Title: Identification and Validation of Cell-free DNA Methylation Biomarkers for Human Brain Cancers

1. Detailed *m*SEPT9 Assay Protocol:

DNA Extraction: DNA was extracted from 5 mL of blood plasma using a modified viral DNA/RNA extraction kit (chemagen AG, Baesweiler Germany). Plasma samples were thawed at room temperature and extracted following the directions of the kit with modifications. Samples were lysed and treated with protease at 56°C for 10 min in a 50 mL Falcon tube. 100 μL of magnetic particles and 15 mL of binding buffer were then added, and binding was performed for 60 min at room temperature on a rotator. Magnetic particles were captured for 4 min, the supernatant discarded and the pellet was resuspended in 3 mL of wash buffer. 1.5 mL of particle solution were transferred to a 2 mL SafeLock, the beads captured and the supernatant discarded. This was repeated to complete the 3 mL transfer. Tubes were briefly centrifuged and the residual wash buffer was removed by pipetting after bead separation. The tubes were then placed in a 56°C dry block for 5 min, 100 μl of elution buffer was added, the tubes incubated at 65°C with shaking on a thermomixer for 15 min, the particles separated on a magnetic stand and the eluted DNA transferred to a 0.5 mL SafeLock tube (Eppendorf). A 5μl aliquot of the DNA sample was transferred to 45 μl of elution buffer for the measurement of genomic DNA.

Bisulfite Conversion: The sample input for bisulfite treatment was 95-100 μl of extracted DNA in elution buffer. The bisulfite reagents (for 25 reactions) were prepared as follows.

Bisulfite solution: Sodium bisulfite (4.71gm) and sodium sulfite (1.13gm) were dissolved in 10 mL of ddH2O in a falcon tube, by vigorous shaking and heating to 50°C if required, and the pH adjusted to 5.4-5.5 with 0.2M NaOH as necessary. DME-radical scavenger solution: 188 mg of 6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid was dissolved in 1.5 mL diethyleneglycoldimethylether (DME), vortexing to ensure an uniform solution.

Bisulfite Reaction: 190 μL of bisulfite solution and 30μl DME-radical scavenger solution were added to the 95-100 μl DNA sample in 0.5 mL SafeLock tubes. The tubes were incubated in a Eppendorf Mastercycler (Eppendorf) according to the following protocol: 5 min 99°C, 25min 50°C, 5 min 99°C, 1h 25min 50°C, 5 min 99°C, 4h 55min 50°C, hold 20°C. This protocol allowed overnight bisulfite conversion.

Bisulfite Purification: Following bisulfite conversion, DNA was purified using a customized kit from chemagen AG. The bisulfite reaction (320 μL) was transferred to a 2 mL SafeLock tube, and 1 μLof polyA (500 ng/μL) and 1.5 mL of binding buffer were added. 10 μL chemagen magnetic particles were added and the sample was mixed by vortexing. The samples were incubated at room temperature on a thermal mixer at a rotation of 1000 rpm for 60 min. Magnetic particles were separated on a magnetic stand, the liquid discarded, the tubes briefly centrifuged and the residual liquid removed following magnetic separation. The particles were washed twice with wash buffer II from the kit, and once with 70% ethanol. Following the ethanol wash, the tubes were centrifuged again, and the residual liquid removed following magnetic separation. The particles were dried by placing open tubes in a 55°C heat block, and 55 μL elution buffer (10 mM Tris pH 7.2) added. Samples were incubated at 55°C for 15 min on a thermal mixer with rotation set to 1000 rpm, placed on a magnetic separator and the eluate containing DNA transferred to a new tube. A 5 µL aliquot of bis-DNA was added to 45 µL of elution buffer for the measurement of a 10 fold diluted sample. This purification method leaves out the desulfonation step following bisulfite conversion, to make the DNA amenable to carry over prevention by UNGase treatment as describe previously.(1) PCR amplification of sulfonated DNA requires an extended activation time to allow desulfonation to occur prior to amplification.

PCR Analysis: The oligonucleotide sequences and assay information are outlined in the Table below.

Supplemental Table 1. Oligonucleotide sequences, concentrations and cycling conditions for the real time PCR assays used in this study.

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| **PCR** | **Forward Primer** | **Reverse Primer** | **Blocker** | **Probe** | **Cycling Conditions** |
| SEPT9  ASSAY conc | aaataatcccatccaacta  0.3μM | Gatt-dS-GtTGtttAttAGttATtATGT  0.3μM | gttattatgttggattttgtggttaatgtgtag-C3  1.0 μM | FAM-ttaaccgcgaaatccgac-BHQ1  0.1 μM | Activation - 95°C 30 min.  55 Cycles: 95°C 10 sec (4.4°C/sec), 56°C 30 sec (2.2°C/sec)  cooling - 40°C 5sec (2.2°C/sec). |
| CFF1  (genomic assay)  ASSAY CONC | TAAGAGTAATAATGGATGGATGATG  0.63 μM | CCTCCCATCTCCCTTCC  0.63 μM | N/A | 6FAM-ATGGATGAAGAAAGAAAGGATGAGT-BHQ-1  0.2 μM | Activation - 95°C 15 min.  50 Cycles: 95°C 10 sec (4.4°C/sec), 58°C 60 sec (2.2°C/sec)  cooling - 40°C 5sec (2.2°C/sec). |
| β-actin  (bisulfite assay)  ASSAY CONC | gtgatggaggaggtttagtaagtt  0.9 μM | ccaataaaacctactcctcccttaa  0.9 μM | N/A | FAM-accaccacccaacacacaataacaaacaca-BHQ1a  0.1 μM | Activation - 95°C 30 min.  50 Cycles: 95°C 10 sec (4.4°C/sec), 57°C 30 sec (2.2°C/sec), 72°C 10sec (4.4°C/sec)  cooling - 40°C 5sec (2.2°C/sec). |

For the *SEPT9* PCR, the reverse primer contained an abasic d-spacer base in the 5th position. The blocker was terminated with a C3 spacer at the 3’ end to prevent extension. The *SEPT9* real time probe used the FAM / BHQ-1 fluorophors dyes. The CFF1 PCR was identical to previous studies. The primers for the β-actin assay were shortened compared with previous studies, but the probe was identical to previous studies (2, 3). Oligonucleotide quality was determined in house by MALDI-TOF prior to use, and the limit of detection for each PCR was evaluated prior to use in studies. The Quantitect Multiplex mastermix from Qiagen was used at a 1x final concentration in all assays. The total PCR reaction volume was 25 μl, using 96 well reaction plates on a Roche LC480 real time thermal cycler. For the *SEPT9* and β-actin PCR reactions, the activation temperature was extended to allow the desulfonation reaction to proceed prior to amplification.

Real time PCR analysis:Total genomic DNA was measured by real time PCR analysis of the diluted genomic aliquot using the CFF1 reaction. Total bisulfite DNA was measured from a 10 µL aliquot of the bisulfite DNA eluate with the β-actin reaction. *SEPT9* methylation was measured in triplicate using 10 µL of the bis DNA eluate per replicate, and in a single measure using 10 µL of the diluted bisulfite DNA eluate.

PCR Assay Prequalification: Prior to the study, the 90% limit of detection (LoD) was determined for the *SEPT9* PCR by analysis of bisulfite treated methylated (Sss1 treated) DNA diluted in a background of bisulfite treated 50 ng peripheral blood leukocyte (PBL) DNA (Roche Applied Science). As illustrated in Data Supplement Figure 1, the dilution series was from 100pg to 3.125 pg in 2 fold steps. We tested 12 replicates for four different PBL backgrounds, for a total of 48 replicates at each concentration. The 90% LoD was defined as the lowest concentration of spiked methylated DNA in a background of 50 ng human genomic DNA for which the measurement values had an area under the ROC curve (AUC) of 0.9 compared with measurements without spiked methylated DNA, and was estimated to be 9.4 pg for the *m*SEPT9 assay. For β-actin, the LoD of 9.7 pg was determined with a dilution series of bisulfite treated methylated (Sss1 treated) DNA, and for the genomic CFF1 PCR the LOD of 4.3 pg was measured with a dilution series of genomic PBL DNA.

2) Characterization of Assay performance with model samples:

Model samples: For workflow development two types of model sample were produced: 1) purified methylated DNA (CpGenome, Millipore) was spiked into plasma negative for methylated *SEPT9*; 2) plasma positive for methylated *SEPT9* was spiked into plasma negative for the *SEPT9* biomarker.

To determine the performance of DNA extraction from plasma, total genomic DNA recovery was measured using the genomic real time PCR assay CFF1. An example experiment from the assay development process using spiked plasma samples is shown in Figure 2. The recovery of total genomic DNA is shown for eight individual samples, in which we measured average DNA recovery of 2.93 ± 1.36 ng/mL of input plasma from a set of surrogate plasma samples in which low levels of plasma containing methylated *SEPT9* were spiked into a *SEPT9* negative plasma background (Figure 2a). Based on these and additional studies (not shown) we demonstrated equivalent recovery of genomic DNA to that measured with the research assay.

For bisulfite conversion and purification, the protocol was tested using surrogate samples as described for genomic DNA extraction above, and as illustrated in the sample experiment in Figure 2a, the recovery of bis-DNA measured using the real time β-actin PCR reaction was 1.6 ± 0.63 ng/mL of starting plasma, a yield in the range of 55% of the total genomic DNA.

To test the recovery of methylated target DNA in the *m*SEPT9 assay, methylated *SEPT9* positive plasma was spiked in a dilution series into a background of methylated *SEPT9* negative plasma (Figure 2b) with the target concentration of *SEPT9* biomarker at less that 10 pg/mL in the 8-fold dilution samples. For each dilution, the PCR positive rate for the original research assay and the new *m*SEPT9 assay was measured in 8 independent spiked samples. The detection rates differed marginally between the two assays at the higher concentrations of the *SEPT9* biomarker, and were identical (50%) at the greatest dilution (Figure 2b). Based on these results and numerous additional experiments (data not shown), for surrogate samples we considered the new assay essentially equivalent to the previously described research assay, and proceeded to validate the assay with clinical samples in a training and test study.

Study Quality Control

Positive controls for DNA extraction were 25ng/mL CpGenome methylated DNA diluted in 5mg/mL bovine serum albumin (BSA), while negative extraction controls were BSA without spiked DNA. Positive controls for bisulfite processing were composed of 10ng fully methylated CpGenome DNA spiked into 90 ng of human genomic DNA prepared from buffy coat cells (Roche Applied Sciences) in a 100 µL volume of elution buffer. Negative bisulfite conversion controls were composed of elution buffer alone

Supplemental Figure 1. The LoD calibration curve for the methylated *SEPT9* real time assay used in the training and test studies. The concentration of spiked methylated DNA in picograms is indicated across the top of the graph. The measured concentration of the methylated spike is indicated on the Y-axis. The dotted blue line indicates perfect identity between expected and observed, the black line connects the observed median values and the dashed red line represents the regression of the observed median values. The dotted black lines show the 90% confidence interval around the median value.

Supplemental Figure 2. Performance of the *m*SEPT9 assay on model DNA samples.

Supplemental Figure 2a. Concentration of total genomic DNA (gray bars) based on the CFF1 PCR assay, and bis-DNA (hatched bars) based on the β-actin PCR assay, calculated per mL of input plasma for eight independent sample pools. Samples were produced by spiking plasma positive for methylated *SEPT9* into methylated *SEPT9* negative plasma

Supplemental Figure 2b. *SEPT9* positive rate in percentage measured for the *SEPT9* FRET based research assay (gray bars) and the new *m*SEPT9 assay (hatched bars). Plasma samples were prepared in a dilution series of methylated *SEPT9* positive plasma spiked into a background of methylated *SEPT9* negative plasma, with a target of less than 10 pg/mL in the 8 fold dilution samples. Each percentage measurement is the aggregate of PCR results for 8 independent spiked DNA pools at the given dilution.

Supplement Figure 3a. Plot of cumulative total bis-DNA measured by β-actin real time PCR, for cancer cases and control patients expressed in ng / mL plasma. Note that the total DNA measurement is essentially identical for controls and cases.

Supplement Figure 3b. Plot of cumulative total bis-DNA measured by β-actin real time PCR, for cancer cases by stage in ng / mL plasma Note that the total DNA measurement is essentially identical for stages I-III but that a number of stage IV cancers show high concentrations.

References:

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3. Grutzmann R, Molnar B, Pilarsky C, Habermann JK, Schlag PM, Saeger HD, et al. Sensitive detection of colorectal cancer in peripheral blood by Septin 9 DNA methylation assay. [Epub ahead of print] Plos One November 20, 2008 as DOI: 10.1371/journal.pone.0003759.