Diagnostics

4

4.1 Virological diagnostics

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The diagnosis of HIV infection is made by the detection of circulating antibodies to HIV. Antibodies are identified by the use of a screening test, usually an enzyme-linked immunosorbent assay (ELISA), followed by definitive diagnosis using a Western Blot assay. In some situations, such as pre-seroconversion or neonatal infection, measurement of HIV antibodies may be unreliable. In these cases, diagnosis of infection may use direct detection of HIV itself – whether by quantification of plasma HIV RNA, HIV viral DNA or HIV antigen (viral protein), or by detection and amplification of virus in a tissue culture. Techniques that quantify viral nucleic acids are most frequently used.

4.1.1 HIV antibody testing

HIV enzyme-linked immunosorbent assay

Because of its rapidity, sensitivity and low cost, the ELISA (also known as the enzyme immunoassay, or EIA) is the standard screening tool for HIV infection.[1] Recombinant or native HIV antigens, fixed in a solid phase, are exposed to and bound by HIV antibodies in test serum. The presence of these antibodies is then detected by a second anti-human antibody, with a sensitivity of more than 99.5%. Most commercially available ELISA kits contain antigens from both HIV-1 and HIV-2 and are therefore able to detect infection with either of these viruses. The ELISA also detects antibodies against a broad range of HIV types and subtypes, and is continually updated to include newly described strains.

ELISA results are generally scored as positive (highly reactive), negative (non-reactive) or indeterminate (partially reactive). The HIV antibodies detected by the ELISA (and the Western Blot assay) appear before neutralising antibodies, which are observed after the start of the decline in plasma viraemia. A positive ELISA value is usually observed within three to six weeks following infection. Very rarely, antibodies may develop up to 12 weeks after infection, or an individual may be unable to produce HIV antibodies, such as in agammaglobulinaemia.

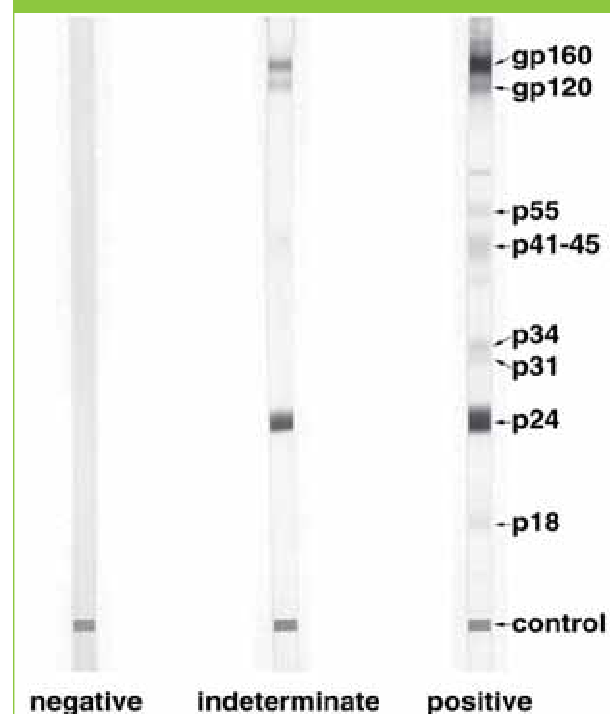
The weeks between infection and seropositivity are termed the window period and are associated with high levels of circulating HIV, and potentially more efficient transmission. Commercial fourth-generation screening assays, which combine antigen and antibody screening may reduce this window period to six days.[2]

False-positive test results are rare and the specificity of the ELISA is above 99.8%. Factors associated with false-positive ELISA results include antibodies to human leukocyte antigen (HLA) class II antigens, other autoantibodies, hepatic disease, recent influenza vaccination and acute viral infections, as well as laboratory errors of procedure or specimen handling. Thus, although a negative ELISA reult, repeated at three months, effectively rules out a diagnosis of HIV infection, a positive or indeterminate ELISA result is not indicative of infection unless confirmed by a positive Western Blot assay.

Western Blot assay

The Western Blot assay detects antibodies in patient sera that react with a number of different viral proteins. These viral proteins are separated into bands of distinct molecular weight using protein gel electrophoresis. After transfer (blotting) to

Figure 4.1 Typical appearance of an evolving HIV-1 Western Blot in HIV infection



Note: Serial strip blots exposed to serum from the same patient over time show the stepwise appearance of reactivity to the major HIV antigens.

Source: Collated specimens provided by Alan Breschkin, Victorian Infectious Diseases Reference Laboratory, VIC. Used with permission.

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a solid material, proteins that are reactive with specific HIV antibodies in test sera can be identified. Antibodies to different structural or functional HIV proteins appear in a defined order. First, antibodies to the structural gag proteins, the precursor (p55), p24 and p17, appear. These are followed by antibodies to the envelope glycoproteins – the precursor gp160, the extracellular gp120, the intracellular gp41 – and then the polymerase components p31, p51 and p66. Antibodies to the smaller regulatory and accessory HIV proteins encoded by Vpr, Vpu, Vif, Rev, Tat and Nef may also be seen.

A Western Blot result is judged to be negative if there is no reaction of the patient's serum with any protein bands at the molecular weights corresponding to these HIV gene products. A patient with a positive or indeterminate ELISA result and a subsequent negative Western Blot result, is concluded to have had a false-positive ELISA result. A positive Western Blot result is defined by the detection of antibodies to all of the three main groups of HIV proteins – envelope (gp160, gp120 or gp41), gag (p24) and polymerase (p66 or p51).

Patterns of reactivity that fall between positive and negative are termed indeterminate (Figure 4.1). Indeterminate Western Blots assays are most commonly caused by the presence of unrelated antibodies that are cross-reactive with HIV proteins – most commonly with p24 and p55. One or more bands on a Western Blot assay are seen in 20% to 30% of individuals with no HIV infection, making the Western Blot assay a poor screening test in comparison with the ELISA. It is possible, although less likely, that an indeterminate Western Blot result is the result of early HIV infection and incomplete evolution of the anti-HIV immune response. This possibility is more likely if there is a high index of clinical suspicion of primary HIV infection. An indeterminate Western Blot assay should be followed up by a repeat Western Blot after four to six months, to exclude an evolving pattern. Additionally, tests for the presence of viral nucleic acid or protein may be used, especially when the index of suspicion is high. A positive result in one or more such assays leads to a presumptive diagnosis of HIV infection, pending the development of a positive Western Blot. Negative results from one or more of these assays and a non-evolving indeterminate blot exclude HIV infection.

HIV-2 infection is rare in Australia and most sera from people with HIV infection will cross-react with HIV-1 Western Blots assays. Specific Western Blots assays can be used to confirm HIV-2 infection and distinguish it from HIV-1.

4.1.2 HIV quantification

HIV RNA quantification (or viral load) is a critical tool in the management of HIV disease. Detection of HIV RNA can help provide a positive diagnosis of HIV infection in certain clinical situations, such as acute or neonatal disease, where standard serological testing is inappropriate or unclear. Quantification of HIV RNA levels allows and predicts the rate of HIV disease progression[3,4] and is the major laboratory tool for monitoring response to antiretroviral therapy.

Nucleic acid quantification

In Australia, there are several assays that are routinely used to quantify the levels of HIV RNA circulating in plasma:

- Reverse transcriptase polymerase chain reaction (RT-PCR) assay
- Roche COBAS™ Amplicor
- Bayer branched-DNA (bDNA) assay, called VERSANT™
- BioMerieux nucleic acid sequence-based amplification (NASBA) technology based assay, called NucliSENS™.

All tests use different technologies but have now achieved a sensitivity capable of detecting 40-400 copies of HIV RNA per mL of plasma. Enhanced sensitivity of detection is achieved by the use of a pre-concentration step in which plasma undergoes ultracentrifugation to precipitate the viral particles.[5,6] In acute HIV infection, plasma HIV RNA levels may be used to confirm the acute retroviral syndrome before the appearance of HIV antibodies reactive with an ELISA or Western Blot assay. Most true-positive results will show very high levels (10^5 to 10^6 copies/mL) of circulating HIV RNA, consistent with uncontrolled primary viraemia. False-positive results occur in less than 10% of cases; these usually involve low RNA levels (less than 10^4 copies/mL) and are not reproducible.[5,7] See chapter 12 for discussion of laboratory diagnosis of primary HIV infection.

(a) Reverse transcriptase polymerase chain reaction (RT-PCR) assay

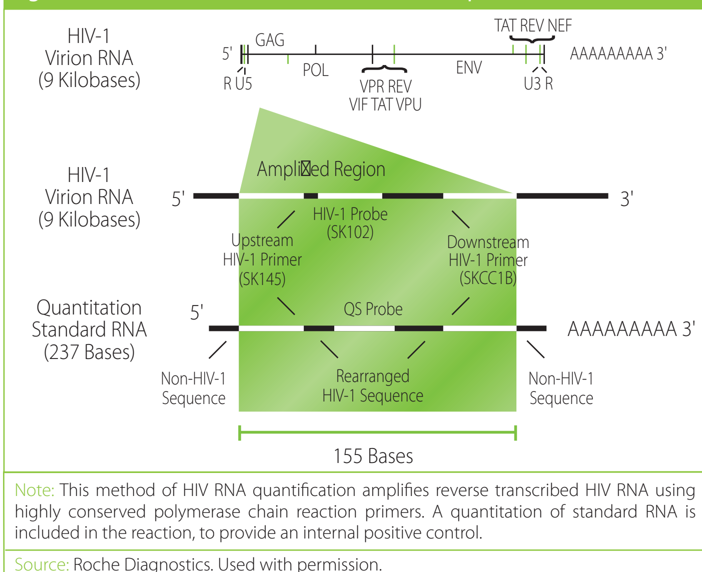
RT-PCR incorporates purification and reverse-transcription of the HIV RNA genome, followed by quantitative PCR using highly conserved primer pairs (Figure 4.2). The bDNA assay captures HIV RNA in the solid phase and signal amplification is produced during successive rounds of hybridisation with multiple oligonucleotides (Figure 4.3). The NucliSENS assay is based on target amplification using NASBA technology. None of these tests has been shown to be reliable in the quantification of subtypes N or O or HIV-2.[8]

Across multiple subtypes, the Roche COBAS™ Amplicor version 1.5 gives a median value of 0.22 $\log_{10}$ higher than VERSANT™ version 3.0. Significantly discordant results that are not explained by HIV-subtype differences are detected between the two assays in 25.7% of cases.[9]

(b) Real time PCR assay

Conventional nucleic acid based quantification methods are being replaced in some laboratories with one of a number of real time PCR assays that have become available. The Roche COBAS™TaqMan™ HIV-1 test combines automated sample preparation for HIV-1 RNA purification using the AmpliPrep, and real-time PCR amplification and detection using the COBAS™

Figure 4.2 The RT-PCR method of HIV RNA quantification



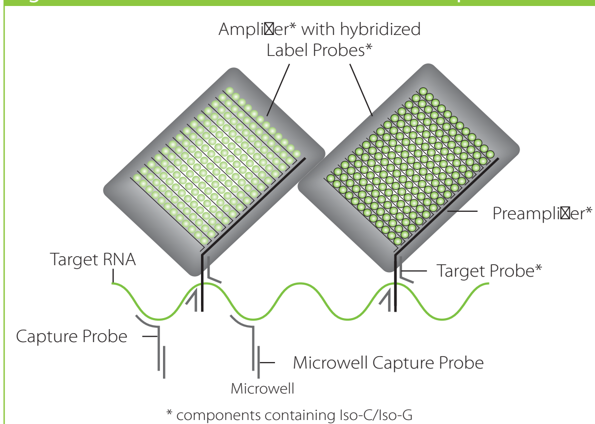
Note: This method of HIV RNA quantification amplifies reverse transcribed HIV RNA using highly conserved polymerase chain reaction primers. A quantitation of standard RNA is included in the reaction, to provide an internal positive control.

Source: Roche Diagnostics. Used with permission.

TaqMan™ or the COBAS™ TaqMan™ 48 analyser. Similar to the Roche COBAS™ Amplicor test, this assay targets a conserved region in HIV-1 gag but uses fluorescently-labelled probes to detect the amplified products in real time. The advantages are a wider dynamic range and greater sensitivity.[10]

The Abbott Real*Time* HIV-1 assay, for use on the m2000 system, utilises a unique partially double-stranded probe that targets the HIV-1 pol. The probe strands are labelled with a fluorophore (reporter) at the 5' end, and a quencher moiety at the 3' end of the shorter, complementary strand. In the presence of target pol sequences, the reporter probe preferentially binds, and

Figure 4.3 The bDNA method of HIV RNA quantification



Note: This method of HIV RNA quantification amplifies multiple oligonucleotide target probes that hybridise with target RNA.

Source: Bayer Diagnostics. Used with permission

upon release of the shorter quencher probe, fluoresces. Assay performance characteristics from Abbott Laboratories show that the assay has a 5-$\log_{10}$ linear range, assay specificity of 100% (n=259), 95% probability of detecting samples with a viral load of 25 copies/mL, and recognition of subtype panels from group M (A-H), group O, and group N.[11,12,]

(c) NASBA

The NucliSENS™ HIV-1 Easy Q assay (bioMerieux) is based on target amplification using NASBA technology and measures the amount of nucleic acid directly by electrochemilum inescence. The assay is sensitive, with a 4 $\log_{10}$ dynamic range (176 to 3 470 000 copies/mL), and shows excellent correlation with the Amplicor HIV-1 Monitor (Standard and Ultrasensitive) tests using subgroup B viruses.[13,14]

(d) Ultrasensitive reverse transcriptase assays

The Cavidi ExaVir Load assay (Cavidi Tech AB) is a low cost, viral load assay that quantifies HIV-1 viral load by measuring the levels of HIV-1 reverse transcriptase in the plasma and converting the results to a value expressed as copies per mL. As it measures RT activity it is able to quantify all subtypes of HIV-1 and HIV-2. Results from the ExaVir assay have been found in a number of laboratories to correlate positively with those from the Amplicor (R=0.84 -0.99)[15,16] Although this assay has been developed primarily for resource limited settings, it is also being investigated as an alternative viral load assay for use in Australia and other developed countries.[17]

The variability in HIV RNA quantification observed between laboratories and between assays dictates that, where possible, all assays of an individual's viral load should be sent to the same laboratory which uses a consistent assay.[18]

(e) HIV DNA PCR

The HIV DNA assay detects both unintegrated and integrated forms of HIV DNA present in circulating peripheral blood mononuclear cells. The PCR primers are commercially available from Roche Laboratories. The main clinical use of this assay is in the qualitative detection of HIV in situations where serological testing is inappropriate (e.g. pre-seroconversion, or neonatal infection where results may be confounded by the presence of maternal antibodies) or where serological testing has been disputed or inconclusive (e.g. indeterminate Western Blot assay result). The HIV DNA PCR test is highly sensitive (greater than 99%, detecting one copy of HIV DNA per 10 000 to 100 000 cells) and specific (98%), but has not replaced serology either as a screening test or as a diagnostic test in isolation. The accuracy of this test for HIV of non-B subtypes has not been determined.

HIV DNA PCR and HIV RNA RT-PCR techniques are commonly used in laboratory research. Amplification may be performed on various genomic regions of interest, and the products used in such analyses as HIV subtype determination, phylogenetic analysis, detection of genetic mutations, and prediction of viral tropism and drug resistance.[19,20] In general, these techniques are experimental and are not used routinely in patient management. Health care professionals should bear in mind that such techniques are available and may help in the diagnosis or treatment of unusual cases. Consideration should be given to discussing individual cases with a laboratory research professional.

HIV antigen testing

An ultrasensitive HIV p24 assay has been developed which directly detects and quantifies HIV p24 antigen. While initially examined for utility as an assay to monitor viral load,[21,22] it is no longer considered to be appropriate for viral load testing due to a lack of sensitivity.[23,24] The p24 assay, however, has been favourably evaluated for use as a paediatric HIV-1 diagnostic tool in resource limited settings.[25]

HIV tissue culture

Tissue culture allows HIV to be expanded and propagated *in vitro* by its culture with donor peripheral blood mononuclear cells (PBMC) in the presence of stimulatory factors such as interleukin 2 (IL-2). Coculturing of patient PBMC or cells from other body compartments suspected to be infected with HIV with stimulated donor PBMC is an alternate method for the detection of very low levels of virus. Culture supernatants are assayed for the presence of p24 or reverse transcriptase, which generally appear over one to two weeks. The efficiency of the process falls dramatically with lower viral load, although it can be improved by using techniques such as the removal of inhibitory CD8 cells from the patient and donor peripheral blood mononuclear cells, stimulation with phytohaemagglutinin or IL-2, or exposure to ultraviolet (UV) radiation. The tissue culture technique is expensive and time-consuming and must be performed only within a laboratory with appropriately certified containment. For viral detection and isolation in clinical situations, tissue culture has now been replaced by the methods outlined above and is never performed as a routine test. However, it remains a mainstay of laboratory practice, where it is used for the maintenance and analysis of viral strains.

Table 4.1 HIV antiretroviral therapy resistance mutations

1. Mutations in the HIV reverse transcriptase gene that confer drug resistance

a. NRTIs

	Thymidine Analog (TAMs)						NRTI Discriminatory						Multi-NRTI Resistance					
	M41	**D67**	**K70**	**L210**	**T215**	**K219**	**K65**	**K70**	**L74**	**V75**	**Y115**	**M184**	**T69**	**Q151**	**A62**	**V75**	**F77**	**F116**
3TC							R	E		I		VI	Ins	M	V	I	L	Y
FTC							R	E		I		VI	Ins	M	V	I	L	Y
ABC	L	N		W	FY		R	E	VI	TMI	F	VI	InsD	M	V	TMI	I	Y
DDI	L	N		W	FY		R	E	VI	TMI		VI	InsD	M	V	TMI	I	Y
TDF	L	N	R	W	FY		R	E		MI	F		Ins	M	V	MI	I	Y
D4T	L	N	R	W	FY	QE	R			TMI			Ins	M	V	TMI	I	Y
ZDV	L	N	R	W	FY	QE				I			Ins	M	V	I	I	Y

b. NNRTIs

	A98	**L100**	**K101**	**K103**	**V106**	**V108**	**E138**	**V179**	**Y181**	**Y188**	**G190**	**P225**	**F227**	**M230**	**P236**	**K238**
NVP	G	I	EP	NS	AM	I	K	DEF	CIV	LHC	ASE	H	LC	L		NT
DLV	G	I	EP	NS	AM	I	K	DEF	CIV	LHC	E	H	C	L	L	NT
EFV	G	I	EP	NS	AM	I	K	DEF	CIV	LHC	ASE	H	C	L		NT
ETV	G	I	EP	NS	AM		K	DEF	CIV	LHC	ASE	H	C	L		

2. Mutations in the HIV protease gene that confer drug resistance

a. Major PI Resistance Mutations

	D30	**V32**	**V33**	**M46**	**I47**	**G48**	**I50**	**I54**	**L76**	**V82**	**I84**	**N88**	**L90**
ATV/r			F	IL	V	V	L	VALM		AF	V	S	M
DRV/r		I	F		VA		V	LM	V	F	V		M
FPV/r		I	F	IL	VA		V	LM	V	F	V		M
IDV/r		I		IL	V			VALM	V	AFTS	V	S	M
LPV/r		I	F	IL	VA		V	VALM	V	AFTS	V		M
NFV	N		F	IL	V	V		VALM		AFTS	V	DS	M
SQV/r						V		VALM		TF	V	S	M
TPV/r		I	F	IL	V			VA		TFSL	V		M

b. Minor PI resistance mutations

	L10	**V11**	**L23**	**L24**	**K43**	**F53**	**Q58**	**A71**	**G73**	**T74**	**N83**	**L89**	**L89**
ATV/r	IVF							VTI	STCA				
DRV/r	IVF	I						VTI	STCA			V	V
FPV/r	IVF	I						VTI	STCA			V	V
IDV/r	IVF			I		L		VTI	STCA				
LPV/r	IVF			I		L		VTI	STCA				
NFV	IVF		I			L		VTI	STCA			V	V
SQV/r	IVF					L		VTI	STCA				
TPV/r	IVF				T		E	VTI	STCA	P	D	V	V

3. Mutations in the HIV integrase gene that confer resistance

	H51	**T66**	**L74**	**E92**	**F121**	**E138**	**G140**	**S147**	**Q148**	**S153**	**N155**	**E157**	**G163**	**R263**	**R263**
RAL			M	Q		AK	AS		HRK		H	Q	R		
EVG	Y	I		Q	Y	K	S	G	HRK	Y	H	Q		K	K

4. Mutations in the HIV envelope gene that confer resistance

	G36	**I37**	**V38**	**Q40**	**N42**	**N43**	**L44**	**L45**
ENF	DEVS	V	EAMG	H	T	DKS	M	M

NRTIs
3TC = lamivudine
FTC = emtricitabine
ABC = abacavir
DDI = didanosine
TDF = tenofovir
D4T = stavudine
ZDV = zidovudine

NNRTIs
NVP = nevirapine
DLV = delavirdine
EFV = efavirenz
ETV = etravirine

PIs
ATV = atazanavir
DRV = darunavir
FPV = fosamprenavir
IDV = indinavir
LPV = lopinavir
NFV = nelfinavir
SQV = saquinavir
TPV = tipranavir

IIs
RAL = raltegravir
EVG = elvitegravir

FIs
ENF = enfuvirtide

/r = ritonovir

mutations in red are associated with reduced susceptibility
mutations in blue are associated with high levels of phenotypic resistance and/or clinical evidence for reduced susceptibility
mutations in yellow indicate drugs that should be contraindicated when the mutation is present

Source: adapted from Standford University HIV Drug Resistance Database. For details of interpreation please refer to http://hivdb.stanford.edu

4.1.3 Drug resistance testing

HIV shows a high rate of genomic evolution due to the error-prone nature of reverse transcriptase (which introduces random sequence changes into newly produced viral RNA),[26,27] the high rate of viral production, and the rapid turnover of productively infected cells.[28] For these reasons HIV strains rapidly develop resistance to antiretroviral monotherapy through the acquisition of mutations in RT, envelope or protease that confer improved

enzymatic function in the presence of the drug. The principle behind combination antiretroviral therapy (cART) is to achieve a low level of viral replication and thus a reduced opportunity for the introduction of advantageous mutations into newly produced viruses. Strains that have mutated to become resistant to one therapeutic agent may also have abnormally lowered replicative capacity (fitness), making the acquisition of mutations to further agents less likely.

The resistance profile of a viral strain can be estimated by examination of the nucleic acid sequence of the target genes for evidence of known resistance mutations, or directly assessed by tissue culture of the virus (full length or cloned into a common viral backbone) in the presence of a panel of antiretroviral drugs. Treatment may then be altered in the knowledge that a patient is carrying a virus resistant to one or more agents. Studies have demonstrated improved virological outcomes in patients whose therapeutic choices have been guided by the use of resistance testing[29-35] and the use of resistance testing is regarded as standard of care in Australia, western Europe and the USA.[36,37]

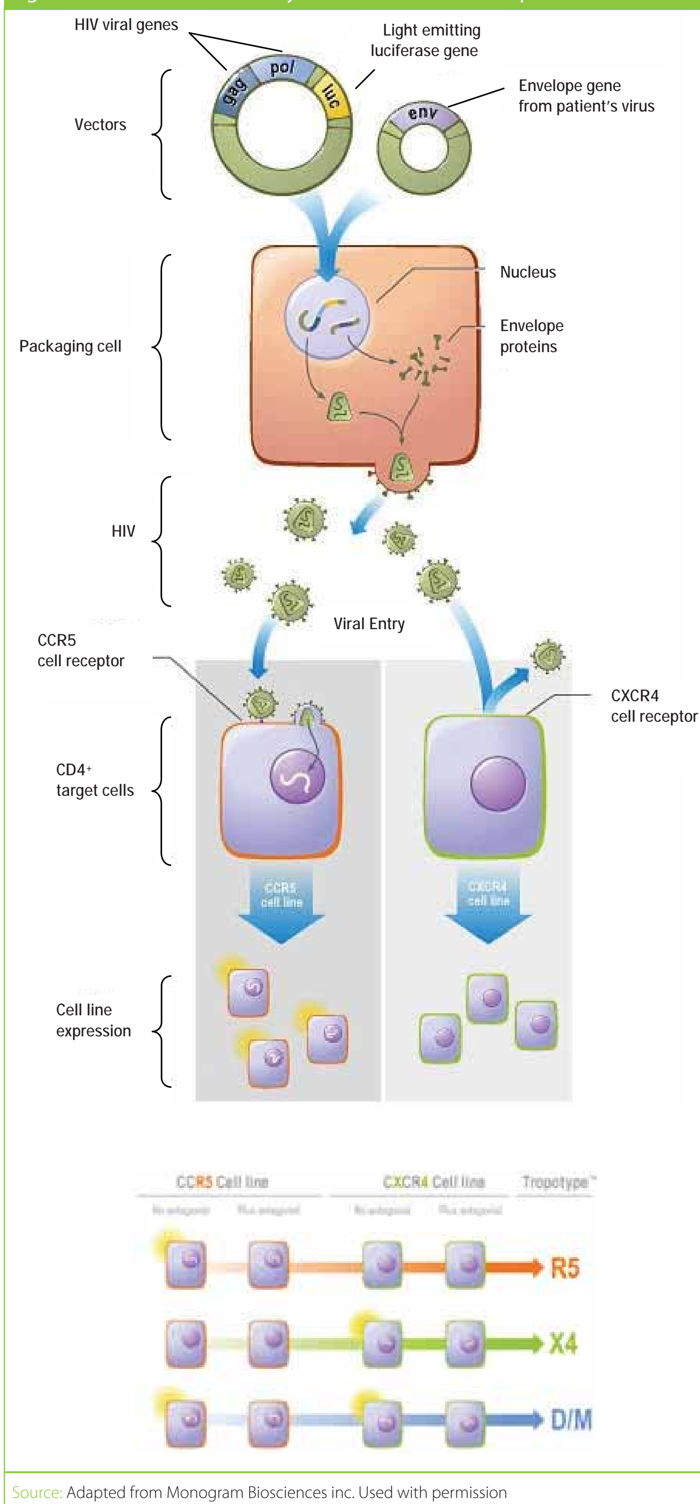
There are three commercially available methods for determining the resistance profile of an HIV strain, although only one, the resistance genotyping assay, is routinely available in Australia.

Resistance genotyping

Assessment of the HIV genotype (i.e. the nucleic acid sequence) involves the isolation of viral RNA followed by the RT-PCR amplification of portions of the target genes. These amplification products are then sequenced using standardised automated DNA sequencing procedures. Ultracentrifugation of a patient plasma samples with a low viral load (1000-2000 copies/mL) will pellet free virus for RNA extraction and sequencing. Test sequences are compared with a wild-type control reference strain (i.e. no prior exposure to antiretroviral agents) for annotating differences in the predicted amino acid sequences. Mutations that have been associated with resistance to antiretroviral therapy are then identified by comparison with a database of known codon (genetic code) mutations. Resistance mutations are usually reported as primary (generally associated with phenotypically detectable drug resistance) or secondary (mutations generated after the primary mutation, and which may confer varying levels of resistance). The nomenclature of mutations is the wild-type amino acid (using the 20 letter amino acid code)/the amino acid position in the protein/the mutant amino acid e.g. for a change from the wild-type methionine residue at position 46 of protease to isoleucine is denoted M46I. Mutations that involve the insertion of amino acids follow similar nomenclature with the additional amino acids following the residue position. Table 4.1 shows HIV resistance mutations to the classes of antiretroviral agents.

Caution must be exercised in the interpretation and use of resistance genotyping data. Two major factors contribute to the complexity of this process. The contribution of minor species and mutational complexity. It is important to realise that genotypic resistance testing gives a snapshot of the dominant viral forms circulating in the plasma at the time of testing. Viral variants with different resistance profiles may circulate at low levels or be present latently in proviral DNA. These may rapidly re-appear and become dominant once the selective pressure of antiretroviral therapy changes. Only minor species that are circulating at substantial levels (above about 20%) may be detected and reported. For this reason, genotypic resistance data must always be interpreted in the light of a thorough history of treatment and previous resistance testing.

Figure 4.4. HIV-1 Trofile™ assay to determine HIV-1 coreceptor use



Source: Adapted from Monogram Biosciences inc. Used with permission

Mutations are associated with drug resistance on the basis of *in vitro* or *in vivo* data. These data are not always clear and the publication of new evidence means the assignment of mutation to phenotype is being continuously updated. The substantial sequence variability between HIV strains means that it can be difficult to distinguish naturally occurring viral variants from selected mutations that confer an evolutionary advantage – particularly if this advantage is small or secondary to the existence of a primary mutation. When in doubt, consultation with the laboratory performing the assay is strongly indicated.

Resistance phenotyping

This test is the gold standard as viral drug resistance is tested directly *in vitro*. However, it remains expensive and time-consuming, despite recent advances. Two commercial versions, of similar accuracy (the Antivirogram™ assay (virco and the PhenoSense™ assay[Monogram Inc.]) offer testing of recombinant viruses generated from patient samples. These offer the advantage of direct measurement of drug susceptibility, without necessitating biological isolation of the virus. These phenotypic tests are not commercially available in Australia.

Virtual phenotyping

This test is provided commercially by the company virco and involves comparison of viral RT, protease and envelope sequences with an extensive proprietary database of sequences from viral strains for which the phenotype has been formally derived. The virco virtual phenotype is not routinely available in Australia.

Detection of coreceptor usage

With the recent development and licensing of maraviroc, a CCR5 antagonist, there is now a need to determine coreceptor usage prior to initiation of either a CCR5 or CXCR4 antagonist. The most commonly used commercial test to determine coreceptor usage is Trofile™, performed by Monogram (Bioscience Inc.).[38,39] In this assay, the envelope (env) gene from patient plasma is amplified by PCR, cloned and then cotransfected with an env-deleted common backbone virus that expresses luciferase. The pseudotyped viruses are then used to infect cell lines that express CD4 and either CXCR4 or CCR5 in the presence and absence of CXCR4 and CCR5 antagonists. Trofile™ has a lower sensitivity currently of 1000 copies/mL and can detect the presence of minority CXCR4-using quasispecies to a level of approximately 1-10%. In 5% of cases no amplification is possible (Figure 4.4). Given that the assay can currently only be performed in the USA, it can take approximately four weeks to receive a result for a specimen collected in Australia.

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4.2 Immunological diagnostics and therapeutic drug monitoring

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4.2.1 Immunological diagnostics

Tables 4.2 and 4.3 summarise the assays used to identify T cell subsets (these are not HIV-specific) and assays used to detect HIV-specific responses, respectively.

4.2.2 Therapeutic drug monitoring

Using therapeutic drug monitoring (TDM) to monitor patient plasma drug levels and to adjust doses for toxicity or treatment of drug-related toxicity is described in chapter 10.

Sample analysis

A whole blood sample collected in a tube with ethylenediaminotetra acetate as anticoagulant is recommended for most drug analyses. The use of gel separator tubes for drug analysis is not recommended as drugs have been shown to bind to the gel. Whole blood samples can be stored overnight at 4°C. TDM of the non-nucleoside reverse transcriptase inhibitor (NNRTI) and protease inhibitors (PIs) is performed using high-performance liquid chromatography (HPLC). Samples are treated with the organic solvent chlorobutane to extract the drug from the plasma. The extract is analysed on a HPLC to separate the drug of interest from endogenous interference. The drug is detected by light absorption in the ultraviolet region and quantitated using known standards. Figure 4.5 shows chromatograms from patients with and without antiretroviral therapy. Peaks are compared with control panels of purified antiretroviral compounds to determine area under the curve and subsequent blood plasma drug concentrations.

Table 4.2 Assays used to identify T cell subsets (not HIV-specific)

Characteristic	Marker	Cell type	Method
Phenotype	CD4 CD8 CD45RA+CD27+62L+ CD45RO	T helper T suppressor Naïve [1] Memory [2]	Flow cytometry
Activation status	CD38+HLA-DR+ HLA-DR	Activated	Flow cytometry
Origin	TRECs [3]	Recent thymic emig-rants	PCR

1 True naïve and memory T cells are identified by expression of other surface markers (including $CD28^{+}$, $CDIIa^{low}$), but the phenotype listed correspond to the most frequently used markers.
2 Memory T cells can be further classifed into central and effector memory on the basis of expression and/or downregulation of CD45RA, CCR7, CD28, and CD27.
3 Interpretation of TREC is most accurate with simultaneous measurement of peripheral lymphocyte proliferation.

TREC = T cell receptor excision circle; PCR = polymerase chain reaction; HLA-DR = Human Leukocyte Antigen DR-1.

Table 4.3 Assays used to detect HIV-specific responses

Assay	CD4	CD8	Quantitative	Functional	Interpretation
Tetramer [1]	√ [2]	√	√		Number of T cells recognising specific HIV-epitopes
ELISPOT [3]	√	√	√	√	Number of T cells capable of producing cytokines following recognition of HIV-antigens
Intracellular cytokine production [4]	√	√	√	√	Number of T cells capable of producing cytokines following recognition of HIV-antigens
CTL ^{51}Cr release assay [5]		√	√	√	Number of CTLs able to kill cells expressing HIV antigens
Lymphoproliferative assay [6]	√			√	Number of CD4 cells able to proliferate following recognition of HIV antigens

1 Tetrameric HLA-peptide complexes detect T cells that express a T cell receptor complex which specifically recognises a given peptide presented by a given human leukocyte antigen molecule. This assay uses flow cytometric methods.
2 Not well developed for CD4 cells.
3 T cells are exposed to HIV antigens. Cells recognising these antigens produce cytokines such as interferon-gamma (IFNgamma). Secreted IFNgamma is captured by specific antibodies coating the well. Cells are then removed by washing and bound IFNgamma is detected by colorimetric techniques.
4 T cells are exposed to HIV antigens. Cells recognising these antigens produce cytokines such as IFNgamma. Cell membranes are then made permeable to permit fluorescent monoclonal antibodies to enter cells, which bind to the cytokine of interest. The cytokine-containing cells are then enumerated by flow cytometry.
5 Autologous B cells are transformed to express HIV antigens on their cell surface. These cells are pulsed with ^{51}Chromium (^{51}Cr) which remains intracellular. CD8 cells are then mixed with autologous B cell targets. HIV-specific cytotoxic T lymphocytes lyse target cells and thus release ^{51}Cr which can then be measured.
6 Lymphoproliferative assay – CD4 cells are mixed with HIV antigen and ^{3}H-thymidine. CD4 cells which recognise HIV antigens proliferate and incorporate ^{3}H-thymidine. The stimulation index reported compares the amount of ^{3}H-thymidine incorporated into cells exposed to HIV antigens compared with those exposed to control antigens.

ELISPOT = enzyme-linked immunospot assay; CTL = cytotoxic T lymphocyte.

Chromatographic assay availability

Currently in Australia, there are no commercially available chromatographic assays for NNRTIs and PIs, although some hospitals have developed in-house assays. Generally, in-house assays vary in three areas:

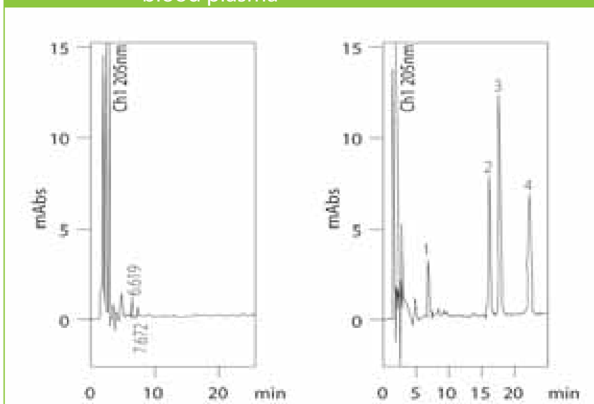
- Extraction procedure
- Chromatographic process (column type and mobile phase)
- Sensitivity (sensitivity depends on the purpose of the assay – TDM or single-dose pharmacokinetics).

Acceptable criteria for assay variability is less than10% (co-efficient of variation) and ±15% at the limit of quantitation (lowest standard). Assay specificity (the ability to separate the drug of interest from other drugs, metabolites and endogenous compounds) is a significant problem. Chromatographic analysis solves this problem by selective extraction procedures, varying the chromatographic process (changing columns and mobile phase) and selective detection mechanisms, such as diode array ultraviolet detection. This technique involves comparing an ultraviolet fingerprint of the chromatographic peak with a library of pure standards (controls). This produces a match with a purity assessment. The gold standard chromatographic system for specificity is the liquid chromatographic mass spectrometer, used by only a few laboratories because of the purchase cost.

Quality assurance system

A mandatory requirement of laboratory accreditation is enrolment in an external quality assurance system. An international program has been established and measures the performance of a particular laboratory against a panel of 30 laboratories.[1] This program is new and only three cycles of assessment have been completed. Preliminary data for the PIs have shown large variability in the performance between laboratories. Mean inaccuracies for all PIs were 25.3% for low concentrations and 17.1% for high concentrations. Enrolment in the international program should alert poorly performing laboratories to their weaknesses. Improvement in performance is expected as the program continues.

Figure 4.5 Chromatograms of drug concentrations in blood plasma



Note: Chromatograms of blank human plasma (left panel) and a standard extracted from human plasma (right panel), showing specific peaks for known antiviral medications.

(1) = indinavir; (2) = ritonavir; (3) = lopinavir; (4) = internal standard; mAbs = milliabsorbant units.

Source: John Ray, St Vincent's Hospital, Sydney. Used with permission.

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