A Guide to HIV-1 Reverse Transcriptase and Protease Sequencing for Drug Resistance Studies

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I. HIV-1 Drug Resistance

A. Introduction

HIV-1 RT and protease sequencing and drug susceptibility testing have been done in research settings for more than ten years to elucidate the genetic mechanisms of resistance to antiretroviral drugs. Retrospective studies have shown that the presence of drug resistance before starting a new drug regimen is an independent predictor of virologic response to that regimen (DeGruttola *et al*., 2000; Hanna and D'Aquila, 2001; Haubrich and Demeter, 2001). Prospective studies have shown that patients whose physicians have access to drug resistance data, particularly genotypic resistance data, respond better to therapy than control patients whose physicians do not have access to the same data (Baxter *et al*., 2000; Cohen *et al*., 2000; De Luca *et al*., 2001; Durant *et al*., 1999; Melnick *et al*., 2000; Meynard *et al*., 2000; Tural *et al*., 2000). The accumulation of retrospective and prospective data has led three expert panels to recommend the use of resistance testing in the treatment of HIV-infected patients (EuroGuidelines Group for HIV Resistance, 2001; Hirsch *et al*., 2000; US Department of Health and Human Services Panel on Clinical Practices for Treatment of HIV Infection, 2000) (Table 1).

There have been several recent reviews on methods for assessing HIV-1 drug resistance (Demeter and Haubrich, 2001; Hanna and D'Aquila, 2001; Richman, 2000) and on the mutations associated with drug resistance (Deeks, 2001; Hammond *et al*., 1999; Loveday, 2001; Miller, 2001; Shafer *et al*., 2000b). This review will detail the use of HIV-1 genotypic resistance testing in research settings where it is used to learn about the mechanisms and clinical significance of drug resistance and in clinical settings where it is used to help guide anti-HIV treatment.

B. Evolution of HIV-1 drug resistance

The evolution of HIV-1 drug resistance within an individual depends on the generation of genetic variation in the virus and on the selection of drug-resistant variants during therapy. HIV-1 genetic variability is a result of the inability of HIV-1 RT to proofread nucleotide sequences during replication (Mansky, 1998). It is exacerbated by the high rate of HIV-1 replication *in vivo*, the accumulation of proviral variants during the course of HIV-1 infection, and genetic recombination when viruses with different sequences infect the same cell. As a result, innumerable genetically distinct variants (quasispecies) evolve in individuals in the months following primary infection (Coffin, 1995).

The HIV-1 quasispecies in an individual undergoes continuous genetic variation, competition, and selection. Development of drug resistance depends on the size and heterogeneity of the HIV-1 population within an individual, the extent to which virus replication continues during drug therapy, the ease of acquisition of a particular mutation (or set of mutations), and the effect of drug-resistance mutations on drug susceptibility and virus fitness. Some mutations selected during drug therapy confer measurable phenotypic resistance by themselves, whereas other mutations increase resistance when present with other mutations or compensate for the diminished replicative activity that can be associated with drug resistance.

Table 1. Expert Panel Recommendations on HIV Drug Resistance Testing

	Recommendations (EuroGuidelines Group for HIV Resistance, 2001; Hirsch *et al*., 2000; US Department of Health and Human Services Panel on Clinical Practices for Treatment of HIV Infection, 2000)
Primary HIV-1 infection	The DHHS and IAS-USA state that resistance testing should be considered. The more recent EuroGuidelines state that testing should be strongly considered, reflecting the increasing rates of primary HIV-1 drug resistance.
Established HIV-1 infection	Not generally recommended by any of the guidelines. Detection of drug resistance is inversely proportional to the time since infection because rates of resistance were lower in the past and because resistant strains tend to be overgrown by susceptible strains that were either co-transmitted at the time of infection or that resulted from back mutations.
First regimen failure	Resistance testing is recommended by all three panels. Patients with virologic failure while receiving drug combinations have virus isolates that are not necessarily resistant to all of the drugs in the combination and because HIV-1 may develop drug resistance by more than one mechanism and each mechanism may have different consequences for cross-resistance.
Suboptimal viral suppression after initiation of HAART	Recommended by DHHS. Not specifically recommended by the IAS-USA and EuroGuidelines committees but probably falls under the "First regimen failure" category.
Multiple regimen failures	Recommend testing to optimize the number of active drugs in the next regimen; exclude drugs to which response is unlikely.
Pregnancy	Recommend testing to optimize maternal treatment and prophylaxis for neonate.
Post-exposure prophylaxis	Addressed by EuroGuidelines which recommends testing but cautions that treatment should not be delayed while waiting for the test result. Rather, the results of the test should be used to modify the treatment.

It has been estimated that every possible single point mutation occurs between 10^4 and 10^5 times per day in an untreated HIV-1-infected individual and that double mutants also occur commonly (Coffin, 1995). It is not known, however, whether multidrug-resistant viruses already exist at low frequencies in untreated persons or if they are generated by residual viral replication during therapy (Ribeiro and Bonhoeffer, 2000). Answers to this question depend on the effective population number of HIV-1 *in vivo*. Some authors have argued in favor of a high effective population number and a deterministic model of HIV-1 evolution (Rouzine and Coffin, 1999); others have argued in favor of a lower effective population number and a stochastic model of HIV-1 evolution (Brown, 1997; Brown and Richman, 1997; Frost *et al*., 2000).

Although HIV-1 drug resistance is usually acquired during anti-HIV drug therapy, drug resistance can also be transmitted between individuals. In the United States and Europe about 10% of new infections are with HIV-1 isolates harboring resistance to at least one of three classes of anti-HIV drugs (Balotta *et al*., 2000; Boden *et al*., 1999; Briones *et al*., 2001; Duwe *et al*., 2001; Grant *et al*., 1999; Harzic *et al*., 1999; Little *et al*., 1999; Salomon *et al*., 2000; Simon *et al*., 2001; Tamalet *et al*., 2000; Yerly *et al*., 1999). Recent studies suggest that transmitted HIV-1 drug resistance is gradually increasing (Little, 2000; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance, 2001).

C. HIV-1 protease

The HIV-1 protease enzyme is responsible for the post-translational processing of the viral Gag and Gag-Pol polyproteins to yield the structural proteins and enzymes of the virus. The enzyme is an aspartic protease composed of two non-covalently associated structurally identical monomers 99 amino acids in length (Figure 1). Its active site resembles that of other aspartic proteases and contains the conserved triad, Asp-Thr-Gly, at positions 25-27. The hydrophobic substrate cleft recognizes and cleaves 9 different sequences to produce the matrix, capsid, nucleocapsid, and p6 proteins from the Gag polyprotein and the protease, RT, and integrase proteins from the Gag-Pol polyprotein (Erickson *et al*., 1999; Miller 2001). The enzyme contains a flexible flap region that closes down on the active site upon substrate binding.

There are six FDA-approved protease inhibitors (PIs): amprenavir, indinavir, lopinavir (manufactured in combination with ritonavir), nelfinavir, ritonavir, and saquinavir. Mutations associated with PI resistance are found at more than 20 different residues of the enzyme (Table 2) (Figure 1). Resistance is mediated by structural changes that reduce binding affinity between the inhibitor and the mutant protease molecule. The effects of non-active site mutations are less obvious and appear to involve other mechanisms: alterations in enzyme catalysis, effects on dimer stability, alterations in inhibitor binding kinetics, or active site re-shaping through long-range structural perturbations (Erickson *et al*., 1999; Miller 2001). The three-dimensional structures of wildtype HIV-1 protease and of several drug-resistant mutant forms bound to various inhibitors have been determined by crystallography (Baldwin *et al*., 1995; Chen *et al*., 1995; Mahalingam *et al*., 1999).

Sequence analysis of drug resistance clones has shown that mutations at several of the protease cleavage sites also contribute to drug resistance (Cote *et al*., 2001; Doyon *et al*., 1996; Mammano *et al*., 1998; Zhang *et al*., 1997) (Table 3). Growth kinetic studies have shown that the cleavage site mutations in some circumstances improve the kinetics of protease enzymes containing drug-resistance mutations and that these mutations appear to be compensatory rather than primary. There have been no reports that changes at cleavage sites alone can cause PI resistance.

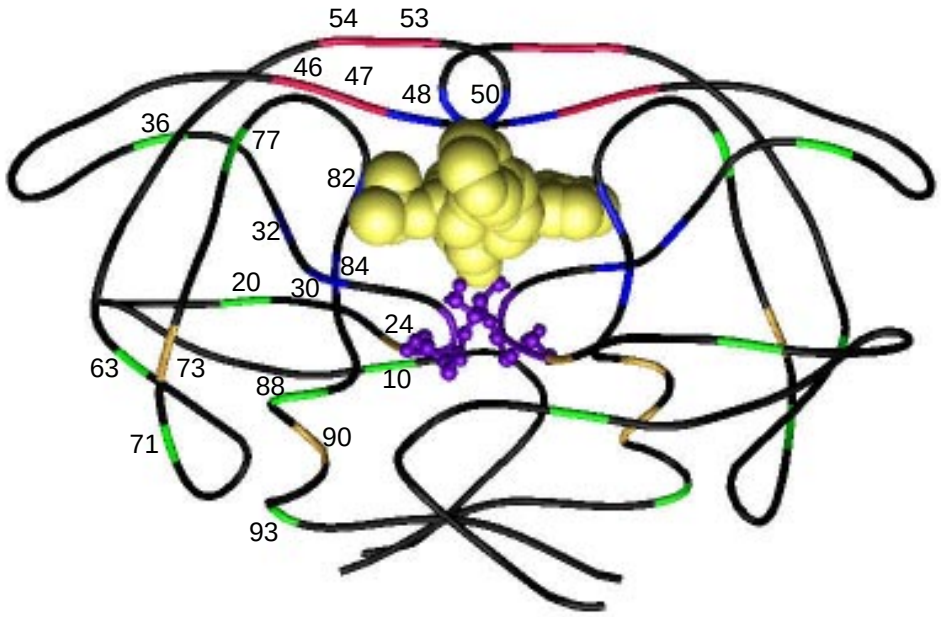

Figure 1. Structural model of HIV-1 protease homodimer labeled with protease inhibitor resistance mutations. The polypeptide backbone of both protease subunits (positions 1–99) is shown. The active site (positions 25–27) is displayed in ball and stick mode. The protease was co-crystallized with a protease inhibitor, which is displayed in space-fill mode.

Table 2. HIV-1 Protease Inhibitor (PI) Drug Resistance Mutations

Codon	Mechanism	Effect on resistance	References
8	Substrate cleft	R8Q/K confers high-level resistance to some of the earliest PIs. It occurs extremely rarely and its effect on current PIs is not known.	Gulnik *et al*., 1995; Ho *et al*., 1994
10	Accessory (polymorphic)	L10I/F/V/R are associated with resistance to all PIs when present with other mutations.	Hertogs *et al*., 2000b; Para *et al*., 2000; Shafer *et al*., 1999b; Zolopa *et al*., 1999b
20	Accessory (polymorphic)	K20R/M/I are associated with resistance to IDV, RTV, LPV, and possibly other PIs when present with other mutations. Variants at this position occur commonly in several non-B subtypes	Hertogs *et al*., 2000b; Shafer *et al*., 1999b, Condra *et al.* 1996, Molla *et al*., 1996
24	Accessory	L24I is associated with IDV and LPV resistance when present with other mutations.	Condra *et al*., 1996; Kempf *et al*., 2000
30	Substrate cleft	D30N causes resistance to NFV. D30N is perhaps the only protease mutations that does not confer cross-resistance to multiple PIs.	Patick *et al*., 1998; Patick *et al*., 1996; Zolopa *et al*., 1999b
32	Substrate cleft	V32I is a substrate cleft mutation that confers resistance to IDV, RTV, and APV. Although it is in the substrate cleft, this mutation has a minimal effect on drug resistance.	Condra *et al*., 1996; Snowden *et al*., 2000
36	Accessory (polymorphic)	M36I/V are associated with resistance to all PIs when present with other mutations. Variants at this position occur commonly in non-B subtypes.	Hertogs *et al*., 2000b; Shafer *et al*., 1999b
46	Enzyme flap	M46I increases resistance to IDV, RTV, NFV, APV, and LPV when present with other mutations.	Condra *et al*., 1996; Partaledis *et al*., 1995; Patick *et al*., 1996; Zolopa *et al*., 1999b
47	Enzyme flap	I47V increases resistance to APV when present with I50V. Its effect on other PIs has not been well-characterized.	Partaledis *et al*., 1995
48	Substrate cleft	G48V causes resistance to SQV. G48V also confers limited cross-resistance to NFV, IDV, and RTV. Its effect on APV and LPV is not known.	Jacobsen *et al*., 1995 Winters *et al*., 1998a
50	Substrate cleft	I50V causes resistance to APV. I50V also contributes resistance to RTV and LPV. Occurs primarily in patients receiving APV as their first PI.	Partaledis *et al*., 1995; Snowden *et al*., 2000; Xu et *al*., 2001; Parkin *et al.*, 2001; Prado *et al.*, 2001
53	Enzyme flap	F53L is a substrate cleft mutation that occurs only in isolates from treated patients. It is associated with resistance to IDV, RTV, LPV, SQV, and possibly NFV and APV.	Kempf *et al*., 2000; Shafer, et *al*., 1999b; Gulnik *et al*., 1995
54	Enzyme flap	I54V/L/T increase resistance to each of the PIs when present with other mutations. I54M occurs in patients receiving APV.	Hertogs *et al*., 2000b; Kempf *et al*., 2000; Snowden *et al*., 2000; Zolopa *et al*., 1999b; Condra *et al*., 1996

Table 2. cont.

Codon	Mechanism	Effect on resistance	References
63	Accessory (polymorphic)	L63P/A/Q/S/H/C/T/I occur commonly in untreated persons but are also associated with resistance to PIs when present with other mutations.	Hertogs *et al*., 2000b; Shafer *et al*., 1999b,Condra *et al*. 1996 Yahi*et al*. 1999
71	Accessory (polymorphic)	A71V/T is associated with resistance to IDV, RTV, SQV, NFV, LPV and probably APV when present with other mutations.	Hertogs *et al*., 2000b; Shafer *et al*., 1999b; Zolopa *et al*., 1999b, Condra *et al*., 1996, Molla *et al*., 1996
73	Accessory	G73S/T/C are associated with resistance to IDV, SQV, NFV and possibly the remaining PIs when present with other mutations.	Hertogs *et al*., 2000b; Shafer *et al*., 1999b, Dulioust *et al*., 1998, Zolopa *et al*., 2001
77	Accessory (polymorphic)	V77I is a common polymorphism that is associated with drug resistance in isolates containing other mutations.	Hertogs *et al*., 2000b; Shafer *et al*., 1999b, Patick *et al*., 1998
82	Substrate cleft	V82A/T/F/S cause resistance to IDV, RTV, and LPV. When present with other mutations, these mutations contribute resistance to NFV, APV, SQV. V82I is a polymorphism that does not appear to be associated with drug resistance.	Condra *et al*., 1996; Falloon *et al*., 2000; Kempf *et al*., 2000; King *et al*., 1995; Molla *et al*., 1996; Shafer *et al*., 1998; Sham *et al*., 1998
84	Substrate cleft	I84V contributes resistance to each of the PIs.	Condra *et al*., 1996; Hertogs *et al*., 2000b; Kempf *et al*., 2000; Molla *et al*., 1996; Patick *et al*., 1996; Snowden *et al*., 2000
88	Accessory	N88D/S/T increase NFV resistance particularly when present with D30N or M46I. These mutations may cause low-level cross-resistance to IDV and RTV. N88S causes hyper-susceptibility to APV.	Patick *et al*., 1998; Ziermann *et al*., 2000
90	Impacts on substrate cleft	L90M causes resistance to SQV and NFV. When present with other mutations it contributes resistance to IDV, RTV, APV, and LPV.	Condra *et al*., 1996; Jacobsen *et al*., 1995; Molla *et al*., 1996; Para *et al*., 2000
93	Accessory (polymorphic)	I93L is a common polymorphism that is associated with drug resistance in isolates containing other mutations.	Shafer *et al*., 1999b, Molla *et al*., 1996, Patick *et al*., 1998

APV: amprenavir
IDV: indinavir
LPV: lopinavir
NFV: nelfinavir
RTV: ritonavir
SQV: saquinavir.

D. HIV-1 reverse transcriptase (RT)

The RT enzyme is responsible for RNA-dependent DNA polymerization and DNA-dependent DNA polymerization. RT is a heterodimer consisting of p66 and p51 subunits (Figure 2). The p51 subunit is composed of the first 450 amino acids of the RT gene. The p66 subunit is composed of all 560 amino acids encoded by the RT gene. Although the p51 and p66 subunits share 450 amino acids, their relative

Table 3. HIV-1 Protease Cleavage Sites

Site	AA	Position	References
gag			
MA/CA	SQNY/PIV	1187–1188	Mammano *et al*., 1998, Cote *et al*., 2001
CA/p2	ARVL/AEA	1880–1881	Mammano *et al*., 1998, Cote *et al*., 2001
p2/NC	ATIM/MQR	1920–1921	Mammano *et al*., 1998), Cote *et al*., 2001
NC/p1	RQAN/FLG	2085–2086	Mammano *et al*., 1998), Cote *et al*., 2001, Zhang *et al*., 1997, Bally *et al*., 2000
p1/p6	PGNF/LQS	2136–2137	Mammano *et al*., 1998, Cote *et al*., 2001, Bally *et al*., 2000, Doyon *et al*., 1996
pol			
TF/PRSF	SV/PQI	2257–2258	Cote *et al*., 2001
PR/RT	TLNF/PIS	2552–2553	Cote *et al*., 2001
RT (p51/p66)	AETF/YVD	3869–3870	Cote *et al*., 2001
RT/IN	RKVL/FLD	4232–4233	Cote *et al*., 2001

MA - Matrix
CA - Capsid
NC - Nucleocapsid
TF - Transframe
PR- Protease
RT- Reverse Transcriptase
IN - Integrase.
Scissile bonds are indicated by the slashes in the amino acid sequence.
Nucleic acid positions in relation to HXB2 (GenBank accession No. K03455) are indicated in the Position column.

arrangements are significantly different. The p66 subunit contains the DNA-binding groove and the active site; the p51 subunit displays no enzymatic activity and functions as a scaffold for the enzymatically active p66 subunit. The p66 subunit has five subdomains including the “fingers”, “palm”, and “thumb” subdomains which participate in polymerization, and the “connection” and “RNase H” subdomains (Huang *et al*., 1998; Kohlstaedt *et al*., 1992).

The nucleoside RT inhibitors (NRTIs) are prodrugs that are triphosphorylated by host cellular enzymes. The triphosphorylated NRTIs then compete with natural deoxynucleoside triphosphates (dNTPs) for incorporation into the newly synthesized DNA chains where they cause chain termination. There are two biochemical mechanisms of NRTI drug resistance. The first mechanism is mediated by mutations that allow the RT enzyme to discriminate against NRTI during synthesis, thereby preventing their addition to the primer DNA chain (Larder and Stammers, 1999; Sarafianos *et al*., 1999a; Sarafianos *et al*., 1999b; Huang *et al*., 1998). The second mechanism is mediated by mutations in RT that increase the rate of hydrolytic removal of the chain terminating NRTI and enable continued DNA synthesis (Arion *et al*., 1998; Arion *et al*., 2000; Boyer *et al*., 2001; Meyer *et al*., 1999; Meyer *et al*., 1998).

The non-nucleoside RT inhibitors (NNRTIs) bind to a hydrophobic pocket in the RT enzyme close to, but not contiguous with, the active site. These compounds inhibit HIV-1 replication allosterically by displacing the catalytic aspartate residues relative to the polymerase binding site (Esnouf *et al*., 1995; Kohlstaedt and Steitz, 1992; Spence *et al*., 1995). The mutations responsible for NNRTI resistance are in the hydrophobic pocket which bind the inhibitors. A single mutation in this pocket may result in high-level resistance to one or more NNRTIs. Resistance usually emerges rapidly when NNRTI are administered as monotherapy or in the presence of incomplete virus suppression suggesting that resistance is caused by the selection of a pre-existing population of mutant viruses within an individual (Havlir *et al*., 1996; Jackson *et al*., 2000; Wei *et al*., 1995).

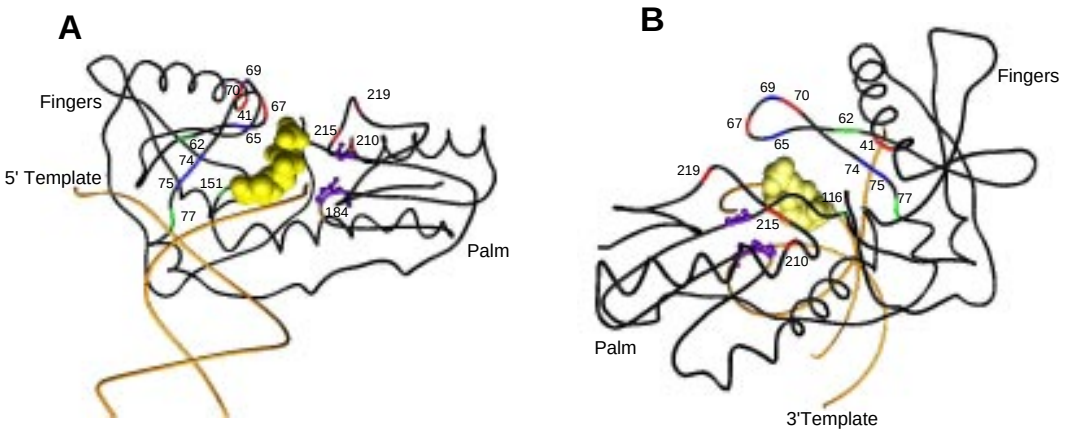

Figure 2. Structural model of HIV-1 reverse transcriptase (RT) labeled with nucleoside RT inhibitor (NRTI) resistance mutations. The polypeptide backbone of the fingers and palm domain (positions 1–235), and DNA primer and template strands are shown. The active site positions (110, 185, 186) are displayed in ball and stick mode. The incoming nucleotide is displayed in space-fill mode. These drawings are based on the structure published by Huang, *et al*., 1998 and are shown in "front" (a) and "back" (b) views.

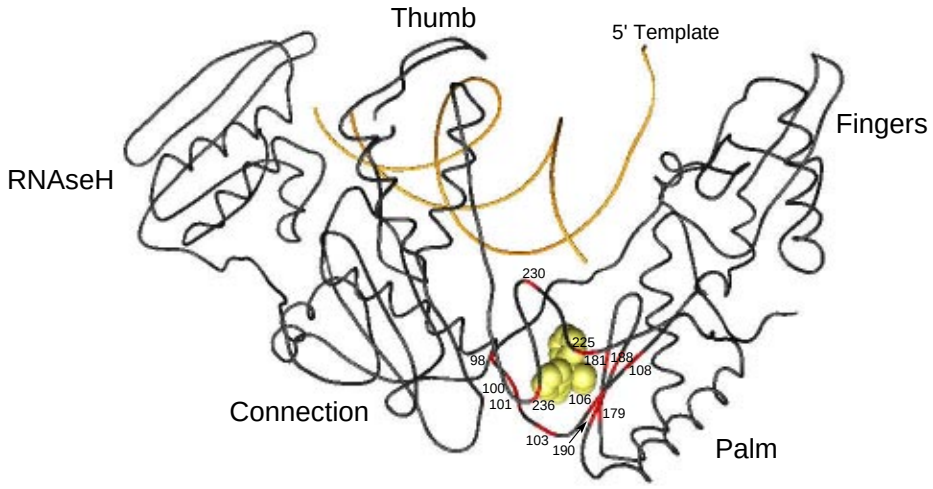

Figure 3. Structural model of HIV-1 reverse transcriptase (RT) labeled with non-nucleoside RT inhibitor (NNRTI) resistance mutations. The polypeptide backbone of the complete p66 subunit (positions 1–560), and DNA primer and template strands are shown. This drawing is based on the structure provided by Kohlstaedt and Steitz, 1992 in which the RT is co-crystallized with nevirapine, which is displayed in space-fill mode. The positions associated with NNRTI resistance are shown surrounding the hydrophobic pocket to which nevirapine and other NNRTIs bind.

Most NRTI and NNRTI drug resistance mutations are in the 5′ polymerase coding regions, particularly in the "fingers" and "palm" subdomains (Figures 2 and 3) (Tables 4 and 5) (Larder and Stammers, 1999; Sarafianos *et al*., 1999b). The earliest three-dimensional structures of HIV-1 RT showed the enzyme bound to an NNRTI (Kohlstaed *et al*., 1992) and bound to a double stranded DNA molecule (Ding *et al*., 1998; Jacobo-Molina *et al*., 1993). In 1998, a new structure showed the interaction between the catalytic complex and the incoming dNTP (Huang *et al*., 1998). Structures of mutant enzymes have also been determined (Ren *et al*., 1998; Sarafianos *et al*., 1999a, Stammers *et al*., 2001).

Table 4. HIV-1 Nucleoside RT Inhibitor (NRTI) Drug-Resistance Mutations

Codon	Mechanism	Effect on Resistance[1]	References
41	Classical AZT[2]	M41L increases AZT resistance when present with T215Y/F.	Kellam *et al*., 1992; Larder *et al*., 1994
44	Accessory[5]	E44A/D occurs with increased frequency in patients receiving multiple NRTI. It has recently been shown to cause low-level 3TC resistance when present with V118I.	Delaugerre *et al*., 2001; Hertogs *et al*., 2000a
62	MNR[4]	A62V is associated with multinucleoside resistance caused by Q151M.	Iversen *et al*., 1996; Shirasaka *et al*., 1995b
65	β2-β3 loop region[3]	K65R causes high-level resistance to DDC and low-to-intermediate levels of resistance to ddI, ABC, and 3TC.	Gu *et al*., 1994a; Gu *et al*., 1994b; Tisdale *et al*., 1997; Zhang *et al*., 1994
67	Classical AZT,[2] β2-β3 loop region [3]	D67N contributes to AZT resistance usually with mutations at codons 70 or 215. D67E/G occurs in heavily treated patients.	Larder and Kemp, 1989
69	β2-β3 loop region[3]	T69D/N/A cause ddC and ddI resistance and may cause low-level D4T resistance particularly when present in isolates with classical AZT resistance mutations. Insertions at this codon are by themselves associated with low level resistance to each of the NRTI. Together with AZT resistance mutations, insertions are associated with moderate-to-high levels of resistance to AZT, ddI, ddC, d4T, and 3TC.	Bloor *et al*., 1998; Fitzgibbon *et al*., 1992; Hertogs *et al*., 1998; Winters *et al*., 1998; Tamalet *et al*., 1998; Winters *et al*., 2001
70	Classical AZT,[2] β2-β3 loop region[3]	K70R causes AZT resistance.	Larder and Kemp, 1989; de Jong *et al*., 1996, Shulman *et al*., 2001
74	β2-β3 loop region[3]	L74V causes ddI, ddC, and ABC resistance. L74V partially suppresses T215Y-mediated AZT resistance.	St. Clair *et al*., 1991; Tisdale *et al*., 1997; Kozal *et al*., 1994a
75	MNR[4]	V75T/M/A causes d4T resistance and may cause low-level ddI and ddC resistance. V75I increases multinucleoside resistance caused by Q151M when present with F77L and F116Y.	Lacey and Larder, 1994 Bloor *et al*., 1998; Iversen, et al., 1996; Shirasaka *et al*., 1995b
77	MNR[4]	F77L increases multinucleoside resistance caused by Q151M when present with V75I or F116Y.	Iversen *et al*., 1996; Shirasaka *et al*., 1995b
115	Accessory[5]	Y115F causes low-level resistance to ABC.	Tisdale *et al*., 1997
116	MNR[4]	F116Y increases multinucleoside resistance caused by Q151M when present with F77L or V75I.	Iversen *et al*., 1996; Shirasaka *et al*., 1995b
118	Accessory[5]	V118I occurs with increased frequency in patients receiving multiple NRTI. It has recently been shown to cause intermediate 3TC resistance when present with E44A/D.	Delaugerre *et al*., 2001; Hertogs *et al*., 2000a

Table 4. cont.

Codon	Mechanism	Effect on Resistance	References
151	MNR[4]	Q151M causes intermediate levels of resistance to AZT, ddI, ddC, d4T, and ABC. Q151M, together with its associated changes at codons 62, 75, 77, and 116 causes high-level resistance to these NRTI and low-level resistance to 3TC.	Iversen *et al*., 1996; Schmit et al., 1998; Shafer *et al*., 1994; Shirasaka *et al*., 1995b; Van Laethem *et al*., 2000
184	Close to active site[3]	M184V/I cause high-level 3TC resistance and low-level ddI, ddC, and ABC resistance. M184V/I partially suppresses T215Y-mediated AZT resistance.	Boucher *et al*., 1993; Gu *et al*., 1992; Larder *et al*., 1995; Schuurman *et al*., 1995; Tisdale *et al*., 1997; Tisdale et al., 1993
210	Classical AZT[2]	L210W increases AZT resistance when present with mutations at position 215.	Harrigan *et al*., 1996; Hooker *et al*., 1996
215	Classical AZT[2]	T215Y/F causes AZT resistance and also limits the effectiveness of d4T, ABC, ddI, and ddC. T215S/C/D represent transitions between T and Y or F.	Kozal *et al*., 1993; Larder, *et al*., 1991; Larder and Kemp, 1989; Rey *et al*., 1998; Yerly *et al*., 1998; Japour *et al*., 1998; Lanier *et al*., 1998; Izopet *et al*., 1998
219	Classical AZT[2]	K219Q/E increase AZT resistance when present with K70R or T215Y/F. K219N/R occur commonly in heavily NRTI-treated patients.	Larder and Kemp, 1989, Larder *et al*., 1991

[1] 3TC: lamivudine, ABC: abacavir, AZT: zidovudine, d4T: stavudine, ddI: didanosine, ddC: zalcitibine.

[2] Classical AZT resistance mutations: Various combinations of these mutations have been shown to mediate ATP and pyrophosphate (PP)-dependent hydrolytic removal (pyrophosphorolysis) of zidovudine monophosphate from a terminated cDNA chain (Arion *et al*., 1998; Boyer *et al*., 2001; Meyer *et al*., 1999; Meyer *et al*., 1998; Meyer *et al*., 2000b), to cause a compensatory increase in RT processivity (Arion *et al*., 1998; Arts *et al*., 1998; Caliendo *et al*., 1996), and to confer cross-resistance to d4T, ABC, and limit the effectiveness of ddI and ddC (Coakley *et al*., 2000; Harrigan *et al*., 2000; Holodniy *et al*., 1996; Izopet *et al*., 1999; Japour *et al*., 1995; Lanier *et al*., 1999; Mayers *et al*., 1999; Montaner *et al*., 2000; Pellegrin *et al*., 1999; Shulman *et al*., 2001).

[3] Mutations at position 184 and mutations in the β2-β3 loop region cause resistance by decreasing affinity of RT for the nucleoside analog. Several of these mutations, including M184V and L74V, interfere with the activity of the classical AZT resistance mutations.

[4] MNR or multinucleoside resistance mutations: Q151M is the primary mutation. Mutations at positions 62, 75, 77, and 116 are secondary. Q151 possibly interacts directly with the 3′-OH of the incoming ddNTP (Sarafianos *et al*., 1999b).

[5] Accessory: The combination of mutations at positions 44 and 118 have been shown to confer low-level 3TC resistance (Hertogs *et al*., 2000a). But the increasing prevalence of these mutations in isolates from heavily treated patients that also have classical AZT resistance mutations, suggests a broader role (Delaugerre *et al*., 2001). G333E is a polymorphism that facilitates AZT resistance in isolates with M184V and multiple classical AZT resistance mutations.

Table 5. HIV-1 Non-Nucleoside RT Inhibitor Drug-Resistance Mutations

Codon	Effect on Resistance	References
98	A98G is associated with low-level resistance to each of the available NNRTIs. A98S is a polymorphism that is not associated with NNRTI resistance.	Petropoulos *et al*., 2000; Bacheler *et al*., 2001
100	L100I causes high-level resistance to EFV and NVP. Its effect on DLV susceptibility is not known. L100I suppresses T215Y-mediated AZT resistance.	Bacheler *et al*., 2001; Byrnes *et al*., 1994; Fujiwara *et al*., 1998; Petropoulos *et al*., 2000
101	K101E is associated with intermediate resistance to the NNRTIs. K101R/Q occasionally occur in patients who have not received NNRTI and have not been associated with resistance to the current NNRTI.	Bacheler *et al*., 2001; Hanna *et al*., 2000a; Petropoulos, et al., 2000
103	K103N causes resistance to NVP, DLV, and EFV. K103R occurs in about 1%-5% of persons receiving NRTI and probably does not cause NNRTI resistance.	Young *et al*., 1995 Bacheler *et al*., 2001; Demeter *et al*., 2000; Hanna *et al*., 2000a; Petropoulos *et al*., 2000; Shulman *et al*., 2000a
106	V106A causes high-level resistance to NVP, intermediate resistance to DLV, and low-level resistance to EFV. V106I occurs occasionally in patients not receiving NNRTIs and causes little if any phenotypic NNRTI resistance.	Bacheler *et al*., 2001; Byrnes *et al*., 1993; Larder *et al*., 1993a; Young *et al*., 1995
108	V108I causes low-level resistance to each of the available NNRTI.	Petropoulos *et al*., 2000; Bacheler *et al*., 2001
179	V179D/E is associated with resistance to the NNRTIs. V179I is probably a polymorphism that is not associated with NNRTI resistance.	Byrnes *et al*., 1993; Kleim et al., 1996; Winslow *et al*., 1996
181	Y181C/I causes resistance to NVP and DLV. It causes low-level resistance to EFV. Y181C partially reverses T215Y-mediated AZT resistance.	Bacheler *et al*., 2001; Byrnes, *et al*., 1994; Byrnes *et al*., 1993; Larder, 1992; Petropoulos, *et al*., 2000
188	Y188L causes high-level resistance to NVP and EFV and intermediate resistance to DLV. Y188C causes high-level resistance to NVP and low-level resistance to NVP and DLV. Y188H causes low-level resistance to each of the NNRTIs.	Byrnes *et al*., 1993 Bacheler, *et al*., 2001; Petropoulos *et al*., 2000
190	G190A/S causes resistance to NVP and EFV but not to DLV. G190E and other mutations at this position may cause resistance to each of the NNRTIs, although these mutations have been associated with decreased HIV-1 replication.	Bacheler *et al*., 2001; Hanna, *et al*., 2000a; Huang *et al*., 2000b; Kleim *et al*., 1994
225	P225H is associated with EFV resistance when present with other NNRTI mutations. It confers hyper-susceptibility to DLV.	Bacheler *et al*., 2001; Pelemans *et al*., 1998
227	F227L is a recently described mutation that is associated with resistance to NVP, DLV, and EFV when present with other NNRTI mutations.	Bacheler *et al*., 2001; Balzarini *et al*., 1998
230	M230L is a recently described mutation that causes high-level resistance to each of the currently available NNRTI.	Huang *et al*., 2000c
234	L234I confers resistance to an experimental NNRTI, AG-1549. Its effect on current NNRTIs is not known.	Fujiwara *et al*., 1998
236	P236L causes DLV resistance. It confers hyper-susceptibilityto NVP.	Dueweke *et al*., 1993

DLV: delavirdine, EFV: efavirenz, NVP: nevirapine

II. Approaches to HIV-1 Drug Resistance Testing

A. Source of HIV-1

Plasma is the main source of virus used for testing HIV-1 drug resistance in clinical settings. The sequence of plasma virus represents the quasispecies most recently selected for by antiretroviral drug therapy because plasma contains only actively replicating virus (Perelson *et al*., 1996). The evolution of HIV-1 sequences in peripheral blood mononuclear cells (PBMC) lags behind that in plasma (Koch *et al*., 1999; Kozal *et al*., 1993; Simmonds *et al*., 1991; Smith *et al*., 1993; Wei *et al*., 1995), and in patients failing therapy, mutations observed in plasma-isolated virus may not become the dominant quasispecies in PBMC until several weeks later.

Other sources of virus for HIV-1 drug resistance studies include resting T-lymphocytes and macrophages, lymph nodes, cerebrospinal fluid (CSF), and genital secretions. Reservoirs of virus harbored in long-lived cells such as resting T lymphocytes and macrophages pose the ultimate obstacle to HIV-1 eradication and have been an area of intense study (Pantaleo *et al*., 1998) (Finzi *et al*., 1997; Furtado *et al*., 1999; Wong *et al*., 1997b; Zhang *et al*., 2000). In patients with complete virus suppression during therapy (plasma HIV-1 RNA <50 copies/ml) there is generally little or no evidence of virus evolution in proviral DNA in long-lived cells (Ramratnam *et al*., 2000; Zhang *et al*., 2000). However, in patients with lapses in therapy, the population of virus within long-lived cells becomes replenished and may evolve drug resistant forms (Finzi *et al*., 1999; Ramratnam *et al*., 2000; Zhang *et al*., 1999).

The concentration of HIV-1 in lymph nodes is usually two to three orders of magnitude greater than the concentration of HIV-1 in plasma (Cavert *et al*., 1997; Chun *et al*., 1997; Embretson *et al*., 1993; Wang *et al*., 2000; Wong *et al*., 1997a). In patients receiving HAART, the concentration of HIV-1 in lymph nodes decreases in parallel with plasma viremia (Cavert *et al*., 1997; Notermans *et al*., 1998; Wong *et al*., 1997a). Several groups have sequenced protease and RT genes of virus recovered from proviral DNA in peripheral and visceral lymph nodes to determine the effect of antiviral therapy on virus in these heavily infected parts of the body (Haddad *et al*., 2000; Wong *et al*., 1997c). Most patients with undetectable levels of virus in the plasma still have detectable virus in lymph nodes (Wong *et al*., 1997a; Wong *et al*., 1997b), but there is no evidence that mutations develop in lymph nodes and yet remain absent from plasma (Dybul *et al*., 2000; Gunthard *et al*., 1998a; Schapiro *et al*., 1998; Erice *et al*., 2001).

It is not known to what extent HIV-1 in the central nervous system (CNS) reflects infection of the brain or simply mirrors what is present in the plasma (Daar, 1998). Some drugs, particularly the PIs, penetrate poorly into the CNS, and CSF virus has been sequenced to determine if the CNS is a sanctuary that permits virus replication during HAART therapy (Cunningham *et al*., 2000; Di Stefano *et al*., 1995; Gunthard *et al*., 2001; Venturi *et al*., 2000). But virologic failure in the CNS is rarely observed in the absence of virologic rebound in the plasma.

HIV-1 in the genital tract has been studied to determine whether specific variants are more likely to be transmitted either sexually or perinatally (Panther *et al*., 2000; Poss *et al*., 1995). In patients with ongoing virus replication, virus levels in genital secretions are usually proportional to virus levels in plasma. HIV-1 variants with genotypic resistance have been reported in genital secretions of both males and females receiving incompletely suppressive anti-HIV therapy (Di Stefano *et al*., 1999; Eron *et al*., 1998; Si-Mohamed *et al*., 2000). As in other latently infected cells, proviral HIV-1 DNA can occasionally be detected in nonspermatazoal mononuclear cells even in patients with plasma HIV-1 RNA levels <50 copies/ml (Gunthard *et al*., 2001; Zhang *et al*., 1998).

B. Phenotypic drug susceptibility testing

Phenotypic drug-susceptibility assays measure drug inhibition of HIV-1 *in vitro*. Two companies have developed standardized assays amenable to high-throughput performance (Virco, Mechelen, Belgium and ViroLogic, South San Francisco, CA, USA) (Hertogs *et al*., 1998; Petropoulos *et al*., 2000).

Both assays amplify the entire protease, much of RT and some of *gag* from HIV-1 RNA extracted from patient plasma. The amplified material is incorporated into a *pol*-deleted recombinant virus construct using either ligation or homologous recombination. A standardized virus inoculum is then used to infect a cell line and virus replication is measured in the presence and absence of a range of concentrations of different antiretroviral drugs. Drug susceptibility is reported as the concentration of drug required to inhibit virus replication by 50% (IC50).

Recombinant virus susceptibility assays have several advantages over older non-recombinant assays. Recombinant virus assays can be done using plasma, whereas non-recombinant assays require the isolation of PBMCs. Recombinant virus assays use PCR to amplify protease and RT, dramatically decreasing the need for virus culture. Finally, recombinant virus assays can be performed under highly uniform conditions because the backbone of the virus construct, which remains constant, can be tailored for replication in the cells used for susceptibility testing.

The use of recombinant viruses for susceptibility testing, however, may not always be optimal. PI resistance is modulated by mutations at *gag-pol* cleavage sites. Four of the nine protease cleavage sites in the recombinant virus come from the patient virus sample, but five come from the laboratory virus construct. If a patient's virus sample contained compensatory mutations at one of these five cleavage sites, the recombinant virus (lacking the compensatory mutations) might give inaccurate drug susceptibility results. The possibility that anomalous results might occur while testing either highly mutant viruses or viruses belonging to non-B subtypes has not yet been studied.

Recombinant and non-recombinant susceptibility tests suffer from the fact that the antiviral activities of NRTIs *in vitro* differ from the antiviral activities of these drugs *in vivo*. Abacavir and didanosine have one hundred times less antiviral activity than zidovudine *in vitro.* Yet abacavir and possibly also didanosine are more potent than zidovudine *in vivo.* Didanosine is weak *in vitro* because it is poorly triphosphorylated to its active form in the activated lymphocytes required for *in vitro* susceptibility testing (Gao *et al*., 1993; Shirasaka *et al*., 1995a). *In vitro* resistance to didanosine and stavudine are often impossible to detect even in patients experiencing virologic rebound while receiving these drugs. The poor triphosphorylation of didanosine may partly explain the difficulty in detecting didanosine resistance; but difficulty in detecting stavudine resistance is related to less well understood properties of the cells used for susceptibility testing (Meyer *et al*., 2000a; Lennerstrand *et al*., 2001).

C. HIV-1 genotypic testing

Genotypic tests are used more commonly in clinical settings because of their wider availability, lower cost, and shorter turnaround time. Genotypic tests provide more insight into the potential for resistance to emerge because they detect mutations present as mixtures, even if the mutation is present at a level too low to affect drug susceptibility in a phenotypic assay and they detect transitional mutations that do not cause resistance by themselves but indicate the presence of selective drug pressure. Genotypic testing has been shown to be clinically useful in four of five prospective randomized studies (Durant *et al*., 1999; Baxter *et al*., 2000; Tural *et al*., 2000, De Luca *et al*., 2000, Meynard *et al*., 2000); whereas phenotypic testing has been shown to be clinically useful in one of four prospective randomized studies (Cohen *et al*., 2000; Meynard *et al*., 2000; Melnick *et al*., 2000; Haubrich *et al*, 2001).

Figure 4 shows the distribution of known drug-resistance mutations within HIV-1 protease and RT. Mutations which may confer resistance to one or more drugs by themselves are represented by tall lines. Accessory mutations that confer resistance only when present with other mutations are represented by short lines. Sequencing for clinical purposes should encompass nearly all of the protease and positions 41–236 of the RT. Mutations at position 283 have been shown to cause low-level resistance to NNRTIs when present with other mutations (Brown *et al*., 2000); G333E is a naturally occurring polymorphism that facilitates zidovudine resistance in isolates from some patients receiving zidovudine and lamivudine that also have multiple zidovudine resistance mutations (Kemp *et al*., 1998) but dual resistance to these drugs usually emerges without this change (Masquelier *et al*., 1999; Shafer *et al*., 1998; Kuritzkes *et al*., 2000).

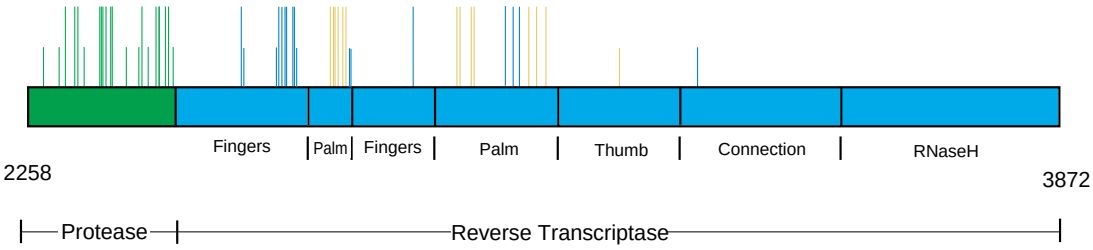

Figure 4. Schematic diagram of showing the distribution of drug resistance mutations within the HIV-1 protease and RT genes. The nucleotide numbers relative to the HXB2 genome are shown (2258–3872). The protease is shown in green and the RT is shown in blue. Tall lines indicate the positions of mutations that confer resistance in the absence of other mutations. Short lines indicate the positions of accessory mutations that confer resistance only when present with other drug-resistance mutations. Protease inhibitor resistance mutations are shown in green; nucleoside RT inhibitor mutations are in blue; non-nucleoside RT inhibitor mutations are in yellow.

D. Clonal and population-based sequencing

The extent of genetic variation within an individual is lowest at the time of initial infection; and shortly after infection, usually only a single variant is detected (Burger *et al*., 1991; Diaz *et al*., 1997; Liu *et al*., 1997; Pang *et al*., 1992; Wolfs *et al*., 1992). Whether or not additional strains are transmitted but remain at levels too low to be detected is not known. The initial HIV-1 quasispecies, however, is more complex in those uncommon cases in which a person is initially infected with viruses from more than one source (Diaz *et al*., 1995; Long *et al*., 2000; Pieniazek *et al*., 1995; Zhu *et al*., 1995).

During the course of infection, virus sequence variation within an individual may range from about 1% to >5% in hypervariable regions of *env* (Brown, 1991; Wolfs *et al*., 1992). Several studies suggest that virus diversity is greater in patients mounting an anti-HIV-1 immune response (Liu *et al*., 1997; Lukashov *et al*., 1995; Shankarappa *et al*., 1999; Wolinsky *et al*., 1996). Genetic variability is usually lower in the plasma virus population compared with the cellular proviral DNA population. In the absence of drug therapy, genetic variability is usually lower in those genes coding for conserved proteins such as protease and RT than in envelope proteins (Imamichi *et al*., 2001; Quinones-Mateu *et al*., 1996).

Direct PCR, or population-based, sequencing is done in clinical settings because it is quicker and more affordable than sequencing multiple clones. Clonal sequencing is performed in research settings to answer questions about the evolution of HIV-1 drug resistance. For both population-based and clonal sequencing, the ability to detect minor variants is inversely related to the proportion of the minor variants within the whole virus population. In direct PCR sequencing, electrophoretic double peaks indicating a nucleotide mixture occur only when the second nucleotide is present in at least 20 percent of the total virus population. (D'Aquila, 2000; Gunthard *et al*., 1998b; Larder *et al*., 1993b; Schuurman *et al*., 1999b; Shafer *et al*., 2000c). With either method, unequal amplification of viral variants present as a mixture may occur because one or more species may be preferentially amplified during PCR due to differences in primer binding (Becker-Pergola *et al*., 2000b).

By sequencing multiple clones, one can measure the frequency of distinct variants within the HIV-1 quasispecies and assess the contribution of individual clones to the evolution of drug resistance. Clonal sequencing may detect dual infection and *in vivo* recombination (Long *et al*., 2000; Pieniazek *et al*., 1995), and can assess the co-linearity of mutations within a viral genome (D'Aquila, 2000; Zhang *et al*., 1997). Sequencing multiple clones from two different tissue samples or from the same tissue at two times enables statistical comparisons between the two virus populations (Imamichi *et al*., 2001; Wong *et al*., 1997c; Zhang *et al*., 2000). Finally, the composition of the HIV-1 quasispecies sheds light on the rates of mutation fixation, the fitness of mutant variants, and the effective size of the virus population *in vivo* (Brown and Cleland, 1996; Brown and Richman, 1997; Goudsmit *et al*., 1997; Goudsmit *et al*., 1996; Gunthard *et al*., 1999; Najera *et al*., 1995; Rodrigo, 1999; Rouzine and Coffin, 1999).

Cloning PCR-amplified genetic material into a plasmid or bacteriophage does not guarantee that each of the recovered clones will contain the sequence of a different virus. If the DNA used for cloning was amplified from a small number of cDNA or proviral DNA molecules, there is a risk that some clones may have been derived from the same initial template and thus be "PCR siblings" rather than DNA from different viruses. To guarantee the recovery of unique clones, some groups perform a limiting dilution of unamplified nucleic acid (viral RNA, cDNA, or proviral DNA) to ensure that only a single target sequence is amplified in each reaction (Brown and Simmonds, 1995); other groups dilute the starting material (but not to a limiting dilution) and then create one molecular clone from each dilution (Bacheler *et al*., 2000; Condra *et al*., 1996).

PCR may introduce errors and may also cause recombination due to template switching (Brown and Simmonds, 1995; Learn *et al*., 1996; Meyerhans *et al*., 1989). *Taq* polymerase is a low fidelity DNA polymerase which lacks proof-reading activity. The error rate for *Taq* is $20–100 \times 10^6$ (Sambrook and Russell, 2001). A 2 kb product amplified by nested PCR (e.g. 30 cycles $\times$ 2) could contain as many as 12 nucleotide changes, some of which will alter the encoded amino acid sequence (Learn *et al*., 1996). Use of a high fidelity thermostable DNA polymerase such as *Pfu* will minimize this problem (Cline *et al*., 1996). Limiting dilution PCR is required to prevent PCR recombination (Brown and Simmonds, 1995).

E. Sequencing by hybridization

The Affymetrix GeneChip® is designed to determine the complete sequence of HIV-1 protease and the first 1200 nucleotides of HIV-1 RT. Affymetrix uses photolithography and light-directed combinatorial chemistry to create precisely positioned and densely packed arrays of oligonucleotide probes on a glass wafer. The wafers are packaged in plastic cartridges that serve as hybridization chambers. Fluorescein-labeled RNA is transcribed from a cDNA sample and hybridized to the probes bound on a glass surface. Binding of the RNA to complementary probes is detected using a laser scanner. The GeneChip® is divided into several thousand segments each containing millions of similar probes designed to interrogate each of the nucleotide positions in a test DNA or RNA molecule. Every nucleotide in the test molecule requires at least four sets of oligonucleotide probes to determine whether that nucleotide is an A, C, G, or T.

The design or tiling of Affymetrix gene chips requires prior knowledge of the most commonly expected polymorphisms in a gene. Because of this requirement, this method of sequencing is also referred to as "re-sequencing". The genetic variability of HIV-1 poses the ultimate challenge to sequencing by hybridization. Several studies comparing GeneChip® and dideoxynucleotide sequencing and have found that dideoxynulceotide sequencing is more reliable at detecting HIV-1 protease and RT mutations (Gunthard *et al*., 1998b; Hanna *et al*., 2000b; Vahey *et al*., 1999; Wilson *et al*., 2000) (Table 6). The current GeneChip® is not capable of detecting insertion or deletions and is unreliable at sequencing viral subtypes other than the subtype B on which the chip tiling has been based. In addition, genomic regions containing clusters of adjacent mutations can interfere with probe hybridization and result in errors. Improved microarrays for sequencing isolates belonging to subtypes A–F and for detecting insertions are under development (Myers *et al*., 2000).

Point mutation assays are inexpensive and have the potential to be highly sensitive for mutations present in only a small proportion of circulating viruses (Servais *et al*., 2001a; Van Laethem *et al*., 1999). The INNO-LiPA® HIV-1 RT Assays (Innogenetics, Ghent, Belgium) is designed to detect NRTI-associated mutations at codons 41, 69, 70, 74, 75, 184, and 215 in the RT gene (Clarke *et al*., 2000; Stuyver *et al*., 1997). The INNO-LiPA® HIV-1 protease assay is designed to detect mutations at codons 30, 46, 48, 50, 54, 82, 84, and 90 in the protease gene (De Smet *et al*., 2000; Servais *et al*., 2001a; Servais *et al*., 2001b). The INNO-LiPA® assays have probes for wildtype and mutant alleles of each codon attached

Table 6. Reproducibility of HIV-1 Protease and Reverse Transcriptase Sequencing

Reference	Comparison	Main Findings
(Demeter *et al*., 1998)	Dideoxynucleotide cycle sequencing of cultured cell pellets in 11 laboratories	99.7% nucleotide concordance. The homogeneity of cultured virus and the fact that RNA extraction and reverse transcription were not required probably contributed to the high concordance.
(Gunthard *et al*., 1998b)	Comparison of dideoxynucleotide and GeneChip sequencing of PCR-amplified DNA	98.8% nucleotide concordance.
(Schuurman, et al., 1999b)	Dideoxy terminator and GeneChip sequencing of mixtures of plasmid clones in 23 laboratories. Mutations at three positions were studied.	20/23 laboratories reliably detected 100% wildtype and 100% mutant codons. Most laboratories detected the mutants present as 50% mixtures. Laboratories were inconsistent at detecting mutations present as 25% mixtures.
(Schuurman, et al., 1999a)	Dideoxynucleotide and GeneChip sequencing of plasma samples seeded with different proportions of plasmid clones.	Detailed results pending. The number of cor rectly identified results did not vary among the three most widely used methods (ABI Kit, homebrew assay with ABI sequencer, VGI kit and sequencer).
(Vahey *et al*., 1999)	Comparison of dideoxynucleotide and GeneChip sequencing	GeneChip sequencing inaccurately tested non-subtype B sequences.
(Shafer *et al*., 2000c)	Dideoxynucleotide sequencing of replicate plasma samples in the same laboratory	99.4% amino acid concordance. Most discordances were partial and were caused by the distribution of genetic variants within the plasma samples.
(Hanna *et al*., 2000b)	Comparison of dideoxynucleotide and GeneChip sequencing of cultured virus stock supernatant.	97.4% concordance. Dideoxyterminator sequencing was more sensitive at detecting drug-resistance mutations.
(Wilson *et al*., 2000)	Comparison of dideoxynucleotide and GeneChip sequencing and Line Probe Assay	96.6% concordance between dideoxynucleotide and GeneChip sequencing.
(Shafer *et al*., 2001)	Comparison of dideoxynucleotide sequencing of 46 plasma samples in two laboratories	99.1% concordance. 90% of discordances were partial defined as one laboratory detecting a mixture and the second laboratory detecting just one of the components of the mixture.

to a nitrocellulose strip. Biotin-labeled RT-PCR product from the patient sample is hybridized to the strip. An avidin-enzyme complex and the enzyme substrate produce a color change on the paper strip where the PCR product has hybridized with a probe. This assay is currently limited because it can only detect a subset of drug resistance mutations and has a 10% rate of uninterpretable results due to poor hybridization, which is particularly likely to occur when uncommon mutations occur at key codons (Puchhammer-Stockl *et al*., 1999; Servais *et al*., 2001a). Other point-mutation assays have been developed but have not been used in clinical settings (Eastman *et al*., 1998; Shafer *et al*., 1996).

III. Dideoxynucleotide cycle sequencing

A. General considerations

The template for HIV-1 protease and RT sequencing is prepared from plasma by RNA isolation, reverse transcription, and PCR amplification. These same procedures are required whether one uses a dedicated kit or an assembly of reagents obtained from separate vendors. Two commercial HIV-1 RT and protease genotyping kits described below are available and are under consideration by the FDA for use in clinical settings (Dileanis *et al*., 2000a; Ruiz *et al*., 2000). These kits have stronger quality control and validation profiles than home brew methods but may be more expensive and less versatile.

B. Preventing sample contamination

There are three major issues related to sample contamination in HIV-1 genotyping: contamination with RNases, cross-contamination of one sample with another, and contamination of PCR reactions with exogenous DNA and PCR products from previous reactions. RNases are present on hair and skin, and can easily contaminate solutions, plasticware, and equipment. Degradation of HIV-1 RNA by RNases can be minimized by wearing gloves, opening and handling samples and solutions behind protective shielding, and adding an RNase inhibitor to reverse transcription reactions (Sambrook and Russell, 2001).

Standard approaches have been adopted to minimize sample cross-contamination and contamination with PCR products from previous reactions (Kwok and Higuchi, 1989). Pre- and post-PCR steps should be performed in separate rooms and there should be no transfer of supplies, reagents or equipment from the post-PCR area to the pre-PCR area. Disposable gowns and gloves should be worn and stored in the pre-PCR area. A separate workstation - laminar flow hood with UV lights and dedicated pipettes - in the pre-PCR room or in another “clean room” should be used for all reagent preparation. Positive displacement pipettors and aerosol-resistant pipette tips should be used for all procedures and pipette tips should be changed between each addition. Reagents should be added to tubes before adding plasma RNA or DNA.

Each set of PCR reactions should include a negative control to be analyzed by agarose gel or sequencing to monitor for contamination. A uracil N-glycosylase (UNG) system can also be used to minimize contamination of PCR reactions with products generated in previous amplifications. In this system, PCR reactions are performed with deoxyuracil triphosphate (dUTP) in place of dTTP. Any contaminating dU-containing PCR products are degraded by UNG prior to PCR during a brief incubation (e.g. 50ºC for 10 minutes). UNG is destroyed at the start of PCR by incubation at a higher temperature (e.g. 93ºC).

C. RNA extraction

HIV-1 RNA extraction typically involves virus concentration, virus disruption, RNA recovery, and RNA purification. Typically, 0.2 ml – 1.0 ml of plasma is ultracentrifuged at $\geq$ 20,000 g for 30 to 60 minutes at 4ºC. The resulting virus pellet, which is invisible, can be used directly or resuspended in nuclease-free water.

Most HIV-1 RNA extraction methods utilize a lysis solution that contains a chaotropic guanidine salt as its active ingredient. Guanidine thiocyanate releases RNA from the virus particle and protects it from degradation by RNases. Lysis solutions may also include phenol, urea, glycogen, carrier RNA, an RNase inhibitor, or dithiothreitol to promote efficient lysis and limit degradation of released RNA. The lysis solution is added to plasma or the concentrated virus pellet. Although the lysed virus is no longer infectious, guanidine thiocyanate is toxic and should be handled appropriately.

The released RNA can then be recovered using alcohol precipitation or by attachment to silica particles or columns. Alcohol precipitation can be performed either directly from the lysis solution (Erali and Hillyard, 1999) or after phenol-chloroform purification steps (Chomczynski and Sacchi, 1987). There are several commercial kits designed to facilitate RNA extraction. The QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) contains a proprietary lysis reagent for virus disruption and a column containing a silica-based resin for RNA recovery and purification (Fischer *et al*., 1999; Fransen *et al*., 1998). The NucliSens Isolation Kit (Organon-Teknika, Durham, NC) is based on the Boom method (Boom *et al*., 1990) that uses a guanidinium thiocyanate-containing lysis reagent for virus disruption and silica particles for RNA recovery (Niubo *et al*., 2000; Verhofstede *et al*., 1996). The AMPLICOR HIV-1 MONITOR™ Test (Roche Diagnostics, Branchburg, NJ) performs HIV-1 quantitation using a 142-bp gag gene segment (Erali and Hillyard, 1999; Fransen *et al*., 1998; Mulder *et al*., 1997). Several laboratories have found that the RNA isolated using this kit can also be used for protease and RT sequencing (Shafer *et al*., 2000c). However, the kit is expensive, making its use as an extraction method practical only for laboratories performing both HIV-1 quantitation and sequencing.

D. Reverse transcription and PCR

Extracted viral RNA must be reverse transcribed to cDNA prior to PCR amplification. Reverse transcription involves incubating the RNA with dNTPs, a commercial RT enzyme, and a DNA primer. RT enzymes such as MMuLV (Moloney murine leukemia virus) and AMV (avian myeloblastosis virus) are most commonly used. RT enzymes that lack RNase H activity (e.g. Superscript, GIBCO BRL, Gaithersburg, MD), have increased thermostability, enabling reverse transcription to be performed at higher temperatures. This may increase yield by disrupting secondary RNA structure. Reverse transcription can be performed with random hexamer primers or, more commonly, a single HIV-1-specific primer. Reverse transcription and PCR can be carried out in one-step or two-step formats.

Following reverse transcription, PCR amplification is necessary to obtain enough target DNA for sequencing. PCR primers should bind to regions of the virus that are conserved and unaffected by drug treatment. *Taq* polymerase is adequate for population based sequencing, because *Taq*-induced errors may not be detected if many molecules were present in the starting nucleic acid preparation. However, sequence analyses of PCR-generated cloned DNA should be performed using an enzyme with proof reading activity, such as, *Pfu* which has an error rate of 1.6×10^6, compared to $20–100 \times 10^6$ for *Taq* polymerase (Cline *et al*., 1996; Sambrook and Russell, 2001).

In highly optimized system, samples with plasma HIV-1 RNA levels >1,000 copies/mL often have enough genetic material for sequencing after only one round of 30–40 PCR cycles. While nested PCR increases DNA yield and the sensitivity and specificity of the amplification, it also increases the effort and cost required for amplification, and the risk of sample cross-contamination.

E. Sequencing

Cycle sequencing using a highly processive, heat stable DNA polymerase allows sequencing reactions to be performed with only 1.0 μg of double-stranded template. Automated sequencers detect fluorescence from one or more dyes to identify A, C, G, and T termination reactions. Fluorescent dye labels can be incorporated into DNA extension products using either 5′- or 3′-dye labeled dideoxynucleotide triphosphates (ddNTPs). With dye terminator labeling, each of the four ddNTPs is tagged with a different fluorescent dye. With dye primer labeling, extension products are identified using primers tagged with four different fluorescent dyes in four separate base-specific reactions. Dye primers produce more consistent peak heights making them better for detecting and quantifying mixtures of bases at a given position. But dye primer sequencing requires four separate sequencing reactions for each primer. In contrast, dye terminators require only one reaction and are also more versatile because unlabeled primers can be used.

The quality of the template DNA (e.g. PCR product) is the most important factor for obtaining high quality sequence data. PCR products should be purified away from excess primers and ddNTPs prior to sequencing, although under some conditions, PCR products can be sequenced without purification if the yield of the desired product is high and the product is specific (produces a single band when analyzed by gel electrophoresis). Cycle sequencing reaction products should be purified to remove unbound fluorescent labels prior to electrophoretic separation of the reaction products. Electrophoretic peaks should be sharp, well-defined, and scaled high in the first several hundred nucleotides sequenced. Noisy data is most commonly caused by a "dirty" DNA template containing more than one priming site or an impurity that inhibits the sequencing reaction. Sequence quality can also be affected by gel-, instrument-, and software-related problems.

F. Applied Biosystems ViroSeq™ HIV-1 Genotyping System

The Applied Biosystems ViroSeq™ HIV-1 Genotyping System includes reagents and protocols for every step of genotyping from RNA extraction to generation of a genotyping report. Specific features of this system are described in Table 7. RNA is extracted from plasma by a modification of the extraction technique in the AMPLICOR HIV-1 MONITOR™ UltraSensitive Method (Roche Diagnostics). RNA is reverse-transcribed with MMuLV RT and a 1.8 kb product is amplified using AmpliTaq Gold DNA polymerase and AmpErase dUTP/UNG, which minimizes the risk of sample cross-contamination. PCR products are purified prior to sequencing. BigDye™ dideoxyterminator sequencing reactions are prepared for six kit-specific primers (an alternate 7th primer is provided). Sequencing reactions are purified using isopropanol or columns and then run on a an ABI Prism® automated sequencer (310, 377, or 3100).

HIV-1-specific analysis software is used to assemble data from each sequencing primer, compare the assembly to a reference sequence, and generate a report of results. Sequences can be saved in the FASTA format. This assay has been successful at sequencing RNA from a majority of patients with plasma HIV-1 RNA levels ≥ 1,000 copies/ml and subtypes A–G (Cunningham *et al*., 2001; Dileanis *et al*., 2000b; Marlowe *et al*., 2000).

G. Visible Genetics TRUGENE™ HIV-1 Genotyping Kit

The Visible Genetics (VGI) TRUGENE™ HIV-1 Genotyping Kit includes reagents for use with dedicated VGI equipment and is specifically designed for sequencing HIV-1 protease and RT. Specific features of this system are described in Table 7. Reagents for RNA extraction are not included, but are available from VGI as a separate kit, TRUPREP™. Extraction using other commercial systems is also validated. The kit employs a combined RT-PCR amplification followed by 4 dye-primer sequencing reactions called CLIP reactions, each with multiple primers.

The 1.3 kb RT/PCR product includes sequences for the entire protease gene and of codons 1–247 of the RT gene. The CLIP sequencing reactions provide sequences for the entire protease gene and codons 40–247 of the RT gene. Sequencing reactions are run using VGI-specific gel preparation and electrophoresis equipment. Each gel provides sequence information on one isolate. Sequencing reactions use two DNA primers, each labeled with a different fluorescent dye, for bidirectional sequencing. The CLIP reactions perform semi-logarithmic amplification of the product while determining its sequence, improving the sensitivity of sequencing samples with low copy numbers (Lee *et al*., 2001). Generic VGI sequence analysis software with HIV-specific modules is used to assemble the sequencing data, compare the results to a reference sequence, and generate a report of the results.

Table 7. HIV-1 Protease and RT Sequencing Kits

	Applied Biosystems ViroSeq™ HIV-1 Genotyping System	Visible Genetics TRUGENE™ HIV-1 Genotyping Kit
Sample requirements		
Plasma volume	500 ul	Depends on method used for extraction
Viral load	>2,000 RNA copies/ml is recommended	>1,000 RNA copies/ml is recommended
RNA isolation		
	Lysis and centrifugation using a modification of the MONITOR Ultrasensitive Method (Roche Diagnostics).	Separate kits can be purchased for RNA extraction.
RT/PCR		
RT	Single primer, MMuLV RT, RNase inhibitor	Single primer, MMuLV RT, RNase inhibitor
PCR	Non-nested, 40 cycles with Ampli-*Taq* Gold DNA polymerase. 1.8 kb product	Non-nested, 27 cycles with proprietary DNA polymerase. 1.3 kb product
Controls	Positive and negative controls included beginning with the RT/PCR step. dUTP/UNG Amperase	Positive and negative controls included beginning with the RT/PCR step.
Sample clean up	Microcon columns	None
Analysis	Agarose gel	None
Equipment needed	9600 or 9700 thermal cycler	Laboratory's preferred thermal cycler
Sequencing		
Method	BigDye™ dideoxyterminators	CLIP bi-directional
Number of primers	6-7 primers, one per reaction	6 reactions, each containing a primer mix
Equipment	9600 or 9700 thermal cycler, ABI 310, 377, 3100	Customized equipment for gel preparation and electrophoresis
Throughput	16 samples per 7 hour run on ABI 377 (96-well). 32 samples per run over 24 hours on ABI 3100	1 sample per 50 minute run. With 4 Long Read Towers, 28 samples in 8 hours
Data analysis system		
Region analyzed	Protease 1-99, RT 1-320	Protease 1-99, RT 40-247
Computer platform	MacIntosh for ABI 377/ABI 310, PC or MacIntosh for ABI3100	PC
Software features	Primer assembly, auto trimming, full view of electropherograms, translation and alignment to reference sequence, quick tab to positions of interest for editing.	Primer assembly, auto trimming, full view of electropherograms, translation and alignment to reference sequence, quicktab to positions of interest for editing. Includes "fingerprinting" comparison to prior runs.
Mutations reported	Resistance mutations, novel variants, insertions/deletions	Resistance mutations, known polymorphisms, novel variants, insertions/deletions
Sequence format	FASTA	FASTA (Ns may need to be deleted for data analysis)
Other reported information	Information about sequence quality and editing	Drug resistance interpretation

H. Sequence reproducibility

Inter-laboratory comparisons of sequence results obtained using cultured PBMCs, uncultured plasma, and prepared mixtures of HIV-1 plasmid clones indicate that dideoxynucleoside sequencing is highly reproducible in experienced laboratories (Table 6). In one study, the sequence concordance among 13 research laboratories performing dideoxynucleotide sequencing on cultured cell pellets was 99.7% at all nucleotide positions and 97% at positions associated with zidovudine resistance (Demeter *et al*., 1998). Sequencing cultured cell pellets is simpler than sequencing clinical samples such as plasma because RNA extraction and reverse transcription are not necessary and because cultured virus is more homogeneous and contains fewer mixtures than uncultured virus (Delassus *et al*., 1991; Kusumi *et al*., 1992). Nonetheless, the high inter-laboratory concordance in this study supports the intrinsic reliability of the dideoxy method for HIV-1 analysis.

Two large multicenter comparisons of sequence results obtained from samples containing mixtures of plasmid clones (ENVA-1) and spiked plasma samples (ENVA-2) have also been performed (Schuurman *et al*., 1999a; Schuurman *et al*., 1999b). These studies found that the ability of the participating laboratories to detect mutations was directly proportional to the percent of mutant plasmid clones within each mixture. Only a minority of laboratories detected mutations in mixtures in which the mutant clones made up less than 25% of the total.

Two clinical laboratories assessed the reproducibility of HIV-1 RT and protease sequencing using plasma aliquots obtained from 46 heavily treated HIV-1 infected individuals. Although both laboratories used sequencing reagents from Applied Biosystems, each used a different in-house protocol for plasma HIV-1 RNA extraction, reverse transcription, PCR, and sequencing. Overall sequence concordance between the two laboratories was 99.0%. But about 90% of the discordances were partial, defined as one laboratory detecting a mixture while the second laboratory detected only one of the mixture's components (Figure 5). Complete discordances were significantly more likely to occur in plasma samples with lower plasma HIV-1 RNA levels. Nucleotide mixtures were detected at approximately 1% of the nucleotide positions, and, in every case in which one laboratory detected a mixture, the second laboratory detected either the same mixture or one of the mixture's components. The high concordance in detecting mixtures and the fact that most discordance between the two laboratories was partial suggest that most discordance was due to variation in sampling the HIV-1 quasispecies rather than to technical artifact.

IV Sequence analysis

A. Sequence editing

Current automated sequencing platforms provide programs for assembling sequences of overlapping primers and for viewing multiply-aligned, equally-spaced electropherograms. Aligned electropherograms should be inspected at positions demonstrating ambiguous base calling (indicating possible nucleotide mixtures), positions associated with drug resistance, and positions with amino acid differences from the consensus subtype B reference sequence. The Applied Biosystems and Visible Genetics HIV-1 genotyping systems provide additional programs with HIV-1-specific features.

Because HIV-1 from any infected individual is genetically heterogeneous, the sequence of most clinical samples will contain positions with more than one electrophoretic peak indicating a mixture of nucleotides. In heavily treated patients, about 1% of positions show evidence of a nucleotide mixture but mixtures of more than two nucleotides at a single position are extremely rare (Shafer *et al*., 2001). Base-calling is affected by the background signal generated in each sequencing reaction. Reagents that reduce background signal will lead to an increased ability to detect the presence of minor variants within a mixture (Zakeri *et al*., 1998). There is a trade-off between calling too many mixtures, some of which may be false positives, and calling too few mixtures. Each laboratory should establish a consistent method for calling mixtures. A mixture rate higher than 2%–3% and the presence of more than two nucleotides at a single position suggests a high degree of sequencing noise.

Protease

Laboratory A \ Laboratory B	A	C	G	T	R	Y	W	M	K	S
A	4897	2	2		22		2	6		
C		2174		1		14		3		1
G	5		3040		17				1	
T			1	3338		6	3			
R	9				43					
Y		10		2		30				
W	3			5			8			
M	3							5		
K			2	2					2	
S			1							2

Reverse Transcriptase

Laboratory A \ Laboratory B	A	C	G	T	R	Y	W	M	K	S
A	13510	1	10		69		3	5		
C		5684	1	6		34		6		2
G	8		6787		59				1	3
T	2	7	1	7903		30	8		5	
R	25		8		70					
Y		7		14		55				
W	12			2			12			
M	4	2						12		
K			3	3					10	
S		1	3							8

Figure 5. Matrices showing the exact numbers of nucleotide concordances and discordances between two laboratories (A - vertical, left and B - horizontal, top) performing dideoxynucleotide sequencing on cryopreserved plasma aliquots from 46 heavily treated HIV-1 infected patients. Exact matches are shown along the diagonal. The numbers of partial discordances are written in black on a grey background and the numbers of complete discordances are written in red on a white background. R (A/G) and Y (C/T) represent transitions. M (A/C), W (A/T), K (G/T), and S (C/G) represent transversions. Data from the protease is shown at top and data from the reverse transcriptase (RT) is shown beneath. One RT sequence had a B and another had an H (not shown). There were no Ns or other highly ambiguous nucleotides. Adapted from (Shafer et al., 2001).

B. Sequence formats

After a sequence has been edited it should be stored in a plain text format (e.g., FASTA, GenBank, GCG). Although less informative than other formats, the FASTA format offers the simplest way of dealing with sequence data in a human- and computer-readable fashion. In this format, the sequence is preceded by a line which begins with the ">" character and which may contain one or more identifiers separated by the "|" character. The FASTA format is compatible with GenBank searches and most sequence analysis programs and is available as an export option in the software for ABI sequencers. Sequences generated with the TRUGENE™ HIV-1 Genotyping System contain "Ns" at the 5′ end and in the region between the non-contiguous protease and RT sequences, which may need to be manually deleted prior to analysis of sequence data with other software systems. There are several other commonly used sequence formats, as well as freely available tools that convert sequence files between different formats (e.g. readseq, ClustalW) (Ouellette, 1998).

C. Data management

A typical sequencing project typically generates a vast amount of raw data. Intelligent management of these data can save many hours during subsequent sequence analyses. All raw data, including sequencing run folders, should be backed up prior to review or manipulation. Electropherograms for each sample, FASTA files, and other reports can be organized into folders. Standardized worksheets can be generated to track dates and details of sample extraction, reverse transcription and PCR, product analysis, and sequencing reactions.

Sequence files should be stored both individually and as part of a sequence alignment in which each sequence is aligned to a consensus reference sequence. Creating a multiple sequence alignment for HIV-1 protease and RT sequences is straightforward because insertions and deletions are rare. The Los Alamos Laboratory HIV Sequence Database website has a tutorial which contains links to free and inexpensive programs for creating and editing multiple sequence alignments (e.g. ClustalW, BioEdit, GeneDoc). Joe Felsenstein's site at the University of Washington and the IUBIO archive each provide a comprehensive set of links to free sequence analysis tools (see Appendix for URLs).

There are also several comprehensive sequence analysis packages, mostly commercial, that allow users to manipulate, analyze, and annotate sequence data (e.g. GCG, DNASTAR, MacVector, the Staden Package, GDE). Some of these packages are expensive and most of their analysis tools are designed to help predict the function of previously uncharacterized sequence data. Although these packages can be useful in the management of HIV-1 protease and RT sequences, many of the programs are not necessary because the structure and function of HIV-1 protease and RT are already known.

In contrast, most sequence laboratories require a laboratory information management system (LIMS) to help meet the challenge of keeping track of many sequence variants, each of which is linked to a variable amount of external data (i.e. the treatment regimen of the person from whom the virus isolate was obtained, the drug susceptibility of the sequenced isolate). Although there are several expensive commercial LIMS under development, most investigators have developed their own LIMS in which the information associated with a sequence is stored in a spreadsheet or database.

D. Sequence analysis for quality control

Laboratory cross-contamination and sample mix-ups are common. Learn *et al* (1996) described two studies in which laboratory contamination or sample mix-up resulted in the publication of erroneous data. Frenkel *et al* (1998) also used similar sequence analyses to demonstrate that reports of transient HIV-1 viremia in perinatally exposed infants were also laboratory artifacts. All new sequences should therefore be analyzed for the presence of laboratory artifacts. Each sequence should be compared to the sequences of other HIV-1 isolates amplified at the same time and the distribution of pairwise genetic distances between these sequences should be examined (Learn *et al*., 1996). Sequences from epidemiologically unrelated individuals should be examined to determine whether the sequences are more closely related to one another than expected. Sequences from the same individual should be examined to determine whether the sequences diverge more than expected.

Phylogenetic trees containing each of the sequences obtained in a laboratory can also help identify closely related sequences. For this purpose, trees are generally created using rapid distance-based methods (e.g. neighbor joining algorithm) rather than by slower character-based methods (e.g. maximum parsimony and maximum likelihood algorithms). Programs for creating neighbor-joining trees can be found in phylogenetic software packages such as PHYLIP (Felsenstein, 1993) and PAUP (Swofford, 1998), as well as, in less comprehensive packages such as TreeCon (Van de Peer and De Wachter, 1997). TreeView is a useful free program for viewing and manipulating tree files on PCs and Macs (Page, 1996).

The Los Alamos Laboratory HIV Sequence Database web site contains tutorials on sequence quality control and on making phylogenetic trees (Kuiken *et al*., 1999). It also contains the following useful pages: (i) GenBank BLAST Search which compares a new sequence to published isolates with highly similar sequences to help exclude contamination with commonly used laboratory HIV-1 isolates, (ii) Neighbor TreeMaker which accepts submitted sequence alignments and creates a phylogenetic tree using the PHYLIP neighbor joining tree program, (iii) SeqPublish which creates a formatted version of a sequence alignment which makes it easier to spot potential problems, and (iv) Vespa which detects signature sequence patterns that should be shared among sequences obtained from the same patient or from the same group of clustered sequences (Korber and Myers, 1992).

Sequence analyses for quality control purposes require an understanding of the expected degree of intra- and inter-patient sequence variability and the use of programs that compute the nucleotide distance between sequence pairs. Among untreated individuals in the United States with subtype B virus,

the mean inter-individual nucleotide divergence in protease and RT sequences is about 3%-4% (Figure 6). Among treated individuals harboring viruses with drug-resistance mutations, the mean inter-individual nucleotide divergence is even higher. Among untreated individuals a nucleotide distance of <1% in the protease or <2% in the RT is highly unusual and should prompt closer examination of the sequences for the possibility of laboratory contamination. Among treated individuals harboring viruses with drug-resistance mutations, a nucleotide distance of <2% in the protease or <3% in the RT should prompt closer examination of the sequences.

In geographic regions where HIV-1 has recently been introduced, the mean nucleotide divergence of protease and RT sequences from different individuals may be low. In cases such as these it may be necessary to use additional approaches to exclude cross-contamination. These additional methods include using Vespa to detect signature mutations that segregate sequences into clusters (Korber and Myers, 1992) and the creation of trees using synonymous nucleotide distances. Synonymous nucleotide distances are likely to diverge at a higher rate than nonsynonymous nucleotides. Because mixtures are so common in sequences of isolates from treated persons, it is essential to know how ambiguous nucleotides are handled by programs used for distance analyses.

Analysis of synonymous and nonsynonymous nucleotide changes provides insight into the extent to which sequence changes are neutral or caused by evolutionary selection. The two most useful programs for estimating synonymous and nonsynonymous nucleotide distances include the SNAP (Los Alamos Sequence Database, (Kuiken *et al*., 1999)) and MEGA (Nei and Gojobori, 1986).

In the Stanford University Hospital Diagnostic Virology Laboratory, a sequence management program compares new sequences to each of the sequences generated within the preceding two months and to all of the previous sequences of viruses from the same person. Each report is accompanied by an internal report containing a histogram showing the nucleotide distances between the new sequence and recent sequences. This allows laboratory contamination to be excluded each time a new sequence is completed. To detect sample mix-up, sequences from the same individual that differ by more than 3% are flagged and followed by a position by position summary of the differences between current and previous sequences showing which differences are at drug resistance positions and which differences are partial sequence differences caused by mixtures.

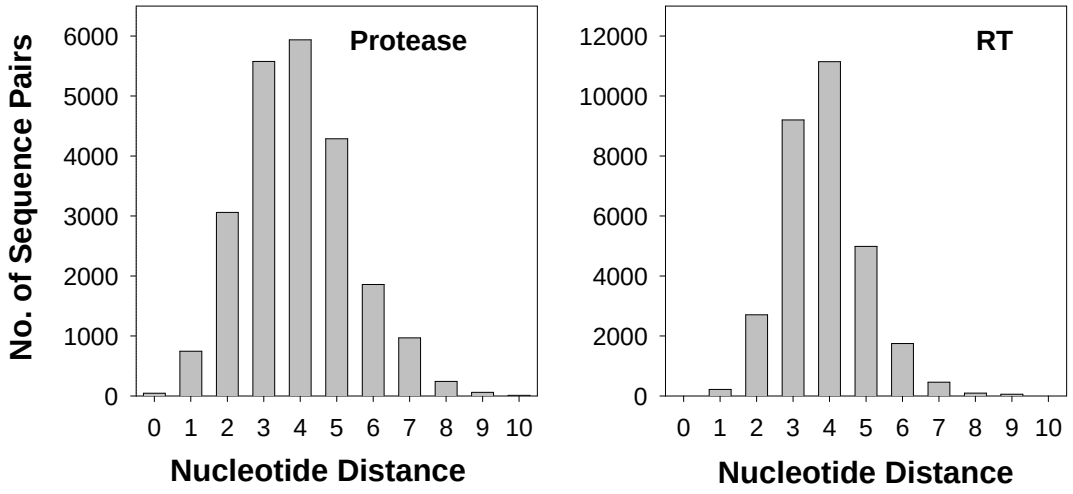

Figure 6. Distribution of pairwise nucleotide distances between subtype B isolates from untreated persons. The figure with protease sequences contains approximately 180 sequences obtained between 1985–1998 and has been adapted from Figure 2 in (Shafer *et al*., 1999b). The figure with RT sequences contains approximately 250 sequences obtained between 1985–2000 and has been taken from data in the HIV RT and Protease Sequence Database through April 2001 (Kantor *et al*., 2001).

E. Sequence analysis of non-B subtypes

It might be expected that the functional constraints on the protease and RT enzymes would prevent their genes from displaying much inter-subtype variation. But several groups have shown that the protease and RT genes of global isolates are sufficiently different from one another to allow subtype grouping (Becker-Pergola *et al*., 2000a; Gonzales *et al*., 2001; Pieniazek *et al*., 2000; Vergne *et al*., 2000; Yahi *et al*., 2001). Although most inter-subtype variation is caused by synonymous nucleotide substitutions, there are subtype-specific amino acid patterns (Becker-Pergola *et al*., 2000a; Cornelissen *et al*., 1997; Gonzales *et al*., 2001; Pieniazek *et al*., 2000; Vergne *et al*., 2000).

In North America and Europe, most HIV-1 isolates belong to subtype B. Subtype B, however, accounts for only a small proportion of HIV-1 isolates worldwide and non-B isolates have been identified with increased frequency, particularly in Europe. The available anti-HIV drugs have been developed by drug screening and susceptibility testing using subtype B isolates, and drug resistance mutations have been identified and characterized almost entirely in subtype B isolates.

A few studies have tested the *in vitro* susceptibility of non-B HIV-1 isolates to antiretroviral drugs. Although Group O isolates often demonstrate intrinsic resistance to the NNRTIs (Descamps *et al*., 1997; Quinones-Mateu *et al*., 1997), most studies have shown that non-B Group M isolates are as susceptible as subtype B isolates to each of the three anti-HIV drug classes (Palmer *et al*., 1998; Shafer *et al*., 1999a; Shafer *et al*., 1997; Tanuri *et al*., 1999). Nonetheless, the number of isolates tested in these studies are too few for these studies to be conclusive. Moreover, subtype G isolates with reduced susceptibility to protease inhibitors (Descamps *et al*., 1998b) and subtype F isolates with reduced susceptibility to some NNRTIs have been described (Apetrei *et al*., 1998).

Protease inter-subtype variation is more likely than RT inter-subtype variation to occur at positions associated with drug resistance. A protease substrate cleft mutation, V82I occurs commonly in subtype G isolates. A few isolates with V82I have been studied *in vitro* and found to have low-level (<5-fold) protease inhibitor resistance (Brown *et al*., 2001; Descamps *et al*., 1998b; King *et al*., 1995) but there is no evidence that V82I has the same clinical significance as other mutations at this position such as V82A/T/F/S (Table 2). Among HIV-1 isolates from untreated persons, resistance-associated variants at positions 10, 20, and 36 occur more commonly in non-B isolates, whereas resistance-associated variants at positions 63, 77, and 93 occur more commonly in subtype B isolates (Gonzales *et al*., 2001).

There is no evidence for novel drug resistance mutations in non-B HIV-1 isolates and it is generally assumed that drug-resistance mutations described in the context of subtype B isolates will exert the same phenotypic effects in all HIV-1 subtypes. In a few reports, mutations characterized in subtype B HIV-1 were also observed in non-B HIV-1 following exposure to antiretroviral drugs (Caride *et al*., 2000; Eshleman *et al*., 2001; Yahi *et al*., 2001; Weidle *et al*., 2001; Adje *et al*., 2001). A study comparing the genotypes and phenotypes of >30 non-subtype B samples found no evidence for natural resistance to current anti-HIV drugs for subtypes A-H (de Bethune *et al*., 1999). Furthermore, in that study, resistance mutations detected in samples from patients with prior antiretroviral treatment were consistent with treatment history and did not differ among subtypes (de Bethune *et al*., 1999).

Inter-subtype genetic variability may complicate HIV-1 genotyping because primers used for reverse transcription, PCR, and sequencing may have a lower rate of annealing to non-B compared with subtype B templates. But the extent to which this occurs has not been studied. Several studies analyzing non-B HIV-1 are listed in Table 8. Primers from these studies may be more useful for sequencing non-B isolates than the primers typically used for sequencing subtype B isolates. Both the Applied Biosystems ViroSeq™ HIV-1 Genotyping System (Dileanis *et al*., 2000a; Eshleman *et al*., 2001; Fontane *et al*., 2000; Marlowe *et al*., 2000) and the Visible Genetics TRUGENE™ HIV-1 Genotyping System (Fontane *et al*., 2000; Lambert *et al*., 2000) have been used for the analysis of some non-B subtypes but the primers used in both commercial systems are proprietary.

Table 8. Non-Subtype B HIV-1 Protease and Reverse Transcriptase Sequencing

Type of study	Reference	Subtypes	Genotyping system
Sequences from untreated individuals	Lambert *et al*., 2000	A-G	TRUGENE™
	Pieniazek *et al*., 2000	A-D, F, H	Home brew
	Becker-Pergola *et al*., 2000a	A, D	PE Biosystems HIV-1 Genotyping System (version 1)
	Cornelissen *et al*., 1997	A-E	Home brew
	Vergne *et al*., 2000	A-D, F, G, J, K, group O	Home brew
Drug susceptibilities of isolates from untreated individuals	Shafer *et al*., 1999a; Shafer *et al*., 1997	C	Home brew
	Palmer *et al*., 1998	A-E	No genotyping
	Descamps *et al*., 1998a	A-H	Not stated
	Tanuri *et al*., 1999	F	Home brew
	Apetrei *et al*., 1998	F	Home brew
	de Bethune *et al*., 1999	A-H	Virco
	Descamps *et al*., 1997	Group O	Home brew
Sequences from treated individuals	Descamps *et al*., 1997	Group O	No genotyping
	de Bethune *et al*., 1999	A-H	Virco
	Eshleman *et al*., 2001	A, D	ViroSeq™
	Ushiro-Lamb *et al*., 2000	A-D, F	TRUGENE™
	Caride *et al*., 2000	A, F	Home brew
	Quinones-Mateu *et al*., 1998	Group O	Home brew
	Yahi *et al*., 2001	A, A-G, C, D, F	ViroSeq™
	Weidle *et al*., 2001	A, C, D	Home brew
Assay performance	Fontane *et al*., 2000	A-J	TRUGENE™, ViroSeq™, Home brew
	Marlowe *et al*., 2000	A-G	ViroSeq™
	Vahey *et al*., 1999	A-F	Affymetrix GeneChip™ HIV PRT 440

V Drug resistance interpretation

A. General approach

The first step in drug resistance interpretation is to generate a list of amino acid differences between the sample sequence and a wildtype reference sequence (usually, the subtype B consensus sequence). The second step is to use the list of mutations to infer drug susceptibility and likelihood of response to specific anti-HIV drugs. Genotypic interpretation is independent of the process of genotyping. Therefore, laboratories doing HIV-1 genotyping can provide physicians with the option of receiving a file

with the sequence data because such data can then be analyzed by interpretation systems other than those used by the sequencing laboratory.

HIV-1 drug resistance is rarely an all-or-none phenomenon and clinicians usually need the answers to the following two questions: (i) Does the genotype suggest that the patient will respond to a drug in a manner comparable to a patient with a wildtype isolate? (ii) Does the genotype suggest that the patient will obtain any antiviral benefit from the drug? The second question distinguishes HIV-1 susceptibility testing from anti-bacterial susceptibility testing. In the case of bacteria, it is usually possible to avoid using any drug with reduced susceptibility against a pathogen. It is also this second question that makes it necessary to include at least two and preferably three levels of resistance (e.g. low-level, intermediate, high-level).

Using HIV-1 sequences to infer drug susceptibility and likelihood of response to therapy is ideally performed by a computerized expert system because few clinicians can possibly remember all of what is known about each drug-resistance mutation. Table 9 contains several of the most commonly used systems for HIV-1 genotypic interpretation. During the next year, these algorithms will evolve and most likely converge through an ongoing process of inter-algorithm comparison and validation using clinical data sets. It is unlikely that algorithms will remain proprietary because there is no precedent for basing important medical decisions on proprietary unpublished data.

An expert system performs reasoning over representations of human knowledge. It consists of a knowledge base and an inference engine. A computer-readable knowledge base will benefit patients, clinicians, and researchers because it is the first step towards creating an expert system and because it will identify gaps in what is known about drug-resistance mutations.

B. Knowledge base for drug resistance interpretation

How do we know what we know about drug-resistance mutations? The initial study of HIV-1 drug resistance typically involves culturing a wildtype HIV-1 isolate in the presence of increasing concentrations of the drug being studied, identifying mutations that allow the virus to continue to replicate, and confirming the phenotypic effect of these mutations in site-directed mutagenesis experiments (Table 10, Source A). While this process is the most rigorous means of demonstrating that a particular mutation confers drug resistance, it has two limitations. First, these studies identify mutations that are sufficient to cause resistance to the drug under evaluation without proving that these mutations are necessary for the development of drug resistance. Second, data from these studies are generally derived from isolates with only one or two drug-resistance mutations and rarely reflect the more complicated patterns of mutations observed in clinical isolates from patients receiving drug combinations.

Three additional types of data must be taken into account before HIV genotyping can be used to the best advantage of patients. These data include correlations between genotype and phenotype of clinical HIV-1 isolates (Table 10, Source B), correlations between genotype and treatment history (Table 10, Source C), and correlations between genotype and clinical outcome (Table 10, Source D).

Data correlating genotype and phenotype of clinical isolates indicate the phenotypic effect of mutations in the genetic context in which they usually occur. Data correlating genotype and treatment history directly identify the mutations the virus used to escape anti-HIV drug suppression *in vivo*. Those data are particularly important for elucidating the mechanisms of resistance to drugs that are difficult to study *in vitro*. Data correlating genotype and the subsequent clinical outcome of patients who receive new treatment regimens identify which drugs are most likely to be effective in patients with specific resistance mutations.

Data correlating genotype with clinical outcome are the most relevant for clinical management of patients with HIV-1 infection. Such data, however, are available for a limited number of clinical scenarios, making it necessary for clinicians to rely on the other three types of available data (Table 10, Sources A–C) for clinical decision making.

Table 9. Algorithms for Interpreting HIV-1 Protease and RT Sequences

Algorithm	Availability	Description
Resistance Collaborative Group (RCG) (DeGruttola *et al*., 2000)	Public	Table of rules developed for a standardized re-analysis of eight published studies linking drug resistance mutations and clinical outcome. Not intended for clinical use.
HIV RT and Protease Sequence Database (Shafer *et al*., 2000a)	Public	Method used at Stanford University Hospital Diagnostic Virology Laboratory. Mutations are assigned drug penalties. Drug penalties are added and drugs are assigned an inferred level of resistance. Drug penalties are hyperlinked to primary data linking mutation and drug. Program can be found at http://hivdb.stanford.edu.
French National Agency for AIDS Research (ANRS) (Rousseau *et al*., 2001)	Public	Table of rules listing mutations conferring genotypic resistance or possible genotypic resistance to anti-HIV drugs.
Retrogram™ (Virology Networks)	Proprietary	Drug-based rules. Updated regularly by an expert panel
GuideLines™ (Visible Genetics)	Proprietary	Drug-based rules. Updated regularly by an expert panel.
VirtualPhenotype™ (Virco; Mechelin, Belgium) (Verbiest, *et al*., 2000)	Proprietary	Pattern matching algorithm that uses a large genotype-phenotype correlative database to infer phenotypic properties based on sequence data..

Table 10. Sources of Data on HIV Drug Resistance Mutations

Source A. Correlation between genotype and phenotype based on laboratory isolates

The pre-clinical evaluation of a new drug often involves culturing a wildtype laboratory HIV-1 isolate in the presence of increasing drug concentrations, and identifying mutations that allow the virus to continue to replicate. Site-directed mutagenesis experiments are done to confirm that the mutations arising during virus passage in the presence of the drug confer drug resistance when introduced into a wildtype virus. Drug resistance mutations identified by this process acquire widespread acceptance, are referred to as "canonical" resistance mutations, and are often considered the predominant mutations responsible for resistance to the drug under evaluation.

Source B. Correlation between genotype and phenotype based on clinical isolates

Laboratory isolates often contain only one or two drug-resistance mutations and rarely reflect the more complicated patterns of mutations observed in clinical isolates from patients receiving combination drug therapy. The complexity of sequences obtained on clinical isolates often precludes site-directed mutagenesis. Instead statistical associations between drug resistance mutations and *in vitro* resistance are required to elucidate the role of specific mutations or mutation patterns in causing drug resistance.

Source C. Correlation between genotype and treatment history

Sequences of HIV-1 isolates from patients failing antiretroviral therapy are crucial observations of HIV evolution that show which virus mutations are most significant *in vivo*. Such data are also essential for elucidating the genetic mechanisms of resistance to drugs that are difficult to test *in vitro*.

Source D. Correlation between genotype and clinical outcome

Data correlating genotype and clinical response to subsequent antiretroviral therapy are the most clinically relevant and the most useful for clinicians who must select anti-HIV drugs for their patients. However, data of this type generally lags several years behind data from sources A–C.

C. Stanford University HIV RT and Protease Sequence Database

The HIV RT and Protease Sequence Database at Stanford University is an online database that contains published data on the first three types of data described above (Table 10, Sources A–C)). Efforts are underway to include data from studies that correlate genotype with clinical outcome (Kantor *et al*., 2001). As of May, 2001, the database contained 18,000 protease and RT sequences from about 3,500 individuals, as well as about 5,000 phenotypes on >1,000 HIV isolates. Users can retrieve sequence sets based on criteria such as previous drug treatment or presence of specific mutations. Users can also retrieve *in vitro* drug susceptibility results on isolates containing specific mutations.

The database website contains two sequence analysis program. The first program, HIV-SEQ, accepts user-submitted RT and protease sequences, compares them to a reference sequence, and uses the differences (mutations) as query parameters for interrogating the sequence database (Shafer *et al*., 2000a). HIV-SEQ allows users to examine new sequences in the context of previously published sequence data which provides two main advantages. Unusual sequence results can be detected allowing the person sequencing the gene to recheck the primary sequence output while it is still on the desktop. Unexpected associations between sequences or isolates can be discovered because the person analyzing a new sequence can immediately retrieve data on isolates sharing one or more mutations with the sequence.

The second program, a drug resistance interpretation program, accepts user-submitted protease and RT sequences and returns inferred levels of resistance to the 15 FDA-approved anti-HIV drugs. Each drug resistance mutation is assigned a drug penalty score; the total score for a drug is derived by adding the scores of each mutation associated with resistance to that drug. Using the total drug score, the program reports one of the following levels of inferred drug resistance: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, high-level resistance. The genotypic interpretation is based on clinical, as well as, phenotypic data.

Mutation scores are derived from published literature linking mutations and anti-HIV drugs, including correlations between genotype and treatment history, genotype and phenotype, and genotype and clinical outcome (Table 10). The program usually assigns the same score to a mutation regardless of whether the mutation is present in pure form or as a mixture. Mutations that cause hypersusceptibility to a drug have a negative drug penalty score. The program generates a score sheet listing each of the drug penalty scores. A listing of all mutation / drug score pairs can be found with the program's release notes (http://hiv-4.stanford.edu/cgi-test/hivtest-web.pl). Clicking on a drug penalty score provides a link to the primary data linking the mutation and drug. In addition to drug-penalty scores, the drug-resistance program generates a list of comments pertinent to the submitted sequence. Some of the comments are standard comments for specific mutations. Other comments are based on known interactions between combinations of mutations present in the sequence.

D. VirtualPhenotype™

Virco (Cambridge, UK and Mechelin, Belgium) uses a genotypic-phenotypic correlative data base to infer phenotypic properties based on sequence data. By performing both genotyping and phenotyping on >18,000 samples, they have generated a large genotype-phenotype database. The VirtualPhenotype™ is an interpretation system that uses the mutations within a sequence as parameters for searching the database for drug susceptibility data on isolates containing matching mutations (Verbiest *et al*., 2000). Over 60 unique amino acid positions are searched. The analysis includes a tabulation of the number of matches in the data base for each drug, and the distribution of phenotypes (fold increase in IC50) for the matching samples. The mean IC50 of the matching samples is interpreted using a biologically defined, drug-specific cut-off value, providing a quantitative prediction of drug resistance. The VirtualPhenotype™ has been shown to have a high correlation with results from Virco's recombinant phenotypic assay, and to be an independent predictor of clinical response to antiretroviral therapy (Graham *et al*., 2001). The main limitations of the VirtualPhenotype™ are that it is based on a proprietary database and that it is entirely dependent on phenotypic data and cannot utilize data from clinical studies (Table 10, sources C and D).

Appendix. Web Resources for HIV Drug Resistance Testing in Clinical and Research Settings

	Web address	Features
Drug resistance data		
Los Alamos National Laboratory HIV Sequence Database	http://hiv-web.lanl.gov	Searchable HIV-related data bases including a database of drug-resistance mutations.
Stanford HIV RT and Protease Sequence Database	http://hivdb.stanford.edu/hiv	A comprehensive database of HIV RT and protease sequences linked to treatment data and phenotypic drug susceptibility data
International AIDS Society-USA	http://hivinsite.ucsf.edu/	Contains the most recently published guide lines (Hirsch *et al*., 2000) along with simplified diagrams of key drug resistance mutations.
Sequence analysis programs		
PHYLIP	http://evolution.genetics.washington.edu/phylip.html	PHYLIP is a free, comprehensive package of programs for phylogenetic analysis of sequence data. This site also a comprehen sive set of links to other sequence analysis tools.
IUBIO archive	http://iubio.bio.indiana.edu/soft/molbio/	A comprehensive set of links to sequence analysis programs.
Los Alamos National Laboratory HIV Sequence Database	http://hiv-web.lanl.gov	Programs for making alignments and phylogenetic trees, estimating synonymous/nonsynonymous substitutions, detecting signature mutations and inter-subtype recombination.
Stanford HIV RT and Protease Sequence Database	http://hivdb.stanford.edu/hiv (link to http://hiv-4.stanford.edu/cgi-bin/hivseq-web.pl and http://hiv-4.stanford.edu/cgi-test/hivtest-web.pl)	Two programs including HIV-SEQ and a drug resistance interpretation program. The release notes for HIV-SEQ contain the consensus B protease and RT sequences.
BLAST	http://ncbi.nlm.nih.gov	Nucleotide and amino acid sequence similarity search
TreeView	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html	Viewing and manipulating tree files
Suppliers of systems for HIV sequence analysis		
Applied Biosystems	http://www.appliedbiosystems.com	ViroSeqTM HIV-1 Genotyping System, reagents and equipment for home-brew assays
Visible Genetics	http://www.visgen.com	TRUGENE TM HIV-1 Genotyping Kit, TRUPREPTM RNA isolation kit
Innogenetics	http://www.innogenetics.com	INNO-LiPA(R) HIV-1 line probe assays
Affymetrix	http://www.affymetrix.com	Affymetrix GeneChip(R) Systems
Companies that provide phenotypic assays		
Virco, Inc.	http://www.vircolab.com	Services include the AntivirogramTM phenotyping assay, VirtualPhenotypeTM, and GENChecTM genotyping assay
ViroLogic	http://www.virologic.com	Services include the PhenoSenseTM phenotyping assay

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