**List of changes based on Editorial comments:**

We have answered, point by point, all comments of editor and reviewers below:

1. We retrieved the most recent version of the manuscript from the ‘file inventory’.

2. Short Abstract does not exceed 50 words.  
  
3. We have included a step on how to prepare the mixture (step 1.2). We added an extra step (step 1.3) and a note regarding formation of polyplexes.  
  
4. In step 1.6 (now 1.7) we have clarified that the solution is added to the plated cells.   
  
5. We have simplified the protocol steps so that they are no more than 3-4 lines of text covering 2-3 actions. We have placed each "Note" on its own separate line. Highlighted text does not exceed 2.75 pages.

6. We have edited Figure 1 and Figure 5, which were substituted in this last resubmission.

**List of changes based on Reviewers comments:**

**Reviewer #1:**   
Minor Concerns:

1. Introduction: We have deleted the definition of fluorescence.  
  
2. In Figure 1, we have corrected the mislabeled axis.

3. Step 3.1: We have defined a "tight" population in terms of CV.   
  
4) In order to further clarify the substrate assay, we have added a schematic of the assay in Figure 5 (now Figure 5b), edited the legend of the figure and edited the description in the results, which should help interpret the results.   
  
**Reviewer #2:**   
Major Concerns:  
The major issue raises the lack of description regarding compensation and flow-cytometry technology. We feel this is not a ‘cytometry only’ based method. We agree with the reviewer that a reference or two needed to be added to guide the reader to the appropriate source for further reading on cytometry. We did add an explanation about compensation in the Discusssion (line 530).

Minor Concerns:  
1. We have edited all text to make it less ‘ idiosyncratic’.

2. We have included in the discussion the usage of bar-coding for other fluorescence-based assay technologies such as high-content imaging.

**Reviewer #3:**   
Major Concerns:  
The reviewer raises a major concern regarding the use of one fluorescent marker expressed utilizing retroviral technology. It feels like the reviewer does not believe that retroviral transduction can produce a reliable source of cells for this goal, a statement we politely disagree with. Our expertise with this technology proves otherwise. The reviewer also mentions that we should focus on optimizing the transduction protocol for simultaneous expression of multiple fluorescent reporters, rather than a single marker at different intensities. While we have mentioned the utility of different fluorescent markers, we have decided to focus and explain in this protocol the use of one marker at different intensities. We have mentioned different markers in step 1.2 and 1.4. We have also mentioned in the first paragraph of the results (line 393-403) that one can utilize different markers rather than one at different intensities. Figure 1 also shows two different markers. Following figures (2 through 4), although they do show one of the markers at two intensities, also include an additional marker as well. See also lines 505-509 in the discussion. Finally, the reviewer mentions that expansion of the protocol in primary cells would significantly improve the quality and usefulness of this work. We feel that this is beyond the scope of the manuscript and protocol. Moreover, retroviral technology and selection of clones in primary cells is cumbersome, expensive and biologically difficult if not impossible to achieve, as descendant of these primary cells will lose their ‘primary’ properties.

Specific Comments and Minor Concerns:

1. We have commented on the usage of primary cells (see above).

2. We have described the optimal confluency for transfection and described the health of the cells on the note below step 1.1.

3. Line 158: We have removed the portion about lentivirus production.   
  
4. Line 160 (now 164): Information about the packaging Phoenix-GP cell line was added, now in a new note.   
  
5. Line 163 (now 168): We have addressed the questions regarding addition of the transfection mixture and clarified the steps. We added a note regarding the replacement of fresh media.

6. Line 169 (now 187): The reviewer raises lack of clarity regarding number of vectors for transfection. We have clarified this point within the two notes following step 1.2 and 1.6. Please be aware that verbiage within the original step 1.3 was moved to the note below step 1.6, decreasing the number of steps. Information regarding packaging constraints was also added.

7. Line 173 (now 192): The reviewer mentions that prior to the filtration through a 0.45μM membrane, an initial centrifugation step should be performed to pellet any non-adherent cells and/or debris. While we have never done this, such a note can be added if requested.   
  
8. Line 180-181 (now 198): We have added a range for the number of cells used for transduction. We also added a note below step 1.5 describing the confluency for adherent cells.   
  
9. Line 184 (now 209): We have mentioned percentage infection rate rather than volume of viral supernatant and left the volume used as example. We also added a note below step 1.6 describing this. Please note that original step 1.6 was divided into two steps 1.6 and 1.7, to adhere to the two-three sentences rule per step.

10. Line 185 (now 208): We have clarified the addition step of polybrene.  
  
11. Lines 232-269 (287-327): The reviewer has doubts as of the ability of retroviral transduction to produce cell lines stably expressing a fluorescent marker at different intensities. We have a well-established experience in doing so and published in Cytometry part A how these cell lines are stable and distinguishable for at least a period of six months. We feel it is beyond the scope of this methods paper to describe the scientific reason behind the difference in expression level utilizing retroviral technology. The matter of fact is that the technology, followed by cell sorting, does allow to purify distinguishable populations. But we did add, to ease the reviewer’s point, a sentence in the discussion to clarify this point. We would like to remind that the last two sentences of the intro stated: ‘This protocol is thus not intended to show the well-established retroviral/lentiviral technology for genetic transfer or the value of fluorescent proteins but rather to show the enhancing power of combining both for multiplexed applications’.  
  
12. Lines 441-443 (now 459-462): Same point as the one above.