Core course
BMS361N
Genetic Engineering

Polymerase chain reaction

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The polymerase chain reaction

- PCR
- cDNA Cloning
- Reverse transcriptase PCR(RT-PCR)
- Rapid Amplification of cDNA Ends(RACE)

Polymerase Chain Reaction (PCR)

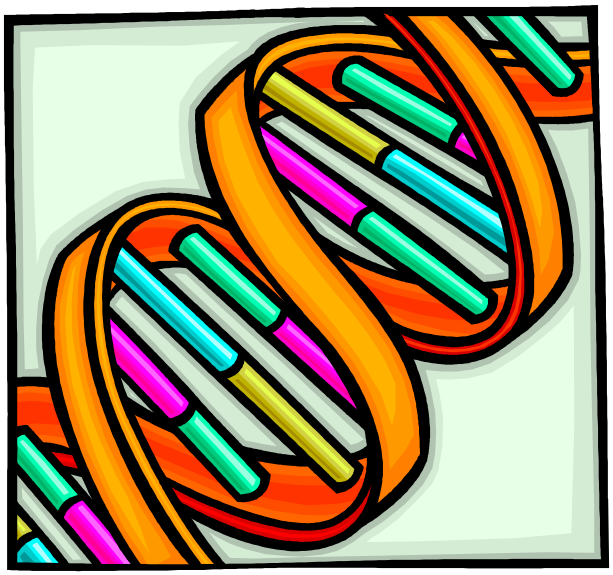
- PCR is a means to amplify a particular piece of DNA
    - *Amplify= making numerous copies of a segment of DNA*
- PCR can make ***billions*** of copies of a target sequence of DNA in a few hours
- PCR was invented in the 1984 as a way to make numerous copies of DNA fragments in the laboratory
- Its applications are vast and PCR is now an integral part of Molecular Biology

DNA Replication vs. PCR

- PCR is a laboratory version of DNA Replication in cells
    - The laboratory version is commonly called "*in vitro*" since it occurs in a test tube while "*in vivo*" signifies occurring in a living cell.

DNA Replication in Cells (*in vivo*)

- DNA replication is the copying of DNA
- It typically takes a cell just a few hours to copy all of its DNA
- DNA replication is semi-conservative (i.e. one strand of the DNA is used as the template for the growth of a new DNA strand)
- This process occurs with very few errors (on average there is one error per 1 billion nucleotides copied)
- More than a dozen enzymes and proteins participate in DNA replication



Key enzymes involved in DNA Replication

- DNA Polymerase
- DNA Ligase
- Primase
- Helicase
- Topoisomerase
- Single strand binding protein

DNA Replication enzymes: DNA Polyerase

- catalyzes the elongation of DNA by adding nucleoside triphosphates to the 3’ end of the growing strand
    - A nucleotide triphosphate is a 1 sugar + 1 base + 3 phosphates
    - When a nucleoside triphosphate joins the DNA strand, two phosphates are removed.
- DNA polymerase can *only* add nucleotides to 3’ end of growing strand

Complementary Base-Pairing in DNA

- DNA is a double helix, made up of nucleotides, with a sugar-phosphate backbone on the outside of the helix.
    - *Note: a nucleotide is a sugar + phosphate + nitrogenous base*
- The two strands of DNA are held together by pairs of nitrogenous bases that are attached to each other via hydrogen bonds.
    - The nitrogenous base adenine will only pair with thymine
    - The nitrogenous base guanine will only pair with cytosine
- During replication, once the DNA strands are separated, DNA polymerase uses each strand as a template to synthesize new strands of DNA with the precise, complementary order of nucleotides.

DNA Replication enzymes: DNA Ligase

- The two strands of DNA in a double helix are **antiparallel** (i.e. they are oriented in opposite directions with one strand oriented from 5’ to 3’ and the other strand oriented from 3’ to 5’
    - *5’ and 3’ refer to the numbers assigned to the carbons in the 5 carbon sugar*
- Given the antiparallel nature of DNA and the fact that DNA ploymerases can only add nucleotides to the 3’ end, one strand (referred to as the **leading strand**) of DNA is synthesized continuously and the other strand (referred to as the **lagging strand**) in synthesized in fragments (called **Okazaki fragments**) that are joined together by **DNA ligase.**

DNA Replication enzymes: Primase

- DNA Polymerase *cannot* initiate the synthesis of DNA
    - Remember that DNA polymerase can *only* add nucleotides to 3' end of an already existing strand of DNA
- **In humans, primase** is the enzyme that can start an RNA chain from scratch and it creates a **primer (a short stretch RNA with an available 3' end)** that DNA polymerase can add nucleotides to during replication.

    Note that the RNA primer is subsequently replaced with DNA

DNA Replication enzymes:

Helicase, Topoisomerase and Single-strand binding protein

- **Helicase** untwists the two parallel DNA strands
- **Topoisomerase** relieves the stress of this twisting
- **Single-strand binding protein** binds to and stabilizes the unpaired DNA strands

PCR: the *in vitro* version of DNA Replication

The following components are needed to perform PCR in the laboratory:

1) DNA (your DNA of interest that contains the target sequence you wish to copy)
2) A heat-stable DNA Polymerase (like Taq Polymerase)
3) All four nucleotide triphosphates
4) Buffers
5) Two short, single-stranded DNA molecules that serve as primers
6) Thin walled tubes
7) Thermal cycler (a device that can change temperatures dramatically in a very short period of time)

PCR

The DNA, DNA polymerase, buffer, nucleoside triphosphates, and primers are placed in a thin-walled tube and then these tubes are placed in the PCR thermal cycler

PCR Thermocycler

The three main steps of PCR

- The basis of PCR is temperature changes and the effect that these temperature changes have on the DNA.
- In a PCR reaction, the following series of steps is repeated 20-40 times

  (note: 25 cycles usually takes about 2 hours and amplifies the DNA fragment of interest 100,000 fold)

Step 1: **Denature** DNA

At 95°C, the DNA is denatured (i.e. the two strands are separated)

Step 2: Primers **Anneal**

At 40°C- 65°C, the primers anneal (or bind to) their complementary sequences on the single strands of DNA

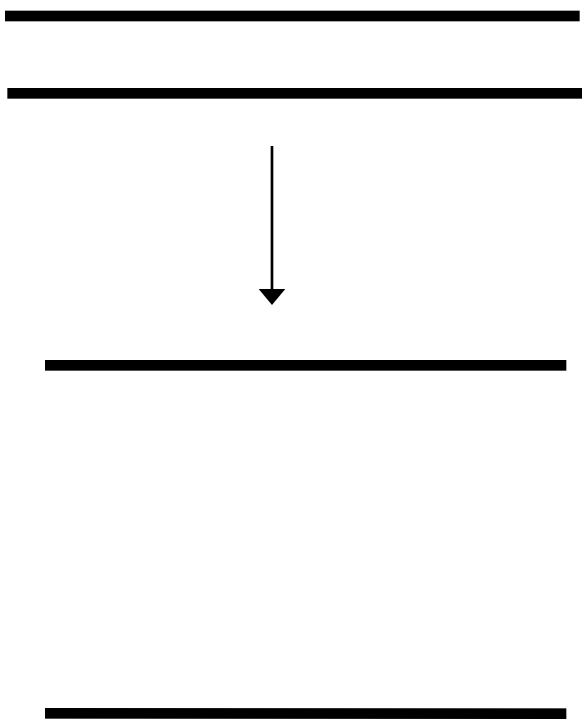
Step 3: DNA polymerase **Extends** the DNA chain

At 72°C, DNA Polymerase extends the DNA chain by adding nucleotides to the 3' ends of the primers.

Heat-stable DNA Polymerase

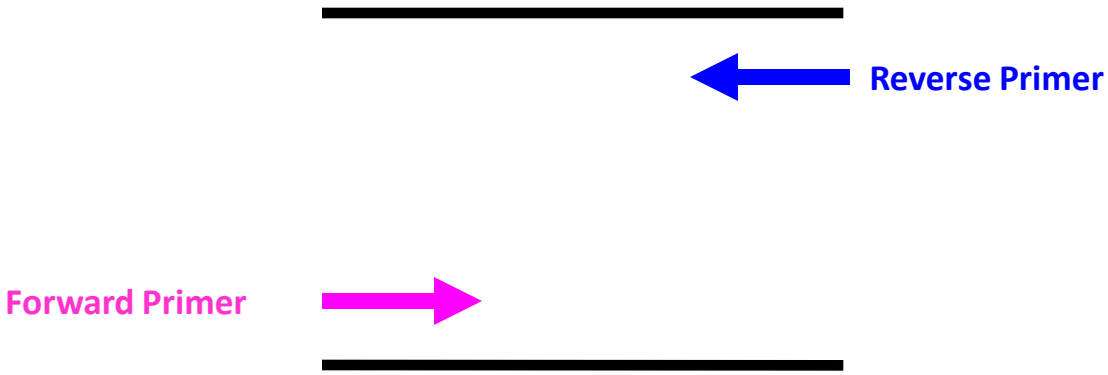
- Given that PCR involves very high temperatures, it is imperative that a heat-stable DNA polymerase be used in the reaction.
    - Most DNA polymerases would denature (and thus not function properly) at the high temperatures of PCR.
- Taq DNA polymerase was purified from the hot springs bacterium *Thermus aquaticus* in 1976
- Taq has maximal enzymatic activity at 75 °C to 80 °C, and substantially reduced activities at lower temperatures.

Denaturation of DNA



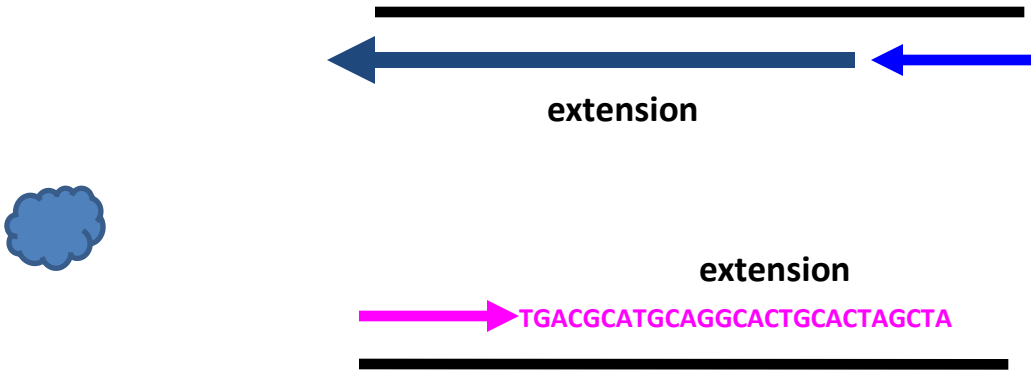
This occurs at 95 ºC mimicking the function of helicase in the cell.

Step 2 Annealing or Primers Binding



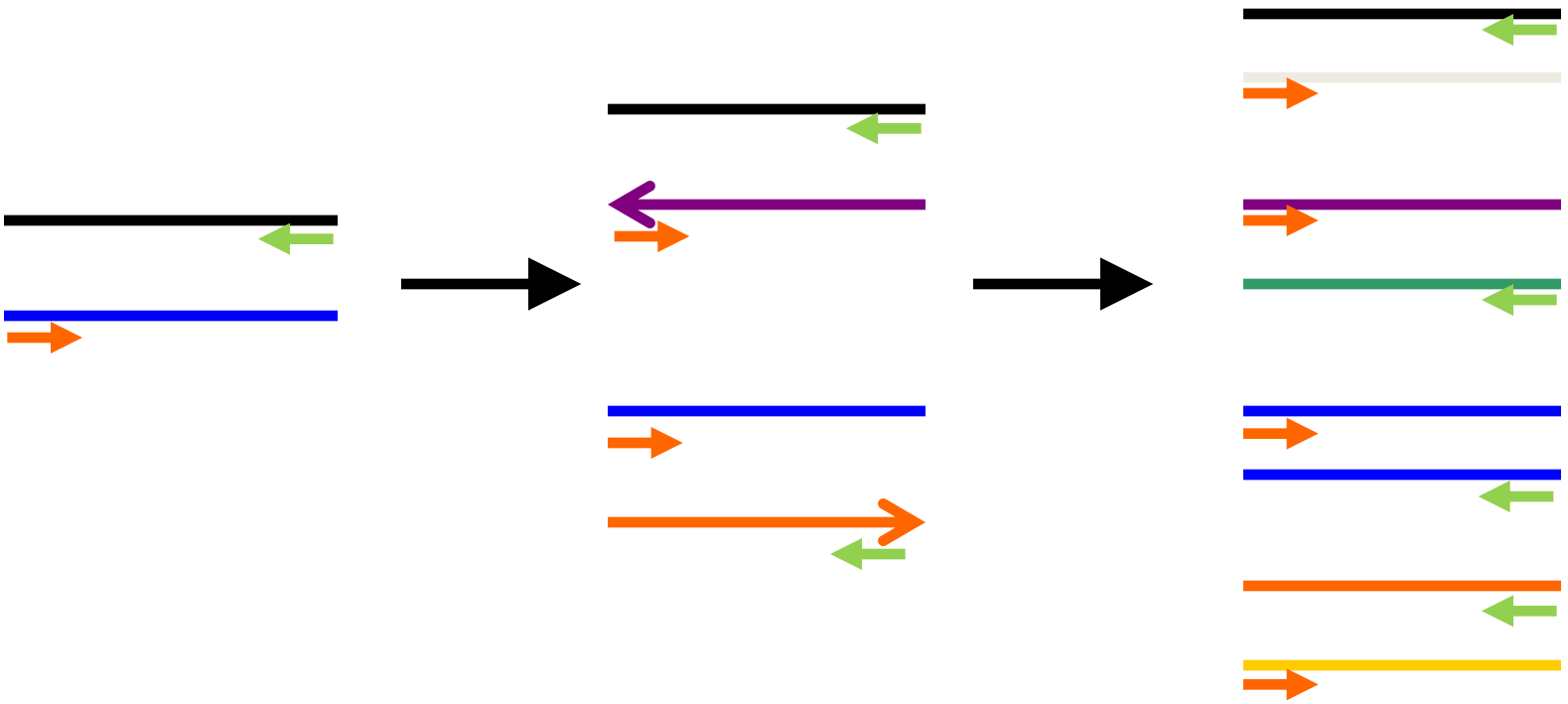
Primers bind to the complimentary sequence on the target DNA. Primers are chosen such that one is complimentary to the one strand at one end of the target sequence and that the *other* is complimentary to the *other* strand at the other end of the target sequence.

Step 3 Extension or Primer Extension



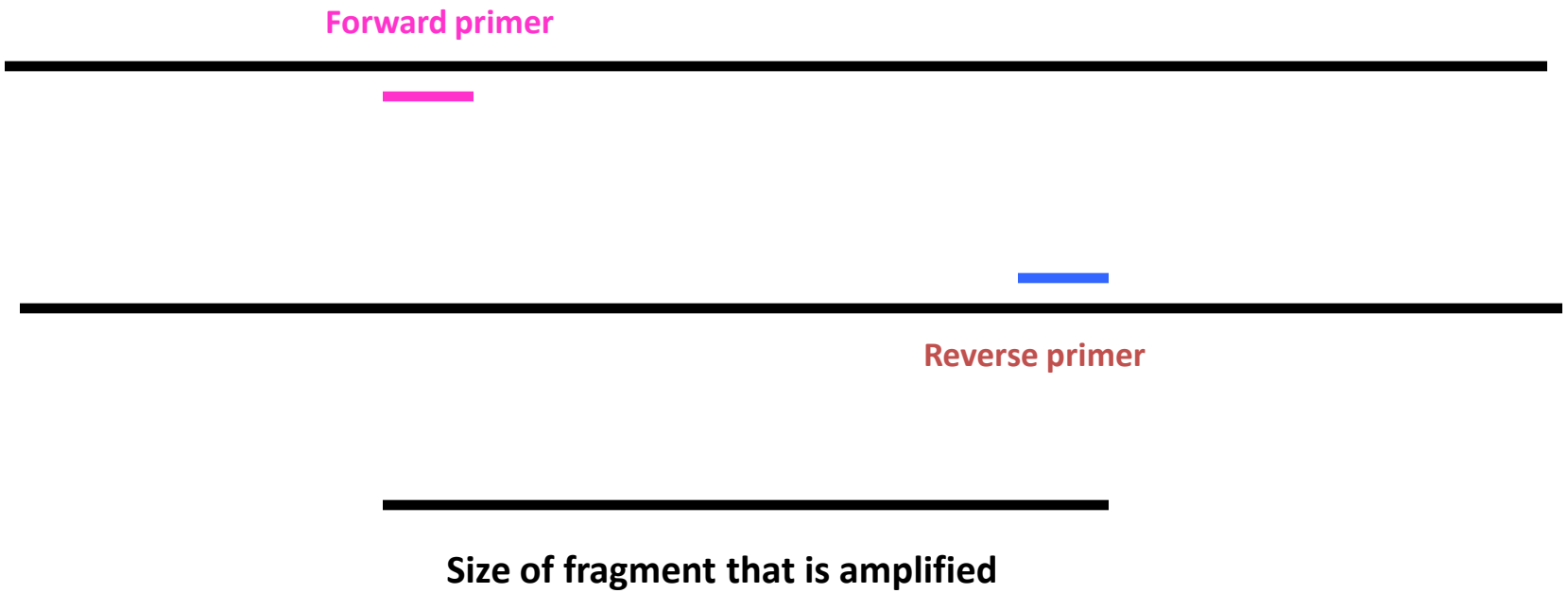
DNA polymerase catalyzes the extension of the strand in the 5-3 direction, starting at the primers, attaching the appropriate nucleotide (A-T, C-G)

- The next cycle will begin by denaturing the new DNA strands formed in the previous cycle



The Size of the DNA Fragment Produced in PCR is Dependent on the Primers

- The PCR reaction will amplify the DNA section between the two primers.
- If the DNA sequence is known, primers can be developed to amplify any piece of an organism's DNA.



The DNA of interest is amplified by a power of 2 for each PCR cycle

For example, if you subject your DNA of interest to 5 cycles of PCR, you will end up with 2^5 (or 64) copies of DNA.

Similarly, if you subject your DNA of interest to 40 cycles of PCR, you will end up with 2^{40} (or) copies of DNA!

PCR has become a very powerful tool in molecular biology

- One can start with a single sperm cell or stand of hair and amplify the DNA sufficiently to allow for DNA analysis and a distinctive band on an agarose gel.
- One can amplify fragments of interest in an organism's DNA by choosing the right primers.
- One can use the selectivity of the primers to identify the likelihood of an individual carrying a particular allele of a gene.

More about Primers

- PCR primers are short, single stranded DNA molecules (15-40 bp)
- They are manufactured commercially and can be ordered to match any DNA sequence
- Primers are sequence specific, they will bind to a particular sequence in a genome
- As you design primers with a longer length (15 → 40 bp), the primers become more selective.
- DNA polymerase requires primers to initiate replication

Selectivity of Primers

- Primers bind to their complementary sequence on the target DNA
  - A primer composed of only 3 letter, ACC, for example, would be very likely to encounter its complement in a genome.
  - As the size of the primer is increased, the likelihood of, for example, a primer sequence of 35 base letters repeatedly encountering a perfect complementary section on the target DNA become remote.

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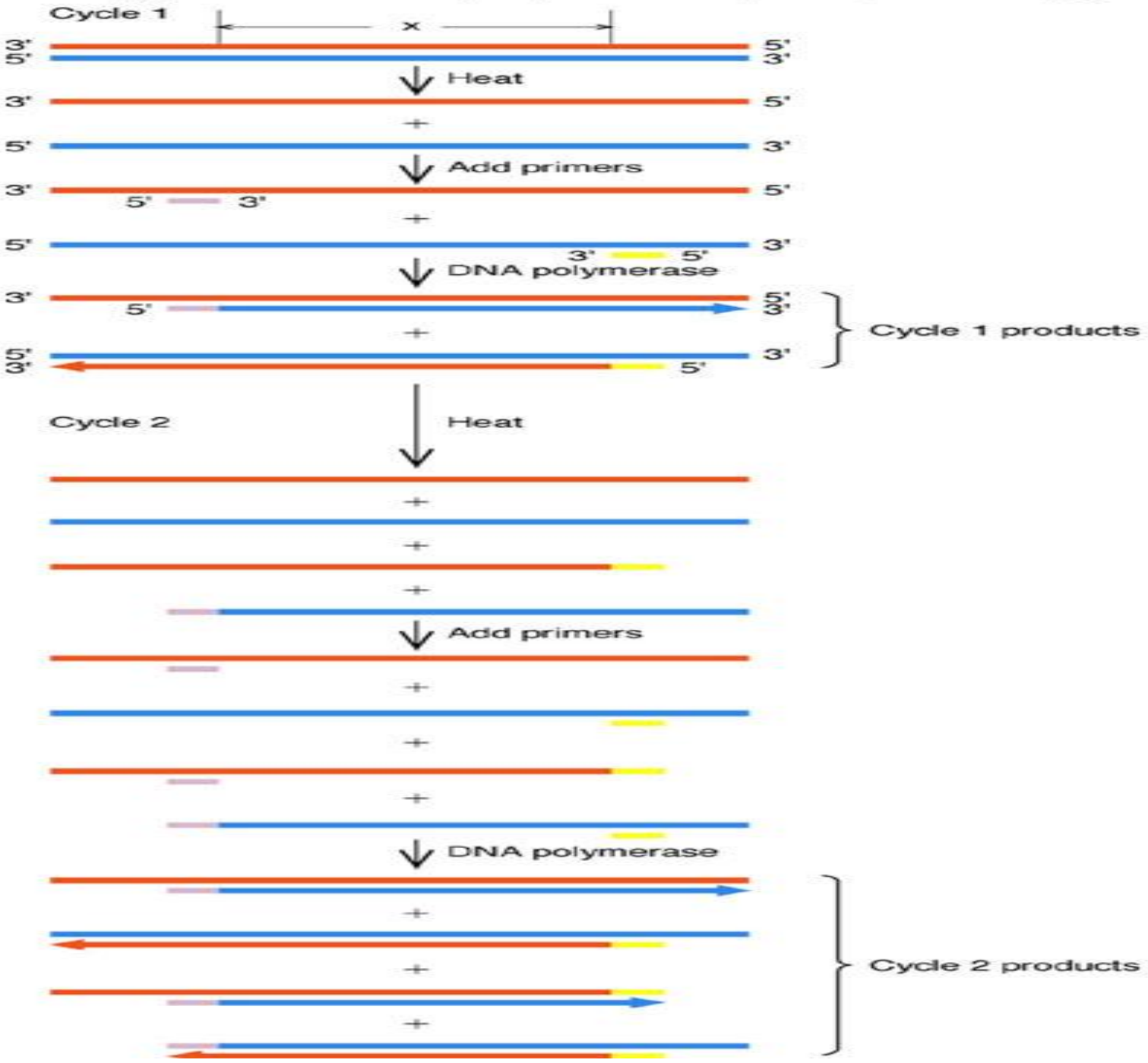
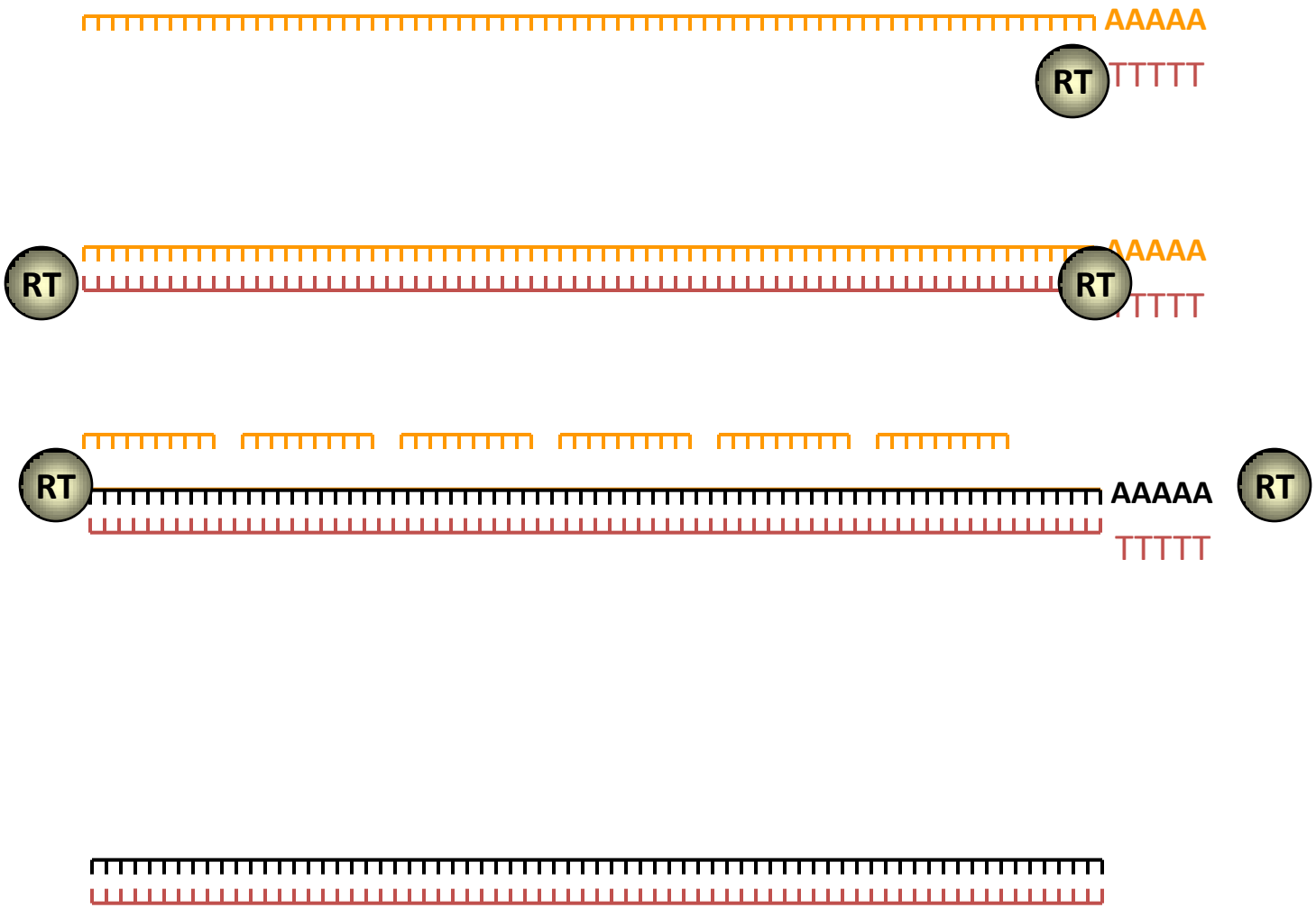




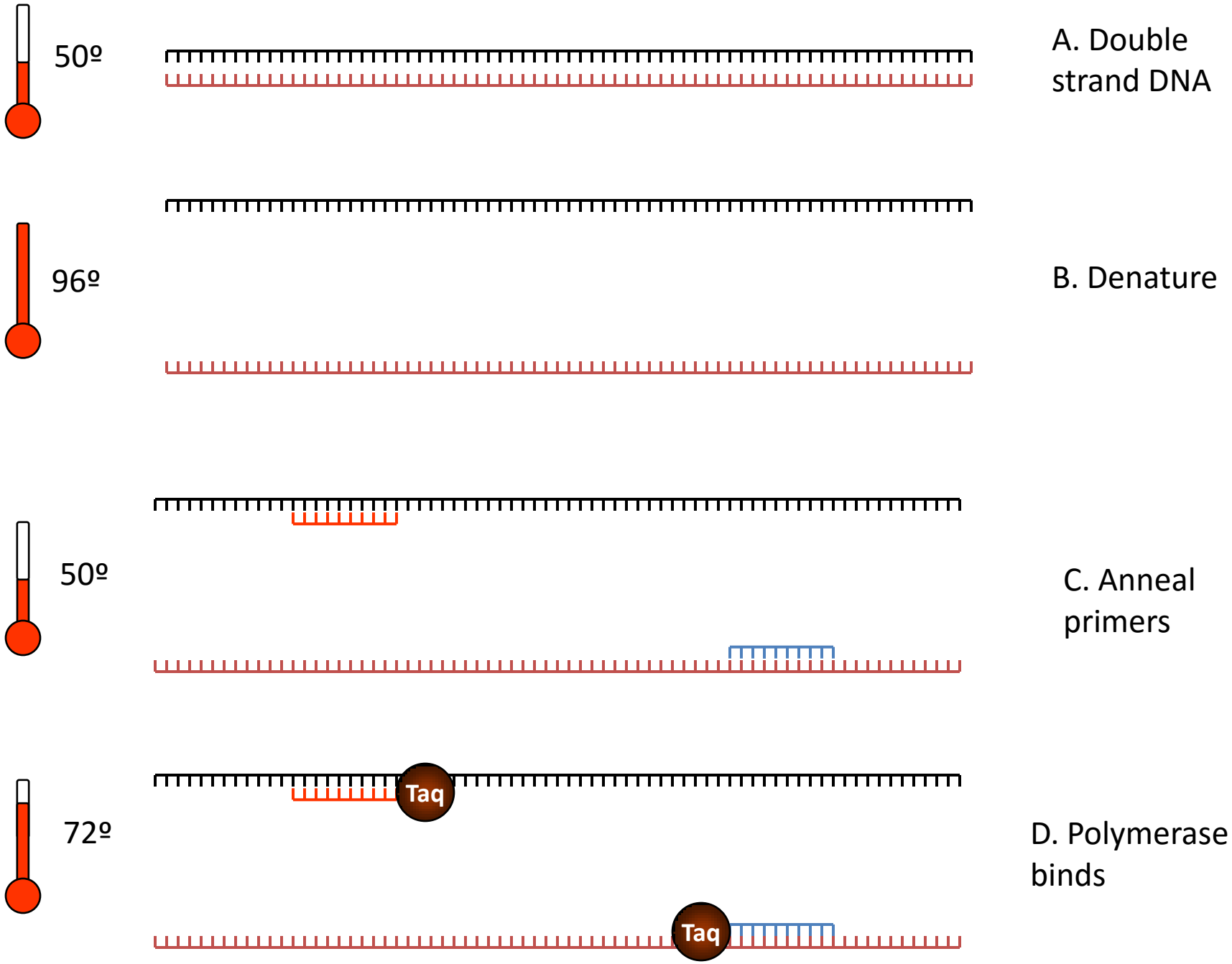
Oligo dT primer is bound to mRNA

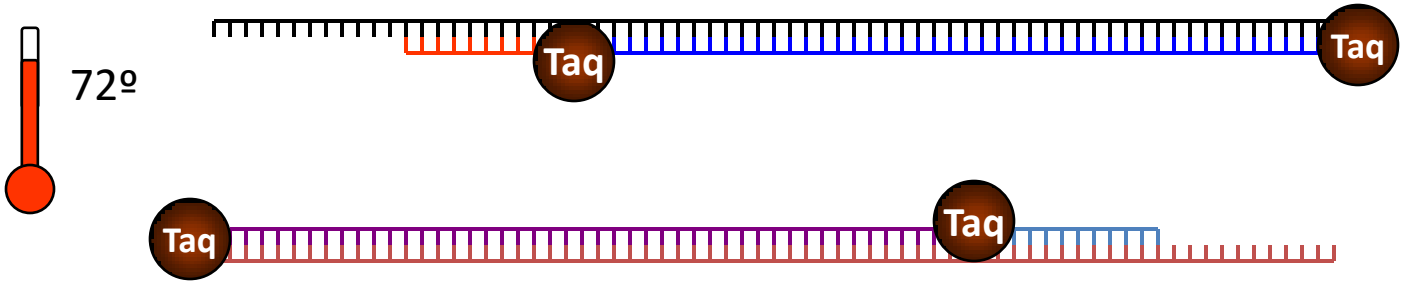
Reverse transcriptase (RT) copies first cDNA strand

Reverse transcriptase digests and displaces mRNA and copies second strand of cDNA

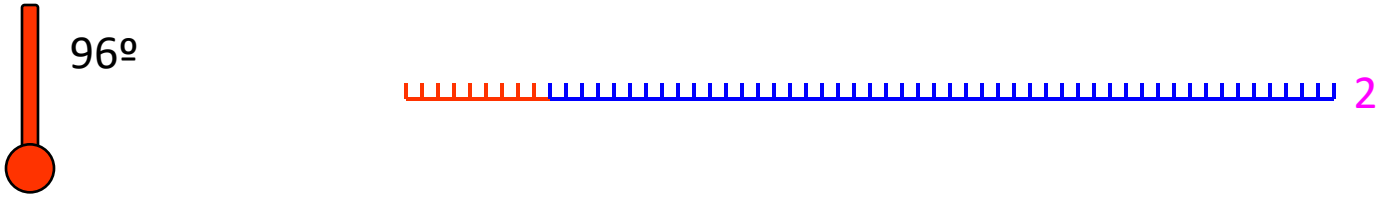
Double strand cDNA

Conversion of mRNA to cDNA by Reverse Transcription

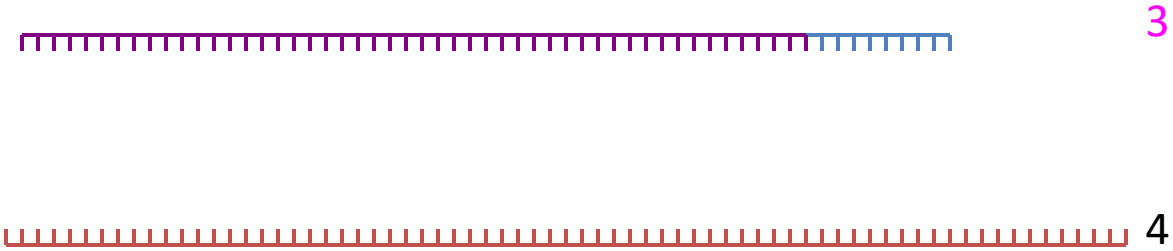

50º
A. Double strand DNA
96º
B. Denature
50º
C. Anneal primers
Taq
72º
D. Polymerase binds
Taq



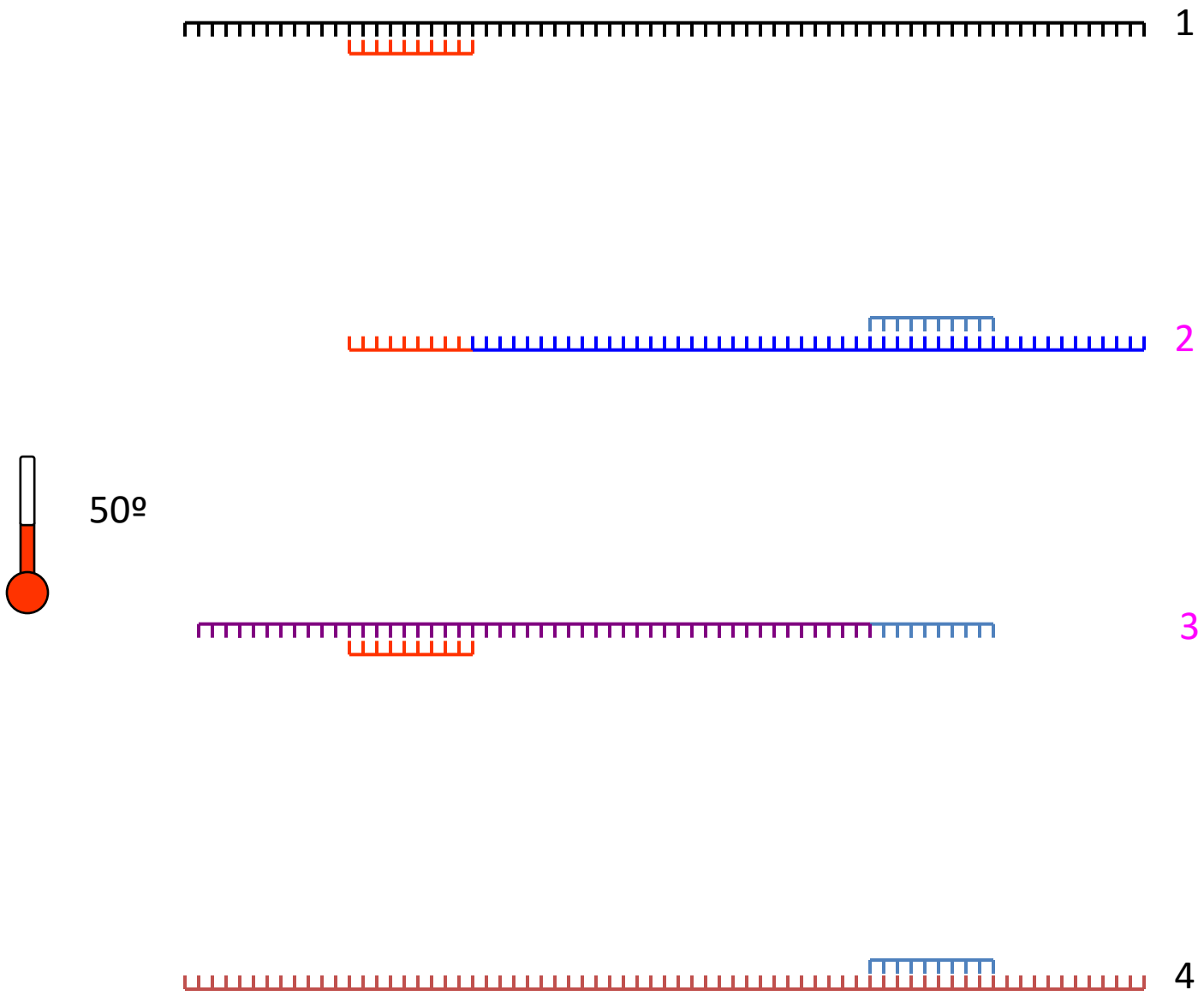
E. Copy strands



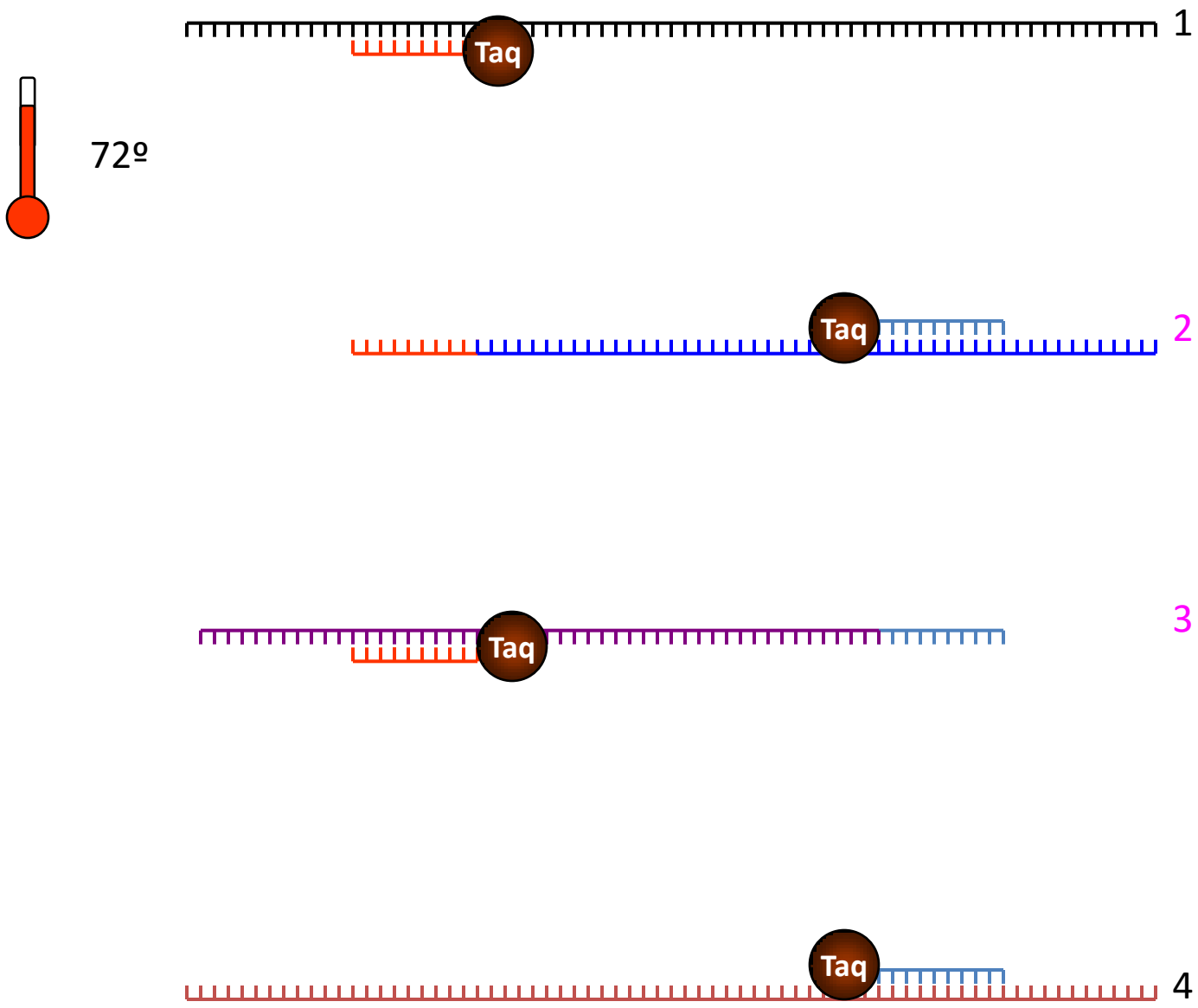
F. Denature

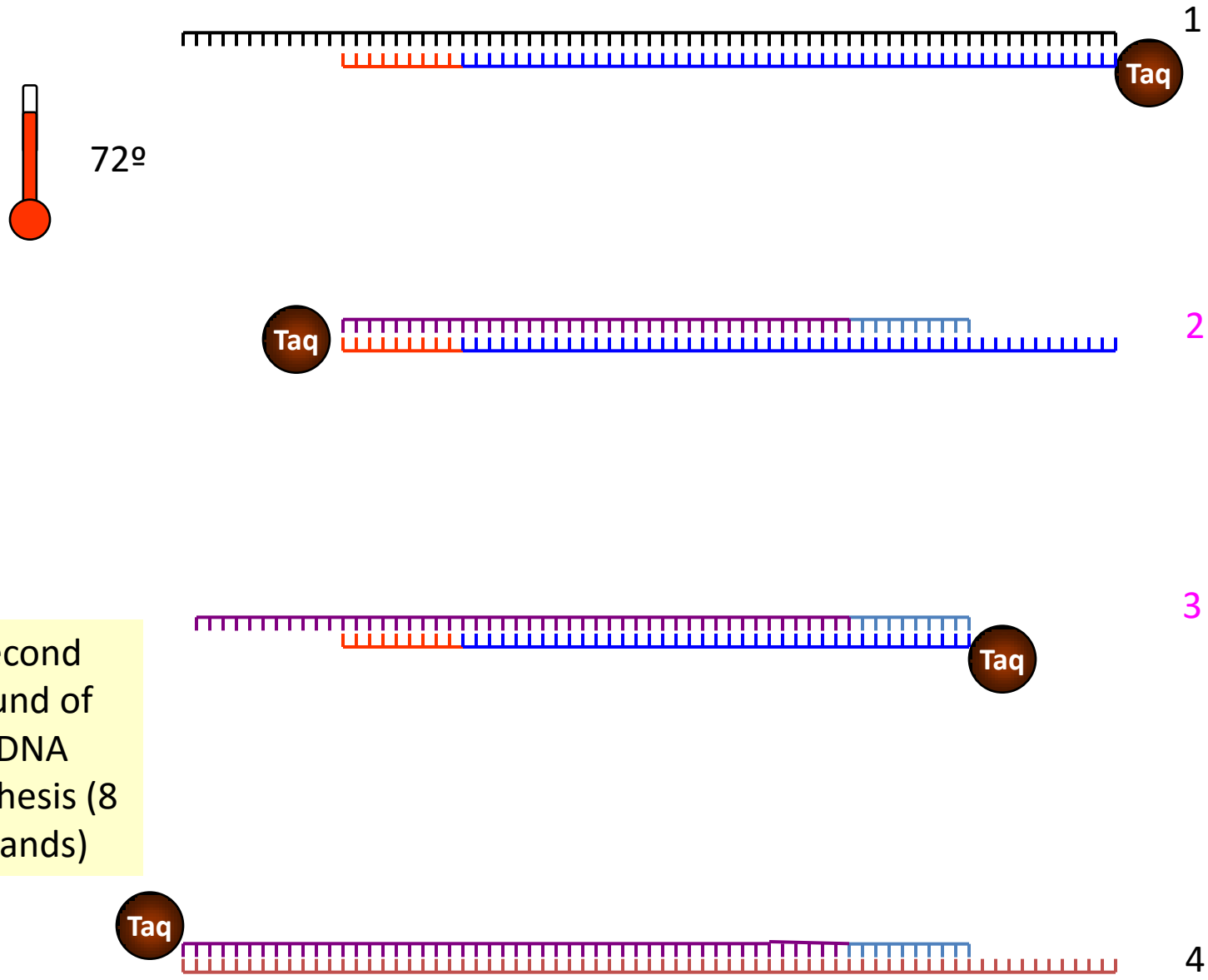


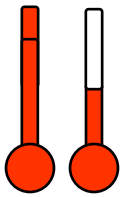
First round of cDNA synthesis (4 strands)

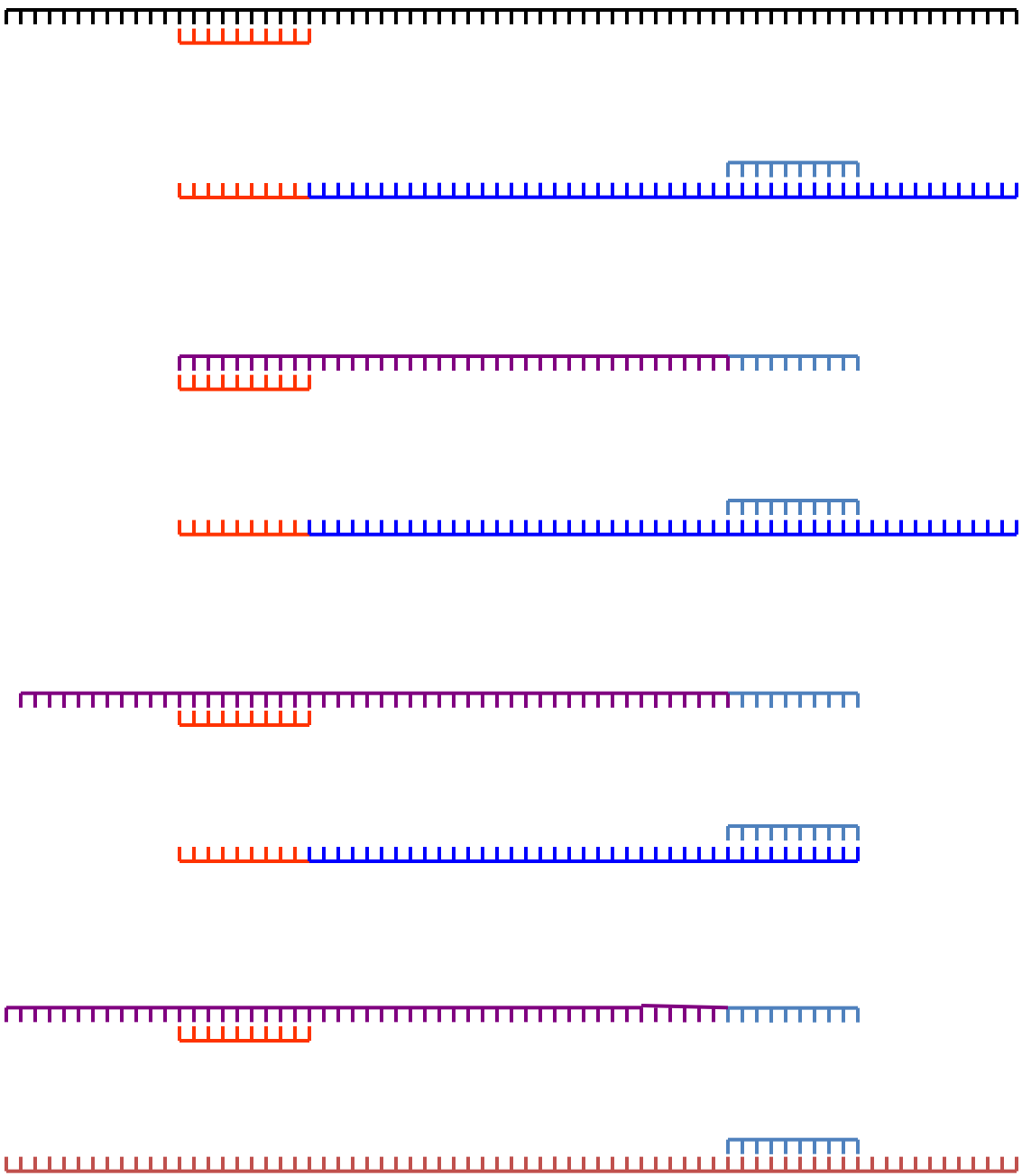


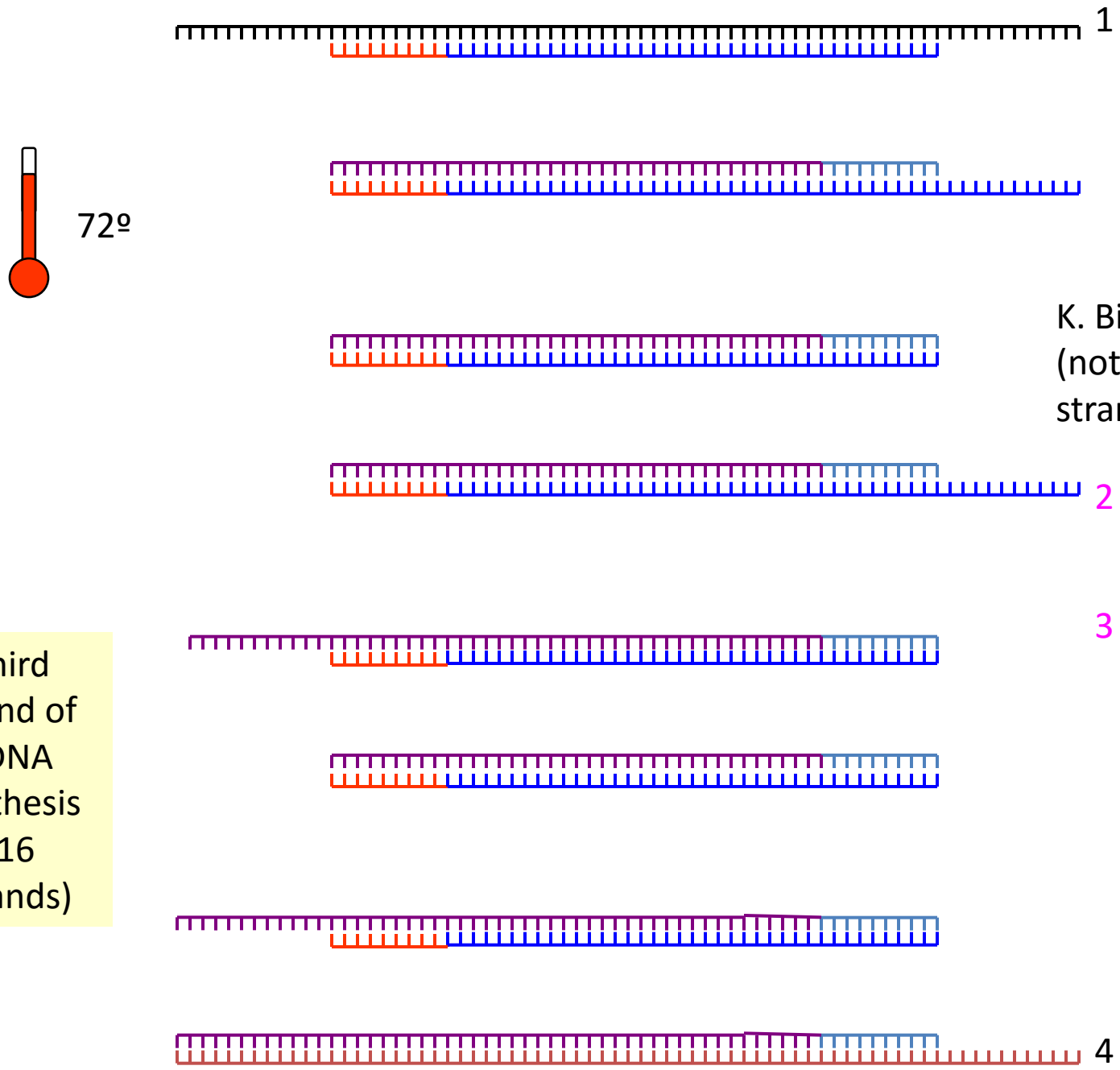
G. Anneal primers

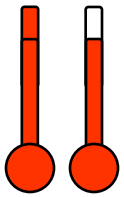

Taq
1
72º
Taq
2
H.
Polymerase
binds
Taq
3
Taq
4

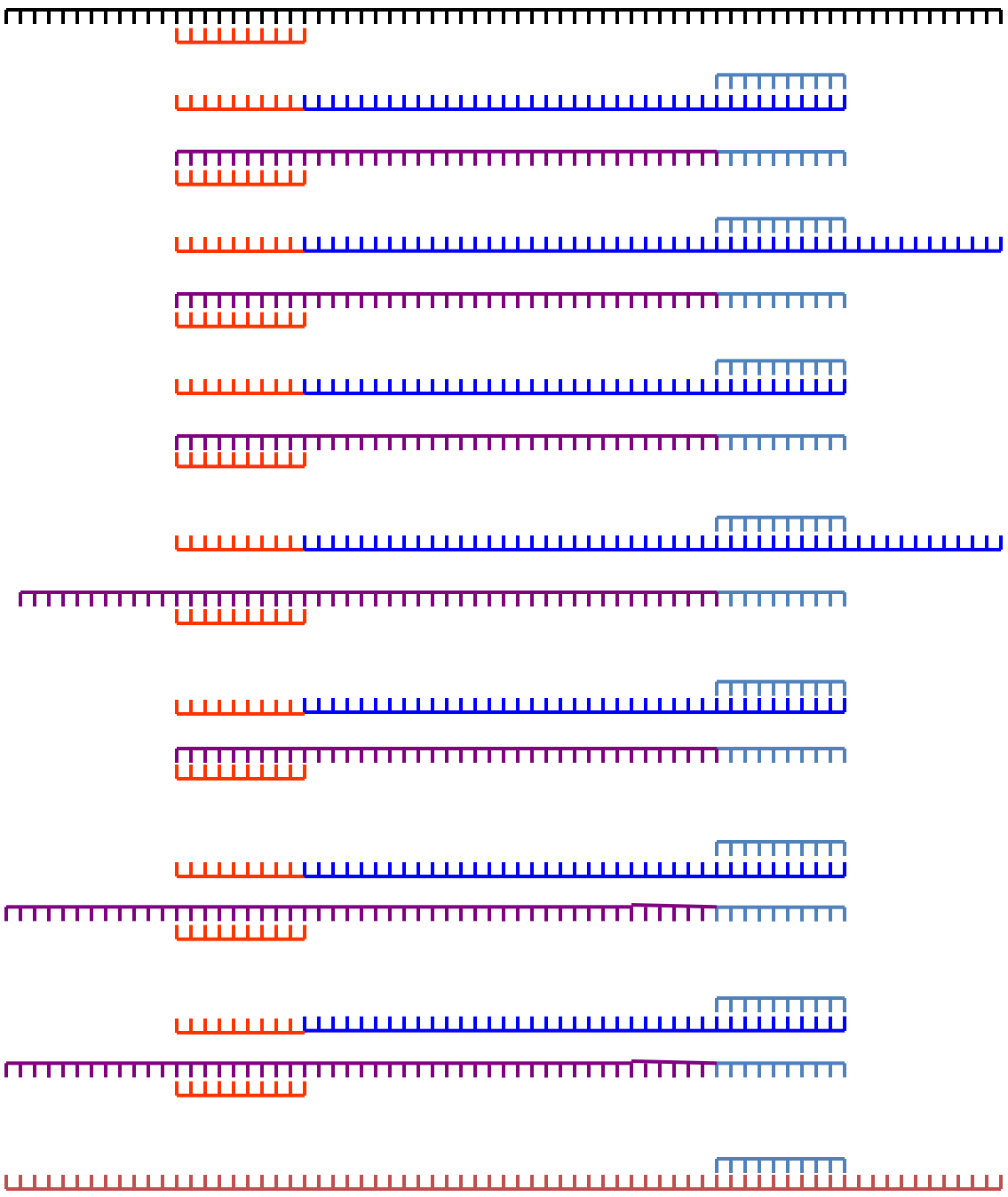

1
Taq
72º
Taq
2
I. Copy strands
3
Taq
Second round of cDNA synthesis (8 strands)
Taq
4

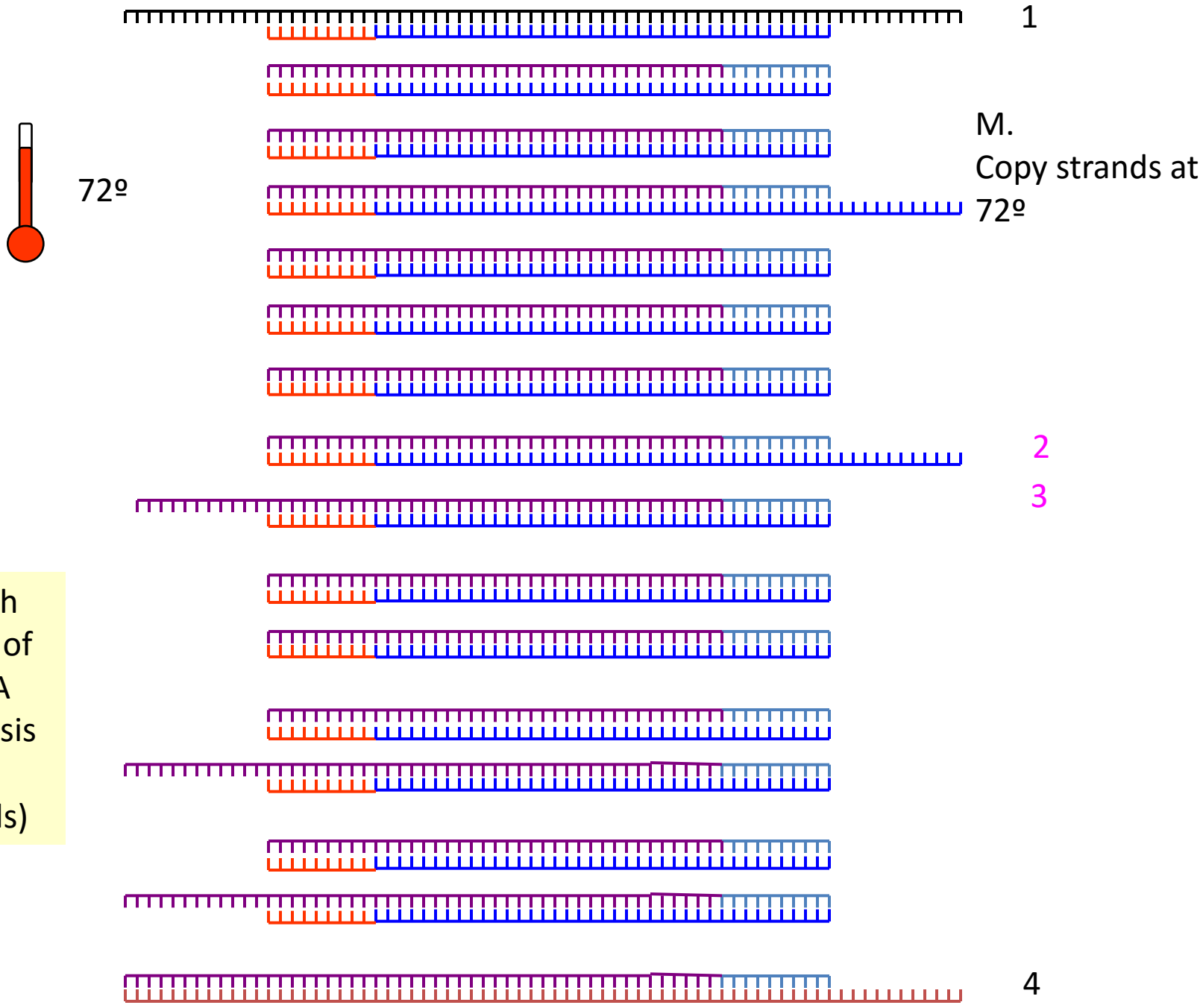



1
J.
Denature at 96º
Anneal primers at
50º
2
3
4


1
72º
K. Bind polymerase (not shown) and copy strands
2
3
Third round of cDNA synthesis (16 strands)
4

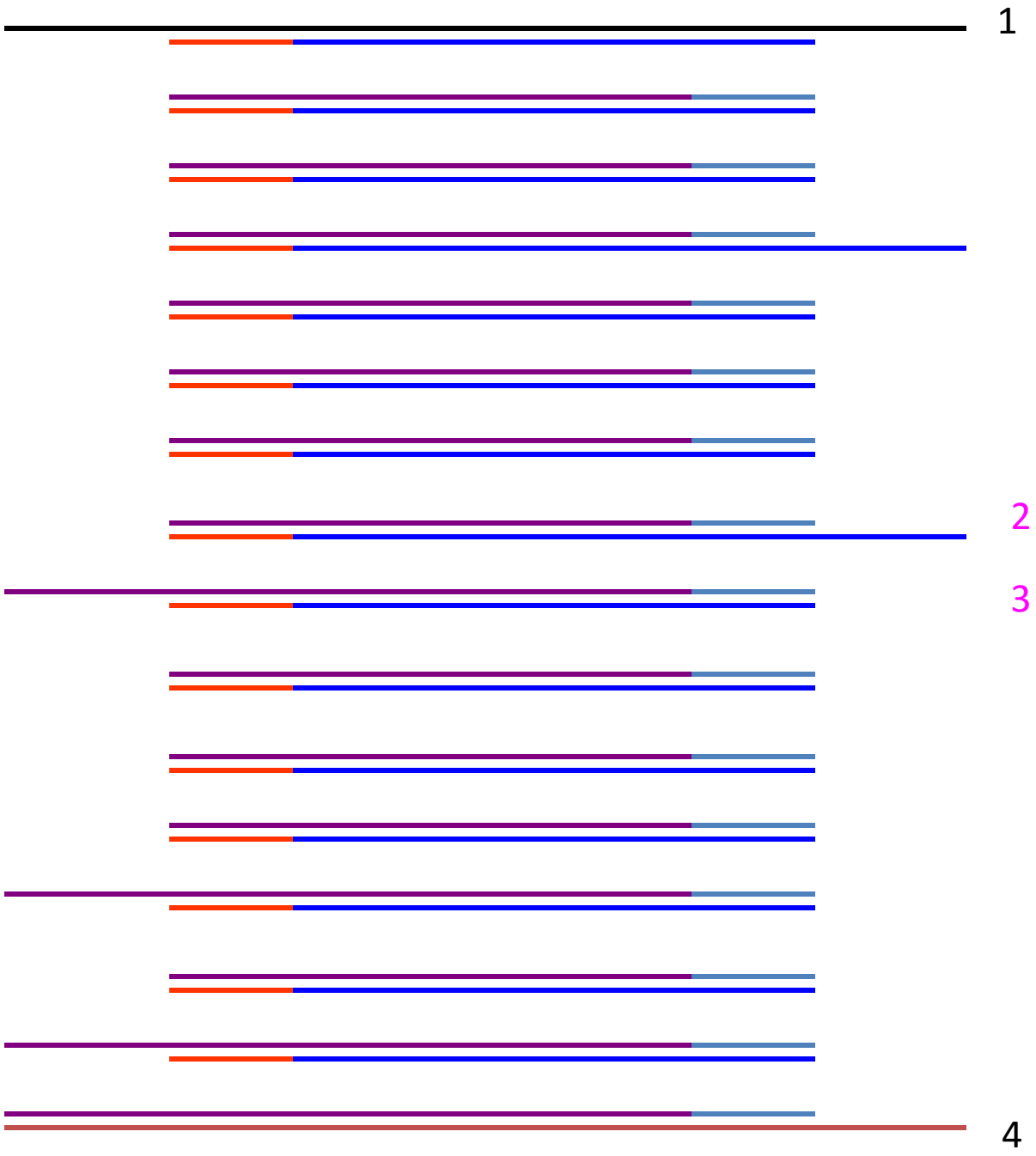



1
L.
Denature at 96º
Anneal primers at 50º
2
3
4

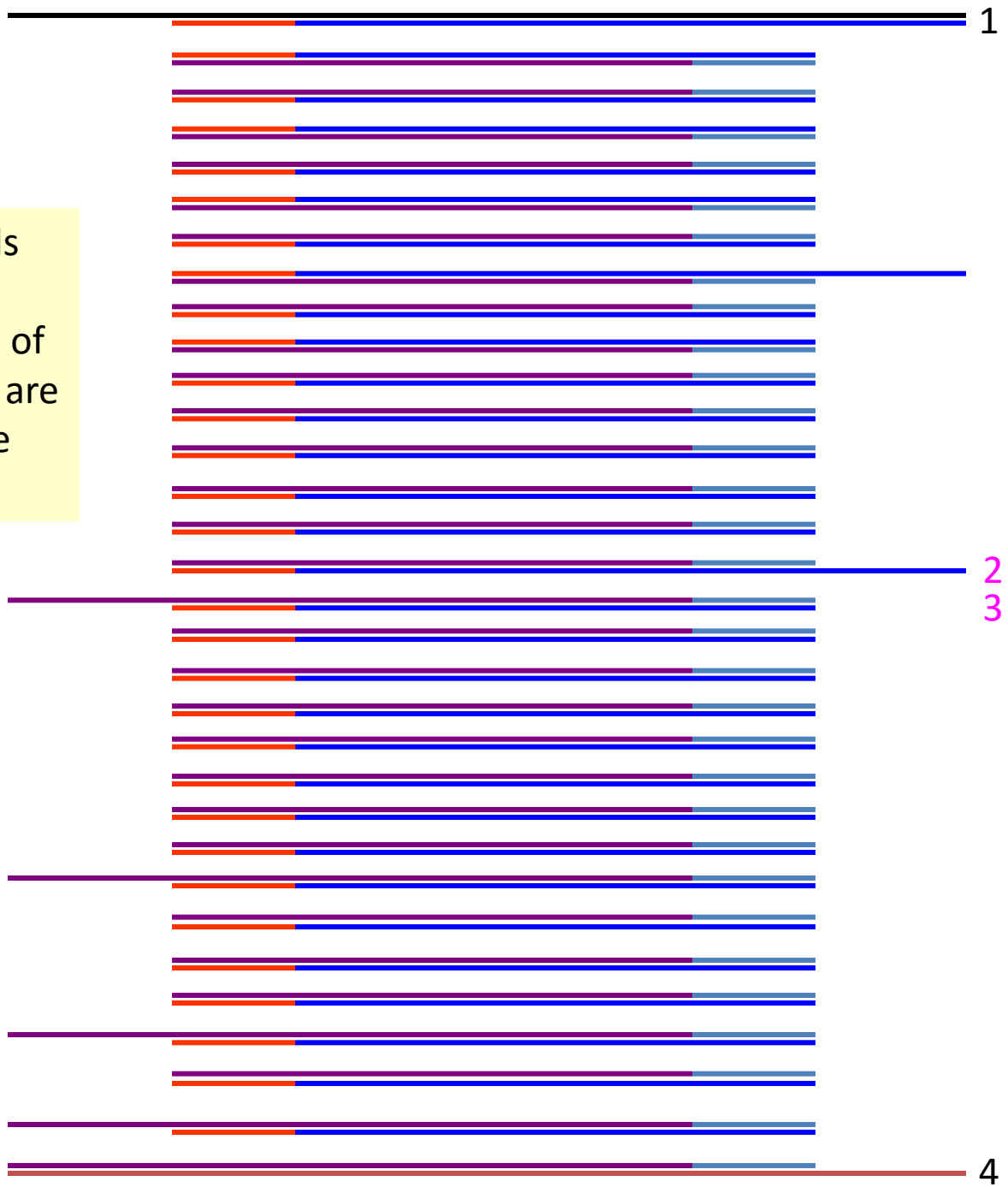


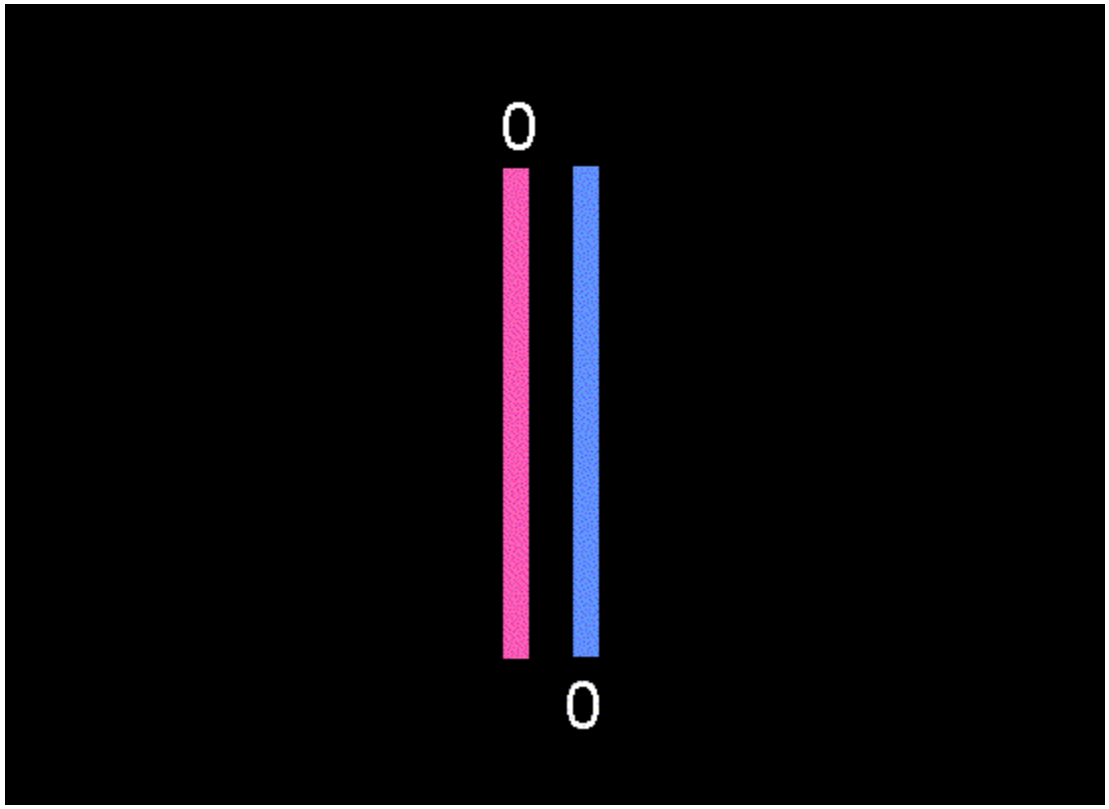
Fourth round of cDNA synthesis (32 strands)

cDNA strands (32) are now shown as lines



After 5 rounds there are 32 double strands of which 24 (75%) are are same size

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The End

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