8 August 2023

# CoV2a1 and CoV2r11 Isoforms

Guide for In-House In-Vitro Diagnostic use

IXILKA
BIOTECH

---

RESEARCH
TECHNOLOGIES

# Applied Science for Biotechnology

Ixilka Biotech S.L.U. is an Artificial Intelligence Driven Research Biotech Company for Human Good focused on Precision Medicine solutions.

Supporting Clinical Research Artificial Intelligence Driven (CRAID) for Collaboratory Open Research Project (CORP), system predicts micro-organic aetiology TARGET that causes a set of symptoms related to physiological and pathological health conditions.

Isolating and synthesizing the target of potential micro-organic aetiologies, experimental functionality in-vitro confirms its clinical use as biomarkers.

After clinical confirmation of the target AI designs synthetic Nanobodies or Peptide Binding Proteins, with high affinity and specificity to those target isoforms.

In a second In-Vitro assay, revealing the biomarker with de-novo synthetic NAb/PBP, is conclusive to validate the use of this isoforms for the development of new In-Vitro Diagnostic methods and biomedicines or immunologic treatments.

# AntiCoV2a1 Project

AntiCov2a1 (initially under the name of NAbS-Project) is the first CORP surged during the start of CoVID-19 pandemic with the aim of detecting the aetiologies behind the severe Covid-19 disease and post-Covid persistent syndrome, denominated Long-Covid or Persistent-Covid.

The CORP team was stated initially by Iñigo Ximeno-Rodríguez (Data Scientist and Software Architect), David Abia-Holgado (Biologist and Bioinformatician), Alexander Padrón-Gonzalez (Immunologist and Medical Advisor), Alejandro Bermejo (Biochemistry and Physician), Juan Carlos Alonso-Gómez (Nephrologist and Immunologist), Raquel Abalo-Delgado (Pharmaceutic), Jose Antonio Uranga-Ocio (Histologist), Jose-Luis Rojo-Alvarez (Telecommunication Engineer) and Mabel Pumar-Armada (Business Strategist).

In the post-theoretical phase of the project other partners were involved, such as Javier García-Palomo (Biochemistry), Francisco Mera-Cordero (Physician),

Sara Notararigo (Clinical Assistant), Jesús Calderon Amigo (Clinical Analyst), Manuel Fuentes-García (Immunologist) and IMG-Pharma company.

## CoV2a1 - The hypothetical SuperAntigen behind severe Covid19

The computational model predicts a parallel mechanism to infection as the main cause of the symptoms that lead to a poor prognosis, which has been identified with a peptide from the SARS-CoV-2 virus, capable of activating cellular cytotoxicity not mediated by antibodies, which is called Cov2a1.

The matrix protein of this peptide has been detected in high proportions in the secretions of all those patients with a poor prognosis and in those who suffer from Multiorgan Inflammation Syndrome and/or Persistent Covid Disease, as it has been reported in several studies.

Bioinformatics processes confirm that the peptide CoV2a1 is loaded through TAP to be exposed in MHC class 1 molecules for multiple HLA-A alleles.

CoV2a1 presents a partial structure homology with different interleukins and a high coupling binding with different transmembrane receptors and specially with those that release inflammatory cytokines and chemokines.

Hypothesis states that CoV2a1 is a superantigen capable of cause hypercytokinemia (also known as cytokine storm) by interreacting with specific interleukin cell receptors (ILRs).

Intracellular NLPR3 detects ARN viroporins inducing pyroptosis as it was confirmed by Xu H, Akinyemi IA, Chitre SA, et al in 2022 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8762580).

Pyroptosis during the infected cell causes the release of all intracellular content to the interstitial compartment, including proteins in process and the epitopes to be presented.

The release of CoV2a1 peptide to the interstitial compartment allows it to interreact with plasmatic cells as lymphocytes and macrophages causing the secretion of inflammatory cytokines what leads to the characteristic interstitial edema in multiple organs during acute infection of SARS-CoV-2 and during recurrent episodes of Long-Covid disease.

When CoV2a1 raises endothelial cell, where Weibel–Palade bodies storage IL-8, this chemokine can be released stimulating angiogenesis and vascular permeability which leads to vasculitis and vasospasm. Depending of the immunologic state of the host other chemokines such as β RANTES (CCL5) can be released.

## In-Vitro Validation of CoV2a1 Isoforms

Simulations predict that CoV2a1 may cause observable effects on humoral fluids at picomolar concentrations (~5 pg/ml) which is in the range of interleukins.

These levels of CoV2a1 in body fluids are reached after the fourth day of infection, regardless of the evolution of the disease up to that day, if the immune system has not developed capable and enough antibodies against that peptide, systemic inflammation will follow and the response of the Immune System will be downregulated, allowing viruses to spread out through the blood and to infect ciliates cells and monocytes, including lymphocytes, facilitating persistence in clots and intracellular vesicles.

Hypothesis states that more than 5 picograms per millilitre of CoV2a1 in blood lymphocytes will be exhausted in a few hours releasing stored interleukins of IL-6 and IL-8.

In order to validate this hypothesis, the system proposes quantifying by immuno-chromatography assay (ICA) the release of these interleukins after exposing whole blood to different concentrations of CoV2a1.

Exposure time for stimulating lymphocytes to release IL-6 was calculated in more than 2 hours. In less than 30 minutes whole blood in contact with oxygen will be coagulated, but anticoagulants such as trisodium citrate or ethylenediaminetetraacetic acid (EDTA) can interfere in the process, thus no anticoagulant should be used, being necessary to reduce the stimulation time until the blood coagulates completely. In order to check how much anticoagulant affects to the results, some samples were duplicated with EDTA.

Number of lymphocytes can vary in each subsample, furthermore that coagulation will prevent osmosis, the value of interest is the amount of events per quantifiable signal detected (Ev/MFI). Given that Bio-Plex Multiplex System with Luminex reader outputted this data, this system was elected.

Overloading the samples with CoV2a1 no more interleukins will be detected than the maximum concentration, calculated around to 10 picograms per millilitre. In fact it is predictable that over the maximum concentration MFI by event will be reduced thus some interferons secreted from monocytes that recognizes the epitope will induce apoptosis of lymphocytes. No individual result is significant, but the number of events per MFI signal detected in sum for each different concentration of Cov2a1.

CoV2a1 was synthetised and aliquoted in 8 different concentrations at the BSL3 facilities of Salamanca University by Javier García-Palomo.

Six donors without previous infection of Covid-19 were selected, and with the support of the "**Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León"**, and with the acceptance of the "**CEIm ÁREA DE SALUD VALLADOLID ESTE"** (Exp. BIO-2022-118) the 2th of September of 2022 the assay was performed by the supervision of Javier García-Palomo and with the

permission of Manuel Fuentes-García for the resources at the facilities of the Salamanca Cancer Research Center (CIC).

After processing raw data, and with the conformity of Alex Padrón-Gonzalez, results plot (figure 1) were fully coincident with the prediction.

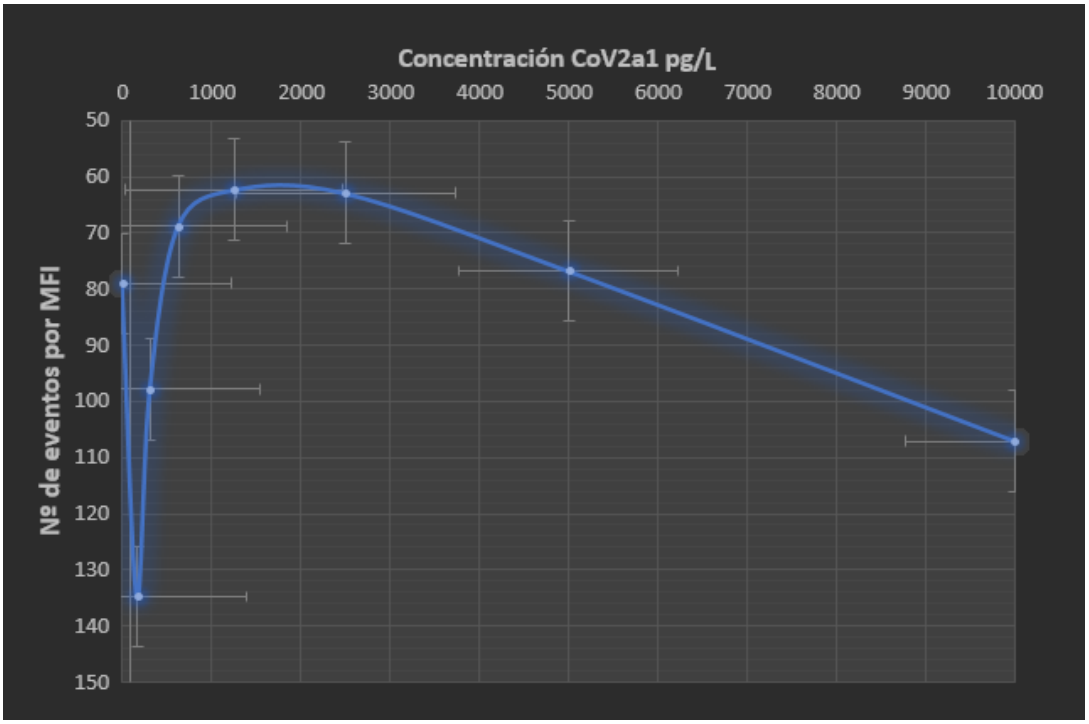


Figure 1. Number of events detected for each MFI signal in sum by CoV2a1 concentration.
Note that less events for each MFI means more interleukins secreted for each cell.

Additionally as it was predicted, anticoagulant reduce considerably the clearance of lectures.

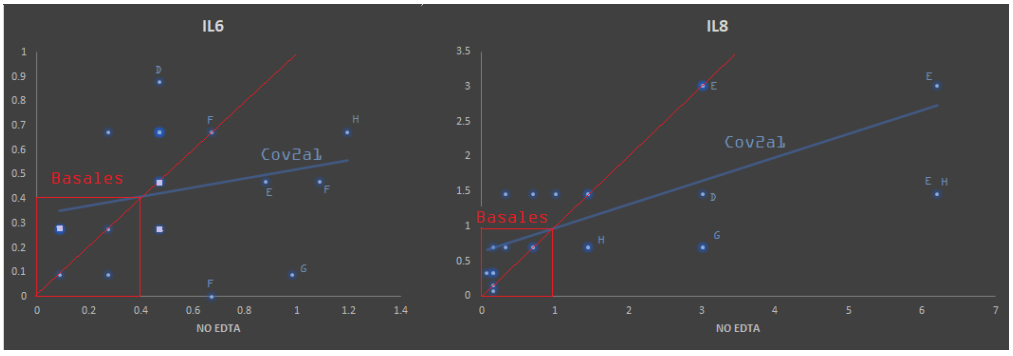


Figure 2. Normalized signal EDTA (Y axis) vs No EDTA (X axis).

## CoV2r11 – In Silico designed Peptide Binding Protein against Cov2a1

After the in-vitro confirmation of the primary aetiology of CoV2a1, as an inducer of hypercytokinemia, the CRAID system developed 12 hyperspecific Peptide Binding Proteins (synthetic Nanobody equivalents) as reagents for its identification as well as for neutralizing the effect of CoV2a1.

In January 2023, de novo synthesis of model 11 (CoV2r11) proposed by CRAID was carried out in two formats, one of which (CoV2r11x) has a fluorescent ATTO-550 additive that will allow this study to proceed to confirm its neutralizing potential and bioavailability.

## In-Vitro validation of CoV2r11x

The CoV2a1 peptide was immobilized in different concentrations in small spots and incubated with different dilutions of CoV2r11x overnight.

After a single wash, image was acquired with ChemiDoc MP Imaging System with Alexa Fluor 555 filter. Raw images were processed with CRAID prototype to enhance wavelength of 600 nm. which is the peak emission for the ATTO-550.

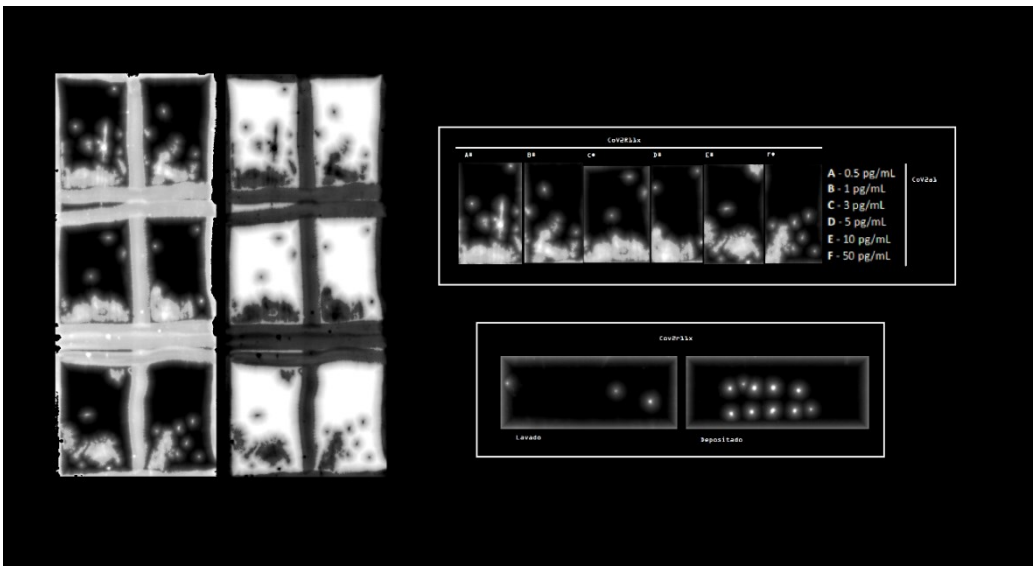


Figure 3. Processed acquired images of microarray affinity assay CoV2a1-CoV211x

The target CoV2a1 can be revealed by CoV2r11x in spots with concentration of 5 picograms per millilitre (and higher) which is the expected concentration of CoV2a1 in blood, as it can be seen in figure 3.

## Fundaments for In-Vitro Diagnostics Development

Due to the results of both experiments, the CoV2a1 and CoV2r11 isoforms have been claimed for use in the laboratory for research purposes, and without loss of generality specifically to perform In-Vitro diagnostic procedures in the scope of Long-Covid.

Detecting CoV2a1 peptide in organic fluids of a patient at concentrations greater than 5 pg/ml is sufficient to conclude a pathological condition related to Post-Covid Hypercytokinemia (PCH) as the primary trigger of Long-Covid Syndrome.

Persistence of the virus, or other mRNA mechanism of replication, is required to endogenous production of CoV2a1 but not always it is released neither detectable in fluids. In addition to periods of latency or low replication, health conditions, SARS-CoV-2 genotype (not all variants traduce CoV2a1 always), specific antibodies or Peptide-Binding Proteins (such as Thymosin Alfa-1) could reduce the detectable volume of CoV2a1. It is advisable to repeat the test several times, preferably in the first days of recurrences to rule out Long Covid Syndrome triggered by hypercytokinemia.

In the acute phase of the viremia detecting CoV2a1 after the fifth day may be stated as a predictor of severity and persistence.

## Guide for In-House In-Vitro Diagnostics Development

Regarding to the article 5.5 of the UE regulation 2017/746, without impairment of other state members limitations, Clinical Laboratories of health institutions, as well as research laboratories, are authorized to produce internally (In-House) any of the claimed uses, including **In Vitro Diagnosis**, as long as there is no equivalent commercialized medical device and while they are not intended to be used for industrial purposes.

Licence for In-House development of diagnostic by using isoforms sequences of CoV2a1 and CoV2r11 can be requested by email to anticov2a1@ixilka.net or by the web-form at the URL https://ixilka.net/inhouse.aspx

All licence granted are subject to the General Confidential Agreement, and the Ixilka Biotech Ethics Compliance.

Applicants must define its own in-house protocol following this basic instructions:

1. Synthesize Cov2a1 and Cov2r11 peptides with a purity higher than 95%. Note that CoV2a1 can release cellular cytokines with only few nanograms inhaled or aspired, so it may be treated as cytotoxic compound in all the process.

2. Label CoV2r11 (as CoV2r11x) with a fluorescence dye linked to cysteine such as Rhodamine 6G (ATTO-550).

3. Proceed in a **Biological Safety Level 2 Plus Containment Facility** (BSL3 conditions preferable) to aliquot CoV2a1 with highly purified water in different dilutions between 1pg/ml and 20 pg/ml, and Cov2r11/x in dilutions between 5 ng/ml and 500 ng/ml.

4. Store dilutions in buffers at -22º centigrade for less than 6 months. It is not convenient to refreeze the dilutions, so it is recommended to dispose dilutions in small buffers.

5. Coat well-plates with different dilutions of CoV2a1 (named CoV2a1-Well). Coatings in complex with A/G protein or Peptide-Binding Proteins, such as Thymosin Alfa-1, should not interfere with CoV2r11x recognition.

6. Incubate different dilutions of CoV2r11x overnight on the Cov2a1-Wells.

7. Wash the Cov2a1-well and proceed to read fluorescence with 550 nm. filter in order to validate functionality.

8. Proceed with plasma samples and different dilutions of CoV2r11x, if CoV2a1 is supernatant in blood it will be bound to Cov2r11x in a few hours, then proceeding with the mixture as in point 6 decrease in fluorescence determine the quantity of Cov2a1.

Alternatively to CoV2a1-Wells, magnetic beads can be used, as well as microarray strips can be performed fixing CoV2a1 in high concentrations in a polymer substrate or similar. It allows reducing the volume quantity of samples to a droplet of blood, making it affordable for portable fluorescence readers.

Ixilka Biotech helps applicants with technical and scientific knowledge during the protocol development and its basic functionality study which must be documented and submitted to the competent Regulatory Authority by the responsible of the Health Institution. Security and reliability of the In-House In-Vitro Diagnostics are the exclusive responsibility of the Health Institution owner.

# Resources and complementary information

- This document would be actualized in the following link:

  https://ixilka.net/publications/AntiCov2a1-IHIVD-Guide.pdf

- Project update:

  https://ixilka.net/publications/AntiCov2a1-Project.pdf

- Artificial Intelligence Driven Open System:

  https://aidros.net