QPCR
POLE Mutation Analysis Kit

Realtime PCR Amplification Kit

Handbook

RUO For research use only. Not for use in diagnostic procedures.

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LOT Batch code

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Notice of Change

Please note the following adaptions compared to the previous handbook version:

Document code	Changes	Date
QPOLHB01v1en	Initial release of the handbook	26.04.2023
QPOLHB01v2en	Spelling correction (in data analysis workflow overview) Addition of P phrases (Warning and Precautions)	23.05.2023

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Product Description

The QPCR POLE Mutation Analysis Kit is a qualitative and comprehensive PCR-based multiplex assay for the detection of 6 single nucleotide mutations (with the ability to differentiate 5 mutations) within the exonuclease domain of POLE genes in human DNA derived from formalin-fixed, paraffin-embedded (FFPE) samples.

The assay must be used by qualified and trained personnel in a professional laboratory environment only.

Results are intended solely for research use and not for diagnostic procedures.

The detection limit for the qualitative analysis is 1 % allele frequency (VAF), which represents 2 % tumor DNA.

The optimal input under standard conditions is 4 ng DNA per reaction.

Summary and Explanation

POLE is a human gene encoding the catalytic subunit (exonuclease domain) of DNA polymerase epsilon. The main function of POLE exonuclease domains (EDMs) is their proofreading activity – an exonuclease function that detects and removes misinserted bases in the daughter strand due to failed complementary pairing with the parent strand [1].

The QPCR POLE Mutation Analysis Kit is a PCR-based multiplex qPCR assay for the detection of 6 mutations, whereby the 5 major POLE mutations are differentiated in DNA samples extracted from FFPE samples. The functionality of the assay is controlled by internal and external controls. Table 1 summarizes the list of POLE mutation targets.

Table 1 List of detected POLE mutations

Assay	AA Change	CDs Mutation
Primer Mix A	A456P	c.1366G>C
	P286R	c.857C>G
	V411L (G>C) and (G>T)	c.1231G>C/ c.1231G>T
Primer Mix B	S297F	c.890C>T
	S459F	c.1376C>T

Materials provided

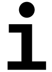
Table 2 QPCR POLE Mutation Analysis Kit content

Reagent	Cap Color		Volume per kit (2 x 50 reactions)	Storage
Nuclease-Free Water	Light blue		1 x 1.5 mL	−25 °C to −15 °C, protected from light
QPCR POLE Master Mix	Black		1 x 500 µL	
QPCR POLE Positive Control	White		1 x 80 µL	
QPCR POLE Primer Mix A	Red		1 x 50 µL	
QPCR POLE Primer Mix B	Yellow		1 x 50 µL	

NOTE

Please note that the packaging size describes the number of testings **without** taking into account the number of required controls or the required excess for pipetting.

NOTE



The kit contains reagents to perform up to 50 tests with each QPCR POLE Primer Mix.

Reagent storage and handling

The kit is shipped on dry ice. The components of the kit should arrive frozen, except the QPCR POLE Master Mix that is stored in a buffer that prevents freezing of the reagent.

Please check for the completeness of the kit upon receipt. Please immediately contact BIOTYPE GmbH if one or more components are not frozen, or if tubes or the packaging have been compromised during the shipment.

Store all components at -25 °C to -15 °C without light exposure and avoid repeated thawing and freezing. Especially the QPCR POLE Primer Mix A and QPCR POLE Primer Mix B must be stored protected from light.

The QPCR POLE Positive Control should be stored separately from PCR reagents.

The expiry date of the kit is indicated on the kit box label.

Material and devices required but not provided

General laboratory equipment

- Desktop centrifuge with a rotor for 2 mL reaction tubes
- Centrifuge with a rotor for microtiter plates
- Vortex mixer
- Calibrated adjustable pipettes with disposal aerosol tight filter tips
- Appropriate* 200 µL 96-well reaction plates with proper optical foil, PCR grade
- Suitable racks for 2 mL tubes
- Disposable powder-free gloves
- Qubit Fluorometer (cat. no. Q33238, Thermo Fisher Scientific)
- PCR Workstation or Clean Bench

*depending on the device manufacturer

NOTE

All material to be used for PCR shall have appropriate quality (DNA free and for molecular biology). Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturers’ instructions and recommendations.

Reagents, kits and consumables

Table 3 Reagents required, but not provided

Reagent	Supplier	Order number
Qubit™ dsDNA HS Assay	Thermo Fisher Scientific	Q32851
Qubit™ dsDNA BR Assay	Thermo Fisher Scientific	Q32850
TE Buffer pH 8.0 (Ambion)	Fisher Scientific GmbH	10742317
QIAamp DNA FFPE Tissue Kit	Qiagen	56404
Deparaffinization Solution	Qiagen	19093
RNase A (100 mg/mL)	Qiagen	19101

Instruments and software

The QPCR POLE Mutation Analysis Kit was verified to be used with the following PCR cycler:

- QuantStudio™ 5 Real-Time PCR System (cat. no.: A28139, Thermo Fisher Scientific)
- QuantStudio™ Design and Analysis Software (v. 1.5.1 or higher)

NOTE

Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.

NOTE

The application of QPCR POLE Mutation Analysis Kit on other instruments than the above-mentioned one needs to be verified in the user responsibility.

Specimens and test samples

The following specimen has been verified with the QPCR POLE Mutation Analysis Kit : DNA samples extracted from formalin-fixed paraffin-embedded

(FFPE) endometrial tissue have been tested for use with the QPCR POLE Mutation Analysis Kit.

In additional, FFPE material spiked with plasmids containing the POLE mutations were tested.

The obtained DNA shall be stored undiluted at -25 °C to -15 °C.

Warnings and Precautions

Attention! Valid for QPCR POLE Master Mix:

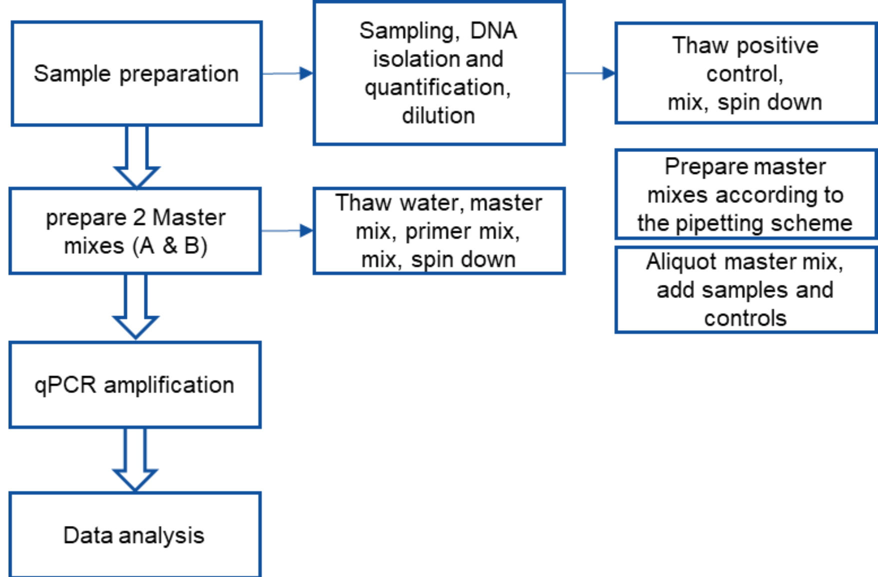
	Warning	**H371**	May cause damage to central nervous system. Route of exposure: Oral.

P101	If medical advice is needed, have product container or label at hand.
P102	Keep out of reach of children.
P103	Read label before use.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P264	Wash thoroughly after handling.
P301+ P312	IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell.
P330	Rinse mouth.
P405	Store locked up.
P501	Dispose of contents/container in accordance with local/regional/national/international regulations.

- Read the instructions carefully before using the product.
- Read the safety data sheets (SDS) for all BIOTYPE products, which are available on request. Please contact the respective manufacturers for copies of the SDS for any additionally needed reagents.
- Kit components of different kit lots must not be mixed.
- Aliquoting the kit components into other reaction vessels is not permitted.
- The use of this product is limited to personnel specially instructed and trained in PCR techniques.
- Before the first use, check the product and its components for:
    - Integrity
    - Completeness with respect to number, type and filling (see chapter Materials provided)
    - Correct labelling
    - Frozenness upon arrival.
- Do not use a kit that has passed its expiration date.
- Discard sample and assay waste according to your local safety regulations.
- All instruments used have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.

Procedure

Overview of the experimental workflow



Sample preparation

DNA extraction

DNA purification should be carried out from macro-dissected FFPE tissue according to the supplier's instructions. It is recommended that DNA be purified with the QIAamp DNA FFPE Tissue Kit using the Deparaffinization Solution and RNase A. Another optional step is the overnight Proteinase K digestion, using 3 FFPE-sections up to 10 µm (surface up to 250 mm²).

The DNA must be quantified directly after the extraction.

NOTE

For sample dilution we recommend the use of 1 x TE (Tris-EDTA)- Buffer, pH value 8.0 and a sample volume > 1.5 µL

NOTE

A long storage of the raw sample material might lead to a fragmentation of the genetic material and therefore, led to an insufficient quality of the material. This can affect the analysis result, e. g. through incomplete profiles. The QPCR POLE Mutation Analysis Kit is designed for use with short PCR products; heavily fragmented DNA will influence the assay perfomance.

DNA quantification and dilution

Quantification of the DNA should be carried out by fluorometric quantitation using the Qubit™ 3.0 Fluorometer. For low FFPE tissue input (e. g., tissue biopsies) use the Qubit™ dsDNA HS Assay according to the manufacturer's protocol. Alternatively, the usage of the Qubit™ ds DNA BR Assay is possible.

NOTE

Set up the QPCR POLE Mutation Analysis Kit using **4 ng DNA** (corresponds to 5 µL template with a concentration of 0.8 ng/µL). Using a DNA amount below 4 ng per reaction will result in low PCR yields and the signal might fall below the target-specific detection limits.

DNA storage

Store the DNA samples at -25 °C to -15 °C. Undiluted DNA samples can be stored for 4 weeks at 2 °C to 8 °C or at -25 °C to -15 °C for long-term storage.

Positive Control (PC) preparation

Thaw the QPCR POLE Positive Control (white cap), homogenize it by gentle vortexing followed by briefly centrifugation.
Apply the QPCR Positive Control included in the kit as positive control PC instead of a sample. No additional dilutions steps are required.

PCR Master mix setup

Remove and thaw the following components from the QPCR POLE PCR Amplification Kit :

- Nuclease-Free Water (light blue cap)
- QPCR POLE Primer Mix A (red cap)
- QPCR POLE Primer Mix B (yellow cap)
- QPCR POLE Master Mix (black cap)

During the PCR master mix setup, it is recommended to keep the QPCR POLE Master Mix in a cooled environment (e. g., on a cooling rack). All frozen components need to be thawed at room temperature (15 °C to 30 °C, ca. 30 min, protected from light) and homogenized by inverting the tubes, pipetting, or gently vortexing. The reagents should be then briefly centrifuged (approx. 10 s).

NOTE

Make sure to analyse each sample with each QPCR POLE Primer Mix to obtain the complete information of the assay. Therefore, prepare two separate PCR master mixes, one for each primer mix.

Prepare the PCR master mix for each primer mix (2 separate master mixes for each sample) according to Table 4 for the total number of samples to be tested, in an appropriately sized microcentrifuge tube, in a dedicated clean area. Include at least one PC and one NTC for each QPCR POLE PCR Mix (A & B) into your calculation.

Example of a plate layout for 46 samples:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
B	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
C	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
D	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
E	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30
F	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
G	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42
H	Sample 43	Sample 44	Sample 45	Sample 46	PC	NTC	Sample 43	Sample 44	Sample 45	Sample 46	PC	NTC
	PCR Master Mix A containing Primer Mix A						PCR Master Mix B containing Primer Mix B					

NOTE

As a rule of thumb, if you are testing fewer than 10 samples, use enough master mix for one extra sample. If you are testing 10 or more samples, use an excess reagent master mix volume of +10 %.

Table 4 PCR master mix reaction setup

	Volume		
Component	**# 1**	**# 10**	**# 50**
Nuclease-Free Water	9 µL	90 µL	450 µL
QPCR POLE Primer Mix A **OR** QPCR POLE Primer Mix B	1 µL	10 µL	50 µL
QPCR POLE Master Mix	5 µL	50 µL	250 µL
DNA template or control sample	5 µL	10 x 5 µL	50 x 5 µL
Total volume	**20 µL**	**200 µL**	**1000 µL**

Gently mix the PCR master mix without generating bubbles followed by brief centrifugation.

NOTE

Do not vortex the PCR master mixes to prevent blubble generation. Mix the PCR master mix gently by inverting or pipetting.

Aliquot 15.0 µL of the PCR master mixes in an appropriate 200 µL PCR plate and briefly centrifuge the closed plate.

Application of DNA templates and controls

Add 5.0 µL of the following sample types to the prepared PCR plate containing the PCR master mixes.

NTC: add 5.0 µL of Nuclease-Free Water instead of a sample.

DNA Sample: add 5.0 µL of the prepared, diluted DNA samples (0.8 ng/µL). Technical replicates are not required for qualitative analysis.

PC: add 5.0 µL of QPCR POLE Positive Control instead of a sample.

NOTE

Use at least one positive control PC and one no template control NTC per PCR master mix (A & B). Otherwise, the run cannot be validated.

Close the PCR plate with a foil suitable for the used qPCR instrument, gently mix and spin down.

PCR amplification

Program the qPCR cycler with the following amplification profile. Make sure to set the ramping to 1.6 °C/s. Perform a “hot start” PCR in order to activate the polymerase and to prevent the formation of non-specific amplification products.

Table 5 PCR protocol

Temperature	Time	Cycles
95 °C	3 min (hot start for activation of the polymerase)	1 x
95 °C	10 s	45 x
62 °C	20 s	

NOTE

If thermal cyclers with rapid heating and cooling steps (> 2 °C/s) are used, **ramping is required to be adjusted to 1.6 °C/s** in order to provide an optimal kit performance.

Define the following properties for the correct signal detection during the plate setup:

Table 6 Properties for the correct signal detection during the qPCR run

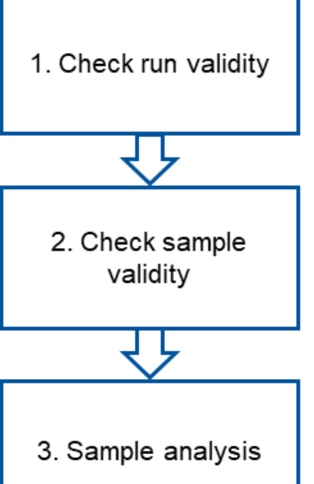
Primer Mix	Target	Reporter (Fluorescence)	Quencher
A	A456P	FAM	none
A	P286R	VIC	none
A	V411L	ROX	none
B	S297F	FAM	none
B	S459F	VIC	none
A & B	POLE IC	Cy5	none

NOTE

For basic information regarding the setup, programming and maintenance of the different qPCR instruments, please refer to the user manual of the respective instrument.

Data Analysis

Workflow overview



- **NTC** no amplification Ct < 35
- **PC** all targets incl. IC Ct 21 - 26

- IC (Cy5 channel) Ct 24 - 28

- Target Ct < 35 is considered positive
- Positive sample : 1 positive target besides IC expected

General settings

- Use the standard curve method without passive reference dye.
- Set the following thresholds in the QuantStudio™ Design and Analysis Software:

Table 7 Specific thresholds used in the data analysis with QuantStudio™ 5 instruments

Primer Mix	Target	Reporter (Fluorescence)	Threshold in ΔRn
A	A456P	FAM	12 000
A	P286R	VIC	10 000
A	V411L	ROX	40 000
B	S297F	FAM	30 000
B	S459F	VIC	20 000
A & B	POLE IC	Cy5	10 000

NOTE

To setup an experiment in the QuantStudio™ Design and Analysis Software, create a template file with the desired samples and the above mentioned parameters. This can be saved and re-used to standardize Analysis settings.

Run validation

- Check if **NTC** shows **no amplification, Ct < 35**
- Check if **PC** shows amplification of all targets (incl. IC) with Ct-Values in a range from **21 - 26**

NOTE

It is recommended to divide the plate evenly with the same layout for PCR Master Mix A and PCR Master Mix B to simplify the workflow. During Run Setup, select the corresponding targets according to the assay performed in each well.

Sample analysis

View the amplification plots for the entire qPCR run. A detailed analysis of raw data depends on the real-time PCR instrument used.

The threshold "baseline noise levels" should either be automatically defined or predefined for specific cycles (e. g. 3 - 15). Use the NTC to determine the respective threshold.

Since the Standard Curve Method is used for presence/absence detection of mutation alleles, the above mentioned thresholds and the cut-off at Cycle 35 are necessary to correctly assign a positive vs. a negative sample.

Information for data export and data processing can be found in the respective manuals of the real-time device manufacturer. Export the "Sample name" and the “Ct values” for subsequent calculations.

NOTE

If other qPCR instruments than the QuantStudio™ 5 are used, the data analysis steps need to be adjusted and the procedure needs to be verified in the users responsibility.

If PC and NTC are valid (see Run validation), continue with the sample data analysis.

The **Internal Control** in the Cy5 channel should have a Ct-Value between **24 - 28**. If this is not the case, consider adjusting the DNA concentration accordingly to measure within the range of highest assay sensitivity and specificity.

In a valid sample, any target with a Ct-Value below the cutoff of $\mathbf{C_t < 35}$ is considered positive.

NOTE

For a POLE positive sample, besides the IC, one positive target is expected. If more than one mutation target is positive, consider repetition of the analysis and/or confirmation of the result with an additional method (e. g. MODAPLEX POLE/POLD1 Analysis Kit or Sanger sequencing).

Troubleshooting

The troubleshooting guide may be helpful for solving any problems that may arise. The BIOTYPE Customer Support is happy to answer any questions about the information and protocols given in these handbook (see Technical Assistance).

Failure	Comments and suggestions
Invalid no template control (NTC)	1. Targets and/or internal controls were detected in NTC. Contamination occurred during the preparation of the qPCR. Repeat the qPCR with new reagents in replicates. If possible, close the PCR tubes directly after addition of the sample to be tested. Ensure that the work space and the instruments are decontaminated at regular intervals. Change gloves occasionally. 2. Check the maintenance interval for all used devices (e. g. pipettes)
Invalid positive control (PC)	1. Incomplete detection of targets, and/or internal controls. Incorrect handling occurred during the preparation of the qPCR and/or positive control. Repeat the qPCR with new reagents in replicates. If possible, close the PCR tubes directly after addition of the sample to be tested. Ensure that the workspace and the instruments are decontaminated at regular intervals. 2. Check the maintenance interval for all used devices (e. g. pipettes) 3. If only the Ct-Value of target V411L is > 28, the primer concentration could be too low. Check the Pipetting scheme to ensure 1 µL QPCR POLE Primer Mix per reaction and 20 µL total reaction volume are used.
The storage conditions for one or more kit components do not comply with the instructions given in Reagent storage and handling	Check the storage conditions and the expiration date (see the kit box label). Please use a new kit if the reagents were stored improperly.

Quality Control

All kit components undergo an intensive quality assurance process at BIOTYPE GmbH. Quality of the test kits is permanently monitored to ensure unrestricted usability. Please contact us if you have any questions regarding quality assurance.

Technical Assistance

For technical advice, please contact our Customer Support Team:

e-mail: support@biotype.de

phone: +49 (0)351 8838 400

References

[1] S. Briggs and I. Tomlinson. "Germline and somatic polymerase ε and δ mutations define a new class of hypermutated colorectal and endometrial cancers." J Pathol. 2013 Jun;230(2):148-53

Limitations of Use

- The procedures in this handbook must be followed, as described. Any deviations may result in assay failure or cause erroneous results.
- Use of this product is limited to personnel specially instructed and trained in qPCR techniques.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test.
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The kit has only been verified using the kits described in chapter Reagents, kits and consumable for DNA extraction and purification.
- Verified for use with an optimum input of 4 ng DNA per reaction.
- Good laboratory practice is required to ensure the performance of the kit.

- Results must be interpreted by a trained professional user.
- Interpretation of results must account for the possibility of false negative and false positive results.
- Do not use expired or incorrectly stored components.

Ordering information

Direct your orders via email to sales@biotype.de.

Product	Packaging size	Order number
QPCR POLE Mutation Analysis Kit	100 reactions (2 x 50 reactions)	35-14900-0100

Trademarks and Disclaimers

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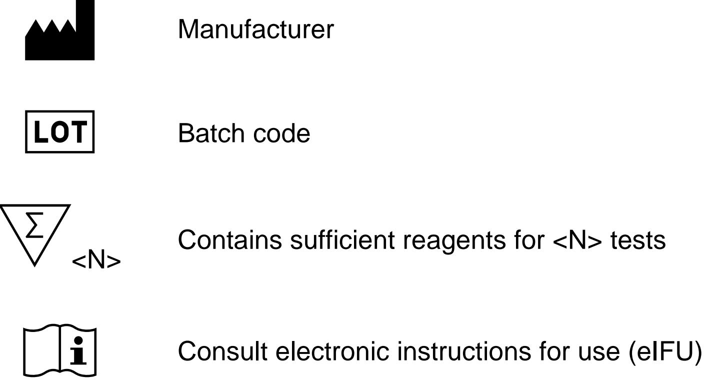
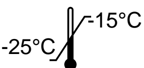
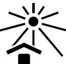
Explanation of Symbols



	Use-by date
	Temperature limit
	Catalogue number
	Keep away from sunlight
	Keep dry

Further marking used in this handbook:

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