Training on New and rapid Tuberculosis diagnostics (first and second line Probe Assay)

Module 4: Identification of the *Mycobacterium tuberculosis* complex by the Molecular Line-Probe Assay

Uganda Supranational Reference Laboratory

Outline

- Various identification kits for MTB complex and non MTB complex
- Line Probe Assay steps
- Major components of a PCR reaction
- Overview of the reverse hybridization process

Different Line Probe Assays that will identify the MTB complex

CM/AS common mycobacteria and additional species

- Species identification of the MTB complex and 30 other Mycobacterium species (2 strips)

MTBC

- Differentiation of the members of the MTB complex

MTBDRplus (multidrug resistant)

- Identification of MTB complex and mutations associated with INH and RIF resistance

MTBDRsl (Extensively drug resistant)

- Mutations associated with fluoroquinolone, aminoglycoside and EMB resistance

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The Line Probe Assay Kits have different primers and probes

Kit components are specific for each of the line-probe assays

- Kit-specific primers
- Kit-specific probes on the strips

Primers from one kit or type of assay cannot be used with strips from another kit

- Invalid results

Line Probe Assay steps

1. DNA extraction
   - Sample
2. Amplification
   - PCR
3. Hybridization
   - Detection

DNA preparation

DNA sample

- Processed sputum sediment
- Culture

Heat-kill bacteria

- Sample heat-killed in culture laboratory before moving to molecular laboratory for DNA extraction

DNA extraction

- Sonication
- Heating (waterbath, heating block, hot air oven)

Polymerase Chain Reaction-PCR

- PCR reaction using cells from AFB isolate
  - Primers are labelled with biotin
  - Biotin-labelled primers are incorporated into the amplified target DNA sequences during PCR
  - More than one primer set is used in the same reaction
    - Several labelled target sequences are amplified at the same time

Hybridization-detection

Reverse hybridization

- Unlabeled probes specific for MTB complex or other mycobacterial species are bound to strips
  - Several target sequence probes can be placed on a strip
- Biotin-labelled target DNA binds to complementary probe on strip

Probe-target complex is detected

- Streptavidin-conjugated reagent detects biotin-labelled probe-target complex

Polymerase Chain Reaction (PCR)

PCR was invented by Kary Mullis in 1983

Kary Mullis on the left receiving the Nobel Prize for Chemistry in 1993

PCR is a technique that takes a small amount of a specific DNA sequence and amplifies it for further testing

It has been called a "molecular photocopier"

Major components of a PCR reaction

Primers – short pieces of DNA needed to start DNA synthesis by binding to specific regions of target DNA

- DNA polymerase requires primers to begin extension

Deoxynucleotides (dNTPs) – building blocks for DNA synthesis

- dATP, dTTP, dCTP, dGTP

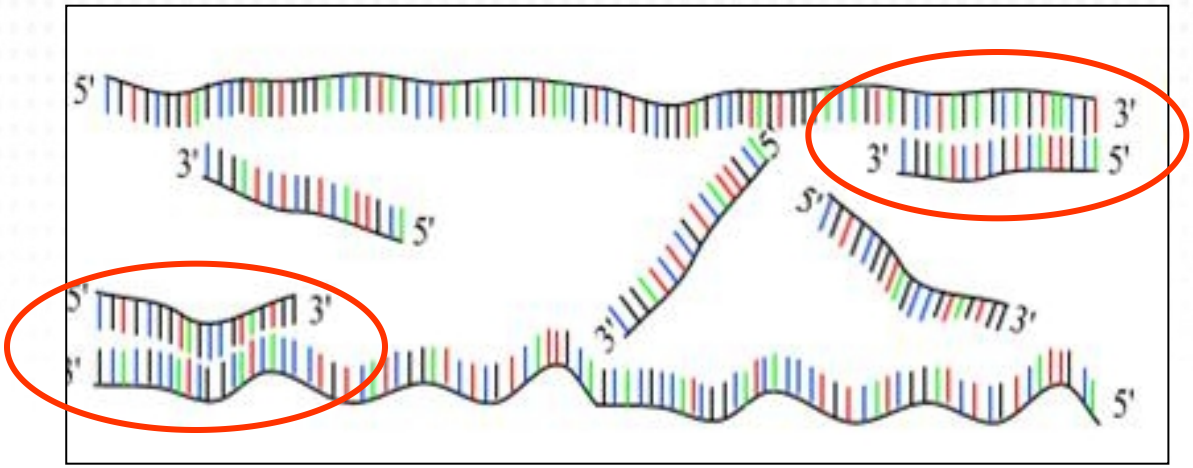
DNA polymerase – enzyme for DNA synthesis

Buffer - provides optimal conditions for the activity of DNA polymerase

PCR performed in a Thermal cycler

PCR machine (Thermocycler)

Primers



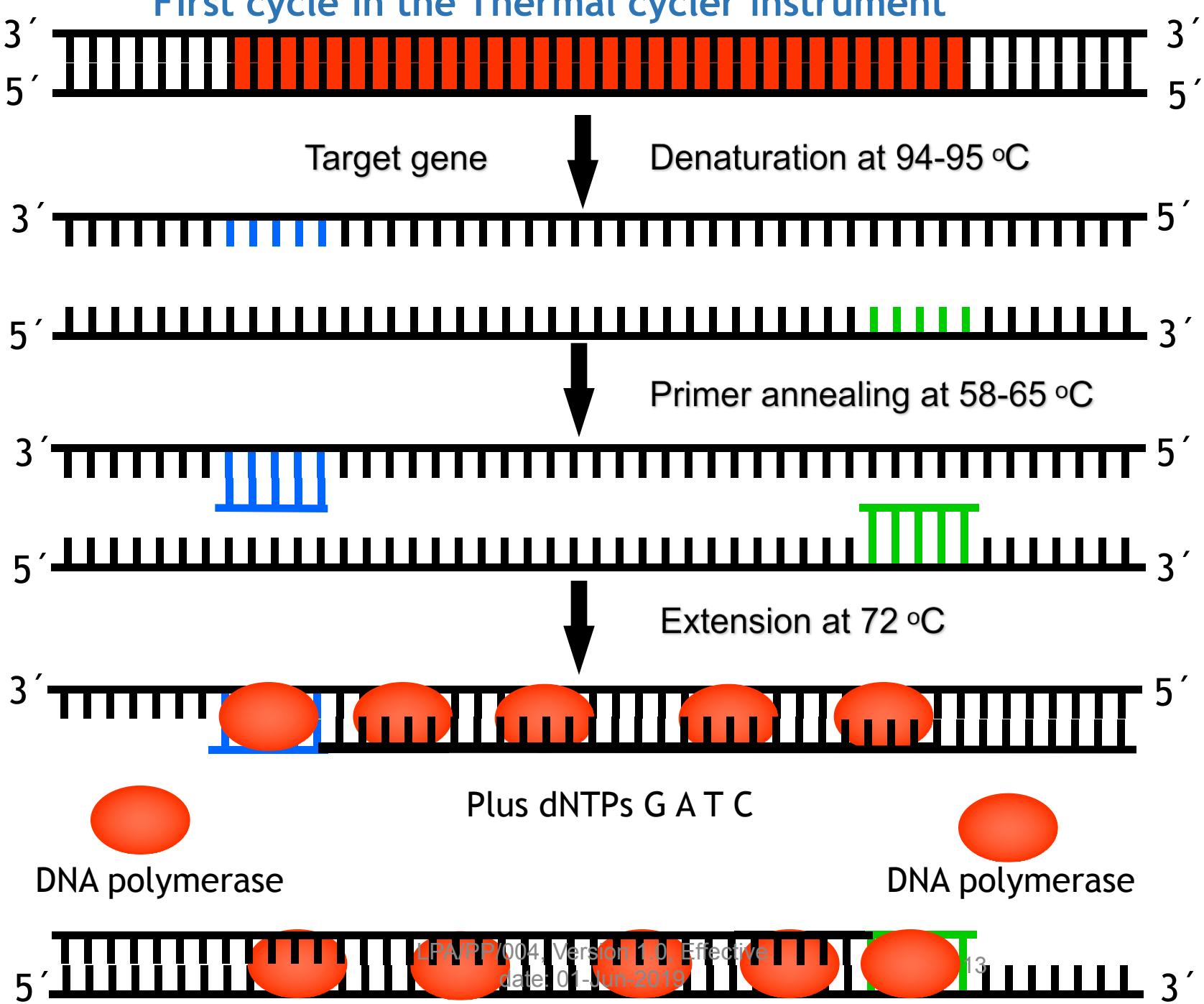
Primers are sequence-specific oligonucleotides that hybridize to the target DNA and serve as a starting point for the DNA polymerase to add nucleotides

Forward and reverse primer set flank the target sequence and allow both DNA strands to be copied simultaneously in both directions.

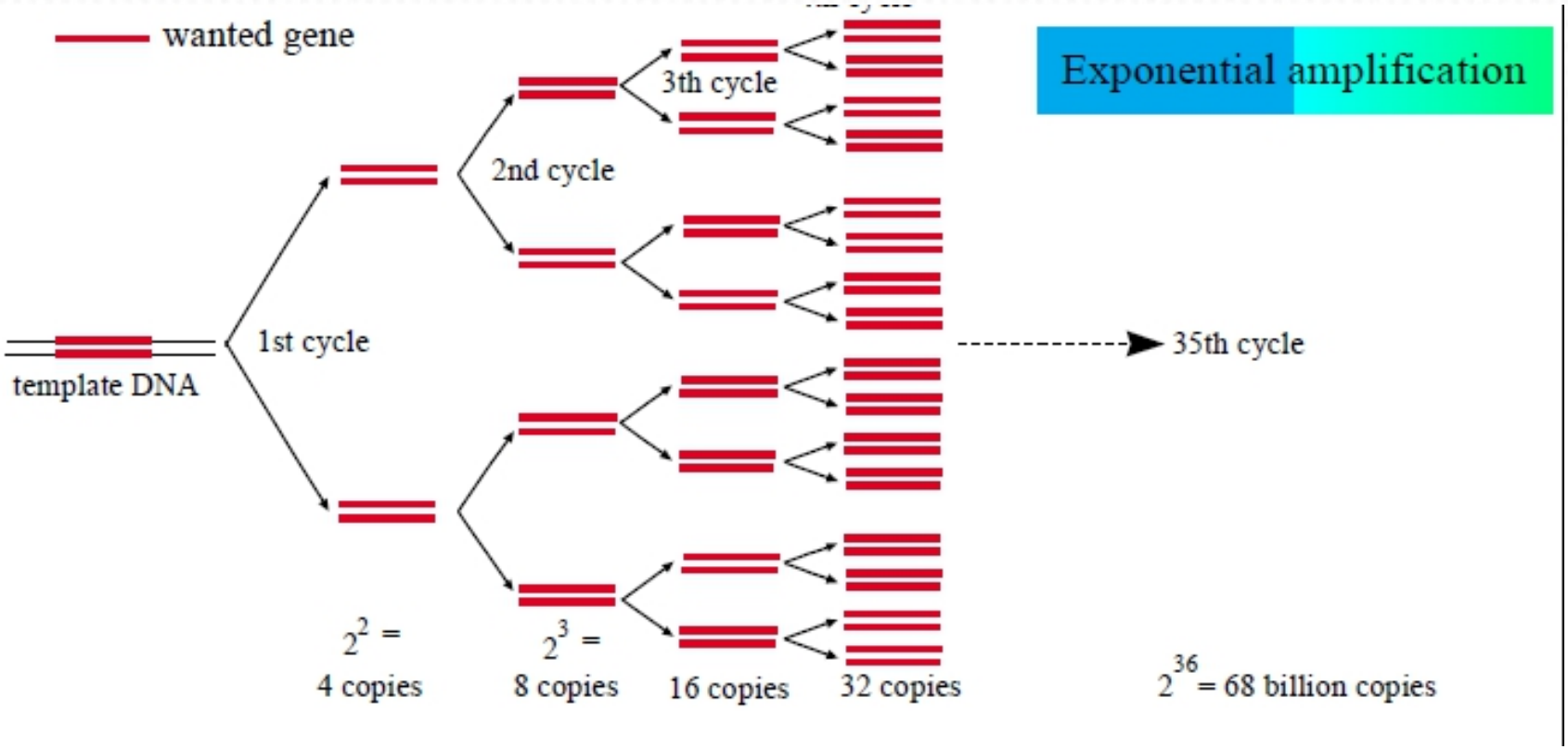
Three steps of PCR

- Denaturation
  - Increase temperature, bonds break between the base pairs, strands separate
- Annealing
  - Decrease temperature, primers bind to complementary sequence on DNA strands
- Extension
  - Increase temperature, DNA polymerase adds dNTPS to strands

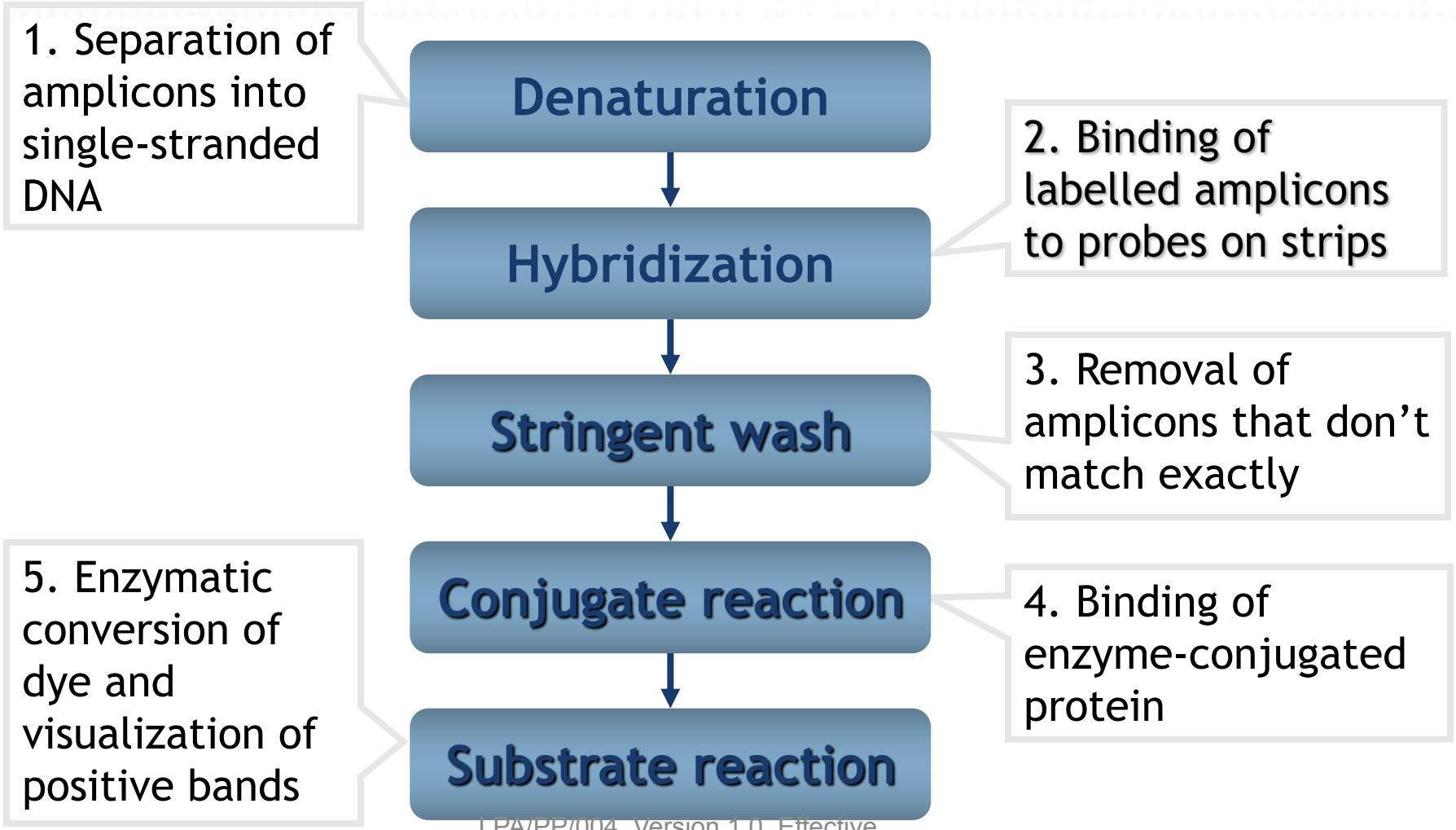
1 cycle = 3 steps


First cycle in the Thermal cycler instrument
3´
5´
3´
5´
Target gene
Denaturation at 94-95 °C
3´
5´
5´
3´
Primer annealing at 58-65 °C
3´
5´
5´
3´
Extension at 72 °C
3´
5´
Plus dNTPs G A T C
DNA polymerase
DNA polymerase
5´
3´

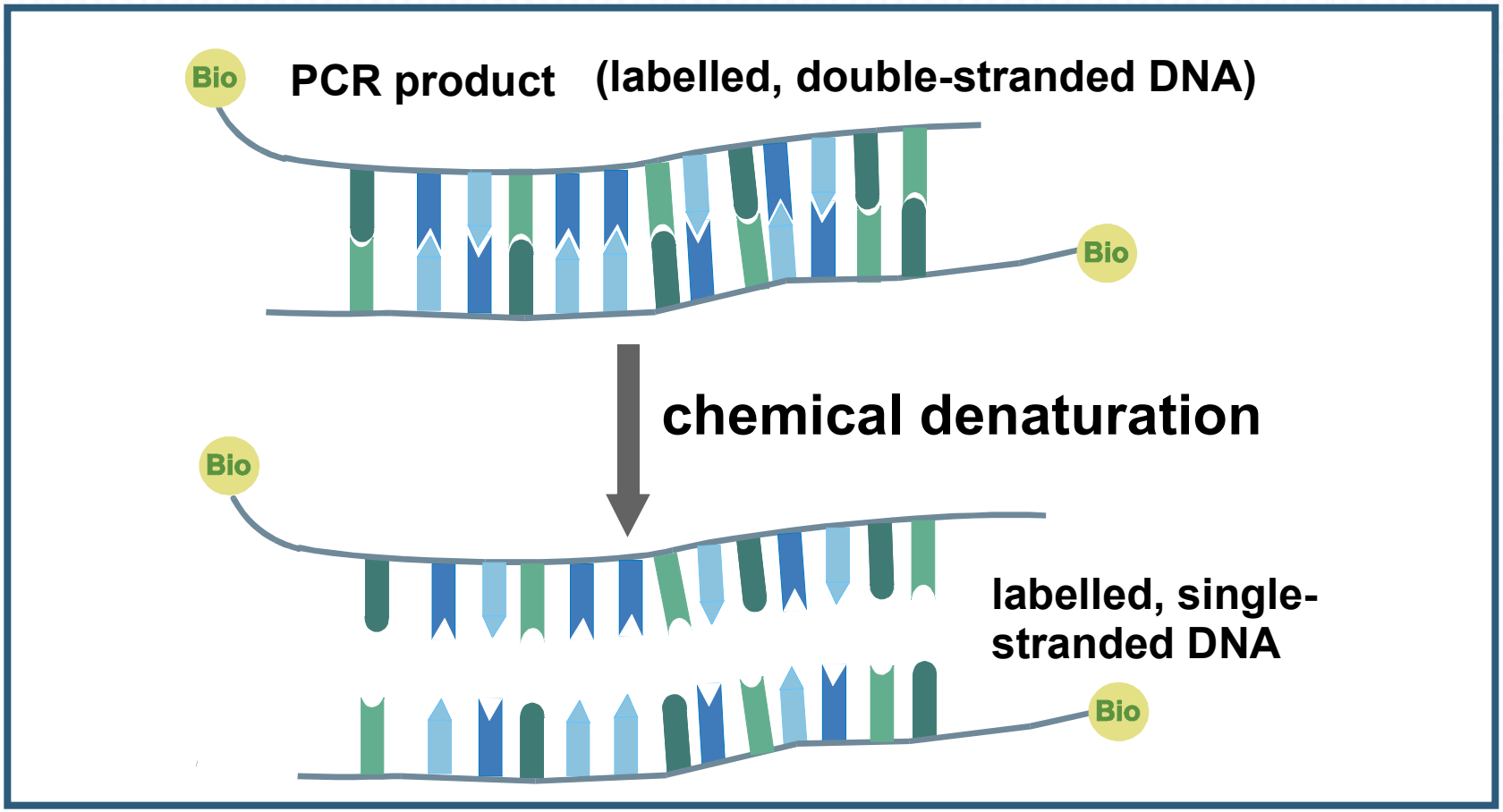
35 Cycles: Billions of copies of target are made



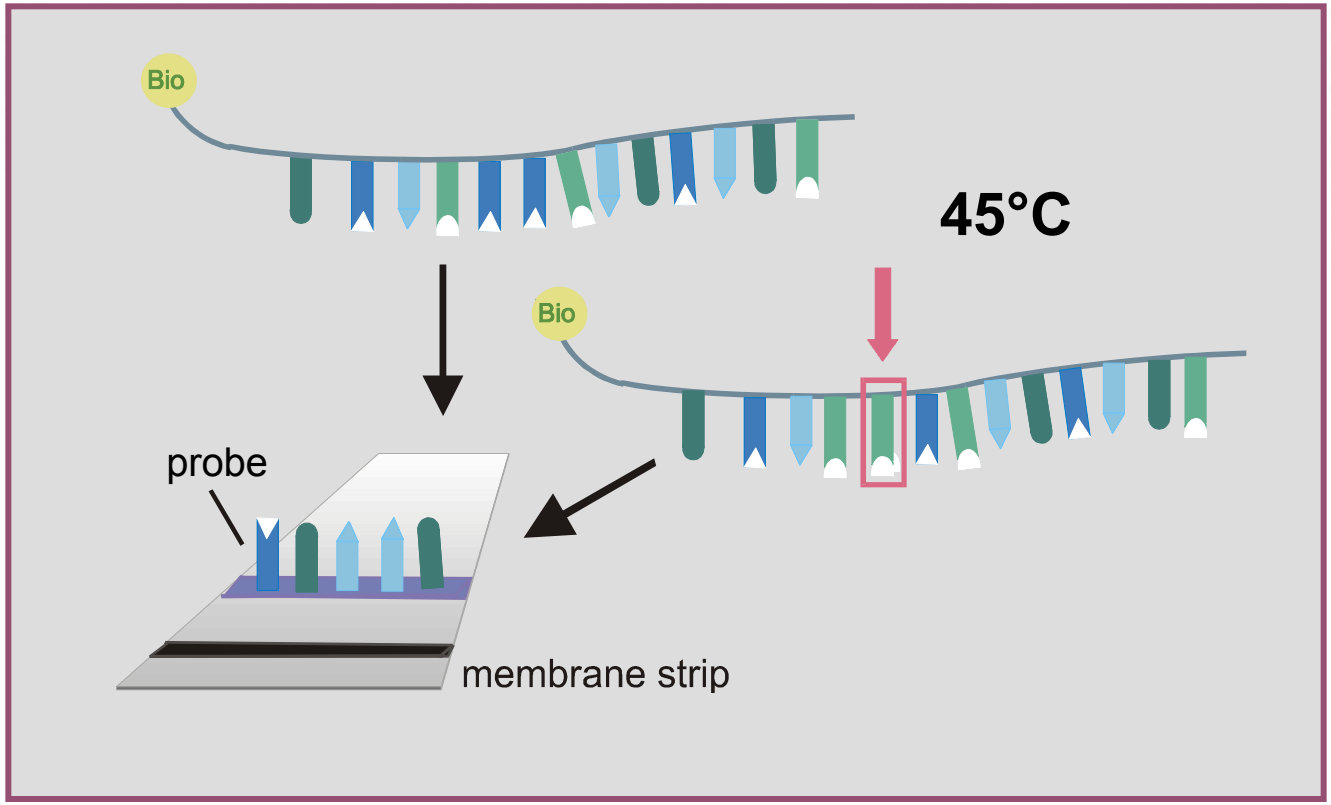
Overview of reverse hybridization



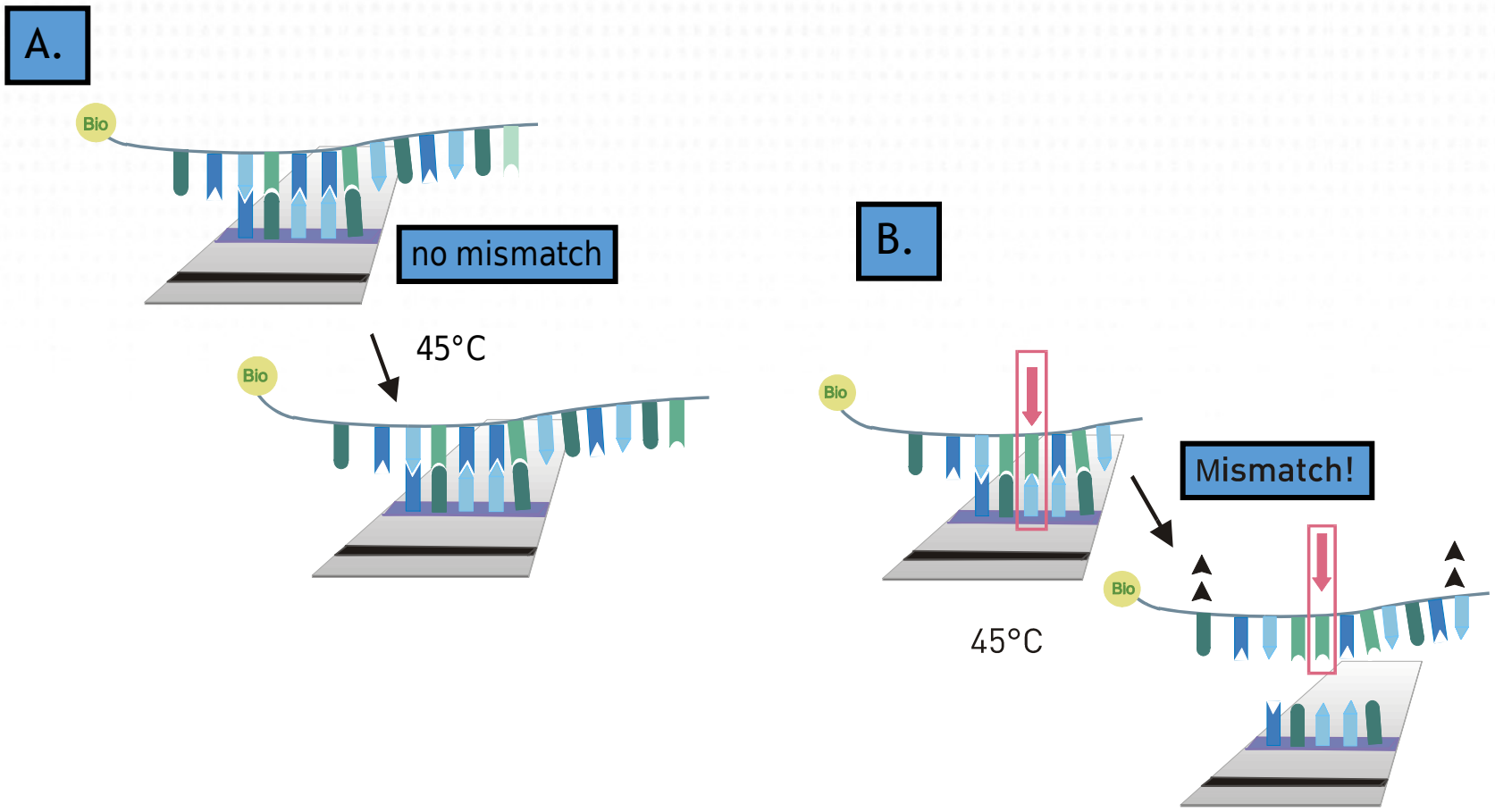
DEnaturation of dsDNA amplicons



Hybridization of ssDNA amplicons



Stringent washing of strips



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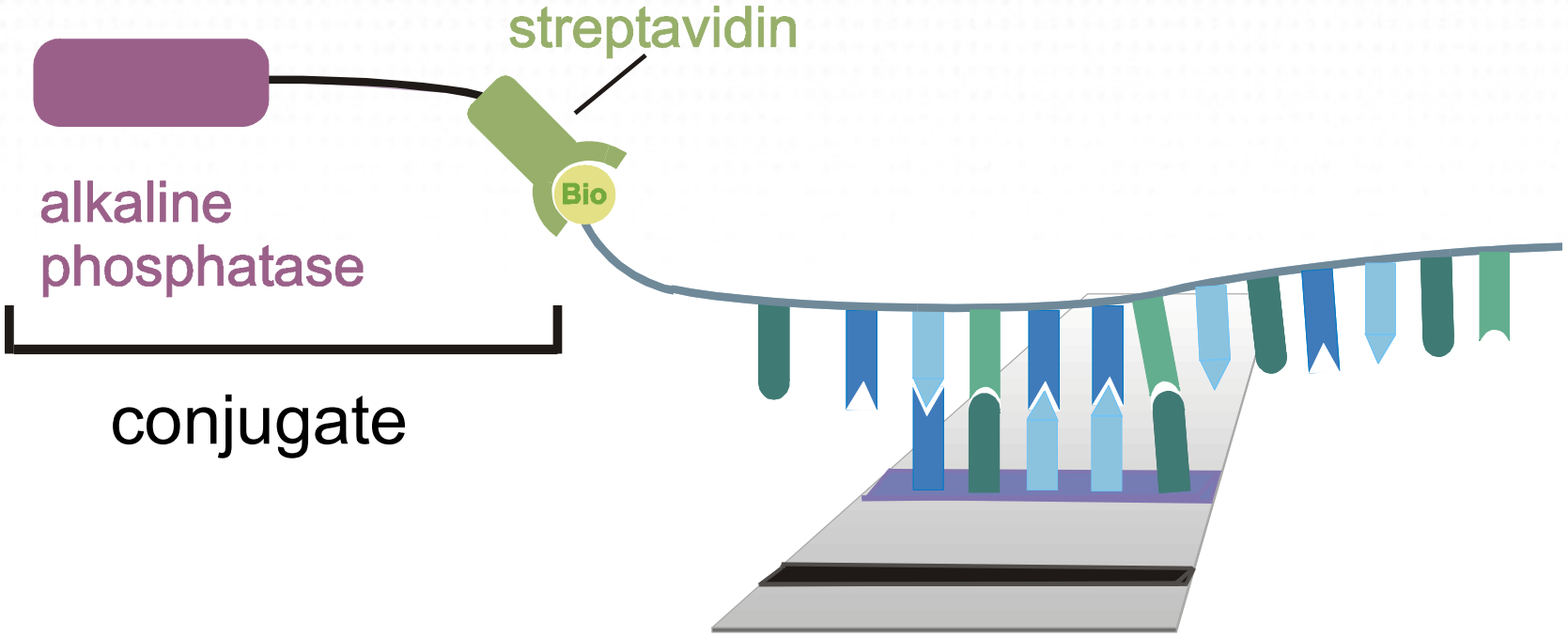
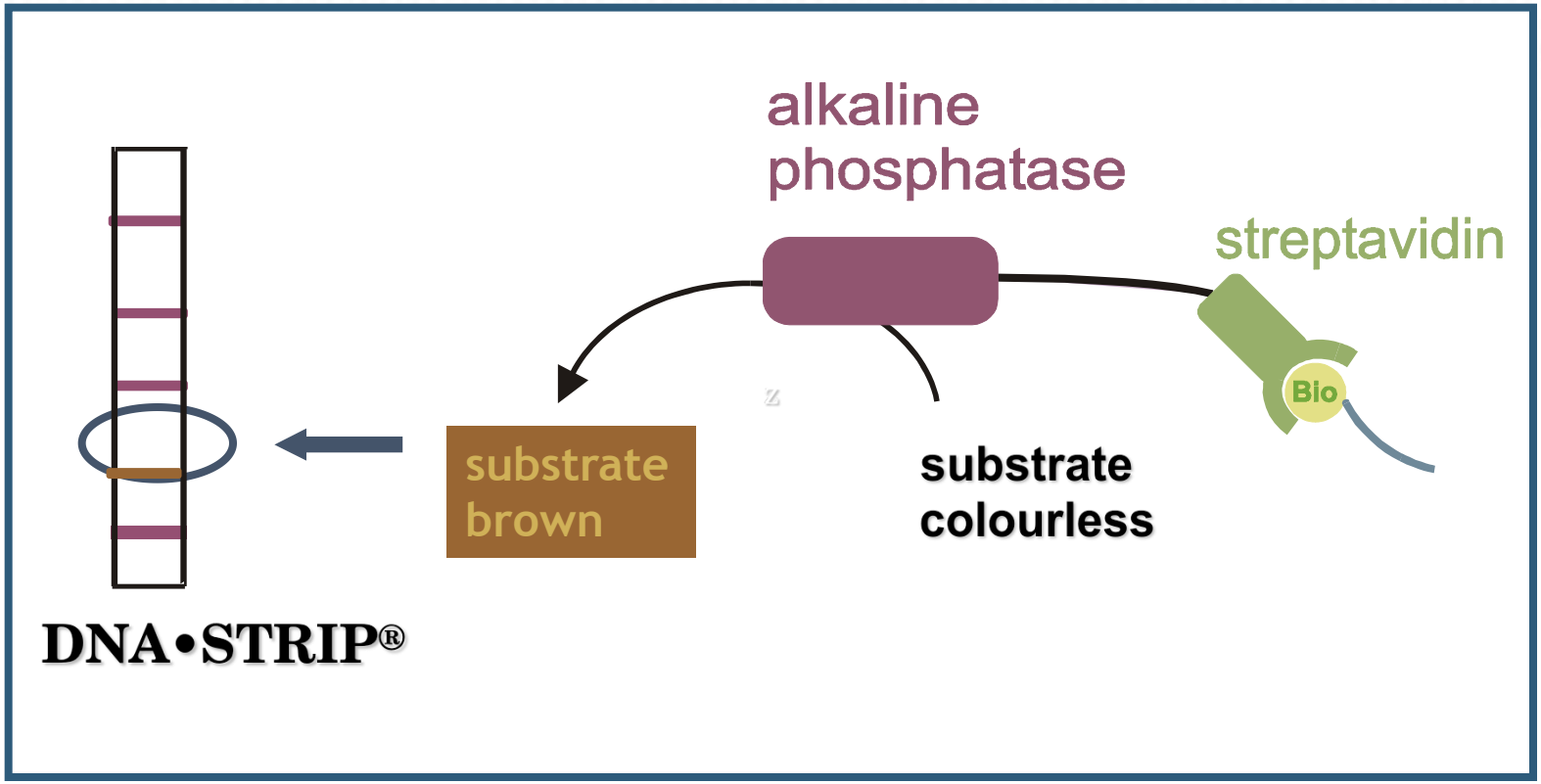
Streptavidin binding to bound Amplicon



Substrate reaction / color development



Quality control of LPA

- The following controls must be included in each PCR run to ensure quality results
  - Positive processing control
    - Well characterized isolate of *M. tuberculosis e.g. H37RV*
  - Negative processing control
  - Negative amplification monitoring control
    - Contamination control

Assessment

1) Identify the 3 LPA steps

2) Name the 3 steps involved in a PCR reaction

3) Name the 5 key steps in reverse hybridization

4) Identify what happens in each of the 5 steps above

Summary

- The LPA can rapidly identify NTMs, MTBC, MDR TB and XDR TB
- The PCR reaction can create billions of DNA copies in just a few hours/cycles.
- The PCR thermal cycler is simply a rapid heating and cooling instrument.
- Reverse hybridization enables the detection and visualization of the PCR reaction.

References

- GLI TB training package http://www.stoptb.org/wg/gli/trainingpackages.asp

Acknowledgments

KOFIH
Korea Foundation for International Healthcare
한국국제보건의료재단