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therascreen[®] BRCA1 /2 NGS FFPE gDNA Kit Handbook Part 1: Experiments

Version 1

For the identification of variants in *BRCA1* and *BRCA2*

IVD

For in vitro diagnostic use

For use with Illumina[®] MiSeqDx[™] platform

CE

REF

875011



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Intended Use

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit (next-generation sequencing) panel is a molecular diagnostic assay intended to be used for the identification of variants in the coding regions of the *BRCA1* and *BRCA2* human genes, in DNA derived from formalin-fixed paraffin-embedded (FFPE) ovarian tumor tissue. The *therascreen* BRCA1/2 NGS FFPE gDNA Kit panel is intended to be used as an aid for the classification of ovarian cancers.

Warning

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit has been validated in combination with the Illumina MiSeqDx platform and with the Biomedical Genomics Workbench software (including a specific analysis workflow).

IMPORTANT: This handbook is provided in two parts. Part 1 contains a summary and explanation, principles of the procedure and the description of the wet lab workflow:

- Genomic DNA extraction
- Target PCR amplification
- Sample pooling and purification
- Library construction
- Cleanup of adapter-ligated DNA
- Fine size selection
- PCR amplification of purified library
- Library cleanup, quantitation and pooling
- Preparation of the pooled library for sequencing
- Setting up and starting the sequencing run
- Troubleshooting guide

Part 2 contains information on data analysis and kit performance:

- Data analysis
 - Installing the analysis workflow
 - Installing the analysis plug-in
 - Exporting Illumina FASTQ files from the MiSeqDx
 - Importing Illumina FASTQ files
 - Sequence analysis
- Interpretation of results
- Troubleshooting guide
- Performance characteristics

IMPORTANT: The workflow was designed and optimized to establish the performance described in Part 1 and Part 2 of this handbook. The instructions for use must be strictly followed. Any deviation from the instructions in Part 1 and Part 2 of this handbook will void QIAGEN responsibility. The entire workflow should be subject to independent verification by the end-user laboratory before being introduced into routine use.

Summary and Explanation

Over 200,000 women are diagnosed worldwide with primary ovarian cancer (OvCa) each year. OvCa has the highest mortality rate of all female gynecological cancers (1, 2). The vast majority of positive diagnoses are of women with advanced stage OvCa and the average five-year overall survival is about 45%.

Approximately 15% of OvCa is attributable to germline mutations in *BRCA1/2* and a smaller percentage can be accounted for by other germline mutations. In contrast, a large fraction of OvCa cases can be attributed to a growing number of somatic aberrations (occurring only in the tumor mass itself) in critical tumor suppressor genes, including *BRCA1/2* (3, 4).

OvCa is a heterogeneous group of distinct diseases. About 90% of all OvCas are epithelial carcinomas and belong to five major histotypes: Mucinous, Endometrioid, Clear Cell, Low-Grade Serous and High-Grade Serous Carcinoma (HGSC). HGSC is the most prevalent, and most deaths (70%) are of patients presenting with advanced-stage (FIGO stage III or IV) HGSC. Although HGSC has distinguishing clinical characteristics from the other subtypes, patients with this histotype still show diverse outcomes even after the same or very similar treatment regimens (5). Further patient segmentation is based on molecular alterations (6).

It is currently estimated that *BRCA1/2* germline mutations occur in around 10–20% of OvCas, and this proportion may be as high as 25% in patients with HGSC (7, 8). *BRCA1/2*-mutated patients show a higher overall survival than *BRCA1/2* wild-type patients (9). In addition, and importantly, patients with *BRCA1/2* somatic alterations show similar prognosis as their *BRCA1/2* germline-mutant counterparts. Somatic mutations rates have been estimated at around 7% in unselected OvCa patients (4).

Standard OvCa therapy consists of cytoreductive surgery followed by platinum-based chemotherapy. Compared to other solid cancers, HGSCs are unusually sensitive to platinum-based chemotherapy and are frequently amenable to retreatment. Although most patients respond to initial treatment, drug resistance emerges, and most patients relapse with a median progression-free survival of only 18 months (10, 11).

Poly (ADP-ribose) polymerase (PARP) inhibition was first introduced in 2005 as a novel cancer-targeting strategy following preclinical work which showed activity in *BRCA*-mutated tumor cells. *BRCA1* and *BRCA2* dysfunction, which results in a lack of homologous recombination, markedly sensitizes cells to PARP inhibition by synthetic lethality. Patients with germline *BRCA1/2*-mutated OvCa have been shown to derive a clinical benefit from treatment with PARP inhibition (12–14). Recent clinical data further indicate efficacy of PARP inhibition in patients with somatic *BRCA* mutations but without germline mutations, thereby greatly expanding the number of patients who could benefit from this treatment (3, 15).

In view of the great significance of *BRCA1/2* mutations for prognosis and treatment, the Association of Gynecologic Oncology (AGO) Austria has recently recommended that women with OvCa be tested for germline *BRCA1/2* mutation and that quality-controlled testing of the tumor material should also be performed (16).

As the majority of clinical OvCa specimens are FFPE tissue, subsequent analysis of DNA extracted from such FFPE tumor samples is qualitatively challenging. Also, unlike the clinically-relevant mutation spectrum of other genes analyzed on FFPE tumor DNA (e.g., *KRAS* or *EGFR*), where the distribution and number of mutations is small, thousands of clinically-relevant variations in *BRCA1/2* have been described. These are distributed widely throughout multiple, large coding regions and intron-exon boundaries (17). In addition, tumor samples are histologically heterogeneous (18) and tumor-specific DNA will contain varying proportions of contaminating DNA from normal cells.

Sanger DNA sequencing methods may not be sensitive enough to detect low-level somatic changes and are difficult to scale for high throughput applications. In this context, NGS offers a solution to this challenging type of analysis for a comprehensive mutation screening of *BRCA1* and *BRCA2* genes. This technology is explicitly supported by AGO to test for those commonly mutated genes in an effort to better stratify the patients (16).

Principle of the Procedure

The *therascreen* *BRCA1/2* NGS FFPE gDNA Kit is a PCR amplicon-based assay using 4 multiplexed primer mixes designed to amplify all coding regions of the *BRCA1* and *BRCA2* genes, including the 20 intronic nucleotides adjacent to each exon. The recommended amount of DNA for each multiplex PCR reaction is 10 ng of purified genomic DNA, for a total of 40 ng.

After the PCR reaction is complete, the 4 reactions of each sample are pooled and purified. Next, the resulting PCR amplicons are bar coded and amplified using a library preparation

method compatible with Illumina MiSeq instrument. Then, using a quantitative PCR-based solution, initial library quantitation is performed before proceeding to library normalization and pooling. Next, a second library quantitation is performed for an accurate normalization of the pooled library. The pooled library is now ready for denaturation and sequencing in Illumina MiSeqDx platform. Sequencing demultiplexed raw data (FASTQ files) are then imported to the Genomics Cancer Research Workbench software for deeper analysis to identify variant positions compared with the reference sequence of the *BRCA1* and *BRCA2* genes.

In the first step, the full coding regions of *BRCA1* and *BRCA2* genes (including at least 20 adjacent intronic nucleotides) are amplified in 4 separate multiplex PCR reactions per sample using HotStarTaq® DNA polymerase in a standard thermal cycler (Figure 1). At the end of the reaction, PCR products are pooled per sample before proceeding to bead purification.

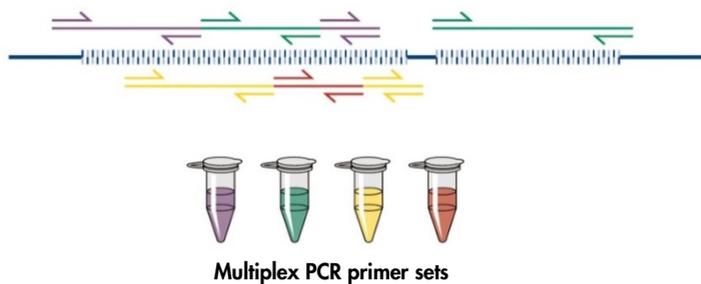


Figure 1. Multiplex PCR-based targeted enrichment scheme. The *therascreen* BRCA1/2 NGS FFPE gDNA Kit uses multiplex PCR-based targeted enrichment technology. The adjacent primer sets are distributed across an appropriate number of pools to minimize nonspecific amplification products.

In a second step, each purified PCR product reaction is individually bar coded by adding Illumina-compatible adapters in both amplicon extremities (Figure 2).

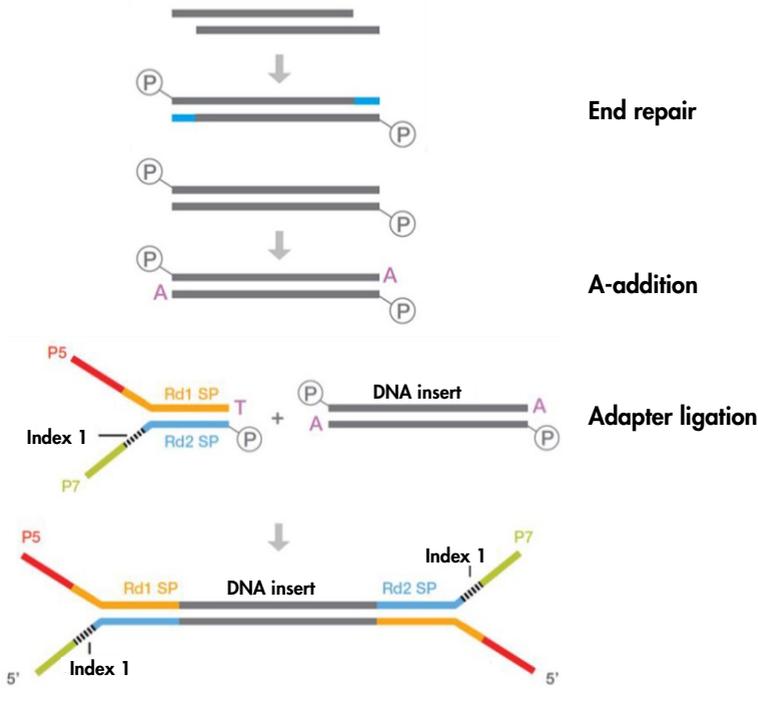


Figure 2. Library preparation and scheme for ligation of adapters. An end-repair step is performed to repair damaged DNA to produce 5' phosphorylated, blunt-ended DNA. The A-addition reaction produces double stranded amplicons with a 3' "A" overhang compatible for ligation to 3' dT overhangs on Illumina adapters.

After reactions are complete, a two-step library size selection reaction is performed: first to remove un-ligated PCR fragments and second to remove large fragments using a bead-based protocol.

The next step increases PCR library quantity. A simplex PCR amplification is performed to specifically amplify the bar coded fragments (Figure 3).

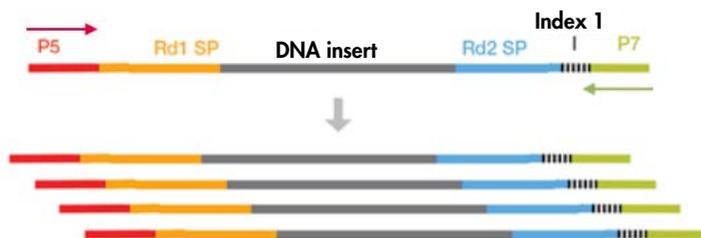


Figure 3. Library amplification scheme. Universal P5 and P7 primers are used to amplify tagged fragments that have adapters on both ends.

Next, each purified amplified library is quantified by qPCR and pooled in equimolar amounts. The pooled library is then quantified a second time before proceeding to sequencing.

Sequencing is performed following the Illumina manufacturer's protocol. FASTQ files are processed with Biomedical Genomics Cancer Research Workbench software with the BRCA1/2 CE-IVD Workflow. A variant call format file is generated for each sample and Biomedical Genomics Cancer Workbench software is recommended for variant interpretation.

To ensure good quality of the results, in-process control criteria are used at different steps of the library preparation and sequencing run (Figure 4). These criteria allow validation of the different steps of the workflow to identify samples that give poor sequencing results or to indicate potential contamination.

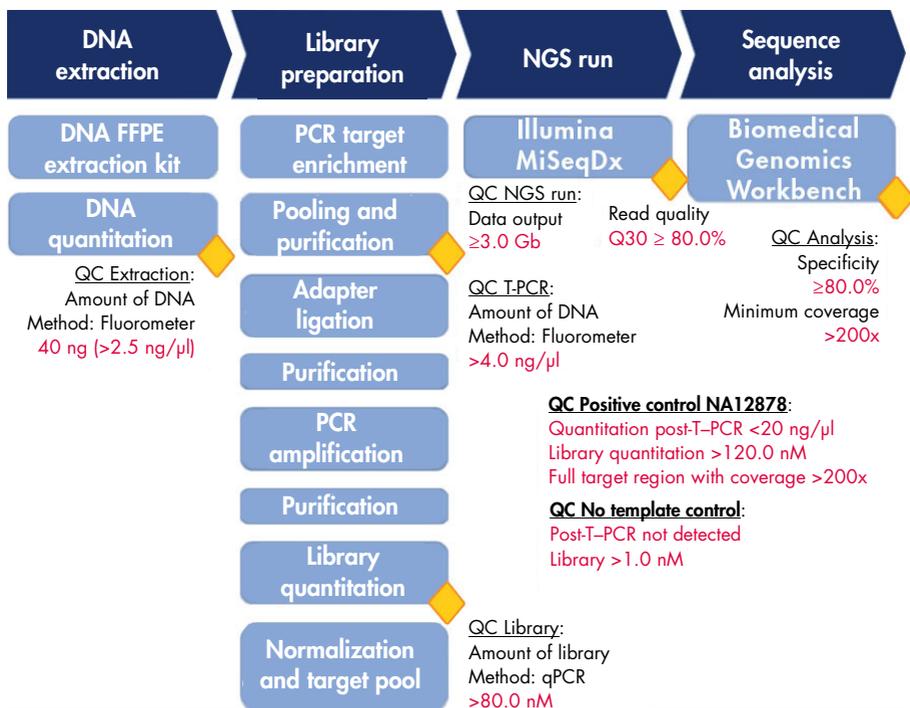


Figure 4. In-process control criteria. Through the sequencing workflow (blue boxes), several in-process control steps are performed (yellow lozenges) to validate the T-PCR, the library preparation and the sequencing run. The final criterion used to ensure good quality variant calling at a given position is the minimum coverage obtained. Specificity relates to the percentage of pair reads aligned to the target region.

Materials Provided

Kit contents

<i>therascreen</i> BRCA1/2 NGS FFPE gDNA Kit	(20)
Catalog number	875011
Number of samples	2 x 10
BRCA Primer Mix 1 of 4*	290 µl
BRCA Primer Mix 2 of 4*	290 µl
BRCA Primer Mix 3 of 4*	290 µl
BRCA Primer Mix 4 of 4*	290 µl
<i>Taq</i> DNA Polymerase (HotStar <i>Taq</i> 5 units/µl)	2 x 85 µl
GR NGS Panel 5x PCR Buffer V2	465 µl
Nuclease-Free Water (clear cap)	1900 µl

* Pooled in 4 tubes of primer pairs are 253 pairs of primers designed to cover the target region. Amplicons are designed with an average of 153 bp; minimum 105 bp and maximum 200 bp.

Materials Required but Not Provided

Required products and reagents

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

Kits are required for:

- Preparation of genomic DNA from FFPE tissue
- DNA quantitation (preferably using a fluorometer)
- PCR sample purification (preferably magnetic bead-based technology)
- Library construction (compatible with Illumina MiSeq instruments)
- Library cleanup (preferably silica gel column technology)
- Fine size selection (preferably magnetic bead-based technology)
- PCR amplification of purified library
- Library quantitation (preferably with qPCR technology)

In addition to kits for genomic DNA extraction, PCR sample purification, library construction, library cleanup, fine size selection and DNA quantitation, the products and reagents listed in Table 1 are required for the *therascreen* BRCA1/2 NGS FFPE gDNA Kit workflow.

Table 1. Required products and reagents

Product/reagent	Provider	Purpose	Number of reactions	Catalog no.
MiSeq Reagent Kit v2 1/2 & 2/2	Illumina	Illumina flow cell, cartridge and buffer for sequencing (151-cycle paired-end)	Seq. run	MS-102-2002
PhiX Control v3	Illumina	To be added in the final library pool and used as control during the sequencing run	N/A	FC-110-3001
NaOH 10N	N/A	DNA denaturation	N/A	N/A
80% ethanol*	N/A	Necessary for purification steps	N/A	N/A
96–100% ethanol*	N/A	Necessary for genomic DNA purification step	N/A	N/A
10 mM Tris-Cl, pH 8.5	N/A	Necessary to dilute the PhiX internal control	N/A	N/A
Tween 20	N/A	Necessary to dilute the PhiX internal control	N/A	N/A
Nuclease-free water	N/A	For DNA dilution	N/A	N/A

N/A = not applicable

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Positive control

- DNA NA12878 from Coriell Institute for Medical Research repository to be used as positive control (catalog.coriell.org)

Consumables

- Nuclease-free aerosol-resistant sterile PCR pipet tips with hydrophobic filters
- 1.5 ml or 2.0 ml LoBind nuclease-free PCR tubes (Eppendorf[®], cat. no. 022431021 or 02243048)
- 12-strip nuclease-free PCR tubes
- 96-well PCR plates compatible with the in-house thermal cycler
- Ice

Equipment

Make sure that instruments used in this procedure have been checked and calibrated according to the manufacturer's recommendations.

General equipment and reagents

- Dedicated pipets (adjustable) (1–10 µl; 10–100 µl; 100–1000 µl)
- Disposable gloves
- Vortex mixer
- Benchtop centrifuge with rotor for 0.2 ml and 2 ml reaction tubes (capable of attaining 15,000 rpm [20,000 x g])
- Fluorometer for quantitation of DNA
- Thermal cycler for target PCR amplification and library quantitation

Sequencing equipment

- Illumina MiSeqDx (Illumina, Inc.; cat. no. DX-410-1001)
- Illumina MiSeq software version 2.5.0.5 or higher
- Illumina Experiment Manager Software version v1.9 or higher

Software for sequence analysis

- Biomedical Genomics Workbench version 2.1.1 from CLC bio (www.clcbio.com)
- CLC Genomics Server 7.0.2 with Biomedical Genomics Extension from CLC bio
- QIAGEN GeneRead Panel Analysis Plugin
Available for download from the **Product Resources** tab of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit product page on the QIAGEN website.
- BRCA 1/2 CE-IVD Workflow
Available for download from the **Product Resources** tab of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit product page on the QIAGEN website.

System requirements recommended by CLC bio (www.clcbio.com/support/system-requirements)

- Windows Vista®, Windows® 7, Windows 8, Windows 10, Windows Server 2008 or Windows Server 2012
Mac OS® 10.7 or later
Linux: Red Hat® 5.0 or later; SUSE® 10.2 or later; Fedora® 6 or later
- 8 GB RAM required ;16 GB RAM recommended
- 1024 x 768 display required;1600 x 1200 display recommended
- Intel® or AMD® CPU required
- Minimum 100 GB free disk space in the default operating system user temp directory
- Minimum 90 GB free disk space required in the CLC_References directory (if you are not connected to a server)

If less free disk space is available, the reference data location can be changed. See resources.qiagenbioinformatics.com/manuals/biomedicalgenomicsworkbenchapplication/current/.

Expand the section **Getting started**, open **Reference data** and click on **Download and configure reference data**.

Special requirements for read mapping

The numbers below give minimum and recommended memory for systems running mapping and analysis tasks. The requirements suggested are based on the genome size.

- Human (3.2 Gb) and mouse (2.7 Gb)
 - Minimum: 6 GB RAM; recommended: 8 GB RAM
- Systems with less memory than specified will benefit from installing the legacy read mapper plug-in (see www.clcbio.com/clc-plugin/read-mapper-legacy-version). This is slower than the standard mapper but adjusts to the amount of memory available.

Special requirements for the 3D Molecule Viewer

System requirements

- A graphics card capable of supporting OpenGL® 2.0
 - Updated graphics drivers
- Please make sure the latest driver for the graphics card is installed.

System recommendations

- A discrete graphics card from either NVIDIA® or AMD/ATI™
- Modern integrated graphics cards (such as the Intel HD Graphics series) may also be used, but these are usually slower than the discrete cards.
- A 64-bit workbench version is recommended for working with large complexes

Warnings and Precautions

For in vitro diagnostic use

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

General precautions

Use of NGS tests requires good laboratory practices, including maintenance and calibration of all equipment used, and compliance with applicable regulations and relevant standards.

- Discard sample and assay waste according to your local safety procedures.
- Reagents provided in the *therascreen* BRCA1/2 NGS FFPE gDNA Kit are optimally diluted. Do not further dilute reagents, as this may result in a loss of performance.
- All reagents supplied in *therascreen* BRCA1/2 NGS FFPE gDNA Kit are intended to be used solely with the other reagents supplied in the same kit. Do not substitute any reagent between *therascreen* BRCA1/2 NGS FFPE gDNA kits, as this may affect performance.
- Do not use expired or incorrectly transported and stored components of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit and kits required but not provided. Always check before use.
- Alteration of incubation times and/or temperatures may result in erroneous or discordant data.
- Caution must be observed to ensure correct sample testing with emphasis on incorrect sample entry, loading error, pipetting error and bar coding error.
- Make sure the samples are handled in a systematic way to ensure correct identification at all times to maintain traceability.

-
- Use extreme caution to prevent cross-contamination.
 - Use extreme caution to prevent PCR product carry-over contamination resulting in a false positive signal.
 - Use extreme caution to prevent contamination by DNase, which may cause degradation of DNA templates.
 - Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials). Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
 - Prepare pre-PCR master mix with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrices (cDNA, plasmid or PCR products) are introduced. Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).
 - Refer to the Illumina MiSeqDx instrument user manual for additional warnings, precautions and procedures. The NGS platform needs to be correctly installed to ensure power supply, and once launched, to ensure no interaction of the user with the platform.
 - Do not open the Illumina MiSeqDx instrument until a run is finished.

Reagent Storage and Handling

Shipping conditions

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit is shipped on dry ice. If any component of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note or the reagents, please contact QIAGEN Technical Services or local distributors (visit www.qiagen.com).

Storage conditions

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit should be stored immediately upon receipt at -15°C to -30°C in a constant-temperature freezer. Caution must be observed to protect qPCR mixes from light.

For storage information relative to the reagents and kits not provided, please refer to the respective handbooks.

Stability

When stored under the specified storage conditions, the *therascreen* BRCA1/2 NGS FFPE gDNA Kit is stable until the stated expiration date.

Once opened, reagents can be stored in their original packaging at -15°C to -30°C until the stated expiration date shown on the packaging. Do not exceed a maximum of 5 freeze-thaw cycles.

For stability information relative to the reagents and kits not provided, please refer to the respective handbooks.

Specimen Handling and Storage

Sample material must be human genomic DNA extracted from FFPE tissue.

Specimens must be transported according to standard pathology methodology to ensure specimen quality.

Store all FFPE blocks and slides at room temperature 15–25°C based on CLSI standard MM13-A (19). Transport conditions of FFPE blocks are similar to storage conditions.

Slides may be stored at ambient temperature for up to 1 month prior to DNA extraction.

FFPE-derived DNA should be either freshly prepared or stored between 4°C and 8°C for short-term storage, or between –15°C and –30°C for long-term storage

Procedure: Part 1

Workflow overview

Parts of the workflow described in the scheme below have been optimized for this procedure, including steps requiring kits and reagents that are not provided.



Please read the following procedure carefully and refer only to Part 1 and Part 2 of this handbook for instructions.

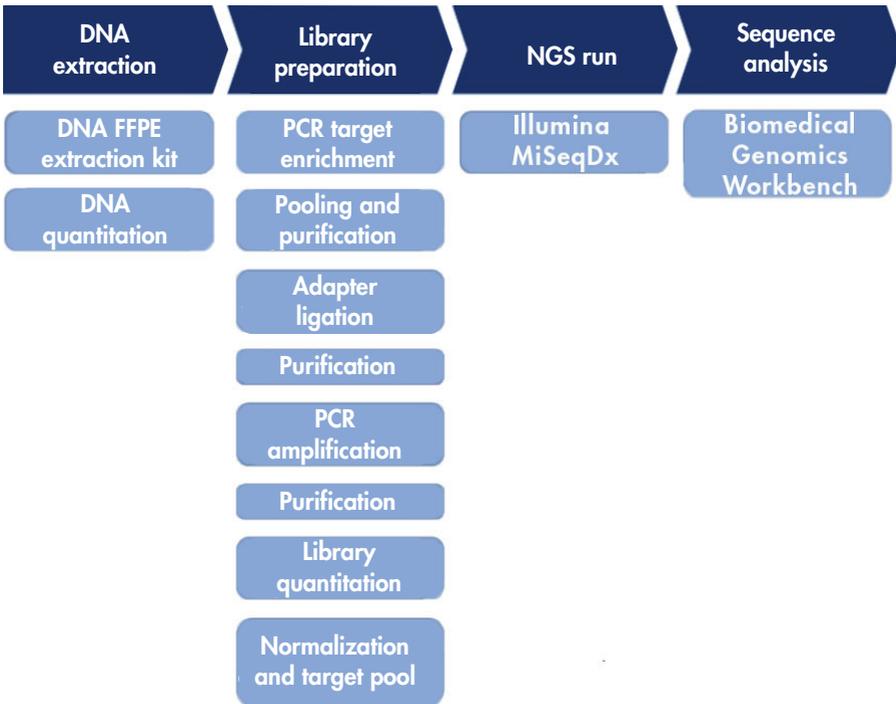


Figure 5. Overview of the NGS workflow.

Genomic DNA extraction

Use a kit to purify genomic DNA from samples prepared from FFPE ovarian cancer specimens.

Genomic DNA quantitation

For accurate DNA quantitation resulting in optimal DNA concentration, we recommend using a fluorometer and an assay kit that allows DNA quantitation across a range of initial sample concentrations from 100 pg/μl to 1000 ng/μl.

The DNA concentration of a sample must be ≥ 2.5 ng/μl to ensure sample quantity for downstream experiments.

If the quantity of DNA is insufficient, extract more tumor tissue, if available (see “Troubleshooting Guide”, page 40).

Protocol: Target PCR setup

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit allows testing of 20 samples in total in two sequencing runs. We recommend performing each sequencing run with 10 samples plus one NA12878 positive control and one nuclease-free water for no template control (NTC). Distribute samples in a 96-well PCR plate according to the plate layout shown in Figure 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	x	x	x	x	x	x	x	x	x	x	x	x
B	x	x	x	x	x	x	x	x	x	x	x	x
Primer Mix 1 of 4 C	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Positive control	NTC
Primer Mix 2 of 4 D	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Positive control	NTC
Primer Mix 3 of 4 E	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Positive control	NTC
Primer Mix 4 of 4 F	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Positive control	NTC
G	x	x	x	x	x	x	x	x	x	x	x	x
H	x	x	x	x	x	x	x	x	x	x	x	x

Figure 6. Layout scheme of the 96-well target PCR plate.

Procedure

1. Thaw reagents on ice.
2. Dilute DNA (including the positive control NA12878) to 2.5 ng/μl with nuclease-free water in a LoBind tube. For each sample, 10 ng (4 μl, 2.5 ng/μl) per PCR reaction is required, for a total of 40 ng.

3. We recommend testing 10 samples plus one NA12878 positive control and one NTC control per sequencing run. Prepare PCR strips or a 96-well PCR plate according to the number of reactions. Label with sample names and pool numbers.
4. Prepare the 4 T-PCR premixes on ice according to Table 2 below. For each sample, 4 T-PCR premixes will be needed. Mix gently by pipetting up and down.

Table 2. Preparation of T-PCR premixes for each primer mix pool

Component	1 reaction +10% extra	12 reactions +10% extra
BRCA Primer Mix 1, 2, 3 or 4 of 4	11 μ l	132 μ l
<i>Taq</i> DNA Polymerase (HotStarTaq, 5 units/ μ l)	1.5 μ l	18 μ l
GR NGS Panel 5x PCR Buffer V2	4.4 μ l	52.8 μ l
Nuclease-Free Water	0.7 μ l	8.4 μ l
Total Volume	17.6 μl	211.2 μl

5. Dispense 16 μ l of T-PCR premix into wells of a 96-well plate according to the plate layout described in Figure 6.
6. Add 4 μ l of each DNA sample into the reaction mix of each PCR according to the plate layout shown in Figure 6. Mix gently by pipetting up and down.

7. Seal the wells before loading into a thermal cycler and program the cycling conditions as indicated in Table 3.

Table 3. Cycling conditions

Time	Temperature	Cycles
15 minutes	95°C	1
15 seconds	95°C	26
150 seconds	60°C	
10 minutes	72°C	1

8. After the reaction is complete, proceed with “Step: Sample pooling and purification”, page 27.

Note: If the samples are to be stored prior to purification, transfer them to a –15°C to –30°C freezer.

Note: The launch of the T-PCR run can be delayed up to 6 hours before starting the PCR reaction if reactions mixes are stored at 2–8°C.

Step: Sample pooling and purification

The 4 T-PCR reactions from the target PCR step are pooled for a unique pool of PCR products per sample. The pool of PCR products is purified, preferably using magnetic bead-based technology, before proceeding to library preparation.

Transfer approximately 25 µl of each sample supernatant to a clean 1.5 ml LoBind tube and determine the DNA concentration of samples, preferably with a fluorometer.

Note: If samples are to be stored prior to library construction, transfer them to a –15°C to –30°C freezer.

Before proceeding to library construction, check DNA samples against the quality control criteria as explained below.

Quality control criteria

Measure the concentrations of the purified pooled PCR-enriched DNA samples, the positive control and the no template control (NTC) using a fluorometer.

- NA12878 positive control concentration must be $>20\text{ng}/\mu\text{l}$ to show that no error has occurred during the T-PCR and purification steps.
- NTC must be “not detected”. Detectable DNA indicates potential contamination of the PCR and/or purification.
- DNA concentration of PCR-enriched DNA samples 1–10 must be $>4\text{ng}/\mu\text{l}$ to ensure sample quality for downstream experiments.

If there is evidence of contamination, or the concentration of sample DNA or positive control is insufficient, see “Troubleshooting Guide”, page 40.

Step: Library construction

Perform the library construction using reagents and adapters compatible with Illumina MiSeq instruments.

Step: Cleanup of adapter-ligated DNA

Cleanup of adapter-ligated DNA using silica gel columns technology removes DNA fragments <150 bp following library construction.

Step: Fine size selection

After library construction, this step allows the removal of large size fragments with magnetic bead-based technology.

Transfer approximately 17 μ l of each supernatant to a clean 1.5 ml LoBind tube.

Note: The median size of the library DNA should be 280 bp.

Step: PCR amplification of purified library

High fidelity amplification ensures that there is sufficient quantity of library DNA for the downstream sequencing step.

Step: Library cleanup

Primers, nucleotides, polymerases and salts are removed from the amplified libraries using silica gel column technology.

Note: A library can be stored in a -15°C to -30°C freezer prior to quantitation.

Step: Library quantitation

Accurate quantitation of amplifiable library molecules is essential for optimal reads quality and efficient data generation. The use of a qPCR library quantitation kit compatible with Illumina libraries is required for this step.

Quality control criteria

- The concentration for the positive control NA12878 must be >120 nM to show that there were no errors causing loss of DNA during the library construction, amplification and purification procedures.
- The concentration for the NTC must be <1.0 nM to show that there was no contamination during the library construction, amplification and purification procedures.
- The concentration of a sample library must be >80 nM to ensure sample quality for downstream experiments.

If there is evidence of contamination, or the concentration of sample DNA or positive control is insufficient, see “Troubleshooting Guide”, page 40.

Step: Library pooling

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit allows testing of 20 tumor samples in two sequencing runs. We recommend performing a sequencing run with 10 samples plus one NA12878 positive control and a no template control (NTC). Libraries of 10 samples and controls are diluted to 4nM and pooled, and then the pool is quantitated. The calculated concentration is used to obtain a final library pool concentration of 14 pM after denaturation.

Library dilution to 4 nM and pooling

Note: Take extreme care when pooling libraries.

Prepare dilutions of 10 library samples, and the NA12878 positive control, to a final concentration of 4 nM in nuclease-free water according to the initial library concentrations calculated in the previous step. Diluted libraries of 10 samples, NA12878 positive control and the negative control are pooled together.

Library pool quantitation

The use of a qPCR library quantitation kit compatible with Illumina libraries is required for this step.

Before proceeding to “Protocol: Preparation of pooled library for sequencing” page 32, check the quantitation of the pool against the quality control criteria as explained below.

Quality control criteria

- The concentration for the NTC must be <1 pM to show that there was no contamination during the pooling procedure.
- The concentration of the pooled library must be approximately 4 nM to ensure pool quality for sequencing.

If the concentration of the pooled library is insufficient, see “Troubleshooting Guide”, page 40.

Protocol: Preparation of pooled library for sequencing

Two procedures are performed in this protocol:

- Denaturation and dilution of the pooled library
- Preparation of PhiX internal control and addition to the pooled library

Note: Detailed descriptions of library denaturation and setting up an Illumina MiSeq run are available in the Illumina *MiSeq System Denature and Dilute Libraries Guide* and *MiSeq System User Guide*.

Reagents required for this protocol

- NaOH 10N
- MiSeq Reagent Kit v2 1/2 and 2/2
- PhiX Control v3
- 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 to dilute PhiX

Important points before starting

- Always prepare freshly diluted NaOH for denaturing libraries. This step is essential to the denaturation process.
- To prevent small pipetting errors from affecting the final NaOH concentration, prepare at least 1 ml of freshly diluted NaOH.
- For best results, begin thawing the reagent cartridge before denaturing and diluting libraries. For instructions, see the *MiSeq System User Guide*.

Things to do before starting

- Prepare freshly diluted NaOH 0.2N.

Combine the following volumes in a microcentrifuge tube:

- Laboratory-grade water (800 μ l)
- Stock 1.0 N NaOH (200 μ l)

The result is 1 ml of 0.2 N NaOH.

- Remove the tube of HT1 (Hybridization Buffer; provided with the kit) from storage at -15° to -30° C and set aside at room temperature to thaw. When thawed, store at 2° to 8° C until you are ready to dilute denatured libraries.

Denaturation and dilution of the pooled library

Procedure

1. Carefully denature the library pool by adding 5 μ l of NaOH 0.2N to 5 μ l of the library pool, vortex briefly, pulse spin and then incubate for 5 minutes at room temperature.

Note: Always use freshly prepared diluted NaOH.

2. Stop the denaturation reaction by adding 990 μ l of prechilled HT1 to the tube containing the denatured DNA.
3. Dilute the denatured DNA to 14 pM using prechilled HT1 in a final volume of 600 μ l, taking into account the initial library pool concentration.

For example, if the concentration of the pooled library was calculated at 20 pM after denaturation, adjust the concentration to 14 pM by pipetting 180 μ l of pre-chilled HT1 into 420 μ l of denatured DNA. Invert the tube several times to mix.

4. Place the denatured and diluted DNA on ice until you are ready to load the library.

Preparation of PhiX internal control and addition to the pooled library

Procedure

1. Dilute the PhiX internal control library to 4 nM by combining the volumes of components shown in Table 4.

Table 4. PhiX dilution to 4nM

Component	Volume
10 nM PhiX internal control library	2 μ l
10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20	3 μ l

2. Denature the 4 nM PhiX internal control library by combining the volumes of components shown in Table 5.

Table 5. PhiX denaturation

Component	Volume
4 nM PhiX internal control library	5 μ l
NaOH 0.2N	5 μ l

3. Pulse spin the tube and incubate for 4.5 minutes at room temperature.
4. Stop the denaturation reaction by adding 990 μ l of pre-chilled HT1.
5. Dilute the 20 pM denatured PhiX internal control library to 12.5 pM by mixing 375 μ l of denatured PhiX 20 pM with 225 μ l of prechilled HT1. Invert the tube several times to mix.
6. Add 6 μ l of 12.5 pM PhiX to the 600 μ l diluted library. Vortex and centrifuge for 1 minute at 1000 x g, and then place on ice.

Proceed to "Protocol: Setting up and starting the sequencing run", page 35.

Protocol: Setting up and starting the sequencing run

Two procedures are performed in this protocol:

- Loading the pooled library into the MiSeq reagent cartridge
- Starting the sequencing run

Loading the pooled library into the cartridge

Note: A detailed description of setting up an Illumina MiSeq run is available in the Illumina *MiSeq System User Guide*.

Reagents required for this protocol

- MiSeq Reagent Kit v2 1/2 and 2/2

Things to do before starting

- Remove the reagent cartridge (provided with the kit) from -15°C to -30°C storage. Place the reagent cartridge in a water bath containing enough room temperature deionized water to submerge the base of the reagent cartridge up to the waterline printed on the reagent cartridge. Do not allow the water to exceed the maximum waterline.
- Allow the reagent cartridge to thaw in the room temperature water bath for approximately one hour or until completely thawed.

Procedure

1. Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge. Make sure that no water has splashed on the top of the reagent cartridge.
2. Invert the reagent cartridge ten times to mix the thawed reagents and then visually inspect that all positions are thawed. Inspect the reagent in position 1 to make sure that it is fully mixed and free of precipitates.

3. Gently tap the cartridge on the bench to reduce air bubbles in the reagents. Place the reagent cartridge on ice or set aside at 2–8°C until you are ready to set up your run. For best results, proceed directly to step 4.
4. Visually check the integrity of the foil seal and then use a clean 1 ml pipette tip to pierce the foil seal over the reservoir on the reagent cartridge labeled “Load Samples”.
5. Pipette 600 µl of the denatured, diluted library pool with PhiX control into the “Load Samples” reservoir (sample position #17 on the cartridge).

Starting the sequencing run

Note: A detailed description of setting up an Illumina MiSeq run is available in the *Illumina MiSeq System User Guide*.

Note: A detailed description of creating and editing sample sheets for Illumina instruments and analysis software is available in the *Illumina Experiment Manager User Guide*.

Things to do before starting

- Clean the Illumina Flow Cell (provided in the MiSeq Reagent Kit v2 1/2) according to the manufacturer’s instructions. The flow cell is a single-use device.
- Illumina recommends creating the sample sheet prior to sample preparation.

Procedure

1. Restart MiSeq with **Research** mode and then select **Sequence** mode.
2. When the **Load Flow Cell** screen appears, load the flow cell.
3. When the **Load Reagents** screen appears, empty the waste bottle. Remove the bottle of MiSeq SBS Solution (PR2) from storage at 2–8°C. Invert to mix, remove the lid and load the PR2 bottle immediately.
4. Select the appropriate sample sheet according to the adapter bar code set selected during the adapter ligation step.

5. Using Illumina Experiment Manager, select **Create Sample Sheet**. In the **Select Category** window, choose **Other** and **FASTQ Only**.
6. In the **FASTQ Only Run Setting** window, select **TruSeqLT**, choose **1 Index Read**, select **Paired End Sequencing**, and **151** for **Cycles Read1** and **Cycles Read2**, and then select **Use Adapter Trimming** and **Use Adapter Trimming Read 2**.
7. Choose adapters according to those selected for the library preparation (see Table 6, below).

Note: We recommend using Illumina Experiment Manager to create a new sample sheet for every sequencing experiment. Do not open or modify the sample sheet before use.

Table 6. Available adapters and bar codes

Generead Adapter I Set A 12-Plex		Generead Adapter I Set B 12-Plex	
Adapter name	Bar code	Adapter name	Bar code
Adapter Bc 1 Illumina	ATCACG	Adapter Bc 13 Illumina	AGTCAA
Adapter Bc 2 Illumina	CGATGT	Adapter Bc 14 Illumina	AGTTCC
Adapter Bc 3 Illumina	TTAGGC	Adapter Bc 15 Illumina	ATGTCA
Adapter Bc 4 Illumina	TGACCA	Adapter Bc 16 Illumina	CCGTCC
Adapter Bc 5 Illumina	ACAGTG	Adapter Bc 18 Illumina	GTCCGC
Adapter Bc 6 Illumina	GCCAAT	Adapter Bc 19 Illumina	GTGAAA
Adapter Bc 7 Illumina	CAGATC	Adapter Bc 20 Illumina	GTGGCC
Adapter Bc 8 Illumina	ACTTGA	Adapter Bc 21 Illumina	GTTTCG
Adapter Bc 9 Illumina	GATCAG	Adapter Bc 22 Illumina	CGTACG
Adapter Bc 10 Illumina	TAGCTT	Adapter Bc 23 Illumina	GAGTGG
Adapter Bc 11 Illumina	GGCTAC	Adapter Bc 25 Illumina	ACTGAT
Adapter Bc 12 Illumina	CTTGTA	Adapter Bc 27 Illumina	ATTCCT

8. Select **Finish** and save the sample sheet file in the desired MiSeq folder.

9. After loading the flow cell and reagents, review the run parameters and perform a pre-run check before starting the run.
10. Review **Experiment Name**, **Analysis Workflow** and **Read Length**. These parameters are specified in the sample sheet.
11. Review the folder locations in the lower-left corner.
If any changes are needed, select **Change Folders**. When the changes are complete, select **Save**, and then select **Next**.
12. Select **Next**. The **Pre-Run Check** screen opens.
In the lower-left corner of the **Review** screen, the current folder locations for recipes, sample sheets, manifests and output folders are listed.
13. The system performs a check of all run components, disk space and network connections before starting the run.
If any items do not pass the pre-run check, a message appears on the screen with instructions on how to correct the error.
14. When all items successfully pass the pre-run check, select **Start Run**.
15. When the run is complete, the **Next** button appears. Review the results on the **Sequencing** screen before proceeding.
Note: The **Sequencing** screen remains viewable until **Next** is selected. After you select **Next**, it is not possible to return to the **Sequencing** screen.
16. Select **Next** to exit the **Sequencing** screen and proceed to a post-run wash.

The post-run wash is the standard instrument wash performed between sequencing runs. Always perform an instrument wash after completing a run. Follow the software prompts to load the wash components and perform the wash. The post-run wash takes approximately 20 minutes. Start the wash directly following the completion of a run. An instrument wash is required before you can set up a subsequent run.

Before proceeding to “Protocol: Data analysis”, page 12 in Part 2, check the output of the sequencing run against the quality control criteria as explained below.

Quality control criteria

- Data output >3 Gb indicates sufficient quantity of reads.
- The percentage of reads with a Q-Score >Q30 must be >80%. A Q-score is a prediction of the probability of a wrong base call.

If the output of the sequencing run is insufficient, see “Troubleshooting Guide”, page 40.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise in the assessment of *BRCA1/2* mutation status using the *therascreen* BRCA1/2 NGS FFPE gDNA Kit. For contact information, see back cover or visit www.qiagen.com.

For troubleshooting information relating to other kits, please refer to the respective kit handbooks.

For troubleshooting information relating to the Illumina MiSeqDx instrument and related software including Biomedical Genomics Workbench and BRCA 1/2 CE-IVD Workflow, please refer to the respective user guides and manuals.

Comments and suggestions

Low yield of target DNA

- | | |
|---|---|
| a) Check the gDNA concentration | We recommend using a fluorometer for gDNA quantitation from FFPE starting material.

The gDNA concentration must be >2.5 ng/μl to ensure sufficient sample quantity for downstream experiments. The kit is optimized for 10 ng of gDNA per target PCR reaction (40 ng total). |
| b) Check concentration of positive control obtained after Target-PCR (T-PCR) pooling and purification | If the concentration of the NA12878 positive control is less than 20 ng/μl, an error could occur during the T-PCR or during the sample pooling and purification step.

Repeat the T-PCR step for all the samples. |

Comments and suggestions

- c) Check concentration of samples after T-PCR pooling and purification
- Sample concentration below the quality control criterion of 4 ng/ μ l may indicate degraded DNA.
- Repeat DNA extraction from the failed sample.

Low library yields

- a) Check the T-PCR yields
- After the T-PCR and before proceeding to the library preparation, concentrations of positive control and samples must be >20 ng/ μ l and >4 ng/ μ l respectively.
- If the concentration of the positive control is too low, repeat the T-PCR step for all samples.
 - If the concentration of a sample is too low, repeat the extraction of the sample DNA.
- b) Check concentration of the positive control after the library preparation and size selections steps
- Quantitate the purified and size-selected libraries using an Illumina-compatible qPCR library quantitation kit. Repeat the library quantitation if quality control criteria are not fulfilled.
- If the concentration of the NA12878 positive control is less than 120 nM, an error may have occurred during library preparation, size selection, PCR amplification or the PCR purification step.
- Restart the library construction from the gDNA for all samples.

Comments and suggestions

- c) Check concentration of the samples after the library preparation and size selections steps
- Quantitate the purified and size-selected libraries using an Illumina-compatible qPCR library quantitation kit. Pay attention to the qPCR quality control criteria described in the protocol.
- If a sample concentration is less than 80 nM, an error may have occurred during the library preparation, size selection, PCR amplification or the PCR purification step.
- Restart the library construction from the gDNA for that sample.

Low output of sequencing data (Total reads <3Gb)

- Check the quantity of library material added to the Illumina sequencing cartridge
- To avoid misreading parts of the targeted *BRCA1/2* region, 3 Gb of total sequencing data output is recommended. If the quality criterion of 3 Gb is not fulfilled, restart the protocol from the library quantitation step.
- Check Illumina flow cell images according to the manufacturer's instructions.
- If the library is overloaded (saturating cluster density), decrease the amount of pooled libraries added to the cartridge.
 - If the clustering density is low, increase the amount of pooled libraries added to the cartridge.

Comments and suggestions

Low sequencing specificity (% of reads aligned to the *BRCA1/2* target region)

Check the mean size of the size-selected and purified libraries

If the quality criterion of 80% specificity is not fulfilled, assess the purification quality by analyzing library fragment size. The mean amplicon size should be about 280 bp.

Restart the protocol from the T-PCR.

Low reads coverage

Check the minimum coverage per amplicon

If the quality criteria of 200x coverage is not fulfilled, we recommend:

- Checking that the 4 target PCR reactions were pooled at equivalent volumes.
- Checking the reads homogeneity in terms of the number of reads obtained per sample for the 10 samples plus the positive control.

Contamination of no template control (NTC)

a) Check NTC after T-PCR

If sample is detected in the NTC, contamination may have occurred during the T-PCR, or during the sample pooling and purification step.

Restart the T-PCR

b) Check the concentration of NTC after the library preparation and size selections steps

If the concentration of NTC is greater than 1 nM, contamination may have occurred during the library preparation, size selection, PCR amplification or the PCR purification step.

Restart the T-PCR.

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Catalog number
	Manufacturer
	Material number
Rn	R is for revision of the Handbook and n is the revision number
	Lot number
	Global Trade Item Number
	In vitro diagnostic medical device
	CE mark for European conformity
	Use by
 <N>	Contains reagents sufficient for N reactions

Symbol	Symbol definition
	Temperature limitation
	Components (i.e., a list of what is included)
	Contains (contents)
	Number (i.e., vials, bottles)
	Caution
	Consult instructions for use
	Keep away from sunlight

Ordering Information

Ordering information for additional required products and reagents may be found in Table 1, page 15.

Product	Contents	Cat. no.
<i>therascreen</i> BRCA1/2 NGS FFPE gDNA Kit CE (20)	For 20 reactions: For the identification of variants in <i>BRCA1</i> and <i>BRCA2</i> with Illumina MiSeqDx platform; BRCA Primer Mix 1, BRCA Primer Mix 2, BRCA Primer Mix 3, BRCA Primer Mix 4, HotStarTaq DNA polymerase, GR NGS Panel 5x PCR Buffer V2, nuclease-free water for NTC	875011

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.

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