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1 **Near-point-of-care assay with a visual readout for**
2 **detection of HIV-1 drug resistance mutations: a proof-of-**
3 **concept study**

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14

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17

18 **Abstract**

19 Human immunodeficiency virus (HIV) infection is a chronic disease that can be treated with
20 antiretroviral (ARV) therapy. However, the success of this treatment has been jeopardized by
21 the emergence of HIV infections resistant to ARV drugs. In low- to middle-income countries
22 (LMICs), where transmission of resistant viruses has increased over the past decade, there is
23 an urgent need to improve access to HIV drug resistance testing. Here, we present a proof-of-
24 concept study of a rapid and simple molecular method to detect two major mutations (K103N,
25 Y181C) conferring resistance to first-line nonnucleoside reverse transcriptase inhibitor
26 regimens. Our near-point-of-care (near-POC) diagnostic test, combining a sequence-specific
27 primer extension and a lateral flow DNA microarray strip, allows visual detection of HIV
28 drug resistance mutations (DRM) in a short turnaround time (4 h 30). The assay has a limit of
29 detection of 100 copies of plasmid DNA and has a higher sensitivity than standard Sanger
30 sequencing. The analytical performance was assessed by use of 16 plasma samples from
31 individuals living with HIV-1 and results demonstrated the specificity and the sensitivity of
32 this approach for multiplex detection of the two DRMs in a single test. Furthermore, this near-
33 POC assay could be easily tailored to detect either new DRMs or DRM of from various HIV
34 clades and might be useful for pre-therapy screening in LMICs with high levels of transmitted
35 drug resistance.

36

37 **Keywords:** HIV-1, drug resistance mutations, rapid testing, lateral flow test, multiplex
38 detection, near POC.

39 **1. Introduction**

40 Human immunodeficiency virus type 1 (HIV-1) remains a major global public health issue. In
41 2019, approximately 38 million people worldwide were living with HIV and 1.7 million were
42 newly infected. According to estimates, 690,000 people died from HIV-related causes in
43 2019. Antiretroviral (ARV) drugs have been widely used to treat and prevent HIV infection.
44 However, acquired HIV drug resistance (ADR) has emerged as the result of the high genetic
45 instability of retroviruses[1] and poses a major threat to ongoing efforts to control the
46 pandemic[2]. Indeed, in cases of ADR, people living with HIV may transmit the drug-
47 resistant virus to a person who might subsequently not respond to first-line ARV regimens;
48 this is known as pretreatment drug resistance. As a consequence, transmission of resistant
49 viruses has increased over the past decade[3, 4], mostly in low- and middle-incomes countries
50 (LMICs).

51 In high-income countries, sequencing of reverse transcriptase (RT) and protease genes is
52 routinely performed by Sanger-based methods in order to diagnose viral mutations after
53 repeated detection of positive HIV viremia and adherence counseling or before the initiation
54 of ARV treatment. However, these techniques do not have the same public health impact in
55 LMICs, both because their availability is restricted to a small number of centralized
56 laboratories nationwide and because they are not affordable. For these countries, there is an
57 urgent need to provide access to a rapid and simple HIV drug resistance (HIVDR) detection
58 assay[2, 5, 6].

59 In the past 10 years, various point-mutation assays have been developed to increase access to
60 diagnosis in the populations that need it. These have included multiplex allele-specific PCR[7,
61 8], solid-phase melting curve analysis[9], ligation on RNA amplification[10], and the pan-
62 degenerate amplification and adaptation assay[11, 12]. Despite their performance, they have
63 not yet been implemented in low-level health-care facilities because of their system

64 complexity: they (i) require sophisticated instruments and (ii) need to be performed by highly
65 trained personnel. The ideal HIV DRM assay would fit the ASSURED (Affordable, Sensitive,
66 Specific, User-friendly, Rapid and robust, Equipment free, and Deliverable) criteria
67 recommended by the World Health Organization (WHO)[13]. Therefore, lowering test
68 complexity is still an issue for the application of these technologies in low-resource
69 settings[14].

70 Point-of-care (POC) tests offer the possibility of providing fast results requiring minimal user
71 intervention to clinicians in remote settings[15, 16]. In the field of classical on-site
72 diagnostics, lateral flow biosensors are the best-established platform for POC testing. The
73 technical advantages of these sensors are that they are self-operating devices that perform
74 rapid assays from a single sample and that results can be obtained by visual readout. Only two
75 membrane-based methods have been reported for HIVDR detection[17-20]. They are both
76 based on the detection of ligated products via a customized lateral flow strip. The most
77 advanced, the oligonucleotide ligation assay, enables highly accurate detection of only one
78 drug resistance mutation (DRM) in a single test. Several monoplex kits have been developed
79 to detect six major DRMs and were recently found to have excellent diagnostic performances
80 in randomized controlled trials, although the public health benefit was mitigated in terms of
81 population viral load control and in a cost-effectiveness analysis [17, 20].

82 Recently, we have developed an approach for rapid multiplex detection of point mutations
83 using a combination of a sequence-specific primer extension (SSPE) and a lateral flow DNA
84 microarray strip[21]. This simple assay allows simultaneous visual detection of multiple
85 nucleic sequences on a single device. This type of strategy has been applied to genetic
86 diseases and cancer[22-24]. In this study, we applied this strategy for the development of a
87 near-POC HIV DRM assay. This assay was designed for its use in decentralized laboratories
88 to improve access to diagnostic tests in LMICs. As a proof-of-concept, the test was developed

89 to detect two major mutations (K103N and Y181C) conferring resistance to nonnucleoside
90 reverse transcriptase inhibitors (NNRTI) included in first-line regimens used in LMICs[2, 25].
91 Herein, we present the analytical performances of the near-POC assay and its evaluation on
92 clinical samples.

93 **2. Material and methods**

94 **2.1 HIV plasmids and clinical samples**

95 The HIV-1 subtype B plasmids containing K103 (wild-type, WT), 103N (mutant, MUT),
96 Y181 (WT), and 181C (MUT) in the RT region were provided by the National Institute of
97 Health (NIH) acquired immune deficiency syndrome (AIDS) reagent program (Table 1).
98 Five plasma specimens from individuals with HIV-1 subtype B were collected, with informed
99 consent, from the Centre Hospitalier Universitaire (CHU) Montpellier (Montpellier, France).
100 These samples harbored viruses resistant to NNRTIs (K103N, Y181C). Eleven additional
101 plasma specimens infected with WT HIV-1 were obtained from blood donor collection
102 (French Blood Agency National Plasma Bank, Tours, France).

103

104 **2.2 Multiplex HIV DRM detection assay**

105 *2.2.1 PCR amplification of HIV regions*

106 Oligonucleotide primers and probes were obtained from Eurogentec (Seraing, Belgium),
107 Table 1). Each PCR reaction contained 0.2 μ M primers (ANRS RT18 forward [5'-GGA AAC
108 CAA AAA TGA TAG GGG GAA TTG GAG G -3'] and RT21 reverse [5'-CTG TAT TTC
109 TGC TAT TAA GTC TTT TGA TGG G -3'] primers), 1X KAPA Taq HotStart buffer, 1.5
110 mM MgCl₂, 1 U of KAPA Taq HotStart (KAPA biosystems, Wilmington, MA), and a
111 plasmid DNA template in a final volume of 50 μ L. PCR was performed in a thermal cycler
112 (Biometra TProfessional, Analytik Jena, Jena, Germany) starting with enzyme activation at

113 95°C for 3 min, followed by 40 cycles of 40 s at 94°C, 40 s at 55°C, and 1 min at 72°C, and a
114 final extension step of 10 min at 72°C.

115 One microliter of plasmid DNA at a defined number of copies (quantification using a
116 BioSpec-nano spectrophotometer, Shimadzu, Kyoto, Japan) was added to the PCR mixture to
117 determine the limit of detection of the assay, whereas 5 µL (10 ng) of each solution of WT
118 plasmids with various proportions of MUT plasmids were used to evaluate its sensitivity.

119

120 *2.2.2 Design of sequence-specific primers*

121 Based on the 2019 WHO HIV Drug Resistance Report[26], we designed primer sequences
122 targeting two major DRMs, K103N and Y181C, and the corresponding WT sequences (Table
123 2) using OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA). Each primer
124 consisted of a sequence specific to the HIV-1 variants and a tag sequence complementary at
125 the 5'-end to the capture probes spotted on the lateral flow membrane (Supplementary
126 Material Table S1).

127

128 *2.2.3 Multiplex SSPE assay*

129 The multiplex extension assay was performed in a 20 µL reaction mixture containing 5 µL of
130 PCR product, 1 X ThermoPol Reaction Buffer, 2 mM MgSO₄, 0.8 U of Vent® (exo-) DNA
131 polymerase (New England Biolabs, Beverly, MA), 200 nM of Y181, 181C, and K103
132 primers, 400 nM of 103N primer, 25 nM of control primer, 2.5 µM each of dATP, dGTP, and
133 dCTP, 1.25 µM dTTP, and 1.25 µM biotin-11-dUTP (Thermo Fisher Scientific, Waltham,
134 MA). The cycling conditions were as follows: initial denaturation 3 min at 95°C, followed by
135 30 cycles of 30 s at 95°C, 15 s at 57°C, 15 s at 72°C, and a final extension step of 3 min at
136 72°C.

137

138 *2.2.4 Construction of lateral flow test strips*

139 The dry-reagent dipstick (5 mm x 80 mm) containing a wicking pad, a glass-fiber conjugate
140 pad, a nitrocellulose membrane, an absorbent pad, and an adhesive backing card was
141 assembled as previously described[21] (Supplementary Material Figure S1). All components
142 were purchased from Merck Millipore (Darmstadt, Germany). The dipstick test was
143 composed of a test zone and a control zone. The five anti-tag capture probes were manually
144 spotted on the membrane in spotting buffer (6X saline-sodium citrate (SSC), 2% methanol,
145 2% sucrose) with a final volume of 0.1 μ L at selected concentrations (Supplementary Material
146 Table S1). Anti-tag oligonucleotides corresponding to the WT sequences were spotted on the
147 left side of the membrane, while oligonucleotides corresponding to the MUT sequences were
148 spotted on the right side. An HIV-1 positive control anti-tag probe (Table 2) was spotted on
149 the upper part of the test zone to verify the amplification process. A solution of 25 μ g/mL
150 biotinylated BSA with a 0.1 μ L volume was loaded in duplicate at the top of the strip to
151 define a control zone. After assembly, the dipstick tests were stored dry in a desiccator cabinet
152 at room temperature.

153

154 *2.2.5 Detection of the DRMs by lateral flow dipstick*

155 The extended products (3 μ L) were applied to the bottom of the membrane, close to the
156 conjugate pad. The wicking pad was then immersed in 50 mL conical tube containing
157 prewarmed developing solution (1X SSC, 10% Tween-20, 0.5% sodium dodecyl sulfate
158 (SDS), and 0.4 M urea) and incubated at 44°C in a hybridization incubator (Techne, Cole-
159 Parmer, Staffordshire, U.K.). The visual readout was completed within 20 minutes. The
160 validity of an assay was determined by the two following criteria: (i) generation of two red
161 dots on the control zone and (ii) the presence of a red dot for the HIV-1 positive control.

162

163 **2.3 Analytical performances of the HIV DRM detection assay**

164 *2.3.1 Determination of the limit of detection*

165 A serial dilution of a plasmid DNA standard with the WT HIV-1 genotype from 10^9 copies to
166 1 copy was realized to evaluate the signal response on WT probes. The multiplex SSPE was
167 performed on each dilution and extended products were analyzed on lateral flow strips.

168

169 *2.3.2 Determination of the assay sensitivity*

170 To determine the analytical sensitivity of the multiplex HIV DRM detection assay for each
171 DRM, solutions of WT plasmids with various proportions of MUT plasmids (0, 5, 10, 20, or
172 50% of MUT allele) were prepared. The sensitivity of the assay was determined based on the
173 visual reading of red spots on the strip for each DRM and repeated 4 times.

174

175 **2.4 HIV DRM detection assay on clinical specimens**

176 The performance of this HIV DRM detection assay was assessed using 16 plasma samples.
177 Viral RNA was extracted from 400 µL of plasma with the MagNA Pure Compact instrument
178 using the Nucleic Acid Isolation Kit I (Roche diagnostics, Switzerland) according to the
179 manufacturer's instructions and eluted in 50 µL elution buffer. A 1163 bp region of the pol
180 gene was amplified by one-step RT-PCR (Qiagen, Germany) in a final volume of 50 µL
181 containing 1X QIAGEN OneStep RT-PCR buffer, 400 nM dNTPs, 0.6 µM of ANRS RT18
182 and RT21, 2 µL QIAGEN OneStep RT-PCR enzyme mix, and 5 µL of RNA extract. RT-PCR
183 consisted of a 30 min reverse transcription step at 50°C and a 15 min Taq polymerase
184 activation at 95°C, followed by 40 cycles of 40 s at 94°C, 40 s at 56°C, and 1 min at 68°C,
185 and a final extension step of 10 min at 72°C.

186 The strips were imaged on a flatbed scanner (perfection V600 photo, Epson, Japan), in 24-bit
187 color (16.7 million colors) with a 1200 dpi resolution. After conversion of 24-bit images into
188 an 8-bit format, scan strips were analyzed with the ImageJ program
189 (<https://imagej.nih.gov/ij/>). The integrated density per spot was calculated as the product of
190 the number of square pixels and mean gray value within the circular feature defining the spot
191 and corrected using automatic threshold. Integrated density data of capture probes were
192 imported into Excel (Microsoft Corp, Redmond, WA) and interpreted semi-automatically
193 using a spreadsheet program.

194

195 **3. Results**

196 **3.1 Principles of the DRM detection assay**

197 The workflow of the assay for HIV DRM detection is presented in Figure 1. After
198 amplification of the region of interest by PCR, a multiplex primer extension is performed
199 using sequence-specific primers (Figure 2). Extension is made possible if the 3'-end primer
200 matches targeted sequences. Biotin-dUTP is incorporated during this step allowing the
201 detection of extended products on the strip. Degenerative nucleotides are introduced
202 downstream to encompass known HIV-1 sequence variations[27, 28].

203 After the multiplex SSPE reaction, samples are loaded onto the lateral flow dipstick and the
204 strip is dipped in the running buffer. As the sample migrates, tagged extended products are
205 captured by anti-tag capture probes immobilized on the nitrocellulose and anti-biotin
206 antibodies conjugated to gold nanoparticles permit the detection of DRMs by the generation
207 of red dots that are visible to the naked eye. The excess of gold nanoparticles is captured at
208 the control zone of the dipstick by immobilized biotinylated BSA. After the assay has been
209 completed, the strips that meet the analysis criteria described in the Material & Methods are
210 considered valid, and genotypes are determined by visual reading of red spots on the strip for
211 each DRM (Supplementary Data Figure S1).

212

213 **3.2 Limit of detection of the DRM detection assay**

214 Strong signals were observed on WT probes on a serial dilution of plasmid DNA standard; the
215 limit of detection was 100 copies but barely visible at 50 copies (Figure 3). Moreover, no
216 specific signals on MUT capture probes were observed.

217

218

219 **3.3 Sensitivity of the assay for DRM detection**

220 As shown in Figure 4, intense and specific signals were visualized on the MUT and WT
221 probes for the 100% WT and 100% MUT plasmids and no nonspecific signals on other
222 capture probes were observed. For the dilutions of MUT plasmids, color intensities of
223 corresponding probes were, as judged by the naked eye, proportional to the percentage of
224 mutated template. A visible signal was observed for solutions with MUT sequences down to
225 10% for K103N and 20% for Y181C.

226 A signal ratio (r) was calculated for each DRM to investigate whether the HIV DRM
227 detection assay was quantitative with the formulae:

$$228 \quad (r) = \text{IMUT} / (\text{IWT} + \text{IMUT})$$

229 where IWT was the integrated density on the WT capture probe and IMUT the integrated
230 density on the MUT capture probe. The data (Table 3) showed an increase in the ratio (r)
231 values but showed that there was not a linear relationship between this increase and the
232 percentage of MUT plasmids.

233

234 **3.4 Evaluation of the performance of the DRM detection assay on clinical specimens**

235 The performance of this HIV DRM detection assay was assessed using 16 plasma. The
236 alignment of the 16 HIV-1 pol sequences showed a variability around the two codons (103
237 and 181). In order to improve SSPE efficiency, we designed new HIV-1 subtype B primers
238 that included wobbles to cover polymorphisms at and adjacent to targeted variants. The
239 design of the primer Y181C proved to be more challenging, due to the relatively frequent
240 variations at codon 179 (V179E, V179I). Therefore, we decided to work on the positive strand
241 instead of the minus strand. The new SSPE primers contained four wobbles for the Y181C
242 mutation (Table 2). For the K103N mutation, only one wobble (R=A, G) was introduced to
243 the 3'-end of the 103N specific primer and a new K103 primer (K103 plasma) was designed

244 with one wobble (Y=C, T) incorporated at the 3'-end, to cover the two existing codons. A
245 visible signal was observed for all samples including the sample #16 with a viral load as low
246 as 692 copies/mL, equivalent to 14 copies of viral RNA in the assay (Figure 5). All five
247 samples with DRMs were detected by the HIV DRM assay. Furthermore, some samples did
248 not contain the HIV WT sequence, as previously determined by Sanger sequencing, and no
249 false positive results were noticed. Scan images of the lateral flow strips analyzed by our
250 computer program yielded the same results (Supplementary Data Figure S2).

251

252 **4. Discussion**

253 We have developed a rapid and simple molecular method to identify two major mutations
254 associated with resistance to first-line NNRTI drugs. The assay fulfills most of the ASSURED
255 criteria[13]. It is affordable: for the detection of two DRMs, the entire cost of the assay,
256 including viral RNA extraction and all reagents, was below \$20, while the cost of the Sanger
257 sequencing assay is estimated to be around \$120[29]. It is sensitive: the lower detection limit
258 was around 20 copies per assay, in contrast with the 200 copies per assay that is usually the
259 cut-off for the Sanger sequencing assay. As a consequence, the HIV DRM duplex assay had a
260 higher variant detection limit – 10 % of the total viral population – than the conventional 20%
261 threshold of the Sanger sequencing method for K103N and Y181C[30, 31]. However, further
262 developments to the multiplex SSPE step are underway to increase the analytical
263 performance, such as evaluation of new DNA polymerases. It is specific: to date, we have not
264 observed a cross reaction either between WT and variant sequences or between sequences
265 from different subtypes. It is user friendly: in particular, the final readout is simple. It is rapid
266 and robust: the time-to-result is estimated to be 4 h 30. Further improvements of the assay are
267 already in progress, including optimization of the one-step RT-PCR to reduce the time-to-
268 result to 3 h. This method is compatible with a one-day “test-and-treat” strategy to achieve the

269 90-90-90 global HIV target set by The Joint United Nations Programme on HIV/AIDS
270 (UNAIDS)[32]. It is not equipment free: the assay still requires a system for nucleic acid
271 extraction, a thermal cycler, and a dry oven, suggesting that a laboratory environment is
272 necessary. However, it obviates the need for complex instruments, which makes its
273 implementation possible in most advanced health-care services with minimal laboratory
274 facilities. It would be deliverable to end users: considering the assay is based on well-known
275 methods and the composition of the kit is fairly simple, there are no anticipated obstacles for
276 manufacturing the test kit.

277 The strategy described herein has several limitations. First, it is the result of a proof-of-
278 concept design and the HIV DRM detection assay simultaneously detected only two DRMs
279 (K103N, Y181C) in a single test. However, the number of variant SSPE primers can be
280 increased, as can the number of probes on the lateral flow DNA microarray. This suggests that
281 the four DRMs recently identified in a meta-analysis of transmitted drug resistance (TDR) in
282 individuals living with HIV-1 suggested that four NNRTI DRMs (K101E, K103N, Y181C,
283 and G190A) could be detected with a single strip [33]. Second, this design cannot apply to all
284 situations worldwide given the heterogeneity of the HIV sequence. However, we
285 demonstrated in this study how flexible the technology is. We were able to tailored primers
286 and probes in order to get a signal. A preliminary deep analysis of the circulating sequences
287 will be compulsory prior proposing new DRM detection assay. Considering the different
288 geographical distribution of the HIV variants, it will make sense to propose one DRM assay
289 for one discrete region, e.g. the one dedicated for austral Africa will be different from the one
290 for South-East Asia. Thirdly, viral resistance is not static and evolved upon selection pressure.
291 Since 2019, NNRTIs drugs are no longer recommended for HIV treatment in LMICs and have
292 been replaced by integrase inhibitors (INSTIs). More precisely, the WHO recommends the
293 shift to a combination comprising dolutegravir (DTG) as the preferred regimen in LMICs.

294 The drug has, so far, a high genetic barrier to resistance but its scaling up is far from being
295 optimal in LMICs and it is probably too early to be capable to capture DTG DRMs by
296 surveillance programs. Nonetheless, as soon as these new DRMs are identified, the DRM
297 detection assay can be modify in response the new situation. The performance of the assay
298 can benefit from additional improvements. The development of a software interface to
299 analyze results and guide nonskilled users which would improve accuracy and traceability of
300 results. Finally, the molecular assay provides an additional information to the clinician, the
301 HIV-1 positive viremia. It is not yet quantitative nor semi-quantitative, but such development
302 could be easily investigated with this simple technology. Together, the assay might be useful
303 for pre-therapy screening in regions with high levels of TDR but also during routine patient
304 follow-up.

305 This approach performed well in this proof-of-concept study. The number of DRMs tested
306 needs to be determined according to the local context, and implementation of the assay in
307 low-level health-care facilities will require further evaluation of its acceptability and efficacy.

308 **Authors' contributions**

309 **Julien Gomez-Martinez:** investigation, methodology, experimentation, data analysis, writing
310 original draft: review; **Vincent Foulongne:** conceptualization, methodology, sequencing;
311 **Didier Laureillard:** conceptualization, funding acquisition; **Nicolas Nagot:**
312 conceptualization, funding acquisition; **Brigitte Montès:** methodology, sequencing; **Jean-**
313 **François Cantaloube:** conceptualization, methodology, funding acquisition; **Philippe Van**
314 **de Perre:** conceptualization, methodology, funding acquisition; **Chantal Fournier-Wirth:**
315 conceptualization, methodology, funding acquisition; **Jean-Pierre Molès:** conceptualization,
316 methodology, supervision, data analysis, funding acquisition, writing original draft: review;
317 **Jean-Charles Brès:** conceptualization, methodology, supervision, data analysis, funding
318 acquisition, writing original draft: review and editing. **Jean-Pierre Molès** and **Jean-Charles**
319 **Brès** contributed equally.

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324 Molecular Clone (p6463-13) (cat# 7401), HIV-1 NL4-3 1392 Infectious Molecular Clone
325 (p7324-1) (cat#7396), HIV-1 Infectious Molecular Clone (p5485) (cat#12229) and HIV-1
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329 **Declaration of competing interest**

330 The authors declare that they have no known competing financial interests or personal
331 relationships that could have appeared to influence the work reported in this paper.

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466

467 **TABLES**

468 Table 1. Multiple sequence alignment between the reference plasmid sequences containing DRMs K103N and Y181C, and the
 469 sequence-specific primers, including the HIV-1 positive control primer, used for the multiplex SSPE.

Plasmid sequences for reverse transcriptase codon 103/Primer sequences																	
		RT codon		102	103	104	105	106	107	108	109	110	111	112	113	114	
Plasmids	WT	p7324-1	5'	AAG	AAA	AAA	TCA	GTA	ACA	GTA	CTG	GAT	GTG	GGT	GAT	GCA 3'	
	WT	p6463-13	5'	AAG	AAA	AAA	TCA	GTA	ACA	GTA	CTA	GAT	GTG	GGT	GAT	GCA 3'	
	MUT	p5485	5'	AAG	AAC	AAA	TCA	GTA	ACA	GTA	CTG	GAT	GTG	GGT	GAT	GCA 3'	
	WT	p16182	5'	AAG	AAA	AAA	TCA	ATA	ACA	GTA	CTG	GAT	GTG	GGT	GAT	GCA 3'	
Primers		R103K	3'		T	TTT	AGT	TAT	TGT	CAT	GAC	CTA	CAC	CCA	CTA	5' Tag	
		R103N	3'		Y	TTT	AGT	TAT	TGT	CAT	GAC	CTA	CAC	CCA	CTA	5' Tag	
Plasmid sequences for reverse transcriptase codon 181/Primer sequences																	
		RT codon		169	170	171	172	173	174	175	176	177	178	179	180	181	182
Plasmids	WT	p7324-1	5'	GAG	CCT	TTC	AGA	AAA	CAA	AAT	CCA	GAA	ATA	GTT	ATC	TAT	CAA 3'
	WT	p6463-13	5'	GAG	CCT	TTC	AGA	AAA	CAA	AAT	CCA	GAA	ATA	GTT	ATC	TAT	CAA 3'
	WT	p5485	5'	GAG	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAG	ATA	GTT	ATC	TAT	CAG 3'
	MUT	p16182	5'	GAG	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAC	ATA	GTC	ATC	TGT	CAA 3'
Primers		F181Y	Tag 5'	G	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAM	ATA	GTI	ATC	TA	3'
		F181C	Tag 5'	G	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAM	ATA	GTI	ATC	TG	3'
Consensus subtype B sequence/Primer sequence – HIV-1 positive control																	
		RT codon		149	150	151	152	153	154	155	156	157					
Plasmids		p7324-1	5'	CTT	CCA	CAG	GGA	TGG	AAA	GGA	TCA	CCA	3'				
		p6463-13	5'	CTT	CCA	CAG	GGA	TGG	AAA	GGA	TCA	CCA	3'				
		p5485	5'	CTT	CCA	CAG	GGA	TGG	AAA	GGA	TCA	CCA	3'				
		p16182	5'	CTT	CCA	CAG	GGA	TGG	AAA	GGA	TCA	CCA	3'				
Primers		HIV-Pos	3'		GGT	GTC	CCT	ACC	TTT	CCT	AGT	G	5'	Tag			

471 **Table 2.** SSPE primers with tag sequences.

HIV variant	Primer sequences ^a
K103	5'- AGG TTG GCA CCA TAG TCT CAT CAC CCA CAT CTA GTA CTG TTA CTG ATT TT-3'
103N	5'- GCT TCC AAG ATA AGA GCC AAT CAC CCA CAT CTA GTA CTG TTA CTG ATT TR-3'
Y181	5'- CAC ATC TAA TAC TTT ATA CAA TTC GCC TTT TAG AAA ACA AAA TCC AGA MAT AGT YAT CTA-3'
181C	5'- TTC TCT TCT TCA CTT TAT ACA AAT GCC TTT TAG AAA ACA AAA TCC AGA MAT AGT YAT CTG-3'
Reverse Y181	5'- CAC ATC TAA TAC TTT ATA CAA TTC CCT ACA TAY AAR TCA TCC AYR TAT TGA T -3'
Reverse 181C	5'- TTC TCT TCT TCA CTT TAT ACA AAT CCT ACA TAY AAR TCA TCC AYR TAT TGA C -3'
K103 plasma	5'- AGG TTG GCA CCA TAG TCT CAT CAC CCA CAT CTA GTA CTG TTA CTG ATT TY-3'
HIV-1 positive control	5'- AGT AGA TGT CCT CAA ATG GGT GAT CCT TTC CAT CCC TGT GG-3'

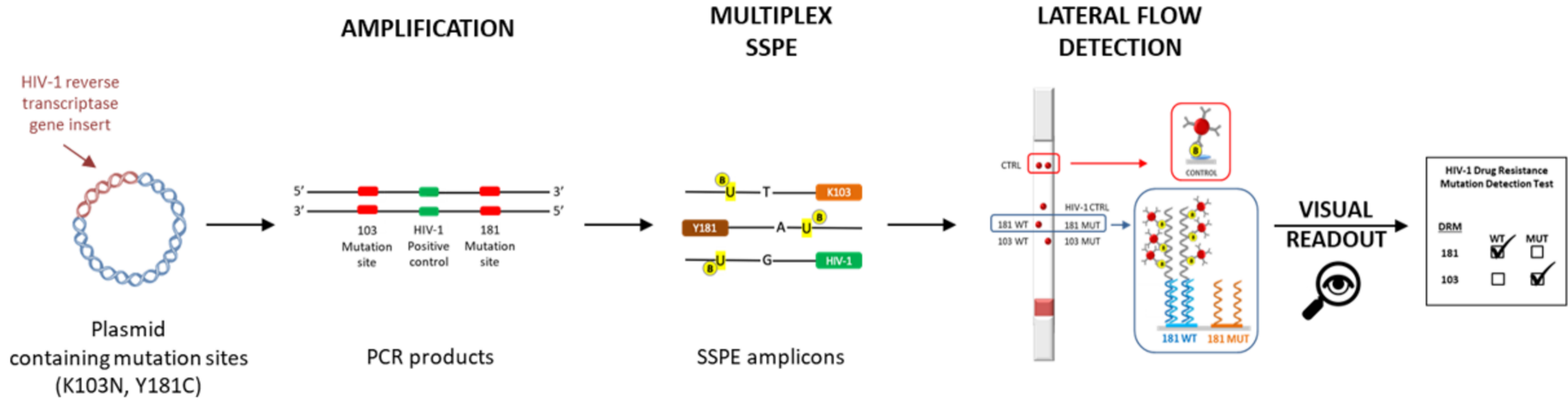
472 ^aBold sequences correspond to the tag sequences of each primer – 3'-end sequence was specific for the mutant or wild type sequence of HIV-1 variant.

473

474 **Table 3.** Ratio values (*r*) for plasmid mixture dilutions.

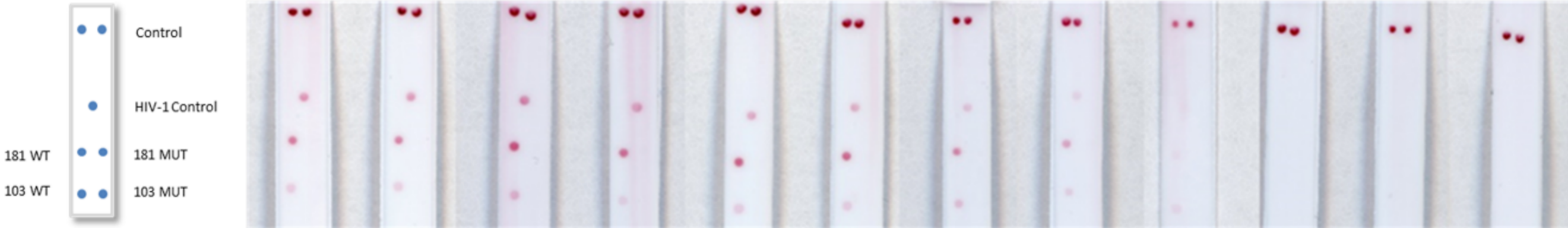
	Percentage of MUT (%)					
	0	5	10	20	50	100
DRM	0	0	0	0	0	0
K103N	0	0	0.23±0.04	0.32±0.04	0.5±0.03	+1
Y181C	0	0	0	0.1±0.06	0.41±0.05	+1

475



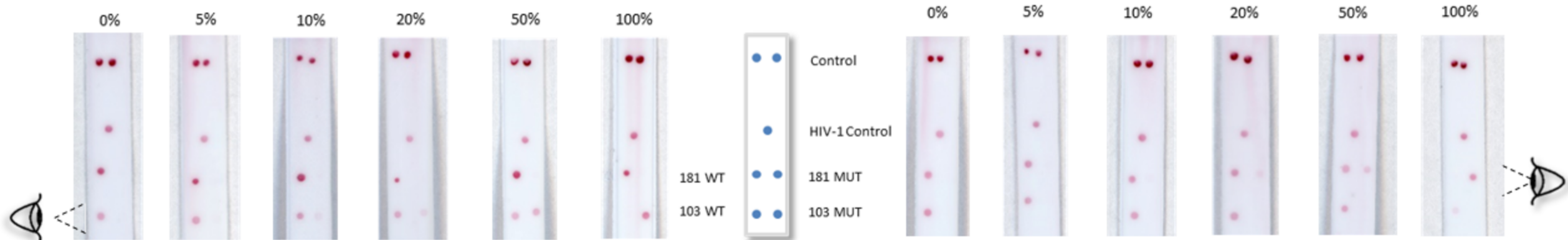
Number of copies

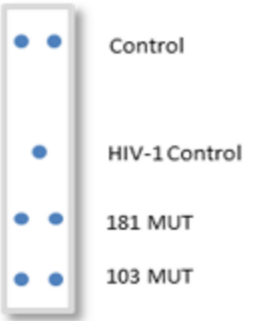

10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 50 10 1 Water



Percentage of 103 MUT

Percentage of 181 MUT



Sample ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16
Viral Load (copies/mL)	68,000	51,000	46,000	68,502	157,315	118,122	123,861	36,934	225,000	27,398	54,737	11,621	2,169	127,300	20,204	692
HIV-1 subtype	B	B	B	C	B	B	B	C	B	B	B	B	B	B	B	CRF11-cpx
																
Detection of DRM	+	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-
	(181 MUT)	(103 MUT)	(103 MUT)						(181 MUT)					(181 MUT)		

