BRCA PLUS

REF: AVG100016 16 TESTS
REF: AVG100048 96 TESTS

USER GUIDE

AVG100_IFU_Rev02_DEC23

CONTENTS

CONTENTS 2

1. AIM OF USE 3

2. KIT CONTENTS 3

3. STORAGE AND PRODUCT STABILITY 4

4. KIT FEATURES 4

5. REQUIRED MATERIAL NOT PROVIDED 4

6. IMPORTANT NOTES AND SAFETY INFORMATION 5

7. OPERATIVE PROTOCOL 5
- 7.1 DNA SAMPLES PREPARATION 5
- 7.2 TARGET ENRICHMENT 5
- FOR ILLUMINA SEQUENCERS GO TO SECTION A (chapters 7.3-7.7) 7
- FOR ION TORRENT SEQUENCERS GO TO SECTION B (chapters 7.8-7.12) 7
- SECTION A: LIBRARY PREP FOR ILLUMINA SEQUENCERS 7.3 8
- PERFORMING END-REPAIR AND dA-TAILING (ER/AT) REACTION *8*
- 7.4 LIGATION OF THE UNIVERSAL ADAPTERS *9*
- 7.5 SAMPLE INDEXING WITH UDI PRIMERS *11*
- 7.6 PURIFICATION OF THE AMPLIFIED LIBRARIES *11*
- 7.7 LIBRARIES NORMALIZATION AND POOLING *12*
- SECTION B: LIBRARY PREP FOR ION TORRENT SEQUENCERS *13*
- 7.9 LIGATION OF AVISEQ BARCODE SCREEN ADAPTERS *14*
- 7.10 AMPLIFICATION WITH BARCODE-AMPLIFICATION PRIMERS *15*
- 7.11 PURIFICATION OF THE AMPLIFIED LIBRARIES *16*
- 7.12 LIBRARIES NORMALIZATION AND POOLING *17*

8. TROUBLESHOOTING 18

9. INFORMATION REGARDING MANUFACTURER AND EUROPEAN REPRESENTATIVE 18

10. SYMBOLS 19

1. AIM OF USE

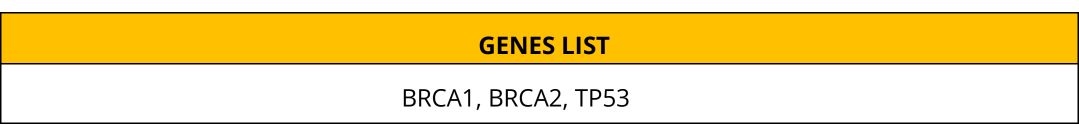
Aviseq BRCA PLUS is a kit for the analysis of the genes listed in Table 1 through a molecular protocol based on Next Generation Sequencing (NGS) technologies.

The kit is validated for germline analysis (SNPs, indels, CNVs) of DNA extracted from body tissues (blood or others) and somatic analysis of DNA extracted from tissue fresh, frozen or FFPE.

Aviseq BRCA PLUS must be used together with the following Avicenna kits (NOT included in the kit) containing the specific adapters, and it's compatible with Ion Torrent and Illumina Sequencer.

For Illumina: AviSeq UDI Primers Set A (ref. AVG406) ,AviSeq UDI Primers Set B (ref. AVG407),AviSeq UDI Primers Set C (ref. AVG408) or AviSeq UDI Primers Set D (ref. AVG409) and Universal Adapter (tube 5) NOT included in the kit.

For Ion Torrent: Aviseq Barcode Screen 1-16 (AVG900) or Aviseq Barcode Screen 1-96 (AVG901) and Barcode Amplification Primer (tube 8) NOT included in the kit.



GENES LIST
BRCA1, BRCA2, TP53

Table 1: List of target genes of the Aviseq BRCA PLUS kit.

2. KIT CONTENTS

Aviseq BRCA PLUS kit contains all reagents required for the preparation of a specific bidirectional library of amplicons designed for the NGS analysis using Illumina or Ion Torrent sequencers.

The kit provides a quantity of reagents for 16 or 96 tests, which can be divided respectively into a maximum of two or six analysis session.

Dividing the kit into more analysis sessions may alter the overall performance of the kit and/or decrease the total number of tests that can be performed.

The kit contains the reagents listed in Table 2:

Tube	Description	Volume 16 tests	Volume 96 tests
A	BRaCA PLUS Pool 1	160 µl	960 µl
B	BRaCA PLUS Pool 2	160 µl	960 µl
C	BRaCA PLUS Pool 3	160 µl	960 µl
Taq	Taq polymerase	13.6 µl	81.6 µl
RB	Reaction Buffer	280 µl	1680 µl
NTP	dNTPs mix 10mM	64 µl	384 µl
1	ER/AT Enzyme	48 µl	288 µl
2	ER/AT Buffer	112 µl	672 µl
3	Ligation Enzymes	80 µl	480 µl
4	Ligation Buffer	400 µl	2400 µl

Table 2: Volume of reagents included in the Aviseq BRCA PLUS kit.

3. STORAGE AND PRODUCT STABILITY

All reagents provided with this kit are ready to use and should be stored at -20°C. The kit, intact and properly stored, will maintain high quality performance capacity until the expiration date marked on each single reagent tube and on the external jar/container.
When the reagents are thawed, they must be kept on ice until use and then stored at -20°C.

In case of damages of the packaging, incorrect transportation and storage condition, the kit should not be used: please contact us at (techsupport@avcna.net).

4. KIT FEATURES

- SPECIFICITY: >99%
- SENSITIVITY: >99%

The residual risk in the use of the kit is related to an incorrect result. The knowledge of the operator and the clinician should anyway reduce the risk as the quality and coverage of the sample should indicate a potential error of the results.

5. REQUIRED MATERIAL NOT PROVIDED

5.1 Generic Material

- Computer with constantly updated and guaranteed secure internet connection.
- Micropipettes calibrated and periodically verified 0.2-2 µl, 2-20 µl, 20-200 µl or 100-1000 µl and relative filter tips.
- Vortex.
- Disposable Gloves without powder.
- Thermocycler calibrated and periodically verified.
- Tubes and Caps or 96-wells plate, as needed, DNase and RNase free.
- Nuclease-free water.
- 1.5 ml tube magnetic separator or 96-wells plate compatible magnetic separator.
- Fresh 70% ethanol.
- Illumina, Ion Torrent sequencers calibrated and periodically verified.
- For Illumina: AviSeq UDI Primers Set A (ref. AVG406) ,AviSeq UDI Primers Set B (ref. AVG407),AviSeq UDI Primers Set C (ref. AVG408) or AviSeq UDI Primers Set D (ref. AVG409) and Universal Adapter (tube 5) NOT included in the kit.
- For Ion Torrent: Aviseq Barcode Screen 1-16 (AVG900) or Aviseq Barcode Screen 1-96 (AVG901) and Barcode Amplification Primer (tube 8) NOT included in the kit.

5.2 Specific Material

The material listed below has been used and validated by Avicenna:

- Qubit™ 2.0 Fluorometer (Invitrogen Cod. Q32866) or Qubit™ 3.0 Fluorometer (Invitrogen Cod. Q33216) or Qubit™ 4.0 Fluorometer (Invitrogen Cod. Q33226) calibrated and periodically verified.
- Qubit™ assay tubes (Invitrogen Cod. Q32856).
- Qubit™ dsDNA HS Assay Kit (Invitrogen, cod. Q32851).
- Magnetic beads for the purification of genomic libraries, AMPure XP (Beckman Coulter, cod. A63880) or MAGTIVIO (magSi-NGSPREP Plus, cod. MDKT00010075).

QUALITATIVE ANALYSIS OF DNA (Optional)

- Agilent 2100 Bioanalyzer system or 2200 TapeStation System calibrated and periodically verified with DNA HS chips and reagent and DNA ScreenTape and reagents respectively.

6. IMPORTANT NOTES AND SAFETY INFORMATION

The user is required to apply the following provisions.
If the device or the results it generates, even in part, is transferred to a third party, the user must inform the end-user about the application of the specific provisions. The manufacturer is committed to constantly checking the possibilities of implementing the procedures, providing support to users.

- The kit is for professional use, it must be used by trained professionals in molecular biology.
- Do not use if package damaged.
- Biological samples and all reagents should be used in properly equipped rooms, clean and clear of potential contaminants. We suggest cleaning working areas frequently using a solution containing sodium hypochlorite 1-5%.
- Always use safety equipment such as laboratory coat, gloves and safety goggles during all steps described in the protocol.
- Check the risks and safety procedures associated with instruments, electricity, chemicals and other resources applied to the use of the device.
- Prepare ways of detecting errors in the operation of the device, evaluating after each usage the quality of the results generated; in case of doubts or anomalies found, the supplier must be promptly contacted for support.
- When the results produced are used in diagnostic or clinical processes, the user is required to consider the possible risks associated with diagnostic errors, to set up control mechanisms and to inform the medical personnel responsible for the diagnostic or clinical processes.
- To avoid contamination of reagents we recommend using DNase/RNase free tubes, filter tips and to pay particular attention to keep all instruments clean and free of contaminants.
- We suggest preparing a unidirectional workflow from the initial phase of DNA isolation following the PCR preparation phase, amplification, and post-amplification phases in order to keep working areas separated for the different phases of the procedure using for each phase dedicated laboratory coats, micropipettes, tubes and filter tips.
- Used reagents and biological samples must be wasted according to legal procedures.
- Verify that transportation and storage have been carried out accordingly to the indication of the manufacturer. In case the customers is suspicious that those have not been followed, consider that the results of the tests may not be correct.

➢ **Stopping point**: every time is present a stopping point you can proceed with the following step, or store the amplified samples at 4 °C for 24 hours or -20 °C for a longer period.

7. OPERATIVE PROTOCOL

7.1 DNA SAMPLES PREPARATION

Use any CE-marked commercial kit to obtain DNA from biological tissues. Determine the starting DNA concentration by fluorometric methods (picogreen/Qubit) for accuracy. Dilute each DNA sample in PCR grade water to a final concentration of 10 ng/µl.

7.2 TARGET ENRICHMENT

The amplification of the target regions is obtained through a PCR reaction performed in 3 reaction tubes. Each reaction tube contains one of the different primer pools. At the end of PCR, all the PCR tubes from each sample will be mixed into a single pool and processed in a single tube.

Refer to the table below for the final quantities of reagents required to prepare each reaction of target enrichment:

Quantity for 1 sample (DNA 10 ng/µl)	Pool 1	Pool 2	Pool 3
Reaction Buffer	3.5 l	3.5 l	3.5 l
dNTPs 10mM	1 µl	1 µl	1 µl
Taq Polymerase	0.2 µl	0.2 µl	0.2 µl
DNA (10 ng/µl)	1 µl	1 µl	1 µl
BRaCA PLUS Pool 1	10 µl	-	-
BRaCA PLUS Pool 2	-	10 µl	-
BRaCA PLUS Pool 3	-	-	10 µl
H_2O	9.3 µl	9.3 µl	9.3 µl
TOTAL	**25 µl**	**25 µl**	**25 µl**

Table 3: Quantity of reagents for each reaction of target enrichment.

Proceed with the preparation of the PCR reaction according to the following steps:

STEP 1: PREPARATION OF THE AmpliMix

In a clean and appropriated work area (e.g., laminar flow hood) prepare the AmpliMIX, the amplification mix containing all the PCR reagents common to all reaction tubes.

a) Thaw the reaction buffer, the dNTPs and the Primers Pool. Vortex and briefly centrifuge. Keep Taq Polymerase in ice (do not vortex).
b) Prepare AmpliMix as described in the following table. The volumes described are for one sample:

AmpliMix	x 1
Reaction Buffer	10.5 µl
dNTPs 10mM	3 µl
Taq Polymerase	0.6 µl
H_2O	28 µl
TOTAL	**42.1 µl**

Table 4: Preparation of the AmpliMix.

STEP 2: PREPARATION OF THE MASTER MIXES

a) Accurately mix the AmpliMix by pipetting (do not vortex), divide the total volume in 3 x 1.5 ml tubes, then add a specific Primers Pool to each tube, as shown in Table 5 below:

	x 1		
MasterMixes	**MMix 1**	**MMix 2**	**MMix 3**
AmpliMix	14 µl	14 µl	14 µl
BRaCA PLUS Pool 1	10 µl	-	-
BRaCA PLUS Pool 2	-	10 µl	-
BRaCA PLUS Pool 3	-	-	10 µl
TOTAL	**24 µl**	**24 µl**	**24 µl**

Table 5: Preparation of the three MasterMixes.

b) For each sample, aliquot 24 µl of MasterMix three times per each PCR reaction tube, change working area then add 1 µl of DNA (10 ng/µl) in each of the 3 PCR tubes per each sample. Spin down briefly

N.B. **During all procedures keep the mixtures on ice when not in use. In case of working with 96-well plates, pay attention to use proper refrigerated blocks. Keep tubes on ice before starting the PCR reaction.**

STEP 3: PCR REACTION

Place PCR tubes in the thermocycler and then start the following thermal profile (Table 6):

Stage	Temperature (°C)	Time	Ramping	Cycles
Stage 1	94	2 min	4°C/s	1
Stage 2	94	30 sec	4°C/s	30
	60	2 min	1°C/s	
	65	2 min	1°C/s	
Stage 3	10	HOLD	4°C/s	1

Table 6: PCR thermal profile.

For each sample, mix together the corresponding PCR product in a unique pool. Mix well by pipetting and then transfer the total volume (about 75 µl) in a new 1.5ml tube.

- **STOPPING POINT**

FOR ILLUMINA SEQUENCERS GO TO SECTION A (chapters 7.3-7.7)

FOR ION TORRENT SEQUENCERS GO TO SECTION B (chapters 7.8-7.12)

SECTION A: LIBRARY PREP FOR ILLUMINA SEQUENCERS

This section will perform end-repair and dA-tailing (ER/AT) of the pooled PCR products obtained from Step 7.2 (TARGET ENRICHMENT), and will then ligate the Universal Adapters to the ER/AT DNA targets.

The following table (Table 7) resumes the volumes of all reagents needed for the ER/AT and Ligation reactions per each sample.

REAGENT	VOLUME per each sample
Library Pool (after target enrichment)	50 µl
ER/AT Buffer (Tube 2)	7 µl
ER/AT Enzyme (Tube 1)	3 µl
Universal Adapters (Tube 5)	5 µl
Ligation Buffer (Tube 4)	25 µl
Ligation Enzymes (Tube 3)	5 µl
TOTAL	**95 µl**

Table 7: Reagents needed for library prep.

7.3 PERFORMING END-REPAIR AND dA-TAILING (ER/AT) REACTION

In a dedicated post-PCR area, process the PCR amplified to add the specific adapters needed for NGS sequencing.

Reagents required:

- ER/AT Enzyme (Tube 1).
- ER/AT Buffer (Tube 2).
- Freshly prepared 70% ethanol
- Magnetic Beads equilibrated at room temperature.

Before starting:

- Remove the required reagents from the freezer and thaw/equilibrate them on ice. Mix all kit components by inverting the tubes (do not vortex), spin down briefly and place back on ice before use.
- Start the thermocycler program as follows (Table 8) with the **heated lid set at 85°C**:

Stage	Temperature (°C)	Time	Cycles
Stage 1	30	HOLD	1
Stage 2	30	5 min	1
Stage 3	65	15 min	1
Stage 4	4	HOLD	1

Table 8: ER/AT thermal profile.

Library purification:

a. Vortex the magnetic beads equilibrate at room temperature.

b. Add 75 µl of resuspended magnetic beads (1X) to each reaction obtained in section 7.2 step 3, and mix by pipetting.
c. Incubate for 5 minutes at room temperature.
d. Pellet the beads on a magnetic rack for at least 1 minute.
e. Keep the tube on the magnetic rack, pipette off and discard the supernatant.
f. Wash the beads by adding 200 µl of freshly prepared 70% ethanol. Incubate for 1 minute, then remove the ethanol.
g. Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic rack, making sure not to disturb the beads pellet.
h. Remove the tubes from the magnetic rack, spin them down and place them back on the magnetic rack. Then remove all the residual ethanol with a P10.
i. Air dry the samples at room temperature for maximum 5 minutes without drying the beads to the point of cracking.
j. Remove the tubes from the magnetic rack and resuspend each pellet in 52 µl of RNase/DNase-free distilled water. Pipet up and down at least 10 times to thoroughly mix the beads suspension with water and incubate 5 minutes at room temperature.
k. Pellet the beads on a magnetic rack for at least 1 minute or until the eluate is clear and colorless.
l. Carefully remove and transfer 50 µl of each eluate containing the DNA purified samples into a 0.2 µl PCR-tube.
m. Prepare the ER/AT Mix according to the following volumes (Table 9) of reagents. Mix well by pipetting.

REAGENT for the ER/AT Mix	VOLUME per each sample
ER/AT Buffer (Tube 2)	7 µl
ER/AT Enzyme (Tube 1)	3 µl
TOTAL	**10 µl**

Table 9: Volumes of reagents for each ER/AT reaction.

n. Add 10 µl of the ER/AT Mix to each sample and mix well by gently pipetting. Spin down the tubes and place them in the thermocycler **(go to stage 2 on thermocycler)**.
o. The finale volume is 60 µl.
p. Once the run is finished, place the tubes on ice and **proceed immediately** with the ligation of the Universal Adapters (point 7.4).

7.4 LIGATION OF THE UNIVERSAL ADAPTERS

Reagents Required

- Library from step 7.3
- Fresh ethanol 70 %
- Water for molecular biology
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- Ligation Enzymes (Tube 3)
- Ligation Buffer (Tube 4)
- Universal Adapters (Tube 5)
- Magnetic beads for the purification of genomic libraries (equilibrated at room temperature for at least 30 minutes)

Before starting:

Thaw and place on ice:

- Ligation Enzymes (Tube 3)
- Ligation Buffer (Tube 4)

- Universal Adapters (Tube 5)

Program the thermocycler as follows with the lid temperature control turned off. Start the program to pre-cool the thermocycler as shown below in Table 10:

Stage	Temperature (°C)	Time	Cycles
Stage 1	20	HOLD	1
Stage 2	20	30 n	1

Table 10: Ligation Thermal profile

STEP 1: LIGATION OF THE UNIVERSAL ADAPTERS

a. Prepare the Ligation Mix per each sample according to the following volumes (Table 11). Mix well by gently pipetting.

REAGENT for the Ligation Mix	VOLUME per each sample
Ligation Buffer (Tube 4)	25 µl
Ligation Enzymes (Tube 3)	5 µl
TOTAL	**30 µl**

Table 11: Volumes of reagents for each Ligation reaction.

b- Without removing the tubes from ice, add 5 µl of Universal Adapters (Tube 5) to each ER/AT sample obtained in point 7.3 and mix thoroughly by gently pipetting about 10 times.
c- Aliquot 30 µl of the Ligation Mix to each sample and mix well by gently pipetting.
d- The final volume is 95 µl.
e- Spin down briefly the tubes and place them in the thermocycler **(go to stage 2 on thermocycler)**.

At the end of the reaction, remove the samples from thermocycler and proceed with the purification step.

STEP 2: PURIFICATION

a. Vortex the tube, then add 76 µl of magnetic beads (0.8X) to each sample from STEP 1 of the Ligation protocol.
b. Incubate for 5 minutes at room temperature.
c. Pellet the beads on a magnetic rack.
d. Keep the tube on the magnetic rack and pipette off and discard the supernatant.
e. Wash the beads by adding 200 µl of freshly prepared 70% ethanol; Incubate for 1 minute, then remove and discard the ethanol.
f. Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic rack, and making sure not to disturb the beads pellet.
g. Remove the tubes from the magnetic rack, spin them down and place them back on the magnetic rack. Then remove all the residual ethanol with a P10.
h. Incubate about 5 minutes at room temperature but do not let the beads dry to the point of cracking.
i. Remove the tube from the magnetic rack and resuspend the pellet in 34 µl of RNase/DNase-free distilled water. Pipet up and down 5 times to thoroughly mix the beads suspension with water.
j. Incubate 5 minutes at room temperature.
k. Pellet the beads on the magnetic rack until the eluate is clear and colorless, for at least 1 minute.
l. Carefully remove and transfer 32 µl of the eluate containing the DNA library into a new 0.2 ml PCR-tube.

7.5 SAMPLE INDEXING WITH UDI PRIMERS

The insertion of the UDI primers takes place through a PCR reaction. For each sample, the final composition of the reaction mix will be as follows (Table 12):

REAGENT	MIX per each sample
Library in H_2O nuclease free	32 µl
Reaction buffer	7 µl
dNTPs	1 µl
Taq Polymerase	0.25 µl
UDI from UDI plate	10 µl
TOTAL	**50.25 µl**

Table 12: PCR MIX preparation for UDI insertion

Proceed with the preparation of the PCR reaction according to the following steps:

STEP 1: AmpliMix PREPARATION

a. Thaw and keep on ice the Reaction buffer, the Taq Polymerase (do not vortex), the dNTPs and the UDI primers plate (refer to page 3).
b. Prepare 500 µl of 70% Ethanol per each sample.
c. Prepare the AmpliMix as described in the following table (Table 13).

AmpliMix	x 1
Reaction buffer	7 µl
dNTPs 10mM	1 µl
Taq Polymerase	0.25 µl
TOTAL	**8.25 µl**

Table 13: AmpliMix volumes per each sample.

d. Aliquot 8 µl of AmpliMix in each 0.2 µl PCR-tube contained the purified library. (cancellare)
e. Add 10 µl from a single well of UDI primers for each sample.
f. Add 32 µl of the purified Library**: the final volume is 50 µl.**
g. Place PCR tubes in the thermocycler and run the following thermal profile (Table 14):

Stage	Temperature (°C)	Time	Ramping	Cycles
Stage 1	94	2 min	4°C/s	1
Stage 2	94	30 sec	4°C/s	10
	60	1 min	1°C/s	
	65	1 min	1°C/s	
Stage 3	10	HOLD	4°C/s	1

Table 14: Thermal profile

➢ **STOPPING POINT**

7.6 PURIFICATION OF THE AMPLIFIED LIBRARIES

a. Add 40 µl of resuspended beads (0.8X) to each sample.

b. Incubate for 5 minutes at room temperature.
c. Pellet the beads on a magnetic rack.
d. Keeping the tubes on the magnetic rack, pipette off and discard the supernatant.
e. Wash the beads by adding 200 µl of freshly prepared 70% ethanol; Incubate for 1 minute, then remove the ethanol.
f. Repeat the wash step twice, for a total of two washes, keeping the samples on the magnetic rack, and making sure not to disturb the beads pellet.
g. Spin down the tubes, place them back on the magnetic rack and remove all the residual ethanol with a P10.
h. Incubate about 5 minutes at room temperature but do not let the beads dry to the point of cracking.
i. Remove the tubes from the magnetic rack and resuspend the pellet in 62 µl of RNase/DNase-free distilled water. Pipet up and down 10 times to thoroughly mix the beads suspension with water and
j. incubate 5 minutes at room temperature.
k. Pellet the beads on a magnetic rack until the eluate is clear and colorless, for at least 1 minute.
l. Carefully remove and transfer 60 µl of each eluate containing the DNA library into a 1.5 ml tube.

7.7 LIBRARIES NORMALIZATION AND POOLING

a) Quantify the libraries obtained with fluorimeter Qubit and kit Qubit dsDNA HS Assay.
b) Create an equimolar pool in a new tube and dilute that at 4nM. Follow the instruction released by Illumina for each specific instrument. It is suggested to spike a 10% of the Phix reagent in the final reaction volume.

SECTION B: LIBRARY PREP FOR ION TORRENT SEQUENCERS

This section will perform end-repair and dA-tailing (ER/AT) of the pooled PCR products obtained from Step 7.2 (TARGET ENRICHMENT), and will then ligate the Aviseq Barcode Screen to the ER/AT DNA targets.

The following table (Table 15) resumes the volumes of all reagents needed for the ER/AT and Ligation reactions per each sample.

REAGENT	VOLUME per each sample
Library Pool (after target enrichment)	50 µl
ER/AT Buffer (Tube 2)	7 µl
ER/AT Enzyme (Tube 1)	3 µl
Barcode Screen plate	5 µl (volume /Barcode Screen
Ligation Buffer (Tube 4)	25 µl
Ligation Enzymes (Tube 3)	5 µl
TOTAL	**95 µl**

Table 15: Reagents needed for library prep compatible with Illumina sequencers.

7.8 END REPAIR AND dA-TAILING (ER/AT) REACTION

Reagents required:

- ER/AT Enzyme (Tube 1)
- ER/AT Buffer (Tube 2)
- Fresh prepared 70% ethanol (500 ml per sample)
- Magnetic Beads for library purification. Equilibrate them at room temperature for at least 30 minutes.

Before starting:

- Remove ER/AT Enzyme (Tube 1) and ER/AT Buffer (Tube 2) from the freezer and thaw them on ice. Mix all kit components by repeated inversions of the tubes before use.
- Program the thermocycler as follow (Table 16) and **set the heated lid to 85°C**. Start the program to pre-cool the thermocycler as shown below in Table 16.

Stage	Temperature (°C)	Time	Cycles
Stage 1	30	HOLD	1
Stage 2	30	5 min	1
Stage 3	65	15 min	1
Stage 4	4	HOLD	1

Table 16: ER/AT thermal profile

Library purification:

a. Add 75 µl of resuspended beads (1X) to the reaction from the end of paragraph 7.2 step 3 and mix by pipetting.
b. Incubate for 5 minutes at room temperature.
c. Pellet the beads on a magnetic rack.
d. Keep the tube on the magnetic rack and pipette off and discard the supernatant.
e. Wash the beads by adding 200 µl of freshly prepared 70% ethanol; Incubate for 1 minute, then remove the ethanol.
f. Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic rack and making sure not to disturb the beads pellet.
g. Remove the tubes from the magnetic rack, spin the down and place them back on the magnetic support. Then, remove all the residual ethanol with a P10.
h. Incubate about 5 minutes at room temperature but do not let the beads dry to the point of cracking.
i. Remove the tube from the magnetic rack and resuspend the pellet in 52 µl of RNase/DNase-free distilled water. Pipet up and down 10 times to thoroughly mix the beads suspension with water.
j. Incubate 5 minutes at room temperature.
k. Pellet the beads on a magnetic rack until the eluate is clear and colorless, for at least 1 minute.
l. Carefully remove and transfer 50 µl of each eluate into a new 0.2 µl PCR-tube.
m. Prepare the ER/AT mix according to the following volume (Table 17) of reagents. Mix well by pipetting.

REAGENT	VOLUME per each sample
ER/AT Buffer (Tube 2)	7 µl
ER/AT Enzyme (Tube 1)	3 µl
TOTAL	**10 µl**

Table 17: Volumes of reagents for each ER/AT reaction.

n. Add 10 µl of the ER/AT Mix to each sample and mix well by gently pipetting.
o. The finale volume is 60 µl.
p. Spin down the tubes and place them in the thermocycler **(go to stage 2 on thermocycler)**.

Once the run is finished, place the tubes on ice and proceed immediately with the ligation of the Aviseq Barcode Screen

7.9 LIGATION OF AVISEQ BARCODE SCREEN

Reagents Required

- Library from step 7.8
- Fresh Ethanol 70% (500 ml per each sample)
- Water for molecular biology
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- Ligation Enzymes (Tube 3)
- Ligation Buffer (Tube 4)
- Aviseq Barcode Screen 1-16 (AVG900) or Aviseq Barcode Screen 1-96 (AVG901)
- Magnetic Beads for library purification. Equilibrate them at room temperature for at least 30 minutes.

Before starting:

Defrost or place on ice:

- **Barcode Screen plate (use a single Barcode Screen for a single sample only)**
- Ligation Enzymes (Tube 3)
- Ligation Buffer (Tube 4)
- Fresh 70% ethanol

- Equilibrate Magnetic Beads at room temperature for at least 30 minutes.

Program the thermocycler as follow with the lid temperature control turned off. Start the program to pre-cool the thermocycler as shown below in Table 18:

Stage	Temperature (°C)	Time	Cycles
Stage 1	20	HOLD	1
Stage 2	20	30 min	1

Table 18: Ligation thermal profile.

STEP 1: LIGATION OF Aviseq Barcode Screen

a. Add 5 µl of Barcode Screen to each sample obtained in point 7.8. Mix thoroughly by gently pipetting and place on ice.
b. Prepare the Ligation Mix according to the following table (Table 19) and mix well by pipetting.

REAGENT	VOLUME per each sample
Ligation Buffer (Tube 4)	25 µl
Ligation Enzymes (Tube 3)	5 µl
TOTAL	**30 µl**

Table 19: Quantity of reagents per each Ligation reaction.

c. Aliquot 30 µl of the Ligation Mix per each sample and resuspend well to homogenize (at least 10 times).
d. The final volume is: 95 µl.
e. Spin down briefly the tubes and place them in the thermocycler **(go to stage 2 on thermocycler)**.
f. At the end of the reaction, remove the tubes from the thermocycler and proceed with the purification step.

STEP 2: PURIFICATION

a. Add 76 µl of resuspended beads (0.8X) to each sample.
b. Incubate for 5 minutes at room temperature.
c. Pellet the beads on a magnetic rack.
d. Keep the tube on the magnetic rack and pipette off and discard the supernatant.
e. Wash the beads by adding 200 µl of freshly prepared 70% ethanol; Incubate for 1 minute, then remove the ethanol.
f. Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic rack, and making sure not to disturb the beads pellet.
g. Spin down the tube and remove all the residual ethanol with a P10.
h. Incubate about 5 minutes at room temperature but do not let the beads dry to the point of cracking.
i. Remove the tube from the magnetic rack and resuspend the pellet in 34 µl of RNase/DNase-free distilled water; pipet up and down 5 times to thoroughly mix the beads suspension with water.
j. Incubate 5 minutes at room temperature.
k. Pellet the beads on a magnetic rack until the eluate is clear and colorless, for at least 3 minutes.
l. Carefully remove and transfer 32 µl of the eluate containing the DNA library into a 0.2 µl PCR-tube.

7.10 AMPLIFICATION WITH BARCODE-AMPLIFICATION PRIMERS

For each sample, the final composition of the reaction mix will be as follows:

REAGENT	VOLUME per each sample
Library in H_2O nuclease free	32 µl
Reaction buffer	7 µl
dNTPs	1 µl
Taq Polimerase	0.25 µl
Barcode Amplification Primer	5 µl
H20	4.75 µl
TOTAL	**50 µl**

Table 20: PCR MIX preparation

Proceed with the preparation of the PCR reaction according to the following steps:

STEP 1: AmpliMIX PREPARATION

a. Thaw the Reaction buffer, dNTPs and Barcode Amplification Primer. Keep Taq Polymerase in ice (do not vortex).
b. Prepare AmpliMix as described in the following table (Table 21):

AmpliMIX	VOLUME per each sample
Reaction buffer	7 µl
dNTPs 10mM	1 µl
Taq Polymerase	0.25 µl
Barcode Amplification Primer	5 µl
H20	4.75 µl
TOTAL	**18 µl**

Table 21: AmpliMIX

c. Dispense 18 µl of AmpliMIX in each 0.2 µl PCR-tube.
d. Add the 32 µl Purified Library: the Final Volume is 50 µl.
e. Place PCR tubes in the PCR machine and run the following thermal profile:

Stage	Temperature (°C)	Time	Ramping	Cycles
Stage 1	94	2 min	4°C/s	1
Stage 2	94	30 sec	4°C/s	10
	60	1 min	1°C/s	
	65	1 min	1°C/s	
Stage 3	10	HOLD	4°C/s	1

Table 22: Library amplification thermal profile

➢ **STOPPING POINT**

7.11 PURIFICATION OF THE AMPLIFIED LIBRARIES

a. Add 40 µl of resuspended beads (0.8X) to each sample.
b. Incubate for 5 minutes at room temperature.
c. Pellet the samples on a magnetic rack.
d. Keep the tube on the magnetic rack and pipette off and discard the supernatant.
e. Wash the beads by adding 200 µl of freshly prepared 70% ethanol; Incubate for 1 minute, then remove the ethanol.

f. Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic rack and making sure not to disturb the beads pellet.
g. Spin down the tube and remove all the residual ethanol with a P10.
h. Incubate about 5 minutes at room temperature but do not let the beads dry to the point of cracking.
i. Remove the tube from the magnetic rack and resuspend the pellet in 32 µl of RNase/DNase-free distilled water; pipet up and down 5 times to thoroughly mix the beads suspension with water.
j. Incubate 5 minutes at room temperature.
k. Pellet the beads on a magnetic rack until the eluate is clear and colorless, for at least 3 minutes.
l. Carefully remove and transfer 30 µl of the eluate containing the DNA library into a 0.2 µl PCR-tube.

7.12 LIBRARIES NORMALIZATION AND POOLING

a. Quantify the libraries obtained with fluorimeter Qubit and kit Qubit dsDNA HS Assay.
b. Create an equimolar pool in a new tube and then follow the ThermoFisher instruction released for each specific sequencer instrument.

8. TROUBLESHOOTING

PROBLEM	POSSIBLE CAUSE	SUGGESTION
Absence of bands on agarose gel after electrophoresis	Wrong PCR thermal profile	Verify the PCR thermal profile and calibration then repeat the PCR reaction
	Mistakes in master mix preparation	Verify PCR mix components and repeat the PCR reaction
	Degraded reagents	Verify expiry date and storing conditions of the products
	Presence of inhibitors	Verify concentration and quality of DNA extracted using a spectrophotometer. If necessary, repeat DNA extraction.
	Low amount of DNA	Verify concentration and quality of DNA extracted using a spectrophotometer. If necessary, repeat DNA extraction.
Absence of bands on agarose gel after electrophoresis	Wrong PCR machine settings	Verify the PCR thermal profile and calibration then repeat the PCR reaction
Presence of fragments with low molecular weight	Mistakes in master mix preparation	Verify PCR mix components and repeat the PCR reaction

In case of incidents or non-conformity of the products, the user has to inform the manufacturer, or the Representative or the distributor, providing all the necessary information to identify the product (Name and LOT) and a detailed description of the problem.

10. SYMBOLS

CE	*According to 98/79/CE Directive*	REF	*Catalogue number*
IVD	*In Vitro Diagnostic Medical Device*	LOT	*Batch code*
	Expiration date		*Temperature limitation*
i	*Consult instruction for use (IFU)*	Σ	*Sufficient for n. tests*
	Manufacturer		*Do not use if package damaged*
EC REP	*Representative*		
	Warning		