Validation of the EntroGen MGMT RT-44 Real-Time PCR Kit as an Alternative to the QIAGEN PyroMark® Q24 CpG *MGMT* Assay: A Platform Comparison Study

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Introduction

MGMT promoter methylation is a key predictive and prognostic biomarker in glioblastoma (GBM). Pyrosequencing is one of the main methods used to evaluate *MGMT* promoter methylation, and provides quantitative, site-specific data. With the discontinuation of the QIAGEN PyroMark® Q24 CpG MGMT assay expected in 2026, alternative platforms are needed. This study evaluates the EntroGen MGMT RT-44 real-time PCR analytical performance and concordance with prior QIAGEN pyrosequencing results to support clinical transition.

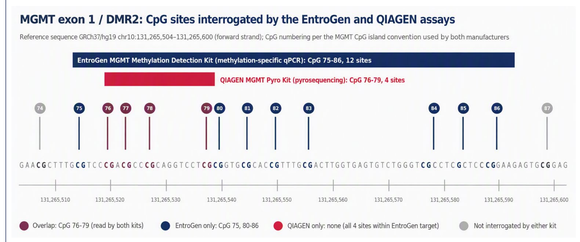


Figure 1. CpG sites in *MGMT* exon 1 / DMR2 interrogated by the EntroGen and QIAGEN assays. The forward-strand reference sequence (GRCh37/hg19, chr10:131,265,504–131,265,600) is shown with CpG dinucleotides numbered 74–87. The EntroGen MGMT Methylation Detection Kit (methylation-specific qPCR) covers CpG 75–86 (12 sites), and the QIAGEN PyroMark® Q24 CpG MGMT(pyrosequencing) covers CpG 76–79 (4 sites).

Materials and Methods

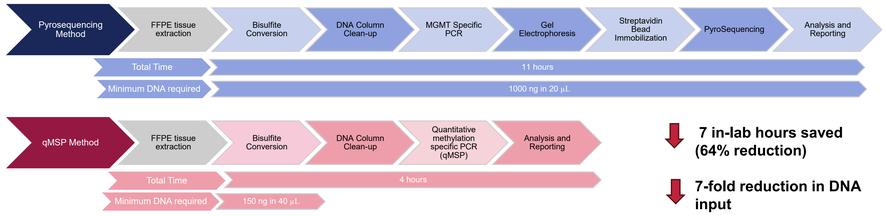


Figure 2. Workflow comparison of the QIAGEN pyrosequencing method compared to the EntroGen qMSP method. Overall, the EntroGen method saves a total of 7 hours of in-lab time (64% reduction) and requires a 7-fold reduction in the DNA input required. While both methods still require a bisulfite conversion and a DNA column clean-up; the EntroGen bisulfite conversion runs for a total of 25 minutes as opposed to the QIAGEN bisulfite kit which requires a 5-hour program.

DNA was bisulfite-converted (EntroGen) and analyzed by real-time PCR using the *Quant-Studio 6*. The EntroGen assay uses methylation-specific primers and probes, with *ACTB* as an internal control. Qualitative concordance and quantitative methylation levels were compared with the *Pyromark Q24*, and reproducibility was assessed across runs and operators.

1. **Limit of Blank (LOB):** 24 blank wells (4 unmethylated FFPE samples x 3 wells x 2 runs) were run to determine the background noise and false-positive threshold.
2. **Limit of Detection (LOD):** Using Zymo Human HCT116 DKO Methylated and Non-Methylated DNA (D5014) controls, a dilution series was created (0 – 5.0%, 56 wells x 2 runs) to determine the lowest methylation levels detected above the LOB (0.23%).
3. **66 GBM formalin-fixed paraffin-embedded (FFPE) samples** that were previously run on the *Pyromark Q24* were selected to be run on the EntroGen assay over 15 QC-passing runs. The sample selection included 35 previously reported positive samples (≥10% methylated, incl. 2 CAP-proficiency samples), 6 indeterminates (7% < methylated <10%) and 25 negatives (≤ 7% methylated incl. 5 CAP).

Pyrosequencing: % Methylated *MGMT* = (%CpG site 76 + %CpG site 77 + %CpG site 78 + %CpG site 79) ÷ 4
qMSP: % methylated *MGMT* = [FAM(*MGMT*) copy number ÷ VIC (*ACTB*) copy number] × 100 × conversion factor (CF)

Reporting Criteria

Pyroseq category	Pyroseq threshold	Total samples, n	qMSP negative, n (%)	qMSP positive, n (%)	Classification agreement
Negative	≤7%	25 (n=2 CAP)	25 (100.0%)	0 (0.0%)	100.00%
Indeterminate	7% < CpG <10%)	6 (n=0 CAP)	5 (83.3%)	1 (16.7%)	Not applicable
Positive	≥10%	35 (n=5 CAP)	1 (2.9%)	34 (97.1%)	97.10%

a.

97.1% — **PPA (34/35)** 95% CI 85.5%–99.5%

100% — **NPA (25/25)** 95% CI 86.7%–100%

0.97 — **Cohen's κ** OPA 98.3% (59/60)

b.

qMSP result	Report
< 0.23%	Unmethylated
0.23 to < 0.44%	Methylated, low level (below LoD; repeat if possible)
≥ 0.44%	Methylated

c.

Table 1. Comparison of pyrosequencing and qMSP methylation classification. qMSP positivity was defined as ≥0.23% methylation. Among indeterminate pyrosequencing samples (7% to <10%; n=6), five (83.3%) were qMSP-negative and one (16.7%) was qMSP-positive. Overall concordance was high, with PPA of 97.1%, NPA of 100%, and Cohen's κ of 0.97. qMSP results were reported as unmethylated (<0.23%), low-level methylated (0.23% to <0.44%), or methylated (≥0.44%).

DNA Quantity

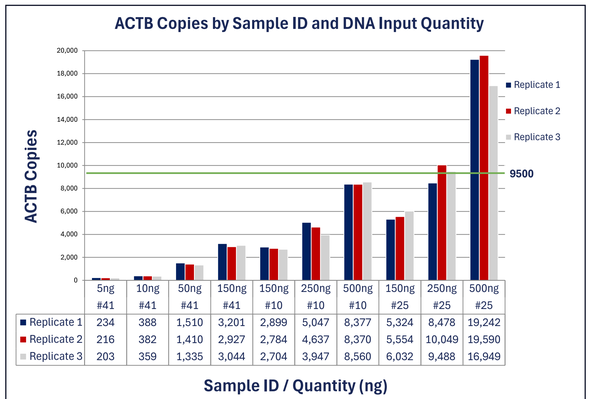


Figure 3. DNA input study featuring three selected samples (1 negative, 2 positives, ID #41, 10, 25).

DNA input optimization was assessed using *ACTB* copy number as a quality metric, with an acceptable range defined as 200-9,500 *ACTB* copies. Across all evaluated samples, 150 ng DNA input consistently generated *ACTB* copy numbers within the target range, demonstrating reliable amplification while avoiding excessive template input.

Limit of Blank and Limit of Detection

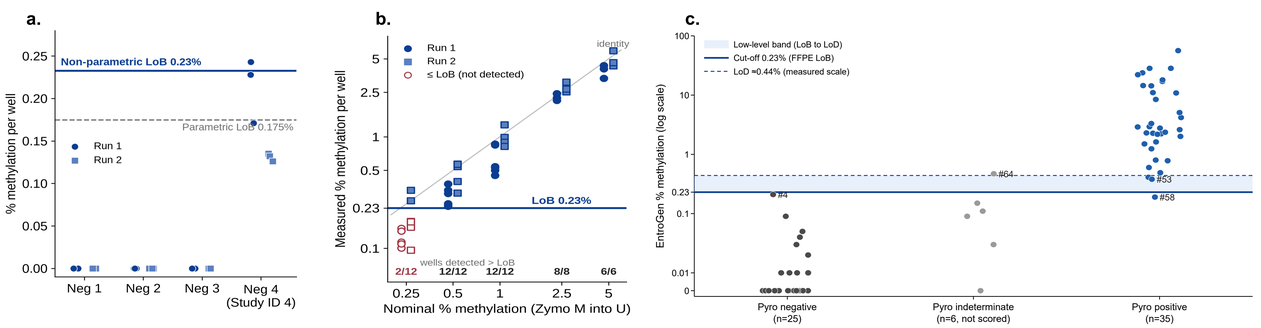


Figure 4. The threshold set for the EntroGen qMSP assay based off the limit of blank (LOB) calculated from 24 wells from 4 negative samples **(Fig 4a),** and the limit of detection (LOD). The LOD was derived from a dilution series of Zymo Methylated and Unmethylated controls **(Fig 4b).** The resulting threshold (0.23%) was then applied to all sample results and gave a positive percent agreement (PPA) of 97.1%, negative percent agreement (NPA) of 100% and Cohen's kappa of 0.97 (**Table 1b, Fig 4c**).

- **LoB**: calculated using the non-parametric method due to non-normal distribution of the values obtained from the wells.
  - LoB → 0.95 × 24 + 0.5 = 23.3
  - 23rd well → 0.228% , 24th well → 0.243%
  - **23.3 well → 0.23%**
- **LoD:** Detection was 2/12 wells at 0.25% and 12/12 at 0.5% and above. A parametric approach was used to obtain the LoD.
  - LoD → LoB + 1.683 × SD at 0.5% = 0.23 + 1.683 × 0.12 = **0.44%**
- **Cut-off:** The reporting threshold was set at the LoB (0.23%), the level that 95% of blank results fall below. It was checked against 60 patient specimens previously classified by pyrosequencing.

Conclusions

- The EntroGen MGMT RT-44 assay is a relative way of measuring *MGMT* methylation as opposed to the absolute measurement through *QIAGEN Pyromark Q24.*
- A limit of blank cut-off of 0.23% gave NPA 100%, PPA 97.1% and κ 0.97, within the empirically optimal range, without being fitted to clinical data.
- Reporting the LoB–LoD interval as low-level methylation keeps sensitivity for true low positives.
- There was strong concordance throughout the 66 samples with a single discordance sample (Study ID 58)

Next Steps:

- Further testing of specimens at low methylation levels to determine the robustness of the cut-off.

References
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[3] Mansouri A, et al. MGMT promoter methylation status testing to guide therapy for glioblastoma. Neuro-Oncology 2019. PMC6374759
[4] Huseyinoglu Z, et al. Optimal qMSP cutoff value for MGMT promoter methylation in glioblastoma. BMC Cancer 2025. PMC12664229