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Chapter 3

Molecular Techniques
Alternatives to PCR, Part I

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Types of Amplification

- **Target Amplification**
  - Polymerase Chain Reaction
  - Transcription-Based Amplification Systems
  - Rolling Chain Amplification
- **Probe Amplification**
  - Ligase Chain Reaction
  - Strand Displacement Amplification
  - Qβ Replicase
- **Signal Amplification**
  - Branched DNA Amplification
  - Hybrid Capture Assays
  - Cleavage-Based Amplification
  - Cycling Probe

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Target Amplification:
Transcription-Based Amplification Systems

- Transcription-Based Amplification Systems (TAS)
  - Usually RNA is the target and product instead of DNA
  - DNA copy is synthesized from target RNA, then transcription of DNA produces millions of copies of RNA
  - Original procedure used heat to denature DNA/RNA hybrids

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Target Amplification:
Transcription-Based Amplification Systems

- Commercial variations
  - Transcription-mediated amplification (TMA)
  - Nucleic acid sequence-based amplification (NASBA)
  - Self-sustaining sequence replication (3SR)

- Variations differ from original in that:
  - RNase H degrades RNA from hybrid instead of heat
  - Reverse transcriptase derived from avian myeloblastosis virus (AMV) has inherent RNase H activity
  - Thus, only 2 enzymes are required: AMV reverse transcriptase and T7 RNA polymerase

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Transcription-Mediated Amplification (TMA)

- Isothermal
- Target amplification of RNA
- DNA is reverse transcribed from RNA target adding RNA polymerase promoter
- RNase H degrades RNA in RNA:DNA duplex
- Second primer binds and reverse transcriptase is used to form double stranded DNA
- RNA polymerase synthesizes RNA from double stranded DNA

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TMA

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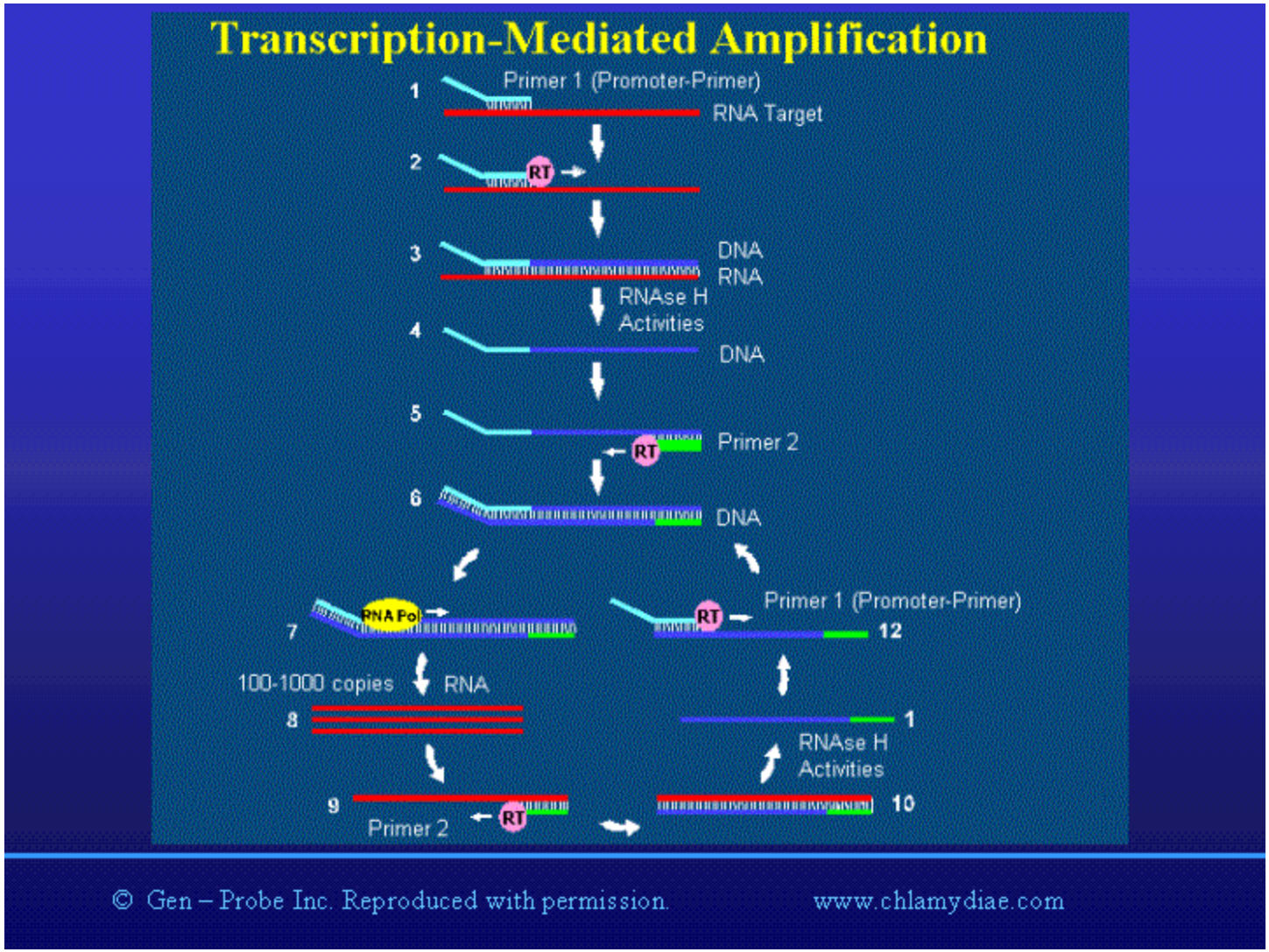

http://www.chlamydiae.com/images/tma/tmaall.GIF. Accessed 6/17/2010.

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Nucleic Acid Sequence Based Amplification (NASBA)

- Isothermal
- Target amplification of RNA (but with modifications, can be performed on DNA target)
- DNA is reverse transcribed from RNA target by Avian Myeloblastosis Leukemia Virus Reverse Transcriptase (AMV-RT)
- RNase H degrades RNA in RNA:DNA duplex
- Second primer with T7 RNA polymerase promoter region binds DNA and AMV-RT is used to form double stranded DNA
- T7 RNA polymerase synthesizes RNA from double stranded DNA

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NASBA

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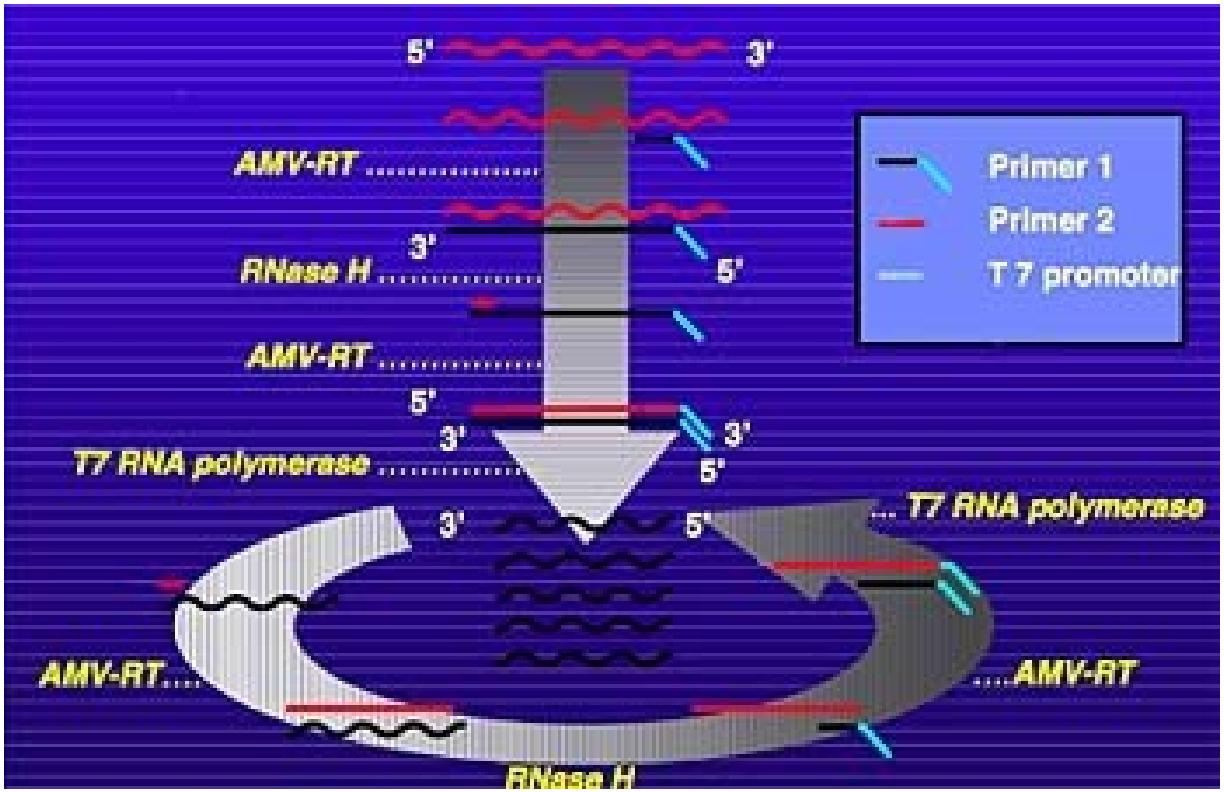

www.marine.usf.edu/microbiology/nasba.shtml. Accessed 6/17/10

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Transcription-Based Amplification Systems

- **Advantages**
  - Isothermal process; no need for thermal cycling
  - Targeting RNA allows for direct detection of RNA viruses
  - More sensitive for detecting bacteria (eg. TB) because each bacterium has multiple copies of RNA, but only one copy of DNA

- **Applications**
  - Detection of M. tuberculosis in respiratory samples
  - Chlamydia trachomatis in genital specimens
  - HIV and cytomegalovirus (CMV) quantitation in blood

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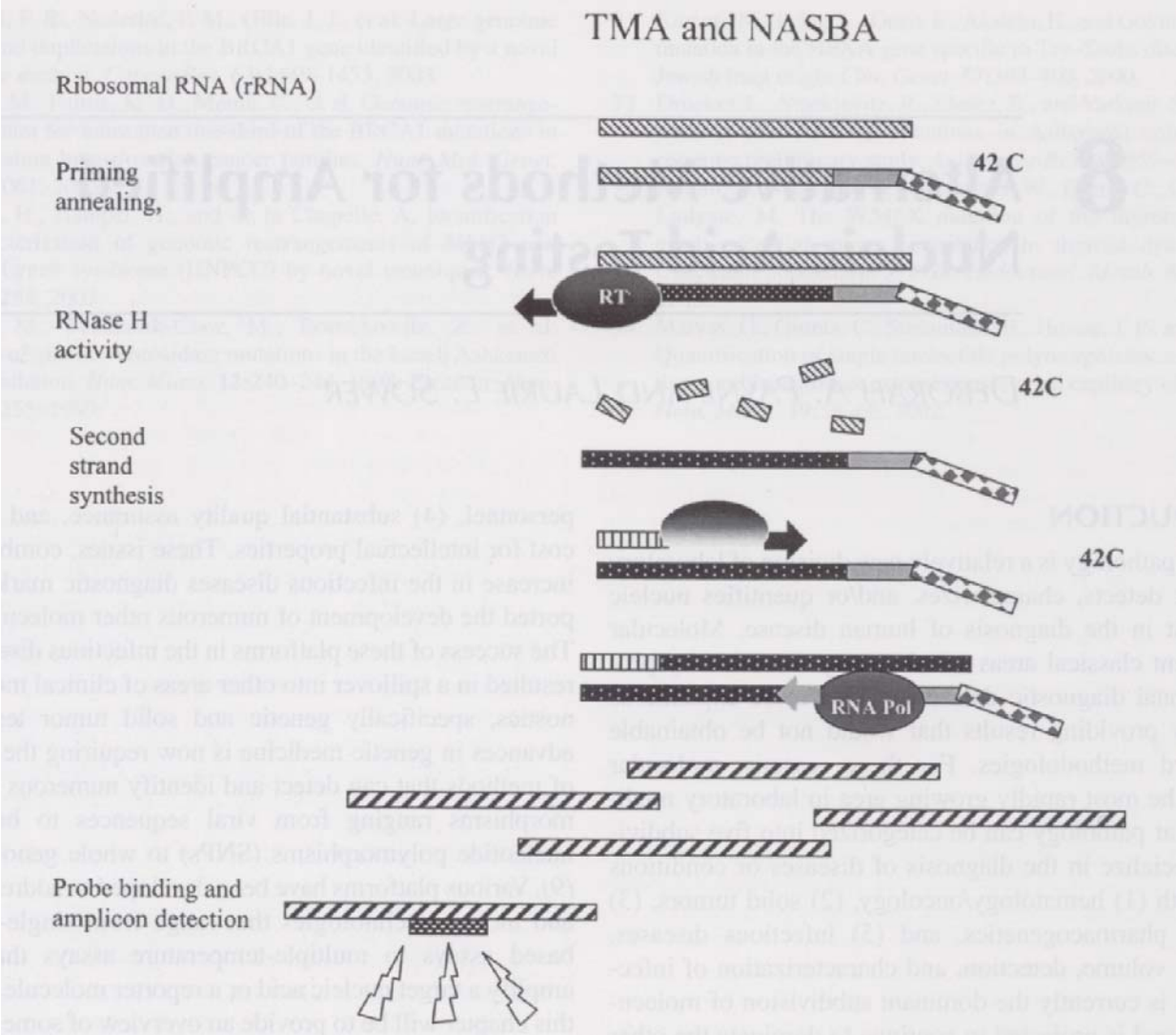
TMA and NASBA both involve:

RNA → RT-PCR →

DNA → RNA polymerase

to make lots more RNA



Coleman, WB and Tsongalis, GJ (eds). Molecular Diagnostics: For the Clinical Laboratorian. 2nd ed. Totowa, NJ: Humana Press, 2006.

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Rolling Chain Amplification (RCA)

- Isothermal
- Whole plasmid genome amplification
- Single strand is nicked, strand is extended upstream on nick in 5′ → 3′ direction
- Strand downstream of nick is displaced as newly synthesized strand rolls around
- Complementary strand is formed from single strand unit

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Rolling Chain Amplification

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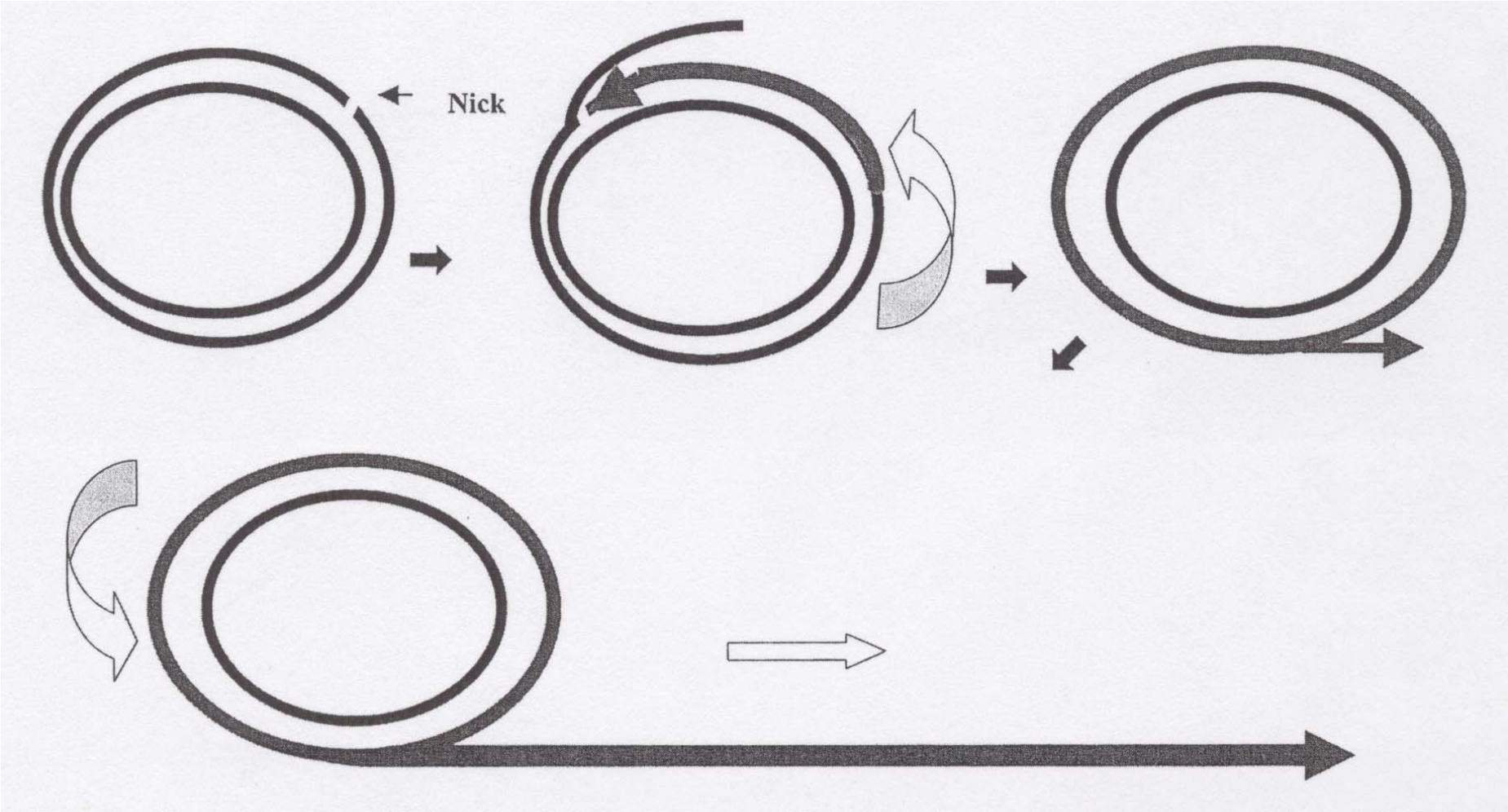

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Multiplex Ligation-Dependent Probe Amplification (MLPA)

- A variation of the multiplex PCR
- Permits multiple targets to be amplified using a single primer pair
- Each probe consists of a two oligonucleotides which recognizes adjacent target sites on the DNA
- One probe oligonucleotide contains the sequence recognized by the forward primer
- Other probe sequence recognizes the reverse primer
- Only when both probe oligonucleotides are hybridized to respective targets, can they be ligated into a complete probe

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Multiplex Ligation-Dependent Probe Amplification (MLPA)

- Advantage of splitting probe into two parts:
  - Only the ligated oligonucleotides are amplified
  - Unbound probe oligonucleotides are not amplified
  - If probes were not split, the primer sequences at either end would cause the probes to be amplified regardless of hybridization to the template DNA
  - Amplification product would not be dependent on the number of target sites present in the sample DNA

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Multiplex Ligation-Dependent Probe Amplification (MLPA)

- Each complete probe has a unique length
- Resulting amplicons can be separated and identified by capillary electrophoresis
- Avoids the resolution limitations of multiplex PCR
- Unlike other techniques (DGGE, DHPLC, SSCA), MLPA is accurate, time-efficient technique to detect genomic deletions and insertions (one or more entire exons)

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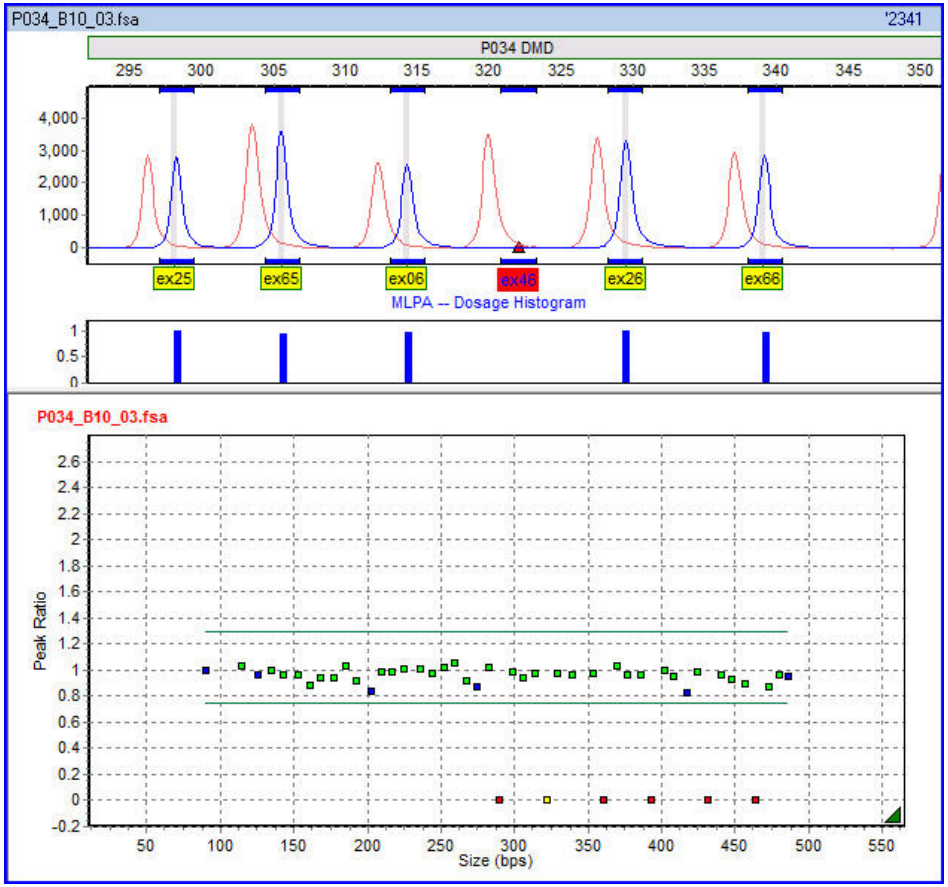
Multiplex Ligation-Dependent Probe Amplification (MLPA)

- Since forward primer used for probe amplification is fluorescently labeled, each amplicon generates a fluorescent peak
- Relative quantity of each amplicon can be determined by comparing peak pattern obtained on a given sample with reference samples
- This ratio is a measure for the ratio in which the target sequence is present in the sample DNA



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Probe Amplification

- Number of target nucleic acid sequences is not changed
- Synthetic probes specific to the target sequences bind to target
- Probes themselves are amplified

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Probe Amplification:
Ligase Chain Reaction (LCR)

- Amplifies primers/probes complementary to target nucleic acid
- Entire target sequence must be known to prepare primers
- Primers bind adjacent to each other, separated by only one base
- DNA ligase is used to connect adjacent primers together
- Ligated primers then serve as template for the binding of more probes
- Can be used to detect point mutations (eg. Sickle cell mutation) and for detecting C. trachomatis and N. gonorrhoeae

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LCR

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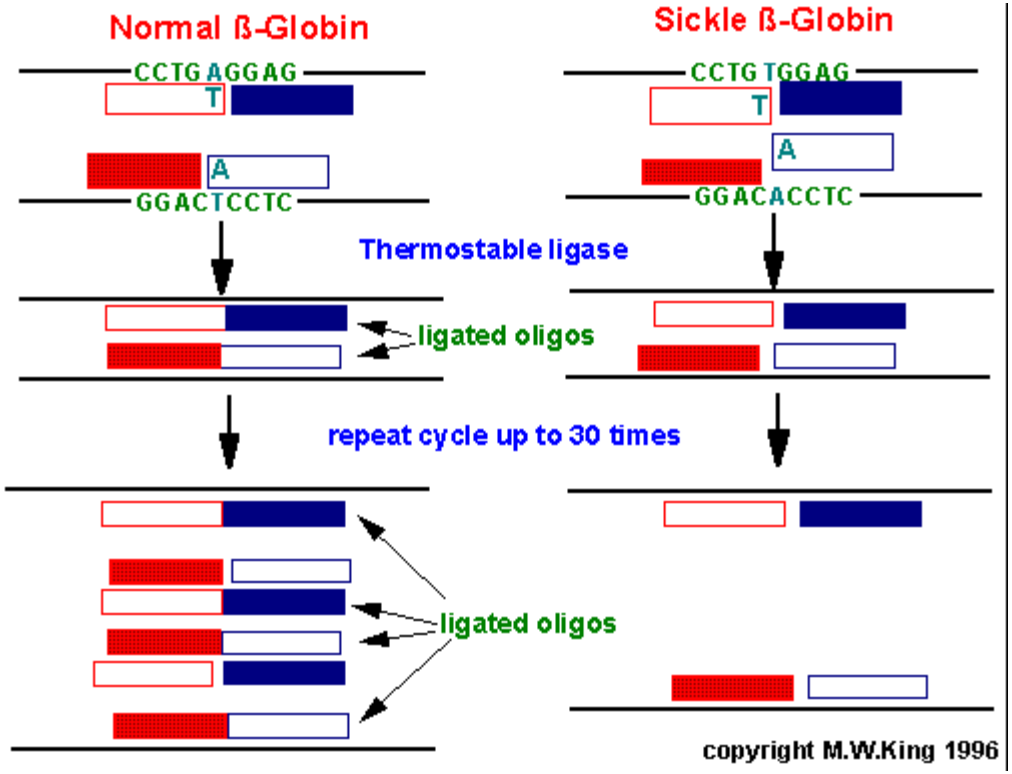

http://www.obgynacademy.com/basicsciences/fetology/genetics/images/lcr.gif. Accessed 6/17/10

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Probe Amplification:
Strand Displacement Amplification (SDA)

- Isothermal (after initial denaturation step, reaction proceeds at one temperature)
- Target amplification of DNA
- Incorporation of primers with restriction site and extension of complementary DNA strand
- Restriction site recognized and nicked by restriction enzyme
- Nicked primer recognized by DNA polymerase and extended to form new strand while displacing original strand
- Used to test for M. tuberculosis, C. trachomatis, and Neisseria gonorrhoeae

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Strand Displacement Amplification

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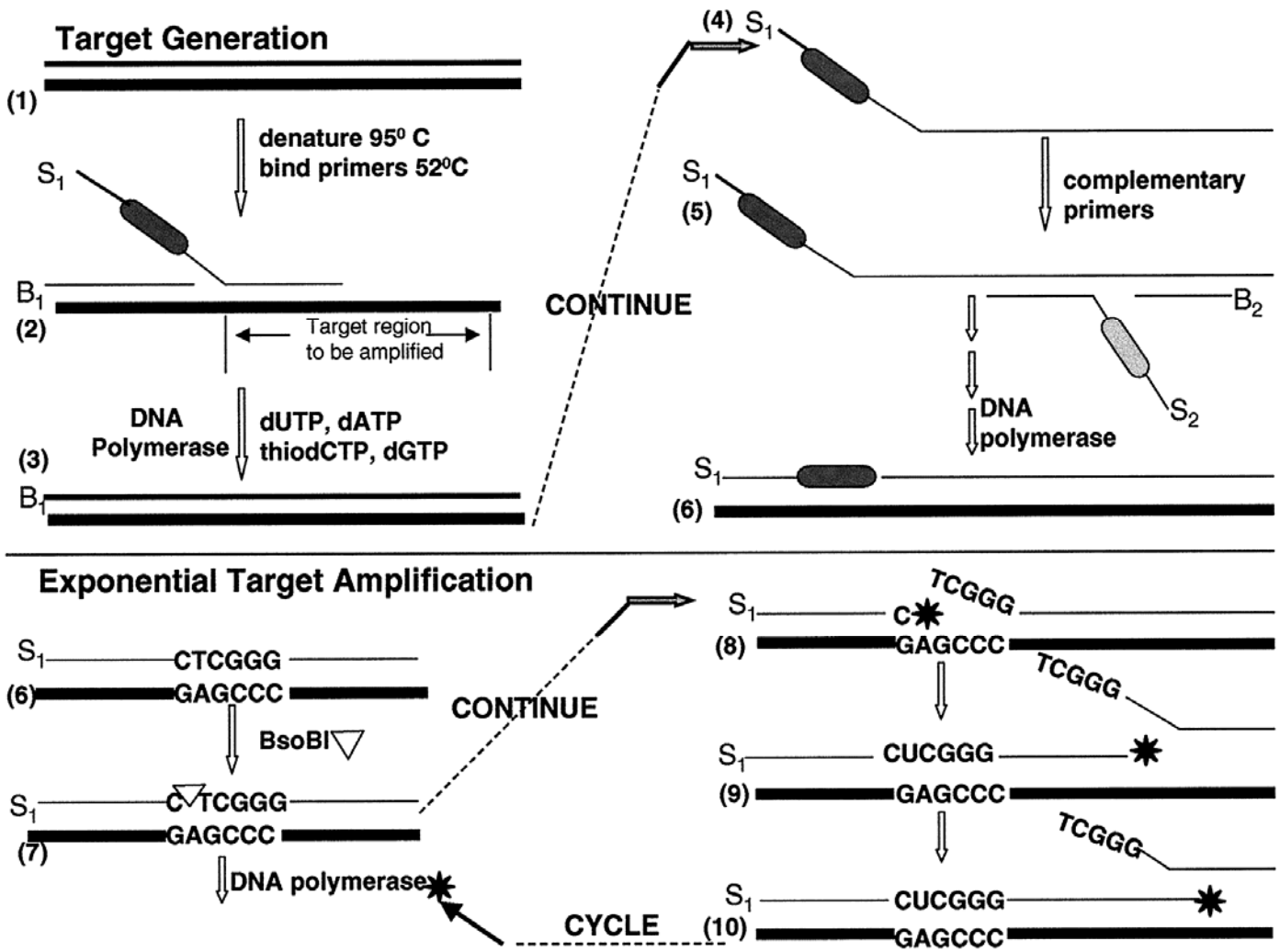

http://www.clinchem.org/cgi/content/full/45/6/777/F1

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Probe Amplification:
Qβ Replicase

- Named for the major enzyme that is used to amplify probe sequences
- Qβ replicase is a RNA-dependent RNA polymerase from bacteriophage Qβ
- Uses reporter probes containing a promoter sequence that is recognized by Qβ replicase.
- Used for detecting infectious organisms
- Assays are not commercially available in United States at this time

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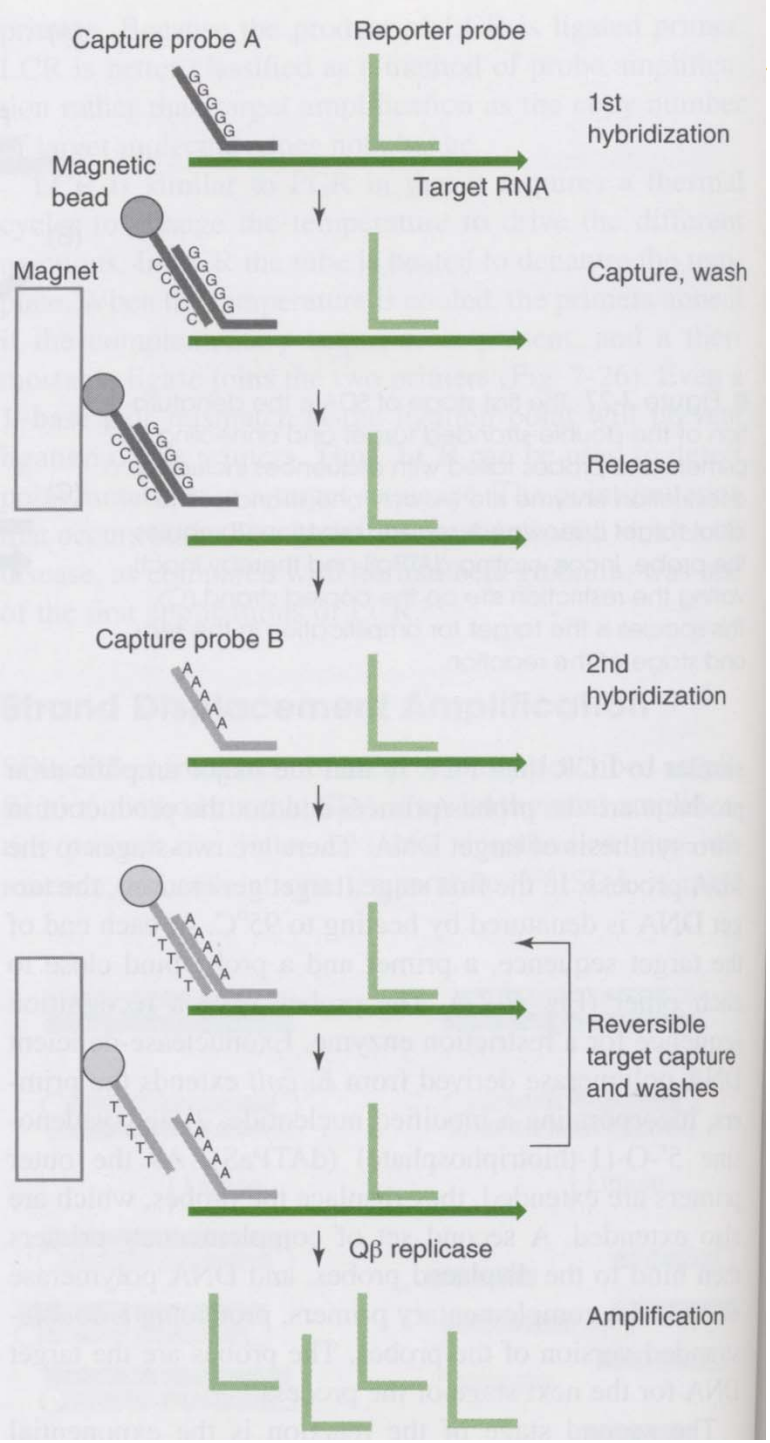
Qβ Replicase

The Qβ replicase method proceeds through a series of binding and washing steps. Probe bound to the purified template is then amplified by Qβ replicase. The resulting RNA can be detected by fluorometry using propidium iodide as a fluorescent label of the synthesized probe or by chromogenic methods.



Buckingham, L and Flaws, ML. *Molecular Diagnostics: Fundamentals, Methods, & Clinical Applications*. Philadelphia: F.A. Davis Company, 2007.

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