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Ultra-fast, high throughput and inexpensive detection of SARS-CoV-2 seroconversion using Ni^{2+} magnetic beads

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ABSTRACT

To monitor the levels of protecting antibodies raised in the population in response to infection and/or to immunization with SARS-CoV-2, we need a technique that allows high throughput and low-cost quantitative analysis of human IgG antibodies reactive against viral antigens. Here we describe an ultra-fast, high throughput and inexpensive assay to detect SARS-CoV-2 seroconversion in humans. The assay is based on Ni^{2+} magnetic particles coated with His tagged SARS-CoV-2 antigens. A simple and inexpensive 96 well plate magnetic extraction/homogenization process is described which allows the simultaneous analysis of 96 samples and delivers results in 7 min with high accuracy.

The COVID-19 outbreak, caused by the novel beta coronavirus SARS-CoV-2, has posed an extraordinary threat to human health with ~3,000,000 deaths reported worldwide by April 2021. No effective medication to treat the disease is yet available. Despite the successful development of efficient vaccines by different laboratories, vaccination is still limited to developed countries and only a small fraction of the world's population has been immunized [1]. COVID-19 vaccination is extremely limited, if any, in developing countries, and social distancing is the only effective measure to reduce the spread of the disease.

Tracking COVID-19 is of key importance to understand and mitigate the spread of the disease. COVID-19 testing can be performed by a variety of methods with the most common being molecular or antigen tests which detect active SARS-CoV-2 infections. Alternatively, an immunological assay can be employed to detect human antibodies reacting against different SARS-CoV-2 antigens [2]. One advantage of immunological COVID-19 tests is that antibodies remain detectable for months after convalescence and thus can be used for epidemiological surveillance studies [3]. Furthermore, with the advance of COVID-19 vaccination, these immunoassays can be applied to identify the fraction of the population that developed IgG antibodies reacting against SARS-CoV-2 antigens, which are likely to reflect the protection after immunization. Since there is no knowledge on how long IgG antibodies raised against SARS-CoV-2 in the context of a natural infection or vaccination will last, large immune surveillance studies will be necessary in near future to depict that issue [4]. Hence, the development of simple, fast and inexpensive COVID-19 immunoassays that can provide quantitative data in high throughput format in response to different antigens will be key to enable such large cohort studies in the population.

We previously described a fast, simple, and inexpensive Ni^{2+} magnetic bead immunoassay which allows detection of human antibodies reactive against the SARS-CoV-2 nucleocapsid protein using a minimal amount of serum or blood [5]. Here we show that such system is amendable to high throughput and can deliver ultrafast results in less

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than 7 min with different SARS-CoV-2 antigens while maintaining low analytical cost.

1. Experimental section

Human samples were collected at Hospital Erasto Gaertner in Curitiba and Secretaria Municipal de Saúde in Guaratuba and Federal University of Paraná in Matinhos. Samples for serological analysis comprised both serum and plasma-EDTA. COVID-19 positive cases were confirmed by the detection of SARS-CoV-2 RNA via real-time RT-PCR from nasopharyngeal sample swabs. The time point of sampling of serum ranged from 1 to 100 days after PCR detection. Among the 63 COVID-positive cases there were 12 convalescents including 2 asymptomatic and 10 mild non-hospitalized cases. All remaining samples were collected within the first 14 days of the hospitalization period. The cohort of 204 negative controls consisted of pre pandemic samples collected in 2018. For the work done in Germany, pre pandemic samples were purchased from Central BioHub GmbH (Henningsdorf, Germany). COVID-19 positive samples were collected from convalescent donors post quarantine and were self-reported PCR-positive for SARS-CoV-2. The Institutional Ethics Review Board CEP/HEG (n# 31592620.4.1001.0098) and CEP/UFPR (n#43948621.7.0000.0102) approved this study. Informed consent was obtained from all participants in this study. All methods were performed in accordance with the relevant guidelines and regulations.

Magnetic beads-based immunoassay. The magnetic bead-based immunoassay was developed using Ni^{2+} magnetic beads as described previously [5]. The recombinant N-terminal 6x His-tagged Nucleocapsid protein of SARS-CoV-2 was expressed from the pLHSARSCoV2-N plasmid using *E. coli* BL21 (λDE3) as host [6]. The cells were grown in 100 ml LB medium at 120 rpm at 37 °C to OD_{600nm} of 0.4. The incubator temperature was lowered to 16 °C, after 30 min, IPTG was added to a final concentration 0.3 mM and the culture was kept at 120 rpm at 16 °C over/night. Cells were collected by centrifugation at 3000×*g* for 5 min. The cell pellet was resuspended in 25 ml of buffer 1 (Tris-HCl pH 8 50 mM and KCl 100 mM). Cells were disrupted by sonication in an ice bath. The soluble fraction was recovered after centrifugation at 20,000×*g* for 10 min and incubated for 5 min on ice with gentle mixing with 10 ml of Ni^{2+} magnetic particles (Promega cat number V8550) pre equilibrated in buffer 1. The beads were washed 2 times with 25 ml of buffer 1 and 2 times with 25 ml of buffer 1 containing 100 mM imidazole. Beads were resuspended in 25 ml of TBST and stored in 0.8 ml aliquots at 4 °C. Starting from 100 ml of *E. coli* culture, coated beads for up to 6000 assays could be prepared and were stored at 4 °C for up to 1 month. In the case of Spike and Spike RBD, 1 mg of His-tagged antigens purified as described previously [7,8] were incubated with 1 ml of nickel magnetic particles in 25 ml of TBST. After 5 min at room temperature with gentle mix the beads were washed with 25 ml of TBST and resuspended in 5 ml of TBST. Loaded beads were stored in 0.8 ml aliquots at 4 °C.

The magnetic bead immunoassay was performed using the 96-sample format with flat bottom plates (Cralplast). The 0.8 ml aliquots of antigen loaded beads were resuspended in 10 ml of TBST containing 1%

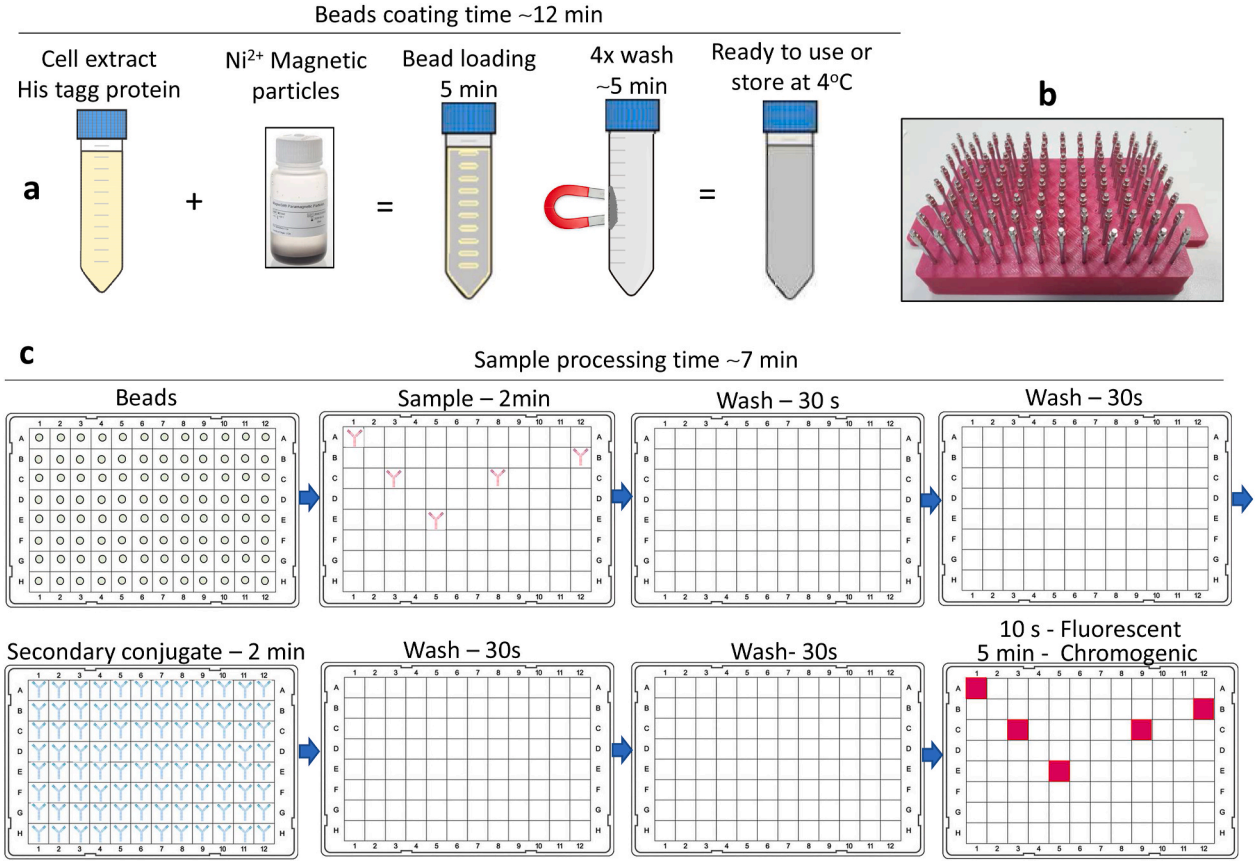

Fig. 1. Steps of the high throughput ultrafast Ni^{2+} magnetic bead immunoassay. a) Diagram of the resin coating/antigen purification process. The soluble crude cell extract containing the 6x His tag Nucleocapsid protein is incubated with commercial Ni^{2+} magnetic particles for 5 min, followed by 4 washes with increasing concentrations of imidazole. After ~12 min the beads are ready to use or storage at 4 °C. b) Photograph of the 96-pin magnetic extractor device used for 96-well plate magnetic bead transfer. c) Diagram of the 8 steps of the magnetic immunoassay. Bead transfer from plate to plate is performed using the magnetic extractor device followed by manual homogenization for the indicated time. In the fluorescence-based assay, the final plate contains TBST buffer, and just a 10s bead homogenization is required before the fluorescence measurements. For chromogenic detection, the final plate contains the HPR chromogenic substrate TMB, and a 5 min incubation is required prior to optical density measurements. The total time required for the fluorescence-based assay is ~7 min.

(w/v) skimmed milk and 0.1 ml of the mixture was distributed in each well of a 96 well plate (Fig. 1). Four micro liters of human serum were diluted in 0.2 ml of TBST 1x skimmed milk 1% (w/v) directly on the wells of a second 96 well plate. When indicated, serum was substituted with 10 μl of freshly collected capillary blood from finger puncture. The magnetic beads were transferred to the sample plate and incubated with the human sample (serum or blood) for 2 min with gentle mix. The beads were captured and loaded into sequential 2 wash steps for 30 s in 1x TBST. The beads were incubated for 2 min with 0.15 ml goat anti-human IgG-HPR (Thermo Scientific) diluted 1:1500 in 1x TBST, followed by a 2-wash step for 30 s in 1x TBST. The beads were transferred to wells containing 0.15 ml of the HPR substrate TMB (Thermo Scientific) and incubated for 5 min. The entire procedure took less than 12 min. When the reactions were complete, the beads were removed and the OD_{650nm} was recorded using a TECAN M Nano plate reader (TECAN) monochromator at bandwidth 9 nm and 25 flashes. For the process operating in the fluorescent detection mode the secondary conjugated used was goat anti-human IgG-PE (Moss Inc.) diluted 1:250 in 1x TBST and beads were transferred and homogenized for 10 s to a final plate containing TBST before fluorescent reading using a TECAN M Nano plate reader (TECAN) operating at fluorescent top reading. Excitation 545 nm (bandwidth 9 nm and 25 flashes) and emission 578 nm (bandwidth 20 nm, integration time 20 μs and Z-position at 20,000 μm).

Magnetic bead extractor device. Details of the construction of the magnetic extractor device and operation are provided in supplementary information and supplementary video.

Data analysis. One COVID-19 positive serum was used as reference throughout the study. Raw data were normalized as % of this reference before applying Receiver Operating Analysis (ROC) using GraphPad Prism 7.0. The cut off signals that were used to discriminate positive *vs* negative IgG samples were established at $\geq$ 13 or 11% using Nucleocapsid or Spike, respectively. Statistical analysis was performed using the *t*-test on GraphPad Prism 7.0.

2. Results and discussion

We have previously described a magnetic particle immunoassay which was successfully applied to track SARS-CoV-2 seroconversion in humans [5]. The assay principle is based on the use of commercially available Ni^{2+} magnetic particles which are coated with the purified, 6x His-tagged Nucleocapsid protein of SARS-CoV-2. The coated beads were used matrix for a process resembling an indirect ELISA. Beads are incubated with serum or blood, washed, incubated with anti-human IgG-HPR, washed, and finally immersed on TMB, a chromogenic HPR substrate. The whole process is performed in 12 min. This immunoassay requires extraction and homogenization of the magnetic beads in different solutions. A major drawback of the system is that it only processes 12 samples at a time. Here we describe a manually operating, inexpensive 96 well plate magnetic extraction/homogenization process which allows high throughput immunological analysis of COVID-19 cases. A 96-sample format magnetic extractor device (Fig. 1b) was built using a plastic piece fabricated on a 3D printer (Figs. S1 and S2). Inox nails were fixed to this base and a set of neodymium magnets were manually added to the top of each nail by magnetic attraction (Fig. S1). This magnetic extractor device is connected to a set of twelve 8× 0.2 ml tubes PCR strips allowing the capture of magnetic bead particles from the solution. The magnetic particles can be transferred to another 96 well plate with the assistance of a set of two fiber glass sticks placed underneath the PCR strips (Fig. S3). When the beads are placed on the next 96 well plate, the magnetic extractor device is lifted and separated from the PCR strips allowing the beads to go into the solution. The operator manually holds down the PCR strips and makes gentle movements back and forwards to allow complete bead homogenization (Fig. S4 and supporting video). The process is repeated until the final incubation step. It is worth mentioning that the process described here is amendable to automation if performed using automated magnetic beads extractor devices which are commercially available. However, to the best of our knowledge, such automated devices operate using deep-well 96 well plates which can considerably increase the cost of the analysis.

This 96-sample format process when combined with our chromogenic magnetic bead COVID-19 immunoassay was able to significantly increase the analytical throughput. The 96-sample assay was employed to analyze SARS-CoV-2 seroconversion by investigating the presence of IgG reactive to the SARS-CoV-2 Nucleocapsid antigen in sera from 204 pre pandemic samples and 63 COVID-19 subjects. The performance obtained resembled that of our previously described assay based on the 12-samples format. Receiver operating characteristic (ROC) analysis revealed an area under curve (AUC) of 0.996. A sensitivity of 97% could be achieved at a cost of 99.5% specificity. It is important to mention that the 96-sample format maintained high intra assay/inter assay reproducibility (Table 1).

Of note, the assay was performed by directly loading soluble *E. coli* extracts containing the 6x His tagged Nucleocapsid protein of SARS-CoV-2 onto the Ni^{2+} magnetic beads. By skipping the step of protein purification prior to loading, we were able to considerably increase the analytical throughput. The entire process took only 12 min saving time and costs for protein purification (Fig. 1a).

We anticipated that the high throughput chromogenic magnetic beads immunoassay could be easily adapted to various His tagged antigens. As a proof of concept, we immobilized a His tagged version of the full-length SARS-CoV-2 Spike protein, which was expressed in eukaryotic cells, onto the Ni^{2+} magnetic beads. The Spike coated beads were then used to determine the presence of reactive IgG in the same cohort of samples. The chromogenic analysis using Spike antigen operated with AUC of 0.99, and a sensitivity of 97% could be achieved at a cost of 99.5% specificity (Table 1 and Fig. 2a). Again, the levels of reproducibility were high within and between different assays (Table 1). A team of operators in Germany independently performed all the steps of the immunoassay, from antigen preparation to testing, and was able to validate the ability of the chromogenic magnetic system using His-tagged versions of either Spike or Spike RBD as antigens to discriminate COVID-19 cases (Fig. S5a). These findings suggest that this simple and easy to implement magnetic particle immunoassay may be universally used with other His tagged antigens to track cases of diseases other than COVID-19.

Even though the chromogenic assay generated data in a short time, we speculated that the overall reaction time could be decreased by changing the detection mode from chromogenic to fluorescent, thus skipping the 5 min incubation step necessary to build up oxidized TMB (3,3′,5,5′-Tetramethylbenzidine). As a proof of concept, we changed the secondary anti-human IgG HPR (horseradish peroxidase)-conjugate to phycoerythrin-conjugate. These changes allowed an ultrafast (7 min) high throughput process which was able to discriminate COVID-19 cases using either Nucleocapsid or Spike as antigens (Fig. 2a and Table 1). The data obtained using the chromogenic and fluorescent system showed excellent correlation using serum (Fig. 2b and c) or blood (Fig. S5b). The AUC, sensitivity, specificity, and reproducibility parameters of the

Table 1
Performance of the assay in different formats.

Assay	AUC[1]	% Sensitivity[2]	Intra-assay CV%[3]	Intra-assay CV%[4]
S Chromogenic	0.990	96.8 (95% CI 89.0–99.6)	1.7	5.8
S Fluorescent	0.993	95.2 (95%CI 86.7–99.0)	1.2	2.4
N Chromogenic	0.996	96.8 (95%CI 89.0–99.6)	1.8	8.9
N Fluorescent	0.991	95.2 (95%CI 86.7–99.0)	2.9	7.4

[1] - AUC indicates the value obtained for the area under the ROC curve.
[2] - Specificity was set to 99.5% (95% CI, 97.7–99.9%) for all assays.
[3] - Intra-assay CV% data obtained running the same sample in four wells of the same plate.
[4] 4 - Inter-assay CV% data of same sample measured in duplicate in 3 different plates.

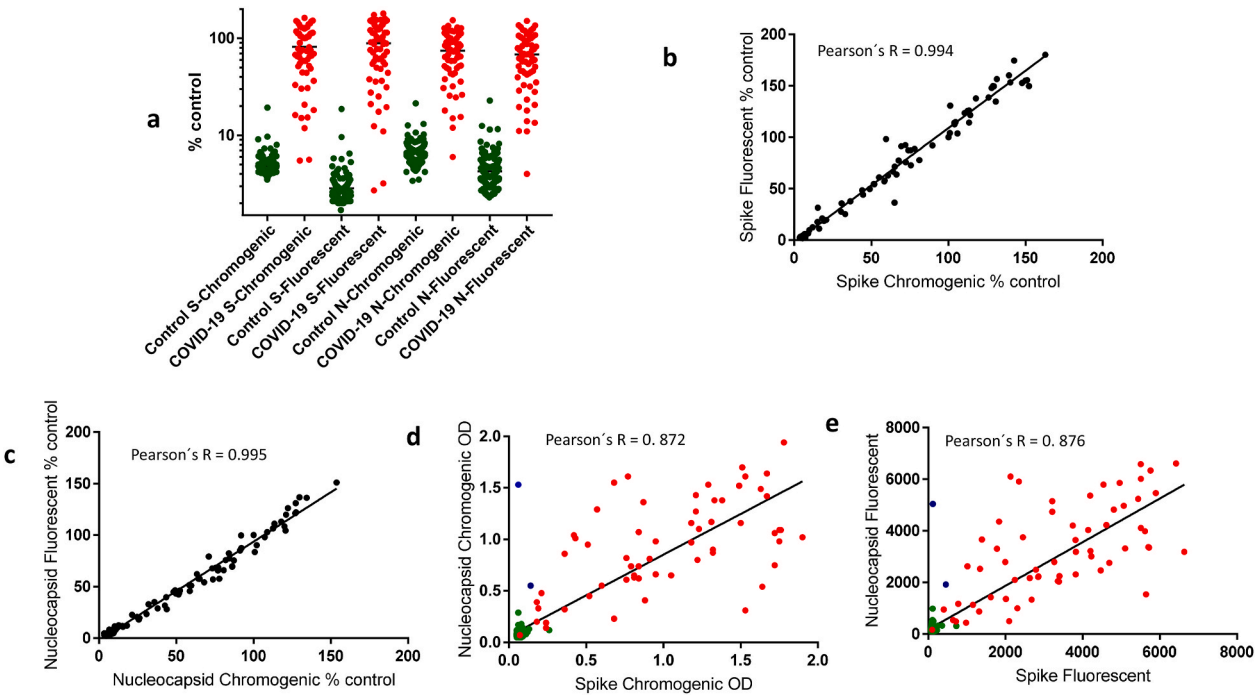

Fig. 2. Performance of the Ni^{2+} magnetic immunoassay in different formats. a) The serum from negative pre-pandemic controls (green) and RT-qPCR COVID-19 positive cases (red) were analyzed using the indicated antigen (S - full length Spike or N - Nucleocapsid) and detection mode. The data was expressed as % of a reference control. b) Correlation between % signal obtained using Spike as antigen with Chromogenic *vs* fluorescence detection. c) Correlation between % signal obtained using Nucleocapsid as antigen with Chromogenic *vs* fluorescence detection. d) Correlation between raw signal obtained using Spike vs Nucleocapsid as antigen in chromogenic and fluorescent formats (e). Samples labeled in blue (in d and e) were collected at the day of hospitalization. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fluorescent assay were in the same range as those obtained with the chromogenic method (Table 1). The cost of the consumables per assay was below 1$ independently of the detection mode used.

There are several debates in the literature with respect to the best antigen to use to detect COVID-19 cases with high sensitivity and specificity [3,9,10]. Among the cohort of 204 pre-pandemic samples examined here, one sample showed significant cross reaction to each antigen (Fig. 2a). Hence, in this study identical specificity (99.5%) was obtained using either Nucleocapsid or Spike as antigens (Table 1). The sensitivity levels were in the similar range (>95%) (Table 1). However, it is worth mentioning that a lower background was observed in the negative cohort using Spike *vs* Nucleocapsid as antigen (Fig. 2a). Hence, it is likely that Spike will better perform to discriminate COVID-19 positive cases in samples with low IgG titers. The same argument holds true with respect to the detection mode. A lower background (% of reference) was obtained in the pre pandemic negative cohort using the fluorescent detection in comparison to the chromogenic (Fig. 1a). The levels of reactive IgG (raw signal) were well correlated using either Nucleocapsid or Spike as antigens (Fig. 2d and e). Two of the samples with high discrepant signals (high for Nucleocapsid and background for Spike) were collected at the day of hospitalization (Fig. 2d and e, samples in blue). Thereby, the divergent data are likely to result from a still immature humoral response.

3. Conclusions

Here we describe significant improvements to our Ni^{2+} magnetic beads immunoassay. Firstly, we show that the system is amendable to high throughput analysis by employing a remarkably simple and low-cost magnetic extractor device and bead homogenization process (Figs. S1–S4 and supporting video). Even though automated magnetic extractor and bead homogenization beads are commercially available, they are unaffordable to most laboratories. Furthermore, they usually require specialized plastics (deep well plates and chip combs) which significantly increases the costs of the analysis. The process described here can be performed in regular 96-well plates and PCR tube strips (Figs. S1–S4). The magnetic extraction/homogenization process device described may be applied to other processes such as purification of SARS-CoV-2 RNA from swab samples, which routinely uses magnetic bead extraction [11]. Secondly, we show that antigen can be purified on the fly by loading the cell extracts directly onto the Ni^{2+} magnetic beads in such way that 6x His tag antigen purification and bead loading occur simultaneously in a 12 min process (Fig. 1a). Thirdly, the magnetic bead immunoassay can be performed in an ultrafast 7 min format just by using an anti-human IgG PE conjugate. Fourthly, we show that the magnetic bead immunoassay can be applied to Nucleocapsid, Spike or Spike RBD carrying a His tag as antigens (Fig. 2a). The use of Spike RBD is of particular interest as it will enables quantification of IgG with potential neutralization activity against SARS-CoV-2.

To the best of our knowledge the method described here is the only COVID-19 immunoassay that uses the principles of the well-established indirect ELISA and delivers ultrafast results in a high throughput and inexpensive format. We believe that the technique described here will be an important tool to understand the levels of immunization to previous infections and/or vaccination in large immunological surveillance studies and accordingly mitigate the enforcement of infection prevention strategies such as social distancing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114360.

Author information

Author Contributions. L.F.H conceived and designed the study. L.F.H, M.S.C and N. M. P collected and analyzed the data in Brazil. L.F.H designed the magnetic extractor device and process with the help of T.P. C.S, M.C.C.H and M.S.C. K.A.S leaded the German team with support from B.W, J.T.A and N.D. F.G.M.R, D.L.Z, M.N.A, J.M.N, L.F.H and R.A.R and contributed samples and reagents. L.F.H wrote the paper.

Notes

Federal University of Paraná UFPR has filed for patent protections for: the magnetic immunoassay process, magnetic COVID-19 immunological test product and magnetic bead extractor device and processing method. All designed product and processes will be freely available for academic and noncommercial users.

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