High Throughput Library Preparation Enhancements for Genotype by Sequencing using Low Pass Whole Genome Screening in Animal and Plant Studies

Abstract and Study Goals:

Low-pass whole genome sequencing is a technique where each base in the genome is sequenced multiple times and is now a preferred tool in agricultural research. Agricultural genomes often have a wider variety of species and are much larger than human genomes. The average depth of coverage is less than 5 times, allowing many genomes to be sequenced at once. Reducing the coverage depth lowers sequencing costs. This method detects known SNP markers and novel content like repeats, non-coding sequences, and transposable elements.

However, the drop in sequencing costs has shifted most expenses to library preparation, including DNA quantification, purification, and balanced pooling, which require significant time, manpower, and resources. One challenge is maintaining even sample representation due to dropout or over-sequencing.

We present improvements in low-pass whole genome sequencing using the iconPCR system. This method provides balanced, pooled libraries at a fraction of the cost by removing over 66% of the manual steps in library preparation. AutoNormalization allows for flexible input DNA amounts, sample-specific amplification, consistent yields, and single-tube SPRI purification. This reduces the need for manual pooling and liquid-handling robots.

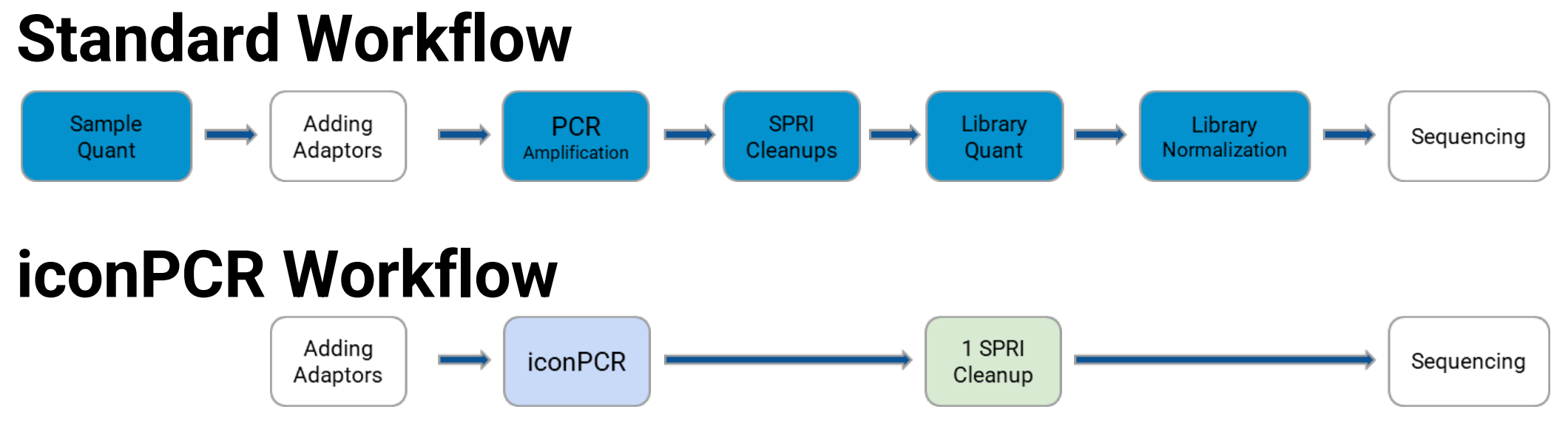
The system uses a 96-well real-time thermocycler, where each well is independently controlled. Libraries are amplified to the target level, and cycling stops once the required concentration is reached. The 96-well plate is combined into a single multiplex pool and purified in one step, reducing labor-intensive processes like purification, quantification, and manual mixing.

Streamlining Library Prep to Match Ultra-High Throughput Sequencing

By implementing iconPCR, this study shows how elimination or drastic reduction in individual library prep steps. A wide range of input mass can be used without labor-intensive steps to manage the variability. Analysis of the amplification plots can identify sub-optimal amp, dropouts and reagent quality issues.

Workflow Improvements:

- Elimination of input mass normalization
- Uses existing consumables and reagents
- Massive reduction in pipetting steps and tips
- Reduced error opportunity
- Single plate amplification vs. 3 different runs
- >90% reduction in hands-on-time for SPRI
- AutoNormalized libraries ready for sequencing



Standard Workflow

Sample Quant → Adding Adaptors → PCR Amplification → SPRI Cleanups → Library Quant → Library Normalization → Sequencing

iconPCR Workflow

Adding Adaptors → iconPCR → 1 SPRI Cleanup → Sequencing

Simplified workflow using standard reagents and consumables

96—plex Droplet-Emulsion PCR (dePCR) Preparation of WGS Libraries

Study Goals:

To assess workflow optimization and library preparation efficiency, a 96-reaction study was designed to evaluate drastic reductions in the hands-on-time and manipulation steps using liquid handling robotics. In this study, DNA from the Genome in a Bottle sample HG006 (NA24694) was prepared in a dilution series from 10 ng to 120 ng input quantities in a 96-well plate using 8 replicates per concentration. A Control plate was prepared and processed through the standard amplification workflow. In parallel, replicate plates were processed using the iconPCR system to identify optimal Auto-Normalization settings.

Control Plate Setup: The Control plate was split by input quantity and amplified in three separate thermocycler runs to enable different levels of amplification.

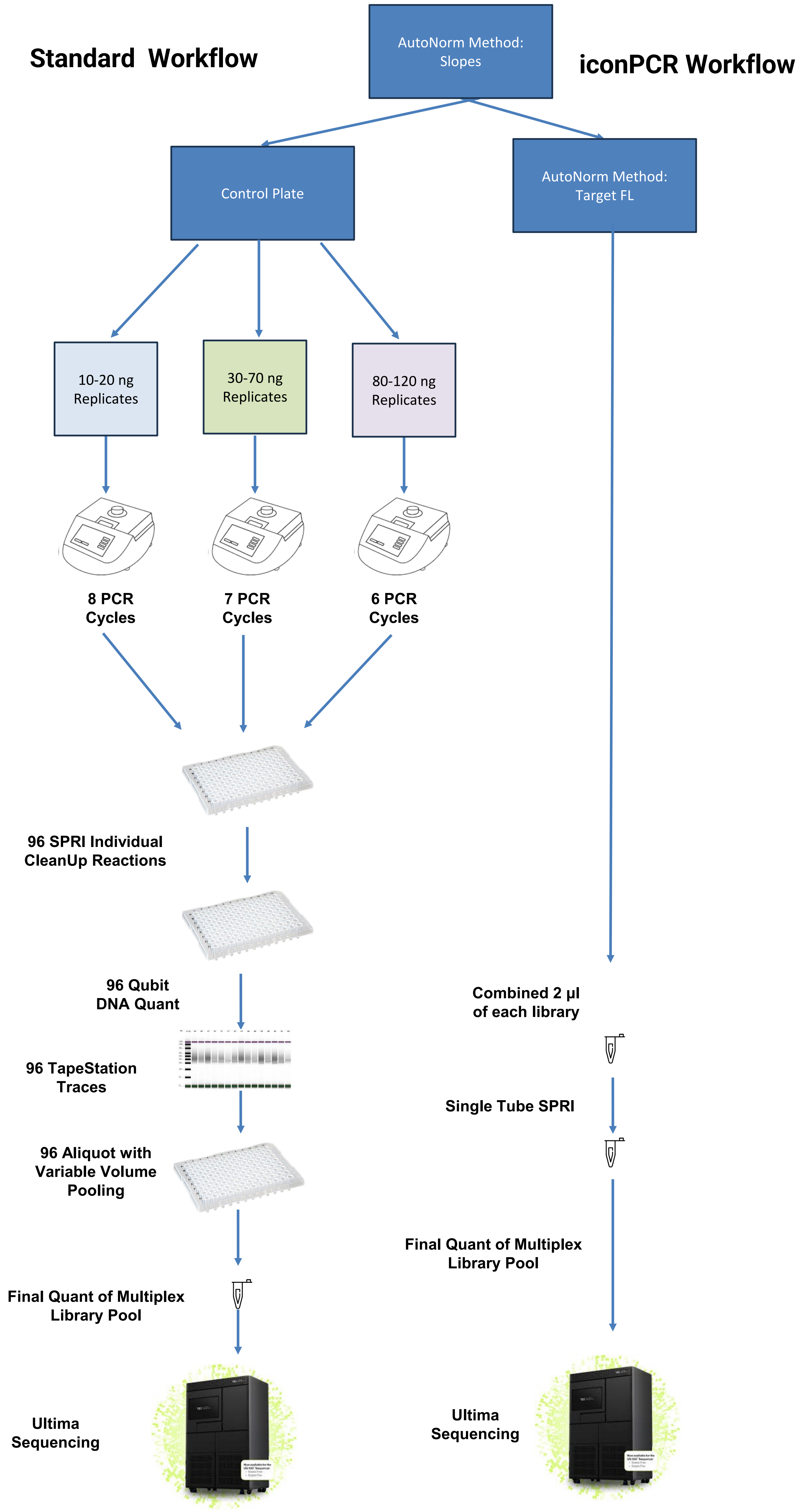
Post-amplification, samples were combined into a single 96-well plate for processing on a Hamilton liquid handling robot.

Control Samples: Input vs. # PCR Cycles

8 PCR Cycles		7 PCR Cycles					6 PCR Cycles				
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng

IconPCR Plate Setup: Using a Determination run, the optimal thresholds were selected for all three AutoNorm methods: Slope, Target Fluorescence (FL) and xBaseline. Three plates were amplified with the different AutoNorm methods to demonstrate as options for variable yields and AN levels. The Target FL method was chosen for sequencing and comparison to the Control.

Library Prep and Sequencing Summary Schematic



Standard Workflow

iconPCR Workflow

AutoNorm Method: Slopes

Control Plate

AutoNorm Method: Target FL

10-20 ng Replicates

30-70 ng Replicates

80-120 ng Replicates

8 PCR Cycles

7 PCR Cycles

6 PCR Cycles

96 SPRI Individual CleanUp Reactions

96 Qubit DNA Quant

96 TapeStation Traces

96 Aliquot with Variable Volume Pooling

Final Quant of Multiplex Library Pool

Ultima Sequencing

Combined 2 µl of each library

Single Tube SPRI

Final Quant of Multiplex Library Pool

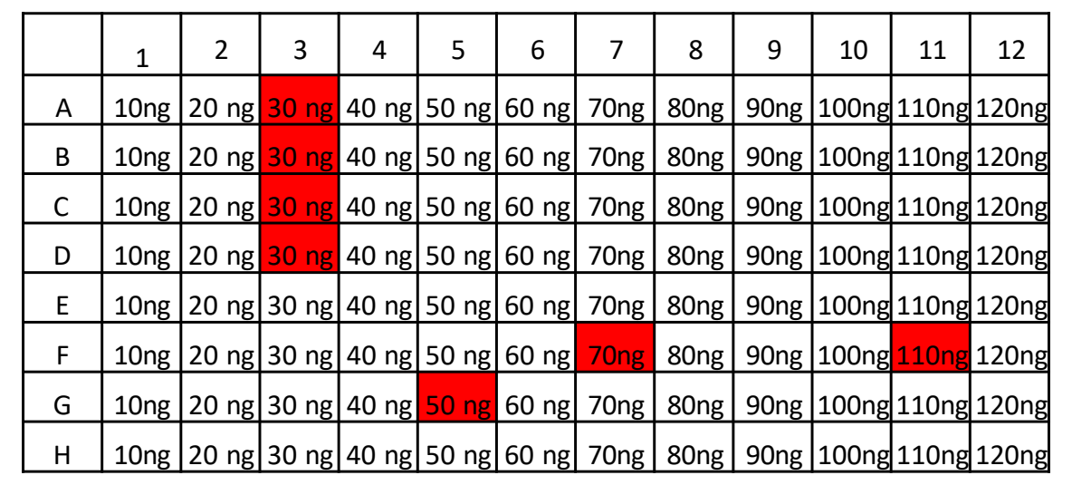
Ultima Sequencing

Simplification Reduces Pipetting Error Opportunity: iconPCR can Rescue Hyper-Variable Challenges

Sample Dropouts:

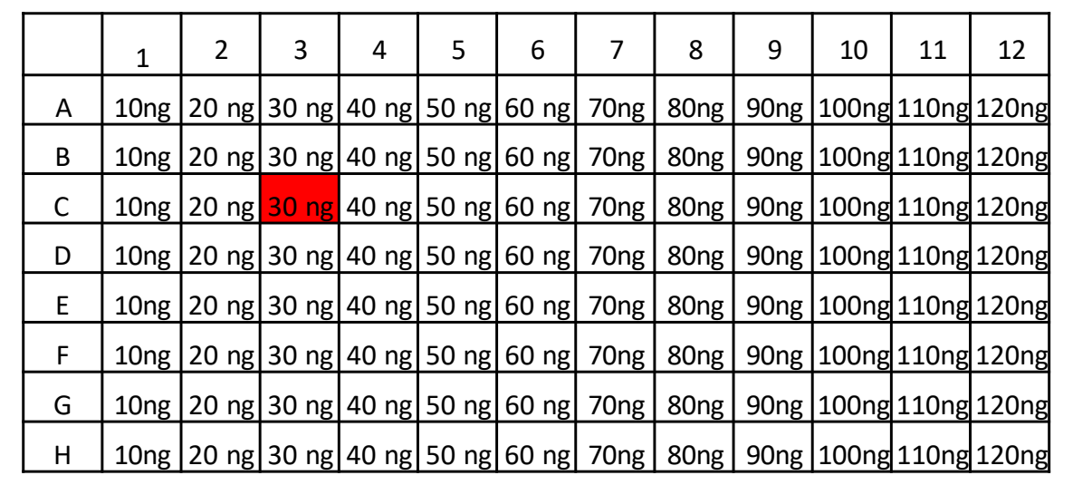
Both the Control and iconPCR stamped plates showed a high degree of variability in the 30-ng replicate column indicating a technical pipetting error. The Control plate recording 4 replicate dropouts in the 30 ng column plus 4 random dropouts across the plate. The iconPCR plate showed high variability however, AutoNorm was able to rescue 3 of 4 dropouts from Column 3 and had no random dropouts across the plate.

Control Workflow



	1	2	3	4	5	6	7	8	9	10	11	12
A	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
B	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
C	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
D	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
E	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
F	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
G	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
H	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng

iconPCR



	1	2	3	4	5	6	7	8	9	10	11	12
A	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
B	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
C	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
D	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
E	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng
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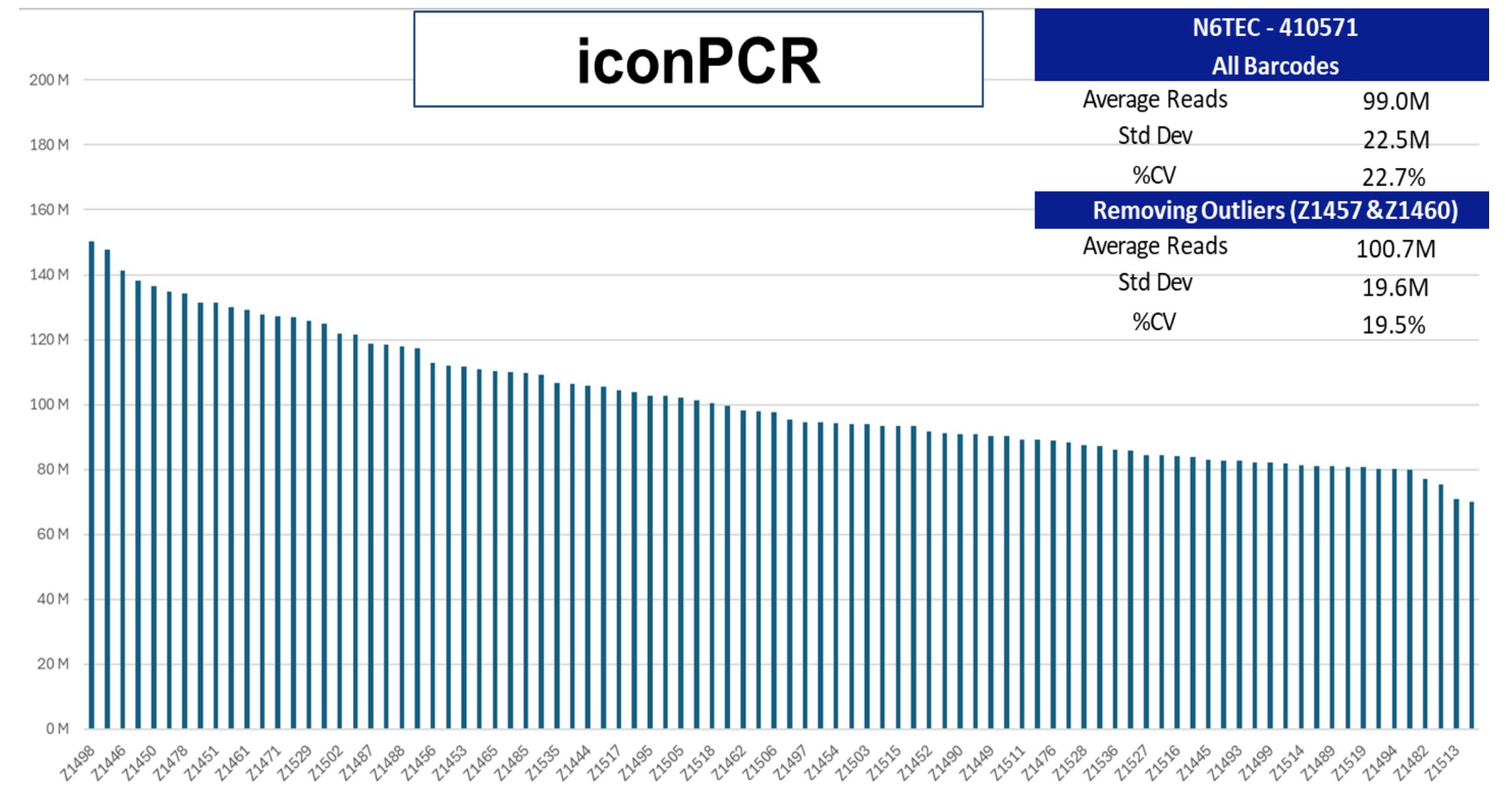
H	10ng	20 ng	30 ng	40 ng	50 ng	60 ng	70ng	80ng	90ng	100ng	110ng	120ng

Library Balancing using Manual Normalization vs iconPCR AutoNormalization

Both the Control multiplex library and the AutoNormalized iconPCR pool were shallow-sequenced using a 2x150 bp sequencing run. The quantity of each library was measured and represented by each barcoded sample index. Average representation and variation (%CV) are shown for both workflows.

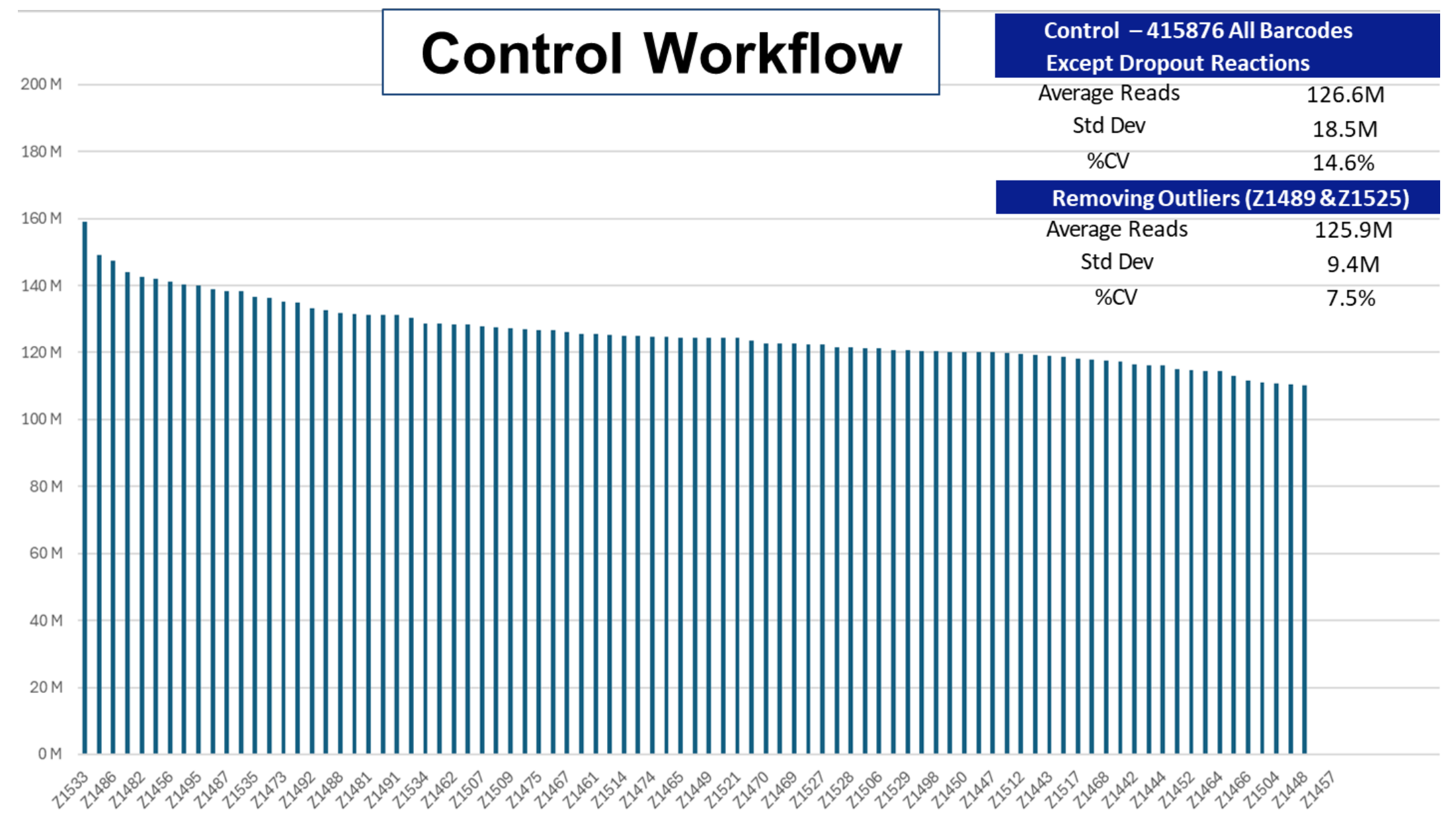


N6TEC - 410571 All Barcodes	
Average Reads	99.0M
Std Dev	22.5M
%CV	22.7%
Removing Outliers (Z1457 &Z1460)	
Average Reads	100.7M
Std Dev	19.6M
%CV	19.5%

Pooling of the AutoNorm Run:

The multiplexed sequencing library was created by pooling 2 µl of each reaction. This method will reflect slight variations within the final concentration and is the most course method of pooling reflecting in up to 1 Ct difference between samples.

Refined balancing can be achieved by using the iconPCR Pooling by Fluorescence (FL) method. This uses the final RFU values from each well to adjust the volume of each sample.



Control – 415876 All Barcodes Except Dropout Reactions	
Average Reads	126.6M
Std Dev	18.5M
%CV	14.6%
Removing Outliers (Z1489 &Z1525)	
Average Reads	125.9M
Std Dev	9.4M
%CV	7.5%

Pooling of the Control Run:

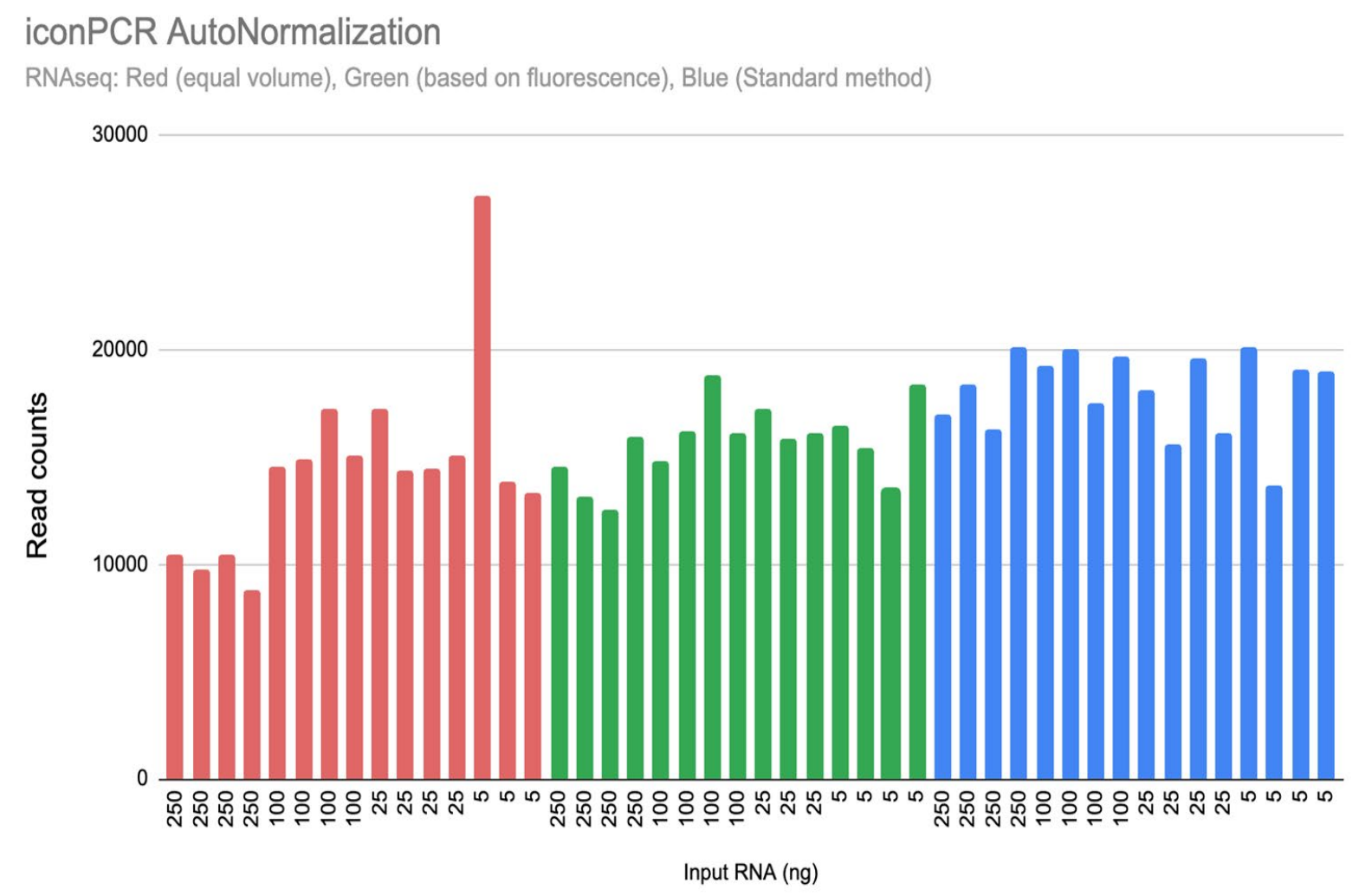
The percent coefficient of variation for the Control run generated a %CV of 7.5% which shows the high precision that reflects the additional time, effort and costs associated with legacy NGS workflows.

Pooling by FL will result in barcode balancing on par with manual pooling

Pooling by Vol versus Pooling by FL (example):

From previous studies, using the final RFU value allows for higher precision for assay types requiring larger sequencing requirements per sample, such as WGS.

For sequencing-light assays, including RNA-seq, 16S and scRNA-seq, Pooling by Volume results provides a simple automation workflow as all samples are aliquoted in the same volume.



Pooling By Vol	Pooling By FL	Individual Purification
13558	14859	18182
4353	1776	1941
30.1%	11.2%	10.7%

Example of Pooling Methods:

A 50X fold dilution series was generated for RNA-seq libraries ranging from 250 ng to 5 ng input. Libraries were amplified using iconPCR AutoNorm and the subsequent material was further processed using the standard purification, quant and pooling of each library (standard) as well as both of n6's recommended methods: Pooling by Vol and Pooling by FL.

Thus, researchers are able to choose between the different methods for the most economical and efficient sequencing option depending on assay type.

Pooling by FL Provides Equal Library Balancing as Manual Method:

Making adjustments to the pooling volume based on the End-point RFU values, the same level can be achieved as the laborious legacy method. N6 provides an analysis tool to directly make recommendations for all methods.

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n6 iconPCR team: Yann Jouvenot, Pranav Patel, Wes Austin, Chris Streck, Gagneet Kaur, n6, Pleasanton, CA