TargetPlex FFPE-Direct DNA Library Preparation For Ion Torrent NGS (cat. no. YST0068)

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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 hundreads of actionable mutations without the need for separate, labor intensive, and time-consuming, pre-analytical FFPE DNA extraction, purification, and isolation steps. The proprietary 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 sensitivite detection of mutations from FFPE research 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 worflow please email info@sensecarebio.com.

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The following reagent kits are required:

• TargetPlex™DNA Library Kit (cat. no. YST0068)

• 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 DNA Library PCR Quantification Kit for Ion Torrent (cat. no. YST0076)

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 libraries 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 balade 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 second 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 program steps as described below.

STAGE	STEP	TEMP	TIME
Enz. Activation	Denature	95°C	10 min.
Cycle	Denature	99°C	15 sec.
(30 - 35 cycles)*	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 and Consumables Required for Step B:

• 10X Digestion Buffer (included)
• Digestion Enzyme Mix (included)
• MagClean Magnetic Bead (SenseCare Bio cat. no. YST020, not included)
• Nuclease-free water
• PCR tube

(Optional) Ensure that the amplified PCR product yield is greater than 120ng DNA by assessing with an Agilent Bioanalyzer 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	20μl
10X Digestion Buffer	4 μl
Digestion Enzyme Mix	2 μl
H_2O	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 Bead by inverting the bottle multiple times.
9. Add 72 μl (1.8X) of resuspended MagClean Magnetic Bead 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 separate 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

Reagents and Consumables 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
H_2O	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 Bead at a low concentration to remove large genomic DNA.

Reagents and Equipment Required for Step D:

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

1. Resuspended MagClean Magnetic 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 supernatant with a pipette. **Caution: Do not discard 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 remove 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 put 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)
- Ion Adaptor Barcode PCR Tube - comes as an 8-well strip (adaptors 1 - 8), (included)
- Nuclease-free water

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

1. Centrifuge the Ion Adaptor Barcode 8-well PCR tubes which contain 1μl tube of the Ion Adapter Barcodes per tube.
2. Carefully open the caps of one Ion Adaptor Barcode PCR Tube.

3. **Transfer 10μl of the Enzymatic Primer Digested Product (Step B) or Purified End-Repaired Product (Step D) to one Ion Adapter Barcode 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.

Ion Torrent Adaptor Ligation Reaction Set-Up

Component	Volume/rxn
Ion Torrent Adaptors	1.0μl (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	2.0μl (step 6)
H_2O	5.0μl (step 6)
Total =	20.0 μl

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 Consumables Required for Step F:

• SenseCare Bio MagClean Beads (cat. no. YST0202, included) or Beckman Coulter Agencourt AMPure XP Beads
• Magnetic stand
• Freshly prepared 70 % ethanol solution
• 2X SenseCare HiFi PCR Master Mix (included)
• 10X Ion Library Amplification Primer Mix (included)
• Nuclease-free water

1. Resuspended AMPure XP beads by inverting the bootle multiple times.
2. Add 20 μl (1.0X) of resuspended AMPure XP 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 separate 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.
4. Add 125 μl of freshly prepared 70% ethanol to the tube while on the magnetic stand. Incubate at room temperature 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 beads, add 50μl of the following mixture of components:

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

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 Consumables Required for Step G:

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

1. Resuspended MagClean Beads by inverting bottle multiple times.
2. Add 25μl (0.5X) of resuspended AMPure XP 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 AMPure XP 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 temperature for 30 seconds, then without disturbing the bead pellet, carefully remove, and discard the supernatant. **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°C to -15°C.

H: Library Quantity and Quality Assurance - For Ion Torrent DNA Libraries

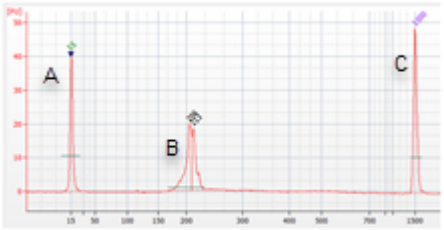
Perform the following procedures for quality control analysis.
Reagents and Consumables Required for Step H:

• 2100 BioAnalyzer Instrument with Bioanalyzer DNA 1000 Chip Kit (Agilent Technologies, Inc., not included)
• DNA Library PCR Quantification Kit – Ion Torrent (SenseCare cat.no YST0076, not included.)
• Qubit 4.0 Fluorometer with the Qubit dsDNA BR Assay Kit (Life Technologies/ ThermoFisher, not included)

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 Focused Lung Panel – Primer Pools (cat. no. YST0115, not included) Barcoded DNA Library for Ion Torrent NGS Sequencing



Bioanalyzer profile of the TargetPlex FFPE-Direct Focused Lung NGS Library
Expected DNA library fragment profile distribution is between 150 - 220 bps on a DNA 1000 Chip. (A = Lower marker B = Expected libraries C = Upper marker)

qPCR Library Quantification Kits (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: Ion Torrent Manifest File

The Ion Torrent 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 Ion Torrent barcode sequences provided.

SenseCare Barcode	Sequence
BC01	CTAAGGTAAC
BC02	TTACAACCTC
BC03	CCTGCCATTCGC
BC04	TGGAGGACGGAC
BC05	TGAGCGGAAC
BC06	CCTTAGAGTTC
BC07	TCCTCGAATC
BC08	AACCTCATTC