

## OPTIMIZATION OF THE MAIN PARAMETERS OF A LATERAL FLOW ASSAY BASED ON THE RECOMBINANT P32 ANTIGEN OF LUMPY SKIN DISEASE VIRUS

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### ABSTRACT

Lumpy skin disease is a highly contagious infectious transboundary disease of cattle, resulting in significant economic losses. The wide and rapid spread of lumpy skin disease to new territories emphasizes the objective need to improve diagnostic methods. The article outlines the findings of lateral flow assay parameter optimization for lumpy skin disease detection using recombinant P32 antigen. It was found that the optimal amount of protein for immobilization on 20 nm colloidal gold particles was 20 µg/mL. Bovine serum albumin at a final concentration of 0.25% was used to stabilize the conjugate. The optimal dilution of the conjugate was 1:2, and the concentration of recombinant protein and anti-species antibodies for application to the membrane was 500 µg/ml and 250 µg/ml, respectively. The colloidal gold conjugate interacted with antibodies and specifically reacted with the recombinant protein. No cross-reaction with other proteins was noticed at the same time. The created express test allowed for the detection of antibodies from immunized animals when the serum was diluted up to 1:200, however, 1:50 was the ideal ratio. The conjugate remained active after 90 days of storage at room temperature. The findings demonstrate the express method's potential for use in serological research for the prevention and control of the spread of lumpy skin disease.

**Keywords:** lumpy skin disease, diagnostics, colloidal gold, P32 antigen, lateral flow assay, vaccination.

### INTRODUCTION

Lumpy skin disease is a transboundary disease and poses a serious threat to livestock, causing significant economic damage both within our country and in the world as a whole [1]. Lumpy skin disease is a transboundary illness that poses a serious threat to livestock and has a large negative economic impact on both our country and the rest of the globe. The risk of lumpy skin disease is related to severe morbidity among unvaccinated cattle reaching 85% and mortality reaching up to 40% in some cases. Historically lumpy skin disease was widespread in Africa, however, in 1988 the disease was first noted in Egypt. The first incidence of lumpy skin disease documented outside of the African continent was in Israel in 1989 [2]. Later, the lumpy skin disease virus spread to some countries in some Middle Eastern, European, and Asian countries [1, 3, 4]. In Kazakhstan, lumpy skin disease was first registered in 2016, in the Atyrau region. In 2016, the Atyrau region in Kazakhstan witnessed the first case of lumpy skin disease. Since lumpy skin disease was first reported in the Russian Federation in 2015, it is likely that the virus was brought into the country from there [5]. Lumpy skin disease is caused by a virus belonging to the *Capripoxvirus* family, which also includes shippox and goatpox viruses [6].

The clinical signs of the disease are fever, formation of nodules on the skin and mucous membranes, lesions in the respiratory and gastrointestinal tracts, and enlargement of superficial lymph nodes [7]. It is believed that blood-sucking insects, such as mosquitoes, flies and ticks, are the main mechanism of transmission of lumpy skin disease virus from sick animals to healthy ones [8]. According to the World Organization for Animal Health (OIE), the virus neutralization test, enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test, and western blot analysis are the primary serological methods used for diagnosing lumpy skin disease [9]. However, all these methods are time-consuming,

and require laboratory conditions, appropriate equipment, and trained personnel. In contrast, such a rapid method as lateral flow assay (LFA) does not require the above conditions. At the same time, LFA is practically not inferior in sensitivity and specificity to ELISA. The analysis can be carried out in the field by a veterinarian or a farmer and get results within 15-20 minutes.

The creation of an express test for detecting antibodies against lumpy skin disease is an urgent task given the rapid spread of the virus, the ongoing importation of animals from other countries, and the widespread distribution of the lumpy skin disease virus in blood-sucking insects in our country [10]. The article outlines the outcomes of developing the key LFA parameters based on a recombinant P32 antigen for the purpose of identifying antibodies in the serum of immunized animals.

### MATERIALS AND METHODS

#### *Ethical approval*

This study was approved by the Ethical Committee of the National Center for Biotechnology, Astana, Kazakhstan (Protocol No. 3 dated 07.08.2020).

#### *Reagents and materials*

The previously obtained recombinant p32 antigen of the lumpy skin disease virus was used for application to the test line. On the control line, anti-species antibodies were used. Colloidal gold was synthesized with chloroauric acid (Sigma-Aldrich, St. Louis, MO, USA), sodium citrate (Appli-Chem, Darmstadt, Germany), and mQ grade water. Sodium chloride, bovine serum albumin (BSA) (PAA Laboratories, Pasching, Austria), phosphate-buffered saline (PBS) (Gibco, Paisley, UK), and recombinant protein G (Invitrogen, Rockford, IL, USA) were used in the work. The strip's construction featured a nitrocellulose membrane of the CNPC-SS12-

L2-H50 type (Advanced Microdevices PVT.LTD, India), a sample pad - PT-R5, a glass fiber membrane for the conjugate - GFB-R4 (India), and an absorbent pad - AP-045 (India).

#### Animals

Rabbits weighing 2 kg and 8–9 months old had been used in the experiment. A vaccine strain against lumpy skin disease of cattle and sheep pox (VNIIZZH strain, Russia) was subcutaneously injected into the withers of animals in a quantity of 200  $\mu$ L at a dilution of 1:100. Immunization was done on days 1, 12, 24, and 36. Days 10, 22, 34, and 46 following immunization were used to collect blood from the ear vein. Complete Freund's adjuvant was added to the first vaccination while incomplete Freund's adjuvant was added to the second vaccination. The use of an adjuvant was omitted from subsequent immunizations. Serum samples were stored at  $-20^{\circ}\text{C}$ .

#### Preparation of colloidal gold solution

A solution of colloidal gold was prepared by the citrate method [11]. The estimated diameter of colloidal gold particles was about 18–20 nm. Briefly, 100 mL mQ grade water was passed through a 0.22  $\mu$ m filter into a sterile 500 mL flask and a sterile magnetic anchor was added. Next, the flask was placed on a magnetic stirrer and the cooling system was connected. One mL of a 1% of a colloidal gold solution was added to the flask and stirred until boiling, after which four mL of a freshly prepared 1% of sodium citrate solution was added and boiled for 15 minutes. The colloidal gold solution was cooled at room temperature protected from light and stored at  $4^{\circ}\text{C}$ .

#### Determination of the optimal concentration of protein G for immobilization on colloidal gold.

Protein G diluted in water quality mQ from 1 to 30  $\mu$ g/mL in an amount of 100  $\mu$ L was added to the tubes. From above, 1 mL of colloidal gold solution was added, mixed, and incubated for 10 min at  $25^{\circ}\text{C}$ . Next, 100  $\mu$ L of 10% NaCl (AppliChem, Darmstadt, Germany) was added, and the incubation procedure was repeated. The optical density of the samples was measured on a microplate spectrophotometer at a wavelength of 580 nm. A dependence graph was constructed using the optical density results.

#### Preparation of colloidal gold conjugate with protein G and determination of its stability.

The optimal concentration of protein G was added to a colloidal gold solution (pH 6.0) and incubated at  $25^{\circ}\text{C}$  for 20 minutes with constant stirring on a shaker. A final concentration of 1%, 0.5%, and 0.25% was then reached by adding 10% BSA. After mixing, the tube was incubated for 30 minutes on a shaker at  $25^{\circ}\text{C}$ . Unbound protein was separated by centrifugation at  $8000 \times g$ , for 40 minutes. The supernatant was removed and the soft pellet was resuspended in PBS, pH 7.4 with the addition of the appropriate concentration of BSA. The resulting conjugate was applied to a glass fiber membrane in different dilutions and left to dry at room temperature. The activity and mobility of the conjugate were determined after 1, 7, 14, 28, 60, and 90 days.

#### Assembly of the test strips

The test and control lines were applied to the nitrocellulose membrane using an XYZ3050 dispenser (BioDot, USA). To determine the optimal parameters, the P32 antigen was applied to the test strip at different concentrations, from 100  $\mu$ g/

mL to 1 mg/mL, and anti-species antibodies were applied to the control strip at a concentration of 100  $\mu$ g/mL to 500  $\mu$ g/mL. The application volume was 2  $\mu$ L per 10 mm of the membrane width. After drying at  $25^{\circ}\text{C}$  for 24 hours, the membrane was cut into strips 4 mm wide using a CM4000 modular cutter (BioDot, USA). Colloidal gold-protein G conjugate was manually applied to a 4 $\times$ 6 mm glass fiber membrane and dried at  $25^{\circ}\text{C}$  for 24 hours. Subsequently, a membrane with a conjugate was applied to the adhesive base of the membrane, and a sample pad was placed on top. An absorbent pad was applied to the adhesive base on the strip's opposite side.

#### Evaluation of the effectiveness of the LFA on laboratory animals

The analysis was carried out at room temperature. The collected strips were immersed in test tubes with blood serum samples in various dilutions (150  $\mu$ L) and left for two minutes in a vertical position. Then the strips were transferred to a horizontal surface and the reaction was recorded after 15–20 minutes. The analysis was carried out by the presence and intensity of the color of the test and control lines. The analysis was evaluated by the presence and color intensity of the test and control bands.

## RESULTS

A bright cherry-colored solution with particles that were around 20 nm in diameter was produced as a result of the synthesis of colloidal gold (Figure 1).

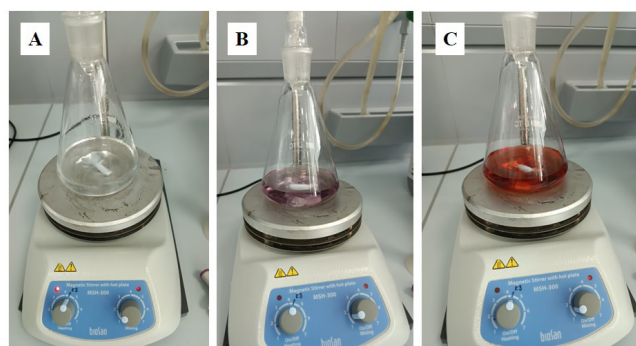


Figure 1. Colloidal gold synthesis process.

A - before adding sodium citrate; B - 1 minute after the addition of sodium citrate;

C - 15 minutes after the addition of sodium citrate.

In order to determine the optimal amount of protein for adsorption on colloidal gold, various concentrations were used, from 0 to 30  $\mu$ g/mL. The reaction was evaluated visually (Figure 2) and by optical density (Figure 3). Figure 2 illustrates how the addition of 10% NaCl leads to the degradation of colloidal gold in tubes with protein concentrations ranging from 2 to 12  $\mu$ g/mL. The graph shows that the optical density increases initially and peaks at a protein concentration of 8  $\mu$ g/mL, then falls and plateaus at a protein G concentration of 20  $\mu$ g/mL.

Colloidal gold-protein G conjugate showed its activity during the entire period of storage at room temperature (Figure 4 A). The figure shows that the conjugate leaves the membrane and binds to the antibodies. It was also found that there was no significant difference when adding different amounts of BSA to the buffer, so further work was carried out with a

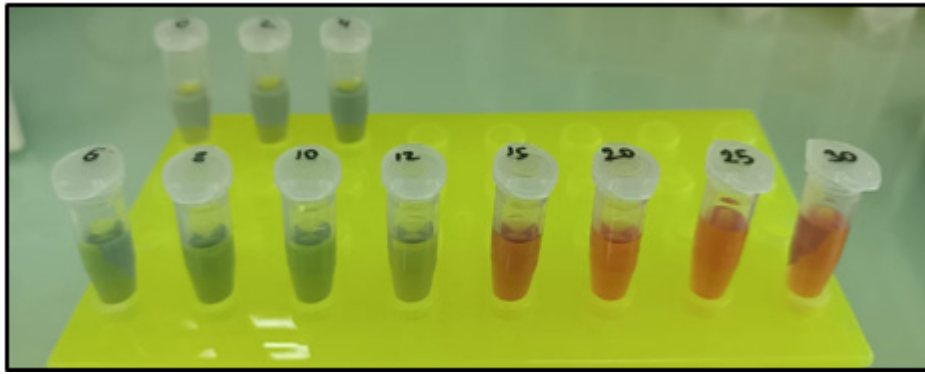


Figure 2. Determination of the optimal concentration of protein G for immobilization on colloidal gold

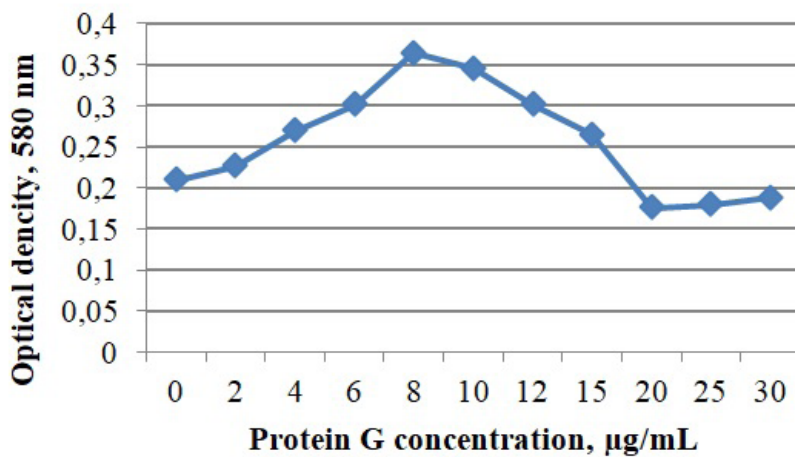


Figure 3. Dependence of protein G adsorption on colloidal gold.

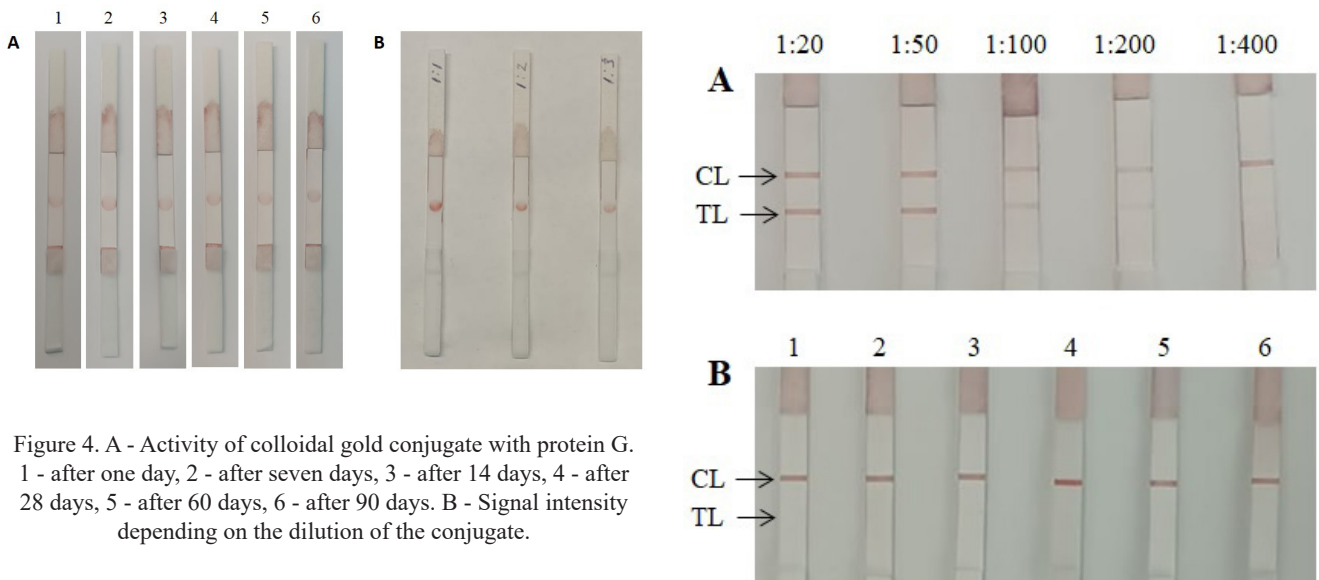


Figure 4. A - Activity of colloidal gold conjugate with protein G. 1 - after one day, 2 - after seven days, 3 - after 14 days, 4 - after 28 days, 5 - after 60 days, 6 - after 90 days. B - Signal intensity depending on the dilution of the conjugate.

concentration of 0.25%. The most optimal dilution of the conjugate was 1:2 (Figure 4 B).

As a result of optimizing the amount of antigen applied to the membrane, it was found that the optimal antigen concentration is in the range of 500-800 µg/ml (Figure 5A). For secondary antibodies, the optimal concentration was in the range of 200-300 µg/ml (Figure 5B).

Figure 5. Optimization of applied proteins on the test and control lines.

A - P32 antigen. B - Secondary antibodies

Optimal concentrations of the antigen and secondary antibodies were used to detect antibodies in the serum of immu-



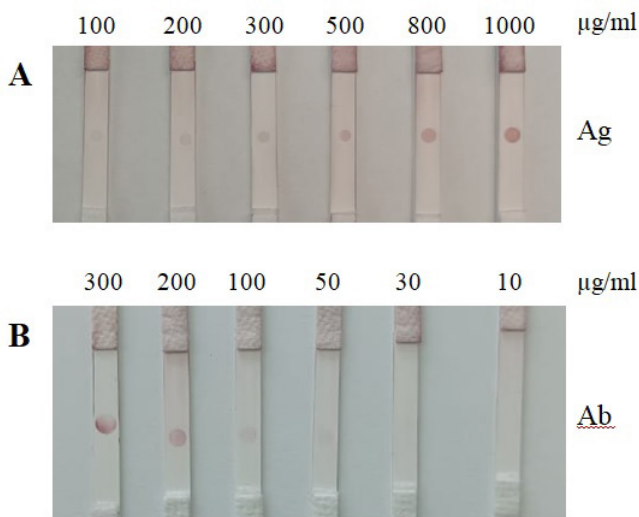


Figure 6. Sensitivity and specificity assessment of the test. A - Dilution of serum of a rabbit immunized with a vaccine strain. B - Determination of cross reaction. 1 - Omp31 protein (brucellosis), 2 - BP26 protein (brucellosis), 3 - p24 protein (leukemia), 4 - 3B protein (foot and mouth disease), 5 - MOMP protein (campylobacteriosis), 6 - negative control, CL - control line, TL - test line

nized animals. The strips were added to different dilutions of blood serum. It was shown that the optimal dilution of serum was 1:50 (Figure 6).

The conjugate specifically bound to the recombinant P32 protein on the test line, as shown in Figure 6. No reaction was observed when utilizing a non-immunized rabbit's serum. Additionally, the reaction was negative when heterologous proteins of pathogens causing diverse animal diseases were used.

## DISCUSSION

Over the years of capripoxvirus spread, numerous serological test systems have been created. An indirect ELISA test based on whole inactivated sheep pox virus was one of the first. The specificity and sensitivity of the test were 97% and 88%, respectively [12]. A novel indirect ELISA has recently been developed and tested to detect antibodies to the lumpy skin disease virus [13]. The authors used purified and inactivated lumpy skin disease virus as an antigen. The results revealed that the home test's sensitivity and specificity, which were 95% and 89.8%, respectively, did not fall short of the commercial test. The difficulty in acquiring a sufficient quantity of the whole virus, however, is the primary issue with creating an ELISA based on it [2]. In addition, special laboratory conditions and trained personnel are required to work with the virus. All this complicates and increases the cost of developing a diagnostic test system.

An alternate strategy was to use the technology for obtaining recombinant antigens. For example, the usage of recombinant structural protein P32 as an antigen, which includes major immunogenic determinants and is present in all capripoxviruses [14]. Based on the P32 antigen of lumpy skin disease virus, an ELISA was developed by analogy with diagnostic tests against sheep pox virus and goat pox virus [15].

Taking into account the rapid spread of the lumpy skin

disease virus, timely and accurate diagnosis will allow localization of the disease in time. LFA based on colloidal gold is widely used both in medicine and veterinary medicine for the detection of pathogens [16, 17, 18] and serological studies [19]. The main advantage of LFA over other methods is the simplicity and speed of the setting. According to the analysis of literature data, only two articles on the use of LFA for the identification of the lumpy skin disease virus have been published thus far. In 2022, an LFA was developed to detect lumpy skin disease virus in pathological material. The sensitivity of the LFA was 72.8%, and the specificity was 88.8% [20]. In another study, a sandwich variant of LFA based on colloidal gold and monoclonal antibodies was used against different epitopes of the P32 protein of the lumpy skin disease virus. By optimizing LFA parameters, the authors managed to achieve high sensitivity and specificity of the test [21].

In contrast to the works described, we developed the LFA based on the recombinant P32 protein to determine the immune response in animals. This test will allow us to identify sick animals or latent virus carriers in the field. In addition, the test will allow for monitoring among post-vaccinated livestock. In the article, we describe the development of optimal test parameters on laboratory animal models. The main parameters include the size of colloidal gold, the concentration of protein G for immobilization on colloidal gold, the pH of solutions, the concentration of antigen and secondary antibodies for application to the membrane, the amount of applied reagents, drying and storage conditions, as well as the quantity of applied reagents.

To synthesize colloidal gold particles with a diameter of ~ 20 nm, 4 ml of sodium citrate was added. When determining the optimal amount of protein for absorption on colloidal gold, it was found that the transition from an unstable state to a stable state is observed at 20 µg/ml. The most optimal concentration of recombinant protein and anti-species antibodies was 500 µg/ml and 250 µg/ml, respectively. It should be noted that the use of an excessive amount of antigen can lead to an increase in molecules that are not bound to the surface, which will adversely affect the analytical properties of the test system. On the other hand, a decrease in protein concentration can result in a low signal level and a reduction in the test's sensitivity [22]. In addition, an excess amount of protein usage will certainly lead to an increase in the cost of the final product. It was found that the conjugate of colloidal gold with protein G remains active for 90 days of storage at room temperature and ambient humidity. Specific conditions need to be met for prolonged storage. The storage temperature should be below 25°C, and the humidity should be less than 20% [23]. Two obvious bands were visible when testing the sera of immunized animals at dilutions of 1:20 and 1:50; at dilutions of 1:100 and 1:200, the test line stained less strongly. No reaction was seen in the test zone when utilizing the heterologous recombinant proteins or the negative control, demonstrating the test's specificity.

The acquired results point to the express method's potential for use in serological studies for lumpy skin disease prevention and control. Future research should, however, focus on determining the test's sensitivity and specificity on field samples taken from sick or vaccinated animals.

## CONCLUSION

In this research, a LFA based on the recombinant P32 antigen was created for the diagnosis of LSD. The main parameters of the test have been optimized. It was found that the colloidal gold conjugate interacted with antibodies and specifically reacted with the recombinant P32 protein. Cross-reactivity with other proteins was not observed. The developed express test made it possible to detect antibodies at a serum dilution of 1:200, but the optimal dilution was 1:50. Despite being stored at ambient temperature for three months, the conjugate maintained its activity. The results can be used for serological diagnosis and field monitoring of the spread of lumpy skin disease.

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## НОДУЛЯРЛЫ ДЕРМАТИТ ВИРУСЫНЫҢ Р32 РЕКОМБИНАНТТЫ АНТИГЕНІ НЕГІЗІНДЕ ИММУНОХРОМАТОГРАФИЯЛЫҚ ТАЛДАУДЫҢ НЕГІЗГІ ПАРАМЕТРЛЕРІН ОҢТАЙЛАНДЫРУ

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### ТҮЙІН

Нодулярлы дерматит - айтарлықтай экономикалық шығынға әкелетін ірі қара малдың өте жұқпалы трансшекаралық ауруы. Нодулярлы дерматит ауруың жаңа аумақтарға кең және жылдам таралуы диагностикалық әдістерді жетілдірудің объективті қажеттілігін көрсетеді. Мақалада нодулярлы дерматит диагностикасына арналған рекомбинантты Р32 антигені негізінде иммунохроматографиялық талдаудың параметрлерін оңтайландыру нәтижелері берілген. 20 нм коллоидты алтында иммобилизациялау үшін ақуыздың оңтайлы мөлшері 20 мкг/мл екені анықталды. Конъюгатты тұрақтандыру үшін соңғы концентрациясы 0,25% бұқаның сарысу альбумині қолданылды. Конъюгаттың оңтайлы сұйылтуы 1:2 болды, ал мембранаға қолдану үшін рекомбинантты протеин және түрге қарсы антиденелердің концентрациясы сәйкесінше 500 мкг/мл және 250 мкг/мл болды. Коллоидты алтын конъюгаты антиденелермен байланысқа түсті және рекомбинантты ақуызбен телімді әрекеттесті. Басқа ақуыздармен айқаспалы реакция байқалған жоқ. Өзірленген экспресс-тест иммунизацияланған жануарлардың антиденелерін қан сарысуындағы сұйылту 1:200 болғанға дейін анықтауға мүмкіндік берді, бірақ оңтайлы сұйылту 1:50 болды. Конъюгат өзінің белсенділігін бөлме температурасында 90 күн бойы сақтады. Алынған нәтижелер нодулярлы дерматиттің таралуының алдын алу және бақылауында серологиялық зерттеулерде қолдану үшін болашағы бар экспресс әдіс екендігін көрсетеді.

**Кілтгі сөздер:** нодулярлы дерматит, балау, Р32 антигені, иммунохроматографиялық талдау, коллоидты талдау, вакцинация.

## ОПТИМИЗАЦИЯ ОСНОВНЫХ ПАРАМЕТРОВ ИХА ТЕСТА НА ОСНОВЕ РЕКОМБИНАНТНОГО Р32 АНТИГЕНА ВИРУСА НОДУЛЯРНОГО ДЕРМАТИТА

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### АННОТАЦИЯ

Нодулярный дерматит представляет собой высококонтагиозное инфекционное трансграничное заболевание крупного рогатого скота, приводящее к значительным экономическим потерям. Широкое и быстрое распространение нодулярного дерматита на новые территории подчеркивает объективную необходимость в совершенствовании диагностических методов. В статье представлены результаты оптимизации параметров иммунохроматографического анализа на основе рекомбинантного Р32 антигена для диагностики нодулярного дерматита. Было установлено, что оптимальное количество белка для иммобилизации на коллоидном золоте размером 20 нм составило 20 мкг/мл. Для стабилизации конъюгата использовали бычий сывороточный альбумин в конечной концентрации 0,25%. Оптимальное разведение конъюгата составило 1:2, а концентрация рекомбинантного белка и антивидовых антител для нанесения на мембрану - 500 мкг/мл и 250 мкг/мл, соответственно. Конъюгат коллоидного золота связывался с антителами и специфически реагировал с рекомбинантным белком. При этом перекрестная реакция с другими белками не наблюдалась. Разработанный экспресс тест позволил выявлять антитела иммунизированных животных до разведения сыворотки 1:200, однако оптимальное разведение составило 1:50. Конъюгат сохранял свою активность на протяжении 90 дней хранения при комнатной температуре. Полученные результаты свидетельствуют о перспективе экспресс метода для использования в серологических исследованиях при профилактике и контроле распространения нодулярного дерматита.

**Ключевые слова:** нодулярный дерматит, диагностика, антиген Р32, иммунохроматографический анализ, коллоидное золото, вакцинация.