Countable Labs.

Direct single-molecule counting of DNA methylation at ultra-low input via a one-step, one-tube methylation-sensitive restriction enzyme (MSRE) workflow.

Ethan Chu[1], Michael Porter[1], Kelly Li[1], Christina Fan[1]

Introduction

DNA methylation is a critical epigenetic mechanism with broad applications in oncology research, including tumor classification, cancer biomarker discovery and residual disease research. Quantification of methylation status in cell-free DNA (cfDNA) is especially demanding given low abundance of cfDNA and the inefficiency and loss resulted from conventional methylation measurement methods, such as bisulfite conversion.

In addition to methylation detection, precise quantification methods are also limited: qPCR relies on relative quantification or standard curves; digital PCR suffers from dead-volume loss and limited partitions; and NGS demands complex library preparation that increases turnaround time and cost.

Here, we demonstrate that single-molecule counting by Countable PCR, coupled with methylation-sensitive restriction enzyme (MSRE), provides a simple, precise, and sensitive approach for direct, absolute quantification of methylated DNA molecules without bisulfite conversion. The hypermethylated *MLH1* promoter locus is used to illustrate this workflow.

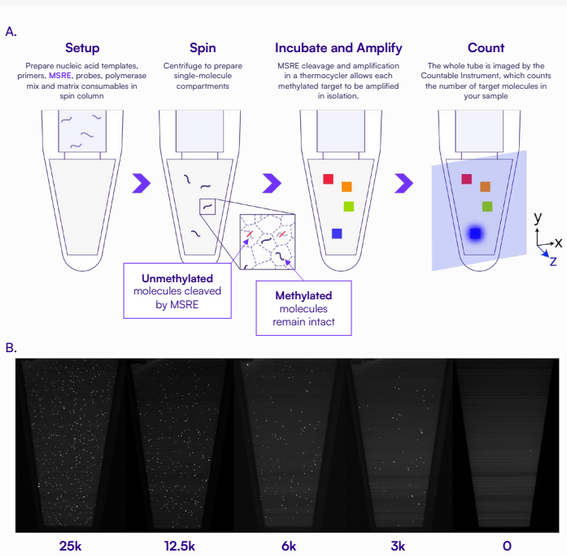


Figure 1. (A) Schematic diagram of Countable PCR workflow. In Countable PCR, target nucleic acid molecules are partitioned into ~30 million compartments within an optically clear matrix. Subsequent MSRE digestion, PCR amplification, and counting all occur within a single tube. The system achieves zero dead-volume sample loss, preserving precious cfDNA, offering a highly sensitive, ultra-low input alternative for epigenetic liquid biopsies. Unlike conventional dPCR, the Countable system provides true single-molecule counting across a wide dynamic range, enabling high multiplex detection of target sequences. (B) Cross-sectional matrix snapshots across the titration range visualize the proportional increase of distinct *MLH1* counts.

Materials and methods

To circumvent bisulfite-induced DNA degradation, we developed a one-step workflow using methylation-sensitive restriction enzymes (MSREs). During an isothermal incubation, HpaII selectively cleaves unmethylated *MLH1* CpG sites, rendering them unamplifiable. Intact methylated targets and the *RPP30* reference gene are then directly co-quantified via Countable PCR single-molecule counting, enabling precise methylation quantification without bisulfite conversion. Percent methylation is then calculated by taking the ratio of counts from *MLH1* to *RPP30*.

Assay performance was evaluated using the Human Methylated & Non-methylated DNA Set (Zymo Research, Cat. No. D5014).

Leveraging the capabilities of the Countable platform, we systematically optimized and characterized the assay across the following parameters:

Assay specificity & multiplexing. Singleplex and 2-plex configurations were compared to confirm the compatibility of the duplex and the absence of cross-reactivity between targets. Countable PCR enables simultaneous co-quantification of *MLH1* and *RPP30* in a single reaction.

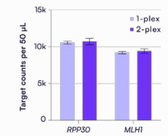


Figure 2. Singleplex and 2-plex configurations yield consistent counts for both *RPP30* and *MLH1*, confirming multiplexing specificity without cross-reactivity.

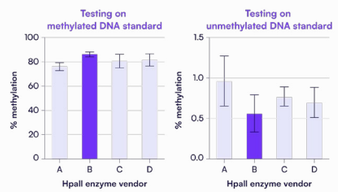


Figure 3. Four-way comparison of HpaII enzyme variants demonstrates that Vendor B achieves the most optimal cleavage profile, yielding the highest signal for methylated DNA and the lowest background from unmethylated DNA.

MSRE selection. Four HpaII enzyme variants across multiple manufacturers and formulations were evaluated for digestion efficiency. The Countable system enables direct enzyme integration into the reaction without post-digestion purification or sample transfer.

Incubation time. Isothermal incubation times ranging from 15 minutes to 16 hours were evaluated. The absolute single-molecule counting of Countable PCR provided the resolution needed to precisely track cleavage efficiency, minimizing unmethylated background while preserving methylated signal.

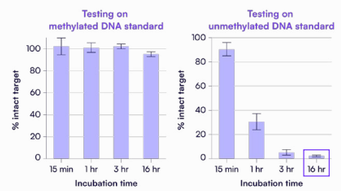


Figure 4. Isothermal incubation time optimization demonstrates that 16 hours yields the lowest unmethylated background while maintaining a stable methylated signal.

Results

As a proof of concept, we demonstrated a one-step, one-tube MSRE workflow for direct single-molecule counting of DNA methylation. The assay enabled sensitive quantification of methylated *MLH1* across a broad input range (5—400 ng) with linearity across the full 0—100% methylation range and an LOD of 0.31%. Titrations were prepared by mixing 100% methylated and 100% unmethylated DNA at varying ratios. Our data suggest this LOD is primarily limited by input sample quality (i.e. low level of residual methylation in unmethylated DNA reference materials) rather than assay performance.

Replicate measurements at the 5 ng lower limit confirmed consistent precision at low input. *RPP30* co-quantification as an internal reference verified target recovery across all input levels, supporting reliable percent methylation calculations.

- LOB was calculated as <counts @0% input> + 1.645*stdev(counts @0 input)
- LOD was calculated as LOB + 1.645*stdev(counts @0.1% input)

Table 1. Target counts and calculated percent methylation (*MLH1*/*RPP30*) for 100% methylated and 100% unmethylated reference samples at both 5 ng and 400 ng inputs. The 5 ng data illustrates the assay's capability for reliable quantification and distinct signal resolution at ultra-low mass inputs. The 400 ng data confirms that the workflow maintains highly efficient MSRE cleavage and accurate target recovery even under elevated DNA loads.

	Expected % methylation	n	*RPP30* counts/50µL	Average	*MLH1* counts/50µL	Average	Measured % *MLH1*/*RPP30*
5 ng	100%	1	1,126	1,106	1,137	1,129	102.13%
		2	1,089		1,139		
		3	1,102		1,111		
	0%	1	1,280	1,241	6	6	0.52%
		2	1,278		4		
		3	1,165		9		
400 ng	100%	1	75,661	73,494	73,549	71,808	97.72%
		2	71,326		70,066		
	0%	1	88,422	93,573	231	218	0.23%
		2	98,724		204		

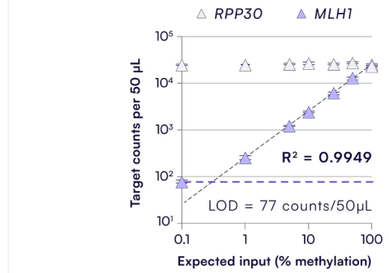


Figure 5. Measured target counts per 50µL of *RPP30* and *MLH1* against the expected sample input. Mean triplicate counts per 50 µL reaction plotted against the expected methylated input (0—100%). Methylated *MLH1* (light purple) demonstrates highly linear quantification (R^2 = 0.9949) across the full dynamic range, with an LOD of 77 counts (horizontal purple dashed line) against a background of about 25,000 *RPP30* counts per 50 µL (gray). Stable *RPP30* co-quantification confirms uniform total DNA recovery independent of methylation status.

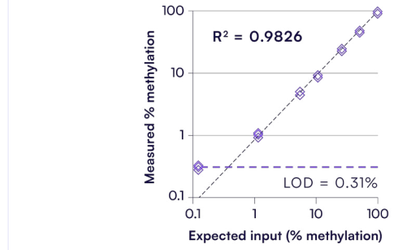


Figure 6. Measured % methylation from *RPP30* and *MLH1* counts against the expected sample input. Measured percent methylation plotted against an expected input *MLH1* methylation between 0.1—100%. The assay demonstrates strong concordance with a best-fit correlation of R^2 = 0.9826 (solid purple), closely tracking the ideal 1:1 response (dashed black). A fractional LOD of 0.31% (dashed purple) confirms sensitivity at ultra-low methylation abundances.

Conclusion

- We developed a bisulfite-free, two-plex Countable PCR assay for direct *MLH1* methylation quantification, utilizing a highly efficient HpaII MSRE digestion protocol.
- This *MLH1* assay achieves strict linearity in both targets (R^2 > 0.98) and ultra-high sensitivity for *MLH1* (LOD = 0.31%, or 77 counts), maintaining precision at inputs as low as 5 ng.
- Using *MLH1* as a model locus, this workflow shows strong potential for extension to additional methylation biomarkers, supporting cfDNA methylation research, including biomarker discovery, tumor classification studies and residual disease research.

[1] Countable Labs, Inc. 1810 Embarcadero Rd. Suite 200 Palo Alto, California 94303, USA

Learn more about how Countable PCR simplifies workflows for epigenetic applications.

Visit countablelabs.com/resources/dna-methylation-single-molecule-counting-low-input

© 2026 Countable Labs, Inc. For Research Use Only. Not for Use in Diagnostic Procedures.