AVICENNA

AviSeq™

Genomic DX

CARDIO

REF: AVG303016 16 TESTS
REF: AVG303096 96 TESTS

USER GUIDE

AVG303_IFU_Rev01_JUN22

CE
IVD

CONTENTS

1. AIM OF USE

AviSeq CARDIO is a kit designed for the characterization of familial cardiovascular diseases, through a molecular protocol based on Next Generation Sequencing technologies (NGS).
The kit is validated for the analysis of DNA extracted from tissues, blood or body fluids.
AviSeq CARDIO must be used together with the specific adapters (UDI) included in the kit.

The gene regions that are analyzed with this kit are indicate in Table 1.

GENES LIST
To take a view of the panel genes consult the following attachment: Cardio_genes_list

Table 1: List of genes analysed with the Kit.

2. KIT CONTENTS

AviSeq CARDIO kit contains all reagents needed to capture genomic regions of interest for sequencing on the Illumina or Nanopore platform.

The kit size is 16 or 96 tests.
Note: the excess volume present in the kit is calculated to allow the subdivision respectively into a maximum of 2 or 12 analysis sessions. Dividing the kits 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 the following Table 2:

BOX A				
			30316A	30396A
Tube	Description	Storage	Volume 16 tests	Volume 96 tests
1	Frag/AT Enzymes	- 20°C	96 µl	576 µl
2	Frag/AT Buffer	- 20°C	64 µl	384 µl
3	Ligation Master Mix	- 20°C	320 µl	1,92 ml
4	Equinox Library Amp Mix (2X)	- 20°C	450 µl	2,4 ml
5	Universal Adapters	- 20°C	80 µl	480 µl
6	HybridizationMix	- 20°C	20 µl	120 µl
7	HybridizationEnhancer	- 20°C	30 µl	180 µl
8	AmplificationPrimers	- 20°C	3 µl	18 µl
9	CARDIO Probe	- 20°C	4 µl	24 µl
10	Blocker Solution	- 20°C	5 µl	30 µl
11	Universal Blockers	- 20°C	8 µl	48 µl
BOX B				
			30316B	30396B
Tube	Description	Storage	Volume 16 tests	Volume 96 tests
12	DNA PurificationBeads	+ 2–8°C	2,1 ml	12,5 ml
13	Binding Buffer	+ 2–8°C	0,8 ml	4,8 ml
14	WASH BUFFER1	+ 2–8°C	200 µl	1,2 ml
15	WASH BUFFER2	+ 2–8°C	0,7 ml	4,2 ml
16	StreptavidinBinding Beads	+ 2–8°C	100 µl	0,6 ml

BOX C				
			30316C	30396C
Plate	**Description**	**Storage**	**Quantity**	**Quantity**
1	Udi primers	- 20°C	16 well	96 well

Table 2: Kit contents.

3. STORAGE AND PRODUCT STABILITY

All reagents supplied with this kit are ready to use. The kit, intact and properly stored, will maintain high quality performance capabilities until the expiration date indicated on each individual reagent tube and on the jar/outer container.

4. KIT FEATURES

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

5. REQUIRED MATERIAL NOT INCLUDED

5.1 Generic Material

- Computer with constantly updated and guaranteed secure internet connection.
- Micropipettes calibrated and periodically verified 0.2-2 µl, 2-20µl, 20-200µl or 100-1000µl and filter tips.
- Vortex.
- Disposable Gloves without powder.
- Thermal cycler calibrated and periodically verified.
- PCR tubes and Caps or 96-wells plate, as needed, DNase and RNase free.
- Dry bath.
- Nuclease-free water.
- 1,5 ml tube magnetic separator or 96-wells plate compatible magnetic separator.
- Fresh 80% ethanol.
- Illumina sequencers calibrated and periodically verified.

5.2 Specific Material

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

- 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).
- Qubit™ dsDNA BR Assay Kit (Invitrogen, cod. Q32850).

QUALITATIVE ANALYSIS OF DNA (Optional)

- Agilent 2100 Bioanalyzer system with DNA reagent kit calibrated and periodically verified.

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.

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

7. LIBRARY PREPARATION

7.1 DNA SAMPLES PREPARATION

Use any commercial kit to obtain DNA from biological tissues. Determine the starting DNA concentration by fluorometric methods for accuracy.

- Use the Thermo Fisher Scientific Qubit dsDNA HS or BR Assay kit to accurately quantify the purified gDNA input.
- **It is not** recommended to measure DNA concentration by absorbance at 260 nm.
- The DNA must be suspended in water for molecular biology, Tris-HCl 10 mM pH 8.0 or EB buffer.
- For genomic samples (gDNA), accurate input quantity is critical to achieve optimal library fragment yield and length.
- The recommended DNA input is:
  - Germinal sample: 100ng of high quality gDNA (minimum input 50ng).
- The reagents are compatible with mass inputs from 1 ng to 500 ng, but may require optimization of the following steps in library preparation for optimal performance.

7.2 DNA FRAGMENTATION, END REPAIR AND dA-TAILING

Perform enzymatic fragmentation of gDNA and subsequent dA-tail and end repair to generate dA-tailed DNA fragments.

Reagents required:
- Genomic DNA (gDNA): 100 ng per sample.
- Molecular biology water.

• Qubit dsDNA HS o BR Assay (or equivalent).
• Frag/AT Enzymes (Tube 1).
• Frag/AT Buffer (Tube 2).

Before starting:
Defrost or place on ice:

• Molecular biology water.
• gDNA.
• Frag/AT Buffer (Tube 2).
• Frag/AT Enzymes (Tube 1).

Make sure the genomic DNA samples are of high quality with an OD 260/280 ratio between 1.8 and 2.0. Use the Qubit system to quantify genomic DNA prior to library preparation.

STEP 1: PREPARE THE THERMOCYCLER, SAMPLES AND REAGENTS

Program the thermal cycler with the following conditions. Set the lid temperature at 105°C. Start the program to pre-cool the thermal cycler as shown below in Table 3.

Note: 20 min at 37°C is the condition for germline samples, to obtain an average fragment size of 300bp (For 2X150 sequencing chemistries).

Refer to the *Technical Note* document to adjust the settings based on the type of sample and chemistry used.

Stage	Temperature (°C)	Time	Cycles
Stage 1	4	HOLD	1
Stage 2	37	20 min	1
	65	30 min	
Stage 3	4	HOLD	1

Table 3: PCR thermal profile.

WARNING: It is not recommended to measure DNA concentration by absorbance at 260 nm.

Mix the gDNA gently. Assay the genomic DNA (gDNA) samples, using the Qubit dsDNA HS or BR Assay kit, to determine their concentration.

Transfer 100 ng of sample in 40 µl of total volume of diluted gDNA sample inside a 0.2 ml PCR-strip or inside a well of a 96-well PCR plate.

Spin to make sure all of the solution is at the bottom of the tube and place on ice.

STEP 2: FRAGMENTATION, END REPAIR, AND dA-TAILING

Vortex the Frag/AT Buffer tube (Tube 2) for 5 seconds. Spin to make sure all solution is at the bottom of the tube.

Invert the Frag/AT Enzymes tube (Tube 1) a minimum of 5 times to homogenize. Spin to make sure all solution is at the bottom of the tube. Prepare the Fragmentation Mix in a 1.5-ml microcentrifuge tube kept on ice as reported in Table 4 below.

Mix thoroughly by gently pipetting, avoiding the formation of bubbles.

REAGENT	VOLUME PER REACTION
Frag/AT Buffer (Tube 2)	4 µl
Frag/AT Enzymes (Tube 1)	6 µl
Total	**10 µl**

Table 4: Quantity of reagents for each reaction.

Add 10µl of Fragmentation Mix to each gDNA sample. Mix thoroughly by gently pipetting, avoiding the formation of bubbles. Seal the plate or cap the strip/tube and place it on ice.

WARNING: Thorough mixing is critical to achieving desired fragment lengths.

Spin and transfer immediately to the pre-cooled thermal cycler. Start the thermal cycler program from stage 2 to stage 3 as shown in Table 3.

WARNING: While the thermal cycler program is running, prepare the reagents for point 7.3: Ligation of Universal Adapters and Purification **(read before beginning)**.

When the thermal cycler program is complete and the block has reached 4°C, remove the samples and place them on ice.

Proceed immediately to point 7.3: LIGATION OF UNIVERSAL ADAPTERS AND PURIFICATION.

7.3 LIGATION OF UNIVERSAL ADAPTERS AND PURIFICATION

In this phase, the universal adapters are ligated to the DNA fragments with dA tail obtained in point 7.2, once purified they are ready for the introduction of the Indexes by PCR.

Reagents Required

- dA-tailed DNA fragments obtained in point 7.2.
- Ethanol.
- Water for molecular biology.
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution).
- Ligation Master Mix (Tube 3).
- Universal Adapters (Tube 5).
- DNA Purification Beads (Tube 12).

Before starting:
Defrost or place on ice:

- Universal Adapters (Tube 5) (can be used for all samples).
- Ligation Master Mix (Tube 3).
- Prepare 1 ml of 80% ethanol for each sample.
- Equilibrate DNA Purification Beads (Tube 12) at room temperature for at least 30 minutes.
- Program the thermal cycler to incubate samples at 20°C with the lid temperature set to the minimum or turned off. Start the program so that the thermal cycler has reached 20°C at the end of sample preparation.

STEP 1: LIGATION OF UNIVERSAL ADAPTERS

Add 5 µl of Universal Adapters (Tube 5) to each tube or well containing the dA-tailed DNA fragments obtained in point 7.2. Mix thoroughly by gently pipetting and place on ice. Invert the Ligation Master Mix (Tube 3) a minimum of 5 times to homogenize.

WARNING: *Do not vortex the Ligation Master Mix.*

Add 20 µl of Ligation Master Mix (Tube 3) to each sample obtained in point 7.2, mix thoroughly by gently pipetting. Seal the plate or cap the strip and spin to ensure all solution is at the bottom of the tube/well.

REAGENT	VOLUME PER REACTION
Universal adapters (Tube 5)	5 µl
Ligation Master Mix (Tube 3)	20 µl
Total	**25 µl**

Table 5: Quantity of reagents for each reaction.

Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, at the end take the samples and proceed with the purification.

Stage	Temperature (°C)	Time	Cycles
Stage 1	20	15min	1

Table 6: Ligation thermal profile.

WARNING: While the thermal cycler program is running, prepare the reagents for point 7.4: Amplification with UDI primers, purification and quality control.

STEP 2: PURIFICATION

a) Vortex the pre-equilibrated DNA Purification Beads (Tube 12) at room temperature until they are well homogenized.
b) Add 60 µl of homogenized DNA Purification Beads (Tube 12) (0.8X) to each sample obtained in the previous step.
c) Incubate the samples for 5 minutes at room temperature.
d) Place the samples on the magnetic support for 1 minute or until the supernatant is clear.
e) The beads form a pellet, leaving a transparent supernatant. Without removing the plate or tubes from the magnetic support, remove and discard the supernatant.
f) Wash the pellet of beads by gently adding 200 µl of fresh 80% ethanol (without disturbing the pellet). 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 support. Carefully remove all remaining ethanol with a 10 µl pipette, making sure not to disturb the beads pellet.
h) Dry the beads pellet on the magnetic support for 5 minutes or until the pellet is dry. Do not desiccate the pellet to the cracking point.
i) Remove the plate or tubes from the magnetic support and add 17 µl of water for each sample. Pipette until completely homogenized.
j) Incubate at room temperature for 2 minutes.
k) Place the plate or tubes on the magnetic support and wait for 3 minutes or until the beads form a pellet.
l) Transfer 15 µl of clear supernatant, containing ligated and indexed libraries, to a new 0.2 ml PCR strip or well of a 96-well plate, making sure not to disturb the beads pellet.
Proceed with point 7.4: AMPLIFICATION WITH UDI PRIMER, PURIFICATION AND QUALITY CONTROL.

7.4 AMPLIFICATION WITH UDI PRIMER, PURIFICATION AND QUALITY CONTROL

In this step, the gDNA libraries containing the adapters are amplified with the UDI primers, purified and quality control (QC) performed to complete the protocol.

Reagents Required

- Ligated libraries and containing the adapters from point 7.3.
- Fresh 80% ethanol.
- DNA Purification Beads (Tube 12) equilibrated.
- Water for molecular biology.
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution).
- Equinox Library Amp Mix (2X) (Tube4).
- UDI primers (BOX C).

Before starting

Defrost or place on ice:

- Equinox Library Amp Mix (2X) (Tube 4).
- UDI Primers (BOX C).

STEP 1: PREPARE THE THERMOCYCLER AND SET UP PCR

Stage	Temperature (°C)	Time	Cycles
Stage 1	98	45 sec	1
Stage 2	98	15 sec	8
	60	30 sec	
	72	30 sec	
Stage 3	72	1 min	1
Stage 4	4	HOLD	1

Table 7: PCR thermal profile.

Add 10 µl of UDI Primer contained in the 96-well plate, to each gDNA library obtained in point 7.3, mix well by gently pipetting.

WARNING: *To mix, invert the Equinox Library Amp Mix tube (2X) (Tube 4) 5 times before use. Do not vortex.*

Add 25 µl of Equinox Library Amp Mix (2X) (Tube 4) to the gDNA library from point 7.3 and mix well by gently pipetting.

Spin the plate or strips and immediately transfer them to the thermal cycler and start the program. At the end of the PCR, remove the samples and proceed with purification.

STEP 2: PURIFICATION

a) Vortex the pre-equilibrated DNA Purification Beads (Tube 12) at room temperature until they are well homogenized.
b) Add 50 µl of homogenized DNA Purification Beads (Tube 12) (1X) to each sample obtained in the previous step.
c) Incubate the samples for 5 minutes at room temperature.
d) Place the samples on the magnetic support for 1 minute or until the supernatant is clear.
e) The beads form a pellet, leaving a transparent supernatant. Without removing the plate or tubes from the magnetic support, remove and discard the supernatant.
f) Wash the pellet of beads by gently adding 200 µl of fresh 80% ethanol (without disturbing the pellet). 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 support. Carefully remove all remaining ethanol with a 10 µl pipette, making sure not to disturb the beads pellet.

h) Dry the beads pellet on the magnetic support for 5 minutes or until the pellet is dry. Do not desiccate the pellet, do not let crack the beads pellet.
i) Remove the plate or tubes from the magnetic support and add 22 µl of water for each sample. Pipette until completely homogenized.
j) Incubate at room temperature for 2 minutes.
k) Place the plate or tubes on the magnetic support and wait for 3 minutes or until the beads form a pellet.
l) Transfer 20 µl of clear supernatant, containing ligated and indexed libraries, to a new 0.2 ml PCR tube or well of a PCR strip or 96-well plate, making sure not to disturb the beads pellet.

STEP 3: QUALITY CONTROL

Quantify samples with the Thermo Fisher Scientific Qubit dsDNA HS or BR Assay kit and verify the size of each library on the Agilent DNA 7500 assay.

Using 100 ng of good quality gDNA in a 20 minutes fragmentation at 37°C and 8 cycles of PCR should result in a final concentration ≥15 ng/µl. Under these conditions, the average fragment length observed is generally between 200 and 400 bp.

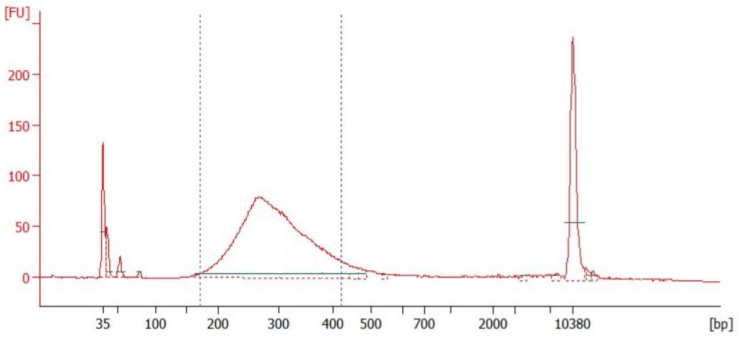

Table 8: Bioanalyzer profile from a purified library.

➢ **STOPPING POINT**

7.5 PRE-CAPTURE CONCENTRATION WITH DNA PURIFICATION BEADS

Before starting:
• Thaw on ice: Probes (Tube 9), Hybridization Mix (Tube 6), Blocker Solution (Tube 10) and Universal Blockers (Tube 11). When thawed, vortex the components for 2 seconds and spin.
• Thaw the Hybridization Enhancer (Tube 7) at room temperature.
• **When thawed, put the Hybridization Mix (Tube 6) to incubate at 65°C.**
• Equilibrate the DNA Purification Beads (Tube 12) at room temperature.
• Prepare 80% ethanol (250 µl/sample).

STEP 1: CALCULATE THE VOLUME IN µL REQUIRED FOR EACH AMPLIFIED INDEXED LIBRARY

NUMBER OF INDEXED SAMPLES PER POOL	QUANTITY OF EACH INDE LIBRARY PER POOL	TOTAL QUANTITY PER POOL
8	187.5 ng	1500 ng

Table 9: Amount of library per pool.

WARNING: *It is important to use the same ng for each sample merged.*

a) Transfer the calculated volume for each indexed library to a new 0.2 ml PCR-tube.
b) **If the total volume of the pooled amplified indexed libraries is ≥25 µl, proceed directly to the next step. If the total volume is <25 µl, add molecular biology water to bring the total volume to 25 µl**.
c) Add the homogenized DNA purification beads (Tube 12) equal to 1.5 times the volume of the pooled libraries (1.5X).
d) Example: For a library pool volume equal to 25 µl, add 37.5 µl of DNA purification beads (Tube 12).
e) Incubate the samples for 5 minutes at room temperature.
f) Place the samples on a magnetic support for 1 minute. The beads will form a pellet leaving a transparent supernatant. Remove and discard the supernatant.
g) Wash the pellet of beads with 200 µl of fresh ethanol at 80%, incubate for 1 minute, then remove the ethanol. Repeat this wash, for a total of two washes, keeping the samples on the magnetic support.
h) Gently remove and discard the remaining ethanol with a 10 µl pipette, taking care not to disturb the pellet of beads.
i) Keeping the samples on the magnetic support, air dry the pellet of beads for 5 minutes or until dry. Do not desiccate the beads to the point of crack the pellet.
j) While the pellet dries, remove the Hybridization Mix (Tube 6) from the incubation at 65°C and mix thoroughly by pipetting slowly until it is homogenized. Allow the hybridization mix to incubate at room temperature until the pellet is ready.
k) Remove the plate or tubes from the magnetic support and add 10 µl of Hybridization Mix (Tube 6) to each sample. Mix by pipetting slowly until the solution is homogeneous.
l) Carefully transfer the entire volume of the beads suspension containing the amplified indexed libraries to a new 0.2 ml PCR strip.

Proceed to the next step immediately.

WARNING: The presence of DNA purification beads in the hybridization reaction has no negative effects

STEP 2: HYBRIDIZATION WITH CAPTURE PROBES
Program the thermal cycler with the following conditions. Set the lid temperature to 85°C.

Stage	Temperature (°C)	Time	Cycles
Stage 1	95	5 min	1
Stage 2	70	HOLD	1

Table 10: Hybridization thermal profile.

To the DNA purification beads suspension containing the amplified indexed libraries add the reagents described in the following Table (Table 11). After adding the last component, carefully mix the entire hybridization reaction by pipetting, making sure not to generate bubbles. Spin to make sure all of the solution goes to the bottom of the tube.

WARNING: Hybridization Mix is a very viscous solution. Pipette slowly to ensure correct volume.

WARNING: It is recommended to load a total of 5 µg of Human Cot-1 DNA for each hybridization reaction.

WARNING: Small white particles may be present in the CARDIO Probe tube, this will not affect the final capture product.

REAGENT	VOLUME
SPRI bead slurry with Amplified indexed Libraries	10 µl
Blocker Solution (Human Cot-1 DNA; [1 µg/µL]) (Tube 10)	2.5 µl
Universal Blockers (Tube11)	3.5 µl
CARDIO Probe (Tube 9)	2 µl

H2O (up to the volume of 40µL)	2 µl
Total	**20 µl**

Table 11: Reagents and volumes for the hybridization reaction.

Add 15 µl of Hybridization Enhancer (Tube 7) above the capture reaction. Spin to remove all bubbles.

Transfer the hybridization reaction to the thermal cycler and start the program. Incubate for 16 hours at 70°C with the setting of the step to infinity.

IMPORTANT: ***Make sure that the tube is well closed to avoid excessive evaporation during the 16 hours of incubation.***

7.6 CAPTURE OF HYBRIDIZED CARDIO PROBES TO STREPTAVIDIN BEADS

Before starting
Preheat the following tubes to 48°C until the precipitate is dissolved:

- Binding Buffer (Tube 13).
- Wash Buffer 1 (Tube 14).
- Wash Buffer 2 (Tube 15).

For each hybridization reaction:

- Equilibrate 400 µl Binding Buffer (Tube 13) at room temperature.
- Equilibrate 100 µl Wash Buffer 1 (Tube 14) at room temperature.
- Leave 350 µl Wash Buffer 2 at 48°C (Tube 15).

Equilibrate the Streptavidin beads (Tube 16) at room temperature for at least 30 minutes.

In preparation for Step 7.7 (Post-Capture Amplification, Purification and Quality Control):
Thaw on ice:

- Equinox Library Amp Mix (2X) (Tube 4).
- Amplification Primers (Tube 8).

Equilibrate DNA Purification Beads (Tube 12) at room temperature for at least 30 minutes.

STEP 1: PREPARE THE STREPTAVIDIN BEADS

a) Vortex the equilibrated Streptavidin Binding Beads (Tube 16) until homogenized.
b) Place 50 µl of Streptavidin Binding Beads (Tube 16) into a 1.5-ml microcentrifuge tube. Prepare a tube for each hybridization reaction.
c) Add 100 µl Binding Buffer (Tube 13) to each tube and pipette to mix.
d) Place the tubes on the magnetic support for 1 minute, then remove and discard the clear supernatant without disturbing the beads pellet. Remove the tubes from the magnetic support.
e) Repeat the wash (Point "c" and "d") twice for a total of three washes.
f) After removing the supernatant from the third wash, add 100 µl Binding Buffer (Tube 13) and resuspend the beads by vortexing until homogeneous.

STEP 2: BIND THE TARGETS

IMPORTANT: *Rapid transfer directly from the thermal cycler at 70°C is a critical step to minimize off-targets.* ***Do not remove the hybridization reaction tubes from the thermal cycler or allow it to cool to less than 70°C before transferring the solution to the washed streptavidin beads. Allowing it to cool to room temperature for less than 5 minutes will result in an increase of up to 10-20% of off-targets.***

a) At the end of the hybridization, immediately transfer the entire volume of the hybridization reaction into the corresponding tube containing the washed Streptavidin beads obtained in step 1. Mix by pipetting and tapping the tube.
b) Mix the hybridization reaction with the Streptavidin beads for 30 minutes at room temperature on the shaker, rocker, or wheel at a speed sufficient to keep the solution mixed.

NOTE: *Do not vortex. Aggressive mixing is not necessary.*

c) Remove the tubes containing the hybridization mixture and the Streptavidin beads from the shaker and spin to make sure that all the solution is at the bottom of the tube.
d) Place the tubes on the magnetic support for 1 minute.
e) Remove and discard the clear supernatant including the Hybridization Enhancer. Do not touch the beads pellet.

NOTE: *A little Hybridization Enhancer may be visible after the removal of the supernatant and during each washing step. It will not affect the final catch product.*

f) Remove the tubes from the magnetic support and add 100 µl Wash Buffer 1 (Tube 14). Pipette to mix.
g) Spin to ensure all solution is at the bottom of the tube.
h) Transfer the entire volume (~ 100 µl) into a new 1.5-ml tube, one for each hybridization reaction. Place the tubes on the magnetic support for 1 minute.

IMPORTANT: *This step reduces the background from non-specific binding to the surface of the tube.*

i) Remove and discard the clear supernatant taking care not to disturb the pellet.
j) Remove the magnetic support tubes and add 100 µl of Wash Buffer 2 (Tube 15) heated to 48° C. Pipette to mix and spin to ensure all solution is at the bottom of the tube.
k) Incubate the tubes for 5 minutes at 48°C.
l) Place the tubes on the magnetic support for 1 minute.
m) Remove and discard the clear supernatant taking care not to disturb the pellet.
n) Repeat the wash (Steps "j" - "m") twice for a total of three washes.
o) After the last wash, use a 10 µl pipette to remove all traces of supernatant. Proceed to the next step immediately. Do not let the beads dry.

NOTE: *Before removing the supernatant, the beads pellet can be briefly spinned to collect the supernatant at the bottom of the tube or plate and place the tubes or plate back on the magnetic support.*

p) Remove the tubes from the magnetic support and add 32µl of water. Pipette to homogenize, then incubate this solution, hereinafter referred to as Streptavidin Binding Beads slurry, on ice.

Proceed with step 7.7: POST-CAPTURE AMPLIFICATION, PURIFICATION AND QUALITY CONTROL.

7.7 POST-CAPTURE AMPLIFICATION, PURIFICATION AND QUALITY CONTROL

Prepare 250 µl of fresh 80% ethanol for each sample of Streptavidin Binding Bead slurry.

STEP 1: PROGRAM THE THERMAL CYCLER

Program the thermal cycler with the conditions listed below in Table 12. Set the lid to 105°C.

Stage	Temperature (°C)	Time	Cycles
Stage 1	98	45 sec	1
Stage 2	98	15 sec	11
	60	30 sec	
	72	30 sec	
Stage 3	72	1 min	1
Stage 4	4	HOLD	1

Table 12: Thermal profile.

If beads are layered in the Streptavidin Binding Beads slurry solution, pipette to homogenize.

Transfer 11 µl of Streptavidin Binding Bead slurry to a 0.2-ml PCR-strip, then transfer the strips on ice and proceed to the next step.

NOTE: *Store the remaining 11 µl of water/Streptavidin Binding Bead slurry at –20°C for future use.*

STEP 2: AMPLIFICATION

Prepare the PCR mixture by adding the reagents listed below (Table 13) to the tubes containing the Streptavidin Binding Bead slurry.

REAGENT	VOLUME PER REACTION
Streptavidin Binding Bead Slurry	11 µl
Amplification Primers (Tube 8)	1.5 µl
Equinox Library Amp Mix (2X) (Tube 4)	12.5 µl
Total	25 µl

Table 13: PCR Mix.

Spin the tubes, transfer them to the thermal cycler and start the program.

At the end of the program, remove the tubes from the block and proceed immediately with the purification step.

STEP 3: PURIFICATION

a) Vortex the DNA Purification Beads (Tube 12) until homogenized.
b) Add 25 µl (1.0X) of homogenated DNA Purification Beads to the tubes obtained in step 1 of point 7.7. Mix by vortexing.

NOTE: *It is not necessary to recover supernatant or remove Streptavidin Binding Beads from the PCR product.*

c) Incubate for 5 minutes at room temperature.
d) Place the tubes on the magnetic support for 1 minute or until the supernatant is clear.
e) DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic support, remove and discard the clear supernatant.
f) Wash the pellet by adding 100 µl of fresh 80% ethanol without disturbing the pellet. Incubate for 1 minute, then remove the ethanol.
g) Repeat the wash for a total of two washes, holding the tubes on the magnetic support.
h) Gently remove the remaining ethanol using a 10 µl pipette, taking care not to disturb the pellet.

NOTE: *Before pipetting, the pellet can be briefly centrifuged to collect the ethanol at the bottom of the plate or tube and then returned to the magnetic stand.*

i) Dry the pellet of beads in the air on the magnetic support for 5 minutes or until the pellet is dry, without desiccate the pellet to the cracking point.
j) Remove the tubes from the magnetic support and add 61 µl of water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Pipette to homogenize.
k) Incubate 2 minutes at room temperature.

l) Place the tubes or plate on the magnetic support for 3 minutes or until the pellet is formed.
m) Transfer 14 µl of clear supernatant containing the enriched library to a new 0.2-ml PCR-strip or 96-well PCR plate, taking care not to disturb the pellets.

STEP 4: QUALITY CONTROL

Quantify samples with the Thermo Fisher Scientific Qubit ds DNA HS or BR Assay kit and verify the size of each library in the Agilent Bioanalyzer High Sensitivity DNA kit.

NOTE: *When using the Agilent Bioanalyzer High Sensitivity DNA Kit, load 1 µl of the final sample. The average fragment length should be 300 bp.*

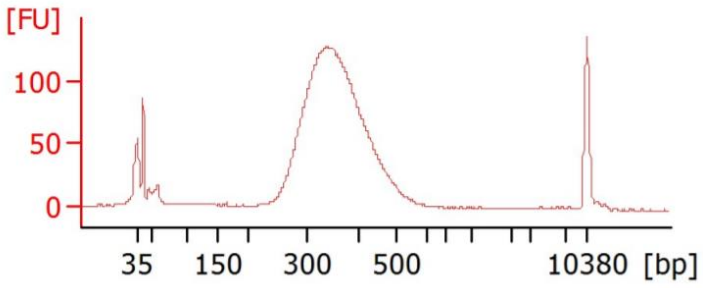

Table 14: Final library profile.

➢ **STOPPING POINT**

NOTE: *If not proceeding immediately, store the spiked library sample at –20°C for up to 24 hours.*

8. TROUBLESHOOTING

PROBLEM	POSSIBILE 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.
Presence of fragments with low molecular weight	Primer residues and / or degradation of adapters, primers dimers, etc.	Eliminate low molecular weight fragments by purification with AMPure XP Beads

9. SYMBOLS

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

Avicenna FZ-LLC
Dubai Science Park. Warehouse Complex ,
Dubai –UAE

EC REP Donawa Lifescience Consulting Srl
Piazza Albania, 1000153 Rome, Italy