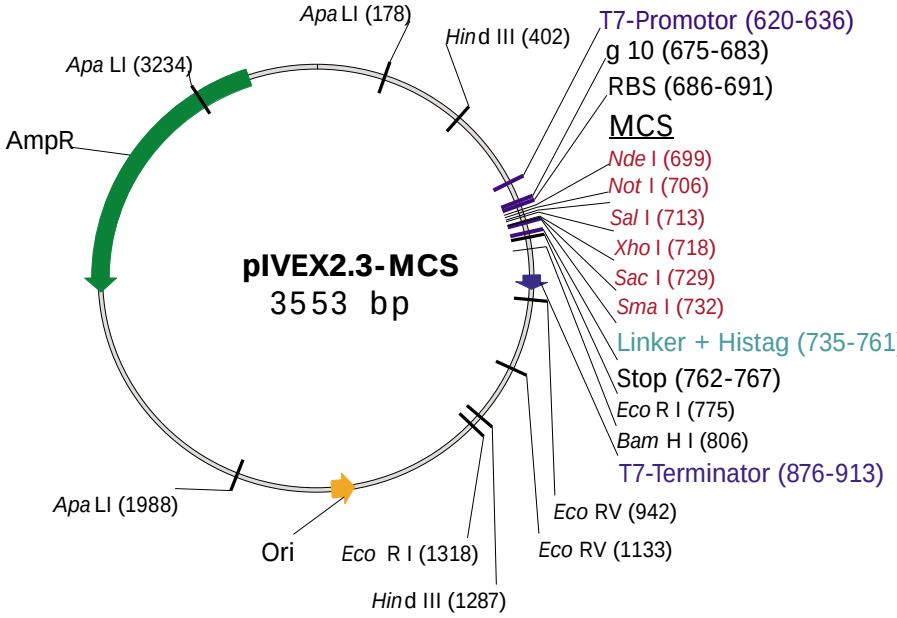
5.2 Vector maps

pIVEX 2.3 vector



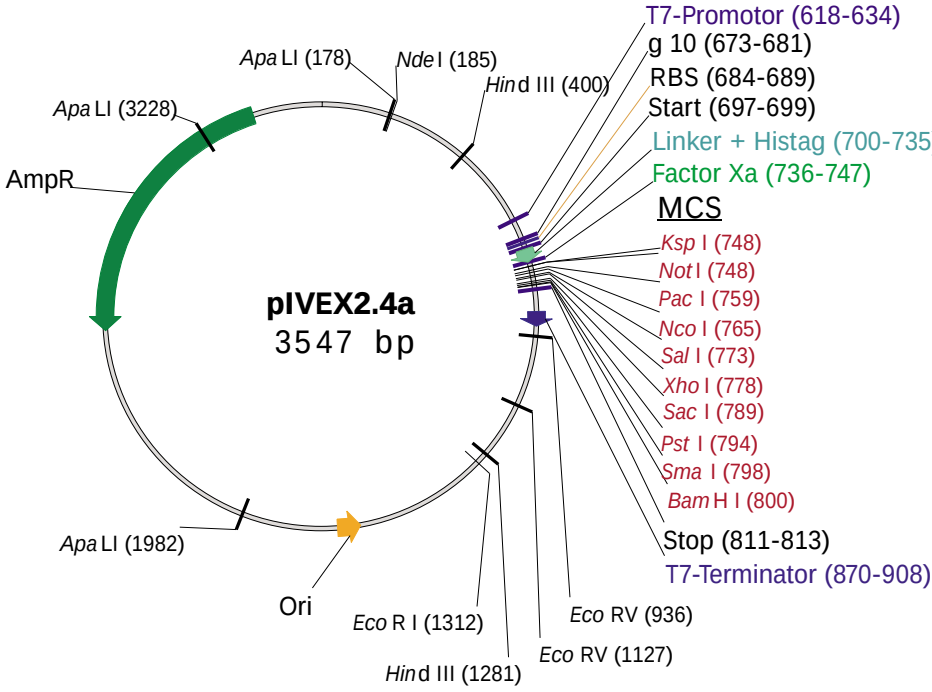

```
                                T7-Promotor
601        TCTCGATCCC  GCGAAATTAA  TACGACTCAC  TATAGGGAGA  CCACAACGGT
           AGAGCTAGGG  CGCTTTAATT  ATGCTGAGTG  ATATCCCTCT  GGTGTTGCCA

                                   g10 ϵ         RBS          NcoI
651        TTCCCTCTAG  AAATAATTTT  GTTTAACTTT  AAGAAGGAGA  TATACCATGG
           AAGGGAGATC  TTTATTAAAA  CAAATTGAAA  TTCTTCCTCT  ATATGGTACC
                                                           Met

                SmaI    Linker      Histag
701        TGTCCCCCGG  GGGGGGTTCT  CATCATCATC  ATCATCATTA  ATAAAAGGGC
           ACAGGGGGCC  CCCCCCAAGA  GTAGTAGTAG  TAGTAGTAAT  TATTTTCCCG
                       GlyGlySer  HisHisHisH  isHisHis**  ****
```

pIVEX2.3-MCS vector



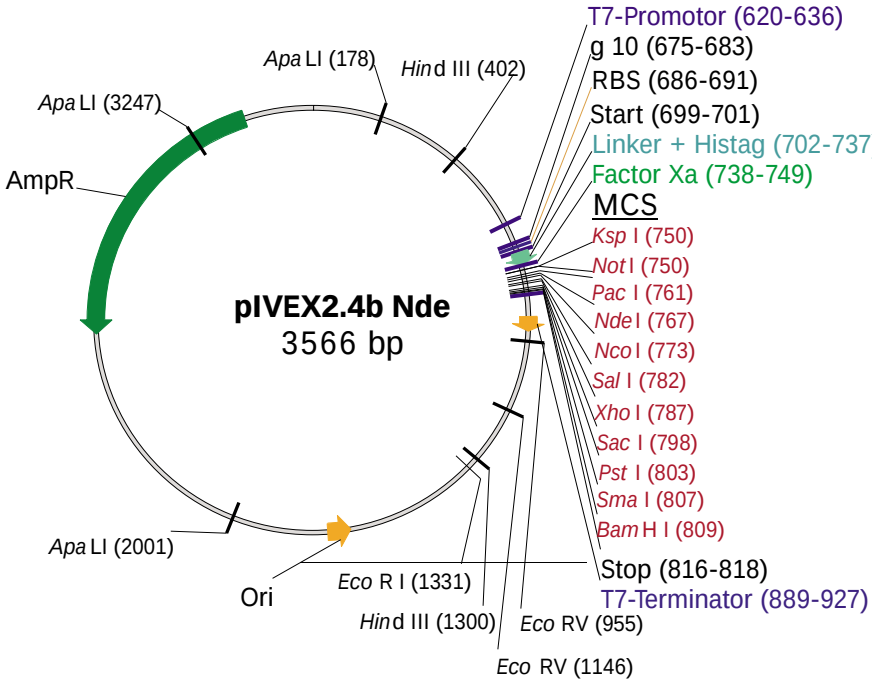

```
                                    T7-Promotor
601     GATCTCGATC  CCGCGAAATT  AATACGACTC  ACTATAGGGA  GACCACAACG
        CTAGAGCTAG  GGCGCTTTAA  TTATGCTGAG  TGATATCCCT  CTGGTGTTGC

                                      g10 ε       RBS         NdeI
651     GTTTCCCTCT  AGAAATAATT  TTGTTTAACT  TTAAGAAGGA  GATATACATA
        CAAAGGGAGA  TCTTTATTAA  AACAAATTGA  AATTCTTCCT  CTATATGTAT
                                                            M

           NotI       SalI XhoI    SacI Sma      Linker     His-Tag
701     TGAGCGGCCG  CGTCGACTCG  AGCGAGCTCC  CGGGGGGGGT  TCTCATCATC
        ACTCGCCGGC  GCAGCTGAGC  TCGCTCGAGG  GCCCCCCCCA  AGAGTAGTAG
        etSerGlyAr  gValAspSer  SerGluLeu      GlyGly  SerHisHisH

                                    EcoRI
751     ATCATCATCA  TTAATAAAAG  GGCGAATTCC  AGCACACTGG  CGGCCGTTAC
        TAGTAGTAGT  AATTATTTTC  CCGCTTAAGG  TCGTGTGACC  GCCGGCAATG
        isHisHisHi  s******
```

pIVEX2.4a vector



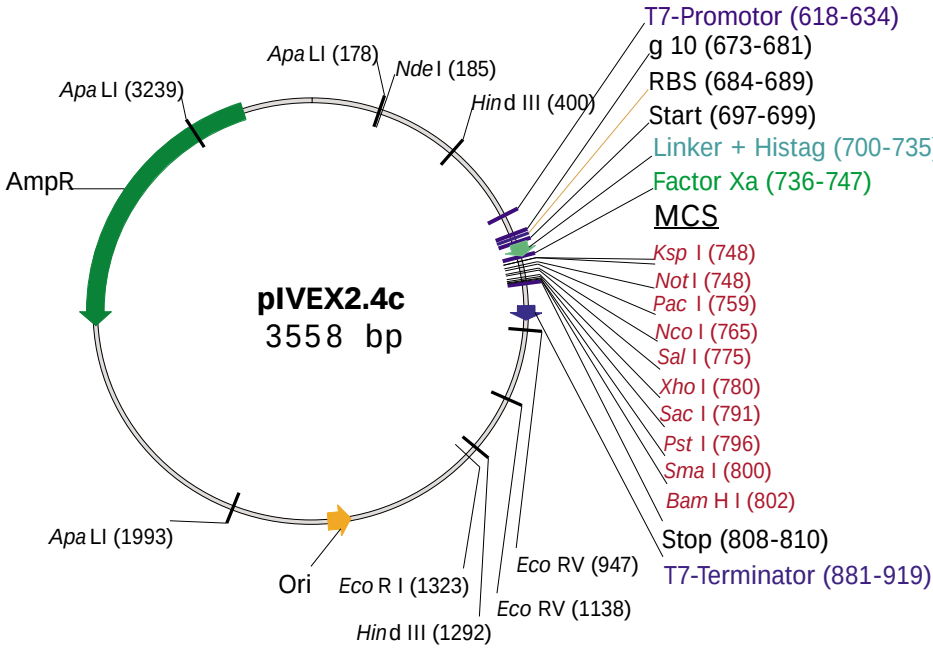

```
                                T7-Promotor
601     TCTCGATCCC   GCGAAATTAA   TACGACTCAC   TATAGGGAGA   CCACAACGGT
        AGAGCTAGGG   CGCTTTAATT   ATGCTGAGTG   ATATCCCTCT   GGTGTTGCCA

                                    g10 ε          RBS
651     TTCCCTCTAG   AAATAATTTT   GTTTAACTTT   AAGAAGGAGA   TATACCATGT
        AAGGGAGATC   TTTATTAAAA   CAAATTGAAA   TTCTTCCTCT   ATATGGTACA
                                                            MetS

        Linker  Histag                                       KspINotI

701     CTGGTTCTCA   TCATCATCAT   CATCATAGCA   GCGGCATCGA   AGGCCGCGGC
        GACCAAGAGT   AGTAGTAGTA   GTAGTATCGT   CGCCGTAGCT   TCCGGCGCCG
        erGlySerHi   sHisHisHis   HisHisSerS   erGlyIleGl   uGlyArgGly

           PacI         NcoI        SalI XhoI      SacI PstI      SmaIBa
751     CGCTTAATTA   AAACCATGGC   AGTCGACTCG   AGCGAGCTCT   GCAGCCCGGG
        GCGAATTAAT   TTTGGTACCG   TCAGCTGAGC   TCGCTCGAGA   CGTCGGGCCC
        ArgLeuIleL   ysThrMetAl   aValAspSer   SerGluLeuC   ysSerProGl

        mHI
801     ATCCGGCTGC   TAACAAAGCC   CGAAAGGAAG   CTGAGTTGGC   TGCTGCCACC
        TAGGCCGACG   ATTGTTTCGG   GCTTTCCTTC   GACTCAACCG   ACGACGGTGG
        ySerGlyCys   ***
```

pIVEX2.4b vector




```
                                T7-Promotor
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

         Linker     Histag                                  KspI
701     GTCTGGTTCT  CATCATCATC  ATCATCATAG  CAGCGGCATC  GAAGGCCGCG
        CAGACCAAGA  GTAGTAGTAG  TAGTAGTATC  GTCGCCGTAG  CTTCCGGCGC
        tSerGlySer  HisHisHisH  isHisHisSe  rSerGlyIle  GluGlyArgG

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

           SmaIBamHI
801     CAGCCCGGGA  TCCGGTAAGA  TCCGGCTGCT  AACAAAGCCC  GAAAGGAAGC
        GTCGGGCCCT  AGGCCATTCT  AGGCCGACGA  TTGTTTCGGG  CTTTCCTTCG
        GlnProGlyI  leArg***
```

pIVEX2.4c vector




```
                          T7-Promotor
601   TCTCGATCCC  GCGAAATTAA  TACGACTCAC  TATAGGGAGA  CCACAACGGT
      AGAGCTAGGG  CGCTTTAATT  ATGCTGAGTG  ATATCCCTCT  GGTGTTGCCA

                                g10 ε         RBS
651   TTCCCTCTAG  AAATAATTTT  GTTTAACTTT  AAGAAGGAGA  TATACCATGT
      AAGGGAGATC  TTTATTAAAA  CAAATTGAAA  TTCTTCCTCT  ATATGGTACA
                                                        MetS

      Linker  Histag                                     KspINotI
701   CTGGTTCTCA  TCATCATCAT  CATCATAGCA  GCGGCATCGA  AGGCCGCGGC
      GACCAAGAGT  AGTAGTAGTA  GTAGTATCGT  CGCCGTAGCT  TCCGGCGCCG
      erGlySerHi  sHisHisHis  HisHisSerS  erGlyIleGl  uGlyArgGly

         PacI        NcoI        SalI Xho I     SacI     PstI SmaI
751   CGCTTAATTA  AAACCATGGC  AAAGTCGACT  CGAGCGAGCT  CTGCAGCCCG
      GCGAATTAAT  TTTGGTACCG  TTTCAGCTGA  GCTCGCTCGA  GACGTCGGGC
      ArgLeuIleL  ysThrMetAl  aLysSerThr  ArgAlaSerS  erAlaAlaAr

      BamHI
801   GGATCCGTAA  GATCCGGCTG  CTAACAAAGC  CCGAAAGGAA  GCTGAGTTGG
      CCTAGGCATT  CTAGGCCGAC  GATTGTTTCG  GGCTTTCCTT  CGACTCAACC
      gAspPro***
```

5.3 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.

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 unefficient 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 Pwo 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.

5.4 References

1 DeVries, J. K. & Zubay G. (1967) "DNA-directed peptide synthesis, II. The synthesis of the α-fragment of the enzyme β-galactosidase" *Proc. Natl. Acad. Sci.* U.S.A. **57,** 1010.
2 Spirin, A. S. et al (1988) "A continuous cell-free translation system capable of producing polypeptides in high yield" *Science* **242,** 1162.
3 Coxon, A. & Bestor, T. H. (1995) Chemistry & Biology **2,** 119.
4 Sambrook et al (1989) "Molecular Cloning - A Laboratory Manual" Second Edition, Cold Spring Harbor Laboratory Press, New York.
5 Ausubel, U. K. et al (1993) "Current Protocols In Molecular Biology" John Wiley & Sons Inc., New York.
6 Ahmed, A. K. et al (1975), *J. Biol. Chem*. **250**, 8477.
7 Odorzinsky, T. W. & Light, A. (1979), *J. Biol. Chem*. **254**, 4291.
8 Rudolph, R. et al (1997) in "Protein Function – A Practical Approach" Creighton, T. E. ed. Oxford University Press Inc. New York, pp 57 - 99.

5.5 Notice for the purchaser

5.6 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.

5.7 Related products

Product	Pack size	Cat. No.
Rapid Translation System RTS 500 Instrument	1 instrument	3 064 859
Expand High Fidelity PCR-System	10 × 250 units	1 759 078
PCR Cloning Kit (blunt end)	1 kit	1 939 645
Agarose Gel DNA Extraction Kit	1 kit	1 696 505
Rapid DNA Ligation Kit	40 reactions	1 635 379
Agarose MP	500 g	1 388 991
Sma I	1000 units	220566
Not I	200 units	1 014 706
Nco I	200 units	835 315
Nde I	200 units	1 040 219
Rca I (=*Bsp* HI)	200 units	1 467 123
BamH I	1000 units	220 612
Xba I	1000 units	674 257
Xma CI (= *Xma* I)	200 units	1743 392
Bsp LU11I	200 units	1 693 743
Pin AI (*Age* I)	200 units	1 464 841
Sgr AI	200 units	1 277 014
Bse AI	200 units	1 417 169
Phosphatase, alkaline, shrimp	1000 units	1 758 250
r-GFP	50 µg	1 814 524
Anti-His_6	100 µg	1 922 416
Anti-His_6-Peroxidase	50 U	1 965 085
Restriction Protease Factor Xa Cleavage and Removal Kit	Kit I 3 × 30 µg Kit II 3 × 100 µg	1 644 777 1 644 785
Complete Protease Inhibitor Cocktail Tablets, EDTA-free	20 tablets (each tablet for 50 ml extract)	1 873 580
Complete Protease Inhibitor Cocktail Tablets, mini, EDTA-free	25 tablets (each tablet for 10 ml extract)	1 836 170

6. Quick reference procedure

Reconstitution of reaction components and preparation of working solutions

Step	Component	Reconstitution/ Preparation of Working Solutions	Solution number
1	*E.coli* Lysate Bottle 1, red cap	Reconstitute with 0.25 ml Reconstitution Buffer (bottle 5). DO NOT VORTEX!	1
2	Reaction Mix Bottle 2, green cap	Reconstitute with 0.8 ml Reconstitution Buffer (bottle 5)	2
3	Feeding Mix Bottle 3, blue cap	Reconstitute with 10.5 ml Reconstitution Buffer (bottle 5)	3
4	Energy Mix Bottle 4, orange cap	Reconstitute with 0.6 ml Reconstitution Buffer (bottle 5)	4
5	Feeding Solution	Add 0.5 ml of solution 4 to solution 3.	7
6	Reaction Solution	Add 0.75 ml of solution 2, 0.05 ml of solution 4 and 5-15 µg of the DNA template (50 µl maximum volume) to solution 1. Mix carefully by rolling or gentle shaking. DO NOT VORTEX!	8

continued on next page

6. Quick reference procedure , continued

Setup of the reaction

Step	Description	Procedure
1	Loading of the Reaction Solution	• Open both lids of the 1 ml reaction compartment (small compartment with larger diameter) • Fill with 1 ml of Reaction Solution (solution 8) using the circular opening and let escape the air through the oval-formed opening by tipping the device slightly. It is not necessary to remove all air bubbles. • Close the lids
2	Loading of Feeding Solution	• Turn the reaction device upside down. • Open the two lids of the feeding chamber (large compartment with smaller diameter) • Fill with 10 ml of Feeding Solution (solution 7) using the circular opening. ***Note:*** Air bubbles should be removed as completely as possible. • Close the lids.
3	Starting the run	• Put the loaded device on the table with the 10 ml feeding compartment down and the 1 ml reaction compartment up. • Make sure, that both stir bars are properly located at the bottom of their respective compartment. • Insert the loaded reaction device into the RTS 500 Instrument. • Set instrument parameters and start run • Check, that both stir bars are rotating.
4	End of run	• Remove the reaction device from the RTS 500 Instrument. • Open both lids of the 1 ml reaction compartment and remove the Reaction Solution through the circular opening.

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