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

Julien Gomez-Martinez, Vincent Foulongne, Didier Laureillard, Nicolas Nagot, Brigitte Montès, Jean-François Cantaloube, Philippe van de Perre, Chantal Fournier-Wirth, Jean-Pierre Molès, Jean-Charles Brès

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

Julien Gomez-Martinez[a], Vincent Foulongne[a,b], Didier Laureillard[a,c], Nicolas Nagot[a], Brigitte Montès[b], Jean-François Cantaloube[a], Philippe Van de Perre[a], Chantal Fournier-Wirth[a], Jean-Pierre Molès[a, §], Jean-Charles Brès[a, §,*].

[a] Pathogenesis and Control of Chronic and Emerging Infections, EFS, Inserm, University of Montpellier, F-34394 Montpellier, France.

[b] Laboratoire de Virologie, Centre Hospitalier Universitaire de Montpellier, Montpellier, France.

[c] Department of Infectious and Tropical Diseases, Centre Hospitalier Universitaire Carémeau, Nîmes, France.

[§] These authors contributed equally to this work.

* corresponding author: Phone: +33(0)4-67-61-64-20; Fax: +33(0)4-67-61-64-56

E-mail: jean-charles.bres@efs.sante.fr

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Material and methods

2.1 HIV plasmids and clinical samples

The HIV-1 subtype B plasmids containing K103 (wild-type, WT), 103N (mutant, MUT), Y181 (WT), and 181C (MUT) in the RT region were provided by the National Institute of Health (NIH) acquired immune deficiency syndrome (AIDS) reagent program (Table 1).

Five plasma specimens from individuals with HIV-1 subtype B were collected, with informed consent, from the Centre Hospitalier Universitaire (CHU) Montpellier (Montpellier, France). These samples harbored viruses resistant to NNRTIs (K103N, Y181C). Eleven additional plasma specimens infected with WT HIV-1 were obtained from blood donor collection (French Blood Agency National Plasma Bank, Tours, France).

2.2 Multiplex HIV DRM detection assay

2.2.1 PCR amplification of HIV regions

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

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 final extension step of 10 min at 72°C.

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

2.2.2 Design of sequence-specific primers

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

2.2.3 Multiplex SSPE assay

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

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 µL 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 µg/mL
150 biotinylated BSA with a 0.1 µL 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 µL) 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

2.3 Analytical performances of the HIV DRM detection assay

2.3.1 Determination of the limit of detection

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

2.3.2 Determination of the assay sensitivity

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

2.4 HIV DRM detection assay on clinical specimens

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

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

3. Results

3.1 Principles of the DRM detection assay

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

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

3.2 Limit of detection of the DRM detection assay

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

3.3 Sensitivity of the assay for DRM detection

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

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

(r)= IMUT / (IWT + IMUT)

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

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

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

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

4. Discussion

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

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

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

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

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

Authors' contributions

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

Acknowledgments

This research was supported by an ANRS grant [No. Contrat Initiation #12387] and Etablissement Français du Sang. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 NL4-3 1499 Infectious Molecular Clone (p6463-13) (cat# 7401), HIV-1 NL4-3 1392 Infectious Molecular Clone (p7324-1) (cat#7396), HIV-1 Infectious Molecular Clone (p5485) (cat#12229) and HIV-1 Infectious Molecular Clone (p16182) (cat#12237) from Dr. Robert W. Shafer. The English content of the manuscript has been revised by Angloscribe, an independent scientific language editing service (Angloscribe, Calvisson, France).

Declaration of competing interest

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

REFERENCES

[1] J.N. Barr, R. Fearns, Genetic Instability of RNA Viruses, Genome Stability (2016) 21-35.

[2] R.K. Gupta, M.R. Jordan, B.J. Sultan, A. Hill, D.H.J. Davis, J. Gregson, A.W. Sawyer, R.L. Hamers, N. Ndembi, D. Pillay, S. Bertagnolio, Global trends in antiretroviral resistance in treatment-naive individuals with HIV after rollout of antiretroviral treatment in resource-limited settings: a global collaborative study and meta-regression analysis, The Lancet 380(9849) (2012) 1250-1258.

[3] R.K. Gupta, J. Gregson, N. Parkin, H. Haile-Selassie, A. Tanuri, L. Andrade Forero, P. Kaleebu, C. Watera, A. Aghokeng, N. Mutenda, J. Dzangare, S. Hone, Z.Z. Hang, J. Garcia, Z. Garcia, P. Marchorro, E. Beteta, A. Giron, R. Hamers, S. Inzaule, L.M. Frenkel, M.H. Chung, T. de Oliveira, D. Pillay, K. Naidoo, A. Kharsany, R. Kugathasan, T. Cutino, G. Hunt, S. Avila Rios, M. Doherty, M.R. Jordan, S. Bertagnolio, HIV-1 drug resistance before initiation or re-initiation of first-line antiretroviral therapy in low-income and middle-income countries: a systematic review and meta-regression analysis, The Lancet Infectious Diseases 18(3) (2018) 346-355.

[4] A.N. Phillips, J. Stover, V. Cambiano, F. Nakagawa, M.R. Jordan, D. Pillay, M. Doherty, P. Revill, S. Bertagnolio, Impact of HIV Drug Resistance on HIV/AIDS-Associated Mortality, New Infections, and Antiretroviral Therapy Program Costs in Sub–Saharan Africa, The Journal of Infectious Diseases 215(9) (2017) 1362-1365.

[5] R.L. Hamers, R. Schuurman, K.C.E. Sigaloff, C.L. Wallis, C. Kityo, M. Siwale, K. Mandaliya, P. Ive, M.E. Botes, M. Wellington, A. Osibogun, F.W. Wit, M. van Vugt, W.S. Stevens, T.F.R. de Wit, Effect of pretreatment HIV-1 drug resistance on immunological, virological, and drug-resistance outcomes of first-line antiretroviral treatment in sub-Saharan Africa: a multicentre cohort study, The Lancet Infectious Diseases 12(4) (2012) 307-317.

[6] P. Phanuphak, S. Sirivichayakul, A. Jiamsakul, S. Sungkanuparph, N. Kumarasamy, M. Lee, T. Sirisanthana, P. Kantipong, C. Lee, A. Kamarulzaman, M. Mustafa, R. Ditangco, T. Merati, W. Ratanasuwan, T. Singtoroj, R. Kantor, Transmitted Drug Resistance and Antiretroviral Treatment Outcomes in Non-Subtype B HIV-1-Infected Patients in South East Asia, Journal of Acquired Immune Deficiency Syndromes 1(66) (2014) 74-79.

[7] G. Zhang, F. Cai, Z. Zhou, J. DeVos, N. Wagar, K. Diallo, I. Zulu, N. Wadonda-Kabondo, J.S.A. Stringer, P.J. Weidle, C.B. Ndongmo, I. Sikazwe, A. Sarr, M. Kagoli, J. Nkengasong, F. Gao, C. Yang, Simultaneous Detection of Major Drug Resistance Mutations in the Protease and Reverse Transcriptase Genes for HIV-1 Subtype C by Use of a Multiplex Allele-Specific Assay, Journal of Clinical Microbiology 51(11) (2013) 3666-3674.

[8] G. Zhang, F. Cai, I.L. de Rivera, Z. Zhou, J. Zhang, J. Nkengasong, F. Gao, C. Yang, Simultaneous Detection of Major Drug Resistance Mutations of HIV-1 Subtype B Viruses from Dried Blood Spot Specimens by Multiplex Allele-Specific Assay, Journal of Clinical Microbiology 54(1) (2016) 220-222.

[9] D.S. Clutter, G. Mazarei, R. Sinha, J. Manasa, J. Nouhin, E. LaPrade, S. Bolouki, P.L. Tzou, J. Hannita-Hui, M.K. Sahoo, P. Kuimelis, R.G. Kuimelis, B.A. Pinsky, G.K. Schoolnik, A. Hassibi, R.W. Shafer, Multiplex Solid-Phase Melt Curve Analysis for the Point-of-Care Detection of HIV-1 Drug Resistance, The Journal of Molecular Diagnostics 21(4) (2019) 580-592.

[10] L. Zhang, J. Wang, M. Coetzer, S. Angione, R. Kantor, A. Tripathi, One-Step Ligation on RNA Amplification for the Detection of Point Mutations, The Journal of Molecular Diagnostics 17(6) (2015) 679-688.

[11] I.J. MacLeod, C.F. Rowley, M. Essex, PANDAA-monium: Intentional violations of conventional qPCR design enables rapid, HIV-1 subtype-independent drug resistance SNP detection, bioRxiv (2019) 795054.

[12] V. Kouamou, J. Manasa, D. Katzenstein, A.M. McGregor, C.E. Ndhlovu, T. Makadzange, Diagnostic accuracy of Pan Degenerative Amplification and Adaptation (PANDAA) assay for HIV-1 drug resistance mutations analysis in low and middle-income countries, Journal of Clinical Microbiology (2020) JCM.01045-20.

[13] R.W. Peeling, K.K. Holmes, D. Mabey, A. Ronald, Rapid tests for sexually transmitted infections (STIs): the way forward, Sexually Transmitted Infections 82(suppl 5) (2006) v1-v6.

[14] C. Dincer, R. Bruch, A. Kling, P.S. Dittrich, G.A. Urban, Multiplexed Point-of-Care Testing – xPOCT, Trends in Biotechnology 35(8) (2017) 728-742.

[15] P.K. Drain, E.P. Hyle, F. Noubary, K.A. Freedberg, D. Wilson, W.R. Bishai, W. Rodriguez, I.V. Bassett, Diagnostic point-of-care tests in resource-limited settings, The Lancet Infectious Diseases 14(3) (2014) 239-249.

[16] P. Yager, G.J. Domingo, J. Gerdes, Point-of-Care Diagnostics for Global Health, Annual Review of Biomedical Engineering 10(1) (2008) 107-144.

[17] N. Panpradist, I.A. Beck, J. Vrana, N. Higa, D. McIntyre, P.S. Ruth, I. So, E.C. Kline, R. Kanthula, A. Wong-On-Wing, J. Lim, D. Ko, R. Milne, T. Rossouw, U.D. Feucht, M. Chung, G. Jourdain, N. Ngo-Giang-Huong, L. Laomanit, J. Soria, J. Lai, E.D. Klavins, L.M. Frenkel, B.R. Lutz, OLA-Simple: A software-guided HIV-1 drug resistance test for low-resource laboratories, EBioMedicine (2019).

[18] N. Panpradist, I.A. Beck, M.H. Chung, J.N. Kiarie, L.M. Frenkel, B.R. Lutz, Simplified Paper Format for Detecting HIV Drug Resistance in Clinical Specimens by Oligonucleotide Ligation, PLOS ONE 11(1) (2016) e0145962.

[19] M.E. Natoli, B.A. Rohrman, C. De Santiago, G.U. van Zyl, R.R. Richards-Kortum, Paper-based detection of HIV-1 drug resistance using isothermal amplification and an oligonucleotide ligation assay, Analytical biochemistry 544 (2018) 64-71.

405 [20] N. Panpradist, I.A. Beck, P.S. Ruth, S. Avila-Rios, C. Garcia-Morales, M. Soto-Nava, D.
406 Tapia-Trejo, M. Matias-Florentino, H.E. Paz-Juarez, S. Del Arenal-Sanchez, G. Reyes-Teran,
407 B.R. Lutz, L.M. Frenkel, Near point-of-care, point-mutation test to detect drug resistance in
408 HIV-1: a validation study in a Mexican cohort, Aids 34(9) (2020) 1331-1338.
409 [21] J. Gomez-Martinez, M. Silvy, J. Chiaroni, C. Fournier-Wirth, F. Roubinet, P. Bailly, J.-
410 C. Brès, Multiplex Lateral Flow Assay for Rapid Visual Blood Group Genotyping, Analytical
411 Chemistry 90(12) (2018) 7502-7509.
412 [22] F. Papanikos, A. Iliadi, M. Petropoulou, P.C. Ioannou, T.K. Christopoulos, E. Kanavakis,
413 J. Traeger-Synodinos, Lateral flow dipstick test for genotyping of 15 beta-globin gene (HBB)
414 mutations with naked-eye detection, Analytica Chimica Acta 727(0) (2012) 61-66.
415 [23] M. Petropoulou, A. Poula, J. Traeger-Synodinos, E. Kanavakis, T.K. Christopoulos, P.C.
416 Ioannou, Multi-allele DNA biosensor for the rapid genotyping of ‘nondeletion’ alpha
417 thalassaemia mutations in HBA1 and HBA2 genes by means of multiplex primer extension
418 reaction, Clinica Chimica Acta 446 (2015) 241-247.
419 [24] N. Fountoglou, M. Petropoulou, A. Iliadi, T.K. Christopoulos, P.C. Ioannou, Two-panel
420 molecular testing for genetic predisposition for thrombosis using multi-allele visual
421 biosensors, Analytical and Bioanalytical Chemistry 408(7) (2016) 1943-1952.
422 [25] Y.W. Weng, I.T. Chen, H.C. Tsai, K.S. Wu, Y.T. Tseng, C.L. Sy, J.K. Chen, S.S. Lee,
423 Y.S. Chen, Trend of HIV transmitted drug resistance before and after implementation of
424 HAART regimen restriction in the treatment of HIV-1 infected patients in southern Taiwan,
425 BMC infectious diseases 19(1) (2019) 741.
426 [26] WHO, HIV drug resistance report 2019, 2019.
427 [27] S.-Y. Rhee, R. Kantor, D.A. Katzenstein, R. Camacho, L. Morris, S. Sirivichayakul, L.
428 Jorgensen, L.F. Brigido, J.M. Schapiro, R.W. Shafer, B.H.I.V.W.G. International Non

Subtype, HIV-1 pol mutation frequency by subtype and treatment experience: extension of the HIVseq program to seven non-B subtypes, AIDS (London, England) 20(5) (2006) 643-651.

[28] Robert W. Shafer, Rationale and Uses of a Public HIV Drug-Resistance Database, The Journal of Infectious Diseases 194(Supplement_1) (2006) S51-S58.

[29] U.M. Parikh, K. McCormick, G. van Zyl, J.W. Mellors, Future technologies for monitoring HIV drug resistance and cure, Current Opinion in HIV and AIDS 12(2) (2017) 182-189.

[30] B.B. Simen, J.F. Simons, K.H. Hullsiek, R.M. Novak, R.D. MacArthur, J.D. Baxter, C. Huang, C. Lubeski, G.S. Turenchalk, M.S. Braverman, B. Desany, J.M. Rothberg, M. Egholm, M.J. Kozal, T.B.C.P.f.C.R.o. AIDS, Low-Abundance Drug-Resistant Viral Variants in Chronically HIV-Infected, Antiretroviral Treatment–Naive Patients Significantly Impact Treatment Outcomes, The Journal of Infectious Diseases 199(5) (2009) 693-701.

[31] S.C. Inzaule, R.L. Hamers, M. Noguera-Julian, M. Casadellà, M. Parera, C. Kityo, K. Steegen, D. Naniche, B. Clotet, T.F. Rinke de Wit, R. Paredes, A. Osibogun, C.L. Wallis, C. Nalubwama, E. Letsoalo, F. Senono, H. Adelabu, H. Kakooza, H. Namata, I. Sanne, I. Nankya, J. Menke, J.M.A. Lange, K.C.E. Sigaloff, K. Mandaliya, M. Hardman, M. Siwale, M. de Jager, M. Dolan, M.E. Botes, M. O'Mello, M. Wellington, M. Mutebi, M. Nakitto, M. Labib, N. Pakker, P. Ondoa, P. Mugyenyi, P. Ive, R. Nakanjako, R. Schuurman, R. Lüthy, S.N. Balinda, S. Akanmu, T.S. Boender, T.A. Adeyemo, T. Rodoye, W.S. Stevens, W. Namala, Clinically relevant thresholds for ultrasensitive HIV drug resistance testing: a multi-country nested case-control study, The Lancet HIV 5(11) (2018) e638-e646.

[32] UNAIDS, 90–90–90 - An ambitious treatment target to help end the AIDS epidemic, 2017.

[33] S.-Y. Rhee, J.L. Blanco, M.R. Jordan, J. Taylor, P. Lemey, V. Varghese, R.L. Hamers, S. Bertagnolio, T.F.R. de Wit, A.F. Aghokeng, J. Albert, R. Avi, S. Avila-Rios, P.O. Bessong,

454 J.I. Brooks, C.A.B. Boucher, Z.L. Brumme, M.P. Busch, H. Bussmann, M.-L. Chaix, B.S.
455 Chin, T.T. D'Aquin, C.F. De Gascun, A. Derache, D. Descamps, A.K. Deshpande, C.F.
456 Djoko, S.H. Eshleman, H. Fleury, P. Frange, S. Fujisaki, P.R. Harrigan, J. Hattori, A.
457 Holguin, G.M. Hunt, H. Ichimura, P. Kaleebu, D. Katzenstein, S. Kiertiburanakul, J.H. Kim,
458 S.S. Kim, Y. Li, I. Lutsar, L. Morris, N. Ndembi, K.P. Ng, R.S. Paranjape, M. Peeters, M.
459 Poljak, M.A. Price, M.L. Ragonnet-Cronin, G. Reyes-Terán, M. Rolland, S. Sirivichayakul,
460 D.M. Smith, M.A. Soares, V.V. Soriano, D. Ssemwanga, M. Stanojevic, M.A. Stefani, W.
461 Sugiura, S. Sungkanuparph, A. Tanuri, K.K. Tee, H.-H.M. Truong, D.A.M.C. van de Vijver,
462 N. Vidal, C. Yang, R. Yang, G. Yebra, J.P.A. Ioannidis, A.-M. Vandamme, R.W. Shafer,
463 Geographic and Temporal Trends in the Molecular Epidemiology and Genetic Mechanisms of
464 Transmitted HIV-1 Drug Resistance: An Individual-Patient- and Sequence-Level Meta-
465 Analysis, PLOS Medicine 12(4) (2015) e1001810.

466

TABLES

Table 1. Multiple sequence alignment between the reference plasmid sequences containing DRMs K103N and Y181C, and the 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	AA**A**	AAA	TCA	GTA	ACA	GTA	CTG	GAT	GTG	GGT	GAT	GCA	3'	
	WT	p6463-13		5'	AAG	AA**A**	AAA	TCA	GTA	ACA	GTA	CTA	GAT	GTG	GGT	GAT	GCA	3'	
	MUT	p5485		5'	AAG	AA**C**	AAA	TCA	GTA	ACA	GTA	CTG	GAT	GTG	GGT	GAT	GCA	3'	
	WT	p16182		5'	AAG	AA**A**	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	T**A**T	CAA	3'
	WT	p6463-13		5'	GAG	CCT	TTC	AGA	AAA	CAA	AAT	CCA	GAA	ATA	GTT	ATC	T**A**T	CAA	3'
	WT	p5485		5'	GAG	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAG	ATA	GTT	ATC	T**A**T	CAG	3'
	MUT	p16182		5'	GAG	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAC	ATA	GTC	ATC	T**G**T	CAA	3'
Primers		F181Y	Tag	5'		G	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAM	ATA	GTI	ATC	T**A**	3'
		F181C	Tag	5'		G	CCT	TTT	AGA	AAA	CAA	AAT	CCA	GAM	ATA	GTI	ATC	T**G**	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 C**AT CAC CCA CAT CTA GTA CTG TTA CTG ATT TT-3'
103N	5'-**GCT TCC AAG ATA AGA GCC A**AT 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 C**AT CAC CCA CAT CTA GTA CTG TTA CTG ATT TY-3'
HIV-1 positive control	5'-**AGT AGA TGT CCT CAA ATG G**GT GAT CCT TTC CAT CCC TGT GG-3'

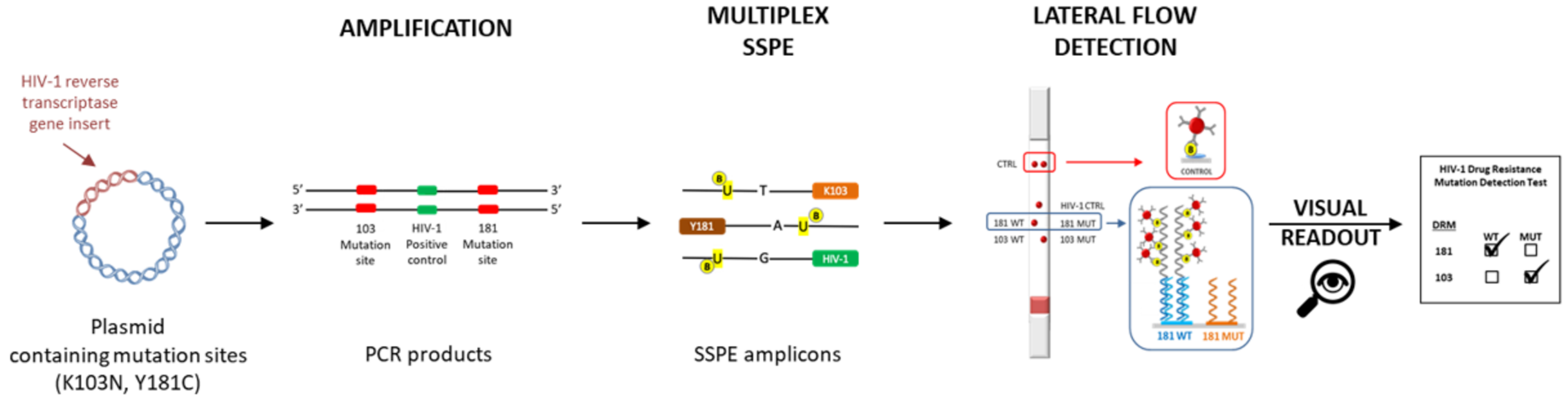
472 [a] Bold sequences correspond to the tag sequences of each primer – 3'-end sequence was specific for the mutant or wild type sequence of HIV-1 variant.

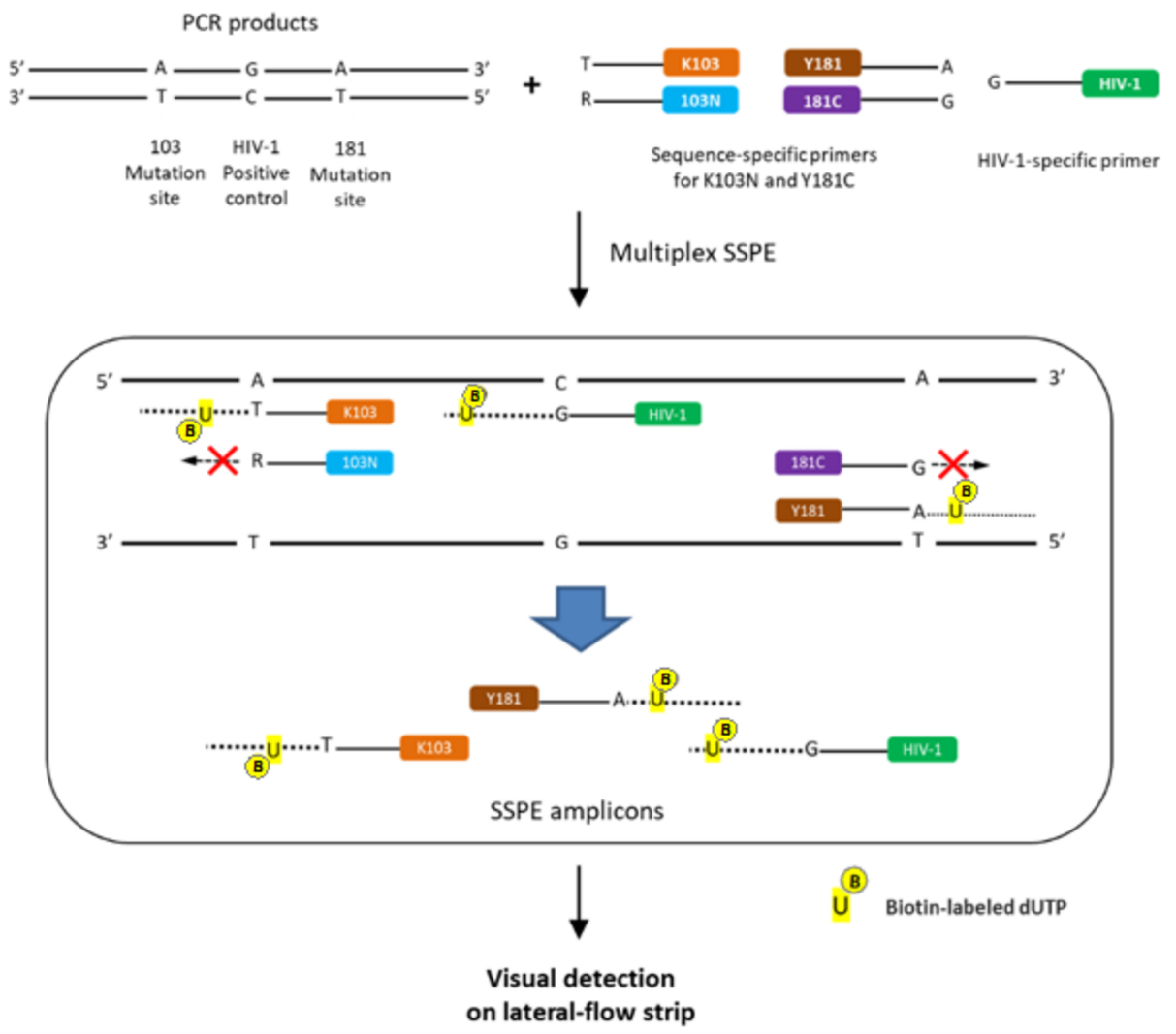
473

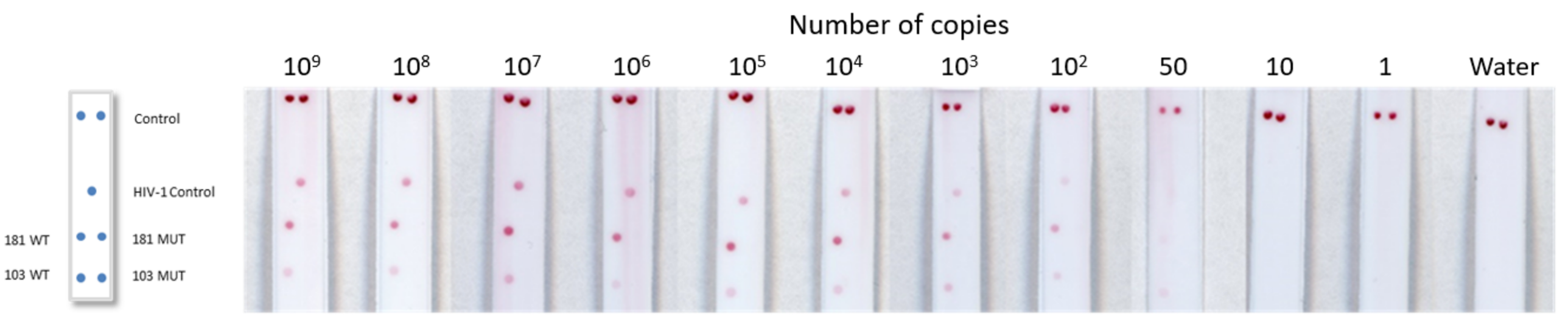
474 **Table 3.** Ratio values (*r*) for plasmid mixture dilutions.

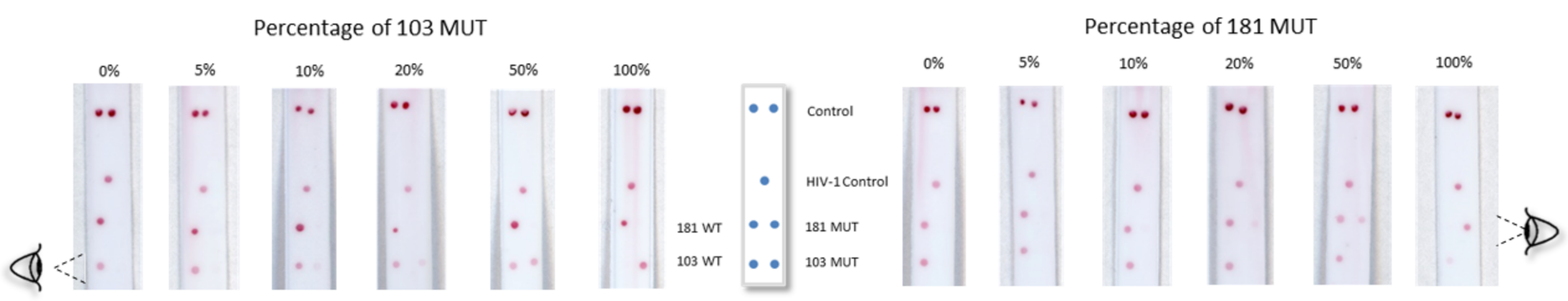
	Percentage of MUT (%)					
DRM	0	5	10	20	50	100
K103N	0	0	0.23±0.04	0.32±0.04	0.5±0.03	+1
Y181C	0	0	0	0.1±.06	0.41±0.05	+1

475

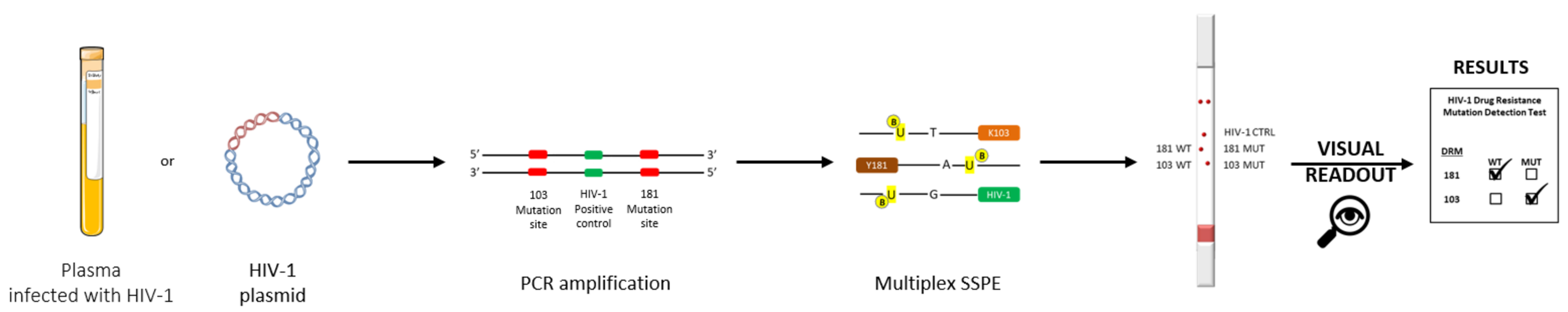

AMPLIFICATION
MULTIPLEX SSPE
LATERAL FLOW DETECTION
HIV-1 reverse transcriptase gene insert
5′
3′
3′
5′
103 Mutation site
HIV-1 Positive control
181 Mutation site
T
K103
Y181
A
G
HIV-1
CTRL
CONTROL
HIV-1 CTRL
181 WT
181 MUT
103 WT
103 MUT
181 WT
181 MUT
VISUAL READOUT
HIV-1 Drug Resistance Mutation Detection Test
DRM
WT
MUT
181
103
Plasmid containing mutation sites (K103N, Y181C)
PCR products
SSPE amplicons


PCR products
5′ A G A 3′
3′ T C T 5′
103 Mutation site
HIV-1 Positive control
181 Mutation site
T K103
R 103N
Y181 A
181C G
Sequence-specific primers for K103N and Y181C
G HIV-1
HIV-1-specific primer
Multiplex SSPE
SSPE amplicons
Biotin-labeled dUTP
Visual detection on lateral-flow strip


Number of copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
50
10
1
Water
Control
HIV-1 Control
181 WT
181 MUT
103 WT
103 MUT


Percentage of 103 MUT
0%
5%
10%
20%
50%
100%
181 WT
103 WT
Control
HIV-1 Control
181 MUT
103 MUT
Percentage of 181 MUT
0%
5%
10%
20%
50%
100%

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)	-	-


or
5′
3′
3′
5′
103
Mutation
site
HIV-1
Positive
control
181
Mutation
site
B
U
T
K103
Y181
A
B
U
B
U
G
HIV-1
181 WT
103 WT
HIV-1 CTRL
181 MUT
103 MUT
VISUAL
READOUT
RESULTS
HIV-1 Drug Resistance
Mutation Detection Test
DRM
WT
MUT
181
103
Plasma
infected with HIV-1
HIV-1
plasmid
PCR amplification
Multiplex SSPE