Supplementary Information

Supplementary Figure 1. Selection of gene targets for monitoring the operational status of the Hh pathway in gliomas. (**A**) *PTCH1* and *GLI1* mRNA levels were measured by quantitative real time PCR in epilepsy specimens and expressed relative to levels of *GAPDH* mRNA. *PTCH1* mRNA was detected in all epilepsy specimens (11/11) and relative expression levels were similar. *GLI1* mRNA was not detected in all of the epilepsy specimens (6/9) and the relative expression levels were more variable. (**B**) NIH 3T3 cells were plated in multi-well plates, induced with Shh-conditioned medium and assayed at various time points for *PTCH1* and *GLI1* mRNA induction. Compared to basal levels, *GLI1* expression was induced to higher levels than *PTCH1* expression at all time points.

Supplementary Figure 2. Dual HPLC system and characterization of LC-MS/MS assay accuracy and precision. (**A**) Dual HPLC system setup. Components were separated by in-line SAX and reverse phase ion pair HPLC. The following steps were taken to improve the selectivity of the assay and minimize high background signals: SCX (Hamilton PEEK PRP-X200, PSDVB, 8x2 mm) and C18 (Phenomenex Security Guard, 4x2.0 mm) cartridges were placed in-line between the injection loop and the SAX (PEEK PRP-X110, PSDVB, 8x3 mm) cartridge to remove cationic and hydrophobic components in the sample; SCX and SAX cartridges were placed in line between pump 2 and the analytical column (Phenomenex Synergi, 4.6 x 150 mm, 4 μm) to remove ionic contaminants in mobile phases 2A and 2B. The additional clean-up cartridges were regenerated between each sample injection by washing with mobile phases 1C and 2C (see below). Mobile phases 1A and 2A were 10 mM TBAA in water/acetonitrile (98.5:1.5, pH 5.1), 1B and 2C were 10 mM TBAA, 0.5 M ammonium acetate in

water/acetonitrile (95:5, pH 5), 2B was 10 mM TBAA in water/acetonitrile (3:7, pH 5) and 1C was 10 mM TBAA in water/acetonitrile (2:8, pH 5). Pump 1 flow was 1000 µl/min, and pump 2 flow was 800 µl/min. The following gradient conditions were used for a 100 µl sample injection- Pump 1: 0-2 min 100% A; 2-6 min linear gradient 100% B; 6-7 min 100% B; 7-9 min linear gradient 100% C; 9-11 min linear gradient 100% A; 11-16 min 100% A; and Pump 2: 0-4 min 2% B; 4-8 min linear gradient 100% B; 8-9 min 100% B; 9-11 min linear gradient 100% C; 11-13 min linear gradient 98% A, 2% B; 13-16 min 2% B. Valve 2 was used to route the ion exchange eluent to waste (0-3.15 min), to the Synergi C18 analytical column (3.15-3.25 min), and back to waste (3.25-16min). Columns were kept at room temperature, and autosampler temperature was 5˚C. The following optimized instrument parameters were applied for the detection of analyte and internal standard: N_2 sheath gas 40 psi; N_2 auxiliary gas 20 psi; capillary temperature 350˚C; spray voltage 4 kV; tube lens voltage 50 V; source fragmentation energy 10 V; and capillary voltage 21 V. Quantitation was determined by MRM (2HG *m/z* 147→129, collision energy 25 V; and $^{13}C_4$-2HG *m/z* 151 → 133, collision energy 25 V) in positive ion mode. The isolation width and scan ranges were 2.0 *m/z* for both transitions. Data acquisition and quantitative spectral analysis were carried out utilizing Thermo Xcalibur v. 2.0.7 SP1 and Thermo LCQuan v. 2.6.0 build 1128, respectively.

(**B**) Characterization of assay accuracy and precision. Accurate quantitation of 2HG in cell lysates and cell growth media was achieved by $^{13}C_4$-2HG calibration and linear ion trap MRM detection over the 2HG concentration range of 0.02-20 µg/ml. The 2HG analyte was found to be stable in media and methanol lysate for at least 48 h at 10˚C. A lower limit of detection of 0.16 pmol on column and a lower limit of quantitation of 2.7 pmol on column were determined. The intrarun (within run) and interrun (between run) accuracy and precision for the

determination of 2HG are shown. The 2HG intrarun absolute %RE was ≤25.66, ≤7.06 and ≤13.14% at the low-, mid- and high-level QC concentrations, respectively. Corresponding %RSD values were ≤14.46, ≤3.54 and ≤5.45%. Interrun accuracy and precision were calculated from the average 2HG concentration from each of the three interday batch runs. Absolute %RE for the interday runs was ≤14.00, ≤6.20 and ≤13.03%, and the corresponding %RSD was ≤4.11, ≤3.49 and ≤6.20%.