

MGMT Methylation Detection:

A MS-MLPA and qMSP comparison

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Trillium Health Partners
Better Together

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DISCLOSURE

STATEMENT

- Sponsorship to present at AMP 2019 conference
- No personal financial interests to disclose

OUTLINE

FOR TODAY'S PRESENTATION

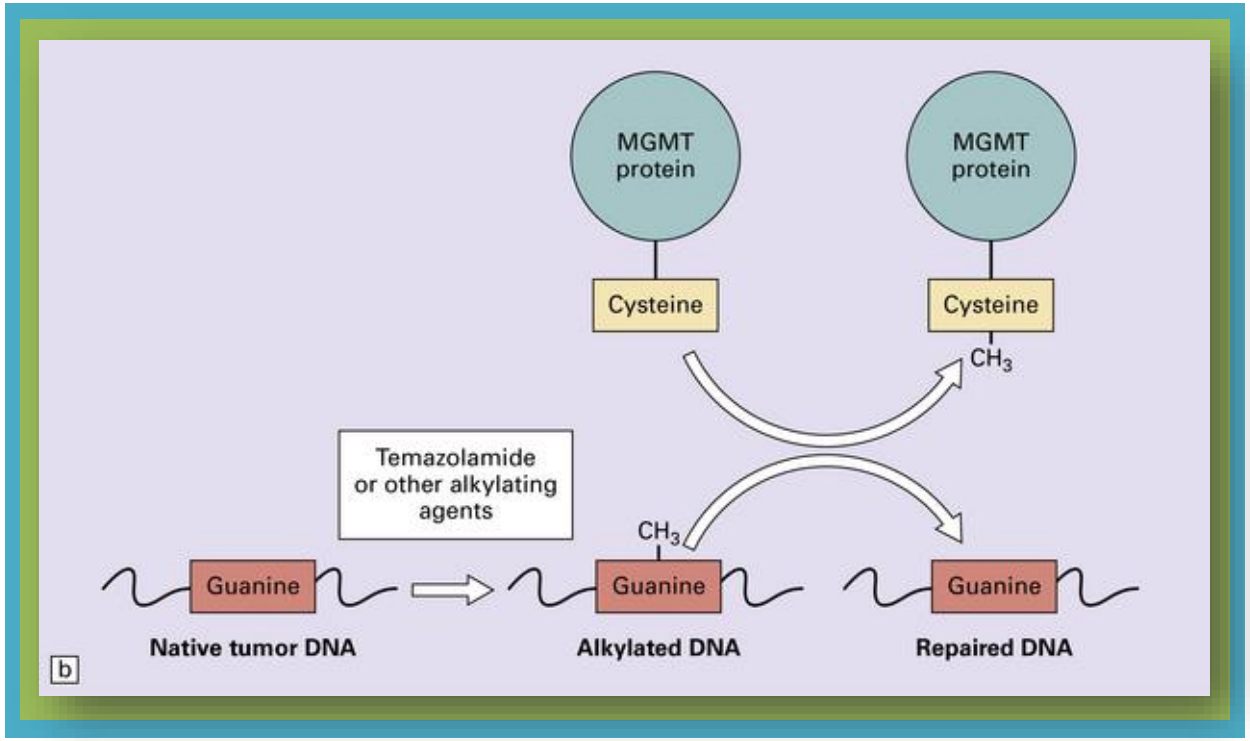
- MGMT Overview
- Testing Methodologies
- Methylation Specific MLPA
- EntroGen Methylation Detection Kit MGMT-RT44
- Validation Goals
- Validation Results
- Conclusion

MGMT PROTEIN

LOCATION & FUNCTION

- O^6-methylguanine DNA methyltransferase
- Enzymatic DNA repair mechanism
- Protein deactivated upon DNA repair

(Vlassenbroeck, 2008)



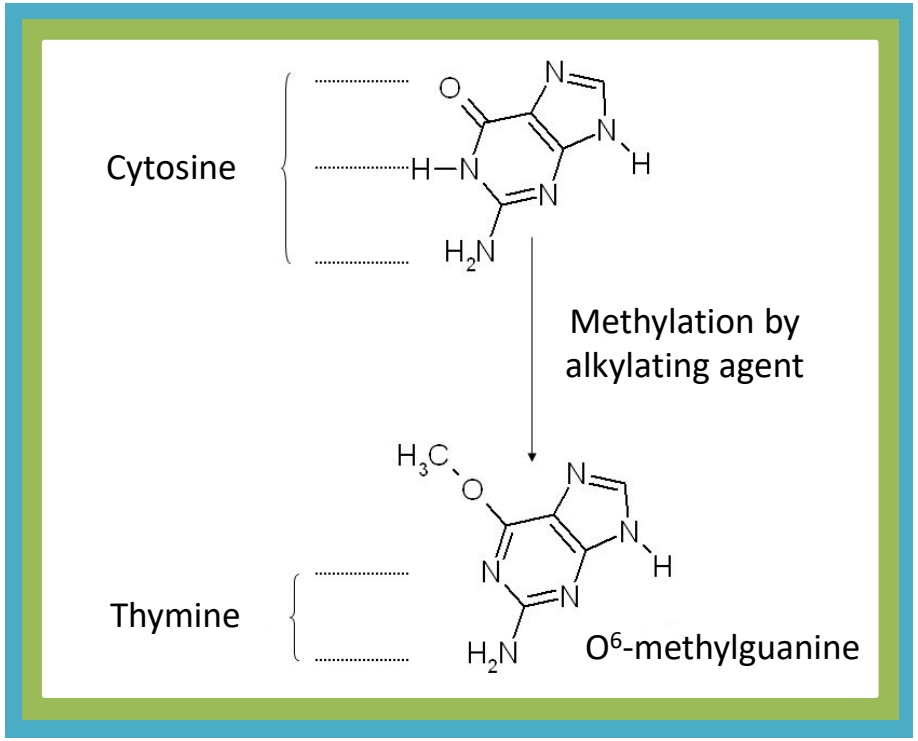
Promoter methylation of MGMT - Retrieved from; https://clinicalgate.com/astrocytic-neoplasms/ (Sept 2019)

MGMT & CANCER

UNMETHYLATED VS METHYLATED MGMT

- Alkylating agents are common cancer treatments
  - Damages DNA by attaching alkyl group to guanine
- Active, unmethylated MGMT negates desired treatment effect by performing DNA repair
- Methylated MGMT reduces enzyme available for DNA repair
  - strong prognosticator and treatment predictor for malignant gliomas(glioblastomas)

(Vlassenbroeck, 2008)



MGMT METHYLATION

SCHEMATIC OF MGMT GENE

Schematic of MGMT gene highlighting the CpG island and known methylation regions (DMR1, DMR2)

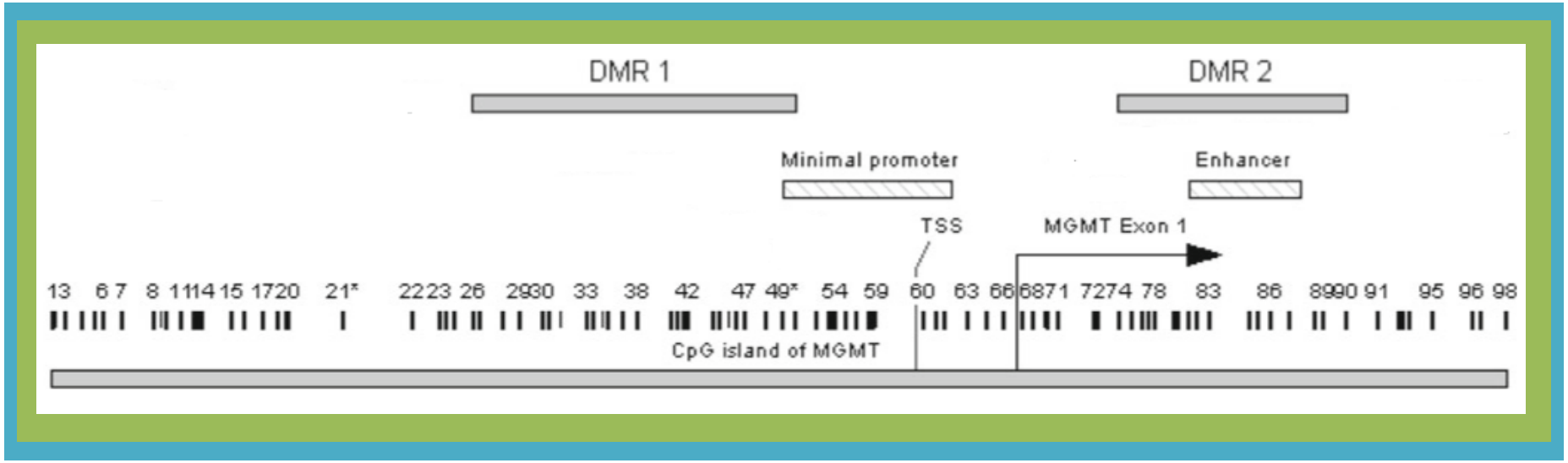
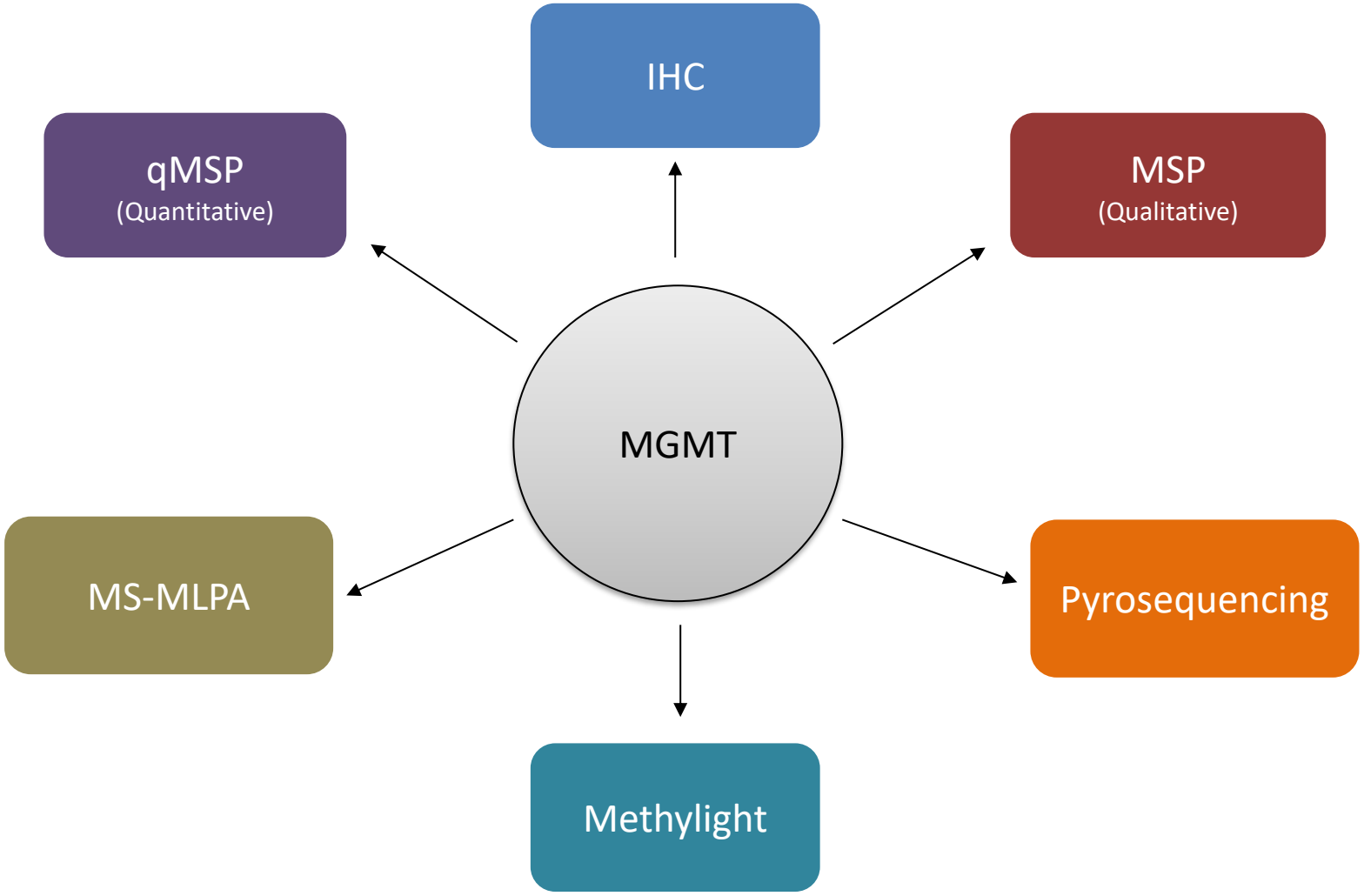


Diagram of MGMT CGI - Modified from Malley, D.S. et al 2011.

DETECTION METHODS

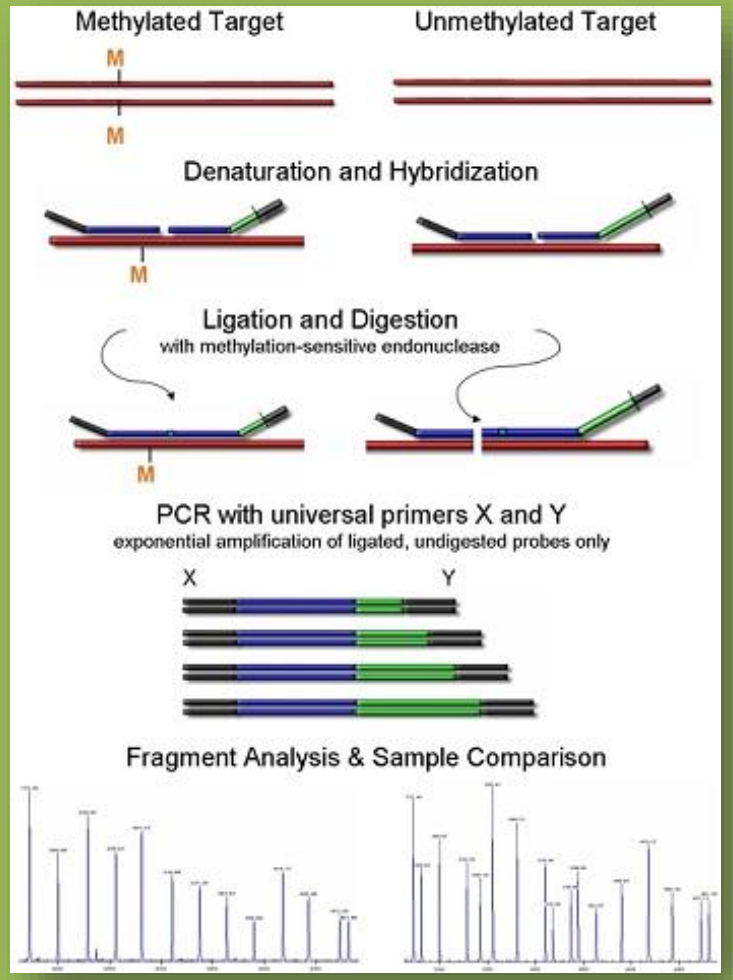
LOCATION & FUNCTION



PREVIOUS THP METHOD

UTILIZING MS-MLPA

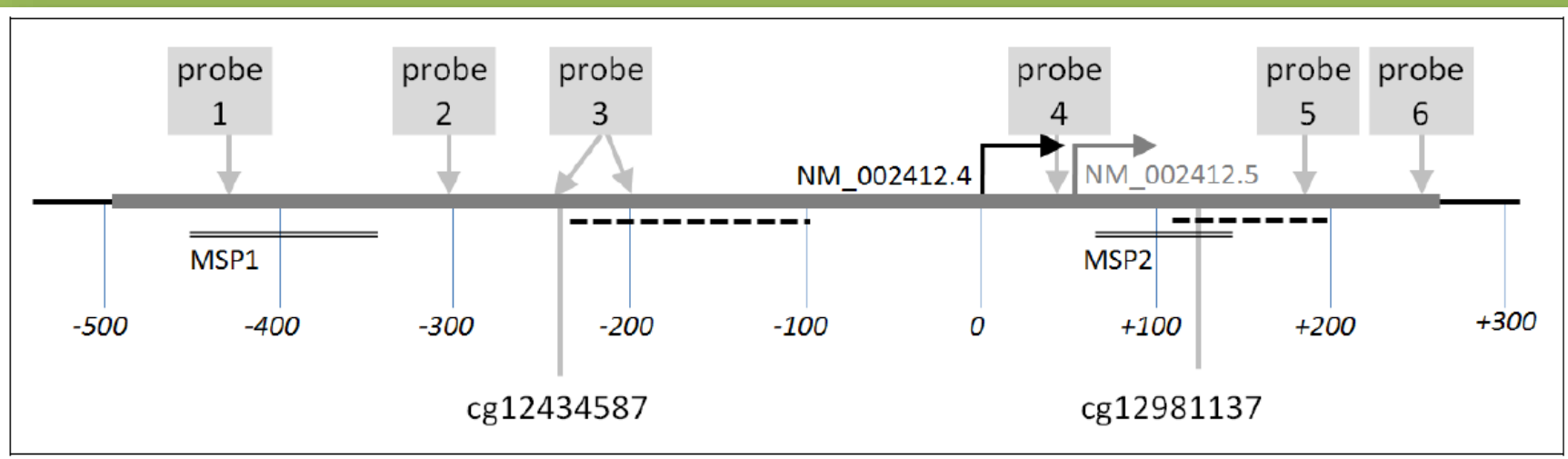
- MS-MLPA @ THP since 2014
- Average 85 samples per year
- Median age 63
- Age range 22-86



MS-MLPA Image from MRC-Holland Website; 19/09/19: https://www.mlpa.com/WebForms/WebFormMain.aspx?Tag=_zjCZBtdOUyAt3KF3EwRZhMUCJLqQzwZq_fiQWQTnAP-0V13AZUzpnKmyAPu7IsFt

MS-MLPA

PROBE LOCATIONS



CpG island in *MGMT* promoter region

Methylation hotspots: (-249 to -103 nt) and (+107 to +196 nt) from NM_002412.4 transcription start site (Qian XC and Brent TP. 1997).

cg12434587 & cg12981137 — CpG sites shown to be important for classification and prognosis (Bady et al. 2012).

MSP1 and MSP2 sites according to van Nifterik KA et al. (2010).

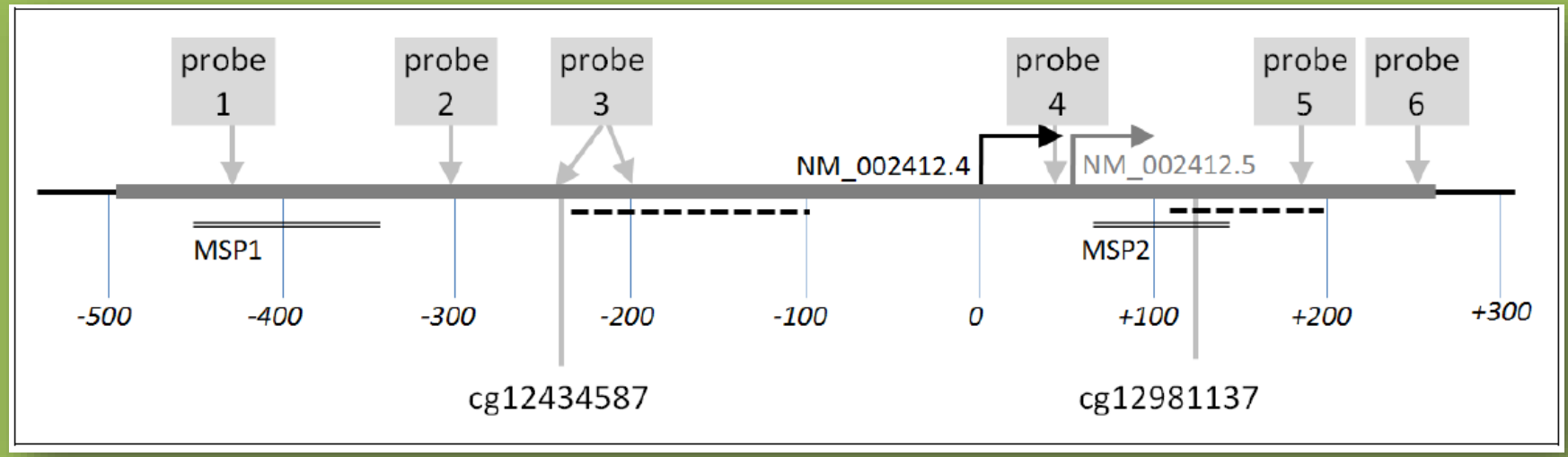
Image adapted from MRC Holland MS-MLPA ME012 Product Description vA1-01

MS-MLPA ANALYSIS

CUT-OFFS & ALGORITHM

MLPA Cut-offs & Algorithm			
Primary Probes	**Probe 2 190nt (>0.12)**	**Probe 3 126nt (>0.12)**	**Probe 4 160nt (>0.12)**
Secondary Probes	**Probe 1 141nt (>0.60)**	**Probe 5 215nt (>0.48)**	**Probe 6 172nt (>0.48)**

Probe Scenarios	Final Result
Positive in primary 3 and or 4, and average of all primary probes >0.1	Methylated
Primary markers are >0, <0.1 but have contiguous support with secondary markers	Adjudicate
Anything else	Unmethylated



CHALLENGES

RUNNING MS-MLPA

- High backgrounds for most of the probes
- Equivocal results and ‘subjective’ judgment calls
- Difficult to establish low, moderate and high qualifiers for methylation status in reports
- Infrequently used in CAP surveys – difficult to assess and understand discrepant results
- Constant need of normal brain tissue as reference samples in each run

Other options easy to implement?

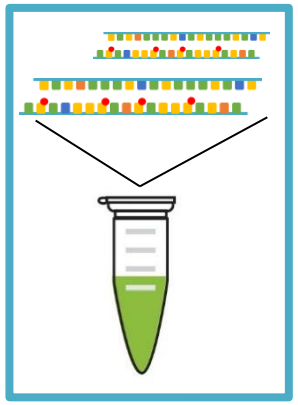
ENTROGEN'S MGMT-RT44

FEATURES & BENEFITS

- Real-time PCR assay – rapid & easy to use
- Semi-quantitative method
- Methylation specific primers targeting clinically relevant DMR2
- Includes standards
- Includes internal control gene for normalization

WORKFLOW

ENTROGEN'S MGMT METHYLATION DETECTION KIT



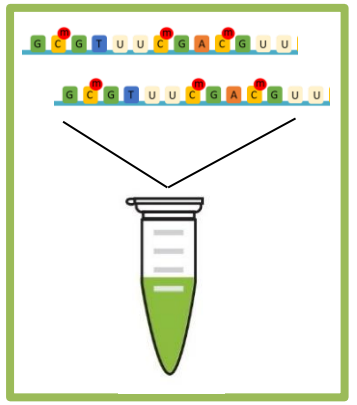
Sample DNA

Bisulfite Conversion

(reagents not included)

DNA Clean-Up

(reagents not included)



Bisulfite treated DNA

PCR set-up



PCR run

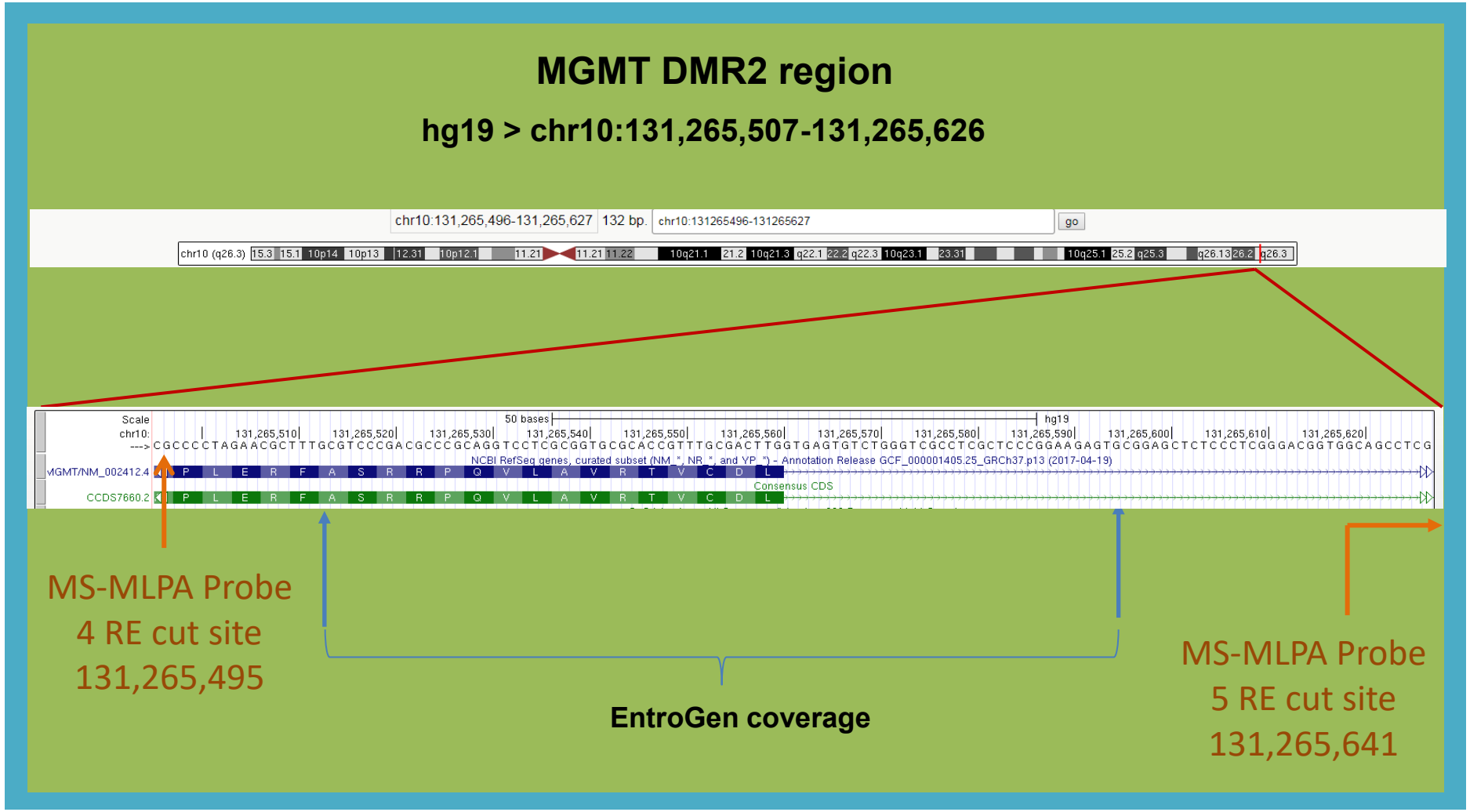


Data Analysis

Results:
-MGMT copy number
-ActB (IC) copy number

MS-MLPA vs qMSP

REGION OF INTEREST



VALIDATION PLAN

ENTROGEN'S MGMT METHYLATION DETECTION KIT

- Comparison to previous MS-MLPA results
- Seven runs total
  - Runs 1-3 processed same samples on separate days
  - Runs 4-6 processed another set of samples on separate days
  - Run 7 to establish assay linearity
- Total Validation
  - Roche LC480
  - 6 dilutions of methylated control (Zymo D5014)
  - 12 Patient samples & 8 reference samples

ACCEPTANCE CRITERIA

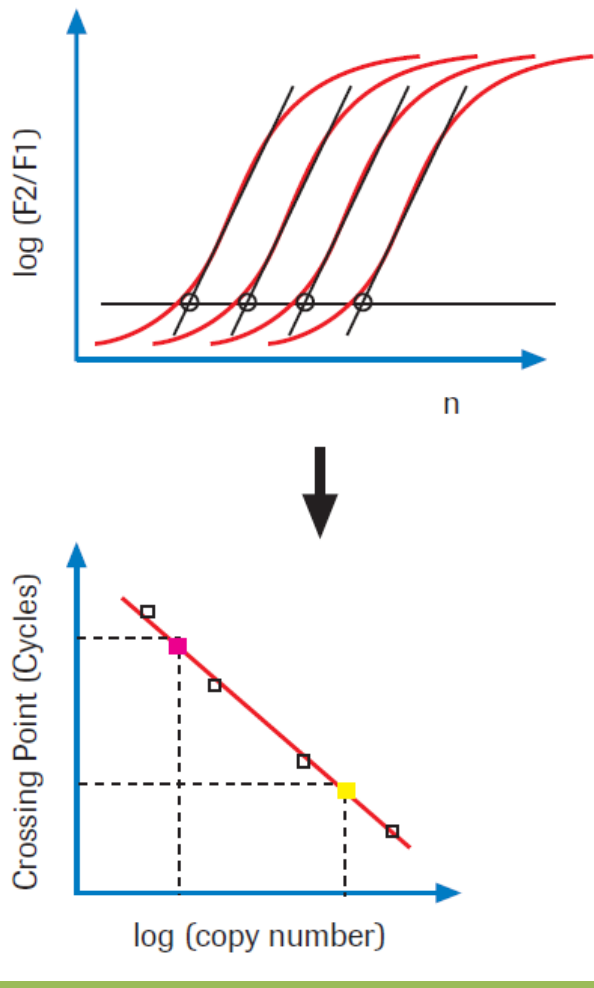
ENTROGEN'S MGMT METHYLATION DETECTION KIT

- Reference samples need to be accurately assessed
- High degree of concordance between patient samples for MS-MLPA and qMSP
- Ease of use and workflow management

VALIDATION GOALS

ENTROGEN'S MGMT METHYLATION DETECTION KIT

- Set parameters for assay on Roche LC480
- Determine values for:
  - Cycle range
  - Noiseband
  - Threshold
- Evaluate LC480 software calculations for variability
- Determine clinical cut-off

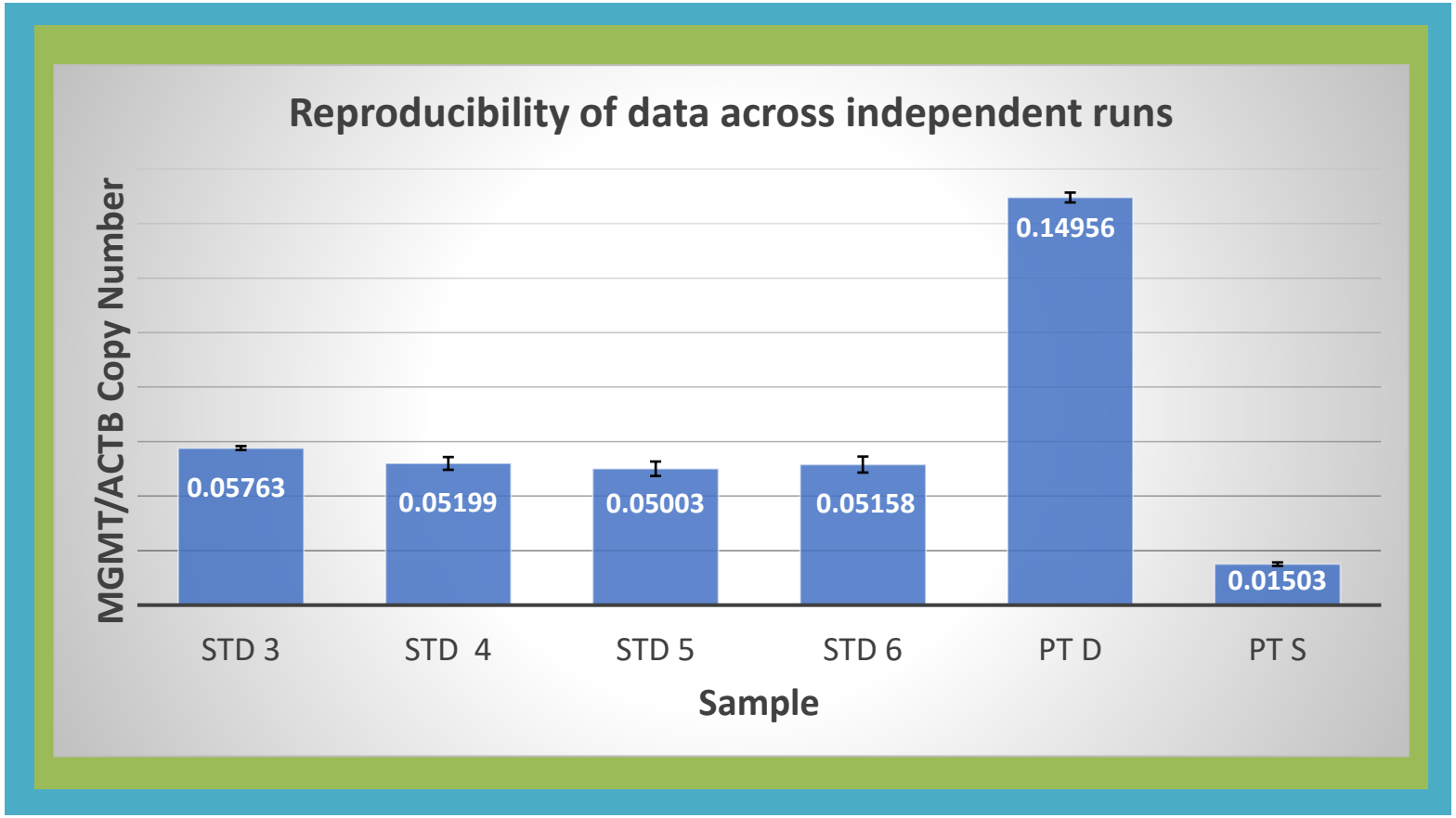


Adapted from Roche LC480 Operator's Manual v1.5

VALIDATION RESULTS

ENTROGEN'S MGMT METHYLATION DETECTION KIT

Intra- / Inter- Run Variability



VALIDATION RESULTS

FINAL PERCENTAGE VARIATION

Intra-Run Percent Methylation Results

	100% Ctrl	75% Ctrl	50% Ctrl	25% Ctrl	10% Ctrl	0% Ctrl	Patient Z	Patient S
Run 3	96.03	63.93	40.82	17.10	5.74	0		
Run 4	120.25	94.01	20.34	30.22	8.56	0	4.61	2.69
Run 5	112.31	82.40	53.30	34.95	9.25	0	6.43	1.26
Run 6	116.88	91.06	63.50	28.07	12.30	0	4.51	1.81
Mean	111.37	82.85	52.54	27.59	8.96	0.00	5.18	1.92
SD	10.73	13.54	9.27	7.56	2.69	0	1.08	0.72

What is the clinically relevant cut-off?!?

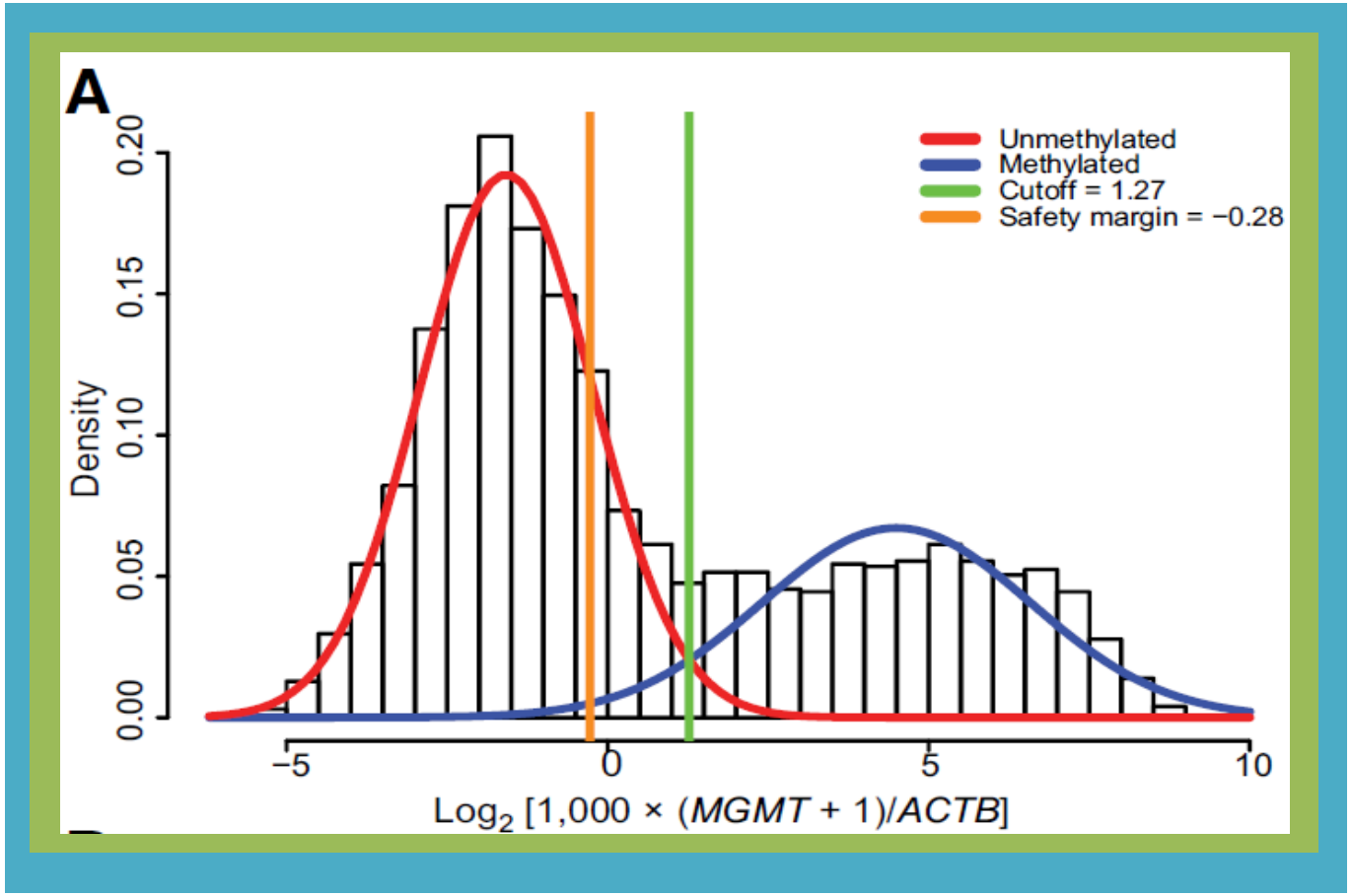
HEGI ET AL. 2019

ESTABLISHING CUTOFFS

- Analysis of 4 randomized trials → 4041 patients
- MGMT methylation assessment by qMSP
- $\log_2(1000 \times \frac{MGMT+1}{ACTB})$
- Unsupervised cut-off: 1.27
- Supervised cut-off: -0.28

ESTABLISHING CUTOFFS

UTILIZING THE GREY-ZONE



Value in utilizing “grey-zone” -0.28>x<1.27

Optimal MGMT methylation cut-off – adapted from (Hegi, 2019)

VALIDATION RESULTS

UTILIZING THE GREY-ZONE

Patient Sample	F	H	N	S	**T**	**V**	W	R	**U**	O	P	Q
Prev MS-MLPA Result	UM	M	UM	M	**UM**	**M**	M	M	**Low M**	UM	M	UM
Hegi et al. Ratio Result	UM	M	UM	M	**M**	**Low M**	M	M	**UM**	UM	M	UM

M = Methylated
UM = Unmethylated

- 100% concordance with reference samples
- High accuracy of patient sample results
  - Data review clarifies apparent discordance of 3 borderline patient samples

CONCLUSION

Determining Factors for Switching Methods

- Hegi et al. 2019 ratio method is easy to implement and concordant with reference samples
- Patient outcome based
- Grey-zone is clinically relevant
- EntroGen MGMT methylation kit provides appropriate output result for clinically relevant analysis
- Highly accurate results for reference material and patient samples
- Reproduceable results
- Consistency between inter- & intra- runs

ACKNOWLEDGMENTS

- EntroGen
- Trillium Health Partners Genetics Laboratory
- Trillium Health Partners Pathologists & Oncologists

REFERENCES

Vlassenbroeck, I. et al. (2008). Validation of real-time Methylation-Specific PCR to determine O6-Methylguanine-DNA Methyltransferase gene promoter methylation in glioma. *Journal of Molecular Diagnostics, 10*(4), 332-337. doi: 10.2353/jmoldx.2008.070169

Malley, D.S. et al. (2011). A distinct region of the MGMT CpG island critical for transcriptional regulation is preferentially methylated in glioblastoma cells and xenografts. *Acta Neuropathologica, 121*, 651-661. doi: 10.1007/s00401-011-0803-5

Hegi, M. E. et al. (2019). MGMT promoter methylation cutoff with safety margin for selecting glioblastoma patients into trials omitting temozolomide: A pooled analysis of four clinical trials. *Clinical Cancer Research, 25*, 1809-1816. doi: 10.1158/1078-0432.CCR-18-3181



Thank You!

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MGMT Methylation: MSPCR vs Pyrosequencing

Data Provided By:

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Carol Beadling, PhD

OHSU Research Assistant Professor

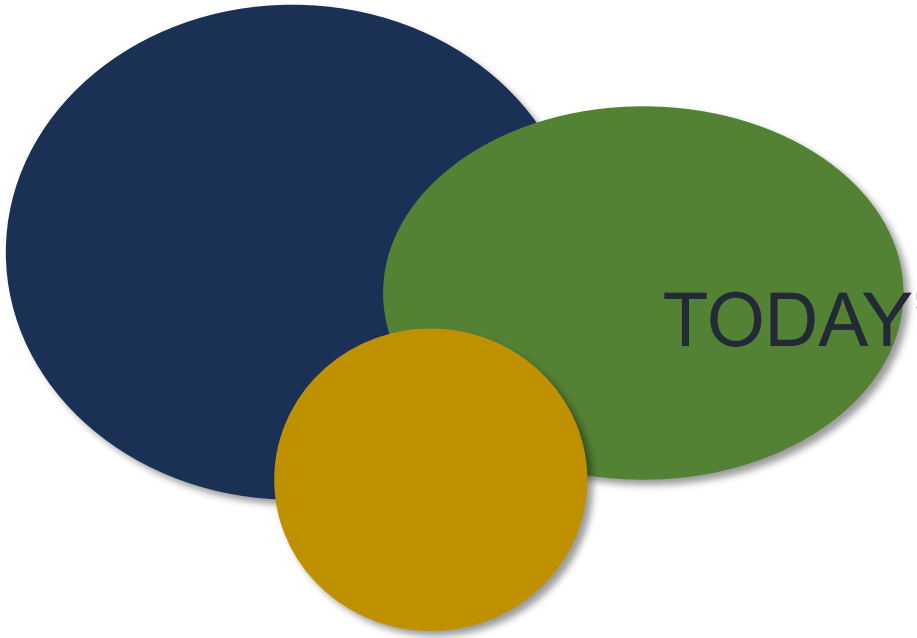
Amy Schilling, BS

OHSU Molecular Technologist

Tanaya Neff, MS

OHSU Laboratory Section Coordinator

TOPIC OF DISCUSSION



TODAY'S DISCUSSION WILL COVER

- MGMT Methylation Detection Kit Technical Overview
- MGMT CpG site coverage for real-time vs. pyrosequencing
- Model distributions to define positive/negative cutoffs
- Correlate classification of samples with previous platform

EntroGen®

Technical Specifications

- Multiplexed, semi-quantitative real-time PCR assay
  - Requires FAM (MGMT) & VIC (ACTB) detection
- Compatible with most real-time PCR instruments
  - Validated on ABI 7500, QuantStudio 5, BioRad CFX96, Roche LightCycler 480

EntroGen®

Workflow

DNA Isolation



Bisulfite Conversion

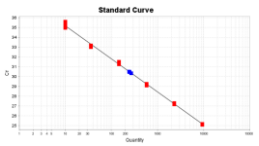


PCR Setup





Data Analysis



Compatible Bisulfite Conversion Kits

EZ DNA Methylation-Lightning Kit
Zymo Research

Processing Time	1.5 hr
Conversion Efficiency	> 99.5 %
Input	100 pg – 2 µg
Input for EntroGen Kit	100-500 ng

EpiTect Fast Bisulfite Conversion Kit
Qiagen

Processing Time	~ 1 hr
Conversion Efficiency	> 99%
Input	1 ng – 2 µg
Input for EntroGen Kit	100-500 ng

Both kits include reagents and consumables for clean-up after conversion

EntroGen®

PCR Plate Setup

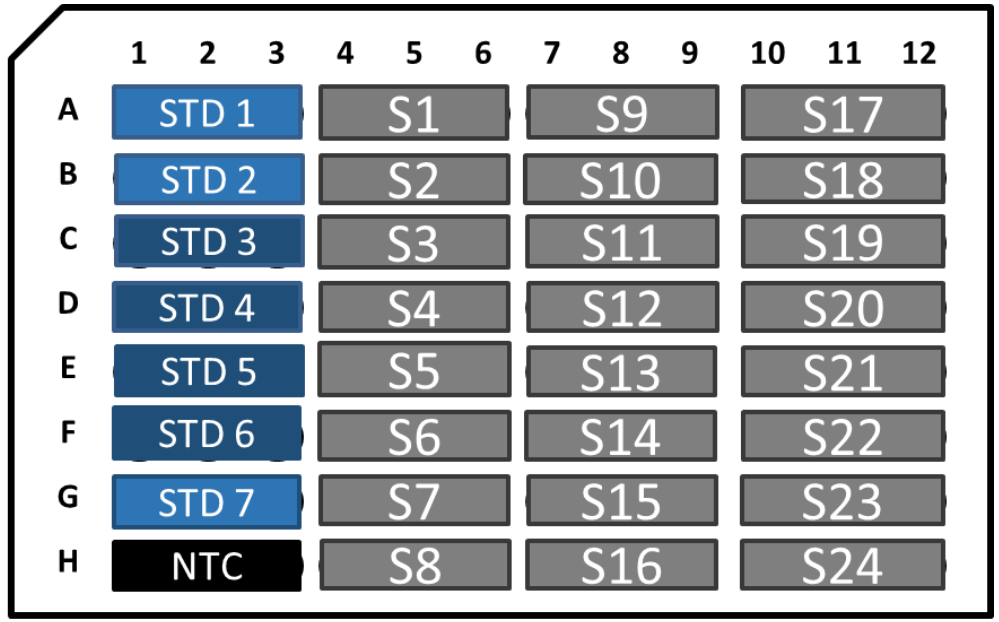
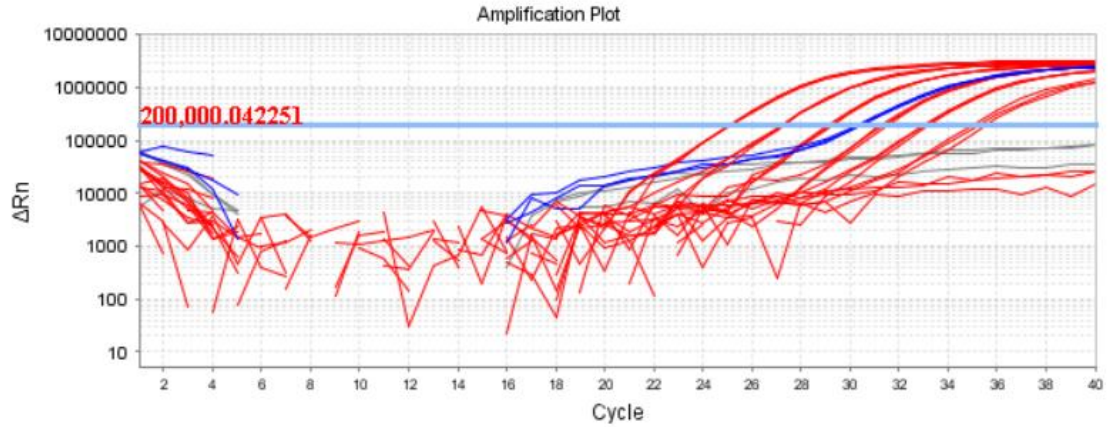
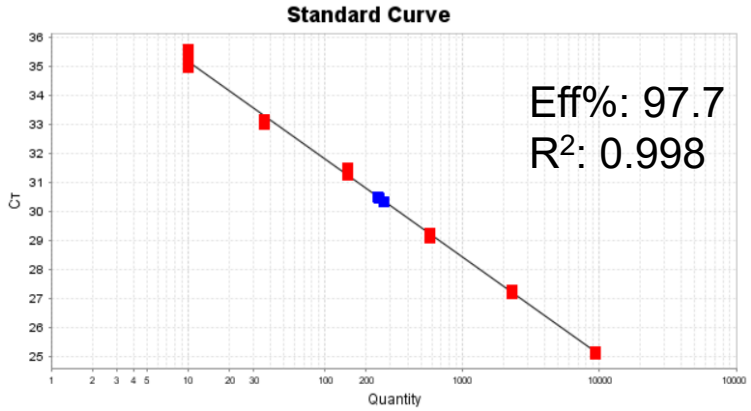


Plate Map Key: MGMT standards | MGMT + ACTB standards | Unknown Samples | No Template Control

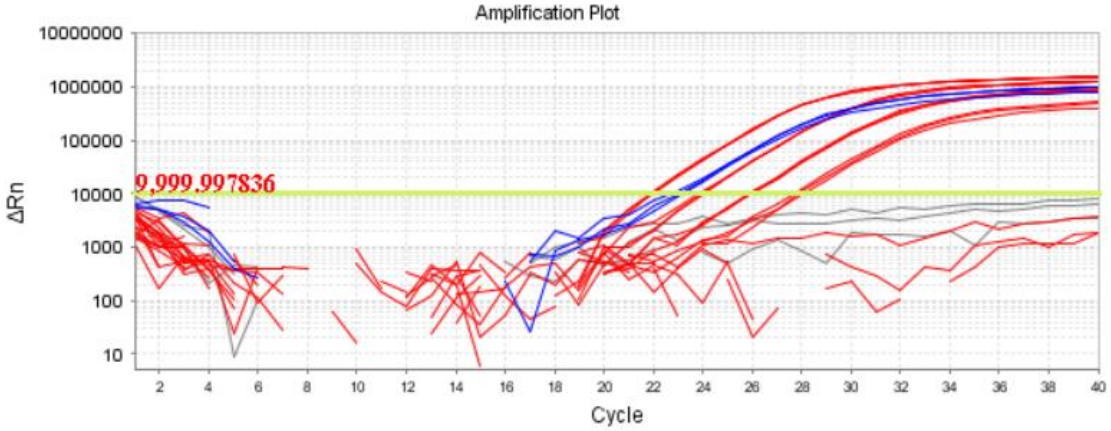
Results

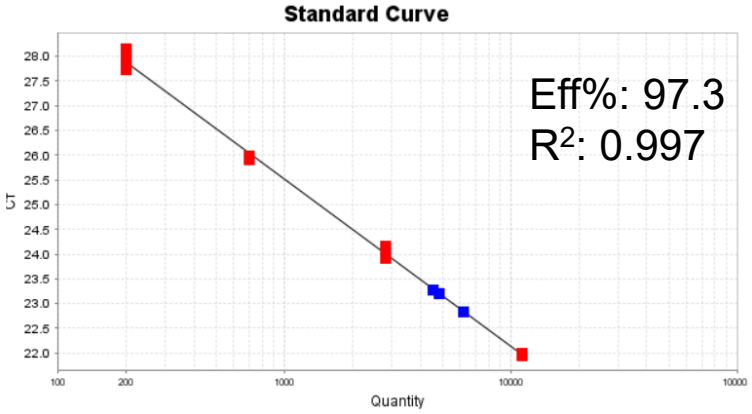
FAM Channel: Methylated MGMT





VIC Channel: Internal Control, ACTB





EntroGen®

Results

$$Methylation\ ratio\ = \frac{(methylated\ MGMT\ copies\ /ACTB\ copies)}{Tumor\ fraction} \times CF$$

CF = Conversion Factor provided by EntroGen to correct for lot-to-lot variation

EntroGen®

Results

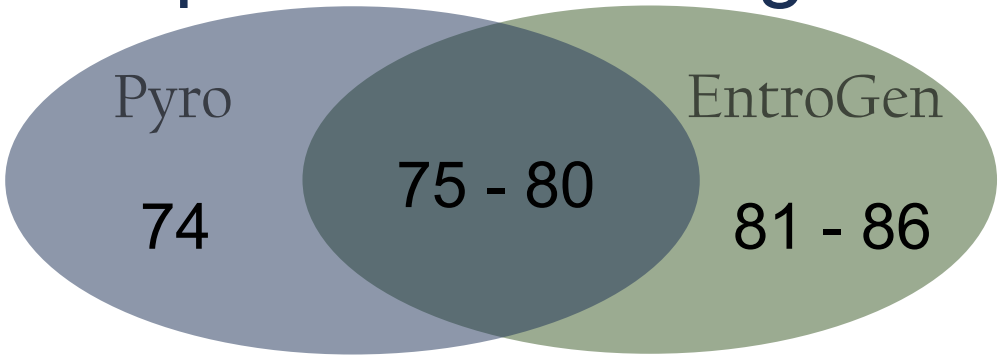
3 ways to calculate Methylation Ratio

- Manually, using copy numbers derived from real-time PCR software
- EntroGen provided macro-enabled Excel worksheet
  - Import comma-delimited Ct values
- EntroGen provided standalone software (EPAS™)
  - Import raw data file from ABI/BioRad instruments

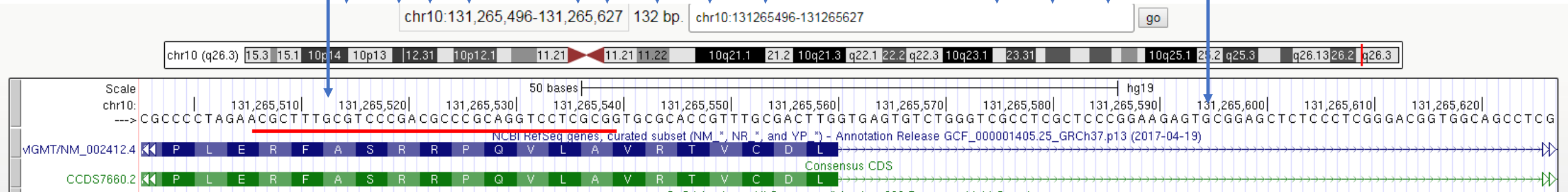
MGMT DMR2 REGION

CpG 73-90
hg19 > chr10:131265496-131265626

CpG Site Coverage



131265513
EntroGen



CpG 73

CpG 74 | CpG 75 | CpG 76 | CpG 77 | CpG 78 | CpG 79 | CpG 80

CpG 90

KDL pyrosequencing assay measures 7 CpG sites

MODELS TO ESTABLISH CUTOFFS

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EntroGen MSPCR Sample Runs	Total (incl. replicate runs)	Unique Samples
All sample runs (includes tumors and controls)	120	88
Tumor sample runs (excludes controls)	93	74
Tumor samples runs with measurable methylation	61	48

- Expect to see distinct grouping of methylated and unmethylated samples
- Let the data distribution show where to draw the cutoff
- Methylated vs unmethylated classifications should match between pyrosequencing and MSPCR, even if percent methylation values do not match

MODELS TO ESTABLISH CUTOFFS

J Mol Diagn. 2008 Jul;10(4):332-7. doi: 10.2353/jmoldx.2008.070169. Epub 2008 Jun 13.

Validation of real-time methylation-specific PCR to determine O6-methylguanine-DNA methyltransferase gene promoter methylation in glioma.

Vlassenbroeck I[1], Califice S, Diserens AC, Migliavacca E, Straub J, Di Stefano I, Moreau F, Hamou MF, Renard I, Delorenzi M, Flamion B, DiGuiseppi J, Bierau K, Hegi ME.

Samples with methylated and unmethylated MGMT segregate into distinct distributions based on methylated MGMT copy number

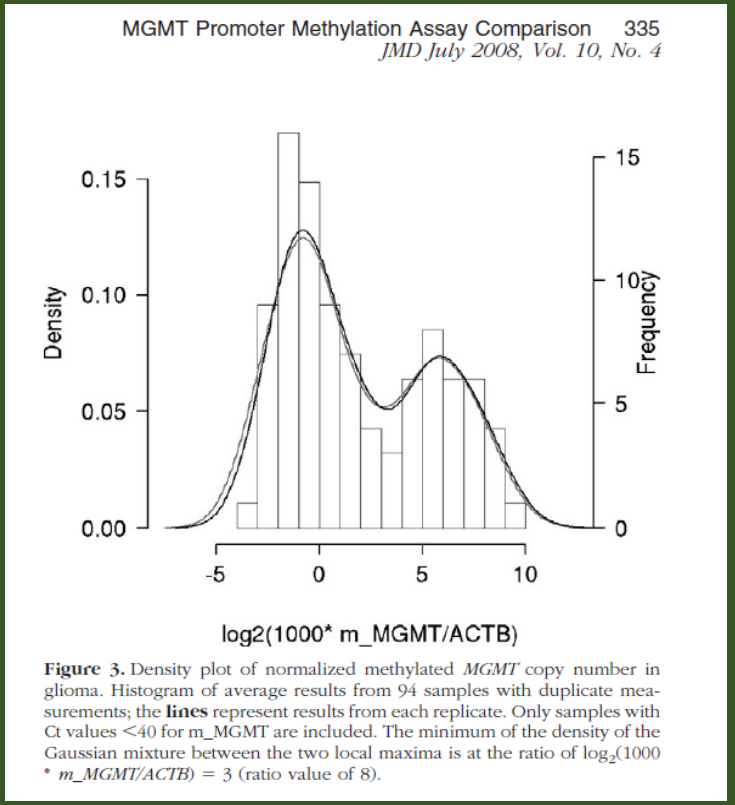


Figure 3. Density plot of normalized methylated *MGMT* copy number in glioma. Histogram of average results from 94 samples with duplicate measurements; the **lines** represent results from each replicate. Only samples with Ct values <40 for m_MGMT are included. The minimum of the density of the Gaussian mixture between the two local maxima is at the ratio of $\log_2(1000 * m_MGMT/ACTB) = 3$ (ratio value of 8).

DISTRIBUTION MODELS

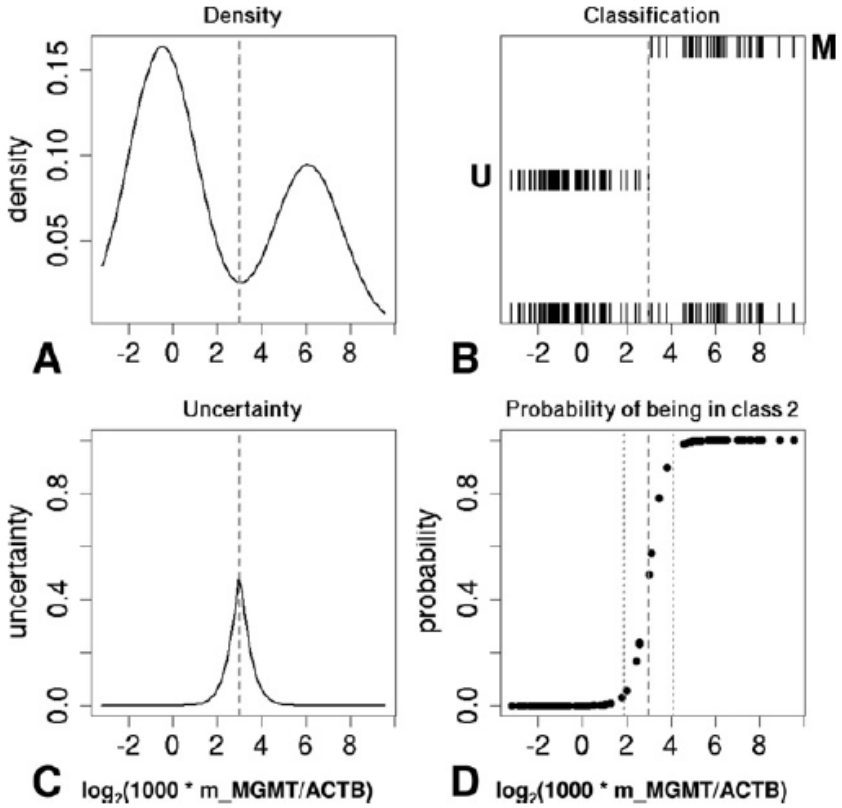
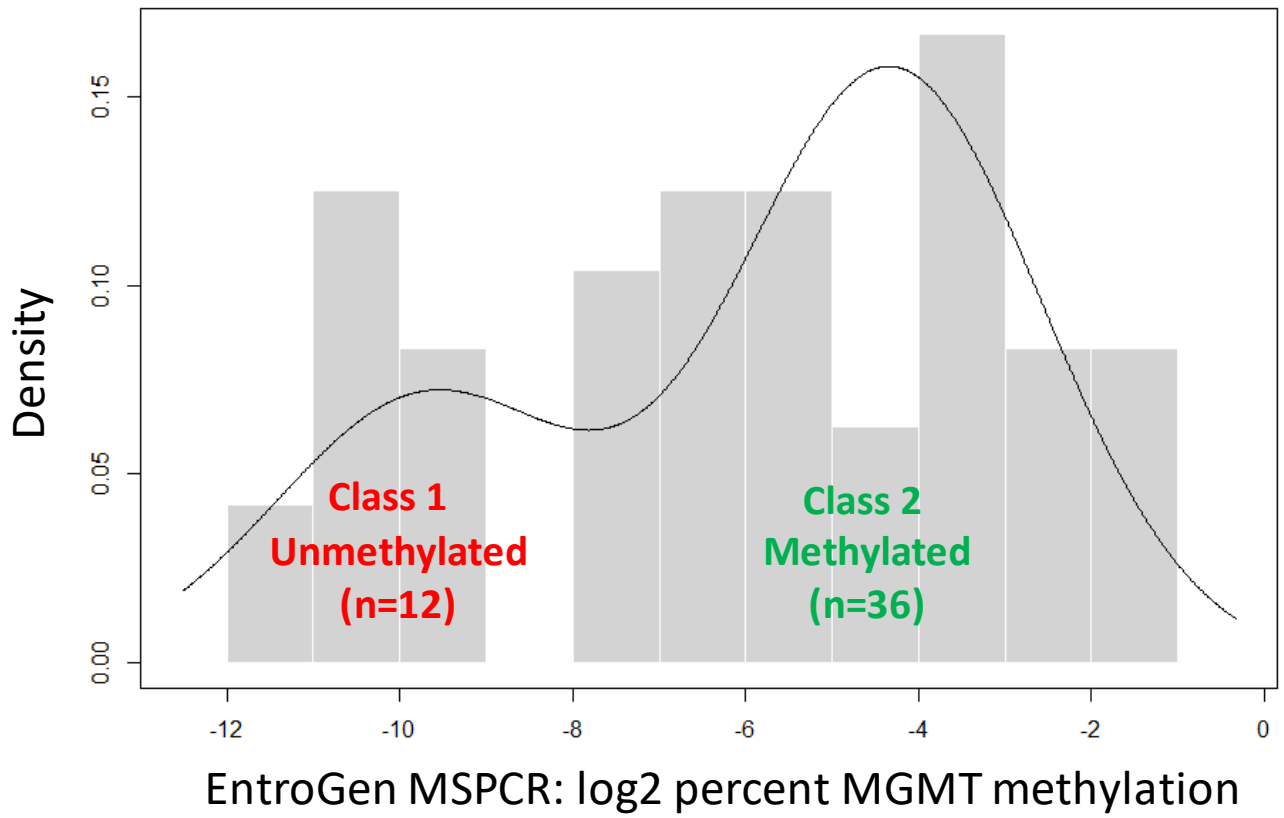


Figure 4. Definition of natural cut-off for methylated *MGMT*. Density (**A**), Classification (**B**), Uncertainty of classification (**C**), and posterior Probability **(D)** of class 2 (promoter methylation) obtained by fitting a mixture model to the average $\log_2(1000 * m_MGMT/ACTB)$. In the classification plot (**B**), all of the data are displayed at the bottom; assignment to the class with highest posterior probability at two different levels is above (**M**, class 2, methylated; **U**, class 1, non-methylated). Uncertainty (**C**) was defined as the smaller of the two posterior probabilities. A gray **dashed line** represents the optimal cutoff according to the selected model ($\log_2$ ratio = 3; ratio value = 8). The region between the gray **dotted lines** (**D**) defines a classification "gray zone"; the thresholds for 95% posterior probability of class 1 and class 2, respectively, are 2 (ratio value = 4) and 4 (ratio value = 16).

Distributions modeled with R mclust

Image from: Validation of real-time methylation-specific PCR to determine O6-methylguanine-DNA methyltransferase gene promoter methylation in glioma Vlassenbroeck, I, et al, J Mol Diagn 2008 Jul

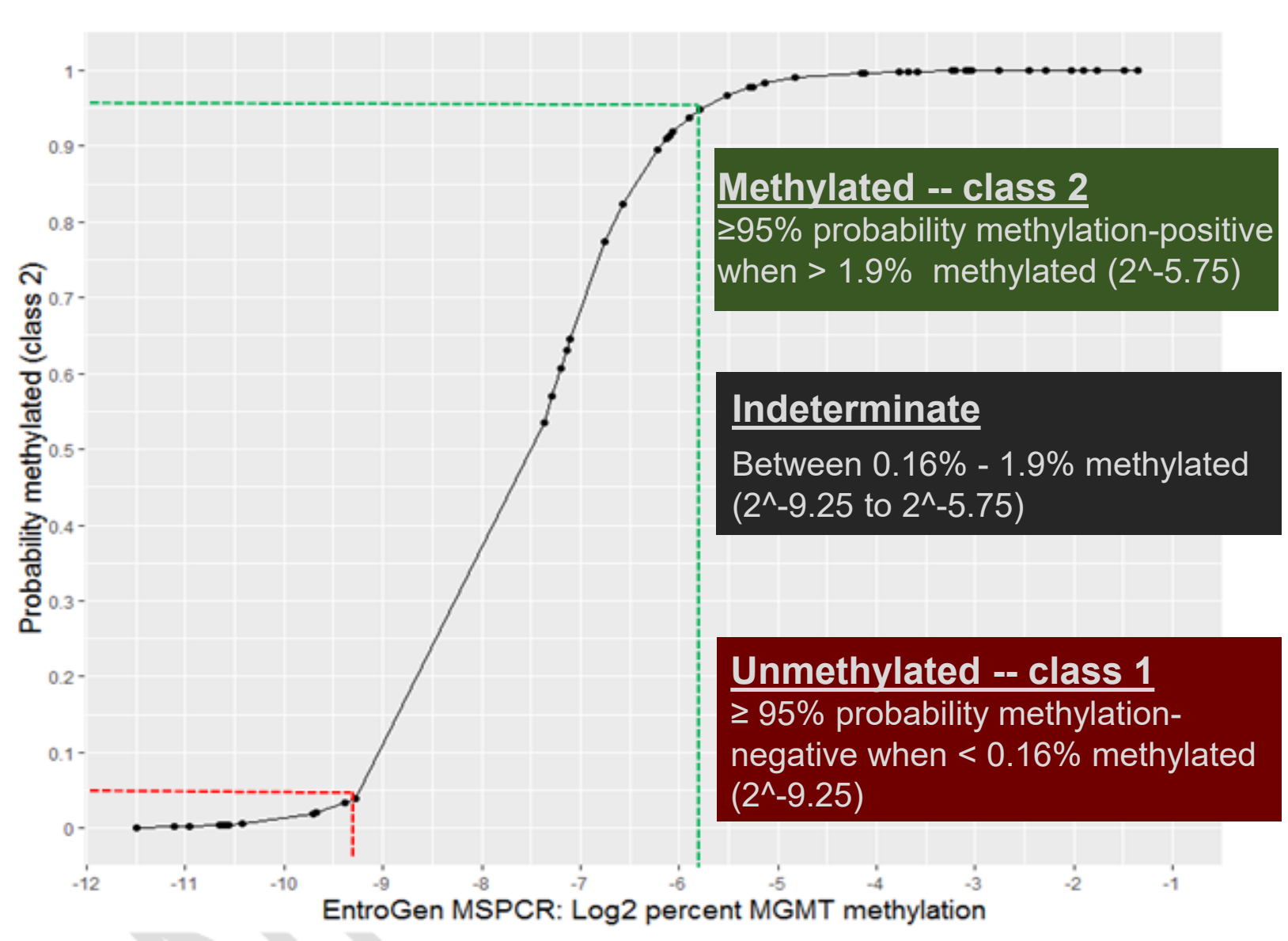
MSPCR BIMODAL DISTRIBUTION MODELS



EntroGen MSPCR density plot: Bimodal distributions of MGMT methylation

- 48 unique samples
- Does not include 26 samples with undetectable MGMT methylation

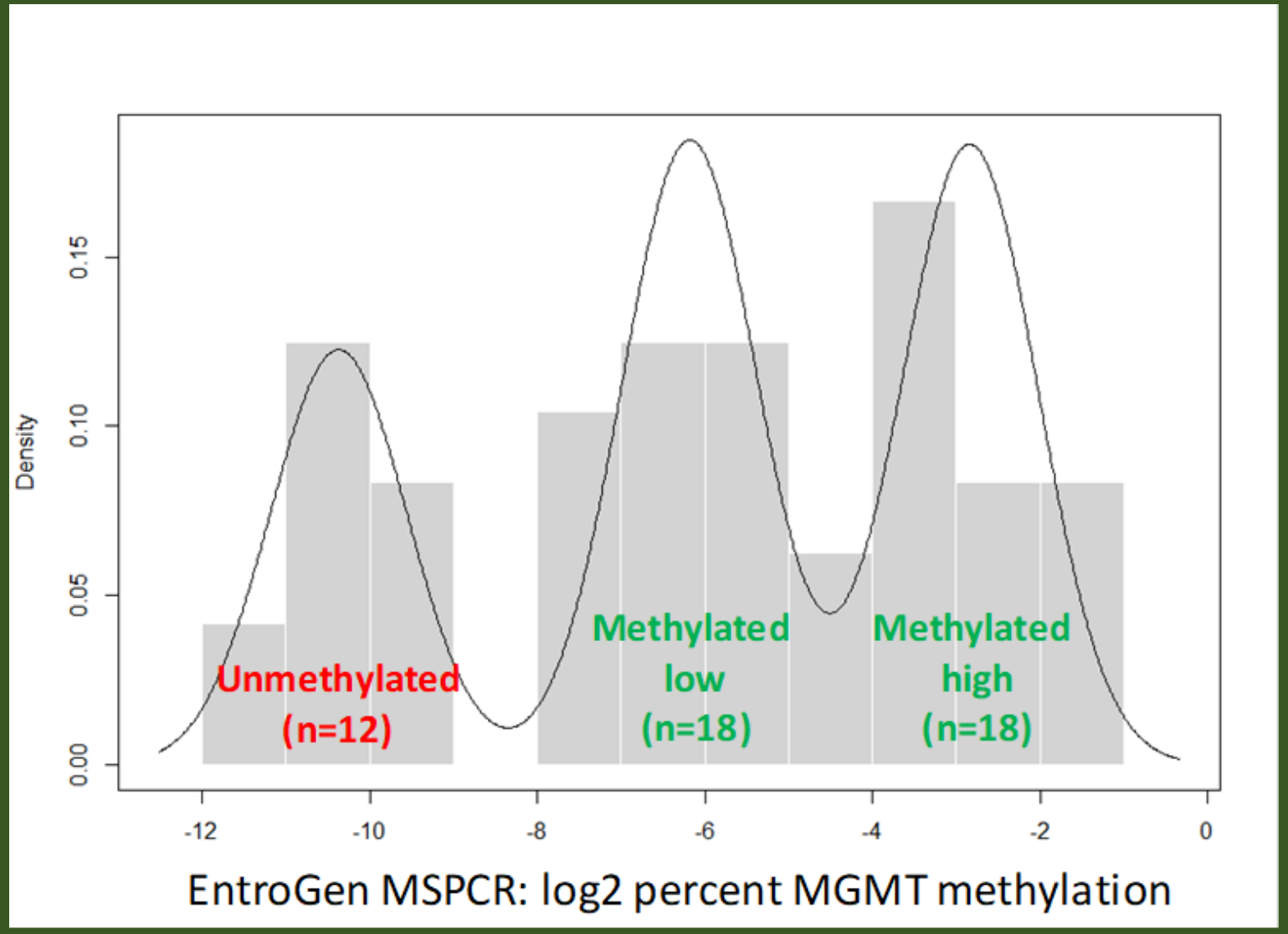
MSPCR: MGMT METHYLATION CUTOFF PROBABILITY



Probability distribution: Bimodal distribution of MGMT methylation

13 out of 48 samples were Indeterminate

MSPCR TRIMODAL DISTRIBUTION MODELS



EntroGen MSPCR density plot: Trimodal distribution model of MGMT methylation

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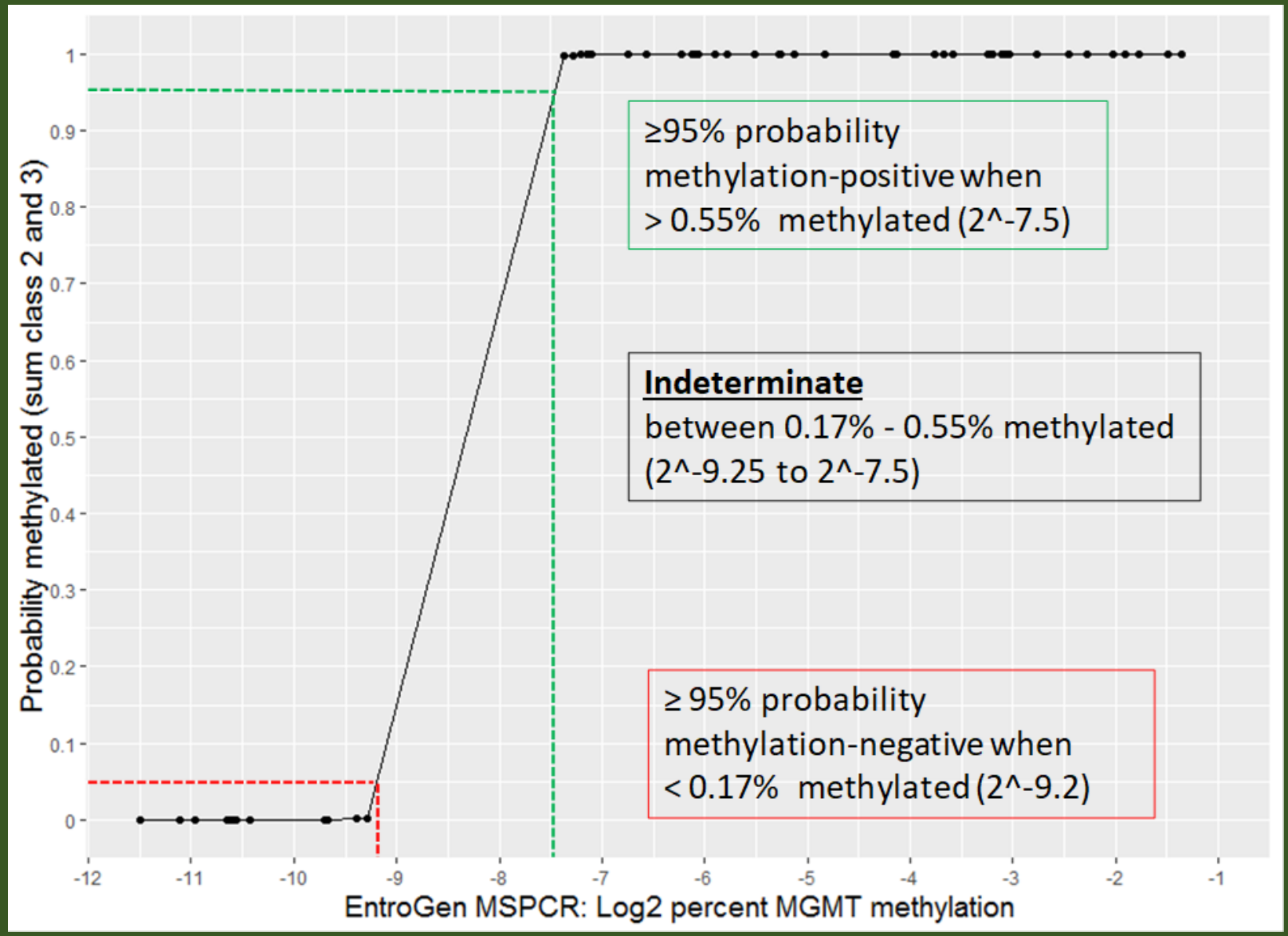
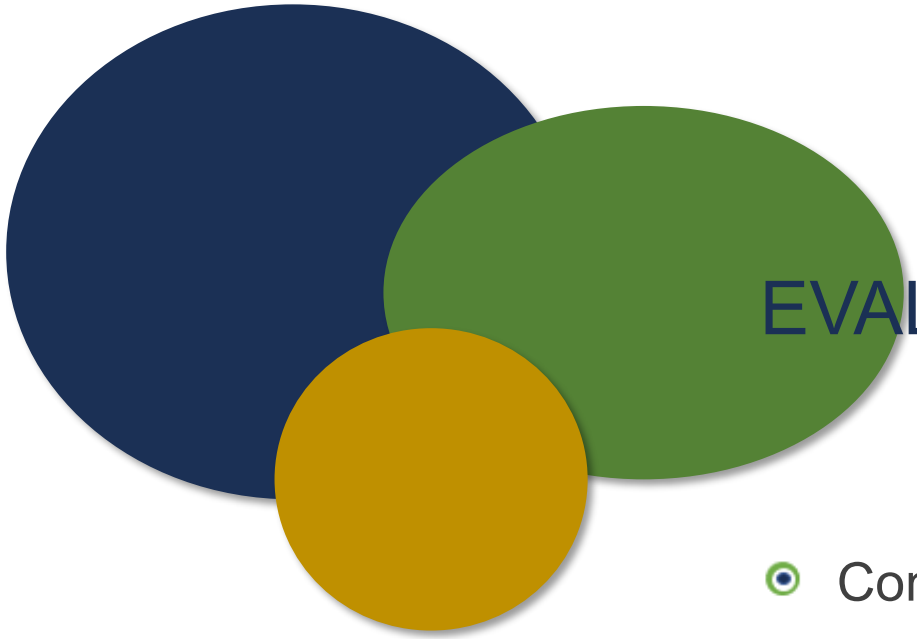
MSPCR: MGMT METHYLATION CUTOFF PROBABILITY



Probability distribution: Trimodal distribution of MGMT methylation

0 out of 48 samples were Indeterminate

CUTOFF VALIDATION

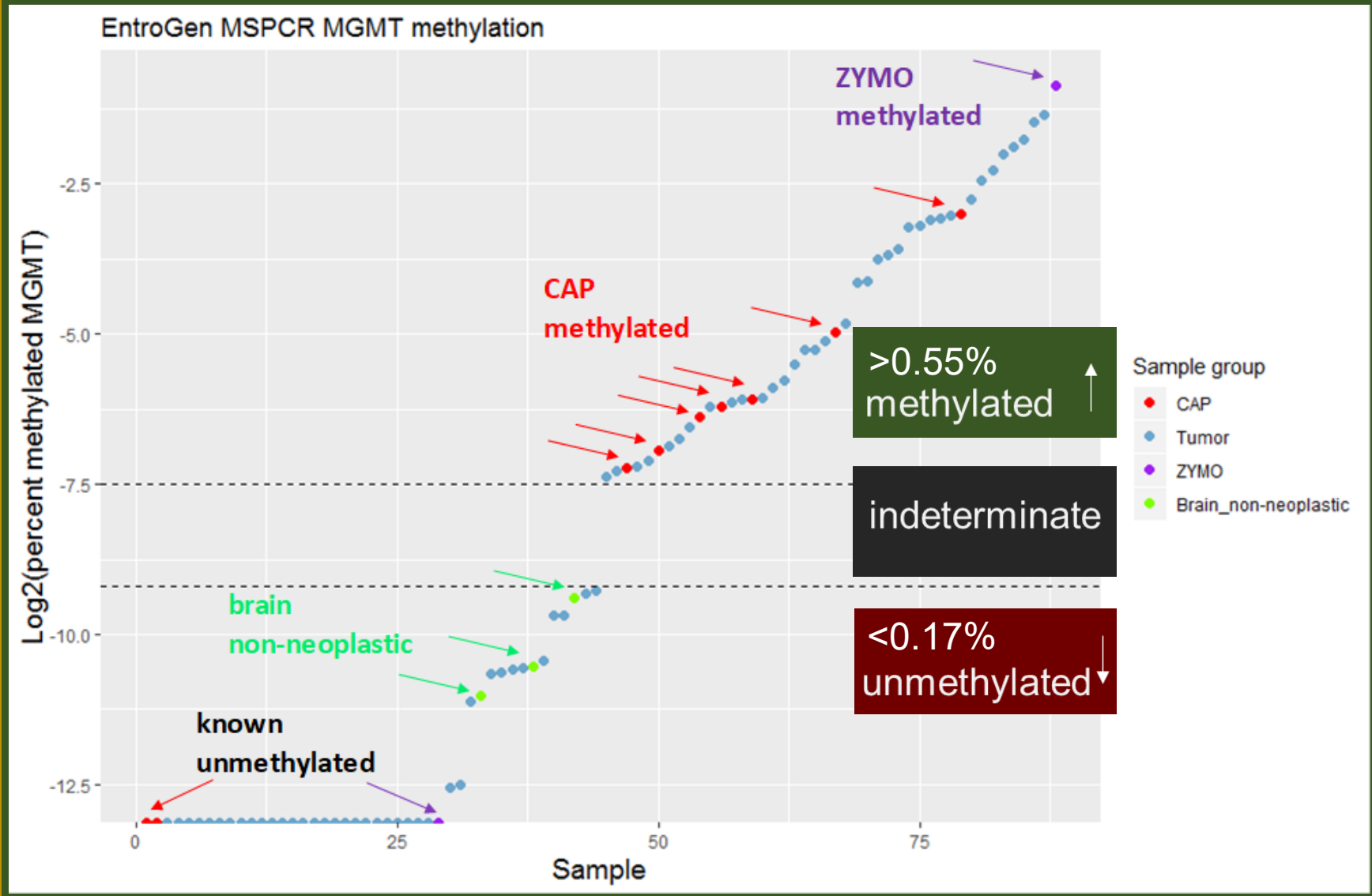


EVALUATE MODEL CUTOFFS

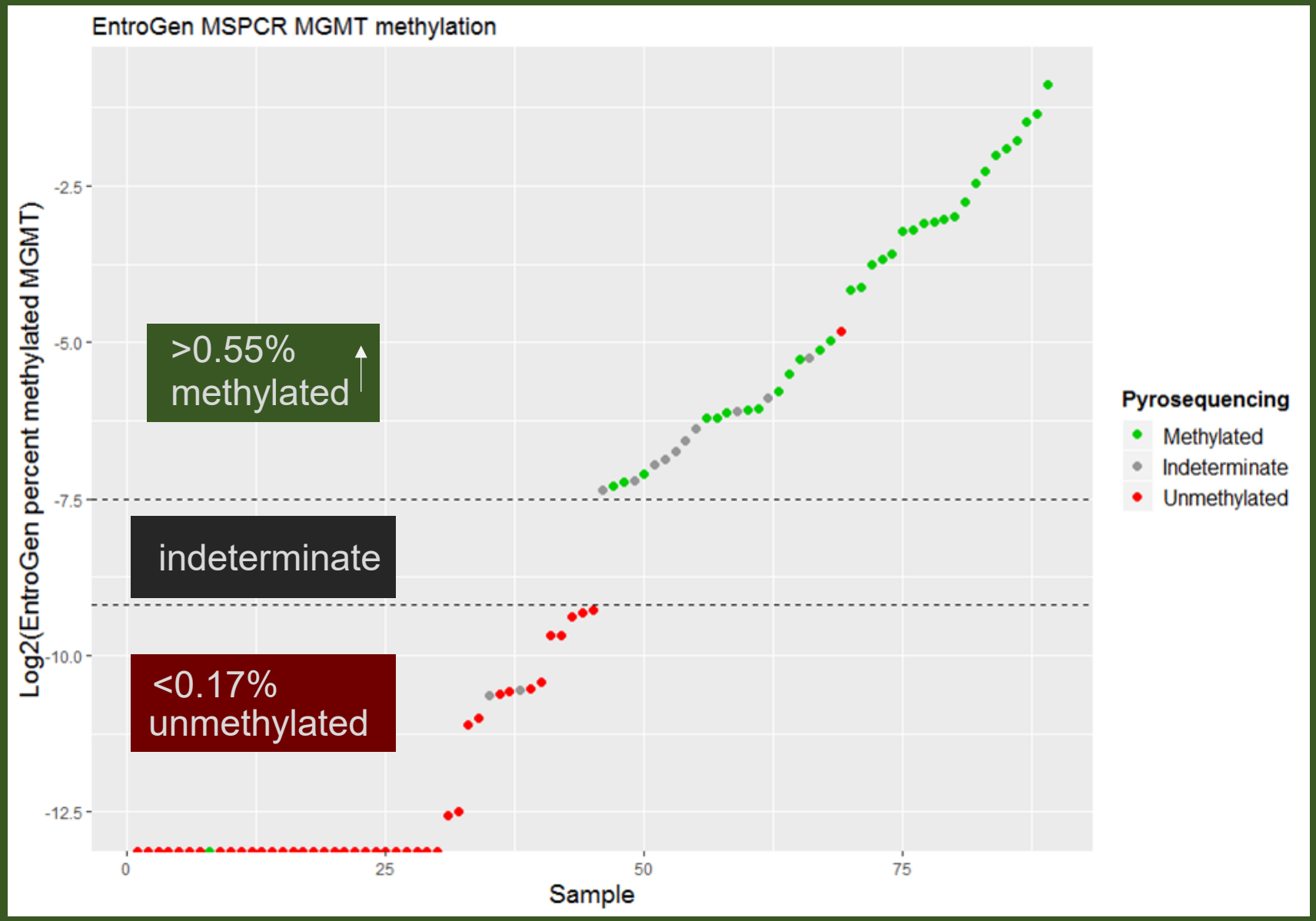
- Control samples with known methylation status
- Clinical samples previously tested with pyrosequencing

Total of 88 unique samples

VALIDATION SAMPLES



CONCORDANCE BETWEEN METHODS



CONCORDANCE BETWEEN METHODS

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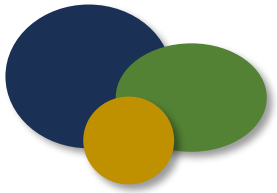
Unique Samples	Pyrosequencing Methylated	Pyrosequencing Indeterminate	Pyrosequencing Unmethylated
EntroGen MSPCR Methylated	33	10	1
EntroGen MSPCR Indeterminate	0	0	0
EntroGen MSPCR Unmethylated	1	2	41

MGMT vs PYROSEQUENCING



97.37% concordance between the two methods

2 DISCORDANT SAMPLES

Sample	MGMT copies	ACTB copies	% MGMT methylation (normalized to ACTB)	EntroGen Mclust model	Pyrosequencing (tumor-corrected)	Pyrosequencing
5	0.00	2125	0.00%	unmethylated	36%	methylated
83	80.9	3871	3.53%	methylated	11%	unmethylated

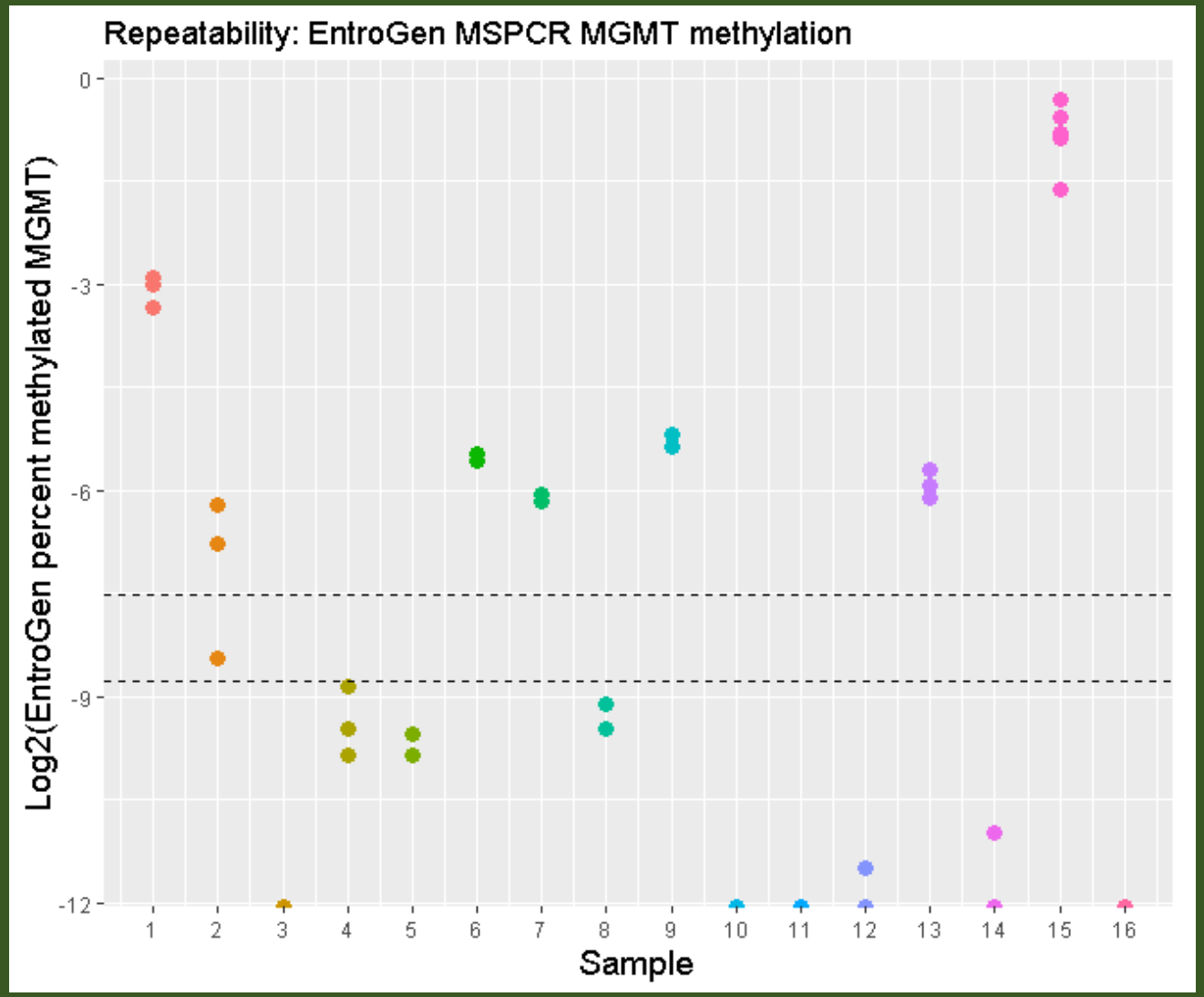
EntroGen mclust model

Methylated > 0.55%
Unmethylated < 0.17%
Indeterminate between 0.17%-0.55%

Pyrosequencing mclust model

Methylated ≥ 25%
Unmethylated ≤ 12%
Indeterminate between 12%-25%

Repeatability: EntroGen MSPCR



0.55%

0.17%

PROPOSED ENTROGEN MSPCR CLASSIFICATION

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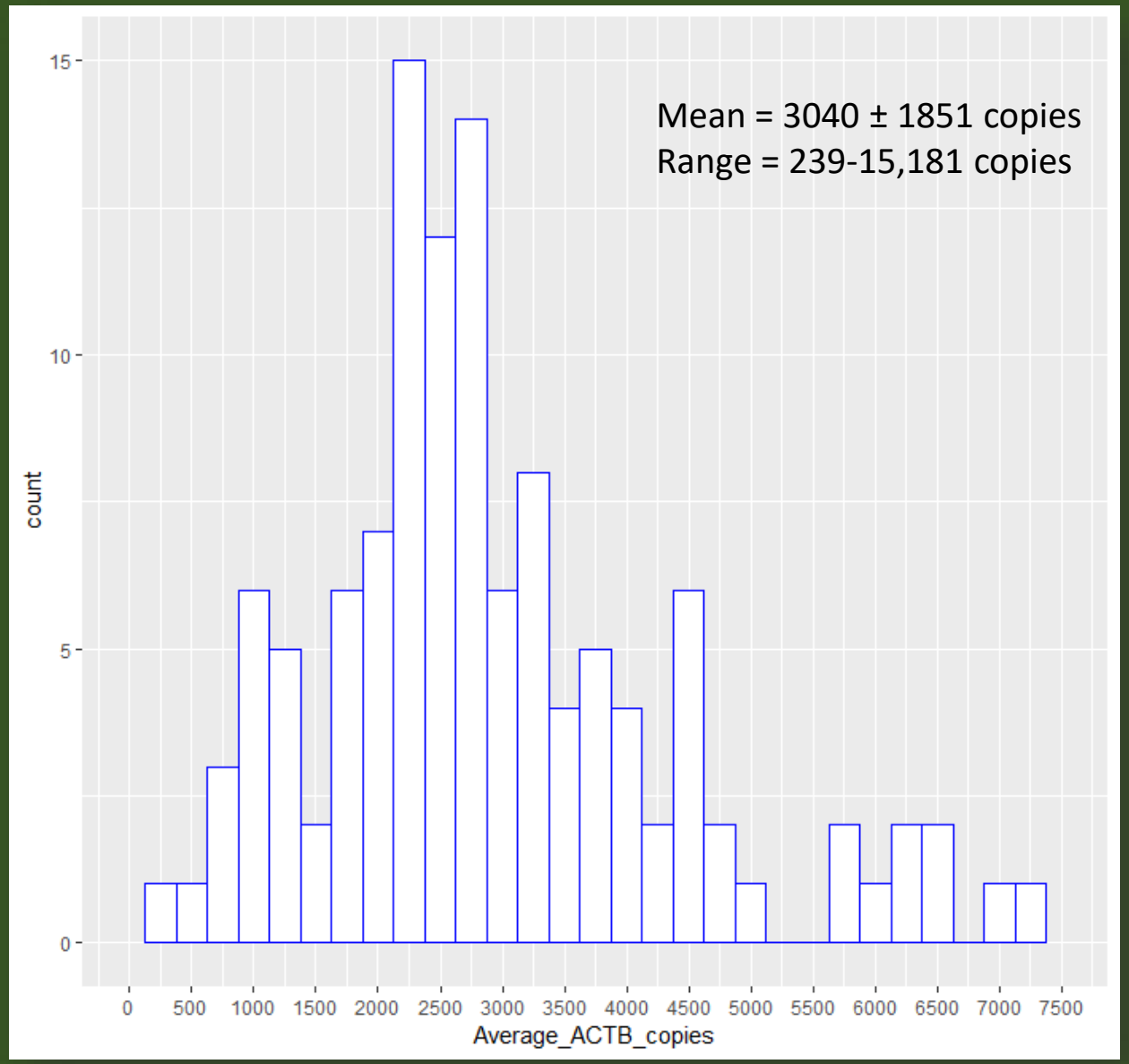
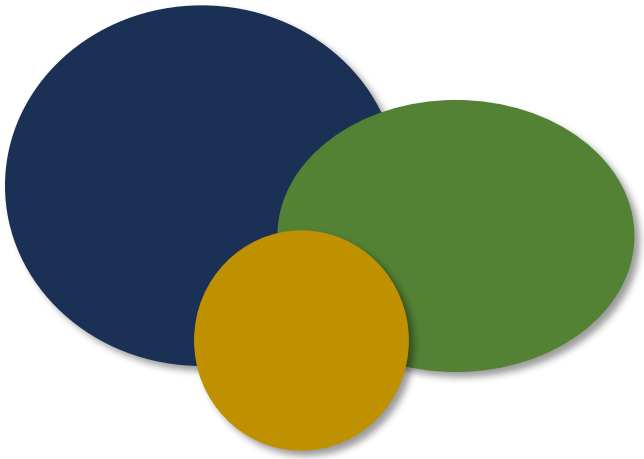
Classification	Results
Unmethylated	<0.17% methylation
Indeterminate	methylation between 0.17-0.55%
Methylated	>0.55% methylation

All classifications require ACTB > 200 copies

DISTRIBUTION OF ACTB COPIES PER SAMPLE



CONCLUSION



FINAL VALIDATION ASSESSMENT

- 97.37% concordance between the two methods
- More efficient workflow (pyro takes 1 full day to run)
- 99.2% (120/121) of samples in range
- Provides appropriate output result for clinically relevant analysis

Sensitivity†	Specificity ††	PPV*	NPV**
97.1%	97.6%	97.1%	97.6%

†Sensitivity: true positive / (true positive + false negative)
††Specificity: true negative / (false positive + true negative)
*PPV: Positive predictive value = true positives / (true positives + true negatives)
**NPV: Negative predictive value = true negatives / (true negatives + false negatives)

KEY TO SUCCESS

Once we discovered that pyrosequencing and MSPCR could not be directly compared, we were able to adopt a new approach that resulted in a high concordance between both assays.

EntroGen®
Predictive • Preventive • Personalized

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FOR PRODUCT INFORMATION