February 2017

therascreen® BRCA1/2 NGS FFPE gDNA Kit Handbook Part 2: Analysis

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

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

R2 MAT 1103449EN

Contents: Part 2

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.

Principle of the Procedure

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 7). These criteria allow validation of the different steps of the workflow to identify samples that give poor sequencing results or to indicate potential contamination.

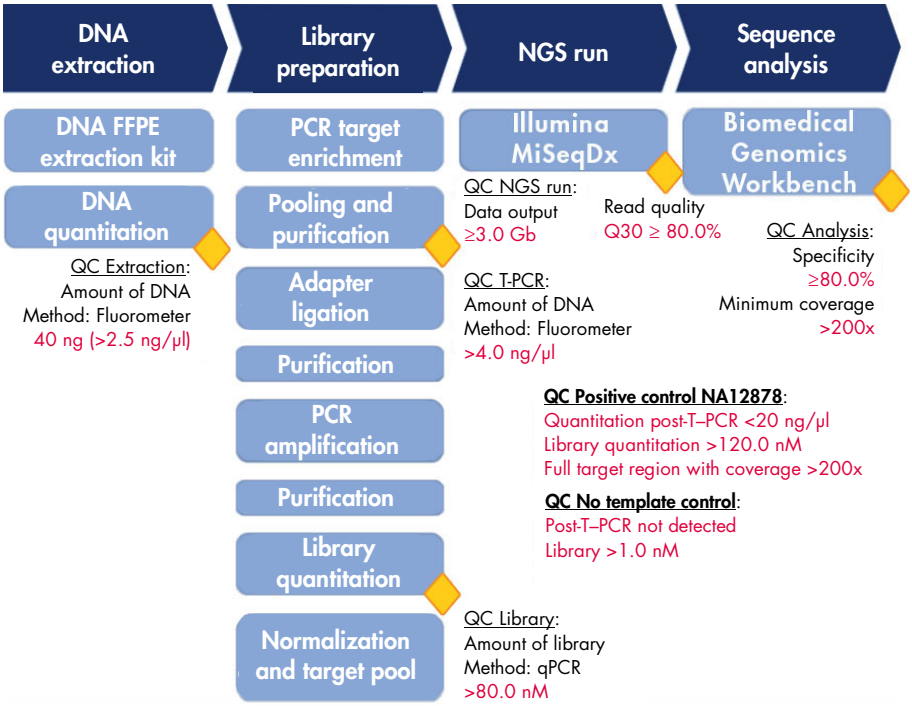


Figure 7. In-process control criteria. Through the sequencing workflow (blue boxes), several in-process control steps are performed (yellow lozenges) to validate the T-PCR, the library preparation and the sequencing run. The final criterion used to ensure good quality variant calling at a given position is the minimum coverage obtained. Specificity relates to the percentage of pair reads aligned to the target region.

Materials Required but Not Provided: Analysis

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

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.

Procedure: Part 2

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.

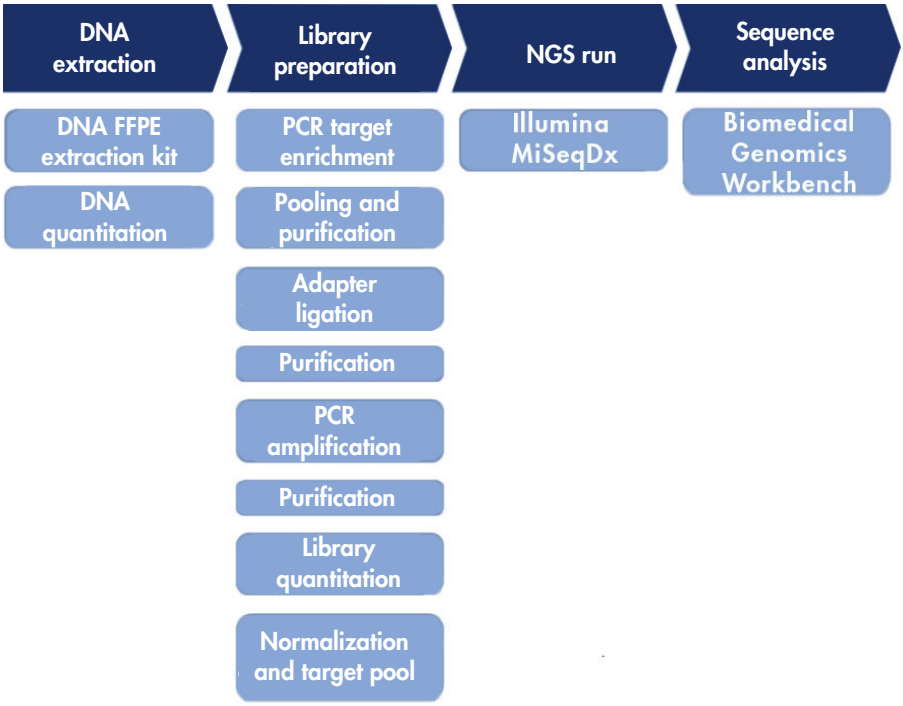


Figure 8. Overview of the NGS workflow.

Protocol: Data analysis

This section includes a description of software installation and analysis of FASTQ files generated during the sequencing.

Products and software required for data analysis:

- Biomedical Genomics Workbench software version 2.1.1 (**www.clcbio.com**)
- CLC Genomics Server 7.0.2 with Biomedical Genomics Extension(**www.clcbio.com)**
- The FASTQ files (two FASTQ files are expected per sample for paired reads)

It is important to use the specific assay analysis workflow to perform the sequence analysis. It is expected that the user performs the CLC software analyses using a non-"admin" user account.

Things to do before starting

- If not already installed, the assay analysis BRCA 1/2 CE-IVD Workflow must be installed prior to sequence analysis. A downloadable version is available from QIAGEN at the **Product Resources** tab of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit product page on the QIAGEN website.
- If not already installed, the QIAGEN GeneRead Panel Analysis Plugin must be installed prior to sequence analysis.

Installing the analysis workflow

There are two options for installing the analysis workflow:

- Local installation (follow "Workflow: local installation process")
- Installation on CLC Genomics Server (skip "Workflow: local installation process" and follow "Workflow: server installation process")

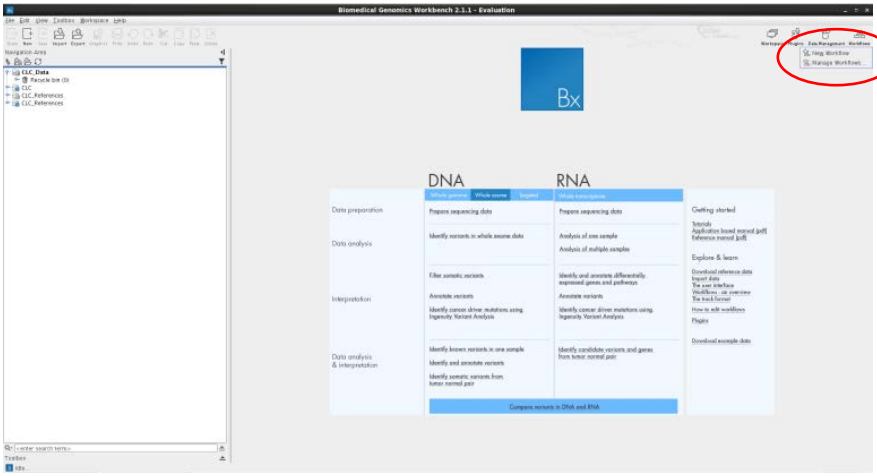
If the assay analysis BRCA 1/2 CE-IVD Workflow is already installed, skip "Workflow: local installation process" and "Workflow: server installation process".

Workflow: local installation process

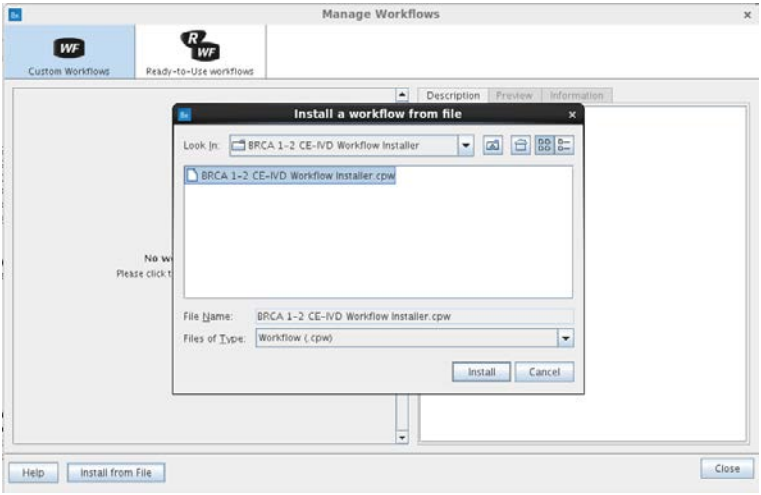
This installation procedure will install the assay analysis BRCA 1/2 CE-IVD Workflow on your local computer where the Biomedical Genomics Workbench software is installed.

Procedure

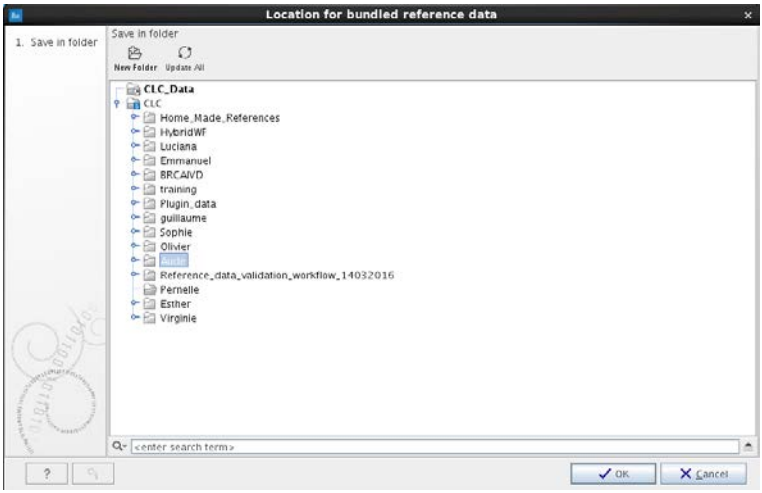
1. Launch the **Biomedical Genomics Workbench** software.
2. Click on **Workflows** and then **Manage Workflows**.



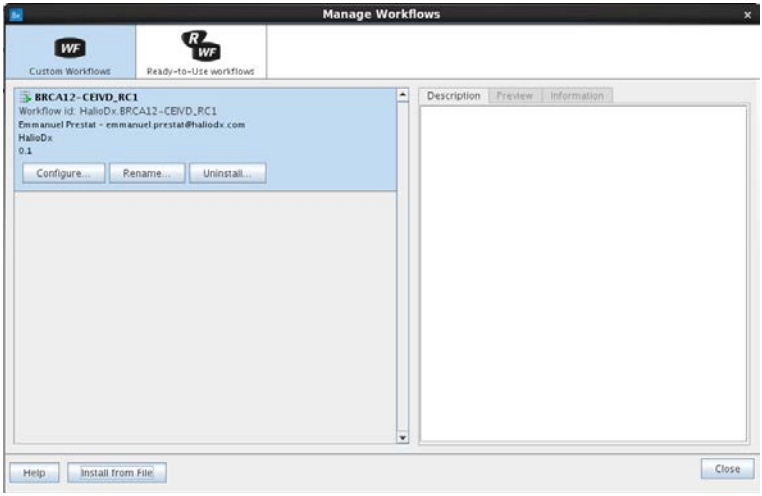
3. Click **Install from File**.



4. Select the workflow file **BRCA 1-2 CE-IVD Workflow Installer.cpw**. Click **Install**.
5. Create a new folder and select it, and then click **OK**.



6. Click **Close**.



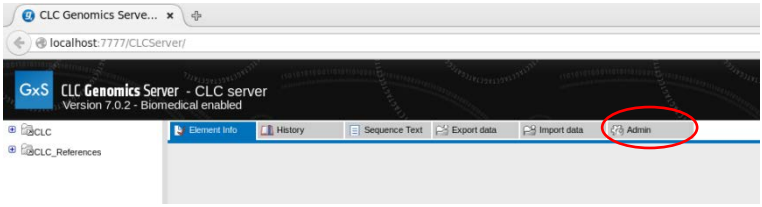
Workflow: server installation process

This installation procedure will install the assay analysis BRCA 1/2 CE-IVD Workflow on the CLC Genomics Server (Biomedical enabled). In the following procedure, we use "serverIP" for the server IP address and assume the CLC Genomics Server port is "7777" (the default).

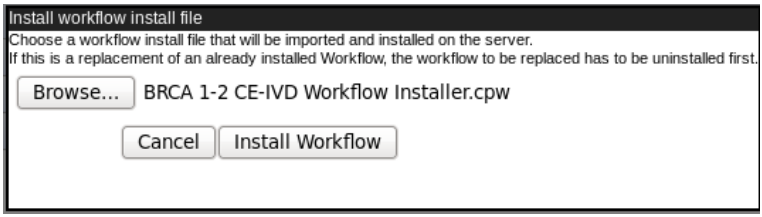
Procedure

1. Using a web browser, connect to **http://serverIP:7777/**.
   Replace "serverIP" with the IP of the server, or use "localhost" if the web browser is run directly from the server.
2. Enter the CLC administrator credentials (by default, login is "root" and password is "default").

3. Click the **Admin** tab.



4. Open **Workflows** and click **Install Workflow...**.



5. Select the workflow file **BRCA 1-2 CE-IVD Workflow Installer.cpw** and then click **Install Workflow**.

Note: When the installation of BRCA 1/2 CE-IVD Workflow is complete, the Biomedical Genomics Workbench software has to be restarted before importing FASTQ files from the MiSeq instrument.

Installing the analysis plug-in

There are two options for installing the analysis plug-in:

- Local installation (follow "Plug-in: local installation process")
- Installation on CLC Genomics Server (skip "Plug-in: local installation process" and follow "Plug-in: server installation process")

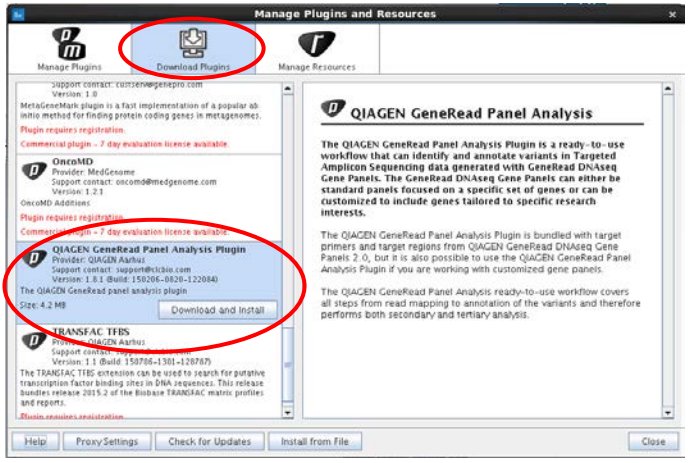
If the assay analysis plug-in is already installed, skip "Plug-in: local installation process" and "Plug-in: server installation process".

Plug-in: local installation process

This installation procedure will install the QIAGEN GeneRead Panel Analysis Plugin on your local computer where the CLC Biomedical Genomics workbench is installed.

Procedure

1. Launch the Biomedical Genomics Workbench software.
2. Click on **Plugins**, and then select **Download Plugins**.



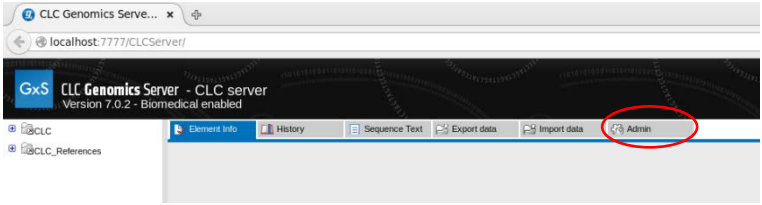
3. Select the QIAGEN GeneRead Panel Analysis Plugin and then click **Download and Install**.

Plug-in: server installation process

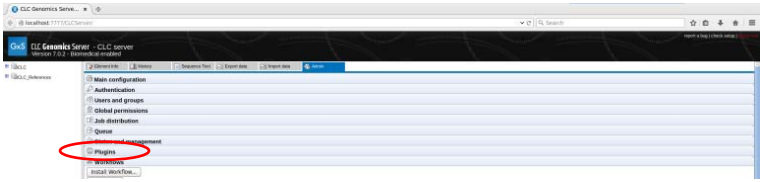
This installation procedure will install the QIAGEN GeneRead Panel Analysis Plugin on the CLC Genomics Server (Biomedical enabled). In the following procedure, we use "serverIP" for the server IP address and assume the CLC Genomics Server port is "7777" (the default).

Procedure

1. Using a web browser, connect to **http://serverIP:7777/**.
   Replace "serverIP" with the IP of the server, or use "localhost" if the web browser is run directly from the server.
2. Enter the CLC administrator credentials (by default, login is "root" and password is "default").
3. Click the **Admin** tab.



4. Open **Plugins**.



5. Go to the **Install new plugin** panel and **Browse…** to the file location.

Install the downloadable version of the GeneRead Panel Analysis Server Plugin (available from the **Product Resources** tab of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit product page on the QIAGEN website).

Exporting Illumina FASTQ files from the MiSeq instrument

FASTQ files stored on the MiSeq computer must be exported from the MiSeq instrument to the destination of your choice (external drive or server) to be available for the Biomedical Genomics Workbench software.

Note: The FASTQ files are located in the following sequencing run folder: **MiSeqAnalysis\RunID\Data\Intensities\BaseCalls**.

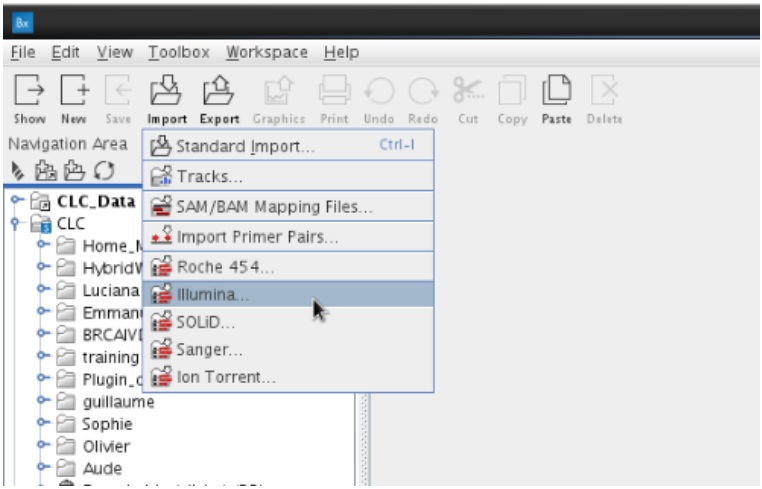
We recommend that the results file not be stored long-term on the NGS platform to avoid confusion between successive runs, and to maintain sufficient free disk space.

Importing Illumina FASTQ files

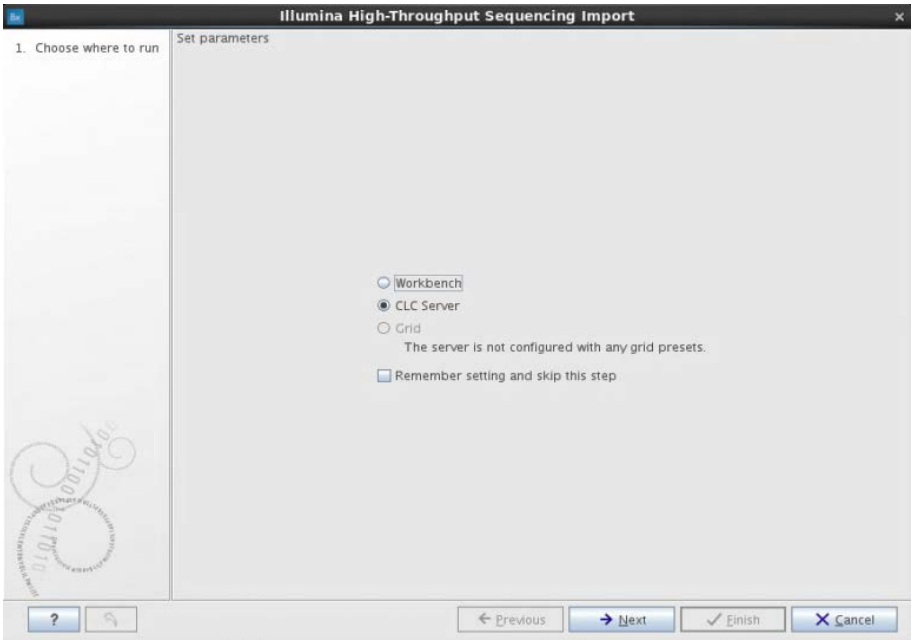
There are two Illumina FASTQ files per sample.

Procedure

1. Open the Biomedical Genomics Workbench software.
2. Click **Import** and select **Illumina** in the menu.

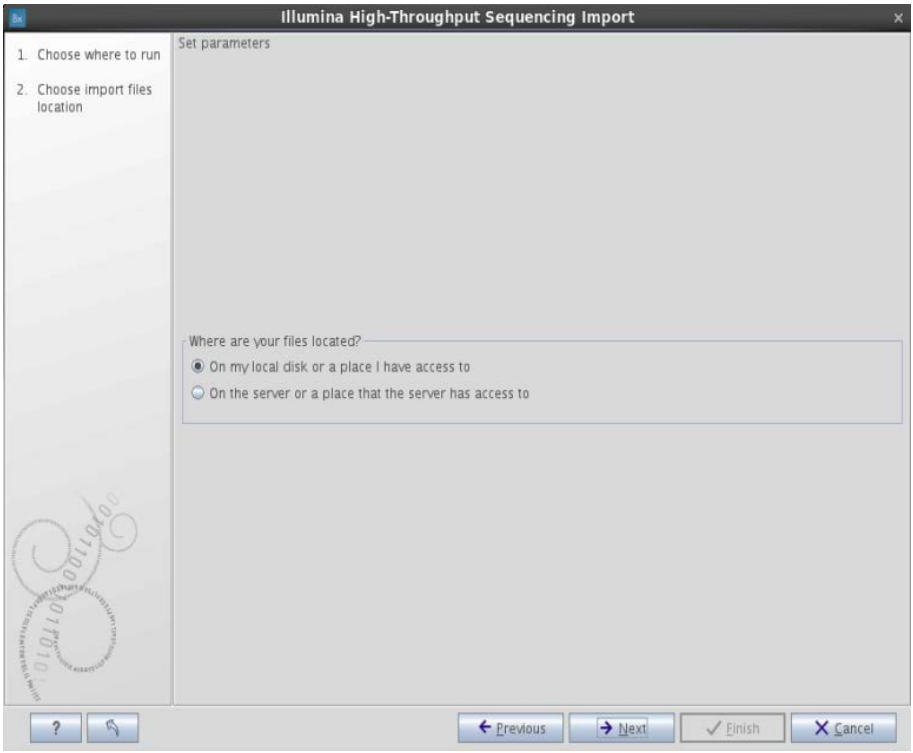


3. Choose where to run by selecting the appropriate option.
   - Select **Workbench** if the BRCA 1/2 CE-IVD Workflow is installed locally (i.e., installed using the "Workflow: local installation process").
   - Select **CLC Server** if the BRCA 1/2 CE-IVD Workflow is installed at the server level (i.e., installed using the "Workflow: server installation process").


Illumina High-Throughput Sequencing Import
1. Choose where to run
Set parameters
Workbench
CLC Server
Grid
The server is not configured with any grid presets.
Remember setting and skip this step
Previous
Next
Finish
Cancel

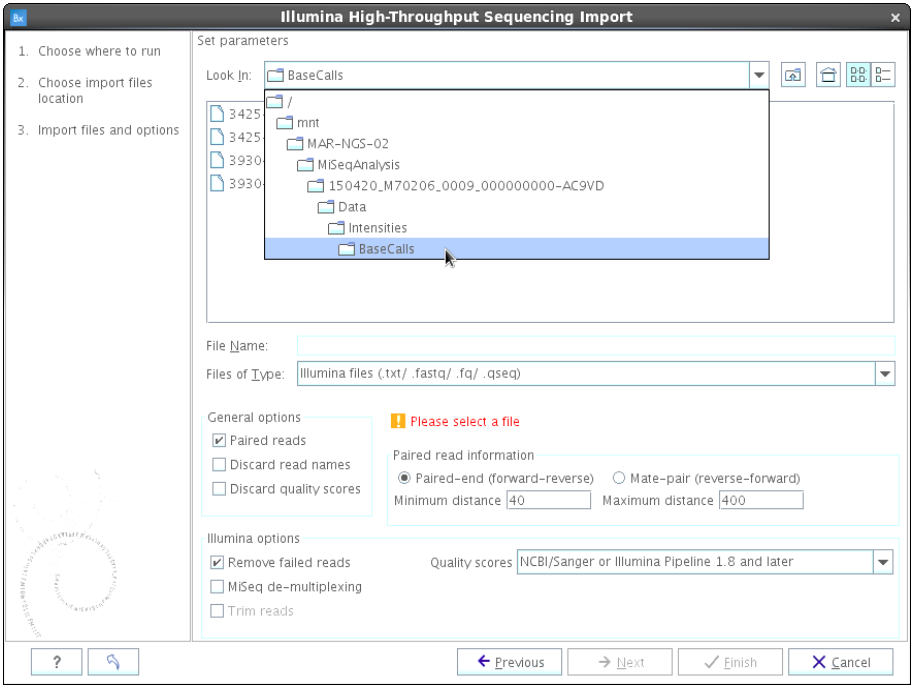
4. Click **Next**.

5. If **CLC Server** was selected in the previous step, the following window will open.



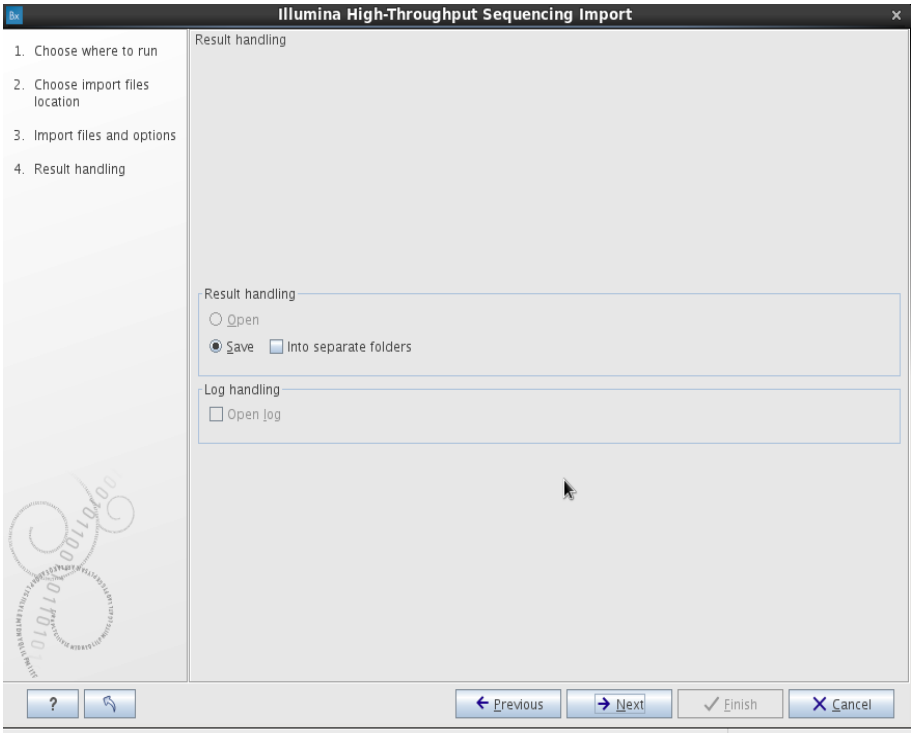
6. Select **On my local disk or a place I have access to** and click **Next**.

7. Select all the FASTQ files to be analyzed from the following path in the MiSeq file: **Analysis/Data/Intensities/BaseCalls**.



8. Select the following settings:
   - Check **Paired reads**
   - Select **Paired-end (forward-reverse)**
   - Fill in the **Minimum distance** field with **40** and **Maximum distance** field with **400**
   - Check **Remove failed reads**
9. Click **Next**.

10. The following window will open. Select **Save** and click **Next**.



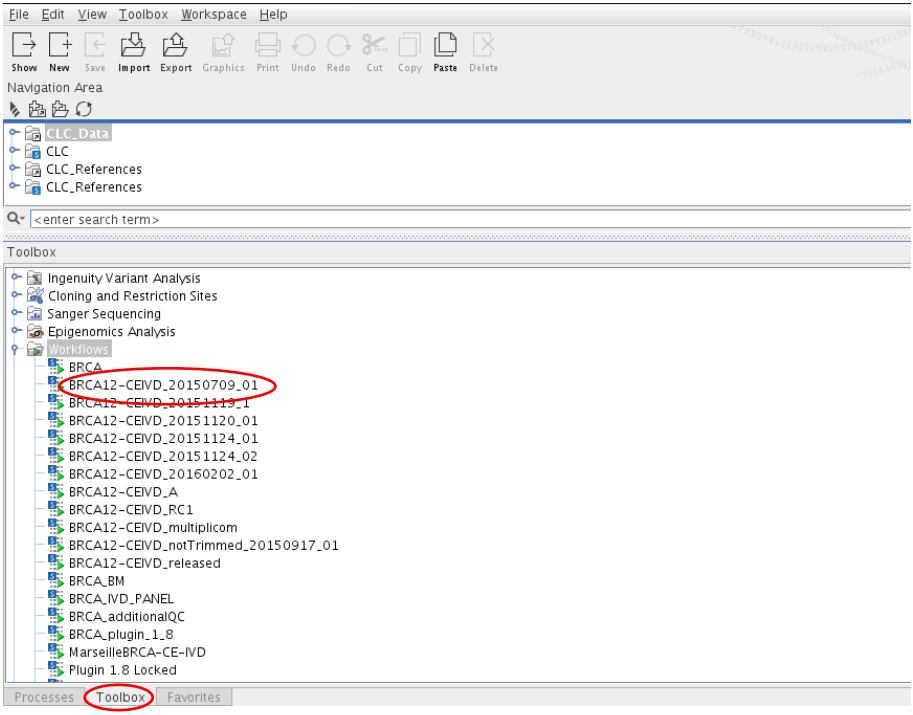
11. Create a folder to store the paired FASTQ files and click **Finish**.

Sequence analysis

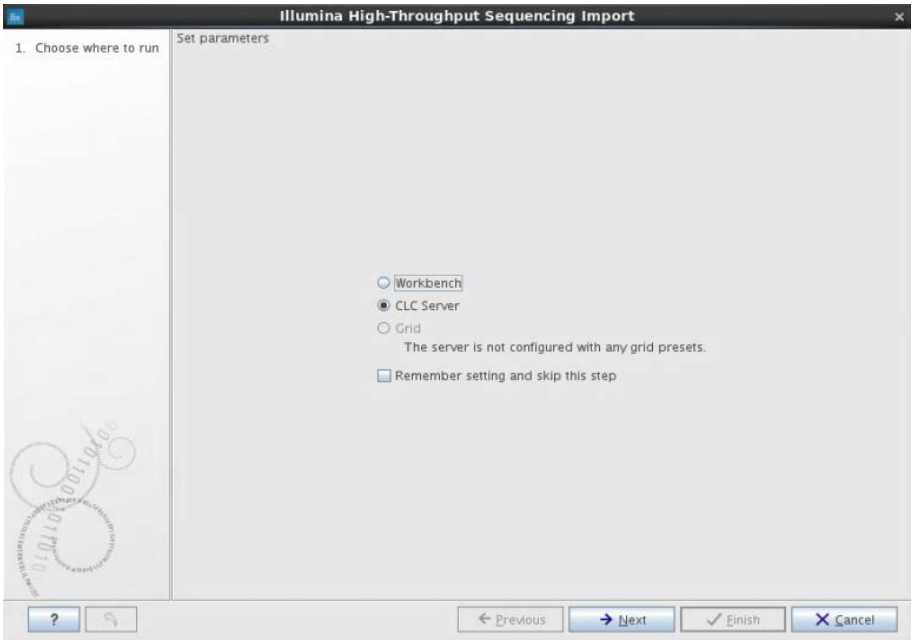
Process the FASTQ files with the assay analysis BRCA 1/2 CE-IVD Workflow that was installed using the "Workflow: local installation process" or the "Workflow: server installation process". Follow the step by step procedure for the paired FASTQ analysis as described below.

Procedure

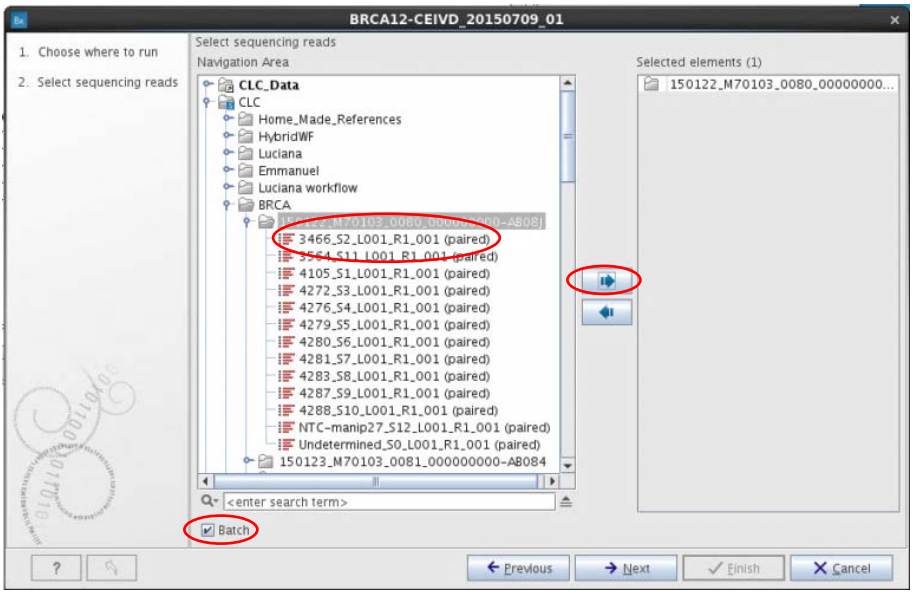
1. Select the **Toolbox** tab and double-click the workflow name.



The following window opens.

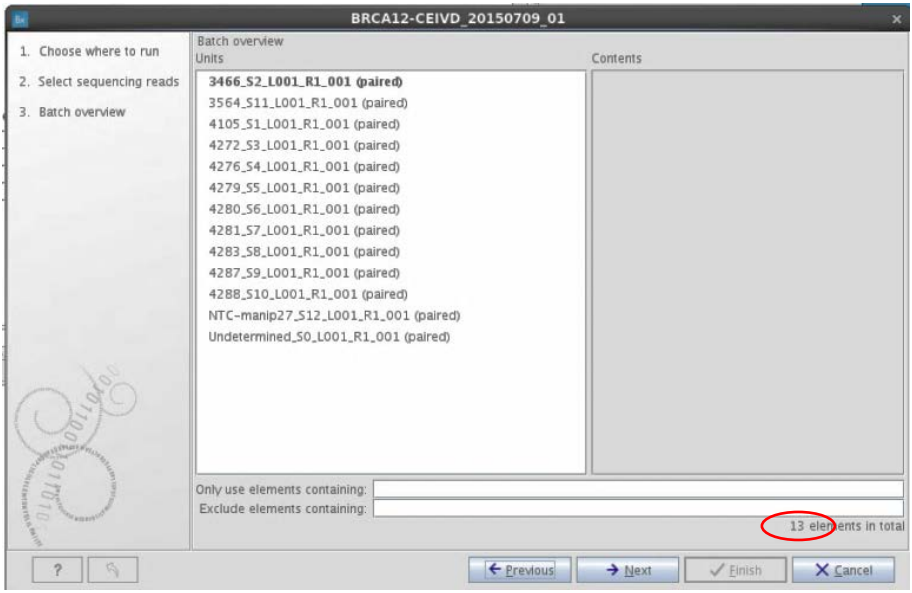


2. Select the applicable option:
   - Select **Workbench** if the BRCA 1/2 CE-IVD Workflow is installed locally.
   - Select **CLC Server** if the BRCA 1/2 CE-IVD Workflow is installed at the server level.
3. Click **Next**.



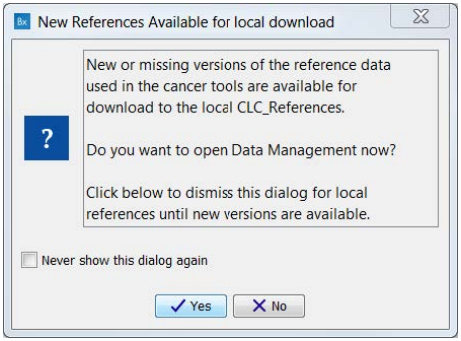
4. Choose the folder that contains the FASTQ files, check **Batch** and click the blue arrow  to select the folder.
5. Click **Next**.

6. Visually confirm that 13 elements are selected in the **Units** panel. Click **Next**.



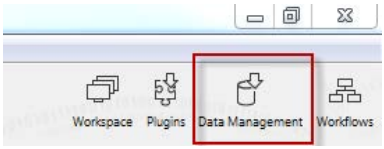
Note: The first time the BRCA 1/2 CE-IVD Workflow is used, you must select the reference data in the folder **CLC_References**.

The first time you open Biomedical Genomics Workbench a dialog box appears with a message that reference data are available for download either to the local or server **CLC_References** repository.

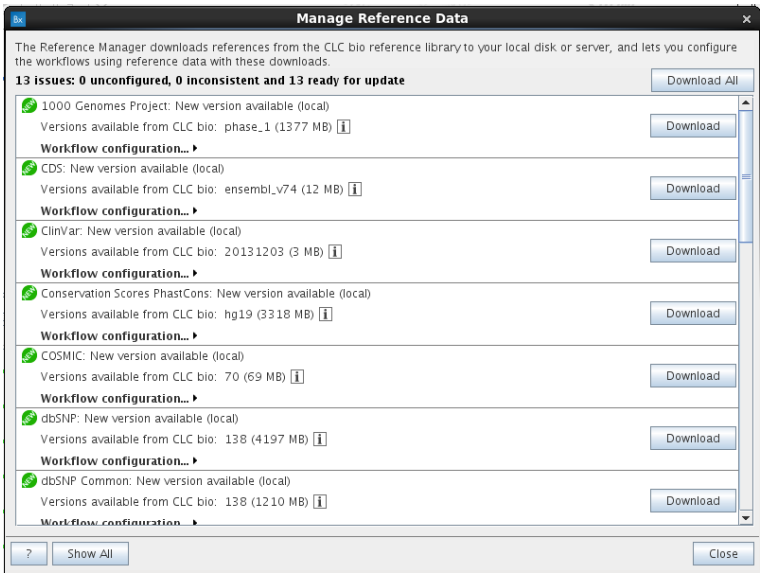


Click **Yes**. This will take you to the **Manage Reference Data** wizard.

This wizard can also be accessed from the upper right corner of the Biomedical Genomics Workbench by clicking on **Data Management**.



To install the reference data, click **Data Management** and download the following reference databases: 1000 Genomes Project, CDS, ClinVar, Conservation Scores PhastCons, Cosmic, dbSNP, dbSNP Common, Genes, HapMap, mRNA, Sequence, Target Primers, Target Regions.



Refer to *Biomedical Genomics Workbench Application Manual*, Section 4.1 "Reference data" for more information.

Select the reference data in **CLC_References** to perform step 7 to step 22 below.

For example, for step 7, the workflow input for CDS must be selected from the following path:

CLC_References/homo_sapiens/cds/ensemble_V74/Homo_sapiens_ensembl_v74_CDS

Note: After first use, the following 16 steps (7–22) only require clicking through screens by clicking **Next** at the bottom of the screen.

7. Click **Next**.

8. Click **Next**.

9. Click **Next**.

10. Click **Next**.

11. Click **Next**.

12. Click **Next**.

13. Click **Next**.

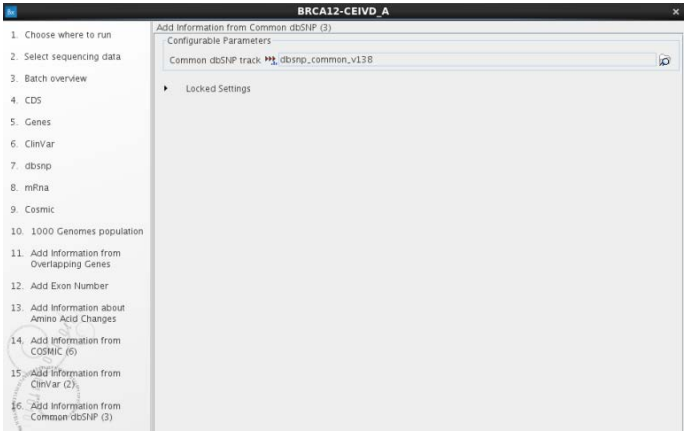
14. Click **Next**.

15. Click **Next**.

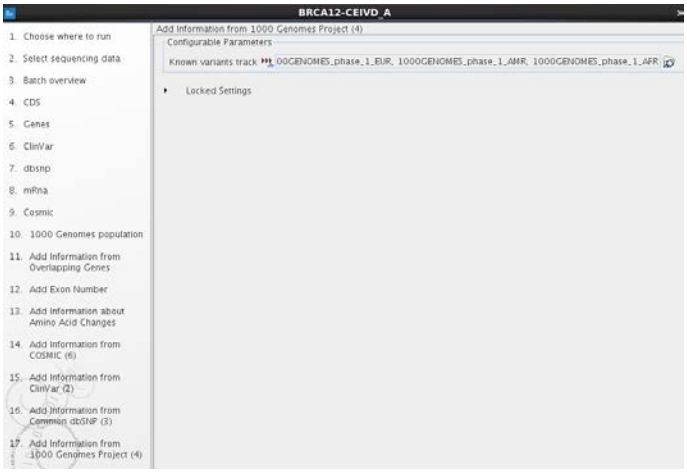
16. Click **Next**.

17. Click **Next**.

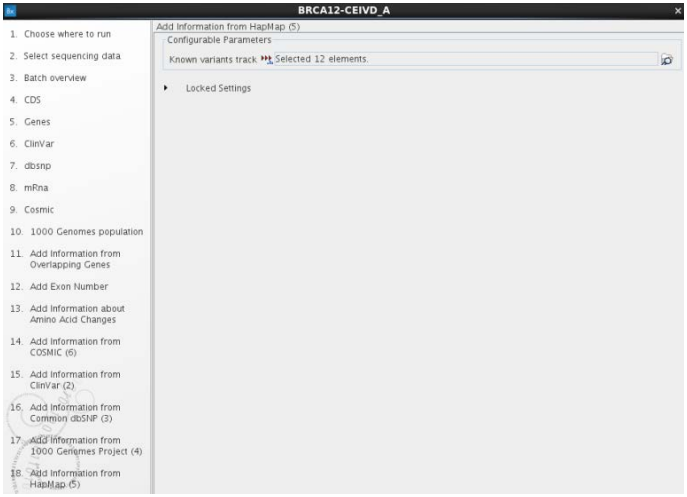
18. Click **Next**.



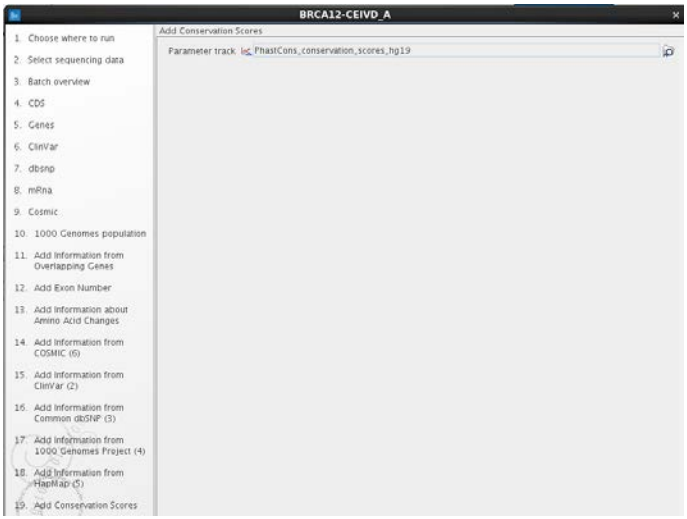
19. Click **Next**.



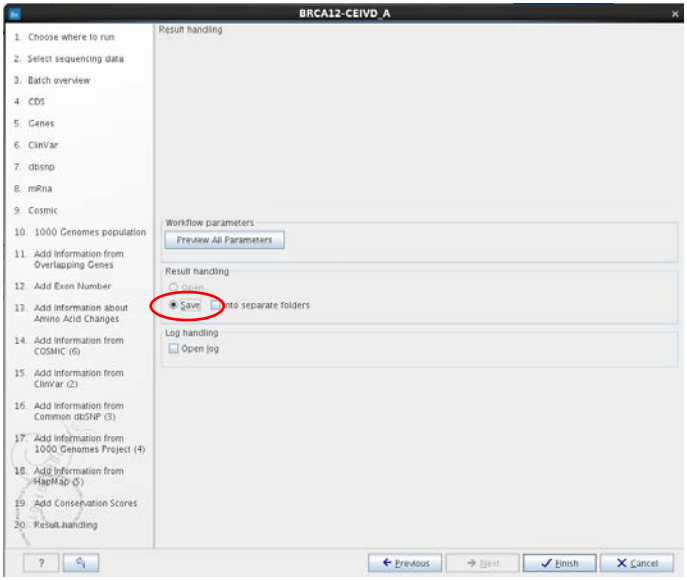
20. Click **Next**.



21. Click **Next**.



22. Click **Next**.



23. Select **Save** and then click **Finish**. The sequence analysis will start.

When the sequence analysis is complete and before proceeding to variant analysis:

- Verify that the minimum number of reads per amplicon for the positive control is >200x. This indicates that there is sufficient homogeneity through the *BRCA1/BRCA2* amplicons.
- Check that the minimum number of reads per amplicon for the samples is >200x.

IMPORTANT: We recommend using only targets with coverage >200 reads for the final analysis. The minimum coverage obtained per amplicon can be found in the "Locally realigned trimmed reads read mapping (coverage) region statistics" report.

The read specificity defines the percentage of reads aligned to the target region. To calculate the sequencing specificity and ensure that it is sufficient, open the "Mapping summary report" (see Table 7) to find the total number of reads then open the "Coverage summary report". Data for the reads aligned onto the target region are shown in the "Targeted regions overview" of the "Coverage summary report" (see Table 8). Read specificity for the example is 92%. We recommend a read specificity >80%.

Table 7. Mapping summary report

Summary statistics					
	Count	**Percentage of reads**	**Average length**	**Number of bases**	**Percentage of bases**
References	25	–	123,827,759.24	3,095,693,981	–
Mapped reads	3,012,232	98.91%	143.92	433,521,188	99.16%
Not mapped reads	33,274	1.09%	110.32	3,670,839	0.84%
Reads in pairs	2,962,962	97.29%	148.38	426,681,289	97.60%
Broken paired reads	49,270	1.62%	138.82	6,839,899	1.56%
Total reads	3,045,506	100%	143.55	437,192,027	100%

Table 8. Targeted regions overview

Reference	Total mapped reads	Mapped reads in targeted region	Specificity (%)	Total mapped reads excluding ignored	Total mapped reads in targeted region excluding ignored	Specificity excluding ignored (%)
1	0	0	–	0	0	–
2	0	0	–	0	0	–
3	0	0	–	0	0	–
4	0	0	–	0	0	–
5	0	0	–	0	0	–
6	0	0	–	0	0	–
7	0	0	–	0	0	–
8	0	0	–	0	0	–
9	0	0	–	0	0	–
10	0	0	–	0	0	–
11	0	0	–	0	0	–
12	0	0	–	0	0	–
13	1,606,916	1,606,916	100.0	1,606,916	1,606,916	100.0
14	0	0	–	0	0	–
15	0	0	–	0	0	–
16	0	0	–	0	0	–
17	1,200,127	1,200,127	100.0	1,200,127	1,200,127	100.0
18	0	0	–	0	0	–
19	0	0	–	0	0	–
20	0	0	–	0	0	–
21	0	0	–	0	0	–
22	0	0	–	0	0	–
X	0	0	–	0	0	–
Y	0	0	–	0	0	–
MT	0	0	–	0	0	–
Total	2,807,043	2,807,043	100.0	2,807,043	2,807,043	100.0

Quality control criteria

- There must be 200x of minimum coverage per amplicon for the NA12878 positive control.

  There is sufficient read depth of coverage between the amplicons to ensure validation of the sequencing run and the sequence alignment.
- There must be 200x of minimum coverage per amplicon for the samples.

  This ensures homogeneity of coverage and the quality of the samples.
- To ensure specificity of the primers, >80% of reads are aligned to the target region for the NA12878 positive control.
- To ensure specificity of the primers, >80% of reads are aligned to the target region for samples.

If these quality control criteria are not met, see “Troubleshooting Guide”, page 42.

Interpretation of Results

For each sample, export the file in the Variant Call Format (VCF file) as described below and submit to the *BRCA1/BRCA2* variants database of your choice to identify the clinical significance of the variants. For variants having a clinical pathological impact, check if the called variant is present in the list of identified false positive variants (see "False positive variants", page 57, and in Table 16 on page **Error! Bookmark not defined.**).

Pay attention to the type of sequence within which a variant is found:

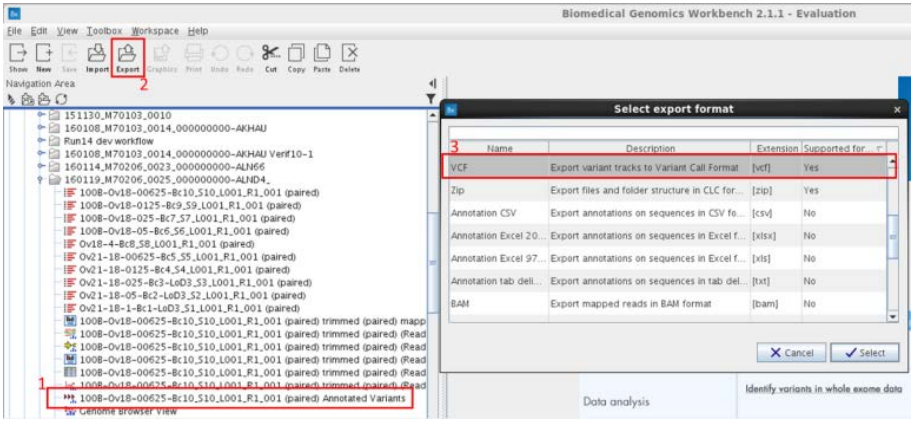
- Intronic sequence
- Exonic sequence
- Adjacent/flanking (= 20 nt before and after the target region)
- Homopolymer
- Nucleotide stretch region

Note: Homopolymers (>6 nucleotides) and nucleotide stretch regions (di- or tri-nucleotide repeats) are sources of false positives. The corresponding variants should be assessed with care. We recommend performing a confirmatory experiment using an alternative sequencing method (e.g., Sanger sequencing).

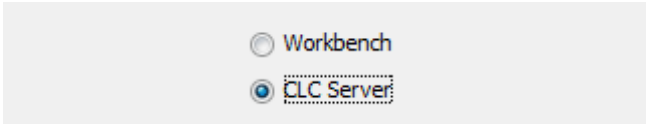
Note: The interpretation of the variants should be performed by a clinical geneticist.

Exporting a VCF file

1. Select the "Annotated Variants" file in the **Navigation Area** panel (1).
2. Click the **Export** button in the toolbar (2).
3. In the **Select export format** window, choose **VCF** (3) and click **Select**.

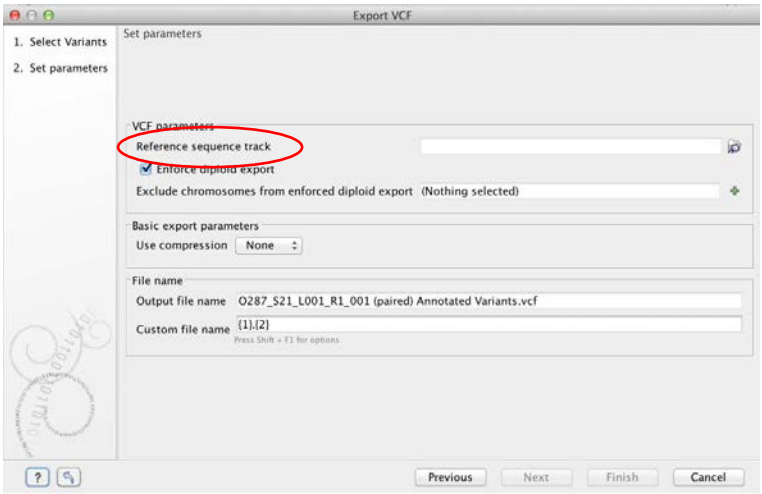


4. If you are logged into a CLC Server, you will be asked whether to run the export job using the Workbench or the CLC Server. Select **Workbench** or **CLC Server**.



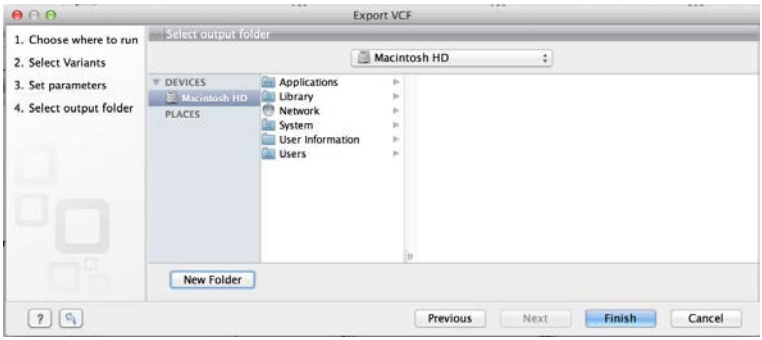
5. Click **Next**.

6. Confirm selection of the data to be exported. The **Export VCF** dialog opens.



Note: For the first use, the **Reference sequence track** must be selected from the following path: **CLC_References/homo_sapiens/sequence/hg19/Homo_sapiens_sequence_hg19**.

7. Click **Next**.



8. Select the export folder and click **Finish**.

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/µl 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/µl and >4 ng/µl 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.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *therascreen* BRCA1/2 NGS FFPE gDNA Kit is tested against predetermined specifications to ensure consistent product quality.

Limitations

The kit is intended for professional use.

The product is to be used only by personnel specially instructed and trained in molecular biology techniques and familiar with this technology.

This kit should be used following the instructions given in this manual, in combination with a validated instrument mentioned in "Materials Required but Not Provided: Analysis", page 7.

Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit is validated only for buffered formalin-fixed paraffin-embedded tissue.

Only the Illumina MiSeqDx has been validated for sample library sequencing.

Any off-label use of this product and/or modification of the components will void QIAGEN's liability.

It is the user's responsibility to validate system performance for any procedures used in their laboratory that are not covered by the QIAGEN performance studies.

Detection of mutations is dependent on sample integrity, the tumor content and amplifiable DNA present in the specimen.

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit together with the analysis workflow is not suited for copy number variation (CNV) analysis.

Any diagnostic results generated with the product must be interpreted within the context of all relevant clinical or laboratory findings.

Performance Characteristics

IMPORTANT: The assay performance characteristics for the *therascreen* BRCA1/2 NGS FFPE gDNA Kit were obtained with the Biomedical Genomics Workbench software reporting tool with the BRCA 1/2 CE-IVD Workflow. The entire workflow should be subject to independent verification by the laboratory end user before being introduced into routine use.

Reportable range of the assay

The design of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit covers the entire coding regions of *BRCA1* and *BRCA2*, plus at least 20 bp flanking each coding exon. Information on coverage by the kit is given in Table 9.

Table 9. Coverage information

Coverage	Number of values	FFPE 82 samples	NA12878 67 samples
Minimum coverage	Minimum	1,863	813
	Median	5,624	4,725
	Mean	5,591	5,187
	Maximum	11,890	14,187
	% of data with coverage ≥200x	100%	100%
Normalized minimum coverage* (equivalent to 3 GB of data per sequencing run)	Minimum	1,142	539
	Median	3,499	3,124
	Mean	3,494	3,416
	Maximum	7,383	9,211
	% of data with coverage ≥200x	100%	100%

* The amplicon coverage per sequencing run was normalized by multiplying the minimum coverage obtained with the kit by the ratio: 3 Gb (minimum sequencing data output recommended)/Run sequencing output (in Gb).

Uniformity of amplification

Among 82 FFPE sample datasets, the data showed that 99.29% of the targeted positions are covered by a number of reads higher than 20% of the mean depth of coverage (95% prediction interval 96.81–100%).

Interfering substances

Reagents from the FFPE DNA extraction kit and potential interfering substances from FFPE samples were tested for potential impact on the performance of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit. No impact of interfering conditions or substances on performance was observed according to DNA and library quantitation, sequencing output or variant calling.

Carryover

Ligation of specific oligonucleotide sequences is used for Illumina cluster generation and the identification of samples. Alternating the use of 2 sets of 12 bar codes between successive runs decreases the risk of contamination.

Samples bar-coded with the first adapter set were used in a sequencing run, Run 1. Two additional sequencing runs were then performed without samples, Run 2 and Run 3, and any remaining first set bar codes were quantified in Run 2 and Run 3 to measure carryover contamination between runs. The same experiment was performed using the second set of bar codes. The run-to-run global percentage of carryover contamination was established by comparing the ratio of the number of reads obtained from Run 1 and Run 3 using both sets of bar codes, considering that the customer uses alternatively the two available sets of bar codes.

The run-to-run carryover, defined as the percentage of reads from the samples of Run 1 that are still detected in Run 3 (without samples), was 0.43% and 0.47% for the first bar code set and the second bar code set, respectively.

On-target reads (assay specificity)

On-target reads is defined as the percentage of reads aligned on the target region among the total reads obtained per sample. The percentage is calculated according the formula:

$$\text{On-target reads (\%)} = \frac{\text{Number of reads aligned to the target region} \times 100}{\text{Total number of reads}}$$

On-target statistics were obtained from 82 data sets generated using 52 FFPE-derived DNA extracts using 3 different kit lots. On average, 94.51% of the reads were aligned to the target region (Table 10).

Table 10. On-target reads (assay specificity)

Average	Minimum	Maximum	Standard deviation
94.51%	87.67%	96.17%	1.4%

Assay precision

The repeatability (within-run precision) and the reproducibility (between-run precision) of the assay were assessed in terms of allele frequency (AF). Two mixed samples were produced to generate a maximum of variants with expected AF between 5 and 15%. These samples were tested in duplicate through 6 sequencing runs, using 2 MiSeqDx platforms (inter-instrument reproducibility), 3 operators (inter-operator reproducibility) and 3 kit lots (inter-lot variability).

The goal was to determine relative percentages of the total variability introduced at each level: lot, operator, instrument, replicates. Analysis of the variance components indicates that the major component of the total variance is repeatability, i.e., it arises from the within-run duplicates (see Table 11; column Sr).

Table 11. Compilation of all variants detected in 36 datasets for 2 samples

Variant Position & Ref allele > Variant allele	Expected AF	Calculated average AF (n=36)	SD*	CV* (%)	Sr*	Si*	So*	Sin*	Stotal*	Reliability†
S1/32913055 A>G	99.9	99.9	0.06	0.06	0.06	0.00	0.00	0.00	0.06	100.00
S2/32913055 A>G	99.9	99.9	0.05	0.05	0.05	0.01	0.02	0.01	0.05	84.18
S1/32929387 T>C	99.9	99.7	0.16	0.16	0.11	0.09	0.12	0.02	0.18	33.41
S2/32915005 G>C	99.9	99.7	0.38	0.38	0.26	0.22	0.29	0.00	0.44	34.25
S2/32929387 T>C	99.9	99.7	0.19	0.19	0.15	0.10	0.12	0.00	0.21	47.81
S1/32915005 G>C	99.9	99.7	0.45	0.46	0.31	0.27	0.33	0.00	0.53	34.18
S2/32906729 A>C	90.3	90.1	0.85	0.95	0.80	0.00	0.00	0.36	0.88	83.49
S1/32906729 A>C	88.6	91.8	0.70	0.76	0.64	0.00	0.34	0.00	0.73	78.30
S1/32936646 T>C	54.5	52.5	1.78	3.40	1.54	0.48	0.69	0.74	1.90	65.44
S2/32936646 T>C	51	50.2	1.90	3.78	1.40	0.18	1.17	1.02	2.09	44.49
S1/32913691 C>T	43.9	46.2	1.68	3.64	1.56	0.00	0.25	0.72	1.73	80.86
S1/41251931 G>A	42.5	43.1	1.31	3.04	1.11	0.00	0.71	0.46	1.40	63.31
S2/32913691 C>T	42.3	44.9	1.27	2.84	1.10	0.00	0.79	0.00	1.35	65.87
S2/41251931 G>A	41	43.5	1.75	4.01	1.39	0.00	1.23	0.33	1.89	54.60
S2/41244000 T>C	14.3	9.6	0.99	10.27	0.99	0.00	0.00	0.00	0.99	100.00
S2/41231516 C>T	14.2	12.9	0.94	7.29	0.65	0.00	0.65	0.50	1.05	39.14
S2/41219780 T>C	14.2	12.2	0.98	8.05	0.91	0.00	0.44	0.00	1.01	81.35
S2/41219804 T>C	14.2	12.2	0.97	7.95	0.91	0.00	0.41	0.00	1.00	83.03

Variant Position & Ref allele > Variant allele	Expected AF	Calculated average AF (n=36)	SD*	CV* (%)	Sr*	Si*	So*	Sin*	Stotal*	Reliability†
S2/41244435 T>C	14.2	11.9	0.66	5.58	0.62	0.00	0.11	0.25	0.68	83.56
S2/41245466 G>A	14.2	11.8	0.96	8.17	0.93	0.00	0.21	0.16	0.97	92.56
S2/41234470 A>G	14.2	11.7	0.57	4.90	0.54	0.00	0.15	0.19	0.59	83.18
S2/41245237 A>G	14.2	11.6	1.12	9.65	1.07	0.00	0.21	0.33	1.14	88.24
S2/41244936 G>A	14.2	11.5	0.87	7.55	0.73	0.00	0.40	0.39	0.92	62.78
S2/41223094 T>C	14.2	11.4	0.65	5.70	0.62	0.00	0.22	0.00	0.66	88.91
S2/41215416 T>C	13.6	9.3	0.78	8.43	0.78	0.00	0.00	0.00	0.78	100.00
S1/32890572 G>A	9.6	6.6	0.72	10.95	0.72	0.00	0.11	0.00	0.72	97.88
S2/32929232 A>G	9.5	12.4	0.76	6.13	0.76	0.00	0.00	0.00	0.76	100.00
S1/32929232 A>G	9.5	6.7	0.68	10.07	0.68	0.00	0.00	0.00	0.68	100.00
S2/32911888 A>G	9.4	9.5	0.60	6.33	0.52	0.13	0.35	0.00	0.64	65.08
S2/32890572 G>A	9.4	9.1	0.88	9.72	0.80	0.08	0.19	0.40	0.92	76.65
S1/32911888 A>G	9.3	6.2	0.45	7.33	0.42	0.00	0.03	0.20	0.47	81.41
S1/32914236 C>T	9.3	5.8	0.60	10.27	0.55	0.00	0.26	0.12	0.62	78.49
S2/32915411 32915414 AATT>-	9.2	9.7	0.76	7.83	0.70	0.00	0.26	0.26	0.79	78.35
S1/32915411 32915414 AATT>-	9.2	5.6	0.51	9.03	0.50	0.00	0.11	0.00	0.51	95.65
S2/32907259 G>A	9.0	9.6	0.53	5.52	0.53	0.00	0.00	0.08	0.53	97.53
S1/41244936 G>A	5.7	7.0	0.70	9.96	0.57	0.00	0.48	0.00	0.75	59.27

Variant Position & Ref allele > Variant allele	Expected AF	Calculated average AF (n=36)	SD*	CV* (%)	Sr*	Si*	So*	Sin*	Stotal*	Reliability†
S1/41219780 T>C	5.6	6.9	0.78	11.23	0.73	0.00	0.00	0.33	0.80	83.21
S1/41219804 T>C	5.6	6.9	0.77	11.20	0.72	0.00	0.00	0.34	0.80	81.37
S1/41245237 A>G	5.6	6.6	0.68	10.39	0.69	0.00	0.00	0.00	0.69	100.00
S1/41244000 T>C	5.6	6.4	0.42	6.54	0.41	0.00	0.04	0.10	0.42	93.21
S1/41223094 T>C	5.5	6.3	0.46	7.29	0.44	0.00	0.18	0.00	0.47	85.03
S1/41245471 C>T	5.4	6.6	0.72	10.90	0.67	0.15	0.28	0.00	0.74	81.60
S1/41234470 A>G	5.4	6.5	0.56	8.61	0.54	0.00	0.09	0.15	0.57	90.40
S1/41245466 G>A	5.4	6.5	0.68	10.55	0.62	0.00	0.35	0.00	0.71	75.93
S1/41231516 C>T	5.4	6.4	0.62	9.81	0.54	0.00	0.35	0.13	0.66	68.51
S1/41244435 T>C	5.4	6.3	0.66	10.61	0.63	0.00	0.24	0.00	0.68	87.22
S2/32913603 G>A	5.3	4.9	0.68	14.02	0.65	0.00	0.25	0.10	0.70	85.68

* SD: standard deviation; CV: coefficient of variation (Stotal/Mean) Sr: SD repeatability (within-lot precision); Si: SD inter-lot; So: SD inter-operator; Sin: SD inter-instrument; Stotal: SD total (within-lab precision).

† Reliability = $(Sr^2/Stotal^2) \times 100$

Limit of Detection (LOD)

Assay cut-off

The assay cut-off for allele frequency was defined as the minimum allele frequency for which 99.9% of the detected variants are true positive variants after removal of false positive variants described in Table 13, Table 14 and Table 16. The variant allele frequency (VAF) cut-off was calculated as 5.75% for 53 previously characterized samples: 13 samples obtained from the Coriell Institute for Medical Research repository and 20 clinical FFPE samples tested in duplicate using 3 kit batches.

To demonstrate that the assay can detect a VAF of 1.5%, 4 individual FFPE DNA tumor-derived samples were mixed at different ratios with one other FFPE DNA tumor-derived sample. To reproduce a reduction in homogeneity between multiplexed samples, sequencing runs were performed with decreasing amounts of sample library material in a range from 4 nM (the recommended amount) to 0.125 nM of library loaded into the cartridge. Total library input was maintained at a constant concentration of 4 nM by increasing the amount of NA12878 positive control DNA.

The assay sensitivity was calculated by probit analysis and the results are reported in Table 12. For a library input of 4 nM, the assay detects variants at a VAF of 1.54%.

Table 12. Assay sensitivity: minimum VAF detected with decreasing library input

Library input (sample)	VAF LOD
4 nM	1.54%
2 nM	1.54%
1 nM	1.54%
0.5 nM	1.93%
0.25 nM	3.16%
0.125 nM	3.88%

Minimum coverage to detect VAF at 5.75%

The minimum coverage (total number of reads) required to call the VAF cut-off of 5.75% was assessed. Four individual FFPE DNA tumor-derived samples were mixed at different ratios with one other FFPE DNA tumor-derived sample to obtain VAF values ranging from 5.25% to 6.25%, with an estimated median VAF of 5.75%. Multiple sequencing runs were performed with two-fold serial dilutions of the same set of mixed samples with library amounts ranging from 4 nM to 0.015625 nM. For example, if 15000x coverage is obtained with a library input of 4 nM, the same variant will be detected 59x with 0.015625 nM input.

Probit analysis was used to determine the LOD for each AF class using a method following guidance in NCCLS EP17-A2 (20). The final value is the coverage needed to detect the AF with 95% confidence. An example graph is shown in Figure 9.

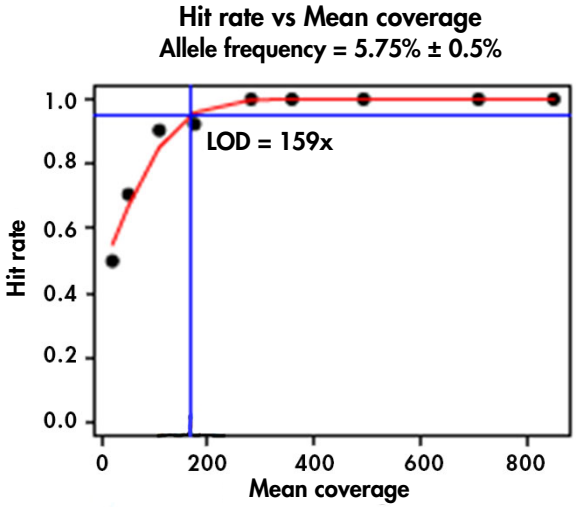


Figure 9. Probit analysis. Hit rate represents the percentage of variants detected according to the coverage.

A minimum of 200 reads per position is required to detect VAF at 5.75%. (The LOD was 159x, rounded up to 200x.)

Accuracy

Accuracy was estimated using previously characterized samples, including DNA samples from Coriell Institute for Medical Research repository and DNA extracted from FFPE ovarian tumor samples.

- Set 1: 13 samples from the Coriell repository fully characterized for the target region (191 variants expected)
- Set 2: 27 samples from the Coriell repository partially characterized for 25 pathogenic variants (27 variants expected)
- Set 3: 20 Samples of DNA extracted from FFPE ovarian tumor samples fully characterized for the target region (570 variants expected)

All expected variants were detected in the Coriell samples (Set 1 and Set 2), including two large deletions of 40 nucleotides in *BRCA1* (BRCA1:c.1175_1214del40 p.Leu392Glnfs).

Accuracy was calculated with the following formula:

$$\text{Accuracy (\%)} = \frac{\text{(Number of true positives + Number of true negatives) x 100}}{\text{Target region (21,150 bases)}}$$

Accuracy for the FFPE samples (Set 3) was calculated at 99.988% using a probit approach to set the allele frequency threshold at 5.75%.

Variant limitations

False positive variants

A list of 47 false positive variants was established for a set of previously characterized samples: 13 samples from the Coriell repository and 20 clinical FFPE samples tested in duplicate; n=53. False positive variants arise from instrument limitation in relation to sequences with homopolymeric tracts >6 nt and/or regions with repeats of di- and tri-nucleotide stretches. False positive variants can also be artifacts arising from primer dimers.

The list of false positive variants for *BRCA1* on chromosome 17 is shown in Table 13. The list of false positive variants for *BRCA2* on chromosome 13 is shown in Table 14. The position of the false positive (FP) is described in the first column (Hg19 coordinates) followed by the reference (REF) and alternative (ALT) nucleotides found. The percentage of data sets in which the FP variant was found (from 53 data sets) is shown in the column "% of datasets (n=53)". The minimum, average and maximum AF percentages from the 53 data sets are shown in the columns "Minimum AF (%)", "Average AF (%)" and "Maximum AF (%)". Variants are classified in 4 categories: "Homopol" (FP from homopolymeric tracts >6 nt), "Primer dimer" (FP from primer dimer artifacts), "Stretch" (FP from di- and tri-nucleotide repeats), and "Other" (FP from polymerase synthesis and/or sequencing errors).

Table 13. List of false positive (FP) variants detected in *BRCA1* on chromosome 17

Region	REF	ALT	Type	% of datasets (n = 53)	Minimum AF (%)	Average AF (%)	Maximum AF (%)	False positive classification
41231323	C	T	SNV	9.4	1.3	2.0	2.5	Other
41231324	G	A	SNV	9.4	1.1	1.4	2.6	Other
41231333	G	C	SNV	7.5	1.0	1.5	1.7	Stretch
41231352	T	C	SNV	9.4	1.4	1.8	2.1	Other
41231370	C	A	SNV	5.7	1.1	1.2	1.3	Other
41231401	C	T	SNV	1.9	1.4	1.4	1.4	Other
41231404	T	C	SNV	11.3	1.2	2.4	3.8	Other
41231419	G	A	SNV	9.4	1.3	2.5	3.4	Other
41242939..41242940	CA	–	Deletion	100	3.1	4.8	6.1	Stretch
41243524	A	C	SNV	92.5	1.1	1.7	2.7	Primer dimer
41244613	G	A	SNV	3.8	2.0	2.1	2.1	Other
41245586^41245587	–	T	Insertion	96.2	1.1	1.4	2.4	Homopol
41245587	T	–	Deletion	100	2.3	3.2	3.9	Homopol
41246532	T	–	Deletion	13.2	1.0	1.1	1.2	Homopol
41246926	A	–	Deletion	94.3	1.0	1.4	2.0	Homopol
41267808	G	–	Deletion	77.4	1.0	1.2	1.5	Homopol
41276152^41276153	–	AT	Insertion	94.3	1.1	1.5	2.0	Stretch
41276153..41276154	AT	–	Deletion	100	1.9	2.7	3.3	Stretch

Table 14. List of false positive (FP) variants detected in *BRCA2* on chromosome 13

Region	REF	ALT	Type	% of datasets (n = 53)	Minimum AF (%)	Average AF (%)	Maximum AF (%)	False positive classification
32893197^32893198	–	T	Insertion	100	4.3	5.6	6.8	Homopol
32893198	T	–	Deletion	100	11.0	12.6	13.5	Homopol
32893198..32893199	TT	–	Deletion	5.7	1.1	1.2	1.3	Homopol
32893318	G	A	SNV	3.8	1.3	1.8	2.2	Other
32900364	T	–	Deletion	75.5	1.0	1.3	1.9	Homopol

Region	REF	ALT	Type	% of datasets (n = 53)	Minimum AF (%)	Average AF (%)	Maximum AF (%)	False positive classification
32905197	T	–	Deletion	18.9	1.0	1.1	1.3	Homopol
32905219^32905220	–	T	Insertion	100	8.8	10.4	11.4	Homopol
32905219^32905220	–	TT	Insertion	96.2	1.0	1.2	1.6	Homopol
32905220	T	–	Deletion	100	19.7	21.1	23.1	Homopol
32905220..32905221	TT	–	Deletion	100	3.8	4.4	5.4	Homopol
32907421	A	–	Deletion	94.3	2.2	3.1	3.9	Homopol
32907535^32907536	–	T	Insertion	100	6.8	7.7	10.0	Homopol
32907535^32907536	–	TT	Insertion	3.8	1.0	1.1	1.1	Homopol
32907536	T	–	Deletion	100	21.4	23.8	26.3	Homopol
32907536..32907537	TT	–	Deletion	100	3.6	4.6	6.2	Homopol
32911074	A	–	Deletion	11.3	1.0	1.2	1.6	Homopol
32911443	A	–	Deletion	9.4	1.0	1.1	1.2	Homopol
32912346	A	–	Deletion	77.4	1.0	1.2	1.5	Homopol
32913559	A	–	Deletion	100	1.0	1.5	2.0	Homopol
32913837	A	–	Deletion	18.9	1.0	1.0	1.1	Homopol
32914828	A	G	SNV	100	2.4	4.1	6.8	Primer dimer
32921032	C	T	SNV	3.8	1.4	1.4	1.4	Other
32953633	A	–	Deletion	17.0	1.0	1.1	1.3	Homopol
32954022^32954023	–	A	Insertion	13.2	1.0	1.1	1.2	Homopol
32954023	A	–	Deletion	100	2.1	3.3	4.1	Homopol
32954303	T	–	Deletion	100	1.5	2.3	2.8	Homopol
32968809	T	–	Deletion	96.2	2.3	2.6	3.2	Homopol
32972287	T	–	Deletion	5.7	1.0	1.1	1.2	Homopol
32972526	G	A	SNV	3.8	1.4	1.4	1.4	Other

False negative variants

Extra base pairs may be added (insertions) or removed (deletions) from the DNA of a gene. The number can range from one to thousands. Collectively, these mutations are called indels. Performance of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit was established based on detection of substitution variants and small indels (≤3 nt). Large indels (>3 nt) were detected but the performance characteristics for detection of these large indels could not be determined.

The *therascreen* BRCA1/2 NGS FFPE gDNA Kit together with this analysis workflow is not suited for copy number variation (CNV) analysis.

Primer binding sites of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit are selected to avoid the presence of variants within the selected regions. This does not exclude the presence of rare variants within a primer binding site leading to incorrect amplification, and masking the presence of potential clinically-relevant mutations. If such a phenomenon is suspected, we recommend performing a confirmatory experiment using an alternative method.

Validation results

The validation study of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit assay was conducted on FFPE samples from ovarian tumor tissue using 2 independent kit lots. This study assessed performance under normal conditions of use: 40 ng input, a threshold variant allele frequency of 5.75%, and 200x coverage.

A total of 171 ovarian FFPE tumor samples was provided and characterized by 3 collaborating laboratories: Curie Institute in France, one in Germany and one in the United Kingdom. The laboratories were free to select the method of their choice to identify the *BRCA 1/2* variants and utilized different platforms, assays, software and algorithms for variant calling.

To further characterize the clinical samples, an external sequencing service provider used the Multiplicom BRCA Tumor MASTR™ Plus Dx assay to deliver results using an independent bioanalytical platform.

Results from all methods were used to classify the variants as follows:

- True positive (TP): variant call based on *therascreen* BRCA1/2 NGS FFPE gDNA Kit data and present in at least one other method
- True negative (TN): no variant call based on results for *therascreen* BRCA1/2 NGS FFPE gDNA Kit and collaborations
- False positive (FP): variant call based on *therascreen* BRCA1/2 NGS FFPE gDNA Kit data but absent in other methods
- False negative (FN): no variant call based on *therascreen* BRCA1/2 NGS FFPE gDNA Kit data but present in at least one other method

To calculate the number of true negative variants, the target region was defined as the intersection between the 3 methods that represents 19612 nucleotides. Three unique variants outside this target region were excluded from the analysis.

After this classification, one false negative variant and 4 false positive variants were observed among the 2130 variants obtained with the 3 methods. The discrepant result for *BRCA1* on chromosome 17 (false negative) is shown in Table 15. Discrepant results for *BRCA2* on chromosome 13 (false positive) are listed in Table 16.

Table 15. Discrepant result according to 3 methods (all variants) for *BRCA1* on chromosome 17

Genomic location GRCh37	REF*	ALT*	Type	*therascreen* kit VAF* (%)	Expected VAF (%)	Clinical significance	Variant classification
41276067	29	–	Deletion	Not detected	71.11	Pathogenic	False negative

* REF: Reference nucleotide; ALT: alternative nucleotide; VAF: Variant allele frequency; SNV: Single nucleotide variation.

Table 16. Discrepant results according to 3 methods (all variants) for *BRCA2* on chromosome 13

Genomic location GRCh37	REF*	ALT*	Type	*therascreen* kit VAF* (%)	Expected VAF (%)	Clinical significance	Variant classification
32906729	A	C	SNV*	99.83	Not detected	Unknown	False positive
32912299	T	C	SNV	34.59	Not detected	Unknown	False positive
32912299	T	C	SNV	71.86	Not detected	Unknown	False positive
32913709	T	–	Deletion	43.18	Not detected	Pathogenic	False positive

* REF: Reference nucleotide; ALT: alternative nucleotide; VAF: Variant allele frequency; SNV: Single nucleotide variation.

The false negative variant, *BRCA1* g.41276067 c.19_47del29, was a large deletion of 29 nucleotides on *BRCA1* from an ovarian FFPE sample. This deletion was previously detected with the *therascreen* BRCA1/2 NGS FFPE gDNA Kit workflow on an FFPE sample from bladder.

Three false positive single nucleotide variants were detected. One was located in the *BRCA2* g.32906729 region and two were located in the *BRCA2* g.32912299 region. The Multiplicom assay exhibited a very low coverage in these regions, respectively 28x, 38x and 0x. Investigation within the BAM sequence alignment files showed that the two variants occurred at the corresponding allele frequencies: 28/28 (100% VAF) and 9/38 (24% VAF) respectively. The discrepancies probably come from an amplicon dropout.

The false positive variant *BRCA2* g.32913709delT is located in the first nucleotide of the amplicon. The corresponding amplification primer hybridizes in a sequence containing a true pathogenic variant g.32913703 del TACT. This deletion was detected by the *therascreen* BRCA1/2 NGS FFPE gDNA Kit assay and its presence could explain the misalignment of the primer leading to the release of this false positive variant. This false positive will never lead to a patient misclassification since it is systematically linked to the detection of the true pathogenic variant.

The results of the study are shown in a contingency table (Table 17) and overall assessment of sensitivity and specificity is shown in Table 18.

- Clinical sensitivity is defined as the positive agreement:
  True positive/(True positive + False negative)
- Clinical specificity is defined as the negative agreement:
  True negative/(False positive + True negative)
- Accuracy is defined as the overall agreement between assay results and the results from collaborations and the third method:
  True positive + True negative/Number of bases in the target region

Table 17. Contingency table with calculated performance of the *therascreen* BRCA1/2 NGS FFPE gDNA Kit

		Results from collaboration and Multiplicom assay (Reference method)		
		Positive	**Negative**	**Total**
Results of *therascreen* BRCA1/2 NGS FFPE gDNA Kit assay	**Positive**	2125 True positive	4 False positive	**2129**
	Negative	1 False negative	3,351,522 True negative	**3,351,523**
	Total	**2126**	**3,351,526**	**3,353,652**

Table 18. Sensitivity, specificity and accuracy among methods

Parameter	Result	95% CI
Sensitivity	99.9530%	99.7382–100%
Specificity	99.9999%	99.9997–100%
Accuracy	99.9998%	99.9996–100%

References

1. WHO, IARC GLOBOCAN. (2012) Cancer incidence and mortality worldwide in 2012. **http://globocan.iarc.fr/**.

2. Siegel, R., Naishadham, D. and Jemal, A. (2013) Cancer statistics. CA Cancer J. Clin. **63**, 11–30.

3. Kanchi, K.L. et al. (2014) Integrated analysis of germline and somatic variants in ovarian cancer. Nature Communications **5**, 3156.

4. Hennessy, B.T. et al. (2010) Somatic mutations in BRCA1 and BRCA2 could expand the number of patients that benefit from poly (ADP ribose) polymerase inhibitors in OvCa. J. Clin. Oncol. **28**, 3570.

5. Gilks, C.B. and Prat, J. (2009) Ovarian carcinoma pathology and genetics: recent advances. Hum. Pathol. **40**, 1213.

6. Kurman, R.J. and Shih, Ie M. (2010) The origin and pathogenesis of epithelial ovarian cancer. A proposed unifying theory. Am. J. Surg. Pathol. **34**, 433.

7. Pal, T. et al. (2005) BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. Cancer **104**, 2807.

8. Risch, H.A. et al. (2001) Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with OvCa. Am. J. Hum. Genet. **68**, 700.

9. Cancer Genome Atlas Research Network. (2011) Integrated genomic analyses of ovarian carcinoma. Nature **474**, 609.

10. Foley, O.W., Rauh-hain, J.A. and Del Carmen, M.G. (2013) Recurrent epithelial OvCa: an update on treatment. Oncology **27**, 288, 298. Review.

11. Yap, T.A., Carden, C.P. and Kaye, S.B. (2009) Beyond chemotherapy: targeted therapies in ovarian cancer. Nat. Rev. Cancer **9**, 167.

12. Audeh, M.W. et al. (2010) Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent OvCa: a proof-of-concept trial. Lancet **376**, 245.

13. Alsop, K. et al. (2012) BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with OvCa: a report from the Australian OvCa Study Group. J. Clin. Oncol. **30**, 2654.

14. Ledermann, J. et al. (2014) Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous OvCa: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. Lancet Oncol. **15**, 852.

15. Burgess, M. and Puhalla, S. (2014) BRCA 1/2-mutation related and sporadic breast and OvCas: more alike than different. Front. Oncol. **4**, 19.

16. Marth, C. et al. (2015) AGO Austria recommendations for genetic testing of patients with OvCa. Wien Klin. Wochenschr. **127**, 652.

17. Casey, G. (1997) The BRCA1 and BRCA2 breast cancer genes. Curr. Opin. Oncol. **9**, 88.

18. Prat, J. (2012) Ovarian carcinomas: five distinct diseases with different origins, genetic alterations, and clinicopathological features. Virchows Arch. **460**, 237.

19. Clinical and Laboratory Standards Institute (CLSI) (2006) *Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods: Approved Guideline*, 1st ed. CLSI Document MM13-A. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

20. Clinical and Laboratory Standards Institute (CLSI) (2012). *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures: Approved Guideline*, 2nd ed. CLSI Document EP17-A2. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
REF	Catalog number
	Manufacturer
MAT	Material number
Rn	R is for revision of the Handbook and n is the revision number
LOT	Lot number
GTIN	Global Trade Item Number
IVD	In vitro diagnostic medical device
CE	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 in Part 1.

Product	Contents	Cat. no.
therascreen BRCA1/2 NGS FFPE gDNA Kit CE (20)	For 20 reactions: For the identification of variants in *BRCA1* and *BRCA2* 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.

This page intentionally left blank

This product is intended for in vitro diagnostic use. QIAGEN products may not be resold, modified for resale or used to manufacture commercial products without written approval of QIAGEN.

Information in this document is subject to change without notice. QIAGEN assumes no responsibility for any errors that may appear in this document. This document is believed to be complete and accurate at the time of publication. In no event shall QIAGEN be liable for incidental, special, multiple, or consequential damages in connection with, or arising from the use of this document.

QIAGEN products are warranted to meet their stated specifications. QIAGEN's sole obligation and the customer's sole remedy are limited to replacement of products free of charge in the event products fail to perform as warranted.

Limited License Agreement for *therascreen* BRCA1/2 NGS FFPE gDNA Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see. **www.qiagen.com**

HB-2197-002 1103449 157014158 02/2017