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

*Ampli*1™ LowPass kit for Illumina®

Whole Genome Library preparation for NGS Illumina® platforms

USER MANUAL

Version 3.0

REF		48 reactions Storage
KI0123	***Ampli*1™ LowPass Kit for Illumina® - Set A**	
KI0041	*Ampli*1™ LowPass Kit for Illumina® - Set A Reagents	-25°C…-15°C
KI0112	*Ampli*1™ LowPass Kit for Illumina® - PEG NACL	+15°C…+25°C
KI0125	***Ampli*1™ LowPass Kit for Illumina® - Set B**	
KI0040	*Ampli*1™ LowPass Kit for Illumina® - Set B Reagents	-25°C…-15°C
KI0112	*Ampli*1™ LowPass Kit for Illumina® - PEG NACL	+15°C…+25°C

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1. Kit Contents

***Ampli*1™ LowPass Kit for Illumina®**, thereinafter *Ampli*1 LowPass Kit, consists of two Sets (Set A ref. **KI0123** and Set B ref. **KI0125**) and each Set provides 48 unique dual-index combinations and allows up to 48 reactions of library preparation.
In addition, each set is composed by two boxes:
- Box 1, consisting of *Ampli*1 LowPass kit reagents,
- Box 2, consisting of PEG NaCl solution.

Box 1 contains *Ampli*1 LowPass kit reagents	
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 [60 µM]	1 x 30 µl

***** *Ampli*1 LowPass Kit **Set A** provides 48 unique dual-index combinations from D701 to D706 and *Ampli*1 index from D501 to D508.
***** *Ampli*1 LowPass Kit **Set B** provides 48 unique dual-index combinations from D707 to D712 and *Ampli*1 index from D501 to D508.

Box 2 contains PEG NaCl solution:	
Reagent Name	**Content**
PEG NaCl solution	1 x 5000 µl

2. Storage and Handling

Upon receipt, store the *Ampli*1 LowPass kit reagents (Box 1/2) at -25°C…-15°C and PEG NaCl solution (Box 2/2) at room temperature (RT, +15°C…+25°C). Read this manual carefully before starting.
Avoid repeated freeze-thawing cycles. *Ampli*1 LowPass kit can undergo up to 6 freeze-thawing cycles without any reduction in performance.
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. It is recommended 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 responsibilities for any injury or damage, which may be caused by the failure of the user to follow said guidelines.

3. 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 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.

4. Technical Assistance

For technical assistance and additional information, contact the Customer Support:
E-mail: ampli1.support@siliconbiosystems.com
Telephone: (+39) 051-9944100, Mon-Fri, 9 am – 18 pm CET +01:00

5. Intended Use & Product Use Limitation

The ***Ampli*1 LowPass Kit for Illumina** is intended 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.

6. Product Description

*Ampli*1 LowPass kit exploits the deterministic nature of *Ampli*1 Whole Genome Amplification products (WGA) [Menarini Silicon Biosystems, Ref. WG001R or KI0030] 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.

7. Additional Required Materials & Equipment

- SPRIselect beads (Beckman Coulter, Ref. B23317 or B23318);
- Invitrogen DynaMag™, Agencourt SPRIplate or similar magnetic rack for 0.2 ml tubes (Recommended: DynaMag™-96 Side, Ref. 12331D or DynaMag™-96 Side Skirted, Ref. 12027D);
- 0.2 ml PCR tubes (Recommended: Axygen® 0.2 mL Maxymum Recovery® thin wall PCR Tubes with Flat Cap; Ref. PCR-02-L-C);
- Dedicate micropipette sets for pre-PCR and post-PCR area;
- Low Retention Filter tips;
- 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, Ref. Q32851/4);
- Agilent 2100 Bioanalyzer (Agilent Technologies);
- Agilent High Sensitivity DNA Kit (Agilent Technologies, Ref. 5067-4626);
- Real-Time qPCR Instrument;
- qPCR-based library quantification assay for Illumina® libraries (recommended: KAPA Library Quantification Kit Illumina Platforms [Roche] product ref. depends on Real-Time qPCR Instrument type];
- NaOH for molecular biology;
- 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; suggested: TE Buffer ThermoFisher, Ref. 12090015);
- -20°C Storage Freezer;
- +4°C Storage Refrigerator.

8. Sample Specifications

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

9. What to Know Before Starting

Laboratory setup

- Use good laboratory practices to minimize cross-contamination of products and reagents.
- Use 0.2 ml PCR tubes, 8-tube strips or 0.2 ml PCR plates (96-well format) according to the labware and sample throughput.
- Verify if 96-Well Tray is needed on the thermocycler to load tubes and 8-tube strips, in order to provide the thermal contact necessary for high-performance thermal cycling.
- To save time all incubation steps of the protocol should be pre-programmed on the thermocycler.
- Verify that the correct cycling protocol is being used prior to starting the thermocycler.
- Use aerosol-barrier pipette tips: Gilson Diamond Filter tips in sterilized TIPACK™ or Eppendorf Dualfilter T.I.P.S., Low Retention PCR Clean are recommended.

Working Area Organization

- Wear nitrile/powder-free gloves for all protocol steps, and use only freshly opened plastic ware (e.g. reaction tubes and pipette tips).
- Perform *Ampli*1 WGA clean up and Barcoding reactions in a separate working area or room from library clean up step and downstream processing.
- Use a separate set of pipettes for *Ampli*1 WGA clean up and Barcoding reactions from those used for library clean up step and downstream processing.
- Before beginning and after use, wash the working surface using 0.5% sodium hypochlorite (10% bleach).

Pipetting recommendations

- In order to compensate volume losses during pipetting, it is suggested to include a 10% excess in each reaction mix.
- Each reaction mix must be prepared at least for 8 samples at the time.
- Prepare the reaction mixes in advance to ensure that magnetic beads do not dry out during purification steps.
- Use fresh 80% ethanol solution (approximately 2 ml per sample), prepared on the same day by diluting absolute ethanol BioUltra with nuclease-free water.
- Store SPRIselect beads and PEG NaCl solution at room temperature and vortex thoroughly to homogenize the components before use.
- 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.
- Be sure to remove all of the ethanol from the bottom of the well as it is a known PCR inhibitor, during the washing step of clean up procedures.
- At no time “with bead” samples should be stored on ice, this might affect DNA binding to

SPRIselect beads.

- Visually check tips to ensure volumes are equivalent if using a multi-channel pipette.
- Touch tip to side of well and slowly dispense reagent on the side of the well/tube to form a droplet. This enables you both to pipette small volumes accurately and to see that you added reagent to the well.
- When performing elution from beads dispense reagents directly on the beads pellet (dispensing with the tip touching the side of the tube/well at an angle and dragging it slightly along the wall where the beads pellet is).
- During purification steps, if beads pellet is difficult to resuspend, extend the incubation at Room Temperature (RT), at least for 5 minutes, and mix frequently (vortexing thoroughly and/or pipetting up and down).
- Before vortexing and spin down samples and reagents, ensure that the tubes and plate-wells are correctly closed.
- Verify that an accurate amount of reagents are dispensed in each tube/well, and prevent excess carryover from droplets adhering to the tip.

Tips and recommendations

- We recommend evaluating the quality of Ampli1 WGA products with Ampli1 QC kit (Menarini Silicon Biosystems, ref. WG-QC4 or KI0027). This assay is a multiplex PCR of four markers indicative of the quality of the Ampli1 WGA product obtained. Positivity to at least 2 markers (GII>=2) has been demonstrated to be predictive of successful genome-wide analysis to detect chromosomal aneuploidies and copy number alteration (CNA).
- When handling barcoded adapters (Ampli1 D7xx, Ampli1 D5xx) open one adapter tube at time to prevent any cross-contamination.
- We recommend to accurately follow Sequencing Guidelines, on Chapter 11. Ampli1 LowPass Kit has been validated on MiSeq® and HiSeq® platforms, in single-read and paired-end sequencing approaches, using a custom Read1 sequencing primer (**Ampli1 SEQ primer).**
- Since paired-end sequencing is not required for the low-pass genome-wide copy-number profiling, it is recommended to use the single-read sequencing approach. In case of paired-end sequencing, the first portion of second read will contain an Ampli1 WGA adapter. Details for removing adapter are specified in Data Processing Recommendations (Chapter 13).

10. *Ampli*1 LowPass Procedure

*Ampli*1™ LowPass kit is a single-tube and three-step method designed to generate Illumina-compatible libraries, with a hands-on time of only 2 hours (Figure 1).

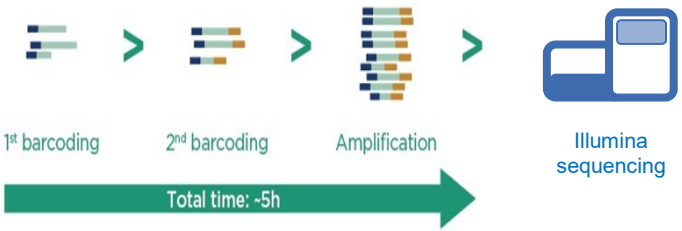

Figure 1. *Ampli*1 LowPass kit workflow.
The *Ampli*1 LowPass workflow, starting from purified *Ampli*1 WGA products, consists of three main steps:
1) First barcoding reaction with *Ampli*1 D7xx index adapter;
2) Second barcoding reaction with *Ampli*1 D5xx index adapter;
3) Final Library Amplification.
Between each reaction step, a purification is required (with SPRI beads or PEG NaCl solution).

All reactions and purifications, from First Barcoding reaction up to Library Clean up, are performed in the same tube; this is facilitated by optimization of clean-up methodology (Figure 2). Following the final elution in Low TE buffer, library is ready for sequencing on Illumina System.

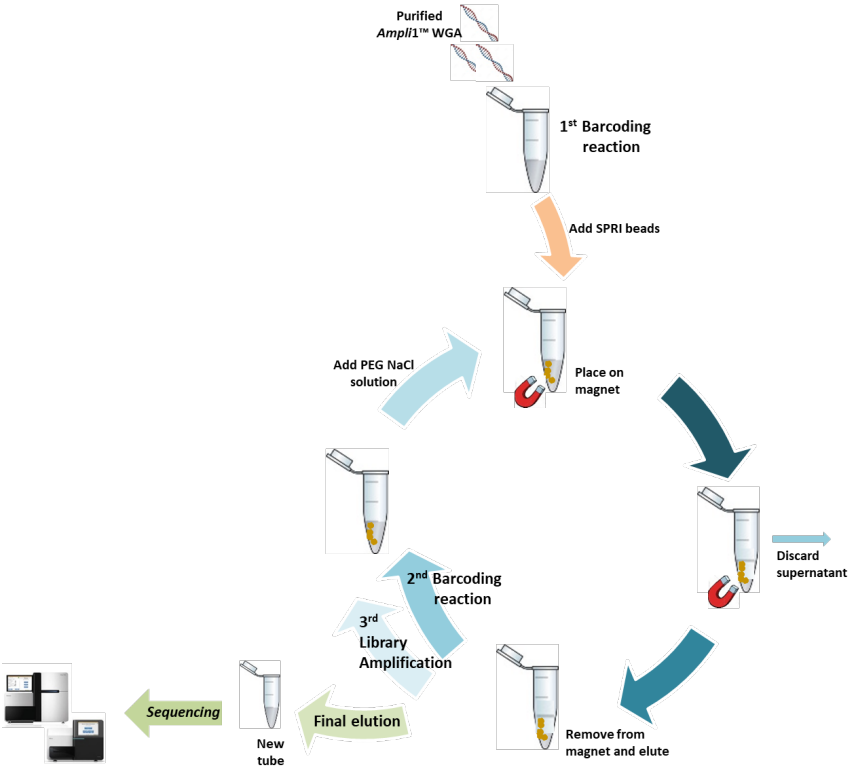

Figure 2. Single-tube Ampli1 LowPass procedure. Representation of single-tube library preparation, based on three-round clean-up methodology.

Step 1 of 7: *Ampli*1 WGA clean up	
	1. Transfer 5 µl of *Ampli*1 WGA sample in a new tube/well plate and add 5 µl of nuclease-free water. ***NOTE**: Choose performing reactions in 0.2 ml PCR tubes or 0.2 ml PCR plates (96-well format) depending on labware and sample throughput.*
Beads Binding	2. Thoroughly shake the SPRIselect Beads bottle to resuspend any magnetic particles that may have settled. 3. Add 18 µl (1.8X) of resuspended SPRIselect Beads to the diluted WGA sample. 4. Mix thoroughly by pipette mixing 10 times (in case of PCR plate) or vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube). ***NOTE**: The colour of the mixture should appear homogeneous after mixing.* 5. Let the mixed samples incubate for 5 minutes at room temperature (RT) for maximum recovery.
Separation	6. Place the tube/PCR plate on the magnetic plate for 2 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step. 7. Carefully remove and discard the cleared solution avoiding disturbing the beads. This step must be performed while the tube/PCR plate is situated on the magnetic plate. ***NOTE**: Take care to not aspirate beads during this step, which have formed a pellet on the side of the tube/well.*
Washing	8. With the tube/PCR plate still on the magnetic plate, add 200 µL of 80% ethanol to each tube/well and incubate for 30 seconds at room temperature. Then, carefully aspirate out the ethanol and discard. 9. Repeat the previous step for a total of two washes. 10. Remove all of the ethanol from the bottom of the tube/well with a P10 pipette and air dry for about 1 minute. ***NOTE**: drying time is dependent on temperature and humidity of the working area. Take care not to over dry the beads (beads pellet should not appear cracked). When observing beads pellet lack of shininess proceed to the next step.* 11. Remove the tube/PCR plate from the magnetic plate.

Elution	12. Elute *Ampli*1 WGA products from beads by adding 22 µl of nuclease-free water directly on the beads pellet (dispensing with the tip touching the side of the tube/well at an angle and dragging it slightly along the wall where the beads pellet is). 13. Mix thoroughly by pipette mixing 10 times (in case of PCR plate) or vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube). 14. Incubate at RT for 2 minutes. ***NOTE**: If beads pellet is difficult to resuspend, extend the incubation at RT (at least for 5 minutes) and mix frequently (vortexing thoroughly or pipetting up and down).*
Transfer	15. Place the tube/PCR plate in the magnetic plate for 5 minutes, then carefully transfer 20 µl of cleared supernatant (cleaned up *Ampli*1 WGA samples) to a new PCR tube/PCR plate.
	**SAFE STOPPING POINT**: Cleaned up *Ampli*1 WGA samples may be stored at +2°C…+8°C overnight (12–16 hours). For longer periods, the cleaned up WGA products may be stored at -25°C…-15°C for up to 90 days. If stored for longer than 90 days, prepare new cleaned up *Ampli*1 WGA products.

Step 2 of 7: First Barcoding reaction

with Ampli1 D7xx index adapter

1. Prepare the First Barcoding reaction mix by adding all components in the order listed in the table below.

   NOTE: First Barcoding reaction mix must be prepared at least for 8 samples at the time.

Reagent Name	Volume per 1 sample [µl]	Volume per 8 samples [µl]
*Ampli*1 PCR Water	6.50	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 First Barcoding reaction mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube.
3. Place on ice the First Barcoding reaction mix until you are ready to use it.

   ***NOTE**: Choose performing reactions in 0.2 ml PCR tubes or 0.2 ml PCR plates (96-well format) depending on labware and sample throughput.*
4. Dispense 9 µl of First Barcoding reaction mix to each pre-labelled tube/well of PCR plate.
5. Add 3 µl of specific *Ampli*1 D7xx to each tube/well.
6. Add 3 µl of cleaned up *Ampli*1 WGA sample to each tube/well.

   ***NOTE**: The final volume of each reaction is 15 µl.*
7. Mix by vortexing and spin down briefly. Place all samples in the thermocycler and run the following First Barcoding reaction program.

First Barcoding reaction - thermocycler program:

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

SAFE STOPPING POINT: First Barcoding reaction samples may be stored at +2°C…+8°C overnight (up to 16 hours).

Step 3 of 7: Prepare reaction mixes

NOTE: *When you are ready to proceed to the following steps (First Barcoding reaction sample clean up and Second Barcoding reaction) prepare the reaction mixes, as follow, to ensure that magnetic beads do not dry out during purification steps.*

Step 3.1: Second Barcoding reaction mix

1. Prepare the Second Barcoding reaction mix by adding all components in the order listed in the table below.

   NOTE: *Second Barcoding reaction mix must be prepared at least for 8 samples at the time.*

Reagent Name	Volume per 1 sample [µl]	Volume per 8 samples [µl]
*Ampli*1 PCR Water	9.50	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 Second Barcoding reaction mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube.
3. Place on ice the Second Barcoding reaction mix until you are ready to use it.

Step 3.2: Library Amplification reaction mix

1. Prepare the Library Amplification reaction mix by adding all components in the order listed in the table below.

   NOTE*: Library Amplification reaction mix must be prepared at least for 8 samples at the time.*

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 Library Amplification reaction mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube.
3. Place on ice the Library Amplification reaction mix until you are ready to use it.

Step 4 of 7: Second Barcoding reaction

First Barcoding reaction sample clean up and Second Barcoding reaction with Ampli1 D5xx index adapter

Beads Binding

1. Ensure SPRIselect beads and First Barcoding reaction samples (prepared in the Step 'First Barcoding reaction with *Ampli*1 D7xx index adapter') are at room temperature.
2. Thoroughly shake the SPRIselect Beads bottle to resuspend any magnetic particles that may have settled.
3. Add 22.5 µl (1.5X) of resuspended SPRIselect Beads to the diluted WGA sample.
4. Mix thoroughly by pipette mixing 10 times (in case of PCR plate)
   or
   vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube).

   NOTE: *The colour of the mixture should appear homogeneous after mixing.*
5. Let the mixed samples incubate for 5 minutes at room temperature (RT) for maximum recovery.

Separation

6. Place the tube/PCR plate on the magnetic plate for 2 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
7. Carefully remove and discard the cleared solution avoiding disturbing the beads. This step must be performed while the tube/PCR plate is situated on the magnetic plate.

   NOTE: *Take care to not aspirate beads during this step, which have formed a pellet on the side of the tube/well.*

Washing

8. With the tube/PCR plate still on the magnetic plate, add 200 µL of 80% ethanol to each tube/well and incubate for 30 seconds at room temperature. Then, carefully aspirate out the ethanol and discard.
9. Repeat the previous step for a total of two washes.
10. Remove all of the ethanol from the bottom of the tube/well with a P10 pipette and air dry for about 1 minute.

    NOTE: *drying time is dependent on temperature and humidity of the working area. Take care not to over dry the beads (beads pellet should not appear cracked). When observing beads pellet lack of shininess proceed to the next step*.
11. Remove the tube/PCR plate from the magnetic plate.

Elution

12. Elute the cleaned up sample from beads by adding 12 µl of Second Barcoding reaction mix directly on the beads pellet (dispensing with the tip touching the side of the tube/well at an angle and dragging it slightly along the wall where the beads pellet is).
13. Add 3 µl of specific *Ampli*1 D5xx to each sample tube/well.

    NOTE: *the final volume of each reaction is 15 µl.*
14. Mix thoroughly by pipette mixing 10 times (in case of PCR plate)
    or
    vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube).
15. Incubate at RT for 2 minutes.

    NOTE: *If beads pellet is difficult to resuspend, extend the incubation at RT (at least for 5 minutes) and mix frequently (vortexing and/or pipetting up and down).*
16. Place all samples in the thermocycler and run the following Second Barcoding reaction program.

    NOTE*: the Second Barcoding reaction must be carried out in presence of SPRIselect beads. Indeed, the Transfer Step in a new PCR tube/PCR plate is not required.*

Second Barcoding reaction - thermocycler program

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

17. Proceed immediately to the following step.

Step 5 of 7: Library Amplification

Second Barcoding reaction sample clean up and Library Amplification reaction.

Beads Binding	1. Ensure PEG NaCl solution and Second Barcoding reaction samples (prepared in the Step 'Second Barcoding reaction with *Ampli*1 D5xx index adapter') are at room temperature. 2. Thoroughly shake the PEG NaCl solution bottle to resuspend. 3. Add 22.5 µl (1.5X) of resuspended PEG NaCl solution to the Second Barcoding reaction sample. 4. Mix thoroughly by pipette mixing 10 times (in case of PCR plate) or vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube). ***NOTE**: The colour of the mixture should appear homogeneous after mixing. If beads pellet is difficult to resuspend, mix frequently (vortexing thoroughly and/or pipetting up and down).* 5. Let the mixed samples incubate for 5 minutes at room temperature (RT) for maximum recovery.
Separation	6. Place the tube/PCR plate on the magnetic plate for 2 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step. 7. Carefully remove and discard the cleared solution avoiding disturbing the beads. This step must be performed while the tube/PCR plate is situated on the magnetic plate. ***NOTE**: Take care to not aspirate beads during this step, which have formed a pellet on the side of the tube/well.*
Washing	8. With the tube/PCR plate still on the magnetic plate, add 200 µL of 80% ethanol to each tube/well and incubate for 30 seconds at room temperature. Then, carefully aspirate out the ethanol and discard. 9. Repeat the previous step for a total of two washes. 10. Remove all of the ethanol from the bottom of the tube/well with a P10 pipette and air dry for about 1 minute. ***NOTE:** drying time is dependent on temperature and humidity of the working area. Take care not to over dry the beads (beads pellet should not appear cracked). When observing beads pellet lack of shininess proceed to the next step.* 11. Remove the tube/PCR plate from the magnetic plate.
Elution	12. Elute the cleaned up sample from beads by adding 15 µl of Library Amplification reaction mix directly on the beads pellet (dispensing with the tip touching the side of the tube/well at an angle and dragging it slightly

along the wall where the beads pellet is).

NOTE: *the final volume of each reaction is 15 µl.*

13. Mix thoroughly by pipette mixing 10 times (in case of PCR plate)
or
vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube).
14. Incubate at RT for 2 minutes.

NOTE: *If beads pellet is difficult to resuspend, extend the incubation at RT (at least for 5 minutes) and mix frequently (vortexing and/or pipetting up and down).*

15. Place all samples in the thermocycler and run the following Library Amplification program.

NOTE: *the Library Amplification reaction must be carried out in presence of SPRIselect beads. Indeed, the Transfer Step in a new PCR tube/PCR plate is not required.*

Library Amplification reaction - thermocycler program

Cycles	Temparature [°C]	Time
	95	4 minutes
1	95	30 sec
	60	30 sec
	72	2 minutes
12	95	30 sec
	60	30 sec
	72	2 minutes (+20sec/cycles)
	72	7 minutes
	10	∞

16. Proceed immediately to the following step.

Step 6 of 7: Library clean up	
Beads Binding	1. Ensure PEG NaCl solution and libraries (prepared in Step 'Library Amplification reaction') are at room temperature. 2. Thoroughly shake the PEG NaCl solution bottle to resuspend. 3. Add 22.5 µl (1.5X) of resuspended PEG NaCl solution to the library. 4. Mix thoroughly by pipette mixing 10 times (in case of PCR plate) or vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube). ***NOTE:*** *The colour of the mixture should appear homogeneous after mixing. If beads pellet is difficult to resuspend, mix frequently (vortexing thoroughly and/or pipetting up and down).* 5. Let the mixed samples incubate for 5 minutes at room temperature (RT) for maximum recovery.
Separation	6. Place the tube/PCR plate on the magnetic plate for 2 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step. 7. Carefully remove and discard the cleared solution avoiding disturbing the beads. This step must be performed while the tube/PCR plate is situated on the magnetic plate. ***NOTE:*** *Take care to not aspirate beads during this step, which have formed a pellet on the side of the tube/well.*
Washing	8. With the tube/PCR plate still on the magnetic plate, add 200 µL of 80%ethanol to each tube/well and incubate for 30 seconds at room temperature. Then, carefully aspirate out the ethanol and discard. 9. Repeat the previous step for a total of two washes. 10. Remove all of the ethanol from the bottom of the tube/well with a P10 pipette and air dry for about 1 minute. ***NOTE:*** *drying time is dependent on temperature and humidity of the working area. Take care not to over dry the beads (beads pellet should not appear cracked). When observing beads pellet lack of shininess proceed to the next step.* 11. Remove the tube/PCR plate from the magnetic plate.
Elution	12. Elute Library fragments from beads by adding 17 µl of LowTE Buffer directly on the beads pellet (dispensing with the tip touching the side of the tube/well at an angle and dragging it slightly along the wall where the beads pellet is).

	13. Mix thoroughly by pipette mixing 10 times (in case of PCR plate) or vortex and briefly spin-down the sample to collect the liquid from the sides of the tube (in case of PCR tube). 14. Incubate at RT for 2 minutes. ***NOTE:*** *If beads pellet is difficult to resuspend, extend the incubation at RT (at least for 5 minutes) and mix frequently (vortexing thoroughly or pipetting up and down).*
Transfer	15. Place the tube/PCR plate in the magnetic plate for 5 minutes, then carefully transfer 15 µl of cleared supernatant (cleaned up library) to a new PCR tube/PCR plate. ***NOTE:*** *These libraries are now ready for sequencing. Refer to the following sections for quantifying, combining and sequencing libraries.*
	**SAFE STOPPING POINT:** Cleaned up libraries may be stored at +2°C…+8°C overnight (12–16 hours). For longer periods, the cleaned up libraries may be stored at -25°C…-15°C for up to 90 days. If stored for longer than 90 days, prepare new libraries.

Step 7 of 7: Quantify and combine *Ampli*1 LowPass Libraries

This section describes two procedures (A and B) for quantifying *Ampli*1 LowPass libraries and guidelines for combining *Ampli*1 LowPass libraries, in order to obtain a final library pool.

A) Quantify libraries by qPCR and Bioanalyzer

This procedure provides instructions for quantifying *Ampli*1 LowPass libraries using the average library size (bp) estimated by Bioanalyzer instrument analysis and the library quantity (nM) determined by qPCR assay.

***NOTE**: This procedure will lead to balanced pooling.*

- Dilute each library 1:5 with LowTE (1 µl of library + 4 µl of LowTE).
- Use Agilent DNA HS Kit and load 1 µl of diluted *Ampli*1 LowPass library on the Agilent® High Sensitivity DNA chip. Run the chip according to manufacturer's instructions.
- From the Agilent® 2100 Bioanalyzer instrument analysis determine the library size distribution using the Bioanalyzer software. If necessary, follow the manufacturing's instruction to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak (Figure 3).
- If the library concentration assayed is >10ng/µL, perform further dilutions of the library.

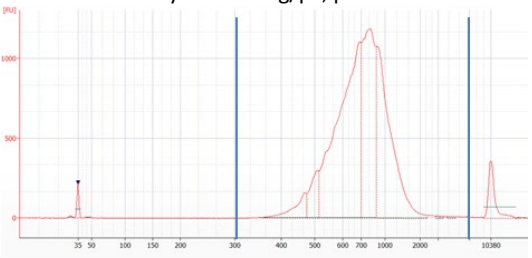

Figure 3. Example of successful *Ampli*1 LowPass library, assessed by Agilent 2100 Bioanalyzer. Typical *Ampli*1 LowPass libraries demonstrate a broad size distribution, with average fragment sizes ranging from 300 bp to 3500 bp in length.

- Determine the concentration of each *Ampli*1 LowPass library by a qPCR assay following the user's manual instruction, adjusting the quantification for the average library size determined by Bioanalyzer assay.
- Each *Ampli*1 LowPass library should be diluted 1:100.000 and analyzed in triplicate.

B) Quantify libraries by Qubit™

This procedure provides instructions for quantifying *Ampli*1 LowPass libraries using the

Qubit™ (Thermo Fisher), assuming that the size range of all each *Ampli*1 LowPass library is on average 700bp.

***NOTE**: This procedure could lead to unbalanced pooling. In alternative to the procedure A, it is suggested to choose this strategy if, based on previous experience, Ampli1 LowPass libraries have similar average size among them.*

- Analyze 2 µl of each Ampli1 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 for more information.
- Calculate the molar concentration for each Ampli1 LowPass library using the following conversion factor:

1ng/µL = 2nM

EXAMPLE: If the concentration of the Ampli1 LowPass library, determined by Qubit™ analysis, is 10ng/µL the molar concentration will be 20nM.

Libraries Combination

Mix together equal amounts of each *Ampli*1 LowPass library to obtain a final library pool.

It is suggested to obtain a final library pool of 2nM or 4nM, according to the Illumina guidelines for denaturing and diluting libraries before sequencing on Illumina System ("MiSeq Systems: Denature and Dilute Libraries Guide"; "HiSeq Systems: Denature and Dilute Libraries Guide"; " NovaSeq 6000 Sequencing System Guide").

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.

It is suggested, in order to avoid suboptimal cluster density, to quantify the final library pool by a qPCR assay following the user's manual instruction.

***NOTE:** Improper final library pool quantification, using other methods (e.g. Bioanalyzer), will lead to suboptimal cluster density, impacting sequencing data.*

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**, supplied in the Box 1 of 2 of *Ampli*1 LowPass kit Reagents). For further details, see Sequencing Guidelines in Table 1.

- The *Ampli*1 SEQ primer is concentrated at 60 µM; this custom primer is needed to sequence the Read 1. Dilute the *Ampli*1 SEQ primer with HT1 buffer (Illumina) prior each sequencing experiment following indications reported in Table 1 below. For further information see "MiSeq System: Custom Primers Guide"; "HiSeq System: Custom Primers Guide" or "NovaSeq Series: Custom Primers Guide".

- *Ampli*1 LowPass libraries sequencing can be performed in single-read (SR) or paired-end (PE). It is recommended to use single-read sequencing approach. However, in case of paired-end sequencing, the first portion of second read will contain an *Ampli*1 WGA adapter. Further details are provided 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).

- Because the *Ampli*1 SEQ custom primer is used for reading Read 1, instead of Illumina sequencing primer, the Illumina PhiX library control cannot be sequenced.

- In order to perform the genome wide copy number profiles data analysis, we suggest to obtain at least 500'000 reads for each library.

- See additional Sequencing Guidelines for the MiSeq® (Chapter 11.1 and 11.2) and HiSeq® (Chapter 11.3) systems.

Table 1. Sequencing Guideline. 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***
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	PE Cluster Kit v4-cBot-HS + SBS Kit v4-HS (250 cycles)	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

* Dilute *Ampli*1 SEQ custom primer [60 µM], as indicated in the Table 1, using HT1 and then load to the reagent cartridge. Refer to Illumina's technical manuals (MiSeq System: Custom Primers Guide; HiSeq System: Custom Primers Guide; NovaSeq Series: Custom Primers Guide) for additional information.

** In the rapid run mode of the HiSeq® 2500 or 1500, using the *TruSeq Rapid Duo cBot Sample Loading Kit* it is allowed to load separately each rapid flow-cell lane with a unique library pool; therefore loading up to 96 libraries per lane and up to 192 libraries per run (Rapid mode).

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** [60 µM] 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.
3. Load 600 µL of diluted *Ampli*1 SEQ primer on the MiSeq®reagent cartridge in the position 18 for Read 1 Custom Primer (see "MiSeq System: Custom Primers Guide).
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 "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 (refer to the Table 2 and Table 3A/3B, 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® 2500/1500 and 2000/1000 systems, according to the manufacturer's protocol.
2. Dilute the ***Ampli*1 SEQ primer** [60 µM] 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 1.
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 (refer to the Table 2 and Table 3A/3B, 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. Refer to Illumina "Indexed Sequencing: Overview Guide", 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 3A) and *Ampli*1 index from D501 to D508 (Table 2).
- *Ampli*1 LowPass Kit Set B provides a set of unique *Ampli*1 index from D707 to D712 (Table 3B) and *Ampli*1 index from D501 to D508 (Table 2).

Table 2.
***Ampli*1 LowPass indexes included both in Set A and Set B**. Fill the Sample Sheet table, in the column Index 2 (I5), with these indexes

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

Table 3A.
***Ampli*1 LowPass indexes included in the Set A.**
Fill the Sample Sheet table, in the column Index 1 (I7), with these indexes

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

Table 3B.
***Ampli*1 LowPass indexes included in the Set B.**
Fill the Sample Sheet table, in the column Index 1 (I7), with these indexes

*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 single-end sequencing, adapter trimming is not necessary.

  In case of paired-end sequencing, the insert of the libraries produced with Ampli1 LowPass Kit for Illumina platforms contains an Ampli1 WGA adapter, with sequence AGTGGGATTCCTGCTGTCAGT at second end of the fragment.
  This adapter can easily be trimmed from the sequences in FASTQ format using software like cutadapt (https://github.com/marcelm/cutadapt).

- **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. Troubleshooting

Table 4. This Table lists helpful Information and possible problems, causes and suggested remedies

Problem	Possible cause	Suggested remedy
Incomplete resuspension of beads after ethanol washes, during clean up steps.	Over-drying of beads.	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
After the Second Barcoding reaction and Library Amplification steps, once you added PEG NaCl solution, beads may appear strongly clumped at the bottom of the tube and do not get easily resuspended.	Over-drying of beads during previous clean-up steps.	At this stage, vortexing thoroughly and also to rub the bottom of the tube and resuspend by pipetting up and down for at least 10 times.
Library yield, lower than expected (lower than 2 nM).	Incorrect quantification or incorrect removal of ethanol during clean up steps. Ultimately, inadequate sample input quality.	Repeat qPCR library quantification; if library yield is again lower than 2 nM, proceed quantifying the *Ampli*1 WGA sample, as suggested below. Quantify (by Qubit™) the cleaned up WGA sample: - if the concentration is higher than 1,5 ng/µl re-prepare the library starting from *First Barcoding reaction* step. - if the concentration is lower than 1,5 ng/µl re-prepare the library starting from *Ampli1 WGA clean up* step.
During the library profiling on the Bioanalyzer, the peak of the lower marker can be exchanged with primers peaks (Figure 4).	Primers carry over after final library clean up.	In this case, the peak of the lower marker had to be set manually on the Bioanalyzer software.
Suboptimal cluster density on sequencing flow-cell.	Improper final library pool quantification, using other methods (e.g. Bioanalyzer).	Quantify the final library pool by a qPCR assay, following the user's manual instruction.

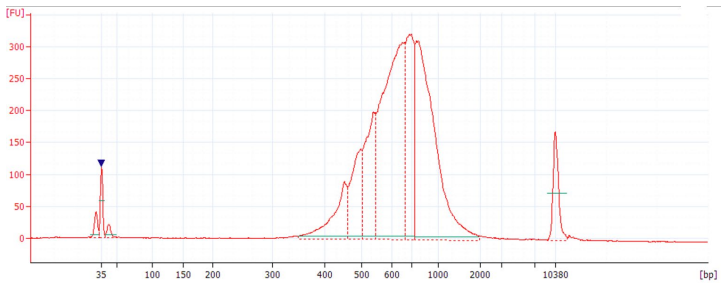

Figure 4. Ampli1 LowPass library with the primers peaks, assessed by Agilent 2100 Bioanalyzer.
In this case, the peak of the lower marker can be wrongly exchanged with primers peaks, therefore, the peak of the lower marker (blue arrow) had to be set manually on the Bioanalyzer software.

15. 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.
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17. 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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