For research use only.
Not for use in diagnostic procedures.
For *in vitro* use only.

*Ampli*1™ LowPass kit

Whole Genome Library preparation for Illumina® platforms

USER MANUAL

Version 2.0

Content version: September 2017

REF WGLPIA, WGLPIB

48 reactions

Store WGLPIA and WGLPIB Box at -25°C…-15°C
Store PEG NaCl solution at 15°C…25°C

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1. Kit Contents

This Hand Book	HB
Reagent Name	**Content**
*Ampli*1™ PCR Reaction Buffer	1 x 1100 µl
*Ampli*1™ PCR Taq Polymerase	1 x 20 µl
*Ampli*1™ PCR Water	2 x 1100 µl
*Ampli*1™ PCR dNTPs	1 x 200 µl
*Ampli*1™ PCR BSA	1 x 250 µl
*Ampli*1™ D7xx *****	6 x 30 µl
*Ampli*1™ D5xx *****	8 x 24 µl
*Ampli*1™ P5 primer	1 x 60 µl
*Ampli*1™ P7 primer	1 x 60 µl
*Ampli*1™ SEQ Primer	1 x 30 µl

***** *Ampli*1™ LowPass Kit **Set A** (ref# WGLPIA) provides 48 unique dual-index combinations from D701 to D706 and *Ampli*1™ index from D501 to D508.
***** *Ampli*1™ LowPass Kit **Set B** (ref# WGLPIB) provides 48 unique dual-index combinations from D707 to D712 and *Ampli*1™ index from D501 to D508.

Reagent Name	**Content**
PEG NaCl solution	1 x 10000 µl

2. Storage and Handling

Upon receipt, store the *Ampli*1™ LowPass Box at -25°C…-15°C and PEG NaCl solution at room temperature (RT, 15°C…25°C). Please read this manual carefully before starting. Transfer enzyme tube to ice just prior to use. Other kit components should be thawed, stored on ice and briefly vortexed before use. Prepare reagent master mixes in advance to ensure the magnetic beads do not over-dry during purification steps. Always add reagents to the master mix in the order specified in the protocol. When stored and handled under these conditions the kit components are stable until the indicated expiration date. Handle and store reagents with the appropriate attention and care, and set up reactions according to good laboratory practices. Menarini Silicon Biosystems SpA recommends that the user follows the Guidelines for Research involving Recombinant DNA Molecules (NIH guidelines) Federal Register, July 5, 1994

(59 FR 34496) and any amendments thereto. Menarini Silicon Biosystems SpA disclaims all responsibility for any injury or damage, which may be caused by the failure of the user to follow said guidelines.

3. Intended Use & Product Use Limitation

The *Ampli*1™ LowPass Kit for Illumina® intended use is to produce barcoded sequencing libraries suitable for genome-wide copy-number profiling starting from *Ampli*1™ WGA Kit products; it is not intended for Whole Genome variant analysis or genome assembly applications.

*Ampli*1™ LowPass sequencing method allows a control-free calling of CNAs and is a high-throughput method to analyze several samples with higher processivity and lower cost than array CGH (aCGH).

The product applications include the characterization and investigation of genomic heterogeneity and evolution in tumor cell populations such as individual circulating and disseminated tumor cells (CTCs/DTCs).

The *Ampli*1™ LowPass Kit is intended for research use and for *in vitro* use only. No claim or representation is made for any intended use to provide information for the diagnosis, prevention or treatment of a disease.

4. Safety Information

When working with chemicals always wear suitable personal protective equipment such as a lab coat, disposable gloves, and protective goggles. For more information please consult the appropriate material safety data sheets (MSDS).

MSDS of each Menarini Silicon Biosystems kits and components are available online at http://www.siliconbiosystems.com/msds-documents.

5. Technical Assistance

For technical assistance and additional information, please refer to Menarini Silicon Biosystems Customer Support:

e-mail: ampli1.support@siliconbiosystems.com

Telephone number: (+39) 051-9944100

(Mon-Fri, 9 am – 18 pm CET +01:00)

6. Additional Required Materials & Equipment

- SPRIselect beads (Beckman Coulter, Product #B23317 or B23318)
- Invitrogen DynaMag™, Agencourt SPRIplate or similar magnetic rack for 0.2 ml tubes (Recommended: DynaMag™-96 Side, Product # 12331D or DynaMag™-96 Side Skirted, Product # 12027D).
- 0.2 ml PCR tubes (Recommended: Axygen® 0.2 mL Maxymum Recovery® thin wall PCR Tubes with Flat Cap; Product #PCR-02-L-C)
- 1.5 ml tubes
- Programmable thermal cycler operating within manufacturer's specifications
- Microcentrifuge for 0.2 ml tubes and 1.5 ml tubes
- Vortex-mixer
- Qubit® 2.0 or 3.0 Fluorometer (Thermo Fisher)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher, Product #Q32851)
- Agilent 2100 Bioanalyzer (Agilent Technologies)
- Agilent High Sensitivity DNA Kit (Agilent Technologies, Product #5067-4626)
- Dedicate micropipette sets for pre-PCR and post-PCR area
- Low Retention Filter tips
- Ethanol BioUltra, for molecular biology, ≥99.8%
- Nuclease-free water (molecular biology grade)
- LowTE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- -20°C Storage Freezer
- +4°C Storage Refrigerator

7. *Ampli*1™ LowPass Description

*Ampli*1™ LowPass kit exploits the deterministic nature of *Ampli*1™ Whole Genome Amplification products (WGA) [Menarini Silicon Biosystems, Product code: WG001R (ROW), WG001U (USA)] to generate sequencing-ready libraries with a single-day and single-tube protocol. *Ampli*1™ LowPass kit consists of a novel streamlined, fragmentation-free and size selection-free method, for the generation of Illumina® compatible libraries, making the process suitable for high-throughput liquid handling automation.

For maximum flexibility, the *Ampli*1™ LowPass kits Set A and Set B can be used to generate up to 96 barcoded libraries suitable for Illumina MiSeq®, HiSeq® 1000/1500, 2000/2500, 3000/4000 and NovaSeq™ sequencing instruments.

8. Sample Specifications

***Ampli*1™ LowPass Kit works exclusively with *Ampli*1™ WGA products.**

9. What to Know Before Starting

1. Working Area Organization

In order to prevent any contamination, it is strongly recommended to:

- Wear nitrile/powder-free gloves for all protocol steps, and use only freshly opened plastic ware (e.g. reaction tubes and pipette tips).
- Dedicate a separate working space and use a separate set of pipettes for the pre-PCR (WGA purifications and PCR reactions set up) and post-PCR steps (barcoded libraries handling).
- Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
- Use barrier tips: Gilson Diamond Filter tips in sterilized TIPACK™ or Eppendorf Dualfilter T.I.P.S., PCR Clean are recommended.

2. Tips and techniques

- For each reaction mix, it is strongly recommended to prepare at least 8 samples at the time.
- In order to compensate for the volume losses during pipetting, it is suggested to include a 10% excess in each reaction mix.
- Prepare the reaction mixes in advance to ensure that magnetic beads do not dry out during purification steps.
- Store SPRIselect beads and PEG NaCl solution at room temperature and vortex thoroughly to homogenize the components before use.
- Use fresh 80% ethanol solution (approximately 2 ml per sample), prepared on the same day by diluting absolute ethanol BioUltra with nuclease-free water.
- During purification steps, SPRIselect beads drying time is dependent on temperature and humidity of the working area. Elution reaction mix/buffer should be added when observing bead pellet lack of shininess. At no time "with bead" samples should be stored on ice, this might affect DNA binding to SPRIselect beads.
- When handling barcoded adapters (*Ampli*1™ D7xx, *Ampli*1™ D5xx) open one adapter tube at a time to prevent any cross-contamination.
- To save time all incubation steps of the protocol should be pre-programmed on the thermal cycler.

3. Recommendations and suggestions

- We recommend evaluating the quality of *Ampli*1™ WGA products with *Ampli*1™ QC kit (Menarini Silicon Biosystems, Product code: WG-QC4). This assay is a multiplex PCR of four markers indicative of the quality of the *Ampli*1™ WGA product obtained. Positivity to at least 2 markers has been demonstrated to be predictive of successful genome-wide analysis to detect chromosomal aneuploidies and copy number alteration (CNA).
- We recommend to accurately follow Sequencing Guidelines, on Chapter 11. *Ampli*1™ LowPass Kit has been validated on MiSeq® and HiSeq® platforms, in single-read and paired-end sequencing approaches, using a custom Read1 sequencing primer (***Ampli*1™ SEQ Primer**). In order to obtain at least 500'000 reads per sample, we suggest multiplexing up to 48 libraries on a MiSeq® Flow-cell and up to 96 libraries for each HiSeq® lane.
- It is very important to select unique dual-index combinations (one *Ampli*1™ D5xx and one *Ampli*1™ D7xx) and meet Illumina® recommended compatibility requirements. When designing low-plexity index pools (less than 12) we suggest to follow MiSeq® multiplexing guidelines, on Chapter 11.1.

10. *Ampli*1™ LowPass Procedure

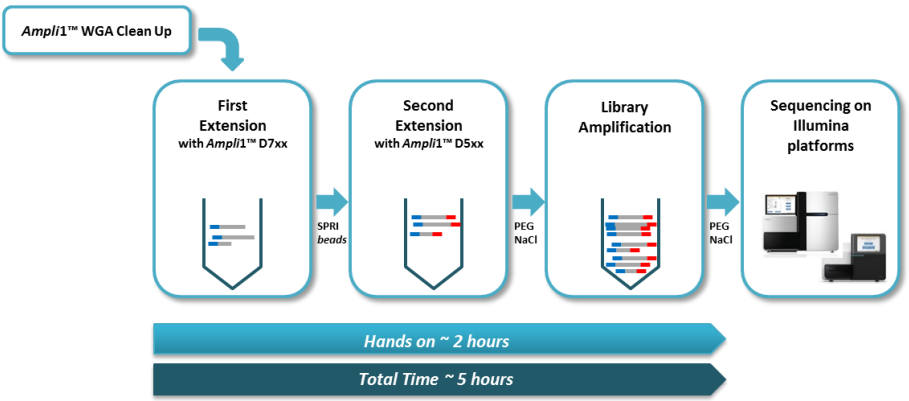

***Figure* 1. *Ampli*1™ LowPass kit workflow.** *Ampli*1™ LowPass kit is a novel three-step method designed to generate Illumina®-compatible libraries, with a hands-on time of only 2 hours.

The *Ampli*1™ LowPass workflow, starting from purified *Ampli*1™ WGA products and consists of three main steps:

1. First Extension (FE) reaction with *Ampli*1™ D7xx index adapter
2. Second Extension (SE) reaction with *Ampli*1™ D5xx index adapter
3. Final Library Amplification (LA)

Between each reaction step, a purification is required (with SPRI beads or PEG NaCl solution). All reactions and purifications, from FE to LA steps, are performed in the same tube; this is facilitated by optimization of clean-up methodology, as depicted in Figure 2. Following final elution in Low TE buffer, library is ready for sequencing on Illumina® platforms.

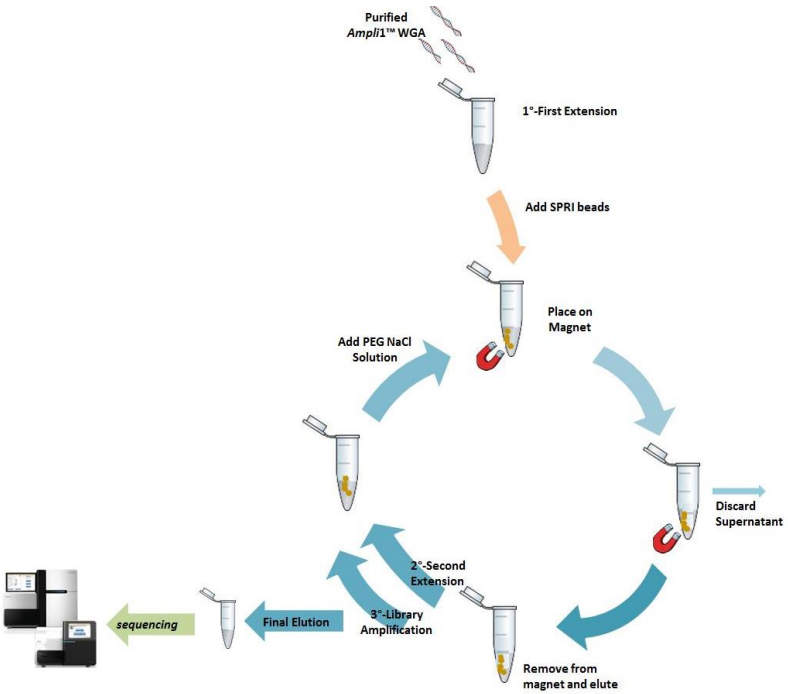

***Figure* 2. Single-tube *Ampli*1™ LowPass procedure.**

Step 1:

***Ampli*1™ WGA clean up**

1. Transfer 5 µl of WGA product in a new 0.2 ml tube and add 5 µl of nuclease free water.
2. Vortex SPRIselect Beads to resuspend.
3. Add 18 µl (1.8X) of resuspended SPRIselect Beads to the diluted WGA product. Mix well by vortexing and briefly spin the sample to collect the liquid from the sides of the tube.
4. Incubate 5 minutes at RT.
5. Place the tube on a magnetic plate to separate the beads from the supernatant. Wait for the solution to clear before proceeding; usually it takes ~5 minutes. Carefully remove and discard the supernatant avoiding disturbing the beads.
6. Add 200 µl of 80% ethanol to the tube while in the magnetic plate. Incubate at RT for 30 seconds, and then carefully remove and discard the supernatant.
7. Repeat the previous step for two washes in total.
8. Remove any last drops of ethanol at the bottom of the tube with a P10 pipette and air dry for about 1 minute. Take care not to over dry the beads.
9. Remove the tube from the magnetic plate. Elute WGA products from beads by adding 22 µl of nuclease free water.
10. Mix well by vortexing and briefly spin down to collect the liquid at the bottom of the tube. Incubate at RT for 2 minutes.

If beads pellet is difficult to resuspend, add nuclease free water and incubate at RT at least for 5 minutes mixing frequently.

11. Place the tube in the magnetic plate for about 5 minutes and carefully transfer 20 µl supernatant to a new PCR tube.

SAFE STOPPING POINT: For short-term storage, keep samples at +2°C…+4°C; For long-term storage, samples shall be kept at -25°C…-15°C.

Step 2:

First Extension (FE) reaction
*Ampli*1™ *D7xx*

1. Prepare the First Extension (FE) reaction mix by adding all components in the order listed in the table below. It is strongly recommended to prepare at least 8 samples at the time and to include a 10% excess to compensate pipetting losses.

Reagent Name	Volume per 1 sample [µl]	Volume per 8 samples [µl]
*Ampli*1™ PCR Water	6.5	52
*Ampli*1™ PCR Reaction Buffer	1.50	12
*Ampli*1™ PCR dNTPs	0.51	4.08
*Ampli*1™ PCR BSA	0.37	2.96
*Ampli*1™ PCR Taq Polymerase	0.12	0.96
Total per reaction	**9**	**72**

2. Once the FE reaction mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube.
3. Dispense 9 µl of FE reaction mix to each pre-labelled tube.
4. Add to each pre-labeled sample tube 3 µl of specific *Ampli*1™ D7xx.
5. Add 3 µl of purified *Ampli*1™ WGA product to each pre-labeled sample tube. The final volume of each reaction is 15 µl.
6. Mix by vortexing and spin down briefly. Place all samples in the thermocycler and run the following FE program.

FE thermocycler program

Cycles	Temperature [C°]	Time
	95	4 minutes
1	95	30 sec
	62	30 sec
	72	3 minutes
	10	∞

SAFE STOPPING POINT: For short-term storage, up to overnight storage, keep samples at +2°C…+4°C.

Step 3:

Second Extension (SE) reaction
*Ampli*1™ D5xx

Prepare the Second Extension (SE) reaction mix in advance to ensure that magnetic beads do not dry out during FE clean up.

Step 3.1: Second Extension (SE) reaction mix

1. Prepare the SE reaction mix by adding all components in the order listed in the table below. Calculate the amount needed for the number of samples you are analyzing; it is strongly recommended to prepare at least 8 samples at the time and to include a 10% excess to compensate pipetting losses.

Reagent Name	Volume per 1 sample [µl]	Volume per 8 samples [µl]
*Ampli*1™ PCR Water	9.5	76
*Ampli*1™ PCR Reaction Buffer	1.50	12
*Ampli*1™ PCR dNTPs	0.51	4.08
*Ampli*1™ PCR BSA	0.37	2.96
*Ampli*1™ PCR Taq Polymerase	0.12	0.96
Total per reaction	**12**	**96**

2. Once the SE reaction mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube, then store it on ice before use.

Step 3.2: FE product clean up

1. Ensure SPRIselect beads and FE samples (prepared in **Step 2**) are at room temperature. Vortex SPRIselect Beads to resuspend.
2. Add 22.5 µl (1.5X) of resuspended SPRIselect Beads to the FE product prepared in **Step 2**. Mix well by vortexing and briefly spin the sample to collect the liquid from the sides of the tube.
3. Incubate 5 minutes at RT.
4. Place the tube on a magnetic plate to separate the beads from the supernatant. Wait for the solution to clear before proceeding; usually it takes ~5 minutes. Carefully remove and discard the supernatant avoiding disturbing the beads.

5. Add 200 µl of 80% ethanol to the tube while in the magnetic plate. Incubate at RT for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat the previous step for two washes in total.
7. Remove any last drops of ethanol at the bottom of the tube with a P10 pipette and air dry for about 1 minute. Take care not to over dry the beads.
8. Remove the tube from the magnetic plate.
9. Elute purified FE product from beads by adding 12 µl SE reaction mix prepared in advance (**Step 3.1**).
10. Add to each FE sample tube 3 µl of specific *Ampli*1™ D5xx; the final volume of each SE reaction is 15 µl.
11. Mix the SE reaction by vortexing and spin down briefly.

If beads pellet is difficult to resuspend, add SE reaction mix and incubate at RT at least for 5 minutes mixing frequently.

Step 3.3: Run the SE reaction

1. Place all samples in the thermocycler and run the following SE program.

Note: SE reaction should be carried out in presence of SPRIselect beads.

SE thermocycler program

Cycles	Temperature [C°]	Time
	95	4 minutes
1	95	30 sec
	60	30 sec
	72	3 minutes
	10	∞

2. Proceed immediately to **Step 4**.

Step 4:

Library Amplification

Prepare the Library Amplification (LA) reaction mix in advance to ensure that magnetic beads do not dry out during SE clean up.

Step 4.1: Library Amplification (LA) reaction mix

1. Prepare the LA reaction mix by adding all components in the order listed in the table below. Calculate the amount needed for the number of samples you are analyzing; it is strongly recommended to prepare at least 8 samples at the time and to include a 10% excess to compensate pipetting losses.

Reagent Name	Volume per 1 sample [µl]	Volume per 8 samples [µl]
*Ampli*1™ PCR Water	10.50	84
*Ampli*1™ PCR Reaction Buffer	1.50	12
*Ampli*1™ P5 primer	1	8
*Ampli*1™ P7 primer	1	8
*Ampli*1™ PCR dNTPs	0.51	4.08
*Ampli*1™ PCR BSA	0.37	2.96
*Ampli*1™ PCR Taq Polymerase	0.12	0.96
Total per reaction	**15**	**120**

2. Once the LA reaction mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube, then store it on ice before use.

Step 4.2: SE product clean up

1. Ensure PEG NaCl solution and SE samples (prepared in **Step 3.3**) are at room temperature. Vortex PEG NaCl solution to mix.
2. Add 22.5 µl (1.5X) of mixed PEG NaCl solution to the SE products prepared in **Step 3.3**. Mix well by vortexing and briefly spin the sample to collect the liquid from the sides of the tube.
3. Incubate 5 minutes at RT.
4. Place the tube on a magnetic plate to separate the beads from the supernatant. Wait for the solution to clear before proceeding; usually it takes ~5 minutes. Carefully remove and discard the supernatant avoiding disturbing the beads.
5. Add 200 µl of 80% ethanol to the tube while in the magnetic plate.

Incubate at RT for 30 seconds, and then carefully remove and discard the supernatant.

6. Repeat the previous step for two washes in total.
7. Remove any last drops of ethanol at the bottom of the tube with a P10 pipette and air dry for about 1 minute. Take care not to over dry the beads.
8. Remove the tube from the magnetic plate.
9. Elute purified SE product from beads by adding 15 µl LA reaction mix prepared in advance (**Step 4.1**).
10. Mix by vortexing and spin down briefly.

If beads pellet is difficult to resuspend, add LA reaction mix and incubate at RT at least for 5 minutes mixing frequently.

Step 4.3: Run the LA reaction

1. Place all samples in the thermocycler and run the following LA program.

Note: LA reaction should be carried out in presence of SPRIselect beads.

LA thermocycler program

Cycles	Temperature [C°]	Time
	95	4 Minutes
1	95	30 Sec
	60	30 Sec
	72	2 Minutes
10	95	30 Sec
	60	30 Sec
	72	2 Minutes (+20 sec/cycle)
	72	7 Minutes
	10	∞

2. Proceed immediately to **Step 5**.

Step 5:

***Ampli*1™ LowPass Library clean up**

1. Ensure PEG NaCl solution and libraries (prepared in **Step 4.3**) are at room temperature. Vortex PEG NaCl solution to mix.
2. Add 22.5 µl (1.5X) of mixed PEG NaCl solution to the library prepared in **Step 4.3**. Mix well by vortexing and quickly spin the sample to collect the liquid from the sides of the tube.
3. Incubate 5 minutes at RT.
4. Place the tube on a magnetic plate to separate the beads from the supernatant. Wait for the solution to clear before proceeding; usually it takes ~ 5 minutes. Carefully remove and discard the supernatant avoiding disturbing the beads.
5. Add 200 µl of 80% ethanol to the tube while in the magnetic plate. Incubate at RT for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat the previous step for two washes in total.
7. Remove any last drops of ethanol at the bottom of the tube with a P10 pipette and air dry for about 1 minute. Take care not to over dry the beads.
8. Remove the tube from the magnetic plate. Elute purified library from beads by adding 17 µl Low TE.
9. Mix well by vortexing and briefly spin down to collect the liquid at the bottom of the tube.
10. Incubate at RT for 2 minutes.

If beads pellet is difficult to resuspend, add Low TE and incubate at RT at least for 5 minutes mixing frequently.

11. Place the tube in the magnetic plate for about 5 minutes and carefully transfer 15 µl supernatant to a new PCR tube.

SAFE STOPPING POINT: For short-term storage, keep samples at +2°C…+4°C; For long-term storage, samples shall be kept at -25°C…-15°C.

Step 6:

Quantify and combine *Ampli*1™ LowPass libraries

This section describes two procedures (6 A or 6 B) for pooling *Ampli*1™ LowPass libraries with unique dual-index combinations to a standard final concentration of 4 nM:

- **Step 6 A:** This procedure combines Ampli1™ LowPass libraries in a final pool using the Ampli1™ LowPass library average size (bp), determined by Bioanalyzer (Agilent Technologies), and the Ampli1™ LowPass library quantity (ng/µL), measured by Qubit® (Thermo Fisher). Therefore, this procedure is expected to give a balanced pool.
- **Step 6 B:** This procedure combines *Ampli*1™ LowPass libraries in a final pool using the *Ampli*1™ LowPass library quantity (ng/µL), measured by Qubit® (Thermo Fisher), assuming that the size range of all individual *Ampli*1™ LowPass library is on average 700bp. This procedure could result in unbalanced library pooling. It is suggested to choose this strategy if, based on your previous experience, *Ampli*1™ LowPass libraries have similar average size.

Step 6 A: Quantify and combine *Ampli*1™ LowPass libraries by Qubit® and by Bioanalyzer

- Analyze 2 µl of each *Ampli*1™ LowPass library using the Qubit® 2.0 or 3.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit, see the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326) for more information.
- Assess library quality by Agilent DNA HS Kit on the 2100 Bioanalyzer. Load 1 µl of *Ampli*1™ LowPass library on the Agilent® High Sensitivity DNA chip. Run the chip according to manufacturer's instructions. Calculate the average size (bp), by Agilent 2100 Bioanalyzer Software, selecting a region in in the "Results table" and edit it on the "Regions tab", as shown in Figure 3. If necessary, follow the manufacture's instruction to perform a region analysis (smear analysis) placing the entire distribution of library fragments within a single region.

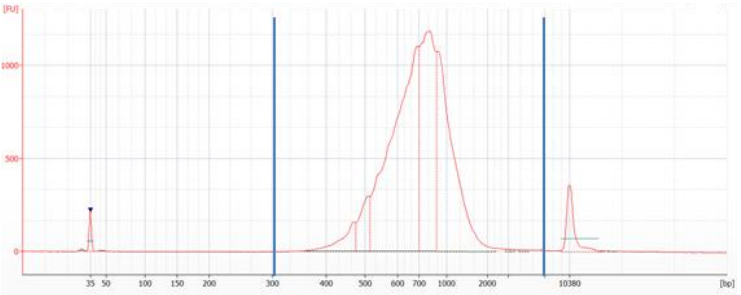

***Figure* 3: Example of successful *Ampli*1™ LowPass library.** The *Ampli*1™ LowPass library expected average size is ~700bp. If the concentration, determined by Bioanalyzer analysis, is > 10ng/µL, repeat the analysis using a 1:5 dilution of the library.

- Calculate the molar concentration using the conversion factor guidelines listed in Table 1 below (the average library size should be round-up to the nearest hundredth).

Average Library size (bp)	Conversion factor
600	**1 ng/µL = 2.5 nM**
700	**1 ng/µL = 2 nM**
800	**1 ng/µL = 1.9 nM**

***Table* 1**. The *Ampli*1™ LowPass library average size (bp) is determined by Bioanalyzer; the *Ampli*1™ LowPass library quantity (ng/µL) is measured by Qubit®.

Example: If the average size of the *Ampli*1™ LowPass library is 800bp, use the conversion factor: 1ng/µL = 1.9 nM. Therefore, if the concentration determined by Qubit® analysis is 10ng/µL, the final molar concentration will be 19nM.

- Mix together equal amounts of each *Ampli*1™ LowPass library to obtain a 4nM pool.
  Example: Dilute all individual *Ampli*1™ LowPass libraries to a 4nM and add 2µL of each *Ampli*1™ LowPass library to a single tube.

IMPORTANT: This procedure is expected to give a balanced pool.

Step 6 B: Quantify and combine *Ampli*1™ LowPass libraries by Qubit®

- Analyze 2 µl of each *Ampli*1™ LowPass library using the Qubit® 2.0 or 3.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit, see the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326) for more information.
- Calculate the molar concentration for each *Ampli*1™ LowPass library using the following conversion factor:

  1ng/µL = 2nM

  Example: If the concentration of the *Ampli*1™ LowPass library, determined by Qubit® analysis, is 10ng/µL the molar concentration will be 20nM.

- Mix together equal amounts of each *Ampli*1™ LowPass library to obtain a 4nM pool.

  Example: Dilute all individual *Ampli*1™ LowPass libraries to a 4nM and add 2 µL of each *Ampli*1™ LowPass library to a single tube.

IMPORTANT: This step assumes that the size range of all individual *Ampli*1™ LowPass libraries is on average 700bp. Choosing this procedure could result in unbalanced library pooling.

11. Sequencing Guidelines

- The final *Ampli*1™ LowPass library pool is ready for direct sequencing on Illumina MiSeq®, HiSeq® and NovaSeq™ platforms, using a custom Read1 primer (***Ampli*1™ SEQ primer**). For further details, see Sequencing Guidelines in Table 2.
- *Ampli*1™ LowPass libraries sequencing can be performed in single-read (SR) or paired-end (PE). In case of paired-end sequencing, the first portion of second read will contain an *Ampli*1™ WGA adapter. To remove the adapter from the raw sequence, proceed trimming as specified in Data Processing Recommendations (Chapter 13).
- *Ampli*1™ LowPass library is designed with Illumina TruSeq HT dual-index adapter sequences; each index is 8 bases in length. For indexes sequence information, see Chapter 12.
- When designing low-plexity index pools (less than 12 libraries) we suggest to follow MiSeq® multiplexing guidelines (Chapter 11.1).
- The sequencing run must be performed using the *Ampli*1™ SEQ primer, supplied in *Ampli*1™ LowPass kit Set A and Set B (ref# WGLPIA and WGLPIB). This custom primer is needed to sequence the Read 1. The *Ampli*1™ SEQ primer is concentrated at 60 µM. Dilute the *Ampli*1™ SEQ primer with HT1 buffer (Illumina) prior each sequencing experiment following indications reported in Table 2 below. For further information see "MiSeq System: Custom Primers Guide, Document # 15041638 v01 or "HiSeq System: Custom Primers Guide, Document # 15061846 v02".
- Because the *Ampli*1™ SEQ custom primer is used for Read 1, instead of Illumina sequencing primer, the Illumina PhiX library control cannot be sequenced.
- The number of combined *Ampli*1™ LowPass libraries that can be accommodated in a single sequencing run depends on the Illumina® system and on the sequencing chemistry chosen (see Sequencing Guidelines in Table 2).
- See additional Sequencing Guidelines for the MiSeq® (Chapter 11.1 and 11.2) and HiSeq® (Chapter 11.3) systems.

***Table* 2. Sequencing Guidelines.** Use the following guidelines to design your sequencing strategy.

Illumina® Sequencing platform	Chemistry	Reagent Kit	Level of multiplexing of *Ampli*1™ LowPass libraries	*Ampli*1™ SEQ custom primer dilution *
MiSeq®	150 SR 75 PE	v3 (150 cycles)	Up to 48	600 µl at 0.5 µM
HiSeq® 2500/1500 and 2000/1000	50 SR	Rapid SR Cluster Kit v2 + Rapid SBS Kit v2 (50 cycles) + TruSeq Rapid Duo cBot Sample Loading Kit	Up to 96/lane**	2 ml at 0.5 µM
		SR Cluster Kit v4-cBot-HS + SBS Kit v4-HS (50 cycles)	Up to 96/lane	5 ml at 0.5 µM (150 µl into each tube of an 8-tube strip for use on the cBot)
	100 PE	Rapid SBS Kit + Rapid SBS Kit v2 (200 cycles) + TruSeq Rapid Duo cBot Sample Loading Kit	Up to 96/lane**	2 ml at 0.5 µM
	125 PE	PE Cluster Kit v4-cBot-HS + SBS Kit v4-HS (250 cycles)	Up to 96/lane	5 ml at 0.5 µM (150 µl into each tube of an 8-tube strip for use on the cBot)
HiSeq® 3000/4000	50 SR	SR Cluster Kit + SBS Kit (50 cycles)	Up to 96/lane	5 ml at 0.5 µM (150 µl into each tube of an 8-tube strip for use on the cBot)
	150 SR	SR Cluster Kit + SBS Kit (150 cycles)		
NovaSeq™ 5000/6000	50 PE	S2 Reagent Kit (100 cycles)	Up to 96/lane	4 ml at 0.3 µM

SR) Single-Read**; PE)** Paired-End

* *Ampli*1™ SEQ custom primer [60 µM] needs to be diluted, as indicated in the table, using HT1 and then loaded to the reagent cartridge. Please refer to Illumina's technical manuals (MiSeq System: Custom Primers Guide, Document # 15041638 v01; HiSeq System: Custom Primers Guide, Document # 15061846 v02) for additional information.

** TruSeq Rapid Duo cBot Sample Loading Kit allows to load separately each rapid flow-cell lane with a unique sample pool and it is only available in the rapid run mode of the HiSeq® 2500 or 1500.

11.1 MiSeq® multiplexing guidelines

It is very important to select appropriate dual-index combinations (one *Ampli*1™ D5xx and one *Ampli*1™ D7xx) such that they are unique and meet Illumina® recommended compatibility requirements.

When designing low-plexity index pools (less than 12) we suggest to take account the following figures that illustrate possible pooling strategies for 8 or 12 libraries generated with *Ampli*1™ LowPass kits.

When combining more than 12 libraries into one pool, many multiplexing are allowed.

Dual-index combinations may also be evaluated for compatibility using Illumina® Experiment Manager Software.

Dual-Indexed–8-plex. Set A *Ampli*1™ LowPass indexes are indicated in light blue;
Set B *Ampli*1™ LowPass indexes are indicated in violet.

*Ampli*1™	D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D501	x	x	x	x	x	x	x	x	x	x	x	x
D502	x	x	x	x	x	x	x	x	x	x	x	x
D503	x	x	x	x	x	x	x	x	x	x	x	x
D504	x	x	x	x	x	x	x	x	x	x	x	x
D505	x	x	x	x	x	x	x	x	x	x	x	x
D506	x	x	x	x	x	x	x	x	x	x	x	x
D507	x	x	x	x	x	x	x	x	x	x	x	x
D508	x	x	x	x	x	x	x	x	x	x	x	x

Dual-Indexed–12-plex. Set A *Ampli*1™ LowPass indexes are indicated in light blue;
Set B *Ampli*1™ LowPass indexes are indicated in violet.

*Ampli*1™	D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D501	x	x	x	x	x	x	x	x	x	x	x	x
D502	x	x	x	x	x	x	x	x	x	x	x	x
D503	x	x	x	x	x	x	x	x	x	x	x	x
D504	x	x	x	x	x	x	x	x	x	x	x	x
D505	x	x	x	x	x	x	x	x	x	x	x	x
D506	x	x	x	x	x	x	x	x	x	x	x	x
D507	x	x	x	x	x	x	x	x	x	x	x	x
D508	x	x	x	x	x	x	x	x	x	x	x	x

11.2 MiSeq® sequencing guidelines

1. Denature and dilute *Ampli*1™ LowPass library pool for sequencing on the Illumina MiSeq® system, according to the manufacturer's protocol.
2. Dilute *Ampli*1™ SEQ primer with HT1 to obtain a final concentration of 0.5 µM, as follows: **5 µL of *Ampli*1™ SEQ primer + 595 µL of HT1 Illumina buffer**.
   600 µL of diluted *Ampli*1™ SEQ primer is loaded on the MiSeq®reagent cartridge in the position 18 for Read 1 Custom Primer (see "MiSeq System: Custom Primers Guide, Document # 15041638 v01).
3. Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below:
   - On the Instrument selection page, select "MiSeq";
   - In the MiSeq Application Selection page, select category "Other" and select application "FASTQ Only";
   - For Single-Read sequencing run:
     on the workflow parameter, select:
   - "TruSeq HT" as the Library Prep Kit
   - Index Reads: "2"
   - Read Type: "Single Read"
   - Cycles Read 1: "151"
   - **Checkbox "Custom Primer for read 1"**
   - For Paired-End sequencing run:
     on the workflow parameter page, select:
   - "TruSeq HT" as the Library Prep Kit
   - Index Reads: "2"
   - Read Type: "Paired End"
   - Cycles Read 1: "76"
   - Cycles Read 2: "76"
   - **Checkbox "Custom Primer for read 1"**
   - Make sure the "Use adapter trimming Read 1" and "Use adapter trimming Read 2" are selected.
   - Fill the sample table with Sample IDs and associated index 1 and index 2 (please refer to the Table 3 and Table 4A/4B, in the Indexing Kit Contents, Chapter 12).

11.3 HiSeq® sequencing guidelines

1. Denature and dilute *Ampli*1™ LowPass library pool for sequencing on the Illumina HiSeq® system, according to the manufacturer's protocol.
2. Dilute the *Ampli*1™ SEQ primer with HT1 at final concentration of 0.5 µM, as follows:
   - For **Rapid** run mode: **16.66 µL of *Ampli*1™ SEQ primer + 1983.33 µL of HT1 Illumina buffer**.
   - For **High Output** run mode: **41.66 µL of *Ampli*1™ SEQ primer + 4958.33 µL of HT1 Illumina buffer**.
3. Dispense the diluted *Ampli*1™ SEQ primer, as indicated in Table 2.
4. Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below:
   - On the Instrument selection page, select "HiSeq";
   - In the HiSeq Application Selection page, select category "Other" and select application "HiSeq FASTQ Only";
   - For Single-Read sequencing run:
     on the workflow parameter, select:
     - "TruSeq HT" as the Library Prep Kit
     - Index Reads: "2"
     - Read Type: "Single Read"
     - Cycles Read 1: "51"
     - **Checkbox "Custom Primer for read 1"**
   - For Paired-End sequencing run:
     on the workflow parameter page, select:
     - "TruSeq HT" as the Library Prep Kit
     - Index Reads: "2"
     - Read Type: "Paired End"
     - Cycles Read 1: "76"
     - Cycles Read 2: "76"
     - **Checkbox "Custom Primer for read 1"**
   - Make sure the "Use adapter trimming Read 1" and "Use adapter trimming Read 2" are selected.
   - Fill the sample table with Sample IDs and associated index 1 and index 2 (please refer to the Table 3 and Table 4A/4B, in the Indexing Kit Contents, Chapter 12).

12. Indexing Kit Contents

The *Ampli*1™ LowPass Kit is designed with Illumina TruSeq HT dual index adapter sequences. Therefore, it is allows to perform single-read (SR) or paired-end (PE) run only with dual-indexing sequencing. Please refer to Illumina® "Indexed Sequencing: Overview Guide" Document# 15057455 v03, for additional information.

*Ampli*1™ LowPass Kit consists of two sets:

- *Ampli*1™ LowPass Kit **Set A** provides a set of unique *Ampli*1™ index from D701 to D706 (Table 4A) and *Ampli*1™ index from D501 to D508 (Table 3).
- *Ampli*1™ LowPass Kit **Set B** provides a set of unique *Ampli*1™ index from D707 to D712 (Table 4B) and *Ampli*1™ index from D501 to D508 (Table 3).

***Table* 3.** *Ampli*1™ LowPass indexes included both in Set A and Set B

***Ampli*1™ D5xx**	**Sequences for Sample Sheet**
D501	TATAGCCT
D502	ATAGAGGC
D503	CCTATCCT
D504	GGCTCTGA
D505	AGGCGAAG
D506	TAATCTTA
D507	CAGGACGT
D508	GTACTGAC

***Table* 4A.** *Ampli*1™ LowPass indexes included in the Set A

***Ampli*1™ D7xx**	**Sequences for Sample Sheet**
D701	ATTACTCG
D702	TCCGGAGA
D703	CGCTCATT
D704	GAGATTCC
D705	ATTCAGAA
D706	GAATTCGT

***Table* 4B.** *Ampli*1™ LowPass indexes included in the Set B

***Ampli*1™ D7xx**	**Sequences for Sample Sheet**
D707	CTGAAGCT
D708	TAATGCGC
D709	CGGCTATG
D710	TCCGCGAA
D711	TCTCGCGC
D712	AGCGATAG

13. Data Processing Recommendations

- **Trimming adapter sequences**
  In case of paired-end sequencing, the insert of the libraries produced with *Ampli*1™ LowPass Kits (ref# WGLPIA and WGLPIB), for Illumina® platforms, contains an *Ampli*1™ WGA adapter at second end of the fragment.
  This adapter can be trimmed from the sequences in FASTQ format using cutadapt software (https://github.com/marcelm/cutadapt) with the following command:

  cutadapt -a ACTGACAGCAGGAATCCCACT -g AGTGGGATTCCTGCTGTCAGT -o output.fastq -e 0.2 input.fastq

  In case of single-end sequencing, adapter trimming is not necessary.

- **Copy Number Alterations (CNA) Analysis**
  FASTQ files can be aligned on a genome sequence using BWA software for obtaining BAM alignments required for computing copy-number profiles.
  Several software packages are available for CNA calling from whole genome sequencing data. Based on our experience we suggest processing data with Control-FREEC software by Boeva *et al* 2011 which can be freely downloaded from http://bioinfo-out.curie.fr/projects/freec/.

14. Patent & Trademark Information

This product is patent pending.
The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product. Menarini Silicon Biosystems SpA products may not be transferred to third parties, resold, modified for resale, used to manufacture commercial products without written approval of Menarini Silicon Biosystems SpA.
*Ampli*1™ is a trademark of Menarini Silicon Biosystems SpA, or its subsidiaries, which may be registered in certain jurisdictions. Other brands and product names are trademarks of their respective holders.

15. Warranty

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16. Annex 1: Chemicals Classification

EU Regulation 1272/08 ("CLP" Regulation) art. 28 sub. 1 and sub. 3, art. 29 sub. 2 and Annex I sub. 1.5.2.1 allow to report in the secondary label only the most important information about chemicals classification.


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