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

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Content

1. Introduction

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.

3. Quality control

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

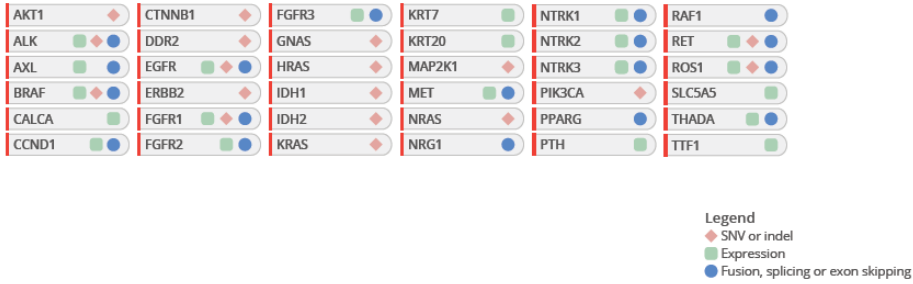
PhiX Control v3 is a reliable, adapter-ligated library used as a control for Illumina sequencing runs. The library is derived from the small, well-characterized PhiX genome, offering several benefits for sequencing and alignment. PhiX Control v3 is provided as a ready-to-use library, and can be utilized 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.

4. 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. Sensitivity and limit of detection (LOD)

Sensitive Mutation Detection

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.

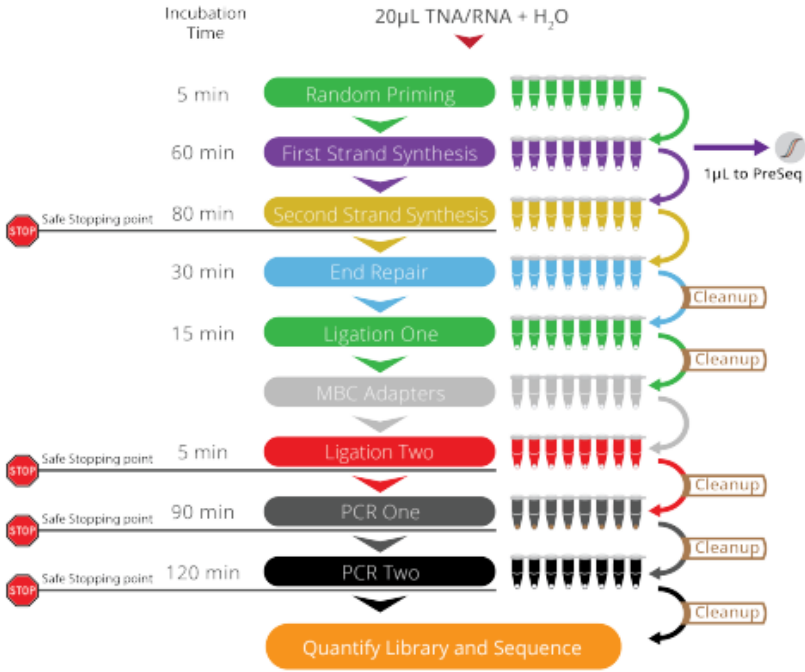
RNA Expression

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

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)	

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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.

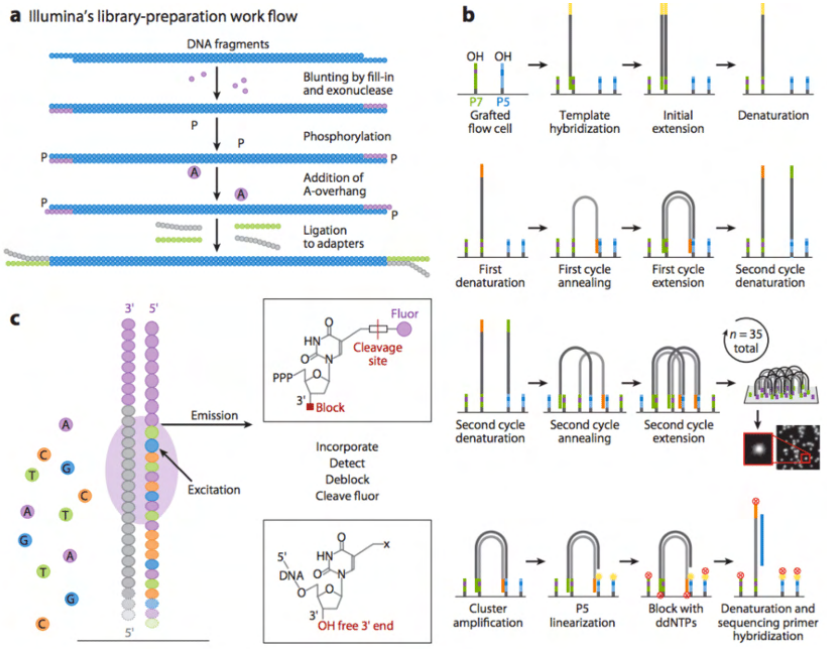
(4) Refer to the table below for amounts of pooled library, PhiX and HT1 Buffer to combine in a new 1.5mL microcentrifuge tube and vortex briefly to mix.

(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.

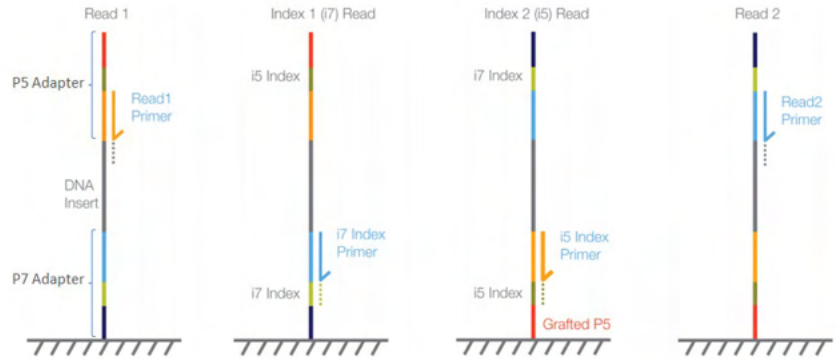
ILLUMINA MISEQ SEQUENCING

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.

7. 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.

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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"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".