AVICENNA

AviSeq™

Genomic DX

RESPASENSE

REF:AVG304016 16TESTS
REF: AVG304096 96 TESTS

USER GUIDE

AVG304_IFU_Rev01_JUN22

CE
IVD

CONTENTS

1. AIM OF USE

RESPASENSE is a kit for the analysis of hotspots in the genomic targets listed in Table 1. Analysis is performed with a molecular protocol based on Next Generation Sequencing (NGS) technologies.

RESPASENSE must be used together with the following AviSeq reagents (not included in the kit) containing the specific adapters and index compatible with Illumina, Ion Torrent or Nanopore sequencer:

Illumina: Index Set (cod. AVG402, cod. AVG403, cod AVG405).

Ion Torrent: Barcode set 1-16 or Barcode Set 17-32 (AviSeq, cod. AVG502 or cod. AVG503).

The kit is validated for germline analysis (SNPs, indel) of DNA extracted from blood and tissues (fresh, frozen).

GENES
SRRM1, NCMAP, CLIC4, RCAN3, NIPAL3, RUNX3, IVNS1ABP, SWT1, IFITM3, IRF7, UNC93B1, OAS1, OAS3, TBK1, PCSK3, ACE1, BSG, DPP9, TICAM1, APOE, IRF3, DPP4, TMEM189, UBE2V1, IFNAR2, IFNAR1, TMPRSS2, GGR, SLC6A20, CCR9, FYCO1, CXCR6, XCR1, LZTFL1, CCR5, TLR3, ABO, ACE2

Table 1: Detail of genes targeted.

2. KIT CONTENTS

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 4 analysis sessions.

Tube	Description	Volume 16 tests	Volume 96 tests
1	Primer Mix Pool 1	20 µl	110 µl
2	Primer Mix Pool 2	20 µl	110 µl
Taq	Hot Start Hi-Fi Taq	12 µl	64 µl
BA	Buffer A	320 µl	1840 µl
BB	Buffer B	320 µl	1840 µl
NTP	dNTPs Mix 10 mM	36 µl	184 µl

Table 2: Kit contents.

3. STORAGE AND PRODUCT STABILITY

All reagents provided with our kits 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 expiry date marked on each single reagent tube and on the external jar/container.
When the reagents are thawed they must be kept on ice throughout the process.

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, 2-20 µl, 20-200 µl or 100-1000 µl and filter tips.
- Vortex.
- Disposable Gloves without powder.
- Thermal cycler 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 or Ion Torrent sequencers calibrated and periodically verified.
- For Illumina sequencers: Index Set (cod. AVG402, cod. AVG403, cod AVG405).
- For Ion Torrent sequencers: Barcode set 1-16 or Barcode Set 17-32 (AviSeq, cod. AVG502 or cod. AVG503).

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).
- 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 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. OPERATIVE PROTOCOL

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. Dilute each DNA sample in PCR grade water to a final concentration of 10 ng/µl.

NB: The efficiency of the amplification process strongly depends on the quality of the DNA. For not fragmented DNA (i.e. fresh tissue, cytological samples…) 10 ng are in general sufficient for a good amplification process.

7.2 TARGET ENRICHMENT

Amplification of the target regions are performed with PCR in 2 reaction tubes, each containing one different primer pools. At the end of PCR, all the PCR reactions will be mixed into a single pool.

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

Multiplex POOL 1 (Mix x 1 reaction)		**Multiplex POOL 2 (Mix x 1 reaction)**	
Primer Mix Pool 1	1 µl	Primer Mix Pool 2	1 µl
Buffer A	4 µl	Buffer A	4 µl
Buffer B	4 µl	Buffer B	4 µl
dNTPs Mix 10 mM	0,4 µl	dNTPs Mix 10 mM	0,4 µl
Hot Start Hi-Fi Taq	0,2 µl	Hot Start Hi-Fi Taq	0,2 µl
DNA	10 ng	DNA	10 ng
H_2O	Up to 20 µl	H_2O	Up to 20 µl
TOT	**20 µl**	**TOT**	**20 µl**

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

The Reaction could be set up in 2 STEPS:

Step 1: Prepare AmpliMIX

In a clean and appropriatedwork area (e.g. laminar workflow) prepare the AmpliMIX,the amplificationmix containing the PCR reagents common to all reaction tubes.

a) Unfreeze Buffer A, Buffer B, dNTPs, Primer Mix Pool 1 and 2. Vortex and briefly centrifuge.Keep Hot Start Hi-Fi Taq (do not vortex).

b) Prepare AmpliMIX as described in the following table:

AmpliMIX	x 1
Buffer A	8 µl
Buffer B	8 µl
dNTPs Mix 10 mM	0,8 µl
Hot Start Hi-Fi Taq	0,4 µl
H_2O	16,8 µl
TOTAL	**34 µl**

Table 4: AmpliMIX for 1 sample.

Step 2: Prepare Master Mixes

a) Accuratelymix theAmpliMIX (do not vortex),then dividethe volumein 2 x 1.5 ml tubesand add a specific Primer Mix Poolto each tube, as to the table below:

MMixes	x 1	
	MMix 1	MMix 2
AmpliMIX	17 µl	17 µl
Primer Mix Pool 1	1 µl	-
Primer Mix Pool 2	-	1 µl
TOTAL	**18 µl**	**18 µl**

Table 5: Preparationof Master Mixes.

b) For each sample, dispense 18 µl of each MasterMix per tube, change working area then add 2 µl of DNA (concentration5 ng/µl) in each of the 2 PCR tubes. Brieflycentrifuge thesamples.

N.B. During all time keep the mixes and reagents on ice when not in use.

Place PCR tubes in the thermocycler and then start the program according to the following thermal profile:

Stage	Temperature (°C)	Time	Ramping	Cycles
Stage 1	98	2 min	4°C/s	1
Stage 2	98	10 sec	4°C/s	27
	60	2 min	1°C/s	
	72	20 sec	1°C/s	
Stage 3	72	5 min	4°C/s	1
Stage 4	10	HOLD	4°C/s	1

Table 6: PCR thermal profile.

At the end of PCR, mix the PCR products deriving from the same sample in a unique tube to obtain a library pool, and then proceed with the protocol.

➢ **STOPPING POINT**

7.3 QUANTIFICATION OF PCR PRODUCTS

Analyse the obtained libraries with a fluorometer Qubit and kit Qubit dsDNA HS Assay.

7.4 QUALITATIVE AND QUANTITATIVE ANALYSIS OF LIBRARIES (OPTIONAL)

A quick estimation of DNA products size may be obtained running samples on agarose gel. However, we strongly recommend using Bioanalyzer 2100 with DNA reagent kit; please refer to procedure of instrument.

INDEXING PROTOCOL

7.5 INDEXING PCR MIX PREPARATION

7.5.1 Defrost, quickly vortex and centrifuge the reagents supplied. Keep the Hot Start Hi-Fi Taq on ice (do not vortex).

N.B. For PCR use Buffer A, Buffer B, dNTPs and Hot Start Hi-Fi Taq contained in panel kit; during all time keep the mixes and reagents on ice when not in use.

For Illumina sequencers: Index Set (cod. AVG402, cod. AVG403, cod AVG405).

For Ion Torrent sequencers: Barcode set 1-16 or Barcode Set 17-32 (AviSeq, cod. AVG502 or cod. AVG503).

7.5.2 For each sample, prepare a mix according to the following scheme.

ILLUMINA	
Master Mix	**x1**
DNA (library)	100 ng
Buffer A	10 µl
Buffer B	10 µl
dNTPs Mix 10 mM	1 µl
Hot Start Hi-Fi Taq	0,2 µl
Index i7 + i5	2 µl
H_2O	Up to 50 µl
TOT	**50 µl**

ION TORRENT	
Master Mix	**x1**
DNA (library)	100 ng
Buffer A	10 µl
Buffer B	10 µl
dNTPs Mix 10 mM	1 µl
Hot Start Hi-Fi Taq	0,2 µl
Barcode Oligo	2 µl
H_2O	Up to 50 µl
TOT	**50 µl**

Table 7: Master mix preparation volumes.

The Reaction could be set up in 2 STEPs

Step 1: Prepare AmpliMIX (example for a library concentrated 10 ng/µl):

AmpliMIX	x1
Buffer A	10 µl
Buffer B	10 µl
dNTPs Mix 10 mM	1 µl
Hot Start Hi-Fi Taq	0,2 µl
H_2O	16,8 µl
TOT	**38 µl**

Table 8: PCR mix preparation volumes.

Step 2: Prepare Master Mixes

For Illumina: for each sample add 2 µl of indexes to AmpliMix, by picking from 96-well plate. (Table 9).

Note: for the conversion table refer to the IFU of each index set kit.

For Ion Torrent: assign to each sample a specific barcode and add to each sample 2 µl of the selected barcode.

ILLUMINA	
Master Mix	**x1**
AmpliMIX	38 µl
Indexs	2 µl
TOT	**40 µl**

ION TORRENT	
Master Mix	**x1**
AmpliMIX	38 µl
Barcode	2 µl
TOT	**40µl**

Table 9: Master Mix preparation.

7.5.3 In a post-PCR area, add 10 µl (100 ng of DNA) of the purified library (point 7.2) Please note that the total volume of the PCR should be 50 µl.

7.5.4 Briefly centrifuge samples.

7.5.5 Keep tubes on ice until placement into the thermocycler.

7.5.6 Place the reaction tubes/plate in the thermal cycler and run the following PCR protocol:

Stage	Temperature (°C)	Time	Ramping	Cycles
Stage 1	98	1 min	4°C/s	1
Stage 2	98	10 sec	4°C/s	15
	54	40 sec	1°C/s	
	72	3 min	1°C/s	
Stage 3	72	2 min	4°C/s	1
Stage 4	10	HOLD	4°C/s	1

Table 11: Thermal-cycler profile.

➢ **STOPPING POINT**

7.6 PCR PURIFICATION

In appropriate post-PCR area, proceed with the library purification:

N.B. Fresh ethanol 70 % is required for a proper wash low molecular weight fragment. Low ethanol concentrations can lead to loss of sample.

a) For each sample, distribute 40 µl (0.8X) of magnetic beads (previously resuspended) in low binding 1.5 ml tubes.
b) Add 50 µl of each amplified library pool; pipet up and down 5 times to thoroughly mix the beads suspension with the DNA.
c) Incubate samples at room temperature for 5 minutes.
d) Place tubes on magnetic support and incubate for 2 minutes or until the mixture appears clear; remove supernatant without touching the pellet.
e) Add 200 µl of fresh ethanol 70% and rotate tubes twice to wash the beads; remove supernatant without touching the pellet.
f) Repeat the washing step.
g) Ensure that all ethanol is removed; keeping tubes on the magnetic support let the beads dry on air for 5 minutes (do not desiccate, do not let crack the beads pellet)
h) Remove the tubes from the magnetic support and elute the DNA in 42 µl of RNase/DNase free distilled water; pipet up and down 5 times to thoroughly mix the beads suspension with the DNA.
i) Incubate 5 minutes at room temperature.
j) Place tubes on magnetic support and incubate for 2 minutes or until the mixture appears clear; carefully collect 40 µl of the supernatant containing the desired amplicons without touching the pellet.

➢ **STOPPING POINT**

7.7 QUALITATIVE AND QUANTITATIVE EVALUATION OF PCR PRODUCTS (OPTIONAL)

A quick estimation of DNA products size may be obtained running samples on agarose gel. However, we strongly recommend using Agilent 2100 Bioanalyzer (refer to manufacturers manual for protocol).

7.8 QUANTITATIVE EVALUATION OF PCR PRODUCTS

Quantify PCR products using a fluorometer, according to the manufacturer instructions, to prepare an equimolar pool of samples for sequencing.

For Illumina: dilute each library according to the Illuminas equencer procedure.

For Ion Torrent: dilute each library according to the Ion Torrent sequencer procedure.

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 concentrationand quality of DNA extracted using a spectrophotometer.If necessary,repeat DNA extraction.
Presence of fragmentswith low molecularweight	Primer residues and / or degradationof adapters, primers dimers, etc.	Eliminate low molecularweight fragmentsby purificationwith 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*

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