Qualify DNA Extracted from FFPE Samples

☐1 [Optional] Make 5 µl aliquots of QCT.
☐2 Place thawed tubes on ice.
☐3 Add 5 µl QCT to 495 µl nuclease-free water.
☐4 Vortex to mix.
☐5 Add 1 µl QCP to 9 µl nuclease-free water.
☐6 Vortex to mix.
☐7 Add 1.5 µl extracted genomic DNA to 148.5 µl nuclease-free water.
☐8 Vortex to mix.
☐9 Determine the plate layout of the qPCR reaction.
☐10 Prepare the SYBR master mix reaction.

Consumable	Per well	Per 48-well plate	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

☐11 Gently invert to mix.
☐12 Set aside on ice away from light.
☐13 Add 8 µl of the master mix to each well.
☐14 Add 2 µl of the QCT dilution, the sample dilutions, or nuclease-free water to each well of the plate.
☐15 Centrifuge at 250 × g for 1 minute.
☐16 Place on the qPCR machine, close the lid, and run the following program.

Procedure	Temperature	Time
Hot Start	50°C	2 min
	95°C	10 min
x40	95°C	30 sec
	60°C	30 sec
	72°C	30 sec

☐17 Make sure that amplification of the NTC occurs at least 10 cycles after QCT amplification.
☐18 Remove outliers from a triplicate group that are > 0.5 Cq different from the rest of the group.
☐19 Exclude replicates exhibiting abnormal amplification curves.
☐20 Subtract the average Cq for the QCT from the average Cq for each sample to yield the ΔCq values for each sample.

Hybridize the Oligo Pool

☐1 Place thawed tubes on ice.
☐2 Preheat a 96-well heat block to 95°C.
☐3 Label a new 96-well PCR plate "HYP_Plate_ID".
☐4 Dilute 10 µl of genomic DNA extracted from FFPE samples according to the following table.

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

☐5 Add 10 µl diluted sample to the left half of the plate.
☐6 Add 10 µl diluted sample to the right half of the plate.
☐7 Add 5 µl FPA to the left half of the plate.
☐8 Add 5 µl FPB to the right half of the plate.
☐9 Add 35 µl OHS3. Gently pipette to mix.
☐10 Seal with a heat sealer or foil seal.
☐11 Centrifuge at 1000 × g at 20°C for 1 minute.
☐12 Place on the heat block and incubate for 1 minute.
☐13 Reduce the heat block to 40°C, and incubate for 14–18 hours.

Remove Unbound Oligos

- ☐1 Assemble the filter plate assembly unit.
- ☐2 Label the filter plate assembly unit FPU.
- ☐3 Prewash the FPU membrane.
- ☐4 Preheat the incubator to 37°C.
- ☐5 Confirm that the heat block has cooled to 40˚C.
- ☐6 Centrifuge at 1000 × g at 20°C for 1 minute.
- ☐7 Transfer the entire volume to the corresponding wells of the FPU.
- ☐8 Centrifuge at 2400 × g at 20°C for 5 minutes.
- ☐9 Wash the FPU.
- ☐10 Repeat the wash.
- ☐11 Discard all the flow-through waste, and then reassemble the FPU.
- ☐12 Add 45 µl UB1.
- ☐13 Centrifuge at 2400 × g for 5 minutes.

Extend and Ligate Bound Oligos

- ☐1 Add 45 µl ELM3.
- ☐2 Seal with a foil seal, and then cover with the lid.
- ☐3 Incubate for 45 minutes.
- ☐4 During incubation, prepare the IAP.

Amplify Libraries

- ☐1 Add 20 µl of 10 N NaOH to 3.98 ml sterile water.
- ☐2 Save the following program as PCR AMP on a thermal cycler with a heated lid.
  - Choose the preheat lid option and set to 100°C
  - 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
- ☐3 Arrange the index primers in the TruSeq Index Plate Fixture.
- ☐4 Label a new 96-well PCR plate IAP and place on the TruSeq Index Plate Fixture.
- ☐5 Add 9 µl of each Index 1 (i7) adapter down each column.
- ☐6 Add 9 µl of each Index 2 (i5) adapter across each row.
- ☐7 Prepare the PMM2/TDP1 PCR master mix.
- ☐8 Invert 20 times to mix. Do not vortex.
- ☐9 Remove FPU from the incubator. Remove foil seal and replace with the filter plate lid.
- ☐10 Centrifuge at 2400 × g for 2 minutes.
- ☐11 Add 25 µl of 0.05 N NaOH. Pipette to mix.
- ☐12 Incubate at room temperature for 5 minutes.
- ☐13 Transfer 22 µl master mix to each well of the IAP plate containing index adapters.
- ☐14 Transfer samples eluted from the FPU plate to the IAP plate.
- ☐15 Centrifuge 1000 × g at for 1 minute.
- ☐16 Transfer the IAP plate to the post-amplification area.

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

☐18 Place on the preprogrammed thermal cycler and run the PCR AMP program.

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.

Clean Up Libraries

☐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.

☐4 Label a new 96-well PCR plate SGP.

☐5 Centrifuge the IAP plate at 1000 × g at 20°C for 1 minute.

☐6 Invert AMPure XP beads 10 times. Vortex vigorously and then invert again 10 times.

☐7 Add 55 µl of AMPure XP beads to the CLP plate.

☐8 Transfer 55 µl PCR product from the IAP plate to the CLP plate.

☐9 Shake at 1800 rpm for 2 minutes.

☐10 Incubate at room temperature without shaking for 10 minutes.

☐11 Place on a magnetic stand until liquid is clear. Keep the plate on the magnetic stand until step 15.

☐12 Remove and discard the supernatant.

☐13 Wash 2 times with 200 µl 80% EtOH.

☐14 Remove residual EtOH.

☐15 Remove the CLP plate from the magnetic stand.

☐16 Air-dry the beads for 5 minutes.

☐17 Add 40 µl EBT.

☐18 Shake at 1800 rpm for 5 minutes.

☐19 Incubate at room temperature without shaking for 2 minutes.

☐20 Place the plate on the magnetic stand for 2 minutes.

☐21 Transfer 40 µl supernatant from the CLP plate to the SGP plate.

☐22 Centrifuge the SGP plate at 1000 × g for 1 minute.

Check Libraries

☐1 Load 1 µl of the resuspended library on an Agilent Technologies 2100 Bioanalyzer using the Agilent DNA-1000.

☐2 Check the size and purity of the sample. The expected final product is a band at ~300–330 bp.

Normalize Libraries

- ☐1 Determine the concentration for all samples.
- ☐2 Label a new midi plate LNP_plate.
- ☐3 Dilute 4 µl of all samples > 20 nM to 4 nM with the EBT buffer.
- ☐4 Dilute at least 20 µl of any samples ≤ 20 nM to 4 nM.

SAFE STOPPING POINT

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

Pool Libraries

- ☐1 If the LNP plate was stored frozen, thaw at room temperature. Pipette to mix.
- ☐2 Centrifuge at 1000 × g at 20°C for 1 minute.
- ☐3 Determine the libraries to be pooled.
- ☐4 In a microcentrifuge tube, add 2 µl of 10 nM PhiX library to 8 µl EBT buffer.
- ☐5 Add 10 µl of 0.1 N NaOH to 10 µl of 2 nM PhiX library to yield 20 µl.
- ☐6 Vortex briefly and then centrifuge at 280 × g for 1 minute.
- ☐7 Incubate for 4.5 minutes at room.
- ☐8 Add 980 µl prechilled HT1 to the 20 µl denatured PhiX library.
- ☐9 Centrifuge at 1000 × g at 20°C for 1 minute.
- ☐10 Determine the samples to be pooled.
- ☐11 If the LNP plate was stored frozen, pipette to mix each library.
- ☐12 Add 10 µl of 1N NaOH to 140 µl EBT buffer. Vortex to mix.
- ☐13 Transfer 5 µl of each 4 nM library from the LNP plate in a PCR 8-tube strip.
- ☐14 Add 15 µl NaOH/EBT solution to each 5 µl of library.
- ☐15 Incubate for 5 minutes at room temperature.
- ☐16 Label a microcentrifuge tube PAL.
- ☐17 Add 10 µl of each library/NaOH/EBT solution to the PAL tube.
- ☐18 Pipette to mix.
- ☐19 Denature and dilute pooled libraries to the loading concentration for the instrument you are using. See the denature and dilute libraries guide for your instrument.

Acronyms

Acronym	Definition
ACD1	Amplicon Control DNA 1
ACP1	Amplicon Control Oligo Pool 1
CLP	Clean-up Plate
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

Acronym	Definition
TDP1	TruSeq DNA Polymerase 1
UB1	Universal Buffer 1