

LEXSinduce 3 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.*

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1. INTRODUCTION

LEXSY – the unique protein expression platform offered by Jena Bioscience combines eukaryotic protein expression/folding/modification with easy handling known from bacterial expression systems. The trypanosomatid protozoan host *Leishmania tarentolae* used in LEXSY was first isolated from the Moorish gecko *Tarentola mauritanica*, is kept in axenic culture, and is not pathogenic to mammals (Biosafety level 1). An illustrated LEXSY overview can be downloaded from our website at http://www.jenabioscience.com/images/b3e879b381/LEXSY_essence.pdf.

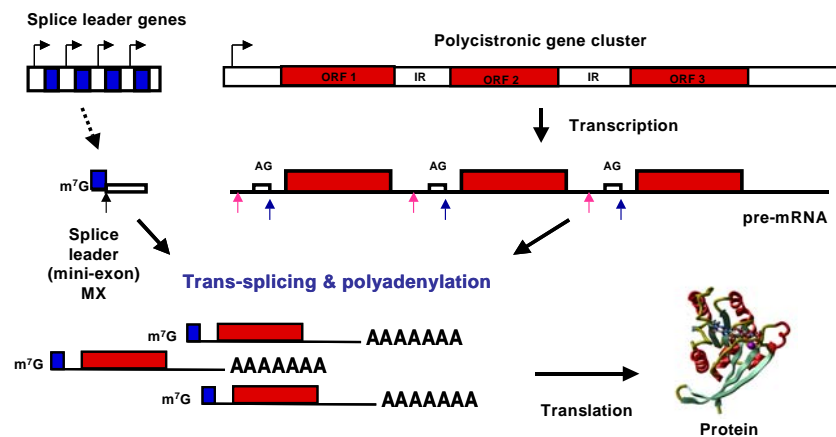


Fig. 1: Transcription in trypanosomatid protozoa.

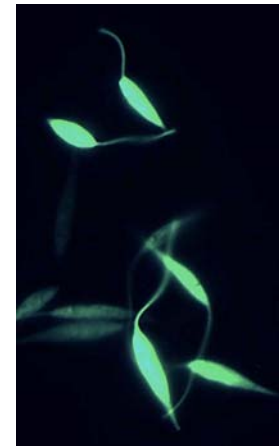


Fig. 2: LEXSY cells expressing green-fluorescent protein

In trypanosomatid protozoa mRNAs are transcribed as polycistronic precursors which are posttranscriptionally processed into individual mRNAs by trans-splicing and polyadenylation within the intergenic regions (Fig. 1; Ref. 1). Regulation of protein expression in these species occurs mainly on the level of RNA and may be influenced by the structure of the intergenic regions (1, 2). On pLEXSY vectors intergenic regions are used which were optimized for expression of heterologous proteins in *L. tarentolae* (3).

For high-level expression target genes are supplied with specific splicing signals provided on the expression vector included in the kit. These constructs are stably integrated into a chromosomal locus of the *Leishmania* host (or, alternatively, can be maintained episomally). The expression vectors are constructed in *E. coli* by standard techniques and introduced into the LEXSY hosts by electroporation.

Jena Bioscience offers two types of LEXSY Kits which contain all components required for engineering and selection of expression strains:

- The **LEXSYcon2 kit** (Cat.-No. EGE-1300) contains one of the **pLEXSY-2** vectors (EGE-231, EGE-232, EGE-233 or EGE-234 with alternative selection marker genes) for **constitutive** cytoplasmic or secretory expression in the **LEXSY host P10**.
- The **LEXSInduce3 kit** (Cat.-No. EGE-1410) contains one of the **pLEXSY_I-3** vectors (EGE-243, EGE-244 or EGE-245) for **inducible** cytoplasmic or secretory expression in the **LEXSY host T7-TR**.

Recombinant LEXSY strains can be cultivated as static or agitated suspension cultures. They grow to high cell densities (5×10^8 cells/ml) in complex media (**LEXSY BHI** or **LEXSY YS**). The cells can be lysed easily in a microfluidizer, by sonication, osmotic shock or detergents.

Numerous cytosolic, membrane-localized and extracellular proteins have been expressed with LEXSY at Jena Bioscience and in customer laboratories with yields up to 300 mg per litre culture. In addition, we have established cultivation of LEXSY strains in laboratory fermentation plants with volumes of 1 – 30 litres. For further details, please visit our website <http://www.jenabioscience.com>.

The **LEXSInduce3 kit** contains one of three expression vectors of choice which differ in the antibiotic resistance marker and the option to monitor expression online by a fluorescence reporter. The vector **pLEXSY_I-neo3** (encoding aminoglycoside 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 cultivation by coexpressed fluorescence. The control vector provided in the kit contains the EGFP gene linked to the respective selection marker.

The **pLEXSY_I-3 vectors** enable inducible expression of target proteins either with or without secretory signal peptide provided on the vectors. For customer convenience the same vector can be used for cloning of ORFs either for cytosolic or for secretory expression. The LmSAP secretory signal peptide encoded on these vectors was derived from the gene for secreted acid phosphatase (*Im sap1*) of *Leishmania mexicana* (7). In-frame fusion of the ORF of a target protein to this signal peptide allows secretory expression in LEXSY hosts, whereas cloning into any of the restriction sites at the 5' end of the signal peptide-encoding sequence will result in cytosolic expression (Fig. 5).

An illustration of the inducible LEXSY can be downloaded from the Jena Bioscience website at http://www.jenabioscience.com/images/b3e879b381/Illustration_inducible_LEXSY.pdf

One main advantage of LEXSY is the mammalian-type posttranslational modification of target proteins, such as glycosylation (Fig. 3), phosphorylation or prenylation. Recombinant human erythropoietin (EPO) purified at Jena Bioscience from LEXSY was biologically active, natively processed at the N-terminus, and N-glycosylated (Ref. 3). The N-glycosylation profile was exceptionally homogeneous, with a biantennary oligosaccharide and the Man₃GlcNAc₂ core structure accounting for >90% of the glycans present. *L. tarentolae* is thus the first described biotechnologically useful unicellular eukaryotic host producing biantennary, fully galactosylated, core- α -1,6-fucosylated N-glycans. This N-glycosylation profile was coincident with the profile of recombinant human Interferon- γ expressed in LEXSY and of LEXSY host Gp63 glycoprotein.

An illustration of LEXSY glycosylation can be downloaded from the Jena Bioscience website at http://www.jenabioscience.com/images/b3e879b381/Illustration_LEXSY_Glycosylation.pdf.

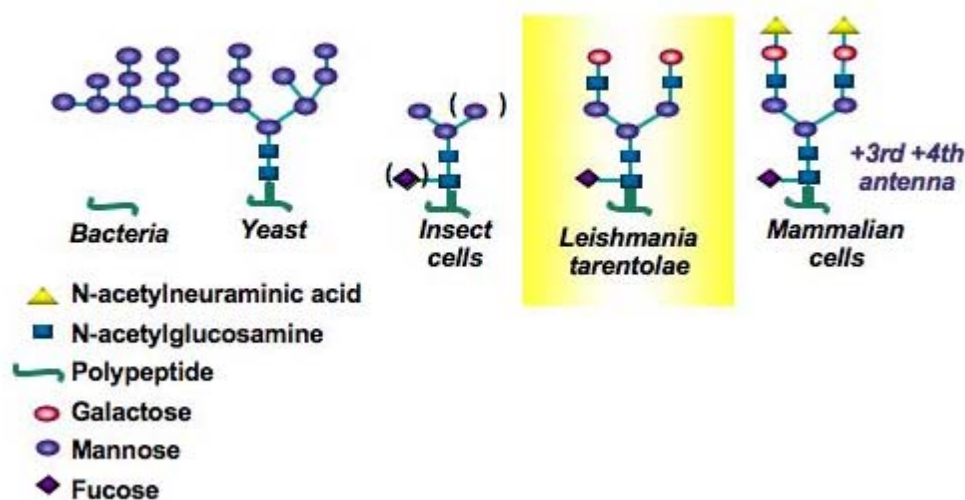


Fig. 3: N-glycosylation of proteins in LEXSY compared to glycosylation in other protein expression systems. Note that the glycosylation patterns obtained in mammalian cells and in LEXSY differ only in the presence of N-acetylneuraminic acid at the ends of the sugar chains in the latter.

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 month. However, we have successfully reactivated LEXSY stocks after 9 years of storage at -80°C. For reactivation protocol refer to chapt. 3.4.

It is recommended to prepare sufficient glycerol stocks (chapt. 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 chapt. 3.2.) Alternatively, the T7TR 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/1116_expression_vectors.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 **A1432**, 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**
 - 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 cultivation medium

LEXSY BHI, Powder for preparation of liquid cultivation medium

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

The antibiotics for host strain maintenance

- **LEXSY NTC and LEXSY Hygro**
 - 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 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* 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

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
 - aliquote upon arrival and avoid frequent freeze-thaw cycles (eventually occurring turbidity upon thawing will not compromise induction)

Additives

- **Hemin** (0.25% porcine Hemin in 50% Triethanolamine)
 - 2 ml, ready-to-use 500x stock solution, steril-filtered
 - store at 4°C in the dark (note, that 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) may be added to avoid bacterial contaminations
 - 5 ml, ready-to-use 200x stock solution, steril-filtered
 - store at -20°C
 - stable for 12 months

2.6. Equipment and materials supplied by customer

- Incubator at 26°C (no CO₂ 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

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

3. CULTIVATION OF LEXSY T7-TR HOST AND EXPRESSION STRAINS

LEXSY T7-TR host and **expression strains** must be cultivated in the dark at 26°C (no CO₂ incubator required) in complex media (**LEXSY BHI**, Cat-No.-ML-411, 412 or **LEXSY YS**, Cat-No. ML-431, 432) or chemically defined media (**Synthetic LEXSY Medium**, Cat-No. ML-103, 107) 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 deionized water and autoclave **exactly** 15 min at 121°C. **Control temperature profile using a reference with the same volume.** Note, that overexposure may result in decomposition of media ingredients (a sign is a dark brown color) which adversely affects growth of LEXSY strains. Proper autoclaved medium is of amber color. Store core medium at room temperature before addition of Hemin and PenStrep (stable for 12 month).

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

3.2. Cultivation conditions

L. tarentolae is an obligate aerobic microorganism. **Standard cultivation 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 x10⁷ 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 concentration 100 µg/ml each).

To prevent genomic instabilities which eventually might occur upon long-term cultivation 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 cultivation for transfection ref. to chapt. 4.4. and for protein expression see chapt. 5.4.

To conveniently monitor the growth of the LEXSY cultures it is advised to 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 (Fig. 14 in Appendix 8.7.).

If you, eventually, 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.

Cultivation may be performed in

- ventilated tissue culture (TC) flasks for suspension cultures, culture volume 10-200 ml
- Erlenmeyer flasks, agitated at approx. 100-140 rpm, culture volume 50-500 ml
- Fernbach flasks, agitated at approx. 75-90 rpm, culture volume 0.5-1 litre
- standard bioreactors, 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 20% glycerol for at least 12 months. However, we recovered viable cells under these conditions after 9 years of storage, without any loss of vitality. Glycerol stocks may be prepared from standard cultivation in **LEXSY BHI medium** for strain maintenance after 3 days (1:20 dilution) or after 4 days (1:50 dilution) at OD 1.4-2, ca. $6-8 \times 10^7$ cells/ml (ref. to **chapt. 3.2.**). Alternatively, stocks can be prepared from 1:10 inoculated cultures after 24 h if TC flasks are incubated flat. Avoid to prepare 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 elongated (Fig. 2) but not of needle-like appearance. Do not use other media than **LEXSY BHI** for cryoconservation.

Glycerol stocks preparation using a cryocontainer

Using this method, refrigeration proceeds continuously with $-1^{\circ}\text{C}/\text{min}$

- Add 1.2 ml autoclaved Glycerol (80% by weight) to a sterile 15 ml Falcon tube
- Withdraw 3.6 ml of culture OD 1.4-2 (ca. $6-8 \times 10^7$ 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 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, refrigeration proceeds in steps of $0^{\circ}\text{C}/-20^{\circ}\text{C}/-80^{\circ}\text{C}$

- Add 1.2 ml autoclaved Glycerol (80% by weight) to a sterile 15 ml Falcon tube
- Withdraw 3.6 ml of culture OD 1.4-2 (ca. $6-8 \times 10^7$ 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
- 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 glycerol stocks prepared prior to stopping respective suspension cultivation.

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. $6-8 \times 10^7$ cells/ml). This usually takes 2 days; wait longer if cells recover more slowly and follow status by microscopy
- Dilute dense culture 1:10 into fresh **LEXSY BHI** and incubate for 3 days. Do not dilute culture of low density. For strain maintenance dilute into fresh **LEXSY BHI** on Monday and Friday each week (see **chapt. 3.2.**).

4. ENGINEERING OF LEXSY 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. The architecture of this expression system is shown in Fig. 4.

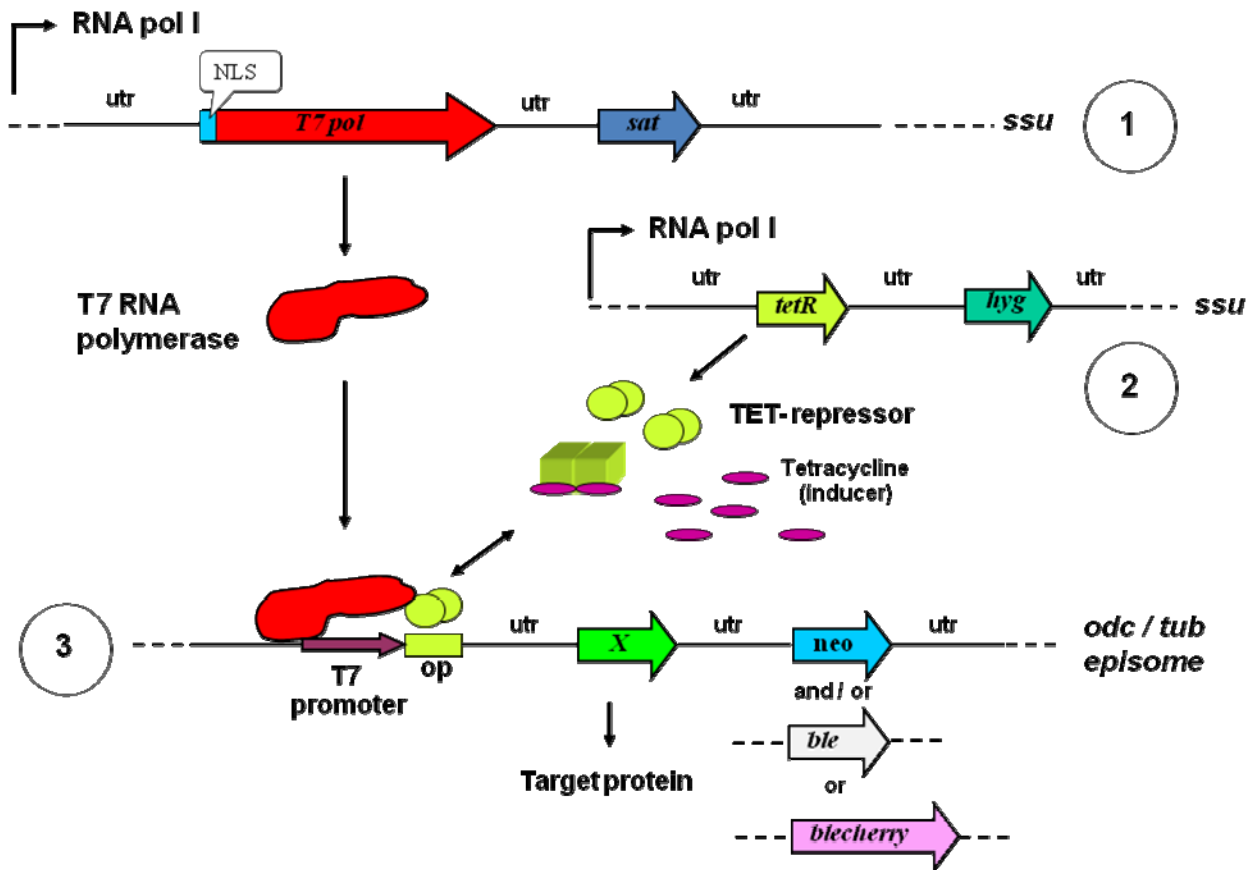


Fig. 4: Components of the inducible LEXSY expression system. Target genes (*X*) are inserted into the expression cassette under the control of T7 promoter with TET operator (TRE) and integrated into the chromosomal ornithine decarboxylase (*odc*) locus (2) of the expression host *Leishmania tarentolae* T7-TR constitutively expressing T7 RNA polymerase (1) and TET repressor (3) under the control of host RNA polymerase I. T7 polymerase (*T7pol*) and TET repressor (*tetR*) genes are stably integrated into the host chromosomal small ribosomal subunit RNA locus (*ssu*). In case of the pLEXSY_I-blecherry3 construct, the marker gene is substituted by a *blecherry* fusion. The utr's are optimized nontranslated gene flanking regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in *Leishmania tarentolae*.

In the first cloning step the target gene is supplied with linker sequences containing restriction sites that allow insertion into the pLEXSY_I-3 vectors downstream of the T7 promoter/TET operator arrangement. These vectors contain optimized non-translated regions flanking the target gene insertion sites, which provide the splicing signals for posttranscriptional mRNA processing. Following construction in *E. coli* the expression plasmid is linearized and integrated into the *odc* locus of the LEXSY host T7-TR by homologous recombination (double crossover) via the 5' and 3' *odc* fragments on the expression plasmid (Fig. 5).

4.1. Amplification of target gene

The **pLEXSY_I-3 vectors** (Fig. 5) allow insertion of the target gene into the cloning sites in a way that proteins are expressed either cytosolically or are secreted. The 5' insertion sites for cytosolic expression are **BglII**, **NcoI**, or **SlaI** and for secretory expression **SaI**, or **XbaI** (in EGE-244 in addition **NarI**). In both cases a stuffer fragment is replaced by the target ORF. At the 3' end of this stuffer fragment the restriction sites for **MspCI**, or **KpnI** yield fusion to a C-terminal His₆ stretch, whereas utilization of the **NotI** cloning site avoids this His₆ 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. All restriction enzymes for cloning into **pLEXSY_I-3** vectors can be purchased from Jena Bioscience (**Cat.-No. EN-106 to EN-143** www.jenabioscience.com).

- Analyse your target gene for internal sites for the restriction enzymes you intend to use. Also, make sure that your target gene does not contain any internal **SwaI** site, since this site will be used for vector linearization prior to transfection. If a **SwaI** site is present however, you may choose to remove it by silent mutagenesis (or by gene synthesis, see below) or to transfect circular DNA. In the latter case clonal selection (ref. to 5.1.) is required since the episomes tend to amplify and to evtl. integrate into the genome in a heterogeneous manner. Non-clonal selection in suspension cultures (ref. to 5.2.) following transfection with circular DNA usually resulted in reduced expression levels
- 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_I-3** vector. We recommend to preserve the sequence immediately in front of the translation start codon as close as possible. The triplet preceding the ATG seems to be important for the expression level of the target protein (Ref. 6). GCC and ACC were found favourable with an EGFP reporter gene in the LEXSY host and are members of the *L. major* consensus pre-ATG triplet. High expression was found also with AAT, ATG, TAA, TGA and TGT but the start and stop codons ATG, TAA and TGA are very rare at this position in the *Leishmania* genome. GTT, GGA, TCG and TCT yielded no product in LEXSY with the same reporter construct. However, there is no evidence, that these considerations are valid for other gene as well
- If you use the **SaI** 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. Therefore, we recommend to include the nucleotide sequence GTC GAC GCT GGC GCC into the 5' primer for amplification. However, the signal peptide cleavage site may vary dependent on the sequence of the fused ORF and we suggest to calculate the cleavage site *in silico* for optimal primer design (<http://www.cbs.dtu.dk/services/SignalP>)
- Amplify the target gene by standard PCR techniques with a high fidelity polymerase (**JBS Cat.-No. PCR-234**), gel-purify the fragment (**JBS Cat.-No. PP-202**) and trim the ends with the appropriate restriction enzymes. Optionally, you may subclone the PCR fragment and generate the gene cassette by restriction of the resulting plasmid. This has the advantage that cleavage by both enzymes can be monitored and sequence confirmation may be performed prior to insertion into the expression vector. Prepare the target gene fragment for ligation
- If you consider gene synthesis, apply the codon usage 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

signal peptide

```

BglIII  NcoI    SmaI                                     Sall      NarI      XbaI
AGA TCT GCC ATG GCC TCG AGG CTC GTC CGT GTG CTG GCC GCC GCC ATG CTG GTT GCA GCG GCC GTG TCG GTC GAC GCT GGC GCC TCT CTA GAC nnnnn
TCT AGA CGC TAC CCG AGC TCC GAG CAG GCA CAC GAC CCG CCG CCG TAC GAC CAA CGT CCG CCG CAC AGC CAG CTG CGA CCG CGG AGA GAT CTG nnnnn
      Met Ala Ser Arg Leu Val Arg Val Leu Ala Ala Ala Met Leu Val Ala Ala Ala Val Ser Val Asp Ala Gly Ala Ser Leu Asp
      ▲ SPCS

NheI    MspCI   KpnI                                     NotI
GCT AGC CTT AAG GGT ACC CAC CAC CAT CAC CAC CAC TAG GCGGCCGC
CGA TCG GAA TTC CCA TGG GTG GTG GTA GTG GTG ATC CGCCGGCG
      Ala Ser Leu Lys Gly Thr His His His His His His ...
  
```

stuffer

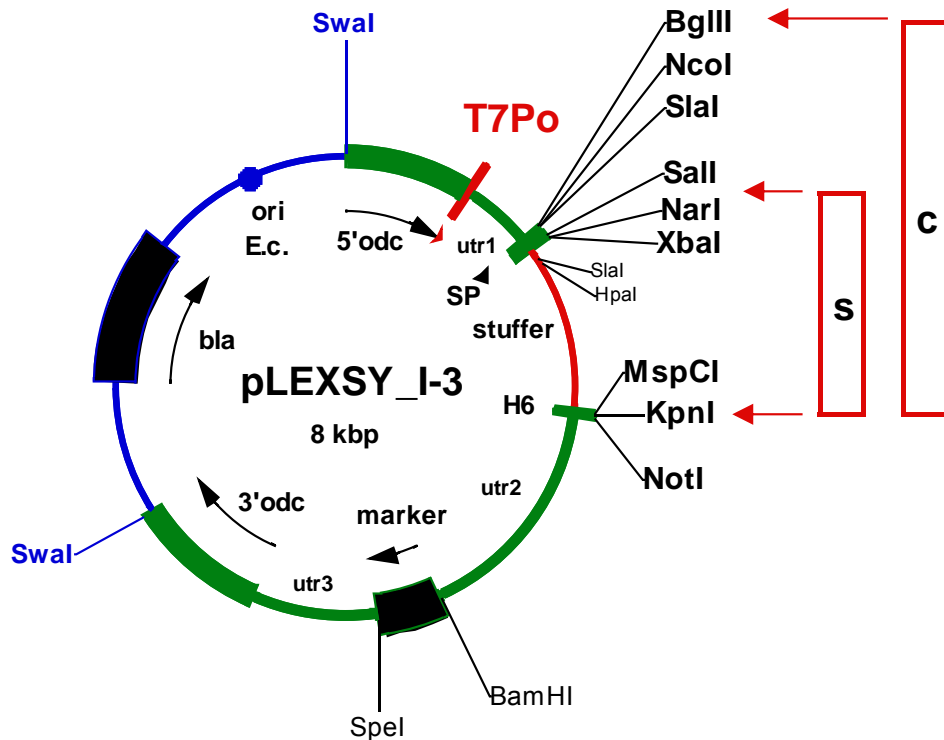


Fig. 5: Map of the **pLEXSY_I-3** vector family with cloning sites for the target genes replacing the 1 kb stuffer fragment. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *SwaI*. Utr1 derived from 0.4k-IR of *L. 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 (7), 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* (aminoglycoside phosphotransferase) genes. Please note, the *NarI* (*EheI*, *KasI*) site indicated in the sequence is unique only in EGE 244 and the *NheI* site is not unique in any of the vectors.

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 (Fig. 5). 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 larger 7 - 8 kbp fragment and prepare it for ligation
- Ligate the vector and the target gene fragment with **T4 DNA ligase (JBS Cat.-No. EN-149)** using standard ligation methods, and transform competent *E. coli* cells which tolerate *Leishmania* sequences as e.g. XL-10, XL-1, Stbl2, Stbl4, SURE etc. with the ligation mix. Include controls for vector linearization, ligation and transformation. For discrimination against the original expression vector you may treat the ligation mix with *HpaI* if your target gene does not contain this site
- Select recombinant *E. coli* clones with ampicillin and screen for the presence of the insert in the plasmids. Directional cloning into **pLEXSY_I-3 vectors** normally yields high rates of positive clones. Insert screening may be performed by 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 LEXSY 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 at nt. 1087-1106 5' of the insert (78-98 bp in front of ATG of **NcoI**). Reverse primer **A264** anneals at nt. 2359-2384 of the vector 3' of the insert (74-100 bp after the stop codon in front of **NotI** restriction site). The primer sequences are shown in Appendix 8.2. of this manual, the DNA sequences of the vectors including description can be downloaded from http://www.jenabioscience.com/cms/en/1/catalog/1116_expression_vectors.html.

4.3. Preparation of the expression plasmid for LEXSY host transfection

- Digest to completion with **SwaI** 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 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 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.

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 (ref. to **chapt. 3.2.**). Do not use the first inoculation culture from a glycerol stock immediately for cultivation for transfection, but passage the culture several times before electroporation as described above. However, for transfection use a culture which has been passaged this way for less than three months (ref. to **chapt. 3.2.**). For maintaining T7 polymerase and TET repressor genes add **LEXSY NTC** and **LEXSY Hygro** from 1000x stocks provided in the kit (final concentration 100 µg/ml each).

- On Friday inoculate *L. tarentolae* pre-culture 1:20 in 10 ml **LEXSY BHI*** medium (**Cat.-No. ML-411**) and incubate in tissue culture (TC) flask upright @ 26°C dark until Monday
- On Monday dilute pre-culture 1:10 in 10 ml **LEXSY BHI*** medium and incubate in TC flask flat @ 26°C o/n
- On Tuesday check cell density of the culture until ca. 6×10^7 cells/ml are reached (OD 1.4)** and ensure by microscopy that the cells are vital and of droplike shape (Fig. 2)
- Spin cells 3 min, 2000g at room temperature and remove ½ volume of supernatant
- Resuspend pellet in remaining medium to get **10⁸ cells/ml** and put on wet ice for 10 min
- Have ready on wet ice in parallel tubes with 0.1 - 5 µg transforming DNA in max. 50 µl water or Tris buffer pH 8.0 and electroporation cuvettes d=2 mm***
- Add 350 µl pre-chilled cells to the tube with DNA and transfer to the electroporation cuvette on wet ice
- Electroporate @ **450V, 450µF** and monitor pulse time (ca. 5-6 msec)****
- Put cuvette back on ice for exactly 10 min
- Transfer electroporated cells with capillary to 10 ml **LEXSY BHI*** in a ventilated TC flask
- Incubate o/n @ 26°C as static suspension culture (ca. 20h, OD 0.3-0.4)
- Proceed with clonal or polyclonal selection (**chapt. 5.1 and 5.2.**)

* contains Hemin, LEXSY NTC, LEXSY Hygro and PenStrep

** if the cell density differs from this value, concentrate cells in the next step in such a way to get **10⁸ cells/ml**. For transfection cultures between OD 1.0-1.8 can be used. Avoid to transfect cells if they are long and thin

*** 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 0.4 ml 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** (Fig. 12 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/1173_lexsy_methods.html.

The protocol for electroporation of LEXSY with a **Nucleofector** is described in Ref. 9.

5. Selection of transgenic LEXSY T7-TR strains

For establishing expression strains we routinely use the two methods described below in parallel. We repeatedly found similar expression levels when comparing cultures derived from clonal (5.1.) or polyclonal (5.2.) selections following transfection with linearized expression cassettes designed for chromosomal integration. However, transfection of circular expression plasmids requires clonal selection, since the episomes tend to amplify and to eventually integrate into the genome in a heterogeneous manner. Polyclonal selection in suspension cultures following transfection with circular DNA usually resulted in reduced expression levels.

5.1. Clonal selection by plating on solid media

Plating of cells after transfection allows selection of genetically defined recombinant clones. 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)**. Optionally, you may place a sheet of nitrocellulose on top of the solidified and dried for 10 minutes agar (see below).

- Withdraw 1 - 4 batches of 2 ml from the transfected 10 ml o/n culture (**chapt. 4.4.**) The remaining culture may be used in parallel for polyclonal selection as described in **chapt. 5.2.**
- Pellet cells for 3 min at 2000g and room temperature
- Transfer the supernatant back to the culture in the TC flask and resuspend the cells in approx. 50 µl of residual medium
- Carefully spread the resuspended cells onto freshly prepared **LEXSY BHI** agar supplemented with the antibiotics **LEXSY NTC**, **LEXSY Hygro** and the resp. selective antibiotic (**LEXSY Bleo** for **pLEXSY_I-ble3** and **pLEXSY_I-blecherry3** or **LEXSY Neo** for **pLEXSY_I-neo3**)
- Optionally, you may streak the cells onto nitrocellulose filters (BA85, 0.45 µm, blotting grade) placed on the surface of the agar. Plating is easier on these membranes than directly on the 1% agar, and swarming of colonies, as evtl. observed on soft agar, is diminished. Moreover, plating on membranes allows colony lifts for testing induction profiles of clonal populations e.g. by fluorescence scanning or specific detection methods for the given target protein*. You may also consider to test both plating techniques for your application
- Seal plates with parafilm and incubate bottom up at 26°C
- 5 – 7 days after plating small, defined colonies begin to appear on a slight background lawn.
- After these colonies have grown up to 1 – 2 mm diameter (approx. 7-9 days after plating), they can be transferred to 0.2 ml of selective growth medium in a 96-well plate using a pipette tip
- After 24 hours incubation at 26°C these clones must be expanded into 1 ml selective medium in a 24-well plate. If the colonies are grown for a longer period on the agar plates (they survive on agar plates for ca. 20 days post electroporation and can reach 5 mm diameter), they may be expanded directly into 1 ml selective medium in 24 well plates bypassing the 96 well format. For speeding up growth, the 24 well plate may be agitated on a microplate shaker located in the 26°C incubator
- After another ca. 48 hour incubation at 26°C the cultures are expanded into 10 ml selective medium in TC flasks and can be used for evaluation.

* In case of **pLEXSY_I-blecherry3** clones colony lift technique can be applied for selection supported by monitoring induction through development of a cherry color of the colonies in daylight. To this end fresh **LEXSY BHI agar** plates are prepared containing the inducer Tetracycline in addition to the selection antibiotics if the colonies arrive at ca. 1-2 mm diameter. Following transfer of the membrane with the colonies the plates are incubated for additional 1-3 days. The development of a cherry color of the colonies is based on co-induction of the *blecherry* gene and indicates that the inducible system is intact. In case there are evtl. singular pale colonies those are excluded from further expansion.

5.2. Polyclonal selection in suspension culture

- As soon as the 10 ml o/n cultures obtained from the transfection experiments (see 4.4.) start to get slightly turbid (OD₆₀₀ 0.4; ca. 10⁷ cells/ml; usually approx. 20 h after electroporation), add the appropriate selective **LEXSY antibiotic(s)** (see 3.2.) to the recommended final concentration(s) from the steril-filtered stocks provided in the kit 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 antibiotic(s) 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 antibiotic(s) 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.

5.3. Confirmation of genomic integration by diagnostic PCR

Integration of the expression cassette into the *odc* locus 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 expression cassette and one primer hybridizing to a *odc* 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.
<i>5'odc - utr1 (aprt)</i>	A1304	A1715	1.1 kbp	60°C
<i>blecherry - 3'odc</i>	A708	P1510	2.7 kbp	60°C
<i>ble - 3'odc</i>	A708	P1510	2.0 kbp	60°C
<i>neo - 3'odc</i>	A1432	P1510	1.7 kbp	60°C

The PCR reactions will result in a characteristic fragment for each vector (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 cultivation / induction conditions and time of harvest for each individual protein.

- Inoculate 10 - 100 ml **LEXSY BHI** supplemented with **Hemin + Pen-Strep** and the respective **LEXSY antibiotics** 1:10 with the expression clone and grow at 26°C as static suspension culture in TC flasks (flat) or as agitated culture in Erlenmeyer flasks (approx. 140 rpm). Avoid higher agitation to prevent shearing of cells, which might reduce yields. For agitation of larger volume use Fernbach flasks (500-700 ml) or fermenters (>1 Liter)
- 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 LEXSY host) 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 the Tetracycline concentration from the titration curve in Appendix 8.6.
- Take aliquots at different time points of cultivation (e.g. OD 1 - 4) 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). This is particularly 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 $V \text{ [ml]} = 2/OD$, 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 gel loading buffer and apply 20 µl per lane on SDS-PAGE
- For **analysis of secretory protein expression** concentrate culture supernatants 50 - 200x with trichloroacetic acid (TCA) as follows:
 - Sediment cells from 5 – 50 ml culture 10 min @ 3000g
 - Add 4 – 40 ml of (steril-filtered) supernatants to 1 – 10 ml 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, then spin 15 min 15.000g at 4°C
 - Remove supernatants completely, resuspend pellet in ca. 1-2 ml 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 20 - 200 µl gel loading buffer (corr. 50 – 200x concentration).
 - Apply 10 - 20 µl sample/slot for SDS-PAGE and Western blotting.

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

7. LITERATURE

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Please, visit <http://www.jenabioscience.com>

8. APPENDIX

8.1. Maps of the pLEXSY_I-3 expression vectors

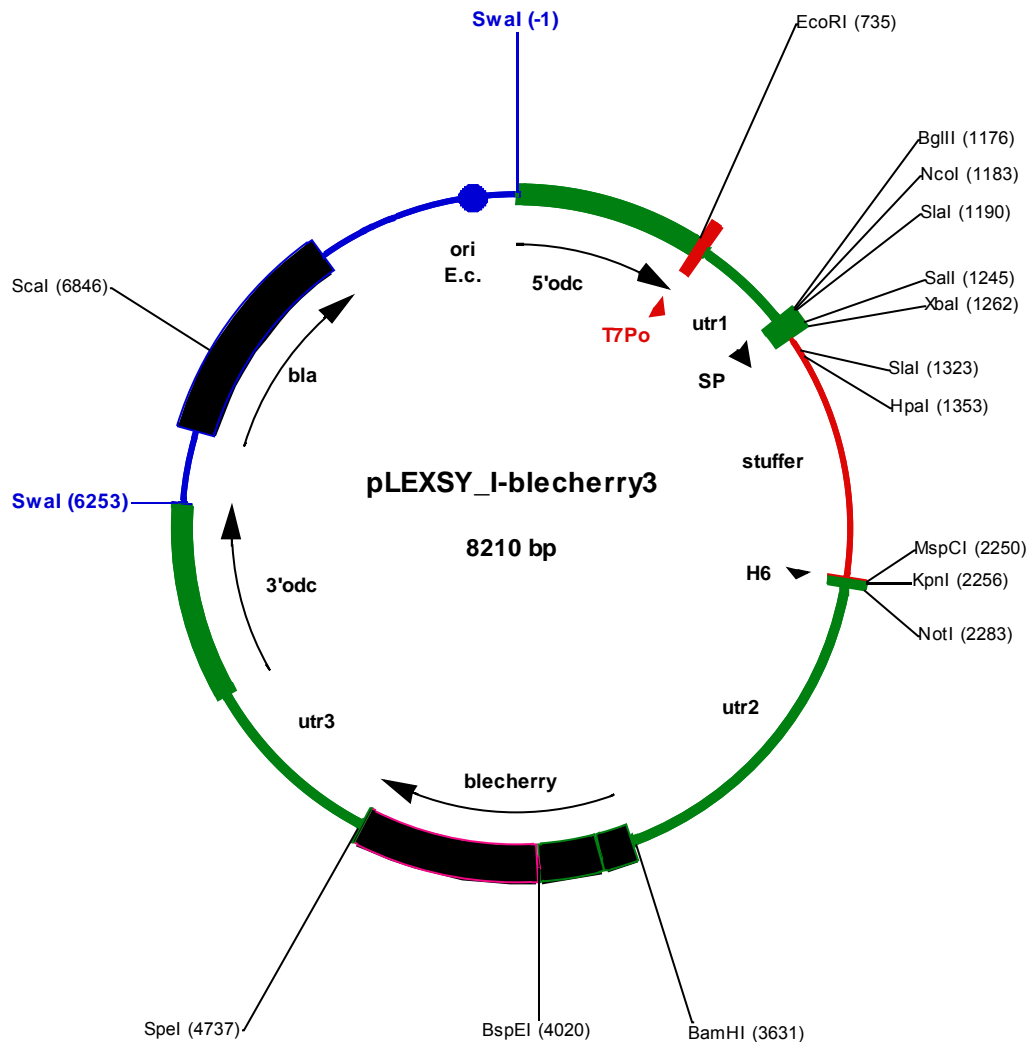


Fig. 6: Map of pLEXSY_I-blecherry3 expression vector (Cat.-No. EGE-243) with cloning sites for the target genes replacing the 1 kb stuffer fragment. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with Swal. Utr1 derived 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 (7) 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. 5.

The DNA sequence of the expression vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/cms/en/1/catalog/1116_expression_vectors.html.

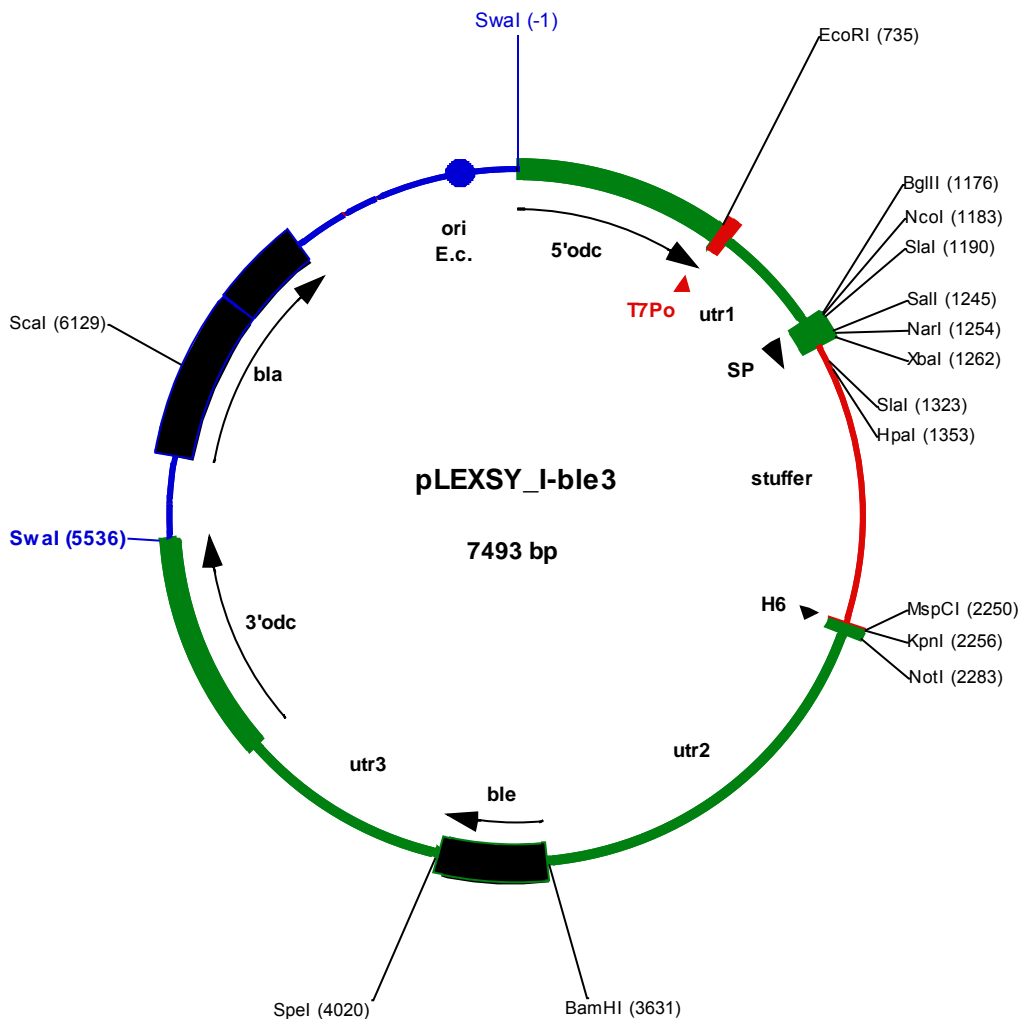


Fig. 7: Map of **pLEXSY_I-ble3** expression vector (**Cat.-No. EGE-244**) with cloning sites for the target genes replacing the 1 kb stuffer fragment. In this plasmid the *NarI* (*EheI*, *KasI*) site can be used for in frame fusion to the secretory signal peptide. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swal*. 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 (7) 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. 5.

The DNA sequence of the expression vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/cms/en/1/catalog/1116_expression_vectors.html.

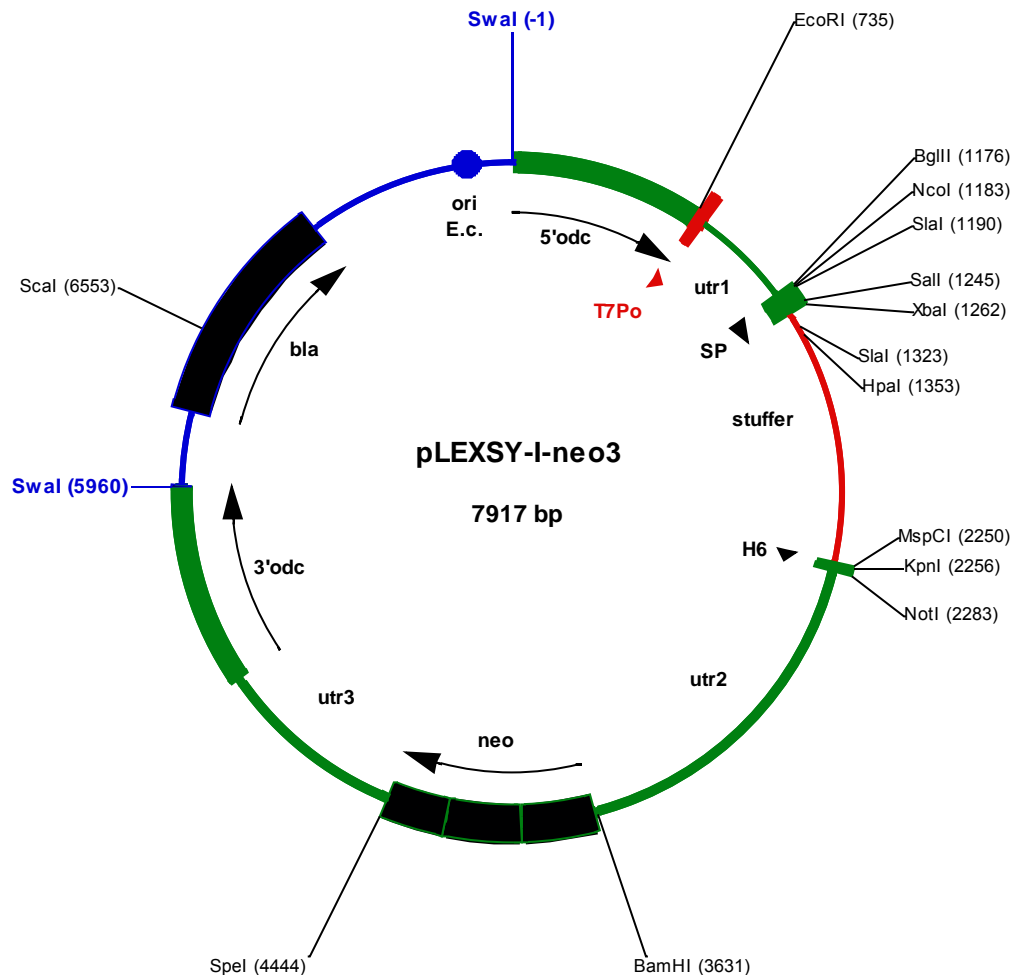


Fig. 8: Map of **pLEXSY_I-neo3** expression vector (**Cat.-No. EGE-245**) with cloning sites for the target genes replacing the 1 kb stuffer fragment. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swal*. Utr1 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 (7) 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 not the antibiotic Neomycin which must not be used for selection in LEXSY. The sequence of the multiple cloning sites is indicated in Fig. 5.

The DNA sequence of the expression vector can be downloaded from the website of Jena Bioscience at http://www.jenabioscience.com/cms/en/1/catalog/1116_expression_vectors.html.

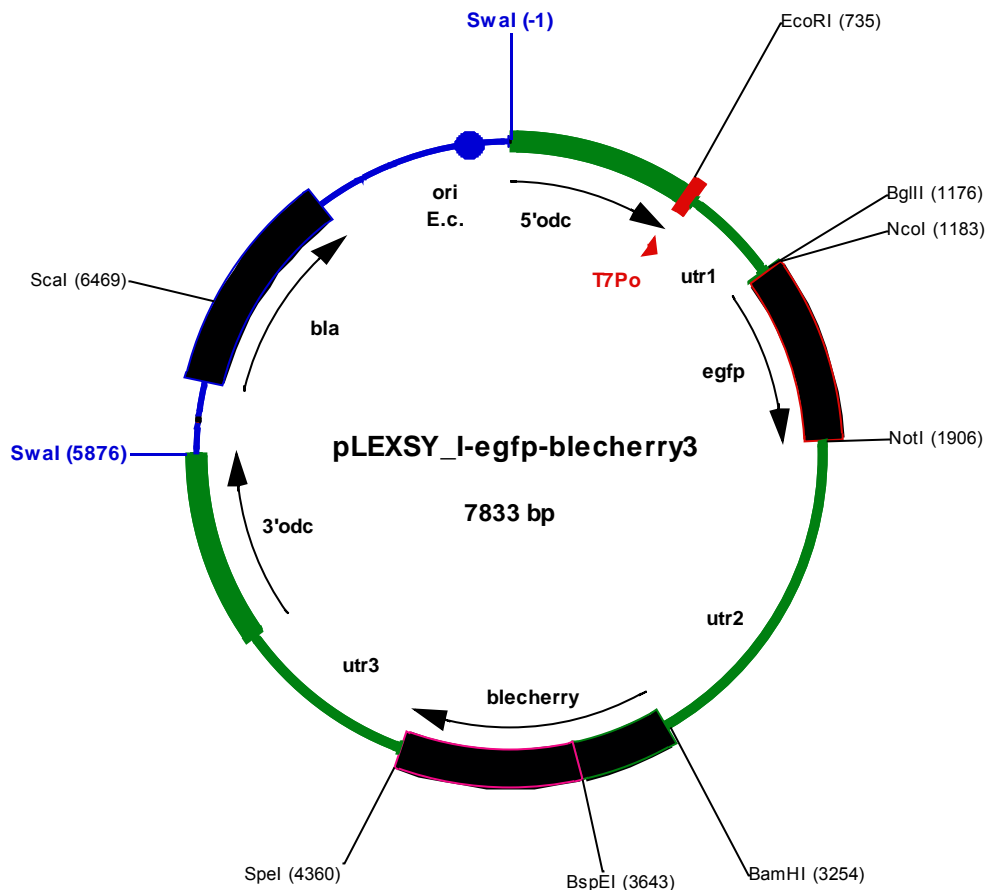


Fig. 9: Map of **pLEXSY_I-egfp-blecherry3** control plasmid (Cat.-No. EGE-246) containing the *egfp* gene which had replaced the 1 kb stuffer fragment of **pLEXSY_I-blecherry3** expression vector. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *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**. *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/cms/en/1/catalog/1116_expression_vectors.html.

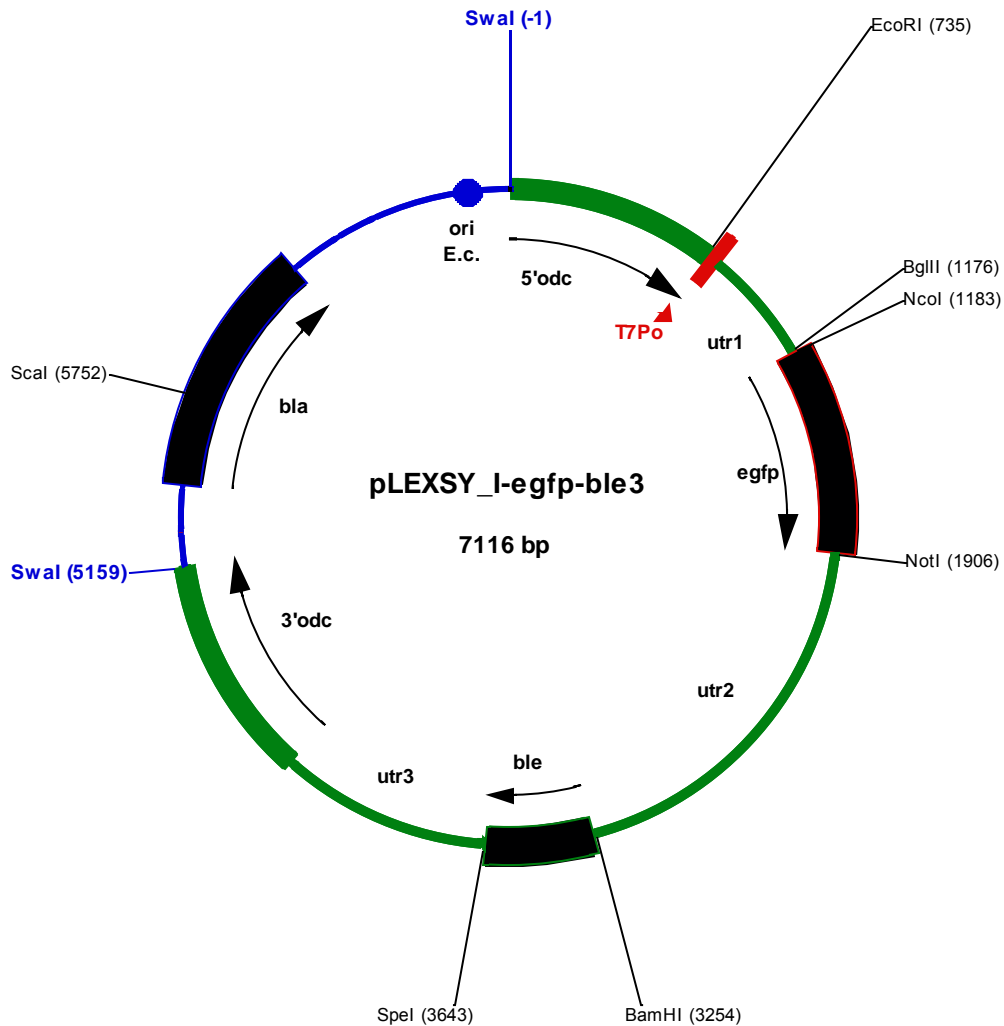


Fig. 10: Map of **pLEXSY_I-egfp-ble3** control plasmid (**Cat.-No. EGE-247**) containing the *egfp* gene which had replaced the 1 kb stuffer fragment of **pLEXSY_I-ble3** expression vector. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swal*. 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/cms/en/1/catalog/1116_expression_vectors.html.

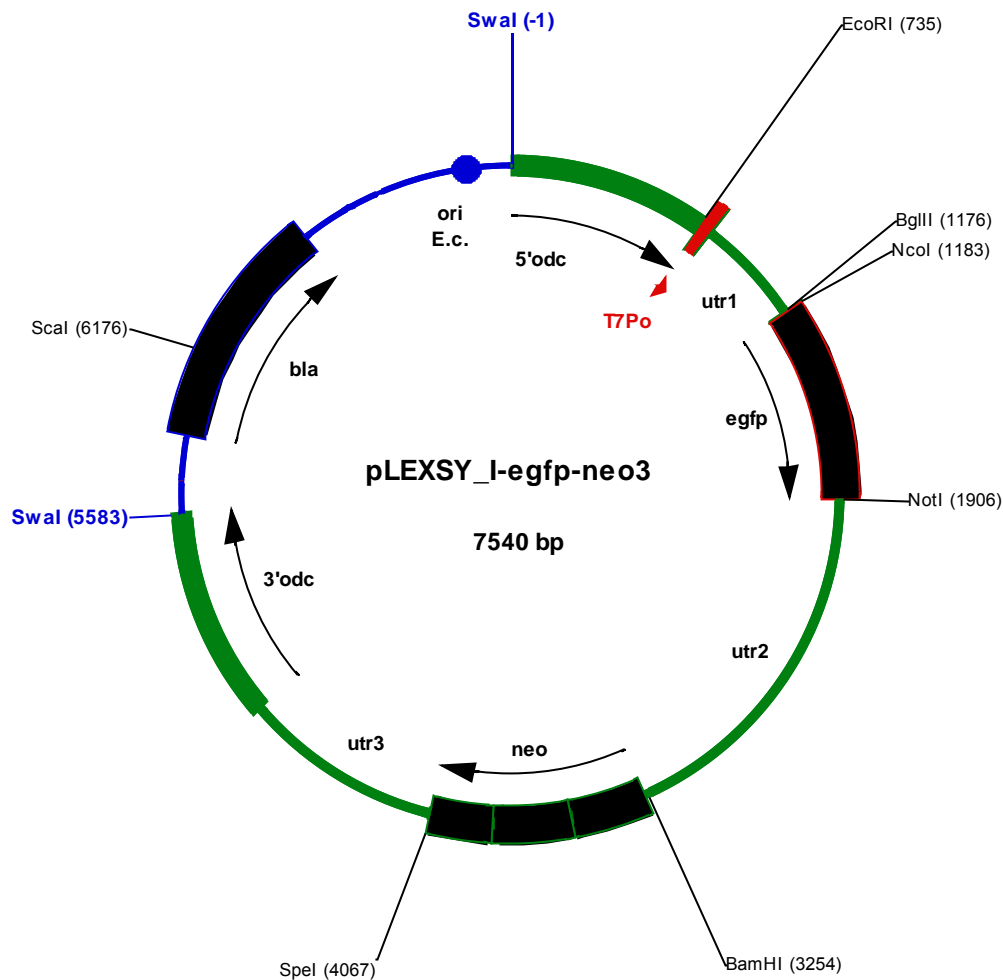


Fig. 11: Map of **pLEXSY_I-egfp-neo3** control plasmid (**Cat.-No. EGE-248**) containing the *egfp* gene which had replaced the 1 kb stuffer fragment of **pLEXSY_I-neo3** expression vector. 5'odc and 3'odc are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swal*. 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 not 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/cms/en/1/catalog/1116_expression_vectors.html.

8.2. Sequences of the primers available for LEXSinduce3 kits

Insert sequencing forward primer P1442	all expression vectors with 5'utr <i>aprt</i>	5'-CCGACTGCAACAAGGTGTAG-3'	PM-110
Insert sequencing reverse primer A264	all LEXSY expression vectors	5'-CATCTATAGAGAAGTACACGTAAAAG-3'	PM-101
<i>odc</i> forward primer A1304	integration diagnostics of all <i>odc</i> expression vectors	5' -TCCGCCATTCATGGCTGGTG-3'	PM-114
<i>aprt</i> reverse primer A1715	integration diagnostics of all expression vectors with 5'utr <i>aprt</i>	5'-TATTCGTTGTCAGATGGCGCAC-3'	PM-111
<i>ble</i> forward primer A708	integration diagnostics of all <i>ble</i> & <i>blecherry</i> expression vectors	5'-GGATCCACCGCATGGCCAAGTTGACCAGTG-3'	PM-107
<i>neo</i> forward primer A1432	integration diagnostics of all <i>neo</i> expression vectors	5'-GCATGGCGATGCCTGCTTGC-3'	PM-108
<i>odc</i> reverse primer P1510	integration diagnostics of all <i>odc</i> 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 **A1432**
- *odc* reverse primer **P1510**

8.3. Preparation of LEXSY BHI agar plates for clonal selection

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

<i>Component</i>	<i>Storage</i>	<i>Amount</i>
2x LEXSY BHI (74 g/L)	room temperature	35 ml
inactivated Fetal Calf Serum (FCS)*	-20°C	10 ml
1M HEPES, pH 7.4	4°C	4 ml
Pen-Strep	-20°C	1 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 drying.

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

8.4. Alternative electroporation protocols

High-Voltage protocol for transfection of LEXSY (Ref. 4)

- On Friday inoculate *L. tarentolae* pre-culture 1:20 in 10 ml **LEXSY BHI*** medium (**Cat.-No. ML-411**) and incubate in tissue culture (TC) flask upright @ 26°C dark until Monday
- On Monday dilute pre-culture 1:10 in 10 ml **LEXSY BHI*** medium and incubate in TC flask flat @ 26°C o/n
- On Tuesday check cell density of the culture until ca. 6×10^7 cells/ml are reached (OD 1.4)** and ensure by microscopy that the cells are vital and of droplike shape (Fig. 2)
- Spin cells 3 min, 2000g at room temperature and remove $\frac{1}{2}$ 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 0.1-5 μ g transforming DNA in max. 50 μ l water or Tris buffer pH 8.0 and electroporation cuvettes d=4 mm***
- Add 450 μ l 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 μ F with 10 sec between pulses (pulse time ca. 0.3 msec)****
- Put cuvette back on ice for exactly 10 min
- Transfer electroporated cells with capillary to 10 ml **LEXSY BHI*** in a ventilated TC flask
- Incubate o/n at 26°C as static suspension culture (ca. 20h, OD 0.3-0.4)

* contains Hemin, LEXSY NTC, LEXSY Hygro and PenStrep

** if cell density differs from this value, concentrate cells in the next step in such a way to get **10^8 cells/ml**. For transfection cultures between OD 1.0-1.8 can be used

*** 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 0.5 ml outside of the linear electric field

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

The standard Low voltage electroporation protocol is described in section 4.4.

Low voltage protocol (BHI)		High voltage protocol (BHI)
<u>Exponentl. decay</u>	<u>Time constant</u>	
d=2mm V=0.4 ml	d=2mm V=0.4 ml	d=4mm V=0.5 ml
set: 450V 450 μ F 1 pulse	set: 450V T=3.5ms 1 pulse	set: 1500V 25 μ F 2 pulses @ 10" interval
get: 5 - 6 ms $\approx 20 \Omega$ ≈ 450 V	get: ≈ 3.5 ms $\approx 20 \Omega$ ≈ 450 V	get: ≈ 0.3 ms

Fig. 12: Overview of parameters of electroporation protocols with BioRad GENE PULSER

8.5. How to grow a *Leishmania* culture

L. tarentolae needs **aerobic** conditions for development. The strains can be maintained as continuous suspension culture with regular dilutions (ref. to **chapt. 3.2.**). All cultivations are performed **at 26°C**. Higher temperatures lower the growth-rates and vitality significantly and *L. tarentolae* will not survive at 37°C.

All growth media should be supplemented with **Hemin (Cat.-No ML-411)** which is essential for *Leishmania*. **Hemin** is light-sensitive, so *Leishmania* must be cultivated 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 life 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, lowering aeration for longer intervals between passages. Don't cultivate *Leishmania* much longer than for 7 days in the same medium without dilution. For cultivation for transfection ref. to **chapt. 4.4.**, for cultivation for protein expression ref. to **chapt. 5.4.**)

Always control appearance and motility of cells by microscopy. Cells of mid-growth phase cultures are of drop-like shape, approx. 15 x 5 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 - 5 h in agitated cultures. They need time to grow or to adapt to new conditions and sometimes they seem to be a bit inactive. Continuous inoculations into fresh medium, regular resuspension of sedimented cells in static suspension cultures in ventilated TC flasks, a dark place and some rest - and they will recover faster than you think.

If you - despite following these instructions - eventually 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.

Don't centrifuge *Leishmania* cultures at high speed > 3000g and don't resuspend cell pellets by rigorous vortexing. The cells are sensitive to these procedures and may lyse. Centrifugation at 2000g is sufficient for sedimentation and makes careful and quick resuspension of cell pellets easier. If required, prolong centrifugation time at 2000g rather than using higher speed.

If you cultivate LEXSY strains in bioreactors be careful with stirring. We found it sufficient to aerate the culture in a 10 L fermentation without stirring for obtaining high cell densities up to 10⁹ cells/ml. If you intend to use a stirrer, avoid high shearing forces.

If you encounter problems with cultivation or for further questions, please, don't hesitate to contact the Jena Bioscience LEXSY team at expression@jenabioscience.com.

8.6. Titration of Tetracycline concentration for modulation of expression level

Routinely, the transgenic T7-TR strains are induced for target protein expression with **10 µg/ml** Tetracycline (final concentration) from the 1000x stock provided in the kit (ref. to **chapt. 5.4.**) albeit nearly full induction is reached already with 1 µg/ml Tetracycline (Fig. 13). 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.

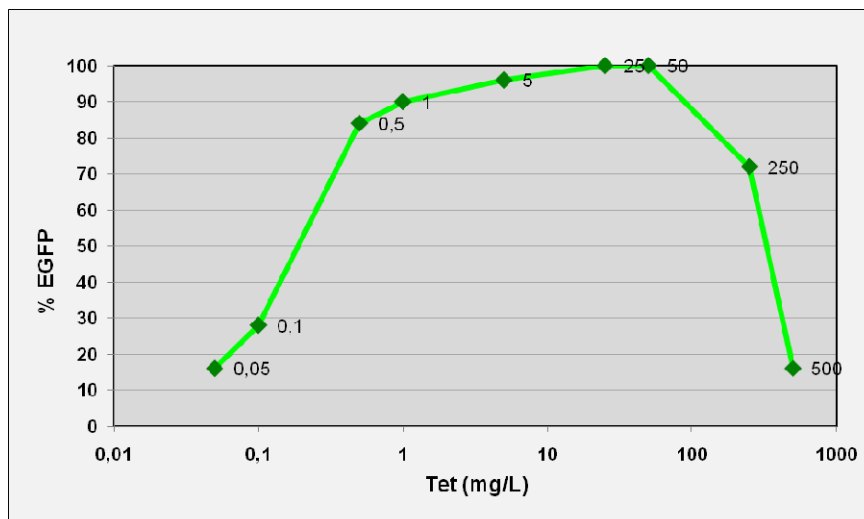


Fig. 13: Dependence of EGFP reporter expression on concentration of Tetracycline in inducible LEXSY. LEXSInduce provides a broad induction plateau from 1 – 50 µg/ml of inducer Tetracycline. Expression rates were estimated 24 h post induction by FACS.

8.7. Correlation of optical density and cell concentration

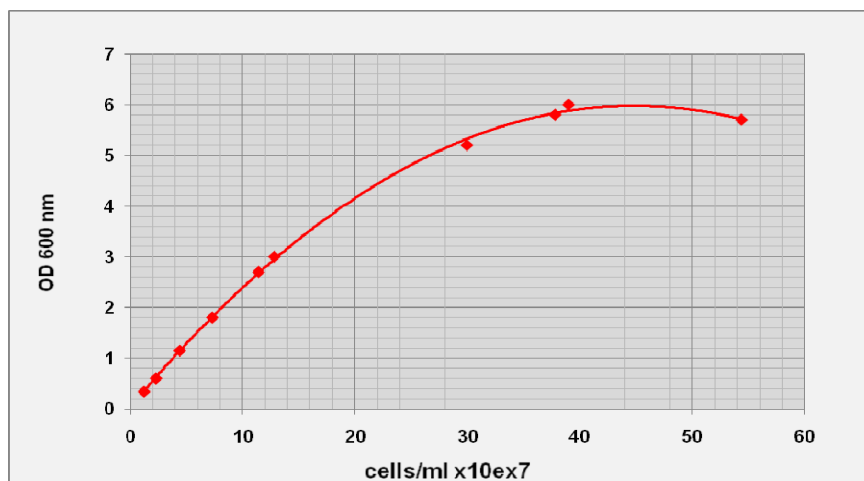


Fig. 14: Calibration of OD readings of spectrophotometer (biowave CO8000) with cell densities determined with a particle counter (Coulter). LEXSY cultures were grown to different stages and OD readings and cell counts were determined from the same sample. The non-linear behaviour at higher OD is due to changes in cell shapes and cell lysis in late stationary cultures.