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Immunoassays

Tools for Sensitive, Specific, and Accurate Test Results

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Immunoassays have become increasingly popular tools for measuring biologic analytes, because they offer sensitive, specific results and are relatively easy to use. In addition, some immunoassay methods are rapid, yield improved precision, and are relatively easy to automate, thus requiring less hands-on involvement.

Measurement of a biologic analyte typically involves two major steps: reaction and detection. These steps may be chemical, physical, biologic, or immunologic. The term immunoassay refers to any assay that, at its core, depends on the binding of antigen and antibody.

Two basic types of immunoassays exist. The competitive immunoassay (type II) relies on the competition between the antigen of interest (the analyte) and a constant amount of a similar but labeled antigen for a limited amount of specific antibody. The noncompetitive or immunometric immunoassay (type I) uses an excess of labeled specific antibody toward the analyte of interest.

In competitive immunoassays, the analyte is labeled, while in immunometric immunoassays, the reagent is labeled. Throughout this article, we assume that the analyte is the antigen while the reagent is the antibody. Although this is true in most cases, the reverse holds true in certain instances. For example, during the measurement of hepatitis B antibodies, the analyte is the antibody and the reagent is the antigen.

In any immunoassay, measurement of the biologic analyte requires a means for distinguishing the bound fraction (antigen-antibody complex) from the unbound fraction (free antigen or free antibody depending on the type of immunoassay). To achieve this, heterogeneous immunoassays require a physical separation step while homogeneous immunoassays do not. After the two fractions can be distinguished, detection of the label in the appropriate fraction can occur.

ABSTRACT *Immunoassays are popular in the clinical laboratory because they can quickly and easily provide precise and accurate test results. We review the principles of immunoassays with emphasis on the advantages of the two basic types (competitive* vs *noncompetitive). We address the methods for distinguishing bound from unbound fractions (homogeneous* vs *heterogeneous immunoassays) and discuss specific labels for signal generation and detection. Special cases of immunoassays not requiring specific labels are reviewed briefly. Finally, examples and illustrations of commonly used automated immunoassays are provided. This is the third part of a continuing education series on chemistry. Other articles focus on rapid immunoassays, immunosensors, and prostate-specific antigen. After reading this article, participants should be able to distinguish competitive from noncompetitive immunoassays, understand the differences between homogeneous and heterogeneous immunoassays, and identify popular, automated immunoassays.*

Labels commonly used in immunoassays include radioisotopes, fluorophores, and enzymes. Signal detection methods include radioactivity, colorimetry, fluorometry, chemiluminometry, and nephelometry. Special immunoassays not requiring specific labels include immunonephelometry, immunoturbidometry, and agglutination.

Competitive (Type II) Immunoassays

In competitive immunoassays, the patient's unlabeled analyte of interest (the antigen) competes with a constant added amount of labeled similar antigen for a limited amount of specific antibody (see "Immunoassays Compared"). Because the two antigens compete for the same antibody, the labeled antigen must react identically to the unlabeled one. In the most common form of this assay, termed "equilibrium assay," the analyte to

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measure and the labeled antigen are added simultaneously to the specific antibody for which they compete: the reaction then is allowed to reach equilibrium following the law of mass action. At equilibrium, the amount of bound, labeled antigen-antibody complex is inversely proportional to the amount of the analyte of interest present in the specimen (see "Immunoassays Compared"). In the rare "sequential assay," the analyte to measure is incubated first with the antibody. After incubation, labeled antigen is added. This helps make the assay more sensitive.

Competitive immunoassays require only a small amount of antibody. These assays became popular because of their high sensitivity (ie, the ability to detect small amounts of analyte); a small amount of analyte is capable of producing a large signal (if the bound fraction is being measured). Their sensitivity is related and inversely proportional to the affinity constant of the antibody. For these assays, complete purification of the antibody is not essential; they remain highly specific regardless, and are not affected significantly by substances that cross-react with the antibody or by the presence of a heterogeneous population of antibody. Any factors that affect the kinetics or the equilibrium constant of the reaction will affect the sensitivity and specificity of the assay, however.

Competitive immunoassays often are slow and require long incubation to reach equilibrium. This problem has been solved by the use of kinetic measurements that assess the initial rate of the reaction thus expediting results. Competitive immunoassays are most effective in measuring small analytes such as drugs or hormones. One example of a competitive immunoassay is traditional radioimmunoassay (RIA).

Understanding Immunoassays

Following is a quick guide to some of the terms used in this article.

Agglutination The visual clumping of particulates.

Chemiluminometry The process of measuring the energy created by means of oxidation of an organic compound and emitted as light when the excited product returns to its stable, neutral state.

Colorimetry The process of measuring light intensity, usually at a particular wavelength.

Competitive (type II) immunoassay An immunoassay in which the patient's unlabeled analyte competes with a constant amount of labeled analyte for a limited amount of reagent.

Enzyme A protein capable of activating a substrate thus catalyzing a reaction.

Fluorometry The process of measuring the light emitted by a fluorophore.

Fluorophore A substance capable of absorbing light at one wavelength and emitting it at a second (larger) wavelength.

Immunoassay Any assay that, at its core, depends on the binding of antigen and antibody.

Nephelometry The process of measuring the scattering of light that occurs when incident light collides with a particulate substance of relatively small size.

Noncompetitive (immunometric, type I) immunoassay An immunoassay in which the patient's analyte is allowed to bind with an excess amount of labeled reagent.

Radioactivity The energy produced as an atomic species with an unstable nucleus decays to a more stable form.

Radioisotope An atomic species with an unstable nucleus capable of producing radiation as it decays to a more stable form.

Turbidometry The process of measuring the decrease in transmitted light that occurs when incident light is blocked (or reflected) as it interacts with a particulate substance of relatively large size.

Noncompetitive (Immunometric, Type I) Immunoassays

Immunometric immunoassays use an excess of labeled antibody toward the analyte of interest (the antigen). A single-labeled antibody may be used (see "Immunoassays Compared"). Additionally, in the immunoassay that is sandwich type, two antibodies, one labeled and one unlabeled but fixed to a solid support (eg, linked to plastic beads, tubes, plate wells), can be used. Following completion of the antigen-antibody reaction, the amount of bound labeled antibody is directly proportional to the amount of analyte present in the specimen (see "Immunoassays Compared").

Immunometric immunoassays are very specific; however, they require large amounts of pure, specific antibodies. This was a drawback prior to the development of monoclonal antibodies. In theory, these assays also are extremely sensitive: ideally the smallest amount of antigen detectable is one molecule. In reality, however, their sensitivity is limited by the technical ability to detect low signals. In contrast with competitive immunoassays, immunometric immunoassays are influenced less by substances or conditions affecting the kinetics of the antigen-antibody reaction because the antibody is present in excess. For the same reason, reactions proceed quickly to completion, making these assays faster. The finite amount of antibody available for binding limits the linear range of these assays, however, and

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occasionally has been responsible for a decreased response with high concentrations of analyte (antigen excess), a phenomenon known as the "high-dose hook effect." Immunoassays that are sandwich-type are limited to the measurement of larger antigens with at least two distinct antibody binding sites, such as tumor markers.

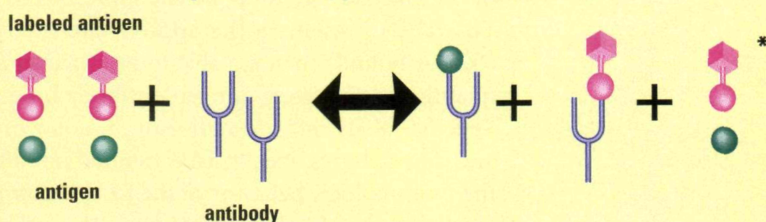
Separation

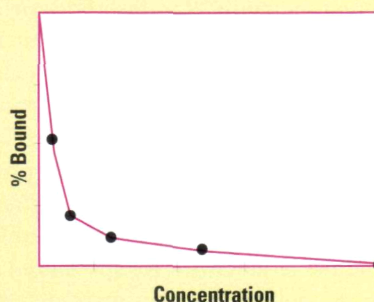
After the antigen and antibody react, the bound and unbound labeled fractions must be distinguished. To achieve this, heterogeneous immunoassays require a physical separation step. Such assays rely on physical, chemical, or immunologic differences such as size, charge, and adsorption to solid surface for the separation; this may occur in either the liquid or solid phase. For instance, in solid phase immunoassays, a simple wash is all that is needed to separate the bound analyte, which is immobilized by the reagent attached to a solid support, from the unbound, which is washed away. These assays require complete separation. In addition, the separation step should not disturb antigen-antibody equilibrium. A potential advantage of heterogeneous immunoassays is the removal (at the time of separation) of substances that may interfere with the measurement. These assays can measure large and small molecules; they also are suitable for use on large samples, thus increasing assay sensitivity. The additional separation step, however, can be more time-consuming, making the test more complicated and possibly less precise. If automated, heterogeneous immunoassays require specially designed, complex, dedicated analyzers.

Homogeneous immunoassays do not require a separation step to distinguish bound from unbound fractions. They rely instead on changes in label characteristics as a result of binding, for example, conformational change or inhibition of an enzyme, or decrease in rotational movement of a fluorophore. These changes usually are due to a size difference between the (small) unbound antigen and the (large) antigen-antibody complex. For this reason, these assays are better designed to measure small analytes. Homogeneous immunoassays are quick, easy to perform, easy to automate, and easily adapt to chemistry analyzers that are widely available. Because separation does not take place, these assays are subject to potential interfering substances present in the sample.

Immunoassays Compared

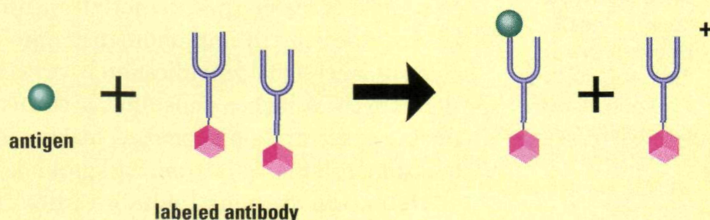
Competitive (Type II) Immunoassay



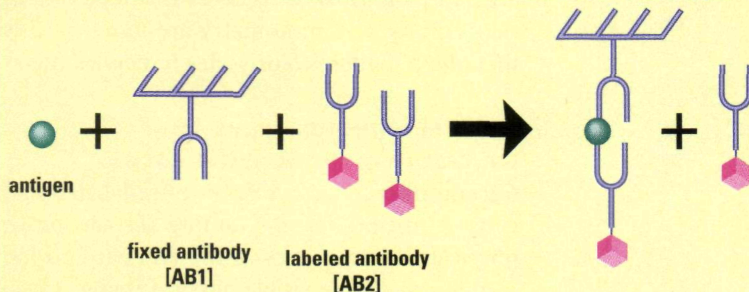


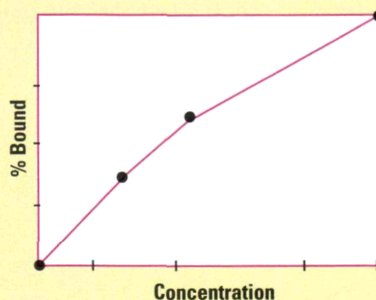
* Concentration of the analyte of interest is inversely proportional to the percentage of bound labeled antigen.

Noncompetitive (Immunometric, Type I) Immunoassay



Immunometric Immunoassay That Is Sandwich Type





\+ Concentration of the analyte of interest is directly proportional to the percentage of bound labeled antibody.

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Labeling and Detection

To quantitate the desired analyte, most immunoassays use specific labels attached to either the antigen (competitive immunoassays) or the antibody (immunometric immunoassays). The labels present in the appropriate fraction (free or bound) then are able to be detected and quantitated following antigen-antibody binding. Specific labels include radioisotopes, enzymes, and fluorophores. Ideally, labels should not affect the immunologic behavior of the labeled component nor should they interfere with antigen-antibody equilibrium. Radioisotopes originally were the label of choice because of their ease of measurement and lack of sensitivity to environmental conditions. Because of the difficulty and expense of dealing with radioactive materials (including disposal problems), the potential health hazards resulting from exposure to radioactivity, the short shelf life of radioactive labels, and the requirement for specialized instrumentation, methods using enzyme or fluorophore labels have become increasingly popular.

The signal generated by the label is essential in determining assay sensitivity. To maximize the signal, and therefore sensitivity, labels with higher signals (greater specific activity), multiple labels (especially in immunometric immunoassays), or label signal amplification have been used alternatively. A higher signal thus is created for a smaller concentration of analyte, making it easier to distinguish the signal from background.

Detection systems include radioactivity, colorimetry, fluorometry, chemiluminometry, and nephelometry, depending on the signal generated by the label. In general, methods that use fluorometry or luminometry are more sensitive than those that use colorimetry or nephelometry.

Test Your Knowledge
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Special Immunoassays That Do Not Require Specific Labels

Certain immunoassays do not use labeled antigens or antibodies, depending instead on the physical characteristics of the antigen-antibody complex or on the visible physical change caused by the antigen-antibody interaction. Such assays exploit, for instance, the fact that the binding of added antibody to the antigen of concern results in the formation of a larger bound, particulate complex that scatters light (immunonephelometry) or blocks light thus increasing the turbidity of the sample (immunoturbidometry). Whenever the antibody agglutinates the antigen, the reaction can be visualized and semiquantitated (agglutination).

Popular, Automated Immunoassays

Enzyme-Multiplied Immunoassay Technique

Enzyme-multiplied immunoassay technique (EMIT) (see "Automated Immunoassays") is a competitive, homogeneous immunoassay that uses an enzyme label and a colorimetry-based detection system. The enzyme label is bound covalently to the antigen at a position close to the active site of the enzyme. During the immunologic reaction, when the enzyme-labeled antigen binds to the antibody, the enzyme's active site is blocked physically and the enzyme is inhibited functionally. In the free form of the enzyme-labeled antigen, however, the enzyme remains functionally active and will act on an added substrate generating a colored product that is measured spectrophotometrically. The resulting color change is proportional to the amount of free-labeled antigen available that, due to the competitive nature of the immunoassay, is proportional to the amount of analyte present in the specimen.

The EMIT assay does not need a separation step for distinguishing bound from free fractions. Kinetic, rather than endpoint measurements of the reaction are being used, thus rendering the method fast and applicable to stat measurements. It also is automated easily. The EMIT assay is used mainly for the detection of drugs (eg, therapeutic drug monitoring, toxicology).

Fluorescence Polarization Immunoassay

Fluorescence Polarization Immunoassay (FPIA) (see "Automated Immunoassays") is a competitive, homogeneous immunoassay that uses a fluorophore label and a polarized, fluorometry-based detection system. In this assay, the (ideally small) antigen is labeled with a fluorophore. The fluorophore-labeled antigen and the patient analyte compete for a limited amount of antibody. Binding of antigen to antibody results in the formation of a large complex with respect to the free antigen. This large complex will become relatively fixed in solution while the unbound free antigen

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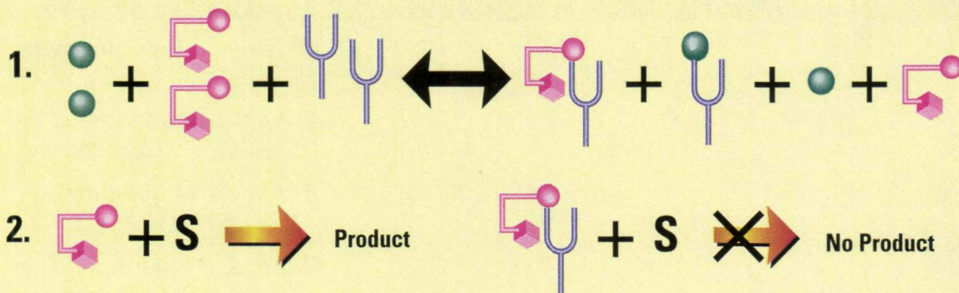
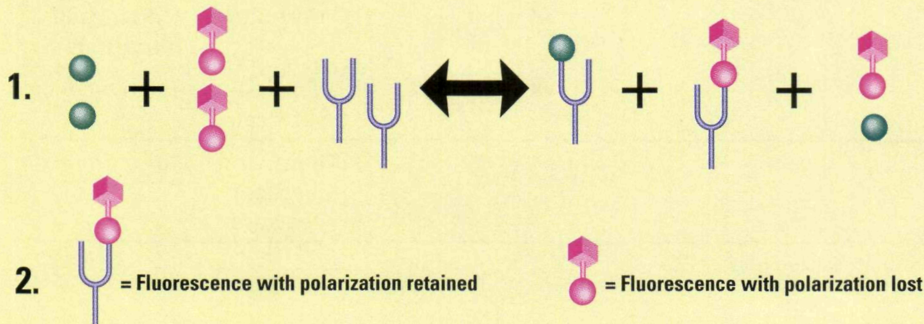
Automated Immunoassays

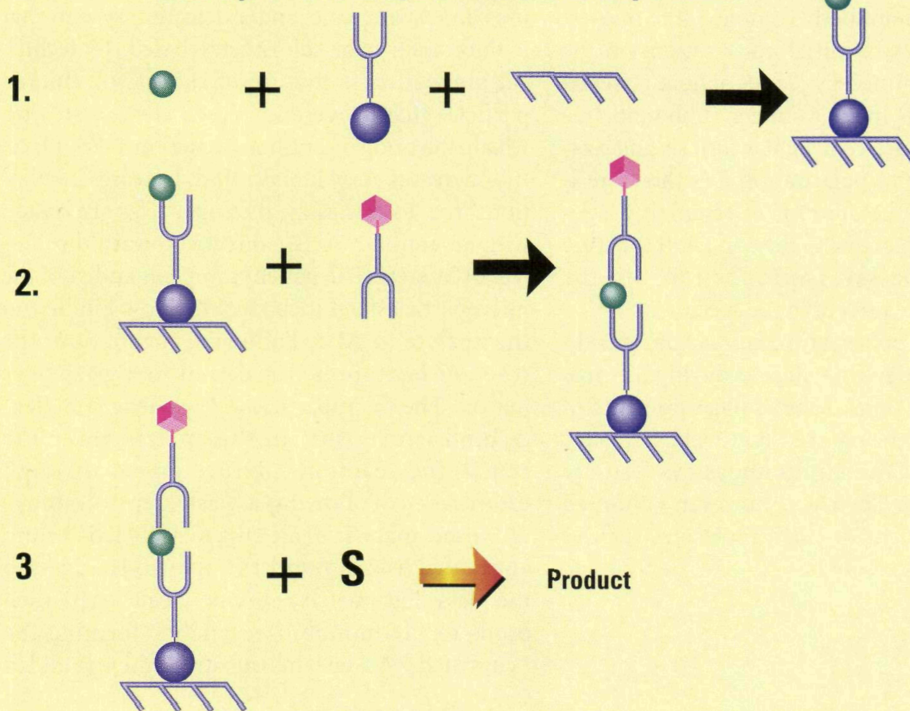
Enzyme–Multiplied Immunoassay Technique (EMIT)



Fluorescence Polarization Immunoassay (FPIA)

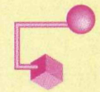


Microparticle Enzyme Immunoassay (MEIA)



Key

antigen



enzyme-labeled antigen

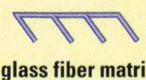
antibody

S

substrate

fluorophore-labeled antigen

antibody-coated microparticle (AB1)



glass fiber matrix

enzyme-labeled antibody (AB2)

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COMMONLY USED IMMUNOASSAYS						
Assay	**Competitive (Type II)**	**Immunometric (Type I)**	**Homo-geneous**	**Hetero-geneous**	**Label**	**Detection (Most Common)**
EIA*—Enzyme immunoassay					Enzyme	Colorimetry or Fluorescence
ELISA—Enzyme-linked immunosorbent assay	X	X		X	Enzyme	Colorimetry or Fluorescence
EMIT—Enzyme-multiplied immunoassay technique	X		X		Enzyme	Colorimetry
MEIA—Microparticle enzyme immunoassay		X		X	Enzyme	Fluorescence or Colorimetry
RIA*—Radioimmunoassay					Radioisotope	Radioactivity
FIA*—Fluorescent immunoassay					Fluorophore	Fluorescence
FPIA—Fluorescence polarization immunoassay	X		X		Fluorophore	Polarized fluorescence
SLFIA—Substrate-labeled fluorescent immunoassay	X		X		Enzyme substrate	Fluorescence
CIA*—Chemiluminescence immunoassay					Chemilumi-nescent reactant	Photometry

*Generic category; encompasses all immunoassay types.

still can rotate freely. The assay uses polarized light as an excitation source. In response, the excited fluorophore will emit fluorescent light. When emitted by a fluorophore stabilized in a large complex (the antigen-antibody complex), the fluorescent light will retain initial polarization; on the other hand, if emitted by a fluorophore attached to the small, freely moving antigen (unbound, free antigen), the fluorescent light will lose polarization. The degree of polarization loss therefore is proportional to the amount of remaining free-labeled antigen that, due to the competitive nature of the immunoassay, is proportional to the amount of analyte present in the specimen.

Fluorescence polarization immunoassays do not require a separation step to distinguish free from bound fractions. Kinetic measurements of the reactions are being used that yield fast, stat-capable results. These immunoassays also are automated easily. Their use, however, is limited to the measurement of small molecules such as drugs and small hormones.

Microparticle Enzyme Immunoassay

Microparticle enzyme immunoassay (MEIA) (see "Automated Immunoassays") is a heterogeneous immunoassay that is "sandwich type." It uses an enzyme label and a detection system that is fluorometry- or colorimetry-based. Its technical innovation is the use of submicron microparticles that provide increased surface area on which the antigen-antibody reaction takes place; this increases assay kinetics and decreases incubation time. In this assay, microparticles are coated with an antibody (AB1) directed toward the analyte of interest. The patient sample is added to the microparticles and incubated to allow binding of the analyte to AB1. Following incubation, the reaction mixture is transferred to a glass fiber matrix. The microparticle-AB1-analyte complexes bind irreversibly to this matrix while the remaining reaction mixture passes through unhindered. Following a wash step to remove unbound materials, an enzyme-labeled second antibody (AB2) toward the analyte is added to the glass fiber matrix; enzyme-labeled AB2 then binds to the immobilized analyte forming the "sandwich." An enzyme substrate then is added

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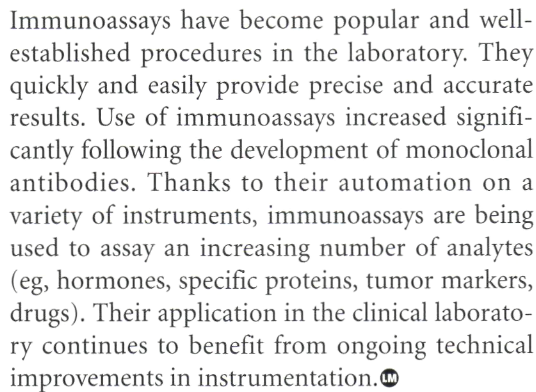
and the product of the enzymatic reaction is detected either fluorometrically or colorimetrically, depending on the substrate used. The measured signal is directly proportional to the amount of analyte in the specimen. One popular MEIA assay uses alkaline phosphatase as the enzyme label and 4-methylumbelliferyl phosphate (MUP) as the substrate. MUP is catalyzed into the fluorescent methylumbelliferone product, which then is measured.

Microparticle enzyme immunoassays require a separation step to distinguish free from bound fractions. This is achieved using a solid-phase, sandwich technique. The method has been automated. Current applications use kinetic measurements (enzyme rate reaction) and yield fast, stat-capable results. These immunoassays are used to quantitate a wide variety of molecules, including polypeptide hormones and tumor markers.

Commonly used immunoassays are shown in the Table.

Conclusion



Immunoassays have become popular and well-established procedures in the laboratory. They quickly and easily provide precise and accurate results. Use of immunoassays increased significantly following the development of monoclonal antibodies. Thanks to their automation on a variety of instruments, immunoassays are being used to assay an increasing number of analytes (eg, hormones, specific proteins, tumor markers, drugs). Their application in the clinical laboratory continues to benefit from ongoing technical improvements in instrumentation.

Suggested Readings

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