Stratech

Jena Bioscience

Building Blocks of Life

LEXSY – Eukaryotic Protein Expression

- Fast growth, high yield, easy handling
- Eukaryotic post-translational modifications
- *In vitro* system available

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ACCCACGAAAGGGAA ATAAGC AACO TTCAGGGAAGAA CTAUAACTGCCAC ACCCACGAAAGGGAA ATAAGC AACO TTCAGGGAAGAA CTAUAACTGCCAC

LEXSY Expression

LEXSY features:

- Correct protein folding – no inclusion bodies
- High growth rate: 6 – 8 h generation time
- Full range of post-translational modifications
- High expression success rates with yields of up to 500 mg per litre of culture

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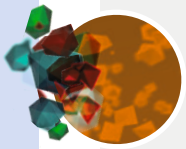
Building Blocks of Life

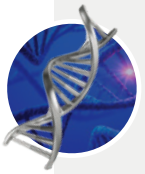
LEXSY Expression

In the field of recombinant protein production, Jena Bioscience has developed its proprietary LEXSY (Leishmania Expression System) technology. It is based on an S1-classified unicellular organism that combines easy handling with a eukaryotic protein folding and modification machinery. Besides everything you need to establish LEXSY in your lab we also offer custom expression of recombinant proteins.



Crystallography & Cryo-EM

For the crystallization of biological macro-molecules – which is often the bottleneck in determining the 3D-structure of proteins – we offer specialized reagents for protein stabilization, crystal screening, crystal optimization, and phasing that can reduce the time necessary to obtain a high resolution protein structure from several years to a few days.

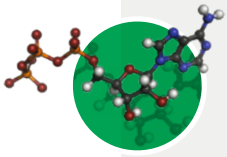


Molecular Biology & Proteins

For applications in the field of Molecular Biology we offer a large selection of single reagents, complete kits and optimized master mixes. This section includes products for DNA or RNA purification, amplification and modification with focus on PCR-related techniques.

Click Chemistry, Probes & Epigenetics

Our Probes & Epigenetics as well as Click Chemistry sections offer innovative reagents for the functionalization, conjugation and labeling (fluorophores, haptens) of (bio) molecules complemented by epigenetic modification analysis tools.



Nucleotides & Nucleosides

In our chemistry division, we have hundreds of natural and modified nucleotides in stock. In addition, with our premade building blocks and in-house expertise we manufacture even the most exotic nucleotide analog from mg to kg scale.

RNA Technologies

Ribonucleic acids (RNAs) are essential for transfer of genetic information and cell regulation. Our RNA Technologies division offers a toolbox for synthesis, labeling, modification, analysis and detection of protein-coding messenger RNAs (mRNAs) as well as non-coding RNAs (ncRNAs) complemented by custom specific services.

Jena Bioscience

Building Blocks of Life

Established in 1998 by a team of scientists from the Max-Planck-Institute of Molecular Physiology (Dortmund), Jena Bioscience utilizes more than 25 years of academic know-how to develop innovative reagents for clients from both research and industry in 100+ countries. To date, Jena Bioscience still remains an owner-operated business.

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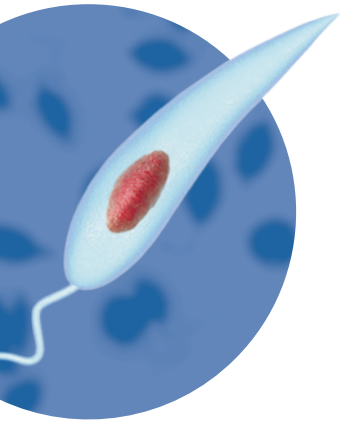


Introduction

The Leishmania expression system LEXSY is the proprietary eukaryotic protein expression platform developed by Jena Bioscience. LEXSY is based on the protozoan host *Leishmania tarentolae* and was designed to combine eukaryotic protein synthesis, folding and modification with prokaryotic growth rates, simplicity and ease of handling.

Leishmania tarentolae is a robust, unicellular, flagellated eukaryotic organism circa 5 x 15 µm in size (Figure 1). It was isolated from lizards *Tarentolae annularis* and *Tarentolae mauritanica* and has been cultivated in axenic culture over decades. It is not pathogenic to mammals and is fully approved for use in biosafety level 1 laboratories (S1).

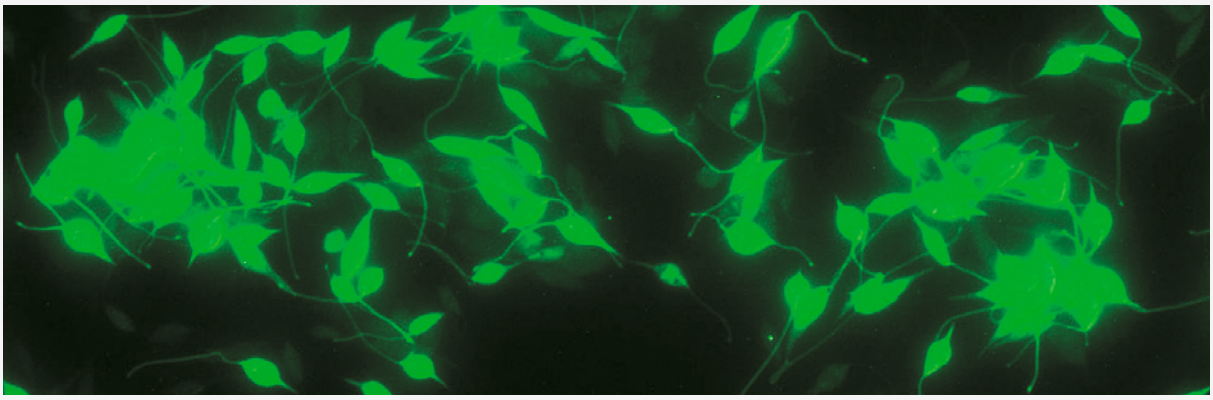


Figure 1

Leishmania tarentolae cells expressing green fluorescent protein

Overcoming limitations of other expression systems

Prokaryotic expression systems such as *E. coli* lack essential components for protein folding and modification and are therefore in many cases not suitable for production of functional proteins of higher organisms. Alternative eukaryotic expression systems based on e.g. mammalian or insect cells, however, have high generation times, require long development cycles and deliver low protein yields resulting in costs that are magnitudes above those of *E. coli*-produced proteins.

LEXSY was developed in order to overcome these limitations and combine the advantages of eukaryotic and prokaryotic expression systems: eukaryotic protein synthesis and folding/modification machinery and low generation times and ease of handling.

LEXSY features:

- **Correct protein folding – no inclusion bodies**
- **High growth rates – 6–8 h generation time**
- **Full range of post-translational modifications, including: mammalian-type N-glycosylation, glypiation, phosphorylation, acetylation, prenylation, myristoylation, ADP-ribosylation, proteolytic processing and oligomerization**
- **High expression success rates with yields of up to 500 mg per litre of culture (Figure 2)**

Protein synthesis and folding/modification machinery

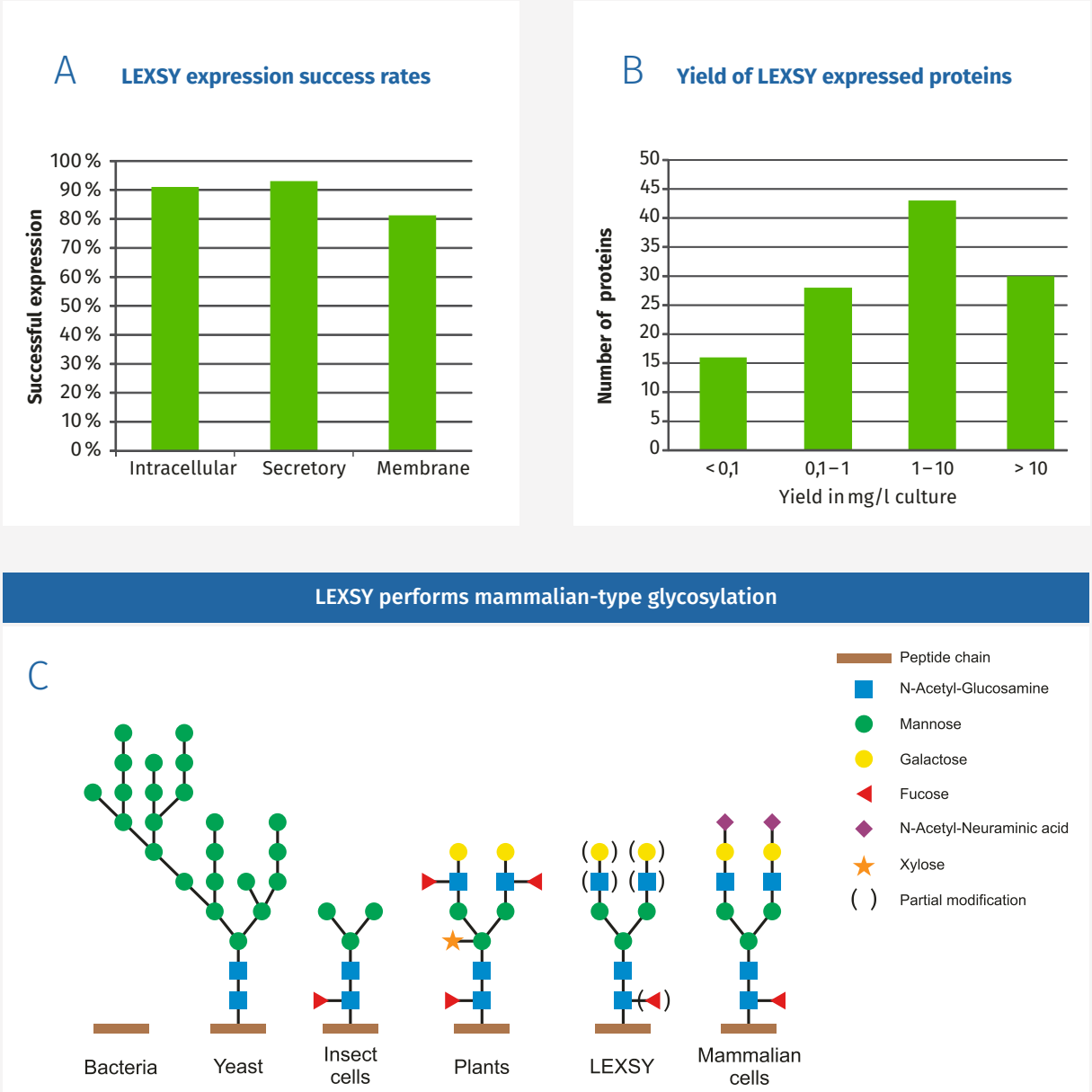


Figure 2

A: *Success rates of LEXSY protein expression: Over 100 proteins have been expressed in LEXSY to date. An overall success rate of more than 90 % makes LEXSY the expression system of choice for difficult to express proteins.*

B: *Yields of LEXSY expression: Over 60 % of all proteins expressed with LEXSY show yields of more than 1 mg/l of culture. Expression levels up to 500 mg/l have been achieved with some proteins.*

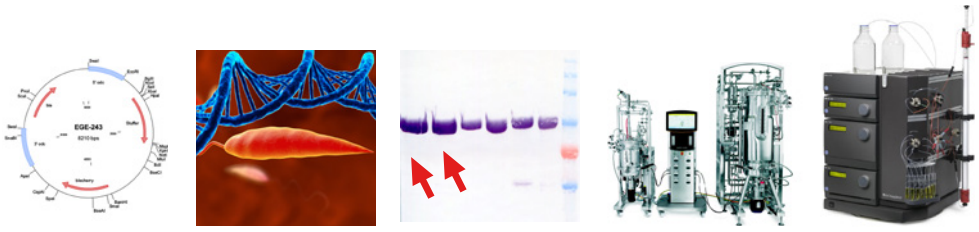
C: *Glycosylation in LEXSY was investigated with human erythropoietin (EPO), human interferon gamma (hu IFNγ),* Toxoplasma gondii *surface antigen SAG1 and host surface glycoprotein GP63. In all cases a biantennary, galactosylated, core-α-1,6-fucosylated N-glycan structure was found that is similar to mammalian-type glycosylation (Breitling* et al. *2002).*

LEXSY features

- Biosafety level 1 (S1, as *E. coli*)
- Easy plasmid generation in *E. coli* shuttle vectors
- High transfection efficiencies using established electroporation protocols
- Cultivation in inexpensive media at 26 °C – no cell culture equipment necessary
- Rapid growth of LEXSY expression strains to high cell densities (10^9 cells/ml)
- Easy harvest and downstream processing (Figure 3).

From gene to protein within six weeks

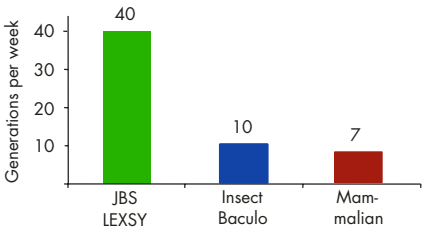
A



Expression plasmid construction	Transfection & selection	Expression evaluation	Scale-up	Protein purification
1 week	2 weeks	1 week	1 week	1 week

LEXSY grows faster than insect and mammalian cells

B



LEXSY grows to higher densities than insect and mammalian cells

C

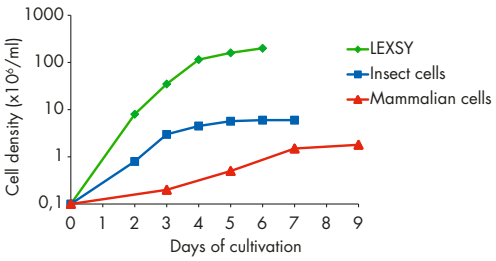


Figure 3

A: *The LEXSY technology enables short evaluation cycles. Target genes are inserted into LEXSY expression vectors and LEXSY host is transfected by electroporation. Recombinant clones are expanded for expression evaluation in small scale suspension cultures (typically 1-10 ml). Up-scaled cultivation is used for protein production and purification. The overall procedure requires typically six weeks from cloning to purified protein.*

B: *Due to fast growth of LEXSY strains in agitated suspension cultures up to 40 generations per week were achieved, whereas with insect or mammalian cells only 10 or 7 generations per week were obtained, respectively.*

C: *LEXSY strains grow to cell densities known from bacterial cultures. For comparison the different host cultures were inoculated at the same density of 10^5 cells/ml and growth was monitored by cell counting. Following routine inoculation at 10^6 cells/ml, agitated laboratory LEXSY cultures reach $3x10^8$ cells/ml within 48 h for optimal harvest of cells and protein purification (not shown). In high density fermentations up to 10^9 cells/ml were obtained for LEXSY cultures (Fritsche* et al. *2007).*

LEXSY Configurations

The LEXSY protein expression technology is available as live cell-based expression system (*In Vivo* LEXSY) and as cell-free system (*In Vitro* LEXSY) (Table 1).

***In Vivo* LEXSY** requires construction of an *L. tarentolae* expression strain, is suitable for cultivation and fermentation in inexpensive media and delivers high yields of recombinant proteins.

***In Vitro* LEXSY** allows protein production directly from a gene of interest (either as a PCR product or sub-cloned into an appropriate DNA vector), enabling fast production of a large number of proteins in parallel. It is not suitable for large-scale protein production.

Table 1

In Vivo versus In Vitro LEXSY

***In Vivo* LEXSY**	***In Vitro* LEXSY**
From gene to protein within approximately 6 weeks	From gene to protein within 2 days or less
Scalable, suitable for production of large amounts of recombinant protein by cultivation in inexpensive media	Small scale protein preparation only
Low costs	High costs

In Vivo LEXSY

In Vivo LEXSY is available in two principle configurations: constitutive or inducible.

The constitutive system is the basic architecture that permits efficient production of a large variety of proteins. It is based on integration of an expression cassette into the chromosomal ssu-locus encoding the tandem 18S rRNA genes (Figure 4). This cluster is transcribed by the endogenous RNA Polymerase I.

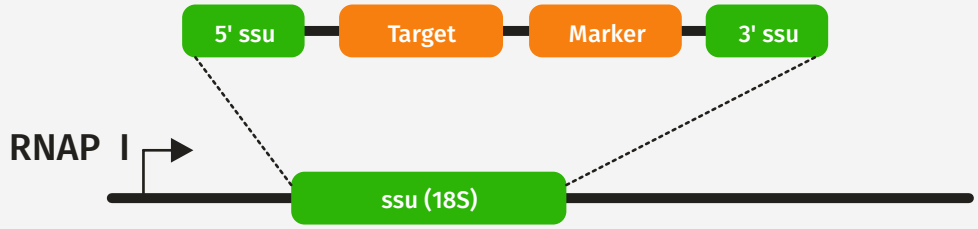


Figure 4

Architecture of constitutive LEXSY. Following transfection, the linearized expression cassette carrying the target gene is integrated into one copy of the 18S rRNA genes (ssu) by homologous recombination. For selection, four alternative antibiotic resistance markers are available.

The **inducible** system (Figure 5) enables tight control of protein expression analogous to the well-known bacterial T7 expression architecture. Expression is switched on by addition of an inducer (tetracycline) and thereby alleviates potential toxicity issues of an expressed protein. Further, it was shown for a number of intracellular proteins that inducible expression achieves 5–10-fold higher yields than constitutive expression.

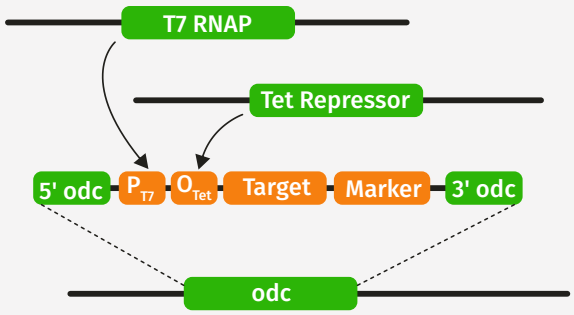


FIGURE 5

Architecture of inducible LEXSY. Engineered LEXSY host T7-TR expresses bacterial T7 RNA polymerase and TET repressor. The transfection target gene is expressed under control of a T7 promoter/TET operator assembly.

Both, the constitutive and the inducible configuration, permit **intracellular or secretory** expression of proteins from the same vector simply by choosing the way of cloning: Secretion is achieved by fusion of the mature part of the target gene to a *Leishmania* signal peptide encoding sequence present on the vector (Figure 6), and is recommended for proteins that undergo Post-Translational Modifications such as disulfide bond formation or glycosylation. LEXSY was shown to yield exceptionally homogeneous mammalian-type N-glycosylation patterns (Breitling *et al.* 2002, Figures 2 and 13).

The first step of the construction of recombinant LEXSY strains – cloning of the target gene into a LEXSY expression vector – is performed in *E. coli*. Cloning of the target gene into the multiple cloning site provides essential non-translated flanking regions already optimized for the LEXSY host. All pLEXSY expression vectors bear compatible cloning sites for insertion/shuffling of expression cassettes and – in addition – a C-terminal Hexa-Histidine tag for protein detection and affinity purification.

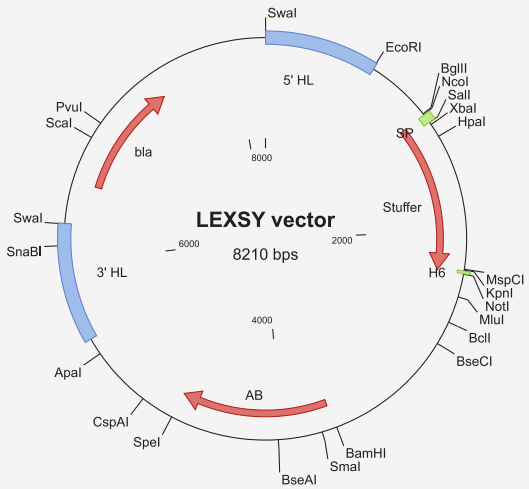


Figure 6

General map of a LEXSY expression vector. 5' HL and 3' HL are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with SwaI. SP designates the signal peptide of L. mexicana *secreted acid phosphatase LMSAP1 (Wiese* et al. *1995) and H6 the hexa-Histidine stretch. Alternative cloning strategies result in cytosolic or secretory expression of the target proteins. AB is the antibiotic resistance cassette for selection in* L. tarentolae*, up to 6 different antibiotics are available.*

In Vivo LEXSY Products

For getting started with *In Vivo* LEXSY, Expression Kits are available that contain all components for construction of expression strains and setup of expression evaluation (Figure 8).

Figure 8

Kit contents of an inducible ***LEXSY expression kit***.

The **constitutive LEXSY Expression Kits** are available with four alternative selection markers (LEXSY NTC, Neo, Hyg, or Bleo selection). The **inducible LEXSY Expression Kits** are available with three alternative selection marker genes (blecherry, ble, or neo) for selection with the antibiotics LEXSY Bleo, or Neo.

Cat.-No.	Amount
LEXSYcon2.1 Expression Kit	
EGE-1310ble	1 Kit
EGE-1310hyg	1 Kit
EGE-1310neo	1 Kit
EGE-1310sat	1 Kit
EGE-1310bsd	1 Kit
EGE-1310pac	1 Kit
LEXSinduce3 Expression Kit	
EGE-1410blecherry	1 Kit
EGE-1410ble	1 Kit
EGE-1410neo	1 Kit
LEXSinduce4 Expression Kit	
EGE-1420blecherry	1 Kit

Each Kit contains the expression vector of choice indicated in the product name. The inducible expression kits contain in addition a control vector with the *egfp* gene inserted into the expression site. For upgrading of the expression kits all LEXSY expression vectors are also available separately. The cloning sites of all pLEXSY vectors are compatible (Figure 6).

LEXSY expression vectors

Product	Cat.-No.	Amount
pLEXSY-ble2.1 integrative constitutive expression vector antibiotic selection of transfectants with LEXSY Bleo	EGE-271	5 µg
pLEXSY-hyg2.1 integrative constitutive expression vector antibiotic selection of transfectants with LEXSY Hygro	EGE-272	5 µg
pLEXSY-neo2.1 integrative constitutive expression vector antibiotic selection of transfectants with LEXSY Neo	EGE-273	5 µg
pLEXSY-sat2.1 integrative constitutive expression vector antibiotic selection of transfectants with Nourseothricin (NTC)	EGE-274	5 µg
pLEXSY-bsd2.1 integrative constitutive expression vector antibiotic selection of transfectants with LEXSY BSD	EGE-275	5 µg
pLEXSY-pac2.1 integrative constitutive expression vector antibiotic selection of transfectants with LEXSY Puro	EGE-276	5 µg
pLEXSY_I-blecherry3 integrative inducible expression vector antibiotic selection of transfectants with LEXSY Bleo and expression monitoring with Cherry fluorescence	EGE-243	5 µg
pLEXSY_I-ble3 integrative inducible expression vector antibiotic selection of transfectants with LEXSY Bleo	EGE-244	5 µg
pLEXSY_I-neo3 integrative inducible expression vector antibiotic selection of transfectants with LEXSY Neo	EGE-245	5 µg
pLEXSY_IE-blecherry4 episomal inducible bicistronic expression vector antibiotic selection of transfectants with LEXSY Bleo and expression monitoring with Cherry fluorescence	EGE-255	5 µg

Recombinant LEXSY cells must be selected in liquid culture (polyclonal selection) or on plates (clonal selection). For convenience **LEXSY Plating Kits** were developed that contain all components required to set up clonal selection. The three different kit formats differ by auxiliary components depending on preferences of customer laboratories.

LEXSY Plating Kits

Product	Cat.-No.	Amount
LEXSY Plating Kit comfort components for solid medium, LEXSY BHI- and fetal-calf-serum-based, with nitrocellulose membranes, spatula, dishes & serological pipettes	ML-451	for 40 plates
LEXSY Plating Kit core components for solid medium, LEXSY BHI- and fetal-calf-serum-based, without nitrocellulose membranes, spatula, dishes & serological pipettes	ML-452	for 40 plates
LEXSY Plating Kit basic components for solid medium, LEXSY BHI-based, without fetal- calf-serum, nitrocellulose membranes, spatula, dishes & serological pipettes	ML-453	for 40 plates

Selection of recombinant LEXSY strains is performed with antibiotics which can also be used for other eukaryotic hosts, provided as powder and/or as sterile ready to go 1.000 x stock solutions.

LEXSY Selection Antibiotics

Cat.-No.	Amount
Nourseothricin (NTC) sterile ready to go 1.000x stock solution, 100 mg/ml	
AB-101S	1 ml
AB-101L	5 ml
AB-101-10ML	10 ml
AB-101-50ML	50 ml
Nourseothricin (NTC) powder (non-sterile)	
AB-102L	1 g
AB-102XL	5 g
AB-102-25G	25 g
AB-102-100G	100 g
LEXSY Bleo sterile ready to go 1.000x stock solution, 100 mg/ml	
AB-103S	1 ml
AB-103L	5 ml
LEXSY Hygro sterile ready to go 1.000x stock solution, 100 mg/ml	
AB-104S	1 ml
AB-104L	5 ml
LEXSY Neo sterile ready to go 1.000x stock solution, 50 mg/ml	
AB-105S	1 ml
AB-105L	5 ml
LEXSY BSD sterile ready-to-go stock solution, 10 mg/ml	
AB-107L	5 × 1 ml
AB-107S	1 ml
LEXSY Puro sterile ready-to-go stock solution, 10 mg/ml	
AB-108L	5 × 1 ml
AB-108S	1 ml

Figure 9

Selection of LEXSY antibiotics.

LEXSY suspension cultures are grown in complex medium. For routine cultivations, transfection, cryoconservation and expression evaluation complex **LEXSY BHI** cultivation medium is used, based on brain and heart extracts.

Complex LEXSY Cultivation Media

Product	Cat.-No.	Amount
LEXSY BHI - Liquid Media Kit		
sterile, brain-heart-infusion based medium, recommended for strain maintenance, electroporation, expression evaluation and cryoconservation	ML-411 S	1 L
	ML-411 L	5 L
LEXSY BHI - Powder Media Kit		
Brain-heart-infusion based medium, recommended for strain maintenance, electroporation, expression evaluation and cryoconservation	ML-412 S	for 1 L
	ML-412 L	for 5 L
	ML-412 XL	for 10 L
	ML-412 XXL	for 50 L

Additives for LEXSY Cultivation Media

Cat.-No.	Amount
Hemin (porcine) sterile 500 × stock solution in 30 % triethanolamine	
ML-108 S	2 ml (for 1 L)
ML- 108 L	10 ml (for 5 L)
ML-108X L	20 ml (for 10 L)
ML-108 XXL	100 ml (for 50 L)
Pen-Strep sterile 200 × stock solution of penicillin and streptomycin	
ML-105 S	5 ml (for 1 L)
ML-105 L	25 ml (for 5 L)
ML-105 XL	50 ml (for 10 L)
ML-105 XXL	250 ml (for 50 L)

Hemin is essential for growth of LEXSY cultures. Addition of Pen-Strep prevents potential bacterial contaminations. Following addition of these components the media are stable for two weeks. If the completed media are to be used after this period, appropriate amounts of additives have to be re-added.

In Vitro LEXSY

Cell-free expression has become a powerful method for production of recombinant proteins and plays a central role in a wide variety of applications such as functional analysis and biochemical characterization of proteins and protein interactions, investigation of protein translation mechanisms, protein engineering, *in vitro* evolution, and structural biology (Katzen *et al.* 2005). Its multiplexed format can be used for production of protein arrays for drug screening and diagnostics (He *et al.* 2007).

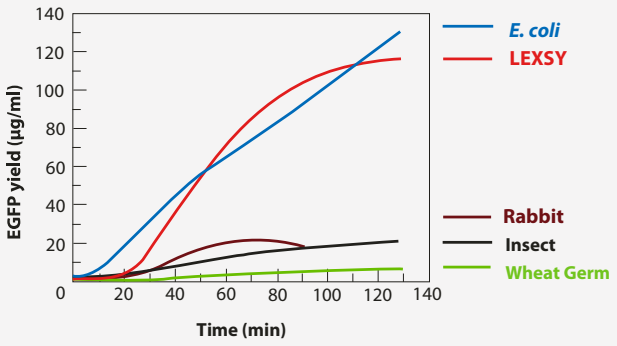
The main advantages of cell-free protein expression are its rapidity of only few hours and its independence of living host-organisms. These features enable very fast generation of results and greatly alleviate typical *in vivo* expression problems caused by toxicity and/or degradation of the protein of interest.

Our ***In Vitro* LEXSY Translation System** is a rapid, convenient, flexible and cost-efficient tool for production of recombinant proteins from DNA templates in a single-tube reaction based on the cell extract of the protozoon *Leishmania tarentolae* (Mureev *et al.* 2009, Kovtun *et al.* 2010 & 2011).

In contrast to *E. coli in vitro* translation, LEXSY contains chaperones for correct folding of proteins of higher eukaryotes (Kovtun *et al.* 2010). Further, compared to insect, rabbit and wheat germ systems, LEXSY yielded significant higher expression levels (Figure 10). Finally, our In Vitro LEXSY Translation System allows efficient suppression of background translation that is often required in other cell-free systems. A simple anti-splice leader oligonucleotide blocks translation of endogenous mRNA.

Figure 10

Coupled transcription-translation of PCR generated EGFP template in LEXSY compared to other commercially available In vitro *translation systems. The EGFP ORF was amplified by overlap-extension PCR and fused individually with the translational leaders according to the instructions of the cell-free systems manufactures. For details refer to Mureev* et al. *2009.*



Two principle versions of *In Vitro* LEXSY are available: Plasmid based or PCR based (Figure 11). The **Plasmid-based *In Vitro* LEXSY Translation** is recommended for high-yield and/or large volume reactions. It is also recommended for open reading frames larger than 2500 bp and requires sub-cloning of the target ORF into the pLEXSY_invitro vector.

The **PCR-based *In Vitro* LEXSY** Translation is rapid and flexible. It utilizes PCR-mediated fusion of the target ORF to a T7 promoter and leader sequence by overlap extension (OE-PCR) technique and does not require any cloning step. Therefore, this approach allows rapid generation of large protein libraries directly from unpurified PCR products.

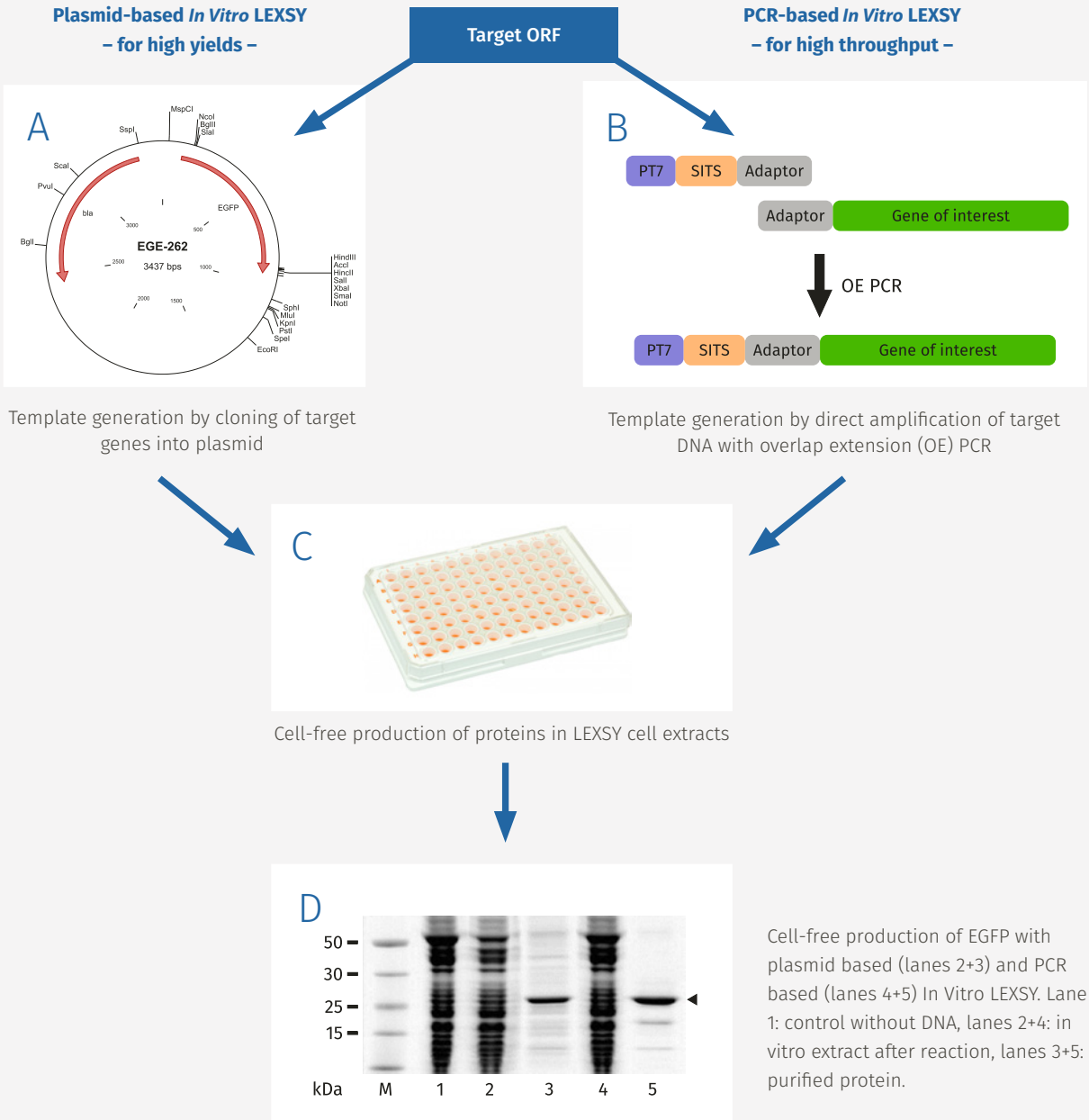


Figure 11

Flow chart of the two principle versions of In Vitro *LEXSY. DNA templates for* in vitro *translation can be generated either by cloning into pLEXSY_in vitro vector* **(A)** *or by a two step-PCR amplification procedure* **(B)**. *The DNA templates are transcribed and translated in a single-tube reaction with LEXSY cell extracts* **(C)**. *The* in vitro *produced proteins can be detected by immuno blotting or after purification* **(D)**. *EGFP = control gene or for N- or C-terminal in-frame fusions to the EGFP gene, bla = ampicillin resistance gene, PT7 = T7 RNA polymerase promoter, SITS = species independent translation sequence, Adaptor = 24 nt DNA sequence (encoding KDIKHVSE peptide) for overlap extension PCR (OE PCR). For more details, refer to the* In Vitro *LEXSY manuals.*

In Vitro LEXSY products

Cat.-No.	Amount
In Vitro LEXSY Translation Kit – 15 reactions for plasmid based cell-free protein synthesis	
EGE-2002-15	1 Kit
In Vitro LEXSY Translation Kit – 15 reactions for PCR based cell-free protein synthesis	
EGE-2010-15	1 Kit
In Vitro LEXSY Translation Cell Extract – 15 reactions for cell-free protein synthesis	
EGE-260	250 µl

Figure 12 shows examples of proteins produced with *In Vitro* LEXSY Translation Kit. Enhanced Green Fluorescent Protein (EGFP) and its fusion proteins can conveniently be detected directly in SDS-PAGE gels by *in situ* fluorescence scanning (A) or isolated by affinity chromatography on a GFP binding matrix for subsequent detection by conventional Coomassie staining (B). For non-fluorescent protein targets Western blotting can be used for visualization.

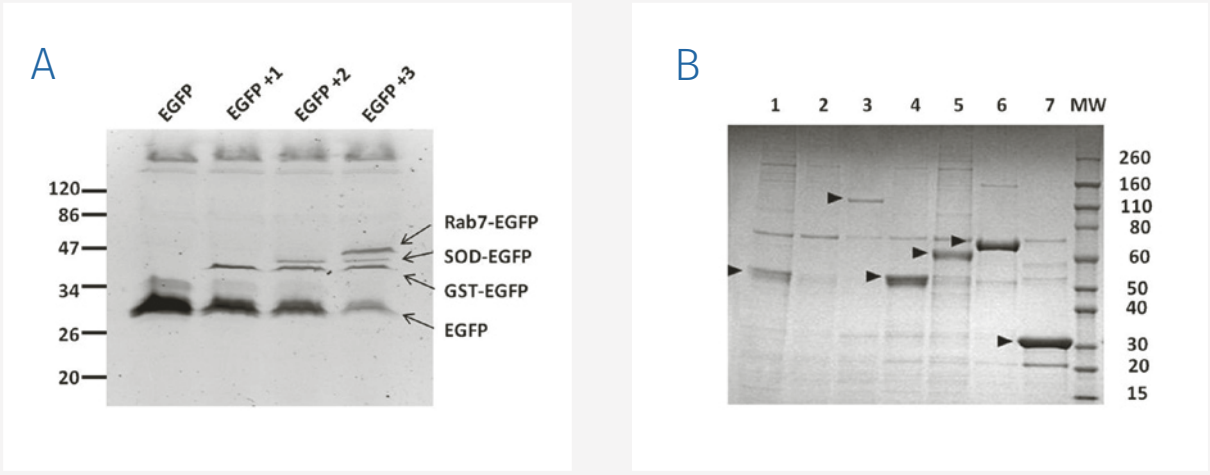


Figure 12

Cell-free expression of EGFP fusion proteins with In Vitro *LEXSY.* **A:** *Simultaneous* in vitro *co-expression of four proteins in a single extract (EGFP and three EGFP fusion proteins, Rab7 = Ras-related small GTPase7, SOD = Cu/Zn superoxide dismutase, GST = Glutathione-S-Transferase). The* in vitro *reactions were resolved on SDS-PAGE and the products detected by* in situ *fluorescence (Adapted from Mureev* et al. *2009). All proteins are present at similar yields indicating suitability of the system for production of heteromeric protein complexes.*

B: *Purification of* in vitro *produced EGFP fusion proteins. 1 = Rab8 (Ras-related small GTPase8)-EGFP, 2 = Cog5 (Complex of Golgi5)-EGFP, 3 = Cog8 (Complex of Golgi8)-EGFP, 4 = Rab1 (Ras-related small GTPase1)-EGFP, 5 = RabGGTß (Geranyl-Geranyl Transferase ß)-EGFP, 6 = MBP (Maltose Binding Protein)-EGFP, 7 = EGFP.* In vitro *reactions and GFP matrix purification were performed as described in the* In Vitro *LEXSY user manual. The purified target proteins were resolved by SDS-PAGE and Coomassie stained. Right lane, molecular size protein marker (kDa). Adapted from Kovtun* et al. *2010.*

Applications and selected examples

Solubility and functionality of recombinant proteins

Incorrect folding and insufficient solubility – resulting in compromised biological activity – are the major shortcomings of prokaryotic protein production systems (Zerbs *et al.* 2009, Makrides 1996). Due to LEXSY's fully eukaryotic protein synthesis, folding and modification machinery most proteins of higher organisms expressed in LEXSY are correctly folded and processed and therefore obtained in a fully functional state (Table 2).

Table 2

Selected examples of LEXSY-expressed proteins with full biological activity

Protein	Localisation	Origin	Reference
Erythropoietin	secreted	human	Breitling *et al.* 2002
Surface Antigen 1 & 2	secreted	*Toxoplasma gondii*	Ebert *et al.* 2007, not publ.
Proprotein Convertase 4	secreted	rat	Basak *et al.* 2008
Laminin-332	secreted	human	Phan *et al.* 2009
Cu/Zn superoxide dismutase	cytosolic	human	Gazdag *et al.* 2010
Tissue Plasminogen Activator	secreted	human	Hemayatkar *et al.* 2010
N-Acetyl Serotonin Methyl Transferase (ASMT)	cytosolic	human	Ben-Abdallah *et al.* 2010
Hydroxynitrile Lyase (MeHNL)	cytosolic	cassava plant	Dadashipour *et al.* 2011
Coagulation factor VII	cytosolic	human	Mirzaahmadi *et al.* 2011

Proprotein Convertase 4 (PC4) is a Ca^{2+} dependent mammalian subtilase (proprotein convertase subtilisin kexin PCSK), which plays a key role in fertilization. Recombinant PC4 could previously be generated only in extremely poor yields using rat GH4C1 or insect Hi5 cells. Using LEXSY, full length and truncated forms of this enzyme were expressed, and soluble, active protein was purified in high yields. Biochemical analysis demonstrated high specific activity, which was superior to PC4 obtained from GH4C1 or Hi5 cells. The substrate specificity found confirmed its biological role and allowed inhibitor design for therapeutic and clinical applications (Basak *et al.* 2008).

Tissue Plasminogen activator (t-PA) is a serine protease with 17 disulfide bonds that need to be correctly formed for the enzyme's biological activity. Different expression systems (yeast, insect cells, transgenic plants) have been tried for production of recombinant human t-PA but yielded unsatisfactory results due to poor secretion, improper folding and hyper-glycosylation. At present, human t-PA is mainly produced at large scale in Chinese hamster ovary (CHO) cells, however, uncontrollable variability in mammalian cell culture processes make development of expressing cell lines laborious and time-consuming. Moreover, high costs of cell culture media and contamination with viruses and prions are additional problems associated with the use of mammalian cells. LEXSY in contrast alleviates these problems and yielded correctly folded t-PA with full biological activity (Hemayatkar *et al.* 2010).

N-Acetyl Serotonin Methyl Transferase (ASMT) is the last enzyme in the melatonin synthesis pathway and possibly involved in autism-related disorders. Attempts to produce human ASMT in recombinant *E. coli* yielded only insoluble and heavily degraded material. In contrast, recombinant ASMT was produced in soluble, active form and purified in milligram amounts when expressed in LEXSY (Ben-Abdallah *et al.* 2010).

Hydroxynitrile lyase from cassava plant Manihot esculenta (MeHNL) is involved in cyanogenesis in this higher plant. Dadashipour et al. (2011) compared expression and features of MeHNL in *E. coli, Pichia pastoris, Leishmania tarentolae* and two cell-free translation systems. While the wild type enzyme formed inclusion bodies when expressed in *E. coli* it could be expressed in soluble form in *L. tarentolae* and *Pichia pastoris*. Moreover, the wild-type and mutant enzyme showed high activity for both proteins (up to 10 U/ml) in the eukaryotic host *L. tarentolae* and *Pichia pastoris*, while those of *E. coli* exhibited about 1 and 15 U/ml, respectively.

Mammalian-type glycosylation

Glycosylation is a major posttranslational modification of a large variety of secreted and membrane proteins. It occurs in more than 50 % of all human proteins (Rich *et al.* 2009) and is often a pivotal factor for folding, function and stability. Glycoproteins account for about 60 % of the therapeutic protein market with annual growth rates of >20 % (Gerngross 2004). Due to the absence of glycosylation pathways in prokaryotes, recombinant glycosylated proteins cannot be produced in e.g. bacteria. Further, glycosylation in most alternative eukaryotic expression hosts such as yeast and insect differs largely from the desired mammalian-type glycosylation (Figure 2c). Despite several improvements including glycoengineering have been reported for these two systems, an expression system with adequate mammalian-type glycosylation is still highly desirable for protein expression in research, diagnostics and pharmaceutical applications.

Glycosylation in LEXSY was thoroughly investigated using recombinant **human erythropoietin (EPO)** as a model. EPO expressed in LEXSY was shown to be efficiently secreted into the culture medium, natively processed at the N-terminus and biologically active. Glycosylation analysis revealed two glycans, a complex mammalian-type biantennary oligosaccharide and the $Man_3GlcNAc_2$ core structure (Figure 13). LEXSY is thus the first biotechnologically useful unicellular eukaryotic system producing biantennary fully galactosylated, core-α-1,6-fucosylated N-glycans. In addition, the N-glycosylation pattern was exceptionally homogenous consisting of only two defined glycoforms, while glycoproteins from other eukaryotes are typically heterogenous multi-glycoform populations (Figure 13). LEXSY-derived homogenous protein preparations are therefore expected to be prone to crystallization and subsequent structure determination.

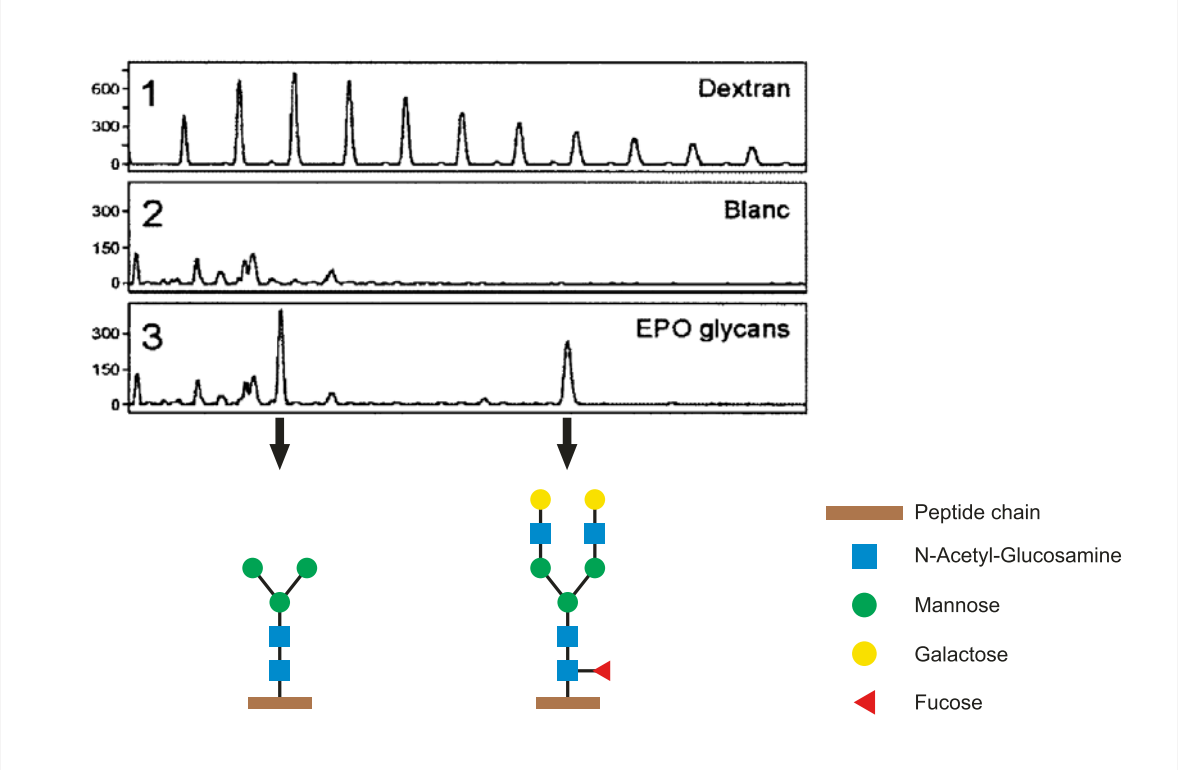


Figure 13

Analysis of recombinant human erythropoietin isolated from culture supernatants of a LEXSY expression strain (for details refer to Breitling et al. 2002).

Expression of complex oligomeric proteins

Many proteins of higher organisms are oligomers consisting of more than one polypeptide chain, and recombinant production of these complexes in an active form often requires simultaneous co-expression of the individual polypeptides. LEXSY allows up to four different antibiotic selection markers to be used for expression of up to four different proteins simultaneously facilitating production of functional oligomers. For example, LEXSY was used to express human laminin-332 (α3β3γ2), a large heterotrimeric glycoprotein and essential component of the epithelial basal lamina that promotes cell adhesion and migration (Phan *et al.* 2009) (Figure 14).

Alternatively, avoiding limitation by availability of selection markers *in vivo*, oligomeric proteins can be obtained using *In Vitro* LEXSY by co-expession of the respective polypeptides in the same extract (Figure 12A).

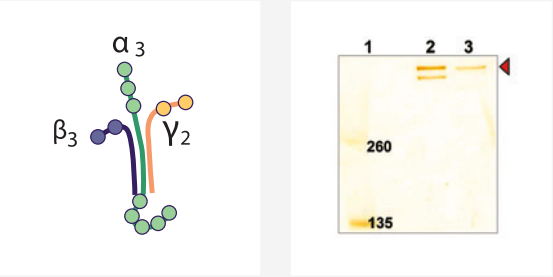


Figure 14

Model of heterotrimeric laminin-332 (left) and Western blot of purified 420 kDa laminin heterotrimer separated under non-reducing conditions. Lane 1: molecular size marker (kDa), 2: laminin from 293-F cells (2 forms), 3: laminin from LEXSY (one defined form) after Phan et al. *(2009).*

Expression of recombinant antibodies

Recombinant production of antibodies with focus on monoclonal antibodies (MAbs) has become a challenging task due to the rapidly expanding pharmaceutical and diagnostic markets. Currently more than 90 MAbs are clinically approved and ca. 400 MAbs are under development in Clinical Phases I-III. The annual demand of the leading 9 MAbs was estimated to be more than 2.200 kg per year (Werner 2011).

LEXSY was evaluated for production of heavy and light chains of human IgG, single chain antibodies and Fc fusions. **Recombinant Fc fusions** were efficiently expressed in LEXSY, completely secreted to the culture medium and one-step affinity purified with Protein A sepharose with yields of ca. 10 mg/L. SDS PAGE analysis demonstrated that the proteins were secreted in the native configuration as dimers (Figure 15).

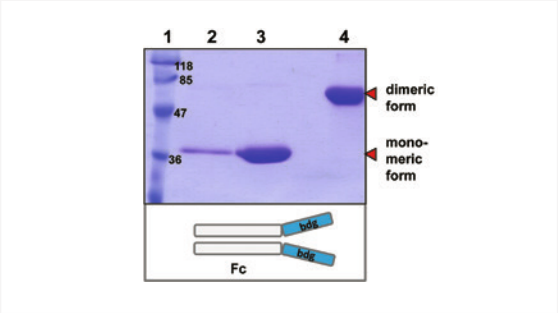


Figure 15

Purification of Fc fusion protein from LEXSY cultivation medium. Lane 1: molecular size marker, 2-3: Protein A sepharose-purified Fc fusion under reducing conditions, 4: like lane 3, non-reducing conditions (JBS not published).

Expression of membrane proteins

Integral membrane proteins are notoriously hard to express in common recombinant expression systems. However, LEXSY was used to produce channelrhodopsin (ChR2) both in its native form and as a slow-mutant. Both variants were expressed with yields in the mg per litre range and the recombinant proteins were crystallized and their crystal structures solved at 2.4 and 2.7 Å, respectively (Figure 16) (Volkov *et al.*, 2017).

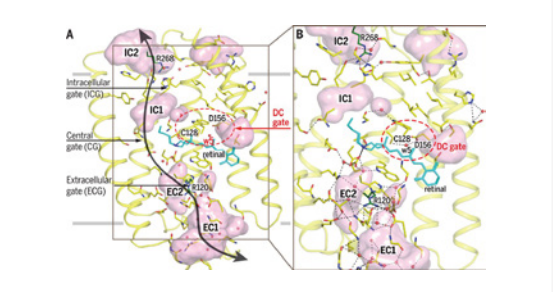


Figure 16

General structure presentation of ChR2, derived from LEXSY-expressed protein. From Volkov et al., *(2017).*

Structural biology: LEXSY proteins for NMR...

The applicability of LEXSY for structural biology was demonstrated by successful **^{15}N-HSQC NMR** analysis of a 28 kDa ^{15}N-Val labeled protein purified from recombinant LEXSY strain grown in a synthetic LEXSY cultivation medium (Figure 17). All 18 Val residues of the *in vivo* labeled protein could be completely assigned in ^{15}N-HSQC NMR spectrum in full agreement with X-ray crystallography (Niculae *et al.* 2006).

Since *Leishmania tarentolae* is auxotrophic for 11 amino acids and can be grown in chemically defined media, multiple options for labeling strategies are available. Alternatively to chemically defined media labeling strategies in complex media were developed (Foldynová-Trantírková *et al.* 2009).

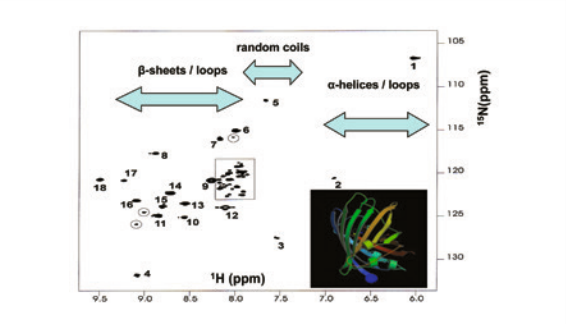


Figure 17

^{15}N-HSQC NMR analysis of ^{15}N-Val labeled EGFP purified from recombinant LEXSY strain. For detailed description refer to Niculae et al. *(2006).*

...and X-ray crystallography

It was shown that LEXSY-expressed proteins can be subjected successfully to crystallography and X-ray analysis. The resolution of a new protein structure was achieved for LEXSY expressed hu Cu/Zn superoxide dismutase SOD1 (Figure 18).

In addition, the exceptionally homogeneous glycosylation pattern of LEXSY-produced proteins can be a remarkable advantage for structural analysis of glycoproteins (see also chapter Mammalian-type glycosylation).

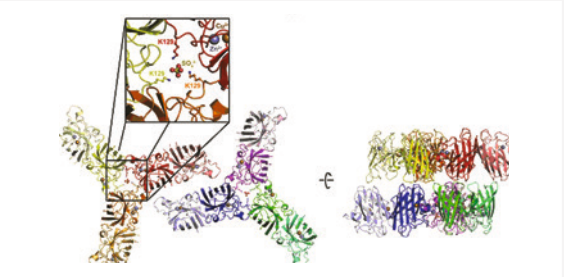


Figure 18

Structure determination of the new P212121 crystal form of LEXSY-produced human Cu/Zn superoxide dismutase (SOD1). The asymmetric unit contains six SOD dimers arranged as two triangular wheels around sulfate ions. The wheels are arranged in a side-to-side fashion (Gazdag et al. *2010).*

LEXSY in Parasitology

Leishmania tarentolae is a close relative to pathogenic Leishmania species as well as to other pathogens such as Trypanosomes, Plasmodium and Toxoplasma (Figure 19). Due to this evolutionary proximity, the LEXSY technology is efficiently expressing parasite proteins with

- High yields
- Correct protein folding
- Native post-translational modifications

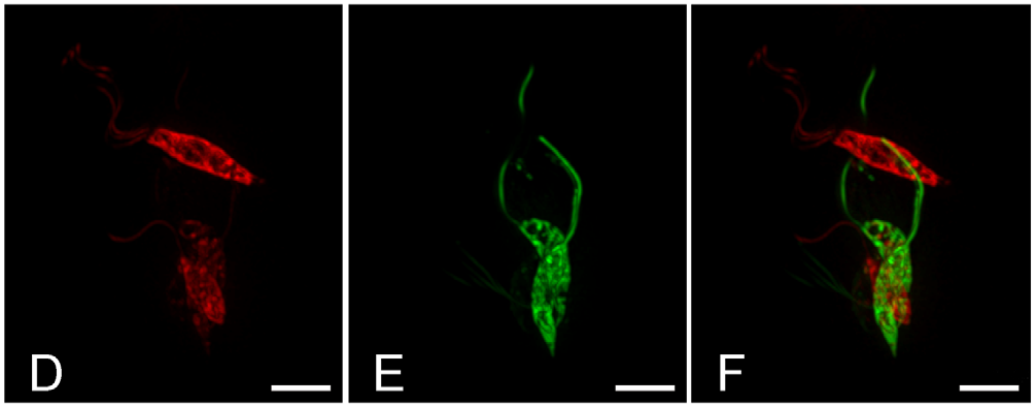


Figure 19

Red and green fluorescent Leishmania donovani cells isolated from Sandfly gut. The cells were engeneered using LEXSY vectors. From Sadlova et al.*, 2011.*

In addition, the expression vectors developed for LEXSY can be used for creation of transgenic strains of other *Leishmania* species including *L. amazonensis, L. donovani, L. infantum, L. major, L. mexicana* and also *Crithidia sp.* as well as the plant parasite *Phytomonas serpens*.

These features of LEXSY enable functional characterization of parasite proteins, investigation of parasite-host interactions, *in vivo* and *in vitro* screening of anti-leishmanial drugs and vaccine development.

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LEXSY Expression