Molecular Biology Toolbox for Impatient Protein Crystallographers

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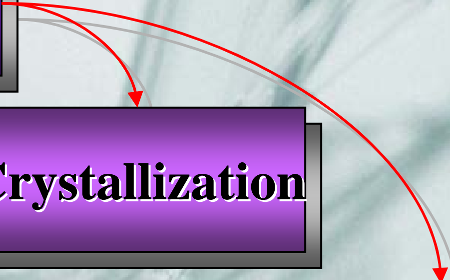
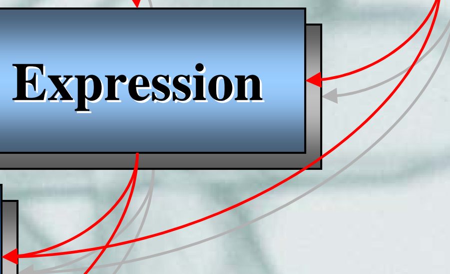
Structure



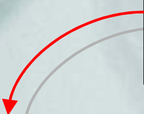
Crystallization

Purification



Expression

Domain

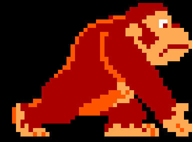


Gene

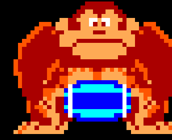
Idea

1UP
000000

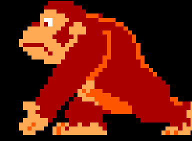
HIGH SCORE
000000



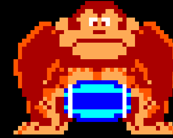
Structure



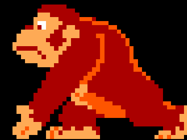
Crystallization



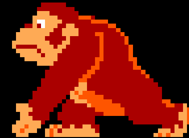
Purification



Expression

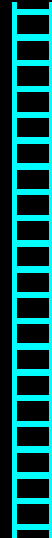


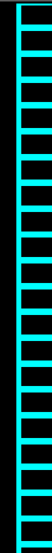
Domain



Gene

Idea





Structure

Crystallization

Purification

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Idea

Expression

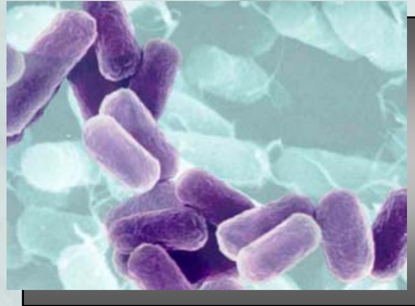
Expression

It would be nice to have a single expression system with an optimum blend of all protein expression features and parameters.

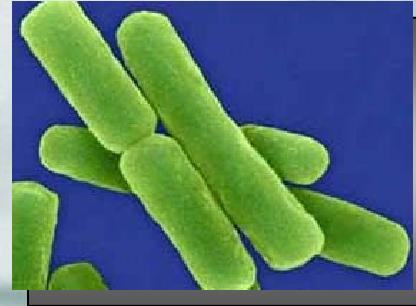
However, no such system is described to-date.

Each expression system offers unique advantages and challenges. It is useful to attempt expression in multiple systems, but the options are usually limited by availability, feasibility, and economy of application.

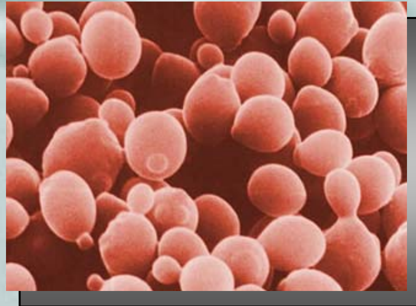
Expression



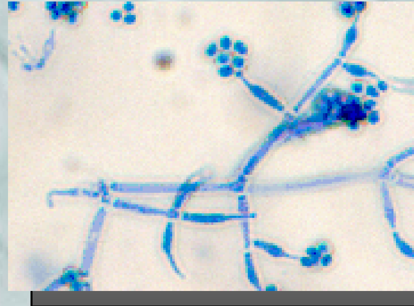
Gram-negative bacteria



Gram-positive bacteria

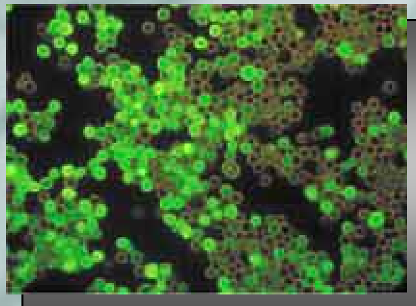


Yeasts

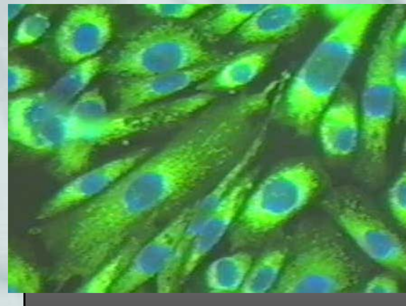


Other Fungi

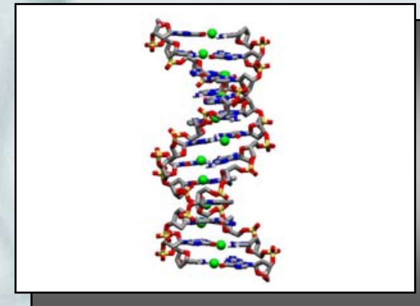
Plants / Plant Cells



Insect Cells



Mammalian Cells



In Vitro

Expression

- Numerous commercial as well as public-domain expression systems are available

- With a partial exception of *in vitro* expression, all recombinant protein expression systems rely on transfer of the gene (or fragment) of interest into a specially designed DNA vehicle, which is subsequently introduced into the expression host.

Recombinant DNA

Important recombinant DNA features

- DNA persistence elements
- Selection markers

- Transcription control elements
- Translation control & enhancement elements
- Localization control features

- Folding enhancement tools
- Tags for purification and detection

Factors influencing the choice of recombinant DNA technologies

- Speed
- Reliability / Fidelity
- Economy

Recombinant DNA

A useful combination of techniques

The tools listed above are necessary and sufficient to satisfy 99% of all recombinant DNA needs of a typical crystallographic lab.

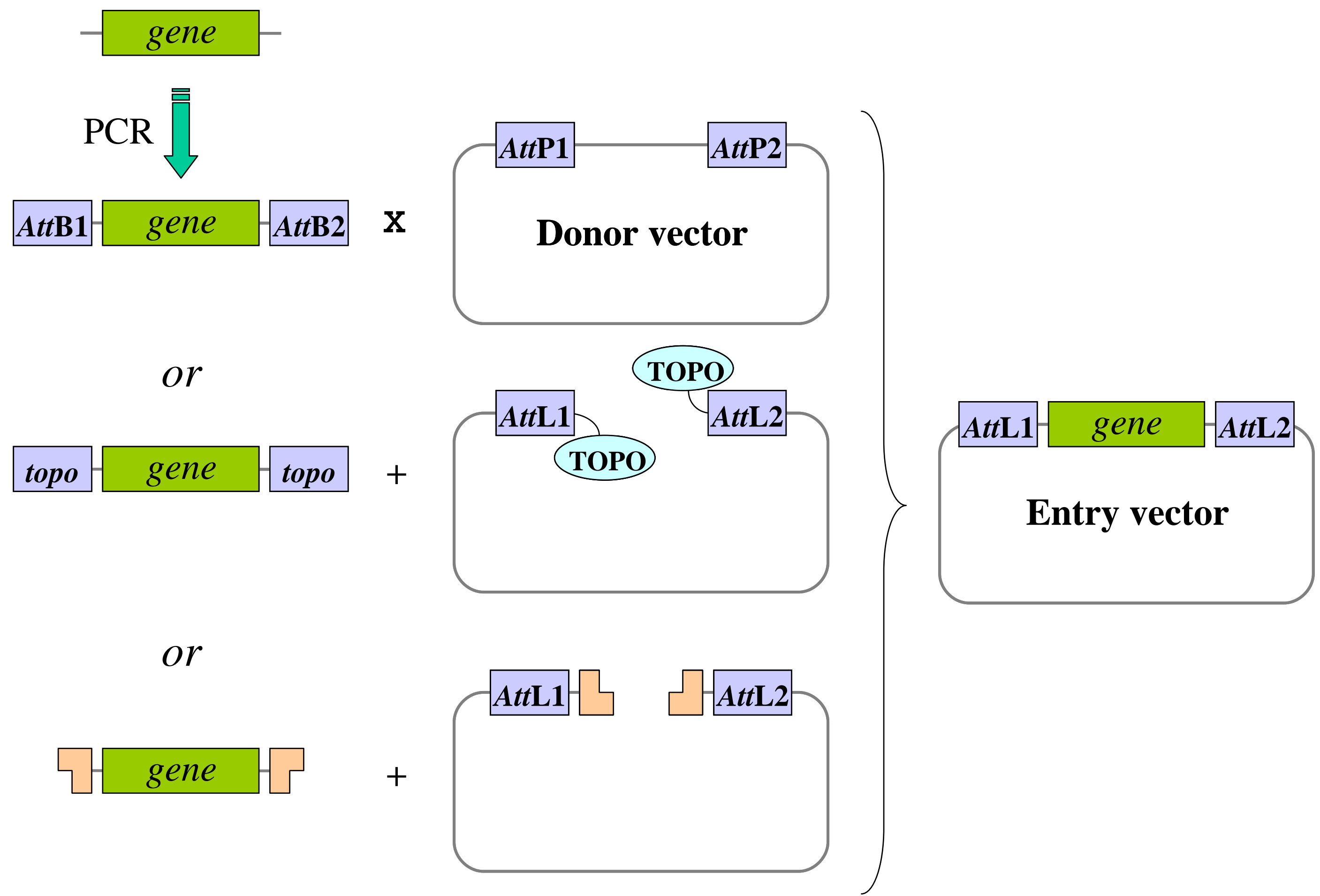
Synthetic DNA

- Increasingly popular and affordable ($0.6–2.4/bp).
- Any DNA sequence can be synthesized with 100% fidelity.
- Often faster than amplification out of cDNA libraries.
- Allow for multiple sequence optimization strategies.
- Moderately fast (2-4 weeks for 1500-bp gene).

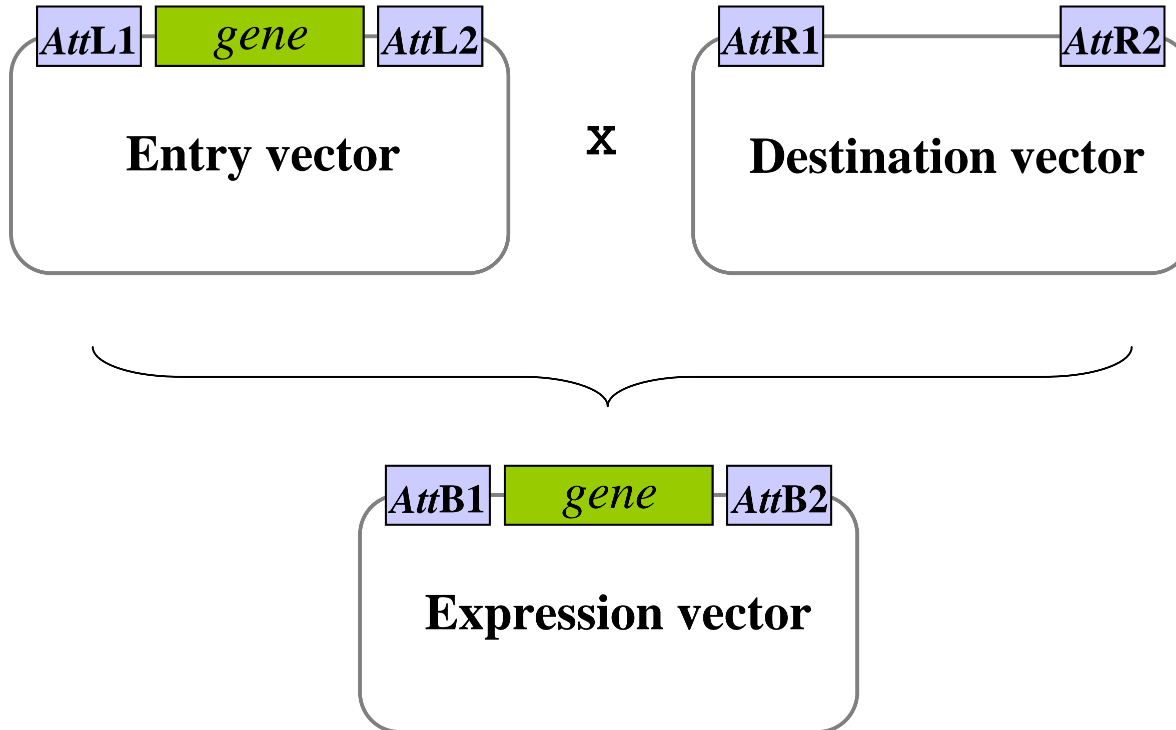
Clone libraries

- Hundreds of thousands of genes in stock.
- Modestly priced ($98-1500 per gene).
- Typically available within 1-3 days.
- “Popular” genes are increasingly available.
- Can (and sometimes do) contain sequence errors.

Recombinant DNA



Recombinant DNA



Gateway© cloning

- Rapid (2-4 days from gene to expression/shuttle vector).
- Extremely simple to perform.
- True-positive cloning with 99% fidelity.
- Universally applicable (no sequence or host restrictions).
- No ligation or restriction digests required.
- Almost any existing vector can be converted into Gateway.
- Numerous Gateway genes and expression vectors available.

Several other cloning systems (LIC, Creator, etc.) offer similar advantages. In our opinion, Gateway is the more convenient system, especially for scientists who are not full-time molecular biologists.

QuikChange© mutagenesis

- Rapid (2 days from template to product).
- Cheap !
- Applicable to nearly any vector 1-30 Kbp in size.
- Allows for deletions of arbitrary length.
- Substitutions and insertions are limited to ~35-60 nt frame.
- Can introduce sequence errors.

QuikChange mutagenesis is one of the most useful extensions of proofreading PCR amplification.

PCR error rates and DNA sequencing

• High-fidelity, proofreading polymerases (e.g. PfuUltra, KOD, etc.) typically produce less than one error per 1000-nt amplicon in 30 cycles.

• The use of DMSO and other chaotropes minimizes likelihood of errors associated with difficult DNA sequences.

• If Gateway cloning is used consistently, there is no chance to introduce errors into the sequence of interest. Therefore there is only need for one sequencing step – namely at the time of the initial PCR reaction

• Synthetic genes arrive pre-sequenced and fully verified. If synthetic gene is used with Gateway typically there is no need to sequence at all.

An example of the complete process

Protein X = *Homo sapiens*, 400 aa, two domains joined by a long linker.

Day1:

- Searched vendor databases for protein X (GeneCopoeia, Invitrogen, OriGene, Molecular Biosciences, etc…). Found several clones with the right sequence, ordered the gene.
- Analyzed domain structure and decided on the fragments to study. Ordered relevant PCR primers (IDT-DNA).

Day 2:

- Primers arrive.

Day 3:

- Gene arrives.
- Performed 2-stage PCR for Gateway Entry vector.
- Performed BP reaction.
- Transformed TOP10 *E. coli* with BP product.

Day 4:

- Picked 3 BP colonies, set up small-scale cultures for DNA prep.

Recombinant DNA

Day 5:

- Prepared plasmid DNA from 3 BP colonies (Entry vector).
- Submitted Entry vector DNA for sequencing.
- Performed LR recombination between an Entry vector and several Destination vectors.
- Transformed expression-grade (BL-21, etc.) cells.

Day 6:

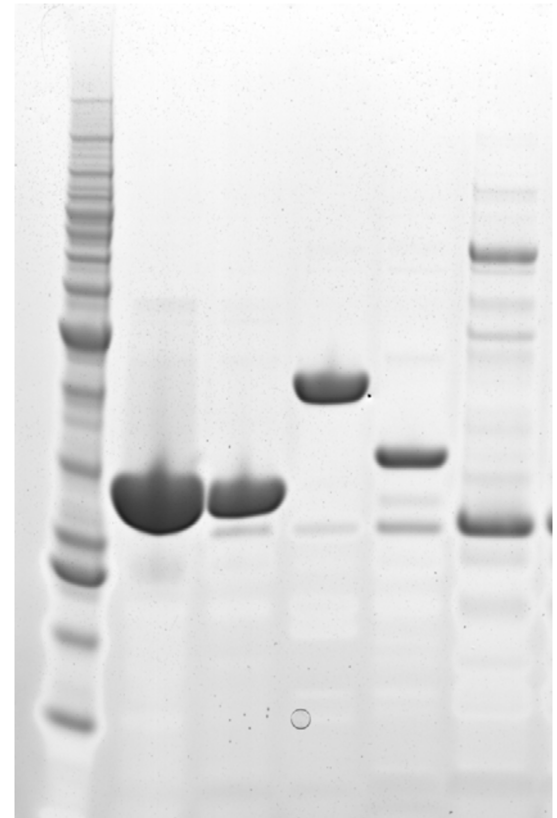
- Picked 2 LR colonies from reaction with each Destination vector, set up small-scale cultures for protein expression studies.

Recombinant DNA

Day 7:

- Analyzed small-scale expression studies.
- MS confirms accurate molecular weight.

Gel: Soluble expression of various X constructs -
E. coli lysates were passed through 50-ul Ni-NTA resin,
eluates loaded on the gel.



Lane 1	- m.w. standards
Lane 2	- His-tagged-TVMV-X vector 1.
Lane 3	- His-tagged-TVMV-X vector 2.
Lane 4	- His-SUMO-TVMV-X.
Lane 5	- His-TRX-TVMV-X
Lane 6	- His-MBP-TVMV-X

Day 8:

- Sequencing results come in (no errors).

Conclusions

- Modern molecular biology techniques allow for ~1 week turn-around time for multiple constructs designed to express protein(s) of interest in several hosts.

- Even very small groups can rapidly produce and evaluate multiple expression constructs, with only a modest investment of money and effort.

Acknowledgements:

Structural biology

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Ken Greis

Protein production group

Cloning group (DNA sequencing)

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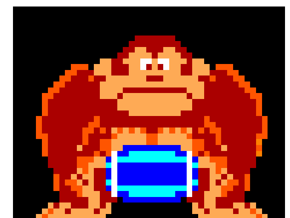
David Waugh

Joseph Tropea



Thank you!