LEXSY –
Eukaryotic Protein Expression

- Fast growth & high yields
- Post translational modifications
- Easy handling & purification

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Jena Bioscience

Jena Bioscience GmbH was founded in 1998 by a team of scientists from the Max-Planck-Institute for Molecular Physiology in Dortmund. 25+ years of academic know how were condensed into the company in order to develop innovative reagents and technologies for the life science market.

Since the start up, the company has evolved into an established global reagent supplier with more than 5500 products on stock and > 3000 customers in 50+ countries. Jena Bioscience serves three major client groups:

- **Research laboratories at universities, industry, government, hospitals and medical schools**
- **Pharmaceutical industry in the process from lead discovery through to pre-clinical stages**
- **Laboratory & diagnostic reagent kit producers and re-sellers**

Our company premises are located in the city of Jena / Germany with a subsidiary in Teltow, in the vicinity of the German capital Berlin.

Jena Bioscience's products include nucleosides, nucleotides and their non-natural analogs, recombinant proteins & protein production systems, reagents for the crystallization of biological macromolecules and tailor-made solutions for molecular biology and biochemistry.

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

In the field of recombinant protein production, Jena Bioscience has developed its proprietary LEXSY technology. LEXSY (*Leishmania* Expression System) is based on a S1-classified unicellular organism that combines easy handling with a full eukaryotic protein folding and modification machinery including mammalian-like glycosylation. LEXSY is primarily used for the expression of proteins that are expressed at low yields or are inactive in the established systems, and expression levels of up to 500 mg/L of culture were achieved.

For the crystallization of biological macromolecules – which is the bottle-neck in determining the 3D-structure of most proteins – we offer reagents and tools for crystal screening, crystal optimization and phasing that can reduce the time for obtaining a high resolution protein structure from several years to a few days.

Our specialized reagents are complemented with a large selection of products for any molecular biology & biochemistry laboratory such as kits for Standard PCR and Real-Time PCR, fluorescent probes, oligonucleotides, cloning enzymes, mutagenesis technologies, and many more…

We combine highest quality standards for all our products (certified according to DIN EN ISO 9001) with individualized customer support. We establish direct lines of communication from clients to our in-house scientists, resulting in productive interactions among people with similar research interests who speak the same language. Furthermore, we offer support programs and attractive discount schemes for young scientists establishing their own labs. If you wish to receive more information, just send an e-mail to **info@jenabioscience.com**.

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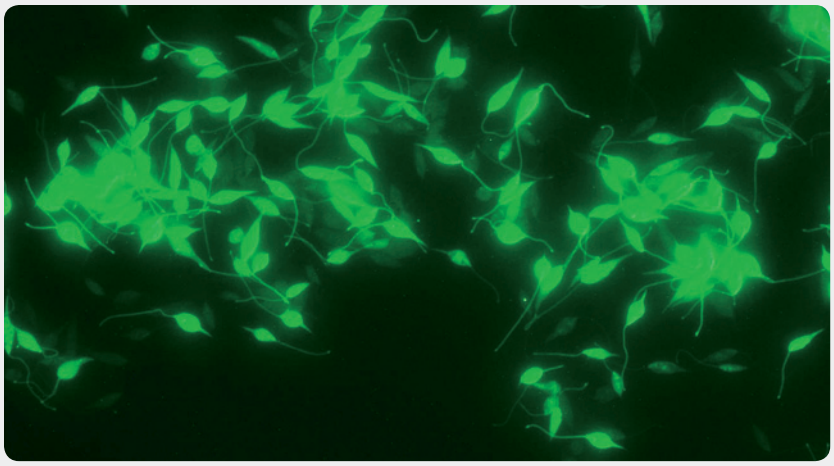
Introduction

The ***L****eishmania* **ex**pression **sy**stem **LEXSY** is the proprietary eukaryotic protein expression platform by Jena Bioscience. LEXSY is based on the protozoan host *Leishmania tarentolae* and was designed to combine eukaryotic protein synthesis and modification with simplicity and ease of handling.



L. tarentolae is a robust, unicellular, flagellated eukaryotic organism of circa 5 x 15 µm 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 mammalians and is fully approved for use in biosafety level 1 laboratories (S1).

Figure 1
Cultured *Leishmania tarentolae* cells expressing green fluorescent protein

LEXSY and all of its components are commercially available world-wide. Licensing is not required for non-profit research institutions such as universities; however, the use of LEXSY and its components for commercial purposes requires a license from Jena Bioscience. For more information and terms of licensing, please contact **expression@jenabioscience.com.**

Overcoming limitations of other expression systems

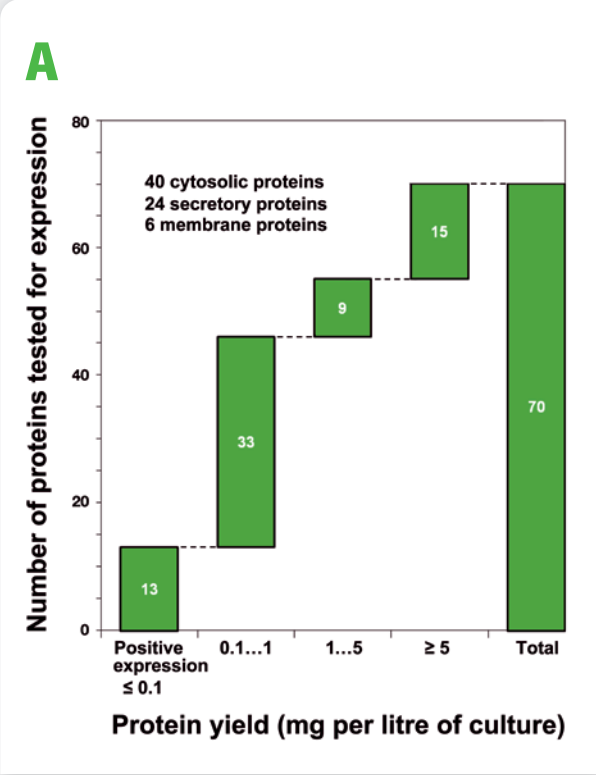
Prokaryotic expression systems such as *E. coli* lack essential components for protein folding and modification and are therefore, in most cases not suitable for production of functional proteins of higher organisms. Alternative eukaryotic expression systems based on e.g. mammalian or insect cells however,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

Hence, LEXSY was developed in order to make use of its eukaryotic protein synthesis and folding/modification machinery and its simplicity and ease of handling.

PROTEIN SYNTHESIS AND FOLDING/MODIFICATION MACHINERY

LEXSY FEATURES:

- **Eukaryotic protein synthesis for correct folding (no inclusion bodies)**
- **Full range of Post-Translational Modifications including mammalian-type N-glycosylation, glypiation, phosphorylation, acetylation, prenylation, myristoylation, ADP-ribosylation, proteolytic processing and oligomerisation**
- **High expression-success rates with yields of up to 500 mg per litre of culture (Figure 2)**



Target protein	Size (kDa)	Yield (mg/L)
Cytoplasmic proteins		
EGFP	28	300
SOD1	16	30
SPEE	35	30
p85 of PI3 kinase	85	3
smmyHC	154	1
Nuclear proteins		
T7 RNA Pol	100	1
Secreted proteins		
MHC II-β	30	500
CRP	23	44
SAG1&2	15/31	10
Fc fusion	39	10
MDP1	45	6
Laminin 332	420 (150+135+135)	0.5
Membrane proteins		
EGFP-Rab7 (mb-associated)	52	12
PDM9 (Type I)	43	0.5
BkrB2-GST (Type III TM7)	55	0.1

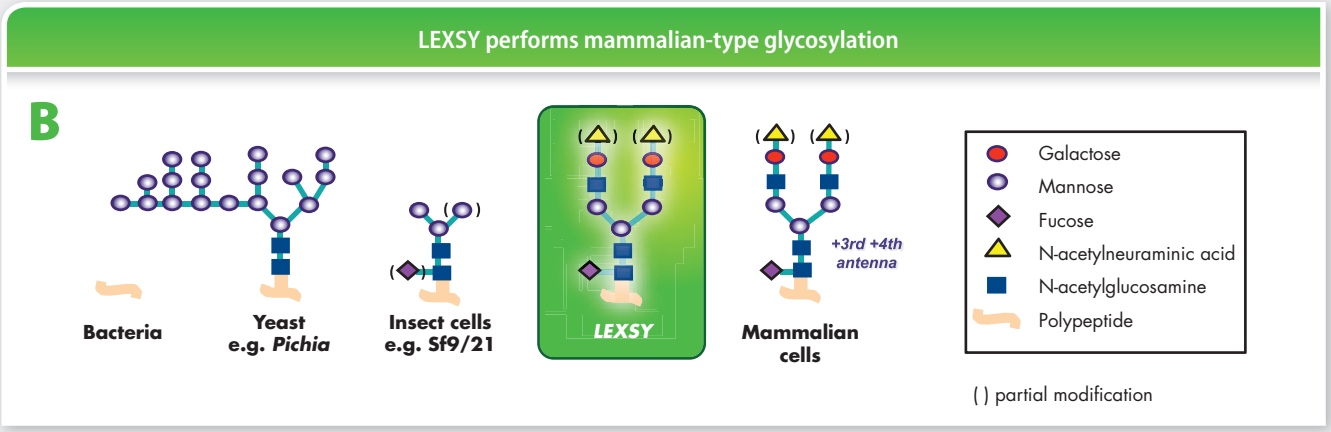


Figure 2

A: Expression of 70 proteins was screened in LEXSY. After cultivation for 2–3 days in suspension culture more than 80% were expressed at > 0.1 mg/L of culture.

Table: Typical examples of LEXSY-expressed proteins clustered by type of protein. SOD1 = human Cu/Zn superoxide dismutase; EGFP = enhanced green fluorescent protein of *A. victoria*; SPEE = human spermidine synthetase; p85 = bovine Phosphoinositide 3-Kinase regulatory subunit α; smmyHC = heavy chain of human smooth muscle myosine; T7 RNA Pol. = RNA polymerase of phage T7 supplied with nuclear localization signal; MHC II-β = human Major Histocompatibility Complex II β subunit (Wienhold *et al.*, not published); CRP = human C-reactive protein of pentaxin family; SAG1/2 = surface antigens of *Toxoplasma gondii* (Fritsche *et al.* 2008); Fc fusion = N-terminal fusion of DNA binding domain to human Fc fragmant (Figure 17); MDP1 = human renal dipeptidase 1; Laminin 332 = large heterotrimeric human laminin glycoprotein α3β3γ2 (Figure 16, Phan *et al.* 2009); EGFP-Rab7 = EGFP fusion of Ras-associated small GTP-binding protein Rab7 (membrane associated by prenylation); BrkB2-GST = GST fusion of human bradykinin receptor B2 (7TM transmembrane protein); PDM9 = human transmembrane protein with EGF-like and two follistatin-like domains 2 (type I membrane protein N out).

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

SIMPLICITY AND EASE OF HANDLING – FROM GENE TO PROTEIN WITHIN SIX WEEKS

LEXSY exerts

- 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).

Figure 3

A: The LEXSY technology enables short evaluation cycles. Target genes are inserted into LEXSY expression vector 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, respectively, were obtained.

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 translation system (*In Vitro* LEXSY) (Table 1).

***In Vivo* LEXSY** requires construction of an *L. tarentolae* expression strain that is suitable for fermentation in inexpensive media and that 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 ultrafast production of a large number of proteins in parallel but which is not suitable for infinite upscale.

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 that are 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.

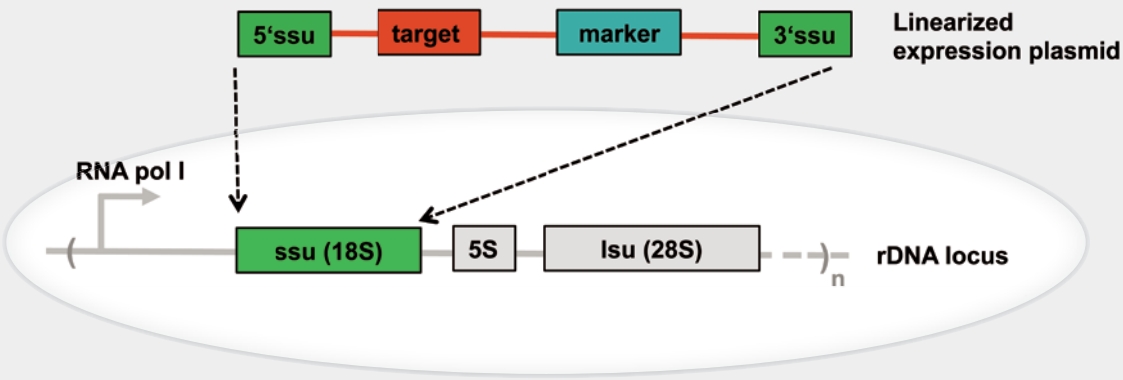


Figure 4

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 (Table 2).

The **inducible** LEXSY (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.

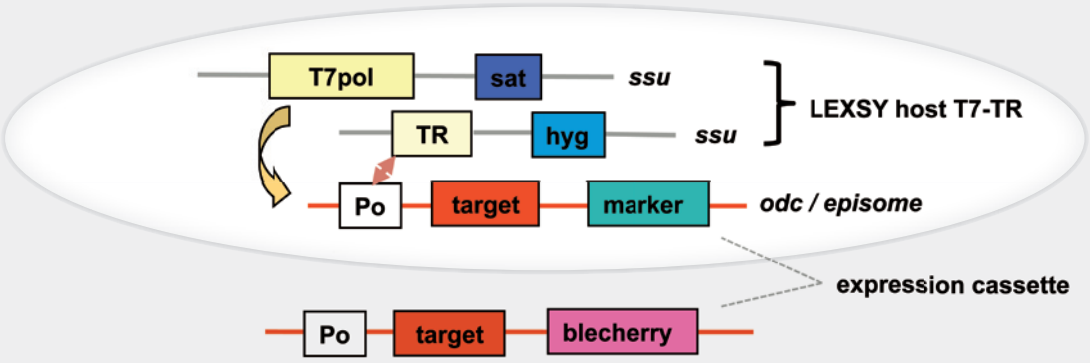


Figure 5
Architecture of inducible LEXSY. Engineered LEXSY host T7-TR expresses bacterial T7 RNA polymerase and TET repressor. Following transfection target gene is expressed under control of T7 promoter-TET operator assembly (more details are given in the text).

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 14).

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 sites 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'Hexa-Histidine tag for protein detection and affinity purification.

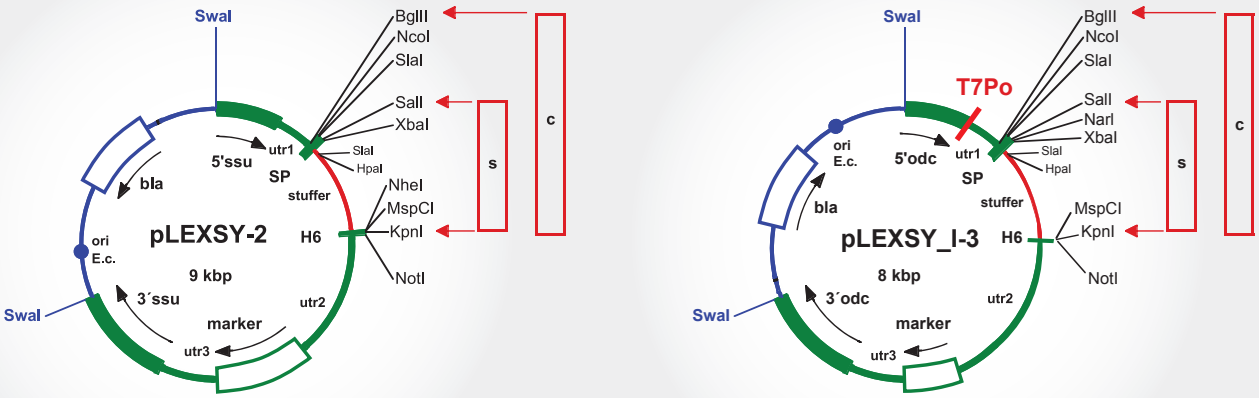


Figure 6
Maps of the integrative **pLEXSY** vector family for **constitutive** (left) or **inducible** (right) expression. 5´ and 3'*ssu* or 5' and 3' *odc* are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. Utr1, utr2 and utr3 are optimized non-translated gene-flanking regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host. 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 (c) or secretory (s) expression of the target proteins. In the constitutive system *sat* (streptothricine acetyltransferase), *neo* (aminoglucoside phosphotransferase), *hyg* (hygromycin phospho-transferase), or *ble* (bleomycin resistance) genes are available as selection markers for selection with the antibiotics LEXSY NTC, -Neo, -Hyg, or -Bleo, respectively. In the inducible system *blecherry*, *ble* and *neo* resistance genes are available for selection with LEXSY Bleo, or -Neo.

Table 2
In Vivo LEXSY configurations

Parameters	Constitutive LEXSY LEXSYcon		Inducible LEXSY LEXSinduce	
	Intracellular	Secretory	Intracellular	Secretory
Typical cultivation time	2–4 days	2–4 days	1–3 days	1–3 days
Number of available selection markers	4[1]	4[1]	2[2]	2[2]

[1] 4 alternative selection antibiotics available (LEXSY NTC, LEXSY Hygro, LEXSY Bleo, LEXSY Neo) – Page 13
[2] 2 alternative selection antibiotics available (LEXSY Bleo, LEXSY Neo)

Furthermore, the inducible LEXSY is available as integrative or episomal version. In the **integrative** version the expression cassette is stably integrated into the chromosomal ornithin decarboxylase *(odc)* locus whereas the **episomal** version makes use of amplification and oligomerisation of expression plasmids maintained extrachromosomally as self-replicating episomes (Kushnir *et al.* 2011).

Finally, the inducible configuration **pLEXSY_I-blecherry** enables efficient screening of high expression clones and online monitoring of induction using Cherry-fluorescence (Figure 7). This was achieved by fusion of the *ble* resistance and *cherry* fluorescence genes.

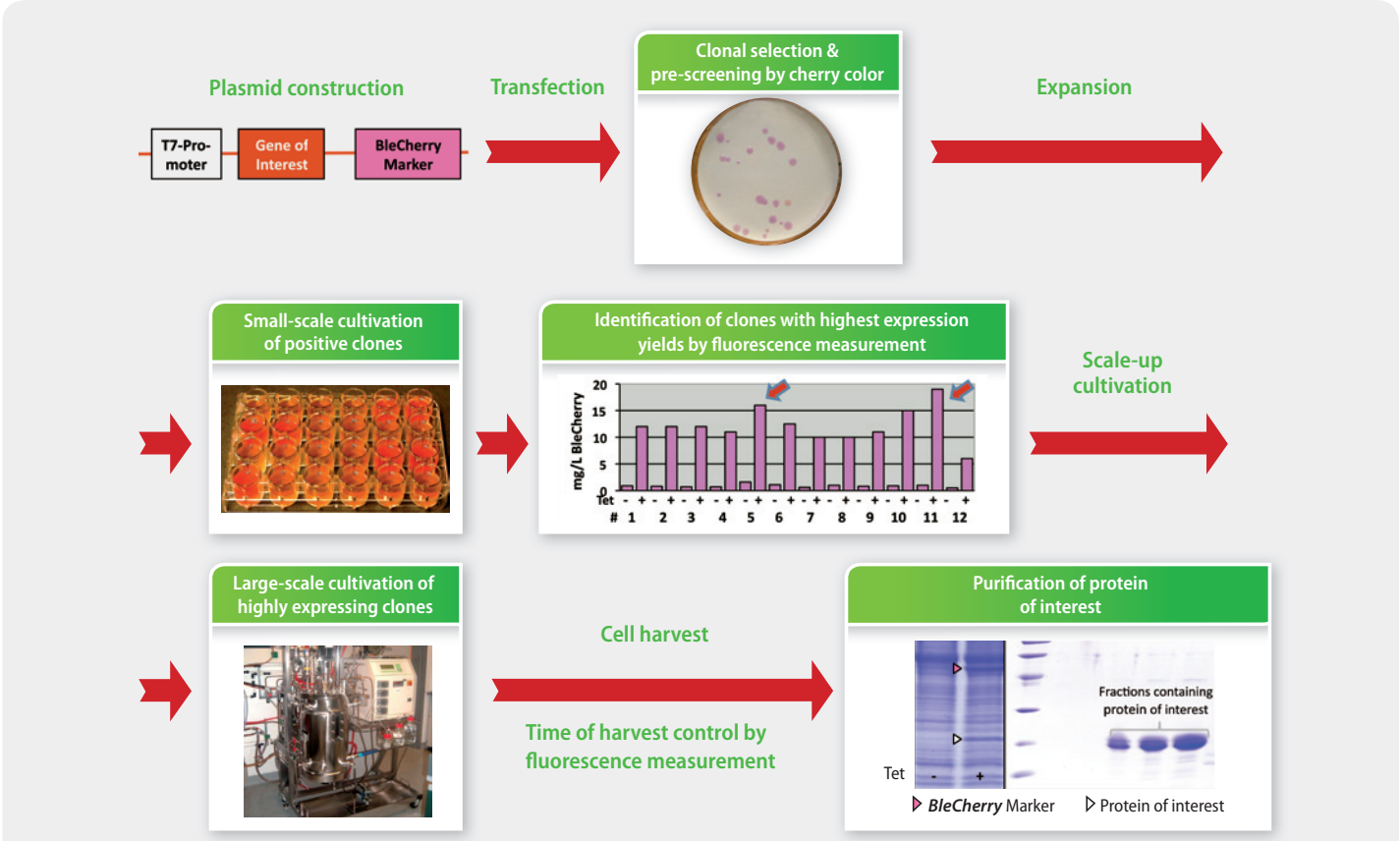


Figure 7
Inducible protein expression with **pLEXSY_I-blecherry** architecture employing coupled expression of target and BleCherry proteins. **Row 1:** LEXSY expression plasmid is constructed by insertion of the gene of interest into the pLEXSY_I-blecherry expression vector (Cat. No. EGE-243 or EGE-251). Following transfection of LEXSY host T7-TR, cells are spread onto nitrocellulose membranes covering selective LEXSY BHI Agar and incubated for ca. 7 days at 26°C. After appearance of recombinant colonies the membrane is transferred onto a fresh LEXSY BHI Agar plate containing the inducer tetracycline and incubated for 1–2 additional days. Recombinant clones are pre-screened by cherry color in daylight. Most intense cherry-colored colonies are expanded by small-scale cultivation in suspension for further evaluation. **Row 2:** Clones with the highest expression yields are identified in multi well plates by fluorescence measurement of samples of the cultures 48 h post induction. Most productive clones are expanded for upscale. **Row 3:** Large-scale fermentation (10–30 L scale) is performed for purification of large amounts of proteins. The optimal time of harvest is determined by online fluorescence measurements of culture samples. At the optimal time point the cells are harvested for downstream processing.

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).

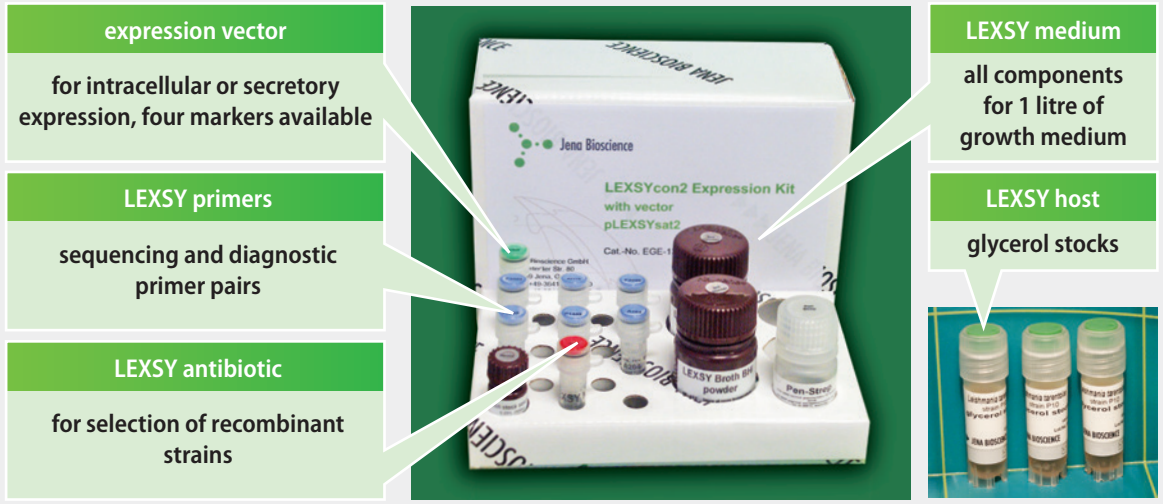


Figure 8

The **constitutive LEXSY Expression Kits** contain LEXSY host strain *L. tarentolae* P10, pLEXSY-2 expression vector, all components for preparation of 1 L of medium including selective antibiotic, primer sets for insert sequencing and diagnostic PCR and a detailed and easy-to-follow manual.

The **inducible LEXSY Expression Kits** contain instead LEXSY host strain T7-TR expressing T7 RNA polymerase and TET repressor and the pLEXSY_I vector. In addition, all single components as well as auxiliary products are available separately.

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.

LEXSY EXPRESSION KITS

Product	Cat. No.	Amount
LEXSYcon2 Expression Kit for constitutive protein expression, contains vector pLEXSY-sat2 or pLEXSY-neo2 or pLEXSY-hyg2 or pLEXSY-ble2	EGE-1300sat EGE-1300neo EGE-1300hyg EGE-1300ble	1 Kit
LEXSinduce3 Expression Kit for inducible protein expression from **integrative** constructs, contains vector pLEXSY_I-blecherry3 or pLEXSY_I-ble3 or pLEXSY_I-neo 3	EGE-1410blecherry EGE-1410ble EGE-1410neo	1 Kit
LEXSinduce4 Expression Kit for inducible protein expression from **episomal** constructs, contains vector pLEXSY_IE-blecherry4	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 available also separately. The cloning sites of all pLEXSY vectors are compatible (Figure 6). This enables convenient transfer of target genes between the LEXSY configurations for expression optimization.

LEXSY EXPRESSION VECTORS

Product	Cat. No.	Amount
pLEXSY-ble2 **integrative constitutive expression vector** antibiotic selection of transfectants with LEXSY Bleo	EGE-231	5 µg
pLEXSY-hyg2 **integrative constitutive expression vector** antibiotic selection of transfectants with LEXSY Hygro	EGE-232	5 µg
pLEXSY-neo2 **integrative constitutive expression vector** antibiotic selection of transfectants with LEXSY Neo	EGE-233	5 µg
pLEXSY-sat2 **integrative constitutive expression vector** antibiotic selection of transfectants with LEXSY NTC	EGE-234	5 µg
pLEXSY_I-blecherry3 **integrative inducible expression vector** antibiotic selection of transfectants with LEXSY Bleo, 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-neo 3 **integrative inducible expression vector** antibiotic selection of transfectants with LEXSY Neo	EGE-245	5 µg
pLEXSY_IE-blecherry4 **episomal inducible expression vector** antibiotic selection of transfectants with LEXSY Bleo, expression monitoring with Cherry fluorescence	EGE-255	5 µg

LEXSY CONTROL VECTORS

Product	Cat. No.	Amount
pLEXSY-egfp-sat2 **integrative constitutive control vector with egfp gene** antibiotic selection of transfectants with LEXSY NTC, expression monitoring with EGFP fluorescence	EGE-235	5 µg
pLEXSY-cherry-sat2 **integrative constitutive control vector with cherry gene** antibiotic selection of transfectants with LEXSY NTC, expression monitoring with Cherry fluorescence	EGE-236	5 µg
pLEXSY-red-sat2 **integrative constitutive control vector with Ds red gene** antibiotic selection of transfectants with LEXSY NTC, expression monitoring with Ds red fluorescence	EGE-237	5 µg
pLEXSY_I-egfp-blecherry3 **integrative inducible control vector with egfp gene** antibiotic selection of transfectants with LEXSY Bleo, expression monitoring with EGFP and Cherry fluorescence	EGE-246	5 µg
pLEXSY_I-egfp-ble3 **integrative inducible control vector with egfp gene** antibiotic selection of transfectants with LEXSY Bleo, expression monitoring with EGFP fluorescence	EGE-247	5 µg
pLEXSY_I-egfp-neo3 **integrative inducible control vector with egfp gene** antibiotic selection of transfectants with LEXSY Neo, expression monitoring with EGFP fluorescence	EGE-248	5 µg

Once constructed, the expression plasmids are used for transfection of the LEXSY host strains by electroporation. Following electroporation, recombinant strains are selected either polyclonally or clonally. Polyclonal selection results from addition of selection antibiotic to the cell suspension whereas clonal selection is achieved by plating of cells onto solid selection media. 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 dependent 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

The plating kits provide components proven to ensure high efficiency of colony formation following plating of transfected LEXSY cells. Figure 9 shows typical plating efficiencies over a broad range of transforming DNA concentrations.

ng DNA per plate	plate 1	plate 2	plate 3	clones per µg of DNA
4	2	2	0	325
20	12	15	6	550
100	63	57	61	600
200	108	113	121	570
1000	>>100	>>100	>>100	n.d.

Figure 9
LEXSY plating efficiencies for an integrative construct. LEXSY host was cultivated at 26°C in suspension culture in LEXSY BHI Medium and electroporated 24 h post inoculation at cell density of 6 x 10^7 cells/ml by low voltage procedure with settings 450 V and 450 µF. Linearized DNA was added at concentrations indicated in the table. Three aliquots of 2 ml were withdrawn from each o/n culture of electroporated cells, sedimented and spread onto LEXSY BHI Agar. Colonies were counted 7 d post plating. For details of the protocol refer to LEXSY manuals.

Selection of recombinant LEXSY strains is performed with antibiotics used also for other eukaryotic hosts. Jena Bioscience offers **LEXSY Selection Antibiotics** which are evaluated for efficient selection of recombinant LEXSY strains and are provided as powder as well as sterile ready to go 1.000 x stock solutions.

LEXSY SELECTION ANTIBIOTICS

Product	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	AB103S	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

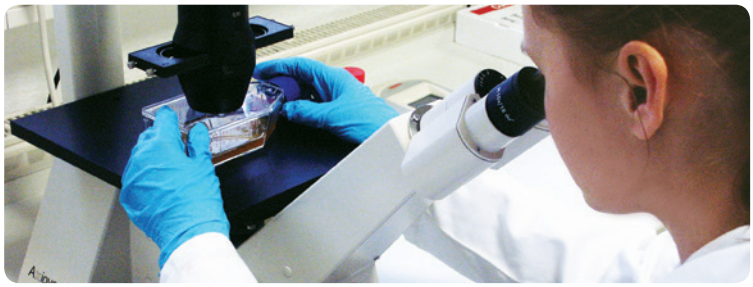
For optimal selection LEXSY NTC, LEXSY Bleo and LEXSY Hygro are used at 100 µg/ml and LEXSY Neo at 50 µg/ml final concentrations in the cultivation medium.

LEXSY suspension cultures are grown in complex or in synthetic media. For routine cultivations, transfection, cryoconservation and expression evaluation Complex **LEXSY BHI** Cultivation Media are used, based on brain and heart extracts. For cultivation in animal-free media Jena Bioscience offers **LEXSY YS** media based on yeast and soy extracts. In both types of complex media cell densities up to 5×10^8 cells/ml are reached in agitated laboratory cultivations.

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-411S	1 L
	ML-411L	5 L
LEXSY BHI - Powder Media Kit Brain-heart-infusion based medium, recommended for strain maintenance, electroporation, expression evaluation and cryoconservation	ML-412S	for 1 L
	ML-412L	for 5 L
	ML-412XL	for 10 L
	ML-412XXL	for 50 L
LEXSY YS - Liquid Media Kit sterile, yeast-soybean based, casein-free medium medium free of animal components	ML-431S	1 L
	ML-431L	5 L
LEXSY YS - Powder Media Kit yeast-soybean based, casein-free for medium free of animal components	ML-432S	for 1 L
	ML-432L	for 5 L
	ML-432XL	for 10 L
	ML-432XXL	for 50 L

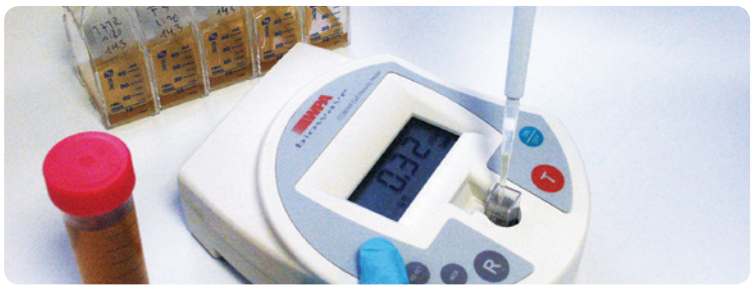
If cultivation of LEXSY strains is required in protein-free defined media, Synthetic LEXSY Cultivation Media are available. They are optimized for high cell densities up to 3×10^8 cells/ml in agitated laboratory cultivations.



SYNTHETIC LEXSY CULTIVATION MEDIA

Product	Cat. No.	Amount
Synthetic LEXSY Medium - liquid, ready-to-grow, sterile, contains Hemin and Pen-Strep, shelf life 2 weeks	ML-103S	1 L
	ML-103L	5 L
Synthetic LEXSY - Liquid Media Kit sterile, with Hemin and Pen-Strep stock solutions, shelf life 6 months	ML-107S	1 L
	ML-107L	5 L

All LEXSY Cultivation Media Kits contain ready-to-go stock solutions of Hemin and PenStrep, which must be added before use. These additives are also available separately.



ADDITIVES FOR LEXSY CULTIVATION MEDIA

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

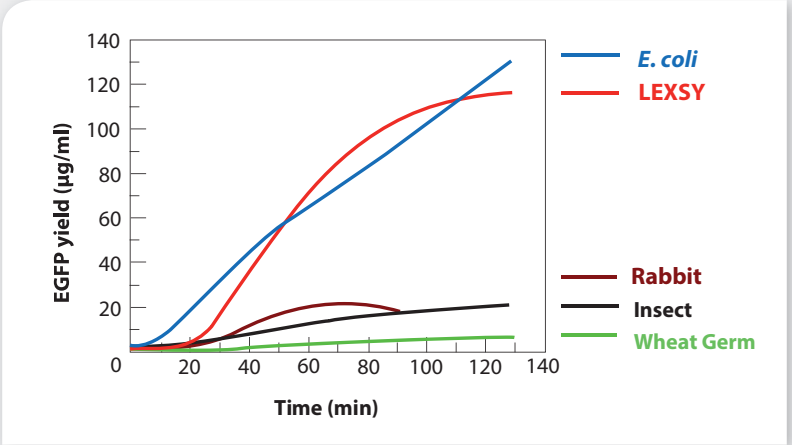
Cell-free expression has become a powerful method for production of recombinant proteins and plays a central role in a wide variety of applications such as functional analysis and biochemical characterization of proteins and protein interactions, investigation of protein translation mechanisms, protein 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 NEW, 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.



Dependent on the way of template preparation two principle versions of *In Vitro* LEXSY are available, that are plasmid based or PCR based versions (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.

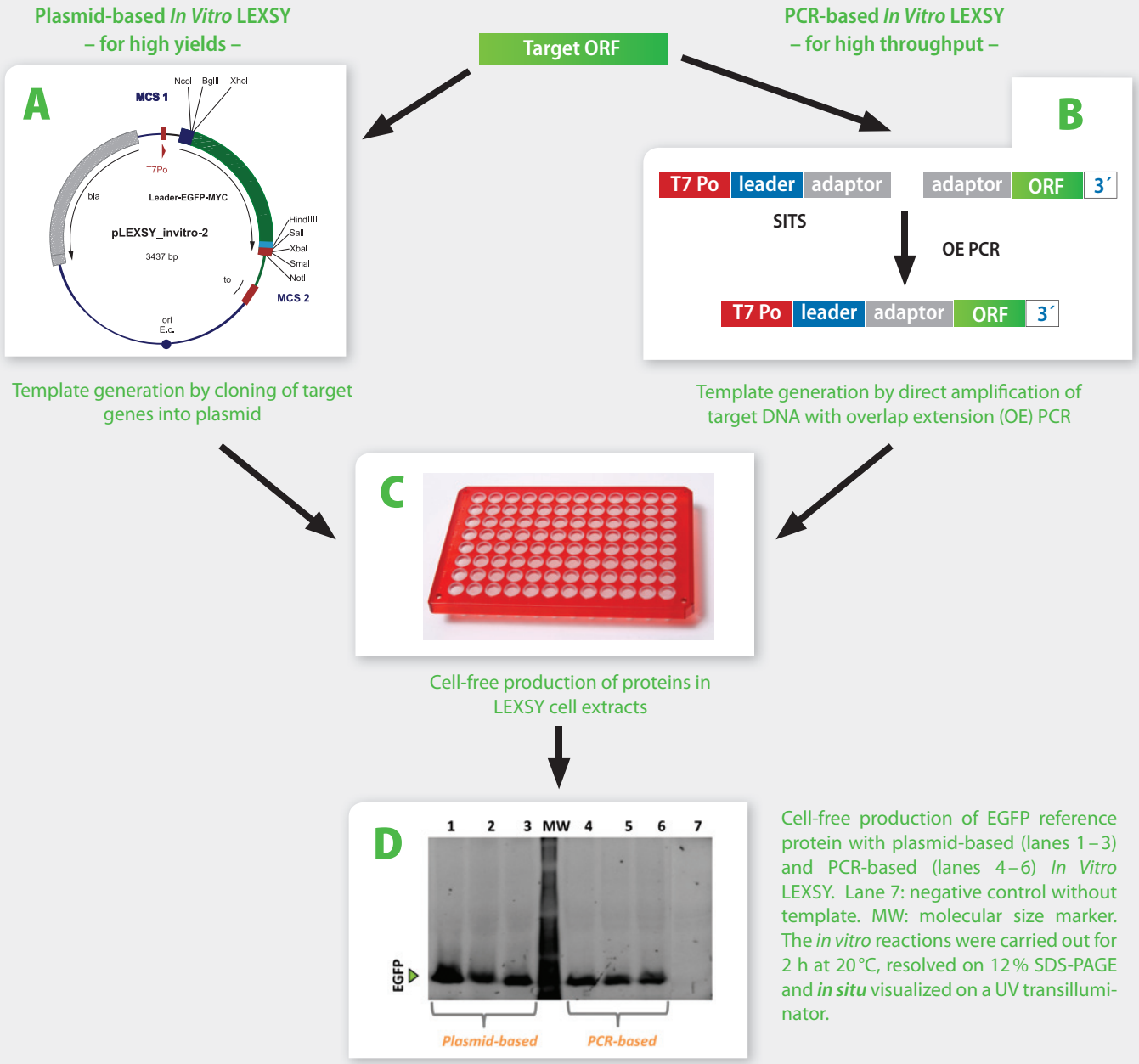


Figure 11

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 fluorescence scanning in case of EGFP fusion proteins (**D**), by Western blotting or other techniques. Abbrevations: T7Po = T7 RNA polymerase promoter, MCS1 & MCS2 = multiple cloning sites for replacement of vector-borne EGFP control gene and for N' or C' in-frame fusions to the EGFP gene resp., Leader = 63 nt poly-TTTTA sequence for generation of unstructured 5' end of template mRNA, Adaptor = 24 nt DNA sequence (encoding KDIKHVSE peptide) for overlap extension PCR (OE PCR), MYC = Myc-tag, to = T7 transcription terminator, bla = ampicillin resistance gene, ori E.c. = replication origin for *Escherichia coli*, SITS = species independent translation sequence consisting of T7Po + leader- + adaptor sequences. For more details, refer to the *In Vitro* LEXSY manuals.

IN VITRO LEXSY PRODUCTS

Product	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.

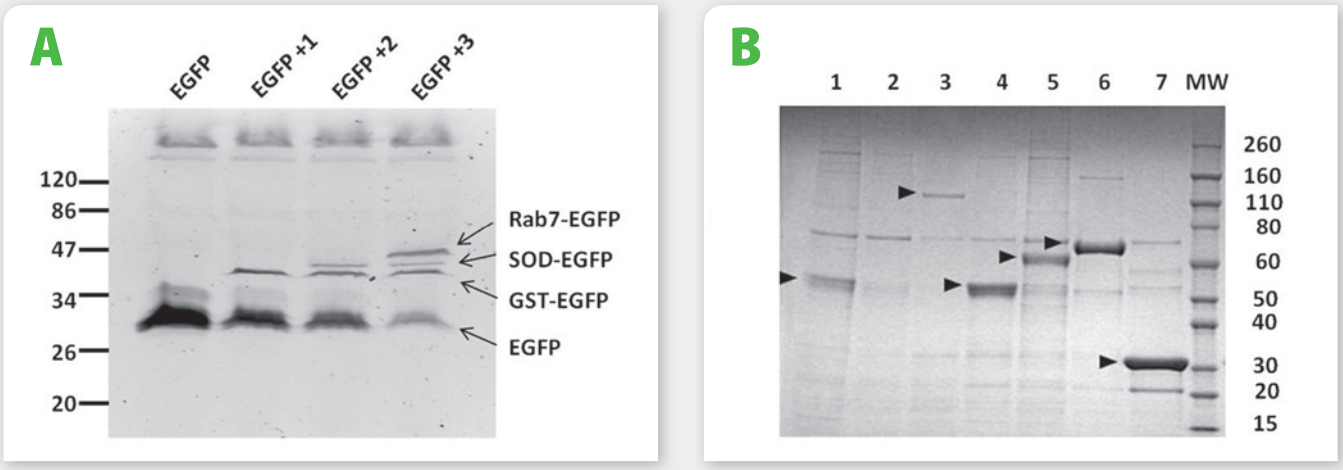


Figure 12

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/modification machinery most proteins of higher organisms expressed in LEXSY are correctly folded and processed and therefore, are obtained in a fully functional state (Table 3).

Table 3
Selected examples of LEXSY-expressed proteins with full biologic 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^{++} 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, transgeneic 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 active (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 2B). 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 fully 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 A & B). LEXSY-derived homogenous protein preparations are therefore expected to be prone to crystallization and subsequent structure determination.

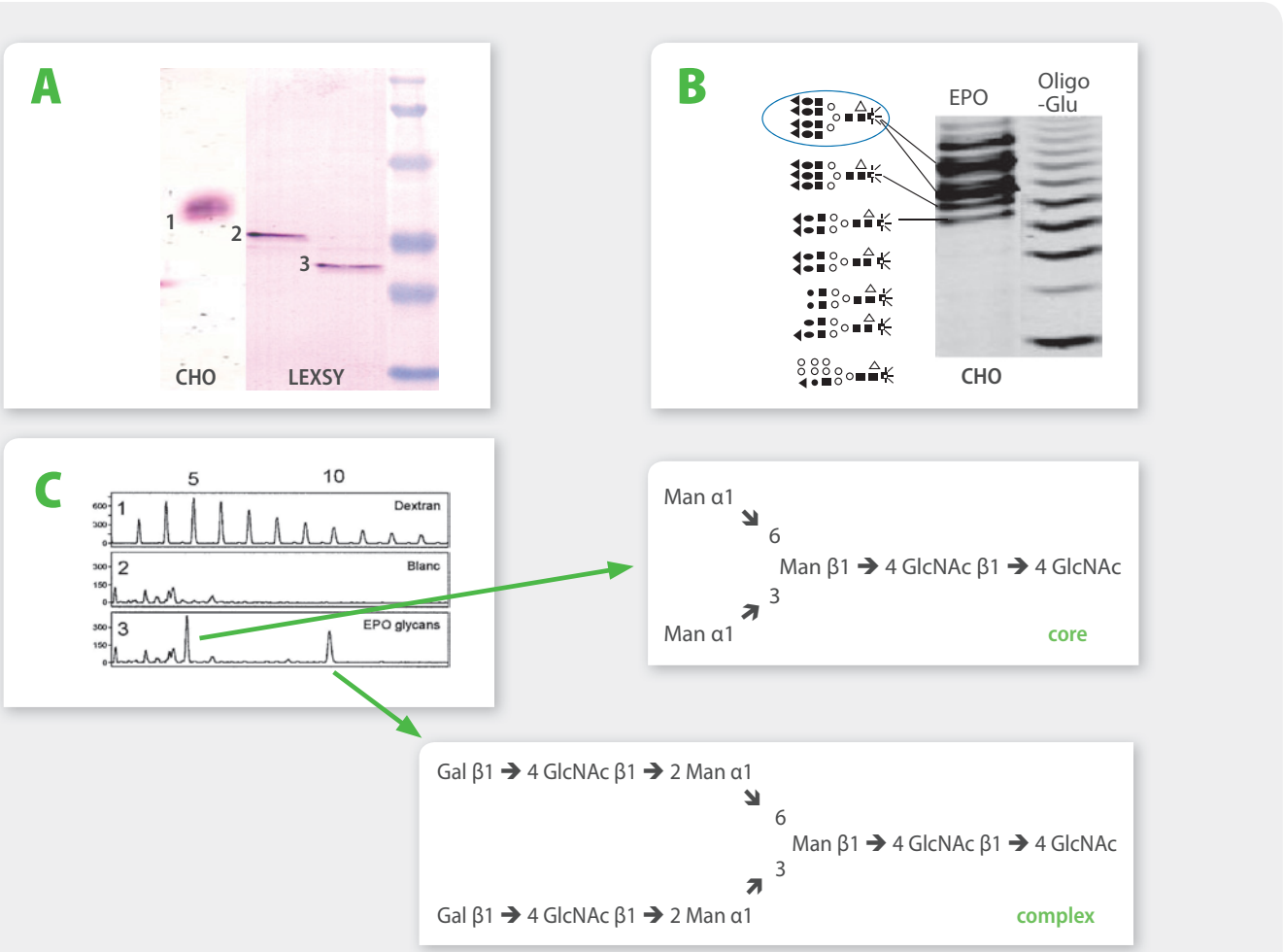


Figure 13

Analysis of recombinant human erythropoietin isolated from culture supernatants of a LEXSY expression strain.

A: Western blot of recombinant human EPO produced in CHO cells (1) and LEXSY secreted EPO before (2) and after (3) de-glycosylation with N-glycosidase F (PNG).

B: Electrophoretic resolution of heterogenous population of CHO-derived EPO. Glycan structures are depicted at the left.

C: Enzymatic resolution of complex and core glycan structures released from LEXSY-produced EPO (for details refer to Breitling *et al.* 2002).

Similar glycosylation profiles were also found in other LEXSY-produced proteins including human interferon-γ (IFN-γ) and host major surface protein GP63 suggesting this to be a common feature of all recombinant glycoproteins produced in LEXSY.

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 employed to express human laminin-332 (α3β3γ2), a large heterotrimeric glycoprotein and essential component of 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).

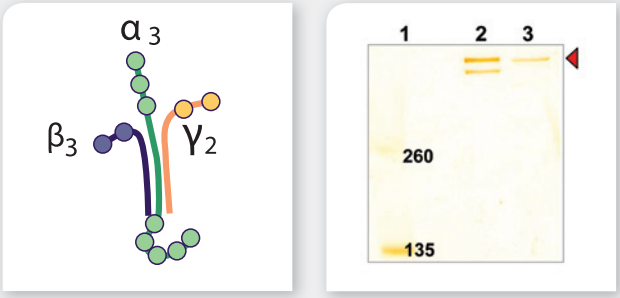


Figure 14
Model of heterotrimeric laminin-332 (left) and Western blot of purified 420 kDa laminin heterotrimer separated under non-reducing conditions. Lanes 1 molecular size marker (kDa), 2 laminin from 293-F cells (2 forms), 3 laminin from LEXSY (one defined form) after Phan *et al.* (2009).

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 20 MAbs are clinically approved and ca. 300 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).

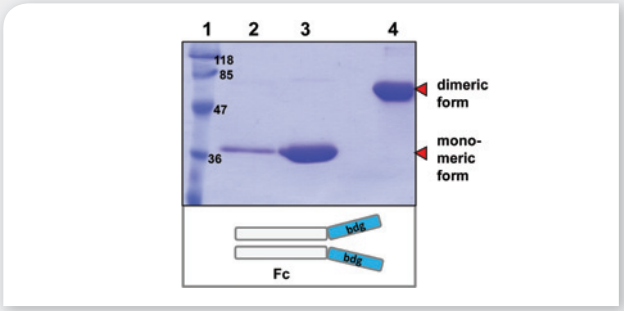


Figure 15
Purification of Fc fusion protein from LEXSY cultivation medium. Lane 1 molecular size marker, 2-3 Protein A sepharose-purified Fc fusion under reducing conditions, 4 dto. non-reducing conditions (JBS not published).

Structural biology: LEXSY proteins for NMR and X-ray crystallography

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 16). 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).

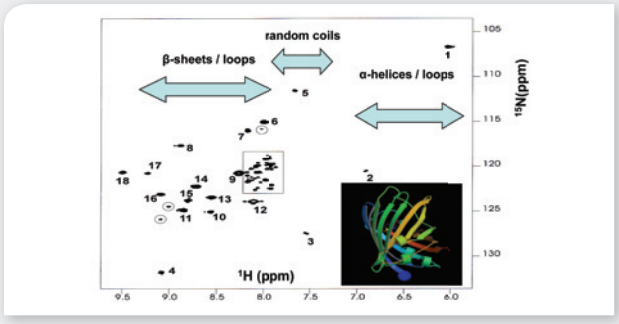


Figure 16
^{15}N-HSQC NMR analysis of ^{15}N-Val labeled EGFP purified from recombinant LEXSY strain. For detailed description refer to Niculae *et al.* (2006).

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 17).

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).

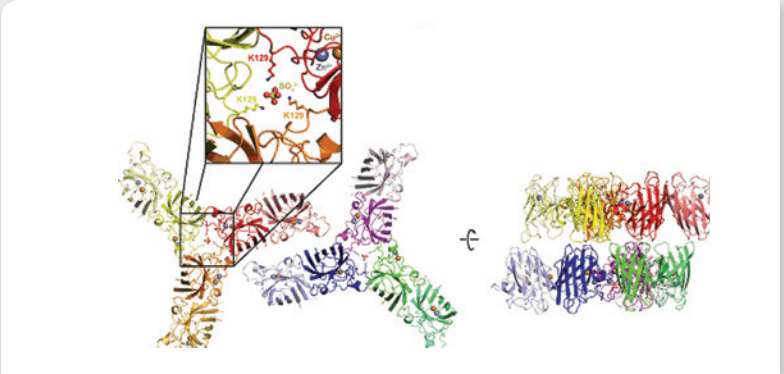


Figure 17

Structure determination of the new $P2_12_12_1$ 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 18). Due to this evolutionary proximity, the LEXSY technology is efficiently expressing parasite proteins with

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

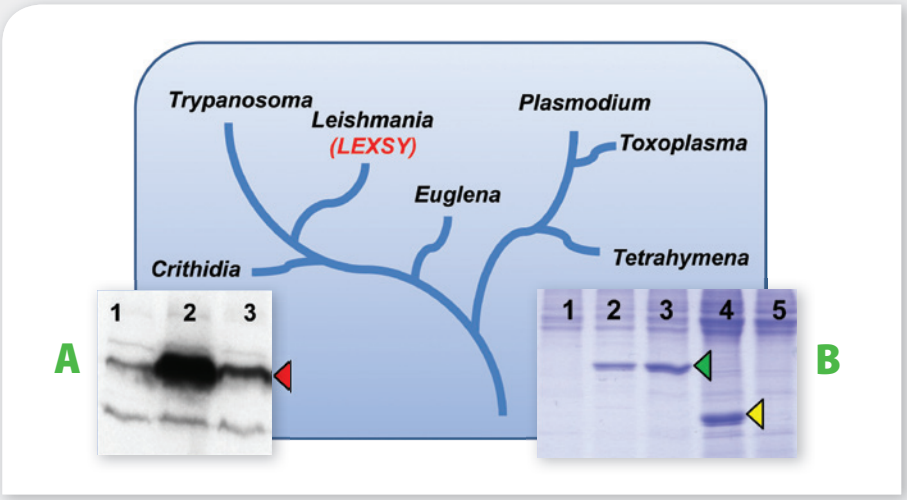


Figure 18

Leishmania tarentolae-based LEXSY is evolutionary closely related to a number of the most common parasites and was used for overexpression and purification of functional parasite proteins.

A: Western blot of 93 kDa J-binding protein (JBP1) of *Leishmania sp.* Lane 1 = host control, lane 2 = induced culture, lane 3 = non-induced culture (Courtesy of S. Vainio, The Netherlands Cancer Institute, Amsterdam).

B: Coomassie stain of immunoreactive surface proteins SAG1 (28 kDa) and SAG2 (15 kDa) of *Toxoplasma gondii*. Lanes 1 and 5 = host controls, lanes 2 and 3 = SAG1, lane 4 = SAG 2 secreted to the culture medium (Courtesy of M. Ebert, FZMB, Erfurt).

For more examples, refer to figure 19 A & D.

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* (Figure 19 B-C).

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.

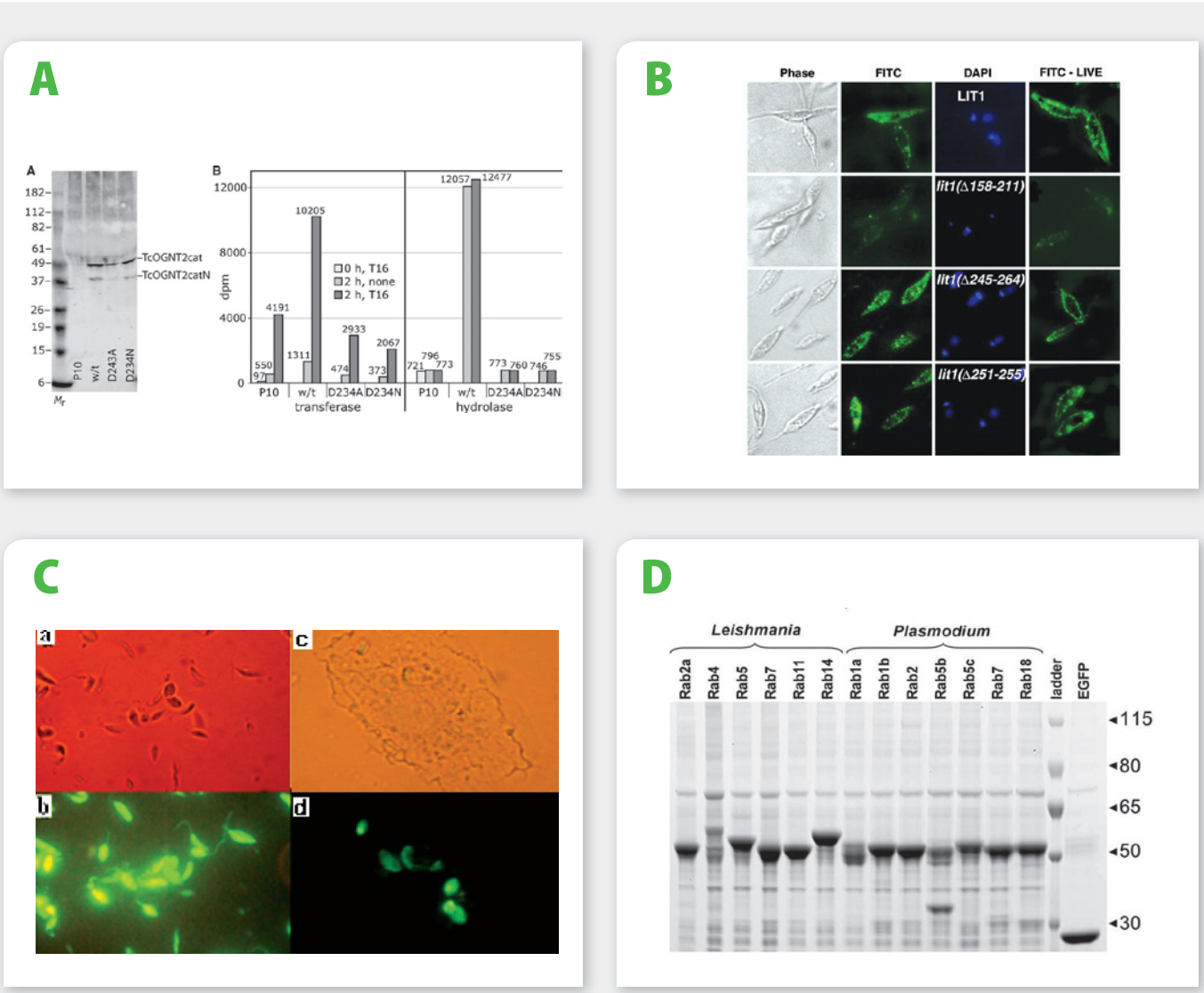


Figure 19

A: Expression and functional analysis of the catalytic domain of α-N-acetylglucosaminyltransferase from *Trypanosoma cruzi* (TcOGNT2cat) in LEXSY by Western blotting (left) and enzymatic activities (right). P10 = non-transfected host strain; wt = P10 expressing wild-type TcOGNT2cat; D234A and D234N = single point mutants (from Heise *et al.* 2009).

B: Subcellular localization of ferrous iron transporter LIT1 expressed in *L. amazonensis* Δlit1 promastigotes using pLEXSY constructs. Immunofluorescence demonstrated different targeting of wild type and mutant proteins to the plasma membrane. LIT1 immunofluorescence = green, parasite DNA = blue, FITC = anti-LIT1 IF on fixed/non-permeabilized promastigotes, FITC-LIVE = anti-LIT1 IF on live promastigotes (from Jacques *et al.* 2010).

C: EGFP imaging in *L. major* reporter strain stably transfected with pLEXSY-egfp construct by Epi-fluorescence microscopy of recombinant *L. major* promastigotes (left) and intracellular amastigotes in bone marrow-derived macrophages (right) (from Bolhassani *et al.* 2011).

D: Expression of protozoon RabGTPases originating from *L. tarentolae* or *P. falciparum* in PCR-based *In Vitro* LEXSY. Coomassie stained SDS-PAGE gel loaded with EGFP-Rab GTPases eluted from a GFP binding matrix. For details see *In Vitro* LEXSY manual (adapted from Kovtun *et al.* 2010).

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