High accuracy DNA sequencing on a small, scalable platform via electrical detection of single base incorporations

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Abstract:

High throughput DNA sequencing technologies have undergone tremendous development over the past decade. Although optical detection-based sequencing has constituted the majority of data output, it requires a large capital investment and aggregation of samples to achieve optimal cost per sample. We have developed a novel electronic detection-based platform capable of accurately detecting single base incorporations. The GenapSys technology with its electronic detection modality allows the system to be compact, accessible, and affordable. We demonstrate the performance of the system by sequencing several different microbial genomes with varying GC content. The platform is capable of generating 1.5 Gb of high-quality nucleic acid sequence in a single run. We routinely generate sequence data that exceeds 99% raw accuracy with read lengths of up to 175 bp. The utility of the platform is highlighted by targeted sequencing of the human genome. We show high concordance of SNP detection on the human NA12878 HapMap cell line with data generated on the Illumina sequencing platform. In addition, we sequenced a targeted panel of cancer-associated genes in a well characterized reference standard. With multiple library preparation approaches on this sample, we were able to identify low frequency mutations at expected allele frequencies.

Introduction:

Soon after the finished version of the human genome was released in 2004 (1), there was an initiative from the National Human Genome Research Institute (NHGRI) to sequence a human genome for $1000 (2). This initiative, in part, ushered in a period of development of a number of different DNA sequencing technologies (3-8). Today, a variety of commercial platforms exist that enable massively parallel, high throughput, and low-cost genomic sequencing, revolutionizing the nature of scientific and biomedical research. Despite continued technical improvements that have reduced the per base cost of DNA sequencing, the majority of sequencing is still carried out on large, expensive instruments with high costs per run (9).

We have developed a novel sequencing-by-synthesis approach that employs electrical detection of nucleotide incorporations. With electrical-based detectors, simple fluidics, no optics, and no robotics or moving parts, our automated sequencer is compact, robust, easy to use, and inexpensive. The instrument detects a steady-state signal, providing several key advantages over current commercially available sequencing platforms and allowing for highly accurate sequence detection. Sequencing is carried out on microfluidic chips that have a scalable number of detectors, supporting applications ranging from targeted sequencing of specific amplicons to genome-scale data collection. We believe that sequencing instruments that are compact, scalable, easy to use, and affordable to purchase and run will permit a more distributed model, in which genomic assays are put back into the hands of individual researchers. Here, we demonstrate the capabilities of the Genapsys sequencing platform in a variety of applications across multiple sample types.

Results:

Detection of single base incorporation via electrical impedance modulation:

An overview of the Genapsys sequencing platform is outlined in Figure 1. Briefly, a library of DNA fragments is clonally amplified with DNA primers conjugated to GenapSys beads. Amplified beads are loaded onto the fluidic chamber on top of a fully integrated Complementary Metal-Oxide Semiconductor (CMOS) sequencing chip. The sequencing chip is then inserted into the GS111 instrument, which uses fluidics to inject reagents that will disperse evenly over the entire chip surface (Figure 2A). The GS111 instrument has fluidics to control reagent injection. Nucleotides are injected one base at a time and incorporations are measured as electrical signals from each sensor that collect on the instrument's Solid-State Device (SSD) storage unit. The incorporation data files are transferred in real time to a secure cloud-hosted server and storage environment. At the end of the run, the final FASTQ base call file becomes available.
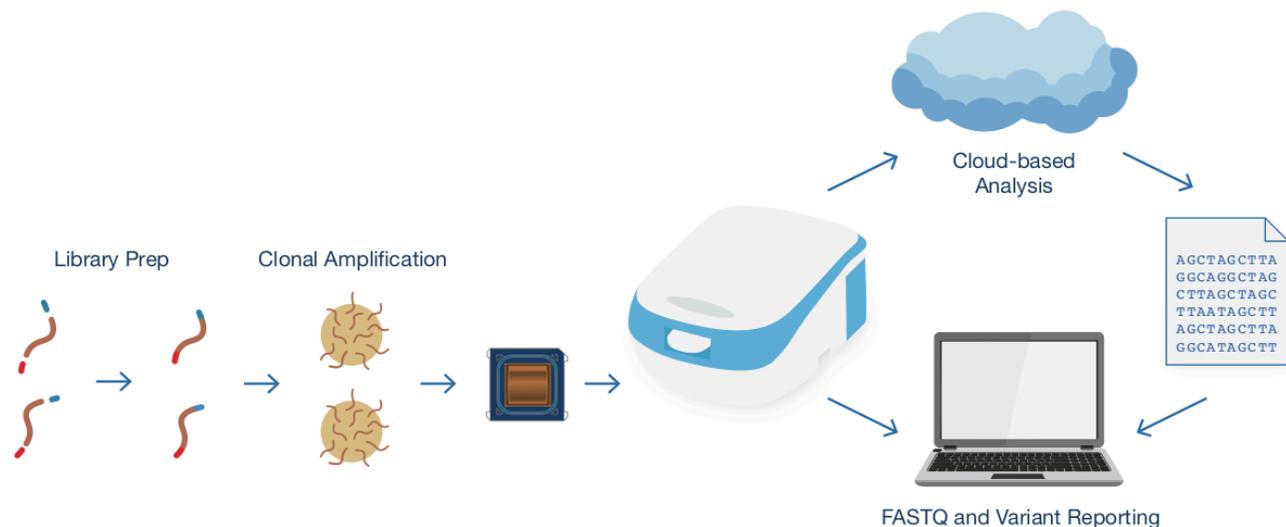

FIGURE 1: Overview of the Genapsys Sequencing Platform
An overview of the process for generating sequence data on the GenapSys sequencing platform. Libraries are clonally amplified onto beads, which are subsequently loaded onto the sequencing chip. Automated sequencing takes place on the GS111 instrument, which utilizes a cloud-based system for instrument control and data analysis, with the ultimate output being a FASTQ file of millions of individual DNA sequences with per-base quality scores.

Detection modality, signal processing and base calling:

An example of the raw signal is shown in Supplementary Figure 1. Total signal of a sequencing bead increases with each successful incorporation (red line). A fraction of the sensors is purposefully loaded with amplified beads containing a sequencing primer blocked from amplification by virtue of a chemical modification at the

primer's 3' end. Data from these sensors are used to normalize and remove any signal drift (blue line). The measured signal does not change significantly when a nucleotide is added that is not a match to the next DNA base in the template strand.   When a nucleotide is incorporated, the measured impedance value of that sensor will jump creating a graph that resembles a staircase. Information on how the signal has changed upon injection of a given nucleotide can be summarized as differential signal (Figure 2B). The magnitude of the differential signal correlates with the number of incorporated nucleotides. Thus, if the template contains more than one of the same consecutive base, the size of the signal change will be larger. Due to the steady-state nature of the impedance metric, the measured values do not significantly change over time. Thus, if desired, multiple measurements can be taken to improve precision. Figure 2B contains a representative example of acquired data showing the distribution of measured differential signal across multiple nucleotide flows for a single template sequence. Flows that are expected to have nucleotide incorporations are highlighted in red. Non-incorporating flows are shown in blue and do not deviate significantly from the baseline. The inferred sequence, including the number of nucleotides in each incorporation are shown below the plot (Figure 2B).
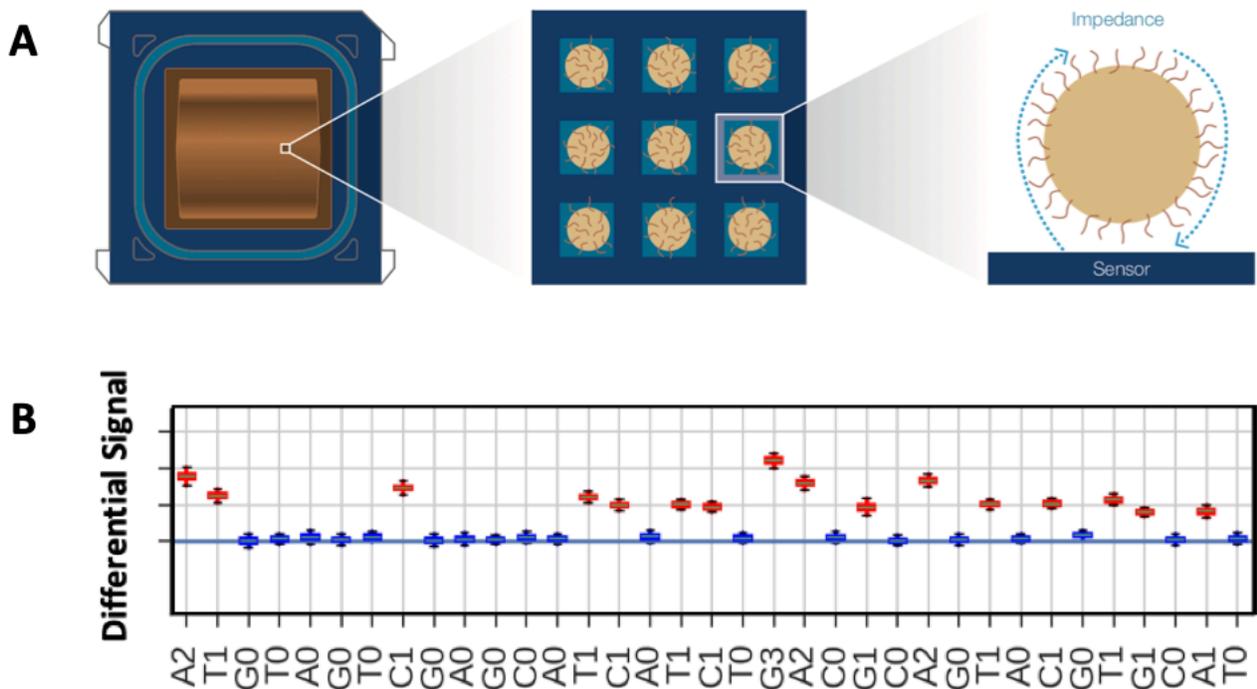

FIGURE 2: Sequencing Chip and Raw Data
(A) The CMOS sequencing chip contains millions of individual sensors, which are each loaded with a clonally amplified bead. The electrodes in each sensor are capable of measuring minute changes in impedance when nucleotides are incorporated opposite the bead-bound templates. (B) Raw data from each sensor is summarized as a differential signal for each nucleotide flow. This box plot represents the distribution of signal for a subset of nucleotide flows for a control template. Red boxes denote incorporating flows and blue boxes are flows with no nucleotide incorporation. The identity of the injected nucleotide and number of incorporated bases is shown below the plot.

For each base, a quality score is predicted using a pre-trained Phred quality table (12). Low quality reads can be filtered based on the average quality score of their base calls. The substitution error rate is particularly low (~ 0.01%) which compares favorably to other sequencing technologies (13-15). A breakout of the error types and a distribution of Q-score relative to position in the read for a typical run is shown in Supplementary Figure 2.

Sequencing of Microbial Genomes:

To test the performance of our platform, we used it to sequence the *E. coli* genome. Results are summarized in Table 1. The sequencing workflow is fully automated on the GS111 instrument with average run times that vary depending on the number of nucleotide flows. The average read length is 141 bp with base call lengths that range from 100 to 175 bp. The distribution of base call lengths is shown in Supplementary Figure 3. Average coverage across the 4.69 Mb *E. coli* genome is 320x (Figure 3A-B), and the sequencing data shows relatively even coverage across the entire *E. coli* genome (Figure 3A). The average accuracy of the reads is 99.9% at 75 bp, more than 99.8% at 100 bp, and remains well above 99% past 150 bp (Figure 3C). More than 97% of the bases have Q-scores that exceed Q20.

Number of Reads	12,066,883
Number of Bases	1,694,270,754
Number of Mapped Reads	10,820,542
Number of Mapped Bases	1,485,017,178
Average Mapped Read Length	141 bp
Average Cumulative Accuracy at Position 100	99.8%
Percentage of Bases with Q>20	97.4%
Percentage of Bases with Q>30	63.9%
Genome Size	4.69 Mb
Average Coverage	319.9

Table 1: Sequencing Statistics (*E. coli*)

To further evaluate the system's performance across a range of sequence contexts and a range of GC contents, we made libraries from additional microbial genomes. Details regarding the genomic DNA samples can be found in Supplementary Table 1. We selected microbial genomes with a range of average GC content. For example, *Campylobacter jejuni* is a low GC organism with an average GC content of about 30%. To address the higher GC content range, we sequenced *Bifidobacterium animalis subsp. Lactis* with an average GC content of 61%. Average coverage of each of these genomes exceeded 800x, with similar read lengths and accuracy statistics to the *E. coli* run. Statistics from the sequencing runs are outlined in Supplementary Figure 4. Figure 3D shows the distribution of sequencing coverage broken down into bins of GC content. For comparison, we also show Illumina data generated from the same genomic DNA. This data shows that the sequencing system is robust and relatively uniform across a wide variety of sequence contexts and GC content. While there is some decrease in normalized coverage at the GC content extremes, the bias is consistent with what is typically observed for sequencing technologies that rely upon amplification (16).
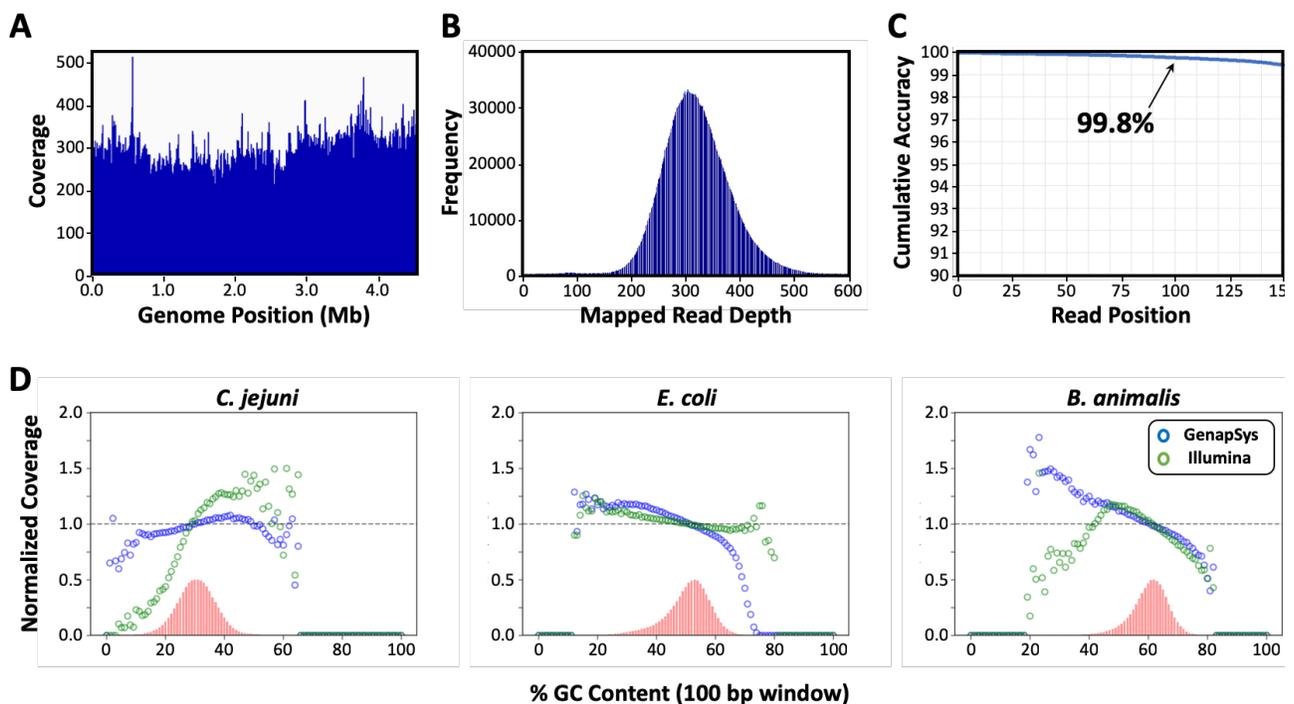

FIGURE 3: Microbial Genome Sequencing Statistics
(A) Coverage plot across the *E. coli* genome. (B) Histogram of mapped read depth for sequencing of the *E. coli* genome. (C) Average cumulative sequencing accuracy by read position. (D) Normalized coverage for sequencing of three different microbial genomes. Coverage is binned by percent GC content in a 100 bp window. The red histogram represents the abundance of each GC content bin in the genome. Blue circles are normalized coverage from the GenapSys platform and green circles are from data generated on the Illumina platform.

SNP Discovery in a Human Exome Sample:

As a demonstration of the technology applied to human samples, we carried out whole exome sequencing of DNA from the extremely well characterized human cell line NA12878. NA12878 is a lymphoblastoid cell line (LCL) prepared from a normal female Caucasian participant of the international HapMap project (17). This DNA sample has served as human reference standard for the Genome in a Bottle (GIAB) Consortium and has been well characterized using a variety of different genomic technologies (18).

Targeted sequencing libraries made from NA12878 genomic DNA were generated via probe-based capture using the IDT xGEN Exome Research Panel (v1.0). The exome library was sequenced to approximately 96x average coverage across the 39 Mb targeted region. Approximately 78% of the sequencing reads mapped to the target region with relatively uniform coverage (Supplementary Table 2). The read length and accuracy are similar to the *E. coli* run detailed above.

Using standard methods, we identified single nucleotide polymorphisms (SNPs) relative to the human genome reference (hg38). When carrying out the variant analysis with a focus on the high confidence regions from the GIAB v3.3.2 consortium, the GenapSys sequencing data of the NA12878 exome generated 20,609 SNP variants. To benchmark the performance of the GenapSys platform for SNP detection, we subjected the same library to Illumina sequencing with an average coverage of approximately 280x. Using the same analysis parameters, the Illumina data identified 21,212 SNPs. The overlap between the two platforms was extremely high. This is illustrated in Figure 4A, where the Venn diagram shows a sensitivity of 96.5% and a precision of 99.4%, with low levels of both false positives and false negatives for the GenapSys data relative to the Illumina data. If we consider the GIAB high confidence call set as ground truth, we maintain high concordance with 95.6% sensitivity and 99.0% precision as shown in Figure 4B. Furthermore, if instead of the high confidence regions, we consider all SNP variants from the Illumina dataset as a comparison, we obtain a sensitivity of 94.8% and precision of 98.3% (data not shown). The ratio of transition to transversion SNPs (TiTv ratio) is 2.85. Overall, this compares very favorably to similar comparisons done between different sequencing platforms (19).
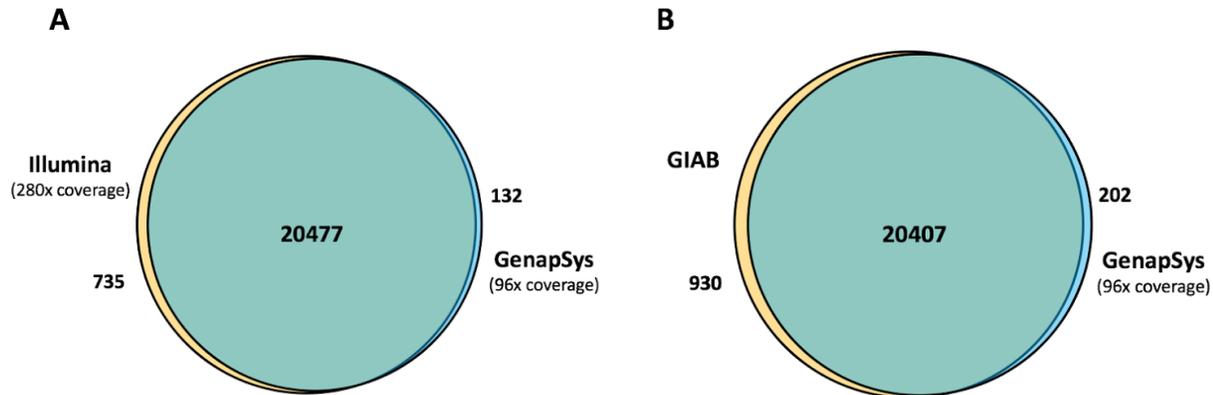
FIGURE 4: Human Exome Variant Concordance
Venn diagrams illustrating the overlap of SNPs identified in the NA12878 Exome sample from GenapSys data. BCFtools was used to call variants. (A) GenapSys SNPs relative to SNPs called from the Illumina dataset using high confidence regions from the GIAB consortium. (B) GenapSys SNPs relative to the GIAB high confidence call set.

In an effort to evaluate other variant calling methods, we employed DeepVariant (20), a deep learning approach, to call SNP variants from the same NA12878 exome dataset. DeepVariant has shown better performance than conventional tools and is, reportedly, more generalizable to the error profiles of different sequencing technologies (20). The results of this method for SNPs when focusing on the same high confidence regions are shown in Supplementary Figure 5. The Venn diagrams show significant overlap between variant calls from the Illumina and GenapSys data for both the test dataset as well as the entire exome with 97.5% sensitivity and 99.8% precision for the latter. As a more generalizable variant calling algorithm, the DeepVariant approach could be preferred for use with GenapSys sequencing data over other commonly used tools such as GATK (21), which is specifically tuned to the particular error modes present in Illumina sequencing data.

Low Frequency Variant Detection in a Targeted Human Cancer Reference Sample:

Quantitation of low frequency somatic mutations in cancer samples requires a sequencing platform with high accuracy. To evaluate the performance of the Genapsys platform for this application, we performed targeted sequencing of a quantitative multiplex reference standard (Horizon Discovery) using a Pan-Cancer probe panel from IDT. The genomic DNA sample is a mixture of genomic DNA from 3 cell lines, HCT116, RKO and SW48, with verified oncology-relevant mutations, with allele frequencies that range from 1% to 24.5%. One sequencing run generates more than sufficient coverage for detection of low frequency alleles. The on-target rate across the

800 Mb panel was more than 62% and read length and accuracy statistics were similar to the *E. coli* runs (Supplementary Table 2).

Table 2 shows the concordance of our SNP variant calls with the expected allelic frequencies for 11 different mutations. The allele frequencies measured by the GenapSys sequencing platform closely match the expected allele frequencies determined by the vendor of the quantitative multiplex reference standard using Droplet Digital PCR. Mutant alleles with frequencies as low as 1.0% were correctly identified. As an example, aligned reads visualized in the IGV browser for EGFR variant L858R are shown in Supplementary Figure 6. There are 925 reads that map to this genomic location and 28 of them show the variant, correlating well to the expected allele frequency of 3.0%. There is a relatively balanced proportion of forward and reverse reads from both the mutant and wild type alleles. As a benchmark, we sent the same library out for sequencing on the Illumina sequencing platform and used the same analysis methods on both sets of sequencing data. The measured allele frequencies are similar. These data demonstrate comparable performance of the GenapSys sequencing platform with well-established methods.

Gene	Variant	Expected	GenapSys	Illumina
EGFR	T790M	1.0	1.0	1.0
EGFR	delE746-A750	2.0	2.5	1.7
EGFR	L858R	3.0	3.0	2.8
KRAS	G12D	6.0	3.5	4.3
PIK3CA	E545K	9.0	3.8	6.6
KIT	D816V	10.0	8.5	6.9
BRAF	V600E	10.5	7.8	8.9
NRAS	Q61K	12.5	10.4	10.6
KRAS	G13D	15.0	14.1	15.4
PIK3CA	H1047R	17.5	14.2	15.2
EGFR	G719S	24.5	23.2	23.3

Table 2: Low Frequency Variant Detection via Hybrid Capture Probe-based Targeted Sequencing

We also investigated low frequency mutation detection with a library preparation approach using multiplex PCR amplicons. The Ion AmpliSeq Hotspot Cancer Panel v2 consists of 207 primer pairs and generates amplicons targeting genomic 'hot spot' regions that are commonly mutated in human cancers. An amplicon library was generated using the AmpliSeq panel with the Horizon HD701 gDNA sample. Sequencing of this library using the GenapSys platform led to the identification of the expected mutations, including low-frequency mutations.

Figure 5 shows the correlation of expected allele frequency to that measured by the GenapSys sequencing platform. The correlation is very high with an R^2 of greater than 0.99.
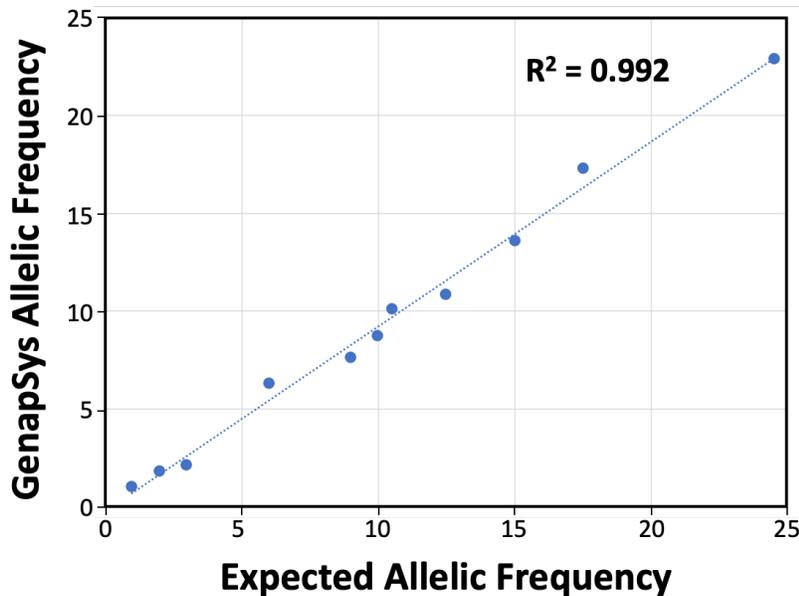

FIGURE 5: Low Frequency Variant Correlation via Amplicon-based Targeted Sequencing
Scatter plot showing high correlation between measured and expected allelic frequency for 11 variants from the HD701 Quantitative Multiplex Reference Standard. The x-axis represents the expected allelic frequency in the HD701 gDNA as measured by ddPCR and the y-axis represents the measured allelic frequency for AmpliSeq Cancer Hotspot Panel v2 targeted sequencing on the GenapSys platform. The Pearson correlation coefficient is 0.992.

Conclusions:

We have demonstrated that the Genapsys sequencing platform is capable of generating very accurate DNA sequence data. The system's architecture, including CMOS-based electronic detection, an absence of moving parts and optics, minimal computational requirements, and simple fluidic controls, allows for an instrument that is compact, scalable, and affordable.

As with many other sequencing technologies, we believe this platform is capable of rapid technological improvements that will increase accuracy, throughput, and speed. In particular, significantly higher sensor densities are feasible and would make dramatic improvements to throughput. Thanks to the instrument's flexible architecture, such enhancements could be realized by modifying the consumables without substantially altering the instrument itself. Therefore, additional sequencing throughput would come without significant additional investment or run time increases, which could dramatically improve the cost per base.

Further improvements to the sequencer and workflow have the potential to improve the performance and usability of the platform. Enhanced nucleotide modification chemistries could increase the signal to noise ratio, improving accuracy and allowing for longer read lengths. Better optimization of flow times could reduce the duration of sequencing runs. Automation and integration of sample preparation prior to sequencing could further increase the ease of use. Our detection architecture is compatible with bead-less techniques, in which the sequencing libraries are clonally amplified directly on the surface of the chip. All steps of the sequencing process could be automated in a single instrument.

In the past two decades, a few DNA sequencing technologies have been developed and made commercially available. Currently, the vast majority of DNA sequence data is generated using optical based approaches. Dramatic improvements in throughput have made it possible, for example, to re-sequence a human genome at a cost approaching $1000 per sample. However, these sequencing runs are carried out on large, expensive instrumentation with high per run costs. Though a reduction in cost per base has been enabled by extremely high output runs and multiplexing of multiple samples into a single run, the instrument price and run price is still out of reach for many individual researchers.

The breakthrough innovation of Genapsys' platform is the steady-state electrical detection of base incorporations. Combined with fully integrated CMOS chips, the system is small, accessible, and affordable. Its flexible architecture allows the system to be highly scalable, with potential throughputs ranging from one million to hundreds of millions of sensors, all on an instrument with the same small footprint and ease of use. The detection modality can also be extended to other types of nucleic acids, proteins, and live single cells using the same platform. We believe our sequencing technology will ultimately extend the reach of the power of genomics to a wider audience and a wider range of applications.

Materials and Methods:

Library Preparation:

Genomic DNA samples were purchased from various commercial sources. A table of genomic DNA samples used in this study can be found in Supplementary Table 1. Sequencing libraries were generated by random shearing of genomic DNA followed by ligation of custom adapters using the Kapa HyperPrep Kit (Roche). Briefly, approximately 1 μg of genomic DNA was acoustically sheared to ~200 bp using a M220 Focused-ultrasonicator (Covaris). Sheared DNA was end repaired, A-tailed, and ligated to custom adapters (Integrated DNA

Technologies) using the Kapa HyperPrep Kit per manufacturer's recommendations. Ligated fragments were purified with AMPure XP beads (Beckman Coulter) and size-selected on a Pippin Prep (Sage Science). Size-selected libraries were PCR amplified using the Kapa HiFi PCR Kit (Roche) and purified using AMPure XP beads. The qualities of final libraries were assayed using a Bioanalyzer High Sensitivity DNA Kit (Agilent).

Target Capture and Multiplex PCR Amplicon Panels:

Targeted sequencing libraries were enriched from standard sequencing libraries using xGen Lockdown Panels (Integrated DNA Technologies). Genomic libraries from the NA12878 cell line (Coriell) were enriched with the xGen Exome Research Panel v1.0, and libraries from the Horizon Quantitative Multiplex Reference Standard gDNA (HD701) were enriched with the xGen Pan-Cancer Panel v1.5. Enrichment was carried out per manufacturer's recommendations using the xGen Hybridization and Wash Kit (Integrated DNA Technologies) and custom xGen Blocking Oligos complementary to our custom adapter sequences. Enriched libraries were subsequently amplified using the Kapa HiFi PCR Kit and purified using AMPure XP beads.

Multiplex PCR Amplicon panels for Cancer were generated using the Ion AmpliSeq Cancer Hotspot Panel v2 (ThermoFisher Scientific). Multiplex PCR was performed on 10 ng of genomic DNA from the HD701 reference standard, followed by partial digestion of primer sequences by the FuPa reagent, according to the manufacturer's recommendations. The amplified products were purified using AMPure XP beads, end-repaired, A-tailed, and ligated to custom adapters as above. Ligated fragments were amplified by PCR and purified using AMPure XP beads, to give rise to the final library.

Clonal Amplification and DNA Sequencing:

Library molecules were clonally amplified onto beads following manufacturer's recommendations (GenapSys). Clonally amplified and enriched beads were mixed with reference beads prior to addition to the sequencing chip. Reference beads are amplified beads that have been annealed with a 3' blocked sequencing primer. Beads were loaded onto the sequencing chip which has an array of sensors, each capable of capturing one bead. GenapSys Sequencing polymerase was injected into the chip and incubated for 5 minutes to allow for binding to the DNA templates per manufacturer's recommendations.

Sequencing begins with electrical calibration of the chip, followed by serial injection of individual nucleotide buffers. For each injection, the chip was incubated for several seconds to allow for nucleotide incorporation, then the electrical signal of individual sensors was captured. The chip was washed, and this process was then

repeated using a different nucleotide. To sequence 150 base pairs, approximately 250-300 nucleotide flows were required.

Signal Processing and Base Calling:

Sequence data was generated using the Genapsys analysis pipeline. The initial step of data analysis was sensor identification where sensors with beads are differentiated from those without a bead based on initial sensor calibration (Supplementary Figure 7A). The population of sensors with bead were further grouped into sequencing and reference based on the characteristics of their measured signal over a predetermined number of nucleotide flows (Supplementary Figure 7B). Reference sensors are those with blocked primer beads and were used to subtract the background noise from the sequencing sensors' signal.

Base calling was carried out using the GenapSys proprietary base calling pipeline. Data from each sequencing sensor for each nucleotide flow was evaluated relative to the reference sensors. The base caller was designed to identify the number of incorporations in the case of homopolymer sequence stretches. For every base call, a set of predictor measures were computed that were used as input into a pre-trained Phred quality table (12) in order to obtain a quality score.

Sequence Alignment and Variant Detection:

The reference genomes were indexed using BWA (v0.7.17) and BWA-MEM was used to align the single-end reads to the reference genome (22). Sequencing reads were aligned to the hg38 reference genome using BWA-MEM. To call variants, BCFtools and DeepVariant (20) were used. The 'mpileup' command in BCFtools (v1.9) was used to generate the genotype likelihoods at each genomic position with sufficient coverage. The BCFtools 'call' command was then used to call the variant sites using default settings. The BCFtools 'isec' command was used for variant evaluation. Additional variant analysis was carried out using the DeepVariant (v0.7.2) approach (20). The pre-trained DeepVariant model from Google was further trained on the GenapSys NA12878 exome dataset with 60% training, 20% cross-validation, and 20% test.

Data Availability:

Sequence data has been deposited into the Sequence Read Archive (SRA) under BioProject accession number PRJNA529876.

Supplementary Material:

Supplementary Figure 1: Steady-State Signal of Individual Base Incorporations

Supplementary Figure 2: Error Types and Q-Score Distribution

Supplementary Figure 3: Base Call Length Distribution

Supplementary Figure 4: Microbial Genomes

Supplementary Figure 5: DeepVariant Analysis of SNPs in NA12878 Exome Data

Supplementary Figure 6: Low Frequency Variant Read Visualization

Supplementary Figure 7: Sensor Identification and Active Sensor Determination

Supplementary Table 1: Genomic DNA Samples

Supplementary Table 2: Targeted Sequencing Statistics

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Author Contributions:

Author Information:

The authors declare competing financial interests: all authors are employees or contractors that work for GenapSys, Inc.

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