AVICENNA

AviSeq™

Genomic DX

COV19

REF: AVG212016 16 TESTS
REF: AVG212096 96 TESTS
REF: AVG212384 384 TESTS

USER GUIDE

AVG212_IFU_Rev01_JUN22

CE
IVD

CONTENTS

1. INTENDED USE

The AviSeq COV19 is intended for use in the genomic analysis of the SARS-CoV-2 virus using next generation sequencing (NGS) technology. The kit must be used together with the following AviSeq Indexing kits depending on sequencing platform (instrument) and application:

For Illumina instruments use:

Index Set A (Ref: AVG400)
Index Set B (Ref: AVG401)
Index Set-16 Test (Ref: AVG404)
Index Set-384 Test (Ref: AVG405)

This kit and the associated protocol has been validated using viral cDNA generated from viral RNA extracted from nasopharyngeal blood swabs and other materials found to contain viral RNA (e.g., blood, tissue). Use of RNA extracted from other sources must be validated by the user. In addition, the user must validate their nucleic acid purification and cDNA synthesis (reverse transcription) protocols in-house. The use of a positive cDNA control is recommended for each analysis session/run. The AviSeq COV19 kit has been validated using the AviSeq COV19 CONT (code AVG212C).

2. KIT CONTENTS

This kit is available in 16, 96 or 384 test formats suitable for a maximum of 4 analysis sessions. Increasing the number of times this kit is used i.e., >4 analysis sessions, may negatively impact the performance characteristics of the kit reagents due to too many freeze-thaw cycles. The total number of successful tests may decrease as a result.

LABEL	Description	Volume 16 tests	Volume 96 tests	Volume 384 tests
1	Primer Mix Pool 1	72 µl	390 µl	1560 µl
2	Primer Mix Pool 2	72 µl	390 µl	1560µl
Taq	Hot Start Hi-Fi Taq	16 µl	86 µl	350 µl
BA	Buffer A	400 µl	2100 µl	8400 µl
NTP	dNTPs Mix 10 mM	40 µl	210 µl	840 µl

Table 1: Details of kit contents and as per the 3 kit formats available

3. STORAGE AND PRODUCT STABILITY

All reagents are ready to use and should be stored at -20° C immediately on receipt. The kit, if intact and properly stored, will maintain a high level of performance quality until the expiry date marked on each reagent tube and on the external packaging. When the reagents are thawed and in use, they must be kept on ice throughout the process. and returned to -20° C storage immediately after use.

4. KIT FEATURES

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

Using high quality DNA at the recommended concentration and following the guidelines of this IFU exactly.

5. REQUIRED MATERIAL NOT INCLUDED

5.1 Generic Material

- Computer with constantly updated and guaranteed secure Internet connection
- Micro pipettes calibrated and periodically verified 0.2-2 µl, 2-20µl, 20-200µl or 100-1000µl and filter tips
- Vortex
- Disposable powder-free gloves and other appropriate PPE
- 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 (Invitrogen Magnetic Stand-96; Cat#: AM10027)
- Fresh 70% ethanol and 5% hypochlorite
- Illumina sequencers calibrated and periodically verified

5.2 Specific Material

The equipment and reagents listed below have been validated for use with this kit and the associated protocol:

- 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)
- For Illumina sequencers: Index set A _96 Test (Ref AVG400) or Index Set B (Ref. AVG401 or Index Set-16 Test (Ref: AVG404) or Index Set-384 Test (Ref: AVG405).

QUALITATIVE ANALYSIS OF DNA (Optional)

- Agilent 2100 Bioanalyzer calibrated and periodically verified, and associated Agilent DNA Reagent Kit

6. IMPORTANT NOTES AND SAFETY INFORMATION

Users must comply with all recommendations listed below. Failure to do so will result in poor results and/or failed runs. If the kit or any component thereof is forwarded to a third party, it is your responsibility to provide the end user with these recommendations. Note: the manufacturer is committed to the highest level of quality assurance at all steps in the procedure and will provide full support to all customers.

- This kit is intended for use by professionals trained in molecular biology. Use by untrained individuals may result in suboptimal outcomes.
- 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 strongly recommend cleaning working areas frequently using a solution containing sodium hypochlorite 3-5% followed by sterile water and 70% Ethanol.
- Always use appropriate personal protective equipment (PPE) such as laboratory coat, gloves and safety goggles during all steps described in the protocol.
- Ensure that you are aware of all safety instructions associated with all equipment and related electrical supply, chemicals and other resources utilized in the performance of the procedures outlined in this IFU.
- Ensure that the quality control procedures in place at each phase of the protocol are adequate to detect any errors, particularly after each usage of the kit. This should include a quality evaluation of the results generated. Contact your local supplier immediately on detecting any errors and/or anomalies in the performance of the kit
- It is essential in a diagnostic and/or clinical setting that users of this kit put strong control and quality assurance procedures in place following a critical evaluation of possible risk factors in the protocol to minimize or eliminate diagnostic errors. Any errors detected must be communicated to the relevant persons immediately.

- 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 highly recommend the lab is designed in 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.
- Reagent and biological waste material must be discarded according to local regulations and following local legal procedures for biological waste disposal.

➢ **Stopping point**: This protocol includes Safe Stopping Points at points indicated. Users can proceed to the next step in the protocol or choose to store the samples/libraries for 24 hours at 4 °C or for a longer period at -20 °C.

7. OPERATIVE PROTOCOL

7.1 cDNA Synthesis / Reverse Transcription

7.1.1 Use a commercial kit to reverse-transcribe the viral RNA and obtain the cDNA. Determine the concentration of the starting cDNA using a validated fluorometric method e.g., Qubit, Picogreen, etc., and following the respective manufacturer's instructions.

The reverse transcription kits listed below have been validated for use with this kit.

- LunaScript RT SuperMix Kit (New England Biolabs code BE3010)
- ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs code E6560S).

NB: The efficiency of the SARS-CoV-2 target amplification and its success is largely dependent on the quality and concentration of cDNA used in the PCR reaction.

7.1.2 Bring the starting cDNA to a concentration of 5 ng / µl in a total volume of 6 µl per reaction (final concentration is 30ng per reaction).

7.1.3 The positive control should be used at a concentration of 5 ng / µl in a total volume of 6 µl per reaction.

7.2 TARGET ENRICHMENT

The SARS-CoV-2 genome is amplified in 2 separate reaction pools containing primer pairs targeting different regions of the viral genome. At the end of the first PCR reaction, both amplicon libraries are pooled for a single amplicon library per sample.

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)**	
Pool 1 COVID	3.6µl	Pool 2 COVID	3.6µl
Buffer A	5µl	Buffer A	5µl
dNTPs Mix 10 mM	0.5µl	dNTPs Mix 10 mM	0.5µl
Hot Start Hi-Fi Taq	0.3µl	Hot Start Hi-Fi Taq	0.3µl
cDNA	30ng	cDNA	30ng
H_2O	Up to 25 µl	H_2O	Up to 25 µl
TOTAL	**25µl**	**TOTAL**	**25µl**

Table 2: quantities of reagents for each target enrichment reaction

The Reaction can be set up in 2 STEPS:

- **Step 1: Prepare AmpliMIX**

In a clean designated work area for master mix preparation (e.g., clean room, within laminar flow, etc.,), prepare the amplification master mix (AmpliMIX) containing all PCR reagents for both the Primer Pools. This will also aid in prevention of multiple freeze-thaw cycles of the PCR reagents.

a) Unfreeze Buffer A, dNTPs, COVID Pool 1 and 2. Vortex and briefly centrifuge. Keep all reagents, once thawed, on ice. Keep the Taq Polymerase on ice (do not vortex but centrifuge briefly).

b) Prepare AmpliMIX as described in the following table:

AmpliMIX	x 1
Buffer A	10 µl
dNTPs Mix 10 mM	1 µl
Hot Start Hi-Fi Taq	0.6 µl
H_2O	19.4 µl
TOTAL	**31 µl**

Table 3: AmpliMIX for 1 amplification of 1 sample using both Primer Pools.

- **Step 2: Prepare Master Mixes**

a) Accurately mix the AmpliMIX (do not vortex), then divide the volume in by dispensing 15.4ul of the AmpliMix into each of the 2 PCR tubes that have been clearly labeled PP1 (Primer Pool 1) and PP2 (Primer Pool 2).

MMixes	x 1	
	MMix 1	MMix 2
AmpliMIX	15,4 µl	15,4 µl
COVID Pool 1	3,6 µl	-
COVID Pool 2	-	3,6 µl
TOTAL	**19 µl**	**19 µl**

Table 4: Preparation of MasterMixes

b) For each sample, dispense 19 µl of each MasterMix per tube as indicated in the table above. Add 3.6ul of the appropriate COVID Primer Pool to the corresponding Master Mix tubes. The volume of Master Mix and Primer Pool will now be 19ul. Once aliquoted, ensure that all reagents are placed back at -20°C storage and move the aliquoted master mix to the DNA addition work area. Ensure that the cDNA has been diluted to 5ng/ul. Vortex and briefly centrifuge all samples and the Positive control before use. Add 6ul of cDNA (total input concentration = 30ng) to each of the 2 PCR tubes (PP1 and PP2) for each sample. Similarly, add 6ul of the Positive Control to the PC marked tube. Pipette mix all sample and PC tubes 10 times, close tubes and briefly centrifuge to concentrate sample to the bottom of the tube. The final volume will be 25 µl for each reaction.

N.B. Keep the mixes and reagents on ice at all times during use and return to -20°C storage immediately after use. DO NOT freeze-thaw >4 times.

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

Stage	Temperature (°C)	Time	Ramping	Cycle
Stage 1	98	30 sec	4°C/s	1
Stage 2	95	15 sec	4°C/s	25
	63	5 min	1°C/s	
Stage 4	10	HOLD	4°C/s	1

Table 5: Thermal cycling conditions for amplicon generation

N.B.:

- **Use the PCR thermal profile of Table 5 for samples previously tested using Real Time PCR (qPCR) and producing a Ct ≤ 25**
- **Increase the number of cycles at Stage 2 of Table 5 to 35 cycles for samples previously tested using Real Time PCR (qPCR) and producing a Ct > 25.**

At the end of the PCR, briefly centrifuge the tubes and combine the amplification products of the 2 tubes thus producing one library pool for each sample. Ensure that each amplicon pool to be combined have the same/ corresponding sample IDs prior to pooling them.

➢**STOPPING POINT**

7.3 QUALITATIVE AND QUANTITATIVE ANALYSIS OF LIBRARIES (Optional)

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

An estimate of the size of the PCR products can be obtained by running the Agilent 2100 Bioanalyzer with the HS DNA Kit. Refer to the manufacturer's instructions for the procedure to follow

8. INDEXING PROTOCOL

a. INDEXING PCR MIX PREPARATION

1. Defrost, quickly vortex and centrifuge the reagents supplied for the indexing procedure. Place all reagents on ice once thawed. Keep the Hot Start Hi-Fi Taq on ice (do not vortex).

N.B. Use Buffer A, dNTPs and Hot Start HiFi Taq supplied in this panel kit for the PCR amplification. Always keep all reagents and the master mix on ice while conducting the Master Mix preparation and aliquoting. Return unused reagents to freezer storage immediately after use.

2. For each sample, prepare a mix according to the following table.

ILLUMINA	
Master Mix	**x1**
cDNA (from purified library)	3 µl
Buffer A	10 µl
dNTPs Mix 10 mM	1 µl
Hot Start Hi-Fi Taq	0.2 µl
Index i7 + i5	2 µl
H_2O	33,8 µl
TOTAL	**50 µl**

Table 6: Master mix preparation volumes

3. The reactions can be set up in two steps.

- **Step 1: Prepare AmpliMIX**:

AmpliMIX	x1
Buffer A	10 µl
dNTPs Mix 10 mM	1 µl
Hot Start Hi-Fi Tac	0.2 µl
H_2O	33.8 µl
TOTAL	**45 µl**

Table 7: PCR mix preparation volumes

- **Step 2: Prepare Master Mix**

<table>
<tr><th>For index set A (Ref. AVG400) and index set B (Ref. AVG401)</th><th>For index set 16 (Ref. AVG404) and index set 384 (Ref. AVG405)</th></tr>
<tr><td>

Prepare Master Mix as per Table 8.1 below

Indexing: assign two indexes to each sample, one from the oligo series 700 (i7) and one from the oligo series 500 (i5).

In order to choose the right combination between index i7 and index i5, please refer to the note "Technote Nextera low plex pooling guidelines Illumina" released by Illumina.

<table>
<tr><th colspan="2">ILLUMINA</th></tr>
<tr><th>Master Mix</th><th>x1</th></tr>
<tr><td>AmpliMIX</td><td>45 µl</td></tr>
<tr><td>Index i5</td><td>1 µl</td></tr>
<tr><td>Index i7</td><td>1 µl</td></tr>
<tr><td>TOTAL</td><td>47 µl</td></tr>
</table>

Table 8.1: Master Mix preparation

4. In the post PCR area **add 3 µl of the library (step 7.2)** and mix well by gently pipetting. Please note that the total volume of the PCR should be 50 µl.

Proceed with step 5 below.

</td><td>

N.B. To avoid contamination of the Indexes in the plate format, in case of puncture, centrifuge and clean the surface of the plate with 70% Ethanol or 70% Isopropanol and ensure evaporation prior to proceeding.

Prepare Master Mix (table 9.2)

Indexing: for each sample add 2 µl of indexes to Ampli Mix, by picking from 96-well plate.

Note: for the conversion table refer to the "Conversion

INDEX" document of each index set kit batch.

<table>
<tr><th colspan="2">ILLUMINA</th></tr>
<tr><th>Master Mix</th><th>x1</th></tr>
<tr><td>AmpliMIX</td><td>45 µl</td></tr>
<tr><td>Indexes</td><td>2 µl</td></tr>
<tr><td>TOTAL</td><td>47 µl</td></tr>
</table>

Table 8.2: Master Mix preparation

4. In the post PCR area **add 3 µl of the library (step 7.2)** and mix well by gently pipetting. Please note that the total volume of the PCR should be 50 µl

Proceed with step 5 below

</td></tr>
</table>

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

Stage	Temperature (°C)	Time	Ramping	Cycle
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 9: Thermal-cycler profile

➢ STOPPING POINT

b. LIBRARY POOLING AND PURIFICATION

In an appropriately dedicated post-PCR area, proceed with the creation of the library pool and purification.

N.B. Use freshly prepared 70% ethanol for proper elimination of low molecular weight molecules. Lower ethanol concentrations may result in sample loss.

a) Transfer 5µl of each library to a new 1.5 ml Low Bind tube. **N.B. A minimum of 4 and a maximum of 16 libraries could be pooled together for a single-tube purification. If preparing <4 samples for a run, please consider purifying each sample separately.**

b) Add magnetic beads equal to 0.8 times the volume of the pooled libraries (0.8X)
Example: For a library pool volume of 80 µl, add 64 µl of magnetic beads.
c) Pipet up and down 5 times to thoroughly mix the beads suspension with the cDNA
d) Incubate samples at room temperature for 5 minutes.
e) Place tubes on magnetic rack and incubate for 2 minutes or until the mixture appears clear; discard supernatant without touching the pellet.
f) Add 200 µl of fresh ethanol 70% and rotate tubes twice to wash the beads; discard supernatant without touching the pellet.
g) Repeat the step (f) above.
h) Ensure that all ethanol is removed; keeping tubes on the magnetic rack let the beads dry on air for 5/10 minutes (do not desiccate)
Note: Insufficient drying may result in ethanol carry-over and possible inhibition of downstream procedures. Prolonged drying will result in poor resuspension of the pellet thus poor DNA recovery and possible failed library preparation.
i) Remove the tubes from the magnetic rack and elute in 42µl of RNase/DNase free distilled water; pipet up and down 5 times to thoroughly mix the beads suspension.
j) Incubate 5 minutes at room temperature.
k) Place tubes on magnetic rack 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 into a clean nuclease-free 0.2ml tube.

➢ STOPPING POINT

c. QUALITATIVE EVALUATION OF PCR PRODUCTS (OPTIONAL)

While a quick and inexpensive estimation of the size distribution of the amplification products can be obtained by running an Agarose gel, this practice is not recommended and may result in sample wastage. We strongly recommend the use of a Bioanalzyer 2100 with DNA reagent kit for a more accurate profile of the DNA fragments in the sample library. Follow the IFU guidelines for the DNA reagent kit exactly.

d. QUANTITATIVE EVALUATION OF PCR PRODUCTS

Quantify the amplification product concentration using an appropriate fluorometer such as the Qubit with the Qubit dsDNA HS assay and following the manufacturer's IFU exactly. Accurate quantification is essential to prepare an equimolar library pool.

Before sequencing: follow the instructions given in Tecnote_C004. It is suggested to spike a 5% of the Phix reagent in the final reaction volume.

9. 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	Mistakes in master mix preparation	Verify PCR mix components and repeat the PCR reaction

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
	Consult instruction for use (IFU)	Σ	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