BIT's 5th Anniversary of
PepCon-2012
The Dedicated Event for Protein/Peptide Professionals
Protein and Peptide Conference
Theme: Powerful Proteins and Peptides
Time: March 23-25, 2012 Venue: Beijing International Convention Center, China

LEXSY

the eukaryotic protein expression platform based on the protozoan organism *Leishmania tarentolae*

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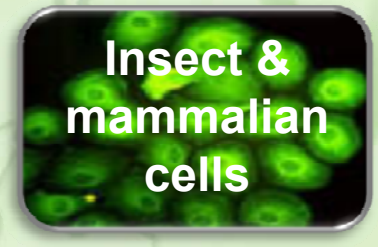
Shortcomings of conventional expression systems require alternative solutions

- Insufficient folding of complex proteins of higher organisms
- Inclusion bodies
- Lack of post-translational modifications

- Posttranslational modifications differ largely from mammalian cells (high mannose)
- Problematic cell disruption

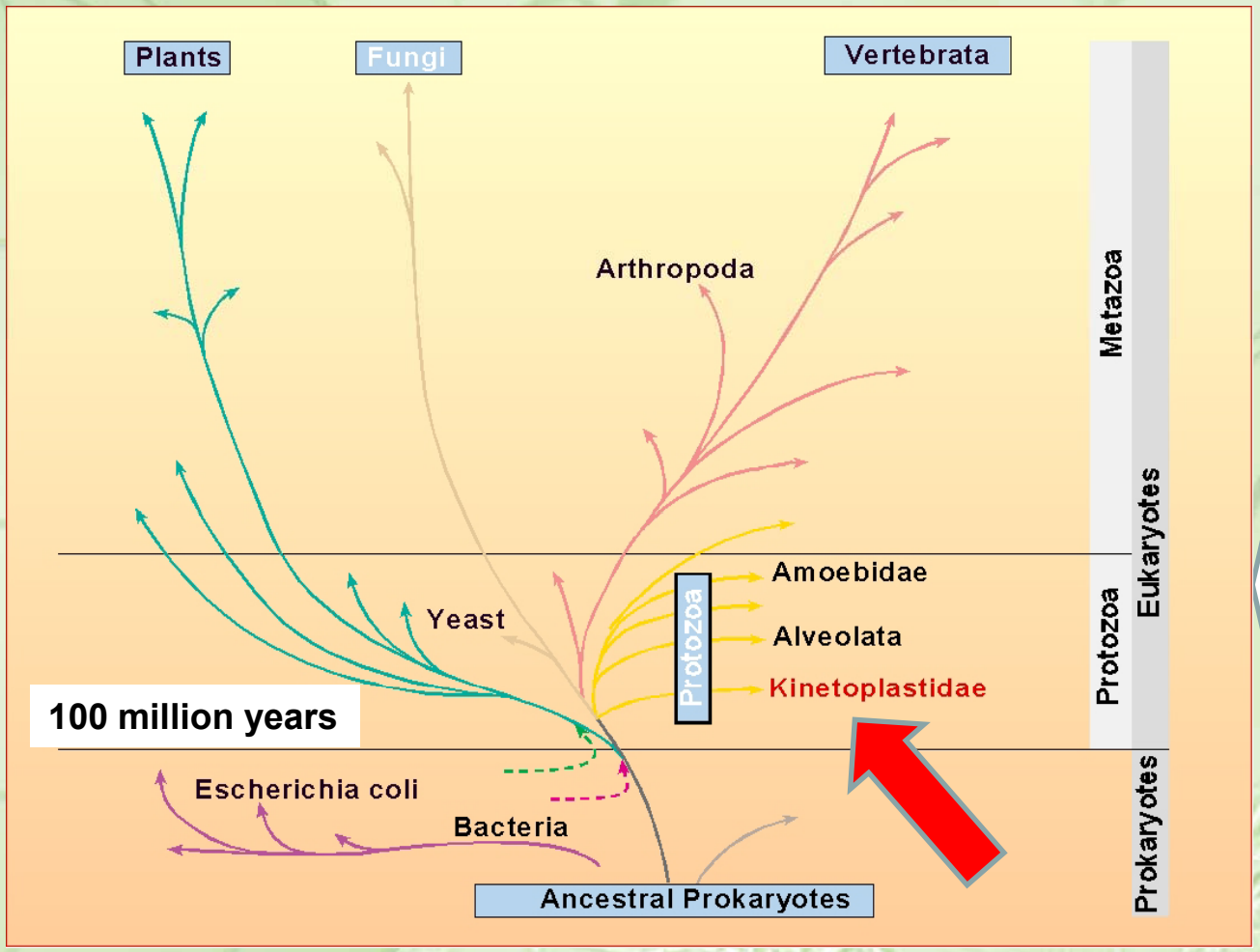


- Laborious construction of over-expressing strains
- Expensive media
- Low growth rates
- Difficult scale-up

- Long developmental cycles
- Complex downstream processing
- Contamination problems (viruses)

We need an expression system with eukaryotic machinery but bacterial robustness

Protozoa link the pro- and eukaryotic world



- **Fully eukaryotic protein folding and modification**
- **molecular genetic manipulation developed**
- **Genome sequencing initiatives**

Promising candidates among *Kinetoplastidae*

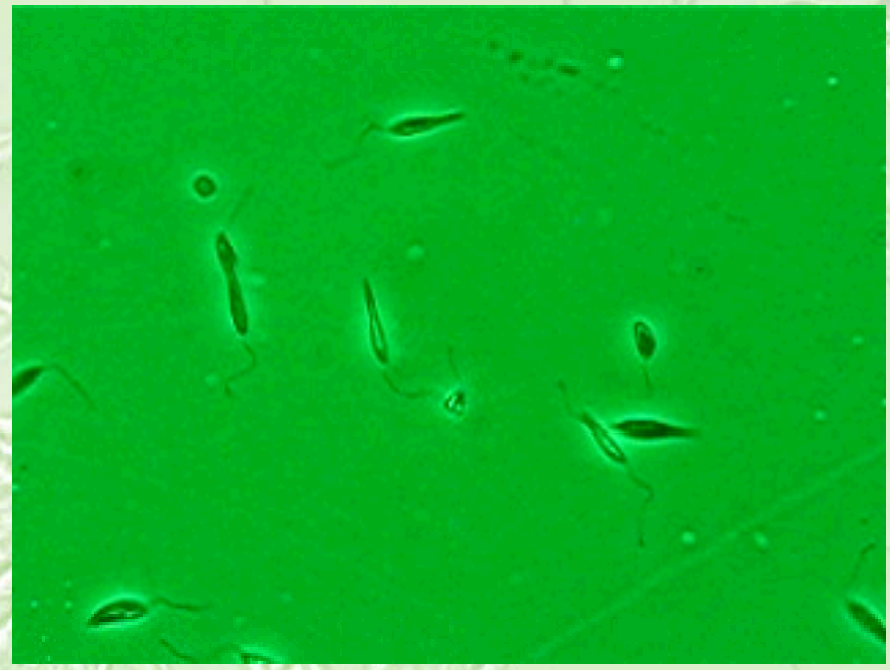
Leishmania tarentolae was chosen as expression host

Host: *Tarentola mauritanica*

- unicellular flagellated protozoa
- parasite of lizard (*Sauroleishmania*)
- not pathogenic for mammalians
- biosafety group 1 organism
- can be easily cultivated *in vitro*
- genome sequence published 2012

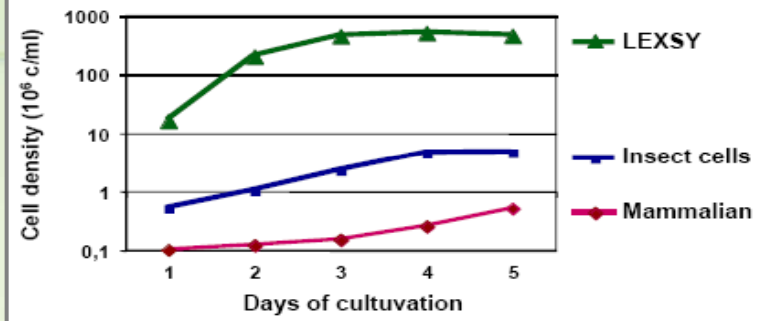
"LEXSY"

(*Leishmania* Expression System)

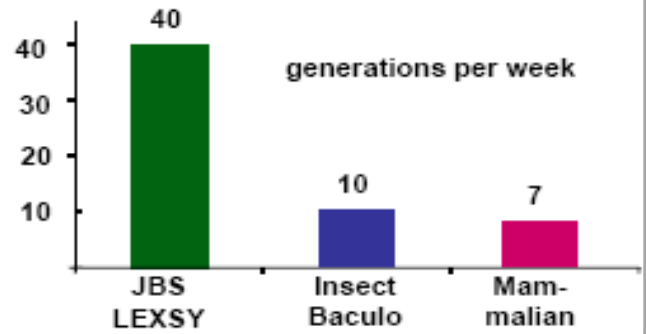


LEXSY combines eukaryotic cellular machinery with bacterial robustness

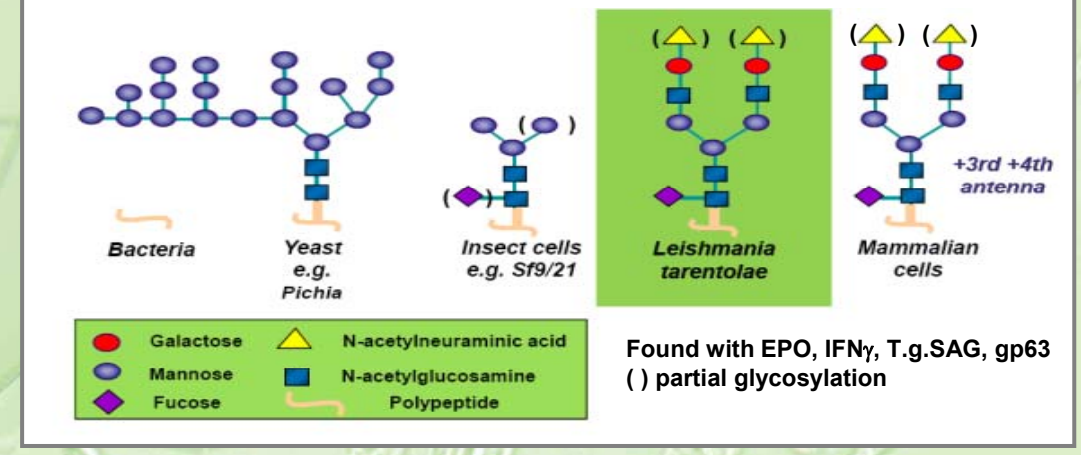
LEXSY grows to higher densities than insect and mammalian cells



LEXSY grows faster than insect and mammalian cells



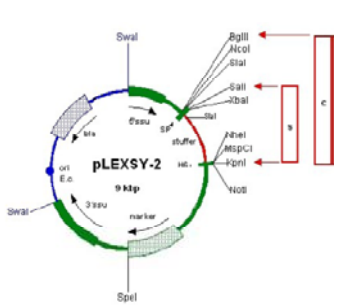
LEXSY performs mammalian-type glycosylation



Found with EPO, IFNγ, T.g.SAG, gp63
( ) partial glycosylation

With LEXSY - in six weeks from gene to protein

gene



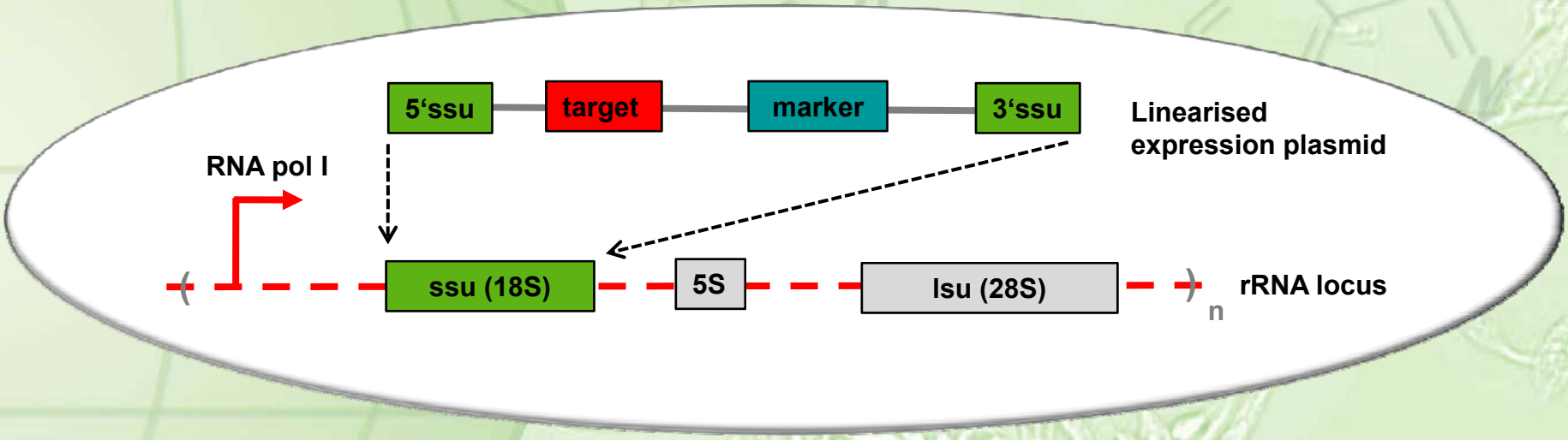
Expression plasmid construction	Transfection & selection	Expression evaluation	Scale-up	Protein purification
Easy cloning in *E. coli* Versatile LEXSY expression vectors	Reliable electropora-tion protocols Clonal or polyclonal selection	Constitutive or inducible Intracellular or secretory Fluoresc. monitoring	Fully adapted to common fermentation technology Up to 100 litres tested	One-step affinity purification Conventional techniques
1 week	2 weeks	1 week	1 week	1 week

protein

LEXSY enables short evaluation cycles

Constitutive expression is the standard application for most proteins

- *ssu* integration (multicopy 18S rRNA cluster)
- transcription by host RNA polymerase I

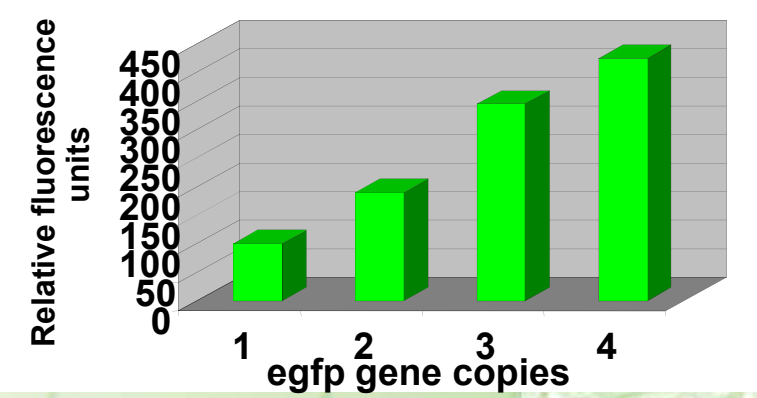


- RNA polymerase I: 10x stronger than RNA Polymerase II
- maximal activity during exponential growth (early product synthesis)
- four selection markers (LEXSY-NTC, -Bleo, -Hyg & -Neo)

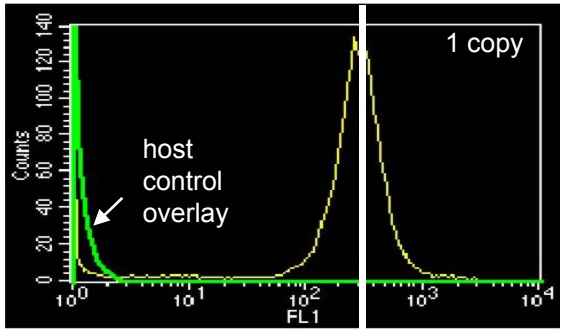
Co-integration of expression cassettes into *ssu* locus

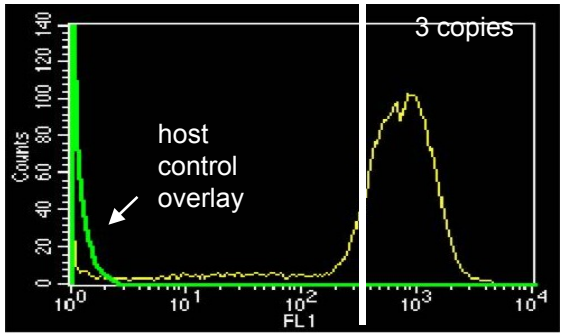
yielded additive effect of protein expression and allowed production of functional multi-subunit proteins

Expression levels

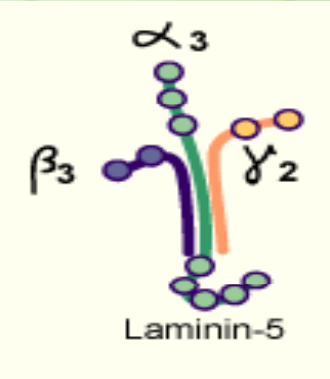


FACS analysis





Also confirmed with eukaryotic target proteins EPO and Cu/Zn SOD



$\alpha 3\beta 3\gamma 2$

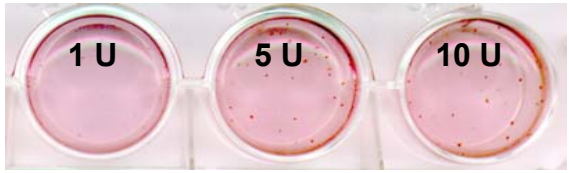
Expression of multi-subunit proteins

Phan *et al.* (2009) **420 kDa** Laminin-332 heterotrimer

Case studie

Human Erythropoietin was exceptionally homogeneously glycosylated in LEXSY

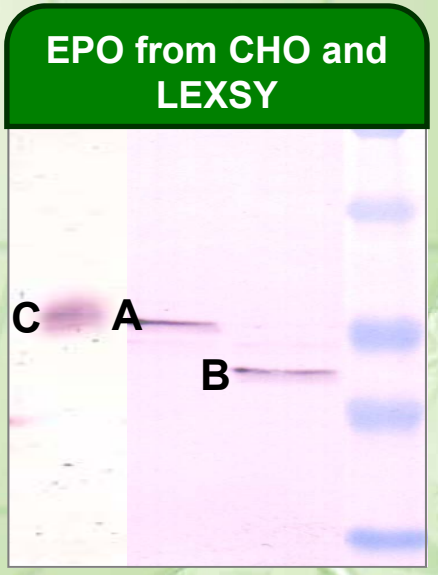
- Completely secreted to the culture medium
- Natively processed at the N-terminus
- Biologically fully active



1.2 x 10^5 U/mg

- Exceptionally homogenously & mammalian-type N-glycosylated

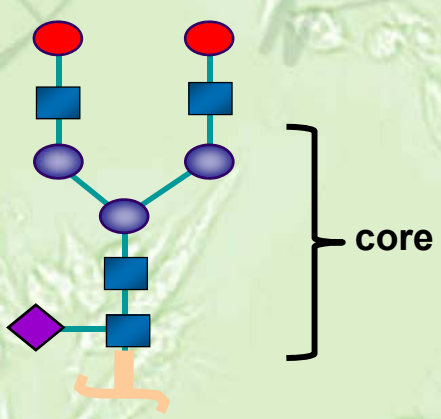
(biantennary fully galactosylated $Man_3GlcNAc_2$core-α-1,6-fucosylated structure)



A: homogenously glycosylated EPO from LEXSY

B: N-deglycosylated EPO from LEXSY

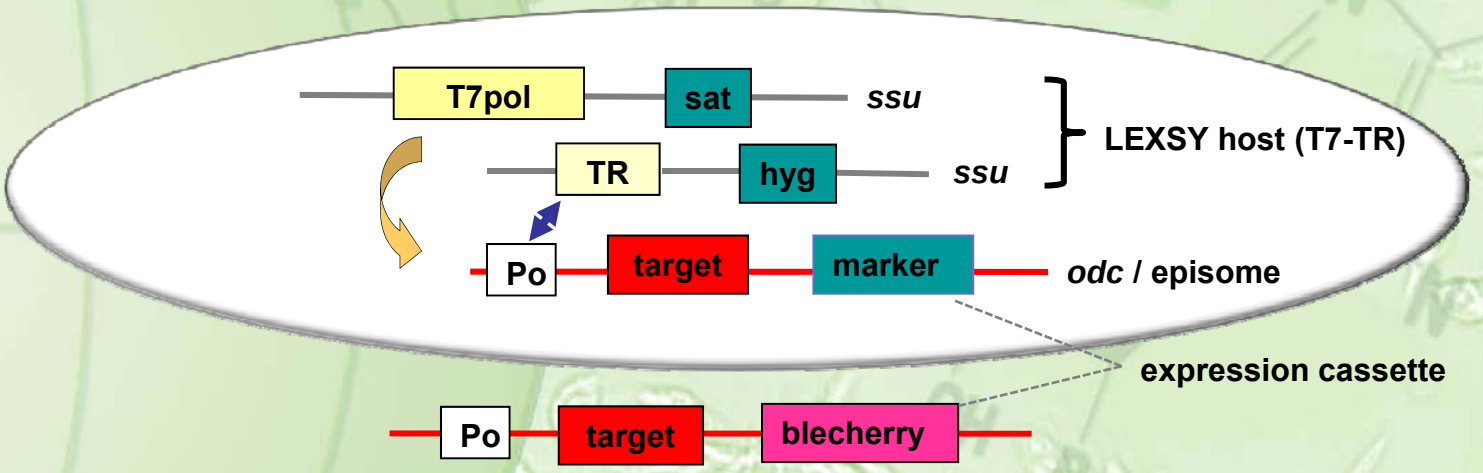
C: heterogenously glycosylated EPO from CHO



- Breitling *et al.* (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expression and Purification* **25**: 209

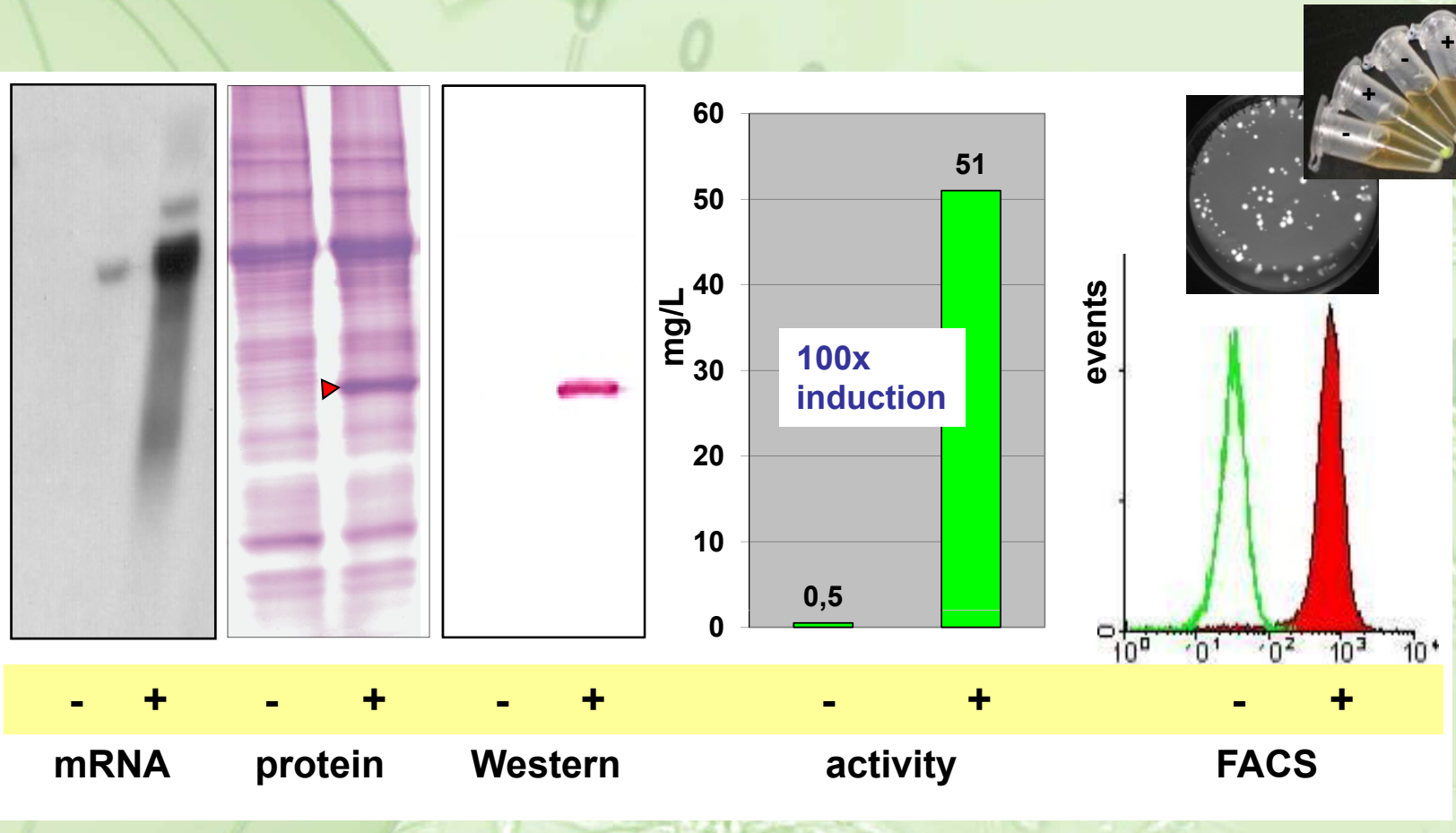
Inducible expression is the application for difficult to express proteins

- *odc* integration or episomal propagation
- transcription by T7 RNA polymerase
- control by TET repressor



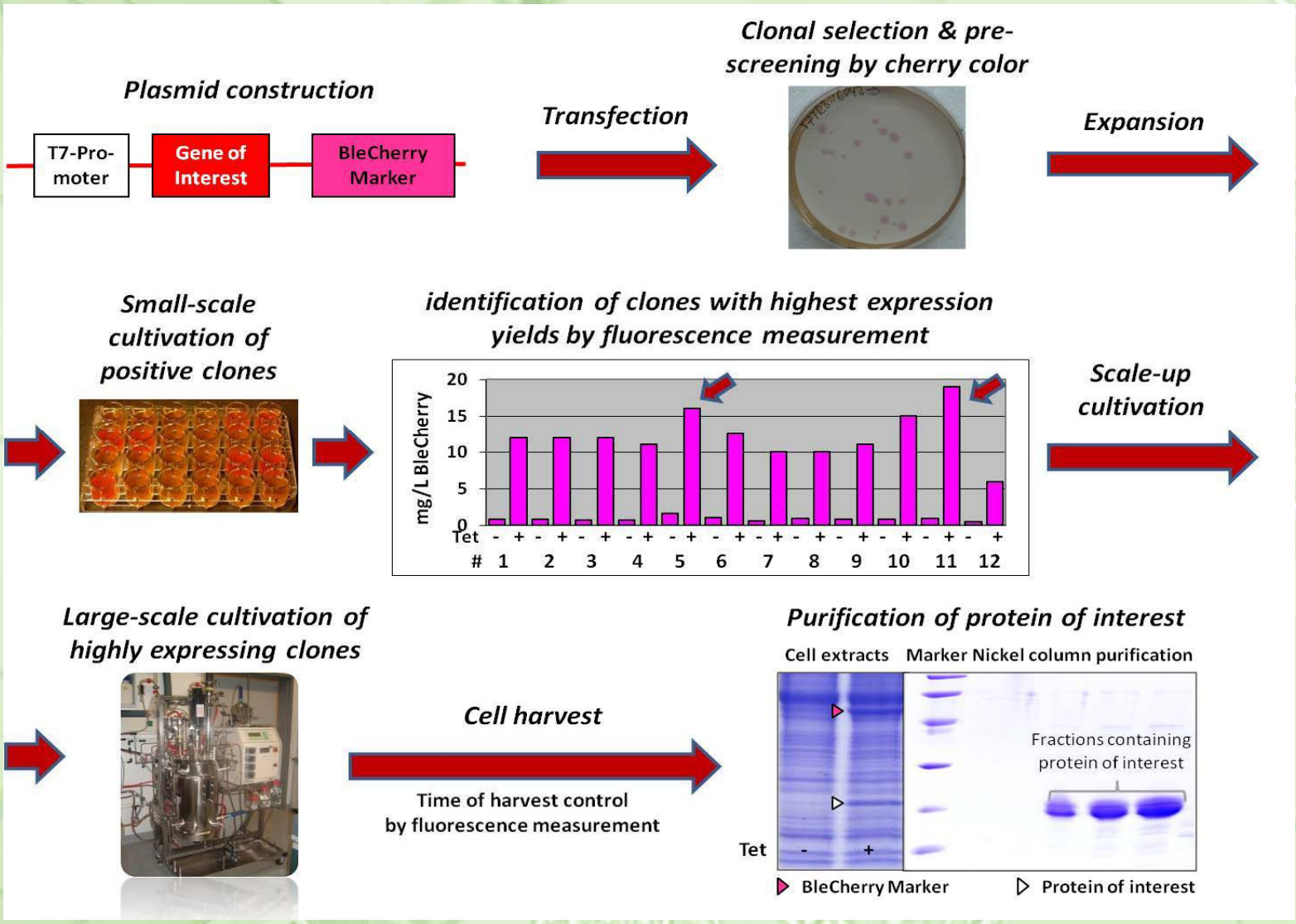
- uncoupling from cellular down-regulation
- much stronger than RNA Pol I
- tetracycline induction at any time during cultivation
- fluorescent marker (blecherry) for in-process monitoring

Efficient expression control in inducible LEXSY from mRNA to protein

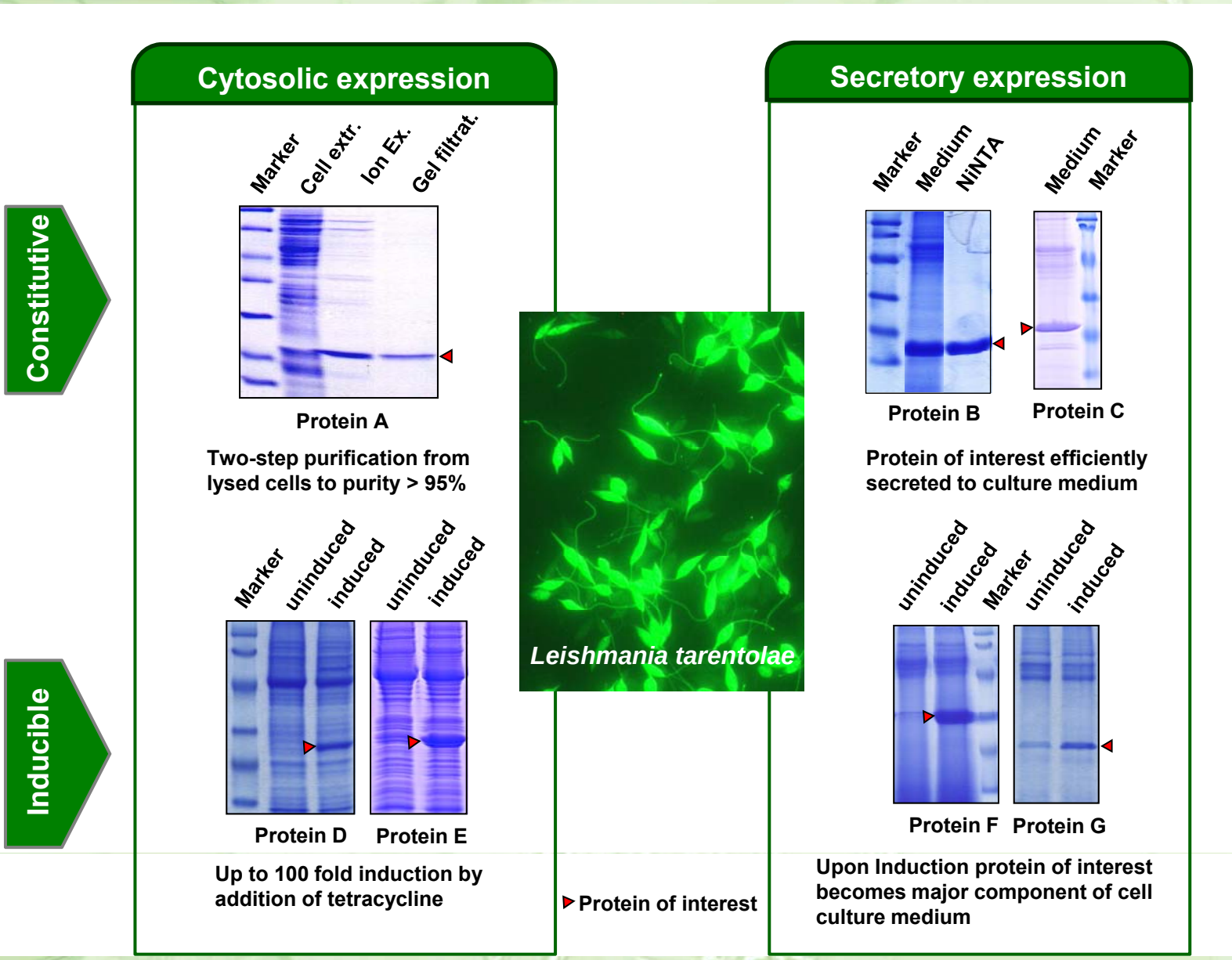


- induction profiles and yields stable over > 500 generations
- all cells induced

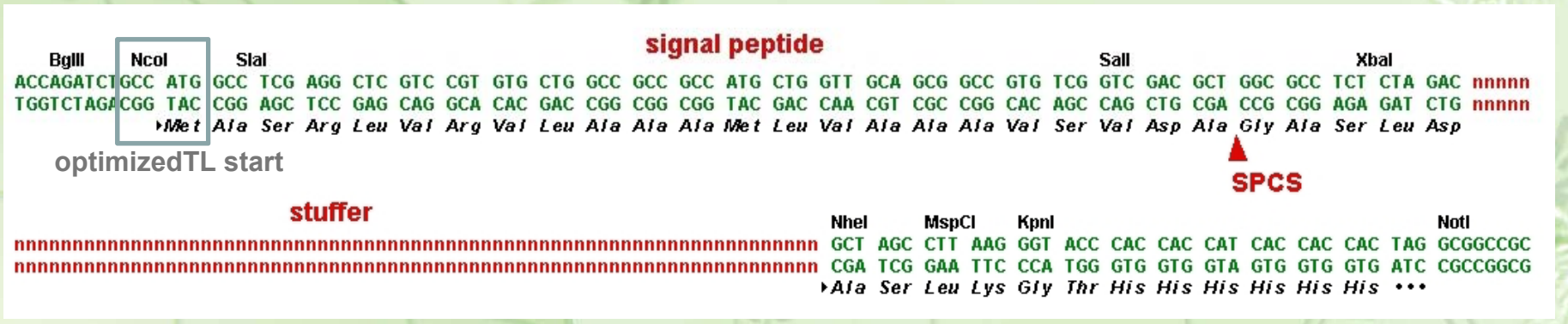
LEXSY BleCherry architecture enables efficient screening and online monitoring of induction



High-level protein expression in all four LEXSY formats



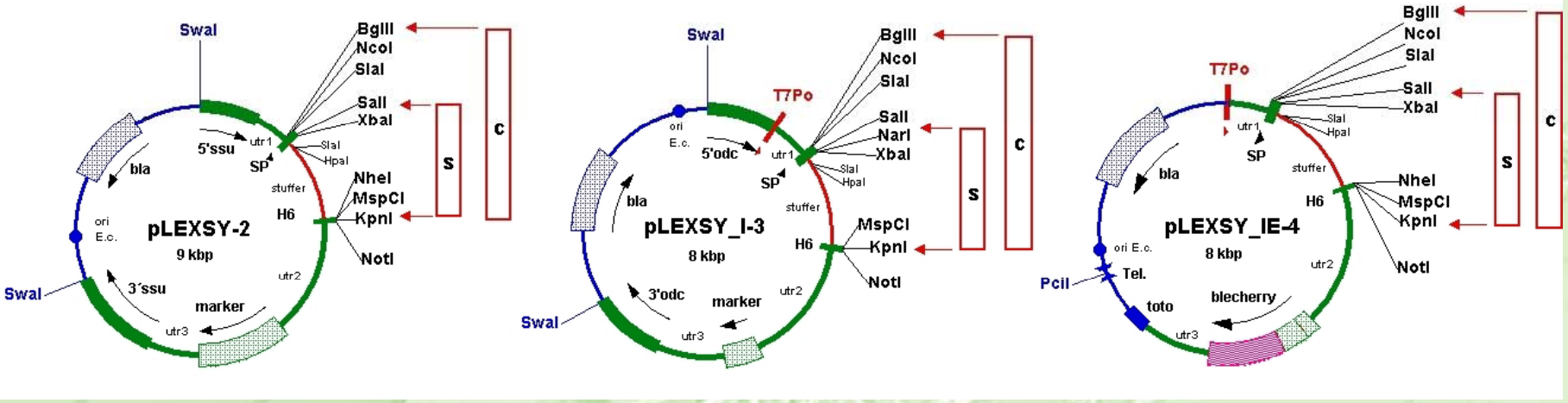
Three LEXSY configurations for intracellular or secretory protein expression



Constitutive integrative

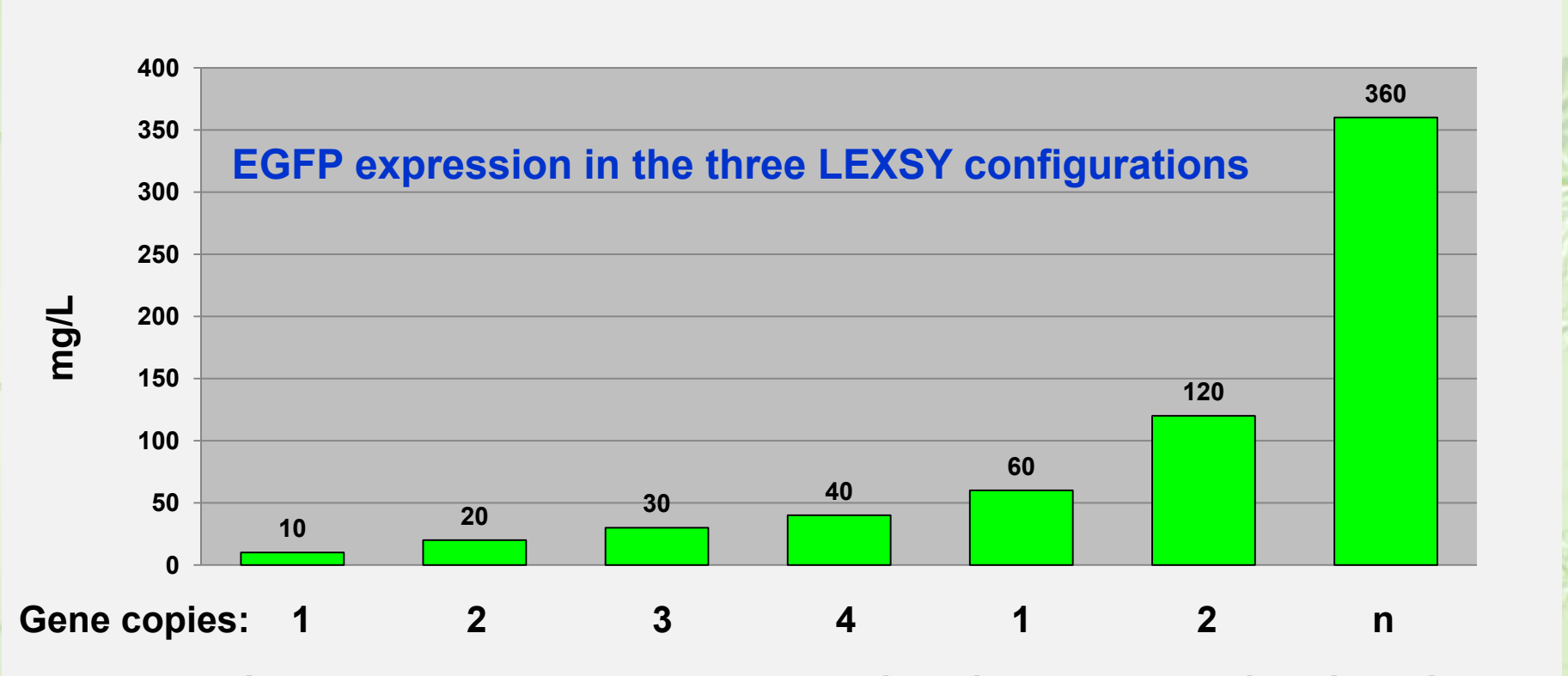
Inducible integrative

Inducible episomal

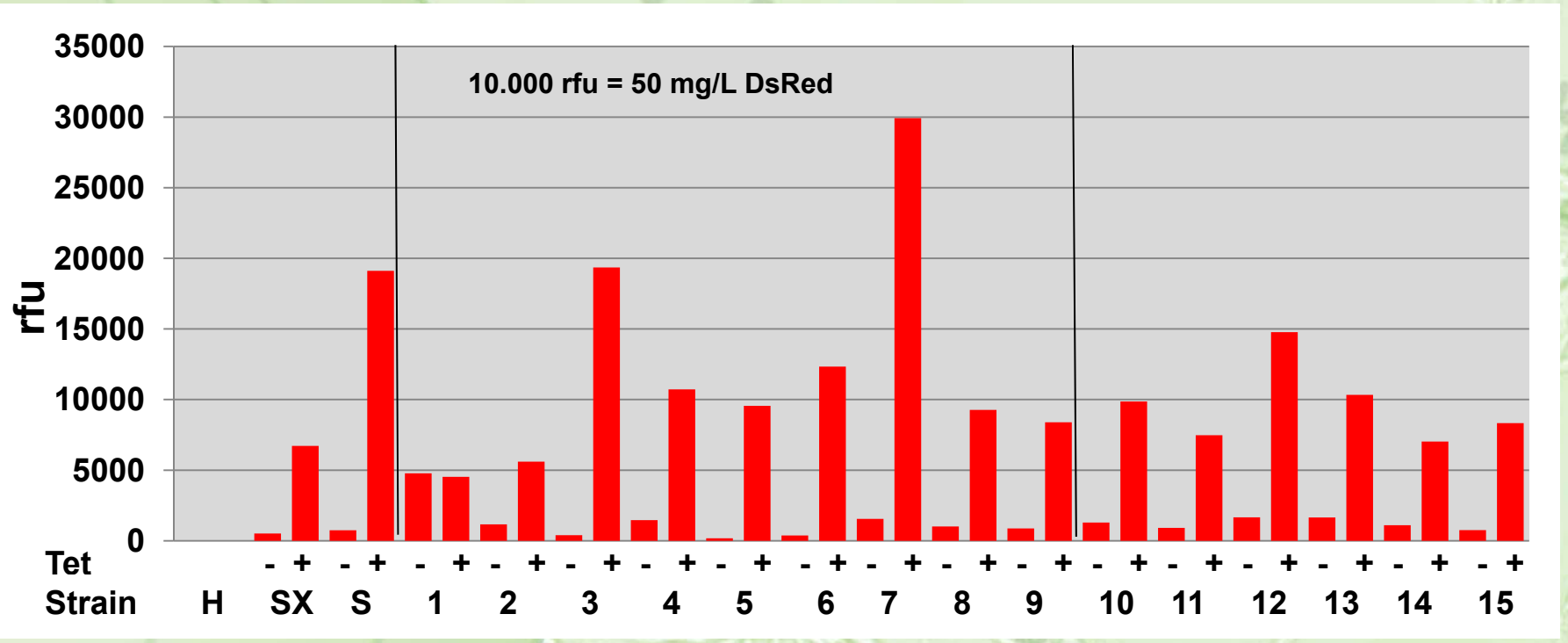


C: integration for cytosolic expression, S: integration for secretory expression
SP: signal peptide of secreted acid phosphatase of *Leishmania mexicana*
SPC: signal peptide cleavage site

Protein expression levels are directly correlated to target gene copy number



Circular inducible episomes delivered the highest yields
but displayed clonal heterogeneity

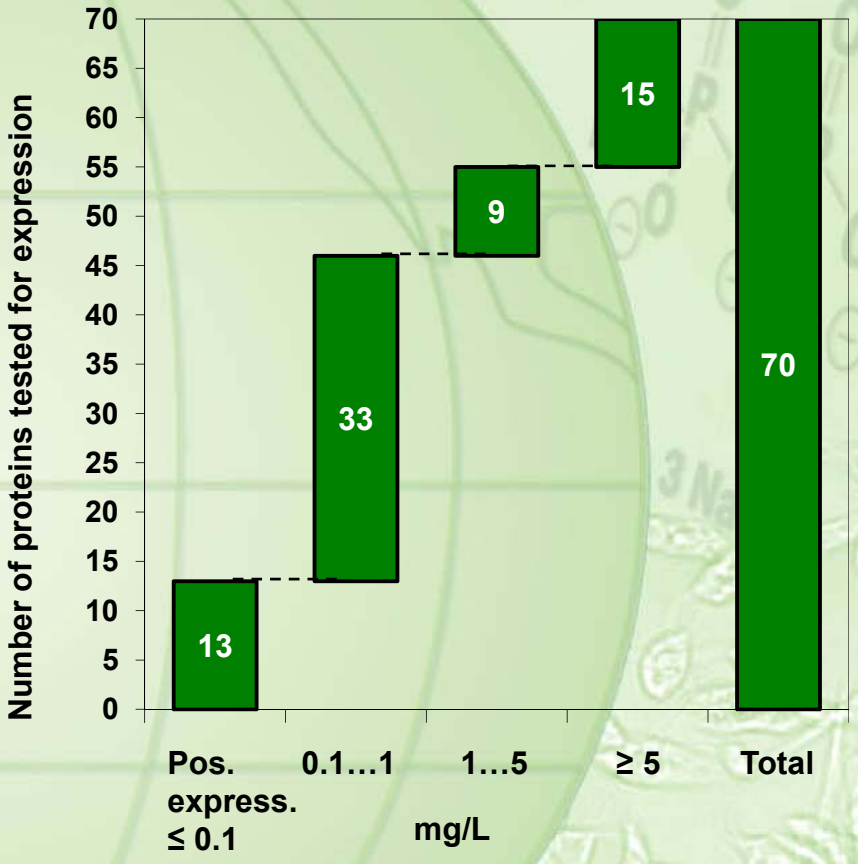


H = LEXSY T7-TR host
SX = polyclonal selection of linear episomes
S = polyclonal selection of circular episomes
1-15 clones

→This problem can be solved by co-expression of blecherry marker

80% of target proteins expressed at > 0.1 mg/L

1/3 > 1 mg/L, yields of up to 500 mg/L reached



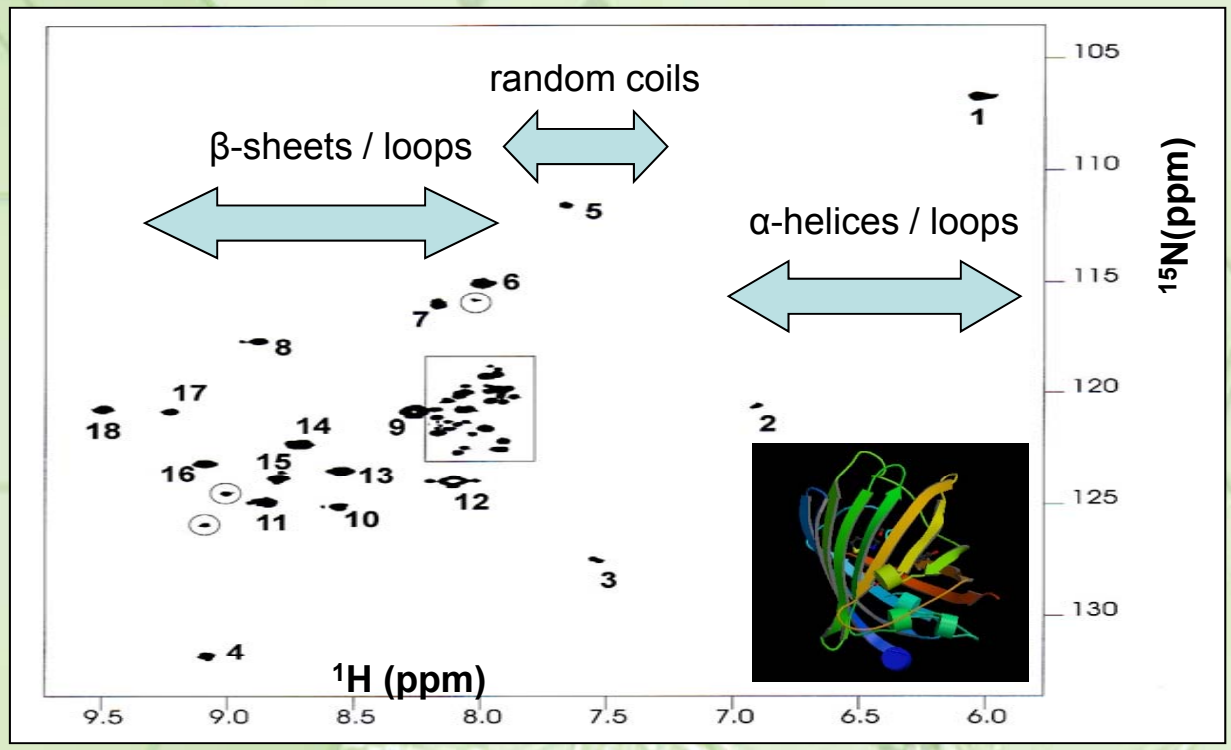
40 cytosolic proteins
24 secretory proteins
6 membrane proteins

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

Case studie

LEXSY enables *in vivo* protein labeling for NMR studies

- complete assignment of all 18 ^{15}N-Val residues in ^{15}N-HSQC NMR of EGFP -



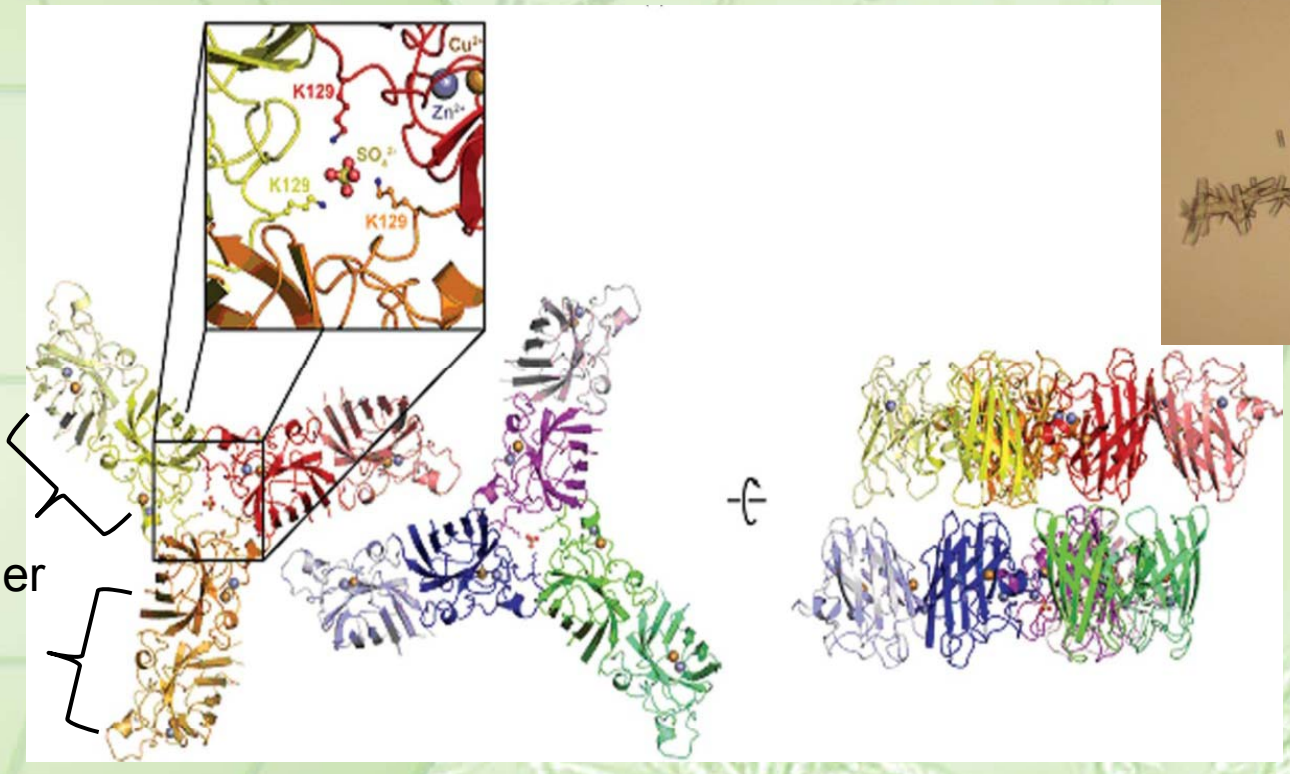
- Niculae *et al.* (2006) Isotopic labeling of recombinant proteins expressed in the protozoan host *Leishmania tarentolae*. *Protein Expression and Purification* **48**: 167
- Foldynová-Trantírková *et al.* (2009) A Cost-effective Amino-acid-type Selective Isotope Labeling of Proteins Expressed in *Leishmania tarentolae*. *Journal of Biomolecular Structure & Dynamics* **26:** 755

LEXSY is auxotrophic for 11 amino acids and can be grown in chemically defined media

Case studie

Successful crystallography and X-ray analysis of LEXSY expressed protein for structural biology

- new protein structure of human Cu/Zn superoxide dismutase -



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.

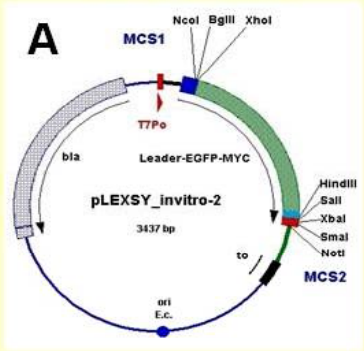
• Gazdag *et al.* (2010) Purification and crystallization of human Cu/Zn superoxide dismutase recombinantly produced in the protozoan *Leishmania tarentolae*. *Acta Crystallographica* **F66:** 871

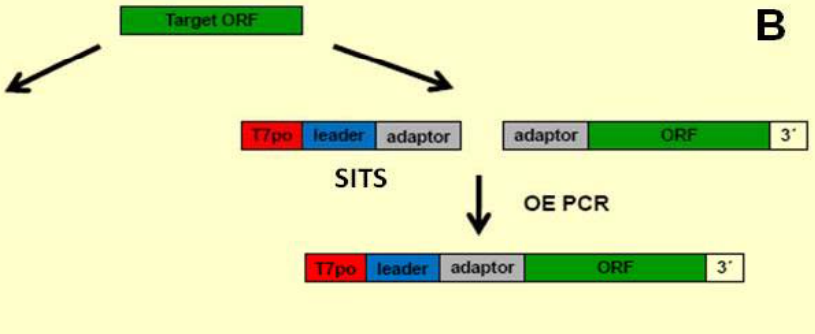
In Vitro LEXSY: Rapid cell-free protein production

Based on cell extracts of *L. tarentolae*

Plasmid based *in vitro* LEXSY
- for high yields -

PCR based *in vitro* LEXSY
- for high throughput -



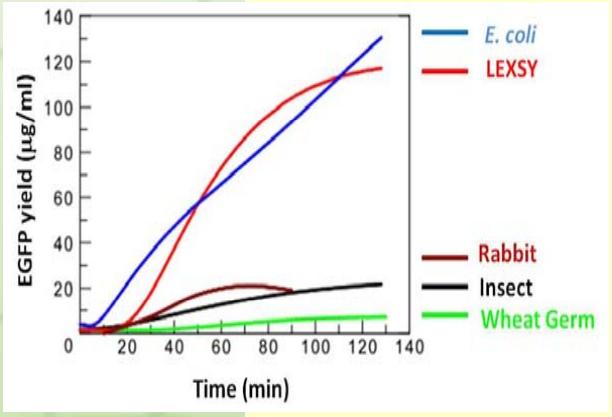


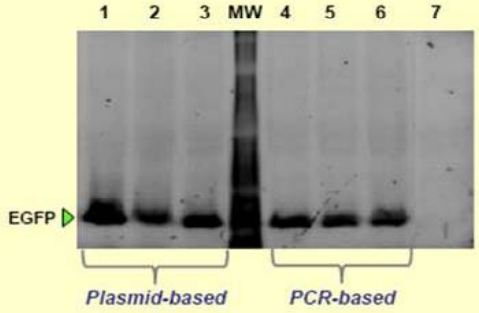
Template generation by cloning of target genes into plasmid vector

Template generation by direct PCR amplification of target DNA by overlap extension (OE) PCR

C

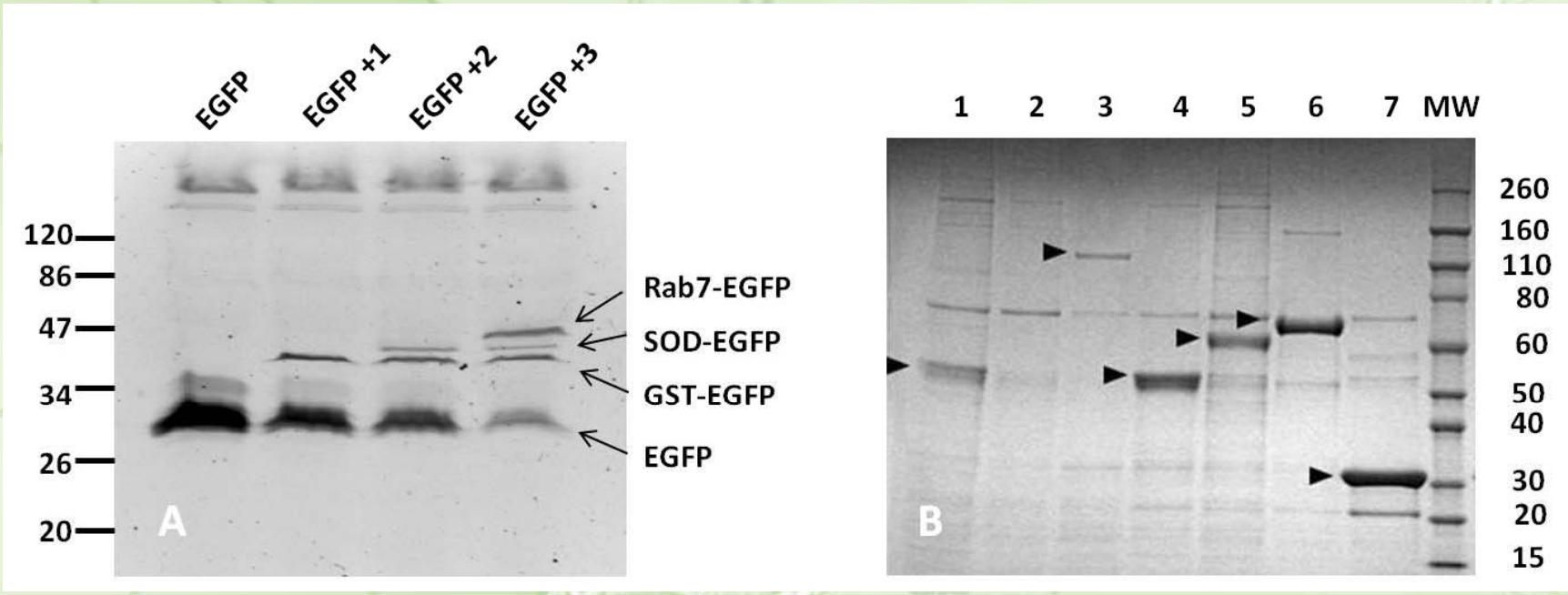
Cell-free production of proteins in transcription-translation coupled LEXSY cell extracts





Cell-free production of EGFP reference protein with plasmid-based (lanes 1-3) and PCR-based (lanes 4-6) *in vitro* LEXSY. Lane 7 negative control without template, MW molecular size marker. The *in vitro* reactions were carried out for 2 h at 20°C, resolved on 12% SDS-PAGE and *in situ* visualized on a UV transilluminator.

In vitro expressed EGFP fusion proteins can be visualised *in situ* and affinity purified



In-gel *in situ* visualisation of EGFP fusion proteins co-expressed in the same cell extract

EGFP-Cap matrix purification of EGFP fusion proteins and detection by Coomassie staining

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

LEXSY - powerful protein expression system

- Robust fast growing unicellular host
- Eukaryotic protein synthesis / folding / modification
- Easy construction and rapid growth of LEXSY expression strains
- Short evaluation times
- Flexible expression solutions
- High yields and simple purification of target proteins
- LEXSY proteins for NMR and X-ray chrystallography
- *In Vitro* LEXSY for rapid and parallel cell-free protein production

Collaborators

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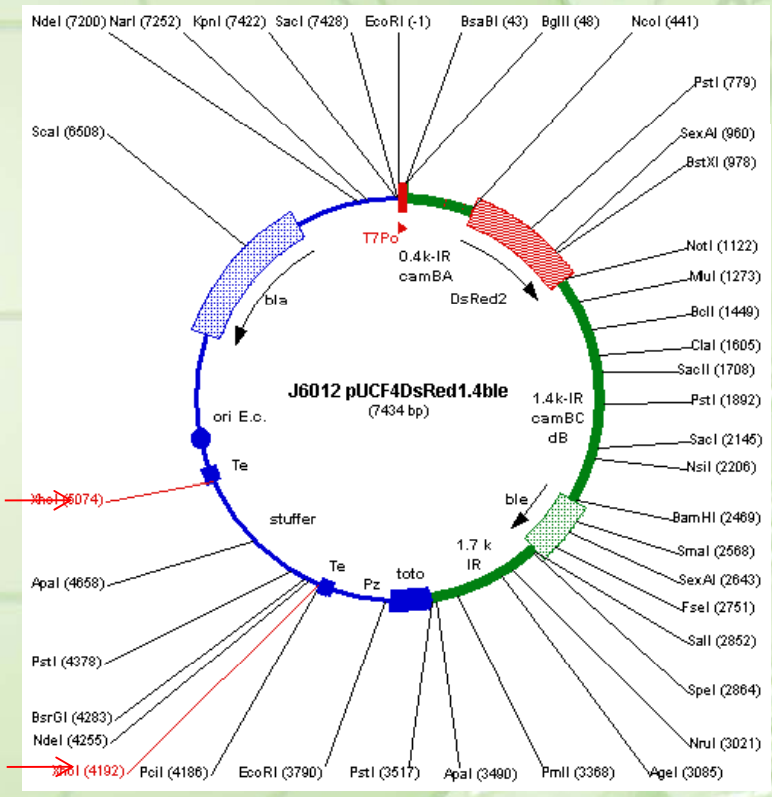
Appendix

Evaluation of inducible episomal DsRed vector J6012



Circular episome

<10% red colonies

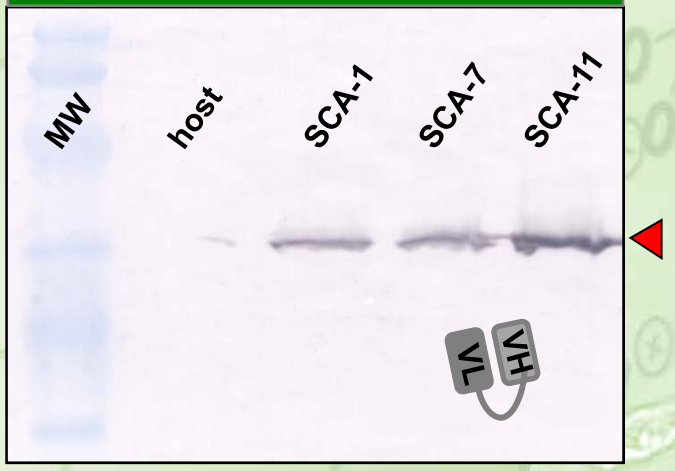
Linear episome
(XhoI treatment)

90% red colonies

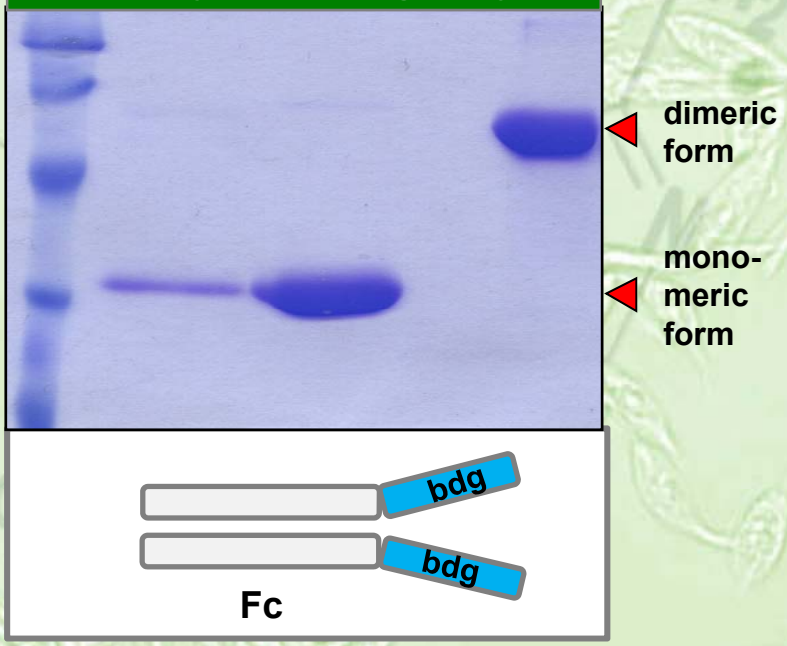
Transfected cells were spread onto NC filters and filters were transfered To LEXSY BHI plates with tetracycline after colonies had appeared. Red color was visible in daylight.

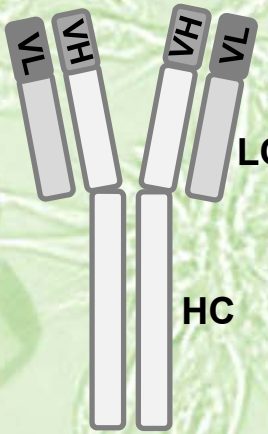
Different antibody constructs were expressed in LEXSY

Single chain antibodies



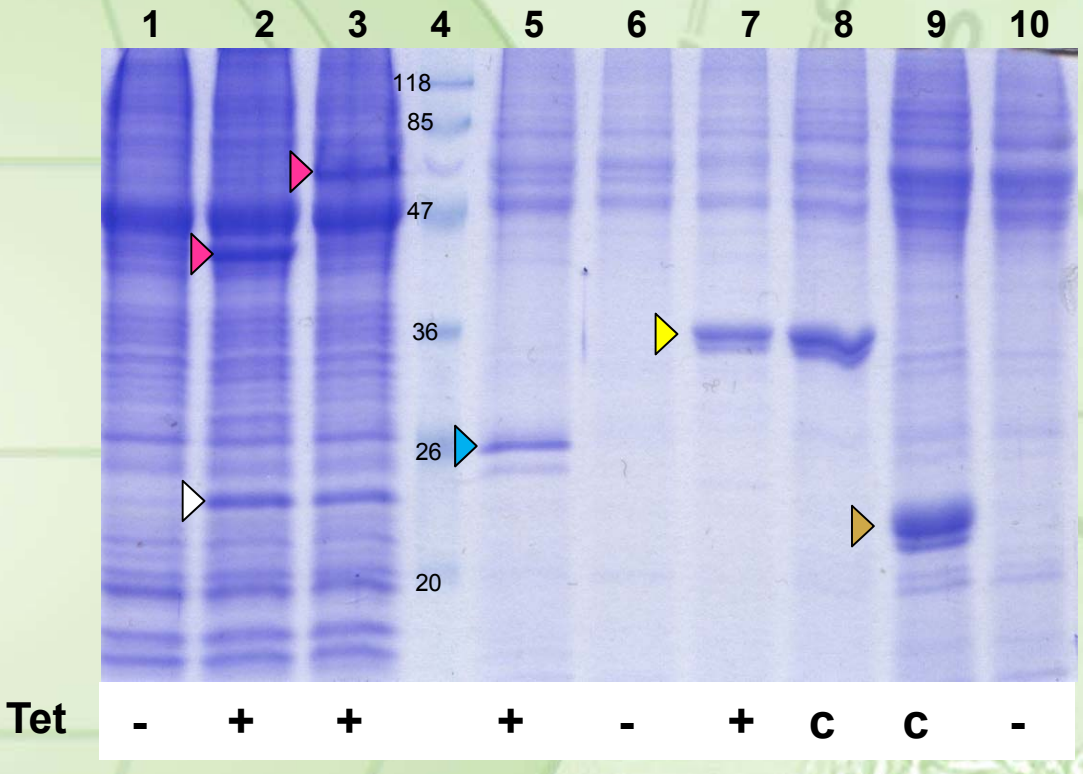
Fc-fusions purified by Prot A affinity chromatography





HC and LC of human IgG were expressed separately in different LEXSY strains

Various target proteins were efficiently expressed in LEXSY

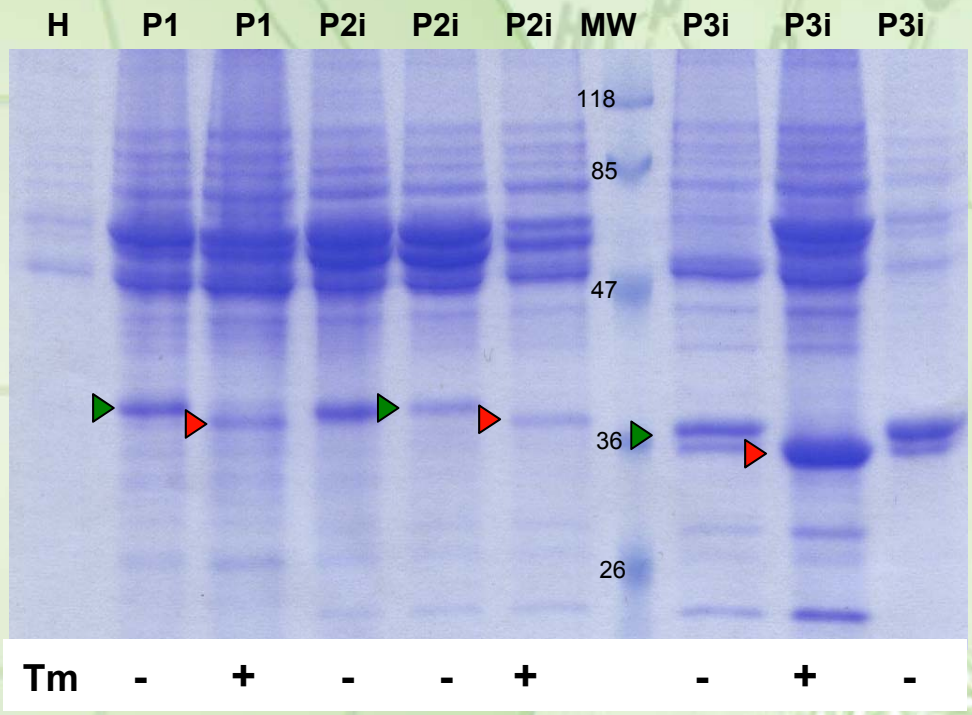


1-3 inducible
2 boiled
3 not boiled
4 MW marker
5-7 inducible (blecherry)
8-9 constitutive
10 host strain

- BleCherry
- SOD1
- PTX fungal toxin*
- SAG1 surface antigene**
- SAG2 surface antigene**

*Pyrenophora tritici-repentis protein ptx,
** Toxoplasma gondii

Glycosylation of secretory target proteins was inhibited *in vivo* by addition of Tunicamycin



Concentrated culture supernatants of LEXSY clones

H	LEXSY host (negative control)
MW	prestained molecular size marker
P1	constitutive secretory expression
P2i	inducible secretory expression
P3i	inducible secretory expression (enzymatic deglycosylation was shown)
Tm	Tunicamycin added to culture at 10 µg/ml

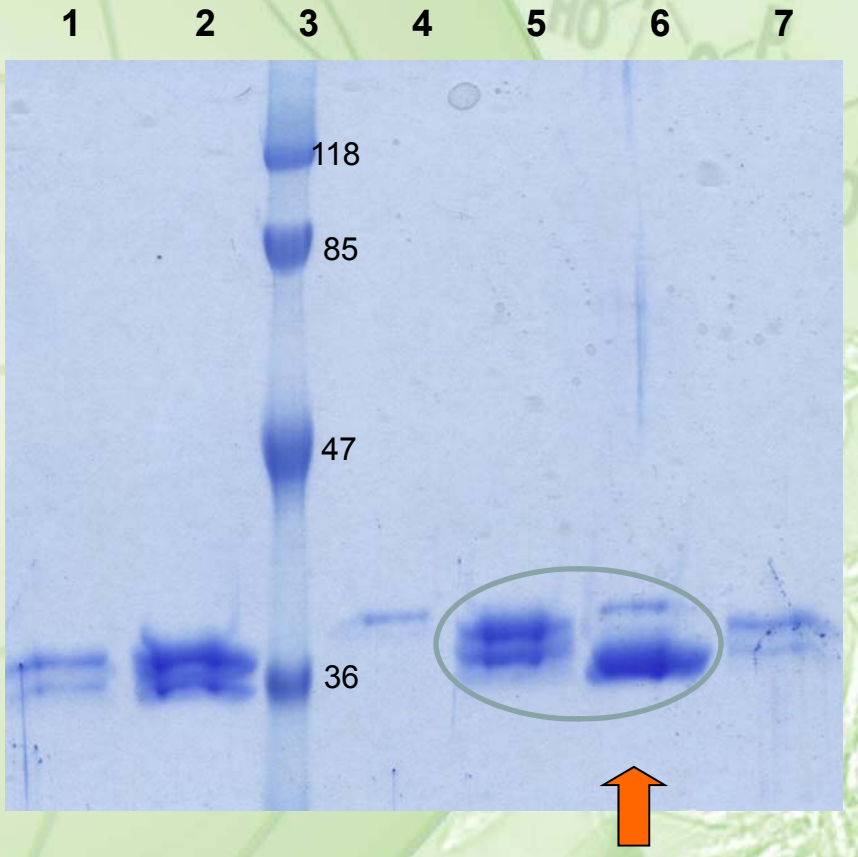
▶ Glycosylated target protein from LEXSY

▶ Non-glycosylated target protein from LEXSY

Mobility shift caused by addition of Tunicamycin to culture indicates glycosylation of target proteins in LEXSY

LEXSY expressed protein *was* deglycosylated *in vitro*

Target protein was affinity-purified from culture supernatant and enzymatically deglycosylated



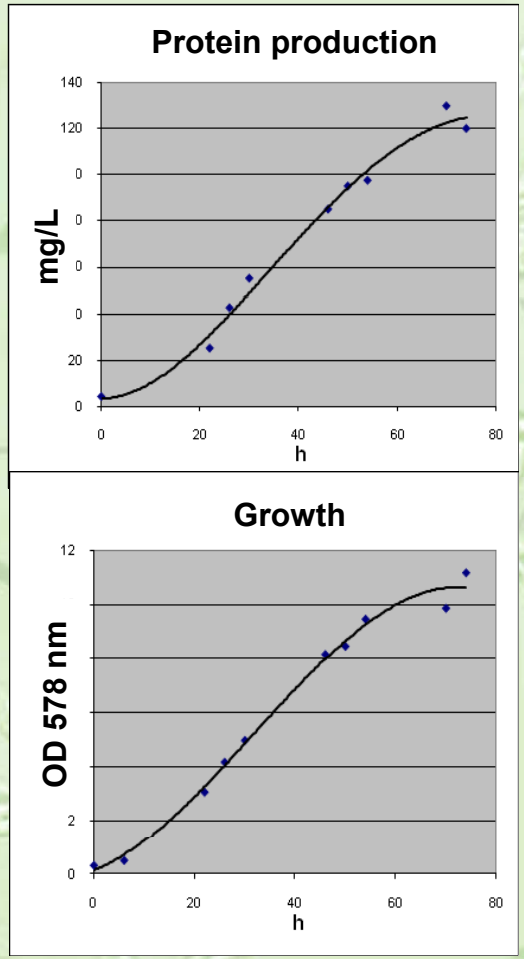
1 imidazol eluted POI
2 concentrated POI in PBS
3 prestained molecular size marker
4 N-Glycosidase F (138 ng = 250U) 36 kDa
5 POI in deglycosylation buffer
6 POI + N-Glycosidase F
7 imidazol eluted POI

POI: target protein of interest

2 glycoforms
consistent with previous findings (EPO)

shift to one band

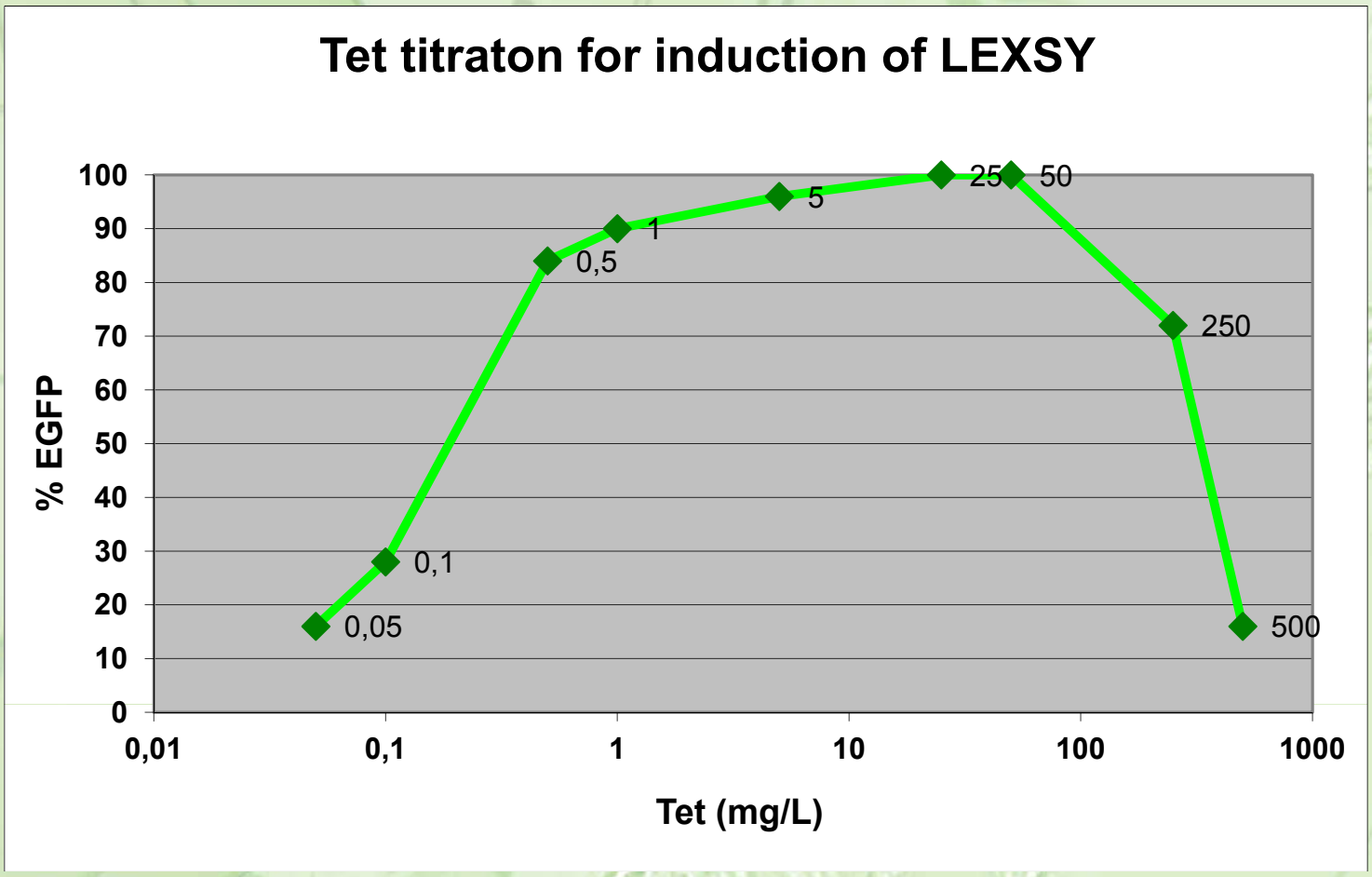
LEXSY is compatible with common fermentation technology



Constitutive protein production parallels growth of expression culture
>120 mg/L of culture reached at 8 x 10^8 cells/ml

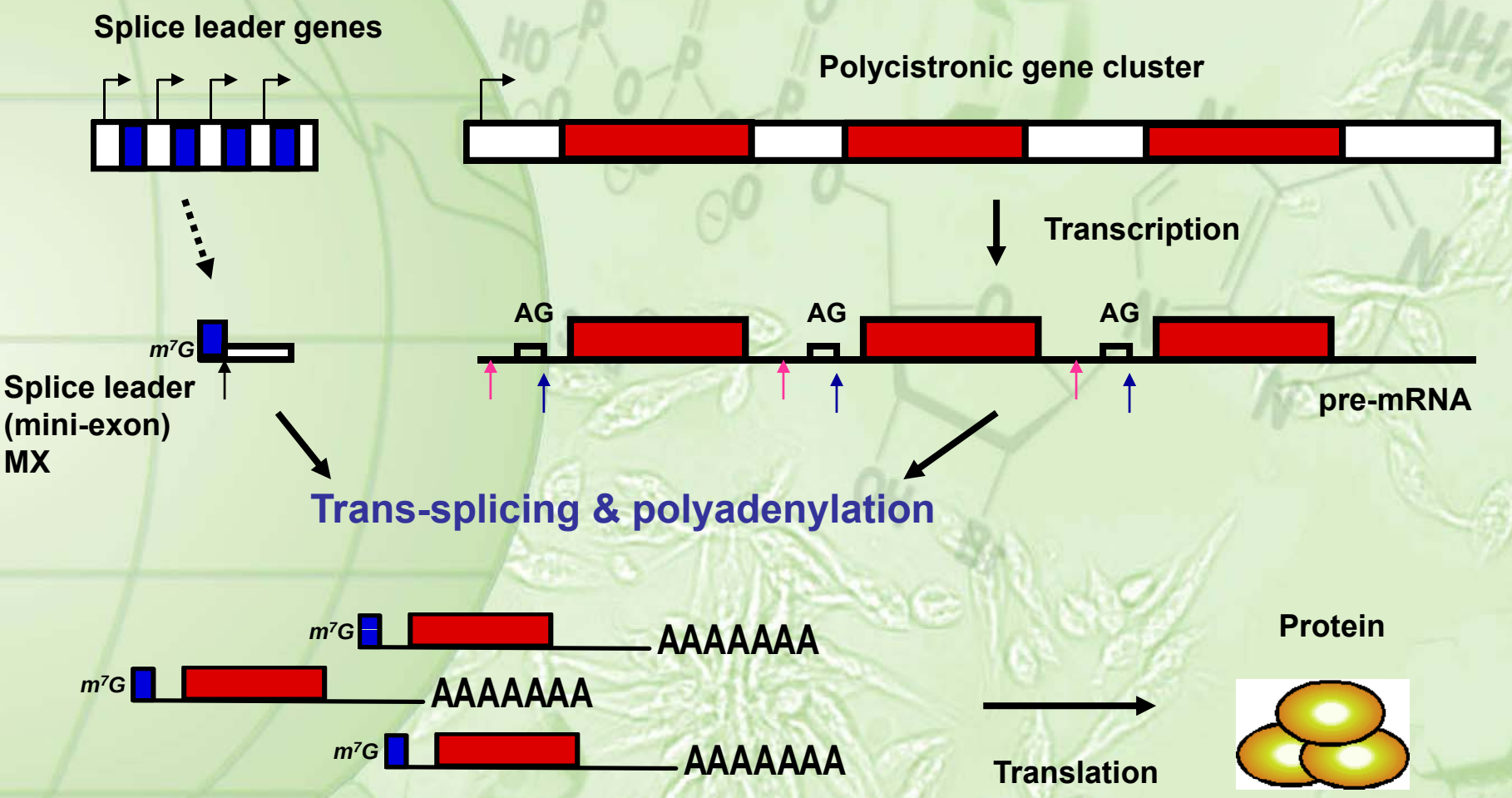
Appendix

Broad induction plateau in inducible LEXSY



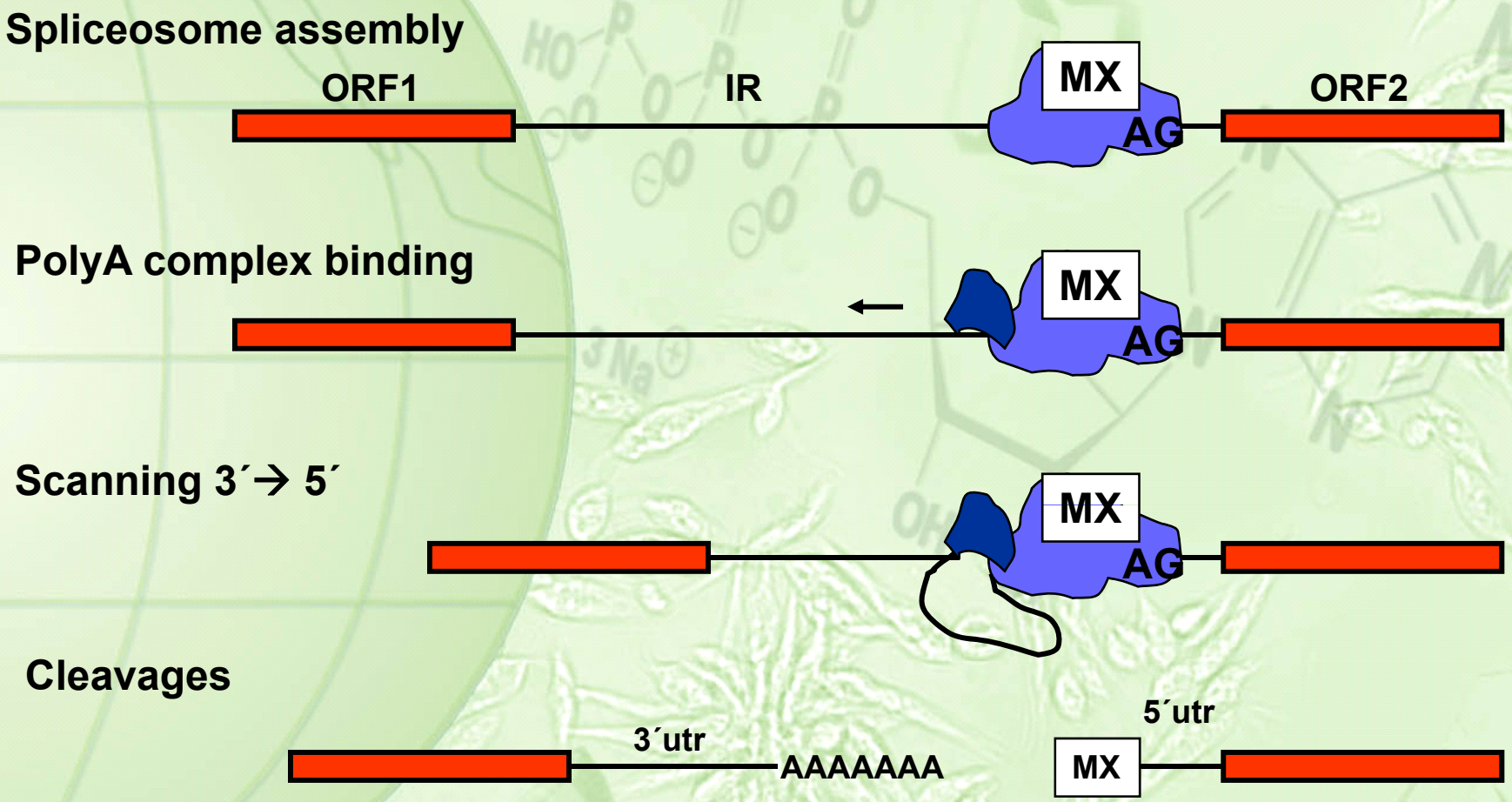
Appendix

Transcription is uncoupled from RNA-processing in *Leishmania*



Appendix

Scanning model for coupling of splice site and poly(A) site selection in *Leishmania*



MX: miniexon

After LeBowitz et al., 1993