Advances in Monitoring and Interventions for Emerging Infectious Diseases

Expert Insights

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Contents

Editorial

Emerging infectious disease is a rising area of focus for global health. According to the World Health Organization, the rate of these emerging infectious diseases is increasing, with some 40 or more new diseases discovered since the 1970s (https://www.bcm.edu/departments/molecular-virology-and-microbiology/emerging-infections-and-biodefense/emerging-infectious-diseases). Research into the underlying biological mechanisms of pathogen entry, replication, host immune response, and post-infection immune status provides crucial early information to identify and develop therapeutic interventions such as vaccines and antibody-based treatments. To achieve these ends, scientists employ technologies such as advanced flow cytometry, ligand-based antibody binding assays, and live cell imaging and analysis to gain meaningful insights.

This booklet provides an overview of recent advances in research for emerging infectious diseases. The studies include descriptions regarding advances in scaffold platforms for identifying novel mimetic ligands for blocking viral binding and the development of novel methods for detection of antibodies from patient samples.

Fernandes *et al.* (2022) in *Protein Science* explore the use of a class of highly stable proteins derived from both human and plants to act as a scaffold to display engineered peptides designed to bind to spike proteins of SARS-CoV-2. Identifying candidate peptides using these scaffolds is a first step in the development of mimetic ligands that can block the binding of viral spike proteins to host cell receptors and lend themselves to be used as therapeutic interventions. Albecka *et al.* (2021) in *The EMBO Journal* present a novel *in vitro* method for detection of antibodies against the nucleoprotein (N) of SARS-CoV-2 from patient serum samples. This method, electroporated-antibody-dependent neutralization assay or EDNA, allows for the direct quantification of N-antibody titres and is the first such assay of its kind developed. Further, this assay could be employed for the potential development and diagnostics for vaccines designed utilizing N-antigens.

Our booklet is rounded out by two publications from Sartorius. First, an infographic on the host-pathogen lifecycle and the solutions that Sartorius offers for research at key steps in that cycle is presented. Second is an overview of recent publications using advanced flow cytometry for infectious diseases research using the iQue® system. From SARS-CoV-2 to HIV studies,

the reader is given a review of recent research utilizing flow cytometry for infectious disease.

In summary, this booklet aims to inform the reader as to recent advances in infectious disease research that can be accomplished using advanced flow cytometry, ligand binding antibody assays, and live-cell analysis approaches offered by Sartorius.

Jeremy Petravicz, PhD
Editor, Current Protocols

Engineering Defensin α-Helix to Produce High-Affinity SARS-CoV-2 Spike Protein Binding Ligands

Fernandes LA, Gomes AA, Guimarães BG, *et al.*

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) entry into human cells occurs through the binding of viral spike (S) glycoprotein to human angiotensin-converting enzyme 2 (ACE2), followed by its proteolytic cleavage by the cellular transmembrane protein serine 2 (TMPRSS2). Therefore, targeting the interaction between the human receptor and the viral protein by competitive binding has been proposed as a strategy to block viral entry into human cells. In fact, studies have shown that soluble ACE2 inhibits SARS-CoV-2 infection. However, the production of soluble ACE2 in a eukaryotic expression system is not a scalable strategy for COVID-19 diagnostic or therapeutic applications.

Current research focuses on developing ACE2 peptide-based therapeutics allowing a more cost- and time-efficient synthesis compared to the whole eukaryotic protein. Since interactions with the receptor-binding domain (RBD) of SARS-CoV-2 S protein mainly involve ACE2 N-terminal α-helix 1, most rationally designed peptidomimetics are based on this α-helical peptide. However, short peptides tend to adopt random-coil conformations, losing their binding ability, and are also easy targets of proteases, which limit their clinical applications.

To generate potent and stable binders of SARS-CoV-2 S protein RBD, Fernandes *et al*. present a novel strategy based on the creation of conformationally constrained α-helices structured on a prokaryotically expressed and highly stable tertiary structure. To design ACE2 α-helix mimetics, the authors propose using defensins, which are small (45-54 amino acids) cysteine-rich, cationic proteins found in animals, plants, and fungi, as scaffolds. Defensins have crucial roles in the innate immune response against pathogens and present a helix-constrained conformation. In addition, the presence of three to five disulfide bonds confers defensins the advantages of protease resistance, extreme temperature tolerance, and broad pH-range stability. In this work, researchers selected the main residues located on the ACER2 α-helix 1 contacting RBD, introduced them into the α-helix of plant and human defensins, and investigated the ability of the engineered proteins to bind the RBD of SARS-CoV-2 S protein.

Design of defensin-based ACE2 mimetics

As a first step, scientists chose a human defensin (PDB 1IRH) that contains an α-helix stabilized by two disulfide bonds to produce a potentially low-immunogenic and stable ACE2 mimic. Using Pymol software, they identified surface-located ACE2 α-helix 1 residues involved in RBD contacts (**Fig. 1a**). Then, they inserted residues D30, K31, H34, and E35 into the human defensin α-helix to produce the engineered protein, called h-deface2 (**Fig. 1b**). This human defensin contains a short α-helix that can accommodate the insertion of only four ACE2 residues.

To overcome this issue, they chose a defensin from the tobacco plant (PDB 6MRY) with a more extended α-helical structure that could accommodate five additional ACE2 residues, which was expected to result in increased affinity. Residues L29, D30, K31, N33, H34, E35, E37, D38, and L39 of ACE2 were introduced into the plant defensin α-helix, leading to the so-called p-deface2 protein (**Fig. 1c**). Following, since recent studies have identified ACE2 mutations E35K and K31N resulting in increased S protein binding, they created a new version of the protein including these mutations (p-deface2-MUT).

The engineered proteins were initially expressed as Trx-fusion peptides to assist disulfide bond formation, with expression showing an elevated yield (>50 mg/L). Additional experiments were conducted to discard the possibility of Trx-tag interference in the binding.

Determination of dissociation constants

Dissociation constant (Kd) calculations using biolayer interferometry were unfeasible since the curves did not follow a single exponential behavior, probably indicating that multiple binding events were occurring. Therefore, the team used dose-response curves from ELISA assays to calculate the apparent Kd values using nonlinear regression Michaelis-Menten curve fits (**Fig. 2**). With this method, the apparent Kd values were 54.4 ± 11.3 nM for h-deface2, 33.5 ± 8.2 nM for p-deface2, and 14.4 ± 3.5 nM for p-deface2-MUT.

The binding specificity of engineered ACE2-defensins was confirmed by mutating several α-helix amino acids to alanine, which disrupted binding to the SARS-CoV-2 S protein.

Additionally, thermostability experiments were performed. The three engineered proteins retained binding activity after heating (with over 60% of signal for the three proteins relative to non-heated proteins even at 95°C). Differential scanning fluorimetry experiments with h-deface2 showed a melting temperature of 70.7°C. Notably, the proteins also retained their total binding activity after lyophilization and reconstitution.

Diagnostic application of ACE2-defensins

To investigate the potential clinical application of the novel mini-proteins to detect SARS-CoV-2 neutralizing antibodies, researchers used an approved neutralizing antibody test based on the principle of competitive ELISA. They modified the assay by replacing immobilized biotinylated-ACE2 with biotinylated p-deface2 protein. Then, HRP-coupled SARS-CoV-2 S protein and test (neutralizing) or control (non-neutralizing) human sera provided with the kit were added. The presence of neutralizing antibodies decreased the signal compared to the control serum, confirming the disrupted interaction between p-deface2 and HRP-coupled S protein. This experiment suggested the utility of p-deface2 for diagnostic applications to discriminate positive and negative SARS-CoV-2 neutralizing serum (**Fig. 3**).

ACE2-mimetics binding to cells expressing SARS-CoV-2 S protein

Next, HEK293 cell lines were stably transfected with plasmids expressing either wild-type (Wuhan-Hu-1) or Delta variant (B.1.617.2) S protein. Expression and cell-surface display of the proteins were confirmed by flow cytometry. Then, biotinylated h-deface2 and p-deface2 were titrated to determine their ideal concentrations for subsequent flow cytometry experiments. A concentration of 0.1 mg/mL was selected for both engineered proteins. The team

Figure 1

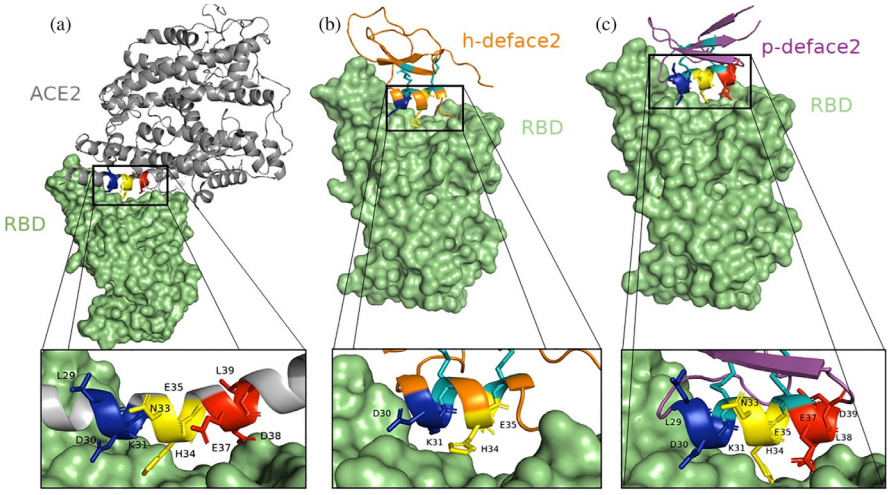


Fig. 1: *Schematic diagram of defensin-based-ACE2 mimetics. (a) 3D structure of SARS CoV-2 RBD/ACE2 complex highlighting the interactions between α-helix 1 and S RBD. (b) Hypothetical model of h-deface2 and RBD complex. The introduced h-deface2 α-helix residues were superimposed to ACE2 surface resides (D30, K31, H34, and E35) to create a hypothetical complex model. (c) Hypothetical model of p-deface2 and RBD complex. The p-deface2 α-helix residues (L29, D30, K31, N33, H34, E35, E37, D38, and L39) were superimposed to ACE2 surface resides to create the hypothetical complex model. Disulfide bonds are shown in cyan. The 3D structure of defensin-based peptides was created using the Phyre2 web portal.*

Figure 2

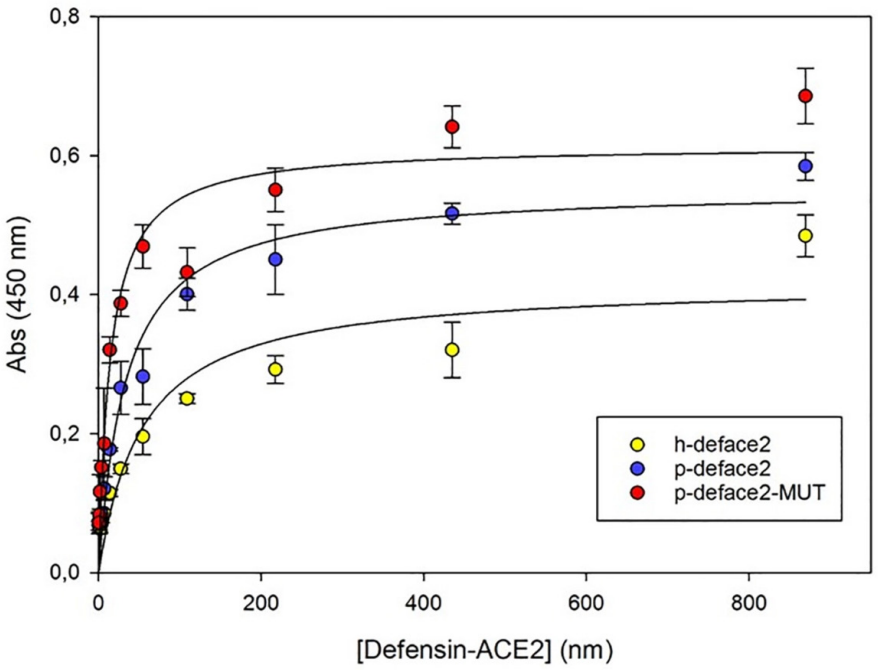


Fig. 2: *ACE2-defensins binding to SARS-CoV-2 S protein: h-deface2, p-deface2, and p-deface2-MUT dose-response curves by direct ELISA. The apparent Kd values were determined using the nonlinear regression Michaelis-Menten curve fit.*

Figure 3

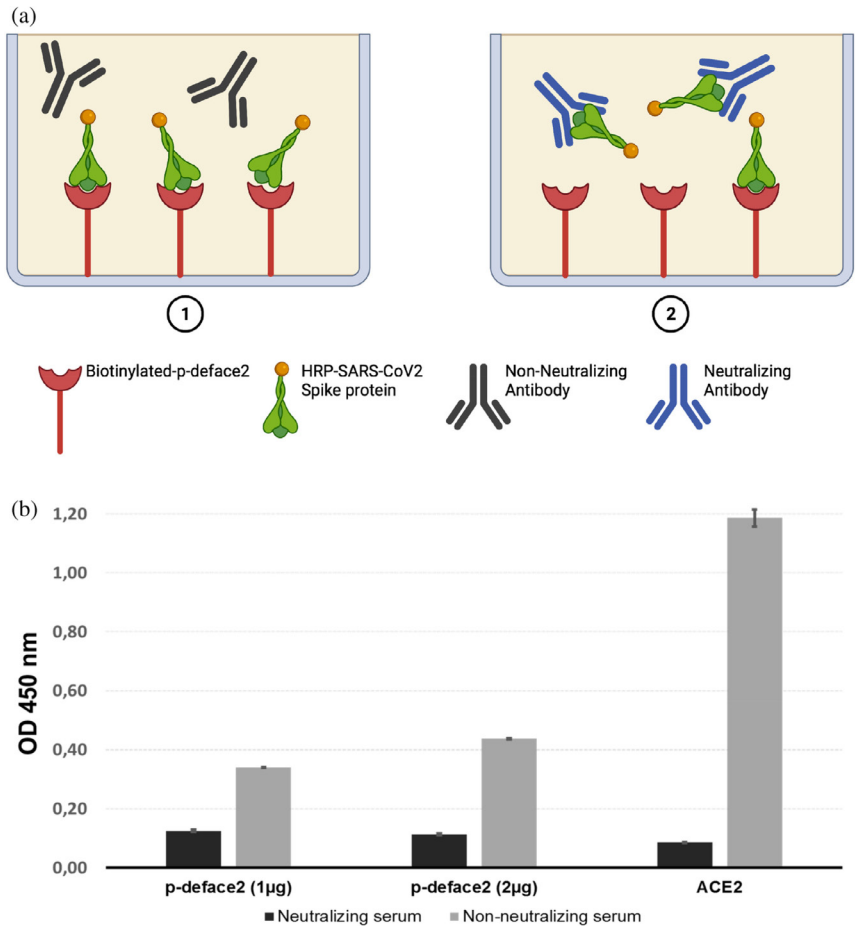


Fig. 3: *Detection of the presence of neutralizing antibodies using defensin-based-ACE2 mimetics. (a) Schematic diagram of competitive ELISA to detect SARS-CoV-2 neutralizing antibodies. In the presence of non-neutralizing antibodies (1), the HRP-coupled S protein binds to the immobilized p-deface-2; while neutralizing antibodies (2) block the HRP-coupled S and p-deface-2 interaction, resulting in decreased signal in the presence of HRP substrate. The figure was created with BioRender.com. (b) Results of the ELISA assay with immobilized biotinylated ACE2 or p-deface2, followed by the addition of HRP-coupled S protein in the presence of neutralizing or non-neutralizing human serum.*

observed higher binding of both proteins to HEK293 cells expressing wild-type and Delta variant S than to control cells. Some background binding was noticed, probably due to the interaction between positively charged amino acids of defensins (that are highly basic proteins) and negatively charged phosphatidyl-lipid moieties on the cell plasma membrane.

Discussion

The engineered proteins presented here demonstrated several advantages: high yield in a bacterial expression system, simple purification to homogeneity, exceptional thermostability, total biological activity upon lyophilization and reconstitution, and potent binding to SARS-CoV-2 S protein. All these are attractive features for their potential use in diagnostic or therapeutic applications.

The apparent dissociation constant of h-deface2 was higher than that of p-deface2, likely because the plant defensin provided a more extended α-helix that accommodated more ACE2 residues, resulting in improved binding. The addition of two mutated residues in p-deface2-MUT further improved the binding (Kd = 14.4 nM), which demonstrates that defensins are versatile scaffolds. Importantly, the engineered proteins were able to bind both Wuhan and Delta variant SARS-CoV-2 S protein, suggesting that they may be potentially suitable against emerging variants or other SARS viruses.

Experimental details

For rational design of defensin-based mimetics, the analysis of ACE2-RBD structure revealed that the majority of RBD contacts involved the surface-located ACE2 α-helix residues L29, D30, K31, N33, H34, E35, E37, D38, and L39. Therefore, scientists positioned ACE2 H34 at the center of the human defensin α-helix on its most solvent-accessible face. Additional residues D30, K31, and E35 were modeled into the helix to produce h-deface2. Similarly, p-deface2 was engineered by positioning H34 at the center of the plant defensin α-helix, and then modeling the additional residues (L29, D30, K31, N33, E35, E37, D38, and L39) at the flanking regions to generate p-deface2. Hypothetical three-dimensional structures of defensin-based peptides were created. All genes were purchased and cloned into pET-32a (+) between the Nco I and Xho I restriction sites. Therefore, all proteins contained a Trx tag to assist disulfide bond formation, followed by a His-tag and an S-tag.

Recombinant pET-32a (+) plasmids were transfected by electroporation into *Escherichia coli* BL21(DE3) pLysS competent cells for protein expression and purification. Cells were lysed and sonicated, and the proteins of interest were purified using sepharose columns. Biotinylated proteins were obtained using a biotin N-hydroxy-succinimide ester reagent.

For direct ELISA assays, 96-well plates were coated with 25/50 ng of SARS-CoV-2 S ECD in 50 µL PBS and blocked with 1% BSA. Next, diluted biotin-coupled defensins were added. Finally, 100 µL of streptavidin-HRP (1:5,000) were added to the wells and incubated for 15 min. Plates were washed, and the reaction was visualized by adding a chromogenic substrate. The reaction was quenched, and absorbance was measured at 450 nm with a plate reader.

For assessment of thermostability, defensin-based ACE-2 mimetics (1 µg) were preincubated at 37, 50, 65, 80, or 95 °C for 5 min, cooled on ice, resuspended, and incubated for 1 h against 50 ng of ECD previously immobilized on polystyrene plates for analysis by ELISA.

For flow cytometry experiments, around 1 million cells were incubated with h-deface2 or p-deface2 labeled with streptavidin-phycoerythrin at the indicated concentration. Propidium iodide was also added to the reaction to estimate cell viability. Finally, cells were washed and analyzed by flow cytometry.

Summary

Soluble ACE2 protein can block the interaction between SARS-CoV-2 S protein and the ACE2 receptor expressed on host cells. Therefore, high-affinity ACE2 mimetic ligands constitute a promising strategy to disrupt this protein–protein interaction for diagnostic and therapeutic applications. Fernandes *et al*. propose using human and plant defensins – small and highly stable proteins containing a solvent-exposed α-helix conformationally constrained by two disulfide bonds – to engineer critical residues on the ACE2 α-helix 1 that interact with SARS-CoV-2 S protein. The engineered proteins, called h-deface2, p-deface2, and p-deface2-MUT, presented remarkable features, including high yield using a bacterial expression system, elevated solubility, exceptional thermostability, and high-affinity binding to the S protein with apparent Kd values in the nM range (54.4 ± 11.3 nM for h-deface2, 33.5 ± 8.2 for p-deface2, and 14.4 ± 3.5 nM for p-deface2-MUT). This work also confirmed the potential clinical applicability of the engineered proteins in a diagnostic assay to detect SARS-CoV-2 neutralizing antibodies. Besides their utility for developing helical ACE2 mimetics, defensins provide promising scaffolds to engineer α-helices in a constrained form to design high-affinity binders for other targets.

Digest of
Fernandes LA, Gomes AA, Guimarães BG, et al. Engineering defensin α-helix to produce high-affinity SARS-CoV-2 spike protein binding ligands. Protein Science 2022;31:e4355. https://doi.org/10.1002/pro.4355

A Functional Assay for Serum Detection of Antibodies Against SARS-CoV-2 Nucleoprotein

Albecka A, Clift D, Vaysburd M, *et al.*

Humoral immune responses against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) result in the production of antibodies against two viral antigens: spike (S) and nucleoprotein (N). These antibodies are widely used to diagnose present or past SARS-CoV-2 infections. Current research has mainly focused on anti-S antibodies, showing that they can neutralize SARS-CoV-2 and constitute an effective antiviral treatment. Besides, the S antigen is the basis of currently approved vaccination strategies.

Conversely, research into anti-N antibodies remains scarce, mainly because of the lack of an assay to measure their ability to block SARS-CoV-2 infection. Although previous evidence suggests that anti-N response may play a role in the immunity to this coronavirus, the potential mechanisms behind this protection remain widely unexplored. Notably, new efforts to develop N-based vaccines or approaches combining S and N could help overcome the emergence of resistant viral strains that are less susceptible to the immunity induced by S-based vaccines. Therefore, this study aimed to develop a new *in vitro* assay – called electroporated-antibody-dependent neutralization assay (EDNA, see scheme in **Fig. 1**) – for rapid quantification of the antiviral activity of anti-N antibodies in SARS-CoV-2 convalescent serum. This assay may provide evidence supporting N as a candidate vaccine antigen and allow the efficient testing of novel N-based vaccines

.

Experimental details

The murine hepatitis virus (MHV) was selected as a model to develop a new assay to measure N-antibody activity, since N antibodies have been shown to play a role in immunity to MHV *in vivo*. Electroporated Vero ACE2/TMPRSS2 cells (stably expressing SARS-CoV-2 entry factors ACE2 and TMPRSS2) were infected with MHV-A59, the cell growth curves were analyzed by imaging, and cell viability was assessed by an ATP luminescence assay. Besides, electroporated Vero ACE2/TMPRSS2 cells were infected with SARS-Cov-2 to assess viral RNA loads by RT-qPCR and production of viral particles by plaque assays.

TRIM21 is a cytosolic Fc receptor and E3 ubiquitin ligase crucial for intracellular antibody-dependent neutralization of non-enveloped viruses. In this work, TRIM21 knockout (KO) L929 cells were generated using CRISPR-Cas9 technology.

Human serum and cell samples were obtained in the context of a study evaluating seroprevalence and immune correlates of protective immunity to SARS-CoV-2 conducted in the UK. Inactivated serum samples were screened for SARS-CoV-2 N and S binding antibodies by Luminex assay and selected for further investigation based on selective binding profiles. In addition, fresh peripheral blood mononuclear cells were isolated from SARS-CoV-2 convalescents, and N-specific T cells were quantified by ELISpot.

Anti-N antibodies mediate intracellular neutralization of MHV

MHV was used as a model to develop an assay to measure N-antibody activity. First, scientists established a system to follow cytopathic MHV infection over time and quantify virus titers using live-cell imaging. Using this approach, the team tested the neutralization capacity of a polyclonal antiserum raised against disintegrated, purified MHV-A59 virions, including antibodies against S and N. The antiserum was added either to the cell media or delivered directly into the cytosol using the so-called Trim-Away technology, where antibodies are electroporated into cells and form a complex with their protein target that is recognized by TRIM21, which uses ubiquitination to recruit the proteasome and mediate complex degradation (**Fig. 1**). Intracellular delivery of the antiserum

reduced viral replication 10-fold more potently than extracellular incubation (**Fig. 2**). This observation suggested the presence of antibodies in serum that can bind to viral proteins post-fusion in the cytosol and neutralize replication.

Following, researchers investigated whether anti-N antibodies were responsible for blocking replication by electroporating L929 cells with serial dilutions of an anti-N monoclonal antibody or a control antibody, followed by challenge with MHV-A59. Electroporation of the anti-N monoclonal antibody completely neutralized viral replication, while the control antibody had no effect. TRIM21 knockout in L929 cells did not affect cell proliferation or cytopathic MHV-A59 infection in the absence of serum or antibody. However, TRIM21 knockout cells electroporated with MHV antiserum or anti-N monoclonal antibody could no longer neutralize MHV-A59, suggesting that TRIM21 plays a crucial role in intracellular neutralization. Following, researchers confirmed that electroporated anti-N antibodies, but not a control antibody, caused degradation of MHV-A59 N protein, consistent with the block to MHV replication caused by N-protein degradation. Altogether, these results confirmed that the proposed electroporation-based method called EDNA can be used to measure N-antibody activity *in vitro*.

EDNA can be used to measure intracellular antibody neutralization of SARS-CoV-2

Next, anti-N antibodies or a control antibody were added with or without electroporation to Vero cells modified to stably express ACE2 and TMPRSS2 and, after 24 h, cells were infected with SARS-CoV-2. When viral replication was assessed by RT-qPCR in lysed cells at 24 h post-infection, experiments showed that electroporation of N antibodies reduced viral RNA by 3-logs. The addition of the same sera to the cell media without electroporation had no impact on replication, indicating that only intracellular N antibodies mediated neutralization. Anti-N sera showed a similar effect on the production of infectious particles, as confirmed by plaque assays. The experiments revealed that intracellular neutralization by N antibodies was dose-dependent.

In addition, the study indicated that intracellular neutralization of SARS-CoV-2 depends on TRIM21, as observed in TRIM21 knockout cells infected with SARS-CoV-2 where TRIM21 expression was reconstituted (**Fig. 3**). In summary, these results confirmed that N antibodies mediate TRIM-21-dependent neutralization of SARS-CoV-2, and that EDNA can quantify N-antibody activity.

Figure 1

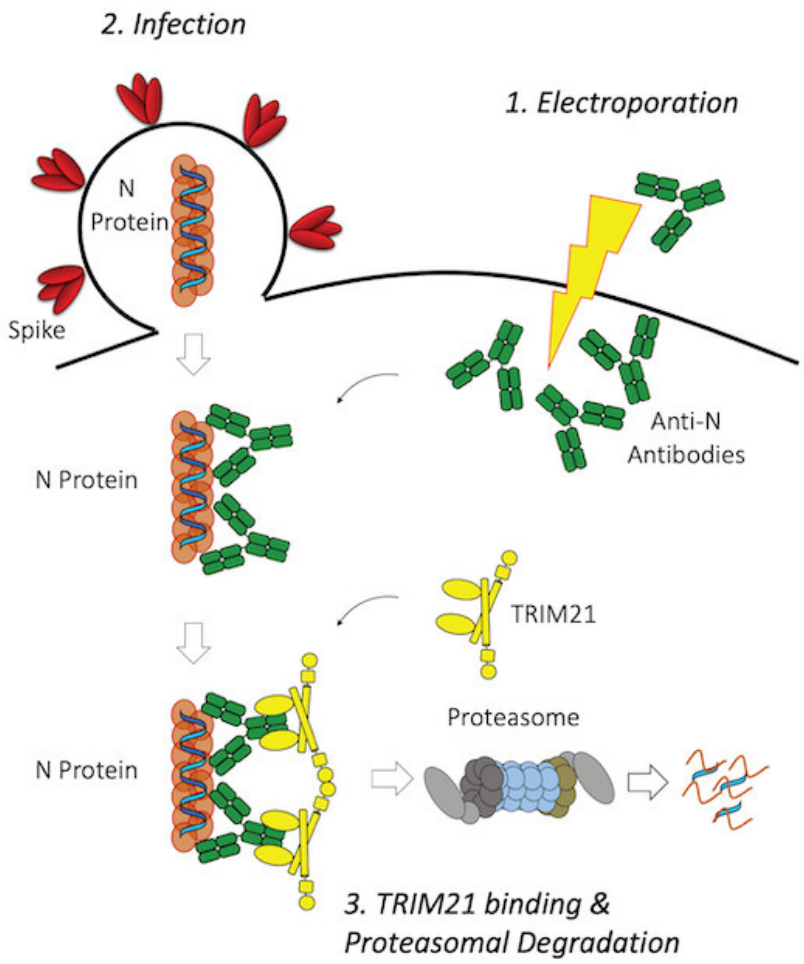


Fig. 1: *Scheme of the electroporated-antibody-dependent neutralization assay (EDNA), a novel in vitro assay that quantifies the antiviral activity of N antibodies in SARS-CoV-2 convalescent sera.*

EDNA provides a functional assay for N antibodies in SARS-CoV-2 convalescent sera

Next, scientists investigated the use of EDNA as an N-antibody neutralization test in sera from four convalescents with confirmed SARS-CoV-2 seropositivity and two seronegative patients. Experiments using a capillary-based protein detection system showed a range of responses among the different sera, with some displaying limited reactivity to any SARS-CoV-2 antigen, others reacting robustly against N but weakly against S, and others showing strong anti-N and anti-S profiles. Although these observations suggested that the assay can measure a good dynamic range in antigen reactivity, further experiments in larger cohorts are needed for confirmation.

Figure 2

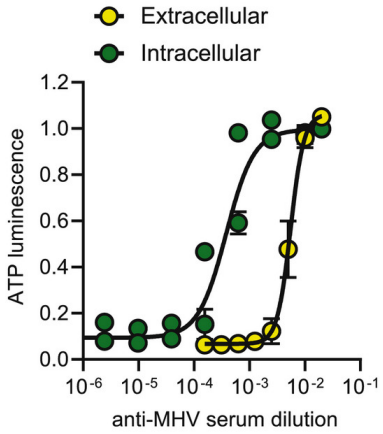


Fig. 2: *Viability of cells infected with MHV-A59 in the presence of intracellular or extracellular antiserum determined by ATP luminescence assay at 48 h post-infection. Increasing doses of antiserum resulted in increased cell survival.*

Figure 3

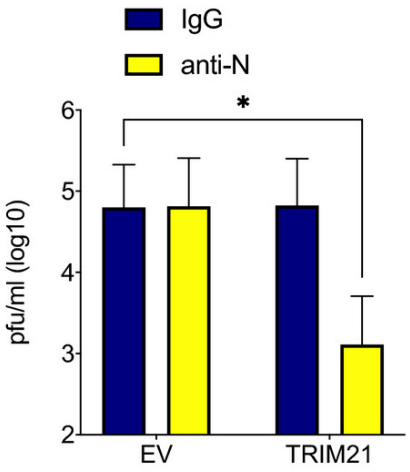


Fig. 3: *Electroporation of anti-N antibodies significantly inhibits SARS-CoV-2 replication only in TRIM-21-reconstituted 293T ACE2/TMPRSS2 TRIM21 KO cells (* P < 0.05). EV, empty vector; IgG, immunoglobulin G (control).*

When serum with strong anti-N response was electroporated into cells later challenged with SARS-CoV-2, infection decreased over 10-fold. On the contrary, sera without anti-N antibodies did not neutralize the infection. Additional experiments confirmed that neutralization activity in the polyclonal sera was dependent upon TRIM21.

Neutralizing anti-N antibodies from convalescents correlate with levels of N-specific T cells

Next, a larger panel of seroconverted human sera was collected, confirming a considerable variation in response strength. Substantial numbers of individuals possessed monodominant responses; this is, strong N or S responses but not both. When cells infected with SARS-CoV-2 were electroporated with convalescent sera, the ability of serum from different individuals to inhibit viral replication intracellularly varied. A trend between neutralization potency and strength of response was observed in N but not in other antigens. Serum possessing a strong S/weak N antibody response was able to neutralize infection extracellularly but not intracellularly, confirming that anti-N antibodies are responsible for the observed intracellular neutralization.

Sera from two of the strongest responders to N protein were selected to perform a titration in order to establish the linear range of the binding assay. After electroporation, similar neutralization curves were observed for both sera, showing that neutralization activity was largely lost once diluted to a chemiluminescent signal <106 (**Fig. 4**). This observation suggested the existence of a functional threshold for intracellular neutralization of SARS-CoV-2.

Figure 4

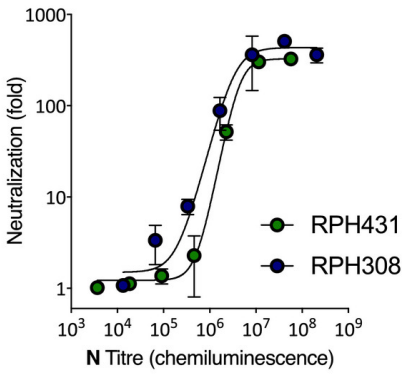


Fig. 4: *Intracellular neutralization of SARS-CoV-2 by titrated sera from two strongly N antibody-positive individuals.*

To understand how anti-N antibodies are imported by cells *in vivo* and provide protection, researchers isolated peripheral blood mononuclear cells from SARS-CoV-2 convalescents and quantified N-specific T cells using ELISpot. After stimulation with an N peptide library, the results of this analysis revealed an over 100-fold range in the number of specific cells (those expressing interferon) between individuals. The number of N-specific T cells and the intracellular neutralizing activity within each individual were modestly correlated, consistently with the hypothesis that N antibodies may contribute to protection against the virus by promoting T-cell immunity. These results confirmed the utility of EDNA to measure the neutralization activity of anti-N antibodies and not just their serum levels.

Discussion

This work shows that the novel *in vitro* assay called EDNA can quantify the activity of N antibodies produced upon SARS-CoV-2 infection, similarly to classical neutralization assays for S-antibody activity. The authors demonstrate that EDNA can be applied to measure N-antibody activity in SARS-CoV-2 convalescent sera, can be used in conjunction with RT-qPCR and plaque assays, and has a large dynamic range. By electroporation, the assay delivers antibodies directly into the cytoplasm before the viral challenge. In this way, EDNA can test the ability of antibodies against antigens usually hidden inside the viral envelope to disrupt infection, even in sera with low anti-S titers.

The experiments with MHV and SARS-CoV-2, and both polyclonal sera and monoclonal anti-N antibodies, indicate that intracellular neutralization is dependent upon TRIM21, a receptor previously shown to mediate antibody-dependent intracellular neutralization. The authors hypothesize that a similar mechanism of degradation of N protein blocks viral replication during EDNA. In fact, intracellular neutralization in SARS-CoV-2 experiments correlated with N antibody but not S antibody titers. However, N antibody binding titers are not always predictive of intracellular neutralizing capacity, as sera with titers differing more than 10-fold showed similar neutralization activity.

Previous work on viruses such as influenza found a synergistic link between N antibodies and N-specific cytotoxic T cells. The authors hypothesize a similar process occurring during SARS-CoV-2 infection, as suggested by the correlation between N-antibody neutralization measured by EDNA and N-specific T cells measured by ELISpot.

One limitation of this method is that antibodies are artificially delivered into the cytosol *in vitro* and, therefore, it may not reproduce cellular uptake and cytosolic import during natural SARS-CoV-2 infection *in vivo*, a mechanism that remains unclear. The current findings need to be validated in larger cohorts and in correlation with patient outcomes.

Summary

The immune response to SARS-CoV-2 results in antibodies against spike (S) and nucleoprotein (N). Although neutralization assays for S antibodies are widely available, assays for N-antibody activity are lacking. In this study, Albecka *et al*. introduce a novel method called electroporated-antibody-dependent neutralization assay (EDNA) for *in vitro* quantification of N-antibody activity in unpurified serum from SARS-CoV-2 convalescents. The results of this work reveal that N antibodies intracellularly neutralize SARS-CoV-2 through the cytosolic Fc receptor TRIM21. Using EDNA to test convalescents sera, this study demonstrates a wide range of response strength, with low N antibody titers showing neutralizing effect in some cases, in contrast to other sera presenting high titers but weak neutralizing activity. In addition, the experiments revealed a

correlation between N antibodies and N-specific T-cell activity within individuals, suggesting that these antibodies may protect against SARS-CoV-2 by fostering T-cell immunity. Although these findings should be validated in larger cohorts and in correlation with patient outcomes, the present study confirms the utility of EDNA to quantify the activity of N antibodies produced upon SARS-CoV-2 infection. Besides, these observations support further research on N-based vaccination strategies against SARS-CoV-2 infection as an alternative to current approaches based only on S.

Digest of
Albecka A, Clift D, Vaysburd M, et al. A functional assay for serum detection of antibodies against SARS-CoV-2 nucleoprotein. The EMBO Journal 2021;40:e108588. https://doi.org/10.15252/embj.2021108588

SARTORIUS

Simplifying Progress

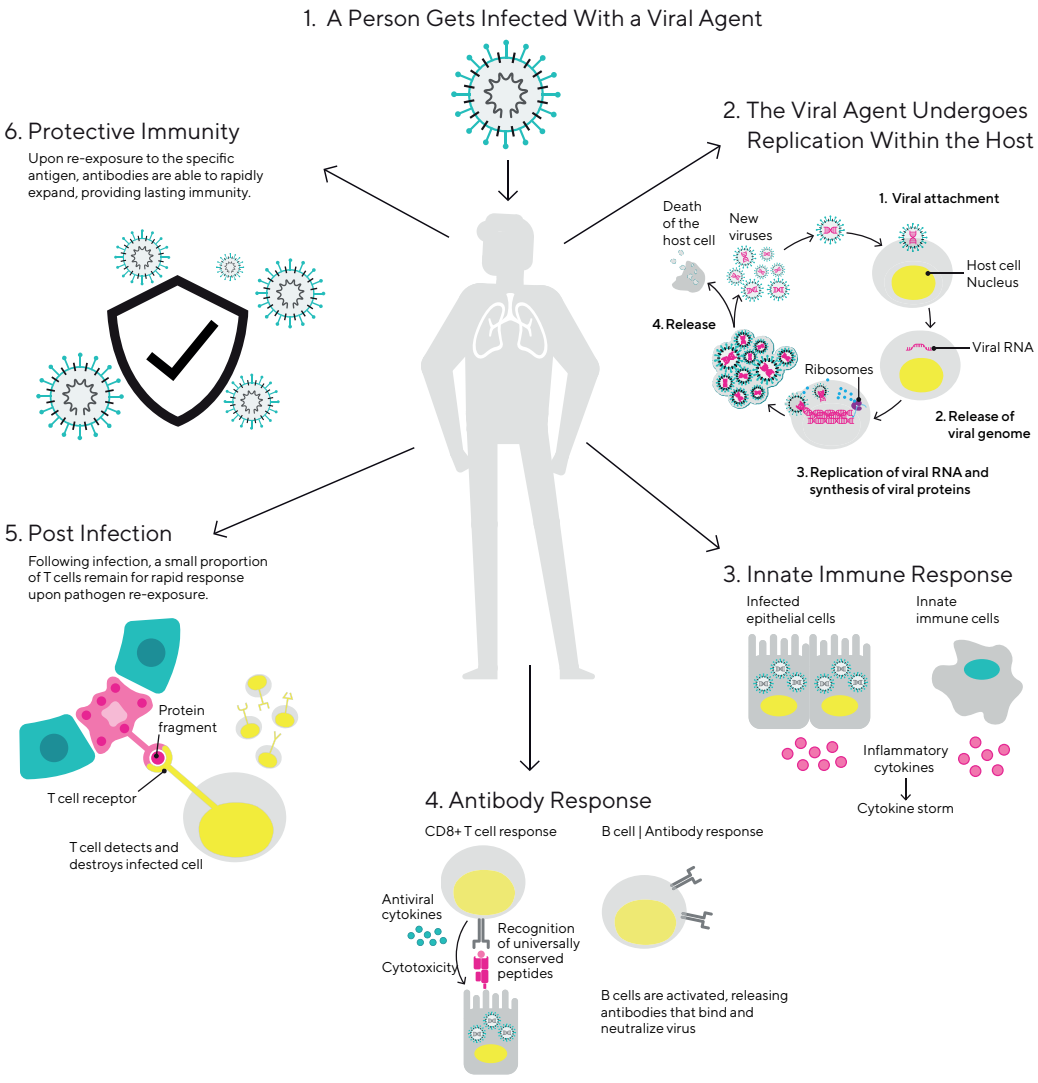
Evaluating Viral Infections Across the Entire Host-Pathogen Life Cycle

Gaining further insights into certain aspects of virology, including infection, transmission, and the mechanisms underlying the body's host immune response, ultimately accelerate the development of novel, efficacious therapeutics and vaccines.

To support these early stage investigations, scientists are using live-cell analysis, advanced flow cytometry, and label-free binding analysis to better understand these dynamic processes and interactions. This infographic highlights the benefits of using advanced cell analysis solutions to study viral pathogenesis and the host immune response to infection.

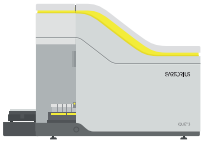
70% of scientists find it valuable to combine live-cell kinetic imaging with flow cytometry, to extract more measurements from a *single* sample.

The Host-Pathogen Cycle



Gain Deeper Insights Into Virology

From onset of infection and the initial immune response to post infection and protective immunity, there is a need to understand this complex biology in order to accelerate research.



Advanced Flow Cytometry Assay

Live-Cell Analysis Assays

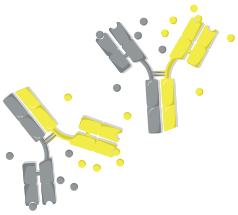
Label-Free Binding Analysis

	Advanced Flow Cytometry Assay	Live-Cell Analysis Assays	Label-Free Binding Analysis
Infection and Replication	▪ NK Killing ▪ T Cell Activation ▪ T Cell Exhaustion ▪ Antibody Titer ▪ Cytokine Profiling	▪ Infection and Replication ▪ Immune Cell Killing ▪ NETosis ▪ Chemotaxis	▪ Virus-Host Receptor Recognition Analysis ▪ Host-Pathogen Interactions ▪ Antibody Titer ▪ Inhibitor Binding Analysis
Antibody Response	▪ T Cell Activation ▪ Antibody Titer ▪ Antibody Screening	▪ Live-Cell Immunocytochemistry	▪ Antibody Titer ▪ Antibody Screening ▪ Epitope Binning
Post-Infection	▪ T Cell Memory ▪ T Cell Exhaustion ▪ Antibody Titer		▪ Antibody Titer
Protective Immunity	▪ Neutralizing Antibodies ▪ Antibody Titer	▪ Proliferation|Viability	▪ Neutralizing Antibody Analysis ▪ Antibody Titer ▪ Epitope Binning

Live-Cell Analysis, Advanced Flow Cytometry, and Label-Free Binding Analysis in Action

Advanced Flow Cytometry Assay

Screening for antibody binding to SARS-CoV-2 proteins was performed on an iQue® system for advanced high throughput flow cytometry. The authors used the assays to demonstrate varied humoral immune responses across COVID-19 patients.[1]



1. Dogan, M., Kozhaya, L., Placek, L., Gunter, C., Yigit, M., Hardy, R., Plassmeyer, M., Coatney, P., Lillard, K., Bukhari, Z., Kleinberg, M., Hayes, C., Arditi, M., Klapper, E., Merin, N., Liang, B.T., Gupta, R., Alpan, O., & Unutmaz, D. (2021, Jan 29). SARS-CoV-2 specific antibody and neutralization assays reveal the wide range of the humoral immune response to virus. *Commun Biol, 4*(1), 129. https://doi.org/10.1038/s42003-021-01649-6 PMID: 33514825; PMCID: PMC7846565.

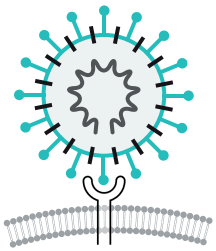
Live-Cell Analysis Assays

Using the Incucyte® S3 Live-Cell Analysis System, researchers monitored cell death and cell-to-cell fusion when using isolated anti-SARS-CoV-2 antibodies from severe COVID-19 convalescent donors.[2]

2. Mor., M., Werbner, M., Alter, J., Safra, M., Chomsky, E., Lee J.C., et al. (2021). Multi-clonal SARS-CoV-2 neutralization by antibodies isolated from severe COVID-19 convalescent donors. *PLoS Pathog, 17*(2), e1009165. https://doi.org/10.1371/journal.ppat.1009165

Label-Free Binding Analysis

Octet® binding assays were used to characterize and validate peptides and single domain antibodies to target SARS-CoV-2 recognition of host receptors.[3,4]



3. Zhang, G., Pomplun, S., Loftis, A.R., Loas, A., & Pentelute, B.L. (2020). The first-in-class peptide binder to the SARS-CoV-2 spike protein. *bioRxiv,* https://doi.org/10.1101/2020.03.19.999318
4. Wu, Y., Li, C., Xia, S., Tian, X., Kong, Y., Wang, Z., Gu, C., Zhang, R., Tu, C., Xie, Y., Yang, Z., Lu, L., Jiang, S., & Ying, T. (2020). Identification of Human Single-Domain Antibodies against SARS-CoV-2. *Cell Host & Microbe, 27,* 891–898. https://doi.org/10.1016/j.chom.2020.04.023

"Integral Molecular is supporting the global response to COVID-19 by employing the processing power of the iQue® in the discovery and characterization of SARS-CoV-2 therapies. Integral Molecular's projects employ three key technologies that rely on high-throughput flow cytometry to assist in the rapid development of therapeutics against COVID-19."
– Integral Molecular

"Within our COVID-19 research, we are looking to develop inflammatory assays using the Incucyte®, specifically around monocyte, macrophage and neutrophil activation. Different drugs will be tested in order to provide information to the clinicians concerning potential subsequent lung tissue scarring and long-term effects on patients, in order to identify optimal drug treatments."
– Dr. John Marwick, Senior Research Fellow, Department of Molecular, Genetic and Population Health Sciences, University of Edinburgh, Scotland

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Publications Review

October 6, 2021

Keywords or phrases:
Flow Cytometry, Infectious Disease, Antibody Screening, Antibody Characterization, Immuno-Oncology, T Cell Characterization, Cytokine Profiling, Phenotypic Screening, Covid, Phagocytosis, Antibody Neutralization, NK Cell Activation, Cellular Cytotoxicity, Antibody Isotype

Advanced Flow Cytometry Applications in Infectious Disease and Immuno-Oncology

Introduction

Time is of the essence. Whether identifying a vaccine candidate that induces production of robust neutralizing antibodies or developing a cancer treatment with life-saving potential, speed matters. Success relies on the ability to rapidly execute a variety of assays, often with small sample volumes, in a biologically relevant, high throughput, reproducible, and cost-effective workflow.

For infectious diseases, assays related to antibody function, neutralization studies and epitope mapping are essential for development of vaccines and therapeutics. In cancer, screening clinical candidates and measuring cellular response is foundational to selecting small molecules and monoclonal antibodies for development, while elucidating T cell phenotype, effector function and secreted cytokines are prerequisites for advanced cell therapies.

Among the technologies used to characterize antibodies, cell phenotype and response, and functional insights is flow cytometry. While flow cytometry is an indispensable tool for analysis of cells and cell-based assays, it is recognized as a difficult technique requiring a great deal of expertise to master. Because the technology is so complex, many researchers choose to route samples to centralized flow cytometry core labs, which further complicates workflows and significantly lengthens the time to results.

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The iQue® advanced high throughput flow cytometry platform was designed to simplify this powerful technique without sacrificing advanced assay capabilities, thus expanding access to a greater number of labs and users. The system enables rapid, high throughput protein analysis, immunophenotyping, functional assessments and profiling, including antibody screening and immune cell activation. Deeper biological insights are enabled by simultaneous measurements of cell-specific parameters—such as phenotypic changes, proliferation rates, and secreted factors—in a single multiplexed assay, using volumes as low as 1-2 µL per well. The iQue® platform utilizes a patented sampling method, which allows for the fastest sample acquisition in the industry. The platform can rapidly process data from multiple assay plates, is compatible with 96, 384, or 1,536-well configurations, and offers continuous plate loading via connection with any automation system. Since this unique sampling method uses just a few microliters of sample, this enables researchers to conserve assay reagents and precious patient-derived samples while retaining material for further downstream characterization studies.

Combined with iQue Forecyt® software, which accelerates the transition from acquisition to analysis within minutes, data can be easily visualized and rapidly interpreted without the need for data extrapolation and export, even for complex biological assays.

This white paper describes use of the iQue® advanced flow cytometry platform for a wide variety of assays essential to the development of therapeutics and vaccines to battle infectious diseases and the advancement of therapies, both conventional and complex, to treat cancer.

Infectious Disease Applications

The emergence of novel pathogens continues driving a remarkable paradigm shift in the speed at which new vaccines and novel therapeutics are developed to fight infectious diseases. Time-to-result in discovery and development is critical. Increasingly powerful high throughput analytical techniques, accessible to drug and vaccine developers at any level of expertise, are available to accelerate workflows to characterize viral biology and the host immune response.

The following research studies demonstrate use of the versatile iQue® advanced flow cytometry platform to enhance throughput and streamline data analysis for a broad range of multiplexed assays including antibody isotyping, neutralization and functional studies, and epitope mapping.

SARS-CoV-2 Studies

The speed at which the COVID-19 pandemic reached all parts of the world made development of effective SARS-CoV-2 vaccines and therapeutics a global health imperative. As an emerging pathogen, all aspects of the virus had to be elucidated along with an understanding of the humoral immune response and rapidly emerging variants with enhanced infectivity.

Mercado, *et al.*, used the iQue® platform to quantify antibody titer for neutralization studies and to gain insights into immune cell function for recombinant, replication-incompetent adenovirus vectors encoding a full-length and stabilized SARS-CoV-2 spike protein.[1] The researchers developed a series of vectors encoding different variants of the SARS-CoV-2 spike protein and evaluated their immunogenicity and protective efficacy. Effector functions, including antibody-dependent neutrophil phagocytosis (ADNP), cellular phagocytosis (ADCP), complement deposition (ADCD) and natural killer activation (ADNKA) assays, were studied. In February 2021, Johnson & Johnson was granted emergency use authorization by the US Food and Drug Administration (FDA) for the Ad26.COV2.S vaccine, which was described in this publication.

In parallel with vaccine development, researchers explored the immunological mechanisms and serological signatures that underlie the different clinical trajectories experienced by COVID-19 patients. Atyeo, *et al.*, used the iQue® platform to assess levels of antigen-specific antibody subclass,

isotype, sialic acid, galactose and Fcγ-receptor binding levels in patients' plasma samples.[2] The platform was also used for functional analysis of plasma samples to quantify ADCP, ADNP, ADCD and ADNKA. The authors observed distinct antibody signatures among individuals with different outcomes.

Dogan, *et al.,* developed SARS-CoV-2-specific antibody and neutralization assays that revealed a wide range of the humoral immune response to virus.[3] The iQue® platform was used in conjunction with a highly sensitive bead-based fluorescent immunoassay from Sartorius for measuring SARS-CoV-2 specific antibody levels and isotypes in COVID-19 patient plasma and serum. This type of highly specific and sensitive assay is essential for understanding the quality and duration of antibody response to SARS-CoV-2 and in evaluating the effectiveness of potential vaccines.

As the COVID-19 pandemic continued to expand, several variants of concern emerged. Understanding the mechanisms leading to increased transmissibility and possible immune resistance was critical to guide intervention strategies. Cai, *et al.,* explored the structural basis for enhanced infectivity and immune evasion of SARS-CoV-2 variants.[4] Using the iQue® platform as an integral part of their workflow, the researchers determined that reshaping of antigenic surfaces of the major neutralizing sites on the spike protein can lead to resistance to some potent neutralizing antibodies and result in enhanced viral fitness and immune evasion.

In addition to vaccines, therapeutics will be essential to control the COVID-19 pandemic and complete protection from SARS-CoV-2 infection may require antibodies that block viral particles attaching to host cells and others to assist in eliminating infected host cells postinfection. Atyeo, *et al.,* note that while significant effort has been invested in identifying antibodies that block infection, the ability of antibodies to target infected cells through Fc interactions may be vital to eliminate the virus.[5]

To explore the role of Fc activity in SARS-CoV-2 immunity, the authors studied the functional potential of a cross-SARS-reactive antibody derived from a SARS-CoV-2 infected individual. The authors explored Fc functional profiling of the original antibody and an engineered version to examine the role of Fc effector function on the response to SARS-CoV-2 infection. ADCP, ADNP, ADCD and ADNKA functional insights were determined using the iQue® platform (Figure 1). Distinct Fc functional profiles resulted in enhancement of disease, pointing to antibody mechanisms of action that may be detrimental when developing antibody therapeutics against the virus.

While approved SARS-CoV-2 vaccines offer robust protection from the virus, the immunologic mechanisms of protection and how boosting alters immunity are not well understood. Alter, *et al.,* profiled the humoral immune response in non-human primates immunized with either a single- or double-dose regimen of the Novavax vaccine (NVX-CoV2373). The researchers used the iQue® platform to perform isotyping assays and derive functional insights including ADCP, ADNP, ADCD, ADNKA. Study results suggested that a single dose may prevent disease, but that two doses may be essential to block further transmission of SARS-CoV-2 and emerging variants.

Ebolavirus Studies

Ebolavirus causes severe disease in humans with an average mortality rate of 50% in past outbreaks. The devastating impact of this pathogen continues to drive development of both vaccines and therapeutic antibodies. In the studies outlined below, the iQue® platform was used to characterize neutralizing antibodies against clinically relevant ebolavirus species.

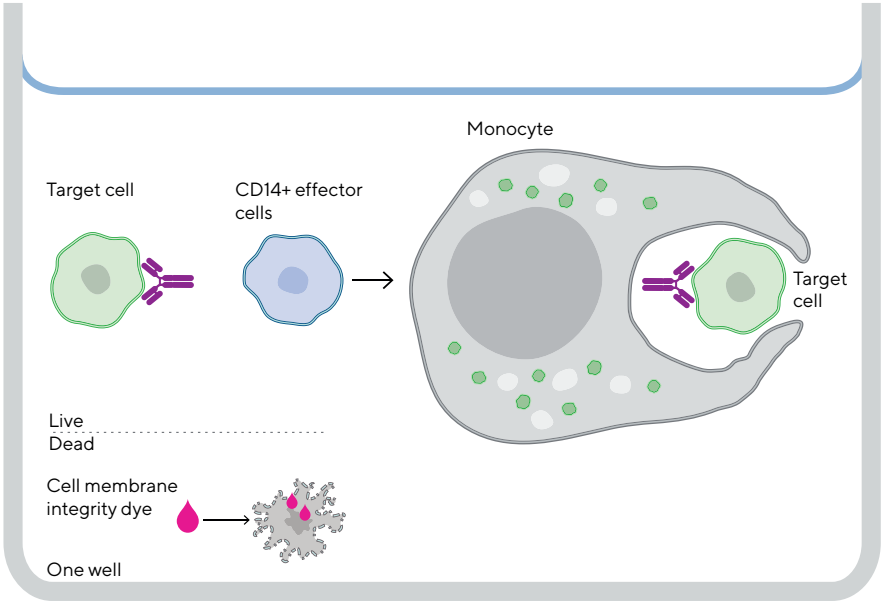
Gilchuck, *et al.,* isolated monoclonal antibodies from survivors of ebolavirus infections and identified a potent antibody which bound an epitope in the viruses' surface glycoprotein base region and effectively neutralized three different strains of the virus.[6] In a subsequent study, Gilchuk, *et al.,* analyzed the antibody repertoire in human ebolavirus survivors to identify a pair of neutralizing monoclonal antibodies that cooperatively bind the ebolavirus glycoprotein base region and glycan cap epitope.[7] The versatile iQue® platform was used in several experiments described in these publications, including evaluation of binding of antibodies to the ebolavirus glycoprotein expressed on the surface of Jurkat cells; the capacity of antibodies to inhibit cleavage of the glycoprotein, which occurs during infection; and binding of antibodies following epitope mapping using an ebolavirus glycoprotein alanine-scan mutation library.

Murin, *et al.,* further explored the mechanism behind activity of broadly neutralizing and synergistic glycan cap antibodies in concert with glycoprotein base-binding antibodies.[8] The iQue® platform was used to analyze synergistic binding of antibodies to cell-surface displayed glycoprotein and binding of antibodies to an ebolavirus glycoprotein alanine-scan mutation library. Results of the study provide the molecular basis for synergy, breadth of reactivity, and virus neutralization by potent glycan cap-directed antibodies and suggest a strategy for design of therapeutic antibody cocktails.

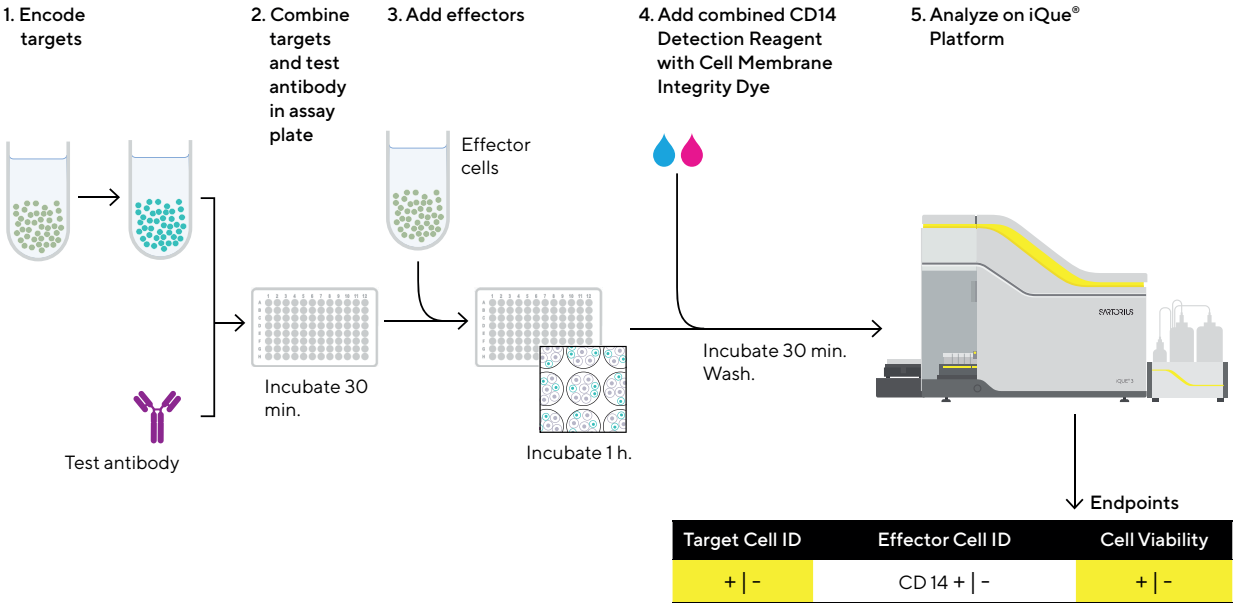
Figure 1

Rapid, Quantitative ADCP Assay Design Using the iQue® Platform

A.



B.



Note. (A) A rapid and quantitative ADCP assay design and workflow using the iQue® platform to investigate mechanisms of action in the field of antibody development is shown. Candidate antibodies can be tested for the ability to bind target cells engineered to display an antigen from a pathogen. The assay then provides a fluorescent readout for phagocytosis by monocytes, in a high throughput and multiparametric manner, and reports on cell viability, along with the ability to multiplex the readout for additional secreted proteins of interest, such as cytokines or effector proteins. (B) The streamlined workflow, with minimal wash steps, on the iQue® platform allows for rapid assay setup, acquisition, and integrated data analysis.

The utility of a protective combination therapy using two multifunctional human antibodies continues to be explored.[9] This study described development of a therapeutic cocktail comprising two broadly neutralizing human antibodies that recognize the ebolavirus glycoprotein and provides preclinical data to support clinical development for a pan-ebolavirus therapy. The iQue® platform was used to assess the binding of antibodies to ebolavirus glycoprotein and measure antibody-dependent cell-mediated cytotoxicity (ADCC).

Zika Virus Studies

Transmission of the Zika virus has been confirmed in dozens of countries with millions of cases of infection. Neutralizing antibodies to the Zika virus offer potential as both prophylactic and therapeutic agents. Long, *et al.,* demonstrated that a highly effective human monoclonal antibody has postexposure therapeutic activity against Zika infectivity in a mouse model.[10] The team used the iQue® platform to measure antibody reactivity to an epitope-mapping library generated using alanine-scanning mutagenesis.

Using Zika as a model virus, Gilchuk, *et al.,* developed and demonstrated an integrated sequence of technologies, including the iQue® platform, designed to enable rapid response for discovery of antiviral antibodies.[11] As an integral part of the workflow, the advanced flow cytometry platform was used for high throughput quantitation of monoclonal antibodies, competition binding analysis, and epitope mapping.

HIV Studies

Several critical challenges remain in the effort to fully understand the nature and progress of human immunodeficiency virus (HIV) infection. Kwon, *et al.,* used the iQue® platform as part of the workflow to define the specific subset of resting CD4+ T cells that harbor intact, replication-competent latent reservoirs of HIV-1 provirus.[12] Greater definition of these subsets would allow more specific targeting of reservoir cells and provide insight into viral persistence.

Another focus of investigation is how antibody effector functions evolve following HIV infection and how the humoral immune response is naturally tuned to recruit antiviral activity of the innate immune system. These questions were addressed by tracking the trajectory of the immune responses following acute HIV infection.[13] The iQue® platform was used in ADCD assays to better define antibody effector functions.

Broad Applicability to Other Infectious Disease Research

In addition to the pathogens described above, the iQue® platform has also been central to deriving insights into the induction of antibody effector functional responses in influenza[14] and Mayaro virus,[15] identifying new sites of vulnerability in the hepatitis C virus via alanine-scanning mutagenesis[16] and reporter virus production, epitope mapping, and neutralization in the study of dengue virus.[17]

The platform is also being used in novel applications such as viral-specific T cells (VSTs), which represent a possible treatment for viral infection after stem cell transplant.[18] To optimize production of VSTs, the authors designed a high throughput assay based on the iQue® platform to fully characterize T cell viability, function, growth, and differentiation. The system was used to measure T cell phenotype and function, including expression of memory markers, and cytotoxicity.

Hagen, *et al.,* have incorporated the iQue® platform into a workflow exploring how gut microbiota can impact responsiveness to vaccination, which has significant implications for increasing vaccine efficacy and improving global health.[19] The authors demonstrated the potential for antibiotic-driven perturbation of the microbiome to influence immune responses to vaccination in healthy adults.

Immuno-Oncology Applications

The versatility and speed of the iQue® flow cytometry system also delivers throughput advantages for the discovery and development of advanced cancer therapeutics including multispecific antibodies, antibody-drug conjugates (ADCs) and adoptive cell transfer as well as small molecules and monoclonal antibodies. The multiplexing capabilities of the platform enable drug candidate screening, phenotyping, functional studies, and cytokine measurements, along with characterization of cell subpopulations for cell therapy. The ability of the iQue® platform to enable assay miniaturization is especially critical for applications in immuno-oncology when the source material is limited, such as patient-derived cells.

Monoclonal and Multispecific Antibody Applications

Comacho-Sandoval, *et al.,* developed and validated an ADCC assay to test the efficacy and potency of biopharmaceutical products using conventional flow cytometry.[20] Based on their experience in the development and validation of bioassays under GLP-cGMP environment, they transferred this ADCC assay to the high throughput iQue® platform in which they were able to evaluate cell membrane permeability, caspase activation, and phosphatidyl serine exposure as characteristics of death on target cells in the same sample with low volume of acquisition. The authors note that these results demonstrated that high throughput technology is suitable for use in control quality environments, and that the automation provided a faster acquisition and analysis of data with precise and accurate results.

Immunotherapies targeting CD20 on the surface of tumor cells are an integral part of the care regimen for patients with non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia. Unfortunately, disease relapse or recurrence occurs in many patients. Resistance of B-cell malignancies to CD20-targeting monoclonal antibodies is rarely a result of a loss of CD20 expression or mutations and as such, this protein remains an attractive target for therapeutic intervention. Preclinical studies have shown that a CD3 x CD20 bispecific antibody can induce T cell activation and T cell-mediated cytotoxicity towards CD20-expressing malignant B cells with high potency.[21] In this study, T cell activation and T cell-mediated cytotoxicity were measured by flow cytometry using the iQue® platform following co-culture with tumor cells.

Recruitment of native T cells by systemic delivery of bispecific "bridging" proteins has become an important modality in the fight against cancer. These molecules typically include a tumor-targeting moiety fused to an anti-CD3 recognition domain for engagement of T cells. This approach brings T cells into the proximity of tumor cells, triggering effector-driven lysis of target cells. Fierle, *et al.,* studied the potential of tumor endothelial marker (TEM-1), a cell surface antigen frequently expressed in the context of tumors, as a target for T cell-redirected immunotherapy to increase the selectivity of targeting compared to classical bispecific antibody formats.[22] The researchers used the iQue® platform for quantitation of effector cytokines produced by these novel trivalent T cell engagers.

Antibody Drug Conjugate (ADC) Applications

ADCs leverage the specificity of monoclonal antibodies to selectively target and deliver potent drugs to antigen-expressing tumor tissues. With this construct, these therapeutics have the potential to minimize the systemic side effects of their cytotoxic warheads. Success of an ADC is dependent on selection of an appropriate target on cancer cells to properly direct the therapeutic while sparing collateral damage to healthy tissue.

Raman, *et al.*, demonstrated the potential of glypican 2 (GPC2) as a possible immunotherapeutic target for a variety of cancers due to several factors including cell-surface location, tumor-specific expression, and tumor dependence.[23] A GPC2 ADC was shown to be efficacious against neuroblastoma and small-cell lung cancer via binding of a tumor-specific conformational-dependent epitope of the core GPC2 extracellular domain. In this study, the iQue® platform was used in the membrane proteome array workflow to determine whether the anti-GPC2 antibody displayed cross-reactivity with more than 6,000 human plasma membrane proteins.

Adoptive Cell-Based Therapy Applications

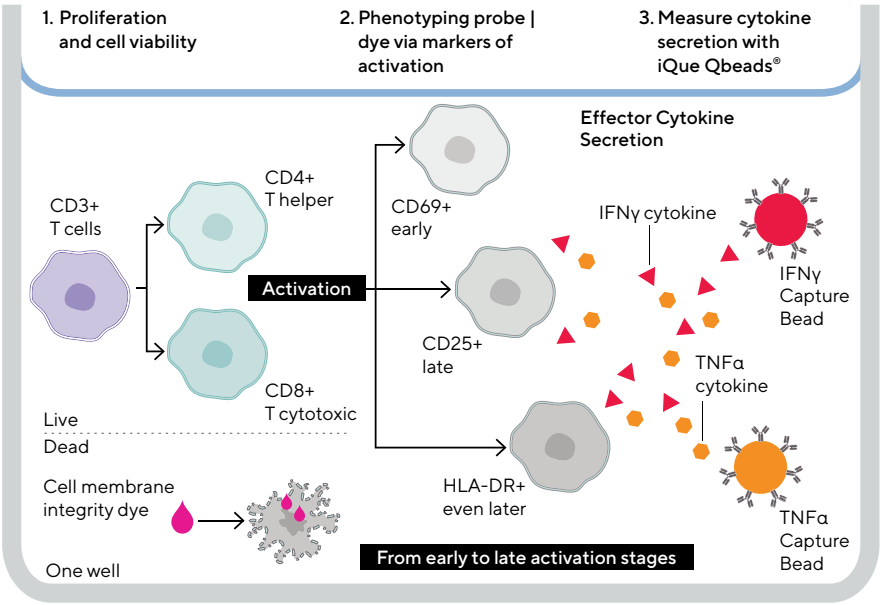
Cell-based therapies represent a remarkable leap forward in treating a wide range of challenging diseases and conditions. While these advanced modalities have delivered impressive clinical outcomes, the percentage of cancer patients that respond to this type of treatment remains frustratingly low. The potential offered by these therapies and the obstacles to their widespread application are driving a significant amount of research focused on understanding the complexity of the immune response.

Rizell, *et al.,* recently reported on the tolerability of ilixadencel, which consists of monocyte-derived, allogeneic dendritic cells stimulated with a combination of

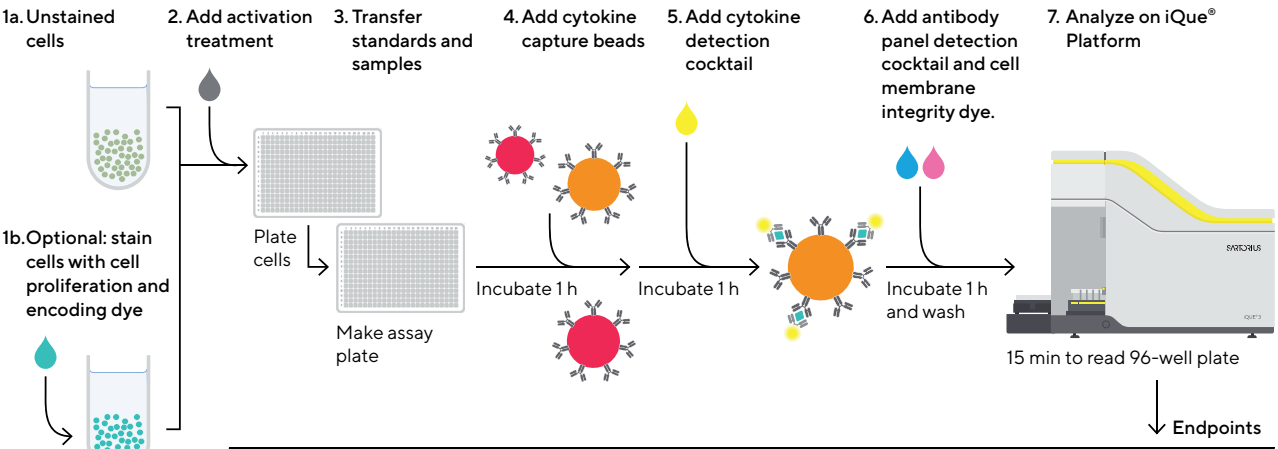
Figure 2

Measuring T Cell Activation Using the iQue® Platform

A.



B.



Basic Cell ID		Cell Surface Activation Markers			Secreted Cytokines		Cell Viability	Cell Proliferation (optional)	Relative Cell Count
		CD69 (early)	CD 25 (late)	HLA-DR (even later)	TNFα	IFNγ			
CD3+	CD4+	+ | -	+ | -	+ | -	+	+ | -	+ | -	+	+
	CD8+	+ | -	+ | -	+ | -	+ | -	+ (Th1)	+ | -	+	+

Note. (A) This describes a multiparametric assay for determining T cell activation state, immunophenotype, cell viability, and proliferation as well as the concentration of the secreted cytokines IFNγ and TNFα. These 11 metrics are collected from a 1-2 µL sample, from a single well on the iQue® platform. (B) The workflow from sample preparation to acquisition takes approximately 3 hours, along with pregated templates for simplified and visual data analysis of plates worth of data (Figure 2B).

proinflammatory factors, administered by intratumoral injection to function as an immune primer in hepatocellular carcinoma.[24] A possible mode of action for intratumorally injected ilixadencel is recruitment of alloreactive CD3+ T cells leading to a mixed leukocyte reaction (MLR) and production of cytokines that induce maturation of bystander dendritic cells. The impact of potentially coadministrated drugs, including anti-PD1 antibodies, on CD3+ T cell activation/proliferation against ilixadencel was investigated using the iQue® platform (Figure 2).

A novel CAR design with exquisite drug sensitivity, robust antitumor responses, and flexibility to enable multiplex antigen targeting or retargeting has been described by Leung, *et al.*[25] The authors believe this approach may further assist the development of safe, potent, and durable T cell therapeutics. The iQue® platform was used in these studies to measure cytokine production by modified T cells to monitor for cytokine-release syndrome, which is associated with many CAR-T responses.

Gene Transfer Applications

Common lentivirus vectors are efficient gene delivery vehicles but offer little specificity. This broad tropism limits their use for targeted gene delivery *in vivo*. To minimize engagement with off-target cells and tissues, a strategy referred to as pretargeting is being leveraged.

The approach defined by Parker, *et al.*, involves the administration of pretargeting molecules such as bispecific antibodies that bind both selected epitopes on target cells and nanocarriers, followed by administration of drug-loaded nanocarriers.[26] In their study, the authors explored how different bispecific formats may impact the efficiency of the pretargeting process. The iQue® platform was used in cell uptake assays to determine if the bispecific antibodies remained on the cell surface or were taken up by the cells. Use of tetravalent bispecifics was shown to be an important feature of pretargeting molecules and provides support for this approach as a promising nanoparticle delivery strategy. In a subsequent study, Parker, *et al.*, sought to improve specificity of lentivirus vectors using a bispecific antibody that binds both the vectors and cell receptors combined with ablation of the native receptor binding of the vector to minimize off-target transduction.[27] Coupling bispecific specificity and ablated native vector tropism synergistically enhanced the selectivity of the targeted gene delivery system. The authors believe that by abrogating the native broad tropism, this redirection strategy may enable lentivirus-based gene delivery *in vivo*, expanding beyond current *ex vivo* applications. Bispecific-mediated viral infectivity and transduction efficiency was measured by flow cytometry using the iQue® platform.

Small Molecule Applications

Small molecules continue to be a mainstay in the discovery and development of novel medicines, even as the drug industry has expanded into new modalities such as cell therapies, gene therapies, RNA interference (RNAi), clustered regularly interspaced short palindromic repeats (CRISPR), and others. The iQue® platform has been integrated into small molecule screening workflows, offering significant speed, throughput, and multiplexing advantages.

While 60% to 70% of acute myeloid leukemia (AML) patients enter complete remission after a standard induction regimen, most relapse within three years; the five-year overall survival rate is only 27%. Given these statistics, identification of novel treatment strategies for AML represents a pressing medical need.

Three recent studies incorporated the iQue® platform into workflows for screening potential therapeutics for AML. Baccelli, *et al.*, identified mubritinib as a strong *in vitro* and *in vivo* antileukemic compound, acting through ubiquinone-dependent inhibition of electron transport chain complex I (ETC1).[28] Kuusanmak, *et al.*, implemented an iQue® flow cytometry-based approach to simultaneously evaluate the *ex vivo* sensitivity of different cell populations in 34 primary AML samples to seven drugs and 27 rational drug combinations.[29] Data demonstrated that different cell populations present in AML samples have distinct sensitivity to targeted therapies. To characterize diversity in drug responses in major hematopoietic cell types, Majumder, *et al.*, developed a high throughput flow cytometry assay using the iQue® platform to enable monitoring of the dose responses of 71 oncology compounds simultaneously on multiple hematopoietic cell populations defined by surface antigen expression.[30] A comparison of drug responses in healthy and neoplastic cells (from patients with either AML, multiple myeloma, or chronic lymphocytic leukemia) showed healthy cell responses to be predictive of the corresponding malignant cell response.

The iQue® platform is also being used in a workflow to screen small molecule libraries for modulation of FOXP3 and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) in human regulatory T (Treg) cells. FOXP3+ Treg cells play an essential role in controlling immune responses in cancer and are actively being explored for their clinical potential. However, expression of FOXP3 in induced Tregs, recognized as the master regulator of Treg cells, is unstable and molecular targets involved in regulating FOXP3 expression and Treg cell function are not well defined. Drug targets capable of regulating FOXP3 expression and its downstream genes, such as CTLA4, have the potential to stabilize the Treg phenotype and function. Ding, *et al.*, have

developed an automated 384-well plate iQue® flow cytometry phenotypic assay measuring protein expression of FOXP3 and CTLA4 in human Treg cells.[31]

Inhibition of the antiapoptotic machinery of cancer cells is a promising therapeutic approach that has driven the development of small molecule BH3 mimetics, which mimic BH3 proteins by antagonizing the prosurvival function of antiapoptotic proteins, inducing apoptosis in cancer cells. To qualify as an authentic BH3 mimetic several criteria must be met including the need to function directly on the mitochondria of a cell of known antiapoptotic dependence, and directly and selectively inhibit the antiapoptotic protein with high-affinity binding. Villalobos-Ortiz, *et al.*, developed a comprehensive biochemical toolkit consisting of BH3 profiling in parallel with high throughput viability testing to validate BH3 mimetic candidates.[32] As part of this workflow, the iQue® system was used for viability testing.

Blake, *et al.*, used an iQue® flow cytometry-based assay to screen a library of more than 800 protein kinase inhibitors and identified compounds that promoted either the stability or degradation of MYC in a KRAS-mutant pancreatic ductal adenocarcinoma (PDAC) cell line.[33] Because stabilization of the MYC oncoprotein by KRAS signaling promotes growth of PDAC, a better understanding of how this stability is regulated may lead to effective therapies for a very challenging cancer.

A recent study incorporated the iQue® platform to help define the antiproliferative mechanisms of deferiprone (DFP), a hydroxypyridinone-derived iron chelator currently in clinical use for iron chelation therapy.[34] The authors found that DFP derives its antiproliferative activity largely from the inhibition of a subset of iron-dependent histone lysine demethylases (KDMs). They also identified new DFP-based KDM inhibitors that are more cytotoxic to cancer cell lines; one lead compound potently inhibited breast tumor growth in murine xenograft models. All flow cytometry-based assays were conducted using the iQue® platform.

Summary

As demonstrated by this collection of infectious disease and oncology applications, the iQue® platform represents a game-changing technology with unmatched throughput, versatility, and multiplex capabilities. When used with iQue Forecyt® software, which enables transition from acquisition to analysis within minutes, data can be easily visualized and quickly interpreted without the need for data extrapolation and export, even for complex biological assays. Whether performing functional studies to understand antibody neutralization or immune cell function in vaccine or oncology discovery and development, the iQue® platform will accelerate and streamline workflows, even for those who are not experts in flow cytometry, leading to actionable results in a compressed timeframe.

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Further Reading

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