# FLOWSCRIPT™ Platform Detects Genotypic and Phenotypic Markers Associated with HPV Infection and Cancer Progression

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## ABSTRACT

Enzo's newest flow cytometry platform, FLOWSCRIPT™, acts as "a snapshot cell query" by allowing the analysis of mRNA transcript expression in individual cells in a mixed cell population. By studying whether a gene or a set of genes is turned on or off, it is possible to obtain clinically relevant information at the single cell level. The first product developed for use with this technology is the FLOWSCRIPT™ HPV E6/E7 Assay. Integration and ultimately overexpression of HPV oncoproteins, E6 and E7, promotes the growth of malignant cells through the inhibition of tumor suppressors and has been linked with increased likelihood of cervical cancer progression.

## INTRODUCTION

The FLOWSCRIPT™ HPV E6/E7 Assay is a flow cytometry-based assay for the detection of mRNAs that precede the expression of the oncogenic proteins, E6 and E7, produced during infection by high risk Human Papilloma Virus (HPV) viruses.[1] The FLOWSCRIPT™ HPV E6/E7 Assay is capable of detecting E6/E7 mRNA transcripts from multiple high risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 82) which together account for over 95% of cervical cancer.[2] The assay employs a novel in situ hybridization technique utilizing cocktails of oligonucleotide probes specific to multiple targets within the E6 and E7 genes to ensure the detection of these transcripts from most known variants of high risk HPV. Fixed and permeabilized cells are hybridized with probes and analyzed by flow cytometry for E6/E7 transcript expression. Each probe contains a fluorescent label and a quenching molecule whereby no signal is observed in the absence of target. During hybridization, the probe anneals to the target sequence, thereby emitting a detectable signal.

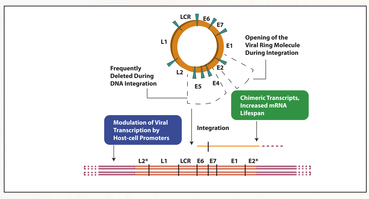



**Diagram 1.** The human HPV genome contains eight open reading frames which include E6, E7, E1, E2, E4, E5, and L2 and L1. "E" or "L" refer to 'early' or 'late' functions, respectively. During the course of cancer development, the viral molecule frequently becomes integrated into host-cell DNA.

## MATERIALS AND METHODS

| CATALOG # | ITEM DESCRIPTION |
|---|---|
| ENZ-GEN300-0100 | FLOWSCRIPT™ HPV E6/E7 Assay Kit |
| ENZ-GEN301-0004 | FLOWSCRIPT™ HPV E6/E7 Positive Control Cells |
| ENZ-GEN302-0004 | FLOWSCRIPT™ HPV E6/E7 Negative Control Cells |
| ENZ-51008-100 | NUCLEAR-ID® Red cell cycle kit (GFP CERTIFIED®) for flow cytometry |

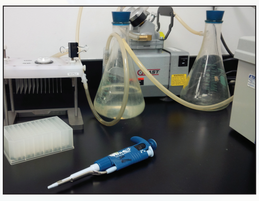
### Other Reagents Used:

- Formaldehyde (37% by weight, methanol stabilized), Molecular Biology Grade
- Flow Cytometer Calibration Beads

### Instrumentation:

Though this assay is currently clinically validated for use on the BD Accuri™ C6, the FLOWSCRIPT™ assay should be compatible with any properly calibrated cytometer with a laser and detector setup capable of exciting and reading emissions from Fluorescein, such as the BD FACSCalibur™, Handyem HPC-150, and Millipore Guava easyCyte™.

**Plate Aspirator:** This method can be validated with the use of a 96-well plate for larger sample batches and automated cytometer platforms. Utilizing an aspiration system such as the V&P Scientific VP 177A-1 aspiration manifold is an efficient way to handle this type of workflow, along with the CSampler function of the BD Accuri C6.



### FIX

- Spin down cells and discard supernatant
- Resuspend with 150µL Fixative solution
- Incubate for 30 minutes at room temperature

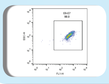
### WASH

- Add 150µL of Buffer B
- Spin samples and discard supernatant
- Wash in 300µL Buffer B
- Repeat wash, discard supernatant

### HYBRIDIZE

- Resuspend sample in 300µL Probe Hybridization Mix
- Incubate at 65°C for 1 hour
- Incubate at 4°C for 1 hour
- DURING INCUBATION, PROTECT FROM LIGHT

### ANALYZE



- Run samples on flow cytometer

## ANALYTICAL SENSITIVITY: LIMIT OF DETECTION

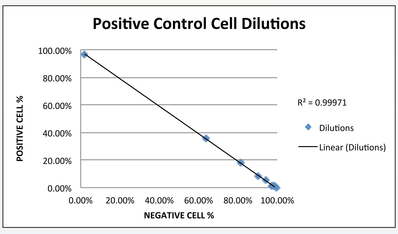



**Figure 1**. The limit of detection (LOD) for the HPV E6/E7 assay was determined by preparing dilutions of the positive and negative control cells (ENZ-GEN301-0004 and ENZ-GEN302-0004). The positive control cell sample was diluted serially (1:2) using negative control cells as the diluent. These dilution values ranged from 100% positive cells, down to 0.39% positive cells.

## ASSAY PRECISION NEAR THE LIMIT OF DETECTION

| Dilution Level | FLOWSCRIPT™ Result | Dilution Level | FLOWSCRIPT™ Result | Dilution Level | FLOWSCRIPT™ Result |
|---|---|---|---|---|---|
| 1.56% | 1.40% | 0.78% | 0.50% | 0.39% | 0.64% |
| 1.56% | 1.70% | 0.78% | 0.80% | 0.39% | 0.30% |
| 1.56% | 1.90% | 0.78% | 1.10% | 0.39% | 0.20% |
| 1.56% | 1.40% | 0.78% | 1.00% | 0.39% | 0.40% |
| 1.56% | 1.60% | 0.78% | 0.80% | 0.39% | 0.50% |
| 1.56% | 1.00% | 0.78% | 1.10% | 0.39% | 0.50% |
| 1.56% | 1.10% | 0.78% | 0.90% | 0.39% | 0.60% |
| 1.56% | 1.50% | 0.78% | 0.80% | 0.39% | 0.70% |
| 1.56% | 1.50% | 0.78% | 1.10% | 0.39% | 0.45% |
| 1.56% | 1.40% | 0.78% | 1.10% | 0.39% | 0.20% |
| Mean = | 1.45% | Mean = | 0.92% | Mean = | 0.45% |
| SD = | 0.26% | SD = | 0.20% | SD = | 0.18% |

**Figure 2:** Assay precision near the LOD was examined by preparing and analyzing multiple replicates at the lowest three levels of the dilution set (1.56%, 0.78%, and 0.39%). 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.

## DETECTION OF E6/E7 IN CELLS AT DIFFERENT PHASES OF THE CELL CYCLE

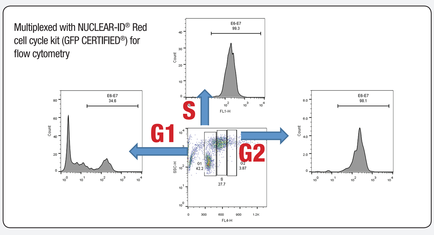



**Figure 3:** Using the FLOWSCRIPT™ HPV E6/E7 mRNA assay, E6/E7 mRNA was labeled using in situ hybridization in human cervical samples.

## CLINICAL APPLICATION

The results obtained from this assay were expressed as the percentage of cells in the analysis gate that over-express mRNA for the two oncoproteins, E6 and E7 combined (Fig 4). FLOWSCRIPT™ HPV Positive and Negative Control Cells were concurrently processed and analyzed during the clinical assay workflow and act as an external control, confirming E6/E7 probe functionality.

To assess the clinical application of the FLOWSCRIPT™ HPV assay, a validation study of over 200 ThinPrep or SurePath clinical specimens was performed by Enzo Clinical Labs. Findings support the claim that E6/E7 testing on cytology samples is a useful discriminating test as there was a strong negative predictive value for ASCUS and LSIL cytology results when correlated to biopsies.

**Representative NILM Sample** | **Representative HSIL Sample**

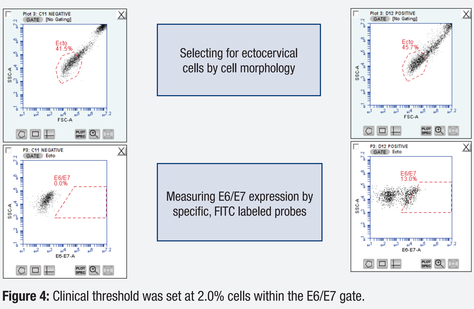



**Figure 4:** Clinical threshold was set at 2.0% cells within the E6/E7 gate.

**Correlation of Cytology and FLOWSCRIPT™ data as a Negative Predictive Indicator**

| CYTOLOGY | NEGATIVE PREDICTIVE VALUE* | SAMPLE SIZE |
|---|---|---|
| ASCUS | 100% | 12 of 12 |
| LSIL | 75% | 3 of 4 |

**Figure 5:** NPV calculated as negative E-TECT results with no evidence of CIN 2 or 3 on biopsies divided by all negative results.

## CONCLUSIONS

- Detection of viral mRNA is indicative of viral activity
- Flow cytometry allows analysis of mixed populations on a cell by cell basis, as opposed to other assays such as qPCR which assess a population as a single result
- FLOWSCRIPT™ 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** of up to 96 samples per run
  - » **Multiplexing capability** to obtain more information (e.g. cell cycle) in a single test
- Detection of E6/E7 mRNA in fixed cell preparation allows for improved diagnosis of the early stages of cervical cancer
- The FLOWSCRIPT™ platform is anticipated to be further utilized for applications related to cancer, immune-mediated disorders, patient monitoring and drug development

## REFERENCES

1. Stanley, Margaret A. Epithelial Cell Responses to Infection with Human Papillomavirus. *Clinical Microbiology Reviews*. American Society for Microbiology. April 2012, vol. 25 no. 2; 215-222. Doi: 20.1128/CMR.0502811
2. Burger EA, Korner H, Klemp M, et al. HPV mRNA tests for the detection of cervical intraepithelial neoplasia: a systematic review. *Gynecologic Oncology*. 2011; 120(3): 430-8.