

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

Ampli1™ OncoSeek Panel for Illumina®

Targeted NGS Amplicon Panel for Illumina® platforms

USER MANUAL

 Version 7

REF

KI0119	<i>Ampli1™</i> OncoSeek Panel for Illumina®	48 reactions
KI0116	<i>Ampli1™</i> OncoSeek Panel for Illumina® - Pre-PCR	Storage
KI0117	<i>Ampli1™</i> OncoSeek Panel for Illumina® - Post-PCR	-25°C...-15°C
KI0118	<i>Ampli1™</i> OncoSeek Panel for Illumina® - PEG NACL	-25°C...-15°C
		+15°C...+25°C



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1. PRODUCT DESCRIPTION & CONTENT

The *Ampli1* OncoSeek Panel offers a targeted NGS assay comprising 60 clinically-relevant oncology-related genes for the simultaneous analysis of SNVs, indels and CNAs.

This kit contains reagents for a rapid preparation of up to 96 barcoded libraries compatible with Illumina platforms.




The *Ampli1* OncoSeek Panel requires exclusively, as starting material, Whole Genome Amplified (WGA) DNA with *Ampli1* WGA Kit (Ref. WG001R or KI0030).

Table 1 and 2 list the key features of *Ampli1* OncoSeek Panel.

Table 1: Main Features of <i>Ampli1</i> OncoSeek Panel for Illumina	
Feature	Specification
Number of amplicons	386
Amplicon size (priming regions excluded)	45-154 (mean=89.2)
Target size	33.6 Kb
DNA quantity input	10 ng of DNA (<i>Ampli1</i> WGA)
Time required	4 hours
Hands-on time	2 hours

Table 2: *Ampli1* OncoSeek Panel for Illumina content and number of amplicons per gene.

ABL1	5	CDK6	10	FBXW7	4	HRAS	2	MLH1	1	PTPN11	2
AKT1	2	CDKN2A	2	FGFR1	12	IDH1	1	MPL	1	RB1	7
ALK	13	CSF1R	2	FGFR2	13	IDH2	2	MSH6	1	RET	5
APC	15	CTNNB1	1	FGFR3	7	JAK2	1	MYC	9	SMAD4	9
AR	10	DDR2	1	FGFR4	9	JAK3	3	MYCN	9	SMARCB1	4
ATM	8	DNMT3A	1	FLT3	4	KDR	5	NOTCH1	3	SMO	5
BRAF	12	EGFR	19	GNA11	2	KIT	22	NRAS	3	SRC	1
CCND1	8	ERBB2	14	GNAQ	2	KRAS	11	PDGFRA	13	TP53	19
CDH1	2	ERBB4	4	GNAS	2	MAP2K1	4	PIK3CA	17	TSC1	1
CDK4	10	EZH2	1	HNF1A	4	MET	15	PTEN	8	VHL	2

-  Genes with contiguous, overlapping coverage;
-  Genes with amplicons for CNA, SNVs and InDel calling;
-  Genes with amplicons for CNA calling only.

***Ampli1*™ OncoSeek Panel for Illumina®**, thereafter *Ampli1* OncoSeek Panel, consists of two reagent-boxes and one PEG NaCl solution-bottle:

- Box 1: Pre-PCR Reagents
- Box 2: Post-PCR Reagents
- Bottle: PEG NaCl Solution

Box 1 of 2: Pre-PCR Reagents

Reagent Name	Cap Color	Content
Reagent P1	green	106 µl
Reagent B1	green	160 µl
Enzyme E1	green	800 µl
TE Pre	white	1200 µl

Box 2 of 2: Post-PCR Reagents		
Reagent Name	Cap Color	Content
Index D50X	blu	33 µl each of D501-D508
Index D70X	blu	44 µl each of D701-D712
Reagent B2	yellow	2 vials of 819 µl
Enzyme E2	yellow	53 µl
Enzyme E3	yellow	53 µl
Enzyme E4	yellow	106 µl
TE Post	white	1200 µl

Bottle: PEG NaCl Solution		
Reagent Name	Cap Color	Content
PEG NaCl solution	white	1 x 20000 µl

2. STORAGE AND HANDLING

Upon receipt, store the Box 1 and Box 2 at -25°C...-15°C. Store the PEG NaCl solution at +15°C...+25°C.

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. TECHNICAL ASSISTANCE

For technical assistance and additional information, contact the Customer Support:

E-mail: customersupport@siliconbiosystems.com

Telephone: (+39)051-9944100, Mon-Fri, 9 am – 18 pm CET +01:00

4. WARNING AND PRECAUTIONS

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>.

5. INTENDED USE

The **Ampli1 OncoSeek Panel for Illumina** is a kit intended for the preparation of Illumina-compatible libraries and sequence of the targeted genes listed below.

- Selected regions of human somatic variants (single nucleotide variants, small indels) in the following genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, DDR2, DNMT3A, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MET, MLH1, MPL, MSH6, NOTCH1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, TP53, TSC1, VHL.
- Selected regions of the following genes ALK, AR, BRAF, CCND1, CDK4, CDK6, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, FGFR4, KIT, KRAS, MET, MYC, MYCN, PDGFRA, PIK3CA, frequently subjected to copy number alterations (CNAs).

The **Ampli1 OncoSeek Panel for Illumina** kit is indicated for *Ampli1* WGA kit products generated from human single cells isolated from blood, after enrichment with CELLSEARCH® CTC Kit and sorting with DEPArray™ System.

6. USE LIMITATION

The *Ampli1* OncoSeek Panel is intended for research use and for *in vitro* use only.

The *Ampli1* OncoSeek Panel for Illumina is not intended to be used with whole genome amplified DNA obtained with kits other than *Ampli1* WGA Kit.

No claim or representation is made for any intended use to provide information for the diagnosis, prevention or treatment of a disease.

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) or 0.2 ml PCR plates (Recommended: PCR Plate 96 Eppendorf twin.tec LoBind, semi-skirted);
- 1.5 ml tubes (major laboratory supplier);
- Programmable thermocycler with adjustable ramp rates, operating within manufacturer's specifications;
- 0.2 ml PCR tubes (Recommended: Axygen® 0.2 mL Maxymum Recovery® thin wall PCR Tubes with Flat Cap; Ref. PCR-02-L-C) or 0.2 ml PCR plates (Recommended: PCR Plate 96 Eppendorf twin.tec LoBind, semi-skirted);
- Vortex-mixer (major laboratory supplier);
- Qubit® 2.0 or 3.0 Fluorometer (Thermo Fisher) and Qubit dsDNA HS Assay Kit (Thermo Fisher);
- Micropipette sets;
- Filter tips (Recommended: Gilson Diamond Filter tips in sterilized TIPACK™ or Eppendorf Dualfilter T.I.P.S., PCR Clean);
- Ethanol BioUltra, for molecular biology, ≥99.8%;
- Nuclease-free water (molecular biology grade);
- Real-Time qPCR Instrument;
- qPCR-based library quantification assay for Illumina libraries (recommended: KAPA Library Quantification Kit Illumina Platforms [Roche] Ref. depends on Real-Time qPCR Instrument);
- -25°C...-15°C Storage Freezer;
- +2°C...+8°C Storage Refrigerator.

8. SAMPLE SPECIFICATIONS

Ampli1 OncoSeek Panel works exclusively with *Ampli1* WGA products.

The *Ampli1* WGA shall have a Genome Integrity Index (GII) ≥ 3 , assessed by *Ampli1* QC kit analysis.

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, pre-program DNA Target Amplification and barcoding reaction step on the thermocycler.
- For DNA Target Amplification step on the thermocycler, set heating ramp rate to 2.6 °C/sec and cooling ramp rate to 1.6 °C/sec; turn lid heating ON and set temperature of the lid to 105°C.
- 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 newly opened kept or sterile packages of plastic ware (e.g. reaction tubes and Dualfilter Low Retention Filter tips). Do not use autoclaved plastic ware.
- Perform *Ampli1* WGA clean up and Barcoding reaction in a separate working area or room from Library clean up step and downstream processing.
- Use a separate set of pipettes for *Ampli1* WGA clean up and Barcoding reaction from those used for Library clean up step and downstream processing.
- Before beginning and after use, wash the working surface with 10% bleach followed by water rinses.

Pipetting recommendations

- In order to compensate volume losses during pipetting, it is suggested to include a 5% excess in each reaction mix.
- 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).

- Before vortex and spin down samples and reagents, ensure that the tubes and plate-wells are correctly closed.
- Verify that accurate amounts 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. WGQC4 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 3 markers ($GII \geq 3$) has been demonstrated to be predictive to obtain a good sequencing quality metrics.
- To maximize efficient use of enzyme reagents, remove enzyme tubes from -20°C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow reagents to reach 4°C prior to pipetting. Attempting to pipette enzymes at -20°C may result in a shortage of enzyme reagents.
- Assemble reagents master mixes on ice before adding to samples. Use 5% excess volume to compensate for pipetting loss.
- Enzyme E1 vial may show solid particles, therefore before using it check for the presence of precipitates. If precipitates are present, allow the vial to reach room temperature and gently vortex to dissolve solid particles.
- After thawing reagents, briefly vortex all reagents except the enzymes in the Barcoding Step (E2, E3, E4 tubes) and spin down in a microcentrifuge.
- Bring SPRIselect® beads and PEG NaCl solution to 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.
- During the washing step of clean up procedures, remove any residual ethanol solution from the bottom of the tube which could affect the enzyme activity.
- At no time “with bead” samples should be stored on ice, this might affect DNA binding to SPRIselect beads.
- When handling D70x or D50x indexes, open one tube at time to prevent any cross-contamination.
- We recommend to accurately follow Sequencing Guidelines on this Instruction for Use (Section 13).
- *Ampli1* OncoSeek Panel has been validated on MiSeq® System (Illumina), using 150 Paired End run sequencing.
- In order to perform data analysis, we suggest to obtain at least 500'000 reads for each *Ampli1* OncoSeek library.

10. PROCEDURE

Ampli1 OncoSeek Panel is designed to generate Illumina-compatible libraries, with a hands-on time of only 2 hours.

The *Ampli1* OncoSeek Panel workflow, starting from cleaned-up *Ampli1* WGA products, consists of three main phases (Figure 1):

- A. DNA Target Amplification,
- B. Barcoding reaction,
- C. Purification.

The final library is ready to be sequenced on Illumina systems after the Library quantification by qPCR (D).

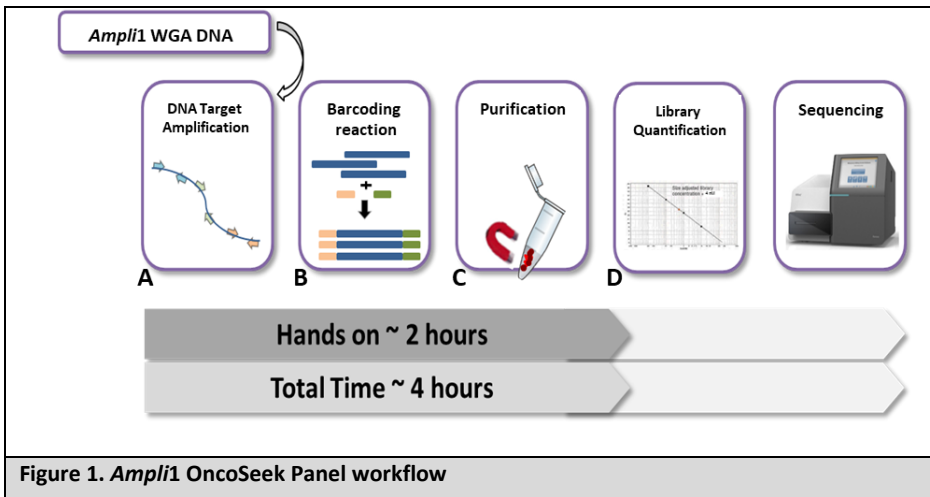


Figure 1. *Ampli1* OncoSeek Panel workflow

Step 1 of 4: *Ampli1* WGA clean up and quantification

1. Transfer 5 µl of *Ampli1* 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 *Ampli1* 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 a magnetic rack until the solution clears and a pellet is formed 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 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 *Ampli1* WGA products from beads by adding 12 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 10 µl of cleared supernatant (cleaned up *Ampli1* WGA samples) to a new PCR tube/PCR plate.

Quantification

16. A Qubit® assay shall be used to quantify cleaned up WGA samples.
Following instructions refer to the Qubit Fluorometer assay:
 - Analyze 2 µl of each sample using the Qubit 2.0 or 3.0 Fluorometer and the Qubit dsDNA HS Assay Kit. Please refer to the Qubit dsDNA HS Assay Kits User Guide (Pub. No. MAN0002326) for more information.
 - The DNA input for DNA Target Amplification step is 10 ng in a final volume of 10 µl. Dilute each *Ampli1* WGA sample input for DNA Target Amplification step with TE Pre (Box 1: Pre-PCR Reagents).



SAFE STOPPING POINT: Cleaned up *Ampli1* 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 *Ampli1* WGA products.

Step 2 of 4: DNA Target Amplification

1. Program the PCR thermal cycler instrument, as follow:

DNA Target Amplification - thermal cycler program		
Cycles	Temperature [°C]	Time
4	98	1 minute
	98	10 sec
	63	5 minutes
	65	1 minute
22	98	10 sec
	64	1 minute
	65	1 minute
	4	∞

NOTE: Set heating ramp rate at 2.6 °C/sec and cooling ramp rate at 1.6 °C/sec; turn lid heating ON and set temperature of the lid at 105°C.

NOTE: Allow the block to reach 98°C before loading samples and confirm lid heating is turned ON.

2. Prepare the master mix **on ice**, by adding all components in the order listed in the table below.

Reagent Name	Volume per 1 sample [µl]
Reagent P1*	2
Reagent B1	3
Enzyme E1	15
Total per reaction	20

* Reagent P1 contains the Ampli1™ OncoSeek Panel primer pool

NOTE: Check Enzyme E1 vial for the presence of precipitates. If precipitates are present, allow the vial to reach room temperature and gently vortex to dissolve solid particles.

3. Once the master mix has been prepared, briefly vortex and spin it down to collect all the volume at the bottom of the tube.
4. Place on ice the master 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.
5. Load 10 µl of diluted Ampli1 WGA product (prepared in Step 1 of 4) into each PCR tube/well of PCR plate.

- 6. Assemble on ice.** Dispense 20 µl of master mix to each pre-labelled tube/well of PCR plate.

NOTE: Final volume of each reaction is 30 µl.

- 7.** Mix by vortexing and spin down briefly.
- 8.** Allow the block of the thermocycler to reach **98 °C** and then load samples.



SAFE STOPPING POINT: Targeted amplification products may be stored at +2°C...+8°C overnight (12–16 hours).

Step 3 of 4: Barcoding reaction

Step 3.1: Prepare Barcoding reaction mix

1. Move samples to post-PCR area and keep the samples (prepared in Step 2 of 4) at room temperature prior to proceeding with the purification step.
2. Prepare the Barcoding reaction mix on ice, with the following components. All components may be master-mixed when running multiple samples in parallel.

Reagent Name	Volume per 1 sample [μl]
Reagent B2	31
Enzyme E2	1
Enzyme E3	1
Enzyme E4	2
Total per reaction	35

3. Place on ice the Barcoding reaction mix until you are ready to use it.

Step 3.2: Purification step

4. Ensure SPRIselect beads and samples (obtained in step 2 of 4) are at room temperature.

Beads Binding

5. Thoroughly shake the SPRIselect beads bottle to resuspend any magnetic particles that may have settled.
6. Add 36 μl (1.2X) of resuspended SPRIselect Beads to each 30 μl of sample (prepared in the 'DNA Target Amplification' step 2 of 4).
7. 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.*

8. Let the mixed samples incubate for 5 minutes at room temperature (RT) for maximum recovery.

Separation

9. Place the tube/PCR plate on the magnetic plate for 2 minutes, until the solution clears and a pellet is formed before proceeding to the next step.

10. 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

11. 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.
12. Repeat the previous step for a total of two washes.
13. 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.
14. Remove the tube/PCR plate from the magnetic plate.
15. Proceed immediately to the Barcoding Reaction step.

Step 3.3: Barcoding reaction step

Elution

16. Elute the cleaned-up sample from beads by adding a unique combination of 5 µl index D50X and 10 µl index D7XX to each sample 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).
17. Add 35 µl of Barcoding Reaction mix to each sample tube/well.
NOTE: Final volume of each reaction is 50 µl.
18. 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).
19. 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).
20. Place all samples in the thermocycler, as follow: **37°C for 20 minutes (Lid heating OFF)**, and run the barcoding reaction.
NOTE: Barcoding Reaction step must be carried out in presence of SPRIselect beads. Indeed, the Transfer step in a new PCR tube/PCR plate is not required.

Step 4 of 4: Purification and final elution

1. Ensure PEG NaCl solution and Barcoding reaction samples (prepared in Step 3 of 4) are at room temperature.

Beads Binding

2. Thoroughly shake the PEG NaCl solution bottle to resuspend.
3. Add 42.5 µl (0.85X) of resuspended PEG NaCl solution to the 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, until the solution clears and a pellet is formed 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 (obtained in the step 4 of 4) from beads by adding 22 μ l of TE Post 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 and/or pipetting up and down).*

Transfer

15. Place the tube/PCR plate in the magnetic plate for 5 minutes, then carefully transfer 20 μ l 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.

11. LIBRARY QUANTIFICATION AND COMBINATION

This section describes the suggested procedure for quantifying and pooling *Ampli1* OncoSeek libraries, with unique dual-index combinations, in order to obtain a final library pool of 2 or 4 nM.

Library quantification

Determine the concentration of each *Ampli1* OncoSeek library by a qPCR assay following the user's manual instruction, adjusting the quantification for the average library size of 243 bp. Each sample should be diluted 1:100.000 and analyzed in triplicate.

NOTE: *Improper library quantification by other methods (e.g. Bioanalyzer or Qubit) will lead to unbalanced pooling and suboptimal cluster density, impacting sequencing data.*

Library combination

Mix together equal amounts of each *Ampli1* OncoSeek library to obtain a 2 or 4 nM pool; according to the Illumina guidelines for denaturing and diluting libraries before sequencing on Illumina System ("MiSeq Systems: Denature and Dilute Libraries Guide").

It is very important to select unique dual-index combinations (one D5xx index and one D7xx index) and meet Illumina recommended compatibility requirements.

Ampli1 OncoSeek Panel has been validated on MiSeq® System (Illumina), using 150 Paired End run sequencing. It is suggested to sequence at least 500'000 paired-end reads per sample, in order to obtain ~1000X read depth with Illumina MiSeq reagent kit V2 (300 cycles).

12. SEQUENCING GUIDELINES

1. Denature and dilute *Ampli1*™ OncoSeek library pool for sequencing on the Illumina MiSeq system, according to the manufacturer's protocol.
2. It is suggested to use a 1% PhiX spike-in as a sequencing control.
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";
 - on the workflow parameter page, select:
 - "TruSeq DNA PCR-free" as the Library Prep Workflow
 - "TruSeq DNA CD indexes (96 indexes)" as Index adapters
 - Index Reads: "2"
 - Read Type: "Paired End"
 - Cycles Read 1: "151"
 - Cycles Read 2: "151"
 - 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 (Table 3 and Table 4).

The kit utilizes Illumina-compatible adapter sequences and has been validated on MiSeq Illumina platform.

Table 3. *Ampli1* OncoSeek Panel D7xx index 1 (i7).

Fill the Sample Sheet table, in the column Index 1 (I7), with these indexes.

D7xx index 1 (i7)	Sequences for Sample Sheet
D701	ATTACTCG
D702	TCCGGAGA
D703	CGCTCATT
D704	GAGATTCC
D705	ATTCAGAA
D706	GAATTCGT
D707	CTGAAGCT
D708	TAATGCGC
D709	CGGCTATG
D710	TCCGCGAA
D711	TCTCGCGC
D712	AGCGATAG

Table 4. *Ampli1* OncoSeek Panel D5xx index 2 (i5).

Fill the Sample Sheet table, in the column Index 2 (I5), with these indexes.

D5xx index 2 (i5)	Sequences for Sample Sheet
D501	TATAGCCT
D502	ATAGAGGC
D503	CCTATCCT
D504	GGCTCTGA
D505	AGGCGAAG
D506	TAATCTTA
D507	CAGGACGT
D508	GTA CTGAC

13. DATA PROCESSING GUIDELINES

Adapter trimming

Ensure that adapter trimming was enabled while setting up the sequencing run (see section 11 for sample sheet creation instructions). If not, repeat FASTQ extraction with adapter trimming.

Ampli1-OncoSeek Data Analysis with MSBiosuite Platform

The *Ampli1*™ OncoSeek Panel is integrated in a complete workflow for single cell molecular characterization comprising Ampli1 WGA Kit and MSBiosuite cloud-based platform (Menarini Silicon Biosystems S.p.a.). To ensure correct data interpretation we recommend to analyze sequencing data produced using *Ampli1*™ OncoSeek Panel for Illumina through the MSBiosuite platform.

MSBiosuite includes the *Ampli1*™ OncoSeek bioinformatic workflow implementing all the steps from alignment, trimming, sequence variants calling and CNAs calling to advanced variant annotation and reporting (powered by N-of-One) into a scalable and flexible platform. The platform (powered by BlueBee) implements clinical-grade data security, global data compliance (HIPAA, GDPR, ISO 27001, ISO 13485) and data residency. Please visit <http://www.siliconbiosystems.com/> for more information.

Alternative data processing with public tools

Warning

- 1) The panel makes use of special patented hybrid primer to allow optimal detection of certain hotspots (EGFR exon 19 deletion; ALK p.F1174L). However, the bioinformatic analysis with standard methods will not detect EGFR wild type allele and ALK p.F1174L mutant allele.
- 2) Copy number analysis is not tested nor supported with available open source tools.

The *Ampli1* OncoSeek Panel is designed with overlapping amplicons that enable contiguous regions of coverage in a single-tube format. Therefore, synthetic primer sequences will be encountered both at the beginning and end of some reads. A trimming step is necessary to remove these primers from overlapping amplicons which may generate false positive calls.

The latest version of Cutadapt (Martin, et al. 2011) is recommended for anchored 5' and 3' trimming of primer sequences from paired-end (PE) FASTQ files. FASTA files of primer sequences of *Ampli1* OncoSeek Panel are provided contacting Menarini Silicon Biosystems technical support at customersupport@siliconbiosystems.com.

Below is an example command line for primer trimming from paired end reads using cutadapt software.

```
cutadapt -n 2 \  
-a file:ampli1_oncoseek_primer3.fa \  
-g file:ampli1_oncoseek_primer5.fa \  
-A file:ampli1_oncoseek_primer3.fa \  
-G file:ampli1_oncoseek_primer5.fa \  
-o sequence1.trim.fastq \  
-p sequence2.trim.fastq \  
sequence1.fastq \  
sequence2.fastq
```

Where:

ampli1_oncoseek_primer3.fa: FASTA file with 3'-end primer sequences

ampli1_oncoseek_primer5.fa: FASTA file with 5'-end primer sequences

sequence1.trim.fastq: FASTQ file with trimmed reads (first mate)

sequence2.trim.fastq: FASTQ file with trimmed reads (second mate)

sequence1.fastq: Input FASTQ file (first mate)

sequence2.fastq: Input FASTQ file (second mate)

After primer trimming step, the resulting fastq files (*.trim.fastq) can be used for downstream analyses (read alignment, variant calling, etc).

Additional Assistance

For further assistance about bioinformatic analysis of results visit the website

www.siliconbiosystems.com or contact technical support at

customersupport@siliconbiosystems.com.

14. PATENT & TRADEMARK INFORMATION

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. *Ampli1* OncoSeek Panel is covered by US patent No. US 9,938,574 B2 and corresponding patent claims outside the US. 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 for the purchaser's own internal research. For information on obtaining additional rights, please contact Menarini Silicon Biosystems. *Ampli1* OncoSeek Panel is to be used solely with any of Menarini Silicon Biosystems products, and is sold by Menarini Silicon Biosystems solely for use in conjunction with such SBS products. *Ampli1*™ 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.

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15. WARRANTY







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16. 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.

17. GLOSSARY OF SYMBOLS

Symbol	Description
	Batch code
	Catalogue number
	Use-by date
	Consult instruction for use
	Manufacturer
	Temperature limit

