

A MODIFICATION-FREE NEGATIVE CONTROL DEFINES THE OPERATING POINTS AT WHICH 5mC AND 6mA CAN BE CALLED FROM R9.4.1 NANOPORE DATA

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Abstract

Nanopore sequencing reads DNA base modifications from the ionic-current signal, but the R9.4.1 chemistry has no vendor model for N6-methyladenine (6mA), and third-party models are applied without an absolute error estimate. We measured the false-positive rate of two 6mA callers and of one all-context 5-methylcytosine (5mC) caller against a negative control that cannot carry either mark: PCR-amplified cDNA, in three independent controls from two cell lines. False-positive rates were resolved per 5-mer context and compared with the specificity that each modification's prevalence demands. For CpG methylation, the measured false-positive rate remained approximately two orders of magnitude below the maximum rate permitted under the stated assumptions, and no context restriction was required. For 6mA, the pooled false-positive rate at the commonly used probability threshold of 0.7 was 23.2-fold higher than the maximum admissible rate. Most of the excess background was contributed by 30 of the 256 adenine-centred contexts, none of which carries a cytosine immediately 5' of the called base; excluding them improves specificity 5.9-fold at threshold 0.95 and brings the caller inside the requirement there. The rank ordering of context-specific false-positive rates was correlated between cell lines (Spearman $\rho = 0.862$), although absolute rates were not identical. Under the

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tested Dorado/NEMO configuration, a second 6mA caller classified 0.63% of adenines as modified in amplified DNA even at threshold 0.99, corresponding to 277 times the admissible false-positive rate and insufficient specificity for rare 6mA detection. Genomic DNA prepared with the FFPE repair step omitted, so that 8-oxo-dG is preserved, inflates apparent 6mA 9- to 15-fold while leaving CpG calls unaffected.

Key words: nanopore sequencing, DNA methylation, N6-methyladenine, false-discovery rate, negative control, mitochondrial DNA

1. Introduction Nanopore sequencing reads base modifications from the ionic-current signal, without chemical conversion or amplification. The approach is mature for 5-methylcytosine (5mC) at CpG dinucleotides, where several callers agree closely and have been benchmarked against bisulphite data [1]. It is not mature for N6-methyladenine (6mA). Independent quantification has attributed most 6mA reported in eukaryotes to bacterial contamination [2]; high-precision mapping in mammalian cells found the residual mitochondrial signal to be independent of the putative methyltransferase and probably artefactual [3]; and the abundance at which the mark is claimed to occur, of the order of four residues per mitochondrial genome [4], is low enough that a caller’s error rate, rather than its sensitivity, becomes the limiting quantity.

This limit is rarely made explicit. For a target of prevalence p detected with sensitivity S at a tolerated false-discovery rate α , the admissible false-positive rate is

$$(1) \quad \text{FPR}_{\max} = \frac{\alpha p S}{(1 - \alpha)(1 - p)}.$$

At a somatic CpG methylation prevalence of approximately 0.7, the stated assumptions permit a false-positive rate of approximately 0.123 among genuinely unmodified sites while maintaining a 5% false-discovery rate. At $p = 4.3 \times 10^{-4}$, the value implied for mitochondrial 6mA [4], the same tolerance admits fewer than one error in forty-four thousand. The two requirements differ by nearly four orders of magnitude. Agreement between modification callers is frequently used as supporting evidence, but it does not provide an independent estimate of the absolute error rate: two callers applied to one set of reads share the flow cell, the pore and the signal, and therefore share their confounders [5].

R9.4.1 chemistry has been withdrawn, so the large body of data acquired with it cannot be regenerated. We therefore asked what can be established about caller error from material already in hand. PCR-amplified cDNA provides an empirical negative control because the polymerase incorporates unmodified deoxynucleotides. Therefore, modification calls detected in this material can be treated as false-positive calls under the assumptions of the experiment. The design derives from the controlled-amplicon approach of BICCI et al. [6]; because that study used a reference-anchored hidden Markov model, whereas the callers

examined here read modification state from the basecalled sequence, we adopted its experimental design and none of its numerical thresholds.

2. Materials and methods. 2.1. Material. Three amplified controls (SQK-PCS111 kit, FLO-MIN106 flow cells; Oxford Nanopore Technologies, Oxford, UK) were used: JAR cDNA on two flow cells seventeen days apart, and JEG-3 cDNA on a third, the last two run on the same day. Native genomic DNA (SQK-LSK109) from the same two choriocarcinoma lines provided the comparison arms; these libraries include the FFPE repair step and are termed Fpg-repaired. A further JAR genomic library, and both adipose-derived mesenchymal stromal cell libraries of Section 3.6, were prepared by the published 8-oxo-dG protocol [7] – the same ligation kit with the FFPE repair step deliberately omitted, so that 8-oxo-dG is preserved – and are termed unrepaired. Mitochondrial genomes were linearised with PvuII, which cuts the revised Cambridge reference sequence [8] once, at position 2074.

2.2. Basecalling. All datasets were basecalled with Guppy 5.0.16 and the Rerio all-context model `res_dna_r941_min_modbases-all-context_v001`, which emits 5mC and 6mA in one pass, is the only all-context 6mA model available for this chemistry and has been used elsewhere in a treated-versus-control design [9]. A second 6mA caller (Dorado 0.3.3 with the third-party NEMO_R9_6mA model) was evaluated in parallel. Configuration identity between every pair of arms was verified field by field from each run’s reported parameter set (version, configuration, model file, chunk size, chunks per runner, minimum q-score, records per file, basecaller and runner counts, device), with no field differing. Only pass reads were retained, and primary alignments with mapping quality ≥ 20 , total soft-clipping ≤ 300 bp and mean read quality ≥ 9.1 were analysed.

2.3. Alignment and extraction. Legacy tags were converted with *modkit update-tags --mode implicit* (modkit 0.6.4). Genomic arms were aligned with *minimap2 -ax map-ont -y* [10] and transcript-derived arms with *minimap2 -ax splice -uf -k14 -y*, to T2T-CHM13v2.0 [11]. Because 85% of amplified-arm reads span a splice junction, projection of modification calls across N CIGAR operations was verified by independent reimplementations; all 659 calls on 23 junction-spanning reads across 11 chromosomes agreed. Per-read calls were extracted with *modkit extract full --mapped-only --kmer-size 5*.

2.4. Context assignment. Reference k-mers are written in reference-strand orientation, whereas the modification lies on the read strand; they were therefore reverse-complemented on the minus strand and case-normalised, the reference being soft-masked. Uncorrected, these two steps alone place half of all observations in the complementary context and inflate the apparent context count from 512 to 8127. Contexts were therefore assigned from the read-derived k-mer, whose central base matches the called base in 100% of rows. Positions implied canonical by implicit-mode marking were retained in the denominator with probability zero: each is an observation of an unmodified base that the caller declined to call.

2.5. Read-end exclusion. The adenine false-positive rate is strongly position-dependent within reads, peaking 50–100 bp from the nearest read end and reaching a plateau beyond 250 bp; cytosine shows no comparable structure. The effect is not uniform across materials: at threshold 0.7 the ratio of the outer zone to the read interior is 2.64 in the amplified control, 1.52 in Fpg-repaired genomic DNA and 1.45 to 0.86 in the samples, and it was not observed with the second caller. This difference is consistent with a caller- or model-specific effect rather than an effect introduced solely by the alignment or filtering procedure. Including the outer zone therefore imports a control-specific defect into the context ranking. Every value reported here excludes positions within 250 bp of a read end, applied identically to every arm; this costs 36% of the control’s observations and 4.8% of the sample arms’, and raises the between-line agreement of the per-context rate from $\rho = 0.739$ to $\rho = 0.862$ (Section 3.3).

2.6. Statistics. Sensitivity could not be measured because no enzymatically modified positive control was available. Equation (1) was therefore evaluated at $S = 1$, the most permissive assumption. Since any lower sensitivity would reduce the admissible false-positive rate further, a context that fails under $S = 1$ would also fail under lower sensitivity values. Contexts with fewer than 200 observations were reported as missing rather than estimated (in the event, no context fell below this in any control); contexts with no observed error were assigned a single-event pseudocount and their specificity treated as a lower bound. Specificity is reported as $Q = -10 \log_{10}(\text{false-positive rate})$. Prevalence was taken as 0.7 for CpG, 0.007 for non-CpG cytosine and 4.3×10^{-4} for mitochondrial 6mA [4], with $\alpha = 0.05$. Agreement between controls was assessed by Spearman rank correlation, and the per-position composition of failing contexts by two-proportion tests with Benjamini–Hochberg correction across 16 comparisons.

3. Results 3.1. False-positive rates vary across sequence contexts. Across 4.05×10^7 adenine, 4.17×10^6 CpG and 3.45×10^7 non-CpG cytosine per-read observations in the first amplified control, the pooled false-positive rate at probability threshold 0.7 was 1.40×10^{-3} for CpG cytosines, 1.22×10^{-3} for non-CpG cytosines and 5.24×10^{-4} for adenines. Measured against equation (1) these behave very differently (Table 1). CpG methylation is detected with about a hundredfold margin at every threshold tested, and all 64 CpG contexts qualify. Non-CpG methylation qualifies only from threshold 0.9 upwards. For 6mA the pooled rate is 23.2 times the admissible value at threshold 0.7, corresponding to an expected false-discovery rate of 55% for per-site calls.

When analysed by sequence context, the elevated adenine false-positive rate was concentrated in a subset of 5-mers (Fig. 1). The median adenine context reaches Q 53.0 at threshold 0.98 against a requirement of Q 46.5, while the 30 worst contexts reach only Q 30 and carry the pooled rate down with them. Restricting the analysis to the 226 qualifying contexts, at a cost of 10.8% of observations, improves the pooled rate at threshold 0.95 from 4.49×10^{-5} to 7.64×10^{-6} , a

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Measured false-positive rate against the specificity that each modification's prevalence requires (equation (1), $\alpha = 0.05$, $S = 1$), and the effect of restricting adenine to the 226 qualifying contexts. Read interiors only; denominators 4171315 (CpG), 34466386 (CpH) and 40471342 (adenine) observations

Mark	Prevalence	Required FPR	Measured, thr. 0.7	Measured, thr. 0.98	Contexts qualifying	Allowlist FPR, thr. 0.95
5mCpG	0.7	1.23×10^{-1}	1.40×10^{-3}	4.79×10^{-6}	64 / 64	not required
5mCpH	0.007	3.71×10^{-4}	1.22×10^{-3}	1.02×10^{-5}	192 / 192	not required
6mA	4.3×10^{-4}	2.26×10^{-5}	5.24×10^{-4}	1.95×10^{-5}	226 / 256	7.64×10^{-6}

Note: The 30 excluded adenine contexts, grouped by motif family: nine carry a 5' CT before the called adenine (*CTATA*, *CTAAG*, *CTAGT*, *CTATT*, *CTATC*, *CTACC*, *CTATG*, *CTAAA*, *CTAGA*); nine contain CG (*CGAAA*, *CGAAG*, *TAACG*, *ATACG*, *CGACG*, *CGATA*, *CGATT*, *GTACG*, *TTACG*); the remaining twelve are *GAA*, *GGAA* or *AGAA* (*AGAAA*, *GAAAG*, *GAAAA*, *AGAAG*, *GAAAT*, *GAAGT*, *GAAGG*, *GGAAA*, *GAAGC*, *GAAGA*, *GAAAC*, *GGAAG*). None carries a cytosine immediately 5' of the called base, against 28% of the 226 qualifying contexts.

5.9-fold gain that brings the caller inside the requirement there rather than only at 0.98 (Table 1).

3.2. The failing contexts lack a 5' cytosine. The 30 failing contexts differ in composition from the 226 qualifying ones, but not in the way the pooled statistics first suggest. None of the 30 carries a cytosine immediately 5' of the called adenine, against 28% of the qualifying contexts ($P_{\text{adj}} = 0.012$), and 47% carry a cytosine two positions 5' against 22% ($P_{\text{adj}} = 0.028$). Measures of adenine content and homopolymer length, which do separate the two sets when all read positions are pooled, do not separate them in read interiors: the longest homopolymer run is 2.00 against 1.85 ($P = 0.076$) and the flanking adenine count 1.10 against 0.99 ($P = 0.34$). Eighteen of the 30 fall into two disjoint motif families – nine carry a 5' CT before the called adenine and nine contain CG – and the remaining twelve are *GAA*, *GGAA* or *AGAA*. The mechanism the composition suggests is the inverse of a homopolymer effect: a cytosine immediately 5' of the called base gives a clean current transition into it, and its absence leaves the adenine poorly localised. No simple predicate reproduces the set, so the allowlist is carried as the explicit list of contexts given in the note to Table 1.

3.3. Context-specific false-positive rates are correlated between cell lines. In two additional amplified controls, the rank ordering of context-specific false-positive rates was strongly correlated between flow cells from the same line (Spearman $\rho = 0.983$) and remained correlated between the two cell lines ($\rho = 0.862$) (Fig. 2). The exclusion list showed substantial, although incomplete, overlap between controls: 25 of the 30 excluded contexts were also among the 30 worst

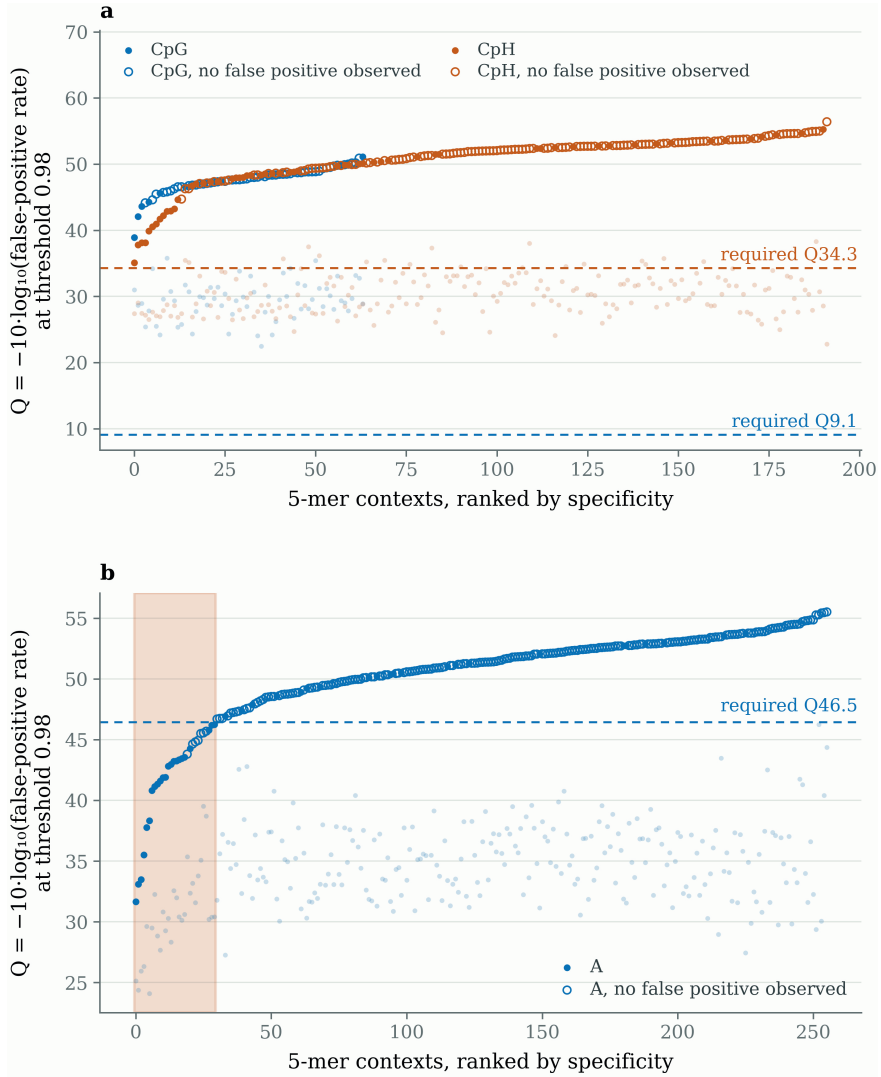


Fig. 1. Per-5-mer false-positive rate of the Guppy 5.0.16 + Rerio all-context model, measured on PCR-amplified cDNA, which cannot carry either modification, at modified-base probability threshold 0.98 and expressed as $Q = -10 \log_{10}(\text{false-positive rate})$, with contexts ranked by specificity. Faint points, the same k-mers at threshold 0.7. Dashed rules, the specificity each mark's prevalence demands at $\alpha = 0.05$ and $S = 1$: Q 9.1 for CpG at $p = 0.7$, Q 34.3 for non-CpG cytosine at $p = 0.007$ and Q 46.5 for 6mA at $p = 4.3 \times 10^{-4}$. Open symbols, contexts in which no false positive was observed, whose Q is a lower bound from a single-event pseudocount. (a) Cytosine: every context clears its requirement – all 64 CpG ($n = 4171315$ observations) and all 192 non-CpG ($n = 34466386$). (b) Adenine: 30 of 256 contexts fall below Q 46.5 ($n = 40471342$). Shading marks those 30 rejected contexts; the 226 unshaded contexts to the right are the allowlist. The rejected contexts reach as low as Q 30 while the median adenine context reaches Q 53.0. The 5-mer restriction applies to sequence context and not to a motif, so the caller remains all-context. All values are computed over read positions ≥ 250 bp from the nearest read end

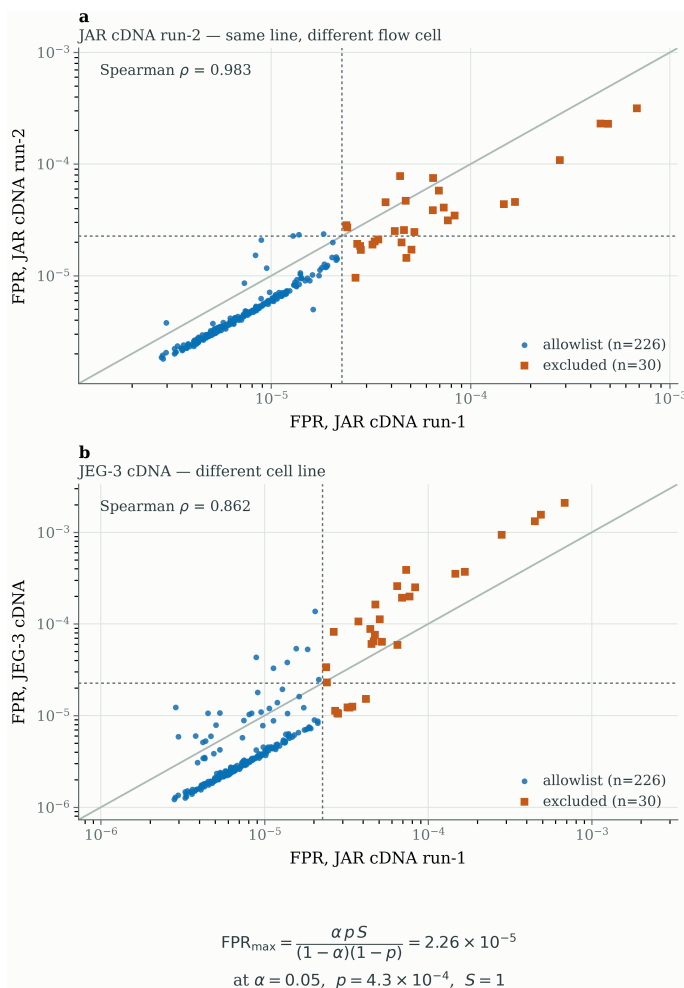


Fig. 2. Per-5-mer false-positive rate for adenine contexts at modified-base probability threshold 0.98, read interiors ≥ 250 bp, plotted for two further amplified controls against JAR cDNA run 1, from which the allowlist was derived. Blue circles, the 226 contexts on the allowlist; orange squares, the 30 excluded. Grey line, identity, not a fit. Dashed rules on both axes, the required specificity given by the equation below the panels. (a) JAR cDNA run 2, the same cell line on a different flow cell and day (212 704 032 observations, 125 772 775 in the read interior). (b) JEG-3 cDNA, a different cell line and mitochondrial haplotype (322 382 960 observations, 193 002 075 in the interior). Reference control: JAR cDNA run 1, 123 935 296 observations, 79 109 043 in the interior. Spearman correlation was $\rho = 0.983$ between the two JAR runs and $\rho = 0.862$ between JAR and JEG-3. When all read positions were included, the corresponding values were 0.936 and 0.739. The overlap between controls was substantial but incomplete: 25 of 30 and 23 of 30 excluded contexts are also among the 30 worst in the other control, while 3 of 226 and 7 of 226 allowlisted contexts exceed the requirement there – the blue points above the horizontal rule – and 10 of the 30 excluded contexts would qualify on a second flow cell of the same line. The median allowlist rate in both additional controls was approximately half of that in the reference control, indicating greater reproducibility of the context ranking than of the absolute background rate

in the second JAR control, and 23 of 30 in the JEG-3 control. Transfer is not perfect in either direction – 3 of 226 and 7 of 226 allowlisted contexts exceed the requirement in the second control, and 10 of the 30 excluded contexts would qualify on a second flow cell of the same line. The median allowlist rate in each of the two additional controls was approximately half of that observed in the first control, indicating that the relative ranking of contexts was more reproducible than their absolute false-positive rates; therefore, background estimates transferred between cell lines should be reported with an uncertainty interval rather than as a single point estimate. Over all read positions, the same two coefficients are 0.936 and 0.739, so it is the between-line agreement that falls below 0.8 while the within-line agreement does not; excluding the outer 250 bp is what carries the between-line value across the conventional 0.8 threshold for a stable ranking, and that is the reason for excluding them.

3.4. A second 6mA caller fails the same test. The Dorado/NEMO combination reports 0.63% of adenines as modified at threshold 0.99 on amplified DNA, and 12.2% at threshold 0.5, that is 277 to 5378 times the admissible value; these figures are genome-wide, over 98 348 102 adenine observations in the read interior. Restricting the same evaluation to the mitochondrial contig, as a first analysis did, changes nothing: the genome-wide rates run 5–17% above the mitochondrial ones and the verdict is identical at every threshold. On mitochondrial sequence, where the treated and untreated arms can be compared with it directly, this caller’s background accounts for 93–97% of what it reports on the untreated arm and for 104–111% of what it reports on the treated one, and its treated-to-untreated ratio is 0.87–0.89, that is, no effect. It did not show a comparable read-end pattern, suggesting that this effect may be specific to the Rerio caller or model rather than being caused solely by the library or alignment procedure. Under the tested configuration, the caller did not provide sufficient specificity for reliable detection of rare 6mA events. This comparison also shows that the proposed criterion distinguishes between caller configurations with substantially different background rates.

3.5. Unrepaired DNA inflates 6mA but not CpG. Genomic DNA prepared by the 8-oxo-dG protocol, in which the repair step is omitted so that the lesion is preserved, yielded 9- to 15-fold higher apparent 6mA than Fpg-repaired genomic DNA from the same line at every threshold (14.8-fold at 0.7; 11.0-fold at 0.99). This experiment does not directly measure 8-oxo-dG. The result is consistent with unrepaired DNA lesions, or another protocol-associated factor, increasing the number of bases classified as 6mA. CpG calls from the same reads did not show analogous inflation but instead showed a moderate reduction (ratio 0.80) at threshold 0.7, which became more pronounced at higher thresholds. This pattern is compatible with reduced call confidence, although the underlying cause was not directly examined. Accordingly, libraries prepared without the repair step should not be used for absolute 6mA estimates with this caller without additional

matched controls. The present results do not indicate comparable inflation of cytosine calls.

3.6. Application. We compared mitochondrial 6mA in adipose-derived mesenchymal stromal cells, untreated and after palmitate. The treated arm gave 0.41, 0.39, 0.36, 0.34, 0.30, and 0.31 times the untreated level at thresholds 0.5, 0.7, 0.9, 0.95, 0.98, and 0.99 (Fig. 3). Across that range the stringency rises a hundred-fold and four hundred times fewer calls survive, yet the ratio hardly moves. The difference is significant at every threshold. At 0.98, for instance, the untreated arm gave 156 calls in 1 824 089 adenine observations and the treated arm 19 in 734 591 ($z = 5.2$, $P = 1.8 \times 10^{-7}$). Both arms are unrepaired material, so the inflation measured in Section 3.5 is common to them and cancels in the ratio. For the same reason no absolute level is quoted, and neither arm is compared with the amplified control's floor, which was measured on repaired material and is not a floor for these libraries.

4. Discussion Three practical conclusions follow. First, the appropriate operating threshold depends both on the characteristics of the caller and on the expected prevalence of the modification. For the caller examined here the operating point can now be stated: probability threshold 0.95 or above, read interiors only, restricted to the 226 qualifying contexts; without the context restriction only from 0.98, and with no margin; at the common default of 0.7 it is 23-fold above the admissible rate. The allowlist at 0.95 holds even if sensitivity is as low as 0.34, the requirement scaling linearly with S . The margins at 0.98 and 0.99 are partly selection, the allowlist having been derived at 0.98, which is why the 0.95 figures are the ones quoted. Second, agreement between callers alone does not establish accuracy, because different callers may be affected by shared features of the sequencing signal and experimental material. Had we considered only the direction of the condition ratios, the two 6mA callers would have appeared to agree because both ratios were below unity. However, the negative-control analysis showed that one caller was detecting almost entirely background signal. A modification-free negative control provides an empirical estimate of background that cannot be obtained from caller agreement alone [5]. Third, false-positive calls were concentrated in a subset of sequence contexts, suggesting that context-based filtering may improve specificity: excluding 30 of the 256 contexts recovers sixfold specificity for 6mA, and the per-context rate proved stable enough between cell lines to be transferred with an interval.

Several limitations of the approach should be considered. An amplified control carries no neighbouring modifications, so it estimates a technology-centric background; it also proved not to be a lower bound for native genomic DNA, which called 6mA 3.2-fold below the amplified arm, most plausibly because the amplified libraries were of lower read quality (median mean q-score 9.14 against 10.25). A cDNA control moreover changes two things at once, amplification and transcript origin, so it differs from the arms it calibrates in read length, primer

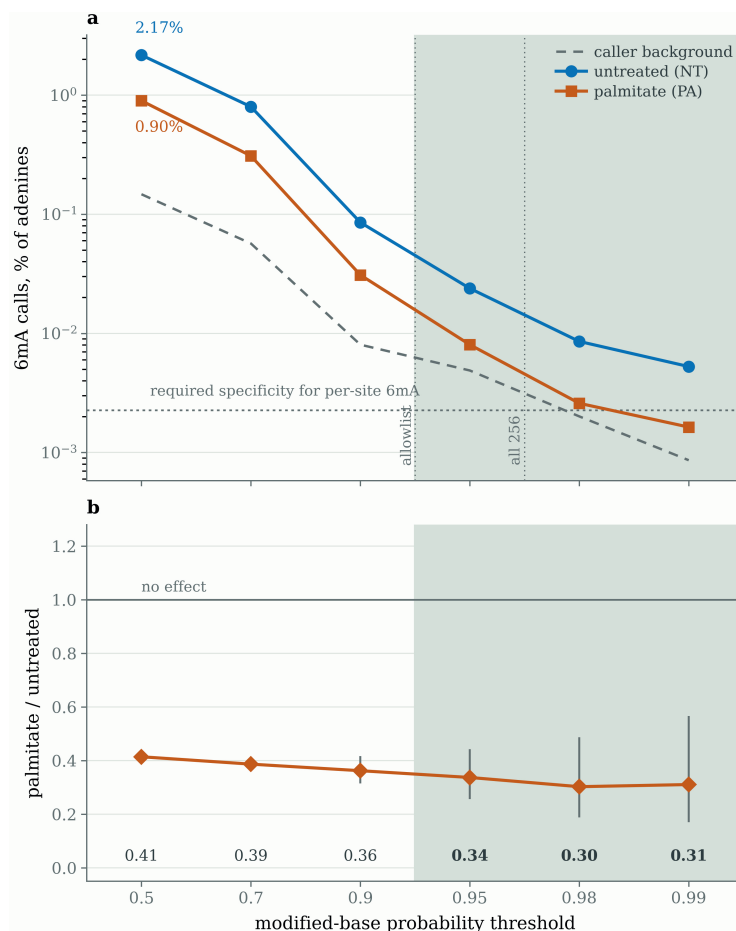


Fig. 3. Mitochondrial 6mA in adipose-derived mesenchymal stromal cells under palmitate. (a) Call rate per adenine in each arm across the probability-threshold sweep. Dashed grey curve, the caller's rate on control-arm reads aligned to chrM in the same read zone ($n = 348\,588$ adenine observations). Horizontal dotted rule, the specificity that per-site 6mA calling would require (2.26×10^{-5}). Shading marks the thresholds at which the caller meets that requirement when restricted to the 226-context allowlist; the two vertical dotted rules give the two boundaries – calls become admissible from threshold 0.95 on the allowlist (left rule) and only from 0.98 if all 256 adenine contexts are used (right rule). The AdMSC libraries were prepared without the Fpg repair step, so they carry unrepaired 8-oxo-dG, whereas the amplified control is a PCR product and can carry no lesion at all; the dashed curve is therefore this caller's rate on lesion-free amplified DNA and is not a floor for these arms. It is drawn to give scale, and no statement of the form “this arm exceeds background” is made. (b) The palmitate-to-untreated ratio, with 95% confidence intervals on the rate ratio from the Poisson counts of modified calls; values in bold are those at thresholds where the caller qualifies. The treated-to-untreated ratio remained below unity across the examined thresholds, ranging from 0.41 to 0.31, although the number of retained calls decreased substantially with increasing stringency. Read positions ≥ 250 bp from the nearest read end throughout; untreated $n = 1\,824\,089$ and palmitate $n = 734\,591$ adenine observations. Panel (b) is presented as an exploratory relative comparison between the two libraries. Since the extent of protocol-associated inflation was not measured independently in each condition, the ratio should be interpreted with some caution

content, splice alignment and k-mer composition; a whole-genome-amplified control, which shares the library route, is the better choice where it is obtainable [12]. Sensitivity was not measured, so all requirements were evaluated under the most permissive assumption. An enzymatically methylated positive control would supply the sensitivity term and convert the requirement from a bound into an estimate.

Although this study examines only R9.4.1 chemistry, the general validation principle may also apply to other nanopore modification-calling workflows. Adenine methylation remains contested in mammalian systems [2,3], while site-specific mitochondrial 6mA has been reported in *Caenorhabditis elegans* by two methods physically independent of nanopore sequencing [13,14], in an organism in which genomic 6mA has been described since 2015 [15]. Studies of rare DNA modifications would further benefit from evaluating each analytical workflow against material that lacks the modification and from relating the measured false-positive rate to the expected abundance of the mark. Such a control can be generated from amplified material and may also be applied retrospectively when suitable sequencing libraries are already available.

Data availability. Code reproducing the figures, analyses, supplementary data, and links to the original raw data are available at <https://github.com/SoryHay/r941-modcall-negative-control>.

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