APPENDICES

Appendix (1):

1. Instruments and solutions:

1.1 Instrument:

-Rotary microtome.

-Oven.

-Coplinjars.

-Staining racks.

-Microtome blade.

-Coated slides.

-Cover glass.

-Water bath.

-Dako pen.

-Thermal cycler.

-Workstation.

-Pipettors.

-Tube rack.

1.2 Solutions:

-Xylene.

-Ethyle alcohol.

-Mayers haematoxylene.

-Distilled water.

-Citrate buffer.

-Peroxidase blocker.

-Anti CDX2 antibodies (primary antibodies).

-Anti human papilloma virus antibodies (primary antibodies).

-Dextran polymer conjugated secondary antibodies and HRP.

-3.3 diaminobenzidinetetrahydrochloridin substrate buffer.

-DPX mounting media.

-PCR mix 16-35.

-Buffer.

-Taq polymerase.

-Mineral oil.

-DNA buffer.

-HPV genotype control type 16.

-DNA extraction kit.

-Detection agarosekit.

Appendix (2):

بسم الله الرحمن الرحيم

Ministry of Health
Khartoum State
Directorate of
Research

التاريخ: 2014 /4/21 م

النمرة وخ/وص/اع /اب

السيد : .....

المحترم

السلام عليكم ورحمة الله وبركاته

الموضوع : الموافقة على تنفيذ بحث

إشارة الى الموضوع أعلاه فقد تمت الموافقة على تنفيذ بحث بعنوان :

The expression of CDX2 and association of HPV in esophageal cancer in Ibn Seina hospital, 2014

و الذي أجيز من إدارة البحوث و يقوم بتنفيذه الباحثة:

محمد محي الدين

الرجاء مساعدة الباحث و تسهيل مهمة جمع البيانات ، لعموم الفائدة.

وجزاكم الله خيرا

د. عبد الرحمن العشا

مدير إدارة البحوث

Appendix (3):

quartett

Cat. No.	2030415870
Lot No.	333313
Quantity	6 ml

Antibody to
CDX2

Host:	**Rabbit**
Immunogen:	Synthetic peptide of human CDX2 protein
Subclass:	IgG
Presentation:	Diluted antibody, in buffer with < 0.1 % sodium azide.
Assay system:	IHC(p,f)
Titre:	Used in an avidin/biotin complex AEC/DAB system vial is sufficient for 60 slides using 100 µl per slide.
Fixatives:	1) NOTOXhisto 2)Formalin
Treatments:	Staining of formalin/paraffin tissues is enhanced by boiling the tissue sections in **ProTaqs Antigen Enhancer I, Cat# 401602092** for 20 min followed by cooling at RT for 20 min.
Specificity:	CDX2 is a caudal-related homeobox transcription factort that is expressed in the nuclei of intestinal epithelium and has a function in proliferation and differentiation of intestinal cells. This antibody detects primary and metastatic colorectal carcinomas, intestinal metaplasia of the stomach and intestinal type gastric cancer.
Reactivity:	Human, others not tested.
Storage:	Store at 2-8 °C. Do not use after expiration date indicated on the vial.

Errors excepted. This data sheet is a general information. The product attribute can diversify with changing Lot No as well as variations in tissue selection, tissue processing, antigen retrieval and detection systems. We do not take responsibility for any possible damage including personal injury, time or effort on economic loss caused by this product. Our warranty is limited to the price paid for the product. The product may only be used by authorized and skilled personnel. Non-application as prescribed in this data sheet leads to loss of all liability.

FOR IN VITRO USE, NOT FOR DIAGNOSTIC USE

Manufacturer:
BIOCYC GmbH & Co. KG, Im Biotechnologiepark TGZ I, 14943 Luckenwalde; certified by
ISO 13485:2003 and AC: 2007, ISO 9001:2008; Registry number: 60018916, 60018917

Distributed by:
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Cat.No.	2160100670
Lot No.	280528
Quantity	7 ml

Antibody to
Papilloma Virus 16 and 18, E6 Early Protein

Host:	**Mouse**
Subclass:	IgG
Immunogen:	Human papilloma virus 18-E6.
Presentation:	Diluted antibody in PBS, pH 7.4, containing < 0.1 % sodium azide.
Assay System:	IHC(p,f)
Titre:	Used in an avidin/biotin complex AEC/DAB system, vial is sufficient for 70 slides using 100 µl per slide.
Fixation:	1)NOTOXhisto, 2)Formalin
Treatments:	None.
Specificity:	Human papilloma virus early proteins, HPV 18 and 16 E6 protein.
Reactivity:	Human, others not tested.
Storage:	Store at 2-8 °C.
Reference:	J. Gen. Virol. (1987) 68, 1351.

Errors excepted. This data sheet is a general information. The product attribute can diversify with changing Lot No as well as variations in tissue selection, tissue processing, antigen retrieval and detection systems. We do not take responsibility for any possible damage including personal injury, time or effort on economic loss caused by this product. Our warranty is limited to the price paid for the product. The product may only be used by authorized and skilled personnel. Non-application as prescribed in this data sheet leads to loss of all liability.

FOR IN VITRO USE, NOT FOR DIAGNOSTIC USE

Manufacturer:
BIOCYC GmbH & Co. KG, Im Biotechnologiepark 1G/2.1, 14943 Luckenwalde, certified by
ISO 13485:2012 and AC: 2012 , ISO 9001:2008; Registry number: 60018916, 60018917

Distributed by
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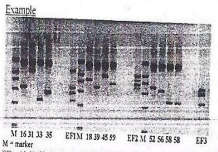
Table 2. Results for controls

Control	Which step of test is controlled	Band 325 bp	Band 425 bp	Band 240 bp	Band 723 bp
Cneg	DNA isolation	No	No	No	No
DNA-buffer	Amplification	No	No	No	No
Internal Control	Amplification	No	No	No	Yes
HPV 16 DNA (+) C	Amplification	Yes	No	No	No
HPV 18 DNA (+) C	Amplification	No	Yes	No	No
HPV 58 DNA (+) C	Amplification	No	No	Yes	No

Example



M = marker
EP1 = 16, 31, 33, 35 Controls Amplification Mix
EP2 = 18, 39, 45, 59 Controls Amplification Mix
EP3 = 52, 56, 58 Controls Amplification Mix

PERFORMANCE CHARACTERISTICS

Analytical specificity: The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific HPV primers and probes.

Analytical sensitivity: The kit HPV High Risk Typing allows to detect HPV DNA in 100% of the tests with a sensitivity of not less than 1000 copies/ml.

Target region: E6, E2, E1, L1 genes

TROUBLESHOOTING

1. Controls analysis do not correspond to the listed above table 2:
   a. The PCR was inhibited.
   b. The reagents storage conditions didn't comply with the instructions.
      - Check the storage conditions
   c. The PCR conditions didn't comply with the instructions.
      - Check the PCR conditions.
2. Positive specific band with Negative Control of extraction.
   a. Contamination during DNA extraction procedure. All samples results are invalid.
      - Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
      - Use only filter tips during the extraction procedure. Change tips between tubes.
      - Repeat the DNA extraction with the new set of reagents.
3. Positive specific band with Negative Control of PCR (DNA-buffer).
   a. Contamination during PCR preparation procedure. All samples results are invalid.
      - Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
      - Pipette the Positive control at last.
      - Repeat the PCR preparation with the new set of reagents.
4. Absence of the Internal Control band in a clinical sample:
   a. Insufficient quantity of clinical material.
   b. The PCR was inhibited.
      - Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
      - Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
   c. The reagents storage conditions didn't comply with the instructions.
      - Check the storage conditions
   d. The PCR conditions didn't comply with the instructions.
      - Check the PCR conditions.

Sacace Biotechnologies Srl
44 Scalabrini str. 22100 Como, Italy

Sacace
BIOTECHNOLOGIES

REF V-25-50F
VER 11.05.18

IVD For *in Vitro* Diagnostic Use

CE

HPV High Risk Typing

Key to symbols used

REF	List Number		Store at +2-8/-20°C
IVD	For *in Vitro* Diagnostic Use		Caution!
LOT	Lot Number	VER	Version
	Expiration Date		Consult instructions for use
Σ	Contains reagents		Manufacturer

NAME

HPV High Risk Typing

INTRODUCTION

Genital infection with HPV is one of the most common sexually transmitted diseases (STDs) of viral etiology worldwide (20% - 46% in different countries in sexually active young women).

Cervical cancer is the second most common cancer in women worldwide, and a compelling body of clinical, epidemiological, molecular, and experimental evidence has established the etiological relationship between some sexually transmitted HPV genotypes and cervical neoplasia throughout the world. Based on the frequency of detection of HPV genotypes from different grades of Cervical Intraepithelial Neoplasia (CIN Grades I – III), HPV genotypes are subdivided into High-risk HPV types (16, 18 31 and 45), Intermediate-risk types (33, 35, 39, 51, 52, 56, 58, 59, and 68), and Low-risk types (6, 11, 42-44).

Several methods have been used to diagnose clinical or subclinical infection with HPVs including clinical observation, cytological screening by Pap smear, electron microscopy, immunocytochemistry, but these methods have some disadvantages such as non-standardization and subjectivity, insufficient sensitivity and low predictable value. The most perspective way of HPV diagnosis is a direct detection of DNA of the human papilloma virus of high carcinogenic risk by the polymerase chain reaction. While the value of the Pap smear in routine screening for cervical displasia is undisputed, it is now known that 99% of cases of cervical carcinoma are caused by infection with twelve genotypes of the human papilloma virus (HPV). Identification of these high-risk genotypes is very valuable in the management of cervical carcinoma, both as a prognostic indicator and as a secondary screening test where results of a Pap smear are inconclusive. Results from the combination of the Pap smear and the HPV DNA test can aid in determining the intervals for screening.

The PCR-based methods have been used successfully for the detection and typing of genital HPV genotypes in clinical specimens such as cervical swabs or scrapes, cervicovaginal lavages, frozen biopsies and formalin-fixed paraffin-embedded tissues.

INTENDED USE

HPV High Risk Typing is an *in vitro* nucleic acid amplification test for qualitative detection and genotyping of *Human Papillomavirus* (16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66) in the urogenital swabs and biopsies.

PRINCIPLE OF ASSAY

HPV High Risk Typing Test is based on three major processes: sample preparation, multiplex amplification of DNA using specific *HPV* primers and detection of the amplified products on agarose gel. Each PCR-mix-1 tube contains primers directed against regions of four *HPV* types and β-globine gene used as Internal Control. If the swab is not correctly prepared (high quantity of mucous or insufficient quantity of epithelial cells) the Internal Control will not be detected.

MATERIALS PROVIDED

- **PCR-mix-1 "16-35"** (primers directed against regions of *HPV 16,31,33,35* and Internal Control (β-globine gene), 0,275 mL
- **PCR-mix-1 "18-59"** (primers directed against regions of *HPV 18,39,45,59* and Internal Control (β-globine gene), 0,275 mL
- **PCR-mix-1 "52-66"** (primers directed against regions of *HPV 52,56,58,66* and Internal Control (β-globine gene), 0,275 mL
- **2,5 x buffer-** 3 x 0,6 mL
- **TaqF Polymerase,** 0,09 mL
- **Mineral Oil,** 8,0 ml
- **Negative Control C-*** 1,2 ml;
- **DNA-buffer (C-),** 0,5 mL
- **Internal Control** (β-globine gene), 0,2 mL
- **HPV Genotype Controls Panel** (types 16, 31, 33, 35, 18, 45, 39, 59, 52, 56, 58, 66), 12 x 0,15 ml

Contains reagents for 55 samples.

**must used in the isolation procedure as Negative Control of Extraction.*

MATERIALS REQUIRED BUT NOT PROVIDED

- Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl, 5-40 µl) with aerosol barrier
- Tube racks

Reagents non provided

- DNA extraction kit (recommended nucleic acid extraction kit: DNA-Sorb-A (Sacace, REF K-1-1/A)
- Detection agarose kit

WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
9. Material Safety Data Sheets (MSDS) are available on request.
10. This kit is designed for use with "DNA-Sorb" extraction kit. It is the user's responsibility if kits other than "DNA-Sorb" are used to perform this DNA extraction.
11. Use of this product should be limited to personnel trained in the techniques of amplification.
12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

STORAGE INSTRUCTIONS

HPV High Risk Typing must be stored at 2-8°C. Store **TaqF Polymerase** at -20°C. The kit can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

HPV High Risk Typing is stable up to the expiration date indicated on the kit label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

HPV High Risk Typing can analyze DNA extracted with **DNA-Sorb-A** (REF K-1-1/A) from:

- *Cervical swabs:*
  - Remove excess mucus from the cervical os and surrounding ectocervix using a cotton or polyester swab. Discard this swab.
  - Insert the Sampling Cervical Brush 1.0-1.5 centimeters into the cervical os until the largest bristles touch the ectocervix. Do not insert brush completely into the cervical canal. Rotate brush 3 full turns in a counterclockwise direction, remove from the canal.
  - Insert brush into the nuclease-free 2,0 ml tube with 0,3 mL of Transport medium (Sacace). Vigorously agitate brush in medium for 15-20 sec.
  - Snap off shaft at scored line, leaving brush end inside tube.
- *Tissue* homogenized with mechanical homogenizer and dissolved in PBS sterile (recommended DNA-Sorb-C REF K-1-6/50 not included in this kit, but can be ordered separately)
- *Liquid-based cytology samples* (Cytoscreen, PreservCyt) (recommended DNA-Sorb-D REF K-1-8/100 not included in this kit, but can be ordered separately)

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

AMPLIFICATION

1. Prepare required quantity of tubes for samples and controls (blue for PCR-mix-1 "16-35", rose for PCR-mix-1 "18-59" and green for PCR-mix-1 "52-66").
2. Prepare for each PCR-mix-1 one new tube and add for each sample **5*(N+1) µl** of **PCR-mix-1, 10*(N+1)** of **2,5 x buffer** and **0,5*(N+1)** of **TaqF Polymerase.**
3. Add **15 µl** of **Reaction Mix** into each sample tube. Add 1 drop (25 µL = of Mineral Oil).
4. Add to appropriate tube **10 µL** of DNA sample obtained after sample preparation.
5. Prepare Controls as follows:
   - Negative Control: add **10 µL** of **DNA-buffer** to the tube labeled $_{Clear\ ampl.}$
   - Internal Control: add **10 µL** of **Internal Control** to the tube labeled $_{Clear}$
   - Positive controls:
     - of PCR-mix-1 "16-35": add **10 µL** of **HPV 16, 31, 33 and 35 DNA (C+)** to the 4 labeled blue tubes;
     - of PCR-mix-1 "18-59" add **10 µL** of **HPV 18, 39, 45 and 59 DNA (C+)** to the 4 labeled rose tubes;
     - of PCR-mix-1 "52-66" add **10 µL** of **HPV 52, 56, 58 and 66 DNA** (C+) to the 4 appropriate green tubes;
6. Close tubes and transfer them into the thermalcycler only when temperature reaches 95°C and start the following program:

Thermocyclers with block temperature adjustment: **"PTC-100"(MJ Research) BioRad, Biometra**				Thermocyclers with active temperature adjustment. **"PE 2400" (Perkin Elmer), Omn-E (Hibaid)** and other			
Step	t°C	Time	Cycles	t°C	Time	Cycles	
1	95°C	Pause		95°C	Pause		
2	95°C	15 min	1	95°C	15 min	1	
3	95°C	30 sec	42	95°C	30 sec	42	
	63°C	40 sec		63°C	30 sec		
	72°C	50 sec		72°C	40 sec		
4	72°C	1 min	1	72°C	1 min	1	
5	10°C	Storage		10°C	Storage		

RESULTS ANALYSIS

Analysis of PCR results is based on the presence or absence of specific bands of amplified DNA in Agarose gel (3%). Mix in the new tube the contents of the 4 tubes with amplified DNA of the 4 Controls (**HPV 16, 31, 33 and 35**). Repeat the same procedure for the controls of PCR-mix-1 "18-59" and PCR-mix-1 "52-66". Add 10-15 µL of amplified products on the agarose gel. The length of specific amplified DNA fragments is:

PCR-mix-1 «16 – 35»			**PCR-mix-1 «18 – 59»**			**PCR-mix-1 «52 – 66»**		
Type	Lenght	IC	Type	Lenght	IC	Type	Lenght	IC
HPV 16	325 bp	723bp	HPV 18	425 bp	723bp	HPV 52	360 bp	723bp
HPV 31	520 bp		HPV 39	340 bp		HPV 56	325 bp	
HPV 33	227 bp		HPV 45	475 bp		HPV 58	240 bp	
HPV 35	280 bp		HPV 59	395 bp		HPV 66	304 bp	