High-Risk HPV Multiplex Real-time PCR Kit, IVD

REF MD04921, 96 reactions

For professional* in vitro *diagnostic use only

CE IVD

EN

Instructions for Use

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NZYtech genes & enzymes
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1. Introduction

Papillomaviruses constitute a diverse group of DNA viruses with the ability to infect both the skin and mucous membranes of humans and animals. The human papillomavirus (HPV) is particularly significant, being implicated in over 99% of cervical cancers worldwide. While more than 200 distinct types of HPV have been catalogued, at least 14 have been classified as high-risk (HR HPV) given their association with the initiation of mucosal lesions that may progress to cervical cancer and other complications[1-3]. Cervical cancer, ranking as the second most common malignant tumour in women, underscores the gravity of HPV-related health concerns globally. Beyond its oncogenic potential, HPV holds the dubious distinction of being the most prevalent sexually transmitted infection on a global scale[4,5].

High-risk HPVs include types 16 and 18, responsible for a substantial ~70% of the most severe, cancerous lesions[6]. Additional types of high-risk, such as HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68, collectively contribute to over 90% of cervical adenocarcinomas[7-10]. The progression to malignancy is frequently linked to the integration of viral DNA into the host cell genome, emphasizing the complex interplay between viral factors and host biology. Notably, coinfection with high-risk HPV subtypes has emerged as a discernible risk factor for an elevated incidence of disease[6].

Today, HR HPV screening or diagnosis mostly involves cytological techniques that have less specificity and sensitivity than molecular techniques[2]. Cervicovaginal cytology or liquid-based cytology are proficient in detecting precursor lesions and indicating cervical cancer following HPV infection[11]. However, while effective in identifying precursor lesions and indicating the presence of cervical cancer after HPV infection, these methods have limitations in distinguishing between different virus types, specifically various high-risk HPV types. A significant advancement in diagnostic precision is evident with real-time PCR assays, providing a rapid and potent methodology for the efficient detection of distinct HR HPV types[8].

2. Intended Use

NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, is a molecular test designed for the rapid qualitative detection of viral DNA from High-Risk Human Papillomavirus (HPV) in human biological samples obtained through cervical cytology by a clinician. The test performs multiplexed amplification of target DNA via real-time Polymerase Chain Reaction (PCR) for 14 high-risk HPV types in a single reaction. The kit specifically identifies HPV16 and HPV18 in two distinct detection channels and reports the remaining 12 high-risk types (HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68) in a pooled result detected in the same channel. The purpose of this test is to assess the presence or absence of the 14 high-risk HPV types. This information, combined with the physician's evaluation of the patient's medical history, other risk factors, and adherence to professional guidelines may be used to guide patient care. The kit is intended for use by laboratory-trained personnel, specifically instructed in real-time PCR techniques and *in vitro* diagnostics.

3. Principles of the Assay

NZYtech´s High-Risk HPV Multiplex Real-time PCR Kit, IVD, includes a comprehensive set of reagents, enzymes and oligonucleotides (primers and probes) for the qualitative detection of HR HPVs using common real-time PCR platforms (refer to **Section 6** for required instrument specifications). This kit enables the identification of various high-risk HPV genome types: HPV16, HPV39 and HPV68 through the amplification of targets located in the E6 gene; HPV18 via the amplification of a target in the E1 gene; HPV31 through the amplification of a target located in the E5 gene; HPV33, HPV35, HPV52 and HPV58 by amplifying targets in the E7 gene and finally, HPV45, HPV51, HPV56, HPV59 and HPV66 through the amplification of targets located in the L1 gene.

NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, is meticulously designed to offer the broadest detection profile possible while maintaining specificity for the 14 high-risk HPV genomes mentioned above. The kit provides a complete set of reagents for the detection of the 14 viral genomes, targeting highly conserved regions of the high-risk HPV types through highly optimized primers and probes. Additionally, it includes an efficient internal control for the detection of the human β-actin gene (ACTB) using specific primers and probe set, to confirm successful sample DNA extraction and the absence of PCR inhibitors. The oligonucleotides are specifically designed for HPV detection and do not show significant homology with other genomes, which reflects the high specificity and detection sensitivity of the test. This design ensures the test's high specificity and detection sensitivity, avoiding the detection of other organisms causing similar infections. The natural evolution of the virus detected by this kit implies that new sequence information will become available overtime, which reflects well-known viral adaptation strategies. Thus, NZYtech periodically revisits viral genomic targets and, if required, will release new versions of this kit.

The High-Risk HPV Multiplex Real-time PCR Kit, IVD, uses the real-time PCR technique for the qualitative determination of DNA, a gold standard in laboratory molecular diagnosis. This highly sensitive and specific methodology ensures accurate detection of various high-risk HPV types. The principle of the High-Risk HPV Multiplex Real-time PCR Kit, IVD, consists of the use of isolated and purified DNA through an extraction system to search for the presence of viral DNA. The extracted DNA undergoes multiplex PCR amplification in a single reaction, employing sets of highly specific primers and probes based on the TaqMan® principle. During amplification, probes selectively anneal to their target genes, and as DNA amplifies, degradation of these probes, flanked by two primers, leads to the separation of the reporter dye from the quencher, increasing fluorescence. To identify the amplification of the 15 specific targets in a single reaction - HPV16, HPV18, twelve HR HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and human ACTB - the specific probes are labelled with different fluorophores, namely Texas Red®, Cy5™, FAM™, and HEX™ reporter dyes, respectively. In addition, the primers/probe sets are provided in optimized concentrations ensuring that amplification of lower abundant nucleic acids is not compromised when other viral targets are present at higher concentrations. This meticulous design guarantees the kit's reliability and accuracy in detecting the specified 14 high-risk HPV genomes.

4. Kit Composition

NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, provides a comprehensive set of reagents sufficient to perform 96 Real-time PCR reactions in a single step.

KIT COMPONENT		VOLUME (PER VIAL)	NUMBER OF TUBES	CAP COLOUR
HPV MMix	NZYSupreme Multiplex qPCR Probe Master Mix (2x)	1050 µL	1	Neutral
HPV PPMix	HPV HR/ACTB Primers & Probe Mix (10x)	205 µL	1	Brown
HPV POS	HPV HR/ACTB Positive Control	105 µL	1	Red
NTC	No-template control	105 µL	1	Neutral

5. Storage, Stability and Handling Conditions

The NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, is shipped refrigerated. All components should immediately be stored at -85 °C to -15 °C upon arrival. When in use, the kit components should be returned to the freezer promptly after use to minimise the time at room temperature.

- Minimise the number of freeze-thaw cycles by storing in working aliquots. If appropriate, kit components may be aliquoted into smaller volumes after thawing. The kit is stable at least through 10 freeze-thaw cycles.
- The HPV PPMix should be stored and protected from light. Particularly, do not expose the HPV MMix to direct sunlight after combining it with the HPV PPMix.
- If the package that protects the kit arrived damaged, please contact NZYtech.
- Beware to the expiry date indicated on the packaging. NZYtech does not recommend using the kit after the expiry date. On this date, the kit must be discarded following the disposal instructions in **Section 8.2.**

6. Materials and Instrumentation Required but Not Provided

- Real-time PCR Instrument that includes Texas Red/JUN, FAM, VIC/HEX/JOE and Cy5 fluorescence channels (at emission wavelengths of 615, 520, 556 and 670 nm, respectively). See in **Section 11** the instrument models for which the kit was validated.
- Equipment and consumables for isolating viral DNA from biological/clinical samples.
- RNase/DNase-free qPCR plasticware: PCR tubes, strips, caps, 96-well plates and adhesive films.
- Pipettors and filter tips (RNase/DNase-free).
- Cooling block.
- Disposable gloves.
- Vortex and centrifuge.

7. Sample Collection and Preparation

The kit is designed for the detection of DNA extracted from cervical samples (liquid base solution for cytology). Several factors, including the biological sample collection procedure, transport, storage, and sample processing time, are of utmost importance to ensure sample integrity and achieve optimal results. For effective collection, it is recommended that samples must be obtained using an endocervical swab/spatula, which should be resuspended in PreservCyt® solution (Hologic Corp.) or Amies medium. Subsequently, specimens should be tightly sealed in appropriate tubes or containers, accurately labelled, and promptly transported to the laboratory. Collected samples are advised to be tested as soon as possible to maintain result accuracy. Failure to adhere to proper sample collection, handling, and/or transport procedures may lead to inaccurate results. Extracted nucleic acids constitute the starting material for the assay with NZYtech' High-Risk HPV Multiplex Real-time PCR Kit, IVD. NZYtech recommends the usage of the magnetic bead technology-based nucleic acid purification kit: NZY Mag Viral RNA/DNA Isolation Kit, IVD (MD0488, NZYtech). This kit has been validated for the extraction of HPV clinical samples and subsequent detection using NZYtech High-Risk HPV Multiplex Real-time PCR Kit, IVD. Please ensure that cross-contaminations did not occur and that DNA samples are suitable in terms of purity, concentration, and nucleic acid integrity. NZYtech' kit contains an internal control that targets human DNA co-purified with viral HPV DNA. Human DNA is amplified with the set of oligonucleotides (primers and probe) from the human β-actin gene. The introduction of internal control is useful in assessing the efficiency of DNA extraction and isolation and/or in detecting the presence of potential inhibitors during sample processing.

8. Precautions and Warnings

Carefully follow the procedures and guidelines provided in this handbook to ensure that the test is performed correctly. Before using the test, check the product's integrity, namely the amount and type of kit components and their correct labelling. As in any analytical testing procedure, good laboratory practices are essential. Any deviation from them may result in assay failure or cause erroneous results. Due to the high sensitivity of the kit, special care must be taken to keep reagents and PCR amplification mixes free from contamination.

8.1. Safety Information

Before using the kit, please consult the Safety Data Sheet (SDS) that is available on the NZYtech website (www.nzytech.com). This kit detection should be performed only by staff trained in the relevant technical and safety procedures in appropriately equipped laboratories. International and national guidelines on laboratory biosafety should be followed in all circumstances.

8.2. Handling and Procedural Requirements

- Only for professional *in vitro* diagnostic use.
- Do not use this kit after the expiration date.
- Do not use the test components if the kit sealing is damaged.
- Do not interchange reagents of different production lots.
- No reagents from other manufacturers should be used along with reagents of this test kit.
- DNase/RNase-free disposable plasticware and pipettes should be used in all procedures.
- Use DNase/RNase-free filter tips throughout the protocol to prevent aerosol and liquid contamination.
- Sample preparation, reaction set-up and amplification should be performed in different working areas. The order of tasks in the lab should be unidirectional. Always wear disposable gloves in each area and change them before entering a different area. If possible, change your coat between different procedures.
- Select specific materials and equipment for each work area and do not transfer them from one area to another.
- Biological samples must be handled as if they are infectious following proper biosafety precautions.
- Always use the NTC - No-template Control provided in the kit.
- Positive controls contain high-copy number templates; they should be opened and processed away from test samples and kit components to avoid cross-contamination.
- Handle post-amplification plates with care and dispose them immediately after the end of the testing; plates should always be discarded into a proper biohazard container after use.
- At the end of each test, clean work surfaces and equipment with a DNA/RNA remover (DNA & RNA Cleaner, MB462, NZYtech).
- Residues of chemicals and preparations are considered hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations.
- All results should be interpreted by a health-care professional in the context of the patient medical history and clinical symptoms.
- This test cannot exclude diseases caused by other pathogens.
- A negative result for any PCR test does not conclusively rule out the possibility of infection.
- A positive result is not a definitive indicator of the presence of high-grade cervical disease or the possibility of cancer development.
- Follow good laboratory practices, wear protective clothing, permanently wear disposable powder-free gloves, wear goggles and a mask. Do not eat, drink, or smoke in the working area.

9. Testing Procedure

Please read the instructions for use carefully before performing the assay. Beware that all pipetting steps and experimental plate set-up should be performed following good Real-time PCR practices. After the plate is poured start immediately to the Real-time PCR protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artefacts that reduce the sensitivity of detection. Before the experiment, start by gently mixing the reaction tubes provided with the finger and centrifuge for five seconds to collect contents at the bottom of the tube. Place tubes on ice. **We strongly recommend pipetting HPV POS last to avoid cross contaminations.**

9.1. Reaction set-up

1. Prepare a Real-time PCR mix enough for the number of tests to be performed with a 5% additional volume for pipetting losses. Proceed according to the table below that specifies the volumes for 1 and n tests (where n corresponds to the total number of reactions).

COMPONENT	**1 TEST VOLUME (µL)**	***n* TESTES * VOLUME + 5% (µL)**
HPV MMix **	10	*n* x 10,5
HPV PPMix	2	*n* x 2,1
FINAL VOLUME	**12**	***n* x 12,6**

** To calculate the total number of reactions needed for each assay, count the number of samples and add two more, to include the Negative and Positive controls.*

*** Please notice that a precipitate in the bottom of the master mix tube may be observed, after multiple freeze/thaw cycles. To ensure optimal performance, please make sure all components are thawed and resuspended prior to use. In this case do not spin the master mix before pipetting.*

2. Pipette 12 µL of the Real-time PCR mix into individual wells according to your real-time PCR experimental plate set-up.
3. For the negative control, add 8 µL of NTC instead of DNA template into the negative control well. The final volume should be 20 µL.
4. For the biological samples, add 8 µL of each DNA sample into the sample wells, according to your experimental plate set-up. The final volume in each well should be 20 µL.
5. For the positive control, add 8 µL of HPV HR/ACTB Positive Control instead of DNA template into the positive control wells. The final volume should be 20 µL.
6. Cover and seal the plate with an appropriate optical adhesive film or caps before proceeding with the Real-time PCR and detection steps.
7. Place the reaction plate in the real-time PCR instrument and run the Real-time PCR protocol according to the section below.

9.2. Programming the real-time PCR instrument

The table below displays a standard protocol optimized on a few Real-time PCR platforms. However, these conditions may be adapted and validated to suit different machine-specific protocols.

Real-time PCR settings

CYCLES	TEMPERATURE	TIME	STEP
1	95 °C	3 min	Polymerase activation
45	95 °C	5 s	Denaturation
	60 °C	30 s	Annealing/Extension *

** Depending on the qPCR equipment select the appropriate detection channels. Fluorogenic data should be collected during this step through channels indicated below.*

Fluorescent Dyes & Detection Channels

TARGETS	FLUORESCENT DYE	DETECTION CHANNELS
HPV16	Texas Red®	Texas Red/JUN
HPV18	Cy5™	Cy5
Other HR HPV	FAM™	FAM
ACTB	HEX™	VIC/HEX or JOE

NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD was validated for the following Real Time PCR Systems: Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5, Applied Biosystems® QuantStudio 5 Dx, Bio-Rad® CFX C1000 Touch and Bio-Rad® CFX Opus. If other equipment is used, the kit should be validated by the user by using previously characterized samples (positive and negative).

10. Data Analysis

10.1. Run Validation Criteria

Data analysis is performed by the software of the instrument. Considering performance differences in different real-time PCR instruments, thresholds for the four fluorescence signals (Texas Red, FAM, VIC and Cy5) are determined automatically by the software with manual adjustments in case this is required. Before analysing samples results, we recommend verifying if the real-time PCR test is valid. Thus, for each plate, please confirm if the results for Positive and Negative controls performed as expected, according to the following criteria:

Positive control (HPV POS): the amplification of Texas Red (HPV16), Cy5 (HPV18), FAM (Other HR HPV) and VIC (ACTB) curves are positive. Positive control is expected to amplify at Ct < 32, in the four channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

Negative control (NTC): no amplification is detected. If the negative control has amplification curves (Texas Red, Cy5, FAM and VIC) with a sigmoidal shape, sample contamination may have occurred. Repeat the test following good qPCR practices.

If the controls are according to the expected, the test is **valid**. Please proceed with the interpretation of the results for the tested samples.
If any of the controls do not exhibit the expected performance, the assay was compromised or executed improperly and should be considered **invalid**. **Please, repeat the test**. If the problem persists, contact the manufacturer.

10.2. Test Results Interpretation

NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, uses the following Ct cut-off values for results interpretation:

CT VALUE	RESULTS INTERPRETATION
Amplification Ct ≤36	Detected (+) → POSITIVE
No Amplification Ct>36	Not detected (-) → NEGATIVE

HPV16 is detected if the Texas Red amplification curve displays a sigmoidal shape with a Ct≤36, regardless of what result is obtained for the ACTB (VIC) assay.

HPV18 is detected if the Cy5 amplification curve displays a sigmoidal shape with a Ct≤36, regardless of what result is obtained for the ACTB (VIC) assay.

Other HR HPV is detected if the FAM amplification curve displays a sigmoidal shape with a Ct≤36, regardless of what result is obtained for the ACTB (VIC) assay.

HPV16, HPV18, Other HR HPV are not detected if Texas Red, Cy5 and FAM curves do not amplify or amplify at Ct>36, while the ACTB (VIC) assay displays a positive sigmoidal curve (Ct≤45).

The test is invalid if the HPV16, HPV18, Other HR HPV and ACTB assays are negative. The test should be repeated with nucleic acids re-purified from the sample.

The following table summarises the interpretation of principal results. Evaluate the overall shape of the amplification curves; only sigmoidal amplification curves are indicative of true amplification.

HPV16 (TEXAS RED/JUN)	HPV18 (CY5)	OTHER HR HPV (FAM)	ACTB (VIC/HEX/JOE)	RESULTS INTERPRETATION
+	-	-	+/-*	HPV16 detected → POSITIVE
-	+	-	+/-*	HPV18 detected → POSITIVE
-	-	+	+/-*	Other HR HPV detected → POSITIVE (any or combinations of 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
+	+	-	+/-*	HPV16 and HPV18 detected → POSITIVE
+	-	+	+/-*	HPV16 and Other HR HPV detected → POSITIVE (16 plus any or combinations of 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
-	+	+	+/-*	HPV18 and Other HR HPV detected → POSITIVE (18 plus any or combinations of 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
+	+	+	+/-*	HR HPV detected → POSITIVE (16, 18, and any or combinations of 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
-	-	-	+	HR HPV not detected → NEGATIVE (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
-	-	-	-	Invalid test, repeat extraction

** Internal Control detection on the VIC channel is not required for positive results on the Texas Red, Cy5, or FAM detection channels. A high concentration/load of detectable DNA in the sample can lead to reduced or absent internal control signal.*

***Note:** Interpretation of results must account for the possibility of false negative and false positive results.*

- False negative results may be caused by:
  - Unsuitable collection, handling and/or storage of samples.
  - Sample degradation.
  - Presence of qPCR inhibitors.
  - Mutations in the genome of viruses.
  - Failure to follow procedures in this handbook.
  - Use of unvalidated receiving kits or real-time PCR platforms.
- False positive results may be caused by:
  - Unsuitable handling of samples containing high concentrations of HR HPV DNA. The high susceptibility of the qPCR method for cross contaminations special care should be taken during DNA isolation.
  - Cross-contamination with the positive control due to it unsuitable handling.
  - Unsuitable handling of amplified product (post-amplification plate).

Negative results do not preclude infection and the test result should not be used as the sole basis for treatment or other patient management decisions. In addition, this test cannot rule out diseases caused by other pathogens.

11. Performance Evaluation

This kit performance was validated for the instruments specified in **Section 9.2** (see above). If other equipment is used, the kit should be validated by the user by using previous characterised samples (positive and negative).

11.1. Expected Results

Typical amplification plots observed for clinical HPV negative samples (Figure 1A) or samples from patients infected with HR HPVs (Figure 1B) are presented in Figure 1.

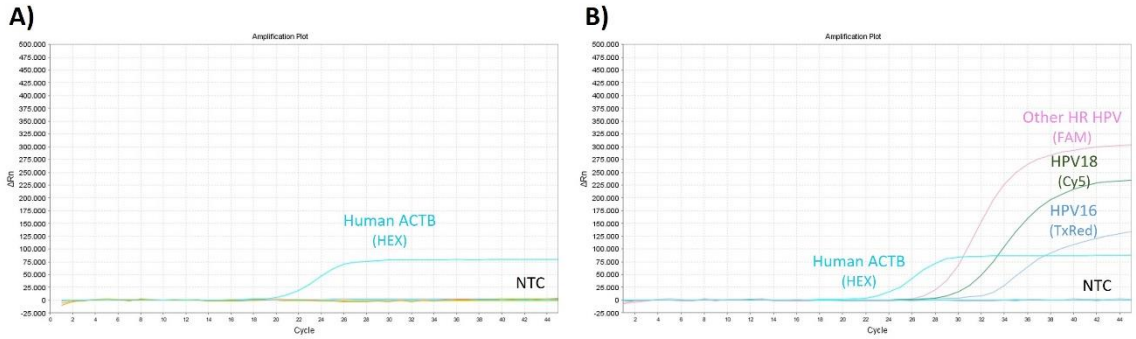


Figure 1. Representative HR HPVs fluorescence curves generated by the High-Risk HPV Multiplex Real-time PCR Kit, IVD, in a clinical HPV negative cervical swab specimen (Figure 1A) and an HPV cervical swab sample from a patient identified as carrier of HR HPVs (Figure 1B). Blue curve: detection of DNA harbouring HPV16 target through the Texas Red channel; Green curve: detection of DNA harbouring HPV18 target through the Cy5 channel; Rose curve: detection of DNA harbouring Other HR HPV targets through the FAM channel; Light blue curve: detection of the human ACTB target through the VIC channel.

11.2. Limit of Detection (LoD) - Analytical Sensitivity

The analytical sensitivity was defined as the lowest concentration of analyte that could be reliably detected with 95% confidence. This was assessed by testing HPV nucleic acids at different copy numbers, particularly, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68, spiked into DNA extracted from negative cervical swab samples, using three different kit batches following typical testing reaction conditions. Tests were repeated over 4 days, and the putative LoD was determined. The LoD confirmation was performed by two different operators, using three kit batches in an experiment with a total of 48 replicates. The data revealed that NZYtech High-Risk HPV Multiplex Real-time PCR Kit, IVD, detects the following LoD value for each HR HPV type, with a confidence ≥95%. All assays were performed using the Applied Biosystems® 7500 FAST Real-time PCR equipment and the analysis was performed using the equipment's software.

HPV TYPE	CONCENTRATION (COPIES/RX)	CONCENTRATION (COPIES/ML)	NUMBER OF POSITIVE TESTED	% POSITIVES	95% CONFIDENCE INTERVAL	
					LOWER	UPPER
HPV16	15	750	47/48	97,9%	89,1%	99,6%
HPV18	15	750	47/48	97,9%	89,1%	99,6%
HPV31	7,5	375	47/48	97,9%	89,1%	99,6%
HPV33	50	2500	48/48	100,0%	92,6%	100,0%
HPV35	10	500	46/48	95,8%	86,0%	98,8%
HPV39	7,5	375	47/48	97,9%	89,1%	99,6%
HPV45	30	1500	46/48	95,8%	86,0%	98,8%
HPV51	25	1250	48/48	100,0%	92,6%	100,0%
HPV52	20	1000	48/48	100,0%	92,6%	100,0%
HPV56	15	750	48/48	100,0%	92,6%	100,0%
HPV58	10	500	47/48	97,9%	89,1%	99,6%
HPV59	40	2000	46/48	95,8%	86,0%	98,8%
HPV66	30	1500	47/48	97,9%	89,1%	99,6%
HPV68	20	1000	46/48	95,8%	86,0%	98,8%

11.3. Analytical Specificity

11.3.1. Cross-Reactivity (Exclusion) and Specificity

Cross-reactivity and inclusivity were evaluated by *in silico* analysis of oligonucleotide probes and primers included in the kit against pathogens related to Human Papillomavirus. Human colonizing pathogens commonly encountered in clinical specimens were also analysed. Assay primers and probes were screened against published genome sequences. Upon *in silico* analysis the assay design was found to detect all fourteen HR HPVs (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68) and exhibited no reactivity with non-related species.

In addition to *in silico* analysis, a panel of bacteria, fungi and virus, including those commonly found in the female urogenital tract, as well as some HPV types classified as low-risk were tested with the High-Risk HPV Multiplex Real-time PCR Kit, IVD. Cross-reactivity of High-Risk HPV Multiplex Real-time PCR Kit, IVD, was assessed using the following pathogens: *Bifidobacterium breve, Proteus mirabilis, Clostridium perfringens, Pseudomonas aeruginosa, Enterobacter cloacae, Staphylococcus aureus, Enterococcus avium, Staphylococcus epidermidis, Enterococcus faecium, Escherichia coli, Fusobacterium nucleatum, Klebsiella oxytoca, Streptococcus pneumoniae, Streptococcus pyogenes, Proteus mirabilis, Neisseria meningitidis, Corynebacterium genitalium* and *Bacteroides fragilis*. Assays were performed using the genomic DNA of the organisms referred to above. Furthermore, wet testing was performed using seven inactive samples that are representative of clinical human specimens, including *Candida albicans* (Z006 strain), *Gardnerella vaginalis* (Z247 strain), *Atopobium vaginae* (Z242 strain), *Trichomonas vaginalis* (Z070 strain), *Candida glabrata* (Z007 strain), *Lactobacillus crispatus* (Z246 strain) and *Candida krusei* (Z009 strain). In addition, low-risk HPV clinical samples (HPV6/11, HPV40, HPV42, HPV43, HPV44, HPV53, HPV54, HPV53/73 and HPV82) were also tested with the High-Risk HPV Multiplex Real-time PCR Kit, IVD. All tests were performed in triplicate using three kit batches. Data confirmed that none of the microorganisms tested generated an amplification signal.

11.3.2. Interfering Substances

The impact of 20 potentially interfering biological and chemical substances that may be present in the sampling area, including human whole blood, antimicrobials, antifungal creams, washing products, or moisturizers, were evaluated for potential interference with the High-Risk HPV Multiplex Real-time PCR Kit, IVD. The assays were performed using negative cervical specimens (stored in ThinPrep PreservCyt® solution) spiked with HPV16 and HPV18 positive specimens at ~2-3x LoD. Potential interfering substances were added to the contrived samples at concentrations representing the highest levels expected in human cervical samples, based on literature data. All extractions/tests were performed in duplicate using one kit batch and compared to data obtained with a control test that contained no interferents. At the concentrations tested, the results revealed that none of the molecules under test affected the sensitivity of the kit. Nevertheless, the BETADINE® vaginal solution might cause interference if used at concentrations higher than 0,04% v/v. The table below resumes the data collected under these experiments.

POTENCIAL INTERFERENT	ACTIVE INGREDIENT	FINAL CONCENTRATION IN THE SAMPLE	INTERFERENCE YES (Y) OR NO (N)		
			HPV16	HPV18	OTHER HR HPVs
Anidulafungin (antifungal)	Anidulafungin	10% v/v	N	N	N
Flucytosine (antifungal)	Flucytosine	10% v/v	N	N	N
Voiconazole (antifungal)	Voriconazole	10% v/v	N	N	N
Amphotericin B (antifungal)	Amphotericin B deoxycholate	10% v/v	N	N	N
Gino-Canesten® (antifungal)	Clotrimazole (10 mg/ml)	10% w/v	N	N	N
Lomexin® (antimicrobial)	Fenticonazole nitrate	10% w/v	N	N	N
Progeffik® (Medicine)	Progesterone (5 mg/mL)	10% v/v	N	N	N
BETADINE® Vaginal (Washing product)	Povidone iodine (100 mg/mL)	0,04% v/v	N*	N*	N*
Washing product	PEG-7 Glyceryl Cocoate, Propylene Glycol	10% v/v	N	N	N
Washing product	Lactic acid	10% v/v	N	N	N
Warm Up Cherry Lubricant	Hydroxyethyl cellulose, Chlorhexidine Gluconate	10% w/v	N	N	N
ClimaCare (Topical product)	Hyaluronic acid, Lactic acid	10% w/v	N	N	N
WOMAN ISDIN (Topical product)	Glycerin, Glyceryl polyacrylate, Polyacrylic acid	10% w/v	N	N	N
Microlax® (Laxatives)	Sodium citrate dihydrate, Sodium lauryl sulfoacetate	5% v/v	N	N	N
Scheriproct® (Anti-Hemorrhoid Ointments)	Prednisolone, Cinchocaine	10% w/v	N	N	N
Urine (human)	-	10% v/v	N	N	N
Whole blood (human)	-	10%; 25% v/v	N	N	N
Plasma (human)	-	10% v/v	N	N	N
Mucus (porcine stomach, type II)	-	0,3% w/v	N	N	N
Zovirax® cream	Acyclovir	7% w/v	N	N	N
Control without any interfering substance	H_2O	5% v/v	N	N	N

** BETADINE® Vaginal solution interfered with the assay when present at concentrations > 0,04% v/v.*

11.4. Precision

Assay precision for the NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD was determined by the repeated testing of positive samples of HPV16, HPV18 and HPV45 representing two viral load levels, 3x LoD and 30x LoD copies per reaction, spiked into DNA extracted from negative cervical samples, using 3 different kit batches, and following typical testing reaction conditions. Precision was evaluated by measuring the Cq average, Cq coefficient of variation and % of replicate detection, as described below for each case. The data is resumed in the tables displayed below.

Precision of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, while detecting HPV16 target gene.

VARIABLE		HPV16 (COPIES/REACTION)	
		3x LOD	30x LOD
REPEATABILITY	n	12	12
	Mean Cq	33,86	30,37
	Coefficient of Variation (%)	0,61	0,79
	% Replicate Detection	100	100
DAILY REPRODUCIBILITY	n	48	48
	Mean Cq	33,65	30,34
	Coefficient of Variation (%)	1,25	0,87
	% Replicate Detection	100	100
LOT-TO-LOT REPRODUCIBILITY	n	36	36
	Mean Cq	33,70	30,37
	Coefficient of Variation (%)	1,06	0,63
	% Replicate Detection	100	100
OPERATOR REPRODUCIBILITY	n	36	36
	Mean Cq	33,79	30,38
	Coefficient of Variation (%)	0,68	0,71
	% Replicate Detection	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	60	60
	Mean Cq	33,74	30,62
	Coefficient of Variation (%)	1,49	1,28
	% Replicate Detection	100	100

Precision of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, while detecting HPV18 target.

VARIABLE		HPV18 (COPIES/REACTION)	
		3x LOD	30x LOD
REPEATABILITY	n	12	12
	Mean Cq	34,31	30,75
	Coefficient of Variation (%)	0,59	0,70
	% Replicate Detection	100	100
DAILY REPRODUCIBILITY	n	48	48
	Mean Cq	34,20	30,81
	Coefficient of Variation (%)	1,13	1,16
	% Replicate Detection	100	100
LOT-TO-LOT REPRODUCIBILITY	n	36	36
	Mean Cq	34,13	30,71
	Coefficient of Variation (%)	0,98	0,67
	% Replicate Detection	100	100
OPERATOR REPRODUCIBILITY	n	36	36
	Mean Cq	34,28	30,78
	Coefficient of Variation (%)	0,82	0,85
	% Replicate Detection	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	60	60
	Mean Cq	33,99	30,81
	Coefficient of Variation (%)	1,56	1,35
	% Replicate Detection	100	100

Precision of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, while detecting HPV45 target.

VARIABLE		HPV45 (COPIES/REACTION)	
		3x LOD	30x LOD
REPEATABILITY	n	12	12
	Mean Cq	34,04	30,35
	Coefficient of Variation (%)	1,28	0,56
	% Replicate Detection	100	100
DAILY REPRODUCIBILITY	n	48	48
	Mean Cq	34,01	30,34
	Coefficient of Variation (%)	1,52	0,73
	% Replicate Detection	100	100
LOT-TO-LOT REPRODUCIBILITY	n	36	36
	Mean Cq	34,03	30,33
	Coefficient of Variation (%)	1,59	0,67
	% Replicate Detection	100	100
OPERATOR REPRODUCIBILITY	n	36	36
	Mean Cq	34,08	30,38
	Coefficient of Variation (%)	1,46	1,03
	% Replicate Detection	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	60	60
	Mean Cq	33,69	30,16
	Coefficient of Variation (%)	2,02	1,17
	% Replicate Detection	100	100

11.4.1. Repeatability

Repeatability was assessed by one operator by analysing 12 replicates of each sample (3x LoD and 30x LoD copies per reaction), accounting for a final number of 24 tests performed per target.

11.4.2. Daily Reproducibility

Daily reproducibility was assessed by one operator by analysing 48 replicates of each sample (3x LoD and 30x LoD copies per reaction), for 4 days, with 12 replicates of each concentration per day (a total of 96 assays per target were performed).

11.4.3. Lot-to-lot Reproducibility

Reproducibility between lots was assessed by one operator through the analysis of 36 replicates of each sample (3x LoD and 30x LoD copies per reaction) using 3 different kit batches with 12 replicates per batch.

11.4.4. Operator Reproducibility

Operator reproducibility was assessed by testing 36 replicates of each sample (3x LoD and 30x LoD copies per reaction), by three different operators with 12 replicates per operator and per viral load, making a total of 24 replicates per operator, including the 3 kit targets.

11.4.5. Inter-instrument Reproducibility

Inter-instrument reproducibility was measured by one operator through the testing 60 replicates of each sample (3x LoD and 30x LoD copies per reaction), in five different qPCR instruments, in a total of 12 tests per equipment.

REAL-TIME PCR EQUIPMENT' MANUFACTURER	REAL-TIME PCR PLATFORM MODEL
Applied Biosystems®	7500 FAST
	QuantStudio™ 5
	QuantStudio™ 5 Dx
Bio-Rad®	CFX C1000 Touch Real-time PCR
	CFX Opus Real-time PCR

11.5. Clinical Evaluation

The clinical performance characteristics of the High-Risk HPV Multiplex Real-time PCR Kit, IVD, were assessed in four distinct groups of cervical samples characterized using isothermal PCR and real-time qPCR techniques. A total of 359 clinical swab samples stored in PreservCyt® solution (Hologic) or Amies medium collected from women with different ages and with a valid result for cytology were included in this clinical study.

The clinical performance of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, was evaluated using 139 cervical samples characterized using a commercial isothermal PCR kit. Nucleic acids were extracted by an external laboratory, and then amplified using the High-Risk HPV Multiplex Real-time PCR Kit, IVD. Data revealed 100% of clinical sensitivity (PPA) for detection of HPV16 and HPV18, and 94% for Other HR HPV. The clinical specificity (NPA) for detecting HPV16 was 95%, for HPV18 was 99% and for Other HR HPV was 100%.

Furthermore, a comparison of the clinical performance of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, and real-time PCR comparator tests was also performed using cervical samples. A total of 220 clinical samples were characterized using three commercial real-time PCR kits. Data revealed that 100% of clinical sensitivity (PPA) for the detection of HPV16 and HPV18, and 83% for Other HR HPV. The clinical specificity (NPA) for detecting HPV16 and HPV18 was 100%, while for Other HR HPV it was 98%.

Data resuming the clinical performance of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD, when compared with isothermal and Real-time PCR comparator kits is presented in the table below.

COMPARATOR ASSAY	TARGET	TP	TN	FP	FN	SENSITIVITY (95% CI)	SPECIFICITY (95% CI)
Isothermal PCR	**HPV16**	10	123	6	0	100% (72-100%)	95% (90-98%)
	HPV18	5	133	1	0	100% (57-100%)	99% (96-100%)
	Other HR HPV	63	72	0	4	94% (86-98%)	100% (95-100%)
Real-Time PCR	**HPV16**	36	183	1	0	100% (90-100%)	100% (97-100%)
	HPV18	22	197	1	0	100% (85-100%)	100% (97-100%)
	Other HR HPV	59	146	3	12	83% (73-90%)	98% (94-99%)

Notes: *TP=True Positive; TN=True Negative; FP=False Positive; FN=False Negative; CI=Confidence Interval.*

12. Quality Control

All components of NZYtech's High-Risk HPV Multiplex Real-time PCR Kit, IVD are tested following the protocols described above. The multiplex real-time PCR system allows the detection of targets described for the identification of HPV16, HPV18, twelve HR HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), as well as human β-actin gene (ACTB). Positive amplifications are observed for target genes, positive control, and internal controls through Texas Red, Cy5, FAM and VIC channels.

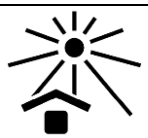
13. Technical Support

For Technical support, please contact our dedicated technical support team by Phone: +351 (0) 21 364 35 14 or Email: info@nzytech.com.

14. Trademarks and Disclaimers

All trademarks that appear in this manual are the property of their respective owners.

15. Explanation of Symbols

IVD	*In vitro* diagnostic medical device		Consult instructions for use
REF	Catalogue number		Manufacturer
LOT	Batch code		Use by
	Temperature limitation		Sufficient for
CONTROL +	Positive control		Keep away from the sun light (primer/probe mix)
CONTROL –	Negative control		

16. Conformity Declaration

Product Name: High-Risk HPV Multiplex Real-time PCR Kit, IVD

Catalogue Number: MD04921

Intended use: High-risk HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) qualitative detection.

Classification: Others (not covered by Annex II or not intended to self-testing) according to the EC Directive 98/79/EC.

Manufacturer: NZYtech - Genes & Enzymes,

Estrada do Paço do Lumiar, Campus do Lumiar

Edifício E, R/C,

1649-038, Lisboa

Portugal

We, NZYtech, Lda – Genes & Enzymes, hereby declare that this product, to which this declaration of conformity relates, conforms with the following standards and other normative documents ISO 9001:2015 and ISO 13485:2016, following the provisions of the 98/79/EC Directive and of the Regulation (EU) 2017/746 on in vitro diagnostic medical devices as transposed into the national laws of the Member States of the European Union.

The product technical file is maintained at NZYtech, Estrada do Paço do Lumiar, Campus do Lumiar - Edifício E, R/C, 1649-038 Lisboa, Portugal.

Joana Brás, PhD

Diretora Técnica

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