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## HIV/AIDS Programme

Strengthening health services to fight HIV/AIDS

# WHO RECOMMENDATIONS ON THE DIAGNOSIS OF HIV INFECTION IN INFANTS AND CHILDREN

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# **WHO RECOMMENDATIONS ON THE DIAGNOSIS OF HIV INFECTION IN INFANTS AND CHILDREN**

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# ACRONYMS AND ABBREVIATIONS

<b>Ab</b>	antibody	<b>HIV</b>	human immunodeficiency virus
<b>Ag</b>	antigen	<b>HR</b>	hazard ratio
<b>ANC</b>	antenatal care	<b>IATT</b>	Inter-Agency Task Team
<b>ARR</b>	absolute risk reduction: the difference in the event rate between the control group (CER) and treated group (EER); $ARR = CER - EER$	<b>Ig</b>	immunoglobulin
<b>ART</b>	antiretroviral therapy	<b>IMAI</b>	Integrated Management of Adolescent and Adult Illness
<b>ARV</b>	antiretroviral	<b>IMCI</b>	Integrated Management of Childhood Illness
<b>AZT</b>	zidovudine	<b>infant</b>	child less than 12 months of age
<b>b-DNA</b>	branched DNA (assay)	<b>LR</b>	likelihood ratio: the likelihood that a given test result would be expected in a patient with the target disorder compared with the likelihood that that same result would be expected in a patient without the target disorder. For a positive test result $LR+ = \text{sensitivity}/(1 - \text{specificity})$ and for a negative test result $LR- = (1 - \text{sensitivity})/\text{specificity}$ .
<b>CDC</b>	Centers for Disease Control and Prevention	<b>MTCT</b>	mother-to-child transmission (of HIV)
<b>CER</b>	control group event rate	<b>NAT</b>	nucleic acid test
<b>CHER</b>	children with HIV early antiretroviral therapy	<b>NASBA</b>	nucleic acid sequence-based amplification
<b>CI</b>	confidence interval: the range around a study's result within which the true value would be expected to lie	<b>NPV</b>	negative predictive value: the proportion of people with a negative test who are free of the disease under study
<b>CRF</b>	circulating recombinant forms	<b>NNT</b>	number needed to treat: the number of patients who need to be treated to prevent one outcome
<b>DBS</b>	dried blood spot	<b>OR</b>	odds ratio
<b>DPS</b>	dried plasma spot	<b>p24 Ag</b>	p24 antigen (assay)
<b>EER</b>	evaluation event rate	<b>PBMC</b>	peripheral blood mononuclear cell
<b>EIA</b>	enzyme immunoassay		
<b>ELISA</b>	enzyme-linked immunosorbent assay		
<b>EQAS</b>	external quality assessment scheme		
<b>FN</b>	false negative		
<b>FP</b>	false positive		
<b>GRADE</b>	grading of recommendations assessment, development and evaluation		

<b>PCR</b>	polymerase chain reaction	<b>Sn</b>	sensitivity: the probability of having a positive test result when the disease is truly present
<b>PEP</b>	post-exposure prophylaxis	<b>SOP</b>	standard operating procedure
<b>PHCW</b>	primary health-care worker	<b>STARD</b>	standards for the reporting of diagnostic accuracy studies
<b>PICO</b>	population, intervention, comparator and outcome	<b>TMA</b>	transcription-mediated amplification
<b>PICOT</b>	population targeted, intervention, comparator, outcome and time	<b>TN</b>	true negative
<b>PITC</b>	provider-initiated testing and counselling	<b>TP</b>	true positive
<b>PMTCT</b>	prevention of mother-to-child transmission (of HIV)	<b>UNAIDS</b>	Joint United Nations Programme on HIV/AIDS
<b>POC</b>	point of care	<b>UNICEF</b>	United Nations Children's Fund
<b>PPV</b>	positive predictive value: the proportion of people with a positive test who have the disease under study	<b>Us p24 Ag</b>	ultrasensitive p24 antigen
<b>QA</b>	quality assurance	<b>WB</b>	western blot
<b>QADAS</b>	quality in diagnostic and screening tests	<b>VL</b>	viral load
<b>RCT</b>	randomized controlled trial	<b>VQA</b>	virology quality assessment
<b>RR</b>	relative risk	<b>WHO</b>	World Health Organization
<b>RT</b>	reverse transcriptase	<b>WITS</b>	Women and infants transmission study
<b>SdNVP</b>	single-dose nevirapine		
<b>Sp</b>	specificity: the probability of having a negative test result when the disease is truly absent		



# 1. EXECUTIVE SUMMARY

Worldwide, in 2008, an estimated 430 000 [240 000–610 000] new infections due to the human immunodeficiency virus (HIV) occurred in children, of which 90% were acquired through mother-to-child transmission (MTCT) of HIV. Of the 430 000 new infections, between 280 000 and 360 000 were acquired during labour and in the pre-partum period. Of the remaining new infections, the majority were acquired during breastfeeding.

In infants<sup>i</sup> who acquire HIV around the time of delivery, disease progression occurs very rapidly in the first few months of life, often leading to death. To enable antiretroviral (ARV) prophylaxis to be given to infants as soon as possible after birth, all infants should have their HIV exposure status known at birth. As not all mothers are given HIV tests, very few HIV-exposed infants are identified and very few infants are known to be gaining access to early diagnosis, the necessary prerequisite to 'timely' initiation of antiretroviral therapy (ART). Currently, only an estimated 15% of HIV-exposed infants needing testing are tested in the first two months of life.

Recently published data confirming dramatic survival benefits for infants started on ART as early as possible after the diagnosis of HIV, prompted a review of the World Health Organization (WHO) paediatric treatment guidelines. In June 2008, new guidance was issued, which recommends prompt initiation of ART in infants diagnosed with HIV infection. In order to identify those infants who will need immediate ART, early confirmation of HIV infection is required. In November 2008, a meeting was convened to review recommendations by WHO for the diagnostic testing of HIV infection in infants and children. The meeting brought together the guideline review group that had developed the initial recommendations in 2005.

The guideline review group followed the grading of recommendations assessment, development and evaluation (GRADE) approach in reviewing the recommendations for early detection of HIV infection according to the current WHO Guidelines for Guidelines. This document contains the new recommendations. The full document, including GRADE evidence profiles and the factors that have been taken into account in the group's decision-making with respect to the strength of the recommendations, is available in annexes which are posted online at <http://www.who.int/hiv/topics/paediatric/en/>

## 1.1 Recommendations

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**Recommendation 1:** It is strongly recommended that HIV serological assays used for the purpose of clinical diagnostic testing have a minimum sensitivity of 99% and specificity of 98% under quality-assured, standardized and validated laboratory conditions.

<18 months of age – used as a screening assay to determine HIV exposure

>18 months of age – used as a diagnostic assay

(Strong recommendation – Moderate quality of evidence)

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<sup>i</sup> An infant is a child under 12 months of age.

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**Recommendation 2:** It is strongly recommended that HIV virological assays used for the purpose of clinical diagnostic testing (usually at or after 6 weeks of age) have a sensitivity of at least 95% and ideally greater than 98%, and specificity of 98% or more under quality-assured, standardized and validated laboratory conditions.

(Strong recommendation – Moderate quality of evidence)

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**Recommendation 3:** It is strongly recommended that HIV virological testing be used to diagnose HIV infection in infants and children below 18 months of age.

(Strong recommendation – High quality of evidence)

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**Recommendation 4:** In infants and children undergoing virological testing, the following assays (and respective specimen types) are strongly recommended for use: HIV DNA on whole blood specimen or DBS; HIV RNA on plasma or DBS; Us p24 Ag on plasma or DBS.

(Strong recommendation – High quality of evidence)

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**Recommendation 5:** It is strongly recommended that all HIV-exposed infants have HIV virological testing at 4–6 weeks of age or at the earliest opportunity thereafter.

(Strong recommendation – High quality of evidence)

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**Recommendation 6:** In infants with an initial positive virological test result, it is strongly recommended that ART be started without delay and, at the same time, a second specimen be collected to confirm the initial positive virological test result. Do not delay ART. Immediate initiation of ART saves lives and should not be delayed while waiting for the results of the confirmatory test.

(Strong recommendation – High quality of evidence)

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**Recommendation 7:** It is strongly recommended that test results from virological testing in infants be returned to the clinic and child/mother/carer **as soon as possible**, but at the very latest within four weeks of specimen collection. Positive test results should be fast-tracked to the mother–baby pair as soon as possible to enable prompt initiation of ART.

(Strong recommendation – High quality of evidence)

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**Recommendation 8:** It is strongly recommended that all infants with unknown or uncertain HIV exposure being seen in health-care facilities **at or around birth** or **at the first postnatal visit** (usually 4–6 weeks), or other child health visit, have their HIV exposure status ascertained.

(Strong recommendation – High quality of evidence)

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**Recommendation 9:** It is strongly recommended that well, HIV-exposed infants undergo HIV serological testing at around 9 months of age (or at the time of the last immunization visit). Infants who have reactive serological assays at 9 months should have a virological test to identify HIV-infected infants who need ART.

(Strong recommendation – Low quality of evidence)

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**Recommendation 10:** It is strongly recommended that infants with signs or symptoms suggestive of HIV infection undergo HIV serological testing and, if positive (reactive), virological testing.

(Strong recommendation – Low quality of evidence)

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**Recommendation 11:** In breastfeeding infants or children, it is strongly recommended that breastfeeding is not discontinued in order to perform any kind of diagnostic HIV test.

(Strong recommendation – High quality of evidence)

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**Recommendation 12:** It is strongly recommended that children 18 months or older, with suspected HIV infection or HIV exposure, have HIV serological testing performed according to the standard diagnostic HIV serological testing algorithm used in adults.

(Strong recommendation – High quality of evidence)

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**Recommendation 13:** In sick infants in whom HIV infection is being considered as an underlying cause of symptoms and signs, and **virological testing is not available**, HIV serological testing and use of the clinical algorithm for presumptive clinical diagnosis of HIV infection is strongly recommended.

(Strong recommendation – Low quality of evidence)

The revised recommendations require national programmes to review their HIV testing algorithms and ensure that clinical care pathways are updated to reflect these revised diagnostic approaches for infants and children. They also require immunization and maternal and neonatal/child health services to develop the capacity to provide diagnostic testing for infants and children.

## 2. INTRODUCTION

The World Health Organization/Joint United Nations Programme on HIV/AIDS/United Nations Children's Fund (WHO/UNAIDS/UNICEF) report on universal access (2008) and the UNAIDS/WHO 2009 epidemiological update estimated that, globally, the number of children below 15 years of age living with HIV increased from 1.6 million [1.4–2.1 million] in 2001 to 2.1 million [1.9–2.3 million] in 2008 (1, 2). Almost 90% of these children live in sub-Saharan Africa. Worldwide, in 2008, an estimated 430 000 [240 000–610 000] new infections occurred in children (3), of which 90% were acquired through mother-to-child transmission (MTCT) of HIV. It is estimated that, of the 430 000 new infections, between 280 000 and 360 000 were acquired during labour and in the pre-partum period. Of the remaining new infections, the majority were acquired through breastfeeding. In 2008, 280 000 [150 000–410 000] children died of AIDS.

Globally, over 4 million [3 700 000–4 360 000] adults and children are now on antiretroviral therapy (ART), but the scaling up of ART has met with less success in infants and very young children as compared with that in older children. Currently, in the majority of children, ART is initiated in those who have developed serious illness as a result of advanced HIV infection, and at an average age of approximately five years. Despite starting ART late, analyses of observational cohorts have shown that the response to ART is very good: survival in children starting ART is about 93–95% at 12 months and 91–92% at 24 months (4–6). However, infants and young children with severe clinical or immunological HIV disease starting ART have worse outcomes on ART (7). In infants who acquire HIV around the time of delivery, disease progression occurs very rapidly in the first few months of life, often leading to death.

Recently published data, confirming dramatic survival benefits for infants started as early as possible after diagnosis of HIV on ART prompted a review of the WHO paediatric treatment guidelines (8–10). The Children with HIV Early Antiretroviral Therapy (CHER) study conducted in South Africa demonstrated that, for infants infected at or around birth but with no signs of immunodeficiency, early mortality was reduced from 16% to 4% by starting treatment early (absolute risk reduction [ARR] 0.12, confidence interval [CI] 0.06–0.20; hazard ratio [HR] 0.24, [CI] 0.11–0.51). As a result, in 2008, WHO revised the recommendations for initiation of ART in infants with HIV infection and now recommends ART to be started as soon as HIV infection is diagnosed (11). However, very few infants are gaining access to early diagnosis, the necessary prerequisite to 'timely' ART. Currently, only an estimated 15% of HIV-exposed infants needing testing are tested in the first two months of life (1, 2). In order to identify those infants who will need immediate ART, early confirmation of HIV infection is required. Therefore, WHO has reviewed recommendations for diagnostic testing in infants and children. These new recommendations are designed to improve programme efforts at early identification of HIV-exposed and -infected children.

## 3. BACKGROUND

Improved assays to detect viral components, such as HIV-1 DNA and RNA, and p24 antigen (p24 Ag), are now available, as well as enhanced technologies for rapid testing for HIV antibodies. In addition, the successful use of filter papers to transfer specimens (dried blood spots [DBS]) from remote sites to more advanced testing laboratories means that early infant testing and subsequent initiation of immediate ART is fast becoming feasible for infants in resource-limited settings.

WHO has previously published recommendations for the diagnosis of HIV infection in infants and children (12, 13), and recommendations for implementation of provider-initiated HIV testing (14). In 2008, a meeting was held to review and update infant HIV treatment guidelines and recommendations for initiating ART in HIV-infected infants (11). The optimal timing of initial testing as part of follow up for HIV-exposed infants was also reviewed.

Immediate initiation of ART, without waiting for clinical or immunological disease progression in infants, is now recommended, and therefore early diagnostic testing should be performed with the aim of identifying as many HIV-infected infants as early as possible. To enable antiretroviral (ARV) prophylaxis to be given to infants as soon as possible after birth, all infants should have their HIV exposure status known at birth. Evidence and experience from national programmes and research on early infant diagnosis mean that new data are now available and WHO has therefore updated recommendations for diagnosis of HIV in infants and young children.

Recommendations on the use of appropriate HIV testing technologies and diagnostic approaches, and clear and simple testing strategies, are necessary to facilitate early infant diagnosis, and contain and efficiently manage public health resources. Diagnostic testing methods including HIV serological (= antibody testing = rapid testing = HIV serology = HIV enzyme immunoassay [EIA]) and virological testing (including HIV RNA, HIV DNA and HIV p24 Ag) were reviewed, as well as approaches to the clinical diagnosis of HIV infection in settings where virological testing is not (yet) available. The timing of testing was reviewed as well as the actions that need to be taken following the availability of results from each of the diagnostic approaches recommended.

**Note: HIV antibody assays** detect antibodies to HIV-1/2. HIV serological testing can be performed using 'rapid test devices', or 'HIV EIA', and is also sometimes referred to as 'HIV serology'. For consistency, this document will use the term HIV serological testing wherever tests for HIV antibody are referred to. **HIV virological assays** detect the presence of HIV nucleic acids in the form of HIV DNA or HIV RNA, or HIV viral antigen such as p24 (p24 Ag). A clinical diagnosis of HIV may be made based on the presenting symptoms and signs. Many of these are nonspecific in infants and younger children, and HIV serological and virological testing is therefore essential to support the clinical diagnosis of HIV.

### 3.1 Objective

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This publication summarizes current knowledge on the methods of diagnosing HIV infection in infants and children and sets out recommendations for practice and policy. Recommendations are designed to improve clinical management of the HIV-exposed and -infected child, and improve programme efforts at early identification of HIV-exposed and -infected children.

WHO HIV case definitions for clinical and surveillance purposes were revised in 2006 (13); HIV cases diagnosed and not previously reported in a country should be reported according to a standard national case definition. Countries are therefore encouraged to develop and regularly review their testing algorithms based on the recommendations provided, in accordance with their prevailing resource situation.

### **3.2 Audience for the guidelines**

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This publication is primarily intended for use by national advisory boards, national reference laboratories, national HIV/AIDS and child health programme managers, and other senior policy-makers who are involved in the planning of national child survival and HIV prevention and care strategies for infants and children in resource-limited countries. They may also be used by child health-care providers, professional bodies advising national programmes and developing treatment and care guidelines. Separate guidance and tools for implementation for national programmes and laboratories is referenced.

### **3.3 Preparatory work**

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Key population, intervention, comparator and outcome (PICO) questions were identified and a series of grading of recommendations assessment, development and evaluation (GRADE) evidence profiles and summary-of-findings tables prepared to guide the development of the recommendations (Table 1) (15).

Existing WHO recommendations were supplemented with draft recommendations developed from the above GRADE profiles for consideration by the guideline review group.

### **3.4 Guideline review group**

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A guideline review group was selected from the expert group that developed the initial WHO recommendations in 2005, with additional HIV laboratory experts, methodological experts and non-HIV GRADE experts in accordance with the WHO procedures for guideline development. These experts reviewed and assessed scientific evidence and experiences, and reviewed the evidence draft recommendations at a consultation in Geneva in November 2008, and then worked remotely to complete the recommendations and guideline. The consultation provided the basis for the present recommendations. The list of guideline group members is given in Annex 6.

### **3.5 Process**

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Potential declarations of interest were assessed prior to developing recommendations and in advance of the guideline meeting. Completed declarations of interest forms were reviewed by the WHO technical staff and all participants were again asked to clarify their potential conflicts of interest with the expert panel at the guideline meeting. Based on the completed forms and the discussions held at the meeting, no guideline group members with conflicts of interest were identified.

For each of the key recommendations, plenary review of the evidence profiles was followed by plenary discussion and assessment of the existing and draft recommendations. Discussions focused on consideration of costs, values, preference, feasibility and the balance of evidence for desirable and undesirable effects (risk–benefit assessment). Consensus was achieved in the plenary sessions on the wording and strength of the recommendations. The factors and decision-making of the guideline group are documented in Annex 3; decisions were agreed upon when a consensus was obtained. There were no areas where the group was unable to reach a consensus. The guideline panel subsequently reviewed and approved the recommendations and annexes. Peer review of the recommendations was undertaken throughout March–June 2009. Suggested modifications and clarifications were discussed by the original guideline group.

**Table 1. Grading of recommendations and levels of evidence**

Strength of recommendation	GRADE evidence profile
<p><b>Strong:</b> The panel is confident that the desirable effects of adherence to the recommendation outweigh the undesirable effects.</p> <p><b>Weak/Conditional:</b> The panel concludes that the desirable effects of adherence to a recommendation probably outweigh the undesirable effects, however, it is only applicable to a specific group, population or setting,</p> <p>or</p> <p>new evidence may result in changing the balance of risk to benefit,</p> <p>or</p> <p>the benefits may not warrant the cost or resource requirements.</p> <p>Further research is required to inform decision-making.</p>	<p><b>High:</b> Further research is very unlikely to change our confidence in the estimate of effect.</p> <p><b>Moderate:</b> Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.</p> <p><b>Low:</b> Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.</p> <p><b>Very low:</b> Any estimate of effect is highly uncertain, and largely according to expert opinion.</p>

Source: WHO Guidelines for guidelines – 2008 GRADE Working group (15, 16)

### 3.6 Review date

Each recommendation made has a date by which it should be reviewed and the full document will be reviewed in 2012.

## 4. KEY RECOMMENDATIONS

1. Performance requirements of commercial and non-commercial HIV assays
  - Serological assays (recommendation 1)
  - Virological assays (recommendation 2)
2. Tests to diagnose HIV infection in infants and children below 18 months of age
  - Virological test required (recommendation 3)
  - Assays recommended (recommendation 4)
  - Timing of virological testing (recommendation 5)
  - Confirmation of virological tests ( recommendation 6)
  - Reporting of results to the caregiver (recommendation 7)
3. Identifying exposure to HIV
  - Testing at or around birth (recommendation 8)
  - Testing at or around 9 months (recommendation 9)
4. Testing symptomatic infants and children less than 18 months of age
  - Testing symptomatic infants (recommendation 10)
5. Testing infants who are breastfeeding
  - Discontinuation of breastfeeding not required prior to testing (recommendation 11)
6. Tests required to diagnose HIV infection in children 18 months or older
  - HIV assays required (recommendation 12)
7. Performance of clinical algorithms where virological testing is not available
  - Use of algorithm with HIV serological testing (recommendation 13)

### 4.1 Performance requirements of commercial and non-commercial HIV assays

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The sensitivity and specificity of a virological or serological HIV assay depends on the assay characteristics and how it is used. The number of false-positive (FP) and false-negative (FN) results depends on the population size, specificity, sensitivity, and also on the HIV prevalence in the population being tested.

Annex 5 provides a short summary of published studies reporting disease prevalence in various populations of children being tested for HIV. Tables 2–5 in the text illustrate the rates of FP or FN results expected when the prevalence of HIV infection in the population undergoing testing is 1%, 5%, 10%, 30% and 50%. (These are the suggested useful ranges of prevalence likely to be found in infants and children undergoing testing in a variety of settings).

If the assay performance is poor, this will increase the number of FP and FN results (see Annex 4 for illustrative examples). Table 3 illustrates the positive predictive value (PPV) and negative predictive value (NPV) of serial testing algorithms based on two or three tests as recommended by WHO for HIV serological testing algorithms.



## Assumptions related to important patient outcomes

The guideline review panel agreed to place high value on the following important patient outcomes:

- False-negative results: infants in whom a diagnosis of HIV has been missed due to FN test results are at high risk for disease progression or death in the first year of life.
- False-positive results: infants incorrectly identified as infected due to FP test results will be started on ART unnecessarily, with the attendant risks of adverse events.
- Quality assurance (QA) programmes are recommended for laboratories performing HIV testing including assessment of the performance of all the assays used.

## Recommendation 1: HIV antibody assays

It is strongly recommended that HIV serological assays used for the purpose of clinical diagnostic testing have a minimum sensitivity of 99% and specificity of 98% under quality-assured, standardized and validated laboratory conditions.  <18 months of age – used as a screening assay to determine HIV exposure  >18 months of age – used as a diagnostic assay	
Quality of evidence:	Moderate, based on tests meeting or exceeding the level of sensitivity of 99% and specificity of 98%, using serial testing at various levels of HIV prevalence (Tables 2–3).
Strength of the recommendation:	<b>Strong</b>
Time to review:	2015

## Discussion

HIV serological assays can be used to screen for HIV exposure in infants, and as a part of a diagnostic testing algorithm in children older than 18 months (see Annex 4 for characteristics of a screening test as recommended by WHO).

To avoid confusion at the facility level, it is recommended that the standard and usual site-specific HIV testing algorithms be followed when testing children. Where serological testing is used to screen for HIV exposure, virological testing is recommended based on one reactive HIV serological test.

The sensitivity and specificity of a given HIV serological assay in infants may not be the same as that reported in adult populations. Additional assessment of the performance characteristics of the available serological assays by age is required in paediatric populations.

Data are scant on the performance of HIV serological assays for oral fluid in paediatric populations.

**Table 2. Likely performance of HIV assays (HIV serological or HIV virological tests) with a sensitivity of 99% and specificity of 98% at various prevalence levels**

Prevalence in population being tested (%)	1	2	5	10	20	30	50
No. of positive results per 10 000 tests (TP+FP)	297	394	685	1170	2140	3110	5050
No. truly infected in 10 000	100	200	500	1000	2000	3000	5000
No. uninfected in 10 000	9900	9800	9500	9000	8000	7000	5000
No. uninfected testing positive/10 000 tested (FP)	198	196	190	180	160	140	100
No. infected testing negative/10 000 tested (FN)	1	2	5	10	20	30	50
No. uninfected testing negative (TN)	9702	9604	9310	8820	7840	6860	4900
No. infected testing positive (TP)	99	198	495	990	1980	2970	4950
PPV (%)	33.3	50.3	72.3	84.6	92.5	95.5	98.0
NPV (%)	100.0	100.0	99.9	99.9	99.7	99.6	99.0

**Table 3. Serial testing using assays with a sensitivity of 99% and specificity of 98%**

Prevalence in the population initially being tested (%)	1-test algorithm PPV (%)	1-test algorithm NPV (%)	2-test algorithm PPV (%)	2-test algorithm NPV (%)
1	33.3	100	96.1	99.5
2	50.3	100	98	99
5	72.3	99.9	99.2	97.4
10	84.6	99.9	99.6	94.7
20	92.5	99.7	99.8	88.8
30	95.5	99.6	99.9	82.2
50	98	98	100	66.4

Note that a second test is performed only on initially reactive specimens.

## Recommendation 2: HIV virological assays

It is strongly recommended that HIV virological assays used for the purpose of clinical diagnostic testing (usually at or after 6 weeks of age) have a sensitivity of at least 95% and ideally greater than 98%, and specificity of 98% or more under quality-assured, standardized and validated laboratory conditions.	
Quality of evidence:	Moderate, based on the likely performance of single or serial testing meeting or exceeding the thresholds of sensitivity of 95% and specificity of 98% at various HIV prevalence levels (Tables 4–5).
Strength of the recommendation:	<b>Strong</b>
Time to review:	2013

Tables 4–5, 7 show the PPV, NPV and likely performance of a range of HIV virological assays.

**Table 4. Likely performance of HIV assays (HIV serological or HIV virological tests) meeting or exceeding thresholds for sensitivity of 95% and specificity of 98% at various HIV prevalence levels**

Prevalence in population being tested (%)	1	2	5	10	20	30	50
No. of positive results per 10 000 tests (TP+FP)	293	386	665	1130	2060	2990	4850
No. truly infected in 10 000	100	200	500	1000	2000	3000	5000
No. uninfected in 10 000	9900	9800	9500	9000	8000	7000	5000
No. uninfected testing positive/10 000 tested (FP)	198	196	190	180	160	140	100
Infected testing negative/10 000 tested (FN)	5	10	25	50	100	150	250
No. uninfected testing negative (TN)	9702	9604	9310	8820	7840	6860	4900
No. infected testing positive (TP)	95	190	475	950	1900	2850	4750
PPV (%)	32.4	49.2	71.4	84.1	92.2	95.3	97.9
NPV (%)	99.9	99.9	99.7	99.4	98.7	97.9	95.1

**Table 5. Serial testing using tests with a sensitivity of 95% and specificity of 98%**

Prevalence in the population initially being tested (%)	1-test algorithm PPV (%)	1-test algorithm NPV (%)	2-test algorithm PPV (%)	2-test algorithm NPV (%)
1	32.4	99.9	95.8	97.6
2	49.2	99.9	97.9	95.3
5	71.4	99.7	99.2	88.7
10	84.1	99.4	99.6	78.8
20	92.2	98.7	99.8	62.3
30	95.3	97.9	99.9	49.1
50	97.9	95.1	100.0	29.2

Source: Calculations courtesy of and checked by J. Schüpbach and M. Penazzato.

## Discussion

The number of FP and FN test results depends on the HIV prevalence in the population being tested and the assay performance, as illustrated in the tables above. In national programmes, the actual performance of virological assays may be lower, further decreasing the accuracy of the assay (and thus increasing the number of FP and FN test results). Therefore, at all times, a confirmatory test is recommended for all test results that are initially positive (see **recommendation 6**).

- It should also be noted that the accuracy of the assay may be affected by the viral subtype (see **recommendation 4**) and the time of testing in relation to the time of acquisition of infection.
- The HIV prevalence in the infant population being tested depends on the MTCT rates, which are dependent on the use and type of prevention of mother-to-child transmission (PMTCT) regimens, and the rate, duration and nature of breastfeeding.
- In infants and children presenting with pneumonia, diarrhoea or other conditions such as severe malnutrition, the prevalence of HIV is usually higher (see Annex 5; large range, can be as high as 70% depending on the presenting condition, country and facility).
- QA programmes are recommended for laboratories performing HIV virological assays (and serological assays) including assessment of the performance of the virological assays.
- For virological assays to be used as point-of-care (POC) tests in peripheral sites, a higher sensitivity but lower specificity may be preferred. Many such POC assays are in development and will be evaluated subsequently. The results should become available over the next few years.

## 4.2 Tests to diagnose HIV in infants and children below 18 months of age

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HIV serological testing is generally used to diagnose HIV infection in adults and children above 18 months of age. Because of the passage of maternal HIV antibody across the placenta to the baby, HIV serological testing in infancy cannot be used to confirm HIV infection in the infant, but does indicate maternal HIV infection and exposure of the infant. In order to diagnose HIV infection definitively in children below 18 months of age, assays that detect the virus or its components (i.e. virological tests) are therefore required.

### Recommendation 3

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It is strongly recommended that HIV virological testing be used to diagnose HIV infection in infants and children below 18 months of age.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2013

**Note:** WHO case definitions for surveillance and clinical purposes are already defined in the previously published guidance (13).

In children younger than 18 months, a diagnosis of HIV infection is based on: a positive virological test for HIV or its components (HIV RNA or HIV DNA or ultrasensitive [Us] HIV p24 Ag) confirmed by a second virological test performed on a separate specimen taken more than 4 weeks after birth. HIV serological testing is not recommended for definitive or confirmatory diagnosis of HIV infection in children until they are 18 months of age.

### Recommendation 4

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In infants and children undergoing virological testing, the following assays (and respective specimen types) are strongly recommended for use: HIV DNA on whole blood specimen or DBS; HIV RNA on plasma or DBS; Us p24 Ag on plasma or DBS.	
Quality of evidence:	<b>High</b> (Table 6)
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

**Table 6. Recommended assays, quality of their supporting evidence and strength of recommendations**

Assay	Quality of evidence (GRADE)	Strength of recommendation on use
HIV DNA whole blood	High	Strong
HIV DNA DBS	High	Strong
HIV RNA plasma	High	Strong – except for infants on ART
HIV RNA DBS	High	Strong – except for infants on ART
Us p24 Ag plasma	High	Strong – except for infants on ART. There is a suggestion that this assay might perform at a lower sensitivity for HIV-1 subtype D. Further research is recommended.
Us p24 Ag DBS	High	Strong – except for infants on ART. There is a suggestion that this assay might perform at a lower sensitivity for HIV-1 subtype D. Further research is recommended.

## Discussion

For GRADE profiles of each of the diagnostic technologies, see Annex 1. The performance of any assay depends on laboratory conditions, procedures and operations. Where in-house assays are being used, they must be standardized and validated by the National Reference Laboratory in-country. The choice of assay may depend on ART use and geographical setting; the predominant viral subtype should also be considered.

- *DNA assays*: good accuracy in whole blood and DBS in almost all circumstances.
- *RNA assays*: good accuracy; however, there are concerns if the infant is on ART because of the reduced amount of detectable RNA.
- *Branched-DNA (b-DNA) assays*: lower specificity (around 97%) and are therefore associated with more FP results.
- *Us p24 Ag*: good accuracy; however, there are concerns if the infant is on ART because of the reduced amount of detectable p24 Ag.

See Tables 3 and 5 for the effect on PPV and NPV of performing a second test.

**Table 7. Scenario assuming the use of one assay that has a sensitivity of 98% and specificity of 99% in a sample size of 10 000**

<b>Prevalence in population being tested (%)</b>	<b>1</b>	<b>2</b>	<b>5</b>	<b>10</b>	<b>20</b>	<b>30</b>	<b>50</b>
No. of positive results per 10 000 tests	197	294	585	1070	2040	3010	4950
No. truly infected in 10 000 (TP)	98	196	490	980	1960	2940	4900
No. uninfected testing positive/10 000 tested (FP)	99	98	95	90	80	70	50
Infected testing negative/10 000 tested (FN)	2	4	10	20	40	60	100
No. of infants who will be treated in order to correctly treat 100 infants with HIV	201	150	119	109	104	102	101
No. of uninfected infants who will receive ART unnecessarily for every 100 infants treated	101	50	19	9	4	2	1

- All the quantitative HIV RNA assays are very sensitive (17), but are currently licensed in most countries only for monitoring of HIV infection.
- The performance of quantitative RNA assays depends on the selected threshold for detection; sensitivity may be reduced when DBS samples are used, as they have a much smaller specimen volume (usually less than 100  $\mu$ l) than that used for plasma.
- p24 Ag assays: Only ultrasensitive (Us) assays should be used. Viral subtype may influence the detection of p24 Ag, especially subtype D, according to one report. There are no concerns with subtypes A, B and C, E (= circulating recombinant forms [CRF] 01\_AE and CRF02\_AG). Theoretical concerns exist about the detection of p24 Ag when the infant is on ART or ARV prophylaxis (18).
- Some of the newer molecular assays may detect both DNA and RNA when whole blood samples are used. These assays might offer advantages in terms of sensitivity and reduced window period for diagnosis because it has been reported that viraemia occurs before viral DNA can be detected.

## Recommendation 5

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It is strongly recommended that all HIV-exposed infants have HIV virological testing at 4–6 weeks of age or at the earliest opportunity thereafter.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

### Discussion

- Early diagnosis of HIV infection enables immediate HIV care and initiation of ART. This significantly reduces mortality and hospitalization.
- After a first positive virological test, an immediate confirmatory test is recommended (see Table 8 and **recommendation 6**).
- If the child is older than 9 months, an HIV serological test is recommended prior to any virological testing, and a virological test should be performed for those with a reactive HIV serological test.
- In the non-breastfed or never-breastfed infant, a negative serological test result at or above the age of 9 months can be used to rule out HIV infection.
- If breastfeeding, the infant may still be at risk for acquiring HIV infection and will need age-appropriate retesting 6 weeks or more after cessation of breastfeeding.

## Recommendation 6

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In infants with an initial positive virological test result, it is strongly recommended that ART be started without delay and, at the same time, a second specimen be collected to confirm the initial positive virological test result. Do not delay ART. Immediate initiation of ART saves lives and commencement of ART should not be delayed while waiting for the results of the confirmatory test.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2013



**Table 8. Preferred second assay for confirmation after an positive first virological assay**

Any of the following first virological assays	Preferred confirmatory second assay	Rationale
<ul style="list-style-type: none"> <li>• HIV DNA whole blood or DBS</li> <li>• HIV RNA plasma or DBS</li> <li>• Us p24 Ag plasma or DBS</li> </ul>	Quantitative HIV RNA (viral load) on plasma specimens taken prior to starting ART	Viral load at start of ART or while on ART is useful for monitoring purposes.  If the infant is already on ART at the time of the test, an undetectable viral load cannot be taken to exclude HIV.

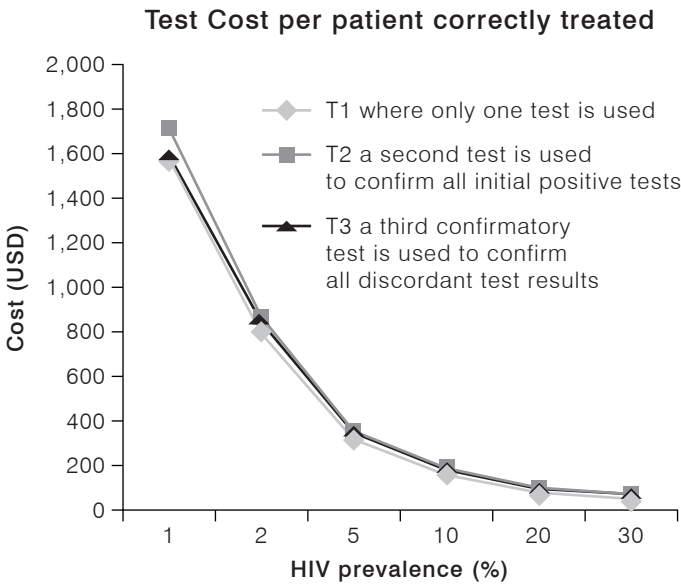
### Discussion

- Confirmatory testing may increase pressure on constrained health systems (cost and capacity), but using two virological tests of at least 98% sensitivity and 98% specificity minimizes FP and FN test results. While a high value is placed on not treating infants unnecessarily (cost of treatment and treatment-related adverse events), the consequence of missing HIV infection may be death.
- Confirmatory testing is particularly important in settings where the prevalence of HIV in the population being tested is less than 5%.<sup>i</sup>
- The volume of the second specimen required to confirm an initial positive test result should be adequate. DBS may therefore not be the best method for specimen collection.
- If the first test is positive and the confirmatory virological test is negative, a third test will be needed to resolve the discordance between the two earlier virological tests. Repeat testing on the stored initial specimen is also suggested.
- Further testing to resolve discordance between an initial positive test and negative confirmatory virological test should be performed using an HIV DNA assay.
- Repeating the same assay on a new specimen will address the majority of laboratory, specimen handling and amplification errors, but may not address all problems, e.g. subtype variation.
- Repeating the assay on the same specimen will address contamination, but may not address sample mislabelling, clerical errors and subtype variation.
- At an HIV prevalence of 2% in the infant population being tested (i.e. a good coverage of PMTCT interventions), 294 positive infants will be identified for every 10 000 virological tests performed, of whom 196 are actually infected, i.e. only two out of three infants who initially test positive are actually infected. One out of three infants starting ART is therefore not infected, and the confirmatory test will be negative. This infant will need to have a negative HIV DNA test result before ART is discontinued.

<sup>i</sup> This is the threshold that WHO recommends, below which serial testing algorithms for clinical purposes should use three assays (for successive reactive results) for a positive diagnosis of HIV infection.

- Where the prevalence of HIV in infants is higher (assuming a prevalence of 30% in the population undergoing testing, such as testing within inpatient provider-initiated testing and counselling [PITC] settings), of 3010 positive tests results that will be identified for every 10 000 assays performed, 2940 infants will be truly infected and 70 infants who are uninfected would start ART, and 60 infants who are infected would be missed due to FN test results. Thus, 3010 repeat tests would be required to find these 70 uninfected infants.
- Analyses of the consequences of confirmatory testing and estimates of direct cost, likely deaths and numbers of children unnecessarily treated based on using one test only (1 test), using a second test to confirm all initial positive results (2 tests), and using the third test to resolve all discordance between the first and second tests (3 tests), are ongoing. Preliminary results in this chart show that using a confirmatory test (T3) does not increase the cost of managing patients at any HIV prevalence.

**Figure 1. Costs of using one, two and three tests for diagnosing HIV at different levels of HIV prevalence**



## Recommendation 7

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It is strongly recommended that test results from virological testing in infants be returned to the clinic and child/mother/carer <b>as soon as possible</b> , but at the very latest within four weeks of specimen collection. Positive test results should be fast-tracked to the mother–baby pair as soon as possible to enable prompt initiation of ART.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

### Discussion

- Delays in specimen handling and return of test results cause critical delays in confirming HIV infection and commencement of life-saving HIV treatment and care, and increase the chances of infected infants dying or being lost to follow-up.
- POC assays are under evaluation and could offer considerable advantages.
- Recent programme evaluation suggests the greatest delays and losses of mother baby pairs occur due to delays on this step.<sup>i</sup>

## 4.3 Identifying exposure to HIV

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### Recommendation 8

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It is strongly recommended that all infants with unknown or uncertain HIV exposure being seen in health-care facilities <b>at or around birth or at the first postnatal visit</b> (usually 4–6 weeks), or other child health visit, have their HIV exposure status ascertained.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

This may be ascertained, preferably in the following order:

1. by determining whether the HIV status of the mother has been assessed in this pregnancy through review of records, or maternal or caregiver questioning (STRONG recommendation);  
or
2. by performing an HIV serological test on the mother after obtaining informed consent if maternal HIV testing has not been done or the HIV status of the mother remains unclear for the duration of the pregnancy (STRONG recommendation); or

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<sup>i</sup> MOH/CDC/Clinton/UNICEF programme evaluations reported to PMTCT IATT Lab Working Group.

3. by recommending HIV serological testing of the infant to detect HIV exposure, if the mother is unavailable or does not consent to maternal HIV testing. Maternal or guardian consent is required for such testing (STRONG recommendation).

## Discussion

- Early screening for HIV exposure facilitates provision of ARV prophylaxis for the HIV-exposed infant, as well as early HIV virological testing and care for the HIV-infected infant.
- If the infant is seen <72 hours after delivery and HIV exposure is identified, post-exposure prophylaxis (PEP), counselling on safe breastfeeding and an HIV virological test at 4–6 weeks is recommended. The effectiveness of PEP is highest if initiated within 24 hours of delivery. In addition, mothers should be counselled on safe infant feeding as per national/local recommendations.
- For infants first seen at 4–6 weeks or the earliest thereafter and in whom HIV exposure is documented, HIV virological testing should be performed (see **recommendations 3, 4 and 5**) and the mother should receive safe infant-feeding counselling. A negative HIV serological test in the mother does not per se exclude HIV exposure; the possibility of very recent incident infection of the mother during this pregnancy should be kept in mind, especially in areas with a high incidence of HIV.
- The value of screening for HIV exposure should be balanced with the opportunity costs, especially in low-prevalence areas (19).
- Whenever HIV testing is performed, consent from the parent or legal guardian should be sought, and the confidentiality of the testing and results ensured. Verbal consent is sufficient.

## Recommendation 9

It is strongly recommended that well, HIV-exposed infants undergo HIV serological testing at around 9 months of age (or at the time of the last immunization visit). Those who have reactive serological assays at 9 months should have a virological test to identify HIV-infected infants who need ART.	
Quality of evidence:	Low
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

## Discussion

HIV serological testing in this population is undertaken to rule out HIV infection.

- At 9 months of age, maternal HIV antibodies may no longer be detectable in some HIV-uninfected but -exposed infants. If the serological test is non-reactive (negative), **HIV**

**infection can be excluded and co-trimoxazole prophylaxis stopped if the infant has not been breastfed for at least 6 weeks prior to the test.**

- A positive (reactive) serological test can be caused by the persistence of maternal antibodies (HIV-exposed, but -uninfected child) or by the presence of the child's antibodies (child HIV infected) and follow up for HIV needs to continue.
- If the infant is breastfeeding, a negative serological test or a negative virological test does not definitively exclude HIV infection because exposure to HIV is continuing.
- Previously untested HIV-exposed infants or infants of unknown HIV status should be tested.
- Combined HIV antigen–antibody assays will also detect maternal HIV antibody, and thus offer no advantages for infant testing compared with standard serological assays, unless it is an assay that distinguishes between antibody and antigen.
- Recently available fourth-generation rapid assays (Determine antigen–antibody combo) have not been evaluated for early infant diagnosis, although they can detect acute infection in adults. The presence of maternal antibodies during the early phase of infection may complicate detection of p24 Ag due to formation of an antigen–antibody complex. Therefore, a proper evaluation is necessary before this or similar other tests can be recommended for early infant diagnosis.
- In children less than 18 months, HIV serological tests perform reliably but may detect persisting maternal HIV antibody. Therefore, in infants less than 18 months of age, a positive HIV serological test confirms HIV exposure but cannot definitively diagnose HIV. HIV serological testing can be used to exclude HIV infection (20, 21) (see **recommendations 8, 9 and 10**).

#### 4.4 Testing symptomatic infants and children less than 18 months of age

##### Recommendation 10

It is strongly recommended that infants with signs or symptoms suggestive of HIV infection undergo HIV serological testing and, if positive (reactive), virological testing.	
Quality of evidence:	Low
Strength of the recommendation:	<b>Strong</b>
Time to review:	2013

##### Discussion

- In sick children who are HIV infected, starting HIV treatment and care early reduces mortality. Therefore, if HIV infection is suspected, the diagnosis should be confirmed as quickly as possible.
- HIV infection is an unlikely cause of signs and symptoms such as fever, cough and poor growth in low HIV-prevalence settings.

- However, in settings where maternal HIV prevalence is high, HIV infection is a more likely cause of such signs and symptoms.
- National programmes are encouraged to identify regions, areas or populations where HIV is most likely to be present.
- If HIV serological testing is positive (reactive), and the clinical algorithm positive (suspected HIV infection; see Annex 2, Tables A–19 to A–21), ART and co-trimoxazole should be started and confirmatory HIV virological testing performed immediately.

## 4.5 Testing infants who are breastfeeding

### Recommendation 11

In breastfeeding infants or children, it is strongly recommended that breastfeeding is not discontinued in order to perform any kind of diagnostic HIV test.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2013

### Discussion

Early diagnosis of HIV infection in breastfed children facilitates early HIV treatment and care.

- An infant or child found to be infected should continue breastfeeding.
- If the child is uninfected or the HIV status not definitively ascertained, the mother should receive safe infant-feeding counselling.
- Confirmatory testing after complete cessation of breastfeeding should be performed at or around six weeks after complete cessation, using the test best suited to the age of the child at the time of testing.

## 4.6 Tests required to diagnose HIV in children 18 months or older

### Recommendation 12

It is strongly recommended that children 18 months or older, with suspected HIV infection or HIV exposure, have HIV serological testing performed according to the standard diagnostic HIV serological testing algorithm used in adults.	
Quality of evidence:	<b>High</b>
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

## Discussion

- In children older than 18 months, maternal HIV antibodies are usually no longer detectable. The presence of HIV antibodies in this population is therefore a quick and reliable means of definitively diagnosing HIV infection.
- The nationally defined serial testing algorithm should be followed.

Confirmation of HIV status facilitates HIV treatment and care of HIV-infected infants and children. In children who continue to be exposed to HIV through breastfeeding, repeat testing should be done only six weeks after cessation of breastfeeding, as this window period reliably indicates the true status of infection.

### 4.7 Performance of clinical algorithms where virological testing is not available

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#### Recommendation 13

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In sick infants in whom HIV infection is being considered as an underlying cause of symptoms and signs, and <b>virological testing is not available</b> , HIV serological testing and use of the clinical algorithm for presumptive clinical diagnosis of HIV infection is strongly recommended (Table 9).	
Quality of evidence:	Low
Strength of the recommendation:	<b>Strong</b>
Time to review:	2012

## Discussion

- For the performance characteristics of the clinical algorithm, with or without HIV serological testing, see the evidence profiles in Annex 2 (Tables A–20 and A–21).
- While the quality of evidence for use of the clinical algorithm is low, the positive likelihood ratio (LR) for use of the clinical algorithm is 10.9, i.e. using the algorithm is nearly 11 times more likely to accurately identify HIV infection than clinical examination alone.

**Table 9. Clinical algorithm to identify severe HIV infection needing ART**

A presumptive diagnosis of severe HIV disease should be made if:	
The child is confirmed as being HIV antibody-positive  <p style="text-align: center;"><b>AND</b></p>	The infant has symptoms of two or more of the following: <ul style="list-style-type: none"> <li>• oral thrush</li> <li>• severe pneumonia</li> <li>• severe sepsis</li> </ul> <p style="text-align: center;"><b>OR</b></p> A diagnosis of any AIDS indicator condition(s) <sup>a</sup> can be made
Other clues that support the diagnosis of severe HIV disease in an HIV-seropositive infant include: <ul style="list-style-type: none"> <li>• recent HIV-related maternal death; or</li> <li>• advanced maternal HIV disease; or</li> <li>• the child's CD4+ count is &lt;20%.</li> </ul>	
Diagnosis of HIV infection should be confirmed with virological testing as soon as possible.	

a AIDS indicator conditions include *Pneumocystis* pneumonia, cryptococcal meningitis, severe wasting or severe malnutrition, oesophageal candidiasis, Kaposi sarcoma, and extrapulmonary tuberculosis.

Clinical criteria for presumptive diagnosis performed with reasonably high sensitivity, specificity and acceptable accuracy in identifying symptomatic HIV-exposed children less than 18 months requiring ART (sensitivity of 68.9% [95% CI 63.62–74.08]; specificity of 81.01% [95% CI 76.57–85.44]). This algorithm performed with maximum accuracy in discriminating between TP and FP results among children aged 9–12 months, and showed the highest sensitivity for identifying the requirement for ART among children above 12 months (22).



## 4.8 Summary tables

**Table 10. Summary of recommended testing approaches**

Category	Test required	Purpose	Action
<b>Well, HIV-exposed infant</b>	Virological testing at 4–6 weeks of age	To diagnose HIV	Start ART if HIV-infected
<b>Infant – unknown HIV exposure</b>	Maternal HIV serological test <b>or</b> infant HIV serological test	To identify or confirm HIV exposure	Need virological test if HIV-exposed
<b>Well, HIV-exposed infant at 9 months</b>	HIV serological test (at last immunization, usually 9 months)	To identify infants who have persisting HIV antibody or have seroreverted	Those HIV seropositive need virological test and continued follow up; those HIV negative, assume uninfected, repeat testing required if still breastfeeding
<b>Infant or child with signs and symptoms suggestive of HIV</b>	HIV serological test	To confirm exposure	Perform virological test if <18 months of age
<b>Well or sick child seropositive &gt;9 months and &lt;18 months</b>	Virological testing	To diagnose HIV	Reactive – start HIV care and ART <sup>a</sup> if under 24 months, or based on national start criteria if 24 months or more <sup>b</sup>
<b>Infant or child who has completely discontinued breastfeeding</b>	Repeat testing six weeks or more after breastfeeding cessation – usually initial HIV serological testing followed by virological testing for HIV-positive child and <18 months of age	To exclude HIV infection after exposure ceases	Infected infants and children <24 months of age, need to start HIV care, including ART <sup>b</sup>

<sup>a</sup> In some countries, specific thresholds/criteria for initiating ART may be applied.

<sup>b</sup> See *Antiretroviral therapy for HIV infection in infants and children*. WHO, 2010

**Table 11. Well child health visits and schedule of HIV testing**

<b>Time</b>	<b>Child health schedule (23)</b>	<b>Rationale</b>
<b>Birth</b>	Vitamin K BCG Polio	HIV serological test of the mother or infant if maternal status unknown
<b>4–6 weeks</b>	Diphtheria, pertussis and tetanus (DPT 1) Polio Hepatitis B Hib	1. HIV serological testing if exposure status not known 2. Virological testing for all HIV-exposed (or HIV serology-positive) infants
<b>10–12 weeks</b>	Polio DPT 2 Hepatitis B Hib	Testing if unwell and HIV suspected If HIV serology positive/ HIV-exposed and not previously had virological test → HIV virological test
<b>14 weeks</b>	Polio DPT 3 Hepatitis B Hib	Testing if unwell and HIV suspected If HIV serology positive/ HIV-exposed and not previously had virological test → HIV virological test
<b>9 months</b>	Measles	HIV serological testing for all HIV-exposed infants followed by HIV virological testing in case of positive serological testing
<b>18 months or older</b>		HIV-exposed and HIV infection status not previously determined → HIV serological testing

## 5. LABORATORY METHODS FOR DIAGNOSIS OF HIV INFECTION IN INFANTS AND CHILDREN

The definitive diagnosis of HIV infection at any age requires diagnostic testing that confirms the presence of HIV. Serological testing identifies HIV antigen and/or antibody generated as part of the immune response to infection with HIV. In children older than 18 months of age, serological testing should be used in the same manner as in adults. However, maternal HIV antibody is transferred to the baby passively during pregnancy and then declines (with a half-life of 28–30 days in non-breastfed infants) (24-29). Infected infants then go on to produce HIV antibody (30); however, most commonly used HIV serological assays cannot distinguish between maternal HIV antibody and HIV antibody produced by the infant, making the interpretation of reactive HIV serological test results difficult (29, 31). In order to diagnose HIV infection definitely in children aged less than 18 months, assays are required that detect the virus or its components (i.e. virological tests). A range of laboratory-based techniques are available, and these are discussed in more detail in the following section.

### 5.1 Laboratory quality assurance

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In order to build laboratory capacity, national HIV/AIDS programmes should invest in a QA programme for all laboratories performing diagnostic testing, and should use existing available services provided by WHO and others including the Centers for Disease Control and Prevention (CDC) to support external quality assessment schemes (EQAS).

Having highly accurate tests does not necessarily guarantee reliable laboratory results. Many processes are involved from the time the specimen is collected and transported to the laboratory, tested and until the results are reported, during which errors can occur. Therefore, ongoing QA within the context of the laboratory quality system, both internally and externally, is essential. Clinicians and staff providing laboratory services need regular communication about the performance of tests to improve and ensure appropriate performance. Well-defined standard operating procedures (SOPs), following nationally defined and validated testing algorithms, are essential for optimal use of all laboratory-based testing.

### 5.2 Commercially versus non-commercially available tests

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Many methods for the detection of nucleic acids are available both commercially and non-commercially (in-house assays). Most of the assays utilized to detect nucleic acids (NAT) are complex, technically demanding or inappropriate for non-specialist diagnostic laboratories. This is especially the case for in-house assays that lack the stringent quality control processes and technical support from the manufacturer that are available to commercial assays. Non-commercial/in-house assays are not recommended for widespread use by national programmes, but may be used where ongoing and functional laboratory QA systems are in place and production is standardized.

The sensitivity and specificity of different assays will vary, and test devices or equipment can be made by one company, but distributed and sold under several brand names. WHO, through the WHO Prequalification of Diagnostics Programme, evaluates the performance of commercially available technologies including serological assays (EIAs and rapid tests), CD4 enumeration

technologies and molecular technologies. Prequalification confers an eligibility to tender with United Nations (UN) agencies and enables bulk purchase. WHO Prequalification is a voluntary process, usually at the request of the manufacturer, and the results of these evaluations are made available online (32). It is recommended that countries use serological assays (rapid tests or EIAs) within a testing algorithm that has been validated in-country by the National Reference Laboratory (or another laboratory designated for this purpose).

### 5.3 Serological testing

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Antibodies to HIV can be measured by a variety of techniques. None of these detect HIV itself, but rather detect an immune response to the virus, and therefore take some time to develop and become reactive (or positive) after HIV infection has been acquired. Antibodies to HIV-1 and HIV-2 are detected by EIA, also known as enzyme-linked immunosorbent assay (ELISA), simple/rapid test devices, and western blot (WB) tests. However, because maternal HIV antibodies (immunoglobulin G [IgG]) are passively transferred across the placenta, HIV serological assays in infants are difficult to interpret. Infants born to HIV-infected women may therefore initially test seropositive, irrespective of their own infection status.

Most commercially available EIAs have a high sensitivity and specificity and are able to detect all subtypes of HIV-1 and HIV-2. A wide range of HIV serological assays are available, and it is therefore important to identify the most suitable assays for a given set of programme circumstances.

General factors that need to be considered in selecting products and equipment for performing serological testing to diagnose HIV infection in infants are similar to those for virological testing and include:

- commercial availability of reagents and equipment
- cost of the tests
- training and availability of staff
- volume and type of specimens required
- number of specimens required to be processed (specimen throughput)
- ongoing and functional laboratory QA
- number of tests required
- need to detect HIV-1 and HIV-2
- location of testing site
- specimen collection and processing
- need for a cold chain in some circumstances.

The major performance and operational characteristics of commercially available serological assays are summarized in WHO reports available on the web site of the department of Essential Health Technologies (32).

WHO describes generic testing strategies based on the purpose of HIV testing and prevalence of disease. A testing strategy describes generically a testing approach for specific needs (for example, transfusion and transplantation safety, surveillance, diagnosis of HIV infection [client-initiated or provider-initiated testing and counselling]), taking into consideration the presumed HIV prevalence in the population being tested.

These strategies outline combinations of serological assays (EIAs and/or rapid assays or any combination of these) used to diagnose HIV status. A **testing algorithm** describes the combination and sequence of specific HIV assays used within a given HIV testing strategy. It has been shown that combinations of EIAs or combinations of rapid assays or mixed combinations of EIAs and rapid assays can provide results as reliable as, and in some instances more reliable than, the traditional EIA/WB combination, and at a much lower cost (33, 34). This document refers to an 'assay' as a specific HIV test kit, whereas a 'test' refers to the actual manipulations of carrying out a test on a specimen. Retesting refers to repeating more than once a test using the same assay and where possible, the same specimen (35). The testing strategies described in this document do not refer to retesting, but rather to carrying out one, two or three tests using different HIV assays.

In a high-prevalence setting (for the population undergoing testing), the first reactive test result should always be confirmed with a second test, using a different assay on the same specimen. Two reactive test results lead to a diagnosis of HIV seropositivity. For specimens that are discordant, i.e. first test is reactive, and the second test non-reactive, the specimen should be tested by a third assay (which must be different from the first and second assays, usually using the same specimen). If the result is non-reactive, the individual is considered HIV seronegative. If the result of the third test is reactive, the individual should be referred for further testing in three weeks.

In a low-prevalence setting (for the population being tested), the first reactive test should be confirmed with a second assay. If the first and second test results are discordant, the individual can be assigned an HIV-seronegative status. When the first and second test results are both reactive, a third different assay must be performed on the same specimen to confirm seropositivity. If the first and second test results are reactive and the third test result is non-reactive, then the individual should be referred for further testing in three weeks. A well-selected combination of different rapid tests has positive and negative predictive values comparable to EIA combinations. Additional information can be found in WHO publications (32).

WHO/UNAIDS describe a generalized HIV epidemic as prevalence of  $\geq 1\%$  in antenatal clinic attendees; however, for the purpose of selecting HIV testing strategies, prevalence thresholds of  $\geq 5\%$  in the target population undergoing testing are suggested as the threshold at which it is recommended as acceptable to use a two-test serial testing strategy for the diagnosis of HIV. The assays chosen should meet or exceed criteria for sensitivity equal to or greater than 99% and specificity equal to or greater than 98%.

Serological testing may be done serially or in parallel. Serial testing refers to the performance of the second test after an initial reactive result. In parallel testing, two different assays are performed at the same time. WHO recommends that serial testing be used except in settings where a very rapid result is urgently required (e.g. in the labour ward). In such cases, parallel testing may be preferred.

### 5.3.1 Specimen collection

Serological assays for venous/capillary whole blood and oral fluid specimens have now been developed and make POC HIV testing feasible using rapid test devices. Serological testing by the EIA format is designed for use on serum or plasma specimens, which requires preparation of the specimen from venous whole blood and use of reagents that often require refrigeration. When using venous/capillary whole blood, the specimen requires no additional processing and so equipment (e.g. a centrifuge) can be avoided.

### 5.3.2 Timing of serological testing in infants

The most recent advances in EIA technology have produced 'combination assays', which allow for the simultaneous detection of p24 HIV antigen and HIV antibodies. This approach has further shortened the window period, i.e. the interval between HIV infection and detectable HIV antigen/antibodies. Rapid tests appear to offer similar performance characteristics but they detect antibody 2–8 days later than third-generation EIAs.

All children born to HIV-infected mothers carry detectable maternal HIV antibody and this declines slowly over the first year of life. The rate of decay of maternal antibody has been ascertained largely by analysis of studies to detect HIV antibody in children who have not been breastfed. The mean and/or median age at the time of seroreversion ranges between 9 and 16 months of age in studies from both developed and developing countries (36). These data indicate that maternal antibody may remain detectable through the first 6 months of life but significant decay occurs by 9–12 months of age. Most HIV-uninfected children do not have detectable antibody at 12 months of age (29).

Similar findings have been observed in studies assessing the decay of passively transferred maternal antibody for measles and hepatitis A. The decay of passively acquired antibodies occurred more rapidly in Nigerian children than German children, resulting in susceptibility to measles in most Nigerian infants by 4 months of age. While the majority of uninfected non-breastfed children will have cleared maternal antibody by the age of 12 months, a small percentage of children do not serorevert until the age of 18 months (25, 37) and, in rare instances, even beyond (38). With the use of more sensitive serological assays, a higher sensitivity in detection of maternal antibody needs to be considered (39). Further analysis and studies are required to determine the pattern of decay of maternal HIV antibody in HIV-exposed infants, particularly in African, Latin American and Asian children.

### 5.3.3 Interpretation of serological test results

Negative HIV serological testing in an infant, following nationally validated testing algorithms, suggests the following:

- The infant is **not** HIV-exposed;  
or
- The infant is HIV-exposed but has seroreverted;  
or

- If the infant has never been breastfed or not breastfed in the past 6 weeks, the infant is HIV uninfected.

If the infant is still breastfeeding, a negative HIV serological test result cannot exclude HIV infection.

Positive or reactive HIV serological testing in an infant suggests the following:

- The infant is HIV-exposed;  
and /or
- The infant may be HIV-infected – the older the infant, the more likely the infant is of being HIV-infected.

For the purposes of testing in children in relation to breastfeeding, the window period required before serological testing can be reliably interpreted after cessation of breastfeeding using rapid tests or EIA is recommended to be 6 weeks. Isolated case reports of false-negative rapid HIV tests in sick children who then go on to being HIV-infected need to be investigated.

#### **5.3.4 Rapid test devices**

Rapid test devices with diagnostic performance comparable to that of traditional EIA methods (i.e. sensitivity >99% and specificity >98%) are currently commercially available. These assays may be particularly appropriate for use in resource-limited settings since they can be performed in clinic or community settings and little equipment is required. Only rapid tests that are validated as part of a national testing algorithm by the National Reference Laboratory (or another laboratory designated for this task) should be used.

Rapid HIV assays can be based on several test formats; these tests are designed for use with individual specimens, and are quick and easy to perform, making them more cost-effective than EIAs in low-throughput laboratories. They can be used with serum/plasma and venous or capillary whole blood. With the latter, application of finger-stick whole blood is a useful specification in the context of resource-limited settings.

Most rapid tests are presented as a kit incorporating the reagents, and do not usually require additional equipment. As the procedures are simpler and involve a limited number of steps, there is a lesser chance of error and they can be carried out following SOPs by health-care workers who have received appropriate training. Test results become available within 10–30 minutes and their interpretation in children over 18 months is generally straightforward. Many rapid tests include an internal quality control that validates the test result (this can either be specimen addition or reagent addition control). Test kits for rapid assays are designed either as a single test or in a multiple test format suitable for a limited number of specimens, which allows for only the desired number of tests to be used at once, without compromising the integrity of the remainder of the test kit. Most rapid HIV test kits can be stored at room temperature (2–30 °C; consult the manufacturers' instructions for use). Further details on rapid HIV serological tests are available at: [http://www.who.int/diagnostics\\_laboratory/publications/Report16\\_final.pdf](http://www.who.int/diagnostics_laboratory/publications/Report16_final.pdf)

### 5.3.5 EIA

Enzyme immunoassay is a common immunological technique that has been adapted for the detection of HIV antibodies. Most EIAs have a high sensitivity and specificity, and are able to detect HIV-1/HIV-2 and HIV variants. The most recent advances in EIA technology have produced 'combination assays', which combine p24 Ag EIAs with traditional antibody EIAs, allowing for the simultaneous detection of HIV antigen and antibodies using a single test. This approach has further shortened the window period, i.e. the interval between HIV infection and detectable HIV antigen/antibodies.

EIAs are manufactured for screening large numbers of specimens (about 90 or more at a time) and are best suited for batch testing (at least 40 specimens per day). This makes them particularly cost-effective for use in centralized surveillance and blood transfusion services and less suitable for lower-throughput facilities. An EIA procedure usually takes two hours, hence high-throughput laboratories are able to report on the EIA results the same day, depending on their batching procedures. Laboratories with limited numbers of specimens will usually wait until they have at least 40 specimens to run the test procedure (½ EIA plate + controls), which can lead to a delay in the availability of results.

EIAs require sophisticated equipment, and are technically demanding; automatic pipettes, incubators, washers, readers and a constant electricity supply must be available. The equipment needs to be regularly maintained to ensure accuracy of the test results. The validity of the test results depends on skilled technicians who are able to prepare correctly the necessary reagents, pipette with accuracy and operate the equipment. However, the requirements are fewer than those for molecular methods of DNA or RNA detection.

The performance characteristics of commonly used serology assays have been previously summarized by WHO; details are available online (32).

#### **Serological HIV testing using commercially available EIAs or rapid assays can be used:**

- to diagnose HIV infection in children from the age of 18 months;
- to identify the HIV-exposed infant;
- to identify those who are not likely to be HIV-infected among infants who were never breastfed or have been weaned for at least 6 weeks, and;
- to identify HIV-exposed children aged 9–18 months who have remained HIV seropositive and who may be HIV-infected and need virological testing.

### 5.3.6 Western blotting

The WB assay consists of a multilayer process similar to that of the EIA. HIV antigens are laid out – from the highest in molecular weight to the lowest – on a strip of nitrocellulose. When a specimen is incubated with the strip, any existing HIV antibodies bind to these HIV antigens. Addition of enzyme leads to an antibody–enzyme complex. In a final step, a chemical is added that changes colour when it comes into contact with the protein–antibody–enzyme layers.



The WB can be positive, negative or indeterminate. Indeterminate results are neither positive nor negative, and usually reflect the beginning of seroreversion at the time of testing, or cross-reactivity. In these rare situations, retesting should be performed three weeks later. WB requires sophisticated equipment, is technically demanding and needs expertise in interpretation. With the improvement in performance characteristics of serological and virological testing, it is no longer essential as a confirmatory HIV test for adults or children.

## 5.4 Virological testing

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HIV infection in infants is diagnosed by detecting the presence of viral nucleic acid (i.e. viral RNA or viral DNA) often called nucleic acid testing (NAT), or viral products such as p24 Ag. For the diagnosis of HIV, NAT includes extraction, amplification and detection of HIV nucleic acids, and may be qualitative or quantitative.

### 5.4.1 Assays

Several techniques allow the diagnosis of HIV infection. Most of these assays rely on the amplification of the genetic material of HIV and can be classified as qualitative (detect the presence of nucleic acids, yes or no) or quantitative (provide a quantification [the amount] of nucleic acid). Polymerase chain reaction (PCR)-based HIV DNA and HIV RNA assays (40) have become the most widely used assays, even in resource-limited settings, for both diagnostic and monitoring purposes (41-46).

A range of methods for detection of RNA also allow for quantification of HIV RNA and are widely used for monitoring the progression of HIV disease and response to ART in children and adults. Quantification of HIV RNA is not a prerequisite for initiating or continuing ART in infants, children or adults. Other methodologies, such as nucleic acid sequence-based amplification (NASBA), b-DNA, and transcription-mediated amplification (TMA) are valuable technologies to detect and/or quantify HIV RNA.

These assays are intended to detect and/or quantify viral RNA from blood cell-free plasma specimens. However, whenever venous or capillary blood specimens are tested using these methodologies, as in the case of DBS on filter paper, the viral DNA present in peripheral blood mononuclear cells (PBMCs) can potentially be co-amplified and detected, contributing to the viral load results. There is reasonable agreement between viral load results from plasma and DBS (47, 48). However, this is not always the case where the plasma viral load is  $\leq 1000$  copies/ml; the DBS specimen can present higher results. More frequently, the limit of detection for DBS is 3000 copies/ml. Such a situation is usually only of concern when total suppression of HIV viral load is sought and DBS specimens are used. Infants generally have much higher viral loads than adults and, in children not on ART, potential discrepancies of viral load results between DBS and plasma specimens should be irrelevant in the context of diagnosis (49).

In settings with limited laboratory facilities, Us p24 Ag detection can be considered as an alternative to DNA or RNA detection for the diagnosis of HIV-1 in infants and children (50-53). This methodology is EIA-based and can be performed in any laboratory using serological techniques. HIV isolation in cell cultures is no longer recommended for the diagnosis of HIV

infection due to the cost, complexity and biosafety concerns. Box 1 provides a brief overview of the methods of detection of HIV nucleic acids. The need for specific equipment, trained personnel, and consistent QA (including external quality assessment and quality control) of laboratory practices limits the availability of molecular-based diagnostic techniques for HIV DNA and RNA. In addition, the reagents and consumables required for performing the assays remain expensive.

### **Box 1. HIV nucleic acid detection**

The most commonly used methodologies to detect nucleic acids are:

- *Polymerase chain reaction (PCR)*: it exponentially amplifies the DNA target by many orders of magnitude by cycling the temperature of the reaction several times. DNA primers define the region of the target sequence to be amplified by a DNA polymerase that duplicates the number of sequences in each cycle of the reaction. In the case of RNA specimens such as HIV, the RNA must be converted to DNA by a reverse transcriptase enzyme in an isothermal reaction before initiating the PCR. This reaction is referred to as RT-PCR.

The PCR assays and NAT in general can be further classified as:

1. *End-point assays*: detection is performed after amplification is completed. Manipulation of the PCR product is required for detection, generating risks of cross-contamination and making this process more labour intensive.
  2. *Real-time assays*: detection is performed as the nucleic acid is being amplified, usually by the binding of fluorescent-labelled probes to the amplified PCR product, which emit a wavelength that is captured by the system. The quantification is performed by comparing the amount of fluorescence of the target sample with standards. This is a closed-tube methodology which requires no downstream manipulation of the PCR product, thus minimizing cross-contamination of specimens and reducing time to generate results.
- *Nucleic acid sequence-based amplification (NASBA)*: it selectively amplifies the target RNA through isothermal production of an intermediate DNA by a reverse transcriptase. This DNA serves as a template for the cyclical amplification of RNA using an RNA polymerase. Fluorescent-labelled probes hybridize to newly synthesized RNA and quantification is determined by comparing the fluorescence of the target with internal standards.

- *Branched-chain DNA (b-DNA)*: it is based on amplifying the signal rather than the target RNA. The several steps of this methodology include the following:
  1. Viral RNA is captured by complementary nucleotide probes (capture) attached to a micro-well plate.
  2. Another set of probes attach to both viral RNA and to preamplifier probes.
  3. The preamplifier probes hybridize to b-DNA (amplifier probe).
  4. Multiple copies of alkaline phosphatase-labelled probes are added to the reaction binding to the b-DNA.
  5. A chemiluminiscent substrate is added to the reaction for detection and colour emission occurs. The amount of light emission is proportional to the amount of viral RNA in each specimen and quantification is based on comparison of the specimens with a standard specimen.
- *Transcription-mediated amplification (TMA)*: this is a qualitative method that can detect either RNA or DNA. It uses the same principle as the NASBA methodology.

General factors that need to be considered in selecting methods and equipment for performing virological testing to diagnose HIV infection in infants include:

- equipment required for assay performance and specimen handling
- commercial availability of equipment and reagents
- availability of maintenance and service of equipment
- training and availability of laboratory staff
- cost of equipment and reagents
- volume of biological specimens required to perform the assay
- number of specimens required to be processed (specimen throughput)
- specimen storage and transport
- level of laboratory capacity
- ongoing and functional laboratory QA
- viral types and subtypes
- specimen collection and processing (including DBS from remote sites)
- use of equipment for other purposes and diagnosis of other infections.

### 5.4.2 HIV DNA testing

Qualitative HIV DNA PCR is currently widely used as the standard method for diagnosis of HIV infection in infants and is the assay against which other assays are usually compared in research settings (54-59). Some of the main characteristics of this assay relevant to infant diagnosis can be summarized as follows:

- HIV proviral DNA is integrated in the cell's genome, and so detection of cell-associated HIV DNA within PBMCs by PCR is one of the most sensitive methods for establishing HIV infection.
- HIV DNA tests are reliable in the presence of ARV exposure for PMTCT or maternal ART. ART does not cure infection and HIV-1 DNA remains detectable in the PBMCs and lymphoid tissue of HIV-infected children who have received ART for several years and have undetectable viral replication as measured by HIV RNA assays (41, 60, 61).
- The current commercially available HIV DNA PCR assays have acceptable sensitivity for detection of most common group M HIV-1 subtypes including A, B, C, D, E, G and H.
- DNA PCR can be performed on specimens of whole blood collected onto filter paper as DBS without significant loss of sensitivity or specificity (62).

### 5.4.3 HIV RNA testing

HIV RNA assays detect HIV viral RNA in venous/capillary whole blood dried on filter paper and plasma using a variety of methodologies. These include RT-PCR, b-DNA, TMA and NASBA. Most of these methodologies are used to quantify RNA and monitor HIV disease progression or response to ART. TMA is used qualitatively (41, 63, 64) and most of these assays can be used as alternatives for early infant diagnosis of HIV. Some characteristics of these assays are as follows:

- RNA detection methods are able to detect a wide range of viral subtypes (65, 66), (67).
- RNA detection in plasma appears as or more sensitive than DNA detection methods in the first few weeks of life (61, 68-70)
- Administration of maternal and infant ART (zidovudine [AZT] or nevirapine [NVP]) for PMTCT is associated with reduced replication of HIV. However, studies in general have shown no loss of sensitivity regardless of infant or maternal ARV prophylaxis (42, 44, 69, 71).
- RNA detection can be performed on plasma or venous/capillary whole blood specimens collected onto filter paper (44).
- RNA detection from whole blood specimens collected onto filter paper is less sensitive than from plasma specimens due to the reduced specimen volume collected on the filter paper.
- The viral load threshold at which specimens collected onto filter paper can be determined is, in theory, proportional to the volume of specimen collected.

Because low viral loads (<5000 copies/ml) during the first year of life are very rare in HIV-infected infants not receiving ART (72), where capacity exists, a viral load assay should be used as the confirmatory test. This information will be useful to determine the clinical status of the child.

#### 5.4.4 Ultrasensitive p24 antigen-based testing

p24 Ag assays measure the HIV core protein p24 found in whole blood, serum or plasma, either in free form or bound by anti-p24 antibody. When antibodies to HIV become detectable, p24 Ag is often no longer demonstrable, probably due to the development of antigen–antibody complexes in the bloodstream. Assays that use ultrasensitive methods, including an antibody–antigen dissociation procedure and signal amplification for p24 Ag allow smaller quantities of p24 Ag to be detected, and have improved the sensitivity of p24 Ag testing.

Technical improvements have enabled the newer immune complex-dissociated Us p24 Ag test to perform almost as well as HIV DNA PCR for the diagnosis of HIV in infants (58). Several of these modified p24 Ag assays have been evaluated in clinical studies and compared with the most sensitive PCR methods available (see Annex 1, Tables A–7 and A–16). Us p24 Ag assay has been validated in countries where HIV subtypes A, B, C, D, C and F predominate (59, 73). Some characteristics of these assays are as follows:

- These tests are less complex and, in some cases, less costly than nucleic acid detection and can be done in laboratory settings that can perform EIA testing;
- Testing can be performed on whole blood, plasma and serum. There is no need for nucleic acid extraction and specimens are generally stable. A minimum requirement for laboratories would be the capacity to perform the EIA technology.
- They can also be performed using venous/capillary whole blood specimens collected as DBS (74).
- Current data suggest that Us p24 Ag can be used up to the age of 18 months, although there is a theoretical concern that the sensitivity may decline with increasing age and might be correlated with viral load.
- There are theoretical concerns about the use of Us p24 Ag detection assays when the mother or infant is on ARV or ART to reduce MTCT. It is advised that alternative tests be used if the infant is on ART.

#### 5.4.5 Specimen collection

Blood (venous or capillary) must be drawn from the infant or young child for all methods of virological testing and serological assays. These assays differ in the volume and type of specimen required. Taking specimens of venous blood from infants and children is more difficult than taking venous blood from adults and should only be conducted by persons trained in the techniques of venous blood collection from children. Specimen collection using heel or finger prick specimens for preparation of DBS (see below) is usually easier than other methods for venesection in infants.

##### ***Volume and type of specimens required***

In general, virological assays require at least 500–1000  $\mu$ l of plasma or serum to enable the test to be performed. Assays using DBS require 25–100  $\mu$ l of whole blood. Generally, rapid assays require 50 to 150  $\mu$ l (some may use as little as 5  $\mu$ l, which is usually preferred) derived from serum/plasma or whole blood specimens. Usually 1–3 ml of venous whole blood is requested by

the laboratory for specimen processing and testing of other clinical markers and patient monitoring.

#### 5.4.6 Use of dried blood spots (DBS)

Blood specimens collected onto filter paper have many advantages for the diagnosis of HIV infection in infants. Capillary whole blood for DBS can be obtained with a sterile single-use lancet by finger stick from older children or by heel stick from infants, which is dropped onto the absorbent specimen collection (filter) paper.

The procedure is less traumatic than venepuncture, uses only a small volume of blood and is reliable for safe specimen collection and delivery at significantly lowered cost. Specimens require thorough air-drying individually for a minimum of four hours prior to packaging or sending for testing, and must then be protected from moisture with a desiccant agent. Once dried, the filter paper can be stored at room temperature for short periods of time and can be more easily transported to centralized testing facilities (75). Dried specimens carry less biohazard risk than liquid specimens. Training is still required to adequately perform specimen collection and handling, and transport is more difficult from remote or rural areas. DBS can be used for virological (HIV DNA or HIV RNA (39, 76) testing and p24 Ag assays (39, 41, 76), some serological testing (75), and HIV drug-resistance testing (41, 48, 74, 76-86). Further details on specimen collection, storage and transport are available online (87).

The time between specimen collection and testing can be important as viral nucleic acids may degrade over time, particularly if stored at high ambient temperature (e.g. during transportation to the laboratory) or high humidity for extended periods. DBS (for HIV DNA and RNA testing) have been used successfully in resource-constrained settings to ease collection and transport of specimens (88). DBS are collected and transported to the diagnostic laboratory by mail, courier or local transport, and the results subsequently returned by e-mail, telephone, SMS, courier, local modes of transport, or computerized information systems. As it is being used as a diagnostic test, results need to be turned around as soon as possible (see **Recommendation 7**).

Use of DBS should be more widely implemented to improve access to virological testing in a range of resource-limited settings. Some national programmes have had good experience with implementing DBS HIV DNA in selected areas (e.g. Botswana, Cameroon, Kenya, South Africa and Uganda).<sup>i</sup> HIV RNA on DBS has been used in Cote d'Ivoire for infant diagnosis and should be further evaluated. Information on standardization of materials, specimen collection protocols, specimen handling and documentation supporting widespread implementation of programmes in clinical settings for HIV NAT testing using DBS can be found online (89, 90).

The use of DBS or dried plasma spot (DPS) specimens for quantification of viral load should take into consideration the specimen volume collected on the filter paper and used in the available assays. The reduced volume of the specimens (approximately 100  $\mu$ l) will decrease the sensitivity of the assay, which is usually calculated as virus copies per millilitre (1000  $\mu$ l). The required specimen volume for each assay should be considered in this light with proper validation of the assay.

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<sup>i</sup> MOH/CDC/Clinton/UNICEP programme evaluations reported to PMTCT IATT Lab Working Group.

### 5.4.7 Interpretation of nucleic acid tests in infants

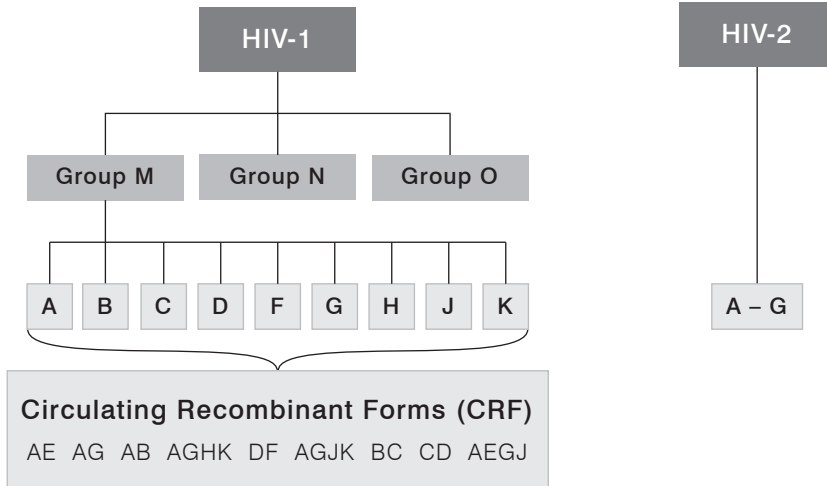
#### *HIV type and subtype*

There are two distinct serotypes of HIV virus: type 1 and type 2. HIV-2 is found largely in west Africa and vertical transmission of HIV-2 is unusual (63). Data on HIV-2 infection in children are scant but it is thought to be rare.

#### **Box 2. Diversity of the human immunodeficiency virus**

HIV is a virus that replicates rapidly in the human host. The reverse transcriptase enzyme responsible for copying the virus' genetic material during replication is error prone and does not have proofreading activity. This and frequent recombination, a characteristic of retroviruses, drives the diversity of HIV genetic material at a high rate. Many of the newly produced viruses are not infectious due to detrimental mutations or recombinations that render the virus non-viable. When a cell is coinfecting with distinct strains of HIV, recombination might occur, generating viruses with hybrid genomes. However, when new hybrid virus strains created by recombination are found in three or more epidemiologically unlinked individuals, these viruses are classified as 'circulating recombinant forms' or CRFs. HIV strains are classified into types, groups, subtypes and CRFs based upon genetic similarities. The classification of subtypes (or clades) and CRFs is a complex issue and subject to change (91).

**Fig. 2. Genetic diversity of HIV**



The diversity of HIV (Fig. 2) means that molecular techniques for identification of HIV DNA and RNA require constant monitoring and assessment. NAT for HIV must be able to detect the predominant strains found within the local target population. Children infected with HIV either during pregnancy, at the time of labour/delivery or through breastfeeding may acquire a variety of HIV subtypes and heterogeneity: coinfection and recombinants are not uncommon.

Not all virological assays detect different types/groups of HIV equally reliably.

Currently available commercial virological tests cannot reliably diagnose HIV-2 infection (meaning in-house methods are the only alternative) and so serological testing is recommended to diagnose HIV-2. Most serological assays are currently manufactured to detect HIV-1 and HIV-2 simultaneously (either discriminatory or combined detection of reactivity) and can be used in the same way that HIV-1 serological testing is used. Despite the variation in HIV subtypes, commercial serological EIAs appear to detect most subtypes and groups of HIV-1 infection (92, 93). Newer rapid assays that reliably detect HIV-1 and HIV-2 have the potential to offer reliable detection of HIV-1 and HIV-2 with accuracy comparable with EIAs (94).

#### 5.4.8 Timing of virological testing

Infants and children can be infected with HIV during pregnancy, during delivery and post partum through breastfeeding, or through sexual or parenteral exposure. Infants infected in utero usually have detectable HIV at birth and progress to disease more rapidly. Infants infected at or around delivery may take a short time to have detectable virus. Therefore, the sensitivity of NAT depends on time of acquiring infection and the timing of the test (95, 96), and the sensitivity of all methods of virological testing are therefore lower at birth. In infants with in utero HIV infection, HIV DNA and RNA can be detected in venous blood specimens obtained within 48 hours of birth. However, in infants with peripartum acquisition of HIV, HIV DNA and RNA are not detected in early venous blood specimens but become detectable at or after 1 to 2 weeks of age (71). By six weeks of age, almost all infants infected prior to, at, or around birth can be identified by NAT or Us p24 Ag testing.

Disease progression is rapid in young HIV-infected infants, thus specimens being used for diagnosis need to be transported and processed as quickly as possible, with positive results returned urgently. It is currently recommended that all virological testing for the purposes of diagnosis on DBS be conducted as soon as possible. The promptness and reliability of the supply chain management and logistics system should be evaluated at each point in the chain to minimize total turnaround time. The turnaround time from specimen collection to results being given should never be longer than four weeks.

Infants and children of HIV-infected mothers who continue to breastfeed are at ongoing risk for acquiring HIV infection and hence negative virological test results are difficult to interpret if the infant is still feeding. **Once breastfeeding is completely discontinued, it is considered that virological tests conducted at least six weeks after discontinuation of breastfeeding are indicative of true HIV infection status, i.e. the window period for virological testing after stopping breastfeeding is up to six weeks.** This is the same for all currently available methods of virological detection (46, 61, 69, 97-100). **Virological testing can be performed if an infant is still breastfeeding.** Positive virological tests in breastfeeding infants indicate infection and



should prompt the usual confirmatory testing, but negative tests cannot be used as evidence of absence of infection.

#### **5.4.9 Reliability of virological tests in the presence of ARV exposure**

There are theoretical concerns about the use of RNA or p24 Ag virological testing in infants who have been administered or are taking ARV prophylaxis for PMTCT or are breastfeeding where the mother is taking ART. Detection of viral RNA and p24 Ag depend on viral replication and ARVs inhibit this. Administration of more potent ARVs or combinations temporarily suppresses HIV RNA levels to low or undetectable levels (101). However, there are currently no data available to suggest that existing recommendations need to be revised. HIV RNA testing has been demonstrated to provide equal or improved sensitivity over DNA testing on plasma specimens (see GRADE profiles) (43). For infants on prophylaxis, some uncertainty remains related to reliability in extended infant prophylaxis.

## 6. NEXT STEPS

### 6.1 Dissemination and implementation

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- IMAI, IMCI and operational manuals will be updated to reflect the revised recommendations.
- The WHO HIV department (Headquarters) will inform WHO regional and country representatives of these revised recommendations.
- The WHO HIV department (Headquarters) will inform key implementing partners, stakeholders and professional groups affiliated to child health and paediatric HIV.
- Technical support to help countries adopt and adapt the revised guidelines will be provided and/or facilitated by WHO in collaboration with various partners.
- The UN Inter-Agency Task Team (IATT) for PMTCT will provide technical support for country adaptation and implementation, and will undertake programme evaluation of national programmes.
- Further guidance on scaling up diagnostic testing for children and infants can be found at: [http://www.who.int/hiv/pub/paediatric/framework\\_2008/en/index.html](http://www.who.int/hiv/pub/paediatric/framework_2008/en/index.html)

### 6.2 Outstanding issues for further research

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- Assessing the performance of HIV RNA plasma and DBS assays in specimens from infants and children receiving ART
- Assessing HIV detection (antibody/antigen) using rapid tests in sick infants and children
- Confirming the performance of RNA and Us p24 Ag assays in different HIV strains
- Defining minimum thresholds for performance of HIV RNA assays when using DBS
- Assessing the optimum timing of virological and HIV serological screening assays
- Assessing the performance of different serological assays in infant populations
- Comparing the performance of HIV serological assays from oral fluid specimens versus venous/capillary blood and serum/plasma in infants
- Assessing the performance of rapid HIV serological assays on oral fluid specimens in infants
- Assessing the performance of clinical/serological algorithms (research is ongoing)
- Estimating the rate of decay of maternal HIV antibody in breastfeeding infants
- Assessing the HIV antibody response at 18 months in infants starting ART immediately
- Assessing the actual performance of HIV serological assays in infants and children in the second year of life
- Assessing the performance of HIV combined antigen/antibody (fourth-generation) assays for diagnosis in breastfeeding infant populations.

### **6.3 New products or technologies required**

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- POC virological assays and CD4 enumeration technologies for percentage
- Delivery of test results by SMS (text messaging) or other innovative technologies.

It is anticipated that the new POC CD4 and virological diagnostic testing technologies will need to be reviewed periodically.

### **6.4 Technical assistance, tools and advocacy required**

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- Packaging and ensuring a supply of related consumables in 'bundles' or 'kits' for use at sites
- Building health system capacity to perform testing, transfer specimens to the laboratory and return results to the site and to patients in a timely fashion. This would secure referral for care and treatment for all infants and children who test positive.
- Scaling up of counselling and testing services for children
- Providing guidance on implementation and operations, including setting up of laboratory services and transport networks for rapid handling of specimens and delivery of results back to mother/carer/child
- Ensuring that QA programmes are functional and well-supported (including external quality assessment and quality control), and providing training to laboratory personnel
- Ensuring a sufficient supply of ART
- Putting in place systems for early identification of HIV-infected infants, their follow up and retention in care.

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