SarcomaFusion

References:

GEP-SF08

GEP-SF16

GEP-SF24

GEP-SF48

ENGLISH

GENEXPATH SarcomaFusion Instruction for Use (IFU)

User precautions.

CE ***In vitro*** **diagnostic medical device according to Directive (EU) 98/79/EC**

IVD

For *in vitro* diagnostic use.

It is for professional use only.

Read all information in this instruction for use (IFU) before use.

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Important precautions.

General recommendations.

- Usable for *in vitro* diagnostic use
- Follow laboratory best practices in terms of handling PCR products (wear disposable overalls and gloves, mark out dedicated zones for pre- and post-PCR, use filter tips).
- Also take precautions to avoid nuclease contamination likely to cause RNA and DNA degradation (use nuclease-free reagents and consumables).
- Ensure that the thermocyclers are in working order and calibrated based on manufacturer recommendations.
- It is particularly important not to substitute reagents not included in the kit, particularly buffers and enzymes used during the reverse transcription, ligation and PCR amplification steps. The incubation temperatures and times, as well as volumes and concentrations, must also be respected.
- The **SarcomaFusion** test kit contains a GAPDH internal positive control. It is strongly recommended to complete it to check that your experiment has been carried out correctly.
- **GENEXPATH SarcomaFusion** reagents are only intended for use with Illumina's MiSeq or NextSeq 500/550 sequencing platforms.
- Safety data sheets are available in the user space.
- If the user lights out errors in the instruction manual: please send an email at contact@genexpath.com
- Any serious incident occurring in connection with the device must be notified to us at contact@genexpath.com.

Icons

Important points and critical steps of the protocol that could compromise result quality.

Steps where the protocol may be suspended.

Intended use

This protocol is intended for **GENEXPATH SarcomaFusion** testing. It is used to prepare sequencing libraries for Illumina's MiSeq or NextSeq 500/550 sequencers.

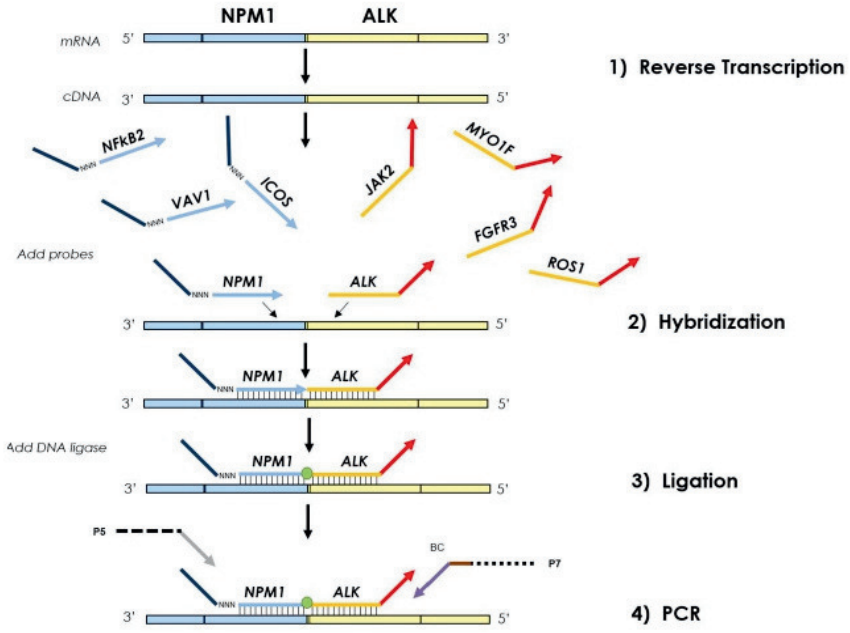
The fastQ files generated using this test contain data about counting sequences corresponding to the potential presence of a fusion transcript, i.e. ligation of two probes and their amplification.

They can be analysed using **GENEXPATH RT-MIS** software, which contains a specific sequence demultiplexing application.

By studying 206 genes, this test can detect fusion transcripts found in more than 80 types of bone and soft tissue tumours.

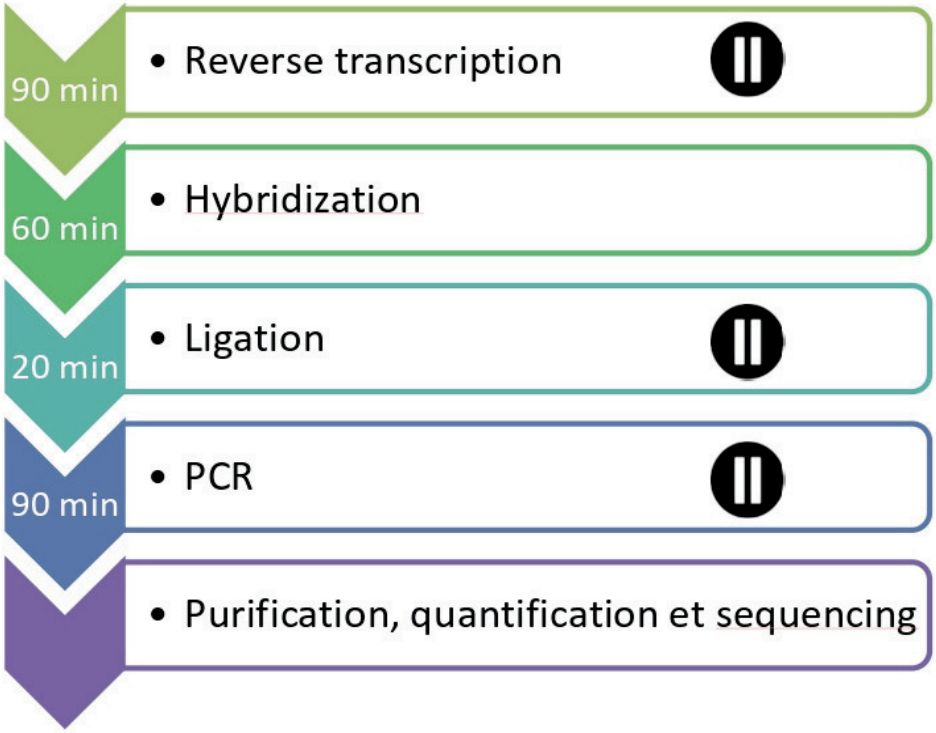
Test principle.

The **GENEXPATH SarcomaFusion** test uses a ligation-dependent RT-PCR method (LD-RT-PCR). This semi-quantitative technique helps detect chromosome translations using specific oligonucleotide probe pairs. A probe pair for a control gene (GAPDH) is included in the test probe mix allowing an internal control of your experiment.



Four steps are sufficient to obtain libraries from a total RNA extraction.

- A reverse transcription (RT) step.
- A hybridization of specific oligonucleotide probes step.
- A ligation step.
- A PCR amplification step.



No purification is required until the libraries are obtained, which limits material losses and ensures this technique has excellent sensitivity. Also, the gene sequences targeted by the probes are particularly short (between 40 and 60 bases), which ensures excellent robustness with regards to RNA degradation.

LD-RT-PCR is therefore a particularly appropriate approach for analysing difficult biological samples like fixed paraffin-embedded tissue biopsies.

For each sample, around 10^5 sequences are sufficient to obtain an analysable expression profile, which helps test a large number of samples simultaneously in the same sequencing FlowCell. **GENEXPATH SarcomaFusion** libraries can also be loaded at the same time as other sequencing libraries, generated by other methods.

Reagents.

Content of GENEXPATH SarcomaFusion reagent kit.

GENEXPATH SarcomaFusion probe mix	GEP-SFPM
GENEXPATH SarcomaFusion barcodes	GEP-BC-xxx
GENEXPATH SarcomaFusion sequencing primer	GEP-SP-001
GENEXPATH SarcomaFusion GAPDH barcodes	GEP-BCC-xxx
GENEXPATH SarcomaFusion GAPDH sequencing primer	GEP-SP-002

XXX: barcode number

On receipt, these reagents should be stored between -25°C and -15°C.
They are ready to use and do not need to be diluted.
The shelf life of the reagents is 1 year.
Return to storage conditions immediately after use.
Do not use reagents after their expiration date stated on the label.

Format of reagent kits sold and quantities:

	Reagent kit - U = number of analyses			
	8U	**16U**	**24U**	**48U**
GEP-SFPM probe mix	30 µL	48 µl	54 µl	108 µL
Barcodes **GEP-BC-xxx (from 001 to 032 depending on number of analyses purchased)** BC=barcode	8 BC	8 BC	12 BC	24 BC
	N°017 to 024	N°001 to 008	N°021 to 032	N°001 to 024
	5 µL/BC	9 µL/BC	9 µL/BC	9 µL/BC
GEP-SP-001 sequencing primers	60 µL	96 µL	144 µL	288 µL
For the internal control				
GAPDH barcodes **GEP-BCC-xxx (from 001 to 032 depending on number of analyses purchased)** BCC=control barcode	8 BCC	8 BCC	12 BCC	24 BCC
	N°017 to 024	N°001 to 008	N°021 to 032	N°001 to 024
	5 µL/BCC	9 µL/BCC	9 µL/BCC	9 µL/BCC
GAPDH sequencing primers **GEP-SP-002**	60 µL	96 µL	144 µL	288 µL

Reagents are supplied in larger quantities than actually required. After the ordered number of analyses has been completed, they should be discarded. If a new order is placed, new reagents will be delivered.

For a reagent kit with more than 8 analyses, each barcode will be used for 2 different analyses.

Reagents not supplied in the reagent kit:

Reagents	Suppliers and references
SuperScript™ VILO™ cDNA Synthesis Kit	Invitrogen, ref 11754250
SALSA MLPA Buffer	MRC Holland, ref SMR33
SALSA Ligase Buffer A	MRC Holland, ref SMR12
SALSA Ligase Buffer B	MRC Holland, ref SMR13
SALSA Ligase 65	MRC Holland, ref SMR20
Red'y' Star PCR Mix	Eurogentec, ref PK-0073-02R

AMPure XP (Magnetic beads)	Beckman Coulter, ref A63880
Qubit® dsDNA HS Assay	Fisher Scientific, ref 10616763
Sequencing reagents	Illumina
TE buffer (10 mM Tris-Acetate pH 8.0, 1mM EDTA)	Variable
Ethanol 100%	Variable
NaOH 1 N	Variable
Tris Buffer 200 mM pH 7	Variable
Nuclease-Free Water	Variable

On receipt and between each use, these reagents should be stored based on supplier recommendations.

Required equipment:

Equipment	Suppliers and references
Thermocycler in pre-PCR zone	Variable
Thermocycler in post-PCR zone	Variable
Qubit® Fluorometer (or equivalent)	Thermo Fisher Scientific, ref Q33238
Qubit® Assay Tubes	Fisher Scientific, ref 12037609
DynaMag™-96 Side Magnet - Magnetic plate (AMPure XP purification)	Thermo Fisher Scientific, ref 12331D
PCR 200 µL plates and tubes	Variable

Before starting.

Biological samples.

The **GENEXPATH SarcomaFusion** test is used to prepare sequencing libraries using total RNA extractions from sarcoma tumour biopsies or tissues (bone and soft tissue tumours).

These samples may be fresh, frozen or formalin-fixed, paraffin-embedded (FFPE).

To extract RNA from fixed tissues, we recommend using the kit Promega Maxwell® RSC RNA FFPE (Promega, ref AS1440 and AS4500).

The amount of RNA to be analysed should be between **50 and 500 ng, in a volume of 2.5 µL**. If the concentration of the solutions to be analysed is too high, this RNA can be diluted in nuclease-free water.

Programming the thermocyclers.

To limit the risks of contamination, use two thermocyclers, one in the pre-PCR zone and one in the post-PCR zone.

Two programmes are required:

- The first is for the first three steps of the protocol: **reverse transcription of RNA to cDNA, hybridization of oligonucleotide probes, and ligation**. It must be run on the thermocycler located in the pre-PCR zone.
- The second is used to amplify ligation products and incorporate the barcodes and adapters required for sequencing. It must be run on the thermocycler located in the post-PCR zone.

- ***Programme 1: Pre-PCR.***

As the reaction volumes are small, ensure that the temperature of the thermocycler's heated lid remains high (95°C) at all steps of the programme to avoid evaporation.

Breaks at 4°C or 54°C are provided between the different programme steps to add the required reagents.

Step 1: Reverse transcription of RNA to cDNA.
- Heated lid: 95°C
- 10 minutes 25°C
- 60 minutes at 42°C
- 5 minutes 85°C
- 4°C infinite

Step 2: Hybridization of probes.
- Heated lid: 95°C
- 2 minutes 95°C
- 60°C infinite (1 hour of hybridization)

Step 3: Ligation.
- Heated lid: 95°C
- 54°C infinite (distribution of ligation mix)
- 15 minutes 54°C
- 5 minutes 98°C
- 4°C infinite

- ***Programme 2: PCR.***

- Heated lid: 95°C
- 6 minutes 94°C
- 35 x (30 seconds 94°C; 30 seconds 58°C; 30 seconds 72°C)
- 4 minutes 72°C

➢ 4°C infinite

Detailed protocol.

Step 1: Reverse transcription.

This step must be completed in the pre-PCR zone.

- ***Required reagents.***

- 5X Vilo reaction mix, 10X super script (SuperScript Vilo cDNA Synthesis Kit), nuclease-free water, total RNA extraction to test (25 to 250 ng/µL).

It is recommended to carry out the entire procedure in 200 µL PCR plates or tubes.

- ***Reverse transcription.***

- Thaw the following reagents, then keep them on ice or in a cooling rack: 5X Vilo reaction mix and 10X super script.

- Prepare a reverse transcription mix. For each sample, mix (for a total volume of 2.5 µL per reaction):

○ 5X Vilo reaction mix	1 µL
○ Nuclease-free water	1 µL
○ 10X super script	0.5 µL

- Distribute this mix in 200 µL PCR tubes (2.5 µL per tube) kept on ice or in a cooling rack:

- Add 2.5 µL of each of the total RNA solutions to the different tubes.

- Vortex, centrifuge briefly.

- Place the tubes in the thermocycler in the pre-PCR zone, and proceed to **step 1 of the Pre-PCR programme** (Reverse transcription of RNA to cDNA).

Then proceed directly to step 2, or keep the ligation products between -25°C and -15°C.

Step 2: Hybridization of probes.

This step must be completed in the pre-PCR zone.

- ***Required reagents.***

- **GENEXPATH SarcomaFusion** probe mix (GEP-SFPM), SALSA MLPA Buffer.

- ***Hybridization of probes.***

  - **At the end of step 1**, when the thermocycler temperature has dropped to 4°C, remove the tubes, centrifuge them briefly, and place them on ice or in a cooling rack.

  - Thaw the Salsa MLPA buffer and the **GENEXPATH SarcomaFusion** probe mix, then keep them on ice or in a cooling rack.

  - Prepare a hybridization mix. For each sample, mix (for a total volume of 3 µL per reaction):

○ Salsa MLPA Buffer	1.5 µL
○ **GENEXPATH SarcomaFusion** probe mix	1.5 µL

  - Vortex, centrifuge briefly.

  - Add 3 µL of this mix to each cDNA tube.

  - Centrifuge briefly.

  - Place the tubes back in the thermocycler.

  - Check the temperature of the heated lid (95°C).

  - Proceed to **step 2 of the pre-PCR programme** (hybridization of probes).

Step 3: Ligation.

This step must be completed in the pre-PCR zone.

- ***Required reagents.***

  - SALSA Ligase Buffer A, SALSA Ligase Buffer B, SALSA Ligase 65, nuclease-free water.

- ***Ligation.***

  - 15 minutes before the end of step 2, thaw SALSA Ligase Buffer A and SALSA Ligase Buffer B and keep them on ice or in a cooling rack.

  - Place the Salsa Ligase 65 enzyme on ice or in a cooling rack.

  - Prepare a ligation mix. For each sample, mix (for a total volume of 32 µL per reaction):

○ Nuclease-free water	25 µL
○ Salsa Ligase Buffer A	3 µL
○ Salsa Ligase Buffer B	3 µL

  - Vortex, centrifuge briefly

○ Salsa Ligase 65	1 µL

- Vortex, centrifuge briefly.
- After 60 minutes of incubation, proceed to **step 3 of the pre-PCR programme (ligation).**
- Lower the temperature of the heated block to 54°C.
- Add 32 µL of the ligation mix directly to each tube, without removing them from the heated block.
- After distributing the mix, proceed to the next step of the programme (15 minutes at 54°C, 5 minutes at 98°C).

At the end of this step, when the temperature of the PCR block drops to 4°C, immediately proceed to step 4 (PCR amplification) or freeze the ligation products (between -25°C and -15°C).

After this step, do not keep products at higher temperatures (e.g. 4°C or room temperature) to avoid non-specific ligations which could result from residual enzyme activity.

Step 4: Amplification and incorporation of barcodes and adapters.

In this step, the ligation products are amplified by PCR thanks to additional tails at the end of the probes. These amplifications are carried out using pairs of primers supplied in the **GENEXPATH SarcomaFusion** barcode tubes (GEP-BC-xxx).

To allow analysis of several samples in the same FlowCell, the PCR 3' primer has a molecular barcode which will be recognised by the demultiplexing algorithm of the **GENEXPATH RT-MIS** platform.

To carry out the internal control with GAPDH probes, two different PCR are completed, so you must double the number of tubes. For a given sample, you must use the same barcode number GEP-BC-xxx and GEP-BCC-xxx for information analysis. So you must add, for each sample, in one tube the barcode GEP-BC-xxx and in the other the associated barcode GEP-BCC-xxx.

- ***Required reagents.***
  - **GENEXPATH SarcomaFusion** barcodes (GEP-BC-xxx), **GENEXPATH SarcomaFusion** GAPDH barcodes (GEP-BCC-xxx), Red'y' Star PCR Mix, nuclease-free water.
- ***Amplification.***
  - Prepare an amplification mix in the pre-PCR zone. For each sample, mix (for a total volume of 18 µL per reaction):
    - Red'y' Star PCR Mix 12.5 µL

- Nuclease-free water 5.5 µL

- Vortex, centrifuge briefly.
- Distribute 18 µL of this amplification mix in the different wells in a PCR plate.
- Add 5 µL of ligation products generated in step 3 to each of the wells.
- Add 2 µL of **GENEXPATH SarcomaFusion** barcode (GEP-BC-xxx or GEP-BCC-xxx depending on test).

Use different GEP-BC-xxx barcodes for each tested sample, but for the same sample, use the same number for GEP-BC-xxx and GEP-BCC-xxx.

- Place the plate in the thermocycler in the post-PCR zone.
- Start **programme 2** (PCR).

At the end of the programme, when the temperature of the thermocycler drops to 4°C, quickly proceed to step 5 (purification) or freeze the amplification products between -25°C and -15°C.

Do not keep these products at higher temperatures for a long period (e.g. 4°C in the thermocycler or at room temperature).

Step 5: Purification and assay of sequencing libraries.

At the end of the amplification step, the sequencing libraries should be purified to eliminate PCR primers and unincorporated nucleotides. This purification uses AMPure XP magnetic beads. The libraries should be assayed via fluorimetry with the Qubit® dsDNA HS kit before loading in the sequencer.

- ***Required reagents.***
  - Ethanol 100%, nuclease-free water, AMPure XP beads, TE buffer (10 mM Tris-Acetate pH 8.0, 1 mM EDTA), Qubit® dsDNA HS Assay.
- ***Step 5.a: Purification of sequencing libraries.***

Ensure that the beads are completely re-suspended before use.

- Purify 25 µL of PCR products with 45 µL of AMPure XP beads (following manufacturer recommendations).
- Elute the purified PCR products in 50 µL of TE buffer.

After purification, the libraries can be stored between -25°C and -15°C before sequencing.

- *Step 5.b: Assay of sequencing libraries.*

  - Assay 10 µL of each sequencing library via fluorimetry using the Qubit® dsDNA HS Assay kit.

Step 6: Dilution, pooling and sequencing of libraries.

After purification, the **GENEXPATH SarcomaFusion** libraries must be diluted, pooled and loaded in the sequencer.

For optimal results, a minimum of 10^5 sequences should be read for each sample.

Unlike most classic sequencing libraries, the reading of molecular barcodes required for demultiplexing **GENEXPATH SarcomaFusion** sequences takes place during read1. These sequences are not demultiplexed automatically by the sequencer, and will be saved in "Undetermined" fastQ files. Demultiplexing is carried out using the specific algorithm provided on the **GENEXPATH RT-MIS** platform.

- *Required reagents.*

  - **GENEXPATH SarcomaFusion** sequencing primer (GEP-SP-001), **GENEXPATH SarcomaFusion** control sequencing primers (GEP-SP-002) (if internal control completed), Illumina sequencing reagents.

- *Sequencing on an Illumina MiSeq sequencer.*

For detailed information about dilution and denaturation of libraries, preparation of the sequencing primer, the injection sheet and the start of sequencing, refer to the Illumina guide to the MiSeq system.

- *Step 6.a: Dilution and pooling of libraries.*

  - Dilute each **GENEXPATH SarcomaFusion** library at a concentration between 2 nM and 4 nM, considering an average amplified fragment size of 150 pb.

  - Pool **GENEXPATH SarcomaFusion** libraries in the equivalent volume.

  - If other libraries are sequenced on the same FlowCell, adjust the concentrations of different pools then combine them to obtain the desired sequence numbers (minimum 10^5 sequences for each **GENEXPATH SarcomaFusion** library).

Example: For a pool of 10 **GENEXPATH SarcomaFusion** libraries requiring 1 M sequences (10^5 sequences for each library), sequenced with a pool of libraries B at the same concentration and requiring 3 M sequences, mix 1 µL of the **GENEXPATH SarcomaFusion** libraries pool and 3 µL of the pool of libraries B.

- *Step 6.b: Denaturation and dilution of the libraries pool.*

  - Denature and dilute the final pool based on recommendations in the Illumina guide to the MiSeq system, to obtain a final load concentration of 8 to 10 pM.

- *Step 6.c: Preparation of the sequencing primers.*

  - If the **GENEXPATH SarcomaFusion** libraries pool is sequenced alone, dilute 3 µL of each **GENEXPATH SarcomaFusion** sequencing primer (GEP-SP-001 and GEP-SP-002, if internal control) in a final volume of 600 µL of HT1 buffer, then place this 600 µL in well 18 of the MiSeq reagent cartridge.

  - If the **GENEXPATH SarcomaFusion** libraries pool is loaded with other libraries sequenced using Illumina sequencing primers, pipette the entire content of well 12 (around 600 µL), add 3 µL of each **GENEXPATH SarcomaFusion** sequencing primer (GEP-SP-001 and GEP-SP-002 if internal control), then place this mix in well 18 of the cartridge.

- *Step 6.d: Preparation of the injection sheet.*

  - If the **GENEXPATH SarcomaFusion** library is sequenced alone, create the injection sheet to generate FASTQ files with 120 cycles in read 1.

  - If **GENEXPATH SarcomaFusion** libraries are combined with other sequencing libraries, generate the injection sheet using the usual parameters, without entering the **GENEXPATH SarcomaFusion** samples.

  - Specify the use of custom during run setup (with Local Run Manager on the Create Run page. In manual run mode, on the Run Setup screen).

In all cases, ensure that read 1 is carried out with a minimum of 120 cycles and the Custom Primer for Read 1 box is ticked.

  - In all cases, the **GENEXPATH SarcomaFusion** library sequences will not be demultiplexed by the sequencer but will be saved in an "Undetermined" FastQ file, which will then be loaded on the **GENEXPATH RT-MIS** platform.

- *Step 6.e: Start of sequencing.*

  - Start sequencing by following the procedure described in the Illumina guide to the MiSeq system.

- *Sequencing on an Illumina NextSeq 500/550 platform.*

For detailed information about dilution and denaturation of libraries, preparation of the sequencing primer, the injection sheet and the start of sequencing, refer to the Illumina guide to the NextSeq system.

- ***Step 6.a: Dilution and pooling of libraries.***

  - Dilute each **GENEXPATH SarcomaFusion** library at a concentration between 0.5 nM and 4 nM, considering an average amplified fragment size of 150 pb.

  - Pool **GENEXPATH SarcomaFusion** libraries in the equivalent volume.

  - If other libraries are sequenced on the same FlowCell, adjust the concentrations of different pools then combine them to obtain the desired sequence numbers (minimum 10^5 sequences for each **GENEXPATH SarcomaFusion** library).

Example: For a pool of 10 **GENEXPATH SarcomaFusion** libraries requiring 1 M sequences (10^5 sequences for each library), sequenced with a pool of libraries B at the same concentration and requiring 3 M sequences, mix 1 µL of the **GENEXPATH SarcomaFusion** libraries pool and 3 µL of the pool of libraries B.

- ***Step 6.b: Denaturation and dilution of the libraries pool.***

  - Denature and dilute the final pool based on recommendations in the Illumina guide to the NextSeq system, to obtain a final load concentration of 0.8 pM to 1 pM.

- ***Step 6.c: Preparation of the sequencing primers.***

  - If the **GENEXPATH SarcomaFusion** libraries pool is sequenced alone, dilute 6 µL of each **GENEXPATH SarcomaFusion** sequencing primer (GEP-SP-001 and GEP-SP002, if internal control) in a final volume of 2000 µL of HT1 buffer, then place this 2 mL in well 7 of the MiSeq reagent cartridge.

  - If the **GENEXPATH SarcomaFusion** libraries pool is combined with other libraries sequenced using Illumina sequencing primers, pipette the entire content of well 20 (around 2 mL), add 6 µL of each **GENEXPATH SarcomaFusion** sequencing primer (GEP-SP-001 and GEP-SP-002 if internal control), then place this mix in well 7 of the cartridge.

- ***Step 6.d: Preparation of the injection sheet.***

  - If the **GENEXPATH SarcomaFusion** library is sequenced alone, create the injection sheet to generate FASTQ files with 120 cycles in read 1.

  - If **GENEXPATH SarcomaFusion** libraries are combined with other sequencing libraries, generate the injection sheet using the usual parameters, without entering the **GENEXPATH SarcomaFusion** samples.

  - Specify the use of custom during run setup (with Local Run Manager on the Create Run page. In manual run mode, on the Run Setup screen).

In all cases, ensure that read 1 is carried out with a minimum of 120 cycles and the Custom Primer for Read 1 box is ticked.

- In all cases, the **GENEXPATH SarcomaFusion** library sequences will not be demultiplexed by the sequencer, but will be saved in the four "Undetermined" FastQ file, which should then be loaded on the **GENEXPATH RT-MIS** platform.

- ***Step 6.e: Start of sequencing.***

- Start sequencing by following the procedure described in the Illumina guide to the NextSeq system.

Step 7: Results analysis.

The sequence files generated by the Illumina sequencing platform (MiSeq or NextSeq) in FastQ format must be analysed using the **GENEXPATH RT-MIS** software available at the following address: https://connect.genexpath.com/.

To help download the FastQ file, it should not be decompressed (fastq.gz).

This software is a comprehensive bioinformatics solution which includes different data processing algorithms. It carries out demultiplexing to assign sequences to each sample. It then precisely identifies the gene expression markers and quantifies them.

The **GENEXPATH SarcomaFusion** test is based on quantification of qualitative markers characterising the presence or absence of chromosome translocations.

GENEXPATH RT-MIS generates concise and transparent reports, from implementation of sequencing reactions to automated analysis of sequencing results.

GENEXPATH RT-MIS requires sequencing files to be loaded in FASTQ format, as well as the list of barcodes used during testing.

GENEXPATH RT-MIS assesses the sequencing quality of each sample by quantifying the number of reads identified and the number of UMI (unique molecular identifiers) detected.

For each sample, **GENEXPATH RT-MIS** generates an analysis report indicating the presence or absence of a fusion transcript, the number of reads and UMI obtained, as well as a bibliography reference corresponding to the transcript (if a fusion has been detected). This data can be downloaded.

GENEXPATH RT-MIS includes a user guide directly accessible online to explain how to use the tool, to describe all generated results and explain the presentation of results.

The company **GENEXPATH** does not permanently store results generated by the software **GENEXPATH RT-MIS**. The data must be downloaded directly after each analysis and saved by the user in their document management system.

Limits of the procedure

- The SarcomaFusion test was developed based on data from the literature to detect the most frequent fusion transcripts in patients with sarcoma. It is intended for FFPE use or frozen samples, possibly obtained from needle biopsies.

- The performance demonstrated in the "Characterization of performance" paragraph has been validated according to the instructions set out above.

- A low amount of RNA or a low quality sample may cause an uninterpretable result.

- Sequencing must be performed with Illumina technology sequencers (Miseq and NextSeq).

Characterization of performances

Analytical performance on reference samples

To demonstrate the analytical performance of the SarcomaFusion test, i.e. its ability to detect translocations, several reference samples were analyzed:
- 4 RNAs extracted from FFPE samples (3 positive and 1 negative)
- 3 RNAs extracted from frozen samples (all positive)
- 2 cell lines (all negative)

Positive samples refer to samples for which fusions were known and validated. These samples were analyzed according to the procedure described in this instruction manual and the results of the SarcomaFusion test are reported in Table 1.

- ***Table 1 : Summary of the results***

samples	expected result / *obtained*	predictive value	
sample 1	EWSR1 exon 7 - CREB1 exon 7 *EWSR1 exon 7 - CREB1 exon 7*	Real positive	6
sample 2	JAZF1 exon 3 - SUZ12 exon 2 *JAZF1 exon 3 - SUZ12 exon 2*	Real negative	3
sample 3	PAX3_7 exon 7 - FOXO exon 2 *PAX3_7 exon 7 - FOXO exon 2*	Positive Predicted value	100%
sample 4	Negative *no fusion detected*	Recall	100%
sample 5	SS18 exon 10 – SSX exon6 SS18 exon 10 – SSX exon6	False positive rate	0%
sample 6	PAX3_7 exon 7 – FOXO exon 2 *PAX3_7 exon 7 – FOXO exon 2*	Sensibility	100%
sample 7	EWSR1 exon 7 – FLI1 exon 6 *EWSR1 exon 7 – FLI1 exon 6*	False positive	0
cell line 1	Negative	False negative	0

	No fusion detected		
cell line 2	Negative *no fusion detected*	Negative predicted value	100%
		Accuracy	100%
		False negative rate	0%
		Specificity	100%

The results demonstrate that the SarcomaFusion test provides high sensitivity and specificity for the detection fusion transcripts in sarcomas.

Analytical performances on a cohort of patients

A study published in 2022 on 158 bone and soft tissue tumor samples (Lanic MD et al., Modern Pathology, 2022) demonstrated the following performance:
Sensitivity = 98.1%
Specificity = 100%
In this article, the authors report that the few abnormalities not detected by the SarcomaFusion test are explained by:
- The presence of rare or complex translocations not covered by the SarcomaFusion test
- The low quality and quantity of RNA of some samples

Repeatability

The repeatability of the SarcomaFusion assay is defined as its ability to accurately quantify an expected fusion transcript. Two tests were carried out:

- A test to test the repeatability of the results of 3 samples within the same run
- A second allowing to test the repeatability of the results of 5 samples on 3 different runs

- ***Repeatability intra-run***

3 analyzed samples in triplicate by the SarcomaFusion test were studied (Figure 1). The count data of each fusion against the replicas is fully comparable.

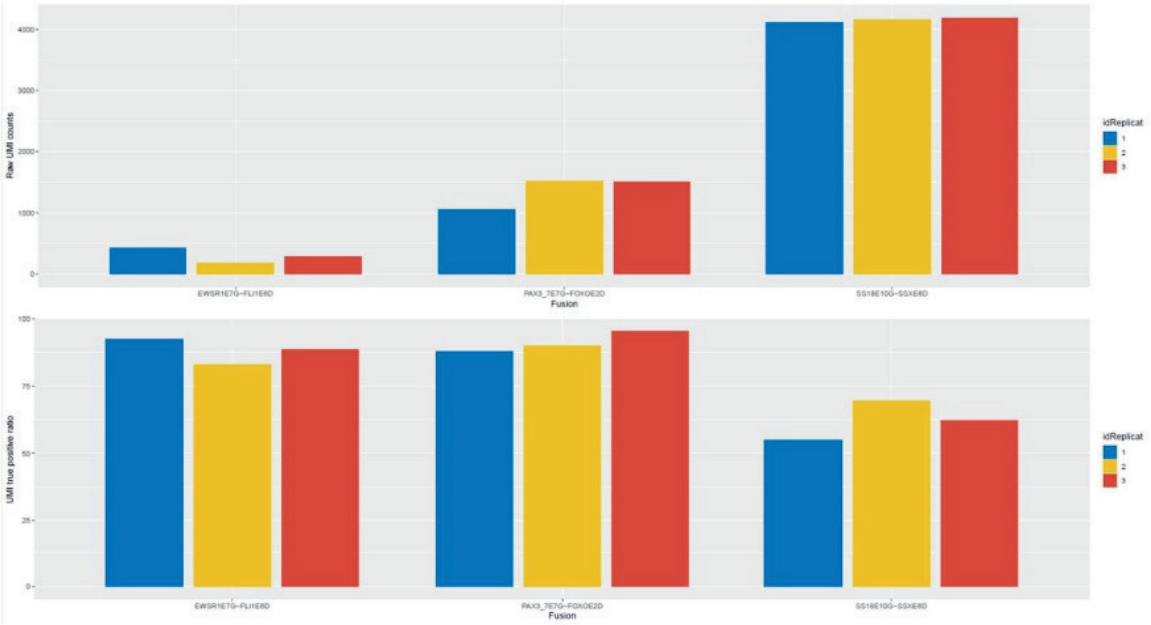


Figure 1: The histograms represent at the top the raw number of UMIs detected and at the bottom the raw number of UMIs related to the total number of UMIs of the sample according to the expected fusion and the replicas.

- ***Repeatability inter-runs***

5 samples analyzed by the SarcomaFusion test were studied on 3 different runs (Figure 2). The count data for each fusion is perfectly comparable.

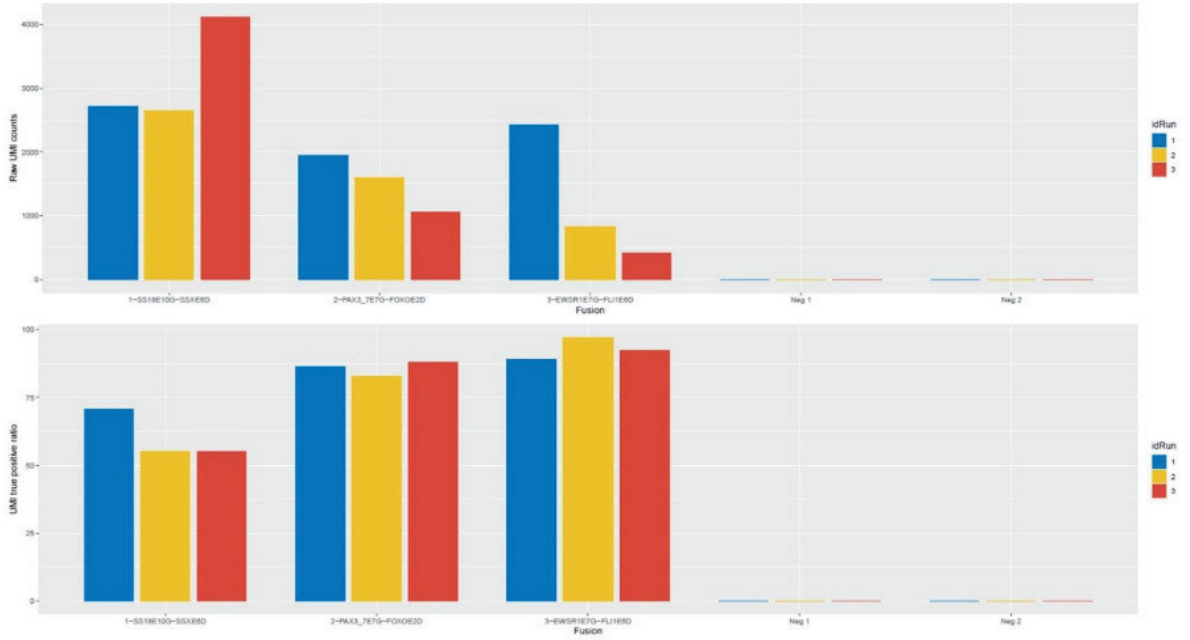


Figure 2: The histograms represent at the top the raw number of UMI detected and at the bottom the raw number of UMI related to the total number of UMI of the sample according to the expected fusion and the run.

Reproducibility

Reproducibility refers to the ability of the SarcomaFusion test to detect translocations between different users under identical conditions.

In order to evaluate this parameter, 5 samples were analyzed by 3 different users:
- 3 positive samples (SS18 exon 10 – SSX exon6, PAX3_7 exon 7 – FOXO exon 2, EWSR1 exon 7 – FLI1 exon 6)
- 2 negative samples (cell lines)

The data, shown in Figure 3, shows reproducible quantification between different users.

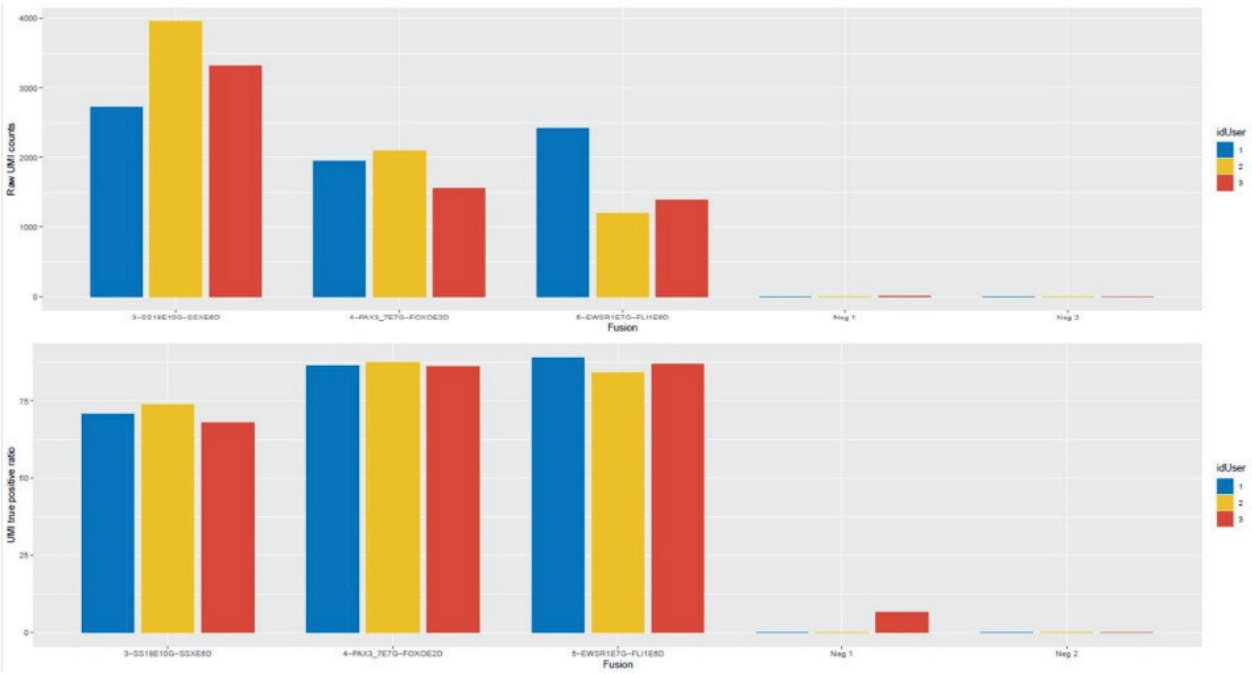


Figure 3: The histograms represent at the top the raw number of UMI detected and at the bottom the raw number of UMI related to the total number of UMI of the sample according to the expected fusion and the user.

Analytical sensibility

The analytical sensitivity of the SarcomaFusion test is defined as its ability to detect translocations as a function of the amount of RNA in the sample and the percentage of tumor cells in the sample.

In order to determine these two sensitivity limits, two serial dilutions were carried out from 2 samples:

- A dilution in water to simulate a drop in the amount of RNA

- A dilution of the sample to be tested in universal RNA in order to simulate a decrease in tumor enrichment

The results are reported in Figure 4.

Dilution of two control samples to initial amounts of 529 and 489 ng RNA in nuclease free water shows that the expected fusions are still detected at 4 ng RNA amounts. Even if the quantification of fusions depends on the tumor enrichment of the sample tested, the limit obtained is well below the recommendations for use of the SarcomaFusion test (between 50 and 500 ng).

The second range of dilutions made from two positive samples and universal RNA shows that the expected anomalies are always detected at 3% of tumor sample. At 0% positive RNA, the test no longer detects any trace of the fusions.

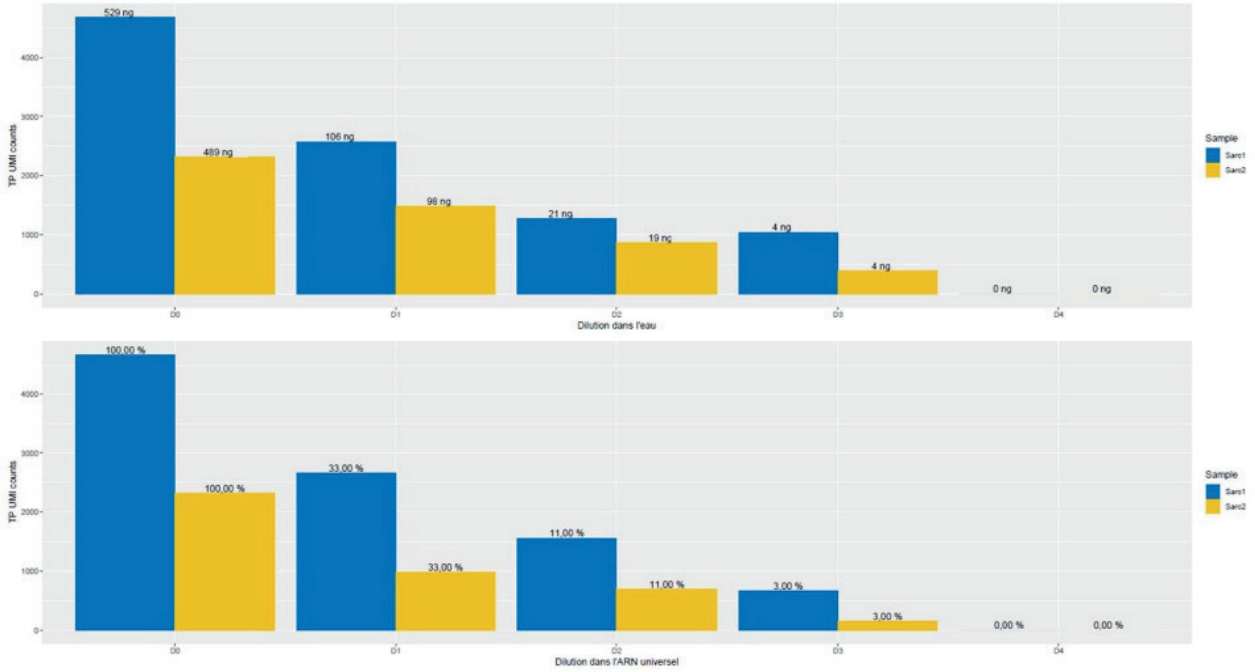


Figure 4: The histograms represent the raw number of UMIs detected from the expected fusions in two samples according to the dilution ranges carried out in water (top) or in universal RNA (bottom).

Bibliography

Detection of sarcoma fusions by a next-generation sequencing based-ligation-dependent multiplex RT-PCR assay. Lanic MD et al., Mod Pathol 2022 (PMID : 35075283).

Table of symbols

Manufacturer	REF Reagent name
Manufacturing date	Temperature limit
Shelf life	Use the instruction manual
LOT Batch code	CE EC marking european conformity
	IVD *in vitro* diagnostic medical device

Notes

GENEXPATH SarcomaFusion reagents are protected by intellectual property rights and cannot be modified, reproduced, sold or transferred without the manufacturer's permission.

Information in this document is likely to change.

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Version 10-12-2025