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TITLE:

Chromogenic In Situ Hybridization as a Tool for HPV-Related Head and Neck Cancer Diagnosis

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KEYWORDS:

chromogenic in situ hybridization, HPV, transcription, semiquantitative analysis, head and neck cancer, E6, E7, RNA, squamous cell carcinoma

SUMMARY:

Human papillomavirus (HPV) RNA chromogenic in situ hybridization is considered to be one of the gold standards for active human papillomavirus infection detection within tumors. It allows the visualization of HPV E6-E7 mRNA expression with localization and semiquantitative evaluation of its signal.

ABSTRACT:

Human papillomavirus (HPV) infection is a major risk factor for a subtype of oropharyngeal squamous cell carcinoma (OPSCC), which tends to be associated with a better outcome than alcohol- and tobacco-related OPSCC. Chromogenic in situ hybridization (CISH) of HPV viral RNA could allow the semiquantitative evaluation of viral transcripts of the oncogenic proteins E6 and E7 and an in situ visualization with a good spatial resolution. This technique allows the diagnosis of an active infection with the visualization of HPV transcription in the tumoral HPV-infected cells.

An advantage of this technique is the avoidance of contamination from nonneoplastic HPV-infected cells adjacent to the tumor. Overall, its good diagnosis performances have it considered to be the gold standard for active HPV infection identification. Since E6 and E7 viral protein interaction with cell proteins pRb and p53 is mandatory for cell transformation, HPV RNA CISH is functionally relevant and acutely reflects active oncogenic HPV infection. This technique is clinically relevant as well since “low” or “high” HPV transcription levels helped the identification of two prognosis groups among HPV-related p16-positive head and neck cancer patients. Here we present the protocol for manual HPV RNA CISH performed on formalin-fixed paraffin-embedded (FFPE) slides with a kit obtained from the manufacturer. Instead of chromogenic revelation, RNA in situ hybridization may also be performed with fluorescent revelation (RNA FISH). It may also be combined with conventional immunostaining.

INTRODUCTION:

HPV RNA CISH is a powerful tool for the detection of active HPV infection, which may prove crucial in benign or malignant lesions in various locations such as the oropharynx or the uterine cervix. The detection of an active HPV infection may support the diagnosis of an HPV-induced lesion and, thereby, influence its treatment and prognosis.

HPV is the most frequent sexually transmitted infection, and over 100 viral genotypes have been described[1]. Schematically, low-risk genotypes such as genotypes 6 and 11 are known to induce genital warts, recurrent respiratory papillomatosis, and other benign lesions, whereas high-risk genotypes such as genotypes 16 and also 18 are responsible for most cervical cancers and anal cancers and play a role in HNSCC oncogenesis in variable proportions as accounted for by regional epidemiological data[2].

Several tools are available for the detection of HPV infection. As a high-risk HPV infection leads to the expression of viral oncogenic proteins E6 and E7[3], the detection of E6 and E7 transcripts is widely viewed as the gold standard for active HPV infection identification[4]. HPV RNA CISH can be performed on FFPE samples that are quite easily obtained from patients suffering from various HPV-related diseases. Its performance has been evaluated in squamous intraepithelial neoplasia in the cervix, the anus, and the vagina, and in invasive squamous cell carcinoma in the cervix, the anus, and the upper aerodigestive tract[5]: it achieves a sensitivity of over 98% among HPV DNA polymerase chain reaction (PCR)-positive cases. This is slightly better than p16 immunostaining (93%) and HPV DNA in situ hybridization (DNA ISH: 97%), which are more commonly used. In another cohort of 57 patients with squamous cell carcinoma (SCC) arising from the head and neck region, the genital region, the skin, and the urinary tract, compared to HPV DNA ISH, HPV RNA CISH achieved better sensitivity (100% versus 88%) and specificity (87% versus 74%)[6].

P16 immunostaining is an indirect marker reflecting cell cycle disruption that may be caused (but not exclusively) by HPV infection[4,7]. This cost-effective test possesses good sensitivity and a negative predictive value and is recommended as a surrogate marker of high-risk HPV infection in oropharynx cancer (OPC) by the College of American Pathologists (CAP) and by the Union for International Cancer Control (UICC)[8].

Though this paper solely focuses on the detection of HPV in HNSCC, HPV RNA CISH is clinically relevant in various other conditions that involve HPV infection. For instance, this technique may improve the accuracy of the diagnosis of low-grade squamous intraepithelial lesions of the cervix (LSIL, formerly known as cervical intraepithelial neoplasia, grade 1 [CIN1]) for morphologically ambiguous cases[9]. Regarding oropharyngeal SCC, HPV RNA CISH allows the identification of HPV-related SCC, labeled as distinct from HPV-unrelated oropharyngeal SCC in the recent eighth edition of the TNM Classification of Head and Neck Cancer (of the Union for International Cancer Control [UICC])[10]. Since HPV-related SCC exhibits a better prognosis with longer survival and enhanced radiotherapy and chemotherapy sensitivity than HPV-unrelated SCC[11–13], the detection of HPV infection may impact patient management[14,15]. Besides, HPV RNA CISH can be used for the diagnosis of HPV-related multiphenotypic sinonasal carcinoma with a higher signal than HPV DNA CISH[16]. Several multivariate analyses suggest that the detection of E6 and E7 transcripts is correlated with a better prognosis in oropharyngeal SCC overall[7,15,17,18] and in the subgroup of p16-positive oropharyngeal SCC[19,20].

Here we present the protocol for manual HPV RNA CISH performed on FFPE slides with a kit obtained from the manufacturer.

PROTOCOL:

The protocol follows ethical guidelines and was approved by the Ethical Committee (Comité-de-Protection-des-Personnes Ile-de-France-II, #2015-09-04).

1. Preparation of the materials

1.1. Preparation of 1x wash buffer

1.1.1. Prepare 3 L of 1x wash buffer by adding 2.94 L of distilled water and one bottle (60 mL) of wash buffer (50x) (see the **Table of Materials**) to a large carboy. Mix well.

NOTE: The 1x wash buffer may be prepared ahead of time and stored at room temperature for up to 1 month.

1.2. Preparation of counterstaining reagents

1.2.1. Prepare 50% hematoxylin.

1.2.1.1. In a fume hood, add 100 mL of Gill's hematoxylin I (see the **Table of Materials**) to 100 mL of distilled water in a staining dish.

NOTE: The 50% hematoxylin staining solution can be reused for up to 1 week.

1.2.2. Prepare 0.02% (w/v) ammonia water (bluing reagent).

1.2.3. In the fume hood, add 1.43 mL of 1 N ammonium hydroxide to 250 mL of distilled water in

a graduated cylinder or another container. Seal the cylinder with paraffin film. Mix its contents well for 3x–5x.

NOTE: For assay quantitation, it is critical to use ammonium hydroxide. The reagents may be prepared ahead of time. Ensure all containers remain covered.

1.3. Preparation of 1x target retrieval reagent

1.3.1. In a large beaker, add 70 mL of 10x target retrieval reagent (see the **Table of Materials**) to 630 mL of distilled water.

1.3.2. Place the beaker on a heating plate with a magnetic stirrer. Cover it with aluminum foil.

1.3.3. Boil its contents at 100 °C for 10–15 min.

NOTE: Do not let it boil for more than 30 min.

1.4. Reagent equilibration

1.4.1. Ensure the hybridization oven is on and at 40 °C. Place wet humidifying paper at the bottom of the tray.

NOTE: A hybridization oven (see the **Table of Materials**) is needed for steps 3.4 to 4.2.

1.4.2. Remove the amplification reagents (AMP1–AMP6, see the **Table of Materials**) from the refrigerator and keep them at room temperature, at least 30 min before the relevant incubation step.

1.4.3. Before each use, warm the target and/or control probes for at least 10 min at 40 °C in the oven or in a water bath or incubator.

2. Adhesion enhancing and deparaffinization in the fume hood

NOTE: Start the protocol with 3–5 µm-thick histological samples mounted on unstained slides.

2.1. In order to enhance adhesion, bake the slides at 60 °C for 1 h or at 40 °C overnight in an oven.

2.2. Charge the slide rack with unstained histological slides. Immerse the slide rack for 5 min in fresh xylene contained in a staining dish, with occasional agitation. repeat with fresh xylene.

2.3. Immerse the slide rack for 3 min in fresh 100% ethanol contained in a staining dish, with constant agitation. Repeat with fresh 100% ethanol.

2.4. Let the slides dry for 2 min at room temperature.

NOTE: Do not reuse deparaffinization reagents for dehydration of the slides after the assay.

3. Tissue pretreatment

NOTE: These steps follow the "standard" pretreatment recommendation according to the manufacturer's instructions for head and neck samples. The timing of sections 3.1 and 3.2 may need to be adjusted depending on the manipulated tissue.

3.1. Blockade of peroxidase activity

3.1.1. Add 4–6 drops of hydrogen peroxide (see the **Table of Materials**) to each slide and incubate them for 10 min at room temperature.

3.1.2. Wash the slides 2x for 2 min in distilled water at room temperature.

3.2. Breakage of RNA/tissue bounds

3.2.1. With a claw, remove the aluminum foil from the boiling 1x target retrieval reagent (TTR1x) from section 1.3 and stop stirring. Immerse the slide rack slowly and very carefully for 15 min. Cover the beaker again with the aluminum foil.

NOTE: Simmering has to persist during this step.

CAUTION: Use the claw to manipulate the aluminum foil and the slide rack so as to avoid burn injuries. Make sure to wear proper personal protective equipment, such as gloves and a lab coat.

3.2.2. With the claw, immediately transfer the hot slide rack to a distilled water bath and wash it for 2 min.

NOTE: Make sure the samples do not cool down in the TTR1x.

3.2.3. Wash the slides in fresh 100% ethanol for 2 min.

3.2.4. Let the slides dry at room temperature for 2 min.

3.3. Barrier creation

3.3.1. With a hydrophobic barrier pen (see the **Table of Materials**), draw a barrier around the sample. Let it dry out at for least 5 min.

NOTE: Allow the barrier to really dry out. If it wears out during the procedure, do not hesitate to draw it again. Avoid touching the tissue with the pen. The protocol can be paused overnight here.

3.4. Protease digestion

3.4.1. Place the slides on the slide rack and add ~4 drops of Protease Plus per sample (see the **Table of Materials**).

3.4.2. Cover the humidity control tray with a lid and insert it into the hybridization oven for 30 min at 40 °C.

NOTE: To prevent evaporation, make sure the turn knob is completely turned to the lock position.

3.4.3. Remove the tray from the oven and remove the slide rack.

3.4.4. One slide at a time, quickly remove any excess liquid and place the slide in a slide rack submerged in a staining dish filled with distilled water.

3.4.5. Wash the slides 2x for 2 min in distilled water at room temperature. Agitate constantly.

4. Running the assay

NOTE: Do not let sections dry out between the incubation steps.

4.1. Hybridization of the HPV probe

NOTE: Ensure the probes are prewarmed to dissolve any precipitation prior to use. For this step, instead of **HPV probe**, peptidyl-prolyl isomerase B (PPIB) for positive control or dihydrodipicolinate reductase (DAPB) for negative control (see the **Table of Materials**) may also be used.

4.1.1. Tap and/or flick the slides to remove any excess liquid and place them in the slide rack. Add ~4 drops of HPV probe to entirely cover each section.

4.1.2. Cover the tray with a lid and insert it into the oven for **2 h** at **40 °C**.

NOTE: To prevent evaporation, make sure the turn nob is completely turned to the lock position.

4.1.3. Remove the tray from the oven and remove the slide rack.

4.1.4. One slide at a time, quickly remove any excess liquid and place the slide in a slide rack submerged in a staining dish filled with 1x wash buffer.

4.1.5. Wash the slides in 1x wash buffer for 2 min at room temperature with constant agitation. Repeat this with fresh 1x wash buffer.

4.2. Hybridization of AMP1, AMP2, AMP3, and AMP4

NOTE: These steps include hybridization in the hybridization oven and AMP1–AMP4 from the purchased kit (see the **Table of Materials**).

4.2.1. Tap and/or flick to remove any excess liquid from the slides and place them in the slide rack. Add ~4 drops of **AMP1** to entirely cover each section.

4.2.2. Cover the tray with a lid and insert it into the oven for **30 min** at **40 °C**.

4.2.3. Remove the tray from the oven and remove the slide rack.

4.2.4. One slide at a time, quickly remove any excess liquid and place the slide in a slide rack submerged in a staining dish filled with 1x wash buffer.

4.2.5. Wash the slides in 1x wash buffer for 2 min at room temperature with constant agitation. Repeat this with fresh 1x wash buffer.

4.2.6. Repeat steps 4.2.1–4.2.5, but use ~4 drops of **AMP2** instead of AMP1 and incubate for **15 min** at **40 °C.** Wash the slides 2x for 2 min, both times in fresh wash buffer.

4.2.7. Repeat steps 4.2.1–4.2.5, but use ~4 drops of **AMP3** instead of AMP1 and incubate for **30 min** at **40 °C.** Wash the slides 2x for 2 min, both times in fresh wash buffer.

4.2.8. Repeat steps 4.2.1–4.2.5, but use ~4 drops of **AMP4** instead of AMP1 and incubate for **15 min** at **40 °C.** Wash the slides 2x for 2 min, both times in fresh wash buffer.

4.3. Hybridization of AMP5 and AMP6

NOTE: These steps do not include hybridization in the oven but hybridization at room temperature. AMP5 and AMP6 come from the purchased kit (see the **Table of Materials**).

4.3.1. Tap and/or flick the slides to remove any excess liquid and place them in the slide rack. Add ~4 drops of **AMP5** to entirely cover each section.

4.3.2. Cover the tray with a lid and incubate for **30 min** at **room temperature**.

4.3.3. One slide at a time, quickly remove any excess liquid and place it in a slide rack submerged in a staining dish filled with 1x wash buffer.

4.3.4. Wash the slides in 1x wash buffer for 2 min at room temperature with constant agitation. Repeat this with fresh 1x wash buffer.

4.3.5. Repeat steps 4.3.1–4.3.4, but use ~4 drops of **AMP6** instead of AMP5 and incubate for **15 min** at **room temperature.** Wash the slides 2x for 2 min, both times in fresh wash buffer.

5. Signal detection with 3,3'-diaminobenzidine

CAUTION: Diaminobenzidine (DAB) is toxic. Follow appropriate precautions and safety guidelines when disposing of and handling this chemical.

5.1. Mix equal volumes of DAB-A and DAB-B (see the **Table of Materials**) in an appropriately sized tube by dispensing the same number of drops of each solution. Make ~120 µL of DAB substrate per section (~2 drops of each reagent/total of 4). Mix it well for 3x–5x.

5.2. Take each slide, one at a time, from the slide rack and tap and/or flick to remove the excess liquid before placing it in the slide rack.

5.3. Pipette ~120 µL of DAB onto each tissue section. Ensure the sections are covered, and incubate for 10 min at room temperature.

5.4. Dispose the remaining DAB according to local regulation and insert the slide into a slide rack submerged in a staining dish filled with tap water.

6. Counterstaining

6.1. Move the slide rack to a staining dish containing 50% hematoxylin staining solution let it rest for 30 s at room temperature. Note that the slides will become purple.

6.2. Immediately transfer the slide rack back to a staining dish containing tap water, and wash the slides 3x–5x by moving the rack up and down.

6.3. Keep repeating the washing step with fresh tap water until the slides are clear, while sections remain purple.

6.4. Replace the tap water in the staining dish with 0.02% ammonia water. Move the rack up and down 2x–3x. Note that the tissue section should turn blue.

6.5. Replace the ammonia water with tap water. Wash the slides 3x–5x.

7. Dehydration

7.1. Move the slide rack to a staining dish containing 70% ethanol in the fume hood and let it rest for 2 min with occasional agitation.

7.2. Move the slide rack to a first staining dish containing 100% ethanol and let it rest for 2 min with occasional agitation.

7.3. Move the slide rack to a second staining dish containing 100% ethanol and let it rest for 2

min with occasional agitation.

7.4. Move the slide rack to a staining dish containing xylene and let it rest for 5 min with occasional agitation.

8. Slide mounting

8.1. Remove the slides from the slide rack and lay them flat with the sections facing up in the fume hood.

8.2. Mount one slide at a time by adding 1 drop of a xylene-based mounting medium to each slide and carefully placing a 24 mm x 50 mm coverslip over the section. Avoid trapping any air bubbles.

8.3. Air-dry the slides for ≥5 min.

9. Sample evaluation

9.1. Examine the tissue sections under a standard brightfield microscope at 20x–40x magnification.

REPRESENTATIVE RESULTS:

As described here, in head and neck squamous cell cancer, a case may be considered positive in the presence of brown punctiform staining in the cytoplasm or in the nuclei of tumor cells. In most studies, the signal is considered as either "positive" or "not detected"[14]. Methods of semiquantification of the signal have been reported but lack standardization between teams. For example, in some studies, the signals were scored as 1+ with 1–3 dots per tumor cell, 2+ with 4–9 dots per tumor cell, or 3+ with 10 dots or more per tumor cell[6]; in post hoc analyses, only 2+ and 3+ signals, which were easier to interpret, were taken into account. In another study, the results were divided into two scores: RNA CISH "high" and RNA CISH "low". RNA CISH "high" score was defined by more than 50% of stained cancer cells, or by staining covering more than 80% of the cell surface (nucleus and cytoplasm) in at least 30% of the cancer cells, as observed with a 20x objective (**Figure 1**)[21].

Regarding positive controls, the PPIB signal should be visible as punctate dots within cell nuclei at 20x–40x magnification. As for negative control slides, one dot to every 10 cells displaying background DAB staining per 20x microscope field is acceptable.

FIGURE LEGEND:

Figure 1: Examples of "low" and "high" RNA CISH staining. (**A** and **B**) RNA CISH "low" score staining in oropharyngeal squamous cell carcinomas. Staining is observed in under 50% of the tumor cells and covers less than 80% of the cell surface. (**C** and **D**) RNA CISH "high" score staining in oropharyngeal squamous cell carcinomas. Staining is observed in more than 50% of the tumor

cells and, in this case, the staining surface exceeds 80% in more than 30% of the tumor cells. This figure has been modified from Augustin et al.[21]

DISCUSSION:

HPV RNA CISH performed with a purchased kit is a powerful tool for the detection of viral transcripts and it indicates active HPV infection. Performed manually, the steps of the protocol are overall easy to follow, and the purchased kit is convenient. This technique allows the staining of 19 histological samples plus one control slide at once, and the assay lasts around 8 h. It is critical not to let the samples dry out between steps unless otherwise mentioned. The pretreatment condition may need to be adjusted depending on the manipulated tissue.

HPV E6-E7 mRNA expression signal is detected with a precise spatial resolution, thereby ruling out any contamination from HPV-infected nonneoplastic cells adjacent to the tumor. Detection of HPV E6 and E7 RNA is functionally relevant since these transcripts are needed for HPV-induced cell transformation through their interaction with the cellular p53 and pRb proteins[4]. Therefore, in precancerous lesions of the uterine cervix, HPV RNA CISH may help discriminate low-grade intraepithelial lesions (LSIL) from high-grade intraepithelial lesions (HSIL) according to the localization of the signal: in most cases of LSIL, abundant diffusely stained nuclei are labeled throughout the epithelial thickness, indicating a productive phase. HSIL exhibit either abundant diffusely stained cell nuclei in the superficial layer, coexisting with strong nuclear and cytoplasmic punctuate signals in the lower layer (in lesions formerly known as CIN2), or strong nuclear staining with cytoplasmic dots throughout the thickness of the epithelium, indicating the transformative phase of HPV infection (in lesions formerly known as CIN3)[22].

Although there is as yet no standard recommendation for the semiquantitative evaluation of the signal, some authors report a clinical relevance since the semiquantitative evaluation of HPV E6 and E7 transcripts has allowed the identification of two prognostic groups among HPV-related HNSCC patients[21]. It has been postulated that the detection of HPV DNA without E6 and E7 transcripts or with only low levels of E6 and E7 transcripts would be functionally irrelevant and that such patients should be pooled with HPV-negative cancer patients[21,23].

Regarding limitations of this procedure, it is postulated that a few nonspecific cross hybridizations might happen in some cases, as hypothesized by Dreyer et al.[23] RNA CISH may not be suitable for discrimination between E6/E7 RNA transcripts and viral DNA, as the protocol includes a 100 °C heat treatment step, which is suspected to allow for viral DNA denaturation[23]. This is supported by the observation of two types of signals in positive cases, namely strong staining mainly localized in tumor cell nuclei, as well as a fine granular signal in the cytoplasm. As the probe used in this protocol specifically binds HPV genotypes 16 and 18, which are involved in a vast majority of HPV-related HNSCC[4], occasional HPV-associated cases may be missed by RNA CISH, either because of certain HPV genotypes that are not included in the probe, or because of mutations or deletions of primer binding sites. Finally, this method requires costly reagents and devices and is not easily accessible in routine practice.

For every sample, a total of three sections are needed: one to perform the HPV assay, one for

the positive control, and one for the negative control. As RNA is a fragile molecule and might deteriorate over time in FFPE samples, the quality of the transcripts has to be checked for every sample, using positive control probes such as PPIB, DNA-directed RNA polymerase II subunit RPB1 (POLR2A), or polyubiquitin-C (UBC), which are human housekeeping genes. Moreover, when there is a semiquantitative approach, the signal must be normalized to the housekeeping gene control probe[21]. The recommended positive control for each tissue may be found in the manufacturer's instructions. However, a recent study confirms that mRNA expression can be robustly studied both in prospective and retrospective samples, showing comparable mRNA levels between samples from 2004 and from 2008. This is in favor of the relative integrity of mRNA over time[24]. The recommended negative control probe used to avoid unspecific staining is the bacterial gene coding for DAPB.

Here the chromogenic in situ hybridization of HPV RNA is described as performed manually. This technique may also be performed with fluorescent labeling and/or combined with conventional immunostaining.

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DISCLOSURES:

The authors have nothing to disclose.

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21. Augustin, J. et al. HPV RNA CISH score identifies two prognostic groups in a p16 positive

oropharyngeal squamous cell carcinoma population. *Modern Pathology*. doi: 10.1038/s41379-018-0090-y (2018).
22. Evans, M.F. et al. HPV E6/E7 RNA In Situ Hybridization Signal Patterns as Biomarkers of Three-Tier Cervical Intraepithelial Neoplasia Grade. *PLOS ONE*. **9** (3), e91142, doi: 10.1371/journal.pone.0091142 (2014).
23. Dreyer, J.H., Hauck, F., Oliveira-Silva, M., Barros, M.H.M., Niedobitek, G. Detection of HPV infection in head and neck squamous cell carcinoma: a practical proposal. *Virchows Archiv*. **462** (4), 381–389, doi: 10.1007/s00428-013-1393-5 (2013).
24. Bingham, V. et al. RNAscope *in situ* hybridization confirms mRNA integrity in formalin-fixed, paraffin-embedded cancer tissue samples. *Oncotarget*. doi: 10.18632/oncotarget.21851 (2017).

Figure

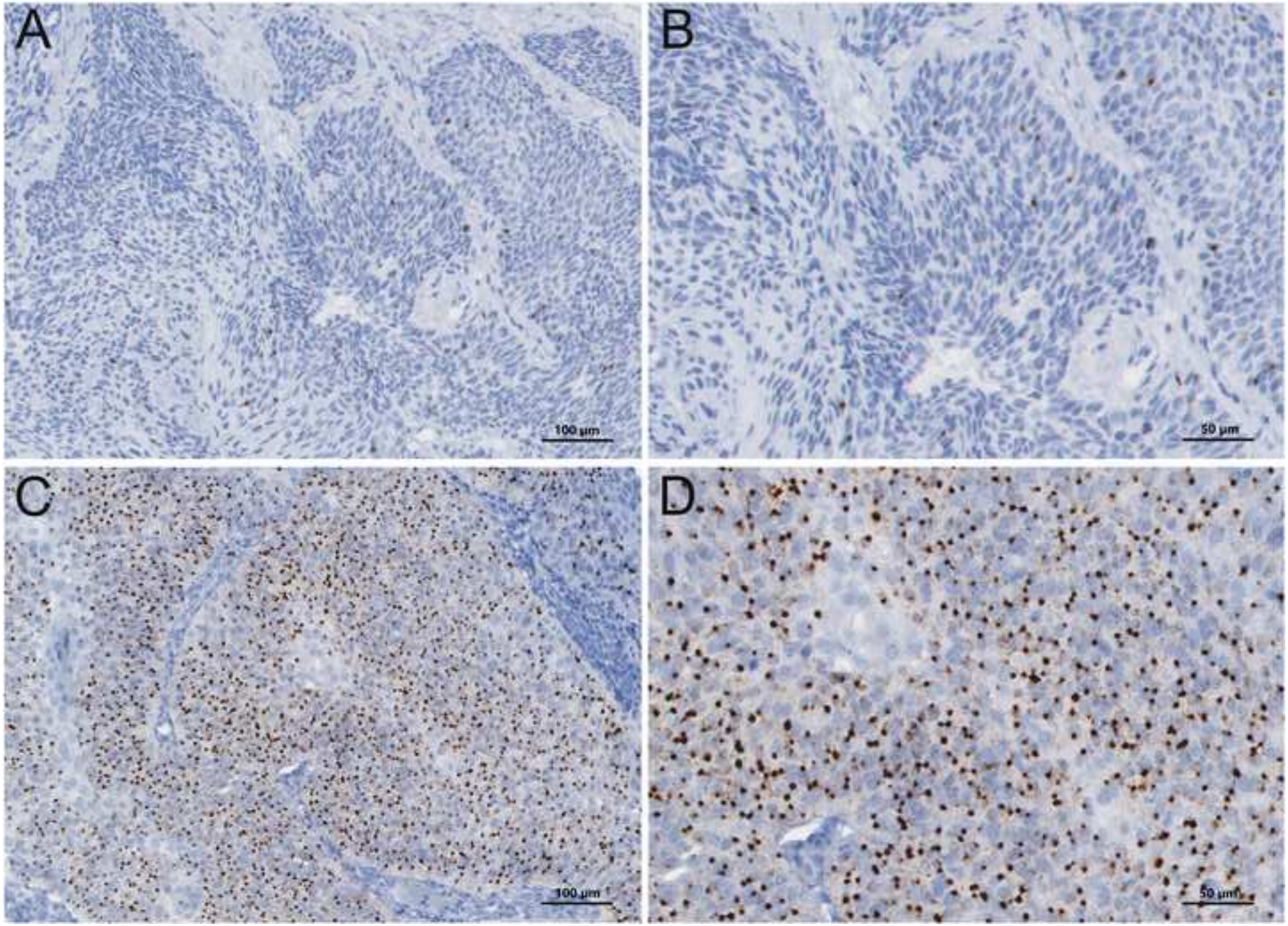

Table of Materials

Click here to access/download;Table of Materials;080219Copie de JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
Hematoxylin solution, Gill No. 1	Merck	GHS132
HybEZ Oven (110v)	Advanced Cell Diagnostics Inc.	321710
HybEZ slide rack	Advanced Cell Diagnostics Inc.	300104
ImmEdge Hydrophobic Barrier Pen	Advanced Cell Diagnostics Inc.	310018
RNAscope 2.5 HD Detection Reagents-BROWN	Advanced Cell Diagnostics Inc.	322310
RNAscope 3-Plex Negative Control Probe	Advanced Cell Diagnostics Inc.	320871
RNAscope 3-Plex Positive Control Probe	Advanced Cell Diagnostics Inc.	320861
RNAscope H202 & Protease Plus Reagent	Advanced Cell Diagnostics Inc.	322330
RNAscope Probe- HPV16/18	Advanced Cell Diagnostics Inc.	311121
RNAscope Target Retrieval Reagents	Advanced Cell Diagnostics Inc.	322000
RNAscope Wash Buffer Reagents	Advanced Cell Diagnostics Inc.	310091

Comments/Description

This kit includes amplification reagents AMP1, AMP2, AMP3, AMP4, AMP5 and AMP6, and detection reagents DAB-A and DAB-B
DAPB
PPIB
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Title of Article: E6 and E7 RNA Chromogenic In Situ Hybridization as a tool for Human Papillomavirus-related Head and Neck Cancer diagnosis. Tips for the use of RNAscope® kit.

Author(s): Sophie OUTH-GAUER1, Jérémy AUGUSTIN1, Marion MANDAVIT2, Ophélie Grad2, Thomas DENIZE 1, Marine NERVO1, Charles LEPINE1, Eric TARTOUR2,3, Cécile BADOUAL1,2

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Article Title:	E6 and E7 RNA Chromogenic In Situ Hybridization as a tool for Human Papillomavirus-related Head and Neck Cancer diagnosis. Tips for the use of RNAscope® kit.
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Paris, January 23rd 2019

Dear Dr Steindel, dear reviewers,

Many thanks for the short review process and for the time and consideration you allocated to our manuscript entitled "**E6 and E7 RNA Chromogenic In Situ Hybridization as a tool for HPV-related Head and Neck Cancer diagnosis**". We are happy to discuss the editorial comments and the reviewers comments and to modify and improve our manuscript according to the concerns and suggestions. All changes in the manuscript are tracked in the MS Word Revision Mode.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues:

Response: Spelling and grammar issues have been addressed thanks to Ms Clark, who is an English teacher.

2. Authors submitted an open access ALA but indicated standard access in the submission questionnaire. Please be consistent.

Response: Sorry for this mistake, we meant to choose standard access.

3. Please revise lines 95-99, 110-113, 202, 204, 206, 225, 227, 229-230, 247-248, 273-274, 291-292, 297-298, 305-306, 311-312, 318-328, 332-333, 335-337 to avoid text from a commercial website (manufacturer's instructions).

7. Title: Please remove "Tips for the use of RNAscope® kit" as JoVE cannot publish manuscripts containing commercial language.

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Response: Commercial mentions have been removed accordingly.

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Response: Please find attached the copyright permission.

5. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

Response: A scale bar has been added.

6. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Response: They have been sorted in the alphabetical order.

8. Please define all abbreviations before use.

Response: All abbreviations have been defined.

9. Please revise the Introduction to include all of the following
a) A clear statement of the overall goal of this method :

Response: see lines 62-63

b) The rationale behind the development and/or use of this technique :

Response: see lines 72-75

c) The advantages over alternative techniques with applicable references to previous studies :

Response: see lines 79-89

d) A description of the context of the technique in the wider body of literature

Response: see lines 94-107

e) Information to help readers to determine whether the method is appropriate for their application

Response: see lines 72-84

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). :

Response: It has been revised.

12. 1.4.1: Please reference Table of Materials for the composition of the Target Retrieval reagent.

Response: see line 145

13. 2.1: Does the slide rack have slides containing samples? Please specify the source of sample. It is otherwise unclear :

Response: see lines 169 and 174.

14. 3.2.1: Does the slide have samples on it?

Response: see line 169

15. Please spell out AMP1/2/3/4/5/6 and DAB and provide their composition. If purchased, please reference the Table of Materials. :

Response: see lines 303, 304, 371 and Table of Materials

16. Please ensure that the highlighted portion identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. :

Response: It has been modified.

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: High lightening has been modified.

19. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. :

Response: see lines 434-441

20. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique. :

Response: see lines 473-478 and 482-489.

Reviewers' comments:

Reviewer #1:

Thank you for the opportunity to review this manuscript presenting suggested modifications for the detection of HPV mRNA in paraffin embedded tissue of oropharyngeal carcinomas. The manuscript is well designed and presented. Publications of these modifications would be useful to other researchers in this field. Interestingly, I had just read their paper (reference 19) on the clinical applications of this technique; and the present manuscript dovetails into it very appropriately. Apart from grammatical/syntax edits, the manuscript is otherwise appropriate for publication.

Response: Thank you very much for your comments. The manuscript has been corrected for grammar and spelling mistakes.

Reviewer #2:

Manuscript Summary:
The manuscript describes chromogenic in situ hybridization assay for HPV detection

Major Concerns:

There are grammatical and language usage errors. For example, "…this technique may also be declined with fluorescent expose and/or combined…" it is not clear what the authors meant "Diagnosing a Human Papillomavirus (HPV) infection may be crucial in several benign or malignant…" perhaps it is better to use the term "Detection" of HPV ….

The manuscript would benefit from review by a native speaker of English language familiar with biomedical literature.

Response: Thank you very much for your comments. The manuscript has been corrected for grammar and spelling mistakes.

The protocol has been adapted from the documentation provided by the manufacturer; however, some of the important steps such as slide preparation (baking) have been omitted.

Response: The baking step has been added to the protocol, please see lines 171-172

It also should be noted that the target retrieval solution step timing needs to be optimized based on the tissue used. The recommended 15 minutes may be too much of too little for different tissue types.

Response: You are absolutely right. This paper is mainly focused on head and neck cancer. However, this point has been stretched out in lines 192-194 and 478.

It should be noted that both cytoplasmic and nuclear signals are considered positive. The attempts to quantify the signal on oropharyngeal squamous cell carcinomas ("low" vs. "high") are meaningless. It should be reported as positive or not detected.

Response: Indeed, both nuclear and cytoplasmic signals are considered positive and this point has been mentioned in lines 435-436. While we agree regarding the lack of recommendation regarding the semi-quantitative approach, we believe in its clinical relevance. We cite two studies with a semi-quantitative approach in head and neck cancers (Augustin et al., 2018; Mendez-Pena, Sadow, Nose, & Hoang, 2017). The study by Augustin and al. shows that this approach is clinically relevant, as it allows the identification of two subgroups of patients with different prognosis. Besides HPV, the literature provides other examples of the clinical relevance of the semi-quantification of the transcription of viruses or genes: among them, high levels of Epstein-Barr virus (EBV) RNA assessed by *in situ* hybridization (EBER) correlate with a better prognosis in EBV-related nasopharyngeal carcinomas (Ke et al., 2014).

It is well known that the RNA preservation in FFPE can significantly affect the extent of the detected signal. Therefore, recommendations to use this assay as quantitative, particularly without normalization to the housekeeping gene control probe may not be valid.

Response: Absolutely. This was not clear enough in the previous manuscript but in has been explicated in lines 511-529. The normalization to the housekeeping gene control probe levels had been done in the study we refer to (Augustin et al., 2018).

We would like to thank you again for your consideration and we look forward to hearing from you.

Best regards,

Sophie Outh-Gauer and Cécile Badoual

SPRINGER NATURE

Title: HPV RNA CISH score identifies two prognostic groups in a p16 positive oropharyngeal squamous cell carcinoma population

Author: Jérémy Augustin et al

Publication: Modern Pathology

Publisher: Springer Nature

Date: Jun 20, 2018

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