1 **Current assays for HIV-1 diagnostics and antiretroviral therapy monitoring: Challenges**

2 **and possibilities**

3

4 *Keywords:* **HIV-1 diagnostic assays, viral load measurement, drug resistance monitoring,**

5 **tropism determination, ultra-deep sequencing**

6

7

8 **Summary**

9 In 2011, there were over 34 million people living with HIV infections, causing a heavy burden

10 to public health sectors. HIV infection is a life-long threat, which cannot be prevented by

11 vaccination and cured by antiretroviral drugs. The infected patients rely on daily antiretroviral

12 therapy to suppress HIV viral replication. Hence, it is important to diagnose HIV infections as

13 early as possible, and to monitor the efficacy of antiretroviral therapy every 3-6 months.

14 Different immunoassays detecting HIV antigens and antibodies have been modified to give

15 better sensitivity and more rapid diagnosis. Several clinical and virological parameters,

16 including CD4+ cell counts, viral load and drug resistance mutations, are also used for

17 treatment monitoring. Many molecular assay optimizations are now being imposed to improve

18 patient care. This review would try to focus on the most updated HIV diagnostic assays, as well

19 as discussing if there will be upcoming possibilities with other advance technologies.

20

21 **Introduction**

22 Nearly three decades ago, the human immunodeficiency virus (HIV) was identified to be the
23 causative agent of the acquired immune deficiency syndrome (AIDS). [1] AIDS progression is
24 associated with a significant decrease in CD4+ cells, causing failure in the immune systems.
25 Based on the World Health Organization statistical data, there were over 34 million people
26 living with HIV infections around the globe till 2011. [201] Great effort has been put into
27 understanding the functions of different viral proteins and the viral pathogenesis inside
28 lymphocytes. The research findings allow scientists to discover HIV antigens and antibodies
29 for detection, antiretroviral drugs for viral inhibition, and vaccines for infection prevention and
30 transmission.

31 To maximize the efficacy of patient care in HIV-infected clinics, HIV detection, viral load
32 measurement and antiretroviral drug resistance monitoring are crucial and can be achieved by a
33 wide range of laboratory tests. Initially, p24 viral proteins were quantified by an enzyme
34 immunosorbent assay test. However, the amount of antigen was at limited level during the
35 stage of acute infection. The assay sensitivity and specificity can be enhanced by the
36 combination use of antibodies Immunoglobulin G and Immunoglobulin M test. [2] Antibodies
37 are readily detected after seroconversion, making them the major targets in enzyme immune
38 assays. Western blot which also detects HIV antibodies, on the other hand, is used as a

39 confirmation diagnostic test globally. The newly developed nucleic-acid based assays have
40 shortened the window period from 4 weeks to 2 weeks. [3] However, the molecular testing is
41 expensive and requires specific diagnostic machines, which is not suitable for the use in remote
42 settings.

43 Zidovudine was the first nucleoside reverse transcriptase inhibitor (NRTI) approved by the
44 Food and Drug Administration (FDA) for HIV treatment since 1987. After a few years of
45 Zidovudine mono-therapy regimen, cases of drug resistance cases were reported. With protease
46 inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) sequentially
47 introduced into the market, the idea of highly active antiretroviral therapy was brought into the
48 HIV clinics in the mid-1990s. [4] Nowadays, 3 more antiretroviral classes (fusion inhibitors,
49 CCR5 antagonists and integrase inhibitors) are on the prescription list, covering over 25 single-
50 or multi-class combinations of antiretroviral drugs.

51 Under antiretroviral drug suppression, the probability of escape mutation occurrence increases
52 due to the fact that HIV uses error-prone reverse transcriptase for viral replication.
53 Consequently, a series of genotypic and phenotypic assays are implemented to deduce drug
54 susceptibility prior to and during the treatment. Besides, two other clinical parameters, CD4+
55 and viral load, are monitored to ensure high treatment efficacy. The CD4+ cell count is treated
56 as a surrogate marker for observing the strength of the immune system, while the number of

57 viral copies is used as a prognostic marker for checking viral activity. The effectiveness of HIV
58 RNA quantitative and qualitative assays have been improved dramatically with molecular
59 assays. In particular, the latest technology of ultra-deep sequencing further increases the
60 sensitivity of qualitative assays by sequencing individual amplicons. [5] Determination of the
61 host genetic polymorphisms has become an extra assessment for antiretroviral drug
62 prescription due to several adverse effects and metabolic interactions.
63 HIV is mainly characterized into HIV-1 group M, N, O and HIV-2. The global pandemic is
64 caused by HIV-1 group M strains while group N and O are very rare. [6] Base on phylogenetic
65 analysis, group M strains are further categorized into 11 subtypes, 58 circulating recombinant
66 forms and many unique recombinant forms. [6, 7] [202] HIV-1 subtype B and C infections are
67 accounted for over 50% of infections worldwide. HIV-2 infections are restricted in the region
68 of Western Africa and thus limited diagnostic development was done. [6, 8] In this review, we
69 will focus on HIV-1 and its current diagnostic assays that are newly utilized to facilitate better
70 detection, shorter turnaround time, and easier to manipulate for diagnosis and antiretroviral
71 therapy (ART) monitoring.
72

73 **HIV-1 detection**

74 HIV-1 can be transmitted vertically by sexual contact, perinatally from mother to child, and

75 through contaminated blood products and needles. Certain groups of people are at high risk,
76 including intravenous drug users, blood products recipients, healthcare workers, sexual
77 workers and the ones who have unprotected sex and multiple partners. HIV-1 treatment is
78 permanent and expensive. It is therefore important to detect HIV-1 in blood samples and
79 individuals as early as possible so as to eliminate any possible infection spread.
80 HIV-1 detection is based on the recognition of viral antigen (p24 antigen test), antibodies
81 (enzyme-linked immunosorbent assay, ELISA), viral proteins (western blot, WB) and nucleic
82 acids (nucleic-acid amplification test, NAAT). During acute infection and before
83 seroconversion, the level of antibodies is very low and only a small amount of detectable
84 antigen is present. HIV-1 detection is usually less accurate within this one-month window
85 period. Hence, shortening the turnaround time is always the major hurdle in upgrading the
86 HIV-1 diagnostic assay. Apart from HIV-1 diagnosis, researchers are interested in identifying
87 recent infection and the prevalence of infection over time. The amount of antibodies will keep
88 rising after seroconversion for about 4 months. Using the detuned assays or
89 sensitive/less-sensitive assays, researchers are able distinguish recent or chronic infections by
90 discriminating antibodies avidity and titer. [9]
91 The current diagnostic algorithm relies on rapid antibody tests or ELISA as a preliminary
92 screening in blood banks, followed by WB confirmation. A modified algorithm, which can

93 shorten the turnaround time and strengthen the sensitivity and specificity, was proposed in the
94 2010 HIV Diagnostics Conference with the devices described in the followings. [10] The 4th
95 generation ELISA, that can simultaneously detect p24 antigens and both anti-HIV-1 and
96 anti-HIV-2 antibodies, are now commonly being used in major resource-rich continents. [11-13]
97 Several FDA-approved or CE-IVD kits are ARCHITECT HIV Ag/Ab Combo assay (Abbott
98 Diagnostics, Germany), Enzygnost HIV integral II (Siemens Healthcare Diagnostics,
99 Germany), GS HIV Combo Ag/Ab EIA (Bio-Rad Laboratories, USA) and VIDAS HIV DUO
100 Ultra (bioMérieux, France). [14-17] In comparing to the traditional double-confirmed results
101 by ELISA and WB, the 4th generation immunoassays can detect 84% of acute HIV infection
102 and are >98% specific and sensitive. [11, 12] They can detect acute infections 7 days earlier
103 than the 3rd generation ELISA (VITROS anti-HIV 1+2 assay, Ortho-Clinical Diagnostics, UK).
104 [18] The NAAT-based qualitative assay, (APTIMA HIV-1 RNA Qualitative assay, Gen-Probe
105 Inc., USA) can further reduce the window period to 26 days before western blot confirmation,
106 due to the high level of viral replication before immune response establishment. [3, 18, 19]
107 Rare false-positive results obtained by the NAAT assay had limited its first-line screening
108 usage in Europe. [20, 21] HIV-1 detection can also be done by rapid tests, which are simple,
109 faster and can be performed without intensive clinical or laboratory settings. The introduction
110 of 2nd generation discriminary rapid tests (Multispot HIV-1/HIV-2 rapid test, Bio-Rad

111 Laboratories, USA) was proven to have comparable results against WB, although

112 contradictory results were also reported. [22, 23] After evaluating both the pros and cons of

113 these new technologies, the 4th generation ELISA assays(such as ARCHITECT Ag/Ab combo),

114 were proposed to be used as the initial screening tool in the US and Europe[10, 21]. Any

115 positive ELISA results will further be confirmed by Western Blot or HIV-1/HIV-2

116 discriminatory assay rapid test. The most sensitive and expensive NAAT tests (e.g. APTIMA)

117 will only be used as a supplementary verification for any discordant detection.

118

119 **Viral load monitoring**

120 HIV-1 infections are considered as a chronic illness, and required non-stop antiretroviral

121 therapy to suppress viral replication continuously. In order to maintain treatment efficacy, viral

122 load, CD4+ counts and drug resistance mutations are monitored closely by different laboratory

123 tests which will be discussed in the followings and summarized in Table 1.

124 Prior to viral load testing, sample preparation and RNA extraction are both crucial procedures

125 for proper downstream processing. Blood samples are first collected in EDTA or plasma

126 preparation tubes (PPT), followed by centrifugation to obtain plasma and/or peripheral blood

127 mononuclear cells. Due to the instability of virus in specimen, storage under -70℃ are

128 necessary. Yet the storage condition is impractical in remote-settings and for shipment after

129 plasma separation. [24] In some resource-limited countries, the use of dried blood spots (DBS)

130 for sample collection has been proven to be able to keep the viral nucleic acid in good condition

131 during transportation. The cost of using filter paper for DBS sampling is much more cost

132 effective than using PPT or EDTA tubes for whole blood collection. [25] Using the Abbott

133 HIV-1 Real-time assay (Abbott Molecular, USA), the RNA quantitative levels had no

134 significant difference between freshly separated plasma or with DBS. In a small study cohort,

135 DBS was 95% sensitive with respect to the real-time assays and the high concordance showed

136 promising future on sample preparation. [25]

137 With a good sample collected, the next step would be viral nucleic acid extraction. Viral RNA

138 extraction requires specialized equipments and sterilized reagents to prevent contaminations

139 and RNA degradation. The procedure involves protein denaturation, RNA capture on solid

140 silica surfaces, inhibitors removal and RNA elution from the silica. RNA becomes less stable

141 after extraction, and requires ultra low temperature storage. Recently, a new device,

142 RNAStable (Biomatrica, USA), was claimed to be able to stabilize RNA in a dry matrix form

143 for at least 3 months under room temperature. [26] Apart from it, the trend of RNA extraction

144 has switched from manual handling to automation in most developed countries. There are 3

145 commonly used CE-IVD marked automated nucleic acid extraction platforms, the Roche

146 COBAS AmpliPrep system (Roche Molecular Diagnostics, Germany), the Abbott *m*2000

147 system (Abbott Molecular, USA) and the NucliSens easyMAG (bioMérieux, France) in the

148 market, which can handle a wide range of biological samples with limited hands-on time. [27,

149 28] These fully automated RNA extraction systems provide standardized extraction protocols,

150 which is important for extreme low-level vireamia measurement. [29]

151 The level of plasma HIV-1 RNA can directly reflect the efficacy of HAART, the possibility of

152 mother-to-child transmission, the odds of drug resistance mutations and the probability of

153 AIDS progression. [30-32] In clinical definition, a successful ART treatment can inhibit viral

154 replication and suppress the viral RNA level to ≤50 copies/ml after 24-week treatment. [33]

155 HIV-1 exists in different genotypes, unique and circulating recombinant forms in isolated

156 continents. [6] A perfect viral load assay is therefore competent in identifying all the diverse

157 genotypes and maintaining high sensitivity for substantial patient care. External Quality

158 Assurance Programs (QCMD, CAP, NATA) are always in place for clinical diagnosis. A

159 10-year evaluation study (2000-2010) on an external quality assurance program in the United

160 Kingdom revealed that end-point assays were gradually replaced by real-time assays. [29] In

161 2010, over 85% of the participating laboratories employed real-time assays for HIV-1 RNA

162 quantification, which demonstrated the lowest coefficient of variation, most rapid turnaround

163 time and highest throughout among the other methods.

164 Currently, there are several CE-IVD marked commercial assays used worldwide, together with

165 some in-house and research assays. These assays are based on nucleic acid sequence-based
166 amplification (NASBA), branched-chain DNA assay (bDNA) and reverse transcription
167 qualitative PCR assay (RT-qPCR). [34, 35] The NucliSENS EasyQ System HIV-1 QT test
168 (bioMérieux, France) is the only assay using the NASBA technology. NASBA provides rapid
169 real-time quantification by amplifying RNA with the use of isothermic heat-stable enzymes.
170 [36] The updated version has allowed better sensitivity towards a range of non-B subtypes. [37]
171 However, the EasyQ system was showed to have lower specificity and limits of detection
172 (176 – 3,470,000 copies/mL) than other real-time PCR assays. [35, 38, 39] On the other hand,
173 the VERSANT HIV-1 RNA 3.0 Assay (Siemens Healthcare Diagnostics, USA) uses the bDNA
174 technology which relies on signal amplification of specific primer and probes binding to the
175 HIV-1 *pol* region. Even though the bDNA assay was demonstrated to give higher diagnostic
176 sensitivity; it performed poor in low viral load measurements and sometimes under estimated
177 the viral RNA level in the specimens. [39] Its dynamic range is comparatively narrow, which is
178 between 75 to 500,000 copies/mL only.

179 For Roche COBAS Amplicor HIV-1 Monitor Test (Roche Molecular Diagnostics, Germany),
180 viral RNA was reverse transcribed into complementary double-stranded DNA, followed by
181 standard PCR. The end-point assay is now gradually replaced by the more sensitive and faster
182 real-time PCR assays. [29] In turns, the Abbott Real-Time HIV-1 system (Abbott Molecular,

183 USA) and the COBAS Taqman HIV-1 Test (Roche Molecular Diagnostics, Germany) are the
184 currently leading technologies for HIV-1 viral load monitoring, with a wider dynamic
185 diagnostic range of 40 - 10,000,000 copies/mL. [35] Both assays allow automated RNA
186 extraction and adopting fluorescence-tagged probes targeting HIV-1 *pol-int* or *gag* gene
187 respectively. These real-time assays apparently provide the best sensitivity and specificity on
188 both B and non-B HIV-1 subtypes. [37]
189 Unfortunately, real-time quantitative assays are not readily available for resource-limited
190 settings. The Cavidi ExaVir Load assay (Cavidi AB, Sweden) and the Ultra-Sensitive p24
191 Antigen Assay (Perkin Elmer Life Sciences, USA) do not require sophisticated laboratory
192 set-up and provide moderate detection limits for viral load monitoring. The former assay
193 estimates the reverse transcriptase activity manually while the later assay simply uses the
194 ELISA approach. [40]
195 New possibilities are now shown to have lower quantitative limits beyond 50 copies/mL in
196 real-time assays. The ultrasensitive VERSANT HIV RNA 1.0 assay (kPCR) (Siemens
197 Healthcare Diagnostic, USA) was used to measure the virological response in a group of
198 ART-experienced patients. The detection limit could reach 3 copies/mL. [41] However, the
199 reproducibility of low vireamia is relatively variable by this assay, as well as the
200 above-mentioned real-time assays by Abbott and Roche. [42] For instance, around 50% of the

201 blips could not be detected in one of the triplicate tests. Although these commercial tests can
202 push the limit of detection to ≤20 copies/mL, the reliability and stability remains a concern. It
203 raised a question whether a single testing is appropriate in the future as biases between different
204 commercial assays at low-level vireamia may affect treatment guidelines. Two or more
205 consecutive viral load measurements should be considered to be more conclusive on treatment
206 monitoring.

207 There have been controversial debates regarding the impact virologic blips; the persistent of
208 HIV-1 RNA low vireamia at different categorized viral load copies will increase the chance of
209 virological failure.[41, 43-46] The existing viruses can escape ART treatment, implying part of
210 the viral population evolved under drug pressure and become drug resistant mutants. Various
211 reasons, including ongoing viral replication, methodological variation or emergence of drug
212 resistant viral particles, may explain the uncertain occurrence of blips. [47] Virological failure
213 was observed in a significant high proportion of ART-experienced patients with viral load over
214 3 copies/mL, suggesting an update revision is required for the future treatment guidelines. [41]
215 The relationship between blips and virological rebound or CD4+ decrease is still under
216 investigation.

217

218 **CD4+ T lymphocyte enumeration**

219 In the last century, CD4+ cell count was used to guide the clinicians on the timing of the

220 initiation of ART. To balance the benefits of early treatment and the economical burden, CD4+

221 cell counts of 500 cells/μL was updated as the standard level for treatment initiation instead of

222 the previous 350 cells/μL. [48] Large collaborative studies had suggested the initiation of ART

223 should be as soon as HIV-1 diagnosis regardless of CD4+ cell counts, which can effectively

224 suppress HIV-1 transmission and AIDS progression. [33, 49] The CD4+ count level is also

225 useful for treatment efficacy monitoring. Flow cytometry counting with fluorescent-labeled

226 monocloncal antibodies is the most widely accepted choice in developed countries for

227 enumeration. The only challenges come from the huge machines and high instrumental cost

228 which makes it not applicable in resource-limited countries. Manufacturers developed various

229 point-of-care CD4 testing devices utilizing limited infrastructure, are currently in-use in

230 remote areas. For instance, the PIMA CD4 Analyzer (Alere, Germany), the Auto 40 System

231 (Apogee Flow Systems, UK) and the PointCare NOW system were shown to have results as

232 good as the traditional flow cytometer. [50-53] The Auto 40 system is as well validated with

233 reference method and assessed with external quality control. [54] Hence, CD4+ counting

234 become possible in rural countries for treatment monitoring.

235

236 **Drug resistance monitoring (PIs, NRTIs and NNRTIs)**

237 HIV-1 infected patients usually have their viral load and CD4+ counts monitored on a 3-month
238 to 6-month basis in developed countries. Virological rebound or treatment failure is defined
239 whenever the viral load is above 200 copies/mL or within the range of 50 to 200 copies/mL in
240 two to three consecutive samples after 6 months of antiretroviral therapy. [33, 55] The failing
241 condition may be due to poor drug adherence, adverse drug effects as well as the emergence of
242 drug resistance mutants. [30] During each round of HIV-1 replication, the error-prone reverse
243 transcriptase increases population dynamics by introducing random mutations into viral
244 population. Certain proportion of the viruses may become fitter and survive under drug
245 selective pressure. These viruses, carrying drug resistance mutations, will gradually
246 accumulate and dominate the major population. Therefore, it is necessary to determine drug
247 resistance mutations or in turns the drug susceptibility at the moment of virological rebound
248 before switching treatment regimen.
249 Phenotypic and genotypic methods are both available commercially for drug resistance
250 monitoring. Apart from clinical uses, both methods are vital for research and drug
251 developments. For example, they can be used to deduce the viral resistance and drug inhibitory
252 mechanisms. Phenotyping estimates the ability of *in vitro* viral entry or replication under drug
253 pressure, with respect to a known susceptible reference strain. The *in vitro* assays require
254 bio-safety class 3 level laboratory setting to handle infectious tissue cultures, cloning,

255 transfection and infection. Although phenotypic assays can provide more insights of the virus,
256 the long turnaround time and expensive running cost restricted the usage to selected clinical
257 cases only. [56] Genotyping, on the other hand, relies on gene amplification and direct
258 sequencing, which can provide results within one week. The analysis of nucleic acid sequences
259 can identify mutations that are established to have known phenotypic drug resistance. [57]
260 However, genotyping cannot predict drug susceptibility directly and is rather difficult to
261 interpret if the viral population is complex or super-infected. The basic principle of
262 phenotyping is to monitor the viral replication and fitness under sequential antiretroviral drug
263 concentrations. [56] This is achieved by direct isolation of viruses from human plasma or
264 peripheral blood mononuclear cells, or by generation of a recombinant virus which carries viral
265 sequences derived from clinical samples and a standard backbone genome. There are two
266 major commercially available phenotyping tools for examining PIs and NRTIs/NNRTIs
267 resistance. The PhenoSense HIV assay (Monogram Biosciences, USA) generates resistance
268 test vectors by inserting the amplified protease (PR) and reverse transcriptase (RT) sequences
269 into a modified HIV-1 NL_{4-3} molecular clone lacking PR and RT regions. The products will
270 then be used to co-transfect human embryonic kidney 293 cell line with a luciferase expression
271 vector to engineer a pseudotyped virus. The 293 cell line is later infected by the pseudotyped
272 viruses under different concentrations of the antiretroviral drugs, and produce luciferase

273 proteins if replication succeeds. Luciferase activity can be measured in a quantification scale,
274 so as to estimate the drug susceptibility. [58] The AntiVirogram (Virco BVBA, Belgium) is
275 slightly different from PhenoSense. The recombinant virus generation procedures are similar.
276 The downstream work relies on culturing the recombinant virus with human T cell line MT4
277 under all available antiretroviral drugs. No molecular cloning step is involved in this
278 phenotyping assay and a panel of recombinant strains will be created to reflect the diversified
279 viral population circulating in the patients. The assay compares the replicating capacity
280 between the wild-type virus and the constructed virus to provide inhibitory concentration (IC_{50})
281 of the antiretroviral drugs. [59] Both assays can readily access the drug susceptibility of patient
282 with viral load over 500 copies/mL. Although there are no significant differences between the
283 two assays for PIs and NNRTIs resistances, it seems that the PhenoSense performs better than
284 the Antivirogram in certain commonly used antiretroviral drugs such as Abacavir, Stavudine
285 and Didanosine. [60]
286 In comparing to phenotypic tests, genotypic tests provide a faster turnaround time and simpler
287 workflow. Current genotypic tests involve direct sequencing of the viral PR and RT region. The
288 protocols adopted by industries and research laboratories are similar; reverse transcription and
289 amplification of the RNA extract, followed by population Sanger sequencing. The Trugene
290 HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics, USA) and the ViroSeq HIV-1

291 Genotyping System (Celera Diagnostics, USA) are both CE-IVD-marked in Europe and
292 approved by the FDA in US. [61, 62] Many other commercial genotyping assays, such as
293 GenoSure MG (Monogram, USA), and less-pricey in-house genotyping assays are also well
294 evaluated worldwide. [63-66] The major limitation of both widely validated kits is that they
295 were designed basing on the HIV-1 subtype B viral genome, whereas their performance on
296 HIV-2 or other HIV-1 genoptypes remains uncertain. A recent study showed that the
297 sequencing primers of the ViroSeq system failed to sequence a panel of diverse subtypes. [67]
298 In particular, 1 out of the 7 sequencing primers failed to sequence over 50% of the included
299 non-B subtype samples. Since non-B subtype HIV-1 are the predominant circulating strains in
300 Asia, Africa and some parts of the European continents [6], the high failure rate of the ViroSeq
301 system on non-B viruses would be a major challenge in the future. It is believed that a modified
302 version of primers will be released in order to provide better coverage to a wide range of
303 genotypes identified recently. Independent laboratories have established various in-house
304 genotyping targeting non-B subtypes, including subtypes A, C, D, CRF01_AE, CRF02_AG.
305 The in-house assays have low sequencing failure rate and are able to achieve over 95%
306 sensitivities and specificities against validated kits. [64, 68, 69] In combining the advantages of
307 both genotyping and phenotyping, A third hybrid approach, the VircoTYPE HIV-1 (Virco
308 BVBA, Beerse, Belgium), is comprised of genotyping technique but with phenotypic analysis.

309 It is a modified version of *Virtual*Phenotype-LM and uses a linear regression modeling with
310 over 80,000 pairs of correlated genotypic and phenotypic samples for accurate drug
311 susceptibility prediction. [59] This approach provides a third option for drug resistance
312 monitoring by obtaining phenotypic information from genotyping only
313 The interpretation of the genetic sequences relies heavily on the most updated knowledge of
314 correlation between mutations and *in vitro* drug susceptibility. There are several HIV-1 drug
315 resistance algorithms available in the Internet, including the Stanford HIV db Program
316 (http://hivdb.stanford.edu/) (Stanford University, USA) and the ANRS database
317 http://www.hivfrenchresistance.org) (Agence Nationale de Recherches sur le Sida, Paris,
318 France). [70, 71] The former database allows the input of a single PR/RT mutation or the
319 PR/RT sequence for drug resistance interpretation on 19 commonly prescribed PR and RT
320 inhibitors while the later provides tables of rules for each class of drug resistance. Moreover,
321 genotypic sequences rely heavily on manual proof-reading and interpretation to the occurrence
322 of mixed viral population or poor sequence quality. This requires several hours of training for a
323 new technical staff and it is difficult to standardize their interpretation level among laboratories.
324 A new automated sequence analysis tool, RECall (http://pssm.cfenet.ubc.ca), does not require
325 manual editing and can identify mixed genetic population has been developed recently. [72]
326 This analysis tool shared over 99% of sequence agreement in comparing to manual editing and

327 will be a solution to tackle to standardization problem mentioned.

328

329 **Drug resistance monitoring (Integrase and fusion inhibitors)**

330 Integrase inhibitors (INI) and fusion inhibitors are the 2 recently FDA-approved antiretroviral
331 drug classes. INI has a relatively low genetic barrier, and more expensive than PIs and
332 NRTIs/NNRTIs. It is only used for patients who had developed multi-classes drug resistant or
333 low tolerance of adverse effects. [33] Drug resistance monitoring is available for INI
334 commercially, yet none of them were approved by the US FDA and CE-IVD marked. The basic
335 principles of genotyping and phenotyping for INI and fusion inhibitors are similar to those of
336 the PIs and RTIs classes. The PhenoSense and GeneSeq Integrase assays (Monogram, USA)
337 are the more commonly used commercially available phenotyping and genotyping assay
338 respectively. [73] Limited evaluation was carried on the ViroSeq and Trugene systems on their
339 capability of integrase drug resistance interpretation. [74, 75] The testing on fusion inhibitor is
340 further limited, which is mainly due to the inconvenient injections of fusion inhibitor.
341 Moreover, natural occurring drug resistance is found in certain HIV-1 subtypes, restricting the
342 susceptibility of this class. [76-78]

343

344 **Tropism identification and drug resistance monitoring (CCR5 antagonist)**

345 HIV-1 tropism is defined by the ability of virus infection with the two major chemokine
346 co-receptors, CCR5 and CXCR4. [79] R5-tropic (R5) virus is previously thought to be the
347 prevalent strain during transmission, while X4-tropic (X4) virus emerges due to AIDS
348 progression at a later disease stage. [80, 81] Recent controversial studies observed a higher
349 percentage of X4 virus in treatment-naïve patients of some subtypes and identified
350 transmission cluster consisted of X4 virus only. [81-83] The transition phase in the viral
351 population implies the possibility of a mixture of R5 and X4 viruses. The importance of
352 tropism identification is growing, due to the first introduction of CCR5 antagonist into salvage
353 therapy in 2007. Treatment guidelines in Europe and USA strongly recommend tropism tests
354 must be done prior to initiation of CCR5 antagonist, as it only suppresses R5 viral entry. [33, 55,
355 84]
356 The successfulness of CCR5 antagonist represents a new antiretrovirals era for scientists. The
357 mechanism seems to be simpler than the traditional drug classes, although the side effects of
358 blocking such co-receptor remain a concern in normal human metabolism. The phenotypic and
359 genotypic tropism tests are therefore aggressively being developed in this decade.
360 The MT-2 assay is the most traditional phenotypic tropism assay. Viruses isolated from patients
361 are used to co-culture with human T cell line MT-2, which express CXCR4 coreceptors only.
362 Syncytia will be formed if the viral isolates are able to infect MT-2 cells, implying the presence

363 of X4- or mixed/dual-tropic (D/M) viruses. [85] The use of MT-2 assay is limited, as it requires
364 specialized laboratory set up and fresh samples for virus isolation. Apart from it, it is
365 impossible to distinguish between virus isolation failure and a pure R5 virus population, due to
366 the lack of a CCR5 coreceptor-expression cell line. To overcome the limitations in MT-2 assay,
367 other single-cycle recombinant virus assays are as well applicable in tropism phenotypic tests.
368 [86] The Enhanced Sensitivity Trofile Assay (ESTA) (Monogram Biosciences, USA) is the
369 current 'gold standard' assay that has been clinically validated the most. [87, 88] The amplified
370 *env* gene is inserted into an expression vector, followed by co-transfecting 293 cell lines with a
371 luciferase-expression vector. Quantification can be done by measuring the luciferase signal
372 after a single round of infection of human primary glioblastoma U87 cell lines, with or without
373 appropriate antagonists. The assay requires at least 1000 copies/ml of viral load to perform and
374 3 ml of fresh sample or frozen plasma that are stored in less than 3 months. As X4 virus usually
375 exists as a minority, the detection limit of ESTA has now being improved greatly from the
376 previous 10% to 0.3% of the total population and is 100% sensitive. [89] The Toulouse
377 Tropism Test (INSERM, France) uses similar approach but with different backbone vector.
378 Both phenotypic tests are highly concordant except the fact that the ESTA assay is more
379 sensitive. [90]
380 Tropism determination can also be done by genotyping the third variable (V3) loop of HIV-1

381 *env* gene. [84, 91, 92] The 35-amino acids region is believed to bind and interact with the
382 co-receptor. The genotypic interpretation is originally based on the net charge and basic amino
383 acids at position 11 and/ 25 of the sequences. [93] Two more advance bioinformatic algorithms,
384 such as Geno2Pheno [co-receptor] (G2P) 1.2
385 (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) and Web PSSM
386 (http://indra.mullins.microbiol.washington.edu/webpssm), are publicly available and provide
387 instant tropism predictions by V3 nucleotides or amino acids sequences respectively. [94, 95]
388 G2P relies on the support vector machine technology trained with a large database of
389 nucleotide sequences and corresponding phenotypes. The interpretation is given in the form of
390 false positive rate, defining the likelihood of mistakenly classifying an R5 virus as X4 instead.
391 Different cut-offs and clinical parameters can be chosen in G2P, depending on the patients'
392 treatment history and the amplification results. [84] Web PSSM is slightly different, as it takes
393 into account of every amino acid at every position, but not insertions and deletions, to
394 determine the probability of an X4 virus. The interpretation is more complex when there is a
395 mixed base pair positions and generate more than one answer, which make it less convenient
396 for clinical practice and evaluation. Many clinical studies had reported a good correlation
397 between Trofile and G2P genotyping data in subtypes B and C. [96, 97] Triplicate V3
398 sequencing is currently recommended, which may have a better chance of detecting the

399 low-level of X4 minority. [98]

400 Several limitations are observed in V3 genotyping. Some heavily-treated patients usually have
401 a mixed viral population in their samples, which creates complication during direct sequencing.
402 Population sequencing detects up to 20% of the minority, which means 20% of the hidden X4
403 virus can grow and dominate under a short period of CCR5 antagonist suppression. New
404 technologies were developed recently to overcome these disadvantages. The denaturing
405 heteroduplex tracking assay (HTA) can detect as low as 0.5% minority strains, which is more
406 sensitive than Sanger sequencing. [99, 100] R5 and X4 viruses may only differ in a single
407 amino acid substitution. Therefore the HTA adopts various techniques to enhance the
408 sensitivity and specificity. The V3 region is first amplified with locked nucleic acids
409 incorporated primers, and annealed by a single-stranded fluorescent probe. The probe consists
410 of V3 R5 consensus so that X4 samples can form heterodupluxes with the probe. A
411 denaturing-gel-electrophoresis can distinguish the variants as DNA homodupluxes migrate
412 faster than DNA heterodupluxes, whose conformation can be, strengthen by formamide. Viral
413 tropic is therefore determined by the migration distance on the gel, and the study successfully
414 detected viral quasispecies in over 50 clones. This technique opens a new door for molecular
415 diagnosis in quantitative analysis and possible automation by the capillary electrophoresis
416 system, another upcoming trend.

417

418 **Ultra-deep pyrosequencing**

419 Direct sequencing is only capable of detecting roughly 20% of the minor viral population,

420 triggering more advance research to lower this detection limit. Ultra-deep pyrosequencing

421 (UDS) technique, provided by the Genome Sequencer FLX (GS-FLX) and Junior (GS-Junior)

422 systems (Roche-454 Life Sciences, Germany), has been developed to enhance the throughput

423 and sensitivity for sequencing. The systems first generate a library by amplifying the target

424 genes with specific fusion primer. Each library fragment will be attached to one bead, followed

425 by emulsifying in a water-in-oil mixture inside microreactors. Emulsion PCR amplification

426 creates millions of fragment copies which are then loaded onto the PicoTiterPlate device for

427 pyrosequencing. [5, 101] The latest version can achieve up to 700 megabases throughputs

428 within 23 hours with read length of 1,000 basepairs.

429 UDS has been extensively evaluated in HIV-1 diagnostic fields. Both pros and cons were

430 reported from many clinical studies. First of all, the cost of running UDS is largely higher than

431 population sequencing and is not as easy accessible as direct sequencing. More importantly, the

432 error rate of UDS is very high comparing to direct sequencing. In turn, the high throughput is

433 the major overwhelming advantage of UDS. Several PIs and RTIs resistance monitoring was

434 carried by UDS lately. [102-104] It seems that UDS is more applicable to

435 treatment-experienced patients, yet more studies are required to support the use of UDS in
436 clinical settings. Besides, the clinical response between the quasispecies and routine Sanger
437 sequences was similar in a recent study. Any extra viral variants observed in quasispecies
438 might actually do not exist in the population nor had loss of replicating ability. [102] Although
439 the study was of a small group of patients, it pointed out that the new technology may not have
440 many implications on clinical evaluation. Moreover, the large amount of data produced in UDS
441 may require a more delicate and detailed database for analysis. Another retrospective study
442 was conducted to show that UDS could predict the virological response more accurate than the
443 triplicate tropism sequencing approach. [105] It included patients enrolled in MOTIVATE and
444 A4001029 studies who were Maraviroc-experienced. Triplicate population sequencing was not
445 able to accurately predict all X4-tropic infections. Any R5 predictions was further deep
446 sequenced by GS-Junior or GS-FLX system, which have a sensitivity of 0.5% detection limit.
447 A significant number of patients who were classified in R5 infection were re-grouped into D/M
448 by UDS. The correlation between Maraviroc-responders from MOTIVATE and A4001029
449 studies was improved. The study showed that the tropism determined by UDS and ESTA were
450 similar, suggesting UDS can potentially replace the necessity of phenotypic assay, and mark it
451 as gold standard instead. The potential of replacing traditional Sanger sequencing by UDS in
452 patient care require further evaluation on the cost and practicality.

453

454 **Host genetics polymorphisms**

455 The close interaction and relationship between virus, antiretroviral drugs and the host cannot
456 be underestimated. It is because a few single nucleotide polymorphisms (SNPs) as well as
457 human leukocyte antigen (HLA) typing are linked to clinical failure or hypersensitivity
458 symptoms in HIV-1 patients. Therefore, understanding the link between pharmacogenomics
459 and metabolism is crucial. The most important example is demonstrated in CCR5
460 polymorphisms. CCR5-△32 has a 32-base deletion in CCR5 genes that will result in truncated
461 malfunctioned protein. CCR5-△32 homozygotes are naturally resistant to R5 infections and
462 heterozygotes are expected to have a slower disease progression than normal. [106] In
463 determining the host status of CCR5 gene, it helps clinicians to have a better idea on the
464 frequency of treatment monitoring. A meta-analysis included over 12,000 genotyped study
465 objects to evaluate the importance of CCR5-△32 heterozygosity. [107] There were no
466 consistent research outcome currently available, as contradicted predictions on the protective
467 behavior of CCR5-△32 remains unclear. The CCR5 level expressed in CCR5-△32
468 heterozygotes can possibly be as high as normal, and the expression level can be affected by
469 other factors apart from genotype.

470 Concerning the relationship of host genetic and drug hypersensitivity, HLA-B*5701 and

471 Abacavir is a well defined example. [108] Abacavir is widely prescribed as the first line
472 treatment regimen. Clinicians observed patients on Abacavir developed serious side effects,
473 such as rash, fever, and these effects disappeared after discontinuing Abacavir treatment. Later
474 it was found that if patients carrying HLA genoptype B*5701 in their alleles, they will have
475 hypersensitivity reaction towards Abacavir. [109] The prevalence of HLA-B*5701 varies
476 greatly around the world, ranging from 8-10% in Caucasians and Thai, to 1 % in Africans, and
477 to nearly 0% in Japanese, Taiwanese and Korean. [110-113] The treatment guidelines indicated
478 that the screening of HLA-B*5701 is compulsory before Abacavir prescription. There are
479 many FDA-approved HLA typing kits, which mostly utilize the direct sequencing techniques
480 or make use of the specific oligonucleotide probes hybridization after PCR. [114, 115] Other
481 SNPs were shown to have association with severe kidney tubular dysfunction in
482 Tenofovir-experienced patients. [116] Tenofovir is a popular first-line NRTI for treating HIV-1
483 infection with tolerable side effects usually. [117] The renal clearance of Tenofovir involves
484 multidrug-resistance protein 2 and 4, which are encoded by the adenosine triphosphate-binding
485 cassette genes *ABCC2* and *ABCC4*. [118, 119] Using the TaqMan SNP Genotyping Assays
486 (Applied Biosystems, CA, USA) two SNPs of the *ABCC2* gene were identified. The CC
487 genotype at position -24 and AA genotype at position 1249, were shown to have strong
488 association with kidney tubular dysfunction in Japanese and European population. [120, 121].

489 The highly polymorphic hepatic cytochrome P450 isoenzyme 2B6 (*CYP2B6*) gene
490 demonstrates the last example of host genetic polymorphisms. This gene participates in many
491 antiretroviral drugs metabolism, Efavirenz and Nevirapine in particular. [122] A SNP at
492 position 516 that changes from guanine to thymidine on the CYP2B6, is widely reported to
493 affect Efavirenz and Nevirapine concentration in plasma. [123] Direct sequencing can detect
494 the SNPs easily, supplemented by pharmacokinetic studies to monitor the concentration of
495 antriretroviral drugs in plasma. A new finding on the high Efavirenz level in hair, measured by
496 liquid chromatorgraphy coupled with tandem mass spectrometry, provides more insights on
497 alternative detection methods. [124, 125] The abovementioned examples elucidated the vital
498 host genetic determinants affecting antiretroviral prescription preferences, together with the
499 evidence on dissimilar disease progression. The cost of patient care after specific genes made
500 known to the adverse side effects. Nonetheless, larger study cohorts are required to reveal the
501 inconsistency in various SNPs and host reactions on virus and antiretroviral drugs.
502

503 **Conclusions & Future Perspectives**

504 After nearly three decades of the discovery of AIDS and HIV viruses, clinicians and scientists
505 have gone through many hurdles in unmasking the mystery of this virus. Current diagnostic
506 assays can detect both HIV antigens and antibodies, providing more rapid and faster detection

507 than before. Viral load and CD4 measurements are crucial for treatment monitoring. Lowering

508 the detection limit to possibly 20 copies/mL in viral load assays, the clinicians are able to

509 identify treatment failure patients at the earliest stage. HIV-1 genotyping is widely accepted as

510 the pre-dominant test to identify drug resistance mutations and tropism, although rare cases

511 require phenotyping tools for detailed analysis.

512 It is not surprised that the detection limits, sensitivity and specificity, costing and turnaround

513 time of all molecular assays will be improved this century with the introduction of new ideas

514 like ultra-deep sequencing and nano-particles assays.[126] Amplicons sequencing allow

515 researchers to identify individual viral mutants that previously undetected in population

516 sequencing. However this technique is highly demanded in budgeting and infrastructure

517 settings, and generates a large amount of data which requires highly-trained technicians and

518 complicated softwares to analyze. Various constraints at resource-limited or point-of-care

519 settings will as well be eliminated with portable devices with lower cost.

520

521 **Executive Summary**

522 **HIV-1 detection**

523 ● HIV-1 diagnostic assays include p24 antigen test, enzyme-linked immunosorbent assays
524 (ELISA), western blot (WB) and nucleic-acid amplification test (NAAT).

525 ● The 4th generation of ELISA and newly developed NAAT could successfully detect HIV
526 antigens and antibodies around 20 days earlier than WB confirmation.

527 **Viral load monitoring**

528 ● Plasma preparation tubes and EDTA tubes are used for sample collection in developed
529 countries, while dried blood spots are used in rural areas.

530 ● RNA extraction is largely facilitated by automated systems to reduce hands-on time and
531 provide standardized protocols.

532 ● Traditional reverse transcriptase polymerase chain reactions are mostly replaced by
533 real-time assays such as nucleic acid sequence-based amplification and branched-chain
534 DNA assay.

535 ● Low vireamia measurement, which may affect treatment guidelines, varies in between
536 assays and laboratories, and requires further evaluation.

537 **CD4+ T lymophocyte enumeration**

538 ● Flow cytometry counting is implemented in developed countries while mobile flow

539 cytometers provide alternative measurements in rural countries.

540 **Drug resistance monitoring (PIs, NRTIs and NNRTIs)**

541 ● When a patient experiences virological rebound or CD4+ decline, viral phenotyping and

542 genotyping is required to estimate drug susceptibility and resistance.

543 ● Phenotyping relies on the cloning of protease and reverse transcriptase sequences into a

544 modified vector, which can be transfected into human embryonic kidney cell line.

545 Luciferase signals are generated when the pseudotyped virus manage to infect the cell line

546 again or co-culture with human T cell line and replicate under different concentrations.

547 ● Genotyping bases on direct sequencing of protease and reverse transcriptase regions and

548 analyzed by commercial phenotyping database or various open-assessed algorithms on

549 internet.

550 **Drug resistance monitoring (Integrase and fusion inhibitors)**

551 ● Phenotyping and genotyping assays are similar to those for PIs, NRTIs and NNRTIs.

552 ● Due to the less common use of integrase and fusion inhibitors, limited clinical validation

553 is available.

554 **Tropism identification and drug resistance monitoring (CCR5 antagonist)**

555 ● HIV-1 virus utilizes CCR5 and/or CXCR4 co-receptor for viral entry. It is compulsory to

556 identify viral tropism before the use of CCR5 antagonist.

557 - Viral tropism can be determined by phenotyping and genotyping, with similar principles
558 in *pol* gene.
559 - Currently, no known CCR5 antagonist drug resistance mutations are identified.

560 **Ultra-deep pyrosequencing**

561 - In comparing to Sanger sequencing, ultra-deep pyrosequencing can detect up to 0.5% of
562 minor variants in viral population.
563 - Clinical studies demonstrated better treatment guidelines by ultra-deep pyrosequencing.
564 - However, the machines and running costs are extremely high that restrict the possibility of
565 routine monitoring in viral load, drug resistance mutations and tropism identification.

566 **Host genetics polymorphisms**

567 - A few single nucleotide polymorphisms and human leukocyte antigen (HLA) typing were
568 shown to have clinical relevance on treatment failure and hypersensitivity reactions in
569 HIV-1 patients.
570 - Examples of CCR5-Δ32, HLA-B*5701, *ABCC2* gene and CYP2B6 are discussed.

571

Table 1. Summary of current diagnostic assays

	Current Technology	Target Sites	Most Common Assays	Manu-facturers	Detection Limits	Ref.
HIV-1 Detection	ELISA (4th generation)	HIV-1 & HIV-2 Ab & p24 Ag	ARCHITECT HIV Ag/Ab Combo assay*	Abbott	p24: < 50 pg/mL Ab: 100% Sensitive 20 days before WB +ve	10-14
		HIV-1 gp41, HIV-2 gp36 Ab & HIV-1 p24 Ag	Enzygnost HIV Integral II	Siemens	p24: >100 pg/mL Ab: 100% Sensitive 14 days before WB +ve	
		HIV-1 gp160, HIV-2 gp36 Ab & p24 Ag	VIDAS HIV DUO Ultra	bioMérieux	p24: >3 pg/mL Ab: >98% Sensitive 20 days before WB +ve	
		HIV-1 gp160, HIV-2 env Ab & HIV-1 p24 Ag	GS HIV Combo Ag/Ab EIA*	Bio-Rad	p24: < 50 pg/mL Ab: 100% Sensitive 19 days before WB +ve	
	NAAT	RNA	APTIMA HIV-1 RNA Qualitative*	Gen-Probe	RNA: >14 cp/mL 95% Sensitive 26 days before WB +ve	16
Viral Load Monitoring	RT-qPCR	RNA *gag* & LTR region	COBAS Tagman HIV-1*	Roche	48 – 10,000,000 cp/mL	31, 33
		RNA *pol*	Abbott Real-time HIV-1*	Abbott	40 – 10,000,000 cp/mL	
	NASBA	RNA *gag*	NucliSENS EasyQ system HIV-1 QT*	bioMérieux	176 – 3,470,000 cp/mL	31-34
	bDNA	RNA *gag*	Versant HIV-1 RNA 3.0 *	Siemens	75 – 500,000 cp/mL	35
	RT-kPCR	RNA *pol/int*	Versant HIV RNA 1.0	Siemens	37 – 11,000,000 cp/mL	37
Drug Resistance Monitoring	Phenotyping (cloning, transfection &infection)	RNA *pol* (PR & RT)	Phenosense	Monogram	≥ 500 cp/mL	53
			Antivirogram	Virco	≥ 500 cp/mL	54
		RNA *pol* (INT)	Phenosense Integrase	Monogram	Limited information available	66
	Genotyping (direct sequencing)	RNA *pol* (PR & RT)	Trugene*	Siemens	≥1,000 cp/mL	56
			Viroseq*	Celera	2,000 – 750,000 cp/mL	57
		RNA *pol* (INT)	GeneSeq Integrase	Monogram	Limited information available	66
			ViroSeq Integrase	Celera		67
Tropism Determination	Phenotyping (cloning, transfection & infection)	RNA *env* (gp160)	Enhanced Sensitivity Trofile Assay	Monogram	100% Sensitive at 0.3% CXCR4, ≥1,000 cp/mL	82
		RNA *env* (gp120 & gp41)	Toulouse	INSERM	100% Sensitive at 0.5% CXCR4, ≥1,000 cp/mL	83
	Genotyping (direct sequencing)	RNA *env* (V3 loop)	In-house only	----	-----	77, 84, 85

Abbreviations: * - FDA approved assays; ELISA – Enzyme-Linked Immunosorbent Assay; NAAT – Nucleic Acid Amplification Test; WB – Western Blot; RT-qPCR – Reverse Transcriptase – qualitative Polymerase Chain Reaction; NASBA – Nucleic Acid Sequence-Based Amplification; Ab – Antibodies; Ag – Antigens; bDNA – Branched-chain DNA assay; RT-kPCR – Reverse Transcriptase – kinetic Polymerase Chain Reaction; cp/mL – copies/mL; +ve – positive; LTR – Long-Terminal Repeats; PR – protease; RT – Reverse Transcriptase; INT - Integrase

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