MOLECULAR METHODS IN DIAGNOSTIC MICROBIOLOGY

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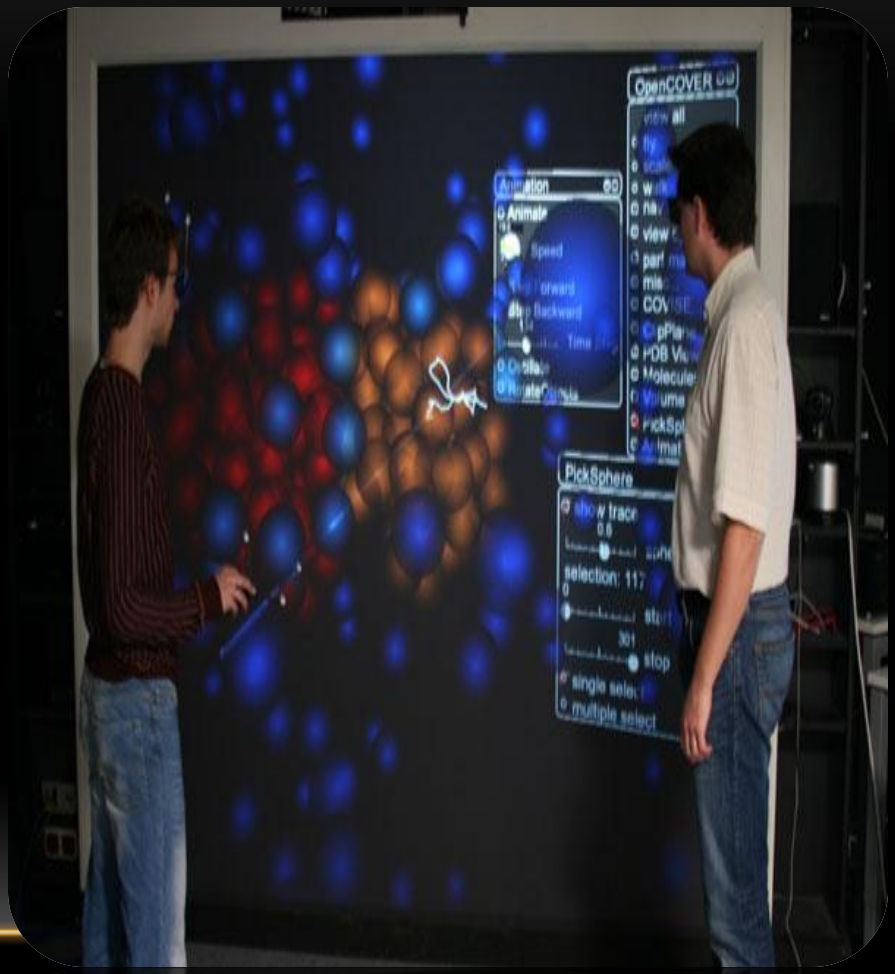
MOLECULAR METHODS IN DIAGNOSIS

- The introduction of molecular methods will not only depend on their performance for each individual microorganism, but also on the clinical relevance of the diagnostic **question asked, the prevalence of the clinical problem and whether the new methods are added to the procedures in use or will replace them**. Therefore no general rules can be proposed, strategies have to be elaborated for each infectious agent or clinical syndrome.

WHEN WE REALLY NEED MOLECULAR METHODS ?

- Molecular diagnosis is most appropriate for infectious agents that are difficult to detect, identify, or test for susceptibility in a timely fashion with conventional methods.



THERE IS AN URGENT NEED FOR MOLECULAR METHODS IN ……

- Strategies concerning the use of molecular diagnostic techniques for the diagnosis of **Mycobacterium tuberculosis, Chlamydia trachomatis, meningo-encephalitis syndrome and respiratory infections,** are need of the time.

NEED FOR NOVEL METHODS IN DIAGNOSIS OF INFECTIONS

- Identification of the infectious agent(s) is essential to provide an accurate diagnosis, appropriately manage patient care and in certain cases reduce the risk of transmission within the community and health care settings. To meet these challenges, innovative technologies have been developed that **detect single pathogens, multiple syndrome related pathogens and genotypic drug resistance**

MOLECULAR METHODS ARE REVOLUTIONIZING

- The use of molecular biology techniques, such as nucleic acid probing and amplification, provides the potential for revolutionizing how we diagnose infecting pathogens and determining the relation between nosocomial isolates. In clinical microbiology, this means that we will be able to detect smaller amounts of **DNA or RNA** of pathogens than is currently possible, that the time required to identify and determine the antimicrobial susceptibility of slow-growing pathogens will be dramatically reduced, and that the diagnosis of nonculturable organisms will become possible.

DIAGNOSTIC MICROBIOLOGY CHANGING FROM PHENOTYPIC METHODS TO MOLECULAR METHODS

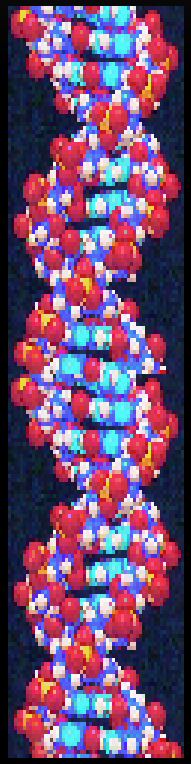
- In hospital epidemiology, the use of such techniques has already provided tests with exceptional discriminatory power. Molecular techniques allow more efficient typing of all pathogens, and permit discrimination between strains of organisms that were previously phenotypically identical or uncharacterizable. **Currently, cost and complexity limit the applicability of these techniques**; however, they are likely to be developed for routine laboratory **use in the next decade, and their impact will be considerable.**

MOLECULAR METHODS ARE NECESSARY IF THE TRADITIONAL METHODS PROVIDE POOR RESULTS ?

- Microscopy gives false positive results -

  - *T.vaginalis, N.gonorrhoeae*
- Intracellular pathogens – *viruses, M.genitalium*
- Low sensitivity – *Chlamydia sp.,Neisseria sp.*
- Seropositivity is common – *Chlamydia sp*.
- Subtyping is mandatory – *HSV, HPV, HCV*
- Microbial growth is slow – *M. tuberculosis*

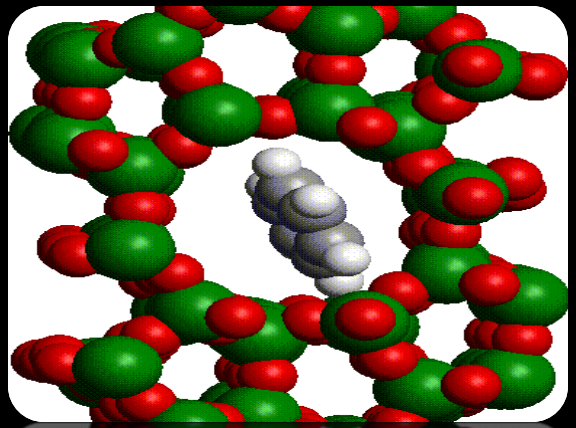
MOLECULAR DIAGNOSTICS – HOW IT WORKS?



- Every organism contains some **unique**,species specific DNA sequences
- Molecular diagnostics makes the **species specific** DNA visible

EMERGING MOLECULAR METHODS IN DIAGNOSIS

- HYBRIDIZATION-
- Direct detection of nucleic acids
- AMPLIFICATION METHODS-
- Target amplification
- Probe amplification
- Signal amplification
- SEQUENCING & ENZYMATIC DIGESTION



MOLECULAR APPLICATIONS IN INFECTIOUS DISEASES

- DNA hybridization - first used to demonstrate relatedness among bacteria
- • Nucleic acid probe technology: for detection of:
- -Antimicrobial resistance genes
- -Presence of organisms – mycobacteria, legionella
- -These methods may require growth
- • Nucleic acid amplification methods – for detection, identification & characterization of organisms Growth is not necessary

Molecular diagnostics is a set of methods to study primary structure (sequence) of DNA

- Hybridization with complementary sequences

-A-A-T-T-C-G-C-G-A-T-G-
- T-T-A-A-G-C-G-C-T-A-C-

- Amplification (synthesis) of species specific sequences

PCR – polymerase chain reaction

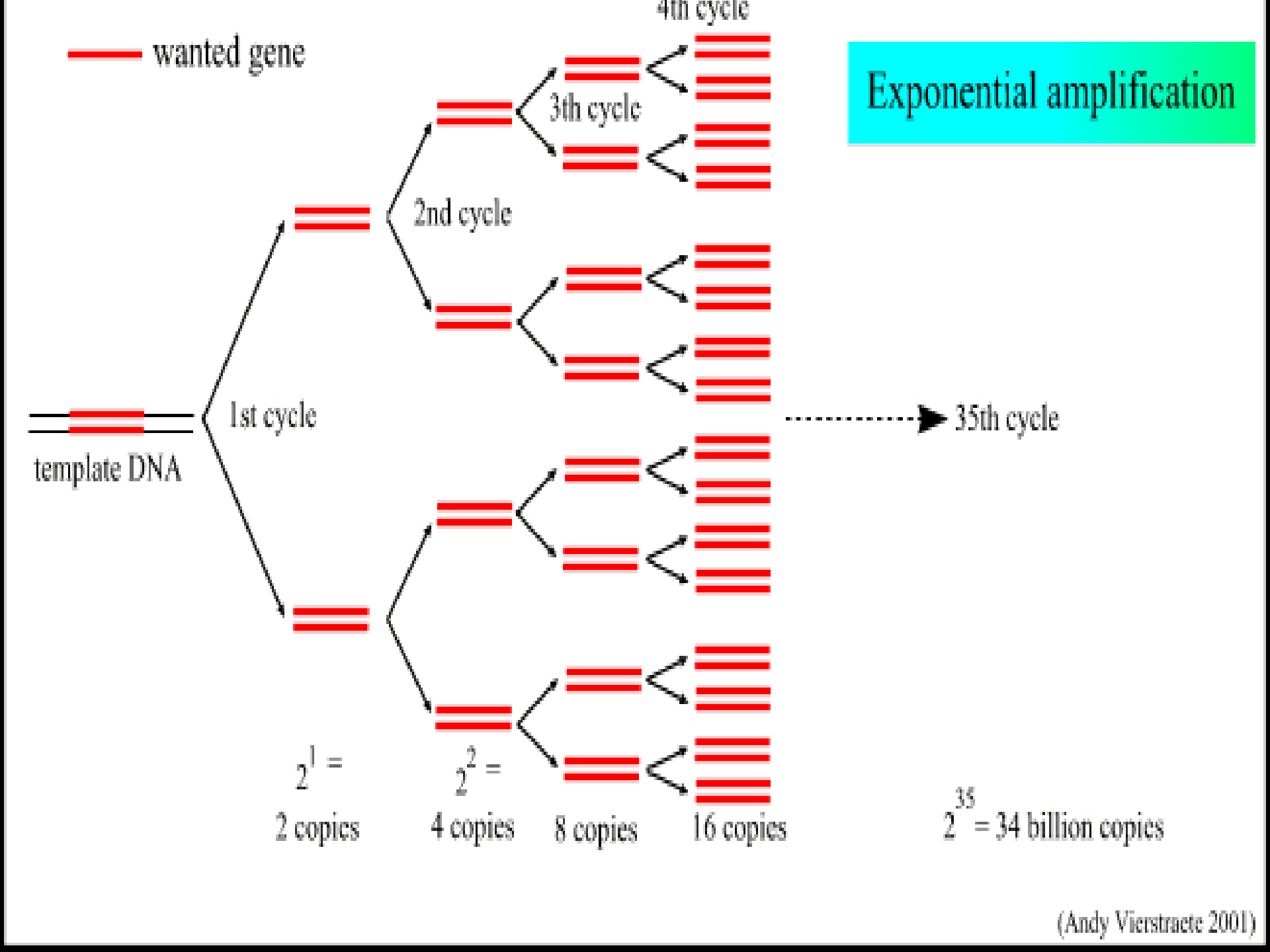
-A-A-T-T-C-G-C-G-A-T-G-

-A-A-T-T-C-G-C-G-A-T-G-

-A-A-T-T-C-G-C-G-A-T-G-

-A-A-T-T-C-G-C-G-A-T-G-

-A-A-T-T-C-G-C-G-A-T-G-



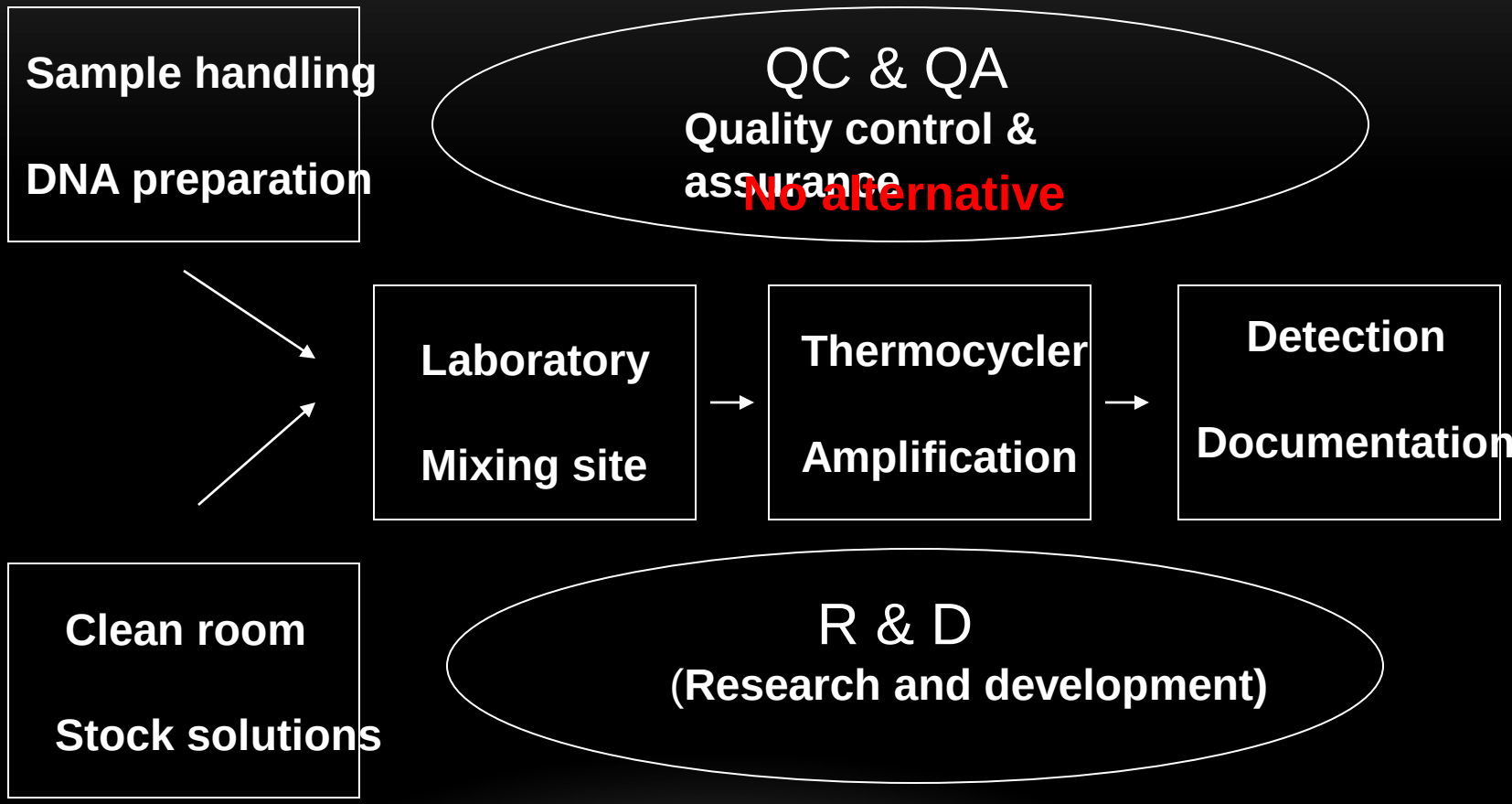
Exponential amplification

(Andy Vierstraete 2001)

ESTABLISHMENT OF A PCR LABORATORY

- To perform PCR for the repetitive detection of a specific sequence, three distinct laboratory areas are required. The **specific technical operations,** **reagents** ,and **personnel considerations**

PCR laboratory



Alternatives: - commercial kits
- robots + kits

Advantages

Molecular methods

- High sensitivity and specificity
- Detects pathogen, not immune response
- Quick results
- High transport toleration

In-house (home-brew) PCR methods

- Cost effective
- High sensitivity
- High quality
- Fast implementation of scientific discoveries
- Customer friendly

R&D is absolutely necessary

USES AND ADVANTAGES IN TESTING BY PCR METHODS

- Clinical diagnostics: detection and quantification of infectious microorganisms, cancer cells and genetic disorders
- Capable of amplifying long targets, up to 6.0 kb
- One-tube system allows rapid, sensitive and reproducible analysis of RNA with minimal risk of sample contamination
- Amplifies products from a wide variety of total RNA or mRNA sources

PREVENTION OF CONTAMINATION IN PCR LABORATORY

- PCR contamination be considered as a form of **infection**. If standard **sterile techniques that would be applied to tissue culture or microbiological manipulations are applied to PCR, then the risk of contamination will be greatly reduced**. Above all else, **common sense should prevail.**

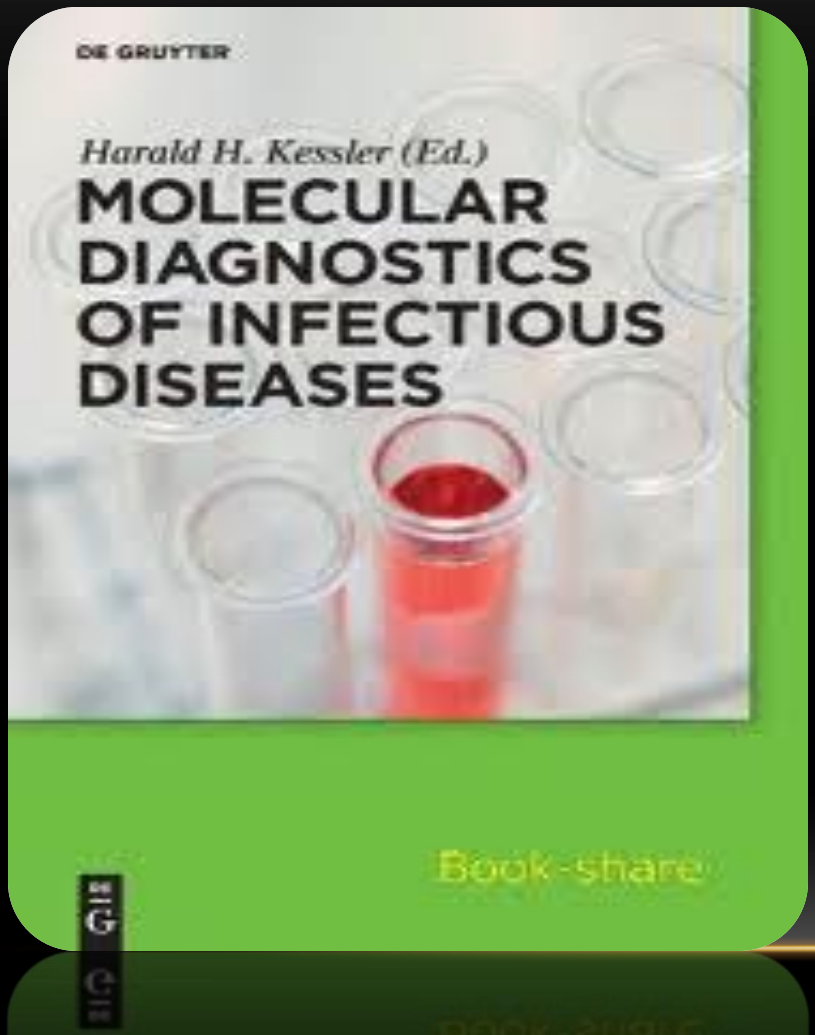
AVOIDING CONTAMINATION

- The single most important source of PCR product contamination is the generation of aerosols of PCR amplicons that is associated with the post-PCR analysis. Methods for eliminating this aerosol range from physical design of laboratories and use of specific pipettes to chemical and enzymatic approaches. The choice of method is often dependent on the frequency of amplification of a target amplicon and the relative amounts and concentrations of the amplicons created by the PCR.

COMMERCIAL KITS SUPPORT THE RESEARCH METHODS

- Commercial kits for the molecular detection and identification of infectious pathogens have provided a degree of standardization and ease of use that has facilitated the introduction of molecular diagnostics into the clinical microbiology laboratory

NUCLEIC ACID PROBES ENTERS IN MOLECULAR BASED DIAGNOSTICS



- The use of nucleic acid probes for identifying cultured organisms and for direct detection of organisms in clinical material was the first exposure that most laboratories had to commercially available molecular tests

MOLECULAR DIAGNOSTICS FOR INFECTIOUS DISEASES

- Molecular Diagnostics for Infectious Disease features emerging and novel technologies, from deep sequencing for microbial diagnostics to rapid molecular methods impacting the detection and control of hospital infections. The program will also feature the use of mass spec for pathogen detection in the clinical setting. These new technologies have the potential to save time, cost, and eventually lives. Some of the challenges to be addressed include clinical adoption and validation, as well as regulatory issues.

BEGINNING NUCLEIC ACID AMPLIFICATION

- Nucleic acid amplification provides the ability to selectively amplify specific targets present in low concentrations to detectable levels; thus, amplification-based methods offer superior performance, in terms of sensitivity, over the direct (non-amplified) probe-based tests. PCR **(Roche Molecular Systems, Branchburg, NJ**) was the first such technique to be developed and because of its flexibility and ease of performance remains the most widely used molecular diagnostic technique in both research and clinical laboratories.

PCR IN CLINICAL MICROBIOLOGY

- Molecular detection has mostly come to the **clinical microbiology** laboratory in the form of PCR technology, initially involving single round or nested procedures with detection by gel electrophoresis.

HELPS RAPID DETECTION

- Polymerase chain reaction (PCR) techniques have led the way into this new era by allowing rapid detection of microorganisms that were previously difficult or impossible to detect by traditional microbiological methods.

ADVANCES ON PCR METHODS

- Fairly recently, a new method of PCR quantification has been invented. This is called "**real-time PCR**" because it allows the scientist to actually view the increase in the amount of DNA as it is amplified.

NEW TECHNOLOGIES – REAL TIME ASSAYS

- The Real Time assays are proving to better technologies

1 Rapid

2 Quantitative measurement

3 Lower contamination rate

4 Higher sensitivity

5 Higher specificity

6 Easy standardization

Now a new gold standard for rapid diagnosis of virus infection in the acute phase samples.

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RT - PCR

- Proving to be

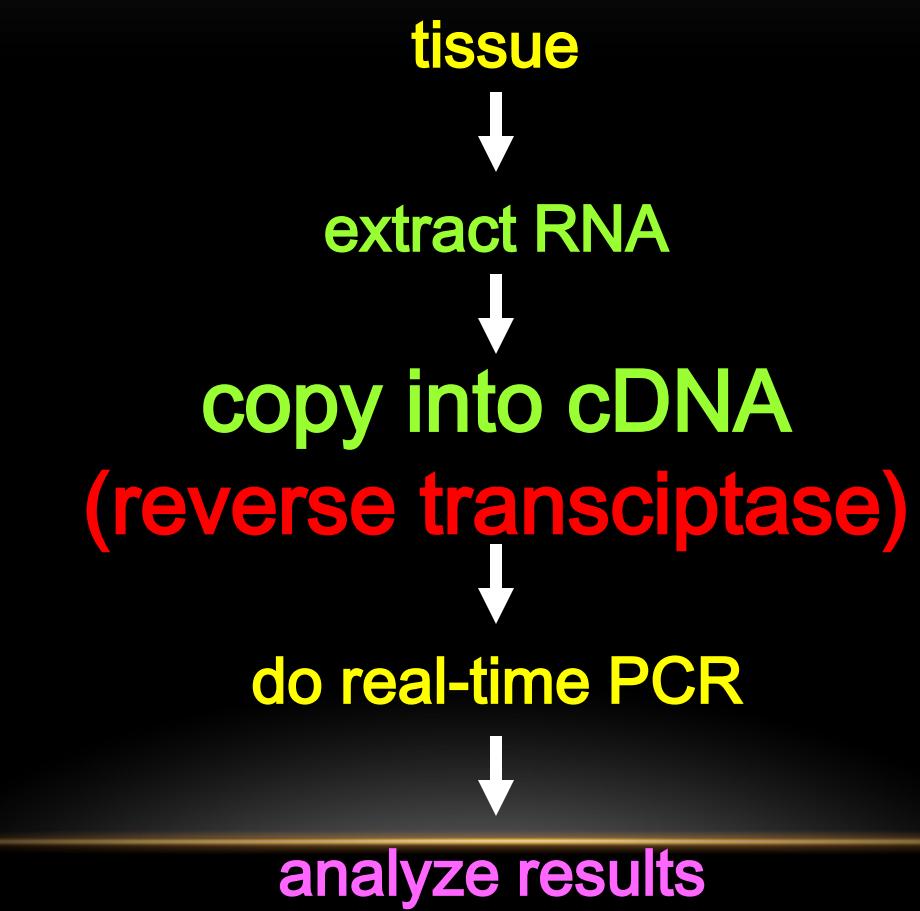
Accurate

Precise

Easy to perform

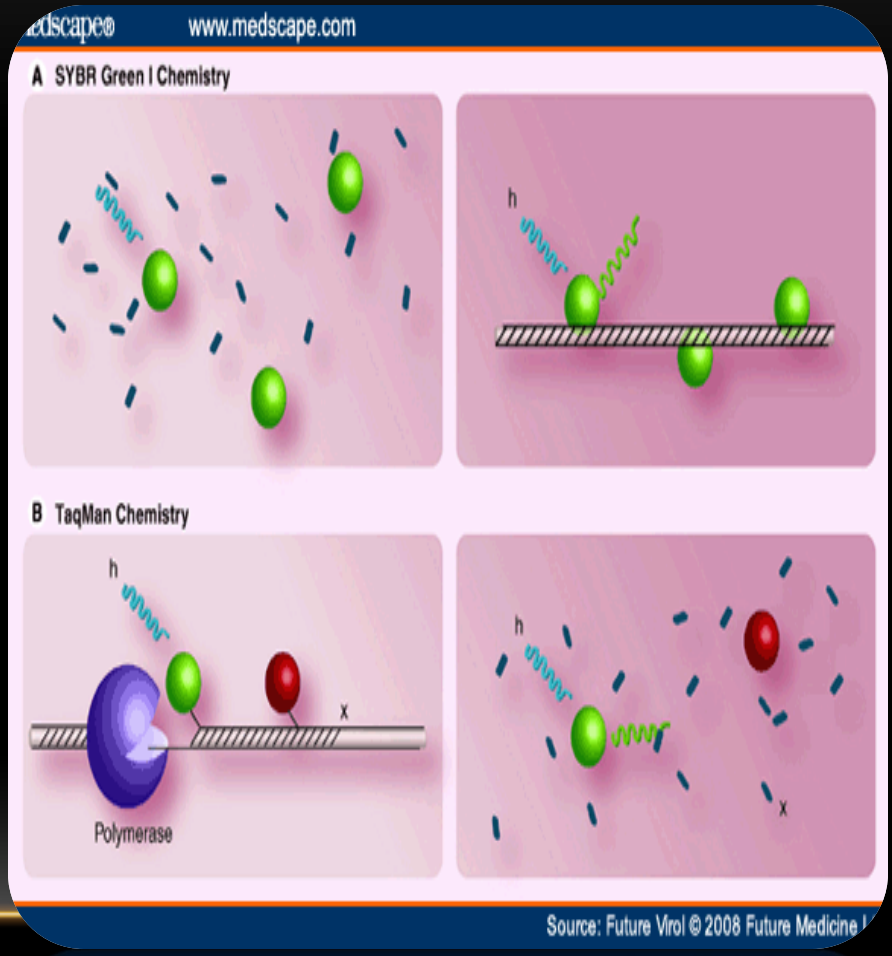
RT PCR technologies are easy to transfer research Laboratory protocols to **Diagnostic Laboratories**

OVERVIEW OF RT - PCR



REAL TIME REPORTERS

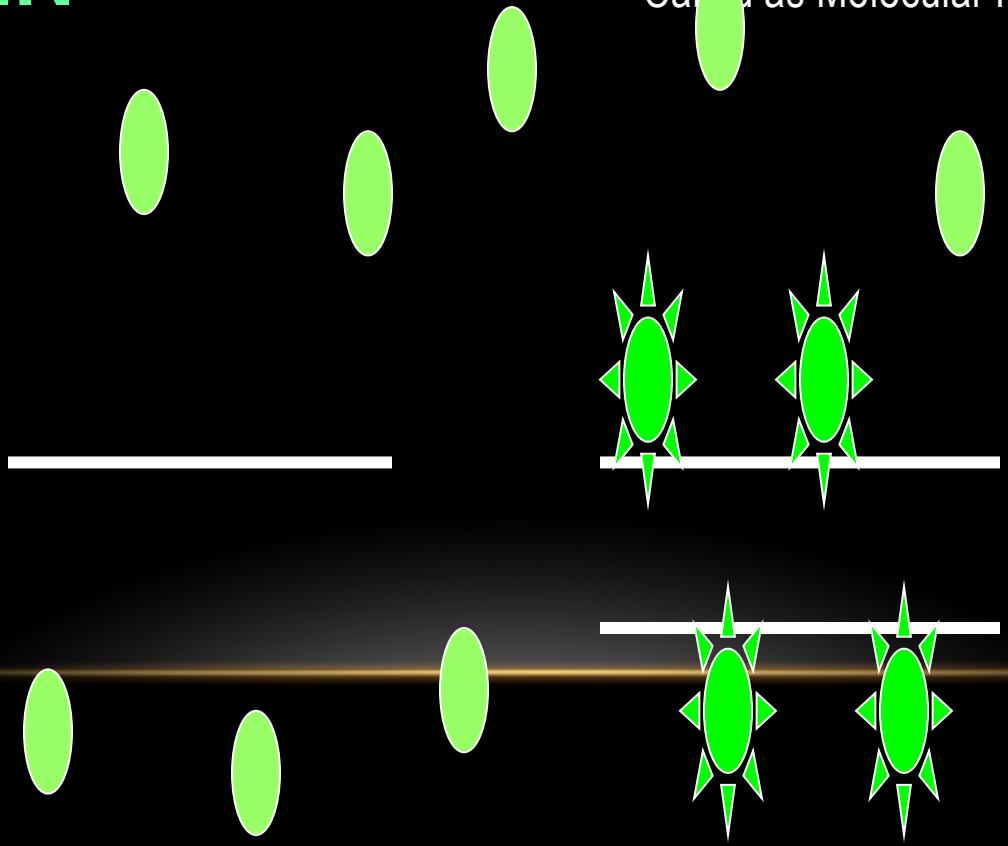
- All real time PCR systems rely upon the detection and quantization of fluorescent reporter, the signal of which increases in direct proportion of the amount of PCR product in a reaction.



REAL TIME PCR
CYBER GREEN

- **USING SYBER® GREEN**

- The simplest and economical format the reporter is the double strand DNA specific dye SYBR ® Green
- Called as Molecular Probe.



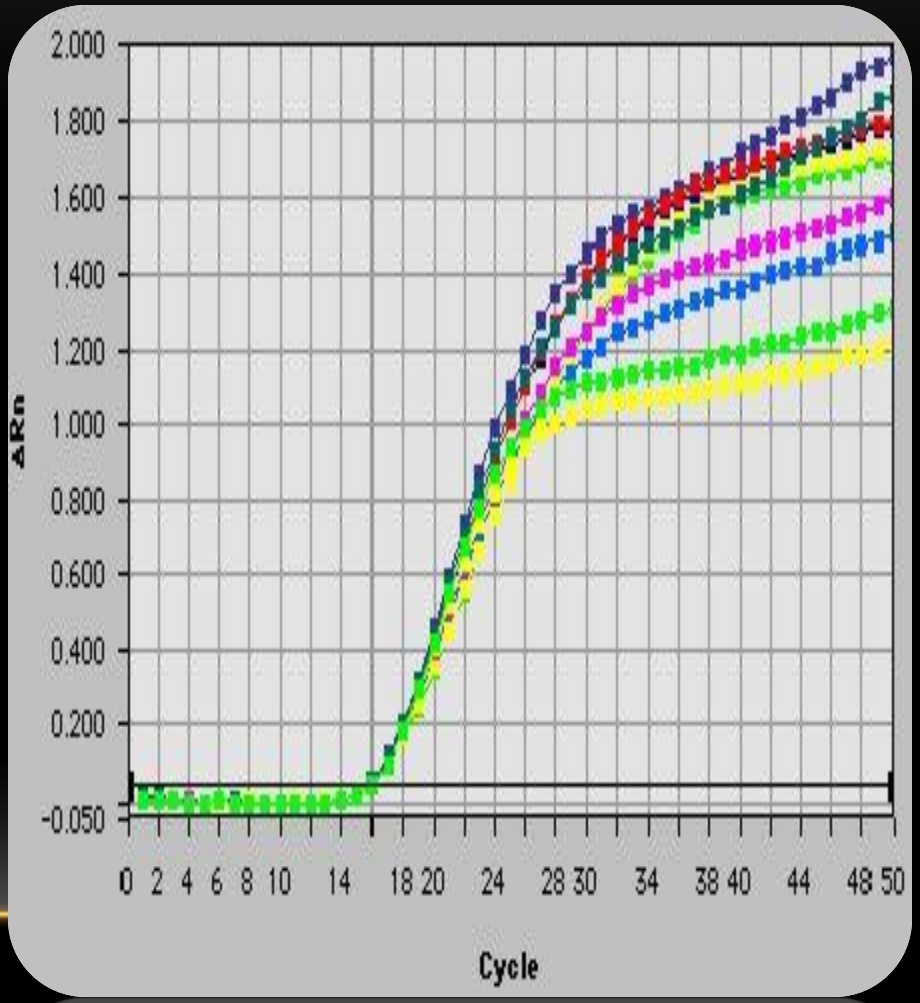
USES OF AUTOMATED RT - PCR

- Several viral infections can be detected in acute phase serum samples.
- Increasing used in for early and accurate detection of all most human viruses including

Measles, Mumps, Herpes simplex viruses, Rota viruses Noro virus,Influenzae virus type A and B, Respiratory Syncitical virus, SARS, Dengue Japanese Encephalitis, Hepatitis B and C, West Nile, Chikungunya,HIV, Avian flu virus,

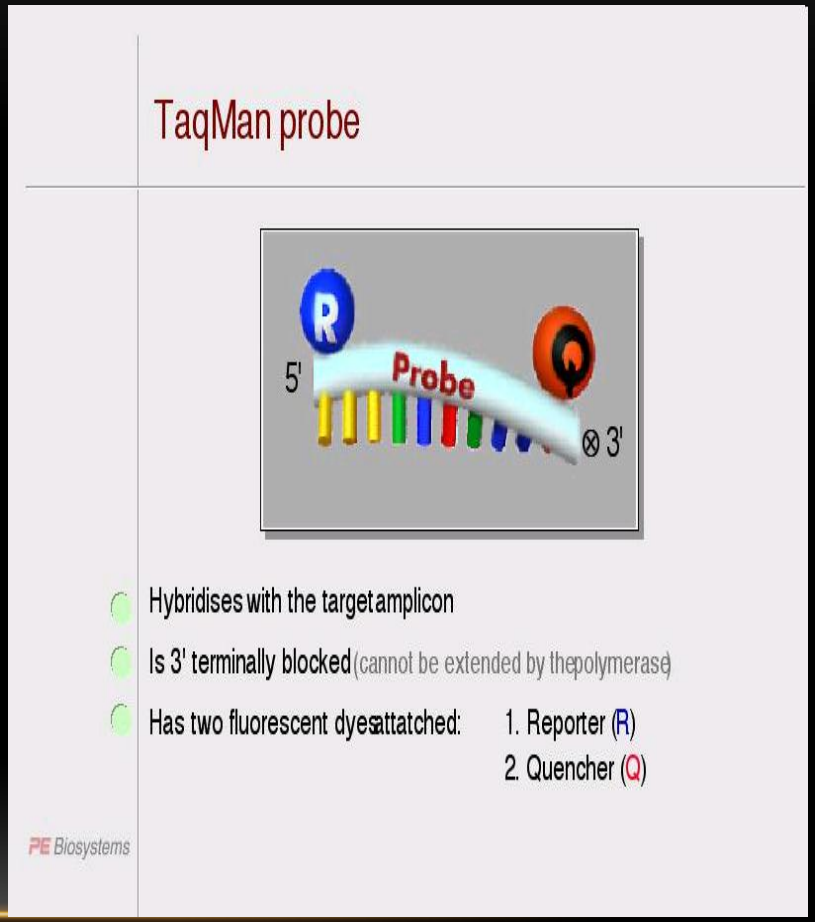
MULTIPLEX PCR IN REAL TIME

- Multiplex real time quantitative RT-PCR assays have been developed for simultaneous detection identification and quantification of **HBV, HCV and HIV-! In plasma and Serum samples.**

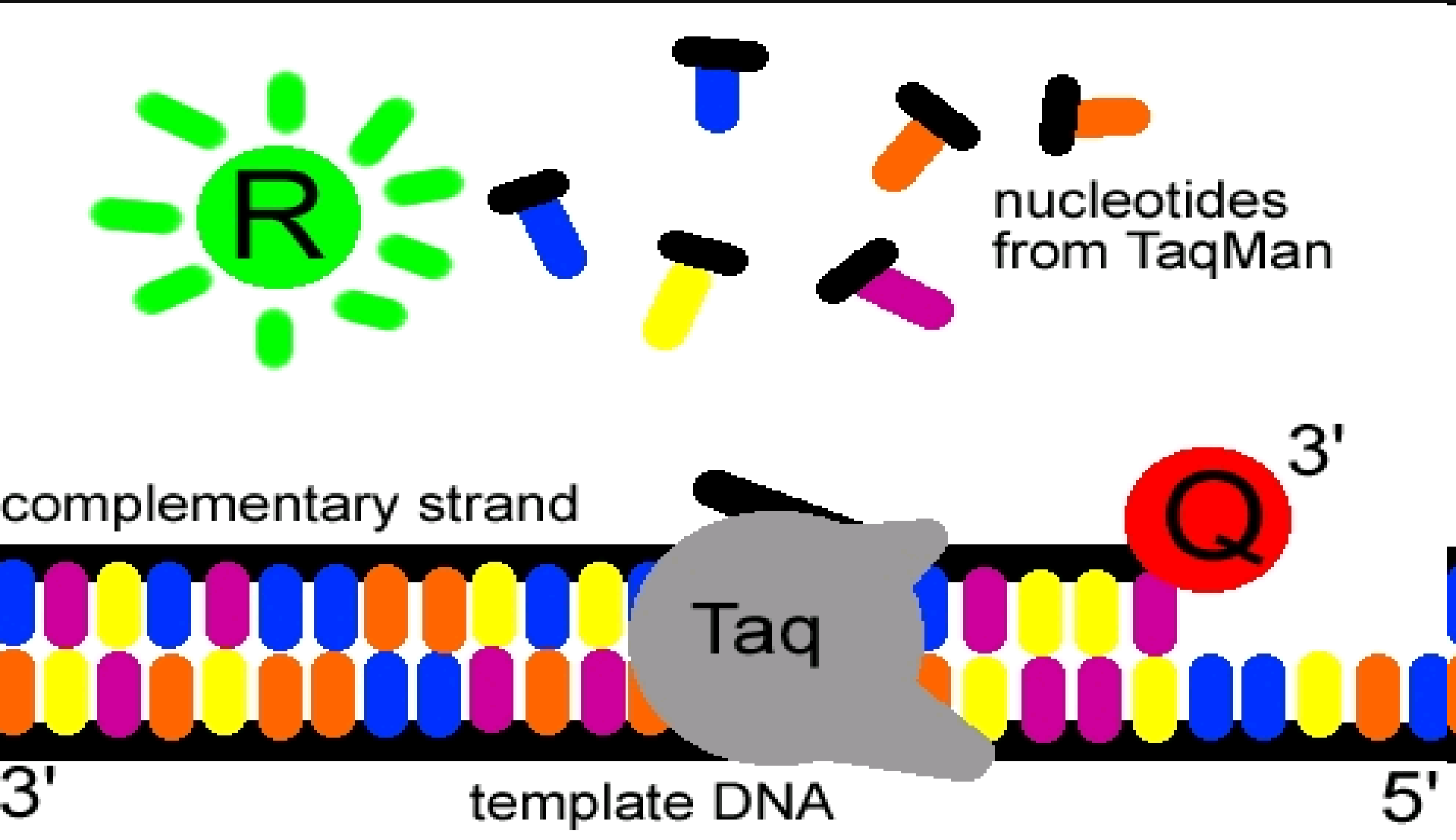


OTHER EMERGING ALTERNATIVES

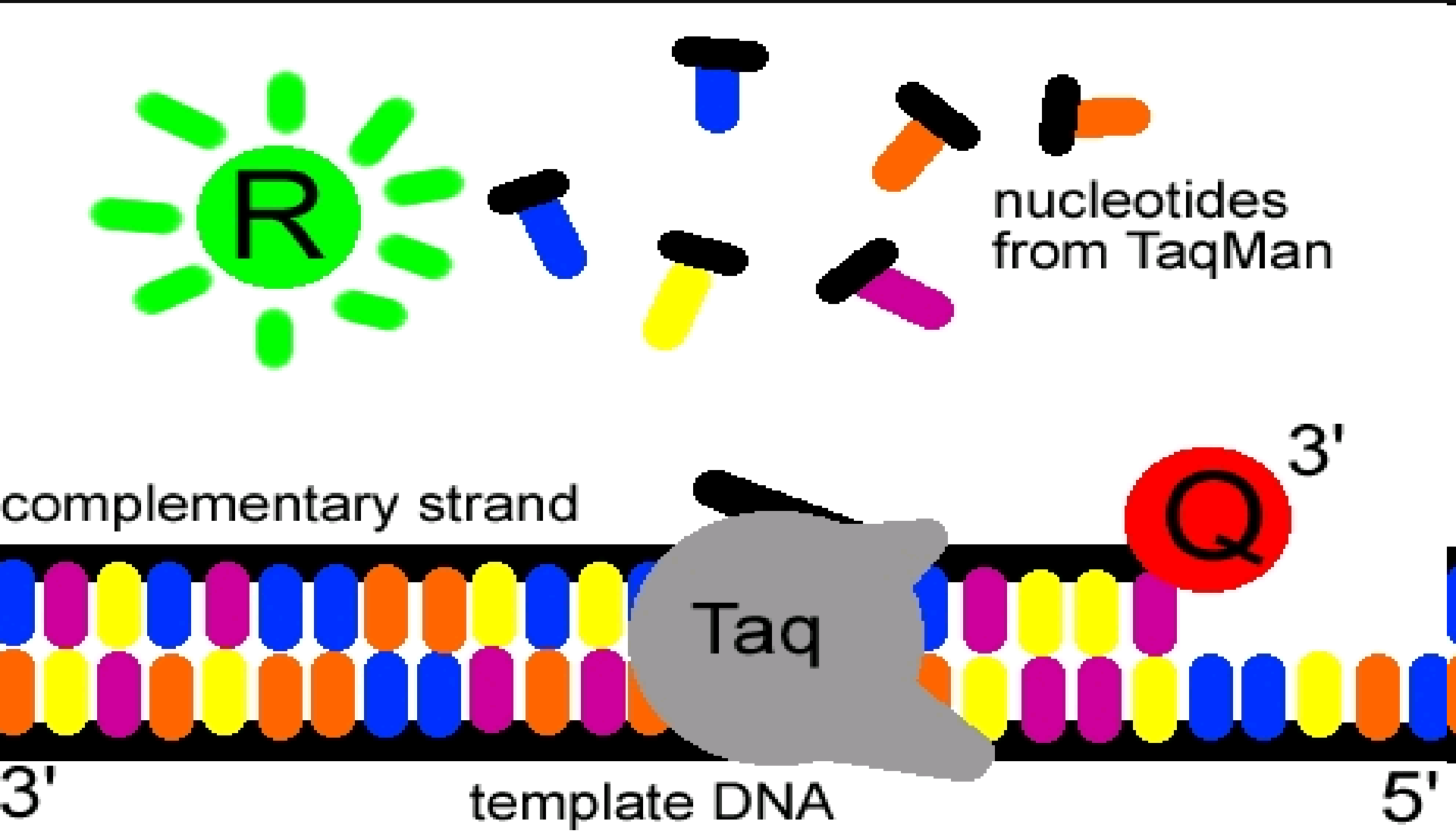
- Two most popular alternatives to SYBR green are **TaqMan®** and **Molecular Beacons**.
- Both technologies depend on hybridization probes relying on fluorescence resonance energy transfer.(FRET) and quantization



TAQMAN®


R
nucleotides
from TaqMan
3'
complementary strand
Q
Taq
3'
template DNA
5'

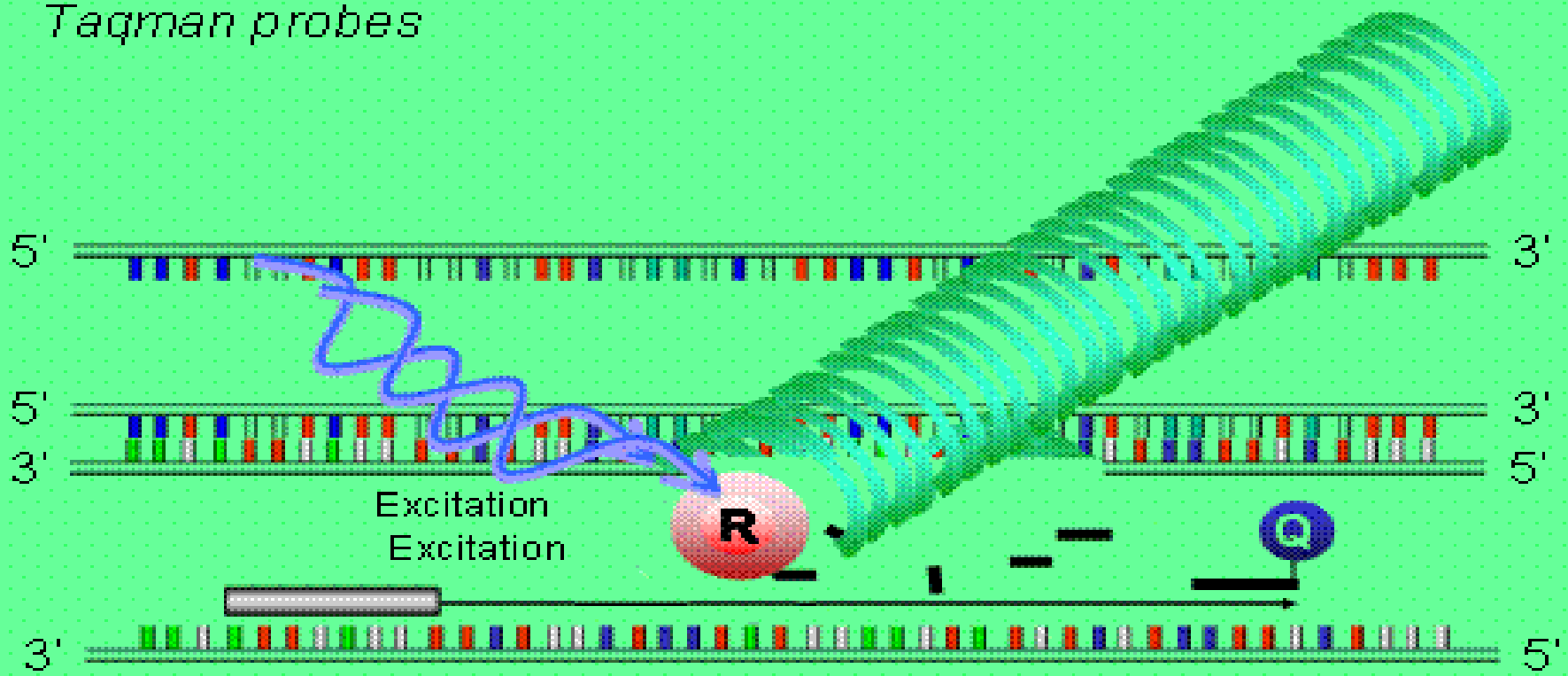
TAQMAN® SEQUENCING



TAQMAN® *PROBES*

Sequence Specific Probes:

Taqman probes



COMMERCIAL KITS AVAILABLE FOR BETTER DIAGNOSIS

- Commercial amplification-based molecular diagnostic systems for infectious diseases have focused largely on systems for detecting *N. gonorrhea*, *C. trachomatis*, *M. tuberculosis*, and specific viral infections (HBV, HCV, HIV, CMV, and enterovirus) . Given the adaptability of PCR, numerous additional infectious pathogens have been detected by investigator-developed or home-brew PCR assays[

ROUTINE DIAGNOSTIC FAILURES CAN BE ADOPTED TO MOLECULAR METHODS

- Organisms that cannot be grown / difficult to grow (HPV, HBV, HCV, HIV, EBV, CMV)
- (i) Fastidious, slow-growing agents
- (M. tuberculosis, Legionella pneumophila)
- (iii) Highly infectious agents (dangerous to culture)
- Francisella tularensis
- *Brucella* species
- Coccidioides immitis

NUCLEIC A PROBE HYBRIDIZATION

Organism	Specimen
Campylobacter spp.	Stool culture
Chlamydia trachomatis	Cervical & urethraswab,urine
Enterobacteriaceae	Blood culture FISH
H. influenza	CSF / TS culture
L. monocytogenes	Culture isolate
M. tb, avium, intracellulare,	Resp specimen culture
M. gordonae, kansasii	
N. gonorrhoeae	Urethral / cervical swab / culture

NUCLEIC ACID PROBE HYBRIDIZATION

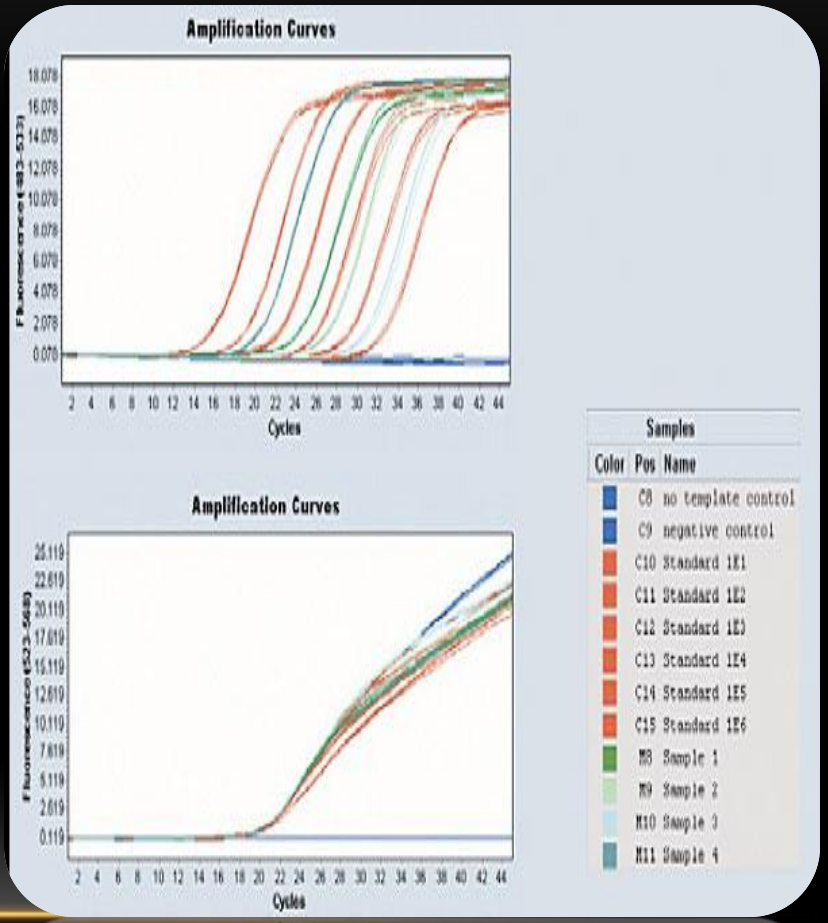
• *P. aeruginosa*	Blood culture FISH
• *S. aureus*	Blood culture FISH (PNA)
• MRSA	Culture isolate
• *Streptococcus* spp	. Blood culture FISH
• *S. pneumoniae*	Culture isolate
• *S. pyogenes*	Throat swab
• Streptococcus Gr	. B Culture isolâtes
• *C. albicans*	Blood culture FISH (PNA)
• *C. immitis*	Culture isolate
• *B. dermatitidis*	Culture isolate

NUCLEIC A PROBE HYBRIDIZATION……

• CMV	Whole blood, WBC HC, ISH
• *H. capsulatum*	Culture isolate
• HBV, HCV	Blood bDNA
• HSV Vesicle fluid	Hybrid Capture
• HIV	Blood bDNA
• HPV	Cervical swab / biopsy HC, ISH
• EB virus	CSF ISH

MOLECULAR METHODS DESIGNED FOR QUANTITATION TOO

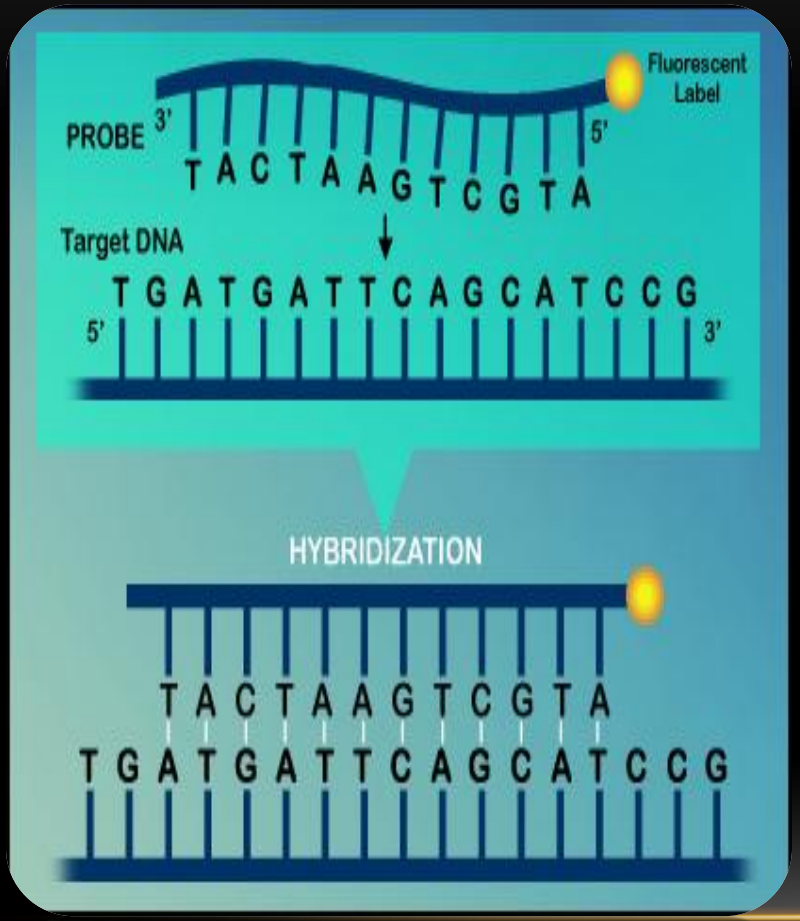
- In addition to qualitative detection of viruses, quantitation of viral load in clinical specimens is now recognized to be of great importance for the diagnosis, prognosis, and therapeutic monitoring for HCV, HIV, HBV, and CMV[Both PCR and nucleic acid strand-based amplification systems are available for quantitation of one or more viruses



SEVERAL METHODS FOR NON CULTIVABLE MICROBES

- Amplification-based methods are also valuable for identifying cultured and non-cultivatable organisms[Amplification reactions may be designed to rapidly identify an acid-fast organism as ***M. tuberculosis, M.lepra*** or may amplify a genus-specific or "universal" target, which then is characterized by using restriction endonuclease digestion, hybridization with multiple probes, or sequence determination to provide species or even subspecies delineation

PROBE HYBRIDIZATION



- Probe hybridization is useful for identifying slow-growing organisms after isolation in culture using either liquid or solid media. Identification of mycobacteria and other slow-growing organisms such as the dimorphic fungi (*Histoplasma capsulatum, Coccidioides immitis,* and *Blastomyces dermatitidis*) has certainly been facilitated by commercially available probes

GENE PROBES ARE USEFUL ..

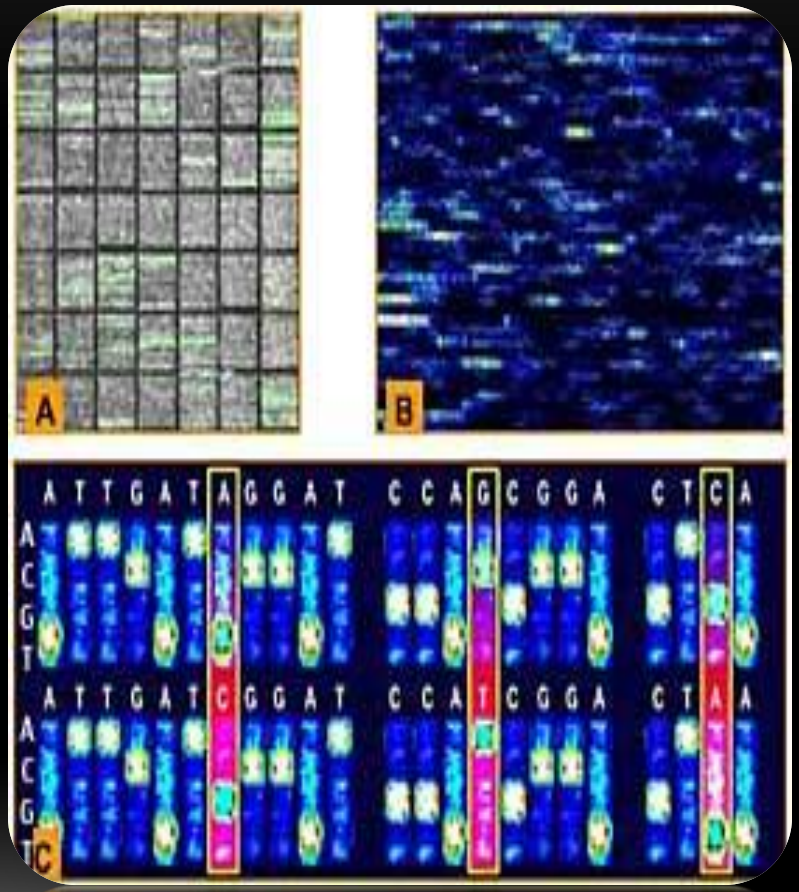
- .All commercial probes for identifying organisms are produced by Gen-Probe and use acridinium ester-labeled probes directed at species-specific rRNA sequences
- Gen-Probe products are available for the culture identification of *Mycobacterium tuberculosis, M. avium-intracellulare* complex, *M. gordonae, M. kansasii, Cryptococcus neoformans*, the dimorphic fungi (listed above), *N. gonorrhea, Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Haemophilus influenza, Enterococcus* spp., *S. agalactiae*, and *Listeria Monocytogenes*.

DETECTING ANTIMICROBIAL-DRUG RESISTANCE

- Molecular methods can rapidly detect antimicrobial-drug resistance in clinical settings and have substantially contributed to our understanding of the spread and genetics of resistance. Conventional broth- and agar-based antimicrobial susceptibility testing methods provide a phenotypic profile of the response of a given microbe to an array of agents. Although useful for selecting potentially useful therapeutic agents, conventional methods are slow and fraught with problems

MOLECULAR METHODS – GENE CHIPS

- Molecular methods may be used to detect specific antimicrobial-drug resistance genes (resistance genotyping) in many organisms Detection of specific point mutations associated with resistance to antiviral agents is also increasingly important Screening for mutations in an amplified product may be facilitated by the use of high-density probe arrays (Gene chips)[.



MOLECULAR METHODS TO DETECT MRSA

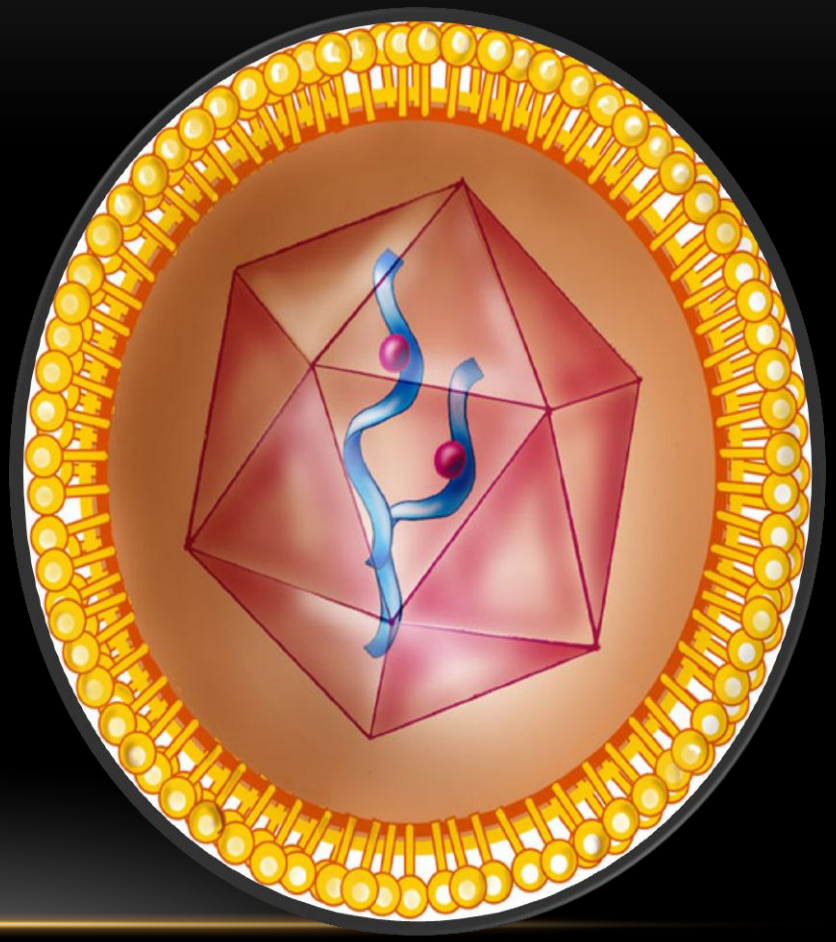
- The most common failing is in the detection of methicillin resistance in staphylococci, which may be expressed in a very heterogeneous fashion, making phenotypic characterization of resistance difficult[. Currently, molecular detection of the resistance gene, *mec A*, is the standard against which phenotypic methods for detection of methicillin resistance are judged[9,15,16

ROUTINE PHENOTYPIC DETECTION OF ANTIBIOTIC RESISTANCE YET CANNOT BE REPLACED

- Despite its many potential advantages, genotyping will not likely replace phenotypic methods for detecting antimicrobial-drug resistance in the clinical laboratory in the near future. Molecular methods for resistance detection may be applied directly to the clinical specimen, providing simultaneous detection and identification of the pathogen plus resistance characterization]

GENOTYPIC DETECTION CARRIES MORE IMPORTANCE IN VIROLOGY

- Likewise, they are useful in detecting resistance in viruses, slow-growing or nonviable organisms, or organisms with resistance mechanisms that are not reliably detected by phenotypic methods[



MOLECULAR METHODS HAVE LIMITATIONS

- However, because of their high specificity, molecular methods will not detect newly emerging resistance mechanisms and are unlikely to be useful in detecting resistance genes in species where the gene has not been observed previously. Furthermore, the presence of a resistance gene does not mean that the gene will be expressed, and the absence of a known resistance gene does not exclude the possibility of resistance from another mechanism. Phenotypic antimicrobial susceptibility testing methods allow laboratories to test many organisms and detect newly emerging as well as established resistance patterns.

Advantages

Molecular methods

- High sensitivity and specificity
- Detects pathogen, not immune response
- Quick results
- High transport toleration

In-house (home-brew) PCR methods

- Cost effective
- High sensitivity
- High quality
- Fast implementation of scientific discoveries
- Customer friendly

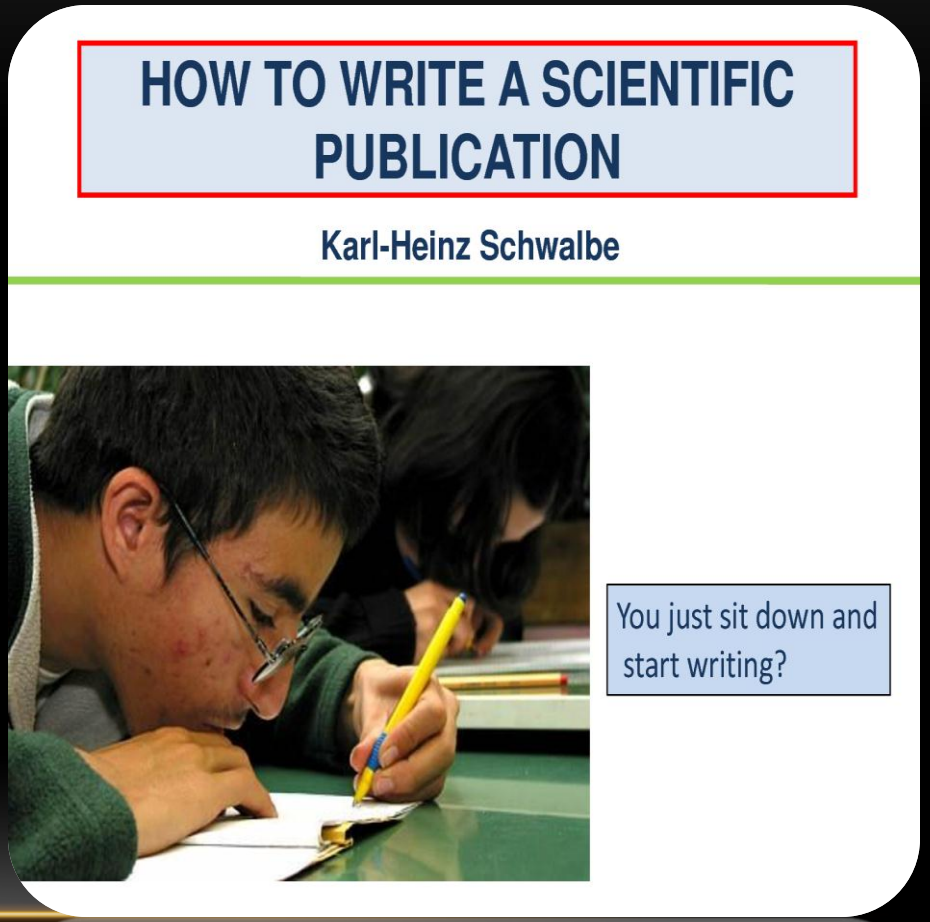
R&D is absolutely necessary

OUR VISION TO FUTURE DIAGNOSIS OF INFECTIOUS DISEASES

- With the ability to test for an unlimited number of potential pathogens simultaneously, next-generation sequencing has the potential to revolutionize infectious diseases diagnostics
- In the microbiology laboratory, this technology will likely replace the traditional “one test, one bug” approach to pathogen diagnostics
- The deep sequence information being generated is rapidly surpassing our capacity to analyze the data and will necessitate the development of highly parallel computational frameworks, such as cloud computing
- In adapting this technology for clinical diagnostics, interpretation of data, appropriate quality control standards, and fulfilling regulatory requirements will be critical
- One powerful application of next-generation sequencing is discovery of novel pathogens that may be associated with acute or chronic illnesses

WHY WE MUST BE FAMILIAR WITH MOLECULAR METHODS

- In Many Developed countries several Diagnostic methods are switched on to Molecular Methods.
- No scientific journal is willing to accept or publish any article without incorporation of Molecular Methods.
- Antibiotic drug resistance is a growing concern, to the world, unless molecular identification is performed on responsible genetic mechanisms no effective scientific conclusions can be drawn to contain the spread.



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