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A high-throughput nanoscale imaging platform for direct visualization and rapid measurement of telomeres at single-molecule resolution

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Abstract

Telomere length is a biomarker of aging, cancer and telomeropathies, with some of the strongest health-related associations occurring at the extremes of the telomere-length spectrum. Critically short telomeres can drive chromosome instability, cellular stress and senescence, yet conventional assays largely provide population-average measurements that obscure these biologically important molecules, and the underlying heterogeneity of individual chromosome ends. In addition, amplification bias associated with highly repetitive telomeric sequences and inter-laboratory variability can limit measurement reproducibility. Although emerging technologies provide higher-

resolution analyses, they often require high DNA input, specialized sample preparation, or complex instrumentation and bioinformatic tools, restricting broader adoption.

Here, we present a high-throughput single-molecule platform that overcomes these barriers through an automated, user-friendly nanoscale imaging workflow compatible with standard genomic DNA preparations. The method combines a unique dual CRISPR-Cas9 telomere boundary labeling strategy with amplification-free magnetic-bead-based enrichment, preserving the native telomere-length distribution while minimizing representation bias. Using only 500 ng of input DNA and 2–4 h of hands-on processing, the assay accurately resolves intact telomeres spanning ~50 bp to >50 kb within the same sample, enabling simultaneous characterization of the full telomere-length spectrum. It also demonstrates high reproducibility across technical replicates and independent operators (ICC up to 0.90). Validation in telomere-length models, replicative aging systems, and clinical biospecimens, together with subtelomeric barcoding for chromosome-arm assignment, establishes a scalable platform for chromosome-resolved telomere profiling, biomarker development, and future clinical implementation in aging, cancer, and telomere biology.

Keywords: telomere measurement, telomere length, subtelomere mapping, nanoscale imaging, single-molecule analysis, chromosome-end profiling, biomarkers, atomic force microscopy

Highlights

- ✓ Amplification-free HS-AFM workflow for single-molecule telomere analysis
- ✓ Low-input genomic DNA requirement
- ✓ Direct base-pair-scale measurement of telomere length
- ✓ Resolution of short-to-long telomeres across a broad range
- ✓ Capture of single-molecule telomere-length heterogeneity
- ✓ Integration of telomere length with chromosome-end identity

Introduction

57 Telomeres are specialized nucleoprotein structures located at the ends of linear
58 chromosomes, where tandem TTAGGG repeats and associated shelterin proteins form
59 a protective chromosome-end cap. This cap prevents chromosome termini from being
60 recognized as DNA breaks, thereby limiting degradation, end-to-end fusion,
61 recombination, and inappropriate DNA damage signaling.¹⁻⁵ Because telomeres shorten
62 progressively with replication and are highly sensitive to oxidative and genotoxic stress,
63 telomere length and integrity serve as dynamic determinants of genome stability, cellular
64 senescence, aging, and disease susceptibility⁶⁻¹¹.

65 Adjacent to telomeres are subtelomeric regions, which are highly variable
66 chromosome-end domains composed of repetitive elements, segmental duplications,
67 telomere-variant repeats, and regulatory or transcribed sequences¹²⁻¹⁸. These regions
68 contribute to telomere maintenance and chromatin regulation through subtelomeric
69 methylation, telomere-position effects, and TERRA-associated transcription, while their
70 repeat-rich architecture also makes them recurrent sites of structural variation and
71 instability^{15,19-22}. Alterations affecting telomere length regulation are primary drivers of
72 telomere biology disorders (TBDs), such as dyskeratosis congenita and idiopathic
73 pulmonary fibrosis, as well as genomic instability in cancer and developmental
74 abnormalities^{9,23-28}.

75 Despite their clinical importance, accurate measurement of telomere length and
76 subtelomeric architecture remains a significant challenge²⁹⁻³³. Conventional telomere-
77 length assays, including Southern blot-based terminal restriction fragment (TRF)
78 analysis and qPCR, provide bulk, average estimates rather than direct single-molecule
79 measurements: TRF reports average telomere-length distributions across cell
80 populations, whereas qPCR measures relative telomere repeat abundance as a telomere-
81 to-single-copy gene ratio^{29-31,34-36}. Although newer fluorescence- and sequencing-based
82 approaches offer single-molecule, chromosome-arm-specific, or allele-resolved
83 measurements, efficient analysis of intact, very long telomeres (>20 kb) from limited-

84 abundance samples remain technically challenging, often requiring complex
85 computational analysis (Table 1)^{31-33,37-41}. To meet the needs for studies of telomere
86 biology, a measurement platform must overcome several critical methodological
87 hurdles:

88 **Sample Integrity and Input:** Efficient preparation of intact long chromosome
89 ends is essential for applications in inherited disease genetics and oncology. Many
90 methods, including long-read sequencing, require high DNA input and are highly
91 impacted by fragmentation during preparation. There is a clear need for a method
92 compatible with low-input (e.g., 500 ng) genomic DNA that preserves the structural
93 integrity of the entirely intact telomeric regions.

94 **Telomere Size Range and Bias:** The ability to extend the range of measurable
95 telomere sizes is required to reflect the actual distribution in a specimen. Non-amplified
96 paths have considerable advantages over PCR-based methods, such as STELA and
97 qPCR, which often fail to capture the full distribution, particularly very long telomeres,
98 due to amplification bias. Moreover, it is important that the measurement is specific for
99 the telomere, without including sub-telomeric regions (which confounds the
100 interpretation of the data).

101 **Chromosome Identification:** Clinical outcomes are often driven by the shortest
102 telomeres rather than the global average. Identifying which specific chromosome arms
103 are losing protection allows for the detection of telomere-driven instability at its earliest
104 stages. This requires the ability to interrogate very large molecules to combine telomere
105 measurement with barcoding of adjacent subtelomeres, allowing identification of the
106 chromosome of origin. Also, if a sentinel short telomere results in epigenetic changes of
107 proximal non-telomeric loci, identifying the chromosome end could facilitate
108 recognition of associated gene expression perturbations.

HS-AFM of barcoded chromosome-end fragments is a promising strategy to overcome these hurdles. AFM provides a complementary route to existing methods by directly visualizing DNA molecules at nanometer-scale resolution. However, implementing a quantitative molecular imaging method for this problem requires solving non-trivial issues regarding throughput and scanning dimensions.

In this work, we present an amplification-free HS-AFM workflow that enables direct, single-molecule profiling of chromosome ends. Our approach overcomes historical limitations through three key innovations:

Targeted Enrichment and Surface Engineering: By modifying protocols used in long-read sequencing to preserve large molecules and employing a spatially directed strategy using a photocleavable linker-magnetic bead system, we achieve efficient concentration of target molecules directly onto the imaging surface. We also developed an automated washing method that preferentially removes contaminants and linearizes surface-captured molecules for measurement.

Automated Large-Area High-Speed Imaging: Traditional fast AFM interrogates small areas rapidly but requires extensive stitching of image tiles to fit large DNA molecules. We developed a novel, large-area fast-scanning AFM that increases the linear dimensions of a single image tile by 24x and the area by 140x compared to previous implementations, maintaining nanometer registration without the slow process of computational tiling. The compact and fully automated imaging system supports walk-away analysis after sample loading.

Direct Sequence Labeling: We adapted a CRISPR-Cas9 DNA labeling strategy to target telomere-subtelomere junctions and incorporated nickase-based strand-displacement barcoding for chromosome-end identification. We demonstrate that this labeling scheme is robust and reproducible within our sample preparation workflow.

We validated this assay across multiple reference cell lines and whole-blood genomic DNA and demonstrated its ability to resolve biologically meaningful differences in telomere-length distributions, while creating a framework for chromosome-end-resolved telomere analysis.

Finally, our approach offers significant practical advantages (Table 1). It does not require an external standard to generate base-pair measurements or telomere ratio values, thereby eliminating a major source of lab-to-lab variability inherent in qPCR, TRF or flowFISH approaches. Furthermore, the workflow is relatively time- and cost-effective (estimated consumable costs are approximately \$15–\$20 per sample), achieving efficiency without the need for complex, computationally intensive bioinformatics pipelines or the high consumable costs associated with nanopore sequencing. Together, these results establish a direct, accessible method for chromosome-end-resolved analysis of telomere and subtelomere architecture.

Results

HS-AFM imaging workflow enables direct single-molecule measurement of intact telomeres

The HS-AFM workflow was designed to preserve the full spectrum of telomere lengths, minimize size-dependent bias, and enable precise end-to-end measurement of individual telomeres. To achieve this, we first evaluated DNA extraction methods for their ability to preserve long genomic DNA molecules and maintain representation of long telomeres during sample preparation (Supplementary Fig. 1 A&B). HS-AFM imaging showed that precipitation-based DNA extraction (Puregene, Qiagen) and high-molecular-weight DNA extraction (Monarch, NEB) preserved long DNA molecules, including fragments approaching megabase scale, whereas column-based extraction (QIAamp, Qiagen) reduced recovery of the longest genomic DNA molecules (Supplementary Fig. 1 A&B). Based on these results, both the Puregene and Monarch

extraction methods were selected for subsequent analyses (Fig. 1, step 1). Extracted genomic DNA was subsequently digested to generate defined telomeric fragments containing adjacent subtelomeric sequence (Fig. 1, step 2). Site-specific restriction digestion was selected over mechanical fragmentation to avoid random DNA shearing that could disrupt the telomere–subtelomere junction. Several restriction enzymes, including those commonly used for terminal restriction fragment analysis, were evaluated (Supplementary Figs. 2–4). Among the enzymes tested, PacI produced a 20–50 kb terminal-fragment profile with sufficient adjacent subtelomeric sequence for downstream analysis (Fig. 1, step 2).

Telomeres were then selectively captured from bulk genomic DNA using a modified oligo-tagging strategy based on the previously described TeloTag approach⁴¹. A modified biotinylated oligonucleotide containing a telomere-complementary sequence and a photocleavable linker was ligated to the native single-stranded 3' overhang, enabling selective capture on streptavidin magnetic beads and subsequent release of telomeric fragments for HS-AFM imaging (Fig. 1, step 3). Enrichment was quantified by qPCR as the relative abundance of telomeric DNA recovered in the bead-bound fraction compared with the unbound fraction (Supplementary Fig. 5 & 6B). Optimization of the genomic DNA-to-oligonucleotide ratio identified 1 µg genomic DNA and 33 nM oligonucleotide as optimal ratio conditions for enrichment (Supplementary Fig. 6A–B). Importantly, exonuclease I pretreatment, which removes the native 3' telomeric overhang, abolished pull-down, demonstrating that capture depends on ligation to the native 3' single-stranded terminus rather than to internal telomeric repeats or nonspecific genomic fragments (Supplementary Fig. 6C). Telomere PNA-FISH provided qualitative support for enrichment specificity, with telomere-positive fragments observed more frequently in the post-enrichment fraction than in bulk input DNA and pre-enrichment PacI-digested samples (Supplementary Fig. 6D).

For precise end-to-end measurement of individual telomeres, we established a unique CRISPR–Cas9 labeling strategy that defines both telomere boundaries (Fig. 1, step 4). Under cleavage-inactive conditions, Cas9 remains stably bound to target DNA, forming a large protein–DNA complex that is readily visualized by HS-AFM and can therefore be used as a programmable molecular marker⁴². Sequence analysis across the HG002 paternal, HG002 maternal, and CHM13 T2T reference assemblies identified a recurrent GC-rich 18-bp motif at the telomere–subtelomere junction of most chromosome arms, which is part of a telomere-associated minisatellite repeat (29-mer) corresponding to a TERRA promoter element^{46–48}, providing a marker for the proximal telomere boundary (Supplementary Fig. 7A, B). To define the distal boundary, a 15-nt version of this motif was incorporated into the terminal oligonucleotide tag, creating a CRISPR-targetable sequence at the chromosome terminus (Supplementary Fig. 7D). Among the Cas9 complexes tested, HiFi Cas9 produced the most robust labeling at both boundary sites, with efficiencies exceeding 96% under optimized conditions (Supplementary Figs. 7 E, F, G and Supplementary Fig. 8).

To maximize image quality and telomere recovery while preserving DNA integrity, we optimized bead selection, removal of residual free oligonucleotides and unbound Cas9 complexes, and UV-release conditions. Among the cleanup methods evaluated, 0.22 μ m dialysis most effectively removed residual free oligonucleotides and unbound Cas9 complexes while preserving intact DNA molecules (Supplementary Fig. 9A). Among five streptavidin magnetic bead types tested, M280 beads provided the best balance between efficient telomere capture and low imaging background (Supplementary Fig. 9B). Telomeric fragments were released from the beads by 365-nm UV cleavage of the photocleavable linker, enabling recovery of captured material under conditions expected to minimize DNA photodamage (Fig. 1, step 5). Control experiments showed that exposure to 365-nm UV for up to 30 min did not produce detectable DNA fragmentation, as assessed by HS-AFM and gel electrophoresis

(Supplementary Fig. 10A). Sequential release experiments further demonstrated that 2 min of UV exposure was sufficient to recover the majority of bead-bound telomeric material (Supplementary Fig. 10B). Under these optimized conditions, 500 ng of genomic DNA yielded sufficient telomere recovery for HS-AFM analysis.

To improve imaging throughput and measurement efficiency, we implemented magnetically directed deposition to concentrate enriched telomeric fragments within the HS-AFM imaging field (Fig. 1, step 5). By increasing the local density of target molecules on the imaging surface, this approach reduced the scan area required for data collection and increased the number of analyzable molecules per image. Following UV release onto mica, controlled buffer flow straightened DNA molecules and facilitated contour tracing for accurate length measurements. This step was performed using an automated washing device to standardize directional buffer flow and improve protocol reproducibility. Depositions of short, long, and mixed DNA populations confirmed that the workflow retained molecules across a broad size range without preferential loss of longer (or shorter) fragments (Supplementary Fig. 10C).

Together, these results establish an amplification-free platform for direct single-molecule measurement of telomeres by nanoscale imaging. By preserving long genomic DNA molecules, selectively enriching telomeres through their native 3' overhang, defining distal and proximal telomere boundaries using dual CRISPR–Cas9 labeling, and enabling quantitative HS-AFM analysis from as little as 500 ng of genomic DNA, the workflow overcomes key limitations of bulk, amplification-based, and sequencing-based approaches.

HS-AFM resolves telomere-length heterogeneity and passage-associated shortening in reference cell models

Having established the HS-AFM workflow, we applied it to a reference panel of human DNA samples spanning long-, intermediate-, and short-telomere states

239 previously reported in the literature^{32,37,49–53} (Supplementary table 1, Supplementary Fig.
240 11C, D). The panel included both lymphoblastoid and fibroblast-derived models,
241 enabling assessment of telomere-length distributions across distinct cellular
242 backgrounds. HS-AFM revealed broad single-molecule telomere-length distributions in
243 every sample, indicating substantial heterogeneity rather than uniform telomere
244 populations (Fig. 2A–C). The expected biological differences among models were
245 readily apparent. The long-telomere models, 1301 and BJ, exhibited the largest
246 distributions, with median telomere lengths of 5.8 kb and 4.9 kb, respectively.
247 Approximately half of the measured telomeres exceeded 6 kb, and 22% were longer than
248 10 kb, whereas telomeres shorter than 500 bp were not detected (Fig. 2A–C). IMR-90
249 and HG002 displayed intermediate profiles, with median telomere lengths of
250 approximately 4 kb and individual telomeres extending up to 25 kb, consistent with
251 previous reports (Supplementary table 1 and Fig. 2A–C). In contrast, progeroid and
252 dyskeratosis congenita (DC) samples showed a marked shift toward shorter telomeres
253 despite their younger ages at collection, with median lengths of 1.7 kb and 1.3 kb,
254 respectively, and ~30% of measured telomeres shorter than 1 kb (Fig. 2A–C). These
255 findings demonstrate that HS-AFM captures both differences in median telomere length
256 and the distributional features that distinguish long-, intermediate-, and telomere-
257 dysfunction states.

258 Technical comparisons supported the robustness of the workflow and highlighted
259 the importance of DNA integrity. Puregene and Monarch extraction methods produced
260 comparable telomere-length distributions, whereas QIAamp extraction
261 underrepresented longer telomeric fragments, consistent with reduced recovery of high-
262 molecular-weight DNA (Supplementary Fig. 11A). Extraction replicates yielding
263 different DNA recoveries generated comparable telomere-length distributions,
264 indicating that variation in extraction yield did not substantially affect HS-AFM
265 measurements (Supplementary Fig. 11B). Independent analyses performed by two

266 technicians also produced broadly comparable telomere-length distributions and size-
267 class assignments (Supplementary Fig. 11B). Reproducibility analysis based on the
268 median telomere length of independent workflow repeats demonstrated high within-
269 condition agreement, with an intraclass correlation coefficient (ICC) of 0.901. Residual
270 repeat-to-repeat variation accounted for only 9.9% of the total variance, indicating
271 strong consistency across independent workflow repeats performed using the same
272 extraction and technician. Together, these findings support the high repeatability and
273 robustness of HS-AFM telomere-length measurements across independent workflow
274 repetitions.

275 We next assessed whether HS-AFM could detect telomere shortening during
276 culture expansion. In IMR-90 fibroblasts, a well-established model of replicative
277 telomere attrition, median telomere length decreased from 3.7 kb at passage 11 (p11) to
278 0.8 kb at passage 44 (p44), corresponding to an estimated loss of ~89 bp per passage
279 (Fig. 2D, E). This value is consistent with a published estimate of 105 ± 17 bp per
280 population doubling by mean TRF analysis in standard IMR-90 cultures⁴³. Consistent
281 with progressive shortening, the fraction of telomeres shorter than 1 kb increased from
282 3.2% to 59%, whereas the fraction longer than 6 kb decreased from 27% to 2.1%. A
283 similar passage-associated redistribution was observed in HG002 cells. Median telomere
284 length decreased from 3.9 kb at p4 to 1.3 kb at p16, corresponding to an estimated loss
285 of ~219 bp per passage (Fig. 2D, E). This change was accompanied by an increase in
286 telomeres shorter than 1 kb from 12% to 40% and a decrease in telomeres longer than 6
287 kb from 28% to 1.3%. The most pronounced shortening occurred in DC patient-derived
288 cells (Fig. 2D, E). Median telomere length declined from 3.4 kb at p1 to 1.3 kb at p4,
289 corresponding to an estimated loss of ~688 bp per passage. This rapid attrition is
290 consistent with the defective telomere maintenance characteristic of dyskeratosis
291 congenita⁴⁴. The proportion of telomeres shorter than 1 kb increased from 14% to 40.7%,
292 whereas the fraction longer than 6 kb decreased from 28% to 6%.

Together, these results demonstrate that HS-AFM resolves biologically distinct telomere-length states and detects dynamic telomere shortening during culture expansion. Across reference models, the abundance of very short telomeres emerged as a particularly sensitive indicator of telomere attrition, capturing distributional changes that were not fully reflected by median telomere length alone.

HS-AFM workflow enables single-molecule telomere profiling from primary biospecimens

We next asked whether the HS-AFM workflow could be applied directly to primary blood-derived DNA, a key requirement for future cohort-based and clinically relevant telomere studies. Starting from only 500 μ L of whole blood, the workflow yielded sufficient material for telomere enrichment, boundary labeling, deposition, and single-molecule HS-AFM measurement (Fig. 3).

Analysis of donors aged 31–90 years revealed broad, donor-specific telomere-length distributions and an overall shift toward shorter telomeres with age (Fig. 3A,B). Average telomere length decreased from 3.7 kb in the 30–35-year group to approximately 3.0 kb at 45 years, 2.4 kb at 56–60 years, and 2.3 kb in donors aged 80–90 years (Fig. 3A,B). This age-associated trend was accompanied by a progressive increase in very short telomeres. The fraction of telomeres shorter than 1 kb increased from 9% in the 30–35-year group to 13% at 45 years, 20% at 56–60 years, and more than 25% in the oldest donors. This pattern was most pronounced in the 90-year-old donor, in whom nearly one-third of measured telomeres were shorter than 1 kb (Fig. 3B). Concomitantly, longer telomere classes became progressively depleted. Telomeres longer than 6 kb represented 27% of molecules in donors aged 30–35 years, compared with 20% at 45 years and approximately 11–12% in donors older than 56 years (Fig. 3B). The fraction of telomeres exceeding 10 kb similarly decreased from an average of 9.3% in the youngest donors to 3.0% in donors aged 80–90 years. Notably, telomeres longer than 50 kb were detected in one 31-year-old donor but were absent from older

individuals (Fig. 3B, C). These findings indicate that age-associated shortening in whole blood is characterized not only by reduced average telomere length, but also by marked remodeling of the underlying single-molecule distribution. A qPCR analysis of the same samples likewise revealed an age-dependent reduction in telomere-associated signal (Supplementary Fig. 11E), consistent with the differences detected using the HS-AFM workflow. Donor-level analysis further revealed substantial inter-individual variation that was not fully captured by average telomere length.

Together, these results demonstrate that single-molecule telomere profiling can be performed from as little as 500 μ L of whole blood. The workflow captures age-associated redistribution of telomere lengths, including the accumulation of sub-kilobase telomeres and depletion of long and ultra-long telomeres, while revealing donor-specific patterns that would be obscured by bulk or median-based measurements alone.

HS-AFM allows simultaneous single-molecule measurement of telomere length and chromosome-end identity

Telomere length varies substantially between chromosome arms, yet most existing methods measure telomeres as a pooled population. To enable chromosome-end-resolved analysis, we developed a subtelomeric barcoding strategy that encodes proximal subtelomeric sequence information on the same DNA molecule used for telomere measurement (Fig. 4A).

Candidate chromosome-end barcodes were identified by analyzing predicted nicking-site distributions across HG002 maternal, HG002 paternal, and CHM13 chromosome-end assemblies using multiple nicking endonucleases (Fig. 4B; Supplementary Fig. 12). Among the enzymes evaluated, Nt.BspQI generated the most informative and readily distinguishable barcode patterns while maintaining spacing compatible with HS-AFM resolution. To convert predicted Nt.BspQI sites into features detectable by HS-AFM, nicked DNA was subjected to strand-displacement synthesis to

generate localized DNA-tail structures at defined genomic positions. Several strand-displacing polymerases were evaluated (Supplementary Fig. 13A), and Klenow Fragment produced the most robust and reproducible DNA-tail formation. Using a linearized pBR322 substrate containing a single Nt.BspQI site at a known position, we optimized strand-displacement conditions and confirmed that DNA-tail structures localized accurately to the predicted site. Labeling efficiency increased with extension time, reaching 95% after 5 min (Supplementary Fig. 12B). HS-AFM resolved strand-displacement products as punctate features consistent with ssDNA, flap-like structures consistent with dsDNA, or mixed structures at the target site (Fig. 4C). The dsDNA-containing structures may arise from secondary synthesis events, including template switching during strand-displacement synthesis. Because Nt.BspQI cleaves a defined strand relative to its non-palindromic recognition sequence, the orientation of each recognition site determines which genomic strand is nicked and the direction of polymerase extension from the nick-associated 3'-OH. Thus, predicted Nt.BspQI site position and orientation together provide positional and directional information that can be incorporated into subtelomeric barcode assignment (Fig. 4B,D).

Subtelomeric DNA-tail barcoding was subsequently combined with CRISPR–Cas9 telomere boundary labeling on HG002 chromosome-end molecules. Individual DNA molecules therefore contained both telomere boundary labels and subtelomeric barcode features, permitting telomere length and chromosome-end identity to be determined from the same molecule. Comparison of observed DNA-tail patterns with predicted Nt.BspQI barcode maps identified molecules corresponding to chromosome ends 18q and 14q (Fig. 4D), demonstrating that chromosome-end-specific subtelomeric signatures can be preserved and decoded on the same molecules used for direct telomere-length measurement.

Together, these results extend the HS-AFM workflow beyond telomere-length measurement by permitting simultaneous single-molecule determination of telomere

length and chromosome-end identity, establishing a framework for chromosome-end-resolved studies of telomere heterogeneity, subtelomeric architecture, and genome instability.

Discussion

In this study, we established an amplification-free HS-AFM workflow for direct single-molecule analysis of telomere-containing DNA molecules. The platform integrates end-preserving genomic DNA preparation, selective enrichment through the telomeric 3' overhang, CRISPR/Cas9-defined measurement boundaries, and subtelomeric barcode mapping. Together, these elements address a central limitation of widely used telomere assays: the inability to directly measure telomeric DNA while preserving chromosome-end context on the same molecule.

A key technical feature of the workflow is selective capture of chromosome-end fragments through ligation to the native telomeric 3' overhang. Loss of enrichment after exonuclease I treatment provides strong evidence that pulldown specificity depends on the presence of the overhang rather than nonspecific bead binding. The workflow was further strengthened by optimization of AFM-compatible bead chemistry, oligonucleotide cleanup, and UV-based release, which together enabled recovery of telomere-enriched DNA with minimal background. These steps are important because molecule integrity, background suppression, and surface deposition directly affect the interpretability of single-molecule AFM measurements.

We present a targeted deposition strategy that optimizes the capture of long DNA fragments and macromolecular complexes for HS-AFM analysis. Unlike standard diffusion-governed methods, this technique concentrates analytes within a minimal scanning field, drastically reducing acquisition time. Integrating this protocol with Cas9 binding allows for the highly precise definition of genomic regions of interest in DNA conformations amenable to feature mapping. Future development of

this strategy may enable a multi-parametric analysis of complex genetic and epigenetic landscapes such as length polymorphisms, epigenetic status, regulatory protein-binding events and non-canonical DNA structures providing a level of structural context that is currently unattainable through bulk biochemical or sequencing-based assays. Beyond nucleic acids and protein–DNA interactions, this targeted deposition strategy is broadly adaptable to diverse macromolecular assemblies. By functionalizing affinity agents—such as antibodies or aptamers—with photocleavable biotin tags, this approach expands the operational scope of HS-AFM from genomics to single-molecule proteomics and multi-omic structural biology.

While Scanning Probe Microscopy (SPM) and its most prominent variant, Atomic Force Microscopy (AFM), have been among the cornerstones of biomolecular imaging for decades, their application was historically constrained by low temporal resolution. Recent engineering advancements in High-Speed AFM (HS-AFM) have mitigated these scanning speed limitations, facilitating the transition of this technology into a viable tool for routine clinical diagnostics. The workflow detailed herein is inherently scalable, featuring a high capacity for end-to-end automation across all procedural phases, from initial sample preparation to high-resolution imaging and subsequent bioinformatic analysis. This modularity may facilitate the seamless integration of the platform into both high-throughput commercial environments and routine clinical diagnostic pipelines

The nickase-mediated labeling strategy described here builds on prior AFM-based approaches in which sequence-specific nicking restriction enzymes were used to introduce topographical labels onto DNA molecules, enabling molecular identification from intramolecular label spacing⁴⁵. The current protocol utilizes sequence-specific enzymatic nicking followed by strand-displacement polymerization, wherein the displaced DNA serves as the molecular label. This streamlined “barcoding” approach enables high-resolution identification of individual DNA molecules and may facilitate haplotype phasing and may facilitate detection of that occur within the sub-telomeric

region, including inversions, translocations, and large-scale insertions or deletions. By bridging the resolution gap between classical cytogenetics and high-throughput sequencing, this protocol could provide a robust physical scaffold for the accurate orientation and independent orthogonal validation of *de novo* genome assemblies.

The use of CRISPR/Cas9 labeling to define telomere boundaries extends AFM from a descriptive imaging tool to a measurement platform. HiFi Cas9 provided the most efficient labeling under the conditions tested, and optimization of reaction chemistry further increased labeling rates to near-saturating levels. This allowed telomere length to be scored between defined molecular landmarks rather than inferred indirectly from bulk signal. Importantly, the resulting measurements resolved expected differences among long-, intermediate-, and short-telomere reference models and captured passage-associated telomere attrition in both lymphoblastoid and fibroblast samples.

The ability to profile whole-blood genomic DNA expands the potential utility of the method beyond cultured cell systems. Current telomere assays used in clinical or translational studies often rely on relative or bulk-average readouts. In contrast, the present workflow provides single-molecule distributions, making it possible to assess shifts in telomere size classes, depletion of long telomeres, or enrichment of critically short molecules. Such distribution-level information may be valuable in settings where average telomere length alone is insufficient to describe biologically meaningful variation.

Long-read sequencing and HS-AFM provide complementary information. Long-read sequencing offers nucleotide-level and allele-resolved analysis, whereas HS-AFM directly measures the physical length of individual telomeric tracts between defined molecular labels and preserves adjacent subtelomeric context on the same molecule. HS-AFM therefore avoids sequence-based repeat-length inference, while long-read sequencing provides base pair level sequence information that HS-AFM does not.^{32,33,40,41}

An additional strength of the platform is preservation of subtelomeric information. Identification of a recurrent telomere–subtelomere junction motif provided a target for boundary labeling, while BspQI-based nickase barcodes created a route toward chromosome-end assignment. The integrated proof-of-principle demonstrates that telomere length and subtelomeric barcode features can be measured on the same molecule. This is particularly relevant because chromosome-end-specific dynamics are increasingly recognized as important in telomere biology, yet they remain difficult to capture with conventional assays.

Overall, our findings establish HS-AFM as a practical framework for direct telomere/subtelomere analysis at single-molecule resolution. By combining selective enrichment, molecular boundary labeling, and subtelomeric mapping, this approach bridges nanoscale structural precision with translational relevance and provides a foundation for studying telomere biology in a chromosome-end-resolved manner.

Methods

Reference Cell Lines

The 1301 lymphoblastoid cell line was purchased from Sigma-Aldrich. HG002, AG03259, and GM01775 lymphoblastoid cell lines were obtained from the Coriell Institute. AG03259 and GM01775 correspond to Hutchinson–Gilford progeria syndrome and dyskeratosis congenita, respectively. BJ fibroblasts were obtained from ATCC, and IMR-90 fetal lung fibroblasts were obtained from the Coriell/NIA Aging Cell Culture Repository.

Lymphoblastoid cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Fibroblast cell lines were maintained in Eagle’s Minimum Essential Medium supplemented with 15% FBS and 1% penicillin–streptomycin under the same conditions. Cells were harvested at approximately 10

479 million cells by centrifugation at 1,200 rpm for 8 min, washed once with phosphate-
480 buffered saline (PBS), and pelleted by a second centrifugation step.

481 Blood Donors

482 A total of 14 individuals were included in this study, comprising four twin pairs
483 and six unrelated singletons ranging in age from 31 to 90 years. Twin participants were
484 recruited through the Mid-Atlantic Twin Registry. Following informed consent under
485 Virginia Commonwealth University Institutional Review Board protocol (Twin ACT
486 (Acquired Chromosomal changes over Time) Study, HM20030175), participants
487 completed health history, dietary, and lifestyle questionnaires and provided peripheral
488 blood samples. Blood collection was performed by a healthcare provider selected by
489 each participant. Samples were shipped overnight to the Jackson-Cook Cytogenetics
490 Laboratory for processing.

491 Genomic DNA preparation

492 Genomic DNA from cultured cell pellets or peripheral blood was extracted using
493 the Monarch® HMW DNA Extraction Kit (New England Biolabs, T3050L), Puregene
494 (Qiagen), or QIAamp (Qiagen) according to the manufacturers' instructions. DNA was
495 eluted in 300 μ L elution buffer and quantified using the Qubit™ dsDNA High
496 Sensitivity Assay Kit (Thermo Fisher Scientific, Q33230), yielding concentrations of
497 approximately 100–300 ng μ L⁻¹.

498 Restriction enzyme selection for telomere preservation

499 To identify restriction enzymes that preserve telomeric and subtelomeric DNA,
500 200-kb chromosome-end intervals were extracted from the T2T-CHM13 v1.1 reference
501 genome and the HG002 maternal and paternal assemblies. Candidate enzymes were
502 evaluated based on cutting frequency, CpG methylation sensitivity, and preservation of

the telomere–subtelomere junction and adjacent subtelomeric sequence. Enzymes examined included PacI, BamHI, PvuI, BspWI, AsiSI, NdeI, EcoRI, HinfI, and RsaI.

Predicted digestion patterns were compared across all three assemblies to identify enzymes that generated large terminal fragments while retaining both telomeric and proximal subtelomeric DNA. Candidate enzymes were further evaluated experimentally by digestion of genomic DNA followed by electrophoretic analysis on 0.6% agarose gels in 1× TAE buffer. Fragment sizes were estimated using a high-molecular-weight DNA ladder.

PacI was selected because it consistently generated large terminal fragments (>50 kb) while preserving subtelomeric telomere-associated sequence and the telomere–subtelomere junction. For subsequent experiments, 0.5 µg genomic DNA was digested with 1 µL PacI (NEB, R0547S) in 1× NEBuffer 4 (BSA-free) in a final reaction volume of 20 µL and incubated at 37°C for 1 h. The resulting fragments were used for telomere adaptor ligation.

Telomere adaptor ligation (TeloTagging)

Photo-cleavable biotinylated adaptor oligonucleotide and phosphorylated telomeric splint oligonucleotides representing all six possible telomeric 3'-overhang permutations were synthesized by Integrated DNA Technologies. All oligonucleotides were resuspended in nuclease-free water at 200 µM. For adaptor annealing, 6 µL photocleavable oligonucleotide and 1 µL of each splint oligonucleotide were mixed with 1.5 µL 10× HiFi Taq Ligase buffer and 6.5 µL nuclease-free water. The mixture was denatured at 99 °C for 1 min and gradually cooled to room temperature at a rate of 1°C min⁻¹. Telomere tagging was performed in 50 µL reactions containing 0.5 µg PacI-digested genomic DNA, 16.5 nM annealed adaptor duplex, 5 µL 10× HiFi Taq DNA Ligase reaction buffer, and 1 µL HiFi Taq DNA Ligase (NEB, M0647S). Reactions were incubated at 65°C for 5 min, followed by 15 cycles of 65°C for 1 min and 45°C for 3

529 min to promote ligation of the adaptor to native telomeric 3' overhangs. Ligated DNA
530 was purified by overnight dialysis against distilled water using a 0.22 µm mixed
531 cellulose ester membrane (MilliporeSigma, HAWP04700).

532 Cas9-mediated telomere labeling

533 Single-guide RNAs targeting the conserved 15-nt telomere–subtelomere junction motif
534 were obtained from Integrated DNA Technologies (IDT) and prepared according to the
535 manufacturer's instructions. For each reaction, Cas9 ribonucleoprotein complexes were
536 assembled by combining 0.2 µL sgRNA (50 µM), 0.2 µL EnGen® Spy Cas9-HF1
537 protein (NEB, E3322S; 20 µM), and 1 µL 10× Cas9 reaction buffer (20 mM HEPES,
538 100 mM NaCl, 0.1 mM EDTA, pH 6.5), with nuclease-free water added to a final
539 volume of 10 µL. The mixture was incubated at room temperature for 10 min to allow
540 ribonucleoprotein complex formation. Ligated genomic DNA was brought to 1× Cas9
541 reaction conditions by addition of 10× Cas9 reaction buffer. Subsequently, 10 µL of the
542 preassembled Cas9 ribonucleoprotein complex was added, and reactions were incubated
543 at 37°C for 1 h to permit binding of Cas9 to the conserved subtelomeric target sequence.
544 DNA cleavage was prevented by the absence of Mg²⁺ from the reaction buffer, which
545 permits target-specific Cas9 binding while preventing Mg²⁺-dependent nuclease activity,
546 as previously described⁴². Optimization of the Cas9-binding conditions is presented in
547 Supplementary Figs. 7 and 8. To stabilize protein–DNA complexes for HS-AFM
548 imaging, glutaraldehyde was added to a final concentration of 1%, followed by
549 incubation at room temperature for 20 min. Crosslinking reactions were quenched with
550 Tris buffer (3 M, pH 7.6) to a final concentration of 750 mM. Excess unbound Cas9
551 ribonucleoprotein complexes were removed by overnight dialysis against 1× Cas9
552 reaction buffer using a 0.22 µm mixed cellulose ester membrane (MilliporeSigma,
553 GSWP02500).

554 Telomere enrichment by streptavidin pulldown

555 Biotinylated, Cas9-labeled genomic DNA was enriched using Dynabeads™ M-
556 280 Streptavidin magnetic beads (Thermo Fisher Scientific, 11205D). For each reaction,
557 a 5 µL bead suspension was washed three times with wash/binding buffer (PBS, 0.01%
558 Tween-20, 0.2 mM EDTA, and 400 mM NaCl) and resuspended in 50 µL of the same
559 buffer. The bead suspension was combined with 50 µL Cas9-labeled DNA and incubated
560 for 60 min at room temperature with gentle rotation (10 rpm) to capture biotinylated
561 telomere molecules.

562 Following magnetic separation, the unbound fraction was removed. Bead-bound
563 DNA was washed once with 500 µL wash/binding buffer and three times with 500 µL
564 TE buffer to remove non-specifically bound DNA. Beads were subsequently
565 resuspended in 10 µL TE buffer for downstream analyses.

566 Telomere molecule imaging

567 Telomere sizing experiments utilized the Precyse system (**ref**
568 **www.eviziadx.com**), an automated high-speed atomic force microscopy (HS-AFM)-
569 based platform for rapid sizing and visualization of DNA molecules. For each sample,
570 3 µL of streptavidin bead-bound telomeric DNA was deposited onto a Precyse 1 × 3-
571 inch mica substrate. A magnet positioned beneath the substrate was used to concentrate
572 bead-associated DNA molecules within a defined imaging region. Following
573 localization, UV illumination was applied to cleave the photocleavable linker and release
574 captured telomere molecules from the magnetic beads.

575 Subsequently, 3 µL of Precyse deposition buffer was added to facilitate adsorption
576 of released DNA molecules to the mica surface and incubated for 5 min. To promote
577 straightening of DNA molecules, 1 mL of Precyse deposition buffer was applied across
578 the substrate in a single direction. Samples were then washed and dried by air jet in an
579 automated washing station and heated at 102°C for 10 min before imaging.

580 For each sample, 28 images were acquired. Individual images covered a 24×48
581 μm field of view with a pixel resolution of 2×2 nm. Typically, more than 500 labeled
582 telomere molecules were imaged within 60 min, and up to eight samples could be
583 analyzed in a single run. Following image acquisition, data were exported as 16-bit TIFF
584 files for downstream processing and telomere length analysis.

585 qPCR-based assessment of telomere enrichment

586 To assess telomere enrichment following streptavidin pulldown, quantitative PCR
587 (qPCR) was performed using primers targeting telomeric repeats and the single-copy
588 albumin gene as a reference control. Samples analyzed included input genomic DNA,
589 the flow-through (unbound) fraction, and the bead-bound (enriched) fraction.

590 Reactions were assembled in 20 μL volumes using SYBRTM Green Master Mix
591 (Bio-Rad, 1725270) according to the manufacturer's instructions. Each reaction
592 contained 10% of the unbound or enriched DNA fraction, 10 μL 2 $\times$ SYBR Green Master
593 Mix, and 1 μL primer mixture. The primer mixture consisted of 16 μL telomere primer
594 pair (20 μM each), 4 μL albumin primer pair (20 μM each), and 40 μL nuclease-free
595 water. Reactions were performed in technical triplicate.

596 *qPCR primer sequences:*

597 Telomere

598 Forward: 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'

599 Reverse: 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'

600 Albumin

601 Forward: 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGaaatgctgcacagaatccttg-3'

602 Reverse: 5'-GCCCCGGCCCGCCGCGCCCGTCCCGCCGgaaaagcatggtcgcctgtt-3'

603 *qPCR cycling conditions*

604 Thermal cycling was performed as follows: 95°C for 5 min; two cycles of 94°C
605 for 15 s and 49°C for 15 s; four cycles of 94°C for 15 s and 62°C for 15 s; 20 cycles of
606 83°C for 15 s, 60°C for 15 s, and 72°C for 15 s with signal acquisition; followed by 30
607 cycles of 94°C for 15 s, 83°C for 15 s, and 87°C for 15 s with signal acquisition. Melt-
608 curve analysis was performed from 95°C with 0.5°C increments and a 5 s hold at each
609 step.

610 Relative telomere enrichment was determined using the $\Delta\Delta C_t$ method, with
611 telomeric signal normalized to albumin and expressed as fold enrichment relative to
612 either the input DNA or unbound fraction.

613 Fluorescence in situ hybridization for telomere enrichment validation

614 To confirm telomere enrichment following streptavidin pulldown, fluorescence in
615 situ hybridization (FISH) was performed using a FITC-labeled telomere-specific peptide
616 nucleic acid (PNA) probe (DAKO, K5327). Four DNA conditions were analyzed:
617 undigested genomic DNA, PacI-digested DNA, DNA prior to pulldown, and DNA
618 following pulldown.

619 DNA samples were deposited onto poly-L-lysine-coated glass slides and air-
620 dried. Samples were fixed in 3.7% formaldehyde for 2 min at room temperature, washed
621 in Tris-buffered saline, pretreated for 10 min, and dehydrated through a graded ethanol
622 series (70%, 90%, and 100%; 2 min each). Following co-denaturation at 80°C for 3 min,
623 slides were hybridized overnight at 37°C with 10 μ L FITC-labeled telomere PNA probe
624 in a humidified dark chamber.

625 Post-hybridization washes were performed in DAKO rinse and wash solution at
626 65°C for 5 min with agitation, followed by ethanol dehydration. Slides were mounted
627 with ProLong™ Gold Antifade Mountant containing DAPI (Thermo Fisher Scientific)
628 and imaged by fluorescence microscopy.

629 Identification of a repeated telomere–subtelomere junction motif

630 To identify conserved sequence features at the telomere–subtelomere junction,
631 chromosome-end sequences from the Telomere-to-Telomere CHM13 v1.1 assembly
632 were analyzed using RepFind. For each chromosome end, a 100-kb interval extending
633 inward from the telomeric terminus was screened for repetitive sequence elements,
634 including short tandem and interspersed repeats. RepFind output tables were manually
635 curated and compared across chromosome ends.

636 A repeated 15-bp sequence motif was identified at 41 of 46 chromosome ends
637 when up to two mismatches were permitted. This motif corresponded to a previously
638 described TERRA promoter-associated 29 bp tandem repeat sequence located near the
639 telomere–subtelomere junction. To assess similarity across genomes, the same motif
640 was queried in the HG002 maternal and paternal assemblies, where it was detected at
641 comparable subtelomeric positions. The 15-bp repeated sequence motif was
642 subsequently used as a telomere–subtelomere junction landmark and target site for Cas9-
643 mediated labeling of telomere-containing molecules.

644 Subtelomeric Nt.BspQI strand-displacement barcoding

645 Predicted Nt.BspQI recognition sites were mapped within chromosome-end
646 intervals from the HG002 maternal, HG002 paternal, and CHM13 assemblies to
647 generate subtelomeric barcode maps. To generate HS-AFM-detectable structural
648 features at these sites, DNA substrates were nicked with Nt.BspQI and subjected to
649 polymerase-mediated strand-displacement synthesis.

650 Initial optimization experiments were performed using pUC19 plasmid DNA
651 containing a single Nt.BspQI site. pUC19 was incubated with Nt.BspQI in NEBuffer
652 r3.1 at 50°C for 1 h, followed by enzyme inactivation at 80°C for 20 min and dialysis
653 against deionized water for 1 h using a 0.1 µm membrane. Nicked pUC19 molecules
654 were subsequently incubated with Phi29 DNA polymerase, Bst-XT WarmStart DNA

655 polymerase, or Klenow Fragment (exo-) in manufacturer-recommended reaction
656 buffers containing dNTPs. Reactions were performed under enzyme-specific incubation
657 conditions, heat-inactivated, dialyzed against deionized water for 1 h using a 0.1 μ m
658 membrane, and analyzed by HS-AFM.

659 Strand-displacement conditions were further optimized using pBR322 DNA
660 containing a single Nt.BspQI site at a defined position. pBR322 was linearized with
661 BamHI, purified using AMPure beads, nicked with Nt.BspQI, and incubated with
662 Klenow Fragment (exo-) at 37°C for 20 sec, 1 min, or 5 min. Reactions were heat-
663 inactivated, dialyzed against deionized water for 1 h using a 0.1 μ m membrane, and
664 analyzed by HS-AFM.

665 For chromosome-end barcoding experiments, telomere-adaptor-ligated HG002
666 genomic DNA was nicked with Nt.BspQI and subjected to strand-displacement
667 synthesis using Klenow Fragment (exo-). Following strand-displacement labeling,
668 molecules were processed by Cas9-mediated labeling, streptavidin enrichment, and HS-
669 AFM imaging as described above. DNA-tail positions detected by HS-AFM were
670 compared with predicted Nt.BspQI site maps derived from the HG002 maternal, HG002
671 paternal, and CHM13 assemblies.

672 Intraclass correlation coefficient

673 Using the HG002 cell line data, we fit a linear mixed-effects model for median
674 telomere length, including technician and unique extraction as random effects.
675 We obtained the variance components for technician, extraction, and residual error from
676 the fitted model. Finally, we used these components to calculate the intraclass correlation
677 coefficient (ICC). This metric quantifies both the correlation between replicates from
678 the same extraction and technician, and the proportion of total variance attributable to
679 the extraction process.

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685 **Competing Interest declaration**

686 Dr. Jason Reed has a financial interest in Evizia, Inc., which has licensed the
687 technology used in this research. Dr. Reed's institution holds an equity interest
688 in Evizia, Inc. The other authors declare no competing interests.

689 **Ethics declaration**

690 The study involving human participants was approved by the Virginia
691 Commonwealth University Institutional Review Board (protocol HM20030175). All
692 participants provided informed consent.

693 **Data Availability**

694 All data supporting the findings of this study are available within the Article and
695 Supplementary Information.

696 **Author Contribution**

697 L.G. conceived and developed the experimental workflow, performed the
698 experiments, optimized the telomere enrichment and labeling strategies, collected and
699 analyzed the data, prepared the figures, interpreted the results, and wrote the original
700 manuscript. A.M. contributed to the development and optimization of the HS-AFM
701 methodology, performed HS-AFM imaging, and contributed to data interpretation and
702 manuscript revision. D.V.L. performed experiments assessing the reproducibility of the
703 workflow and contributed to data collection. D.G. provided expertise in AFM imaging
704 and scanning and contributed to the development and optimization of the imaging

705 methodology. H.C.R. provided expertise in telomere and subtelomere biology,
706 contributed to the development of the subtelomeric chromosome-arm assignment
707 strategy, and critically reviewed and revised the manuscript. K.J.A. performed and
708 advised on the statistical analyses, including assessment of assay reproducibility,
709 contributed to interpretation of the statistical results, and reviewed and revised the
710 manuscript. J.R. contributed to the development and optimization of the HS-AFM
711 approach, provided technical expertise and supervision for nanoscale imaging,
712 contributed to data interpretation, and reviewed and revised the manuscript. C.J.C.
713 conceived and supervised the study, contributed to experimental design and
714 interpretation of the results, provided resources and funding, and critically reviewed and
715 revised the manuscript. All authors read and approved the final manuscript.

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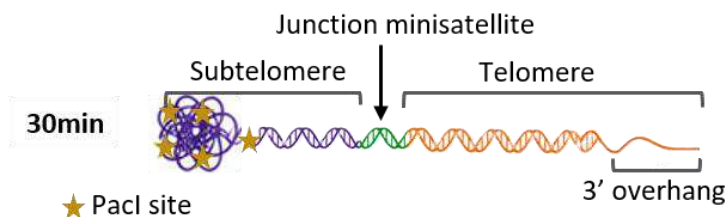
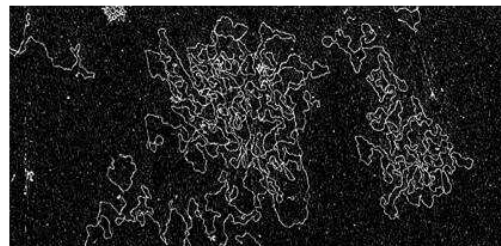
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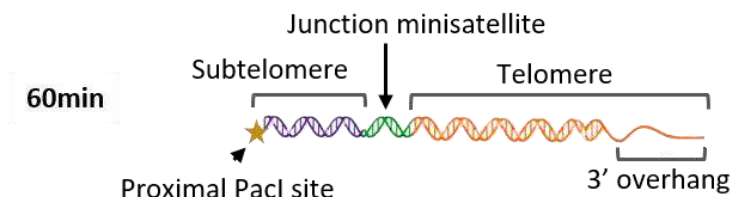
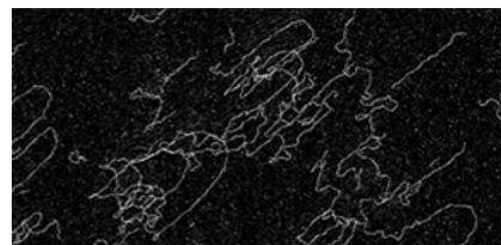
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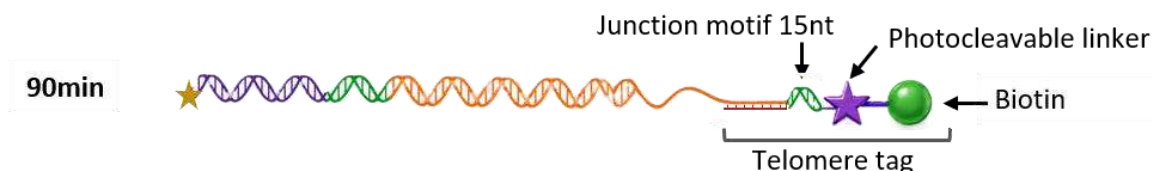
Step 1: High molecular weight genomic DNA extraction (up to Mb) 500ng



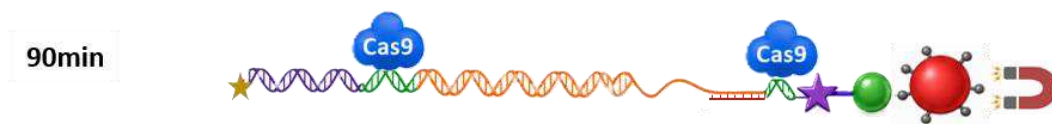
Step 2: Enzymatic fragmentation (~50kb)



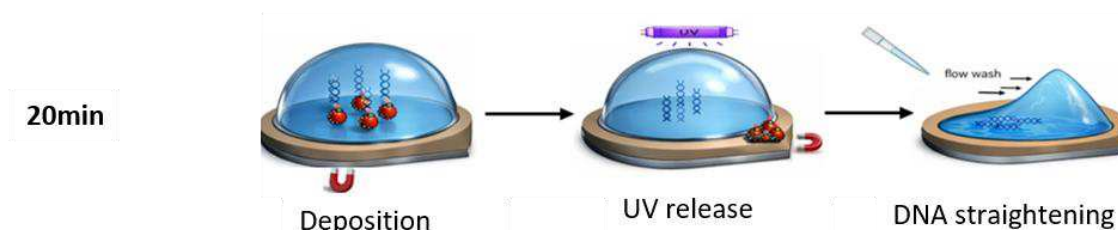
Step 3: 3' overhang telomere-tag



Step 4: Telomere boundary labeling and capture



Step 5: Deposition and UV release onto mica



Step 6: High-speed AFM imaging and analysis

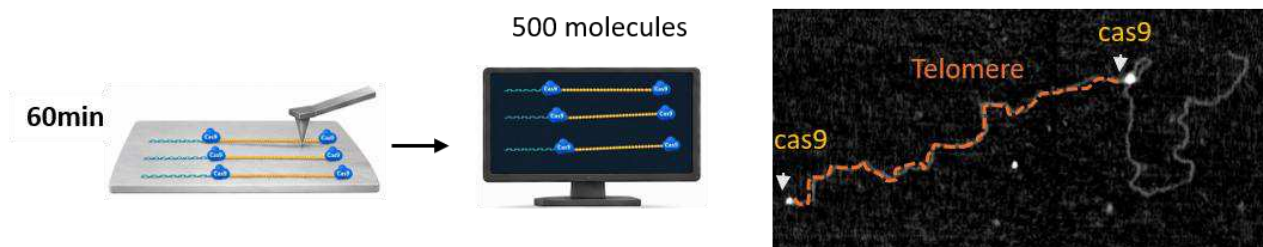
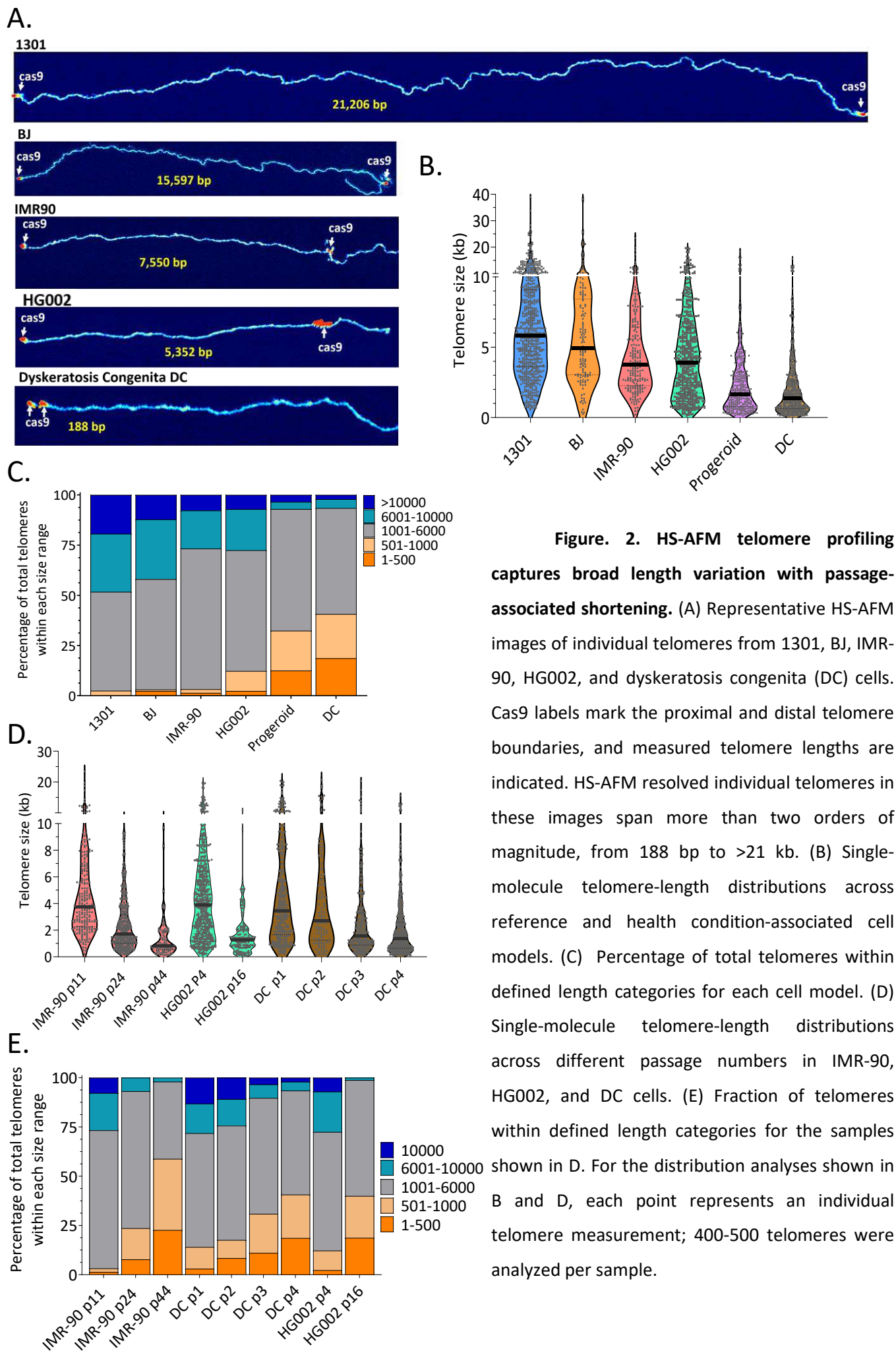


Figure 1. HS-AFM imaging workflow enables direct single-molecule measurement of intact telomeres. High-molecular-weight genomic DNA (500 ng) is enzymatically fragmented to generate chromosome-end molecules containing telomeric and subtelomeric regions. A telomere-specific tag is ligated to the native 3' overhang, followed by telomere boundary labeling and capture using sequence-specific Cas9 markers and biotin-streptavidin enrichment. Captured molecules are deposited onto mica, released by UV cleavage, straightened by buffer flow, and imaged by high-speed atomic force microscopy. This workflow enables direct visualization and measurement of individual telomere molecules.

Method	Primary Output	Sample Requirement	Sample Input Amount	Resolution	Subtelomere Information	Critically Shortest Telomere Detection	Consumable cost per sample (USD)
Q-FISH	Signal intensity per individual chromosome end	Intact metaphase cells	0.5mL blood	~1 kb (<i>single molecule</i>)	Limited (<i>chromosome-arm mapping via karyotyping</i>)	Limited (<i>signal-to-noise threshold</i>)	\$50-150
Flow-Fish (<i>Clinical Diagnostic "Gold Standard"</i>)	Average telomere length per cell across a population	Intact single-cell suspensions	1 million cells	~1 kb (<i>bulk average</i>)	No	No (<i>measures cell-population mean</i>)	\$50-100
qPCR	Relative telomere-to-single-copy gene* (T/S) ratio	Genomic DNA	50ng DNA	Bulk relative metric (<i>no bp resolution</i>)	No	No (<i>measures bulk molecule ensemble mean</i>)	\$2-5
TRF (<i>Historical Reference Benchmark</i>)	Density distribution of telomeric restriction fragment lengths	High-molecular-weight genomic DNA	10ug DNA	0.5–1 kb (<i>bulk DNA ensemble</i>)	Portion included in measurment (<i>adds non-telomeric offset</i>)	No (<i>short fragments lost in gel background noise</i>)	\$20-50
Long-read sequencing	Direct single-molecule sequence readout	High-molecular-weight genomic DNA	10-40ug DNA	Single base pair	Full sequence mapping and variant repeat profiling	Yes (<i>exact single-molecule readout down to 0 bp</i>)	\$80-140
HS-AFM	Single-molecule DNA contour length	High-molecular-weight genomic DNA	500ng DNA	~3 bp/pixel (<i>single-molecule spatial</i>)	Physical contour and probe-based mapping	Yes (<i>direct single-molecule nanometer-scale imaging</i>)	\$15-20
* Standard (unique copy gene) varies among labs							

Table 1. HS-AFM addresses key limitations of conventional telomere length measurement methods. Q-FISH, Flow-FISH, qPCR, TRF, long-read sequencing, and HS-AFM were compared based on measurement type, resolution, ability to provide subtelomeric information, detection of the shortest telomeres, sample input, and estimated cost per sample. Compared with existing approaches, HS-AFM uniquely combines amplification-free single-molecule telomere measurement with chromosome-end specificity, preservation of proximal subtelomeric context, and broad dynamic range. The reported cost per sample represents consumable costs only and does not include instrumentation, infrastructure, personnel, data-analysis, or reference-laboratory overhead.



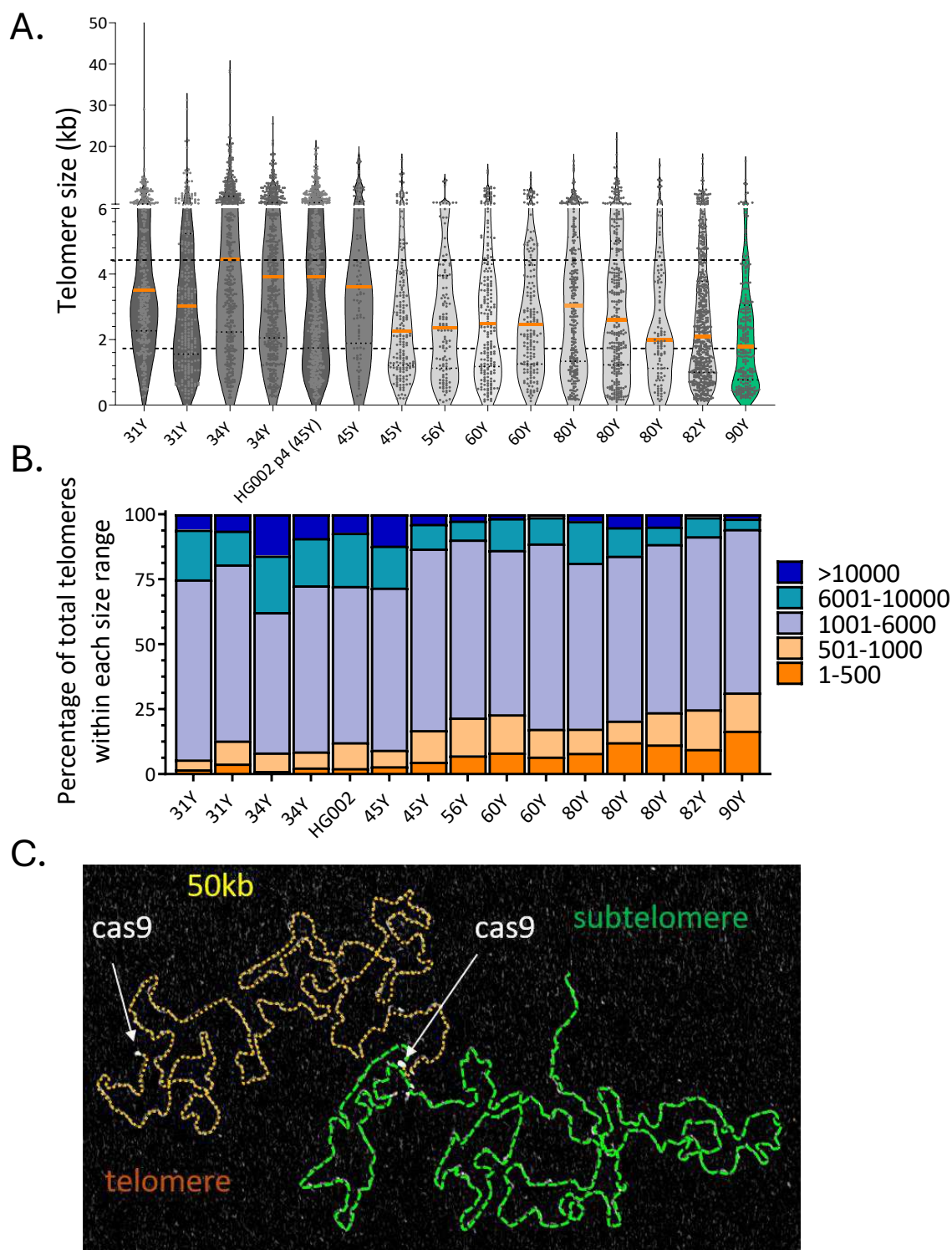


Figure 3. HS-AFM enables single-molecule telomere profiling from primary blood-derived biospecimens. (A) Single-molecule telomere-length distributions measured by HS-AFM in peripheral blood DNA obtained from donors aged 31–90 years. Each point represents an individual telomere measurement; 350–500 telomeres were analyzed per donor. Orange lines indicate median telomere length for each donor. The upper and lower dashed horizontal lines indicate the highest and lowest median telomere lengths observed across the donors, respectively. (B) Percentage of total telomeres within defined length categories for each donor. Age-associated telomere shortening is accompanied by accumulation of short telomeres and depletion of long and very long telomere classes. (C) Representative HS-AFM image of an ultra-long telomere (>50 kb) identified in a 31-year-old donor. Cas9 labels mark the telomere boundaries, enabling direct measurement of the telomeric tract (orange) relative to the adjacent subtelomeric region (green).

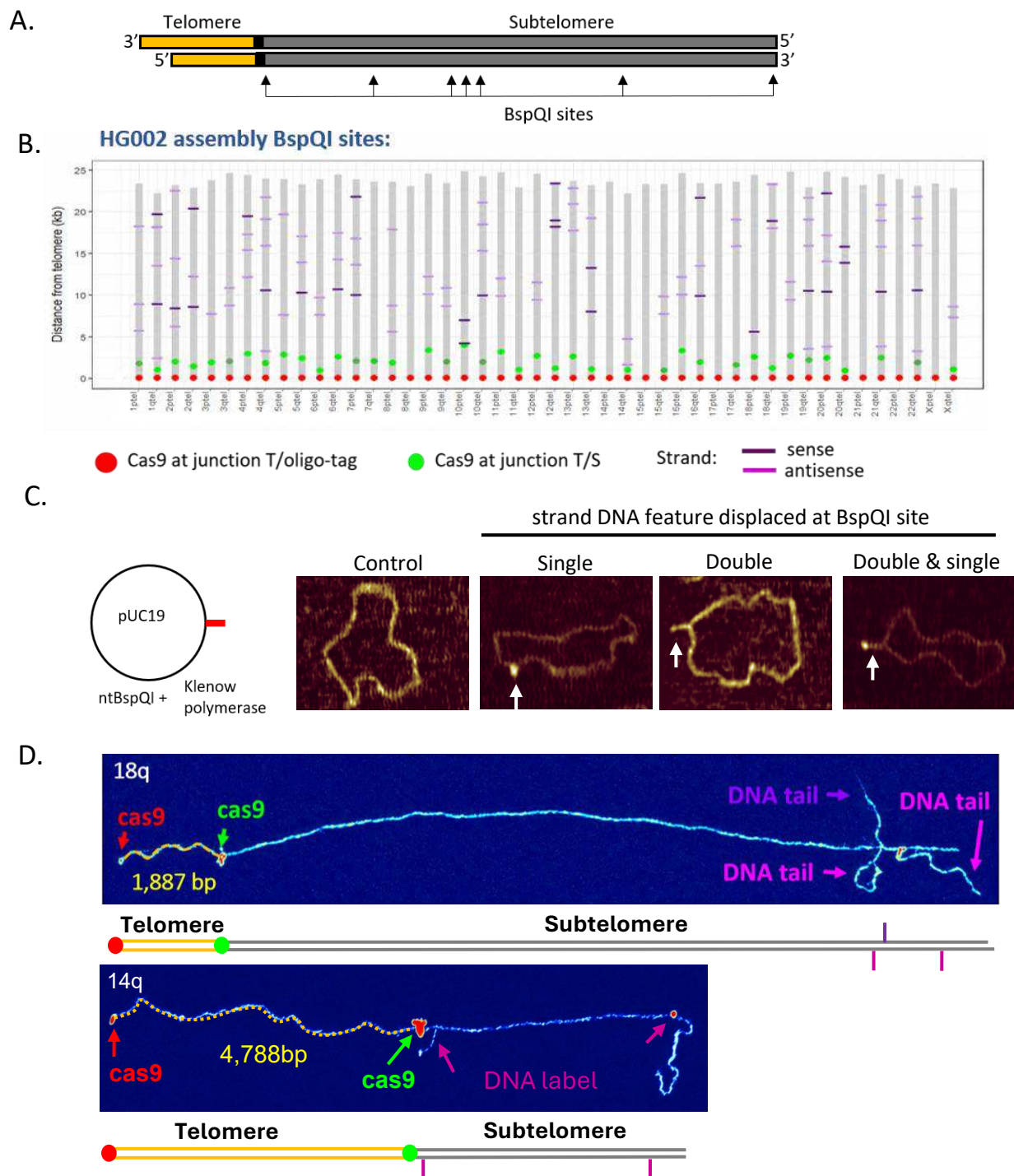


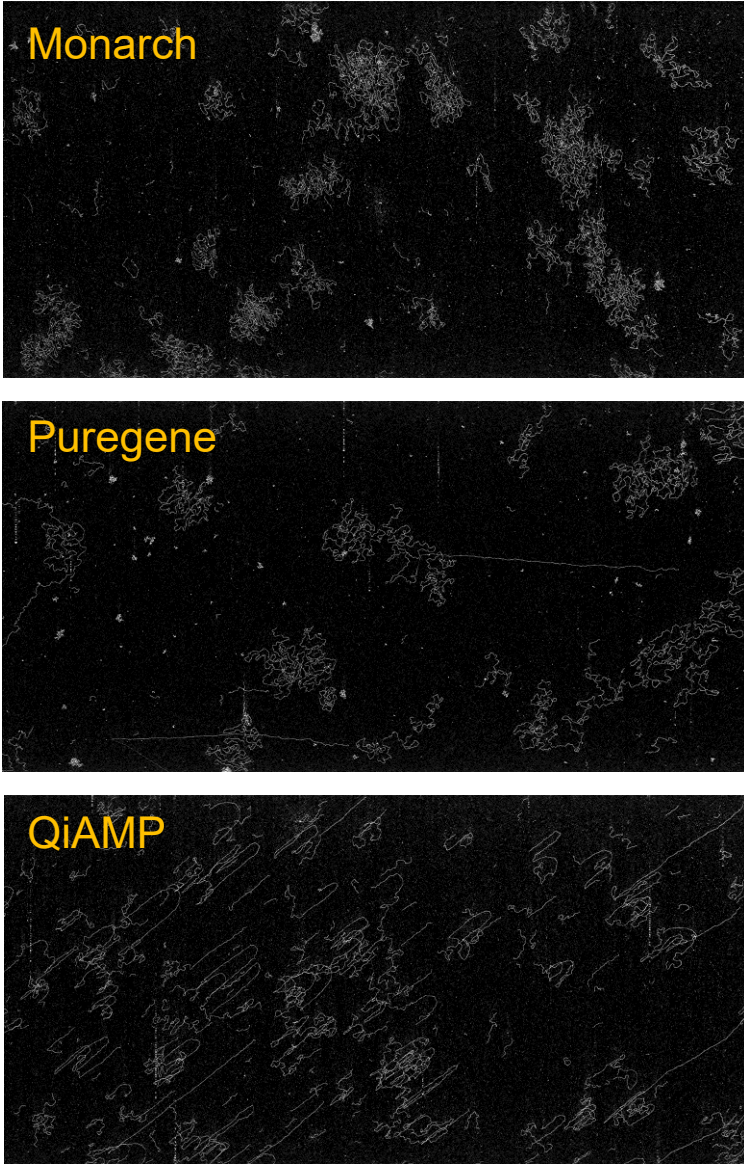
Figure 4. HS-AFM permits simultaneous single-molecule measurement of telomere length and chromosome-end identity. (A) Schematic of the subtelomeric barcoding strategy. Predicted Nt.BspQI sites within proximal subtelomeric regions are converted into DNA-tail barcode features by nicking and strand-displacement synthesis, enabling chromosome-end identification on the same molecule used for telomere-length measurement. (B) Predicted Nt.BspQI barcode architecture across HG002 chromosome ends. The positions and strand orientation of Nt.BspQI sites relative to the telomere boundary generate chromosome-end-specific barcode signatures compatible with HS-AFM resolution. Red and green circles indicate the distal and proximal Cas9 boundary labels, respectively. Purple bars denote Nt.BspQI nicking sites on the sense (dark purple) or antisense (lighter pink/purple) strand. (C) Conversion of Nt.BspQI sites into HS-AFM-detectable molecular features. Following Nt.BspQI nicking and Klenow-mediated strand-displacement synthesis, localized DNA-tail structures were generated at predicted positions. Representative HS-AFM images show punctate single-stranded DNA labels, double-stranded flap labels, and mixed strand-displacement products. Arrows indicate DNA-tail structures generated at the Nt.BspQI site. (D) Representative HG002 chromosome-end molecules combining subtelomeric barcoding with CRISPR–Cas9 telomere boundary labeling. Comparison of observed DNA-tail patterns with predicted Nt.BspQI barcode maps identified molecules consistent with chromosome ends 18q and 14q. Cas9 labels define telomere boundaries, DNA-tail features provide subtelomeric barcode information, and telomere lengths are indicated for each molecule.

Cell line	TRF	Flow-FISH	TeSLA/ STELA	Optical mapping	Long-read sequencing	HS-AFM	Measurement references
1301	~80 kb	~25 kb	–	–	–	mean 6.9 kb (range: 0.5–40kb) passage 4	TRF/Flow-FISH: [49] HS-AFM: This study
IMR90*	8.5 kb (early passage) 5.7 kb (late passage)	–	Chr17: ~1.52 kb	7.2 kb (passage 17) 4.5 kb (passage 45) 4 kb (senescent)	IMR90 E6E7: 4.2 kb (passage 66) 2.7 kb (passage 106)	mean 4.7kb (range 0.2–23kb) passage 11 mean 2.3 kb (range 0.16–9.3kb) passage 24	TRF: [50] Chr17/STELA: [51] Optical mapping: [37] Long-read: [32] HS-AFM: This study
HG002	4–6 kb	–	–	–	~4–5 kb	mean 4.5 kb (range 0.1–20kb) passage 4 mean 1.69 kb (range 0.079–11kb) passage 16	TRF/long-read: [32] HS-AFM: This study
BJ	6.4 kb (early passage) 4.9 kb (senescent)	–	4.7 kb	–	–	mean 6.3 kb (range 0.25–38kb) passage 4	TRF: [52] TeSLA: [53] HS-AFM: This study

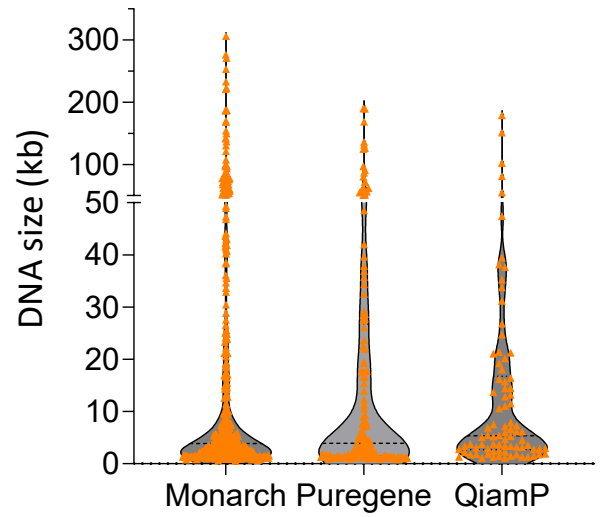
* Note: The long-read sequencing values were measured in IMR90 E6E7 cells, an E6/E7-immortalized derivative that is distinct from the parental IMR90 cells used for the TRF, chromosome 17 STELA, optical-mapping, and HS-AFM measurements.

Abbreviations: TRF, terminal restriction fragment (include a portion of subtelomeric region); Flow-FISH, flow cytometry–fluorescence in situ hybridization; TeSLA, telomere shortest length assay; STELA, single telomere length analysis; HS-AFM, high-speed atomic force microscopy.

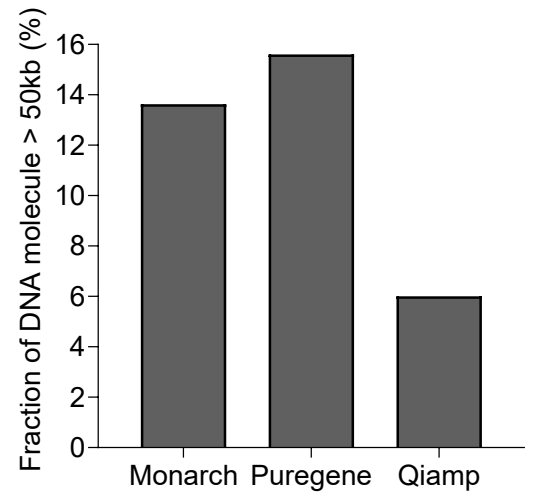
A.



B.



C.

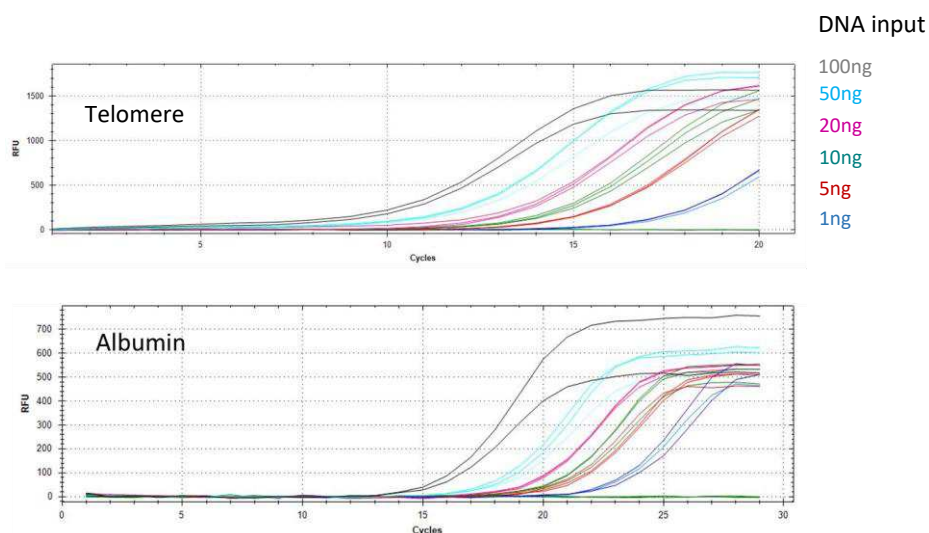


Supplementary Fig 1. Monarch and Puregene extraction methods yield greater recovery of long genomic DNA molecules than QIAamp. (A) Representative HS-AFM images of genomic DNA extracted using the Monarch, Puregene, and QIAamp methods. (B) Length distributions of individual genomic DNA molecules measured by HS-AFM following extraction with each method. Violin plots show the distribution of molecule lengths, with Monarch and Puregene preserving long DNA molecules, including fragments exceeding 50 kb, whereas QIAamp showed reduced recovery of the longest molecules. (C) Percentage of DNA molecules >50 kb recovered using each extraction method, demonstrating greater retention of long genomic DNA molecules by Monarch and Puregene compared with QIAamp

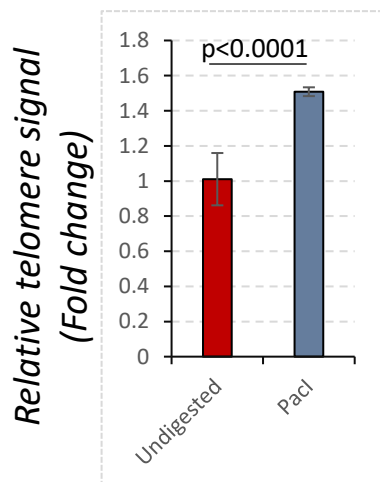


Supplementary Fig 2. CHM13 chromosome-end restriction-site maps. Genome-wide *in silico* restriction-site maps of CHM13 chromosome ends used to evaluate enzymes for end-preserving fragmentation. Analysis of predicted terminal-fragment profiles identified *PacI* as the optimal enzyme for generating 10–50 kb telomere-containing terminal fragments while retaining sufficient adjacent subtelomeric sequence for downstream analysis.

A.

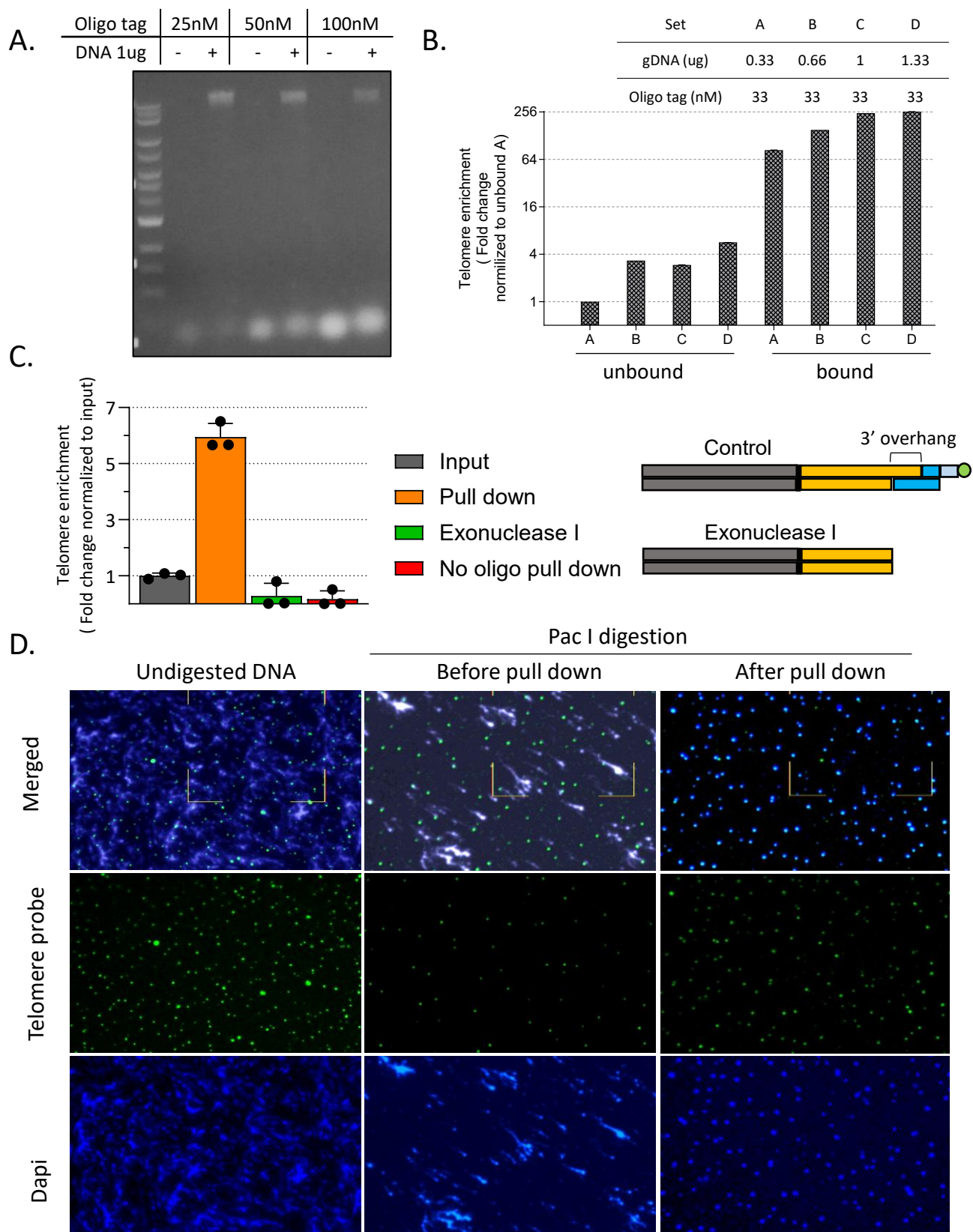


B.

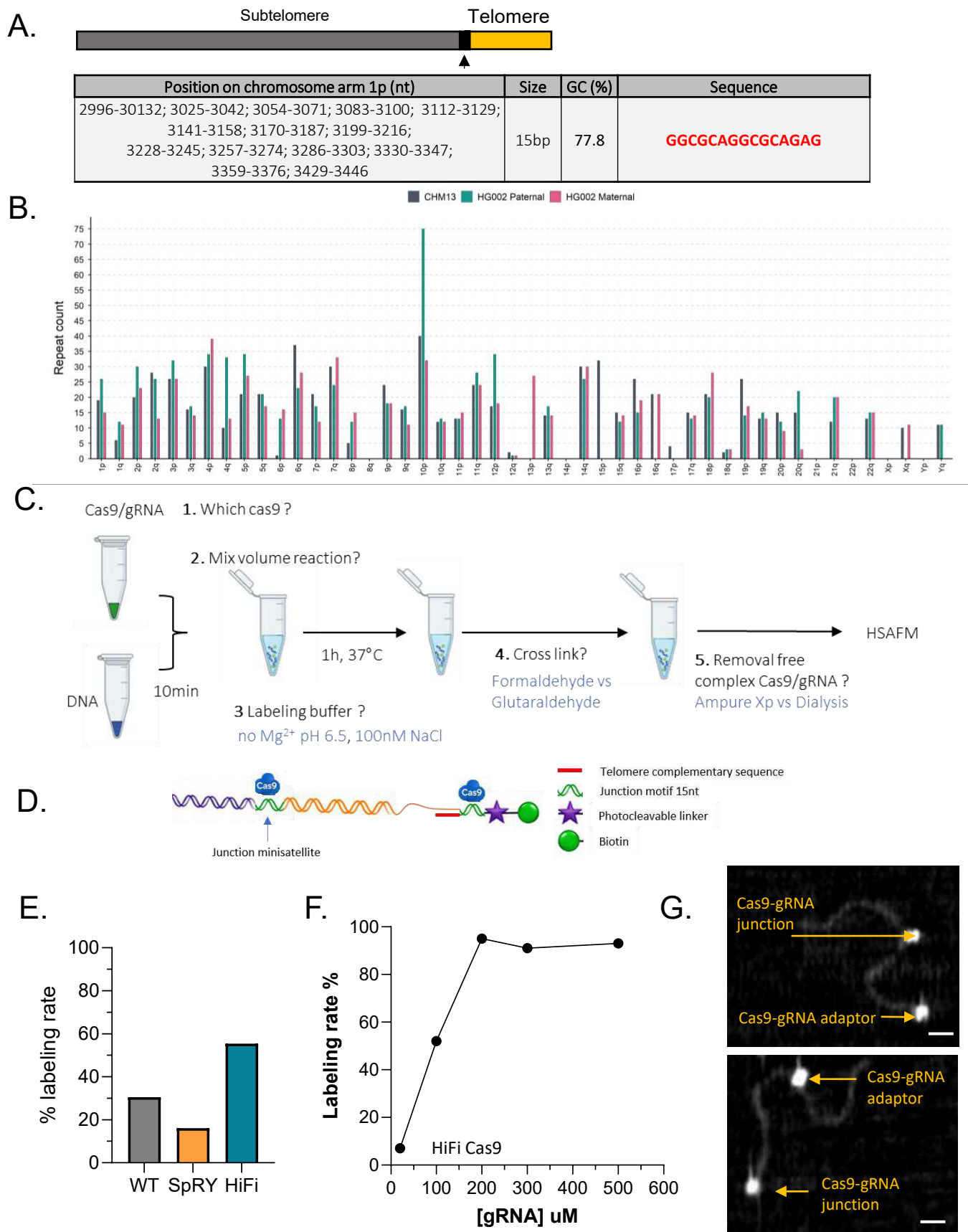


Supplementary Fig. 5. Assessment of qPCR performance and the effect of Pacl digestion.

(A) Representative telomere and albumin qPCR amplification curves across increasing DNA inputs (1–100 ng). (B) Relative telomere signal in undigested and Pacl-digested genomic DNA, expressed as fold change relative to the undigested condition ($P < 0.0001$, two-tailed paired Student's t-test). Data are mean $\pm$ s.d. from three biological replicates analyzed in technical triplicate.

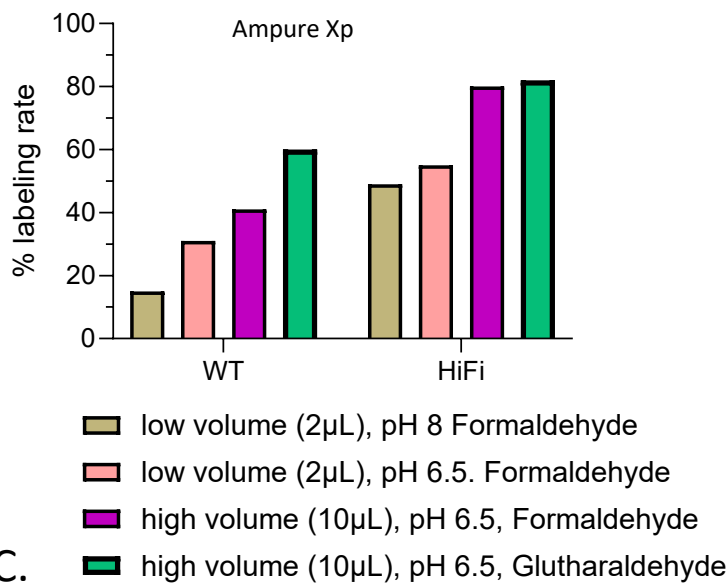


Supplementary Fig 6. Specific capture of telomeric fragments (A) Gel-based assessment of ligation of the biotinylated telomere-tag oligonucleotide to genomic DNA using increasing oligonucleotide concentrations (25, 50, and 100 nM) in the presence or absence of genomic DNA. (B) qPCR analysis of telomere enrichment in bound and unbound fractions across different genomic DNA and oligonucleotide input conditions, identifying conditions that maximize telomere capture efficiency. (C) qPCR analysis of telomere enrichment in input, pull down, exonuclease I-treated, and no-oligo control samples. Removal of the native 3' telomeric overhang by exonuclease I abolished enrichment, demonstrating that capture depends on ligation to the native chromosome-end overhang. A schematic of the exonuclease I treatment strategy is shown. (D) Representative telomere PNA-FISH images of undigested genomic DNA, PacI-digested DNA before pulldown, and the enriched fraction after pulldown. Telomere signals were detected predominantly in the enriched fraction, consistent with the specificity of telomere capture.

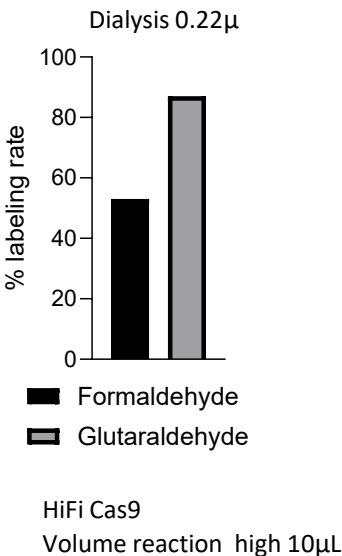


Supplementary Fig 7. CRISPR-Cas9 labeling of telomere boundaries. (A) Sequence, genomic positions, and GC content of the recurrent GC-rich minisatellite motif identified at telomere-subtelomere junctions. (B) Frequency of motif occurrence across chromosome ends in the CHM13, HG002 paternal, and HG002 maternal genome assemblies. Motif searches allowed up to two mismatches outside the PAM-proximal region. (C) Workflow for optimization of CRISPR-Cas9 labeling conditions for HS-AFM analysis. (D) Schematic of dual-boundary labeling using Cas9-gRNA complexes targeting the telomere-subtelomere junction motif and the terminal adaptor sequence. (E, F) Comparison of Cas9 variants and gRNA concentrations for boundary labeling. Labeling efficiencies were calculated from analysis of 500 synthetic DNA molecules containing target site for each condition. (G) Representative HS-AFM images showing Cas9-gRNA complexes bound to the telomere-subtelomere junction motif and terminal adaptor sequence.

A.



B.

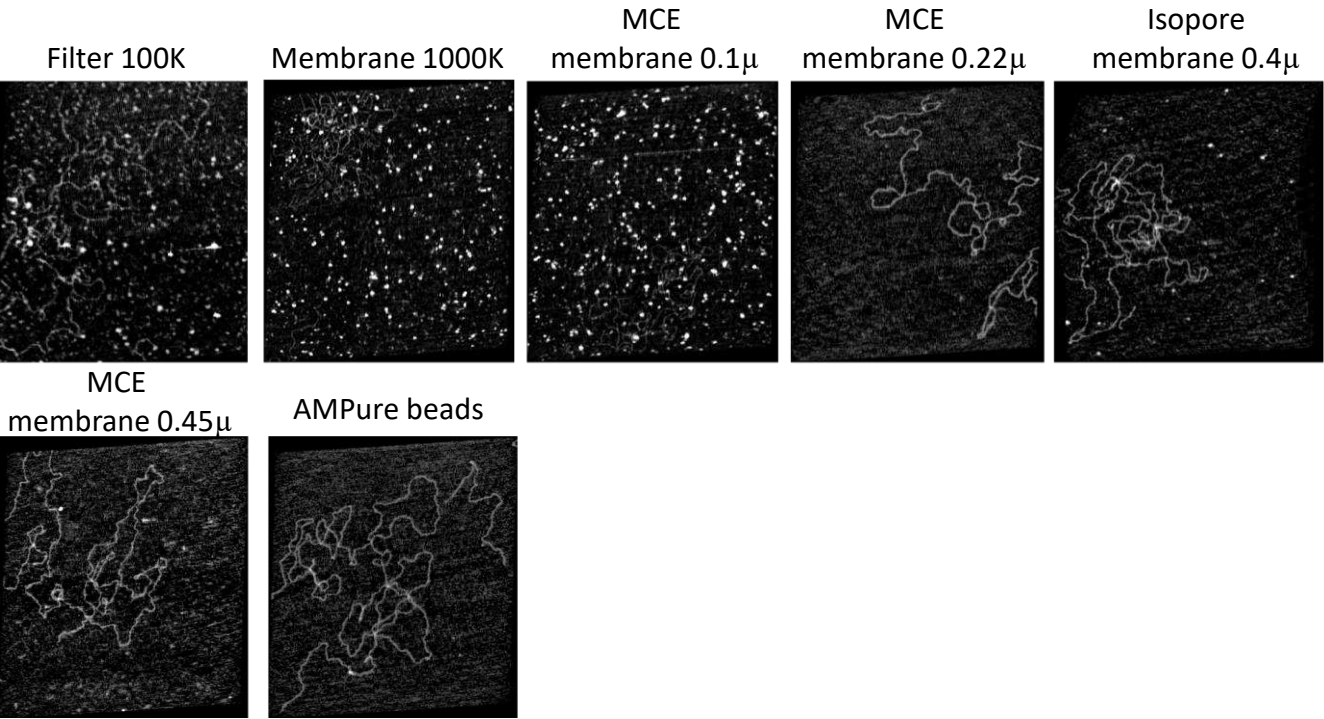


C.

Conditions			Cas9 HiFi/ gRNA labeling	Comments
gRNA	Annealing protocol	Concentration at annealing	Labeling rate (%)	
Two-parts	One step protocol	8 µM	89.3	
Two-parts	Two-steps protocol	8 µM	81.4	
Two-parts	Optimized	8 µM	93.1	
Two-parts	Optimized	25 µM	91.7	
Two-parts	Optimized	50 µM	92.5	
Single-part	No annealing	-	67.4	Some aggregation

Supplementary Fig 8. Optimization of CRISPR–Cas9 boundary labeling conditions. (A) Comparison of Cas9 labeling efficiencies under different reaction volumes, buffer conditions, and crosslinking reagents following cleanup with AMPure XP beads. WT Cas9 and HiFi Cas9 were evaluated under low-volume (2 µL) and high-volume (10 µL) reaction conditions using formaldehyde or glutaraldehyde fixation. (B) Effect of crosslinking reagent on HiFi Cas9 labeling efficiency following 0.22 µm dialysis cleanup. Labeling efficiencies obtained with formaldehyde and glutaraldehyde fixation are shown. (C) Summary of guide RNA preparation and labeling conditions for HiFi Cas9. Labeling efficiencies were determined from analysis of 500 synthetic DNA molecules containing target site. Different annealing protocols and gRNA concentrations were evaluated to identify conditions maximizing labeling efficiency while minimizing aggregation. The optimized two-part gRNA protocol achieved >90% labeling efficiency.

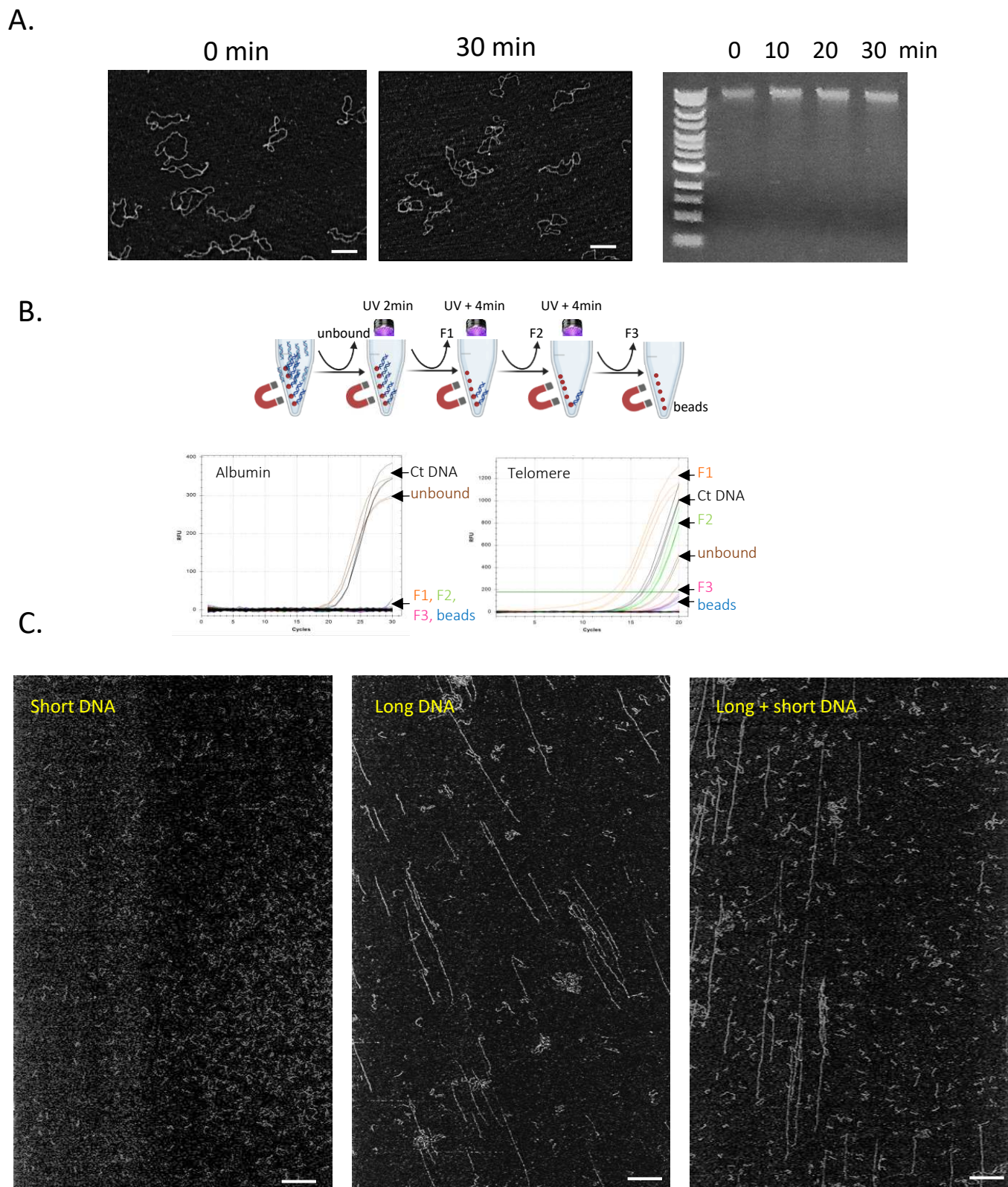
A. Mix DNA 0.250μg + 100nM oligo + ligation reaction



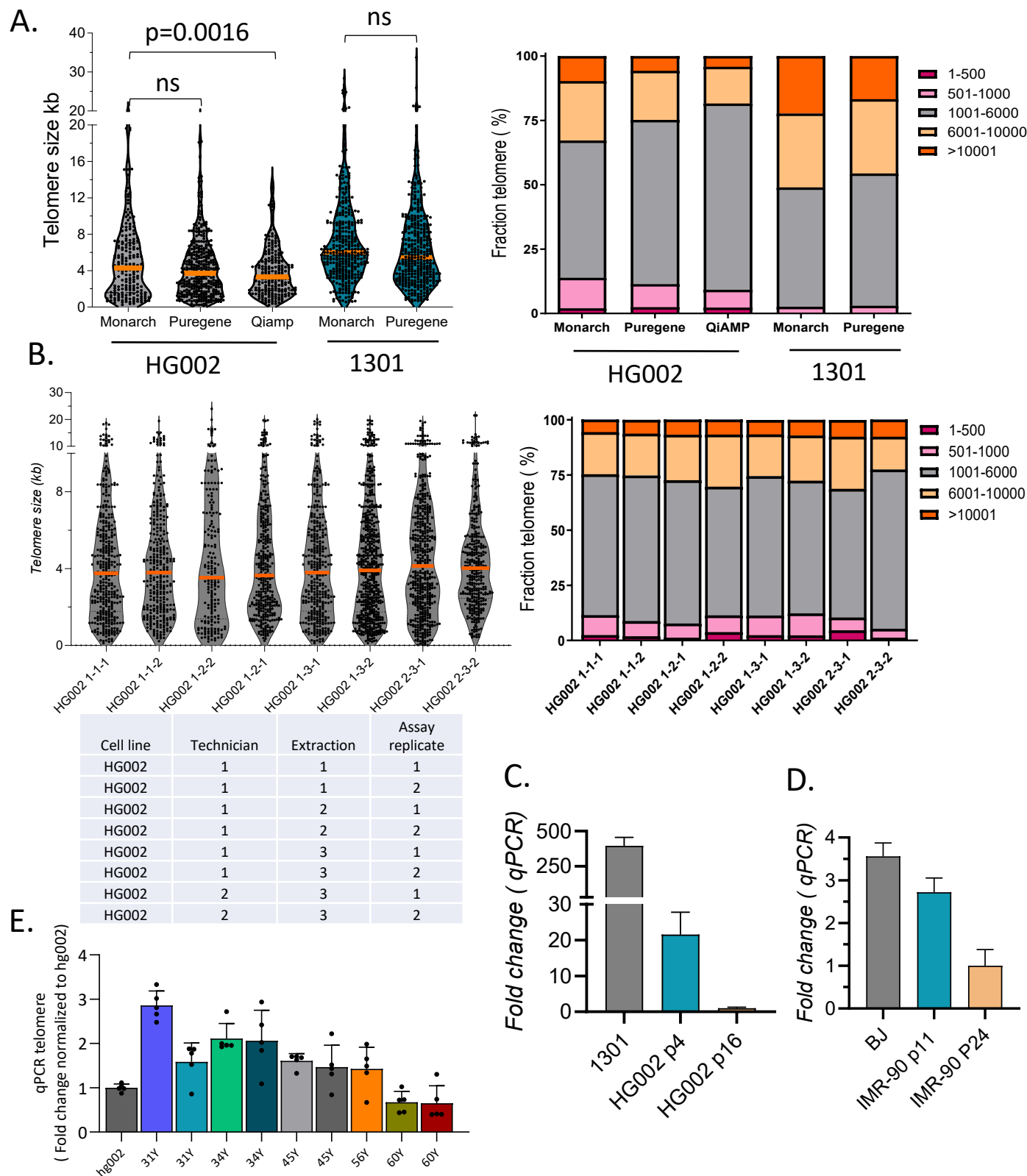
B.

Beads type	NEB	T1	M280	M270	C1
Comment	Size: 1μ Hydrophobic Background associated with beads footprint Good pull down at high DNA concentration (2μg)	Size: 1μ Hydrophobic Good pull down at high DNA concentration (2μg)	Size: 2.8μ Hydrophobic Good pull down at low DNA concentration (100ng)	Size: 2.8μ Hydrophilic High background	Size: 1μ Hydrophilic High background
Image					

Supplementary Fig 9. M280 streptavidin magnetic beads and 0.22 μm dialysis provide optimal conditions for HS-AFM analysis. (A) Representative HS-AFM images of ligation reactions following cleanup with different filtration and purification methods, including 100 kDa filtration, 1000 kDa membrane filtration, 0.1 μm and 0.22 μm MCE membrane filtration, 0.4 μm and 0.45 μm membrane filtration, and AMPure bead purification. The 0.22 μm membrane most effectively removed residual free oligonucleotides while preserving intact DNA molecules. (B) Comparison of streptavidin magnetic bead types for telomere capture and subsequent HS-AFM imaging. Representative images are shown for NEB, T1, M280, M270, and C1 beads. M280 beads provided the best balance between efficient pulldown and low imaging background, whereas M270 and C1 beads generated substantial background signal.

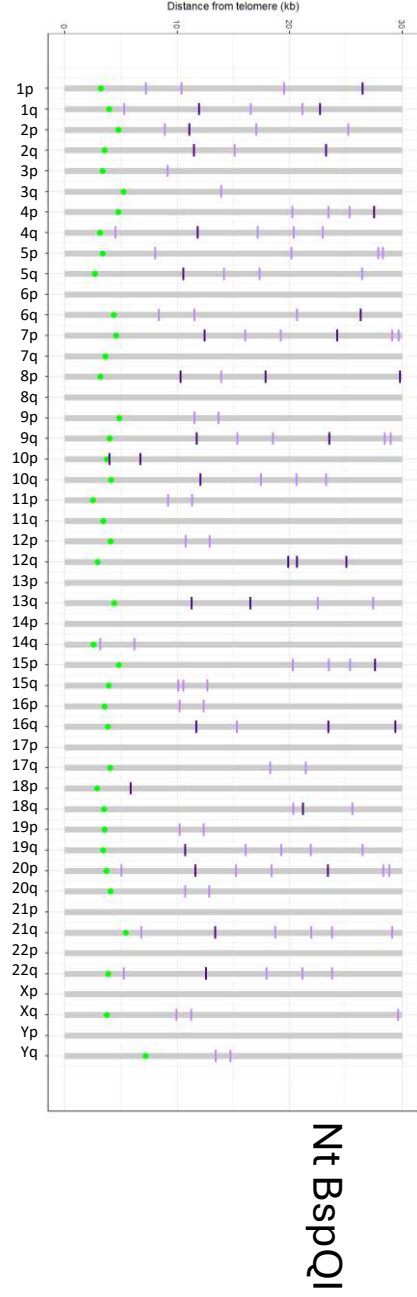
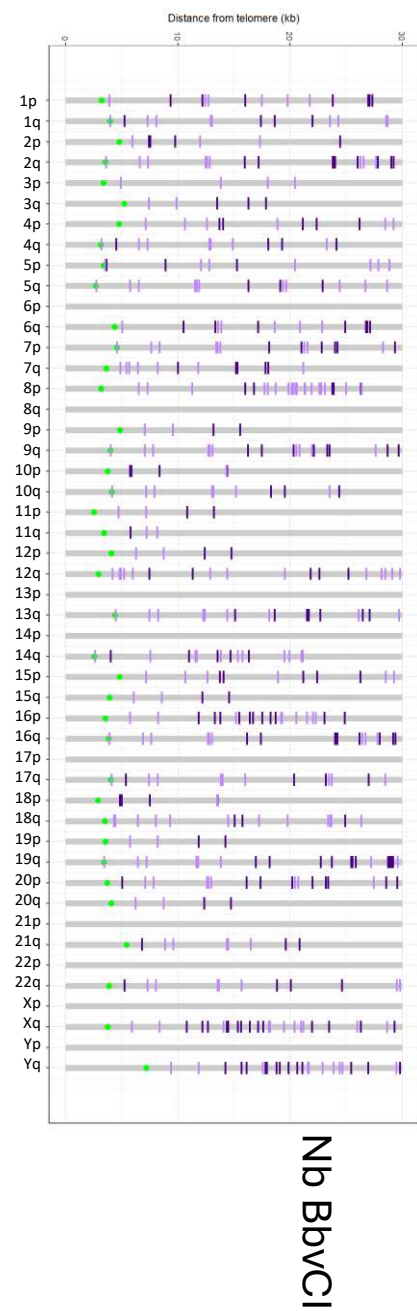
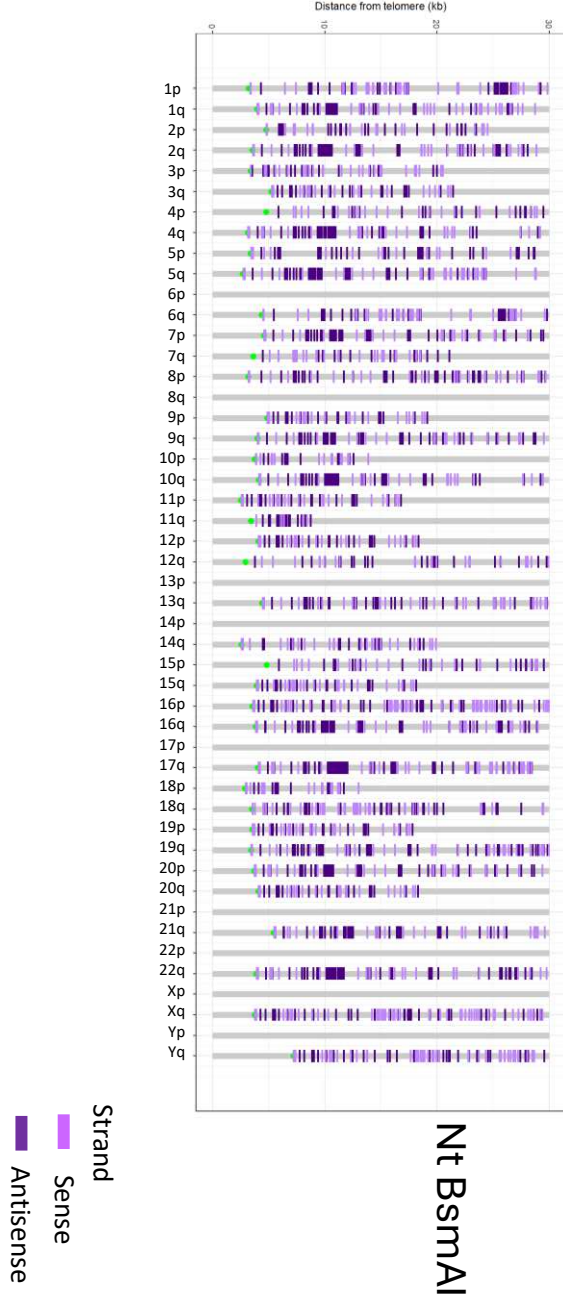


Supplementary Fig 10. Magnetically directed deposition retains DNA molecules across a broad size range for unbiased telomere size analysis. (A) Assessment of DNA integrity following exposure to 365-nm UV light for up to 30 min. Representative HS-AFM images (0 and 30 min) and agarose gel electrophoresis (0, 10, 20, and 30 min) showed no detectable DNA fragmentation after UV exposure. (B) Sequential release of telomeric fragments following photocleavage of the linker. Schematic of the release workflow and qPCR analysis of albumin and telomere signals in released fractions demonstrate that the majority of telomeric material was recovered during the initial UV-release steps. (C) Representative HS-AFM images of short DNA, long DNA, and mixed DNA populations following magnetically directed deposition and release onto mica. Comparable recovery of short and long DNA molecules confirms retention across a broad size range without preferential loss of longer molecules.

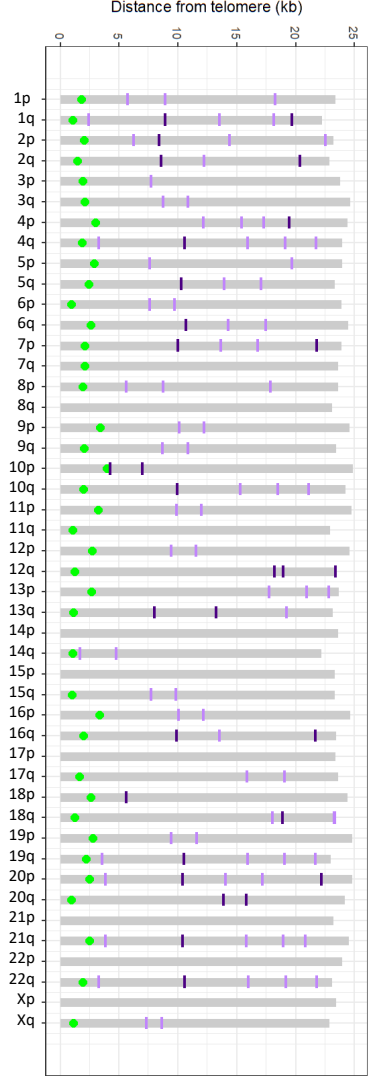


Supplementary Fig 11. HS-AFM measurements are robust to DNA extraction yield, operator analysis, and biological variation. (A) Comparison of telomere-length distributions and telomere size categories in HG002 and 1301 cells following DNA extraction with Monarch, Puregene, or QIAamp. Monarch and Puregene produced comparable telomere-length profiles, whereas QIAamp underrepresented longer telomeric fragments. (B) Telomere-length distributions (left) and size-category profiles (right) obtained from independent HG002 DNA extractions and workflow replicates performed by two technicians. No significant differences in telomere-length distributions were detected among extractions, workflow replicates, or technicians. For A and B, each point represents an individual telomere measurement, with approximately 400–500 telomeres analyzed per condition[CJC1.1]. (C) Relative telomere abundance measured by qPCR in reference cell models spanning long-, intermediate-, and short-telomere states. (D) Relative telomere abundance measured by qPCR in BJ and IMR-90 cells at different passages, demonstrating reduced telomere content with culture expansion. (E) Relative telomere abundance measured by qPCR in peripheral blood DNA samples from individuals of different ages, normalized to HG002. Telomere abundance was higher in younger individuals and generally decreased with age. Data are presented as mean $\pm$ s.d. from three biological replicates, each assayed in technical triplicate.

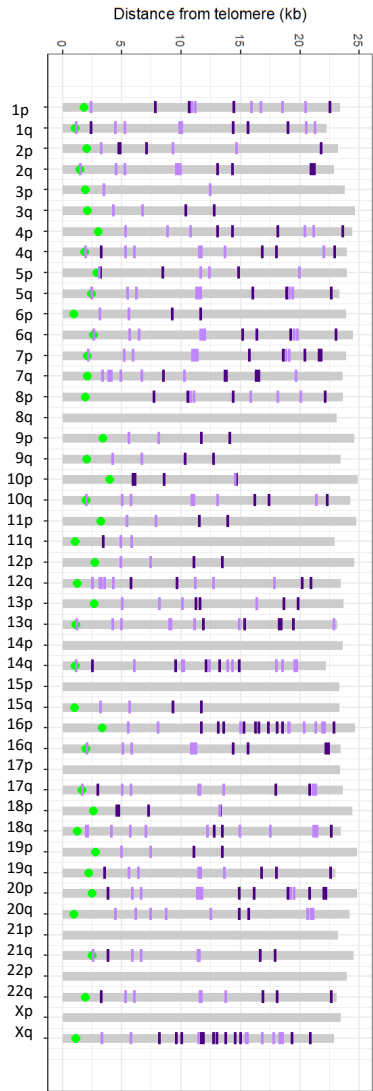
A. CMH13 assembly



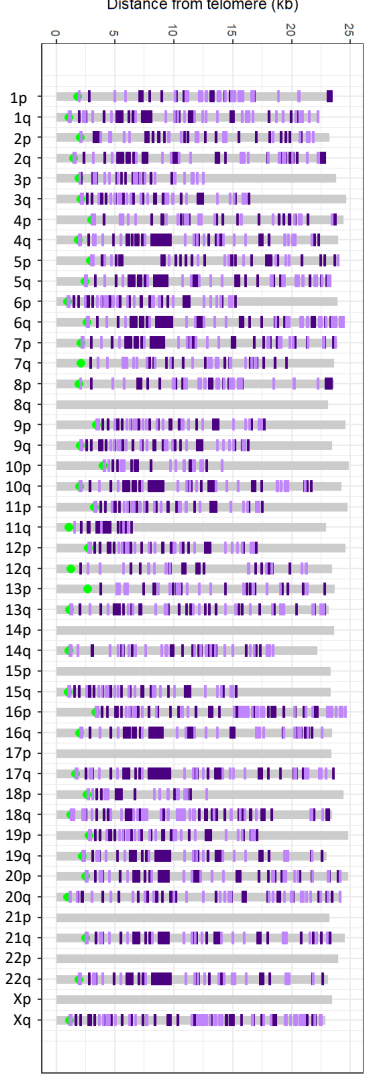
B. HG002 maternal assembly



Nt BspQI



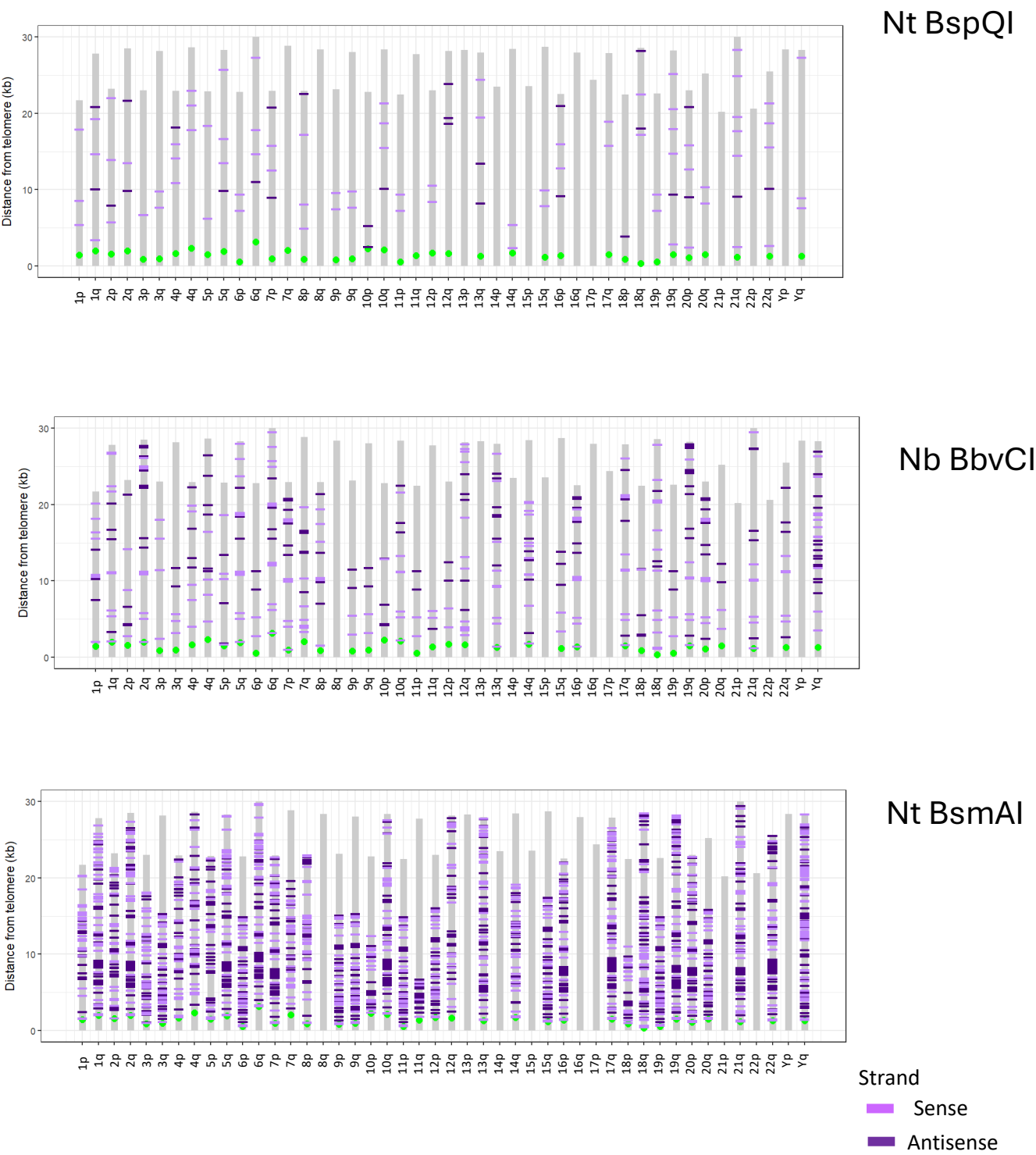
Nb BbvCI



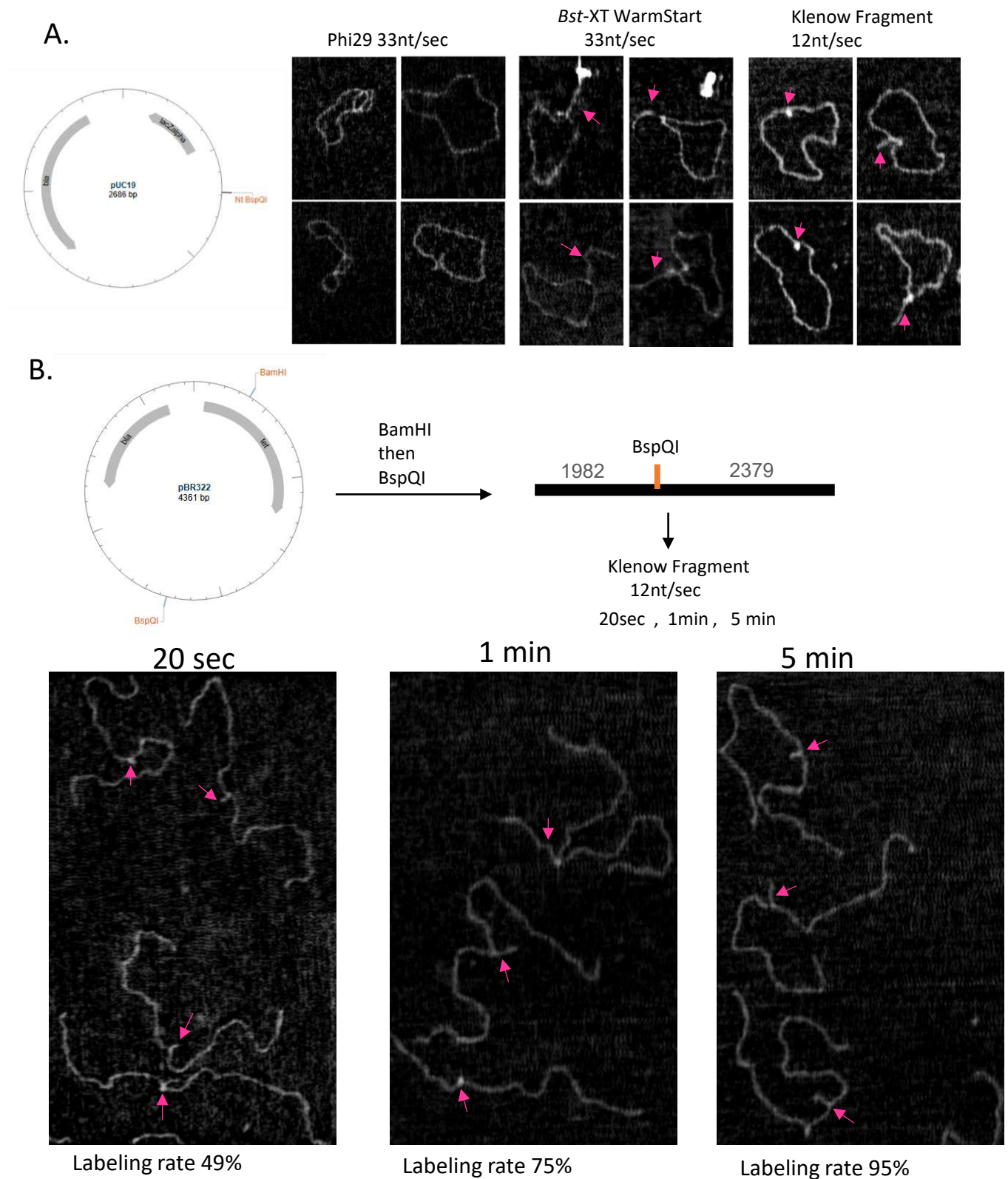
Nt BsmAI

Strand
Sense
Antisense

C. HG002 paternal assembly



Supplementary Figure 12 Nt.BspQI produces distinct chromosome-end barcode signatures across human genome assemblies. (A–C) Predicted Nt.BspQI, Nb.BbvCI, and Nt.BsmAI nicking-site distributions across chromosome ends in the CMH13 (A), HG002 maternal (B), and HG002 paternal (C) assemblies. Chromosome ends are arranged along the x-axis, and distances from the telomere boundary are plotted on the y-axis. Green markers denote telomere boundaries, while purple markers indicate predicted nicking sites, colored according to strand orientation (sense and antisense). Gray vertical bars represent the subtelomeric regions analyzed for barcode generation.



Supplementary Figure 13. Klenow-mediated strand displacement generates robust DNA-tail labels detectable by HS-AFM. (A) Evaluation of polymerases for generation of HS-AFM-detectable DNA-tail structures at nicking sites. A pUC19 plasmid containing a single Nt.BstNBI nicking site was subjected to polymerase-mediated strand-displacement synthesis using Phi29 DNA polymerase, *Bst*-XT WarmStart DNA polymerase, or Klenow Fragment. Representative HS-AFM images are shown for each condition. DNA-tail products generated at the nicking site are indicated by magenta arrows. Phi29 produced few detectable tail structures, whereas *Bst*-XT WarmStart and Klenow Fragment generated readily visualized DNA tails. Polymerization rates for each enzyme are indicated above the images. (B) Optimization of Klenow-mediated strand-displacement labeling. Linearized pBR322 substrate containing a single Nt.BspQI nicking site at a defined position was generated by BamHI digestion followed by Nt.BspQI nicking. The nicked substrate was subjected to strand-displacement synthesis with Klenow Fragment for 20 s, 1 min, or 5 min. Representative HS-AFM images are shown below, with DNA-tail products indicated by magenta arrows.