Cloning in eukaryotes (part 1)

Saccharomyces cerevisiae

1) Types of vectors

2) Integrative gene deletion and whole genome screens

3) TAR cloning

Guide to readings:

1) *Yeast as a model organism 2011*

2) *Glaever et al. 2002*. Creation of an ordered library of barcoded yeast deletion strains.

3) *TAR cloning 2008.* A protocol describing how to use the transformation-associated recombination cloning protocol in yeast

4) *Use of TAR to reconstruct SARS CoV2.* A recent description of construction of synthetic virus from sequence info

Saccharomyces cerevisiae: a model eukaryote

1) Biochemistry and cell biology similar between yeast and "higher" eukaryotes

   -- many gene homologs between yeast and humans, eg. Cell cycle (cancer) genes

2) It is easy to convert it from diploid to haploid (by sporulation) and back to diploid (by mating), so you can look at mutations haploid and diploid

3) Excellent genetic tools are available

4) Very easy to work with in the laboratory

Yeast and genomics

- The yeast genome was sequenced in 1996
- It became a model system for inventing and testing tools for studying entire eukaryotic genomes/transcriptomes/proteomes etc.
- The functions of many eukaryotic gene families were first discovered in yeast
- http://www.yeastgenome.org/

Transformation using yeast

- Electroporation, or chemical competence (Lithium chloride/PEG treatment)

- Selection:
  - nutritional markers
    - His3, Leu2, Trp1: amino acid biosynthetic genes
    - Ura3 – nucleotide biosynthetic gene
    - these require auxotrophic yeast strains

  or

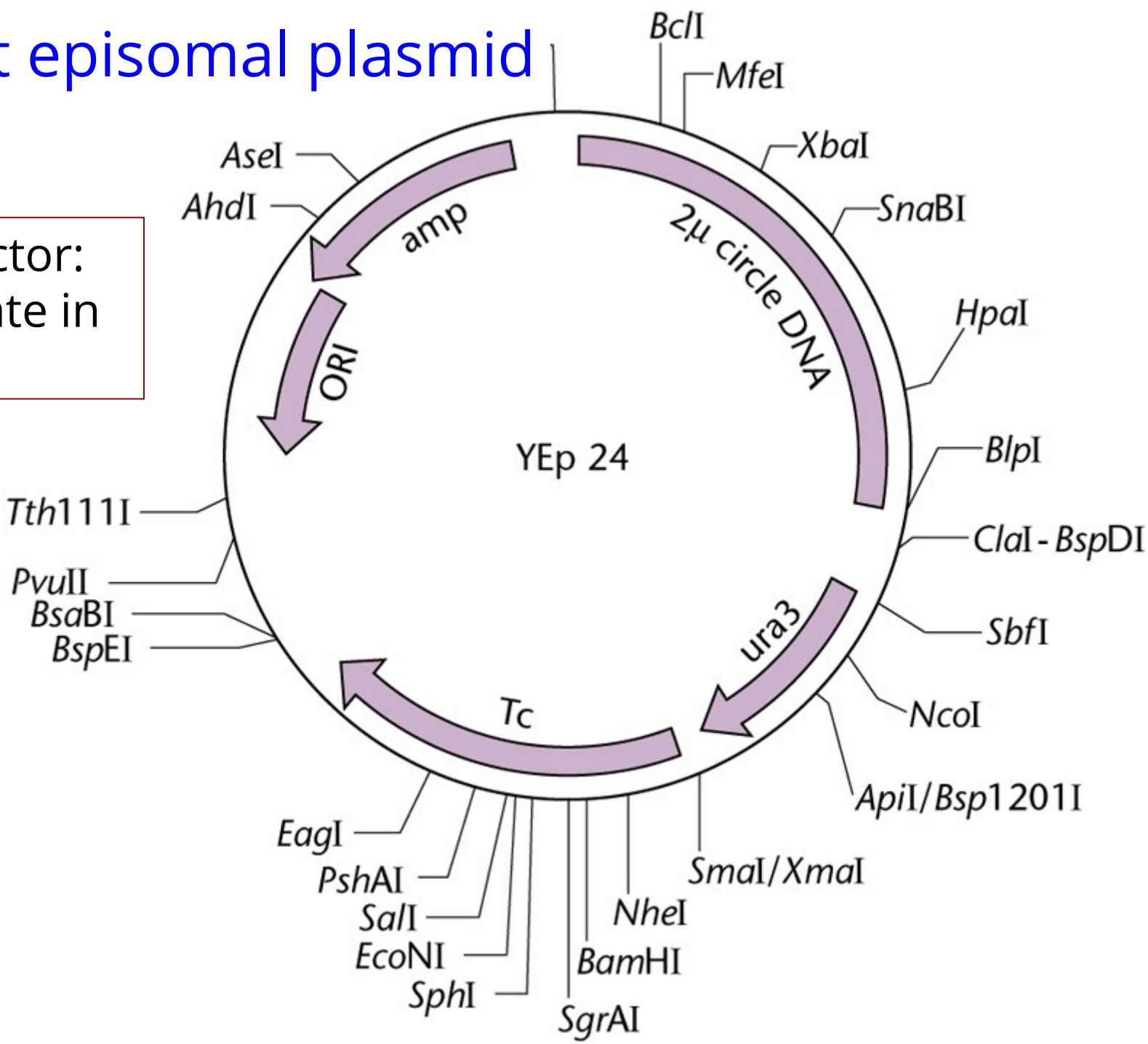
  - Aminoglycoside (ribosome inactivating) antibiotic resistance (kanamycin)

Yeast Episomal plasmid: high copy number plasmid

- Contains naturally occuring “2 micron circle” origin of replication
- High copy number: 50-100/cell

A yeast episomal plasmid

Shuttle vector: can replicate in *E. coli*, too



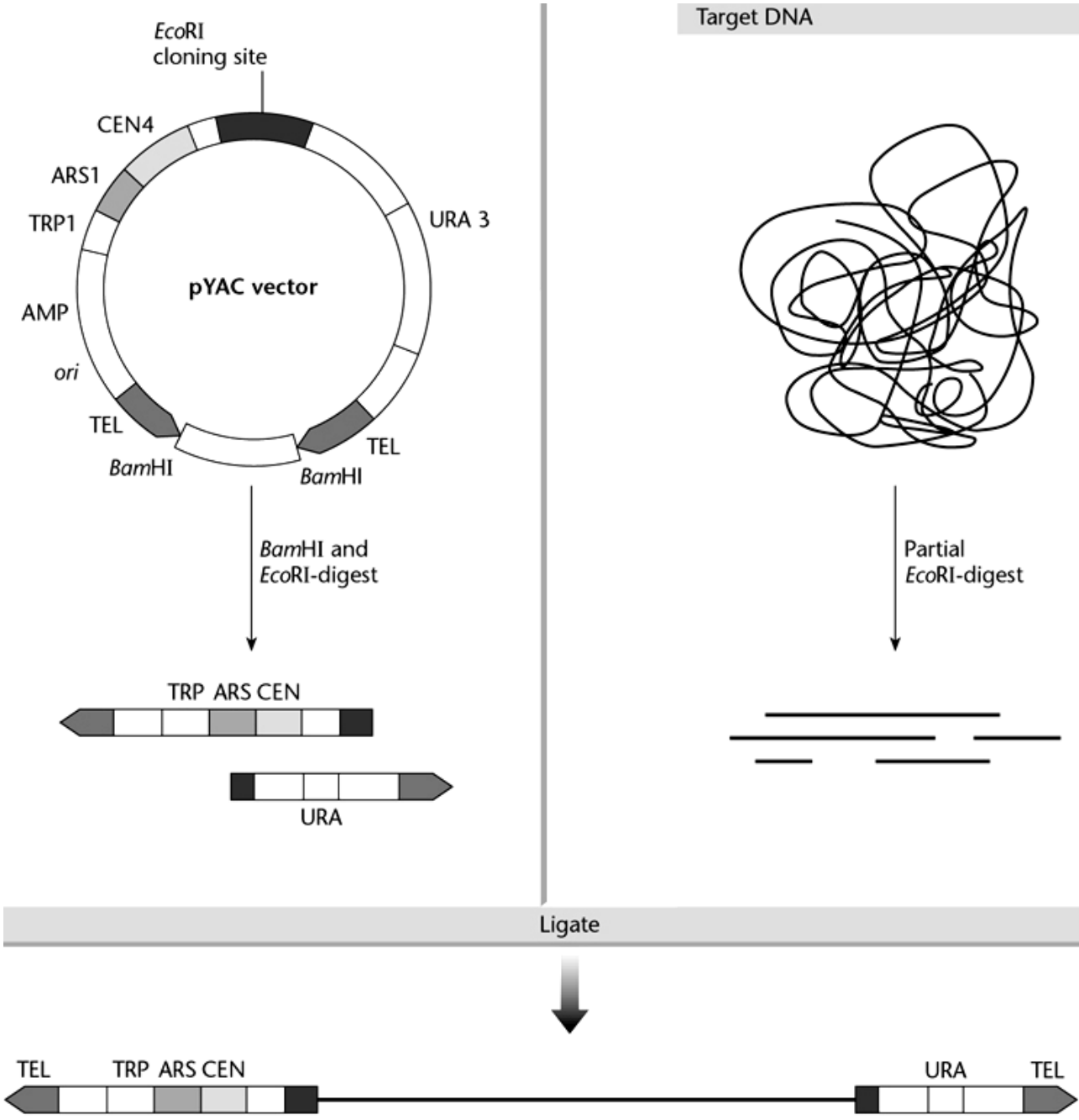
Yeast Centromeric plasmid: low copy number plasmid

- Yeast Centromeric plasmid
- Contains yeast *ars* (autonomously replicating sequence) for replication
- Contains yeast centromere for proper segregation to daughter cells

- Very low copy number, ~1 per cell (good for cloning genes that are toxic or otherwise affect cell physiology)

YAC: yeast artificial chromosome

- Replicates as chromosome: centromere and telomeres
- Clone very large pieces of DNA



Yeast integrative plasmid: homologous recombination

- No yeast replicon in the plasmid
- Can transform but cannot replicate
- Requires integration into chromosome for propagation
- Genes on the chromosome can be manipulated/deleted

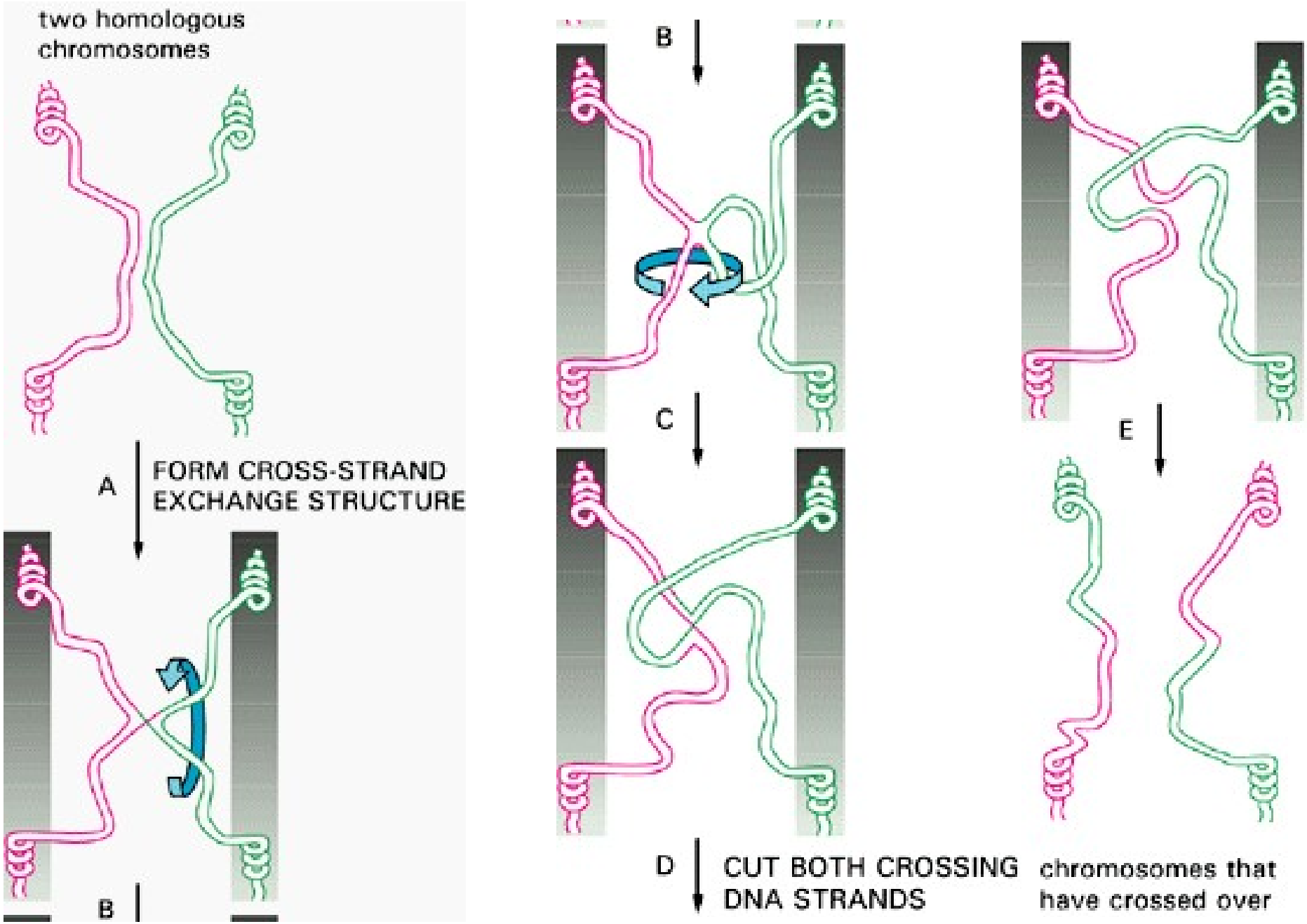
DNA can be integrated by recombination

- If transformed DNA has no way to replicate and has homology to chromosome, it can be integrated by homologous recombination

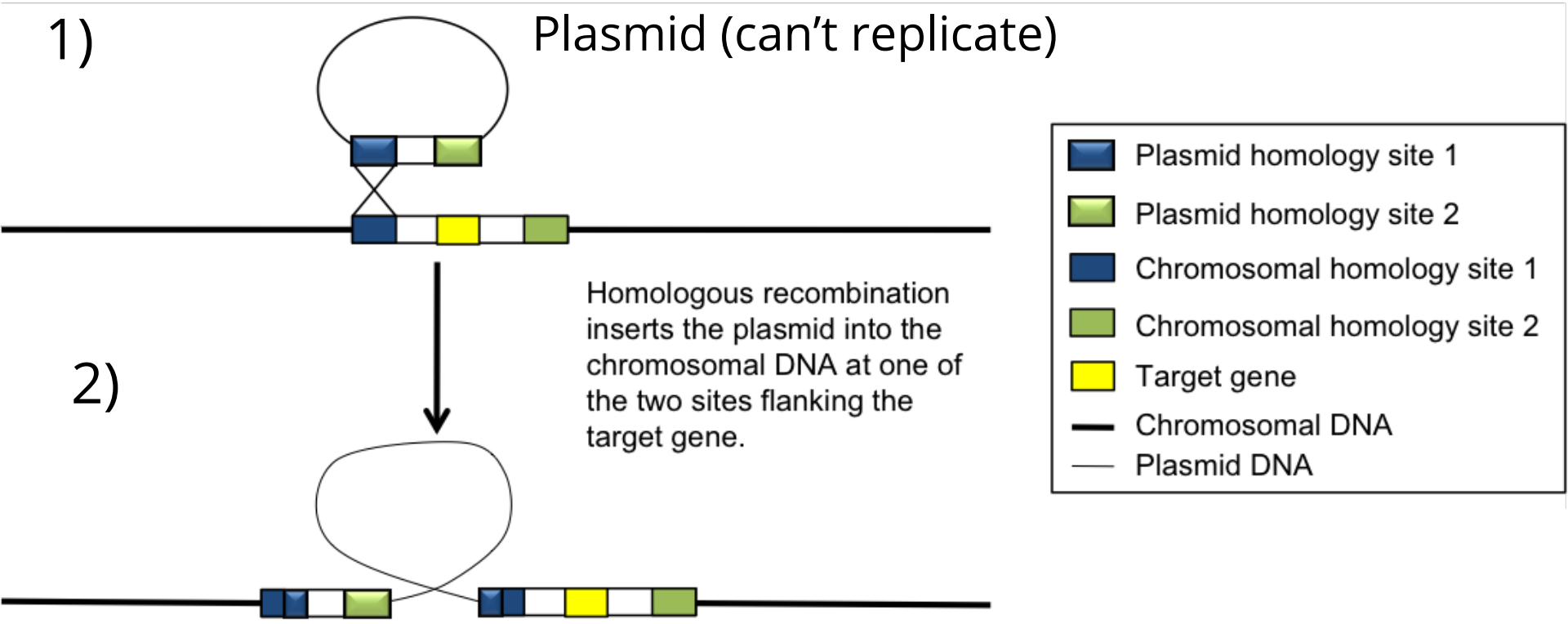
- Two pieces of DNA with the same (or similar) sequence: RecA protein (bacteria) or Rad51 (eukaryotes) causes strand exchange between homologous sequences

- Homologous recombination occurs with a highly predictable frequency: in a transformation experiment with a reasonable length of homologous DNA, ~1/1000 transformed cells will recombine the transformed DNA

Homologous recombination: portrait of a single cross-over

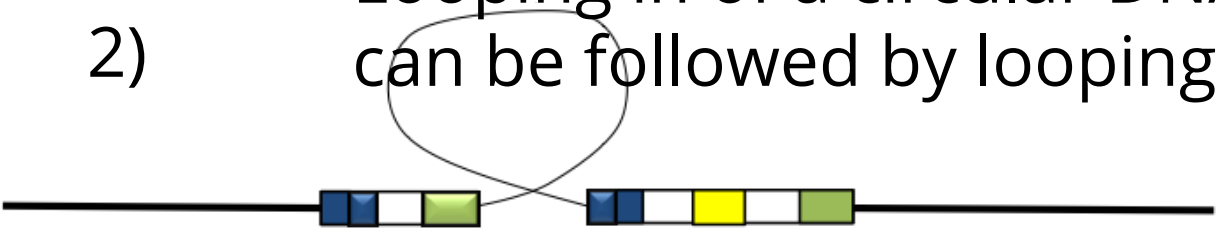


Looping in of a circular DNA by recombination can be followed by looping out



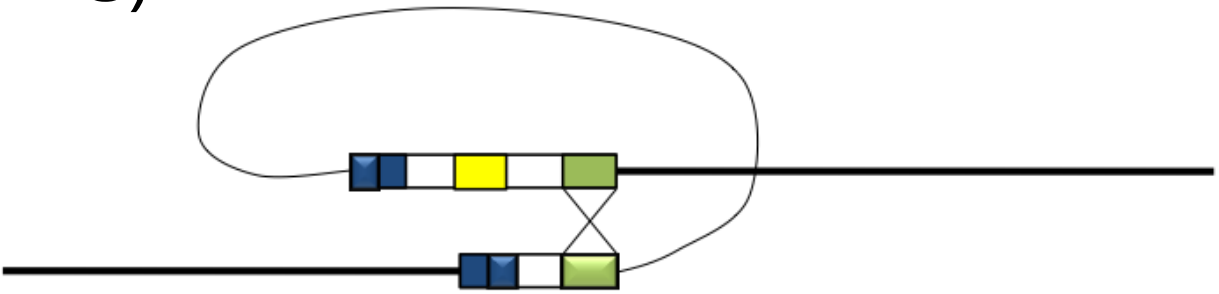
Looping in of a circular DNA by recombination can be followed by looping out

2)



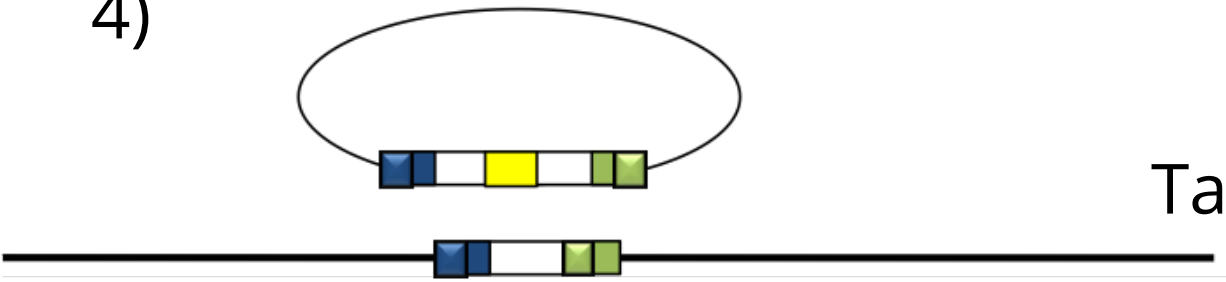
The DNA realigns itself to undergo recombination at the other homology locus.

3)



Homologous recombination removes the plasmid from the chromosomal DNA along with the target gene.

4)

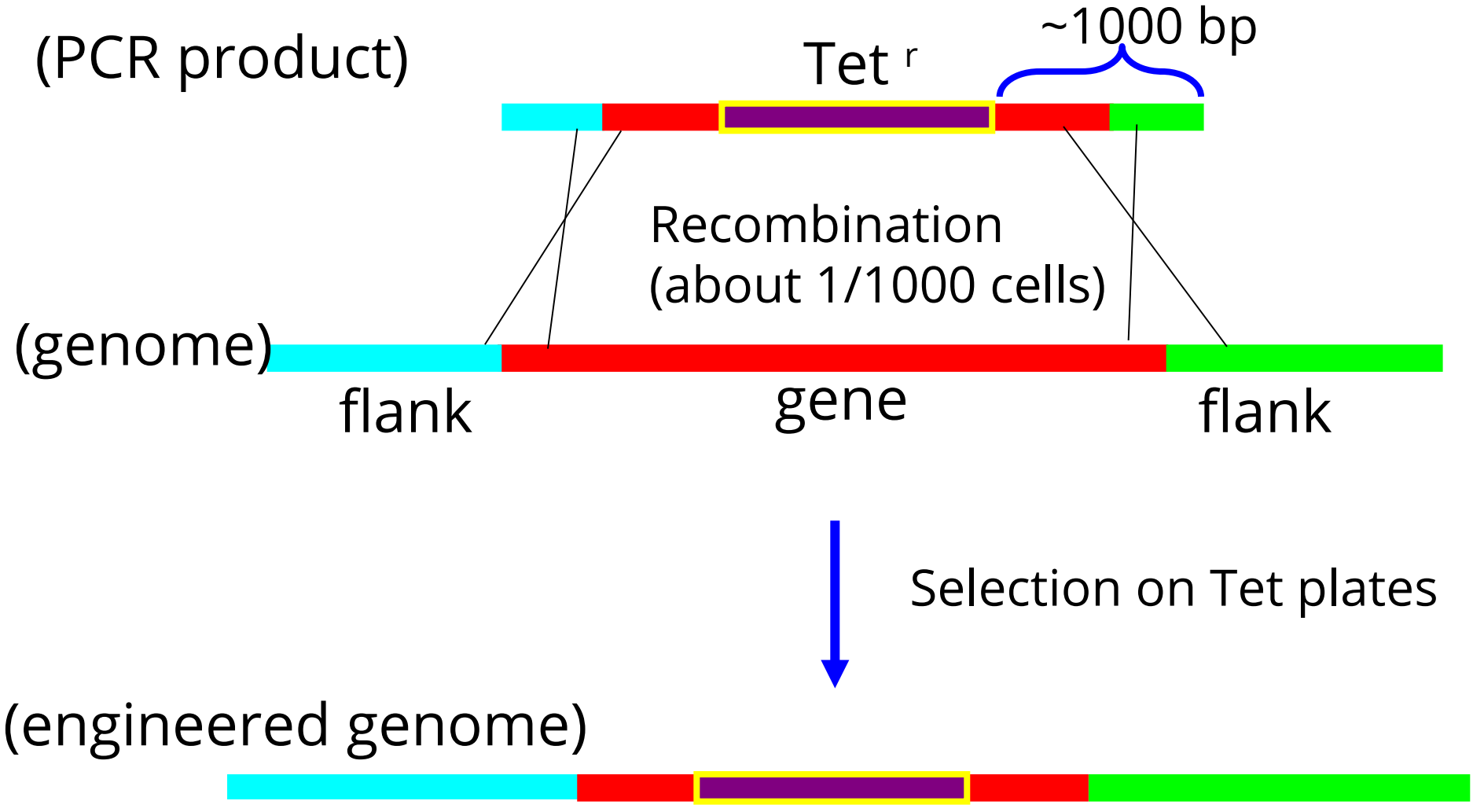


Target gene deleted

Homologous recombination could occur at the other locus in either of step one and step three, leading to four total possible paths, two of which produce the desired removal. Screening is required in order to determine in which cells the knockout was successful.

http://sites.tufts.edu/innateimmunity/knock-out-and-silencing/

Homologous recombination (double crossover) to knock out a gene

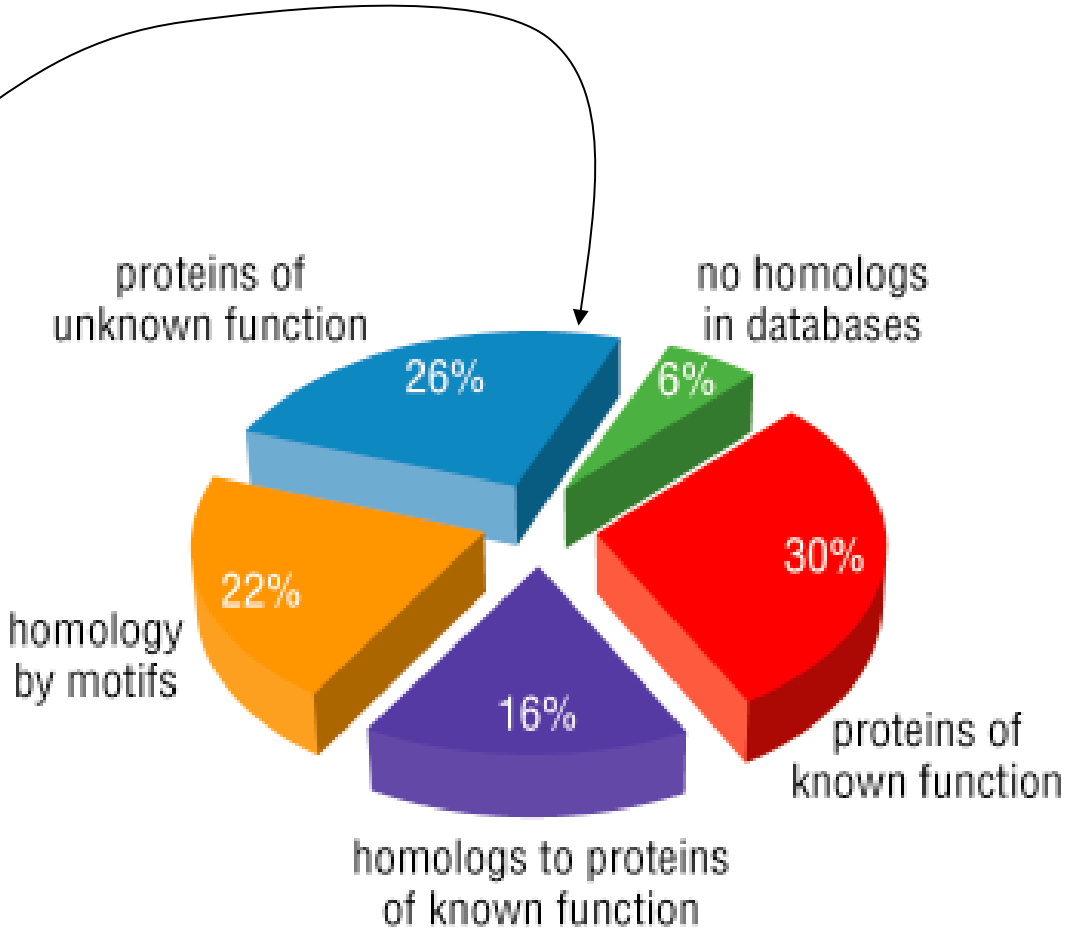


Homology: the function is only inferred

Genome Sizes of Representative Organisms

Organism	Genome size (base pairs)	Number of genes
Mycoplasma genitalium	45.8×10^5	483
Methanococcus jannaschii	1.6×10^6	1,783
Escherichia coli	4.6×10^6	4,377
Pseudomonas aeruginosa	6.3×10^6	5,570
Saccharomyces cerevisiae	1.2×10^7	6,282
Caenorhabditis elegans	1.0×10^8	19,820
Drosophila melanogaster	1.8×10^8	13,601
Arabidopsis thaliana	1.2×10^8	25,498
Homo sapiens	3.3×10^9	~30,000 (?)

Large percentages of coding proteins cannot be assigned function based on homology



Gene function: phenotypes of knockouts

All yeast genes were knocked out -- what what can this tell us about their functions?

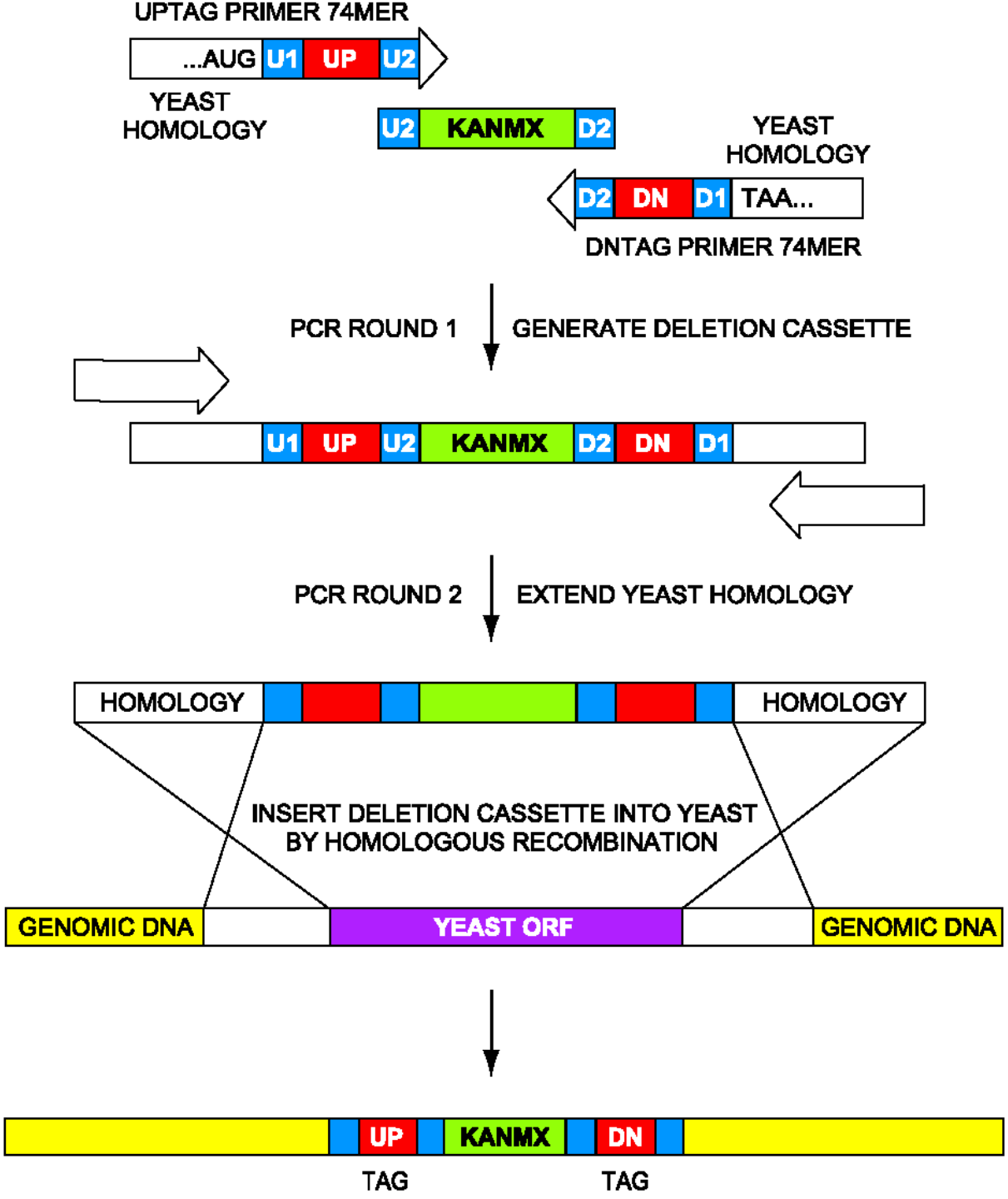
Use “bar codes” for tracing yeast knockout strains…

"Functional profiling of the *Saccharomyces cerevisiae* genome." Glaever et al. *Nature* 2002

"We systematically constructed a nearly complete collection of gene-deletion mutants (96% of annotated open reading frames, or ORFs) of the yeast Saccharomyces cerevisiae. DNA sequences dubbed 'molecular bar codes' uniquely identify each strain, enabling their growth to be analysed in parallel and the fitness contribution of each gene to be quantitatively assessed by hybridization to high-density oligonucleotide arrays."

"We failed to delete 215 genes for unknown reasons; about 62% of these are questionable ORFs that have no known biological function."

Knockouts with tags



Example of gene knock-out screen

Which genes are required for growth with galactose as the sole carbon source?

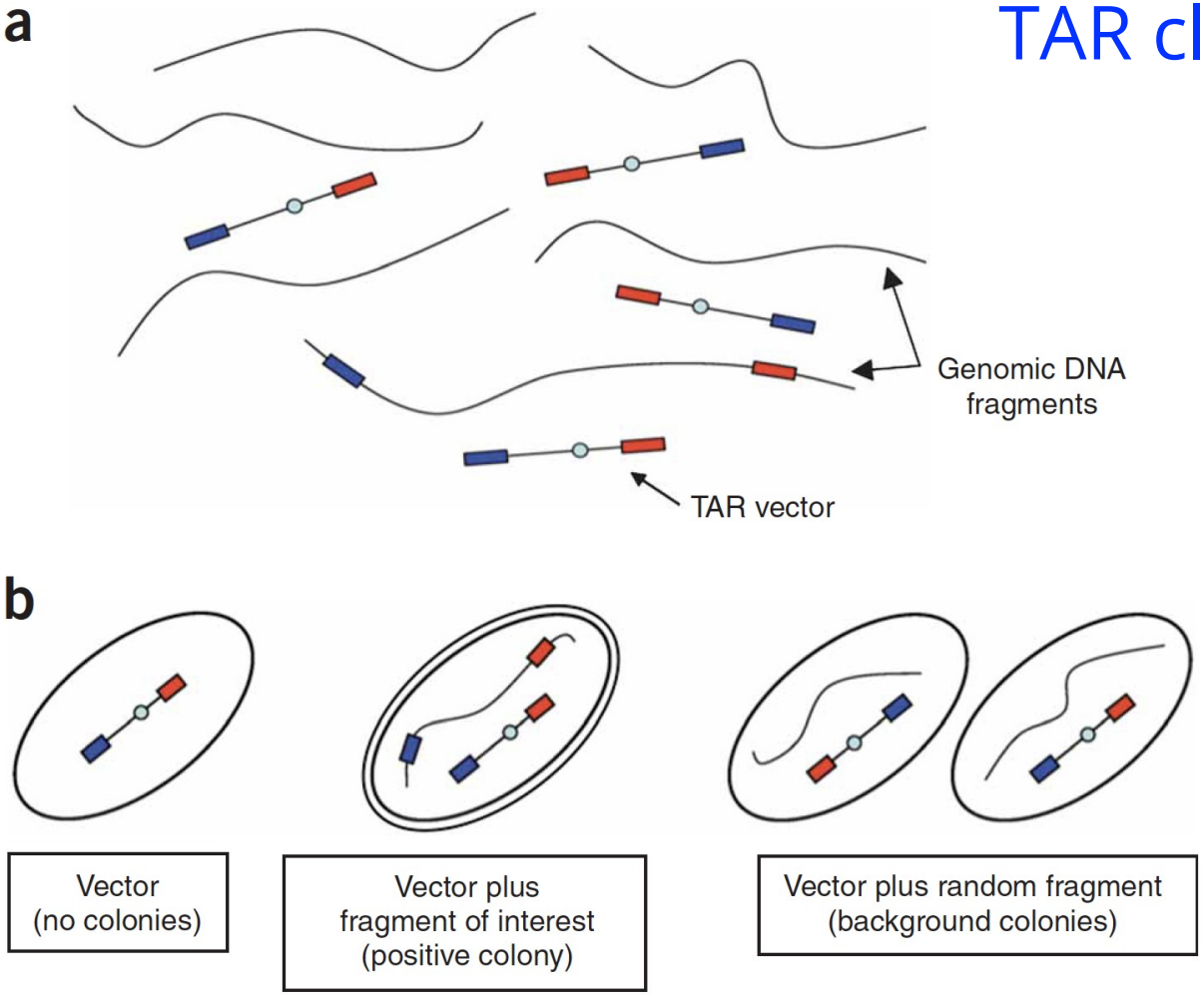
1. Pool collection of viable gene knock-outs
2. Grow on galactose minimal medium
3. Strains with knock-outs of genes essential for growth on galactose will die, removed from population
4. Amplify and label “bar codes”, probe a microarray containing all bar codes--which ones disappear when galactose is sole carbon source?

- 10 new genes identified in galactose metabolism

Cloning in yeast:
TAR (Transformation-associated recombination)

- Free DNA ends are good substrates for homologous recombination in yeast
- Any chromosomal fragment up to 300 kb in length can be isolated in yACs within weeks and with high efficiency
- This means you can clone entire eukaryotic genes, with introns, exons, and control regions, with relative ease

TAR cloning

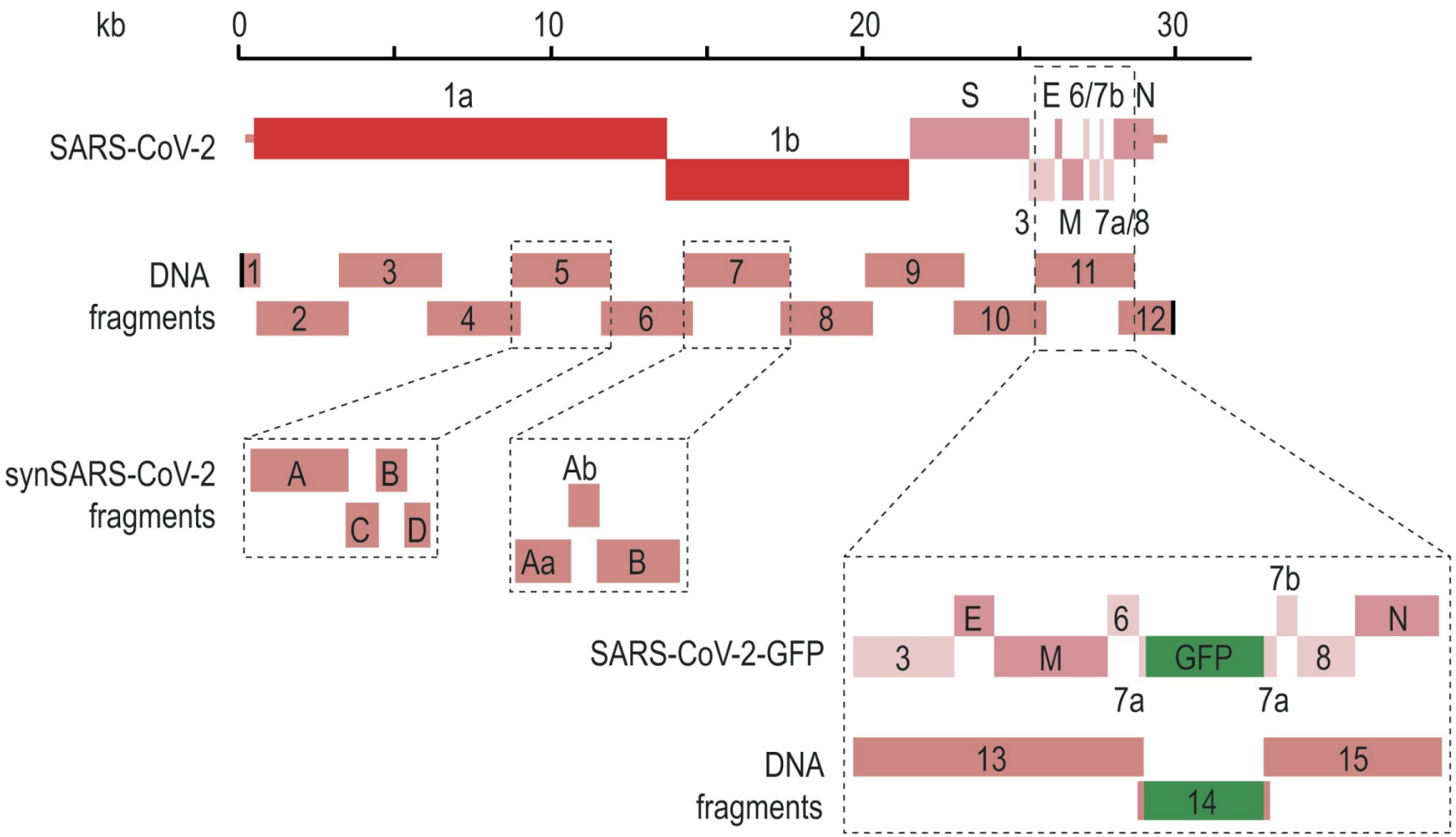


- Fragment genome
- Transform yeast with fragments along with TAR circular yAC vector containing ‘hooks’ for homologous recombination
- Check transformants with PCR primers in middle of desired clone fragment

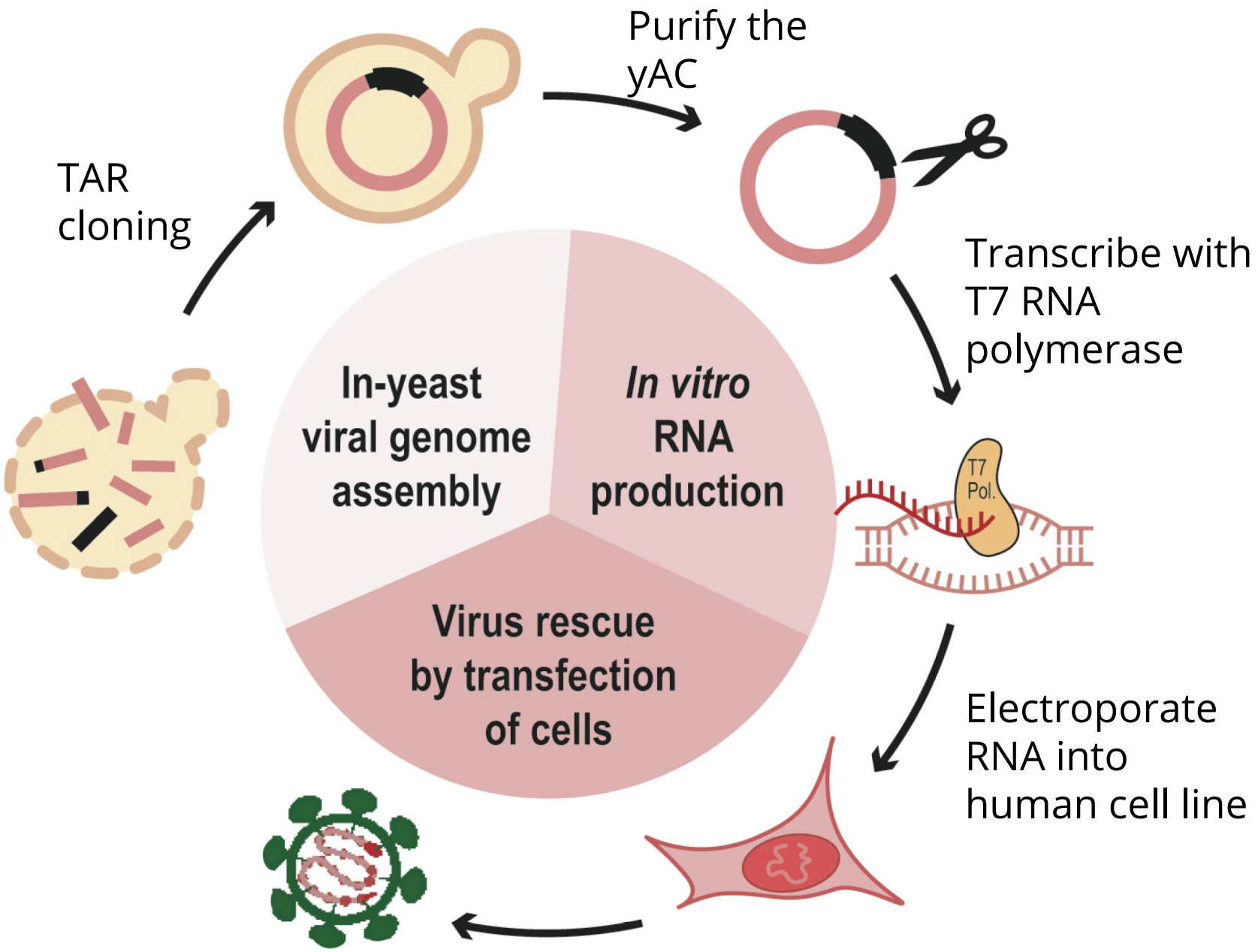
Reconstruction of the SARS CoV 2 genome using TAR cloning

- The size of the genome (~30 kilobases) makes it difficult to clone and maintain in E. coli
- TAR cloning permits one step transformation/assembly from numerous smaller fragments
- A GFP expressing version of the virus was created to assist in screening of antivirals–easy to track presence/absence of virus

DNA fragments for assembly of SARS CoV 2 and variants



Reviving virus from DNA fragments



Cloning in *S. cerevisiae* (cloning in eukaryotes, part 1)

1) Types of vectors

2) Integrative gene deletion and whole genome screens