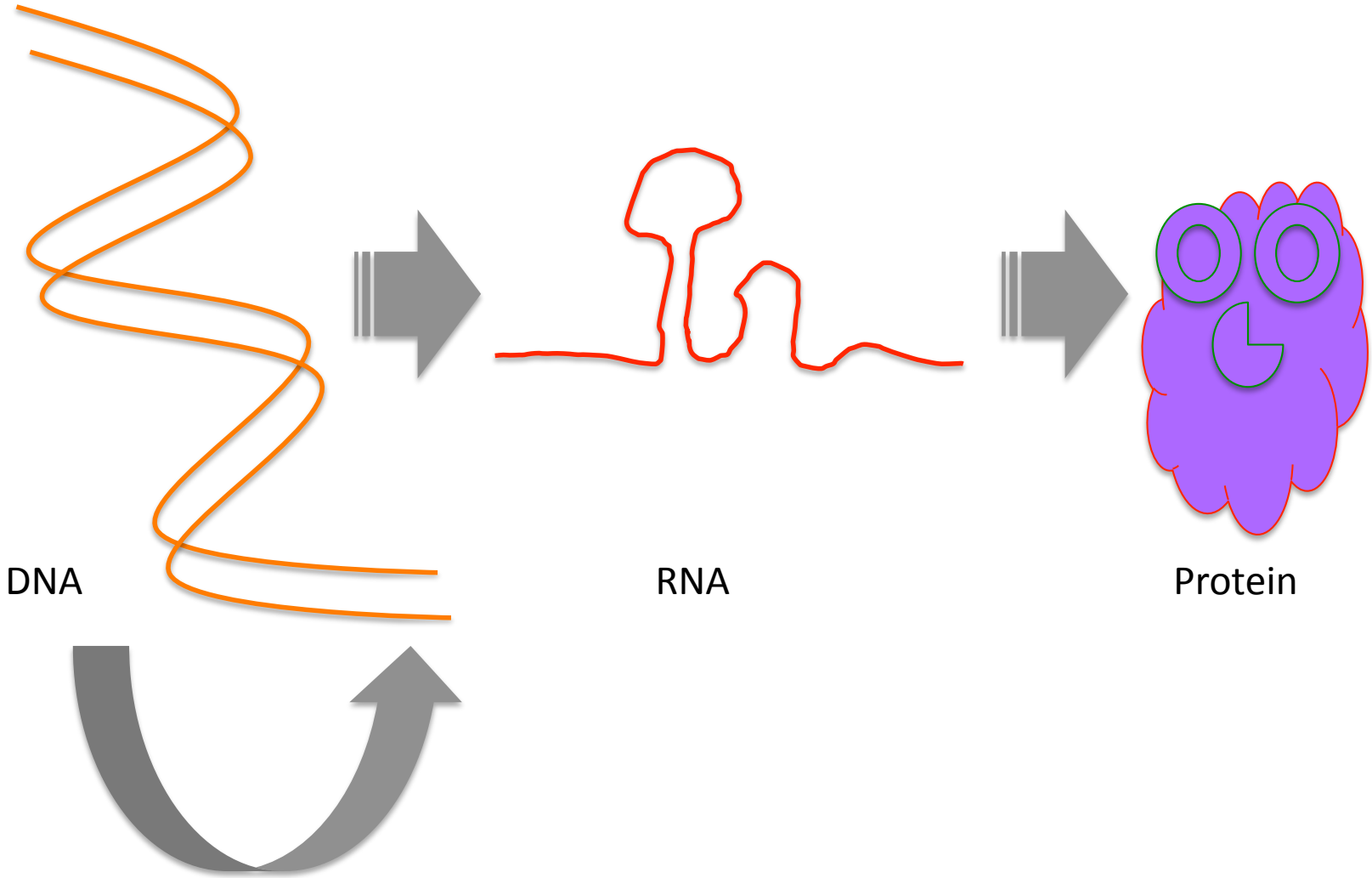
Biotechnology Seminar 2010

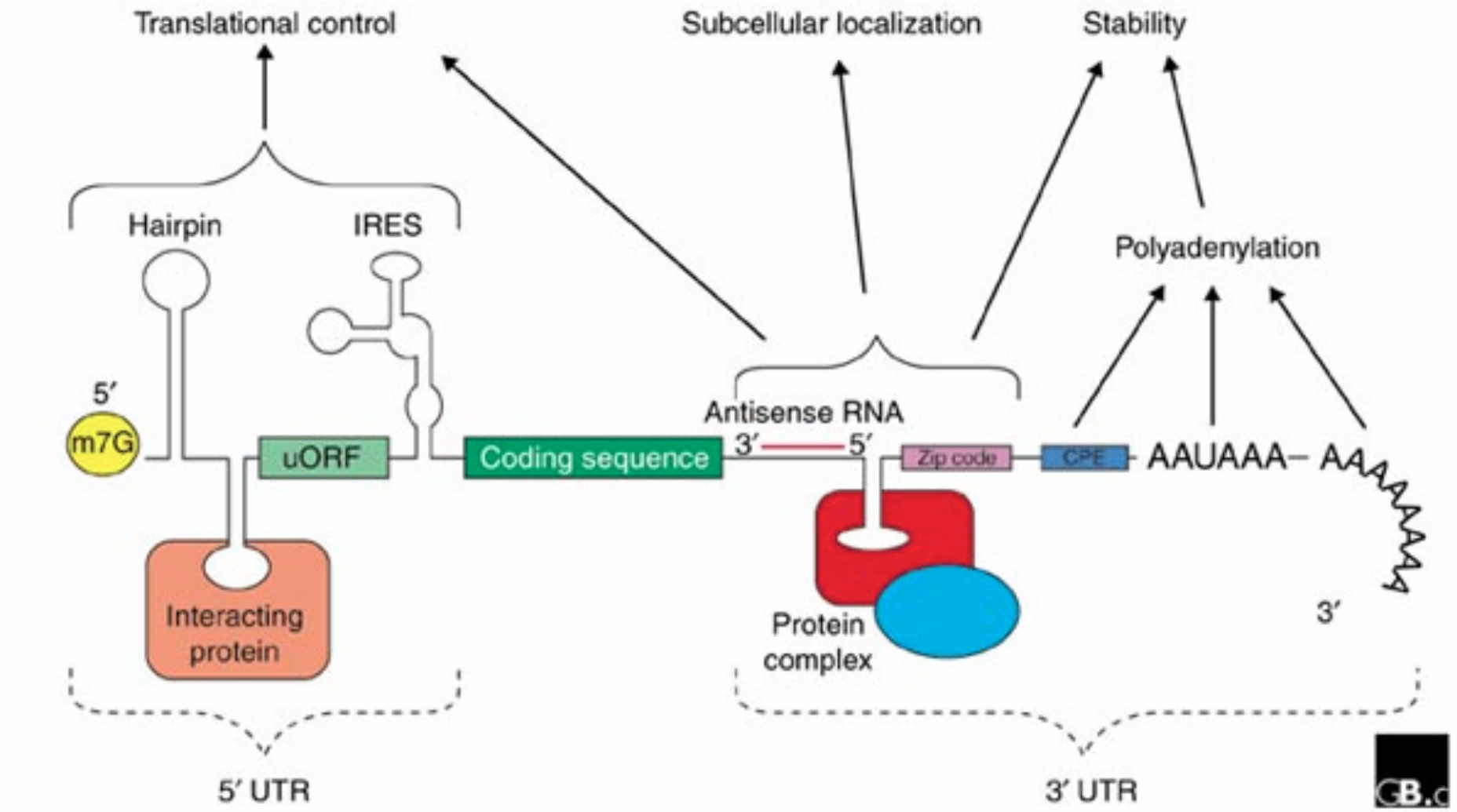
Expression Systems

The Dogma



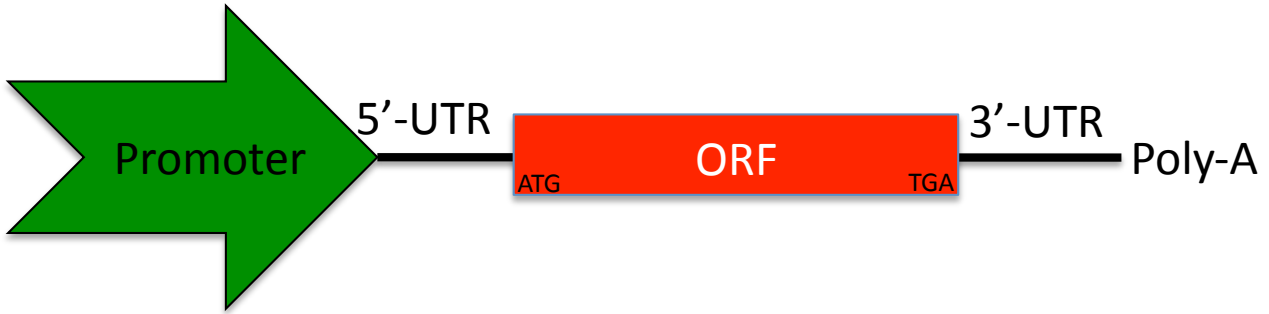
Francis Crick, 1956: The Central Dogma of Biology

Regulation of Gene expression



Mignone et al. Genome Biology 2002 3:reviews0004.1

Expression Vectors



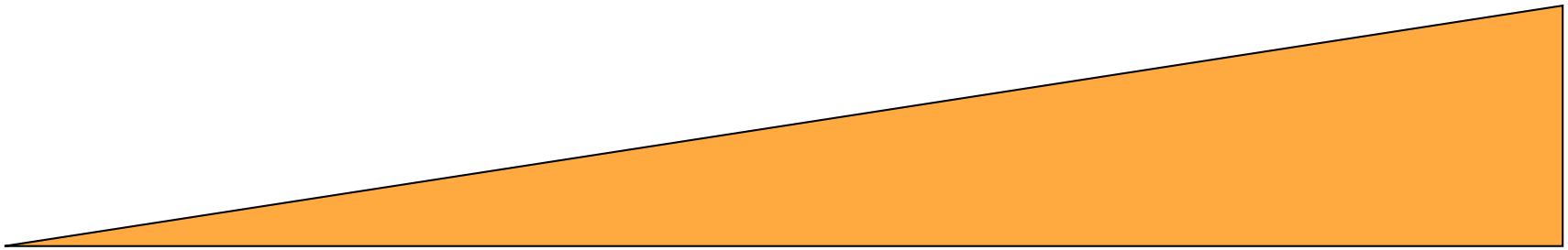
Elements required for cDNA expression:

1. Promoter;
2. Open Reading Frame (ATG start - stop codon);
3. Poly-Adenylation site;

Regulatory elements:

1. Untranslated region (miRNA, 5-UTR modules);
2. Kozak sequence;
3. Splice sites;
4. Post-transcriptional elements;
5. Secondary structure;

Common promoter activities



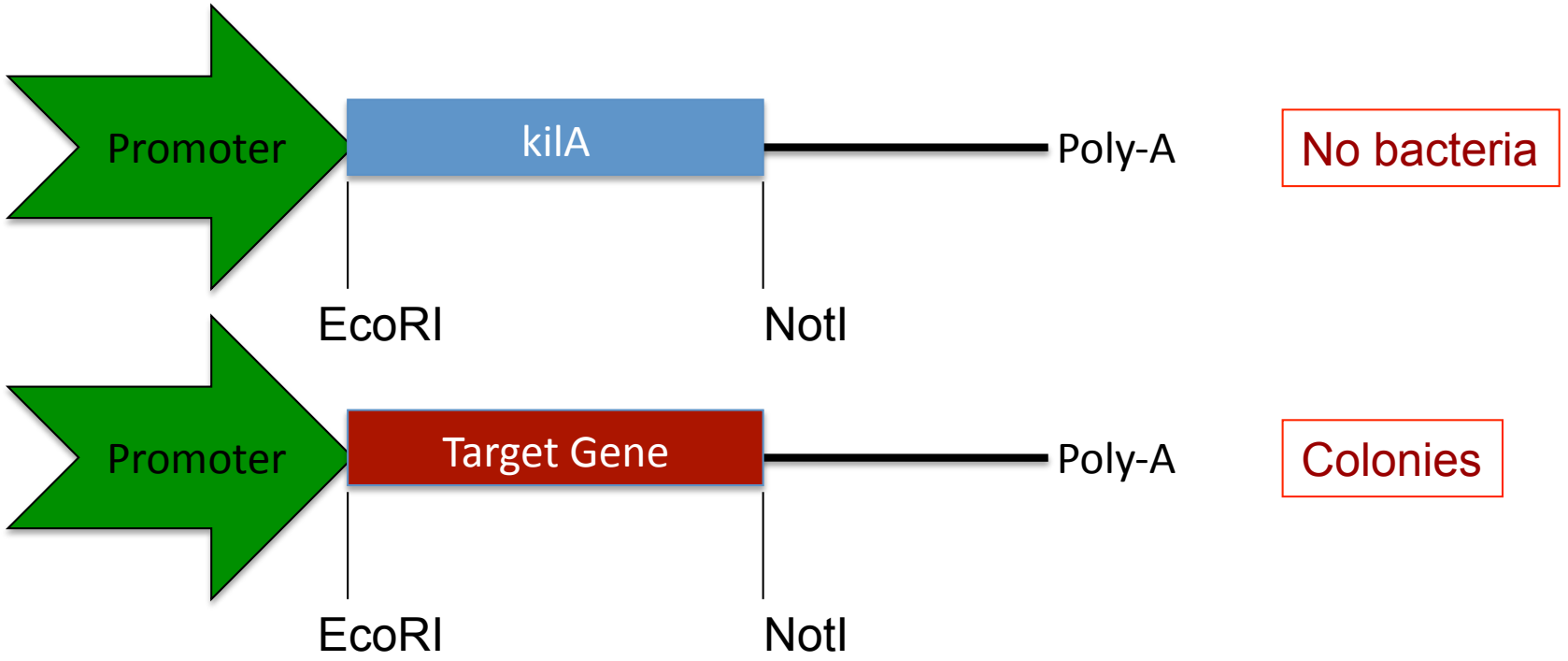
LTR	TK PGK	SV40	EF1A bActin[1]	CMV[2]

[1] mosaic expression in transgenic mouse;
[2] dependent on E1A expression;

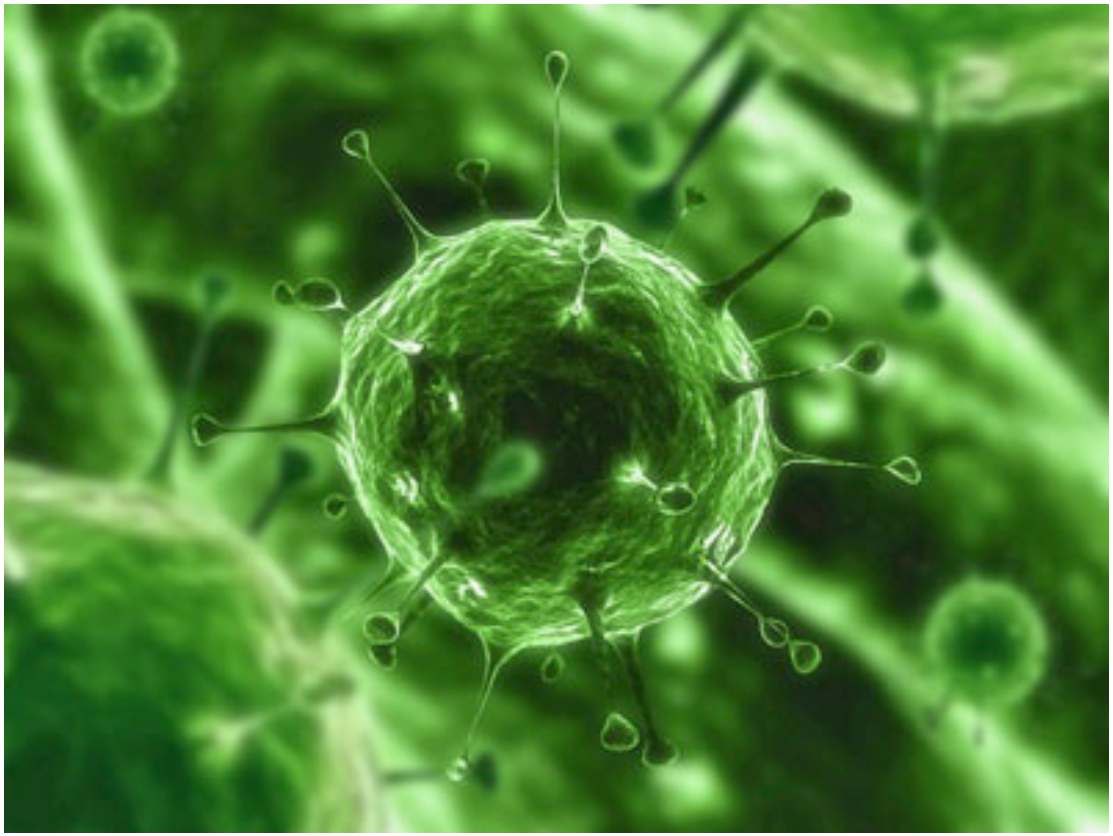
Facilitating Cloning

The broad host range bacterium RK2 encodes several kil genes that are lethal to E.Coli, e.g. kilA (Figurski J Bacteriol. 1983);

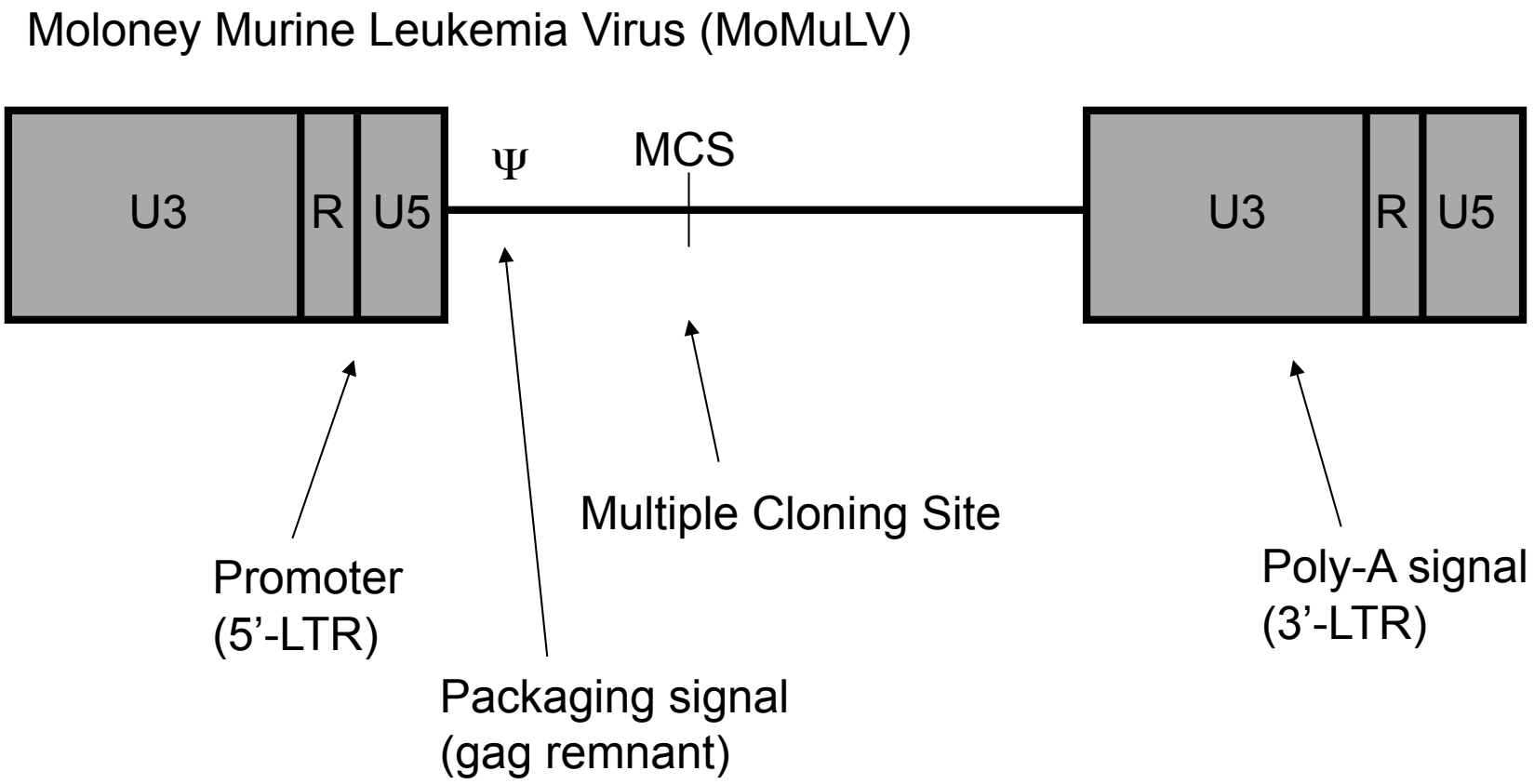
kilA in expression vectors facilitates easy cloning:



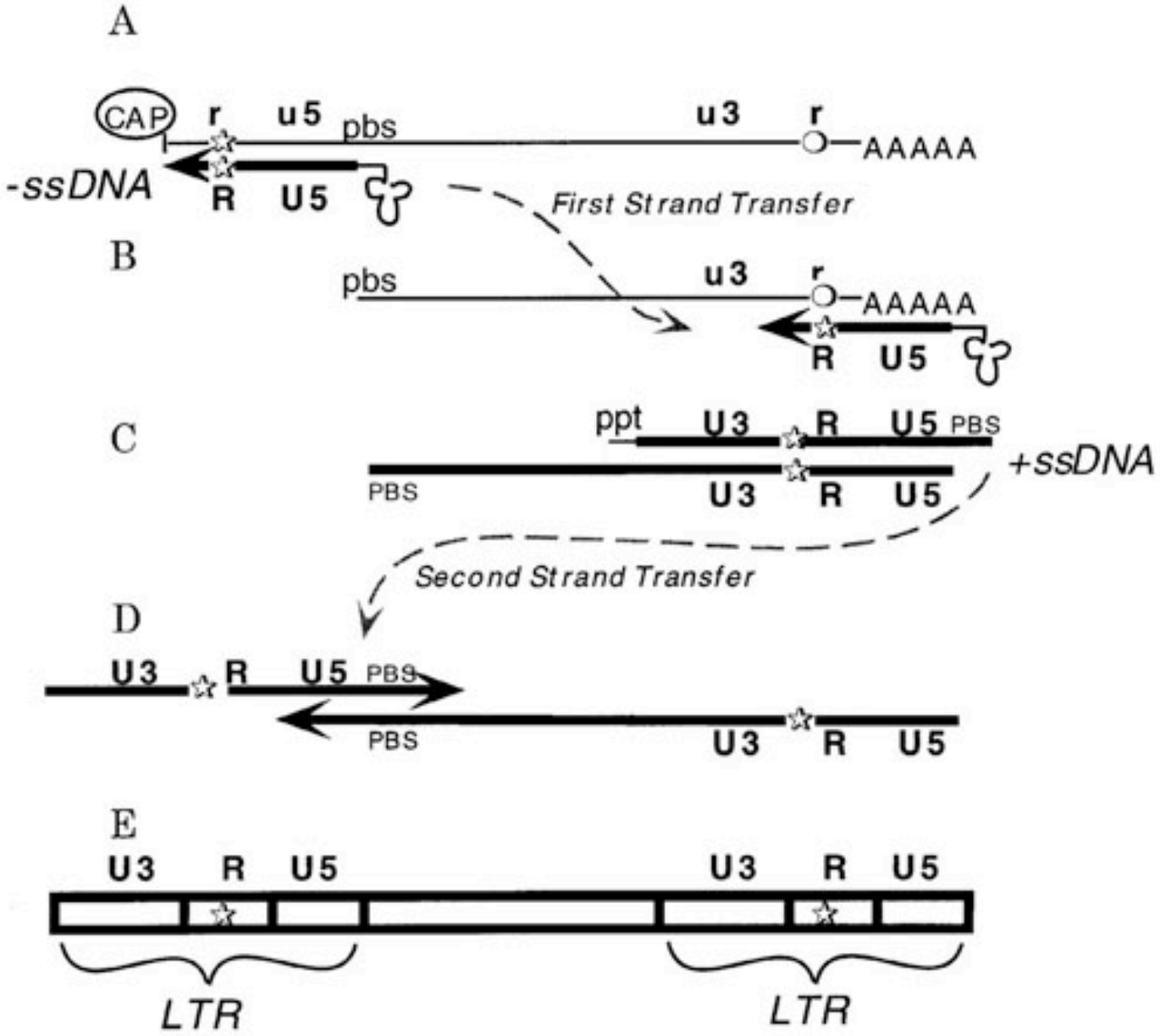
Viruses require efficient organization of genetic information –
IRES, 2A sequences, Frameshifting, Post-Transcriptional Regulatory Elements



Retro- and Lentiviral Expression systems



Retrovirus replication



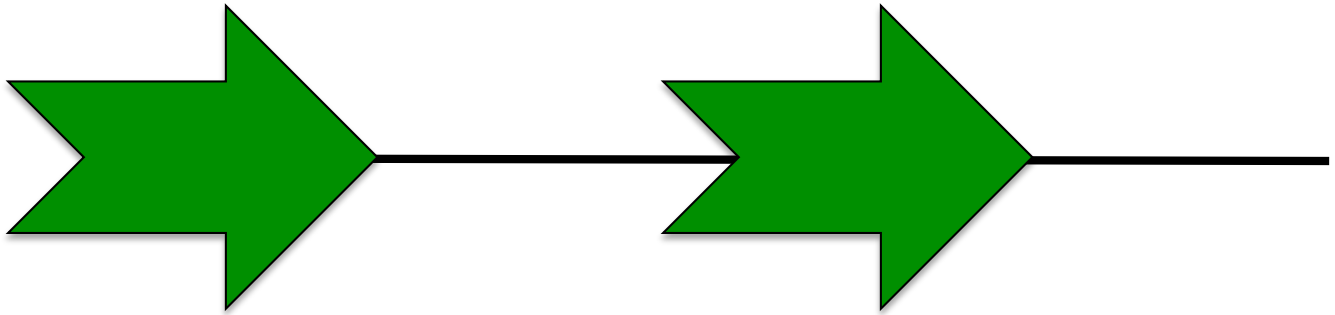
A history on Retroviral Expression Vectors

Restrictions in retrovirus expression
Chances and Pitfalls

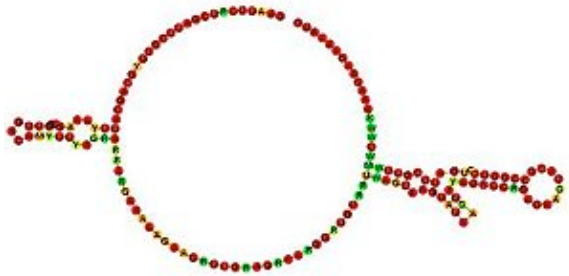
1. Stable Integration into host genome;
2. Random integration (?);
3. Can be titrated (MOI ~1.0);
   Multiplicity of infection - approximation of particle number
4. Lower expression than standard promoter such as EF1A, CMV (more physiological?);
5. Can infect broad range of host organisms and cells with high efficiency (VSVG pseudotyping);
6. Low expression in stem cells can be overcome (pSF1b, pMOWS);
7. Export of unspliced RNA requires PRE - be careful when using introns in expression constructs! 55 nt rule (before Exon junction would result in reduction of RNA)Nagy and Maquat, TIBS 1998

Bicistronic Expression

1. Sequential Promoter

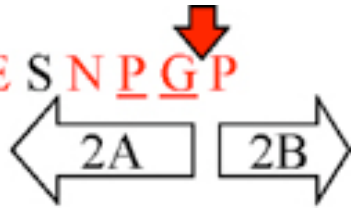


2. Internal Ribosomal Entry Sites (IRES)

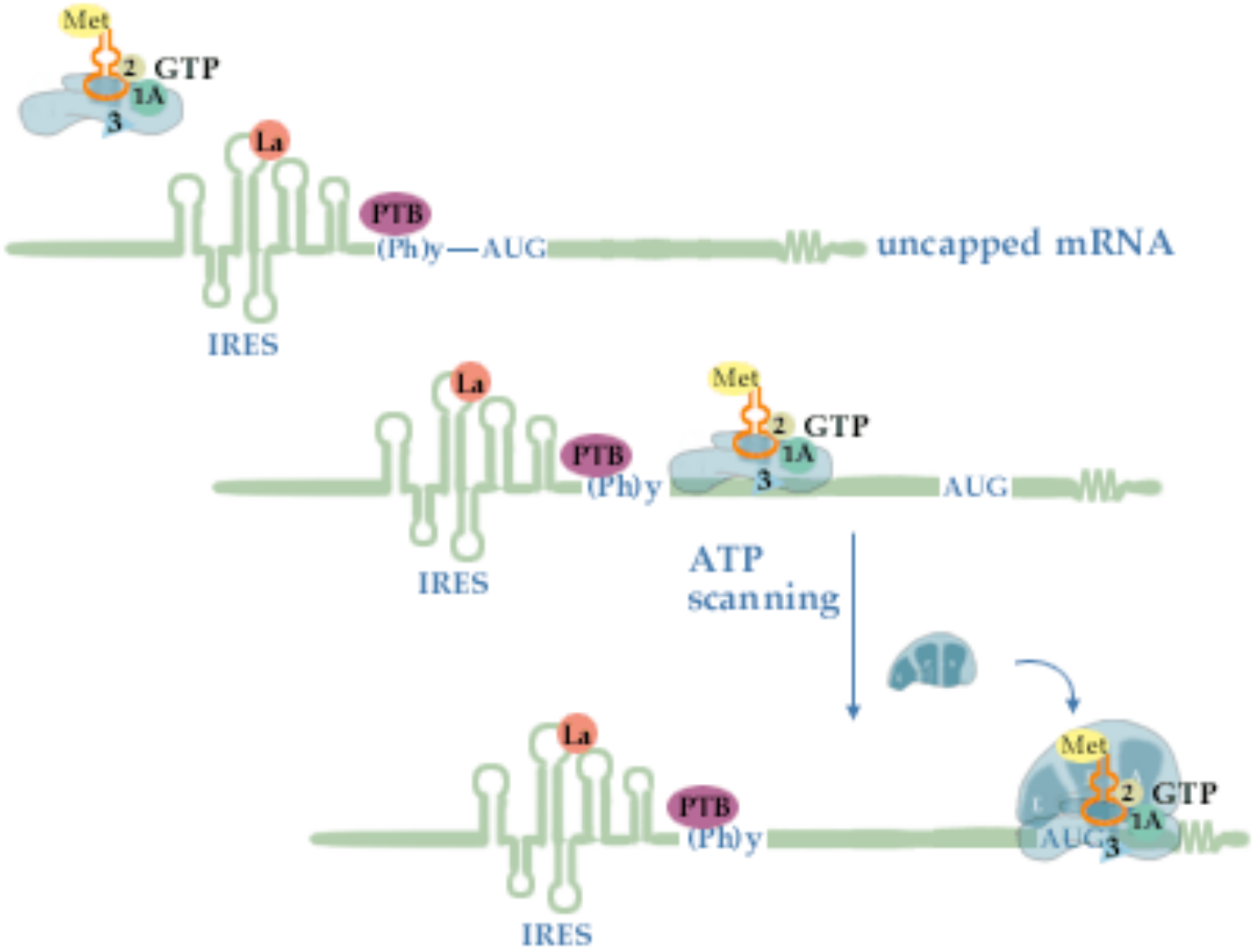


3. Self-Cleaving Peptides

Protein A - V K Q T L N F D L L K L A G D V E S N P G P - Protein B

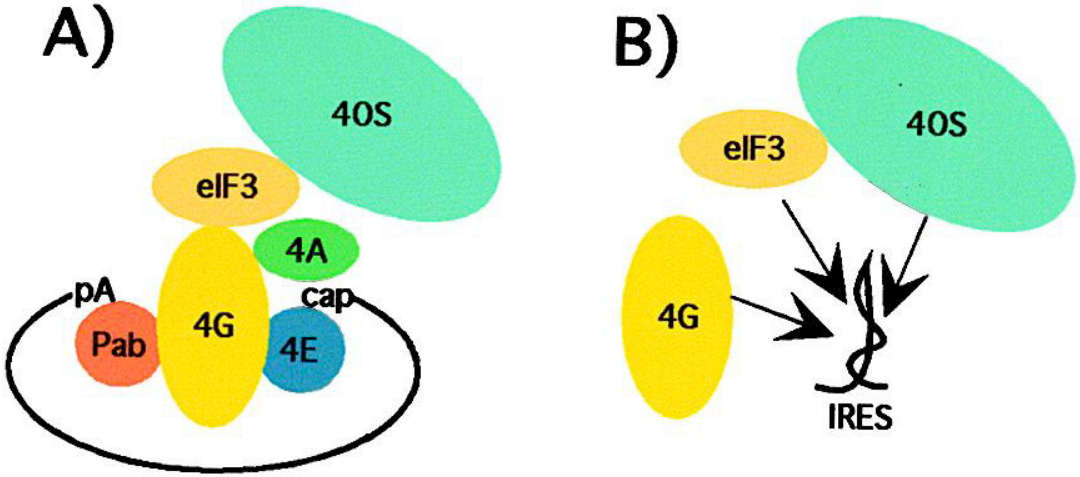


Internal Ribosomal Entry Sites



Cap-independent translation initiation;

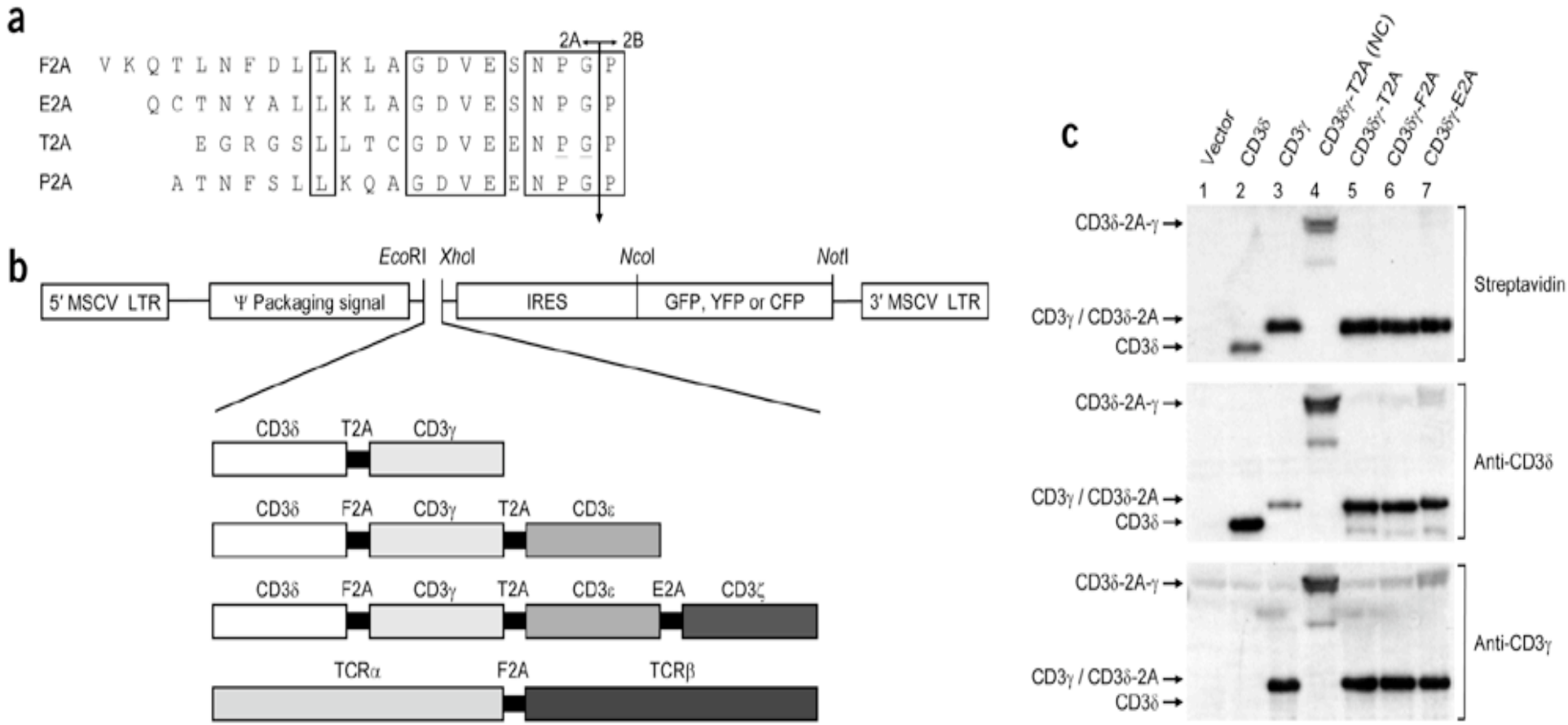
Internal Ribosomal Entry Sites
Cell Cycle Dependence



Cap-dependent translation in G1/S phase;
Cap-independent in G2/M phase;

(Pyronnet et al. Mol Cell 2000)

Self-Cleaving Peptides



(Szymczak et al. Nature Biotechnol. 2004)

Alternative Genetic Decoding
Stop codon by-passing

Seleno-Cysteine is encoded by UGA (stop codon) in conjunction with the selenocystein insertion sequence (SECIS; a 60-nt stem loop).

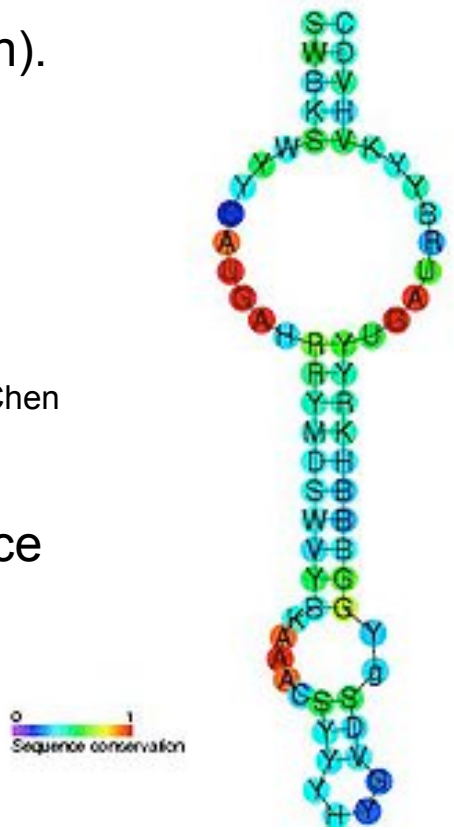
(Zinoni et al. PNAS 1987)

Pyrrolysine is encoded by UAG (stop codon). Although this is not found in eukaryotes, it can be used for functionalization of mammalian proteins: e.g. site-specific incorporation of fluorophores (“click-chemistry”).

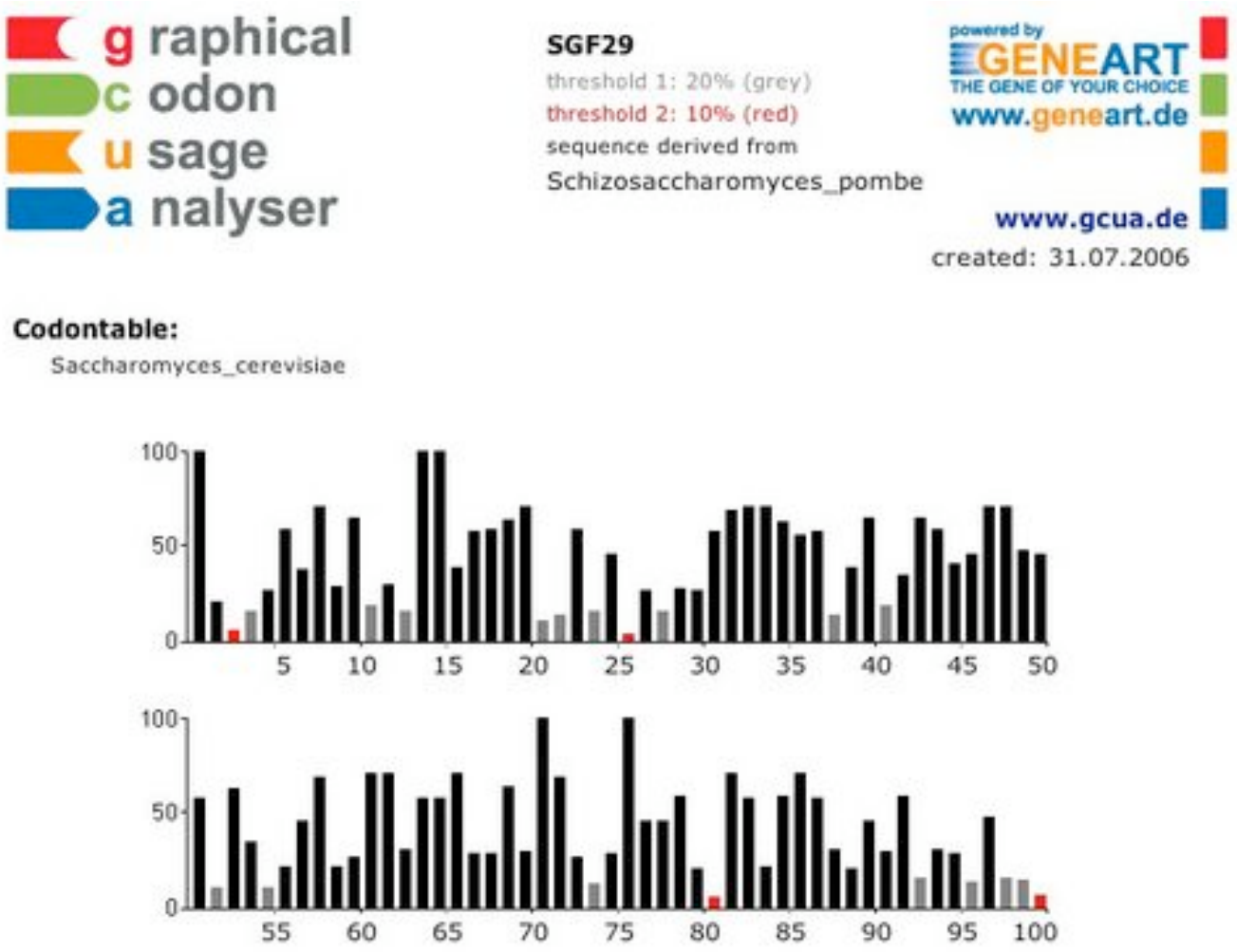
(Hao et al. Science 2002; Fenkner et al. Angew. Chemie 2009; Chen et al. Angew. Chemie 2009)

Compounds such as Gentamycin can induce premature termination read-through; PTC124 is selective for PMTC;

(Welch et al. Nature 2007)



Codon Usage



Codon Usage in various organisms vary. “Humanization” of genes is used to boost expression levels (e.g. in antibody production, gene synthesis).
Other applications: Codon optimized HIV proteins might be useful as vaccines.

Selection marker I

supF

tRNA^{TYR}

suppresses UAG amber (stop) codon;

supF plasmids require complementation of an amber mutant in the Amp and Tet resistance gene on the p3 episome; (MC1061/p3 and selection in Tet/ Amp);

Puromycin

inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes;

fungi and gram-negative are resistant due to low permeability ot puro;

Neomycin, G418, Kanamycin

Neo, Kana reacts with prokaryotic 30S ribosomal subunits;

G418 reacts with eukaryotic ribosomal subunits;

Neomycin phosphotransferase inactivates all of the aminoclysocides;

therefore: NeoR gene can be used in both prokaryotes and eukaryotes;

attention: neo can inactivate wormannin!

Gentamycin can result in read-through of stop codons (Howard et al. Nature Med. 1996)

Selection marker II

Gancyclovir

negative selection;
synthetic analogue of 2'-deoxy-guanosine; phosphorylated by HSV-TK and then blocks incorporation of dGTP into DNA;
bystander effects;

DHFR

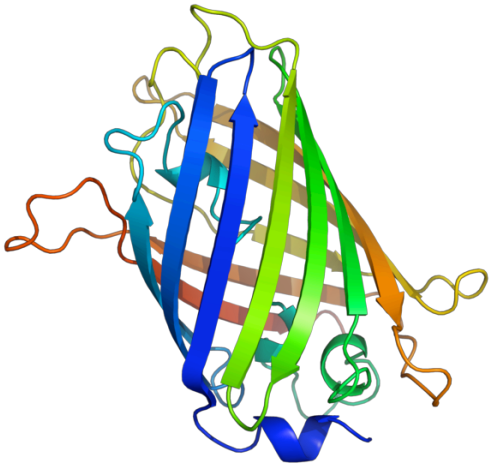
metabolic enzyme in folic acid synthesis as well as purine synthesis;
methotrexate inactivates DHFR and kills cells;
Littlefield, Biedler, Schimke, Fred Alt and others found gene amplification with this system:
mechanism unknown, but leads to amplification of genes in the surrounding sequence (used in biotechnology industry for producing large amounts of a useful protein; best: Gene_IRES_DHFR);

Zeocin, bleomycin, etc.

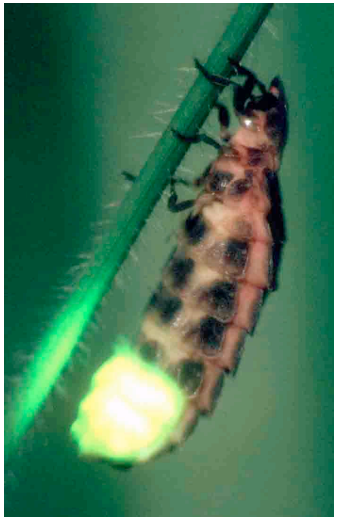
please do not use!
cleaves DNA, but resistant clones may still have some non-toxic DNA damage!

Reporter Genes

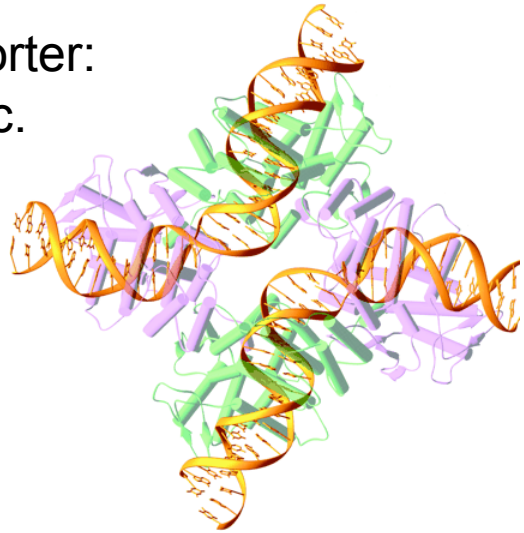
Fluorescent proteins



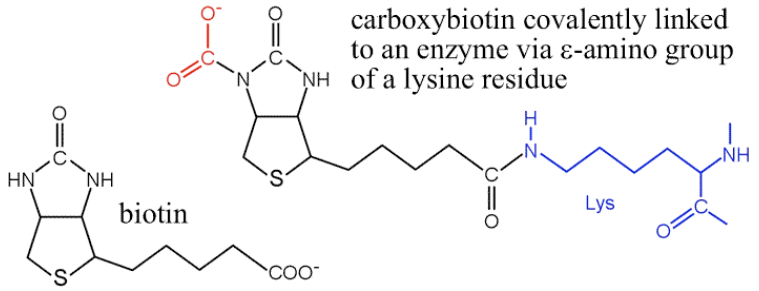
Enzyme:
- Luciferase
- Alkaline Phosphatase



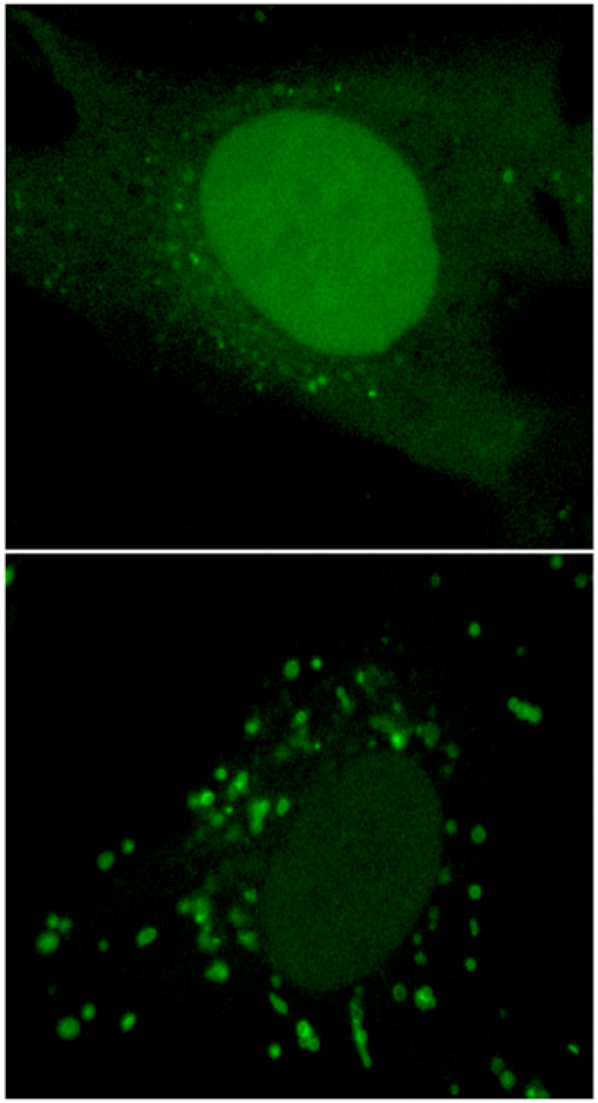
Indirect reporter:
Cre, Dre, etc.



Other:
BirA (Biotin Ligase),
Streptags, etc.



Inducible Reporter switches



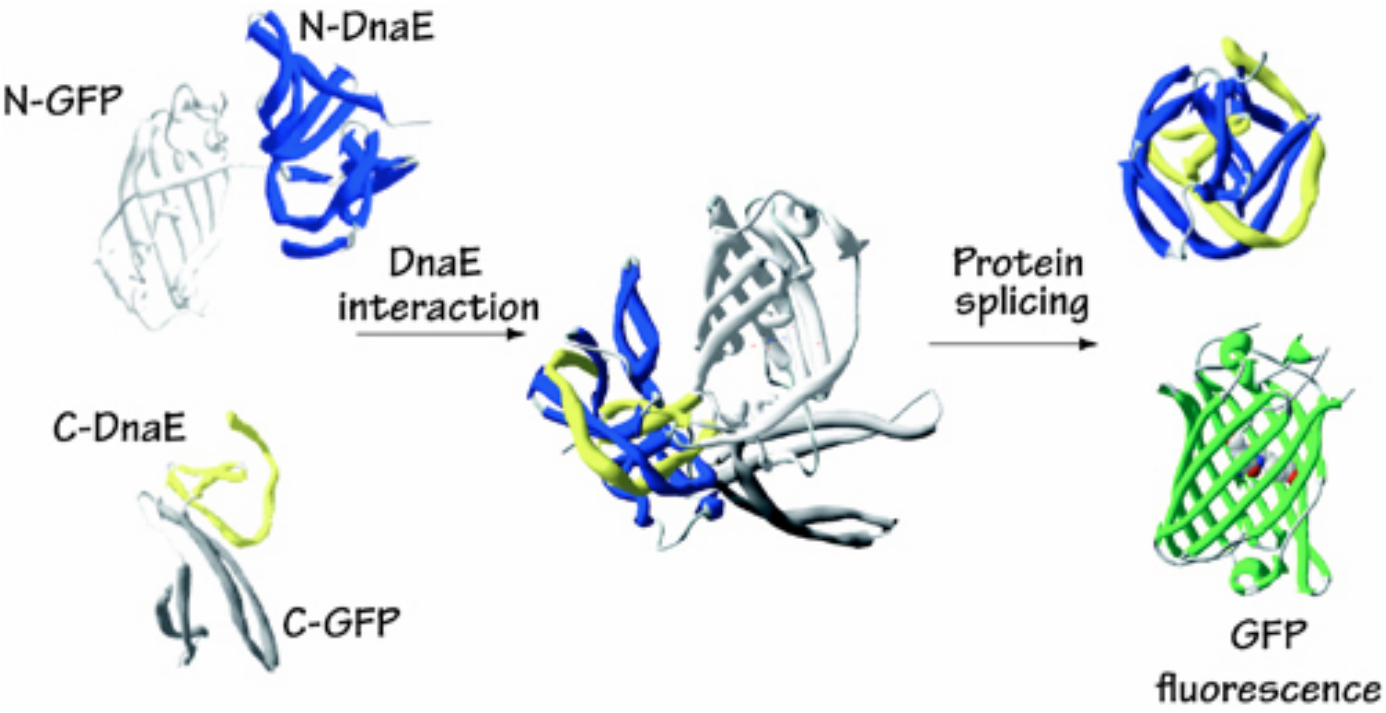
Photoactivatable GFP
Photoswitchable FP

e.g. tracking of autophagosomes

(Jennifer Lippincott-Schwartz, NIH)

lippincottschwartzlab.nichd.nih.gov/images/

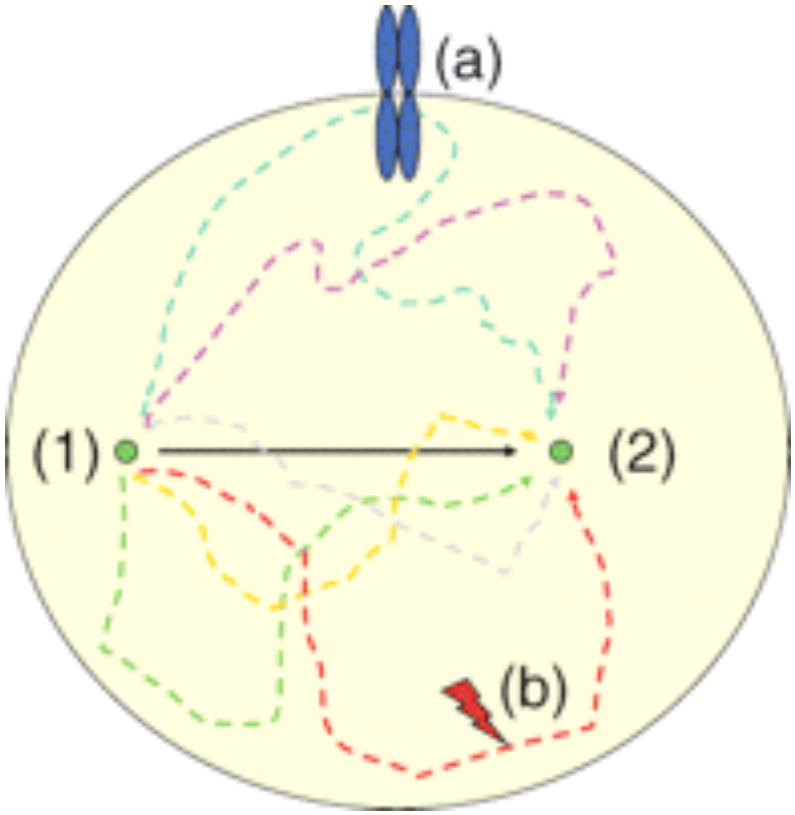
Split Reporter Constructs (GFP, LUC)

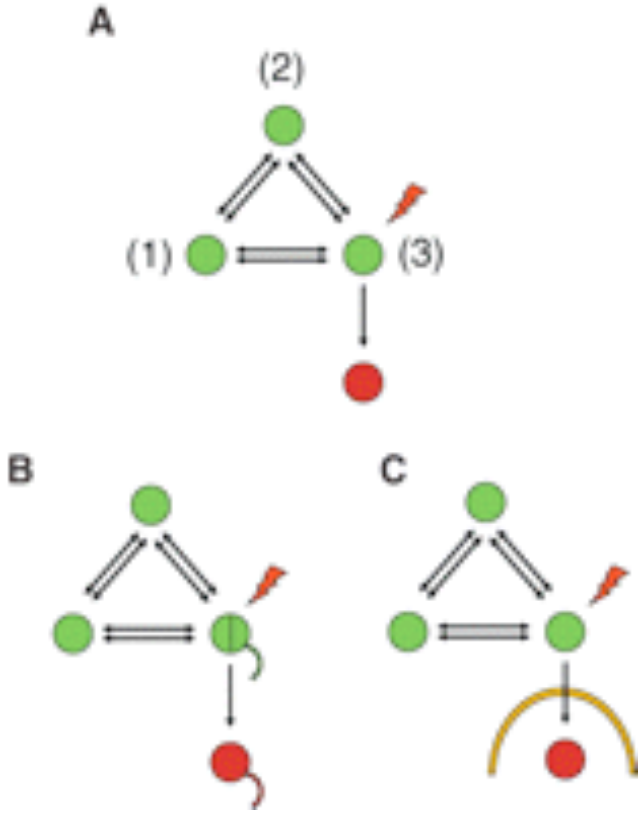


T. Ozawa, The University of Tokyo

Split Gaussia Luciferase - Remy and Michnick, Nature Methods 2008

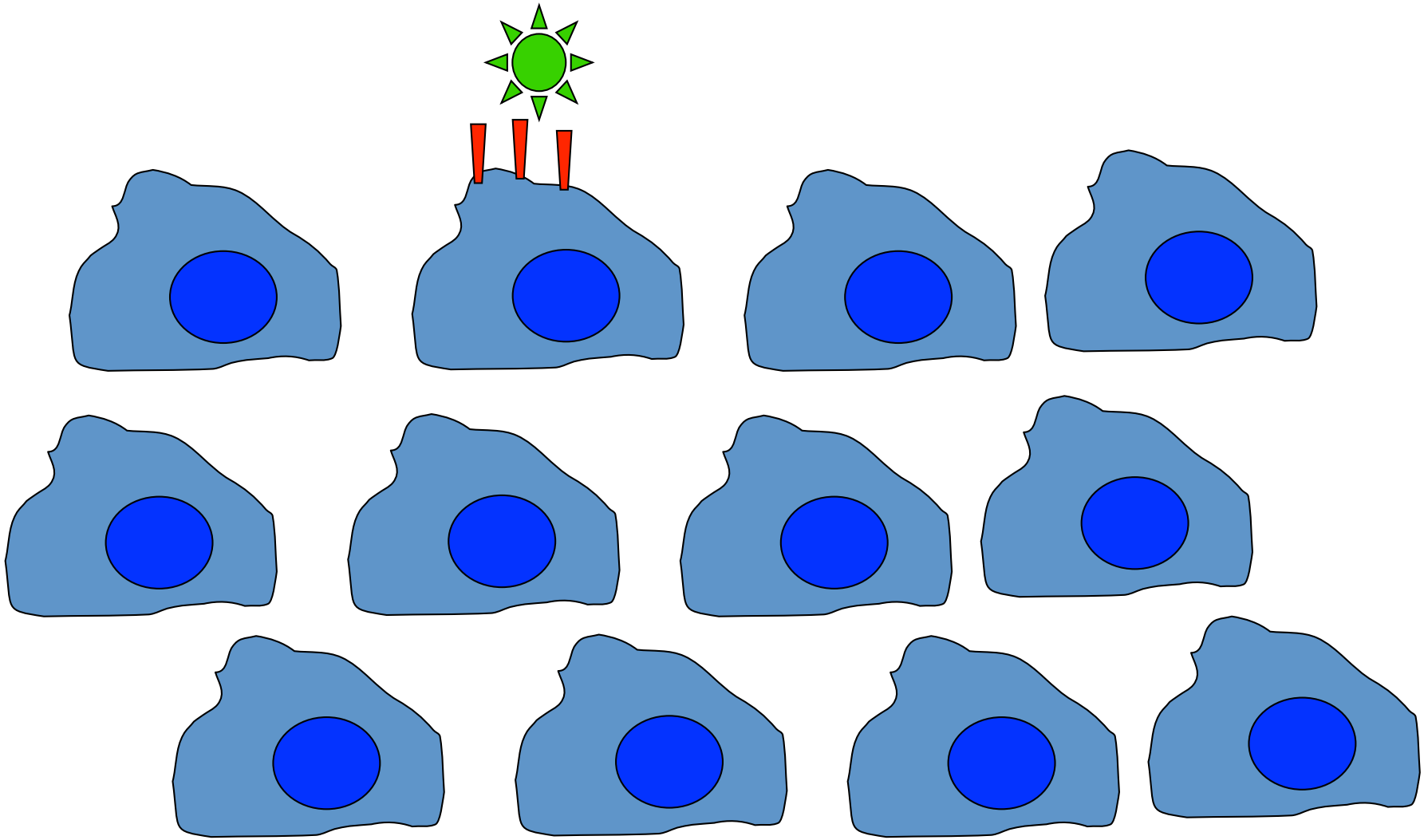
Reverse Reporter





Ketteler, J Biomol Screen 2010

Expression Cloning



cDNA expression to identify ligands, binding partners, enzymes, virus receptors, etc.

The Future in Expression Vectors:

1. Safe integration;
2. Tunable expression (systems biology, mathematical modelling);
3. Multicistronic without footprint;
4. Non-integration (Virosomal);
5. Inducible expression systems;
6. In vivo expression systems;

Lab expression constructs

Transient expression: pEAK12, 14 (EF1A; Puromycin)
pEAK13, 15 (chicken beta-actin; Puromycin)
Retrovirus: pMOWS (+/- Puromycin)

Tagged constructs:

- Flag pEAK14 HindIII Flag EcoRI Gene NotI
- Flag (secreted) pEAK15 HindIII SP Flag NheI Gene NotI
- GFP (N-term) pEAK12 HindIII GFP EcoRI Gene NotI
- GFP (N-term) pMOWSdSV BamHI GFP EcoRI Gene NotI
- GFP (N-term) pMOWS GFP BamHI Gene NotI
- GFP (C-term) pEAK12 HindIII Gene EcoRI GFP NotI

Bicistronic constructs:

- T2A (to be inserted by PCR or Adapter)
- IRES pOS BamHI Gene1 EcoRI IRES GFP NotI
- Dual promoter pMOWSdSV BamHI Gene1 EcoRI NotI SV40 XhoI Gene2 SalI

Promoter constructs

- Luciferase SpeI Promoter XbaI FLUC NotI

How to design expression constructs

1. Large genes, with template (>300 nt) PCR
2. Small genes (<300 nt) Gene synthesis, PCR
3. Very small elements (<80 nt) Adapter cloning

4. Point mutations Overlap Extension PCR
5. Random mutations GeneMorph (Stratagene)
6. Deletions Overlap Extension PCR
7. Fusions Overlap Extension PCR
8. shRNA hairpins Adapter cloning

Adapter Cloning

1. Design sense and antisense Oligo; (e.g. EcoRI - Insert - BamHI)

```
5′-AATTCAAGCTTatgaaggcccatttgataaG-3′
    3′-GTTCGAAtacttccgggtaaactattCCTAG-5′
```

2. Phosphorylate each Oligo;

   T4 Polynucleotide Kinase (PNK; New England Biolabs)

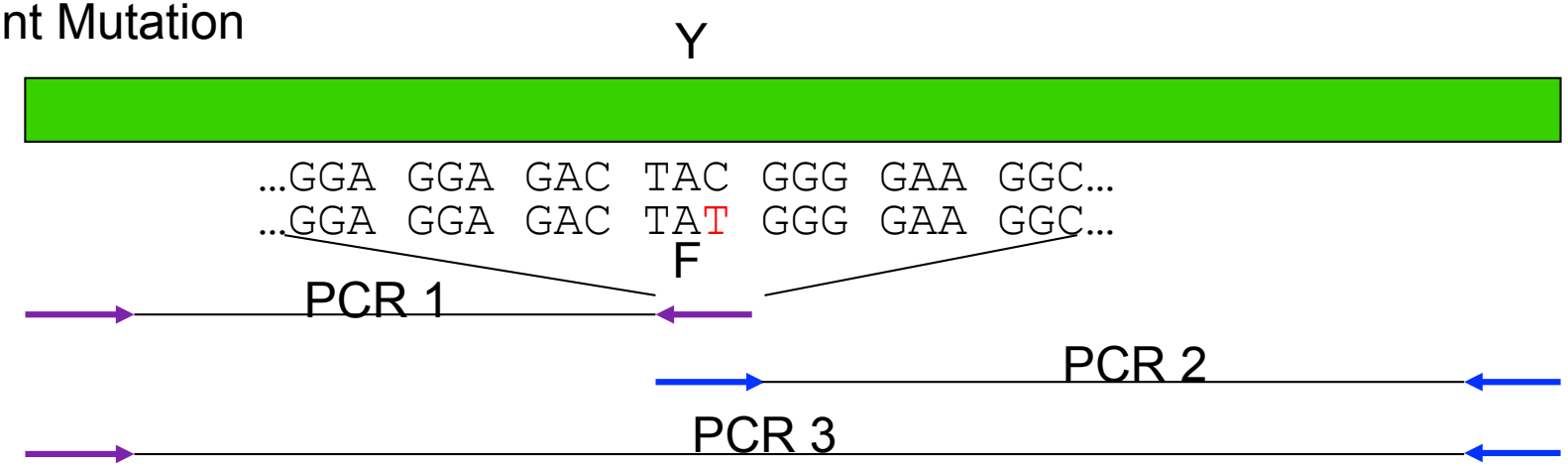
3. Anneal both Oligos;

   Mix and heat at 95C; then cool down slowly;

4. Ligate into Vector;

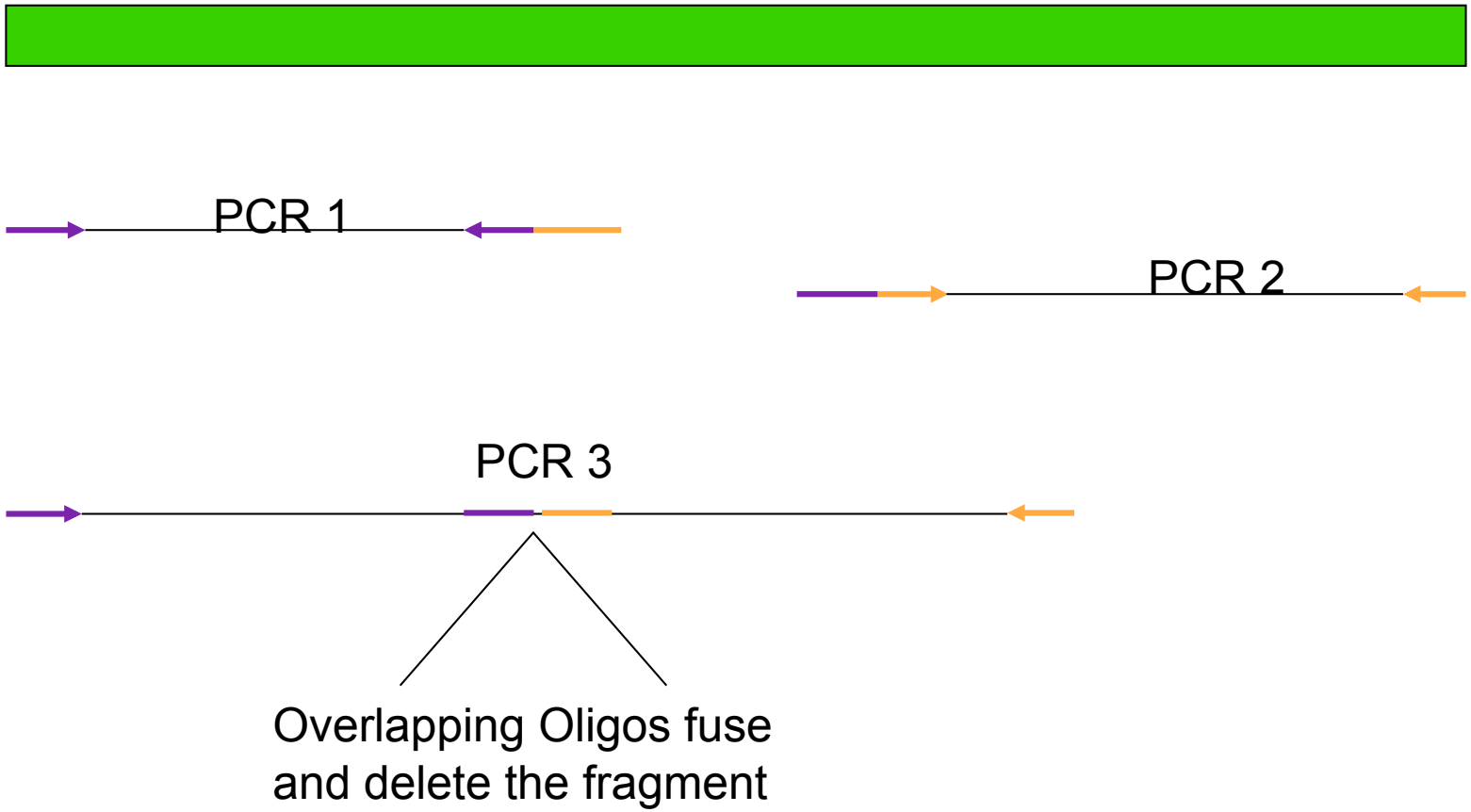
Overlap Extension PCR

1. Point Mutation



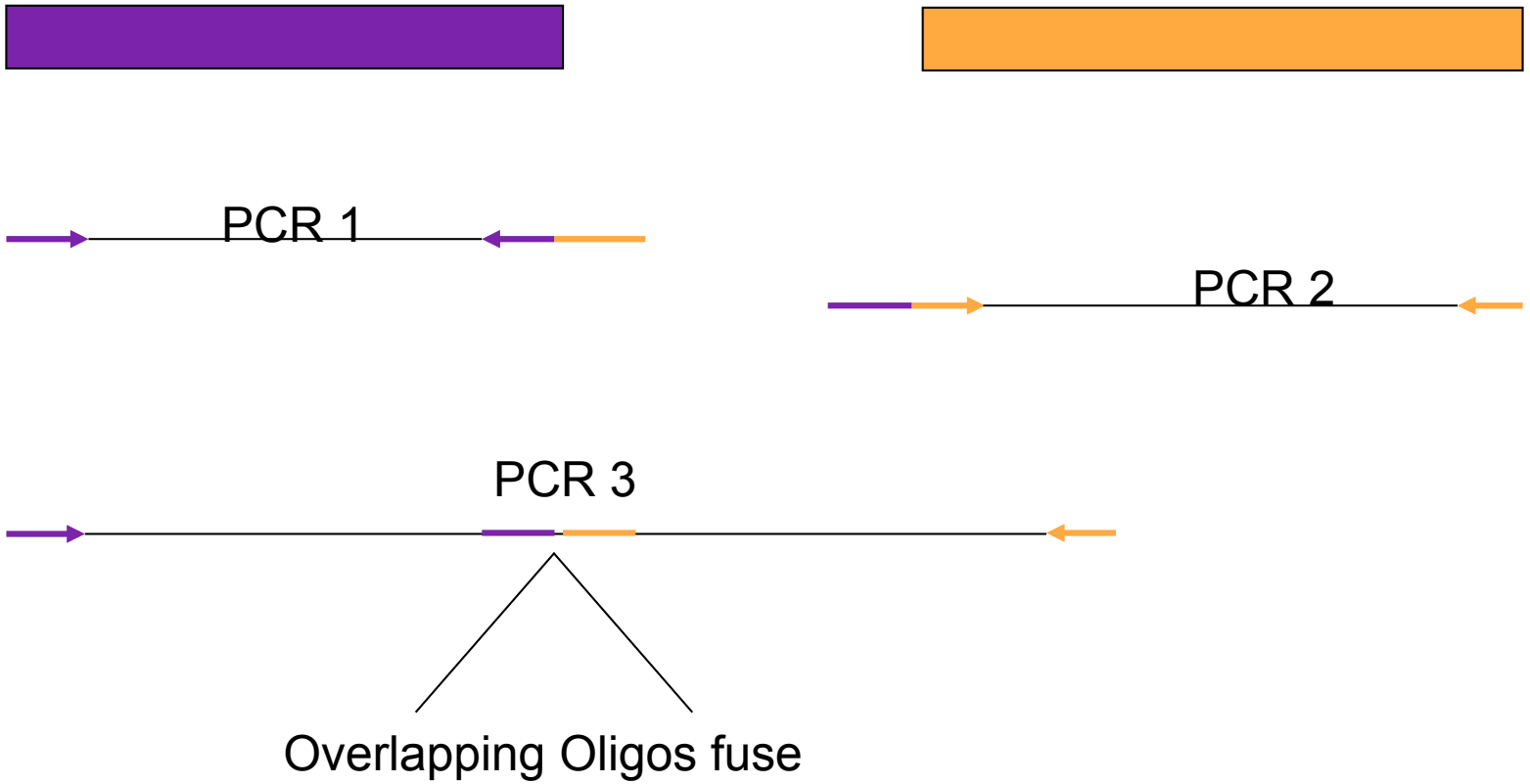
Overlap Extension PCR

2. Deletion



Overlap Extension PCR

3. Fusion



Overlap Extension PCR

3. Fusion

Alternative Method I: Translational Fusion by Restriction digest But leaves restriciton site between the fragments! (BamHI = GlySer; EcoRI = GlnPhe)

Alternative Method II: Type II restriction enzymes.

BsmBI `5′-…CGTCTCNNNNN…-3′`

`3′-…GCAGAGNNNNN…-5′`

“NNNN” can be matched to the fusion sequence, therefore not leaving any trace of the cloning process.

There are other enzymes of this family (BbsI, etc.)…

Where to get genes/genomic elements

Gene
NCBI - Nuceotide search - IMAGE clone number
Open Biosystems - http://www.openbiosystems.com/
Addgene - http://www.addgene.org/
Origene - http://www.origene.com/

Genomic element - BAC
Genome Browser - Gene/Genome search - BAC number - BACPAC resources
http://genome.ucsc.edu/
http://bacpac.chori.org/