Development of COVID-Plex MFIA® for Detection of Antibodies against SARS-CoV-2 (COVID-19)

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Purpose and Background

Severe acute respiratory syndrome-related coronavirus (SARS-CoV) is a strain of coronavirus that naturally infects humans, bats, and several Asian mammals. It is an enveloped positive-sense, single-stranded RNA virus that enters its host cell by binding to the ACE2 receptor. It is a member of the genus *Betacoronavirus* and subgenus *Sarbecoronavirus*.[1]

Two strains of the virus have caused outbreaks of SARS in humans. SARS-CoV-1 caused an outbreak in 2002-2004 and SARS-CoV-2 caused a pandemic of coronavirus in 2019-2020, also referred to as coronavirus disease 2019 (COVID-19). There are hundreds of SARS-CoV strains known to infect different non-human species.[2-4] Coronaviruses are considered zoonotic and the subgenus *Sarbecoviruses* specifically are gaining a notorious history of jumping from animals to humans.

There has been no report of natural infection of laboratory animals, but precautionary testing may be important for some models. Transmission from inoculated cats and ferrets to naive cohorts has been reported.[5] Rodents and other small research animals are not likely to be infected through natural transmission, although infection through high titer inoculation of research animals, including mice, cats, ferrets, and NHPs, has been demonstrated.[2, 6-11]

The risk of NHPs and other lab animals, including mice and rats, in US colonies becoming infected with sarbecoviruses is minimal. Studies show rhesus might be naturally susceptible to the virus that causes COVID-19, but the prevalence of sarbecovirus including COVID-19 in macaques and other NHPs is currently unknown. Mice and rats do not share virus receptor similarity with humans and are highly unlikely to become naturally infected. As bats are a natural reservoir for sarbecoviruses, screening of bats used in research could be important. Few cases of pet and zoo animals, including cats, dogs, and lions, getting infected from their owners or caretakers have been reported; therefore, animals undergoing SARS-CoV-2 research should be screened to check the baseline data/background.

Serum or plasma from immunocompetent animals can be used for SARS-CoV-1 and SARS-CoV-2 antibody detection using ELISA.[12-13] For SARS-CoV-2, there are commercial antibody assays available in the market utilizing truncated (S1 and S2) spike proteins or nuclear protein (NP). However, none of these kits use a combination of spike and NP proteins. To assist in routine colony health surveillance, Charles River Research Animal Diagnostic Services (RADS) has developed an assay on the Multiplexed Fluorometric Immunoassay® (MFIA®) platform to detect SARS-CoV-2 (COVID-19) antibodies in serum utilizing both spike-full length (spike-FL) and NP proteins based on Wuhan strain sequence.[14-15]

MFIA® is a serologic assay in which an array of uniquely colored microbeads are coated with specific antigens and act as the solid phase of the immunoassay.[14-15] Each unique color can be used to bind a unique antigen, thus multiple antibody determinations can be achieved using a single sample in a single test well. When a serum sample is added to a well containing the antigen-coated microbeads, specific antibodies to any of the specific antigens, if present, will bind to the antigen on the

microbeads. After a washing step, a biotinylated anti-species immunoglobulin is added. If a specific antibody is bound in the first step, the biotinylated anti-species immunoglobulin will be bound.

A final addition of a fluorescent reporter molecule, streptavidin-phycoerythrin conjugate, is performed, and then the microbead contents of the wells are placed in a fluorometer. This device then analyzes each bead, identifying the unique color of each bead and simultaneously measures any fluorescent signal. This data is compiled and analyzed by computer and a Mean Fluorescent Index (MFI) is calculated for each antigen on each serum sample. An MFI cutoff value is established, and samples are classified as either negative, borderline, or positive based on the MFI.

The purpose of this qualification is to perform MFIA® on SARS-CoV-2 (COVID-19) antibody positive and negative sera, and to demonstrate that COVID-Plex MFIA® is sensitive and specific. We have used a MagPlex® magnetic bead-based multiplex panel with two SARS-CoV-2-specific recombinant antigens, spike-FL, and NP in the current study. Serum or plasma antibody levels were examined in different species including non-human primates (rhesus or cynomolgus) and rodents from cohorts of well-characterized specific pathogen-free (SPF) colonies. Additionally, convalescent sera from SARS-CoV-2 (COVID-19) PCR positive humans was used. COVID-Plex field trials were conducted at four different facilities, including Charles River Laboratories, using sera from SPF NHPs and macaques experimentally infected with SARS-CoV-2.

Materials and Methods

SARS-CoV-2-specific recombinant spike-full length (spike-FL) and nuclear proteins (NP) were individually coupled to MagPlex® magnetic microspheres (beads) for analysis. In addition, four seasonal coronavirus strain-specific spike full-length protein coupled beads (229E, OC42, HKU1, and

NL63, causing the common cold) were added to the panel to check cross reactivity with SARS-CoV-2-specific spike-FL.16-17 internal control beads, including wild-baculovirus infected SF+ cell lysate as tissue control, and species-specific IgG and anti-IgG as sample and system suitability controls were also in the panel.

The MFIA procedure was described elsewhere[15] and performed following this online manual.

For each assay, the net median fluorescence intensity signal (MFI) was calculated by subtracting the tissue control (TC) from the antigen (AG) MFI. Net scores were calculated from net MFIA values based on cutoffs and formulas as described in the online MFIA manual (see link above). In summary, values of < 1.5 and ≥ 3 were classified as negative and positive, respectively; net signals between these cutoffs were called equivocal/indeterminate. A sample was considered SARS-CoV-2 (COVID-19) antibody positive if both COVID-specific spike-FL and NP antigen coupled beads are individually positive (i.e., antibodies against both COVID-specific beads were present).

Standard positive assay control (immune sera) was convalescent serum from humans infected by SARS-CoV-2 (confirmed positive by PCR). Standard negative assay control sera (species-specific non-immune) were serum pools collected from SARS-CoV-2 antibody-free animals (samples collected prior to 2019).

Briefly, diagnostic sensitivity and specificity of the COVID-Plex assay were evaluated. Assay sensitivity was measured by analyzing convalescent sera from known SARS-CoV-2 PCR positive humans and 32 experimentally infected macaques. In addition, assay specificity was measured by analyzing 322 sera from historically known negative SPF NHP colonies.

COVID-Plex field trials were performed by examining antibody levels in a total of 715 macaque sera or plasma samples from known SARS-CoV-2 negative colonies at four different North American facilities, including Charles River.

Results Summary

Assay Cutoffs and Interpretation Guidelines

Immune response was initially evaluated by testing several hundred negative macaque sera (before performing this study) to aid in developing scoring/interpretation criteria for the COVID-Plex assay. Based on this screening of COVID-positive and negative sera, assay cutoffs were determined, and the following interpretation guidelines were developed. A sample was considered SARS-CoV-2 positive if antibodies against both spike-full length (spike-FL) and nuclear proteins (NP) are present (i.e., sample is individually positive on both protein-coupled beads).

Diagnostic Sensitivity, Specificity, and Repeatability

To determine COVID-Plex assay sensitivity, 50 convalescent phase sera from known SARS-CoV-2 PCR positive humans were analyzed. In addition, sera from 32 macaques experimentally inoculated with whole virus were also tested during field trials. Specificity was calculated comparing the total number of expected negative reactions for the 322 known negative NHP macaque (rhesus and cynomolgus) sera samples.

Sensitivity of the COVID-Plex assay was 96.3% with three negative convalescent sera also testing negative by commercial kits using spike proteins, suggesting an absence of seroconversion. Specificity of the assay was 99.7% with one false positive reaction (tested negative by a commercial SARS-CoV-2 spike protein ELISA kit).

	Sensitivity	Specificity
	Positives*	Negatives
#	79	321
Total Samples Tested	82	322
%	96.3%	99.7%

*Three negatives were human convalescent sera which were also negative on commerical kits utilizing spike proteins suggesting possible absence of seroconversion.

Table 1. Sensitivity and specificity of COVID-Plex for SPF NHPs.

Specificity of the COVID-Plex assay for rabbits was assessed by testing sera from three different SPF rabbit colonies with a total of 341 samples. As expected, none of the rabbits were found to be SARS-CoV-2 positive (i.e., 100% specificity observed for rabbits).

Beads	# Tested	# Positive**	# Borderline**	# Negative
Spike-FL	341	4	1	336
NP	341	1	0	340
SARS-CoV2 (COVID-Plex)*	341	0	0	341

*A sample is considered SARS-CoV2 positive if antibodies against both spike-full length (spike-FL) and nuclear proteins (NP) are present (i.e. sample is individually positive in both proteins).

**Four spike-FL positive samples and one borderline scoring sample had elevated scores on spike beads for all seasonal coronavirus strains (229E, NL63, OC43 and HKU1) suggesting possibile cross reaction due to antibodies against anyone of these strains.

Table 2. Specificity of COVID-Plex for SPF Rabbits.

A very high specificity for the COVID-Plex assay was also observed for other species including 355 mice, 271 rats, 50 guinea pigs (GP), and 21 hamsters. In screening for these species, no SARS-CoV-2 positives were observed (i.e., 100% specificity for all species). Overall, only three NHP false positives (specificity in Table 1) were observed out of a total of over 1400 sera tested from six different research species SPF colonies for SARS-CoV-2 (COVID-19) antibodies.

Field Testing

We conducted a multi-site evaluation of the COVID-Plex bead panel to further evaluate diagnostic specificity. Each site analyzed sera from known negative macaques (previously tested by commercial kits) – site A (n = 155), site B (n = 167), site C (n = 322), and site D (n = 72). Results from all sites were combined to calculate the overall diagnostic specificity of the COVID-Plex assay, which was 99.6%.

LAB	# Tested	# Positives	% Specificity
A	155	0	100.0%
B	166	2	98.9%
C	322	1	99.7%
D	72	0	100.0%
Total	**715**	**3**	**99.6%**

Table 3. A sample was considered SARS-CoV-2 positive if antibodies against both COVID-specific spike-full length (spike-FL) and nuclear proteins (NP) are present (i.e., sample is individually positive on both proteins).

Conclusion

The risk of SARS-CoV-2 (COVID-19) infection in research animals is very low. However, infection in a small number of pet cats and dogs as well as zoo animals have been reported, possibly contracting infection from their owners or caretakers. Therefore, animals undergoing SARS-CoV-2 research need to be evaluated for baseline antibody reaction prior to involvement in testing.

Evaluation of over 1000 negative and positive samples determined the appropriate individual COVID-Plex antigen cutoffs and facilitated the development of the scoring and interpretation guidelines for the panel. Results from the qualification of the COVID-Plex panel with spike-FL and NP antigen coupled beads show a very high sensitivity and specificity. The positive samples used for screening were convalescent phase sera from SARS-CoV-2 PCR positive humans and experimentally inoculated animals with SARS-CoV-2 virus. Overall diagnostic sensitivity and specificity of the COVID-Plex bead panel were found to be 96.3% and over 99% respectively (Table 1-3).

In conclusion, we have validated a blood-based multiplex panel which is highly sensitive and specific for routine screening of SARS-CoV-2 (COVID-19) in lab research animals, including NHPs, rabbits, mice, rats, guinea pigs, and hamsters based on their antibody profiling. The data demonstrates that the COVID-Plex assay can be used for routine monitoring of NHPs and other species colonies, and can be performed in a user-friendly and high throughput format.

However, we have seen cross reaction on COVID-19-specific spike beads in SPF animals in different species with a higher rate in SPF NHPs due to possible cross reaction with seasonal coronavirus (causing the common cold) antibodies. Nearly all sera giving positive scores with spike-FL beads also gave positive scores on four beads coupled to different seasonal coronaviruses. These animals with higher baseline COVID-19 spike reactions may not be used in SARS-CoV-2 studies, especially if the studies involve only the SARS-CoV-2 spike protein (vaccine studies based on spike proteins).

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