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Corresponding Author:	Damian FJ Purcell, Ph.D. University of Melbourne School of Ecosystem and Forest Sciences Melbourne, VIC AUSTRALIA
Corresponding Author's Institution:	University of Melbourne School of Ecosystem and Forest Sciences
Corresponding Author E-Mail:	dfjp@unimelb.edu.au
Order of Authors:	Georges Khoury Damian FJ Purcell, Ph.D.
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Dear Dr Wu,

We would like to resubmit a revised version of our manuscript entitled “High throughput *in vitro* assessment of HIV latency reversing agents on viral RNA transcription and splicing” for publication in JoVE.

We have addressed all the points raised by the editorial team, and included all requested modifications in the text. A copy of the point-by-point response is added below.

We hope that the manuscript in its current revised form is suitable for publication in JoVE.

We thank you in advance for your consideration and shall be looking forward to hearing from you.

Yours faithfully,
Prof. Damian F.J. Purcell

Response to Editor:

Editorial comments:

1. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Lipofectamine 2000, CellTiter 96, etc.

We removed the remaining commercial language/company names of reagents left such as Lipofectamine (lipid reagent), CellTiter (wash buffer), Live/DEAD fixable dead cell stain Near-IR (NIR) (viability dye), CST beads and rainbow 8-peak (calibration beads), FlowJo (flow cytometry data analysis software)... from the manuscript.

In addition, all commercial products have been referenced in the Table of Materials and Reagents.

2. Step 4.12: Please ensure that all text is written in imperative tense.

The sentence was written in imperative tense – lines 300-303, page 7 as follows:

“4.12. Analyze the data using a flow cytometry data analysis software. Exclude cell debris and clump (doublets) based on forward and side scatter then eliminate the dead cells using the viability dye stain (negative population). Identify the cells expressing EGFP and DsRed (Figure 4), as well as the percentage of spliced product DsRed/(DsRed + EGFP) (Figure 5).”

TITLE:

High Throughput *In Vitro* Assessment of Latency Reversing Agents on HIV Transcription and Splicing

AUTHORS AND AFFILIATIONS:

Georges Khoury¹, Damian F J Purcell¹

¹Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia

Corresponding Author:

Damian F J Purcell

dfjp@unimelb.edu.au

Tel: (03)-8344 6753

Email Addresses of Co-authors:

Georges Khoury (georges.khoury@unimelb.edu.au)

KEYWORDS:

HIV, high throughput, fluorescent reporter, transcription, splicing, EGFP-DsRed

SUMMARY:

A high throughput protocol for functional assessment of HIV efficient reactivation and clearance of latent proviruses is described and applied by testing the impact of interventions on HIV transcription and splicing. Representative results of the effect of latency reversing agents on LTR-driven transcription and splicing are provided.

ABSTRACT:

HIV remains incurable due to the existence of a reservoir of cells that harbors stable and latent form of the virus, which stays invisible to the immune system and is not targeted by the current antiretroviral therapy (cART). Transcription and splicing have been shown to reinforce HIV-1 latency in resting CD4⁺ T cells. Reversal of latency by the use of latency reversal agents (LRAs) in the “shock and kill” approach has been studied extensively in an attempt to purge this reservoir but has thus far not shown any success in clinical trials due to the lack of development of adequate small molecules that can efficiently perturb this reservoir. The protocol presented here provides a method for reliably and efficiently assessing latency reversal agents (LRAs) on HIV transcription and splicing. This approach is based on the use of an LTR-driven dual color reporter that can simultaneously measure the effect of an LRA on transcription and splicing by flow cytometry. The protocol described here is adequate for adherent cells as well as the cells in suspension. It is useful for testing a large number of drugs in a high throughput system. The method is technically simple to implement and cost-effective. In addition, the use of flow cytometry allows the assessment of cell viability and thus drug toxicity at the same time.

INTRODUCTION:

Despite effective long-term antiretroviral therapy, HIV persists in a latent state as an integrated provirus in memory CD4⁺ T cells¹. The chromatin structure of the HIV-1 5' long terminal repeat

(LTR) promoter and epigenetic modifications such as histone methylation and deacetylation by DNA methyltransferases (DNMT) and histone deacetylases (HDAC) are important mechanisms leading to transcriptional repression and thus post-integration latency^{2,3}. A large variety of latency reversing agents (LRAs) has been investigated for their efficacy to induce virus production *in vitro* and *in vivo* from latently infected resting CD4⁺ T cells⁴⁻⁸. Among the LRAs tested, HDACi (HDAC inhibitors) and BET bromodomain inhibitors (BETis) induce chromatin decondensation and release of the positive transcription elongation factor b (P-TEFb) respectively, leading to subsequent relieve of the transcriptional repression at the 5'LTR and activation of HIV expression⁹⁻¹³. However, the magnitude of reactivation achieved by these LRAs was limited as only a modest increase in cell-associated unspliced HIV mRNA (US RNA), indicative of viral transcription, was observed *ex vivo*^{14,15}. Importantly, these LRAs also failed to induce a reduction in the frequency of latently infected cells.

HIV expression may be further restricted by inefficient splicing¹⁶ as well as defects in nuclear export of multiply spliced HIV RNA (MS RNA)¹⁷. Thus, identifying new classes of LRAs that are more potent and can affect distinct aspects of virus production post-integration are needed. In addition, the development of novel assays that help defining the optimal compounds to efficiently reverse latency is required.

Here, a protocol is presented, which utilizes a high-throughput approach for functional assessment of the impact of interventions on HIV LTR-driven transcription and splicing. In brief, a new LTR-driven dual color reporter system pLTR.gp140/EGFP.RevΔ38/DsRed (**Figure 1**) is used to assess HIV reactivation by flow cytometry. In this fluorescent reporter, the expression of unspliced HIV mRNA (4 kb) leads to enhanced green fluorescent protein (EGFP) expression, while the expression of spliced mRNA (2 kb) would lead to *Discosoma sp.* red fluorescent protein (DsRed) protein expression. Briefly, we used a fluorescent Env-EGFP fusion protein, gp140unc.EGFP, where the coding sequence of EGFP was placed in frame with an un-cleaved and truncated form of the envelope (Env). Changes were introduced to ablate the cleavage site preventing the dissociation of Env into gp120 and gp41-EGFP, and to truncate the gp160 protein prior to the transmembrane domain creating a soluble Env analogue, which facilitates the correct folding and expression of EGFP. Upon the expression within a cell, Rev localizes to the nucleus where it mediates the nuclear-cytoplasmic export of the 4 kb *env* mRNA via the interaction with the rev responsive element (RRE). The truncation of Env does not compromise the RRE, which lies between gp120 and gp41, and the A7 3' splice site. In this system, splicing at HIV-1 splice donor 4 (SD4) and splice acceptor 7 (SA7) results in the production of a 2 kb mRNA encoding a non-functional Rev protein truncated at amino acid 38 fused to DsRed fluorescent protein, RevΔ38-DsRed. Briefly, DsRed was inserted into the 2nd exon of Rev at amino acid 38 by overlap extension¹⁸. To facilitate the nuclear export of unspliced mRNA, a mammalian expression vector encoding Rev (pCMV-Rev^{NL4.3}) was co-transfected with the fluorescent reporter construct (**Figure 2**). This unique reporter construct described here is useful in high-throughput assessment of HIV transcription and splicing, without the need to use viral vectors.

PROTOCOL:

NOTE: Procedures for cloning, transformation and sequencing are discussed elsewhere^{18,19}. The protocols herein begin from the transfection of the mammalian expression vectors (**Figure 3**).

1. Transfection of HEK293T Cells with Dual Color Reporter Construct

1.1. Cultivate HEK293T cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL) in a 5% CO₂ incubator at 37 °C. After thawing, passage HEK293T cells 2-3 times before using them in transfection experiments. This would give the cells time to recover from the thawing procedure.

CAUTION: Mycoplasma contamination of cell cultures remains a serious problem. Good laboratory practice and routine testing of cell cultures are essential to decrease the risk of mycoplasma contamination and avoid diffusion²⁰.

1.2. Split the cells 1:2 one day before seeding and transfer them into fresh DMEM medium. Cultivate the cells for 24 h in a 5% CO₂ incubator at 37 °C.

1.3. The day before transfection, remove the medium from the flask by aspiration and wash the cells with Dulbecco's phosphate buffered saline (DPBS) solution free of calcium (Ca²⁺) and magnesium (Mg²⁺) to remove traces of serum.

1.4. Dispense 5 mL of trypsin-EDTA solution (0.05% - 10 mM in PBS) into the culture vessel and place it at 37 °C in a 5% CO₂ incubator for 2 to 5 min.

1.5. When the cells are detached, add 5 mL of DMEM supplemented with 10% (v/v) FBS to further inhibit the trypsin activity which may damage the cells.

1.6. Resuspend gently by pipetting the cell suspension up and down to break up the clumps.

1.7. Dilute the cells 1:10 in trypan blue stain (0.4%).

1.8. Count the live cells that do not take up trypan blue using a microscope (10X objective) and a haemocytometer.

1.9. Calculate the number of viable cells per milliliter by taking the average of the cell count and multiplying it by 10,000 and by the dilution factor (1:10) from the trypan blue stain.

1.10. Plate 2×10^4 cells in 100 µL of DMEM medium supplemented with 10% FBS without antibiotics in a 96-well flat-bottom plate for 24 h.

1.11. For each well, dilute 400 ng of pLTR.gp140/EGFP.RevD38/DsRed, 20 ng of pCMV-Rev^{NL4.3} and 100 ng of pCMV-Tat101^{AD8}-Flag DNA in 50 µL of serum free medium. Mix gently. For experiments without Tat, use a matched empty Tat vector pcDNA3.1 (-).

NOTE: In addition, the use of endotoxin-free DNA is highly recommended. For that, prepare endotoxin-free DNA using a midi or maxiprep nucleic acid purification kit. Determine the DNA purity by measuring the 260/280 OD ratio, which should be between Step 1.7 and 1.9.

1.12. Mix the lipid reagent gently before use, then dilute 0.65 μL in 50 μL of serum free medium. Mix gently and incubate for 5 min at room temperature.

1.13. Combine the diluted DNA with the diluted lipid reagent.

1.14. Mix gently and incubate for 20 min at room temperature. The solution may appear cloudy.

NOTE: Proceed to step 1.15. within 30 min.

1.15. Dispense 100 μL per well of the lipid reagent-DNA complexes drop-wise on top of the cells.

1.16. Mix gently by rocking the plate back and forth.

1.17. Incubate the plate for 5 h in a 5% CO_2 humidified incubator at 37 $^{\circ}\text{C}$.

1.17.1. Each reaction mix is sufficient for a single well (96-well) transfection. Adjust the amount and volume of components according to the number of drug and combination that would be tested, and accounts for pipetting variations. All conditions are usually tested in triplicates.

1.17.2. Transfect additional wells with 100 ng of CMV-driven EGFP and DsRed-Express DNA plasmid for compensation purposes.

2. Treatment of Transfected HEK293T Cells with Latency Reversing Agents

NOTE: Prior to each assay, determine the physiological condition of each LRA by measuring the viability of the cells after exposure to high and low dose of the drug with cell proliferation assay.

2.1. Dilute each LRA to the appropriate working concentration with growth medium (*e.g.*, 1 μM for JQ1(+)).

CAUTION: Reconstitute the lyophilized drugs with the appropriate solvent to a concentration of 10 mM. Store all stock LRAs at -80 $^{\circ}\text{C}$ in a single use aliquot (5 μL each) in order to avoid repeated freezing-thawing cycles.

2.2. Carefully aspirate transfection medium with a multichannel and replace with 100 μL medium containing appropriate LRA (Table 1). Add medium very gently alongside the wall of

the well to avoid detaching the transfected HEK293T cells, which are known to easily detach from the culture plate surface.

2.3. Incubate the plate for 48 h in a 5% CO₂ humidified incubator at 37 °C.

3. Staining of Transfected Cells with Fixable Viability Dye for Flow Cytometry Analysis

CAUTION: Removing dead cells and debris is essential to eliminate false positives and to obtain results of the highest quality.

3.1. Detach the cells into the media using 100 µL of phosphate-buffered saline (PBS) per well and by gently pipetting up-and-down (~5 times) with a multichannel, while avoiding frothing of the media. If needed, detach the cells from the plate using 35 µL per well of trypsin-EDTA solution (0.05% - 10 mM in PBS) and incubating 5 min at 37 °C, before neutralizing with medium containing serum.

3.2. Transfer the cells to a 96-well V-bottom plate.

3.3. Spin the cells for 5 min at 500 x g at 4 °C, then carefully aspirate the medium/PBS without touching the cells.

3.4. Wash the cells at least 1 time with 200 µL of protein/serum free PBS.

3.5. Centrifuge at 500 x g for 5 min at 4 °C, then discard the supernatant by tilting the plate and removing the wash buffer without touching the cells.

3.6. Prepare a working solution of fixable viability dye by diluting the viability dye 1:1000 in protein/serum free PBS. Prepare 50 µL of the diluted stain per well.

NOTE: Viability dyes are available in a range of colors suitable for use with blue, red and violet lasers. Prepare a stock solution of fixable viability dye by resuspending one vial of the lyophilized dye (component A) with 50 µL anhydrous DMSO (component B). Store at -20 °C in a single use aliquot (1 µL each), protected from the light.

3.7. Add 50 µL of the diluted viability dye to each well and mix the cells by pipetting up and down with a multichannel.

3.8. Stain for 10-15 min at 4 °C, protected from the light.

3.9. Wash 1-2 times with 150 µL of wash buffer (PBS with 1% bovine serum albumin and 2 mM EDTA).

3.10. Fix the cells with 100 µL of freshly prepared 1% formaldehyde in wash buffer for 10-15

min at 4 °C in the dark.

CAUTION: Formaldehyde is very toxic. Prepare 1% formaldehyde solution in a fume hood to avoid inhalation while wearing gloves and safety glasses for protection.

3.11. Wash the cells 1-2 times with 100 µL of wash buffer.

3.12. Centrifuge at 500 x g for 5 min at 4 °C. Discard the supernatant.

3.13. Resuspend the cell pellet in 70 µL of wash buffer.

NOTE: The protocol can be paused here. Fixed cells could be stored at 4 °C in the dark for analysis the next day on the flow cytometer.

4. EGFP and DsRed Measurements by Flow Cytometry and Data Analysis

NOTE: Analyze HIV transcription (% EGFP) and splicing (% DsRed) on a flow cytometer. Filter the sample before the run with a 70 µm cell-strainer or 100 µm nylon mesh to avoid clogging up the nozzle.

4.1. Start up the cytometer and computer at least 10 min prior to use to ensure lasers warm up.

4.2. Prior to running samples and commencing data acquisition, fill the sheath tank with 0.9% saline solution and ensure that a sodium hypochlorite tablet is added to the waste tank.

4.3. Check the flow cell for air bubbles.

4.4. Verify that the detectors and filters are appropriate for the experiment.

NOTE: For EGFP, use of blue laser (488 nm) and 530/30 bandpass, while DsRed Express is optimally detected using the yellow laser (561 nm) and 610/20 bandpass. A blue laser (488 nm) and 610/20 bandpass can also be used to detect DsRed expression.

4.5. Check the cytometer performance and sensitivity across detectors by running respectively calibration beads.

4.6. Adjust the forward (FSC-1) and side (SSC-A) scatter voltages with unstained sample so that the main population is on-screen and clearly discernable.

NOTE: FSC (Forward-scattered light) is a measurement of light diffraction in a flat angle, which depends on the volume of the cell (cell surface area or size), while SSC (side-scattered light) is a measurement of light diffraction in a right angle, which is proportional to cell granularity and internal complexity.

4.7. Perform manual or automatic compensation by using the single-stained samples ensuring minimal spillover of EGFP+ population into the DsRed detector and vice-versa.

CAUTION: For compensation purposes, use a sample of cells expressing each of the single color fluorescent protein as well as cells singly stained with the fixable viability dye. Do not use FITC and PE compensation beads for EGFP and DsRed fluorescent proteins compensations due to different spectral characteristics. Compensation controls should be bright enough to resolve positive and negative population.

4.8. Create plots and set the gates using fluorescence minus one (FMO) controls.

4.9. Acquire and record a minimum of 10,000 viable cell events per sample. Run samples at medium or low speed to avoid doublets passing through the laser intercept. Use pulse geometry gating such as SSC-A versus SSC-H to eliminate doublets. Check the stability of the run by plotting time versus SSC-A to see how even the flow is during the run.

4.10. At the end of the run, clean the flow cytometry fluidics with concentrated cleaning agents then water for 5 min each.

4.11. Shutdown the system, discard the waste and re-fill the sheath tank after acquisition.

4.12. Analyze the data using a flow cytometry data analysis software. Exclude cell debris and clump (doublets) based on forward and side scatter, then eliminate the dead cells using the viability dye stain (negative population). Identify the cells expressing EGFP and DsRed (**Figure 4**), as well as the percentage of spliced product DsRed/(DsRed + EGFP) (**Figure 5**).

4.13. Determine the effect of the LRA on HIV transcription and splicing by comparing untreated cells and the cells exposed to individual or a combination of LRAs (**Figure 5**).

REPRESENTATIVE RESULTS:

Representative results are shown in **Figure 5** for the expression of HIV-1 unspliced (EGFP) and spliced (DsRed) products following treatment with bromodomain inhibitor JQ1. Both JQ1(+) and Tat significantly increased the percentage of cells expressing EGFP (2.18 and 4.13 FC over DMSO respectively; n=3) indicative of unspliced transcripts. Moreover, JQ1(+) significantly increased the percentage of cells expressing DsRed (46.6 FC over DMSO) as well as the proportion of spliced product (7.37 FC over DMSO) to a similar level as Tat (59.6 and 5.83 FC over DMSO, respectively) confirming the ability of JQ1(+) to turn on HIV transcription and splicing. On the other hand, treatment with the stereoisomer control JQ1(-) abolishes JQ1(+) effect on HIV transcription and splicing confirming the success of the protocol.

In a recent publication, we have shown by RNA analysis an accumulation of HIV spliced transcripts following JQ1(+) treatment²¹. In fact, our data revealed consistent changes in the levels of unspliced and spliced transcripts that were mirrored by the EGFP and DsRed protein expression in this model following treatment with JQ1(+). These results indicate that EGFP and

DsRed expressing cells reflect the ability of the bromodomain inhibitor JQ1(+) to induce HIV transcription and splicing.

In summary, we validated the use of the pLTR.gp140/EGFP.RevΔ38/DsRed reporter in a high-throughput set-up for the assessment of the effect of LRAs on HIV-1 transcription and splicing. Sub-optimal amount of LRA or increased toxicity could lead to skewed results. Optimizing the amount of drug used while preserving cell viability can improve the accuracy of the results.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the model used to determine the effects of latency reversing agents on LTR-driven transcription and splicing. The LTR construct expresses either unspliced envelope (Env) protein fused to enhanced green fluorescent protein (EGFP) or spliced D38rev protein with DsRed. This figure has been reprinted from Khoury *et al.*²¹.

Figure 2: Constructs design. (A). pLTR.gp140/EGFP.RevΔ38/DsRed splicing reporter allows the expression of envelope fused to EGFP (gp140-EGFP) and non-functional Rev protein truncated at amino acid 38 fused to DsRed fluorescent protein (RevΔ38-DsRed). **(B).** pCMV-Rev^{NL4.3} allows the expression of Rev protein. **(C).** pCMV-Tat101^{AD8}-Flag allows the expression of Tat101(AD8) protein with a C-terminal flag-tag. The expression plasmids are constructed using PCR overlaps and restriction digest. All vector maps were created using *SnapGene* software, version 4.1.9.

Figure 3: Assay design for rapid assessment of HIV latency reversing agents. The current method for the assessment of the effect of LRAs on HIV-1 transcription and splicing includes i) seeding of HEK293T cells one day prior to ii) transfection with expressions vectors followed by iii) LRA treatment. iv) Cells are analyzed by flow cytometry 48 h post-treatment. Using this protocol, 32 (in triplicate) to 96 conditions could be tested per plate.

Table 1: Representative example of the plate set-up and controls needed. Columns 1 to 3 correspond to negative (-) controls with no drug and solvent only (DMSO 1:5000) as well as positive (+) controls such as Tat (100 ng), PMA/PHA = phorbol myristate acetate/phytohaemagglutinin (10 nM PMA, 10 µg/mL PHA), JQ1 (+/-) (1 µM), VOR = vorinostat (0.5 µM), and PAN = panobinostat (30 nM). Unknown LRAs are tested in triplicate over a range of concentrations (15.625 to 1000 nM). For compensation purposes, cells expressing each of the single-color fluorescent protein (EGFP+ and DsRed+) as well as cells stained with the viability dye and unstained cells are included in each run.

Figure 4: Gating strategy used in the flow cytometry analysis. The first step in the gating strategy is based on the forward and side scatter, which allows the distinction of the cells of interest based on the size and granularity properties of these cells. It is recommended that this gating be as generous as possible to include all events, while removing cellular debris and dead cells, which are found at the bottom left corner of the density plot. Then remove doublets from the dataset using the pulse geometry gating, SSC-A versus SSC-H, and further narrow on the live cells using the viability dye. Finally, a dump channel (violet) and EGFP or DsRed are used to define to cells of interest. The use of fluorescence minus one (FMO) controls are critical in

defining the population of interest and addressing the issues of spillover from another fluorochrome in the channel of interest. After acquisition, data are analyzed using flow cytometry data analysis software. This figure has been reprinted in adapted form Khoury *et al.*²¹.

Figure 5: Representative results of the effect of bromodomain inhibitors on HIV transcription and splicing. (A). Examples of two-parameter dual color fluorescence EGFP versus DsRed density plots from untransfected cells (- Tat), cells transfected with 100 ng of pCMV-Tat101^{AD8}-Flag (+ Tat) and cells treated with DMSO, JQ1 (+) or JQ1 (-). **(B).** The mean percentage (%) of cells expressing EGFP, DsRed or spliced product (DsRed/DsRed+EGFP) from 3 independent experiments is shown. Comparisons of each condition to DMSO were made using the 2-way ANOVA test. Only statistically significant comparisons are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The black lines represent the mean \pm SEM. DMSO (1:5000), JQ1 (+) and JQ1(-) (1 μ M).

DISCUSSION:

Given the difficulty in measuring virus reactivation *ex vivo*, a wide range of *in vitro* models were developed over the time in order to study HIV latency including latently infected T cell-lines (J-Lats, ACH2, U1), primary models of latent infection of resting (O'Doherty, Lewin, Greene and Spina models) or pre-activated CD4+ T cells (Sahu, Marini, Planelles, Siliciano, Karn models) with single round or replication competent reporter viruses²². To model the physiological conditions of HIV latency in resting CD4+ T cells, dual fluorescent reporter systems ensued such as the RGH (Red-Green-HIV) reporter developed by Ivan Sadowski's group with an LTR-driven Gag-eGFP marker and a CMV-driven mCherry in place of Nef²³. A similar dual color reporter virus, DuoFluo, was developed by E. Verdin laboratory with an LTR-driven EGFP expression and an mCherry fluorescent marker driven by an EF1 α promoter²⁴. In this system, the EGFP was inserted at the 3' end of the provirus in place of Nef. The deletion in the envelope in both these systems prevents multiple rounds of infections. Moreover, the combination of the two fluorescent proteins allows the detection of productively (eGFP+ mCherry+) and latently (eGFP-mCherry+) infected cells. However, several shortcomings of the dual color reporter viruses were noticeable in CD4+ T cells as the direct infection of latently infected cells is largely dependent on the cellular activation state of the T-cells^{23,24}. In fact, very low rate of infection was observed using these two reporter viruses in resting CD4+ T cells: 0.1% for RGH²³ and 0.2% for DuoFluo²⁵. In addition, as the CMV promoter has been shown to be expressed at lower levels in non-activated cells²⁶⁻²⁸, an inactive CMV or EF1 α promoter would lead to underestimation of the latently infected populations.

We have generated and characterized a new HIV reporter vector to study the establishment of latency and virus reactivation in a high throughput assay. Several advantages to our screening method include i) cost effectiveness with the ability to assess transcription and splicing simultaneously, ii) the ability to test a large number of drugs or combinations in a single experiment, iii) quick assessment of the effect of LRAs by flow cytometry (30-45 min typical read time for 96 well plate independent of the number of fluorescent parameters), iv) high dynamic range, v) suitable for automated high throughput transfection and flow cytometry

using robot arms and HTS platform, vi) fast curing of data using a flow cytometry workspace template, vii) optimal for suspension and adherent cells, viii) simplicity of the model and adaptability to luminescence and plate reader measurements (dual luciferase Firefly and Renilla), and ix) scalability (96- and 384-well plate formats).

The propensity of an LRA or a combination of LRAs to activate HIV LTR-driven transcription and splicing thus EGFP and DsRed fluorescent proteins expression could be examined by flow cytometry using our HIV splicing reporter. However, before testing the synergy of novel LRAs, it is highly recommended to determine the physiological conditions of each LRA by measuring the viability of the cells after exposure to a range of concentrations of the single drug. Therefore, including a marker of live and dead cells is essential to eliminate false positives and to obtain results of the highest quality. Another important aspect of flow cytometry analysis is the compensation controls or FMO. It is highly recommended to use singly stained samples ensuring minimal spillover of EGFP+ population into the DsRed and vice-versa. Moreover, it is essential to account for autofluorescence of the cells by including unstained cells. Finally, a regular control of the cytometer performance and sensitivity across the detectors is critical mostly when measuring samples for a study spanning a long period of time.

Although not discussed in the protocol section, there are several limitations of our splicing reporter system. For a more thorough discussion, please see our previous publication (Khoury *et al.*, 2018). Export of the partially or unspliced variants of HIV mRNA is facilitated by the viral protein Rev and its association with the Rev Responsive Element (RRE) in these mRNA species²⁹⁻³². The overexpression of Rev in our system allowed us to circumvent the major block of nuclear retention of multiply spliced and unspliced mRNAs observed in latently infected T cells¹⁷ and focus on understanding how the LRAs induce transcription and splicing thus virus reactivation. In addition, given that our system requires the transfection of 3 plasmids, we chose HEK293T cells because the high transfection efficiency of these cells. Owing the different temporal and spatial availability of cellular factors in T cells compared to a cancer cell line, it is important to validate the results of the splicing reporter acquired in HEK293T cells in T-cell lines and primary cells, which might provide great technical challenges due to their limited transfectability. Therefore, the use of alternative DNA delivery reagents for transfection such as DMRIE-C, which has been shown to be particularly effective for the transfection of suspension cells (*e.g.*, Jurkat), or nucleofection methods such as Amaxa nucleofector or Neon transfection system that have been proven to facilitate efficient and reliable delivery of single or multiple plasmids to difficult-to-transfect cells including primary CD4+ T cells. Alternatively, it would be interesting to test this reporter system in the context of full-length virus in a primary cell model of latency using the aforementioned transfection methods. In fact, the differences in the availability of host transcription, elongation and splicing factors in a rCD4+ T cell may affect the capacity of an LRA to reactivate the latent provirus. Finally, our model does not address whether the LRA can affect transcription and splicing of bystander cells, and whether it can induce replication competent virus. However, we would suspect that by measuring an increase in cell-associated HIV US and MS RNA, this would lead to the production of replication competent virus in the culture supernatant. This could be confirmed by conducting the gold standard quantitative virus outgrowth assay (QVOA)³³.

Due to the complex nature of latency and to ensure efficient virus reactivation, a multifaceted combinatorial strategy may be required³⁴. Potential treatment needs to stimulate multiple pathways including transcription and splicing, which has been previously shown to play essential roles in the establishment of latency^{16,35-37}. Our LTR-driven splicing reporter would allow a high throughput screening of drugs that can optimally target both steps, transcription and splicing, increasing the chance of finding molecules that can synergize efficiently, which would lead to a lower dose levels and thus toxicity of each drug.

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DISCLOSURES:

The authors have nothing to disclose.

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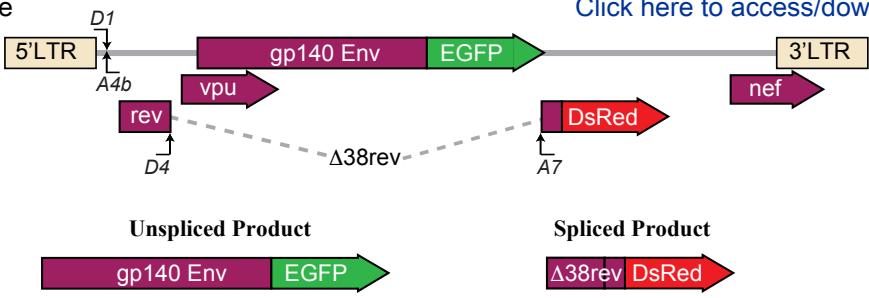


Figure 1

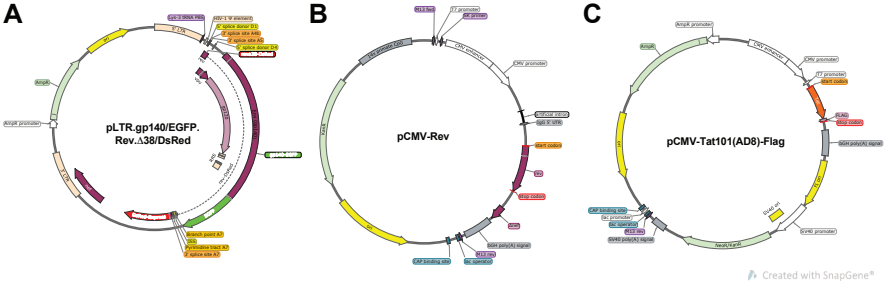
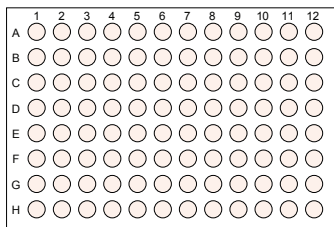


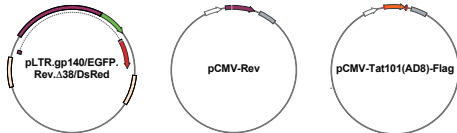
Figure 2

Day 1

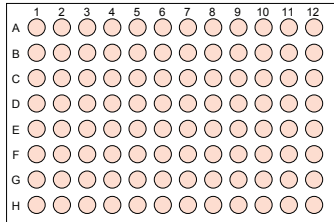


Seeding of HEK293T cells

Expression vectors

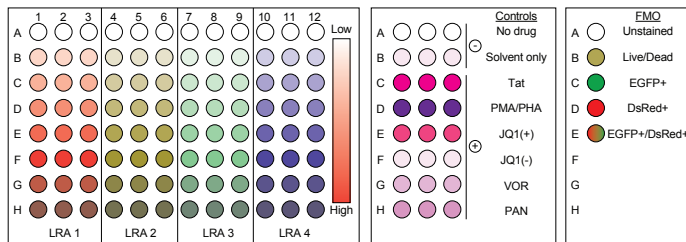


Day 2



Transfection

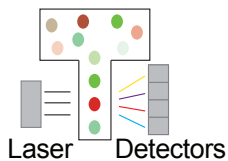
↓ 4-5 h at 37 °C



LRA treatment

↓ 48 h at 37 °C

Day 4



FACS analysis

Figure 3

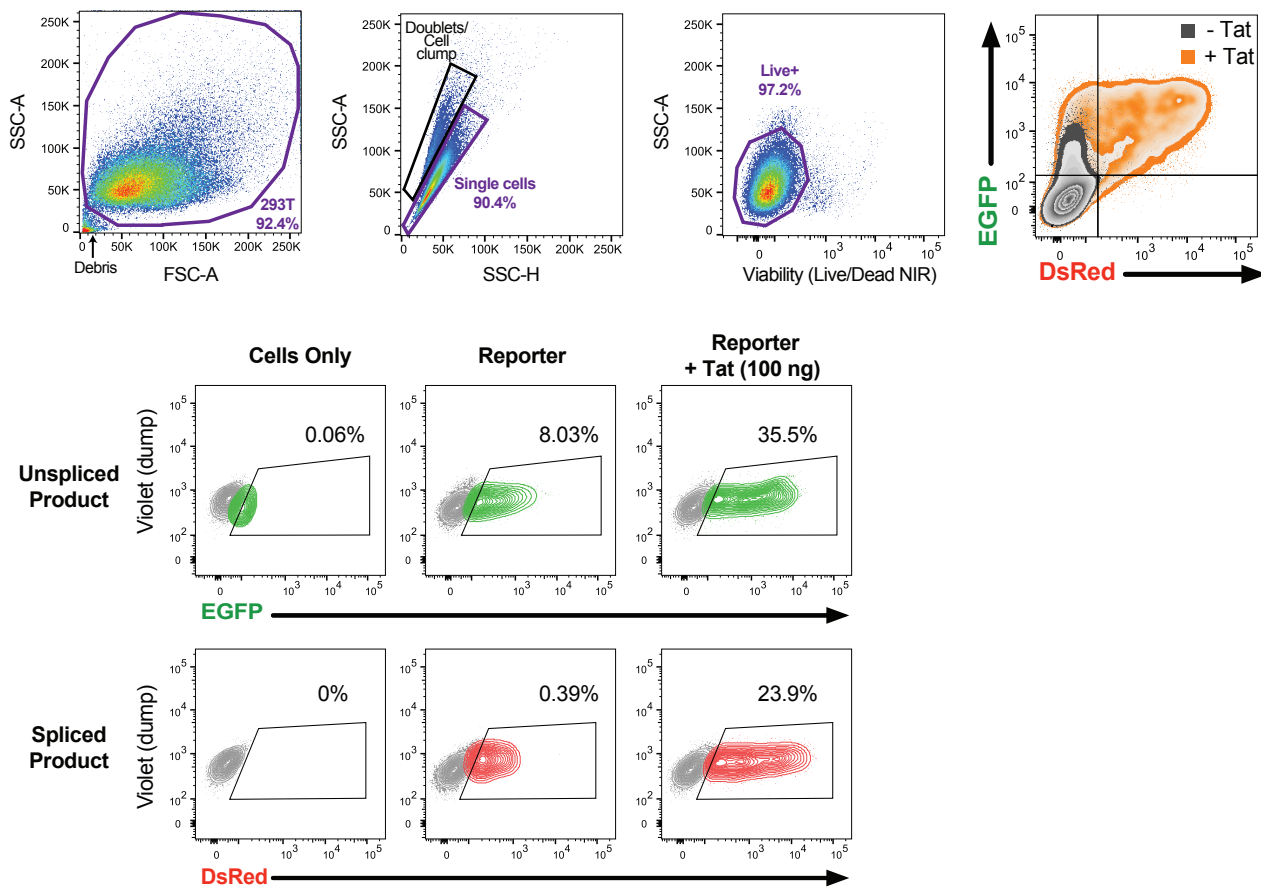


Figure 4

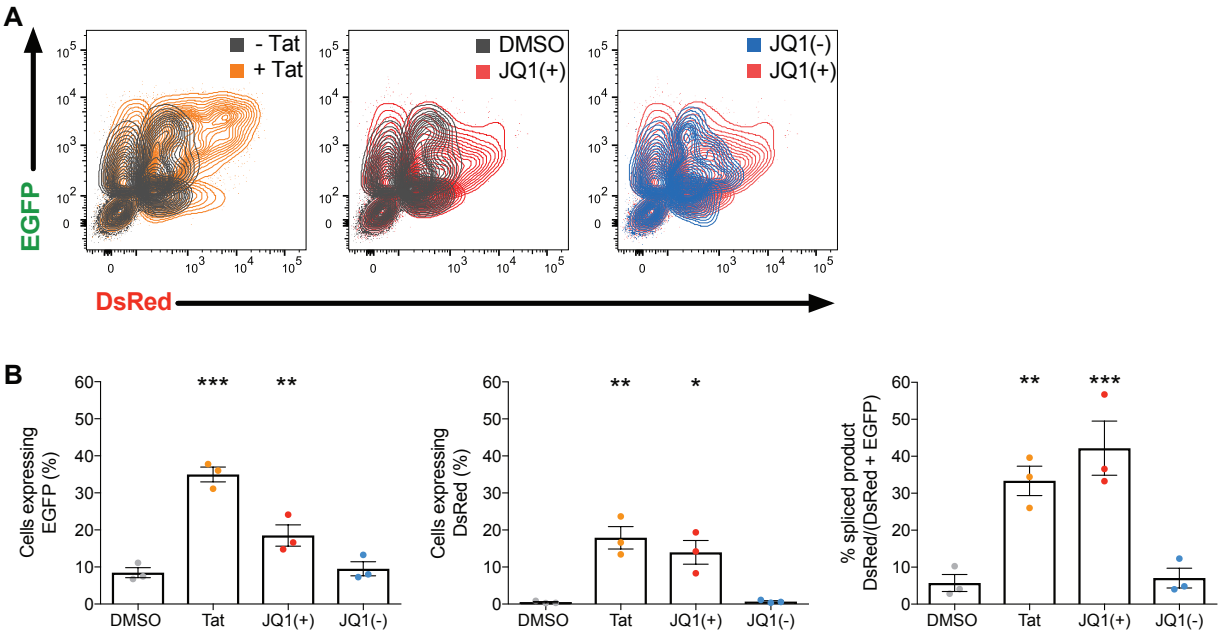


Figure 5

Controls				Tested Compounds								
	1	2	3	4	5	6	7	8	9	10	11	12
A	-	No drug		1000			1000			1000		
B		Solvent only		500			500			500		
C	+	Tat		250			250			250		
D		PMA/PHA		125			125			125		
E		JQ1(+)		62.5			62.5			62.5		
F		JQ1(-)		31.25			31.25			31.25		
G		VOR		15.625			15.625			15.625		
H		PAN		0			0			0		
				LRA1 (nM)			LRA1 (nM)			LRA1 (nM)		

Compensation Controls	
Unstained	
L/D stained	
EGFP+	
DsRed+	
EGFP+	DsRed+



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Cell culture			
HEK293T cells (Human Embryonic Kidney cells)	ATCC	CRL-3216	Replicates vectors carrying the SV40 region of replication.
Dulbecco's Modified Eagle's Medium (DMEM 1x + GlutaMAX-I)	Gibco	10569-010	+ 4.5 g/L D-Glucose + 110 mg/L Sodium Pyruvate
Fetal Bovine serum	Gibco	10099-141	Origin Australia
Penicillin-Streptomycin	Sigma	P4458	
Dulbecco's phosphate buffered saline (DPBS), no calcium, no magnesium	Gibco	14190-136	
Trypan blue Stain, 0.4%	Gibco	15250	
Trypsin-EDTA (0.05%), phenol red	Gibco	25300054	
Lipofectamine 2000	Invitrogen	11668-019	Lipid transfection reagent
Opti-MEM I (1x) reduced serum medium	Gibco	31985-070	Serum free medium
NucleoBond Xtra Maxi	Marcherey-Nagel	740414.50	
pEGFP-N1 plasmid	Clontech (TaKaRa)	6085-1	Expression of EGFP in mammalian cells, CMV _{IE} promoter.
pDsRed-Express-N1	Clontech (TaKaRa)	632429	Expression of DsRed-Express in mammalian cells, CMV _{IE} promoter.
pLTR.gp140/EGFP.RevD38/DsRed	Addgene	115775	
pCMV-Rev ^{NL4.3}	Addgene	115776	
pCMV-Tat101 ^{AD8} -Flag	Addgene	115777	
Dimethyl sulfoxide (DMSO)	Millipore	67-68-5	
JQ1(+)	Cayman Chemical	11187	Stock at 10 mM in DMSO; working concentration 1 µM
JQ1(-)	Cayman Chemical	11232	Stock at 10 mM in DMSO; working concentration 1 µM
Phorbol Myristate Acetate (PMA)	Sigma-Aldrich	16561-29-8	Stock at 100 µg/mL in DMSO; working concentration 10 nM
Phytohaemagglutinin (PHA)	Remel	HA15/R30852701	Stock at 1 µg/µL in PBS; working concentration 10 µg/mL
Vorinostat (VOR)	Cayman Chemical	10009929	Stock at 10 mM in DMSO; working concentration 0.5 µM
Panobinostat (PAN)	TRC	P180500	Stock at 10 mM in DMSO; working concentration 30 nM
CellTiter 96 AQueous One Solution Cell Proliferation Assay	Promega	63581	
Venor GeM Classic	Minerva Biolabs	11-1100	Mycoplasma Detection Kit, PCR-based
Flow cytometry reagents			
LSR Fortessa	BD Biosciences		Flow cytometer (4 lasers-blue, red, violet and yellow)
LSR II	BD Biosciences		Flow cytometer (3 lasers-blue, red and violet)
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Life Technologies	L34976	Viability dye: for 633 or 635 nm excitation, 400 assays. Component A and B are both provided in the kit.
Bovine Serum Albumin	Sigma	A2153	
EDTA 0.5M pH8	Gibco	15575-038	
Formaldehyde Solution 37/10 (37%)	Chem-Supply	FA010	
BD FACS Diva CS&T Research Beads	BD Biosciences	655050	Calibration beads
Sphero Rainbow Calibration Particles (8 peaks)	BD Biosciences	559123	3.0 - 3.4 mm
Sheath solution	Chem-Supply	SA046	90 g NaCl in 10 L water
HAZ-Tabs	Guest Medical	H8801	Chlorine release tablets for disinfection
Decon 90	Decon Laboratories	N/A	Concentrated cleaning agents of flow cytometer. Working solution Decon 90 5%.
Sodium Hypochlorite (12-13% Solution)	Labco	SODHYPO-5L	Concentrated cleaning agents of flow cytometer. Working solution bleach 1%.
7x	MPBio	IM76670	Concentrated cleaning agents of flow cytometer. Working solution 7x 1%.
Materials			
Tissue culture flasks (75 cm ² , canted neck, cap vented)	Corning	430641U	
Tissue culture plates (96 well flat bottom with lid)	Costar	3599	
Tissue culture plates (96 well V-bottom without lid)	Costar	3896	
Centrifuge tubes (10 mL)	SARSTEDT	62.9924.284	100x16 mm
Centrifuge tubes (50 mL)	CellStar	227261	30x115 mm
Microcentrifuge tubes (1.5 mL)	Corning Axygen	MCT-150-C	
Serological Pipette (25 mL), sterile	Corning	CLS4489-200EA	
Serological Pipette (10 mL), sterile	Corning	CLS4488-200EA	
Serological Pipette (5 mL), sterile	Corning	CLS4487-200EA	
Reagent reservoirs (50 mL), sterile	Corning	CLS4470-200EA	
5 mL Round-Bottom polystyrene test tube, with cell-strainer cap	Corning	352235	12 x 75 mm style, 70 mm
Nylon Mesh	SEFAR	03-100/32	100 mm
Titertube Micro test tubes, bulk	BIO-RAD	2239391	microfacs tubes
5 mL Round-Bottom polystyrene test tube, without cap	Corning	352008	12x75 mm style
Snap Caps for 12x75 mm Test Tubes	Corning	352032	
Counting chamber, Neubauer improved double net ruling, bright-line (Haemocytometer, LO-Laboroptik)	ProSciTech	SVZ4NIOU	x3 large squares of 1 mm ² ; Depth 0.100 mm; volume 0.1 mL; area minimum 0.0025 mm ²
Coverslips (Menzel-Gläser)	Grale Scientific	HCS2026	20 x 26 mm
Microscope	Nikon TMS	310528	
Centrifuge 5810R refrigerated	Eppendorf	5811000487	with rotor A-4-81 including adapters for 15/50 mL conical tubes
FLUOstar Omega microplate reader	BMG Labtech	N/A	Plate reader for cell proliferation assay. Filter 490 nm.
Softwares			
FACS Diva	BD Biosciences		Flow cytometer data acquisition and analysis program, version 8.0.1
FlowJo	FlowJo	FlowJo 10.4.2	Flow cytometer data analysis program, FlowJo Engine v3.05481
Omega	BMG Labtech		FLUOstar multi-user reader control, version 5.11
Omega - Data Analysis	BMG Labtech	MARS	FLUOstar data analysis, version 3.20R2
Microsoft Excel	Microsoft	Excel:mac 2011	version 14.0.0
Prism	GraphPad	Prism 7	version 7.0c



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CORRESPONDING AUTHOR:

Name: DAMIAN PURCELL

Department: Microbiology & Immunology, Peter Doherty Institute

Institution: University of Melbourne

Article Title: Highly throughput in vitro assessment of HIV latency reversing agents on viral RNA transfer to and splicing

Signature: [Signature] Date: 6 / 7 / 18

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Response to reviewers:**Reviewer #1:**

Major Concerns:

no

Minor Concerns:

For the protocol, a positive control is always important, for example use a known latency reverse agent as a positive control. To screen agents, assessing cytotoxic of an agent is equal important as assessing its activity. In the analysis step, to count and compare the total live cell number, can be a rough indicator of cytotoxicity.

We have now included in the discussion section the critical steps within the protocol including the cytotoxicity of an LRA and the cell viability measurement (see lanes 423-434, page 10).

Reviewer #2:

The manuscript is clear and sufficiently detailed, below some suggestions for improvement:

- the Env-GFP fusion not only detects transcription of unspliced HIV RNA, but also depends on translation of Env. This process requires export of unspliced RNA, which is a peculiar process that may impact viral reactivation as suggested recently (Yedavalli, Kula, Sarracino). Please clarify better in the text if the mutations introduced to promote folding affect the process and how this also contributes to the assay that would be capable of detecting transcription, splicing and export/translation of viral RNA.

Clarifications were included in the introduction – lines 80-83, page 2 as follows:

“Upon expression within a cell, Rev localizes to the nucleus where it mediates the nuclear-cytoplasmic export of the 4 kb env mRNA via interaction with the rev responsive element (RRE). The truncation of Env does not compromise the RRE, which lies between gp120 and gp41, and the A7 3' splice site.”

And in lines 87-89, pages 2-3 as follows:

“To facilitate the nuclear export of unspliced mRNA, a mammalian expression vector encoding Rev (pCMV-Rev^{NL4.3}) was co-transfected with the fluorescent reporter construct (Figure 2).”

- The method requires transfection of 293T cells with 3 plasmids, which is a source of variability. Please comment on how robust is the method (i.e. what is the variability of the output in multiple parallel experiments), include statistical analysis.

As shown in Figure 5 – panel B, to account for the variability in transfection efficiency each condition is ran in triplicate and the mean \pm SEM of the 3 independent experiments are represented. Our results showed consistent data across replicates and experiments: both Tat and JQ1(+) significantly increased the levels of cells expressing EGFP (4.1 and 2.2 FC over DMSO respectively) and DsRed (59.6 and 46.6 FC over DMSO respectively, 2-way ANOVA test), while no significant change was detected following JQ1(-) treatment (1.1 and 1.8 FC over DMSO respectively). See lines 312-320, page 8 - ‘Representative results’ section.

- The method requires several washes. This in my experience is a great source of variability particularly in 96 wells formats. See comment above. Is there way to reduce the number of passages?

See comment above.

- Indicate the commercial source of products (i.e. 293T cells, Lipofectamine etc).

Details have been added in the enclosed “Table of Materials”.

- Pag2line104 - for how long you keep the 293T in culture? In my experience it is better to change batch after a while for optimal performance.

We agree with the reviewer. For optimal performance, the HEK293T cells are kept in culture for only 10-12 passages.

- Pag2line109 - specify that routine testing for mycoplasma has to be performed. Not only new cell cultures.

The sentence was corrected; see lanes 108-110, page 3:

“CAUTION: Mycoplasma contamination of cell cultures remains a serious problem. Good laboratory practice and routine testing of cell cultures are essential to decrease the risk of mycoplasma contamination and avoid diffusion²⁰.”

- Pag2line122 - wouldn't be better to clarify the concentration of the stock and fix the volume? It is unclear what you mean by 'keep the volume small'.

We agree. The sentence was deleted.

- Pag3line135 - step 2.6 ??

We apologise for the confusion, we meant to the next step “step 1.8.” (now step 1.15., see line 155, page 4).

Reviewer #3:

Major Concerns:

1. The protocol is only of use if the dual reporter construct is readily available (eg NIH AIDS reagent program). Is this the case? A link to the reagent should be added to the manuscript.

The HIV splicing reporter (pLTR.gp140/EGFP.RevD38/DsRed), Rev and Tat expression plasmids (pCMV-RevNL4.3 and pCMV-Tat101AD8-Flag) were deposited to the non-profit plasmid repository "addgene" rendering these constructs accessible for other researchers around the world. The catalogue numbers were included in the Table of Materials.

2. The choice of cell line used for screening (i.e. HEK293T cells) should be discussed early on in the protocol. Which other alternative cell lines do the authors recommend (apart from primary cells which provide great technical challenges due to their limited transfectability)? For example, how would the protocol be adapted if Jurkat cells were used (eg. TransIT-Jurkat transfection reagent from Mirus Bio LLC)?

A clarification of the technical challenges and the alternative methods for transfecting suspension cells such as the use of DNA delivery reagents like DMRIE-C or nucleofection methods (Amaxa nucleofector and Neon transfection system) were included in the 'discussion' section (see lanes 443-454, page 11).

3. The protocol lacks a description of the various controls required for screening; i.e. compensation controls for FACS, LRA treatment controls with solvent only, no treatment controls, tat transfection control etc. Addition of a comprehensive table of required controls would be very helpful in planning experiments.

4. The protocol lacks details for different LRA stock and working concentrations (only JQ1 is mentioned). Addition of a table with a few common LRAs, which can be used as positive controls if new reagents are tested, is lacking. The recent publication by Khoury et al provides for example data on HDACis. In Figure 3, the authors show that LRAs should be tested in a dilution series; details on the recommended LRA dilutions should be added to the protocol.

We thank the reviewer for these excellent suggestions (3. and 4.). We have now included in "Table 1" a representative example of the plate set-up and the controls needed (LRAs and compensation controls) as well as the amount of LRAs to be used in these assays. Details of few common LRAs, which can be used as positive controls, such as HDACi (VOR, Pan) or bromodomain inhibitor (JQ1) have also been included in "Table 1" legend and "Table of materials" (stock and working concentrations, solvent diluent and catalogue numbers).

5. In the discussion, the authors focus on other dual fluorescent reporter systems. These systems however were designed with a different aim, ie. to mark latent cells, which lack LTR-driven transcription by introduction of a constitutive promoter-driven reporter within the HIV-coding sequence (Sadowski and Verdin reporters). A discussion about the advantages/disadvantages of the presented reporter is lacking, i.e. what is the relevance for an LRA if it induces transcription but not splicing?

A detailed description of the advantages (lanes 411-421, page 10) and disadvantages (lanes 436-461, page 10-11) of our HIV splicing reporter were included in the discussion section. Moreover, the advantages of targeting both transcription and splicing were presented in lanes 58-67, page 2 (introduction) and lanes 463-469, page 11 (discussion).

Minor Concerns:

1. The titles of the four sections should be more specific. i.e. '1 Transfection' -> '1 Transfection of HEK293T cells with dual color reporter construct'.

The titles were adapted as follows:

- 1- Transfection of HEK293T cells with dual color reporter construct*
- 2- Treatment of transfected HEK293T cells with latency reversing agents*
- 3- Staining of transfected cells with fixable viability dye for flow cytometry analysis*
- 4- EGFP and DsRed measurements by flow cytometry and data analysis*

2. The labeling of Figure 3 should be improved to provide a more comprehensive one-look overview of the protocol. eg. add names of expression vectors, add required control transfection reactions, specify the term 'seeding' etc

We thank the reviewer for the suggestion. We have included an improved version of “Figure 3” with all the elements requested.

3. The grammar of the text needs proofreading; eg.

- a. Line 29: immune system and IS not targeted
- b. Line 36: is based on the use OF an LTR-driven
- c. Line 59: as well as defectS in
- d. Line 65: utilizes A high-throughput
- e. Line 75: propensity of LRAs to
- f. Line 77: access -> assesses
- g. Line 267: at medium or low SPEED to
- h. Line 377: access -> assess

Corrected. In addition, a thorough proofreading of the text was completed.

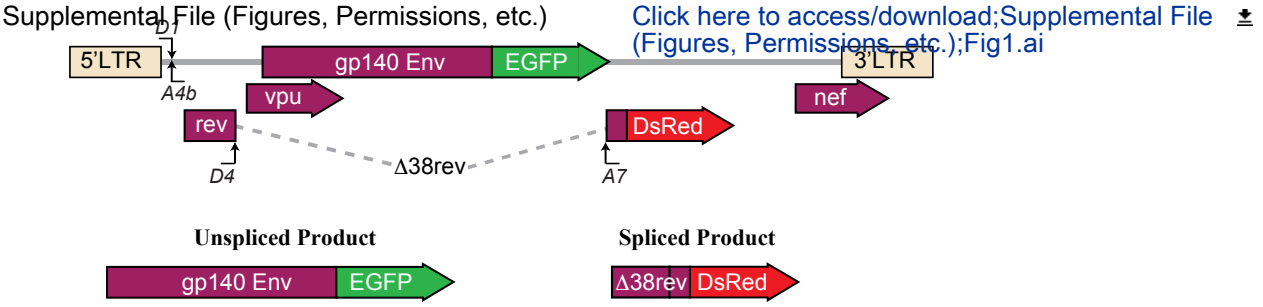


Figure 1

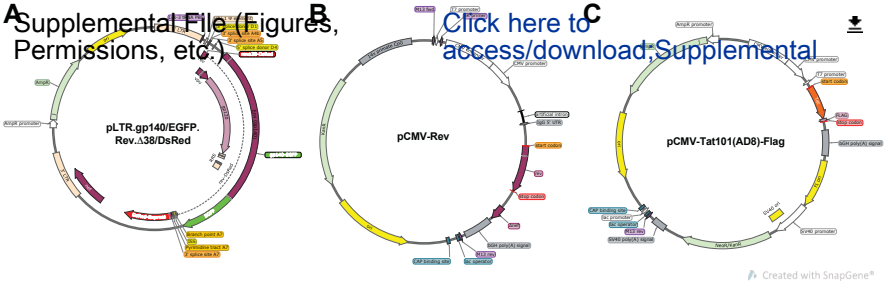
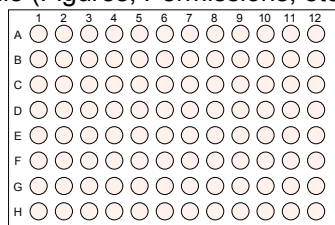


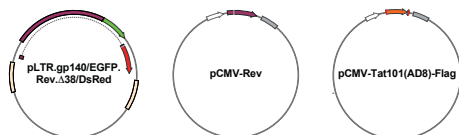
Figure 2

Day 1

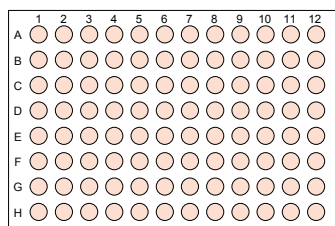


Seeding of HEK293T cells

Expression vectors

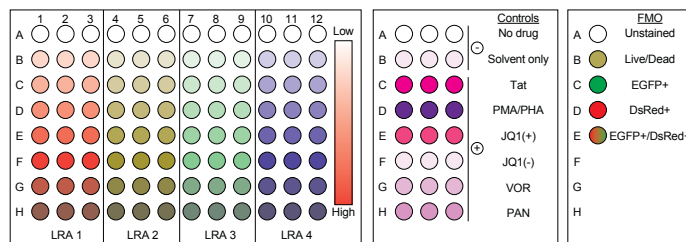


Day 2



Transfection

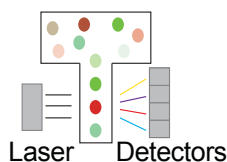
4-5 h at 37 °C



LRA treatment

48 h at 37 °C

Day 4



FACS analysis

Figure 3

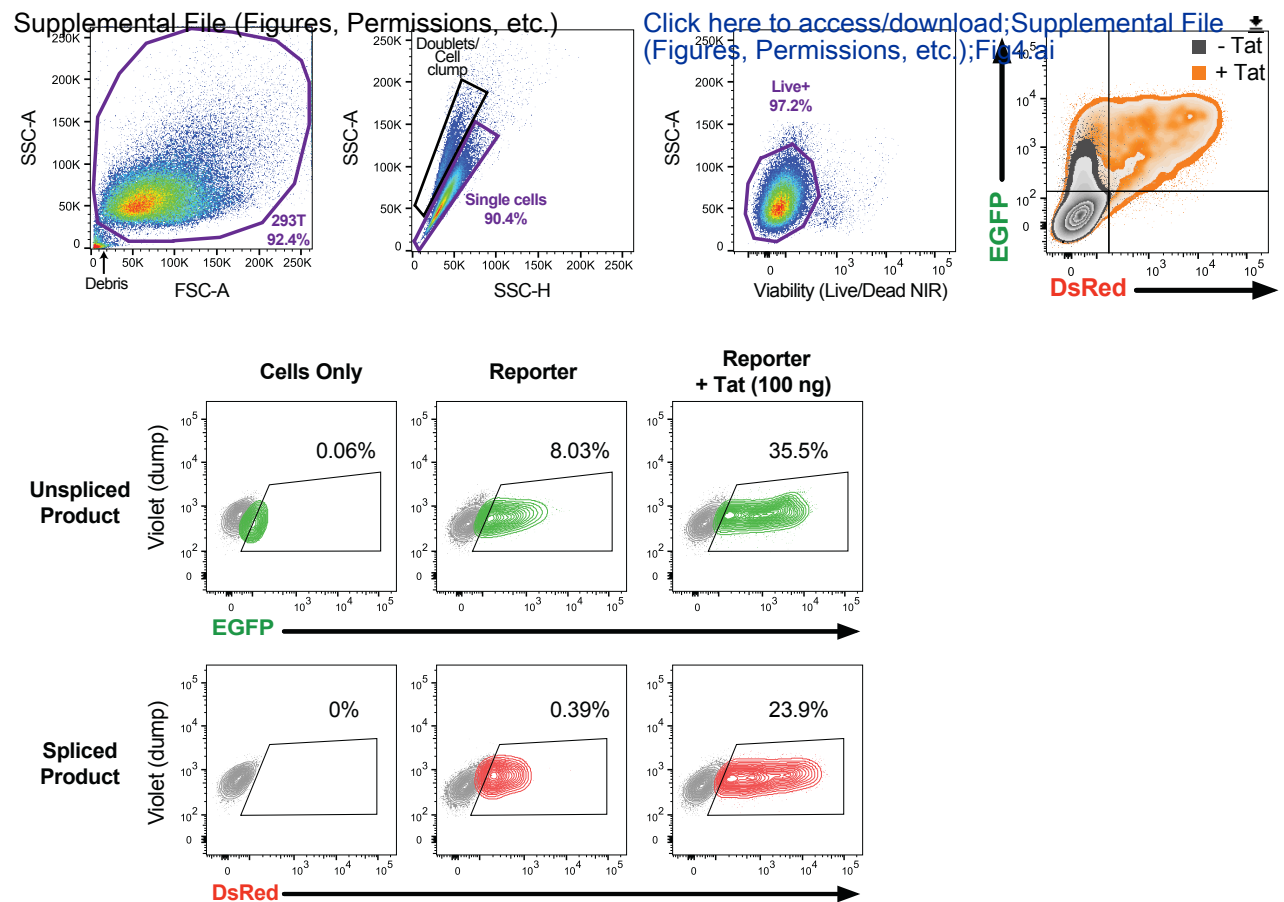


Figure 4

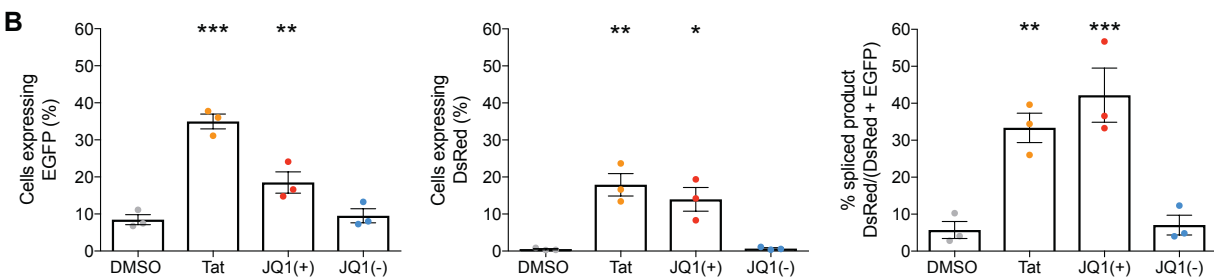
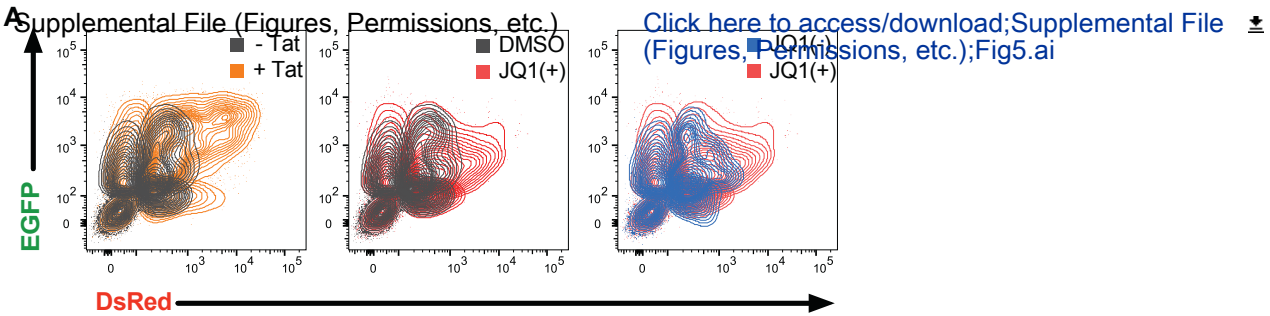


Figure 5