BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY
APPLICATION NOTE

In Situ Hybridization: The Importance of Ultrapure Water for RNA Technologies

By FRAUKE NITZKI and ELMAR HERBIG

Introduction

In situ hybridization (ISH) for localization of DNA/RNA hybrids in cytological preparations was first described in 1969 by Gall and Pardue.[1] This method enables mRNA transcripts to be detected in tissue sections. Unlike expression analyses based on polymerase chain reactions, the exact localization of the target transcripts can be identified within the tissue.

Principle of *In Situ* Hybridization

ISH represents an alternative to immunohistochemical staining if adequate antibodies are not available, and is employed in diverse areas of research. This type of hybridization uses specific nucleic acid fragments (probes) that are complementary to the target sequence in order to detect specific transcripts. Such probes may consist of DNA or of RNA. Meanwhile, RNA probes are frequently utilized. Such probes are commonly labeled with the molecule digoxigenin (DIG), which normally occurs in the plant species *Digitalis purpurea*. DIG labeling enables the specifically bound probes in a tissue section to be visualized by enzyme-conjugated anti-DIG antibodies. For this purpose, after incubation of tissue sections with antibody, the appropriate substrate is pipetted onto the sections and converted by an immunoenzymatic reaction into a visible colorant (Figure 1). This method can be used to analyze the activity of specific genes for research projects or diagnostic procedures. Further practical information on ISH is provided in Wilcox's overview article.[2]

This article discusses the results of the ISH that was carried out as part of a cancer research project. The analyzed skin samples were obtained from genetically modified mice. Based on targeted manipulation of the tumor suppressor gene *Patched (Ptch)* by homozygous knockout, the *Ptch*-knockout mice developed basal cell carcinomas.[3] These skin tumors are the ones that most commonly occur in humans. They frequently exhibit increased activity of the signaling pathway that is regulated by *Ptch*. Inactivation of this important component in this mouse model pathologically activates the signaling pathway. This results in an increased expression of the target gene *Gli1*—a transcription factor that activates a variety of other genes—in tumor cells, and expression of *Gli1* can be detected using ISH.

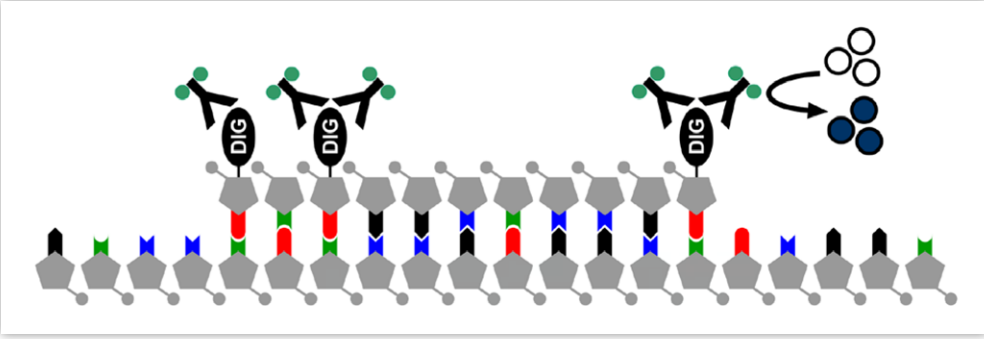

FIGURE 1. Schematic diagram of the general procedure of ISH.
The mRNA transcript (lower strand) is detected by a complementary probe (upper strand) that specifically binds to mRNA. DIG coupled to the probe is identified by an anti-DIG antibody (Y). The enzyme alkaline phosphatase (green dots) is bound to the antibodies and causes the colorless substrate (white circles) to be converted into a blue, water-insoluble product (blue circles).

Production of Ultrapure Water

The arium® pro VF system (Figure 2) has been designed to produce ultrapure water from pretreated drinking water by removing contaminants that are still present in this potable water feed. Production of ultrapure water requires continuous recirculation and a constant water flow rate, which is achieved using a built-in pump system with controlled pressure. The conductivity of the water is measured at the feed water inlet and directly at the downstream port (product water outlet).

The arium® pro VF system used in the studies described in this paper is a predecessor model with the same technical specifications as the redesigned system shown in Figure 2, and works with two different filtration cartridges. These are filled with a special active carbon adsorber and mixed-bed ion exchange resins in order to deliver ultrapure water with a low total organic carbon (TOC) content (*i.e.*, organically bound carbon). In addition, the system has an integrated UV lamp that has a bactericidal and an oxidizing effect at wavelengths of 185 nm and 254 nm, respectively. Moreover, the ultrapure water system has a built-in ultrafilter module used as a crossflow filter. The ultrafilter membrane incorporated in this filter retains colloids, microorganisms, endotoxins, RNA and DNA, and removes RNases, which is essential to performing ISH.

A 0.2 µm final filter installed at the water outlet serves to remove particulates and bacteria during dispensing of the ultrapure water produced. The process that the unit employs to purify water is depicted in Figure 3.

FIGURE 2. The arium® pro VF ultrapure water system.

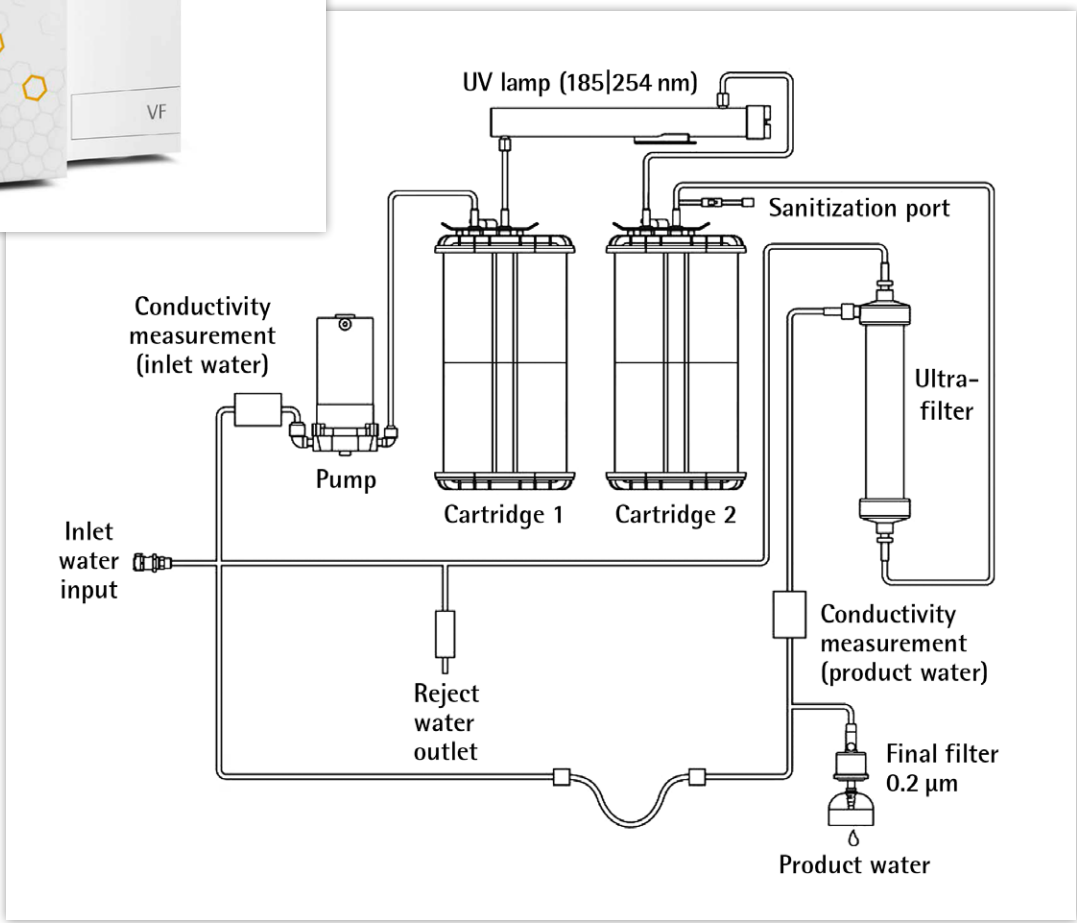

FIGURE 3. Schematic flow diagram of the arium® pro VF ultrapure water system.
In this diagram, the valves and their controllers have been omitted for better clarity of the functions.

The Importance of Ultrapure Water

The ultrapure water quality produced with the arium® pro VF technology is used for highly sensitive applications including analyses involving ISH in human genetics laboratories. The use of pure RNase-free water, chemicals, and materials in this sensitive detection method is extremely important to prevent degradation of the RNA probe. For the study presented in this paper, the most stringent requirements for water purity were achieved by using the Sartorius arium® pro VF system. Results obtained in this research study are described below.

Materials and Methods

Our experience has shown that all reagents used for ISH experiments, as well as water, must have consistently high quality (*i.e.*, free of biological contaminants such as organisms, DNases, RNases, endotoxins, *etc.*). Tap water contains varying levels of biological contaminants and chemicals, depending on its source and regional regulatory purity standards. Diethyl pyrocarbonate (DEPC) is normally added to the buffers and solutions to inactivate RNase. However, this treatment is costly and time-consuming, and laboratory staff must observe particular safety precautions when working with DEPC, as it is considered to be slightly hazardous. Also, DEPC is not effective in removing secondary contaminants from certain solutions, such as those containing Tris. The use of DEPC for decontamination should be avoided whenever possible, and ultrapure water free of contaminants should be employed instead.

Tissue samples were taken from tumorous skin tissue in mice and embedded in paraffin. The paraffin-embedded tissue samples were then sectioned using a microtome. Following this, the paraffin-embedded sections were deparaffinized and rehydrated, and the tissue was permeabilized with proteinase K. To prevent unspecific binding due to differences in electric charge, the tissue samples were incubated with acetic acid anhydride. The *Gli1*-specific DIG-labeled RNA probe was incubated on the tissue sections overnight at 59°C. To remove any unspecifically bound probe, a stringent washing protocol was performed. In this procedure, the tissue sections were repeatedly washed at 63°C in a solution containing formamide, and the unbound probes were removed by incubation with RNase A.

A DIG-specific antibody (Roche) was used to detect the DIG-labeled probe. First, the sections were treated with I-Block™ (Tropix/Life Technologies) to block any unspecific antibody binding sites. Then anti-DIG-antibody was added and the sections were incubated overnight at 4°C. On the following day, the sections were washed to remove any unbound antibody. For detection of the probe, the enzyme alkaline phosphatase (that was bound to the antibody) was used. Therefore, NBT/BCIP* substrate was applied to the sections. Alkaline phosphatase causes conversion of the substrate into a blue chromogenic product. The sections were examined under a light microscope, analyzed, and photographed.

*Nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt

Results

Figure 4A shows the result of hematoxylin-eosin (HE) staining and ISH for the *Gli1* transcript of a basal cell carcinoma. HE staining allows for the identification of tumor cells located below the epidermis, in the dermis. By this staining method, the cell nuclei and the cytoplasm are dyed purple-blue and pink, respectively. In contrast to this method, ISH enables the detection of a specific transcript, in this case, *Gli1*.

The results of ISH with the specific *Gli1* probe are shown in Figure 4B. The specific probe, which is usually designated as an "antisense" probe, is complementary to the mRNA of *Gli1*. A "sense" probe (Figure 4C) is used as a negative control. It has the same sequence as the mRNA

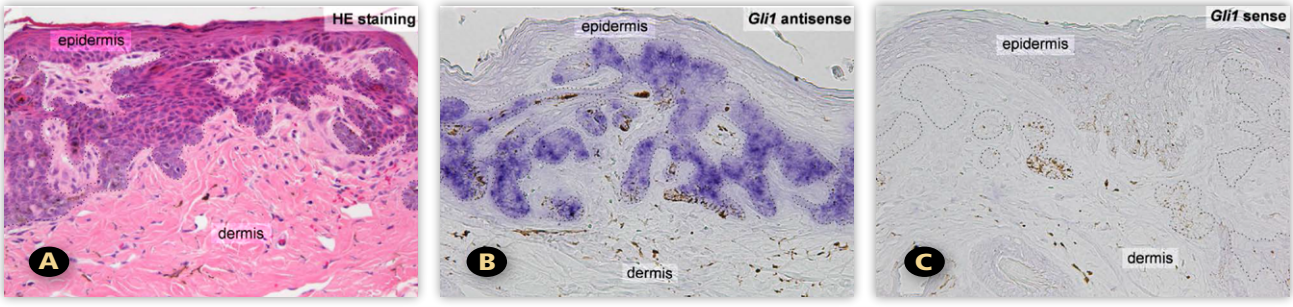

FIGURE 4. HE staining and ISH of basal cell carcinomas: (A) HE-stained tissue; **(B)** the results of *Gli1* ISH (antisense) and; **(C)** the negative control (sense). Tumor cells are marked by a dotted line.

and therefore, cannot bind to the *Gli1* transcript. As shown in Figure 4B, *Gli1* is expressed in the tumor cells and not in the epidermis or dermis.

By contrast, tumor cells are not stained when the unspecific sense probe is used (Figure 4C). Based on these results, it can be assumed that the *Ptch* signaling pathway exhibits strongly increased activity in tumor cells. Contamination with RNases from non-purified water or reagents would have led to degradation of the probes, and detection of *Gli1* with the antisense probe would have been impossible.

Discussion

The ISH method introduced in this paper serves to localize transcripts within a tissue. Normally, the conventional labor- and cost-intensive ISH protocol takes several days, and is performed in various ways in different laboratories. In principle, DNA probes can be used instead of RNA probes to detect mRNA transcripts. The advantages of DNA probes are their higher stability compared with RNA and thus, their easier handling as they cannot be degraded by RNases. However, RNA-RNA hybrid duplexes are more stable than are RNA-DNA hybrid duplexes[4], and for lab staff experienced in handling RNA-RNA probes, are therefore preferable to RNA-DNA. To prevent RNases from degrading the RNA probe, a careful work method is necessary, and all buffers and solutions should be prepared with RNase-free ultrapure water whenever possible.

Ultrapure water produced using the arium® pro system is characterized by the consistently high quality of all water specifications required, such as conductivity/resistivity, total organic carbons, RNases/DNases, and endotoxins. Particularly in the case of endotoxin levels, it was recently demonstrated that ultrapure water produced by the arium® pro VF exhibited exceptionally low concentrations (<0.001 EU/mL)[5], which were well below the typical limits.

Note

This article was originally published by *GIT Labor-Fachzeitschrift*, March 2013, pages 157-159.

Acknowledgements

The authors' special thanks go to the following persons for their proofreading of the German-language manuscript and for their constructive discussions about this subject: Dr. Anja Uhmann, Institute of Human Genetics, Faculty of Medicine, Working Group on Tumor Genetics, at the Georg-August University of Goettingen, Germany; Ms. Stephanie Schweizer and Ms. Alexandra Scholz, both of Sartorius Stedim Biotech GmbH, Goettingen, Germany; and Dr. Herbert Bendlin, based in Ransbach-Baumbach, Germany.

References

[1] Gall JG, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc Natl Acad Sci, USA*, 1969; 63(2): 378-383.

[2] Wilcox JN. Overview of *in situ* hybridisation methodology. *Workshop – Visualizing Neoplasia. Meeting of the Histochemical Society*, Emory University, Winship Cancer Institute, Division of Hematology/Oncology, Atlanta, GA, USA (2000).

[3] Zibat A *et al*. Time-point and dosage of gene inactivation determine the tumor spectrum in conditional *Ptch* knockouts. *Carcinogenesis*, 2009; 30(6): 918-926. PMid:19321799.

[4] Sugimoto N *et al*. Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. *Biochem*, 1995; 34: 11211-11216.

[5] Schmidt K, Herbig E. Weniger ist mehr–Quantitative Endotoxin Bestimmung von Reinstwasser. *Laborpraxis*, 2012; 5:36.Jhg.

About the Authors

Dr. Frauke Nitzki, Institute of Human Genetics, Working Group for Tumor Genetics
Faculty of Medicine, Georg-August University of Goettingen, Germany
Email: fnitzki@gwdg.de

Dr. Elmar Herbig, Sartorius Lab Instruments GmbH, Goettingen, Germany
Email: elmar.herbig@sartorius.com