Multiplex unleashed with Hyperplex PCR™

Validating the 100-Plex Breakthrough

Aplex BIO

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Abstract

The simultaneous, targeted analysis of multiple nucleic acid (NA) sequences is increasingly relevant in biotechnological and biomedical innovations. For infectious and genetic disease diagnostics, gene expression analysis, environmental surveillance, food analysis and beyond, two primary families of NA detection methods exist: (1) PCR-based (quantitative PCR, digital PCR) and (2) next-generation sequencing (NGS)-based. These methods lie at the extreme ends of the efficiency-performance tradeoff spectrum. PCR-based methods offer highly sensitive and fast analysis but are typically limited to five or fewer targets per reaction due to multiplexing constraints, while NGS-based methods can measure thousands of targets with single-nucleotide specificity but with slower turnaround times and higher costs. This creates a gap in bioanalytical methods for detecting 10-100 RNA/DNA targets, with reliable NGS-grade specificity and dPCR-grade sensitivity.

Hyperplex PCR™ (hpPCR™), developed by Aplex Bio, addresses this gap by allowing multiplexing of 100+ targets per tube/well with sequencing-grade specificity, rapid panel customization, and up to single molecule sensitivity. The process remains streamlined, comparable to a qPCR reaction, followed by epifluorescence microscopy imaging-based read-out for same-day results. The use of 8 standard fluorescence filter cubes allows simultaneous distinction of over 100 Nanopixel™ codes, providing precise identification and molecular counting of discrete rolling circle amplification products.

Here serving as a technical demo of a single-tube 100-plex hpPCR assay, we targeted 50 human 1-3 bp indels using 100 padlock probes and 50 primer pairs with amplicon lengths ranging from 107 to 170 bp. Testing the assay with synthetic ssDNA targets, we demonstrate a code identification accuracy of 99.96%, enabling SNP discrimination below a frequency of 0.1%. Specifically, the data shown here for this panel highlights fit-for-purpose performance for several applications such as genetic disease diagnostics and prognostics, pharmacogenomics, personalized medicine and forensic applications. Furthermore, given the simultaneous high linearity and quantitative performance, other challenging applications can be approached, such as cancer mutation profiling and prenatal genetic testing.

The next generation PCR technology – Hyperplex PCR™

Hyperplex PCR™ (hpPCR™) upgrades the standard PCR amplification with 3 key performance improvements compared to legacy quantitative PCR assays. These are (1) increased probing specificity by replacing hybridization-based probes (e.g. TaqMan, molecular beacons) with ligation-based padlock probes (PLPs) which facilitate systematic single nucleotide resolution; (2) increased sensitivity by re-amplifying successfully ligated PCR into single macro-molecules with rolling circle amplification (RCA), enabling molecular counting and; (3) unprecedented multiplexity in a single reaction by probing the RCA products with optically encoded nanoparticle probes designed and synthesized by Aplex Bio – Nanopixels™ - which enable the discrimination of 100+ specific codes resorting to fluorescence microscopy imaging. The four main steps of hpPCR are schematized in Figure 1. The first step comprises a standard end-point multiplex PCR amplification using off-the-shelf enzyme mixtures optionally including reverse transcriptase for 1-step RT-PCR and dUTP/UDG to prevent amplicon-derived cross contamination. The amplicons are then targeted by a panel of PLPs which are subsequently re-amplified by RCA upon successful ligation to the matching templates. The PLPs are uniquely barcoded per target type so that each corresponding RCP type will be equally barcoded with hundreds to thousands of copies of the complementary sequence. Finally, the combination of RCA and Nanopixel probing enables an unprecedented degree of multiplexing from a single sample by specifically targeting the oligo barcodes added to the PLP design by a highly optimized set of optically encoded labels, wherein each RCA product is specifically decorated with tens to hundreds of nanoparticles.

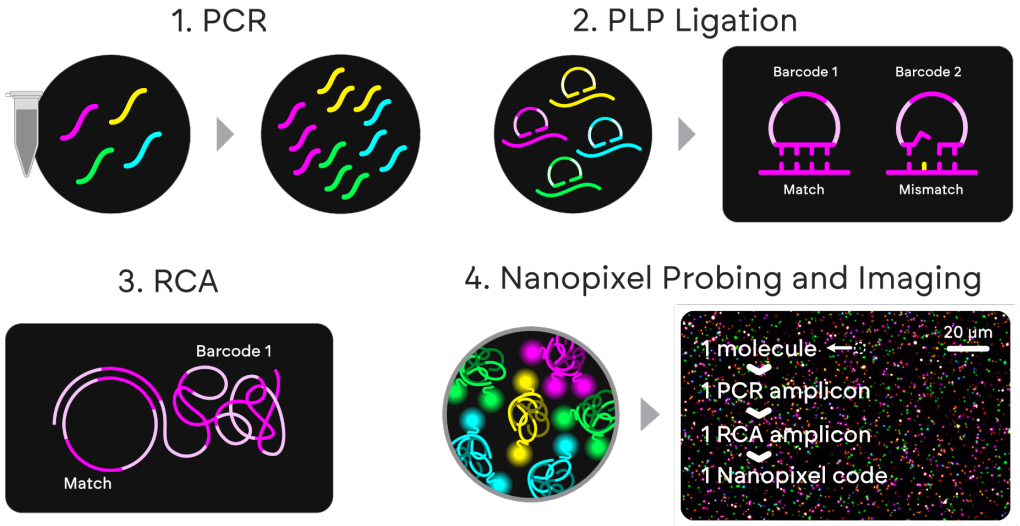


Figure 1. Schematics of the hpPCR assay workflow. After PCR amplification, PLP ligation, RCA and Nanopixel probing, each RCA amplicon corresponds to a single PCR amplicon molecule labelled by a specific Nanopixel code. The number of RCA products of a specific code are proportional to the titers of a certain target PCR amplicon. More than 100 color codes can be detected simultaneously in a single test.

Nanopixels™ - 100-plex per well and beyond

The working principle of the Nanopixel labelling is schematized in Figure 2-A. The Nanopixel labelling solution comprises a combination of nanoparticle labels and an organic fluorophore-based external reference (ER). The purpose of the external reference is to ensure that all measured signals correspond to real RCA products and not to any residual nanoparticle aggregates (nanoparticle signal only) and/or single nanoparticles, since all real RCA products are co-labeled with both nanoparticles and organic fluorophore signal. Each nanoparticle is coated with an oligo complementary to the barcode of each set of padlock probes targeting each sequence of interest. The Nanopixel library thus includes 100 nanoparticle types with unique optical codes which can be used for any set of up to 100 target types via padlock probe barcoding, meaning that the set of 100 Nanopixel probes is universal for any target panel, only the padlock probe 5' and 3'- arm sequences are modified. The working principle of the Nanopixels is based on three layers of fluorescence signal encoding: (1) a core with an internal reference (IR) signal fluorescent in channel C8, (2) a second layer comprising a mixture of dyes fluorescent in two fluorescence channels C1 and C2 and (3) a third layer comprising one fluorophore type fluorescent in one of the channels C3 to C7. The C1/C2 layer encoding is referred to as Intensity modulation (C1 mod and C2 mod), while the C3-C7 encoding is referred to as color modulation (Color – C3-C7 - mod). The optical encoding of each Nanopixel type can be visualized according to the schematics in Figure 2-B. Each nanoparticle type of each of the five color mods C3-C7 contains one out of 20 C1/C2 intensity mod codes, resulting in 100 total combinations. Remarkably, this optical encoding strategy is intrinsically expandable with higher dimensions of intensity mod beyond the current version utilizing only C1-C2, and beyond 100-plex.

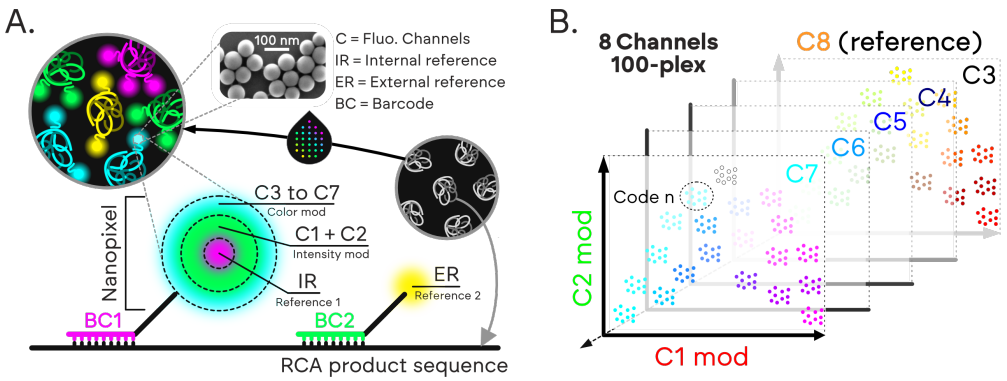


Figure 2. Schematics of the Nanopixel optical encoding (A) and expected 100-plex readout outcome using 8 fluorescence channels C1-C8 (B).

100-plex hpPCR panel for Human identification

To demonstrate the potential of a 100-plex hpPCR analysis, we demonstrate here a Human identification panel (in development) based on targeting 49 common mutations (1-3 bp indels) having a frequency of 40-60% in all ancestral groups and a 50th male/female sex marker based on Y-chromosome identification. Indels are short DNA sequences that are either inserted or deleted at specific locations in the genome. These variations are abundant across the human genome and exhibit a high degree of polymorphism, making them valuable markers for human identification. Unlike short tandem repeats (STRs), which depend on repetitive DNA sequences, Indels offer a simpler and more robust system for analysis directly compatible with PLP-based genotyping. Furthermore, the binary nature of Indel markers (presence or absence) allows for straightforward genotyping, reducing the potential for ambiguity in results. One of the primary advantages of using Indels in human identification is their applicability to degraded DNA samples. Indels are typically short in length, which makes them less prone to fragmentation and more likely to be amplified successfully in PCR-based methods. This attribute is particularly useful in forensic investigations where DNA samples may be highly degraded due to environmental exposure. The summarized hpPCR workflow and probe design strategy is schematized in Figure 3. The assay includes a multiplex PCR step with 50 primer pairs (average amplicon length of approximately 130 bp), carried out using a standard thermal cycler, followed by a 100-plex PLP probing step and 100-plex fluorescence microscopy Nanopixel readout.

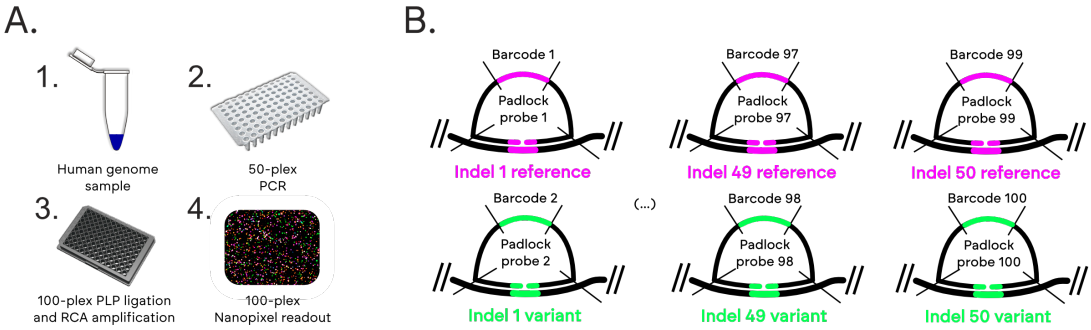


Figure 3. Overview of the 100-plex hpPCR Human ID assay concerning (A) assay steps and (B) PLP design.

Nanopixel probing enables >99.9% accurate 100-plex identification of RCA products

To demonstrate the Nanopixel probing performance, the assay was run by spiking ssDNA targets in solution with a sequence matching the 100 targets with lengths ranging from 100 to 168 bp. The readout of all 100 targets in the C1/C2 intensity modulation scatter plot is shown in Figure 4-A, which contains the expanded 100 targets visualized by separating the intensity modulated map into each of the color modulated codes C3 to C7. To demonstrate the read-out quality of the intensity modulation of the Nanopixel probes, five targets were added at a time, each set sharing the same C1/C2 code out of 20 total code regions (Figure 4-C). In all cases, all 100 PLPs and all 100 Nanopixel types were used for probing. Each dot of the scatter plots represents a single RCP found with a certain measured C1/C2 intensity mod. The visible clusters and total counts in each of the 20 top plots represent the expected C1/C2 intensity for each set of 5 targets. The number "other codes" refers to the sum of signals found in all non-specific 19 C1/C2 code regions. Considering the measured counts in all spiked target combinations, it is possible to obtain an estimation of the target identification accuracy as high as 99.96% considering a pair of specific/non-specific targets, meaning that the Nanopixel probing accuracy enables the detection of rare target fractions (e.g. Variant Allele Frequency measurements) below 0.1% abundance assuming sufficient probing specificity.

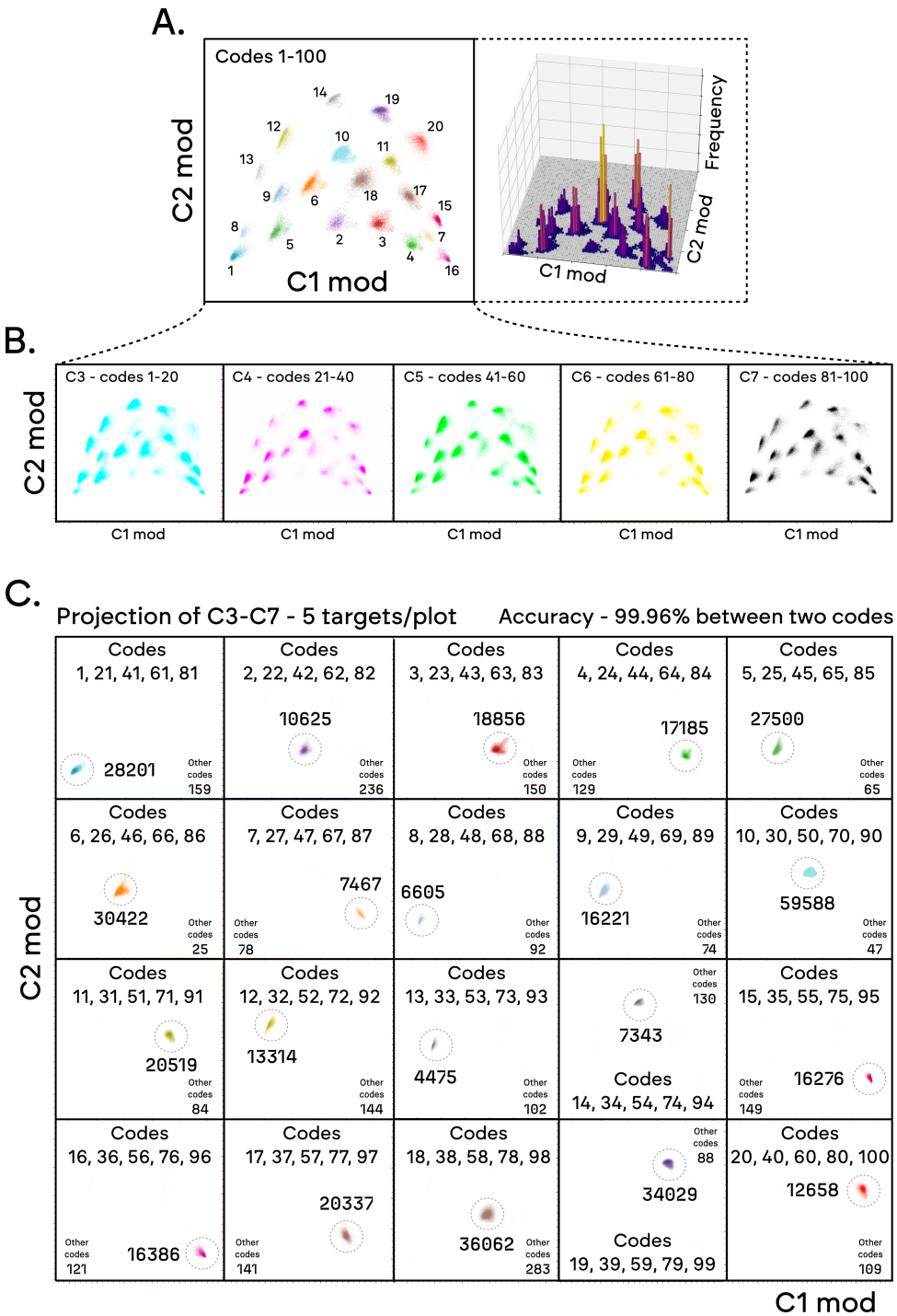


Figure 4. 100-plex target detection and accuracy of readout using intensity modulated Nanopixel probes. (A) C1/C2 code region scatter plot obtained for the readout of 100 targets, highlighting each of the 20 intensity modulated code regions. 3D histogram shows an alternative visualization of the data highlighting the frequency of counts found within each position in the C1/C2 code space. (B) Deconvolution of the intensity and color modulated codes highlighting the counts of all 100 targets measured simultaneously. (C) C1/C2 Intensity mod maps after Nanopixel probing of solutions containing targets 1-100 spiked in sets of 5 each sharing a common intensity modulated code C1/C2. For all results, all 100 PLPs and all 100 Nanopixel types were used to probe the input targets. The data in all plots in A-C correspond to RCPs counted in a total imaging area of approximately 3 x 3 mm^2. The positions in the C1/C2 plot vary according to the C1/C2 intensity mod measured in each RCP. Within each plot, the number of expected (correct C1/C2 code region) and non-expected (sum of remaining 19 C1/C2 code regions) RCP counts are provided per set of 5 targets. An average accuracy of 99.96% was obtained for the identification of the correct code vs the average counts of a non-specific code.

Exceptional specificity enabled by Padlock probes

The 100-plex hpPCR Human ID assay relies on the single nucleotide specificity provided by PLP ligation using high fidelity ligase enzymes [1-3]. The results shown in Figure 5-A demonstrate probe specificity as combinatorial 50-plex assays comprising all 50 PLPs of each Indel type in the presence of the corresponding complementary or non-complementary targets. In all 4 cases, all 100 Nanopixel probes were added in the labelling. The probing specificity calculated as the percent ratio of expected to non-expected counts in the presence of complementary or non-complementary targets of each set of 50 PLPs – shown in Figure 5-B as black bars – was on average above 99%, and for most cases limited by the Nanopixel probing specificity according to the results in Figure 4. These measurements validate the fit-for-purpose performance of the 100-plex PLP probing to accurately identify a panel of select human indels.

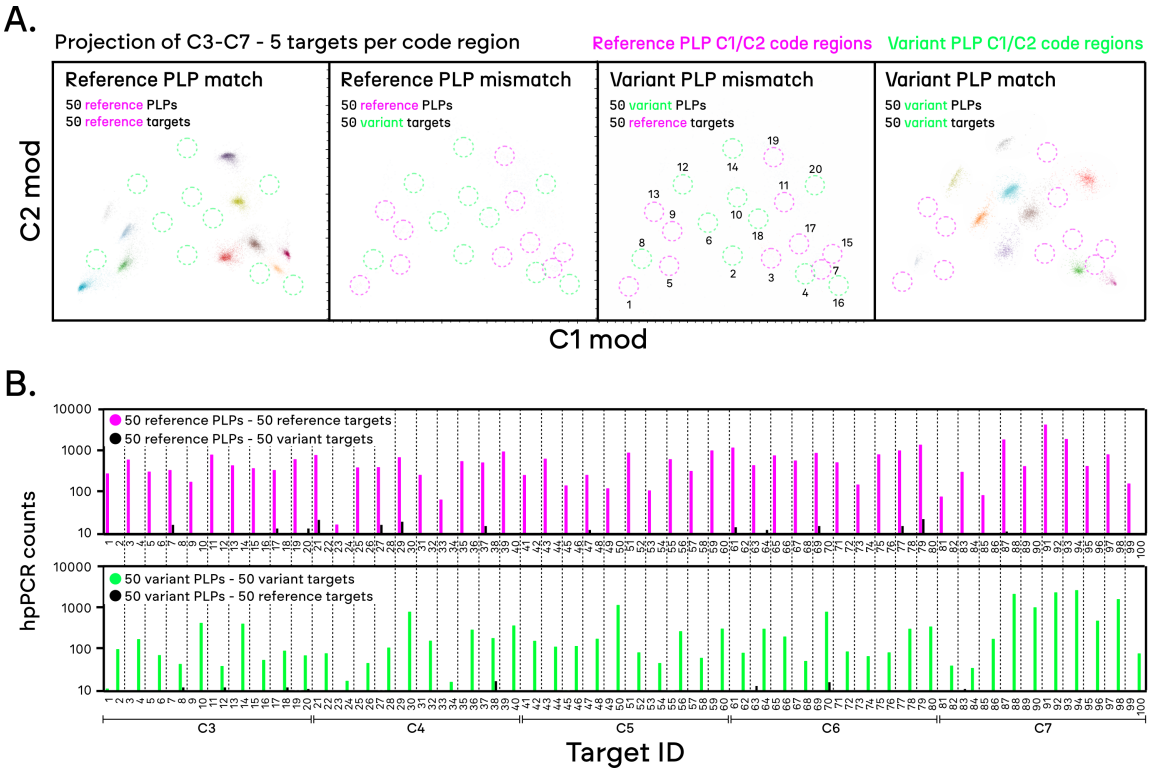


Figure 5. 100-plex genotyping of 50 pairs of reference and variant human Indels. (A) C1/C2 intensity modulation plots obtained after spiking four combinations of 50 targets and 50 PLPs in on-target or off-target configurations. "Reference" and "variant" refer to each of the two Indel types at each variant locus. Each colored set of circles (C1-C2 code regions 1 to 20) corresponds to expected locations for RCP codes of "reference" or "variant" Indel sequences. Each C1-C2 code region 1 to 20 contains five C3-C7 targets of the reference sequence for odd number codes and variant sequence for even number codes. The set of all pairs of targets (2n-1) + 2n until 99+100 comprise the total 50 pairs of reference/indel probes. (B) Quantification of specific (red or green bars) and non-specific (black bars) signals obtained in each of the 4 tested combinations of 50 PLPs and 50 target sequences.

1 million + counts per well and internal controls enable high quantification precision

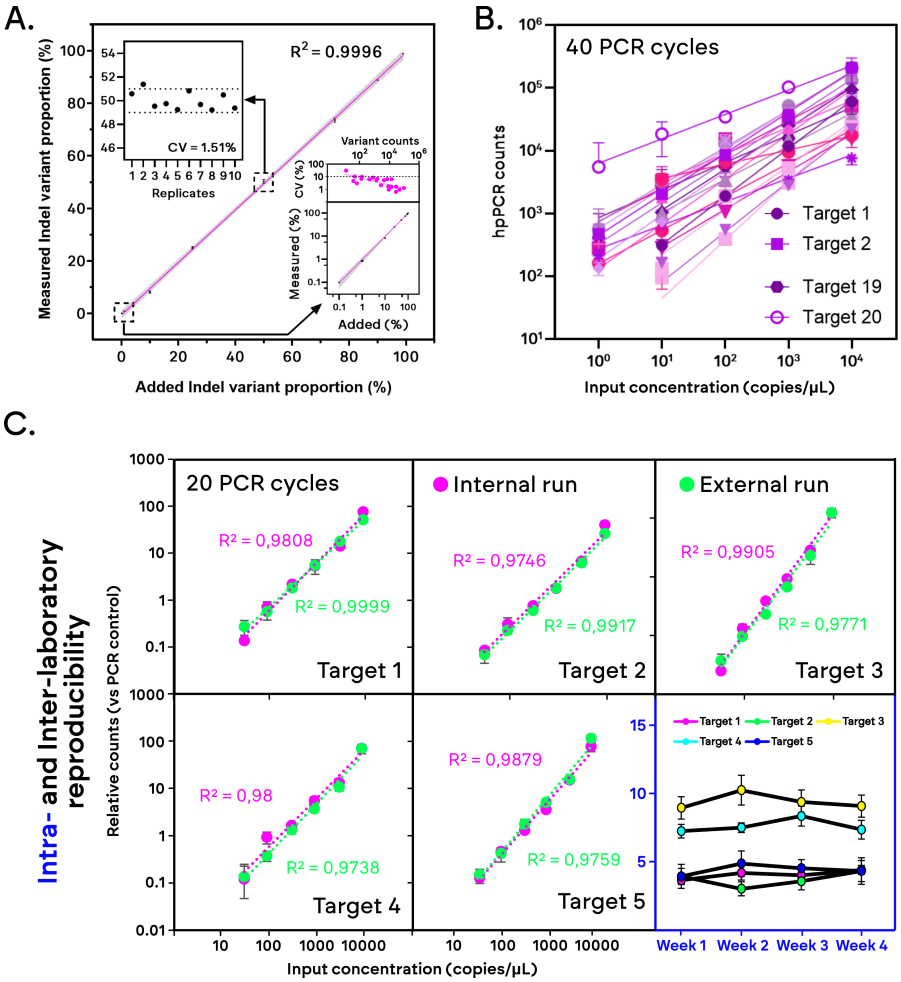
To enable high multiplexity, particularly combined with high precision and sufficient quantitative dynamic range, it is first imperative to provide a sufficient total number of RCA product signals. Typically, up to around 1,000,000 counts can be obtained in a 4 x 4 mm imaging area, roughly equivalent to the available surface per well in a standard 96-well plate format and limited on the upper end by the maximum signal crowding allowing accurate readout of codes from individual RCPs. The sections above focused on the specific application of 100-plex to achieve a binary detection of 100 Indel targets, which is fit-for-purpose for human identification. Here, we use the same targets to further demonstrate the quantitative performance of the assay, further expanding its applicability to other high impact and challenging bioanalytical assays such as cancer diagnostics via quantification of mutation frequency, pharmacogenomics, transplantation monitoring and non-invasive neonatal diagnostics. For this demonstration, we focus primarily on the most technically challenging subset of 20 targets (C1-C2 code region), which are discriminated using only the novel intensity modulated codes enabled by the Nanopixel probes.

The results in Figure 6-A show the relative quantitative performance (i.e. variant proportion) for one pair of reference-variant targets, including an analysis of the impact of the total number of measured counts of the lowest fraction (e.g. variant) on the measurement precision as CV (%). The data was obtained spiking a total of 2400 copies in solution at different reference/variant sequence proportions ranging from 0.1% variant to 99% variant, analyzed with 20 cycles of amplification and resulting in approximately 100.000 counts within the imaging area. Under these conditions, the CV for all measured conditions down to 0.1% variant was below 7%. By modulating the total imaging area, it was concluded that at least about 60 counts for the lowest fraction, i.e. 0.1% variant with 60.000 counts total are required for a CV below 10%. Such observation extrapolates to a total of about 3 million total counts being required

to achieve the 100-plex analysis of 50 mutations with a sensitivity down to 0.1%. The results in Figure 6-B show the performance of the assay in terms of absolute quantification and sensitivity for serially diluted inputs of 20 targets in a 20-plex assay. For most tested primer pairs, an absolute sensitivity below 1 copy/µL was obtained with a dynamic range up to 4 logs.

The results in Figure 6-B further highlight two important features of hpPCR: (1) extremely low copy inputs below can generate hundreds or thousands of measurable counts, thus maximizing the sensitivity of PCR assays beyond digital PCR and (2) due to differences in PCR amplification and PLP ligation yield, a calibration of input copies vs hpPCR counts is needed to provide absolute quantification. Remarkably and importantly, regardless of intrinsic PCR amplification and ligation yield of each set of primers and PLP, the correlation of counts vs input copies remains linear. This observation highlights that common concerns related to PCR bias become negligible in cases where the assay is sufficiently sensitive to cope with a wide range of PCR efficiencies.

Furthermore, despite the requirement of a calibration curve to achieve quantification and unlike qPCR, hpPCR provides a different type of absolute quantification in which several internal assay controls can be used to ensure assay repeatability taking advantage of the high degree of multiplexing. These controls can include (1) exogenous universal PCR controls to normalize for variability in thermocycler equipment and matrix interference, (2) endogenous PCR controls for relative normalization, (3) ligation-only controls added during the ligation step to control surface capture yield. Both types of controls 1 and 3 are added by default in hpPCR. The value of such controls is demonstrated in Figure 6-C, highlighting the intra- and inter-laboratory reproducibility of a 6-plex assay including a universal PCR control. When the hpPCR counts are normalized to a universal PCR control, no statistically significant differences (t-test, p-value > 0.05) were observed for (1) all 5-targets measured in two different laboratories in Sweden by two different users and (2) measurements performed in the same lab, by the same user weekly over the course of 4 weeks.



Added variant proportion (%)	Measured variant proportion (%)	CV (%)	n
0,1	0,097	4,05	3
1	0,848	6,94	3
10	8,155	5,49	3
25	25,048	1,61	3
50	50,248	1,51	10
75	73,620	1,09	3
90	88,813	0,08	3
99	98,799	0,04	3

Table 1. Analytical Parameters of hpPCR for variant proportion detection and quantification. Summary of dilution series data in Figure 6-A for reference and variant ssDNA targets, highlighting variant proportions, assay replicates and CV values.

Figure 6. Analytical performance of hpPCR for mutation frequency and absolute target quantification. (A) Dilution series of mixture of a pair of "reference" and "variant" pre-PCR synthetic target ssDNA at different proportions ranging from 0.1% variant to 99% variant proportion. For 50%, 10 independent hpPCR assays were performed, while the remaining points were measured in triplicate. The shaded area corresponds to 99% confidence interval. All measurements down to 0.1% mutant proportion had a CV below 7% when approximately 100.000 total counts are obtained for the pair of targets. The total input of reference and variant target sequences was 2400 copies (1200 copies each for 50%) and 20 amplification cycles were used for the hpPCR assay. The CV(%) vs variant probe counts highlight that approximately 60 total counts for the lowest target fraction are required to provide a CV below 10%, i.e., approximately 60.000 counts per target pair for a sensitivity of 0.1% VAF. (B) Dilution series of 20 ssDNA synthetic target sequences measured simultaneously with 40 cycles of amplification, each corresponding to a different C1/C2 intensity modulated code. A total of 2 µL sample in 10 µL PCR mix were used. Error bars correspond to ±SD (n=2). (C) Intra- and Inter-laboratory hpPCR reproducibility using a 6-plex tester assay. Five targets vary in concentration ranging from 30 to 9000 copies/µL and the 6th target is used as a PCR universal control kept constant at 1000 copies/µL. All results as plotted as the hpPCR counts of each target divided by the hpPCR counts measured for the universal PCR control. 20 cycles of amplification were used for these measurements. Error bars correspond to ±SD (n=3). The intra-lab reproducibility plot in blue refers to the same readout of all targets spiked at 900 copies/µL measured weekly against the PCR universal control over the course of 4 weeks.

The quantitative performance of the assay was further evaluated for the expanded 100-plex assay. The measurement precision is shown in Figure 7-A for 100 targets measured simultaneously, followed by a log2 dilution series (Figure 7-B). For 20 targets with a common C3-C7 color code, we further demonstrate a titration of variant/reference proportion input where the nearest neighbor C1/C2 intensity modulated codes varied from 2 to 100% abundance (Figure 7-C). These results show comparable performance to the lower plex analysis demonstrated in Figure 7, highlighting the precision of Nanopixel encoding via the negligible interference from neighboring codes in the output readout.

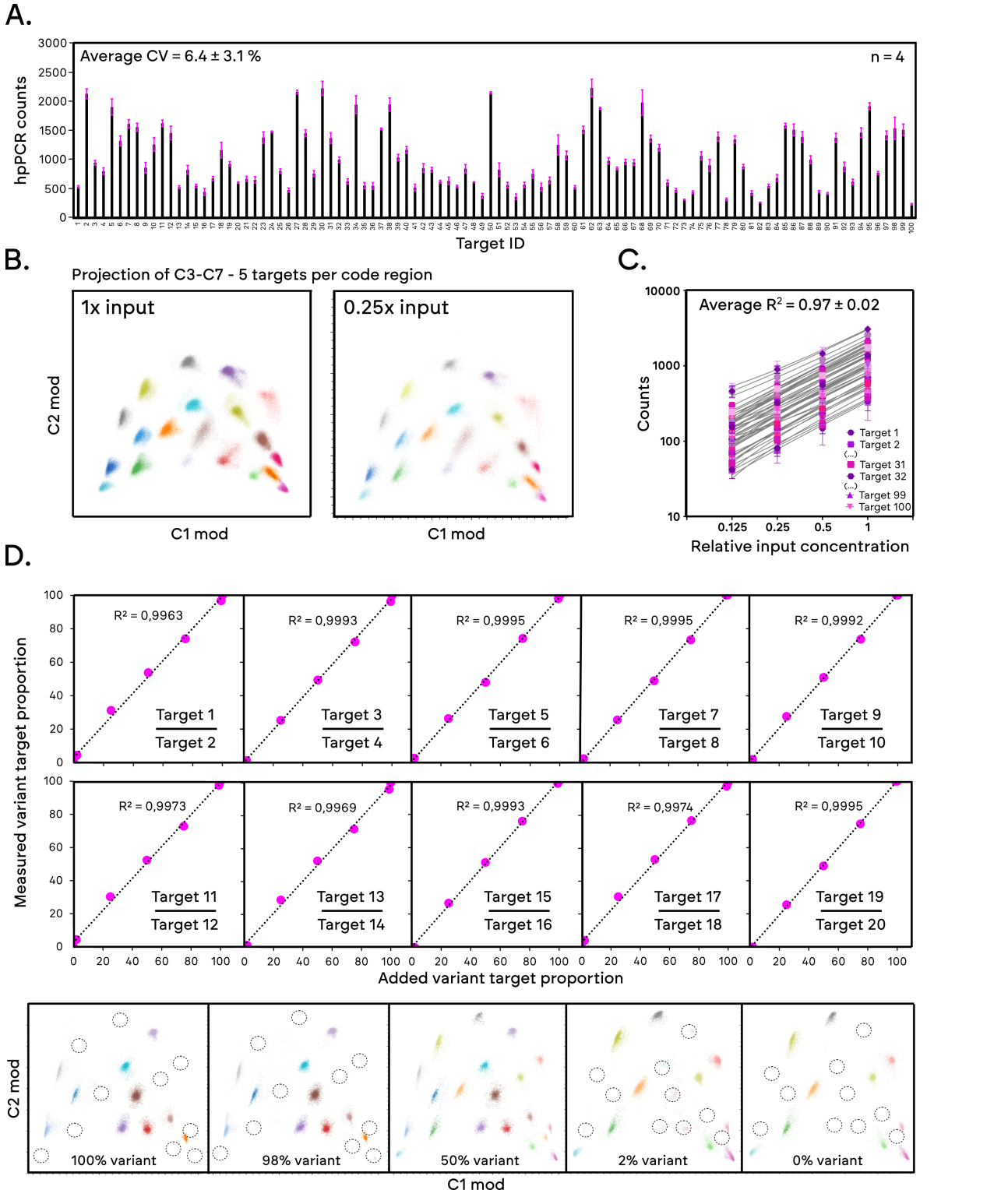


Figure 7. 100-plex hpPCR repeatability and quantification precision. (A) Repeatability of 100-plex detection of 100 synthetic ssDNA target sequences. Replication refers to independent technical repeats of hpPCR. Error bars refer to ±SD (n=4) with an average CV of 6.4%±3.1%. (B and C) C1/C2 intensity modulation plots and hpPCR counts measured for a log2 dilution series of all targets. (D) Dilution series of 10 reference-variant target pairs highlighting the quantification precision of mutant proportion and the detection of rare targets shown as C1/C2 intensity modulated plots, wherein the dotted circles highlight regions of absent targets or containing residual counts corresponding to 2% of reference or variant target proportion.

Conclusions

The data compiled in this white paper demonstrates the following key performance features enabled by Hyperplex PCR (hpPCR) as a next-generation PCR technology:

- Target identification accuracy of more than 99.96% measuring 100 targets simultaneously in a single reaction tube.
- Point-mutation probing specificity enabling mutation frequency quantification down to below 0.1% with high precision below 2% CV.
- Possibility of adding multiple internal controls to neutralize PCR-derived bias and intrinsic variability, ensuring intra and inter-lab reproducibility.
- High linearity and single copy sensitivity with minimal optimization efforts.
- High quantification precision and 4-log+ dynamic range of pre-PCR target copy input enabled by having more than 1 million counts per well.

References

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