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EliGene® Lung NGS

REF 90067-NGS **(for 24 samples)**

Kit components:

2 x 240 µl **LUNG NGS Mix**
1 x 20 µl **PC LUNG NGS**
1 x **Instruction for Use**

Storage and shelf life after first opening:

All components of the kit must be transported and stored at -20 °C. Kit and remaining MasterMixes must be stored at -20 °C in a dark.

Intended use

EliGene® Lung NGS is intended for multiplex PCR of target regions of EGFR gene (exons 18, 19, 20, 21) allowing detection of polymorphisms included in the amplified regions.

Principle of the method

EliGene® NGS kits enable multiplex PCR amplification of all required target regions of the gene(s) of interest. The recommended amount of DNA for each multiplex PCR reaction is between 20 and 100 ng of DNA derived from formalin-fixed paraffin-embedded (FFPE) material or from blood. Next, the resulting amplicons are barcoded with MID sequences, and mixed per sample. This sample is quantified and sequenced using a NGS instrument Illumina for massively parallel sequencing (MPS) according to the manufacturer's instructions. The resulting sequence reads are subsequently analyzed by comparison with the reference sequence of the targeted gene(s) to identify variant positions.

Introduction

EliGene® NGS kits are used for one or more multiplex PCR reactions resulting in amplification of target regions of one sample (Figure 1A). For amplification is used a ready-to-use mastermix containing a hot-start DNA polymerase. The resulting amplicons of each multiplex are diluted 100 fold. In the second step, a second round of PCR is performed using EliGene® Adaptor-IL NGS kit, enabling tagging of all the amplicons to incorporate MIDs and p7 and p5 adaptors required for Illumina MiSeq Sequencing (Figure 1B). The resulting tagged amplicons from are mixed per individual applying a predefined assay specific mixing scheme. Each amplicon library is subsequently purified from small residual DNA fragments shorter than 150bp and the DNA concentration is determined using a Real-Time PCR with concentration standards. Next, these purified and individually tagged amplicon libraries are pooled equimolar, resulting in an amplicon pool or sequencing sample, which is then further processed using custom sequencing primers from EliGene® Adaptor-IL NGS kit and sequenced on the Illumina MiSeq. During the sequencing run, different sequence reads are generated on the same cluster in the process of bridge PCR. The positions of the individual custom sequencing primers supplied by EliGene® NGS kit as well as the Illumina sequencing reagents are indicated and clarified in Figure 1C.

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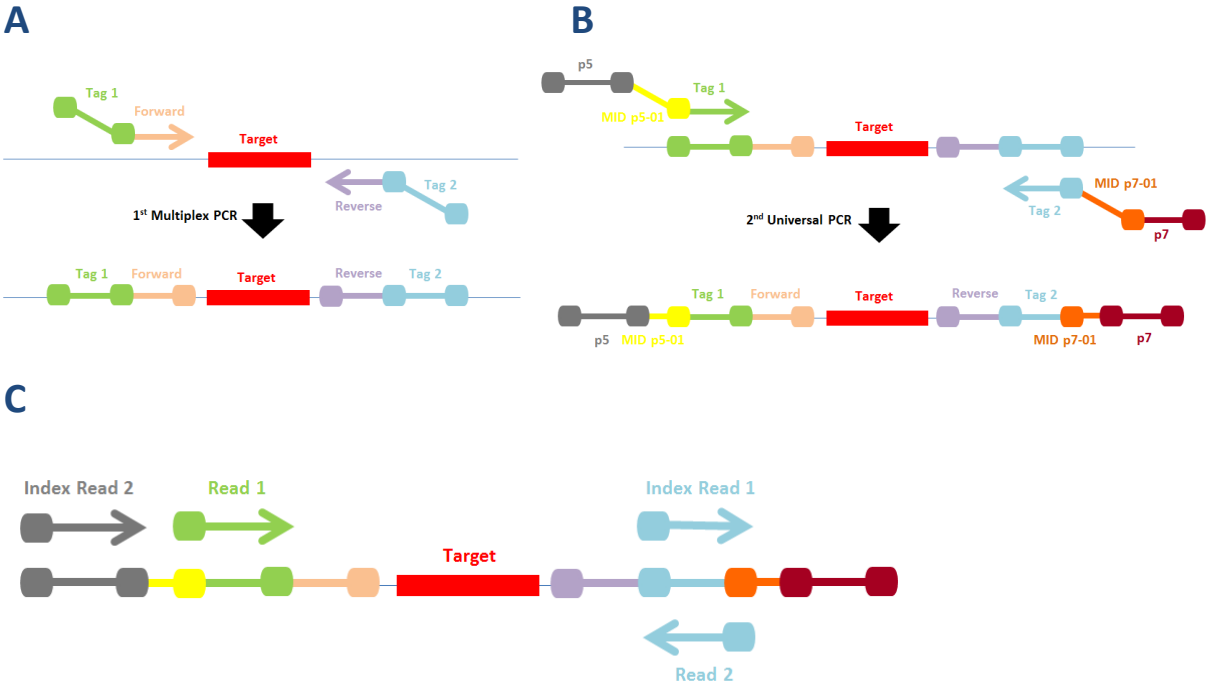

Figure 1. *Layout of the process resulting in multiplex PCR of target regions (A), incorporation of MIDs and p7 and p5 adaptors (B) and structure of resulting product after the sequencing on Illumina MiSeq instrument (C)*

Primary sample collection, handling and storage

Clinical material:	Recommended DNA isolation procedure:
Blood	Manual: EliGene® Urine Isolation Kit (ELISABETH PHARMACON) Automatic: Prepito **Zephyrus Magneto** (ELISABETH PHARMACON)
FFPE material	Manual: EliGene® FFPE Tissue DNA Isolation Kit (ELISABETH PHARMACON)

Blood:

Manual isolation:

Add 10 µl of Proteinase K to the sample and then continue according to the standard protocol of EliGene® Urine Isolation Kit (ELISABETH PHARMACON) for DNA isolation from blood. Isolated DNA use immediately for the detection or store it hours to one week at 4 °C or freeze DNA at -20 °C for longer period than one week.

Automatic isolation:

Isolate DNA from the sample by using MAGNETO **BodyFluid DNA/RNA isolation kit** according to protocol for blood samples with elution to 50 µl of Elution buffer.

FFPE material:

Isolate DNA from the sample according to protocol of EliGene® FFPE Tissue DNA Isolation Kit.

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Material and equipment required but not provided

Equipment	Recommendations/Comments
Sequencing Instrument and associated reagents for sequencing	Illumina MiSeq
Analysis software for read counts and variant calling of the generated sequencing data	BaseSpace, MiSeq Reporter, Variant Caller Studio (Illumina)
Illumina sequencing reagents	MiSeq Reagent Nano Kit, v2 (500 cycles), MiSeq Reagent Kit v2 (500cycle), MiSeq® Reagent Kit v3 (600 cycle)
TE Buffer	10 mM TRIS, 1 mM EDTA, pH=8.0
Thermal cycler	With a ramp rate of 1°C/s, temperature accuracy: ± 0.3°C (50-95°C), temperature non-uniformity across all zones < 0.5°C.
Real-time PCR cycler	With SYBR Green detection.
Quantitative PCR (qPCR) kit for Illumina library	Elisabeth Pharmacon, EliZyme™ Library Quantification Kit
Agarose gel or sensitive digital alternative	2% agarose gel, 1 x TBE buffer, DNA dye, electrophoresis bath or Agilent 2100 Bioanalyzer with Agilent High Sensitivity DNA Kit
GeneRead Size Selection Kit (50)	Qiagen, cat. no. 180514
Vortex	
Centrifuge(s)	1.5 ml tubes, 12 000 x g
Sterile automatic pipette 5–20 µl and disposable tips	Sterile-packed, aerosol-resistant disposable pipette tips, DNA/RNA free, DNase/RNase free
Plastic material (strips, plates, tubes)	Sterile, DNase/RNase free
Sterile rack	DNA/RNA free, DNase/RNase free
Laboratory safety gloves	

Preparation of reagents

- To avoid the contamination keep all tubes closed and follow the instructions.
- All reagents have to be completely thawed, vortexed and centrifuged before use.

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Preparation of master reaction mix

Warning: The following procedure should be strictly followed and performed by qualified personnel; any deviation from the prescribed protocol can lead to erroneous results.

The reagents in this kit are given for multiplex PCR of target sequences of analyzed genes. The recommended amount of DNA for each multiplex PCR reaction is between 20 and 100 ng of DNA derived from formalin-fixed paraffin-embedded (FFPE) material or from blood.

1. Remove LUNG NGS Mix tube from the freezer and allow complete thawing.
2. After thawing, vortex each vial thoroughly and centrifuge before use.
3. Prepare PCR mix for selected number of samples according to table below.

LUNG NGS Mix	DNA sample (20-100 ng)	PCR water
20 µl	1-5 µl	Add to achieve volume of 25 µl

4. Vortex and spin down the resulting PCR reaction mix and run the following thermal cycling profile:

PCR cycling profile:

95°C	15 min	
94°C	30 s	
60°C	90 s	35x
72°C	90 s	
72°C	5 min	

Set the ramp rate of the PCR machine between 1 and 2°C/s

Do not store the amplified products at 4°C: continue immediately with the workflow, otherwise immediately store amplified products at -20°C

Results reading

The successful process of multiplex PCR may be verified by an agarose electrophoresis using a 2% agarose gel and TBE buffer. Successful amplification of the multiplex PCR is detected as a clearly visible 4 bands in following lengths: EGFR (Ex18) 299 bp, EGFR (Ex19) 259 bp, EGFR (Ex20) 365 bp, EGFR (Ex121) 313 bp.

Control process

Use of control samples

EliGene® Lung NGS kit uses an artificial sequence of human DNA as positive control to verify the functionality of the amplification mix and PCR cycler. It is strongly recommended to use PCR water as negative control for each set of samples to detect possible contamination in the laboratory, which could affect the results of the analysis.

Reference material

To control all steps of the procedure, including DNA isolation, library preparation and sequencing you may use reference material positive for selected variation in target area. There are no commercial positive controls available.

Troubleshooting

In case of incorrect amplification, there may be a problem in the reaction mix, of the PCR cycler or the kit is after the expiration date.

Performance characteristics

Analytical performance characteristics:

EliGene® Lung NGS kit is intended for multiplex PCR of target sequences in gene EGFR (exons 18, 19, 20, 21) allowing the detection of polymorphisms in the amplified regions. As initial material is used DNA isolated from blood or FFPE material.

Analytical sensitivity is 20 ng of DNA in reaction mix.

Analytical specificity of method is 100%. Analytical specificity of the method was validated by searching in the DNA databases.

Clinical specificity

Diagnostic performance characteristics:

Measuring interval

The kit enables the detection of ≥ 20 ng DNA molecules in reaction mix.

Internal control of quality

As an internal control of quality the amplification process of selected regions is used.

Limitation of the examination procedure

The sensitivity of kit depends on handling with specimen (isolation and amplification of DNA). It is strictly recommended to use isolation kits and procedures mentioned above.

Biological reference intervals

Not applicable information for this kit.

Warning

Unused content of the tube with MasterMix is stable for 2 weeks at -20 °C. Do not freeze tubes with MasterMix more than 5 times! Do not mix components of the kits of different lots.

Warnings and general precautions

- Handle and dispose of all biological samples as if they were capable of transmitting infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must autoclaved at 121 °C for one hour before disposal.
- Handle and dispose of all reagents and all assay materials as if they were capable of transmitting infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear suitable protective clothing and gloves and protect eyes/face.
- Never pipette solutions by mouth.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose of leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the kit before running the assay.
- Follow the instructions provided with the kit while running the assay.
- Do not use the kit after the expiry date.
- Only use the reagents provided in the kit and those recommended by the manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from other manufacturer´s kit.

Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar safety box. Tubes containing different samples must be never opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled under PCR box or laminar flow box. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.

- Amplification products must be handled in such way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components of the kit

- The tubes containing mix (LUNG NGS Mix) are disposable and therefore must be used once only in the preparation of the reaction mixture.
- These mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P281 Use personal protective equipment as required.

Literature

De Leeneer K, De Schrijver J, Clement L, Baetens M, Lefever S, et al. (2011) Practical Tools to Implement Massive Parallel Pyrosequencing of PCR Products in Next Generation Molecular Diagnostics. PLoS ONE 6(9): e25531. doi:10.1371/journal.pone.0025531

Symbols

	Catalog number
	Upper limit of temperature
	Batch code
	Use by (last day of month)
	*in vitro* diagnostic medical device
	Fulfilling the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests
	Attention, consult instructions for use
	Manufacturer

Manufacturer

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