Product Manual

FLOWSCRIPT® HPV E6/E7 Assay Kit

Catalog #: ENZ-GEN300-0100

for 100 flow cytometry tests

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NON- INFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

TRADEMARKS

ENZO is a registered trademark of Enzo Biochem, Inc.

FLOWSCRIPT is a trademark of Enzo Biochem, Inc.

BD Accuri and BDFACSCalibur are trademarks of Becton, Dickinson and Company.

Guava is a registered trademark of Merck KGaA, Darmstadt, Germany.

easyCyte is a trademark of Merck KGaA, Darmstadt, Germany.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Product Manual

TABLE OF CONTENTS

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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.

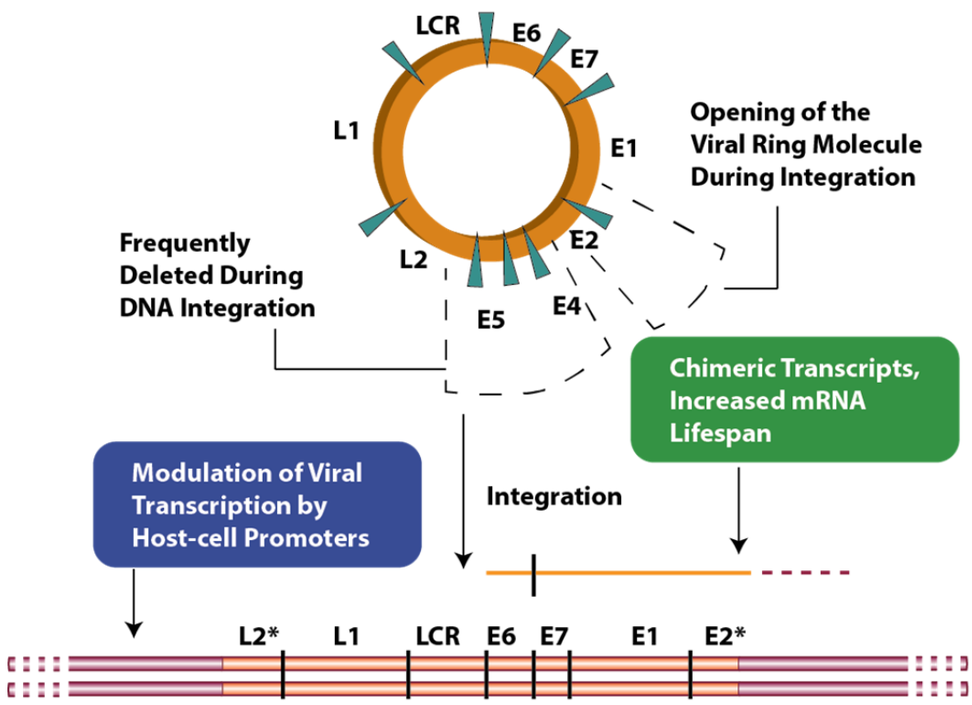


Diagram 1. The human HPV genome contains eight open reading frames which include E6, E7, E1, E2, E4, E5, and L2 and L1. “E” or “L” refer to 'early' or 'late' functions, respectively. During the course of cancer development, the viral molecule frequently becomes integrated into host-cell DNA.

SAFETY WARNINGS & PRECAUTIONS

Handle with care

- Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

MATERIALS SUPPLIED

The reagents provided in the kit are sufficient for 100 assays.

Reagent	Quantity	Part No.
FLOWSCRIPT® HPV E6/E7 Assay Kit	100 tests	ENZ-GEN300-0100
Buffer A (Fixation Buffer)	120 ml	Included in kit, not sold separately
Buffer B (Hybridization Buffer)	2 x 125 ml	Included in kit, not sold separately
HPV E6/E7 Probe Cocktail	50 µl	Included in kit, not sold separately

ACCESSORY COMPONENTS SOLD SEPARATELY

Reagent	Quantity	Part No.
FLOWSCRIPT® HPV E6/E7 Positive Control Cells	4 ml	ENZ-GEN301-0004 Sold separately
FLOWSCRIPT® HPV E6/E7 Negative Control Cells	4 ml	ENZ-GEN302-0004 Sold separately

WORKFLOW

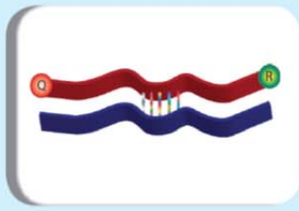
FIX

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

WASH

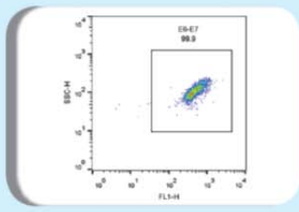
- Add 150µL of Buffer B
- Spin samples and discard supernatant
- Wash in 300µL Buffer B
- Repeat wash, discard supernatant

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

Avoid freeze / thaw cycles

STORAGE

Upon receipt of the kit, immediately store the HPV E6/E7 Probe Cocktails at -20°C. Buffer A and Buffer B can be kept at 2-8°C.

STABILITY

The HPV E6/E7 Probe Cocktail is stable for 6 months at -20°C from date of receipt. Buffer A and Buffer B are stable for 6 months at 2-8°C.

OTHER MATERIALS NEEDED

The following supplies are required but not supplied with the assay kit and the control cells.

EQUIPMENT

- Microcentrifuge
- Micropipettes (P10, P20, P200, P1000)
- Water bath or plate warmer (65±1°C)
- Flow cytometer with a 488 nm laser
- Data acquisition and analysis software
- 4°C refrigerator
- -20°C freezer

CONSUMABLES

- 1.5ml polypropylene microcentrifuge tubes
- 15ml and 50ml polypropylene conical tubes
- Disposable DNase-free pipet tips
- Disposable/sterile serological pipets (5 ml, 10 ml, 50 ml)
- Optional: Disposable cell filters (100 µm nylon mesh)
- Optional: Polystyrene U-bottom 96-well plates

REAGENTS

- Formaldehyde (37% by weight, methanol stabilized), Molecular Biology Grade (Example: Sigma cat# F1635-500ml)
- Optional: Flow Cytometry Calibration Beads

METHODS AND PROCEDURES

A. REAGENT PREPARATION

Important! *Prior to use, remove the required components from storage to allow them to come to room temperature. The following reagents must be prepared within 30 minutes of use.*

1. Fixative Solution (1% formaldehyde in **Buffer A**)

Dilute sufficient amount of ~37% formaldehyde in **Buffer A** to 1% final concentration for **N+1** reactions, where **N** is the number of samples. Refer to **Table 1** for reagent volume calculations.

Select a tube size that is of sufficient volume to contain the required amount of solution.

Table 1. Preparation of Fixative Solution

Reagent	Volume per reaction	Total volume required
37% Formaldehyde	4.05 µl	4.05 µl x (N+1)*
Buffer A (Fixation Buffer)	145.95 µl	145.95 µl x (N+1)*

* ***N*** *is the number of samples per assay run. Excess reagent (one reaction volume) is included to allow for any pipetting variance.*

2. Probe Hybridization Mix

a. After thawing, vortex the **HPV E6/E7 Probe Cocktail** and briefly spin to bring contents to the bottom of the tube.

b. Dilute the required amount of **HPV E6/E7 Probe Cocktail** for **N+1** reactions, where **N** is the number of samples. Refer to **Table 2** for reagent volume calculations.

Select a tube size that is of sufficient volume to contain the required amount of Probe Hybridization Mix.

c. Gently mix by pipetting the contents of the tube up and down a few times. **Do not vortex.**

Table 2. Preparation of Post Hybridization Mix

Reagent	Volume per reaction	Total volume required
HPV E6/E7 Probe Cocktail	0.5 µl	0.5 µl x (N+1)*
Buffer B (Hybridization Buffer)	300 µl	300 µl x (N+1)*

** **N** is the number of samples per assay run. Excess reagent (one reaction volume) is included to allow for any pipetting variance.*

B. CELL FIXATION

1. Transfer 300 µl each of **FLOWSCRIPT® HPV E6/E7 Positive Control Cells** (ENZ-GEN301-0004) and **FLOWSCRIPT® HPV E6/E7 Negative Control Cells** (ENZ-GEN302-0004), in duplicate, and 300 µl of each test sample (in Thin Prep™ or SurePath™) into separate 1.5 ml polypropylene microcentrifuge tubes, or to separate wells of a polystyrene U-bottom 96-well plate.

   NOTE: *The positive and negative control cells are provided at 10^5 cells/ml.*

2. Centrifuge at 1000 x *g* for 6 minutes and discard supernatant, being careful not to disturb cell pellet.
3. To each sample tube, add 150 µl of **Fixative Solution** (from step A-1) and mix well by pipetting up and down.
4. Incubate the tubes at room temperature (15-25°C) for 30 minutes.
5. Add an additional 150 µl of **Buffer B** to each sample and centrifuge the sample tubes at 1000 x *g* for 6 minutes. Discard supernatant, being careful not to disturb the cell pellet.
6. Re-suspend cells in 300 µl of **Buffer B**, by pipetting up and down, and centrifuge the sample tubes at 1000 x *g* for 6 minutes a second time. Discard the supernatant without disturbing the cell pellet.
7. Repeat step 6 for an additional wash step.

   NOTE: *Be sure to remove all supernatant from sample prior to proceeding to the hybridization step. Take care not to disturb the cell pellet.*

C. PROBE HYBRIDIZATION

1. Add 300 µl of **Probe Hybridization Mix** (from step A-2) into each fixed sample and mix carefully by pipetting up and down.

   NOTE: *All steps from this point on should be carried out protecting samples from light.*

2. Incubate the tubes in a 65°C water bath for 1 hour. If using 96-wheel plate, incubate in 65°C plate warmer.
3. After 1 hour incubation at 65°C, incubate the samples at 4°C for 1 hour.
4. The samples are ready for analysis by flow cytometry.

D. CELL ANALYSIS BY FLOW CYTOMETRY (GENERAL RECOMMENDATIONS)

This assay has been validated for use on the following instruments: BD Accuri™, BD FACSCalibur™, and Guava® easyCyte™. The FLOWSCRIPT® HPV E6/E7 Assay should be compatible with any properly calibrated cytometer with a laser and detector setup capable of exciting and reading emissions from fluorescein, results should be read in the appropriate channel.

Use the gating setting determined for the positive and negative control cells. There should be a minimum 5000 events in the analysis gate. Thresholds for the positivity and sensitivity must be determined by the laboratory. The procedures outlined below serve only as guide in establishing a threshold for analysis and may be used at the discretion of the user.

1. Flow Cytometer Gate Setup

a. Instrument setup will require a set of negative control cells with no probe added, and a set of negative and positive control cells each with probe added as outlined in section C (Probe Hybridization), each in duplicate. Acquisition should be run on the slow setting.

b. During the first run, set forward and side scatter to log with sufficient voltage so that control cells appear just in the top right corner. Create a “Control” gate to include these cells (Figure 1).

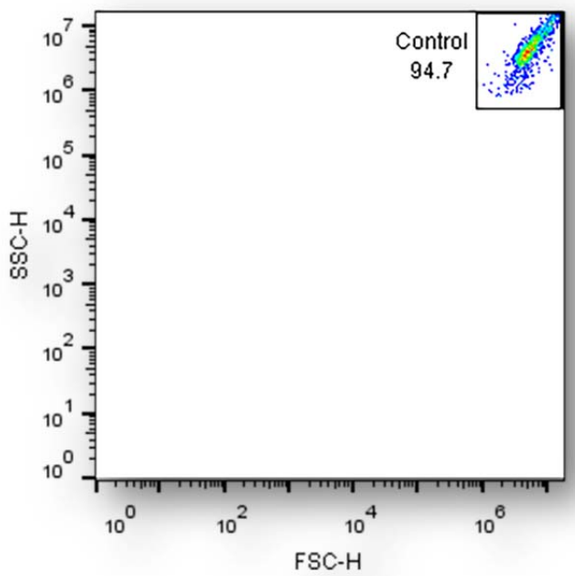


Figure 1. Control cell gate using forward and side scatter.

c. Create a plot dependent on the "Control" gate, with SSC on the Y axis and the FITC channel on the X axis. Using unstained negative control cells, adjust the FITC detector voltages in log view so that each plot shows the cell population at the lowest MFI possible with a small buffer between the population and plot axis (Figure 2).

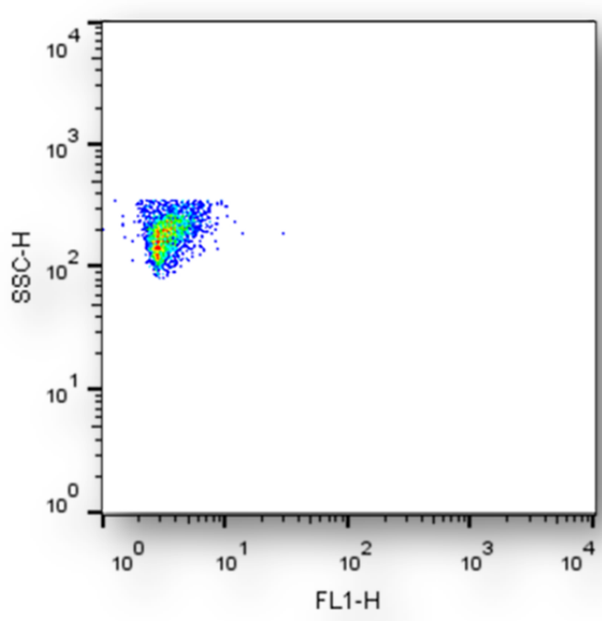


Figure 2. Using the negative control cells, FITC voltage is adjusted so that the cell population shows a low MFI along the X-axis.

d. Next, run negative control cells stained with probes. Cells should still appear with a low MFI in the FITC plot

e. Create a new gate in the FITC plot to the right of the negative stained cell population labeled “E6/E7” (Figure 3).

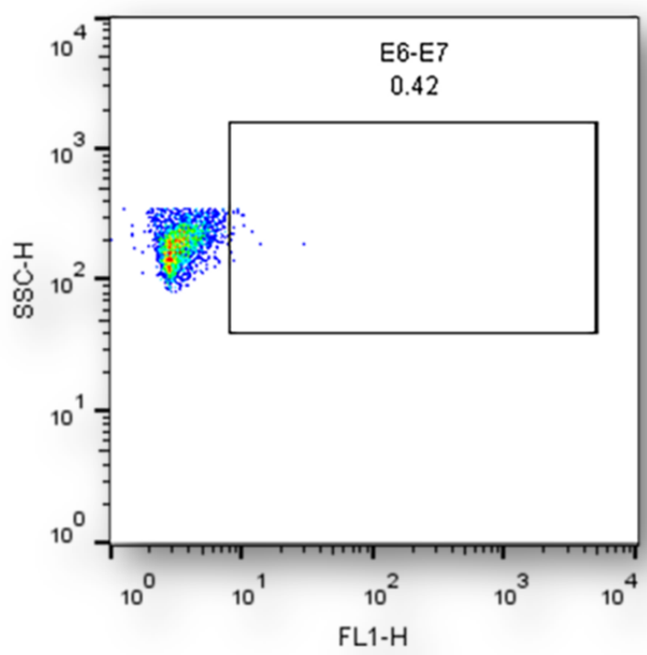


Figure 3. Gate for detection of E6/E7 overexpression in control cell populations.

f. Set cytometer to record 5000 events in the “Control” gate.

Record the duplicate negative control cell samples which have been stained with probes. Less than 5.0% of events should appear in the “E6/E7” gate. If more than this appears in the positive gate, adjust the gate to the right until <5.0% are positive.

g. Record the duplicate positive control cells which have been stained with probes. Greater than 95.0% should appear in the “E6/E7” gate. Negative and positive controls should have a separation of at least two standard deviations of the Negative control cell peak.

2. **Acquisition of Control Cells**

   a. Future runs of the FLOWSCRIPT® HPV E6/E7 assay can be performed using the settings determined in section D-1 (Flow Cytometer Gate Setup). Run control cells before running the samples to ensure settings validity. Please ensure control cells are not past their expiration date before running validation.

   b. Prepare Negative and Positive control cells in duplicate as outlined in the hybridization protocol.

   c. Run Negative control cells to ensure <5.0% appears in the “E6/E7” gate.

   d. Run Positive control cells to ensure >95.0% appear in the “E6/E7” gate.

   e. If either sample runs outside of the designated percentage, rerun the Flow Cytometer Gate Setup.

   NOTE: *This analysis must be done prior to running unknown sample types.*

3. **Acquisition of Unknown Samples**

   a. Mix samples well by pipetting before aliquoting for fixation and hybridization steps.

   b. If samples show significant debris, it is recommended to filter using a 100µm nylon mesh before preparation.

   c. Samples should be prepared in duplicate or triplicate as outlined in the Cell Fixation (section B) and Probe Hybridization (section C) protocols.

   d. A minimum of 5000 events should be collected in the “Control” gate.

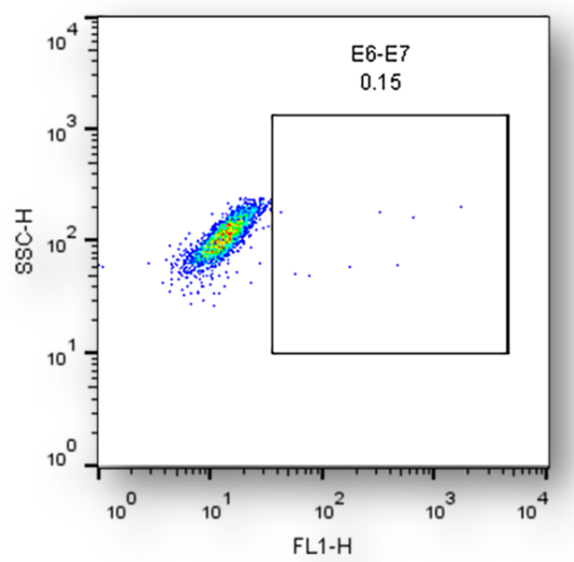
   e. The user should establish a threshold for positivity based on analysis of samples and correlation assessment.

EXPECTED RESULTS

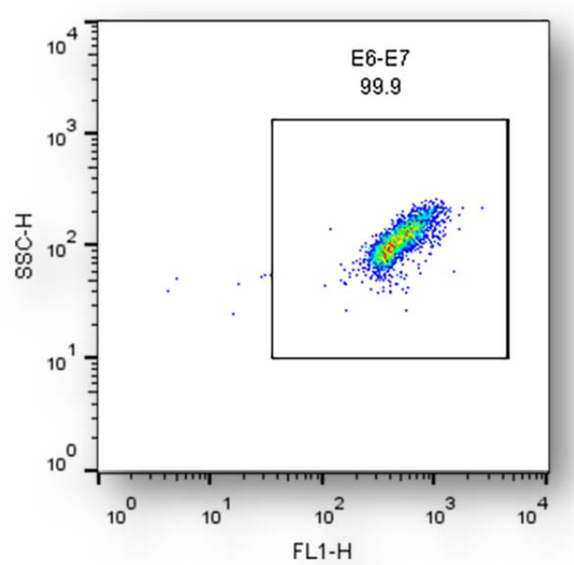
The results are expressed as the percentage of cells in the analysis gate that over-express mRNA for oncoproteins E6 and E7.

1. The positive control cells that over-express oncoprotein E6/E7 mRNA in the analysis gate should be >95.0%
2. The negative control cells that over-express oncoprotein E6/E7 mRNA in the analysis gate should be < 5.0 %.
3. The gated population should have the following appearance:

Negative Population Example



Positive Population Example



FREQUENTLY ASKED QUESTIONS

Q. How does autofluorescence of the cells affect analysis?

A. Some highly autofluorescent cell types may potentially appear as false positives. However, the fixation step in the FLOWSCRIPT® HPV E6/E7 Assay protocol helps to minimize cell autofluorescence ensuring accurate results. Measuring fluorescence in a dot plot against SSC, instead of using histograms, can also help to better visualize samples with high autofluorescence properties. If there is concern that one of the samples may contain autofluorescent cells, it is suggested to run cells which have been treated with the FLOWSCRIPT® HPV E6/E7 Assay protocol but without probes added as a control. If these cells fall within the positive gate, the gating parameters for that cell type would need to be changed.

Q. I am not detecting any fluorescent signal with my probe set. Why is my RNA target not detected?

A. There are a number of reasons for failure to detect signal. First, make sure that the flow cytometer is functional and all PMTs are working by using fluorescent beads. The HPV E6/E7 probe functionality may be validated with FLOWSCRIPT® HPV E6/E7 Positive Control Cells (ENZ-GEN301-0004). Be sure to use control cells and probes which have not passed their expiration date. If the control cells are not showing as positively stained, make sure the temperature of the water bath or plate heater used during hybridization is at least 65°C using a thermometer. If control cells show a positive signal, but samples expected to be positive do not, review the cell or tissue collection procedure and fixation protocols to ensure minimal mRNA degradation in the samples.

Q. How many cells should I use per sample for the FLOWSCRIPT® HPV E6/E7 Assay?

A. It is recommended to collect at least 5000 events in the “Control” gate to ensure statistical significance. Homogeneous populations such as cell lines grown *in vitro* may require as few as 5×10^4 cells per tube to achieve adequate counts, while mixed cell populations such as tissue samples may require significantly higher cell numbers per tube. Always make sure to prepare more cells than needed as some cells may be lost during wash steps.

Q. I have other microfuge tubes available in the lab, including FACS tubes. Can I use the FLOWSCRIPT® HPV E6/E7 Assay with other microfuge or FACS tubes?

A. Other microfuge tubes and FACS tubes should be compatible with FLOWSCRIPT® HPV E6/E7 Assay. Cells may adhere to some materials more than others, affecting cell loss. Please make sure that all tubes or plates used during hybridization are not sensitive to heat up to 100°C.

Q. How critical is it to incubate cells at 65°C during hybridization?

A. The FLOWSCRIPT® HPV E6/E7 assay is designed to be carried out at 65°C to ensure the most efficient hybridization of probes to the mRNAs of interest. Low fluorescence, when running the samples, may be an indication of an inaccurate temperature reading from the water bath or plate heater. Temperatures too far above or below 65°C may prevent efficient hybridization, and adjusting temperature as much as 5°C may help increase the signal.

Q. Is the FLOWSCRIPT® HPV E6/E7 Assay compatible with antibody staining?

A. The FLOWSCRIPT® HPV E6/E7 Assay should be compatible with extracellular and intracellular antibody staining. Select fluorophore-conjugated antibodies to use with this assay which are able to be multiplexed with a FITC-labeled antibody. Cells can be stained as normal with extracellular antibodies before the Cell Fixation step in this protocol, or with **intracellular** antibodies before the Probe Hybridization step.

Q. What cell types are compatible with FLOWSCRIPT® HPV E6/E7 Assay?

A. The FLOWSCRIPT® HPV E6/E7 Assay has been validated for use with cervical cell lines, ecto- and endo- cervical tissue samples, and negative controls such as T cell lines. The assay should be compatible with any cell type easily placed in a single cell suspension.

CITATIONS

1. Schiffman, Mark, et al. "Human papillomavirus testing in the prevention of cervical cancer." Journal of the National Cancer Institute 103.5 (2011): 368-383.
2. Schiffman, Mark, et al. "Human papillomavirus and cervical cancer." The Lancet 370.9590 (2007): 890-907.

NOTES

NOTES

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com