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Clinica Chimica Acta 363 (2006) 48 – 60

EUROPEAN JOURNAL OF INTERNAL MEDICINE

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Review

Real-time assays with molecular beacons and other fluorescent nucleic acid hybridization probes

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Received 3 April 2005; received in revised form 22 April 2005; accepted 27 April 2005
Available online 18 August 2005

Abstract

Background: A number of formats for nucleic acid hybridization have been developed to identify DNA and RNA sequences that are involved in cellular processes and that aid in the diagnosis of genetic and infectious diseases.
Methods: The introduction of hybridization probes with interactive fluorophore pairs has enabled the development of homogeneous hybridization assays for the direct identification of nucleic acids. A change in the fluorescence of these probes indicates the presence of a target nucleic acid, and there is no need to separate unbound probes from hybridized probes.
Conclusions: The advantages of homogeneous hybridization assays are their speed and simplicity. In addition, homogeneous assays can be combined with nucleic acid amplification, enabling the detection of rare target nucleic acids. These assays can be followed in real time, providing quantitative determination of target nucleic acids over a broad range of concentrations.

Keywords: Nucleic acid hybridization; Real-time gene amplification assays; Fluorescence energy transfer; Molecular beacons; Fluorescent nucleic acid hybridization probes

Contents

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doi:10.1016/j.cccn.2005.04.037

1. Introduction

Nucleic acid hybridization is a process whereby a DNA or RNA strand forms an ordered series of hydrogen bonds with its complement, creating a duplex structure. A nucleic acid can identify its complement in a large population of unrelated nucleic acid sequences. The hybrids that are formed are the strongest and most specific macromolecular complexes known. By selecting nucleic acid sequences that are complementary to a target sequence, nucleic acid probes can be designed for the detection of any gene. The applications range from the estimation of similarity between species to the detection of single nucleotide polymorphisms. Nucleic acid hybridization is used to explore complex cellular pathways, for the diagnosis of genetic and infectious diseases and to provide information on the storage, transfer and expression of genetic information in living cells. Aided by the sequencing of the human genome, the development of novel nucleic acid probes has focused on speed, reliability and accuracy in the identification of nucleic acids. In addition, advances in research on pathogenic infections and the characterization of microbes have resulted in a rise in the demand for molecular diagnostic assays.

2. Early nucleic acid hybridization formats

In 1961, in order to answer the question: "Is the RNA synthesized after infection with T2 bacteriophage complementary to T2 DNA?", Spiegelman and Hall developed the basis of a technique now known as nucleic acid hybridization. In their study, DNA isolated from single-stranded T2 bacteriophage was mixed with RNA from T2-infected *Escherichia coli*, and the hybrids were isolated by equilibrium-density gradient centrifugation [1]. The next year, Bolton and McCarthy developed the first simple solid-phase hybridization method [2]. In their method, denatured DNA was immobilized in agar and hybridized to radioactively labeled RNA. In the subsequent washing step, RNA not hybridized to other nucleic acids was removed. The bound RNA was then eluted from the agar by lowering the salt concentration of the elution solution. The radioactivity of the eluent was then shown to be proportional to the fraction of the RNA bound to complements. This method was optimized further by using nitrocellulose membranes as supports on which the hybrids were immobilized [3–5]. The first applications of nucleic acid hybridization were limited to the detection of abundant target nucleic acids. In addition, the preparation of large amount of nucleic acid probes, which is necessary to carry out the hybridization experiments, was difficult and time consuming. In 1975, after the discovery of restriction endonucleases [6] and molecular cloning [7], it became possible to measure both the quantity and the size of specific hybridized DNA and RNA sequences. DNA samples incubated with restriction enzymes were fractionated by gel electrophoresis and then transferred to a nitrocellulose membrane. DNA restriction fragments, containing a specific nucleic acid sequence, were identified by hybridization with labeled nucleic acid probes. This technique, called "Southern blotting", was further developed by and named after Ed Southern [8]. An analogous filter hybridization technique, where RNA is separated by gel electrophoresis, transferred to a membrane and identified by hybridization with a labeled nucleic acid probe, is termed "Northern blotting" [9]. The introduction of sequencing technologies enabled the analysis of the exact composition of the nucleic acids used, increasing the specificity of nucleic acid hybridization [10,11]. Besides being useful in filter hybridization, nucleic acid probes are useful for the detection of nucleic acids within cells and tissues. In these methods, referred to as in situ hybridization, the nucleic acid probe is labeled with a reporter molecule and the sites of hybridization are visualized by microscopy. As with filter hybridization, the earliest phase of in situ hybridization relied on autoradiographic detection of abundant sequences [12,13]. Autoradiographic detection, however, was limited by poor resolution and also lacked the ability to distinguish more than one nucleic acid target simultaneously. In addition, the technique is often time consuming, requiring several weeks before results can be obtained. To overcome these limitations, non-autoradiographic detection methods were developed. The earliest non-autoradiographic techniques used antibodies to recognize RNA–DNA hybrids or used avidin to detect bound biotin-labeled nucleic acid probes [14–16]. Later, methods for the chemical modification of nucleotides and for the recognition of these modified nucleotides by fluorescent molecules, gold particles or enzymatic reporter molecules were developed, increasing assay sensitivity [17]. The introduction of fluorescent dyes as labels for nucleic acid probes, such as derivatives of fluorescein, rhodamine, or Texas red, provided high resolution with the light microscope. One of the biggest advantages of fluorescent probes is that they enable multicolor fluorescence in situ hybridization to detect multiple nucleotide sequences simultaneously [18].

3. Amplification of targets

In clinical diagnostic applications, the total amount of target nucleic acid in a sample is often very low, and therefore the signal generated by the hybridization of specific probes is often not detectable. For example, since only a few copies of the viral pathogen HIV-1 in blood can cause AIDS, it is desirable to have the ability to detect them soon after infection. In order to overcome the limitation in sensitivity of nucleic acid probes, schemes for target amplification have been developed.