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Method Article

Modification and optimization of the inhibition of HIV-1 cell-to-cell transmission assay

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ABSTRACT

HIV-1 infection is caused by cell-free and cell-associated viruses. Currently most of the assays used to screen potential HIV-1 entry inhibitors focus on the inhibition of cell-free viruses. One assay that is widely employed is the TZM-bl neutralization assay that uses pseudotyped viruses. However, a study by Abela et al. showed that many inhibitors that potently inhibit cell-free HIV-1 in this assay can be less effective against the cell-to-cell transmission of the virus. These researchers then designed a method to screen entry inhibitors for activity against cell-associated HIV-1, using pseudotyped viruses. The main limitation of this method, however, was that it can only be reliably employed against viruses that cannot infect target cells as cell-free virion in the absence of a polycation supplement such as DEAE (diethylaminoethyl). Thus, in the current study we provide modifications to this method that solves the problem and makes it possible to study entry inhibitors against cell-to-cell infection of both polycation depend and independent viruses. The main modification involves the introduction of the relative light unit (RLU) vs. virus producing 293-T cells / corresponding supernatants graph. This graph is used to select a virus input that only allows for the detection of cell-associated viruses infection.

- The method is a modification of the cell-to-cell transmission assay published by Abela et al.
- The method allows for the study of the inhibition of cell-to-cell transmission of both polycation dependent and independent HIV-1 pseudoviruses.

ARTICLE INFO

Method name: Modified inhibition of the cell-to-cell transmission assay
Keywords: HIV-1, TZM-bl-cells, Cell-to-cell transmission, Inhibition, Relative-light-unit, Virus expressing, 293-T-cells, Supernatants
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Specifications Table

Subject Area:	Agricultural and Biological Sciences
More specific subject area:	Virology
Method name:	Modified inhibition of the cell-to-cell transmission assay
Name and reference of original method:	Cell-cell transmission using envelope pseudotyped virus particles [1]
Resource availability:	Plasmid encoding HIV-1 envelope Plasmid encoding HIV-1 backbone (pSG3ΔEnv or equivalent) Fugene 6 transfection reagent (Roche, Basel, Switzerland) Phosphate buffered saline (Thermo fisher scientific, MA, USA) Dulbeco Modified Eagle Medium (DMEM) (Thermo fisher scientific, MA, USA) Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA) TZM-bl cells (NIH Reference and Reagent Program, catalog 8129). These cells are engineered to express large amount of CD4 receptor, as well as CCR5 and CXCR4 co-receptors. They also express luciferase under the control of HIV-1 long terminal repeat (LTR). 293-T cells (American Type Culture Collection, Catalog number CRL-3216), highly transfectable cell-line derived from human embryonic kidney cells. Luminometer Flat bottom 96 well plates (Corning incorporated, NY, USA) Flat bottom 96-well black plate (Corning incorporated, NY, USA) Tissue culture dish (Corning incorporated, NY, USA) CO_2 Incubator

Method details

Rational

Many laboratories around the world routinely screen HIV-1 entry inhibitors using the TZM-bl neutralization assay that consists of first incubating the virus with the compound followed by the addition of TZM-bl cells [9]. After 48 h the inhibition of the virus infection is determined by measuring cells luminescence that is proportional to HIV-1 infection. One of the main advantages of this assay is that it can use pseudoviruses instead of infectious viruses; as well as it is relatively easy to use and reliable. However, the TZM-bl neutralization assay only measures the inhibition of cell-free viruses; while HIV-1 also spreads through cell-to-cell transmission that plays an important role during the sexual transmission of the virus [6,7,12]. Recently Abela et al. developed a relatively easy method that can be routinely used to determine entry inhibitors activity against the cell-to-cell transmission of the virus [1]. This method is based on using 293-T cells transfected with plasmids expressing HIV-1 pseudotyped virus. These 293-T cells used as donor cells, are then incubated with the inhibitor before addition of TZM-bl cells, or target cells. With this method if the virus used cannot infect cells as a free-virion in the absence of a polycation, the observed infection is entirely attributed to cell-associated viruses. Consequently, the method's limitation is that it can only be reliably used for such viruses. For those that can infect as cell-free virion in the absence of a polycation, the observed infection will be the product of these viruses and cell-associated ones; therefore, making the measurement of the inhibition of cell-to-cell transmission impossible. Here we report on the modification of this method that makes it possible for the activity of entry inhibitors against both polycation dependent and independent viruses to be determined. Our modification involves the introduction of the relative light unit vs. virus producing 293-T cells / corresponding supernatants graph at the beginning of the assay. This graph enables the determination of the virus input range that only allows for the detection of cell-associated viruses infection, regardless of the fact that a polycation is or is not used in the assay.

Major equipment

- Luminometer
- Flat bottom 96 well plates (Corning incorporated, NY, USA)
- Tissue culture dishes (Corning incorporated, NY, USA)

- Flat bottom 96-well black plate (Corning incorporated, NY, USA)
- CO_2 Incubator

Reagents

- Plasmid encoding HIV-1 envelope
- Plasmid encoding HIV-1 backbone (pSG3ΔEnv or equivalent)
- Fugene 6 transfection reagent (Roche, Basel, Switzerland)
- Phosphate buffered saline (Thermo fisher scientific, MA, USA)
- Dulbeco Modified Eagle Medium (DMEM) (Thermo fisher scientific, MA, USA)
- Fire fly luciferase substrate (Promega, Madison, WI, USA)
- TZM-bl cells (NIH Reference and Reagent Program, catalog 8129)
- 293-T cells (American Type Culture Collection, Catalog number CRL-3216)

Method

This protocol begins with the transfection of 293-T cells with the viral backbone plasmid pSG3ΔEnv and a plasmid encoding HIV-1 envelope [1,13]. To be precise, 2×10^6 293-T cells should first be cultured in a tissue culture dish in 10 mL of the growth medium i.e. DMEM containing 10% fetal bovin serum (FBS), at 37 °C and 5% CO_2 (all incubations were done under these conditions). After 24 h the transfection is performed by adding 4 µg each of pSG3ΔEnv and the envelope plasmid, using the Fugene 6 transfection reagent (Roche, Basel, Switzerland), followed by 48 h incubation. Then virus producing cells are trypsinized after washing with 6 mL of phosphate buffered saline (PBS). Next, count cells and perform the same dilution series of 293-T cells in two different flat bottom 96-well plates. The highest concentration of 293-T cells, in the dilution series, that we recommend is 2×10^5 cells/well/150 µL. However, higher or lower concentrations can be used. This should be followed by 48 h incubation of the virus producing cells. Then remove the supernatants from each wells of one of the plates and wash cells gently twice, with 100 µL of PBS/well, without disturbing them from the surface of the well. Add 3×10^4 TZM-bl cells/well/250 µL of growth medium. With the second plate, remove the supernatants from each well and transfer to the corresponding wells of a third 96-well flat bottom plate followed by the addition of 3×10^4 TZM-bl cells/well/100 µL. Incubate the two plates containing TZM-bl cells for 48 h.

After the incubation remove 150 µL from each well of the two plates and add 100 µL/well of the firefly luciferase substrate (Promega, Madison, WI, USA). This should be followed by 2 min incubation at room temperature to allow the cells to completely lyse. After mixing, transfer 150 µL from each well to the corresponding wells of a 96-well black plate. Using a luminometer read the two black plates luminescence. Plot the RLU vs. the number of 293-T cells for both plates in the same graph (an example of the graph is shown in Fig. 1) i.e. the RLU vs. virus producing 293-T cells / corresponding supernatants graph. Note here that one plot will be for the 293-T cells (shown in black in Fig. 1) and the other plot (shown in red) will be for the corresponding supernatants. Choose the number of 293T-cells input for the inhibition of cell-to-cell transmission assay in the region highlighted by shading in Fig. 1. This is the region where the RLU of the supernatants is not above background while that of 293-T cells is. Note that different viruses will have different donor cells input range. This being said, when selecting the virus producing cells input be careful not to use too high a number of 293-T cells lest that you end up with too many cells per well during the inhibition of cell-to-cell transmission assay. Because this will result in cells being over confluent and not growing optimally. We recommend that the amount of 293-T cells input be between 5×10^3 and 1×10^4 cells/well. We observed that for most viruses tested such input gave an RLU of ± 10,000 after 48 h (an example for the virus QH0692.42 is shown in Fig. 2), which is similar to the one aimed for by Abela et al. [1].

Next add the selected number of 293-T cells in the wells of a flat bottom 96-well plate and incubate with the test sample's dilution series for an hour before addition of TZM-bl cells, at 3×10^4 cells / well. The plate template is illustrated in Fig. 3. The final volume in all the wells should be 250 µL. Then place the cells in the incubator for 48 h and read luminescence as explained above. Use

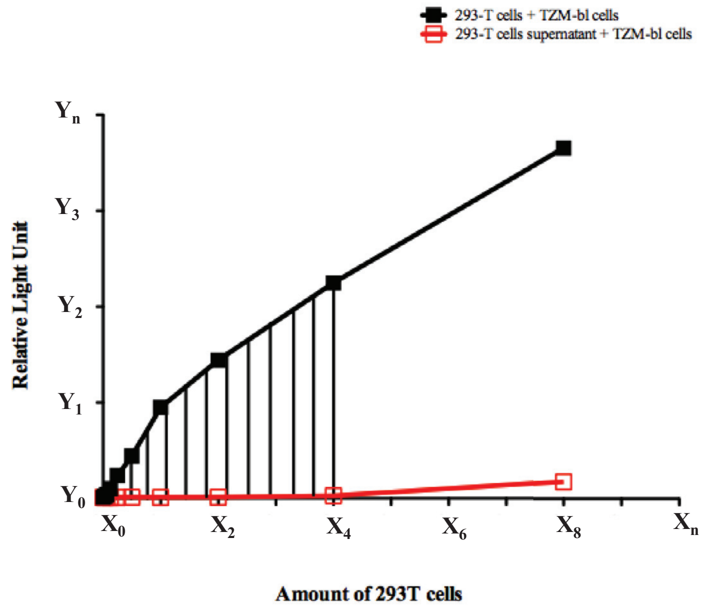

Fig. 1. *Illustration of RLU vs. virus producing 293-T cells / corresponding supernatants graph.* In black is the 293-T cells titration plot and in red is the corresponding supernatants plot. The shading highlights the zone where the virus producing 293-T cells input for the cell-to-cell transmission assay should be selected, in order to eliminate the possibility of cell-free viruses contributing to the observed infection.

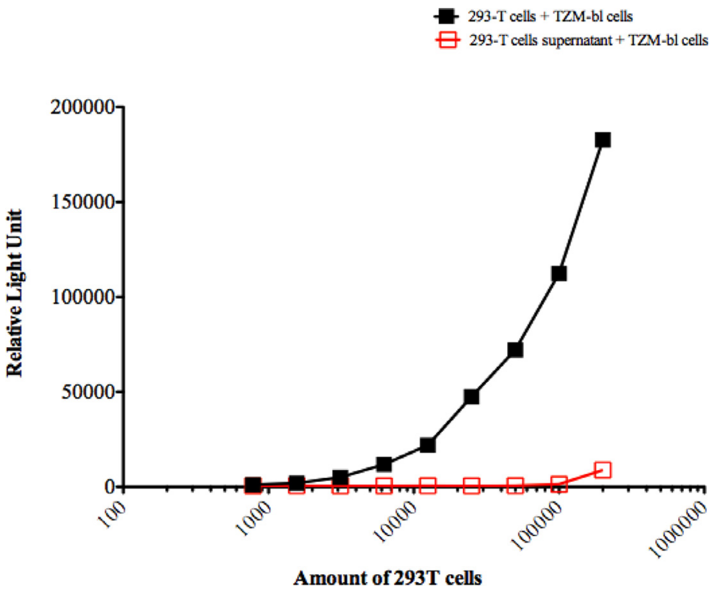

Fig. 2. *Graph used in the determination of QH0692.42 input.* A dilution series of 293-T cells expressing the pseudotyped virus QH0692.42 or supernatants from each of these dilutions were incubated with TZM-bl cells. After 48 h infection was determined by measuring the RLU.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells	Sample dil 8 + donor and acceptor cells
B	TZM-bl cells	TZM-bl cells + 293T cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells	Compound dil 7 + donor and acceptor cells
C	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells	Sample dil 6 + donor and acceptor cells
D	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells	Sample dil 5 + donor and acceptor cells
E	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells	Sample dil 4 + donor and acceptor cells
F	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells	Sample dil 3 + donor and acceptor cells
G	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells	Sample dil 2 + donor and acceptor cells
H	TZM-bl cells	TZM-bl cells + 293T cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells	Sample dil 1 + donor and acceptor cells
			Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	

Fig. 3. *The 96-well flat bottom plate setup for the inhibition of HIV-1 cell-to-cell transmission assay.* The column 1 is for background reading; column 2 for the virus control reading; and the rest of the plate is where dilution series of different test samples are carried out.

the data obtained to determine the sample's inhibitory activity. One way to do this is by calculating the 50% inhibitory concentration (IC_{50}).

Protocol steps

- Culture 2 × 10^6 293-T cells in a tissue culture dish in 10 mL of the growth medium (note that all tissue cultures are done at 37 °C and 5% CO_2)
- After 24 h transfect the cells with 4 µg each of pSG3ΔEnv and the envelope plasmid and place in the incubator for 48 h
- Trypsinize the cells, count and perform a dilution series of the 293-T cells in two flat bottom 96 well plates. The cells should be cultured in 150 µL/well
- After 48 h incubation, remove the supernatants in all the wells of one plate and add 3 × 10^4 TZM-bl cells/well/250 µL of growth medium.
- Remove the supernatants in all the wells of the second plate, transfer to the corresponding wells of a third flat bottom 96 well plate, and add TZM-bl cells at 3 × 10^4 cells/well/100 µL
- Incubate the plates containing TZM-bl cells for two days
- Determine the level of HIV-1 infection in each well of the two plates by luminescence
- Plot the RLU vs. number of 293-T cells for both plates in the same graph
- Choose the virus input i.e. number of virus producing 293-T cells in the region of the graph where the RLU of the supernatants is not above background
- Add the selected amount of 293-T cells in the wells of a flat bottom 96-well plate and incubate with the test sample dilution series for an hour
- Add 3 × 10^4 TZM-bl cells/well bringing the total volume in each well to 250 µL
- Culture the cells for 48 h and determine the IC_{50} of the test sample after reading luminescence

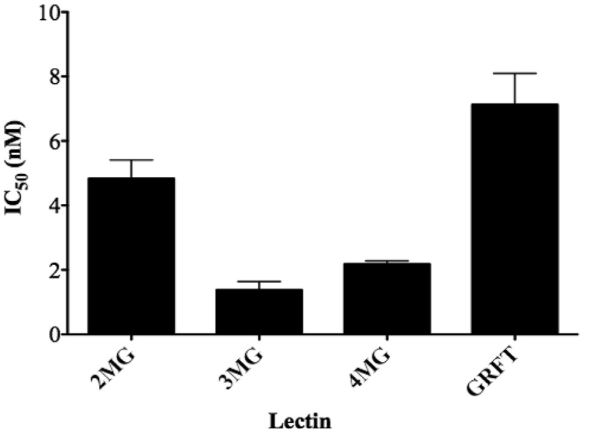

Fig. 4. *Inhibition of the cell-to-cell transmission of QH0692.42 with GRFT and its derivatives.* The 293-T cells expressing HIV-1 were first incubated with the lectins and then co-cultured with TZM-bl cells. The inhibition of infection was determined by measuring luminescence after 48 h. Bars represent the mean plus standard deviation of three independent experiments.

Validation

To validate this method we determined the inhibitory activities of the anti-HIV-1 lectin griffithsin (GRFT) and its derivatives. GRFT is a lectin that binds mannose-rich glycan on HIV-1 envelope and it is a homodimer composed of two domain swapped monomers [3,5,10,14,15]. The derivatives used are called tandemers made of monomeric GRFT linked in tandem repeats of two, three, and four units called 2MG, 3MG, and 4MG, respectively [11]. We chose GRFT and its derivatives for the validation of the method given that we published a number of papers on their inhibitory activity against the virus [2,3,11]. Furthermore, we previously showed that GRFT can inhibit the transfer of the virus from a cell expressing the DC-SIGN receptor to a susceptible target cell i.e. we knew this compound had the potential to inhibit the cell-to-cell transmission of HIV-1 [4]. We tested the four lectins activity against the cell-to-cell transmission of the HIV-1 subtype B known as QH0692.42 that is part of a panel of viruses that are commonly used to test envelope inhibitors [3,8]. The determination of the virus expressing 293-T cells input was performed as explained in the method. The RLU vs. virus producing 293-T cells / corresponding supernatants graph for QH0692.42 is shown in Fig. 2. GRFT, 2MG, 3MG, and 4MG IC_{50} for the inhibition of cell-associated HIV-1 transfer to TZM-bl cells are given in Fig. 4. GRFT inhibited the cell-to-cell transmission of HIV-1 with an IC_{50} value of ~ 7 nM, followed by 2MG with ~ 5 nM, and 3MG and 4MG were the most potent with IC_{50} values of less than 2 nM. These results could be repeated in assays peformed in different days indicating the method's reliability. Our method was also used to determine the four lectins inhibition of cell-to-cell transmission of CAAN5342.A2 and CAP206.8 [2]. lastly, the inhibitory potency of GRFT and its derivatives followed the same trend as that observed for the inhibition of cell-free viruses [11].

Conclusion

The protocol reported here is a modification of the method developed by Abela et al. for studying the inhibition of cell-to-cell transmission of HIV-1 [1]. The key limitation of the Abela et al. method is that it can only be used reliably for viruses that are unable to infect susceptible cells as free-virion in the absence of a polycation. We modified this method making it reliable to study both viruses that are dependent on a polycation and those that are not. Furthermore, we used 293-T cells as virus donor cells and TZM-bl cells as virus target cells. However, we believe our method can be adapted to accommodate other suitable donor and target cells.

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Declaration of Competing Interest

None.

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