Molecular DNA techniques and their immunological applications 1.

Katalin Szabó and Ferenc Boldizsar

PhD course

PCR

Real time quantitative PCR

Cloning

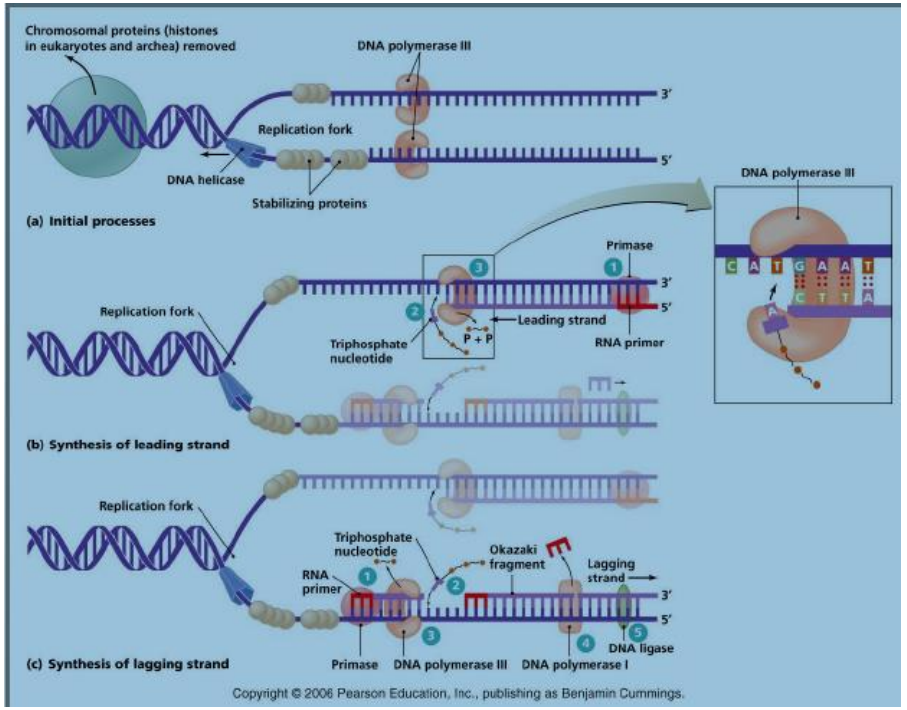
Monoclonal Antibodies for therapy

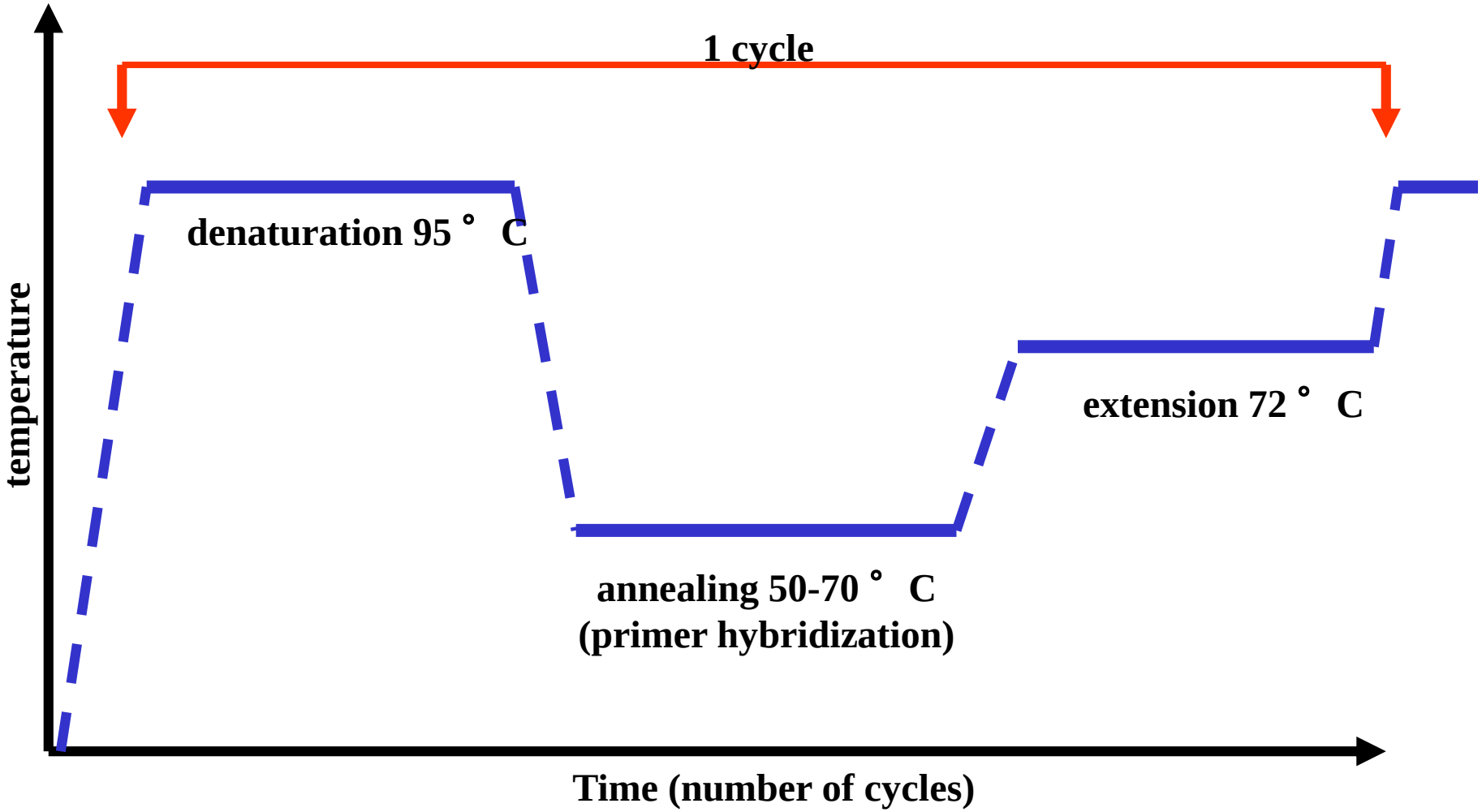
Phage display

PCR

Kary B. Mullis (1983)
and Michael Smith (1985)
Nobel-price in Chemistry 1993.

Def.: amplification of a specific DNA sequence during cyclic temperature changes




1 cycle
denaturation 95 ° C
extension 72 ° C
annealing 50-70 ° C
(primer hybridization)
temperature
Time (number of cycles)

PCR

Nature. 1986 Nov 13-19;324(6093):163-6.

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA.

Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes.

Abstract

Allelic sequence variation has been analysed by synthetic oligonucleotide hybridization probes which can detect single base substitutions in human genomic DNA. An allele-specific oligonucleotide (ASO) will only anneal to sequences that match it perfectly, a single mismatch being sufficient to prevent hybridization under appropriate conditions. To improve the sensitivity, specificity and simplicity of this approach, we used the polymerase chain reaction (PCR) procedure to enzymatically amplify a specific segment of the beta-globin or HLA-DQ alpha gene in human genomic DNA before hybridization with ASOs. This in vitro amplification method, which produces a greater than *10(5)-fold increase* in the amount of target sequence, permits the analysis of allelic variation with as little as 1 ng of genomic DNA and the use of a simple 'dot blot' for probe hybridization. As a further simplification, PCR amplification has been performed directly on crude cell lysates, eliminating the need for DNA purification.

PMID: 3785382 [PubMed - indexed for MEDLINE]

PCR

Science. 1988 Jan 29;239(4839):487-91.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA.

Cetus Corporation, Department of Human Genetics, Emeryville, CA 94608.

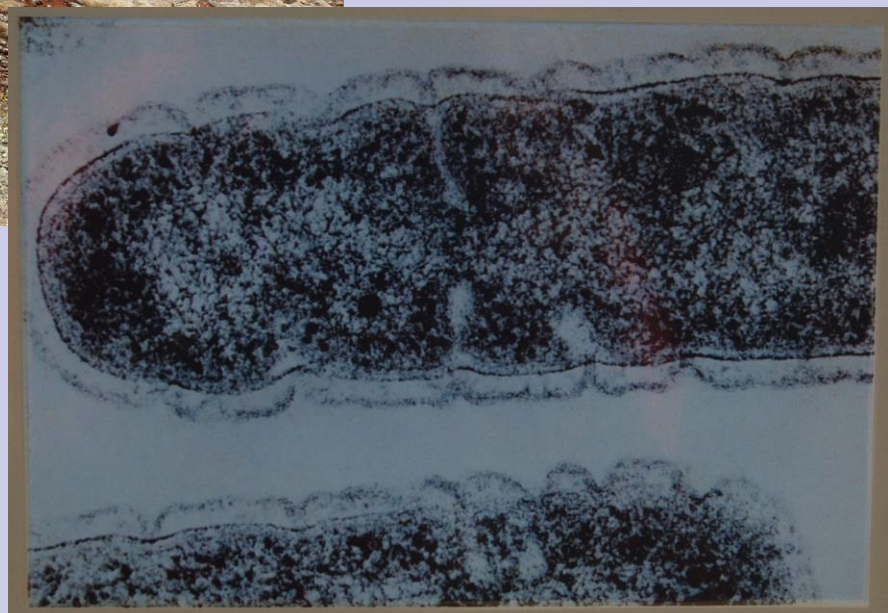
Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase.

Abstract

A thermostable DNA polymerase was used in an in vitro DNA amplification procedure, the polymerase chain reaction. The enzyme, isolated from Thermus aquaticus, greatly simplifies the procedure and, by enabling the amplification reaction to be performed at higher temperatures, significantly improves the specificity, yield, sensitivity, and length of products that can be amplified. Single-copy genomic sequences were amplified by a factor of more than 10 million with very high specificity, and DNA segments up to 2000 base pairs were readily amplified. In addition, the method was used to amplify and detect a target DNA molecule present only once in a sample of 10(5) cells.

PMID: 2448875 [PubMed - indexed for MEDLINE]

1969: Thomas D. Brock and Hudson Freeze
 - Thermus aquaticus
1976: Taq polymerase



Hot water bacteria have a value beyond beauty. *Thermus aquaticus* (shown above), found in some of the park's hot water runoff channels, produces an enzyme used in DNA "fingerprinting" and testing for the virus that causes AIDS.

PCR

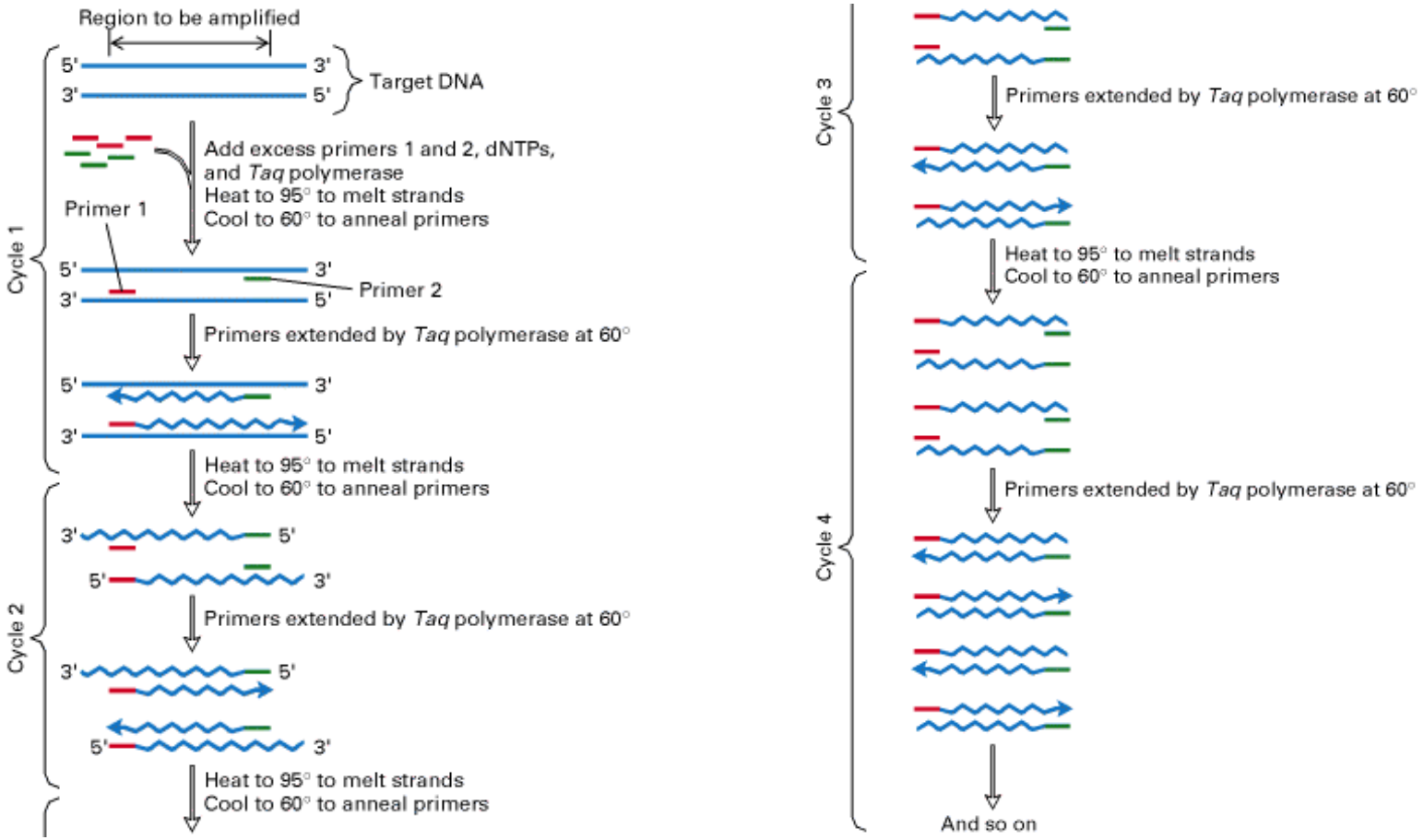


Figure 7-38. The polymerase chain reaction. (In: H. Lodish, A. Berk, L. Zipursky, P. Matsudaira, D. Baltimore, J. Darnell: Molecular Cell Biology 4th Ed.)

PCR

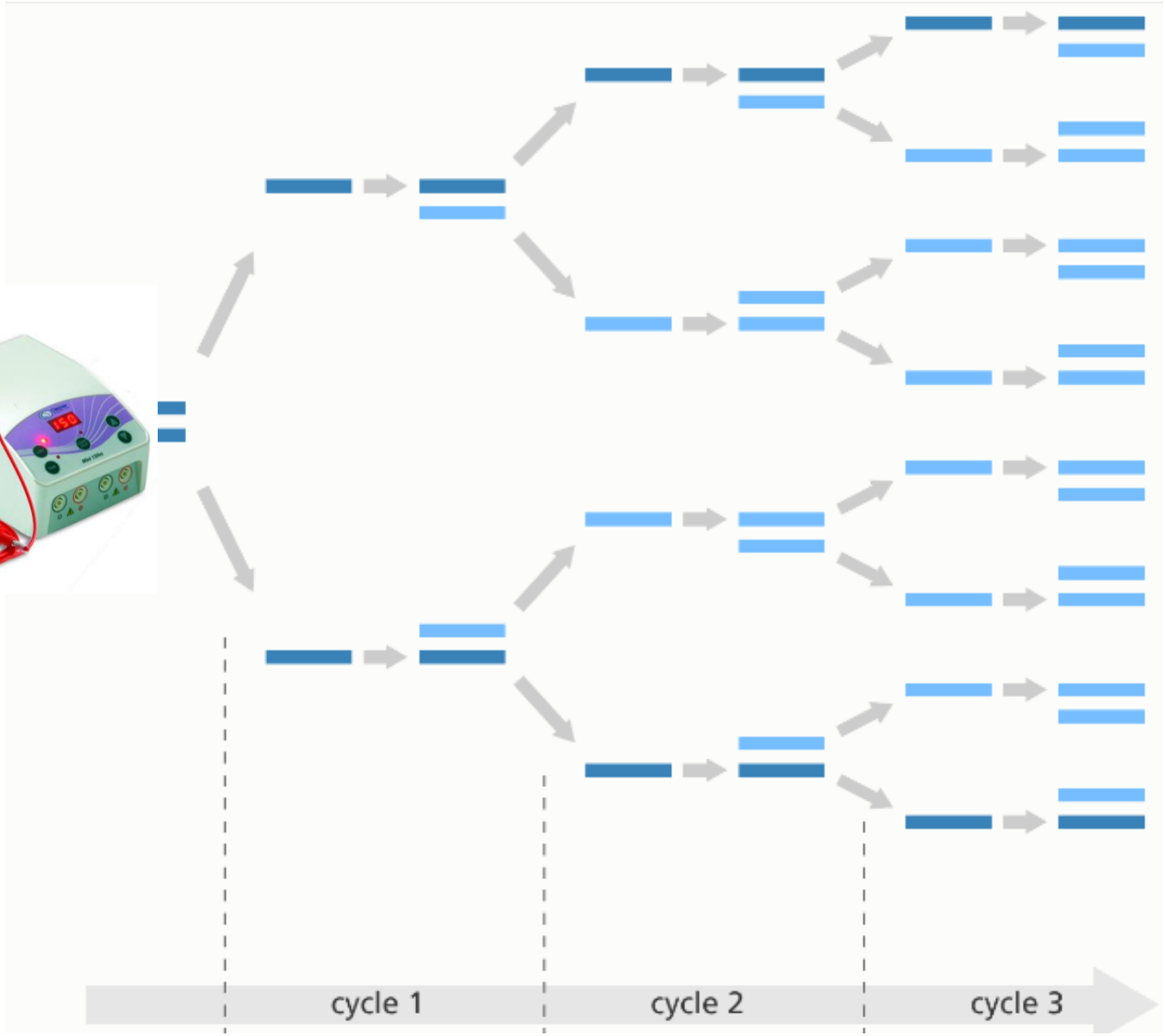
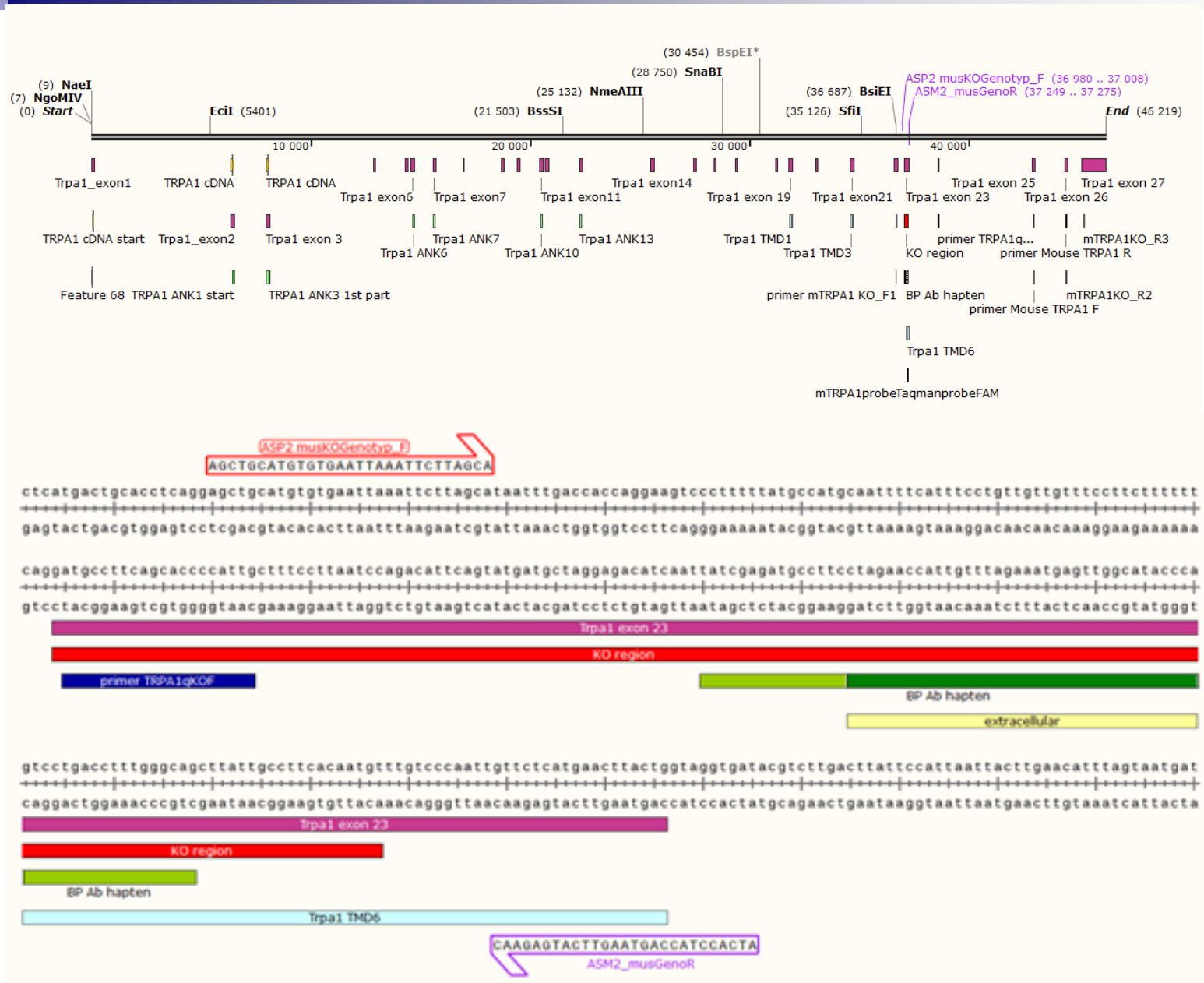


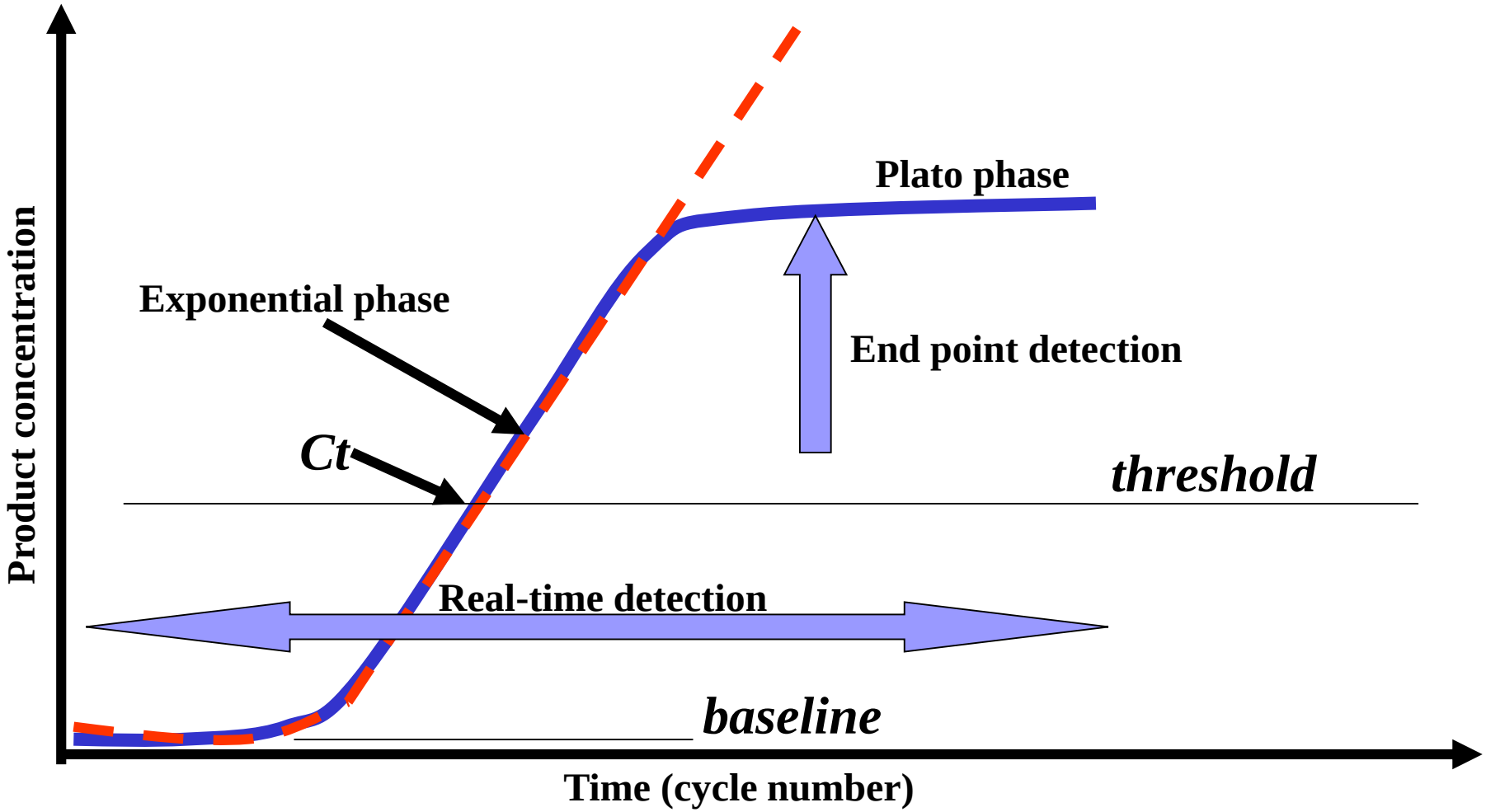
Illustration showing how the polymerase chain reaction (PCR) produces lots of copies of DNA. Image credit: Genome Research Limited



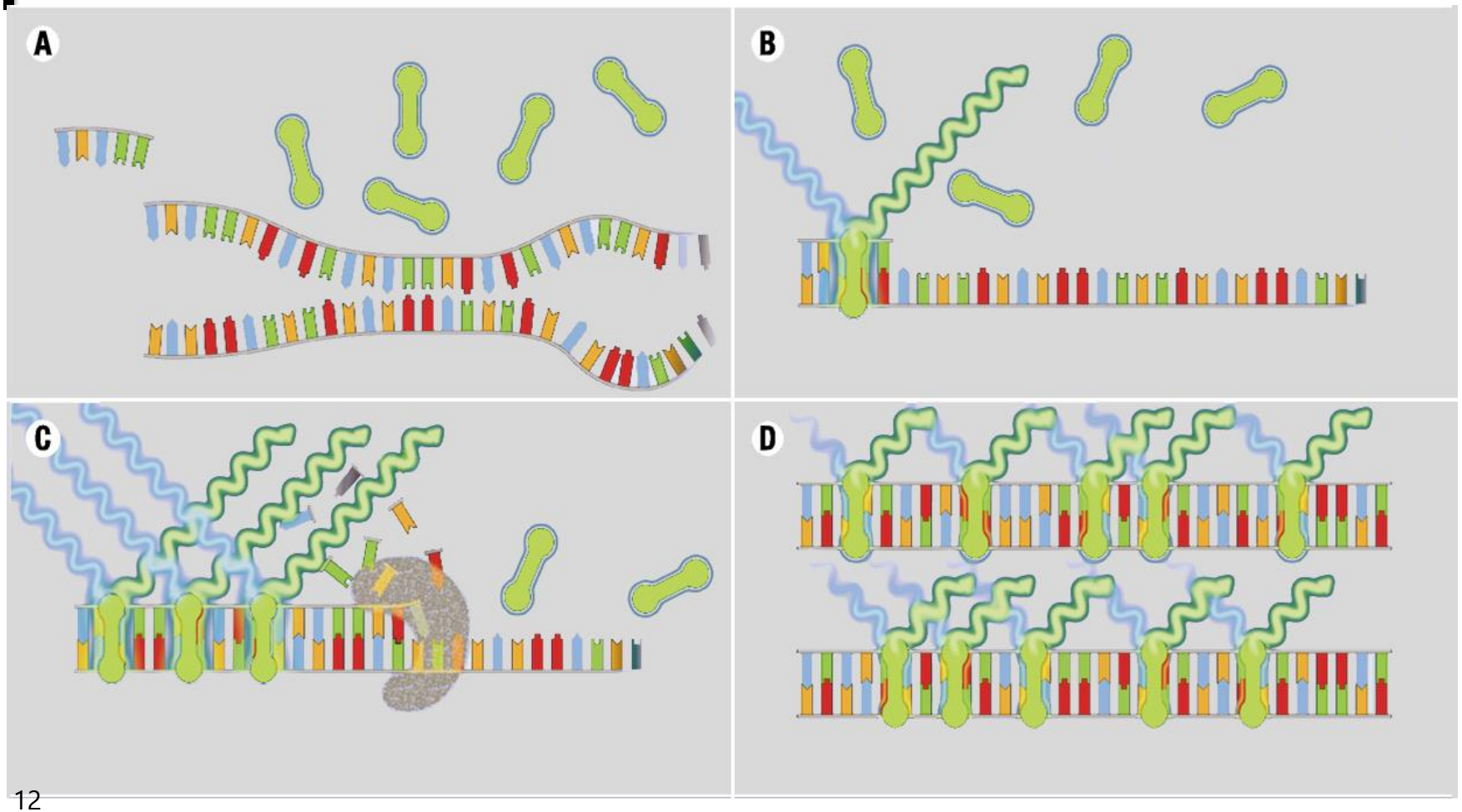
Snapgene genomic

Snapgene cDNA

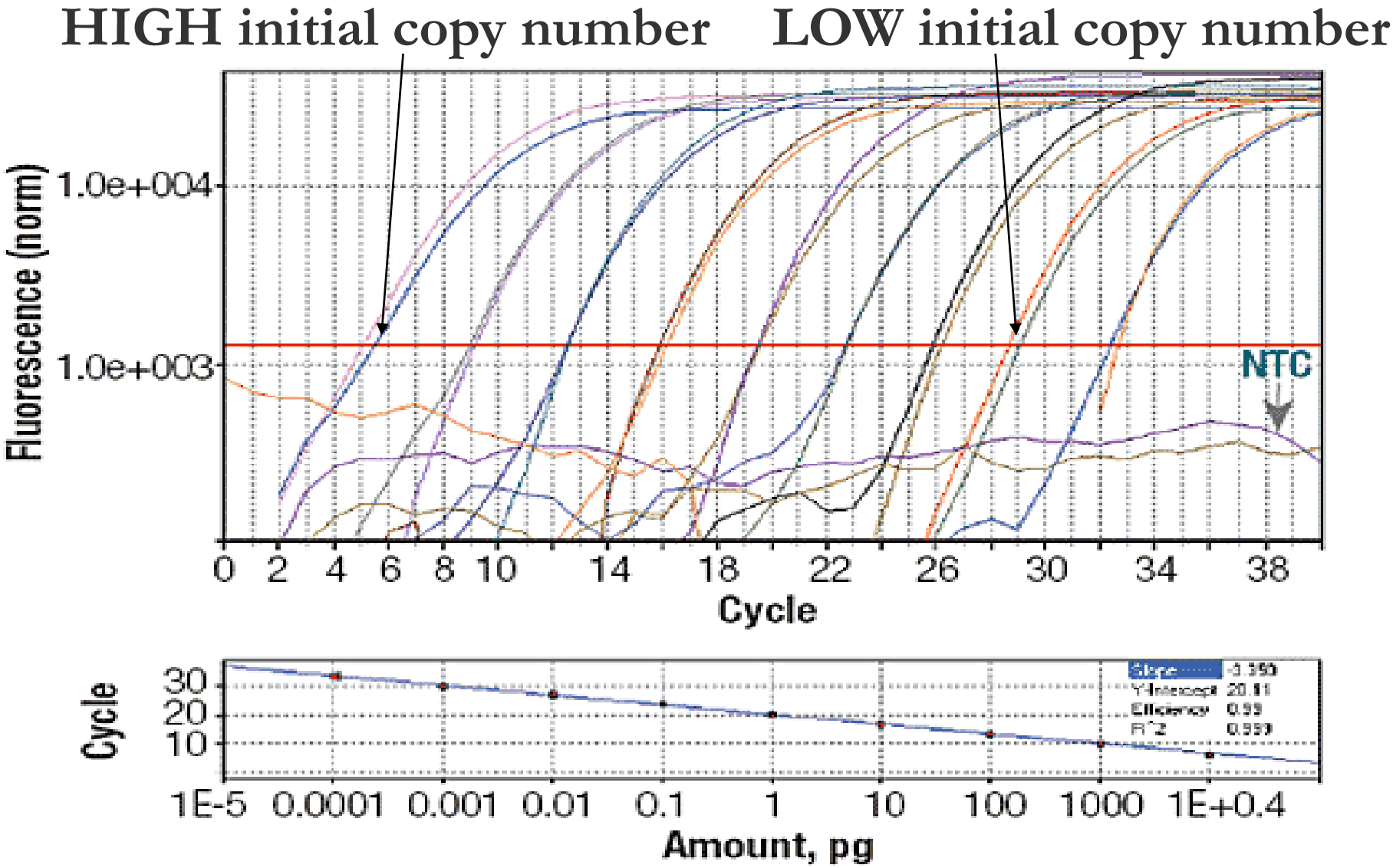
PCR Real Time detection



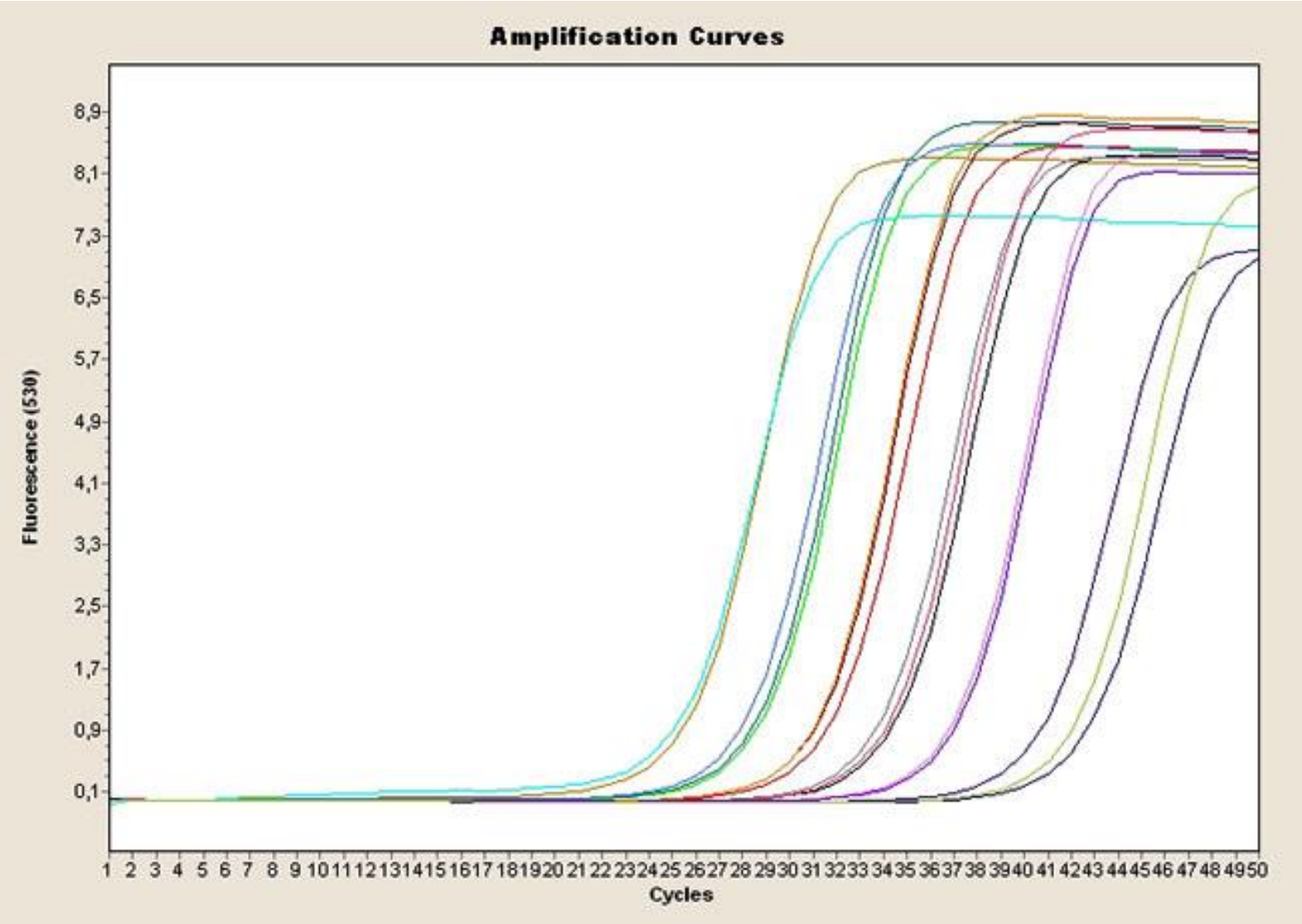
Real-time (quantitative) PCR - SYBR Green I



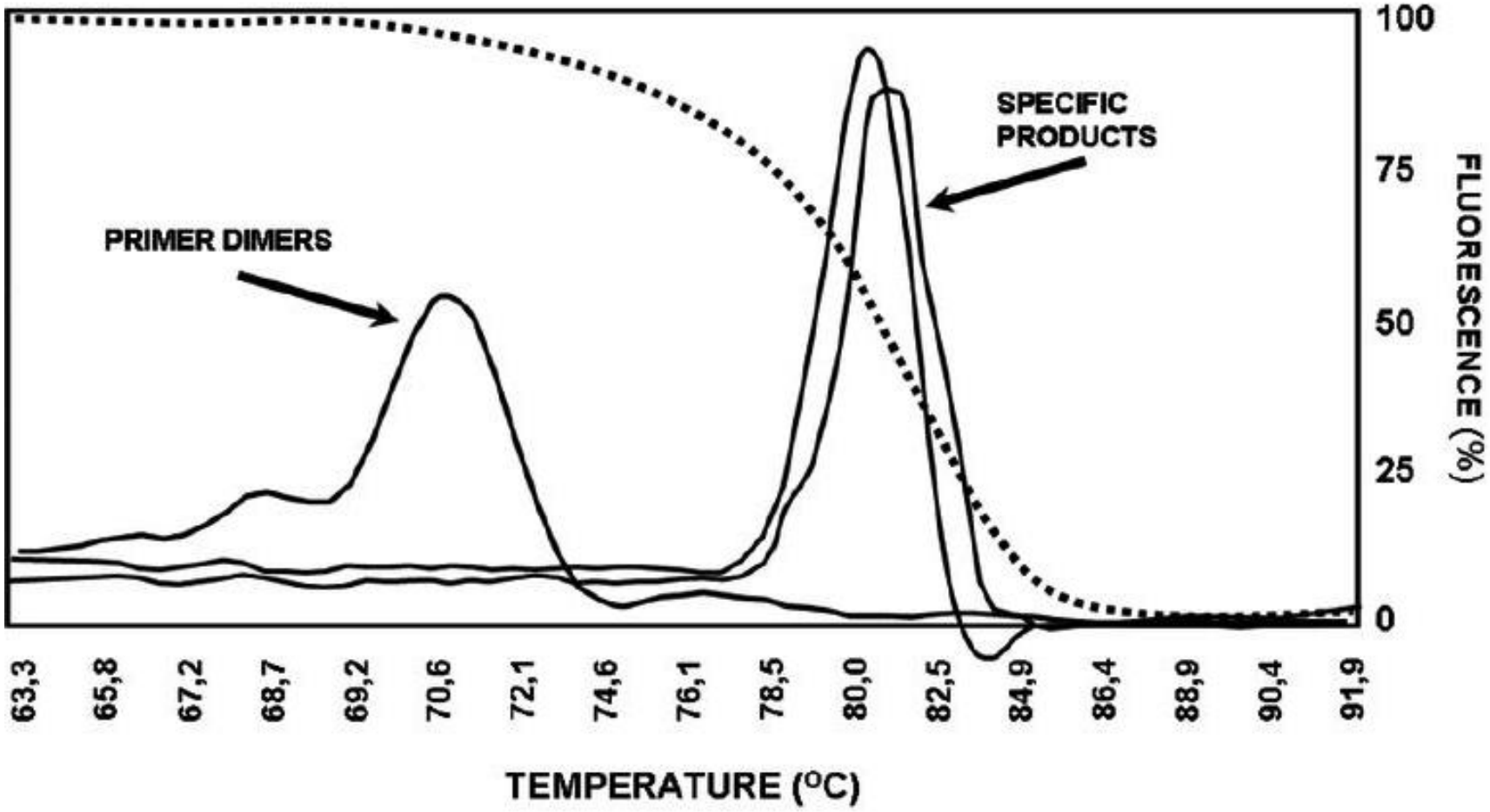
PCR Real Time detection



Real-time (quantitative) PCR

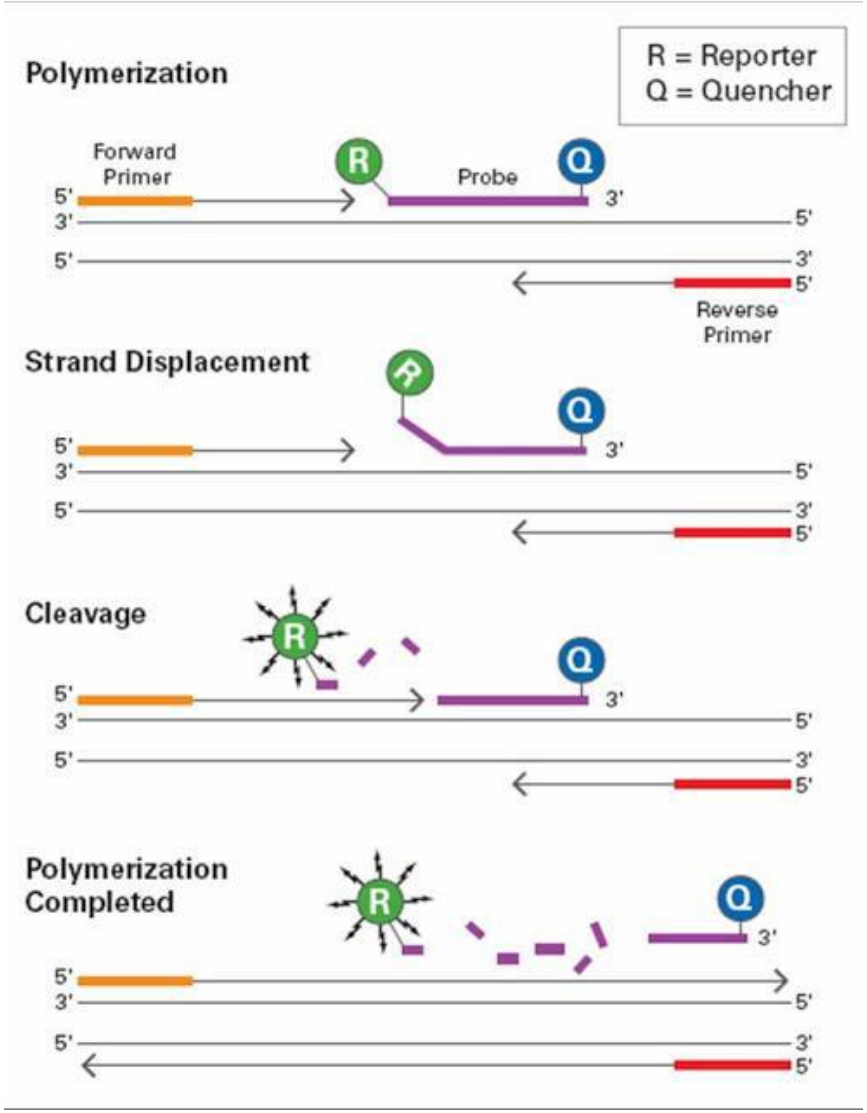


qRT-PCR product specificity



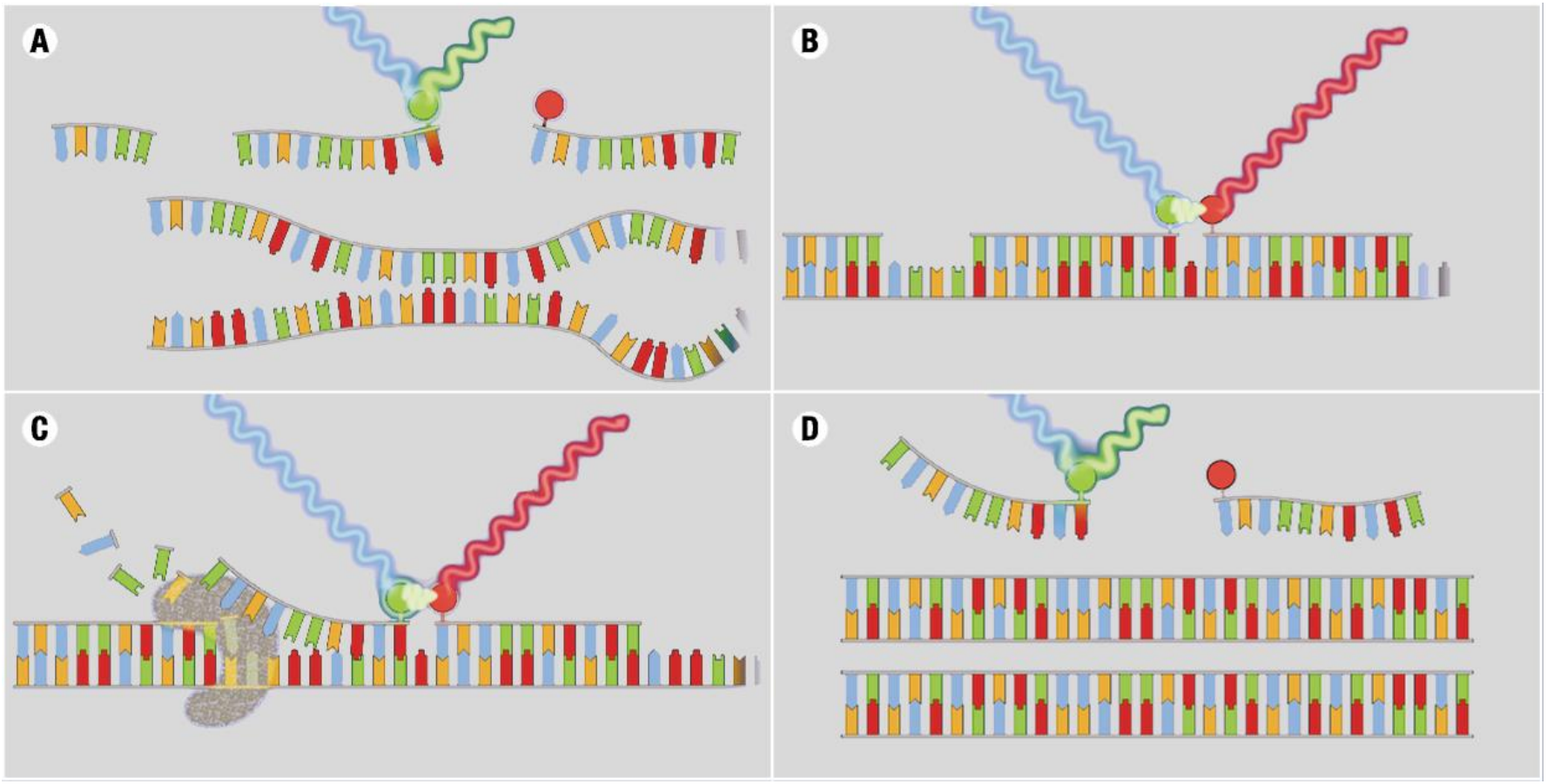
Real-time (quantitative) PCR -

Sequence spec. oligonucleotide probe Taqman

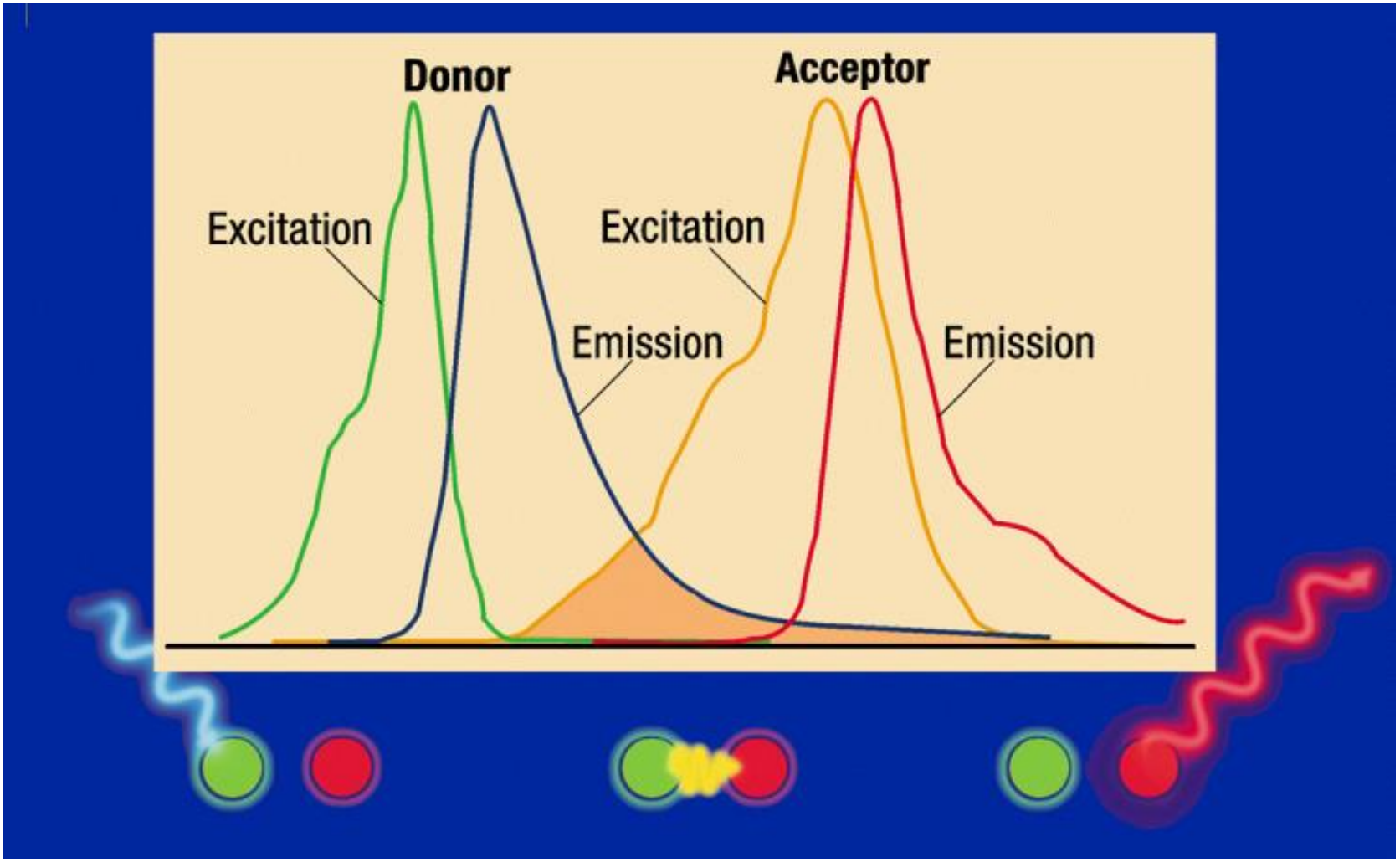


Real-time (quantitative) PCR -

Sequence spec. oligonucleotide probe - FRET

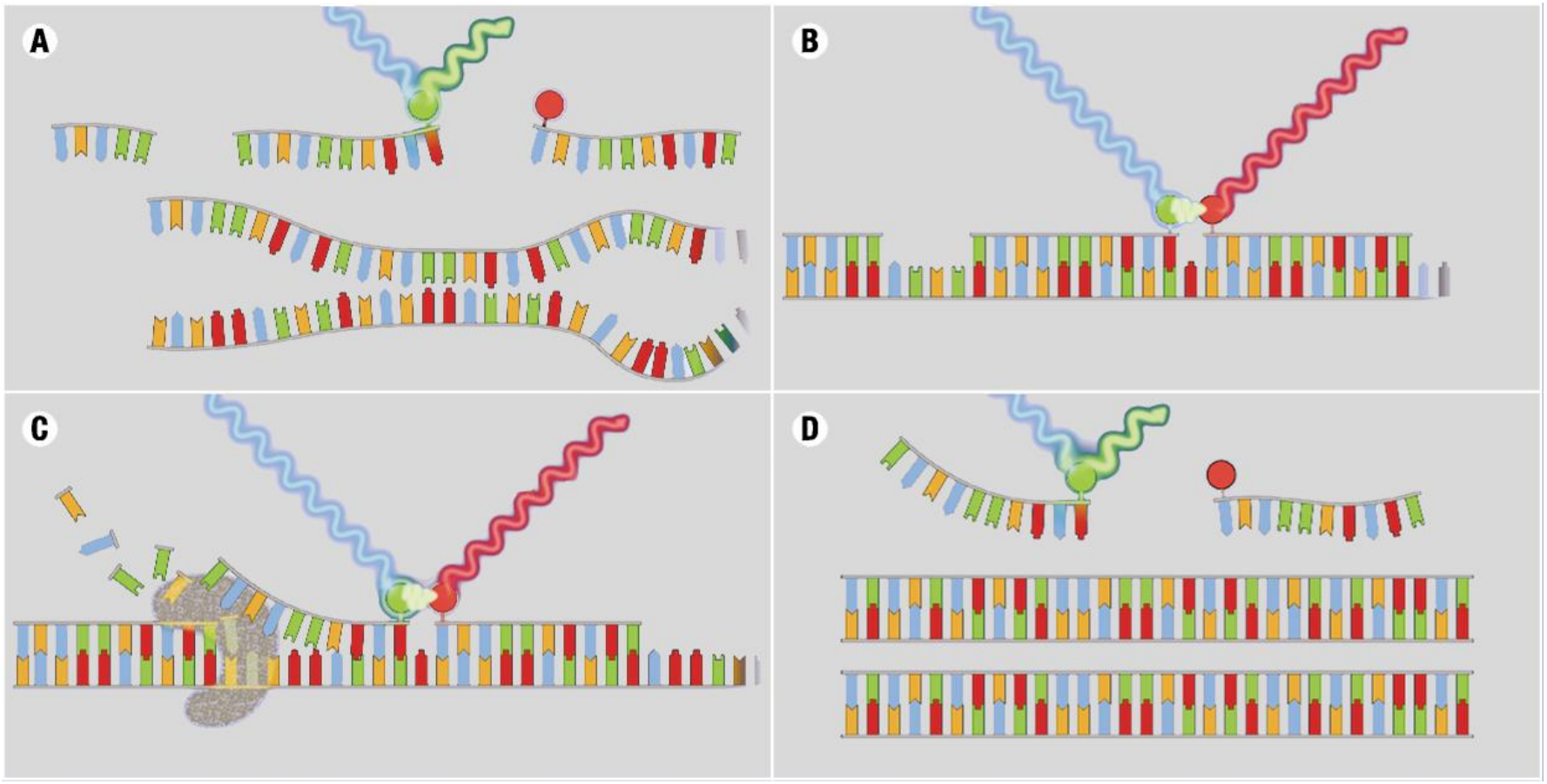


FRET
Fluorescence Resonance Energy Transfer



Real-time (quantitative) PCR -

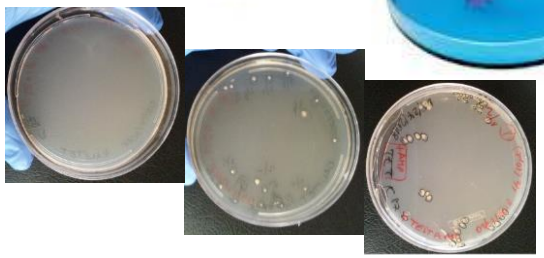
Sequence spec. oligonucleotide probe - FRET

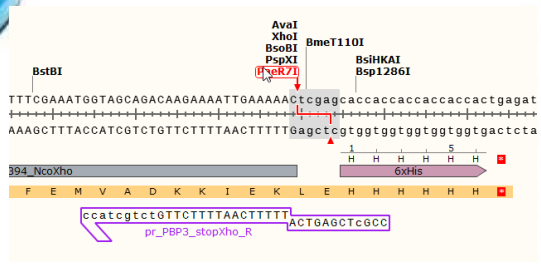


PCR

Real time quantitative PCR

Cloning (antigen, Ab, aptamers…..)





Monoclonal Antibodies for therapy

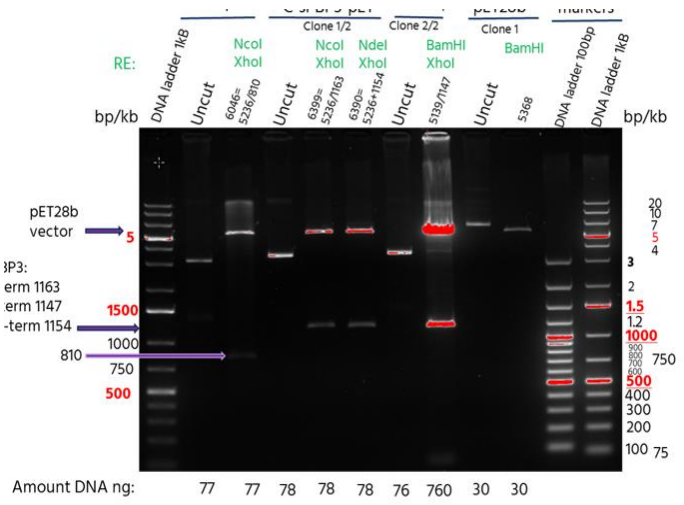
Phage display

Antigen production

- Choosing haptene (protein, domain, mutant)
- Choosing sequence (SNP,species, codon opt.)
- Choosing expression system (*glycosylation, phosphorylation, laboratory, licences, experts, risks, budget)*

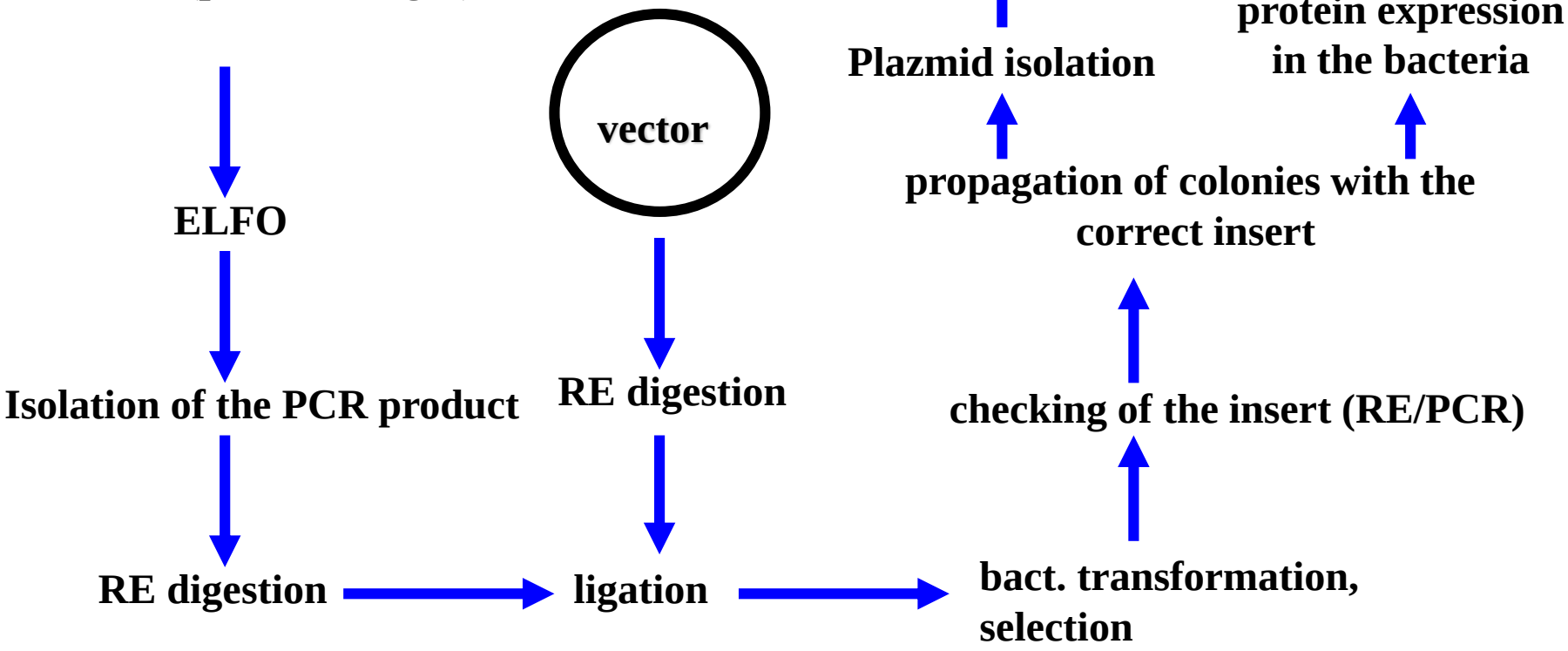
→

Cloning Strategies



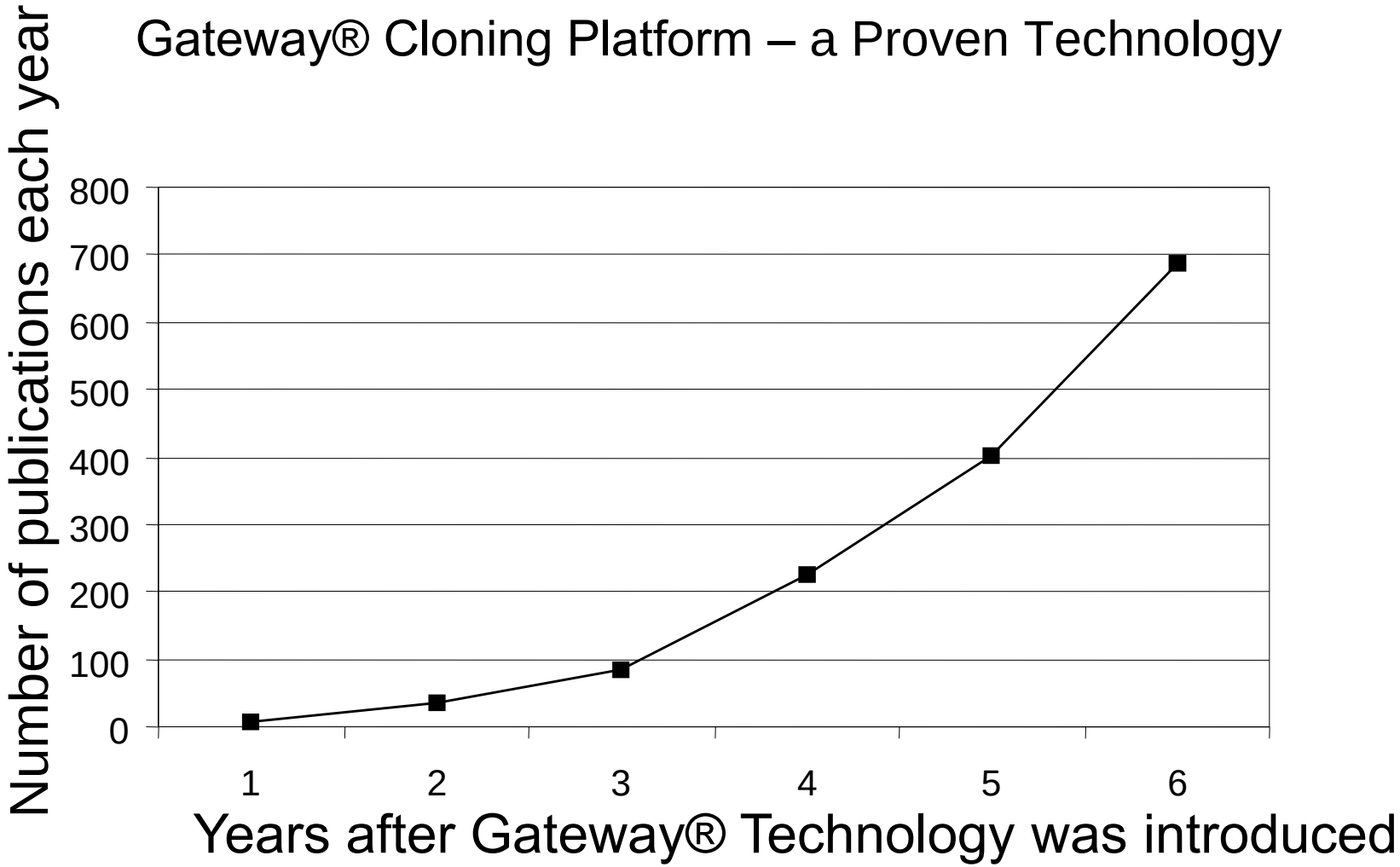
Cloning

-Template (isolate RNA, DNA, buy
-Full length gene PCR
-Restriction sites at both ends of the PCR product: sticky end, correct orientation (primer design!)



" Recombineering" techniques

Gateway® Cloning Platform – a Proven Technology



First published: Hartley et al. 2000; Walhout et al. 2000; Reboul et al. 2001

John M. Watson, et al. FEBS Letters –

"The Gateway® System seems to have become a universal standard in genomic research and there are many gene and gene fragment libraries in these Entry vectors."

Introduction to the Gateway® System

Contents

Defining Gateway® technology.

Advantages of Gateway® cloning.

Ways to enter the Gateway® system.

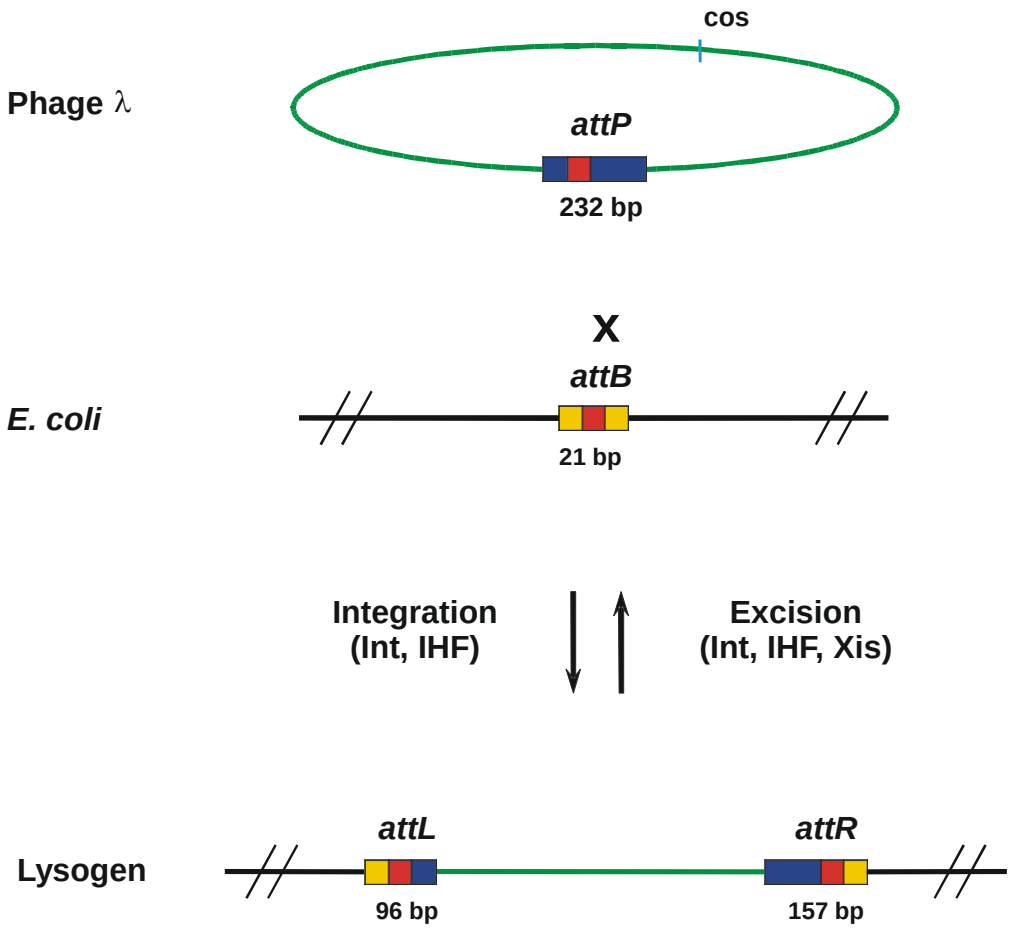
How to obtain a Gateway® expression clone.

The Gateway® Cloning System

- recombinational cloning technique
- based on highly specific integration/excision of λ phage into/out E. coli genome & switch lytic/lysogenic pathways
- developed for cloning multiple variable size DNA fragments in parallel
- major components:
- DNA recombination sequences (*att* sites)
- proteins that mediate the recombination

Reece-Hoyes and WalhoutReece-Hoyes JS, Walhout AJM. Gateway Recombinational Cloning. Cold Spring Harb Protoc. 2018 Jan 2;2018(1)

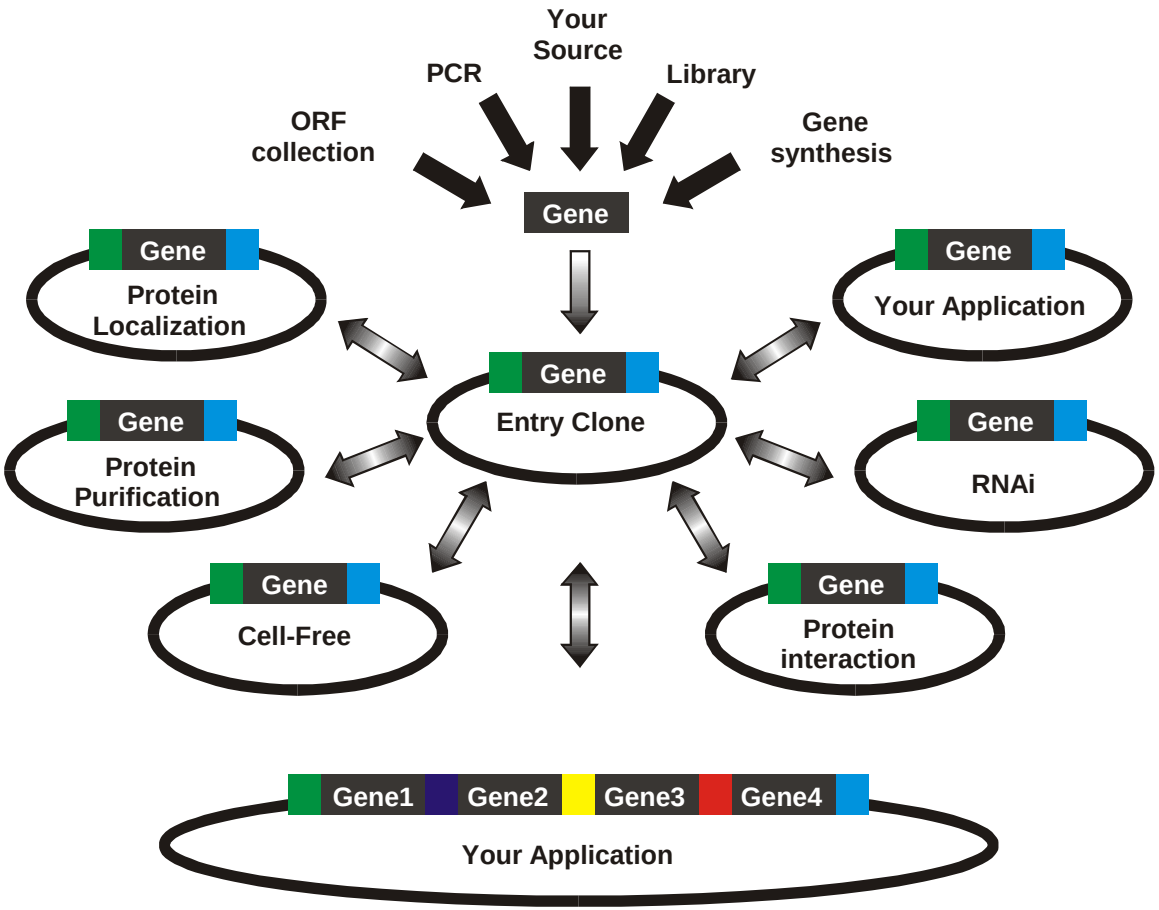
Phage lambda recombination in *E. coli*



The Gateway® System relies on five sets of specific and non cross-reacting *att* sequences

The specificity is given by the 7 nucleotides of the core region

Advantages of Gateway® cloning

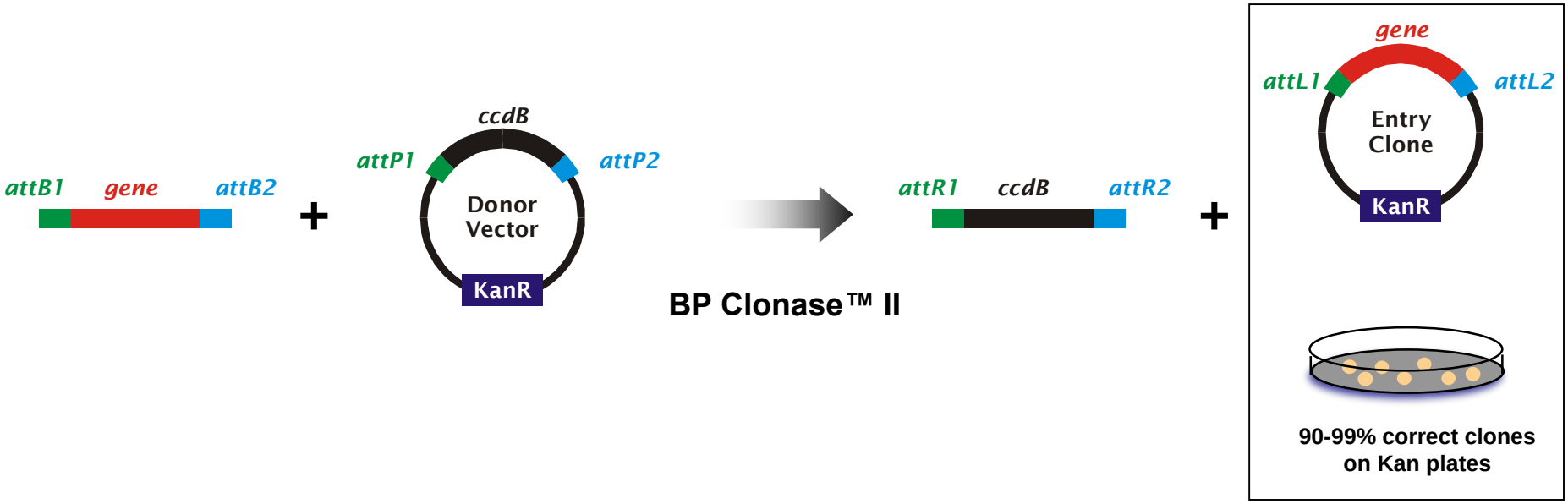


- Directional cloning
- Maintains reading frame
- No restriction enzymes
- No ligation
- 1 hour, room-temperature reaction with >99% efficiency
- No re-sequencing
- Compatible with automation
- Reversible reactions

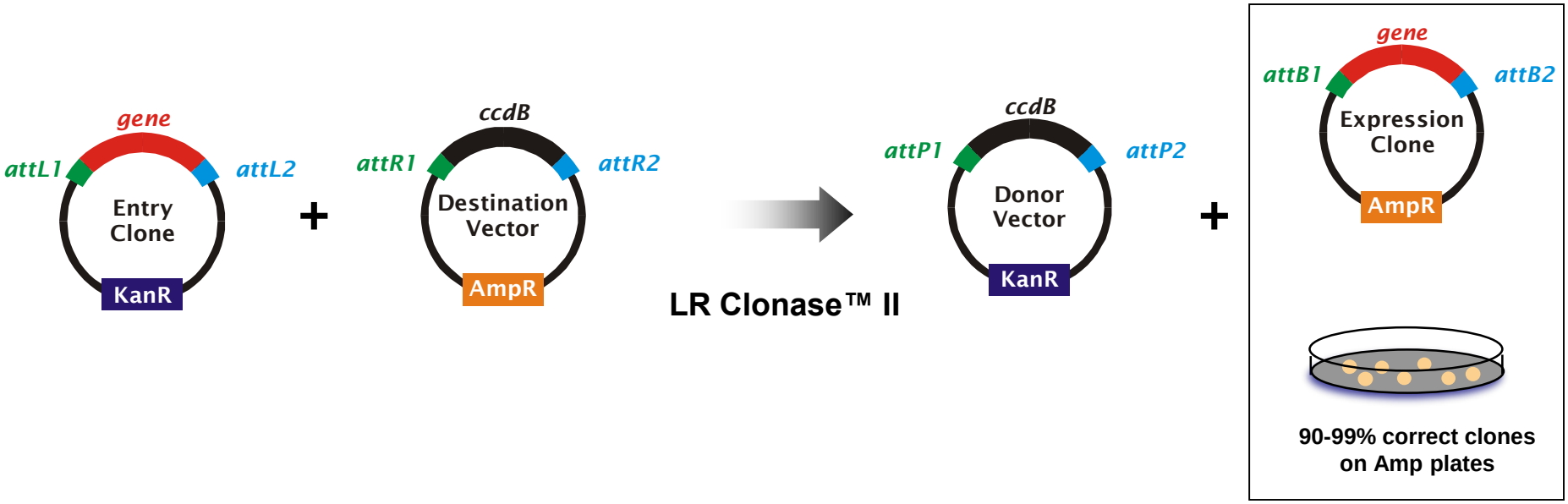
Key Benefits of the Gateway® Technology

- Efficiently and easily shuttle insert DNA from one expression plasmid to another
- Simplify the cloning workflow and save time
- Create expression clones without using restriction enzymes and ligase
- Utilize Ultimate™ ORF clones, a pre-made Gateway® collection
- Simultaneously clone, in a specific order and orientation, up to 4 DNA fragments into one plasmid

Building a Gateway® Entry Clone



Obtaining a Gateway® Expression Clone



Policy for the use and distribution of Gateway® Technology

covered under Limited Use Label License
distribution of Gateway® entry clones as follows:

- **entry clones**, containing *att*L1 and *att*L2 sites, may be **generated by academic and government researchers** for the purpose of scientific research. Such clones may be **distributed for scientific research** by **non-profit organizations and by for-profit organizations** without royalty payment to Invitrogen.

- **expression clones**, containing *att*B1 and *att*B2 sites, may be **generated by academic and government researchers for the purpose of scientific research**. Such clones may **be distributed for scientific research by academic and government organizations** without royalty payment to Invitrogen. Organizations **other** than academic and government may also distribute such Gateway® expression clones **for a nominal fee payable** to Invitrogen.

THANK YOU FOR YOUR ATTENTION

Glossary of terms used in Gateway® cloning

***att* site –** A defined length of DNA that constitutes a recombination site. There are 4 classes of att sites called *attB*, *attP*, *attL*, and *attR*.

***ccdB* gene** – A counterselectable gene that allows for negative selection of unwanted by-product plasmids after recombination.

Donor (pDONR) Vector – A vector with *attP* sites flanking a counterselectable gene that recombines with a gene of interest flanked by *attB* sites.

BP reaction – A recombination event between *attB* and *attP* sites catalyzed by BP Clonase™ II

Entry (pENTR) clone – A vector that contains your gene of interest flanked by *attL* or *attR* sites.

LR reaction – A recombination event between *attL* and *attR* sites catalyzed by LR Clonase™ II

Destination (DEST) Vector – An application-geared vector with *attR* sites flanking a counterselectable gene that will recombine with one or more entry clones.

MultiSite Gateway® Technology – A system that allows simultaneous assembly of multiple DNA fragments into a single destination vector