Spatial Localization of Specific Nucleic Acid Sequences in Cells and Tissues with Novel LoopRNA Technology

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INTRODUCTION

in situ hybridization (ISH) is a method used for the precise spatial localization and detection of specific nucleic acid sequences in formalin-fixed, paraffin-embedded (FFPE) cells and tissue samples with deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) probes. The official birth of this technique can be dated back to 1969, when Yale researcher Joseph Gall and graduate student Mary Lou Pardue published the milestone paper showing that radioactive copies of a ribosomal DNA sequence can detect the complementary DNA in the nucleus of a frog egg [1]. Since then, advancements in molecular biology increased the versatility and the sensibility of this method, becoming a fundamental tool for laboratories, both research and clinical.

The first generation of probes used for ISH were based on isotopic labeling technologies. Due to potential radioactive exposure, non-isotopic label alternatives have been developed. Two common non-isotopic tags used to generate ISH probes are the vitamin biotin and the plant steroid digoxigenin. Biotinylated probes, which are used as the first non-isotopic labels, were easy to work with and cost-effective to generate, in contrast to isotopic variants. However, some tissue samples express endogenous levels of biotin and can result in high-background or false positive results. In order to resolve the non-specific binding of endogenous biotin, the probes can be labeled with digoxigenin, a steroid found exclusively in the *Digitalis purpurea* plant and is widely used in animal cell or tissue samples. Digoxigenin circumvents the previously mentioned background staining with biotin. The use of biotin or digoxigenin also enables the probes to be detected with chromogenic solutions and results can be visualized under the light microscope. Both biotin-labeled probes and digoxigenin-labeled probes can be retained in tissue samples for an extended time as long as they are stored in appropriate conditions.

Today, quantitative and qualitative analysis of DNA, RNA and protein biomarkers are more and more prominent in research and clinical practice, providing crucial information in diagnosis, prognosis, and therapy guidance [2]. The detection of single or low copy targets have been the most challenging with ISH and can be easily missed as some techniques are restricted to highly expressed genes, therefore RT-PCR is generally considered the gold standard for gene expression analysis in clinical diagnostics, as well as in any research field. Nonetheless, RT-PCR requires the sample homogenization for nucleic acid extraction, and consequently loses any spatial information concerning gene expression.

In order to overcome these limitations, continuous improvements on ISH probes and techniques have been introduced over the years, in an attempt to increase sensitivity. As the sensitivity of probes and detection improve, ISH has been gaining a larger role in diagnostic pathology. Both DNA and RNA ISH probes can detect DNA integrated in cells, however, RNA ISH probes can also detect active mRNA.

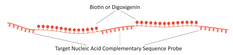
This study introduces AMPIVIEW™ RNA ISH probes, which combine the precision of targeted, sequence-specific RNA probes with the superior sensitivity of LoopRNA technology for the detection of not only infection with viral DNA and RNA such as human papillomavirus (HPV) and SARS-CoV-2, but also the expression of endogenous genes and mRNA such as HER2/neu.

AMPIVIEW™ RNA probes are designed with the regions pairing to the target sequence, interspersed with multiple RNA spacer segments, which are not complementary to the target. The spacers are designed with bases that can be labeled with biotin or digoxigenin. Upon hybridization with the target nucleic acid, the labeled spacer segment loop out and labels can be recognized by a reporter system. The presence of multiple molecules of biotin or digoxigenin, easily accessible for the reporters thanks to the loops, allow for a strong one step amplification of the signal, thus assuring excellent sensitivity (Figure 1). Since the hybridization does not need to undergo multiple amplification steps, the protocol is much faster and easier compared to commercially available probes.

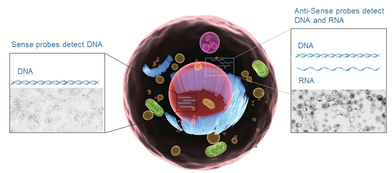
Nucleic Acid Target

Select target nucleic acid (DNA or RNA) sequence from any species, any genome.

AMPIVIEW™ RNA Probes

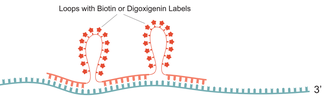


Analyze sequence of the target nucleic acid and design the probes. Probes are produced with conjugated labels such as biotin or digoxigenin between complementary bases.



Probes can be designed as sense probes to detect DNA only or antisense probes to detect both DNA and RNA.

Hybridization



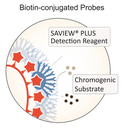
During hybridization, AMPIVIEW™ RNA probes form loops exposing biotin or digoxigenin labels. Labels can be detected with antibodies against biotin or digoxigenin and amplified with IHC detection reagents.

FIGURE 1. Simplified diagram of AMPIVIEW™ RNA probes design for in situ hybridization.

Concerning the detection, post-hybridization washes are followed by a standard IHC protocol, which can be direct or indirect detection [3]. Biotinylated probes can be detected with a streptavidin-based nanopolymer detection solution (direct or one-step detection) such as SAVIEW® PLUS (Figure 2A). The indirect or two-step protocol requires an antibody (anti-biotin or anti-digoxigenin) linker combined with a nanopolymer detection reagent such as POLYVIEW® PLUS (Figure 2B).

Detection

A. SAVIEW® PLUS AP or HRP reagent is a strepavidin-based nanopolymer solution for the amplification and detection of biotin-labeled AMPIVIEW™ RNA probes on tissues and cells. Coupling of streptavidin directly to HRP or AP enzymes results in high sensitivity detection reagents with much easier protocols. This one-step detection system enables faster staining with significantly lower background. SAVIEW® PLUS, combined with HIGH-DEF® chromogens, delivers sharp and crisp stainings.



B. POLYVIEW® PLUS AP or HRP reagent is a biotin-free nanopolymer solution, when combined with anti-biotin or DIGX® anti-digoxigenin linkers, produces consistent and reproducible detection of AMPIVIEW™ RNA probes. Short polymers attach to linkers, yielding a high intensity of active reporters. This increases the sensitivity and specificity, signal intensity and speed of ISH and IHC. POLYVIEW® PLUS, combined with HIGH-DEF® chromogens, delivers sharp and crisp stainings.

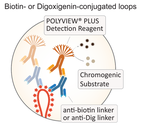


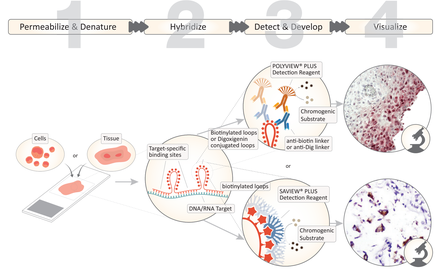
FIGURE 2. Detection systems compatible with AMPIVIEW™ RNA probes

REFERENCES

1. Pardue, M.L; Gall, J.G. "Molecular Hybridization of Radioactive DNA to the DNA of Cytological Preparations," PNAS, vol. 64, no. 2, pp. 600-604, 1969.
2. Wang . F. et. al., "RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues.," Journal of Molecular Diagnosis, vol. 14, no. 1, pp. 22-29, 2021.
3. La Rocca, G. et al. "Recent improvements in in situ hybridization for the detection of HPV infections in clinical samples.," World Cancer Research Journal, 2020.

WORKFLOW and METHODS

AMPIVIEW™ ISH Workflow



METHODS

Immunohistochemistry (IHC)
Detection of specific antibodies were performed manually with POLYVIEW® PLUS AP or HRP detection solutions and corresponding HIGHDEF® chromogens and counterstains according to manufacturer's instructions.

***in situ* Hybridization (ISH)**
AMPIVIEW™ RNA Probes
Detection of AMPIVIEW™ RNA probes were performed manually with POLYVIEW® PLUS AP or HRP detection solutions and corresponding HIGHDEF® chromogens and counterstains.

Detection of RNAscope® RNA probes were performed manually according to manufacturer's instructions.

RESULTS

SENSE vs ANTISENSE PROBES

Signal comparison between sense probes (target DNA) and antisense probes (target DNA and RNA) showed that the antisense probes against HPV 16/18 yielded stronger signals in SiHa (1,3 copies of HPV) cells. Specificity was determined with the lack of detection of HPV 16/18 in C33 (no HPV copies) cells (Figure 3).

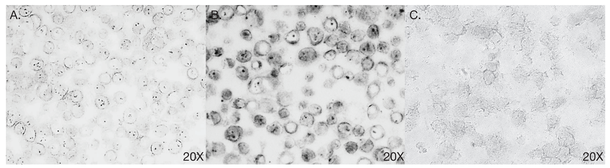


FIGURE 3. Detection of HPV 16/18 with A. AMPIVIEW™ HPV 16/18 sense RNA probes, B. AMPIVIEW™ HPV 16/18 antisense RNA probes in SiHa cells. C. Detection of HPV 16/18 with AMPIVIEW™ HPV 16/18 antisense RNA probes in in C33 cells.

HPV Detection

Probes are specific for HPV 16/18/31/33/51, 6/11 and 16/18 genotypes. (Figure 4).

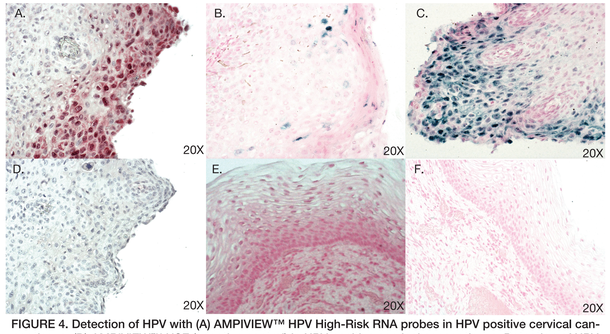


FIGURE 4. Detection of HPV with (A) AMPIVIEW™ HPV High-Risk RNA probes in HPV positive cervical cancer tissue. (D) AMPIVIEW™ NSF (negative control) in HPV positive cervical cancer tissue. Detection of HPV with AMPIVIEW™ HPV 6/11 RNA probes in (B) HPV positive tissue and (E) HPV negative tissue. Detection of HPV with AMPIVIEW™ HPV 16/18 RNA probes in (C) HPV positive tissue and (F) HPV negative tissue.

RESULTS

ISH vs IHC

Results obtained with AMPIVIEW™ HPV high-risk RNA probes in cervical cancer tissue specimens were confirmed by the detection of p16 by IHC (Figure 5).

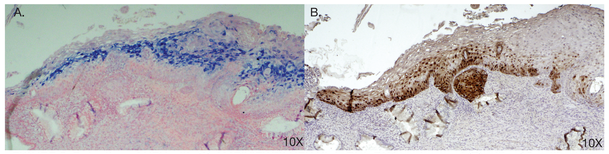


FIGURE 5. Detection of HPV with (A) AMPIVIEW™ HPV High-Risk RNA probes and (B) with p16 antibody in HPV positive cervical cancer tissue specimens.

Results obtained with AMPIVIEW™ HER2 RNA probes in breast cancer tissue specimens were confirmed by the detection of HER2 by IHC (Figure 6).

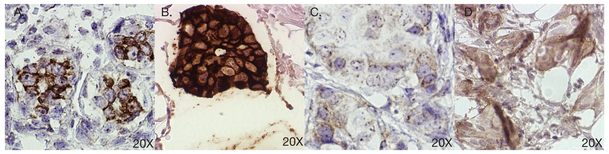
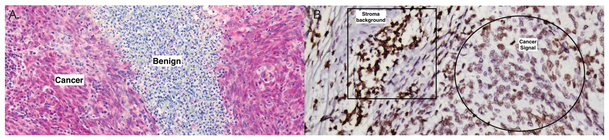


FIGURE 6. Detection of HER2 overexpression in HER2 positive breast cancer tissue with (A) AMPIVIEW™ HER2 antisense RNA probes and (B) HER-2 antibody. Detection of HER2 expression in HER2 negative breast cancer tissue with (C) AMPIVIEW™ HER2 antisense RNA probes and (D) HER-2 antibody.

High Specificity

AMPIVIEW™ RNA probes demonstrates high specificity with low background compared to competitor's product.



A. HPV high-risk — type 16, 18, 31, 33, 51 (red) detected with AMPIVIEW™ HPV High-Risk RNA probes in cervical cancer tissue. B. Competitor's high-risk HPV probes were tested under the same conditions.

CONCLUSION

AMPIVIEW™ RNA probes are a powerful tool for histopathological examination by ISH techniques for research and potential diagnostic purposes, and can aid in further understanding the pathophysiology and distribution of nucleic acids, both DNA and RNA of infectious agents or endogenous genes in cells and tissue specimens.

AMPIVIEW™ RNA probes are carefully crafted with the precision of sequence specific probes, powered by Enzo's LoopRNA ISH™ technology, to deliver superior sensitivity. In addition, the design of the probes make them adaptable to any workflow (manual or automated) and compatible with existing IHC detection systems and ISH setups. This innovation also enables the visualization of gene expression in the context of tissue architecture and analysis with a light microscope.

AMPIVIEW™ RNA probes can be designed to detect any gene and transcript of interest with virtually unlimited potential.