In Vitro Efficacy of Chloroquine (CQ) Against the Novel Coronavirus SARS-CoV-2

David Meyerhoff, Jeff Whyte, and Jeffrey Adamovicz (PI)

Overview

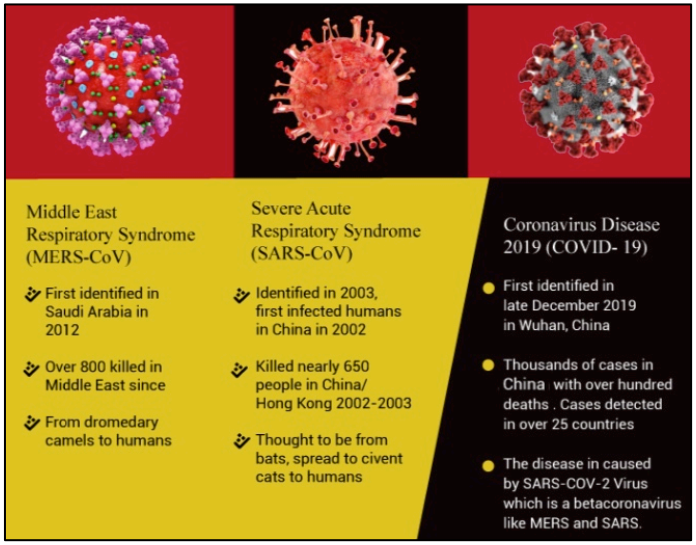
Coronaviruses are named for the crown-like spikes on their surface. There are seven coronaviruses that can infect humans.

Common human coronaviruses:

229E (alpha coronavirus)
NL63 (alpha coronavirus)
OC43 (beta coronavirus)
HKU1 (beta coronavirus)

Coronaviruses that have crossed over from animals to humans are:

- **MERS-CoV** (Middle East Respiratory Syndrome - MERS)
- **SARS-CoV** (Severe Acute Respiratory Syndrome - SARS)
- **SARS-CoV-2** (2019 Novel Coronavirus - SARS-CoV-2, COVID-19)



Chloroquine as a potential treatment for COVID-16

- The COVID-19 pandemic caused by SARS-CoV-2 is having serious consequences on health and the economy worldwide.
- Chloroquine (CQ) is an established drug used in the treatment of malaria.
- The antiviral efficacy of CQ has been reported previously, including against coronaviruses.
- CQ could be integrated into current treatment strategies while novel treatments are awaited.

Oscanoa, et al. (2020). A pharmacological perspective of chloroquine in SARS-CoV-2 infection: An old drug for the fight against a new coronavirus? Int J Antimicrob Agents 56, 106078

Laboratory for Infectious Disease Research
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In Vitro Efficacy of Chloroquine (CQ) Against the Novel Coronavirus SARS-CoV-2

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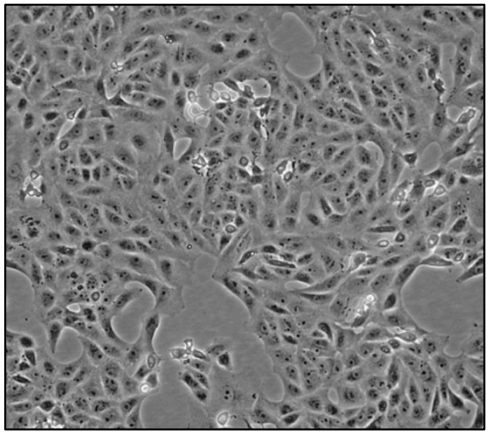
Experimental Aims

Goal: Determine the effectiveness of Chloroquine in inhibiting SARS-CoV-2 infection in vitro.

Test system: Vero E6 cell line.

- The Vero cell line was initiated from the kidney of a normal adult African green monkey on March 27, 1962, by Y. Yasumura and Y. Kawakita (Ammerman, et al. 2008).
- Vero E6 is a **continuous cell line** (can be replicated through many cycles of division and not become senescent).
- Vero E6 cells are **interferon-deficient**; unlike normal mammalian cells, they do not secrete interferon alpha or beta when infected by viruses
- This characteristic allows Vero E6 to be used as host cells for growing viruses.



Uses include:

- Measurement of virus replication in the presence or absence of a research pharmaceuticals.
- The growth of viral stocks for research purposes.

Ammerman, et al. (2008). Growth and maintenance of Vero cell lines. Curr Protoc Microbiol. Appendix 4E.

In Vitro Efficacy of Chloroquine (CQ) Against the Novel Coronavirus SARS-CoV-2

David Meyerhoff, Jeff Whyte, and Jeffrey Adamovicz (PI)

The Laboratory for Infectious Disease Research (LIDR) at the University of Missouri

- State-of-the-art facilities to support research on infectious diseases.
- **Goal**: Develop novel approaches to prevent and treat diseases caused by some of our most deadly pathogens, including **SARS-CoV-2 (COVID-19**).
- 10,000 net sq. feet of biosafety level **BSL-2** and **BSL-3** laboratories.
- Assist health officials in the event of an infectious disease outbreak in the United States.
- Designed to protect researchers and the environment from any release of biohazards.
- Access is strictly controlled, limited to a small number of trained MU faculty, staff and students.
- One of 13 structures as part of **the National and Regional Biocontainment Laboratory Network.** Partial federal support from the **National Institute of Allergy and Infectious Diseases (NIAID)**.

In Vitro Efficacy of Chloroquine (CQ) Against the Novel Coronavirus SARS-CoV-2

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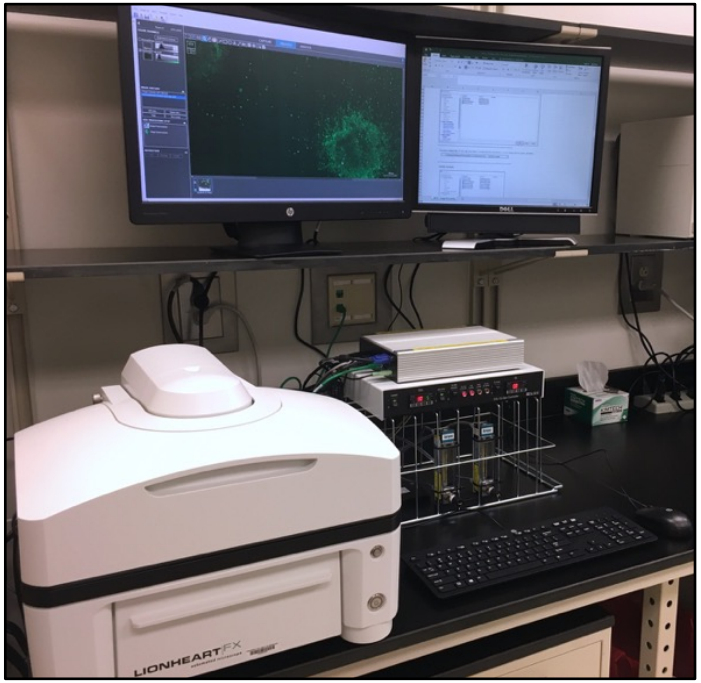
Lionheart FX Automated Microscope (BioTek)

- 4x, 20x, 40x, and 60x magnification with auto-focus and auto-exposure
- Brightfield, phase contrast, and fluorescence imaging (10 colors, 4 simultaneously)
- Temperature (up to 40°C), CO_2/O_2 gas, and humidity control for kinetic assays (tested at LIDR to 96 hours)
- Vessel adapters (multiwell plates, microscope slides, flasks (25 and 75 cm^2), and dishes (35 and 60 mm)
- Powerful Gen5 software for image capture and quantitative data analysis



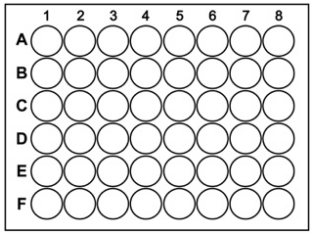
Experiment #1: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**2-fold dilution series**) in infection medium – **19 h exposure**.

Objective:

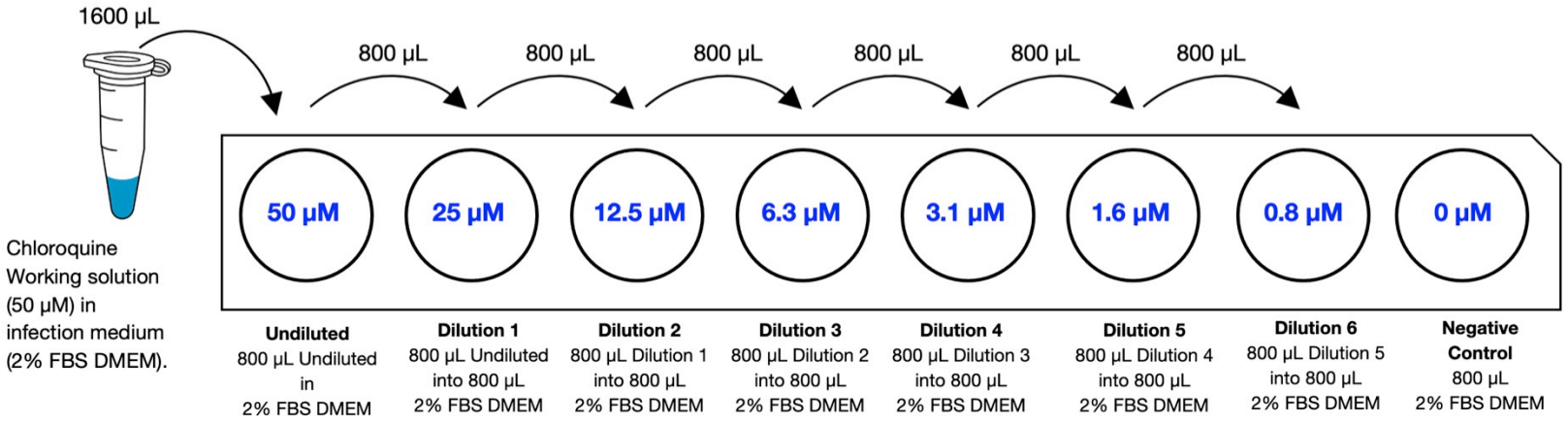
- **Pilot study to establish the optimal CQ concentrations to minimize toxicity to Vero E6 cells.**
- **These established CQ concentrations will be used to test effectiveness against SARS-CoV-2 infection.**

Brief Experimental Methods



- **03-01-2021**: **Preparation of Vero E6 Cells (Immunology Lab - BSL-2):**
  - Seeded **Vero E6** cells (ATCC #CRL-1586) in a 48-well plate at 65,000 cells/well.
  - 250 µL/well of complete **growth medium** (DMEM +**10%** FBS +1 mM GlutaMax +10 µg/mL gentamicin).
  - Cells incubated for 20 hours (37°C, 5% CO2, humidified environment) until 70-80% confluent.

- **03-02-2021: Chloroquine addition to Vero E6 cells (Immunology Lab - BSL-2)):**
  - Preparation of 50 mM chloroquine stock solution in 1X PBS (syringe filtered - 0.22 µm). Aliquots (500 µL) stored at -20°C.
  - Working solution of chloroquine (50 µM) prepared in **infection medium** (DMEM +**2%** FBS +1 mM GlutaMax +10 µg/mL gentamicin).
  - **Two-fold** dilution series of chloroquine in infection medium:



- Growth medium removed from Vero E6 48-well plate and replaced with 250 µL/well chloroquine dilutions in triplicate.

Experiment #1: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**2-fold dilution series**) in infection medium – **19 h exposure**.

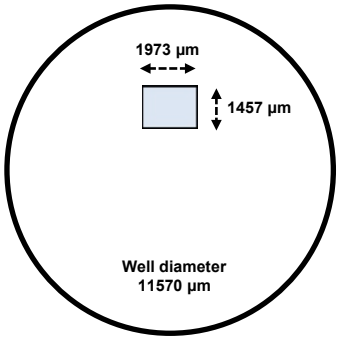
48-Well Plate Experimental Design

- Image capture from rows **A** to **C** for this pilot study.
- Chloroquine concentrations for triplicate wells of Vero E6.

	1	2	3	4	5	6	7	8
A	50 µM	25 µM	12.5 µM	6.3 µM	3.1 µM	1.6 µM	0.8 µM	0.0 µM
B	50 µM	25 µM	12.5 µM	6.3 µM	3.1 µM	1.6 µM	0.8 µM	0.0 µM
C	50 µM	25 µM	12.5 µM	6.3 µM	3.1 µM	1.6 µM	0.8 µM	0.0 µM
D								
E								
F								

Lionheart FX

- **Imaging Chamber**
  - 37°C, 5% CO_2
  - humidified
- **Image Acquisition**
  - 4X Objective
  - Phase Contrast
- **Kinetic Image Capture**
  - **19-hour duration**
  - Capture every 15 min
  - Single tile dimensions:
    - 1973 µm x 1457 µm

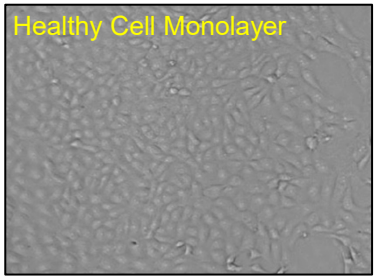


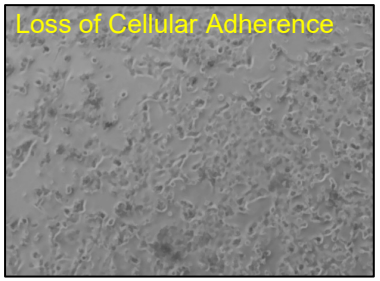
- **Gen5 Software Image Processing (per well)**
  - Image Preprocessing and Deconvolution
  - Cellular Analysis - **Cytopathic Effects (CPE):**
    - **CPE** (rounding and loss of adherence) analyzed per well at each frame over the **19-h** kinetic assay duration.

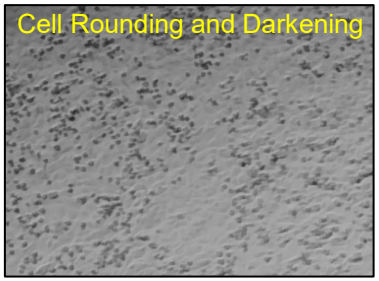
Experiment #1: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**2-fold dilution series**) in infection medium – **19 h exposure**.

Lionheart Gen5 Cellular Analysis for Cytopathic Effect (CPE) of Chloroquine.

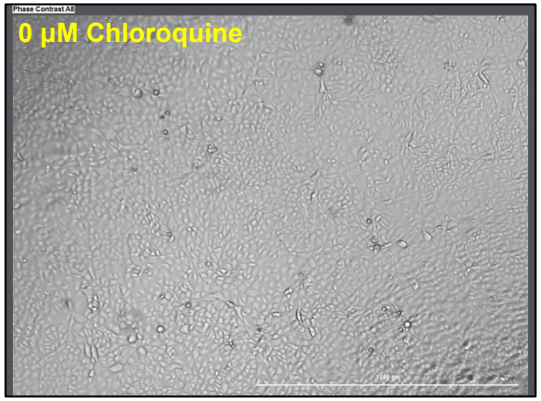


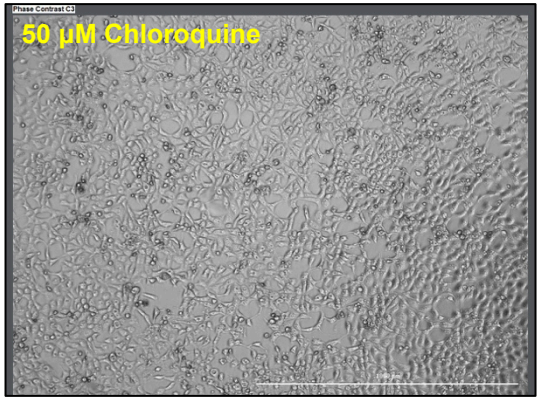




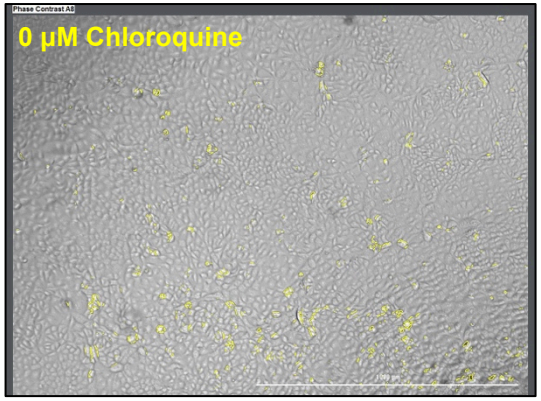
Example Micrographs from Experiment #1 – 19-hour CQ exposure (Phase Contrast 4X).

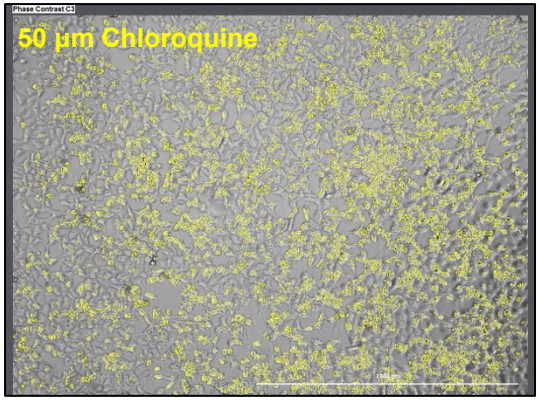
Unmasked Images





Masked Images
CPE in yellow



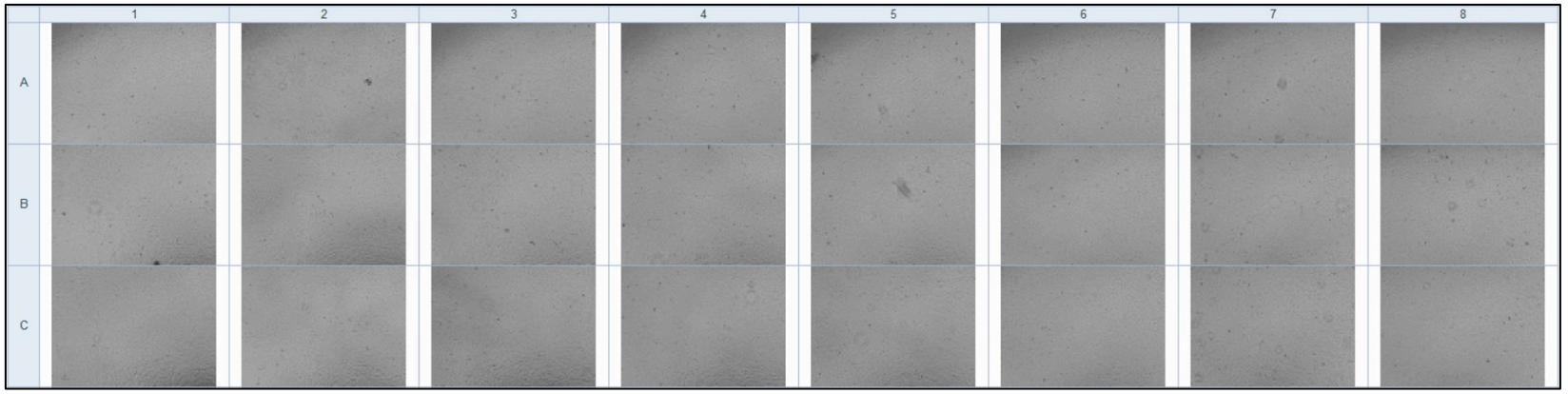


Experiment #1: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**2-fold dilution series**) in infection medium – **19 h exposure**.

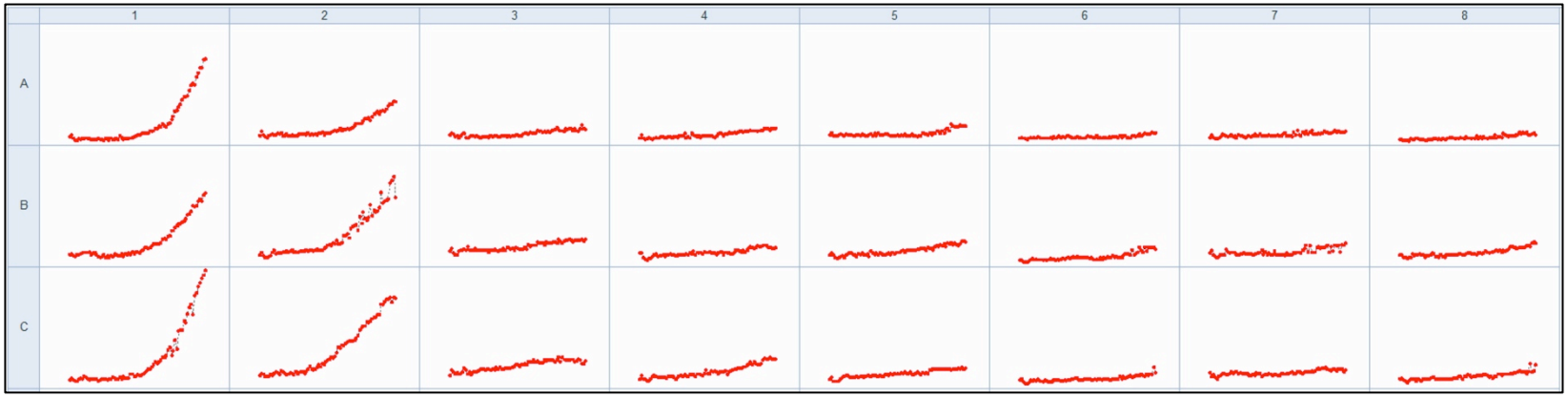
Micrographs per well at end of 19-hour acquisition.

Chloroquine Concentration:	50 µM	25 µM	12.5 µM	6.3 µM	3.1 µM	1.6 µM	0.8 µM	0 µM
	Undiluted	Dil -1	Dil -2	Dil -3	Dil -4	Dil -5	Dil -6	Neg Control



Cell count graphs per well over 19-hour acquisition (one image capture every 15 min).

Chloroquine Concentration:	50 µM	25 µM	12.5 µM	6.3 µM	3.1 µM	1.6 µM	0.8 µM	0 µM
	Undiluted	Dil -1	Dil -2	Dil -3	Dil -4	Dil -5	Dil -6	Neg Control



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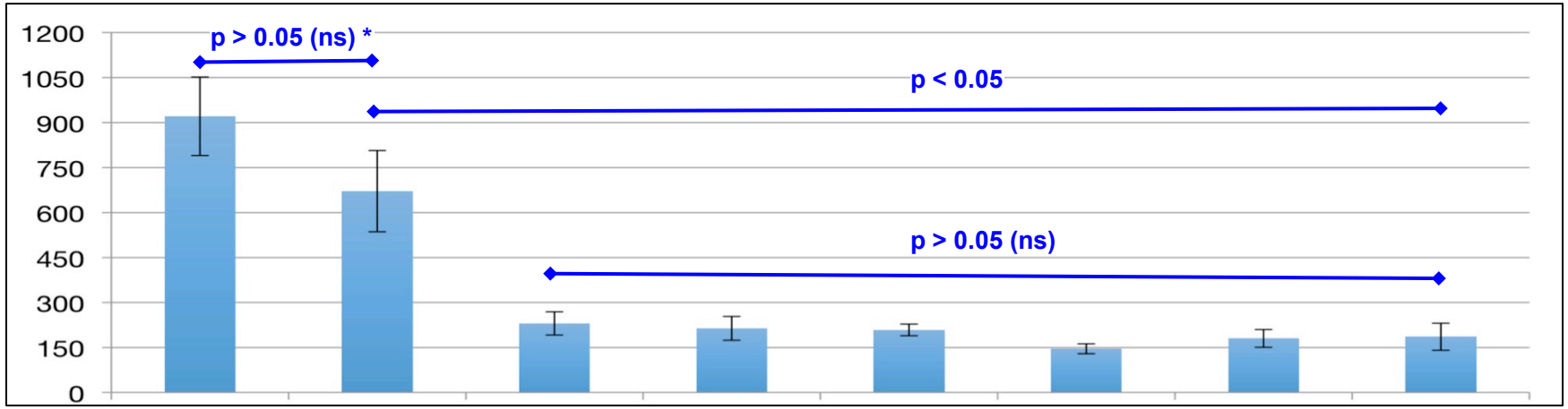
Cell count per well at end of 19-hour acquisition.

Chloroquine Concentration:	50 µM Undiluted	25 µM Dil -1	12.5 µM Dil -2	6.3 µM Dil -3	3.1 µM Dil -4	1.6 µM Dil -5	0.8 µM Dil -6	0 µM Neg Control
A	856	429	153	163	184	116	132	96
B	734	688	268	187	247	173	234	231
C	1173	897	270	292	195	149	175	231



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*** One-way ANOVA with post-hoc Tukey HSD Test**

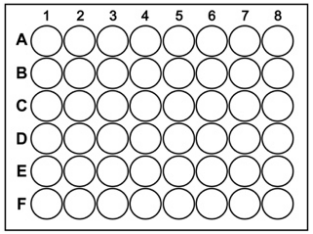
Experiment #2: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**10-fold dilution series**) in infection medium – **48 h exposure**.

Objective:

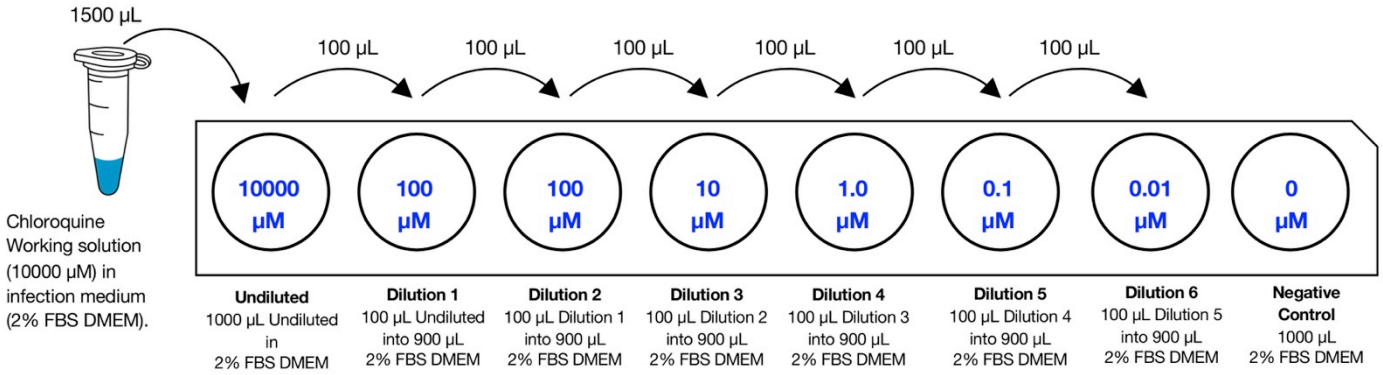
- **Extend dilution range of CQ based on Vero E6 toxicity results form Experiment #1.**
- **Increase duration to 48 hours based on published test conditions for other SARS-CoV-2 antiviral compounds[1].**

Brief Experimental Methods



- **03-07-2021**: **Preparation of Vero E6 Cells (Immunology Lab - BSL-2):**
  - Seeded **Vero E6** cells (ATCC #CRL-1586) in a 48-well plate at 65,000 cells/well.
  - 250 µL/well of complete **growth medium** (DMEM +**10%** FBS +1 mM GlutaMax +10 µg/mL gentamicin).
  - Cells incubated for 20 hours (37°C, 5% CO2, humidified environment) until 70-80% confluent.

- **03-08-2021: Chloroquine addition to Vero E6 cells (Immunology Lab - BSL-2)):**
  - Working solution of chloroquine (50 µM) prepared in **infection medium** (DMEM +**2%** FBS +1 mM GlutaMax +10 µg/mL gentamicin).
  - **10-fold** dilution series of chloroquine in infection medium:



- Growth medium removed from Vero E6 48-well plate and replaced with 250 µL/well chloroquine dilutions in triplicate.

[1] Holwerda, M., V'kovski, P., Wider, M., Thiel, V., and Dijkman, R. (2020). *Identification of an Antiviral Compound from the Pandemic Response Box that Efficiently Inhibits SARS-CoV-2 Infection In Vitro.* Microorganisms 8.

Experiment #2: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**10-fold dilution series**) in infection medium – **48 h exposure**.

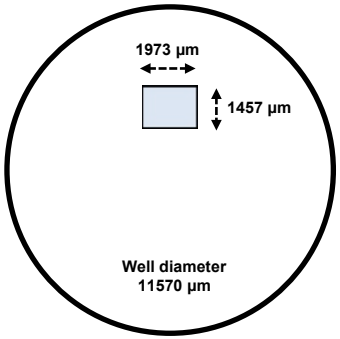
48-Well Plate Experimental Design

- Image capture from rows **A** to **C** for this study.
- Chloroquine concentrations for triplicate wells of Vero E6.

	1	2	3	4	5	6	7	8
A	10000 µM	1000 µM	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	0.0 µM
B	10000 µM	1000 µM	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	0.0 µM
C	10000 µM	1000 µM	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	0.0 µM
D								
E								
F								

Lionheart FX

- **Imaging Chamber**
  - 37°C, 5% CO_2
  - humidified
- **Image Acquisition**
  - 4X Objective
  - Phase Contrast
- **Kinetic Image Capture**
  - **48-hour duration**
  - Capture every 10 min
  - Single tile dimensions:
    - 1973 µm x 1457 µm



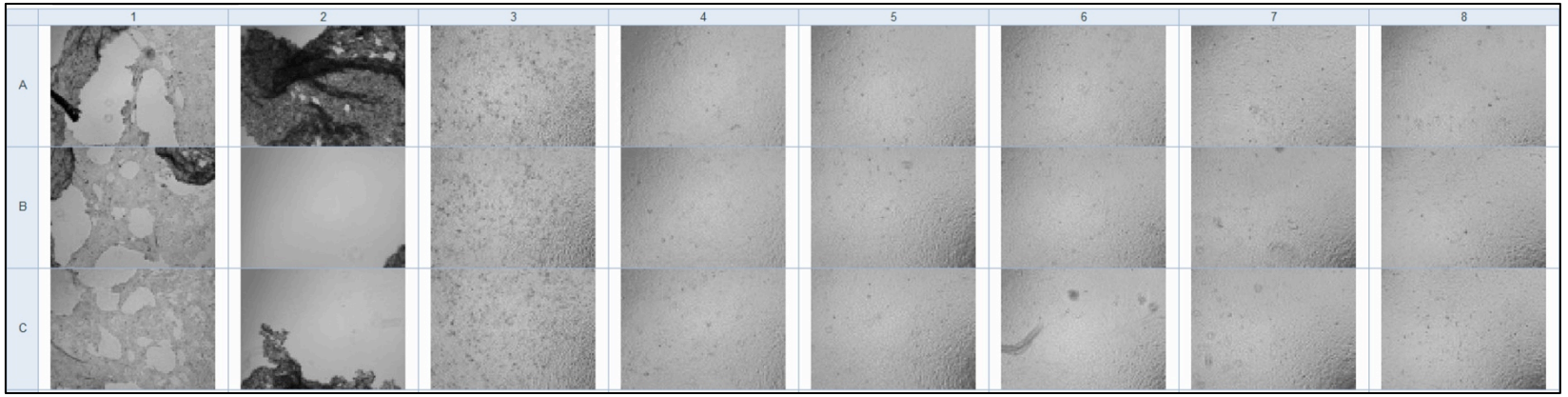
- **Gen5 Software Image Processing (per well)**
  - Image Preprocessing and Deconvolution
  - Cellular Analysis - **Cytopathic Effects (CPE):**
    - **CPE** (rounding and loss of adherence) analyzed per well at each frame over the **48-h** kinetic assay duration.

Experiment #2: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**10-fold dilution series**) in infection medium – **48 h exposure**.

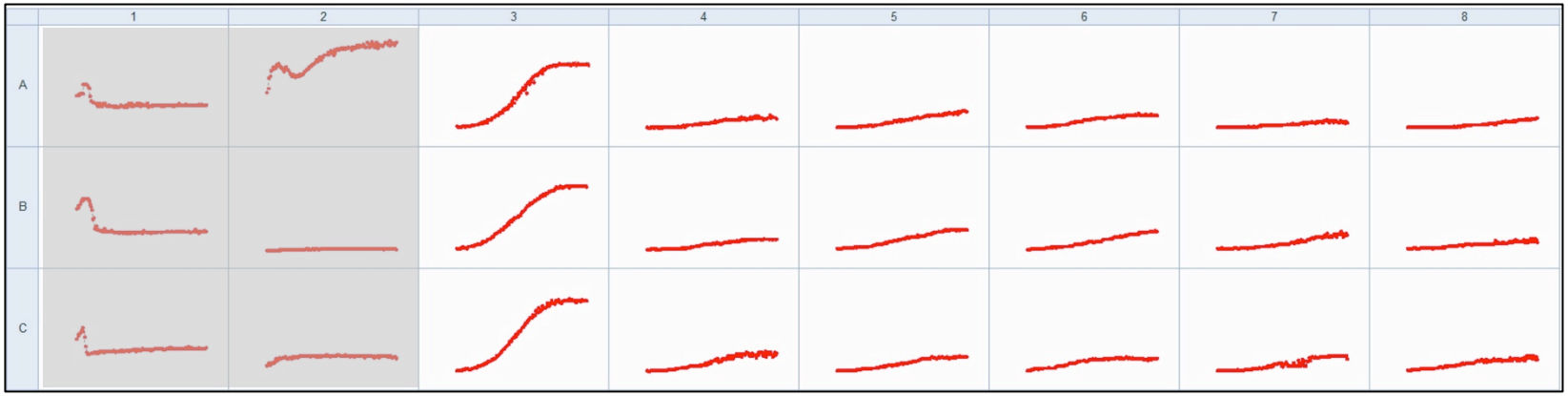
Micrographs per well at end of 48-hour acquisition.

Chloroquine Concentration:	10000 µM	1000 µM	100 µM	10 µM	1.0 µM	0.1 µM	0.01 µM	0.0 µM
	Undiluted	Dil -1	Dil -2	Dil -3	Dil -4	Dil -5	Dil -6	Neg Control



Cell count graphs per well over 48-hour acquisition (one image capture every 10 min).

Chloroquine Concentratio	10000 µM	1000 µM	100 µM	10 µM	1.0 µM	0.1 µM	0.01 µM	0.0 µM
	Undiluted	Dil -1	Dil -2	Dil -3	Dil -4	Dil -5	Dil -6	Neg Control

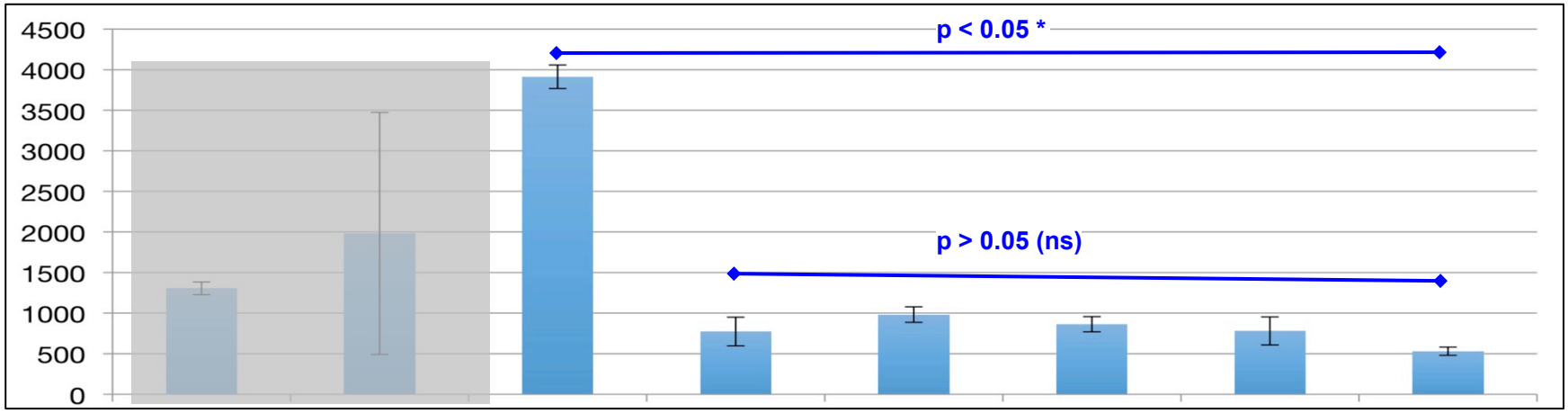


Experiment #2: Vero E6 Exposure to Chloroquine (CQ) – Lionheart FX Kinetic Assay - Experimental Overview

Vero E6 dosing with chloroquine (**10-fold dilution series**) in infection medium – **48 h exposure**.

Cell count per well at end of 48-hour acquisition.

Chloroquine Concentration:	10000 µM Undiluted	1000 µM Dil -1	100 µM Dil -2	10 µM Dil -3	1.0 µM Dil -4	0.1 µM Dil -5	0.01 µM Dil -6	0.0 µM Neg Control
A	1384	4919	3811	549	907	797	435	522
B	1149	65	3730	651	1171	1048	947	448
C	1383	963	4199	1121	868	744	959	623



* One-way ANOVA with post-hoc Tukey HSD Test

Experiment #3: Vero E6 Exposure to Chloroquine (CQ) and SARS-CoV-2 - Experimental Overview

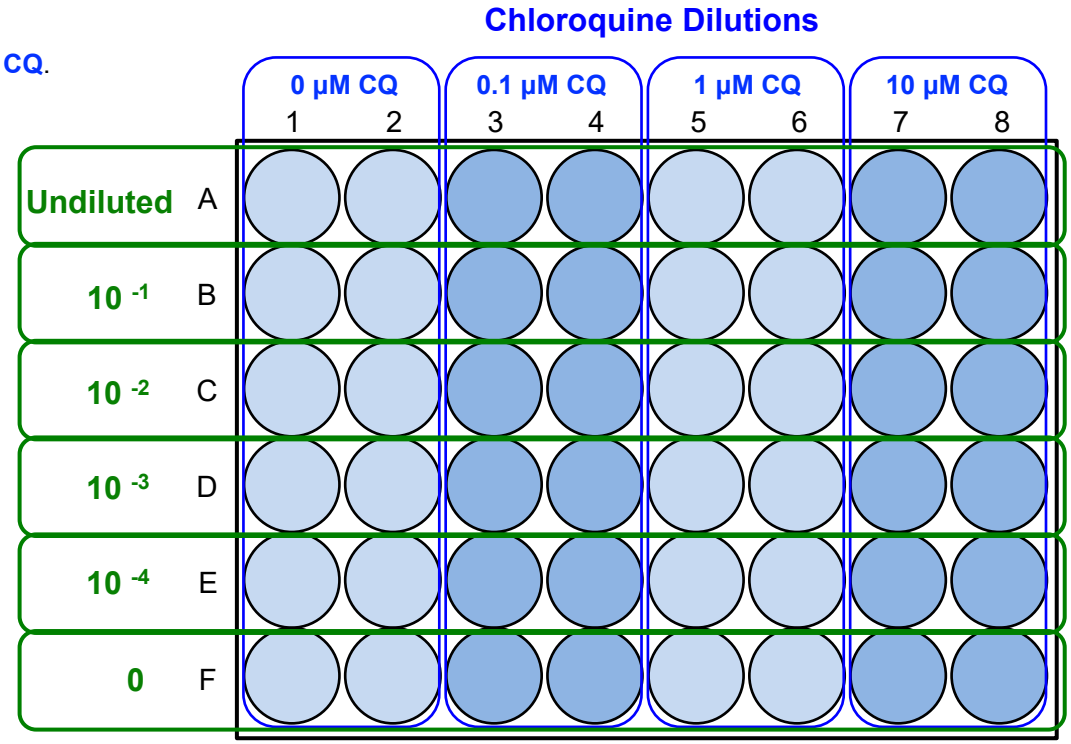
Objective:

- **Determine effectiveness of CQ at reducing CPE in Vero E6 cells infected with SARS-CoV-2.**
- **Expose Vero E6 to CQ concentrations determined to have minimal toxicity to cells based on Experiments #1 and #2.**
- **Toxicity of 10 µM CQ concentration was not significantly different from negative control (0 µM).**
- **Infect cells with 10-fold dilution series of SARS-CoV-2 at each CQ concentration and measure CPE at 48 and 96 hours.**

Brief Experimental Methods

- **04-04-2021: Preparation of Vero E6 Cells (Immunology Lab - BSL-2):**
  - Seeded **Vero E6** cells in a 48-well plate (65,000 cells/well; 250 µL/well of **growth medium**, incubated 20 hours until 70-80% confluent.
- **04-05-2021: Chloroquine addition to Vero E6 cells (Immunology Lab - BSL-2)):**
  - Cells exposed to **10-fold** dilution series of chloroquine in infection medium: **10 µM, 1 µM, 0.1 µM, and 0 µM** in duplicate (125 µL/well).
- **04-05-2021: SARS-CoV-2 infection of Vero E6 cells (Virology Lab – BSL-3):**
  - **10-fold** dilution series **SARS-CoV-2** virus in infection medium (125 µL/well) added to existing medium with **CQ**.
  - Images captured with EVOS microscope at 48- and 96-hours post-infection.
  - **SARS-CoV-2 Isolate** (BEI Resources) *SARS-Related Coronavirus 2, Isolate USA-WA1/2020 Catalog No. NR-5228.*

SARS-CoV-2 Dilutions
(3.16E+05 $TCID_{50}$/mL)



Experiment #3: Vero E6 Exposure to Chloroquine (CQ) and SARS-CoV-2 - Experimental Overview

Vero E6 Image Capture and $TCID_{50}$ Analysis

- **Image Capture with Life Technologies EVOS XL Digital Imaging System Microscope.**
  - 4X magnification digital images acquired for each well (TIF format).

$TCID_{50}$ Assay

A measure of infectivity (viral replication in cells) to obtain a titer for a given virus stock.

Titer: A given number of infectious viral units per unit volume.

Infectious Unit: The smallest amount of virus that produces cytopathic effects (CPE).

$TCID_{50}$: The median tissue culture infectious dose. Defined as the dilution of a virus required to infect 50% of a given cell culture.

Scoring of CPE: Each individual well of a multiwell plate is designated **positive** or **negative** based on the presence or absence of CPE (dark cells, loss of adherence), respectively.

$$\text{Infection rate} = \frac{\text{number of cumulative positive units}}{\text{number of cumulative positive units} + \text{number of cumulative negative units}}$$

$TCID_{50}$ Methods adapted from the following publications:

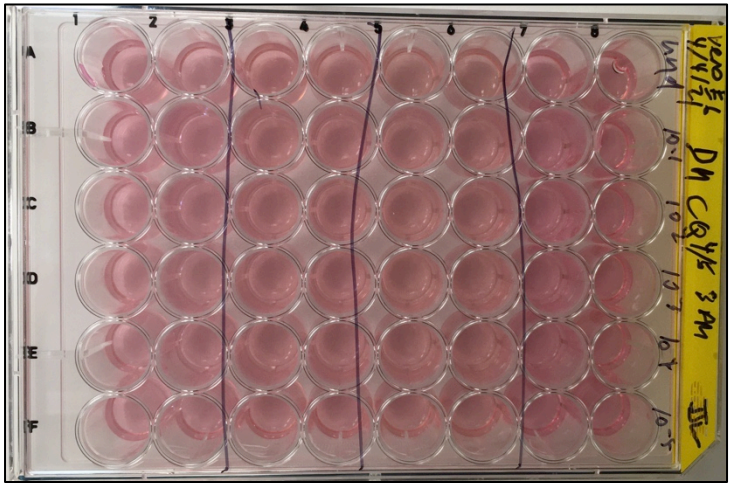
Lei, C., Yang, J., Hu, J., and Sun, X. (2020). *On the Calculation of TCID50 for Quantitation of Virus Infectivity*. Virologica Sinica.

Reed, L.J. and Muench, H. (1938). *A simple method of estimating fifty percent endpoints*. Am J Hyg 27, 493497.

Experiment #3: **Vero E6 Exposure to Chloroquine (CQ) and SARS-CoV-2 - Experimental Overview**
Plate images and scoring sheets (04/07/21) and (04/09/21).

Wednesday 04/07/21

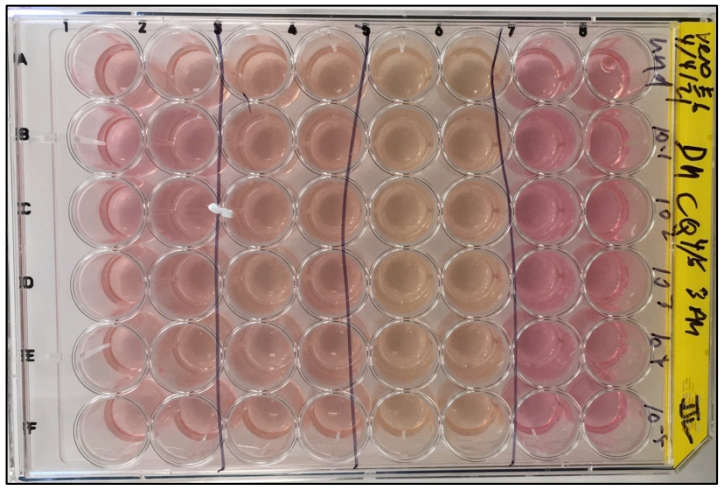
0 µM CQ | 0.1 µM CQ | 1 µM CQ | 10 µM CQ



SARS-CoV-2		0 µM CQ		0.1 µM CQ		1 µM CQ		10 µM CQ	
		1	2	3	4	5	6	7	8
Undiluted	A	+	+	+	+	-	+	+	+
10^{-1}	B	+	+	+	+	+	+	+	+
10^{-2}	C	+	+	-	+	+	+	+	+
10^{-3}	D	+	+	-	-	+	+	+	+
10^{-4}	E	+	-	-	-	+	+	+	+
0	F	-	-	-	-	+	+	+	+

Wednesday 04/09/21

0 µM CQ | 0.1 µM CQ | 1 µM CQ | 10 µM CQ



SARS-CoV-2		0 µM CQ		0.1 µM CQ		1 µM CQ		10 µM CQ	
		1	2	3	4	5	6	7	8
Undiluted	A	+	+	+	+	-	+	+	+
10^{-1}	B	+	+	+	+	+	+	+	+
10^{-2}	C	+	+	+	+	+	+	+	+
10^{-3}	D	+	+	+	-	+	+	+	+
10^{-4}	E	+	+	-	-	+	+	+	+
0	F	-	-	-	-	+	+	+	+

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Experiment #3: Vero E6 Exposure to Chloroquine (CQ) and SARS-CoV-2 - Experimental Overview
$TCID_{50}$ Values (04/07/21) and (04/09/21).

SARS-CoV-2 Infection Date	Imaging/ Scoring Date	Infection Duration (h)	Chloroquine Concentration (µM)	SARS-CoV-2 $TCID_{50}$/mL
05-Apr	07-Apr	48 hours	10.0 µM CQ	8.00E+05
05-Apr	07-Apr	48 hours	1.0 µM CQ	1.42E+06
05-Apr	07-Apr	48 hours	0.1 µM CQ	**8.00E+02**
05-Apr	07-Apr	48 hours	0.0 µM CQ	8.00E+04
05-Apr	09-Apr	96 hours	10.0 µM CQ	8.00E+05
05-Apr	09-Apr	96 hours	1.0 µM CQ	8.00E+05
05-Apr	09-Apr	96 hours	0.1 µM CQ	**8.00E+03**
05-Apr	09-Apr	96 hours	0.0 µM CQ	2.53E+05

$TCID_{50}$ Analysis

- The lowest $TCID_{50}$ based on CPE was determined to be in Vero E6 cells treated with **0.1 µM CQ**.
- Compared to cells not treated with **CQ (0 µM)**, the "protective" effect of **0.1 µM CQ** against **SARS-CoV-2** was a **two-order-of-magnitude reduction in CPE**.

In Vitro Efficacy of Chloroquine (CQ) Against the Novel Coronavirus SARS-CoV-2

David Meyerhoff, Jeff Whyte, and Jeffrey Adamovicz (PI)

Conclusions

- Chloroquine at less than 12.5 μM was not toxic to Vero E6 cells over 48 hours.
- Chloroquine at 0.1 μM provided a protective effect against SARS-CoV-2 cytopathic effects.

Future Directions

- Moving from the Vero E6 (monkey cell line) to a human cell line model could more accurately reflect chloroquine's ability to protect from SARS-CoV-2 in vitro.
- Ultimately, in vivo (animal model) studies are required to determine how chloroquine exerts its effects in a physiological system.
- More investigation into the mechanism of action of chloroquine is required to truly make an informed, definitive statement about its efficacy as a clinical treatment for COVID-19.

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