DAM methylation and cGAS-STING

Added fuel to the inflammatory fire

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Hyper-stimulatory N⁶-methyladenine (m6A) residual SV40 plasmid DNA in mRNA vaccine

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Abstract

Many mRNA vaccine production pipelines rely on *Escherichia coli* to replicate pla DNA templates used in the in vitro transcription of modified RNA. However, *E.* DNA methylation patterns differ substantially from those of humans. In *E. coli*, I methylation is primarily mediated by DNA adenine methyltransferase (Dam), wh introduces N⁶-methyladenine (m6A) within GATC motifs, whereas human methy occurs predominantly at cytosines in CpG dinucleotides. Some *E. coli* strains alsc

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Cytosolic DNA that lacks CpG methylation can potently activate Toll-like recept (TLR9), while m6A-modified DNA has been shown to stimulate the cGAS–STIN(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 residua plasmid DNA has been detected in finished vaccine material, it is likely that this bears bacterial-type methylation patterns that could be immunostimulatory thro TLR9 and cGAS–STING signaling. To investigate this possibility, we applied Oxf Nanopore sequencing to examine the methylation status of plasmid DNA presen Pfizer lot FL8095.

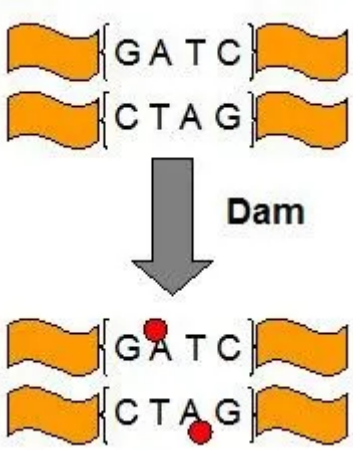


Figure 1. Red dots reflect methylation of **N⁶-methyladenine** in the palindromic sequence GATC (image source- https://2009.igem.org/Team:Imperial_College_Lc M3/DamMethylation)

Methods

We purified 600µl of Pfizer lot FL8095 using 6µl of 10% Triton X. After a quick v samples were heated at 95°C for 2 minutes and then spun at 15,000 RPM for 5 mi Samples are placed on ice to harden the top oil layer and 2.5µl of RNaseA (Monaı 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 proto using 600µl of Ampure, 600µl of 100% Isopropanol and 12µl 1M MgCl2. Samples

mixed and allowed to bead bind for 10 minutes. A magnet is used to separate the 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 Centrifugatior

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

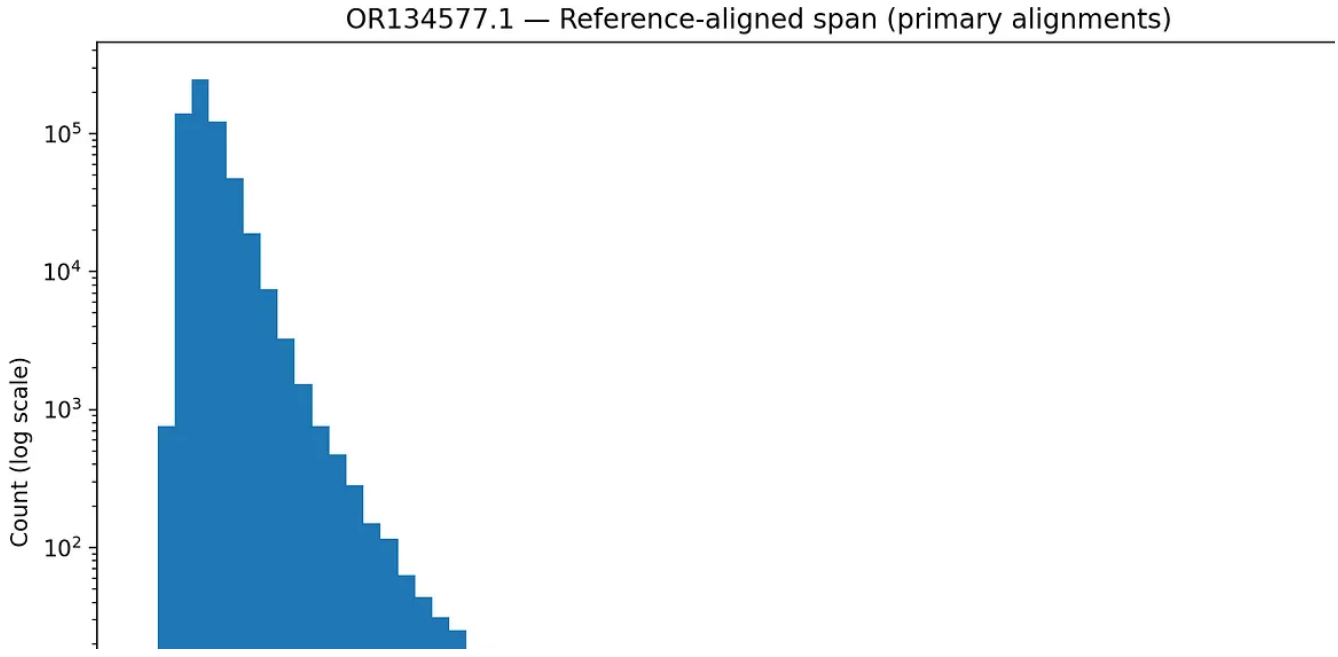
These libraries were loaded onto an Oxford Nanopore MinION Mk1D using thei R10.4.1 flow cells. Sequencing reads were base called with the Dorado base caller

the dna_r10.4.1_e8.2_400bps_sup@v5.2.0_6mA@v1 model. Minimap2 was used tc 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 tl Eam1104I site.
To accurately detect reads spanning the linearization junction, the reference w concatenated (i.e., duplicated end-to-end) so that alignments could occur acros Eam1104I cut site.
Mapping reads to the terminal ends of a single-copy reference would otherwise r in minimal alignments because most read mappers do not handle circular referer 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 palindro sequence. Top strand is Grey and bottom strand is Green. Dam methylation can observed (Figure 4-7) and varies across the plasmid (Figure 8).



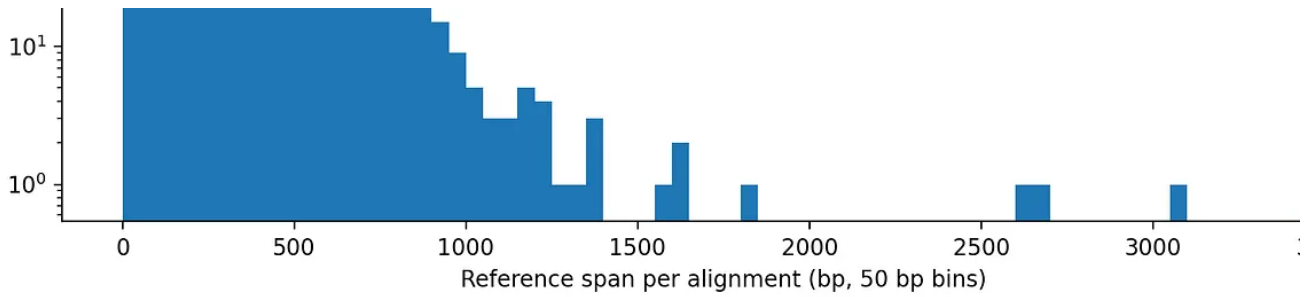


Figure 3. Aligned ONT Read length distributions. Oxford Nanopores cannot thr circular DNA through the pores and cannot be measured with these methods.

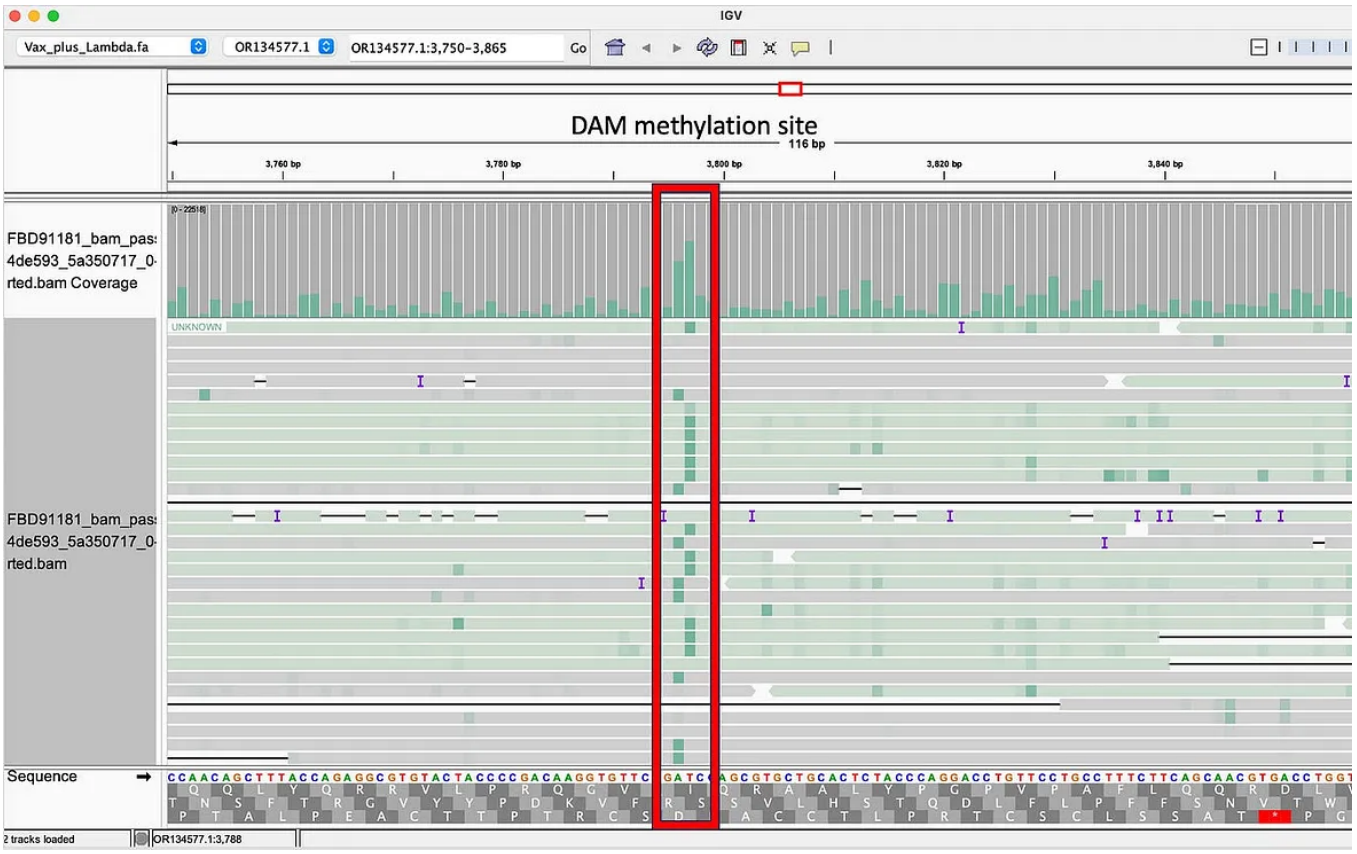
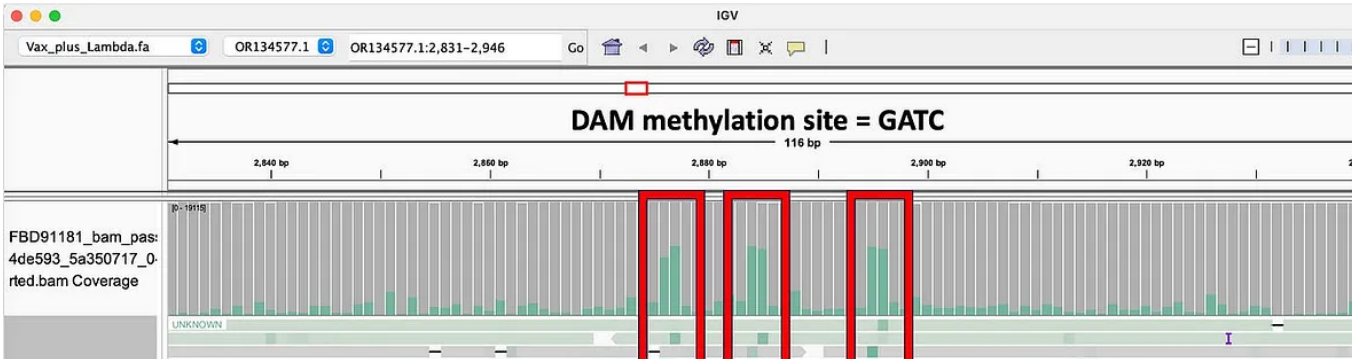


Figure 4. IGV display of Dam methylation (GATC) in Plasmid DNA sequence fro Pfizer lot FL8095



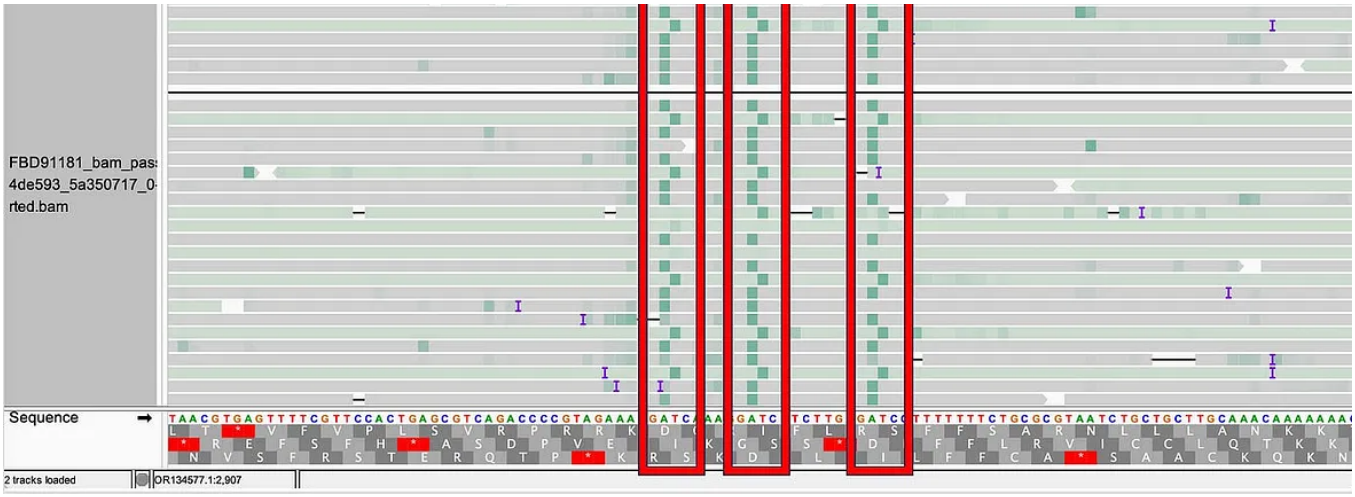
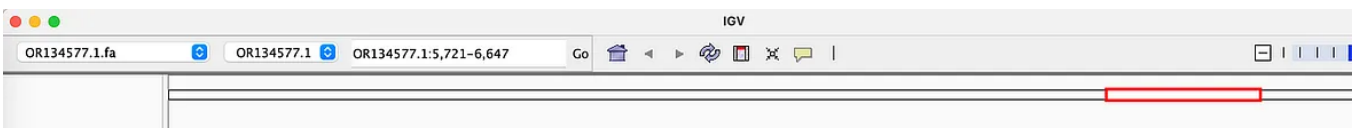


Figure 5. Tandem GATC sites heavily methylated

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



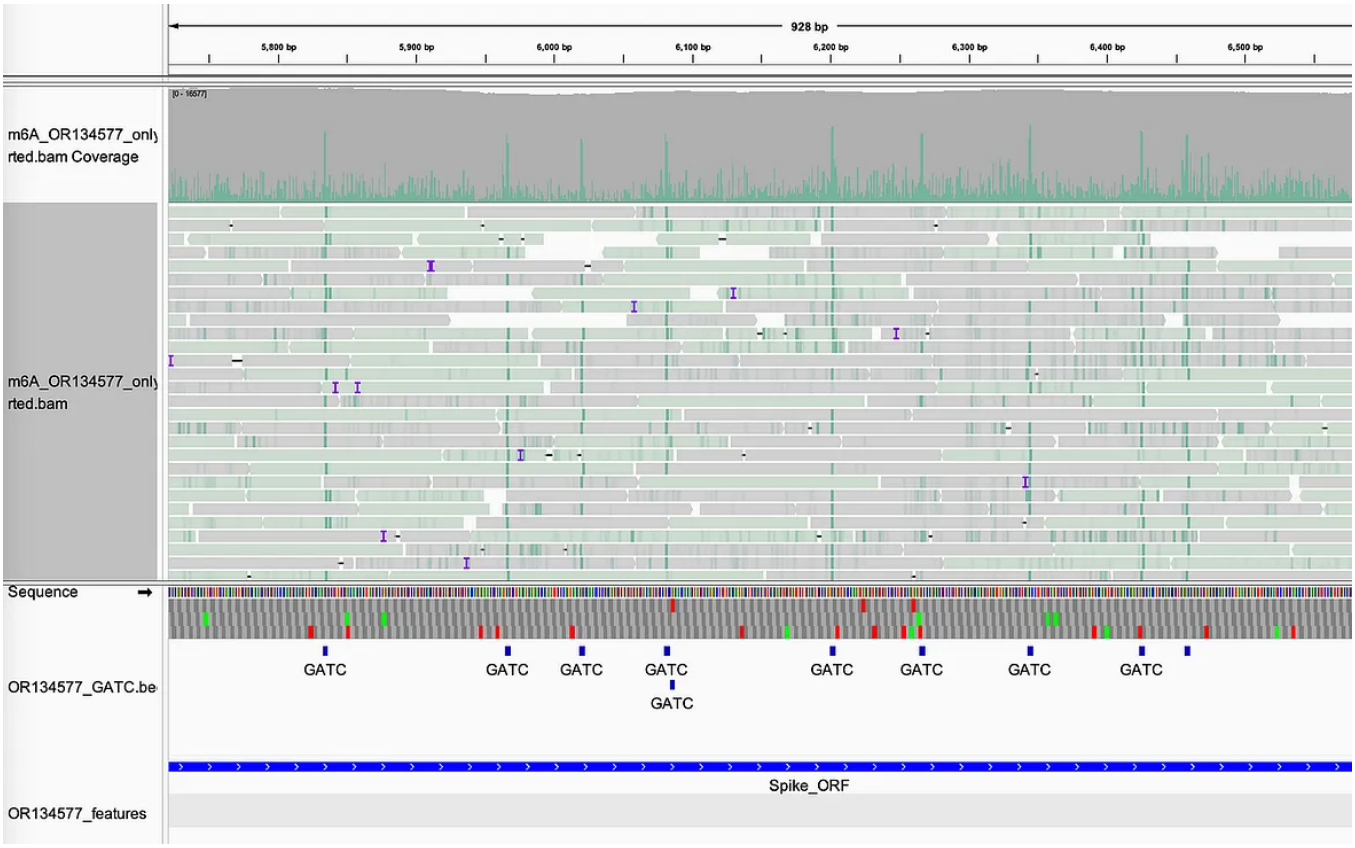


Figure 7. The spike ORF is densely methylated.

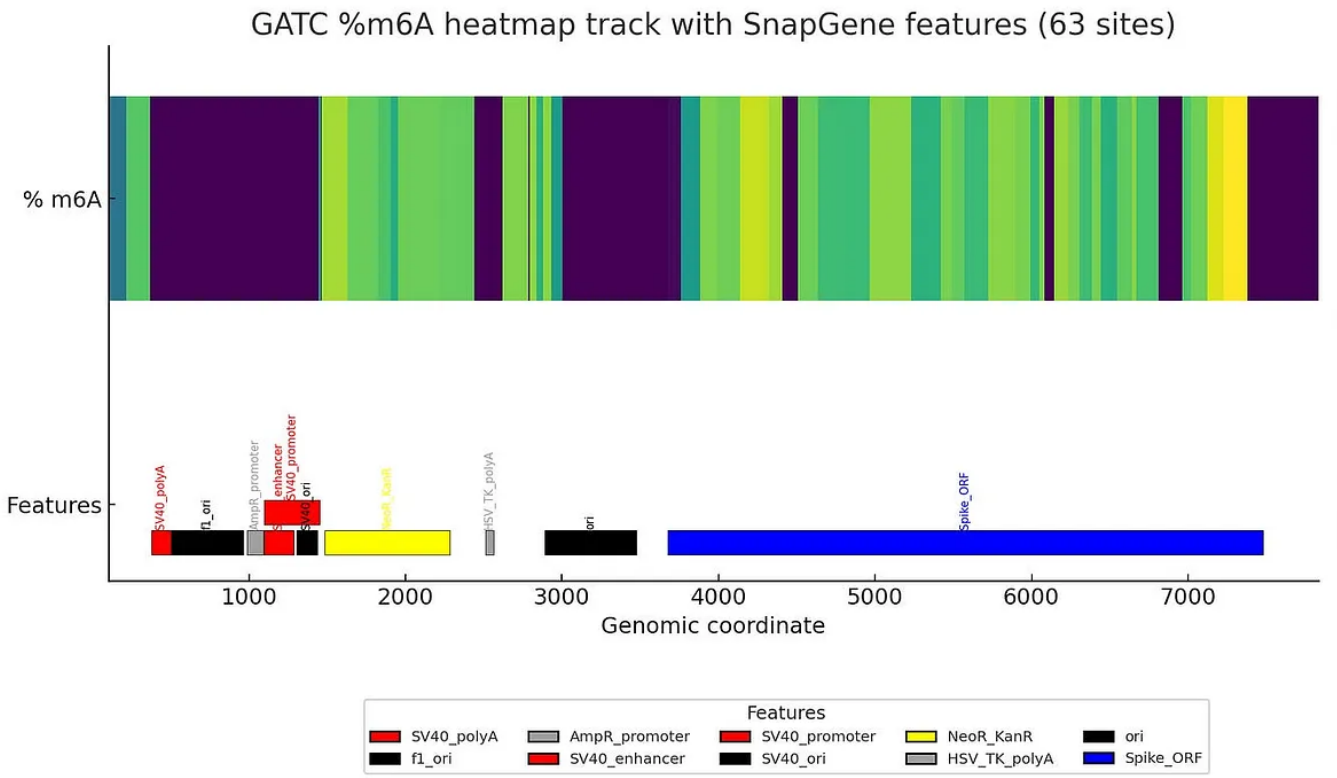


Figure 8. Methylation Heatmap across the plasmid demonstrate hypomethylatio
SV40 components while hypermethylation of Neo/Kan and Spike.

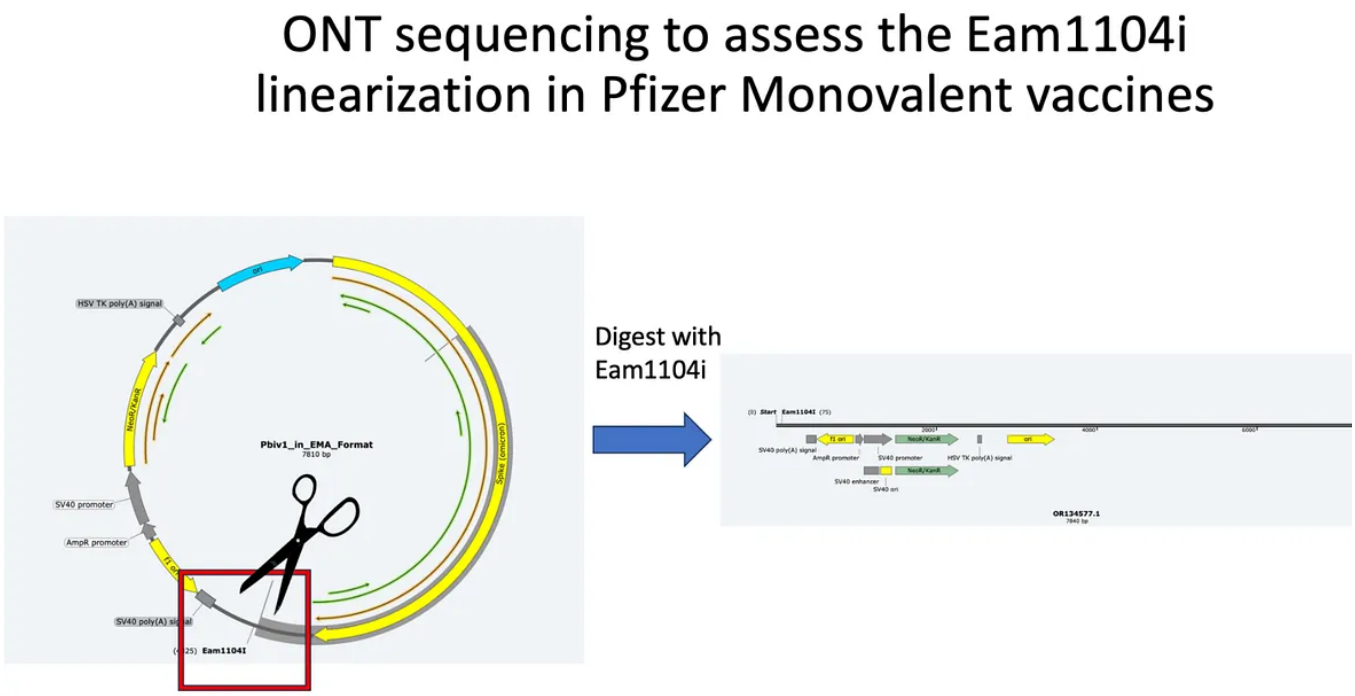


Figure 9. Oxford Nanopore sequencing workflow illustrating the Eam1104I diges strategy for linearization of the Pfizer/BioNTech plasmid prior to RNA transcrip

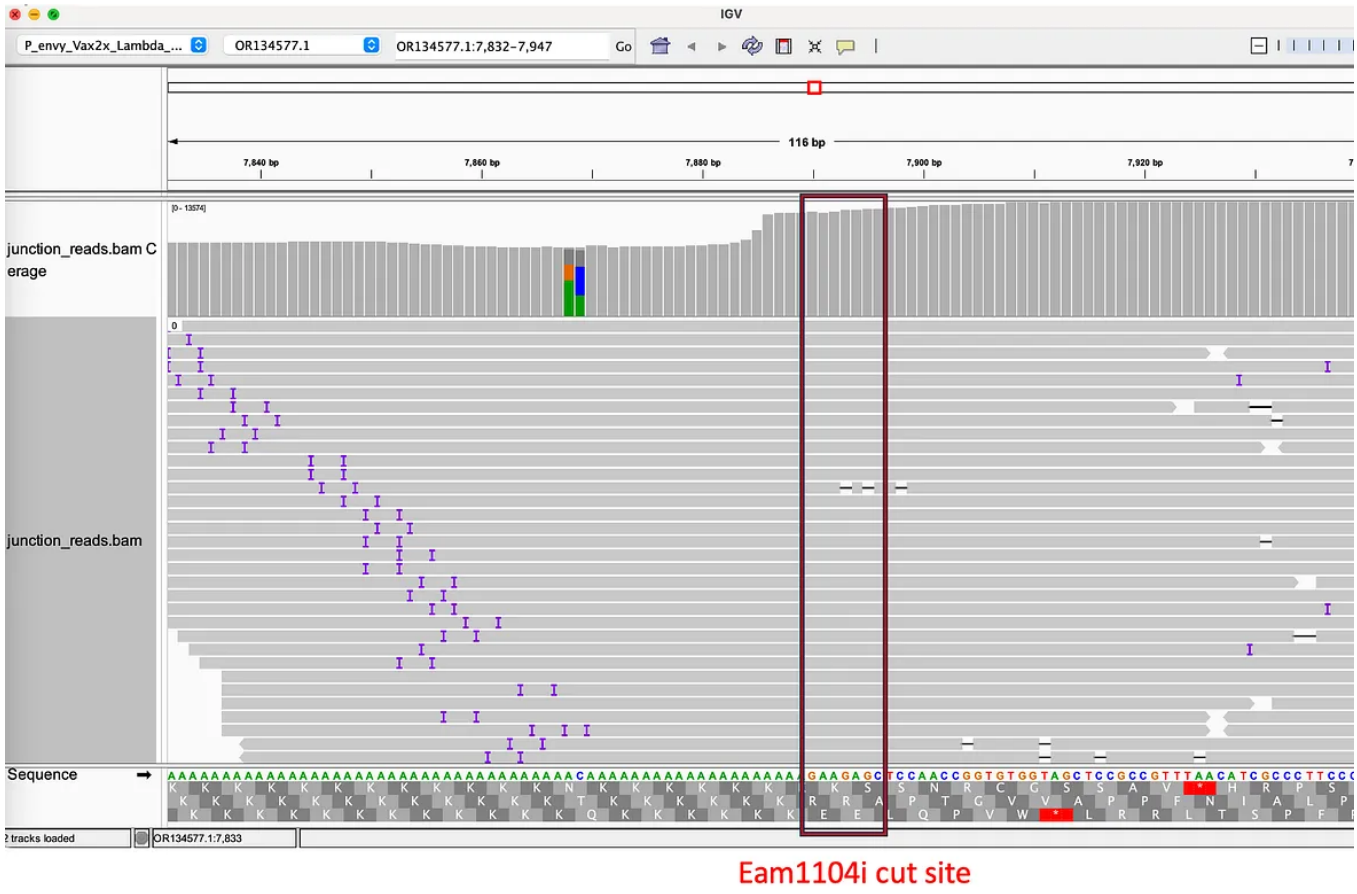


Figure 10. IGV visualization of Oxford Nanopore reads spanning the Eam1104I c site, showing read-through across the expected junction and incomplete plasmid linearization.

Evidence of Incomplete Linearization at the Eam1104I Site

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

The presence of circular plasmids is not expected to arise *post-digestion*, as host li are absent during the in vitro transcription preparation. However, once injected mammalian tissue, host ligases could potentially re-circularize linear plasmid fragments that retain complementary 5′ overhangs. This raises a theoretical conc 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 nor cleavage site. Because Oxford Nanopore platforms cannot process circular DNA, exact abundance of full-length circular plasmids cannot be directly measured wit additional enzymatic linearization of residual DNA. Nevertheless, these findings demonstrate that incomplete digestion and low-level persistence of circular form cannot be excluded in the vaccine DNA template.

Discussion

These data firmly establish that Pfizer/BioNtech plasmid manufacturing did not Dam knock out *E.coli* strains. DNA containing N^6-methyladenine (m6A) is increa recognized as an immunostimulatory signal when detected by the host innate im 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 detecti foreign DNA and triggering type I interferon production (Li & Chen, 2016).

Promoter and enhancer regions are often sensitive to DNA methylation, and clas studies with **cytosine methylation** have shown that methylation within the SV40 regulatory region can reduce promoter-driven gene expression (Bryans et al., 199 Although those studies did not examine adenine methylation, they provide prece that methyl groups within regulatory sequences can influence chromatin accessi or promoter activity. In the context of **m6A** (Dam methylation), it remains plausil that methylation at GATC sites in the SV40 region could alter transcription facto binding or chromatin state, although direct evidence in this system is lacking.

Recent single-molecule chromatin profiling of plasmids demonstrated that prom identity strongly influences nuclear import and chromatinization (Mallory et al., These findings suggest that epigenetic and structural features of plasmids togeth 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. c* strain. This selective lack of methylation suggests that the bacterial host does no target these regulatory elements for m6A modification, thereby potentially prese 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 imı system via cGAS–STING.

Beyond the SV40 regulatory region itself, bacterial methylation state has been sh to influence the biological performance of plasmid DNA in mammalian systems. et al. (2011) reported that *dcm*⁻ plasmids, which lack cytosine methylation at CCV motifs, exhibited enhanced transgene expression in human cells but paradoxicall **reduced immunogenicity.** This highlights that plasmid methylation patterns—b adenine (Dam) and cytosine (Dcm) sites—can have measurable downstream effec expression and innate immune activation. Their observation that plasmid methyl 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 D stress blunts type I interferon output and shifts the transcriptional program towa pro-survival and immunosuppressive signaling. In this context, residual plasmid bearing m6A marks could, if persistently sensed, promote maladaptive inflamma and possibly contribute to oncogenic risk through chronic STING engagement.

It has previously been reported that differential gene expression analyses betwee vaccinated and unvaccinated cohorts revealed altered expression of genes in the STING pathway (Lee et al., 2022; McKernan, 2025). In these RNA-seq datasets, re 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- protocols employ DNase I or Actinomycin D to suppress DNA contamination, ye vaccine-derived plasmid DNA signals persist even under those conditions—cons with the potential for stable, immunostimulatory DNA capable of engaging the c STING axis.

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

Conclusions

These data demonstrate that Pfizer/BioNTech plasmid manufacturing did not en Dam-deficient *E. coli* strains. Residual plasmid DNA in mRNA vaccine preparati has been independently confirmed by several groups (Speicher et al., 2025; Kamn 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 a persistent agonist of the cGAS–STING pathway, contributing to innate immun 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. Alth the frequency of these events is likely very low, such forms could theoretically un re-circularization *in vivo* via host ligase activity. Future vaccine manufacturing sh therefore consider both residual DNA load, its methylation context, and the completeness of linearization—potentially through the use of **dideoxy capping** o blunt-end digestion—to minimize immunostimulatory or replication-competent risk.

Acknowledgments

I thank Phillip Buckhaults for inspiring this line of inquiry and sharing evidence 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 Pytho code for extracting methylation signals from BAM files and helped to organize th manuscript and generate Figure 8.

Conflict of Interest

The author is affiliated with Medicinal Genomics, a company engaged in sequen and genomic analysis of plant and microbial DNA. No external funding influence 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--SOTF1NVphHQSE

Python code:

https://mega.nz/file/ZJhzTSAS#Ts1j7myPlUSpYzE6JsDdUBEb1EZKlx_Aw2_jsjvc

Python output for Figure 8.

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

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