



Agilent NGS FFPE QC Kit

**qPCR-based Quantification
and Qualification of
FFPE-derived DNA Samples
for NGS Target Enrichment**

Protocol

Version D0, December 2015

**For Research Use Only. Not for use in diagnostic
procedures.**



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In this Guide...

This guide provides an optimized protocol for use of the Agilent NGS FFPE QC Kit to quantify and qualify FFPE-derived DNA samples by qPCR prior to use in NGS applications.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment and overview of the workflow) that you should read and understand before you start an experiment.

2 Procedures

This chapter describes the steps to qPCR-analyze the FFPE-derived DNA samples and provides guidelines for implementing the findings in downstream NGS steps.

3 Reference

This chapter includes reference information, including kit contents and troubleshooting.

What's New in Version D0

- Support for use with HaloPlex HS Target Enrichment System (see [“Modifications for the HaloPlex HS Workflow”](#) on page 30)

What's New in Version C0

- Updated guideline for acceptable amplification efficiency to 85–110% (see [page 26](#))
- New recommendation to limit freeze-thaw cycles to five for DNA Standards 1–5 and to aliquot the DNA Standard solutions provided with 96-reaction kits (see [page 14](#))

What's New in Version B0

- Updated product labeling statement

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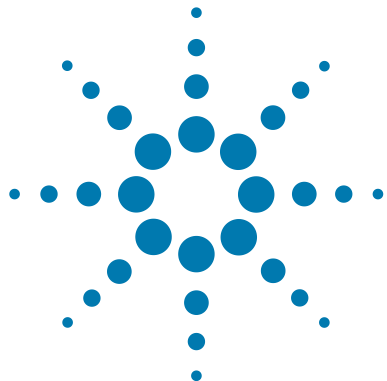
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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.



Procedural Notes

- For optimal consistency, mix gDNA sample solutions well on a vortex mixer then spin the sample tube briefly to collect the liquid.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
 - 1 Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
 - 2 Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
 - 3 Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
 - 4 Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- When preparing frozen reagent stock solutions use the following guidelines:
 - 1 Thaw buffer solutions at room temperature.
Thaw all other reagents (such as enzymes, primers, and nucleotides) on ice.
 - 2 Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
 - 3 Store all components on ice or in a cold block until use, unless otherwise instructed in the protocol.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents

Description	Vendor and part number
Agilent NGS FFPE QC Kit	Agilent
16 reactions	p/n G9700A
96 reactions	p/n G9700B
Qubit BR dsDNA Assay Kit	Life Technologies
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

Required Equipment

Table 2 Required Equipment

Description	Vendor and part number
AriaMx Real-Time PCR System, or equivalent	Agilent p/n G8830A
AriaMx-compatible plasticware:	
96-well plates	Agilent p/n 401490
Optical strip caps	Agilent p/n 401427
Qubit Fluorometer	Life Technologies p/n Q32857
Qubit Assay Tubes	Life Technologies p/n Q32856
DNA LoBind Tubes, 1.5-ml PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Microcentrifuge	General laboratory supplier
P20, P200 and P1000 pipettes	Pipetman or equivalent
Vortex mixer	
Ice bucket	
Sterile, nuclease-free aerosol barrier pipette tips	

Overview of the Workflow

Agilent NGS FFPE QC Kit provides a qPCR-based system for determining the quality and amplifiable quantity of prepared genomic DNA samples derived from formalin-fixed paraffin-embedded (FFPE) human tissues. The Agilent NGS FFPE QC Kit sample quantification and qualification workflow is summarized in [Figure 1](#).

The Agilent NGS FFPE QC Kit is recommended for use in combination with Agilent's NGS target enrichment systems including SureSelect^{XT} Target Enrichment kits (using the Low Input DNA protocol) and HaloPlex HS Target Enrichment kits. Both target enrichment systems are suitable for FFPE samples after making minor protocol modifications based on sample quality, as outlined in this manual. Information on the SureSelect and HaloPlex HS platforms can be found at www.agilent.com/genomics.

The kit helps optimize performance of FFPE-derived DNA samples in NGS applications in the following ways:

- The population of DNA that is intact enough to allow amplification in the FFPE-derived DNA sample is precisely quantified by qPCR. Quantification is done by standard curve analysis, using pre-diluted DNA standards and Primer Set A, which targets a single-copy DNA region of the human genome and produces a 42-bp amplicon.
- The quality of the DNA in the FFPE-derived sample is determined with a DNA integrity score. This analysis is done by comparing qPCR results using Primer Set A to results using Primer Set B, which targets the same DNA region but produces a 123-bp amplicon, resulting in a ΔCq integrity score for the sample. This integrity score is normalized using the provided Human Reference DNA sample, to generate the normalized DNA integrity score ($\Delta\Delta Cq$).
- Guidelines are provided for how to modify Agilent's SureSelect^{XT} Low Input Target Enrichment protocol or the HaloPlex HS protocol according to the $\Delta\Delta Cq$ value for each sample.
- Guidelines are also provided for recommended sequencing depth modifications for FFPE-derived samples with various $\Delta\Delta Cq$ values.

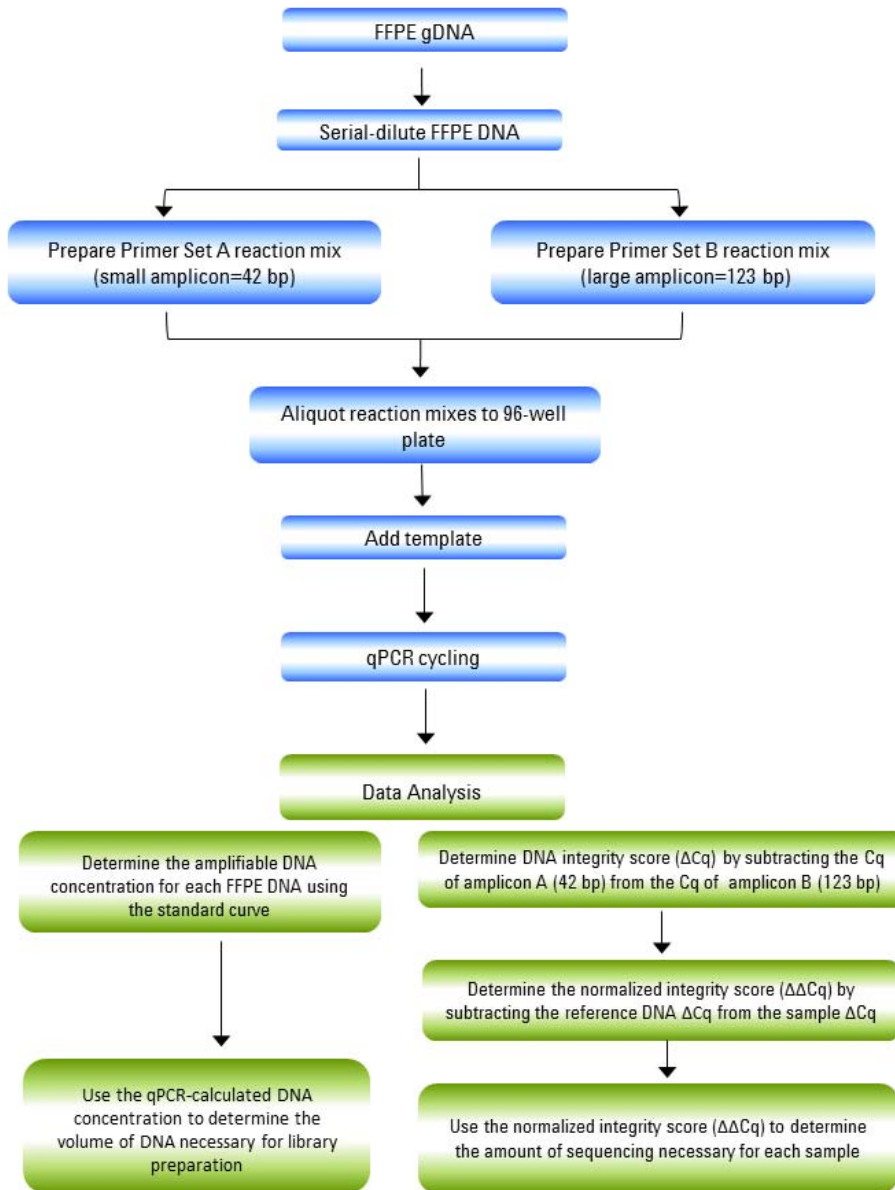


Figure 1 Overall sample qualification workflow.

Preprotocol Considerations

Preparing the FFPE-derived genomic DNA samples as template

Before starting the protocol, purify gDNA samples from FFPE tissue sections. Agilent recommends the Qiagen QIAamp DNA FFPE Tissue Kit (Qiagen p/n 56404) and Qiagen Deparaffinization Solution (Qiagen p/n 19093) for this step.

DNA sample preparation instructions for qPCR (see [page 20](#)) begin with determination of initial sample concentration using the fluorometry-based Qubit dsDNA BR Assay. Using a fluorometry-based method to measure concentration prior to preparing the qPCR samples is important to ensure that the template concentration falls within the quantitative range of the standard curve.

High concentrations of FFPE DNA may be inhibitory to qPCR reactions. Be sure to dilute the FFPE DNA samples to 125 pg/ μ l, as directed on [page 20](#), for use as qPCR template.

Preventing template cross-contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

Limiting freeze-thaw cycles for the DNA Standards

The provided DNA Standards should be subjected to five or fewer freeze-thaw cycles, to ensure optimal amplification efficiency during standard curve generation. When using a 96-reaction kit, after the first thawing of DNA Standards 1–5, subaliquot each DNA Standard solution into single-use aliquots. The plate setup suggested on [page 15](#) requires 12 μ l of each DNA Standard per run.

Recommended Plate Setup for qPCR

To ensure accurate quantification while optimizing throughput, use the following scheme (summarized in [Table 3](#) on page 15) for the qPCR plate:

- Analyze all five of the provided DNA Standards 1–5 amplified with Primer Set A (in triplicate) to generate a standard curve for each run. The range of DNA concentration covered by the standard curve is 2500 pg/μl down to 9.77 pg/μl.
- Analyze 8 FFPE DNA samples per plate, with amplification using Primer Set A and Primer Set B in separate wells, performed in triplicate for each sample/primer combination.
- Analyze the provided Reference DNA with amplification using Primer Set A and Primer Set B in separate wells, performed in triplicate.
- Include no-template control (NTC) reaction in triplicate for each Primer Set A and B.

Table 3 Recommended plate setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1 Primers A	Std 1 Primers A	Std 1 Primers A	FFPE 1 Primers A	FFPE 1 Primers A	FFPE 1 Primers A	FFPE 1 Primers B	FFPE 1 Primers B	FFPE 1 Primers B	Ref DNA Primers B	Ref DNA Primers B	Ref DNA Primers B
B	Std 2 Primers A	Std 2 Primers A	Std 2 Primers A	FFPE 2 Primers A	FFPE 2 Primers A	FFPE 2 Primers A	FFPE 2 Primers B	FFPE 2 Primers B	FFPE 2 Primers B	NTC Primers B	NTC Primers B	NTC Primers B
C	Std 3 Primers A	Std 3 Primers A	Std 3 Primers A	FFPE 3 Primers A	FFPE 3 Primers A	FFPE 3 Primers A	FFPE 3 Primers B	FFPE 3 Primers B	FFPE 3 Primers B	No amp	No amp	No amp
D	Std 4 Primers A	Std 4 Primers A	Std 4 Primers A	FFPE 4 Primers A	FFPE 4 Primers A	FFPE 4 Primers A	FFPE 4 Primers B	FFPE 4 Primers B	FFPE 4 Primers B	No amp	No amp	No amp
E	Std 5 Primers A	Std 5 Primers A	Std 5 Primers A	FFPE 5 Primers A	FFPE 5 Primers A	FFPE 5 Primers A	FFPE 5 Primers B	FFPE 5 Primers B	FFPE 5 Primers B	No amp	No amp	No amp
F	Ref DNA Primers A	Ref DNA Primers A	Ref DNA Primers A	FFPE 6 Primers A	FFPE 6 Primers A	FFPE 6 Primers A	FFPE 6 Primers B	FFPE 6 Primers B	FFPE 6 Primers B	No amp	No amp	No amp
G	NTC Primers A	NTC Primers A	NTC Primers A	FFPE 7 Primers A	FFPE 7 Primers A	FFPE 7 Primers A	FFPE 7 Primers B	FFPE 7 Primers B	FFPE 7 Primers B	No amp	No amp	No amp
H	No amp	No amp	No amp	FFPE 8 Primers A	FFPE 8 Primers A	FFPE 8 Primers A	FFPE 8 Primers B	FFPE 8 Primers B	FFPE 8 Primers B	No amp	No amp	No amp

Use of the Reference Dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Although using the Reference Dye is optional, it is required for optimal results with some instruments, including the recommended Agilent AriaMx instrument.

The 1 mM stock of Reference Dye needs to be diluted using PCR-grade water prior to adding it to the qPCR reactions. Use the dilution guidelines below:

- If using an Agilent qPCR instrument (AriaMx, Mx3000P or Mx3005P Real-Time PCR system), dilute the Reference Dye 1:500 in nuclease-free water to a concentration of 2 μ M. Using this dilution results in a Reference Dye concentration of 30 nM in the final qPCR reactions.
- If using the ABI StepOnePlus instrument, dilute the Reference Dye 1:50 in nuclease-free water for a final concentration of 20 μ M. Using this dilution results in a Reference Dye concentration of 300 nM in the final qPCR reactions.
- If using the Bio-Rad CFX96 instrument, omit the Reference Dye from the qPCR reactions.

Keep all solutions containing the reference dye protected from light. The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the same day for setting up additional assays.

Data acquisition with a real-time PCR instrument

The instrument should be set to collect SYBR Green I data in real-time at the annealing/extension step of each cycle. Consult the manufacturer's instruction manual for the instrument and software version you are using for more information on setting up this mode of data acquisition.

Dissociation Curve analysis

Including a dissociation segment in the qPCR thermal profile is recommended but not required. When running the FFPE QC assays using the recommended Agilent AriaMx instrument and experiment subtype **Quantitative PCR-DNA Binding Dye Including Standard Melt**, a dissociation segment is automatically included in the thermal profile.

Dissociation curves for samples analyzed using the AriaMx instrument are shown in [Figure 2](#) below.

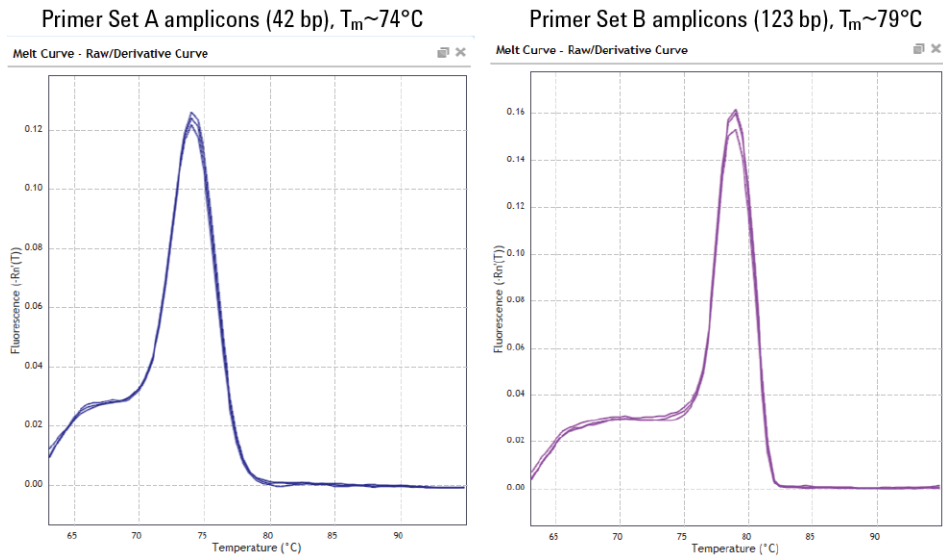
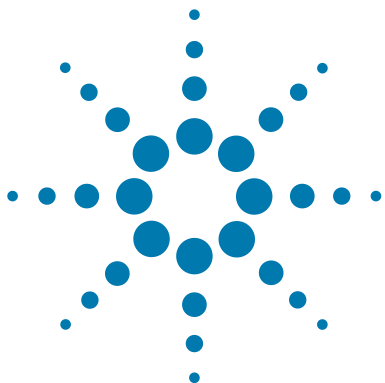


Figure 2 Dissociation curves for qPCR wells analyzed using the AriaMx platform.

1 Before You Begin
Dissociation Curve analysis



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This chapter contains instructions for quantifying the concentration of amplifiable DNA in FFPE-derived DNA samples and for determining the normalized DNA integrity score for each sample.



Amplify FFPE DNA Samples using qPCR

Step 1. Prepare the FFPE-derived genomic DNA samples

- 1 Use the Qubit dsDNA BR Assay to estimate the initial concentration of each gDNA sample. Follow the instructions for the instrument.
- 2 Dilute each gDNA sample with nuclease-free water to 2 ng/ μ l in a 1.5-mL LoBind tube.

Vortex the dilution tubes thoroughly then spin briefly to collect the liquid. Store on ice.
- 3 In a fresh 1.5-mL LoBind tube, transfer 5 μ l of each gDNA sample dilution from [step 2](#) into 75 μ l of nuclease-free water for a final concentration of 125 pg/ μ l.

Vortex thoroughly then spin briefly to collect the liquid. Store on ice.
- 4 Record the dilution factor used for each sample for future use. The total dilution factor is product of the variable dilution factor used in [step 2](#) \times 16 (constant factor used in [step 3](#)).

Step 2. Prepare the qPCR reactions

- 1 If using the Reference Dye, prepare a dilution of the 1 mM Reference Dye stock using PCR-grade water according to [Table 4](#), below.

Table 4 Reference Dye Dilution

qPCR Platform	Dilution to Prepare	Volume of Dilute Dye Needed per 8-Sample Plate
Agilent AriaMx, Mx3000P, or Mx3005P Instrument	1:500	23.7 μ l
ABI StepOne Plus Instrument	1:50	23.7 μ l

NOTE

Keep all solutions containing the reference dye protected from light. The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the same day for setting up additional assays.

- 2 Thaw the 2 \times Brilliant III SYBR Green QPCR Master Mix and keep the tube on ice while setting up the reactions. *SYBR Green is light sensitive; solutions containing the master mix should be protected from light whenever possible.*
- 3 Prepare the Primer Set A (Quantification) reaction mix by combining the components in [Table 5](#) *in order*. Prepare a single reagent mixture for all reactions (plus at least one reaction volume excess).

Vortex the mixture well, then spin the tube briefly to collect the liquid. Keep on ice.

Table 5 Quantification Reaction Mix (Primer Set A)

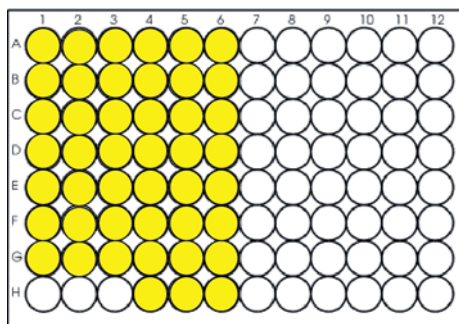
Reagent	Volume for 1 reaction	Volume for 8-sample plate (includes excess)
2 \times Brilliant III SYBR [®] Green QPCR Master Mix	10 μ L	470 μ L
Primer Set A, 42 bp	1 μ L	47 μ L
Diluted Reference Dye (from step 1) [*]	0.3 μ L	14.1 μ L
Nuclease-free, PCR-grade water	4.7 μ L	220.9 μ L
Total	16 μL	752 μL

^{*} If you are not using the Reference Dye, add an equivalent volume of nuclease-free water.

2 Procedures

Step 2. Prepare the qPCR reactions

- 4 Place 16 μL of the Primer Set A Reaction Mix prepared in [step 3](#) into the appropriate individual plate wells or reaction tubes. For 8-sample runs using the recommended plate setup shown on [page 15](#), place the 16- μL aliquots in the shaded wells of Column 1-6 shown below. Keep on ice.



- 5 Prepare the Primer Set B (Qualification) reaction mix by combining the components in [Table 6](#) *in order*. Prepare a single reagent mixture for all reactions (plus at least one reaction volume excess).

Vortex the mixture well, then spin the tube briefly to collect the liquid. Keep on ice.

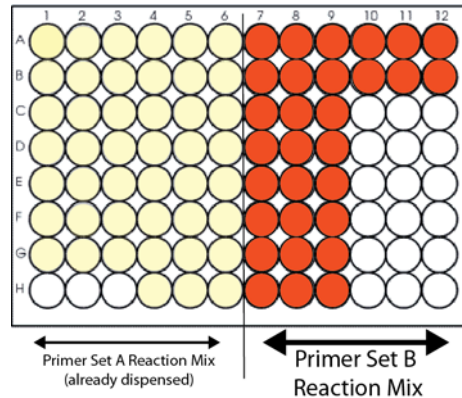
Table 6 Qualification Reaction Mix (Primer Set B)

Reagent	Volume for 1 reaction	Volume for 8-sample plate (includes excess)
2× Brilliant III SYBR [®] Green QPCR Master Mix	10 μL	320 μL
Primer Set B, 123 bp	1 μL	32 μL
Diluted Reference Dye (from step 1) [*]	0.3 μL	9.6 μL
Nuclease-free, PCR-grade water	4.7 μL	150.4 μL
Total	16 μL	512 μL

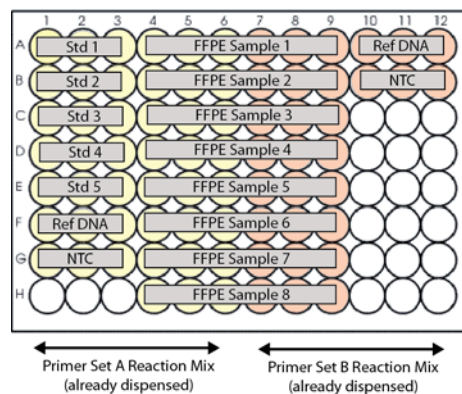
* If you are not using the Reference Dye, add an equivalent volume of nuclease-free water.

Step 2. Prepare the qPCR reactions

- Place 16 μl of the Primer Set B Reaction Mix prepared in [step 5](#) into individual plate wells or reaction tubes. For 8-sample runs using the recommended plate setup shown on [page 15](#), place the 16- μl aliquots in the wells shown in the dark-shaded wells of Column 7-12 shown below. Keep on ice.



- Add 4 μl of the appropriate DNA sample to each well to bring the final reaction volume to 20 μl in all wells. For the no-template control (NTC) reactions, add 4 μl of nuclease-free water in place of the DNA. If using the plate setup in [page 15](#), add the DNA samples as shown below.



- Seal the plate wells with strip caps, then vortex the plate to mix the reactions. Spin the plate briefly to collect the liquid and release any bubbles from the bottoms of the wells.

2 Procedures

Step 3. Run the qPCR amplification

Step 3. Run the qPCR amplification

- 1 Set up the qPCR experiment in the qPCR system software. For Agilent's AriaMx instrument, use experiment subtype **Quantitative PCR-DNA Binding Dye Including Standard Melt**. If using a different instrument, set up the appropriate experiment type for real-time detection of SYBR Green fluorescence, reporting of quantification cycle (C_q) values, and subsequent standard curve analysis.
 - a Assign well information as needed to generate a standard curve from the wells containing the DNA Standards. Enter the amount of template DNA in each Standard well, as shown in [Table 7](#) below.

Table 7 Amount of template in standards wells

DNA Description	Concentration	Amount DNA/qPCR reaction
DNA Standard 1	2500 pg/μl	10,000 pg
DNA Standard 2	625 pg/μl	2,500 pg
DNA Standard 3	156.25 pg/μl	625 pg
DNA Standard 4	39.06 pg/μl	156.25 pg
DNA Standard 5	9.77 pg/μl	39.06 pg

- b Set up the thermal profile shown in [Table 8](#) for Agilent AriaMx, ABI StepOne Plus, and BioRad CFX96 instruments or as shown in [Table 9](#) for Agilent Mx3000P and Mx3005P instruments. A dissociation segment is automatically added to the thermal profile when using Agilent's AriaMx instrument as directed.

Set the instrument to report and detect fluorescence at each cycle during the 63°C annealing/extension step.

Table 8 PCR Cycling Protocol for **Agilent AriaMx, ABI StepOne Plus, or BioRad CFX96**

Number of Cycles	Temperature	Duration
1	95°C	3 minutes
40	95°C	10 seconds
	63°C	20 seconds

Table 9 PCR Cycling Protocol for **Agilent Mx3000P or Mx3005P**

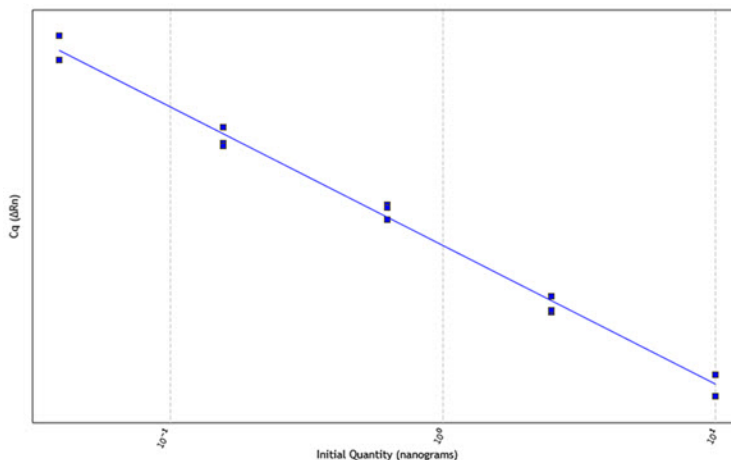
Number of Cycles	Temperature	Duration
1	95°C	3 minutes
40	95°C	10 seconds
	63°C	25 seconds

- 2 Place the plate in the qPCR instrument and run the PCR program.

Analyze the Data

Step 1. Quantify the amplifiable DNA in FFPE samples using the Standard Curve

- 1 In the qPCR analysis software, generate a standard curve (plot of initial DNA quantity versus C_q) using wells assigned to the provided DNA Standards. Verify that the standard curve has an R-squared value >0.98 and an amplification efficiency between 85% and 110%. An example is shown below.



- 2 From the standard curve analysis, get the amount of amplifiable DNA (nanograms) in each FFPE sample well.
- 3 Calculate the concentration of amplifiable DNA (ng/μl) in each FFPE DNA sample:
 - a Divide the amount determined using the standard curve by 4 μl (volume added to each qPCR reaction).
 - b Multiply by the dilution factor recorded for each sample in [step 4](#) on [page 20](#).

For FFPE samples with significantly-degraded DNA, this qPCR-determined concentration of amplifiable DNA is used to calculate the required DNA input volume for SureSelect^{XT} or HaloPlex HS sample preparation.

Step 2. Determine DNA integrity scores for FFPE samples

DNA integrity scores are based on the relative amplification of DNA from a given sample using Primer Set A, which produces a 42-bp amplicon, compared to Primer Set B, which produces a 123-bp amplicon from the same locus. The difference in quantification cycle number, or ΔCq when amplifying using Primer Set B versus A, is used to assign a numerical value to the DNA integrity for each sample.

- 1 Determine ΔCq results for the provided Reference DNA by subtracting the Cq determined for Primer Set A from the Cq determined for Primer Set B.

NOTE

It is normal for the Reference DNA to have a slightly negative ΔCq . The larger amplicon may bind more of the SYBR Green dye and allow signal detection at an earlier cycle compared to the smaller amplicon, even though the amplicon copy number is the same.

- 2 Determine ΔCq results for each FFPE DNA sample by subtracting the Cq determined for Primer Set A from the Cq determined for Primer Set B.
- 3 Find the normalized DNA integrity score ($\Delta\Delta Cq$) for each FFPE DNA sample by subtracting the Reference DNA ΔCq from the sample ΔCq .

Use this $\Delta\Delta Cq$ DNA integrity score to identify any NGS protocol modifications appropriate for a given FFPE sample. See [page 28](#) through [page 31](#) for recommended protocol modifications.

Implement NGS Target Enrichment and Downstream Sequencing Modifications

FFPE-derived DNA samples may be used in Agilent's NGS Target Enrichment and downstream sequencing workflows after making minor protocol modifications. The modifications appropriate for the SureSelect workflow and for the HaloPlex workflow are described in separate sections below.

Modifications for the SureSelect Workflow

Step 1. Implement SureSelect Protocol Modifications

FFPE-derived DNA samples may be used in the SureSelect^{XT} Library Preparation and Target Enrichment protocols after making minor protocol modifications. Protocol modifications that should be applied to all FFPE samples are summarized in [Table 10](#). Additional protocol modifications may be appropriate, depending on the quality of the FFPE sample DNA. Modifications based on the normalized integrity score ($\Delta\Delta Cq$ values) are summarized in [Table 11](#). For the complete SureSelect^{XT} protocol, go to www.genomics.agilent.com and search for document part number G7530-90000 (version B.2 or later). Use the low input (200 ng) library preparation protocol option.

Table 10 SureSelect^{XT} protocol modifications for all FFPE samples

Protocol Step and Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
Duration of DNA Shearing	6 minutes	4 minutes
Dilution of SureSelect Adaptor Oligo Mix for Ligation reaction	Use 1:10 dilution of SureSelect Adaptor Oligo Mix	Use undiluted SureSelect Adaptor Oligo Mix
Amount of prepared library added to Hybridization reaction	750 ng	500–750 ng (use maximum available in range)
Amount of captured DNA bead suspension added to post-capture PCR	14 μ l	30 μ l (decrease the amount of water added to post-capture PCR by 16 μ l to compensate for greater volume of captured DNA)

Table 11 SureSelect^{XT} protocol modifications based on DNA integrity score

Protocol Step and Parameter	non-FFPE Samples	FFPE Samples		
		$\Delta\Delta Cq \leq 1$	$\Delta\Delta Cq$ between 1 and 4	$\Delta\Delta Cq > 4$
DNA input for Library Preparation	200 ng, based on Qubit Assay	200 ng, based on Qubit Assay	100 to 200 ng of amplifiable DNA, based on qPCR quantification	100 to 200 ng of amplifiable DNA, based on qPCR quantification
Pre-capture PCR cycle number	10 cycles	10 cycles	10 cycles	13 cycles

Step 2. Implement Downstream Sequencing Modifications

- 1 After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines in [Table 12](#) below to determine the amount of extra sequencing output required for FFPE DNA samples, based on the $\Delta\Delta Cq$ DNA integrity score.

For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with $\Delta\Delta Cq$ score of 1 requires 150–200 Mb of sequencing output to achieve the same coverage.

Table 12 Recommended sequencing augmentation for FFPE-derived DNA samples

$\Delta\Delta Cq$ value	Recommended fold increase for FFPE-derived sample
<0.5	No extra sequencing output
between 0.5 and 2	Increase sequencing allocation by 1.5× to 2×
between 2 and 3.5	Increase sequencing allocation by 3×
between 3.5 and 5	Increase sequencing allocation by 4× to 5×
>5	Increase sequencing allocation by 6× to 10×

- 2 For all FFPE sample-derived libraries, set up the sequencing run to include adapter trimming.

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Modifications for the HaloPlex HS Workflow

To do this step, use the IEM Sample Sheet Wizard. When prompted by the wizard, select the **Use Adapter Trimming** and **Use Adapter Trimming Read 2** options. This enables the MiSeq Reporter software to identify the adaptor sequence and trim the adaptor from reads.

Modifications for the HaloPlex HS Workflow

Step 1. Implement HaloPlex HS Protocol Modifications

FFPE-derived DNA samples may be used in Agilent's HaloPlex HS Target Enrichment protocol after making minor protocol modifications. Specifically, the DNA input amount should be modified based on the normalized integrity score ($\Delta\Delta Cq$ values) as summarized in [Table 13](#). For the complete HaloPlex HS for Illumina protocol, go to www.genomics.agilent.com and search for document part number G9931-90000.

Table 13 HaloPlex HS protocol modifications based on DNA integrity score

Protocol Step and Parameter	non-FFPE Samples	FFPE Samples		
		$\Delta\Delta Cq \leq 1.3$	$\Delta\Delta Cq$ between 1.3 and 1.5	$\Delta\Delta Cq$ between 1.5 and 2.5*
DNA input for Library Preparation	50 ng, based on Qubit Assay	50 ng, based on Qubit Assay	50 ng of amplifiable DNA, based on qPCR quantification	100 ng of amplifiable DNA, based on qPCR quantification

* FFPE samples with $\Delta\Delta Cq$ values >2.5 may be used for HaloPlex HS Target Enrichment, but enrichment results and sequencing performance may be impacted. For best results, use the same protocol modifications provided for samples with $\Delta\Delta Cq$ values between 1.5 and 2.5.

Step 2. Implement Downstream Sequencing Modifications

Determine the amount of extra sequencing output required for FFPE DNA samples.

NOTE

The recommendation for non-FFPE samples is to collect sequencing output equivalent to 200× the Total Sequenceable Design Size.

- 1 Find the Total Sequenceable Design Size for your probe in the HaloPlex Design Report.
- 2 Use the guidelines in [Table 14](#) to determine the recommended amount of sequencing output for each HaloPlex HS-enriched FFPE sample based on the $\Delta\Delta Cq$ DNA integrity score.

Table 14 Recommended sequencing augmentation for FFPE-derived DNA samples

$\Delta\Delta Cq$ value	Recommended amount sequencing output for FFPE-derived sample
≤ 1.3	300–600× Total Sequenceable Design Size
between 1.3 and 1.5	600–1200× Total Sequenceable Design Size
between 1.5 and 2.5*	1200× Total Sequenceable Design Size

* FFPE samples with $\Delta\Delta Cq$ values >2.5 may be used for HaloPlex HS Target Enrichment, but enrichment results and sequencing performance may be impacted. For best results, use the same protocol modifications provided for samples with $\Delta\Delta Cq$ values between 1.5 and 2.5.

For example, if the Total Sequenceable Design Size is 1 Mb, an FFPE sample with $\Delta\Delta Cq$ score of 1.4 requires 600–1200 Mb of sequencing output to achieve coverage similar to that expected for an intact DNA sample with 200 Mb of sequencing output.

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Modifications for the HaloPlex HS Workflow



3 Reference

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This chapter contains reference information, including component kit contents and troubleshooting guidelines.



Kit Contents

Each Agilent NGS FFPE QC Kit contains the following components:

Table 15 Kit Contents

Reagent	Quantity	
	16 Reactions	96 Reactions
2× Brilliant III SYBR [®] Green QPCR Master Mix	1 tube	5 tubes
Reference Dye, 1 mM	1 tube	1 tube
Primer Set A, 42 bp	1 tube	1 tube
Primer Set B, 123 bp	1 tube	1 tube
DNA Standard 1 (2500 pg/μl)	1 tube	1 tube
DNA Standard 2 (625 pg/μl)	1 tube	1 tube
DNA Standard 3 (156.25 pg/μl)	1 tube	1 tube
DNA Standard 4 (39.06 pg/μl)	1 tube	1 tube
DNA Standard 5 (9.77 pg/μl)	1 tube	1 tube
Reference DNA	1 tube	1 tube

Storage Conditions

Store all components at -20°C upon receipt.

Once the 2× Brilliant III Ultra-Fast SYBR[®] Green QPCR Master Mix has been thawed, it can be stored at 4°C for up to one month or returned to -20°C for long term storage.

The 2× QPCR Master Mix and Reference Dye are light sensitive; protect these solutions from light whenever possible.

For 96 reaction kits, once DNA Standards 1–5 have been thawed, subaliquot each DNA Standard solution into single-use aliquots. The DNA Standards should be subjected to five or fewer freeze-thaw cycles for optimal amplification efficiency during standard curve generation.

Troubleshooting Guide

If little or no increase in fluorescence is detected during cycling

- ✓ Ensure that the sample was diluted to 125 pg/μl using the quantification and dilution steps outlined on [page 20](#). If unsure, make a fresh dilution of the sample before repeating the qPCR assay.
- ✓ Gel-analyze the PCR product to determine whether there was detectable amplification.
- ✓ FFPE samples may contain PCR inhibitors. Check for PCR inhibition by adding an aliquot of the sample to a validated PCR assay. Alternatively, check for inhibition by repeating the qPCR, using a dilution series of the FFPE sample; inhibition is indicated by delayed Cq values for samples with higher concentrations.
- ✓ Verify that the correct dilution of the Reference Dye was used.
- ✓ Ensure that the qPCR thermal cycling program on [page 24](#) was entered correctly. In particular, verify that the initial 95°C incubation step was performed for exactly 3 minutes. The full 3-minute duration is required to activate the DNA polymerase, but exceeding this 3-minute duration can adversely affect performance.

If the R-squared value of the standard curve is <0.98, or the amplification efficiency is not between 85% and 110%

- ✓ Follow the storage condition instructions for the 2× Brilliant III SYBR® Green QPCR Master Mix. (see [page 34](#)). Multiple freeze-thaws, excessive exposure to light, and long-term storage at temperatures higher than -20°C can all impact the performance of the master mix.
- ✓ If you are not using an Agilent instrument for qPCR, the cycling parameters provided in the protocol may need to be optimized for your qPCR instrument. Try optimizing the annealing temperature and extension time to improve the quality of the standard curve.

If replicate reactions for a single sample yield significantly different Cq values

- ✓ If one of the replicate reactions failed to amplify, the data from the failed replicate can be discarded. To calculate the concentration, use only the data from the replicates that amplified.
- ✓ If all replicates amplified, but they yielded significantly different Cq values, repeat the qPCR.

If Cq values obtained from an FFPE sample dilutions is not within the range covered by the standard curve

- ✓ If Cq value is too high, repeat the qPCR with a more concentrated sample dilution.
- ✓ If Cq value is too low, repeat the qPCR with a less concentrated sample dilution.

If qPCR-calculated DNA concentration values for a sample vary in different experiments

- ✓ Vortex the genomic DNA sample and each dilution very thoroughly before each pipetting step.
- ✓ To ensure accuracy and precision during DNA serial dilution steps, use P200 or smaller volume pipettes that have been properly calibrated.

If Cq value for Primer Set A (42-bp amplicon) is higher than Cq value for Primer Set B (123-bp amplicon)

- ✓ It is normal for the Reference DNA to have a slightly negative ΔCq (see [page 27](#)). Other high-quality DNA samples, including unusually intact FFPE-derived DNA samples may also have a slightly negative ΔCq . Use the $\Delta\Delta Cq$ value to normalize the data for all samples.
- ✓ Verify that the correct primer pair was added to each reaction mixture by analyzing dissociation curves for the qPCR reaction products. See [page 17](#) for more information.

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In This Book

This document describes how to use the Agilent NGS FFPE QC Kit to quantify and qualify FFPE-derived DNA samples.

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