BioVendor Group NGS

ENG

Instructions for Use:
epicGEN Solid Cancer & MSI Kit

Catalogue number:
RDEG0001

For research use only

BioVendor R&D®

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
	ENG.001.A
New edition	

1. INTENDED USE

The epicGEN Solid Cancer & MSI Kit is designed to generate targeted libraries for analysis by next-generation sequencing (NGS) from a wide input range of double-stranded DNA (1 ng to 1 µg). This manual describes a workflow for the analysis of selected regions of interest used for genotyping of oncological samples.

1.1 Abbreviations

ctDNA	Circulating tumor DNA
dsDNA	Double-stranded DNA
FFPE	Formalin-Fixed Paraffin Embedded tissue
gDNA	genomic DNA
MSI	Microsatellite instability
NGS	Next Generation Sequencing
PCR	Polymerase chain reaction

2. STORAGE, EXPIRATION

Store the individual parts of the kit as indicated on the label. Under these conditions, all components are stable for the expiration date indicated on the outer packaging.

Protect kit components from light.

Do not use the kit after the expiration date.

3. INTRODUCTION

The epicGEN Solid Cancer & MSI Kit enables efficient preparation of NGS sequencing libraries from dsDNA for sequencing on Illumina® platforms. The procedure involves enzymatic DNA fragmentation, NGS library construction, and subsequent hybridization of labelled probes with target DNA regions (Figure 1).

NGS libraries prepared by this procedure are compatible with Illumina® and GeneMind sequencers. For sequencing on other platforms (e.g. MGI, AVITI), use appropriate adaptor conversion kit.

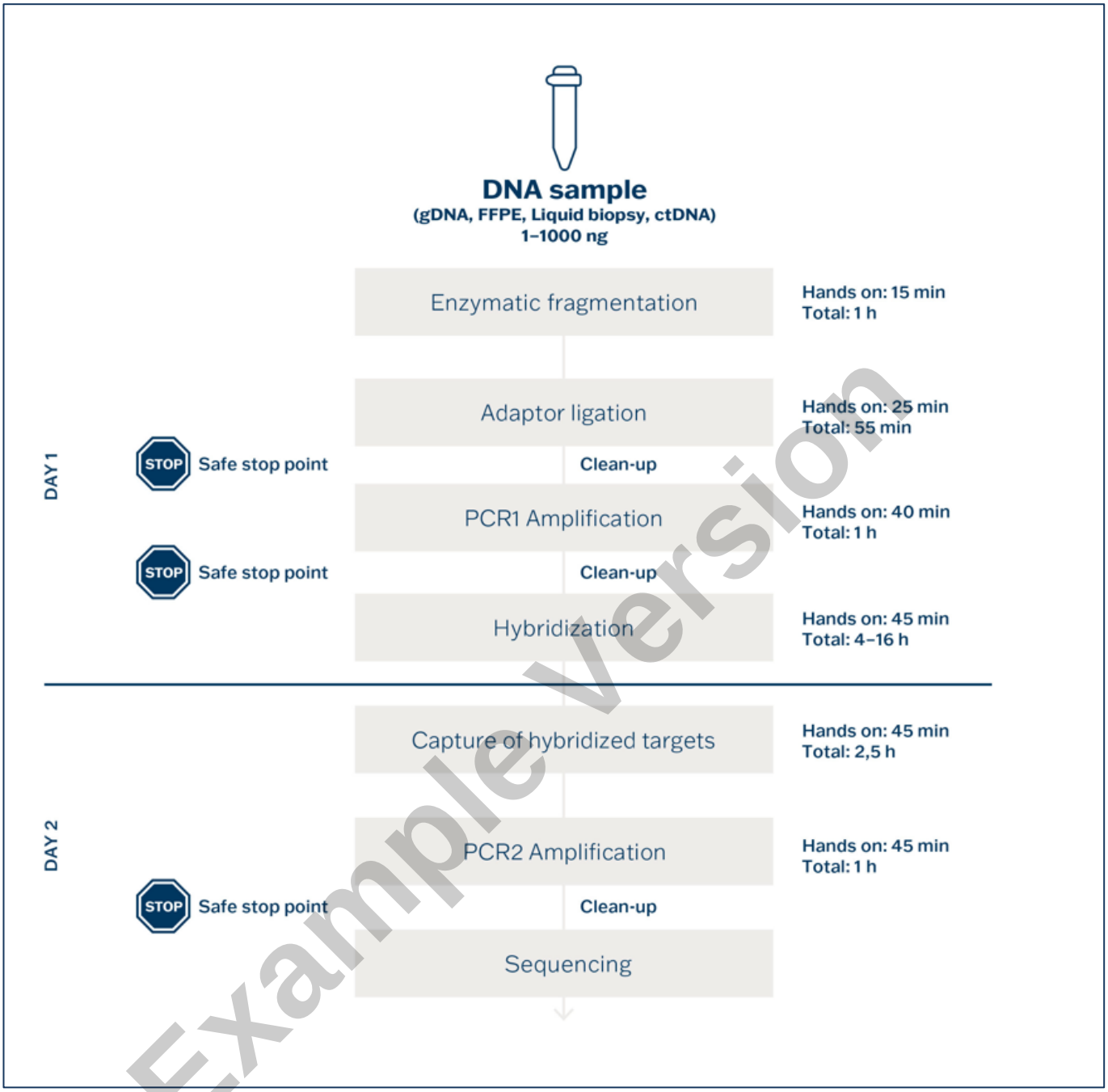


Figure 1: epicGEN protocol scheme

4. TEST PRINCIPLE

The epicGEN Solid Cancer & MSI Kit is designed to genotype cancer-relevant regions of selected genes (Table 1) and determine the degree of microsatellite instability (MSI) (Table 2). The principle of the evaluation is based on the detection of variants with a frequency higher than 1%.

4.1 Overview of genes contained in epicGEN Solid Cancer & MSI Kit

AKT1	ALK	ARAF	ARID1A	ARID1B	ATM	ATR
BRAF	BRCA1	BRCA2	CDK12	CDKN2A	DDR2	EGFR
ERBB2	ESR1	FAT1	FGFR1	FGFR2	FGFR3	CHEK1
IDH1	IDH2	KEAP1	KRAS	MAP2K1	MET	MTOR
NF1	NRAS	NTRK1	NTRK2	NTRK3	PALB2	PIK3CA
PIK3R1	POLE	PTEN	RB1	RET	STK11	TP53

Table 1: Coding regions of genes

BAT25	BAT26	BAT40	CAT25	D18S58	D2S123	D5S346
MONO27	NR21	NR22	NR24	NR27	TGFBR2	

Table 2: MSI loci

5. PRECAUTIONS

For professional use only by trained personnel in a suitable laboratory environment.

The components of the epicGEN Solid Cancer & MSI Kit do not contain infectious material.

Specimens for testing with the epicGEN Solid Cancer & MSI Kit must be handled as potentially infectious material and standard precautions must be followed.

Do not drink, eat or smoke in the area where biological material is handled.

6. TECHNICAL RECOMMENDATIONS

Before and after each test, the working environment must be decontaminated with suitable RNase and DNase removers as well as standard disinfectants. Working in an inappropriate environment can lead to contamination of kit components.

Thaw individual kit components just before use. Work on ice or using cooling racks.

Mix reagents by gentle vortexing and short centrifugation before use.

Perform PCR preparation and post-amplification steps in separate laboratory areas.

Avoid contamination of samples and reagents. For this reason, use disposable tips for each sample and reagents.

Dispose of used and unused material in accordance with valid legislation.

7. REAGENT SUPPLIED

Part of the protocol	Packaging	Reagents	Storage
Library preparation	Box1	epicGEN Fragmentation Buffer	−20 °C
		epicGEN Fragmentation Enhancer	−20 °C
		epicGEN Fragmentation Enzyme	−20 °C
		epicGEN Ligation Buffer	−20 °C
		epicGEN Ligation Enzyme	−20 °C
		epicGEN Adaptors	−20 °C
		epicGEN PCR1 Master Mix	−20 °C
		TE Buffer	−20 °C
	Plate1	epicGEN UDI primers	−20 °C
Hybridization	Box2	epicGEN Hybridization Buffer	−20 °C
		epicGEN Hybridization Buffer Enhancer	−20 °C
		Human Cot DNA	−20 °C
		epicGEN 2X Bead Wash Buffer	−20 °C
		epicGEN 10X Wash Buffer 1	−20 °C
		epicGEN 10X Wash Buffer 2	−20 °C
		epicGEN 10X Wash Buffer 3	−20 °C
		epicGEN 10X Stringent Wash Buffer	−20 °C
		NF Water	−20 °C
		epicGEN PCR2 Master Mix	−20 °C
		epicGEN Primer Mix	−20 °C
		epicGEN Blockers TS	-20 °C
		epicGEN Solid Cancer & MSI panel	−20 °C
	Box3	epicGEN Streptavidin Beads	4 °C

Table 3: Composition of the epicGEN Solid Cancer & MSI Kit.

8. RECOMMENDED MATERIAL (NOT SUPPLIED)

Reagents	Supplier	Catalog number
Absolute Ethanol (100%)	Various supplier	Various
Magnetic beads -		
-SPRIselect™ purification beads	Beckman Coulter	B23317/B23318/B2331
-Agencourt® AMPure® XP-PCR purification beads	Beckman Coulter	A63880/A63881
Digital capillary electrophoresis -		
-Experion™ DNA 1K Analysis Kit	Bio-Rad	700-7107
-High Sensitivity DNA Kit	Agilent	5067-4626
-High Sensitivity D1000 ScreenTape®	Agilent	5067-5584
DNA quantification kit-		
-Qubit™ dsDNA HS Assay Kit or equivalent	Thermo Fisher Scientific	Q32851/Q32854
-Qubit™ dsDNA BR Assay Kit or equivalent	Thermo Fisher Scientific	Q32850/Q32853
Nuclease-Free water		
PCR tubes, 0.2 ml	Various suppliers	Various
96-well, low-bind PCR plates	Various suppliers	Various
Low-bind DNA tubes, 1.5 ml	Various suppliers	Various
Filter tips with range 2–1000 µl	Various suppliers	Various
Microseal® B PCR Plate Sealing Film, adhesive, optical	Bio-Rad	MSB1001
Digital electrophoresis-		
-Experion Electrophoresis Station or equivalent	Bio-Rad	700-7010
-2100 Electrophoresis Bioanalyzer or equivalent	Agilent	G2939BA
-2200 TapeStation System/4200 TapeStation System or equivalent	Agilent	G2965AA/G2991AA
Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33226
Magnetic stand-		
-Magnetic Separator Plate	Permagen	MSP750
-Magnetic PCR Strip Magnetic Separator Rack	Permagen	MSR812
2 PCR thermal cyclers	Various suppliers	Various
Microcentrifuge	Various suppliers	Various
Vortex	Various suppliers	Various

Table 4: Material not supplied with the kit

9. REAGENTS PREPARATION

Always store reagents at −20 °C, except TE Buffer, which can be stored at room temperature. The enzymes in this kit are temperature sensitive. Take care during storage and handling. **For more efficient enzymatic activity of the reagents, remove the tubes with enzymes from −20 °C and place them on ice for at least 10 minutes before use.**

After thawing on ice, briefly vortex the reagents (except for the epicGEN Ligation Buffer) and spin in a microcentrifuge before opening.

Thaw Ligation Buffer (for Ligation Master Mix) at room temperature. epicGEN Ligation Buffer is viscous and requires special handling during pipetting. Pipette the buffer slowly to achieve an accurate volume.

When making Master Mixes, adjust reagent volumes as needed, and use an extra 10% volume to compensate for pipetting loss. Add reagents in the order specified in the protocol. Store Master Mixes on ice until use.

Always use magnetic beads (SPRISelect® or equivalent) after their equilibration to room temperature.

9.1 Risk of contamination

To reduce the risk of DNA and NGS library contamination, physically separate the laboratory space and equipment into pre-PCR and post-PCR sections.

To prevent cross-contamination, we recommend the following steps:

- Clean the lab area with 0.5% sodium hypochlorite (10% bleach).
- Use pipette tips with a filter.
- Always change pipette tips between each sample.

9.2 Size-selection during cleaning with magnetic beads

This protocol was optimized with SPRISelect® magnetic beads (Beckman Coulter), but it can also be used with Agencourt® AMPure® XP (Beckman Coulter). When using other magnetic beads, the conditions for DNA binding may vary.

10. SAMPLE PREPARATION

10.1 DNA quantity and quality requirements

This kit allows to work with a wide range of input DNA from **1 ng - 1 µg**.

The kit allows input of DNA with different quality (high quality gDNA, FFPE DNA, ctDNA)

Quantify the DNA prior to entering the protocol using Qubit™ or similar fluorometric method.

10.2 Fragmentation parameters

The fragmentation times given in this protocol are valid for FFPE quality samples. For higher quality (gDNA) samples, the fragmentation time may need to be optimised.

10.3 EDTA in elution buffer

Enzymatic reactions are sensitive to high concentrations of EDTA, which is a common component of elution buffers in the final steps of DNA extraction or purification processes. High concentrations of EDTA, (>1 mM) in standard TE buffer will slow enzymatic fragmentation, resulting in larger insert sizes.

Under standard enzymatic fragmentation conditions (0.1 mM EDTA TE), the use of 1.5 µl of epicGEN Fragmentation Enhancer (included in the protocol) is required.

If DNA is dissolved in standard TE with 1 mM EDTA, perform a buffer exchange using a column or magnetic beads (3x SPRIselect® is recommended for minimal DNA sample loss). Alternatively, you can adjust the amount of epicGEN Fragmentation Enhancer used in the enzymatic fragmentation step to a maximum of threefold to achieve the desired fragment length (up to 4.5 µl of epicGEN Fragmentation Enhancer per reaction).

11. ASSAY PROCEDURE

11.1 Enzymatic fragmentation

Note: Keep the Fragmentation Master Mix and samples on ice until they are placed in the thermocycler to protect against excessive fragmentation. Enzymes are active at room temperature and can fragment DNA to undesirable fragment lengths.

1. Transfer DNA samples **(1 ng – 1 µg)** into a clean PCR tube and make up to a total volume of **19.5 µl** using a **TE Buffer**. Then place the tube on ice.
2. Set the Fragmentation program on the thermocycler according to Table 5:

Set the lid heating to 70 °C		
Step	**Temperature**	**Time**
Cooling	4 °C	∞
Fragmentation	32 °C	10 min
Inactivation	65 °C	30 min
Cooling	4 °C	< 1 hour

Table 5: Fragmentation program

Note: Do not leave the final cooling for more than 1 hour.

3. Run the **Fragmentation program** on the thermocycler (without samples).
4. Prepare the **Fragmentation Master Mix** according to Table 6:

Reagents	**Volume per 1 reaction**
epicGEN Fragmentation Buffer	3 µl
epicGEN Fragmentation Enhancer	1.5 µl
epicGEN Fragmentation Enzyme	6 µl

Table 6: Fragmentation Master Mix

5. Vortex the Fragmentation Master Mix, spin briefly and store on ice until use.
6. Add **10.5 µl of Fragmentation Master Mix** to each sample (**19.5 µl**), vortex and spin briefly. The final reaction volume is **30 µl**.
7. Place the samples in a pre-cooled thermocycler and run the "Fragmentation" step at 32 °C.
8. Prepare the Ligation Master Mix during fragmentation.

11.2 Ligation of adaptors

Important: For DNA input <500 ng, dilute epicGEN Adaptors according to Table 7. Dilution of adaptors is important to avoid the formation of adaptor-dimers. **Adaptor dilution can be adjusted for optimal results.**

DNA input	Adaptors dilution
≥500 ng	No dilution
100 ng	10x
10 ng	20x
1 ng	30x

Table 7: Adaptor Dilution

1. Set the **Adaptor Ligation Program** on the thermocycler:

Set the program without the heating lid		
Step	**Temperature**	**Time**
Ligation	20 °C	20 min
Cooling	4 °C	∞

Table 8: Adaptor Ligation program

2. Prepare the **Ligation Master Mix** according to Table 9:

Reagents	Volume per 1 reaction
epicGEN Ligation Buffer	12 µl
epicGEN Ligation Enzyme	4 µl
epicGEN Adaptors	5 µl
TE Buffer	9 µl

Table 9: Ligation Master Mix

Note: epicGEN Ligation Buffer is very viscous, pipette slowly

3. After the Fragmentation Program, briefly spin the samples and add **30 µl of Ligation Master Mix** to each sample.
4. Vortex and briefly spin the samples.
5. Place the samples in a thermocycler and run **the Adapter Ligation** program. The total reaction volume is **60 µl**.
6. After the program is complete, proceed immediately to the Post-ligation clean-up (step 11.3).

11.3 Post-Ligation Clean-up

Note: Allow the magnetic beads (SPRISelect®) to reach room temperature (at least 30 minutes) before purification.

1. Prepare fresh 80% Ethanol
2. Vortex SPRISelect® until the magnetic beads are fully homogenized
3. Add **48 µl of SPRISelect®** (0.8x ratio) to each sample.
4. Vortex and briefly spin the samples down (be careful not to allow the beads to settle to the bottom of the tube).
5. Incubate for **5 minutes** at room temperature
6. Place the samples on the magnetic stand and incubate until the supernatant is clear (approx. 2 minutes).
7. Remove the supernatant. Be careful not to disturb the pellet.
8. Add **180 µl of 80% Ethanol** to the samples. Leave the samples on the magnetic stand.
9. Incubate for **30 seconds**, then remove the supernatant.
10. Add **180 µl of 80% Ethanol** to the samples.
11. Incubate for **30 seconds**, then remove the supernatant. Leave the samples on the magnetic stand.
12. Remove residual ethanol with a 10 µl pipette and leave on the magnetic stand until the pellet dries (approx. 2 minutes).
13. Remove the samples from the magnetic stand and add **20 µl of TE Buffer**.
14. Vortex the samples until the pellet is dissolved and incubate for **2 minutes** at room temperature.
15. Briefly spin and place samples on a magnetic stand.
16. Incubate until the supernatant is clear (approx. 2 minutes).
17. With the samples still on the magnetic stand, transfer the supernatant to a new tube. Be careful not to disturb the beads when pipetting.

Safe stop point. At this step, samples can be stored overnight at −20 °C.

11.4 PCR amplification

1. Set **the PCR1 Amplification** program on the thermal cycler according to Table 10:

Set the lid heating to 105 °C.			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98 °C	45 s	1
Denaturation	98 °C	15 s	4-15 (according to Table 11)
Annealing	60 °C	30 s	
Extension	72 °C	30 s	
Final extension	72 °C	60 s	1
Cooling	4 °C	∞	1

Table 10: PCR1 Amplification program

DNA input	**Number of cycles**
1000 ng	4
500 ng	7
100 ng	9
10 ng	12
1 ng	15

Table 11: Recommended number of PCR cycles

2. Add **5 µl of epicGEN UDI Primers** to each sample. Use a unique index pair for each sample.
3. Add **25 µl of PCR1 Master Mix**. The total reaction volume is **50 µl**.
4. Vortex and spin the samples.
5. Place in a thermocycler and run the PCR1 Amplification program.
6. When the program is complete, proceed immediately to Post-PCR purification.

11.5 Post-PCR purification

Note: Allow the magnetic beads (SPRISelect®) to reach room temperature (at least 30 minutes) before purification.

1. Prepare fresh 80% Ethanol
2. Vortex SPRISelect® until the magnetic beads are fully homogenized
3. Add **90 µl of SPRISelect®** (1.8x ratio) to each sample.
4. Vortex and briefly spin the samples down (be careful not to allow the beads to settle to the bottom of the tube).
5. Incubate for **5 minutes** at room temperature
6. Place samples on magnetic stand and incubate until supernatant is clear (approx. 2 minutes).
7. Remove the supernatant. Be careful not to disturb the beads.
8. Add **180 µl of 80% Ethanol** to the samples. Leave the samples on the magnetic stand.
9. Incubate for **30 seconds**, then remove the supernatant.
10. Add **180 µl of 80% Ethanol** to the samples.
11. Incubate for **30 seconds**, then remove the supernatant. Leave the samples on the magnetic stand.
12. Remove residual ethanol with a 10 µl pipette and leave on the magnetic stand until the pellet is dry (approx. 2 minutes).
13. Remove the samples from the magnetic stand and add **22 µl of TE Buffer**.
14. Vortex the samples until the pellet is dissolved and incubate for **2 minutes** at room temperature.
15. Spin briefly and place samples on the magnetic stand.
16. Incubate until the supernatant is clear (approx. 2 minutes).
17. With the samples still on the magnetic stand, transfer **20 µl** of supernatant to a new tube. Be careful not to disturb the beads while pipetting.

Safe stop point: At this step, samples can be stored overnight at −20 °C.

In this step, the libraries are ready for quantification using a fluorometric measurement (e.g. Qubit™ Fluorometer).

Recommendation: Check libraries using capillary gel electrophoresis (e.g. 2200 TapeStation System) to verify correct fragment length. The average length should be in the range of 250-350 bp.

11.6 Hybridization

To continue with the protocol, we recommend combining samples into plexes (max. 8 samples per plex for 1 hybridization reaction). Use **500 ng of DNA** from each sample.

Important: If you are preparing libraries from various sources of material (e.g. gDNA, ctDNA, FFPE, etc.), always combine only samples of the same type into a plex. Otherwise, the representation of the samples may not be equimolar in the plex and can result in insufficient depth of coverage of respective sequences.

1. Set **Hybridization** and **Wash programs** on the thermocycler:

Set the lid heating to 100 °C		
Stop	**Temperature**	**Time**
Denaturation	95 °C	30 s
Hybridization	65 °C	4 – 24 h

Table 12: Hybridization program

Set the lid heating to 70 °C		
Step	**Temperature**	**Time**
Hybridization	65 °C	∞

Table 13: Wash program

Note: This procedure uses magnetic beads to concentrate the samples prior to hybridization. An alternative method is concentration using the SpeedVAC system. This procedure is described in **Appendix 1.**

2. Prepare the **Hybridization Master Mix** according to Table 14 and keep on ice:

Reagents	**Volume per 1 reaction**
epicGEN Hybridization Buffer	9.5 µl
epicGEN Hybridization Buffer Enhancer	3 µl
epicGEN Blockers TS	2 µl
epicGEN Solid cancer & MSI panel	4.5 µl
Total	19 µl

Table 14: Hybridization Master Mix

Note: Bring SPRISelect® to room temperature (at least 30 minutes).

3. Combine samples into plexes (max. 8 samples per plex) that will be hybridized together in 1 reaction. From each sample, use a volume equivalent to **500 ng of DNA** (measured by the Qubit™). For larger volumes, use 1.5 ml LoBind tubes.
4. Add **7.5 µl of Human Cot DNA** to each sample
5. Add **1.8x** volume of **the SPRISelect®** (e.g., for a sample volume of 100 µl, add 180 µl SPRISelect®).
6. Vortex and incubate at room temperature for **10 minutes**.
7. Transfer samples to a magnetic stand and incubate for **2 minutes** or until supernatant is clear.
8. Remove the supernatant. Keep the samples still on the magnetic stand.
9. Add **80% Ethanol**. Chose the volume accordingly so that the magnetic beads are completely submerged below the surface.
10. Incubate for **30 seconds** on the magnetic stand.
11. Remove the supernatant and add fresh **80% Ethanol**.
12. Incubate for **30 seconds** on the magnetic stand
13. Remove the supernatant and remove the remaining ethanol with a 10 µl pipette.
14. Allow the magnetic beads to dry (approx. 2 minutes).
15. Add **19 µl of Hybridization Master Mix** prepared in step 2. chap. 11.6.
16. Vortex to dissolve the pellet.
17. Incubate for **5 minutes** at room temperature.
18. Spin briefly and place samples on the magnetic stand. Incubate until the supernatant is clear.
19. Transfer **17 µl** of the supernatant into a new PCR tube. Avoid transferring magnetic beads to the new tube.
20. Spin briefly and place in the thermocycler.
21. Start the **Hybridization Program**. Allow hybridization to run for at least 4 hours. Continue with the samples in step 2. chap. 11.9.

Note: For optimum hybridization efficiency, allow the program to run overnight. However, do not exceed 24 hours.

11.7 Preparation of hybridization buffers

Note: a minimum of 4 positions in the thermocycler per each sample is required to capture and wash hybridization targets. For more than 24 samples, 2 thermocyclers should be used.

1. Prepare **1X Wash Buffers** according to Table 15:

Stock reagents	Volume per 1 reaction (10% reserve included)		
	Buffer	NF Water	Total
epicGEN 2X Bead Wash Buffer	165 µl	165 µl	330 µl
epicGEN 10X Wash Buffer 1	27.5 µl	247.5 µl	275 µl
epicGEN 10X Wash Buffer 2	16.5 µl	148.5 µl	165 µl
epicGEN 10X Wash Buffer 3	16.5 µl	148.5 µl	165 µl
epicGEN 10X Stringent Wash Buffer	33 µl	297 µl	330 µl

Table 15: Preparation of 1X Wash Buffers

Note: If the epicGEN 10X Wash Buffer 1 forms clots or is foamy, heat the stock solution to 65 °C and vortex it until it clears.

1X Working buffer solutions can be stored at room temperature for up to 4 weeks.

2. For each hybridization reaction, prepare the following reagents that will be placed in the thermocycler during the process (3 positions in the thermocycler for 1 sample):

Reagents	Volume
1X Wash Buffer 1	110 µl
1X Stringent Wash Buffer	160 µl
1X Stringent Wash Buffer	160 µl

Table 16:Reagent volume for Heated wash

Tip: Use a new PCR strip for each reagent and discard it after use.

Note: Do not discard the remaining 1X Wash Buffer 1. It will be used in the Room temperature Wash step.

3. Prepare the **Resuspension Master Mix** according to Table 17:

Reagents	Volume per 1 reaction
epicGEN Hybridization Buffer	8.5 µl
epicGEN Hybridization Buffer Enhancer	2.7 µl
NF Water	5.8 µl

Table 17: Resuspension Master Mix

Note: Keep the Resuspension Master Mix on ice until it is used.

11.8 Preparation of Streptavidin Beads

1. Bring **epicGEN Streptavidin Beads** to room temperature (at least 30 minutes) and vortex thoroughly.
2. Pipette **50 µl of epicGEN Streptavidin Beads** into a PCR tube for each hybridization reaction.
3. Add **100 µl of 1X Bead Wash Buffer** (from step 1 chap. 11.7) and pipette 10 times to completely homogenize the solution.
4. Transfer samples to a magnetic stand and incubate until the supernatant is clear.
5. Remove the supernatant without disturbing the pellet.
6. Repeat steps 3-5 two more times for a total of 3 washes.
7. Add **17 µl of Resuspension Master Mix** from step 3 chap. 11.7 to the beads.
8. Vortex to completely homogenize the solution and spin briefly.
9. Leave the resuspended **epicGEN Streptavidin Beads** at room temperature until step 4. chap. 11.9.

11.9 Capture of hybridized targets

1. Start the **Wash Program** on the thermocycler (step 1, chap. 11.6). When the temperature reaches 65 °C, add the 1X wash buffers prepared in step 2 chap. 11.7. into the thermocycler.
2. At the end of the **Hybridization Program** (step 1 chap. 11.6), remove the samples from the thermocycler and spin briefly.
3. Start the **Wash Program** immediately on the thermocycler.

Note: At this moment there are 2 thermocyclers running on the same **Wash program**. In the first thermocycler there are incubated 1X Wash Buffers, in the second, there will be incubated the samples. In case of lower number of samples (<24), samples and 1X Wash Buffers can be kept in the same thermocycler.

4. Add the **epicGEN Streptavidin Beads** from step 9, chap. 11.8 to the samples.
5. Vortex and spin briefly.
6. Place samples in the thermocycler with active Wash Program.
7. Incubate for **45 minutes**. Remove the samples every 10-12 minutes and vortex lightly to prevent beads from settling to the bottom of the tube.

11.10 Heated Wash

Note: During the entire Heated Wash process, keep the 1X Wash Buffers in the thermocycler at 65 °C.

1. After the 45-minute incubation, remove samples from the thermocycler and spin down.
2. Add **100 µl** of preheated **1X Wash Buffer** to each sample and pipette 10 times. Avoid bubble formation in the tube. Close the lid of the thermocycler with Wash buffers and keep the **Wash Program** running.
3. Place the samples on the magnetic stand and incubate until the supernatant is clear.
4. Remove the supernatant and remove the samples from the magnetic stand.
5. Add **150 µl** of preheated **1X Stringent Buffer** and pipette 10 times. Avoid bubble formation in the tube.
6. Incubate the samples for **5 minutes** in the thermocycler with the **Wash Program** active.
7. Place the samples on a magnetic stand and incubate until the supernatant is clear.
8. Remove the supernatant and remove samples from the magnetic stand.
9. Add **150 µl** of preheated **1X Stringent Buffer** and pipette 10 times. Avoid bubble formation in the tube.
10. Incubate samples for **5 minutes** in the thermocycler with active **Wash Program**.
11. Place samples on a magnetic stand and incubate until the supernatant is clear.

Note: At this step you can turn off the thermocycler with the Wash Program.

11.11 Washing at room temperature

1. Remove the supernatant and add **150 µl** of 1X **Wash Buffer 1**.
2. Vortex until the beads are completely homogenized.
3. Incubate for **2 minutes** at room temperature. After the incubation is complete, spin briefly.
4. Place samples on magnetic stand and incubate until supernatant is clear.
5. Remove the supernatant, remove the samples from the magnetic stand and add **150 µl of 1X Wash Buffer 2**. Vortex samples until the beads are completely homogenized.
6. Incubate for **2 minutes** at room temperature. After incubation is complete, spin briefly.
7. Place samples on a magnetic stand and incubate until the supernatant is clear.
8. Remove the supernatant, remove samples from the magnetic stand and add **150 µl of 1X Wash Buffer 3**. Vortex samples until the beads are completely homogenized.
9. Incubate for **2 minutes** at room temperature. After incubation is complete, spin briefly.
10. Place on magnetic stand and incubate until supernatant is clear.

11. Remove the supernatant. Use a 10 µl pipette to remove any residual buffer (if necessary, briefly spin, place back on the magnet and remove any residual buffer).
12. Add **20 µl NF Water** and vortex until the beads are completely homogenized. Spin briefly.

Note: Leave the magnetic beads in the sample until the next step. Do not discard them.

11.12 PCR Amplification 2

1. Prepare the **PCR2 Amplification Master Mix** according to Table 18:

Reagents	Volume per 1 reaction
epicGEN PCR2 Master Mix	25 µl
epicGEN Primer Mix	1.25 µl
NF Water	3.75 µl

Table 18: PCR2 Amplification Master Mix

2. Set the **PCR2 Amplification program** on the thermocycler according to Table 19:

Set the lid heating to 105 °C.			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98 °C	45 s	1
Denaturation	98 °C	15 s	14
Annealing	60 °C	30 s	
Extension	72 °C	30 s	
Final extension	72 °C	1 min	1
Cooling	4 °C	∞	-

Table 19: PCR2 Amplification Program

Note: The number of PCR cycles can be adjusted. If the number of samples in the plex is less than 8, add additional cycles (4-plex: +1 cycle; 1-plex: +2 cycles).

3. Add **30 µl of Amplification Master Mix** to each sample, vortex and spin briefly. The total reaction volume is **50 µl**.
4. Place samples in the thermocycler and run **PCR Amplification Program 2**.

11.13 Clean-up

Note: Bring SPRISelect® to room temperature (at least 30 minutes).

1. Prepare fresh 80% Ethanol. You will need a minimum of 250 µl for each sample.
2. Add **75 µl of SPRISelect®** (1.5x ratio) to each sample.
3. Vortex and briefly spin down the samples (be careful not to allow the beads to settle to the bottom of the tube).
4. Incubate for **5 minutes** at room temperature
5. Place samples on the magnetic stand and incubate until the supernatant is clear (approx. 2 minutes).
6. Remove the supernatant. Be careful not to disturb the beads.
7. Add **125 µl of 80% Ethanol** to the samples.
8. Incubate for **1 minute** on the magnetic stand.
9. Remove the supernatant and add **125 µl of 80% Ethanol**.
10. Incubate for **1 minute** on the magnetic stand.
11. Remove the supernatant and remove any residual ethanol with a 10 µl pipette and leave on the magnetic stand to dry the beads.
12. Remove the samples from the magnetic stand and add **22 µl of TE Buffer**.
13. Vortex the samples until the pellet is dissolved and incubate for **5 minutes** at room temperature.
14. Briefly spin and place samples on the magnetic stand.
15. Incubate until the supernatant is clear (approx. 2 minutes).
16. Transfer **20 µl** of the supernatant to a new PCR tube.

11.14 Quality control of libraries

1. Measure the concentration of the libraries using the Qubit™ dsDNA HS Assay kit, or an equivalent fluorometric-based quantification method. Concentrations of properly prepared libraries must be at least 2 ng/µl.
2. Check the correct length of the library fragments using a digital gel electrophoresis (e.g. Agilent 2200 TapeStation®, or similar). The average fragment length is in the range of 250-350 bp.

11.15 Sequencing

Dilute and denature epicGEN libraries according to the manual of the respective Illumina sequencer. The recommended read depth is 4 millions paired-end reads (2 millions clusters) per sample. Recommended sequencing run configuration:

Read	Cycles
Read1	151
Index1	8
Index2	8
Read2	151

Table 20: Sequencing run configuration

epicGEN libraries are compatible with all Illumina® and GeneMind sequencers. For sequencing on AVITI and MGI instruments, use the appropriate sequencing adaptor conversion protocol.

12. APPENDIXES

12.1 Appendix 1 – Concentration of samples prior to hybridization using the SpeedVAC system

1. Combine samples into plexes (max. 8 samples per plex) that will be hybridized together in 1 reaction. From each sample, use a volume equivalent to 500 ng of DNA (as measured on the Qubit). For larger volumes, use 1.5 ml LoBind tubes.
2. Prepare **Blocker Mix** for each reaction according to Table 21:

Reagents	Volume per 1 reaction
Human Cot DNA	5 µl
epicGEN Blockers TS	2 µl

Table 21: Blocker Mix

3. Add **7 µl of Blocker Mix** to each sample.
4. Place the samples in the SpeedVAC system and allow to dry completely.

Safe Stop Point: Dried samples can be stored long-term at −20 °C.

5. Prepare the **Hybridization Master Mix** according to Table 22:

Reagents	Volume per 1 reaction
epicGEN Hybridization Buffer	8.5 µl
epicGEN Hybridization Buffer Enhancer	2.7 µl
epicGEN Solid Cancer & MSI panel	4 µl
NF Water	2 µl
Total	17 µl

Table 22: Hybridization Master Mix

6. Vortex and spin down the **Hybridization Master Mix**.
7. Add **17 µl of Hybridization Master Mix** to each dried sample.
8. Vortex until the DNA is completely dissolved and spin briefly.
9. Incubate for **5 minutes** at room temperature.
10. Vortex briefly and spin down. If you are working in 1.5 ml LoBind tubes, transfer the samples to a PCR strip.
11. Place the samples in the thermocycler and run the **Hybridization program** (step 1 chap. 11.6). Leave the hybridization running for at least **4 hours**. Continue with the samples in step 2, chap. 11.9.

Note: For optimal hybridization efficiency, let the program run overnight. No longer than 24 hours.

Continue the protocol with step **11.7 Preparation of hybridization buffers**.

12.2 Appendix 2 – Index sequences

Position in the plate	i7 index	i5 index Forward[1]	i5 index Reverse Complement[2]
A1	CTGATCGT	ATATGCGC	GCGCATAT
B1	ACTCTCGA	TGGTACAG	CTGTACCA
C1	TGAGCTAG	AACCGTTC	GAACGGTT
D1	GAGACGAT	TAACCGGT	ACCGGTTA
E1	CTTGTCGA	GAACATCG	CGATGTTC
F1	TTCCAAGG	CCTTGTAG	CTACAAGG
G1	CGCATGAT	TCAGGCTT	AAGCCTGA
H1	ACGGAACA	GTTCTCGT	ACGAGAAC
A2	CGGCTAAT	AGAACGAG	CTCGTTCT
B2	ATCGATCG	TGCTTCCA	TGGAAGCA
C2	GCAAGATC	CTTCGACT	AGTCGAAG
D2	GCTATCCT	CACCTGTT	AACAGGTG
E2	TACGCTAC	ATCACACG	CGTGTGAT
F2	TGGACTCT	CCGTAAGA	TCTTACGG
G2	AGAGTAGC	TACGCCTT	AAGGCGTA
H2	ATCCAGAG	CGACGTTA	TAACGTCG

[1] sequences for MiSeq, MiniSeq Rapid Reagent kit, NovaSeq v1.0 Reagent kit
[2] sequences for iSeq 100, NextSeq, MiniSeq Standard reagent kit, NovaSeq v1.5 Reagent kit

Table 23: Index sequences

13. RESULTS EVALUATION

To evaluate sequencing data, use the GENOVESA software, available online at ngs-genovesa.biovendor.group.

GENOVESA Genomics module

GENOVESA is a cloud-based all-in-one solution for analysing raw sequencer data (FASTQ files) with technical and application support.

The software allows:

- advanced quality control of sequencing data
- automatic alerts for regions with low coverage
- simple filtering of relevant variants
- customization options
- store patient data and variants in an internal database
- one-click report

14. KIT LIMITATIONS

- The epicGEN Solid Cancer & MSI Kit is validated on DNA from tumor tissue fixed in FFPE blocks.
- Genotyping results are affected by sample quality. Proper collection procedure, transport, DNA isolation and storage of samples is important for testing.
- The results of genotyping should be evaluated by an expert in health care professional.
- The epicGEN Solid Cancer & MSI Kit is designed for genotyping DNA samples using NGS technology.
- A negative result does not exclude mutations below the detection limit of the method.

All instructions in this document should be followed when performing the test. Failure to follow them may affect the quality and reliability of the results.

15. FAQ

1. How many samples do I need to prepare together?

The kit is optimized to work with 8-fold samples. There are enough reagents in the kit for 2 hybridization reactions optimized for 8 samples.

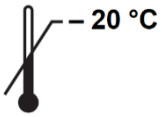
2. Is it possible to hybridize different number of samples than 8 in one reaction?

Yes, it is possible. If you prepare fewer samples, there is no risk of reducing the performance of the kit (but you will use up the hybridization chemistry sooner). If you prepare more than 8 samples in a single hybridization reaction, the resulting coverage of all targets may not be optimal and some of them may not achieve the desired coverage at all.

3. Is the kit designed for samples with lower quality DNA? (e.g. FFPE, ctDNA, ...)

Yes, the protocol is adapted to work with both lower quality samples and high quality gDNA.

16. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Use by date
	Upper limit temperature
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 16 tests

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