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TruSight® Tumor 26 Reference Guide

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support.illumina.com/custom-protocol-selector.html

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Revision History

Document	Date	Description of Change

Material # 20000848
Document # 15042911 v01
	September 2015	• Updated to new library prep style.
		• Rebranded to TruSight Tumor 26.
		• Corrected Consumables list in Library Denaturing and Pooling section—Removed 1X TE and replaced with EBT to match protocol.
		• Corrected Box 2 and Box 3 in TruSight Tumor 26 Kit.
		• Updated Consumables list:
		  • Added Deparaffinization Solution, QIAamp DNA FFPE Tissue Kit, and KAPA SYBR FAST qPCR Master Mix (2X) (Universal).
		  • Removed 100x TE Buffer.
		  • Corrected PhiX control kit name to PhiX Control v3 and corrected the catalog number.

Part # 15042911 Rev. A	May 2013	Initial release.

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Overview

Chapter 1

Introduction

TruSight Tumor 26 takes a deeper view of variation in solid tumors including lung, colon, melanoma, gastric, and ovarian. This step enables clinical researchers to look beyond point mutations within hot spots in single genes for a more comprehensive view of somatic variation. TruSight Tumor 26 provides amplicon-based library preparation reagents, DNA QC, sample indexes, and oligos targeting identified regions of interest. Sufficient reagents are supplied for 48 samples and the indexes provided enable sample indexing of 4 samples per sequencing run. TruSight Tumor 26 harnesses the paired-end read capability, speed, and high data quality of the MiSeq System, providing on-instrument variant-calling software and cloud-based annotation and filtering software.

The TruSight Tumor 26 protocol offers:

- **High Accuracy, Low-Frequency Variant Detection**—Highly accurate somatic variant analysis at limit of detection below 5% allele frequency across 174 amplicons with 1000x minimum coverage of each region Optimized for formalin-fixed, paraffin-embedded (FFPE) tissues.
- **Optimized for Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues**—Exceptional sample success rate with minimal DNA input for accurate base calling even in degraded FFPE samples.
- **Deep Coverage of Variants Involved with Solid Tumors**—Coverage of exon coding regions for analysis of molecular heterogeneity in highly relevant content selected from CAP and NCCN guidelines and late stage clinical trials.

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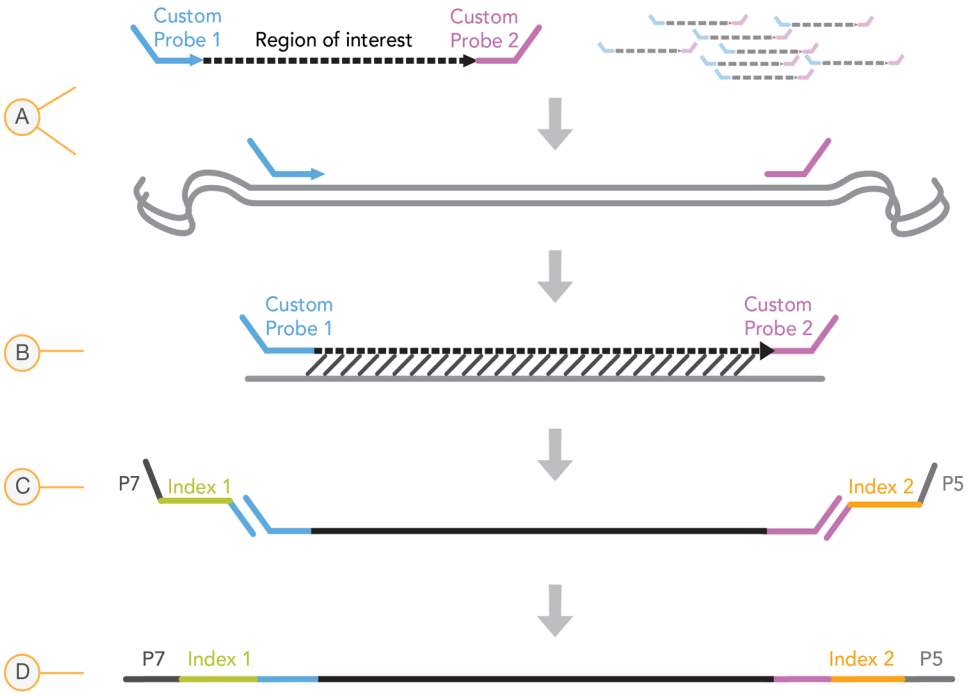
How Does the Assay Work?

For each amplicon, 2 pairs of oligos are designed. One pair is complementary to one strand and another pair to the opposite strand. In separate wells of a 96-well plate, these oligos hybridize to the genomic DNA. Extension and ligation then form DNA templates consisting of the regions of interest flanked by universal primer sequences. Using indexed primers supplied with the kit, DNA templates are then amplified using PCR. The library products are then pooled into a single tube, and sequenced on the MiSeq System.

Figure 1 How the TruSight Tumor 26 Assay Works



A Hybridization of custom oligonucleotide probes
B Extension and ligation
C Addition of indexes and sequencing adapters by PCR
D Final amplicon ready for sequencing on the MiSeq System

DNA Input Recommendations

Formalin-fixed, paraffin-embedded (FFPE) human tissues are a valuable source of material for molecular analysis and clinical studies. Several processes and protocols now exist for the extraction and purification of nucleic acids from FFPE samples. The assays used to evaluate DNA and RNA have evolved from simple monoplex PCR to higher plexity products. As a result, the quality and amount of nucleic acid extracted from FFPE material becomes more critical to the success of these assays.

The TruSight Tumor 26 assay can be used to generate sequencing libraries that are highly multiplexed at both the target and sample level. The high level of assay complexity is enabled by combining an oligo extension-ligation process with universal PCR. Both of these reactions require a largely intact DNA template that can be denatured. The process of preparing FFPE samples negatively impacts DNA quality by fragmenting, cross-linking, and otherwise damaging DNA through various chemical modifications. As a result, it is essential to assess the extent of the damage and, where possible, improve the procedures for fixation of tissue extraction of DNA from FFPE. This adjustment can partially compensate for damage and improve results from the TruSight Tumor 26 assay with FFPE DNA.

DNA Extraction Recommendations

Illumina recommends using the *QIAGEN Supplementary Protocol: Purification of Genomic DNA from FFPE Tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution*. This protocol extracts the highest amount of amplifiable DNA from an FFPE tissue block, with the following modifications:

- Extract gDNA from 8 separate 5 µm FFPE tissue sections per extraction
- Deparaffinize with 320 µl of QIAGEN Deparaffinization Solution
- Lyse samples with 40 µl of Proteinase K in a thermal mixer at 56°C overnight at 1000 rpm to improve genomic DNA yields
- Digest with Proteinase K in a thermal mixer overnight at 1000 rpm
- Decrease elution volume to 30 µl to maximize DNA concentration

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Additional Resources

The following documentation is available for download from the Illumina website.

Resource	Description
TruSight Tumor 26 Protocol Guide (document # 1000000001444)	Provides only protocol instructions. The protocol guide is intended for experienced users. For new or less experienced users, see the TruSight Tumor 26 Reference Guide.
TruSight Tumor 26 Checklist (document # 1000000001445)	Provides a checklist of the protocol steps. The checklist is intended for experienced users. For new or less experienced users, see the TruSight Tumor 26 Reference Guide.
IEM TruSight Quick Reference Card (part # 15048138)	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.

Visit the TruSight Tumor 26 Kit support pages on the Illumina website for additional documentation, software downloads, and best practices.

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Protocol

Introduction

This chapter describes the TruSight Tumor 26 protocol.

- Review Best Practices before proceeding. See *Additional Resources* on page 5 for information on how to access TruSight Tumor 26 Best Practices on the Illumina website.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- If you are pooling, record information about your samples before beginning library preparation for later use in data analysis.
  - Use IEM to create and edit well-formed sample sheets for Illumina sequencing systems and analysis software. See *Additional Resources* on page 5 for information on how to download IEM software and documentation from the Illumina website.
- As a troubleshooting aid, ACD1 (Amplicon Control DNA), and ACP1 (Amplicon Control Oligo Pool) have been included in this kit. Using ACD1 instead of gDNA and ACP1 instead of FPA and FPB in the TruSight Tumor 26 assay can help narrow down issues arising from gDNA sample prep or primer contamination. Libraries made with these controls cannot be sequenced along side other TruSight Tumor 26 libraries as they require longer cycles.

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Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Foil seals are effective at -70°C to 105°C, and suitable for skirted or semiskirted plates.
- Microseal 'A' adhesive film is effective for thermal cycling and easy to cut when using fewer than 96 wells.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
- When multiple plates are used in a step, such as a plate 1 and a plate 2, transfer volumes from the existing plate 1 to the new plate 1. Transfer volumes from the existing plate 2 to the new plate 2.

Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.
  - To pellet beads, centrifuge at 280 × g for 1 minute.

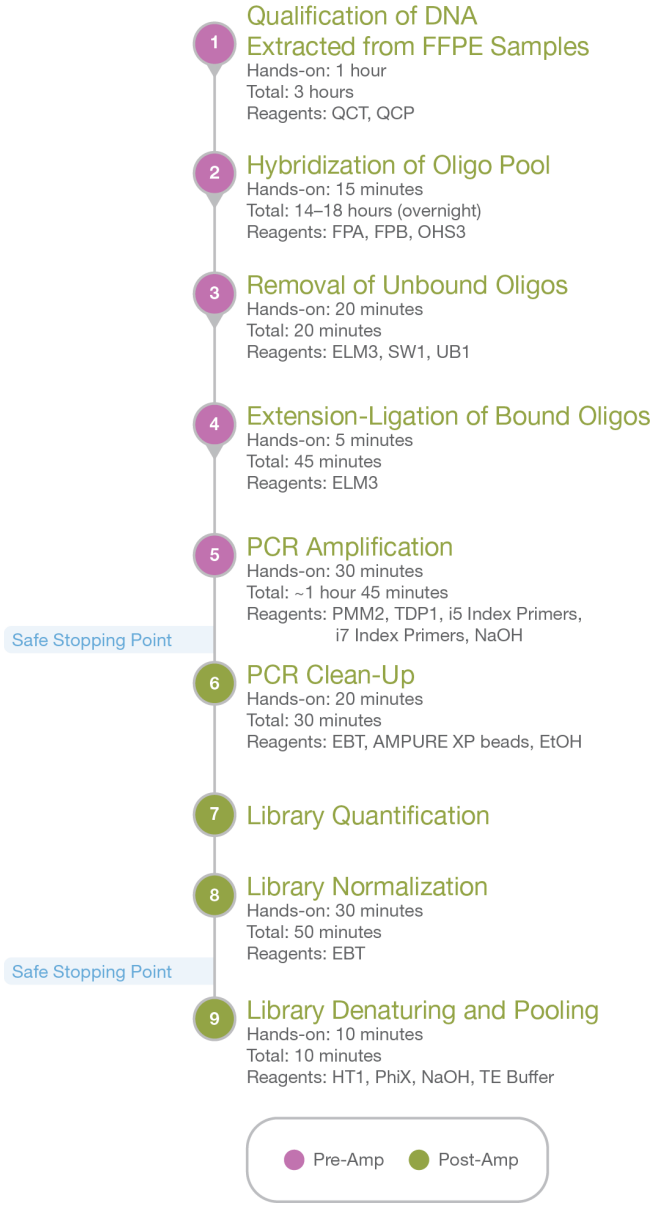
Handling Beads

- Pipette bead suspension slowly.
- When mixing, mix thoroughly.
- To avoid sample loss, confirm that no beads remain in pipette tips after resuspension and mixing steps.
- When washing beads:
  - Use the appropriate magnet for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnet until the instructions specify to remove it.
  - Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

TruSight Tumor 26 Workflow

The following diagram illustrates the workflow using the TruSight Tumor 26 Kit. Safe stopping points are marked between steps.

Figure 2 TruSight Tumor 26 Workflow



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Qualification of DNA Extracted from FFPE Samples

During this step, a qPCR reaction determines the amplifiability of your FFPE-extracted gDNA samples. By comparing the amplifiability of FFPE DNA relative to that of the QCT non-FFPE reference gDNA, a ΔCq value is calculated for each sample. The ΔCq value is then used to predict sample performance in the TruSight Tumor 26 assay. The exact amount of FFPE DNA input varies according to the quality of the extracted DNA.

Consumables

- QCT (Quality Control Template)
- QCP (Quality Control Primer)
- Genomic DNA
- 48 or 96-well plate
- Adhesive seal (dependent on qPCR machine)
- KAPA SYBR FAST qPCR Master Mix (2X) (Universal)
- Nuclease-free water
- PCR 8-tube strips (if using QCT for the first time)

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
QCT	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
QCP	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
Genomic DNA	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
KAPA SYBR FAST qPCR Master Mix (2X) (Universal)	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.

2 If using QCT for the first time, aliquot 5 µl of QCT into different PCR tube strips for long-term storage to avoid freeze-thawing.

3 Place thawed tubes on ice.

Procedure

1 Add 5 µl of QCT to 495 µl of nuclease-free water in a microcentrifuge tube.

2 Vortex the dilution to mix the sample.

3 Add 1 µl of QCP to 9 µl of nuclease-free water in a microcentrifuge tube.

NOTE
Make a larger dilution if qualifying more than one genomic DNA sample.

4 Vortex the dilution to mix the sample.

5 Add 1.5 µl of QIAGEN-extracted genomic DNA to 148.5 µl of nuclease-free water in microcentrifuge tubes to make a 100-fold dilution.

6 Vortex the dilutions to mix the samples.

7 Determine the plate layout of the qPCR reaction.
For 10 samples, use the following layout.

	1	2	3	4	5	6
A	QCT	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9
B	QCT	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9
C	QCT	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9
D	NTC*	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10
E	NTC*	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10
F	NTC*	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10

NTC: No template control. Illumina recommends using nuclease-free water.

8 Prepare the SYBR master mix reaction as follows.
The master mix contains extra volume.

Consumable	µl per well	µl per 48 well plate	µl per 96 well plate
KAPA SYBR FAST qPCR Master Mix (2X) (Universal)	5.0 µl	275 µl	550 µl
Diluted QCP	1.0 µl	55 µl	110 µl
Nuclease-free water	2.0 µl	110 µl	220 µl

9 Mix gently but thoroughly.

10 Place the reaction mix on ice and protect it from light until use.

11 Add 8 µl of the master mix to each well of the plate.

12 According to your plate layout, add 2 µl of the QCT dilution, the sample dilutions, or nuclease-free water to each well of the plate.

13 Seal the plate using an appropriate seal for your qPCR machine.

14 Centrifuge the plate at 250 × g for 1 minute.

15 Make sure that the seal is free of any liquid or dust.

16 Place the plate on the qPCR machine, then close the lid and run the following program.

Procedure	Temperature	Time (minutes)
Hot Start	50°C	2
	95°C	10
x40	95°C	30
	60°C	30
	72°C	30

17 Confirm that the instrument captures images after the 72°C step.

NOTE
Set the Cq threshold to a value that avoids inaccurate measurements due to background (100 RFU on the Bio-Rad 396CFX System).

18 After the final step, the thermal cycler analyzes the quantified libraries. Make sure that amplification of the NTC occurs at least 10 cycles after QCT amplification.

19 Make sure that there is good amplification for the QCT and remove outliers from a triplicate group that are > 0.5 Cq different from the rest of the group.

NOTE
Four or more outliers per plate indicate technical errors.

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20 Exclude replicates exhibiting abnormal amplification curves.
For more information, see Illumina sequencing white paper, *Generating Sequencing Libraries Using DNA from FFPE Samples*.

21 Subtract the average Cq for the QCT from the average Cq for each sample to yield the ΔCq values for each sample.

Hybridization of Oligo Pool

During this step, a custom pool containing upstream and downstream oligos specific to your targeted regions of interest is hybridized to your genomic DNA samples.

WARNING

This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Handle used reagents as chemical waste and discard in accordance with the governmental safety standards for your region. For environmental, health, and safety information, see the SDS for this kit at support.illumina.com/sds.html.

NOTE

Illumina does not support the use of gDNA samples giving a delta Cq value of > 4.

Consumables

- [Optional] ACD1 (Amplicon Control DNA1)
- [Optional] ACP1 (Amplicon Control Oligo Pool 1)
- FPA (TruSight Tumor Oligo Pool A)
- FPB (TruSight Tumor Oligo Pool B)
- OHS3 (Oligo Hybridization for Sequencing 3)
- Genomic DNA
- 96-well skirted PCR plate
- [Optional] Adhesive aluminum foil seal (if a heat sealer is not available)
- Troughs

About Reagents

- Using ACPD1 and ACP1 enables Illumina Technical Support to troubleshoot in the event you need assistance. Illumina technical support recommends including control samples in your assay periodically to establish baselines and monitor overall performance.
- If you are using controls, use ACD1 instead of gDNA and ACP1 instead of FPA and FPB.
- ACP1 is specific for Homo sapiens and does not work with DNA from other species.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
[Optional] ACD1	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
[Optional] ACP1	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
FPA	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
FPB	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.
OHS3	-25°C to -15°C	Thaw at room temperature. If precipitate is observed, incubate at 37°C for 10 minutes and vortex for 1 minute. Repeat as needed until precipitate is no longer visible.

Item	Storage	Instructions
Genomic DNA	-25°C to -15°C	Thaw at room temperature for up to 30 minutes.

2 Place thawed tubes on ice.

3 Set a 96-well heat block to 95°C.

4 Label a new 96-well PCR plate "HYP_Plate_ID".

Procedure

1 Dilute 10 µl of genomic DNA extracted from FFPE samples.
Use the following table to determine the fold dilution required for each calculated delta Cq.

Delta Cq	-2.5 to -1.5	-1.5 to -0.5	-0.5 to 0.5	0.5 to 1.5	1.5 to 4
Dilution	16x	8x	4x	2x	No dilution

NOTE
If preparing libraries from the control DNA in parallel with FFPE DNA samples, dilute 5 µl of ACD1 with 45 µl of TE Buffer. Add 10 µl to each of 2 control wells for FPA and FPB, and/or 2 control wells for ACP1.

2 Add 10 µl of each diluted sample to wells on the left half of the HYP plate, starting with column 1. Then, repeat this process on the right half of the HYP plate, starting with column 7.

3 Using a multichannel pipette, add 5 µl of FPA to all sample-containing wells on the left half of the HYP plate. Then, add 5 µl of FPB to all sample-containing wells on the right half of the HYP plate.

NOTE
If preparing libraries from ACD1, add 5 µl of FPA to 1 control well and 5 µl of FPB to the second control well. If preparing libraries using ACP1, add 5 µl of ACP1 to 2 additional control wells of ACD1.

4 Using a multichannel pipette, add 35 µl of OHS3 to each sample in the HYP plate. Gently pipette to mix.

NOTE
Make sure that there are no crystals or precipitate visible in the OHS3.

5 Seal the HYP plate with a heat sealer or an aluminum foil seal.

6 Centrifuge at 1,000 × g at 20°C for 1 minute.

7 Place the HYP plate in the 95°C heat block and incubate for 1 minute.

8 Change the temperature of the same heat block to 40°C, and incubate for 14–18 hours.

NOTE
Moving the plate from the 95°C heat block to another preheated block set to 40°C can adversely affect hybridization.

Remove Unbound Oligos

This process removes unbound oligos from genomic DNA using a filter capable of size selection. Two wash steps using SW1 ensure complete removal of unbound oligos. A third wash step using UB1 removes residual SW1 and prepares samples for the extension-ligation step.

Consumables

- ELM4 (Extension-Ligation Mix 4)
- SW1 (Stringent Wash 1)
- UB1 (Universal Buffer 1)
- Filter plate with lid (keep spare filter plates as general lab supplies)
- Adapter collar (reusable)
- Midi plate
- Troughs

WARNING

This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Handle used reagents as chemical waste and discard in accordance with the governmental safety standards for your region. For environmental, health, and safety information, see the SDS for this kit at support.illumina.com/sds.html.

WARNING

This set of reagents contains ß-mercaptoethanol. Perform the following procedure in a hood or well-ventilated area.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ELM3	-25°C to -15°C	Thaw at room temperature for ~20 minutes.
SW1	2°C to 8°C	Set aside at room temperature.
UB1	2°C to 8°C	Set aside at room temperature.

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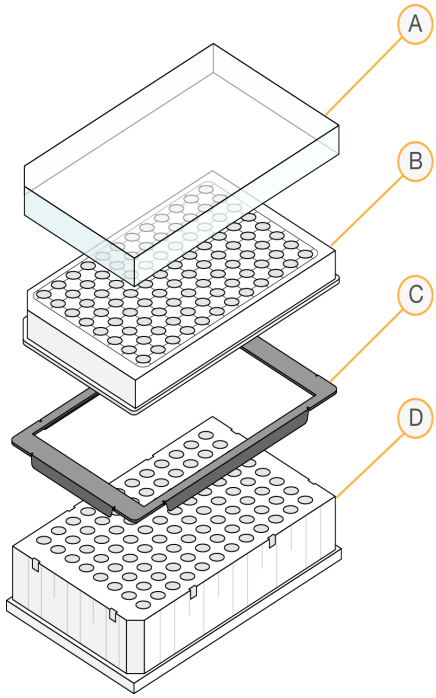
2 Assemble the filter plate assembly unit (FPU) in the following order, from top to bottom:

Figure 3 Filter Plate Unit Assembly



A Lid
B Filter plate
C Adapter collar
D midi plate

3 Apply the FPU barcode plate sticker to the filter plate.

4 Prewash the FPU plate membrane, as follows.

NOTE
Prewash only the wells to be used in the current assay. If using a previously opened filter plate, use only unused wells. Do not reuse wells that have been used in a previous assay.

a Using a multichannel pipette, add 50 µl of SW1 to each well.
b Cover the FPU plate with the filter plate lid.
c Centrifuge the **FPU** at 2,400 × g at 20°C for 5 minutes.

5 Preheat the incubator to 37°C.

6 After the overnight incubation, confirm that the heat block has cooled to 40°C.

NOTE
If the heat block fails to cool to 40°C overnight, repeat library preparation.

Procedure

NOTE
Cover the FPU plate with the filter plate lid during each centrifugation step.

1 Remove the HYP plate from the heat block and centrifuge at 1,000 × g at 20°C for 1 minute to collect condensation.

2 Using a multichannel pipette set to 60 µl, transfer the entire volume of each sample to the corresponding prewashed wells of the FPU plate.

3 Centrifuge the FPU at 2,400 × g at 20°C for 5 minutes.

4 Wash the FPU plate, as follows.

   a Using a multichannel pipette, add 50 µl of SW1 to each sample well.
   b Centrifuge at 2,400 × g for 5 minutes.

5 Repeat the wash as described in the previous step.

6 Discard all the flow-through waste in a hazardous waste container, and then reassemble the FPU.
The same midi plate can be reused for the rest of the pre-amplification process.

7 Using a multichannel pipette, add 45 µl of UB1 to each sample well.

8 Centrifuge the FPU at 2,400 × g for 5 minutes.

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Extension-Ligation of Bound Oligos

This process connects the hybridized upstream and downstream oligos. A DNA polymerase extends the upstream oligo through the targeted region, and is ligated to the 5′ end of the downstream oligo using a DNA ligase. The ligation step forms products containing the targeted regions of interest flanked by sequences required for amplification.

Consumables

- ELM3 (Extension-Ligation Mix 3)
- Adhesive aluminum foil seal
- Troughs

Procedure

1. Using a multichannel pipette, add 45 µl of ELM3 to each sample well of the FPU plate. The extension-ligation reaction takes place on the filter plate membrane.
   If you use care to avoid cross-contamination, changing tips between columns is not required.
2. Seal the FPU plate with adhesive aluminum foil, and then cover with the lid.
3. Incubate the entire FPU plate unit assembly in the preheated 37°C incubator for 45 minutes.
4. During incubation, prepare the IAP (Indexed Amplification Plate) as described in the following section.

PCR Amplification

In this step, the extension-ligation products are amplified using primers. These primers add index sequences for sample multiplexing (i5 and i7) as well as common adapters required for cluster generation (P5 and P7).

Consumables

- PMM2 (PCR Master Mix 2)
- i5 primers (A501–A508)
- i7 primers (A701–A712)
- TDP1 (TruSeq DNA Polymerase 1)
- 0.05 N NaOH (freshly prepared from 10 N NaOH)
- 96-well skirted PCR plate
- Microseal 'B' adhesive film
- Troughs

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
PMM2	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then briefly centrifuge.
Index primers (i5 and i7)	-25°C to -15°C	Thaw at room temperature. Vortex each tube to mix, and then briefly centrifuge.

2 To prepare fresh 0.05 N NaOH, add 20 µl of 10 N NaOH to 3.98 ml of sterile water.

3 Arrange the index primers in the TruSeq Index Plate Fixture, as follows:

- Index 1 (i7) adapters (A701–A712) in columns 1–12
- Index 2 (i5) adapters (A501–A508) in rows A–H

Collect all liquid in the bottoms of the tubes by holding them in place in the rack and tapping it against the bench.

NOTE

If fewer than 48 samples (96 reactions) are being prepared or alternate index combinations are being employed, the Index Plate Fixture is not needed. The indexes can then be added to the appropriate wells of the IAP plate manually.

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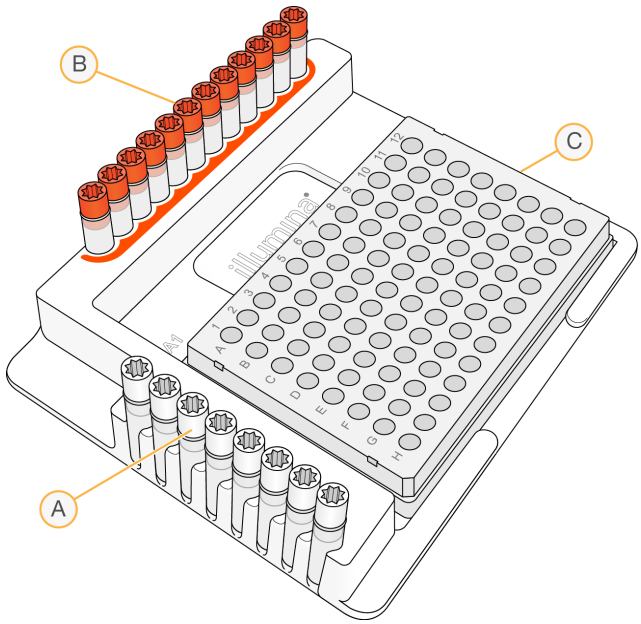
Figure 4 TruSeq Index Plate Fixture



A i5 primers (white caps)
B i7 primers (orange caps)
C IAP plate

4 Label a new 96-well PCR plate IAP (Indexed Amplification Plate) and place the plate on the TruSeq Index Plate Fixture.

5 Add 9 µl of each Index 1 (i7) adapter to each row.

6 Add 9 µl of each Index 2 (i5) adapter to each column.

NOTE
To avoid index cross-contamination, discard the original index caps and apply the new caps provided in the kit. After use, remove all index primer tubes from the working area.

7 Prepare the PMM2/TDP1 PCR master mix according to the number of reactions.
- For 96 reactions, add 60 µl of TDP1 to 2.58 ml of PMM2.
- If preparing fewer reactions, use the following calculation:
  Number of reactions × (0.625 µl TDP + 26.875 µl PMM2).

NOTE
Do not pipette volumes of < 5 µl of TDP1.

8 Invert the PMM2/TDP1 PCR master mix 20 times to mix. Do not vortex.

Procedure

1 When the 45 minute extension-ligation reaction is complete, remove the FPU plate from the incubator. To ensure the reaction supernatant drains into the waste plate, remove the aluminum foil seal and replace with the filter plate lid.

2 Centrifuge the FPU plate at 2,400 × g for 2 minutes.

3 Using a multichannel pipette, add 25 µl of 0.05 N NaOH to each sample well on the FPU plate. If necessary, gently pipette to mix.

4 Incubate the FPU plate at room temperature for 5 minutes.

5 During incubation, use a multichannel pipette to transfer 22 µl of the PMM2/TDP1 PCR master mix to each well of the IAP plate containing index primers.

6 Transfer samples eluted from the FPU plate to the IAP plate, as follows.

NOTE
Slightly tilt the FPU plate to ensure complete aspiration and to avoid air bubbles.

a Set a multichannel P20 pipette to 20 µl and pipette to mix the contents in the first column of the FPU plate.
b Transfer 20 µl from the FPU plate to the corresponding column of the IAP plate.
c Gently pipette to mix.
d Transfer the remaining columns from the FPU plate to the IAP plate in a similar manner.
e After all the samples have been transferred, discard the waste collection midi plate of the FPU. Store the metal adapter collar.

7 Centrifuge the IAP plate at 1,000 × g at room temperature for 1 minute.

8 Transfer the IAP plate to the post-amplification area.

9 On the thermal cycler, set the reaction volume to 60 µl and the temperature ramp speed to maximum.

10 Perform PCR on a thermal cycler using the following program:
- 95°C for 3 minutes
- 27 cycles of:
  - 95°C for 30 seconds
  - 62°C for 30 seconds
  - 72°C for 60 seconds
- 72°C for 5 minutes
- Hold at 10°C

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Verify Library Preparation (Optional)

1. After PCR, combine 5 µl of amplified product with 15 µl of DEPC/DI H20.
2. Run on a 4% TBE agarose gel along with 100 bp ladder to confirm the presence of the 300–330 bp library product. Alternatively, the products can be run on a Bioanalyzer. If generating libraries with ACP1, expect the product to be present at 350–380 bp.

PCR Clean-Up

This process uses AMPure XP beads to purify the PCR products from the other reaction components.

Consumables

- EBT (Elution Buffer with Tris)
- AMPure XP beads
- 96-well midi plates
- Freshly prepared 80% ethanol (EtOH)
- Microseal 'B' adhesive film
- Troughs

Preparation

1. Bring the AMPure XP beads to room temperature.
2. Prepare fresh 80% ethanol from absolute ethanol.
3. Label a new midi plate CLP_Plate_ID (Clean-up Plate).
4. Label a new 96-well PCR plate SGP (Storage Plate).

Procedure

1. Centrifuge the IAP plate at 1,000 × g at 20°C for 1 minute to collect condensation.
2. Invert AMPure XP beads 10 times. Vortex vigorously and then invert again 10 times.

   NOTE
   Immediately proceed to the next step to avoid settling of the beads.

3. Using a multichannel pipette, add 55 µl of AMPure XP beads to each well of the CLP plate.

   NOTE
   The ACD1/ACP1 control can be processed using the same conditions as your CAT.

4. Using a multichannel pipette set to 60 µl, transfer 55 µl PCR product from the IAP plate to the CLP plate.
5. Shake the CLP plate at 1,800 rpm for 2 minutes.
6. Incubate at room temperature without shaking for 10 minutes.
7. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared. Keep the plate on the magnetic stand until step 11.
8. Using a multichannel pipette set to 100 µl, carefully remove and discard the supernatant.

   NOTE
   Delays during this step can lead to bead clumping following removal of the supernatant. Proceed immediately to next step when all supernatant is removed.

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9 Wash 2 times, as follows.

   a Using a multichannel pipette, add 200 µl freshly prepared 80% EtOH. Avoid disturbing the beads.
   b Incubate on the magnetic stand for 30 seconds or until the supernatant appears clear
   c Carefully remove and discard all supernatant from each well.

10 Using a multichannel pipette and fine pipette tips, remove residual EtOH from each well.

11 Remove the CLP plate from the magnetic stand and allow the beads to air-dry for 5 minutes.

12 Using a multichannel pipette, add 40 µl of EBT to each sample.
If you use care to avoid cross-contamination, changing tips is not required.

13 Shake the CLP on a microplate shaker at 1,800 rpm for 5 minutes.
After shaking, if any samples are not resuspended, gently pipette or tap on the bench to mix, and then repeat this step.

14 Incubate at room temperature without shaking for 2 minutes.

15 Place the plate on the magnetic stand for 2 minutes.

16 Transfer 40 µl of the supernatant from the CLP plate to the SGP plate.

17 Centrifuge the SGP at 1,000 × g for 1 minute.

NOTE
Store the SGP plate at room temperature during the library quantification and normalization steps. After library normalization, store the SGP plate at -25°C to -15°C until ready to quantify and normalize any remaining samples.

Library Quantification

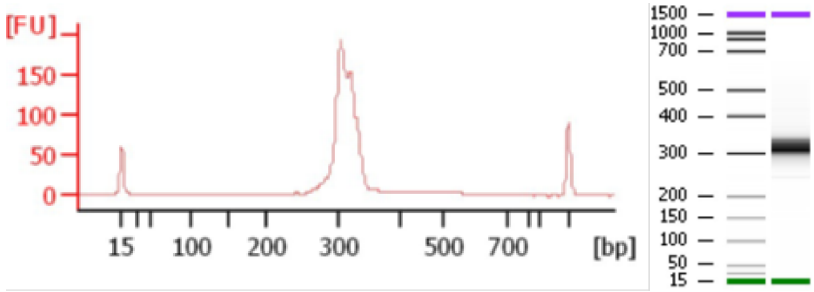
To achieve the highest quality of data on the Illumina MiSeq sequencing platform, it is important to create optimum cluster densities. This step requires accurate quantification of DNA libraries. Illumina recommends quantifying libraries generated from FFPE samples using the Agilent Technologies Bioanalyzer 2100.

Procedure

1 Load 1 µl of the resuspended library on an Agilent Technologies 2100 Bioanalyzer using the Agilent DNA-1000.
Refer to the Agilent DNA Kit Guide (Part Number G2938-90014) and Agilent DNA 1000 Kit Quick Start Guide (Part Number G2938-90015) for complete instructions on using the Agilent Technologies 2100 Bioanalyzer.

2 Check the size and purity of the sample. The expected final product is a band at ~300–330 bp, as in Figure 5.
If generating libraries using ACP1, the expected final product is present at ~350–380 bp. Expect this concentration to correlate with the relative library intensities previously observed on the agarose gel. For more information, see *Verify Library Preparation (Optional)* on page 23.

Figure 5 Representative DNA Sample Prep Library Size Distribution, 300–330 bp



NOTE
If > 10% of the library product is present in the 150–250 bp range, Illumina recommends repeating the *PCR Clean-Up* procedure. Use 40 µl of product and 32 µl of AMPure XP beads for the repeat procedure.

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Library Normalization

This process normalizes the quantity of each library to ensure more equal library representation in your pooled sample.

Consumables

- EBT (Elution Buffer with Tris)
- Microseal 'B' adhesive film
- Midi plate

Procedure

1. From the Agilent Bioanalyzer run, determine the concentration for all samples. Add all concentrations (in terms of nM) for all peaks in the 300–330 bp range (or 350–380 bp for ACP1 libraries) and record the values.
   The expected concentration range is 4–300 nM.
2. Label a MIDI plate LNP_plate (Library Normalization Plate).
3. Dilute 4 µl of all samples > 20 nM to 4 nM with the EBT buffer.
   For example, if a sample is 254 nM, add 4 µl of library to 250 µl of EBT buffer to give 4 nM.
4. For any samples ≤ 20 nM, dilute so that the volume of the final 4 nM working stock is at least 20 µl.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Library Denaturing and Pooling

In preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in Hybridization Buffer, and heat denatured before sequencing on the MiSeq System. PhiX is used as an internal control for sequencing.

Consumables

- EBT (Elution Buffer with Tris)
- HT1 (Hybridization Buffer)
- 2 µl 10 nM PhiX Library
- Laboratory-grade water
- 2.5 L ice bucket
- Microcentrifuge tubes (screw-cap recommended)
- PCR 8-tube strip
- Stock 1.0 N NaOH (diluted to 0.1 N NaOH)

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
HT1	-25°C to -15°C	Thaw at room temperature, and then place in an ice bath to chill.

2. If the LNP plate was stored frozen, thaw at room temperature.
3. Set a heat block with a microcentrifuge tube insert to 96°C.
4. To prepare a fresh dilution of 0.1 N NaOH, add 100 µl stock 1 N NaOH to a microcentrifuge tube containing 900 µl laboratory-grade water.

NOTE
Using freshly diluted NaOH is essential to denature samples for cluster generation on the MiSeq. Preparing a volume of 1 ml prevents small pipetting errors from affecting the final NaOH concentration.

5. Invert the tube several times to mix.

Prepare PhiX Control Library

1. In a microcentrifuge tube, add 2 µl of stock 10 nM PhiX library to 8 µl EBT buffer to yield 10 µl of 2 nM PhiX library.
2. Add 10 µl of 0.1 N NaOH to 10 µl of 2 nM PhiX library to yield 20 µl of 1 nM PhiX library.
3. Vortex the 1 nM PhiX library briefly to mix, then spin at 280 × g for 1 minute.
4. Incubate the PhiX library for 4 minutes 30 seconds at room temperature to denature. Make sure that incubation time does not exceed a maximum of 5 minutes.
5. Add 980 µl of prechilled HT1 to the 20 µl denatured PhiX library to make 20 pM PhiX library.

NOTE
The denatured 20 pM PhiX library can be stored up to 3 weeks at -25°C to -15°C as single-use aliquots. After 3 weeks, cluster numbers tend to decrease.

Prepare Samples for Sequencing

1 Centrifuge the LNP plate at 1,000 × g at 20°C for 1 minute to collect condensation.

2 Determine the samples to be pooled for sequencing.

3 If the LNP plate was stored frozen, use a P200 multichannel pipette to mix each library to be sequenced.

Library Denaturing and Pooling

When sequencing TruSight Tumor 26 libraries on the MiSeq System, Illumina recommends sequencing 4 tumor samples (8 libraries total) per run when using v2 chemistry. If sequencing a different number of samples, adjust the following procedure accordingly.

NOTE
Two libraries represent each sample and are generated from the TruSight Tumor Oligo Pools (FPA and FPB). The FPA and FPB libraries for each sample must be run together on the same flow cell for the MiSeq software to analyze the results for each sample.

NOTE
Control libraries generated from ACD1 with ACP1 must be pooled and run separately from those libraries prepared with FPA and FPB, as they require a longer MiSeq run of 151 cycles.

1 Add 10 µl of 1N NaOH to 140 µl EBT buffer. Vortex the solution.

2 Transfer 5 µl of each 4 nM library to be sequenced from the LNP plate to its own tube in a PCR 8-tube strip.

NOTE
After use, the sealed LNP plate can be stored at -25°C to -15°C for up to 7 days.

3 Add 15 µl of the NaOH/EBT solution to each 5 µl of library and incubate for 5 minutes at room temperature.

4 Label 1 microcentrifuge tube PAL (Pooled Amplicon Library).

5 Add 10 µl of each of the 8 library/NaOH/EBT solutions into the PAL tube.

6 Pipette to mix.
Make sure that the pooled libraries are mixed well.

7 Label 1 microcentrifuge tube DAL (Diluted Amplicon Library).

8 In the DAL tube, mix 792 µl of HT1 with 8 µl of 20 pM PhiX library. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.

9 Add 8 µl from the PAL tube to the DAL tube containing HT1 and PhiX. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.

10 Vortex the DAL tube at top speed.

NOTE
You can make and save additional DAL from the remaining 72 µl of unused PAL. Store the additional DAL at -25°C to -15°C for up to 3 days. Longer storage can lead to suboptimal cluster densities.

11 Centrifuge the DAL tube at 1,000 × g at 20°C for 1 minute to collect contents.

12 Incubate the DAL tube in a heat block at 96°C for 2 minutes.

13 After the incubation, invert the DAL tube to mix. Incubate immediately in the ice-water bath for 5 minutes, then transfer contents to the template position in the MiSeq reagent cartridge.

NOTE
The heat denaturation and cooling steps must occur immediately before loading the DAL into the MiSeq reagent cartridge to ensure efficient template loading onto the flow cell.

14 Proceed to library sequencing as instructed in the *MiSeq System User Guide*.

Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all the required consumables and equipment.

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Acronyms

Acronym	Definition
ACD1	Amplicon Control DNA 1
ACP1	Amplicon Control Oligo Pool 1
CLP	Clean-up Plate
DAL	Diluted Amplicon Library
EBT	Elution Buffer with Tris
ELM3	Extension Ligation Mix 3
FPA	TruSight Tumor Oligo Pool A
FPB	TruSight Tumor Oligo Pool B
FPU	Filter Plate Unit
HT1	Hybridization Buffer
HYP	HYbridization Plate
IAP	Indexed Amplification Plate
LNP	Library Normalization Plate
OHS3	Oligo Hybridization for Sequencing Reagent 3
PAL	Pooled Amplicon Library
PMM2	PCR Master Mix 2
QCP	Quality Control Primers
QCT	Quality Control Template
SGP	Storage Plate
SW1	Stringent Wash 1
TDP1	TruSeq DNA Polymerase 1
UB1	Universal Buffer 1

TruSight Tumor 26 Kit Contents

The TruSight Tumor 26 Kit contains the following components and is shipped on dry ice unless specified otherwise. When you receive your kit, store the kit components at the specified temperatures and in designated pre-amplification and post-amplification areas.

Kit Name	Catalog #	# Samples
TruSight Tumor 26	FC-130-2001	48
TG* TruSight Tumor 26	TG-130-2001	48

NOTE

*TG-labeled consumables include features intended to help reduce the frequency of revalidation. They are available only under supply agreement and require you to provide a binding forecast. Contact your account manager for more information.

TruSight Tumor 26 Kit

Box 1 - Pre-Amplification

Quantity	Reagent	Description	Storage Temperature
1	ACD1	Amplicon Control DNA 1	-25°C to -15°C
1	ACP1	Amplicon Control Oligo Pool 1	-25°C to -15°C
1	OHS3	Oligo Hybridization for Sequencing Reagent 3	-25°C to -15°C
1	ELM3	Extension Ligation Mix 3	-25°C to -15°C
1	PMM2	PCR Master Mix 2	-25°C to -15°C
1	TDP1	TruSeq DNA Polymerase 1	-25°C to -15°C
1	SW1	Stringent Wash 1	2°C to 8°C
1	UB1	Universal Buffer 1	2°C to 8°C

WARNING

This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Handle used reagents as chemical waste and discard in accordance with the governmental safety standards for your region. For environmental, health, and safety information, see the SDS for this kit at support.illumina.com/sds.html.

Box 2 - Post-Amplification

Quantity	Reagent	Description	Storage Temperature
1	HT1	Hybridization Buffer	-25°C to -15°C
4	EBT	Elution Buffer with Tris	Room temperature

Box 3 - TruSight Tumor Oligo Set, Pre-Amplification, Store at -25°C to -15°C

Quantity	Reagent	Description
1	QCP	Quality Control Primers

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Quantity	Reagent	Description
1	FPA	TruSight Tumor Oligo Pool A
1	FPB	TruSight Tumor Oligo Pool B
1	QCT	Quality Control Template

TruSight Tumor 26 Index Kit

Box 1, Pre-Amplification, Store at -25°C to -15°C

Quantity	Description
8	i5 Index Primers, A501 to A508
12	i7 Index Primers, A701 to A712

Box 2, Pre-Amplification, Store at Room Temperature

Quantity	Description
32	i5 Index Tube Caps, White
48	i7 Index Tube Caps, Orange

Additional Required Components, Pre-Amplification, Store at Room Temperature

Consumable	Catalog #
TruSeq Custom Amplicon Filter Plate with Lid	FC-130-1006
TruSeq Index Plate Fixture and Collar Kit (reusable)	FC-130-1007

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
PhiX Control v3	Illumina, FC-110-3001
10 N NaOH (prepare from tablets or use a standard solution)	General lab supplier
96-well skirted PCR plates, 0.2 ml, polypropylene	Bio-Rad, Part # MSP-9601
96-well storage plates, 0.8 ml (midi plates)	Fisher Scientific, Part # AB-0859 Fisher Scientific, Part # AB-0765
Adhesive aluminum foil seal	Beckman Coulter, Part # 538619
Agarose gel (2% or 4%)	General lab supplier
Agencourt AMPure XP, 60 ml kit	Beckman Coulter, Part # A63881/A63880
Conical tubes, 15 ml	General lab supplier
Deparaffinization Solution	QIAGEN, part # 19093
DNA 1000 Kit for Bioanalyzer	Agilent 5067-1504 (for 300 samples)
DNA molecular weight markers	General lab supplier
Eppendorf microcentrifuge tubes (screw top recommended)	General lab supplier
Ethanol, 200 proof for molecular biology	General lab supplier
Ice bucket	General lab supplier
KAPA SYBR FAST qPCR Master Mix (2X) (Universal)	KAPA Biosystems
Microseal 'B' adhesive seals	Bio-Rad, Part # MSB-1001
PCR 8-tube strips	General lab supplier
Solution basin, PVC, nonsterile (trough)	Labcor, Part# 730-001
QIAamp DNA FFPE Tissue Kit	QIAGEN, Part # 56404

Equipment

Pre-PCR Equipment

Consumable	Supplier
37° incubator	Forced Air Oven, VWR International or comparable
Heat block, 96-well	SciGene, Hybex Microsample Incubator for PCR plate Note: This model is recommended for this assay. Passive cooling, as opposed to active cooling performed in a PCR thermal cycler, is recommended for maximum target enrichment specificity and uniformity.
Tabletop centrifuge	General lab supplier (Plate centrifuge that attains designated speeds of protocol)

NOTE
Use a dedicated set of pipettes, pipette tips, vortexer, and centrifuge during pre-amplification steps.

Post-PCR Equipment

Consumable	Supplier
Post-PCR plate shaker	Q Instruments BioShake iQ high-speed thermoshaker (part # 1808-0506) or Q Instruments BioShake XP high-speed lab shaker (part # 1808-0505)
Tabletop centrifuge	General lab supplier (plate centrifuge that attains designated speeds of protocol)
Gel electrophoresis supplies and apparatus	General lab supplier
Bioanalyzer System	Agilent Technologies
Heat block for 1.5 ml centrifuge tubes	General lab supplier
Magnetic stand-96	Invitrogen DynaMag™-96 Side Skirted

NOTE
Use a dedicated set of pipettes, pipette tips, vortexer, heat block, and centrifuge during post-amplification steps.

Thermal Cyclers

The following table lists the recommended settings for the Illumina recommended thermal cycler, as well as other comparable models. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the TruSight Tumor 26 protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad (no longer available for purchase)	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

NOTE

The gDNA qPCR evaluation was optimized on the Illumina Eco Real-Time PCR System and the Bio-Rad CFX396 System. If using other machines, verify the protocol before use.

Index Sequences

Use the following sequences for entry on your MiSeq system sample sheet:

i7 Index PCR Primer	Index Sequence
A701	ATCACGAC
A702	ACAGTGGT
A703	CAGATCCA
A704	ACAAACGG
A705	ACCCAGCA
A706	AACCCCTC
A707	CCCAACCT
A708	CACCACAC
A709	GAAACCCA
A710	TGTGACCA
A711	AGGGTCAA
A712	AGGAGTGG
A501	TGAACCTT
A502	TGCTAAGT
A503	TGTTCTCT
A504	TAAGACAC
A505	CTAATCGA
A506	CTAGAACA
A507	TAAGTTCC
A508	TAGACCTA

Material # 20000848
Part # 15042911 v01

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

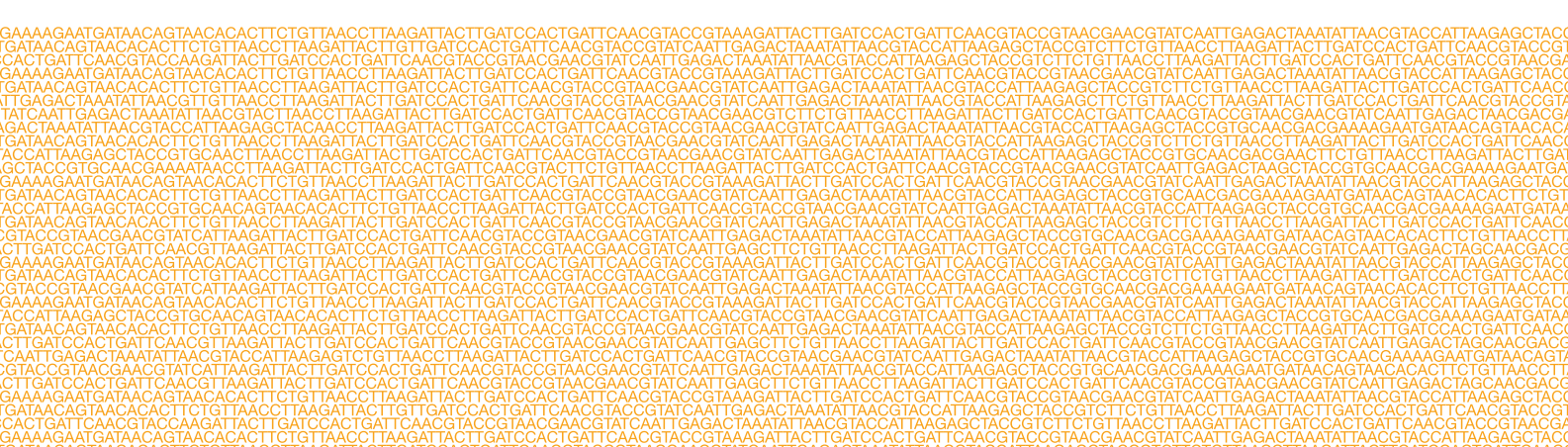
Website	www.illumina.com
Email	techsupport@illumina.com

Table 2 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.



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