USER GUIDE

HOLOGIC®
Diagenode

MicroPlex Library Preparation Kit v3

High Performance Library Preparation for Illumina® NGS Platforms

48 rxns

Cat. No. C05010001

V2 03_2025

Content

! Please read this manual carefully before starting your experiment

Introduction

The MicroPlex Library Preparation Kit v3 combined with Hologic Diagenode's Dual Indexes for MicroPlex v3 (available separately) generates indexed libraries with multiplexing for up to 384 samples for sequencing on Illumina NGS platforms. Once purified and quantified, the resulting dual indexed libraries are ready for Illumina NGS instruments using standard Illumina sequencing reagents and protocols.

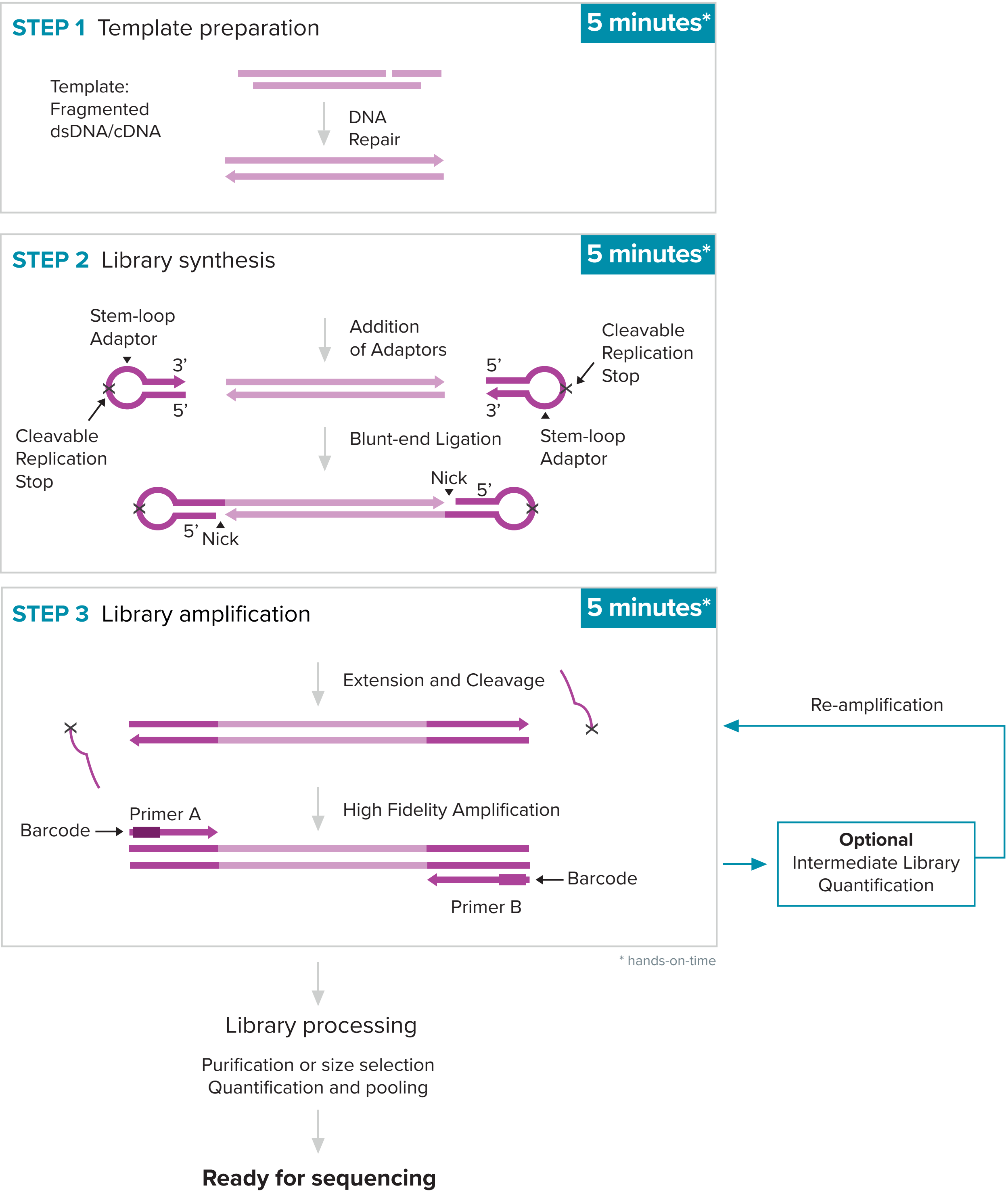
Generated libraries are compatible with **single-end or paired-end** sequencing. MicroPlex chemistry is specifically developed and optimized to generate DNA libraries with high molecular complexity from the lowest input amounts. Only **50 pg to 50 ng** of fragmented **double-stranded DNA** is required for library preparation. The entire **three-step workflow** takes place in a single tube or well in about **2 hours**. **No intermediate purification steps and no sample transfers** are necessary to prevent handling errors and loss of valuable samples. The kit provides excellent results for

- DNA-seq (high-coverage, deep sequencing: de novo sequencing, whole genome resequencing, whole exome sequencing, cell-free plasma DNA sequencing)
- Enrichment techniques: ChIP-seq
- RNA-seq (cDNA)

The MicroPlex Library Preparation Kit v3 can be used manually or with automation using the IP-Star Compact Automated System. The corresponding protocols are included in this manual.

Kit Method Overview

The MicroPlex Library Preparation Kit v3 is based on patented MicroPlex technology (Figure 1). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, MicroPlex uses stem-loop adapters to construct high quality libraries with a fast and efficient workflow:



Step 1. Template Preparation provides efficient repair of the fragmented double-stranded DNA input.

In this step, the DNA is repaired and yields molecules with blunt ends.

Step 2. Library Synthesis enables ligation of MicroPlex patented stemloop adapters.

In the next step, stem-loop adaptors with blocked 5' ends are ligated with high efficiency to the 5' end of the genomic DNA, leaving a nick at the 3' end. The adaptors cannot ligate to each other and do not have singlestrand tails, both of which contribute to non-specific background found with many other NGS preparations.

Step 3. Library Amplification enables extension of the template, cleavage of the stem-loop adaptors, and amplification of the library. Illuminacompatible dual indexes are also introduced using a high-fidelity, highlyprocessive, low-bias DNA polymerase.

In the final step, the 3' ends of the genomic DNA are extended to complete library synthesis and Illumina-compatible dual indexes are added through a high-fidelity amplification. Any remaining free adaptors are destroyed. Hands-on time and the risk of contamination are minimized by using a single tube and eliminating intermediate purifications.

Obtained libraries are purified, quantified and sized. The libraries pooling can be performed as well before sequencing (Figure 1).

Kit Materials

The MicroPlex Library Preparation Kit v3 contains sufficient reagents to prepare up to the specified number of reactions. Enough buffers and enzymes are provided for four uses or freeze-thaw cycles. Contents of the MicroPlex Library Preparation Kit v3 are not interchangeable with other Hologic Diagenode products. The dual indexes compatible with this kit have to be purchased separately (listed in the paragraph: Required materials not provided).

Table 1. *MicroPlex Library Preparation kit v3*

Name	Cap colour	µl/rxn	48 rxns
Dual Template Preparation Buffer	Red	2	105 µl
Dual Template Preparation Enzyme	Red	1	50 µl
Dual Library Synthesis Buffer	Yellow	1	50 µl
Dual Library Synthesis Enzyme	Yellow	1	50 µl
Dual Library Amplification Buffer	Green	25	1260 µl
Dual Library Amplification Enzyme	Green	1	50 µl
Nuclease-Free Water	Clear	4	500 µl

SHIPPING AND STORAGE: The MicroPlex Library Preparation Kit v3 is shipped on dry ice. The kit should be stored at −20°C upon arrival.

The volumes of buffers and enzymes mentioned above are the minimum volumes required to complete 48 reactions. However, an excess is included in each tube to cover pipetting loss.

Required Materials not Provided

- Barcoded primers

The following validated barcoded primers (sold separately) should be used with the Microplex Library Preparation Kit v3:

Table 2. *Validated barcoded primers*

Product	Cat. No.	Number of indexes
24 UDI for MicroPlex v3 - Set I	C05010008	24
24 UDI for MicroPlex v3 - Set II	C05010009	24
24 Dual indexes for MicroPlex Kit v3	C05010003	24

Barcoded primers can be used not only for high-level multiplexing (up to 48 samples) but also for low-level multiplexing of a small number of samples. It is important to select unique index combinations that meet Illumina-recommended compatibility requirements. Please refer to Illumina's technical manuals (Index Adapters Pooling Guide, Illumina Document # 1000000041074 v06) for additional information.

- Low binding aerosol barrier tips
- Freshly prepared 80% (v/v) ethanol
- Agencourt® AMPure® XP beads (Beckman Coulter, Cat. No. A63880)
- Magnetic rack:
  - Compatible with 0.2 ml PCR tubes if working with tubes or strips (eg. Hologic Diagenode DiaMag 0.2ml , Cat. No. B04000001)
  - Alternatively: 96 well plate magnetic rack – if working with PCR plates
- Low TE buffer pH 8.0 (10 mM Tris, 0.1 mM EDTA, pH 8.0), molecular grade
- Centrifuge
- Thermal cycler

NOTE: *Use a thermal cycler equipped with a heated lid that can handle 50 µl reaction volumes. Set the temperature of the heated lid to 101°C – 105°C to avoid sample evaporation during incubation and cycling. We recommend a ramp rate of 3°C/s – 5°C/s; higher ramp rates are not recommended and could impact the quality of the library.*

- 0.2 ml PCR tubes

  NOTE: *Select appropriate tubes that are compatible with the thermal cyclers used. Use appropriate caps or sealing films and seal thoroughly to eliminate evaporation during cycling conditions. Evaporation could reduce robustness and reproducibility of the reactions.*

- BioAnalyzer® (Agilent) or Fragment Analyzer® (Advanced Analytical) (for library quantification and sizing)
- High sensitivity DNA kit from Agilent or High Sensitivity NGS Fragment Analysis kit (35 bp-6000 bp) from Advanced Analytical Qubit 2.0 Fluorometer® (Life Technologies) or QuantiT™PicoGreen® dsDNA Assay Kit (Life Technologies) (for library quantification). Optional: KAPA® Library Quantification Kit – Illumina (Kapa Biosystems), specific to a real time PCR system used) and real time PCR system can be also used.

Additional supplies if working with IP-Star Compact:

- IP-Star Compact Automated System (Hologic Diagenode, Cat. No. B03000002)
- 200 µl tube strips (8 tubes/strip) + cap strips (Hologic Diagenode, Cat. No. C30020002)
- Tips (box) (Hologic Diagenode, Cat. No. C30040021)
- Tips (bulk) (Hologic Diagenode, Cat. No. C30040020)
- 2 ml microtube (Hologic Diagenode, Cat. No. C30010014)
- Medium reagent container (Hologic Diagenode, Cat. No. C30020003)
- 96 well microplates (Hologic Diagenode, Cat. No. C30080030)

Quality Control

The MicroPlex Library Preparation Kit v3 is functionally tested using Next Generation Sequencing (NGS) to ensure product quality and consistency.

Safety Information

Follow standard laboratory safety procedures and wear a suitable lab coat, protective goggles and disposable gloves to ensure personal safety as well as to limit potential cross contamination during the sample preparation and subsequent amplification reactions.

Remarks Before Starting

DNA format

Fragmented double-stranded DNA (gDNA or cDNA), chromatin immunoprecipitates (ChIP), degraded DNA from sources such as FFPE, plasma, or other biofluids are suitable. This kit is not for use with single-stranded DNA (ssDNA) or RNA.

Input DNA amount

Input DNA in the range of 50 pg to 50 ng can be used as starting material. However, for the highest sequencing data quality it is recommended to use as much DNA as possible. For deep whole genome sequencing (WGS) and whole exome sequencing (WES) using human gDNA, FFPE, or plasma DNA, greater than 10 ng of input DNA is recommended to achieve a highly diverse library. For sequencing samples with reduced complexity, such as cDNA, immunoprecipitated DNA from ChIP, bacterial DNA, or targeted genomic regions, lower input amounts (picogram levels) can be used.

Fragment Size

The optimal DNA fragment size is less than 1,000 bp. The MicroPlex Library Preparation Kit v3 is a ligation-based technology and adapters added during the process result in an approximately 140 bp increase in the size of each DNA template fragment. Library molecules with shorter inserts (200 – 300 bp) tend to cluster and amplify more efficiently on the Illumina flow cell. Depending on the application and requirements, the AMPure purification step following the final step (library amplification) can be replaced with AMPure size-selection step to remove unwanted large fragments.

Input Volume

The maximum input sample volume is 10 µl. If a sample is in a larger volume, the DNA must be concentrated into 10 µl or less. Alternatively, the sample may be split into 10 µl aliquots, processed in separate tubes, and the corresponding products pooled prior to the purification step preceding sequencing.

Input Buffer

Input DNA must be eluted or resuspended in a **low-salt and low-EDTA buffered solution**. The preferred buffer is low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0). The concentrations of Tris and EDTA must not exceed 10 mM and 0.1 mM, respectively. Avoid phosphate containing buffers.

Positive and Negative Controls

If necessary, include appropriate positive and negative controls in the experimental design to help verify that reactions proceed as expected. If the experimental samples contain any carryover contaminant(s) in the buffer, the downstream reactions may be impacted, and inclusion of controls would help elucidate such problems. A suitable positive control (reference DNA) is Bioruptor-sheared purified genomic DNA (200 – 300 bp) of comparable input amount. Always prepare fresh dilutions of reference DNA. Include a negative control (No Template Control, NTC) with low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) or nuclease-free water. The positive control and experimental samples should perform equivalently, while the NTC should not amplify.

Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared at each workflow step based on the number of reactions to be performed. Transfer the enzymes to ice just prior to use and centrifuge briefly to collect contents at the bottom of the tube. Thaw the buffers, vortex briefly and centrifuge prior to use. Keep all the components and master mixes on ice. Once the master mix is prepared, thoroughly mix the contents several times with a pipette while avoiding introduction of excessive air bubbles and briefly centrifuge prior to dispensing into the PCR plate or tube(s).

Indexing Reagents

The Indexing Reagents consist of amplification primers containing Illumina-compatible indexes. Index sequences, multiplexing and index pooling guidelines can be found in the Primer Indexes for MicroPlex kit v3 manual.

Library yield and selection of the optimal number of cycles for library amplification

The requirements for a final library concentration depend on a sequencer and may vary between different sequencing service providers. The usual range is between **5-20 nM** in a final volume **10-15 µl** but we recommend inquiring with your sequencing platform.

The number of PCR cycles required at Step 3 of the protocol (Library Amplification) is dependent on the amount of input DNA, quality, fragmentation size and a thermal cycler. Note that an over-amplification could result in higher rate of PCR duplicates in the library.

When working with DNA sample of known quantity or quality, use the **Amplification Guide** for selecting the number of PCR cycles.

When working with DNA samples of unknown quantity and/or quality (such as DNA from FFPE tissue or environmental sample, low input samples below quantification limit etc.), amplify samples for **12 cycles** and perform an intermediate quantification of unpurified libraries as described at OPTIONAL INTERMEDIATE LIBRARY QUANTIFICATION STEP. Depending on the yield, libraries can be re-amplified for few additional cycles to achieve a desired yield or purified.

Library purification or size selection

Purification using Agencourt AMPure® XP (Beckman Coulter) is the preferred method because sequence complexity is conserved. Do not use silica-based filters for purification. The optimal library size is dictated by the sequencing application and a sequencer specification. The final library size corresponds to the initial size of DNA fragments plus approximately 140 bp due to the ligated adapters. Most part of applications will not require a size selection if the initial DNA fragment size is less than 1,000 bp. In this case, amplified products should be purified by Agencourt AMPure XP (Beckman Coulter) to get rid of primers and adaptors (See section "Library processing, option A"). If the size selection is required, follow the protocol described in the section "Library processing, option B" to get rid of primers/adaptor and fragments above 600 bp.

Library Quantification

There are several approaches available for library quantification including real-time PCR, UV absorption, fluorescence detection, or sizing and quantification using the Agilent Bioanalyzer or Fragment Analyzer (Advanced Analytical). It is important to understand the benefits and limitations of each approach.

Real-time PCR-based approaches (such as the KAPA Library Quantification Kit from Kapa Biosystems) quantify the library molecules that carry the Illumina adapter sequences on both ends and, therefore reflect the quantity of the clustering competent library molecules. This approach assumes a relatively uniform size of sheared or fragmented starting gDNA inserts used for library construction. Quantification by PCR can be done on unpurified libraries.

The Agilent Bioanalyzer system or Fragment Analyzer (Advanced Analytical) provide sizing and quantification information about the library analysed, but not about the clustering competency. Quantification can be done both on unpurified or purified samples. In a case of unpurified samples, a region corresponding to libraries should be limited in order to discriminate between primers/adaptors and a library itself.

UV absorption/fluorescence detection-based methods (i.e., Nanodrop® (Thermo Scientific), Qubit®2.0 Fluorometer (Life Technologies), or QuantiT™ PicoGreen® dsDNA Assay Kit (Life Technologies) simply quantify total nucleic acid concentration. These methods do not discriminate adapter presence and offer no information about the size of the library molecules. They can be used only on purified libraries. We better recommend fluorescence-based assays than spectrophotometric measurements (e.g. NanoDrop) due to higher specificity and sensitivity.

PROTOCOL #1

MANUAL PROCESSING

PROTOCOL FOR LIBRARY PREPARATION

STEP 1

Template Preparation

1.1 Dispense **10 µl** of fragmented doubled-stranded DNA into each PCR tube or well of a PCR plate compatible with your thermal cycler.

NOTE: *Positive control reactions using reference DNA: If necessary, assemble reactions using 10 µl of a reference gDNA (e.g., Bioruptor-fragmented DNA, 200-300 bp average size) at an input amount comparable to that of the samples.*

NOTE: *Negative control reactions (NTCs): If necessary, assemble NTCs with 10 µl of nuclease-free water or TE buffer (e.g., 10 mM Tris, 0.1 mM EDTA, pH 8.0).*

1.2 Prepare **Template Preparation Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Component	**Volume for 1 sample**
Dual Template Preparation Buffer **RED CAP**	2.0 µl
Dual Template preparation Enzyme **RED CAP**	1.0 µl

1.3 Add **3 µl** of the **Template Preparation Master Mix** to each 10 µl sample from step 1.1 above. Mix thoroughly with a pipette.

1.4 Tightly cap the tube(s) or seal the PCR plate using an appropriate sealing film.

1.5 Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each tube or well.

1.6 Place the tube(s) or plate in a thermal cycler with heated lid set to 101°C – 105°C. Perform the **Template Preparation Reaction** using the following settings:

Template Preparation Reaction	
Temperature	**Time**
22°C	25 min
55°C	20 min
4°C	Hold for ≤ 2 hours

1.8 After the thermal cycler reaches 4°C, remove the plate or tube(s) and centrifuge briefly.

1.9 Continue to Library Synthesis Step in the same tube(s) or plate.

STEP 2

Library Synthesis

2.1 Prepare Library Synthesis Master Mix as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Component	**Volume for 1 sample**
Dual Library Synthesis Buffer **YELLOW CAP**	1.0 µl
Dual Library Synthesis Enzyme **YELLOW CAP**	1.0 µl

2.2 Add 2 µl of the Library Synthesis Master Mix to each tube or well. Mix thoroughly with a pipette.

2.3 Tightly cap the tube(s) or seal the PCR plate using an appropriate sealing film.

2.4 Centrifuge briefly to collect the contents to the bottom of each well or tube.

2.5 Return the tube(s) or plate to the thermal cycler with heated lid set to 101°C – 105°C.

2.6 Perform Library Synthesis Reaction using the following settings:

Template Preparation Reaction	
Temperature	**Time**
22°C	40 min
4°C	Hold for ≤ 30 minutes

2.7 After the thermal cycler reaches 4°C, remove the tube(s) or plate and centrifuge briefly.

2.8 Continue to the Library Amplification Step in the same tube(s) or plate maintained at 4°C.

STEP 3

Library Amplification

NOTE: *The indexes compatible with this kit have to be purchased separately (see: Required materials not provided) . Select the right index for MicroPlex kit v3 and follow the guidelines from this kit regarding index handling.*

3.1 Prepare the Dual Indexing Reagents (available separately) as follows:

- Remove the Dual Indexing Reagents from freezer and thaw for 10 minutes on the bench top.
- Spin the Dual Indexing Reagents in a table top centrifuge to collect contents at the bottom of the well.
- Thoroughly wipe the Dual Indexing Reagent Tubes foil seal with 70% ethanol and allow it to dry.

3.2 Prepare the Library Amplification Master Mix as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until use.

Component	**Volume for 1 sample**
Dual Library Amplification Buffer **GREEN CAP**	25.0 µl
Dual Library Amplification Enzyme **GREEN CAP**	1.0 µl
Nuclease-Free Water **CLEAR CAP**	4.0 µl

3.3 Add **30 µl** of the **Library Amplification Master Mix** to each tube containing 15 µl of Library Synthesis Reaction Product.

3.4 Add **5 µl** of the appropriate **Dual Indexing Reagent** (available separately) to each tube.

3.5 Mix thoroughly with a pipette. Avoid introducing excessive air bubbles.

3.6 Seal the tube(s) tightly and centrifuge briefly to collect the contents to the bottom of each well or tube.

3.7 Return the tube(s) to thermal cycler with heated lid set to 101°C – 105°C.

3.8 Perform **Library Amplification Reaction** using the cycling conditions in the tables below.

CAUTION: *Ensure that the thermal cycler does not have a denaturing step programmed until Stage 3.*

	Stage	Temperature	Time	Number of Cycles
Extension & Cleavage	1	72°C	3 min	1
	2	85°C	2 mini	1
Denaturation	3	98°C	2 min	1
Addition of Indexes	4	98°C	20 s	4
		67°C	20 s	
		72°C	40 s	
Library Amplification	5	98°C	20 s	5 to 16*
		72°C	50 s	
Hold	6	4°C	Hold	1

** If working with **DNA samples of known quantity or quality**, please use the **Amplification Guide** below to select the required number of amplification cycles.*

Amplification Guide (for stage 5)	
DNA Input (ng)	**Number of Cycles**
50 ng	6 - 8
20 ng	7 - 8
10 ng	7 - 8
5 ng	7 - 9
2 ng	8 - 10
1 ng	11 - 12
0.2 ng	14 - 15
0.05 ng	15 - 16

*If working with **DNA samples of unknown quantity and/or low quality** (eg. FFPE extracted DNA), amplify for **12 cycles** and perform an **INTERMEDIATE LIBRARY QUANTIFICATION OF UNPURIFIED LIBRARY** to estimate the library yield . If the desired yield is not achieved, the libraries can be re-amplified for few additional cycles.*

3.9 Remove the tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.

3.10 Proceed to the Library Purification by Agencourt AMPure XP in the section “Library processing” (recommended for samples of known quantity or quality) or to the Intermediate Library Quantification option shown below (recommended for samples with unknown quantity and/or low quality).

Optional Intermediate Library Quantification

(recommended for samples with unknown quantity and/or a low quality)

Quantify **unpurified** libraries using the BioAnalyzer, the Fragment Analyzer or similar devices. This intermediate quantification allows estimating the library yield and enabling an additional re-amplification if needed. You will need a High Sensitivity NGS Fragment Analysis kit (1 bp-6,000 bp) from Advanced Analytical if using Fragment Analyzer or High Sensitivity DNA kit from Agilent if using BioAnalyzer. Follow the manufacturer's instruction for kit handling and protocol.

NOTE: *The quantification of unpurified library by qPCR (e.g. using KAPA® Library Quantification Kit – Illumina (Kapa Biosystems), is also possible at this step while it will increase the total protocol duration.*

1. Take an aliquot of unpurified libraries: **1 µl** if the BioAnalyzer will be used or **2 µl** if the Fragment Analyzer will be used. Keep remaining libraries at 4°C .
2. Load the aliquot of unpurified library on the Fragment Analyzer or BioAnalyzer and run the analysis.
3. Estimate the library yield using a region selection option to discriminate between unincorporated primers/adaptors and libraries themself as it is shown in the Figure 2 below.

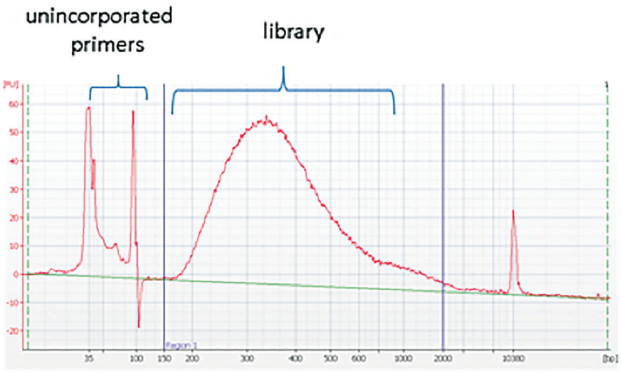


Figure 2. BioAnalyzer trace of unpurified library. *A region corresponding to the amplified library is selected (blue bars) to estimate the yield.*

4. If the library has a sufficient yield, proceed to the **Library Purification or size selection step** in the section: **Library processing**. If the library has a low yield, the remaining library can be further **re-amplified** for 2 to 3 additional cycles to achieve the desired yield. Spin down the tube or plate containing the library and return the plate or tube(s) to a thermal cycler.

5. Use the following cycling settings:

Number of Cycles	Temperature	Time
2 - 3 cycles	98°C	20 s
	72°C	50 s
1 cycle	4°C	Hold

NOTE: *Higher yields may come at the expense of reduced sequencing quality, therefore it is important to avoid an over-amplification.*

NOTE: *MicroPlex libraries can be further amplified with no extra reagents added after storage at 4°C for up to 6 hours or -20°C for up to 7 days.*

PROTOCOL #2

MANUAL PROCESSING

LIBRARY PROCESSING

Library Purification or Size Selection

Most part of applications will not require a size selection if an initial DNA fragments size is less than 1,000 bp. Amplified products should be purified by Agencourt AMPure XP beads (Beckman Coulter) as described at **Option A.** Mixing the sample and AMPure XP beads at a 1:1 ratio is critical to get rid of primers and adaptors.

If your application requires a size-selection, please refer to the **Option B.**

You will need

- Freshly prepared 80% (v/v) ethanol
- DiaMag 0.2ml magnetic rack compatible with 0.2 ml tubes (Hologic Diagenode, Cat. No. B04000001)
- Low TE buffer pH 8.0

Option A: Library Purification using AMPure® XP beads

1. Carefully resuspend the AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.

2. Precisely estimate a library volume and add an equal volume of AMPure XP beads to get a final 1:1 volume ratio. Mix by pipette 8 – 10 times until the mixture is homogeneous.

3. Incubate at room temperature for 5 minutes.

4. Place the tube on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes).

5. Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.

6. Wash the beads pellet 2 times as follows:

- With the tubes on the magnet, add 150 µl of 80% ethanol without disturbing the bead pellet and wait for 30 seconds.
- Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
- Perform a brief low speed spin (~2000 g) to collect all droplets remaining on the tube walls.

Repeat the wash as described above 1 time and proceed to the next step.

7. Leaving the tube cap open, let dry the beads on the magnet for 5 minutes.

8. Elute DNA by resuspending the beads in **20 µl of 1x Low TE buffer**, pH 8.0.
9. Place the tube on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes).
10. Without disturbing the pellet, carefully aspirate and transfer the **supernatant containing purified libraries** to a new tube.
11. Quantify purified libraries using the method of your choice (for more information refer to Remarks before starting).

Option B: Library size selection using AMPure XP beads

1. Carefully resuspend the AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.
2. Precisely estimate library volume and adjust it to **50 µl** using nuclease-free water.
3. Add **32.5 µl of AMPure XP beads** to each sample (to get a final volume ratio of AMPure beads: library of 0.65). Mix by pipette 8 – 10 times until the mixture is homogeneous.
4. Incubate at room temperature for 10 minutes.
5. Place the tube on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes). **Transfer the supernatant** to new tube for further processing.
6. Add to the supernatant **12.5 µl** of **room temperature AMPureXP beads** each. Mix by pipette 8 – 10 times until the mixture is homogeneous.
7. Incubate at room temperature for 10 minutes.
8. Place the tube (or a plate) on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes). Carefully aspirate by pipette and **discard the supernatant** without disturbing the pellet.
9. Wash the beads pellet 2 x times as follows:

- With the tubes on the magnet, add **150 µl** of **80% ethanol** without disturbing the bead pellet and wait for 30 seconds.
  - Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
  - Perform a brief low speed spin (~2000 g) to collect all droplets remaining on the tube walls.

  Repeat the wash as described above 1 time and proceed to the next step.

10. Leaving the tube cap open, let dry the beads on the magnet for 5 minutes.

11. Elute DNA by resuspending the beads in **15 µl** of **1x Low TE buffer**, pH 8.0.
12. Place the tube on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes).
13. Without disturbing the pellet, carefully aspirate and transfer the **supernatant containing purified libraries** to a new tube.
14. Quantify purified libraries using the method of your choice (for more information refer to Remarks before starting).

Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer to Appendix). The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform. The total molarity is the sum of all the individual libraries' molarities in the final volume, e.g. if you add 5 µ l of a 10 nM library to 5 µ l of a 20 nM library, you have 10 µ l of a 15 nM pool. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of each library.

AUTOMATED PROCESSING

PROTOCOL FOR LIBRARY PREPARATION

Protocol for library preparation using IP-Star Compact Automated System

The MicroPlex Library Preparation Kit v3 has been validated on IP-Star Compact Automated System (Diagenode, Cat. No. B03000002). The below protocol provides flexibility to prepare 1 to 48 libraries in one run starting with **50 pg–50 ng** of DNA. The whole protocol of library preparation takes approximately 1h 30 minutes. It allows you to prepare up to 96 libraries per day with 2 runs. At the end, you will recover ligated products ready for amplification. Amplified libraries can be further processed on IP-Star Compact - for libraries purification or size selection.

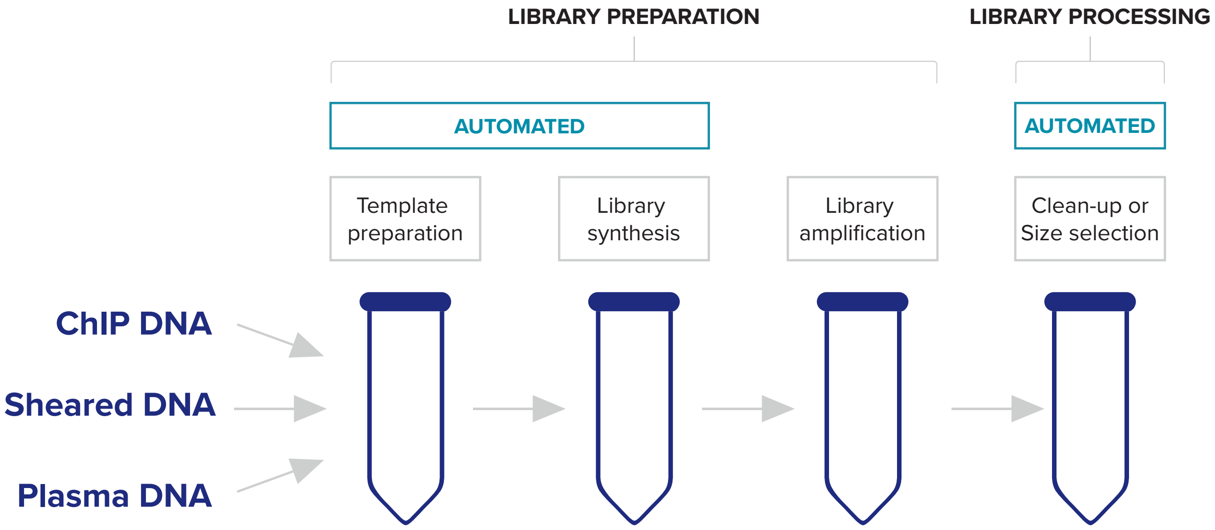
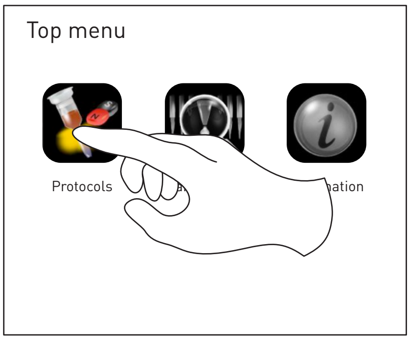
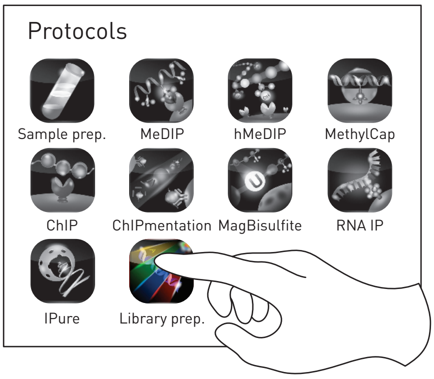


Figure 3. *Microplex Library preparation workflow using IP-Star Compact Automated System.*

IP-Star Setup

1. Switch ON the IP-Star Compact.
2. Select “Protocols” icon and then “Library prep” category.
3. Select “MicroPlex_Library_Preparation” protocol:





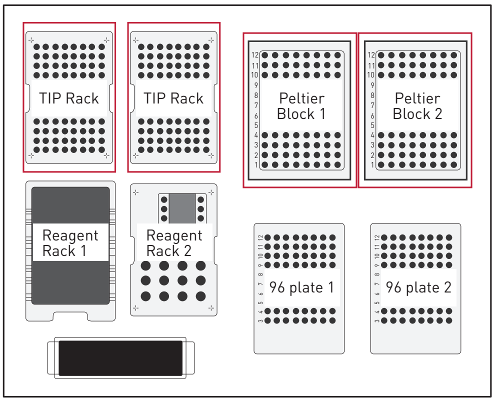
4. Setup the exact number of samples that you want to process by pressing the black box.

   NOTE: *The Peltier Block 1 is now cooling down to 4°C to keep the enzymes and reagents cold.*

If you plan to run between

1 and **8** samples → “**MicroPlex_Library_Preparation_08**”
9 and **16** samples → “**MicroPlex_Library_Preparation_16**”
17 and **24** samples → “**MicroPlex_Library_Preparation_24**”
25 and **32** samples → “**MicroPlex_Library_Preparation_32**”
33 and **40** samples → “**MicroPlex_Library_Preparation_40**”
41 and **48** samples → “**MicroPlex_Library_Preparation_48**”

5. Setup all the plastics on the platform according to the screen layout (press the relevant module to see detailed information).



6. Fill **TIP Rack 1** (and 2 if processing more than 8 samples) with tips according to the screen.
7. Fill **Peltier Blocks 1 and 2** with 200 µ l tube strips according to the screen.

STEP 1&2

Template Preparation & Library Synthesis

NOTE: *Allow the reagent from MicroPlex Library Preparation kit v3 to come at 4°C. Work on ice from this point.*

2.1 Prepare the following mixes.

Template Preparation pre-mix:

	Number of samples						
	1	**8**	**16**	**24**	**32**	**40**	**48**
Dual Template Preparation Buffer **RED CAP**	2 µl	16 µl	32 µl	48 µl	64 µl	80 µl	96 µl
Dual Template Preparation Enzyme **RED CAP**	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
TOTAL	**3 µl**	**24 µl**	**48 µl**	**72 µl**	**96 µl**	**120 µl**	**144 µl**

NOTE: *10 µl of DNA will be added later for each sample.*

Library Synthesis pre-mix:

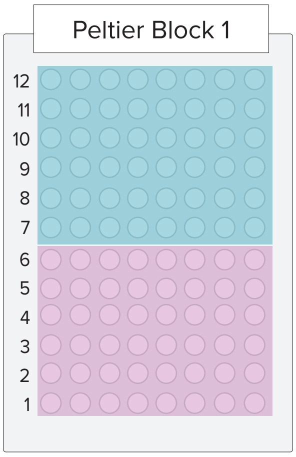
	Number of samples						
	1	**8**	**16**	**24**	**32**	**40**	**48**
Dual Library Synthesis Buffer **YELLOW CAP**	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
Dual Library Synthesis Enzyme **YELLOW CAP**	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
TOTAL	**2 µl**	**16 µl**	**32 µl**	**48 µl**	**64 µl**	**80 µl**	**96 µl**

Library Amplification pre-mix:

	Number of samples						
	1	8	16	24	32	40	48
Nuclease-Free Water **CLEAR CAP**	4 µl	32 µl	64 µl	96 µl	128 µl	160 µl	192 µl
Dual Library Amplification Buffer **GREEN CAP**	25 µl	200 µl	400 µl	600 µl	800 µl	1,000 µl	1,200 µl
Dual Library Amplification Enzyme **GREEN CAP**	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
TOTAL	**30 µl**	**240 µl**	**480 µl**	**720 µl**	**960 µl**	**1,200 µl**	**1,440 µl**

2.2 Fill the **Peltier Block 1** with **Library Synthesis pre-mix** and **Library Amplification pre-mix** according to the screen layout.

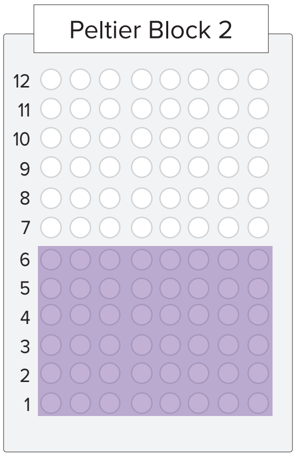
2.3 Fill the **Peltier Block 2** with the **Template Preparation pre-mix** according to the screen layout.



Strip tubes 🟥

Well 7-12	
Library Amplification pre-mix	30 µl
◦ Nuclease Free Water	4 µl
• Library Amplification Buffer	25 µl
• Library Amplification Enzyme	1 µl

Well 1-6	
Library Synthesis pre-mix	2 µl
• Library Synthesis Buffer	1 µl
• Library Synthesis Enzyme	1 µl



PCR tube 🟪

Well 1-6	
DNA	10 µl
Template Preparation pre-mix	3 µl
• Template Preparation Buffer	2 µl
• Template Preparation Enzyme	1 µl

Samples 41-48
Samples 33-40
Samples 25-32
Samples 17-24
Samples 9-16
Samples 1-8

2.4 Add **10 µl of DNA** to each tube containing the Template Preparation pre-mix (according to the screen layout).

2.5 Check the proper insertion of the racks and consumables.

2.6 Close the door and press “Run” to start.

2.7 MicroPlex is running. The “Remaining time” calculation will give you an estimation of the processing time of your experiment.

STEP 3

Library Amplification

NOTE: *The dual indexes compatible with this kit have to be purchased separately (see: Required materials not provided). Select the right Dual index for MicroPlex kit and follow the guidelines from this kit regarding index handling.*

Prepare the Dual Indexing Reagents (available separately) as follows:

- Remove the Dual Indexing Reagents from freezer and thaw for 10 minutes on the bench top.
- Spin the Dual Indexing Reagents in a table top centrifuge to collect contents at the bottom of the well.
- Thoroughly wipe the Dual Indexing Reagent Tubes or Indexing Reagent Plate foil seal with 70% ethanol and allow it to dry.

3.1 Recover your samples on the Peltier Block 1 in lane 7 to 12 according to the screen layout.

3.2 Press “OK” and “Back” until the homepage appears on the screen.

3.3 Add 5 µl of the appropriate Dual Indexing Reagent (available separately) to each sample. Final volume of your sample is 50 µl.

3.4 Mix 4x with a pipette set to 50 µl. Avoid introducing excessive air bubbles.

3.5 Seal the tube(s) tightly and centrifuge briefly to collect the contents to the bottom of each well or tube.

3.6 Return the tube(s) to thermal cycler with heated lid set to 101°C – 105°C.

3.7 Perform Library Amplification Reaction using the cycling conditions in the tables below.

CAUTION: *Ensure that the thermal cycler does not have a denaturing step programmed until Stage 3.*

	Stage	Temperature	Time	Number of Cycles
Extension & Cleavage	1	72°C	3 min	1
	2	85°C	2 mini	1
Denaturation	3	98°C	2 min	1
Addition of Indexes	4	98°C	20 s	4
		67°C	20 s	
		72°C	40 s	
Library Amplification	5	98°C	20 s	5 to 16*
		72°C	50 s	
Hold	6	4°C	Hold	1

** If working with **DNA samples of known quantity or quality**, please use the **Amplification Guide** below to select the required number of amplification cycles.*

Amplification Guide (for stage 5)	
DNA Input (ng)	**Number of Cycles**
50 ng	6 - 8
20 ng	7 - 8
10 ng	7 - 8
5 ng	7 - 9
2 ng	8 - 10
1 ng	11 - 12
0.2 ng	14 - 15
0.05 ng	15 - 16

*If working with **DNA samples of unknown quantity and/or low quality** (eg. FFPE extracted DNA), amplify for **12 cycles** and perform an **INTERMEDIATE LIBRARY QUANTIFICATION OF UNPURIFIED LIBRARY** to estimate the library yield . If the desired yield is not achieved, the libraries can be re-amplified for few additional cycles.*

3.8 Remove the tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.

3.9 Proceed to the Library processing section (recommended for samples of known quantity or quality) or to the Intermediate Library Quantification option shown below (recommended for samples with unknown quantity and/or low quality).

Optional Intermediate Library Quantification (recommended for samples with unknown quantity and/or a low quality)

Quantify **unpurified** libraries using the BioAnalyzer, the Fragment Analyzer or similar devices. This intermediate quantification allows estimating the library yield and enabling an additional re-amplification if needed. You will need a High Sensitivity NGS Fragment Analysis kit (1 bp-6,000 bp) from Advanced Analytical if using Fragment Analyzer or High Sensitivity DNA kit from Agilent if using BioAnalyzer. Follow the manufacturer's instruction for kit handling and protocol.

NOTE: *The quantification of unpurified library by qPCR (e.g. using KAPA® Library Quantification Kit – Illumina (Kapa Biosystems), is also possible at this step while it will increase the total protocol duration.*

1. Take an aliquot of unpurified libraries: **1 µl** if the BioAnalyzer will be used or **2 µl** if the Fragment Analyzer will be used. Keep remaining libraries at 4°C .
2. Load the aliquot of unpurified library on the Fragment Analyzer or BioAnalyzer and run the analysis.
3. Estimate the library yield using a region selection option to discriminate between unincorporated primers/adaptors and libraries themself as it is shown in the Figure 2 below.

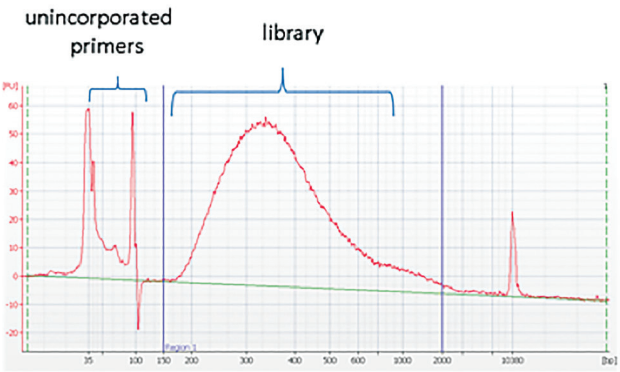


Figure 2. BioAnalyzer trace of unpurified library. *A region corresponding to the amplified library is selected (blue bars) to estimate the yield.*

4. If the library has a sufficient yield, proceed to the **Library Purification or size selection step** in the section: **Library processing**. If the library has a low yield, the remaining library can be further **re-amplified** for 2 to 3 additional cycles to achieve the desired yield. Spin down the tube or plate containing the library and return the plate or tube(s) to a thermal cycler.

5. Use the following cycling settings:

Number of Cycles	Temperature	Time
2 - 3 cycles	98°C	20 s
	72°C	50 s
1 cycle	4°C	Hold

NOTE: *Higher yields may come at the expense of reduced sequencing quality, therefore it is important to avoid an over-amplification.*

AUTOMATED PROCESSING

LIBRARY PROCESSING

Library processing using IP-Star Compact Automated System

Library Purification or Size Selection

Most part of applications will not require size selection if initial DNA fragment size is less than 1,000 bp. Amplified products should be **purified** by Agencourt AMPure XP beads (Beckman Coulter) as described at **Option A**. If your application requires a size-selection, please refer to the **Option B**, which allows to perform **size selection** from 250bp to 500bp just by changing the amount of beads.

You will need

- Freshly prepared 80% (v/v) ethanol
- Low TE buffer pH 8.0
- Agencourt AMPure XP beads (Beckman Coulter)

Option A: Library Purification using AMPure XP beads

Use the room temperature AMPure XP beads for the clean-up.

1. Select "Protocols" icon and then "IPure" category.

2. Select "AMPure_XP_Purification" protocol:

3. Setup the exact number of samples that you want to process by pressing the black box.

If you plan to run between

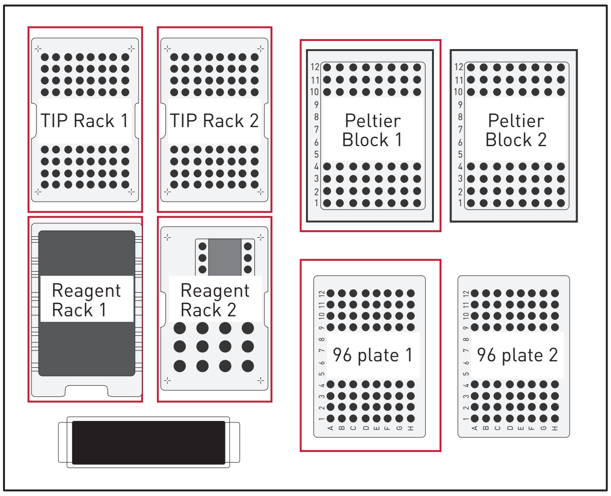
1 and **8** samples → "**AMPure_XP_Purification_08**"
9 and **16** samples → "**MPure_XP_Purification_16**"
17 and **24** samples → "**AMPure_XP_Purification_24**"

NOTE: *The Peltier Block 1 is now cooling down to 4°C to keep your samples cold.*

4. Set up all the plastics on the platform according to the screen layout.

- Fill **TIP Rack 1** (and 2 if processing more than 8 samples) with tips according to the screen.
- Fill **Reagent Rack 1 & 2** with reagent containers according to the screen.

- Fill **96 plate 1** with a 96-well microplate.
- Fill **Peltier Block 1** with 200 µl tube strips according to the screen



5. Fill the robot with all reagents.

- **Peltier Block 1**:
  Put your samples in row 1 - if processing 1 to 8 samples
  - in row 2 - if processing 9 to 16 samples
  - in row 3 – if processing 17 to 27 samples

- 96-well microplate:
  Distribute **room temperature AMPure XP Beads**:
  - in row 1 - if processing 1 to 8 samples
  - in row 5 - if processing 9 to 16 samples
  - in row 9 - if processing 17 to 24 samples

The amount of beads must be 1x the volume of sample (e.g. for 45 µ l of samples use 45 µl of beads)

NOTE: *Resuspend the beads with pipetting up and down several times before dispense them.*

- Fill the container of the **Reagent Rack 1** with freshly prepared 80% ethanol according to the screen.
- Fill the container of **Reagent Rack 2** with Low TE buffer pH 8.0 (“Resuspension Buffer” on the screen) according to the screen.
- Check the proper insertion of the racks and the consumables.

6. Close the door and press “Run” to start.

7. Purification is running. The “Remaining time” calculation will give you an estimation of the processing time of your experiment.

8. After the run, recover your samples on the upper row of the Peltier Block 1 as shown on the screen layout. The final volume of each sample is 20 µl.

9. Press “OK” and “Back” until the homepage appears on the screen. Press “Shutdown” and wait until the screen is black before switching off the IP-Star.

NOTE: *Remove all the plastics from the IP-Star platform, empty the waste shuttle, and clean the inner side of the IP-Star with 70% ethanol.*

Option B: Library size selection using AMPure XP beads

NOTE: *Use room temperature AMPure XP beads for the size selection.*

1. Select “Protocols” icon and then “Library prep.” category.

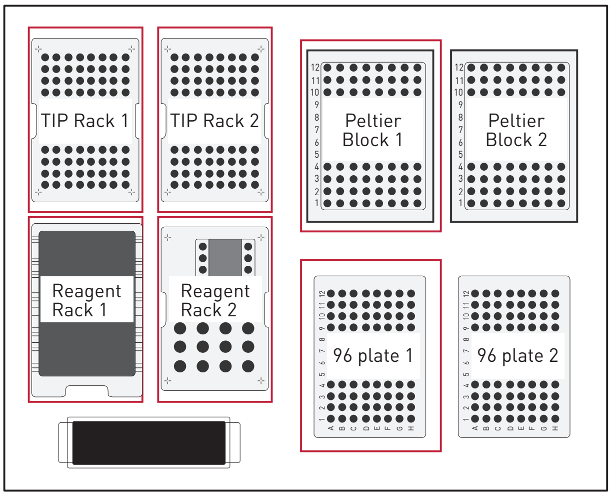
2. Select “AMPure_XP_Size_Selection” protocol:

3. Setup the exact number of samples that you want to process by pressing the black box.

   NOTE: *The Peltier Block 1 is now cooling down to 4°C to keep your samples cold.*

4. Setup all the plastics on the platform according to the screen layout.

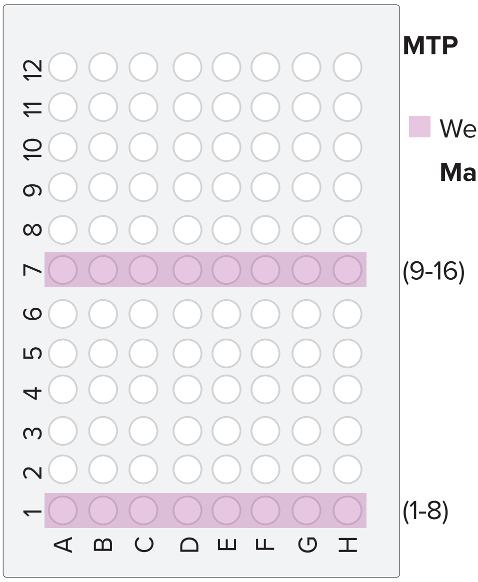
   - Fill TIP **Rack 1** (and 2 if processing more than 8 samples) with tips according to the screen.
   - Fill **Reagent Rack 1 & 2** with reagent containers according to the screen.
   - Fill **96 plate 1** with a 96 well microplate.
   - Fill **Peltier Block 1** with 200 µl tube strips according to the screen.



5. Fill the robot with all reagents.

- Fill **20 µl of samples** in lane 1 (and 2 if processing more than 8 samples) of the Peltier Block 1.
- Add **80 µl of nuclease-free water** ("Resuspension buffer" on the screen) to each sample to have a final volume of 100 µl.
- Distribute **AMPure XP Beads** in lane 1 on 96 **Plate** 1 (and 7 if processing more than 8 samples) according to the required size following recommendations from the table shown below (and on the screen).

NOTE: *Resuspend the beads with pipetting up and down several times before dispensing them.*



🟪 Well 1 & 7

Magnetic beads:

Ampure XP	Final size
110 µl	250 bp
100 µl	280 bp
90 µl	325 bp
80 µl	400 bp
70 µl	500 bp

- Fill the container of the Reagent Rack 1 with freshly prepared 80% ethanol according to the screen.
- Fill the container of Reagent Rack 2 with Low TE buffer pH 8.0 ("Resuspension Buffer" on the screen) according to the screen.

6. Check the proper insertion of the racks and the consumables.

7. Close the door and press "Run" to start.

8. Library size selection is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.

9. After the run, recover your samples on the upper row of the Peltier Block 1. The final volume is 20 µl for each sample.

10. Press “OK” and “Back” until the homepage appears on the screen. Press “Shutdown” and wait until the screen is black before switching off the IP-Star.

NOTE: *Remove all the plastics from the platform, empty the waste shuttle and clean the inner side of the IP-Star with 70% ethanol.*

Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer to the Appendix). The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform. The total molarity is the sum of all the individual libraries’ molarities in the final volume, e.g. if you add 5 µ l of a 10 nM library to 5 µ l of a 20 nM library, you have 10 µ l of a 15 nM pool. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of each library.

FAQ

Can I use the available Illumina primers and validate them with the MicroPlex Kit?

Although the final flanking sequences of MicroPlex are the same as those used by Illumina, the PCR primers are not identical and part of them is supplied with the buffer. For this reason Illumina primers will not work as substitute.

The BioAnalyzer profile of purified library shows the presence of low molecular weight peaks (primers/adaptors) in the samples. Should I re-purify the samples or they can be used directly to the sequencing? If the second purification is recommended, which ratio sample/AMPure beads should I use?

You can do a second round of purification using 1:1 ratio of AMPure beads to sample and this should get rid of the majority of the dimers.

I am going to use the MicroPlex Library Preparation Kit v3 on ChIP samples . Our thermocycler has ramp rate 1.5°/s max while the protocol recommends using a ramp rate 3 to 5°/s. How would this affect the library prep?

We have not used a thermocycler with a ramp rate of 1.5 °C, which seems faster than most of thermocyclers. Too fast of a ramp rate may affect the primer annealing and ligation steps.

What is the function of the replication stop site in the adapter loops?

The replication stop site in the adaptor loops function to stop the polymerase from continuing to copy the rest of the stem loop.

I want to do ChIP-seq. Which ChIP-seq kit can I use for sample preparation prior to Microplex Library Preparation Kit v3?

In our portfolio there are several ChIP-seq kits compatible with Microplex Library Preparation Kit v3. Depending on your sample type and target studied you can use the following kits: iDeal ChIP-seq Kit for Transcription Factors (Cat. No. C01010055), iDeal ChIP-seq Kit for Histones (Cat. No. C01010051), True MicroChIP kit (Cat. No. C01010130), Universal Plant ChIP-seq Kit (Cat. No. C01010152). All these kits exist in manual and automated versions.

Is Microplex Library Preparation Kit v3 compatible with exome enrichment methods?

Microplex Library Preparation Kit v3 is compatible with major exome and target enrichment products, including Agilent SureSelect®, Roche NimbleGen® SeqCap® EZ and custom panels.

What is the nick that is mentioned in the kit method overview?

The nick is simply a gap between a stem adaptor and 3’ DNA end, as shown on the schema in the kit method overview.

Related Products

Product	**Cat. No.**
iDeal ChIP-seq kit for Transcription Factors	C01010055
Auto iDeal ChIP-seq Kit for Transcription Factors	C01010172
iDeal ChIP-Seq Kit for Histones	C01010051
Auto iDeal ChIP-seq Kit for Histones	C01010171
True MicroChIP Kit	C01010132
iDeal FFPE Kit	C01010190
Universal Plant ChIP-seq Kit	C01010152
Auto Universal Plant ChIP-seq kit	C01010153
Bioruptor Pico	B01060010
24 UDI for MicroPlex v3 - Set I (48 rxns)	C05010008
24 UDI for MicroPlex v3 - Set II (48 rxns)	C05010009
24 Dual indexes for MicroPlex Kit v3	C05010003

Technical Support

For technical support contact support.diagenode@hologic.com

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Fragment Analyzer® is registered trademark of Advanced Analytical.

Revision history

Version	Date of modification	Description of modifications
Version 3 03_2025	March 2025	Manual rebranding + Remove obsolete references.

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