MODIFIED MICRO TEST TUBES AS A PROMISING BASIS FOR IMMOBILIZATION OF ANTIBODIES FOR IMMUNOCAPTURE ON THE EXAMPLE OF SARS-COV-2

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The solid-phase immunocapture with antibodies is an important tool used in immunology studies, but conventional polystyrene plates are prone to deformation during thermal cycling and cross-contamination of samples, which reduces accuracy and reproducibility, when molecular genetic testing methods are included in the study. The development of alternative solutions, such as modified polystyrene-coated polypropylene tubes, makes it possible to eliminate these limitations. The study aimed to create a new approach to SARS-CoV-2 immunocapture involving the use of modified test tubes and to assess its efficacy. Monoclonal antibodies P2C5 and R107, as well as inactivated strains GK2020/1 (Wuhan) and hCoV-19/Russia/MOW-PMVL-51/2021 (Omicron) were used for analysis. Immobilization of antibodies, sorption of viral particles, and RNA extraction were accomplished using modified test tubes, standard plates, and uncoated test tubes. The key findings showed that the polystyrene-coated modified test tubes ensured better immunocapture compared to the plates ($p < 0.0001$), especially when using the P2C5 antibody effective against various SARS-CoV-2 lineages, including Omicron. The R107 antibody showed limited specificity, not exceeding that of the control group with bovine serum albumin. The cross-contamination analysis revealed contamination of 14 samples out of 288 in the plates, while no contamination of samples was reported for modified test tubes. Thus, modified test tubes used for high-precision molecular testing have some advantages, since these decrease the risk of cross-contamination and improve immunocapture efficacy.

Keywords: immunocapture, monoclonal antibodies, RT-PCR, modified surface, cross-contamination, SARS-CoV-2

Funding: the study was supported by the Russian Science Foundation grant No. 23-15-20035, https://rscf.ru/project/23-15-20035/

Author contribution: Rubalsky EO, Abdrakhmanova RA, Baeva GR, Rubalskaya TS — experimental procedure involving micro test tubes; Shcheblyakov DV, Favorskaya IA — preparation of monoclonal antibodies; Rubalsky EO, Lazko MV, Poroyskiy SV, Gushchin VA — study design. The authors contributed to the search for and analysis of information, preparation of the draft, and manuscript writing equally.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Astrakhan State Medical University (protocol No. 7 dated 22 February 2023).

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Received: 29.11.2024 **Accepted:** 19.12.2024 **Published online:** 28.12.2024

DOI: 10.24075/brsmu.2024.068

МОДИФИЦИРОВАННЫЕ МИКРОПРОБИРКИ — ПЕРСПЕКТИВНАЯ ОСНОВА ДЛЯ ИММОБИЛИЗАЦИИ АНТИТЕЛ ИММУНОЗАХВАТА НА ПРИМЕРЕ SARS-COV-2

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Иммунозахват антителами на твердой фазе является важным инструментом в иммунологических исследованиях, но традиционные полистироловые планшеты подвержены деформации при термоциклировании и перекрестной контаминации образцов, что снижает точность и воспроизводимость при включении в исследование молекулярно-генетических методов. Разработка альтернативных решений, таких как модифицированные полипропиленовые пробирки с полистироловым покрытием, позволяет устранить эти ограничения. Целью исследования было создать и оценить эффективность нового подхода к иммунозахвату SARS-CoV-2 с использованием модифицированных пробирок. Для анализа использовали моноклональные антитела P2C5 и R107, а также инактивированные штаммы ГК2020/1 (Ухань) и hCoV-19/Russia/MOW-PMVL-51/2021 (Омикрон). Иммобилизацию антител, сорбцию вирусных частиц и выделение РНК проводили с применением модифицированных пробирок, стандартных планшетов и пробирок без покрытия. Основные результаты показали, что модифицированные пробирки с полистироловым покрытием обеспечивали лучший иммунозахват по сравнению с планшетами ($p < 0{,}0001$), особенно при использовании антител P2C5, эффективных против различных генетических линий SARS-CoV-2, включая Омикрон. Антитела R107 продемонстрировали ограниченную специфичность, не превышающую уровень контрольной группы с бычьим сывороточным альбумином. Анализ перекрестной контаминации выявил ее наличие в 14 из 288 образцов в планшетах, тогда как в модифицированных пробирках контаминации образцов не было. Таким образом, использованные для высокоточных молекулярных исследований модифицированные пробирки имеют преимущества, так как обеспечивают снижение риска перекрестной контаминации и улучшение эффективности иммунозахвата.

Ключевые слова: иммунозахват, моноклональные антитела, ОТ-ПЦР, модифицированная поверхность, перекрестная контаминация, SARS-CoV-2

Финансирование: исследование выполнено за счет гранта Российского научного фонда № 23-15-20035, https://rscf.ru/project/23-15-20035/

Вклад авторов: Е. О. Рубальский, Р. А. Абдрахманова, Г. Р. Баева, Т. С. Рубальская — экспериментальная часть с модифицированными микропробирками; Д. В. Щебляков, И. А. Фаворская — подготовка моноклональных антител; Е.О. Рубальский, М. В. Лазько, С. В. Поройский, В. А. Гущин — дизайн исследования. Все авторы внесли равный вклад в поиск и анализ информации, подготовку проекта и редактирование рукописи.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБОУ ВО Астраханский ГМУ Минздрава России (протокол № 7 от 22 февраля 2023 г.).

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Статья получена: 29.11.2024 **Статья принята к печати:** 19.12.2024 **Опубликована онлайн:** 28.12.2024

DOI: 10.24075/vrgmu.2024.068

Immunocapture based on specific antibodies is a key phase of many immunological laboratory testing methods. Polystyrene microplates that ensure the possibility of simultaneous processing of multiple samples are conventionally used for implementation of this method. Polystyrene usually can passively (in non-specific manner) immobilize almost all large molecules having accessible hydrophobic fragments, such as antibodies, on its surface [1]. However, this material is prone to deformation at temperatures above +70 °C (above +60 °C when heated over a long time) [2]. This is a serious technical issue of the use of additional molecular genetic phases of analysis, such as thermal nucleic acid extraction and thermal cycling (for example, in immuno-PCR [3]), which limits the widespread practical use of such methods. Polycarbonate strip tubes gathered in the 96-well plate that have been proposed as an alternative to polystyrene plates are resistant to heat and have high sorption capacity [4].

However, the use of plates is associated with high risk of cross-contamination [5, 6]. The risk can be leveled using various methodological approaches, such as the use of automated sample preparation systems. The use of conventional polypropylene test tubes for molecular genetic testing might potentially decrease the risk of contamination when working in accordance with the principle of "one open test tube". However, the use of test tubes is limited by low protein sorption capacity of polypropylene, including adsorption of antibodies [7], which prevents their use for high-sensitivity methods, such as immuno-PCR, that require stable immobilization of immunocapture antibodies [4].

The development of such methods, as immunomolecular assays, can significantly increase sensitivity and specificity of molecular genetic testing, which is especially important for the diagnosis and monitoring of viral infections showing high mutational variance of the pathogen, such as SARS-CoV-2 virus [8]. It should be noted that such reactions have not yet received wide practical application. To date, no studies of the COVID-19 causative agent by immuno-PCR have been reported in scientific literature. This is likely to be associated with complexity of the design and implementation of the reaction phases. That is why it is necessary to optimize their key components and overcome technical issues inherent to the existing reaction formats for successful practical use of such combination approaches [4, 9].

In this regard, we have proposed original polystyrene coating of polypropylene test tubes. This solution makes it possible to combine advantages of both materials: high sorption capacity of polystyrene and heat resistance of polypropylene, with the reduced risk of cross-contamination.

The study aimed to develop a simple production method and perform testing of new test tubes for immunocapture ensuring effective immobilization of primary antibodies and designed for universal use in molecular genetic testing on an example of SARS-CoV-2.

METHODS

SARS-CoV-2 strains and antibodies

The well-characterized samples of positive control of the strain GK2020/1 (GISAID identifier: EPI_ISL_421275, variant B.1.1.1, Wuhan) and strain hCoV-19/Russia/MOW-PMVL-51/2021 (GISAID identifier: EPI_ISL_12748382, variant B.1.1.529+BA.* of the Omicron lineage) of the SARS-CoV-2 coronavirus chemically inactivated using glutaraldehyde in the final concentration of 0.01% and then incubated at +4 °C for 24 h were used as an antigen.

To assess the effectiveness of primary antibody immobilization (immunocapture) we used the P2C5 experimental monoclonal antibodies against RBD of the coronavirus spike protein (produced at the Immunobiotechnology Laboratory of the Gamaleya National Research Centre for Epidemiology and Microbiology) having the broadest spectrum of activity covering inter alia certain variants of the Omicron lineage [10]. Furthermore, we tested the R107 monoclonal antibody (Hytest, Russia) showing specific activity mainly against RBD of the B.1.1.1 virus variant.

Assessing the effectiveness of immunocapture involving the use of polystyrene ELISA plates

To optimize immobilization of antibodies, we used the FEP-101-896 96-well flat bottom polystyrene immunological plates (Guangzhou Jet Bio-Filtration Co., Ltd., China). We added 100 µL of antibodies in a concentration of 10 µg/mL dissolved in the 0.5 M carbonate buffer (pH = 9.5) to each well of the plate, covered the plates with film and incubated at a temperature of +37 °C for 30 min. We used 100 µL of phosphate-buffered saline (PBS, pH = 7.4) supplemented with 2% bovine serum albumin (BSA) as a blocking buffer with incubation at +37 °C for 30 min. The washing buffer consisted of PBS (pH = 7.4) supplemented with 0.05% Tween 20.

After triple washing of the wells with 300 µL and incubation for 45 s, these were added 50 µL of the SARS-CoV-2 samples inactivated and preliminarily diluted with PBS to the concentration of 1×10^6 copies/mL and incubated at +37 °C for 30 min. Then washing was performed, followed by RNA extraction using the RIBO-prep kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) and quantitative RT-PCR.

RNA was extracted in accordance with the modified manufacturer's protocol: we added 50 µL of TE buffer to the wells of the polystyrene plate, then added 155 µL of the lysing solution, covered the wells with film for ELISA plates and incubated at +65 °C for 10 min. Then the content of the polystyrene plate wells was transferred to micro test tubes, and subsequent phases of RNA extraction were implemented in accordance with the manufacturer's instructions. Quantitative RT-PCR was conducted using the SARS-CoV-2-PCR reagent kit (MedipalTech, Russia) in combination with the samples of the chemically inactivated GK2020/1 strain used as a calibrator. All the experiments were conducted in ten replicates (n = 10).

Assessing immunocapture involving the use of modified micro test tubes

The 1.5 mL sterile micro test tubes made of transparent polypropylene, free from DNAses and RNAses (SPINWIN Tarsons, India) and polystyrene from the FEP-101-896 immunological plates (Guangzhou Jet Bio-Filtration Co., Ltd., China) dissolved in acetone were used as components for production of modified micro test tubes. A total of 50 µL of polystyrene solution were aseptically applied to the bottom of the test tubes and dried in a fume hood. Mechanical stability of the resulting polypropylene layer was visually estimated after the vortex mixing of the added 1 mL of water at 2500 rpm for 2 min by twenty-fold pipetting with the pipette tip touching the micro test tube bottom and centrifugation at 15,000 g for 15 min. A total of 100 µL of antibodies in the carbonate buffer (10 µg/mL) were added to the micro test tubes modified by this method and incubated at +37 °C for 30 min with the lids closed. After blocking the remaining antibody-free surface with

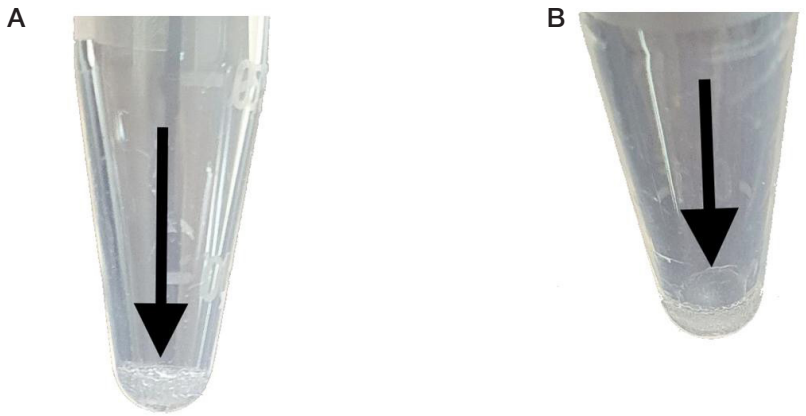

Fig. 1. Polypropylene micro test tube after modification with polystyrene: side view **(A)** and ≈45° view **(B)**

PBS supplemented with 2% BSA and washing (similar to that of plates), 50 µL of SARS-CoV-2 samples were added. Further washing, RNA extraction, and RT-PCR were performed in the same way, as when using polystyrene plates. All the RNA extraction phases were implemented in modified micro test tubes.

The non-modified polypropylene test tubes were used as controls. All the experiments were conducted in ten replicates (n = 10).

Assessing cross-contamination

To assess cross-contamination, 50 µL of the GK2020/1 (variant B.1.1.1) and hCoV-19/Russia/MOW-PMVL-51/2021 (Omicron) strains were added in a staggered manner to the 96-well plates and modified test tubes with the immobilized P2C5 antibody. After incubation, washing, and RNA extraction, genotyping was performed by RT-PCR. To detect the GK2020/1 strain, we conducted sequence-specific RT-PCR with original primers Wu_fw1mod 5'-CGTGGTTCCATGCTATACATG-3' and Wu_rv1mod 5'-CGTCCCTGTGGTAATAAACAC-3' in the ready-made reaction mixture OneTube RT-PCR SYBR (Evrogen, Russia) involving real-time detection in the SYBR-Green channel. The strain of the Omicron lineage was detected using the AmpliTest SARS-CoV-2 VOC v.3 kit (Centre for Strategic Planning and Management of Biomedical Health Risks of FMBA of Russia, Russia) in accordance with the manufacturer's instructions. Three plates and 288 test tubes were used for the experiment. The experiment was conducted in the laminar airflow in the class II A2 biosafety cabinet (LamSystems, Russia).

Statistical analysis

The data were analyzed using the GraphPad PRISM v.10.4.0 software (Graphpad Software, Inc., USA). Distribution was estimated using the Shapiro–Wilk test. Two-way ANOVA with Tukey's test of additivity was used for multiple comparisons of parametric data; the significance level was set as $p < 0.05$ (confidence interval (95%).

RESULTS

Modified micro test tubes

A polypropylene micro test tube after modification with polystyrene is presented in Fig. 1. The modified micro test tubes obtained were mechanically stable during both vortex mixing and mixing by pipetting. Centrifugation also had no visible effect on the polystyrene layer integrity.

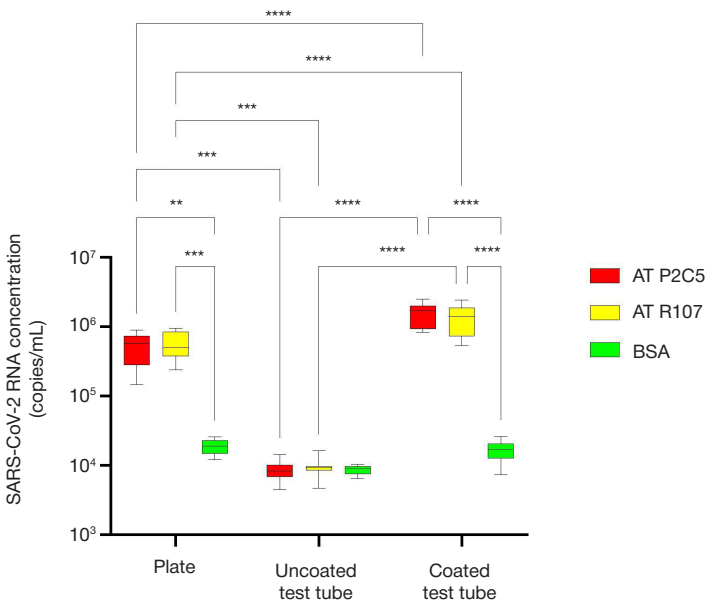

Fig. 2. The GK2020/1 strain immunocapture results. The P2C5 monoclonal antibody is highlighted in *red*, R107 is highlighted in *yellow*, control group with BSA is highlighted in green. ** — $p < 0.01$; *** — $p < 0.001$; **** — $p < 0.0001$

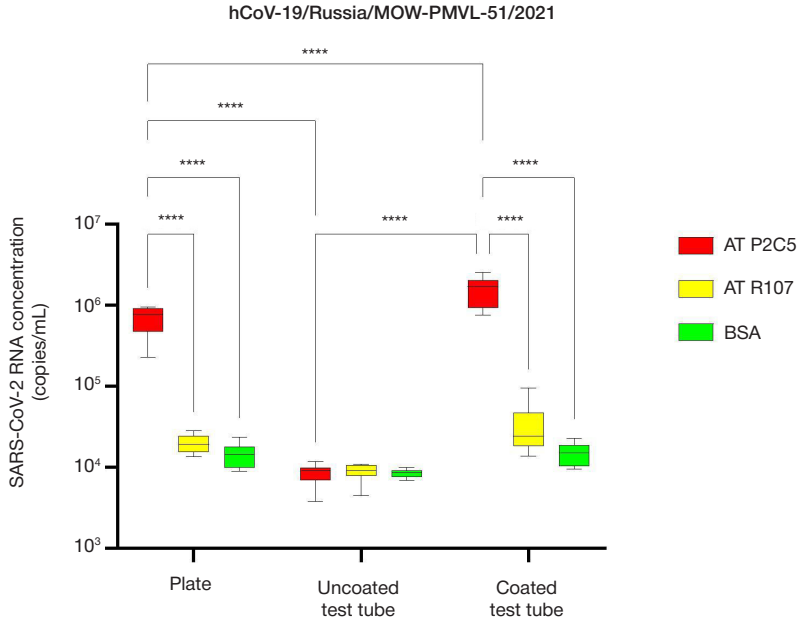

Fig. 3. The hCoV-19/Russia/MOW-PMVL-51/2021 strain immunocapture results. The P2C5 monoclonal antibody is highlighted in *red*, R107 is highlighted in *yellow,* control group with BSA is highlighted in green. **** — $p < 0.0001$

Immunocapture of inactivated SARS-CoV-2

When using the GK2020/1 strain (variant B.1.1.1) as an antigen, effective immunocapture was reported for both P2C5 and R107 antibodies (Fig. 2). The quantity of virus bound by antibodies turned out to be significantly higher when using modified test tubes compared to polystyrene plates ($p < 0.0001$). The number of SARS-CoV-2 RNA copies in the control groups, where BSA was used, was significantly lower, than the number reported for the groups with antibodies when using both plates and modified test tubes. When using the test tubes without coating, the equally low quantities of coronavirus RNA were determined in all groups ($p > 0.05$), which suggests the lack of specific antigen immunocapture, regardless of the use of antibodies.

When using the hCoV-19/Russia/MOW-PMVL-51/2021 strain (Omicron lineage), effective immunocapture was reported for the P2C5 antibody only (Fig. 3). The R107 antibody specific for RBD primarily of the Wuhan SARS-CoV-2 variant showed no significant differences from the control group with BSA. Thus, the strain of the Omicron lineage showed the ability to escape the R107 neutralizing antibody, which is consistent with the data of other researchers on the decreased efficacy of some monoclonal antibodies against new SARS-CoV-2 variants [11, 12]. The modified test tubes ensured better results, than polystyrene plates, for both B.1.1.1 and Omicron lineages.

Cross-contamination

Cross-contamination was found in 14 samples out of 288 when using polystyrene plates (Fig. 4). Contamination was represented by mixing of RNA of the GK2020/1 and hCoV-19/Russia/MOW-PMVL-51/2021 strains, which confirms the risk of material transfer between the wells under such conditions. No cross-contamination was reported for modified test tubes.

DISCUSSION

The findings show that polystyrene-coated modified polypropylene test tubes offer significant advantages over conventional polystyrene plates, including reduced risk of cross-contamination and improved immunocapture efficacy. These data are in line with the studies showing that polystyrene plates represent a good material for immobilization of antibodies, but their use is limited due to low heat resistance and the risk of contamination [2, 5, 6].

The differences in efficacy of the P2C5 and R107 antibodies reported when working with different SARS-CoV-2 strains are consistent with the data on significant mutations in RBD of the Omicron lineage, which reduce the efficacy of antibodies specific for the B.1.1.1 (Wuhan) virus variant [13, 14]. It is assumed that such mutations lead to conformational RBD alterations, thereby preventing binding of antibodies,

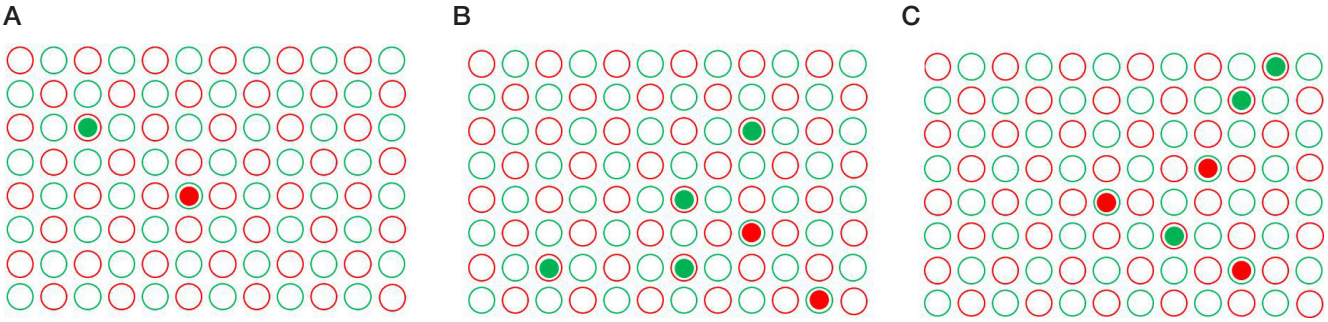

Fig. 4. Cross-contamination detected when extracting SARS-CoV-2 RNA in the 96-well plates. The wells depicted as *red circles* contain the GK2020/1 strain (variant B.1.1.1). The wells depicted as *green circles* contain the hCoV-19/Russia/MOW-PMVL-51/2021 strain (variant B.1.1.529+BA.*Omicron lineage). *Red dots* in *green circles* indicate contamination of the hCoV-19/Russia/MOW-PMVL-51/2021 strain with the GK2020/1 strain. *Green dots* in *red circles* indicate contamination of the GK2020/1 strain with the hCoV-19/Russia/MOW-PMVL-51/2021 strain. **(A–C)** Three sequential tests performed by three different operators

as previously reported for first-generation antibodies against SARS-CoV-2 [15]. High efficacy of P2C5 covering a broad range of variants, including Omicron, confirms their versatility and relevance for monitoring of new strains.

The lack of cross-contamination in modified test tubes can be explained by the lack of direct contact between samples and minimization of airborne transfer. Similar conclusions were drawn in the studies, in which other closed systems were used for molecular diagnosis [16].

Despite positive results, it is necessary to further optimize the conditions of antibody immobilization and RNA extraction in order to improve reproducibility of the method when using modified test tubes. Furthermore, for widespread use of the tubes, testing on other pathogens is necessary to confirm versatility of the method. Further testing and improvement of the test tubes can cover the following areas: study of the suitability and compatibility of polypropylene and polystyrene of various grades; reduction of non-specific signal through the use of polypropylene tubes with low adhesive capacity; polystyrene application automatization.

In general, modified test tubes represent a promising basis for combined immunological and molecular genetic testing requiring high sensitivity and accuracy, especially under conditions of high variability of pathogens, such as SARS-CoV-2.

CONCLUSIONS

The polystyrene-coated modified polypropylene test tubes have shown high immunocapture effectiveness and the lack of cross-contamination, being superior to conventional polystyrene plates. Such findings confirm that the goals of the study have been achieved. The P2C5 antibody have shown versatility when used against various SARS-CoV-2 lineages, including Omicron, which makes these promising for the diagnosis of new variants of the COVID-19 causative agents. Further research should be focused on testing the test tubes with other pathogens and improving their functional characteristics. Possible areas to use the polystyrene-coated modified polypropylene test tubes include clinical diagnosis and development of highly sensitive methods for virus detection, selection, and identification.

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