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 – lanes 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 lanes 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 ± 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 lanes 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 diffusion[20]."

- 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.