Joint Operational Programme
Romania-Republic of Moldova 2014-2020

Guide about the technical procedure, interpretation and incidence of the driver mutations - protocol for DNA extraction from FFPE tissue and from liquid biopsies and EGFR genotyping and NGS

This project is funded by the European Union

Romania-Republic of Moldova
ENI-CROSS BORDER COOPERATION

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A project implemented by Regional Institute of Oncology Iasi

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PMSI Institute of Oncology

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Content

1. Introduction

Real-time PCR analysis is a highly sensitive method based on Taq-Man technology for the detection of somatic mutations in exons 18, 19, 20 and 21 of the EGFR gene in human DNA samples. This method amplifies the mutated sequences in the mutated samples, as well as wild-type DNA, using fluorescent probes for detection.

Each amplification reaction contains a set of primers for the detection of EGFR mutations as well as primers for endogenous control. The primers for the endogenous control amplify a gene to check the quality of the reagents used and whether the reaction contains enough DNA for amplification.

Mutations that can be detected by real time PCR

1. • **T790M**
2. • **48 deletions of the 19th exon (without the possibility of distinction between them)**
3. • **L858R**
4. • **L861Q**
5. • **S768I**
6. • **G719X (G719A, G719S, G719D and G719C) (without the possibility of distinction between them)**
7. • **insertions in the 20th exon: 2319-2320 insCAC and 2310-2311 insGGT (without the possibility of distinction between them)**
8. • **insertions in the 20th exon: 2307-2308 insGCCAGCGTG (ins9)**

Principle of the method: TaqMan technology uses a fluorescent labeled probe that attaches specifically to the area of mutated DNA. A probe is fluorescently labeled at the 5 'end and at the 3' end has a fluorescence inhibiting compound. At each amplification cycle the allele-specific probe is attached to the DNA strand and the generation of amplicons by sense (FW) and antisense (RW) primers causes the emission of the fluorescent signal by degrading the probe (hydrolysis) and removing the reporter fluorochrome from the quencher, in the presence of HotStart polymerase. Thus, at each amplification cycle, fluorescent molecules are released that determine the exponential intensification of the fluorescent signal. If the specific probe does not attach to the DNA, the probe will not be hydrolyzed, as it does not emit fluorescence.

FusionPlex Comprehensive Thyroid and Lung Kit from ArcherDX, is a targeted NGS panel to detect gene fusions, SNV/indels, splicing and gene expression. This panel is expertly designed to cover relevant exons in 34 genes when used in combination with the VariantPlex CTL Panel. Libraries are created by using the FusionPlex assay in conjunction with the Archer MBC Adapters.

This panel complements the Archer VariantPlex® CTL Panel for comprehensive mutation profiling of fusions, CNVs and variants.

Studies are performed from RNA extracted from several types of starting sample. Using Archer's proprietary Anchored Multiplex PCR (AMP™)-based enrichment, fusions of all genes in this kit can be identified in a single sequencing assay, even without prior knowledge of fusion partners or breakpoints.

The Archer® FusionPlex® Lung Kit is focussed to detect EGFR vIII and MET exon 14 skipping events along with prominent ALK, BRAF, FGFR, NRG1, NTRK, RET, and ROS1 fusions and select point mutations in 14 key gene targets associated with lung cancer.

2. Terminology

Genotyping is the process of determining the DNA sequence, called a **genotype**, at specific positions within the genome of an individual. Sequence variations can be used as

markers in linkage and association studies to determine genes relevant to specific traits or disease.

Sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine.

NGS is a massive parallel **sequencing** reaction that generates hundreds of megabases to gigabases of nucleotide **sequence** reads in a single instrument run. This has enabled a drastic increase in available **sequence** data and fundamentally changed genome **sequencing** approaches in the biomedical sciences.

NGS library is a collection of similarly sized **DNA** fragments with known adapter sequences added to the 5' and 3' ends. A **library** corresponds to a single sample and multiple **libraries**, each with their own unique adapter sequences, can be pooled and sequenced in the same sequencing run.

Real-time polymerase chain reaction (**real-time PCR**) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR) and semi-quantitatively (i.e., above/below a certain amount of DNA molecules) (semi-quantitative real-time PCR).

3. Quality score (Q score)

Determination of DNA concentration and quality

To determine the concentration and purity of a solution containing DNA, the absorbance of an undiluted aliquot of 2 µl is measured at 260 nm and 280 nm in a spectrophotometer (**Eppendorf, 1mm Helma Lp cuvette**).

Concentration (ng / µl) = absorbance (260nm) x 50 x dilution factor

A double-stranded DNA sample with a concentration of 50 ng / µl at a wavelength of 260 nm, has a specific absorbance of 1 for an optical path length of 10 mm.

An $A_{260/280}$ ratio of pure nucleic acids is 1.8-2.0.

DNA samples extracted from formalin-fixed tissue and embedded in paraffin cannot be correctly quantified by UV spectrometry. The values calculated from the optical density measurement at 260 nm will substantially overestimate the actual concentration of DNA present. Only the use of appropriate fluorometric quantization platforms (eg Invitrogen Qubit) will allow reliable quantification of DNA from FFPE tissue.

QUALITY CONTROL

Internal control

The quality of the mutation analysis of the EGFR gene is ensured by:

• Carrying out the DNA extraction control by simultaneously processing a paraffin block sample with the test samples.

• A negative control (Nuclease free extraction / water control) and a positive control provided by the kit are used to prepare the amplification mixes for the detection of EGFR mutations.

• The amplification mix of the RT52-EGFR kit contains an endogenous control that allows checking the quality of the DNA used in the reaction.

• A thorough understanding of the described procedure, as well as precise laboratory equipment and techniques are required to obtain reliable results. Only properly trained personnel can use RT52-EGFR for in vitro human diagnosis.

• RT52-EGFR components are not used after the expiration date printed on the outside of the kit box. Do not mix reagents from different batches.

• Avoid microbial contamination and cross-contamination of reagents or samples by using sterile disposable pipette tips throughout the procedure. Do not change the lids between bottles.

Determination of RNA concentration and quality

To determine the concentration and purity of a solution containing DNA, the absorbance of an undiluted aliquot of 2 µl is measured at 260 nm and 280 nm in a spectrophotometer (**NanoDrop**).

Concentration (ng / µl) = absorbance (260nm) x 40 x dilution factor

An RNA sample with a concentration of 40 ng / µl at a wavelength of 260 nm, has a specific absorbance of 1 for an optical path length of 10 mm.

An $A_{260/280}$ ratio of pure nucleic acids is 1.9-2.0.

However, **amplifiable RNA** samples extracted from formalin-fixed tissue embedded in paraffin cannot be **correctly** quantified by UV spectrometry. The values calculated from the optical density measurement at 260 nm will substantially overestimate the actual concentration of RNA present. Only the use of appropriate fluorometric quantization platforms (eg Invitrogen Qubit) will allow reliable quantification of RNA in FFPE tissue.

PhiX Control - NGS

PhiX Control v3 is a reliable, adapter-ligated library used as a control for Illumina sequencing runs. The library is derived from the small, well-characterized PhiX genome, offering several benefits for sequencing and alignment. PhiX Control v3 is provided as a ready-to-use library, and can be utilized in diverse applications to add value to your workflow and increase confidence in your results. The PhiX library provides a quality control for cluster generation, sequencing, and alignment, and a calibration control for cross-talk matrix generation, phasing, and prephasing. It can be rapidly aligned to estimate relevant sequencing by synthesis (SBS) metrics such as phasing and error rate.

POST-EXAMINATION PROCESSES

Checking the results

The results are verified throughout the analytical process by the person in charge of the analysis.

Checking patient results includes:

- analysis of positive, negative and without template internal control results
- browsing parameter by parameter in terms of their concordance and compliance with existing medical information both on the referral note and in the laboratory database.

Access to the database is limited due to the use of identification passwords

4. Mutations

Real-Time PCR mutations

Exon	EGFR Mutation Group	EGFR Nucleic Acid Sequence	COSMIC ID6
Exon 18	G719X	2156G>C	6239
		2155G>A	6252
		2155G>T	6253
Exon 19	Ex19Del	2240_2251del12	6210
		2239_2247del9	6218
		2238_2255del18	6220
		2235_2249del15	6223

		2236_2250del15	6225
		2239_2253del15	6254
		2239_2256del18	6255
		2237_2254del18	12367
		2240_2254del15	12369
		2240_2257del18	12370
		2239_2248TTAAGAGAAG>C	12382
		2239_2251>C	12383
		2237_2255>T	12384
		2235_2255>AAT	12385
		2237_2252>T	12386
		2239_2258>CA	12387
		2239_2256>CAA	12403
		2237_2253>TTGCT	12416
		2238_2252>GCA	12419
		2238_2248>GC	12422
		2237_2251del15	12678
		2236_2253del18	12728
		2235_2248>AATTC	13550
		2235_2252>AAT	13551
		2235_2251>AATTC	13552
		2253_2276del24	13556
		2237_2257>TCT	18427
		2238_2252del15	23571
		2233_2247del15	26038
Exon 20	S768I	2303G>T	6241
	T790M	2369C>T	6240
	Ex20Ins	2307_2308ins9GCCAGCGTG	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
		2311_2312ins9GCGTGGACA	13428
		2309_2310AC>CCAGCGTGGAT	13558
Exon 21	L858R	2573T>G	6224
		2573_2574TG>GT	12429
	L861Q	2582T>A	6213

NGS - mutations

Targeted RNA-seq to identify oncogenic driver mutations from low-quality RNA

- Detect known and novel gene fusions

- Confirm key point mutations (SNVs/indels)

- Capture RNA abundance & expression imbalance

NGS sequencing targets the following genes: AKT1, ALK, AXL, BRAF, CALCA, CCND1, CTNNB1, DDR2, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, GNAS, HRAS, IDH1, IDH2, KRAS, KRT20, KRT7, MAP2K1, MET, NRAS, NRG1, NTRK1, NTRK2, NTRK3, PIK3CA, PPARG, PTH, RAF1, RET, ROS1, SLC5A5, THADA, TTF1.

Known and Novel Fusions

Anchored Multiplex PCR (AMP™) chemistry relies on MBC adapters for target amplification. These partially-functional adapters are ligated to cDNA fragments and contain a universal primer binding site that permits amplification of both known and unknown genomic regions of interest. This approach generates libraries with random start sites and varying lengths, increasing library complexity and retaining sample heterogeneity. AMP chemistry can capture both 5' and 3' fusions, including novel fusions that would be missed by opposing primer-based methodologies.

5. Variant allele frequency (VAF) and limit of detection (LOD)

Sensitivity of the method

Real-time PCR genotyping has a detection limit of up to 1% mutant alleles in a wild-type DNA background.

Mutation	Limit of detection
T790M	1%
Exon 19 deletions	1 %
L858R	1 %
L861Q	1 %
S768I	1.5 %
G719X	3.2 %
Exon 20 insertions	2 %

Sensitive Mutation Detection by using NGS technology

Archer FusionPlex NGS assays show sensitive detection of translocations and point mutations. TPM3:NTRK1 fusion-containing RNA was serially diluted into fusion-negative background RNA and libraries were prepared using the Archer FusionPlex CTL Assay using 100ng total input. After sequencing, fusions were called based on sequencing reads spanning the fusion breakpoint down to **0.39% fusion-containing RNA**. A minimum of 5 supporting unique start sites are required for fusion calls.

RNA Expression

Relative RNA abundance can be determined for select genes, because molecular barcodes are ligated to input material prior to amplification. Knowing RNA abundance helps with tissue of origin identification, expression signature-based differentiation of diffuse large B-cell lymphoma (DLBCL) subtypes and relative expression level detection in critical genes.

Relative RNA abundance can be used to measure CD274 (PD-L1) expression levels across 4 FFPE expression standards (Horizon® CD274 (PD-L1) Reference Standard cat# HD787) of varying PD-L1 status (left panel) and across different DLBCL subtypes (right panel).

6. Technical protocol

DNA extraction from FFPE

DNA extraction is performed from the tissue selected from the area indicated by the pathologist.

Procedure for selecting the tissue of interest:

This procedure is performed in an area outside the molecular biology unit (without strong air currents), in order to eliminate the risk of contamination with any biological product of the PCR amplification zone.

Procedure:

In the case of section samples made on slides, prepare the work table as follows: clean the work surface with 70% alcohol before processing each sample. Prepare: HE blades with the area of interest surrounded, the blades from which the sample will be collected and a properly labeled 1.5 ml tube.

Disposable sterile utensils will be used to scrape the tissue of interest: scalpel blades. Pipette 1 ml of xylene into a 1.5 ml sterile Eppendorf tube. Take a little of this xylene and pipette it into the open lid of the tube.

Take the hematoxylin-eosin-colored tissue section slide and place under a non-stained tissue section slide. Through transparency, the area designated by the pathologist as a tumor will be observed on the blade with uncolored tissue. With a thin-tipped marker, the tumor area is surrounded on the blade with uncolored tissue.

Take a scalpel and soak it in the xylene from the lid of the Eppendorf tube. Gently scrape the designated area using a scalpel. From time to time the scraped material is collected and introduced into the Eppendorf tube. Repeat the procedure for another 3-5 sections of uncolored tissue.

When all the tumor material has been collected, carefully close the Eppendorf tube without spilling the xylene from the lid and shake manually.

In the case of section samples in 1.5ml or 2ml tubes, add 1ml xylene over the section / sections and shake manually.

• The tissue fixed in paraffin and suspended in xylene is incubated at 60°C, for 15 minutes, with stirring at 450 rpm on the thermoblock, then centrifuged for 3 minutes at room temperature, at 13,500 rpm; the supernatant is removed with a semi-automatic pipette of 20-200 µl. Repeat the washing with xylene.

• Rehydration with alcohol: add 1 ml of absolute ethanol and vortex, centrifuge for 3 minutes at 13,500 rpm at room temperature. Remove the supernatant with a 20-200 µl semi-automatic pipette. Repeat washing with alcohol.

• With the lid open, allow the tubes to dry on the thermoblock for 10 minutes at 60° C or centrifuge for 10 minutes at room temperature in a vacuum centrifuge.

NOTE: *Each patient will be treated individually.*

NOTE: *Like all procedures for handling biological samples and extracting nucleic acids, the procedure will be performed by an* ***executor*** *and confirmed by a* ***witness****, who will sign on the* ***processing and extraction sheet*** *the conformity of the annotation of the tubes and their handling.*

ReliaPrep™ FFPE gDNA Miniprep System extraction protocol

The ReliaPrep™ FFPE gDNA Miniprep System kit uses optimized incubation conditions to partially break the cross-links formed during the process of formalin fixation and paraffin embedding of the tissue, without the need for overnight digestion. This system also includes a method of deparaffining that is not based on xylene or other volatile solvents.

ReliaPrep™ FFPE gDNA Miniprep System - 100 reactions
Kit components:
• 50ml Mineral Oil
• 20ml Lysis Buffer (LBA)
• 2 × 1.1ml Proteinase K (PK)
• 32.5ml BL Buffer
• 30ml Wash Solution
• 1ml RNase A
• 15ml Elution Buffer
• 2 packages Collection Tubes (50 tubes/pack)
• 2 packages ReliaPrep™ FFPE Binding Columns (50 columns/pack)
Storage conditions: at room temperature.

Preparation before use
Preparing 1X Wash Solution
Materials not supplied in the kit: 95–100% ethanol
Add 120 ml of 95-100% ethanol to the flask containing 30 ml of concentrated Wash Solution and label the flask.

Deparaffining using mineral oil
• Centrifuge the tube in which the sample was collected to collect the tissue and to avoid spreading it when opening the tube in the extraction area.
• Add 300 µl of mineral oil over the sample.
• Incubate at 80° C for 1 minute.
• Vortex for mixing.
Lysis of the sample
• Add 200µl of Lysis Buffer over the sample.
• Centrifuge at 10,000 × g for 15 seconds. Two phases will form, a lower (aqueous) phase and an upper (oily) phase.
• Add 20µl of Proteinase K directly to the lower phase; mix the lower phase by pipetting.
• Incubate at 56° C for one hour.
• Incubate at 80° C for one hour.
• Cool the sample for 5 minutes at room temperature. Centrifuge briefly to collect any droplets formed under the tube cap.
RNase treatment
• Add 10µl of RNase A directly over the sample lowered. Mix the lower phase by pipetting.
• Incubate at room temperature (20–25° C) for 5 minutes.
Nucleic acid binding
• Add 220µl of BL Buffer over the lysed sample.
• Add 240µl of ethanol (95–100%).
• Swirl briefly for mixing.

• Centrifuge 10,000 × g for 15 seconds. Two phases will form, a lower (aqueous) phase and an upper (oily) phase.

• For each sample, place a column in the collecting tube and number it accordingly.

• Fully transfer the lower (aqueous) phase of the sample, including any precipitate that has formed, into the column and collection tube assembly and close the column cap. Discard the remaining mineral oil.

Note: The mineral oil is inert and will not interfere with the extraction procedure if some of it is transferred to the column.

• Centrifuge at 10,000 × g for 30 seconds.

• Discard the liquid from the collecting tube and reinsert the column into the collecting tube.

• Immediately proceed with washing the column and eluting.

Column washing and elution

• Add 500µl of 1X Wash Solution (with added ethanol) over the column and close the column lid.

• Centrifuge at 10,000 × g for 30 seconds.

• Discard the liquid from the collector tube and reinsert the column into the same collector tube.

• Add 500µl of 1X Wash Solution (with added ethanol) over the column and close the column lid.

• Centrifuge at 10,000 × g for 30 seconds.

• Discard the liquid from the collecting tube and reinsert the column into the same collecting tube.

• Open the column cover and centrifuge the column/tube assembly at 16,000 × g for 3 minutes to dry the column.

• Transfer the column to a 1.5 ml microcentrifuge tube (not supplied), and discard the collecting tube.

• Add 30–50µl of Elution Buffer over the column and close the column cover.

• Centrifuge at 16,000 × g for 1 minute. Remove and discard the column.

• Close the lid of the microcentrifuge tube, and keep the eluted DNA at -20° C.

NucleoSpin FFPE DNA extraction protocol

The NucleoSpin FFPE DNA kit is designed to purify total DNA from paraffin-fixed samples (FFPE). After deparaffining, the tissue is subjected to the action of proteinase K, so the DNA is released into solution. The release of DNA from different bonds is done by incubation at a high temperature in the presence of the decross-link buffer and precipitation is done with absolute alcohol. The entire lysate is loaded onto a NucleoSpin FFPE DNA Column and the DNA is attached to the silicon membrane. After two washes, the salts, metabolites, macromolecular cellular components are removed and the DNA is eluted under conditions of low ionic strength. The DNA obtained, with dimensions of 100-300 bp, is suitable for PCR and other enzymatic reactions.

Kit components:

Paraffin Dissolver	125ml
Lysis Buffer FL	8 ml
Decrosslink Buffer D-Link	30 ml
Wash Buffer B5 (concentrate)	50ml
Proteinase K (lyophilized)	75 mg

Proteinase K Buffer PB	8ml
Elution Buffer BE (5 mM Tris/HCl, pH 8.5)	13ml
NucleoSpin DNA FFPE Columns (green ring) and collection tubes	250
Collection tubes 2ml	500
User manual	1

Preparation before use:

• Proteinase K: add the volume of Proteinase Buffer PB, 3.35ml for the 250-reaction kit, to dissolve the lyophilized Proteinase K. Proteinase K is stable at -20°C for 6 months.

• Wash Buffer B5: add the indicated volume of 96-100% ethanol, 200ml, over Buffer B5 Concentrate. Wash Buffer 5 is stored at room temperature (18-25°C) for up to 1 year.

Non-supplied kit materials: xylene, ethanol (96-100%), 1.5-2ml tubes for elution

DNA extraction from the FFPE sample

1. Add 100 µl lysis Buffer FL over the dewaxed tissue and homogenize by pipetting.
2. Add 10 µl of Proteinase K, vortex vigorously (5 seconds), short spin.
3. Incubate overnight at room temperature with stirring 450 rpm in the thermoblock.
4. Vortex, spin down, add 100 µl Decrosslink Buffer D-Link.
5. Vortex vigorously (5 seconds) and incubate the tubes at 90° C for 30 minutes.
6. Vortex the tubes (5 seconds), spin down and allow to cool to room temperature for 2 minutes.
7. Add 200 µl of 96% ethanol and vortex the tubes vigorously (2x5 seconds).
8. For each sample, prepare a NucleoSpin FFPE DNA Column (green color) with a 2ml collecting tube.
9. Load the entire sample into the column and centrifuge for 30 seconds at 2000 x g.
10. Discard the collector tube. The column is inserted into a new collecting tube.
11. Add 400 µl Buffer B5 and centrifuge for 30 seconds at 11000 x g.
12. Discard the collecting tube. The column is inserted into a new collecting tube.
13. Add 400 µl Buffer B5 and centrifuge for 2 minutes at 11000 x g.
14. Discard the collecting tube and place the column in a 1.5 ml Eppendorf tube.
15. Pipette 50 µl of Buffer BE into the center of the column membrane, leave to incubate for 1 minute.
16. Centrifuge for 30 seconds at 11000 x g.
17. Pipette 50 µl of Buffer BE into the center of the column membrane, incubate for 1 minute.
18. Centrifuge for 30 seconds at 11000 x g.
19. The column is thrown. The extracted DNA is now in the Eppendorf tube. Transfer the DNA to a 1.5 ml Eppendorf tube.
20. Quantify the extracted DNA. The DNA tube is labeled and stored at 4° C, -20° C or -80° C.

RecoverAll Total Nucleic Acid Isolation Kit

MATERIALS PROVIDED WITH THE KIT AND STORAGE CONDITIONS

Amount	Component	Storage
16 mL	Digestion Buffer	room temp
60 mL	Wash 1 Concentrate ***Add 42 mL 100% ethanol before use***	room temp
60 mL	Wash 2/3 Concentrate	room temp

	Add 48 mL 100% ethanol before use	
80	Collection Tubes	room temp
40	Filter Cartridges	room temp
19.2 mL	Isolation Additive	room temp
5 mL	Elution Solution	any temp
160 µL	Protease	–20°C
240 µL	10X DNase Buffer	**–20°C**
160 µL	DNase	**–20°C**
400 µL	RNase A	**–20°C**

Prepare Wash Solutions

a. Add **42 mL** of ACS grade **100% ethanol** to the bottle labeled **Wash 1 Concentrate**. Mix well.
b. Add **48 mL** of ACS grade **100% ethanol** to the bottle labeled **Wash 2/3 Concentrate**. Mix well.
c. Cap the wash solution bottles tightly to prevent evaporation.
d. Mark the labels to indicate that the ethanol has been added.

DEPARAFFINIZATION

(1) Assemble FFPE sections (two 10 µm)

a. Cut **10 µm** sections from FFPE tissue blocks using a microtome.
b. Place the sections of tissue slices in a 1.5 mL microcentrifuge tube

(2) Add 1 mL 100% xylene, mix, and incubate for 3 min at 50°C

a. Add **1 mL 100%** xylene to the sample.
b. Vortex briefly to mix.
c. Centrifuge briefly to bring any tissue that is stuck to the sides of the tube down into the xylene.
d. Heat the sample for **3 min** at **50°C** to melt the paraffin.

(3) Centrifuge for 2 min at maximum speed, and discard the xylene

a. Centrifuge the sample for **2 min** at room temperature and **maximum speed** (Hettich – **14,500 rpm**) to pellet the tissue.
b. (***Optional***) If the sample **does not form a tight pellet**, **recentrifuge** for an additional **2 min**. If a **tight pellet still does not form**, then **proceed with caution in step c**.
c. Remove the xylene without disturbing the pellet. Discard the xylene.

NOTE: *At this step, the tissue is usually clear and can be difficult to see. If the pellet is loose, you may need to leave some xylene in the tube to avoid removing any tissue pieces.*

(4) Wash the pellet twice with 1 mL 100% ethanol

a. Add **1 mL** of **100% ethanol** (room temperature) to the sample and **vortex to mix**. - *The tissue should turn opaque.*
b. **Centrifuge** the sample for **2 min** at room temperature and **maximum speed** (Hettich – **14,500 rpm**) to pellet tissue.
c. **Remove** and discard the **ethanol without disturbing the pellet**. The ethanol will contain trace amounts of xylene, and must be discarded accordingly.
d. **Repeat** steps **a–c** above to wash a second time with **1 mL** of **100% ethanol**.
e. Briefly centrifuge again to collect any remaining drops of ethanol in the bottom of the tube. Remove as much residual ethanol as possible without disturbing the pellet.

(5) Air dry the pellet

Air dry for 15–45 min at room temperature.

PROTEASE DIGESTION

(1) Add Digestion Buffer and Protease

a. Use the following table to determine the appropriate amount of Digestion Buffer needed for your tissue sample.

Sample size	Digestion Buffer per sample
≤ 40 µm	100 µL
40–80 µm	200 µL

b. Add **Digestion Buffer** to each sample.
c. Add **4 µL Protease** to each sample.
d. Swirl the tube gently to mix and to immerse the tissue. If tissue sticks to the sides of the tube, use a pipet tip to push it into the solution, or briefly centrifuge to bring the tissue down into the solution.

(2) Incubate for 16 hr at 50°C

a. Incubate the sample in heat blocks for **16 hr** at **50°C.**

Most sample mixtures will clarify after 16 hr. If the sample does not clarify, it may be heavily oxidized and therefore somewhat resistant to protease digestion. Samples that do not clarify may have slightly lower yields and smaller DNA fragments.

Increasing the incubation time at 50°C to up to 48 hr usually results in recovery of DNA with slightly increased functionality. This may be beneficial in downstream applications requiring larger PCR amplicons, or in sequencing or methylation analysis.

NUCLEIC ACID ISOLATION

(1) Prepare Isolation Additive/ethanol mixture

Combine the indicated amounts of Isolation Additive and ethanol, according to the volume of Digestion Buffer used in your sample.
For **multiple samples**, prepare a master mix for all samples plus ~5% overage.

	Volume of Digestion Buffer	
	100 µL	200 µL
Isolation Additive	120 µL	240 µL
100% ethanol	275 µL	550 µL
Total	395 µL	790 µL

(2) Add Isolation Additive/ethanol and mix

a. Add the appropriate volume of **Isolation Additive/ethanol mixture** to each sample.
b. **Mix by pipetting** up and down.
Some samples may appear white and cloudy after mixing.

(3) Pass the mixture through a Filter Cartridge

a. For each sample, place a **Filter Cartridge** in one of the **Collection Tubes** supplied.
b. Pipet up to **700 µL** of the **sample/ethanol mixture** (from step 2) onto the **Filter Cartridge** and close the lid.
To prevent clogging of the filter, avoid pipetting large pieces of undigested tissue onto the Filter Cartridge

c. **Centrifuge** at **10,000 x g** (Hettich – **10,000 rpm**) for **30 sec** to pass the mixture through the filter.

NOTE*: Do not centrifuge Filter Cartridges at relative centrifugal forces greater than 10,000 x g; higher forces may damage the filters*.

d. **Discard** the flow-through, and **re-insert** the **Filter Cartridge** in the **same Collection Tube**.
e. **If necessary**, **repeat steps a–d** until all the sample mixture has passed through the filter.

(4) Wash with 700 µL of Wash 1

a. Add **700 µL** of **Wash 1** to the **Filter Cartridge**.
b. **Centrifuge** for **30 sec** at **10,000 x g** (Hettich – **10,000 rpm**) to pass the mixture through the filter.
c. **Discard** the flow-through, and **re-insert** the **Filter Cartridge** in the **same Collection Tube**.

(5) Wash with 500 µL of Wash 2/3, and then centrifuge to remove residual fluid

a. Add **500 µL** of **Wash 2/3** to the **Filter Cartridge**.
b. Centrifuge for **30 sec** at **10,000 x g** (Hettich – **10,000 rpm**) to pass the mixture through the filter.
c. **Discard** the flow-through, and **re-insert** the **Filter Cartridge** in the **same Collection Tube**.
d. **Spin** the assembly for an additional **30 sec** to remove residual fluid from the filter.

NUCLEASE DIGESTION AND FINAL NUCLEIC ACID PURIFICATION

NOTE: ***Preheat Elution Solution or nuclease-free water to 95°C.***

(1)Add 60 µL RNase mix and incubate for 30 min at room temp

a. Combine the following solutions to make the RNase mix (a master mix can be used if there is more than one sample).

Amount (per reaction)	Component
10 µL	RNase A
50 µL	Nuclease-free Water

b. Add **60 µL** of the **RNase mix** to the **center** of each **Filter Cartridge**.
c. Cap the tube and **incubate** for **30 min** at **room temp** (22–25°C).

(2)Wash with 700 µL of Wash 1

a. Add **700 µL** of **Wash 1** to the **Filter Cartridge**.
b. **Incubate** for **30–60 sec** at room temperature.
c. Centrifuge for **30 sec** at **10,000 x g** (Hettich – **10,000 rpm)**.
d. **Discard** the flow-through, and **re-insert** the **Filter Cartridge** in the **same Collection Tube**.

(3)Wash twice with 500 µL of Wash 2/3, then centrifuge to remove residual fluid

a. Add **500 µL** of **Wash 2/3** to the **Filter Cartridge**.
b. Centrifuge for **30 sec** at **10,000 x g** (Hettich – **10,000 rpm)**.
c. Discard the flow-through, and re-insert the Filter Cartridge in the same Collection Tube.
d. **Repeat** steps **a–c** to wash a second time with **500 µL** of **Wash 2/3**.
e. Centrifuge the assembly for **1 min** at **10,000 x g** (Hettich – **10,000 rpm)** to remove residual fluid from the filter.

a. Transfer the Filter Cartridge to a fresh Collection Tube.

(4)Elute with 60 µL Elution Solution or nuclease-free water at room temp	b. Apply **60 µL** of **Elution Solution** or nuclease-free water (**preheated to 95°C**) to the ***center of the filter***, and close the cap. c. Allow the sample to **sit** at **room temperature** for **1 min**. d. Centrifuge for **1 min** at **maximum speed** (Hettich – **14,500 rpm**) to pass the mixture through the filter. *The eluate contains the DNA* e. Store the nucleic acid at –20°C or colder.

LIQUID BIOPSY (LB) DNA EXTRACTION
COBAS cfDNA SAMPLE PREPARATION KIT

Note: Only K2 EDTA Plasma samples are to be used with the cobas® cfDNA Sample Preparation Kit. **Reagent preparation and storage**

Prepare working reagents as shown in the table below prior to using the kit for the first time. Use a 5-mL serological pipette to dispense the water. Use 25-mL serological pipettes to dispense the ethanol. If the Proteinase K has already been reconstituted and frozen, thaw a sufficient number of aliquots to process the number of specimens to be run.

Reagents	Reconstitution / Preparation
Proteinase K (**PK**)	Reconstitute **PK** by adding 4.5 mL of sterile water to the vial using a sterile, disposable 5-mL serological pipette. Mix by inverting the vial 5 to 10 times. Aliquot 1.1 mL of reconstituted **PK** into 1.5-mL microcentrifuge tubes and store at -20°C for up to 30 days or until the expiration date, whichever comes first. If the **PK** has already been reconstituted and frozen, thaw sufficient number of aliquots to process the number of specimens to be run (250 µL of reconstituted **PK** is required for each specimen).
Wash Buffer I (**WB I**)	Prepare working **WB I** by adding 15 mL of absolute ethanol to the bottle of **WB I**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB I** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.
Wash Buffer II (**WB II**)	Prepare working **WB II** by adding 50 mL of absolute ethanol to the bottle of **WB II**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB II** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.

All solutions stored at 15 - 30°C should be clear. If precipitate is present in any reagent, warm the solution to 37°C until the precipitate dissolves. Do not use until all precipitate has been dissolved.

1. Label a 15-mL conical tube for each plasma sample and a negative control. Sterile water can serve as a negative control and can be processed the same way as samples.
2. Vortex plasma then transfer 2 mL of each plasma sample or negative control (sterile water) to a separate 15-mL tube.

Note: A minimum of 2 mL of plasma is required to process a sample with the cobas® cfDNA Sample Preparation Kit.

3. Add 250 µL **PK** to each tube.
4. Add 2 mL of **DNA PBB** to each tube.
5. Mix the specimen tubes containing **DNA PBB/PK** by inverting 3 to 5 times.
6. Incubate each tube at room temperature (15°C to 30°C) for 30 minutes.

Note: During the incubation, prepare the required number of HPEA FT by labeling each HPEA FT with proper identification on the cap of each HPEA FT.

Note: Each specimen will need one HPEA FT, three collection tubes (CT) and one elution tube (1.5-mL microcentrifuge tube).

Note: During the incubation, label the required number of elution tubes (1.5-mL microcentrifuge tubes) with specimen identification information.

7. Add 500 µL isopropanol and mix lysate by inverting 3 to 5 times.
8. Transfer all of the lysate into the appropriately labeled **HPEA FT**.
9. Using table top centrifuge, centrifuge **HPEA FT** at 4,000 x g for 5 minutes.
10. After centrifugation, remove the **HPEA FT** from the 50-mL conical collection tube. Place the **HPEA FT** onto a **CT**. Remove the larger locking clip by twisting and pulling it away from the assembly.
11. Remove the smaller locking clip from underneath the filter tube (**FT**) cap by pushing it up so that the seal is broken on both sides of the cap and then pulling it away from the assembly.
12. Remove the **HPEA** from the **FT** by tilting the extender away from the cap side of the FT.
13. Discard the flow-through from the **HPEA FT** into chemical waste and properly dispose of the unit.
14. Label the filter cap appropriately.
15. Add 500 µL working **WB I** to each **FT**.

Note: Preparation of working WB I is described in the table in the Reagent preparation section.

16. Use benchtop microcentrifuge for the rest of the protocol.
17. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.
18. Place each **FT** onto a new **CT**. Discard the flow-through in each **CT** into chemical waste and properly dispose of old **CT**.
19. Add 500 µL working **WB II** to each **FT**.

Note: Preparation of working WB II is described in the table in the Reagent preparation section.

20. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.
21. Place each **FT** onto a new **CT**. Discard the flow-through from the old CT into chemical waste and properly dispose of the old **CT**.
22. Centrifuge **FT/CT** units at 16,000 x g – 20,000 x g for 1 minute to dry the filter membrane.
23. Place the **FT** onto an elution tube (1.5-mL RNase/DNase-free microcentrifuge tube) pre-labeled with specimen identification information. Discard any flow-through in each **CT** into chemical waste and properly dispose of the old **CT**.
24. Add 100 µL **DNA EB** to the center of the **FT** membrane without touching the **FT** membrane.
25. Incubate **FT** with elution tube at room temperature (RT: 15°C to 30°C) for 5 minutes.
26. Centrifuge FT with elution tube at 8,000 x g for 1 minute to collect eluate into the elution tube (pre-labeled 1.5-mL RNase/DNase-free microcentrifuge tube). The eluate is the DNA stock. Vortex eluate prior to use.
27. Discard the FT. Close the caps on the elution tubes.
28. DNA stock is ready for PCR tests after vortexing. Store DNA stock according to instructions in the **Processed sample storage and stability** section.

MagMAX Cell-Free DNA Isolation Kit

MagMAX™ Cell-Free DNA Isolation Kit is designed for isolation of circulating DNA from cell-free human plasma, serum, and urine samples. The kit uses Dynabeads MyOne SILANE technology and extraction chemistry, ensuring reproducible recovery of high-quality cell-free DNA (cfDNA) that is suitable for a broad range of applications, including sequencing, genotyping, and qPCR.

Kit contents and storage

Contents	Amount	Storage
MagMAX Cell-Free DNA Magnetic Beads	1.5 mL	2 – 8°C
MagMAX Cell-Free DNA Lysis/Binding Solution	125 mL	15 – 30°C
MagMAX Cell-Free DNA Wash Solution	100 mL	
MagMAX Cell-Free DNA Elution Solution	5 mL	

Prepare cell-free plasma samples

1. Centrifuge the blood samples at 2000 × g for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube.
3. Centrifuge the plasma samples at 16,000 × g for 10 minutes at 4°C.

Lyse the plasma samples (without PK) and bind the cfDNA to the beads

1. Prepare the Binding Solution/Beads Mix according to the following table and mix thoroughly

Reagents	Plasma volume			
	1 mL	2 mL	4mL	10 mL
MagMAX Cell Free DNA Lysis/Binding Solution	1.25 mL	2.5 mL	5 mL	12.5 mL
MagMAX Cell Free DNA Magnetic Beads	15 µL	30 µL	60 µL	150 µL
Total Volume	1.265 mL	2.53 mL	5.06 mL	12.65 mL

2. Add the appropriate volume of plasma sample.
3. Thoroughly mix the plasma sample and the Binding Solution/Beads Mix by swirling or by inverting the tube 10 times.
4. Shake vigorously for 10 minutes on a vortex with tube adaptor or the microtiter plate shaker (speed 7 or higher) to bind the cfDNA to the beads.
5. Place the tube on the appropriate DynaMag Magnet for 5 minutes or until the solution clears and the beads are pelleted against the magnet.
6. Carefully discard the supernatant with a pipette.
7. Keep the tube on the magnet for another minute and remove the residual supernatant with a pipette.

Wash with Wash Solution

1. Resuspend the beads in 1 mL of MagMAX Cell Free DNA Wash Solution.
2. Transfer the bead slurry to a new non-stick 1.5-mL microcentrifuge tube and save the lysis/binding tube.
3. Place the microcentrifuge tube containing the bead slurry on the DynaMag-2 Magnet for 20 seconds
4. Collect and use the supernatant of the bead slurry to rinse the saved lysis/binding microcentrifuge tube.

5. Transfer any residual beads to the tube containing the bead slurry and discard the lysis/binding tube.
6. Leave the tube on the DynaMag-2 Magnet for an additional 2 minutes, or until the solution clears and the beads are pelleted against the magnets.
7. Remove the supernatant with a 1-mL pipette.
8. Keeping the tube on the DynaMag-2 Magnet, tap the magnet stand on the benchtop 5 times, then remove any residual liquid with a 200-µL pipette.
9. Remove the tube from the DynaMag-2 Magnet, add 1 mL of MagMAX Cell Free DNA Wash Solution, then vortex for 30 seconds.
10. Place the tube on the DynaMag-2 Magnet for 2 minutes, or until the solution clears and the beads are pelleted against the magnets.
11. Remove the supernatant with a 1-mL pipette.
12. Keeping the tube on the DynaMag-2 Magnet, tap the magnet stand on the benchtop 5 times, then remove any residual liquid with a 200-µL pipette.

Wash twice with 80% ethanol

1. Remove the tube from the DynaMag-2 Magnet, add 1 mL of 80% ethanol, then vortex for 30 seconds.
2. Place the tube on the DynaMag-2 Magnet for 2 minutes, or until the solution clears and the beads are pelleted against the magnets.
3. Remove the supernatant with a 1-mL pipette
4. Keeping the tube on the DynaMag-2 Magnet, tap the magnet stand on the benchtop 5 times, then remove any residual liquid with a 200-µL pipette.
5. Repeat step 1–step 3 for a second wash with 80% ethanol.
6. Keeping the tube on the DynaMag-2 Magnet, air dry the beads for 3–5 minutes.
7. Keeping the tube on the DynaMag-2 Magnet, tap the magnet stand on the benchtop 5 times, then remove any residual liquid with a 200-µL pipette.

Elute the cfDNA

1. Add MagMAX Cell Free DNA Elution Solution to the tube according to the following table

Reagents	**Plasma volume**			
	1 mL	**2 mL**	**4 mL**	**10 mL**
MagMAX Cell Free DNA Elution Solution	15 µL	30 µL	50-60 µL	100-150 µL

2. Vortex for 5 minutes using a vortex adapter
3. Place the tube on the DynaMag-2 Magnet for 2 minutes, or until the solution clears and the beads are pelleted against the magnets.
   The supernatant contains the purified cfDNA.

Determination of DNA concentration and quality

To determine the concentration and purity of a solution containing DNA, the absorbance of an undiluted aliquot of 2 µl is measured at 260 nm and 280 nm in a spectrophotometer (**Eppendorf, 1mm Helma Lp cuvette**).

Concentration (ng / µl) = absorbance (260nm) x 50 x dilution factor

A double-stranded DNA sample with a concentration of 50 ng / µl at a wavelength of 260 nm has a specific absorbance of 1 for an optical path length of 10 mm.

An $A_{260/280}$ ratio of pure nucleic acids is 1.8-2.0.

However, amplifiable DNA samples extracted from formalin-fixed tissue and embedded in paraffin cannot be correctly quantified by UV spectrometry. The values calculated from the optical density measurement at 260 nm will substantially overestimate the actual concentration of DNA present. Only the use of appropriate fluorometric quantization platforms (eg Invitrogen Qubit) will allow reliable quantification of DNA in FFPE tissue.

In Vitro Amplification (real time-PCR) kit-Roche Diagnostics

All real-time PCR reagents are stored at -20°C and DNA at 4°C / -20°C.

For RT52-EGFR use the DNA concentration must be adjusted to 5-10ng / µl.

All steps until the start of the program in AB7500 are performed at 0-4°C (on ice or cold stand).

An ice PCR mix is prepared for each sample (calculated with a 5% surplus):

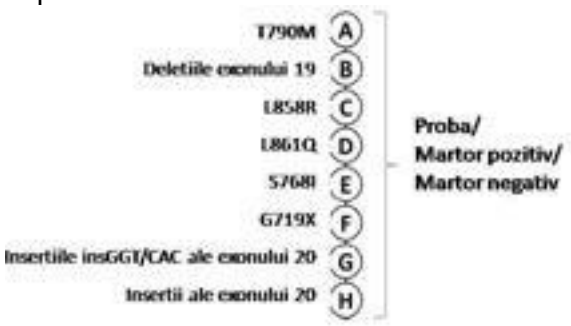
• 126µl Reaction Master Mix 2x

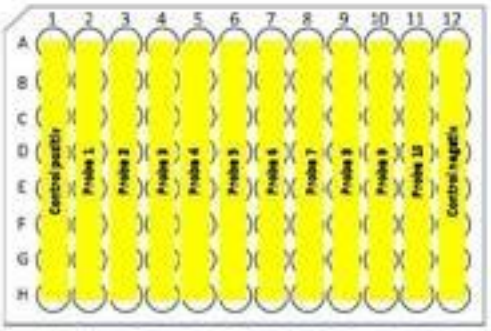
• 8.4 µl 10ng / µl DNA template

• 58.8 Nuclease-Free water.

The same mix is prepared for the positive control provided by the kit as well as for the negative control (Nuclease-Free water) to validate the amplification reaction.

The mixture is vortexed, centrifuged briefly and then distributed 23 µl per well in an A-H column for each sample.





Add 7 µl of each primer per sample mix, homogenize several times by pipetting.

After distributing the primers, the prepared plate is sealed with optical foil (in the case of strips it is covered with specific covers) and centrifuged briefly.

The amplifier board is inserted into the real time PCR AB7500.

Preparation of the amplification program:

1. To create the amplification program, create a new experiment: **Create a new experiment: File> New Experiment> Advanced Setup**

2. In the **Experiment Proprieties** section, name the amplification program, select the instrument type, **Quantitation-Comparative CT experiment**, **TaqMan® Reagents** and **Standard.**

3. Setting the parameters for amplification involves: defining the amplification targets (mutations) and the fluorescence channel on which the amplification is read: endogenous

control is read on the VIC channel and mutations on the FAM, samples are defined, **Passive Refer Dye** is selected as "**None**".

Setting the amplification thermal conditions:

• denaturation: 95°C, 10 minutes,

• 40 cycles: 95°C for 15s, 58°C for 30s.

4. Select the reading on the FAM and VIC channels at the hybridization and extension step at 58°C.

In Vitro Amplification (real time-PCR) TRUPCR® EGFR Kit

The TRUPCR® EGFR Kit is an in vitro diagnostic test intended for the qualitative detection of 32 somatic mutations in exons 18-21 (Table 1) of epidermal growth factor receptor (EGFR) gene from tumor tissue DNA (fresh, frozen or formalin fixed paraffin-embedded tissue) or liquid biopsy. Results are intended to aid the clinician in identifying patients with lung cancer who may benefit from treatment with EGFR tyrosine kinase inhibitors.

Table 1: Details of EGFR Detectable mutations:

EGFR Exon 18	**EGFR Exon 19**
• G719S (2155G>A)	• E746_A750del(2235_2249del15)
• G719C (2155G>T)	• E746_A750del(2236_2250del15)
• G719A (2156G>C)	• L747_P753>S(2240_2257del18)
EGFR Exon 20	• L747_A750>P(2239_2248TTAAGAGAAG>C)
• T790M (2369C>T)	• E746_S752>V (2237_2255>T)
• S768I (2303G>T)	• L747_T751del (2240_2254del15)
• C797S (2389T>A)	• L747_S752del (2239_2256del18)
• C797S (2390G>C)	• E746_T751>A (2237_2251del15)
• V769_D770insASV (2307_2308insGCCAGCGTG)	• L747_T751del (2239_2253del15)
	• L747_T751>P (2239_2251>C)
• D770_N771insG (2310_2311insGGT)	• L747_E749del (2239_2247del9)
• H773_V774insH (2319_2320insCAC)	• E746_E749del (2235_2246del12)
EGFR Exon 21	• L747_P753>Q (2239_2258>CA)
• L858R (2573T>G)	• L747_T751>S (2240_2251del12)
• L861Q (2582T>A)	• E746_S752>A (2237_2254del18)
	• L747_A750>P (2238_2248>GC)
	• E746_S752>D (2238_2255del18)
	• E746_T751>I(2235_2252>AAT)
	• L747_T751>Q(2238_2252>GCA)
	• E746_T751del (2236_2253del18)

The epidermal growth factor receptor (EGFR) is a family member of Receptor tyrosine kinases, expressed on the surface of epidermal cells and plays a central role in transmitting signals that promote cell growth and proliferation. Overexpression or overactivation of EGFR is linked to a number of cancers, including lung cancer, anal cancers and glioblastoma multiform. Additionally, excessive activation of EGFR has been shown to be associated with advanced stages of cancer and a poor prognosis. The TRUPCR® EGFR Kit detects most

prevalent mutations described to date in the EGFR gene, including T790M and C797S, the presence of which correlates with resistance to first, second and third line of tyrosine kinase inhibitors. Detecting somatic mutations in EGFR gene may provide a useful strategy to predict the response to the tyrosine kinase inhibitors in efforts to increase the survival rate of lung cancer patients receiving targeted therapy.

Principle

The TRUPCR® EGFR Kit is based on allele specific amplification and is achieved by ARMS PCR. Taq DNA polymerase is extremely effective at distinguishing between a match and a mismatch at the 3'-end of a PCR primer. Specific mutated sequences can be selectively amplified, even in samples where the majority of the sequences do not carry the mutation as:

- When the primer is fully matched, the amplification proceeds with full efficiency.
- When the 3'-base is mismatched, no efficient amplification occurs.

The kit is designed to selectively amplify mutant specific sequences in samples that contain a mixture of wild-type and mutated DNA. In real-time PCR, the fluorescent signal is generated from the presence of an oligonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5' end and a quencher molecule on its 3' end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Fluorescence resonance energy transfer (FRET). The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. As a result, the fluorescent dye and quencher dye are separated, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of DNA copies in a sample and its value allows qualitative comparisons of analyzed and control samples. The detection is achieved in multiplex reaction using fluorescent probes labelled with FAM, VIC /HEX and ROX/TEXAS RED. The TRUPCR® EGFR Kit comprises of five separate PCR amplification reaction mixes: where mutation-specific reactions (exons 18, 19, 20, and 21 of the EGFR gene) and a reference (wild type control in exon 2 without any known polymorphism/mutation) is amplified simultaneously in each tube.

LIST OF DETECTABLE MUTATIONS BY TRUPCR® EGFR KIT

No	Mutation	Nucleotide Change Detection	Remarks
1	EGFR G719x	G719S (2155G>A)	It detects 3 mutations but does not distinguish between them
		G719C (2156G>T)	
		G719A (2156G>C)	
2	EGFR T790M	T790M (2369C>T)	
3	EGFR S768I	S768I (2303G>T)	
4	EGFR ex20ins	V769_D770insASV (2307_2308insGCCAGCGTG)	It detects 3 mutations but does not distinguish between them
		D770_N771insG (2310_2311insGGT)	
		H773_V774insH (2319_2320insCAC)	
5	EGFR C797S	C797S (2390G>C)	It detects 2 mutations but does not distinguish between them
		C797S (2389T>A)	
6	EGFR L858R	L858R (2573T>G)	
7	EGFR L861Q	L861Q (2582T>A)	
8	EGFR 19-Del (Deletion/Insertion)	E746_A750del(2235_2249del15)	It detects 20 mutations but does not distinguish between them.
		E746_A750del(2236_2250del15)	
		L747_P753>S(2240_2257del18)	
		L747_A750>P(2239_2248TTAAGAGAAG>C)	
		E746_S752>V (2237_2255>T)	
		L747_T751del (2240_2254del15)	
		L747_S752del (2239_2254del18)	
		E746_T751>A (2237_2251del15)	
		L747_T751del (2239_2253del15)	
		L747_T751>P (2239_2251>C)	
		L747_E749del (2245_2246del12)	
		L747_P753>Q (2239_2258>CA)	
		L747_T751>S (2240_2251delL2)	
		E746_S752>A (2237_2254del18)	
		L747_A750>P (2248_2248>GC)	
		E746_S752>D (2238_2255del18)	
		E746_T751>I(2245_2252>AAT)	
		L747_T751>Q(2248_2252>GCA)	
		E746_T751del (2246_2254del18)	
9	EGFR Reference	Detects EGFR region without any polymorphism/mutation.	

The Kit contains amplification reagents for performance of 24/48 amplification reactions. Thaw and handle reagents on ice. Do not freeze/thaw Kit vials repeatedly. In case of frequent use, we recommend to aliquot the contents of the vials into 10 reactions each. This will also rule-out kit/ reagent contamination.

Reagent	Description	Volume in µL 24 reactions	Volume in µL 48 reactions
Multiplex Master Mix	Mix for Real time PCR	600 µL x 2	600 µL X 4
EGFR PPM 1	Primer and probe mix for 19-Del Mutation and EGFR Reference detection	120 µL x 1	120 µL x 2
EGFR PPM 2	Primer and probe mix for T790M Mutation and EGFR Reference detection	120 µL x 1	120 µL x 2
EGFR PPM 3	Primer and probe mix for S768I, G719x Mutation and EGFR Reference detection	120 µL x 1	120 µL x 2
EGFR PPM 4	Primer and probe mix for L858R, C797S Mutation and EGFR Reference detection	120 µL x 1	120 µL x 2
EGFR PPM 5	Primer and probe mix for ex20ins, L861Q Mutation and EGFR Reference detection	120 µL x 1	120 µL x 2
EGFR Negative Control	Nuclease free Water	1500 µL x 1	1500 µL x 2
EGFR Positive Control	Mix of all mutation Positive control	250 µL x 1	250 µL x 2

PCR PROTOCOL FOR APPLIED BIOSYSTEM 7500 SERIES, AND QUANTSTUDIO SERIES REAL-TIME PCR SYSTEMS

1. REACTION PREPARATION:

a) Thaw Multiplex Master Mix and all mutation Primer Probes mixes to ambient temperature (15- 25°C).

b) Vortex all 5 Primer Probe Mixes.

Note: Do not vortex Multiplex Master Mix instead mix the content by gentle tapping.

c) Briefly centrifuge the Multiplex Master Mix and Mutation Primer Probes mixes to collect entire volume at the bottom of the tube.

d) Prepare the reaction mix as follows:

Note: Each sample must be amplified separately in 5 Tubes.

Reagent	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Multiplex Master Mix	10 µl	10 µl	10 µl	10 µl	10 µl
EGFR PPM 1	5 µl	-	-	-	-
EGFR PPM 2	-	5 µl	-	-	-
EGFR PPM 3	-	-	5 µl	-	-
EGFR PPM 4	-	-	-	5 µl	-
EGFR PPM 5	-	-	-	-	5 µl
Total	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**

e) Mix the above prepared Reaction Mix by gentle tapping/pipetting.

Note: Avoid vortexing the reaction Mix.

f) Transfer 15 µl of the above prepared Reaction Mix in 0.2 ml PCR tubes/Strip tube and close the tubes.

g) Add 5 µl Nuclease free Water in all five Non template Control (NTC) tubes.

h) Add 5ng (Fluorometer) or 15ng (Spectrophotometer) (maximum sample volume 5µl*) of each DNA to the sample tubes and makeup the volume to 20 µl by Nuclease free water (EGFR Negative control).

i) Add 5 µl of EGFR Positive Control (PC) in all five PC tubes.

j) Mix the reaction by pipetting up and down.

k) Centrifuge the tube/Strips to collect entire volume at the bottom of the tube.

l) Place the PCR tubes/Strip tubes in real time Instrument.

m) Follow the Real time PCR conditions as described under the heading program set up

* In case of low DNA yield add upto 7 µl of DNA per tube, so in the case total reaction volume will be 22 µl

2. PROGRAM SET-UP

Define the following setting for Temperature Profile and Dye Acquisition

Step	Temperature, °C	Time	Dye Acquisition	Cycles
1	94	10 min	-	1
2	94	15 sec	-	10
	68	30 sec	-	
3	94	15 sec	-	40
	60	60 sec	YES	

Passive Reference Dye – None

3. CHANNEL SELECTION

Define the following setting for channel selection

Tube No.	Target	Reporter			Quencher
		FAM	VIC/HEX	ROX/TEXAS RED	
1	19-Del/Reference	19-Del	Reference	-	None
2	T790M/Reference	T790M	Reference	-	None
3	S768I / G719x/ Reference	S768I	Reference	G719x	None
4	C797S /L858R/ Reference	C797S	Reference	L858R	None
5	ex20ins/ L861Q/ Reference	ex20ins	Reference	L861Q	None

4. MUTATION ANALYSIS

The PCR cycle at which the fluorescence from a particular reaction crosses a threshold value is defined as the threshold cycle value (Ct value). Ct values indicate the quantity of specific input DNA. Low Ct values indicate higher input DNA levels and high Ct values indicate lower input DNA levels.

a. THRESHOLD: For Applied Biosystem instruments the recommended threshold range for FAM and ROX/TEXAS RED channel is 10000 to 50000, and for VIC/HEX channel range is 3000 to 15000. However absolute value varies from instrument to instrument depending upon instrument's age, model and calibration. Please get in touch with our tech-support team for initial threshold setting.

b. EGFR REFERENCE ANALYSIS (FOR DNA ASSESSMENT)

	VIC/HEX	Results
Reference in DNA Samples	12≤ Ct ≤ 26	Proceed with the analysis of the sample
	Ct<12	Excess amount of DNA is present. Samples must be diluted with Nuclease free Water so that Ct values falls in the range indicated above.
	Ct>26	If Sample is positive (if its delta Ct is less than or equal to the cutoff value) report it as positive.
		If sample is negative (if its delta Ct is more than the cutoff value) Proceed with a new DNA extraction to obtain higher concentration of DNA template or DNA of higher quality.

c. MUTATION ANALYSIS: If reference in each tube passed as per above criteria then analyse the samples and note down the threshold cycle (Ct) for Reference and mutation in each tube. Calculate ΔCt as per given formula

ΔCt = Ct Mutation* – Ct Reference

*If there is no amplification in mutation tubes consider the Ct as the last cycle i.e., 40.

To identify the sample status follow table given below

Compare ΔCt values of the samples with those reported in the below table. Samples are classed as mutation positive when the ΔCt is less than or equal to the cutoff ΔCt value of the assay. Above this value, the sample may either contain less than the percentage of mutation able to be detected by the TRUPCR® EGFR KIT (beyond the limit of the assays), or the sample is wild type.

Cutoff ΔCt value of the mutations

Tube No.	Target	FAM	ROX
1	19-Del	12	-
2	T790M	9	-
3	S768I / G719x	11	11
4	C797S /L858R	11.8	13.5
5	ex20ins/ L861Q	13	14

Note: *Above Delta Ct values are valid only if 5ng (Fluorometer) or 15ng (Spectrophotometer) of purified genomic DNA per well is used

GENERAL RECOMMENDATIONS FOR ANALYSIS OF THE SAMPLE

1. Mutation positive: If ΔCt less than or equal to the cutoff ΔCt value for that assay.

2. Mutation Negative: If ΔCt more than the cutoff ΔCt value for that assay, the sample may either contain less than the percentage of mutation able to be detected by TRUPCR® EGFR Kit (beyond the limit of the assays), or the sample is mutation negative.

3. A tumor may contain more than one mutation. In such instances, more than one mutation will be reported.

NOTE

1. The users must be trained and familiar with real time PCR technology prior to the use of this kit.

2. Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings.

3. It is the user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by the TRUPCR performance studies.

4. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

In Vitro Amplification (real time-PCR) castPCR method

Prepare the PCR mix and the PCR plate (Mutation detection experiments)

In a mutation detection experiment, run the test sample with a mutant allele assay(s) and corresponding gene reference assay. Technical replicates are not required. The amount of test sample gDNA used should be 20 ng. The volume of gDNA sample should not be greater than 20% of the total reaction volume. Prepare the PCR mix and the PCR plate as follows:

1. For each sample, calculate the total number of reactions required.
2. Calculate the total volume required for each reaction component:

volume for 1 reaction × total no. of reactions + 10%

Component	Volume for 1 reaction
	20- µL reaction (96-well plate)
TaqMan Genotyping Master Mix, 2X	10 µL
Prepared gDNA sample	2-4 µL
Nuclease-free water	Add water to 18.0 µL
Total volume of super mix	18 µL

3. Label a 1.5-mL microcentrifuge tube, add all components to the labeled tube, cap the tube, then vortex the tube briefly to mix the components.
4. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.
5. Add 18 µL volume of super mix to each tube:
6. Add 2 µL volume of TaqMan Mutation Detection Assay (mutant allele or gene reference assay) to each tube.
7. Close the caps
8. Centrifuge the tubes briefly to spin down the contents and eliminate air bubbles.

Set up the plate document or experiment and start the run

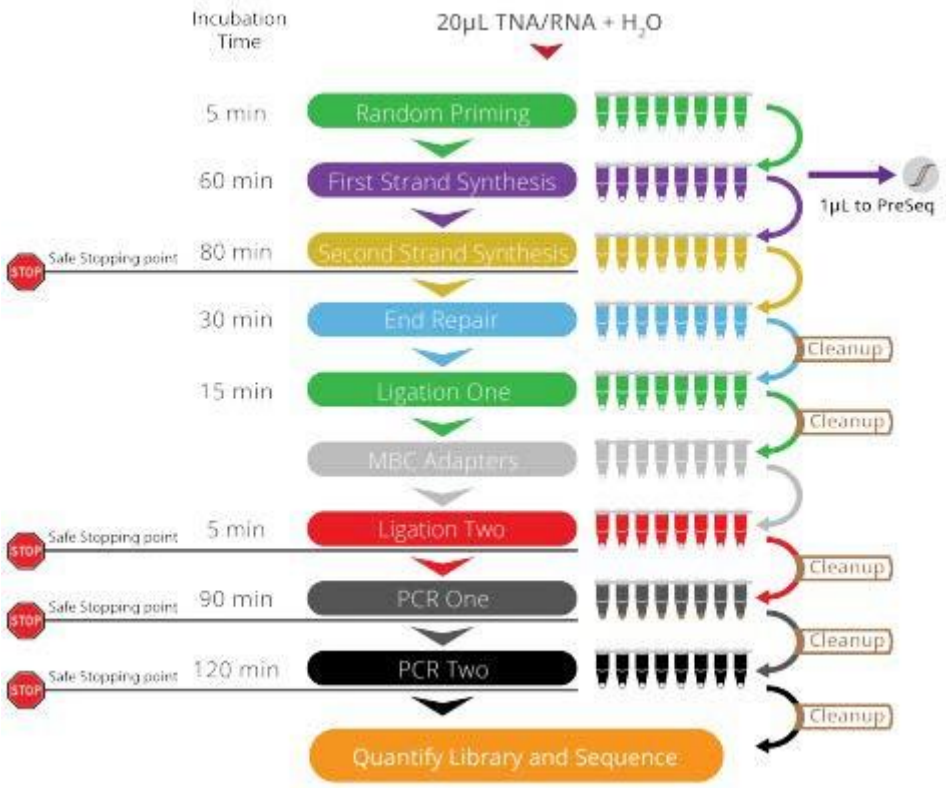
In the real-time PCR system software:

1. Select the experiment type: **Absolute Quantitation or Quantitation - Standard** Curve.
2. For each tube that contains a reaction, apply a sample name, assay name, and target or detector name.
3. Set the following thermal-cycling conditions
   - Run mode – **Standard**
   - Sample volume – 20 µL (96-well plates)
   - Thermal-cycling profile – See the table below

Stage	Temp.	Time (mm:ss)	Cycles	Data collection
1	95°C	10:00	1	No
2	92°C	00:15	5	No
	58°C	01:00		No
3	92°C	00:15	40	No
	60°C	01:00		FAM

4. Load the reaction plate into the real-time PCR instrument, then start the run.

NGS Archer FusionPlex – working protocol



Important Precautions

• Archer reagents are provided as individually lyophilized reaction pellets in 0.2mL PCR tube strips.
• Allow pouches to reach room temperature (20°C to 25°C) before opening in order to prevent moisture condensation on tubes.
• Always centrifuge tubes briefly before opening to pull contents down.
• Detach the required number of reaction tubes using a clean razor blade and return any unused portion to the pouch with desiccant packet, reseal and store at 2°C to 8°C. It is recommended to use the remaining reactions within 4 weeks after opening.
• For MBC Adapters and Second PCR tubes remember to label prior to returning unused portions to storage.

Dissolve, mix and spin down:

o Never touch the lyosphere with the pipette tip.
o Add sample/reagents to pellet in tubes while on ice.
o Allow at least 5 seconds for pellets to dissolve.
o Pipette up and down 8 times to mix after the lyosphere has dissolved.
o Briefly centrifuge and return to ice before proceeding.

Input Nucleic Acid

• Input nucleic acid (TNA or RNA) in *EDTA-free* buffer or Ultra-Pure Water is the optimal starting template for Archer AMP Library Preparation. Do not use EDTA-containing buffers.
Note: Some FusionPlex panels contain gene-specific primers designed for sample tracking that target non-expressed DNA sequence (intronic or intergenic). When using SEX_ID and SNP_ID-based sample tracking, TNA should be used as the starting template.

• Use the maximum allowable input mass (ng) whenever possible. Higher input quantities enable more sensitive variant and fusion detection: 20 – 250ng of RNA for FusionPlex (somatic mutation detection)
• If using total nucleic acid (TNA), DO NOT pretreat with DNase. DNA found in total nucleic acid can act as an internal control, verifying assay performance in the absence of RNA.
• If using FFPE sample types, is recommend extracting TNA using Promega ReliaPrep, Agencourt FormaPure or Promega Maxwell RSC RNA FFPE Kit with the following modifications to the published manufacturer protocol:

Promega ReliaPrep™:
o After step 6B Sample Lysis 5, incubate for 1 hour at 80°C
o At step 7 Column Washing and Elution 9, elute in a minimal elution volume of 40µL using Ultra Pure Water (SA0213)
o Do not use water baths

Agencourt® FormaPure®:
o After step 5, incubate for 1 hour at 80°C
o At step 23, elute in a minimal elution volume of 40µL using Ultra-Pure Water (SA0213)
o Do not use water baths

Promega Maxwell® RSC RNA FFPE Kit:
o Skip DNase I preparation (Optional)
o Skip DNase I treatment of samples (Optional)

Reagents to Prepare Before Starting
• Make fresh 10mM Tris-HCl pH 8.0 by mixing 30µL 500mM Tris-HCl, pH 8.0 (SA0020) with 1470µL Ultra-Pure Water (SA0213).
  o 10mM Tris-HCl pH 8.0 is appropriate for use for up to one week after mixing
• Make fresh 70% ethanol by adding 14mL 100% ethanol to the bottle labeled Ultra-Pure Water for Ethanol Dilution (SA0022).
  o 70% ethanol is appropriate for use for up to one week after mixing
  o Tightly close the bottle cap to minimize evaporation when not in use
• Make fresh 5mM NaOH
  o If working from 1M NaOH, add 5µL of 1M NaOH to 995µL of Ultra Pure Water to yield 5mM final NaOH
  o If working from 5M, add 10µL of 5M NaOH to 990µL of Ultra Pure Water to yield 50mM NaOH. Mix well and briefly spin down. Take 100µL of 50mM NaOH and combine with 900µL of Ultra Pure Water to yield 5mM NaOH. Mix well and briefly spin down.

THERMAL CYCLER PROTOCOLS

Random Priming 2.0	**Step**	**Temperature (ºC)**	**Time (min)**	
	1	65	5	
	2	4	Hold	

First Strand cDNA Synthesis	Step	Temperature (ºC)	Time (min)	
	1	25	10	
	2	42	30	
	3	80	20	
	4	4	Hold	

Second Strand cDNA Synthesis	Step	Temperature (ºC)	Time (min)	
	1	16	60	
	2	75	20	
	3	4	Hold	

End Repair	Step	Temperature (ºC)	Time (min)	
	1	25	30	
	2	4	Hold	

Ligation Step 1	Step	Temperature (ºC)	Time (min)	
	1	37	15	
	2	4	Hold	

Ligation Step 2	Step	Temperature (ºC)	Time (min)	
	1	22	5	
	2	4	Hold	

First PCR Reaction	Step	Temperature (°C)	Time	Cycles
	1	95	3 min	1
	2	95	30 sec	15*
	3	65*	5* min (100% ramp rate)	
	4	72	3 min	1
	5	4	Hold	1

Second PCR Reaction	**Step**	**Temperature (°C)**	**Time**	**Cycles**
	1	95	3 min	1
	2	95	30 sec	20**
	3	65*	5* min (100% ramp rate)	
	4	72	3 min	1
	5	4	Hold	1
*Refer to product insert for panel-specific parameters				
** If you regularly experience library yields higher than 200nM you can decrease cycle number				

Molecular Barcoding, Sample Indexing, and Multiplexed Sequencing

Molecule-level barcoding (or unique molecule identifier tagging) and sample-level barcoding (also known as index tagging) are both incorporated during Archer MBC ligation. Molecular barcodes are an integral component of the Archer Analysis software suite, (visit https://archerdx.com/technology-platform/analysis/ for details). Sample barcodes (i.e. index tags) allow pooled libraries to be sequenced simultaneously thereby enabling maximum sequencing throughput and data demultiplexing during downstream bioinformatics analysis.

Sample Multiplexing

1) In order to efficiently utilize the throughput of the MiSeq (or other Illumina sequencing platform) as well as prevent low index diversity within your sequencing run, multiple samples should be sequenced simultaneously. Samples can be identified through a combination of two unique nucleotide sequences (see below for more details), which are subsequently read during the sequencing process. The unique nucleotide sequence is often termed an “index”.
2) The FusionPlex reagents for Illumina utilize a combination of two indices to distinguish between samples. Index 2 is added during Ligation Step 2 and is embedded in the Archer MBC Adapters for Illumina (p5/i5 index). Index 1 is added in Second PCR and is embedded in MiSeq Index 1 Primers (p7/i7) within the Second PCR reaction pellets.
3) In order to maintain appropriate coverage depth, it is recommended that users determine the maximum number of samples that can be run on a MiSeq flow cell (assuming 12 million reads per run using MiSeq reagents v2 and 25 million reads per run using MiSeq reagents v3). In general, larger panels with more targets will require higher sequencing coverage depth and should be run with fewer samples.

Barcode Diversity

1) The Illumina sequencers will work best when index diversity within a run is high. For example, if eight samples are included in a run, and the user chooses to use only one MBC Adapter paired with eight different MiSeq Index 1 Primers, the run may fail due to low

barcode diversity. In this example it is best to use eight different Archer MBC Adapters paired with eight different MiSeq Index 1 Primers.
2) If using more than 48 MBCs, refer to http://archerdx.com/mbc-adapters for adapter compatibility

Step 1: Random Priming 2.0

1) Pre-heat the thermal cycler to 65°C with heated lid option on.
2) Place an appropriate number of Random Priming 2.0 (SA0194) reaction tubes on ice.
3) Combine the appropriate amounts of Ultra-Pure Water (SA0213) and purified total nucleic acid or RNA (20-250ng) in new PCR tubes.

Component	**Reaction Mix**
Ultra-Pure Water (SA0213)	20 – X µL
Purified Total Nucleic Acid or RNA	X µL
(Total)	(20 µL)

4. Transfer **20**µL TNA/Water mix to the Random Priming 2.0 reaction tubes.
a) Dissolve, mix and spin down (see **Working with Lyophilized Reaction Pellets** section above)
b) Return tubes to ice
5) After the program has reached 65°C, transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:
a) Use a heated lid (≥100°C)
b) Place samples in the thermal cycler, close the lid and start program
c) After the program has reached 4°C, place tubes on ice for at least 2 minutes

Random Priming 2.0 Thermal Cycler Protocol

Step	**Temperature (°C)**	**Time (min)**
1	65	5
2	4	Hold

Step 2: First Strand cDNA Synthesis

Place an appropriate number of First Strand cDNA Synthesis (SA0002) reaction tubes on ice.
2) Spin down the Random Priming 2.0 mixture and transfer **20**µL to the First Strand cDNA Synthesis tube(s).
a) Dissolve, mix and spin down
b) Return tubes to ice
3) Transfer reactions to a thermal cycler and initiate an incubation using the following program
and guidelines:
a) Use a heated lid (≥100°C)

First Strand cDNA Synthesis Thermal Cycler Protocol

Step	**Temperature (°C)**	**Time (min)**
1	25	10
2	42	30
3	80	20

4	4	Hold

b) After the program has reached 4ºC, briefly spin down reactions and place on ice.
4) Make diluted cDNA samples for PreSeq RNA QC assay.
a) For each sample, pipette **9**µL of Ultra-Pure Water (SA0213) into a new PCR tube.
b) Pipette **1**µL of each First Strand cDNA Synthesis reaction into the water and pipette up and down to mix.
c) Keep on ice for use in Step 4 (PreSeq RNA QC assay).

Step 3: Second Strand cDNA Synthesis

1) Place an appropriate number of Second Strand cDNA Synthesis (SA0003) reaction tubes on
ice.
2) Add **21**µL of Ultra-Pure Water (SA0213) to each tube containing the **19**µL of First Strand cDNA Synthesis reaction.
a) Pipette up and down to mix
3) Pipette **40**µL of each First Strand reaction into the Second Strand cDNA Synthesis tubes.
a) Dissolve, mix and spin down
b) Return tubes to ice
4) Transfer reactions to a thermal cycler and initiate an incubation using the following program
and guidelines:

a) Use a heated lid (≥100°C)

Second Strand cDNA Synthesis

Step	Temperature (°C)	Time (min)
1	16	60
2	75	20
3	4	Hold

b) Start the program and pause once block has reached 16ºC
c) Place samples in the thermal cycler; close the lid and resume program
d) While sample(s) are incubating, proceed to RNA PreSeq QC assay (step 4)
e) After the program has reached 4ºC, briefly spin down reactions and place on ice

Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C. It is recommended to review the qPCR results from the PreSeq RNA QC assay at this time to determine predicted sample success.

Step 4: PreSeq RNA QC Assay

1) Thaw 10X VCP Primer Mix (SA0126) at room temperature.
2) Prepare sufficient qPCR reaction mix for
a) Duplicate reactions of each diluted cDNA sample
b) One No Template Control (NTC) made from **10**µL Ultra-Pure Water (SA0213)

Component	Part Number	Reaction Mix (n=1)	Reaction Master Mix (n=20)
iTaq SYBR Green Supermix	Not Supplied	5µL	100µL
10X VCP Primer Mix	SA0126	1µL	20µL

Diluted cDNA sample or NTC	-	4µL	-
(Total)	(10µL)	(120µL)	

`

3) Pipette 6µL of the reaction mix into each assigned well of a qPCR plate/tube.
4) Pipette 4µL of the diluted cDNA samples or NTC into assigned wells/tubes containing reaction mix.
a) Mix slowly to avoid introducing bubbles, cap or seal the reactions and spin down
5) Transfer reactions to a thermal cycler and initiate a run using the following program:

PreSeq RNA QC Assay qPCR Instrument Protocol

Step	Temperature (°C)	Time (Sec)	Cycles
Activation	95	20 (20*)	1
Denaturation	95	3 (15*)	35
Primer Annealing & Extension	60	30 (60*)	
Melt-curve gradient	60-95	0.5°C/sec increment	1

*Times in ( ) are for standard cycling.

Step 5: End Repair

1) Place an appropriate number of End Repair (SA0204) reaction tubes on ice.
2) Pipette **40**µL of the Second Strand cDNA Synthesis product into the End Repair tubes.
a) Dissolve, mix and spin down
b) Return tubes to ice
3) Transfer reactions to a thermal cycler and initiate an incubation using the following program
and guidelines:
a) Heated lid off

End Repair Thermal Cycler Protocol

Step	Temperature (°C)	Time (min)
1	25	30
2	4	Hold

b) Place samples in the thermal cycler; close the lid and start program
c) When the run has completed, briefly spin down reactions and place on ice.

Reaction Cleanup after End Repair

1) Completely resuspend AMPure Beads by vortexing.
2) Add **2.5**X volume (**100**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure a
homogenous mixture.
4) Incubate for **5** minutes at room temperature (20°C to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes **or until beads are fully pelleted** against the tube wall.
7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If

the pellet becomes dislodged from the magnet and a portion is drawn into the pipette tip, return contents to the tube and repeat magnet incubation step).
8) With tubes still on the magnet, add **200**µL of 70% ethanol.

9) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tubes.
10) Remove and discard the supernatant.
11) Repeat steps 8-10 for a total of **two washes** in 70% ethanol.
12) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant
residue and allow tubes to dry for **5** minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
13) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes.

Step 6: Ligation Step 1

1) Place an appropriate number of Ligation Step 1 (SA0196) reaction tubes on ice.
2) Transfer **20**µL of purified DNA from Reaction Cleanup after End Repair step 14 into Ligation
Step 1 tubes. (It is acceptable for a small amount of AMPure beads to be transferred).
a) Dissolve, mix and spin down
b) Return tubes to ice
3) Transfer reactions to a thermal cycler and initiate an incubation using the following program
and guidelines:
a) Use a heated lid (≥100°C)

Ligation Step 1 Thermal Cycler Protocol

Step	Temperature (°C)	Time (min)
1	37	15
2	4	Hold

b) After the program has reached 4ºC, briefly spin down reactions and place on ice.

Reaction Cleanup after Ligation Step 1

1) Completely resuspend AMPure Beads by vortexing.
2) Add **2.5**X volume (**50**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure
even mixing.
4) Incubate for **5** minutes at room temperature (20°C to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes **or until beads are fully pelleted** against the tube wall.
7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipette tip, return contents to the tube and repeat magnet incubation step).
8) With tubes still on the magnet, add **200**µL of 70% ethanol.

9) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tubes.
10) Remove and discard the supernatant.
11) Repeat steps 8-10 for a total of **two washes** in 70% ethanol.
12) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant
residue and allow tubes to dry for **5** minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
13) Elute DNA by resuspending beads in **42**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes.

Step 7: MBC Adapter Incorporation

1) Label MBC Adapter tubes with the sample index tag letter (A, B, or C) and number (1-48) from the MBC Adapters pouch label
a) Use a permanent laboratory marker and orient lid hinges to the back as illustrated:
***Important* As this step incorporates your index tag for sample-level tracking, be sure to**
record which MBC adapter is being used for each sample. Unused tubes must be labeled before returning to the pouch.
2) Place an appropriate number of MBC Adapter reaction tubes on ice.
3) Add **40**µL of the purified cDNA sample from Reaction Cleanup after Ligation Step 1, Step 6.
Avoid pipetting AMPure beads into this reaction. If minute amounts of AMPure beads were carried over, simply place MBC Adapter tubes on magnet for one minute and transfer all liquid to the next tubes while MBC Adapter tubes remain on the magnet.
a) Dissolve, mix and spin down.
4) Immediately proceed to Step 8 Ligation Step 2.

Step 8: Ligation Step 2

1) Place an appropriate number of Ligation Step 2 (SA0197) reaction tubes on ice.
2) Transfer the entire volume of each purified DNA sample from Step 7 MBC Adapters to Ligation Step 2 tubes.
a) Dissolve, mix and spin down
b) Return tubes to ice

3) Transfer reactions to a thermal cycler and initiate an incubation using the following program
and guidelines:
a) Heated lid off

Ligation Step 2 Thermal Cycler Protocol

Step	Temperature (°C)	Time (min)
1	22	5
2	4	Hold

b) After the program has reached 4ºC, briefly spin down reactions and place on ice.
Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C.

Reaction Cleanup after Ligation Step 2

Prepare Ligation Cleanup Beads:

1) Completely resuspend Ligation Cleanup Beads (SA0210) by vortexing.
2) For each reaction, pipette **50**µL of Ligation Cleanup Beads into new 0.2mL PCR tubes.
3) Place tube(s) on the magnet for 1 minute or until the beads are pelleted.
4) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipette tip, return contents to the tube and repeat magnetic pelleting step).
5) Pipette **50**µL of Ligation Cleanup Buffer (SA0209) into each tube to resuspend beads.

Ligation Cleanup Procedure:

Caution: Vortex PCR tubes with fingers firmly placed on all lids as the detergent may compromise sealing of lids.

1) Pipette the entire volume of Ligation Step 2 reaction into the tubes with Ligation Cleanup Beads and Buffer.
2) Mix samples by vortexing.
3) Incubate reactions at room temperature for **5** minutes.
4) Mix samples by vortexing.
5) Incubate reactions at room temperature for **5** minutes.
6) Briefly spin down tubes.
7) Place tubes on the magnet for **1** minute **or until beads are fully pelleted** against the tube wall.
8) Carefully pipette off and discard supernatant (**100**µL) without disturbing the beads.
9) Wash beads **two times** with Ligation Cleanup Buffer.
a) Resuspend beads in **200**µL Ligation Cleanup Buffer by vortexing, briefly spin down, and place back on magnet for **1** minute.
b) Once slurry has cleared, discard supernatant.
10) Wash beads once with Ultra-Pure Water (SA0213):
a) Resuspend beads in 200µL of Ultra-Pure Water by vortexing, briefly spin down and place back on magnet.
b) Once slurry has cleared discard supernatant.

c) Take care to ensure that all supernatant has been removed from beads.
11) Elute DNA from ligation cleanup beads:
a) Resuspend ligation cleanup beads in 18µL of 5mM NaOH.
b) Transfer beads to thermal cycler and incubate at 75°C for 10 minutes then cool to 4°C.
i) Use a heated lid
c) After sample has reached 4°C, briefly spin down and transfer to the magnet.

Step 9: First PCR

1) Place an appropriate number of First PCR (SA0109) reaction tubes on ice.
a) Label tubes by sample number
2) Pipette **2**µL of **GSP1** into each First PCR tube.
a) Spin down and return tubes to ice
3) Pipette **18**µL of supernatant from Step 11 above into appropriately labeled First PCR tube.
a) Dissolve, mix and spin down
b) Return tubes to ice
4) Transfer reactions to a thermal cycler and immediately initiate a run using the following program and guidelines:
a) Use a heated lid (≥100°C)

First PCR Reaction Thermal Cycler Protocol

Step	Temperature (°C)	Time	Cycles
1	95	3 min	1
2	95	30 sec	15*
3	65*	5* min (100% ramp rate)	
4	72	3 min	1
5	4	Hold	1

**Refer to product insert for panel-specific parameters.*

b) After the program has reached 4ºC, briefly spin down reactions and place on ice. It is also acceptable to leave tubes in the thermal cycler at 4°C overnight.

Reaction Cleanup after First PCR

See Important Precautions section above for guidance on working with AMPure XP beads.

1) Completely resuspend AMPure Beads by vortexing.
2) Add **1.2**X volume (**24**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure a
homogenous mixture.
4) Incubate for **5** minutes at room temperature (20°C to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes **or until beads are fully pelleted** against the tube wall.

7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipette tip, return contents to the tube and repeat magnet incubation step).
8) With tubes still on the magnet, add **200**µL of 70% ethanol.
9) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tube(s).
10) Remove and discard supernatant.
11) Repeat steps 8-10 for a total of **two washes** in 70% ethanol.
12) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant
residue and allow tubes to dry for **3** minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
13) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes.
15) Transfer **20**µL of purified solution to a new 0.2mL PCR tube and store reactions as indicated
below or proceed directly to Step 10: Second PCR
Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C

Step 10: Second PCR

1) Place an appropriate number of Second PCR (SA0110) reaction tubes on ice.
Important: **The Index 1 (P7) index tag is incorporated during this step.**
a) Use a permanent marker to label the tubes 1 to 8 from left to right as shown below. (See Molecular Barcoding, Sample Indexing & Multiplexed Sequencing in the Before Getting Started section).
b) Unused tubes must be labeled before returning to the pouch.

Index 1 (P7) sequence table

Sample Number	Illumina Index 1 P7/i7 Sequence
1	TAAGGCGA
2	CGTACTAG
3	AGGCAGAA
4	TCCTGAGC
5	GGACTCCT
6	TAGGCATG
7	CTCTCTAC
8	CAGAGAGG

2) Pipette **2**µL of GSP2 into each Second PCR tube.

a) Record which P7 index sequence is used with which sample.
3) Pipette **18**µL of First PCR cleanup elution into each Second PCR tube.
a) Dissolve, mix and spin down
b) Return tubes to ice
4) Transfer reactions to a thermal cycler and initiate a run using the following program and guidelines:
a) Use a heated lid (≥100°C)

Second PCR Reaction Thermal Cycler Protocol

Step	Temperature (°C)	Time	Cycles
1	95	3 min	1
2	95	30 sec	20**
3	65*	5* min (100% ramp rate)	
4	72	3 min	1
5	4	Hold	1

**Refer to product insert for panel-specific parameters.*
*** If you regularly experience library yields higher than 200nM you can decrease cycle number*
b) After the program has reached 4ºC, briefly spin down reactions and place on ice. It is also acceptable to leave tubes in the thermal cycler at 4°C overnight.

Reaction Cleanup after Second PCR

See Important Precautions section above for guidance on working with AMPure XP beads.
1) Completely resuspend AMPure Beads by vortexing.
2) Add **1.2**X volume (**24**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure a
homogenous mixture.
4) Incubate for **5** minutes at room temperature (20ºC to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes **or until beads are fully pelleted** against the tube wall.
7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipette tip, return contents to the tube and repeat magnet incubation step).

8) With tubes still on the magnet, add **200**µL of 70% ethanol.
9) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tube(s).
10) Remove and discard the supernatant.
11) Repeat steps 8-10 for a total of **two washes** in 70% ethanol.
12) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant
residue and allow tubes to dry for 5 minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
13) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes.

15) Transfer **18**µL of the purified solution to a new 0.2mL PCR tube. Stop or proceed directly to
Quantify, Normalize and Sequence.
a) Be sure to avoid transferring beads to the fresh tube.
Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C.

Quantify, Normalize, and Sequence

Quantify

1) Quantify the concentration of each library using the KAPA Universal Library Quantification Kit
a) The recommended average fragment length of FusionPlex libraries for the sizeadjustment calculation is 200bp.
b) Archer libraries are very concentrated. You will need to dilute libraries 1:10,000-1:250,000 for quantification with KAPA qPCR.

Normalize

1) After quantification, pool libraries at equimolar concentrations and load the sequencer according to manufacturer instructions. For reference sample sheets and additional recommendations, visit our website at http://archerdx.com/mbc-adapters and http://archerdx.com/support/faqs.

Sequence

1) Loading recommendations are provided below. **The final loading concentration must be optimized by each user.**
a) For MiSeq, use the read level sequence in the table below.

Level	Read Length
(R1) Read 1	151
(R2) Index Read 1	8
(R3) Index Read 2	8
(R4) Read 2	151

i) In addition, a reference sample sheet is available for download at: http://archerdx.com/mbc-adapters. Fill out the sample sheet according to the MiSeq protocol.
b) Load sequencing libraries with **5%** PhiX, prepared as follows:
i) Dilute and denature PhiX to 10pM according to the Illumina protocol.
Note: The amount of PhiX depends on the complexity and diversity of the final library pool. A higher concentration of PhiX is recommended for libraries prepared from low input masses resulting in low complexity libraries.
ii) Begin with a **4nM** pool of your barcoded libraries:

(1) Combine **10**µL of the 4nM library pool with **10**µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for **5** minutes at room temperature.
(2) Add **10**µL 200mM Tris pH 7.0 and vortex briefly to mix.
(3) Add **970**µL ice-cold Illumina Hyb buffer and vortex briefly to mix. This makes 40pM library.
(4) Refer to the table below for amounts of pooled library, PhiX and Hyb Buffer to combine in a new 1.5mL microcentrifuge tube and vortex briefly to mix.
(5) Load the entire volume (1.0mL) of the final pool into the appropriate well of the MiSeq cartridge.

MiSeq Loading Guidelines

Desired concentration of final pool (pM)	**13**	**14**	**15**	**16**	**17**	**18**
1) Denature pooled libraries:						
Pooled 4nM libraries (µL)	10	10	10	10	10	10
0.2N NaOH (µL)	10	10	10	10	10	10

2) Incubate for 5 minutes

3) Neutralize and Dilute to 40pM						
Pooled libraries + NaOH from Step 1 (µL)	20	20	20	20	20	20
200mM Tris pH 7.0 (µL)	10	10	10	10	10	10
Hyb Buffer (µL)	970	970	970	970	970	970

4) Dilute library to desired loading concentration (pM)	**13**	**14**	**15**	**16**	**17**	**18**
40pM Libraries from step 2 (µL)	325	350	375	400	425	450
10pM Denatured PhiX (µL)	68	74	79	84	89	95
Hyb Buffer (µL)	607	576	546	516	486	455

5) Load Final Pool into Cartridge						
Final pool (µL)	1000	1000	1000	1000	1000	1000

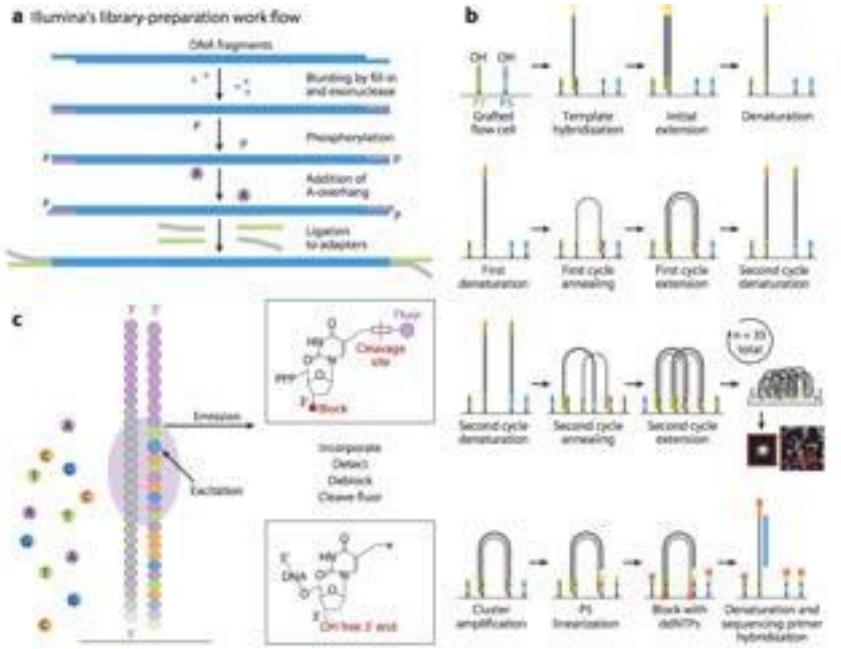
c) For NextSeq, load sequencing libraries with 20% PhiX, prepared as follows:
i) Dilute and denature PhiX to 20pM according to the Illumina protocol
ii) Begin with a 4nM pool of your barcoded libraries:
(1) Combine **10**µL 4nM library pool with **10**µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at ambient room temperature.
(2) Add **10**µL 200mM Tris pH 7.0 and vortex briefly to mix.
(3) Add **970**µL ice-cold HT1 buffer and vortex briefly to mix. This makes 40pM library.
(4) Refer to the table below for amounts of pooled library, PhiX and HT1 Buffer to combine in a new 1.5mL microcentrifuge tube and vortex briefly to mix.
(5) Spin down and load the entire volume (1.3mL) of this final pool in 20% PhiX into the appropriate well of the NextSeq cartridge.

ILLUMINA MISEQ SEQUENCING

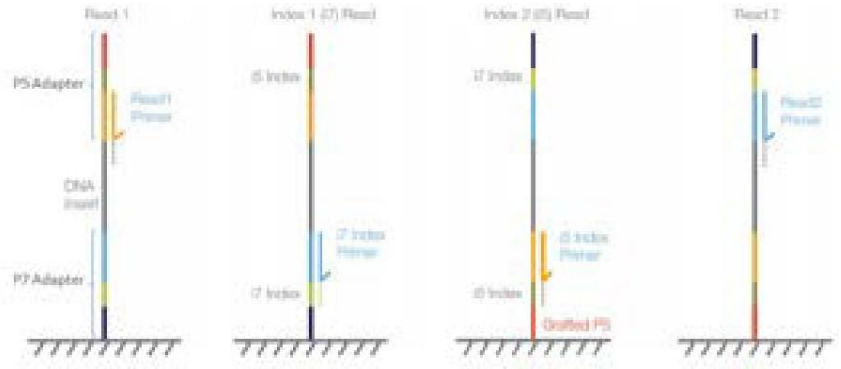
Automatic sequencing is performed in the MiSeq Sequencer (in the sequencing cip called "flowcell") according to the following processes:

1. "cluster generation" by "bridge amplification"
2. cutting and removing the reverse strand
3. sequencing by synthesis of the forward strand,

4. sequencing the code on the reverse strand
5. sequencing the code on the reverse strand
6. "cluster generation" by "bridge amplification"
7. cut the forward strand
8. sequencing by synthesis of the reverse strand



https://www.researchgate.net/figure/a-Illumina-library-preparation-b-Generation-of-clusters-with-bridge-amplification_fig1_272019619



https://support.illumina.com/bulletins/2016/04/adapter-trimming-why-are-adapter-sequences-trimmed-from-only-the--ends-of-reads.html

Analysis

Analyze data with Archer Analysis using either a local software installation or Archer Unlimited. Visit our website at http://analysis.archerdx.com/ for more information. FusionPlex assays may also require a one-time upload of a GTF file (a text file, in GTF format, which directs the software on how to analyze data from the panel). Additionally, if the RNA SNP/InDel pipeline is chosen, there is also an option to select a target mutation file (a text file, in VCF format, which lists specific variants of interest). This file also requires a one-time

upload. Both of these files can be obtained by contacting tech@archerdx.com. Demultiplex NextSeq libraries according to recommendations in FAQs: https://support.archerdx.com/.
Analysis platform produces highly confident gene mutation detection from RNA, DNA and ctDNA sources. The simple user interface paired with in-line visualization enables powerful and clear reporting. Since Analysis allows for third party integration and deploys securely to the cloud or on a server, it is likely to fit within your lab infrastructure.

ION TORRENT NGS USING Ion AmpliSeq RNA Fusion Lung Cancer Research Panel - working protocol

FUSION GENES LIBRARY PREPARATION

RNA: Reverse transcribe and set up target amplification

Reverse transcribe RNA

1. If RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.
2. For each sample, add the following components into a single tube. Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
5X VILO Reaction Mix	2 µL
10X SuperScript Enzyme Mix	1 µL
Total RNA (1–100 ng)	≤ 7 µL
Nuclease-free Water	to 10 µL
Total volume per tube	10 µL

3. Close the tubes, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
4. Load the tubes in the thermal cycler, then run the following program to synthesize cDNA.

Temperature	Time
42°C	30 min
85°C	5 min
10°C	Hold

STOPPING POINT Samples can be stored at 10°C for up to 16 hours in the thermal cycler. For longer periods, store at –20°C.

5. Gently tap the tubes on the bench to ensure reactions are at the bottom of the wells, or if possible, centrifuge the tubes to collect any droplets. Proceed to the next step:

Prepare cDNA target amplification reactions

1. For Ion AmpliSeq RNA Fusion Lung Cancer Research Panel, open the caps of tubes and add the following components to each cDNA synthesis reaction. Prepare a master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq RNA Fusion Lung Cancer Research Panel	4 µL
Nuclease-free Water	2 µL
Total volume per well (includes 10 µL from cDNA synthesis)	**~ 20 µL**

2. Close the tubes caps (strip caps).

Amplify the targets

To amplify target regions, run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 minutes
22 Cycles	Denature	99°C	15 seconds
	Anneal and extend	60°C	4 minutes
Hold	-	10°C	Hold

Cycle number recommendations in the preceding table are based on 10-ng DNA input. Adjust cycle number from the preceding table for lower or higher DNA input:

Amount of DNA/RNA starting material	Adjustment to cycle number
1 ng (300 copies)	+3
10 ng (3,000 copies)	0
100 ng (30,000 copies)	–3

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer term, store at −20°C.

Partially digest amplicons

IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or a cold block, then quickly proceed to incubation.

---

1. Ccentrifuge briefly to collect the contents at the bottom of the wells (tubes), then open the caps.
2. **Add 2 µL of FuPa Reagent** (brown cap) to each amplified sample. The total volume is ~22 µL.
3. Close the caps, vortex thoroughly, then centrifuge to collect droplets.
4. Load the tubes in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 minutes
55°C	10 minutes
60°C	20 minutes
10°C	Hold (for up to 1 hour)

5. Centrifuge briefly to collect the contents at the bottom of the wells (tubes).

STOPPING POINT Store plate at –20°C for longer periods.

Ligate adapters to the amplicons and purify using Ion Xpress adapters

For each barcode X selected, prepare a mix of Ion P1 Adapter and Ion Xpress Barcode X at a final dilution of 1:4 for each adapter. Store diluted adapters at –20°C.

Component	Volume
Ion P1 Adapter	2 µL
Ion Xpress™ Barcode X[1]	2 µL
Nuclease-free Water	4 µL
TOTAL	8 µL

Perform the ligation reaction

IMPORTANT! If there is visible precipitate in the Switch Solution, vortex or pipet up and down at room temperature to resuspend.

1. Briefly centrifuge the tubes to collect the contents.
2. Carefully open the caps, then add the following components in the order listed to each well (tube) containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and adapters before adding to digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Xpress barcode adapter mix, diluted (for barcoded libraries))	2 µL
3	DNA Ligase (blue cap)	2 µL
-	**Total volume** (including ~22 µL of digested amplicon)	**~30 µL**

3. Close the caps, vortex thoroughly, then briefly centrifuge to collect droplets.
4. Load the tubes in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 24 hours)

STOPPING POINT Samples can be stored overnight at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

1. Briefly centrifuge the tubes to collect the contents in the bottom of the wells.

2. Carefully open the caps, then add 45 µL (1.5X sample volume) of Agencourt AMPure XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the tubes in a magnetic rack such as the DynaMag-12 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol.
6. Remove the tubes from the DynaMag-12 and gently pipet up and down five times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.
7. Repeat step 5 and 6 for a second wash.
8. Ensure that all ethanol droplets are removed from the wells. Keeping the tubes in the magnet, air-dry the beads at room temperature for 5 minutes.

Quantify the amplified library using the Qubit 3.0 Fluorometer

Ion AmpliSeq libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification. Amplify the library using Platinum PCR SuperMix HiFi, then purify. Quantify the library using a Qubit 3.0 Fluorometer. After quantification, determine the dilution factor that results in a concentration of ~100 pM, which is appropriate for template preparation using an Ion template kit.

Amplify the library

1. Remove the tubes with purified libraries from the magnetic rack, then **add 50 µL of Platinum PCR SuperMix HiFi** and **2 µL of Library Amplification Primer Mix** to each bead pellet.
2. Close de caps, vortex thoroughly, then centrifuge briefly to collect droplets.
3. Place the tubes back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each tube to a new tube without disturbing the pellet.
4. Close the caps and load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

STOPPING POINT Samples can be stored at –20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt AMPure XP Reagent:

- **First round at 0.5X bead-to-sample-volume ratio**: High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant**.
- **Second round at 1.2X bead-to-original-sample-volume ratio**: Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads**.

First-round purification

1. Centrifuge briefly to collect the contents at the bottom of the wells (tubes), then open the caps.
2. **Add 25 µL (0.5X sample volume) of Agencourt AMPure XP Reagent** in each tubes containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the tubes in a magnet such as the DynaMag 12 Side Magnet for at least 5 minutes, or until the solution is clear.
5. Carefully transfer the supernatant from each tube to a new tube without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 4 above, add **60 µL (1.2X original sample volume) of Agencourt AMPure XP Reagent**. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the tubes in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet**.

4. **Add 150 µL of freshly prepared 70% ethanol** to each tube, then move the tubes side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.
5. Repeat step 4 for a second wash.

6. Ensure that all ethanol droplets are removed from the wells (tubes). Keeping the tubes in the magnet, air-dry the beads at room temperature for 2−5 minutes. **Do not overdry**.
7. Remove the tubes from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads.
8. Colse the caps, vortex thoroughly, then centrifuge to collect droplets.
9. Incubate at room temperature for at least 2 minutes.
10. Place the tubes in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant using Qubit 3.0 Fluorometer.

IMPORTANT! The supernatant contains the desired amplicons. **Do not discard!**

Qubit Fluorometer: Quantify the library and calculate the dilution factor

1. Determine the amplified library concentration.
   a) Make a 1:200 working dilution of Qubit dsDNA HS reagent using the Qubit dsDNA HS Buffer.
   b) Combine 10 µL of the amplified Ion AmpliSeq library with 190 µL of dye reagent, mix well, then incubate for at least 2 minutes.
   c) Prepare each Qubit standard as directed in the user guide.
   d) Measure the concentration on the Qubit Fluorometer.
2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM:

Average amplicon size	Concentration in ng/mL (~100 pM)
140 bp	9

3. Dilute library to ~100 pM, combine, then proceed to template preparation, or store libraries as described below.

(OPTIONAL) COMBINE AMPLICON LIBRARIES

Combine libraries prepared with one panel for equal depth of coverage

You can prepare barcoded libraries from different samples using IonCode or Ion Xpress Barcode Adapters. Multiple uniquely barcoded libraries can be combined during the Equalizer process, or after diluting each individual library to a 100-pM concentration.

For example, if 16 libraries prepared with the same Ion AmpliSeq DNA or RNA panel are combined in a single templating and sequencing reaction:

1. Dilute all individual libraries to 100-pM concentration.
2. Add 10 µL of each of the 16 libraries to a single tube.
3. Mix the combined libraries and proceed to templating and sequencing.

Store libraries

You can store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

ION SPHERE PARTICLES (ISP) PREPARATION

Set up the Ion OneTouch 2 Instrument

Install the Ion OneTouch Recovery Tubes and Ion OneTouch Recovery Router

1. Dispense 150 µL Ion OneTouch Breaking Solution into each of two Ion OneTouch Recovery Tubes.
2. Insert the Recovery Tubes containing Ion OneTouch Breaking Solution into the two centrifuge positions

3. Install the Ion OneTouch Recovery Router into the center slot of the centrifuge.
4. Close the centrifuge lid.

Install the Ion OneTouch Amplification Plate

Remove the used Cleaning Adapter, insert the plate, and pull the handle to close the heat block. Thread the disposable tubing through the catch and pinch valve.

Install the disposable injector

Insert the disposable injector, then confirm automatic placement of the disposable injector above the router by briefly pressing then releasing the spring-loaded top of the Injector Hub.

You should hear a click.

Install the Reagent Tubes

1. Install the **Ion OneTouch Oil** on the left front port. Invert the Ion OneTouch™ Oil bottle (450-mL size) 3 times, then fill the Reagent Tube half-full with Oil. Install the Reagent Tube. Minimize bubbles.
2. Install the **Ion OneTouch Recovery Solution** on the right front port. Invert the bottle of Recovery Solution 3 times, then fill the Reagent Tube one quarter-full with Recovery Solution. Install the Reagent Tube. Minimize bubbles.

Empty the waste container

Appropriately dispose of waste.

Prepare the amplification solution

1. Prepare the reagents as follows:

Reagents	Preparation
Ion PGM Hi-Q View Reagent Mix	1. Allow the tube of reagent mix to come to room temperature before use. 2. Vortex the solution for 30 seconds, then centrifuge the solution for 2 seconds. 3. Keep the reagent mix at room temperature during use. Store unused thawed reagent mix at 2 ºC to 8 ºC.
Ion PGM Hi-Q View Enzyme Mix	Centrifuge the enzyme for 2 seconds and place on ice.
Ion PGM Hi-Q View ISPs	Place the suspension at room temperature.

2. Depending on your library type and concentration, dilute the library as shown in the following table. Use the library dilution within 48 hours of preparation.

	Ion AmpliSeq DNA Library	Ion AmpliSeq RNA Library	gDNA Fragment or Amplicon Library	Ion Total RNA-Seq Library
Library concentration	100 pM	100 pM	100 pM	100 pM
Volume of library	2 µL	4 µL	6.5 µL	5 µL

Volume of Nuclease free Water	23 µL	21 µL	18.5 µL	20 µL
Total volume of diluted library to add to the amplification solution	**25 µL**	**25 µL**	**25 µL**	**25 L**

a. Vortex the diluted library for 5 seconds, then centrifuge for 2 seconds.
b. Place the diluted library on ice.

3. Prepare the Ion PGM Hi-Q View ISPs:
   a. Vortex the ISPs at maximum speed for 1 minute to resuspend the particles.
   b. Centrifuge the ISPs for 2 seconds.
   c. Pipet the ISPs up and down to mix.
   d. ***Immediately*** proceed to the next step.
4. To a 2-mL tube (violet cap) containing 800 µL of Ion PGM Hi-Q View Reagent Mix, add the following components in the designated order. Add each component, then pipet the amplification solution up and down to mix:

Order	Reagent	Cap color	Volume
1	Nuclease-free Water	-	25 µL
2	Ion PGM Hi-Q View Enzyme Mix	Brown	50 µL
3	Diluted library (not stock library)	-	25 µL
4	Ion PGM Hi-Q View ISPs	Black	100 µL
-	**Total**	**-**	**1000µL**

5. Vortex the complete amplification solution prepared in step 4 at maximum speed for 5 seconds.

IMPORTANT! Proceed immediately to "Fill and install the Ion OneTouch Reaction Filter."

Fill and install the Ion OneTouch Reaction Filter

IMPORTANT! We recommend filling the Ion OneTouch Reaction Filter in a room dedicated to pre-PCR activities or a controlled pre- PCR hood. Do not use a reaction filter assembly from any other template preparation kit.

---

1. Pipet 1000 µL of the amplification solution, prepared in the previous procedure, through the sample port of the Ion OneTouch Reaction Filter.
2. Pipet 850 µL of Ion OneTouch Reaction Oil (25-mL size) through the sample port.
3. Change the tip and pipet an additional 850 µL of Ion OneTouch™ Reaction Oil through the sample port.
4. Invert then install the filled Ion OneTouch Reaction Filter into the three holes on the top stage of the Ion OneTouch 2 Instrument.

Start the run

1. After installing the Ion OneTouch Reaction Filter, touch Next to start the run.

IMPORTANT! Remember to add 150 µL of Ion OneTouch Breaking Solution to each Recovery Tube before starting the run. On the reminder screen, confirm and touch **Yes** to advance:

Note: To cancel a run, touch **Abort**, then touch **Yes**.

2. Remove the samples ≤16 hours after starting the run. If you touched Next on the centrifuge screen to centrifuge samples at the end of the run, proceed ***immediately*** to "**Recover the template-positive ISPs**".

Recover the template-positive ISPs

1. At the end of the run, follow the screen prompts to centrifuge the sample.

IMPORTANT! If you removed the Reaction Tubes at the end of the run before the Ion OneTouch™ 2 Instrument had centrifuged the sample or have not processed the sample after 15 minutes, centrifuge the sample on the instrument. On the home screen of the instrument, touch **Open Lid**, touch **Final Spin**, then follow the screen prompts to centrifuge the sample.

---

2. Immediately after the centrifuge has stopped, on the instrument display, touch **Open Lid**. Wait until the lid clicks open, then remove and discard the Ion OneTouch Recovery Router.
3. Remove both Ion OneTouch Recovery Tubes from the instrument, then put the two tubes in a tube rack.
4. Remove all but 100 µL of the Recovery Solution from each Recovery Tube.

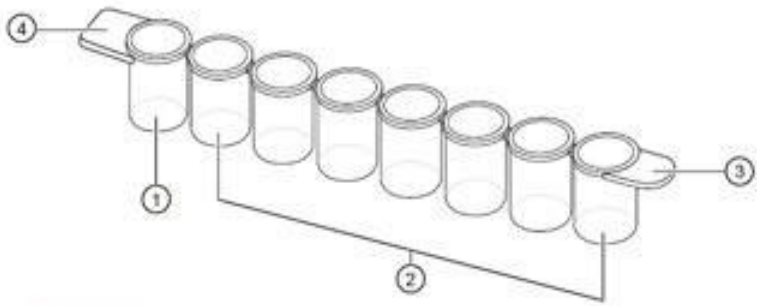
STOPPING POINT Add 500 µL of Ion OneTouch Wash Solution to each Recovery Tube and pipet up and down to disperse the ISPs. Combine the suspension from each Recovery Tube into one new labeled 1.5-mL Eppendorf LoBind Tube. Store the ISPs at 2°C to 8°C for up to 3 days. Before enrichment, centrifuge the ISPs at 15,500 × g for 2.5 minutes. Carefully remove all but 100 µL of supernatant. With a new tip, pipet up and down to resuspend the ISPs. Proceed to step 6.

---

5. Process the ISPs:
   a. Add 500 µL of Ion OneTouch Wash Solution to each Recovery Tube.
   b. Pipet up and down to disperse the ISPs, then combine the suspension from each Recovery Tube into one new labeled 1.5-mL Eppendorf LoBind Tube

      Note: If a precipitate is present, incubate the tube at 50°C for 2 minutes to dissolve.4

   c. Centrifuge the ISPs for 2.5 minutes at 15,500 × g.
   d. Use a pipette to remove all but 100 µL of the Wash Solution from the tube. Withdraw the supernatant from the surface and on the opposite side from the pellet.
6. Obtain an 8-well strip from the Ion OneTouch ES Supplies Kit. Ensure that the square-shaped tab of an 8-well strip is on the left:



① Well 1
② Wells 2–8
③ Rounded tab
④ Square-shaped tab

7. Pipet the ISPs up and down 10 times to mix, then transfer the suspension into Well 1 of the 8-well strip.
8. Retain an aliquot of the unenriched Ion PGM Hi-Q View OT2 Kit from Well 1 for **quality assessment**. Assess the quality of the unenriched, template-positive ISPs using the Qubit 3.0 Fluorometer.

9. Enrich the template-positive ISPs with the Ion OneTouch ES. You may start the enrichment procedure while the Ion OneTouch 2 Instrument cleaning is in progress.

IMPORTANT! Do not store the recovered, template-positive ISPs at –30°C to −10°C. Do not store the ISPs in Ion PGM OT2 Recovery Solution (see step 4 of this procedure).

Enrich the template-positive Ion PGM Hi-Q View ISPs

Determine if a residual volume test is necessary

Condition	Action
First use of the instrument and during monthly maintenance	Perform a residual volume test (see "Ion OneTouch ES Instrument installation, setup, and maintenance" in the Ion OneTouch™ 2 System User Guide Pub. No. MAN0014388).
Routine use and residual volume in Well 1 and Well 8 is >5.0 µL	
Routine use and residual volume in Well 1 and Well 8 is ≤5.0 µL	Operate the instrument without performing the residual volume test. Proceed to "Prepare reagents then fill the 8-well strip".

Prepare reagents then fill the 8-well strip

Prepare Melt-Off Solution

Prepare fresh Melt-Off Solution by combining the components in the following order:

Order	Component	Volume
1	Tween Solution	280 µL
2	1 M NaOH	40 µL
-	**Total**	**320 µL**

IMPORTANT! Prepare Melt-Off Solution as needed, but appropriately dispose of the solution after 1 day.

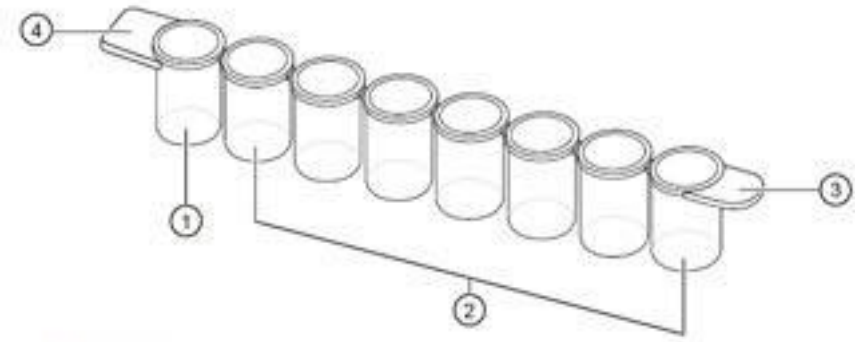
Wash and resuspend the Dynabeads MyOne Streptavidin C1 Beads

1. Vortex the tube of Dynabeads MyOne Streptavidin C1 Beads for 30 seconds to resuspend the beads thoroughly, then centrifuge the tube for 2 seconds.
2. Open the tube, then use a new tip to pipet the dark pellet of beads up and down until the pellet disperses. Immediately proceed to the next step.

3. Transfer 13 µL of Dynabeads MyOne Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind Tube.
4. Place the tube on a magnet such as a DynaMag-2 magnet for 2 minutes, then carefully remove and discard the supernatant without disturbing the pellet of Dynabeads MyOne Streptavidin C1 Beads.
5. Add 130 µL of MyOne Beads Wash Solution to the Dynabeads MyOne Streptavidin C1 Beads.
6. Remove the tube from the magnet, vortex the tube for 30 seconds, then centrifuge for 2 seconds.

Fill the 8-well strip

1. Confirm that the template-positive ISPs from the Ion OneTouch 2 Instrument are in Well 1 of the 8-well strip, as previously prepared.



① Well 1
② Wells 2–8
③ Rounded tab
④ Square-shaped tab

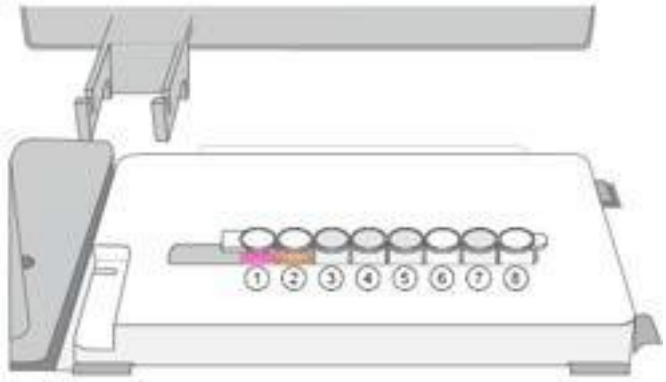
2. If you have not done so already, assess ***the quality of the unenriched, template-positive ISPs*** using the Qubit 3.0 Fluorometer.
3. Fill the remaining wells in the 8-well strip as follows (see the figure in step 4):

Well number	Reagent to dispense in well
Well 1[1]	*Entire* template-positive ISP sample (100 µL; prepared in step 1 of this procedure)
Well 2	130 µL of Dynabeads MyOne Streptavidin C1 Beads resuspended in MyOne Beads Wash Solution (prepared in "Wash and resuspend the Dynabeads MyOne Streptavidin C1 Beads")
Well 3	300 µL of Ion OneTouch Wash Solution
Well 4	300 µL of Ion OneTouch Wash Solution
Well 5	300 µL of Ion OneTouch Wash Solution
Well 6	Empty
Well 7	300 µL of freshly-prepared Melt-Off Solution (prepared in "Prepare Melt-Off

	Solution")
Well 8	Empty

[1] Well closest to the square-shaped tab

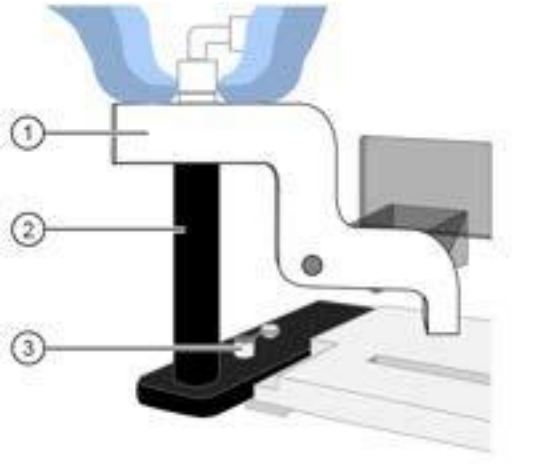
4. Confirm that the square-shaped tab is on the left, then insert the filled 8-well strip into the slot of the Tray, and push it all the way to the right:



Prepare the Ion OneTouch ES

Before every enrichment performed on the Ion OneTouch ES Instrument, install a new PCR collection tube and a new Eppendorf LoRetention Dualfilter P300 pipette tip.

1. Add 10 µL of Neutralization Solution to a new 0.2-mL PCR tube.
2. Insert the open 0.2-mL PCR tube containing Neutralization Solution into the hole in the base of the Tip Loader, as shown in the figure in step 4.
3. Place a new tip in the Tip Loader. Remove the Tip Arm from the cradle, then align the metal fitting of the Tip Arm with the tip.
4. Keeping the fitting on the Tip Arm vertical, firmly press the Tip Arm down onto the new tip until the Tip Arm meets the Tip Loader. Hold the Tip Arm to the Tip Loader for ~1 second to ensure proper installation of the tip.



5. Lift the Tip Arm *straight* up to pull the installed tip from the Tip Loader tube.
6. Return the Tip Arm to the cradle

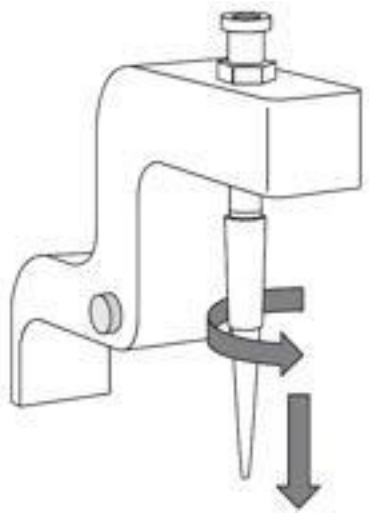
IMPORTANT! Ensure that the back/bottom end of the Tip Arm is not resting on top of the thumb screw, causing the Tip Arm to tilt forward.

Perform the run

Before starting the run:

- Confirm that a new tip and open 0.2-mL PCR tube have been loaded in the Ion OneTouch ES Instrument, and that the 8-well strip is correctly loaded.
- Ensure that Well 1 (ISP sample) is the left-most well and the 8-well strip is pushed to the right-most position in the slot.

1. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run. Do not introduce bubbles into the solution.
2. If needed, power on the Ion OneTouch ES Instrument, then wait for the instrument to initialize. The screen displays "rdy". The Tip Arm performs a series of initialization movements and returns to the home position (~5 seconds).
3. Press **Start/Stop**. The screen displays "run" during the run. The run takes ~35 minutes.
4. At the end of the run, the instrument displays "End" and beeps every 60 seconds. Press the **Start/Stop** button to silence this alarm, then reset the Ion OneTouch ES Instrument for the next run. The instrument can be left on between runs.
5. Immediately after the run, securely close, then remove the PCR tube containing the enriched ISPs.
6. Mix the contents of the PCR tube by gently inverting the tube 5 times.
7. Remove the used tip: with the Tip Arm in its cradle, twist the tip counterclockwise (as viewed from above), then pull it downward to remove and discard the tip.



IMPORTANT! Improper removal of tips can loosen the metal tip adapter fitting on the Tip Arm and affect instrument operation.

8. Remove, then discard the used 8-well strip.

Sequence or store the template-positive ISPs

- Proceed to sequencing using the Ion PGM Hi-Q View Sequencing Kit
  or
- Store the enriched ISPs at 2°C to 8°C for up to 3 days.

8. Tumor type

Non–small-cell lung cancer (NSCLC) is a heterogeneous disease composed of unique molecular subsets with distinct clinical outcomes.

Multiple randomized studies have established the superiority of molecularly targeted therapies versus chemotherapy for the treatment of EGFR-mutant and ALK-positive NSCLC.

In other molecular subsets, single-arm studies confirm that treatment with targeted therapies can induce durable responses.

As drugs that target these molecular drivers are approved for first-line treatment, genotyping in newly diagnosed NSCLC is considered the standard of care.

Activating mutations in the gene encoding EGFR occur primarily in **NSCLC**, and result in constitutive activation of the kinase activity of the EGFR protein, thereby contributing to the oncogenic process.

The prevalence of these mutations in unselected cases of NSCLC is approximately 10% - 30%.

However, these mutations occur more frequently, but not exclusively, in non-smoking/light-smoking female patients of Asian ancestry with adenocarcinoma histologies.

The most common EGFR mutations in NSCLC include a variety of deletions in exon 19 and the substitution mutation L858R in exon 21; these mutations collectively constitute approximately 85% of EGFR mutations observed in NSCLC.

The EGFR test is used as a companion diagnostic test for TKI, a compound that reversibly inhibits the kinase activity of EGFR, preventing autophosphorylation of tyrosine residues associated with the receptor and thereby inhibiting further downstream signaling that promotes cell survival and proliferation.

Erlotinib binding affinity for EGFR exon 19 deletion or exon 21 L858R mutations is higher than its affinity for the wild-type receptor.

Clinical trials have shown that patients with advanced NSCLC and with exon 19 deletion mutations or L858R substitution mutation in exon 21 that were treated with Tarceva as first-line treatment, are likely to experience clinical benefit compared to patients treated with chemotherapy.

Osimertinib, an irreversible inhibitor of both EGFR TKI-sensitizing and T790M resistance mutations in advanced NSCLC, inhibits the kinase activity of EGFR, which inhibits

a cascade of intracellular downstream signaling events that promote cell proliferation, survival, and angiogenesis.

Clinical trials have shown that patients with advanced non-squamous NSCLC with an EGFR TKI-sensitizing mutation and have progressed following therapy with a first-generation EGFR TKI and who have developed a T790M resistance mutation in exon 20 that were treated with Osimertinib are likely to experience clinical benefit.

8. Conclusions

- The protocols for nucleic acids extraction from FFPE samples have been optimized, both in terms of thickness and number of paraffin section as well as the subsequent steps in order to obtain a sufficient number of functional fragments for PCR and NGS application.
- The castPCR method provided great consistency with the results obtained by NGS and at the same time has a high enough sensitivity to detect genetic mutations on a high wild allelic variant background.

Iași Regional Institute of Oncology, as a beneficiary, launches the project "Network of excellence for diagnosis and research in lung cancer", acronym LUNGNEX-RD - Project funded under the ROMD Program 2014-2020, through the European Neighborhood Instrument (ENI) and co-financed by the project partners, based on the contract.

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This project is funded by the European Union

"The European Union is made up of 28 Member States who have decided to gradually link together their knowhow, resources and destinies. Together, during a period of enlarge- ment of 50 years, they have built a zone of stability, democra- cy and sustainable development whilst maintaining cultural diversity, tolerance and individual freedoms. The European Union is committed to sharing its achievements and its values with countries and peoples beyond its borders".