Inhibition of the histone demethylase LSD1 blocks α-herpesvirus lytic replication and reactivation from latency

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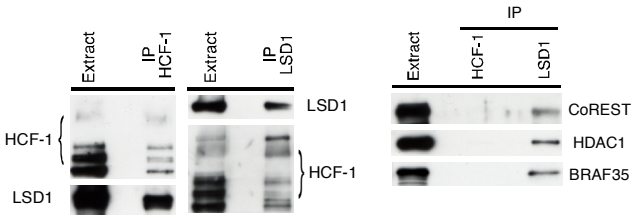
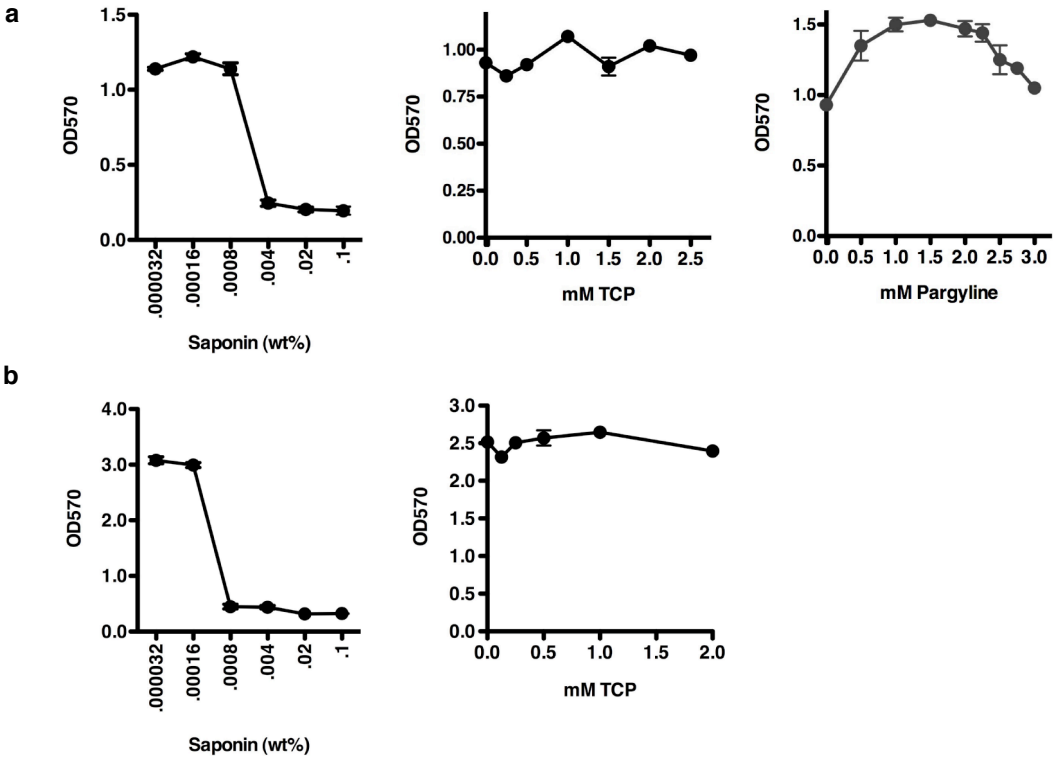


Supplementary Figure 1 The HCF/LSD1 complex is distinct from the CoREST/LSD complex. Western blot of epitope tagged HCF-1 or LSD1 immunoprecipitates probed for HCF-1, LSD1, and components of the repressive LSD1 CoREST complex (CoREST, HDAC1, and BRAF35).



Supplementary Figure 2 MAOI MTT cytotoxicity analyses. HeLa cells (2 x 10^4 per sample) were treated with the indicated concentrations of Saponin (cytotoxic control), TCP, or Pargyline for 6 hrs (a) or 24 hrs (b). Cell viability was measured using MTT assays (Bioassay Systems, #CQMT-500). MTT (3-(4,5-Diemetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is cleaved to form a purple precipitate by active mitochondrial reductase enzymes, thereby measuring cell proliferation, viability, and metabolic activity[1,2]. Assays were performed according to the manufacturer's recommendations using a SpectraMax Plus plate reader.

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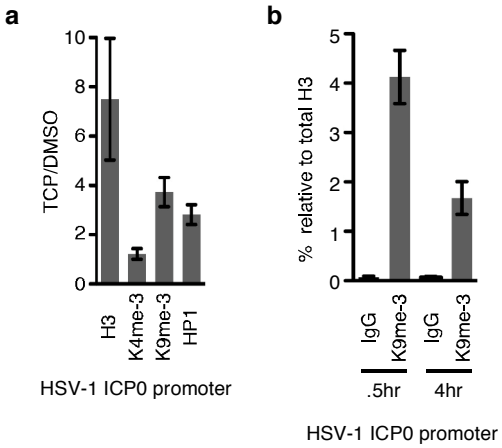
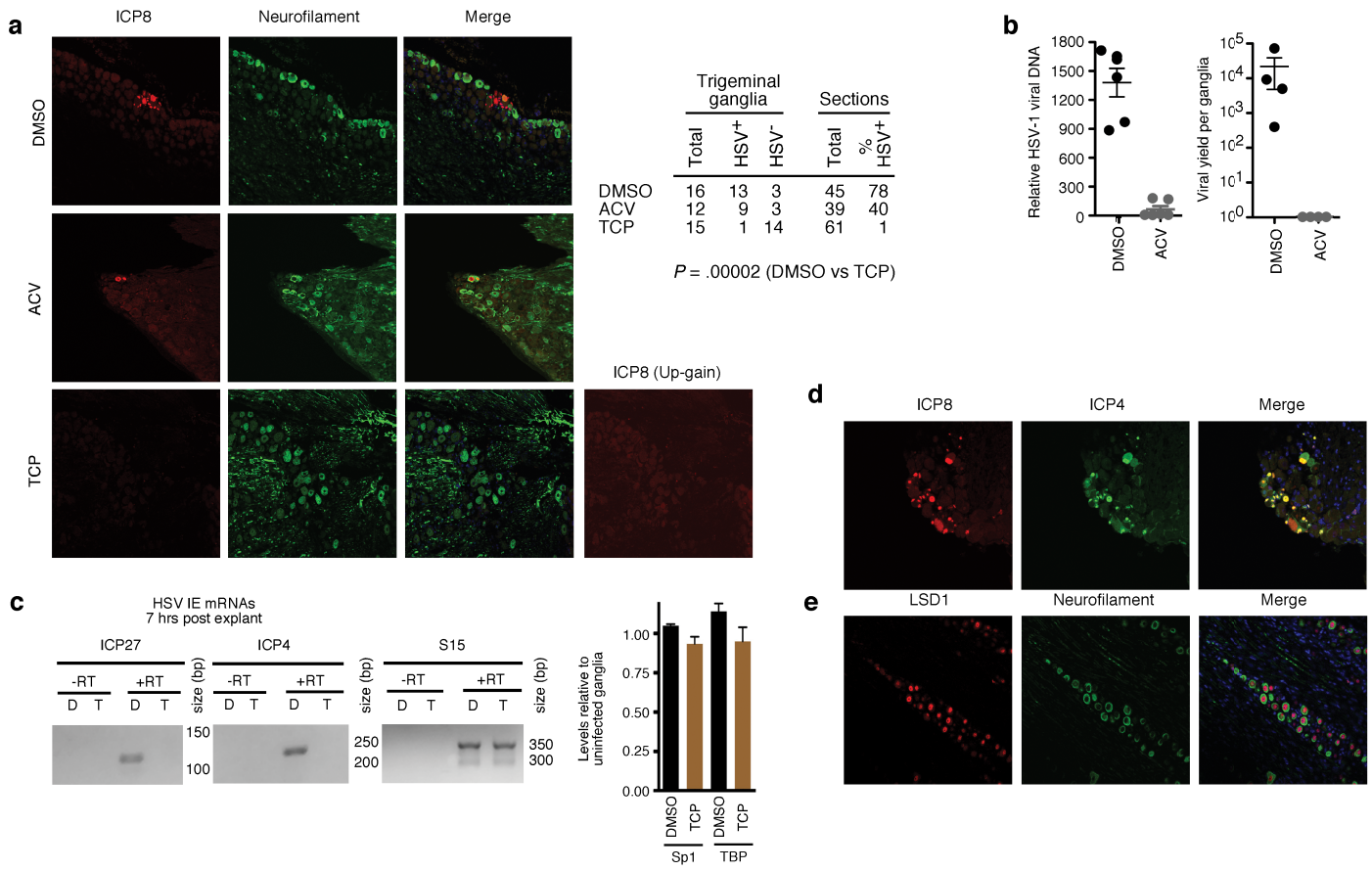
[2]Supino, R. MTT assays. *Methods Mol Biol* **43**, 137-149 (1995).



Supplementary Figure 3 Inhibition of LSD1 results in increased H3K9 trimethylation and HP1 occupancy on the ICP0 HSV IE promoter. (**a**) ChIP assay showing H3K9 trimethylation and HP1γ occupancy on the HSV IE0 promoter in the presence of 2 mM TCP. The results are represented as ratios of occupancy in TCP treated cells to that in DMSO treated cells and are normalized to the ratio of H3 levels at the cellular GAPDH promoter. (**b**) ChIP assay showing H3K9 trimethylation on the HSV ICP0 promoter at various times post infection. Signals are represented as the percent of input relative to total histone H3.



Supplementary Figure 4 TCP inhibits the initiation of HSV reactivation in neurons of explanted trigeminal ganglia. (**a**) A second representative example (refer to **Fig. 4e**) of immunofluorescent staining of HSV latently infected trigeminal ganglia explanted for 48 hrs in the presence of control DMSO, ACV (100 uM), or TCP (2 mM). ICP8, HSV single stranded DNA binding protein. The results of immunofluroescent staining is summarized in the table (right). (**b**) qPCR of viral DNA load (left) and viral yield (right) from paired explanted ganglia of HSV-1 latently infected mice in the absence (DMSO) or presence of ACV (100 uM) for 48 hrs. Paired DNA samples were normalized to cellular GAPDH and quantities are relative to DNA isolated from HSV-1 infected HeLa cells. The data is a control for the efficiency of ACV inhibition of HSV-1 DNA replication and lytic spread in the experiments presented in **Fig 4**. (**c**) HSV IE mRNAs (ICP4 and ICP27) are readily detected in ganglia explanted for 7 hrs in the presence of control DMSO (D) but not TCP (T) as determined by nested PCR analysis. S15 is used as an internal control to normalize DMSO and TCP samples. The levels of Sp1 and TBP controls are shown relative to levels in untreated mock-infected ganglia. -RT, +RT denote the absence or presence of the reverse transcriptase in the cDNA synthesis reaction. (**d**) Representative examples of trigeminal ganglia explanted in the presence of DMSO for 48 hrs and co-stained for HSV ICP8 (viral DNA replication component) and ICP4 (viral immediate early protein 4). Colocalization of the two viral antigens confirms the specificity of viral antigen staining. (**e**) LSD1 is expressed in neurons of sensory ganglia as demonstrated by immuno-fluorescent staining of Mock-infected and HSV-latently infected ganglia with anti-LSD1 and anti-neurofilament N200 antibodies.

Supplementary Methods

Cell culture and viral infections. HeLa, BS-C-1, HEK293, NIH3T3, Vero, MeWo and VZV (Ellen) stocks were obtained from American Type Culture Collection. MCF7-pTR-7/pTER/LSD1 cells inducibly expressing LSD1 RNAi were a kind gift of X. Chen (University of California at Davis)[1]. HSV (strain 17) infections were done by exposing uninfected cells to HSV infected cell lysates in DMEM medium containing 1% fetal bovine serum. At 1 hr post infection, the medium was replaced with DMEM containing 10% serum and incubation was continued. VZV infections were done by directly overlaying VZV infected BS-C-1 cells onto naive cells. For treatment with monoamine oxidase inhibitors, cells were pretreated with the indicated concentration of drug for 4-5 hrs prior to infection and maintained throughout infection. Pargyline (P8013), Tranylcypromine (P8511), and Acyclovir (A4669) were obtained from Sigma.

Reporter assays. For VZV model reporter assays, 2-4 x 10^4 cells were transfected with 1 ug control RNAi, GFP RNAi (Origene TR30003), control vector, HCF-1[2], LSD-1, or LSD-2 shRNA constructs (Origene TI365146 and TI365147, respectively) using Fugene 6 according to the manufacturer's recommendations. 48 hours post transfection, the cells were cotransfected with the VZV reporter construct, RL-CMV internal control, and increasing amounts of pCMV-IE62. 24 hours later, luciferase activity was measured as described below. For the HSV ICP0 reporter, cells were cotransfected with shRNAs, reporter and pRL-CMV internal control. 72 hours post transfection, the cells were infected with 1 pfu HSV-1 for 3 hrs. For assessment of the impact of exogenously expressed LSD1 on the ICP0 promoter-reporter activity, cells were cotransfected with

ICP0 reporter and equivalent amounts of plasmids expressing control LacZ, wild-type LSD1, or LSD1 (K661A) catalytic mutant[3,4]. 24 hrs post transfection, cells were infected as described above. Luciferase reporter activity was measured and analyzed as described[2] using the Dual-Luciferase Assay Kit (Promega) in a Berthold luminometer. All activity units were normalized by protein concentration and the activity of the internal control vector.

Chromatin Immunoprecipitations and qPCR. Chromatin immunoprecipitations from extracts of control, HCF-1 depleted, and LSD1 depleted cells were done essentially as described[5]. HCF-1 depletions utilized either HCF-1 shRNA[2] or siRNA (Qiagen #SI04332755) as indicated in the appropriate figure legend. Samples were analyzed, in triplicate, by qPCR using ABI Sybr Green PCR Master Mix on a ABI PRISM 7900HT with the following conditions: 95 ºC for 10 min followed by 40 cycles of 95 ºC-15 sec; 57 ºC-15 sec; 72 ºC-30 sec. Results were analyzed using ABI SDS 2.3 software. For each experiment, the ChIP data shown is representative of at least two independent experiments. For depletions and MAOI treatments, the results were normalized to the ratio of the levels of total histone H3 at the cellular GAPDH or actin promoters. In some VZV infection experiments, HCF-1 was only partially depleted (52%) to prevent impacts on cell cycle progression[6] and changes in the levels of the viral IE62 activator[2]. Primer sets are listed below.

qRT-PCR. Oligo dT primed cDNA was produced from total RNA using RNAqueous-4PCR and RETROscript (Ambion) according to the manufacturer's recommendations.

cDNA was quantitated by qPCR. Primer sets are listed below. For the detection of viral IE and cellular mRNAs post explant, 8 ganglia from HSV latently infected or mock infected Balb/c mice were explanted in the presence of DMSO, 100 uM Acyclovir or 2 mM TCP for the indicated times. cDNAs were prepared from total RNA and amplified by qPCR (cellular Sp1, TBP controls) or nested PCR (viral IE) using the primer sets listed below. cDNA samples were normalized to one another according to the level of the cellular control Sp1 mRNA. To insure linearity and sensitivity of nested PCR reactions, samples were amplified in parallel with dilutions of cDNA prepared from 3T3 cells infected with HSV at $6.4x10^{-5}$ pfu per cell for 4 hrs. PCR products were resolved in agarose gels and quantitated using a Kodak 4000MM Image Station relative.

Antibodies. Antibodies used in this study: IE62[5]; HCF-1[7]; Normal rabbit IgG, H3K4-trimethyl, H3K9-monomethyl, H3K9-dimethyl, HP1γ, CoREST (Millipore/Upstate Biotechnologies: 12-370, 05-745, 07-450, 05-690, 07-455, respectively); FLAG-M2, V5 (Sigma-Aldrich); HA (Roche), Tubulin, TBP, Sp1 (Santa Cruz Biotechnologies: SC-1904, SC-273, SC-59); MLL1, Set1, RbBP5, BRAF35 (Bethyl Laboratories: BL1408/1289, BL1193, BL766, A301-097A-1, respectively); Histone H3, LSD1, H3K9-trimethyl, HDAC1, H3K9-monomethyl, H3K9-dimethyl (Abcam: ab1791, ab17721-ChIP/ab37165-Western, ab8898, ab7028, ab9045, ab1220, respectively); ICP0, ICP27, ICP4 (Goodwin Institute: GICR1112, GICR1113, GICR1101); ICP8 (gift of William Ruyechan, University at Buffalo, SUNY); and Neurofilament 200 (Sigma N0142).

Coimmunoprecipitations. 2.5 X 10^6 HEK293 cells were transfected with 9.6 ug pHA-LSD or pFLAG-LSD and 14.4 ug pHCF-FLAG or pHCF-V5 expression plasmids using Lipofectamine 2000 (Invitrogen). 48 hours later, nuclear extracts were incubated with FLAG-M2 agarose beads (Sigma) at 4 ºC for 1 hr in NP-40 buffer [50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40, 5% glycerol, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, Complete protease inhibitor]. For endogenous coimmunoprecipitations, antibodies were prebound to Protein G Dynabeads and incubated with HeLa cell nuclear extracts in NP-40 buffer overnight. Immunoprecipitates were washed 5 times with binding buffer, eluted in SDS sample buffer, and resolved in 4-20% Tris-Glycine gels. Western Blots of resolved extracts and immunoprecipitates were developed using Pierce SuperSignal West Dura.

LSD1 depletion-addbacks. For depletion-addback experiments, HeLa cells were transfected with control (Qiagen #1027280) or LSD1-specific siRNAs (Qiagen Hs_AOF2_5/#SI02780932 and Hs_AOF2_6/#SI0281177) using Hiperfect (Qiagen) according to the manufacturer's recommendations. 48 hrs post transfection, cells were transfected with pUC or plasmids expressing control (GFP), siRNA-resistant FLAG tagged LSD1 proteins [wild-type LSD1, LSD1 (K661A) catalytic mutant, or LSD1 lacking the amine oxidase domain (Δ aa 420-852)] using Fugene HD (Roche). 24 hrs later, cells were infected with 0.1 pfu/cell HSV-1 for 2 hrs. Equivalent amounts of infected cell lysates were subjected to quantitative western blot analyses using antibodies to ICP4, LSD1, FLAG, and cellular control TBP. Signals were quantitated using a Kodak 4000MM Image Station and Kodak MI software. siRNA-resistant LSD1

expression constructs were produced by introduction of 5 nucleotide substitutions in the appropriate siRNA target region using the Quik Change Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations.

Statistical analyses of TCP inhibition of HSV reactivation.

Non-paired ganglia timecourse statistic analysis:

Comparisons were made between control (DMSO) and TCP treated ganglia on day 2 and day 4 post explant. Each comparison utilized a one-sided Mann-Whitney U test followed by application of the Dunn's post hoc adjustment to the p-values. Significant differences were found between DMSO and TCP on day 2 ($p = 0.0043$) and on day 4 ($p = 0.0011$). Both of these differences are still significant after applying the Dunn's adjustment as both are less than the alpha level ($\alpha = 0.025$). The Mann-Whitney tests used an exact p-value for small sample sizes. n=6 for each sample set.

Paired ganglia statistical analysis:

Each data point was the result of a single ganglia divided and treated in the presence and absence of TCP. Therefore, a Wilcoxon signed rank test was used to assess differences between each treated and untreated sample. The significant difference was $p = 0.0002$. This test used an exact p-value for small sample sizes with an α level of 0.05. n=16 for each sample set.

TCP titration statistical analysis:

To determine at what concentration TCP would produce a significant reduction in viral load, a Kruskal-Wallis test with Dunn's post hoc comparison was used. The K-W test was appropriate due to the comparison of five samples of data that are not normally

distributed. Significant differences were found between the five groups ($p = 0.0007$, K-W = 19.39). To determine which groups are significantly different, a Dunn's post hoc comparison was done. Assessment of each concentration of TCP as compared to the control (DMSO) demonstrated that 1.0, 1.5, and 2.0 mM data sets showed significant differences ($p < 0.05$) while 0.5 mM did not. Comparing each concentration of TCP against the next higher concentration demonstrated that 1.0 mM was significantly better at repressing viral yields than 0.5 mM. No significant difference was found between 1.0 and 1.5 or 1.5 and 2.0 mM data sets. *n*=5 for control, 0.5, 1.0, and 2.0 mM; *n*=6 for 1.0 mM.

TCP reversal statistical analysis:

Viral yields from ganglia treated without TCP for 2 days, treated with TCP for 2 days, or treated with TCP for 2 days followed by incubation in the absence of drug for 3 days were compared using the Kruskal-Wallis test followed by Dunn's post hoc comparisons. The K-W test was appropriate due to the comparison of three samples of data that are not normally distributed. Significant differences were found between the groups ($p = 0.0007$, K-W = 10.3). A significant difference was demonstrated between the TCP and TCP-R data sets using Dunn's post hoc comparison ($\alpha = 0.025$). *n*=4 for TCP; *n*=6 for TCP-R.

IF statistical analysis:

HSV-1 latently infected trigeminal ganglia (16 DMSO/Control, 15 ACV, and 15 TCP) and control Mock-infected (4 Mock) were fixed at 48 hrs post explant. Additionally, control latently infected ganglia (4 ganglia, Infected-time 0) were removed and immediately fixed without incubation. Generally 3-5 DMSO or control ganglia and 5 ACV or TCP sections, distributed throughout each ganglia, were stained and scored by

confocal microscopy. Ganglia were scored as HSV^+ when any section exhibited ICP8 neuron-specific staining. For DMSO treated ganglia, 74% of the sections scored positive while for ACV, 40% of the sections scored positive. Of the 15 TCP treated ganglia, only one section of one ganglia exhibited a single $ICP8^+$ neuron (1% of total sections). No section of Mock-infected ganglia or control Infected-time 0 ganglia exhibited any $ICP8^+$ neurons. A Fischer's Exact Test demonstrated that there was a significant difference between DMSO, ACV, and TCP treated ganglia ($p = .00002$). The Odds Ratio indicated that the odds of viral reactivation were:

1.4444 times higher for the DMSO than the ACV treated samples

60.6667 times higher for the DMSO than the TCP treated samples

42.0000 times higher for the ACV than the TCP treated samples

Primers. The sequence of primer sets used in these studies (ChIP PCR/qPCR, cDNA qRT-PCR, and cDNA nested RT-PCR) are listed. For the HSV ICP0 promoter, primer set 1 (promoter-distal) was used to determine occupancy of HCF-1, Set1, LSD1, histone H3, and H3K9-trimethyl whereas primer set 15 (promoter proximal) was used to determine occupancy of H3K4-trimethyl. F, forward; R, reverse.

ChIP primers

VZV IE model promoter

5'R ACTAGCAAAATAGGCTGTCCCCAG
3'R CCTTTCTTTATGTTTTTGGCGTC

VZV IE62 promoter (genomic)

5‘P GAAATAGACACCGGGCGTACATC
3’P GAATTTAGACGTACCCGAGTTTTCC

VZV IE62 coding (genomic)

5‘C GTTGCAGACGATCATGTGGTTTC
3’C GTCGCGAGGGTGCTCTCG

HSV ICP0 promoter - distal

P1-5' CGCGGGTCGCTCAATGAAC
P1-3' GCCCGGCCCCCGATT

HSV ICP0 promoter - proximal

P15-5' CCCTGGCCCGACAGTCTG
P15-3' CAGGCCGGCGGGTACTC

GAPDH-pr

GPr5' CGGACTGCAGCCCTCCC
Gpr3' CCTTCCCAGTTTCCGACTGTCC

Actin-pr

APr-F TGGCTCAGCTTTTTGGATTC
APr-R GGGAGGATTGGAGAAGCAGT

RT-PCR primers

ICP0 F-CCCACTATCAGGTACACCAGCTT
R-CTGCGCTGCGACACCTT

ICP27 F-GCATCCTTCGTGTTTGTCATTCTG
R-GCATCTTCTCTCCGACCCCG

ICP4 F-TGCTGCTGCTGTCCACGC
R-CGGTGTTGACCACGATGAGCC

IE62 F-TGGACGAGGCGGCACATAG
R-AGGGCGTGGCGGCAAAACAC

s15 F-TTCCGCAAGTTCACCTACC
R-CGGGCCGGCCATGCTTTACG

TBP F-TGACCCCCATCACTCCTGC
R-CGTGGTTCGTGGCTCTCTTATC

Actin F-TGGCTCAGCTTTTTGGATTC
R-GGGAGGATTGGAGAAGCAGT

Sp1 F-TCAGAACCCACAAGCCCAAAC
R-TGCCAGCAGGAATGGAAGC

Nested RT-PCR primer sets

ICP4 Primary set
GCGAGCAGCCCCAGAAACTC
ACGACGATAACCCCCACCC

ICP4 Secondary/Nested
ICP4GGACAGCAGCAGCACGCC
ATCCCCGACCCCGAGGACG

ICP27 Primary
CCCCAGGACCCCATTATCG
TTCTCTCCGACCCCGACACCAAGG

ICP27 Secondary/Nested
GCTGGATAACCTCGCCACG
CAGAATGACAAACACGAAGGATGC

Primers for detection of viral and cellular DNA

GAPDH CGGACTGCAGCCCTCCC
CCTTCCCAGTTTCCGACTGTCC

HSV gE GGGAGCACCACATAACCGACC
GGCAAAGTCAACACAACAACGC

Supplementary References

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