LEXSY in vitro Translation Kit

Cat#: orb532691 (User Manual)

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. For target mRNA generation the heterologous T7 RNA polymerase was added to the extracts. 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

- 1 vial with 250 ml 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 pLEXSY_invitro-2 vector

- 5 µg in 20 ml of 10 mM Tris HCl pH 8.0 (= 107 nM); may vary, see label
- store at -20°C

2.3 Sequencing primers (see Appendix 6.4 for sequences)

· **PM-116** forward sequencing primer H4132
· **PM-101** reverse sequencing primer A264
- 50 mM in 50 ml 10 mM Tris HCl 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.

3 Protocol section

3.1 Template preparation by cloning into pLEXSY_invitro-2 vector

The map and features of the **pLEXSY_invitro-2** vector are provided in Appendix 6.1-6.2 This vector contains the EGFP gene which can be used as a positive control for transcription/translation reactions. The vector incorporates the species independent translation initiation sequence (**SITS**) upstream of the EGFP ORF. **SITS** consists of a poly-TTTTA 5' UTR 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 synthesis in the LEXSY *in vitro* system.

The **pLEXSY_invitro-2** vector offers three cloning strategies:

1. replacement of vector EGFP control gene by target gene
2. fusion of target gene to N' of vector EGFP gene
3. fusion of target gene to C' of vector EGFP gene

To clone the target protein coding sequence into the **pLEXSY_invitro-2** vector:

· Perform PCR amplification of the gene of interest with primers containing the chosen cloning sites of MCS1 and/or MCS2, e.g. NcoI x NotI. **Make sure that insertion is in frame with SITS**. For incorporation of affinity tags into the C-terminus of the recombinant proteins, include the appropriate sequences into the reverse primer. For N' EGFP fusions insert the target gene into the MCS1 (NcoI-XhoI polylinker) of the **pLEXSY_invitro-2** vector. For C' EGFP fusions insert the target gene into the MCS2 (HindIII-SmaI polylinker)

· Open the **pLEXSY_invitro-2** vector with the appropriate restriction enzymes and clone the PCR product into the vector by standard techniques

· Prepare plasmid DNA with a column based kit. We recommend to add RNAse inhibitor or phenol-chloroform purify the template prior to the *in vitro* reaction

· Adjust the plasmid concentration to approx. 200 nM. To do this, measure the DNA concentration spectrophotometrically and calculate its molar concentration using the formula below

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

· Use the DNA as a template for the *in vitro* translation reaction at final concentration of 20 nM as described below.

3.2 Cell-free translation

The typical **reaction volume** for translation is 20 ml. The yield of *in vitro* synthesized EGFP in the system programmed with the EGFP control plasmid **pLEXSY_invitro-2** is approx. 200 mg/ml. The amount of synthesized EGFP protein in 10 ml 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 between protein yield and folding efficiency. Temperature increase improves the total protein yield but usually decreases the fraction of active protein (Appendix 6.3). We recommend performing standard translation reactions at 20-27°C.

Positive and negative control reactions with pLEXSY_invitro-2 vector or without exogenous DNA resp. should be included into each experiment.

Standard translation protocol

· Thaw the LEXSY cell 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 with liquid nitrogen or on dry ice and store at –80 °C. Avoid more than two freeze-thaw cycles.
· Assemble the translation reaction mixes in final volume of 20 ml 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 reaction (µl)	Volume per positive control reaction (µl)	Volume per negative control reaction (µl)
Nuclease-free water	variable	2	6
Template DNA (20 nM final concentration)	variable	4	0
LEXSY cell extract	14	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 resulting from in frame fusions to EGFP can be detected directly on the SDS-PAGE by *in situ* fluorescence scanning. The **pLEXSY_invitro-2** vector also includes a sequence for the myc-tag that can be detected by Western blotting with specific antibodies.

For detection of EGFP control protein and EGFP fusion proteins
· Mix 10 ml of translation mix with 10 ml 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. 1 for typical results).

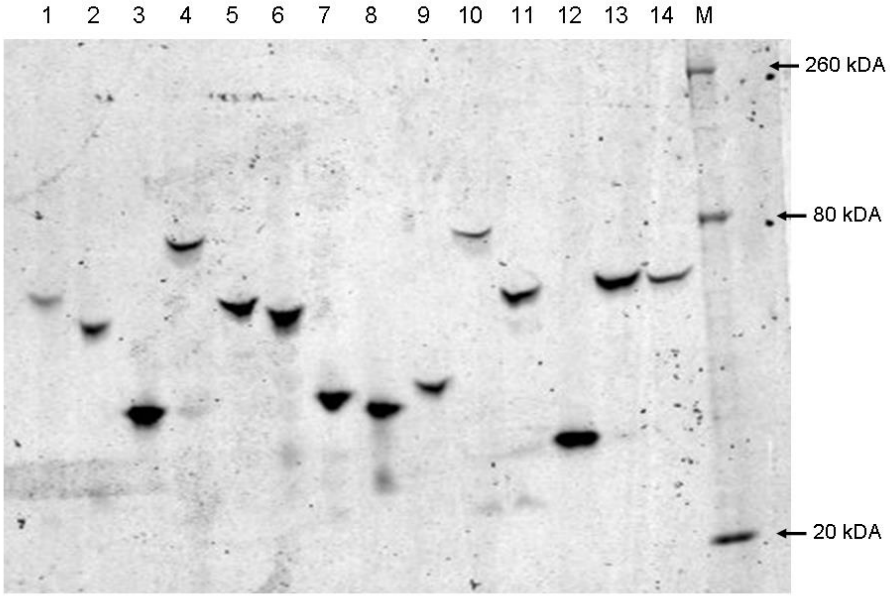

Fig. 1: Visualization of EGFP-tagged translation products resolved on a SDS-PAGE gel by fluorescent scanning. DNA templates encoding a set of C' EGFP tagged proteins (PP2B phosphatases (lanes 1, 2), eIF1a (lane 3), eIF4G (lane 4), eIF4a (lanes 5, 6), eIF4e (lane 7), eIF5a (lane 8), eIF6 (lane 9), PABP (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 ml 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 with 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 a matrix that displays picomolar affinity to GFP protein. See Fig. 2 for typical purification results.

To isolate GFP-tagged proteins from translation mixture
· Increase the ionic strength in the sample by adjusting NaCl concentration to 150 mM

· Add 10-20 ml of GFP binding 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 ml of 1x SDS-loading buffer and boil for 5 minutes. The protein can be eluted from the matrix by lowering the pH. Add 50 ml 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

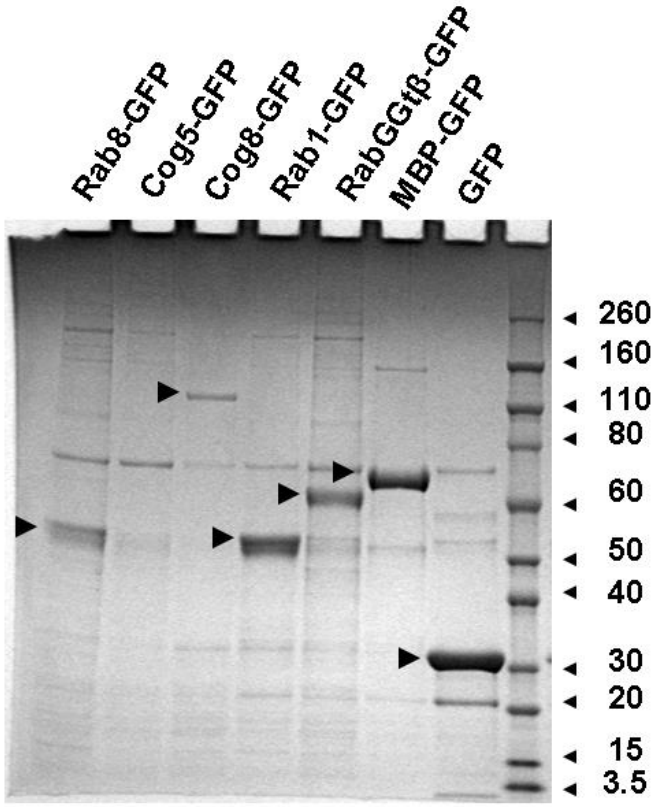

Fig. 2: SDS-PAGE analysis of *in vitro* translated proteins isolated with a GFP binding resin (Coomassie staining). Each translation reaction mixture (150 ml) was programmed with 20 nM of ethanolprecipitated DNA template. The products were isolated with 20 ml of GFP binding beads, eluted with 20 ml of SDSloading 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. 3 for comparison of Ni- and Co-NTA technologies).

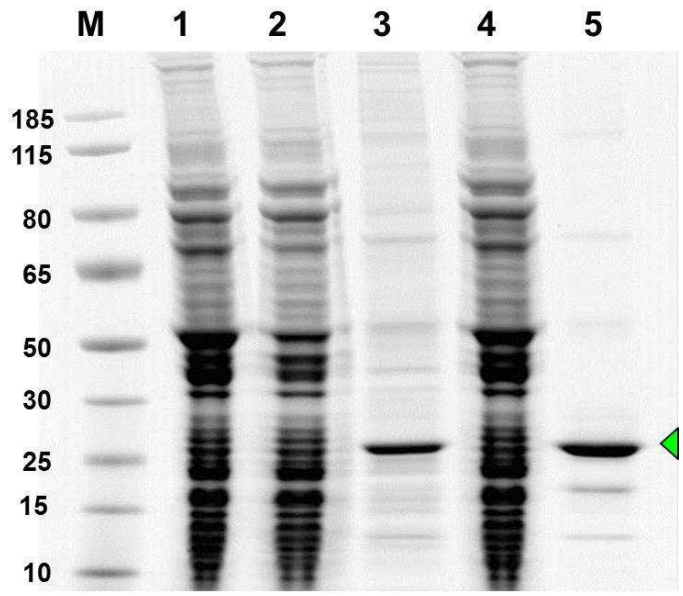

Fig. 3: SDS-PAGE analysis of an *in vitro* translated protein isolated with Ni- or Co-NTA matrices (Coomassie staining). Lane 1 = total extract, lanes 2 & 4 = flow-through, lanes 3 & 5 eluates. Lanes 2 & 3 = Ni-NTA, lanes 4 & 5 = Co-NTA. Green triangle = eluted target protein.

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

5 Literature

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

6 Appendix

6.1 Map of vector pLEXSY_invitro-2

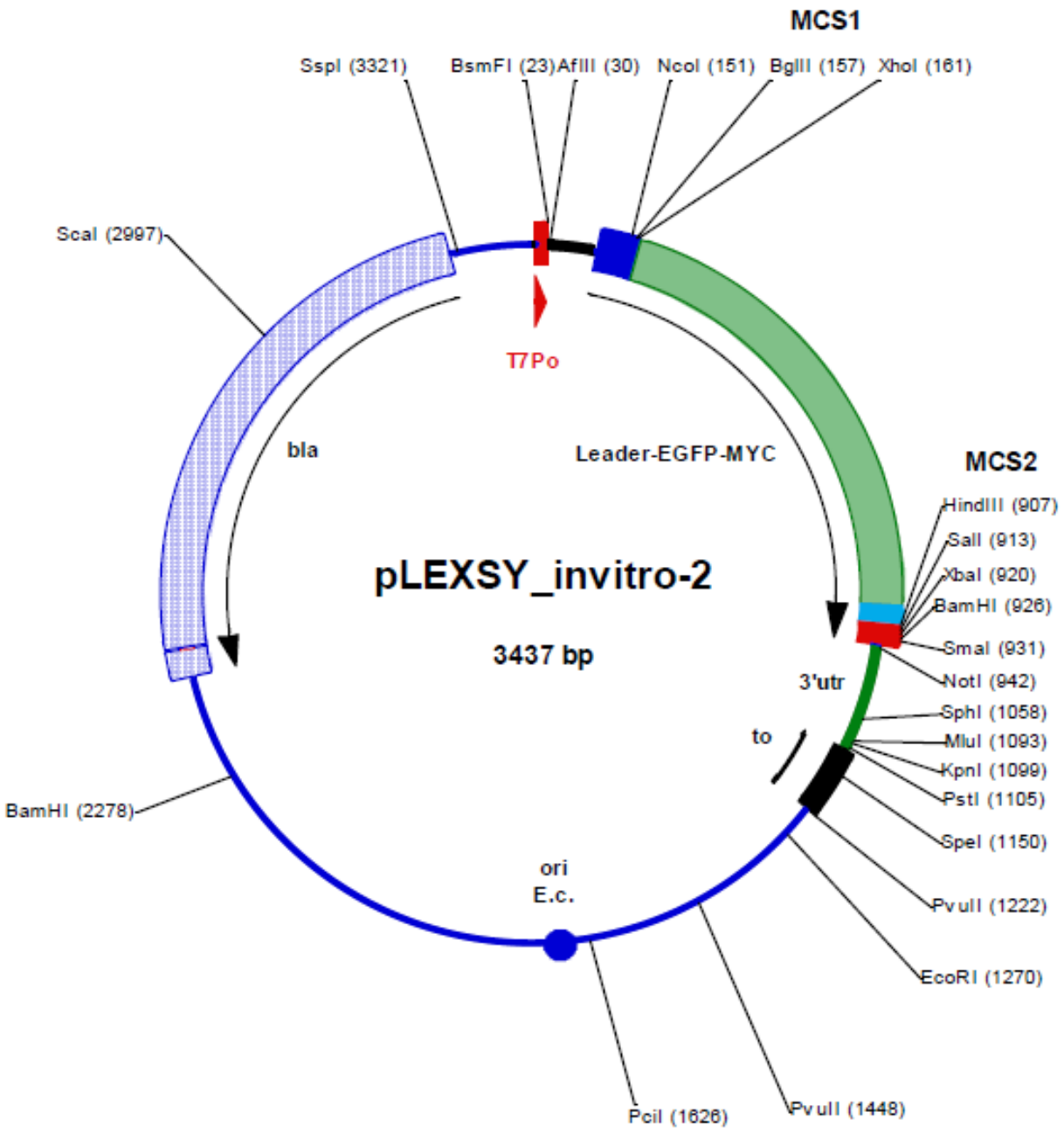

Fig. 4: **Map of pLEXSY_invitro-2 expression vector** with cloning sites for target gene in frame fusions (see Appendix 6.2 and 6.3 for sequence and description). 3' utr is derived from 1.4k-IR *camCB* of *L. tarentolae*. MCS1 and MCS2 are multicloning sites for replacement of vector EGFP control gene or N' or C' fusions resp. SITS is species independent translation sequence (Mureev *et al.,* 2009 and Appendix 6.2.). See text for details.

6.2 Architecture of SITS and EGFP gene

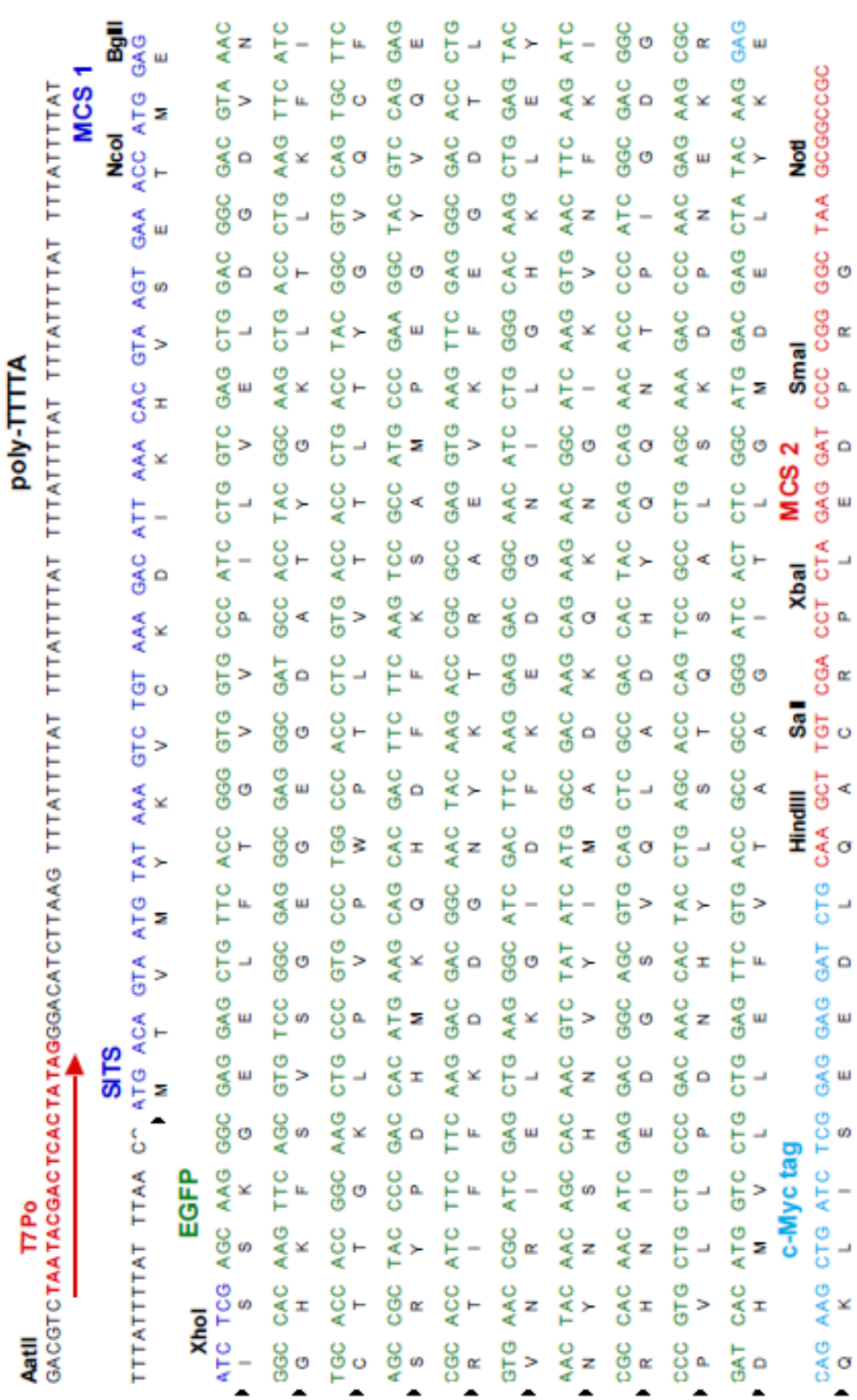

Fig. 5: DNA sequences of SITS and EGFP gene. MCS1 and MCS2 are multicloning sites for replacement of vector EGFP control gene or N' or C' fusions resp. SITS is species independent translation sequence (Mureev *et al.,* 2009).

6.3 Influence of temperature on *in vitro* EGFP synthesis

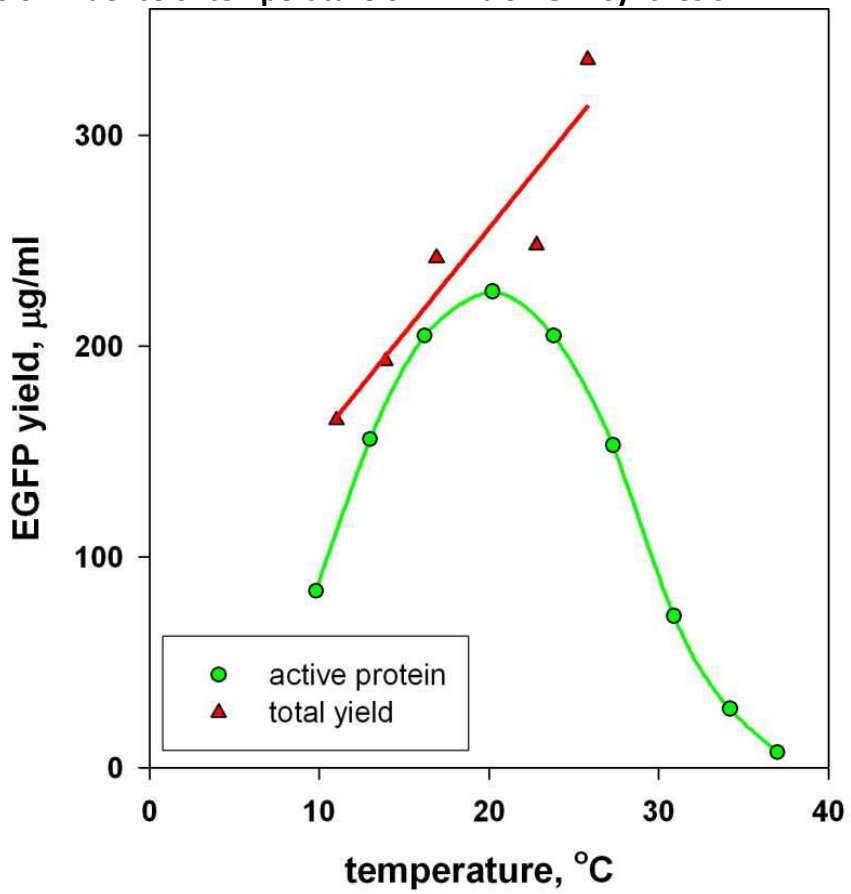

Fig. 6: Influence of temperature on EGFP synthesis and folding efficiency in the LEXSY cell-free translation system. The yield of total full-sized EGFP was evaluated by Western blotting; the yield of properly folded EGFP was evaluated with direct fluorescence measurement.

6.4 Sequences of the primers for LEXSY *in vitro* Translation Kit

Insert sequencing forward primer **H4132**	Anneals to SITS leader sequence	5'-CATGACAGTAATGTATAAAGTC-3'
Insert sequencing reverse primer **A264**	Anneals to 3' utr 1.4k camCB	5'-CATCTATAGAGAAGTACACGTAAAAG-3'