

RETROVIROLOGY: ASSAYS, RESISTANCE AND MUCH MORE....

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ICAP-COLUMBIA UNIVERSITY
CLINICAL UNIT WEBINAR, APRIL 21ST 2011



ICAP

Global. Health. Action.

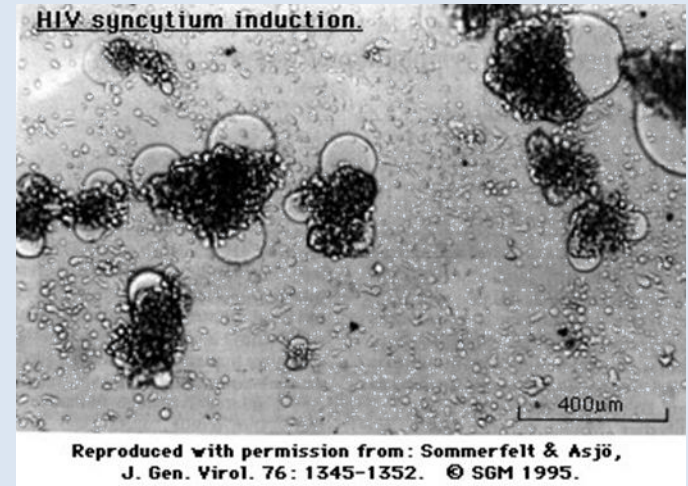
COLUMBIA UNIVERSITY
Mailman School of Public Health

PRESENTATION OUTLINE

- HIV-1 VIRAL LOAD ASSAY & ITS CLINICAL USE
- RECENT ADVANCES IN VIRAL LOAD TECHNOLOGY
- HIV'S GENETIC DIVERSITY & ITS IMPLICATION FOR MOLECULAR ASSAYS
- HIV DRUG RESISTANCE ASSAY & ITS CLINICAL USE

HIV-1 VIRAL LOAD ASSAY & ITS CLINICAL USE

WHAT IS VIRAL LOAD?

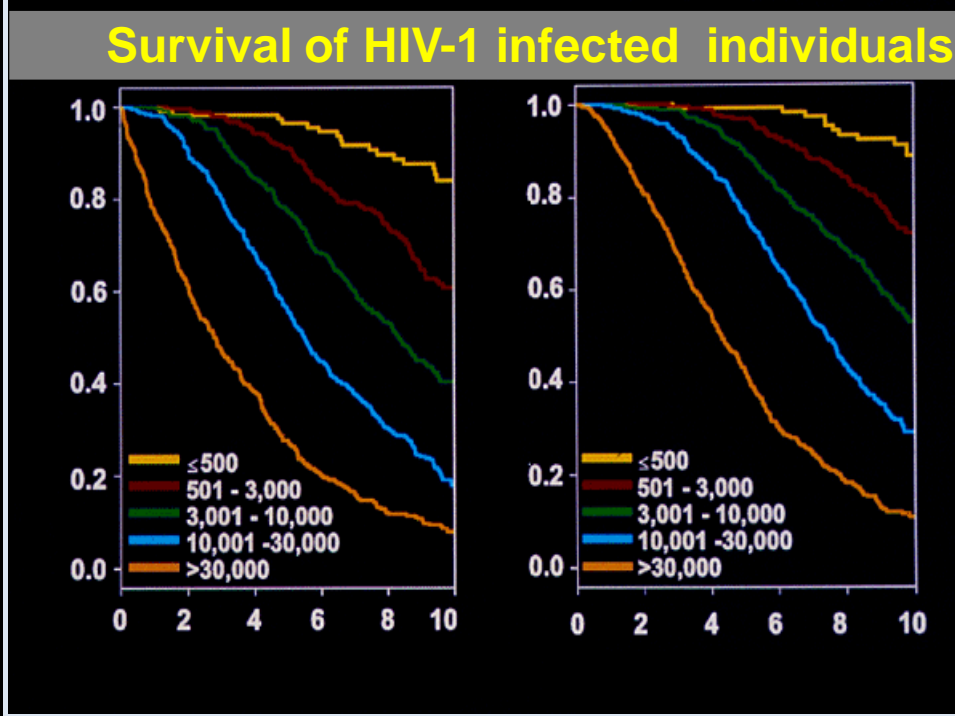
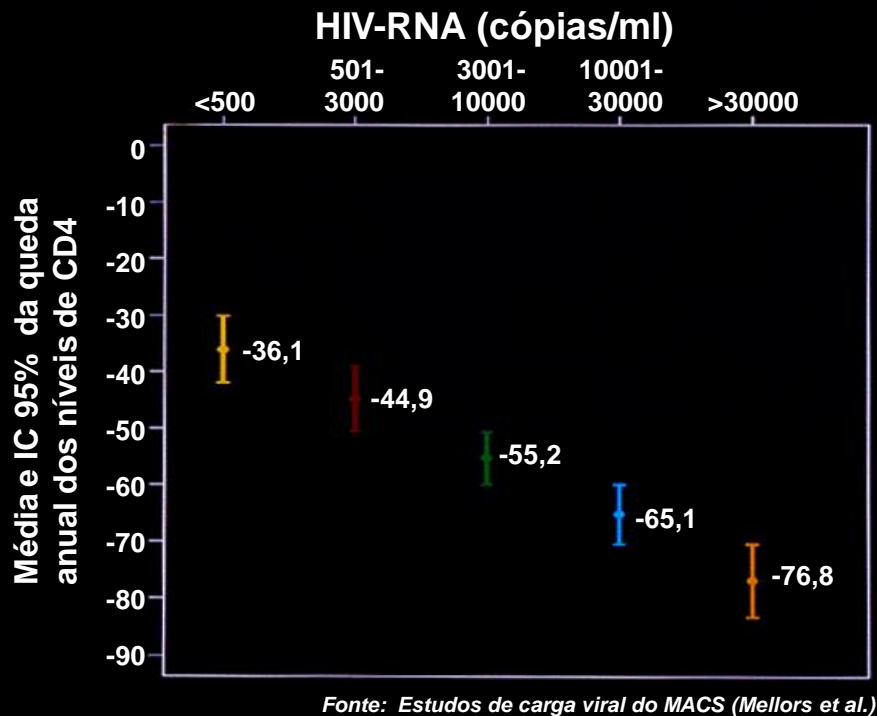


- VL is an excellent thermometer of HIV replication inside the human immune system.
- VL is a good parameter to measure the pathogenic potential of HIV, e.g. Higher VL, faster progression to AIDS.
- High VL is associated with higher vertical HIV transmission as well sexual transmission.

DEVELOPMENT OF AIDS

The CD4 count measures the level of immunodeficiency of a HIV+ individual

The Viral Load measures the speed the HIV+ patient loses CD4 cells/year



HOW TO MEASURE VIRAL LOAD?

- Previously p24 antigen in plasma is used as proxy indicator of VL.
- The best way is to measure VL is to measure the amount of full-length viral RNA present in the plasma.

METHOD STEPS

1- RNA isolation

2- Amplification

- Classical PCR : RNA is switched to DNA with Reverse Transcriptase (RT-PCR), then cycles of hybridization (60°C) and elongation (72°C)
- NASBA : isothermal amplification (61°C)
- bDNA : after reverse transcription of RNA to DNA, nucleotides are branched to DNA – during overnight - on the bottom of a micro titration plate

METHOD STEPS

3- Detection

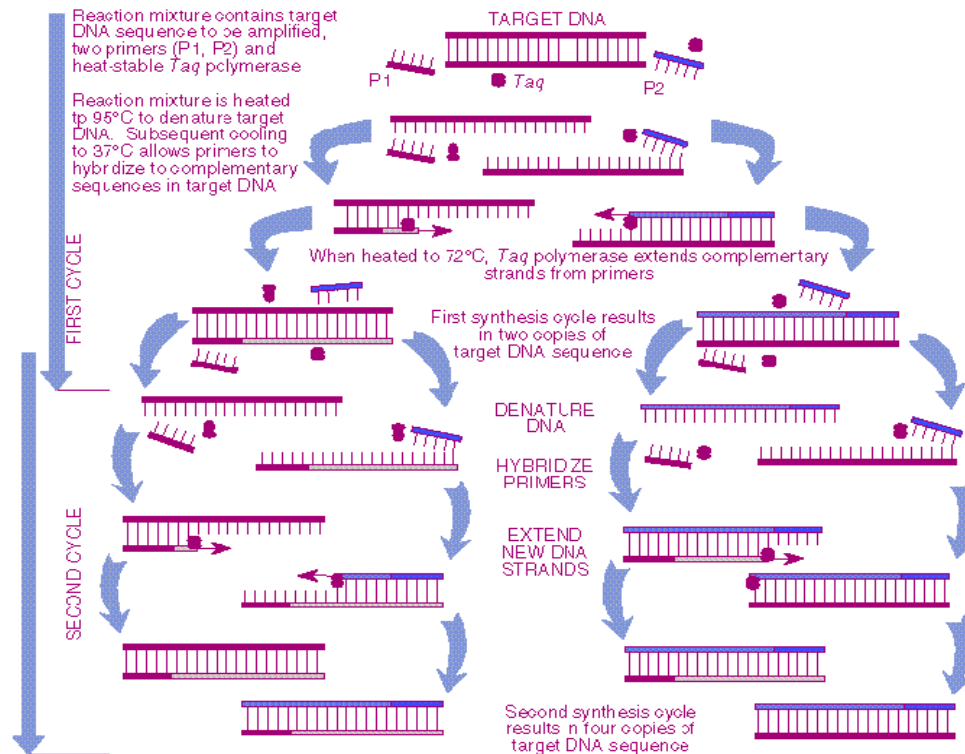
- End point PCR : optical reading measured in a separate reader (tubes are opened)
- NASBA : electroluminescence measured every 30 seconds (amplification and detection integrated)
- bDNA : sandwich chemoluminescence measured in the microtitration plate
- Real time PCR : electroluminescence measured at each cycle (thermocycler and reader integrated)

PCR

1

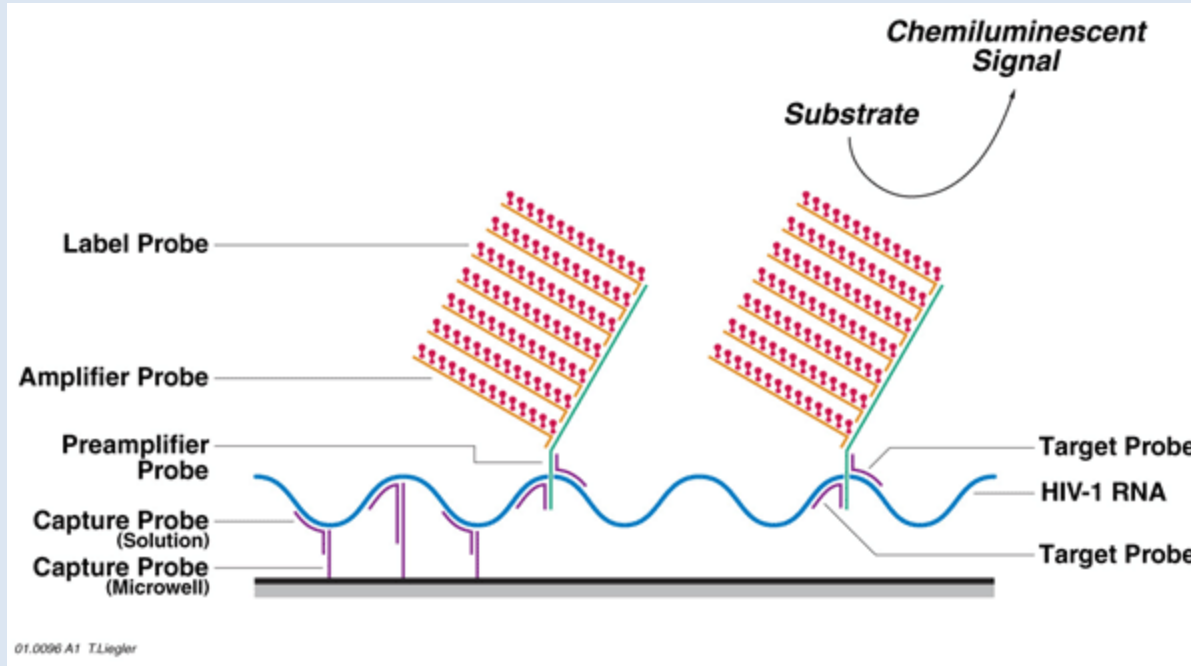
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DNA Amplification Using Polymerase Chain Reaction

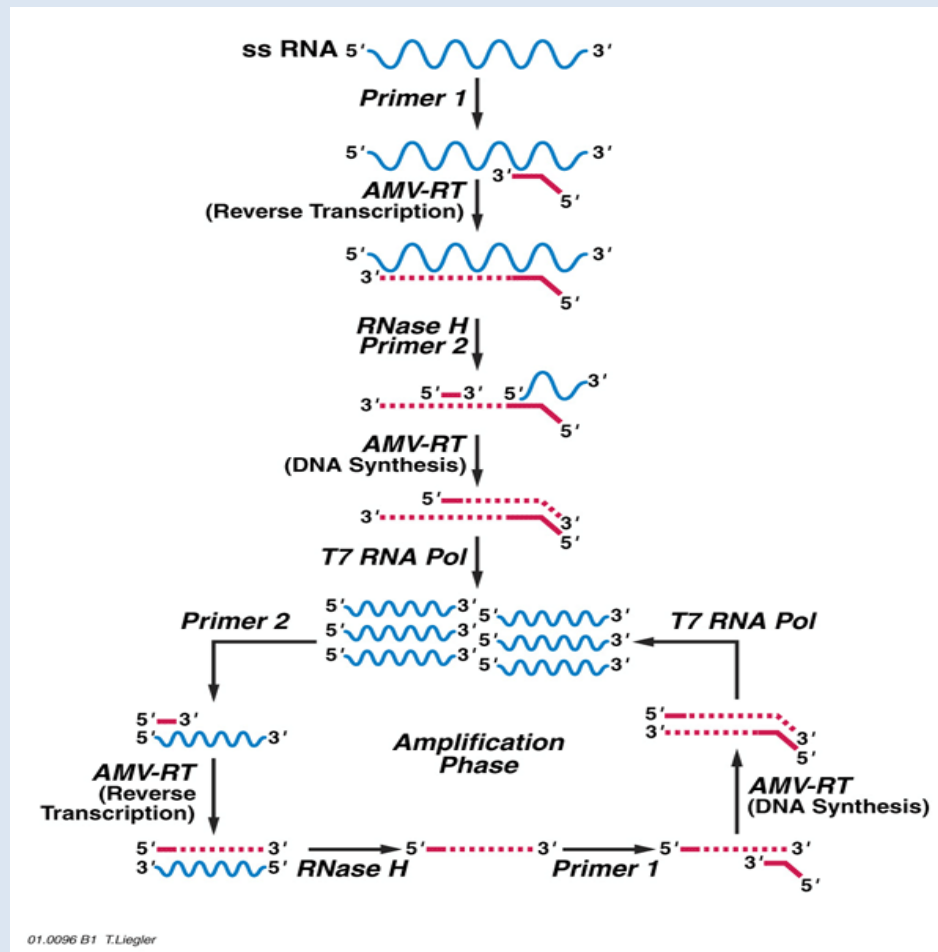


Source: *DNA Science*, see Fig. 13.

B-DNA (BRANCH-DNA) TECHNOLOGY SIGNAL AMPLIFICATION NOT RNA



NASBA TECHNOLOGY (ISOTHERMAL AMPLIFICATION)



TREATMENT FAILURE

- Clinical failure:
 - New or recurrent WHO stage 4 condition and certain WHO stage 3 conditions (e.g., PTB, severe bacterial infections). VL ideal for differentiation from delayed IRIS.
- Immunological failure:
 - Adult: a) fall of CD4 count to pre-treatment baseline (or below); or b) 50% fall from the on-treatment peak value (if known); or c) persistent CD4 level below 100 cells/mm³ (for 6 months).
 - Children: least 24 weeks on ART, in a treatment adherent child: child ≥ 2 years to < 5 years of age CD4 count of < 200 cells/mm³ or $< 10\%$ CD4+; child ≥ 5 years of age CD4 count of < 100 cells/mm³
- Virologic failure:
 - Adult: plasma VL above 5,000 c/ml (in a patient that has taken ART for more than 6 months and drug adherence has been satisfactory).
 - Children: a persistent viral load over 5000 copies/ml in a child who has been on treatment for > 24 weeks and is adherent to their (first-line) ART regimen.

INTERPRETATION OF VIRAL LOAD ASSAY RESULT

- There is evidence to support a VL threshold of 5000–10 000 copies/ml to define failure in an adherent patient with no other reasons for an elevated VL (e.g. drug-drug interactions, poor absorption, intercurrent illness).
- This range of values is associated with higher rates of clinical progression and immunological deterioration in some cohort studies (1, 2).

1- Murri R, Lepri AC, Cicconi P, Poggio A, Arlotti M, Tositti G, et al. Is moderate HIV viremia associated with a higher risk of clinical progression in HIV-infected people treated with highly active antiretroviral therapy: evidence from the Italian cohort of antiretroviral-naïve patients study. *J Acquir Immune Defic Syndr* 2006;41(1):23-30.

2- Cozzi Lepri A, Phillips AN, d'Arminio Monforte A, Castelli F, Antinori A, de Luca A, et al. When to start highly active antiretroviral therapy in chronically HIV-infected patients: evidence from the ICONA study. *AIDS* 2001;15(8):983-90.

GOAL OF VL TEST IN OUR SETTINGS

- To detect unsuppressed virus, leading to adherence or other interventions (WHO Guideline, 2010 Revision)
- To distinguish treatment failure from another cause of decreasing CD4 count
- Recommended viral load testing schedules:
 - Current guidelines suggest measuring baseline (pre-treatment) viral load. A drug is “working” if it lowers viral load by at least 90% within 8 weeks.
 - The viral load should continue to drop to less than 50 copies within 6 months.
 - VL testing can also be used on a targeted basis

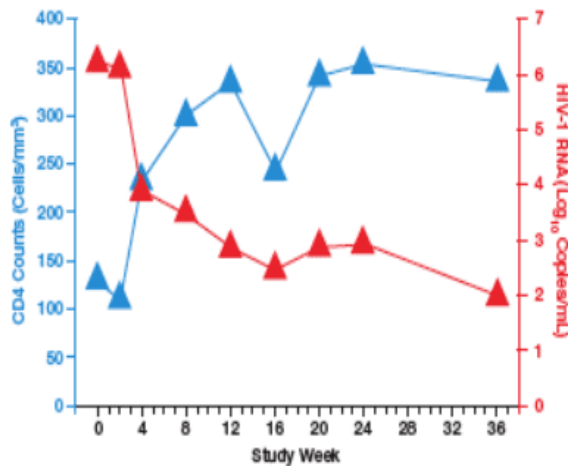
CHALLENGES WITH VIRAL LOAD TESTING

- Specimen management (cold chain, transportation logistics, etc).
- Cost: equipment, reagent, maintenance, skilled man power where human resources are limited already.
- Quality assurance programs in place.
- False positive and negative results due to bad lab procedures.

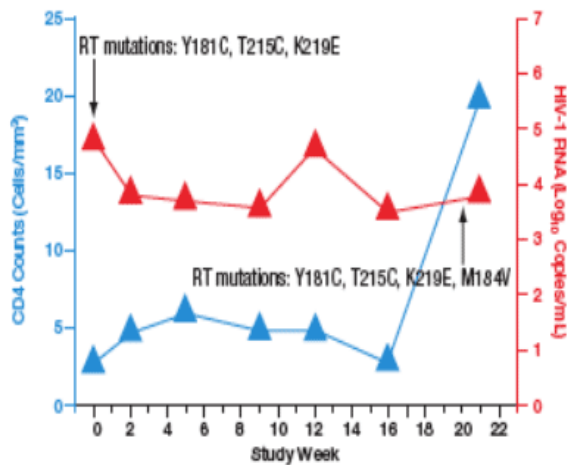
INTERPRETATION OF VIRAL LOAD ASSAY RESULT: CLINICAL, IMMUNOLOGIC & VIROLOGIC FAILURES ...

LPV/r

a Patient 1 (LPV/r): No genotypic mutations at BL or VF; significant diarrhea.



b Patient 2 (LPV/r): Missed visits.



- VL is a good test to monitor ARV therapy
- It can predict a good clinical response more faster than CD4.
- It can also predict therapeutical failure more faster than CD4.

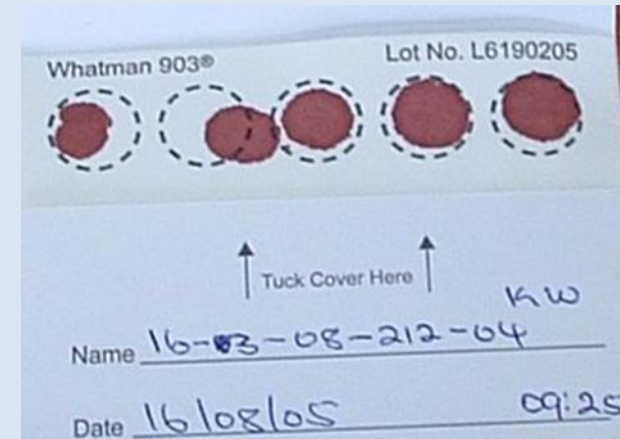
CAN HIV GENETIC VARIATION IMPACT ON VL MEASUREMENTS?

- Although we have a huge genetic variation of HIV in Africa the commercial kits were tested against a large panel of non-B HIVs.
- Nevertheless HIV-2 VL is not detected by commercial tested. We know that HIV-2 is indigenous from west Africa and very rarely found in other countries. In Mozambique HIV-2 accounts solely for 0,4% of HIV infections.

RECENT ADVANCES IN VIRAL LOAD TECHNOLOGY

1- FILTER PAPERS FOR VIRAL LOAD

- RNA is more fragile than DNA
 - Spots with low levels of VL need to be kept frozen or tested within one week.
- 2 spots are usually used (2 x 50 μ l)
 - sensitivity divided by 10 or 20 (limit of detection goes to 2000-5000 copies/ml).
- Elution of dried spots is labor intensive, prone to contamination and time-consuming (automation is a good option)
- **Plasma versus blood spots** : still controversial findings
 - overestimation of DBS because of proviral load (DNA/RNA)?
 - inhibitors remain in DBS
- Some studies has shown that VL preformed in DBS can be an useful tool in resource limited countries.



2- AUTOMATION OF SAMPLE EXTRACTION



m2000 RealTime System Abbott, USA

Automatic Extraction

Can test in 8 hs 92 samples using
600ul of plasma

Manual Extraction

Can test in 8 hs 21 samples using
600ul of plasma

3- POC VIRAL LOAD IN THE PIPELINE

- SAMBA :
 - NASBA based
 - Biochemical step and detection is mastered
 - Heating device not finalised ?
 - Still need plasma separation
 - Clinical trial is pending
- INVERNESS : real time PCR with portable machine and device for blood spot extraction

HIV'S GENETIC DIVERSITY & ITS IMPLICATION FOR MOLECULAR ASSAYS

HIV GENETIC DIVERSITY

- HIV has a large genetic variability and two viruses can be causative agent of AIDS (HIV-1 and HIV-2).
- RT has no proofreading mechanisms and poses a mutations rate of 1 error per 10,000 nucleotides synthesized.
- Huge replication burden (10^{10} particles daily). Most of them are lost and a small amount survive.
- HIV-2 can differ from HIV-1 in 50% of the amino acid of the main genes (gag, pol, and env).
- HIV-2 is less pathogenic and less transmissible by horizontal and vertical routes.

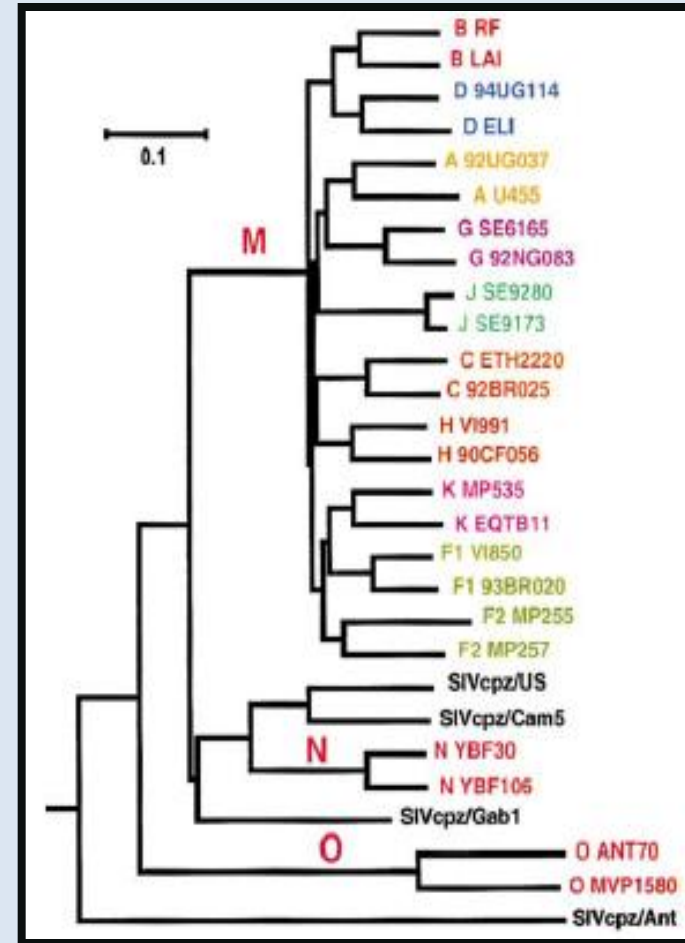
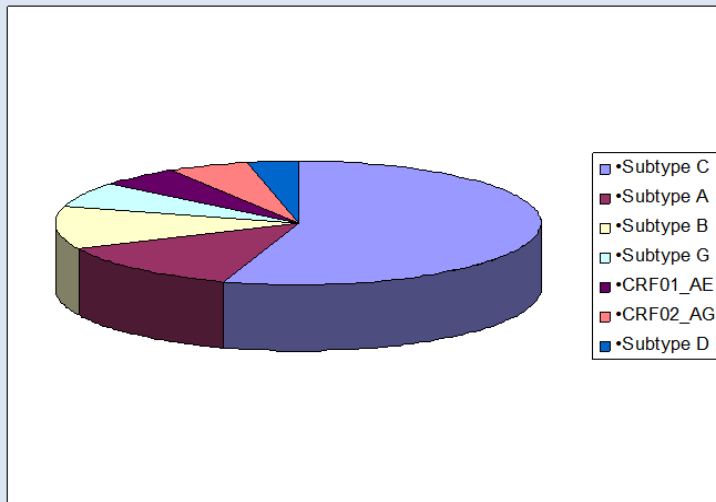
HIV quasispecies



HIV population is a quasispecies. It is like a popcorn bag , it is impossible to have two identical however, they are all popcorns.

HIV-1 GENETIC DIVERSITY

- This huge genetic diversity had led to attempts to classify HIV-1 into discrete units such as:
 - Groups (M, N, O)
 - Subtypes (M group: A, B, C, D, F, G, H, J, K)
 - Circulating recombinant forms (CRF) (39 and counting)
 - Unique recombinant forms (URF) (numerous)



HIV-1 SUBTYPES

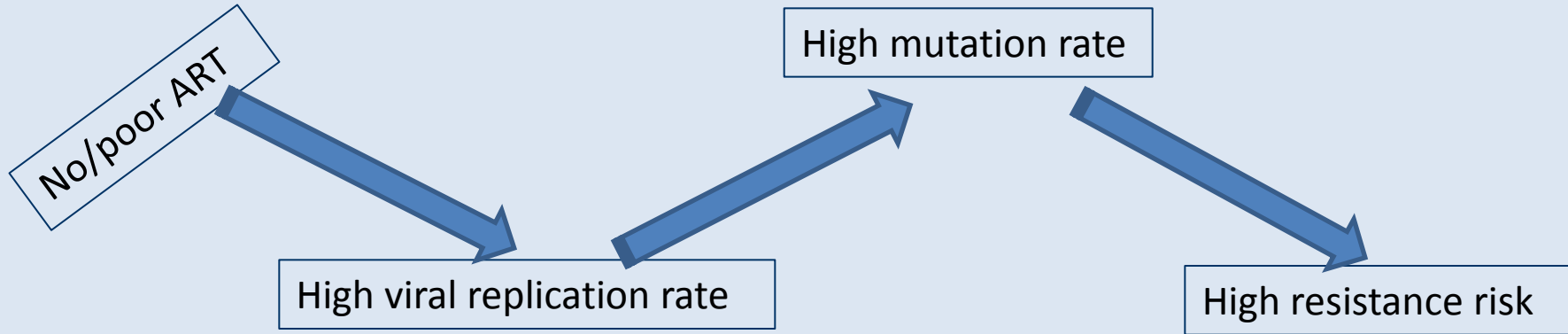
- Subtypes can differ:
 - 10 – 12% in *Pol* sequence (RT, Protease, Integrase)
 - 30% in *Env* sequence
- Substantial intra-subtype variation also exists
 - Sub-subtypes have been defined (A1/A2, F1/F2)
 - Identical subtypes from geographically distinct areas have been reported to differ in some specific position (e.g. RT A98S in subtype G)
- Therefore genetic diversity should be seen more as a spectrum, with subtypes providing general arbitrary boundaries.
- Some HIV subtype such as C can spread quickly and overgrow other subtypes in an epidemic.

HIV DRUG RESISTANCE ASSAY & ITS CLINICAL USE

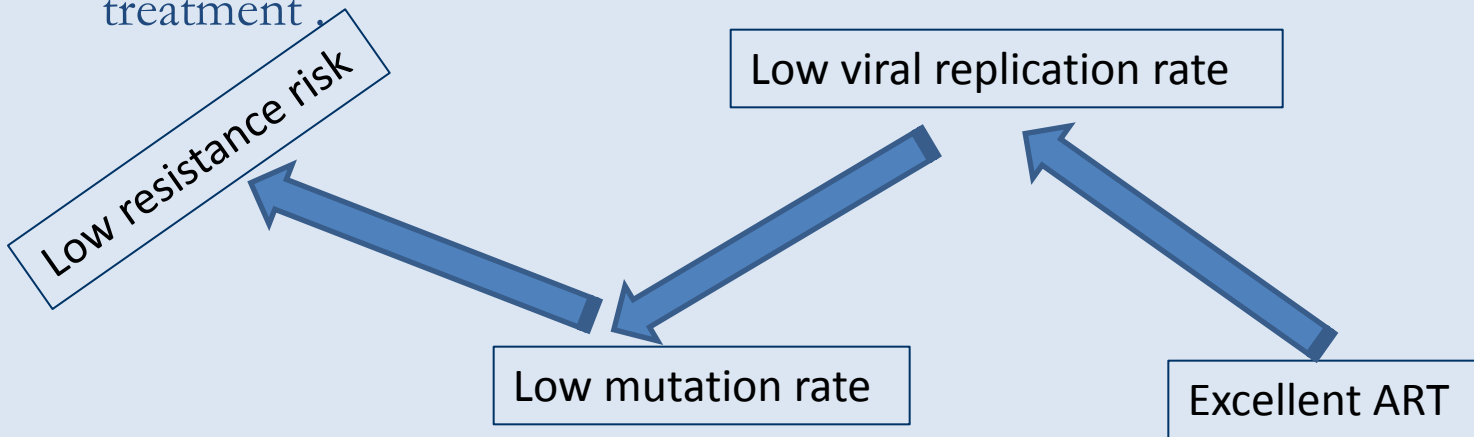
TYPES OF HIV RESISTANCE

- **Primary or transmitted drug resistance:** Drug resistance in previously untreated persons.
 - A virus with drug resistance mutations was transmitted either directly, or through one or more intermediates, from a person with acquired drug resistance.
- **Acquired or secondary drug resistance:** Drug resistance developing in a person who has received antiretroviral therapy.
 - Acquired drug resistance results from the generation of genetic variation in the population of viruses within a person followed by the selection of drug-resistant variants during ART.
- **Main message here:** HIV resistance is not generated by the drug!! The drug just select the existing variants generated during infection.

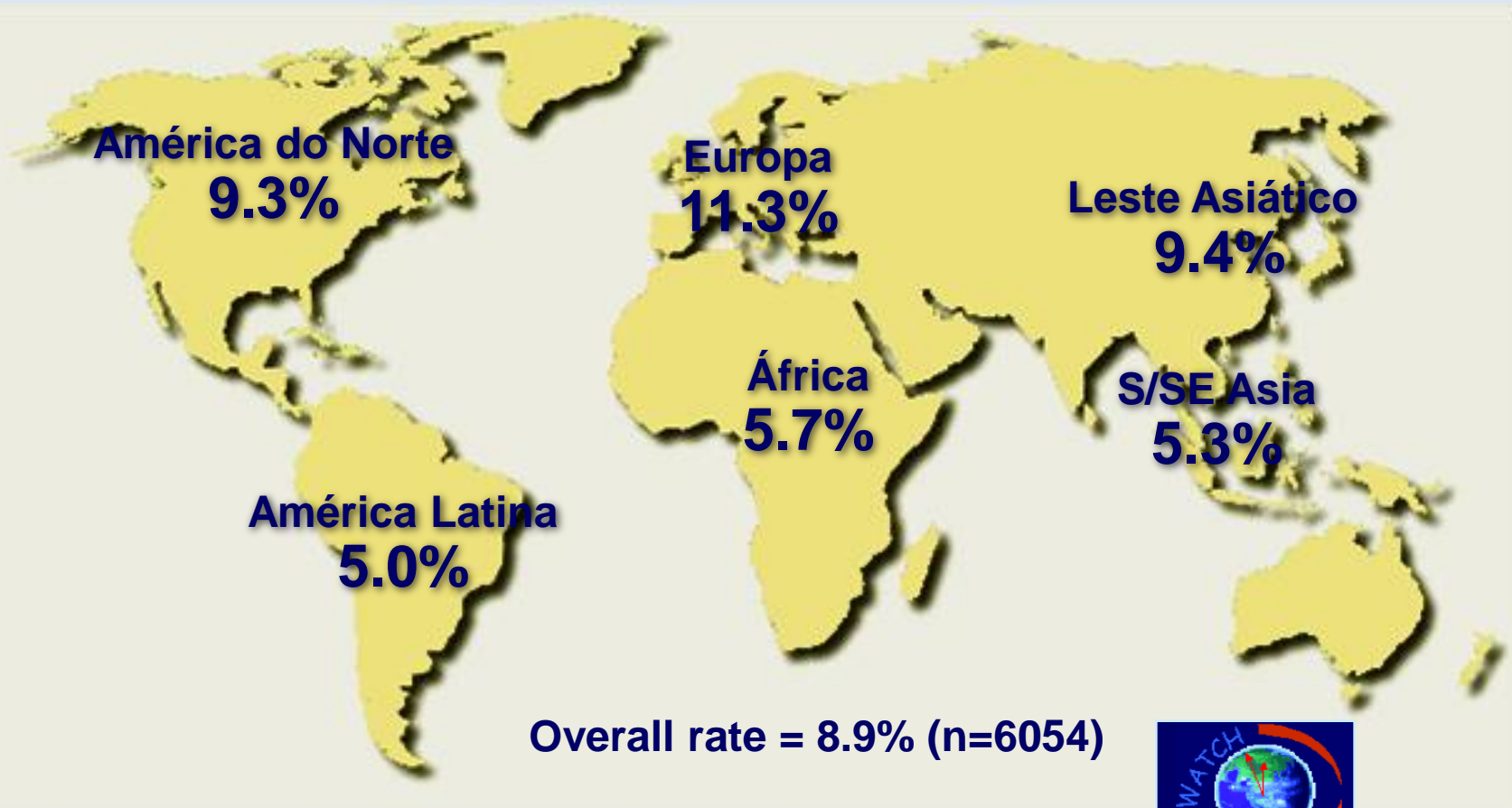
WHY HIV GET RESISTANT TO ARV?



- The main message here is: “ HIV needs to replicate to accumulate mutations.”
- The best way to prevent DR is keeping the VL undetectable during ARV treatment.



PREVALENCE OF TRANSMITTED DRUG RESISTANCE?

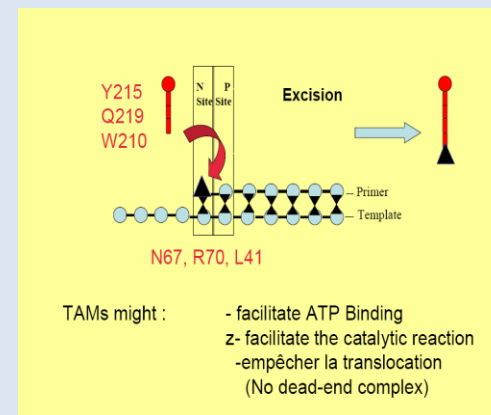
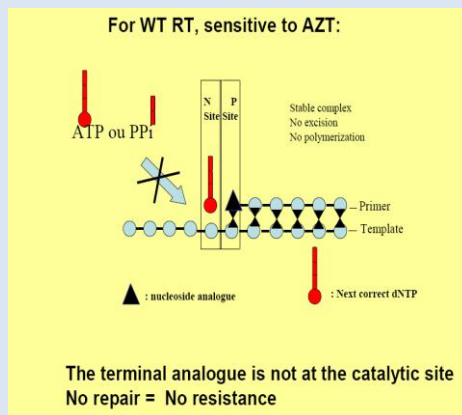


Bowles E et al., 4th EHDRW, Monte Carlo, March 2006, #



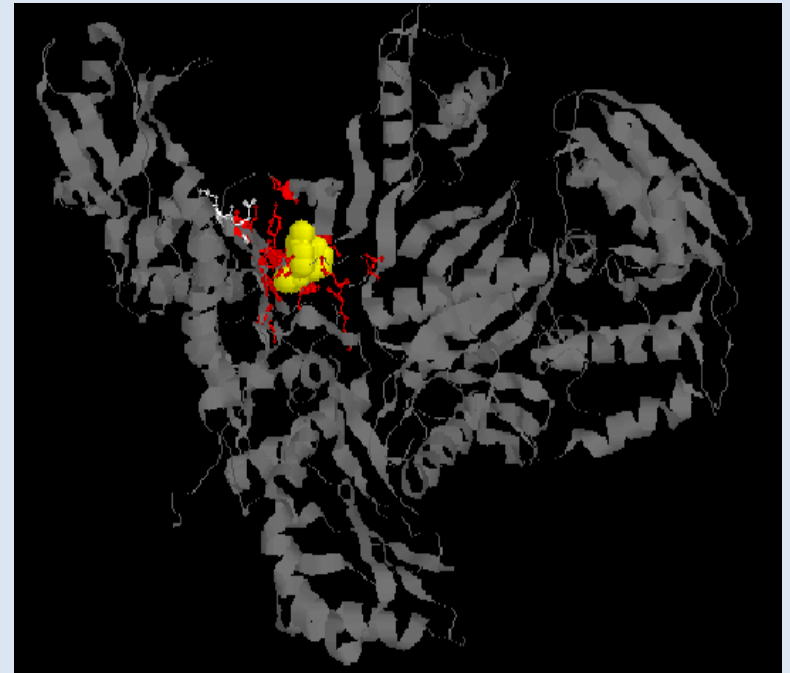
MECHANISM OF ARV DRUG RESISTANCE: RESISTANCE TO NRTIs

- **Discrimination:** mutations (e.g., M184V, K65R, Q151M) that occur at or near the drug-binding site of the reverse transcriptase gene, resulting in increased drug discrimination by this gene.
- **Excision:** of the chain-terminating nucleoside analogue monophosphate (drug), pyrophosphorolysis: a reverse transcriptase reaction that removes the chain-terminating residue and reinstates reverse reaction of DNA polymerization



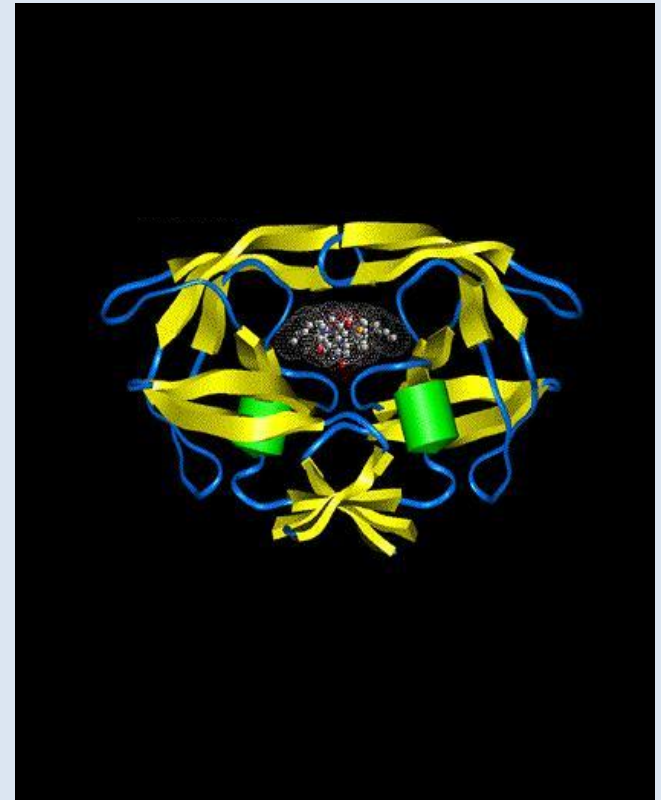
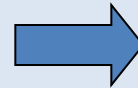
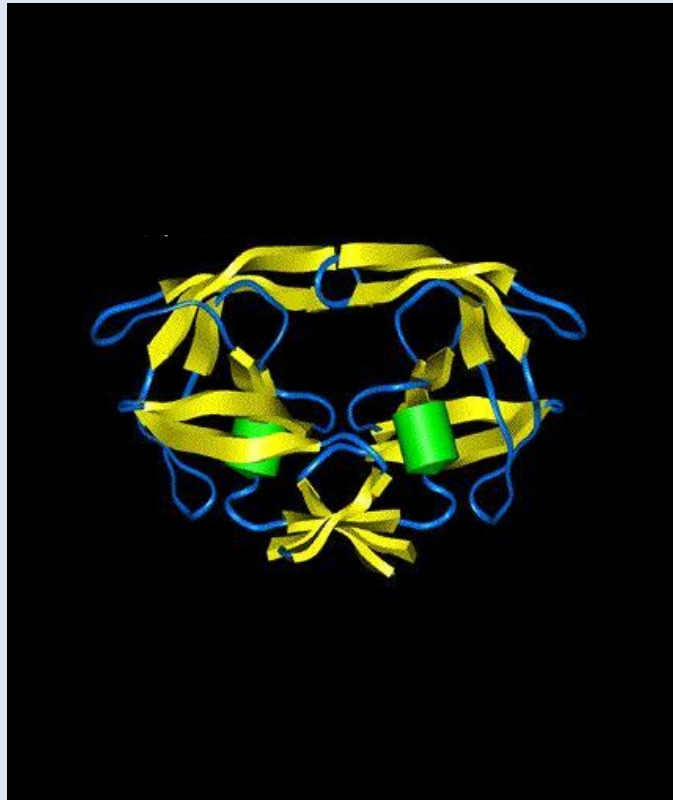
MECHANISM OF ARV DRUG RESISTANCE: RESISTANCE TO NNRTIs & PIs

- Resistance to NNRTIs:
 - Mutations resulting in altered the structure of the complex between reverse transcriptase and NNRTIs

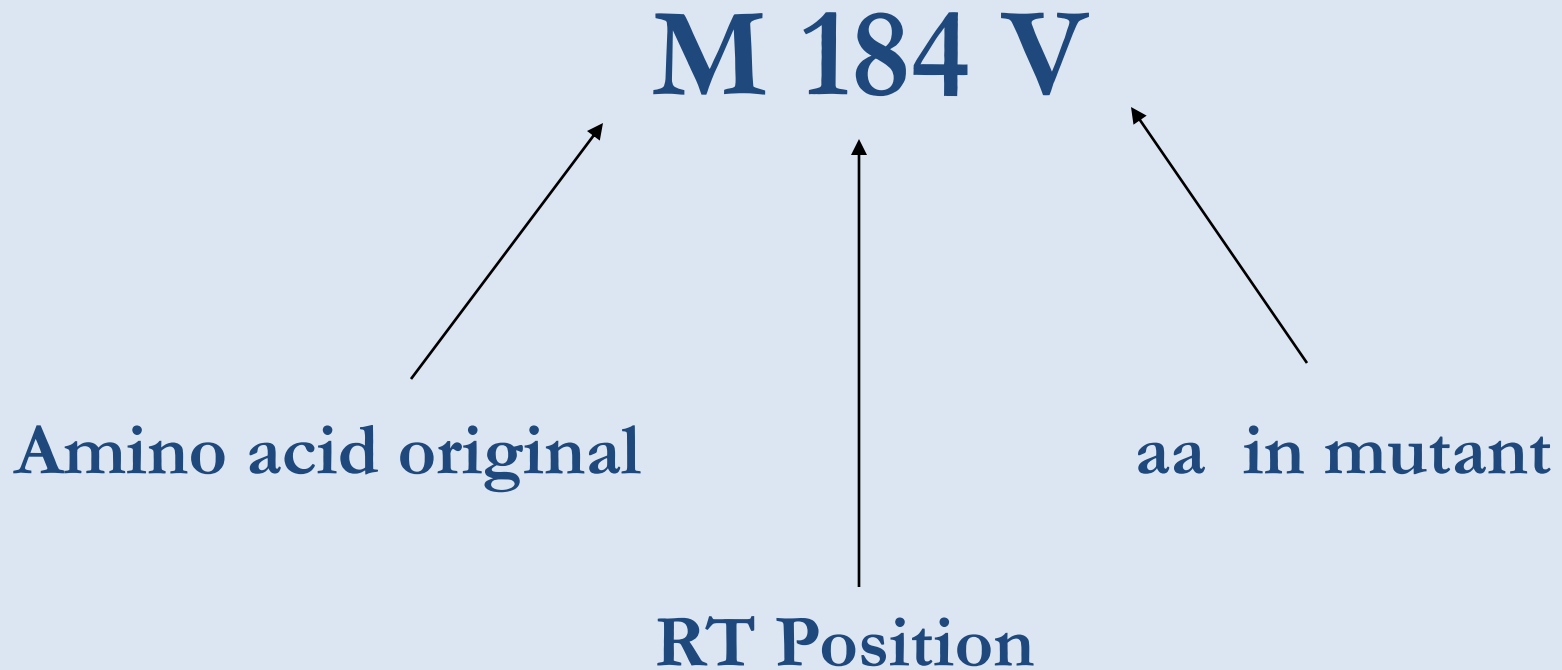


- Resistance to PIs:

- Mutations in HIV's protease gene resulting in changing the actual structure of the enzyme and reduced binding of drug



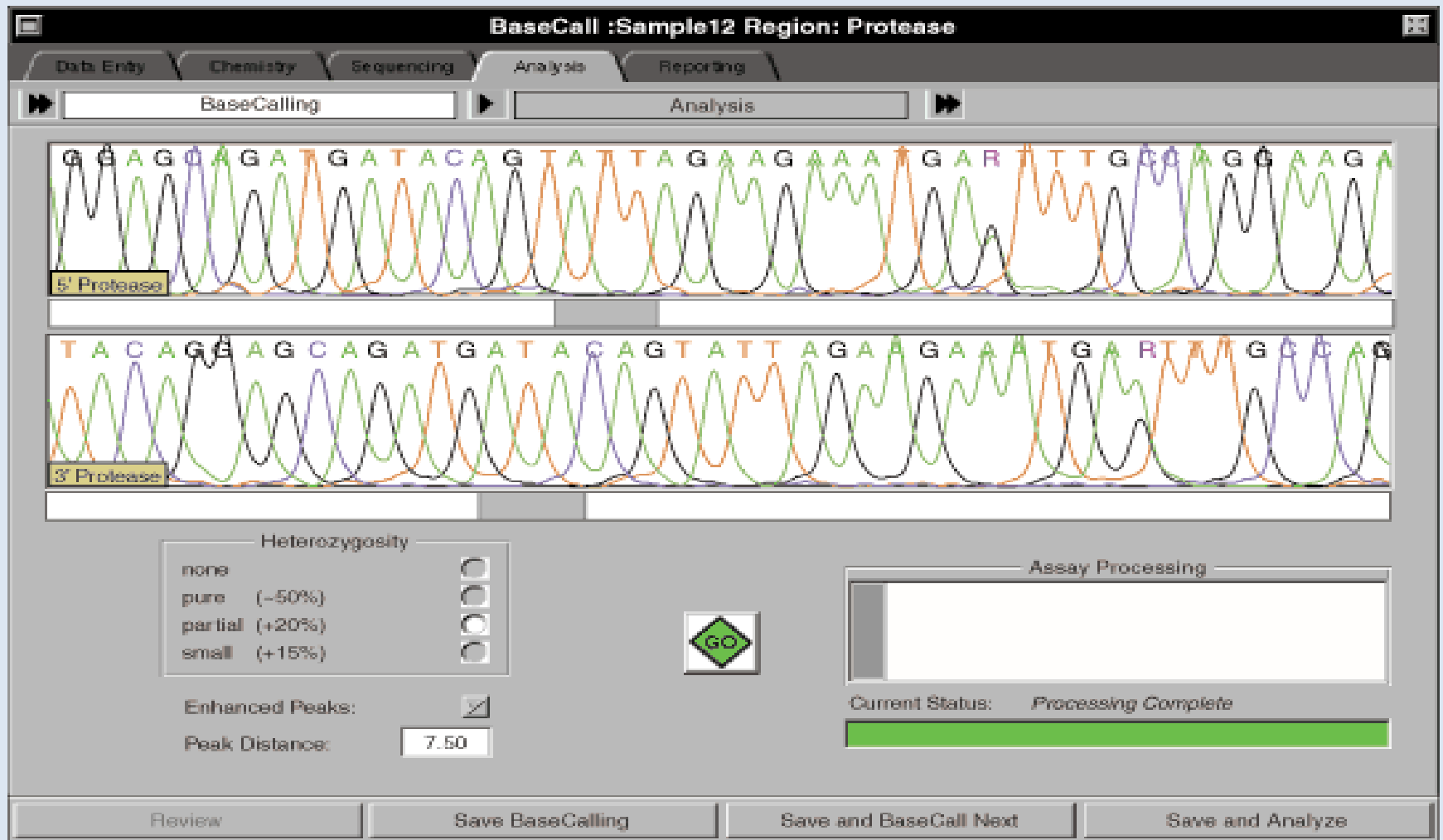
Naming the HIV mutations



HOW TO MEASURE HIV DRUG RESISTANCE

- Genotyping- It sequence the HIV pol region and try to correlate the mutations with the drug resistance phenotype.
- Phenotypic assays – It measure the ability of HIV to growth in the presence of ARV.
- Virtual Phenotype- Correlates the phenotyping data and the mutations.

GENOTYPIC HIV DR ASSAY



REPORTING OF DRUG RESISTANCE ASSAY: GENOTYPING

RT Resistance Mutations: M41L, M184V, L210LW, T215Y

RT Other Mutations: K102Q, K122E, I178L, R211K

Nucleoside RT Inhibitors		Non-Nucleoside RT Inhibitors	
AZT	Intermediate resistance	EFV	Susceptible
DDI	Intermediate resistance	DLV	Susceptible
DDC	Intermediate resistance	NVP	Susceptible
D4T	Low-level resistance		
ABC	Intermediate resistance		
3TC	High-level resistance		

RT Comments

- M41L increases AZT resistance when present with T215Y/F.
- M184V/I cause resistance to 3TC and low level resistance to ddI, ddC, and ABC.
- L210W increases AZT resistance when present with T215Y or T215F.
- T215Y/F AZT resistance. T215S/C/D represent transitions between T and Y or F.
- The presence of multiple AZT resistance mutations at codons 41, 67, 70, 210, 215, and 219 is associated with low-level d4T and ABC resistance and a decreased virologic response to d4T and ABC-containing regimens.
- M184V partially reverses AZT and possibly d4T resistance caused by other mutations. AZT mutations in this isolate include: M41L, L210LW, T215Y.

REPORTING OF DRUG RESISTANCE ASSAY: PHENOTYPING

PhenoSENSE GT™
REPLICATION CAPACITY
COMBINATION HIV DRUG RESISTANCE ASSAY

biosciences
monogram

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Bronx, NY 10451
USA

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[Redacted]	DOB	11/26/1960	Patient ID/Medical Record #	3094032	Gender	M	Monogram Accession #	11-105204	
	Date Received	02/01/2011 11:52	Date Reported	02/16/2011 10:07	Mode	F,M,W	Report Status	FINAL	
	Reference Lab ID/Order #								
	HIV-1 Subtype: AG								

	DRUG		PHENOSENSE™ SUSCEPTIBILITY				Evidence of Susceptibility		Net Assessment
	Generic Name	Brand Name	Cutoffs (Lower - Upper)	Fold Change	Increasing Drug Susceptibility	Decreasing	Pheno Sense	Gene Seq	
NRTI	Abacavir	Ziagen	(4.5 - 6.5)	5.05			P	N	Partially Sensitive
	Didanosine	Videx	(1.3 - 2.2)	1.49			P	N	Resistant
	Emtricitabine	Emtriva	(3.5)	>MAX			N	N	Resistant
	Lamivudine	Epivir	(3.5)	>MAX			N	N	Resistant
	Stavudine	Zerit	(1.7)	0.79			Y	Y	Sensitive
	Zidovudine	Retrovir	(1.9)	0.31			Y	N	Sensitive
	Tenofovir	Viread	(1.4 - 4)	0.28			Y	Y	Sensitive
NRTI Mutations			L74I, M184V, T215F						
NNRTI	Delavirdine	Rescriptor	(6.2)	38			N	N	Resistant
	Efavirenz	Sustiva	(3)	2.50			Y	N	Sensitive
	Etravirine	Intelence	(2.9 - 10)	0.23			Y	N	Sensitive
	Nevirapine	Viramune	(4.5)	191			N	N	Resistant
NNRTI Mutations			K103N, E138E/K, Y181C						
PI	Atazanavir	Reyataz	(2.2)	0.63			Y	Y	Sensitive
		Reyataz / r†	(5.2)	0.63			Y	Y	Sensitive
	Darunavir	Prezista / r†	(10 - 90)	0.84			Y	Y	Sensitive
	Fosamprenavir	Lexiva / r†	(4 - 11)	0.38			Y	Y	Sensitive
	Indinavir	Crixivan / r†	(10)	0.46			Y	Y	Sensitive
	Lopinavir	Kaletra	(9 - 55)	0.47			Y	Y	Sensitive
	Nelfinavir	Viracept	(3.6)	0.53			Y	Y	Sensitive
	Ritonavir	Norvir	(2.5)	0.38			Y	Y	Sensitive
	Saquinavir	Invirase / r†	(2.3 - 12)	0.49			Y	Y	Sensitive
Tipranavir	Aptivus / r†	(2 - 8)	0.77			Y	Y	Sensitive	
PI Mutations			I13V, K20I, M36I, L89M						

▮ Lower Clinical Cutoff (in bold) ▮ Hypersusceptibility
 ▮ Upper Clinical Cutoff (in bold) ▮ Cutoff
 ▮ Biological Cutoff
 ■ Sensitive ■ Partially Sensitive
 ■ Resistant

Y Evidence of Drug Sensitivity
 P Evidence of Partial Drug Sensitivity
 N Evidence of Drug Resistance

For more information on interpreting this report, please visit www.MonogramHIV.com or call Customer Service at 800-777-0177 between the hours of 6:30am to 5:00pm PST Monday through Friday.

Final Remarks

- All new technologies need to be evaluated before placing them in resource limited settings.
- VL can be a useful tool to help clinicians to follow up patients on ART.
- In resource limited settings a simple POC VL testing will help to spread this important lab test .
- HIV drug resistance genotyping due to its complexity will still be used as a research/survey tool in resource limited settings.