Cellomics Technology
innovative tools for genetic engineering

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The general protocol for determining the integration events and gene expression using Real-time quantitative PCR

For detection of the WPRE sequence using real-time PCR using Taqman probe: forward primer (1277F), 5'-CCGTTGTCAGGCAACGTG-3'; reverse primer (1361R), 5'-AGCTGACAGGTGGTGGCAAT-3'; probe (1314P), 5'-FAM-TGCTGACGCAACCCCCACTGGT-TAMRA-3'. You do not need the probe if you do not perform real-time PCR with Taqman probe.

For detection of human albumin (please using primers for different species if the target cells from other species), the sequences of the primers and probe used were as follows: forward primer, 5'-TGAAACATACGTTCCCAAAGAGTTT-3'; reverse primer, 5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3'; probe (1314P), -FAM-TGCTGAAACATTCACCTTCCATGCAGATAMRA-3'. As mentioned before, you do not need the probe.

For detection of human *β*-actin (please using primers for different species if the target cells from other species), the sequences of the primers and probe used were as follows: forward primer, 5'-GCGAGAAGATGACCCAGATC-3'; reverse primer, 5'-CCAGTGGTACGGCCAGAGG-3'; probe, 5'-FAM-CCAGCCATGTACGTTGCTATCCAGGC-TAMRA-3', as mentioned before you may not need the probe.

For the PCR, 12.5 *μ*l of universal PCR master mix (Applied Biosystems), an 800 n*M* concentration of each primer, and 200 n*M* probe were combined and adjusted to a total volume of 20 *μ*l with RNase-free water. Finally, either cDNA or genomic DNA was added to each reaction and the total reaction volume was adjusted to 25 *μ*l. Standard conditions were used for the PCR (2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 sec at 95°C and 1 min at 60°C). For each PCR, a no-template reaction should be included as negative control. Each DNA or cDNA sample should be tested in duplicate, and the mean values should be calculated. Duplicate values varied by no more than 15% from the mean. Ten-fold serial dilutions of plasmid constructs of known concentration and containing the relevant sequences (WPRE, albumin, or *β* -actin) should be prepared so as to create standard curves for quantification of unknown samples. All dilutions should be made in the presence of *Escherichia coli* 16S ribosomal RNA (20 ng/ml) in order to increase the stability of the plasmid dilutions.

For assessment of proviral DNA copies, or lentiviral integration events, genomic DNA should be isolated from target cells transduced with 4-fold serial dilutions of concentrated lentiviral vector, using an Easy-DNA kit (Invitrogen). DNA from approximately $5x10^5$ transduced target cells should be resuspended in 100 *μ*l of distilled H_2O and treated with RNase (40 *μ*g/ml) for 30 min at 37°C. Five microliters, or 5% of the total DNA isolated, was used as the template for each qPCR. Lentiviral integration events should be calculated by normalizing the number of WPRE molecules measured by qPCR to the number of target cells, as quantified by the copy number of albumin molecules detected by qPCR on the same genomic DNA sample. DNA titers should be subsequently calculated by taking the means of titer calculations based on qPCR measurements of WPRE copy number obtained from target cells transduced with four different dilutions of viral supernatant.

For assessment of lentiviral gene expression at the mRNA level, total RNA should be isolated from approximately $5x10^5$ transduced target cells, using RNeasy columns. A DNase step should be included in the procedure to reduce the chance of vector plasmid or genomic DNA contamination. Purified RNA should be resuspended in RNase-free water and 1 *μ*g of total RNA should be subjected to a single round of RT to generate cDNA as described above. Expression levels of WPRE message detected by qPCR should be normalized, using *β*-actin mRNA as a control.