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The DOI for this manuscript is doi: 10.5858/arpa.2026-0018-OA

The final version of this manuscript will replace the New Article version at the above DOI once it is available.

Assessment of Distributive DNA Methylation Profiling for Central Nervous System Tumor Classification Between Laboratories in the United States and Brazil

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• **Context.**—Implementation of machine learning–based central nervous system (CNS) tumor classifiers remains limited, particularly in low- and middle-income countries, where many centers lack the infrastructure, expertise, or financial resources required for clinical deployment. Distributive testing offers a practical pathway for broader adoption, allowing centers without in-house capabilities to access validated classification pipelines. Understanding sample and data quality and classifier performance in the local patient population is a critical initial step in determining the feasibility of this approach.

Objective.—To evaluate whether methylation data generated in a Brazilian site could support tumor classification, using a US-based clinical-grade methylation classifier.

Design.—We conducted retrospective and prospective analyses of preanalytic parameters, methylation testing quality metrics, and classifier outputs on data generated in Brazil. Quality metric distributions from study samples

were compared with in-house clinical data. Classifier performance was assessed by reviewing concordance with histopathology and available ancillary testing information.

Results.—A total of 151 unique CNS tumor samples, representing major tumor families, were evaluated. Preanalytic metrics of the study samples were comparable to those from the US-based laboratory. Some differences in methylation testing metrics reflected instrumentation variation. Confident classifications matching histologic diagnosis were achieved in 141 of 151 samples (93.4%), with ancillary testing and quality control data supporting the classifier call in 6 additional cases. Classifier-histopathology concordance showed no significant correlation with preanalytic or methylation metrics.

Conclusions.—Samples processed in Brazil met US-comparable quality standards, supporting robust classifier performance and feasibility of distributive methylation testing.

(*Arch Pathol Lab Med.* doi: 10.5858/arpa.2026-0018-OA)

In October 2020, the Pathology Department at St. Jude Children's Research Hospital (SJCRH), Memphis, Tennessee, launched a clinical-grade DNA methylation

profiling assay using the Illumina Infinium MethylationEpic array (Illumina, Inc, San Diego, California) and an in-house—developed machine learning model for the purposes of classifying central nervous system (CNS) tumors and detecting pan-genomic copy number variation. This assay is used for routine neuropathology diagnostics and has shown strong technical and diagnostic performance at our center.

Drawing from our experience, we hypothesized that a distributive model for DNA methylation testing—where sample processing and methylation data generation occur at local treatment centers, data analysis (classification) is performed at SJCRH, and results are returned to local clinical pathology teams for final interpretation and decision-making—could provide a faster and more cost-effective pathway for clinical test implementation in low- and middle-income countries (LMICs). This strategy would circumvent many of the obstacles that hinder clinical deployment and maintenance of DNA methylation profiling in resource-limited settings, including the need to assemble, curate, and maintain large, high-quality, and diverse data sets for model development; the requirement for specialized bioinformatics and machine-learning expertise; the substantial cost of implementing clinical-grade classifiers; and the logistical

Accepted for publication June 23, 2026.

Two supplemental digital content files are available for this article. See text for hyperlinks.

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The authors have no relevant financial interest in the products or companies described in this article.

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and technical demands of deploying, updating, and sustaining models over time. From a clinical management perspective, the distributive testing approach could expand access to a diagnostic method that improves accuracy, enhances reproducibility, and supports clinically relevant molecular classification of CNS tumors.

However, because the preanalytic and analytic stages of testing (including both wet- and dry-bench processes) are inherently interdependent, evaluating the wet-bench workflow alongside the informatics pipeline is essential to ensure acceptable end-to-end test performance.

This study was conducted to assess the quality of samples and methylation data generated at a Brazilian cancer hospital and to assess the performance of our clinical methylation CNS tumor classifier on samples collected and processed at that center. Understanding preanalytic and data characteristics—and how the classifier generalizes to the local patient population—is an essential first step when evaluating the feasibility of implementing distributive DNA methylation profiling as an adjunct method for CNS tumor classification at an international site.

MATERIALS AND METHODS

International Study Site Selection

The Barretos Cancer Hospital (currently known as Hospital de Amor, HA) is a private nonprofit cancer hospital located in Barretos, São Paulo State, Brazil. Established in 1967, the hospital has several affiliated centers across different Brazilian states and focuses on pediatric and adult underserved populations.¹ The center was invited to participate in this pilot study, based on the following considerations: (1) the hospital is a St. Jude global affiliate since 2013²; (2) their pathology department has a neuropathologist on staff; (3) their clinical molecular laboratory is equipped with a sequencing instrument compatible with DNA methylation testing; (4) they had some previous experience of running DNA methylation arrays in the research setting; (5) they did not have an in-house classifier for CNS tumors—methylation data were being analyzed by using an open source/research use-only method (<https://app.epignostix.com/>); and (6) laboratory leadership was considering adopting clinical-grade methylation array testing for CNS tumor classification.

A distributive testing approach was considered for implementation of DNA methylation profiling at HA rather than local deployment of the SJCRH classifier, because the Brazilian hospital did not have bioinformatics personnel in place to operate and maintain the classifier locally.

Study Objectives

This study was conducted under both local and SJCRH Institutional Review Board (IRB)—approved research protocols (IRB No. 24-1683), without the return of clinical results. The study was structured into 3 objectives.

Objective 1.—Evaluation of quality metrics and classifier performance on existing methylation profiling data from CNS tumor samples tested according to HA's procedures.

The goal of this retrospective analysis was to assess the quality of samples and data generated from CNS tumors at HA and to evaluate the results of SJCRH's classifier on locally generated patient data compared with local histologic and ancillary diagnostic determinations.

Objective 2.—Assessment of sample quality, data quality, and classifier performance on CNS tumor samples tested at HA, using the SJCRH methylation assay analytical protocols.

The goals of this analysis were to assess concordance in quality control (QC) metrics between the 2 centers, as a measure of HA's technical quality and laboratory proficiency, and to evaluate the

performance of the classifier on samples processed at HA, using SJCRH's clinically validated methylation testing procedures.

Objective 3.—Prospective assessment of sample quality, data quality, and classifier performance on CNS tumor samples obtained at HA and regional centers in parallel with local standard diagnostic testing (eg, histology, immunohistochemistry, and molecular genetic analysis).

The goal of this analysis was to assess methylation QC data and test results generated at HA under real-world conditions, using SJCRH's clinically validated methylation testing procedures.

Sample Cohorts

All samples and data included in this study were de-identified and coded prior to analysis. Sample characteristics for each objective are summarized below.

Objective 1.—Forty-five CNS tumor samples were tested across 6 different runs. Of those, 20 were fresh/frozen tissue and 25 were formalin-fixed paraffin-embedded (FFPE) tissue specimens. Thirty-nine samples were obtained from HA, and 6 were provided by its affiliate centers.³

Objective 2.—Twenty-five FFPE CNS tumor samples were obtained from HA's pathology archives (of which 6 were referrals from HA's affiliate centers in Brazil) and 25 FFPE CNS tumor samples were previously profiled by SJCRH's clinical-grade methylation testing. Criteria for sample selection are listed in Supplemental Table 1 (see [supplemental digital content 1](#) containing 1 table and 3 figures). The selected samples were well characterized histopathologically and supported by orthogonal ground truth data, including immunohistochemistry and molecular genetic testing. FFPE scrolls and hematoxylin-eosin slides from each of the 25 samples selected at SJCRH were sent to HA for methylation testing. Samples were tested across 8 independent methylation runs: 7 samples/chip/run + 1 control sample (male; CAT No. G1471, Promega, Fitchburg, Wisconsin). Two samples obtained from HA (HA5 and HA21) and 1 sample from SJCRH (SJ23) were tested in duplicate in the same run (to assess intrarun reproducibility), and 3 samples (1 from HA [HA26] and 2 from SJCRH [SJ11 and SJ15]) were tested across different runs for interrun reproducibility. Runs were designed to include samples from both centers to account for operator variability.

Objective 3.—Fifty-six FFPE CNS tumor samples were collected prospectively during 8 consecutive weeks from in-house HA patients (n = 44) and from patients from other centers in Brazil (n = 12) that met criteria for molecular diagnostic testing. The samples included in this assessment reflected the range of clinical sample distribution and characteristics that would meet criteria for methylation testing at HA. Samples referred from affiliated centers to HA for neuropathology consultation that met criteria for routine molecular and DNA methylation profiling were also included. Data from this experiment should allow for enough variability of tumor types and preanalytic characteristics to inform the objectives of this analysis. A control sample (male; CAT No. G1471, Promega) was included in each run.

Study Process Overview

DNA methylation was performed at HA on bisulfite-converted genomic DNA samples, using the Illumina Infinium MethylationEPIC BeadChip arrays (version 2.0)⁴ and scanned in a NextSeq 550 (Illumina). A step-by-step comparison of the methylation array testing protocols used at SJCRH and HA is provided in Supplemental Table 2 (see [supplemental digital content 2](#)). The main differences were the DNA input amount: 500 ng at HA versus 100 to 250 ng at SJCRH, the type of Illumina instruments used for chip scanning (NextSeq 550 at HA and iScan [Illumina] at SJCRH), and the operators performing the test. The HA operator received on-site training at SJCRH, including instruction in sample processing, assay execution, and documentation procedures, to ensure competency with the assay workflow and to facilitate implementation of the SJCRH testing protocol at HA.

Table 1. Quality Metrics Assessed for Central Nervous System Samples and Methylation Array Data in the Study

QC Metric	Expected Value
Preanalytic metrics	
Tumor purity, %	≥70 (≥55% acceptable)
DNA concentration	≥250 ng total input (≥100 ng acceptable; 75 ng: minimum DNA input amount)
Mean fragment size (gDNA) ^a	≥500 bp (≥100 bp acceptable)
DIN ^a	≥1.5 (≥1 acceptable)
A ₂₆₀ :A ₂₈₀ ^a	~1.8 (no specific acceptability cutoff)
Methylation profiling metrics	
Bisulfite conversion values	>1
Median intensity value	≥21
Density plot of β values	Bimodal distribution with 2 peaks of β values at 0 and 1
Median intensity of methylated and unmethylated probes	Query samples plots above dotted line

Abbreviations: DIN, DNA integrity number; gDNA, genomic DNA; QC, quality control.

^a Mean fragment size distribution of gDNA and DIN are indicators of DNA quality/integrity, while the A₂₆₀:A₂₈₀ ratio evaluates DNA purity.

Intensity Data (IDAT) files and de-identified metadata, including run-level preanalytic QC metrics and available ancillary testing information, were securely transferred to a password-protected OneDrive (Microsoft, Redmond, Washington) folder at SJCRH. The IDAT files were transferred in batches approximating the sizes expected for Illumina methylation array runs (eg, 7–8 samples/chip; 23–24 samples/run) to simulate real-world testing conditions more closely. The files were analyzed through SJCRH's methylation CNS tumor classifier (MLPNET 4.0), which is a kernel machine model that requires 75 to 500 ng DNA input (preferably 100–250 ng) and at least 55% (ideally ≥70%) tumor purity for reliable performance. Following preprocessing and QC assessment of IDAT files at SJCRH, using the *minfi* package (v.1.28.4),⁵ which generates the methylation testing quality metrics, the classifier produces predicted class memberships and calibrated classification scores for each tumor class and family represented in the model. The class or family with the highest calibrated score is interpreted as the final classification assignment. Score thresholding is used to filter out unreliable inferences. Classifications are considered valid for clinical reporting only when their calibrated scores are 0.9 or greater. Tumor types not represented in the reference training cohort typically receive subthreshold scores, and only predictions at or above the threshold are considered valid.

The SJCRH study team compared results from the classifier analysis (tumor classifications and confidence scores) to ancillary testing information, including histopathology, immunohistochemistry, and molecular assays (fluorescence in situ hybridization, targeted DNA sequencing, and fusion sequencing) provided by HA to assess the technical performance of the classifier on data generated from their patient population at their site. Methylation array classifications using the Epignostix Brain classifier v12.8 (Heidelberg Epignostix GmbH, Heidelberg, Germany) were obtained for all study and control samples as an independent comparator and to aid in the interpretation of complex or borderline samples. Classifier results were shared with HA through a password-protected OneDrive folder and during biweekly conference calls, but they were not used to guide clinical management of any patient. Any discrepancies between classifier findings and ancillary testing results were reviewed and reconciled during the biweekly meetings.

Quality Metric Evaluation

Preanalytic Metrics.—Two preanalytic metrics were used to assess the quality of Objective 1 samples and methylation array data generated at HA: tumor purity (≥70%) and DNA concentration (total input ≥100 ng) (Qubit Fluorometer, Thermo Fisher Scientific, Waltham, Massachusetts). For samples evaluated under Objectives 2 and 3, additional indicators of DNA quality and integrity were assessed, including mean fragment size distribution of

genomic DNA (≥500 bp), DNA integrity number (DIN) (>1.5), and A₂₆₀:A₂₈₀ ratio (~1.8) (Table 1). DIN is a standardized numerical indicator of DNA quality, ranging from 1 for highly degraded DNA to 10 for highly intact DNA, and provides a more objective assessment of DNA fragmentation than mean fragment size distribution alone, particularly in challenging specimens such as FFPE tissue. Mean fragment size distribution and DIN were evaluated with the TapeStation System (Agilent Technologies, Inc, Santa Clara, California), and A₂₆₀:A₂₈₀ ratio was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). Postresection tissue handling and paraffin block preparation were not formally evaluated as part of this study.

Methylation Testing Metrics.—Methylation testing quality was evaluated by using bisulfite conversion values (acceptable >1) and qualitative array QC metrics, including median intensity (≥21), β-value density plot (expected bimodal peaks near 0 and 1, corresponding to unmethylated and methylated loci), and median intensities of methylated and unmethylated probes, with acceptable samples plotting above the reference dotted line (*minfi* package v.1.28.4⁵) (Table 1).

For both preanalytic and methylation testing metrics, samples failing to meet the preestablished acceptance criteria were flagged but taken through the entire analytical process, with the goal of assessing robustness of the classifier performance and to inform possible output patterns generated by suboptimal data.

Quality Metric Data Analysis.—The tumor purity, DNA concentration, mean fragment size distribution, DIN, A₂₆₀:A₂₈₀, bisulfite conversion values, and methylation testing qualitative QC data were summarized to evaluate distribution. These metrics were plotted with the R package *ggplot2* 4.0.1. The distributions of HA study samples were compared with SJCRH clinical laboratory samples to evaluate the similarity across centers. For preanalytic variables (DNA concentration, mean fragment size distribution, DIN, and A₂₆₀:A₂₈₀), the SJCRH reference clinical cohort comprised available data from 758, 790, 824, and 824 samples, respectively collected between November 2022 and June 2025. Bisulfite conversion and median intensity values from study samples were compared with QC data from 210 SJCRH clinical samples analyzed across 14 independent MethylationEpic BeadChip (v2.0) array runs, with *P* values calculated by using the Wilcoxon rank sum test. Density plot of β values and median intensity of methylated and unmethylated probes for the study samples were compared with 6 control samples that are routinely used in SJCRH clinical testing. For the β value distributions, all probes for all samples were pooled, and the pooled distribution was plotted.

Classifier Performance Evaluation

A summary of classification results and calibrated scores for each of the 173 study samples (Objective 1: 45 study samples;

Table 2. Cohort Characteristics and Tumor Distribution Across All Study Objectives (N = 151)

Tumor Family	Number of Samples	Percentage of Samples
Low-grade glioma	33	22
High-grade glioma	30	20
Embryonal	33	22
Medulloblastoma	27	18
Embryonal tumor with multilayered rosettes	4	3
Atypical teratoid/rhabdoid tumor	2	1
Meningioma	25	17
Choroid plexus tumor	11	7
Ependymoma	9	6
Schwannoma	2	1
Other	8	5
Pituitary neuroendocrine tumor	3	2
Other rare tumors	5	3
Mesenchymal nonmeningothelial tumors	2	40
Chordoma	1	20
Craniopharyngioma	1	20
Papillary tumor of pineal region	1	20

Objective 2: 56 study samples + 8 controls; Objective 3: 56 study samples + 8 controls) was compiled and reviewed. For each sample, methylation classifier results were evaluated through comparison against available clinical diagnostic information (histopathology, immunohistochemistry, molecular testing, and cytogenetics) to assess technical performance (accuracy, recall, and specificity) of the classifier and classification agreement between centers (for the 25 Objective-2 samples shared between SJCRH and HA). Diagnostic accuracy evaluation assessed high-confidence classifications (calibrated score ≥ 0.9) for concordance with expected histopathologic diagnosis.

To enable meaningful analysis of the classification data, individual tumor types were mapped to 8 categories (Table 2). For samples receiving scores below the reporting confidence threshold (no call/subthreshold), QC metrics were carefully reviewed to

differentiate between appropriately unclassifiable cases (tumor entities absent from classifier training data sets, normal tissue, or inflammatory microenvironment) and unexpectedly unclassifiable cases suggesting technical or sample quality issues.

Association between quality metrics and classifier performance was calculated with Wilcoxon rank sum test and Kruskal-Wallis test.

RESULTS

Tumor Types Represented in the Study

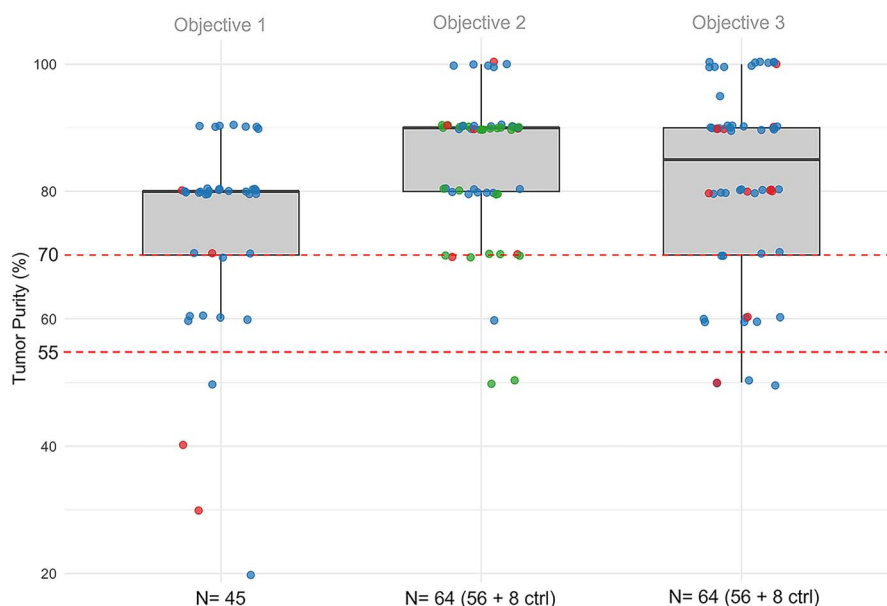
The study analyzed 151 unique CNS tumor samples, representing major CNS tumor families. Cohort characteristics and tumor distribution are summarized in Table 2.

Quality Metric Performance

Preanalytic Metrics.—Across all 3 study objectives, tumor purity ranged from 20% to 100% (Figure 1). Of the 9 tumors with tumor cell content below 55%, only 1 Objective-1 sample, histologically consistent with a pleomorphic xanthoastrocytoma, was classified as low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma. The other 8 tumors obtained confident classifications that matched the histologic and ancillary test results. The classifier's robust performance on low-tumor purity samples likely reflects the breadth of the medulloblastoma cases in its training cohort and the presence of defining genetic alterations in 2 tumors (histone H3 lysine 27 and *IDH* [isocitrate dehydrogenase] mutations), which tend to increase classification yield.

DNA input ranges for all study samples were comparable to those of SJCRH clinical samples included in the analysis (Figure 2, A). For Objective 1 testing, 500 ng of DNA was used, as recommended by the manufacturer. Objectives 2 and 3 testing, conducted following SJCRH's clinically validated wet-lab protocols, targeted 100 to 250 ng of DNA, with a minimum acceptable input of 75 ng. Only 4 (Objective 3) study samples originating from HA and affiliate centers obtained DNA inputs below 100 ng (Figure 2, A). These samples had DNA input amounts of 90 ng or greater, above the minimum acceptable level of 75 ng.

Figure 1. Tumor purity estimates for central nervous system tumor samples across study objectives. Tumor purity estimates for samples analyzed under Objectives 1 to 3. Boxplots show medians and interquartile ranges; individual points represent purity estimates for each sample. Red, blue, and green dots indicate samples from Hospital de Amor (HA) affiliate centers, HA, and St. Jude Children's Research Hospital, respectively. Dashed red lines denote expected purity thresholds for reliable methylation classification ($\geq 70\%$; $\geq 55\%$ acceptable).



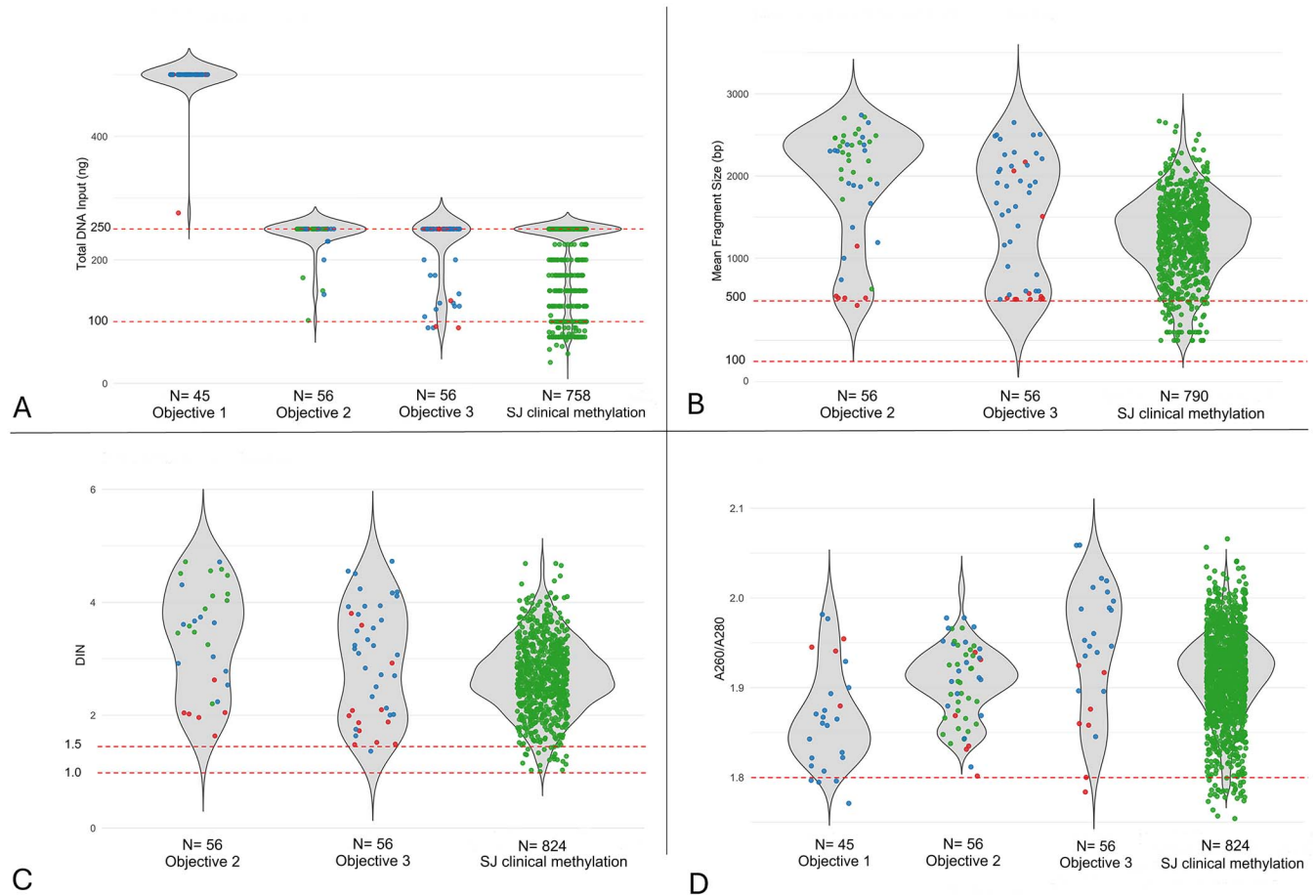


Figure 2. Preanalytic DNA quality metrics for study and reference samples across objectives. Violin plots display the distribution of (A) DNA input amount, (B) mean fragment size distribution of genomic DNA, (C) DNA integrity number (DIN), and (D) A260:A280 purity ratios for samples processed under Objectives 1 to 3, alongside St. Jude Children's Research Hospital (SJ) clinical methylation reference samples. Red, blue, and green dots represent samples from Hospital de Amor (HA) affiliate centers, HA, and SJ, respectively. Dashed red lines indicate expected or acceptable thresholds for each metric. HA samples exhibited distribution patterns comparable to those of SJ clinical samples.

DNA mean fragment sizes and DIN distribution in the study samples were comparable to or exceeded those observed in SJCRH clinical samples (Figure 2, B and C). These metrics, which indicate the average physical length of nucleic acid fragments, were not measured for Objective 1 samples, as these had already been generated at HA for a prior research study.

The A₂₆₀:A₂₈₀ ratio measures DNA purity, with an optimal value ~1.8; deviations indicate potential impurities. However, as this metric did not affect methylation classifier performance during assay validation, no specific acceptability cutoff was established. For this study, the A₂₆₀:A₂₈₀ distribution of study samples was compared to SJCRH samples and found to be comparable (Figure 2, D).

No significant differences in quality metrics were observed between HA and its affiliated centers.

Methylation Testing Metrics.—SJCRH-processed samples showed higher bisulfite conversion (Supplemental Figure 1) and median intensity values (Supplemental Figure 2, A through F) than HA-tested study samples (Kruskal-Wallis $P < .001$). These differences likely reflect differences in scanner instruments between sites (iScan at SJCRH versus Next-Seq 550 at HA). This is further supported by the observation that SJCRH samples sent to HA for methylation array testing exhibited low median methylated and unmethylated probe

intensities (Supplemental Figure 2, C; blue dots below the diagonal dashed line), despite having previously passed this QC metric in the SJCRH clinical laboratory. The β value distributions of the study samples closely resembled those of the SJCRH reference control samples, despite the lower peak amplitudes observed in HA and affiliate center samples (Supplemental Figure 3, A through D), indicating that bisulfite conversion was effective even in the context of reduced bisulfite conversion metrics (Supplemental Figure 2, A through F). Noteworthy, samples from affiliated sites showed a slightly skewed β value distribution (Supplemental Figure 3, D). Given that their overall quality metrics were acceptable, the specific reason for this pattern cannot be definitively determined.

Classifier Performance

Objective 1 Cohort.—Confident classifications (calibrated score ≥ 0.9) matching the histologic neuropathology diagnosis were achieved in 38 of the 45 study samples (84%) (Table 3), with all control samples correctly classified.

Review of the ancillary testing and QC data demonstrated that the classifier calls were correct or explainable in 6 of those 7 discordant samples (Table 4). One unclassified sample was histologically consistent with a transitional meningioma, which is represented in MLPNET 4.0. The reason for its subthreshold

Table 3. Summary of Quality Metrics and Tumor Classification Results Across Study Objectives

Quality Metrics	Samples Meeting QC, No. (%)	Tumor Family (Histology)	Number of Samples	Classification Agreement, No.	Classifier Call for Discordant Classifications	Total Percentage Agreement, No. (%)
Objective 1 (N = 45)						
Tumor purity	40/45 (89)	Embryonal	19	16	Other (2) Embryonal (1)	38/45 (84)
DNA concentration	45/45 (100)	Meningioma	17	16	Subthreshold (1)	
Bisulfite conversion	35/45 (78)	HGG	5	5	NA	
		LGG	2	1	LGG (1)	
Median intensity	20/45 (44)	Ependymoma	2	0	Embryonal (1)	
Density of β values	45/45 (100)				Subthreshold (1)	
Median intensities of meth and unmeth probes	20/45 (44)					
Objective 2 (N = 50 unique, N = 56 total)						
Tumor purity	54/56 (96)	Embryonal	6	6	NA	55/56 (98)
DNA concentration	56/56 (100)	Meningioma	2	2	NA	
gDNA	56/56 (100)	HGG	15	15	NA	
DIN	56/56 (100)	LGG	16	15	Subthreshold (1)	
Bisulfite conversion	20/56 (36)	Ependymoma	7	7	NA	
Median intensity	14/56 (25)	CPT	4	4	NA	
Density of β values	56/56 (100)	Schwannoma	2	2	NA	
Median intensities of meth and unmeth probes	14/56 (25)	Other	4	4	NA	
Objective 3 (N = 56)						
Tumor purity	53/56 (95)	Embryonal	11	11	NA	48/56 (86)
DNA concentration	52/56 (93)	Meningioma	6	6	NA	
gDNA	56/56 (100)	HGG	15	13	Subthreshold (2)	
DIN	56/56 (100)	LGG	15	10	Subthreshold (2) HGG (2) Other (1)	
Bisulfite conversion	22/56 (39)	Ependymoma	1	1	NA	
Median intensity	10/56 (18)	CPT	7	7	NA	
Density of β values	56/56 (100)	Other	1	0	Subthreshold (1)	
Median intensities of meth and unmeth probes	10/56 (18)					

Abbreviations: CPT, choroid plexus tumor; DIN, DNA integrity number; gDNA, genomic DNA mean fragment size; HGG, high-grade glioma; LGG, low-grade glioma; meth, methylated; NA, not applicable; QC, quality control; unmeth, unmethylated.

classification is unclear. However, tumors that fall near the boundaries of a classifier's decision space, whether because of limited representation in the training set, model constraints, or inherent rarity, are more likely to yield uncertain or inconsistent classification results. SJCRH's MLPNET 4.0 and Epignostix classifications were concordant in 40 of 45 tested samples (89%). Among these cases, the assigned classifications were consistent between the 2 classifiers, although 4 tumors had Epignostix-calibrated scores below the reportable threshold of 0.9, and 1 meningioma sample had an MLPNET 4.0-calibrated score below 0.9 (Table 4). In these otherwise concordant cases, subthreshold scores may reflect reduced classifier confidence related to classifier-specific characteristics or biologic heterogeneity rather than true classification discordance.

Objective 2 Cohort.—All samples except 1 (SJ2) received confident classifications (calibrated score ≥ 0.9) consistent with the histologic neuropathology diagnosis (Table 3).

SJ2 was 1 of the 25 samples provided by SJCRH to HA for this study phase. It was originally classified by a previous version of SJCRH's classifier (MLPNET1.0) as low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma with a calibrated score of 0.99. MLPNET4.0 generated the same classification but below reportable threshold (0.77). MLPNET1.0 used a different architecture than MLPNET4.0 (neural network versus kernel machine, respectively) and demonstrated lower specificity and higher recall, which likely accounts for the observed findings. Histologically, this tumor was diagnosed as a ganglioglioma, CNS World Health Organization grade 1, and the Epignostix classifier also failed to produce an above-threshold classification. This case may therefore represent an edge case for multiple classifiers. All other samples showed concordant classifications between the MLPNET 4.0 and Epignostix classifiers.

Table 4. Summary of Histologic Diagnoses, MLPNET4.0 Classifier Calls, and Ancillary Testing for Samples With Histology-Classifier Discordance Across Study Objectives

Histologic Diagnosis	MLPNET4.0 Classifier Call	Ancillary Test Results	Agreement of MLPNET4.0 With Ancillary Testing Results
Objective 1			
Medulloblastoma, NOS	ETMR	C19MC amplification Unclassified result on NanoString nCounter medulloblastoma Epignostix brain classifier v12.8 call: ETMR (subthreshold)	Yes
Medulloblastoma, NOS	Ewing sarcoma	<i>EWSR1::CREM</i> fusion detected by targeted fusion sequencing panel Epignostix brain classifier v12.8 call: Ewing sarcoma (subthreshold)	Yes
Medulloblastoma, NOS	Papillary tumor of the pineal region	Unclassified result on NanoString nCounter medulloblastoma subtyping assay Heterogeneous positivity for CK8/18 IHC Epignostix brain classifier v12.8 call: papillary tumor of the pineal region	Yes
Meningioma	Meningioma (subthreshold)	Epignostix brain classifier v12.8 call: meningioma	Partial
Pleomorphic xanthoastrocytoma	Low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma	Epignostix brain classifier v12.8 call: supratentorial pilocytic astrocytoma (subthreshold) Estimated tumor purity ~40%. MLPNET4.0 classification is explainable from sample's low tumor purity. Similar "misclassification" patterns have been observed in validation studies conducted at our institution	No
Anaplastic ependymoma	Medulloblastoma, SHH	Classified as medulloblastoma, SHH on NanoString nCounter medulloblastoma subtyping assay Epignostix brain classifier v12.8 call: medulloblastoma, SHH	Yes
Anaplastic ependymoma	Unclassified (subthreshold)	Epignostix brain classifier v12.8 call: neuroepithelial tumor, <i>PLAGL1</i> -fused <i>PLAGL1</i> -fused tumors are not included in the MLPNET4.0 classifier reference training cohort, therefore it is not expected to classify with a reportable score	Yes
Objective 2			
Ganglioglioma	Low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma (subthreshold)	Epignostix brain classifier v12.8 call: pilocytic astrocytoma, hemispheric (subthreshold) MLPNET1.0 call: low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma	Partial
Objective 3			
Glioblastoma	Unclassified (subthreshold)	Epignostix brain classifier v12.8 call: unclassified (subthreshold) Estimated tumor purity: 60%	Yes
Diffuse pediatric-type high-grade glioma, MAPK-altered	Unclassified (subthreshold)	Epignostix brain classifier v12.8 call: unclassified (subthreshold) Estimated tumor purity: 60% <i>BRAF</i> V600E variant detected by targeted sequencing	Yes
Low-grade astrocytic glioma, NOS	Unclassified (subthreshold)	Epignostix brain classifier v12.8 call: unclassified (subthreshold)	Yes
Low-grade astrocytic glioma, NOS	Unclassified (subthreshold)	Epignostix brain classifier v12.8 call: unclassified (subthreshold) <i>IDH1</i> and <i>IDH2</i> pathogenic variants not detected by targeted sequencing	Yes

Table 4. Continued

Histologic Diagnosis	MLPNET4.0 Classifier Call	Ancillary Test Results	Agreement of MLPNET4.0 With Ancillary Testing Results
Low-grade astrocytic glioma, NOS	Glioblastoma, <i>IDH</i> -wild type	Epignostix brain classifier v12.8 call: unclassified (subthreshold) <i>IDH1</i> R132H IHC: negative <i>TERT</i> c.-124C>T (C228T) promoter mutation detected by targeted sequencing	Yes
Low-grade astrocytic glioma, NOS	Glioblastoma, <i>IDH</i> -wild type	Epignostix brain classifier v12.8 call: unclassified (subthreshold) <i>TERT</i> c.-124C>T (C228T) promoter mutation detected by targeted sequencing	Yes
Pilocytic astrocytoma	Control tissue/ reactive tumor microenvironment	Epignostix brain classifier v12.8 call: control tissue/reactive tumor microenvironment (subthreshold) <i>BRAF</i> duplication by FISH (suggestive of <i>KIAA1549::BRAF</i> fusion)	Partial
Intracranial mesenchymal tumor with <i>FET::CREB</i> fusion	Unclassified (subthreshold)	Epignostix brain classifier v12.8 call: unclassified (subthreshold) <i>EWSR1::CREM</i> fusion detected by targeted fusion sequencing panel This tumor is not included in MLPNET4.0 and Epignostix classifiers. Therefore, it is not expected to classify above reportable threshold	Yes

Abbreviations: *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; C19MC, chromosome 19 microRNA cluster; CK8/18, cytokeratin 8/18; *CREB*, cAMP responsive element binding protein; *CREM*, cAMP responsive element modulator; ETMR, embryonal tumor with multilayered rosettes; *EWSR1*, EWS RNA-binding protein 1; *FET*, FET RNA-binding protein family; FISH, fluorescence in situ hybridization; *IDH*, isocitrate dehydrogenase; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; NOS, not otherwise specified; *PLAGL1*, PLAG1-like zinc finger 1; SHH: sonic hedgehog; *TERT*, telomerase reverse transcriptase.

All samples tested in duplicate, both within and across runs, yielded concordant results consistent with the expected histologic diagnosis.

Objective 3 Cohort.—The classifier produced confident classifications that matched the histopathologic diagnosis in 48 of the 56 study samples (86%) (Table 3).

Of those, 2 samples that were histologically consistent with low-grade tumors were classified as glioblastoma, *IDH* wild type (Table 4). Both samples had acceptable tumor purity (70%) and met other quality metrics. Despite adequate tumor cell content, 1 sample—histologically consistent with a pilocytic astrocytoma—was classified by methylation profiling as control tissue/reactive tumor microenvironment. The causes of these misclassifications are unclear; however, in clinical practice they would be unlikely to affect patient management, as methylation-based classifications are interpreted in conjunction with clinical information, histopathology, and ancillary laboratory data to formulate an integrated diagnosis.

The remaining 5 discordant results reflected subthreshold classifications in samples with low-to-borderline tumor purity, a tumor type not represented in the classifier's training set (intracranial mesenchymal tumors with *FET::CREB* [*FET::responsive element binding protein*] fusion), and cases with unclear contributing factors (Table 4). All control samples were correctly classified.

MLPNET 4.0 and Epignostix classifications were concordant in 43 of 56 study samples (77%) and in 4 of 8 control samples (50%). In the discordant cases, the Epignostix classifier yielded subthreshold scores for classifications that otherwise aligned with the MLPNET 4.0 calls, supporting the robustness of the SJCRH classifier in this data set.

Association Between Quality Metrics and Classifier Performance

No statistically significant correlation was observed between SJCRH classifier and histopathology concordance and any preanalytic (tumor purity, DNA input, batch ID, sample provenance) or methylation testing metric (bisulfite conversion), consistent with classifier robustness and the good quality of the samples and data generated by HA (data not shown).

DISCUSSION

This study represents a focused assessment of the feasibility of distributive DNA methylation profiling between a US-based reference center (SJCRH) and a single international site (HA) from a low- and middle-income country. The primary objective was to evaluate whether the sample and data quality generated at HA were sufficient to support accurate tumor classification using the SJCRH methylation classifier, particularly across a range of tumor types encountered in this patient population. While the study did not assess economic considerations or the comprehensive implementation of a clinical-grade methylation profiling platform at HA, it provides a framework for understanding the potential for international distributive testing and the factors that influence its success. The future establishment of such a platform at HA would require careful planning by the molecular diagnostics laboratory leadership, taking into account workflow design, cost, reimbursement, data governance requirements, operational considerations, and regulatory factors.

Our findings demonstrate that DNA extracted and processed at the international site met quality standards

comparable to those observed in SJCRH clinical samples. Importantly, this quality was sufficient to support robust classifier performance, with high accuracy and recall in the evaluated samples. Minor differences in methylation testing metrics were observed, attributable to instrumentation differences between sites; however, these differences did not compromise classifier performance. These findings not only demonstrate the robustness of the classifier but also provide important evidence that site-specific quality improvements were not required for HA to generate acceptable data. Because Objectives 1 and 2 relied on samples already present in their biobank, the results reflect routine specimen processing and workflow practices, without pretest coaching or operational changes. Despite this, HA consistently achieved acceptable preanalytic quality, indicating that the classifier performs reliably under real-world laboratory conditions and that, for this site, modification of specimen processing procedures was not necessary.

The utility of DNA methylation profiling in neuropathology is well established, enhancing diagnostic accuracy and enabling clinically relevant molecular subtyping.^{6–14} For centers that do not have an in-house classifier or the resources to develop one, a distributive testing model offers a viable alternative. Such an approach is recognized and accepted by the College of American Pathologists, even in cross-national contexts, provided that quality and procedural requirements are met.

Although limited to a single international site, this study offers meaningful insight into the feasibility of distributive DNA methylation testing and outlines practical considerations for specimen handling, quality review, and classifier performance across diverse patient populations. Importantly, beyond serving as a case example, this work also establishes a framework for how such evaluations can be conducted at future sites and with future assays. Together, these findings suggest that, with appropriate oversight and quality assurance, international implementation of methylation-based tumor classification is feasible and can support reliable diagnostic outcomes.

This work was partially supported by the Brazilian Ministry of Health (MoH), National Council for Scientific and Technological

Development – CNPq, Brazil, and Brazilian National Program of Genomics and Precision Health – Genomas Brasil (MS-SECTICS-Decit/CNPq No. 16/2023 – INOVACRIANÇA – Inovação terapêutica em crianças com tumores sólidos: uma jornada de precisão), and MCTI/CNPq No. 16/2024.

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