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Jena Bioscience

15 Reactions LEXSY *in vitro* Translation

Cell-free protein expression kit based on *Leishmania tarentolae*

for PCR-based template generation

Cat. No. EGE-2010-15

FOR RESEARCH USE ONLY.

NOT INTENDED FOR HUMAN OR ANIMAL DIAGNOSTIC

OR THERAPEUTIC USE

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Content

1	**Introduction**	**3**
2	**Kit components and storage conditions**	**3**
2.1	**LEXSY cell extract**	**3**
2.2	**SITS fragment**	**3**
2.3	**Primer 1 (H4134)**	3
2.4	**Nuclease-free water**	**3**
2.5	**Equipment and materials supplied by user**	**3**
3	**Protocol section**	**4**
3.1	**PCR based template preparation**	**4**
3.2	**Cell-free translation**	**6**
3.3	**Detection of synthesized proteins**	**7**
3.4	**Isolation of synthesized proteins**	**8**
4	**Licensing information**	**10**
5	**Literature**	**10**
6	**Appendix**	**11**
6.1	**Influence of temperature on *in vitro* EGFP synthesis**	**11**
6.2	**DNA sequences of the primers for LEXSY *in vitro* Translation Kit**	**11**

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1 Introduction

The LEXSY *in vitro* translation system is a rapid, convenient, flexible and cost efficient tool to produce recombinant proteins for biochemical, biophysical and structural analysis (Mureev *et al.,* 2009, Kovtun *et al.,* 2010 & 2011). Its key component is the transcription-translation linked cell extract of the eukaryotic protozoan host *Leishmania tarentolae,* which can be programmed by DNA templates encoding the user's gene of interest.

The LEXSY cell extract for *in vitro* translation contains functional ribosomes and all essential components of the eukaryotic translation and folding machinery. Heterologous T7 RNA polymerase was added to the extracts for target mRNA generation. To ensure a low background the translation of endogenous host mRNAs is efficiently blocked by an antisense oligonucleotide making use of the unique gene organization of *Leishmania* (LeBowitz *et al*., 1993).

2 Kit components and storage conditions

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

2.1 LEXSY cell extract (Cat. No. EGE-260)

- 1 vial with 250 µl of frozen LEXSY cell extract (sufficient for 15 reactions)
- store at -80°C, stable for at least 12 month without loss of activity
- freeze unused aliquots in liquid nitrogen and store at -80°C
- avoid more than two freeze-thaw cycles

2.2 SITS fragment (for sequence ref. to Appendix 6.2.)

- 243 bp double stranded DNA fragment containing T7 promoter and SITS element
- 15 ng/µl in 40 µl of 10 mM TrisHCl pH 8.0 (95 nM); may vary, see label
- store at -20°C

2.3 Primer 1 (H4134) (for sequence ref. to Appendix 6.2.)

- forward primer for overlap extension PCR
- 50 µl of 10 µM in 10 mM TrisHCl pH 8.0
- store at -20°C

2.4 Nuclease-free water

- 1.2 ml
- store at -20°C

2.5 Equipment and materials supplied by user

- Standard molecular biology equipment and reagents for PCR, cloning, DNA and protein handling, including gene specific primers for primary PCR amplification of target ORF
- Incubators at 20°C - 37°C
- Bench-top centrifuge at room temperature and 4°C
- Cooling and freezing capacities at +4°C, -20°C and -80°C
- Liquid nitrogen equipment

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3 Protocol section

3.1 PCR based template preparation

The first step of *in vitro* translation protocol is the preparation of template DNA with T7 promoter and a species independent translation initiating sequence **SITS** (Mureev *et al.,* 2009). There are two alternative protocols for template DNA preparation:

- The **PCR–based template preparation** is rapid and flexible. It utilizes PCR-mediated fusion of the target ORF to 5' T7 promoter and SITS by overlap extension (OE) technique (Horton *et al.* 1989). This approach allows rapid generation of large protein libraries directly from unpurified PCR products.

- The **plasmid–based template preparation** is recommended for high-yield and/or large volume *in vitro* translation reactions. It is also recommended foropen reading frames larger than 2500 bp and requires cloning of the target ORF into the pLEXSY_invitro-2 vector provided in the plasmid-based LEXSY cell-free translation kit (JBS, **Cat. No. EGE-2002-15**).

In both configurations **SITS** consists of a poly-TTTTA 5' UTR (Appendix 6.2) fused to a sequence forming three stem-loop structures in mRNA (Mureev *et al.,* 2009). Translation of this sequence results in the addition of 17 amino acids to the N' terminus of the target protein. This configuration ensures the highest yield of recombinant protein synthesized in the LEXSY *in vitro* system.

The flow chart of PCR based template preparation by overlap extension PCR technique (OE-PCR) is shown in Fig. 1.

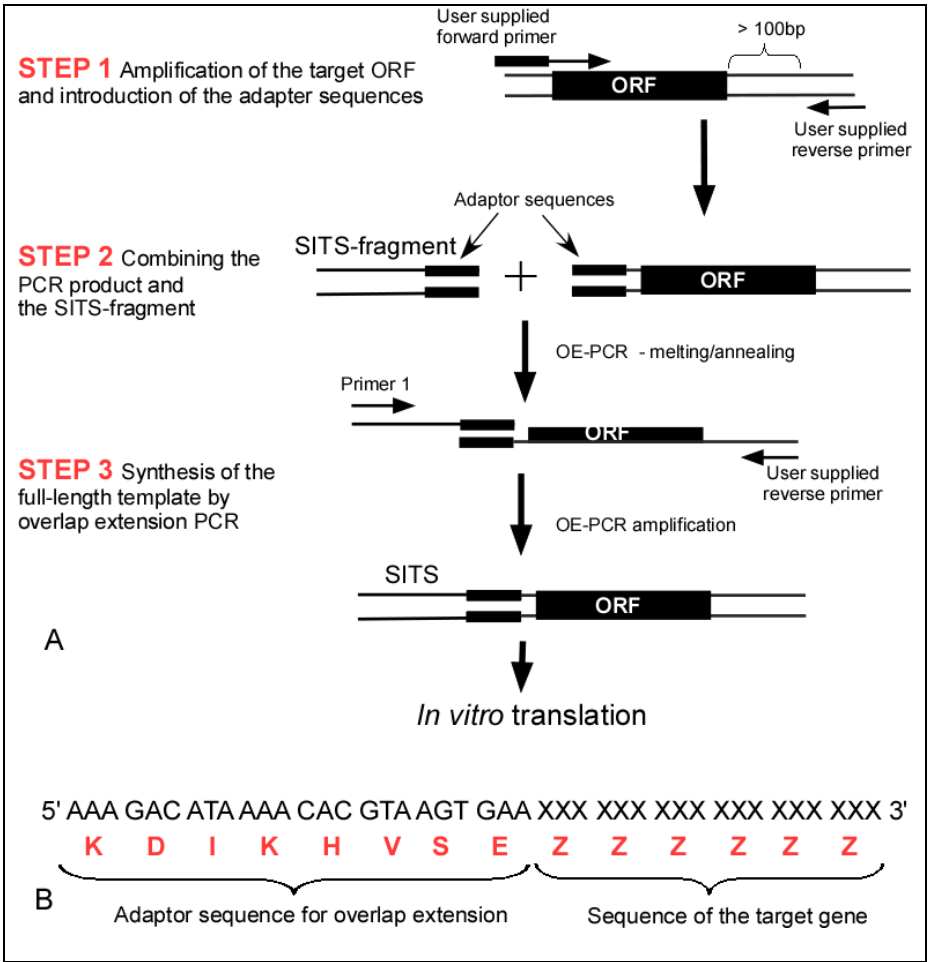

Fig. 1: Preparation of template by overlap extension PCR (OE-PCR) (A) Flow chart of template construction by OE-PCR. (B) Design of the user supplied forward primer. The XXX denotes in frame nucleotide triplets of the target ORF.

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Standard amplification protocol

- Design a gene-specific forward primer with 5' adaptor sequence as shown in Fig. 1 B and a reverse primer annealing 100-150 bp 3' of the target ORF stop codon of the donor construct used.

- PCR amplify your template with the designed primers. (Fig.1 STEP1). This will fuse your ORF to the 5' adaptor sequence for subsequent OE-PCR. Assemble the PCR reaction mixture in a volume of e.g. 50 µl and use high fidelity DNA polymerase (Jena Bioscience, **Cat. No. PCR-204/207**) with template concentration of typically 1 ng/µl, primer concentration of 250 nM and 30 cycles.

- Perform OE-PCR with the purified SITS fragment provided in the kit and the **PCR-STEP1** product as the overlapping templates (Fig.1, STEPS 2 and 3). The PCR products from STEP1 can be used without prior purification*. For OE-PCR Jena Bioscience Taq polymerase (**Cat. No. PCR-202**) is recommended since the reaction buffer is compatible with the LEXSY cell-free translation reaction and allows subsequently direct translation of the crude OE-PCR mixture. For set-up of the OE PCR reaction and thermal cycling conditions see tables 1 and 2.

Reagent	Stock concentration	Final concentration	Volume for 50 µl
water			ad 50 µl
PCR buffer	10x	1x	5 µl
dNTPs	10 mM	0.2 mM	1 µl
forward primer 1 provided (H4134)	10 µM	0.5 µM	2.5 µl
user generated reverse primer	10 µM	0.5 µM	2.5 µl
user generated PCR-STEP1 fragment	1x	1/20 diluted crude reaction or 5 nM purified fragment*	2.5 µl or varying
SITS fragment provided	95nM	5 nM	2.6
Taq DNA polymerase	5 U/µl	2.5 U/100 µl	0.25 µl

Tab. 1: OE-PCR reaction mixture using Taq Pol (Jena Bioscience Cat.-No. PCR-202)

Cycle step	Temp.	Time	Number of cycles
Initial denaturation	95 °C	3 min	1
Denaturation	94 °C	30 sec	30
Annealing	50 °C	30 sec	
Extension	72 °C	1 min per 1kb	
Final extension	72 °C	5 min	1

Tab. 2: OE-PCR reaction conditions using Taq Pol (JBS Cat.-No. PCR-202)

*** *Optionally:*** in some cases gel purification of the user generated PCR-STEP1 fragment is recommended to increase the yield of the OE-PCR product. In this case concentration of template and primers in PCR-STEP1 reaction can be enhanced. Measure the concentration of the purified fragment spectrophotometrically, calculate its molar concentration using the formula below and use the purified fragment at 5 nM concentration in OE PCR instead of the non purified as shown in table 1.

$$\frac{conc(ng/\mu l)}{length(N,bp)\times 0.68}\times 1000 = conc(nM)$$

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- Analyse the PCR products obtained in the PCR-1 and OE-PCR on an agarose gel (see Fig. 2 for examples of typical PCR products). Approximately 200 nM OE-PCR product is required as template for subsequent *in vitro* translation as described below.

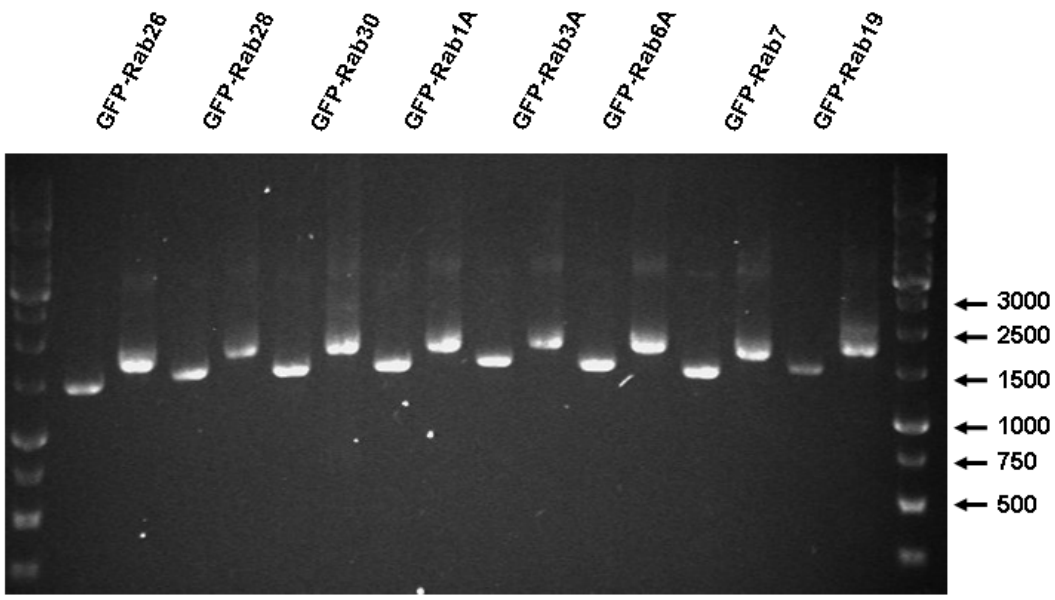

Fig. 2: Electrophoretical analysis of PCR-STEP1 fragments (resp. left lane) and corresponding full-length DNA templates obtained in OE-PCR (resp. right lane). For each GFP-Rab encoding template 2 µl of each PCR reaction mixture were resolved on 0.8 % TAE agarose gel.

3.2 Cell-free translation

The typical **reaction volume** for translation is 20 µl. The yield of *in vitro* synthesized EGFP in the system programmed with the EGFP template is approx. 200 µg/ml. The amount of synthesized EGFP protein in 10 µl of loaded reaction is sufficient to be clearly visible on a SDS-PAGE gel viewed under unfiltered UV light of a transilluminator (ATTENTION! Do not boil the sample - this step will destroy the EGFP chromophore).

The incubation time of *in vitro* translation reactions is typically 2 hours. The user must take into account that some polypeptides need additional time to fold into active protein. After translation the sample can be stored at 4 °C for several hours or overnight for protein maturation. The low protease activity of the extracts allows extended incubation of many translated proteins in the translation mixture at 4 °C without detectable protein degradation.

The incubation temperature of the translation reaction is a compromise in-between protein yield and folding efficiency. Temperature increase improves the total protein yield but usually decreases the fraction of active protein (Fig. 6 Appendix 6.1). We recommend performing standard translation reactions at 20-27°C.

A control reaction without exogenous DNA should be included into each experiment.

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Standard translation protocol

- Thaw the **LTE** extract on ice and keep on ice before use

  Note: keep all reagents on ice during pipetting of the reaction mixes. Thaw the extracts immediately before use and start the reactions within 10 minutes after thawing. Freeze unused extracts in liquid nitrogen and store at –80 °C. Avoid more than two freeze-thaw cycles.

- Assemble the translation reaction mixes on ice according to the table below. Add the LEXSY cell extract in the last step and mix well by pipetting up and down avoiding air bubbles.

- Incubate translation reaction mixes at 20-27°C for 2 hours.

Transcription-translation reaction set-up

Component	Volume per one reaction (µl)	Volume per control reaction (µl)
Nuclease-free water	variable	6.0
Template DNA (20 nM final concentration)	variable	0
LEXSY cell extract	14	14

3.3 Detection of *in vitro* synthesized proteins

Translation of target proteins can be detected by SDS-PAGE and Western blotting. Fusion proteins incorporating EGFP can be detected directly in the SDS-PAGE gel by *in situ* fluorescence scanning.

For detection of EGFP control protein and EGFP fusion proteins

- Mix 10 µl of translation mix with 10 µl of 2X SDS loading buffer. **Do not boil the sample!**

- Resolve the sample on a 12% PAGE gel. **Do not subject the gel to staining or fixation!**

- Visualize EGFP by fluorescence scanning (EGFP Ex/Em 488/507 nm) or view it on the UV transilluminator (see Fig. 3 for typical results).

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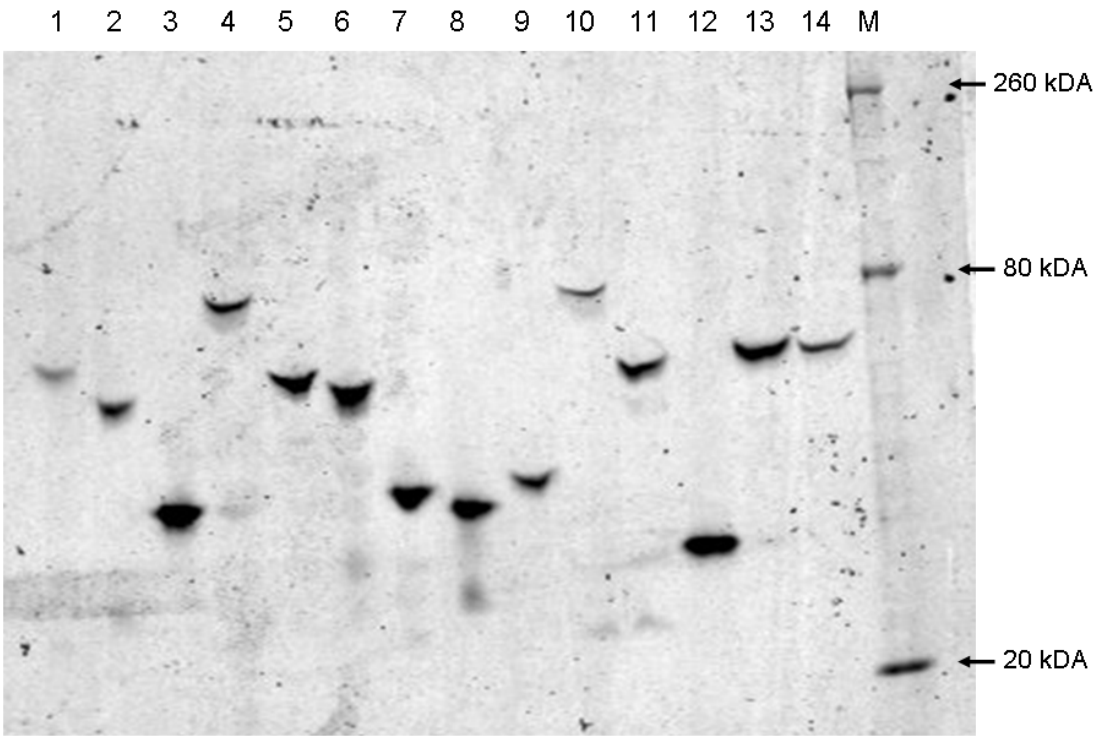

Fig. 3: Visualization of EGFP-tagged translation products resolved on a SDS-PAGE gel by fluorescent scanning. DNA templates encoding a set of C' EGFP tagged proteins (PP2B phosphatases (lanes 1, 2), eIF1a (lane 3), eIF4G (lane 4), eIF4a (lanes 5, 6), eIF4e (lane 7), eIF5a (lane 8), eIF6 (lane 9), PABP (lane 10), eEF2 kinase (lane 11), eIF SUI (lane 12), eIF2α (lane 13), DHH1 (lane14)) were translated in the LEXSY *in vitro* translation system and 5 µl of end point reaction mixture were resolved on a 4-12% PAA gel. The EGFP-fluorescence was detected on a scanner with 488 nm excitation laser and 520 nm emission filter.

3.4 Isolation of *in vitro* synthesized proteins

Recombinant proteins produced in the LEXSY *in vitro* translation kit can be easily isolated by affinity chromatography. Below we provide a protocol for one-step isolation of GFP tagged proteins using **GFP-Cap matrix** that displays picomolar affinity to GFP protein. See Fig. 4 for typical purification results.

To isolate GFP-tagged proteins from translation mixture

- Increase ionic strength in the sample by adjusting NaCl concentration to 150 mM
- Add 10-20 µl of GFP-Cap S resin to the sample and incubate with gentle rotation for 20 minutes at 4°C. Do not allow beads to settle
- Centrifuge 2 min, 2000 x g, 4°C. Discard the supernatant
- Wash resin 2-3 times with washing buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA). Sediment beads and discard the supernatant as described above
- To analyze the bound protein, resuspend the resin in 20 µl of 1x SDS-loading buffer and boil for 5 minutes. The protein can be eluted from the matrix by lowering the pH. Add 50 µl of 0.1 M Gly-HCl pH 2.2 to the resin and incubate for 20 seconds. Pellet the beads at 2000 x g for 30 seconds and collect supernatant with eluted protein. Neutralize the supernatant with 1/10 volume of 1 M Tris base.

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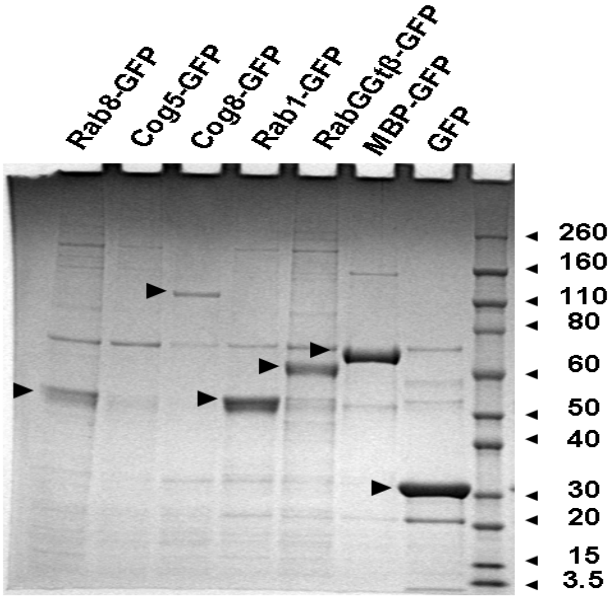

Fig. 4: SDS-PAGE analysis of *in vitro* translated proteins isolated with the GFP-Cap resin (Coomassie staining). Each translation reaction mixture (150 µl) was programmed with 20 nM of ethanol-precipitated DNA template. The products were isolated with 20 µl of GFP-Cap beads, eluted with 20 µl of SDS-loading buffer and resolved on 4-12% PAGE. Target proteins listed on top of the gel.

Proteins harbouring a HexaHistidine-tag can be purified by standard chelating metal affinity chromatography (see Fig. 5 for comparison of Ni- and Co-NTA technologies).

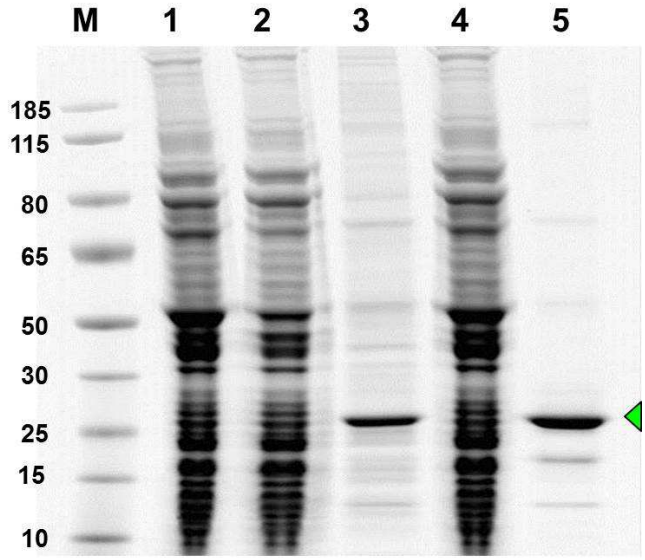

Fig. 5: SDS-PAGE analysis of an *in vitro* translated protein isolated with Ni- or Co-NTA matrices (Coomassie staining). Lane 1 = total extract, lanes 2 & 4 = flow-through, lanes 3 & 5 eluates. Lanes 2 & 3 = Ni-NTA, lanes 4 & 5 = Co-NTA. Green triangle = eluted target protein.

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4. Licensing information

Purchase of the **LEXSY *in vitro* Translation Kit** includes a non-exclusive and non-transferable license for non-commercial research. Commercial use of the **LEXSY *in vitro* Translation Kit**, however, requires separate licensing.

Commercial use includes but is not limited to:

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

Please contact us at expression@jenabioscience.com.

5. Literature

Horton *et al*. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77:** 61

Kovtun *et al.* (2010) Towards the Construction of Expressed Proteomes Using a *Leishmania tarentolae* Based Cell-Free Expression System. *PLOS one* **5:** e14388

Kovtun *et al.* (2011) *Leishmania* cell-free protein expression System. *Methods* **55:** 58

LeBowitz *et al*. (*1993)* Coupling of polyadenylation site selection and trans-splicing in *Leishmania*. *Genes & Dev*. **7**: 996

Mureev *et al.* (2009) Species-independent translational leaders facilitate cell-free expression. *Nature Biotechnology* **27:** 747

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

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6 Appendix

6.1 Influence of temperature on *in vitro* EGFP synthesis

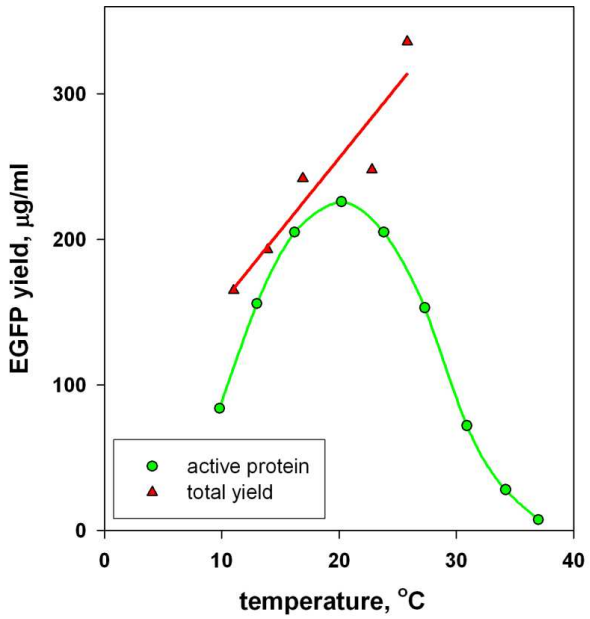

Fig. 6: Influence of temperature on EGFP synthesis and folding efficiency in the LEXSY cell-free translation system. The yield of total full-sized EGFP was evaluated by Western blotting; the yield of properly folded EGFP was evaluated with direct fluorescence measurement.

6.2 Sequences of the SITS-fragment and forward oligonucleotide primer for OE-PCR

SITS-fragment	5'GGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAA CAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAT ACGACTCACTATAGGGACATCTTAAGTTTATTTTATTTTATTTTATTTTATTTTAT TTTATTTTATTTTATTTTATTTTATTTTATTTAACCATGACAGTAATGTATAAAGT CTGTAAAGACATTAAACACGTAAGTGA-3'	
Primer 1 forward primer for OE-PCR H4134	5' GGGTTATTGTCTCATGAGCGG-3'	**Cat. No. PM-117**

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