BASIC METHODS IN MOLECULAR BIOLOGY

1. Manipulations with DNA

- mutations, deletions, …
- genomic DNA vs. **cDNA**
- restriction endonucleases and DNA modifying enzymes
- DNA a RNA polymerases
- synthetic oligonucleotides-short ss DNA (primers for polymerases,...)
- methods of manipulation with DNA (cloning of DNA molecules in plasmids, sequencing, polymerase chain reaction = PCR, mutagenesis)
- genomic libraries vs. cDNA libraries
- computer methods for the analysis of DNA/RNA sequence/structure

2. Analytical methods to detect lesions in DNA (mutations etc,..)

3. Analytical methods to detect gene expression and transkription

- detection of mRNA level in the cell (e.g. by real-time PCR)
- study of DNA-protein (or protein-protein) interactions
- modification of chromatin (position of nucleosomes, acetylation and methylation of histones in promoter regions)
- cDNA microarrays

MANIPULATIONS WITH DNA:

- DNA cloning in plasmids (restriction endonucleases)

- Sequencing

- Polymerase Chain Reaction (PCR)

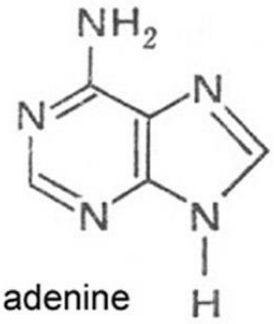
ISOLATION OF DNA, RNA:

Phenol extraction

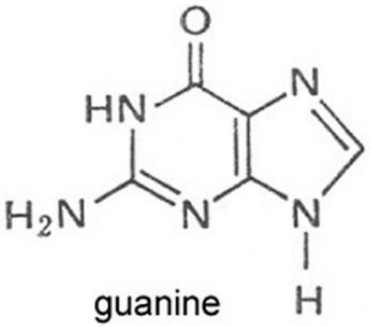
Ethanol precipitation

CONVERSION OF mRNA TO cDNA (complementary DNA)

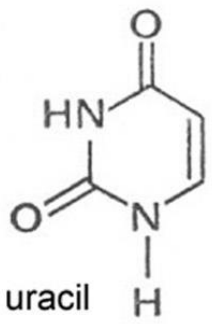
Enzyme reverse transcriptase



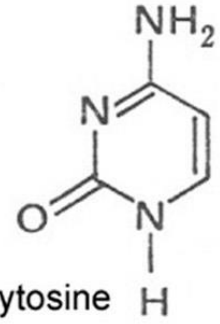
adenine



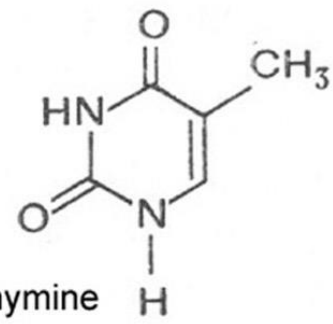
guanine



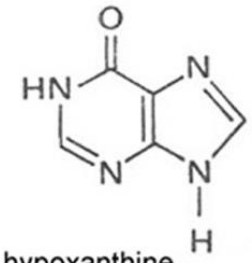
uracil



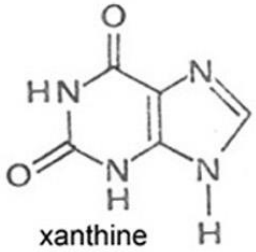
cytosine



thymine



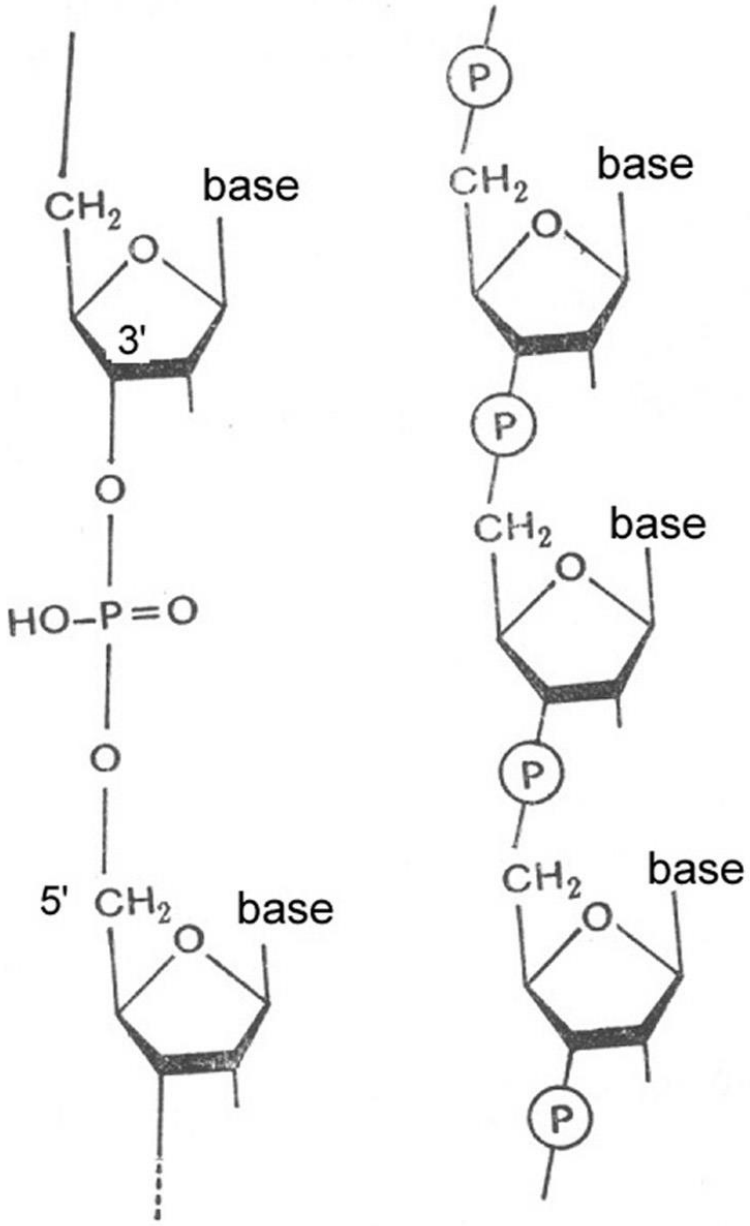
hypoxanthine



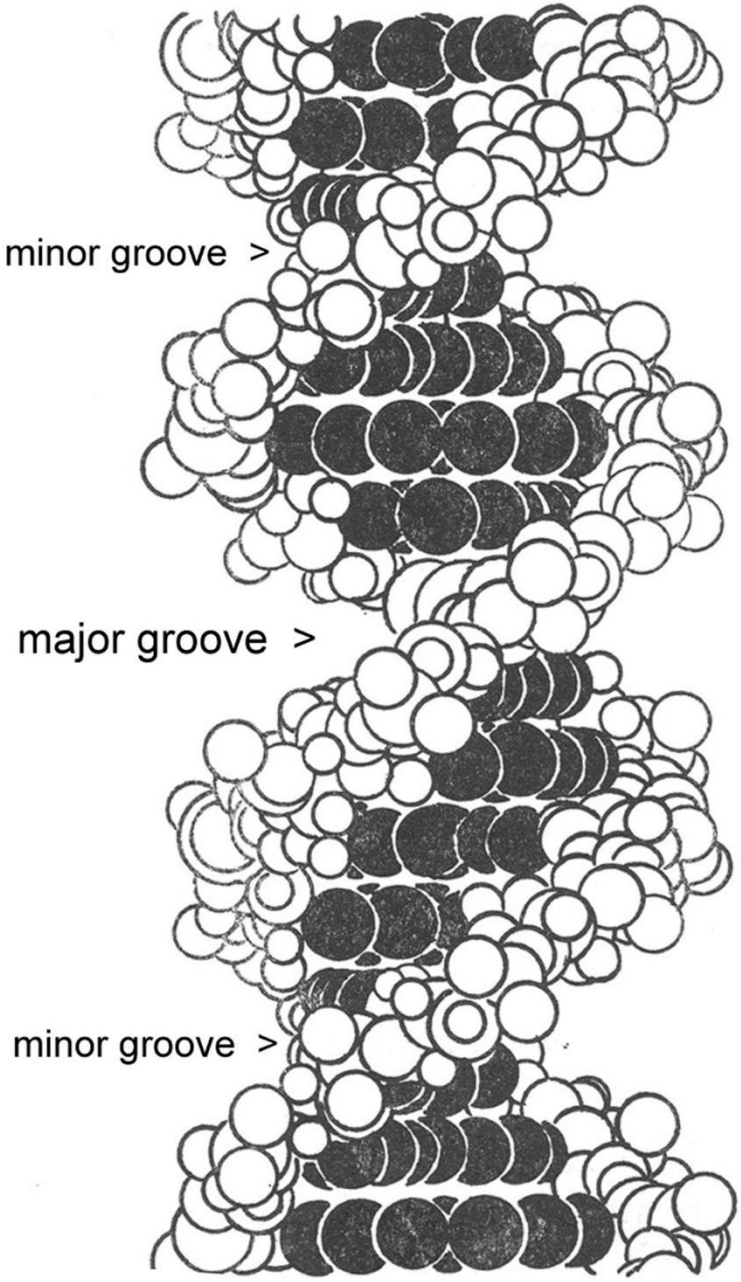
xanthine

in DNA: A, G, C, T	(with 2'-deoxyribose)	dA, dG, dC, dT
in RNA: A, G, C, U	(with ribose)	

DNA structure: phosphodiester bonds



Double helix of DNA



~ 10 base pairs
(3.4 nm)

DNA can be easily:
- isolated as a pure nucleic acid, free of proteins and RNA
- cleaved at specific sites with restriction enzymes and recombined
- sequenced

Types of DNA:
- genomic (nuclear): high molecular weight DNA (> 100 kb long)
- cDNA (copy of messenger RNA)

linear (genomic DNA, DNA of some DNA viruses, cleaved circular DNA) or circular (plasmids, *E.Coli* chromosome,…)

DNA double strand:
5´... T C G C G C T A A A C T C C C T ...3´ = upper strand, the same sequence as in mRNA
3´... A G C G C G A T T T G A G G G A ...5´

when these strands are separated, they have different nucleotide composition
and can be separated (e.g. by electrophoresis under non-denaturing conditions)

or

5´... T C G C G C T A A A C T C C C T ...3´

(the complementary strand is usually not shown)

in RNA: 5´... U C G C G C U A A A C U C C C U ...3´

DNA	RNAs
STRUCTURE:	
2´-deoxyribose	**ribose**
- thymine	**- uracil**
- double helix, higher order structures in the nucleus	**- single strand with a secondary structure**
FUNCTION:	
- storage of genetic information	**- role in the expression of genetic information**
Basic processes in which they participate:	
- replication, transcription (ssDNA as template)	**- transcription, translation**
Localization in the cell:	
- nucleus, (mitochondria)	**- nucleus, cytoplasm, (mitochondria)**

Formation of hybrids:
DNA x DNA
DNA x RNA
RNA x RNA

Isolation of pure nucleic acids – DNA and/or RNA

Cell extract ~ Disintegration of cellular compartments

↓

+ sodium dodecylsulphate ~ PROTEIN DENATURATION

↓

+ phenol/chloroform ~ PHASE SEPARATION

↓

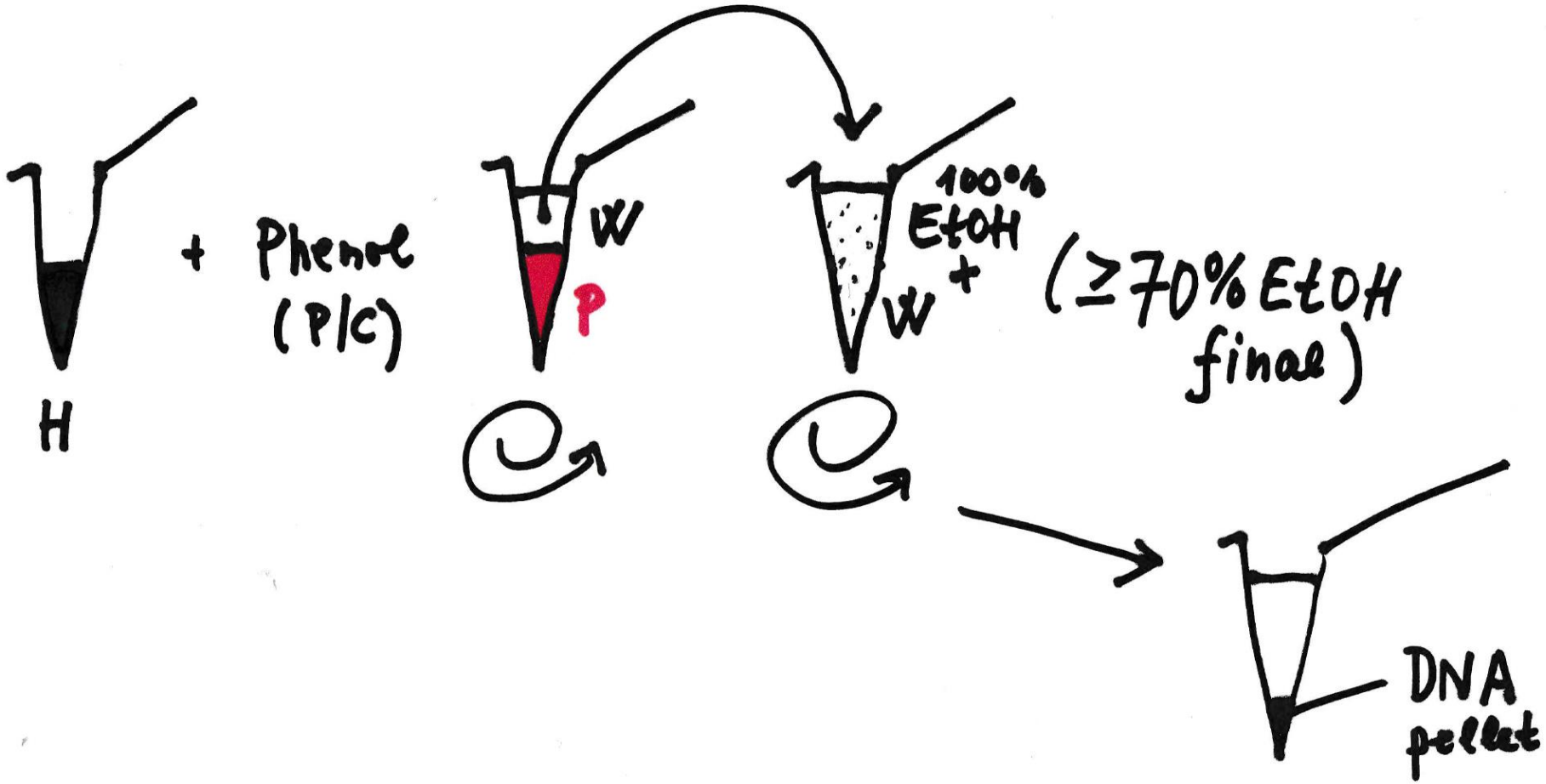
DNA and RNA in the water phase

↓

Ethanol precipitation (both DNA and RNA precipitate in the presence of higher salt concentration and 70% final conc. of ethanol)

The method works in a range of nucleic acid concentrations and molecular weight.

ISOLATION OF DNA:



Q: Can RNA also form a duplex?

Q: What is the difference in the stability of pure DNA and RNA

CLONING OF DNA INSERTS INTO PLASMIDS

Plasmid: circular DNA, replicates autonomously in bacteria, requires origin of replication and resistance to an antibiotic

Insert: any fragment of ds DNA

Ends of plasmid backgroud and insert are important:
Must be compatible (sticky) or blunt

Cleavage by restriction endonucleases

Eco RI: 5' extension

$^{5'}$NNNNNNNN G A A T T C NNNNNNNN $^{3'}$
$^{3'}$NNNNNNNN C T T A A G NNNNNNNN $^{5'}$

$^{5'}$NNNNNNNN G $^{3'}$..... $^{5'}$A A T T C NNNNNNNN $^{3'}$
$^{3'}$NNNNNNNN C T T A A $^{5'}$..... $^{3'}$G NNNNNNNN $^{5'}$

Pst I: 3' extension

$^{5'}$NNNNNNNN C T G C A G NNNNNNNN $^{3'}$
$^{3'}$NNNNNNNN G A C G T C NNNNNNNN $^{5'}$

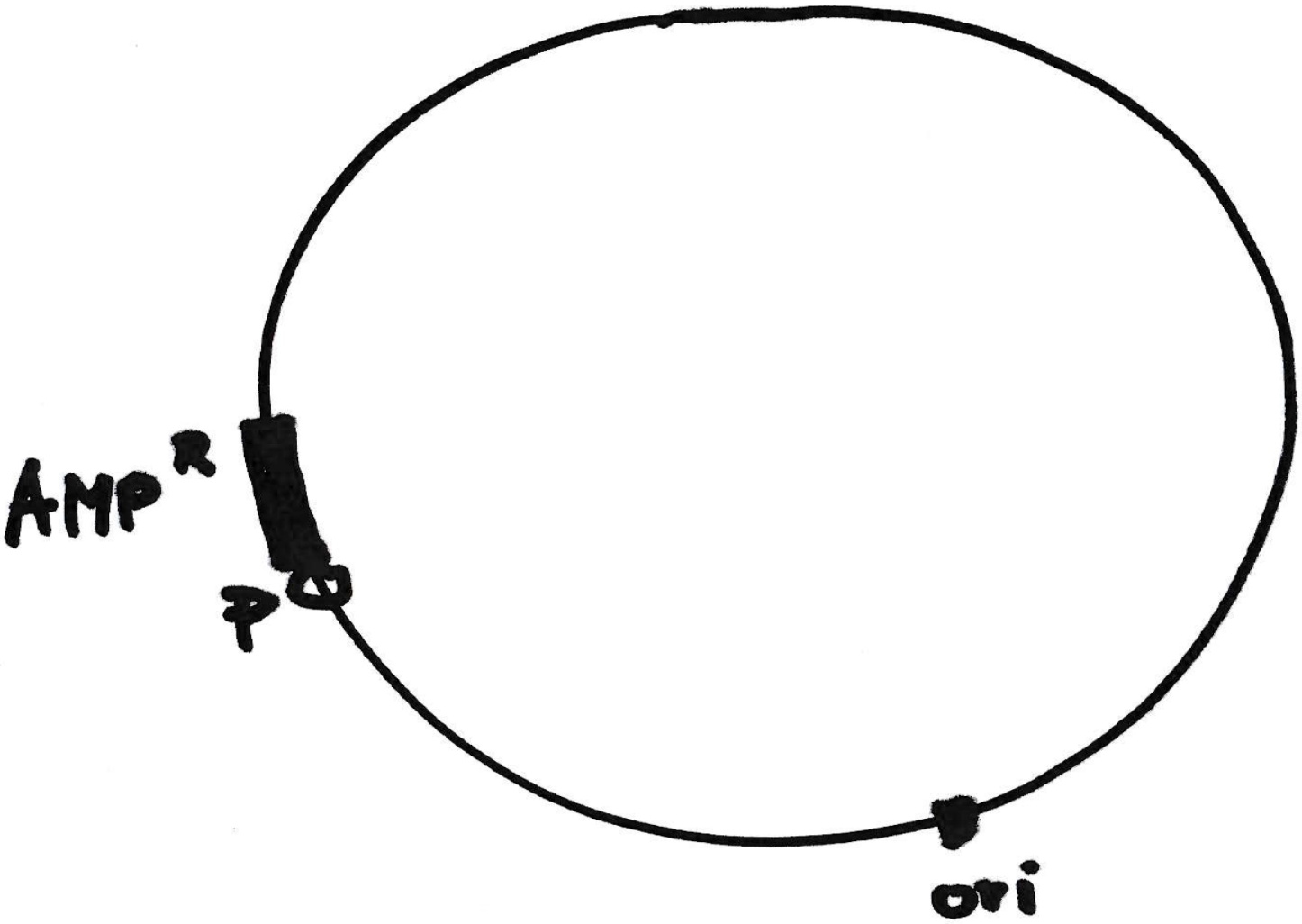
$^{5'}$NNNNNNNN C T G C A $^{3'}$..... $^{5'}$G NNNNNNNN $^{3'}$
$^{3'}$NNNNNNNN G $^{5'}$..... $^{3'}$A C G T C NNNNNNNN $^{5'}$

Dra I: blunt end

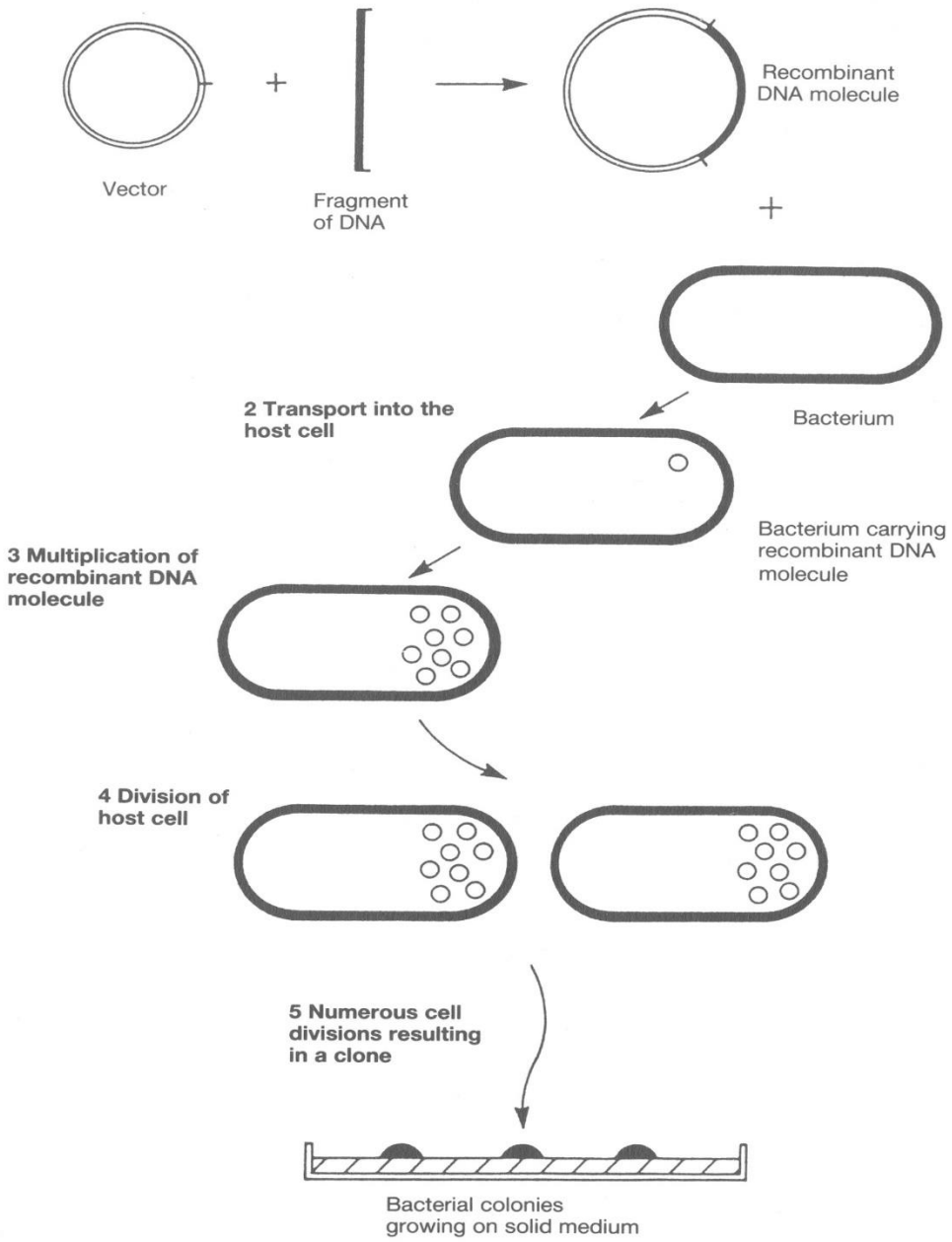
$^{5'}$NNNNNNNN T T T A A A NNNNNNNN $^{3'}$
$^{3'}$NNNNNNNN A A A T T T NNNNNNNN $^{5'}$

$^{5'}$NNNNNNNN T T T $^{3'}$..... $^{5'}$A A A NNNNNNNN $^{3'}$
$^{3'}$NNNNNNNN A A A $^{5'}$..... $^{3'}$T T T NNNNNNNN $^{5'}$

PLASMID:



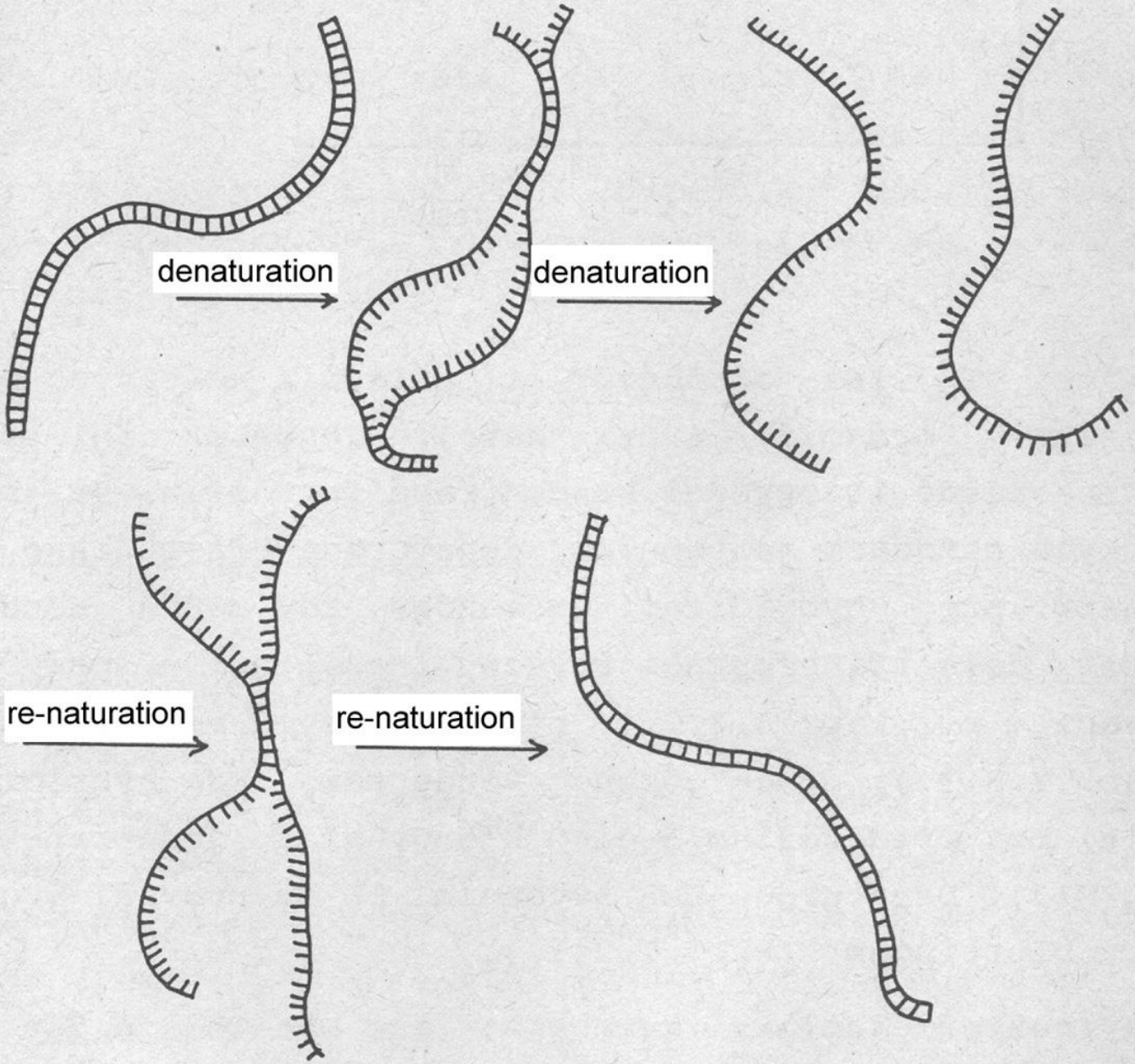
CLONING IN PLASMIDS - CONSTRUCTION OF RECOMBINANT DNA MOLECULE



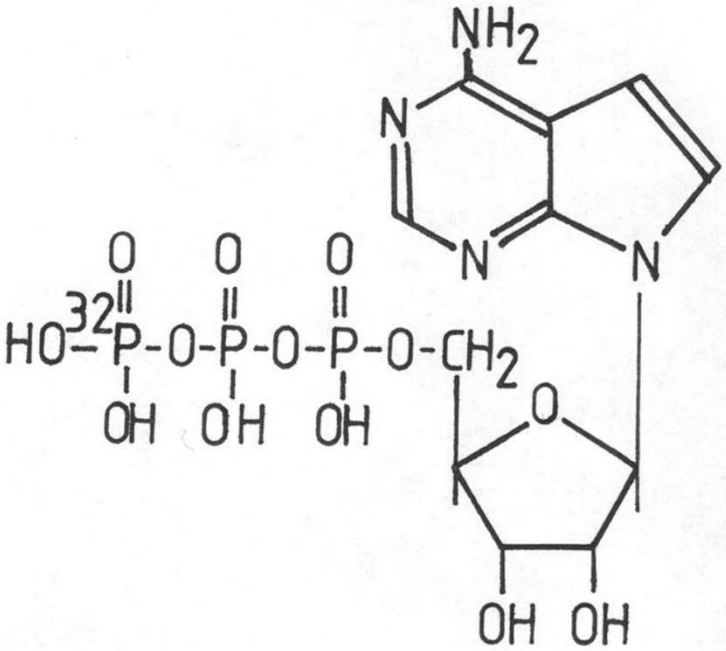
Detection of nucleic acids:

Hybridization,
“probing”,
Types of probes, labeling of probes

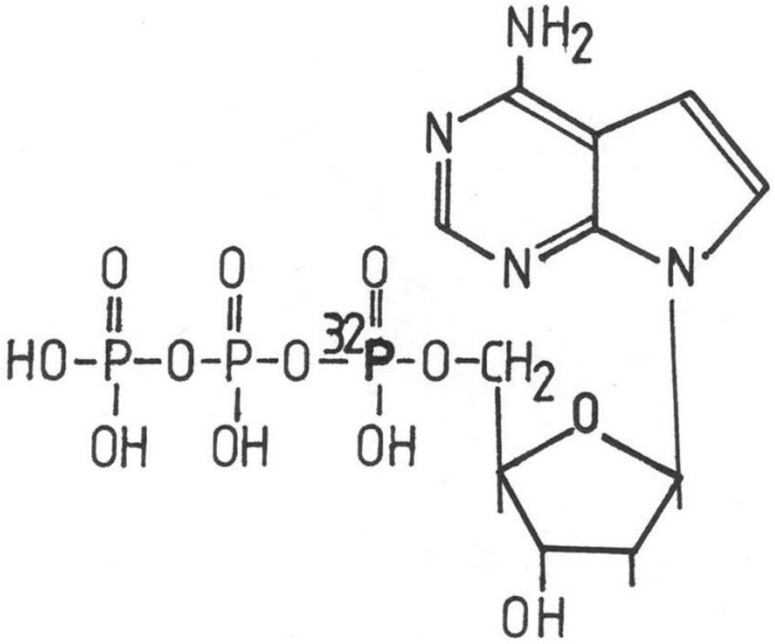
Denaturation of the DNA double helix



Radioactive phosphates in NTP/dNTP



γ-^{32}P-ATP



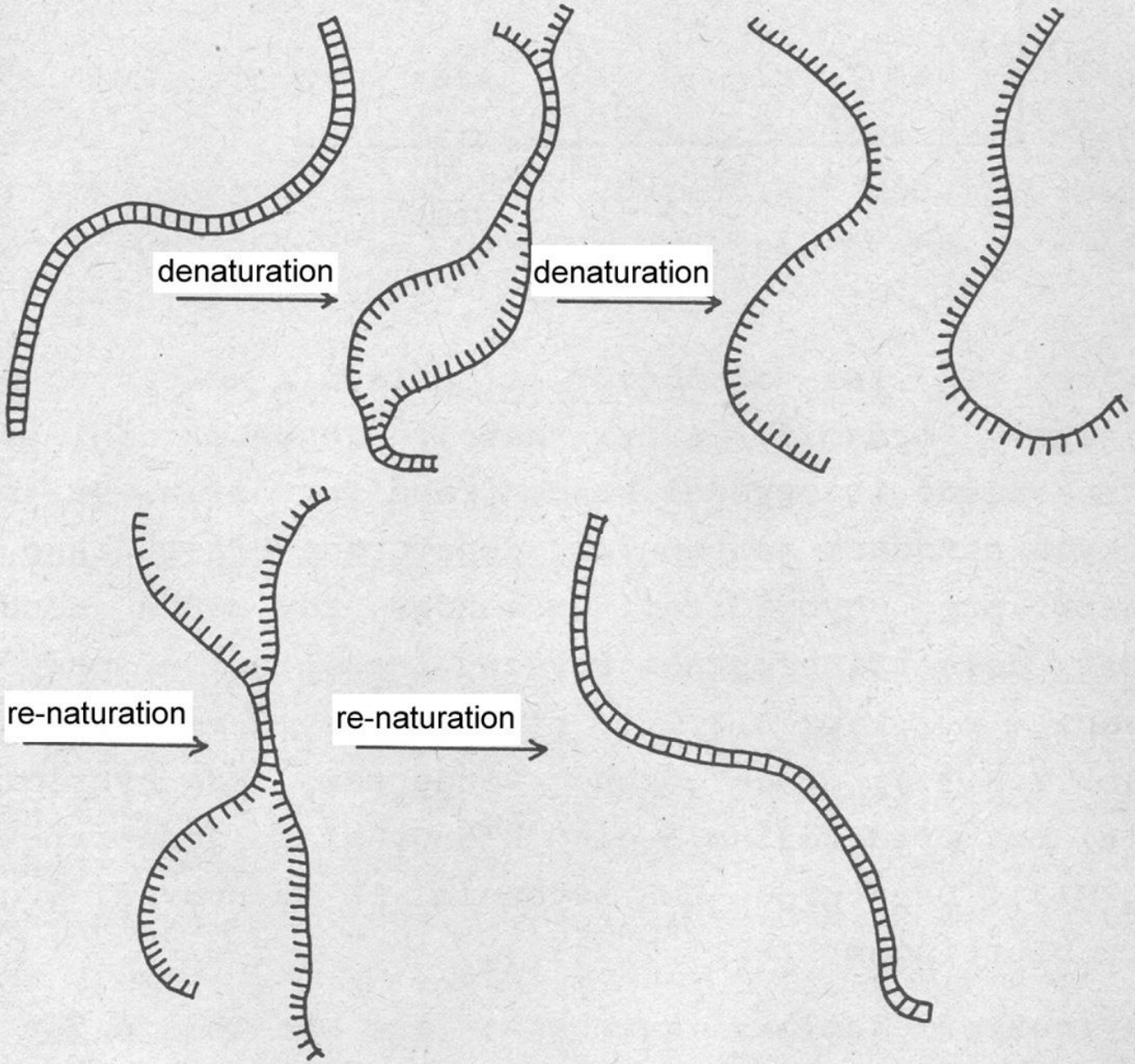
α-^{32}P-dATP

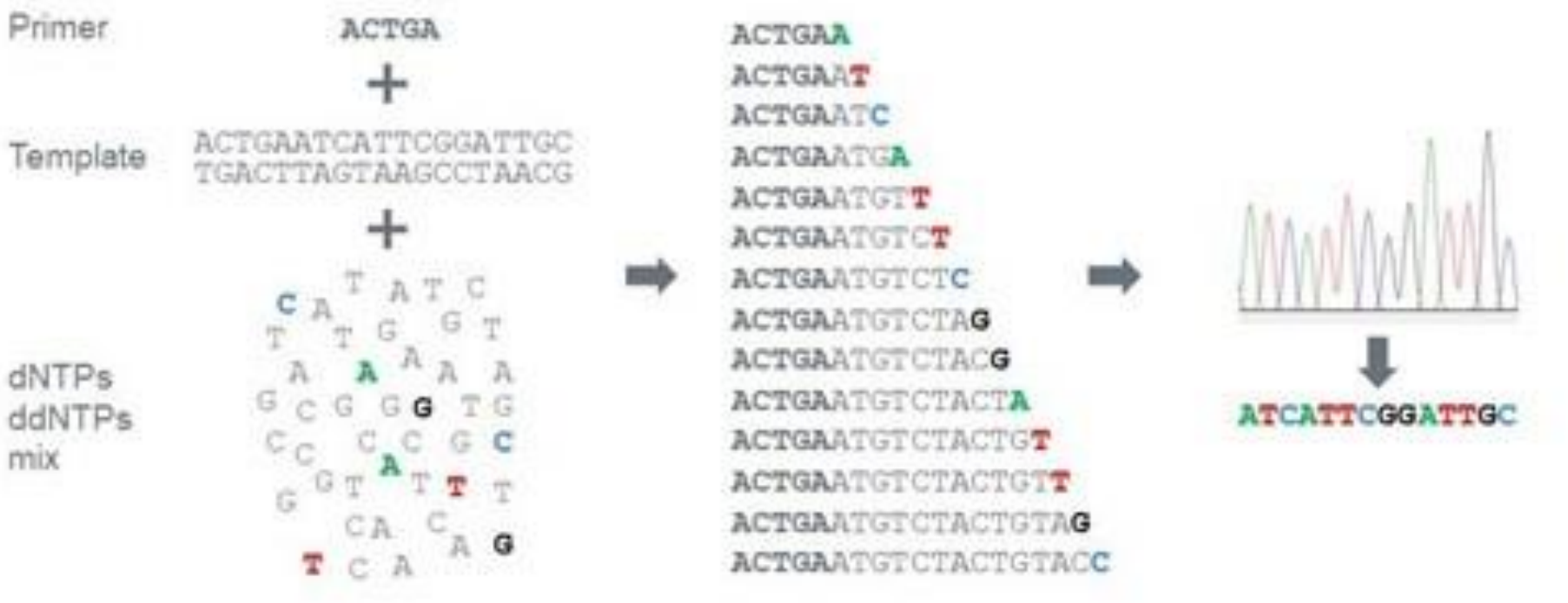
Polymerase chain reaction (PCR)

(~ “cloning” without bacteria, in the test tube)

Use: DNA diagnostics, forensic medicine, research

Denaturation of the DNA double helix



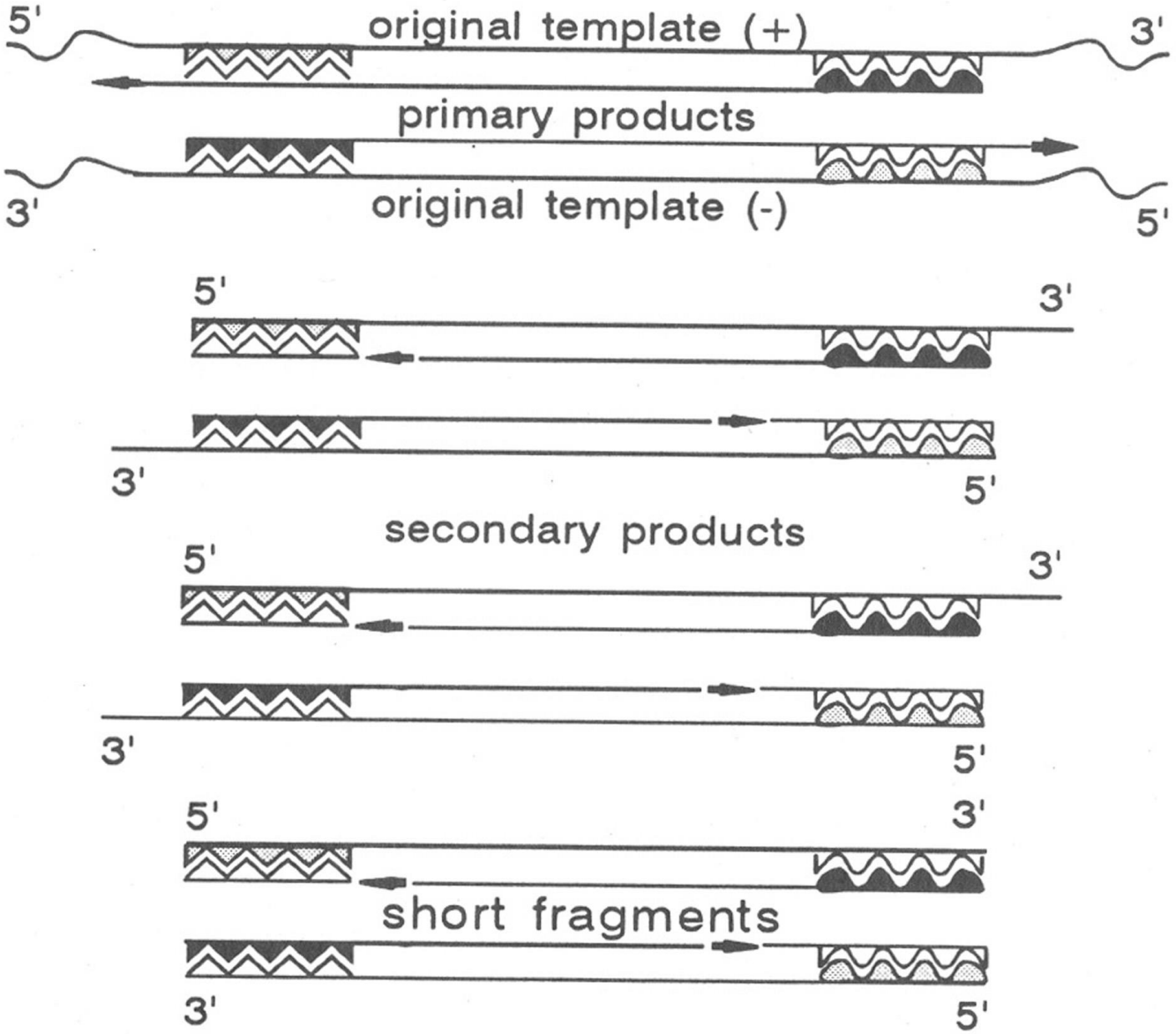


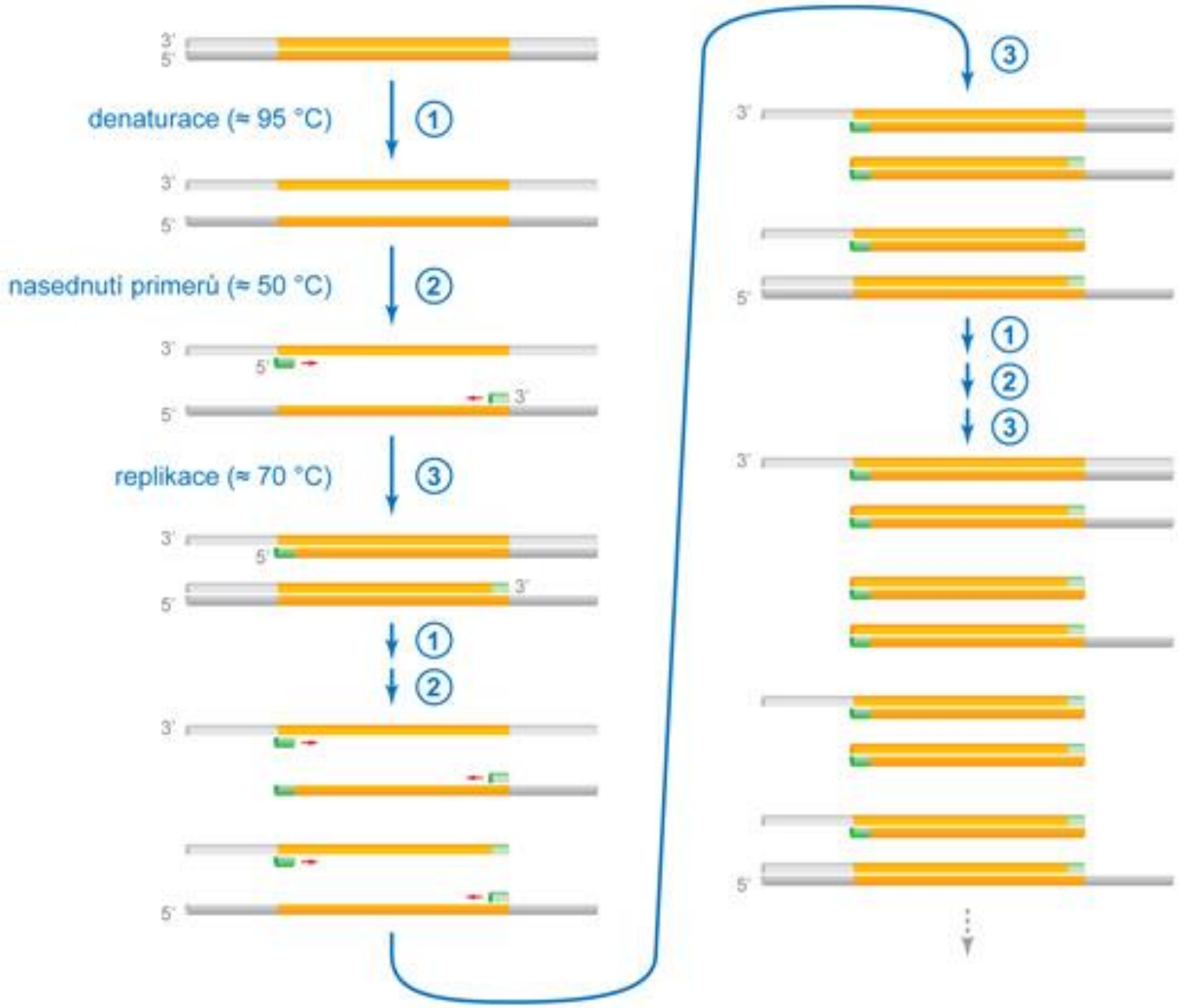
Nucleotides are added like in a normal PCR, and **fluorescently labelled ddNTPs** stop the reaction statistically after every nucleotide

Fragments are **separated by size** through a capillary and fluorescence of each molecule is detected

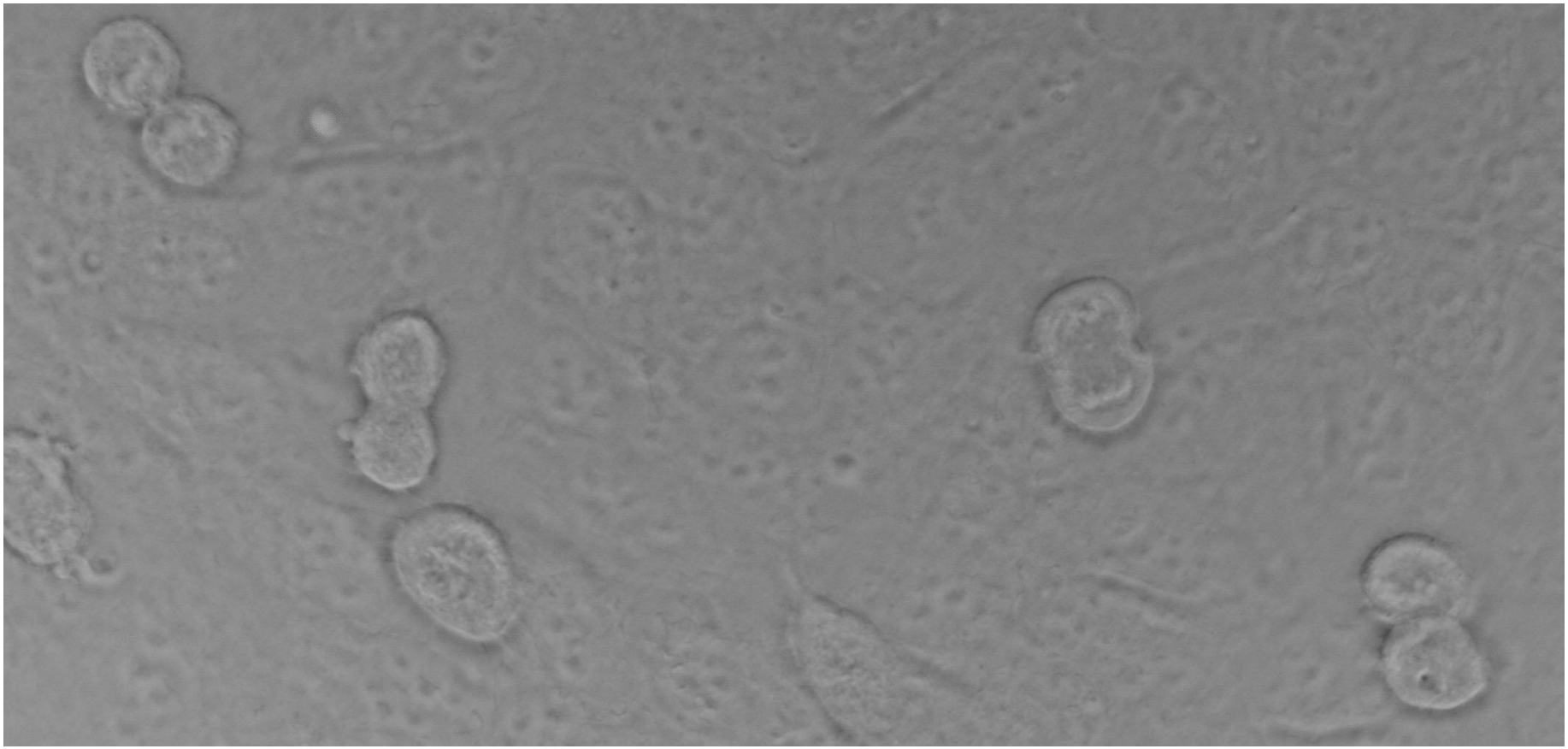
The **basecaller** translates peaks into the sequence of the template

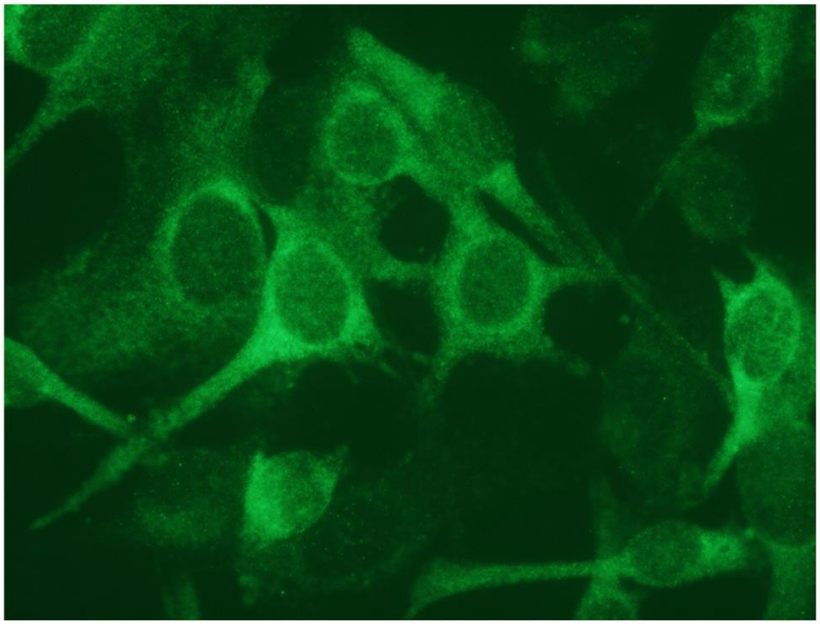
Principle of PCR

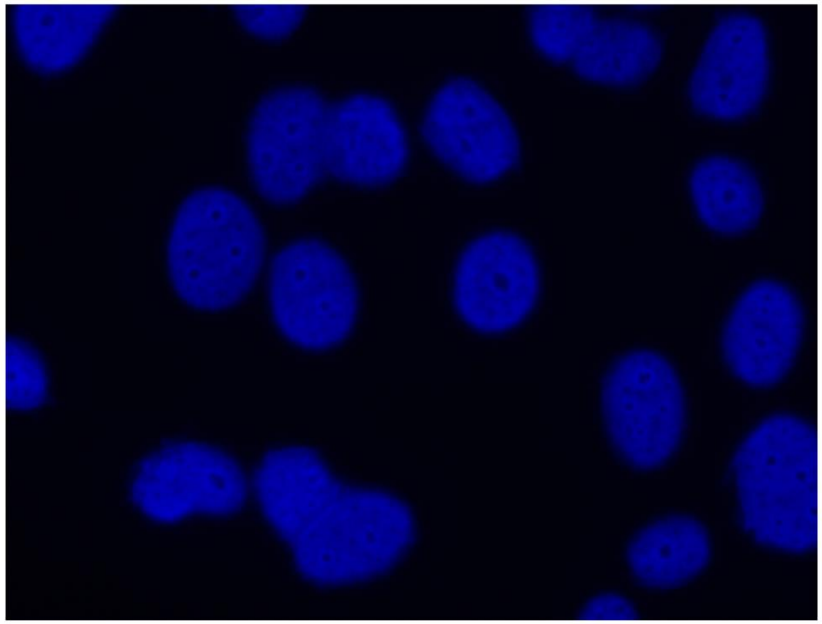



3'
5'
denaturace (≈ 95 °C)
1
nasednutí primerů (≈ 50 °C)
2
replikace (≈ 70 °C)
3

CELL CULTURE METHODS

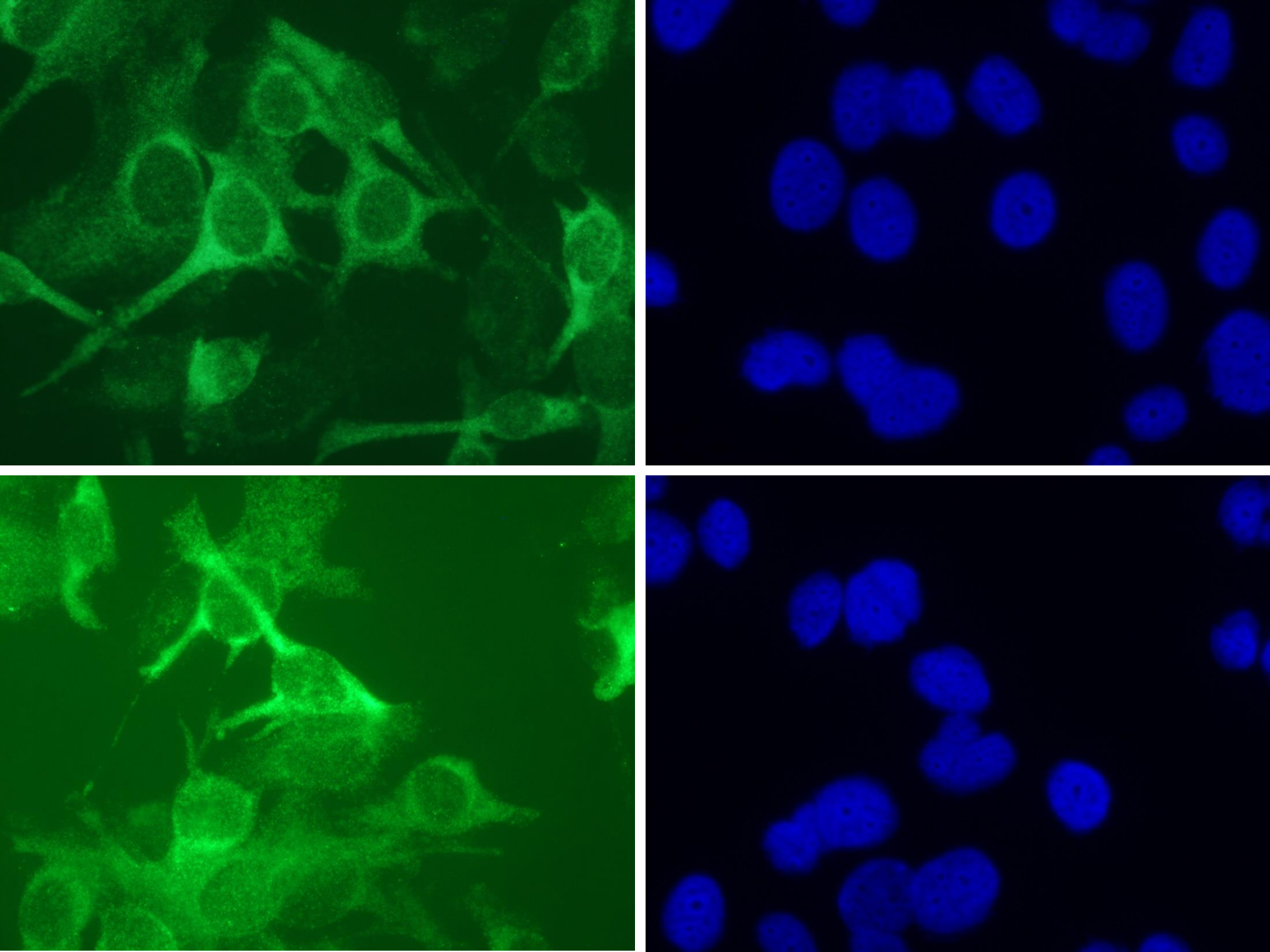


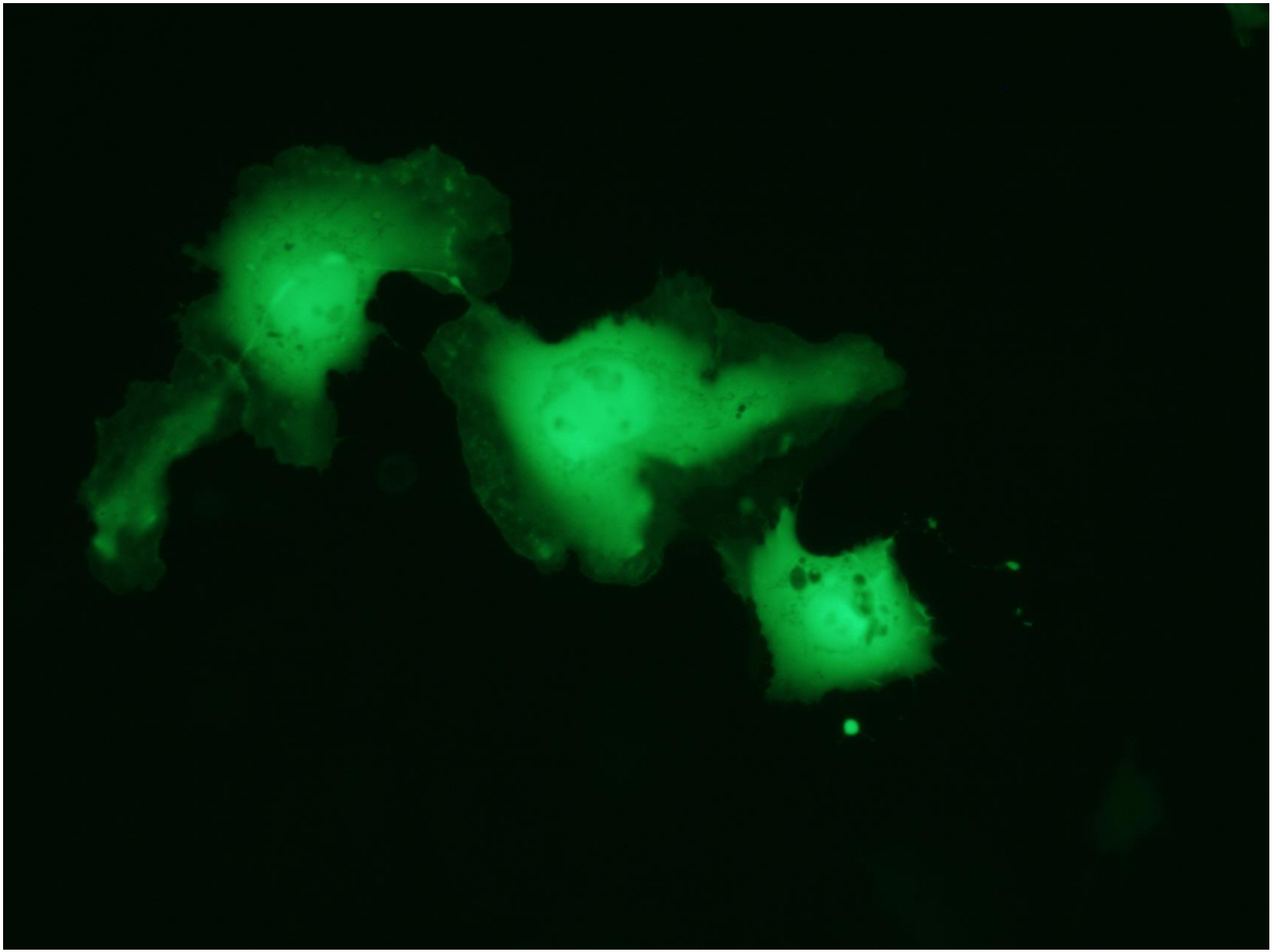


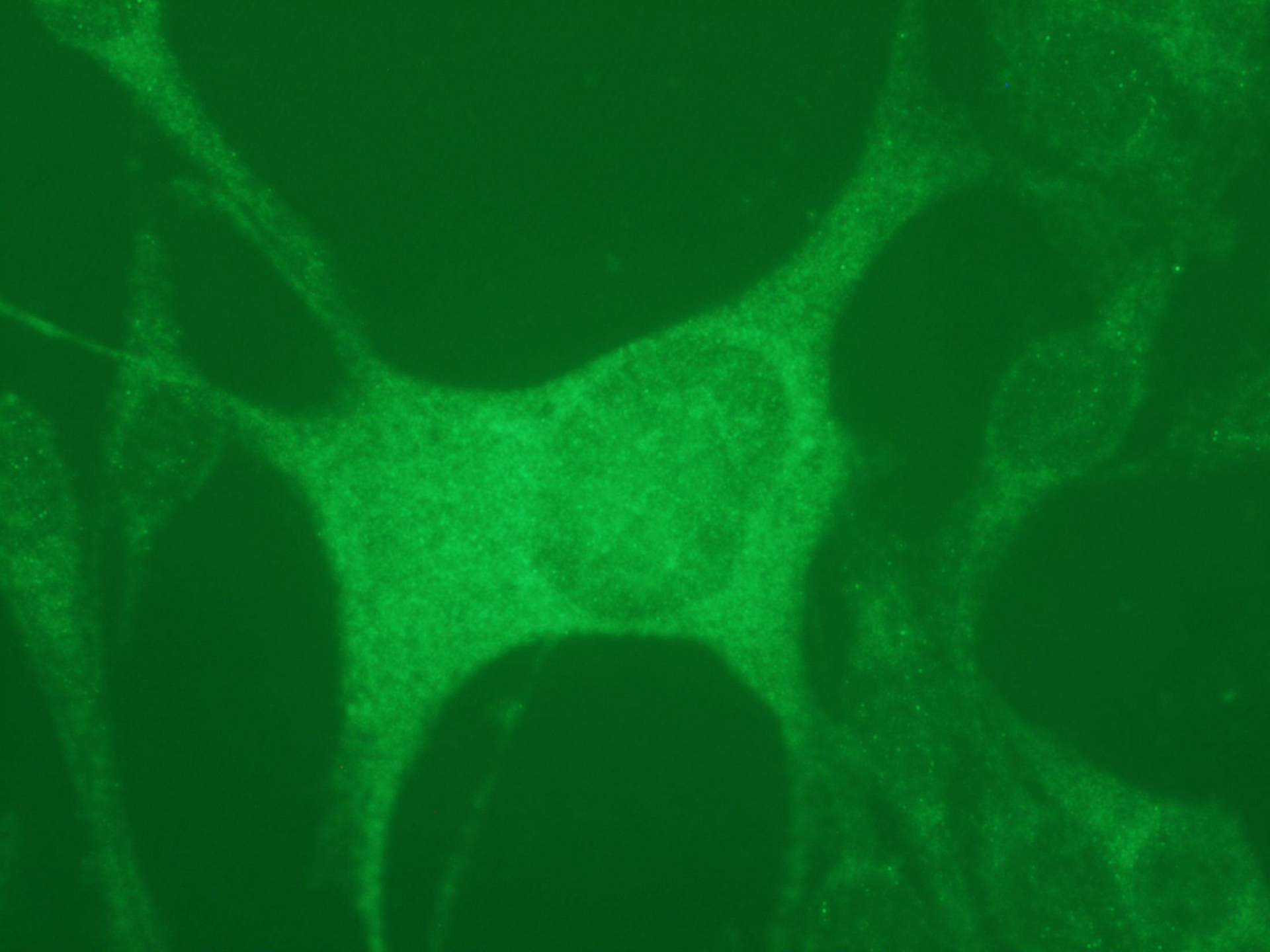


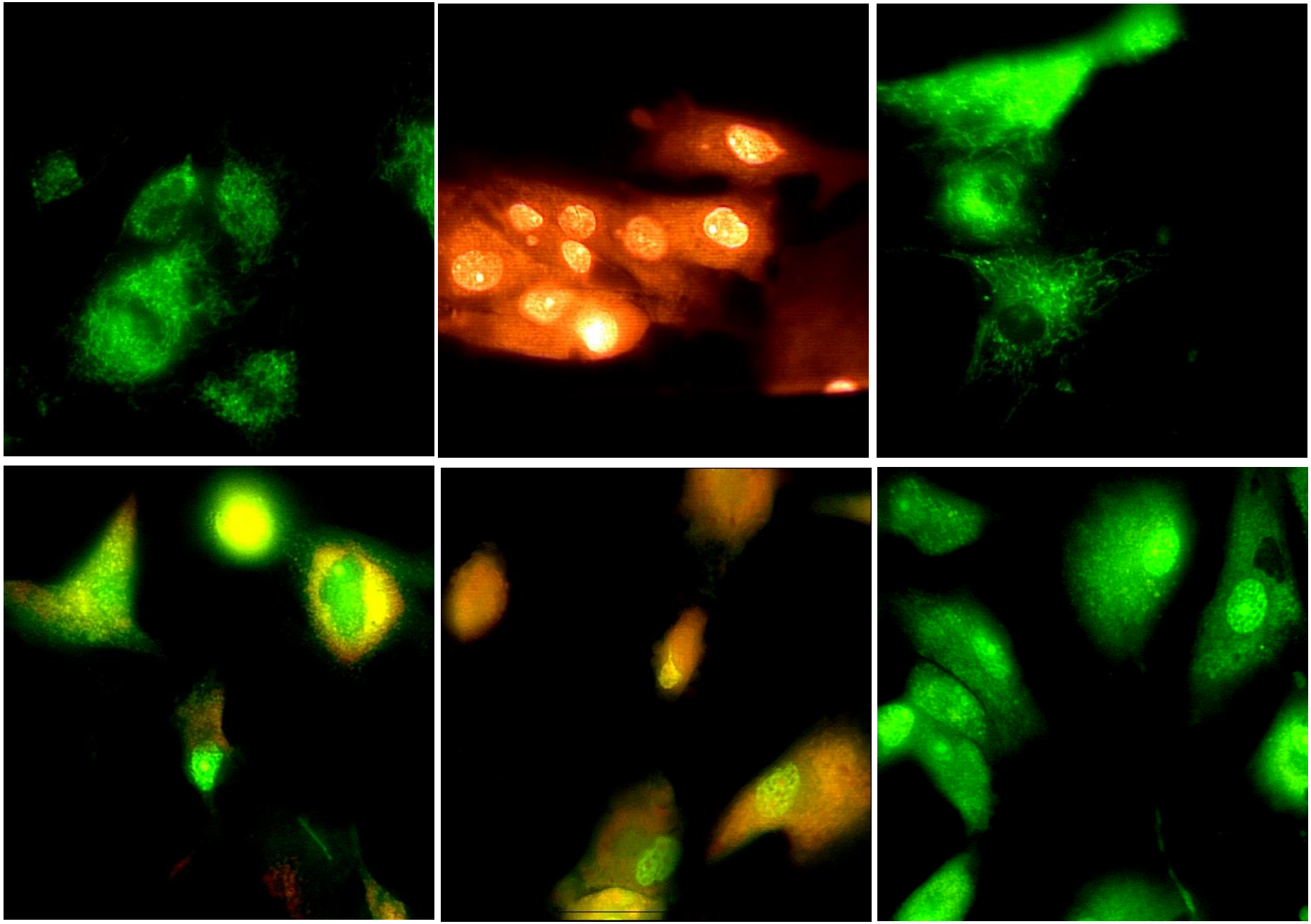
Imunofluorescence

DAPI









FLOW CYTOMETRY

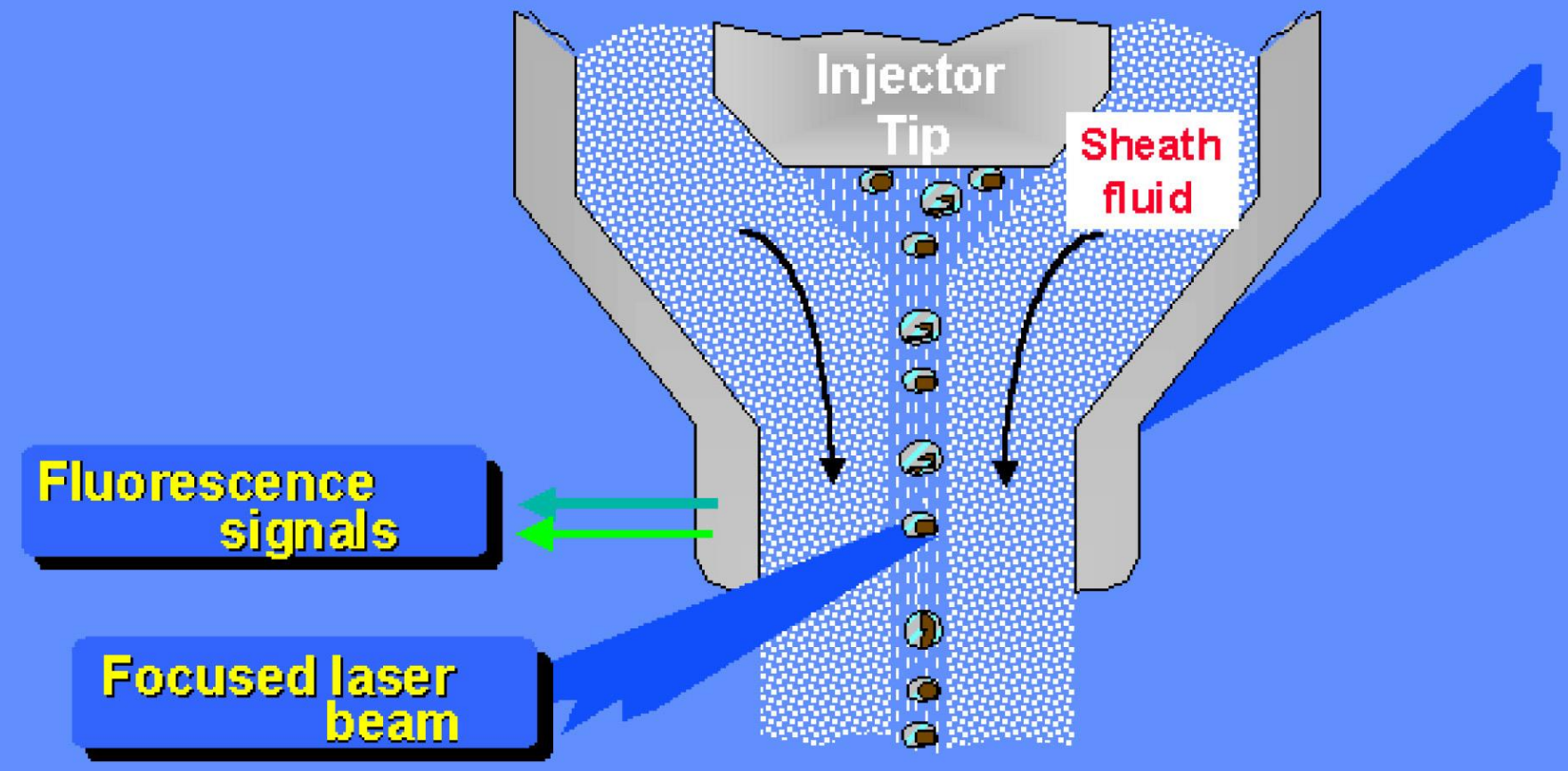
FACS (Fluorescence Activated Cell Sorting)
:

Forward Scatter (FSc)
~ particle size

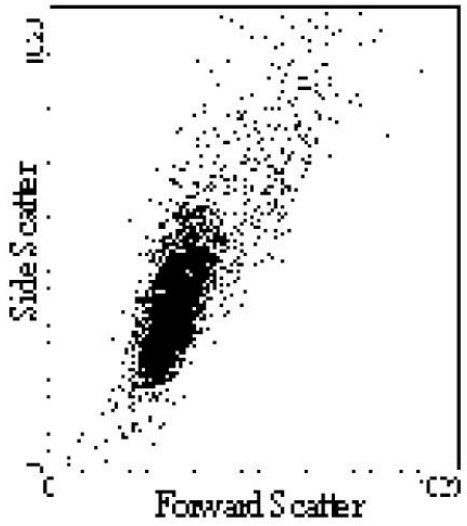
Side (Orthogonal) Scatter (SSc)
~ Cell surface, granularity

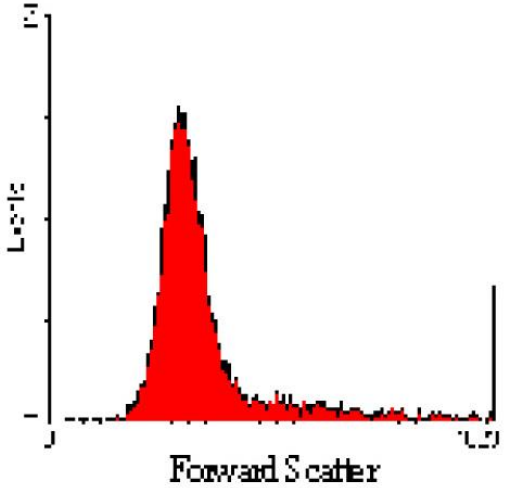
Fluorescent Labeling - emitted light

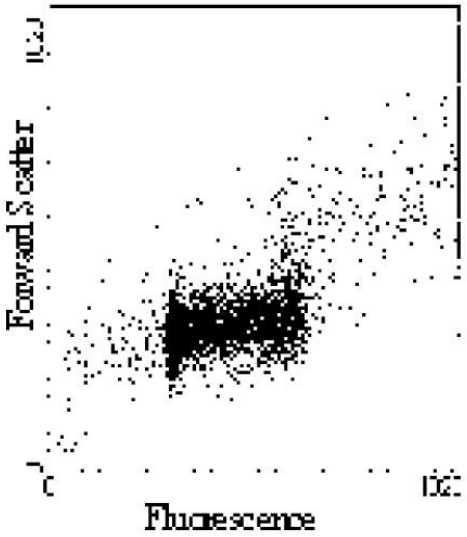
Flow Cell

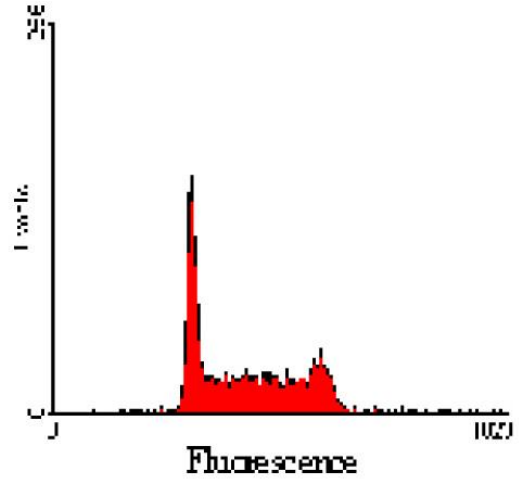

Injector Tip
Sheath fluid
Fluorescence signals
Focused laser beam

DNA Analysis

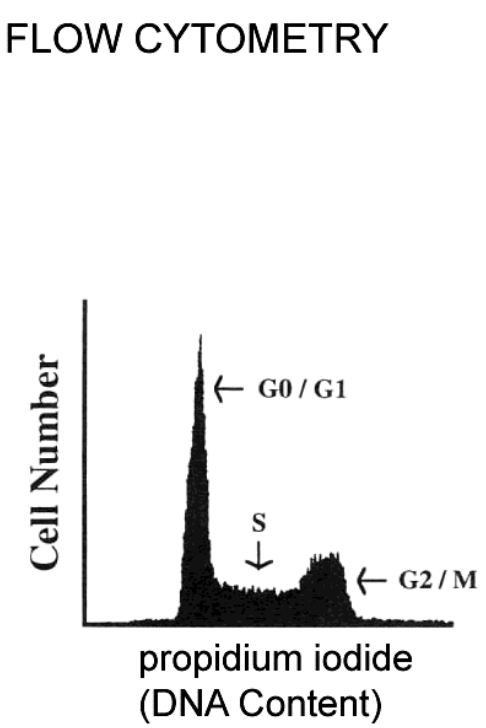


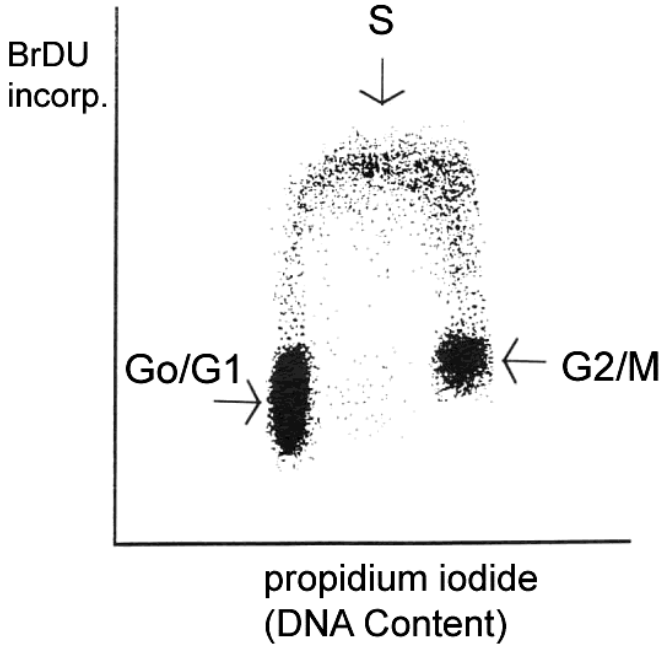


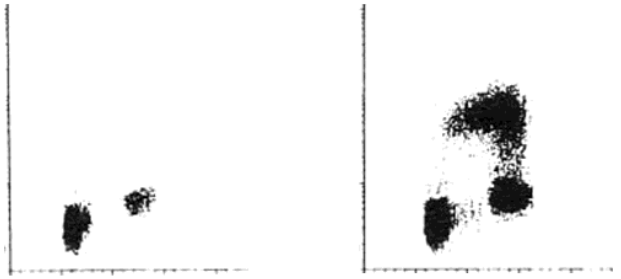


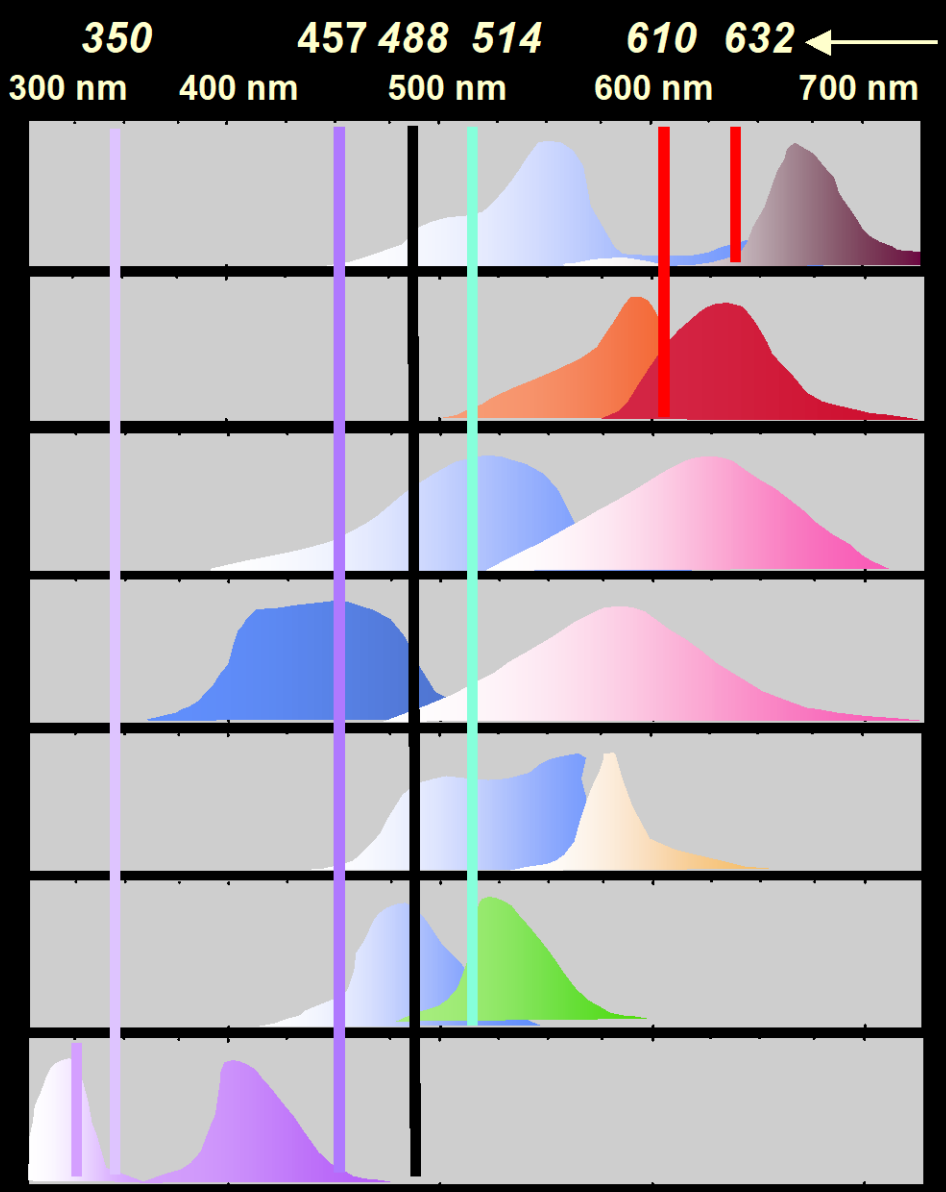


Analýza fází buň. cyklu pomocí průtokové cytometrie





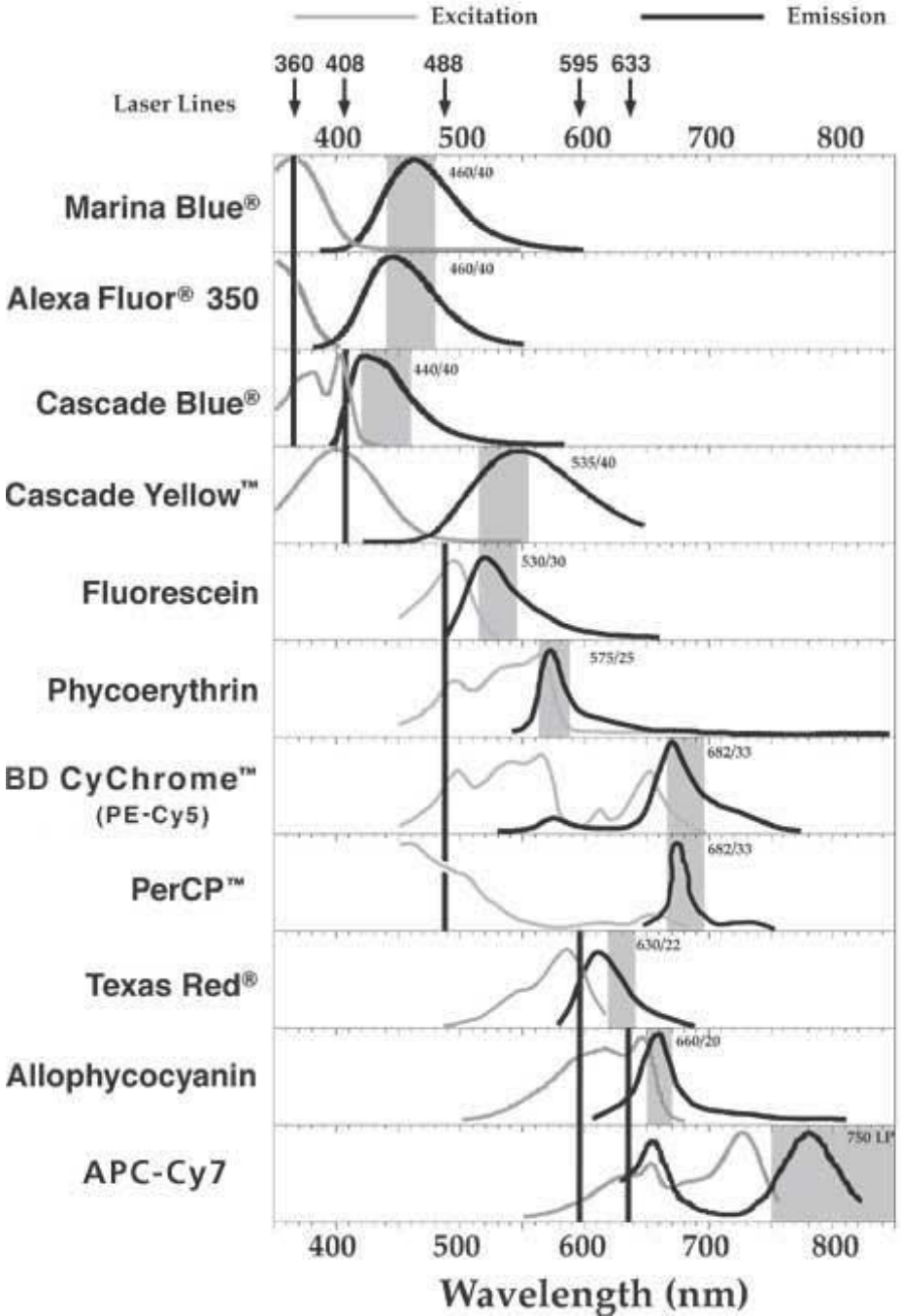




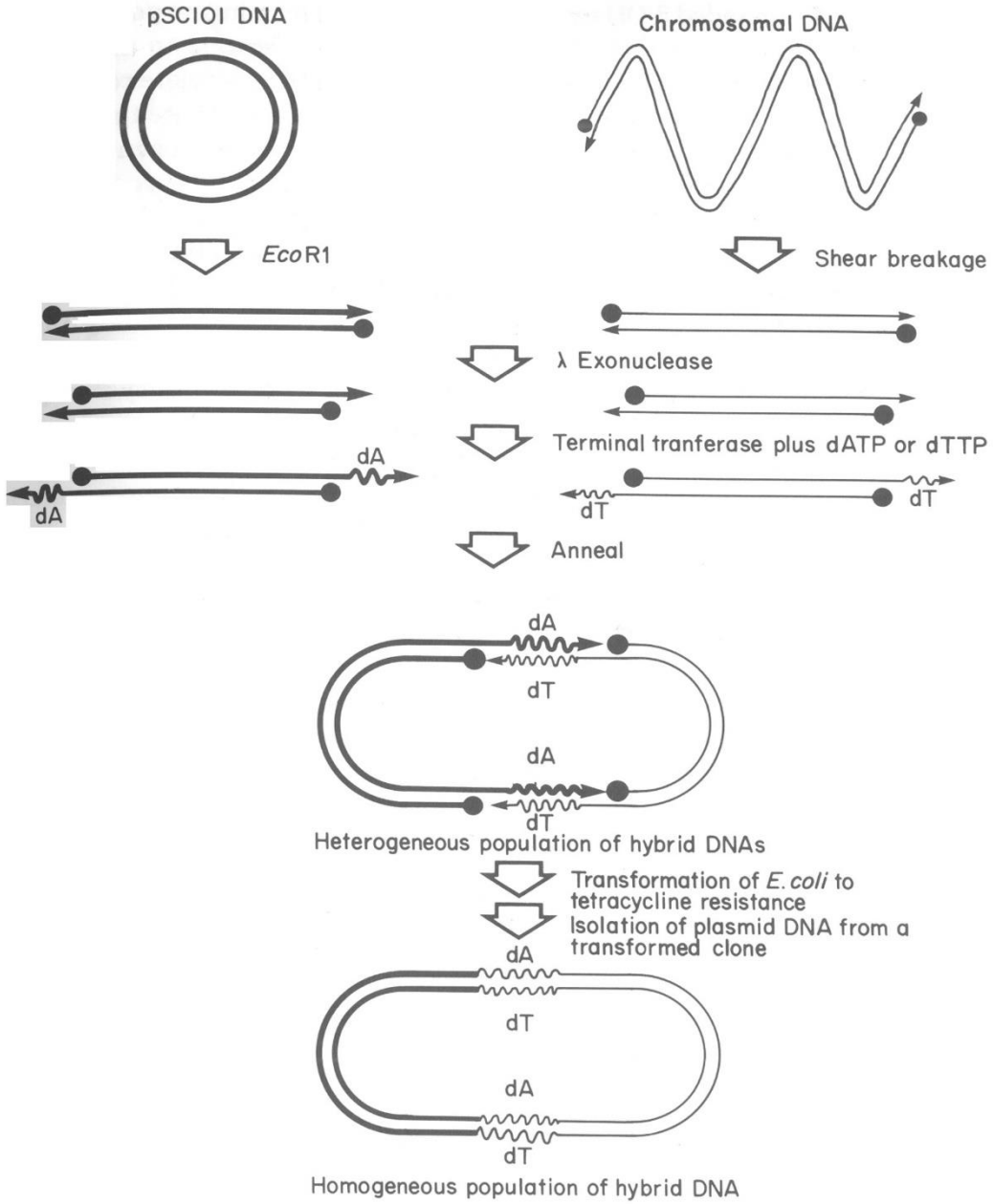
Common Laser Lines

- PE-TR Conj.
- Texas Red
- PI
- Ethidium
- PE
- FITC
- cis-Parinaric acid

Fluorochrome Dyes Used in Flow Cytometry



Cloning by using homopolymer tails



The homopolymer tailing technique.