Adenovirus rapid test development: Digital processing of results to optimize test protocols and components

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Abstract—An immunochromatographic rapid test for the determination of adenovirus in feces was developed. The main stages of the research process are shown: selection of specific components, protocol optimization, and testing in the sample matrix. We have shown the effectiveness of using digital processing of the results of immunochromatographic analysis to select the optimal test components. The detection limit was 30 ng/mL and the analysis time is less than 15 minutes.

**Keywords**—**lateral flow assay, rapid test, adenovirus, digital processing**

# 1. Introduction

Immunochromatographic analysis is a leading format among immunotechniques due to its simplicity, accessibility, and rapid results. Immunochromatographic test systems are used in many areas: environmental monitoring [[1](#_ENREF_1), [2](#_ENREF_2" \o "Byzova, 2018 #5053)], agriculture, food quality control [[3-5](#_ENREF_3)], etc. They are also in demand in medical diagnostics, first of all for prediagnostic purposes. However, the development of such simple and quick analysis requires the consideration of complex factors. Because the test is qualitative, a direct assessment of the effectiveness of the analysis is difficult. The results obtained should be transformed into quantitative data. The digital processing of the results (conversion of the intensity of dye coloration at the analytical zone of the test into a numerical value) can overcome this limitation.

In this paper, using the example of the adenovirus test, we show the main stages in the development of an immunochromatographic test kit. Adenovirus causes one third of all viral infections. It occurs in adults, but occurs especially often in young children: there is 1 case per 1,000 children under 4 years of age [[6](#_ENREF_6)]. Often it manifests in the form of respiratory and intestinal pathologies [[7-12](#_ENREF_7)]. After an acute infection, the pathogen can be found in stool for several months, which causes the endemicity of this disease. Traditionally, enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction kits are used for the analysis of adenovirus. Both approaches are effective, but they require highly qualified specialists, measurements must be done under laboratory conditions, and the tests can last over 2 hours. Despite the high demand for the test and the existence of commercial kits, there are practically no examples in the scientific literature of the successful development of immunochromatographic analysis for this antigen.

# 2. Materials and methods

## 2.1. Materials

Sodium azide, Tween-20, Tris, and chloroauric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA; sial.com). Adenovirus antigen (Tonsil 99) was from Bialexa (Moscow, Russia; bialexa.ru). Bovine serum albumin (BSA) was from Boval Company (Cleburne, TX, USA; bovalco.com). Mouse monoclonal antibodies against adenovirus (clones 1AD, 7AD, 8AD) were provided by Bialexa and Artron BioResearch (Burnaby, B.C., Canada; artronbio.com) (clones A46-Ab1 and A46-Ab2). Goat anti-mouse polyclonal antibodies were from Arista Biologicals (Allentown, PA, USA; aristabiologicals.com). All other reagents were of analytical grade purity or greater. To compare the efficiency of the developed test system, we used rapid test kit VIKIA Rota-Adeno, provided by bioMérieux SA (Marcy l'Etoile, France; [www.biomerieux.com](file:///C:\Users\Worker\Downloads\www.biomerieux.com)). Deionized water, 18 MΩ·cm at 25°C (Simplicity Millipore, Billerica, MA, USA; www.millipore.com), was used for the preparation of all solutions.

## 2.2. Fabrication of immunochromatographic test strips

The immunochromatographic test strips were fabricated from sample pad (GFB-R7L) and nitrocellulose membrane (CNPC-SS12), and an absorbent pad (AP045) was obtained from Advanced Microdevices (Ambala Cantt, India; mdimembrane.com). A conjugate pad, GFDX203000, was obtained from Merck-Millipore (Darmstadt, Germany; merckmillipore.com). The monoclonal antibodies against adenovirus and goat anti-mouse antibodies were dissolved in phosphate-buffered saline (PBS) to concentrations of 1 and 0.5 mg/mL, respectively, then applied to nitrocellulose membranes (CNPC-SS12, 12 µ) fixed on a plastic support at a rate of 0.1 µL/mm, using an IsoFlow dispenser (Imagene Technology, Hanover, NH, USA; imagenetechnology.com). In addition, antibodies conjugated with gold nanoparticles (GNPs) were dissolved in 1% BSA and 1% sucrose (TBSU) + 0.05% Tween 20 with D520 = 3.0 and applied to the glass fiber conjugate pad (3.2 µL/mm). The membranes were dried overnight at room temperature and 20% humidity. Then master lists (membrane composites) were cut into 3.5-mm-wide test strips by using an Index Cutter-1 (A-Point Technologies, Gibbstown, NJ, USA) and then stored at 20-22°C in a sealed package containing silica gel [[13](#_ENREF_13), [14](#_ENREF_14)]. All membranes (including the working pad, sample, and conjugate pads) were collected on a plastic cover.

## 2.3. Preparation and validation of tested samples

For the characterization of stool samples, the commercial kit Adenovirus-antigen-ELISA-BEST (Novosibirsk, Russia; [vector-best.ru](http://vector-best.ru/)) was used. To prepare the samples in the buffer-model solution, PBS with 0.05% Tween-20 (PBST) was used, into which adenovirus antigen (Tonsil 99) was introduced at concentrations of 30-2,000 ng/mL.

To study the matrix effect, feces samples were used. The absence of an adenovirus antigen was confirmed using the Adenovirus-antigen-ELISA-BEST kit. The mixture for analysis was prepared using a special plastic bottle with a sampling stick (FOB tube). The ratio of 100 µg of feces per 1 mL of the PBST solution with pre-introduced adenovirus antigen at concentrations of 30-2,000 ng/mL was maintained for the sample solution. Before analysis, the solution was stirred by shaking the vial for 1-2 min. The application of the sample to the test was carried out through the built-in lid-dropper of the bottle. Three to four drops of solution were applied to each test, which was 100-130 μL.

## 2.4. Immobilization of antibodies on GNPs

GNPs with a concentration of 50 µg/mL and a diameter of 30 nm (A580 = 1) were obtained according to the method of Frens [[15](#_ENREF_15), [16](#_ENREF_16)]. The pH of the GNP solution was adjusted to between 8.5 and 9.0 with potassium carbonate, followed by the addition of antibodies (10 µg/mL of GNP solution) diluted in 10 mM Tris buffer, pH 8.5. The resulting mixture was incubated for 45 min at room temperature, following which a 10% aqueous solution of BSA (VGNP:VBSA = 40:1) was added and the mixture was stirred vigorously for 15 min. The GNPs were pelleted by centrifugation at 15,000 × g for 15 min at 4°C. The precipitate was collected and resuspended in 10 mM Tris buffer, pH 8.5, containing TBSU and 0.05% sodium azide. The obtained solution was stored at 4°C.

## 2.5. Drop test

Each tested clone is applied to the working membrane in a volume of 1 µL (until a drop of 2 mm in diameter is formed on the nitrocellulose membrane) and then dried for 30 minutes at low humidity and a temperature of 25°C. The working membrane is attached to a plastic back card, with the upper edge in contact with the absorbing membrane, and the lower one lowered into the solution of the conjugate of specific antibodies with a colored marker (in PBST). The experiment was carried out in two variants for each pair of antibodies, with and without the addition of 1 μg/mL of antigen.

## 2.6. Analysis and digital processing of results

A mixture of adenovirus in PBST or adenovirus in PBST with stool samples (100 μl) was applied to test strips. Each test strip was incubated for 15 min after which the result of the analysis was evaluated either visually or using digital processing. For statistical processing all the measurements were performed in triplicate. A calibration curve was made as described above.

Processing of digital images of test strips was carried out using the Total Lab program (Nonlinear Dynamics, United Kingdom): the analyzed area was captured, capturing less than 90% of the working membrane area. The intensity of the colored zone(s) and the background level were determined inside this area. Based on the values of the integrated staining intensity of the analytical zone, obtained for different concentrations of adenovirus, a calibration curve was constructed. Based on the dependence of the signal on the concentration of antigen in the sample, the detection limit of the antigen corresponding to a 10% inhibition of binding was determined (IC10) [[17](#_ENREF_17)].

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# 3. Experiment and Result

A typical immunochromatographic test strip is a composite of several membranes. Antibodies are immobilized on the working membrane in the control and analytical zones. The bottom of the working membrane is in contact with the membrane for the sample and the membrane for the conjugate (specific antibodies conjugated with colored marker particles). The upper part is adjacent to the membrane to absorb the sample. The liquid sample passes through the entire composite of membranes, where it sequentially interacts first with labeled antibodies and then with immobilized antibodies in the analytical zone. In the presence of an antigen sandwich complex, "immobilized antibody-antigen-antibody on the surface marker" is formed. The accumulation of such complexes in the analytical zone leads to the appearance of visually detectable coloration. In the absence of antigens, the complex is not formed and the color does not appear.

To achieve the required analytical parameters, we must perform the following stages of development:

1. Choosing pairs of specific antibodies

2. Studying pairs using immunochromatography

3. Testing the effect of the sample matrix

4. Adjusting the results of testing the influence of the matrix

5. Comparative analysis with a commercial test system

## Selection of antibody pairs

Based on the drop-test results, the optimal pairs were selected. The selection criterion was the presence of a high level of a specific signal (formation of a bright crescent-shaped color) in the complete absence of nonspecific binding (see Fig. 1).

The drop-test method was used to screen antibodies from different manufacturers: clones 8AD, 3AD, and 17AD (Bialexa) and A46-Ab1 and A46-Ab2 (Artron).



Figure 1. Drop test for the selection of the optimal antibody pairs.

According to the results of the preliminary screening of all clones of antibodies, the following pairs were detected that effectively form a sandwich complex in the presence of the analyte, and do not interact with each other in its absence:

(a) Bialexa clone 3AD as labeling and 8AD as coating antibodies

(b) Artron clone A46-Ab1 as labeling and A46-Ab2 as coating antibodies

(c) Artron clone A46-Ab2 as labeling and A46-Ab1 as coating antibodies

## The study of selected pairs of antibodies in the immunochromatography

The next step was to test selected combinations of antibodies on the assembled test strips. All reagents are applied automatically and dried, and the test strip itself is assembled into a composite that includes a sample membrane, a conjugate, a working membrane with control and analytical zones, and an absorbent membrane. A series of dilutions of the antigen in the concentration range of 30-2,000 ng/mL in PBST buffer solution is used to check the assembled test strips. The results of the experiment are presented in Fig. 2.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **A** |  |  |  |  |  |  |  |  |
| **B** |  |  |  |  |  |  |  |  |
| **C** |  |  |  |  |  |  |  |  |
|  | 2000 | 1000 | 500 | 250 | 125 | 62 | 31 | 0 |
|  | Adenovirus concentration in PBST, ng/mL | | | | | | | |

Figure 2. Checking the selected antibody pairs for the complete immunochromatography system: (a) Bialexa clone 3AD as labeling and 8AD as coating antibodies; (b) Artron clone A46-Ab1 as labeling and A46-Ab2 as coating antibodies; (c) Artron clone A46-Ab2 as labeling and A46-Ab1 as coating antibodies (the upper line is the control zone, the lower line is the analytical one). PBST, phosphate-buffered saline with 0.05% Tween-20.

The digitization of the staining intensity of the rapid test’s analytical zones was carried out for a more accurate comparison. The data are presented in Fig. 3.



Figure 3. Concentration dependences of the color intensity in the test zone of the test strip: (a) Bialexa clone 3AD as labeling and 8AD as coating antibodies; (b) Artron clone A46-Ab1 as labeling and A46-Ab2 as coating antibodies; (c) Artron clone A46-Ab2 as labeling and A46-Ab1 as coating antibodies.

The detection limit was 30 ng/mL for Artron antibodies and 60 ng/mL for Bialexa antibodies. For all pairs of antibodies, non-specific staining wasn’t observed in the absence of antigens. However, Artron clone A46-Ab2 as labeling and A46-Ab1 as coating antibodies showed a higher staining intensity of the analytical zone of the test strip; therefore, this pair of antibodies was chosen for further work.

## Testing the effect of the sample matrix

After selecting a pair of antibodies, the next step was to test the effect of the sample matrix on the test system. Feces are heterogeneous in structure and composition, and can affect the membranes and the fluid flow rate as a consequence of changing the parameters of the analytical test. To work with samples, we used fecal vials that allowed us to quickly and easily carry out both sampling (Fig. 4-1) and extraction (Fig. 4-2), and apply the samples to a test strip (Fig. 4-3) without additional manipulations and devices.



Figure 4. Using a fecal vial for immunochromatographic analysis of adenovirus.

Test strips were tested for the determination of antigen in the sample matrix solution. As can be seen from the results (Fig. 5), the matrix leads to the appearance of a nonspecific background signal, which may be due to a change in the viscosity of the solution, the kinetics of immunochemical interaction, or the presence of components that cause nonspecific binding.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |
| 2000 | 1000 | 500 | 250 | 125 | 62 | 31 | 0 |
| Adenovirus concentration in feces, ng/mL | | | | | | | |

Figure 5. Testing the analytical system in a sample matrix solution (clone A46-Ab2 as labelling and A46-Ab1 as coating); the upper line is the control zone, the lower line is the analytical one.

To overcome these factors, the following changes were made to the protocol for building the test. To overcome the viscosity of the sample and the kinetics of the reaction, we used a replacement of the working membrane with a variant with a higher flow rate (MDI CNPC, 12-15 µ). To reduce non-specific binding, we applied an additional amount of detergent to the composition of the solution of antibody conjugate with colored particles (0.01% Tween-20) and protein into the analytical zone on the working membrane (BSA at a concentration of 5 mg/mL). It is known that this presence in the analytical zone may reduce non-specific binding [[18](#_ENREF_18)]. The result was a variant of the test system with no background signal and a detection limit of 30 ng/mL (Fig. 6).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |
| 2000 | 1000 | 500 | 250 | 125 | 62 | 31 | 0 |
| Adenovirus concentration in feces, ng/mL | | | | | | | |

Figure 6. Detection of adenovirus using the developed test system (CNPC 15-µ working membrane, BSA and Tween-20); the upper line is the control zone, the lower line is the analytical one. BSA, bovine serum albumin.

## Comparative analysis with a commercial test system

The final step in creating a test system for determining adenovirus infection in feces was a comparison with commercial rapid tests already on the market. The VIKIA Rota-Adeno test provided by bioMérieux SA was chosen as a reference. By determining the number of concentrations of antigen in the matrix (Fig. 7), it was found that the detection limit for both test systems is 30 ng/mL. However, the developed fast tests had a higher staining intensity of the analytical zone.



Figure 7. Comparison of the developed test system (a) with a commercial analogue (b) for determining adenovirus.

# 4. CONCLUSION

A highly sensitive immunochromatographic test was developed, with which it is possible to determine the presence of adenovirus in feces in 15 minutes. The detection limit of the test was 30 ng/mL. The main stages of development of an immunochromatographic test were explained: quick selection of pairs of specific reagents, evaluation of the test efficiency, the method of sampling feces, and approaches for optimizing the immunoassay in a complex sample. The efficiency of digital processing for the immunochromatographic test’s development is shown. The work was financially supported by a Grant of the President of the Russian Federation for state support of young Russian scientists—PhD No. MK-2075.2017.4 (agreement No. 14.W01.17.2075-MK).

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