

Towards error-free HIV diagnosis: guidelines on laboratory practice

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Summary: *Error can have multiple causes. These guidelines set out the most common reasons for inaccuracies in HIV testing and indicate how they can be avoided. Emphasis is placed on laboratory procedures as during over 15 years experience they, rather than the kits and reagents, have proved to be the most frequent source of error.*

Key words:
guidelines
HIV
laboratory diagnosis
laboratory procedures

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In their original form these notes were drafted following a meeting of the Public Health Laboratory Service AIDS Diagnosis Working Group on 11th March 1992¹. They have now (December 2003) been brought up to date and endorsed by its successor group, the Health Protection Agency HIV Laboratory Diagnosis Forum.

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Key points to remember

- Section 1* Other than unlinked anonymous testing, patients should only be tested for HIV infection with their informed consent, and requests to the laboratory should be signed by the person taking clinical responsibility, to whom the result should be issued.
- Section 2* There are many possible opportunities for error in the diagnostic process – these, not the assay kits, are the most common sources of error (see table 1).
- Section 3* Any screening test may give rise to some weak reactions. In low-risk populations many of these will not be true HIV-positive results. Weak positives in combo tests, unreactive in anti-HIV-only tests, should be tested for p24 Ag and/or HIV RNA to ensure the specimen is not from a seroconverting patient.
- Section 4* In England and Wales the prevailing approach is to employ at least two different tests following the initial reactive screening test (or an additional screening test with a line immunoassay). The golden rules are: 1) caution with weak reactions in some or all assays; 2) always examine follow-up specimen – it is a critical safeguard against errors, especially against the misidentification of specimens.
- Section 5* Most commercial screening assays are designed to detect both HIV-1 and HIV-2, but infections with HIV-2 must be distinguished from those with HIV-1 (prognosis, treatment and epidemiology are different). Infections with some rare strains of HIV-1 may be poorly recognised by some screening tests. Submit clinically suspicious anti-HIV negative specimens for more detailed analysis.
- Section 6* The HIV-1 pandemic involves subtypes of HIV-1 and recombinants between subtypes. Genetic analysis can identify subtype, suggesting the regional origin of infection and (at a higher level of resolution) even direct connections between individuals sharing the same strain. However these analyses have ethical and consensual implications.
- Section 7* Where necessary, adequately sensitive tests for anti-HIV can be done on body fluids other than serum (e.g. oral fluid, urine). This is useful for testing children, needle-phobic patients and for epidemiological purposes. However, these specimens are far less satisfactory for detection of virus and its components.
- Section 8* After an exposure to HIV it may take at least six months to rule out transmission, depending on the dose and route of transmission. Post exposure prophylaxis (PEP) may prolong that interval.
- Section 9* Same-day testing has valuable clinical uses. However, it is only a means of generating a negative result (based on a sensitive screening assay). Positive reactions must be reported provisionally and they should lead to examination of a further specimen.
- Section 10* Anti-HIV assays are the cornerstone of diagnosis. Other HIV assays have specialised roles.
- Section 11* HIV diagnosis in infancy requires close liaison between the paediatric and laboratory teams, and good communication between clinician and parents. It may take several months to exclude HIV transmission from mother to baby. Breastfeeding increases the risk of transmission.
- Section 12* The end point reactivity of an anti-HIV positive specimen often exceeds one in a million; cross contamination is therefore a constant threat to test accuracy.
- Section 13* Important decisions about the distribution of resources for HIV care are based on numbers of reports to CDSC.
- Section 14* The clinical team (junior members as well as seniors) need to understand the meaning of the reports so that there is no room for ambiguity.
- Section 15* In contrast to the first section, where the principles set out are well established, virological monitoring and anti-viral resistance testing is still a developing area.
- Section 16* HIV-1 quantification, though imprecise, is an important means of monitoring the success of antiviral treatment. For each patient keep to a single commercial assay product and be sure that laboratory and clinical teams agree on the interpretation of the results.
- Section 17* HIV-1 quantification should be repeated at least six-monthly in the untreated, and according to a more frequent agreed schedule in treated patients.
- Section 18* HIV treatment involves close liaison between the clinical team and the laboratory with shared objectives and a common understanding of what tests are needed and how they should be interpreted. Frequent contacts are necessary to agree treatment strategies.
- Section 19* Reliable results can only be obtained on appropriate specimens in good condition.
- Section 20* Every opportunity should be taken to monitor and improve the quality of HIV testing. This should include IQC, IQA and participation in EQA.

Note: At the end of each of the 20 sections a key point is highlighted. While these do not cover every issue in the text they emphasise what the authors of this report believe to be the most important issues. They are incorporated into the 'key points to remember' section above.

Introduction

There is quite properly the highest level of public and professional expectation of accuracy in HIV testing. This expectation can only be met by supporting (and paying for) rigorous quality standards, validated confirmatory tests, and a spirit of cooperation and lively self-criticism among the practitioners of diagnostic testing. The Health Protection Agency (formerly the Public Health Laboratory Service - PHLS) holds twice-yearly meetings of its HIV Laboratory Diagnosis Forum at which diagnostic procedures are reviewed and, where necessary, updated. The Forum has endorsed this overview of good practice and the possible sources of error in HIV testing and proposed preventive and remedial measures. It is based on more than fifteen years of laboratory experience and continuous improvement measures.

The laboratory diagnosis of HIV infection

1. Consent to test for HIV infection

This must be the first consideration in the testing process. Except for unlinked anonymous surveillance, patients should only be tested for HIV infection with their informed consent. This means that they should have understood the technical shortcomings of the test, e.g. the possibility that a later test may be positive if a negative one follows only shortly after an exposure, as well as the possible personal, social and medical implications of a positive result. There are highly beneficial outcomes such as expert clinical management and access to treatment, but also negative ones, e.g. possible harm to future relations with sexual partners, friends and employers, and effect on life assurance prospects, and on freedom of international movement. Though the General Medical Council (GMC) has acknowledged that laboratories cannot be expected to check that every specimen they receive has been collected with consent to test, and have stated that this is the clinician's responsibility, laboratories still have a general duty of care in this respect. Requests sent to the laboratory for HIV testing should be signed by the person who is taking clinical responsibility and who is the person to whom the result will be reported. Unsigned requests will lead to a delay in reporting while the correct paperwork is obtained. Delay can also arise if there is no named responsible medical practitioner to whom, or adequate address to which, to send the report.

There are heavy professional penalties for any clinician who seeks to test without patient consent. If the laboratory suspects that this may have happened senior staff should make representations to the responsible clinician, if possible before any laboratory test is done. In a recent case before the General Medical Council, a practitioner was severely reprimanded and found guilty of serious professional misconduct for ordering HIV tests without explicit consent².

Remember: Other than unlinked anonymous testing, patients should only be tested for HIV infection with their informed consent, and requests to the laboratory

should be signed by the person taking clinical responsibility, to whom the result should be issued.

2. HIV diagnosis: getting procedures right

Most mistakes in HIV laboratory diagnosis arise from procedural errors such as mislabelling, misplacing specimens in a rack or microplate, cross contamination due to carry over on a pipette tip or by splash, faulty transcription of results or bad communication. There are nine stages at least at which these errors can arise, as listed in table 1. As long as the assays used have been evaluated with favourable results beforehand, the stages of the process, and not the assay kits, are the most common sources of error. They therefore deserve most attention when attempts are being made to improve laboratory practice. Furthermore, they should be kept under continuous review as changes of staff and other developments can reintroduce errors even after they have been corrected.

Remember: There are many possible opportunities for error in the diagnostic process – these, not the assay kits, are the most common sources of error (see table 1).

3. The initial anti-HIV test

The variety of 'screening' tests now available is wide. It ranges from conventional 96-well microplate enzyme immunoassay (EIA) kits to fully automated, manufacturer-dedicated, random access analysers. It also includes simple or rapid tests, typically based on flow-through or lateral flow technology, and various dipstick and agglutination tests. Within this range can be found test kits suitable for almost all circumstances. With few exceptions HIV screening tests available in the UK are designed to detect both HIV-1 and HIV-2 antibodies, and most have been modified to improve the sensitivity of detection of infection with the outlier group of HIV-1, group O. During the 1990s most manufacturers converted from second generation (indirect) assays based on recombinant and/or synthetic antigens that predominantly detected anti-HIV of the IgG isotype to third generation (immunometric) assays capable of detecting anti-HIV of any isotype. As a result of their added sensitivity for IgM anti-HIV, important at the early stages of seroconversion, the third generation tests offer about a one-week advantage in

TABLE 1 Error-prone stages of the HIV diagnostic process

• Specimen collection	• Misinterpretation of test results
• Specimen log-in	• Transcription to the report
• Separation of serum/plasma	• Communication of the report to clinician
• Transfer into test rack/plate	• Interpretation of the report by clinic staff or by the patient
• Reading test results	

early detection compared to their second generation predecessors. The advent of fourth generation assays, usually in the form of a third generation combined anti-HIV-1/-2 assay with an integral test for HIV p24 antigen has been shown to close the 'diagnostic window' by a further five to seven days.

A summary of the key features of a selection of HIV screening tests may be found in tables 2 and 3 for EIAs and automated processors, and in table 4 for simple/rapid test devices. Each of the mainstream assay kits has been evaluated by the Microbiological Diagnostics Assessment Service (MiDAS) and the code number of each of its reports is given in tables 2 and 4. (The reports are available from the Medicines and Healthcare products Regulatory Agency [MHRA], details of which may be found through their website [www.mhra.gov.uk]) The choice between these assays depends on the skills and equipment available, the workload and pattern of work, and the cost. In competent hands the sensitivity and specificity of most of the assays available are similar and adequate, and each may therefore be used singly as the basis for a negative report. The initial assay should be able to detect both anti-HIV-1 and anti-HIV-2, and preferably p24 Ag. Should local circumstances have led to a decision to use a test sensitive only to anti-HIV-1 the clinician should be made aware, as there may be reasons (e.g. previous residence in West Africa or Portugal) why an assay that is also sensitive to anti-HIV-2 is essential.

The only advantage of initially doing two screening assays rather than one is as a check of procedure. For this check to be fully valid the second test should whenever possible be done on serum taken from the original specimen container. The value of this check is that it reduces the risk of false results due to technical errors such as failure to add a specimen to a well, confusion between tubes or a labelling or clerical mistake. All screening tests are prone to producing occasional weakly-reactive results that very often do not prove to be consistent with HIV infection. Care must be taken not to cause alarm if a false positive, i.e. unconfirmable, reaction is found. Use of the 'combo' anti-HIV+Ag tests leads to the further complication that reactive specimens must also be checked for p24 antigen should the presence of anti-HIV not be confirmed. When screening low-prevalence populations such as antenatal patients the great majority of screen-reactive specimens will not be confirmed to contain anti-HIV and, if using a fourth generation assay, they will need to be tested for p24 Ag, even though it will be found only rarely. When testing for p24 Ag it must be remembered that it, too, is prone to false positivity, which must be ruled out by a neutralisation test. The procedure is relatively expensive and time consuming, and to complete all these tests typically requires in excess of 0.5 mL of specimen. On occasion, tests for HIV RNA may be needed.

Remember: Any screening tests may give rise to some weak reactions. In low-risk populations many of these

will not be true HIV-positive results. Weak positives in combo tests, unreactive in anti-HIV-only tests, should be tested for p24 Ag and/or HIV RNA to ensure that the specimen is not from a seroconverting patient.

4. Confirming an initial positive reaction

When a laboratory is testing specimens from a high-HIV-prevalence population, it may be justified to pass straight from an initial positive reaction in the screening test to the assays that form the confirmatory test algorithm. However, in many laboratories a substantial proportion of specimens reactive in the screening test will not be reproducible or will not go on to give results consistent with HIV infection. In such circumstances, it is recommended that the specimen is first retested in duplicate in the screening assay kit from the original specimen container (figure 1). Only for specimens whose reactivity is reproducible need confirmatory testing proceed.

Specimens from HIV-infected individuals typically give rise to strong, and often maximum, signals in most commercial screening assays whereas falsely reactive specimens infrequently do. However, this is not a reliable basis on which to make a diagnosis of HIV infection, and further testing is essential, employing several different tests carefully selected to minimise the possibility of each additional test being prone to the same false-positive effect as gave rise to the false reaction in the initial screening test. This may be achieved by choosing assays with regard to the diversity of their antigen source (viral lysate, recombinant protein, peptide), and assay format (see table 2), though it should be understood that even this approach is not infallible. Ongoing audit of the output of any confirmatory algorithm, and checking that the final result is not at odds with patients' clinical and behavioural characteristics is a key element. In many countries laboratories employ a two-test algorithm that examines repeatedly EIA screen reactive specimens by Western blot, but in England and Wales the prevailing approach has been, and remains, to employ at least two different tests following the initial reactive screening test, as recommended by the World Health Organisation³, or an additional screening test with a line immunoassay (LIA). This approach has been called the 'alternative confirmatory strategy', and the underlying principle has been substantiated by a number of independent studies^{2,4,5}.

Whichever approach is employed, there are two golden rules: first, to apply a cautious interpretation to specimens that give weak reactions in some or all assays employed, including weak, or few, bands on Western blot; second, whatever the final interpretation of applying the algorithm to a first specimen, always examine a follow-up specimen. Strong reactivity in all tests employed and/or strong reactivity with most antigens in Western blot or LIA is, therefore, a basis for a positive report on that specimen. Should atypically weak reactions be observed, however, further testing by a specialist

TABLE 2 A guide to HIV screening assays (October 2003)

Assay type and name	Manufacturer/distributor	Product number	Format*	Volume of specimen required (µL)	Approximate time to test 90 samples (minutes)	MDA evaluation report number†
Combined antigen - antibody (fourth generation)						
AxSYM® HIV Ag/Ab Combo	Abbott-Murex Diagnostics	2G83-20	A (MEIA)	194**	125	03132
Enzygnost HIV Integral	Dade-Behring	OQT011/OQT021	M (EIA)	100	150	01141
GREENSCREEN® PLUS HIV Ag-Ab	Bio-Rad Laboratories Ltd	72375/6	M (EIA)	75	150	00080
Murex HIV Ag/Ab combination	Abbott-Murex Diagnostics	7G79	M (EIA)	100	140	01123
VIDAS HIV DUO (VIDAS-30 analyser)	Biomerieux	30114	A (ELFA)	200	320	99/96
Vironostika® HIV Uni-Form II Ag/Ab	Organon Teknika/Biomerieux	84138/39/40	M (EIA)	50	120	99/51
Immunometric (third generation)						
Access® HIV-1/2 NEW	Beckman Coulter/BioRad	34020/34025/34029	A (ChLIA)	210**	180	02097
AxSYM® HIV-1/2 gO assay	Abbott-Murex Diagnostics	3D41	A (MEIA)	76**	125	00075
Biotest anti-HIV TETRA ELISA	Biotest AG	8070078	M (EIA)	150	180	00015
DETECT-HIV* (v.2) EIA	BioChem Pharma	RHD-202B/RHD-200B	M (EIA)	150	180	2000/15
Enzygnost® Anti-HIV 1/2 Plus	Dade-Behring	OQFK G12	M (EIA)	100	120	98/49
Genscreen® HIV 1/2 EIA version 2	Bio-Rad Laboratories Ltd	72278/9	M (EIA)	75	120	99/46
IMx® HIV-1/HIV-2 III Plus	Abbott-Murex Diagnostics	8C98	A (MEIA)	150	255	97/57
Murex HIV-1.2.0	Abbott-Murex Diagnostics	9E25	M (EIA)	50	120	98/54
Ortho® HIV-1/HIV-2 Ab-capture ELISA Test System	Ortho-Clinical Diagnostics	932380	M (EIA)	150	180	97/49
PRISM® HIV O Plus	Abbott-Murex Diagnostics	3D34	A (ChLIA)	100**	465†	00065
Vironostika® HIV Uni-Form II plus O	Organon Teknika/Biomerieux	84017/8	M (EIA)	50	120	97/49
Vitros ECI anti-HIV 1+2	Ortho-Clinical Diagnostics	124-1850	A (ChLIA)	80**	200	99/60
Wellcozyme HIV 1+2	Abbott-Murex Diagnostics	9E45	M (EIA)	25	150	97/77
Antiglobulin/indirect (second generation)						
Detect HIV (Biochem Immunosystems Inc)	Biotest Diagnostics	380019	M (EIA)	5	120	99/01

* A = automated; M = microplate; B = bead; EIA = enzyme immunoassay; ELFA = Enzyme linked fluorescent assay; MEIA = microparticle enzyme immunoassay; ChLIA = chemiluminescent immunoassay.

** An additional void volume is required depending on which sample vessels are used.

† Time to test 800 samples (PRISM is a 'closed' system intended for large-scale blood donation screening)

‡ MDA reports are available free of charge to NHS, NBS and PHLS. Contact MDA at www.medical-devices.gov.uk; tel: 0207 972 8181; fax: 0207 972 8105

laboratory is warranted. They will employ additional tools, such as Western blot and tests for p24 Ag, IgM anti-HIV, and viral nucleic acids to determine whether the individual from whom the specimen was collected is truly infected with HIV.

Testing of a follow-up specimen is a critical safeguard against error

First, it provides a check that no procedural error has occurred, for instance mislabelling of the specimen. When this happens it is likely that two, not one, erroneous results will have occurred because a negative result will have been issued for someone else whose own specimen would have been anti-HIV positive. Second, when results on the first specimen have been ambiguous a later specimen will usually clarify whether this was due to primary infection, in which case the reactions will typically have evolved, or false reactivity, indicated by lack of significant change in the reactions seen. When the first specimen was unambiguously anti-HIV positive a single strongly reactive test on a follow-up specimen may be considered sufficient to confirm the diagnosis. Until that second specimen has been checked, misidentification of the first specimen remains a possibility.

Other pitfalls arise from the use of fourth generation tests (where, as noted above, both components need to be checked) and the application of screening tests to populations where the strong expectation is of a negative result. Reactivity on these specimens needs very careful scrutiny, unhurried by inappropriate 'turn-around' targets.

Remember: In England and Wales the prevailing approach is to employ at least two different tests following the initial reactive screening test (or an additional screening test with a line immunoassay). The golden rules are: 1) caution with weak reactions in some or all assays; 2) always examine a follow-up specimen – it is a critical safeguard against errors, especially against the misidentification of specimens.

5. Recognition of HIV-2 infection

An essential part of confirmatory testing is to distinguish between HIV-1 and HIV-2 infections. The prognosis and the indications for, and choices of, antiviral treatment are different, as are the epidemiological implications.

HIV-1 infection can be assumed if one of three reactive screening assays used is a competitive assay incorporating only HIV-1 antigen and its reactivity is not unexpectedly low. However, in the case of a weak or negative reaction either HIV-2 infection or a recent HIV-1 seroconversion may be suspected. A 'line assay' such as Liatek/Innolia or Pepti-LAV may then be used to discriminate between HIV-1 and HIV-2 infection. Rapid test devices (RTDs) which incorporate separate type-specific reaction spots may also provide an indication of the infecting HIV type. The cost of using Western blots to distinguish HIV-1 from HIV-2 is

unjustifiably high and the results are often not conclusive; so either Liatek/Innolia should be used, or the specimen be sent to a confirmatory laboratory for tests that will discriminate between types. Suspected anti-HIV-2 positive, dually reactive and other specimens with anomalous results can be referred to the Virus Reference Department (VRD) where they will be tested by a variety of discriminatory methods and by native antigen-based Western blot. In the UK it is important to notify VRD and the Communicable Disease Surveillance Centre of cases of suspected HIV-2 infections as some other methods of HIV surveillance in use (e.g. screening of dried blood spots collected from infants) are insensitive to anti-HIV-2. As a result it may be falsely assumed that HIV-2 infection is very rare in the UK. Less common than HIV-2 are the HIV-1 variants, e.g. 'outliers', that may not be recognised by all tests for HIV-1. If clinical features of HIV infection, including AIDS, are present, the possibility of a variant HIV infection should always be borne in mind, even if a screening assay is negative. Laboratory investigations for these are also available at the VRD.

Remember: Most commercial screening assays are designed to detect both HIV-1 and HIV-2, but infections with HIV-2 must be distinguished from those with HIV-1 (prognosis, treatment and epidemiology are different). Infections with some rare strains of HIV-1 may be poorly recognised by some screening tests. Submit clinically suspicious anti-HIV negative specimens for more detailed analysis.

6. HIV-1 subtyping

HIV-1 displays great genetic heterogeneity and has been divided into subtypes on the basis of phylogenetic analysis. Within the HIV-1 type, a major (M) group of subtypes, e.g. A, B, C and D together with 'circulating recombinant forms' (CRFs), e.g. AE, AG and BF, have been described, and outlier groups 'O' and 'N'. While distinguishing HIV-1 from HIV-2 can usually be achieved by serological means, subtyping can reliably be done only by genetic analysis, for which there are several approaches. Most robust is sequencing and phylogenetic analysis of portions of at least two of the main HIV-1 genes. This is because sequencing results are definitive, whereas other methods such as heteroduplex mobility assay and hybridisation with subtype-specific probes provide more limited information. Sequence analysis of the env, pol, gag and other genes of the infecting virus provides greater discrimination and is more able to show that a recombinant virus is present. It may also reveal mutations associated with resistance to antiviral drugs (see below). Only limited information can be obtained by testing for antibodies to subtype-specific peptide antigens. The latter tests are generally only reliable when used to distinguish infection with the B subtype (hitherto the most common in USA and Western Europe) from infections with other subtypes⁶. This would be expected as typing by gene sequencing is a direct test on the infecting virus,

TABLE 3 A guide to antigens, antibodies, conjugates and substrates used in HIV screening assays

Assay type and name	Solid phase coating						Conjugate				Enzyme (indicator)	Substrate (activator)	
	HIV-1 pep rec	HIV-1 (O) rec	HIV-1 (O) pep	HIV-2 rec	HIV-2 pep	Antibodies	HIV-1 pep rec	HIV-1 (O) pep	HIV-2 rec	HIV-2 pep			Antibodies
Combined antigen - antibody (fourth generation)													
AxSYM® HIV Ag/Ab Combo	●	●	●	●	●	mAbs ● ^a	●	●	●	●	mAbs ● ^b	Streptavidin/POD	TMB
Enzygnost HIV Integral	●	●	●	●	●	pAb ● ^a	●	●	●	●	2 mAb ● ^b	Peroxidase labelled avidin and purified HIV 1/2 antigens	TMB
GREENSCREEN® PLUS HIV Ag-Ab	●	●	●	●	●	mAb ● ^a	●	●	●	●	mAb ● ^b	HRP	TMB
Murex HIV Ag/Ab combination	●	●	●	●	●	mAb ● ^a	●	●	●	●	mAb ● ^b	HRP	TMB
VIDAS HIV DUO (VIDAS-30 analyser)	●	●	●	●	●	mAb ● ^a	●	●	●	●	● ^c	Phosphatase labelled streptavidin	4-MUP
Vironostika® HIV Uni-Form II Ag/Ab	●	●	●	●	●	mAb ● ^a	●	●	●	●	mAb ● ^b	HRP	TMB
Immunometric (third generation)													
Access® HIV-1/2 NEW	●	●	●	●	●		●	●	●	●		AP	Lumi-phos 530
AxSYM® HIV-1/2 gO assay	●	●	●	●	●		●	●	●	●		Phosphatase labelled anti-biotin	4-MUP
Biotest anti-HIV TETRA ELISA	●	●	●	●	●		●	●	●	●		HRP	TMB
DETECT-HIV ^a (v.2) EIA	●	●	●	●	●		●	●	●	●		Streptavidin/POD	TMB
Enzygnost® Anti-HIV-1/2 Plus	●	●	●	●	●		●	●	●	●		POD	TMB
Genscreen® HIV-1/2 EIA version 2	●	●	●	●	●		●	●	●	●		HRP	TMB
IMx® HIV-1/HIV-2 III Plus	●	●	●	●	●		●	●	●	●		Phosphatase labelled anti-biotin	4-MUP
Murex HIV-1.2.0	●	●	●	●	●		●	●	●	●		HRP	TMB
Ortho® HIV-1/HIV-2 Ab-capture ELISA Test System	●	●	●	●	●		●	●	●	●		HRP	OPD
PRISM® HIV O Plus	●	●	●	●	●		●	●	●	●		Acridinium labelled anti-biotin	Hydrogen peroxide solution
Vironostika® HIV Uni-Form II plus O	●	●	●	●	●		●	●	●	●		HRP	TMB
Vitros ECI anti-HIV 1+2	●	●	●	●	●		●	●	●	●		HRP	Luminol derivative
Wellcozyme HIV 1+2	●	●	●	●	●		●	●	●	●		AP	NADP + amplifier
Antiglobulin/indirect (second generation)													
Detect HIV (Biochem Immunosystems Inc)	●	●	●	●	●		●	●	●	●	● ^e	HRP	TMB

● = present; ? = no information; mAb = monoclonal antibody; pAb = polyclonal antibody; Ag = antigen; AP = alkaline phosphatase; HRP = horseradish peroxidase; POD = peroxidase; TMB = 3, 3', 5, a. anti-HIV1 p24
 b. labelled anti-HIV-1 p24
 c. VIDAS HIV DUO conjugate, 1: anti-human IgG conjugate, 2: biotin labelled anti-p24
 d. contains env10 which has high homology and cross reaction with group O antigens
 e. anti-human IgG

whereas serotyping analyses the antibody response of the host against only a small peptide, typically less than 20 amino acids long, derived from the V3 crown of the outer envelope glycoprotein of each major HIV-1 subtype.

Epidemiologically, certain subtypes are common in particular regional foci of HIV-1 infection, for instance subtype A in East and Central Africa, subtype C in south east and southern Africa and the CRFs AE in Thailand and AG in West Africa. Subtypes may also be typical of particular groups at risk, e.g. subtype B in men who have sex with men in western countries and CRF AE in drug users in south east Asia. Subtyping has also been used to demonstrate the diversity of HIV-1 strains present in the UK.⁷

There is little evidence that disease progression is influenced by the infecting subtype. However, genetic variability may affect drug susceptibility and the nature of antiretroviral drug resistance. The same sequence derived from the pol region for the purposes of investigating possible ART drug resistance may also be used to provide an indication of the infecting subtype. Subtyping is not a routine confirmatory test, but with the introduction of rapid automated sequencing it has become easier both to look for heterogeneity in national HIV epidemics, resolve outbreaks by careful phylogenetic analysis and guide antiretroviral drug therapy.

In a recent legal case in Scotland a patient was disadvantaged by disclosure of the use of genotyping to link his infection with that of a contact. This use of genetic analysis must only be allowed with the consent of the clinician and of the patient. Recent court decisions indicate that care must be taken not to attempt to link infections in different patients, e.g. by sequencing studies without prior patient consent and ethical approval.

Remember: The HIV-1 pandemic involves subtypes of HIV-1 and recombinants between subtypes. Genetic analysis can identify subtype, suggesting the regional origin of infection and (at a higher level of resolution) even direct connections between individuals sharing the same strain. However, these analyses have ethical and consensual implications.

7. Alternatives to blood sampling (capillary blood, oral fluid, urine)

There are both clinical and epidemiological contexts in which it is impractical to collect samples of venous blood for HIV testing, and there is now more than a decade of experience of using 'non-invasive' tests based on saliva and urine samples. They have been used both in surveillance and diagnostic studies. Another approach has been the 'minimally invasive' one of collecting finger-prick capillary blood spots onto filter papers, which then rapidly air dry. The serum is then eluted from the spots, which may also be stored for many months before being tested for anti-HIV. Account must be taken of the dilution factor involved in the elution, but otherwise these tests are

as accurate as tests on other blood samples. Blood spot testing has been particularly valuable when applied to the 'Guthrie' cards collected from newborns. For these the anti-HIV test on the infant's blood acts as a surrogate for a test for HIV infection on the mother.

Non-invasive testing has been used on needle-phobic patients, on children and, because of its extra convenience and acceptability, to test insurance applicants and in 'look back' studies. Tests on saliva (or more strictly 'oral fluid') demand the use of an efficient collection device (e.g. Orasure, Oracol) and an IgG class-specific capture assay (GACELISA HIV 1 plus 2) that was devised and validated specifically for this purpose⁸. It is imperative that the collection device is properly used if false-negative anti-HIV results are to be avoided, so that either the patient must be instructed carefully how to take the specimen and/or be supervised while it is taken. A check for the presence of a minimum quantity of total IgG is also advised⁹. Anti-HIV tests on urine¹⁰ do not present collection difficulties except that it must be established that the specimen derives from the patient named on the specimen bottle. If the urine specimen is stored before testing it must be at 4°C; it must not be stored frozen. The two suitable assays are GACELISA HIV 1 plus 2 and GACPAT (details available from VRD). These alternatives to serum specimens were devised primarily for epidemiological studies and tests on urine in particular do not attain the highest level of sensitivity and so are inferior diagnostically. However, for the patient who will not or cannot give a blood sample they have a role.

Only limited investigation of HIV recovery from specimens other than blood have been done, and neither for virus isolation, nor p24 antigen detection, nor for gene amplification, can any alternative diagnostic specimen be recommended. Blood collected into EDTA solution is preferred.

Remember: Where necessary, adequately sensitive tests for anti-HIV can be done on body fluids other than serum (e.g. oral fluid, urine). This is useful for testing children, needle-phobic patients and for epidemiological purposes. However, these specimens are far less satisfactory for detection of virus and its components.

8. Testing after exposure to HIV

A common request to the laboratory is to test an individual who may have recently been exposed to HIV. Data to support good practical guidance on this are few, and mostly originate from studies performed in the USA, often employing HIV screening tests of inferior sensitivity to those available in the UK. Such guidance needs also to take into account post-exposure prophylaxis (PEP), which may delay the appearance of markers of HIV infection.

A study of 81 seroconverting blood donors was conducted by Busch et al., in which statistical modelling was employed to estimate the potential reduction in widow-phase blood donations by

TABLE 4 A guide to simple and rapid HIV screening assays (October 2003)

Assay type and name	Manufacturer/distributor	Product number	Format*	Volume of specimen required (µL)	Approximate time to test 10 samples (minutes)	MDA evaluation report number ^d
Agglutination						
Capillus [®] HIV-1/HIV-2	Cambridge Diagnostics Ltd	6048G/6058G	slide agglutination	10	10/33 ^b	MDA98/27
Serodia [®] HIV-1/2	Mast Diagnostics	9260	microplate agglutination	25	150	MDA98/27
Flow-through/immunocentration^c						
Abbott Testpack [®] HIV-1/HIV-2	Abbott Diagnostics Ltd	1A83-21	cartridge membrane EIA	1	15	MDA98/27
Bionike AQ [®] Rapid HIV Test	Gamidor Ltd	GDA200201		50	6	MDA98/27
HIV Chek [®] 1+2 System 3 Test Kit	Ortho Clinical Diagnostics	940647		50	6.5	MDA98/27
Multispot HIV-1/HIV-2	Biorad Ltd	72269		35	22	MDA98/27
Recombigen [®] HIV-1/HIV-2 RTD	Cambridge Diagnostics Ireland Ltd	96053		20	15	MDA98/27
SUDS HIV 1+2 Test	Abbott Diagnostics Ltd	VK74/75		50	17	MDA98/27
Lateral Flow/immunochromatographic						
Abbott Determine [®] HIV-1/2	Abbott Diagnostics Ltd	7D23-12/-13		50	5	MDA98/76
HIV-1 and -2 Doublecheck ^a	Quest Biomedical	60332000		150	21	MDA98/27
SeroCard HIV	Trinity Biotech plc	1200100		80	14	MDA98/27
Uni-Gold [®] HIV	Trinity Biotech plc	1206500		50	13	MDA98/27
Dipstick						
Immucomb [®] II HIV-1 and -2 BiSpot	Quest Biomedical	60432002		50	50	MDA98/27
Sero-strip HIV-1/HIV-2	SDS International Ltd	SH-0010		1	4.5	MDA98/27

a. SL = slide; MP = microplate; B = bead; EIA = enzyme immunoassay; ELFA = Enzyme linked fluorescent assay; MEIA = microparticle enzyme immunoassay; ChLIA = chemiluminescent immunoassay.

b. 10 minutes by eye; 33 minutes by digital reader

c. Time to test 800 samples (PRISM PRISM[®] is a 'closed' system intended for large-scale blood donation screening)

d. MDA reports are available free of charge to NHS, NBS and PHLS. Contact MDA at www.medical-devices.gov.uk; tel: 0207 972 8181; fax: 0207 972 8105

introducing third generation anti-HIV and stand-alone p24 Ag screening tests¹¹. It was concluded that the mean durations of the 'residual infectious window' were 11 days for HIV RNA PCR, 16 days for HIV DNA PCR and p24 Ag, and 22 days for third generation anti-HIV assays. The term 'residual infectious windows' is not clearly defined in this paper, in particular whether the calculations include a brief period between infection and becoming infectious. However, in a subsequent review of data from several sources, an idealised model was proposed that includes an initial period, typically of one to two weeks, during which HIV replication may be limited to the local mucosal and lymphatic tissues, prior to general dissemination¹². From this we may conclude that seroconversion in a third generation assay would, in about 50% of cases, occur about one month after exposure, and four to eight days earlier (i.e. three to four weeks post exposure) if using a fourth generation assay. In this review the authors also cite cases confirmed by the Centers for Disease Control and Prevention of two exposed healthcare workers (HCW) who took more than six months to seroconvert, presumably harbouring virus in a latent or low-level replicative state in tissues proximal to the site of entry. It is also stated that approximately 5% of HCW take six months or more to seroconvert.

In view of the above evidence, there seems no rapid way of excluding the possibility that transmission has occurred after an HIV exposure. Although the likelihood of being infected but screen-test negative declines over several months, the observation that delayed seroconversions do occasionally occur must be taken into account. Add on to that the potential inhibitory effect of PEP on viral replication and the host's immune response, and one has to conclude that there is no wholly secure alternative to following up for at least six months. Clearly, waiting for six months to test is untenable, and an approach should be adopted that strikes a balance between certainty that transmission has not happened and the need to be able to reassure the patient as soon as possible. A nucleic acid test (see below) may hasten a positive finding after exposure to HIV, but it is not a substitute for anti-HIV testing after a 'safe' interval. We would suggest testing using a sensitive fourth (or third) generation screening test immediately after the exposure and then: at one to two months, at three to four months, and six months. Should suspicious clinical signs and symptoms develop, an immediate test, including for HIV RNA, is indicated. Some time and care will be needed to explain the reasons underlying the need for follow-up testing and to communicate without undue alarm an appropriate level of residual risk of infection, despite negative results, prior to completing the six-months follow up.

Remember: After an exposure to HIV it may take at least six months to rule out transmission, depending on the dose and route of transmission. 'PEP' may prolong that interval.

9. Speed of testing

Laboratories are under pressure to report test results rapidly, and in some localities a same-day service is provided, at least for seronegative patients. This is possible because a carefully performed single assay which gives a negative reaction is generally a basis for a confident negative report, making same-day reporting feasible in most instances. However, same-day reporting of a positive result is generally inappropriate. It must have been explained during counselling prior to the test that a second specimen and further testing may be needed, and that the need for this additional test does not necessarily carry an implication of HIV infection. Without the opportunity to test a second sample an unqualified positive result should not be given. This is especially important for same-day testing as difficulties may arise in communicating results speedily but correctly. Careful attention must be given to preserving confidentiality, e.g. if telefaxing or telephoning results when same-day testing is offered.

As regards speed of reporting, laboratories undertaking confirmatory testing should aim to report a result based on three assays within five working days. However, circumstances that give rise to a need for extra tests may entail further delay. It is expensive and inimical to accuracy to do special HIV tests on a 'one-off' basis, and when confirmatory laboratories do have to batch these tests there may be a delay in reporting a result. In such circumstances consideration should be given to issuing a provisional report, perhaps by telephone. As long as the conversation is confidential it is good practice to telephone positive HIV results to the requesting clinician. It is important that the clinician fully understands the meaning of a provisional report and the importance of additional confirmatory studies.

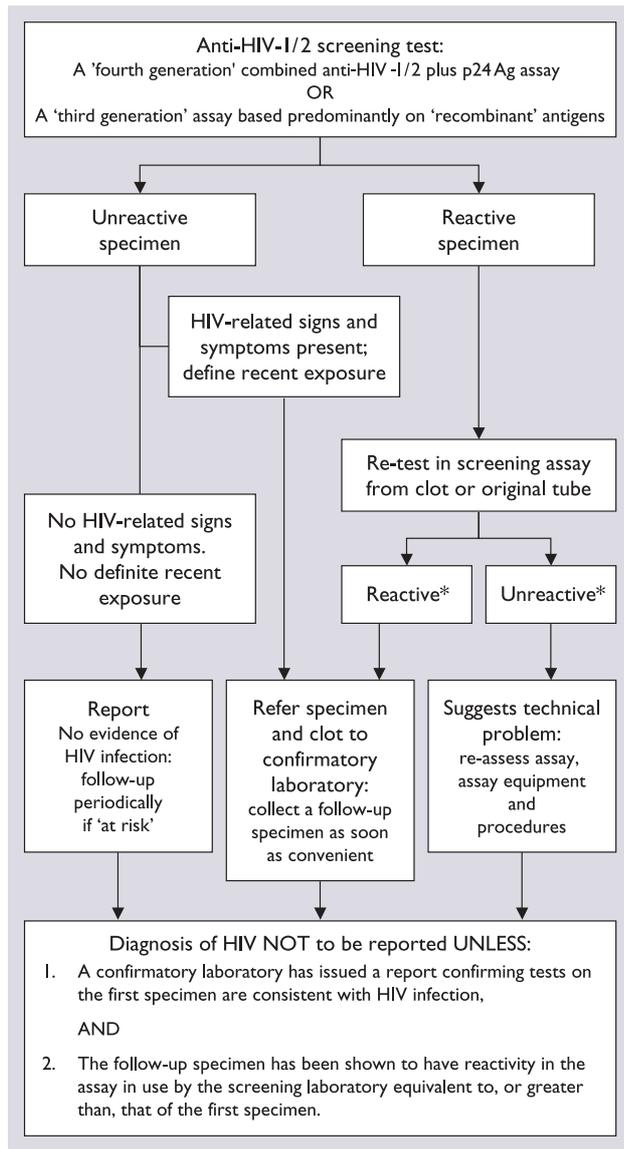
Remember: Same-day testing has valuable clinical uses. However, it is only a means of generating a negative result (based on a sensitive screening assay). Positive reactions must be reported provisionally and they should lead to examination of a further specimen.

10. Status of other HIV assays

Experience has shown that HIV culture and 'stand-alone' tests for p24 antigen are of limited diagnostic value. They may be insensitive and/or non-specific, and they are expensive compared with the standard serological screening tests. In selected circumstances such as HIV diagnosis in infancy (see below) amplification of HIV proviral DNA by PCR is necessary, and tests for HIV p24 antigen and specific IgA anti-HIV may be helpful. There may also be a role for p24 Ag testing and/or RT-PCR detection of HIV RNA in patients with a suspected acute HIV seroconversion illness, though tests on a second specimen a week or so later will often resolve such cases.

Remember: Anti-HIV assays are the cornerstone of diagnosis. Other HIV assays have specialised roles.

FIGURE 1 HPA HIV screening algorithm



* Correction: Labels are in correct positions here. They were switched in the original printed publication.

11. Diagnosing HIV infection in infancy

HIV can be passed from mother to infant, either during gestation, labour, or breastfeeding. Most transmissions occur due to natural trauma in the course of labour. Short-term treatment with antiretrovirals late in pregnancy and to the newborn usually prevents perinatal infection, which otherwise occurs in 15%-30% of pregnancies. Withholding breastfeeding further reduces the risk of transmission, and unless this is done the benefit of peri-partum antiretroviral drug treatment may be lost.

These preventive interventions have important implications for laboratory diagnosis. Because maternal antibody crosses the placenta in pregnancy and may be persistently detectable in the infant for 18 months, and sometimes longer, measurement of changes in its titre over time is a slow way of diagnosing infection in the infant. This is especially so as endogenous production of anti-HIV, reflecting infection in the child is difficult to distinguish from passive maternal antibody until the child is several months old.

Nowadays, HIV diagnosis in infancy is based very largely on detection of the HIV genome by PCR. It is preferable to test for HIV proviral DNA because plasma HIV RNA levels may be suppressed by antiviral treatment and the not infrequent occurrence of borderline quantitative HIV RNA results can confuse the diagnostic status of the child. Tests for HIV proviral DNA require an unseparated EDTA anticoagulated blood specimen to permit extraction of DNA from the white cell fraction. In view of the high genetic diversity of HIV infections in UK heterosexuals, a concern is whether the virus to which the baby is exposed is detectable by the PCR being employed. As a result it is now also recommended to obtain a single whole blood specimen from the mother, preferably before term, to demonstrate that the virus she carries is detected by the PCR that will be applied to specimens from the child. If it is not, PCR employing alternative primers should be employed, and its ability to detect the infecting virus must be confirmed. This alternative PCR test must then be applied to specimens from the child, requiring a mechanism to flag which PCR is to be applied. This service is available through VRD.

In nearly all cases when mother-to-child HIV transmission occurs a positive diagnosis will be possible within the first three months of life, and in the case of *in utero* transmission, often immediately postnatally. Consequently, the first test should be performed in the perinatal period; however, it should not be done on a cord blood specimen because of the likelihood of maternal blood contamination. Should tests on a specimen be consistent with HIV infection of the child, the test should have been repeated on a freshly collected specimen before a definite positive result is given. When diagnostic tests (PCR, p24 Ag) are negative, and especially if antiretroviral therapy has been used, the tests should be repeated at one, three and six months; further tests for antibody alone at 12 and 18 months should be considered, though they may not be essential. When breastfeeding has been continued it is necessary to extend the testing period to several months after breastfeeding has stopped.

The laboratory diagnosis of HIV infection in infants is therefore complicated. It requires close liaison with the paediatrician and it may be difficult to rule out infection in a treated infant until many months after treatment has been discontinued. Although there may be strong parental pressure to rule out infection early, the necessary interval must be allowed. Further guidance is available in a booklet that is available on request from Virus Reference Department, and advice on management of HIV-infected pregnant women has been published¹³, and is available on the British HIV Association's website¹⁴.

Remember: HIV diagnosis in infancy requires close liaison between the paediatric and laboratory teams,

and good communication between clinician and parents. It may take several months to exclude HIV transmission from mother to baby. Breastfeeding increases the risk of transmission.

12. Cross contamination of specimens

Experience over the last 15 years has shown that cross contamination between specimens is one of the more common causes of false positive serological diagnosis¹⁵. It is also potentially a problem with tests based on amplification of the HIV genome. Both serological and molecular assays have end points at very high dilutions for some positive specimens, and whenever the physical possibility exists e.g. containers too close together or, jolts in transit, carry over between specimens may occur. This carry over may for instance arise through carelessness in transport, poor handpipetting technique, or during the operation of badly designed or maintained automatic analysers and pipettors using fixed, non-disposable probes. Sometimes a specimen may have been cross contaminated in another laboratory before being sent to the virology laboratory for HIV testing. Thus, the chance juxtaposition of a negative specimen to a strongly positive specimen can easily lead to reactivity in the negative one, and these reactivities will, to the unwary, indicate an HIV infection. The problem is exacerbated if carry over affects the original specimen as opposed to an aliquot of it. In this case confirmatory tests will appear to support the false-positive reaction(s) so that an erroneous positive report will be issued, and a false-positive diagnosis will only be avoided if the guidance to test a follow-up specimen is followed.

Remember: The end point reactivity of an anti-HIV positive specimen often exceeds one in a million; cross contamination is therefore a constant threat to test accuracy.

13. Reporting to Communicable Disease Surveillance Centre (CDSC)

Confirmed positive laboratory diagnoses of HIV infection should be reported confidentially to CDSC (in Scotland to the Scottish Centre for Infection and Environmental Health, SCIEH). This is essential to maintain surveillance of the UK HIV epidemic, the shape of which is continuing to change in respect of risk group, country of infection and origin, survival and other demographic features. The information is used for resource allocation and planning, and it is potentially against the interests of those who are HIV infected to withhold this information.

The reports should be sent with the patient's name and other personal identifiers removed, other than the name of the clinic, the unique patient number and 'soundex' code, their gender, and the date of birth (these are used to recognise and remove duplicate reports from the database). If reports still bearing personal identifiers are received in CDSC or SCIEH the patients' names are replaced by 'soundex' codes,

in conformity with the principles of the Caldicott Report on Patient Confidentiality (1997)¹⁶, i.e. no high-level personal identifiers are kept.

Remember: Important decisions about the distribution of resources for HIV care are based on numbers of reports to CDSC.

14. Wording of reports

It is particularly important that reports on HIV tests should be carefully considered and formulated, and clearly expressed. The form of words should have been discussed in advance with local clinical teams so that there is no scope for ambiguity as to their meaning.

The importance of the clinician understanding a provisional report was referred to in section nine. The clinician should also realise the importance of a follow-up specimen for an initially positive specimen.

Remember: The clinical team (junior members as well as seniors) need to understand the meaning of the reports so that there is no room for ambiguity.

Virological monitoring of HIV-infected patients

15. Introduction

The involvement of the diagnostic virology laboratory now stretches beyond the initial identification of the infected patient. The introduction of highly active antiretroviral drug therapy (HAART) and the development and validation of tests that guide the physician's decisions about when to initiate HAART, and when and how to adjust it, have added to the laboratory agenda. Laboratories are called on to measure plasma concentrations of HIV RNA and to detect emerging drug resistance in those apparently failing therapy. The reader is referred to the frequently updated guidelines on the management of HIV patients published by the British HIV Association¹⁴, but the following observations focusing on laboratory issues may also be helpful.

Remember: In contrast to the first section, where the principles set out are well established, virological monitoring and anti-viral resistance testing is still a developing area.

16. Quantification of plasma HIV RNA concentration ('viral load')

The quantification of plasma HIV-1 RNA is used to predict disease progression,^{17,18} to assist in deciding when to initiate antiretroviral therapy and monitoring the response to it,¹⁹ and to detect possible therapeutic failure²⁰. During primary HIV infection, peak plasma concentrations well in excess of 10⁶ copies per mL may be seen²¹. Once the interactions of the host response and the virus have equilibrated the HIV RNA levels resolve to a viral set point²², and only later in disease as immune function breaks down, does the viral load climb again. The level of the set point has been shown to be predictive of rapidity of progression

to HIV-related disease^{17,18}. The CD4⁺ lymphocyte cell count is a strong indicator of immune function and, in an established infection, of the level of damage already wrought by HIV on the immune system²³. Consequently, it is important both to establish the viral set point and the CD4⁺ lymphocyte level soon after diagnosis. This will determine optimal patient management, e.g. whether the offer of HAART may be deferred and how intensive clinical follow up should be.

The concentration of HIV in the plasma of an infected person can be sustainably lowered by successful combination antiretroviral drug treatment. HIV RNA concentration is measured by a variety of assays which depend either on amplification of a target within the virus genome (PCR; NASBA) or on amplification of the assay signal generated by hybridisation of specific probes to that genome (see below). Plasma HIV RNA is measured semi-quantitatively, and concentrations of between ten and over a million copies of the virus genome per mL may be detected, depending on the particular kit and protocol employed. For acceptable accuracy and correct interpretation of the result it is important to use the same assay kit for successive tests and to understand that even three-fold variations (i.e. about 0.5 log₁₀) are within the bounds of natural experimental variation. However, results that consistently show a rise or a fall of at least that magnitude may be clinically significant¹⁹.

Measurements of HIV RNA concentration have become far more important, as well as more accurate and better controlled, since highly-active combination antiretroviral treatment was introduced. This has been achieved mainly by using products with better reproducibility. Nevertheless, consistent testing requires frequent quality control and performance assessment, as well as fidelity to a single quantification assay. A range of frozen and freeze-dried plasma-based control samples is becoming available to monitor the reproducibility of estimates of viral load, especially measurements at low concentrations. These controls are an important means of monitoring inter-assay consistency, and purchasers must be prepared to bear the extra costs involved in using them.

As previously mentioned, the commercially available assays in use as part of routine viral load monitoring have differing methodologies: namely branched DNA (bDNA) amplification, which is a signal amplification method; reverse transcription polymerase chain reaction (RT-PCR), a thermal cycling method; and nucleic acid sequence-based amplification (NASBA), an isothermal amplification method. Other methods, such as ligase chain reaction, are in development. Current guidelines indicate the need for optimal sensitivity, particularly when monitoring patients on HAART. It is recommended, therefore, that an 'ultra-sensitive' protocol be used, and these typically provide a lower limit of detection of no more than 50 copies/ml of HIV RNA. It is important to

consider the accuracy and variability of HIV load measurements as both inter- and intra-assay variability and biological variability contribute to total variation²⁴. HIV-1 subtype variation can also affect the accuracy of virus quantification²⁵. It is therefore important that clinicians are aware of the assay used, and that they ensure that for each patient the same assay is used on successive occasions. The wording of reports of these assays should convey the inherent biological and technical variation in these assays.

At present, there is no commercial assay available for the determination of HIV-2 RNA plasma concentrations.

Remember: HIV-1 quantification, though imprecise, is an important means of monitoring the success of antiviral treatment. For each patient keep to a single commercial assay product and be sure that laboratory and clinical teams agree on the interpretation of the results.

17. Frequency of monitoring of HIV-1 RNA quantification

- Plasma HIV RNA concentration should be determined at the time of diagnosis. This may be achieved by collecting an EDTA blood specimen both for confirmatory HIV serology (see section 4) and for viral load measurement;
- The frequency of follow-up viral load monitoring of untreated patients will be guided by results on the initial specimen, CD4 count and clinical findings. In those with high-CD4 counts and undetectable HIV RNA, and without significant clinical findings, we would suggest follow-up measurements every three to six months;
- Once HAART has been initiated, monitoring should be undertaken at one-to-two-month intervals for the first six months of therapy, or until the viral load becomes undetectable, in order to identify early non-responders. Subsequently, monitoring at three-month intervals is sufficient. The use of an ultra-sensitive assay protocol with a limit of detection of ≤ 50 copies/ml is recommended.

Remember: HIV-1 quantification should be repeated at least six-monthly in the untreated, and according to a more frequent agreed schedule in treated patients.

18. Genotypic drug resistance testing

Although sometimes transmitted²⁶, antiretroviral drug resistance generally arises during ongoing viral replication in the presence of sub-optimal therapeutic drug concentrations. The associated selective pressure on HIV leads to the emergence of resistance. Replication while on therapy often arises because of non-adherence to drug regime, but may also result from sub-optimal pharmacokinetics or inappropriate prescribing practice. With appropriately frequent monitoring of viral load therapeutic failure should be detected before its clinical manifestations. While

failure may be due to the infecting virus developing resistance to one or more of the drugs given, other causes should also be investigated. Genotypic drug resistance testing is a complex and expensive procedure. It has been subject to controlled clinical trials, which demonstrate, overall, the clinical utility of this approach²⁷.

Because HIV infection generates an archive of variants present throughout an infection, including those resistant to previous therapy, by laying down proviral DNA in susceptible tissues, detailed knowledge of previous antiretroviral drug treatment and a good understanding of interactions between available drugs are necessary to ensure optimal therapeutic management. Defined point mutations, and combinations of them, have been associated with reduced susceptibility to particular drugs, and these have been used to devise both proprietary and open access databases against which to align patients' sequences. This allows the user to identify key resistance mutations, and the database typically assigns a predicted level of drug resistance. In view of the complexity it is recommended that such investigations are restricted only to specialist virology laboratories with experience in viral genome sequencing.

Although phenotypic methods for assessing HIV drug resistance also exist, the most usual method undertaken within diagnostic laboratories is sequence-based genotypic testing. All genotypic methods involve initial RT-PCR amplification of the infecting virus followed by sequencing or hybridisation approaches (such as DNA chips or line probe assay). Sequencing is the most commonly used approach, either in the form of in-house automated sequencing or using commercial kits which contain the standard reagents for PCR and sequencing reactions. The commercial genotyping kits also usually incorporate software packages to identify from the sequences generated mutations associated with resistance to each of the antiretroviral drugs available, and these packages typically propose an interpretation of the findings. The field of antiretroviral resistance is ever changing, and it is important that those software systems are continually reviewed and updated.

We would draw the readers' attention to the following important aspects of resistance genotyping:

a. *When to perform drug resistance testing*

Testing will mostly be undertaken at times of suspected therapeutic failure, ideally using an aliquot of the same plasma sample used for the viral load determination. In light of data showing evidence for transmission of HIV drug resistance, testing may also be warranted before starting therapy, and is now recommended by the British HIV Association. Evidence is now emerging that patients infected with a resistant virus appear to respond sub-optimally to therapy in the absence of baseline resistance testing to guide most appropriate therapy²⁸.

b. *Virus quantification before resistance testing*

Most currently available in-house and commercial antiretroviral drug resistance assays require the presence of at least 1,000-5,000 copies of HIV RNA/mL. It is therefore important that the viral load within the sample is known before undertaking the resistance assay, and it is best if the resistance test is undertaken on the same sample sent for viral load determination. Alternatively, a new sample can be sent from the patient following an initial viral load determination.

c. *Drug history*

It is essential to know both current and previous drug therapy in order to interpret results. Following cessation of therapy, wild type virus rapidly emerges within plasma, and therefore it is best only to undertake testing on drug-experienced patients at the time of drug failure.

d. *Controls*

Standard controls should be applied to sample extraction and PCR aspects of the assay, with negative extraction controls and PCRs being run for at least every four clinical samples (ideally every other sample). In addition, a positive control with a low viral load should be included in each run to check on the sensitivity of the RT-PCR reactions.

e. *Sample handling*

There are multiple steps within these assays and great care must be taken to avoid sample switching at any stage. Only a small number of samples should be handled at any one time (e.g. five). It is a good idea to colour code each sample and use only tubes of that colour throughout the procedure.

f. *Internal and external quality assurance measures*

It is important that samples with a previously defined sequence are run within the assay. The high cost of these procedures may preclude this being done for every run, but this should be undertaken at a frequency of at least one per twenty clinical specimens. Some have argued that this control should be a mixture of clones to allow an assessment of the reproducibility of detection of mixtures of genotypes. However, others may feel that the use of cloned material in a routine setting is not advisable because of the risk of environmental contamination. An alternative is to run a sample that has previously been sequenced. External quality control schemes are currently provided by Quality Control for Molecular Diagnostics²⁹.

g. *Reporting the results of antiviral resistance testing*

i. *Identification of key mutations*

Commercial sequencing assays incorporate software that identifies key mutations. It is important that this software is updated at very regular intervals since new drug-resistance-associated mutations are continually being reported. Alternatively, sequences generated can be assessed for key mutations by reference to international websites (e.g. <http://hivdb.stanford>).

edu/hiv/ and <http://hivweb.lanl.gov/>). Reliable interpretation and appropriate reporting are dependent on a specific knowledge of the patient's drug history. This also allows another check on sampling errors that may have occurred during the procedure.

ii. *Clinical link*

Clinics for which resistance testing is undertaken should be consulted as to the most appropriate form of reporting. In many cases the identification of resistance mutations alone may not be sufficient to inform clinical decision making. It is preferable for virologists to maintain close contact with the HIV physicians managing the patients being tested (e.g. through regular meetings or ward rounds) in order that maximum benefit is obtained from resistance results, and interpretative expertise is developed.

Remember: HIV treatment involves close liaison between the clinical team and the laboratory with shared objectives and a common understanding of what tests are needed and how they should be interpreted. Frequent contacts are necessary to agree treatment strategies.

19. Nature of specimens for HIV RNA quantification and genotypic resistance testing

The ideal sample for HIV RNA studies is EDTA plasma. It is best that these samples are separated as soon as possible after being taken from the patient, and preferably within six hours. For samples sent to a laboratory distant from that directly serving the clinic, a plasma sample is preferable to whole blood. A study undertaken by the PHLS HIV Diagnosis Forum that investigated inter-laboratory and inter-kit variation and the effect, if any, of mailing specimens, demonstrated no reduction in plasma HIV RNA concentration even after several days in transit³⁰. The quantification assays are generally performed on fresh specimens, though plasma samples stored at -20°C or colder may be used as long as they have not been frozen and thawed more than once. When looking for genotypic markers of resistance this is sometimes done retrospectively, in which case a specimen taken for other purposes and stored under less stringent conditions may suffice. In this case the issue is whether any 'sequenceable' RNA can be recovered, not precise quantification.

Apart from the causes of false results outlined in table 1, nucleic acid amplification methods are also particularly prone to misleading results due to contamination by products of previous amplifications. To minimise this risk it is advisable to adopt a system that physically separates, in a strict one-way process, amplification reagent preparation from nucleic acid extraction and amplification, and from post-amplification analysis. Failure to impose this discipline will lead to false positive quantification

results and incorrect antiretroviral drug resistance profiles.

Remember: Reliable results can only be obtained on appropriate specimens in good condition.

20. Quality systems in molecular HIV investigations

It is assumed that all laboratories providing clinical HIV services will be accredited by Clinical Pathology Accreditation (CPA) or similar. The CPA standards are available from their website³¹. The same generic standards that are applied to serological diagnosis must also be applied to the provision of molecular services, and the following points are emphasised here:

- Standard operating procedures should be in place for all aspects of molecular tests;
- Staff performing the procedures must be adequately trained;
- Procedures should include appropriate internal controls whose performance should be described. The procedures will be assessed for their validity against them;
- The laboratory should participate in an External Quality Assurance (EQA) scheme. For HIV RNA quantification several schemes are available, including one run by UK NEQAS³². EQA schemes for resistance genotyping are available from Quality Control for Molecular Diagnostics²⁹. Members of the HPA HIV Diagnosis Forum have also been piloting a scheme.
- Laboratories should run their own Internal Quality Assurance (IQA) scheme. Typically, this entails examining a predefined proportion (say 2-5%) of specimens a second time. The laboratory's IQA scheme coordinator selects appropriate specimens, relabels and submits them for examination, and then receives the report and checks the results and interpretation against the original. Discrepancies indicate the need for review and possible remedial measures.
- Results should be reviewed together with previous results (if any), relevant clinical information including CD4 count, country of origin/infection, and treatment details. Any possible discrepancies are discussed with the relevant clinical team. In the case of HIV RNA quantification a further sample should be requested for testing by the same and an alternative assay if the viral load result is thought to be inconsistent with this information.

Remember: Every opportunity should be taken to monitor and improve the quality of HIV testing. This should include IQC, IQA and participation in EQA.

Conclusions

It is clear that diagnostic laboratories, working in collaboration with HIV confirmatory centres, can offer accurate, rapid and economical diagnostic HIV testing, mostly based on commercial screening assays for

anti-HIV. Furthermore, the manufactured quality of these assays is now so high that any substantial further improvements in laboratory performance are likely to come from better quality systems rather than use of any different selection of laboratory assays.

In the UK, confirmatory serological and molecular diagnostic tests are available from the Virus Reference Department, Specialist and Reference Microbiology Division, Colindale, and some other specialist laboratories. Detailed molecular epidemiological studies of suspected transmission clusters are also available from VRD. Quantification of plasma HIV RNA concentration has become a routine assay in many virology departments. The investigation of suspected antiviral drug resistance is available through the laboratory network, working in conjunction with the Antiviral Susceptibility Reference Unit, HPA Birmingham.

The HPA HIV Laboratory Diagnosis Forum keeps HIV testing practice under twice yearly review and will update these notes. Through its chairman, it welcomes comments and criticisms, as well as sight of data that support or challenge the information in, and assumptions implicit in, this guidance. These feedbacks are regarded as an important means by which diagnostic accuracy can be maintained and improved.

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Current members of the Health Protection Agency HIV Laboratory Diagnosis Forum

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Selected references

It would be a daunting task to reference these guidance notes fully and the list would soon become dated. Instead selected references are given. Though the statements in these notes are therefore not fully substantiated, they do represent a consensus of the views of the HIV Laboratory Diagnostic Forum, as of early 2003. Further information and advice can be obtained from the Virus Reference Department, from the Antiviral Susceptibility Reference Unit, and from other laboratories represented at the Forum.

- PHLS AIDS Laboratory Diagnosis Working Group. Towards error free HIV diagnosis: notes on laboratory practice. *PHLS Microbiology Digest* 1992; **9**: 61-4.
- Nkengasong J, Van Kereckhoven I, Vercauteren G, Piot P, van der Groen G. Alternative confirmatory strategy for anti-HIV antibody detection. *J Virol Meth* 1992; **36**: 159-70.
- Joint United Nations Programme on HIV/AIDS (UNAIDS) - WHO. Revised recommendations for the selection and use of HIV antibody tests. *Weekly Epidemiol Rec* 1997; **72**: 81-8.
- Groen G van der, Kerckhoven I van, Vercauteren G, et al. Simplified and less expensive confirmatory HIV testing. *Bull WHO* 1992; **69**: 747-52.
- Urassa WK, Bredberg-Raden U, Mbena E, Palsson K, et al. Alternative confirmatory strategies in HIV-1 antibody testing. *J AIDS* 1992; **5**: 170-6.
- Murphy G, Belda FJ, Pau CP, Clewley JP, Parry JV. Discrimination of subtype B and non-subtype B strains of human immunodeficiency virus type 1 by serotyping: correlation with genotyping. *J Clin Microbiology* 1999; **37**: 1356-60.
- Parry JV, Murphy G, Barlow KL, Lewis K, Rogers PA, Belda FJ, et al. National surveillance of HIV-1 subtypes for England and Wales: design, methods and initial findings. *J AIDS* 2001; **26**: 381-8.
- Connell JA, Parry JV, Mortimer PP, Duncan J. Novel assay for the detection of immunoglobulin G anti-human immunodeficiency virus in untreated saliva and urine. *J Med Virol* 1993; **41**: 159-64.
- Mortimer PP, Parry JV. Salivary testing for HIV infection. *BMJ* 1992; **305**: 1093.
- Connell JA, Parry JV, Mortimer PP, Duncan RJS, McLean KA, Johnson AM, et al. Preliminary report: accurate assays for anti-HIV in urine. *Lancet* 1990; **335**: 1366-9.
- Busch MP, Lee LL, Satten GA, Henrard DR, Farzadegan H, Nelson KE, et al. Time course of detection of viral and serologic markers preceding human immunodeficiency virus type 1 seroconversion: implications for screening of blood and tissue donors. *Transfusion* 1995; **35**: 91-7.
- Busch MP, Satten GA. Time course of viremia and antibody seroconversion following human immunodeficiency virus exposure. *Am J Med* 1997; **102**: 117-24.
- Lyall EGH, Blott M, de Ruiter A, Hawkins D, Mercy D, Mitchla Z, et al. Guidelines for the management of HIV infection in pregnant women and the prevention of mother-to-child transmission. *HIV Medicine* 2001; **2**: 314-34.
- British HIV Association website [homepage on internet]. London: BHIVA; 2004 [cited 18 February 2004]. Available at <http://www.bhiva.org>
- Mahoney A, Parry JV, Mortimer P. Cross-contamination and 'confirmed' positive anti-HIV results. *Lancet* 1991; **338**: 953-4.
- Department of Health. The Caldicott Committee. Report on the Review of Patient-Identifiable Information. London: DoH; 1997. Available at <http://www.dh.gov.uk/assetroot/04/06/84/04/04068404.pdf>
- Mellors JW, Rinaldo CR, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* 1996; **272**: 1167-70.
- Mellors JW, Muñoz A, Giorgi JV, Margolick JB, Tassoni CJ, Gupta P, et al. Plasma viral load and CD4⁺ lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med* 1997; **126**: 946-54.
- Hughes MD, Johnson VA, Hirsch MS, Bremer JW, Elbeik T, Eric A, et al. Monitoring plasma HIV-1 RNA levels in addition to CD4⁺ lymphocyte count improves assessment of antiretroviral therapeutic response. *Ann Intern Med* 1997; **126**: 929-38.
- O'Brien WA, Hartigan PM, Daar ES, Simberkoff MS, Hamilton JD. Changes in plasma HIV RNA levels and CD4⁺ lymphocyte counts predict both response to antiretroviral therapy and therapeutic failure. *Ann Intern Med* 1997; **126**: 939-45.
- Daar ES, Moudgil T, Meyer RD, Ho DD. Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N Engl J Med* 1991; **324**: 961-4.

22. Ho DD. Viral counts count in HIV infection. *Science* 1997; **272**: 1124-5.
23. Fahey JL, Taylor JM, Detels R, Hofmann B, Melmed R, Nishanian P, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1 infection. *N Engl J Med* 1990; **322**: 166-72.
24. Giles RE, Perry KR, Parry JV, Mortimer PP, Andrews NJ, Cox A, et al. *Evaluation of three methods for quantification of HIV 1 RNA in plasma. Medical Devices Agency Evaluation Report #MDA/98/42*. London: The Stationery Office; 1998. ISBN 1 85839 916 5.
25. Swanson P, Soriano V, Devare SG, Hackett J. Comparative performance of three viral load assays on human immunodeficiency virus type 1 (HIV-1) isolates representing group M (subtypes A to G) and group O: LCx HIV RNA quantitative, AMPLICOR HIV-1 MONITOR version 1.5, and Quantiplex HIV-1 RNA version 3.0. *J Clin Microbiol* 2001; **39**: 862-70.
26. Pillay D, Cane PA, Shirley J, Porter K. Detection of drug resistance associated mutations in HIV primary infection within the UK. *AIDS* 2000; **14**: 906-8.
27. Hirsch MS, Brun-Vézinet F, Clotet V, Conway B, Krutzkes DR, D'Aquila RT, et al. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society-USA Panel. *Clin Infect Dis* 2003; **37**: 113-28.
28. Little SJ, Holte S, Routy JP, Daar ES, Markowitz M, Collier AC, et al. Antiretroviral-Drug Resistance among Patients Recently Infected with HIV. *N Engl J Med* 2002; **347**: 385-94.
29. Quality Control for Molecular Diagnostics. [homepage on internet]. [cited 18 February 2004]. Available at <http://www.qcmd.org>
30. Skidmore SJ, Zuckerman M, Parry JV. Accuracy of plasma HIV RNA quantification: a multicentre study of variability. *J Med Virol* 2000; **61**: 417-22.
31. Clinical Pathology Accreditation. CPA New Standards London: CPA; 2003. Available at <http://www.cpa-uk.co.uk>
32. UKNEQAS [homepage on internet]. London: UKNEQAS; 2004 [cited 18 February 2004]. Available at <http://www.ukneqasmicro.org.uk>