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**Karl Adler · Thomas Erickson · Mark Bobrow**

# High sensitivity detection of HPV-16 in SiHa and CaSki cells utilizing FISH enhanced by TSA

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**Abstract** Detection of integrated human papillomavirus type 16 (HPV-16) DNA in SiHa and CaSki cells was used as a model system to demonstrate sensitivity and resolution of a well defined target. Using 293- to 1987-base polymerase chain reaction (PCR)-synthesized probes to the E6 and E7 open reading frames of HPV-16, several fluorescent in situ hybridization (FISH) detection methods, enhanced with tyramide signal amplification (TSA), were compared. The synthetic probes were biotin labeled by a nick translation method and the hybridized probes were detected by various fluorescent TSA methods using cyanine 3 tyramide, biotinyl tyramide and a biotin TSA Plus reagent. High sensitivity detection in SiHa cells was demonstrated using a 619-base probe to detect two single copies of integrated HPV-16 DNA. In CaSki cells, which contain up to 600 copies of HPV-16 DNA, a 293-base probe was used for detection. The results of these comparisons show that with refinement of TSA methods and reagents, increasing levels of high sensitivity detection can be achieved and that these methods allow subnuclear localization as well.

## Introduction

Improvements in fluorescent in situ hybridization (FISH) detection techniques have allowed localization of single copy DNA using smaller and more defined probes. These methods, although sensitive, are cumbersome in nature, requiring many incubation steps or specialized equipment. Tyramide signal amplification (TSA), first described by Bobrow et al. (1989), has recently been shown to be effective in in situ hybridization (Kerstens et al. 1995; Raap et al. 1995; van Gijlswijk et al. 1997). Both fluorescent and chromogenic methods provide for rapid and facile detection utilizing an expanding number of TSA reagents in a broad number of applications.

K.E. Adler · T.J. Erickson · M.N. Bobrow (✉)
NEN Life Science Products, 549 Albany St.,
Boston, MA 02118, USA
Tel. +1 617–350–9132; Fax +1 617–695–6358

In situ PCR has been credited with single-copy detection for a variety of applications and has been used to detect integrated human papillomavirus type 16 (HPV-16) DNA in SiHa cells (Nouvo et al. 1991; Zehbe et al. 1992; O'Leary et al. 1994). Two newly developed TSA substrates, cyanine 3 tyramide and biotin TSA Plus, were applied to this well defined target analyte to investigate the lower limits of sensitivity. Utilizing biotin-labeled probes of defined length, ranging from 293 to 1987 bases, several detection approaches were attempted for HPV-16 FISH. The TSA detection protocols were compared to detection with biotinylated probe and streptavidin-Cy3.

## Materials and methods

### Synthesis of HPV-16 probes

Cell lysate was prepared from CaSki cells by lysing cells in distilled $H_2O$ at 95°C for 10 min. The lysate was treated with proteinase K (50 μg/ml) for 30 min at 55°C, and then heated to 95°C for 5 min. PCR, using the primers listed in Table 1, was performed using KlenTaq polymerase (Clontech) in 200 μl reactions. The reaction was cycled 30 times (Perkin Elmer thermal cycler) using a profile of 94°C for 2 min, 50°C for 1 min, 72°C for 2 min, first cycle; additional cycles were at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min. Each PCR product was purified in a 2% agarose gel, electroeluted, and ethanol precipitated.

Probes were biotinylated using a nick translation kit (NEN Life Science Products). The resulting probes were then coprecipitated

**Table 1** Primers for probe synthesis

| Primer | Product size (bases) |
|---|---|
| 5′ primer GCA AGC AAC AGT TAC TGC GAC GT<br>3′ primer ATT CCA TAA TAT AAG GGG TC | 293 |
| 5′ primer GCA AGC AAC AGT TAC TGC GAC GT<br>3′ primer GTG TGC CCA TTA ACA GGT CTT C | 619 |
| 5′ primer GCA AGC AAC AGT TAC TGC GAC GT<br>3′ primer ATA CCC GCT GTC TTC GCT TTC AA | 1065 |
| 5′ primer GCA AGC AAC AGT TAC TGC GAC GT<br>3′ primer TTT GCT TCC AAT CAC CTC CAT CA | 1987 |

with tRNA, human cot-1 DNA, and salmon sperm DNA. Each pelleted probe was then dissolved (estimated probe concentration of 5 ng/μl) in hybridization buffer composed of 50% formamide, 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.2), 10% dextran sulfate, 0.05 M sodium phosphate, pH 7.2.

### Cell fixation

SiHa and CaSki cells were grown on slides and fixed in 4% paraformaldehyde in PBS using methods adapted from previous reports (Lawrence et al. 1989) and fixed cells were stored at 4°C in 70% ethanol.

### Hybridization method and TSA detection

Slides were denatured and dehydrated in graded ethanol solutions. Probes were hybridized at 37°C for 2 h in a humid chamber. Stringency wash was twice in 0.2× SSC buffer at 50°C for 15 min each. The slides were blocked with TNB [0.1 M TRIS, 0.15 M NaCl, 0.5% blocking reagent (NEN Life Science Products), pH 7.4] for 30 min at room temperature. Streptavidin-horseradish peroxidase (HRP) (1:500, 300 μl/slide; NEN Life Science Products) in TNB was added to the slides and incubated at room temperature for 30 min and then the slides were washed 3 times in TNT (0.1 M TRIS, 0.15 M NaCl, 0.05% Tween-20, pH 7.4) for 5 min each.

TSA substrates, diluents, and fluorescent conjugates were from NEN Life Science Products, unless otherwise noted. TSA substrates were diluted in amplification diluent, incubated for 20 min at room temperature, and the slides were then washed 3 times with TNT buffer for 5 min each. For indirect detection (deposition of biotin in the TSA reaction), the slides were incubated with streptavidin Texas Red for 30 min in TNB and then washed 3 times with TNT. Cell nuclei were stained with Hoechst 33342 dye and mounted with Vectashield (Vector Laboratories) mounting media.

Detection of hybridized biotinylated probe without TSA amplification was performed by incubating streptavidin-Cy3 (Jackson ImmunoResearch) in TNB buffer for 30 min. The slides were then washed, counterstained, and mounted as described above.

### Fluorescence microscopy

Fluorescence microscopy was performed on a Ziess Axiophot using appropriate filters. Images were recorded using a Xillix CCD camera with exposures from 0.2 to 2 s.

## Results

HPV-16 probes, synthesized using PCR, were separated in a 2% agarose gel (Fig. 1) and found to migrate as expected for their respective sizes: 293, 619, 1065, and 1987 bases.

Detection of integrated HPV-16 in SiHa cells with increasing probe size from 293 to 1987 bases with direct fluorescent cyanine 3 tyramide is shown in Fig. 2. The number of positive cells observed using the smallest probe (293 bases) was low and difficult to distinguish from background. Visual detection of positive cells was clear using a 619-base probe, although the number of positive cells was noticeably higher with the larger probes (1065 and 1987 bases). Signal intensity was generally proportional to probe size, and resulted in small punctate deposition, especially with the cyanine 3 tyramide.

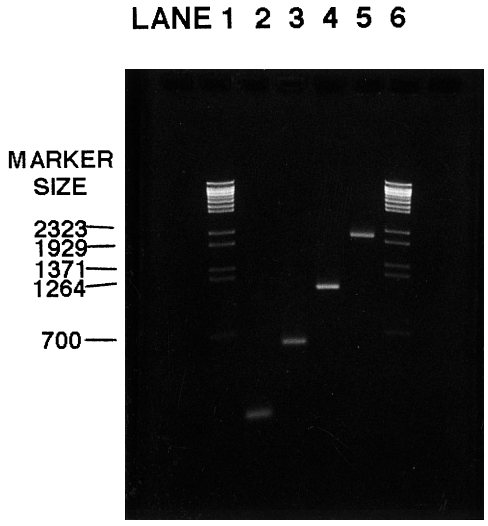


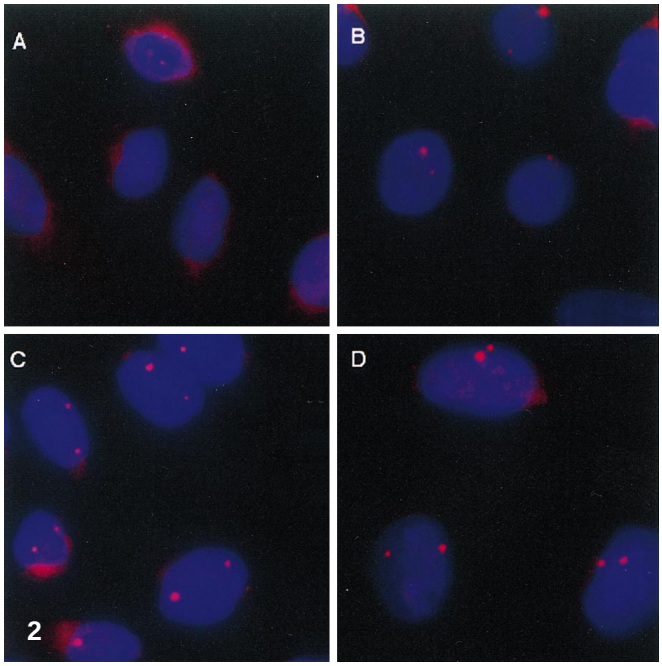

**Fig. 1** Gel analysis of PCR-synthesized human papillomarvirus type 16 (HPV-16) probes, *Lanes 1, 6* markers: *BstE*II digest of lamba DNA, *lane 2* 293-base product, *lane 3* 619-base product, *lane 4* 1065-base product, *lane 5* 1987-base product

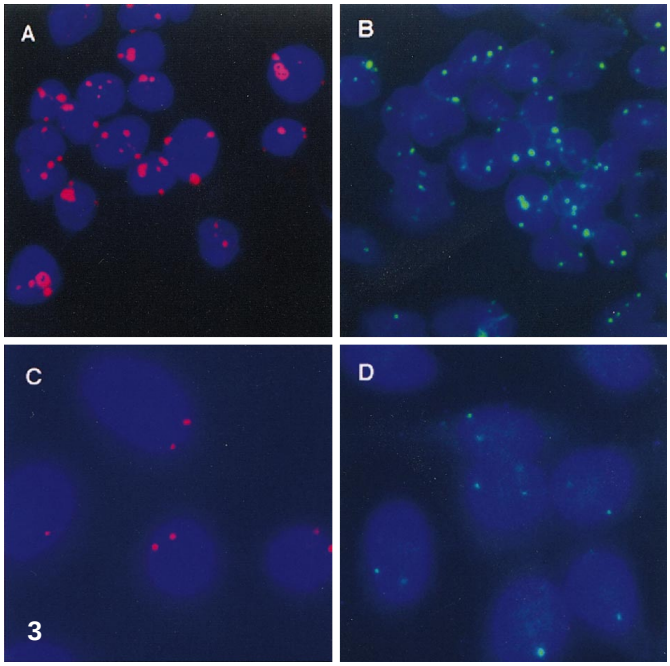
The comparison of detection with cyanine 3 tyramide to fluorescein tyramide is shown in Fig. 3. In CaSki cells, both reagents provided good detection of HPV-16 with the 293-base probe. Using larger probes resulted in brighter signals (data not shown). Because there is a high level of integrated HPV-16 (600 copies), both reagents gave acceptable signal intensities, although sixfold greater exposure times were required with the fluorescein tyramide to capture an image of equivalent intensity.

In SiHa cells, where there are two single copies of HPV-16, cyanine 3 tyramide gave a much brighter, reproducible, and more stable signal than fluorescein tyramide. Even with larger probes (1065 and 1987 bases), using fluorescein tyramide resulted in weak signals which faded significantly.

Detection of HPV-16 using a 1065-base probe, and comparing direct detection with cyanine 3 tyramide to indirect detection with biotinyl tyramide and biotin TSA Plus reagent is shown in Fig. 4. Greatest detection of HPV-16 was achieved with cyanine 3 tyramide (Fig. 4A) and biotin TSA Plus (Fig. 4B) followed by streptavidin Texas Red. Biotinyl tyramide followed by streptavidin Texas Red (Fig. 4C) was used successfully but resulted in inferior signal to noise ratios compared to the other methods. The resulting signal was more diffuse using biotinyl tyramide and biotin TSA Plus as compared to cyanine 3 tyramide. Attempted detection of HPV-16 with the largest biotinylated (1987 base) probe and streptavidin-Cy3 (Fig. 4D) resulted in no detectable signal over background.

Control hybridizations were performed to demonstrate detection was specific for the integrated HPV-16 DNA. Control slides probed with and without denaturation, with RNAse digestion, and with DNAse digestion demonstrated that the nuclear signal observed was specifically from the integrated HPV-16 DNA. Hybridiza-



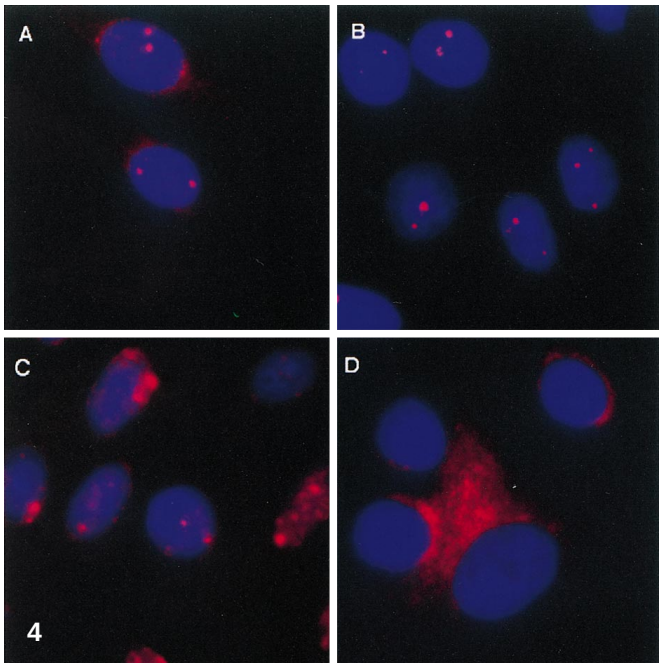




**Fig. 2A–D** Detection of HPV-16 in SiHa cells with different length probes. 1000× magnification. Integrated HPV-16 DNA hybridized and detected with Cyanine 3 tyramide using (**A**) 293-base probe, (**B**) 619-base probe, (**C**) 1065-base probe, (**D**) 1987-base probe

**Fig. 3A–D** Comparison of detection by Cyanine 3 tyramide and fluorescein tyramide. HPV-16 detection in CaSki cells (400× magnification) hybridized with a 293-base probe and detected with (**A**) Cyanine 3 tyramide (exposure 0.2 s) and (**B**) fluorescein tyramide (exposure 1.2 s) HPV-16 detection in SiHa cells (1000× magnification) hybridized with 293-base probe and detected with (**C**) Cyanine 3 tyramide (exposure 0.2 s) and (**D**) fluorescein tyramide (exposure 2 s)

**Fig. 4A–D** Comparison of Tyramide Signal Amplification (TSA) detection by Cyanine 3 tyramide, biotin TSA Plus reagent, biotinyl tyramide, and conventional fluorescent in situ hybridization. HPV-16 detection in SiHa cells hybridized with 1065-base probe. **A** Cyanine 3 tyramide, **B** biotin TSA Plus reagent, **C** biotinyl tyramide, and **D** biotin 1065-base probe followed by streptavidin-Cy3

tion of HPV-16 probes to a HPV-16-negative epidermal cell line, Detroit 551, resulted in no signal.

## Discussion

High sensitivity FISH DNA detection with TSA was demonstrated using probes as small as 619 bases to detect two single copies of integrated HPV-16 DNA in SiHa cells. Visualization of integrated HPV-16 in SiHa cells was observed using two TSA detection methods, direct fluorescence deposition with the TSA substrates, cyanine 3 tyramide and fluorescein tyramide, and indirect detection methods using biotinyl tyramide and biotin TSA Plus reagent. The highest signal to background ratio was observed using cyanine 3 tyramide and biotin TSA Plus. Cyanine 3 tyramide was used to detect HPV-16 in SiHa cells with probes as small as 619 bases. Results with a 293-base probe were not unambiguously positive, although with continued method improvements it is likely that this level of detection is achievable. Method refinements to be considered include alternative labeling methods for small probes (<300 bases), such as PCR incorporation of labeled nucleotide, multiple rounds of TSA (biotinylated probe→ streptavidin HRP→ biotinyl tyramide→ streptavidin HRP→ cyanine 3 tyr-

amide), and use of antibody hapten binding pairs such as biotin: anti-biotin HRP.

The cyanine 3 tyramide was observed to have the best sensitivity detection and well resolved signal, and the newly developed biotin TSA Plus reagent was found to outperform the biotinyl tyramide. The biotin TSA Plus reagent will likely find many applications where superior performance is required, and will extend the useful applications for TSA.

In CaSki cells where there is a high copy number of HPV-16, strong signal intensities were seen using either fluorescein tyramide or cyanine 3 tyramide. In SiHa cells, at the limit of detectability, cyanine 3 tyramide is clearly the better performing directly fluorescent reagent.

Detection of single copy sequences with small PCR-synthesized probes demonstrates the potential of new TSA reagents for high sensitivity detection. With conventional FISH techniques, using PCR-synthesized probes and TSA detection enhancement, the ability to easily make and detect specific sequences of interest is available. The results of these comparisons show that, with refinement of these methods and reagents, increasing levels of detection can be achieved which rival or exceed those which are attainable by alternative methods.

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