plexWell™ Troubleshooting Guide

Applicable to plexWell 96, plexWell 384, plexWell LP 384, plexWell Plus 24, and plexWell WGS 24 Library Preparation Kits

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Please contact support@seqwell.com for more information or assistance with questions not covered in this guide.

1 Little or no library recovered

Sub-category	Potential cause	Solution
Samples	Insufficient input	Review pre-library quantification data. If samples were diluted significantly, re-quantify a subset of samples to ensure that the dilution factor was appropriate. Always use a PicoGreen™-based method to quantify input DNA (not a NanoDrop™)
	Poor sample quality	Check that samples are not degraded. The majority of the DNA should be >500 bp.
	Sample impurity	Significant carryover of RNA or ssDNA from extraction can impact tagmentation even when dsDNA is present. This typically occurs when the amount of RNA or ssDNA is greatly in excess of the dsDNA content. Repurify the starting samples to remove RNA and ssDNA content.
	Inhibitor in sample buffer	The tagmentation reaction is enzymatic. A high concentration of EDTA, denaturing or inhibiting contaminants in your input DNA could impact protocol success.
Hardware	Issue with thermocycler	Confirm that your thermocycler was programmed correctly and is temperature cycling accurately.
Library fragment size is outside of expected range, yield is low, and/ or library concentrations determined by qPCR do not match results from other DNA quantification methods (e.g. PicoGreen, Bioanalyzer or TapeStation)	Missed/failed step	A missed or incorrect step in the protocol can result in no library recovery. An incorrect buffer sequence during the bead cleanup is a common place for this to happen. Ensure you are following the protocol exactly and there was no confusion about which reagents were used at each step.

Little or no library recovered (continued)

Sub-category	Potential cause	Solution
Library fragment size is outside of expected range, yield is low, and/ or library concentrations determined by qPCR do not match results from other DNA quantification methods (e.g. PicoGreen™, Bioanalyzer or TapeStation) (continued)	Low recovery during purification Steps	Purification with MAGwise™ Paramagnetic Beads is a critical component of the library preparation process. Failure to recover the expected amount of DNA during Sample Barcoding (SB) purification or Pool Barcoding (PB) purification will affect library yield, sizing, and quality. Always ensure that MAGwise Beads are equilibrated to room temperature and the beads are thoroughly resuspended before pipetting them. Always mix MAGwise Beads thoroughly with sample. Adhere to recommended incubation and elution times. Always use freshly prepared 80% ethanol for bead washes. Do not air-dry beads. Thoroughly resuspend beads during elution step.
	Library fragments removed during purification	Check the unpurified, amplified material to see if any library fragments were present. If the unpurified material is largely made up of small fragments (<400 bp), most of the library will be removed by a 0.75X purification. To recover short fragments, modify the final purification ratio to 0.8 (for fragments ≥300 bp) or to 1 (for fragments ≥200bp) volume equivalents of MAGwise Beads.
	Library amplification failure	This is typically the result of (i) incorrect programming of the thermocycler (e.g. skipping the fill-in step), (ii) failure to add (the appropriate) amplification primers, or (iii) substituting the recommended amplification reagent for a different polymerase. Confirm that the thermal cycling program used contains the fill-in reaction, and correct cycling parameters. Check unpurified library to confirm the presence of primers.
No Library after final purification (but library present in unpurified QC check)	Incorrect volume of MAGwise Beads used	Confirm the ratio of MAGwise Beads used relative to the volume of the library to be purified. If too little MAGwise Beads are used, the entire library will be lost during cleanup.

2 Completed library fragment size is outside of target range

Sub-category	Potential cause	Solution
Unpurified library contains fragments of the right length, but purified library is too large	Incorrect volume of MAGwise™ Beads used during final purification	The final purification step removes small fragments from the unpurified library. Increase the volume equivalent of MAGwise Beads to recover more of the smaller fragments.
Unpurified and purified library contains only long fragments	Too much sample DNA	The ratio of DNA recovered from the SB purification, as well as the amount of PB reagent used, are critical to generating library fragments of the correct size. If the average DNA input per sample is too high, this ratio will be incorrect. Confirm your sample input amount. Consider an in-process QC following SB purification to ensure that the DNA recovered is in the correct range for the plexWell™ kit that you are using. Refer to the appropriate plexWell User Guide for specific details.
	Too little PB reagent used	The ratio of DNA recovered from the SB purification, as well as the amount of PB reagent used, are critical to generating the correct size library fragments. Make sure you used the correct volume of PB reagent. Since the PB reagent is viscous, always pipette it slowly, dispense fully into the reaction and mix the PB reaction thoroughly.
	Tagmentation reaction failed	Ensure that all TAG reactions are thoroughly mixed prior to incubation and the incubation was performed with the correct parameters.
Unpurified library is short and little library is recovered after final purification	SB Purification recovered too little DNA	The ratio of DNA recovered from the SB purification, as well as the amount of PB reagent used, are critical to generating the correct size library fragments. If the average DNA input per sample is too high, this ratio will be incorrect. Confirm your sample input amount. Consider an in-process QC following SB purification to ensure the DNA recovered is in the correct range for the plexWell kit that you are using. Refer to the appropriate plexWell User Guide for specific details.

Completed library fragment size is outside of target range (continued)

Sub-category	Potential cause	Solution
Unpurified library is short and little library is recovered after final purification	Too much PB reagent used	The ratio of DNA recovered from the SB purification and the amount of PB reagent used is critical to generating the correct size library fragments. Make sure you used the correct volume of PB reagent. Since the PB reagent is viscous, always pipette it slowly, dispense fully into the reaction and mix the PB reaction thoroughly.

3 Sample Sheet/Run Setup and configuration

Sub-category	Potential cause	Solution
Sample Sheet setup	Sample Sheet header information incorrect	Configure the Illumina® Sample Sheet as you would for a Nextera® XT run to ensure that the proper header information and adapter sequences.
Run configuration	Dual index sequencing is recommended	Configure the plexWell™ sequencing run as a dual-indexed sequencing run, using a template for Nextera XT libraries. If only one index (i5) is being used, the sequencing run can be configured as a single-index sequencing run.

4 Sequencing run quality issues

Sub-category	Potential cause	Solution
Cluster density (non-patterned flow cell)	Sub-optimal loading concentration	The amount of DNA loaded onto the flow cell varies by sequencing platform and is key to sequencing performance. The single most important factor for success is accurate quantification of the library. A qPCR-based method is the best for determining library concentration. Apply a size adjustment as described in the quantification kit user guide. If qPCR is unavailable, a PicoGreen™-based method may be substituted. Use a 2-fold serial dilution series to generate a standard curve from at least four points. Convert from ng/µl to nM using the average fragment length (determined with an electrophoretic method). Standards, sample replicates and control samples help to ensure that quantification values are correct. Determining the optimal loading concentration for your sequencer for a new library prep kit will require optimization.
		Check calculations for pooling and diluting libraries prior to loading the sequencer. Quantify the final pool by qPCR.
	Suboptimal denaturation reaction	Confirm that the correct denaturation protocol for the specific sequencing platform was followed.
Low Pass Filter (PF) rate (normal cluster density) on non-patterned flow cell	Sequencer or Sequencing Kit issue (most common)	The most common causes for low PF rates include sequencer-related issues (e.g. defective fluidics or optics), or a bad flow cell (dead tiles). Contact Illumina® technical support for assistance.
	Library issue (less common)	If sequencer performance is ruled out as an issue, contact support@seqwell.com for assistance with troubleshooting.

Sequencing run quality issues (continued)

Sub-category	Potential cause	Solution
Low PF rate with patterned flow cell	Suboptimal loading concentration	plexWell™ libraries typically meet the number of PF clusters specified by Illumina® for a given sequencing platform/kit. The PF for plexWell libraries may differ than that for other library types). Determine if the low PF rate was the result of under- or over-loading by checking the % occupancy. Occupancy <80% indicates under-loading. Adjust the loading concentration up for the next run. Occupancy >95% indicates over-loading. Adjust the loading concentration down for the next run
Low Q30 score even with optimal loading concentration	Sequencer or Sequencing Kit issue	If the Q30 scores are low despite optimization of loading concentration, contact Illumina technical support to troubleshoot the run.

5 Demultiplexing

Sub-category	Potential cause	Solution
Indices weren't present after demultiplexing	Incorrect sequence was used for i5 index	Some sequencing platforms read the i5 index in the forward direction, whereas others read it in the reverse direction. Illumina® has termed these Workflow A and Workflow B, respectively. Make sure that the i5 index sequence was specified correctly for the sequencing platform used.
Low-quality i5 read	Low diversity in i5	If only one PB was used, demultiplex using the i7 index only
High % of non-demultiplexed reads	Sequencing quality or Sample Sheet issue	Contact support@seqwell.com for assistance.

6 Sample bias within a pool/per sample CV is out of range

Sub-category	Potential cause	Solution
Samples within a pool have unequal read count distribution or greater than expected CV	Suboptimal pooling	Uneven read counts are typically caused by pooling incorrect volumes of samples. Only use calibrated pipettes and ensure careful transfer with no loss of sample. Check that there are no effects by row or column where one row or column is under or over represented.
	Differences in sample buffer composition	Extraction method or storage buffer can impact tagmentation efficiency. If there is a trend between sample source, consider a buffer exchange or additional cleanup.
	Input concentration of samples outside of range	Samples with an extreme high or low input concentration can result in outliers with respect to % read count. Confirm that the input concentration of outliers was within concentration range specifications for the plexWell™ kit that was used.

7 Other data quality issues

Sub-category	Potential cause	Solution
High duplication rate and/or low library diversity	Suboptimal amount of library loaded on sequencer	This would typically only apply when patterned flow cells are used. Use the maximum amount of library recommended for the particular sequencer and flow cell. Lower input can result in higher duplication rates.
	Low library yields	Does library meet expected yield (concentration) for the kit used? If so, contact support@seqwell.com for assistance to troubleshoot library diversity. If library QC metrics (concentration and sizing) differ from expected results for the library preparation kit used, refer to sections related to low library yield and/or suboptimal fragment length.
	Mismatch of kit complexity and project needs	Review Product Selection Guide. Contact support@seqwell.com for assistance.
Desired sample coverage not achieved	Desired coverage not compatible with sequencing run output	Review Product Selection Guide and the expected data yield from the sequencing format used. Contact support@seqwell.com for assistance. .
	Less output from sequencing run than expected	Consult sections related to sequencing run performance. Contact Illumina® support.

Revision History

Revision. no.	Revision date	Revision details
v20210105	5-Jan-2021	First version