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REPLI-g® FFPE Handbook

For direct whole genome amplification of DNA from formalin-fixed, paraffin-embedded (FFPE) tissue

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Contents

Kit Contents

REPLI-g FFPE Kit	**(25)**	**(100)**
Catalog no.	**150243**	**150245**
Number of 50 µl reactions	**25**	**100**
REPLI-g Midi DNA Polymerase	25 µl	100 µl
REPLI-g Midi Reaction Buffer	725 µl	2 x 1.45 ml
FFPE Enzyme	25 µl	100 µl
Ligation Enzyme	25 µl	100 µl
FFPE Buffer	200 µl	800 µl
FFPE Lysis Solution, 10x	1.9 ml	2 x 1.9 ml
Proteinase K	250 µl	2 x 250 µl
Handbook	1	1

Storage

The REPLI-g FFPE Kit is shipped on dry ice. With the exception of Proteinase K and FFPE Lysis Solution, the kit should be stored immediately upon receipt at –20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, this product can be kept at least 6 months after shipping without showing any reduction in performance. Proteinase K and FFPE Lysis Solution should be stored at room temperature (15–25°C). Proteinase K is stable for at least one year after delivery when stored at room temperature. For storage longer than one year, or if ambient temperatures often exceed 25°C, we suggest storing Proteinase K at 2–8°C.

Product Use Limitations

The REPLI-g FFPE Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the REPLI-g FFPE Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of REPLI-g FFPE Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

The REPLI-g FFPE Kit contains DNA polymerase, buffers, and reagents for whole genome amplification (WGA) of DNA from formalin-fixed, paraffin-embedded (FFPE) tissue — prior DNA purification is not necessary. Genotyping and DNA sequence analysis of clinical samples can be limited by the small amount of sample available. The REPLI-g FFPE Kit allows uniform amplification of whole genomic DNA from FFPE tissue. The DNA yields obtained depend on the reaction conditions (see Table 1) and quality of the template DNA.

Note: Tissue fixation directly affects the integrity of genomic DNA and thereby determines the suitability of genomic DNA for WGA. The REPLI-g FFPE Kit is best suited for use with genomic DNA with average DNA fragments >500 bp in length.

Table 1. Typical DNA Yields

Reaction Conditions	Amplification time (hours)	Typical DNA yield (µg per 50 µl reaction)
Standard reaction	2	≤ 10
High-yield reaction	8	≤ 40

Principle and procedure

The REPLI-g FFPE Kit contains reagents for two different reactions: a processing reaction which prepares fragmented DNA from paraffin-embedded tissue for amplification, and the actual amplification reaction.

The processing reaction uses novel buffers and enzymes to prepare fragmented DNA from paraffin-embedded tissue for WGA. The processing reaction results in ligation of DNA fragments in a random order. Due to the nature of the ligation reaction, DNA fragments are not assembled in the order in which they would have originally existed in the organism (see Figure 1). However, this does not affect the detection of nucleic acid sequences such as polymorphisms in downstream applications.

After the processing reaction has finished, a master mix containing REPLI-g Midi DNA Polymerase is added. An isothermal WGA reaction lasting 2 or 8 hours is then carried out. Once amplified, the DNA is suitable for immediate use in most downstream genotyping assays without further purification.

The REPLI-g FFPE Kit provides highly uniform amplification across the entire genome — depending on the quality of the DNA in the starting tissue and the number of genome equivalents used in the reaction (refer to Appendix C for more information on assessing DNA quality). This kit is not suitable for use with

DNA fragments <500 bp in length or when using a small number of genome equivalents (i.e., <500). The DNA quality and degree of fragmentation depends on a number of factors, including the time of formalin fixation, storage conditions, and the purification method used.

The DNA amplified by the REPLI-g FFPE procedure is highly suited for use in real-time PCR (e.g., QuantiFast® Kits), end-point PCR (ideally using amplicons significantly smaller than the average fragment size of the starting material), and SNP genotyping. Best results are obtained with amplicons of approximately 100 bp or smaller in size.

For more information about REPLI-g technology, including useful background information on WGA, visit our dedicated WGA resource page at www.qiagen.com/goto/WGA.

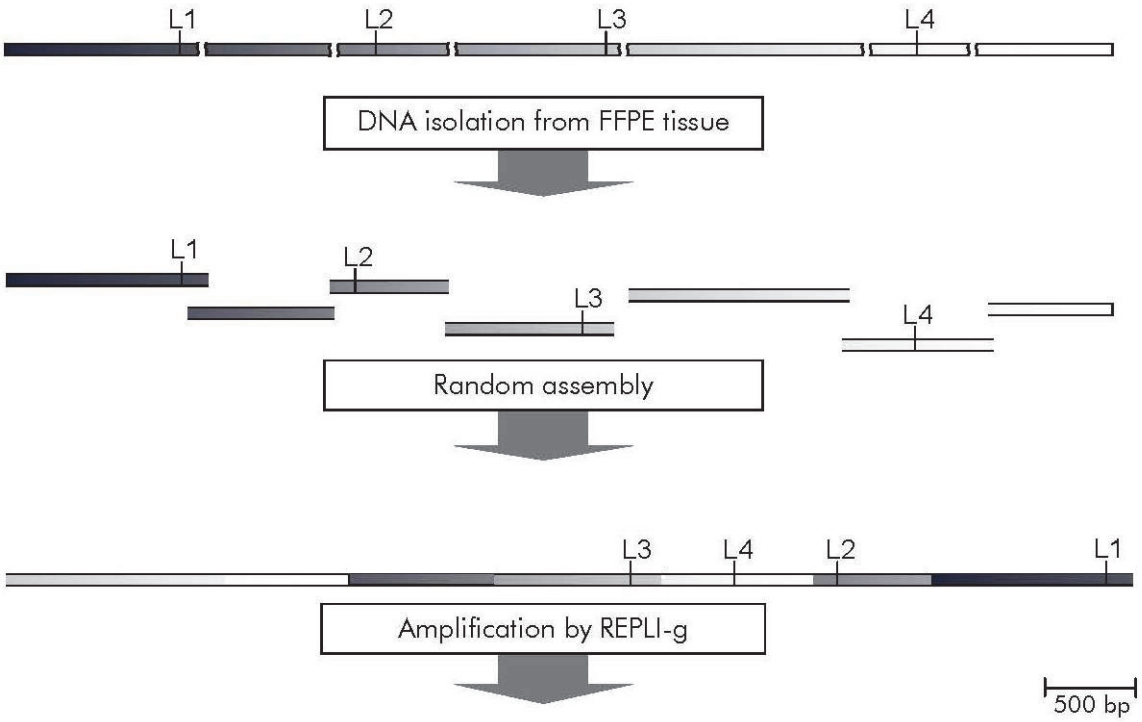
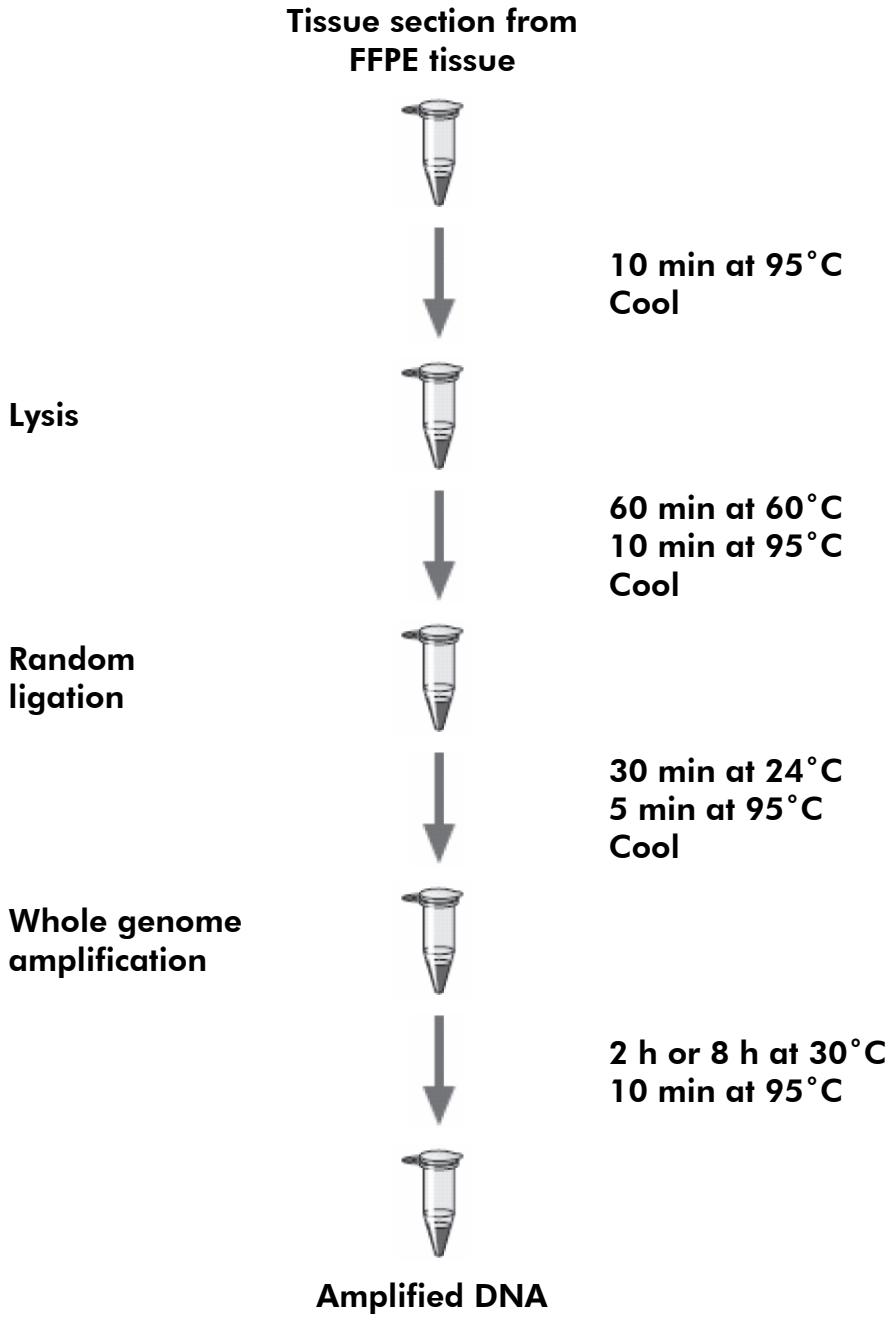


Figure 1. Random DNA ligation. Formalin fixation and paraffin embedding cause DNA fragmentation. During the REPLI-g FFPE procedure, these small fragments are randomly ligated and then amplified. L: Locus.

Amplification Procedure



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Microcentrifuge tubes
- Microcentrifuge
- Thermal cycler, water bath, or heating block
- Vortexer
- Pipets and pipet tips
- Ice
- Nuclease-free water
- A microtome

Important Notes

Starting material quality

The success of downstream applications depends on the quality of the FFPE sample starting material. In the REPLI-g FFPE procedure, DNA fragments are randomly assembled and ligated prior to amplification (Figure 1, page 8). Although the amplified DNA is of high molecular weight, it consists of very small randomly ligated sequence entities. The size of these entities depends on the original DNA fragment size in the FFPE tissue sample.

Formalin fixation also affects the DNA quality by cross-linking DNA molecules with other biomolecules in the tissue. After preparing DNA from FFPE tissue, some of these crosslinks remain. Residual crosslinks can inhibit downstream enzymatic reactions and amplification of affected sites.

We recommend using the procedure detailed in Appendix C determine the quality of the DNA in FFPE tissue section samples and to estimate the success rate of the REPLI-g FFPE whole genome amplification reactions with the tested samples. After this assessment, the DNA in the lysed tissue sections can be directly amplified using the REPLI-g FFPE Kit without prior purification.

Protocol: Direct Amplification of DNA from FFPE Tissue Sections

This protocol allows direct whole genome amplification (WGA) of DNA from formalin-fixed, paraffin–embedded (FFPE) tissue without the need for prior DNA purification. For WGA of purified DNA isolated from FFPE tissue, see Appendix B, page 20.

Important points before starting

- The thickness of the tissue section may vary between 10 and 40 µm. One tissue section is sufficient for one REPLI-g reaction. Depending on the size of the tissue within the section, a fraction of a tissue section may be sufficient. The size of the tissue portion used should exceed 1 cm in diameter. For smaller tissue portions, use thicker sections or two or more sections. Refer to the "Important Notes" for more information regarding starting material.
- If paraffin embedded tissue is limited or if the tissue section contains only a small portion of tissue (<1 cm in diameter), the section should be trimmed to eliminate most of the paraffin on the section. A smaller volume of FFPE Lysis Solution (1x) (<100 µl) should be added in step 3. The volume of FFPE Lysis Solution (1x) should be sufficient to completely cover the tissue section.
- If possible, the starting material for DNA amplification should be freshly cut sections of FFPE tissue. If the sample surface has been exposed to air, discard the first 2–3 sections.
- All enzymes (i.e., ligation enzyme, FFPE enzyme, and REPLI-g Midi DNA Polymerase) should be thawed on ice just before reaction setup. All other components can be thawed at room temperature.
- We recommend using the procedure detailed in Appendix C determine the quality of the DNA in FFPE tissue section samples and to estimate the success rate of the REPLI-g FFPE whole genome amplification reactions with the tested samples. After this assessment, the DNA in the lysed tissue sections can be directly amplified using the REPLI-g FFPE Kit without prior purification.

Things to do before starting

- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- For increased speed and convenience, all incubation steps of the protocol can be preprogrammed on a thermal cycler.

Procedure

1. **Cut one tissue section from a paraffin-embedded tissue block on a microtome and transfer the tissue section into a microcentrifuge tube.**

   Note: Ensure that the chosen tissue section contains a portion of tissue at least 1 cm in diameter. The thickness of the tissue section may vary between 10 and 40 µm. Two or more sections should be used if the tissue portion is less than 1cm in diameter or if the tissue section is less than 10 µm thick.

   If the sample surface has been exposed to air, discard the first 2–3 sections. Ideally, the tissue section should roll up on itself for better transfer of the tissue section into a reaction vial. This can usually be achieved by cutting the tissue very slowly.

2. **Prepare 1x FFPE Lysis Solution according to Table 2. Mix and centrifuge briefly.**

Table 2. Preparation of FFPE Lysis Solution (1x)

Component	Volume/reaction
FFPE Lysis Solution (10x)	10 µl
Nuclease-free Water	90 µl
Total volume	**100 µl**

3. **Add 100 µl of FFPE Lysis Solution (1x) to the tissue section from step 1. Mix and centrifuge briefly.**

   Note: A smaller volume (as little as 20 µl) of FFPE Lysis Solution (1x) can be used for trimmed tissue sections (see "Important points before starting", page 12). The whole tissue section must be submerged in the FFPE Lysis Solution (1x). Larger volumes are not recommended due to dilution effects.

4. **Incubate the sample at 95°C for 10 min to melt the paraffin.**
5. **Cool down the sample to room temperature.**

   Note: At this stage a thin layer of paraffin and tissue forms on the surface of the liquid portion of the sample.

6. **Add 2 µl of Proteinase K. Mix and centrifuge briefly.**
7. **Incubate for 60 min at 60°C and then for a further 10 min at 95°C.**
8. **Transfer 10 µl of the lysed tissue section into a new microcentrifuge tube.**

   IMPORTANT: Avoid carryover of paraffin to the microcentrifuge tube.

9. **Prepare a FFPE master mix on ice according to Table 3. Vortex and centrifuge briefly.**

   IMPORTANT: Add the FFPE master mix components in the order listed in Table 3. The master mix should be kept on ice and used immediately after preparation.

Table 3. Preparation of FFPE master mix

Component	Volume/reaction
FFPE Buffer	8 µl
Ligation Enzyme	1 µl
FFPE Enzyme	1 µl
Total volume	**10 µl**

10. **Add 10 µl FFPE master mix to 10 µl DNA from the lysed tissue in step 8. Mix and centrifuge briefly.**
11. **Incubate the samples at 24°C for 30 min.**

    Note: At this stage, DNA fragments prepared from tissue sections are ligated randomly to form DNA of high molecular weight.
12. **Stop the reaction by incubating at 95°C for 5 min and cool down to 4°C using a thermal cycler or ice.**
13. **Prepare a REPLI-g master mix according to Table 4.**

    IMPORTANT: Add the master mix components in the order listed in Table 4. The master mix should be kept on ice and used immediately upon addition of the REPLI-g Midi DNA Polymerase.

Table 4. Preparation of REPLI-g master mix

Component	Volume/reaction
REPLI-g Midi Reaction Buffer	29 µl
REPLI-g Midi DNA Polymerase	1 µl
Total volume	**30 µl**

14. **Add 30 µl of the REPLI-g master mix to the denatured DNA prepared in step 12. Mix and centrifuge briefly.**
15. **Incubate at 30°C for either 2 h (standard reaction) or 8 h (high-yield reaction).**

16. **Stop the reaction by incubating at 95˚C for 10 min.**

    Note: During the amplification reaction, the solution becomes turbid. The turbidity indicates the presence of high DNA concentrations.

    If the amplified DNA will be quantified using PicoGreen® reagent, please note that the reagent only binds double-stranded DNA. Therefore, quantify the DNA before proceeding with the 95°C incubation, or remove an aliquot (taken after step 15 and cooled to 4°C) for later quantification. If the DNA was quantified after denaturation at 95°C using PicoGreen, multiply the yield by a factor of 2 to compensate for the use of double-stranded DNA.

17. **Store the amplified DNA at –20°C until required for downstream applications.**

    If performing PCR analysis, see guidelines in Appendix A, page 19.

    Note: The amplified DNA should be treated as genomic DNA (i.e., minimize the number of freeze–thaw cycles). Storage of nucleic acids at low concentration over a long period of time may result in acid hydrolysis. We therefore recommend storing the amplified DNA at a concentration of at least 50 ng/µl.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Solution becomes turbid during amplification	
High DNA concentration	The turbidity (caused by high concentration of DNA) will not affect the downstream assay.
Reduced or no high-molecular-weight product in agarose gel in some or all REPLI-g FFPE amplified samples	
Reaction temperature too high	Check the incubator for correct reaction temperature (30°C) during REPLI-g reaction.
DNA yields of approximately 10 µg (2 h protocol) or 40 µg (8 h protocol) but no positive result in downstream assay (e.g., PCR)	
a) Poor DNA quality	DNA embedded in the tissue section is too fragmented. Check DNA quality as recommended in Appendix C. Use a larger amount of tissue or purified genomic DNA for the REPLI-g FFPE reaction. If DNA purified from FFPE sections was used in the REPLI-g FFPE reaction, increase the DNA template amount to 300 ng.
b) PCR amplicon too large	The ideal PCR amplicon size is ≤100 bp.
c) PCR cycling conditions not optimal	For end-point PCR, we recommend cycling conditions described in Table 6, page 20. A reduction of the PCR extension time and/or temperature may increase the yield of specific PCR products. Use larger tissue sections for the REPLI-g FFPE reaction.

	Comments and suggestions
DNA yields of approximately 10 µg (2 h protocol) or 40 µg (8 h protocol) in negative (no-template) controls and positive result in downstream assay (e.g., PCR)	
DNA is generated during REPLI-g reaction by contaminating DNA templates	Decontaminate all laboratory equipment, and take all necessary precautions to avoid contamination of reagents and samples with extraneous DNA. If possible, work in a laminar-flow hood. Use sterile equipment and barrier pipet tips only, and store amplification chemistry and DNA templates in separate locations.
Allele dropout observed in genotyping assay but DNA yield is approximately 10 µg (Mini) or 40 µg (Midi)	
Genomic DNA template is too degraded	Do not use DNA template with fragment sizes <500 bp, or <500 genome equivalents. Check DNA quality as recommended in Appendix C, page 21. Use a larger amount of tissue section for the REPLI-g FFPE reaction or reduce the volume of FFPE Lysis Solution when using trimmed tissue sections. If DNA purified from FFPE sections was used in the REPLI-g FFPE reaction, increase the DNA template amount to 300 ng.
Multiple bands in end-point PCR	
Nonspecific amplicons are produced during PCR	For end-point PCR, we recommend the QIAGEN Fast Cycling PCR Kit and the cycling conditions described in Table 6, page 20. A reduction of the PCR extension temperature and/or time may increase the number of specific products.
Multiple melting curve peaks in real-time PCR	
Nonspecific amplicons are produced during PCR	For real-time PCR, we recommend QIAGEN QuantiFast PCR Kits. A reduction of the PCR extension temperature and/or time may increase the number of specific products.

Comments and suggestions	
Downstream application results not optimum	
Sensitive downstream applications may require DNA cleanup after amplification using the REPLI-g FFPE Kit	Contact QIAGEN Technical Services for DNA cleanup recommendations suitable for your application.

Appendix A: PCR of DNA Amplified with the REPLI-g FFPE Kit

Real-time PCR

If analyzing the amplified DNA by real-time PCR, we recommend dilution with water or TE buffer (see Table 5). Use 2–3 µl of the diluted DNA in each real-time PCR reaction.

For reliable PCR results, we recommend using QuantiFast Kits. For high PCR sensitivity, reduce the length of the PCR amplicon to a size much smaller than the average fragment size of the starting template. The lower the amplicon size, the higher the PCR sensitivity. Optimal results are observed with amplicons of ≤100 bp in size.

Table 5. Dilution of amplified DNA for end-point or real-time PCR

Reaction Conditions	Dilution
Standard reaction (2 hour amplification)	1:20 (e.g., add 2 µl DNA to 38 µl water)
High-yield reaction (8 hour amplification)	1:100 (e.g., add 2 µl DNA to 198 µl water)

End-point PCR

If analyzing the amplified DNA by end-point PCR, we recommend dilution with water or TE buffer (see Table 5). Use 2–3 µl of the diluted DNA in each end-point PCR reaction.

In rare cases, secondary PCR fragments may coamplify with the specific fragment. Secondary fragments are usually larger in size and can be avoided by reducing the extension time.

For reliable results, we recommend the QIAGEN FastCycling PCR Kit with the cycling conditions given in Table 6. For high PCR sensitivity, reduce the length of the PCR amplicon to a size much smaller than the average fragment size of the starting template. The lower the amplicon size, the higher the PCR sensitivity. Optimal results are observed with amplicons of ≤100 bp in size.

Table 6. Cycling conditions for end-point PCR

Step	Time	Temperature	Additional comments
Initial denaturation	5 min	95°C	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	5 s	96°C	Note: If using an Eppendorf Mastercycler® ep S in block mode the denaturation should be performed at 98°C.
Annealing	5 s	60°C*	An annealing temperature >60°C is not recommended. Use a temperature approximately 5°C below T_m of primers.
Extension		68°C	
Number of cycles: 30–40			
Final extension	1 min	72°C	

* For existing primer–template pairs, use the previously defined optimized annealing temperature. However, an annealing temperature of 60°C works for most primer-template pairs and should generally not be exceeded.

Appendix B: Whole Genome Amplification of DNA Isolated from FFPE Tissue

This protocol allows whole genome amplification (WGA) of DNA that has been previously purified from FFPE tissue (e.g., using the QIAamp® DNA FFPE Tissue Kit, cat. no. 56404). For optimal results, we recommend using the protocol for direct WGA from FFPE tissue (page 12).

Important points before starting

- This protocol is optimized for WGA from >100 ng genomic DNA purified from FFPE tissue. The template DNA should be suspended in TE buffer. Larger amounts of starting material may be necessary as the copy number of DNA isolated from FFPE tissue can be highly reduced (see Appendix C,

page 21). DNA amounts between 100–300 ng can be used to compensate for extreme DNA degradation.

- All enzymes (i.e., ligation enzyme, FFPE enzyme, and REPLI-g Midi DNA Polymerase) should be thawed on ice just before reaction setup. All other components can be thawed at room temperature.

Things to do before starting

- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- For increased speed and convenience, all incubation steps of the protocol can be preprogrammed on a thermal cycler.

Procedure

B1. Add >100 ng DNA to a microcentrifuge tube. Adjust the volume to 10 µl using TE buffer.

The amount of template DNA should be >100 ng. Smaller amounts (<100 ng) of starting material can be used if the DNA is of sufficient quality.

Larger amounts of starting material (up to 300 ng) can be used to compensate for extreme DNA degradation.

B2. Incubate for 5 min at 95°C and cool down to 4°C using a thermal cycler or ice.

B3. Continue with step 9 of protocol "Direct Amplification of DNA from FFPE Tissue Sections" (see page 14).

Appendix C: Assessing the Quality of DNA in FFPE Tissue Samples

This protocol allows you to determine the quality of the DNA in FFPE tissue section samples and to estimate the success rate of the REPLI-g FFPE whole genome amplification reactions with the tested samples.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- REPLI-g Human Control Kit (cat. no. 150090)
- QuantiTect® Primer Assay (cat. no. QT00234220)
- QuantiFast SYBR® Green PCR Kit (cat. no. 204052)

- Real-time cycler
- Microcentrifuge tubes
- Real-time PCR tubes or plates
- Microcentrifuge
- Thermal cycler, water bath, or heating block
- Vortexer
- Pipets and pipet tips
- Nuclease-free water
- A microtome

Important points before starting

- The thickness of the tissue section should be adjusted to 10 µm. One tissue section is sufficient for the determination of DNA quality by this real-time PCR assay and can be used afterwards for the REPLI-g FFPE reaction.
- If the tissue section contains only a small portion of tissue (<1 cm in diameter), the section should be trimmed to eliminate most of the paraffin on the section.
- If possible, the starting material for DNA amplification should be freshly cut sections of FFPE tissue. If the sample surface has been exposed to air, discard the first 2–3 sections.
- Use the REPLI-g Human Control DNA (cat. no. 150090) as a standard for non-fragmented DNA in this procedure.
- The QuantiFast SYBR Green PCR Kit has been developed for use in a two-step cycling protocol, with a denaturation step at 95°C and a combined annealing/extension step at 60°C.
- The PCR must start with an initial incubation step of 5 minutes at 95°C to activate HotStarTaq *Plus* DNA Polymerase.
- The protocol has been developed for 96-well block cyclers.

Things to do before starting

- For increased speed and convenience, the lysis of tissue sections can be performed in a preprogrammed thermal cycler.

Procedure

C1. Cut a 10 µm tissue section from a paraffin-embedded tissue block on a microtome and transfer the tissue section into a microcentrifuge tube.

Note: If the sample surface has been exposed to air, discard the first 2–3 sections.

Ideally, the tissue section should roll up on itself for better transfer of the tissue section into a reaction vial. This can usually be achieved by cutting the tissue very slowly.

C2. Prepare 1x FFPE Lysis Solution according to Table 7. Mix and centrifuge briefly.

Table 7. Preparation of FFPE Lysis Solution (1x)

Component	Volume/reaction
FFPE Lysis Solution	10 µl
Nuclease-free Water	90 µl
Total volume	**100 µl**

C3. Add 100 µl of FFPE Lysis Solution (1x) to the tissue section from step C1. Mix and centrifuge briefly.

Note: The whole tissue section must be submerged in the FFPE Lysis Solution (1x).

C4. Incubate the sample at 95°C for 10 min to melt the paraffin.

C5. Cool sample to room temperature.

Note: A thin layer of paraffin and tissue will form on the surface of the liquid portion of the sample.

C6. Add 2 µl of Proteinase K to the sample. Mix and centrifuge briefly.

C7. Incubate the sample for 60 min at 60°C, followed by 10 min at 95°C.

C8. Cool the sample to room temperature.

C9. Thaw 2x QuantiFast SYBR Green PCR Master Mix, primers, Control DNA, and RNase-free water. Ensure the individual solutions are well-mixed prior to use.

C10. Prepare a reaction master mix according to Table 8.

Due to the PCR hot start, it is not necessary to keep the samples on ice during reaction setup or while programming the real-time cycler.

Table 8. Preparation of reaction mix

Component	Volume/reaction	Final Concentration
2x QuantiFast SYBR Green PCR Master Mix	12.5 µl	1x
10x QuantiTect Primer Assay	2.5 µl	1x
RNase-free Water	8 µl	
Total volume	**23 µl**	

C11. Mix the reaction mix thoroughly and dispense 23 µl into PCR tubes or plates.

C12. Dilute REPLI-g Human Control DNA to 5 ng/µl using RNase-free water (e.g., add 5 µl REPLI-g Human Control DNA (10 ng/µl) to 5 µl RNase-free water).

C13. Add 2 µl diluted REPLI-g Human Control DNA (5 ng/µl) or 2 µl lysed tissue section containing the FFPE DNA (from step C8) to the individual PCR tubes or wells containing 23 µl reaction mix.

Note: Carefully mix the lysed tissue section by pipetting before adding the lysed tissue section to the PCR reaction. Do not vortex the lysed tissue sections to avoid a carryover of paraffin to the PCR reaction.

C14. Program your real-time cycler according to the program outlined in Table 9.

Data acquisition should be performed during the combined annealing/ extension step.

C15. Place the PCR tubes or plates in the real-time cycler and start the cycling program.

C16. Optional: Perform melting curve analysis of the PCR product to assess PCR product purity and verify primer specificity.

Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow instructions provided by the supplier.

Table 9. Real-time cycler conditions

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial activation step	5 min	95°C	Maximal/ fast mode	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
Two-step cycling:				
Denaturation	10 s	95°C	Maximal/ fast mode	
Combined annealing/ extension	30 s	60°C	Maximal/ fast mode	Perform fluorescence data collection
Number of cycles	**40**			

Analysis

C1. Determine the "threshold cycle" (C_T value) for the non-fragmented REPLI-g Human Control DNA and the lysed tissue section.

C2. Calculate the difference between the C_T values for both reactions using the following formula:

C_T value (lysed tissue section) – C_T value (REPLI-g Human Control DNA) = Delta C_T

For example, if the C_T values for REPLI-g Human Control DNA and lysed tissue sections were determined to be 21 and 24, respectively, the Delta C_T is calculated to be 3 (24–21=3).

C3. Refer to Table 10 to determine the predicted success rate of REPLI-g FFPE WGA reactions with DNA from the lysed tissue sections.

Note: The classification of DNA quality is based on empirical data which is summarized in Table 10. The quality of DNA in lysed tissue sections determined with this procedure does not necessarily guarantee successful REPLI-g FFPE reactions.

Table 10. Correlation between Delta C_T and DNA quality in lysed tissue sections for the REPLI-g FFPE WGA reaction.*

	REPLI-g FFPE success rate		
Delta C_T	Good	Intermediate	Poor
0 – 3	80%	20%	0%
3 – 6	50%	40%	10%
>6	10%	25%	65%

* If the calculated Delta C_T value is 3, about 80% of the DNA from the sample is of good quality, and successful amplification is possible.

C4. After determining the predicted success rate of the REPLI-g FFPE WGA reaction, the lysed tissue section can be used directly for REPLI-g FFPE reactions (see step 9 of protocol "Direct Amplification of DNA from FFPE Tissue Sections" on page 14).

Note: The lysed tissue section can be stored up to 1 week at –20°C. Alternatively, prepare a fresh lysed tissue section from the same tissue block directly prior to the REPLI-g FFPE reaction.

Appendix D: Determination of DNA Concentration and Yield

Quantification of DNA yield

A 50 µl REPLI-g FFPE Kit reaction typically yields approximately 10 µg (standard protocol) or 40 µg (8 h protocol) of DNA regardless of the amount of template DNA, allowing direct use of the amplified DNA in most downstream genotyping experiments. However, if a more accurate quantification of DNA is required, it is important to use a DNA quantification method specific for double stranded DNA, since REPLI-g FFPE Kit amplification products contain unused reaction primers. PicoGreen reagent displays enhanced binding to double stranded DNA and may be used, in conjunction with a fluorometer, to quantify the double-stranded DNA product. Refer to Appendix E for a detailed procedure.

Quantification of locus representation

Locus representation for each sample can be quantified by real-time PCR. Contact QIAGEN Technical Services or visit our website at www.qiagen.com for a protocol.

Appendix E: Quantification of REPLI-g Amplified DNA with PicoGreen

This protocol is designed for quantification of double stranded REPLI-g amplified DNA using PicoGreen reagent.

IMPORTANT: When working with hazardous chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and reagents to be supplied by user

- Quant-iT™ PicoGreen dsDNA reagent (Invitrogen, cat. no. P7581)
- TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0)
- Human genomic DNA (e.g., Promega, cat. no. G3041)
- 2 ml microcentrifuge tube
- 96-well plates (suitable for use in a fluorescence microplate reader)
- Fluorescence microplate reader (e.g., TECAN® Ultra)

Procedure

E1. In a 2 ml microcentrifuge tube, make a 1:150 dilution of PicoGreen stock solution in TE buffer; each quantification reaction will require 20 µl. Cover the microcentrifuge tube with aluminum foil or place it in the dark to avoid photodegradation of the PicoGreen reagent.

For example, to prepare enough PicoGreen working solution for 100 samples, add 13.3 µl PicoGreen to 1986.7 µl TE buffer.

IMPORTANT: Prepare the PicoGreen/TE solution in a plastic container, as the PicoGreen reagent may adsorb to glass surfaces.

E2. Prepare a 16 µg/ml stock solution of genomic DNA in TE buffer.

E3. Make 200 µl of 1.6, 0.8, 0.4, 0.2, and 0.1 µg/ml DNA standards by further diluting the 16 µg/ml genomic DNA with TE buffer.

E4. Transfer 20 µl of each DNA standard in duplicate into a 96-well plate labeled "A" (see figure below).

Note: The 96-well plate must be suitable for use in a fluorescent microplate reader.

96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H			1.6	0.8	0.4	0.2	0.1	1.6	0.8	0.4	0.2	0.1

Gray squares: genomic DNA standard (µg/ml).

E5. Place 2 µl of each REPLI-g amplified DNA sample for quantification into a second 96-well plate, and add 198 µl TE buffer to make a 1:100 dilution. Store the remaining REPLI-g amplified DNA at –20°C.

E6. Place 5 µl diluted REPLI-g DNA (from step E5) into an unused well of 96-well plate "A" and add 15 µl TE to make a 1:400 dilution.

If using the 8 h protocol for the WGA reaction, place 2 µl diluted REPLI-g DNA (from step E5) into an unused well of 96-well plate "A" and add 18 µl TE to make a 1:1000 dilution.

The 1:100 dilutions can be stored at –20°C and used for future downstream sample analysis.

E7. Add 20 µl PicoGreen working solution (from step E1) to each sample (amplified DNA and DNA standards) in 96-well plate "A". Gently shake the plate on the bench top to mix the samples and reagent.

E8. Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells.

E9. Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescence filters (excitation approximately 480 nm; emission approximately 520 nm).

To ensure that the sample readings remain in the detection range of the microplate reader, adjust the instrument's gain so that the sample with the highest DNA concentration yields fluorescence intensity near the fluorometer's maximum.

Calculation of DNA concentration and yield

E1. Generate a standard curve by plotting the concentration of DNA standards (µg/ml) (x-axis) against the fluorescence reading generated by the microplate reader (y-axis). Plot an average of the fluorescence recorded for each DNA standard of the same concentration.

E2. Use the standard curve to determine the concentration (µg/ml) of the diluted REPLI-g amplified DNA sample. This is achieved by plotting the fluorescence reading of the sample against the standard curve and reading the DNA concentration on the x-axis.

Note: The calculation of DNA concentration depends on the standard curve and the determination of the slope. For accurate results, the standard curve should be a straight line. Any deviation from this may cause inaccuracies in the calculation of REPLI-g amplified DNA concentrations.

E3. Multiply the value determined in step E2 by 400 (standard protocol) or 1000 (8 h protocol) to give the concentration of undiluted sample DNA (as the sample DNA measured by PicoGreen fluorescence had been diluted 1:400 or 1:1000, respectively).

E4. To determine the total amount of DNA in your sample, multiply the concentration of undiluted sample DNA (µg/ml) (step E3) by the reaction volume in milliliters (i.e., for a 50 µl reaction, multiply by 0.05).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
REPLI-g FFPE Kit — for whole genome amplification of DNA from FFPE tissues		
REPLI-g FFPE Kit (25)	DNA Polymerase, Buffers, and Reagents for 25 x 50 µl whole genome amplification reactions	150243
REPLI-g FFPE Kit (100)	DNA Polymerase, Buffers, and Reagents for 100 x 50 µl whole genome amplification reactions	150245
Related products		
REPLI-g Mini and Midi Kits — for highly uniform whole genome amplification from small or precious samples		
REPLI-g Mini Kit (25)*	DNA Polymerase, Buffers, and Reagents for 25 x 50 µl whole genome amplification reactions (typical yield 10 µg per reaction)	150023
REPLI-g Midi Kit (25)*	DNA Polymerase, Buffers, and Reagents for 25 x 50 µl whole genome amplification reactions (typical yield 40 µg per reaction)	150043
QIAamp FFPE Tissue Kit — for purification of genomic DNA from formalin-fixed, paraffin-embedded tissues		
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
RNeasy® FFPE Kit — for purification of high yields of usable RNA from FFPE tissue sections		
RNeasy FFPE Kit (50)	For 50 preps: 50 RNeasy MinElute Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74404

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
QuantiFast SYBR Green PCR Kit — for fast, quantitative, real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiFast SYBR Green PCR Kit (80)*	For 80 x 25 µl reactions: 1 ml 2x Master Mix (contains ROX dye), 2 ml RNase-Free Water	204052
QuantiFast Probe PCR Kits — for fast, quantitative, real-time PCR		
QuantiFast Probe PCR Kit (80)*	For 80 x 25 µl reactions: 1 ml 2x Master Mix (contains ROX dye), 2 ml RNase-Free Water	204252
QuantiTect Whole Transcriptome Kit — for preparation of cDNA from total RNA by whole transcriptome amplification		
QuantiTect Whole Transcriptome Kit (25)*	For 25 x 50 µl reactions: T-Script® Enzyme and Buffer, Ligation Enzymes, Reagent, and Buffer, and REPLI-g DNA Polymerase and Buffer	207043
Qproteome® FFPE Tissue Kit — for isolation of full-length proteins from formalin-fixed tissues		
Qproteome FFPE Tissue Kit (20)*	For 20 protein preparations from formalin-fixed, paraffin-embedded tissue samples: Extraction Buffer, Collection Tubes, Collection Tube Sealing Clips	37623
QIAGEN Fast Cycling PCR Kit — for fast and specific PCR on any thermal cycler		
QIAGEN Fast Cycling PCR Kit (200)*	For 200 x 20 µl reactions: 2 x 1 ml QIAGEN Fast Cycling PCR Master Mix, 10x CoralLoad® Fast Cycling Dye, Q-Solution®, RNase-Free Water	203743
HotStar Taq® *Plus* DNA Polymerase — for highly specific hot-start PCR without optimization		
HotStar Taq *Plus* DNA Polymerase (250)*	250 units HotStar Taq *Plus* DNA Polymerase, 10x PCR Buffer, 10x CoralLoad PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$	203603

* Larger kit sizes available; please inquire.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Notes

Notes

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