Hyperstimulatory N⁶-methyladenine (m6A) in residual SV40 plasmid DNA in mRNA vaccines.

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Abstract

Many mRNA vaccine production pipelines rely on *Escherichia coli* to replicate plasmid DNA templates used in the in vitro transcription of modified RNA. However, *E. coli* DNA methylation patterns differ substantially from those of humans. In *E. coli*, DNA methylation is primarily mediated by DNA adenine methyltransferase (Dam), which introduces N⁶-methyladenine (m6A) within GATC motifs, whereas human methylation occurs predominantly at cytosines in CpG dinucleotides. Some *E. coli* strains also express Dcm methyltransferase, which methylates CCWGG sequences (CC[A/T]GG), further distinguishing bacterial from mammalian epigenetic marks.

Cytosolic DNA that lacks CpG methylation can potently activate Toll-like receptor 9 (TLR9), while m6A-modified DNA has been shown to stimulate the cGAS–STING pathway, leading to the induction of type I interferons and other inflammatory mediators.

Because the Pfizer mRNA vaccine plasmids are propagated in *E. coli*, and residual plasmid DNA has been detected in finished vaccine material, it is likely that this DNA bears bacterial-type methylation patterns that could be immunostimulatory through TLR9 and cGAS–STING signaling. To investigate this possibility, we applied Oxford Nanopore sequencing to examine the methylation status of plasmid DNA present in Pfizer lot FL8095.

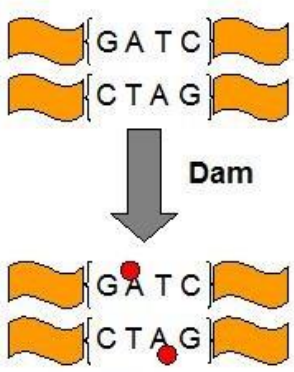

Figure 1. Red dots reflect methylation of N⁶-methyladenine in the palindromic sequence GATC (image source- https://2009.igem.org/Team:Imperial_College_London/M3/DamMethylation)

Methods

We purified 600µl of Pfizer lot FL8095 using 6µl of 10% Triton X. After a quick vortex samples were heated at 95°C for 2 minutes and then spun at 15,000 RPM for 5 minutes. Samples are placed on ice to harden the top oil layer and 2.5µl of RNaseA (Monarch RNaseA -NEB) is added to the bottom layer and incubate at 37°C for 10 minute.

Purification of the small DNA was accomplished with a modified Ampure protocol using 600µl of Ampure, 600µl of 100% Isopropanol and 12µl 1M MgCl2. Samples are mixed and allowed to bead bind for 10 minutes. A magnet is used to separate the beads and they are washed twice with 1ml 70% EtOH. DNA is eluted in 30µl of ddH20.

Figure 2. Pfizer/BioNtech FL8095 sample after TritonX, Heat and Centrifugation.

DNA Sequencing libraries were constructed with Oxford Nanopores ONT ligation sequencing assay V14. Two modifications were made to the default protocol to better capture small DNA. The Ampure step after the End Repair step was increased from 60µl to 90µl. The Ampure step after the Ligation step was increased from 40µl to 100µl.

These libraries were loaded onto an Oxford Nanopore MinION Mk1D using their R10.4.1 flow cells. Sequencing reads were base called with the Dorado base caller using the dna_r10.4.1_e8.2_400bps_sup@v5.2.0_6mA@v1 model. Minimap2 was used to align the reads to NCBI reference OR134577.1.

Assessment of Eam1104I linearization:
Oxford Nanopore reads were aligned to the Pfizer/BioNTech plasmid reference (GenBank accession OR134577.1) to assess the completeness of linearization at the Eam1104I site.
To accurately detect reads spanning the linearization junction, the reference was concatenated

(i.e., duplicated end-to-end) so that alignments could occur across the Eam1104I cut site. Mapping reads to the terminal ends of a single-copy reference would otherwise result in minimal alignments because most read mappers do not handle circular reference continuity.
Reads traversing the Eam1104I junction were then visualized in IGV to confirm incomplete linearization events.

Results

591,304 reads with primary alignments to the Pfizer reference (OR134577.1) were generated. DAM methylation can be seen on both strands of the GATC palindromic sequence. Top strand is Grey and bottom strand is Green. Dam methylation can be observed (Figure 4-7) and varies across the plasmid (Figure 8).

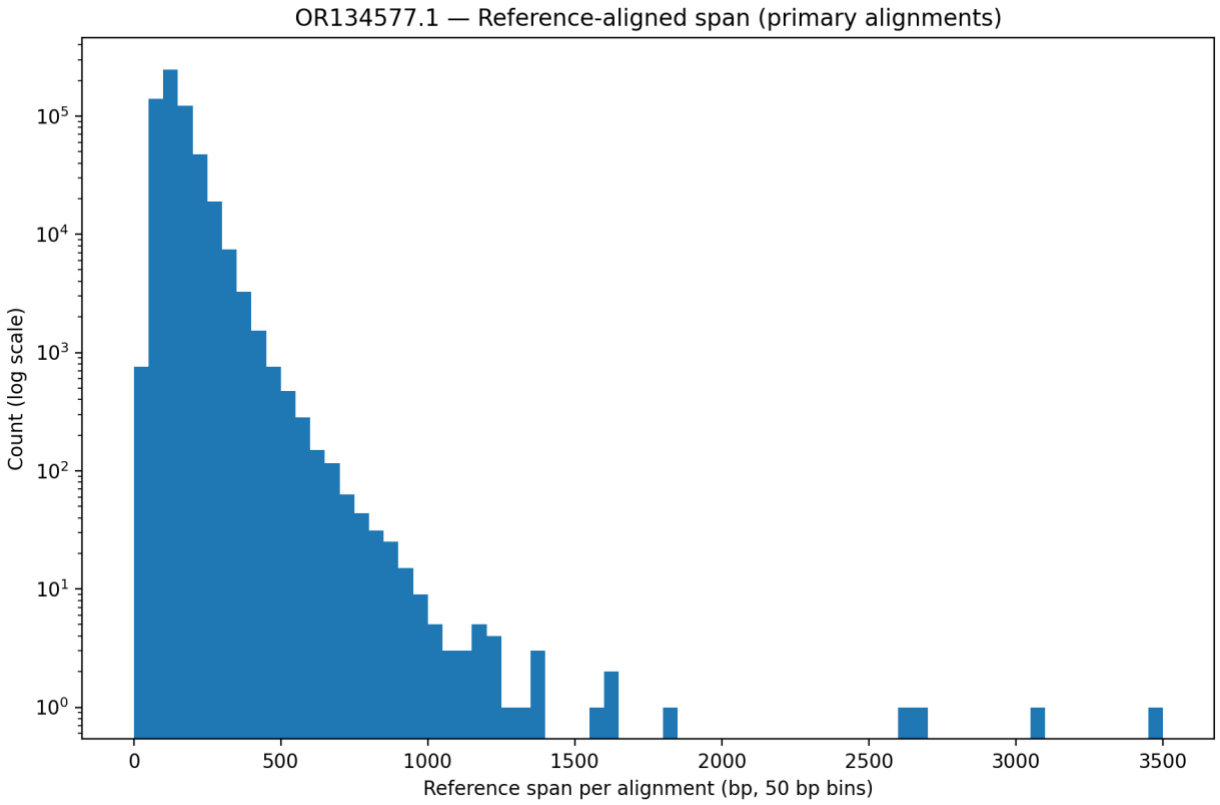

Figure 3. Aligned ONT Read length distributions. Oxford Nanopores cannot thread circular DNA through the pores and cannot be measured with these methods.

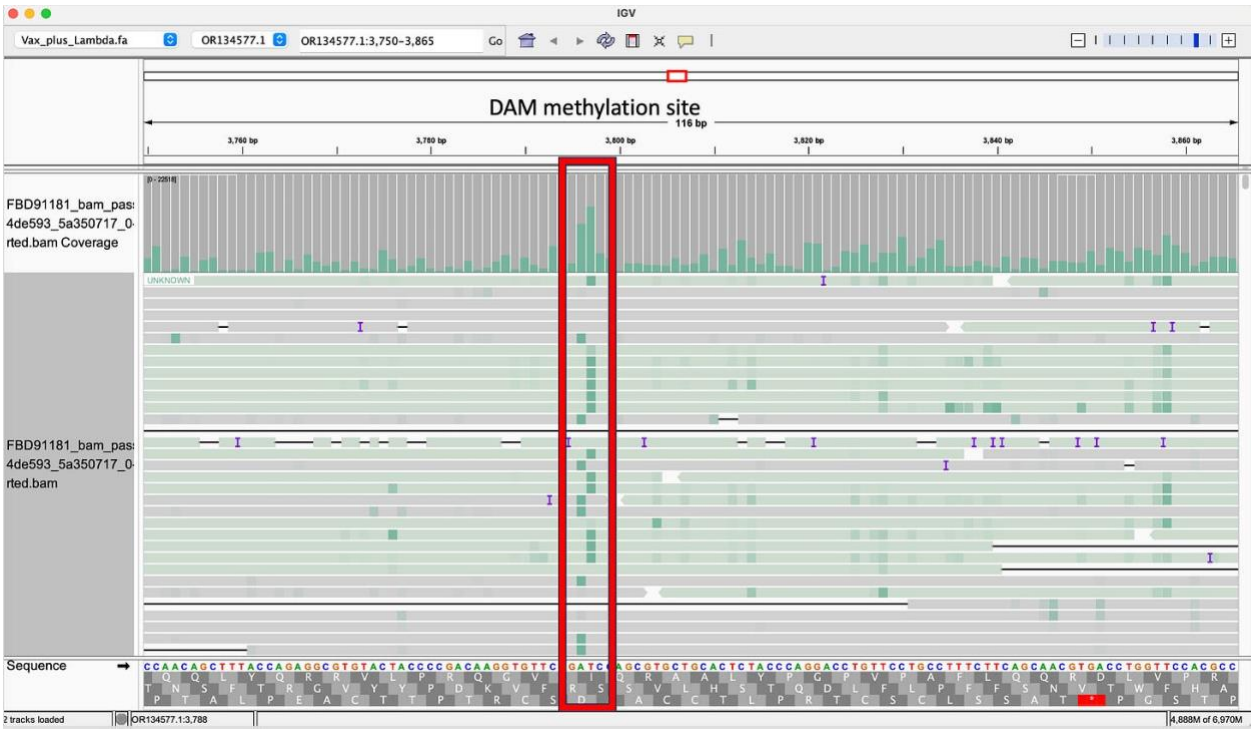

Figure 4. IGV display of Dam methylation (GATC) in Plasmid DNA sequence from Pfizer lot FL8095

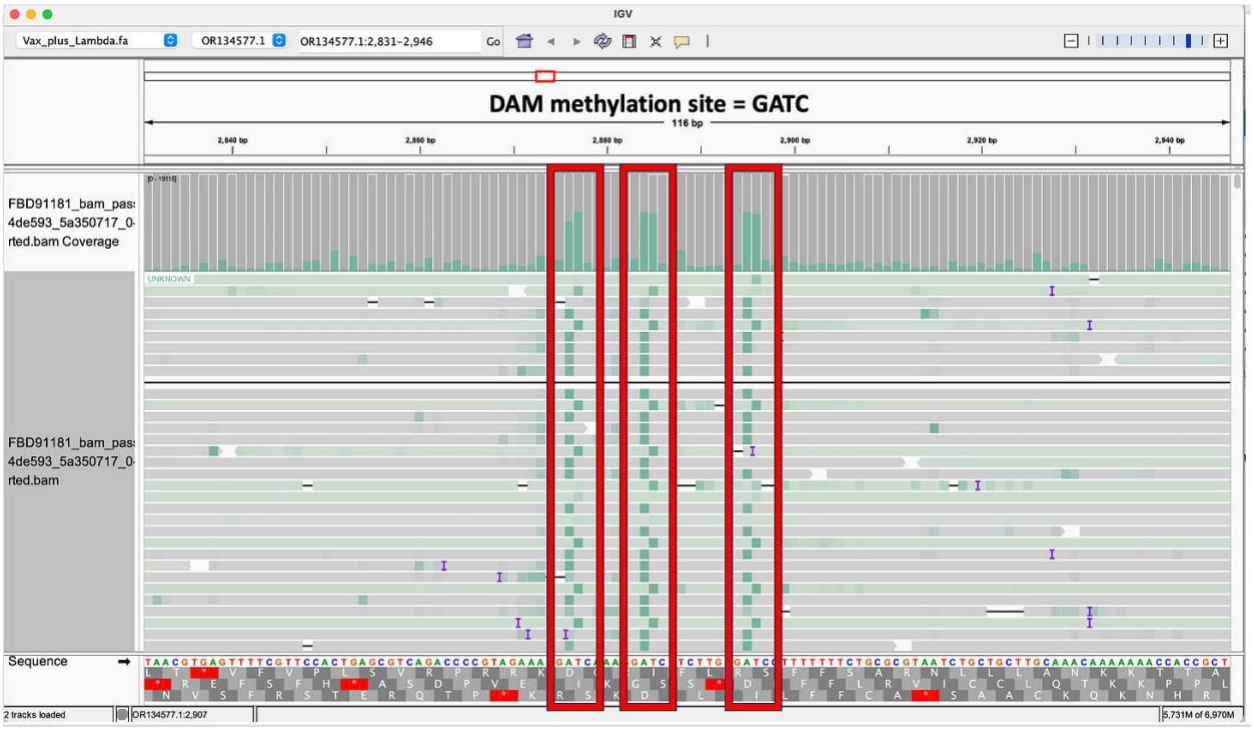

Figure 5. Tandem GATC sites heavily methylated

Figure 6. This strain of *E.coli* does not methylate the SV40 promoters.

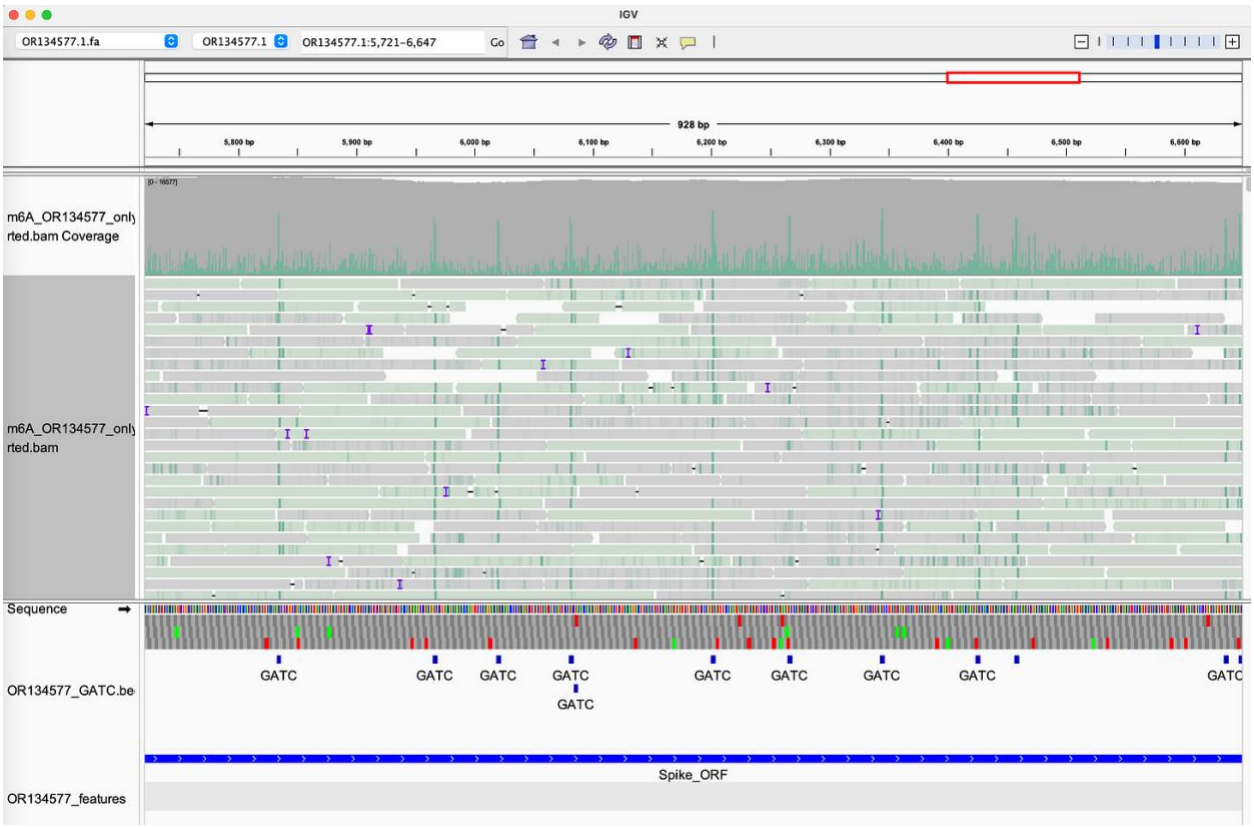

Figure 7. The spike ORF is densely methylated.

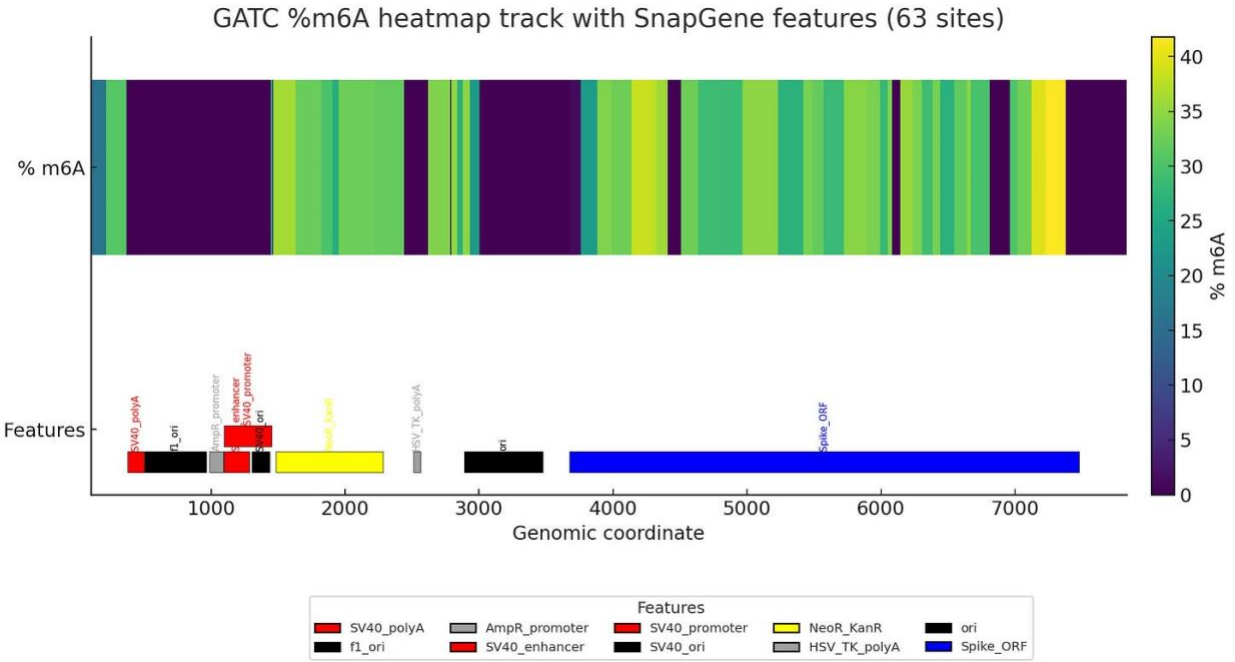

Figure 8. Methylation Heatmap across the plasmid demonstrate hypomethylation of SV40 components while hypermethylation of Neo/Kan and Spike.

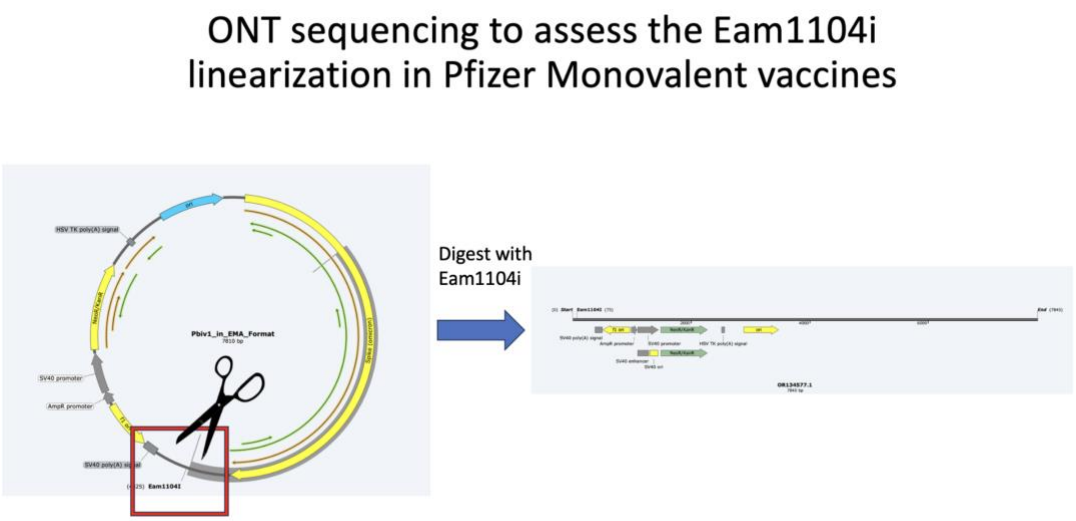

Figure 9. Oxford Nanopore sequencing workflow illustrating the Eam1104I digestion strategy for linearization of the Pfizer/BioNTech plasmid prior to RNA transcription

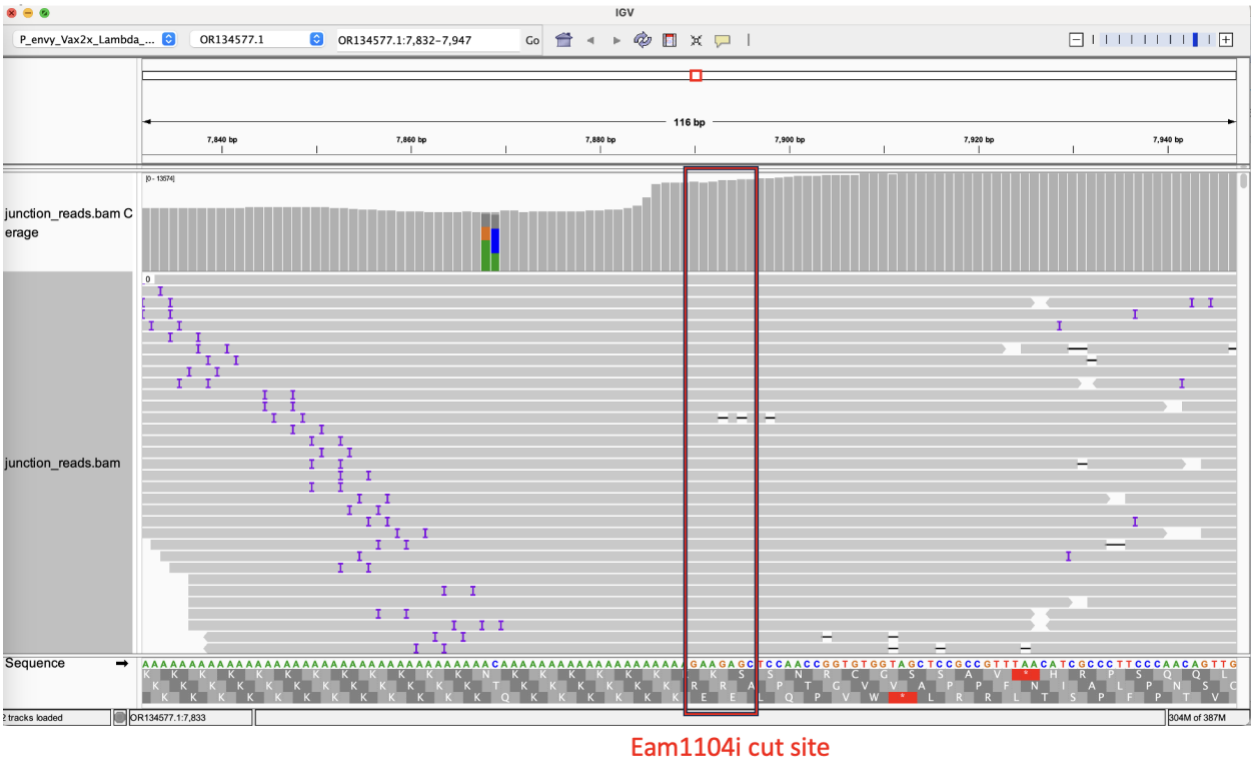

Figure 10. IGV visualization of Oxford Nanopore reads spanning the Eam1104I cut site, showing read-through across the expected junction and incomplete plasmid linearization.

Evidence of Incomplete Linearization at the Eam1104I Site

Oxford Nanopore sequencing revealed multiple reads traversing the Eam1104I linearization site used in the production of Pfizer/BioNTech's mRNA vaccine plasmids. This finding indicates incomplete digestion during the in vitro linearization step, suggesting that a small fraction of plasmid molecules may have remained circularized (Figure 9). Reads spanning the expected cut junction (Figure 10) demonstrate continuity across both cleavage positions, consistent with residual intact plasmids persisting in the final formulation.

The presence of circular plasmids is not expected to arise *post-digestion*, as host ligases are absent during the in vitro transcription preparation. However, once injected into mammalian tissue, host ligases could potentially re-circularize linear plasmid fragments that retain complementary 5′ overhangs. This raises a theoretical concern that rare, replication-competent plasmids could reform in vivo.

The longest continuous read spanning the Eam1104I site exceeds 3,400 bases, confirming that long residual fragments survive purification and traverse the nominal cleavage site. Because Oxford Nanopore platforms cannot process circular DNA, the exact abundance of full-length circular plasmids cannot be directly measured without additional enzymatic linearization of residual DNA. Nevertheless, these findings demonstrate that incomplete digestion and low-level persistence of circular forms cannot be excluded in the vaccine DNA template.

Discussion

These data firmly establish that Pfizer/BioNtech plasmid manufacturing did not utilize Dam knock out *E.coli* strains. DNA containing N^6-methyladenine (m6A) is increasingly recognized as an immunostimulatory signal when detected by the host innate immune system. Recent studies have shown that m6A-modified DNA can act as a potent activator of the cGAS–STING pathway, enhancing interferon responses and amplifying downstream inflammatory signaling (Balzarolo et al., 2021). The cGAS–STING system itself is the primary cytosolic DNA sensor, responsible for detecting foreign DNA and triggering type I interferon production (Li & Chen, 2016).

Promoter and enhancer regions are often sensitive to DNA methylation, and classical studies with cytosine methylation have shown that methylation within the SV40 regulatory region can reduce promoter-driven gene expression (Bryans et al., 1992). Although those studies did not examine adenine methylation, they provide precedent that methyl groups within regulatory sequences can influence chromatin accessibility or promoter activity. In the context of m6A (Dam methylation), it remains plausible that methylation at GATC sites in the SV40 region could alter transcription factor binding or chromatin state, although direct evidence in this system is lacking.

Recent single-molecule chromatin profiling of plasmids demonstrated that promoter identity strongly influences nuclear import and chromatinization (Mallory et al., 2025). These findings

suggest that epigenetic and structural features of plasmids together govern transcriptional accessibility.

The data here indicate that while the plasmid backbone is broadly methylated at GATC sites, the SV40 promoters and enhancers remain unmethylated in this *E. coli* strain. This selective lack of methylation suggests that the bacterial host does not target these regulatory elements for m6A modification, thereby potentially preserving their full transcriptional activity. At the same time, pervasive m6A methylation elsewhere in the plasmid may act as a hyper-stimulatory signal for the innate immune system via cGAS–STING.

Beyond the SV40 regulatory region itself, bacterial methylation state has been shown to influence the biological performance of plasmid DNA in mammalian systems (Carnes et al., 2010). Vito et al. (2011) reported that *dcm*$^-$ plasmids, which lack cytosine methylation at CCWGG motifs, exhibited enhanced transgene expression in human cells but paradoxically reduced immunogenicity. This highlights that plasmid methylation patterns—both at adenine (Dam) and cytosine (Dcm) sites—can have measurable downstream effects on expression and innate immune activation. Their observation that plasmid methylation status should be standardized early in development underscores the relevance of epigenetic state to plasmid-based vaccine and gene therapy vectors.

Importantly, chronic activation of cGAS–STING signaling has been linked to oncogenic processes. Kwon et al. (2020) review evidence showing that while acute pathway activation is protective—promoting antiviral and antitumor immunity—persistent cGAS–STING stimulation can drive noncanonical NF-κB signaling, inflammation, genomic instability, and tumor progression. Sustained cytosolic DNA stress blunts type I interferon output and shifts the transcriptional program toward pro-survival and immunosuppressive signaling. In this context, residual plasmid DNA bearing m6A marks could, if persistently sensed, promote maladaptive inflammation and possibly contribute to oncogenic risk through chronic STING engagement.

It has previously been reported that differential gene expression analyses between vaccinated and unvaccinated cohorts revealed altered expression of genes in the cGAS-STING pathway (Lee et al., 2022; McKernan, 2025). In these RNA-seq datasets, reads corresponding to plasmid DNA sequences from the vaccine were detected in the Sequence Read Archive (SRA). Although Illumina sequencing lacks methylation detection capability, the presence of these plasmid-derived reads supports prior observations of residual DNA in vaccinated individuals. Importantly, most RNA-seq protocols employ DNase I or Actinomycin D to suppress DNA contamination, yet vaccine-derived plasmid DNA signals persist even under those conditions—consistent with the potential for stable, immunostimulatory DNA capable of engaging the cGAS–STING axis.

Taken together, these findings suggest a dual effect: enhanced immunostimulatory capacity from widespread m6A modification, coupled with potentially retained high transcriptional activity of SV40 regulatory elements due to their unmethylated status. This interplay may have important implications for the design of plasmid backbones in mammalian expression systems, where both immune recognition and promoter strength must be carefully balanced.

Conclusions

These data demonstrate that Pfizer/BioNTech plasmid manufacturing did not employ Dam-deficient *E. coli* strains. Residual plasmid DNA in mRNA vaccine preparations has been independently confirmed by several groups (Speicher et al., 2025; Kammerer et al., 2024; König et al., 2024; Wang et al., 2024). These studies demonstrate that linearized or nicked plasmid fragments can persist post-purification and retain biological activity. Combined with the present methylation data, these findings suggest that bacterially methylated DNA bearing N^6-methyladenine (m6A) could act as a persistent agonist of the cGAS–STING pathway, contributing to innate immune stimulation or chronic inflammatory states.

In addition, Oxford Nanopore reads traversing the Eam1104I cut site revealed evidence of incomplete plasmid linearization, indicating that small amounts of circular or partially digested plasmid DNA may remain in the final product. Although the frequency of these events is likely very low, such forms could theoretically undergo re-circularization *in vivo* via host ligase activity. Future vaccine manufacturing should therefore consider both residual DNA load, its methylation context, and the completeness of linearization—potentially through the use of dideoxy capping or blunt-end digestion—to minimize immunostimulatory or replication-competent DNA risk.

Acknowledgments

I thank Phillip Buckhaults for inspiring this line of inquiry and sharing evidence of Dam/Dcm methylation of Pfizer/BioNtech plasmid DNA (unpublished personal communication). Stephen McLaughlin for accelerating Dorado base calling on an amazon GPU and subsequent BAM file generation. ChatGPT5.o generated Python code for extracting methylation signals from BAM files and helped to organize the manuscript and generate Figure 8.

Conflict of Interest

The author is affiliated with Medicinal Genomics, a company engaged in sequencing and genomic analysis of plant and microbial DNA. No external funding influenced the conclusions of this work.

Data Availability

BAM file:
https://mega.nz/file/UYZhFToC#WD6Srp8BniZ8CCwYFvCIZgCdINiIYFsM6_KxblWH-ys

OR134577 features BED file:

https://mega.nz/file/IEpWhAwZ#IluheOf0H4UAnnQ3A7a3DST_AFaBQgErJOEDSI3fHCE

OR134577 GATC BED file:

https://mega.nz/file/cEpgjKrb#8o0aXrpROY9KbzAjVYZ6CET3Y--SOTF1NVphHso-QSE

Python code:

https://mega.nz/file/ZJhzTSAS#Ts1j7myPlUSpYzE6JsDdUBEb1EZKlx_Aw2_jsjvd8kA

Python output for Figure 8.

https://mega.nz/file/hNpAWbrA#KxQCPFjoRHzcDYns0cs1d7lVw7gHYuxHa_Nny2oB9Ew

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