IFTA AG
Certified QMS according to
DIN EN ISO 9001:2000-12
Reg. No. IC 03214 034

Jena Bioscience

LEXSinduce3 Expression Kit

For inducible expression of recombinant proteins in *Leishmania tarentolae*

contains one of the pLEXSY_I-3 vectors

pLEXSY_I-blecherry3 (Cat.-No. EGE-243)

pLEXSY_I-ble3 (Cat.-No. EGE-244)

pLEXSY_I-neo3 (Cat.-No. EGE-245)

Cat.-No.: EGE-1410

FOR RESEARCH USE ONLY.

NOT INTENDED FOR HUMAN OR ANIMAL DIAGNOSTIC

OR THERAPEUTIC USE.

Jena Bioscience GmbH | Löbstedter Str. 71 | 07749 Jena, Germany | Tel.:+49-3641-6285 000 | Fax:+49-3641-6285 100
http://www.jenabioscience.com

Last update: Nov 18, 2015

1 INTRODUCTION 3

2 KIT COMPONENTS AND STORAGE CONDITIONS 4

2.1 LEXSY host T7-TR 4
2.2 pLEXSY_I-3 expression vectors 4
2.3 pLEXSY_I-3 control plasmid with EGFP gene 4
2.4 Primers for diagnostic PCR and sequencing 4
2.5 Ingredients for 1 litre of culturing medium 5
2.6 Equipment and materials supplied by customer 6

3 CULTURING OF LEXSY T7-TR HOST AND EXPRESSION STRAINS 6

3.1 Preparation of LEXSY BHI medium 6
3.2 Culturing conditions 6
3.3 Storage of LEXSY host and recombinant strains by cryoconservation 7

4 ENGINEERING OF LEXSY T7-TR EXPRESSION STRAINS 8

4.1 Amplification of target gene 9
4.2 Insertion of target gene into pLEXSY_I-3 expression vector 11
4.3 Preparation of the expression plasmid for LEXSY host transfection 11
4.4 Transfection of the LEXSY host strain T7-TR by electroporation 12

5 SELECTION OF TRANSGENIC LEXSY T7-TR STRAINS 13

5.1 Clonal selection by plating on solidified media 13
5.2 Polyclonal selection in suspension culture 14
5.3 Confirmation of genomic integration by diagnostic PCR 15
5.4 Evaluation of target protein expression 15

6 LICENSING INFORMATION 16

7 LITERATURE 17

8 APPENDIX 18

8.1 Maps of the pLEXSY_I-3 expression vectors 18
8.2 Sequences of the primers available for LEXSinduce3 kits 24
8.3 Preparation of LEXSY BHI agar plates for clonal selection 25
8.4 Alternative electroporation protocols 26
8.5 How to grow a *Leishmania* culture 27
8.6 Titration of Tetracycline concentration for modulation of expression level 28
8.7 Correlation of optical density and cell concentration 29

Jena Bioscience GmbH • Loebstedter Str. 71 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

1 INTRODUCTION

LEXSY – the unique protein expression platform offered by Jena Bioscience combines eukaryotic protein expression/folding/modification with robustness and easy handling. The unicellular protozoan host *Leishmania tarentolae* used in LEXSY was isolated from the Moorish gecko *Tarentola mauritanica* and kept in axenic culture over decades. It is not pathogenic to mammalians and fully approved for use in biosafety level 1 (S1) laboratories.

LEXSY features:

- Eukaryotic protein synthesis including chaperone system for correct folding (no inclusion bodies)
- Full range of Post-Translational Modifications including mammalian-type N-glycosylation, glypiation, phosphorylation, acetylation, prenylation, myristoylation, ADP-ribosylation, proteolytic processing, and oligomerisation
- High expression-success rates with yields of up to 500 mg per litre of culture.

LEXSY is available in two principle configurations that are **constitutive** (Breitling *et al.* 2002) or **inducible** (Kushnir *et al.* 2005). In both configurations target proteins can be expressed either **intracellularly**, or be **secreted** into the culturing medium. An illustrated overview on LEXSY configurations available, features and applications can be downloaded from our website at http://www.jenabioscience.com/images/b3e879b381/Lexsy_brochure_web.pdf.

This kit contains one of the three expression vectors of choice for inducible production of recombinant proteins from chromosomally integrated expression constructs which differ in the antibiotic resistance marker and the option to monitor expression online by a fluorescence reporter (Figure 1). The vector **pLEXSY_I-neo3** (encoding aminoglucoside phosphotransferase) allows selection with the antibiotic **LEXSY Neo** whereas **pLEXSY_I-ble3** (encoding bleomycin resistance gene) permits selection with the antibiotic **LEXSY Bleo.** The vector **pLEXSY_I-blecherry3** facilitates selection with the antibiotic **LEXSY Bleo** and, in addition, offers the possibility to monitor induction during culturing by coexpressed fluorescence. The control vector provided in the kit contains the EGFP gene linked to the respective selection marker.

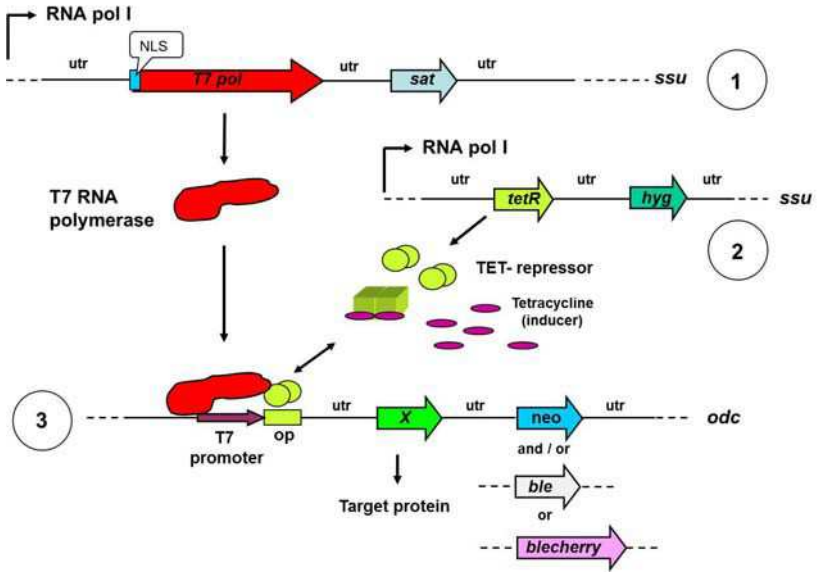


Figure 1: Architecture of the inducible integrative LEXSY expression system. Target genes (*X*) are inserted into the expression cassette under control of T7 promoter with TET operator (op) localised on the integrative LEXSY expression vector (3) and introduced into the engineered expression host *Leishmania tarentolae* T7-TR constitutively expressing T7 RNA polymerase (1) and TET repressor (2). The *blecherry* gene facilitates selection with the antibiotic LEXSY Bleo and, in addition, offers the possibility to screen most productive clones and monitor induction during culturing by co-expressed Cherry fluorescence. The utr´s are optimized non-translated gene-flanking regions providing the splicing signals for post-transcriptional mRNA processing for expression of target and marker genes in *L. tarentolae*. *Ssu* is the chromosomal 18S rRNA integration locus for T7 RNA polymerase and TET repressor genes, *odc* is the chromosomal ornithine decarboxylase integration locus for the expression construct.

Jena Bioscience GmbH • Loebstedter Str. 71 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

2 KIT COMPONENTS AND STORAGE CONDITIONS

The kit is shipped on dry ice. Upon arrival the kit components should be stored at the appropriate temperature as indicated below and on the labels.

2.1 LEXSY host T7-TR

The kit contains three vials with 1.6 ml each of frozen glycerol stocks of **LEXSY host T7-TR**. **Do not freeze-thaw the stocks!** These stocks can be stored at -80°C for at least 12 months. However, we have successfully reactivated LEXSY stocks after >10 years of storage at -80°C. For reactivation protocol refer to chapter 3.4.

We recommend preparation of sufficient glycerol stocks (chapter 3.3) in the initial phase of the project since a **new suspension culture of the T7-TR host should be started from a glycerol stock every three months** (see chapter 3.2.) to ensure strain identity. Alternatively, the T7-TR host can be reordered from Jena Bioscience.

2.2 pLEXSY_I-3 expression vector

one of the vectors

- **pLEXSY_I-blecherry3** (Cat.-No. EGE-243) or
- **pLEXSY_I-ble3** (Cat.-No. EGE-244) or
- **pLEXSY_I-neo3 (**Cat.-No. EGE-245)
  - 5 µg in 10 mM TrisHCl pH 8.0
  - store at -20°C

2.3 pLEXSY_I-3 control plasmid with EGFP gene

one of the vectors

- **pLEXSY_I-egfp-blecherry3** (Cat.-No. EGE-246) or
- **pLEXSY_I-egfp-ble3** (Cat.-No. EGE-247) or
- **pLEXSY_I-egfp-neo3 (**Cat.-No. EGE-248)
  - 5 µg in 10 mM TrisHCl pH 8.0
  - store at -20°C

For vector maps see Appendix 8.1. The DNA sequences can be downloaded from the website of JBS at http://www.jenabioscience.com/cms/en/1/catalog/1856_vectors_and_primers.html.

2.4 Primers for diagnostic PCR and sequencing

one of the marker-specific primers

- ***ble*** forward primer **A708,** for **pLEXSY_I-ble3** and **pLEXSY_I-blecherry3** or
- ***neo*** forward primer **H4662,** for **pLEXSY_I-neo3**

and the primers

- Insert sequencing forward primer **P1442**
- Insert sequencing reverse primer **A264**
- 5`utr (***aprt***) reverse primer **A1715**
- ***odc*** forward primer **A1304**
- ***odc*** reverse primer **P1510**

Jena Bioscience GmbH • Loebstedter Str. 71 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

– 50 µl at 50 µM in 10 mM TrisHCl pH 8.0
– store at -20°C

For primer sequences see Appendix 8.2.

2.5 Ingredients for 1 litre of culturing medium

***LEXSY BHI**, Powder for preparation of liquid culturing medium* (Cat. No. ML-412S)

- 37 g (2 x 18.5 g for 500 ml each)
- store at ambient temperature
- stable for 12 months.

Additives

- **Hemin** 0.25% porcine Hemin in 50% Triethanolamine (Cat. No. ML-108S)
  - 2 ml, ready-to-use 500x stock solution, filter sterilized
  - store at 4°C in the dark (Hemin is light sensitive)
  - stable for 12 months.
- **Pen-Strep** 10.000 units of penicillin (base) and 10.000 µg of streptomycin (base)/ml as penicillin G sodium salt and streptomycin sulfate in 0.85% saline (Cat. No. ML-105S) may be added to avoid bacterial contaminations
  - 5 ml, ready-to-use 200x stock solution, filter sterilized
  - store at -20°C
  - stable for 12 months.

The antibiotics for host strain maintenance

- **LEXSY NTC** (Cat. No. AB-101S) and **LEXSY Hygro** (Cat. No. AB-104S)
  - 1 ml each, ready-to-use 1000x stock solutions, 100 mg/ml, filter sterilized
  - store at -20°C
  - stable for 12 months.

one of the selection antibiotics

- **LEXSY Bleo** (Cat. No. AB-103S) for **pLEXSY_I-ble3** and **pLEXSY_I-blecherry3**
  - 1 ml, ready-to-use 1000x stock solution, 100 mg/ml, filter-sterilized
  - store at -20°C
  - stable for 12 months, or
- **LEXSY Neo*** (Cat. No. AB-105S) for **pLEXSY_I-neo3**
  - 1 ml, ready-to-use 1000x stock solution, 50 mg/ml, filter-sterilized
  - store at -20°C
  - stable for 12 months.

  * Please note, that **LEXSY Neo** (JBS Cat.-No. AB-105) **is <u>not</u> the antibiotic Neomycin** which must not be used for selection in LEXSY.

inducer of the T7-TR system

- **LEXSY Tet (Tetracycline)**
  - 1 ml, ready-to-use 1000x stock solution, 10 mg/ml, filter sterilized
  - store at -20°C
  - stable for 12 months
  - aliquot upon arrival and avoid frequent freeze-thaw cycles (eventually occuring turbidity upon thawing will not compromise induction)

Jena Bioscience GmbH • Loebstedter Str. 71 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

2.6 Equipment and materials supplied by customer

- Incubator at 26°C (no CO_2 incubator required), inverse (or standard) microscope
- Electroporation device, e.g. BioRad GENEPULSER II with PULSE CONTROLLER II and CAPACITANCE EXTENDER PLUS or GENE PULSER Xcell with PC and CE Modules, or Multiporator, or Nucleofector
- Cooling and freezing capacities at +4°C, -20°C and -80°C
- Standard molecular biology equipment for PCR, cloning and protein analysis.

3 CULTURING OF LEXSY T7-TR HOST AND EXPRESSION STRAINS

The standard culturing medium for strain maintenance, transfection, cryoconservation and expression evaluation is **LEXSY BHI Medium (**Cat. No. ML-411, 412). It must be supplemented with **Hemin**, which is essential for *Leishmania*. There is no need to add sera to complex media. Addition of fetal calf serum will not enhance growth of *L. tarentolae* in complex media. To prevent bacterial infections, Penicillin and Streptomycin (**Pen-Strep**) may be added.

3.1 Preparation of LEXSY BHI Medium

Dissolve 37 g/l **LEXSY BHI** powder in de-ionized water and autoclave **exactly** 15 min at 121°C. Control temperature profile with a thermosensor in a reference flask with the same volume liquid. Note that overexposition may result in decomposition of media ingredients (e.g. indicated by a dark brown color) which adversely affects growth of LEXSY strains. Properly autoclaved medium is of amber color. Store core medium at room temperature before addition of Hemin and PenStrep (stable for 12 months).

Add to 500 ml **LEXSY BHI Medium**

- 2.5 ml of 200x **Pen-Strep** stock solution
- 1.0 ml of 500x **Hemin** stock solution (final concentration 5 µg/ml)
- store at 4°C in the dark and use within 2 weeks after supplementation. If the completed media are to be used after this period, appropriate amounts of additives have to be re-added.

3.2 Culturing conditions

All culturing is carried out **at 26°C** in the **dark** under **aerated** conditions (no CO_2 incubator required). **Hemin** must be added to all culturing media.

Standard culturing for strain maintenance is performed as continuous static suspension cultures in ventilated tissue culture (TC) flasks positioned upright with regular successive dilutions into **LEXSY BHI Medium**. Best results are obtained with dilutions at early stationary phase (OD 1.4-2, ca. 6-8 $x10^7$ cells/ml under these conditions). We find it convenient to dilute 10 ml cultures 1:50 on Monday and 1:20 on Friday. Avoid repeated successive dilution of cultures of lower cell densities as this may reduce growth. However, occasional higher dilutions of stationary cells at e.g. 1:100 will not adversely affect subsequent growth. Do not use agitation for strain maintenance (ref. to Appendix 8.5). For maintaining T7 polymerase and TET repressor genes in the T7-TR host genome add **LEXSY NTC** and **LEXSY Hygro** from 1000x stocks provided in the kit (final concentrations 100 µg/ml each).

To prevent genomic instabilities upon long-term culturing of T7-TR host it is recommended, not to exceed successive passages for more than three months. Instead, a **new culture of the T7-TR host should be started from a glycerol stock every three months.**

For **culturing for transfection** refer to chapter 4.4 and for **protein expression** see chapter 5.3

Jena Bioscience GmbH • Loebstedter Str. 71 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

To conveniently monitor the growth of the LEXSY cultures calibrate the OD readings of your spectrophotometer at a defined wavelength between 550 and 600 nm to the cell densities at different time points during growth of a suspension culture by taking OD readings and counting cells from the same sample e.g. in a particle counter or in a Neubauer chamber after immobilisation in 3% (final concentration) formalin. Dependent on the spectrophotometer and wavelength used, this correlation may be different from the data specified in this manual (Figure 12 in Appendix 8.7).

In case you encounter growth problems with the host or LEXSY expression strains, centrifuge cells 3 min at 2000 x g, resuspend pellet carefully in fresh medium and continue incubation.

Culturing may be performed in

- ventilated tissue culture (TC) flasks for suspension cultures, culture volume 10-100 ml
- Erlenmeyer flasks, agitated at approx. 140 rpm, culture volume 50-200 ml
- Fernbach flasks, agitated at approx. 90 rpm, culture volume ca. 0.1-0.5 litre
- standard bioreactors, culture volume 1-100 litres.

3.3 Storage of LEXSY host and recombinant strains by cryoconservation

The LEXSY host and LEXSY expression strains may be stored at -80°C in 10-20% glycerol for at least 12 months. However, we recovered viable cells under these conditions after >10 years of storage, without any loss of vitality. Glycerol stocks may be prepared from standard culturing in **LEXSY BHI Medium** for strain maintenance after 2-3 days (1:20 dilution) or after 3-4 days (1:50 dilution) TC flasks upright, at OD 1.4-2, ca. 6-8 x10^7 cells/ml (refer to chapter 3.2). Alternatively, stocks can be prepared from 1:10 inoculated static cultures after 24 h if TC flasks are incubated flat. Avoid preparation of stocks from cultures not dense enough or from late stationary phase cultures. Prior to conservation, check vitality of cells by microscopy. Cells should be motile and drop-like (Figure 10, Appendix 8.5) but not of needle-like appearance. Do not use other media than **LEXSY BHI Medium** for cryoconservation.

Glycerol stocks preparation using a cryocontainer

Using this method, freezing proceeds continuously with -1°C/min

- Add 1.2 ml autoclaved Glycerol (80% by weight per volume) to a sterile 15 ml Falcon tube
- Withdraw 3.6 ml of culture OD 1.4-2 (ca. 6-8 x10^7 cells/ml)
- Mix with glycerol and distribute 3 x 1.6 ml each to sterile cryovials
- keep 10 min at room temperature
- transfer to a cryocontainer at 4°C containing fresh isopropanol
- keep 10 min at 4°C
- transfer to –80°C over night
- distribute to storage box for long term storage.

Glycerol stocks preparation by stepwise cool-down protocol

Using this method, freezing proceeds in steps of 0°C/-20°C/-80°C

- Add 1.2 ml autoclaved Glycerol (80% by weight per volume) to a sterile 15 ml Falcon tube
- Withdraw 3.6 ml of culture OD 1.4-2 (ca. 6-8 x10^7 cells/ml)
- Mix with glycerol and distribute 3 x 1.6 ml each to sterile cryovials
- keep 10 min at room temperature
- keep 1 h on wet ice

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

- keep o/n at –20°C
- transfer to –80°C for long term storage.

Both protocols are tolerated well by *L. tarentolae*. However, to avoid loss of strains it is recommended to check the reactivation of one sample of the prepared batch of glycerol stocks prior to stopping respective suspension culturing of parent culture.

Glycerol stocks reactivation

- Thaw frozen glycerol stock on ice (ca. 20 min)
- Inoculate the **entire content** of the vial into 10 ml of **LEXSY BHI Medium** with appropriate antibiotic(s). Motile cells can be observed immediately after inoculation by microscopy
- Incubate as static suspension culture in ventilated TC flask (flat) dark at 26°C until culture gets turbid (OD 1.4-2; ca. 6-8 x10^7 cells/ml). This usually takes 2 days; wait longer if cells recover more slowly and follow status by microscopy
- Dilute dense culture 1:10-1:20 into fresh LEXSY BHI Medium and incubate for 3 days. Do not dilute culture of low density. For strain maintenance dilute into fresh LEXSY BHI Medium on Monday and Friday each week (see chapter 3.2).

4 ENGINEERING OF LEXSY T7-TR EXPRESSION STRAINS

This kit was designed for inducible expression of target proteins following integration of the expression cassette into the chromosomal ornithine decarboxylase (*odc*) locus of the ***Leishmania tarentolae* T7-TR** recipient strain which constitutively expresses bacteriophage T7 RNA polymerase and TET repressor under the control of host RNA polymerase I from the 18SrRNA (*ssu*) locus. The principle architecture of this expression system - well known from bacteria - is shown in Figure 1 (page 3).

In the first cloning step the target gene is supplied with linker sequences containing restriction sites that allow insertion into the **pLEXSY_I-3** vector (Figure 2) downstream of the T7 promoter/TET operator arrangement. This vector contains optimized non-translated regions flanking the target gene insertion sites, which provide the splicing signals for posttranscriptional mRNA processing. Dependent on the cloning strategy, target proteins are destined either for intracellular or for secretory expression (Figure 2). Secretory expression is achieved by fusion of the target ORF to the *Leishmania* signal peptide coding region present on the vector. For cloning traditional ligase-dependent or alternative ligase-free methods may be followed. The ligase-free **CyClone** technology of Jena Bioscience (JBS Cat.-No. PP-110) offers the advantage of online design and ordering of primer pairs for insert amplification with vector-compatible overhangs using the free Primer Designer software of Jena Bioscience (http://www.jenabioscience.com/cms/en/1/catalog/1856_vectors_and_primers.html).

Following plasmid construction in *E. coli* the LEXSY host T7-TR is transfected with the linearized expression construct by electroporation. Clonal or polyclonal selection strategies can be applied for establishment of recombinant strains.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

4.1 Amplification of target gene

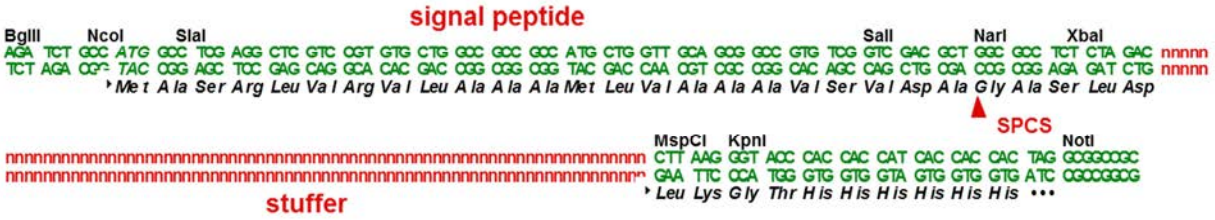
The **pLEXSY_I-3 vectors** allows insertion of target genes into the cloning sites in a way that the corresponding proteins are expressed either intracellularly or are secreted (Figure 2). The 5' insertion sites for intracellular expression are ***Bgl*II**, ***Nco*I**, or ***Sla*I** and for secretory expression ***Sal*I**, or ***Xba*I**. In both cases a stuffer fragment is replaced by the target ORF. At the 3'end of this stuffer fragment the restriction sites for ***Msp*CI**, or ***Kpn*I** yield fusion to a C-terminal 6xHis stretch for affinity purification, whereas utilization of the ***Not*I** cloning site avoids this stretch (e.g. for target genes without or with a different affinity tag). This versatility allows cloning strategies for most ORFs without tedious removal of internal restriction sites or gene synthesis. In addition, the ligase-free **CyClone** technology of Jena Bioscience (JBS Cat.-No. PP-110) permits an alternative approach, which is independent on internal insert restriction sites. This approach is essential if internal insert restriction sites interfere with cloning or plasmid linearization sites.

All enzymes for construction of pLEXSY expression plasmids can be purchased from Jena Bioscience http://www.jenabioscience.com/cms/en/1/browse/109_molecular_biology.html).

- Analyse your target gene for internal sites of the restriction enzymes you intend to use for cloning. Also, make sure that your target gene does not contain any internal ***Swa*I** site, since this site will be used for vector linearization prior to transfection. If one of these sites is present however, you may choose to remove it by silent mutagenesis, avoid it by *de novo* gene synthesis or use the **CyClone** technology of Jena Bioscience (JBS Cat.-No. PP-110)

- Design a forward and reverse primer pair for amplification of your target gene with linker sequences containing the selected restriction sites allowing integration into the pLEXSY vector. In case of the **CyClone** technology use online primer design and ordering software (http://www.jenabioscience.com/cms/en/1/catalog/1856_vectors_and_primers.html)
- We recommend to preserve the sequence immediately in front of the translation start codon as close as possible if using the ***Bgl*II** cloning site. The triplet preceding the ATG seems to be important for the expression level of the target protein (Lukeš *et al.* 2006). **GCC** and **ACC** were found favourable with an EGFP reporter gene in the LEXSY host and are members of the consensus pre-ATG triplet. The triplets GTT, GGC, TCG and TCT yielded no product in LEXSY with the same reporter construct
- If you use the ***Sal*I** site for fusion to the signal peptide: Please note, that the codons following this restriction site encode the amino acids at which the signal peptide is cleaved off during secretion (Figure 2). Therefore, it is necessary to include the nucleotide sequence GTC GAC GCT GGC GCC … into the 5' primer for amplification of target gene. However, the signal peptide cleavage site may vary dependent on the sequence of the fused ORF and wesuggest to predict the cleavage site *in silico* for optimal primer design (http://www.cbs.dtu.dk/services/SignalP). See also Klatt & Konthur 2012
- Amplify the target gene with a high fidelity polymerase (JBS Cat.-No. PCR-237), gel-purify the fragment (JBS Cat. No. PP-202), trim the ends with the appropriate restriction enzymes and prepare the fragment for ligation. In case of the **CyClone** technology no restriction is required
- If you consider gene synthesis, apply the codon bias of *Leishmania tarentoae* (http://www.kazusa.or.jp). Whenever your budget allows, we recommend gene synthesis with *L. tarentolae* optimized codon usage. However, there are numerous examples of high expression of genes with native (e.g. human) codon bias and using synthetic genes does not guarantee always higher expression levels than using native genes.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100



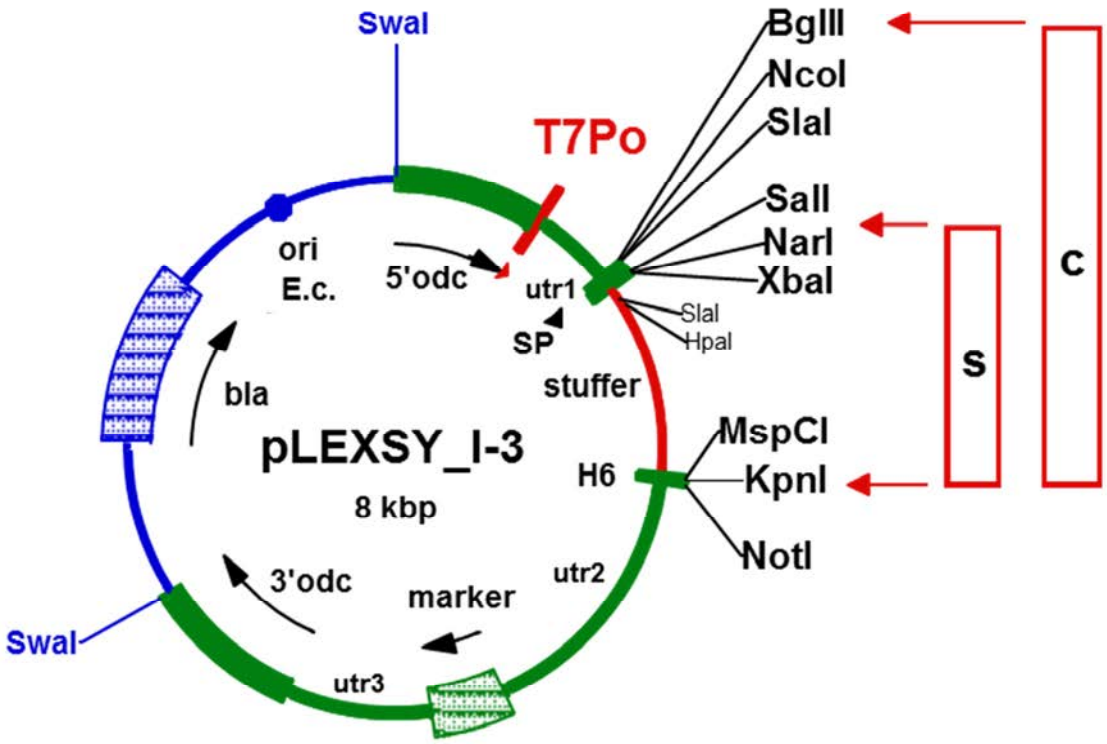


Figure 2: Map of the **pLEXSY_I-3** vector family with cloning sites for the target genes replacing the 1 kb stuffer fragment**. 5´odc** and **3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with ***SwaI***. **Utr1** derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. **SP** designates the signal peptide of *L. mexicana* secreted acid phosphatase LMSAP1 (Wiese *et al.* 1995), **SPCS** signal peptide cleavage site and **H6** the hexa-Histidine stretch. Alternative cloning strategies result in cytosolic **(c)** or secretory **(s)** expression of the target protein. As markers are available the ***ble*** (bleomycin resistance), ***blecherry*** (bleomycin resistance and fluorescence monitoring) and ***neo*** (aminoglucoside phosphotransferase) genes. Please note, the *Nar*I (*Ehe*I, *Kas*I) site indicated in the sequence is unique only in **pLEXSY_I-ble3** (Cat.-No. EGE-244). The DNA sequences including description of all pLEXSY_I-3 vectors can be downloaded from the website of JBS at http://www.jenabioscience.com/cms/en/1/catalog/1856_vectors_and_primers.html.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

4.2 Insertion of target gene into pLEXSY_I-3 expression vector

The **pLEXSY_I-3 vectors** allow directional insertion of the target gene cassette by replacement of a 1 kbp stuffer fragment (Figure 2). This is of advantage compared to insertion into a multiple cloning site since vector cleavage by both restriction enzymes can be monitored by the appearance of the stuffer fragment.

- Digest the expression vector provided in the kit with the appropriate restriction enzymes
- Gel-isolate the large 6.5-7.2 kbp fragment and prepare it for ligation
- Ligate vector and target gene from chapter 4.1 with T4 DNA ligase (JBS Cat.-No. EN-149). For discrimination against the original expression vector you may treat the ligation mix with *Hpa*I after ligation reaction if your target gene does not contain this site. Alternatively, perform **CyClone** reaction if ligase-free method was chosen (JBS Cat.-No. PP-110)
- Transform competent *E. coli* cells which tolerate *Leishmania* sequences as XL-10, DH10B, Stbl2, Stbl4, SURE, DH5α etc. Include controls for vector linearization, ligation and transformation
- Select recombinant *E. coli* clones with ampicillin at 30°C and screen for the presence of the insert in the plasmids. Insert screening may be performed by colony PCR or restriction analysis of recombinant plasmids isolated from a small number of cultures grown o/n in 1 - 3 ml LB with ampicillin at 30°C. We recommend incubation of all *E. coli* strains with pLEXSY plasmids **at 30°C and not at 37°C** for plasmid stability reasons
- Prepare at least 10 µg plasmid DNA from a positive clone for sequence confirmation, restriction and subsequent transfection. Usually, it is sufficient to isolate plasmid DNA with a commercial kit from 50 ml o/n culture grown at 30°C.

Confirm plasmid identity by restriction and sequence analysis of the insert and of the vector/insert fusions using the forward **P1442** and reverse **A264** sequencing primers included in the kit. Both primers are proved for function in cycle sequencing protocols. Forward primer **P1442** anneals 78-98 bp in front of ATG of ***Nco*I**. Reverse primer **A264** anneals 74-100 bp after the stop codon in front of ***Not*I** restriction site.The primer sequences are shown in Appendix 8.2 of this manual, the DNA sequence of the vector including description can be downloaded as .txt file from the LEXSY section of our website at http://www.jenabioscience.com/cms/en/1/catalog/1856_vectors_and_primers.html.

4.3 Preparation of the expression plasmid for LEXSY host transfection

- Digest to completion with ***Swa*I** ca. 10 µg of the obtained expression plasmid containing the target gene. This treatment will generate a 2 kbp fragment representing the *E. coli* part and a larger fragment representing the linearized expression cassette with the target gene to be integrated into the chromosomal *odc* locus of the LEXSY T7-TR host. As positive control you may use the EGFP control plasmid provided in the kit
- For best performance, gel-isolation of the expression cassette with an Agarose Gel Extraction Kit (JBS Cat.-No. PP-202) is recommended (but optional)
- If the digested plasmid is used without fragment purification, enzymes and buffer salts may be removed with a PCR Purification Kit (Cat.-No. PP-201). Alternatively, precipitate the digest with ethanol, wash with 70% ethanol and redissolve in max. 50 µl of sterile double distilled water or 10 mM Tris pH 8.0 per transfection. Control the quality of the digest and the DNA concentration by gel electrophoresis. This preparation is now ready for transfection.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

4.4 Transfection of the LEXSY host strain T7-TR by electroporation

For efficient transfection it is recommended to prime the LEXSY host by successively transferring the cells to fresh medium at 1:20 to 1:50 dilutions twice a week (refer to chapter 3.2). Do not use the first inoculation culture from a glycerol stock immediately for transfection, but passage the culture several times before electroporation as described above. However, use for transfection a culture which has been passaged this way for less than three months (refer to chapter 3.2). For maintaining T7 polymerase and TET repressor genes always add **LEXSY NTC** and **LEXSY Hygro** from 1000x stocks provided in the kit to the culturing medium (final concentration 100 µg/ml each).

- On Friday inoculate *L. tarentolae* pre-culture 1:20 in 10 ml **LEXSY BHI** medium supplemented with Hemin, LEXSY NTC, LEXSY Hygro and PenStrep (refer to chapter 3.1) and incubate in tissue culture (TC) flask upright @ 26°C dark until Monday
- On Monday dilute pre-culture 1:10 into 10 ml of same medium and incubate in TC flask flat @ 26°C o/n
- On Tuesday check cell density of the culture until approx. 6 x 10^7 cells/ml are reached (OD 1.4)* and ensure by microscopy that the cells are vital and of droplike shape (Figure 10, Appendix 8.5)
- Spin cells 3 min, 2000g at room temperature and remove ½ volume of supernatant
- Resuspend pellet in remaining medium to get 10^8 cells/ml and put on wet ice for 10 min
- Have ready on wet ice in parallel tubes with 1-10 µg transforming DNA in max. 50 µl of water or 10 mM Tris buffer pH 8.0 and electroporation cuvettes d=2 mm**
- Add 350 µl pre-chilled cells to the tube with DNA and transfer all 400 µl to the electroporation cuvette on wet ice
- Electroporate @ 450V, 450 µF and monitor pulse time (ca. 5-6 msec)***
- Put cuvette back on ice for exactly 10 min
- Transfer electroporated cells with a capillary to 10 ml LEXSY BHI Medium supplemented with Hemin, LEXSY NTC, LEXSY Hygro and PenStrep in a ventilated TC flask
- Incubate o/n @ 26°C flat as static suspension culture (ca. 20h to OD 0.3-0.4)
- Proceed with clonal or polyclonal selection (chapters 5.1 and 5.2).

* if the cell density differs from this value, concentrate cells in the next step to get 10^8 cells/ml. For transfection, cultures between OD 1.0-1.8 can be used. Do not transfect cells if they are long and thin by microscopy.

** use electroporation cuvettes with long electrodes. The entire volume of 0.4 ml must be between the electrodes. Do not use electroporation cuvettes with short electrodes leaving most of the volume outside of the linear electric field.

*** using BioRad GENEPULSER II with PULSE CONTROLLER II and CAPACITANCE EXTENDER PLUS or GENE PULSER Xcell with PC and CE Modules. The resistance of the sample is 20 Ohms. With GENE PULSER Xcell you may alternatively use the **Time constant protocol** with the **settings 450 V and 3.5 ms** (Figure 9 in Appendix 8.4). Appendix 8.4 describes also an alternative **High voltage protocol** for transfection of LEXSY.

The protocol for electroporation of LEXSY with a **Multiporator** can be downloaded from the website of JBS at http://www.jenabioscience.com/cms/en/1/browse/1888_lexsy_methods.html.

The protocol for electroporation of LEXSY with a **Nucleofector** is described in Vainio *et al.* 2009.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

5 SELECTION OF TRANSGENIC LEXSY T7-TR STRAINS

For establishing expression strains you have the option of clonal and/or polyclonal selection. We repeatedly found similar expression levels when comparing cultures derived from clonal (chapter 5.1) or polyclonal (chapter 5.2) selection strategies following transfection with linearized expression cassettes designed for chromosomal integration. It is **not necessary** to add the inducer tetracycline to the selection medium since background transcription allows sufficient expression of the marker gene to confer antibiotic resistance to the emerging recombinant clones/polyclonal population.

5.1 Clonal selection by plating on solidified media

For plating, LEXSY BHI agar plates are always freshly prepared on the day of plating as described in Appendix 8.3. For customer convenience, all components required for preparing LEXSY BHI agar plates are included in our **LEXSY Plating Kit** (Cat. No. ML-451).

5.1.1 Clonal selection of T7-TR strains with pLEXSY_I-blecherry3 constructs

- Cover the solidified and aspirated LEXSY BHI agar supplemented with the antibiotics **LEXSY NTC**, **LEXSY Hygro** and **LEXSY Bleo** with nitrocellulose sheets (BA85, 0.45 m, blotting grade)*
- Pellet the cells from the transfected 10 ml o/n culture (chapter 4.4) for 3 min at 2000g and room temperature
- Remove the supernatant and resuspend the cells in approx. 50-100 µl of residual medium
- Carefully spread the resuspended cells onto the nitrocellulose sheets on top of the freshly prepared selective LEXSY BHI agar*
- Seal plates with parafilm and incubate bottom up at 26° for 5–7 days until small, defined colonies begin to appear
- Let them grow up to 1–2 mm diameter (approx. 7-9 days after plating)
- For **pre-screening** of high expression clones, transfer the nitrocellulose sheets on top of freshly prepared LEXSY BHI agar supplemented with the antibiotics LEXSY NTC, LEXSY Hygro, LEXSY Bleo and 10 µg /ml **LEXSY Tet** (tetracycline inducer)
- Incubate for 1-2 additional days
- Choose the best expressing clones visually in daylight by highest intensity of cherry color of the colonies (Figure 3)**
- Transfer the selected colonies with a pipette tip into 1 ml of medium supplemented with the antibiotics **LEXSY NTC**, **LEXSY Hygro** and **LEXSY Bleo** in 24 well format and incubate as static or agitated*** suspension culture. This should be done within three weeks post plating

* If you decide not to use the filter-lift technique, plate directly onto the surface of the agar medium and screen best expressing clones by fluorescence measurement of small scale suspension cultures expanded form these colonies. Alternatively, colony lifts can be conducted also after colonies did grow up on the agar surface.

** The development of cherry color of the colonies is based on co-expression of the *blecherry* gene which is co-transcribed with the target gene by T7 RNA polymerase and indicates that the inducible system is intact. The intensity of cherry color is in direct correlation to expression level of co-expressed target gene

*** Agitated suspension culturing in 24 well format may be performed on a microplate shaker with high amplitude (e.g. DESAGA TPM2).

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

5.1.2 Clonal selection of T7-TR strains with pLEXSY_I-neo3 and pLEXSY_I-ble3 constructs

Pellet the cells from the transfected 10 ml o/n culture (chapter 4.4) for 3 min at 2000g and room temperature

Remove the supernatant and resuspend the cells in approx. 50-100 µl of residual medium

- Carefully spread the resuspended cells onto the freshly prepared selective LEXSY BHI agar
- Seal plates with parafilm and incubate bottom up at 26° for 5–7 days until small, defined colonies begin to appear

Let them grow up to 1–2 mm diameter (approx. 7-9 days after plating)

Transfer the selected colonies with a pipette tip into 1 ml of medium supplemented with the antibiotics **LEXSY NTC**, **LEXSY Hygro** and **LEXSY Bleo** (for **pLEXSY_I-ble3** derivatives) or **LEXSY Neo** (for **pLEXSY_I-neo3** derivatives) in 24 well format and incubate as static or agitated suspension culture. This should be done within three weeks post plating

5.2 Polyclonal selection in suspension culture

- As soon as the 10 ml o/n cultures obtained from the transfection experiments (chapter 4.4) start to get slightly turbid (OD_{600} 0.4; ca. 10^7 cells/ml; usually approx. 20 h after electroporation), add the appropriate selective LEXSY antibiotics to the recommended final concentrations from the filtersterilized stocks provided in the kit (chapter 2.5) and continue incubation as static suspension culture at 26°C. **Don´t let the cultures overgrow before selection, since it will take longer to kill non-recombinant cells!**

- Follow the status of the cultures microscopically and visually until you start seeing the difference to the cells electroporated without DNA under the same conditions (negative control). Recombinant cells are motile, of drop-like shape and grow as a "cloudy" suspension culture whereas the cells in the negative control begin to die during the selection period and appear as spherical or irregular forms without flagella under the microscope. Visually, however, the negative control may also appear as a turbid suspension

- It usually takes one consecutive transfer into fresh medium with the appropriate antibiotics at 1:10 inoculation rate to get a turbid culture of the antibiotic-resistant recombinant cell line and a clear negative control (absence of growth). This passage should be performed within 7 days after first drug addition, usually at day 5. Do not wait for a longer period to passage the culture even if the negative control also got turbid

- if you feel that the transfected cultures do not start to grow under selection within the first week post electroporation, centrifuge cells 3 min at 2000g, resuspend pellet carefully in fresh medium with selective antibiotics and continue incubation. Also, you may combine 1:10 dilution with medium renewal of the remaining culture at day 5 of selection

- If at the time of first addition of selective antibiotic the cultures were already too dense, the negative control may not be dead after the first 1:10 passage. In this case another (or even more) subsequent 1:10 dilution(s) into fresh medium with selective antibiotic may be needed to get a turbid recombinant culture and a clear negative control

An illustration of the selection procedure can be downloaded from our website at http://www.jenabioscience.com/images/b3e879b381/Illustration_LEXSY_Transfection_Selection.pdf.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

5.3 Confirmation of genomic integration by diagnostic PCR

Integration of the expression cassette into the *odc* locus of chromosome 12 of *L. tarentolae* can be confirmed by diagnostic PCR using genomic DNA of transgenic strains as template. For this purpose primer pairs including one primer hybridizing within the plasmid-borne expression cassette and one primer hybridizing to a chromosomal *odc*-flanking sequence not present on the plasmid are used. A set of primers for such diagnostic PCRs as well as for insert sequencing are included in each kit. The primer sequences are shown in Appendix 8.2 of this manual.

- Prepare genomic DNA from 2 ml of a dense culture (OD approx. 2-3) by conventional phenol/chloroform extraction or with a commercial kit (e.g. **Cat-No. PP-206, 208**). There is no need to add Lysozyme or Proteinase K during purification

- Perform diagnostic PCR with the primer pairs and under the conditions outlined in the table below

Control region	Forward primer	Reverse primer	Fragment size	Annealing temp.
5'odc - utr1 (aprt)	A1304	A1715	1.1 kbp	60°C
blecherry - 3'odc	A708	P1510	2.7 kbp	60°C
ble - 3'odc	A708	P1510	2.0 kbp	60°C
neo - 3'odc	H4662	P1510	2.4 kbp	60°C

- The PCR reactions will result in a characteristic fragment for each vector (see table), which is not observed in control reactions where the template is the expression plasmid or genomic DNA from the LEXSY host. Additional diagnostic PCR reactions including target gene-specific primers may be performed.

5.4 Evaluation of target protein expression

Expression of the target protein in recombinant LEXSY strains may be evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of cell extracts or, in case of secretory expression, aliquots from supernatants. For obtaining optimal expression we recommend to check different culturing /induction conditions and time of harvest for each individual protein.

Maintain selected recombinant clones from 5.1/5.2 as continuous 10 ml suspension cultures as described in chapter 3.2. Always add selective LEXSY antibiotics **LEXSY NTC**, **LEXSY Hygro** and **LEXSY Bleo** (for **pLEXSY_I-ble3** derivatives) or **LEXSY Neo** (for **pLEXSY_I-neo3** derivatives) to these cultures. Antibiotics may be omitted from larger cultures destined for downstream processing.

- Inoculate 10-100 ml of **LEXSY BHI** medium 1:10 with the expression clone and grow at 26°C in TC flask (flat) as static suspension culture or as agitated culture on a microplate shaker with high amplitude (e.g. DESAGA TPM-2). Alternatively, grow culture in Erlenmeyer flasks at approx. 140 rpm. For culturing of larger volumes use Fernbach flasks (200-700 ml) or fermenters (>1 litre)

- Induce the T7 driven transcription at the time of inoculation (if the target protein is tolerated by the LEXSY host) or at higher cell densities (if the target protein could be harmful for the LEXSY host). Standard induction is performed with 10 µg/ml tetracycline (final concentration) from the 1000x stock provided in the kit. If you intend to tune the transcription rate you may choose tetracycline concentrations from the titration curve in Appendix 8.6.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

- Take aliquots at different time points of culturing (e.g. OD 2 - 6) for estimation of optimal harvest time. For the proteins tested we found harvest of agitated cultures ca. 48h post inoculation/ induction and of static cultures ca. 72h post inoculation/induction optimal. In case of **pLEXSY_I-blecherry3** strains you may monitor induction by measurement of fluorescence at 590 nm (excitation) and 620 nm (emission) in a plate reader. This is particular useful if larger volumes are cultivated. For fluorescence estimation it is sufficient to load 100-200 µl of suspension culture to the microtiter plate
- For **analysis of intracellular expression** calculate volume of aliquot V [ml] = 2/OD, e.g. withdraw 4 ml culture @ OD 0.5 or 2 ml culture @ OD 1.0 or 1 ml culture @ OD 2.0 etc.
    - Sediment cells 5 min at 3.000g
    - Resuspend pellet in 0.2 ml of gel loading buffer and apply 20 µl per lane on SDS-PAGE for Coomassie staining and/or Western blotting
- For **analysis of secretory protein expression** concentrate culture supernatants 100x with trichloroacetic acid (TCA) as follows:
    - Sediment cells from 10 ml of culture 10 min @ 3000g
    - Add 8 ml of (steril-filtered) supernatant to 2 ml of 50% ice-cold TCA to a final concentration of 10%. Sterilfiltration of supernatants prior to TCA precipitation avoids carry-over of cells and is optionally
    - Leave on ice for 30 min, spin 15 min 15.000g at 4°C
    - Remove supernatant completely and collect for safe waste disposal
    - Resuspend pellet in 1 ml of 80% acetone and transfer to an Eppendorf tube (the acetone-wash is performed to remove residual TCA)
    - Spin 15 min 15.000g at 4°C, aspirate supernatant and resuspend pellet in a final volume of 80 µl gel loading buffer (corresponding to 100x concentration)
    - Apply 20 µl sample/slot for SDS-PAGE and Western blotting

For purification of target proteins use appropriate culturing in larger volumes as described in chapter 3.2 and use affinity or conventional techniques.

6 LICENSING INFORMATION

Purchase of the **LEXSY Expression Kits** includes a non-exclusive and non-transferable license for non-commercial research. Commercial use of the **LEXSY expression system**, however, requires separate licensing. Commercial use includes but is not limited to:

- the use of any protein or other substance produced by LEXSY as reagents in screening to discover and/or promote candidate compounds for sale to a customer, distributor, wholesaler or other end user in therapeutic, diagnostic, prophylactic, and/or veterinary areas
- the manufacture, sale or offer to sell of any product containing proteins or other substances produced by LEXSY
- the large-scale production of recombinant protein pharmaceuticals
- "Contract research" to any third party or "Contract manufacturing" for any third party that has not been granted a license to use LEXSY

Please contact us at expression@jenabioscience.com.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

7 LITERATURE

Beverley SM, Clayton CE (1993) Transfection of *Leishmania* and *Trypanosoma brucei* by electroporation. *Methods in Molecular Biology* **21:** 333-348

Breitling R, Klingner S, Callewaert N, Pietrucha R, Geyer A, Ehrlich G, Hartung R, Müller A, Contreras R, Beverley S and Alexandrov K (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expression and Purification* **25:** 209-218

Klatt S and Konthur Z (2012) Secretory signal peptide modification for optimized antibody-fragment expression-secretion in *Leishmania tarentolae*. *Microbial Cell Factories* **11:** 97

Kushnir S, Gase K, Breitling R and Alexandrov K (2005) Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*. *Protein Expression and Purification* **42:** 37-46

Lukeš J, Paris Z, Regmi S, Breitling R, Mureev S, Kushnir S, Pyatkov K, Jirků M, and Alexandrov K (2006) Translational initiation in *Leishmania tarentolae* and *Phytomonas serpens* (*Kinetoplastida*) is strongly influenced by pre-ATG triplet and its 5' sequence context. *Molecular & Biochemical Parasitology* **148**: 125-132

Robinson KA and Beverley SM (2003) Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. *Molecular & Biochemical Parasitology* **128**: 217-228

Vainio S, Genest PA, Ter Ried B, van Luenen H and Borst P (2009) Evidence that J-binding protein 2 is a thymidine hydroxylase catalyzing the first step in the biosynthesis of DNA base J. *Molecular & Biochemical Parasitology* **164:** 157-161

Wiese M, Ilg T, Lottspeich F and Overath P (1995) Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in *Leishmania mexicana* secreted acid phosphatase. *EMBO Journal* **14:** 1067-1074

Please, visit http://www.jenabioscience.com for further literature on LEXSY.

8 APPENDIX

8.1 Maps of the pLEXSY_I-3 expression vectors

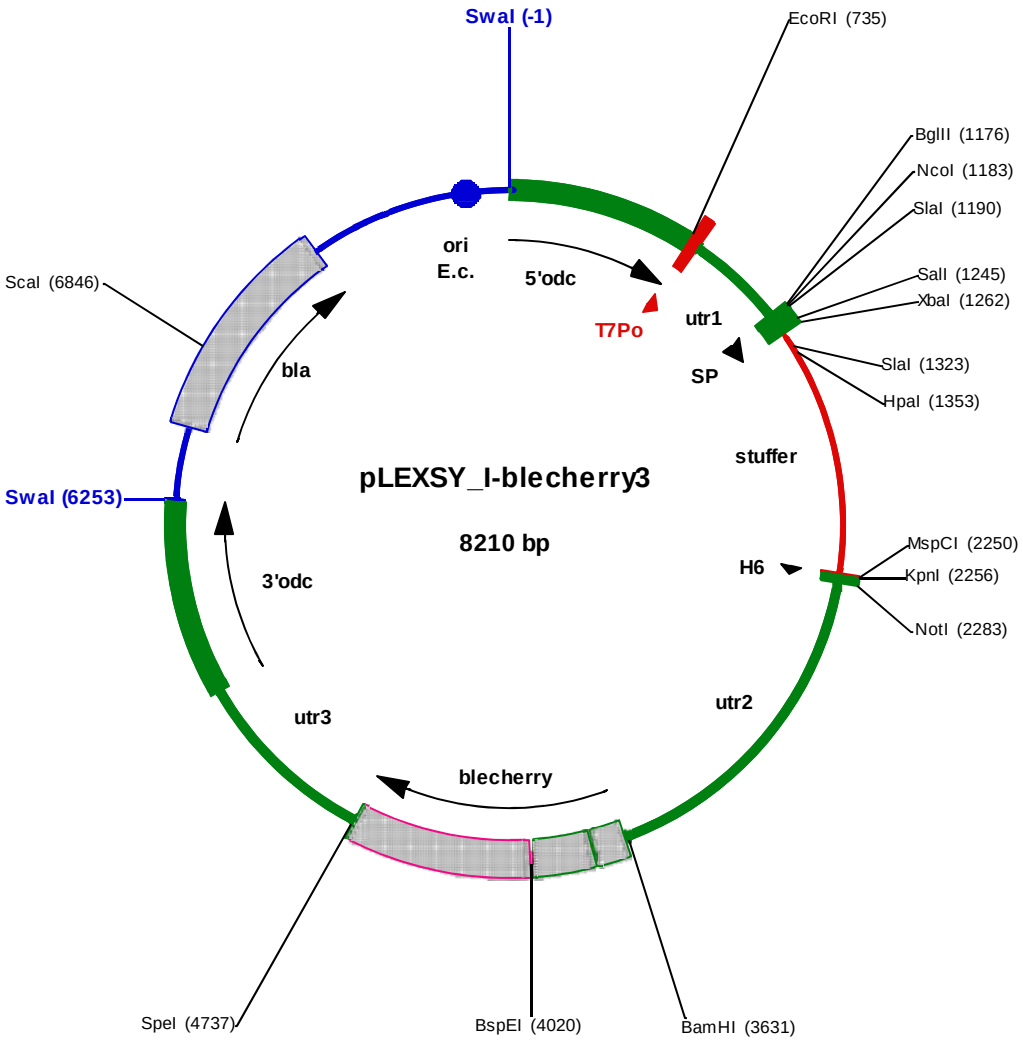


Figure 3: Map of **pLEXSY_I-blecherry3** expression vector **(Cat.-No. EGE-243)** with cloning sites for the target genes replacing the 1 kb stuffer fragment. **5´odc** and **3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. **Utr1** derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. **SP** designates the signal peptide of *L. mexicana* secreted acid phosphatase LMSAP1 (Wiese *et al.* 1995) and **H6** the hexa-Histidine stretch. ***Blecherry*** is the gene for the fusion protein conferring **LEXSY Bleo** resistance and cherry fluorescence which can be measured at 590 nm (excitation) and 620 nm (emission). The sequence of the multiple cloning sites is indicated in Fig. 2.

The DNA sequence of the expression vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/images/103bb272b3/pLEXSY_I-blecherry3_EGE-243_sequ_descript.txt.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

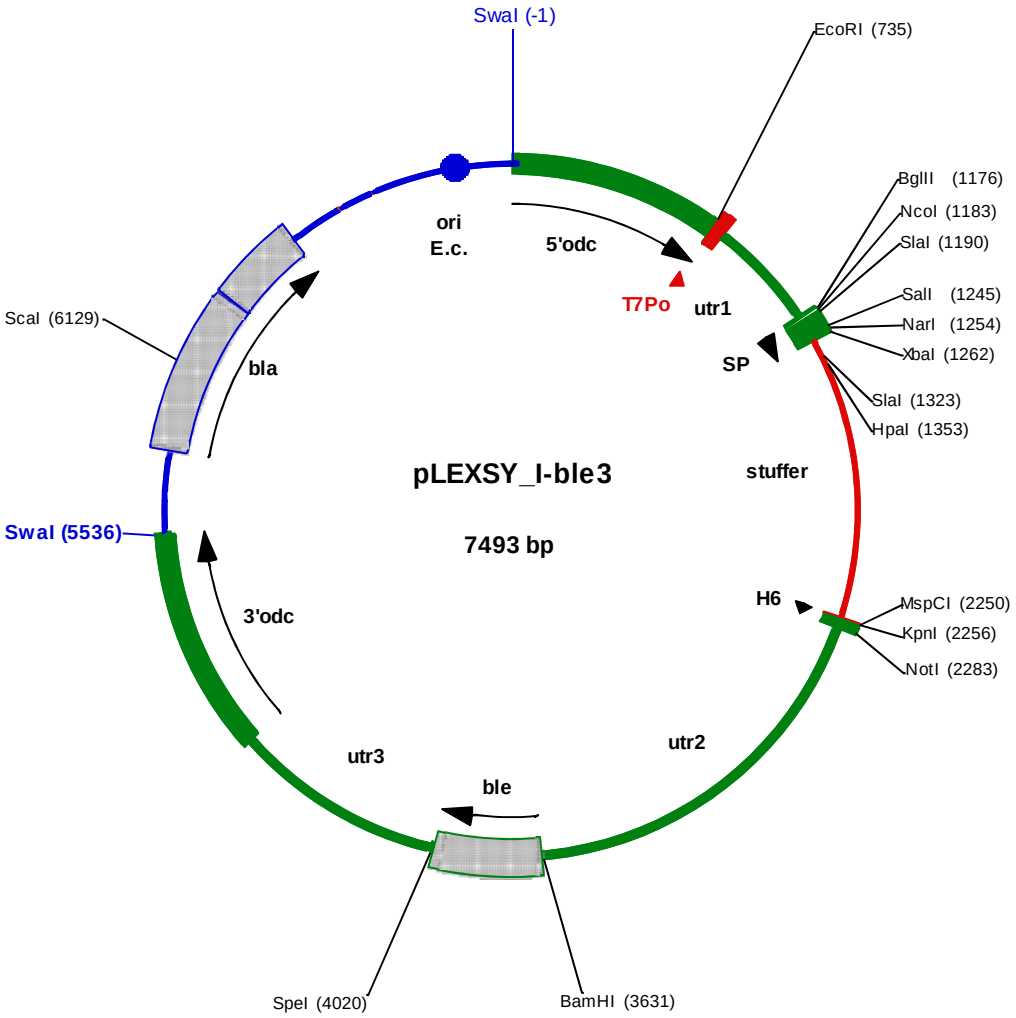


Figure 4: Map of **pLEXSY_I-ble3** expression vector **(Cat.-No. EGE-244)** with cloning sites for the target genes replacing the 1 kb stuffer fragment. In this plasmid the ***NarI*** (***EheI, KasI***) site can be used for in frame fusion to the secretory signal peptide. **5´odc** and **3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. **Utr**1 derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. **SP** designates the signal peptide of *L. mexicana* secreted acid phosphatase LMSAP1 (Wiese *et al.* 1995) and **H6** the hexa-Histidine stretch. The ***ble*** marker gene is the *bleomycin resistance* gene from *S. hindustans* conferring **LEXSY Bleo** resistance to the transgenic LEXSY clones. The sequence of the multiple cloning sites is indicated in Fig. 2.

The DNA sequence of the expression vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/images/103bb272b3/pLEXSY_I-ble3_EGE-244_sequ_descript.txt.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

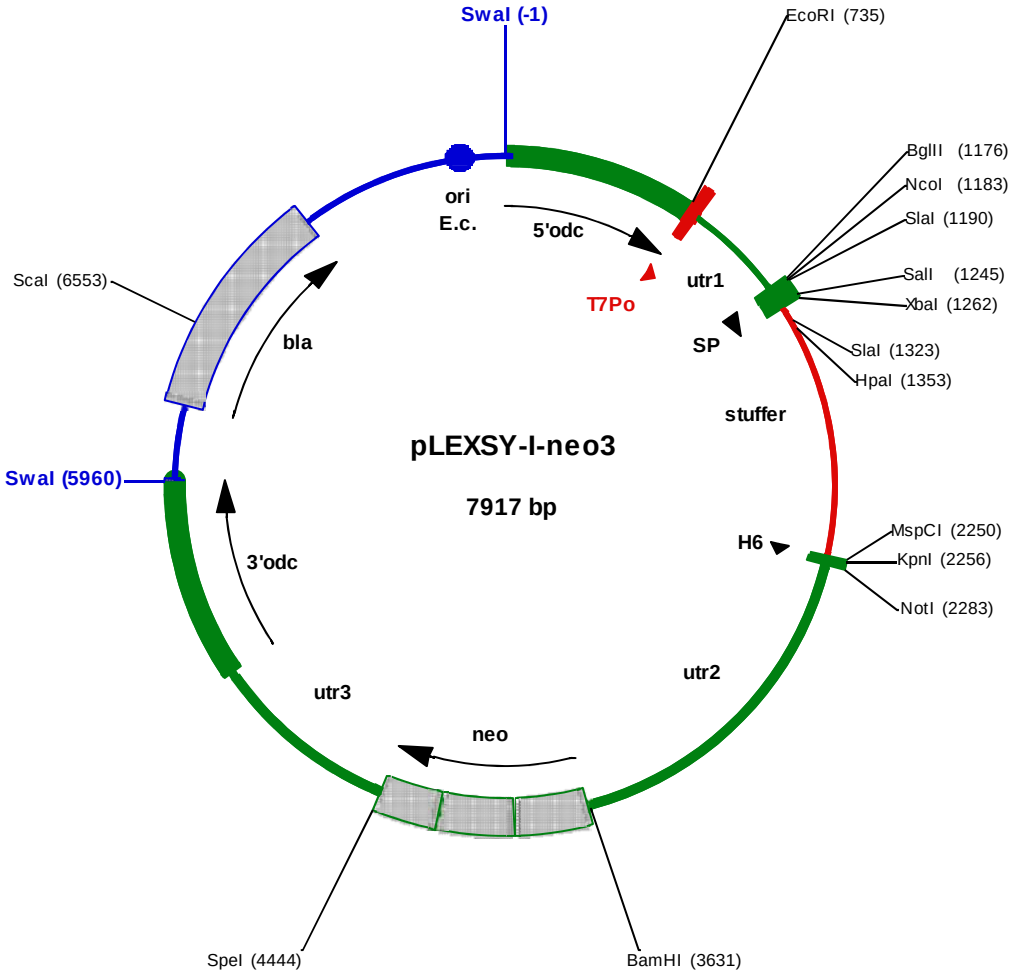


Figure 5: Map of **pLEXSY_I-neo3** expression vector **(Cat.-No. EGE-245)** with cloning sites for the target genes replacing the 1 kb stuffer fragment. **5´odc** and **3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. **Utr1** derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. **SP** designates the signal peptide of *L. mexicana* secreted acid phosphatase LMSAP1 (Wiese *et al.* 1995) and **H6** the hexa-Histidine stretch. The ***neo*** marker gene is encoding the aminoglycoside phosphotransferase 3' (I) syn. APH(3') conferring **LEXSY Neo** resistance to the transgenic LEXSY clones. Please note, that **LEXSY Neo** (JBS Cat.-No. AB-105) **is <u>not</u> the antibiotic Neomycin** which must not be used for selection in LEXSY. The sequence of the multiple cloning sites is indicated in Fig. 2.

The DNA sequence of the expression vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/images/103bb272b3/pLEXSY_I-neo3_EGE-245_sequ_descript.txt.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

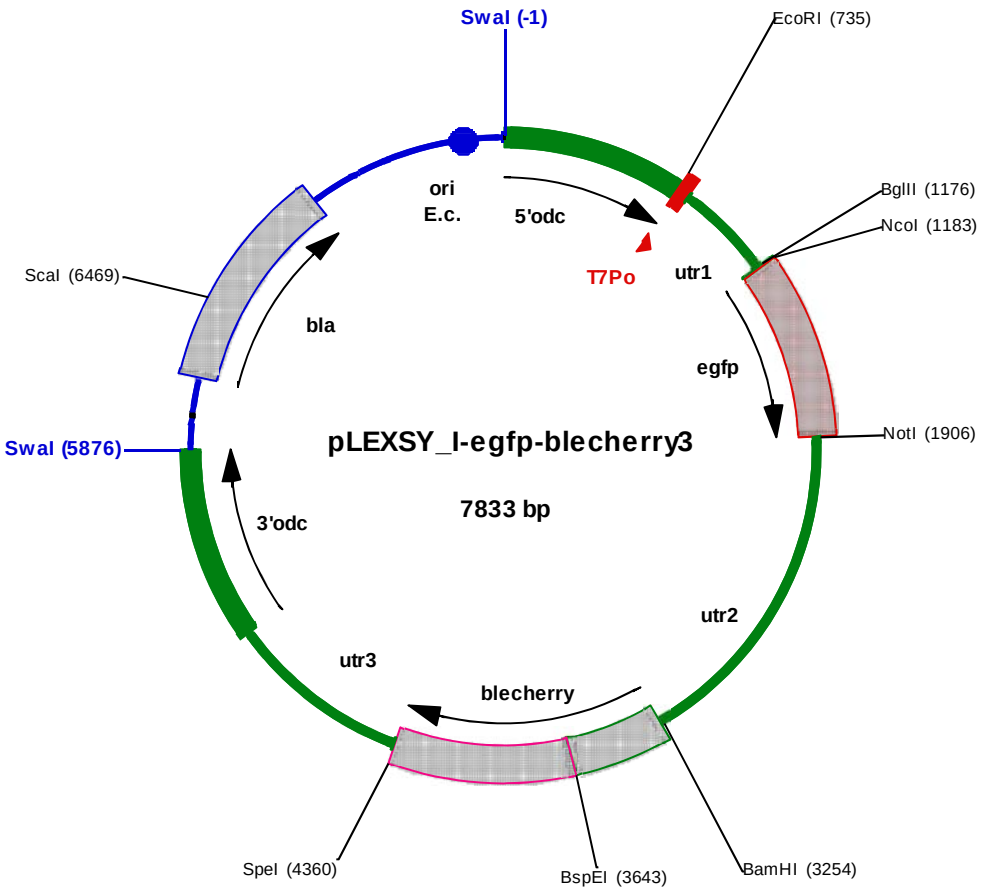


Figure 6: Map of **pLEXSY_I-egfp-blecherry3** control plasmid **(Cat.-No. EGE-246)** containing the ***egfp*** gene which had replaced the 1 kb stuffer fragment of **pLEXSY_I-blecherry3** expression vector. **5´odc and 3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. **Utr1** derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. ***Blecherry*** is the gene for the fusion protein conferring **LEXSY Bleo** resistance and fluorescence. Recombinant LEXSY strains transfected with this control plasmid confer green and cherry fluorescence and appear as gray colonies in daylight. Green fluorescence can be measured at 485 nm (excitation) and 520 nm (emission) whereas cherry fluorescence can be measured at 590 nm (excitation) and 620 nm (emission). Determination of both fluorescences can serve as a measure for the correlation of expression of monitor and target with this vector family.

The DNA sequence of the control vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/images/103bb272b3/pLEXSY_I-egfp-blecherry3_EGE-246_sequ_descript.txt.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

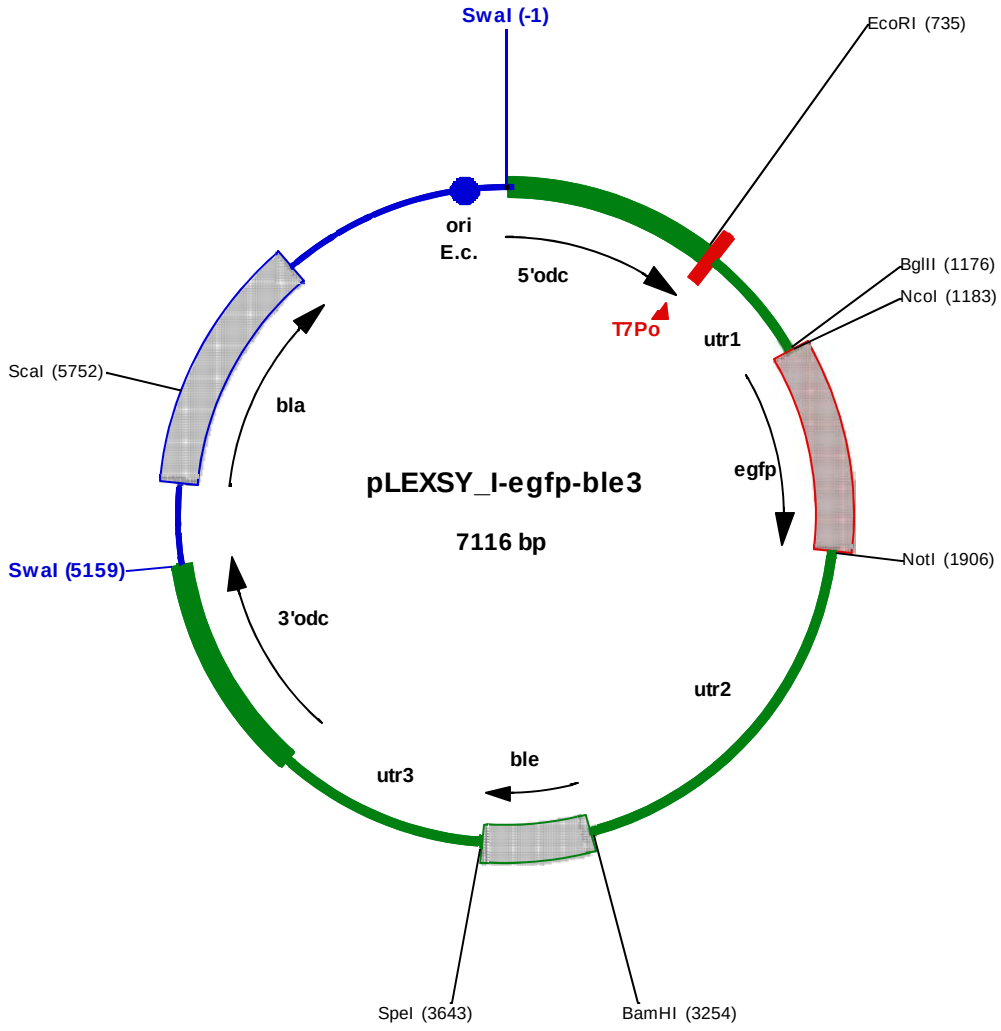


Figure 7: Map of **pLEXSY_I-egfp-ble3** control plasmid **(Cat.-No. EGE-247)** containing the ***egfp*** gene which had replaced the 1 kb stuffer fragment of **pLEXSY_I-ble3** expression vector. **5´odc** and **3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. **Utr1** derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. The ***ble*** marker gene is the *bleomycin resistance* gene from *S. hindustans* conferring **LEXSY Bleo** resistance to the transgenic LEXSY clones. Recombinant LEXSY strains transfected with this control plasmid confer green fluorescence which can be measured at 485 nm (excitation) and 520 nm (emission).

The DNA sequence of the control vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/images/103bb272b3/pLEXSY_I-egfp-ble3_EGE-247_sequ_descript.txt.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

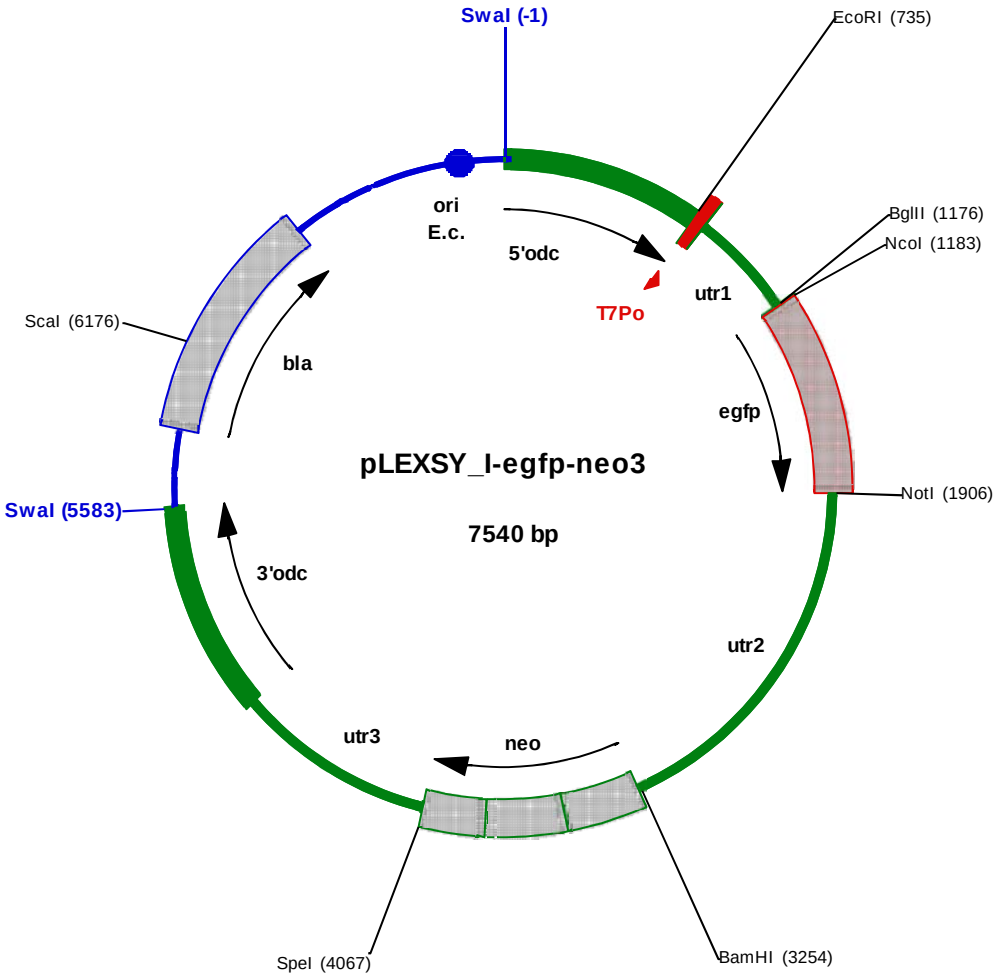


Figure 8: Map of **pLEXSY_I-egfp-neo3** control plasmid **(Cat.-No. EGE-248)** containing the ***egfp*** gene which had replaced the 1 kb stuffer fragment of **pLEXSY_I-neo3** expression vector. **5´odc** and **3´odc** are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. **Utr1** derived from 0.4k-IR of *L. tarentolae aprt*, **utr2** from 1.4k-IR *camCB* and **utr3** from 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host T7-TR. The ***neo*** marker gene is encoding the aminoglycoside phosphotransferase 3' (I) syn. APH(3') conferring **LEXSY Neo** resistance to the transgenic LEXSY clones. Please note, that **LEXSY Neo** (JBS Cat.-No. AB-105) **is <u>not</u> the antibiotic Neomycin** which must not be used for selection in LEXSY. Recombinant LEXSY strains transfected with this control plasmid confer green fluorescence which can be measured at 485 nm (excitation) and 520 nm (emission).

The DNA sequence of the control vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/images/103bb272b3/pLEXSY_I-egfp-neo3_EGE-248_sequ_descript.txt.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

8.2 Sequences of the primers available for LEXSinduce3 kits

Insert sequencing forward primer **P1442**	all expression vectors with 5'utr *aprt*	5′-CCGACTGCAACAAGGTGTAG-3′	**PM-110**
Insert sequencing reverse primer **A264**	all LEXSY expression vectors	5′-CATCTATAGAGAAGTACACGTAAAAG-3′	**PM-101**
odc forward primer **A1304**	integration diagnostics of all *odc* expression vectors	5′ -TCCGCCATTCATGGCTGGTG-3′	**PM-114**
aprt reverse primer **A1715**	integration diagnostics of all expression vectors with 5'utr *aprt*	5′-TATTCGTTGTCAGATGGCGCAC-3′	**PM-111**
ble forward primer **A708**	integration diagnostics of all *ble* & *blecherry* expression vectors	5′-GGATCCACCGCATGGCCAAGTTGACCAGTG-3′	**PM-107**
neo forward primer **H4662**	integration diagnostics of all *neo* expression vectors	5′- GGATCCAATATGGGATCGGCCATTG -3′	**PM-118**
odc reverse primer **P1510**	integration diagnostics of all *odc* integration vectors	5′-GTGCACCCATAGTAGAGGTGC-3′	**PM-115**

Which primers are included in which kit?

LEXSinduce3 kit with **pLEXSY_I–ble3** or **pLEXSY_I–blecherry3**

- Insert sequencing forward primer **P1442**
- Insert sequencing reverse primer **A264**
- *odc* forward primer **A1304**
- *aprt* reverse primer **A1715**
- *ble* forward primer **A708**
- *odc* reverse primer **P1510**

LEXSinduce3 kit with **pLEXSY_I–neo3**

- Insert sequencing forward primer **P1442**
- Insert sequencing reverse primer **A264**
- *odc* forward primer **A1304**
- *aprt* reverse primer **A1715**
- *neo* forward primer **H4662**
- *odc* reverse primer **P1510**

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

8.3 Preparation of LEXSY BHI agar plates for clonal selection

For 4 plates prepare 50 ml medium and bring to 37°C

Component	*Storage*	*Amount*
2x LEXSY BHI (74 g/L)	room temperature	36 ml
inactivated Fetal Calf Serum (FCS)*	-20°C	10 ml
1M HEPES, pH 7.4	4°C	4 ml
Pen-Strep	-20°C	0.5 ml
Hemin (0,25% in 50% Triethanolamine)	4°C	0.2 ml
Selective antibiotic(s), if necessary		

*Inactivated for 20 min at 56°C (or 1h at 52°C)

- Autoclave or melt (microwave) 50 ml 2% BACTO-Agar (DIFCO) and keep at 55°C
- Pour the medium into the warm agar, mix gently to avoid air bubbles, and distribute 25 ml per plate with a serological pipette (air bubbles may be removed with the pipette)
- Dry the plates after solidifying for 10 min **open** under the laminar flow
- Use the freshly prepared plates immediately, at least on the same day
- Optionally, cover the surface of the plates with nitrocellulose membrane after aspiration.

Refer to chapter 5.1 for clonal selection.

For your convenience, the **LEXSY Plating Kit** (Cat.-No. ML-451) is available from Jena Bioscience, containing all components for the preparation of 40 LEXSY BHI agar plates.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

8.4 Alternative electroporation protocol

High-Voltage protocol for transfection of LEXSY (after Robinson *et al.* 2003)

- On Friday inoculate *L. tarentolae* pre-culture 1:20 in 10 ml **LEXSY BHI** medium supplemented with Hemin, LEXSY NTC, LEXSY Hygro and PenStrep (refer to chapter 3.1) and incubate in tissue culture (TC) flask upright @ 26°C dark until Monday
- On Monday dilute pre-culture 1:10 in 10 ml medium and incubate in TC flask flat @ 26°C o/n
- On Tuesday check cell density of the culture until approx. 6 x 10^7 cells/ml are reached (OD 1.4)* and ensure by microscopy that the cells are vital and of droplike shape
- Spin cells 3 min, 2000g at room temperature and remove ½ volume of supernatant
- Resuspend pellet in remaining medium to get 10^8 cells/ml and put on wet ice for 10 min
- Have ready on wet ice in parallel tubes with 1-10 µg transforming DNA in max. 50 µl of water or Tris buffer pH 8.0 and electroporation cuvettes d=4 mm**
- Add 450 µl pre-chilled cells to the tube with DNA, mix and transfer to the electroporation cuvette on wet ice
- Pulse 2 times at 1500 V, 25 µF with 10 sec between pulses (pulse time ca. 1.2 msec)***
- Put cuvette back on ice for exactly 10 min
- Transfer electroporated cells with capillary to 10 ml LEXSY BHI medium in a ventilated TC flask
- Incubate o/n at 26°C as static suspension culture (ca. 20h, OD 0.3-0.4).

* if the cell density differs from this value, concentrate cells in the next step to get 10^8 cells/ml. For transfection, cultures between OD 1.0-1.8 can be used. Do not transfect cells if they are long and thin by microscopy

** use electroporation cuvettes with long electrodes. The entire volume of 0.5 ml must be between the electrodes. Do not use electroporation cuvettes with short electrodes leaving most of the volume outside of the linear electric field

*** using BioRad GENEPULSER II with PULSE CONTROLLER II and CAPACITANCE EXTENDER PLUS or GENE PULSER Xcell with PC and CE Modules

Low voltage protocol		High voltage protocol
Exptl. decay	**Time constant**	
d=2mm V=0.4 ml	d=2mm V=0.4 ml	d=4mm V=0.5 ml
set: 450V 450 µF 1 pulse	set: 450V T=3.5ms 1 pulse	set: 1500V 25 µF 2 pulses @ 10" interval
get: 5 - 6 ms ≈20 Ω ≈450 V deliv.	get: ≈3.5 ms ≈20 Ω ≈450 V deliv.	get: ≈1.2 ms ≈50 Ω ≈1500 V delivered

Figure 9: Overview of parameters of electroporation protocols with BioRad GENE PULSER. The standard Low voltage electroporation protocol is described in section 4.4

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

8.5 How to grow a *Leishmania* culture

- *L. tarentolae* needs **aerobic** conditions. The strains can be maintained as continuous suspension culture with regular dilutions (refer to chapter 3.2). All culturing is performed at **26°C**. Higher temperatures lower the growth-rates and vitality significantly and *L. tarentolae* will not survive at 37°C in axenic suspension culture

- All growth media should be supplemented with **Hemin** which is essential for *Leishmania.* Hemin is light-sensitive, so *Leishmania* must be cultured in the dark. After completion with Hemin the medium must be stored in the dark at 4°C. For optimal growth and vitality the completed medium should be used within 2 weeks. However, if this shelf live is exceeded, it is possible to re-add Hemin (and PenStrep) and to use this medium for 2 more weeks

- For **maintaining** LEXSY strains for transfection and analysis it is convenient to grow static suspension cultures in 10 ml **LEXSY BHI medium** in ventilated tissue culture (TC) flasks. Don´t use agitated cultures for strain maintenance since cells will age much faster. It is not necessary or growth-promoting, to add serum to the BHI medium

- Best results are obtained with inoculations during early stationary phase. Avoid repeated successive dilution of cultures of low cell densities as this may drop growth. However, occasional higher dilutions of stationary cells at e.g. 1:100 will not adversely affect subsequent growth. It is convenient to dilute 10 ml cultures 1:50 on Monday and 1:20 on Friday and to incubate TC flask upright, this way lowering aeration for achieving longer intervals between passages. Don't culture *Leishmania* much longer than for 7 days in the same medium without dilution. For culturing for transfection refer to chapter 4.4, for culturing for protein expression refer to chapter 5.4

- Always control appearance and motility of cells by microscopy. Cells of mid-growth phase cultures are of drop-like shape (Fig. 10), approx. 15x5 µm in size with one flagellum at the flat end, and motile. **These cells are most efficient for transfection and plating on solid media**. Mid-growth phase cultures always contain subpopulations of non- or less motile cells and of cells of different shape. Don't hesitate to transfect, plate or preserve a culture with drop-like cells containing such subpopulations. Cells of older cultures get longer and thinner (needle-like shape) and remain motile. Enhanced motility may result from nutrient deprivation or other limitations and must not necessarily be a sign of mid growth culture stage. Also, bacterial, fungal or other contaminations may be identified by microscopy

- Keep patient, esp. if you are used to working with bacteria. *Leishmania* cells are protozoans with regular doubling times of 7 h in static suspension cultures and 4 - 6 h in agitated cultures. They need their time to grow or to adapt to new conditions

- If you - despite following these instructions - encounter growth problems with the host strain, sediment cells 3 min at 2000g, resuspend pellet carefully in fresh growth medium and continue incubation in ventilated TC flasks. This approach was very helpful in rescuing cultures esp. after transfection

- It is not necessary to centrifuge *Leishmania* cultures at high speed >10.000g. Centrifugation at 2000-3000g is sufficient for sedimentation of cells and makes gentle and quick resuspension of cell pellets easier. However, *Leishmania* cells will survive centrifugation at 20.000g without lysis

- If you culture LEXSY strains in bioreactors be careful with stirring to avoid shear stress. We found it sufficient to aerate the culture in a 10 L fermentation with low speed stirring at 100-300 rpm or even without stirring for obtaining high cell densities up to 10^9 cells/ml. However, *L. tarentolae* was found resistant to shear stress at rotor speeds of more than 500 rpm with turbines consisting of two angular paddles, whereas 700 rpm damaged the cells.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

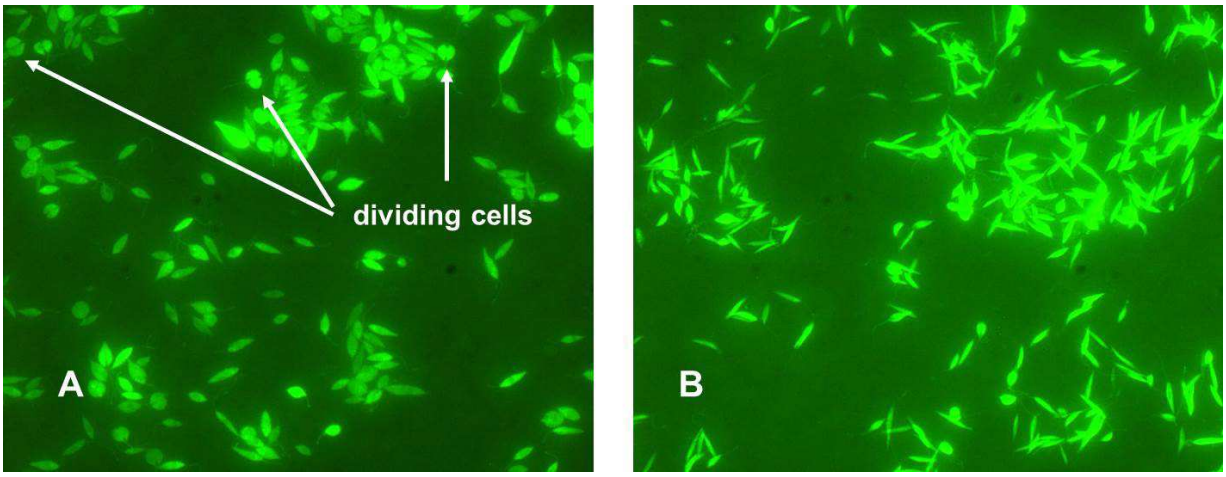


Figure 10: Microscopic image of LEXSY cells expressing green fluorescent protein. **A:** exponentially growing culture, suitable for electroporation and cryoconservation **B**: stationary culture, do not use for electroporation.

8.6 Titration of tetracycine concentration for modulation of expression level

Routinely, the transgenic T7-TR strains are induced for target protein expression with **10 µg/ml** of tetracycline (final concentration) from the 1000x stock provided in the kit (refer to chapter 5.4) albeit nearly full induction is reached already with 1 µg/ml tetracycline (Figure 11). For reducing or tuning expression levels add less than 1 µg/ml. Tetracycline concentrations >50 µg/ml will result in growth inhibition of LEXSY strain.

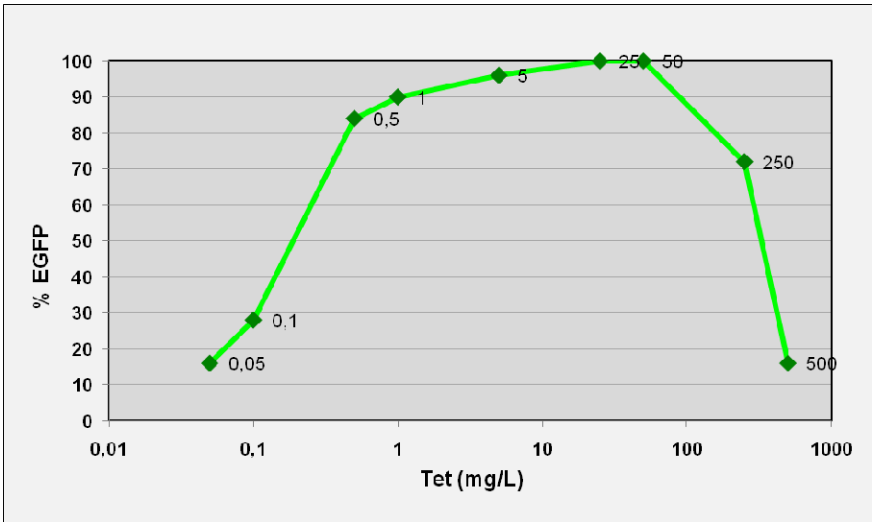


Figure 11: Dependence of EGFP reporter expression on concentration of tetracycline in inducible LEXSY. LEXSinduce provides a broad induction plateau from 1 – 50 µg /ml of inducer tetracycline. Expression rates were estimated 24 h post induction by FACS.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100

8.7 Correlation of optical density and cell concentration

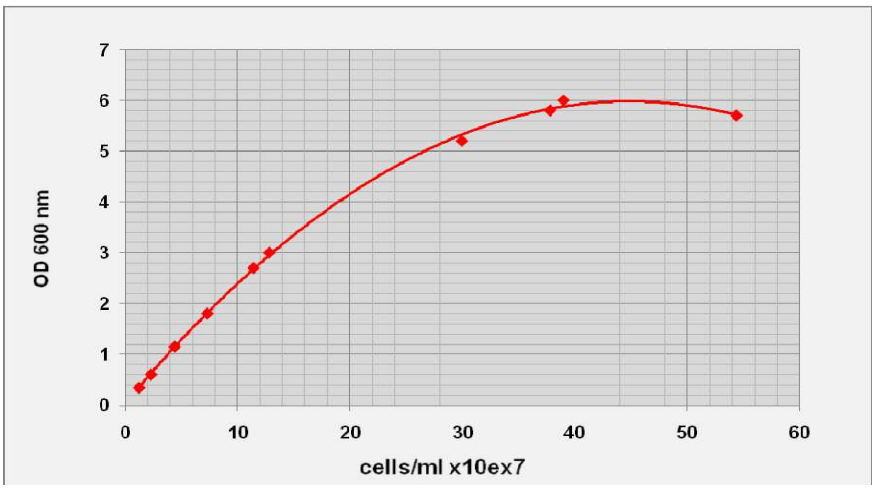


Figure 12: Calibration of OD readings of spectrophotometer (biowave CO8000) with cell densities determined with a particle counter (Coulter). LEXSY cultures were grown to different stages and OD readings and cell counts were determined from the same sample. The non-linear behaviour at higher OD is due to changes in cell shapes and cell lysis in late stationary cultures.

Jena Bioscience GmbH • Loebstedter Str. 80 • D-07749 Jena • Germany • e-Mail: info@jenabioscience.com
Tel.: +49-3641-6285000 • Fax: +49-3641-6285100