



FFPE-Derived DNA Quality Assessment

**In Preparation for HaloPlex
Target Enrichment**

Protocol

Version A, March 2013

**Research Use Only. Not for use in Diagnostic
Procedures.**



Agilent Technologies

Notices

© Agilent Technologies, Inc. 2013

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

G9900-90050

Edition

Version A, March 2013

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

Technical Support

For technical product support, contact your local Agilent Support Services representative.

For US and Canada, call (800) 227-9770 (option 3,4,4). For other countries, find your support center telephone numbers at www.agilent.com/chem/contactus.

Or send an e-mail to:
SureSelect.Support@agilent.com

Notice to Purchaser

Research Use Only. Not for use in diagnostic procedures.

Warranty

The material contained in this document is provided “as is,” and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A **WARNING** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a **WARNING** notice until the indicated conditions are fully understood and met.

In this Guide...

This guide describes a protocol for qualification of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues for target enrichment using the Agilent HaloPlex system.

1 Before You Begin

This chapter contains information (such as required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the PCR-based DNA qualification protocol and includes guidelines for data analysis and suggested HaloPlex protocol modifications for FFPE-derived DNA samples.

Content

1 Before You Begin

Required Reagents 8

Required Equipment 9

Safety Notes 9

2 Protocol

Qualification of DNA Samples for HaloPlex Target Enrichment 12

Step 1. PCR-amplify GAPDH targets from FFPE-derived DNA and Reference DNA samples 13

Step 2. Purify the amplicon DNA 15

Step 3. Measure amplicon yields from test and Reference DNA samples 17

Step 4. Analyze amplicon yield ratio and identify protocol modifications 18



1 Before You Begin

Required Reagents 8

Required Equipment 9

Safety Notes 9

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



1 Before You Begin
Required Reagents

Required Reagents

Table 1 Required Reagents for FFPE DNA Sample Analysis

Description	Vendor and part number
Reference DNA	need information
Herculase II Fusion Enzyme with dNTPs (100 mM; 25 mM for each nucleotide), 200 reactions*	Agilent p/n 600677
Primer 105 FWD (hGAPDH Region 1–105 bp–Forward Primer): 5'-GGCTGAGAACGGGAAGCTTG-3'	General laboratory oligonucleotide supplier; HPLC purified
Primer 105 REV (hGAPDH Region 1–105 bp–Reverse Primer): (5'-ATCCTAGTTGCCTCCCAAA-3')	General laboratory oligonucleotide supplier; HPLC purified
Primer 236 FWD (hGAPDH Region 2–236 bp–Forward Primer): (5'-CGGGTCTTTGCAGTCGTATG-3')	General laboratory oligonucleotide supplier; HPLC purified
Primer 236 REV (hGAPDH Region 2–236 bp– Reverse Primer): (5'-GCGAAAGGAAAGAAAGCGTC-3')	General laboratory oligonucleotide supplier; HPLC purified
10 mM Tris-HCl, pH 7.5–8.5, molecular biology grade	General laboratory supplier
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer	
100 assays, 2-1000 ng	Life Technologies p/n Q32850
500 assays, 2-1000 ng	Life Technologies p/n Q32853
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881

* Also available separately as Herculase II Fusion DNA Polymerase, 40 reactions (Agilent p/n 600675) and 100 mM dNTP Mix (Agilent p/n 200415, sufficient for 1000 HaloPlex enrichment reactions).

Required Equipment

Table 2 Required Equipment for FFPE DNA Sample Analysis

Description	Vendor and part number
2100 Bioanalyzer Platform and Consumables	
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Thermal Cycler	Agilent SureCycler 8800, p/n G8800A or equivalent
Thermal cycler-compatible 0.2-mL strip tubes or 96-well plates	See manufacturer's recommendations
Magnetic separator compatible with strip tubes or 96-well plates	Agencourt SPRIPlate Super Magnet Plate p/n A32782, or equivalent
Benchtop microcentrifuge	VWR p/n 93000-196, or equivalent
P10, P20, and P200 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Qubit 2.0 Fluorometer	Life Technologies p/n Q32866
Qubit assay tubes	Life Technologies p/n Q32856
Ice bucket	General laboratory supplier
Vortex mixer	General laboratory supplier

Safety Notes

CAUTION

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

1 Before You Begin

Safety Notes



2 Protocol

Qualification of DNA Samples for HaloPlex Target Enrichment 12

Step 1. PCR-amplify GAPDH targets from FFPE-derived DNA and Reference DNA samples 13

Step 2. Purify the amplicon DNA 15

Step 3. Measure amplicon yields from test and Reference DNA samples 17

Step 4. Analyze amplicon yield ratio and identify protocol modifications 18

This section contains a PCR-based protocol for FFPE-derived DNA sample quality assessment and suggested modifications to the HaloPlex target enrichment protocol for FFPE-derived DNA samples.



Qualification of DNA Samples for HaloPlex Target Enrichment

Results of target enrichment using the HaloPlex system are influenced by sample DNA integrity. Since DNA purified from formalin-fixed paraffin-embedded (FFPE) tissues can be highly degraded, it is useful to qualify the FFPE DNA samples prior to target enrichment in order to assess sample suitability for enrichment and to identify sequencing protocol modifications that may improve performance.

You can assess the suitability of your FFPE-derived DNA samples for HaloPlex target enrichment using the multiplex PCR-based qualification assay described here. In this protocol, each FFPE DNA sample is used as template for PCR amplification of two independent GAPDH amplicons. Yield of amplicons from the FFPE DNA template is measured and compared to yield of amplicons from an intact reference DNA template such as a HapMap DNA sample. The resulting sample-to-reference yield ratio serves as a quantitative indicator of DNA integrity which can be used to predict sample performance in HaloPlex target enrichment.

Step 1. PCR-amplify GAPDH targets from FFPE-derived DNA and Reference DNA samples

- 1 Use the Qubit dsDNA BR Assay or PicoGreen staining kit to determine the concentration of your FFPE tissue-extracted DNA and the Reference DNA samples.

Follow the manufacturers instructions for the kits and instruments.

- 2 Dilute the each DNA sample to a final DNA concentration of 5 ng/ μ L in 10 mM Tris-HCl, pH 7.5–8.5.

Store the DNA samples on ice.

- 3 Prepare each of the four hGAPDH PCR primers listed in [Table 1](#) on page 8 at a final DNA concentration of 24 μ M.
- 4 Prepare the multiplex PCR master mix on ice by combining the reagents in the following table. Prepare enough master mix for the number of FFPE samples to be analyzed, plus one or more reference DNA samples, plus one reaction excess. Include the appropriate high-quality reference DNA sample(s) in each PCR amplification assay.

Table 3 Preparation of PCR master mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Nuclease-free water	31.9 μ L	414.7 μ L
5X Herculase II Reaction Buffer	10 μ L	130 μ L
dNTPs (100 mM, 25 mM for each dNTP)	0.6 μ L	7.8 μ L
Primer 105 FWD (24 μ M)	1.25 μ L	16.25 μ L
Primer 105 REV (24 μ M)	1.25 μ L	16.25 μ L
Primer 236 FWD (24 μ M)	1.25 μ L	16.25 μ L
Primer 236 REV (24 μ M)	1.25 μ L	16.25 μ L
Herculase II Fusion DNA Polymerase	0.5 μ L	6.5 μ L
Total	48 μL	624 μL

- 5 Mix the master mix components by gentle vortexing, then distribute 48- μ L aliquots to fresh 0.2-mL reaction tubes.

2 Protocol

Step 1. PCR-amplify GAPDH targets from FFPE-derived DNA and Reference DNA samples

- 6 Add 2 μL of the appropriate 5 ng/ μL DNA sample to each tube.
- 7 Mix by gentle vortexing and then spin briefly to collect the liquid.
- 8 Place the amplification reaction tubes in a thermal cycler and run the program in [Table 4](#), using a heated lid.

Table 4 PCR program for FFPE DNA quality assessment

Segment	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	26	95°C	30 seconds
		60°C	60 seconds
		72°C	60 seconds
3	1	72°C	5 minutes
4	1	4°C	Hold

Step 2. Purify the amplicon DNA

In this step, the DNA amplicons are purified using AMPure XP beads.

- 1 Let the AMPure XP beads come to room temperature for at least 30 minutes.
- 2 Prepare 400 μL of 70% ethanol per sample, plus excess, for use in [step 8](#).
- 3 Mix the AMPure XP bead suspension well, until the suspension appears homogeneous and consistent in color.
- 4 Add 100 μL of the homogenous bead suspension prepared in [step 3](#) to each 50- μL PCR sample. Vortex thoroughly.

Using this bead-to-sample volume ratio is imperative to ensure optimal purification results.

- 5 Incubate samples for 5 minutes at room temperature with continuous shaking.

Make sure the samples are properly mixing in the wells during the 5-minute incubation.

- 6 Spin briefly to collect the liquid, then place the tubes in the magnetic plate. Wait for the solution to clear (approximately 5 minutes).
- 7 Keep the tubes in the magnetic plate. Carefully remove and discard the cleared solution from each tube using a 100- μL pipette set to 100 μL . Do not touch the beads while removing the solution.
- 8 Continue to keep the tubes in the magnetic plate while you add 200 μL of 70% ethanol into the tubes.

Use fresh 70% ethanol for optimal results.

- 9 Wait for 30 seconds to allow any disturbed beads to settle, then remove the ethanol using a 200- μL pipette set to 200 μL .
- 10 Repeat [step 8](#) and [step 9](#) once for a total of two washes.
- 11 Remove any residual ethanol with a 20- μL volume pipette.
- 12 Air-dry the tubes with open lids at room temperature until the residual ethanol completely evaporates.

Make sure all ethanol has evaporated before continuing.

- 13 Remove tubes from the magnetic plate and add 40 μL of 10 mM Tris-HCl to each sample.

2 Protocol

Step 2. Purify the amplicon DNA

- 14** Mix thoroughly by pipetting up and down 15 times using a 100- μ L pipette set to 30 μ L.
- 15** Incubate for 2 minutes at room temperature to allow elution of DNA.
- 16** Put the tube in the magnetic plate and leave for 2 minutes or until the solution is clear.
- 17** Remove the cleared supernatant (approximately 40 μ L) to a fresh tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, samples may be stored at -20°C prior to analysis. Avoid subjecting the stored DNA samples to multiple freeze-thaw cycles.

Step 3. Measure amplicon yields from test and Reference DNA samples

Use a Bioanalyzer High Sensitivity DNA Assay kit and the 2100 Bioanalyzer with 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the reagent kit guide for general Bioanalyzer instrument and assay setup instructions.

- 1 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μL of purified amplicons from the test sample and 1 μL of purified amplicons from the Reference DNA sample for the analysis.
- 2 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 3 Analyze the electropherogram for each sample.
 - Check that the electropherogram shows one peak with average size of 105 bp ($\pm 10\%$) and a second peak with average size of 236 bp ($\pm 10\%$).
 - Determine the concentration of both GAPDH amplicons (105-bp and 236-bp) in the sample by integration under each of the two peaks.

See [Figure 1](#) for sample Bioanalyzer electropherograms.

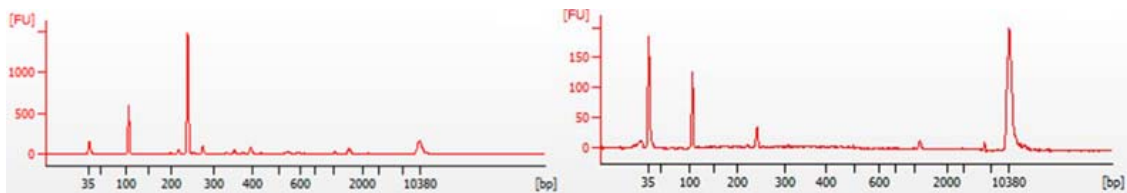


Figure 1 Example of 2100 Bioanalyzer system electropherograms for an intact reference DNA sample (left) and an FFPE-derived DNA sample (right).

Step 4. Analyze amplicon yield ratio and identify protocol modifications

Use the yield values determined in Step 3 to calculate the test-to-reference yield ratios for each amplicon. The average yield ratio may then be used as a quantitative measure of FFPE-derived DNA quality in order to predict performance in the HaloPlex target enrichment workflow. Suggested adjustments to the HaloPlex target enrichment protocol are provided.

- 1 Calculate the yield ratio for the 105-bp amplicon according to the following formula:

$$105\text{-bp ratio} = 105\text{-bp yield FFPE DNA} / 105\text{-bp yield reference DNA}$$
- 2 Calculate the yield ratio for the 236-bp amplicon according to the following formula:

$$236\text{-bp ratio} = 236\text{-bp yield FFPE DNA} / 236\text{-bp yield reference DNA}$$
- 3 Calculate the Average Yield Ratio by averaging the yield ratio values calculated for the 105-bp (from [step 1](#)) and 236-bp (from [step 2](#)) amplicons.
- 4 Use the guidelines in the table below to design modifications to the HaloPlex target enrichment protocol and subsequent sequencing protocol based on the apparent integrity of the FFPE-derived DNA sample. See “[Typical Results](#)” on page 19 for examples of post-enrichment Bioanalyzer profiles for FFPE-derived DNA samples of each category.

Sample Integrity Category	Average Yield Ratio	Recommended DNA Input (ng) in HaloPlex protocol	Recommended additional sequencing
A	>0.2 (>20%)	200–500	1×–5×
B	0.05 to 0.2 (5% to 20%)	500–1000	5×–10×
C	<0.05 (<5%)	1000–2000	10×–100×

NOTE

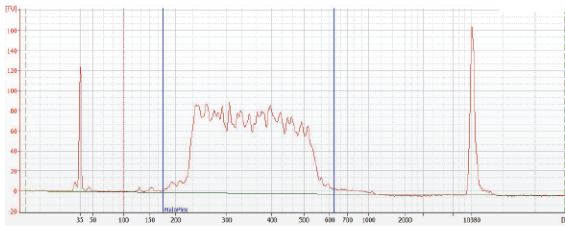
Intact DNA samples are enriched using the HaloPlex protocol at 200 ng of input DNA per reaction.

Step 4. Analyze amplicon yield ratio and identify protocol modifications

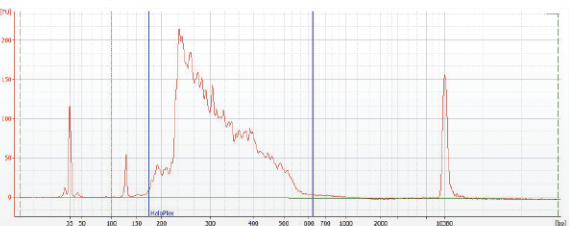
Typical Results

Bioanalyzer profiles of the HaloPlex target-enriched libraries produced from different categories of FFPE-derived DNA samples are shown below. The lower abundance of long DNA fragments in FFPE-derived DNA samples results in deviations from the typical Bioanalyzer profile for intact DNA.

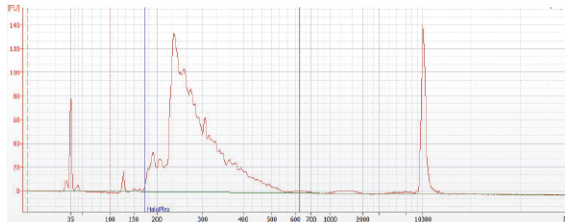
Intact DNA



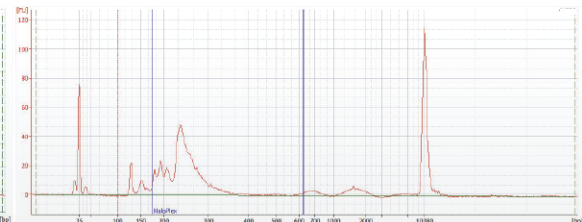
FFPE Category A



FFPE Category B



FFPE Category C



www.agilent.com

In This Book

This guide contains information to run the FFPE-Derived DNA Quality Assessment protocol in preparation for target enrichment using the HaloPlex system.

© Agilent Technologies, Inc. 2013

Version A, March 2013



G9900-90050



Agilent Technologies