

WID[®]-easy PCR Kit

Realtime PCR Amplification Kit

Instructions For Use

	This product has been registered as an IVD within GB (England, Scotland, Wales)
	In-vitro Diagnostic
	OE00103
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Notice of Change



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

Document code	Changes	Date
REC-020 V1.0	Initial release of UKCA - IVD Instructions For Use	December 2024
REC-020 V2.0	Update to PMR calculation section Addition to Limitation for Use section Update to references Update to Manufacturer address Update to Section: Reagent, Storage and Handling	April 2025
REC-020 V3.0	Update to section 10 and Limitation of Use	May 2025
REC-020 V4.0	Update to Table 3 Reagents required but not provided Update to Table 9 expected Cq-ranges for controls	March 2026

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Intended Use

The WID®-easy PCR Kit consists of PCR reagents used to determine the qualitative methylation state of *GYPC* and *ZSCAN12* gene regions from cervicovaginal fluid in women aged 45 years and over presenting with abnormal bleeding, which can act as an aid in the diagnosis of endometrial cancer.

This assay is not to be used for direct diagnosis.

Product Description

The WID®-easy PCR Kit is a relative-quantitative PCR-based multiplex assay for the detection of DNA methylation in *GYPC* and *ZSCAN12* gene regions as a Percentage of Methylated Reference (PMR) value via qPCR measurement of Cq-values. Bisulfite-converted DNA isolated from human cervicovaginal samples is analyzed.

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

Summary and Explanation

DNA methylation is a modification of the DNA which is essential in the epigenetic regulation of gene expression. It involves the addition of a methyl group to the 5th position of the pyrimidine ring of the cytosine nucleotide in the regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide (CpG) in the linear sequence of bases along its 5' → 3' direction. These CpG sites occur with high frequency in genomic regions called CpG islands, which are frequently located in the promoter region of genes. During carcinogenesis, several of these CpG islands become methylated (1, 2, 3), including CpG islands of the *ZSCAN12* and *GYPC* genes in endometrial and cervical cancer (4, 5, 6, 7, 8). The WID®-easy PCR assay allows the detection of hypermethylation of the genes *ZSCAN12* and *GYPC* on bisulfite-converted DNA isolated from cervicovaginal specimens using *COL2A1* as an internal control for human DNA input and efficacy of bisulfite-conversion.

Table 1 List of detected methylation sites

Assay	Gene	Methylation site
Primer Mix A	ZSCAN12	Chr6: 28367535-28367599
Primer Mix B	GYPC	Chr2: 127413797-127413902
Primer Mix B	COL2A1	Chr12: 48381229-48381320

Materials provided

Table 2 Kit content

Reagent	Cap Color		Volume per kit (100 reactions, 2x 50)	Storage
Nuclease-Free Water	Blue		1 x 1.5 mL	-25 °C to -15 °C, protected from light
Master Mix	Black		1 x 1 mL	
Positive Control	White		1 x 60 µL	
Primer Mix A	Red		1 x 50 µL	
Primer Mix B	Yellow		1 x 50 µL	
Standard 1	Green		1 x 60 µL	
Standard 2	Green		1 x 60 µL	
Standard 3	Green		1 x 60 µL	
Standard 4	Green		1 x 60 µL	

NOTE

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

NOTE

The kit contains reagents to perform up to 50 reactions with each Primer Mix.

Reagent storage and handling

The WID®-easy PCR Kit is shipped on dry ice. The kits components should arrive frozen, except the Master Mix, which is stored in a buffer that prevents freezing of the reagent.

Please check the completeness of the kit upon receipt. If one or more components are not frozen or if tubes or packaging have been compromised during shipment, please immediately contact the legal manufacturer or UK Responsible Person.

Store all components at -25 °C to -15 °C without light exposure and avoid repeated thawing and freezing for more than five cycles. Primer Mix A and Primer Mix B must be protected from light.

The Positive Control and the Standards 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 or 384-well reaction plates with proper optical foil; PCR grade. PCR plates need to be calibrated on your instrument.
- Suitable racks and cooling racks for 2 mL tubes

- Disposable powder-free gloves
- Qubit Fluorometer (cat. no. Q33238, Thermo Fisher Scientific) or similar.
- PCR Workstation or Clean Bench

* Depending on the device manufacturer

NOTE

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All materials to be used for PCR shall be of appropriate quality (DNA-free and for molecular biology). Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer's instructions and recommendations.

Reagents, kits, and consumables

Table 3 Reagents required but not provided

Reagent	Supplier	Order number
Standard genomic DNA extraction kit	NA	NA
EZ DNA Methylation-Lightning Kit	Zymo Research	D5030-E or D5031-E or D5046-E or D5047-E
Qubit™ dsDNA BR Assay or QuantiFluor ONE dsDNA System	Thermo Fisher Scientific or Promega	Q32850 or E4871
TE Buffer pH 8.0 (Ambion) or Nuclease-Free Water	Fisher Scientific GmbH or Thermo Fisher Scientific	10742317 or AM9932

Instruments and software

The WID®-easy PCR Kit was validated to be used with the following calibrated PCR instrument:

- QuantStudio™ 5 and 7 Real-Time PCR System (cat. no.: A28139, Thermo Fisher Scientific) with QuantStudio™ Design and Analysis Software (v. 1.5 and above)

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 the WID®-easy PCR Kit on instruments other than the above-mentioned ones are at users risk and must be validated before use.

Specimens and test samples

The following specimen has been verified with the WID®-easy PCR Kit: Genomic DNA isolated from human cervicovaginal samples after bisulfite conversion. Please refer to the limitations of use section.

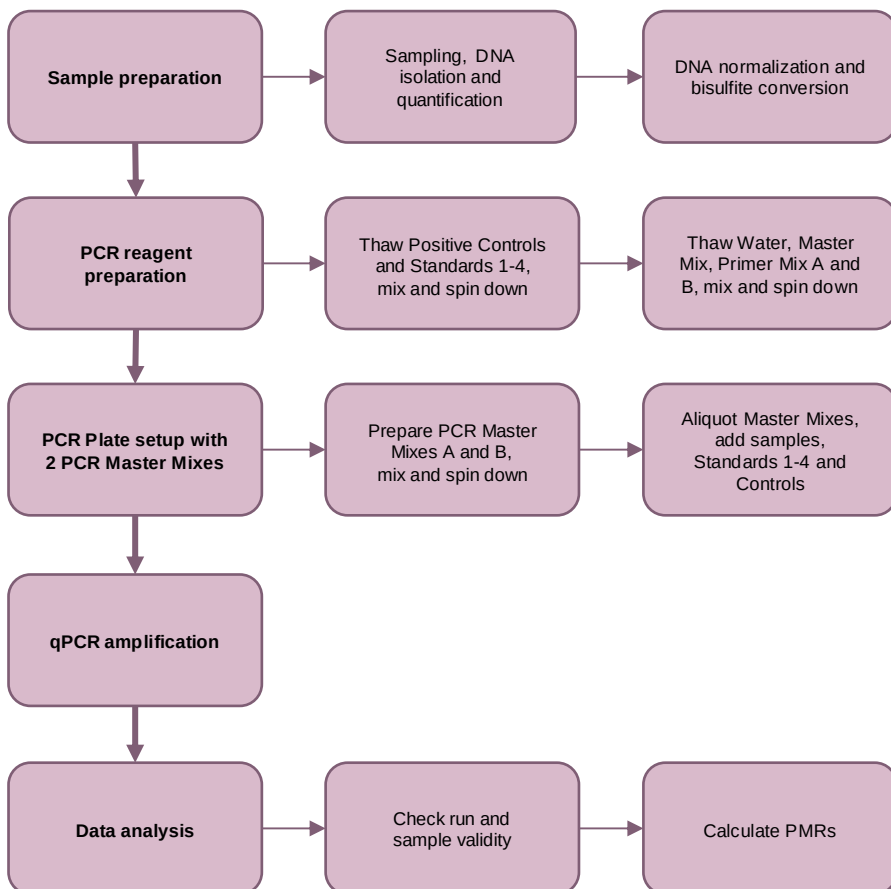
The obtained unmodified genomic DNA shall be stored undiluted at -25 °C to -15 °C.

Warnings and Precautions

- Read the instructions carefully before using the product.
- Read the Safety Data Sheets (SDS) for all 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.
- Kit components shall not be aliquoted into secondary reaction vessels.
 - The use of this product is limited to personnel suitably educated and trained medical staff specially instructed and trained in PCR techniques.
 - This kit is not for self-testing purposes or for direct diagnosis
 - It is recommended that a separate laboratory area is used for PCR amplification to reduce amplicon contamination of reagents
 - Appropriate Personal Protective Equipment must be used by laboratory workers
- 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 samples, reagents and assay waste via laboratory waste according to your local safety regulations.
 - All instruments used must be installed, calibrated, checked, and maintained according to the manufacturer's instructions and recommendations.

Procedure

Overview of the experimental workflow



Sample preparation

DNA extraction

DNA extraction and purification should be carried out from human cervicovaginal samples according to the supplier's instructions using a standard genomic DNA isolation method, which can be bead- or column-based.

The DNA must be quantified directly after the extraction. Make sure to use high-quality DNA.

DNA quantification and dilution

Quantification of the DNA should be carried out by a fluorometric quantification method using the Qubit™ 3.0 Fluorometer or a similar fluorescent-based instrument. Use the Qubit™ dsDNA BR Assay or a similar product according to the manufacturer's protocol. Dilute the DNA to a final concentration of 10 ng/μL. Using a DNA concentration lower than 10 ng/μL is not recommended for further processing. Repeat DNA extraction or sampling if needed.

NOTE



For sample dilution we recommend the use of 1x TE (Tris-EDTA)-Buffer, pH value 8.0 or nuclease-free H₂O and a sample volume > 1.5 μL.

DNA storage

Store the unmodified 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. If using stored DNA, quantify the DNA again before normalization and bisulfite conversion.

Bisulfite conversion

Bisulfite conversion should be carried out using the Zymo Research EZ DNA Methylation-Lightning Kit (bead- or column-based) according to the manufacturer's instructions using 200 ng DNA (equals 20 μL of 10 ng/μL

diluted DNA) as input amount. Elute with 50 μL Nuclease-Free Water to reach a final concentration of 4 ng/ μL (corresponds to 20 ng within 5 μL volume per PCR reaction). Using a bisulfite-converted DNA amount below 20 ng per PCR reaction will result in low PCR yields, and the signal might fall below the target-specific detection limits, which can lead to inconclusive PCR results.

NOTE

Wash buffer used during bisulfite conversion may have potential interference on amplification performance. Therefore, wash steps within the bisulfite conversion assay must be followed.

NOTE

Bisulfite-converted DNA is not suitable for long-term storage and should be used immediately after conversion. Storage of unmodified genomic DNA or the sample material is recommended instead. If immediate processing is not possible, bisulfite-converted DNA can be stored for up to 7 days at -80°C .

Standard 1-4 and Positive Control preparation

Thaw the Standard 1-4 (green cap) and the Positive Control (PC; white cap), and homogenize it by gentle vortexing followed by brief centrifugation.

Provided Standards (Standard 1-4) and Positive Control (PC) must be assessed in duplicates per each PCR run using 5 μL of reagent per well and replicate. No additional dilution steps are required. We recommend keeping standards and PC on ice during qPCR preparation.

PCR Master Mix setup

Remove and thaw the following components from the WID®-easy PCR Kit:

- Nuclease-free water (blue cap)

- Primer Mix A (red cap)
- Primer Mix B (yellow cap)
- Master Mix (black cap)

During the PCR Master Mix setup, it is recommended to keep the reagents 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. 20 min, protected from light) and homogenized by inverting the tubes (Master Mix) or gently vortexing (Primer Mix). The reagents should be then briefly centrifuged (approx. 10 s).

NOTE

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Make sure to analyze each sample with each Primer Mix to obtain the complete information of the assay. Therefore, prepare two separate PCR Master Mixes, one for each Primer Mix. It is recommended to analyse samples in duplicates with both Primer Mixes.

Prepare a PCR Master Mix for each Primer Mix (2 separate PCR Master Mixes for each sample in technical duplicates) 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 two reactions for the PC, the No Template Control (NTC; water as sample), as well as each Standard 1-4 (STD 1-4) for each PCR Master Mix (A & B) into your calculation. Samples, STD 1-4, NTC and PC, must be analyzed in duplicates.

Example of a plate layout for 18 samples is outlined:

	PCR Mix A						PCR Mix B					
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S5	S9	S13	S17	STD1	S1	S5	S9	S13	S17	STD1
B	S1	S5	S9	S13	S17	STD1	S1	S5	S9	S13	S17	STD1
C	S2	S6	S10	S14	S18	STD2	S2	S6	S10	S14	S18	STD2
D	S2	S6	S10	S14	S18	STD2	S2	S6	S10	S14	S18	STD2
E	S3	S7	S11	S15	NTC	STD3	S3	S7	S11	S15	NTC	STD3
F	S3	S7	S11	S15	NTC	STD3	S3	S7	S11	S15	NTC	STD3
G	S4	S8	S12	S16	PC	STD4	S4	S8	S12	S16	PC	STD4
H	S4	S8	S12	S16	PC	STD4	S4	S8	S12	S16	PC	STD4

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, assign all tested targets to corresponding wells.

NOTE

If testing fewer than 10 reactions, use enough PCR Master Mix for one extra sample. If testing 10 or more reactions, use an excess reagent PCR Master Mix volume of +10%.

Table 4 PCR Master Mix reaction setup

Component	Volume		
	# 1 rxn	# 10 rxn	# 50 rxn
Nuclease-Free Water	4 µL	40 µL	200µL
Primer Mix A OR Primer Mix B	1 µL	10 µL	50 µL
Master Mix	10 µL	100 µL	500 µL
PCR Master Mix volume	15 µL	150 µL	750 µL
Bisulfite-converted DNA template or Positive Control or Standard or No Template Control	5 µL	10 x 5 µL	50 x 5 µL
Total volume	20 µl	10 x 20 µl	50 x 20 µl

Gently vortex the PCR Master Mix without generating bubbles, followed by brief centrifugation.

Application of PCR Master Mixes and DNA templates and controls

Aliquot 15 µL of the PCR Master Mix per well in an appropriate, calibrated 96-well or 384-well PCR plate.

Add 5 µL of the following sample types to the prepared PCR plate containing the PCR Master Mixes:

NTC: add 5 µL of Nuclease-Free Water instead of a sample.

DNA Sample: add 5 µL of the prepared, diluted bisulfite-converted DNA samples (4 ng/µL). Technical replicates are required for quantitative analysis.

PC: add 5 µL of Positive Control instead of a sample.

Standard 1-4: add 5 µL of Standard 1-4 instead of a sample.

NOTE



Use at least two technical replicates for each sample/component per PCR Master Mix (A & B). Otherwise, the run cannot be validated.

Close the PCR plate with an optical adhesive foil suitable and validated for your qPCR instrument, gently mix and spin down.

PCR amplification

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

Table 5 PCR protocol

Temperature	Time	Cycles
95 °C	1 min (hot-start for activation of the polymerase)	1 x
95 °C	15 s	45 x
60 °C	30 s	

NOTE

If qPCR instruments with rapid heating and cooling steps (> 2 °C/s) are used, **ramping is required to be adjusted to 1.6 °C/s** 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

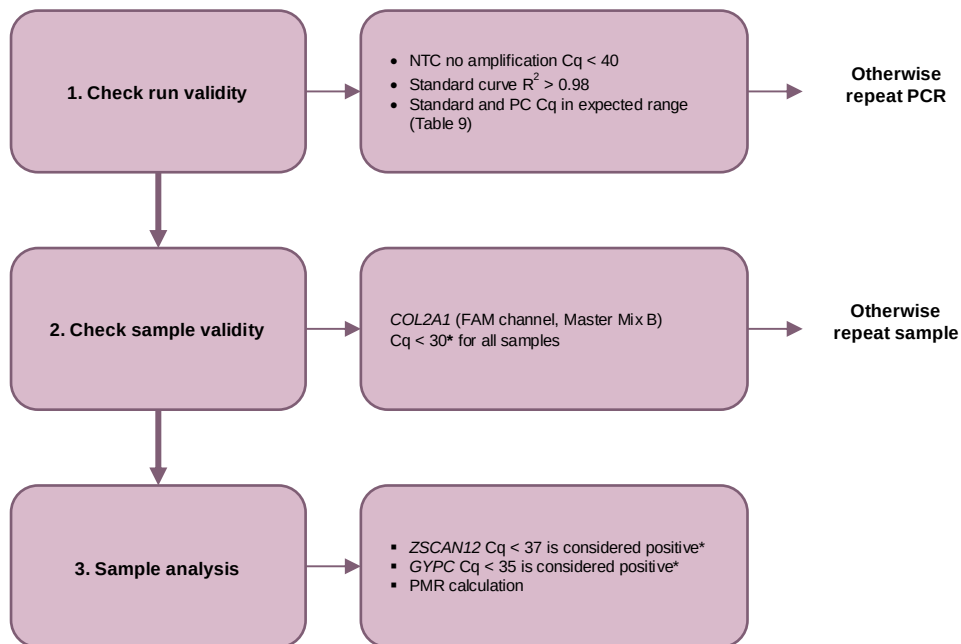
Primer Mix	Target	Reporter (Fluorescence)	Quencher
A	ZSCAN12	FAM	Dark quencher
B	GYPC	Cy5	Dark quencher
B	COL2A1	FAM	Dark quencher

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



- * The COL2A1 cutoff value for determining a positive sample depends strongly on device-specific parameters for baseline and threshold and may be adjusted according to your setup. Ideally, samples should be in a Cq range between Cq > 25 and Cq < 30 if 20 ng input material is used. ZSCAN12 and GYPC cutoff values are also device-specific and should be evaluated during technical verification using blank material (negative samples). It is not recommended to run the assay without Cq cutoff values. Contact customer support in case of questions.

General settings

- Use the standard curve method; for instruments that have a passive reference dye option, select ROX as reference dye.

Table 7 Standards used in the data analysis

Sample	Quantity [copies per reaction]
Standard 1	100,000
Standard 2	10,000
Standard 3	1,000
Standard 4	100

Table 8 Specific thresholds used in the data analysis (with ROX as reference dye)

Primer Mix	Target	Reporter (Fluorescence)	Threshold in ΔRn	Baseline
A	ZSCAN12	FAM	0.22*	1-16*
B	GYPC	Cy5	0.04*	1-18*
B	COL2A1	FAM	0.10*	6-20*

- * These thresholds and baselines were used during assay verification with the above-mentioned instrument and software version and need to be adjusted for different instruments and softwares. Device-specific adjustments need to be done during tech transfer; see troubleshooting. Baseline and Threshold should be adjusted in a way that Cq values for Standards and PC are in the expected Cq ranges (Table 9).

NOTE



To set up an experiment in the QuantStudio™ Design and Analysis Software, create a template file with the desired samples and the above-mentioned parameters (adjusted to your instrument). This can be saved and re-used to standardize analysis settings.

Run validation

- Check if **NTC** shows **no amplification**
- Check if **PC** and **Standard 1-4** show amplification of all targets with Cq-values in the expected ranges

Table 9 expected Cq-ranges for controls*

Sample	ZSCAN12	GYPC	COL2A1
PC	26 - 28.5	23 - 26	26 - 29
Standard 1	20 - 23	18 - 21	21 - 23
Standard 2	23 - 26.5	21 - 24	25 - 27
Standard 3	26 - 29.5	25 - 27.5	28-31
Standard 4	29.5 - 33	28 - 31	31-35
Slope	-3.7 - -3.1	-3.6 - -3.1	-4.2 - -3.1

- * All Cq values and slope depend on device-specific parameters for baseline and threshold. Both may slightly differ in your setup, but ideally, Cq values should be in the expected range after threshold and baseline adjustment. Contact customer support in case of questions.

NOTE



Compliance with above mentioned Cq and slope ranges for Standards and PC is important for correct sample analysis and final PMR ranges.

Sample analysis

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

The "baseline noise levels" should either be automatically defined or predefined for specific cycles (e.g., 3 to 15). Use the NTC to determine the respective baseline. Set the thresholds for all targets according to your previously defined settings (see Run validation).

Verify that Standard and PC mean Cqs and slopes are within the above-mentioned ranges (Table 9). If Standards, PC, NTC and slope are valid (see Run validation), continue with the sample data analysis.

Information for data export and data processing can be found in the respective manuals of the real-time instrument manufacturer. Export the raw data (information should at least include the "sample name", "Cq-values" and "quantity", as well as the respective means (optional) for subsequent calculations).

NOTE



If other qPCR instruments than the QuantStudio™ 5 or 7 are used, the data analysis steps need to be adjusted, and the procedure needs to be verified in the user's responsibility. Contact the tech support if you need further information.

Data analysis/verification

For samples, the *COL2A1* in the FAM channel should have a Cq-value below 30, if this is not the case, the sample must be repeated with re-quantified and adjusted bisulfite-converted DNA. Increasing the input BC-DNA amount in the PCR reaction to 40 ng/reaction is recommended.

In a valid sample, *ZSCAN12* with a Cq value below the cutoff of $Cq < 37$ and/or *GYPC* with a Cq value below the cutoff of $Cq < 35$ is considered a positive PCR amplification. If the Cq value is above the Cq cutoff, it is deemed as negative and set to 'undetermined' (no amplification).

Both technical replicates need to have a target amplification below or above the above-mentioned/pre-defined Cq cutoffs. If replicate Cq values in a sample are above the respective cutoff (Cq_1 and $Cq_2 > 35$ for *GYPC* or > 37 for *ZSCAN12*), they are deemed as 'undertermined' (no amplification) and the PMR of the respective target is zero. If a sample shows an inconclusive result (one technical replicate $< Cq$ cutoff, the 2nd technical replicate $> Cq$ cutoff) for one of the two targets, the qPCR needs to be repeated with a higher input amount of BC-DNA (40 ng).

Cq cutoff levels for *COL2A1*, *ZSCAN12* and *GYPC* can be adjusted during tech transfer but must be validated.

Contact customer support in case of questions.

Table 10 example PCR results and interpretation

		COL2A1 Cq1	COL2A1 Cq2	GYPC Cq1	GYPC Cq2	ZSCAN12 Cq1	ZSCAN12 Cq2
before cutoff was applied	Sample 1	30.2	30.9	33.2	32.9	35.2	35.5
after cutoff was applied		Undet.	Undet.	33.2	32.9	35.2	35.5
conclusion	reference COL2A1 > Cq cutoff → repeat qPCR						
before cutoff was applied	Sample 2	28.1	27.8	35.2	34.7	36.9	36.5
after cutoff was applied		28.1	27.8	Undet.	34.7	36.9	36.5
conclusion	sample needs to be repeated due to undetermined Cq value of GYPC						
before cutoff was applied	Sample 3	28.1	27.8	33.9	34.7	37.1	36.5
after cutoff was applied		28.1	27.8	33.9	34.7	Undet.	36.5
conclusion	sample needs to be repeated due to undetermined Cq value ZSCAN12						
before cutoff was applied	Sample 4	28.1	27.8	36.1	35.9	37.1	37.3
after cutoff was applied		28.1	27.8	Undet.	Undet.	Undet.	Undet.
conclusion	no PCR amplification in both targets; $\Sigma PMR=0$						
before cutoff was applied	Sample 5	28.1	27.8	33.2	32.9	35.2	35.5
after cutoff was applied		28.1	27.8	33.2	32.9	35.2	35.5
conclusion	positive PCR amplification in both targets; ΣPMR calculation						

PMR calculation

Most qPCR analysis software tools allow the automatic calculation of the target quantities when the standard curve is properly set up. If this should not be the case in your software, you should manually calculate the quantity Q for all samples and both Positive Control replicates with the following formula (1).

$$Q = 10^{\frac{C_q(\text{target}) - \text{Intercept}(\text{target Std curve})}{\text{Slope}(\text{target Std curve})}} \quad (1)$$

Next, calculate the Percentage of Methylated Reference (PMR) per sample with formula (2) and (3), where $\bar{Q}_{S \text{ ZSCAN12}}$, $\bar{Q}_{S \text{ GYPC}}$ and $\bar{Q}_{S \text{ COL2A1}}$ refer to mean quantity values of one sample, while $\bar{Q}_{PC \text{ ZSCAN12}}$, $\bar{Q}_{PC \text{ GYPC}}$ and $\bar{Q}_{PC \text{ COL2A1}}$ refer to the mean quantity values obtained from the Positive Control.

$$PMR_{ZSCAN12} = \frac{\frac{\bar{Q}_{S \text{ ZSCAN12}}}{\bar{Q}_{S \text{ COL2A1}}}}{\frac{\bar{Q}_{PC \text{ ZSCAN12}}}{\bar{Q}_{PC \text{ COL2A1}}}} * 100 \quad (2)$$

$$PMR_{GYPC} = \frac{\frac{\bar{Q}_{S \text{ GYPC}}}{\bar{Q}_{S \text{ COL2A1}}}}{\frac{\bar{Q}_{PC \text{ GYPC}}}{\bar{Q}_{PC \text{ COL2A1}}}} * 100 \quad (3)$$

Finally, calculate the sum of both PMR values according to formula (4).

$$\sum PMR = PMR_{ZSCAN12} + PMR_{GYPC} \quad (4)$$

Clinical laboratories should verify the assay's performance characteristics within their own laboratory setting to determine the $\sum PMR$ value.

Performance characteristics should include, but is not limited to, accuracy, precision, analytical sensitivity, analytical specificity, and any

other relevant parameters necessary to ensure suitability for the intended use in the laboratory's specific environment and with their patient population.

Guidance to Σ PMR interpretation and clinical performance data, according to Evans *et al.*, can be found in the 'Clinical Performance' section below. Note the Σ PMR may differ from laboratory to laboratory.

Performance Evaluation

Analytical specificity

Non-bisulfite-converted human DNA was tested with the assay. There is no amplification on human DNA for *ZSCAN12* and *GYPC*.

Analytical sensitivity and Limit of Detection (LOD)

The analytical sensitivity of the WID®-easy PCR Kit is defined as the concentration (copies/μL of the eluate) of bisulfite-converted DNA molecules of the target regions of *ZSCAN12* or *GYPC* that can be detected with a positivity rate of 95 %. The analytical sensitivity was confirmed by analysis of dilution series of artificial DNA (gBlock fragments) of the methylated targets *ZSCAN12* and *GYPC*. The confirmation was carried out on five different days with 6 replicates per concentration.

For the detection of *ZSCAN12* and *GYPC*, the analytical sensitivity is < 1 copies/μL with a 95 % CI (Confidence Interval).

Precision

The precision of the WID®-easy PCR Kit was determined as inter-assay variability (variability between different experimental setups) and inter-lot variability (variability between different production lots).

For the inter-assay variability, the study was run on three different setups (three instruments, three operators) on five days and in five replicates. For the analysis, the SD (Standard Deviation) and Coefficient of Variation (% CV) of repeatability, of reproducibility, and of within-laboratory precision were calculated as per the CLSI EP05 (4.6.2) guidelines. With regard to the C_q-value results, the % CV of repeatability, reproducibility, and within-laboratory precision did not exceed 5 %, indicating that the performance of the assay is not affected by the testing procedure.

For the inter-lot variability, two different production lots were tested at two different cDNA concentrations. The SD and coefficient of variation (% CV) were calculated as per the CLSI EP05 (4.6.2) guideline. The *ZSCAN12* and *GYPC* assays show a % CV < 5 % for all samples, including positive control and standards. In conclusion, the assay performance is not affected by different kit lots.

Limit of blank

This study followed CLSI EP-17-A2, Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures.

The LOB (Limit of Blank) was performed using at least two kit lots with eight replicates measured on three different days; in total, 120 wells were measured. The LOB for *ZSCAN12* is Cp 43.0 and for *GYPC* is Cp 42.4.

Linear range

The linear range of the WID®-easy PCR Kit was evaluated by analyzing a logarithmic dilution series of cDNA for both targets *GYPC* and *ZSCAN12* using concentrations ranging from 10 to 100,000 copies/reaction, and each dilution was analyzed in four replicates.

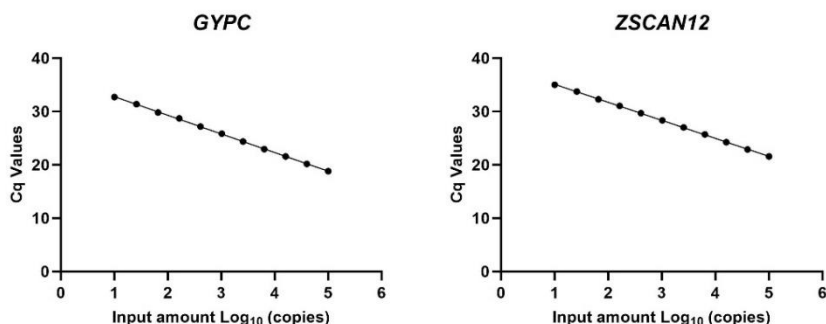


Figure 1: Linear regression of the dilution series plotted against the Log₁₀ concentrations for the *ZSCAN12* (left) and *GYPC* (right).

The linear range was defined for concentrations from 10 to 100,000 copies for both assays. The linearity coefficient was calculated at $R^2 = 0.9998$ for *ZSCAN12* and $R^2 = 0.9991$ for *GYPC*. The measured slopes were -3.376 for the *ZSCAN12* assay and -3.492 for the *GYPC* assay.

Clinical Performance

The Σ PMR can be used to provide guidance on the risk level of endometrial cancer in women 45 years and over, presenting with abnormal uterine bleeding (10). However, the Σ PMR value is to be used as an aid to diagnosis only and should not be used as a final diagnosis.

In the study of Evans *et al* 2023 a cutoff of Σ PMR ≥ 0.3 , determining high risk of endometrial cancer was used in 399 women aged 45 years and older referred for abnormal uterine bleeding (10). 378 patients had sufficient DNA in their cervicovaginal fluid sample to successfully run the WID®-easy PCR assay. In this patient population the clinical performance, compared to the standard of care (sonography and histological tests), is presented in Table 11. Full details can be found in the study of Evans *et al.* 2023 (10).

Table 11. Clinical performance of the WID®-easy PCR assay in 45 years and over, presenting with abnormal uterine bleeding with a Σ PMR ≥ 0.3 Evans *et al.* 2023 (10).

Clinical Performance	WID®-easy PCR Kit
Sensitivity (95% CI)	90.9% (62.3–98.4)
Specificity (95% CI)	97.3% (95.1–98.5)
Positive predictive value (PPV) (95% CI)	50.0% (29.9–70.1)
Negative predictive value (NPV) (95% CI)	99.7% (98.4–100.0)

Troubleshooting

The troubleshooting guide may be helpful for solving any problems that may arise. Customer support is happy to answer any questions about the information and protocols given in this 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. Where possible, close the tubes directly after adding reagents. Ensure that the workspace and the instruments are decontaminated at regular intervals. Change gloves occasionally. 2. Check the maintenance interval for all used devices (e.g., pipettes) 3. To avoid contamination, work in a qPCR working hood and keep gBlock Standard and PC separate from other reagents
Invalid Positive Control (PC) or Standards	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 adding the sample to be tested. Ensure that the workspace and the instruments are decontaminated at regular intervals. 2. Check the maintenance interval for all devices used (e.g., pipettes, PCR cycler). Make sure that all devices that are used are calibrated (including PCR plates). 3. Check the Master Mix calculation and preparation protocol for errors. High C_q-values indicate low Primer Mix and Master Mix concentrations in the reaction. 4. Double-check your qPCR loading.

Cq-values of Positive Control / Standard are below the valid range	Check the Cq-threshold in the analysis view of your qPCR analysis software. It should be above the baseline, and the threshold must be set in the linear phase of the amplification plot. For target <i>GYPC</i>, it is of great importance to determine your qPCR instrument-specific threshold with a blank and a positive sample as low as possible but still safely above the background to ensure maximum assay sensitivity. Please contact the technical support if any assistance is required for this step.
Cq-values of Internal Control <i>COL2A1</i> are above the valid range (Cq > 30)	1. <i>COL2A1</i> Cq values > 30 can have the following reasons: i) too low BC-DNA input in qPCR, ii) inefficient bisulfite conversion, iii) bacterial DNA contamination in your sample 2. Repeat the DNA quantification input to ensure a minimum of 20 ng bisulfite converted DNA per reaction. Adjust the sample concentration if possible (e.g. 40 ng instead of 20 ng per reaction). 3. If the concentration in the gDNA (genomic DNA) sample is sufficiently high, repeat the analysis directly after a fresh bisulfite conversion step. 4. Make sure that the bisulfite conversion is performed using the Zymo EZ DNA Methylation-Lightning Kit according to manufacturer's protocol. 5. If the problem persists for a new sample, repeat the sample collection (possible bacterial contamination).
The storage conditions for one or more kit components do not comply with the instructions given in <u>Reagent storage and handling</u>	Check the storage conditions and the expiration date (see the kit box label). Use a new kit if the reagents were stored improperly. Make sure to not leave the kit components on room temperature for too long. Keep reagents on ice during qPCR preparation.

Quality Control

All kit components undergo an intensive quality assurance process at Sola Diagnostics GmbH. The 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: service@wid-easy.info

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Limitations of Use

- The procedures in this handbook must be followed as described. Any deviations may result in assay failure or cause erroneous results.
- This assay is not to be used directly for diagnosis
- 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 consumables for DNA extraction and purification.
- The kit has only been verified for use with an optimum input of 20 ng bisulfite-converted 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.
- Do not collect samples: during the menstrual cycle or within 24 hours after sexual intercourse without a condom.
- Sample collection for the WID®-easy test should take place without the use of lubricant (or with minimal lubricant) and *before* any other procedures (e.g. taking a sample for another test like a PAP smear, inserting another substance into the vagina or near the cervix like acetic acid or Lugol iodine, transvaginal sonography).
- Conditions that restrict the drainage of tumor DNA from the uterine cavity into the vagina, such as large (endo)cervical polyps or (endo)cervical fibroids, may affect the sensitivity of the test.
- The performance of this assay must be verified by the end user's laboratory before clinical application. Field performance may vary depending on laboratory-specific procedures, sample collection,

handling, and equipment. It is the responsibility of the user to ensure compliance with local regulatory and accreditation requirements.

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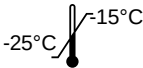
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Explanation of Symbols

	Manufacturer
	Batch code
	In-vitro diagnostic
	UKCA mark (United Kingdom Conformity Assessed)
	United Kingdom Responsible Person
	Contains sufficient reagents for <N> tests
	Consult electronic instructions for use (eIFU)
	Use-by date

	Temperature limit
	Catalogue number
	Keep away from sunlight
	Keep dry

Further marking used in this handbook:



Useful tips



Attention, be sure to follow this notice!

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