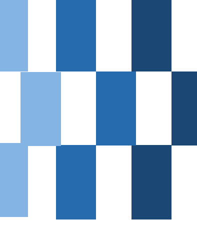
Chapter 4

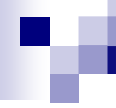
Recombinant DNA Technology



5. Plasmid Cloning Vectors

Plasmid

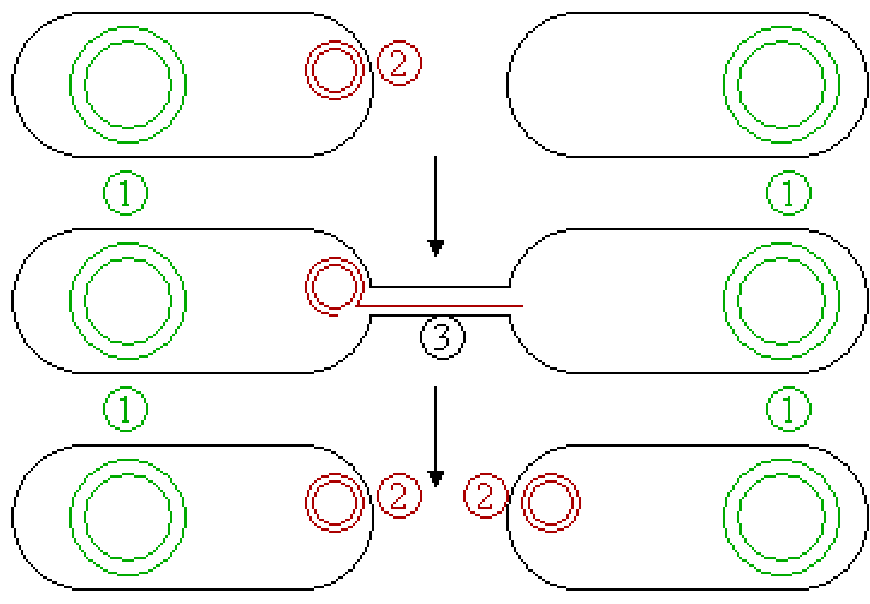
- Plasmids
  - Self replicating
  - Double-stranded
  - Mostly circular DNA (<1 kb~ > 500 kb)
    - Linear : *Streptomyces*, *Borrelia burgdorferi*
  - Replicon
    - Maintained in bacteria as independent extrachromosomal entities : own replication origin
  - Generally dispensable



Types of plasmids

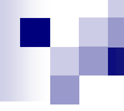
- F plasmid (conjugative)
  - Carry genes to transfer the own plasmid to another cell
- R plasmid
  - Carry antibiotics resistant gene
- Degradative plasmid
  - Carry specific genes for the utilization of unusual metabolites
- Cryptic plasmid
  - No apparent functional coding genes

Conjugation



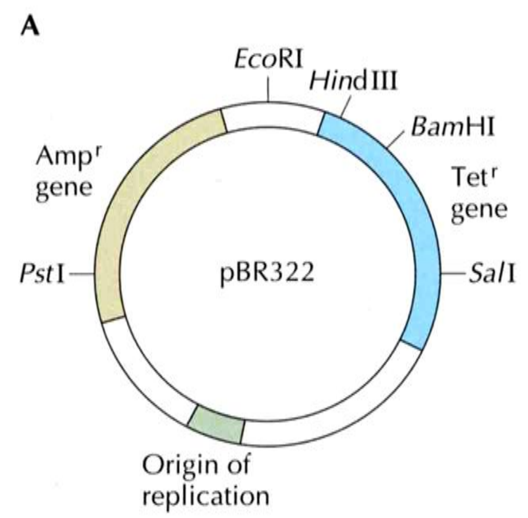
Plasmid

- Plasmid copy number
  - High-copy-number (relaxed)
    - 1~ 100 copies/cell
  - Low-copy-number (stringent)
    - 1~4 copies /cell
- Plasmid incompatibility
  - Inability of two different plasmids to coexist in the same cell
    - Same mechanism of replication and partitioning control
  - Incompatibility (Inc) group
    - Groups of plasmids which are mutually incompatible
- Host range of plasmid
  - Narrow-host-range plasmid
    - Specific replication origin
  - Broad-host-range plasmid



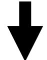
Features for high-quality cloning vector

- Small size
  - Decrease in transformation efficiency with plasmids larger than 15 kb
- Choice of unique restriction endonuclease recognition sites for cloning DNA
- Selectable genetic markers

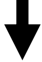


Evolution of Cloning Vectors

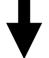
Natural plasmids, e.g. Col E1, pSC101



pBR322 (Bolivar and Rodriguez, 1977) : combination of natural plasmids, Amp^R, Tet^R markers, 4.3 kb

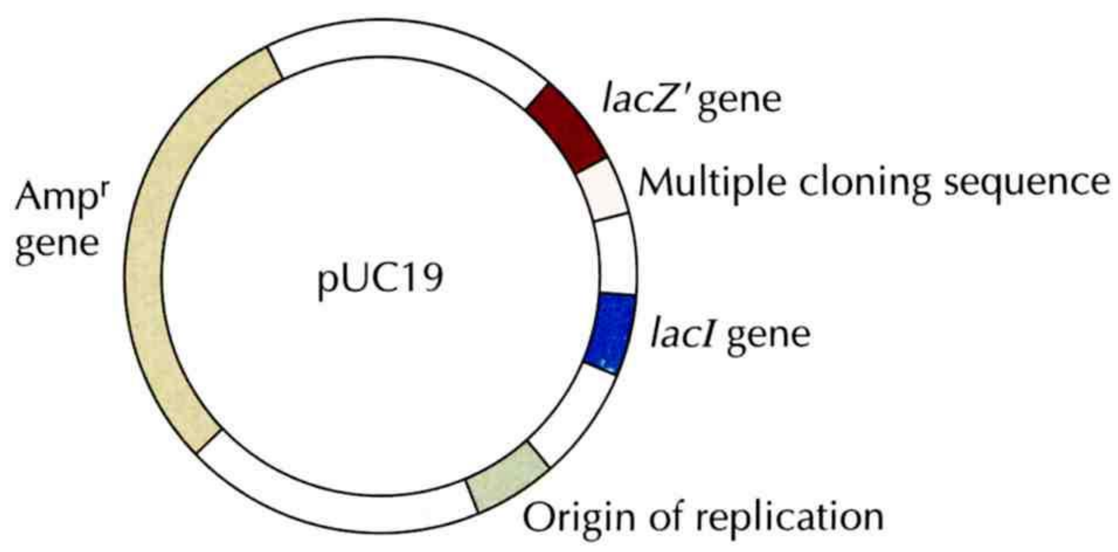


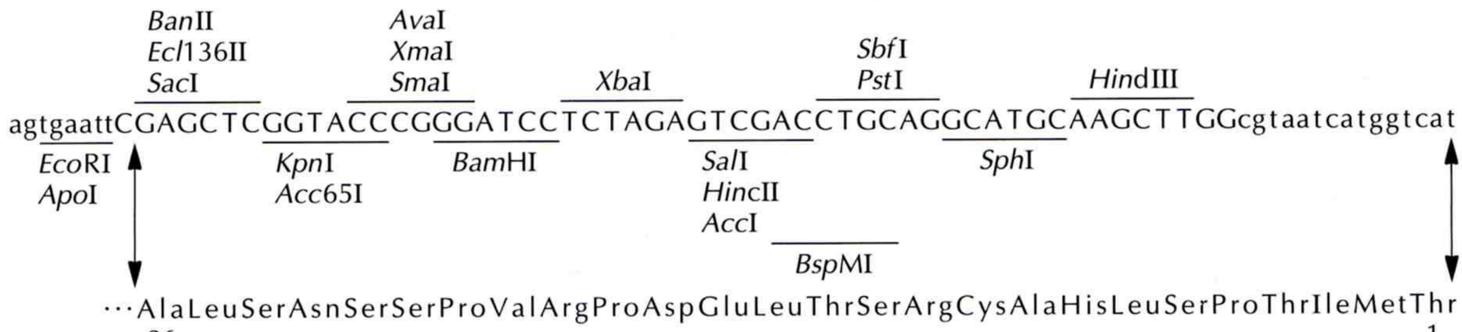
pBR322 derivatives : more unique enzyme sites, other markers

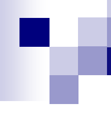


pUC vectors : multiple cloning sites (MCS) into lacZ' encoding α-peptide of of β-galactosidase

pUC19 Plasmid Vector





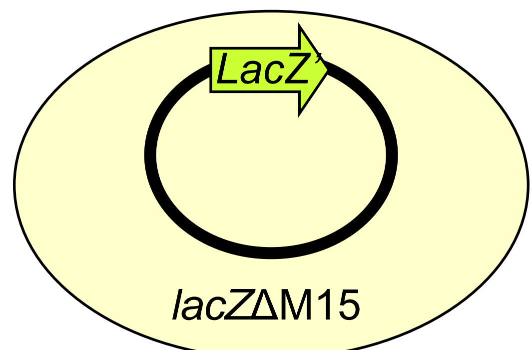


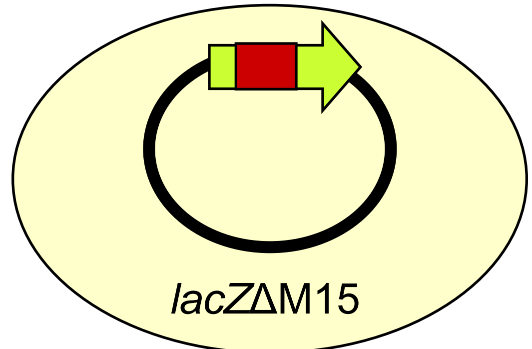
α complementation

lacZ Δ M15 lacks amino acids 11-41
- No tetramer formation of β-galactosidase
- Complemented by N-terminus of LacZ (α- peptide)

pUC vector provide α-peptide

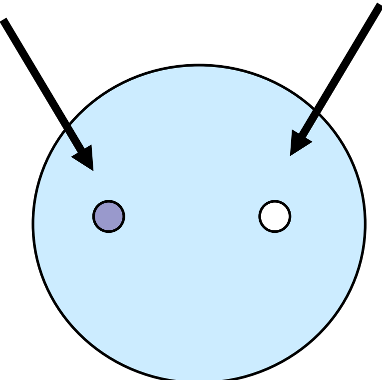
pUC vector with insert prevent α-peptide production





***E. coli* DH5α**

Contain *lacZ*ΔM15 mutation

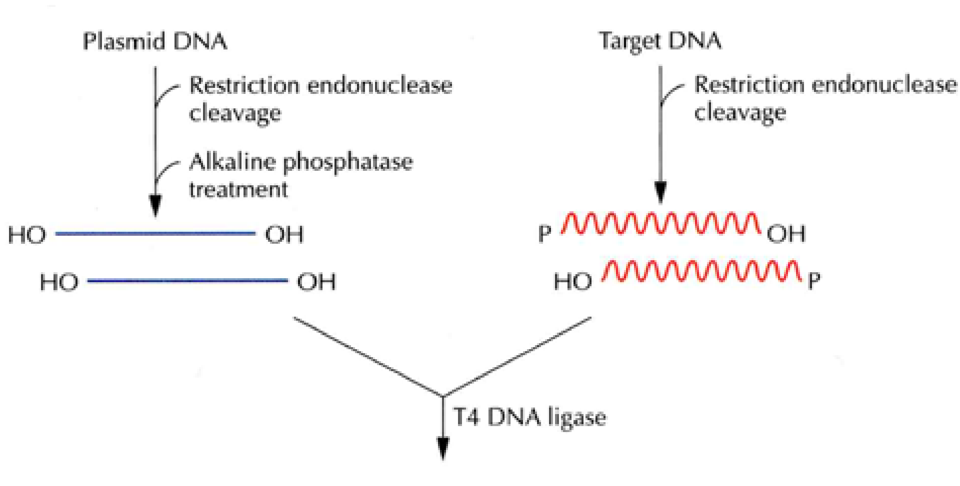


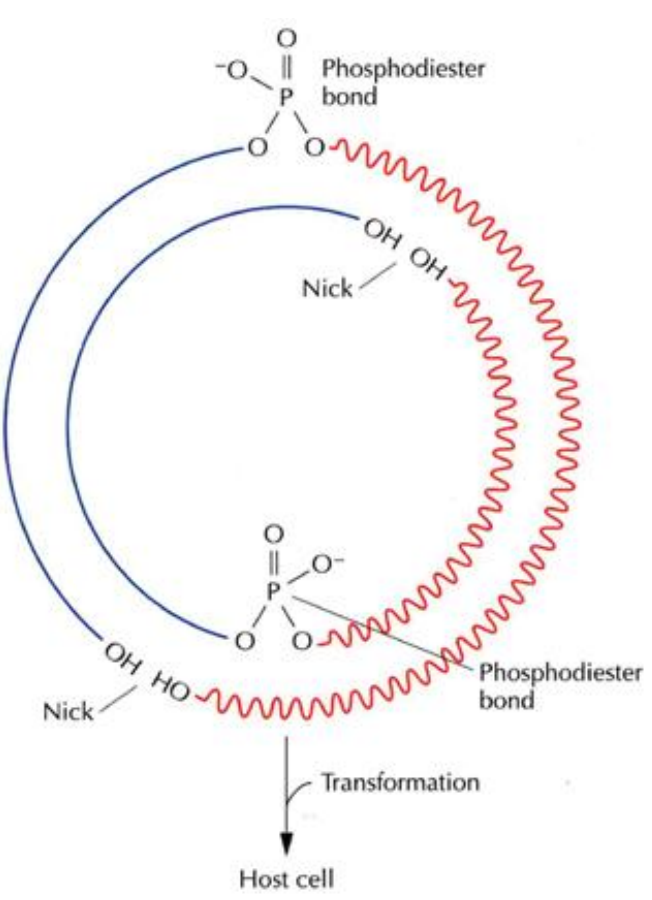
- X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase)

- IPTG (isoproyl-β-D-thiogalactopyranoside) for the induction of lac operon

Cloning Foreign DNA into a Plasmid Vector

Alkaline phosphatase treatment of plasmid DNA to prevent self ligation



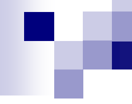


Transformation and Selection

- Transformation
  - Introduction of purified DNA into “competent” bacterial cells
- Competency
  - Natural: Active uptake under high cell density or starvation conditions
    - Gene transfer between bacterial species
    - Source of nutrient
  - Induction of competency of *E. coli*
    - Treatment with cold $CaCl_2$ → heat shock at 42°C for 2 min
    - Wahing cells with low salt buffer → electroporation

Transformation

- Transformation frequency
  - # of transformed cells/ total cells
- Transformation efficiency
  - # of transformed cells/ amount of DNA
- Host cells for cloning
  - $RecA^-$
    - No recombination between DNA molecules
  - Deletion of endA1
    - No endonuclease production



Selection of Transformed Cells

- Cloning into BamHI site of pBR322
- Selection for clones containing insert DNA in the plasmid
  - Transformation and selection on plated medium containing ampicillin
  - Selection for tetracyclin-sensitive clones
- Confirmation of the plasmid

6. Creating and Screening a Library

Making a Gene Library

- Creating a library
  - Subdividing genomic DNA into clonable elements and inserting them into host cells
  - A complete library
    - Library containing all of the genomic DNA of the source organism
- DNA fragmentation by partial digestion
  - Low concentration of restriction enzyme
  - Shortened incubation time
  - Library size
    - > 3 time the amount of DNA in the genome
      - $4X\ 10^6$ genome, 1000 bp insert → require 12,000 clones

Determination of Library Size

- Human Genome size: 2.8×10^6 kb
- Size of random DNA fragment: 20 kb

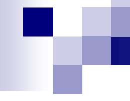
f: ratio of the length of the average insert to the size of the entire genome

$20/2.8 \times 10^6$

N : Number of independent recombinants

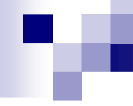
P: Probability of including any DNA in a random library of N

$$N = \frac{\ln(1-P)}{\ln(1-f)} = \frac{\ln(1-0.95)}{\ln(1-7.14 \times 10^{-6})} = 4.2 \times 10^5$$



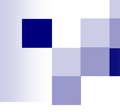
Screening Strategies

- Sequence-dependent screening
  - : Use Hybridization of homologous sequences
- Screening of expression library
  - Screening by Immunological Assay
  - Screening by protein activity

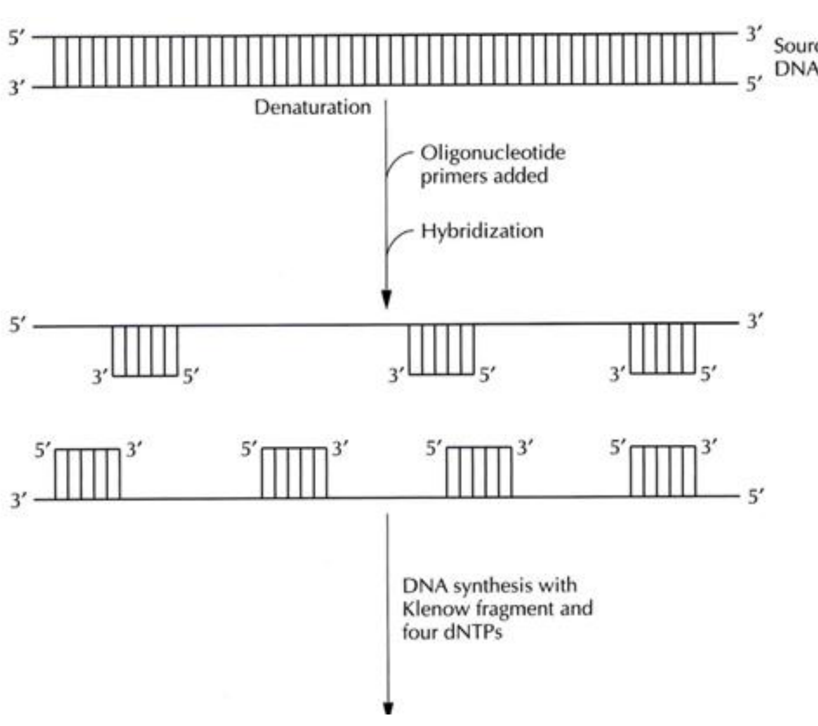


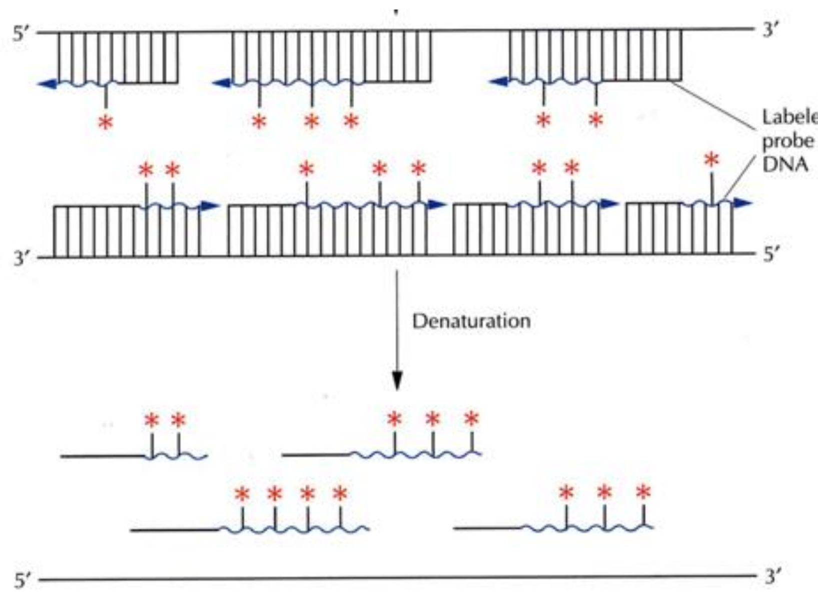
Screening by DNA Hybridization

- Denaturation
  - Breaking base pairing by heating of alkaline treatment
- Renaturation
  - Annealing of denatured strands by slow cooling
- DNA hybridization
  - Attachment of denatured ss target DNA on membrnae (nitrocellulose, nylon)
  - Annealing with labeled single stand probe (100 to 1000nt)

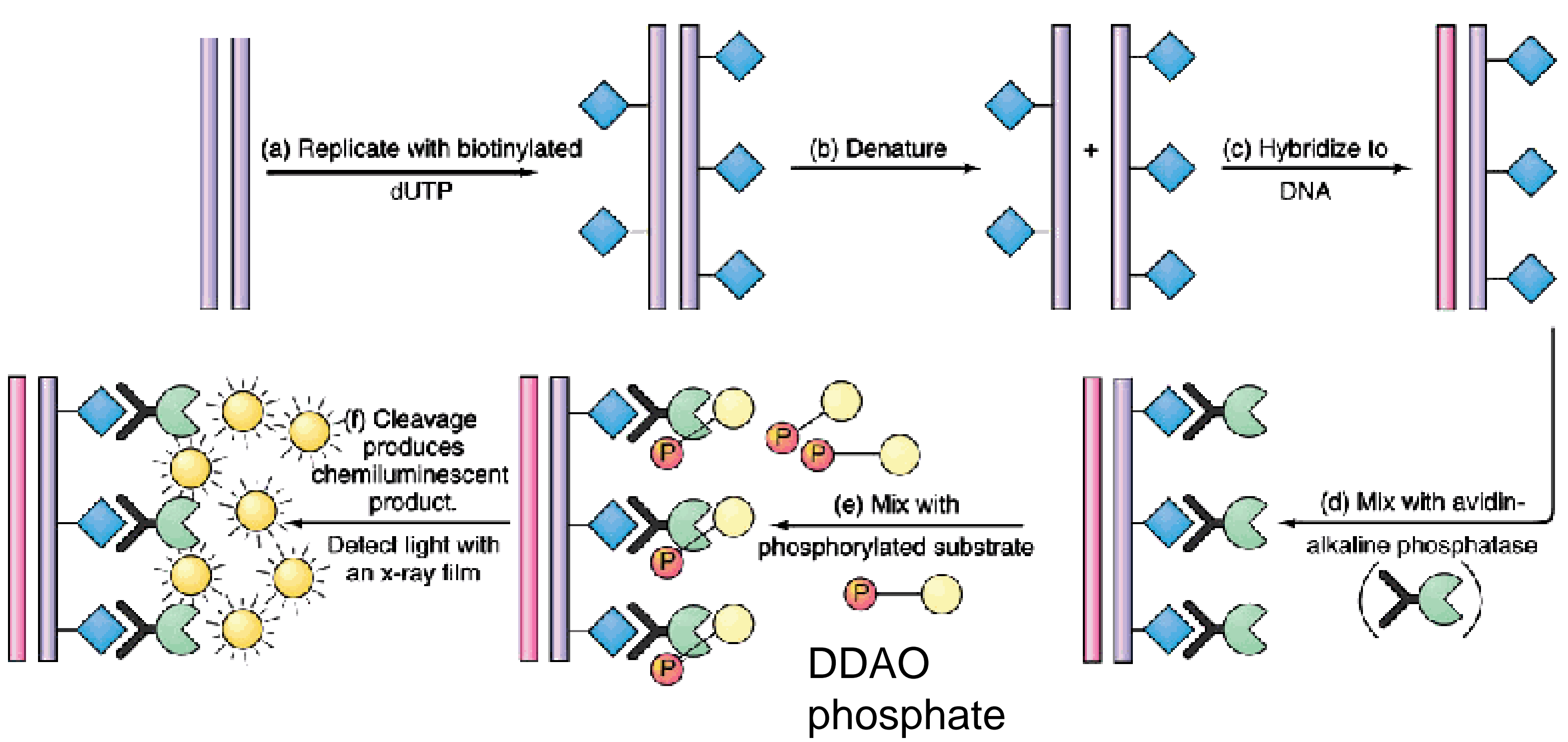


Labeling of DNA Probes Using Random Primers

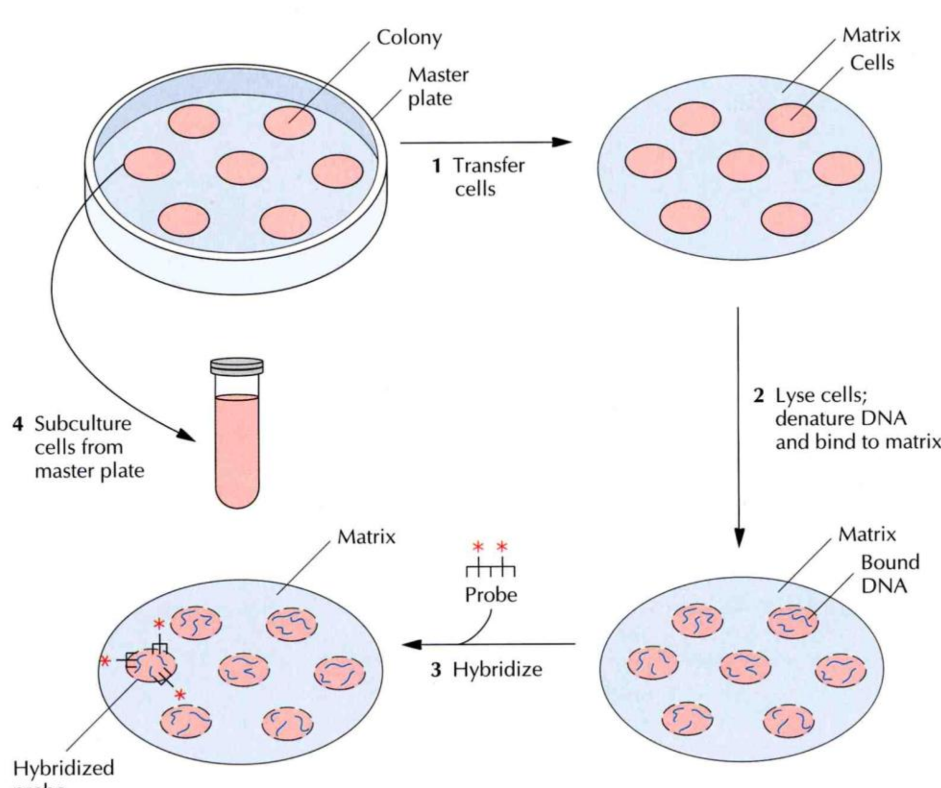




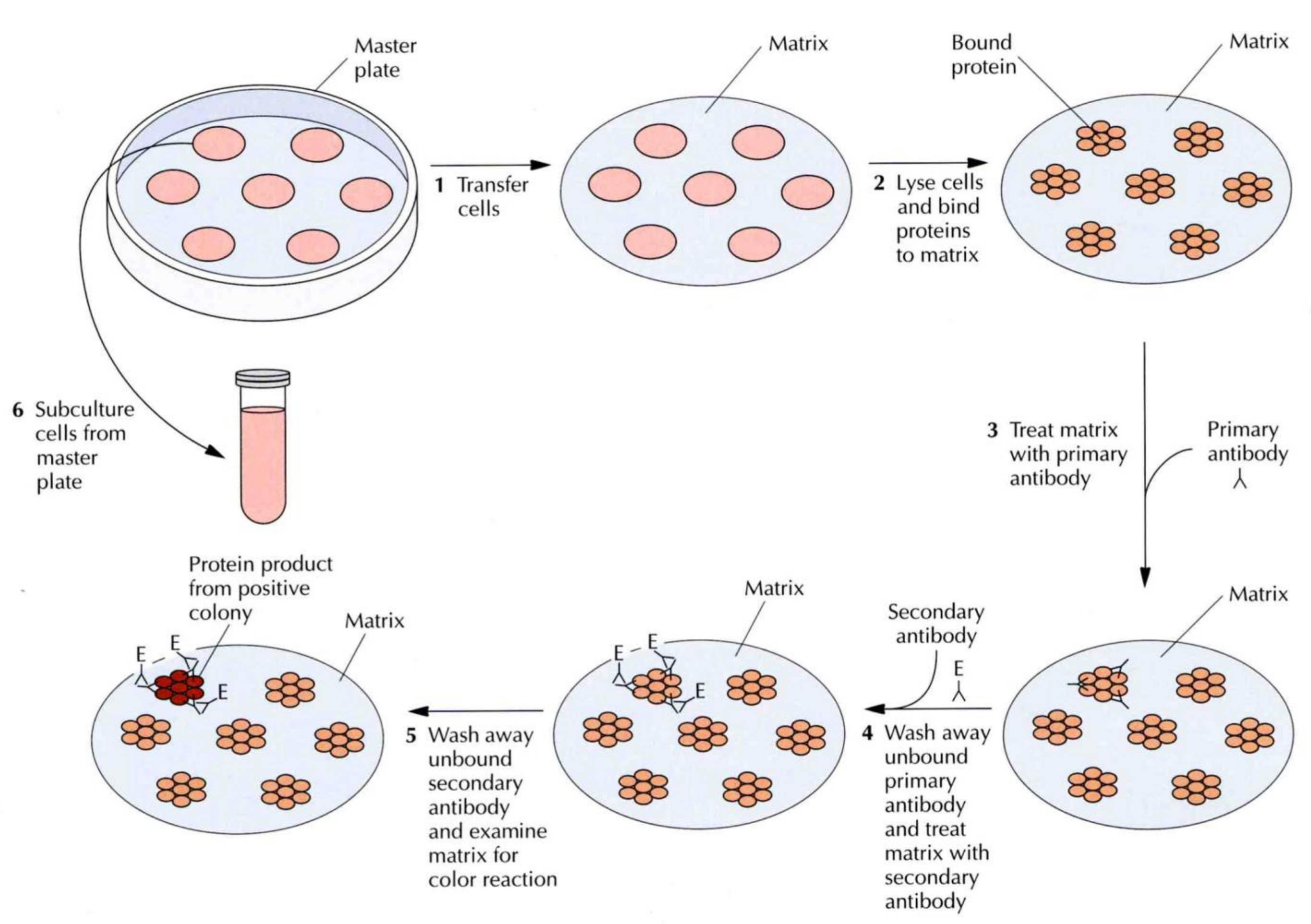
Non-radioactive Detection: Chemiluminescence



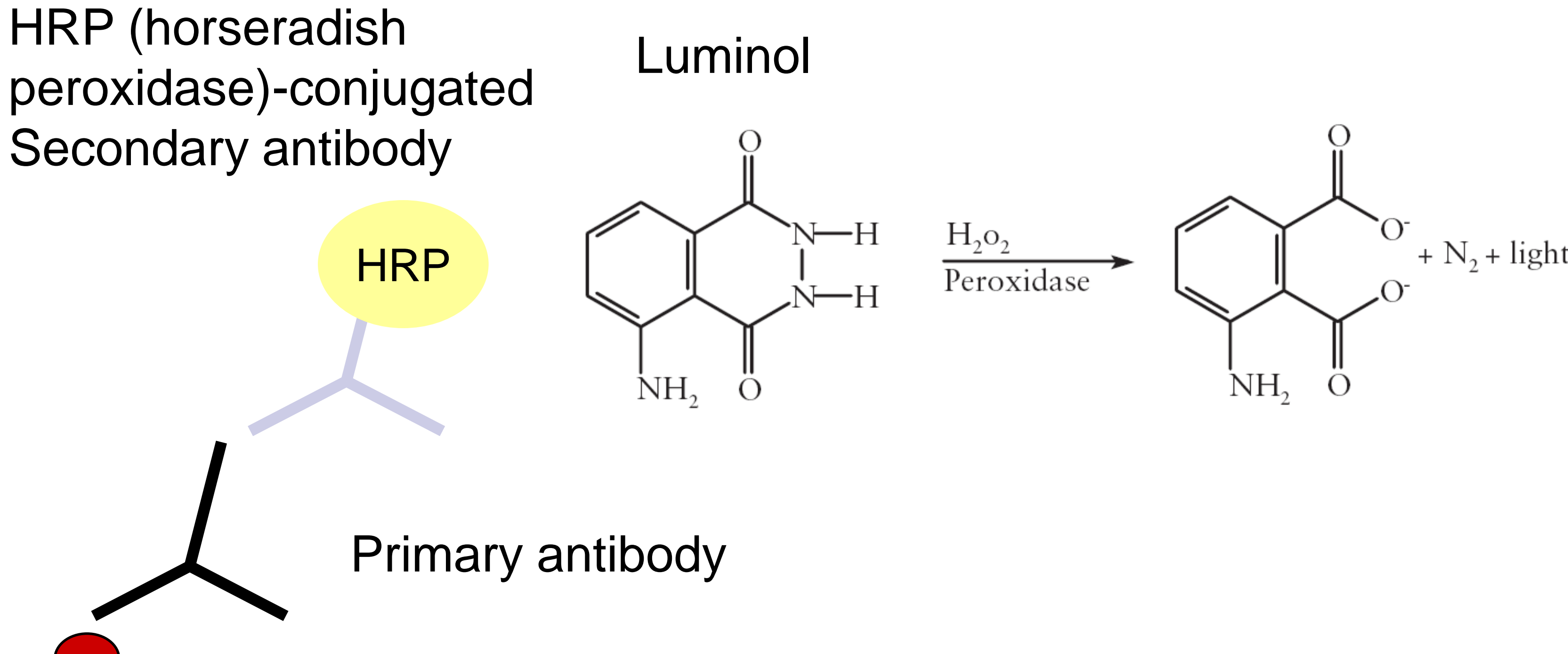
Screening a Library by Colony Hybridization

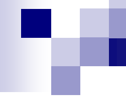


Screening by Immunological Assay



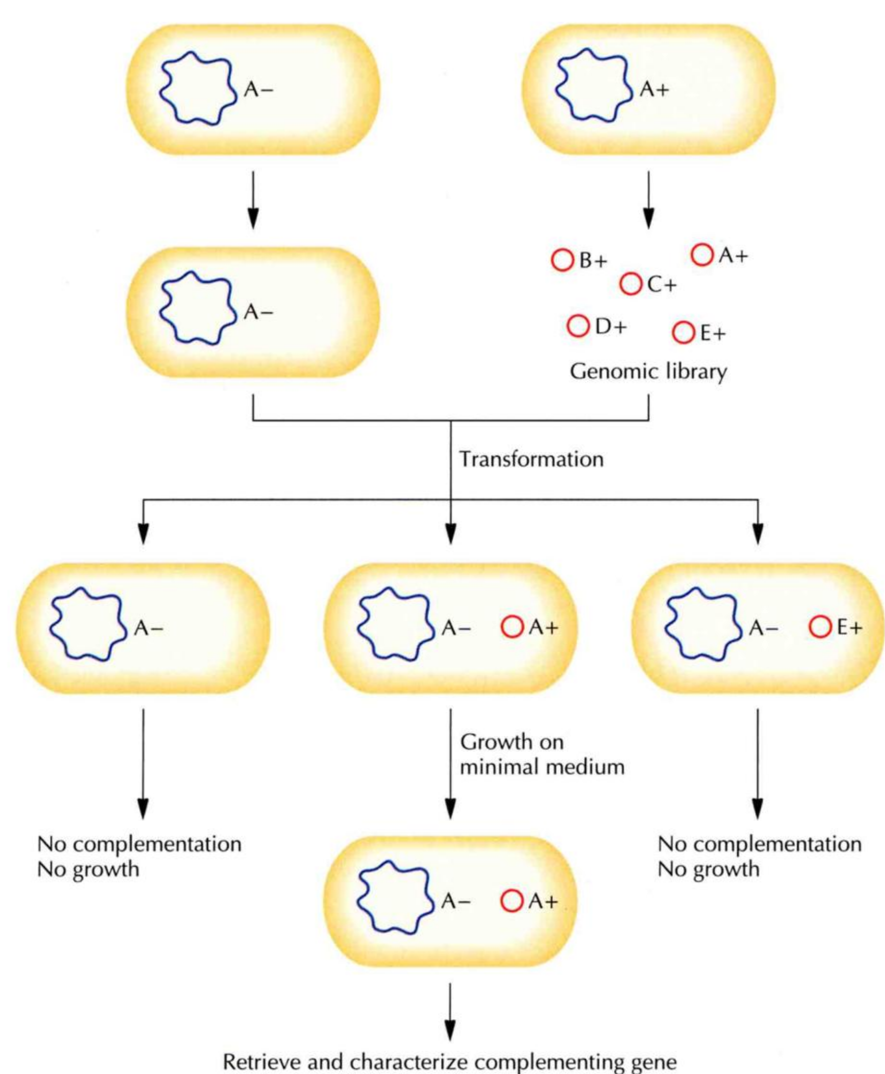
Detection of Ag-Ab reaction by chemiluminescence





Screening by Protein Activity

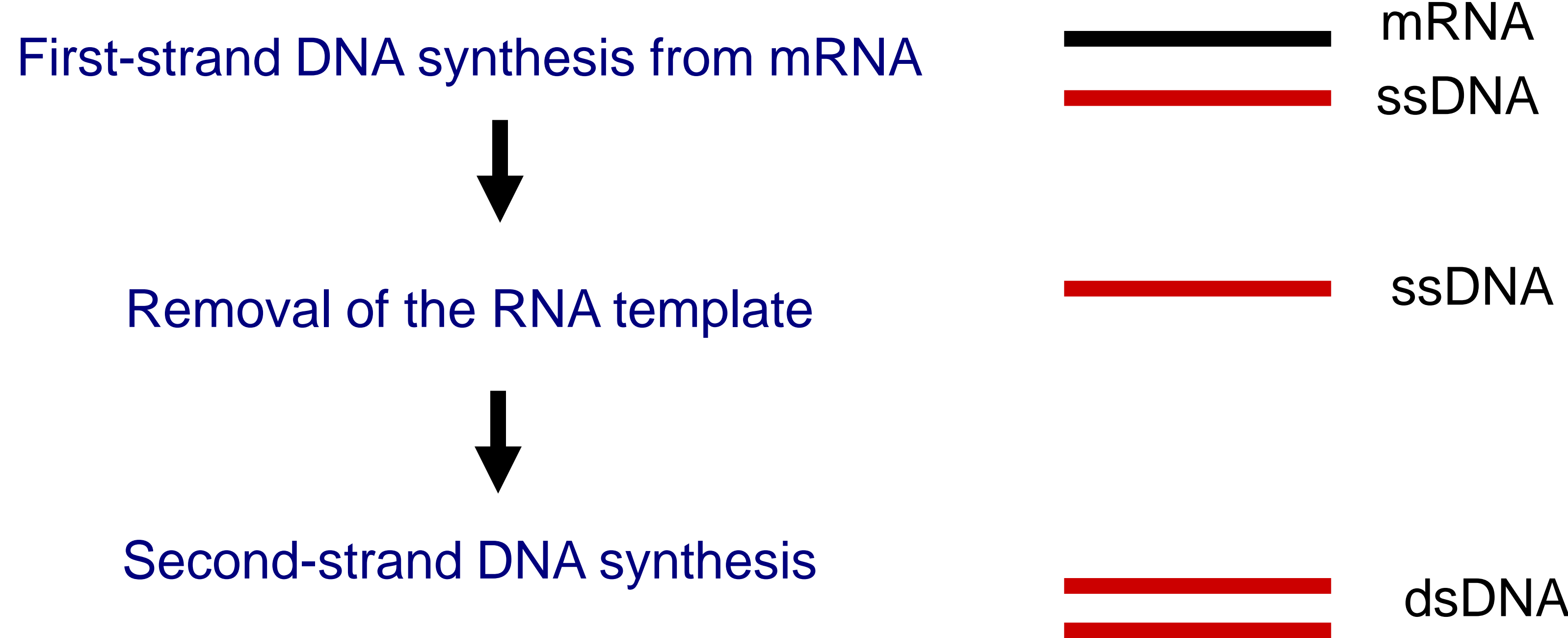
- Screening by enzyme activity assay
  - e.g. lipase: grow cells in the presence of trioleoglycerol and fluorescent dye rhodamine
    - Orange fluorescence halos
- Functional (genetic) complementation
  - Complementation of mutant growth on specific medium



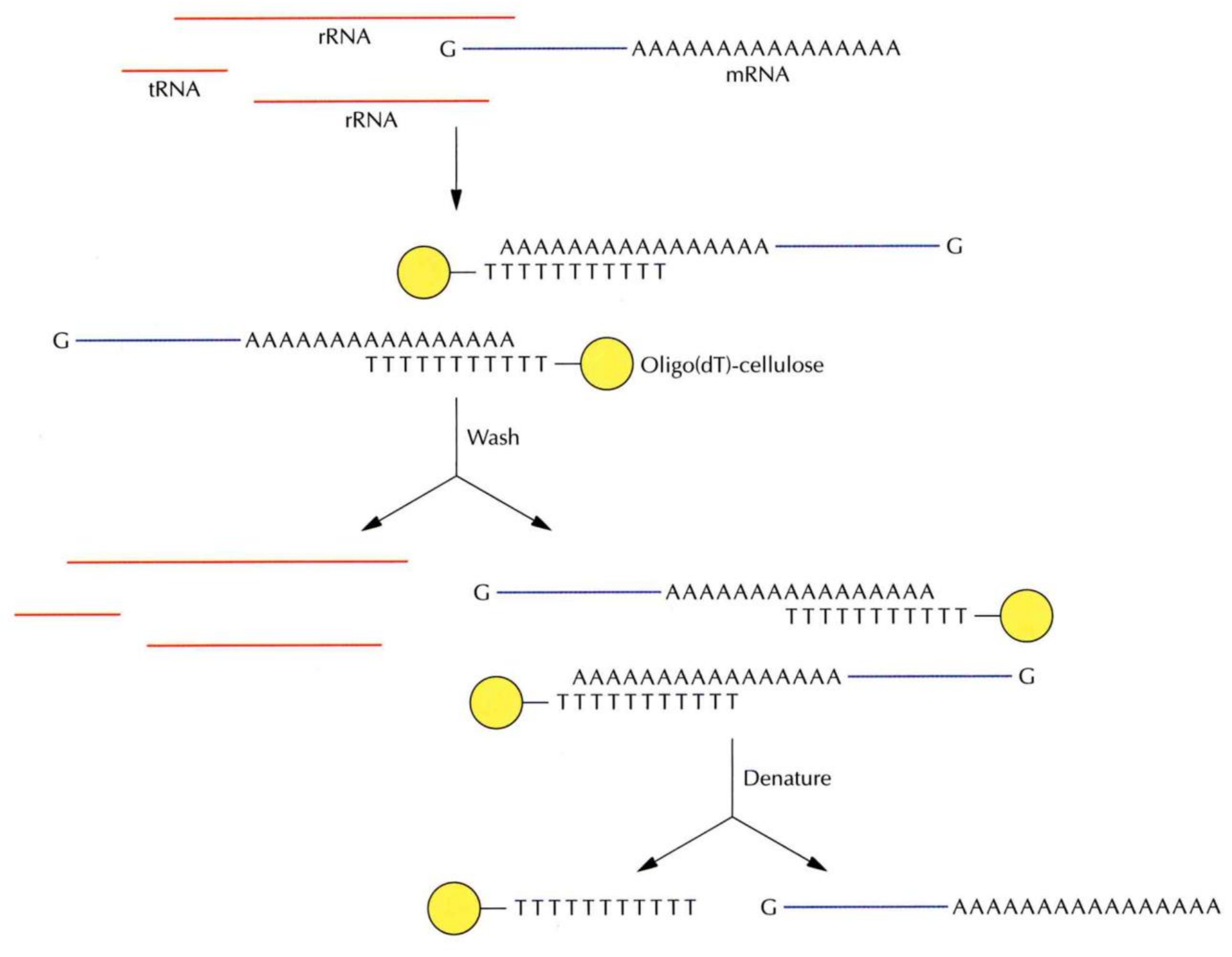
7. Cloning cDNA

Cloning DNA Sequences that Encode Eukaryotic Protein

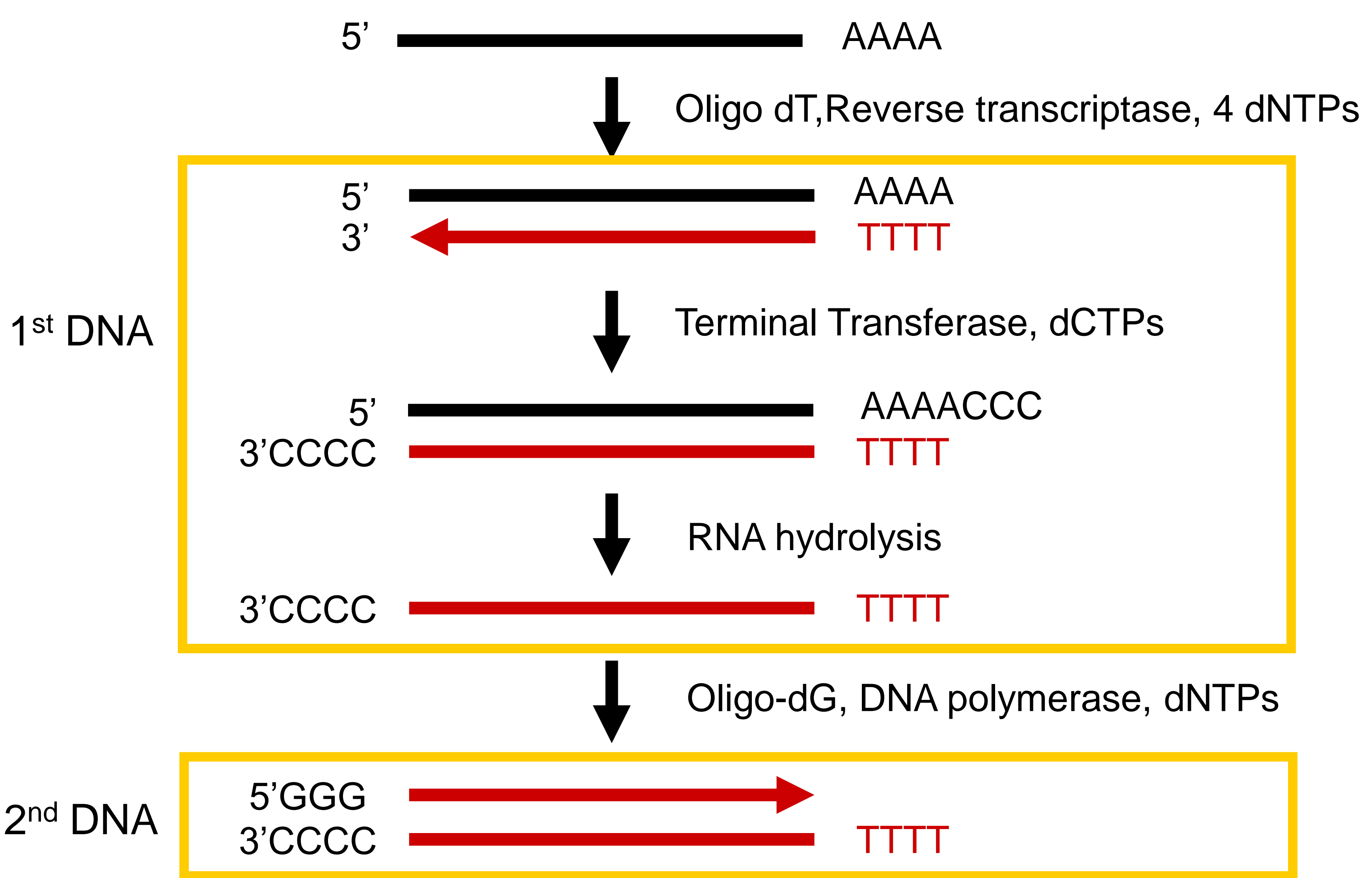
- Generation of cDNA library



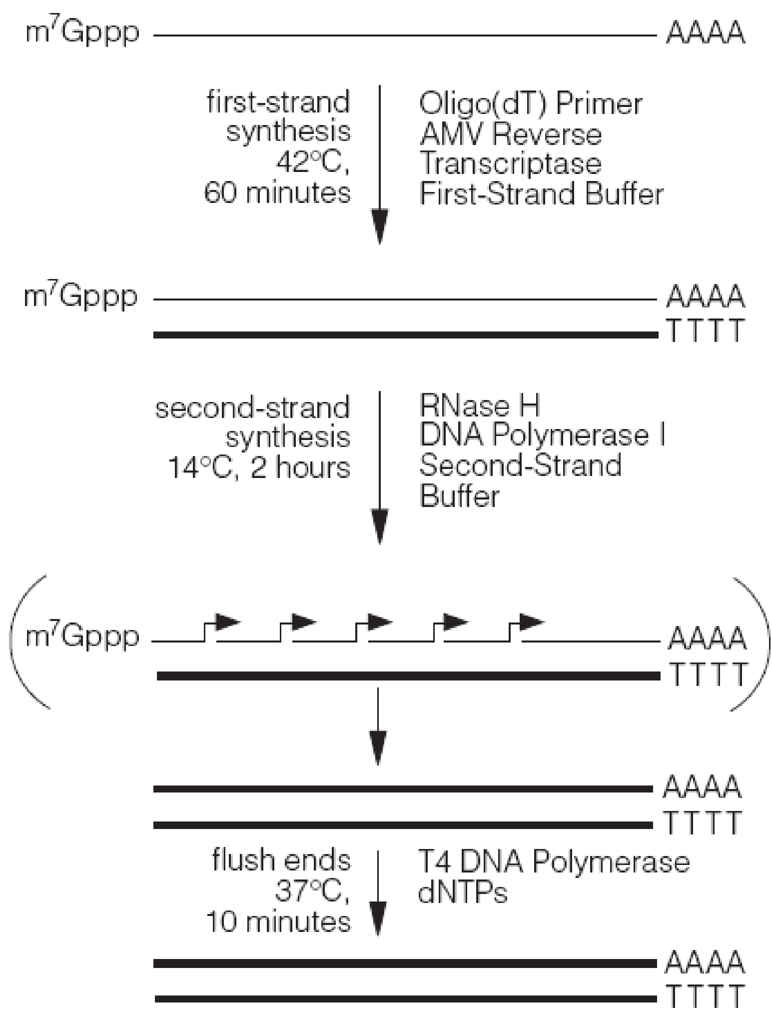
Isolation of Polyadenylated mRNA

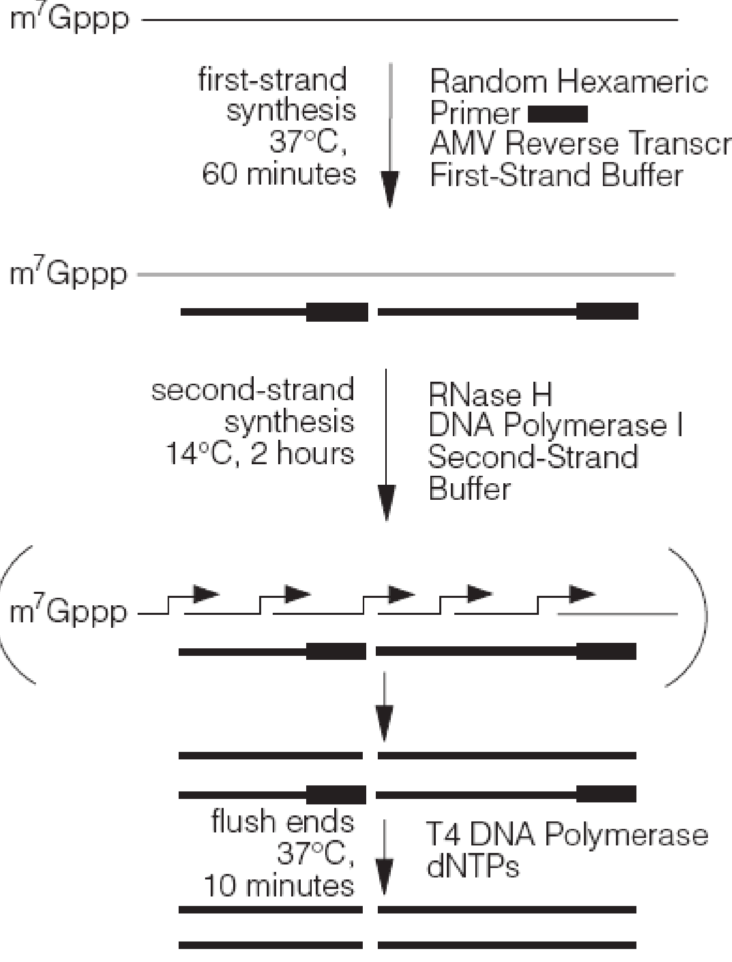


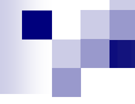
cDNA Cloning Strategies-1



cDNA Cloning Strategies



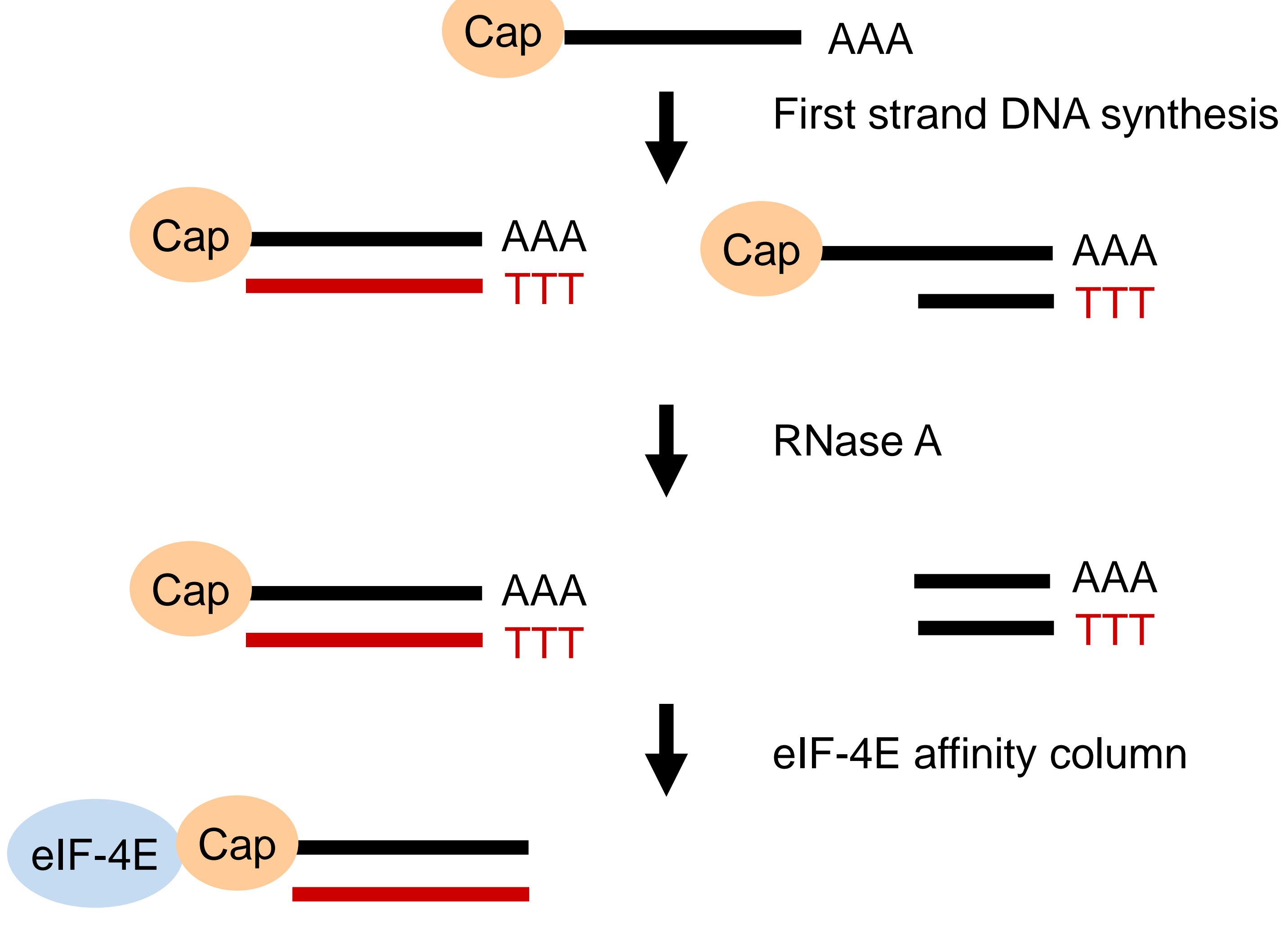




Limitation of cDNA Cloning Strategy

- 3’ end bias for oligo-dT hybridization
  - Use random primer: produce smaller fragments
- Difficult to isolate full length clones
  - Reverse transcriptase from AMV or MMLV
    - Limited processivity of reverse transcriptase
    - Intrinsic RNase H activity

Obtaining Full Length cDNA: Selection of 5' mRNA ends



Obtaining Full Selection of 5’ mRNA ends : Oligo-capping