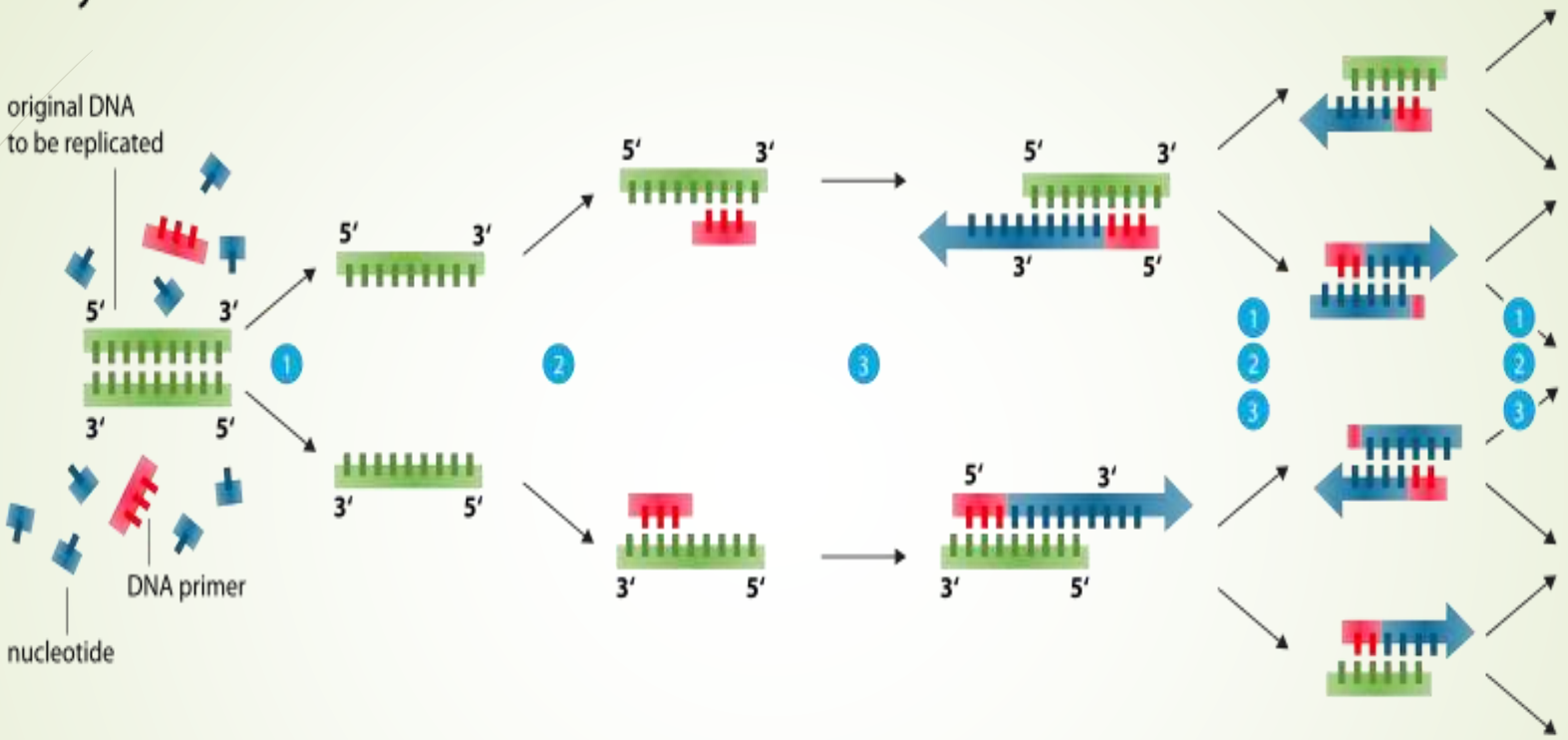
Molecular techniques

Molecular techniques

Polymerase chain reaction

- Technology used in molecular biology to amplify single copy of DNA to millions of copies.
- Method relies on thermal cycling.
- Repeated heating and cooling.
- DNA melting, Anealing, Elongation.
- DNA template, primers, Taq polymerase, dNTPs, buffer, Mg^{2+}

Polymerase chain reaction - PCR

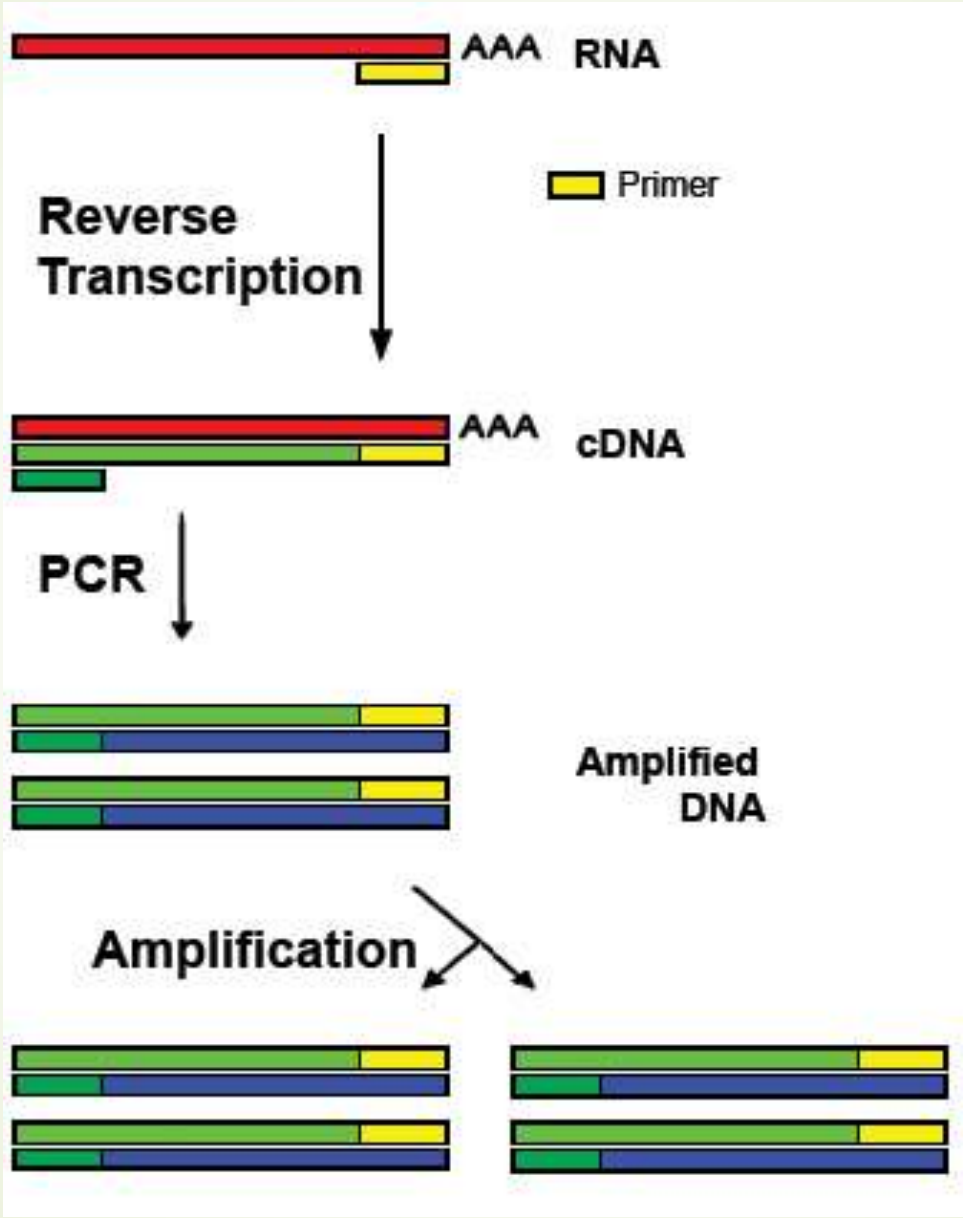


Conventional PCR

- Initiation step – 94-96˚C for 1- 9 min.
- Denaturation step – 94-98˚C for 20-30sec
- Annealing step – 50-65˚C for 20-40 sec
- Extension step - 72˚C for 1min
- Final extension - 72˚C for 5- 15 min

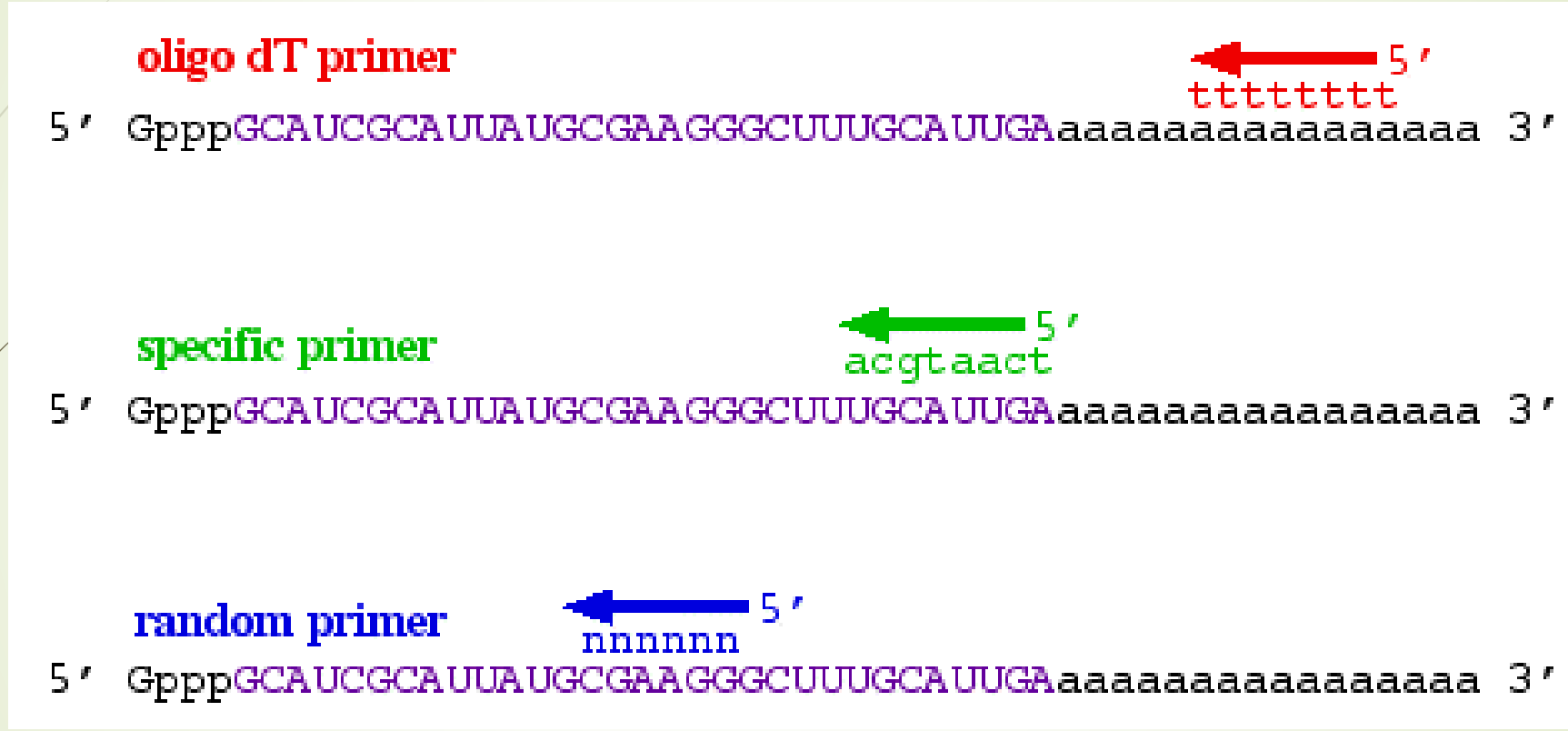
RT-PCR

- Used to deduct RNA expression.
- RNA template first converted to cDNA using reverse transcriptase enzyme.
- cDNA used for further amplification by PCR.
- Often confused with qPCR

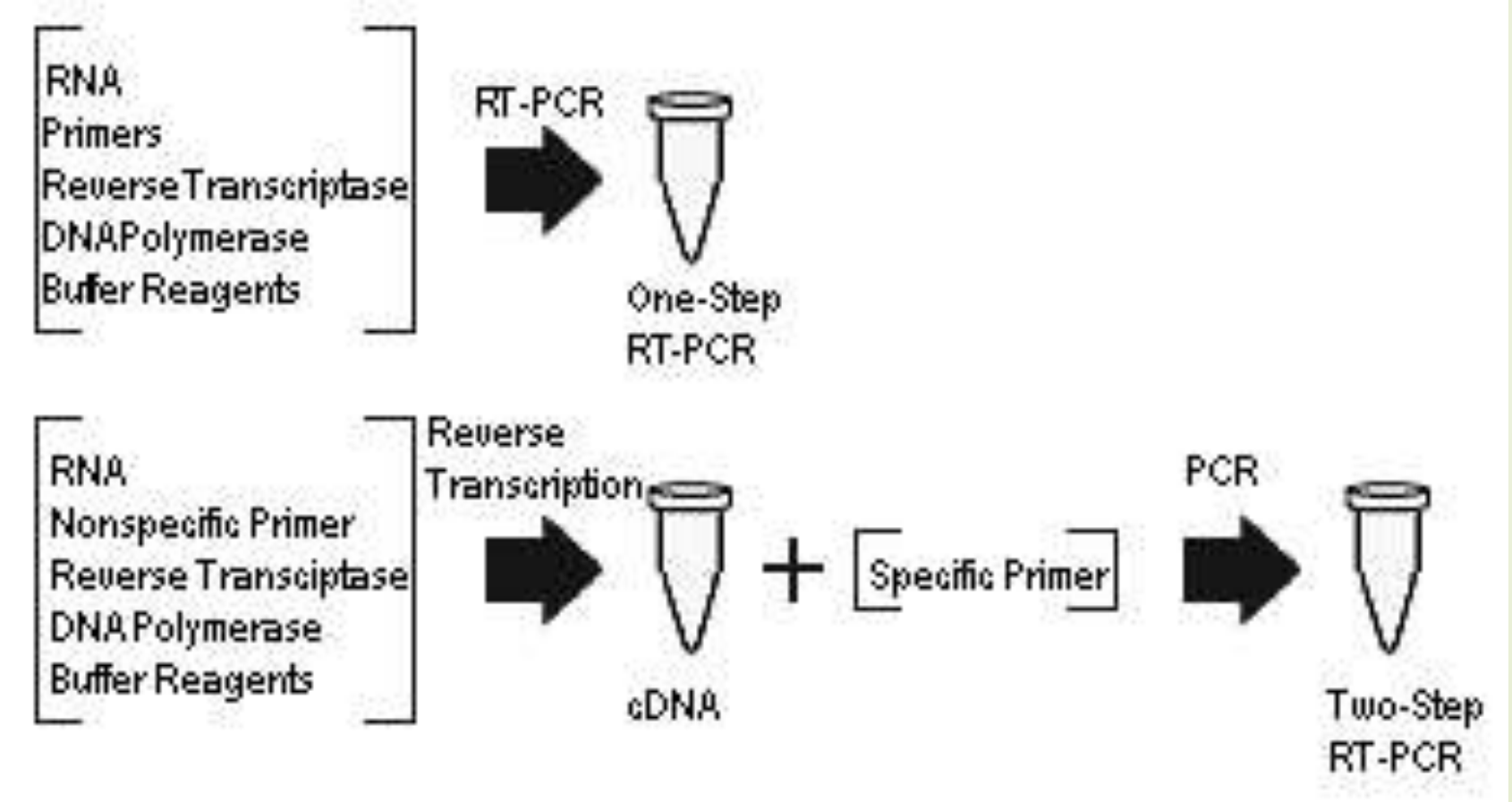

AAA
RNA
Primer
Reverse
Transcription
AAA
cDNA
PCR
Amplified
DNA
Amplification

Primers for cDNA synthesis

- Oligo dT Primer - If the mRNA has a poly-A 3' tail.
- Specific Primer - produce cDNA from a subset of all mRNA.
- Random Primer - produce cDNA from all mRNAs

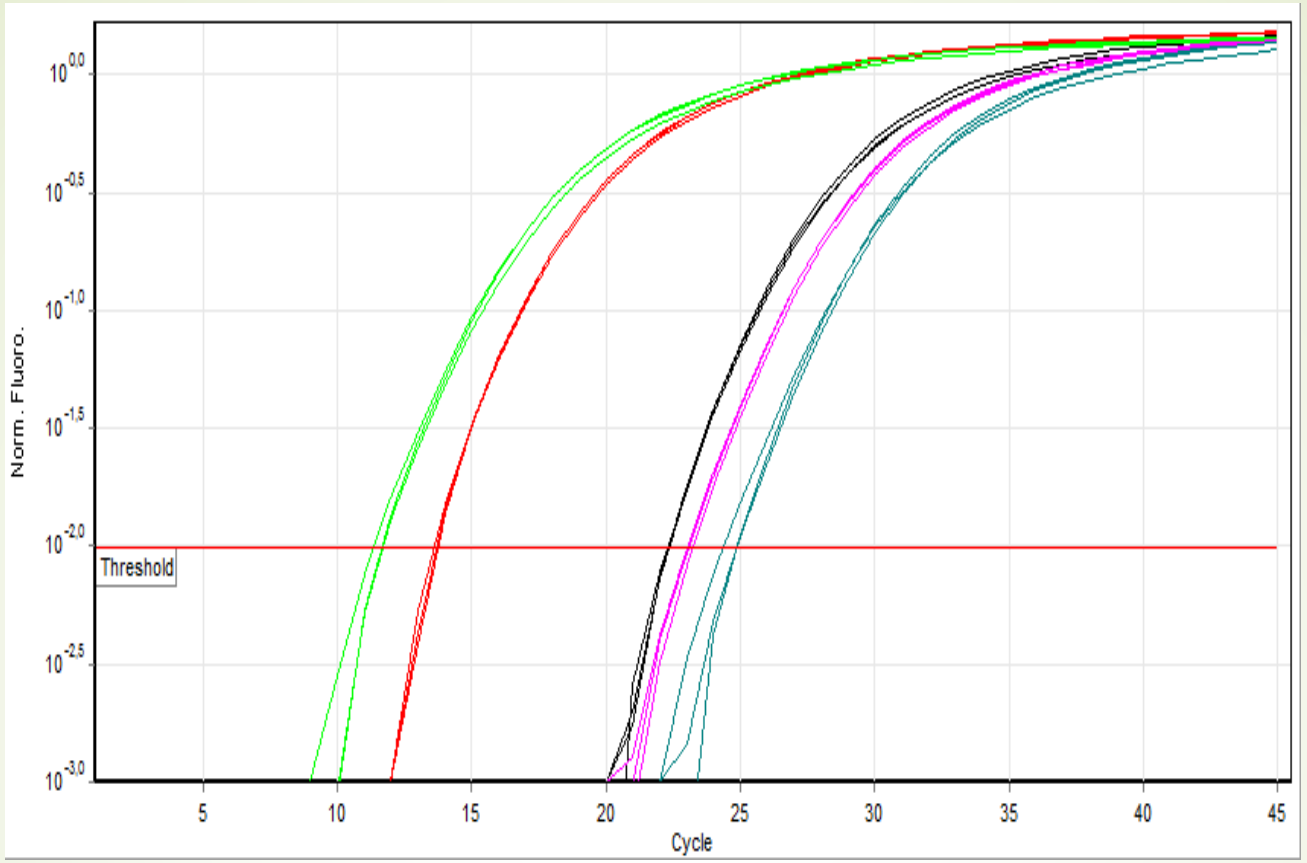

oligo dT primer
5′
tttttttt
5′ GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3′
specific primer
5′
acgtaact
5′ GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3′
random primer
5′
nnnnnn
5′ GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3′

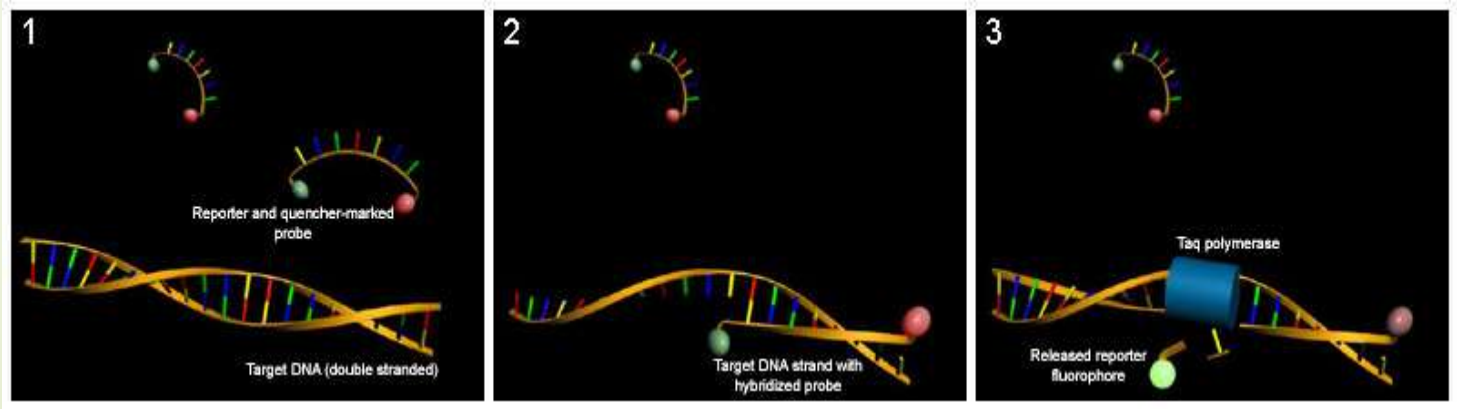
One step and two step assay



Real time PCR

- Amplify and quantify the DNA
- DNA is detected as the reaction progresses in "real time".
- Conventional PCR product of the reaction is detected at its end
- Two methods of deduction
- Non -specific fluorescent dyes that intercalate with any double-stranded DNA
- Sequence -specific DNA probes

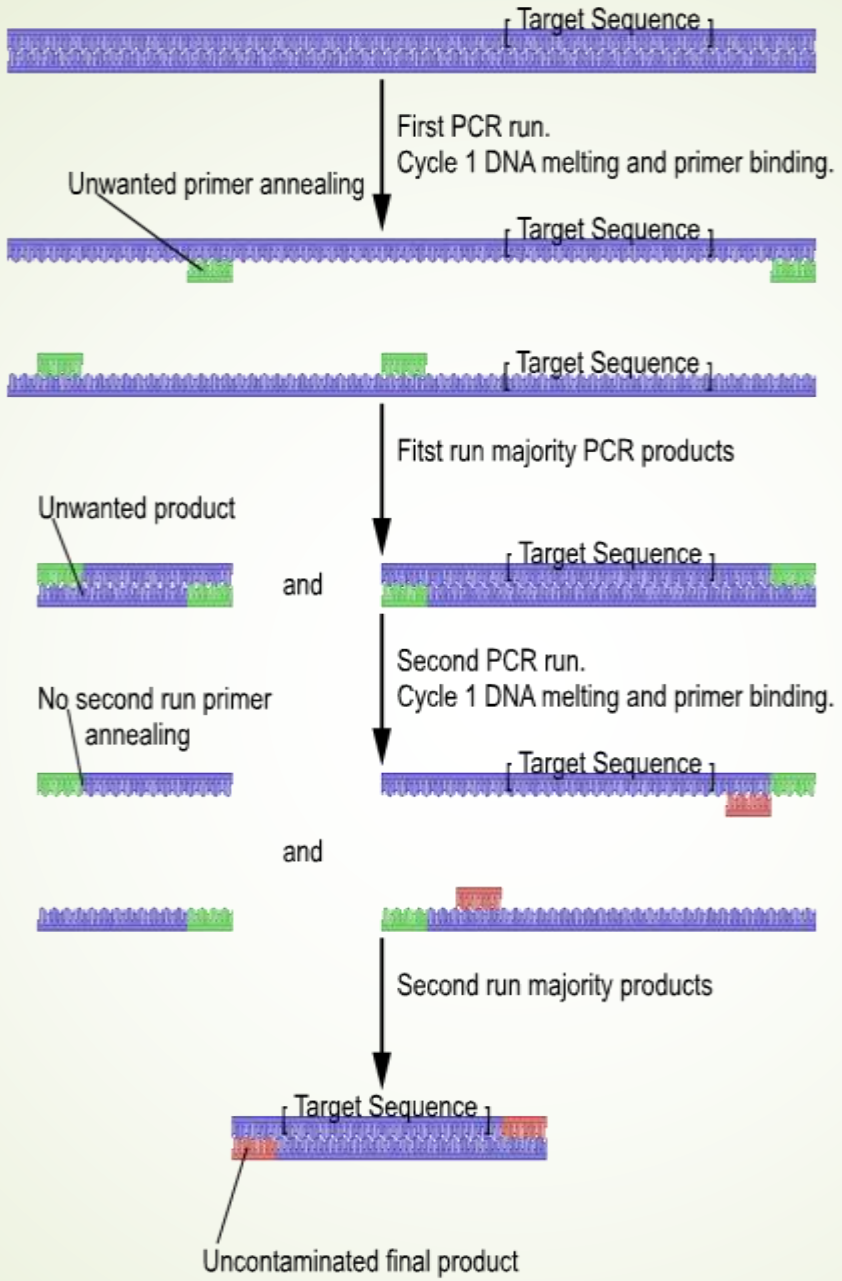

Norm. Fluoro.
Cycle
Threshold
5
10
15
20
25
30
35
40
45


1
Reporter and quencher-marked probe
Target DNA (double stranded)
2
Target DNA strand with hybridized probe
3
Taq polymerase
Released reporter fluorophore

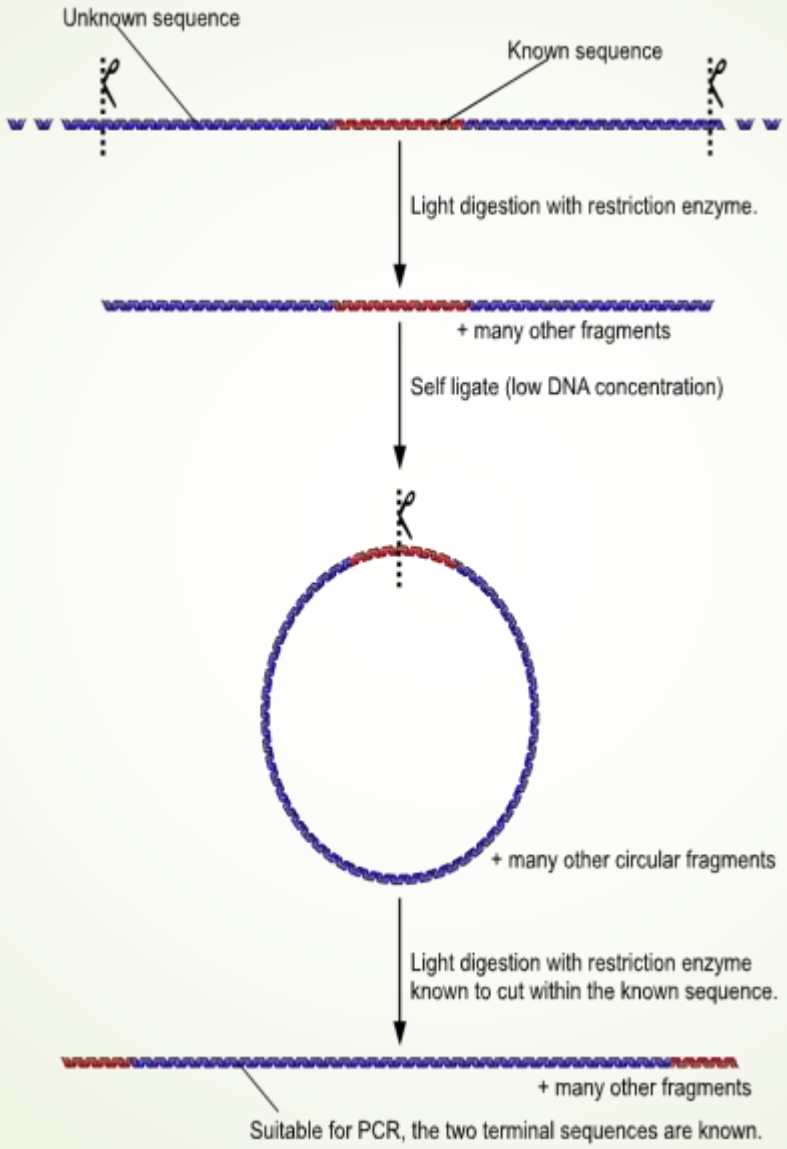
Nested PCR

- Successful in specifically amplifying long DNA products.
- Used to increase the specificity of DNA amplification.
- Two sets of primers are used.
- In the first PCR, one pair of primers is used to generate DNA products.

- Which may contain products amplified from non-target areas.
- Products from the first PCR are then used as template in a second PCR.
- Two different primers whose binding sites are located (nested) within the first set


Target Sequence
First PCR run.
Cycle 1 DNA melting and primer binding.
Unwanted primer annealing
Target Sequence
Target Sequence
Fitst run majority PCR products
Unwanted product
and
Target Sequence
Second PCR run.
Cycle 1 DNA melting and primer binding.
No second run primer annealing
Target Sequence
and
Second run majority products
Target Sequence
Uncontaminated final product

Inverse PCR


Unknown sequence
Known sequence
Light digestion with restriction enzyme.
+ many other fragments
Self ligate (low DNA concentration)
+ many other circular fragments
Light digestion with restriction enzyme
known to cut within the known sequence.
+ many other fragments
Suitable for PCR, the two terminal sequences are known.

Multiplex PCR

- Amplify more than one target simultaneously
- Variation in the PCR product size
- Overlap

Hot start PCR

- Modified form of Polymerase chain reaction
- Avoids a non-specific amplification of DNA by inactivating the taq polymerase at lower temperature.
- dsDNA is denatured by heating the sample at higher temperature
- Temperature is suddenly reduced to 55℃
- At this stage primer and Taq-polymerase were added
- Specific antibodies are used to block this Taq-polymerase at annealing temperature

- when the temperature raises for amplification to 72℃.
- Antibody detaches from Taq-polymerase and the amplification with greater specificity starts.
- In conventional PCR, the Taq DNA polymerase is active at room temperature and even on ice.
- when all the reaction components are put together, nonspecific primer annealing can occur due to these low temperatures.
- This nonspecific annealed primer can then be extended by the Taq DNA polymerase, generating nonspecific products and lowering product yields.

ARMS PCR

- Amplification refractory mutation system
- Specific priming.
- In this method a primer complementary to specific mutation with common primer .
- Another normal primer with common primer is used.
- PCR product in first method shows presence of mutation.
- No PCR product in the second method shows the presence of mutation.

Insitu hybridization PCR

- localized expression of particular gene in cell or tissues

Applications

- Cystic fibrosis and beta thalassemia

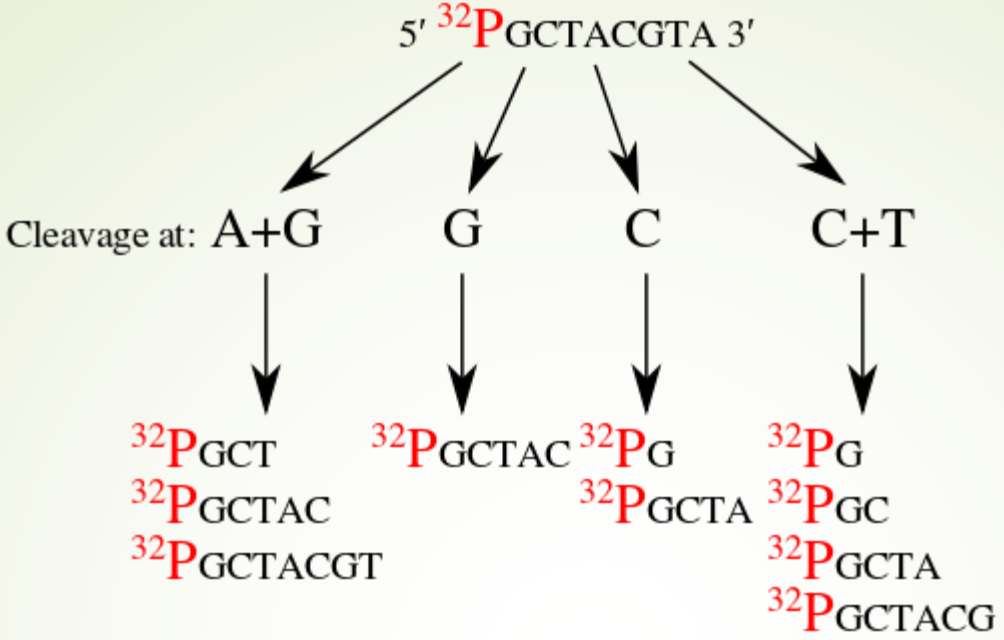
Sequencing methods

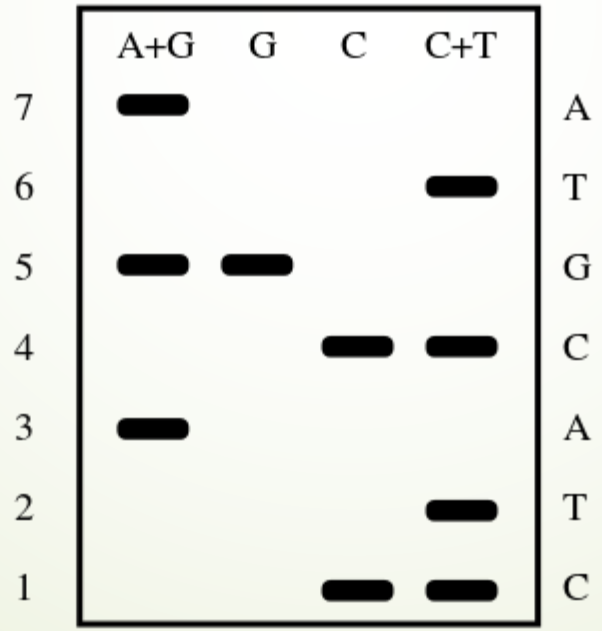
- Sangers method (chain termination method

Maxam-Gilbert's method

- Based on nucleobase-specific partial chemical modification of DNA.
- Subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides.
- Radioactive labeling at one 5′ end of the DNA fragment using gamma-^{32}P ATP.
- Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T).
- The purines (A+G) are depurinated using formic acid

- The guanines (some extent the adenines) are methylated by dimethyl sulfate.
- The pyrimidines (C+T) are hydrolysed using hydrazine.
- The addition of salt (sodium chloride) to the hydrazine reaction inhibits the reaction of thymine for the C-only reaction.
- Modified DNAs may then be cleaved by hot piperidine
- A series of labeled fragments is generated first "cut" site in each
- Electrophoresed in acrylamide gels for size separation



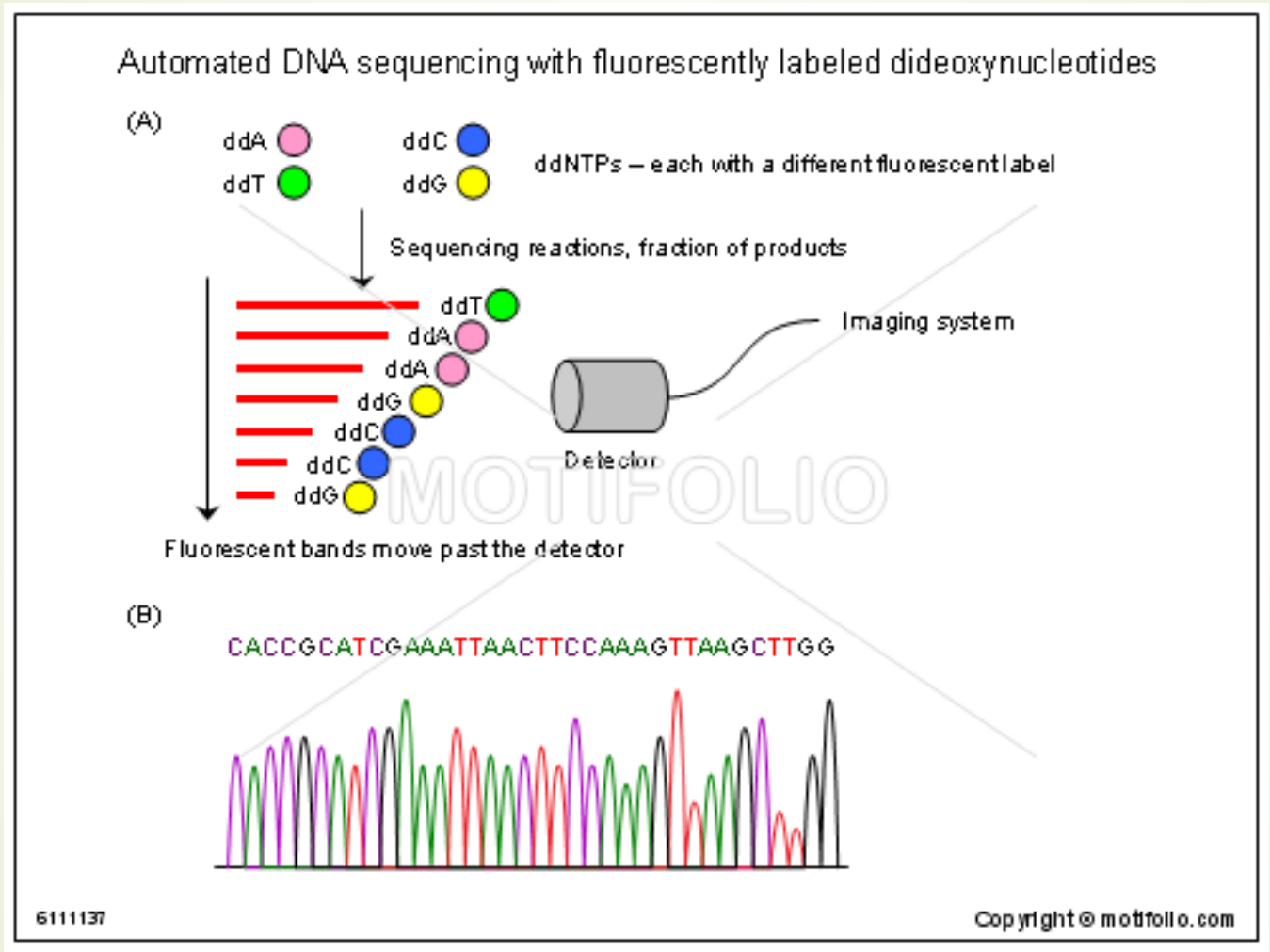


Sequencing Gel

Automated sequencing

- Automated DNA sequencing utilizes a fluorescent dye to label the nucleotides instead of a radioactive isotope.

- A laser is used to stimulate the fluorescent dye


Automated DNA sequencing with fluorescently labeled dideoxynucleotides
(A)
ddA
ddC
ddT
ddG
ddNTPs – each with a different fluorescent label
Sequencing reactions, fraction of products
ddT
ddA
ddA
ddG
ddC
ddC
ddG
Imaging system
Detector
Fluorescent bands move past the detector
(B)
CACCGCATCGAAATTAACTTCCAAAGTTAAGCTTGG
6111137
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Pyrosequencing

- Method of DNA sequencing based on the 'sequencing by synthesis' principle.

Step 1

A sequencing primer is hybridized to a single stranded, PCR amplified, DNA template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5´ phosphosulfate (APS) and luciferin.

Step 2

The first of four deoxyribonucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribonucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.

Step 3

ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate (APS). This ATP drives the luciferase mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a Pyrogram™. The height of each peak (light signal) is proportional to the number of nucleotides incorporated.

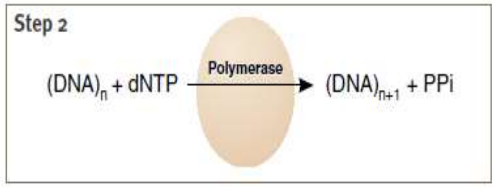
Step 4

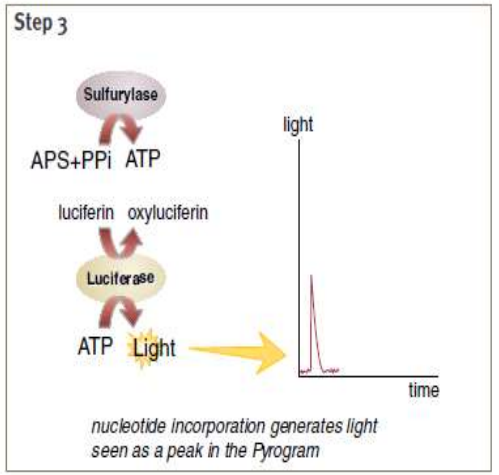
Apyrase, a nucleotide degrading enzyme, continuously degrades ATP and unincorporated dNTPs. This switches off the light and regenerates the reaction solution. The next dNTP is then added.

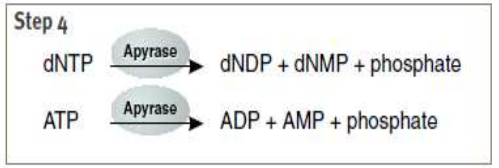
Step 5

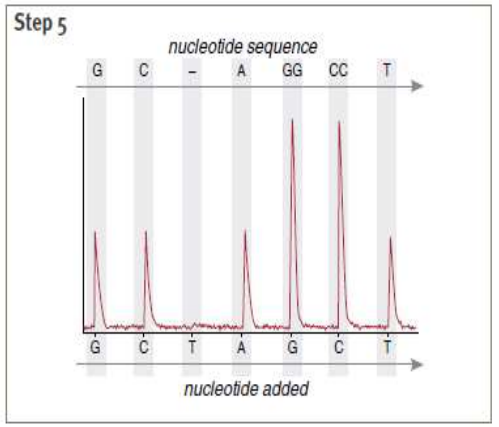
Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase.

As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram.









DNA finger printing

- Obtaining a sample of cells, such as skin, hair, or blood cells, which contain DNA.
- DNA is extracted from the cells and purified.
- DNA was then cut at specific points along the strand with proteins known as restriction enzymes.
- Were sorted by placing them on a gel.
- Sorted double-stranded DNA fragments were then subjected to a blotting

- Split into single strands and transferred to a nylon sheet.
- Autoradiography in which they were exposed to DNA probes—pieces of synthetic DNA that were made radioactive .
- Piece of X-ray film was then exposed to the fragments, and a dark mark was produced at any point where a radioactive probe had become attached


Crime scene
Suspect 1
Suspect 2
Suspect 3