Development of a rapid cell-fusion-based phenotypic HIV-1 tropism assay

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Abstract

Introduction: A dual split reporter protein system (DSP), recombining Renilla luciferase (RL) and green fluorescent protein (GFP) split into two different constructs (DSP7,7 and DSP9,11), was adapted to create a novel rapid phenotypic tropism assay (PTA) for HIV-1 infection (DSP-Pheno).

Methods: DSP7,7 was stably expressed in the glioma-derived NP-2 cell lines, which expressed CD4/CXCR4 (N4X4) or CD4/CCR5 (N4R5), respectively. An expression vector with DSP9,11 (pRE11) was constructed. The HIV-1 envelope genes were subcloned in pRE11 (pRE11-env) and transfected into 293FT cells. Transfected 293FT cells were incubated with the indicator cell lines independently. In developing the assay, we selected the DSP7,7-positive clones that showed the highest GFP activity after complementation with DSP9,11. These cell lines, designated N4R5-DSP7,7, N4X4-DSP7,7 and DSP7,7 were used for subsequent assays.

Results: The env gene from the reference strains (Bal for R5 virus, NL4-3 for X4 virus, SF2 for dual tropic virus) subcloned in pRE11 and tested, was concordant with the expected co-receptor usage. Assay results were available in two ways (RL or GFP).

The assay sensitivity by RL activity was comparable with those of the published phenotypic assays using pseudovirus. The shortest turnaround time was 5 days after obtaining the patient’s plasma. All clinical samples gave positive RL signals on R5 indicator cells in the fusion assay. Median RLU value of the low CD4 group was significantly higher on X4 indicator cells and suggested the presence of more dual or X4 tropic viruses in this group of patients. Comparison of representative samples with Geno2Pheno [co-receptor] assay was concordant.

Conclusions: A new cell-fusion-based, high-throughput PTA for HIV-1, which would be suitable for in-house studies, was developed. Equipped with two-way reporter system, RL and GFP, DSP-Pheno is a sensitive test with short turnaround time. Although maintenance of cell lines and laboratory equipment is necessary, it provides a safe assay system without infectious viruses. With further validation against other conventional analyses, DSP-Pheno may prove to be a useful laboratory tool. The assay may be useful especially for the research on non-B subtype HIV-1 whose co-receptor usage has not been studied much.

Keywords: HIV-1; co-receptor; tropism; co-receptor usage; fusion; chemokine receptor.

Introduction

A new class of drugs to combat HIV-1 infection emerged in 2007 with the marketing approval of maraviroc, a small molecule that binds specifically to the CCR5 co-receptor to block viral attachment and entry [1]. While entry inhibitors are a welcome addition to the antiretroviral arsenal, one problem with this new class of drugs is that treatment is effective only against viruses with the specified co-receptor usage. HIV-1 tropism may be examined genotypically or phenotypically. Genotypic tropism assay (GTA) is based on DNA amplification and sequencing of the third variable (V3) region of the envelope glycoprotein gp120, shown by genetic mapping to be the major determinant of HIV-1 tropism [7–10]. GTA has advantages of platform portability, low cost and rapid turnaround time [11]; however, the interpretation of the sequences is complicated because of the high variability [12]. The assay used in association with maraviroc treatment is the phenotypic tropism assay (PTA) Trofile™ (Monogram Biosciences Inc., CA, USA), a CD4 cell culture assay using replication-defective pseudoviruses [13]. Although Trofile™ is
considered the gold standard, a simpler and effective PTA would be useful.

Here, we describe a novel, cell-fusion-based PTA that uses a dual split reporter protein system (DSP) [14,15] to measure HIV-1 tropism by both Renilla luciferase (RL) activity and green fluorescent protein (GFP) activity. We validated the DSP-Pheno assay using HIV-1 reference strains and applied the assay to test clinical samples from patients with HIV-1 infection.

Methods

Approval of the study and recombinant DNA experiments

Plasma samples from HIV-1-positive patients attending the hospital affiliated with the Institute of Medical Science, the University of Tokyo (IMSUT) were collected and kept frozen until use. Patients provided written informed consent, and the study was approved by the Institutional Review Board of the University of Tokyo (approval number 20-31). Recombinant DNA experiments used in this work were approved by the Institutional Review Board (approval number 08-30), and by the review board in the Ministry of Education, Culture, Sports, Science and Technology (MEXT; approval number 23-1927).

Cell lines

Cell lines N4, N4X4 and N4RS are derived from the human glioma NP-2 cell line and stably express CD4, CXCR4 and CCR5, respectively [16,17]. NP-2-derived cell lines were grown in M10 medium (modified Eagle’s medium (MEM; Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 units/ml of penicillin and 0.1 mg/ml of streptomycin). 293FT cells inactivated foetal bovine serum (FBS), 100 units/ml of penicillin and 0.1 mg/ml of streptomycin. All cell cultures were maintained at 37°C in a humidified 5% CO2 incubator.

Reference viral envelopes

The plasmids encoding reference HIV-1 envelopes with well-characterized co-receptor usage were obtained from NIH AIDS Research & Reference Reagent Program (NIH ARRRP: Germantown, MD, USA), NL4-3 and LAI represented X-4 tropic viruses; BaL represented R5-tropic viruses; and SF2 represented dual (RSX4)-tropic viruses. The co-receptor usage of these laboratory strains has been published [13,18–22].

Construction of DSP expression plasmids

The DSP system utilizes a pair of chimeric reporter proteins, DSP1 and DSP2, each of which is a fusion of split green fluorescent protein (spGFP) and split Renilla luciferase (spRL) [15]. DSP1 fuses the N-terminal region of RL (amino acids 1–229) to the N-terminal region of GFP (amino acids 1–157), with a linker sequence separating the two regions. DSP2 has the complementary structure, with the C-terminal region of GFP (amino acids 158–231), fused to the C-terminal region of RL (amino acids 230–311), also separated by a linker sequence. When both reporter proteins are present in the same cell, they each recover full activity.

To generate plLenti-DSP1, plasmids, we first amplified an attB-flanked DSP1 fragment (1251 bp) using pDSP1 as a template and attB-flanked primers (attB1-DSP1-F (5′-GGGAGACAGTTTTGACCAAAAGCAAGCTGGCTGCTACCAACAAAGCTGGCTCAAGGTTG-3′) and attB2-DSP1-R (51-mer, 5′-GGGACACCTTGGTACAAGGAAGCTGGCTGGCTAGTACCTGGTGGCGG-3′)). Sequential amplicons were transferred to pDONR-221 and pLenti6.3/V5-DEST (Invitrogen) using the ViraPower™ HiPerform™ Lentiviral Gateway® Expression System (Invitrogen) according to the manufacturer’s protocol. Constructs were verified by sequencing.

An expression vector, pRE11 (Figure 1), was constructed for the co-expression of DSP8 and HIV-1 env by multiple rounds of PCR and subcloning. Source plasmids were pRES2- AcGFP1 (Clontech), pmOrange (Clontech), pDSP8 [15] and pmirGLO (Promega). pRE11 incorporated multiple cloning sites under the PGK promoter for the insertion of HIV-1 env (Shown as 5′-XbaI-Xhol-3′ in Figure 1b). Necessary restriction enzyme cleavage sites used for construction, including multiple cloning sites (XbaI-MluI-Swal-Agel-Xholl), were created using synthetic oligonucleotides and PCR. A CMV promoter drives pDSP8 directly. The same CMV promoter expresses mOrange with a nuclear localization signal that serves as a marker for successful transfection via internal ribosomal entry site (IRES). All PCR fragments were confirmed by sequencing.

Generation of pRE11-env strains

Full-length HIV-1 env was prepared by PCR amplification from clinical plasma samples as described [23]. Viral RNA was extracted from 140 μl of patient’s plasma by QIAamp Viral RNA Mini kit according to the manufacturer’s recommendation (QIAGEN, Hilden, Germany). One-step RT-PCR using SuperScript III and High Fidelity Platinum® Taq DNA polymerase (Invitrogen) was carried out in five separate 15-μl reactions to minimize the bias created by PCR. The reaction mixture contained 2 μl of RNA template, 7.5 μl of 2 × reaction buffer, 0.3 μl of 5 mM MgSO4, 0.3 μl of each 10 μM of forward primer (Env-1F, 25-mer, 5′-TAGAGCCTGTGAAGGATCGGAGA-3′) and reverse primer (Env-3Rmix, equimolar mixture of 30-mer, 5′-TGCTGTATCGTCTTGCTAGTTGATGCTCTCAATA-3′ and 30-mer, 5′-TGCTGTATCGTCTTGCTAGTTGATGCTCTCAA-3′), 0.6 μl of
Figure 1. A cell-fusion-based phenotypic tropism assay for HIV-1: DSP-Pheno. (a) Schematic representation of DSP-Pheno assay system. (b) Schematic representation of pRE11, an expression vector for HIV-1 env and DSP8/C11. pRE11 encodes also mOrange with a nuclear localization signal as an indicator of transfection. (c) NP-2-derived clones stably expressing DSP1/C17 (N4-DSP1/C17, N4X4-DSP1/C17 and N4R5-DSP1/C17) were selected by the high GFP expression after direct transfection of pDSP8/C11. The expression of CD4/co-receptors was reconfirmed by appropriate monoclonal antibodies and FACS analysis.
SuperScript III and High Fidelity Platinum® Taq DNA polymerase, 0.25 μl of RNase OUT and 3.75 μl of nuclelease-free water with the final volume of 15 μl/reaction. The one-step RT-PCR condition was 55°C for 30 minutes, 94°C for 2 minutes followed by 30 cycles of 94°C for 20 seconds, 55°C for 30 seconds, 68°C for 4 minutes, then extension at 68°C for 5 minutes. The fragment by the first-round amplification extended from NL4-3 reference position of 5853-8936. Products from five independent reactions were combined. Four microliters of the mixed first-round PCR products were employed EnvB-2F-Xba (41-mer, 5' -TAGCTCTAGAAGCGCTTACCTGATCAGGACGAGAG-3') and EnvB-4R-Xho (41-mer, 5' -TAGCCTGAGAAGCGTAGCAGAACGCGTCTTAGGCATCTCCTAAGGAGAG-3') and EnvB-3F-Xba (41-mer, 5' -TACCTTACAGAAGCGCTTACCTGATCAGGACGAGAG-3'). The second PCR was carried out according to the standard 50-μl PCR protocol of the Platinum® PCR SuperMix High Fidelity as described above. The fragments amplified by the second PCR extended from NL4-3 reference position of 5957–8817. After digestion with Xba I and Xho I, about 3-kb PCR products were purified by 1.2% agarose gel and QiAquick gel extraction kit (Qiagen). The purified products were inserted into pRE11 at XbaI and XhoI sites, resulting in HIV-1 env expression plasmid (pRE11-envbulk) from each patient. pRE11-envbulk, representing a quasispecies of env population from each patient, was followed by transfecting into E. coli JM109. For bulk analysis, transfected JM109 was expanded to 25 ml, followed by QIAGEN Plasmid Midi Kit (Qiagen) for DNA extraction.

Cell-fusion assay
On the day before transfection, 500 μl aliquots of 293FT cells in DMEM supplemented with 10% FBS (D10) were seeded in 24-well tissue culture plates at a density of 2.8 × 10⁴ cells/well and incubated overnight to 70–80% confluency. The cells were then transfected with pRE11 at XbaI and XhoI sites, resulting in HIV-1 env expression plasmid (pRE11-envbulk) according to the manufacturer’s protocol (Roche). On the same day, 100 μl aliquots of N4-DSP1-7, N4X4-DSP1-7, and N4R5-DSP1-7 cells in MEM supplemented with 10% FBS (M10) were seeded in a 96-well tissue culture plate, optical bottom plate (NUNC, Thermo Fisher Scientific Inc., NY, USA) at a density of 1 × 10⁴ cells/well and incubated at 37°C. Forty-eight hours after transfection, the medium of transfected 293FT cells was removed by aspiration and replaced with 1 ml of PBS (Sigma) at RT. Transfected 293FT cells were resuspended by gentle pipetting.

To start the cell-fusion assay, 150 μl/well of transfected cells were overlaid onto N4-DSP1-7, N4X4-DSP1-7, and N4R5-DSP1-7 cells. The cells were incubated for fusion at 37°C in a humidified 5% CO₂ incubator for 6 hours, and then analyzed by automatic image capture using an In Cell Analyzer 1000 (GE Healthcare). Four fields/well of image were captured through red, green and bright field channels, and fused cells were identified by the presence of two or more red nuclei surrounded by a green area (cytoplasm). Immediately after image capturing, EnduRen™ Live Cell Substrate (Promega) was added to each well, and luciferase activity was measured three times using a Glomax 96 microplate luminometer (Promega), according to the manufacturer’s instructions. The mean luciferase activity, recorded as relative light unit (RLU), was the average of three measurements per well. The experiments were conducted in triplicates and repeated independently at least three times.

To test the co-receptor specificity, 2 μM/well of the appropriate inhibitor was added to the cells 90 minutes prior to the cell-fusion assay (CXC4 inhibitor AMD3100 (Sigma) to N4X4-DSP1-7 cells and CCR5 inhibitor maraviroc (Sigma) to NARS-DSP1-7 cells).

Genotyping

pRE11-envbulk were sequenced in both the 5’ and 3’ directions using population-based sequencing on the ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator V3.1 (Applied Biosystems) with forward primer E110 (5’ -CTGTTAATGCGAGCTCTGCAAGA-3’) and reverse primer E115 (5’ -AGAAATCCCTCCCTCCAATATTAA-3’). The V3 nucleotide sequences were submitted to the Geno2Pheno (co-receptor) algorithm (http://corceceptor.bioinf.mpi-inf.mpg.de) setting the false positive rate (FPR) at 10%.

Results

Construction of DSP1-7 and DSP8,11 expression vectors

We inserted DSP1-7 or DSP8,11 sequences into blasticidin-resistant lentivirus vectors and then infected NP-2/CD4 (N4), NP-2/CD4/CXCR4 (N4X4) and NP-2/CD4/CCR5 (NARS) cells with the recovered pseudoviruses, selecting for blasticidin-resistant clones. We screened 49, 51 and 43 lentivirus-infected and blasticidin-resistant clones from N4, N4X4 and NARS, respectively, for high levels of DSP1-7 or DSP8,11 expression following super-transfection with the complementary plasmid (pDSP8,11 or pDSP1-7, respectively). From each of the cell lines, we selected the blasticidin-resistant and DSP1-7-positive clone that showed the highest GFP activity after complementation with DSP8,11. These cell lines, designated N4-DSP1-7, N4X4-DSP1-7 and NARS-DSP1-7, were re-evaluated for their expression of CD4, CXCR4 and CCR5 on the cell surface (Figure 1c).

Using this approach, we obtained N4- and NARS-cells expressing high levels of DSP8,11, but were unable to obtain a stable N4X4 cell line expressing DSP8,11 (data not shown). To circumvent this problem, we decided to generate 293FT cells transiently expressing both DSP8,11 and the HIV-1 env protein and develop a cell-fusion assay system using those cells together with the NP-2-derived cells stably expressing DSP1-7 (Figure 1a). Thus, we constructed the expression vector pRE11, containing the DSP8,11 expression cassette and cloning sites for insertion of HIV-1 env sequences under the control of the PGK promoter (Figure 1b).

Validation of the cell-fusion assay using the env gene from laboratory HIV-1 strains

We validated the DSP assay system (DSP-pheno) using pRE11 constructs engineered to contain env sequences from reference strains with known co-receptor usage. The env reference constructs, which also contained the DSP8,11 expression cassette, were the following: pRE11-HXB2, pRE11-LAI, and pRE11-NL4-3 (X4 strains); pRE11-BaL (R5 strain); and pRE11-SF2 (dual strain). The cell-fusion assays were performed with N4X4-DSP1-7 or NARS-DSP1-7 cells in
combination with 293FT cells transiently expressing one of the pRE11-env constructs. In all the assays, both RL and GFP activities were restored only when cells expressing the appropriate env and co-receptor combinations were co-cultured (Figure 2a and b). Co-culture of N4X4-DSP1,7 or N4R5-DSP1,7 in combination with the 293FT cells transiently expressing the pRE11-env constructs of discordant tropism served as a negative control for expression of RL activities.
The absence of RL activities on N4-DSP 1-7 confirmed the importance of co-receptors for the fusion.

**Assay detection thresholds and sensitivity for minor populations**

To evaluate assay sensitivity in identifying minor variants within a single sample, we mixed pRE11-NL4-3 (X4) and pRE11-BaL (R5) in varying ratios and measured RL activities and GFP signals (Figure 3a and b). Both methods of detection identified X4 viruses more readily than R5 viruses. Based on luciferase activity, the presence of approximately 0.3% X4 viruses gave values significantly higher than background (0% X4), while R5 viruses had to comprise approximately 5% of the mixture for the signal to be detectable over background (Figure 3a). Similarly, based on GFP signals, X4 viruses comprising as little as 0.1% of the mixture could be detected, while detection of R5 viruses had a minimum threshold of approximately 1% (Figure 3b).

**Validation of the chemokine receptor specificity using the CXCR4 inhibitor AMD3100 and CCR5 inhibitor maraviroc**

293FT cells expressing env from reference strains NL4-3 (X4) or BaL (R5) were co-cultured with N4X4-DSP1-7 or N4R5-DSP1-7 cells in the absence or presence of AMD3100 or maraviroc (Figure 4a and b). In the absence of inhibitors, RL activities of

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**Figure 3. Assay sensitivities for minor populations.** pRE11-NL4-3 (X4) and pRE11-BaL (R5) were mixed at indicated ratios. The total plasmid concentration in the mixture was adjusted to 100 ng/µl. The mixture was transfected to 293FT cells and fused to the indicator cells. (a) RL activities. Black columns show the RL activities on X4 indicator (N4X4-DSP1-7), while white columns on R5-indicator (N4R5-DSP1-7). Small bars at the top of each column indicate the mean RLU ± SD from three independent experiments. (b) GFP activities. Left panel shows activities of the mixture with indicated ratio on X4 indicator (N4X4-DSP1-7), while right panel on R5-indicator (N4R5-DSP1-7).
the matched co-culture were high (Figure 4a). In the presence of AMD3100, the RL activity of the co-culture of 293FT cells expressing NL4-3-derived enV with N4X4-DSP1-7 cells was reduced by 83%. The RL activity of the co-culture of 293FT cells expressing BaL-derived enV with N4X4-DSP1-7 cells was low in the absence of AMD3100 and was not affected significantly by its presence. The RL activity of the co-culture of 293FT cells expressing BaL-derived enV with N4R5-DSP1-7 was reduced by 81% in the presence of maraviroc. The RL activity of the co-culture of 293FT cells expressing NL4-3-derived enV with N4R5-DSP1-7 was low regardless of the presence or absence of maraviroc. The results indicated that DSP-Pheno could be used as an assay for entry inhibitors.

Cell-fusion assay of clinical samples
To evaluate assay performance using clinical samples, we selected plasma samples from 101 treatment-naı ¨ve, HIV-1-positive patients, whose infection with clade B viruses had been confirmed (data not shown). The patient population was classified into two groups based on CD4 T cell count. The low CD4 group consisted of 57 patients with CD4 T cell counts <350 cells/μl; median 228 (range 2–350) cells/μl, and median viral load was 4.77 (range 2.97–6.62) log 10 copies/ml (Figure 5a and b). The high CD4 group consisted of 44 patients with CD4 cell counts >350 cells/μl; median 442 (range 351–843) cells/μl, and median viral load was 4.04 (range 1.60–5.41) log 10 copies/ml. The viral load differences

Figure 4. Inhibition of cell fusions by entry inhibitors. Two μM/well of CXCR4 inhibitor AMD3100 or CCR5 inhibitor maraviroc were added into N4X4-DSP1-7 and N4R5-DSP1-7 cells 90 minutes prior to cell-fusion assay using enV derived from reference strains. (a) RL activities. Columns show the mean RLU ± SD from 5 independent experiments. Black columns, RL activities of enV derived from X4 reference strain (NL4-3); white columns, RL activities of enV derived from R5 reference strain (BaL). Results from X4-indicator (N4X4-DSP1-7) (lanes 1–4) and R5-indicator (N4R5-DSP1-7) (lanes 5–8). A, AMD3100; M, maraviroc. Presence or absence of inhibitor indicated by + or −, respectively. (b) GFP activities. Green fluorescence in the left panel of each pair shows successful cell fusions; red spots in the right panels show the successful transfection. Reference strains, indicator cells and inhibitors used are shown in the figure.
between the two groups were statistically significant by the Mann–Whitney U test (p < 0.001). Aliquots of viral envelope DNA from each plasma sample were used to construct pRE11-envbulk for transfection into 293FT cells. The plasma viral load necessary for the assay was roughly 3.00 log 10 copies/ml for subtype B viruses, although we could amplify the env gene in a patient with 1.60 log 10 copies/ml.

We used the laboratory strain, BaL as the R5 control and NL4-3 as the X4 control to define the cut-off values. We examined BaL on N4X4-DSP1-7 cells and NL4-3 on N4R5-DSP1-7 cells. We defined the cut-off value tentatively as the mean value ± 2SD based on 3 determinations in 12 independent experiments for each combination of negative control and indicator cell. Mean luciferase activities of the patients’ plasma samples. Lanes 1 and 5, CD4 < 350 group; lanes 2 and 6, CD4 > 350 group; lanes 3 and 7, R5 controls (BaL); lanes 4 and 8, X4 controls (NL4-3). Dashed red lines are the cut-off value, that is, the mean value ± 2SD based on 3 determinations in 12 independent experiments for each combination of negative control and indicator cell.

Samples from all patients gave positive RL signals on R5 indicator cells (N4R5-DSP1-7) in the fusion assay, which suggested that the bulk of virus in each patient was able to use CCR5 as the co-receptor (Figure 5c, lanes 5 and 6). Median RLU value of the low CD4 group was significantly higher than that of the high CD4 group on R5 indicator cells (p < 0.0001). Mean RLU value of the low CD4 group was also higher significantly on X4 indicator cells (p = 0.0097) and 26/57 (46%) of low CD4 cases versus 15/44 (34%) of high CD4 cases gave positive RL signals (Figure 5c, lanes 1 and 2). Higher fusion activities on both indicator cells are compatible with higher viral loads in patients with lower CD4 T cell counts and may suggest more dual or X4 tropic (dual/X4) viruses in this group of patients.

To compare the result with conventional GTA, we selected 10 samples each from dual/X4 [N4X4-DSP1-7 (+), N4R5-DSP1-7 (+)] group by DSP-Pheno; Lane 2, 10 samples from R5 [N4X4-DSP1-7 (−), N4R5-DSP1-7 (+)] group by DSP-Pheno. For dual/X4 and R5 group, five patients each from CD4 < 350 and CD4 > 350 groups were chosen. Dashed line indicates the cut-off value as 10% of FPR.
Although there were four samples with discordant result in dual/X4 samples, FPR of these samples were low (range: 14.7–23.6%).

Discussion
We developed a quick, safe and sensitive HIV-1 PTA utilizing double split proteins (DSP-Pheno) and validated the specificity of the assay using laboratory strains with known co-receptor usage. We recognize several limitations of this preliminary study, but the results nevertheless are promising. We assayed bulk envelope genes amplified from plasma from HIV-1-infected patients, rather than cloned envelope genes, and our sample only included subtype B HIV-1. Future studies are necessary to demonstrate the usefulness of the DSP-Pheno.

One caveat of the DSP-Pheno assay is that it is a cell-fusion system, and cell–cell fusion may differ in significant details from virus–cell fusion. For example, recent studies have shown that HIV-1 virions carry fewer surface glycoproteins than previously assumed [24]. The DSP-Pheno assay uses neuroglyoma cell-derived NP-2 cell lines with overexpressed CD4 and co-receptors. Although these NP-2-derived cell lines have been characterized extensively [16,17], some unknown cell surface molecules may be involved in the fusion process. The DSP-Pheno assay is a gag-free system and requires only the assembly of reporter proteins pre-formed in the fusion partner, but infection by a retrovirus requires that the entire gag particle pass through the fusion pore. Careful comparison between DSP-Pheno and in-house pseudoviral assay or GTA using clonal clinical isolates is under way.

GFP portion is necessary as a module of DSP to compensate weak self-association of split RL [15]. Although RL would be more suitable for quantitative assay, GFP may prove single clear positive fusion in the sample with very low RL readout. This feature of DSP-Pheno incorporating two different assays may be useful for certain scientific purposes.

Although several issues remain to be clarified, DSP-Pheno has multiple advantages over the conventional pseudoviral PTA: (i) the turnaround time for DSP-Pheno is short, with results available in as few as 5 days, starting from patient’s plasma; (ii) DSP-Pheno is a virus-free assay that does not require a special biosafety facility, making it particularly appealing for in-house use; and (iii) the RL assay in DSP-Pheno has high sensitivity and specificity and compares favourably with the best pseudoviral PTA published in the detection of minor X4 populations using laboratory strains. Trofile™ (Monogram Biosciences Inc., CA, USA) is currently the only commercially available PTA approved for clinical use, and the latest version, “Enhanced Trofile™,” detects X4 minor populations present in concentrations as low as 0.3% [25]. A pseudoviral PTA described by Soda and colleagues had 1% detection threshold for X4 viruses [16]. Although the RL assay in DSP-Pheno could detect X4 laboratory strains present in concentrations as low as 0.3%, further studies are needed to apply the assay for the clinical use. DSP-Pheno may also be useful for the comparison with of GTA to improve the algorithm for the co-receptor usage of non-B subtypes.

Conclusions
We described a new cell-fusion-based, high-throughput PTA for HIV-1, which would be suitable for in-house studies. Equipped with a two-way reporter system, RL and GFP, DSP-Pheno is sensitive and offers a short turnaround time. Although maintenance of cell lines and laboratory equipment for the assay is necessary, it provides a safe assay system without infectious viruses. With further validation against other conventional analysis, DSP-Pheno may prove to be a useful laboratory tool. The assay may be useful especially for the research on non-B subtype HIV-1 whose co-receptor usage has not been studied much.

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Competing interests
AI has received grant support from Toyama Chemical Co. Ltd., Astellas, VIIV Healthcare KK, MSD KK, Baxter through the University of Tokyo. AI has received speaker’s honoraria/payment for the article from Eiken Chemical Co. Ltd., Astellas, Toyama Chemical Co. Ltd, Torii Pharmaceutical Co. Ltd., Takeda Pharmaceutical Co. Ltd. and MSD.

For the remaining authors, there are no competing interests.

Authors’ contributions
PT, NH and AI planned the experimental design. PT and NH did the experiments. NK, ZM and HH provided the materials. ZM, HH, AK-T and GFG joined the discussion. TF, TK, HN, MK and AI were responsible for the patient care and provided clinical information. PT, NH and AI wrote the article. PT and NH contributed equally to the work.

Acknowledgements
This work was supported in part by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for Program of Japan Initiative for Global Research Network on Infectious Diseases (10005010) (AI); Global COE Program (Center of Education and Research for Advanced Genome-Based Medicine – For personalized medicine and the control of worldwide infectious diseases) of MEXT (FO6) (AI); JSPS KAKENHI (25293226) (AKT); JSPS KAKENHI (24790437) (NH); Grants for AIDS research from the Ministry of Health, Labor, and Welfare of Japan [H24-AIDS-IIPPAN-008] (AKT); Grants for AIDS research from the Ministry of Health, Labor, and Welfare of Japan [H25-AIDS-IIPPAN-006] (NH); Research on international cooperation in medical science, Research on global health issues, Health and Labour Science Research Grants, the Ministry of Health, Labor, and Welfare of Japan [H25- KOJII-SITEI-001] (AI)

The authors thank Ms. Barbara Rutledge for discussion and editing English.

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