



Supplemental Material to:

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Epigenetic biomarkers of T-cells in human glioma

Epigenetics 2012; 7(12)

<http://dx.doi.org/10.4161/epi.22675>

<http://www.landesbioscience.com/journals/epigenetics/article/22675>

Supplementary table S1A: Results of qMSP assays for glioma cell lines

	<i>CD3Z</i>	<i>FOXP3</i>
Glioma cell line	demethylation	demethylation
H4	0.02%	0.14%
A172	0.03%	0.06%
T98G	0.30%	0.06%
U138	0.07%	0.28%
U87	0.40%	0.00%
Mean	0.16%	0.11%
Std Dev	0.17%	0.11%

Supplementary table S1B: Results of qMSP assays for brain autopsy samples

	<i>CD3Z</i>	<i>FOXP3</i>
Brain tissue	demethylation	demethylation
Autopsy 1	0.09%	0.04%
Autopsy 2	0.10%	0.03%
Mean	0.10%	0.03%
Std Dev	0.01%	0.01%

Supplementary Table S2A. Logistic regression of glioma risk by control-derived *CD3Z* demethylation tertile

<i>CD3Z</i> demethylation tertile	Crude		Adjusted*	
	OR (95% CI)	p-value	OR (95% CI)	p-value
1st (lowest)	5.5 (2.5, 13.2)	6.06E-05	5.6 (2.5, 13.4)	6.42E-05
2nd (middle)	0.7 (0.2, 2.1)	0.52	0.7 (0.2, 2.0)	0.46
3rd (highest)	Reference		Reference	

*Unconditional multivariate model controlling for age, gender and smoking

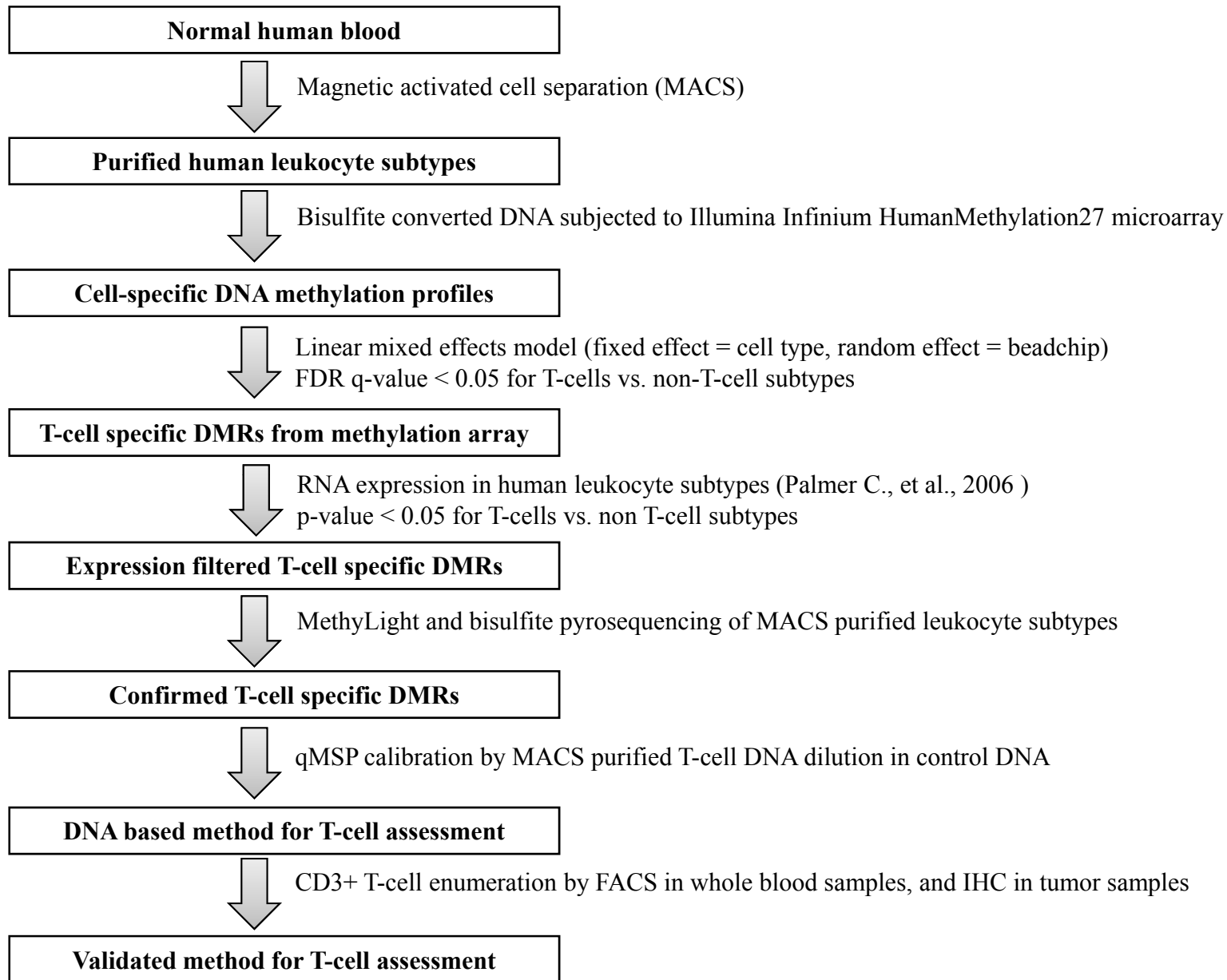
Supplementary Table S2B. Logistic regression of glioma risk by control-derived *FOXP3* demethylation tertile

<i>FOXP3</i> demethylation tertile	Crude		Adjusted*	
	OR (95% CI)	p-value	OR (95% CI)	p-value
1st (lowest)	5.9 (2.6, 14.6)	5.31E-05	6.7 (2.8, 17.7)	4.29E-05
2nd (middle)	1.1 (0.4, 3.1)	0.84	1.2 (0.4, 3.5)	0.74
3rd (highest)	Reference		Reference	

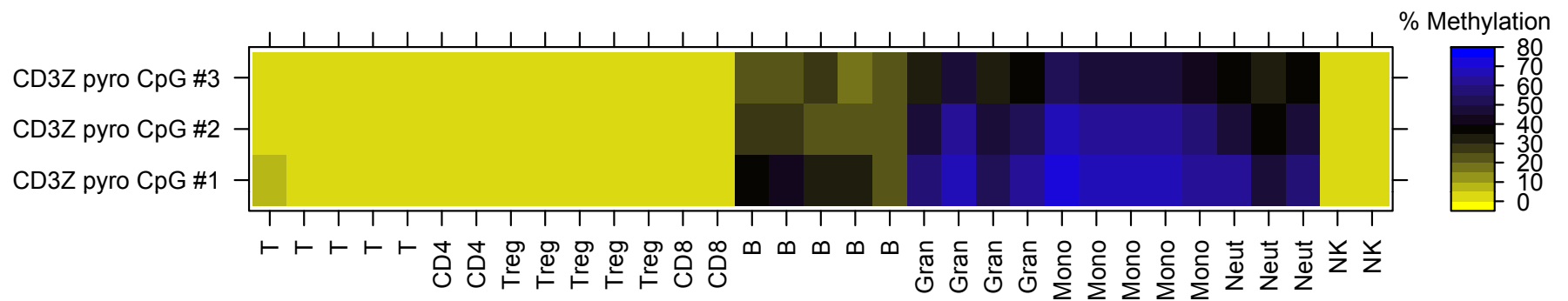
*Unconditional multivariate model controlling for age, gender and smoking

Supplementary Table S3: Oligonucleotide primer and probe sequences used for MethyLight and qMSP assays

Oligonucleotide Name	Sequence (5' to 3')
C-LESS Fwd	TTGTATGTATGTGAGTGTGGGAGAGA
C-LESS Rev	TTTCTTCCACCCCTTCTCTTCC
C-LESS Probe	(6FAM) CTCCCCCTCTAACTCTAT (MGB,NFQ)
<i>CD3Z</i> Fwd	GGATGGTTGTGGTGAAAAGTG
<i>CD3Z</i> Rev	CAAAAACCTCCTTTTCTCCTAACCA
<i>CD3Z</i> Probe	(6FAM) CCAACCACCACTACCTCAA (MGB,NFQ)
<i>FOXP3</i> Fwd	GGGTTTTGTTGTTATAGTTTTTG
<i>FOXP3</i> Rev	TTCTCTTCCTCCATAATATCA
<i>FOXP3</i> Probe	(6FAM) CAACACATCCAACCACCAT (MGB,NFQ)

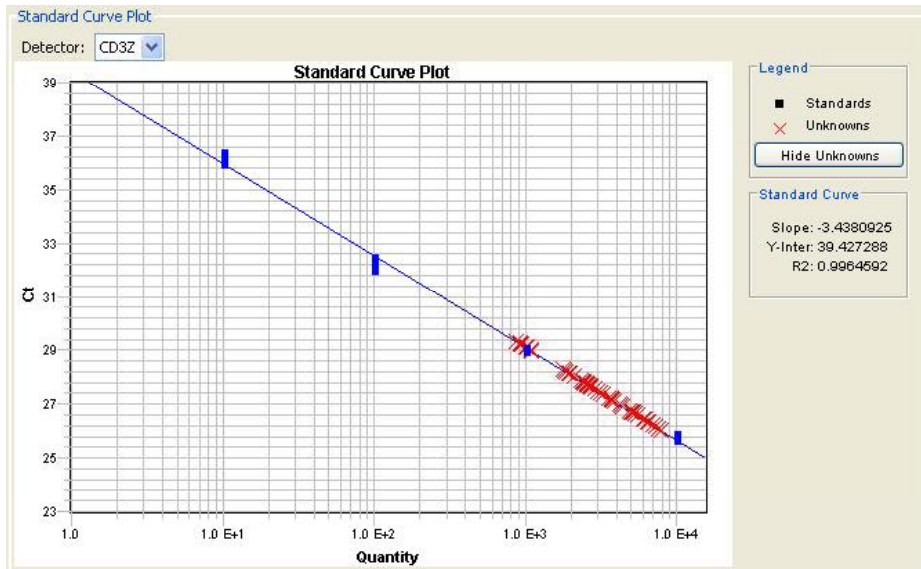


Supplementary Figure S1: T cell-specific epigenetic biomarker development workflow

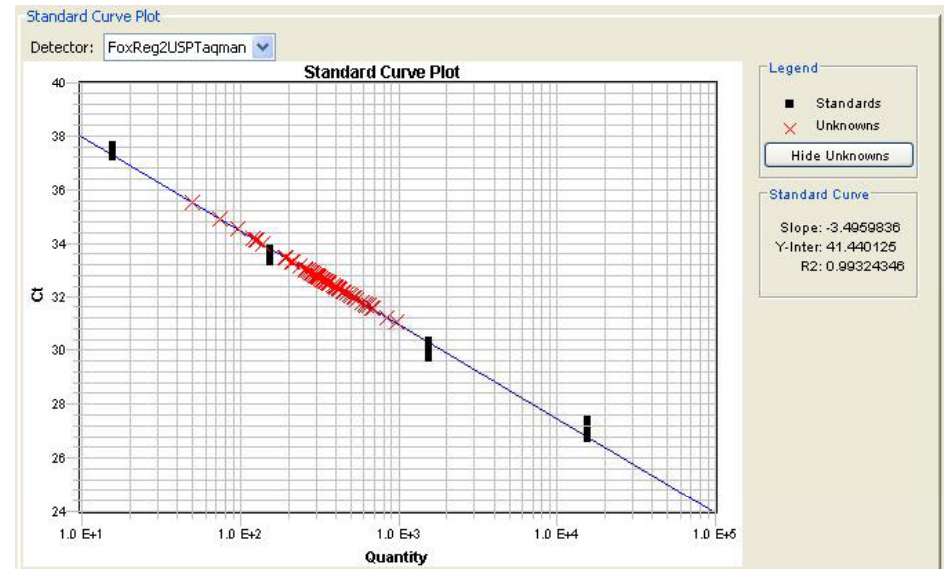


Supplementary Figure S2: Bisulfite pyrosequencing of MACS sorted human leukocyte subsets. Heatmap of DNA methylation at three CpG loci in the *CD3Z* gene assessed by bisulfite pyrosequencing. Abbreviations: B = B lymphocytes, Gran = Granulocytes, Neut = Neutrophils, Mono = Monocytes, NK = CD56+ Natural killer cells, Nkdim = CD16+CD56dim natural killer cells, NKbr = CD16-CD56bright natural killer cells, NK8+ = CD8+CD56+ natural killer cells, NK8- = CD8-CD56+ natural killer cells, NKT = CD3+CD56+ natural killer T-cells, T = CD3+ T lymphocytes, CD8 = CD3+CD8+ T lymphocytes (cytotoxic T-cells), CD4 = CD3+CD4+ T lymphocytes (helper T-cells), Treg = CD3+CD4+CD25+FOXP3+ regulatory T-cells

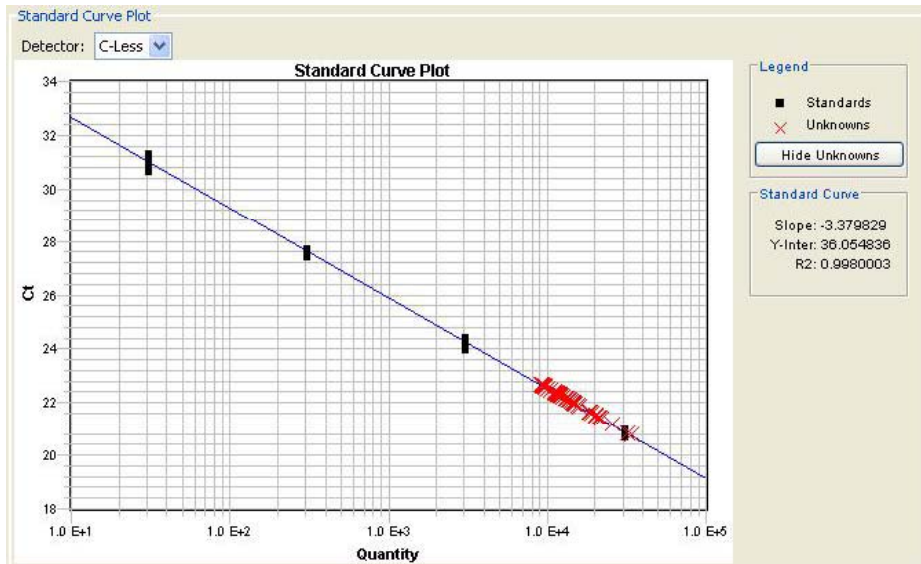
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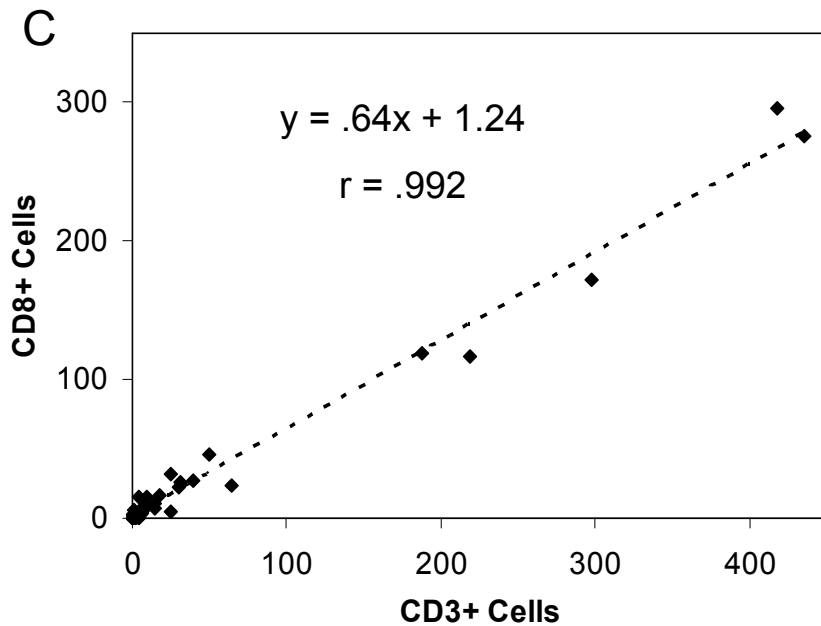
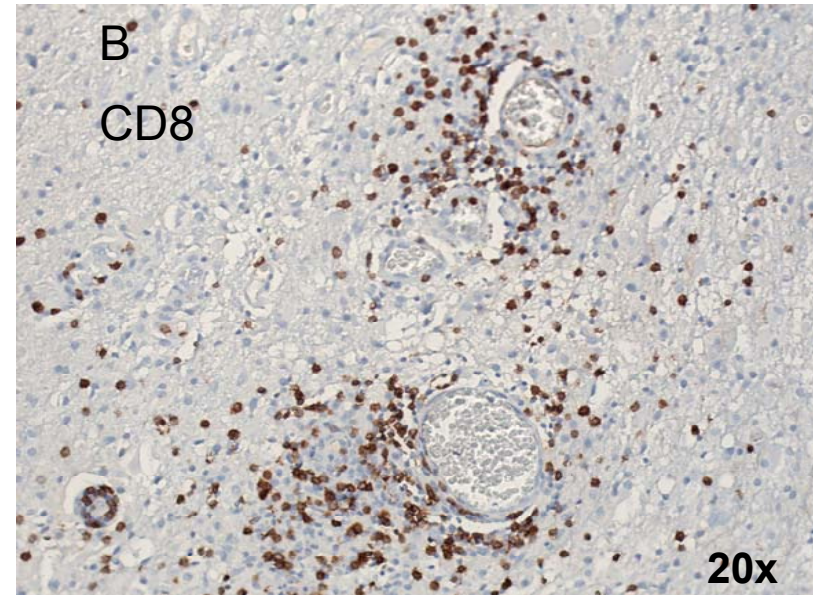
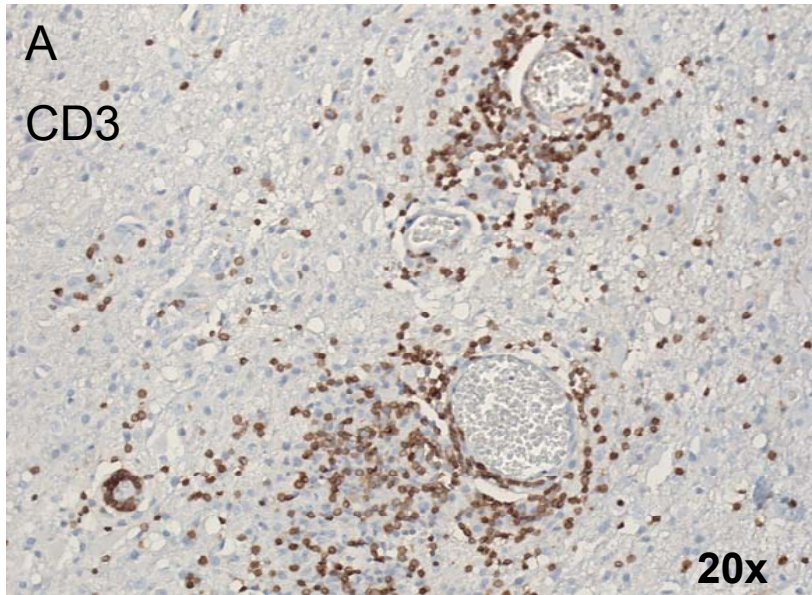
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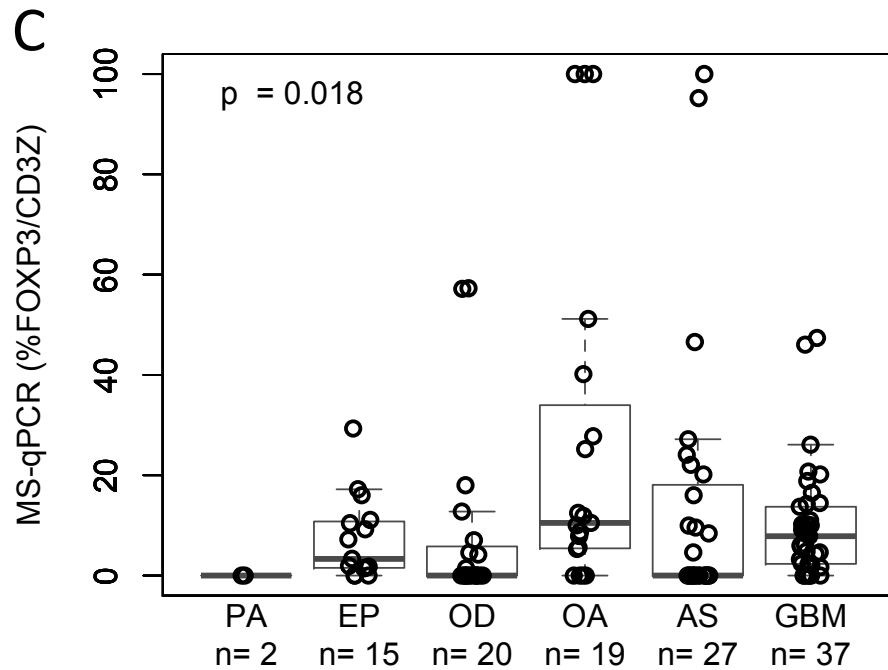
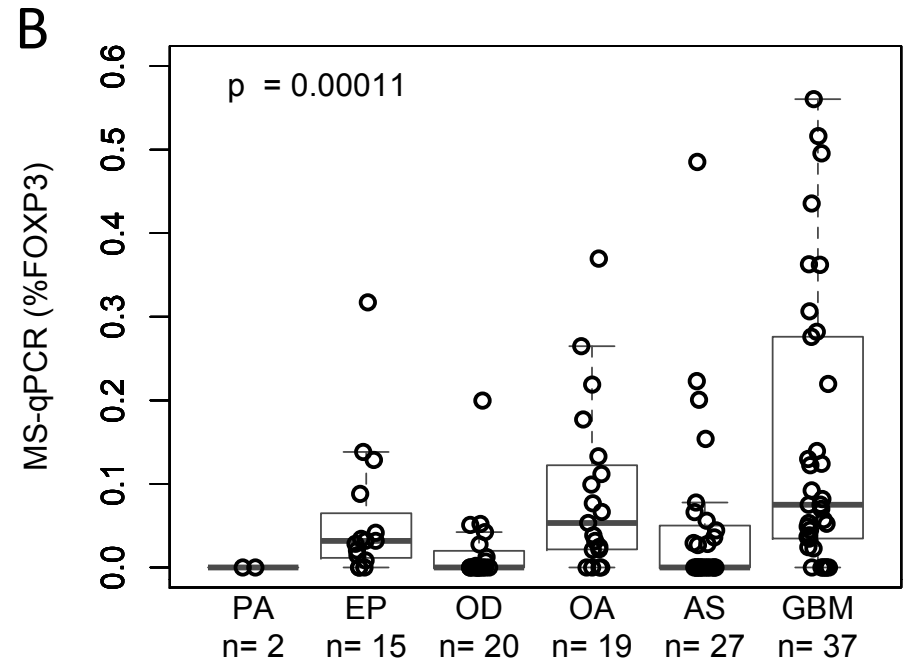
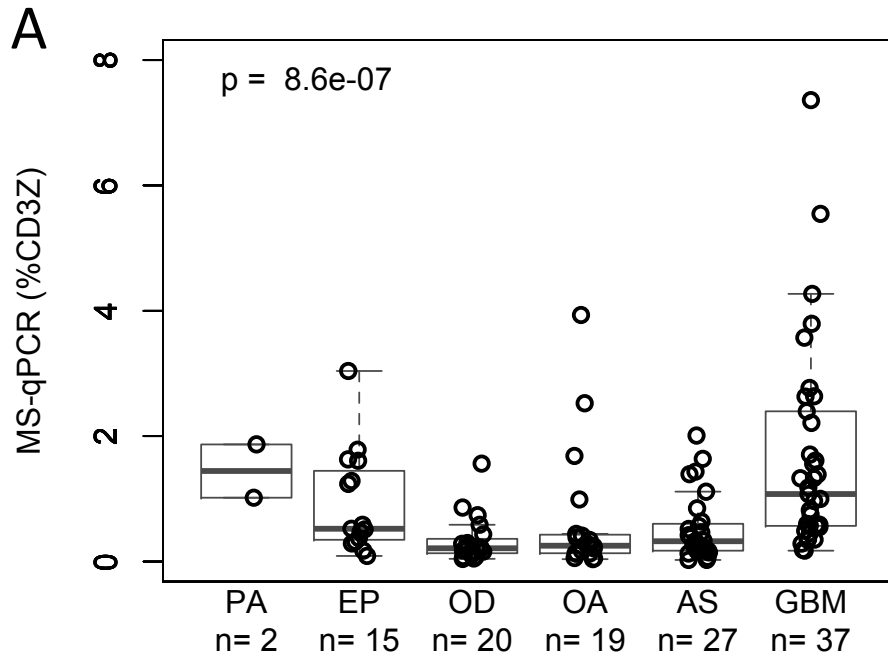
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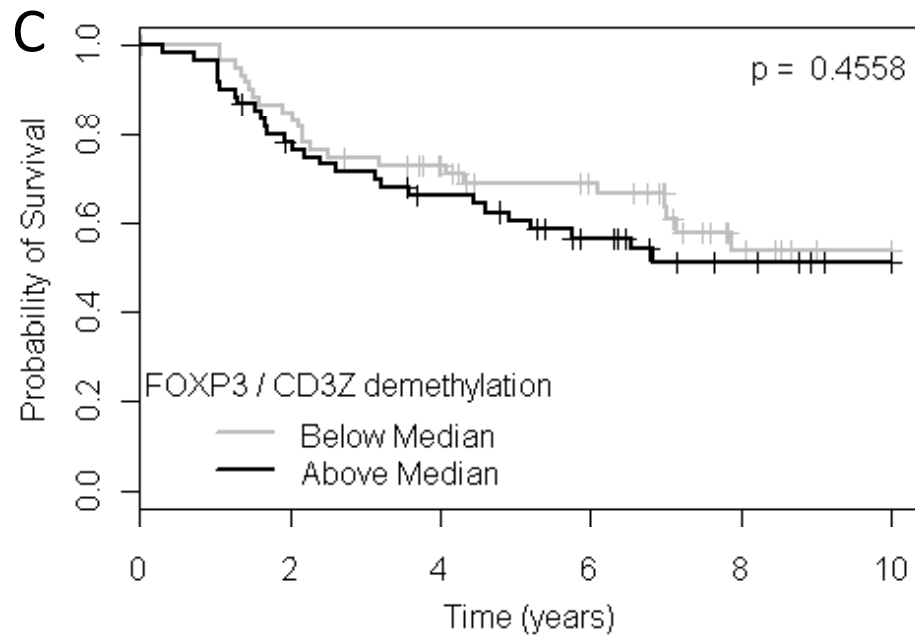
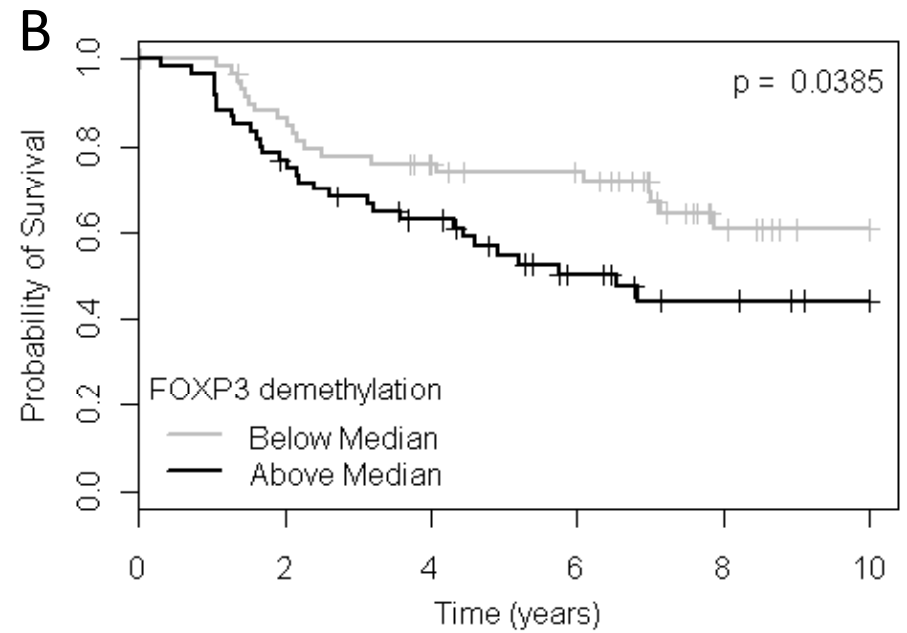
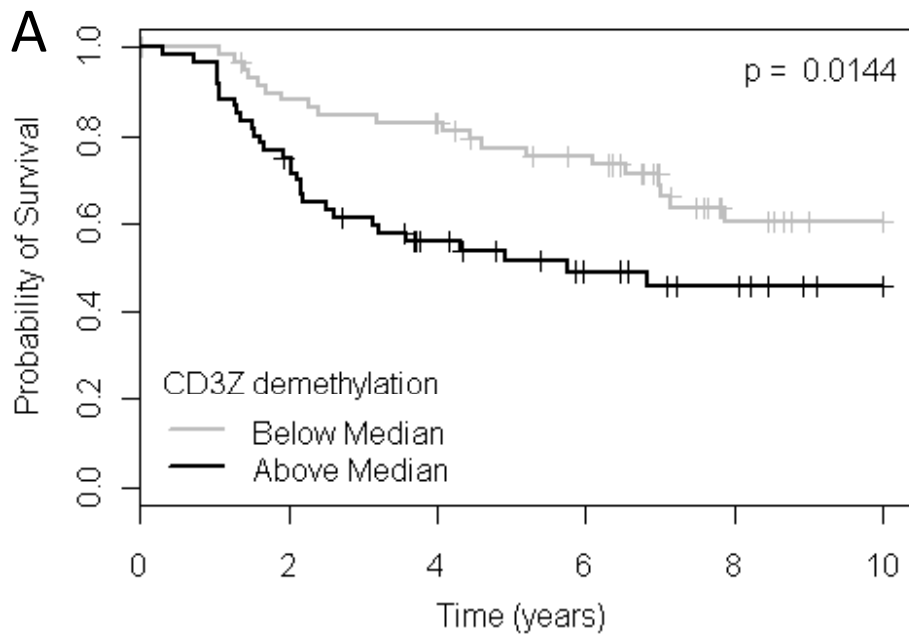
Supplementary Figure S3: Examples of four-point standard curves used in qMSP experiments. A. T-cell calibration curve for *CD3Z* demethylation assay containing bisulfite converted DNA from MACS purified CD3+ T-cells log-diluted in bisulfite converted methylated control DNA. B. Treg calibration curve for *FOXP3* demethylation assay containing bisulfite converted DNA from MACS purified CD4+CD25+ Tregs log-diluted in bisulfite converted methylated control DNA. C. C-less standard curve for measurement of total DNA input containing normal blood gDNA log-diluted in TE buffer. Plots made using Applied Biosystems SDS (v1.4) software. Red x's represent human blood samples from the UCSF San Francisco Adult Glioma Study.



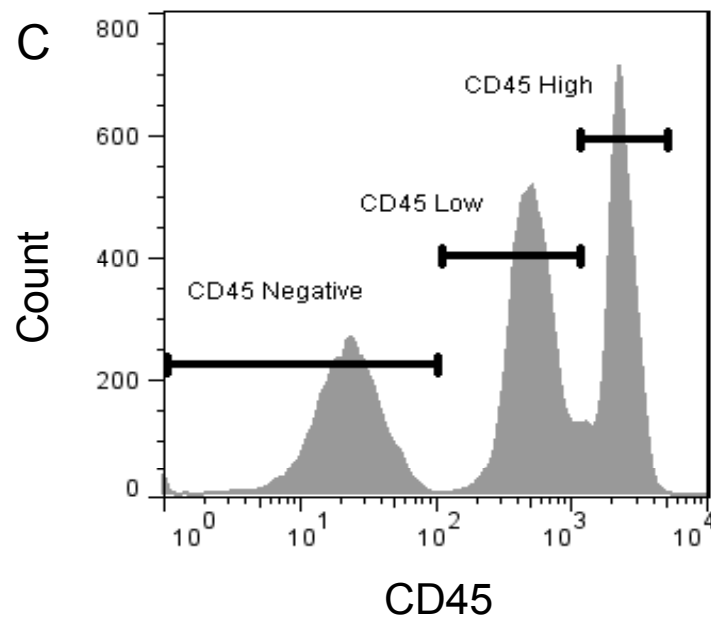
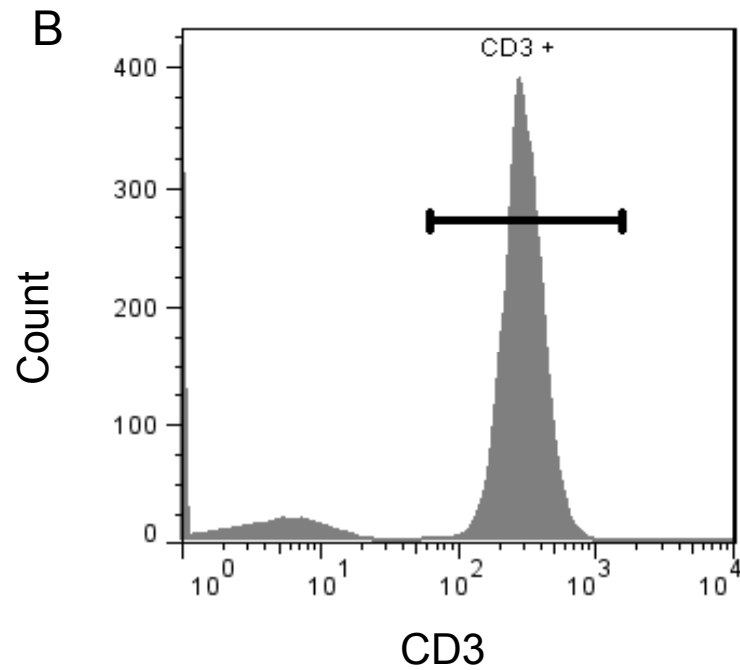
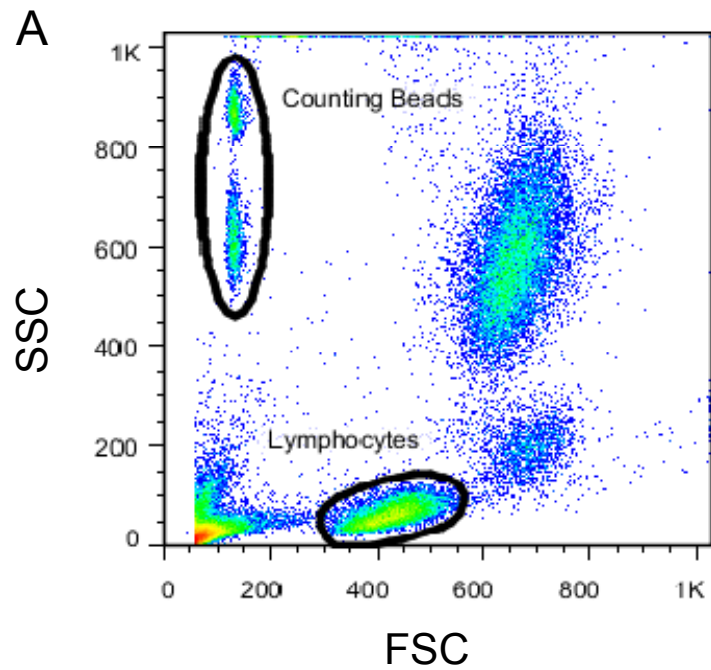
Supplementary figure S4: IHC staining of a representative GBM specimen. A, CD3 staining, Average number of cells positive = 418. B, CD8 staining, Average number of cells positive = 296. C, Correlation of CD3 and CD8 staining, Pearson $r = .992$



Supplemental figure S5: Levels of T-cell and Treg infiltrates in glioma tissues stratified by histological subtype. A, T-cell levels, determined by *CD3Z* demethylation, in solid glioma samples stratified by tumor histology. B, Treg levels, determined by *FOXP3* demethylation, in solid glioma samples stratified by tumor histology. C, Treg percent of T-cells, determined by ratio of *FOXP3* to *CD3Z* demethylation, in solid glioma samples stratified by histology. Abbreviations: PA = Pilocytic Astrocytoma, EP = Ependymoma, OD = Oligodendroglioma, OA = Oligoastrocytoma, AS = Astrocytoma, GBM = Glioblastoma multiforme. Kruskal-Wallis one-way analysis of variance by rank test p-values shown.



Supplementary figure S6: Kaplan Meier analysis of glioma survival stratified by median level of T-cell and Treg infiltrates. A, Survival time by T-cell levels, determined by *CD3Z* demethylation. B, Survival time by Treg levels, determined by *FOXP3* demethylation. C, Survival time by Treg percent of T-cells, determined by ratio of *FOXP3* to *CD3Z* demethylation. Log Rank p-values shown



Supplementary Figure S7: Flow cytometry analysis of CD3+ T-cells and total leukocytes in whole blood from glioma cases and controls. A, Forward and side scatter plot of representative blood sample showing gating for lymphocytes and counting beads. B, Lymphocyte subpopulation showing CD3+ gating. C, CD45 gating on all non-bead events. CD45+ low and high cells were added in order to count total CD45+ cells