DNA AMPLIFICATION

- This lecture presents multiple methods for replicating a specific DNA sequence.

- Goal: Reactions where the product grows exponentially with the number of cycles.

- Main classes of methods:

  (1) Thermal-Cycling: PCR

  (2) Isothermal: many methods

- Note: If RNA is to be detected, usually first transform to DNA

DNA AMPLIFICATION BY Polymerase Chain Reaction (PCR)

- The Polymerase Chain Reaction (PCR) is method to replicate a specific DNA sequence.

- is an in-vitro technique for amplification of a region of DNA whose sequence is known or which lies between two regions with known sequences (these are called **primers**).

- PCR iteratively cycles between different temperatures to achieve amplification.

- **The product grows exponentially with the number of cycles** (roughly as $2^{t/2}$ where t is the number of cycles)

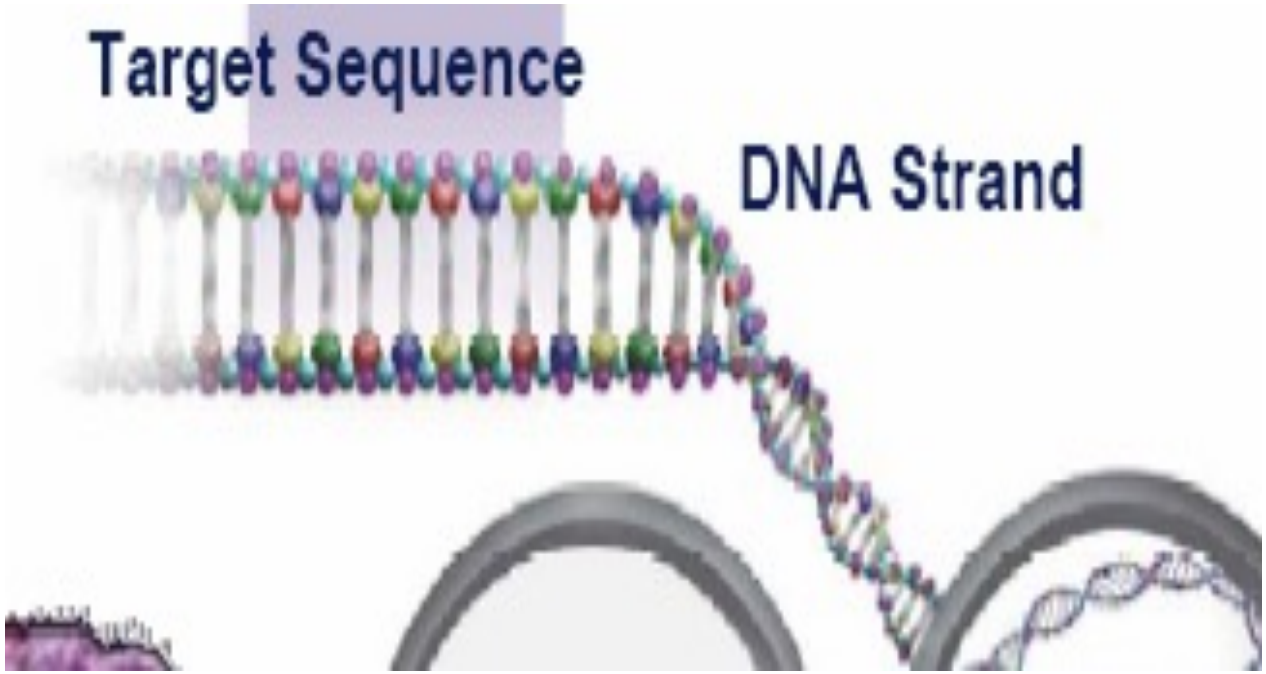
HISTORY OF PCR

- 1966, Thomas Brock discovered Thermus Aquaticus, a thermostable bacteria in the hot springs of Yellowstone National Park
- 1983, Kary Mullis postulated the concept of PCR (Nobel Prize in 1993)
- 1985, Saiki publishes the first application of PCR (beta-Globin)
- 1985, Cetus Corp. Scientists isolate Thermostable Taq Polymerase (from T.Aquaticus), which revolutionized PCR

PCR Reagents

- Target DNA strand to be amplified(template)
  - Usually DNA
  - Can be RNA if an extra step is added
- Two Primers (may be specific or random)
- Thermostable polymerase
- Other Materials:
  - buffer
  - dNTPs
  - Mg^{2+}

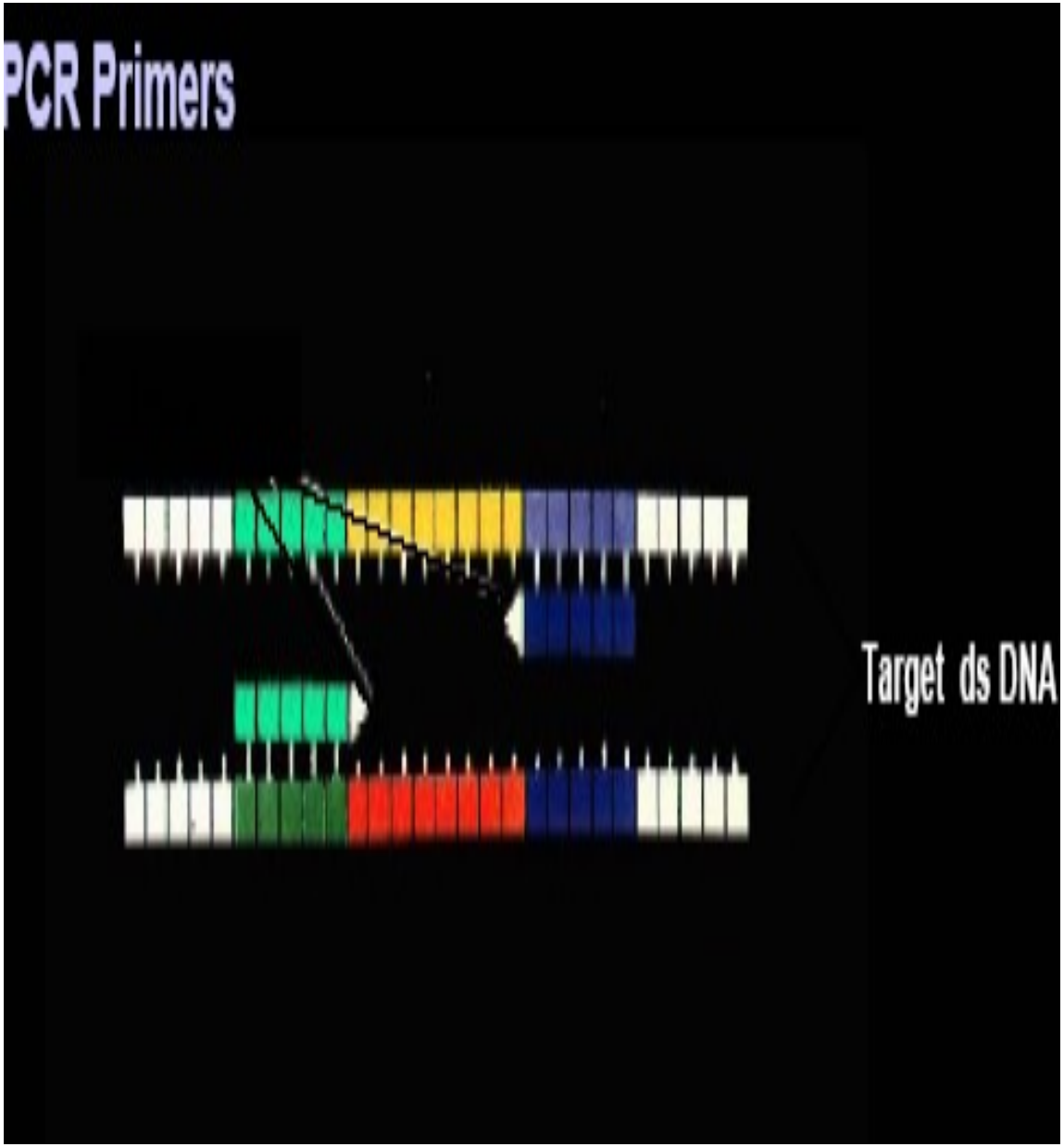
DNA template



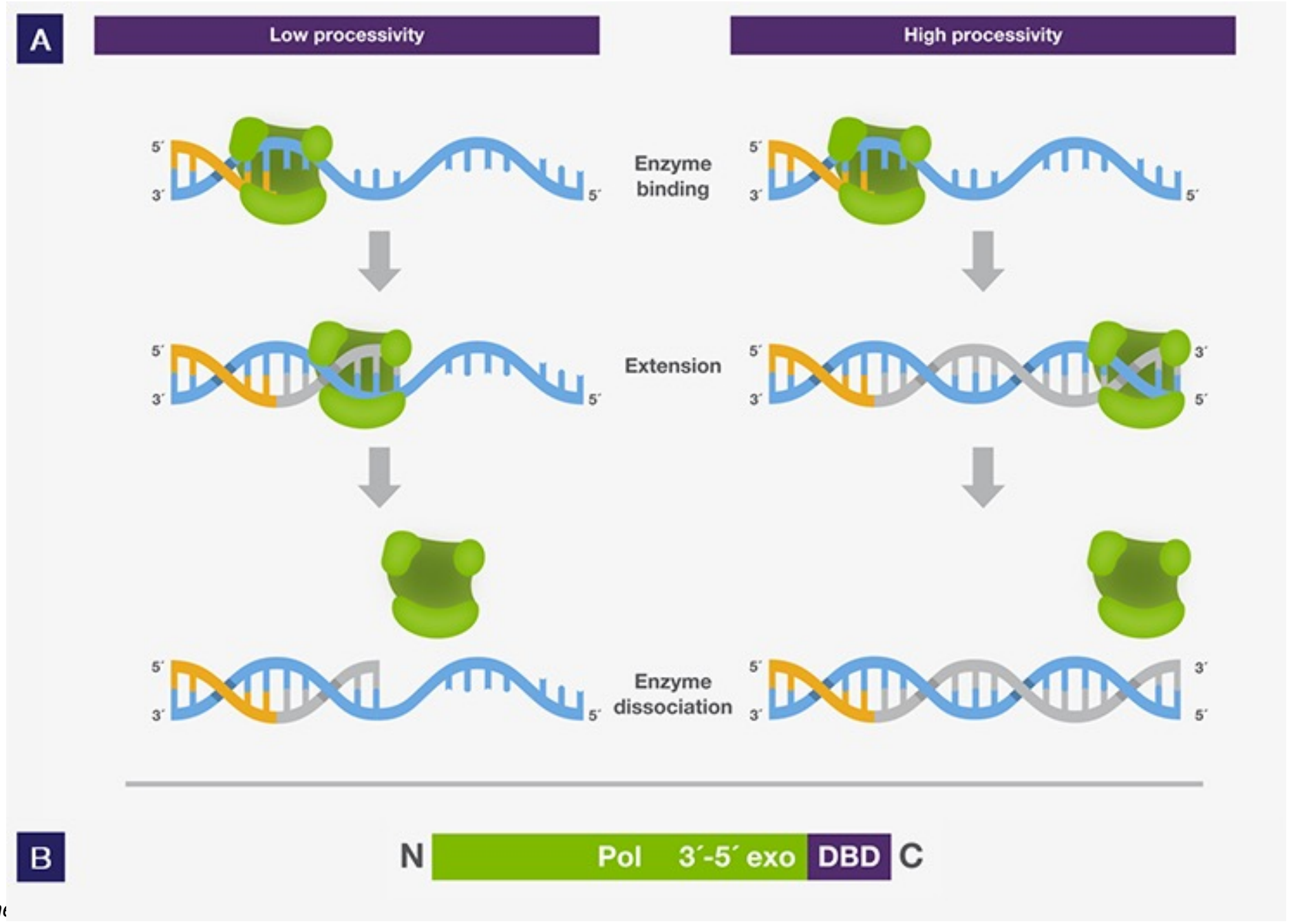
- DNA containing region to be sequenced
- Size of target DNA to be amplified : up to 3 Kb

PCR Primers

- 2 sets of primers
- Generally 20-30 nucleotides long
- Synthetically produced
- complimentary to the 3’ ends of target DNA
- not complimentary to each other



Polymerization by Polymerase Enzyme



Thermostable Polymerase Enzyme

- Usually Taq Polymerase or anyone of the natural or Recombinant thermostable polymerases
- Stable at T^0 up to 95^0 C
- High processivity
- Taq Pol has 5'-3' exo only, no proofreading

Thermostable Polymerases

- *Taq*: *Thermus aquaticus* (most commonly used)
  - Sequenase: *T. aquaticus* YT-1
  - Restorase (*Taq* + repair enzyme)
- *Tfl*: *T. flavus*
- *Tth*: *T. thermophilus* HB-8
- *Tli*: *Thermococcus litoralis*
- Carboysothermus hydrenoformans (RT-PCR)
- *P. kodakaraensis* (Thermococcus) (rapid synthesis)
- *Pfu*: *Pyrococcus furiosus* (fidelity)
  - Fused to DNA binding protein for processivity

Performing PCR

- Assemble a reaction mix containing all components necessary for DNA synthesis.
- Subject the reaction mix to amplification via PCR Cycles.
- Analyze the product of the PCR reaction (the amplicon).

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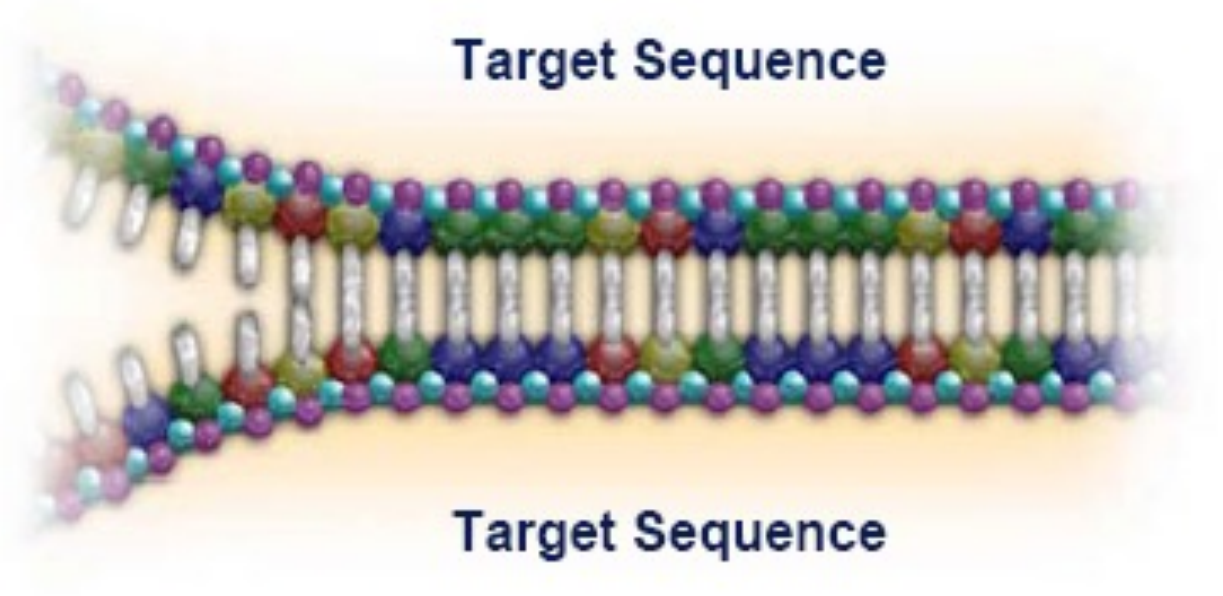
The PCR Cycle

- Comprised of 3 steps:
- (1) Denaturation of DNA at 95^0C
- (2) Primer hybridization (called annealing) at 40-50^0C
- (3) DNA synthesis (Primer extension) at 72^0C

The PCR Cycle

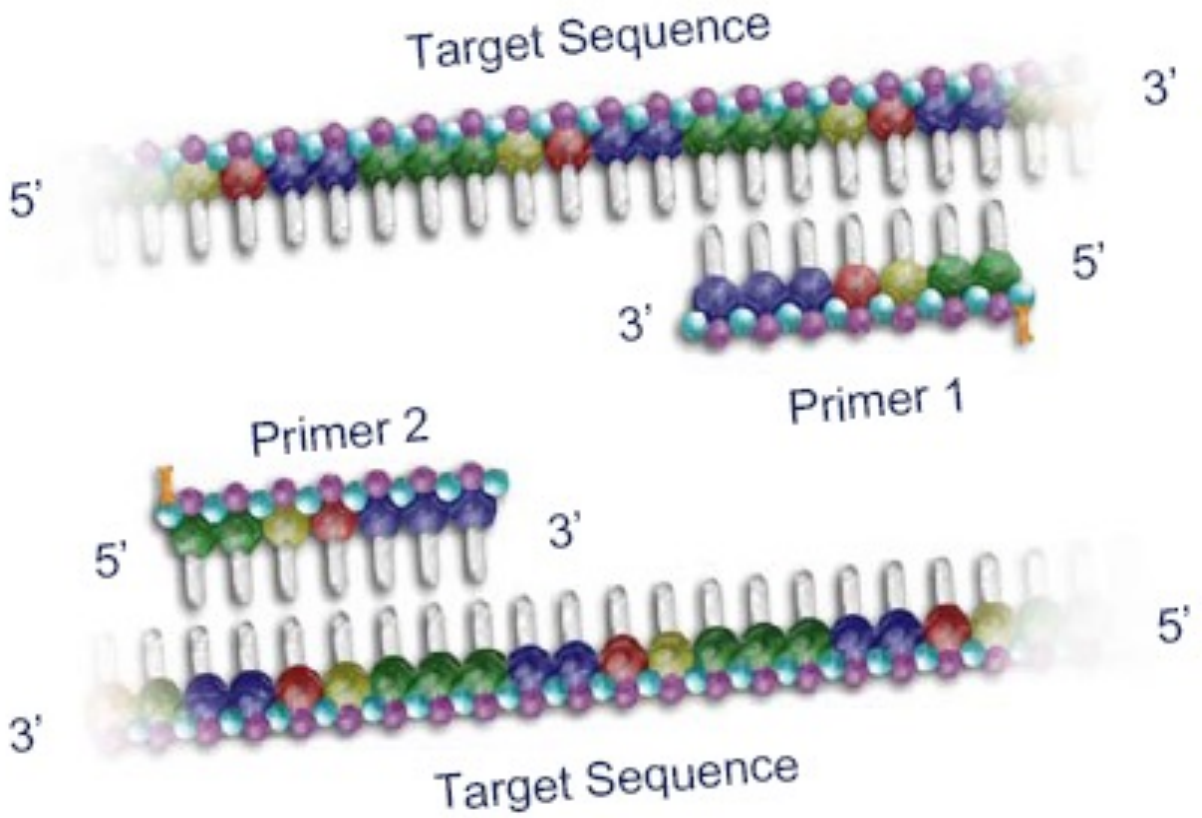
- (1) Denaturation of target (template)
  - Usually 95^{o}C
- (2) Annealing of primers
  - Temperature of annealing is dependent on the G+C content
  - May be high (no mismatch allowed) or low (allows some mismatch) stringency
- (3) Primer Extension – gives synthesis of new product strand

PCR Cycle - Step 1 - Denaturation Template DNA by Heat (95°C)



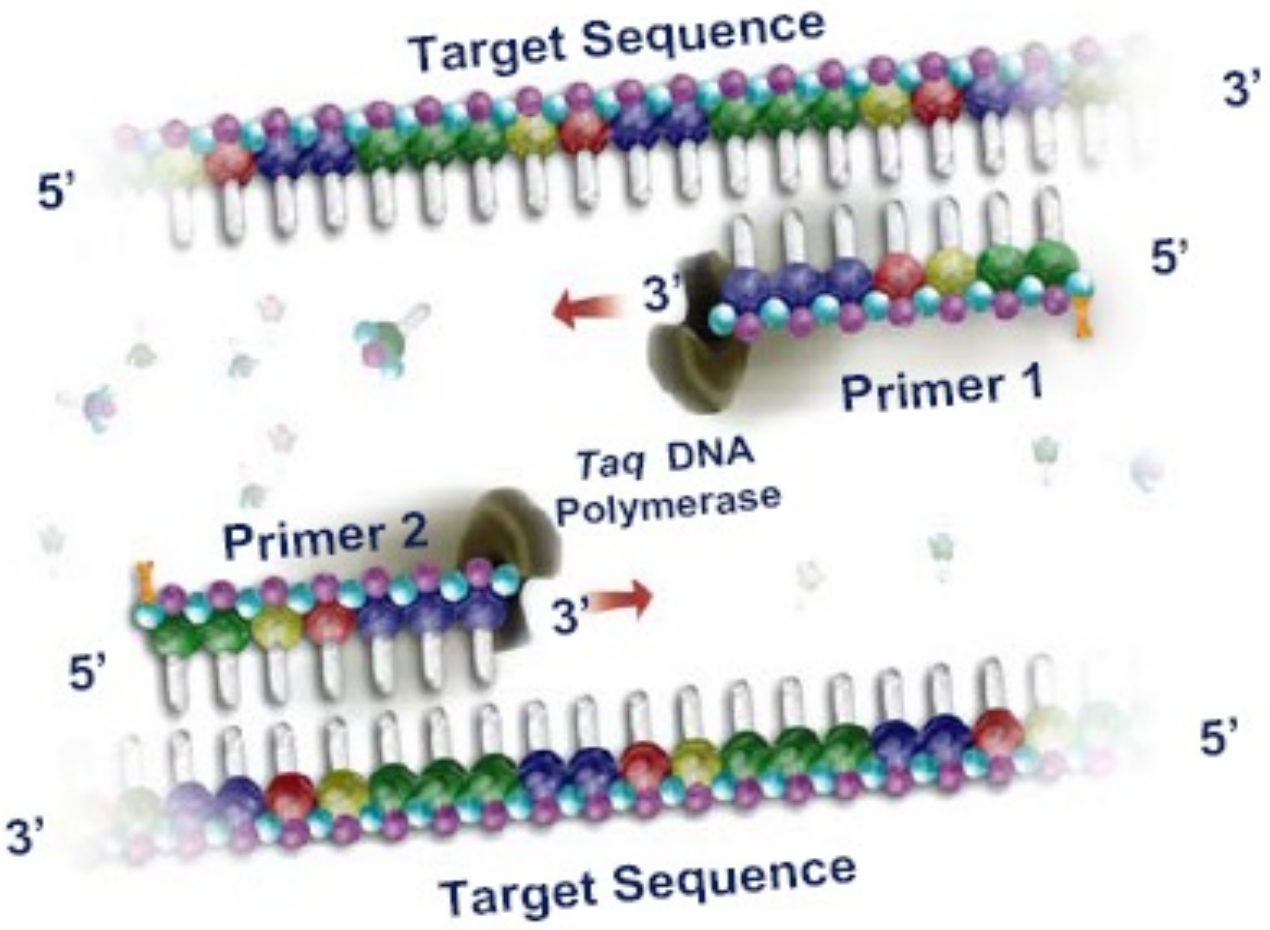
PCR Cycle - Step 2 –

Temperature is lowered (T_m) and primers anneal to target sequences



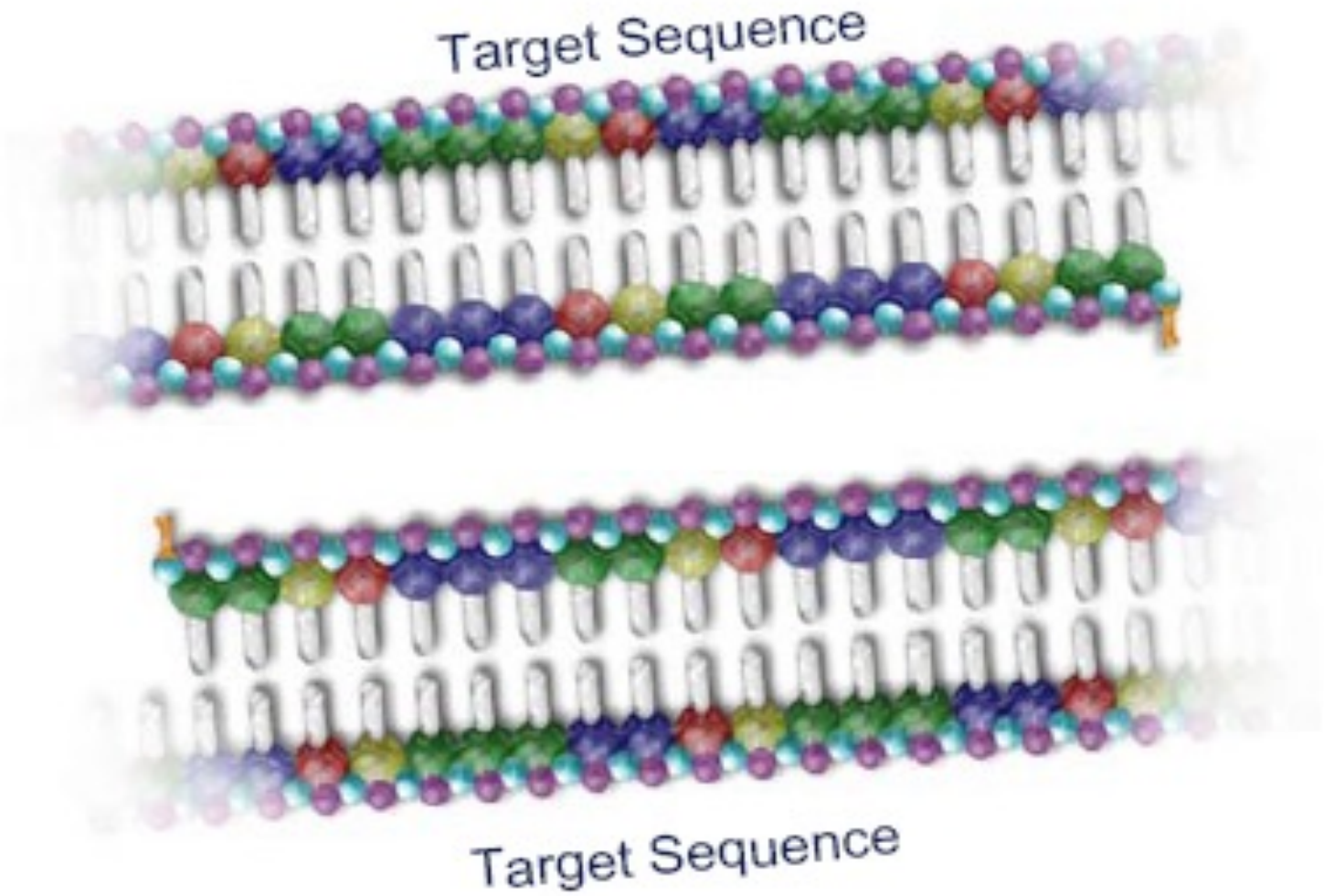
PCR Cycle - Step 3 -

At 72 °C *Taq* DNA polymerase catalyses primer extension as complementary nucleotides are incorporated

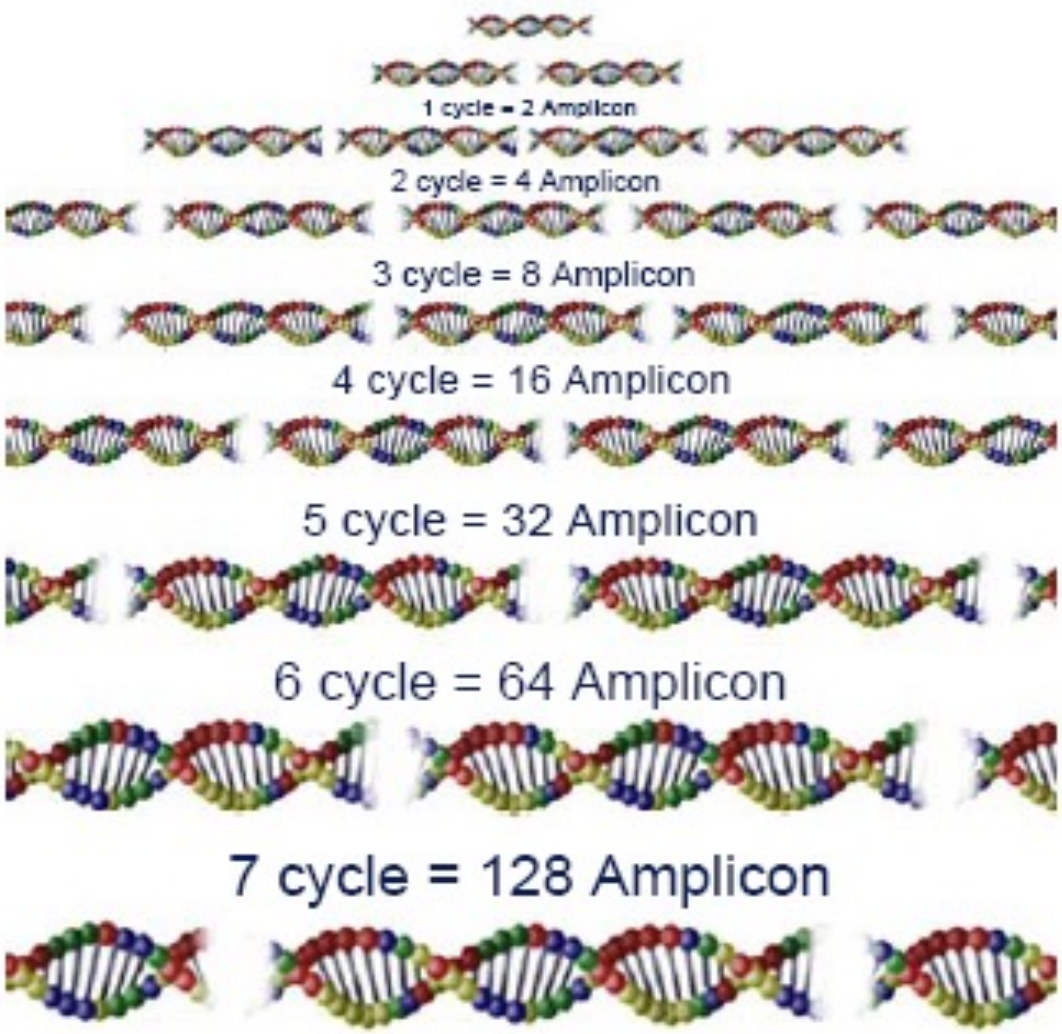


End of the 1st PCR Cycle –

Results in two copies of target sequence

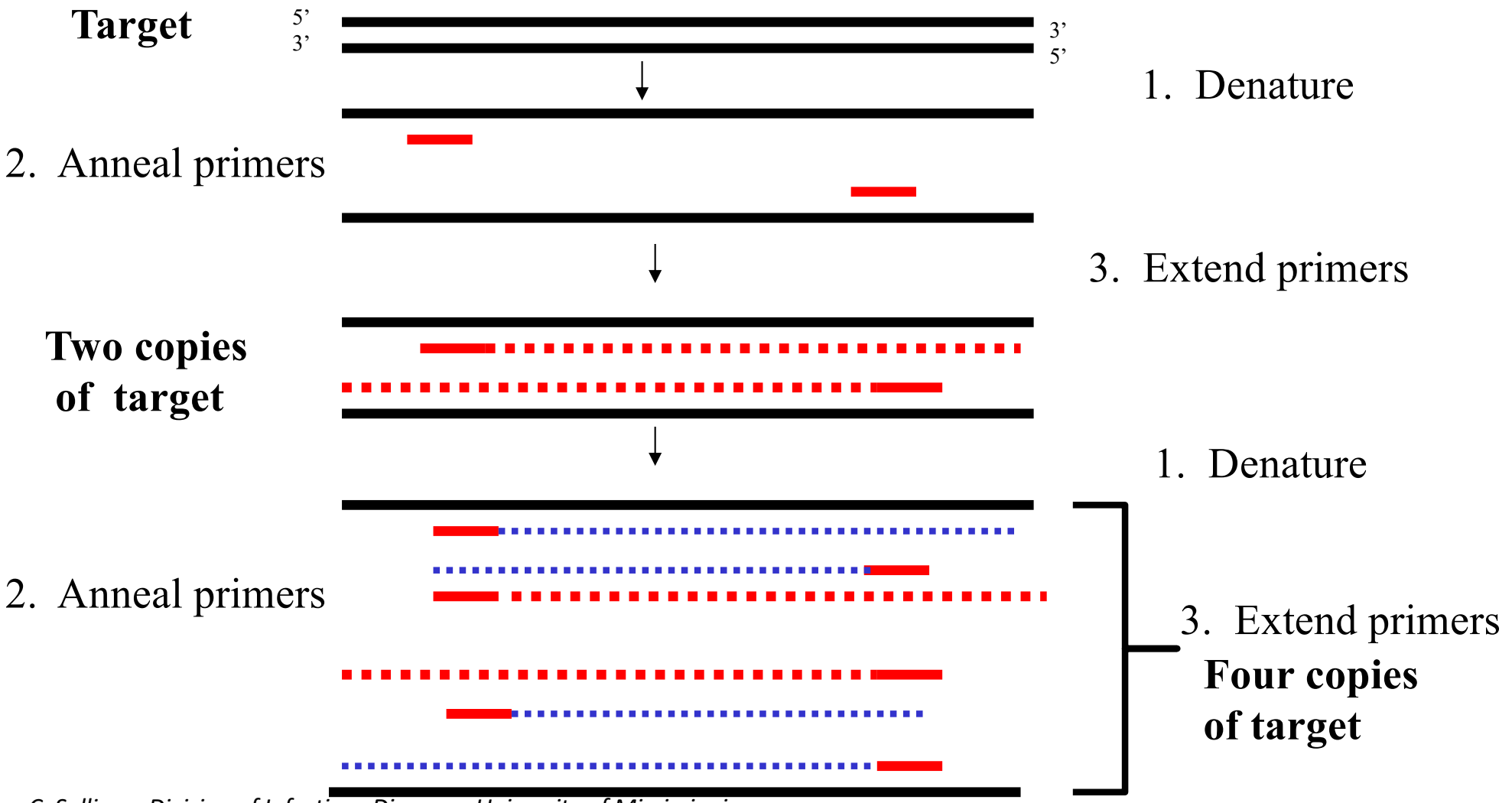


Target Amplification



No. of Cycles	No. Amplicon Copies of Target
1	2
2	4
3	8
4	16
5	32
6	64
20	1,048,576
30	1,073,741,824

AMPLIFICATION BY PCR



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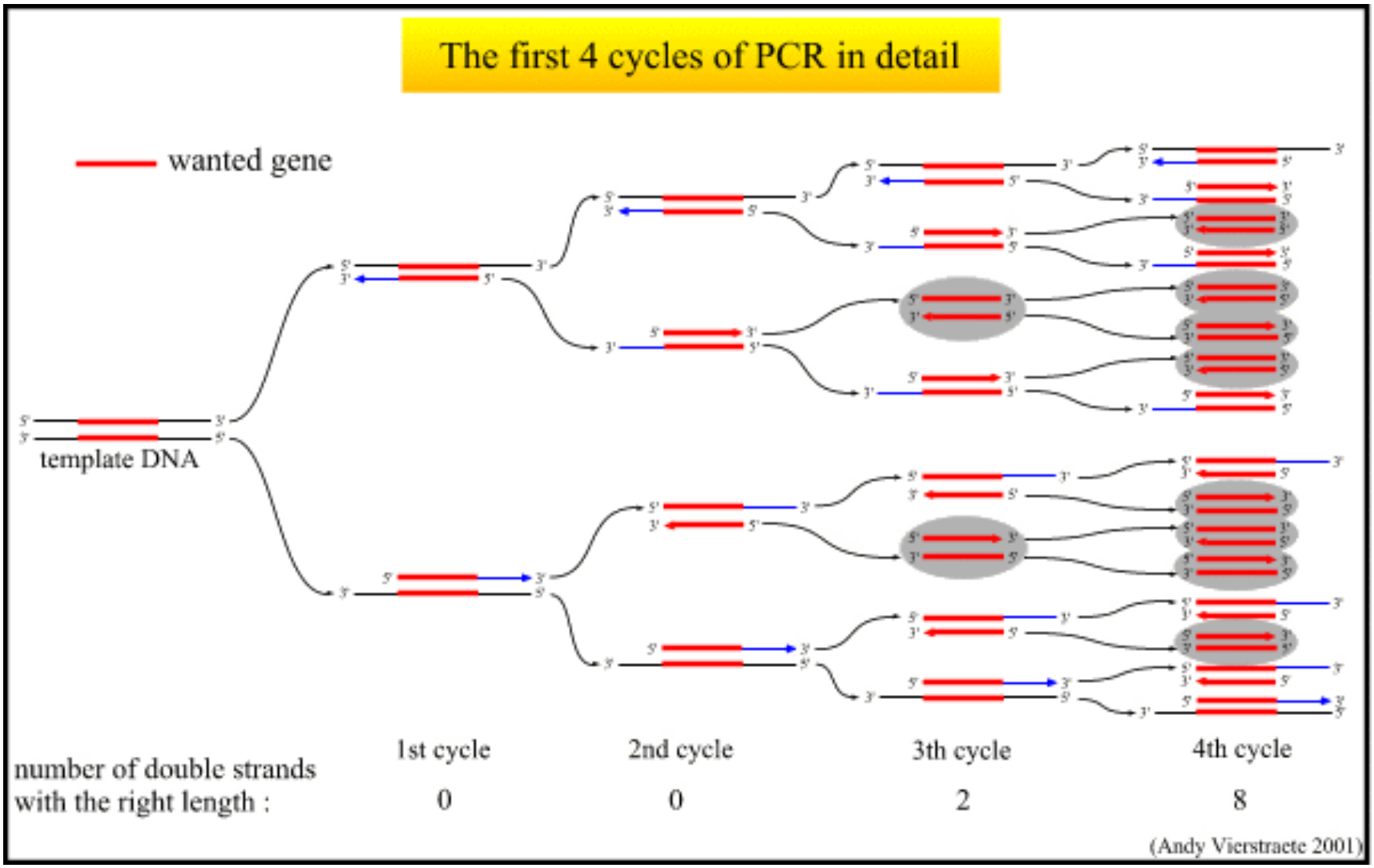
PCR: First 4 Cycles



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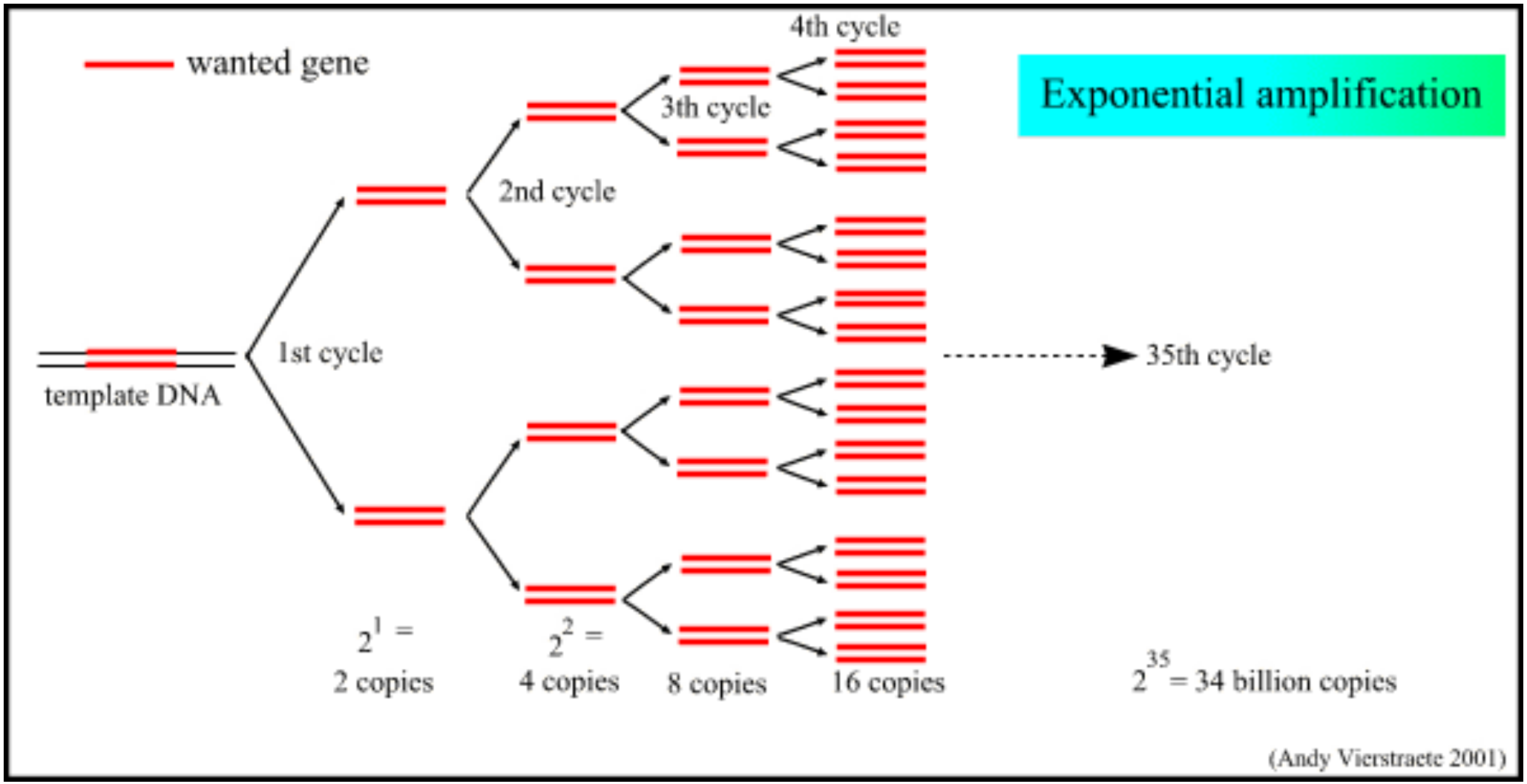
PCR: Completed Amplification Cycle



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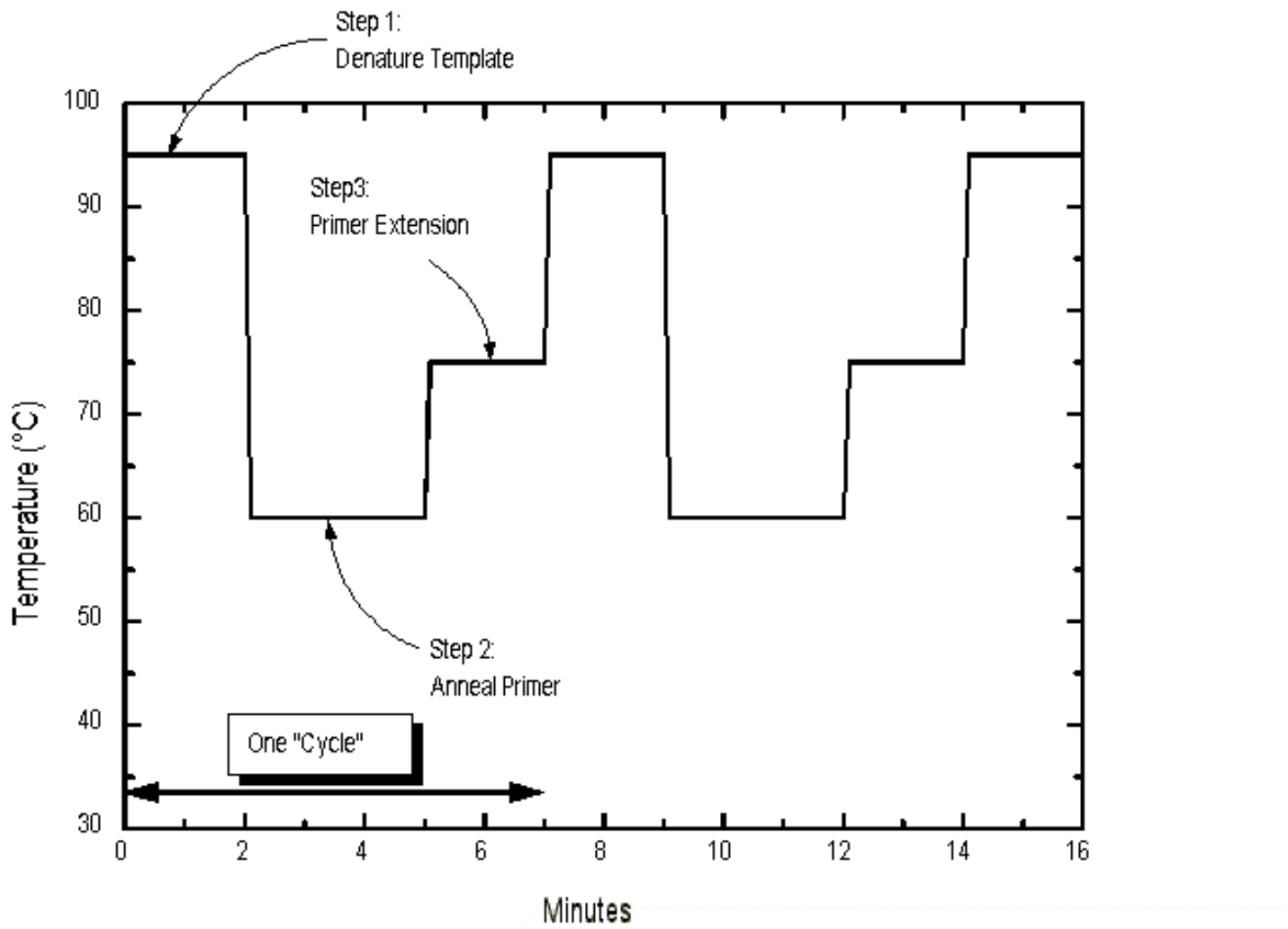
Standard Thermocycle of PCR

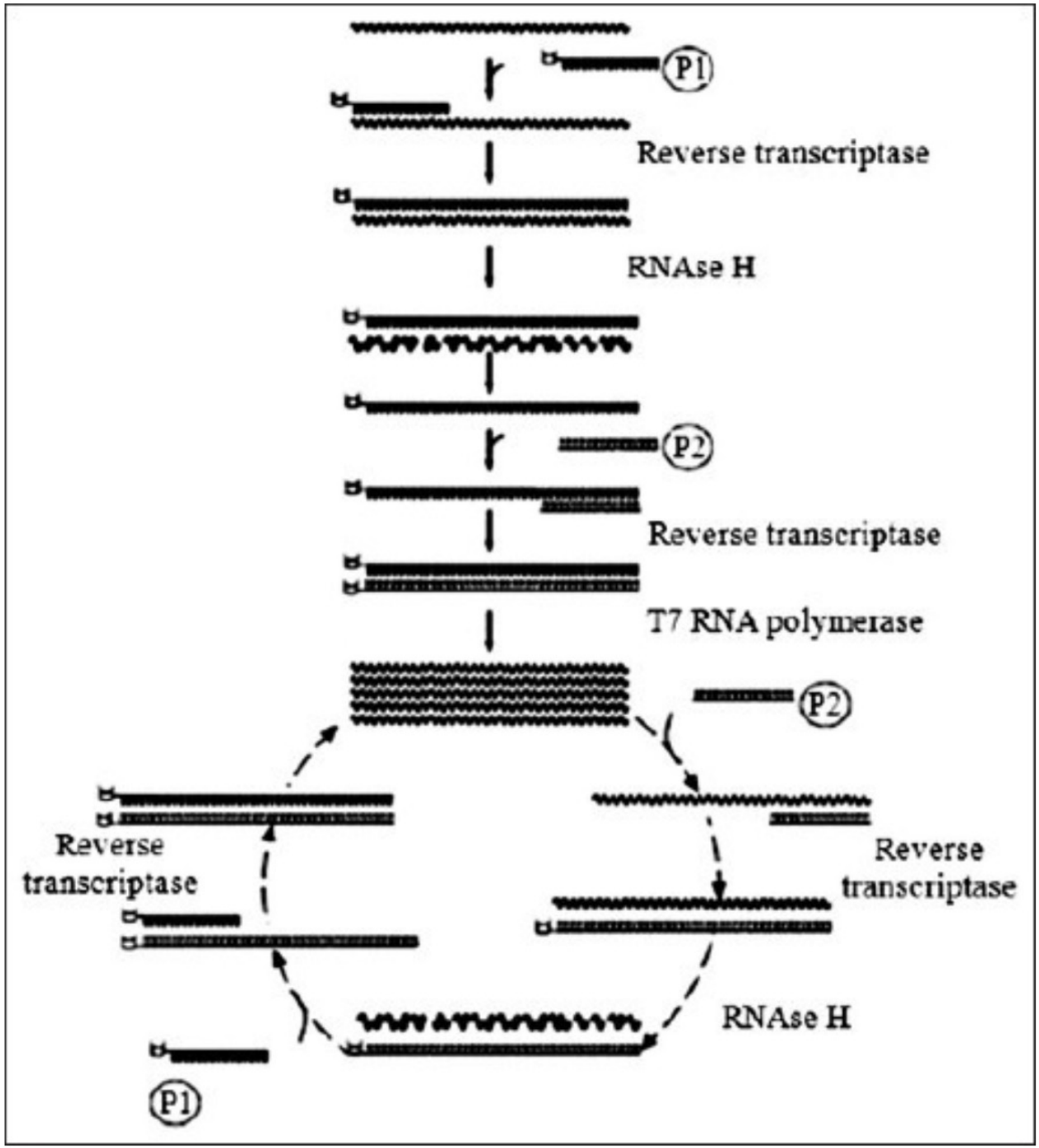


RT-PCR

- Reverse Transcriptase PCR
- Uses RNA as the initial template
- RNA-directed DNA polymerase (rTh)
- Yields ds cDNA

.

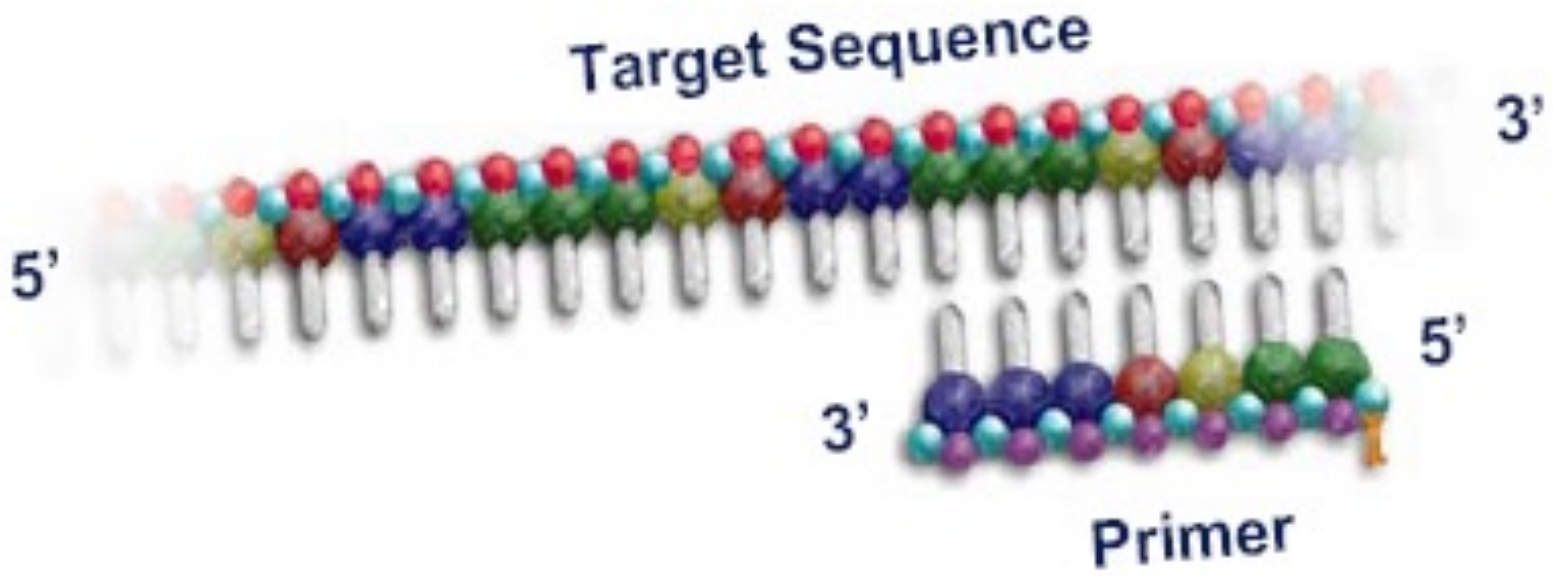
If RNA is to be detected, first transform to DNA:



Principles of nucleic acid sequence based amplification

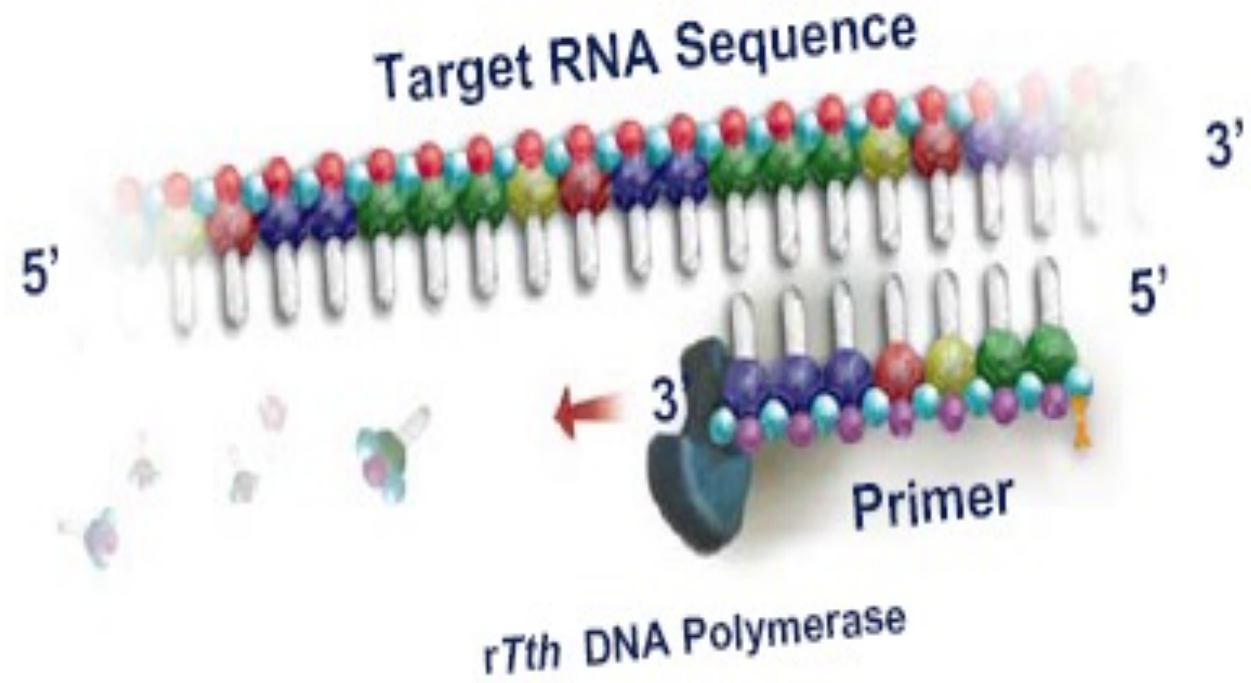
Reverse Transcription - Step 1 –

Primer Anneals to Target RNA Sequence



Reverse Transcription - Step 2 –

r*Tth* DNA Polymerase also has RT activity Catalyses Primer Extension by Incorporating Complementary Nucleotides

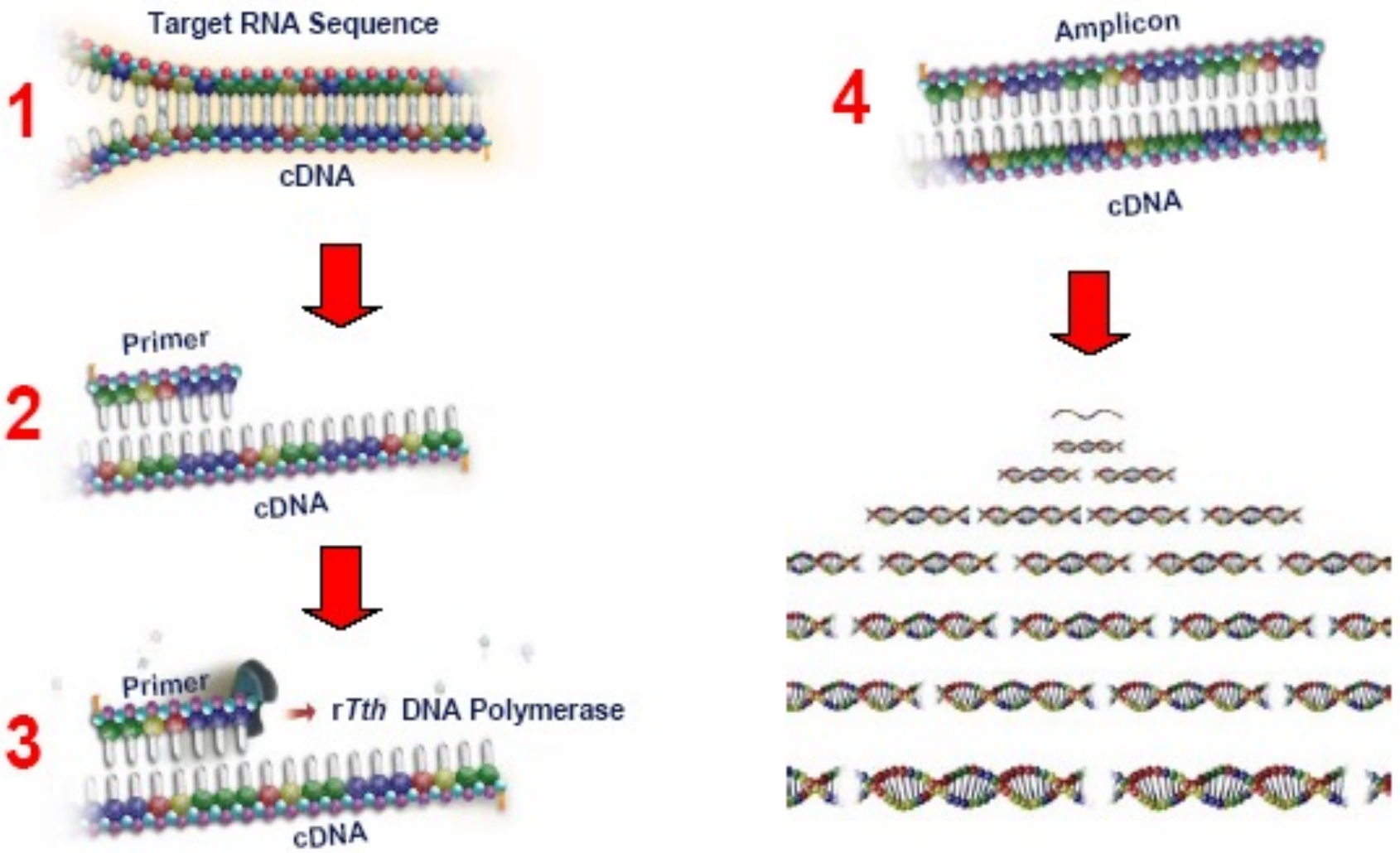


PCR Step 1 - Denaturation by Heat

PCR Step 2 - Annealing of Primer to cDNA

PCR Step 3 - rT*th* DNA Polymerase Catalyses Primer Extension

End of 1st PCR Cycle - Yields a Double-Stranded DNA Copy (Amplicon) of the Target Sequence



Catherine Bangeranye (Hunter CUNY)

Detection of amplification products

- Gel electrophoresis
- Sequencing of amplified fragment
- Southern blot
- etc...

PCR Primers

- Primers are single-stranded 18–30 b DNA fragments complementary to sequences flanking the region to be amplified.
- Primers determine the specificity of the PCR reaction.
- The distance between the primer binding sites will determine the size of the PCR product.

Primer Design

- Types of primers
  - Random
  - Specific
- Primer length
  - Annealing temperature
  - Specificity
- Nucleotide composition

Primer Design

- Not containing inverted repeat sequences to avoid formation of internal structures
- 40-60% GC content preferred for better annealing
- avoid repeated Gs longer than GGGG

Primer Design

- Melting Temperature: T_m of forward primer = T_m of reverse primer
- Tm of primers can be calculated to determine annealing T^0
- Tm= .41(%G+C) + 16.6log(J^+) + 81.5 where J^+ is the concentration of monovalent ions

- For short (14–20 bp) oligomers: Melting Temperature:

$$T_m = 4° (GC) + 2° (AT)$$

Avoiding Misprimes

- Use proper annealing temperature.
- Design primers carefully.
- Adjust monovalent cation concentration.
- Use hot-start: prepare reaction mixes on ice, place in preheated cycler or use a sequestered enzyme that requires an initial heat activation.
  - Platinum *Taq*
  - Ampli*Taq* Gold
  - HotStar*Taq*

A Standard PCR Reaction Mix

0.25 mM each primer

0.2 mM each dATP, dCTP, dGTP, dTTP

50 mM KCl

10 mM Tris, pH 8.4

1.5 mM $MgCl_2$

2.5 units polymerase

10^2 - 10^5 copies of template

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50 ml reaction volume

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PCR Cycle: Temperatures

- Denaturation temperature
  - Reduce double stranded molecules to single stranded molecules
  - 90–96°C, 20 seconds
- Annealing temperature
  - Controls specificity of hybridization
  - 40–68°C, 20 seconds
- Extension temperature
  - Optimized for individual polymerases
  - 70–75°C, 30 seconds

Combinations Of Cycle Temperatures

TEMP	FOR	COMMENTS
94-60-72	Perfect, long primers	Higher temp can be used; maximum annealling temp
94-55-72	Good or perfectly matched primers between 19-24 nt	Standard conditions
94-50-72	Adequate primers	Allows 1-3 mismatches/20 nt
94-48-68	Poorly matched primers	Allows 4-5 mismatches/20 nt
94-45-65	Unknown match, likely poor	Primers of questionable quality, long-shot PCR
94-37-65	Hail Mary	Uncontrolled results

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Amplification Reaction

- Amplification takes place as the reaction mix is subjected to an amplification program.
- The amplification program consists of a series of 20–50 PCR cycles.

Automation of PCR

- PCR requires repeated temperature changes.
- The thermal cycler changes temperatures in a block or chamber holding the samples.
- Thermostable polymerases are used to withstand the repeated high denaturation temperatures.

PCR thermocycler

Gradient PCR thermocycler

Real-time PCR thermocycler (qPCR)

Digital PCR

Product Cleanup

- Gel elution
  - Removes all reaction components as well as misprimes and primer dimers
- Solid phase isolation of PCR product (e.g., spin columns)
- DNA precipitation

Contamination Control

- Any molecule of DNA containing the intended target sequence is a potential source of contamination.
- The most dangerous contaminant is PCR product from a previous reaction.
- Laboratories are designed to prevent exposure of pre-PCR reagents and materials to post-PCR contaminants.

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Contamination of PCR Reactions

- Most common cause is carelessness and bad technique.
- Separate pre- and post-PCR facilities.
- Dedicated pipettes and reagents.
- Change gloves.
- Aerosol barrier pipette tips.
- Meticulous technique
- **10% bleach, acid baths, UV light**
- **Dilute extracted DNA.**

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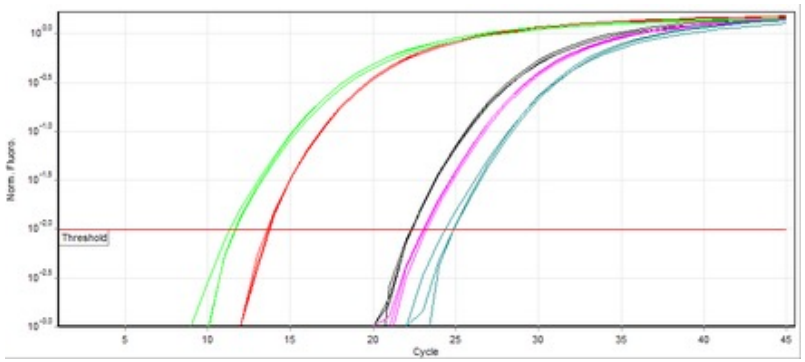
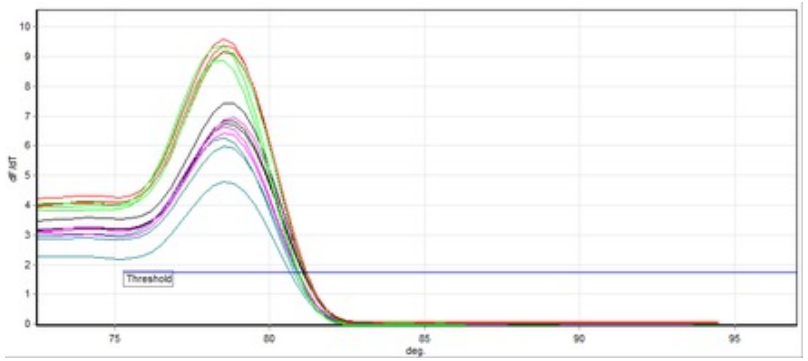
Real-Time (or Quantitative) PCR

- Monitors the quantity of amplification of a targeted DNA strand during the PCR
- Methods used for the detection of PCR products in real-time PCR:
  - (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA and
  - (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence



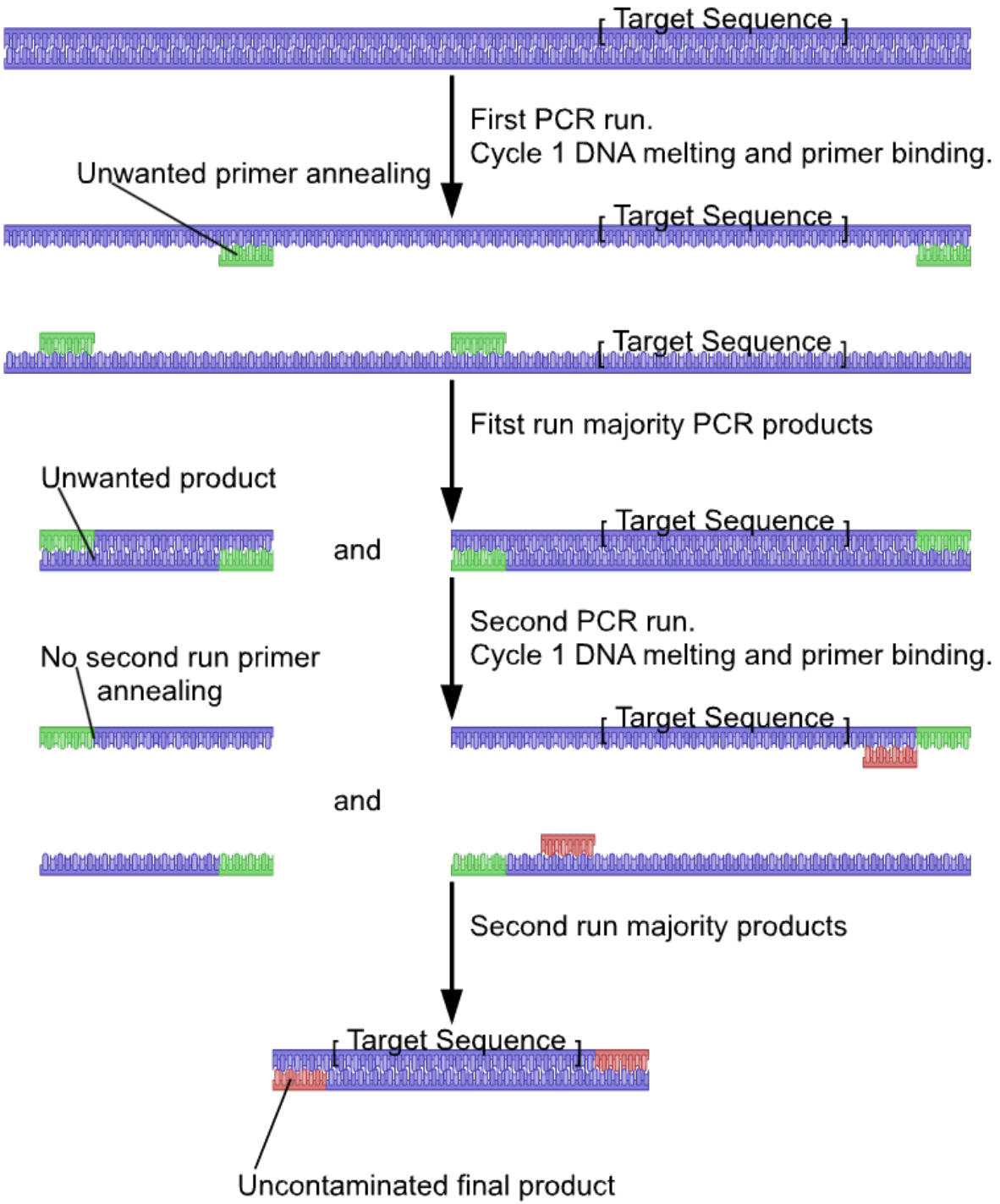
- fluorescence chart produced in real-time PCR



Melting curve produced at the end of real-time PCR

Nested PCR

- **Repeat PCR using a second pair of inner primers.**
- **Reduces primer matching errors.**



Isothermal PCR Amplification Protocols

Isothermal : Do not require changing the reaction.

- So do not require themocyclers.

- Usually very fast.
- Usually use a strand displacement polymerase.

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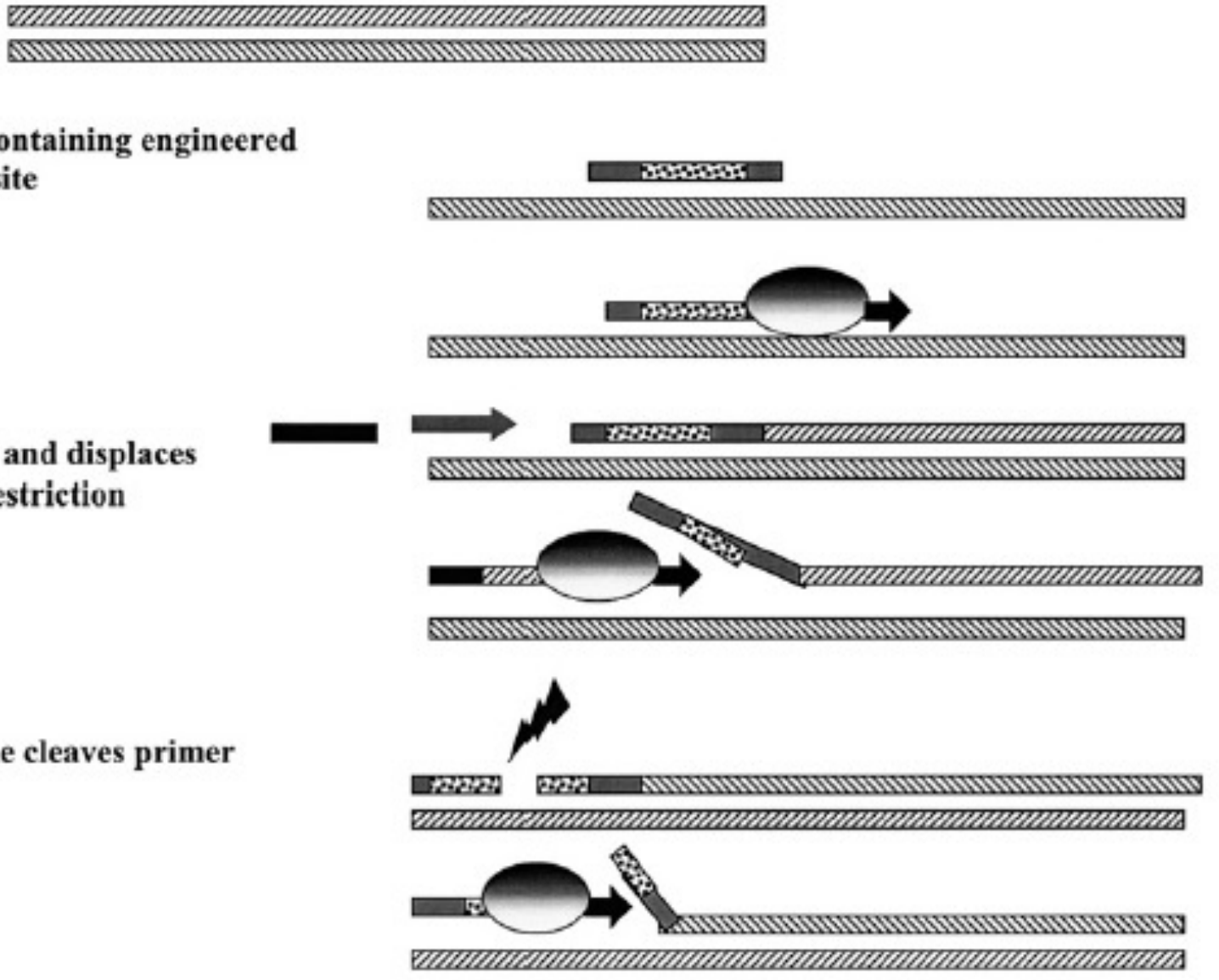
Strand Displacement Polymerase



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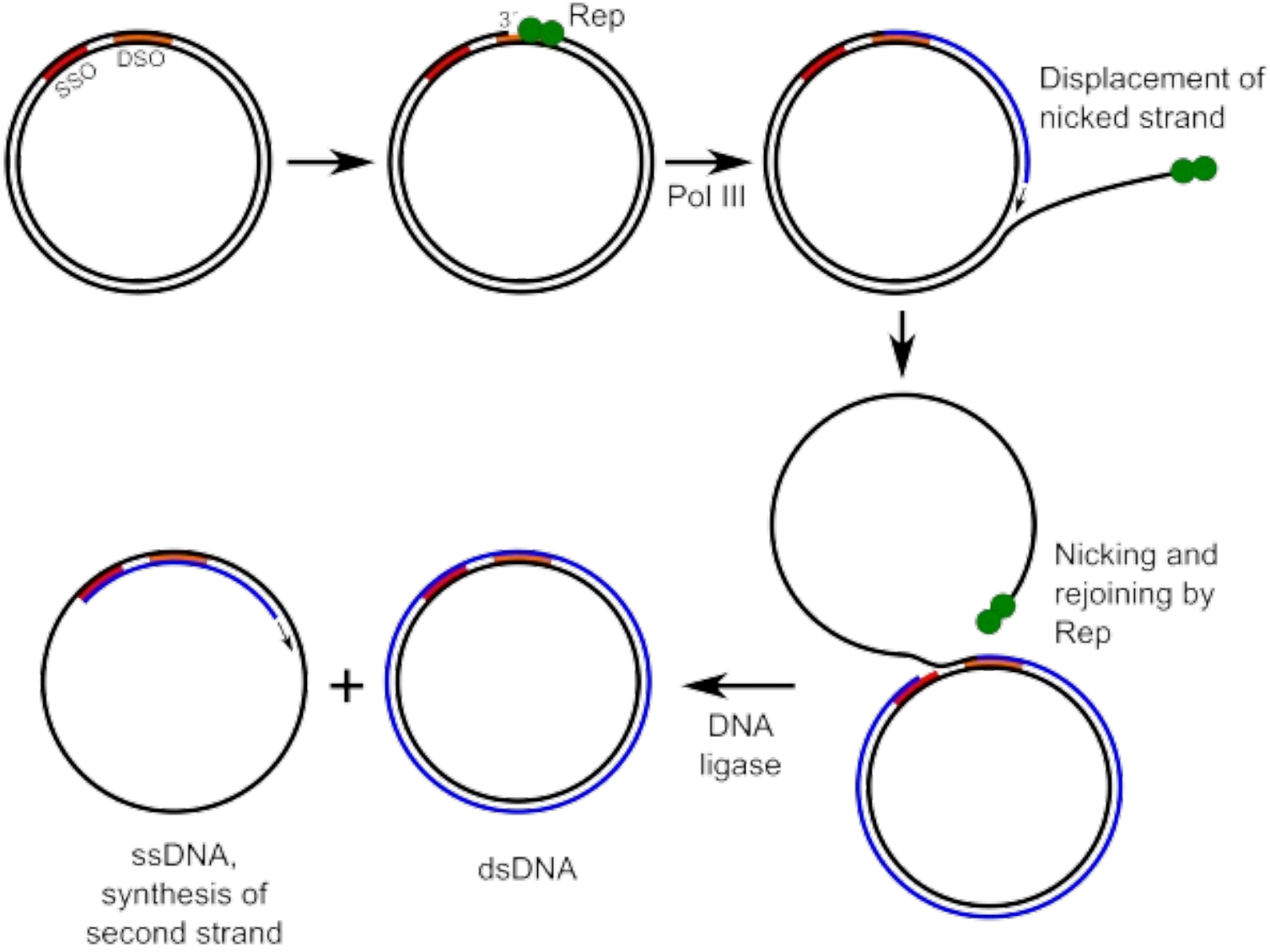
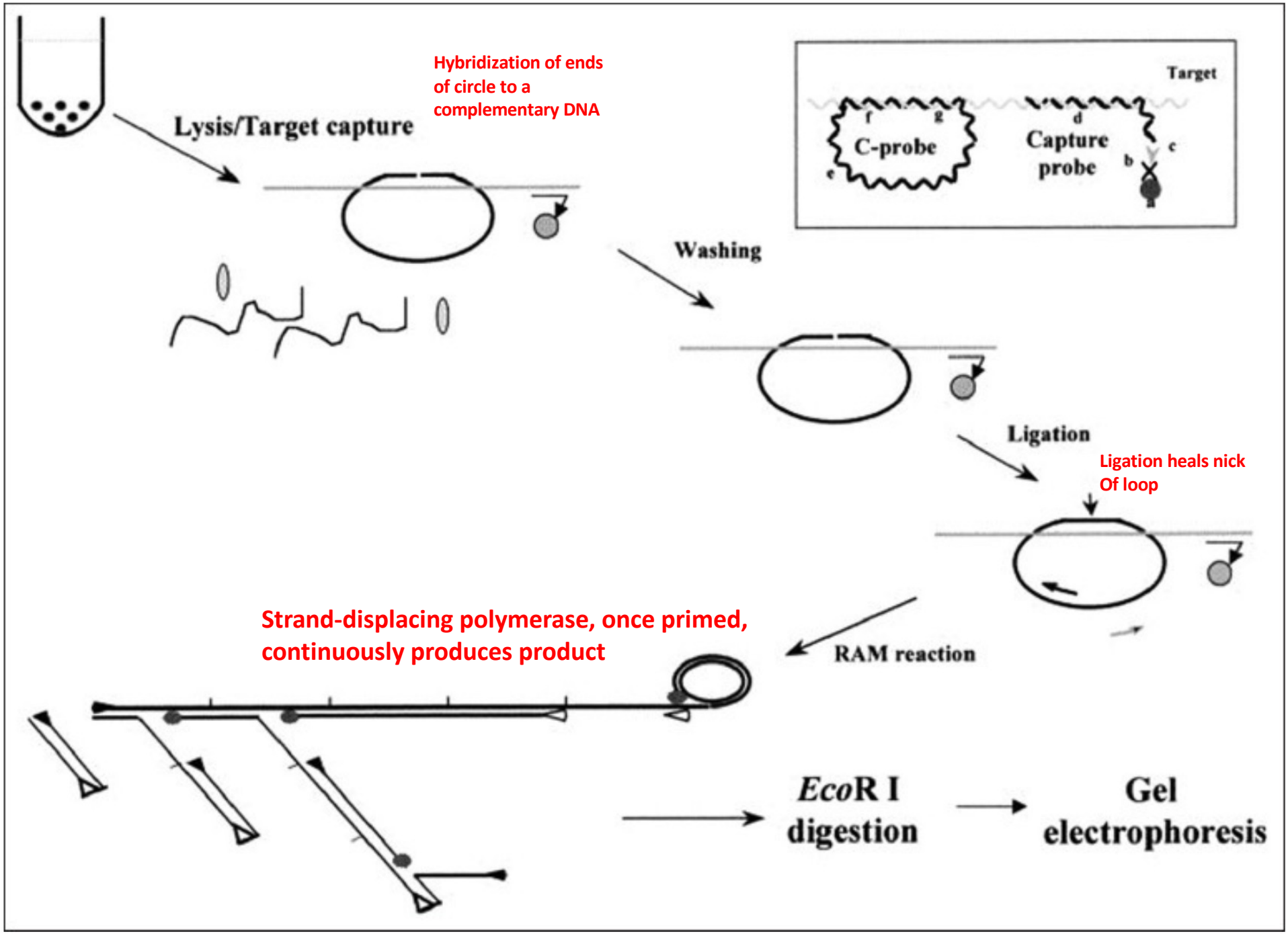
Rolling Circle PCR: An Isothermal PCR Amplification Protocol:

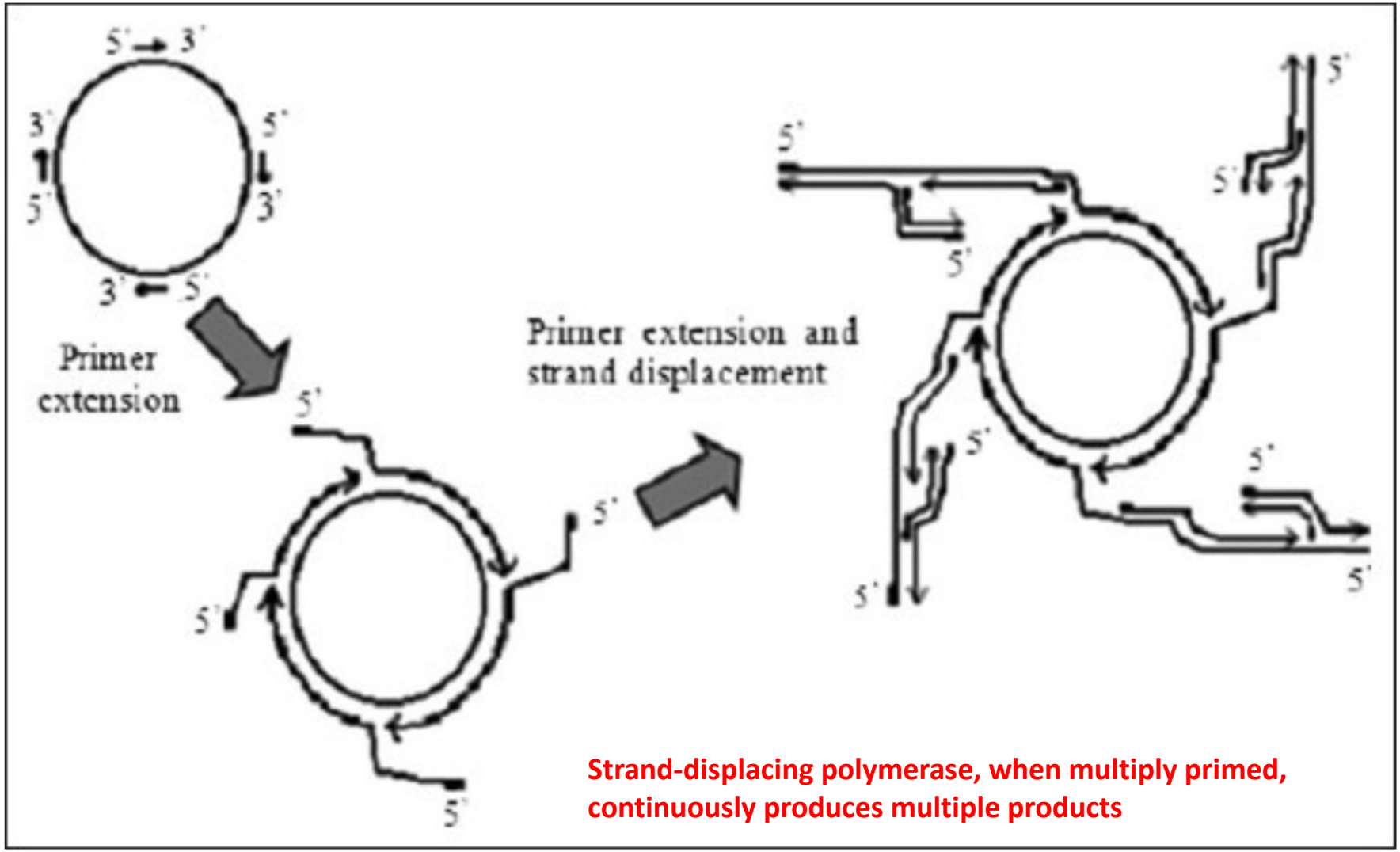
- Circular dsDNA (double-stranded) is synthesized
- Circular dsDNA is "nicked" by a nicking enzyme.
- The 3' end is elongated using "unnicked" DNA
- as leading strand (template)
- 5' end of ssDNA product is displaced, forming ssDNA product



Isothermal PCR Amplification Protocol: Rolling Circle PCR



Isothermal PCR Amplification Protocol: Rolling Circle PCR



multiply-primed rolling circle amplification

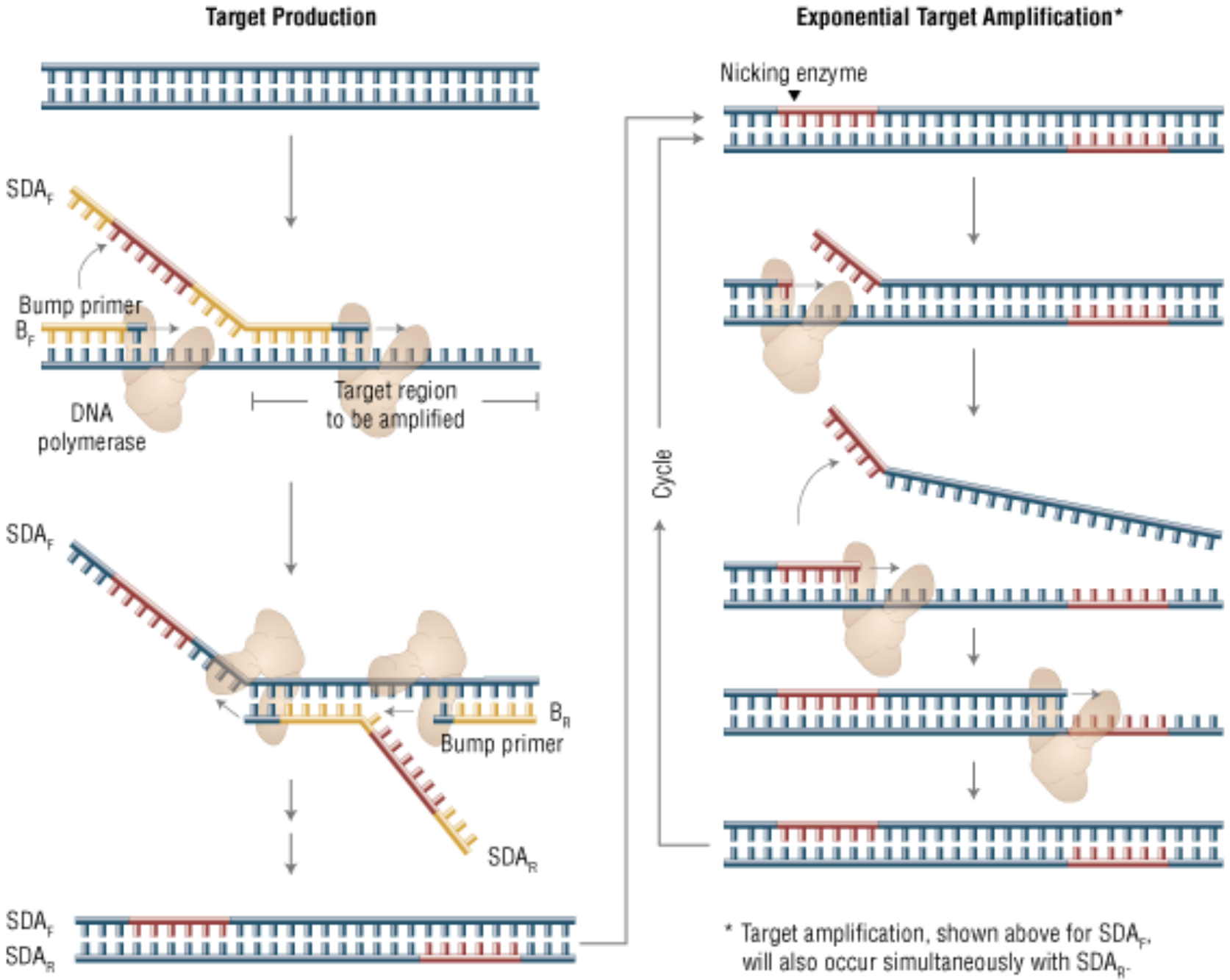
Isothermal PCR Amplification Protocol:

Strand displacement amplification (SDA)

- Nicks are created by a strand-limited restriction endonuclease or nicking enzyme at a site contained in a primer.
- Uses a strand-displacing DNA polymerase, typically *Bst*, to initiate replication at nicking sites, so target DNA is regenerated with each polymerase displacement step, resulting in exponential amplification.
- Used in clinical diagnostics.

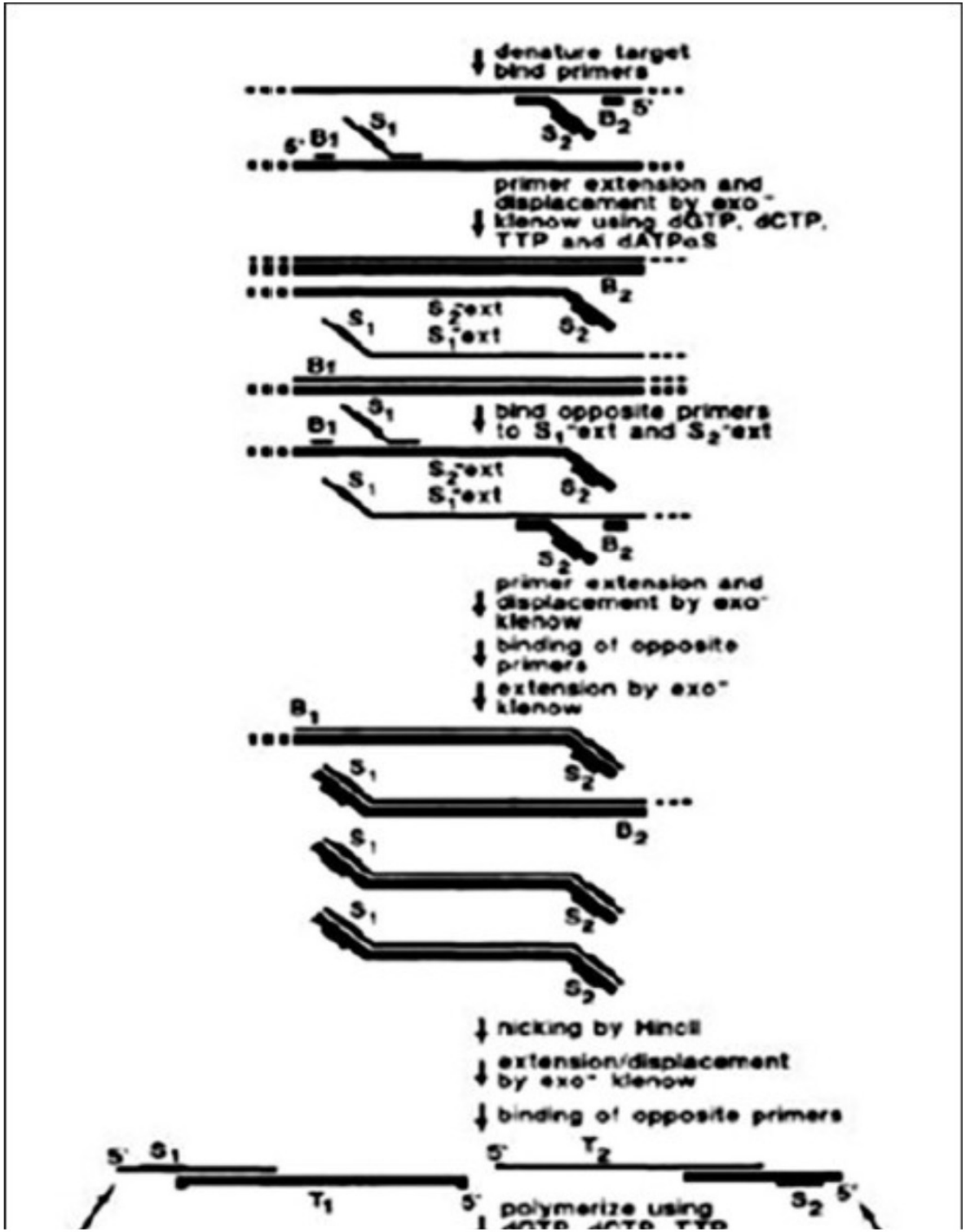
New England BioLabs Website

Strand displacement amplification (SDA)



New England BioLabs Website

Strand displacement amplification (SDA)



Isothermal PCR Amplification Protocol:

Nicking enzyme amplification reaction (NEAR)

- Uses a strand-displacing DNA polymerase initiating replication at a nick created by a nicking enzyme,
- Produces many short nucleic acids from the target sequence.
- Used for pathogen detection in clinical and biosafety applications.

New England BioLabs Website

Isothermal PCR Amplification Protocol:

Loop-mediated isothermal amplification (LAMP)

- A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification.
- LAMP is rapid, sensitive
- magnesium pyrophosphate produced during the reaction can be seen by eye, making LAMP well-suited for field diagnostics.

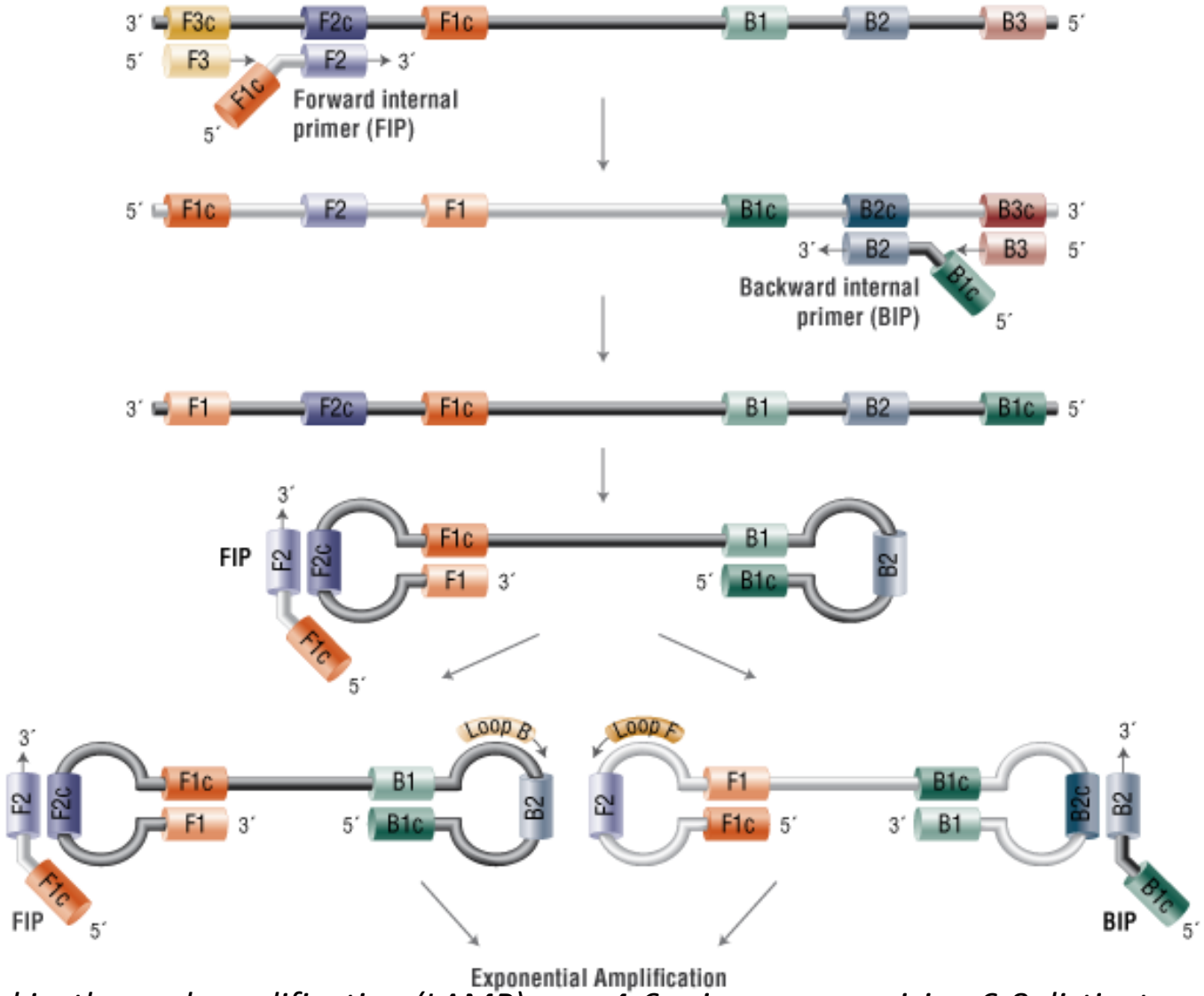
New England BioLabs Website

Loop-mediated isothermal amplification (LAMP)

- **Amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions, employing a DNA strand-displacing polymerase and two primer pairs.**
- **An inner primer containing sequences of the sense and anti-sense strands of the target DNA initiates LAMP.**
- **The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA.**
- **This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure.**
- **In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA and a new stem–loop DNA with a stem twice as long.**
- **The cycling reaction continues with accumulation of 109 copies of target in less than an hour.**
- **The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand.**

Isothermal PCR Amplification Protocol:

Loop-mediated isothermal amplification (LAMP)



Loop-mediated isothermal amplification (LAMP) uses 4-6 primers recognizing 6-8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification

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Initiation of loop-mediated isothermal amplification (LAMP)

When the target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60-65°C, the following reaction steps proceed:

STEP1: As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA.

STEP2: Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP.

STEP3: The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand.

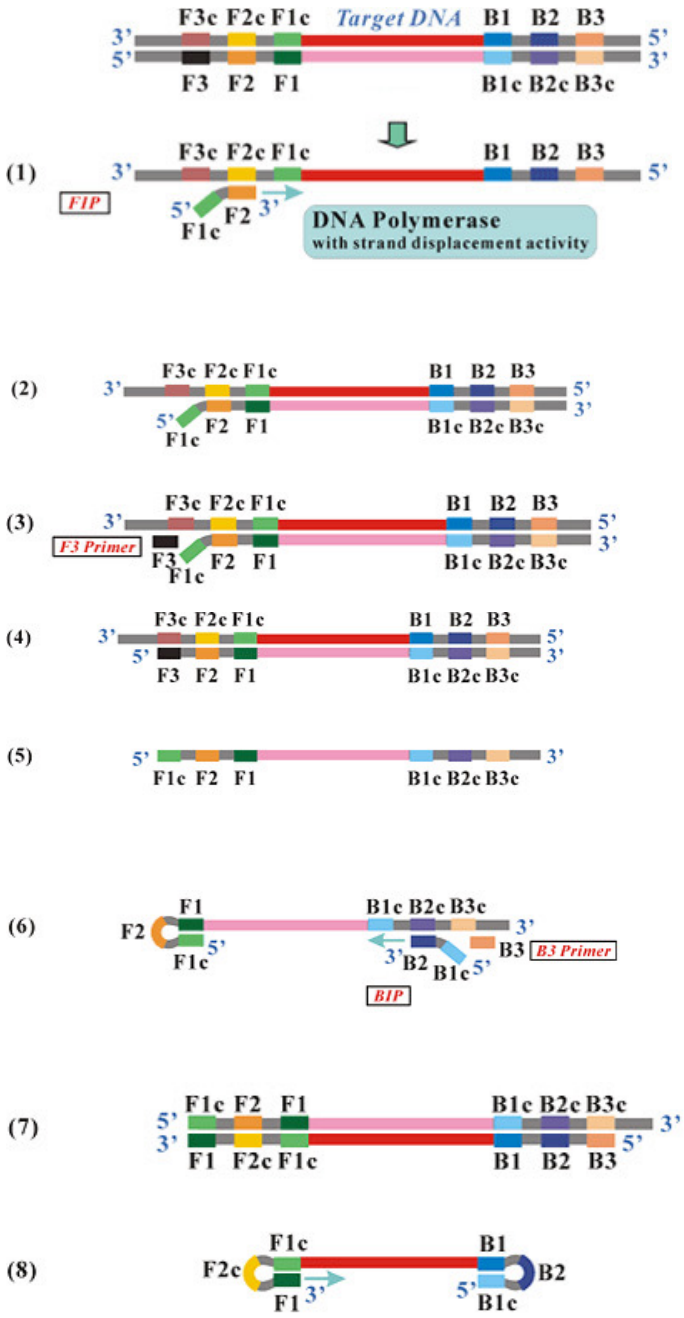
STEP4: A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand.

STEP5: The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, this released single strand forms a stem- loop structure at the 5' end because of the complementary F1c and F1 regions.

STEP6: This single strand DNA in Step (5) serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in Step (5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer.

STEP7: Double stranded DNA is produced through the processes described in Step (6).

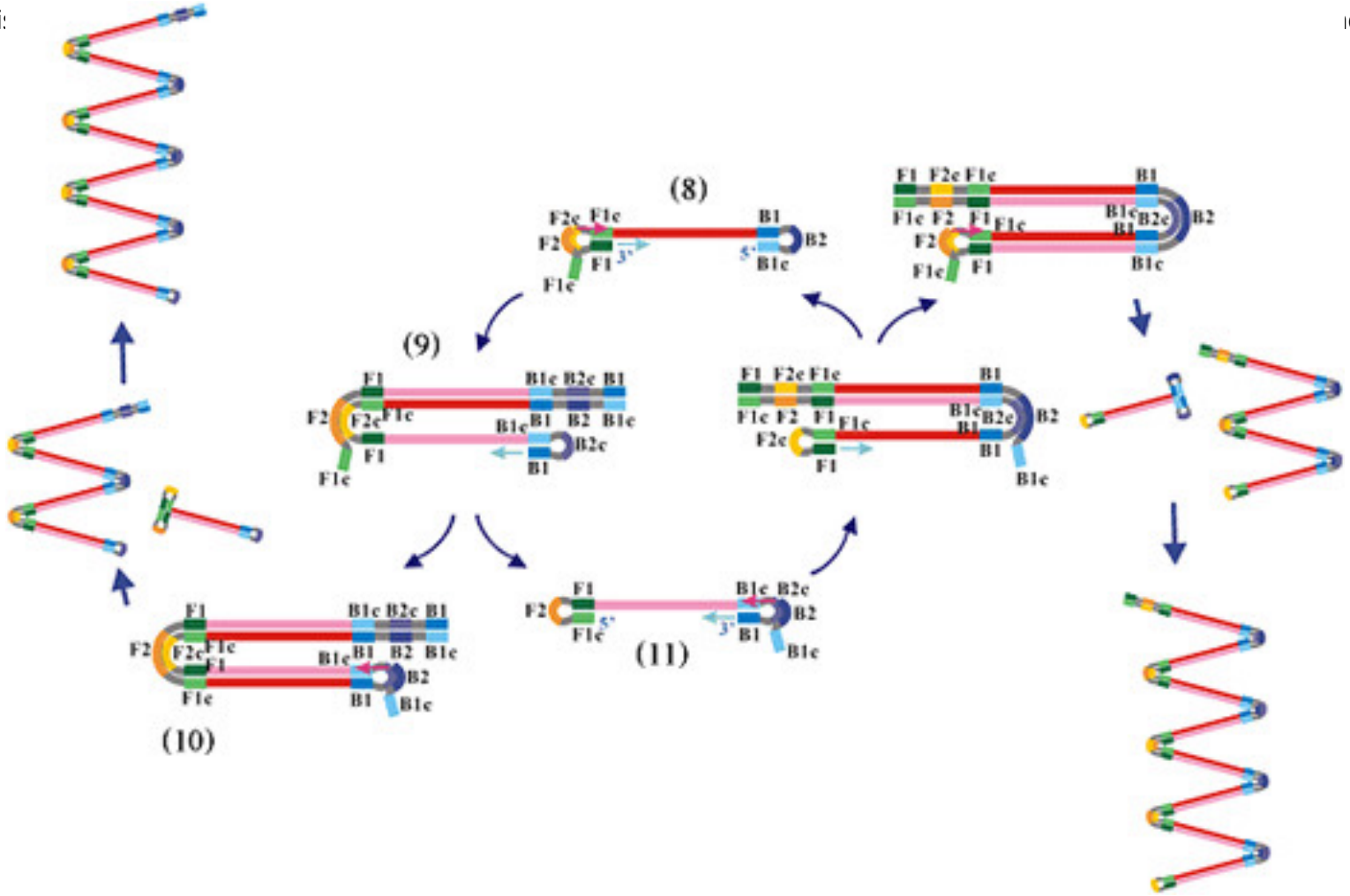
STEP8: The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP method (LAMP cycling). **The above process can be understood as producing the starting structure for LAMP cycling.**



Further Details of loop-mediated isothermal amplification (LAMP), Cont

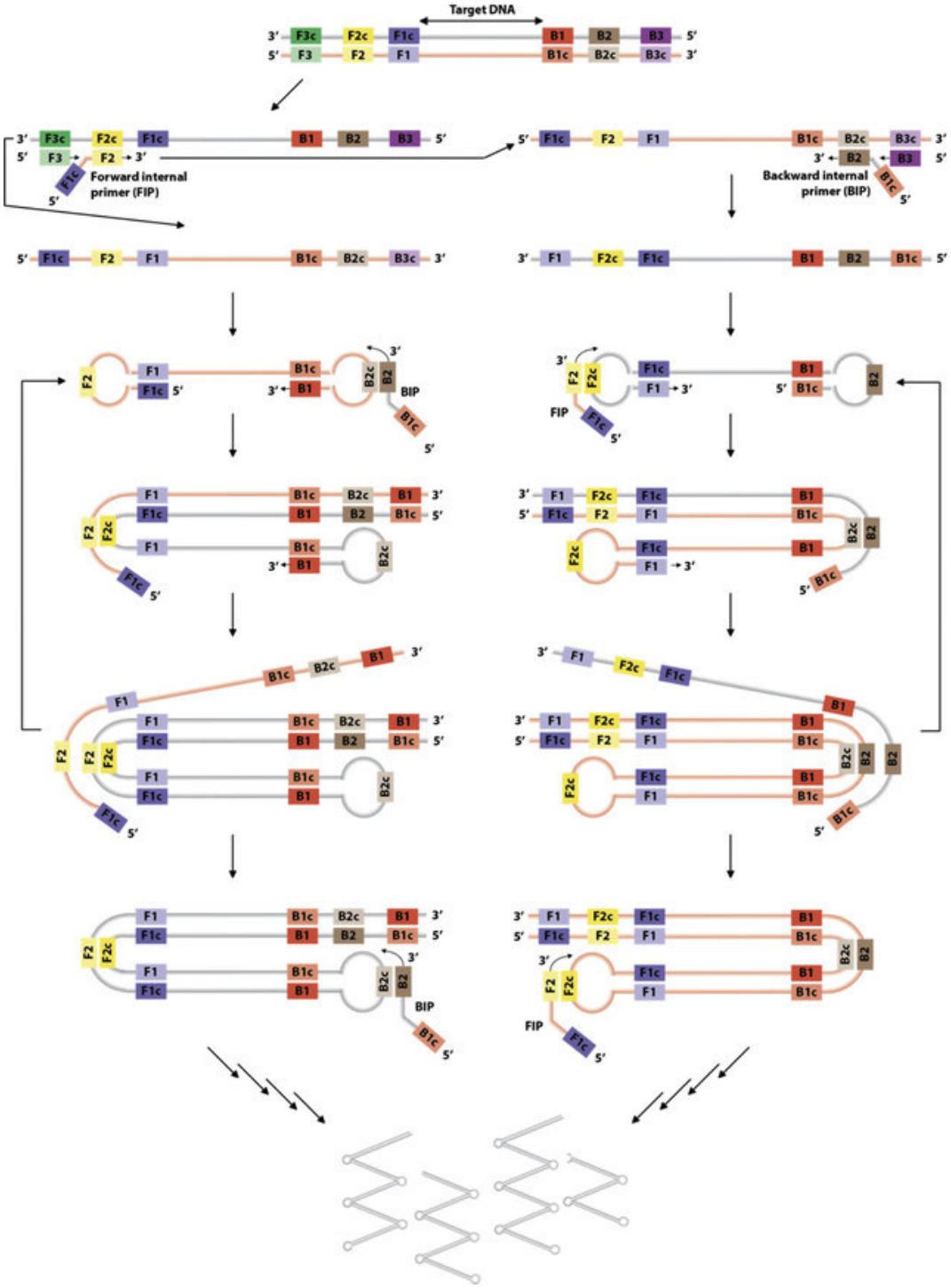
Basic principle (8) - (11) (Cycling amplification step)

A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (Step (9)). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (Step (11)). This structure is the 'turn over' structure of the structure formed in Step (8). Similar to the Steps from (8) to (11), structure in Step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to Steps (9) and (10) as well as the same structure as Step (8) are produced. With the structure produced in Step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of thi e strand are formed.



[Eiken GENOME SITE]

Loop-mediated isothermal amplification (LAMP)



Loop-mediated isothermal amplification (LAMP)

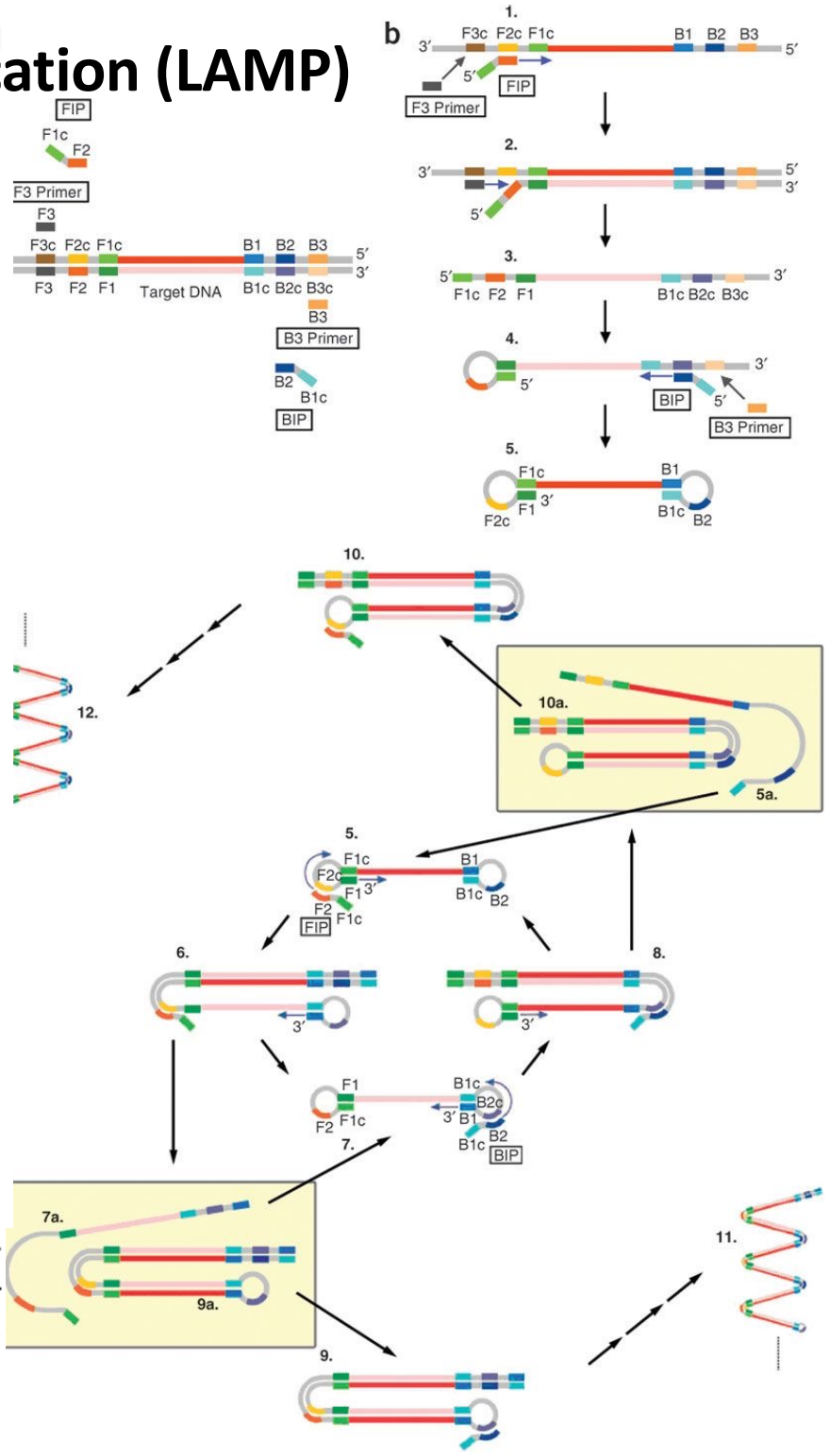


Fig. 1. LAMP assay (a) primer design of the LAMP reaction. For ease of explanation, six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c, and B3 from the 5′ end. As c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence. (b) Starting structure producing step. DNA synthesis initiated from FIP proceeds as follows. The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 3′ end (structure 3). DNA synthesis proceeds with the single-strand DNA as the template, and BIP and B3 primer, in the same manner as described earlier, to generate structure 5, which possesses the loop structure at both ends (dumbbell-like structure). (c) Cycling amplification step. Using self-structure as the template, self-primed DNA synthesis is initiated from the 3′ end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5, and structure 5 is produced from structure 8 in a reaction similar to that which led from structures 5–7. Structures 9 and 10 are produced from structures 6 and 8, respectively, and more elongated structures (11, 12) are also produced. (Tomita *et al.*, 2008).

Arnost Cepica, Animal Health Research Reviews · April 2015

Isothermal Amplification Protocol:

Loop-mediated isothermal amplification (LAMP)

Animations of LAMP:

- **Brief Overviews of LAMP (view suggested):**

https://www.youtube.com/watch?v=L5zi2P4lggw

https://www.youtube.com/watch?v=RkSqI_mB-8E

- **Details of LAMP (view suggested):**

https://www.youtube.com/watch?v=ZXq756u1msE

- **Details & Primer Design & Experimental Instructions for LAMP:**
  https://www.youtube.com/watch?v=GJkvQqDufh0

Loop-mediated isothermal amplification (LAMP)

Basic LAMP Protocol Design Guidelines:

- Reaction Temperature ~ 65C
- If (F1, F1c) are the stem of each loop in LAMP:

(1) The length of the stem (F1, F1c) of each loop in LAMP to form hairpins is ~ 25 nt

=> Hairpin stem have higher melting temperature than reaction (65 C) so loop is immediately formed.

(2) Each amplification primer for (F1, F1c) is also ~ 25 nt.

=> FIP/BIP has a melting temperature (~60C - 65C) near to reaction temperature

Loop-mediated isothermal amplification (LAMP)

Basic Primer Design Guidelines

Multiple Characteristics Influence Primer Performance

- Primers are specified 5′ to 3′, left to right.
- 40-60% GC Content
- Amplicon ≤280 base pairs
- Avoid runs of 3 or more of one base, or dinucleotide repeats (e.g. ACCC or ATATATAT), both can cause mis-priming. Runs of 3 or more Gs (AGGG may cause issues with synthesis and HPLC purification.
- Primer pairs should have similar Tms with a maximum difference of 5°C and should not be complementary to each other.

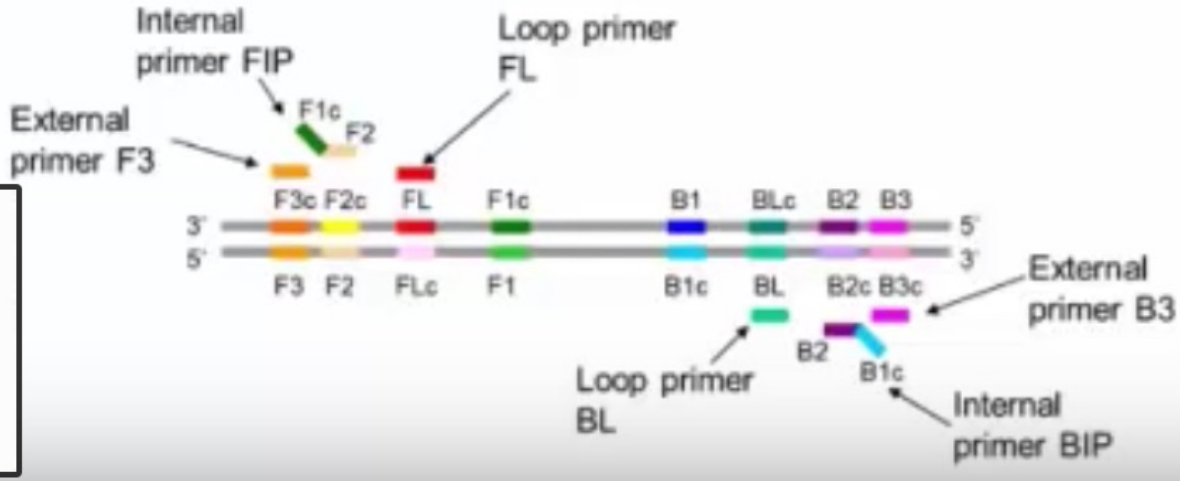
Loop-mediated isothermal amplification (LAMP)

Primer Design Overview

Key Factors Include Tm, Length and Distances

Primer(s)	Length (mer)	Tm (°C)
F3/B3	15-25	55-63
F2/B2	15-25	55-63
F1c/B1c	15-25	60-68
FL/BL	15-22	64-66

Distances	
(F2/B2)	120-160nt
Loop (F1C-F2)	40-60nt
F2-F3	0-60nt
F1c-B1C	0-100nt



Suggested Polymerases for LAMP

LavaLAMP™ Enzymes vs. Bst-like Enzymes

Enzymes have Different Temperature Optima

PrimerExplorer (http://primerexplorer.jp/lampv5e/index.html)
LAMP Designer (http://www.premierbiosoft.com/isothermal/lamp.html)

Both software were designed with Bst or Bst like enzyme in mind

Bst and Gsp DNA polymerase works best around 60°C-65°C

LavaLAMP enzymes works best around 68°C-74°C

Optical Output of LAMP

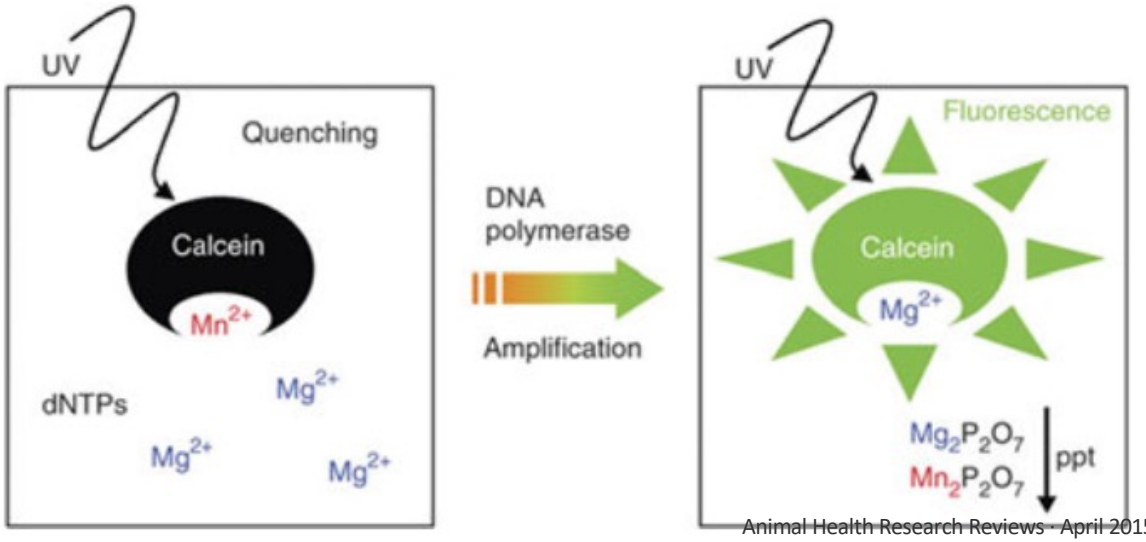


Fig. 2. The calcein dye detection method of the LAMP product. The dye, calcein, binds manganese ions that quench fluorescence. DNA polymerase LAMP results in the production of pyrophosphate that binds with the calcein bound manganese ions as well as magnesium ions resulting in two detection methods that indicate that the LAMP reaction was successful: fluorescent emissions from calcein and/or the production of manganese phosphate/ that forms a precipitate that is visually detected. The presence of magnesium ions in the buffer system of the LAMP system will enhance calcein fluorescence. (Tomita *et al.*, 2008).

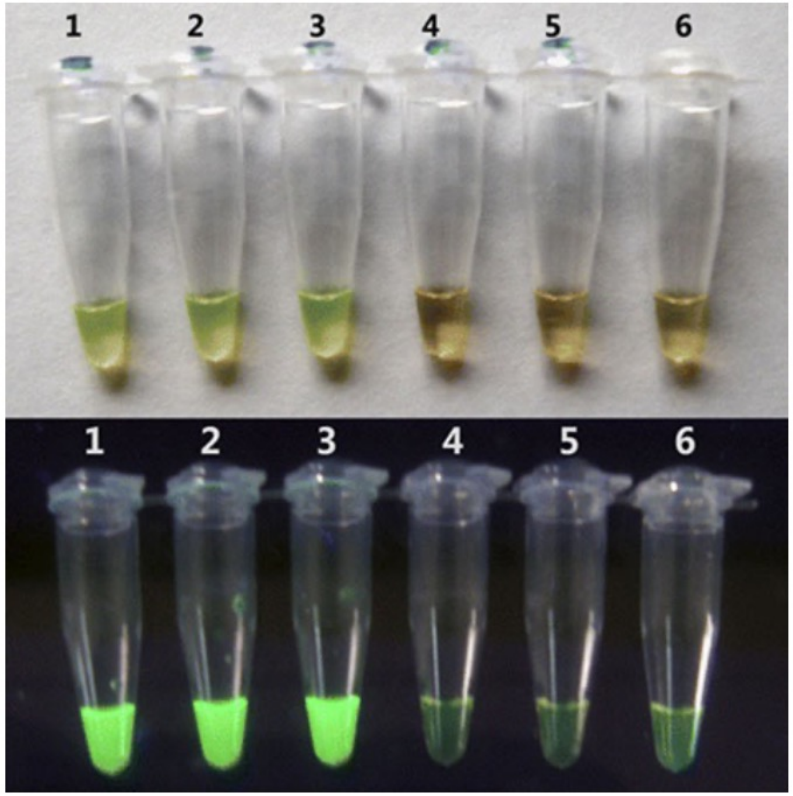
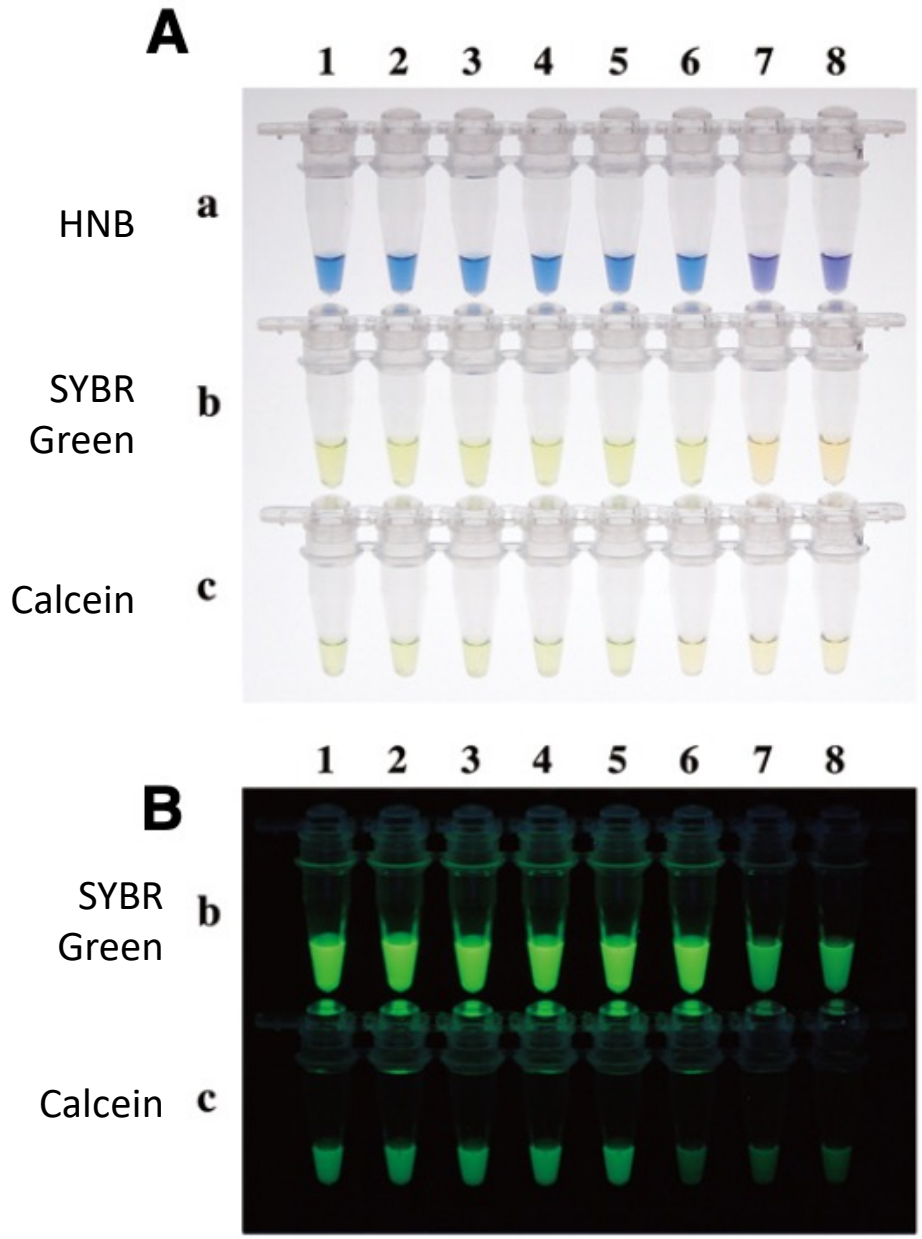
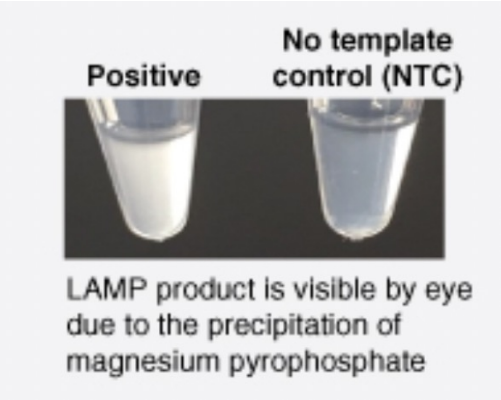


Fig. 3. Visual detection RT-LAMP with a fluorescent detection reagent. Ten-fold serial dilutions of viral RNA (lanes 1–6, 1–0.00001 PFU per reaction mixture, respectively) were detected. (a) Evaluation under normal light. The color of positive samples changed from orange to green, whereas the color of negative samples and the negative control remained orange. (b) Evaluation under UV light. Positive samples were bright green under UV light, while negative samples or the negative control remained weakly green. (Jiang *et al.*, 2011)

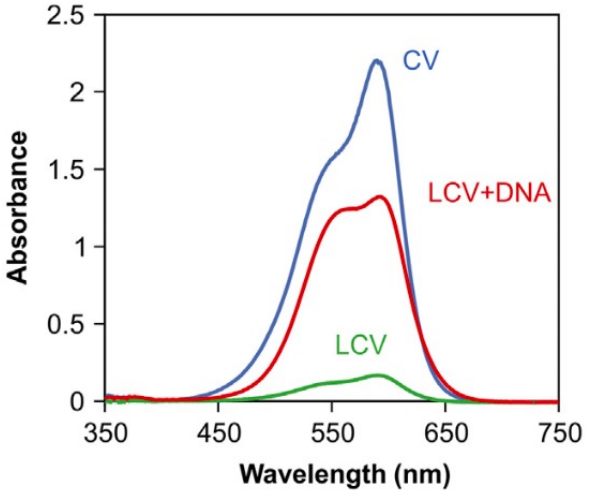


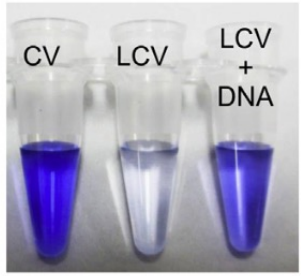
Goto, Motoki, et al. "Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue." *Biotechniques* 46.3 (2009): 167-172.

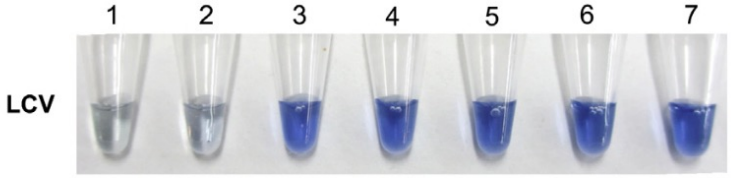
Turbidity



Leuco triphenylmethane dyes

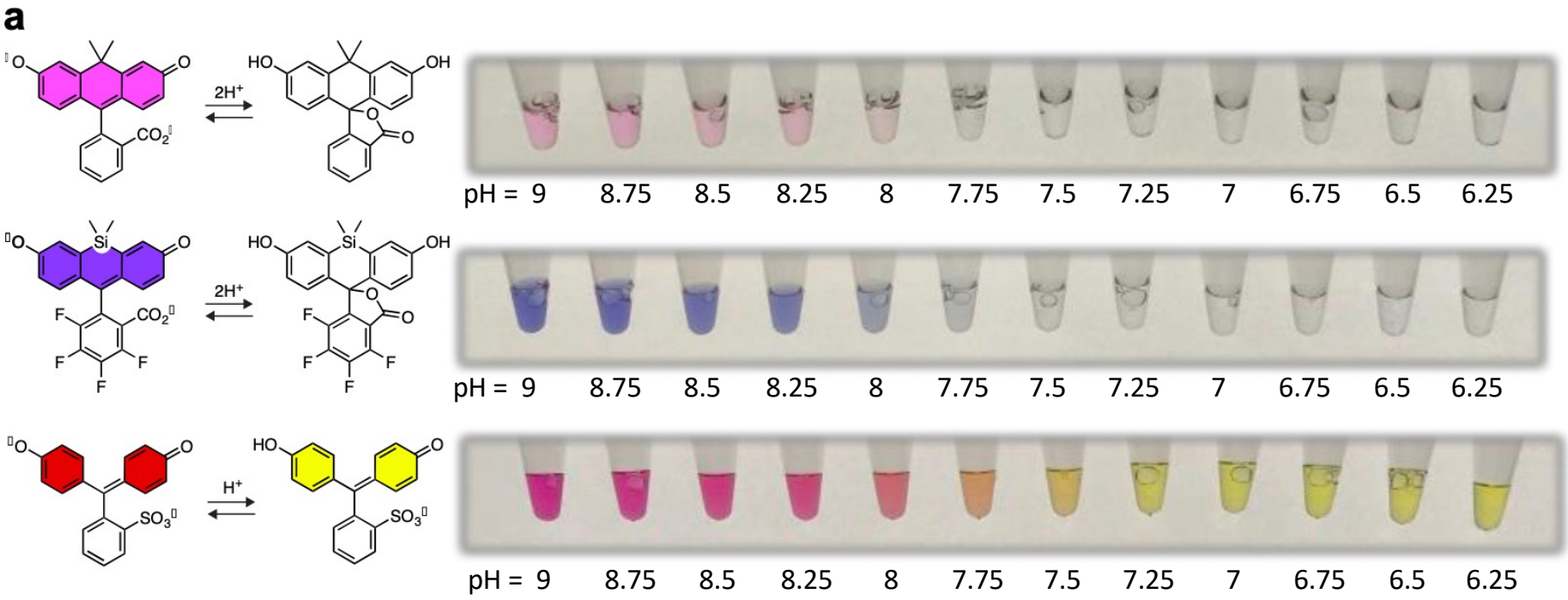


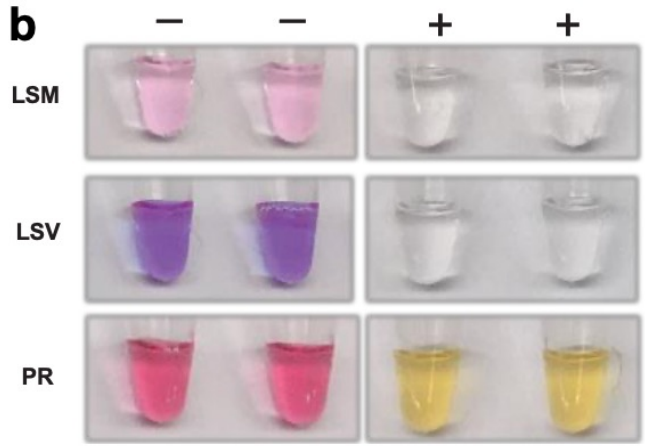


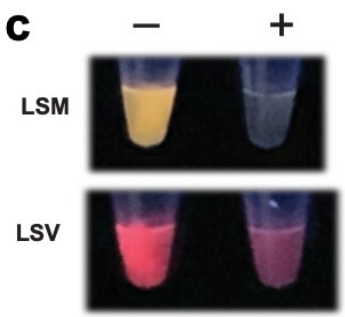


Miyamoto, Shigehiko, et al. "Method for colorimetric detection of double-stranded nucleic acid using leuco triphenylmethane dyes." Analytical biochemistry 473 (2015): 28-33.

pH-based colorimetric readout







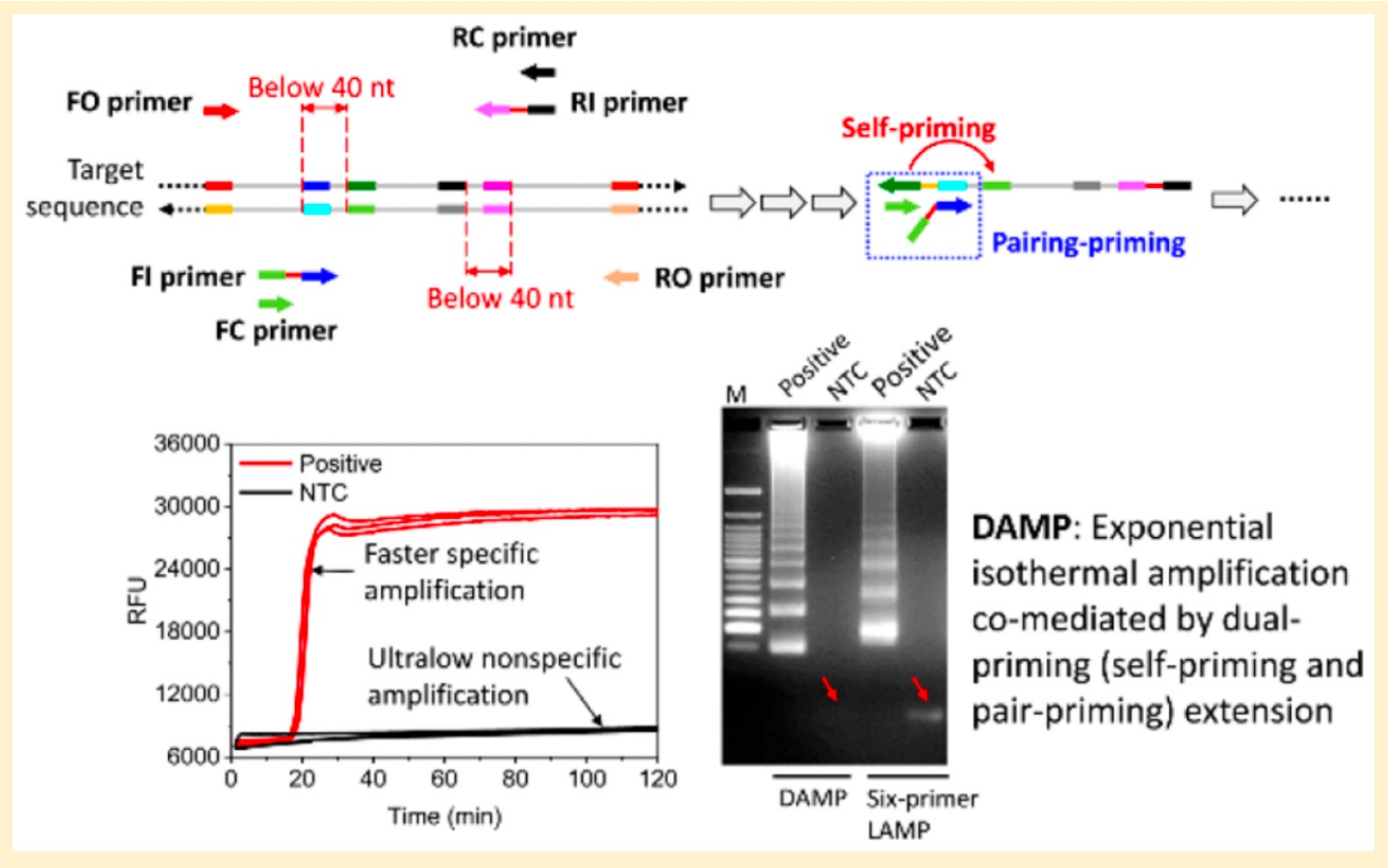
Phenol red

Brown, Timothy A., et al. "Direct detection of SARS-CoV-2 RNA using high-contrast pH-sensitive dyes." medRxiv (2021): 2020-12.

https://www.neb.com/products/m1800-warmstart-colorimetric-lamp-2x-master-mix-dna-rna#Product%20Information

Isothermal Amplification Protocol:

Dual-Priming Isothermal Amplification (DAMP)



Ding, Xiong, et al. "Dual-Priming isothermal amplification (DAMP) for highly sensitive and specific molecular detection with ultralow nonspecific signals." Analytical chemistry 91.20 (2019): 12852-12858.

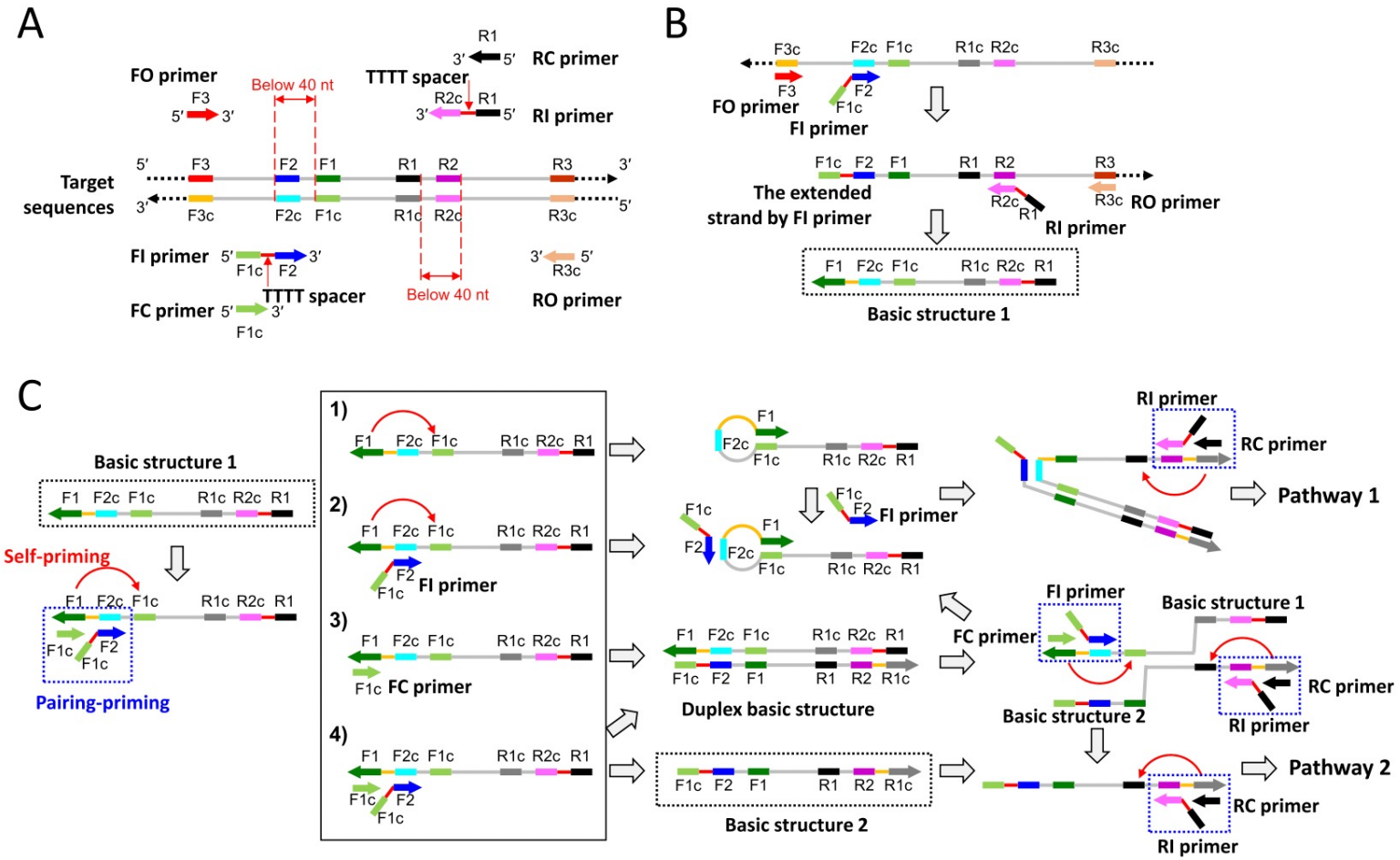


Figure 1. Principle of the DAMP assay. (A) Primer design of the DAMP method: FO, forward outer primer; RO, reverse outer primer; FI, forward inner primer; RI, reverse inner primer; FC, forward pairing-competition primer; and RC, reverse pairing-competition primer. (B) Basic structure producing step. (C) Cycling amplification step. More details of pathways 1 and 2 are given in Figure S3.

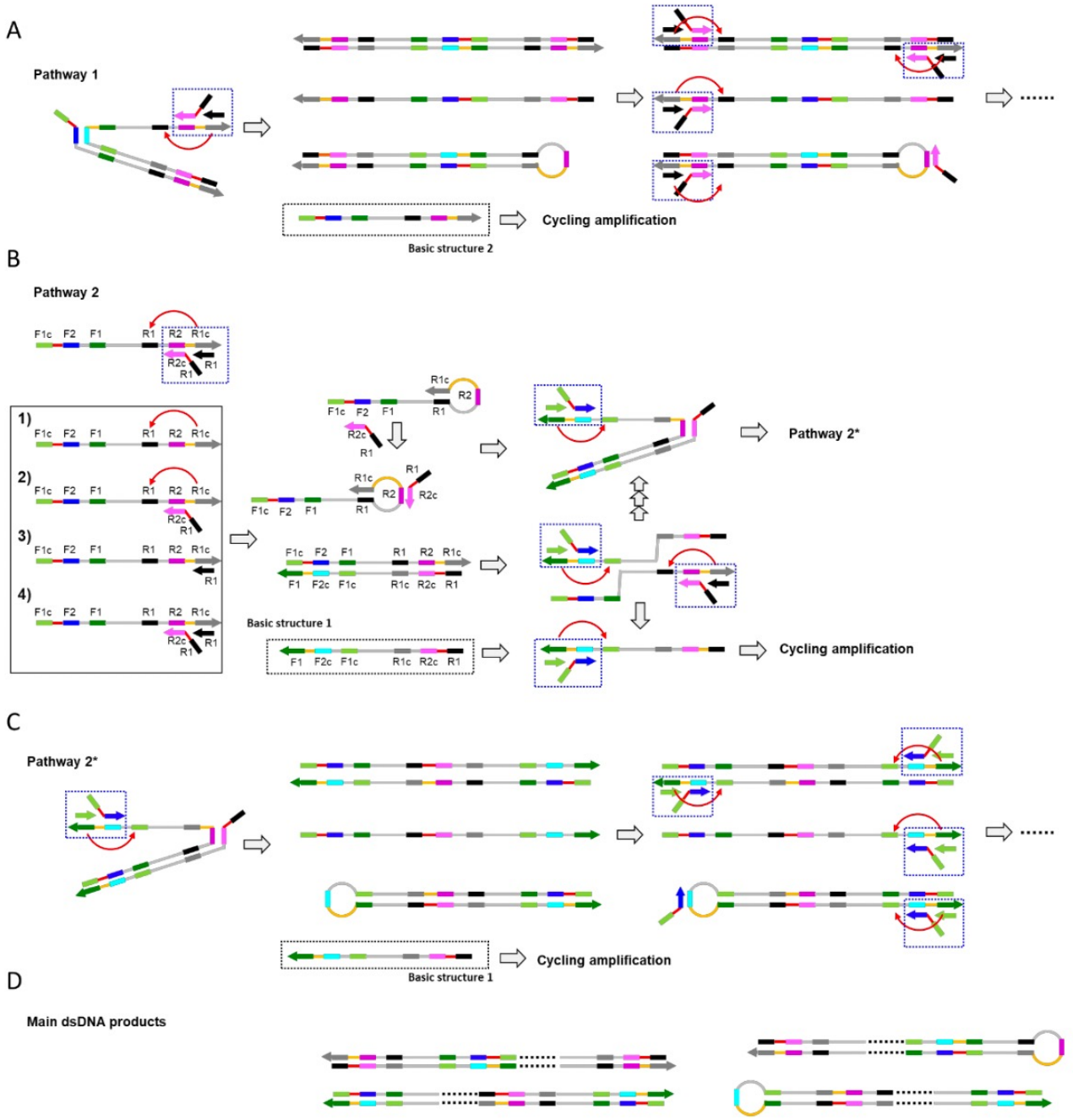
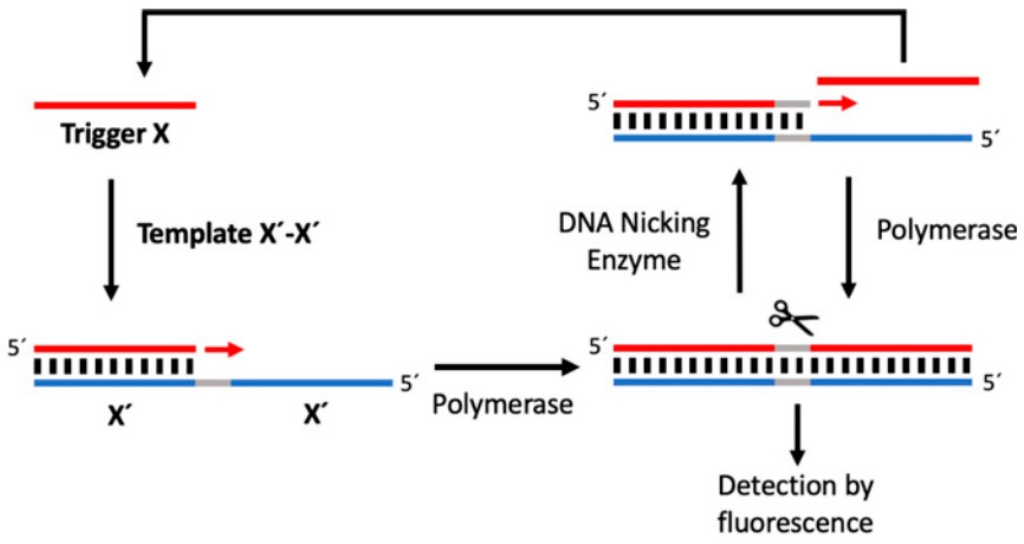


Figure S3. Continued cycling amplification step for DAMP reaction includes: (A) Pathway 1, (B) Pathway 2, and (C) the complementary Pathway 2 (Pathway 2*). (D) The main dsDNA products in DAMP reactions.

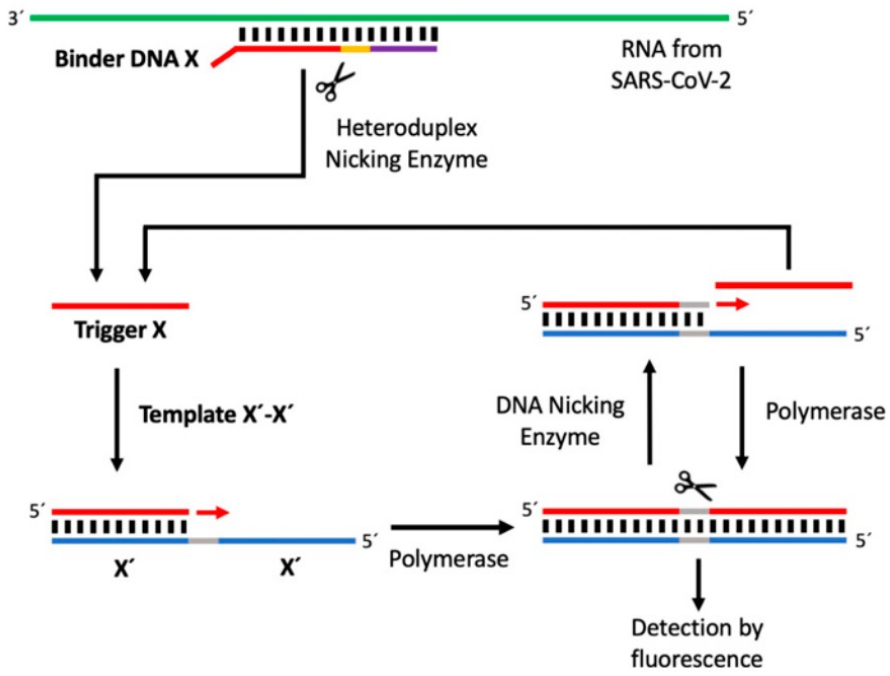
Isothermal Amplification Protocol:

Exponential Amplification Reaction (EXPAR)

A Exponential Amplification Reaction (EXPAR)



B Reverse Transcription-Free EXPAR (RTF-EXPAR)



(A) Reaction scheme for EXPAR:
Trigger X anneals to Template X′-X′ and is extended by a DNA polymerase (Bst 2.0 polymerase); the top strand of the newly formed duplex DNA is then cut by a nicking enzyme (Nt.BstNBI); the released DNA (which is displaced by DNA polymerase in a subsequent extension reaction) is identical to Trigger X and is therefore able to prime another Template X′-X′.

(B) Reaction scheme for RTF-EXPAR:
Binder DNA X anneals to viral RNA; the DNA strand of the DNA:RNA duplex is cut by the restriction endonuclease BstNI, which acts as a nicking enzyme by cutting the DNA strand only; the released DNA strand is Trigger X, which is then amplified by EXPAR.

Isothermal PCR Amplification Protocol:

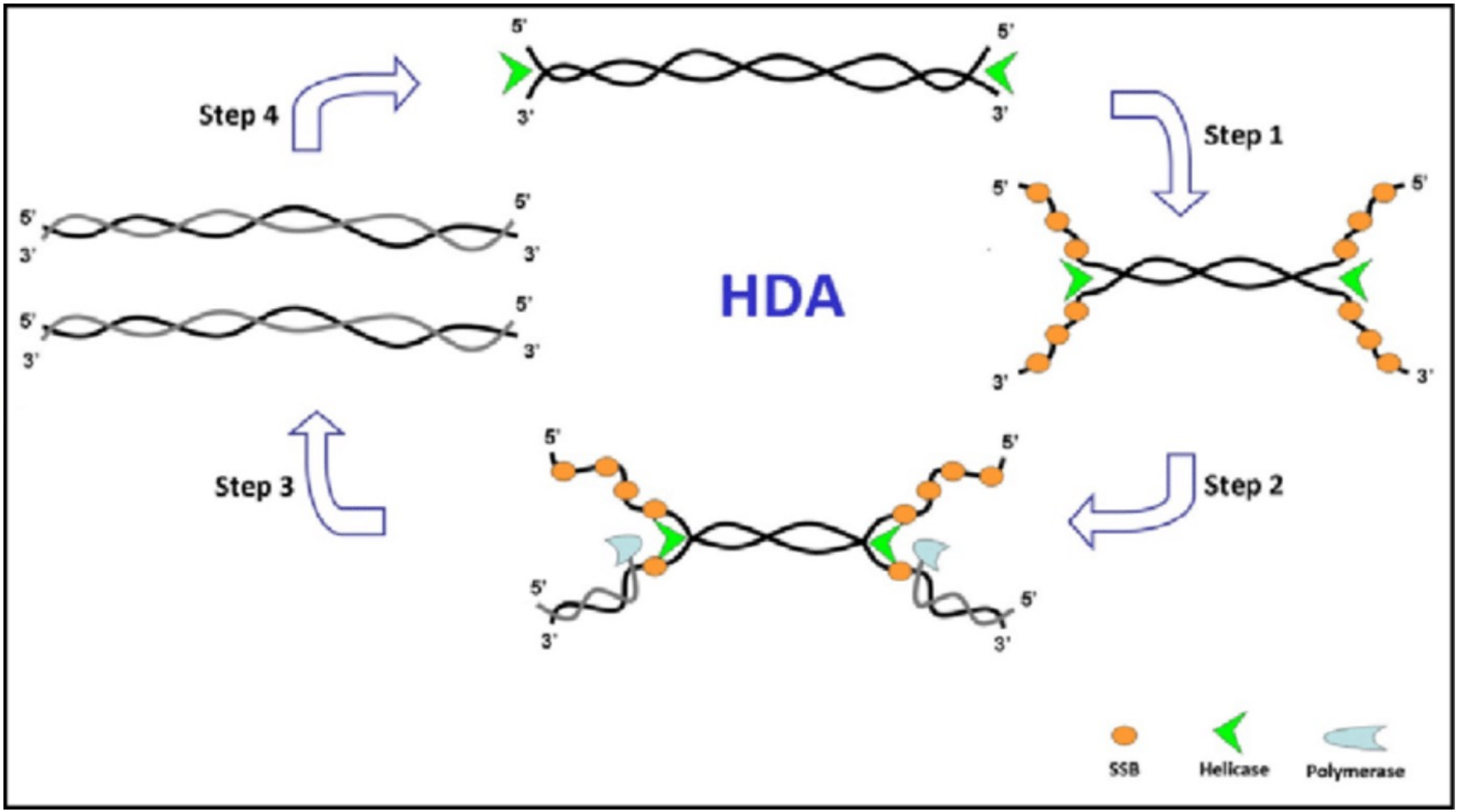
Helicase-dependent amplification (HDA)

- Employs the double-stranded DNA unwinding activity of a helicase to separate strands, enabling primer annealing and extension by a strand–displacing DNA polymerase.
- Like PCR, this system requires only two primers.
- Used in diagnostic devices and FDA-approved tests.

New England BioLabs Website

Isothermal Amplification Protocol:

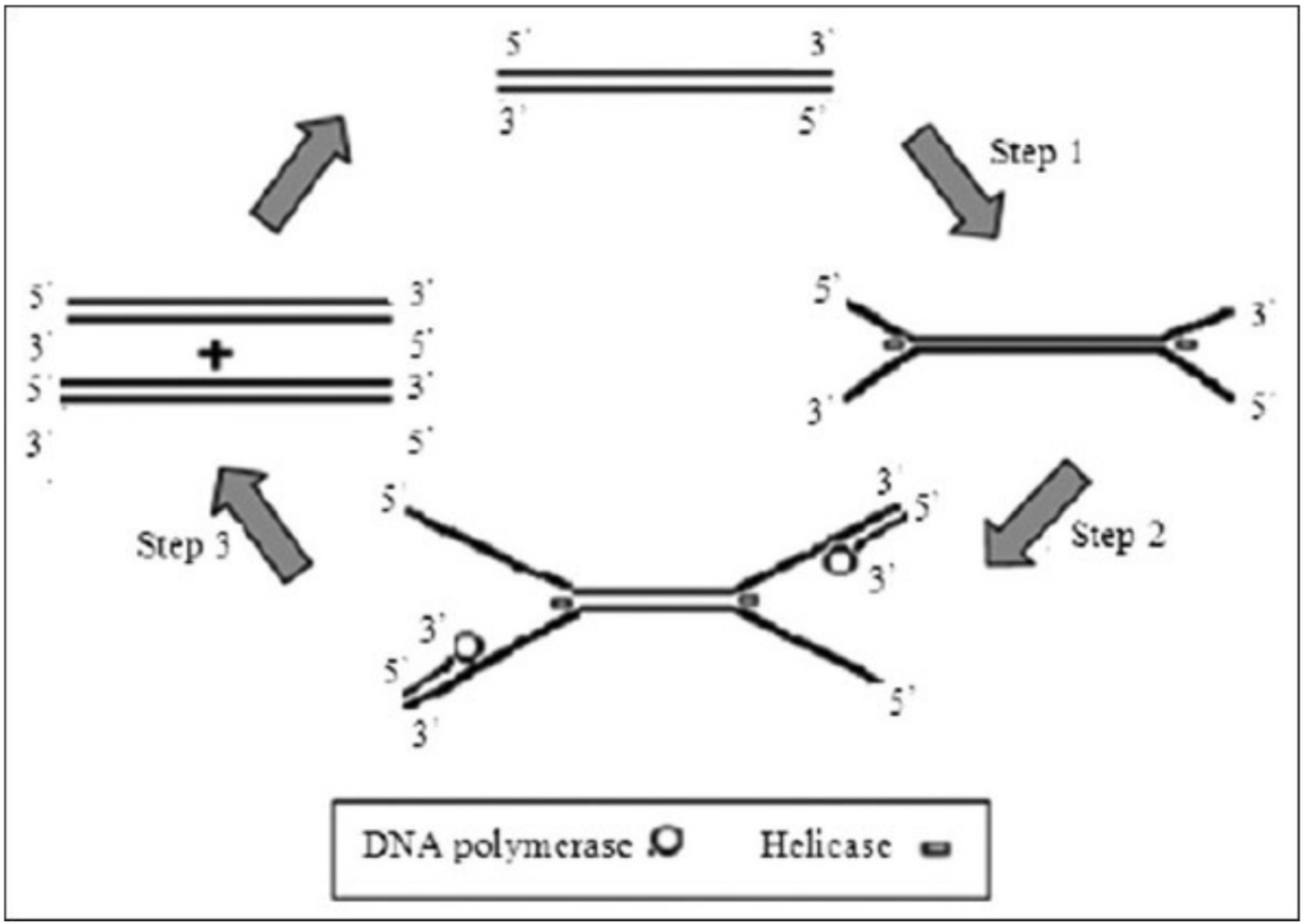
Helicase-dependent amplification (HDA)



Schematic representation of helicase-dependent amplification (HDA) amplification process.

Isothermal PCR Amplification Protocol:

Helicase-dependent amplification (HDA)



Helicase-dependent amplification process. Step 1: The helicase unwinds deoxyribonucleic acid (DNA) duplexes. Step 2: The primers anneal to the singl stranded DNA. Step 3: The primers extended by DNA polymerase; one duplex is amplified and converted to two duplexes. The double-stranded DNAs are separated by helicase and this chain reaction repeats itself

Isothermal PCR Amplification Protocols Compared:

Method	Amplification time	Reaction volume	Target	Detection limit [a]	Ref.
LAMP	within 1 h	25 µL	hepatitis B virus (HBV) DNA	50 copies/25 µL	[50,51]
	within 15 min	10 µL	prostate-specific antigen gene	23 fg/µL	[52]
	within 1 h	5 µL	Pseudorabies virus (PRV) DNA	10 fg	[53]
	within 1 h	[b]	λDNA	two molecule	[54]
	1 h 35 min	35 µL	*E. coli* genomic DNA	24 colony forming units (CFU)/mLl 48 CFU/mL	[55]
HDA	2 h	150 µL	N. gonorrhoeae genomic DNA Methicillin resistant *S. aureus* genomic DNA	1 ng 250 pg	[74]
	0.5 h	~5 µL/192 nL	BNI-1 fragment of SARS cDNA	0.01 ng/µL	[76]
	0.5 h	25 µL	*E. coli* genomic DNA	10 CFU	[78]
RCA	within 65 min	10 µL	Genomic DNA for *V. cholerae*	25 ng	[87]
	4 h	2 pL	pIVEX2.2EM-lacZ plasmid	0.07 pg/µL	[90]
	2.5 h	pL	Human-malaria-causing Plasmodium parasites	less than one parasite/µL	[91]
MDA	10–16 h	60 nL	*E. coli* genomic DNA	[b]	[99]
RPA	within 20 min	10 µL	mecA gene of *Staphylococcus aureus*	less than 10 copies	[102]
	1 h	9 nL	Methicillin-resistant *Staphylococcus aureus* genomic DNA	300 copies/mL	[106]
NASBA	within 2 h	10 nL	Human papillomavirus (HPV)	1.0 ìM	[118]
	2,5 h	80 nL	Artificial human papilloma virus (HPV) 16 sequences SiHa cell line samples	10^{-6} ìM 20 cells/µL	[119]
	0,5 h	2 µL	*E. coli* tmRNA	100 cells in 100 iL	[121]
	2–3 h	30 iL	Water pathogens	10^5 CFU/mL	[122]

[a] The lowest detected concentration is shown when the detection limit is not reported; [b] Not available.

Isothermal PCR Amplification Protocols Compared:

Parameter	PCR	LAMP	3SR	SDA	LCR	NASBA	RCA
High sensitivity	<10	<10	10	10	<10	10	<10
High specificity	+	+	+	+	+	+	+
Allow quantification	+	+	±	±	−	±	−
Live versus dead microorganisms	+	+	+	−	−	±	−
Commercial availability	+	−	±	±	−	−	−
Linear dynamic range	6-7	6	7	ND	ND	7	ND
Multiplexity	+	−	+	−	+	+	+
No. of enzymes	1	1	2-3	2	2	2-3	1
Primer design	Simple	Complex	Simple	Complex	Simple	Simple	Complex
Tolerance to biological compounds	−	+	−	−	−	−	−
Need to template denaturation	+	−	+	+	+	+	−
Denaturing agents	Heat	Betaine	Rnase H	Restriction enzymes; bumper primers	Helicase	Rnase H	Ø29 DNA polymerase
Product detection method	Gel electrophoresis, ELISA, real time	Gel electrophoresis, turbidity, real time	Gel electrophoresis, ELISA, real time, ECL	Gel electrophoresis, real time	Gel electrophoresis, real time	Gel electrophoresis, ELISA, real time, ECL	Gel electrophoresis, real time

PCR: Polymerase chain reaction, LAMP: Loop-mediated isothermal amplification, SR: Sequence replication, SDA: Strand displacement amplification, LCR: Ligase chain reaction, NASBA: Nucleic acid sequence based amplification, RCA: Rolling circle amplification, DNA: Deoxyribonucleic acid, ELISA: Enzyme-linked immunosorbent assay, ECL: Electrochemiluminescent