**ABSTRACT**

While testing for high-risk Human Papilloma Virus (HR-HPV) DNA detection is recognized as an essential screening tool for detecting significant precursor lesions (CIN-2+), it is not a very specific test, given the ubiquitous nature of HPV infections and the fact that most HPV infections are either transient or regres. Even established high-grade CIN (CIN-3+) lesions have a low risk of progressing to cancer. The low HPV (15-25%) of HR-HPV can lead to many unnecessary biopsies. Enzo Clinical Labs E-TECT assay (FLOWSCRIPT™ HPV E6/E7 Assay Kit, ENZ-GEN300C-0100) from Enzo Life Sciences is a flow cytometry-based test for the detection of HR-HPV which indicates the expression of the oncogenic E6 and E7 proteins produced during integrated infection by high-risk HPV viruses. The assay employs a novel in situ hybridization technique utilizing a cocktail of oligonucleotide probes specific to multiple targets within the E6 and E7 genes to ensure the detection of these genes from HPV species. This homogeneous assay can be performed in less than 4 hours and is specific for the most prevalent high-risk HPV genotypes. Validation studies performed with E-TECT™ analysis found a strong negative predictive value (NPV) for ASCUS and LSIL cytology results when followed by biopsy. As such, this assay can be used to help guide providers in assessing the risk of progression in cervical or anal HPV-related lesions. Results can aid in the management of those with positive HR-HPV results and/or NILM, ASCUS and LSIL cytology findings. For this group of patients, E6/E7 results may help to guide whether co-cytology or follow-up screening can be the preferred or acceptable courses of action: thereby preventing unnecessary emotional distress when receiving abnormal cervical and HPV test results.

**INTRODUCTION**

The E-TECT™ test is a flow cytometry-based assay for the detection of mRNAs that indicate the expression of the oncogenic E6 and E7 proteins produced after integration of high-risk Human Papilloma Viruses into cervical cells. By utilizing liquid pipet samples and FlowCyt™ and SureFit™, E-TECT™ data offers a less invasive and more specific methods of evaluating cervical cancer risk, compared to histological biopsy by utilizing flow cytometry, a larger population of cells can be interrogated than by traditional slide-based pap smear, effectively increasing its sensitivity as part of the screening procedure for oncological pathlogy. E-TECT™ employs a novel in situ hybridization technique utilizing a cocktail of oligonucleotide molecular beacons specific to multiple targets found within the High-Risk HPV E6 and E7 genes. In the E-TECT test, they enable the detection of these genes from most known variants of HPV. Extensive probe development of High-Risk HPV (HR-HPV) probe cocktail, they are known to detect HPV Genes to ensure the detection of these genes from HPV species. This homogeneous assay can be performed in less than 4 hours and is specific for the most prevalent high-risk HPV genotypes. Validation studies performed with E-TECT analysis found a strong negative predictive value (NPV) for ASCUS and LSIL cytology results when followed by biopsy. As such, this assay can be used to help guide providers in assessing the risk of progression in cervical or anal HPV-related lesions. Results can aid in the management of those with positive HR-HPV results and/or NILM, ASCUS and LSIL cytology findings. For this group of patients, E6/E7 results may help to guide whether co-cytology or follow-up screening can be the preferred or acceptable courses of action: thereby preventing unnecessary emotional distress when receiving abnormal cervical and HPV test results.

**MATERIALS AND METHODS**

**Materials**

- **Cell Lines:**
  - ENZ-GEN302-0004 FLOWSCRIPT™ HPV E6/E7 Negative Control Cells
  - ENZ-GEN300C-0100 FLOWSCRIPT™ HPV E6/E7 Assay Kit

**Methods**

- **Fixation:**
  - Transfer 300µl of each sample into separate 1.5ml polypropylene microcentrifuge tubes, or to separate wells of a polyethylene U-bottom 96 well plate
  - Centrifuge at 1000 x g and discard supernatant
  - To each sample tube, add 300µl of Fixation Buffer (prepared as 1% Formaldehyde) and mix well by pipetting and inverting
  - Incubate the tubes at 15-30°C for one hour
  - Centrifuge at 1000 x g and discard supernatant

- **Hybridization:**
  - Incubate the tubes at 15-30°C for one hour
  - To each sample tube, add 300µl of Hybridization Buffer (prepared as 1% Formaldehyde) and mix well by pipetting and inverting
  - Incubate the tubes at 65°C for 30 minutes
  - Centrifuge at 1000 x g and discard supernatant

- **Analysis:**
  - Flow cytometry was performed with a BD Accuri C6 flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the data was collected using BD Canto2 software. The samples were analyzed with slow flow rate (14 µL/min, and core size: 10 µm). Sample gating was set with respect to cell morphology and the average standard deviation (SD) regarding these results was 0.21% gated cells. This assures assay reproducibility, even at levels below the 2.0% clinical cut-off.

**PERMOSPERMALIZATION:**

- Resuspend cells in 300µl of Permopolitanization Buffer and mix by pipetting and inverting
- Incubate the tubes at 15-30°C for 30 minutes
- Centrifuge at 1000 x g and discard supernatant

**HYBRIDIZATION:**

- Probe Hybridization Mix: For each sample, pipet 0.5µl of HPV probe cocktail into 300µl of Hybridization Buffer (prepare as n+1)
- Resuspend cells in 300µl of prepared Probe Hybridization Buffer mix, and mix by pipetting and inverting
- Incubate the tubes at 65°C for 30 minutes
- Incubate the tubes at 4°C for one hour
- Samples are then ready for flow cytometric analysis

**DETECTION AND ANALYSIS**

**Flow cytometry**

Flow cytometry was performed with a BD Accuri C6 flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the data was collected using BD Canto2 software. The samples were analyzed with slow flow rate (14 µL/min, and core size: 10 µm). Sample gating was set with respect to cell morphology and the measurements were stopped after 1000 events were counted in the respective gate. The gated events were then plotted by FL1 vs. SSC for E6/E7 mRNA analysis, detecting FITC. The cellular events fluorescing at greater than a threshold, correlating the overexpression of E6/E7 with other forms of clinical diagnostic practices, such as histopathology.

Though this assay is currently clinically validated for use on the BD Accuri C6, the FLOWSCRIPT™ HPV E6/E7 mRNA assay should be adaptable for any properly calibrated cytometer with a laser and detector setup capable of exciting and reading emissions from fluorochromes.

**ANALYTICAL SENSITIVITY: LIMIT OF DETECTION**

**Positive Control Cell Dilutions**

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**CONCLUSIONS**

- Detection of viral mRNA is indicative of viral activity. 

- E-TECT™ flow cytometric analysis of HPV E6/E7 mRNA has many advantages:
  - Specific detection of integrated HPV related to cervical disease
  - Small sample volume requirement for analysis
  - High throughput testing – up to 96 samples per run

**REFERENCES**