RESEARCH ARTICLE

Agreement between an in-house replication competent and a reference replication defective recombinant virus assay for measuring phenotypic resistance to HIV-1 protease, reverse transcriptase, and integrase inhibitors

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Background: Although clinical management of drug resistance is routinely based on genotypic methods, phenotypic assays remain necessary for the characterization of novel HIV-1 inhibitors, particularly against common drug-resistant variants. We describe the development and assessment of the performance of a recombinant virus assay for measuring HIV-1 susceptibility to protease (PR), reverse transcriptase (RT), and integrase (IN) inhibitors.

Methods: The system is based on the creation of replication-competent chimeric viruses through homologous recombination between patient or laboratory virus-derived PCR fragments and the corresponding NL4-3 vector where the whole Gag-PR, RT-RNaseH or IN coding regions has been deleted through inverse PCR. The susceptibility to nucleoside (NRTIs) and non-nucleoside (NNRTIs) RT inhibitors and to IN inhibitors (INIs) is calculated through a single-round infection assay in TZM-bl cells, while protease inhibitor (PI) activity is determined through a first round of infection in MT-2 cells followed by infection of TZM-bl cells with MT-2 supernatants.

Results: The assay showed excellent reproducibility and accuracy when testing PI, NRTI, NNRTI, and INI susceptibility of drug-resistant clones previously characterized through the reference pseudoparticle-based Phenosense assay. The coefficient of interassay variation in fold change (FC) resistance was 12.0%-24.3% when assaying seven drug/clones pairs in three runs. FC values calculated by the Phenosense and in-house for 20 drug/clones pairs were in good agreement, with mean $\pm$ SD ratio of 1.14 $\pm$ 0.33 and no cases differing by more than twofold.

Conclusions: The described phenotypic assay can be adopted to evaluate the antiviral activity of licensed and investigational HIV-1 drugs targeting any of the three HIV-1 enzymes.

KEYWORDS

drug resistance, HIV-1, HIV-1 inhibitors, phenotypic assay

1 | INTRODUCTION

Highly active antiretroviral therapy (HAART) has significantly improved the expectancy and the quality of life of people infected with

the human immunodeficiency virus type 1 (HIV-1).¹ To date, around 30 compounds have been approved for clinical practice, mostly now combined as single pill coformulations to increase the convenience of treatment administration, while other investigational drugs are

under preclinical or clinical development.² The major goal of HAART is the stable suppression of viral replication, but factors such as suboptimal adherence to therapy, drug-drug interactions, and side effects can compromise the efficacy of an antiretroviral regimen, eventually leading to the development of drug resistance.³ Current guidelines indeed recommend the monitoring of drug resistance both at HIV diagnosis and at treatment failure, as the determination of the resistance profile is a necessary step to guide treatment decisions.⁴

Due to extensive accumulation of data correlating HIV mutations with changes of drug susceptibility *in vitro* and *in vivo*, genotypic testing is the recommended approach to monitor for HIV drug resistance in clinical practice. The HIV genomic regions of interest are sequenced and susceptibility to the different drugs is inferred based on updated algorithms translating the mutational pattern into clinical activity.⁵ Since genotype interpretation algorithms by definition consider only well characterized resistance mutations, complex and/or unusual mutational patterns can be occasionally difficult to translate into clinically useful information. As opposed to genotypic testing, phenotypic assays allow direct measurement of drug susceptibility of any viral strain, independently from the complexity of the mutational pattern, by evaluating the replication of the viral strain in the presence of serial dilutions of drugs in cell culture.⁶ Unfortunately, phenotypic assays are not amenable to routine diagnostics due to the higher cost and turnaround time compared to genotypic assays, the need of higher level expertise and biosafety level 3 facilities when replication-competent virus must be handled.

However, phenotypic assays remain essential in the determination of the antiviral activity and definition of the resistance profile of investigational drugs. Two highly standardized large-scale commercial phenotypic services have been used to characterize most of the currently licensed antiretrovirals during preclinical development and/or clinical trials.^{7,8} Other small-scale phenotypic assays have been reported mainly in the academia⁹⁻¹² and it is important to establish how these compare to the two reference commercial assays. We have developed a recombinant virus assay based on the production of chimeric viruses through homologous recombination in eukaryotic cells by cotransfecting the PCR fragment of interest and the corresponding member of a family of deleted pNL4-3-derived vectors. The PCR insert may derive from a clinical sample or from a laboratory adapted virus or clone. In this paper, we report the reproducibility and the accuracy of this system using clones with resistance mutations already characterized with the commercially available Phenosense assay (Monogram Biosciences, South San Francisco, CA, USA).⁸

2 | MATERIALS AND METHODS

2.1 | Overview of the method and performance analysis

The assay separately considers the gag-protease (PR), reverse transcriptase (RT)-RNaseH, and integrase (IN) region. The region

of interest is PCR amplified starting from any suitable source, including a clinical sample, a laboratory adapted virus, clone, or site-directed mutant. The amplification product is inserted into a pNL4-3-based correspondingly deleted vector by homologous recombination upon cotransfection into 293FT cells. The chimeric virus is harvested from the supernatant, expanded in MT-2 cells and titrated in TZM-bl cells through the HIV promoter-driven expression of β -galactosidase.¹³ Susceptibility to nucleoside (NRTIs) and non-nucleoside (NNRTIs) RT inhibitors and to IN inhibitors (INIs) is then measured during a single-round infection of TZM-bl cells in the presence of drug. Susceptibility to PR inhibitors (PIs) is measured through a first round of infection in MT-2 cells in the presence of PIs followed by infection of TZM-bl cells with MT-2 supernatants. Drug susceptibility was expressed as the drug concentration inhibiting 50% of virus replication (IC_{50}). Every assay run includes drug susceptibility testing with the reference wild-type NL4-3 virus. This clone is used to normalize the IC_{50} value of the test virus and derive the resistance fold change (FC) as the ratio between the test virus IC_{50} and the reference virus IC_{50} . Figure 1 shows the steps of the whole procedure.

The interassay reproducibility of the system was evaluated by triplicate testing of two different NRTIs, NNRTIs, and PIs, and one INI, each against one reference drug-resistant virus, accounting for a total of seven triplicate IC_{50} determinations. For these experiments, the reference drug-resistant viruses were directly used following transfection of plasmids into susceptible cells.

The accuracy of the system with respect to the reference Phenosense assay was evaluated by single testing of two different NRTIs, NNRTIs, and PIs against three reference drug-resistant viruses and single testing of one INI against two reference drug-resistant viruses, accounting for a total of 20 single IC_{50} determinations. For these experiments, the reference plasmids were used as templates to obtain the PCR fragment to be recombined with the corresponding linearized deleted vector to evaluate the whole assay procedure.

2.2 | Cell lines and plasmids

293FT cells (Thermo Fisher Scientific, Waltham, MA, USA) were cultured in DMEM high glucose with L-glutamine, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 300 μ g/mL geneticin. TZM-bl cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. MT-2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. All cell culture media and relative reagents were obtained from EuroClone (Pero (MI), Italy). TZM-bl and MT-2 cells were obtained from the AIDS Reagent Program (Division of AIDS, NIAID, NIH). The wild-type reference HIV-1 plasmid pNL4-3 and the HIV-1 plasmids carrying drug resistance mutations already characterized in the Phenosense Assay (Table 1) were also obtained from the AIDS Reagent Program.

FIGURE 1 Overview of the in-house recombinant virus phenotypic drug resistance assay. (A) Schematic representation of the deleted regions in the different plasmids used for generation of the recombinant virus. (B) Production of viral stocks upon transfection of 293FT cells, starting from infectious molecular clones or paired vector and insert DNA. (C) Schematic workflow of the MonoCycle and BiCycle assays for IC_{50} determination

2.3 | Antiviral drugs

The PIs atazanavir (ATV) and darunavir (DRV), the NRTIs tenofovir disoproxil fumarate (TDF) and abacavir (ABC), the NNRTIs efavirenz (EFV) and rilpivirine (RPV), and the INI raltegravir (RAL) were obtained from the AIDS Reagent Program.

2.4 | Generation of vector and insert for production of chimeric virus through homologous recombination

A family of pNL4-3-based deleted vectors was obtained through inverse PCR following cloning of a pNL4-3 fragment comprising the region to be deleted into the shuttle vector pCR2.1 (Thermo Fisher Scientific). Inverse PCR was run using Expand Long Range DNA polymerase (Roche Diagnostics, Basel, Switzerland) as suggested by the manufacturer, with primers bearing a unique *SacII* restriction site at

the 5' end to allow circularization of the deleted amplified product through *SacII* digestion and T4 ligation (see Table S1). Following confirmation of the desired deletion in the shuttle vector, the deleted region was moved back to the original pNL4-3 backbone to obtain the deleted vector of interest.

Target-specific PCR protocols were set up to create a Gag-PR, RT-RNaseH, or IN PCR fragment overlapping by about 100–200 bp at the 5' and 3' end of the corresponding linearized deleted vector. The plasmids carrying the infectious clones with drug resistance mutations were used as templates to generate the Gag-PR, RT-RNaseH, or IN insert regions through PCR. Since the assay could also be used with clinical samples requiring maximum sensitivity, nested amplification primers (Eurofins Genomics, Ebersberg, Germany) (Table 2) were designed to match to highly conserved regions, based on the HIV-1 group M alignments retrieved from the HIV sequence database of the Los Alamos National Laboratory (<http://hiv.lanl.gov>). All the outer PCRs were performed in a volume of 50 μ L consisting of 0.5 U

TABLE 1 Drug resistance mutations contained in the reference infectious clones

Clone ID ^a	Resistance mutations				References
	PIs	NRTIs	NNRTIs	INIs	
11804	Major: V32I, M46I, I47V, I84V Minor: L10V, K20T, Q58E	None	None	None	Varghese et al. ²⁵
11808	Major: V32I, I54V, I84V, L90M Minor: L10F, V11I, K20T, L33F, E35G, A71I, G73S, L89V	None	None	None	
12465	Major: M46I, I54V, V82L, L90M Minor: L10I, K20I, Q58E, A71V, T74P	None	None	None	
7396	None	M41L, D67N, T69N, K70R, T215F, K219E	None	None	Johnston et al. ²⁶
7401	None	M41L, D67N, M184V, L210W, T215Y	None	None	
7402	None	M41L, D67N, T69D, L210W, T215Y	K103N, E138Q	None	
12225	None	M41L, L210W, T215Y	E138G, H221Y, F227L, M230L	None	Balamane et al. ²⁷
12231	None	M41L, T215F	K103N, V179F, Y181C	None	
12237	None	None	V106I, Y181C, G190A, H221Y	None	
11843	None	None	None	T97A, N155H	Reuman et al. ²⁸
11844	None	None	None	L74M, T97A, Y143C, S230R	

^aAIDS Reagent Program catalogue number. PIs, protease inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; INIs, integrase inhibitors.

Q5 Hot Start High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), 100 µmol/L dNTPs (Promega, Madison, WI, USA), 10 µL 5× Q5 reaction buffer (New England Biolabs), 0.1 µmol/L of each primer, and 10² copies of the plasmid as the template. All the inner PCRs were carried out in a volume of 30 µL consisting of 0.3 U Q5 Hot Start High Fidelity DNA polymerase, 100 µmol/L dNTPs, 6 µL 5× Q5 reaction buffer, 0.1 µmol/L of each primer, and 2 µL of the outer PCR product.

2.5 | Transfection, expansion, and titration of recombinant viruses

The day before transfection, 1.5 million 293FT cells were seeded in a T25 flask in a total volume of 8 mL of growth medium. For the production of recombinant viruses to be used in the accuracy experiments, four replicates of the inner PCR product were coprecipitated with 10 µg of the corresponding SacII-linearized NL4-3 deleted

TABLE 2 Primers used for the amplification of target HIV-1 regions and relative thermal cycler protocols

Primer ID	Sequence (5'-3')	Coordinates on HXB2	Sense/antisense	Target region	Thermal profile ^a
P207	GCCTCAATAAAGCTTGCCTTGA	522-543	Sense	Gag-PR, outer PCR	58°C 30 s, 72°C 110 s, 98°C 10 s (28 cycles)
LR53	AACCCATCCAAAGGAATGGAGGTT	3220-3243	Antisense		
P264	TGTGTGCCCGTCTGTTGTGTGACT	557-580	Sense	Gag-PR, inner PCR	61°C 30 s, 72°C 90 s, 98°C 10 s (32 cycles)
LR51	TGTGGTATTCCTAATTGAACTTCCC	2812-2836	Antisense		
P535	GARAGRCAGGCTAATTTTTAGGGA	2071-2095	Sense	RT-RNaseH, outer PCR	59°C 30 s, 72°C 100 s, 98°C 10 s (28 cycles)
P216	TGTCCTGTTTCTGCTGGRATAACYTCTGC	4485-4513	Antisense		
PRO3	CCCCACCAGAAGAGAGCTT	2159-2177	Sense	RT-RNaseH, inner PCR	61°C 30 s, 72°C 90 s, 98°C 10 s (32 cycles)
P215	CCTTCTAAATGTGTACAATCTARTTGCCA	4410-4438	Antisense		
P508	AGCAGGRTATGTTACTGACAGAGGAAG	3911-3937	Sense	IN, outer PCR	59°C 15 s, 72°C 60 s, 98°C 10 s (28 cycles)
P511	TATGGAGACTCCMTGACCCAA	5281-5301	Antisense		
P509	AGAAGTAAACATAGTAACAGACTCACA	4022-4048	Sense	IN, inner PCR	57°C 15 s, 72°C 50 s, 98°C 10 s (32 cycles)
P512	GACTCCMTGACCCAAATGCCA	5275-5295	Antisense		

^aAll amplification profiles started with denaturation at 98°C for 30 s and ended with a final extension at 72°C for 120 s. PR, protease; RT, reverse transcriptase; IN, integrase.

TABLE 3 Interassay reproducibility of IC₅₀ determination using reference drug-resistant infectious clones already characterized with the Phenosense assay

Drug class	Drug	Virus ID ^a	Assay run						Reference FC (Phenosense)	
			1		2		3			
			IC ₅₀ (CI 95%)	FC	IC ₅₀ (CI 95%)	FC	IC ₅₀ (CI 95%)	FC		
PIs	ATV	114	0.7 (0.5-1.0) nmol/L	100.4	0.6 (0.4-1.1) nmol/L	86.5	0.4 (0.2-0.7) nmol/L	79.5	88.8 (12.0)	90
		11808	70.3 (14.3-222.0) nmol/L		51.9 (24.0-112.1) nmol/L		31.8 (9.8-103.2) nmol/L			
	DRV	114	0.6 (0.2 - 1.8) nmol/L	80.5	0.4 (0.2-0.7) nmol/L	85.5	0.6 (0.3-1.3) nmol/L	108.3	91.4 (16.2)	127
		11808	70.3 (22.3-105.2) nmol/L		34.2 (27.3-42.8) nmol/L		65.0 (24.4-173.0) nmol/L			
NRTIs	ABC	114	8.0 (6.0-10.7) μmol/L	10.0	8.9 (6.8-11.7) μmol/L	9.6	3.9 (2.9-5.4) μmol/L	6.2	8.6 (24.3)	7.4
		7401	79.6 (46.8-135.3) μmol/L		85.2 (53.9-134.5) μmol/L		24.2 (13.8-42.5) μmol/L			
	TDF	114	16.0 (12.1-21.0) nmol/L	1.6	8.7 (6.1-12.2) nmol/L	1.5	11.5 (7.9-16.7) nmol/L	2.0	1.7 (15.6)	1.5
		7401	26.2 (13.3-51.6) nmol/L		13.4 (6.7-26.8) nmol/L		22.7 (10.0-51.6) nmol/L			
NNRTIs	RPV	114	0.7 (0.5-1.1) nmol/L	3.6	0.8 (0.2-3.3) nmol/L	3.3	0.9 (0.6-1.3) nmol/L	4.8	3.9 (20.4)	2.3
		12231	2.5 (1.3-4.6) nmol/L		2.6 (0.6-10.7) nmol/L		4.3 (1.0-18.0) nmol/L			
	EFV	114	2.3 (1.6-3.3) nmol/L	96.8	3.1 (2.1-4.6) nmol/L	83.2	1.0 (0.2-5.2) nmol/L	129.2	103.1 (22.9)	90
		12231	222.6 (103.4-479.2) nmol/L		258.0 (123.8-537.7) nmol/L		129.2 (71.78-232.3) nmol/L			
INIs	RAL	114	1.8 (1.5-2.3) nmol/L	95.9	0.8 (0.6-1.0) nmol/L	106.5	0.6 (0.4-0.9) nmol/L	80.7	94.4 (13.7)	116
	11843	172.7 (75.6-394.5) nmol/L		85.2 (48.0-151.4) nmol/L		48.4 (21.0-112.2) nmol/L				

^aAIDS Reagent Program catalogue number. Virus with ID 114 is the HIV-1 reference wild-type NL4-3. Pls, protease inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; INIs, integrase inhibitors; ATV, atazanavir; DRV, darunavir; ABC, abacavir; EFV, efavirenz; RAL, raltegravir; FC, fold change; CI, confidence intervals; CV, coefficient of variation.

FIGURE 2 Dose-response curves for calculating the inter-assay reproducibility of drug susceptibility testing, expressed as fold change (FC) with respect to the wild-type reference virus. Numbers indicate the respective molecular clone as indicated in Table 1. R1, R2, and R3 indicate the replicate experiment done. ABC, abacavir; ATV, atazanavir; DRV, darunavir; EFV, efavirenz; RAL, raltegravir; RPV, rilpivirine; TDF, tenofovir disoproxil fumarate

vector and resuspended in 144 μ L of 0.1 $\times$ TE buffer. Alternatively, for interassay reproducibility experiments, 5 μ g of plasmid encoding the infectious clones with resistance mutations were dissolved with 0.1 $\times$ TE buffer in a final volume of 144 μ L. The solution containing the DNA was mixed with 16 μ L of 2.5 mol/L CaCl_2 and then gently added to 160 μ L of 2 $\times$ HEPES Phosphate Buffer (0.28 mol/L

NaCl, 50 mmol/L HEPES, 1.5 mmol/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.1). The solution was incubated at room temperature for 30 minutes, then added to the cells in the presence of 3 mL of fresh medium. Following overnight incubation, the medium was replaced with 8 mL of DMEM without geneticin. After 48 hours the medium was centrifuged at 300 g for 10 minutes and 3 mL of supernatant were

TABLE 4 Accuracy of IC₅₀ determination using reference drug-resistant infectious clones already characterized with the Phenosense assay

Drug class	Drug	Virus ID ^a	IC ₅₀ (CI 95%)	FC	Reference FC (Phenosense)
PIs	ATV	114	0.6 (0.3-1.2) nmol/L	NA	NA
		11804	15.6 (4.5-54.6) nmol/L	26	33
		11808	46.7 (12.1-180.7) nmol/L	77.8	90
		12465	103.8 (28.3-380.3) nmol/L	173	110
	DRV	114	0.3 (0.1-0.7) nmol/L	NA	NA
		11804	8.9 (2.8-28.2) nmol/L	29.6	25
		11808	28.9 (10.5-79.7) nmol/L	96.3	127
		12465	0.7 (0.2-1.8) nmol/L	2.3	3.5
NRTIs	ABC	114	9.0 (7.6-10.6) μmol/L	NA	NA
		7396	29.2 (15.7-54.3) μmol/L	3.2	4.2
		7401	50.0 (24.4-102.5) μmol/L	5.6	7.4
		7402	45.3 (23.7-86.3) μmol/L	5.0	8.4
	TDF	114	12.0 (8.5-17.1) nmol/L	NA	NA
		7396	74.1 (48.6-112.9) nmol/L	6.2	8.1
		7401	28.3 (20.5-39.1) nmol/L	2.4	1.5
		7402	39.5 (31.2-50.0) nmol/L	3.3	5.9
NNRTIs	RPV	114	3.4 (2.1-5.5) nmol/L	NA	NA
		12225	59.8 (35.6-100.3) nmol/L	17.6	18
		12231	9.5 (5.2-17.4) nmol/L	2.8	2.3
		12237	12.8 (8.4-19.4) nmol/L	3.8	3.5
	EFV	114	1.9 (0.8-4.1) nmol/L	NA	NA
		12225	27.4 (16.4-45.7) nmol/L	14.4	15
		12231	146.1 (104.5-204.4) nmol/L	76.9	90
		12237	40.5 (24.9-66.0) nmol/L	21.3	26
INIs	RAL	114	0.7 (0.5-1.0) nmol/L	NA	NA
		11843	110.3 (57.1-213.0) nmol/L	157.6	116
		11844	3.5 (1.7-7.6) μmol/L	5000	>200

^aAIDS Reagent Program catalogue number. Virus with ID 114 is the HIV-1 reference wild-type NL4-3. PIs, protease inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors; INIs, integrase inhibitors; ATV, atazanavir; DRV, darunavir; ABC, abacavir; TDF, tenofovir; RPV, rilpivirine; EFV, efavirenz; RAL, raltegravir; FC, fold change; CI, confidence intervals; NA, not applicable.

used to infect 2 million MT-2 cells for virus expansion. Viral growth in MT-2 cells was monitored every 48-72 hours and the supernatant was harvested and stored in 1-ml aliquots at -80°C when large syncytia induced by viral replication were observed. Viral titer was then calculated in TZM-bl cells by detecting β-galactosidase expression at 48 hours postinfection. Briefly, serial fourfold dilutions of the virus stock were added to 2×10⁴ cells in a 96-well plate. After 48 hours, cells were washed twice with cold PBS and fixed with the fixation buffer (0.25% glutaraldehyde, pH 7.4 in PBS) for 10 minutes at room temperature. Subsequently, each well was washed twice with cold PBS and the cells were stained with freshly prepared staining buffer (1 mol/L MgCl₂, 0.5 mol/L K₄Fe(CN)₆·3 H₂O, 0.5 mol/L K₃Fe(CN)₆, 20 mg/mL X-Gal in dimethylformamide and 10× PBS buffer) for 2-20 hours at 37°C. The dark blue infected cells or cell colonies were counted with an inverted microscope and the TCID₅₀ was calculated based on wells with 20-100 blue cells.

2.6 | DNA sequencing

DNA sequencing was performed to validate several steps during setup of the procedure, including verification of the absence of unwanted mutations following inverse PCR to generate the deleted plasmid vectors and confirmation of the viral RNA sequence of recombinant viruses after expansion in MT-2 cells. For the latter, RNA was extracted from cell culture supernatant using the EZ1 Advanced XL instrument (Qiagen, Hilden, Germany) and the EZ1 DSP Virus Kit (Qiagen). A quantity of 20 μL of the RNA extract was reverse-transcribed with ImPromil™ Reverse Transcriptase (Promega) as suggested by the manufacturer, then cDNA was amplified with Q5 high fidelity polymerase as described before. Standard Sanger sequencing was done using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the reactions were run on an ABI PRISM 3130XL Genetic

FIGURE 3 Assessment of the accuracy of Mono/BiCycle assays compared to Phenosense assay. (A) Correlation between log-transformed FC values obtained with the Mono/BiCycle assays and the reference Phenosense assay on a panel of drug-resistance viruses. (B) Bland-Altman plot showing the relationship between the average FC for the Phenosense and Mono/BiCycle assays and the ratio of the FC values obtained by the two systems

Analyzer (Applied Biosystems). Sequence traces were analyzed using the SeqMan Pro module (v7.1.0) included in the Lasergene package (DNASTAR, Madison, WI, USA).

2.7 | Estimation of susceptibility to anti-HIV-1 drugs through MonoCycle and BiCycle assays

The MonoCycle assay was developed to measure susceptibility to NRTIs, NNRTIs, and INIs. The assay consisted in the quantification of luciferase activity after one cycle of infection in TZM-bl cells in the presence of serial dilutions of the drugs. To determine the IC_{50} of each recombinant virus, TZM-bl cells were seeded in a 96-well plate at the concentration of 30 000 cells per well and infected with 300 TCID₅₀ of virus in the presence of fivefold serial dilutions of the drugs. After 48 hours, the cells were lysed adding 50 μ L/well of Glo-Lysis Buffer (Promega) and the lysate was transferred to a luminescence plate. A quantity of 50 μ L of Bright-Glo Luciferase Reagent (Promega) was added to each well and luminescence was measured in a GloMax Multi luminometer (Promega). Relative Luminescence Units detected in each well were elaborated with GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). Since the inhibitor effect of PIs cannot be detected with one cycle of infection in TZM-bl cells, we optimized a two-round infection assay named BiCycle Assay consisting in a first round of infection where 60 000 MT-2 cells/well were

infected with 600 TCID₅₀ in the presence of serial fivefold dilution of the drugs. After 72 hours, 50 μ L of supernatant from each well were used to infect TZM-bl cells and the determination of the IC_{50} values was performed as described for the MonoCycle assay.

3 | RESULTS

3.1 | Creation of pNL4-3 Δ GAG-PR, pNL4-3 Δ RT-RNaseH, and pNL4-3 Δ INT

Three modified versions of the reference pNL4-3 plasmid were created by deleting the region involved in drug resistance, namely the entire Gag and PR (plasmid pNL4-3 Δ GAG-PR, coordinates of the deletion on HIV-1 HXB2 reference strain 770-2598), the whole RT and RNaseH (pNL4-3 Δ RT-RNaseH, HXB2 deletion 2495-4283), and the integrase region (pNL4-3 Δ INT, HXB2 deletion 4161-5100). The sites of deletion and the HIV-1 coding sequences of the deleted plasmids were checked by DNA sequencing and no unwanted mutations were introduced by inverse PCR.

3.2 | Interassay reproducibility of drug susceptibility testing

The interassay reproducibility of drug susceptibility testing was evaluated through the determination of the IC_{50} of two most commonly used NRTIs, NNRTIs, and PIs, and one representative INI. The IC_{50} was calculated using one virus for each class of drugs, selected among the infectious clones with moderate or high-level resistance as determined by the reference Phenosense assay (Table 3 and Figure 2). Each virus-drug pair was tested in three independent assays and the fold change (FC) values were calculated with respect to the IC_{50} values obtained with the wild-type NL4-3 strain. The coefficient of FC variation ranged from 12.0% to 24.3% (mean $\pm$ SD, 17.9 $\pm$ 4.7) indicating that the measurement of phenotypic susceptibility was highly reproducible among independent runs. In addition, the mean FC values calculated by the in-house assay were remarkably close to the paired FC values calculated with the Phenosense assay, with a mean $\pm$ SD Phenosense to in-house ratio of 0.98 $\pm$ 0.26.

3.3 | Assay accuracy with recombinant viral strains

To evaluate the accuracy of the whole assay starting from generation of the PCR product, recombinant viruses were created through homologous recombination between the deleted vectors and the corresponding PCR fragments amplified from the drug-resistant clones. Three recombinant viruses carrying PI, NRTI, or NNRTI resistance mutations, and two carrying INI resistance mutations were tested in a single run with the same drugs used in the interassay reproducibility testing (Table 4). FC values calculated in this experiment were again very close to those reported with the Phenosense assay, with mean $\pm$ SD Phenosense to in-house ratio of 1.14 $\pm$ 0.33 and no cases differing by more than twofold, implying that the additional steps of amplification and recombination did not impact the accuracy of

the in-house assay. The Spearman rho for the correlation between log-transformed FC values was 0.97 ($P < .0001$) and Bland-Altman plot showed no significant assay bias with increasing or decreasing resistance levels (Figure 3). In all cases, the FC values obtained with our assay classified the viral isolates at the same drug susceptibility level as the original Phenosense assay.

4 | DISCUSSION

HIV drug resistance has been a major issue since the introduction of antiretroviral therapy. Once drug resistance mutations have been well defined, genotypic assays can effectively assist treatment decisions in most clinical settings.³ However, phenotypic methods remain instrumental when the resistance profile must be characterized for investigational compounds or better clarified for newly introduced drugs. Indeed, development of any new drug must take into account its activity against circulating drug-resistant strains.

The phenotypic assay described here is based on the production of recombinant viruses between any derived PCR fragment and a specific deleted HIV vector. While the PCR fragment can originate from a clinical sample or a laboratory isolate or clone, the family of vectors developed allow separate testing of functionally complete regions, including the whole Gag-PR, the whole RT-RNaseH and the IN coding sequences. Of note, this assay is unique in the ability to test the entire Gag together with PR and the entire RT together with RNaseH. Coevolution of Gag and PR *in vivo* has been consistently reported and recombinant viruses carrying clinically derived Gag-PR may have different PI susceptibility and replication capacity compared to their counterparts with the PR gene only.¹⁴⁻¹⁶ Moreover, recombinant viruses with clinically derived Gag can be used to determine the natural susceptibility to upcoming maturation inhibitors or other compounds targeting gag gene products.¹⁷ Likewise, although most NRTI/NNRTI resistance mutations occur at the RT active site and the NNRTI binding pocket, several polymorphisms or treatment associated mutations found in the C-terminal RT domain and in the RNaseH region have been associated with a decrease in the susceptibility to RT inhibitors and/or restoring of viral fitness.¹⁸⁻²¹

With regard to the two leading phenotypic antiretroviral resistance assay, our system is more similar to the Antivirogram Assay⁷ which is based on generation of replication-competent virus through homologous recombination than to the Phenosense assay,⁸ which is based on generation of pseudovirus through cloning. Homologous recombination does not require the selection of positive clones carrying the PCR insert before transfection of viral DNA into the cell line used for viral production. A potential drawback of this approach is the unknown number of recombination events occurring following the cotransfection of the insert and vector pair, questioning the representation of the quasispecies nature of the PCR insert when starting from clinical specimens. While the use of plasmids as a starting material was not suitable to analyze this issue, we could demonstrate a high rate of successful recombination events with clinically derived PCR products, irrespective of the virus subtype and the length of PCR fragments

(data not shown). The other difference between our system and the Phenosense assay is virus expansion in MT-2 cells as opposed to a single pseudovirus-based replication cycle. This step was introduced to obtain concentrated viral stocks suitable for infection of TZM-bl cells in the drug susceptibility assay. While virus expansion can theoretically generate new variants through stochastic evolution *in vitro*, we did not observe any sequence change when comparing the input DNA with the output virus generated through 72 hours of replication in MT-2 cells. Since the assay was primarily conceived for preclinical evaluation of investigational compounds, an extensive use of the system with clinical samples would require an exhaustive analysis of the possible impact of virus diversification both in the starting material and during virus expansion. However, it is reassuring that the Antivirogram system,⁷ based on the same virus recombination approach, showed excellent inter-assay reproducibility data with clinical samples.

Despite methodological differences, it is notable that the in-house system and the Phenosense assay yielded highly comparable results in the 20 parallel tests done with drug-resistant isolates. In addition, triplicate testing of seven different isolates showed excellent precision of the in-house system, again also yielding high correlation with the Phenosense fold-resistance values. The interassay CV for the in-house system (17.9 ± 4.7) was indeed comparable to that reported for the Phenosense assay (25.3 ± 8.0)⁸ and for the Antivirogram assay (15.6 ± 6.5),⁷ although different numbers of viruses and replicates were used to determine the precision of the three systems. While we could not test any sample previously tested by the Antivirogram assay, the Phenosense and Antivirogram systems have been shown to correlate well to each other.²² Previously published academic small-scale methods for phenotypic resistance testing have been not usually tested in comparison with reference systems. One exception is the system published by Puertas et al.²³ for measuring resistance to PIs, which also includes partial cloning of the Gag region. The assay correlated well with FC values obtained by the Antivirogram system, although with a lower correlation with respect to what we obtained comparing our method to the Phenosense assay. Other phenotypic resistance methods have only been validated by comparing the FC values measured to those expected based on estimates derived from similar mutational patterns.^{9,24} Given the public availability of the drug-resistant clones provided by the AIDS Reagent Program, any in-house assay should be conveniently validated with these standards to generate data which can be used across different studies.

In conclusion, the phenotypic method reported here was shown to estimate HIV-1 drug susceptibility with high interassay reproducibility and accuracy. The possibility to use the MonoCycle and BiCycle version and a family of different vectors make the system suitable for preclinical evaluation of antiviral activity of investigational HIV-1 inhibitors targeting all the enzymatic steps of HIV-1 life cycle.

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SUPPORTING INFORMATION

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