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American Society of Cytopathology Core Curriculum in Molecular Biology

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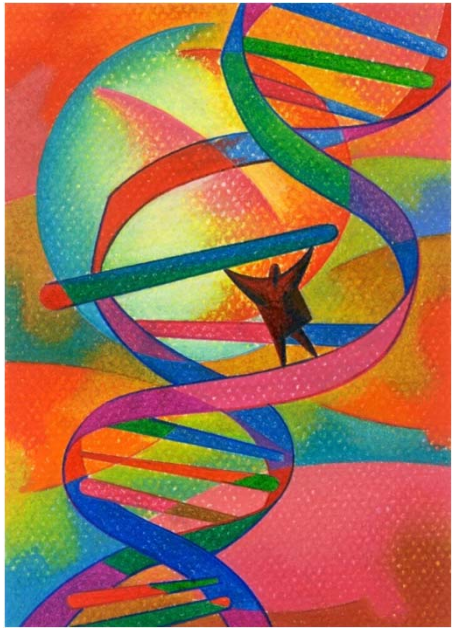

American Society of Cytopathology Core Curriculum in Molecular Biology

Chapter 3

Molecular Techniques
Alternatives to PCR, Part II

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Signal Amplification

- Number of target sequences does not change
- Large amounts of signal are bound to target sequences present in the sample
- Are inherently better at quantitating the amount of target sequences present in a sample

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Signal Amplification:

Branched DNA Amplification (bDNA)

- Target nucleic acid can be DNA or RNA
- Target nucleic acid anchored to solid phase through binding of specific capture probes
- Additional extender probes bind to target nucleic acid
- Extender probes bind to amplifier probes
- Amplifier probes bind to alkaline phosphatase enzyme which can bind to a substrate called Dioxetane to produce chemiluminescence measured by Luminometer
- Used for HIV and HCV viral load detection

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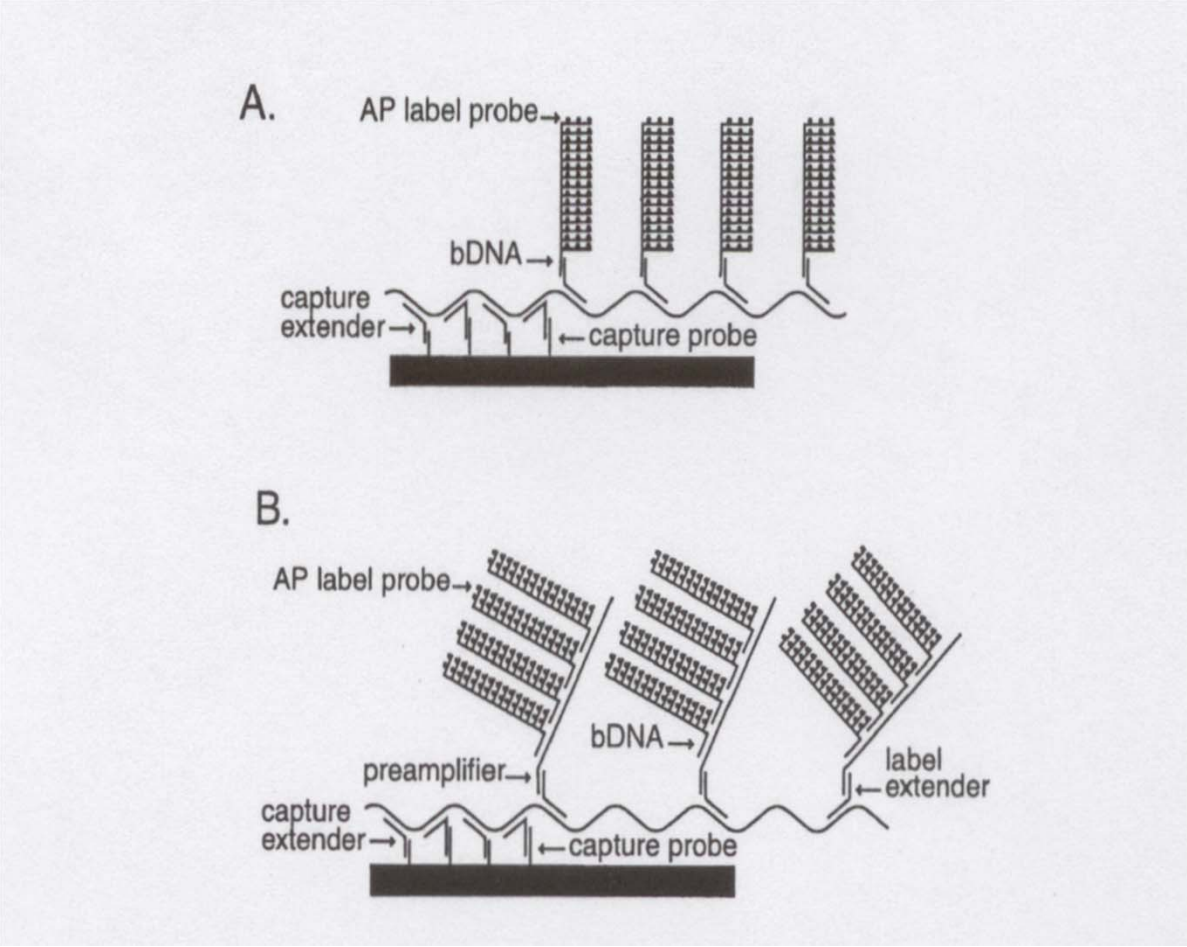
Branched DNA Amplification

A. First generation assay

B. Second and third generation assays. The preamplifier (heavy lines) is unique to the second and third generation assays.



http://nar.oxfordjournals.org/cgi/reprint/25/15/2979.pdf Accessed 6/17/10

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Advantages of bDNA

- Low risk of carryover contamination
- Multiple capture and extender probes can be used that detect slightly different target sequences which occur in viruses (Hepatitis C and HIV)
- Multiple genotypes of same virus can be detected by same system
- Multiple probes binding to same target increases specificity
- Qualitatively and quantitatively detects Hepatitis B and C viruses and HIV-1

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Signal Amplification:
Hybrid Capture Assays

- Used extensively for the detection of HPV (Digene Diagnostics)
- Available also for detection of Hepatitis B and CMV
- RNA probes bind to DNA targets
- DNA:RNA hybrids are captured by hybrid specific antibodies
- Another hybrid specific antibody conjugated to alkaline phosphatase detects captured hybrids
- Chemiluminescence is measured

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Hybrid Capture Assays

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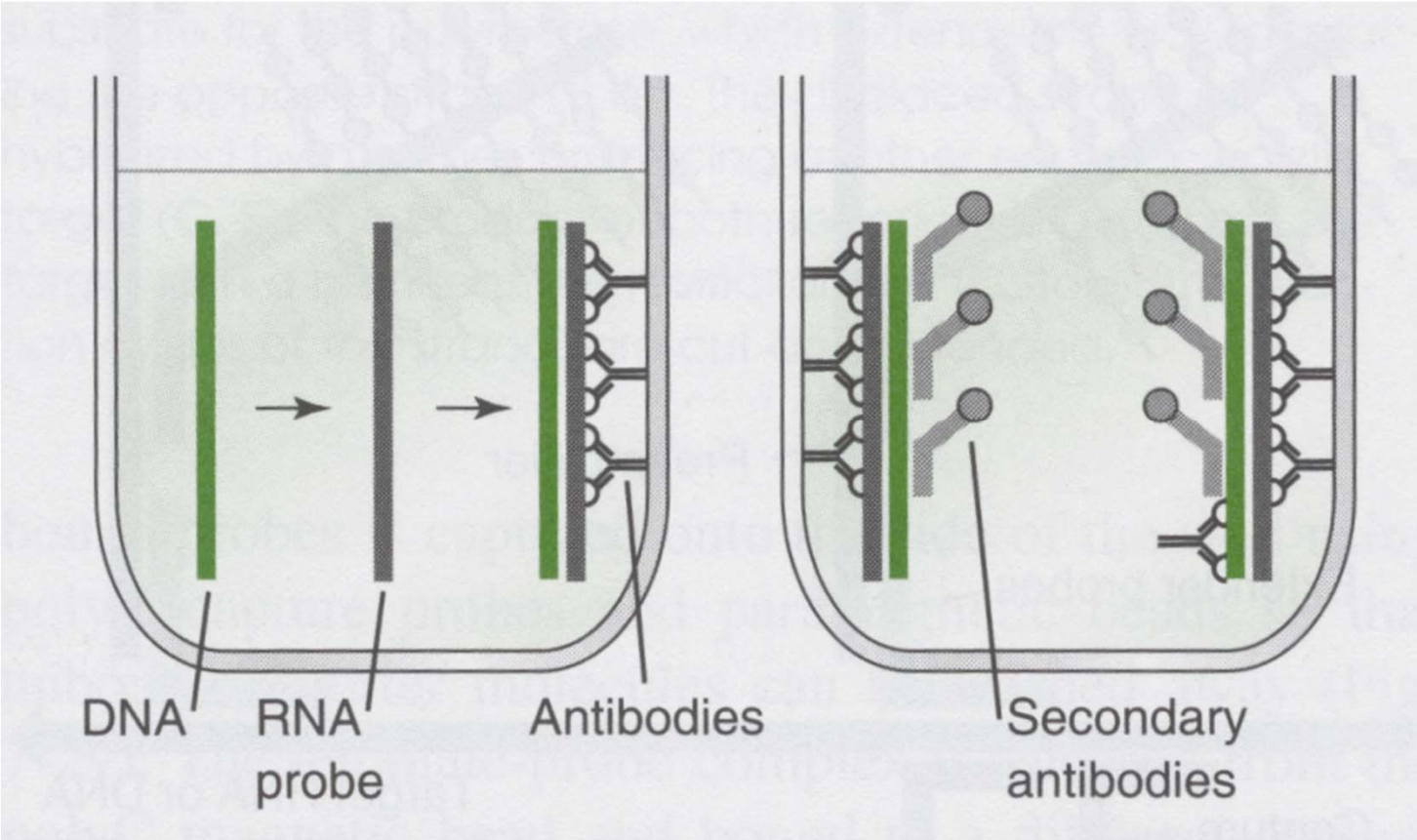

Hybrid capture starts with hybridization of the RNA probe to the denatured DNA target. The RNA:DNA hybrid is then bound by hybrid-specific immobilized antibodies. A secondary antibody bound to alkaline phosphatase generates signal in the presence of a chemiluminescent substrate (right).

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

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Signal Amplification:
Cleavage-Based Amplification

- Uses series of probes that bind to target and overlap
- Cleavase is an enzyme, isolated from bacteria, that recognizes overlapping sequences of DNA and makes a cut ("cleaves") in the overlapping piece
- Basis for "Invader Assay" or "Cervista" (Third Wave Technologies/Hologics)
- Used in detecting HPV and factor V Leiden mutation

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Cleavage-Based Amplification

- Isothermal
- Two reactions
  - Specifically detects single base changes with Invader oligo
  - Generically produces readout and signal amplification
- Binding of the Invader oligo allows for recognition by the Cleavase enzyme, thus releasing the 5‘ arm needed for signal amplification

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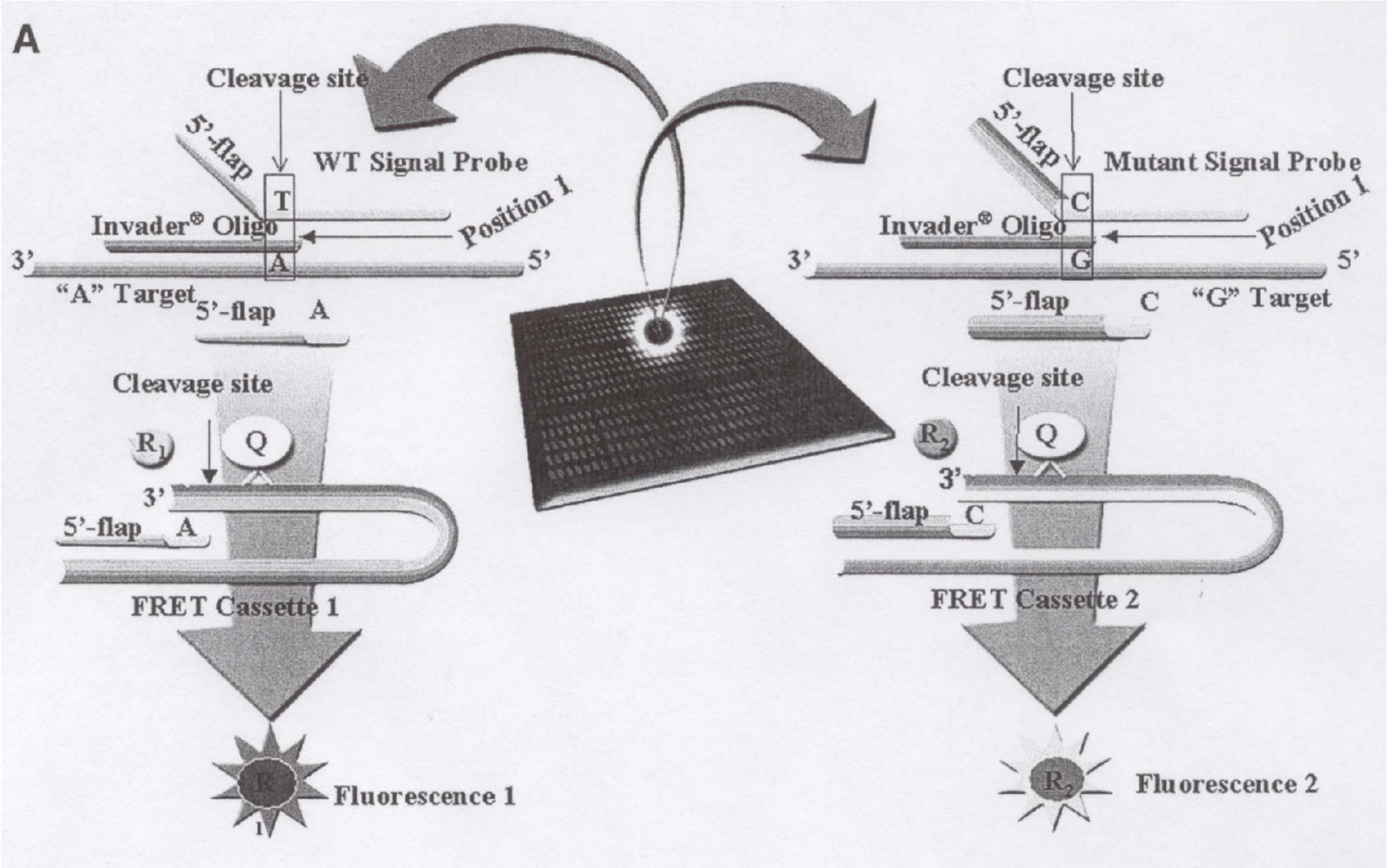
Cleavage-Based Amplification



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

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Cleavage-Based Amplification

- Target nucleic acid is mixed with invader and signal probes
- Probes bind with the 5’ end of the signal probe overlapping with the invader probe
- Cleavase recognizes overlap and cleaves signal probe, which can act as an invader probe in next step

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Cleavage-Based Amplification (con't)

2nd Step:

- A FRET probe is added that has sequences complementary to cleaved signal probe
- The 5' end of FRET probe has a reporter molecule located close to a quencher molecule, thus, the intact FRET probe does not produce a signal
- Signal probe (now invader probe) binds to FRET probe which produces an overlapping region recognized by Cleavase
- When Cleavase cuts FRET probe in overlapping region, the reporter molecule is released from quencher and produces a signal
- Amount of signal can be quantitated and related directly to amount of target molecules in sample.

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Signal Amplification:

Cycling Probe

- Target sequences are detected using a synthetic probe
- Probe consists of sequences of DNA-RNA-DNA
- Probe binds to target nucleic acid
- RNase H cleaves RNA from the middle of probe
- DNA portions are now released from probes
- Template is free to bind to additional probe molecules
- When probe is digested, reporter and quencher dye are separated
- Reporter dye releases fluorescence which is measured

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Cycling Probe

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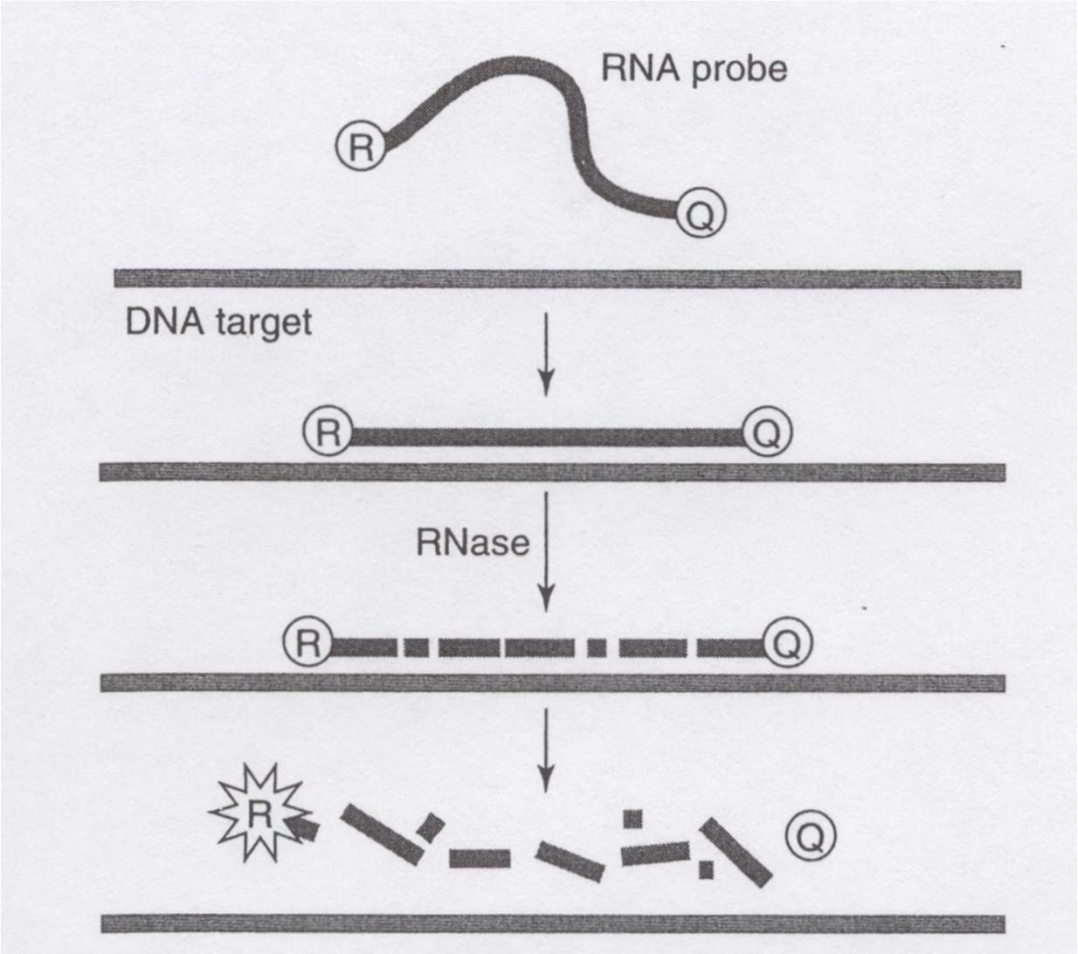
Cycling probe produces fluorescence only when the RNA probe binds to the DNA template. The RNA:DNA hybrid formed by the probe bound to the template is a substrate for RNase H, which digests the RNA probe and releases the reporter dye (R) from the vicinity of the quencher (Q).



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

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Signal Amplification:

Cycling Probe

- Alternatively, presence of chimeric probes remaining when target sequences are not present can also be measured
- Used to detect genes associated with antimicrobial resistance in bacteria (eg. methicillin resistance (mecA) in Staphylococcus aureus and vancomycin resistance (vanA and vanB) in Enterococcus

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