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Ways to Reach Lower Detection Limits of Lateral Flow Immunoassays

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Abstract

This chapter considers factors influencing sensitivity of lateral flow immunoassay and modern developments that are focused on reaching lower detection limits. The existing variety of proposed approaches is classified in accordance with the “big five rules” for these assays, including proper sample, receptor, interaction, response, and output. The solutions for rapid extraction of target analytes and preventing negative influence of extractants are considered. Role to antibodies affinity and specificity is characterized. Potential of alternate bioreceptor molecules is discussed. Immunoreactants’ compositions, concentrations, and locations on the test strip are characterized as factors determining assay parameters. The existing variety of labels is compared in terms of their optical and alternate registration. Tools to modulate a sequence of analytical reactions and to form aggregates of the detected labels are considered. The discussed approaches are illustrated through developments of test strips for detection of mycotoxins, veterinary drugs, and other analytes.

Keywords: immunochromatography, test strips, nonequilibrium interactions, increasing assay sensitivity, nano-sized labels, signal amplification, digital optical measurements

1. Introduction

The history of lateral flow immunoassay (LFIA, immunochromatography) began in the 1980s. The first solved task was to transfer pregnancy tests from a specialized laboratory directly to the point of sample collection [1]. The test strip developed for this purpose fully complied with the requirements for nonlaboratory diagnostics, and its basic principles remain to this day.

The overall design of the immunochromatographic test strip is shown in **Figure 1**. It is a composite of several membranes of different structures and porosities, fixed on a support. The bundling of the test strip can vary, so it makes sense to consider its design based on what analytical tasks are being performed on its different sites.

- A. Typically, the lower portion of the test strip contains a **sample pad**. It ensures the absorption of sample components, in which the presence of the target analyte is checked.
- B. The following is a section with immunoreagents that are washed out during the analysis and move upward along with the components of the sample. As a rule, a **conjugate pad** forms this zone. It contains a conjugate of antibodies against the target analyte with a nanodispersed label—particles of colored latex, colloidal gold, and so on.

The next two sections are located on the main **working membrane** of the test strip.

- C. First, there is a zone along which the movement of the absorbed components of the sample and the washed immunoreagents continues. During this movement, immune reactions occur, and specific intermolecular complexes are formed.
- D. Next, a mixture of reacted and unreacted molecules enters the **binding area** with immobilized immunoreagents. Depending on whether the target analyte was present in the sample and in what amount, binding of labeled immune complexes occurs in certain areas (in the traditional case, with the formation of narrow colored lines). Usually, additional reagents are located here to control the functionality of the test system.
- E. The upper part of the test strip with the **final pad**, usually structurally similar to the sample pad, ensures the further movement of the reaction mixture under the action of capillary forces and the washing of unreacted components from the underlying areas. These processes allow the label's binding to be evaluated correctly.

Membrane components of the test strip are fixed on a **plastic support** and partially overlap with each other for effective fluid movement.

Depending on the tasks to be performed, additional reagents can be used in the test strip, and some of the membranes can be added, combined, or eliminated. However, at the same time, the general design and principle of conducting analytical interactions during the movement of reagents along membranes is preserved.

Simplification of the analysis is achieved by refraining from additional processing and incubation enhancing the signal as well as by visual (device-free) evaluation of the results. Because of this, traditional LFIA, meeting the needs of practice in simplicity and speed, is generally considered inferior to alternative immunoassays (such as ELISA) in sensitivity.

At first, this restriction was not critical. Test systems made it possible to control target compounds in diagnostically demanded concentration ranges, which was enough for their mass application. The implementation of standard LFIA protocols for the detection of new compounds was viewed as an exclusively technological task for manufacturing companies, uninteresting in the scientific sense. In this regard, the number of publications on LFIA in the late 1990s to early 2000s was relatively small. It was believed that the all main methodological problems of LFIA had already been solved.

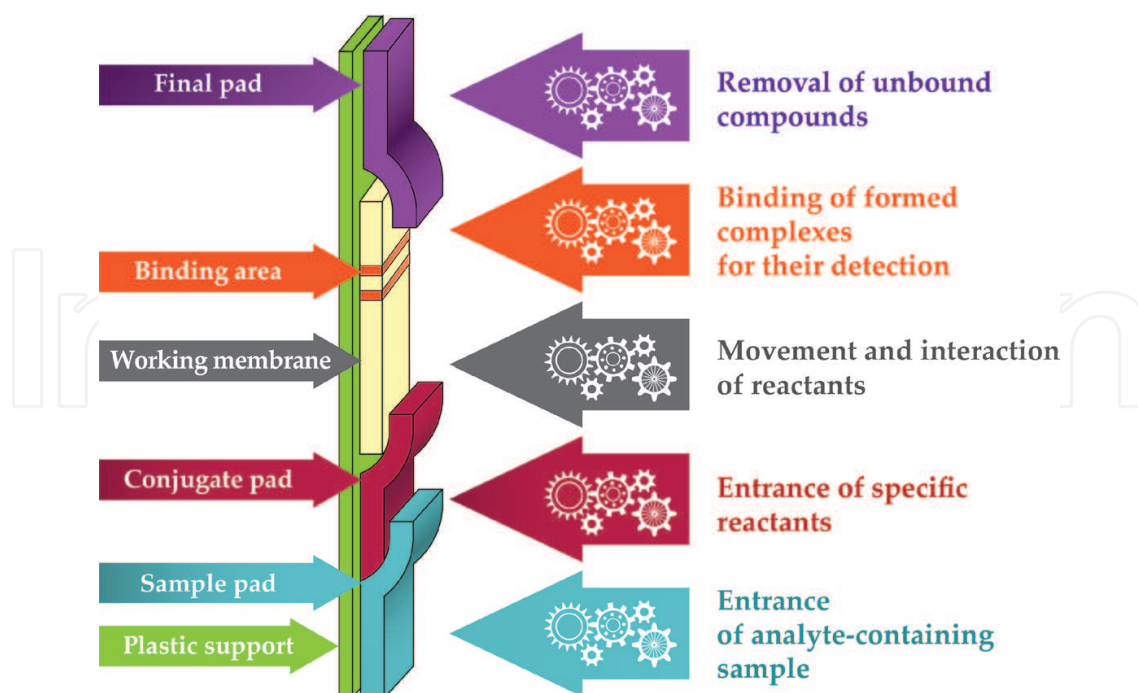


Figure 1. Components of the immunochromatographic test strip and their functions.

2. “Big Five Demands” for new solutions in LFIA

However, the application of LFIA did not stop at the control of the formed row of objects. This method actively developed (especially in the last decade) and covered an increasing number of analytes. What were the reasons for this?

- As applied to medicine: the general trend toward the diagnosis at the site of the requirement (point-of-care (POC)): (i) the use of tests for quick decisions outside the clinic; (ii) the provision of mass screening tests by rapid and inexpensive diagnostic tools; and (iii) providing the doctor with information for decision-making during the time of communication with the patient without transporting samples to the laboratory.
- With regard to other areas of application: interest in promptly obtaining information about mass consumption products, for example, about the quality of raw materials coming to food enterprises and the end products being sent to the trading network.
- Biosafety control is extremely important in modern society. The conclusion about the presence of a toxin should be given promptly and directly at the testing site.

Taking into account this expansion of controlled analytes and types of tested samples, tasks were frequently encountered for which highly sensitive detection was required but not provided by traditional analytic formats.

During the last decade, the development of LFIA modifications has been intensified, allowing highly sensitive analysis, while maintaining the basic merits of the analysis—the rapidity, ease of implementation and interpretation of the results. These developments are systematized in

a number of recent reviews that characterize the general trends in the development of LFIA, its application in different practical spheres, and the most successful methodological decisions [2–8].

On the one hand, this progress is accompanied by the expansion of the assortment of commercial tests and the more active application of LFIA for solving a variety of practical problems. On the other hand, a significant part of new developments remains at the level of single publications and approbation using the example of a single analyte, without realistic assessment of their advantages and limitations. From such isolated examples, it remains unclear how much gain in sensitivity will be achieved if we apply the proposed approach to the new analytes and what conditions must be used for this. A simple demonstration of the minimum detectable concentrations in traditional and modified LFIA leaves open the question of how correctly all the conditions for the analyses were selected, including the concentration and composition of the immunoreagents. It is also unclear which of the approaches for reducing sensitivity can be combined and whether this combination leads to a multiplication of results improvements achieved for each of these approaches individually.

Of course, general theoretical arguments are not enough to answer these questions. Further studies of many research teams are needed. However, it is important to evaluate new developments with the use of a grounded concept to understand (i) what changes are introduced into the traditional LFIA protocol and for what purpose; (ii) by what criteria are the new LFIA protocols assessed and compared with existing ones. Such ordering is the subject of this review. We did not attempt to form a limited list of developments that are most widely represented in recent publications. Our goal was to create a general classification within which different existing and future developments can be characterized.

The structure of the immunochromatographic test system considered (**Figure 1**) allows us to identify groups of problems that should be solved to ensure high-sensitivity, as well as other practically significant characteristics of the analysis (productivity, selectivity, etc.).

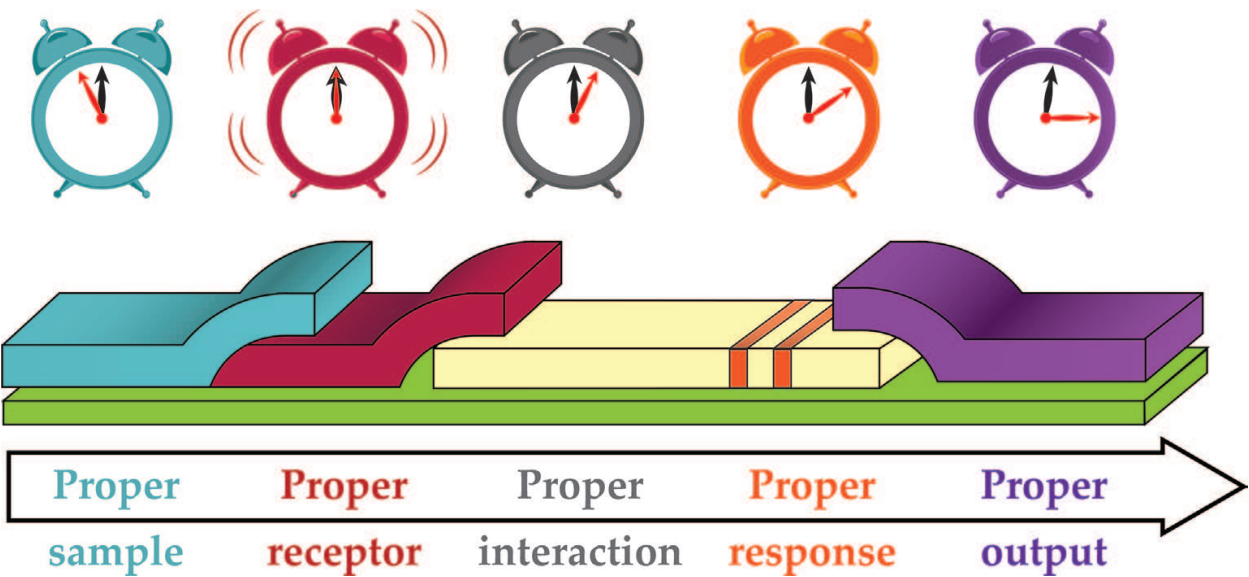


Figure 2. Compounds of immunochromatographic test and “big five demands” associated with them.

We matched each element of the test system and the reagent or process used at this element. Therefore, the choice of the most appropriate (proper) actions during the analysis includes

- A. Choice of the sample preparation method—**proper sample**. A procedure should be chosen whereby a liquid containing the target analyte will be obtained from the initial liquid, semisolid, or solid matrix and used for contact with the immunochromatographic test strip.
- B. The choice of receptor molecules used to selectively bind the target analyte—**proper receptor**. It is necessary to establish which antibodies and their derivatives or alternative compounds will interact with the target analyte during the analysis, and what their derivatives will be and by what methods they should be obtained for inclusion in the test system.
- C. Choice of the conditions for interaction of reagents during the analysis—**proper interaction**. Optimum quantities (concentrations) of reagents, duration of interactions leading to the formation of detectable complexes, and conditions (composition of the medium) for carrying out these interactions should be chosen.
- D. Choice of the registered response of the test system—**proper response**. It is necessary to decide with the help of which label the complexes formed during the analysis will be detected and their quantities will be estimated. Which way of registration will be applied? What additional reagents and actions will be used to increase the response of the test system?
- E. Choice of the procedure for processing the measurement results—**proper output**. It should be suggested how the detected signal will be transformed into a decision about the presence and content of the target analyte and how the most informative results will be obtained.

These five groups of requirements (“big five demands”, **Figure 2**) make it possible to simply and uniquely classify the methodical solutions proposed for the improvement of the LFIA protocols.

This review will be based on our results (from the Laboratory of Immunobiochemistry in the A.N. Bach Institute of Biochemistry of the Federal Centre of Biotechnology of the Russian Academy of Sciences, Moscow, Russia) and on examples from the literature that will be ordered and characterized in accordance with this classification.

3. Proper sample for LFIA

Some types of liquid samples, characterized by the LFIA method, do not require sample preparation: urine, blood serum, natural and drinking water, milk and juices. Their analysis can be initiated by contacting the test strip with the sample as is. To accelerate the movement of the fluid (blood serum and milk), the sample can be diluted immediately before analysis [9]. However, in most cases, the analysis should be preceded by sample preparation.

The main difficulty of sample preparation is the need for a short period to destroy the matrix structures that interfere with the analyte molecules contained in it to interact with antibodies.

Actions that separate matrix components that interfere with analysis, or to destroy these components, are also reasonable. Such complex types of matrices may be tested as tissues of organisms, food and agricultural products, soil, and so on. Sample preparation is extremely important to easily detect the target compounds in these matrices.

The requirements for sample preparation were studied in detail with respect to other analytical methods—liquid and gas chromatography, enzyme immunoassay, and so on. However, the accumulated research results cannot be transferred to LFIA without further development. The main advantage of LFIA—rapidity—cannot be lost because of the long (lasting several hours) extractions recommended in many chromatographic techniques. Work with samples cannot begin from complex procedures that require expensive equipment.

An additional feature of sample preparation for LFIA is that many analytes are extracted efficiently only with organic solvents and water-organic mixtures, but not with aqueous-salt solutions. (Such situations are usually associated with the hydrophobicity of the compounds and their surroundings in the samples.) However, these solvents inactivate antibodies; it means that the extract cannot be directly used as is as a sample for LFIA. As a result, the extracts are either significantly diluted (which is accompanied by a loss in sensitivity), or by means of additional steps, the analyte is transferred to another medium.

The complexity described above determines the tasks that should be solved for effective sample preparation—see their summation in **Figure 3**. In **Figure 3** and the following ones, we depict

- **Strategic tasks** that require complex and risky time-consuming and labor-intensive work in the framework of research projects and
- **Tactical tasks**, which can be solved by varying several known parameters when finalizing the final product.

With respect to proper samples, the success of the developments offered directly by test system manufacturers should be noted. Alexeter Technologies (United States) uses special adhesives placed at the beginning of the test strip, which allow one to collect target molecules of the analyte from a large surface area by simple contact. In many cases, portable homogenizers and low-speed centrifuges are proposed for completing the analytical laboratory. In the case of the 4MycoSensor test systems (Unisensor, Belgium), mycotoxins are extracted from the ground grain in a special Mycobuffer on a shaker for 3 min (5 min for corn). Similar solutions are offered by other manufacturers. A special aqueous two-phase system for the concentration of protein analytes, containing polyethylene glycol, potassium phosphate, and phosphate-buffered saline, was used by Chiu et al. [10]. With its help, a 100-fold reduction in the detection limit was achieved. Concentration of samples combined with dialysis was used by Tang et al. [11] on the examples of myoglobin detection (fourfold signal growth) and nucleic acid of HIV (10-fold growth). Mosley et al. [12], using the examples of *Chlamydia trachomatis* and human immunoglobulin M analyses, showed that the formation of an aqueous two-phase system on the test strip by applying a PEG-potassium phosphate and UCON-50-HB-5100-potassium phosphate obtained a 10-fold reduction in detection limits. In Jue et al. [13], micellar two-phase systems were used for this purpose, which reduced the detection limit of bacteriophage M13 by a factor of 10. An original solution based on

concentrating the analytes in an electric field was proposed by Kim et al. [14]. Using a conventional 9 V battery and commercial tests for choriogonadotropin, they acquired a 25-fold concentration of the target compound.

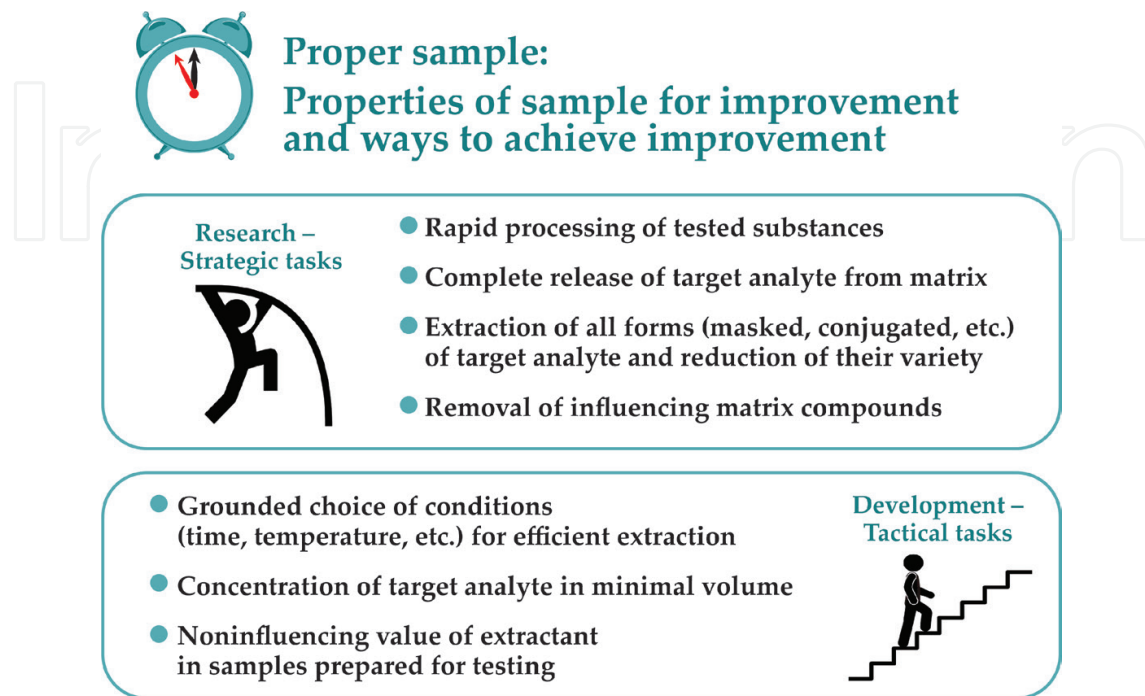


Figure 3. Main research and development tasks to obtain proper samples for LFIA.

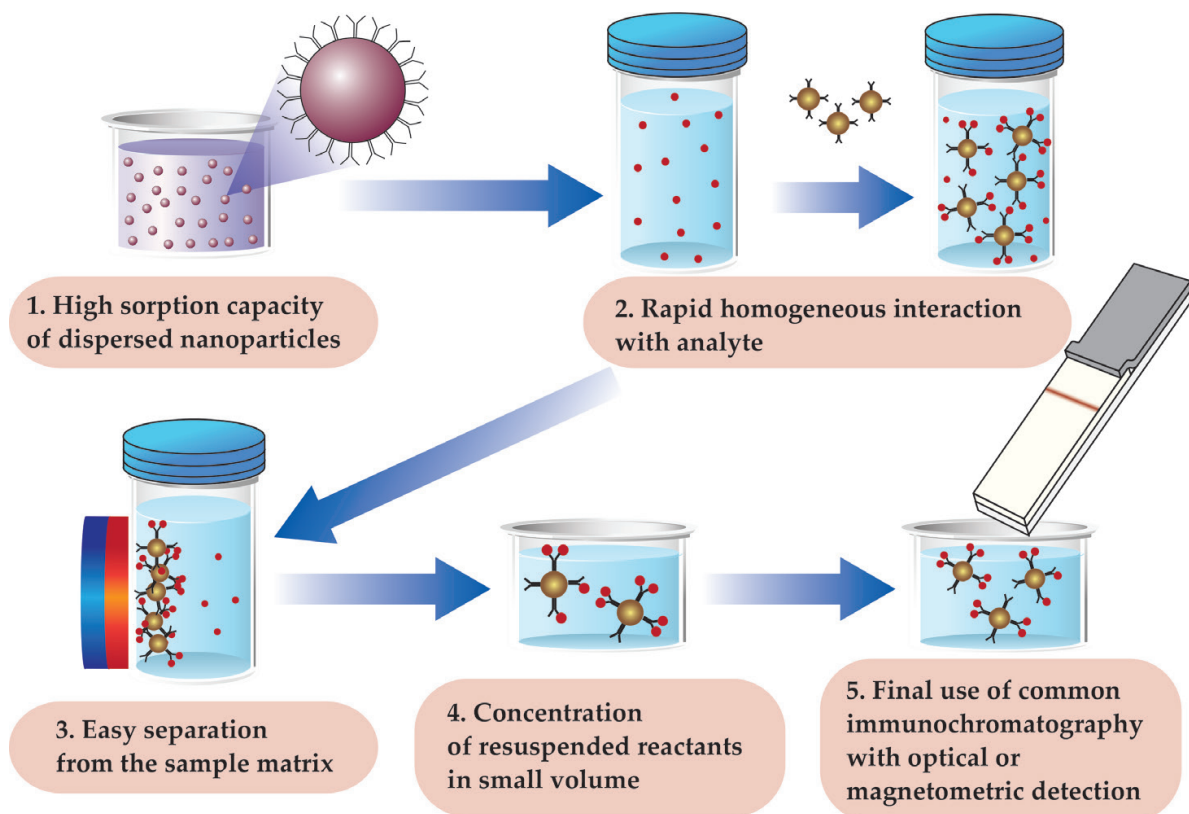


Figure 4. Advantages of magnetic immunosorbents application in LFIA.

Efficient approaches for sample preparation are pseudo-homogeneous analytical techniques, where a dispersed carrier with immobilized receptor molecules is added to a large volume of tested samples. This carrier quickly and efficiently, without diffusion restrictions, captures the analyte from the entire volume of the sample, and then the carrier is separated from the solution rapidly. Note that when the separated carrier is then redissolved in a small volume, the analyte is not only concentrated but also cleared from the organic solvent, thus excluding the influence of this solvent on LFIA. Antibodies, immobilized on a carrier, are often more stable to the denaturing influence of organic substances than free antibodies. According to the data of Urusov et al. [15], when working with magnetic immunosorbents, the content of methanol in the test sample can be increased from 10 to 30%.

The use of particles of iron oxide and other carriers with magnetic properties is extremely promising for immunochromatography because of the simple and rapid separation of the carrier by contact with a permanent magnet. The principle of such an analysis is shown in **Figure 4**, and approaches to the production of magnetic immunosorbents are systematized in the review [16].

Liu et al. [17] showed that the combination of magnetic concentration and immunochromatography yields a 25–50-fold gain in the detection limit of aflatoxin M1 in milk compared to the variants in which magnetic or gold nanoparticles are used as conventional labels. A 40-fold gain in the detection limit was demonstrated by Lu et al. [18] upon the detection of *Listeria monocytogenes*. In Petrakova et al. [19], using the examples of zearalenone and T-2 toxin, the authors showed that magnetic nanoparticles can be used as directly detectable optical markers. Razo et al. [20] combined the use of magnetic immunosorbents to bind analytes, potato virus X, and functionalized gold nanoparticles, which, thanks to the biotin-streptavidin reaction, provide the formation of aggregates of two kinds of nanoparticles. This analysis was 32 times more sensitive than the nonenhanced one. As a whole, the described gains in sensitivity with the use of magnetic immunosorbents did not exceed two orders of magnitude. A greater concentration requires a significant increase in the consumption of immunoreagents and/or time for binding the analyte.

Concentration can also be achieved if LFIA is preceded by a stage with a transverse flow of large volumes of samples through a small volume of a membrane with antibodies or other binding reagents applied to it (immunofiltration). Such analyses usually complete the detection of binding results directly in the filtration zone [21, 22]. Note that the use of LFIA for control of toxicants in solid foods is associated with a certain restriction. To correctly determine the content of the unevenly distributed analyte, several samples of large volumes are selected from different parts of the tested object and combined for subsequent extraction [23, 24]. However, the small volume of liquid absorbed by the test strip allows only a small part of the analyte molecules present in the extract to be taken into account (even with magnetic concentration). Immunofiltration concentration will overcome this limitation and come close to obtaining the proper samples for highly sensitive analyses.

4. Proper receptor for LFIA

The basic requirements for antibodies used in LFIA are related to their affinity and selectivity. However, the topic of which characteristics of antibodies provide the most sensitive analysis

requires additional clarification. Immune reactions during immunochromatography are carried out in the kinetic regime. Therefore, it is unimportant whether the detectable complexes will dissociate for hours or days. Their number is determined primarily by the kinetic constants of the association, which for receptors that are the same in structure and antigens that are similar in size vary within a limited range. In the case of competitive LFIA, the dependence of the number of complexes formed on the analyte concentration in the sample is determined primarily by the affinity of antibodies to the free analyte. Effective binding to a competitor modified by the analyte will interfere with the highly sensitive detection of the free analyte in the sample. In other words, the binding of antibodies to the analyte-protein conjugate should be somewhat worse than with the native analyte. The influence of the characteristics of immunoreagents on the sensitivity of analysis is considered in detail in works devoted to the mathematical modeling of LFIA [25–30].

Given the above limitations, the affinity of antibodies is an important characteristic that affects their analytical use. However, the possibility of natural production of antibodies with more and higher binding to the analyte is limited. This is because an increase in the half-life of an antigen complex with B-cellular receptors greater than the endocytosis time of the complex is not supported by the selection of the corresponding B-cellular receptor lines [31]. The cases of “infinite affinity” of antibodies are rare exceptions for the analytes that form covalent bonds after the immune interaction [32]. An additional way to increase affinity is the genetic modification (directed design) of the active center of antibodies. The use of these methods in routine development is still very limited, despite confirmations of their effectiveness [33].

As far as specificity is concerned, an important problem is which series of structurally close compounds should be detected using this antibody to solve practical problems. Two kinds of situations are possible: (i) it is necessary to recognize a single compound possessing biological activity, in contrast to its analogs and metabolites and (ii) information is required on the total content in the sample of a significant number of homologous compounds. For the second, class-specific assay, it is desirable that affinity of antibodies to homologous compounds correlate with their biological activities, but this is not always possible. In some cases, regulatory documents establish maximum residue levels (MRLs) on the basis of the sum of concentrations of structurally similar toxicants, without correction factors, taking into account their biological activity. Therefore, class-specific analysis usually requires the detection of the maximum number of compounds of this class with at least 10–15% cross-reactivity with respect to the maximum [8].

Additional practically important characteristics of antibodies are the values of their stability under storage and in the course of the assay. The stability may be effectively enhanced by chemical modification of antibodies as well as by addition of protective agents that are common for drying of different immunoreactants.

The strategic tasks for improving receptors for immunoassays are summarized in **Figure 5**. However, in the final development of test systems, commercially available antibodies are usually used, and there is no possibility of directed production of new, improved antibodies. This is the reason for the interest in the use of receptor compounds of a different nature as a substitute for traditional immunoglobulins.



Proper receptor:

Properties of receptor for improvement and ways to achieve improvement

Research – Strategical tasks



- Cheapness
- High affinity
- Better binding to free analyte as compared to conjugated one
- Specificity to analyte's functional form
- Desired list of recognized structural analogs (either narrow or wide specificity)
- Stability in various media
- Batch-to-batch reproducibility

- Modification (immobilization, labeling) without inactivation
- Oriented immobilization
- Best composition of conjugated preparations
- Absence of steric hindrance for interaction

Development – Tactical tasks



Figure 5. Main research and development tasks to obtain proper receptors for LFIA.

Thus, the single-domain antibodies produced by *Tylopoda* and sharks are characterized by significantly greater stability under different conditions [34]. Interest is caused by so-called protein scaffolds of a non-antibody nature that also combine conservative basic structure with hypervariable segments providing receptor functions [35]. However, their analytic application is a matter of the future. The development of test systems based on aptamers—receptor oligonucleotides, selected from random libraries—is being actively pursued. Aptamers are significantly cheaper and more stable reagents in comparison with antibodies, and their properties are well reproducible. Limitations in affinity typical of many of the known aptamers are overcome by improving the selection procedures and subsequent directed design which is a much simpler process than for antibodies [35]. The possibilities of using aptamers in membrane test systems are shown in a number of works and summarized in recent reviews by Jauset-Rubio et al. [36], Chen et al. [37], and Dhiman et al. [38]. For developments on the use of nanobodies, see Tang et al. [39], who presented LFIA for the simultaneous determination of aflatoxin B1 and zearalenone.

Requirements for proper receptors also include its effectiveness after immobilization on a membrane or on the surface of a marker nanoparticle label. Physical adsorption and random covalent coupling may be accompanied by significant loss of antibody reactivity. Therefore, systems are needed in which the oriented immobilization of antibodies is realized through the chemical conjugation of IgG in areas remote from the active center, or by their indirect binding with a preformed antibody-binding layer. In the role of this layer, staphylococcal protein A, streptococcal protein G, or (strept)avidin (reactive with biotinylated antibodies) can act. Approaches to the oriented immobilization of antibodies are systematized in a number of recent reviews [40–43].

Filbrun et al. proposed a procedure for chemical modification of the lysine residues of antibodies before conjugation with gold nanoparticles and showed that it provided conjugates

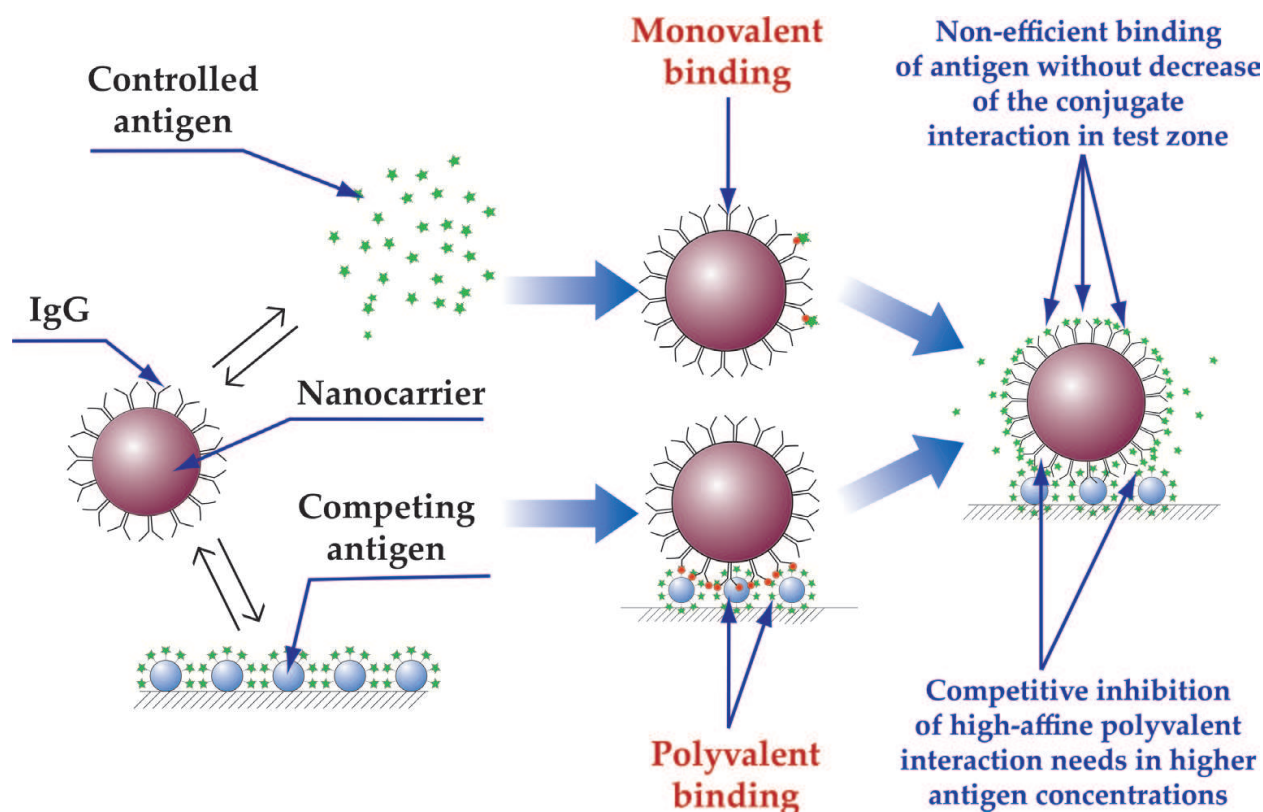


Figure 6. Limitations in the use of common antibody-nanoparticle conjugates in competitive LFIA.

that were stable over a wide pH range [44]. Bauer et al. [45] developed a technique for the preparation and use of antibody conjugates modified with histidine-rich peptides (called “capture and release” antibody reagents). These preparations are affine to metal surfaces and magnetic particles and so may release immobilized antibodies when necessary. The benefits of oriented binding of antibodies to magnetic nanoparticles through modification of antibodies’ carbohydrate components were shown by Puertas et al. using the example of LFIA for choriogonadotropin [46]. A comparison of methods of immobilization for receptors in bacteriophage-based LFIA is given in the works of Kim et al. [47, 48]. In particular, article [48] discussed the use of in vivo-biotinylated peptide for oriented immobilization of receptor molecules on a test strip.

The composition of conjugates of antibodies with nanoparticles also plays an important role. Although the increase in valencies for immune interaction is accompanied by an increase in conjugates’ affinity [49], structural changes of antibodies or steric restriction of their availability to interact with antigens may occur in parallel. With adsorption immobilization of antibodies (i.e. the widespread approach for LFIA purposes), their excessive loading causes the formation of additional layers, the molecules in which can dissociate during the analysis, and preventing the formation of a detectable labeled complex. Additional complications are associated with the use of antibody-nanoparticle conjugates having high surface density in competitive LFIA (**Figure 6**). Such conjugates can form high-affine polyvalent complexes in the analytical zone, which impede competitive interaction with the monovalent analyte from the sample. Further, the resulting complexes contain a significant number of unreacted antibodies and can bind analyte molecules without weakening the detected signal [50]. Therefore, the composition of the conjugate should be selected in relation to the features of each

analytical system as well as other variable parameters—see the list of tactical tasks in **Figure 5**. Describing the development of LFIA for aflatoxin M1 [51], Anfossi et al. found that the lowering the ratio between antibodies and gold nanoparticles caused improvement in the assay sensitivity. The proposed change was to decrease amount of antibodies used for immobilization twice as compared with saturating conditions and by that way to lower the limit of detection, too, almost twice with a minimal weakening of the staining.

5. Proper interaction for LFIA

Because LFIA is a fast analysis, all the processes that should be performed during the time of reagents' movement along the test strip and proper conditions for the interaction of these reagents are necessary (**Figure 7**).

- **First**, the interacting molecules should be in a state corresponding to their high reactivity in a medium without blocking and inactivating components.
- **Second**, the reagents should be included in the stream in accordance with the order and quantities that will ensure a highly sensitive detection of the analyte.
- **Third**, the movement of the reagents should be accompanied by their effective mixing with minimal nonspecific binding to membranes.
- **Fourth**, the location of the immobilized reagents on the test strip and the rate of movement of the soluble reagents (both given parameters depend on the composition of the reaction medium) should allow the time for the formation of detectable complexes to be increased.

These general requirements remain little studied. Studies of the localization of reagents and immune complexes in a 3D membrane structure are limited [52, 53]. A significant variation in reaction media causes problems with mobility and nonspecific sorption of reagents on commercial membranes, the structure and coating of which are established by manufacturers. The developer can only compare several membranes and select reagents that affect the release of dried components and the speed of the flow. An example of such recommendations is provided by Lee et al. [54]. The contribution of fast nonspecific processes of formation of the so-called “protein corona” on the surface of gold nanoparticles to the effectiveness of immune interactions in LFIA is described in a recent paper by de Plug et al. [55]. Choi et al. [56] characterized the effects of temperature and humidity on the analytical characteristics of test systems and somewhat unexpectedly found that the transition to room temperature, conditioned by the requirements of point-of-care diagnostics, may be accompanied by a deterioration in sensitivity. In their work, the analysis at 37–40°C and relative humidity beyond 60% was three times more sensitive. Posthuma-Trumpie et al. [57] focused on the effects of the composition of solutions used in the manufacture of test systems on the analysis parameters. Interesting opportunities for further development are provided by the use of so-called nanomotors for enhanced reagent mixing, which has so far been described only for other types of immunoassays [58, 59].

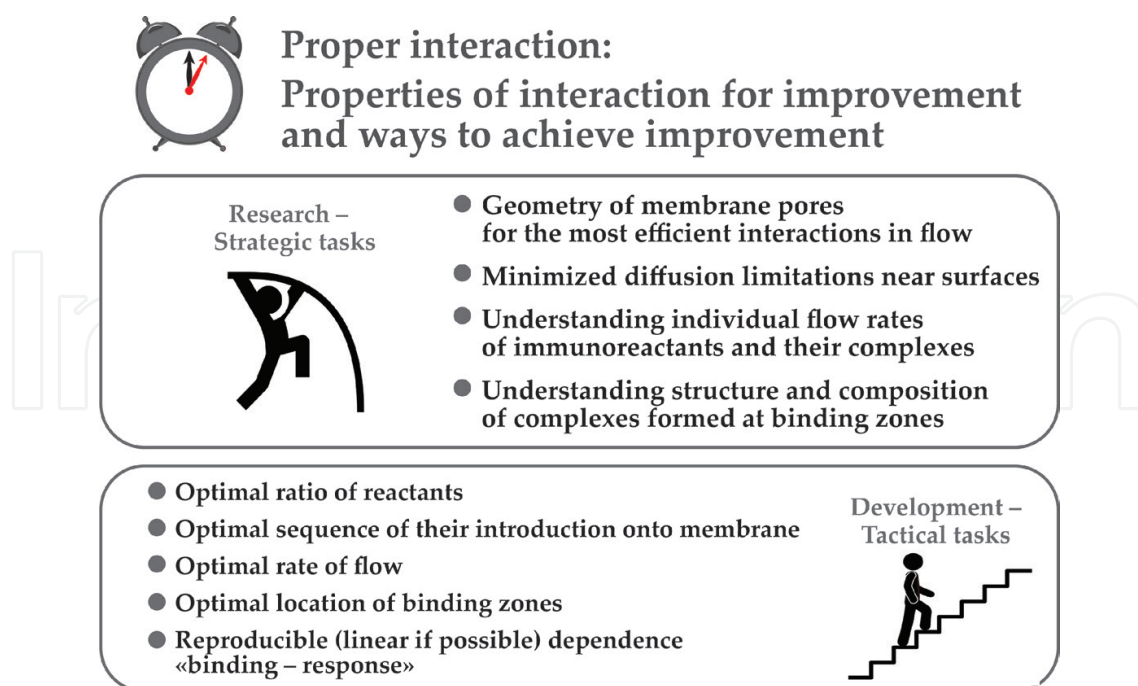


Figure 7. Main research and development tasks to obtain proper interaction for LFIA.

More accessible tools are the choice of concentrations of reagents applied to the test strip and their locations. By varying these parameters, it is possible to provide extremely sensitive detection or to select the threshold of discrimination between positive and negative samples (cut-off level) that meets the regulatory requirements for the maximum permissible level of contamination. A number of works have been published with analyses of the individual effects of these parameters on the analytical characteristics [60, 61] and with the application of multiparametric optimization procedures [62]. Hsieh et al. [52] described a general scheme for the consideration of various factors in the course of LFIA optimization.

In Zvereva et al. [63], the possibility to change the cut-off level by varying the composition of the hapten-protein and the antibody-(gold nanoparticles) conjugates is considered. Using an example of competitive LFIA of chloramphenicol, it was shown that by reducing the load of immunoreagents on carriers, it was possible to shift the detection limit by two orders of magnitude. For sandwich analysis, Liu et al. [64] showed theoretically and experimentally the optimality of the antibody: the nanoparticle ratio was equal to 30:1, but the universality of these recommendations requires further study.

Fu et al. proposed the use of a two-dimensional paper network to control the sequence of interactions in LFIA and, using the example of choriogonadotropin, showed the gain achieved in sensitivity [65]. Similar problems were solved in Rivas et al. [66] using wax-printed pillars as delay barriers (three-fold gain for human IgG detection) and Choi et al. [67] by incorporating agarose into the test strip to achieve flow control (10-fold gain for detecting dengue viral RNA). A sponge shunt was applied by Tang et al. [68] to reduce the fluid flow rate during LFIA (10-fold signal enhancement in nucleic acid testing of Hepatitis B virus). Liu et al. [69] considered the use of a pencil made from polyethylene glycols for the application of reagents

to control the rate of their subsequent release. Shin et al. [70] developed a rotary device for this purpose, the rotation of which makes it possible to initiate a reaction and then sequentially introduce into the system the necessary reagents. The volume of reagents introduced into the system during analysis can be controlled by the vertical flow immunoassay method proposed by Oh et al. [71] and successfully implemented by them for the detection of C-reactive protein. For the same antigen, Rey et al. [72] described an approach to managing the kinetics of interactions that allowed exclusion of the so-called hook effect (falsely low results for very high concentrations of the analyte). The existing variety of approaches to controlling the order of interaction of reagents in test systems is summarized in Jeong et al. [73].

The position of the binding zone influences the degree of equilibrium reached for the reactions occurring during the flow of reactants along the test strip. Moving these zones along the test strip, we can adjust the assay sensitivity. Theoretical aspects of this approach were considered by Ragavendar et al. [74]. However, despite successful overlapping of monotests in multi-tests with a sequential arrangement of binding zones [75, 76], general practical recommendations for ensuring a highly sensitive detection of all analytes have not yet been formulated.

Because synchronous movement in the flow of antigen, antibody, and immune complex molecules is difficult to provide, an alternative is to start the analysis with a quick (several minutes) preincubation of the analyte molecules in the sample with the free or labeled antibodies that are specific to analyte. A number of commercial systems operate on this principle, such as tests for antibiotic control in food produced by Bioo Scientific, United States, and Nankai Biotech, China. Developing this idea, it is possible to implement universal test strips without compounds specific for a concrete analyte. The combination of such test strips with specific reagents added during the incubation stage with the sample allows adaptation of the consumption of test strips to the tasks being solved. Such strips are manufactured by D-r Fuke, Germany, for the detection of immunoglobulin E against various allergens: a complex of immobilized streptavidin, a biotinylated allergen from a preincubation mixture, specific immunoglobulins E, and colloidal gold-labeled anti-species antibodies is detected in the analytic zone of these tests.

The problem of the polyvalence of antibody-nanoparticle conjugates in competitive LFIA noted in the previous section can be solved by replacing the conjugate of analyte-specific antibodies with gold nanoparticles by a combination of native specific antibodies and labels conjugated with anti-species antibodies. It gives possibility to vary the content of antigen-binding sites and the marker independently and therefore combine the high-sensitivity of competitive immunodetection (requiring a low content of specific antibodies) and the intensity of the detected signal (achieved with a high label content). This principle was implemented in our developments in the immunodetection of mycotoxins and demonstrated gains in sensitivity from one to three orders of magnitude [50, 77, 78].

Note that the implementation of competitive analysis in LFIA involves another problem. Visual out-of-laboratory diagnostics makes it possible to distinguish only assay results consisting of the presence or absence of a colored line in the analytical zone. For a visible disappearance of color, the sample must contain a sufficient number of analyte molecules to block all binding sites for labeled specific antibodies (**Figure 8**). In this respect, analysis formats with a direct dependence of the detected signal on the analyte content are preferred. For these

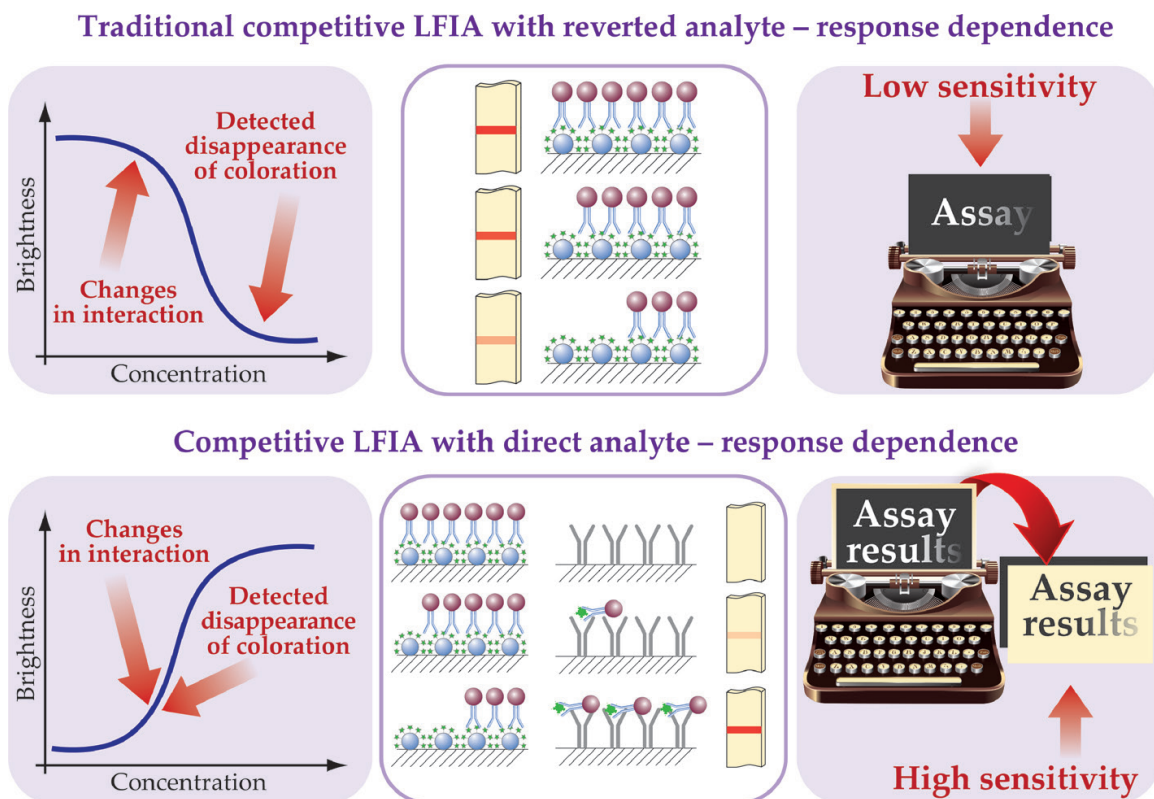


Figure 8. Limitations of competitive immunoassay and one of the ways to overcome them.

formats already small concentrations of the analyte ensure the coloration of the analytical zone in contrast to the absence of color in the absence of the analyte (see **Figure 8**).

However, the implementation of such an analysis for low molecular monovalent antigens is not an easy task. Its solutions for various types of immunoassay are summarized in the reviews of Fan and He [79] and Liu et al. [80]. Unfortunately, many of these approaches, such as idiometric assay [81] and immunoassay using anti-metatype antibodies [82] require the production of antibodies not simply against the target analyte but against more complex antigenic structures, which limits their widespread use. A more universal idea is to use quenching of fluorescence caused approaching between donor and acceptor in the binding zone of the test strip. Such pairs can be two kinds of nanoparticles attached to different immunoreagents. Thus, Shi et al. [83] successfully used for this purpose quantum dots and gold nanoparticles in the analysis of ractopamine, Anfossi et al. [84]—quantum dots and gold or silver nanoparticles in the analysis of fumonisin, and Jiang et al. [85]—ruthenium-doped silicon nanoparticles and silver nanoparticles in the analysis of ochratoxin A. Another perspective approach is open sandwich immunoassay (OSI). The given assay is based on the association of the separated VH and VL chains of the antibody and reinforcement of this association after addition of the target antigen [86]. This approach with the use of so named Quenchbodies is implemented in different versions, mainly with fluorescent detection [87, 88], and it seems promising for LFIA.

In our works, two types of immunoassay for low molecular compounds with direct analyte-signal dependence are described. They do not require special reagents. In Urusov et al. [89],

Passport of immunochromatographic label					
Theory			Practice		
Potential of the label (quantitative)			Applicability of the label (qualitative)		
1	Detection limit of the label (from a certain volume)	Value?	6	Cost and availability	Good/ Bad?
2	Detection limit of the label (from a certain area)	Value?	7	Simplicity of conjugation	Good/ Bad?
3	Maximal quantity of labels that may be conjugated to immunoreagent	Value?	8	Stability during storage	Good/ Bad?
4	Change of registered signal in conjugate	Value?	9	Possibility of quantitative registration using serially manufactured devices	Good/ Bad?
5	Attenuation of registered signal in the assay system	Value?	10	Signal of the label does not modulated by compounds of samples	Good/ Bad?

Figure 9. Characteristics of labels that determine their applicability and competitive potential in LFIA.

an assay was described in which labeled antibodies in the absence of the antigen in the sample completely bind in the first zone to the immobilized analyte. The appearance of the analyte in the sample blocks some of the antigen-binding sites of the antibodies and allows them to reach the second binding zone on the test strip, ensuring the appearance of staining (see **Figure 9**). For the case of deoxynivalenol detection, the proposed approach is 60 times more sensitive than the traditional LFIA. In Berlina et al. [90], an analysis of the food colorant Sudan was described based on the use of two conjugates of gold nanoparticles with (i) antibodies specific to Sudan and (ii) Sudan-ovalbumin conjugates. In the absence of Sudan, the conjugated Sudan-ovalbumin was coated with antibodies on the surface of the gold nanoparticle. So the interaction with the anti-mouse IgG in the test area is prevented. The added Sudan displaced the Sudan-ovalbumin causing the binding of labeled anti-Sudan antibodies in the test area and the appearance of coloration.

6. Proper response for LFIA

The response of the immunochromatographic system is the recorded signal of the label (its color or other parameters), which reflects the formation of a specific immune complex and allows for highly sensitive detection of the target analyte. Therefore, the question of proper response for LFIA is first and foremost a question of choosing a label.

The variety of molecular or colloidal labels that can be used in LFIA is extremely large [91, 92]. According to Goryacheva et al. [92], compounds such as gold nanoparticles of various shapes

and sizes, carbon nanoparticles, selenium nanoparticles, iron oxide nanoparticles, fluorescent dyes, fluorescent dye-doped nanoparticles, quantum dots, infrared emitters, up-converting emitters, nanoparticles with long-lived emission, liposomes, and enzymes may be used for this purpose. There are many articles that demonstrate the advantages of a new marker on the example of the detection of one randomly chosen analyte. However, the question of correct comparison of different labels remains open. Indeed, the differences between test systems depend not only on the label but also on the affinity of the antibodies, the regimen of intermolecular interaction, and the correctness of the choice of reactant content. Therefore, the gain achieved for one analyte does not necessarily persist after the transition to another analyte.

In this situation, it is justified to have “passports” of analytical labels, which are determined by their own properties and can be taken into account when implementing various analytical systems. The proposed list of such parameters is summarized in **Figure 9**.

Note that along with single-valued quantitative parameters reflecting the physical properties of a label, a number of qualitative parameters must be taken into account. Unfortunately, to date, researchers do not have universally recognized quantitative characteristics of existing labels and rules for a priori evaluation of proposed labels. Therefore, when deciding on responses (**Figure 10**), we are forced to follow the data of disparate comparisons of labels in different experimental developments.

Even within the framework of the use of gold nanoparticles, the developer has the opportunity to choose preparations of different sizes and shapes. The well-known recommendation [93] on the preferable use of spherical gold nanoparticles with an average diameter of 30–40 nm is confirmed by published experimental comparisons [49]. Serebrennikova et al. [94] showed the advantages of high-branched gold nanoparticles (“nanoflowers”) as optical markers—a fivefold decrease in the detection limit of prolactin. These patterns were confirmed by Xu et al. [95], and the preferable use of long-tip (13–15 nm) nanoflowers was stated. Ji et al. [96], using gold nanoflowers, reached the detection limit of aflatoxin B1, equal to 0.32 pg./ml.

Optical markers for immunochromatography of different chemical natures are compared in a number of works. The possibilities of using carbon nanoparticles described in Van Amerongen et al. [97, 98] and Liu et al. [99], using the example of salbutamol detection, also showed the advantages of colloidal carbon compared to colloidal gold and nanogold-polyaniline-nanogold microspheres. For ractopamine detection, Hu et al. [100] showed the advantages of time-resolved fluorescent nanobeads compared with fluorescent submicrospheres, quantum dots, and colloidal gold. Effective integration of palladium nanoparticles and horseradish peroxidase with a 10-fold gain in sensitivity as compared to colloidal gold in the detection of *Listeria monocytogenes* was described by Tominaga [101]. The possibilities of high-sensitivity LFIA using graphene oxide and carboxylated graphene oxide as optical markers were shown by Yu et al. [102].

Of great interest are fluorescent markers. In many respects, this is due to the fact that with the correct choice of the wavelengths of excitation and emission, it is possible, by increasing the intensity of the exciting light, to proportionally increase the response in the practical absence (in contrast to the colorimetry) of the nonspecific signal. The gain in sensitivity achieved in



Proper response: Properties of response for improvement and ways to achieve improvement

Research – Strategic tasks



- Choose the label with lowest detectable quantity
- Minimize nonregistered binding of the label
- Choose the tool to detect the label

- Select best parameters of the label (size, composition, modification)

- Amplify signal from the label

- Minimize nonspecific binding of the label

Development – Tactical tasks



Figure 10. Main research and development tasks to obtain proper responses for LFIA.

this case is one or two orders of magnitude [103, 104]. The use of fluorescent markers in LFIA is summarized in the reviews of Pyo and Yoo [105] and Gong et al. [106]. A comparison of the analytical capabilities of quantum dot nanobeads, large-sized (50–600 nm) particles with impregnated quantum dots was given in Duan et al. [107].

Additional capabilities of high-sensitivity analysis are achieved by the registration of energy transfer with the spatial convergence of two labels—fluorescence resonance energy transfer (FRET). Systems using fluorescein isothiocyanate and gold nanoparticles were developed by Wang et al. for the detection of cancer embryonic antigens [108]. Other variants of fluorescent LFIA were also described, for example, registration of background fluorescence quenching in Chen et al. [109], silver nanoparticle-based fluorescence quenching in Jiang et al. [85], and quenching of the fluorescence of quantum dots by gold and silver nanoparticles in Anfossi et al. [84]. (See also Section 5 with their consideration as examples of competitive immunoassays with a direct dependence of the detected signal on the analyte content.)

Extremely promising is the use of surface-enhanced Raman spectroscopy (SERS) for detection of optical labels. SERS signals are based on the increase of optical absorption for reporter molecules by orders of magnitude after their immobilization on the surface of nanoparticles. The possibility of such highly sensitive analyses is demonstrated in the works of Sanchez-Purra et al. [110], Fu et al. [111], and Marks et al. [112]. Clarke et al. [113] described the combination of SERS registration with rapid vertical flow technology as an additional means of increasing sensitivity. In Maneeprakorn et al. [114], SERS detection with 4-aminothiophenol as a signal reporter lowered the detection limit by 300 times compared to traditional LFIA. In Cho et al. [115], the transition to SERS based on silver-intensifying gold nanoparticles led to a 1000-fold decrease in the detection limit. Blanco-Covian et al. [116] proposed the use of a combination of Au @ Ag core-shell nanoparticles and rhodamine B isothiocyanate in LFIA, which allowed them to perform highly sensitive

detection of pneumolysin with a detection limit of 1 pg/ml, recording the surface-enhanced resonance Raman scattering (SERRS).

Note that optical recording methods allow us to evaluate only labels that are in the upper layers of the test strip and are not shielded by membrane fibers. The loss of the optical signal depends on the properties of the material but is usually estimated [93] as about one order of magnitude. In this regard, the work of Jacinto et al. [117] is extremely interesting. They offer an electromagnetic relocation of reporter particles for amplifying an optical signal and describing the fourfold reduction in the detection limit of human chorionic gonadotropin.

This restriction is excluded for analytical methods in which registration of a label is based on other physical principles. Thus, Wang et al. [118] developed the Thermal Contrast Amplification Reader for the registration of gold nanoparticles, which, for systems of influenza and malaria diagnostics and detection of *Clostridium difficile*, showed eight times lower detection limits as compared to an optical reader. Zao et al. [119] improved the detection limit by two orders of magnitude for photoacoustic analysis compared to colorimetric measurements. The magnetic properties of the nanodispersed label in LFIA were recorded by Barnett et al. [120], Chen et al. [121], Lago-Cachon et al. [122], and other authors. Several variants of LFIA with electrochemical detection are presented in the literature, the most recent of which (the work of Zhao et al. [123]) is based on the use of a serial glucometer as a registrar. Just recently, Lin et al. proposed LFIA of myoglobin based on pressure measurement for oxygen generated by platinum nanolabels from hydrogen peroxide [124].

The capabilities of high-sensitivity detection in LFIA are not limited to the choice of a label. Additional reserves provide **amplification of the recorded signal**, which can be provided by

- treatment of the test strip with additional reagents that enhance the coloration or other detectable parameters;
- aggregation of label particles, thereby increasing their number, attached to a single immune complex;
- or initiation by the label of additional reactions, leading to the generation of the detected signal.

The existing variety of developments in this area is summarized in a review of Shan et al. [125]. The systems that implement the aggregation of several types of functionalized nanoparticles cause particular interest. Such approaches are described, for example, by Choi et al. [126] with a 100-fold gain in sensitivity for the detection of troponin I using two kinds of gold nanoparticles; by Razo et al. [20] with the generation of an optical signal by complexes of iron oxide nanoparticles (also used as a concentrating agent) and gold nanoparticles with a 32-fold decrease in the detection limit of potato virus X; by Taranova et al. [127] with a 30-fold gain in the analysis of procalcitonin due to biotin-streptavidin aggregation of gold nanoparticles; by Shi et al. [128] with complexation of gold nanoparticles of two sizes in the analysis of imidaclothiz; by Zhong et al. [129] with the formation of two layers of antibody conjugates with gold nanoparticles in the detection of melamine; and by Shen et al. [130] with aggregation of

gold nanoparticles using polyamidoamine dendrimer, which lowered the detection limit of rabbit immunoglobulin G 20 times.

The growth of the size of gold nanoparticles with the help of the catalyzed reaction of their surface between HAuCl_4 and NH_2OH was examined by Bu et al. [131] as a means of amplification for LFIA. The layered build-up of gold nanoparticles was described by Li et al. [132]. Anfossi et al. [133] and Panferov et al. [134, 135] considered the possibilities of silver enhancement (restoration of the silver salt on the surface of a gold nanoparticle with an increase in its size) in LFIA. In a study by Rodriguez et al. [136], the optimal regimes of silver and gold enhancements were determined to enhance the signal from the gold nanoparticles. Enzymatic amplification using alkaline phosphatase was studied by Panferov et al. [137] for LFIA of potato virus X and by Kim et al. [138] for LFIA of C-reactive protein. A feature of the latest development was the use of a water-swellaable polymer for the accumulation of a colored product. An original polymerization-based amplification approach for enhancing staining was described by Lathwal and Sikes [139].

The basic requirement for amplification approaches is the maintenance of low laboriousness of analysis. Variants using additional reagents, although considered in development, should be finally transformed into devices of dry chemistry, in which all components of the test strip are applied to its membranes.

7. Proper output for LFIA

The generation of a signal reflecting the formation of immune complexes during LFIA is not the final stage of the analysis. The analysis is only completed when a diagnostically meaningful conclusion is made on the basis of this signal.

Effective use of LFIA is possible only when it is combined with modern means for documenting, storing, and processing information. In the absence of these tools, the advantages of rapid and high-performance nonlaboratory diagnostics are lost because of time-consuming processing and description of test results. Of fundamental importance is the transition from a subjective yes-no evaluation of results to automatic quantitative registration and the formation of databases that integrate the results of mass screenings or information on the dynamics of the state of patients (objects). Such systems will allow rapid collection of various indicators “at the time of request,” contributing to an accurate diagnosis. Taking into account the foregoing, **Figure 11** summarizes the requirements for proper output in LFIA.

In some cases, the developer does not need to achieve maximum sensitivity but to fix the threshold that separates the positive and negative results in accordance with the regulatory requirements for MRLs. This allows the composition of conjugates used in the analysis discussed above to be varied [63]. A qualitative “yes-no” analysis can be transformed into a semiquantitative one with a change in the number of colored bands corresponding to several threshold levels. To do this, depletion of the conjugate can be used when interacting

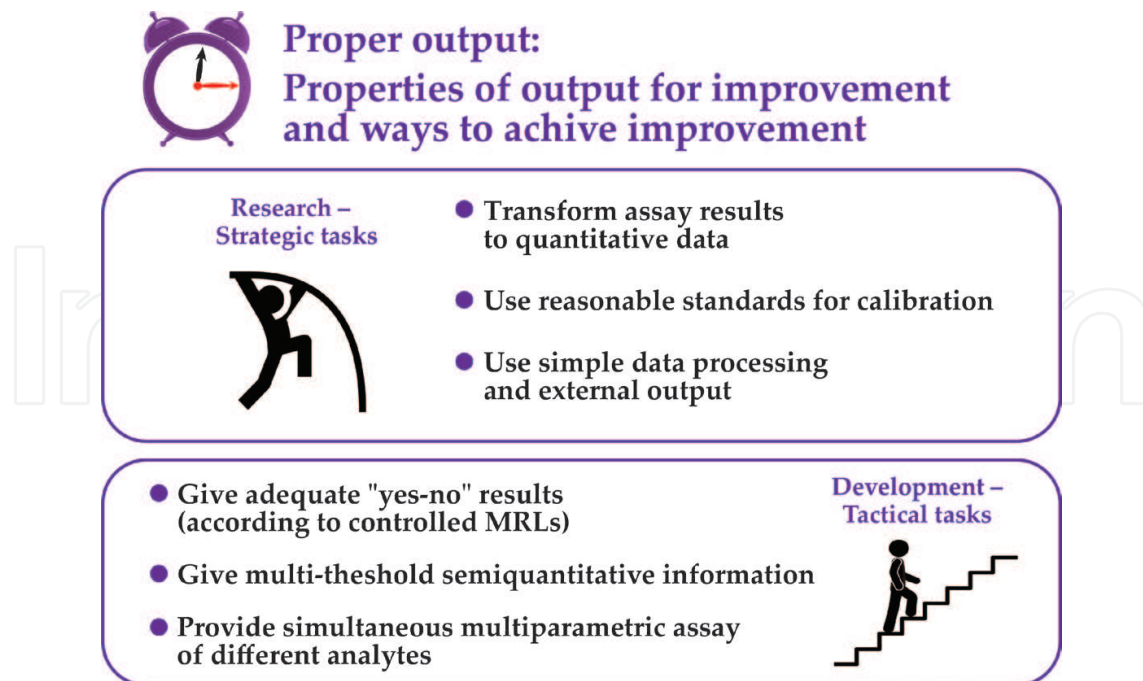


Figure 11. Main research and development tasks to obtain proper output for LFIA.

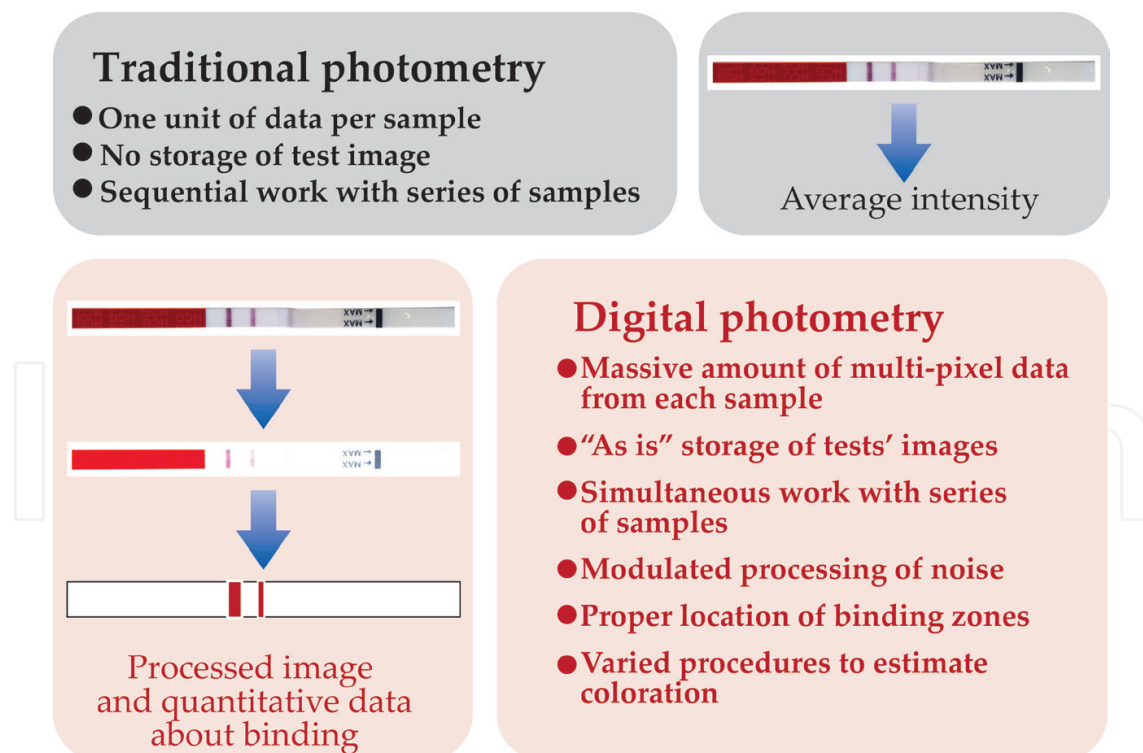


Figure 12. Comparison of traditional and digital photometry as a means for registration and processing of immunochromatographic data.

with several consecutive identical binding zones. Additional opportunities arise when using antibodies with different affinities, varying the surface density of the reagents applied in the binding zones and the distance between these zones and the beginning of the test strip. An example of an appropriate development with three thresholds of potato X virus concentrations corresponding to the degree of plant infection was described by Panferov et al. [140].

Initially, attempts were made to create detectors for membrane tests that recorded the total intensity of the staining (brightness of the reflected light) in certain sections of the test strip using a row of light-emitting diodes and individual systems of signal transformation for each diode. However, such detectors were extremely cumbersome. Blatt et al. [141] proposed a device made from 28 photosensitive sensors located along the test strip. Nowadays, the dominant means of detecting the results of LFIA, allowing a full-color image of the test strip to be received, are digital cameras. This technology is based on the use of inexpensive portable detectors or household recording devices—such as a mobile phone camera [142]. Serially produced cameras record images with a resolution of up to 2400 dpi, which corresponds to the size of an individually characterized section of less than $1\ \mu\text{m}^2$. **Figure 12** summarizes the advantages of digital photometry in LFIA.

Trends in the transformation of LFIA from the visual to the instrumental method are summarized by Cheung et al. [5]. Reviews by Quesada-Gonzalez and Merkoci [143] and Zarei [144] present the current state of analytical technologies based on the use of mobile phones/smart-phones. At the same time, a significant number of manufacturers of test systems offer portable detectors that are adapted to work with their own products [8]. Of the original solutions, mention should be made of Feng et al. [145], in which the registration tool for LFIA was Google Glasses. In recent years, a number of companies have introduced cloud technologies into practice, where external servers receive data about testing results via standard communication devices and store and process this information. Thus, since 2017, Abbott has proposed a set of tools named i-STAT Alinity for distant diagnostics. Special cartridges allow 14 parameters of blood composition by bio- and immuno-chemical techniques to be controlled.

An extremely important means of increasing informativeness, although not related to an increase in sensitivity, is to conduct a multiplex analysis—that is, detection of the presence and level of several analytes using a single test strip. Data on the control of several analytes can be discriminated in space (by the position of binding zones) or by signals (by using different labels). Quantum dots are an effective tool for multi-analysis with different signals. The use of conjugated quantum dots with different spectral characteristics allows one to perform highly sensitive diagnostics with simultaneous detection of, for example, three antibiotics (“traffic light” in Taranova et al. [146]) or four mycotoxins (“rainbow” in Foubert et al. [147]).

Because the number of binding zones that can be sequentially located on one test strip while preserving the rapidity of the analysis and the reliability of the information obtained for each analyte is limited (usually no more than five zones), the transition to “two-dimensional immunochromatography” is promising—see **Figure 13**. This approach, combining the advantages of immunochromatographic tests and immunochips, is based on the formation of an ordered two-dimensional array of points with immunoreagents of different specificity on

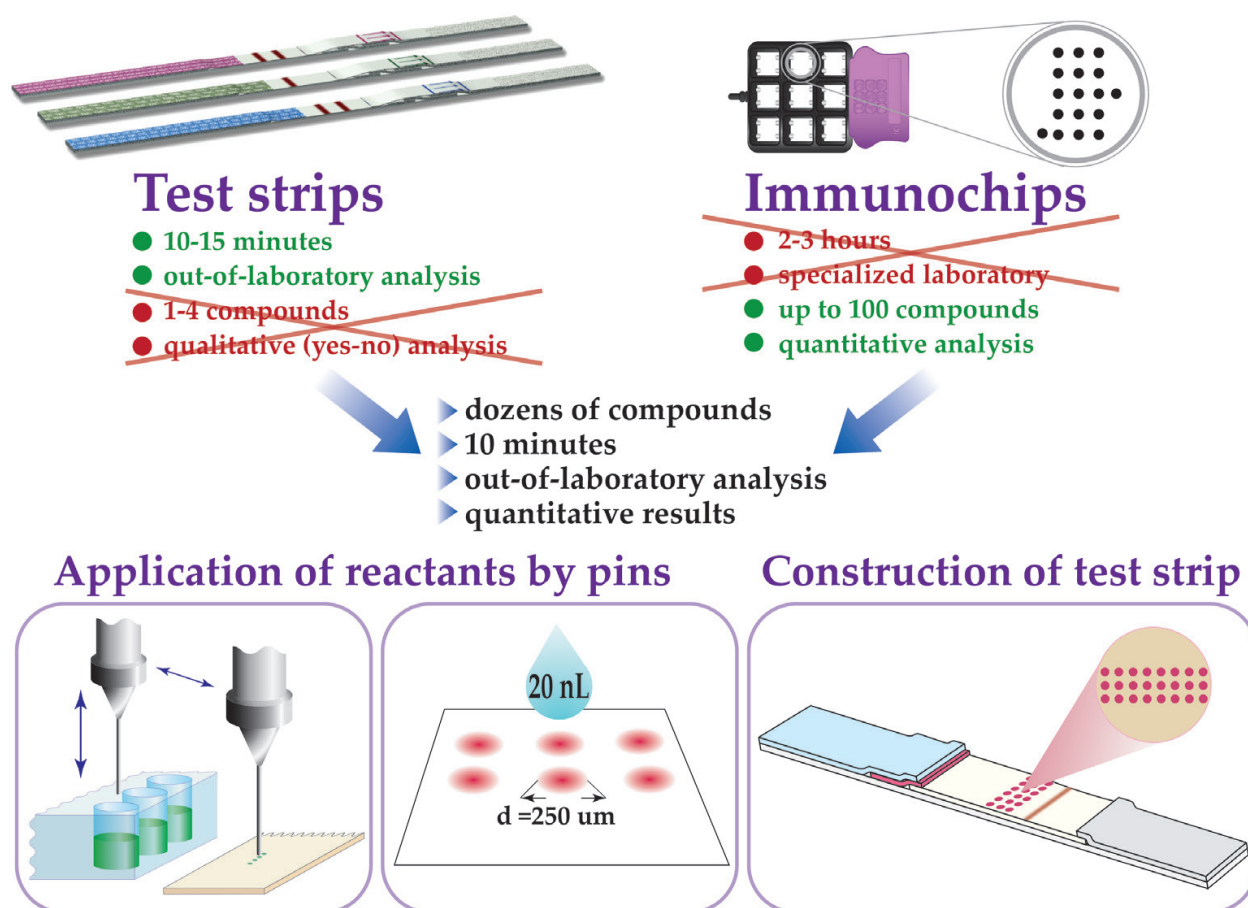


Figure 13. Concept of 2-D immunochromatography.

the membrane of a test strip. In such systems, interaction occurs in several dozens of binding zones. Due to this, the 2D immunochromatography increases the information content of LFIA results and reduces the consumption of reagents and materials for one analysis.

Examples of test systems based on the principle of “two-dimensional immunochromatography” are presented in the works of Taranova et al. [104] on the detection of drugs and Safenkova et al. [148] on the detection of phytopathogens. General approaches to multizonal LFIA were discussed in Hu et al. [149], and the current state of the development of multiplex immunoassays was discussed in Li et al. [150].

8. Further perspectives of LFIA

It would be reasonable to summarize the presented review of LFIA developments using two outcomes—strategic (research) and tactical (development) outcomes.

We may identify the following main tasks, the solutions of which are extremely important regardless of the specific analyte and type of tested samples that are of interest to the developer: To create an effective test, the developer must

- Obtain the most concentrated sample
- Select the membranes that ensure rapid movement of reagents and high intensity of staining when working with this matrix
- Select the optimal label
- Use conjugates of immunoreagents having optimal composition
- Choose the optimal location of the reagents applied to the test strip
- Find the optimal ratio of immunoreagents, combining a sufficient level of label binding and a low detection limit for the analyte.

Considering the strategic situation of the development of LFIA, we should expect test systems of the future to implement high-performance and informative analyses integrated with the tools for collection, storage, and processing of information. With the development of molecular biological methods for the production of modified and new receptors, bioanalytical systems will be able to effectively discriminate various structurally close compounds and, on the basis of their levels in the sample, make more informative diagnostic conclusions.

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