Current Molecular Diagnosis

NSW State Reference Laboratory for HIV/AIDS &
Molecular Diagnostic Medicine Laboratory, SydPath

St Vincent's Hospital Sydney

Trends in molecular diagnostics

- Detection of target genes of interest
- Quantification
- ***Infectious diseases***
  - **HIV**
  - **Hepatitis C & B**
  - **TB / MAC**
  - **Cytomegalovirus**
  - **Herpes simplex**
  - **Varicella zoster**
  - **CT/GC**
  - **HPV**
- Profiling mutations associated with disease outcome
  - Hepatitis C genotype
  - HIV drug resistance genotype
  - Host genetic factors
  - Thrombophilia
  - CyP450 – drug metabolism
  - HLA type


Key developments

Technology

- Uptake in diagnostic arena
- Alternative methods to PCR – SDA, TMA, LCR, NASBA, bDNA
- Availability of Analyte Specific Reagents (ASR)
- Trend to real time or kinetic formats
- Automation
- Contamination and inhibitor control

Key developments

Diagnosis

- Herpes simplex virus – detection and differentiation
- Cytomegalovirus – mRNA or qDNA
- Human papillomavirus (HPV) – ♀♂
- HCV / HIV primary/neonatal infection – Tx applications
- Bacterial sexually transmitted diseases

Key developments

Monitoring

- Cytomegalovirus - response to therapy / relapse
- HIV drug resistance testing
- Hepatitis C RNA quantification and genotype
- Improved technology –sensitivity / specificity / efficiency

Personalized clinical management

? What is driving commercial NAT platform development

- Reduce sources of error
- Reduce tedious processes
- Time to result
- Improved analytical range
- Limit of detection
- Improve specificity

Source of error

Pre-amplification

- Specimen integrity
- Nucleic acid extraction
- Reagent preparation and stability
- Volumetric precision / accuracy

Amplification

- Conserved target sequence – primer design
- Equipment – closed tube, calibration
- Contamination control, competition in low template samples

Post-amplification

- Volumetric precision / accuracy
- End point detection – fragment size vs enzymatic colorimetric probe hybridisation

Uncertainty of measurement

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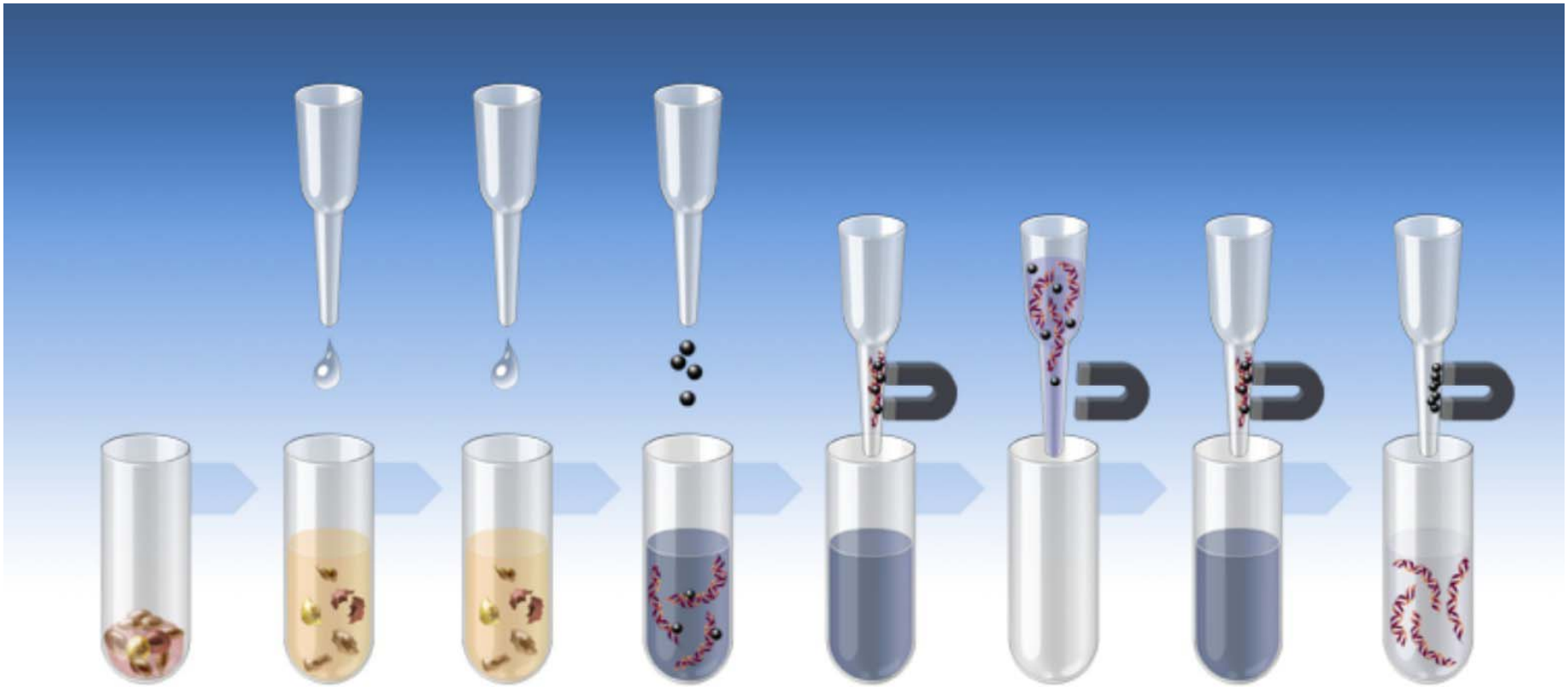
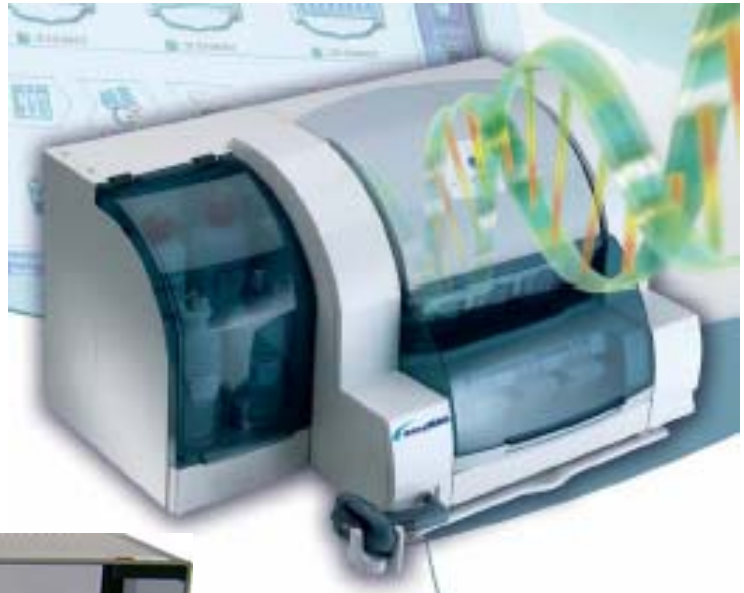
Pre amplification - extraction

- Volumetric errors amplified
- Tedious – manual, repetitive
- Specimen integrity

MagNA Pure LC

Isolation Principle - DNA





NucliSens® easyMAG™

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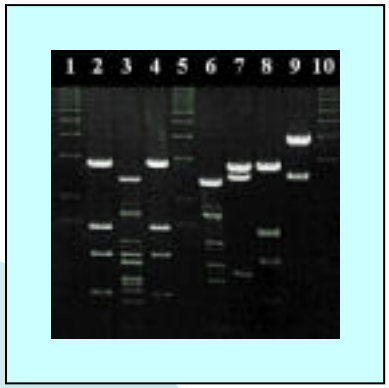
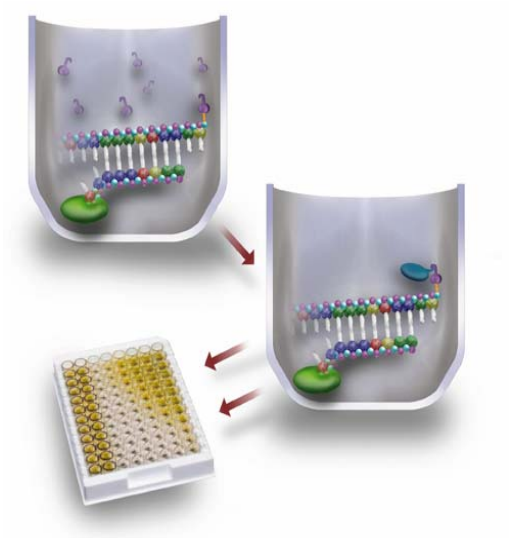
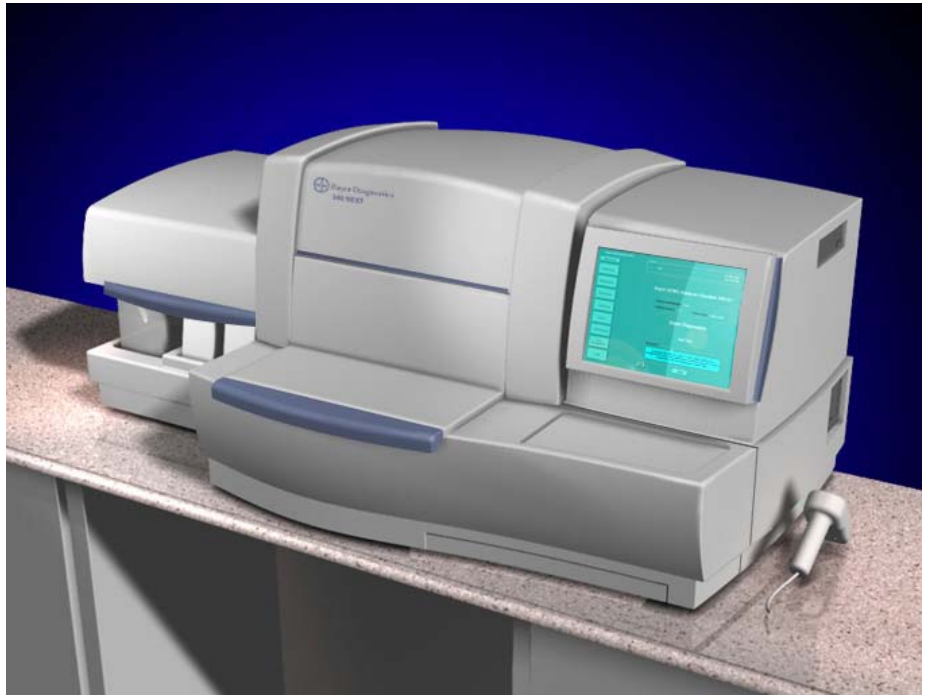
Post amplification & detection
Endpoint detection



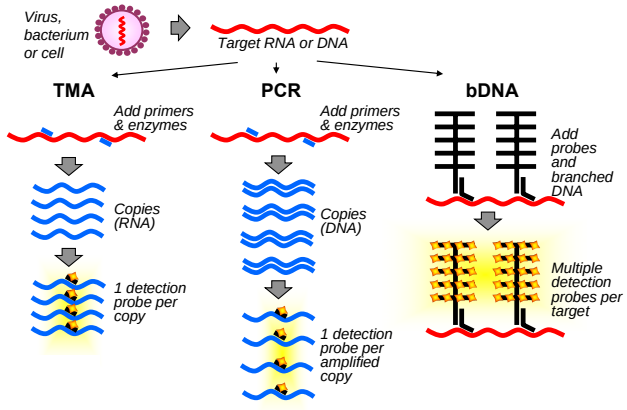


- Volumetric error
- Fragment size vs. probe hybridisation
- Time to result
- Automation calibration issues
- Result calculations

Signal amplification - bDNA



Comparison of Amplification Methods



HIV, HBV, HCV, CMV
Standard curve
Amplify signal of label – no amplicon issues
Overnight
High throughput
Limited extraction

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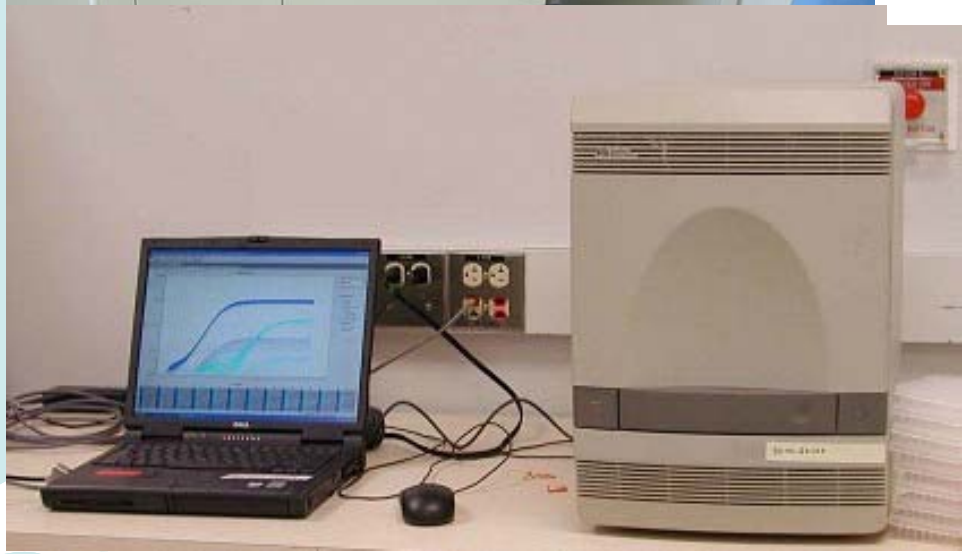
Kinetic / real time product detection

Real time PCR



Cepheid GeneXpert® System

Cepheid | Sample in. Answer out.™

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GeneXpert Cartridge

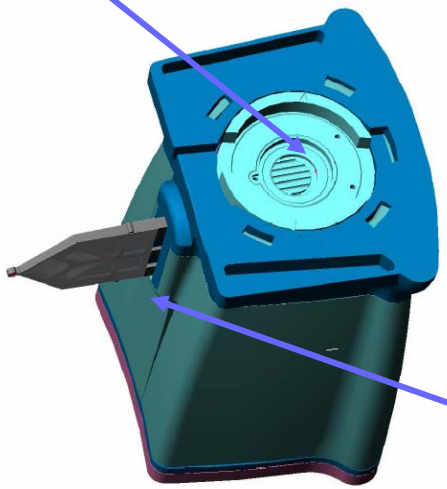
Cepheid | Sample in. Answer out.™

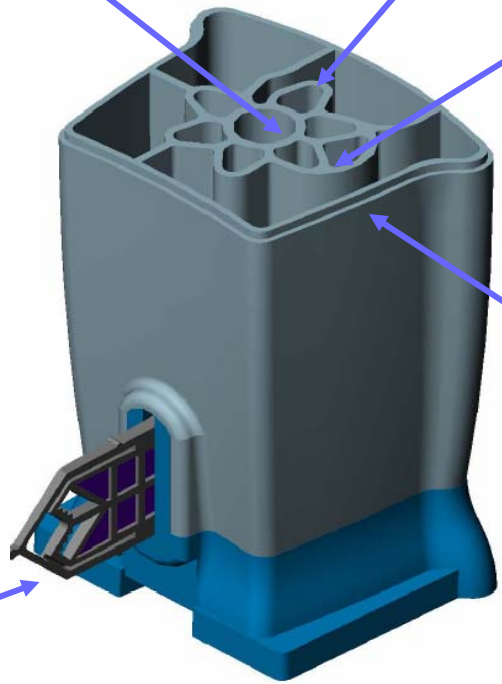
Small volume chambers

Large volume chambers

Syringe Barrel

Filter capture/concentrator for bacteria





Waste chamber

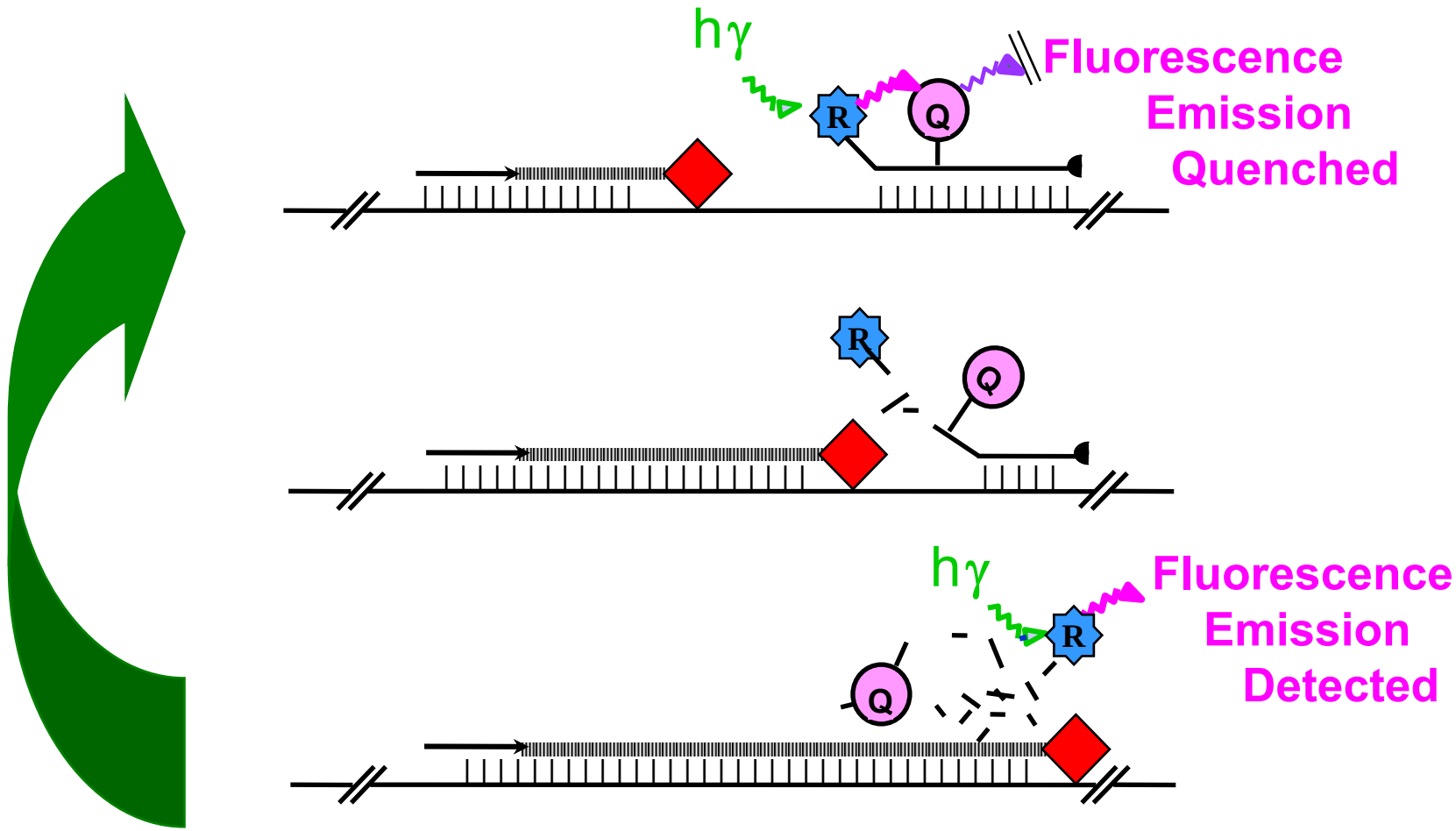
PCR Tube

Solid Support-Active Site

Liquid Handling

Real Time PCR with 5' Nuclease Assay

Product detection during amplification



HIV viral load tests

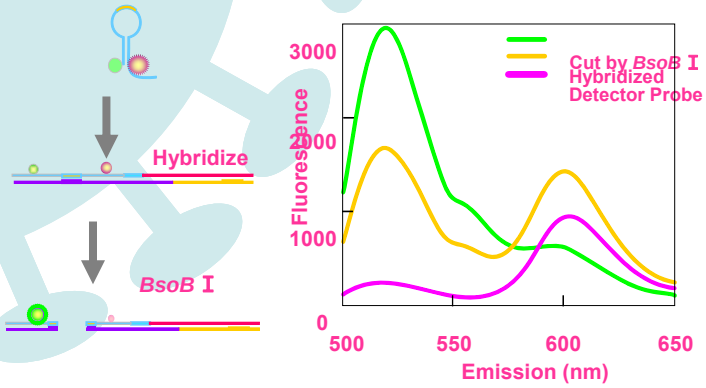
Manufacturer	Principle	Results	Availability	Analytical range
Roche	RT-PCR *(gag)* (COBAS HIV MONITOR v1.5)	copies/mL (6 hours to result)	Widely	<50 – 100,000 <400 – 750,000
Bayer	HIV Branched DNA 3.0 (bDNA) *(pol)*	copies./mL (results 2x less than Roche) (36 hours to result)	NSW, Vic	<50 – 800,000
Biomerieux	HIV-1 QT NASBA *(gag)*	Copies/mL (6 hours to result)	NSW	<400 – 1,000,000 <80 – 500,000
Roche	Real time (Taqman) *(gag)*	Copies/mL (4-6 hours to result)	New (no sites)	<40 – 10,000,000
Biomerieux	EasyQ HIV-1 real time TMA *(gag)*	Copies/mL & IU/mL (4-5 hours to results)	New (no sites)	<40 – 10,000,000
Abbott	Celera Realtime PCR m2000 *(pol integrase)*	copies/mL	Evaluation	<40 – 10,000,000
Artus	Realtime PCR – Rotorgene	Copies/mL	Evaluation	<40 – 10,000,000

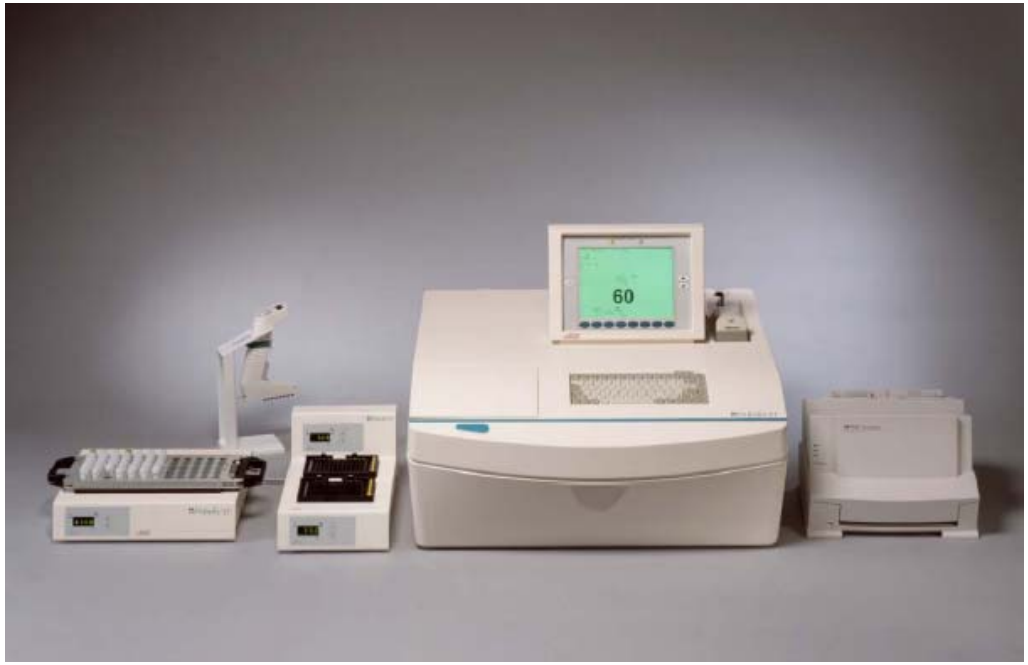

Strand displacement amplification

Exo-Bst DNA polymerase
5’-3’ polymerase activity
Lacks 5’-3’ exonuclease
Initiates DNA replication at single stranded nick

Simultaneous displacement of the nicked single strand (nick translation)

BsoBI restriction endonuclease
Recognition site in SDA primer containing α-thio-substituted nucleotides





FRET detector probe containing BsoBI recognition sequence and anchor sequence

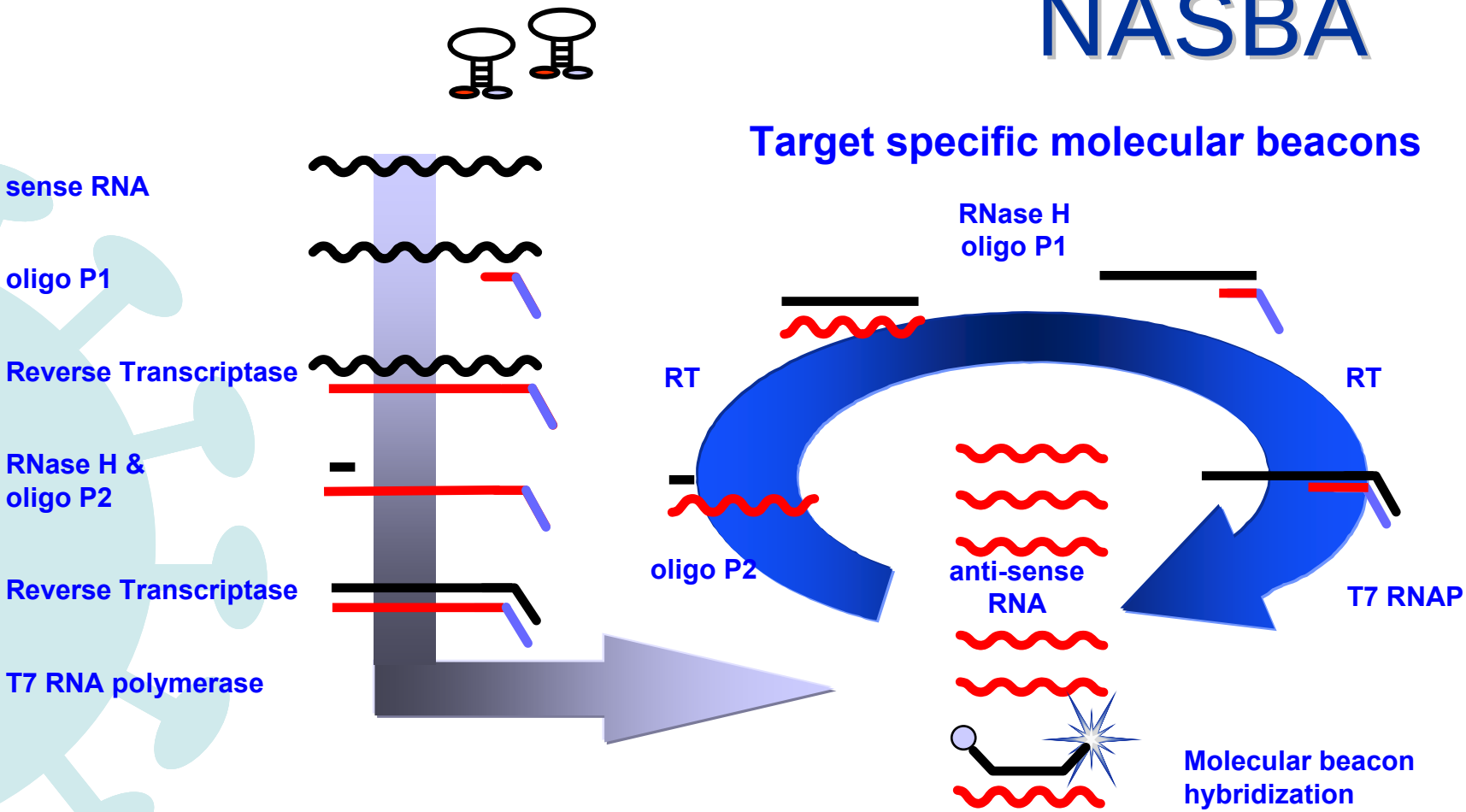
Flourescein 5’/rhodamine 3’ dyes

Isothermal Target Amplification
NASBA

Qualitative format

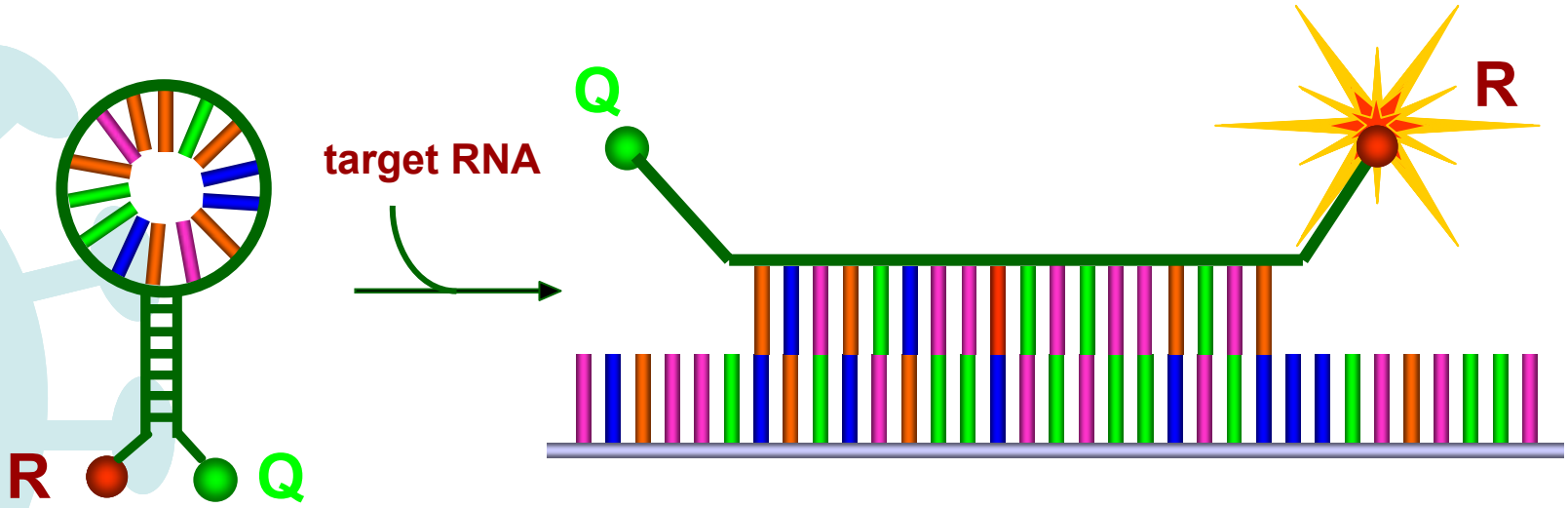
Quantitative format

Real-time Detection in NASBA



Molecular Beacon detection

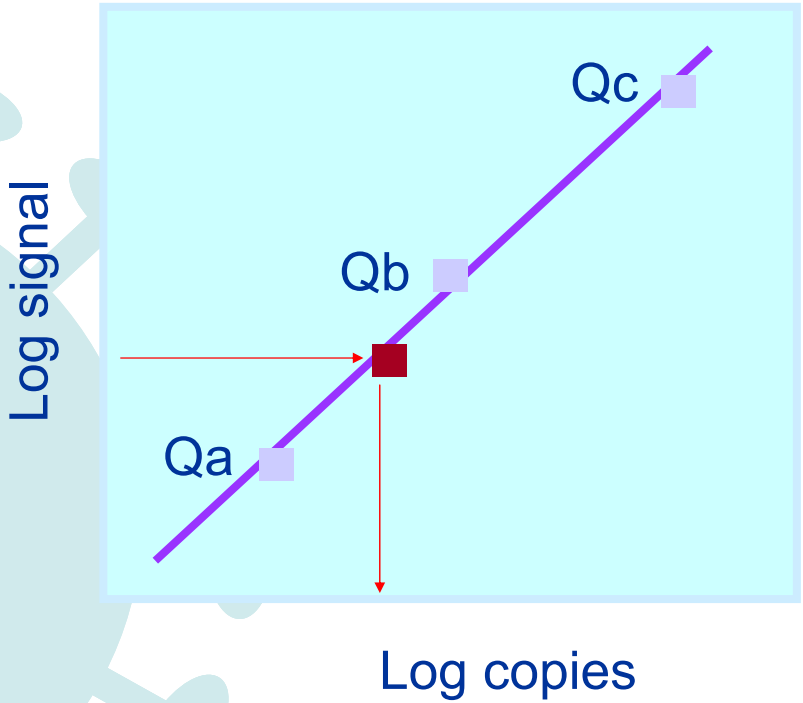
Detection of RNA with molecular beacon



Fluorescence signal increases with increasing RNA levels

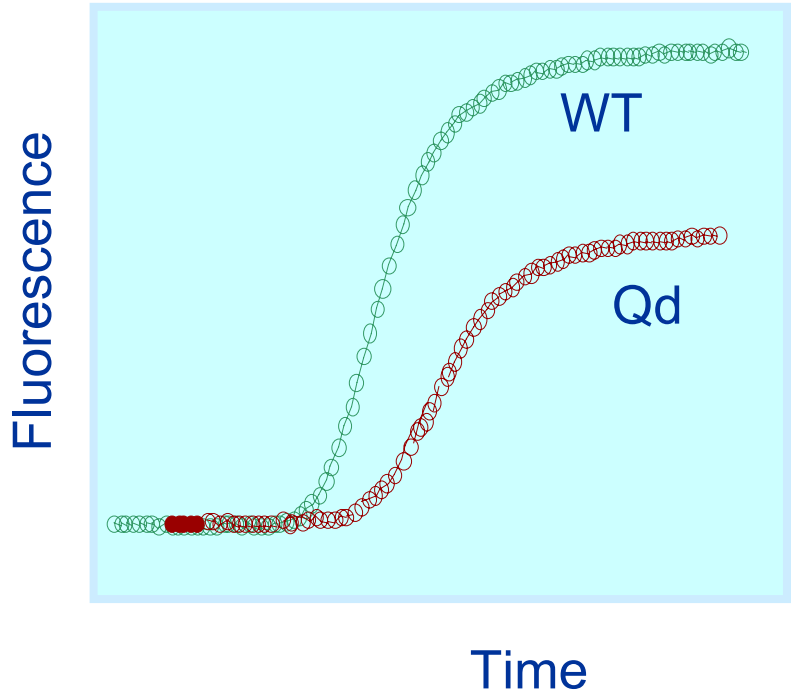
NASBA Calibrators & Quantitation

NucliSens HIV-1 QT



End-point measurement (WT and 3 calibrators)

NucliSens EasyQ HIV-1

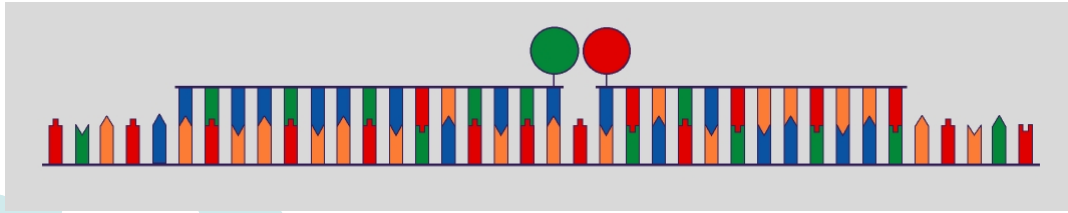


Kinetic measurement (WT and 1 calibrator)

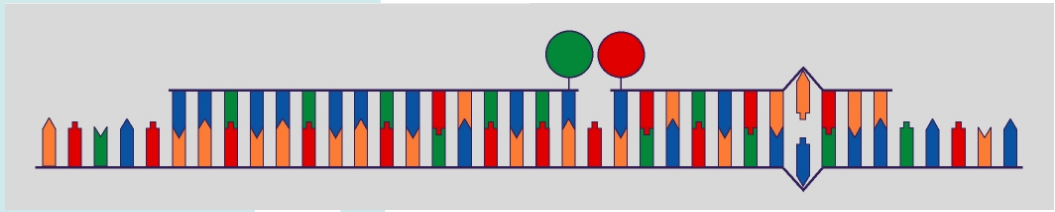
Mutation detection and product analysis

Mutation detection probe design

perfect match



mismatch



Anchor Probe **Mutation Probe**

T_m varies by GC composition

Low GC High GC

temperature

T_m is influenced by:

- **Salt concentration**
- **$MgCl_2$ concentration**
- **SYBR Green I concentration**

T_m of Mutation Probe approx. 5 °C lower than T_m of Anchor Probe

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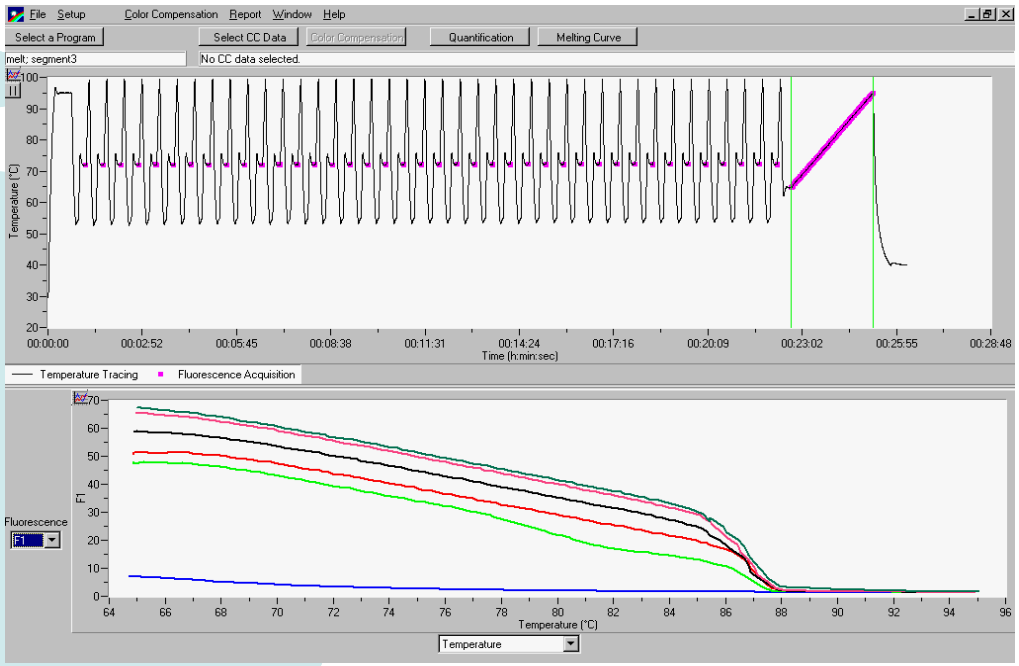
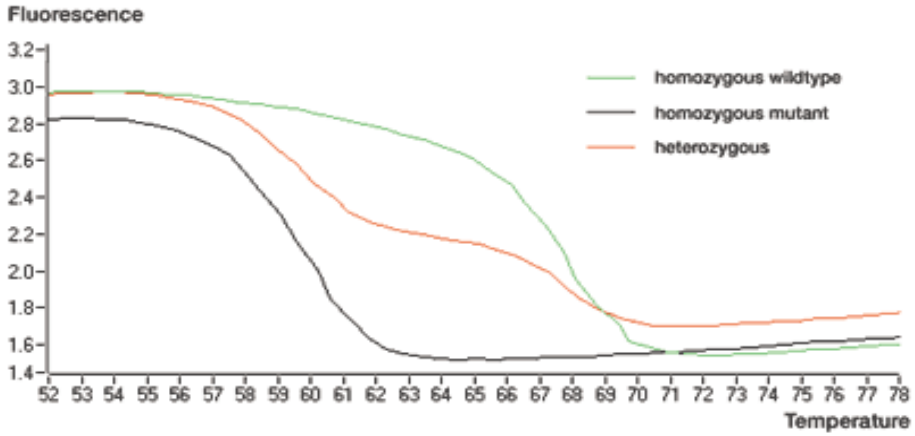
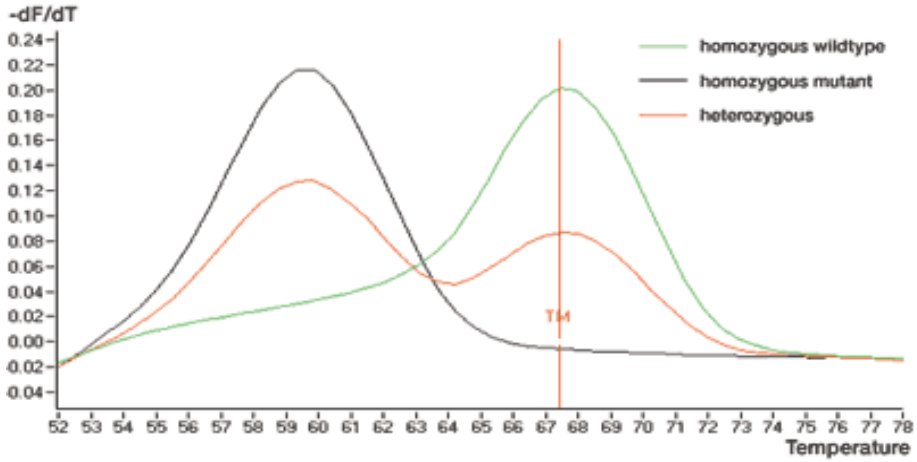
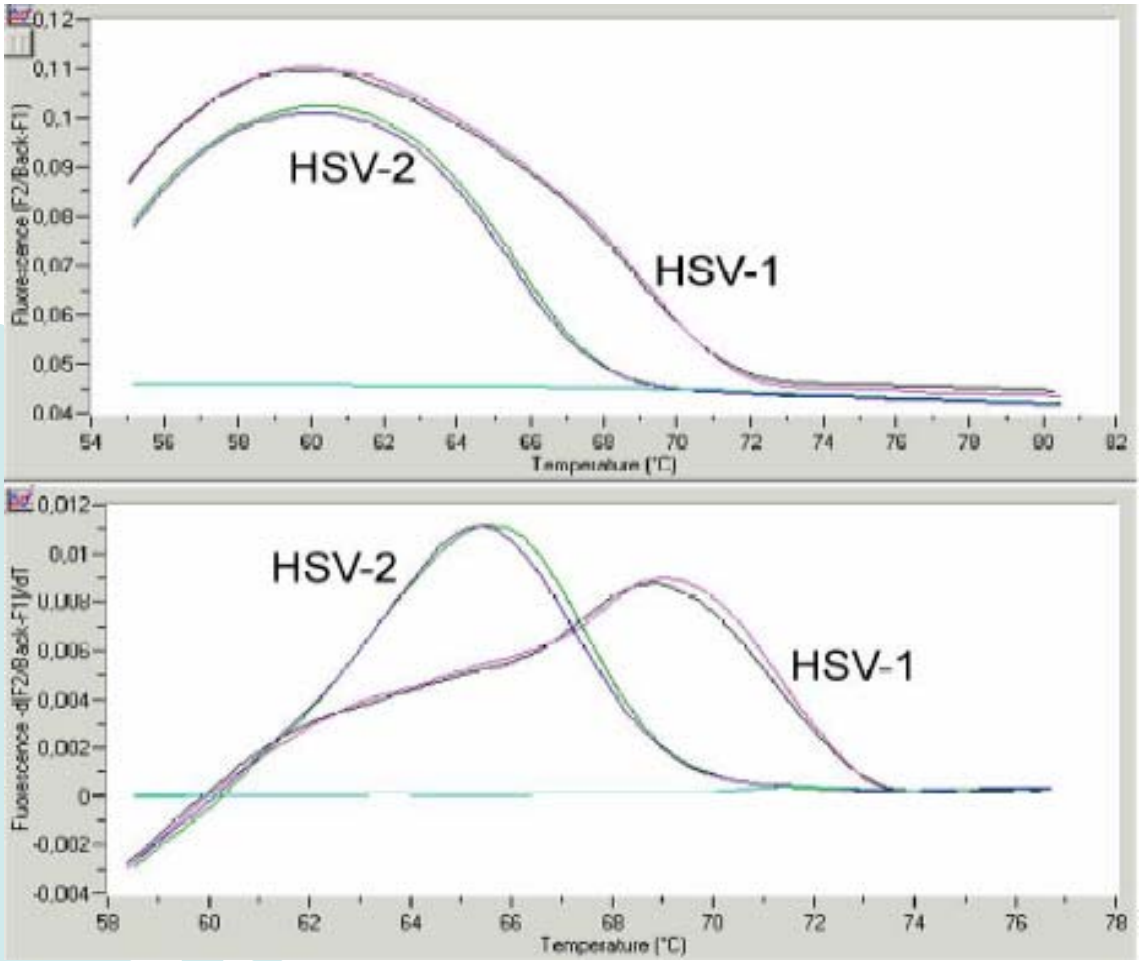
Product analysis – melting curve

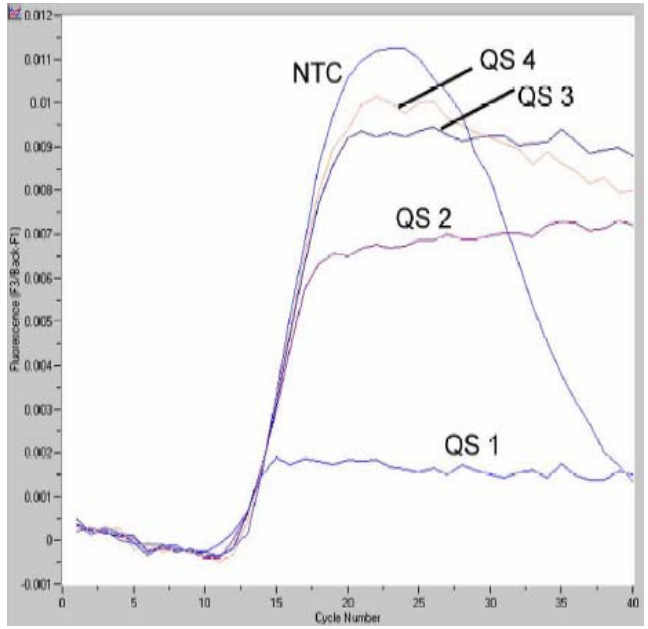






Herpes simplex type differentiation





IC detection

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Selected Applications

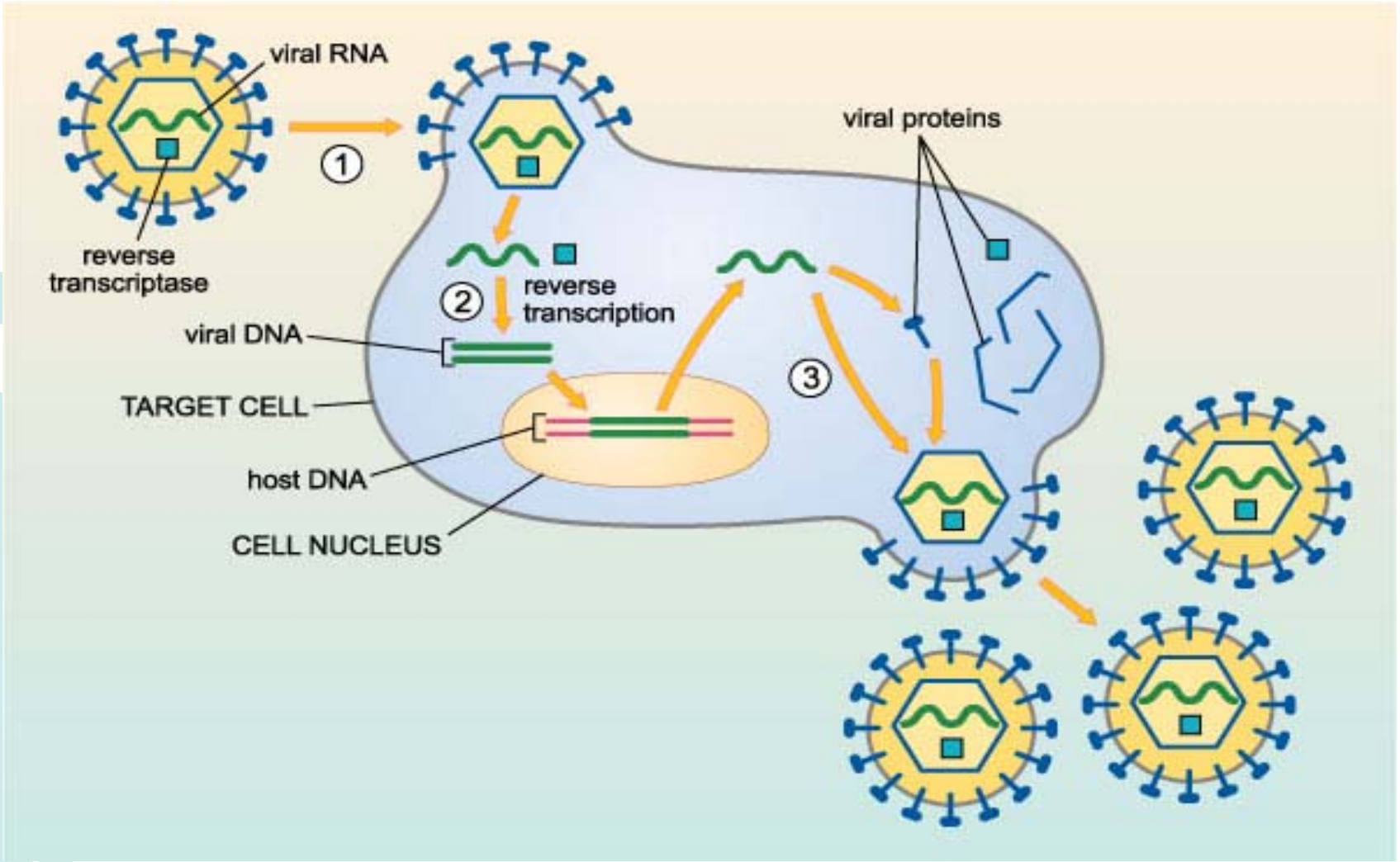
HIV Testing
Direct Detection of Virus

- **p24 antigen detection – serology**
  - p24 only assays – qualitative and quantitative
  - p24 in combination with antibody
  - Serum
- **Virus isolation - culture**
- **Nucleic acid detection - (NAT)**

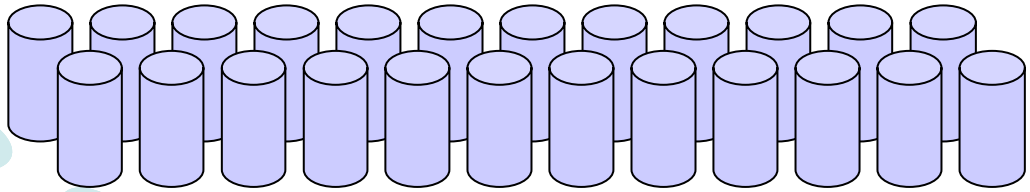
HIV DNA or RNA ?

DNA **qualitative – proviral (cellular)**
resolution of inconclusive serology
diagnosis in infants - maternal antibodies

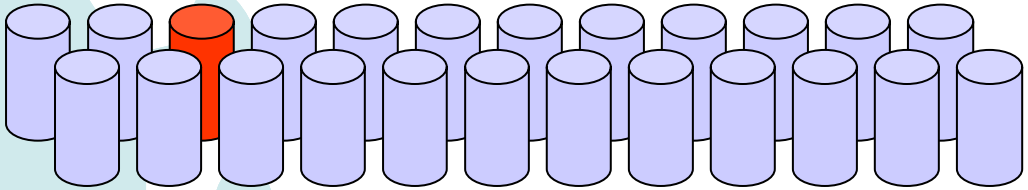
RNA **quantitative – serial viral load**
drug resistance monitoring
subtyping



viral RNA
1
reverse transcriptase
2
reverse transcription
viral DNA
TARGET CELL
host DNA
CELL NUCLEUS
viral proteins
3

Minipool NAT testing in blood donors

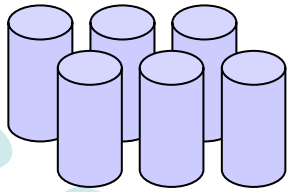


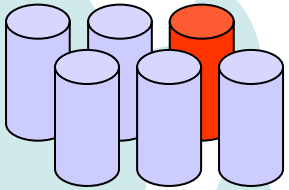
24 x negative results



Re-test ALL Individual samples

High risk population screening





6 x negative results

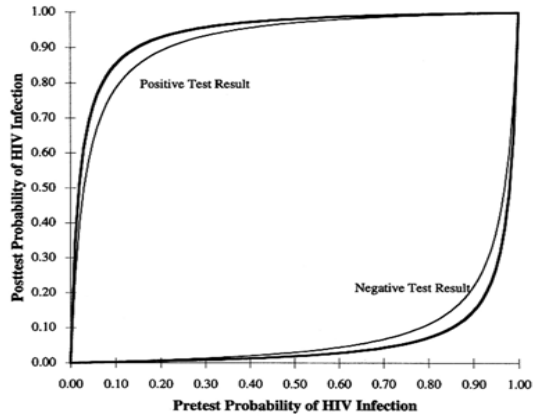
Re-test ALL Individual samples

Neonatal HIV diagnosis

- Serologic assays
  - Maternal antibodies persist up to 18 months postpartum
  - Antibody tests not helpful in newborn
  - Sero-reversion (pos → neg) in serial samples
  - HIV-1 p24 antigen limited value – complexed by Ab
- Virologic assays
  - Virus culture from PBMC
  - Maternal HIV-1 RNA in obstetric setting is useful in predicting risk of perinatal transmission
  - HIV DNA and RNA useful in infant
    - Detection in infant is diagnostic for perinatal HIV infection
    - Useful in timing of transmission (in utero, intrapartum, post partum)
    - Monitoring response to therapy in infected infant


Neonatal Diagnosis Qualitative DNA PCR

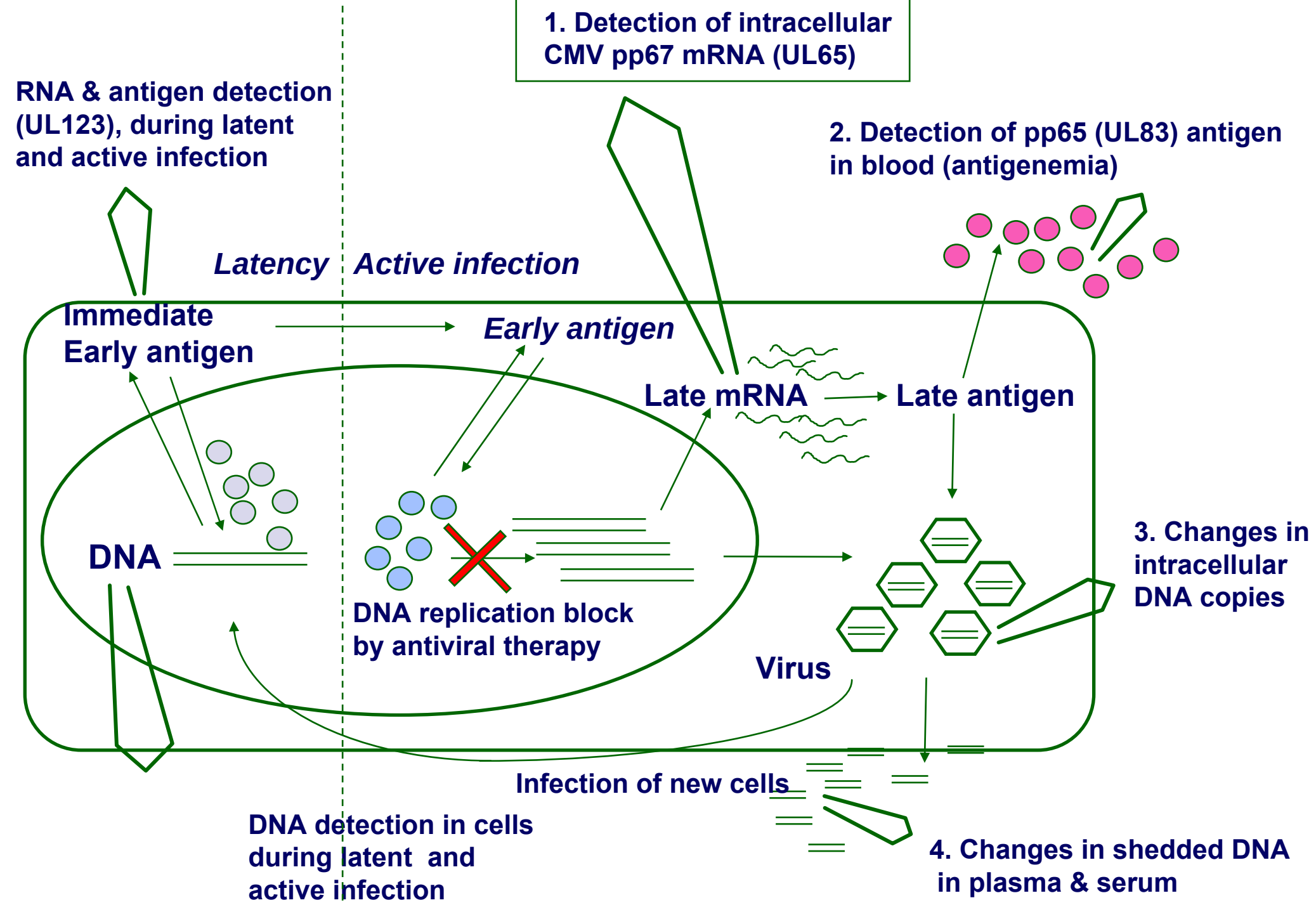
- **Detects HIV proviral DNA in peripheral blood mononuclear cells**
- **Most often recommended as preferred virologic test**
- **Sensitivity varies from 50% in the first month to >96% after 1 month** ***(Zaman MM etal Clin Infect Dis 2002; 34:417-18)***
- **Meta analysis of 96 studies using DNA PCR in infants reported 91.6% median sensitivity and 100% median specificity in early diagnosis** ***(Owens DK etal JAMA 1996;275:1342-48)***
  - **38% (29-46% 90%CI) were detectable at 48hrs**
  - **93% (76-97% 09%CI) detectable at 14 days**



Neonatal Diagnosis
Qualitative DNA PCR

- False positive and false negative rate of 1.8% observed
- Follow up confirmatory PCR testing required

- Limited commercial availability
- Version 1.0 test failed to detect non-B clade infection
- Version 1.5 RUO available on special access
- Quantitative real time assay in development



1. Detection of intracellular CMV pp67 mRNA (UL65)
RNA & antigen detection (UL123), during latent and active infection
2. Detection of pp65 (UL83) antigen in blood (antigenemia)
Latency
Active infection
Immediate Early antigen
Early antigen
Late mRNA
Late antigen
DNA
DNA replication block by antiviral therapy
3. Changes in intracellular DNA copies
Virus
Infection of new cells
DNA detection in cells during latent and active infection
4. Changes in shedded DNA in plasma & serum

Potential Utilities

Transplantation

- Replacement of culture & antigenemia
- Pre-emptive treatment strategies

AIDS

- Rapid confirmation of CMV disease
- identification of high risk patients = therapy
- CSF – CNS involvement
- Replacement of culture

Anti-natal / Pre-natal Screening

- Confirmation of serology

CMV Nucleic Acid Tests

- mRNA detection – intermediate transcript
  - Test performed on leukocytes
  - CMV is a ubiquitous DNA virus, persistent in leukocytes (latent)
  - Latent or abortive infection clinically not relevant
  - Active infection is the deciding factor for initiation of anti-CMV therapy
  - exclusively expressed during active replication
- CMV DNA quantification
  - Plasma / serum = lytic disease > shedding
  - Monitor fold changes in serial samples
  - Response to therapy
  - Prediction of reactivation – pre-emptive therapy


Personalized Medicine

HCV Infection

Real time HCV assay