TargetPlex FFPE-Direct DNA Library Preparation For Illumina NGS (cat. no. YST0146)

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This guide explains how to prepare up to 96 barcoded TargetPlex™ FFPE-Direct™ next-generation sequencing (NGS) DNA libraries from formalin-fixed paraffin embedded (FFPE) samples directly from 5 - 15 um thick tissue sections, a cytology smear on a glass slide, or 10ng of genomic DNA.

Powered by TargetPlex's™ Noise Canceling Technology and the FFPE-Direct™ workflow, this rapid, addition-only, 3.5 hour NGS DNA library workflow is ideal for the analysis of hundreds of actionable mutations without the need for separate, labor intensive, and time-consuming, pre-analytical FFPE DNA extraction, purification, and isolation steps. The propri- etary primers and unique primer-pair tiling design enables high genomic target coverage and high detection sensitivity and specificity.

The TargetPlex™ Noise Canceling Technology is designed to remove target amplification by-products, which significantly impedes downstream NGS sequencing efficiency due to increased background noise. The FFPE-Direct™ workflow will save you significant time and enable higher target enrichment, leading to more sensitive detection of mutations from FFPE re- search samples than ever before possible. Typically, lower sequencing depth is required when using this technology compared to standard multiplex PCR enrichment workflows, allowing for more sample barcoding per sequencing run. If you have any question about this workflow please email info@sensecarebio.com.

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The following reagent kits are required:

- TargetPlex™ DNA Library Kit for Illumina (cat. no. YST0146)

- SenseCare Bio TargetPlex™Primer Pool Panel, composed of one or more Primer Pools (PP) Several pre-defined or custom primer pool panels are available for purchase on the www.sensecarebio.com website.

Reagents provided in these kits are used to amplify targeted genomic regions directly from FFPE slides or gDNA and add adapter barcode sequences to the amplicons.

The following reagent kit is recommended:

- SenseCare Bio Illumina DNA Library PCR Quantification Kit (cat. no. YST0065)

Reagents provided in this kit allows for accurate DNA library quantification downstream of library preparation.

Input Requirements:

- Recommended input is 1/4 to 1/2 tissue section on a slide per library preparation.
- Recommended input is 1 cytology smear slide per library

preparation.

- Recommended input is 10 ng gDNA per primer pool.

Preparation of Highly Degraded FFPE Samples:

Watch YouTube Video for a Demo on the procedure at https://youtu.be/jYpIrkigD-g

Highly degraded FFPE can still provide high-yield DNA li- braries although the protocol may need to be adjusted with additional PCR amplification cycles.

A: FFPE-Direct PCR Target Enrichment

This step uses PCR to amplify target regions of the DNA from FFPE tissue directly.

Reagents and Consumables Required for Step A:

- 2X Ultra HiFi PCR Master Mix (included)
- 5X TargetPlex Primer Pool 1 (not included)
- 5X TargetPlex Primer Pool 2 (not included)
- 100μl or 200μl pipette tip and a razor blade or Rainin wide-orifice pipette tips (Rainin Item Number 30389248)

• FFPE tissue (5 – 15 micron thick section on a standard glass slide, cytology smear, or 10ng of gDNA per primer pool.)
• Nuclease-free water
• 1.5 ml Eppendorf (or equivalent) tube
• 96-well PCR plate compatible with your thermal cycler
• MicroAmp Clear Adhesive Film

Set Up FFPE-Direct for PCR amplification.

1. Prepare PCR Mix by adding the following components into a 1.5mL Eppendorf tube and mix well.

Component	Volume per sample
2X Ultra Hi Fi PCR Master Mix	30μl
Nuclease-free water	18μl
Total per sample =	48 μl

2. Using a 100μl or 200μl pipette attached with a wide-orifice tip (if this is not available you can be prepared a tip by cutting a standard 200μl tip approximately 3-5 mm up from the tip-end using sharp scissors or razor blade. The orifice diamater should be at least 3 mm. (Alternatively, purchase Rainin pipette tip item number 30389248).
3. **Pipette all the 48μl of the PCR mix onto at least half of the interested area of the FFPE tissue area on the slide.**
4. **Using a circular motion of the pipette tip, scrape the FFPE tissue off of the slide so that small bits of the FFPE can be seen suspended into the PCR mix.**
5. Transfer 16uL of the PCR Mix containing the FFPE bits into one clean PCR well.
6. Transfer a second 16uL of the PCR Mix containing the FFPE bits into a second clean PCR well.
7. Add 4μl of 5X NSCLC Primer Pool 1 (PP1) into the first PCR wells.
8. Add 4μl of 5X NSCLC Primer Pool 2 (PP2) into the sec- ond PCR well. **Note: A 96-well PCR plate may be used if multiple FFPE samples are tested.**
9. Close the PCR tube or seal the 96-well PCR plate with a MicroAmp Clear Adhesive Film and briefly centrifuge.
10. Proceed to thermal cycling by running the following pro- gram steps as described below.

STAGE	STEP	TEMP	TIME
Enz. Activation	Denature	95°C	10 min.
Cycle (30 - 35 cycles)*	Denature	99°C	15 sec.
	Extension	60°C	4 min.
Hold	Stop	10°C	Infinity

***Note: Use more PCR cycles (i.e. up to 35 cycles) for highly degraded FFPE samples.** Do not leave samples on Hold in the thermal cycler for more than 4 hours.

11. After PCR themal cycling is complete, brifly centrifuge the PCR products to bring the condensate to the bottom of the well.

B: Enzymatic Primer Digestion

Reagent Required for Step B:

• 10X Digestion Buffer (included)
• Digestion Enzyme Mix (included)
• Nuclease-free water
• PCR tube

(Optional) Ensure that the amplified PCR product yield is greater than 120ng DNA by assessing with an Agilent Bio- analyzer or Qubit 4.0. **Note that less than 120ng yield may not be sufficient for primer digestion and may require repeating Step A.**

1. Combine Primer Pool 1 (PP1) with Primer Pool 2 (PP2) PCR products by pipetting PP2 products into PP1's PCR well. Note that either well would suffice.
2. Transfer only 20μl of the combined PCR products into a new PCR tube or new 96-well Plate for enzymatic digestion.
3. Combine all the following components into the PCR tube and mix thoroughly.

Component	Volume/Sample
PP1 and PP2 Combined PCR Products	20 μl
10X Digestion Buffer	4 μl
Digestion Enzyme Mix	2 μl
Nuclease-Free Water	14 μl
Total =	40.0 μl

5. Close the PCR tube or seal the 96-well PCR plate with MicroAMP clear adhesive film.
6. Incubate the reaction on a thermal cycler to allow primer digestion.
7. Place the tube in a thermal cycler and run the following program.

STAGE	TEMP	TIME
Hold	20°C	30 min
Hold	70°C	10 min
Hold	10°C	Infinity

8. Resuspended MagClean Magnetic beads (available from SenseCare Bio, YST0202) by inverting the bottle multiple times.

9. Add 72 μl (1.8X) of resuspended MagClean beads and mix well by pipetting up and down at least 10 times.
10. Incubate for 5 minutes at room temperature.
11. Place the tube on an appropriate magnetic stand to sep- arate the beads from the supernatant. After the solution is clear (3 minutes), carefully discard the supernatant with a pipette. **(Caution: Do not discard the BEADS as they contain the DNA library.)**
12. Add 125 μl of freshly prepared 70% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the

supernatant.

Note: freshly prepared 70 % ethanol solution is required to ensure best wash.

13. Repeat Step (12) one additional time.
14. Completely remove the residual ethanol, and allow the beads to air dry for 5-7 minutes while the tube is on the magnetic stand with the lid open. Do not allow the beads to completely dry as the beads may flake off.
15. Elute the DNA target from the beads with 12 µl of nuclease-free water. Mix well by pipetting up and down, and put the tube on the magnetic stand until the solution is clear.
16. Transfer the supernatant to a clean PCR tube (i.e the eluted DNA library) .

DO NOT STOP NOW. Immediately proceed to Step C to End-Repair (optional) or if end-repair is skipped, proceed immediately to Step E to Adaptor Ligation.

C: End-Repair (NOTE: THIS IS AN OPTIONAL STEP - USE ONLY FOR HIGHLY DEGRADED FFPE SAMPLES)

The end-repair step is optional and is intended for highly degraded FFPE samples. If this step is skipped proceed to Adaptor Ligation Step E.

Reagent Required for Step C:

- 5X End-Repair Buffer (included)
- End-Repair Enzyme Mix (included)
- Nuclease-free water
- PCR tube

1. Combine the following end-repair components:

Component	Volume/Sample
Digested PCR Product (Step B)	10 µl
5x End-Repair Buffer	4 µl
End-Repair Enzyme Mix	0.2 µl
Nuclease Free-Water	5.8 µl
Total =	20 µl

2. Pipet up and down to thoroughly mix.
3. **Incubate at room temperature for 20 minutes.**
4. **DO NOT STOP NOW.** Immediately proceed to Step D: Purify End-Repair Product.

D: Purify End-Repair Product

Using MagClean magnetic beads at a low concentration to remove large genomic DNA.

Reagents and Equiptment Required for Step D:

- SenseCare Bio MagClean Beads (cat. no. YST0202, not included)
- Magnetic stand
- Freshly prepared 70 % ethanol solution

• Nuclease-free water

1. Resuspended MagClean beads by inverting the bottle multiple times.
   (a) Add 36μl (1.8X) of resuspended MagClean beads to 20μl from Step C and mix well by pipetting up and down at least 10 times.
   (b) Incubate for 5 minutes at room temperature.
   (c) Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (3 minutes), carefully discard the su- pernatant with a pipette. **Caution: Do not dis- card the beads as they contain the DNA library.**
   (d) Add 125 μl of freshly prepared 70% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully re- move and discard the supernatant. **Note: freshly prepared 70 % ethanol solution is required to ensure best wash.**
   (e) Repeat Step (d) one additional time.
   (f) Completely remove the residual ethanol, and allow the beads to air dry for 5-7 minutes while the tube is on the magnetic stand with the lid open. Do not allow the beads to completely dry as the beads may flake off.
   (g) Elute the DNA target from the beads with 12 μl of nuclease-free water. Mix well by pipetting up and down, and place the tube on the magnetic stand until the solution is clear.
   (h) Transfer the supernatant to a clean PCR tube (i.e. contains the eluted DNA library).

E: Adaptor Ligation

This step allows the ligation of the sequencing adaptors to the DNA library.

Reagents Required for Step E:

• 10X Ligation Buffer (included)
• Ligation Enzyme Mix (included)
• Illumina Adaptor Indexes in PCR Tubes - comes as an 8-well strip, (included)
• Nuclease-free water (not included)

In a PCR tube, combine the reagents as indicated in the table below. **Note: Add the components in the order listed.**

1. Centrifuge the Illumina Adaptor Index 8-well PCR tubes which contain 1μl tube of the Ilumina Adapter Indexe per tube.
2. Carefully open the caps of one Illumina Adaptor Indexes PCR Tube.
3. **Transfer 10μl of the Enzymatic Primer Digested Product (Step B) or Purified End-Repaired Product (Step D) to one Illumina Adapter Index Tube.**

4. Mix by pipetting up and down several times well.
5. **Record the Barcode ID used with your sample. IMPORTANT**
6. Add the remaining components (10x Ligation Buffer, Ligation Enzyme Mix, nuclease-free water) as shown in the table below. Mix well.

Illumina Adaptor Index Ligation Reaction Set-Up

Component	Volume/rxn
Illumina Adaptor Index	1.0 µl (step 1)
Product from Step B or Step D	10.0 µl (step 3)
10X Ligase Buffer	2.0 µl (step 6)
Ligase Enzyme Mix	2.0 µl (step 6)
Nuclease-Free Water	5.0 µl (step 6)
Total =	20.0 µl

4.

7. Cover the tube and centrifuge the contents.
8. Place the tube in a thermal cycler and run the following programmed steps.

STAGE	STEP	TEMP	TIME
Hold	Ligation	25˚C	30 min.
Hold	Enzyme Denature	72˚C	5 min.
Hold	Stop	4˚C	infinity

9. **DO NOT STOP.** Immediately proceed to Step F: Amplify DNA Library once the reaction reaches the 4 ˚C Stop step.

F: Amplify DNA Library

This step removes residual library adaptors, ligation buffers and prepares the DNA library for final PCR amplification.

Reagents and Equiptment Required for Step F:

- SenseCare Bio MagClean Beads (cat. no. YST0202, not included) Beads or Ampure XP (Agencourt)
- Magnetic stand
- Freshly prepared 70 % ethanol solution
- 2X SenseCare HiFi PCR Master Mix (included)
- 10X Illumina Library Amplification Primer Mix (included)
- Nuclease-free water

1. Resuspended MagClean beads by inverting the bottle multiple times.
2. Add 20 µl (1.0X) of resuspended MagClean beads to 20 µl of the adaptor ligated PCR library from Step D. Mix well and incubate for five (5) minutes at room temperature.
3. Place the tube on an appropriate magnetic stand to sep- arate the beads from the solution. After the solution is clear (at least 3 minutes).**(Caution: Do not discard the BEADS as they contain the DNA library of interest.)** Carefully remove the supernatant and discard.

Add 125 µl of freshly prepared 70% ethanol to the tube while on the magnetic stand. Incubate at room tem- perature for 30 seconds, and then carefully remove and discard the supernatant.

5. Repeat wash step (Step 4).
6. Completely remove the residual ethanol, and air-dry beads for 5 minutes while the tube is on the magnetic stand with the tube's lid open.
7. To each well containing a magnetic bead, add 50µl of the following mixture of components:

Component	Volume/rxn
2x SenseCare HiFi PCR Master Mix	25 µl
10X Illumina Library Amp Primer Mix	5 µl
Nuclease-free water	20 µl
Total =	50 µl

8. Run on a thermal cycler with the following programmed steps.

STAGE	STEP	TEMP	TIME
Hold	Activation/Denaturation	98˚C	2 min.
Cycle	Denaturation	98˚C	15 sec.
(× 10 - 14)*	Annealing/Extension	64˚C	1 min.
Hold	Stop	10˚C	infinity

***Note: Use more PCR cycles (i.e. up to 14 cycles) for highly degraded FFPE samples.**

G: Purify Final DNA Library

Use magnetic beads to purify sequencing-ready DNA amplicon libraries.

Reagents and Equipment Required for Step G:

- SenseCare Bio MagClean Beads (cat. no. YST0202, not included)
- Magnetic stand
- Eppendorf LoBind PCR tubes or plates
- Freshly prepared 70% ethanol solution
- Nuclease-free water or Low TE buffer
- MicroAmp Clear Adhesive Film

1. Resuspended MagClean beads by inverting bottle multiple times.
2. Add 25μl (0.5X) of resuspended MagClean beads to the 50μl of library and mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Place the tube on the magnetic stand to separate the magnetic beads from the supernatant.
5. After the solution is clear (3 minutes), **carefully pipette the supernatant approximately 75μl containing the desired amplicon library to a new well.** Small amounts of bead carryover do not affect performance.

6. Add 60µl MagClean beads (0.8 X) to each well containing the transferred supernatant 75µl.
7. Pipette up and down to briefly mix solutions.
8. Incubate at room temperature for 5 minutes.
9. Place on the magnetic stand and wait until the liquid is clear (3 minutes).
10. Without disturbing the beads, remove and discard the supernatant. **Note: The amplicon library is captured on the beads.**
11. Add 125µl of freshly prepared 70% ethanol to the tube while on the magnetic stand. Incubate at room tem- perature for 30 seconds, then without disturbing the bead pellet, carefully remove, and discard the super- natant. **Note freshly prepared ethanol solution is required to ensure best performance.**
12. Repeat previous 70% ethanol wash (Step 11).
13. Use a 20 µl pipette to remove and discard all residual EtOH from each well. **Note: This is a critical step.**
14. Air dry the magnetic beads for 5 minutes while the tube is on the magnetic stand with lid open. **Do not allow the beads to completely dry as the beads may flake off.**
15. Elute the DNA library from the beads with 20µl of nuclease-free water or Low TE buffer. Mix thoroughly by pipetting up and down.
16. Incubate at room temperature for 5 minutes.
17. Place on the magnetic stand and wait until the liquid is clear (3 minutes).
18. Transfer the supernatant (approximately 20µl) containing the DNA library to a clean Eppendorf LoBind PCR plate or tube for storage.
19. Ensure the plate is well sealed and store at -25˚Cto - 15˚C.

H: Library Quantity and Quality Assurance - For Illumina DNA Libraries

Perform the following procedures for quality control analysis.

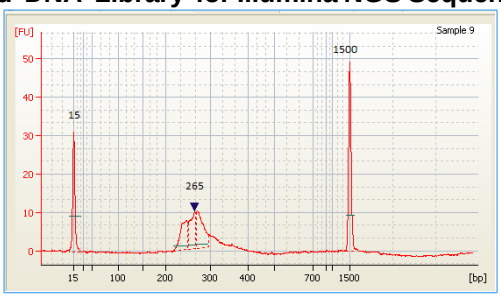
Reagents and Equipment Required for Step H:

• 2100 BioAnalyzer Instrument with Bioanalyzer DNA 1000 Chip Kit (Agilent Technologies, Inc.)
• qPCR Library Quantification Kit for Illumina (SenseCare Bio cat no. YST0065, not included.)
• Qubit 4.0 Fluorometer with the Qubit dsDNA BR Assay Kit (Life Technologies/ ThermoFisher)

Bioanalyzer (Use for accurate DNA library fragment quality control.)

1. Analyze 1µl library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.
2. See related product user guide for details.

Example of a final TargetPlex™ FFPE-Direct™ DNA library from the TargetPlex™ Lung Cancer Panel – Primer Pools (cat. no. YST0115, not included) Barcoded DNA Library for Illumina NGS Sequencing



TargetPlex™ Library Bioanalyzer expected DNA fragment profile range is between 220 - 320 bps on a DNA 1000 Chip.

qPCR Library Quantification Kit (Use for accurate DNA Library quantification analysis.)

1. Quantify 1 µl library by qPCR using SenseCare TaqMan or SYBR Green Real-Time PCR kits.
2. See related product user guide for details.

Qubit 4.0 (Use for accurate DNA library yield analysis.)

1. Analyze 1µl library using the Qubit Fluorometer with the Qubit dsDNA BR Assay Kit.
2. For fluorometric methods, calculate the molarity (nM) of the library using the following formula: ng/uL*1,000,000 /660/210 = nM
3. See related product user guide for details.

I: Illumina Manifest File

The Illumina Manifest file can be obtained by emailing info@sensecarebio.com or gianluca.roma@sensecarebio.com or downloading it from the product specific webpage.

J: Ion Torrent Barcodes Sequences

Below are the eight Illumina Indexes sequences provided.

Illumina Indexes:

Index_ID	I7_Index_ID	index	I5_Index_ID	index2
A01	N701	TCGCCTTA	S502	CTCTCTAT
B01	N701	TCGCCTTA	S503	TATCCTCT
C01	N701	TCGCCTTA	S505	GTAAGGAG
D01	N701	TCGCCTTA	S506	ACTGCATA
E01	N701	TCGCCTTA	S507	AAGGAGTA
F01	N701	TCGCCTTA	S508	CTAAGCCT
G01	N701	TCGCCTTA	S510	CGTCTAAT
H01	N701	TCGCCTTA	S511	TCTCTCCG