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# Ready Reporter Virus™ Kit—Guide to Imaging Readout on the Agilent BioTek Cytation 5

## Summary

The protocols we provide for Ready Reporter Virus (RRV) kits are based on flow cytometric readout; however, RRV neutralization assays are also compatible with high-content imagers, such as the Agilent BioTek Cytation. This guide provides our recommendations on instrument specifications, assay setup, imaging acquisition, and analysis for the Cytation.

This guide provides information on:

- LED/filter cube sets that have been tested with the RRV kit.
- Laboratory consumables and assay setup are adapted to remove agitation of cells.
- Recommendations on image analysis for infected cell counting.

High-content imagers offer a rapid and powerful means of reading out same-day RRV neutralization assays. This guide is intended for use with RRV kits that use HEK-293T–derived target cells (see **Table 1** below).

**Table 1. Compatible RRV kits and protocols to use with this guide.**

| Compatible Kits | Compatible Protocols |
|---|---|
| Ready Reporter Virus™ Kit SARS-CoV-2 D614G<br>Ready Reporter Virus™ Kit SARS-CoV-2 JN.1<br>Ready Reporter Virus™ Kit Influenza A, H5N1 Texas/37/2024<br>Ready Reporter Virus™ Kit Influenza A, H5N1 Vietnam/1194/2004 | C24 SARS-CoV-2 RRV Infectivity of HEK-293T hsACE2 Target Cells_V02<br>C25 Neutralization of SARS-CoV-2 RRV Infection of HEK-293T hsACE2 Target Cells_v02<br>I18 Influenza A (H5N1) RRV Infectivity of HEK 293T Target Cells_V01<br>I19 Neutralization of Influenza A (H5N1) RRV Infection of HEK-293T Target Cells_v01 |

## Introduction

This guide is for users of the Ready Reporter Virus (RRV) kit who have experience with the Agilent BioTek Cytation 5 automated microscopy platform. It may be used alongside our standard RRV infectivity and neutralization protocols for readout by fluorescence microscopy. With appropriate adaptation, RRV kit results are highly comparable between flow cytometric and imaging readouts (see **Appendix Figure 1**).

## RRV Kit Overview

Ready Reporter Virus (RRV) kits use a pseudovirus system that models authentic viral entry in the absence of viral genetic material. Each kit includes non-replicative, pseudotyped reporter virus particles that display relevant viral envelope proteins, along with permissive target cells pre-loaded with reporter dye. When the viral particles fuse with the cell membrane, they deliver an enzyme reporter. The enzyme triggers a change in the fluorescent reporter substrate inside the infected cell (see **Appendix Figure 2**). This fluorescent signal allows you to quantify viral infectivity or neutralization by microscopy.

## Assay Plate Setup

Infectivity or neutralization assay setup for imaging readout largely follows the protocols for readout by flow cytometry, with adaptations listed in **Table 2**. Recommended serial dilutions for neutralizing samples, RRV particle input, and Target Cell seeding density do not need to be adapted unless improvements are empirically determined.

**Table 2. Adaptations to assay plate setup.**

| Protocol Step | Protocol Component | Adaptation for Imaging Readout |
|---|---|---|
| Plastics | 96-well U-bottom assay plate | 96-well Nunc optical-bottom plate |
| Neutralization Setup | Plate shaking or mixing at each hour during 3 hour incubation | Do not shake or mix plate. Prior to imaging, spin down plates at 1500 rpm for 2 minutes. |

## Data Collection by Imaging

As during assay setup, plate shaking should not be enabled for readout by microscopy. Viable Target Cells fluoresce at 520 nm. Infected Target Cells fluoresce at both 520 nm and 447 nm. Therefore, acquiring and analyzing infection is performed with the Em. 409 nm/Ex. 447 nm channel (see **Appendix Figure 3**). This guide describes acquisition and analysis using readily available catalog parts developed for mTagBFP imaging; custom LED and filter cube sets may further minimize background signal.

*Recommended Filter Cube Sets*

| Channel Name | Excitation | Emission | Reporter |
|---|---|---|---|
| mTagBFP | 409 nm (1225009 \| Agilent) | 447 nm (1225115 \| Agilent) | Infected Target Cell |
| GFP | 409 nm (1225001 \| Agilent) | 520 nm (1225101 \| Agilent) | Target Cell |

**Note on fixing samples:** Fixation is a common step in sample preparation prior to microscopy. Fixation of RRV samples is not recommended prior to imaging because it causes reduced signal.

**Note on DAPI staining:** DAPI staining is a common step in sample preparation prior to microscopy to observe nuclear morphology, count total cells, and guide autofocus on high-content imagers. DAPI staining is not compatible with the fluorescent reporter in the RRV kit. The GFP channel readily detects all Target Cells and can be used for total cell counts or to guide autofocus.

## Acquisition and Analysis with the Cytation 5 and Gen5 Software

*Image Acquisition: Principles and Suggested Settings*

| Gen5 Menu | Gen5 Setting | Suggested Value |
|---|---|---|
| Procedure | Plate Type | Nunc 96 well optical bottom plate |
| Procedure | Actions | Image |
| Imaging Step-Inverted Imager | Magnification | 4X |
| Imaging Step-Inverted Imager | Channels | (1) GFP, (2) TagBFP |
| Focus options… | | For Channel 1: Auto<br>For Channel 2: Focal height from first channel (+ offset): 0 |
| Experiment | Load Plate | Use Lid |

*Image Analysis: Principles and Suggested Settings*

| Gen5 Menu | Gen5 Setting | Suggested Value |
|---|---|---|
| Data Reduction | Image Analysis | Cell Counting |
| Cell Counting | Channel | TagBFP |
| Cell Counting | Threshold | Value: determine for your instrument using Line Profile<br>Background: Dark<br>✓Split touching objects |

| | | |
|---|---|---|
| Cell Counting | Object selection | Min. object size: 12 µm<br>Max. object size: 20 µm |
| Cell Counting | Advanced detection options… | ✓Background flattening<br>Rolling ball diameter: 20 µm<br>Image smoothing strength: 2 |

See **Appendix Figure 4** for representative images

## Frequently Asked Questions

**Can I use *only* Agilent Biotek Cytation imager to read the Ready Reporter Virus kit neutralization assay?**

No. The principles in this guide can be applied for kit readouts on other high-throughput imagers. This guide is intended to support the Cytation specifically because it is commonly used and has been directly tested in our laboratory.

**Why do I observe some GFP channel fluorescence in cells that also have BFP channel fluorescence?**

This is a normal effect due to incomplete dye cleavage. Even infected cells may have some residual fluorescence in the 520 nm emissions channel.

**Can I read Ready Reporter Virus kits using flow cytometry?**

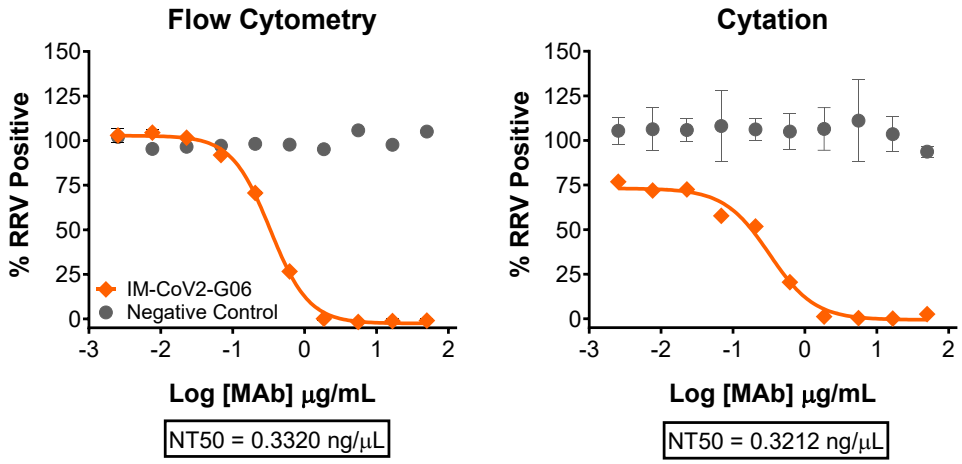
Yes. Flow cytometry is a preferred method for reading RRV kit neutralization assays. Details regarding the use of flow cytometry are available in our standard protocols.

**Are GFP Reporter Virus Particles compatible with imagers?**

Yes. Many users have successfully used imaging readout with our traditional Reporter Virus Particles (RVPs). While we have not created a guide on using RVPs with a microscopy readout, we suggest using lower cell densities than those recommended for flow cytometry readouts. Having fewer cells present supports more effective cell segmentation to generate counts of infected cells. For personalized support, please reach out to our team.

# Appendix

*Figure 1. Comparison of Flow Cytometry- and Imaging-Based Readout*




SARS-CoV-2 D614G RRVs were incubated with neutralizing antibody (IM-CoV-2G06) or negative control antibody for 1 hour prior to infecting HEK-ACE2 Target Cells for 3 hours. Infected Target Cells were measured by flow cytometry (left) or imaging on the Cytation 5 (right). NT50 values were determined from lines of best fit to neutralization data.

*Figure 2. Schematic of RRV Infection and Readout*

Excitation and emission spectra in an uninfected (green box, top) or infected (blue box, bottom) Target Cell. Representative images of overlaid 520 nm and 447 nm fluorescence.

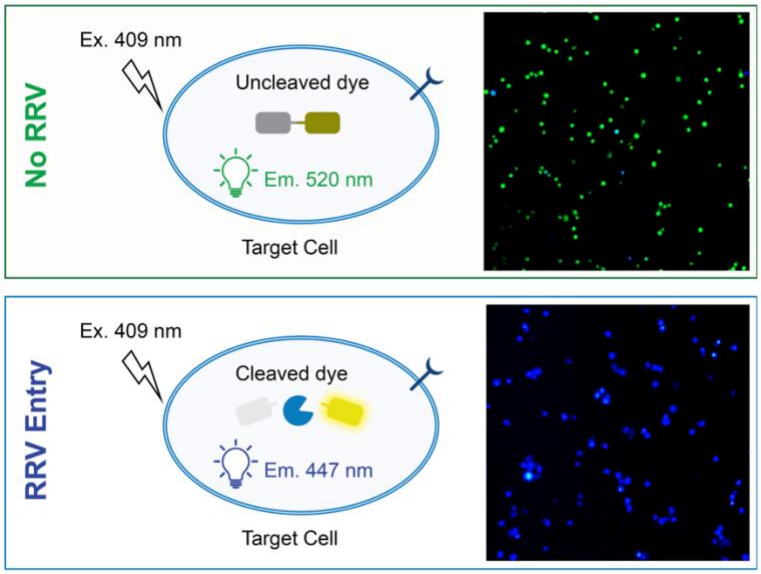


*Figure 3. Representative Images of Target Cell Fluorescence in RRV Infectivity Assay*

SARS-CoV-2 D614G RRV infection of HEK-ACE2 Target Cells (catalog #RRK-702). Images of uncleaved dye (Em. 520 nm), cleaved dye (Em. 447 nm), and merged channels. Images were taken at 1 hour post-infection (top row) and at 4 hours post-infection (bottom row).

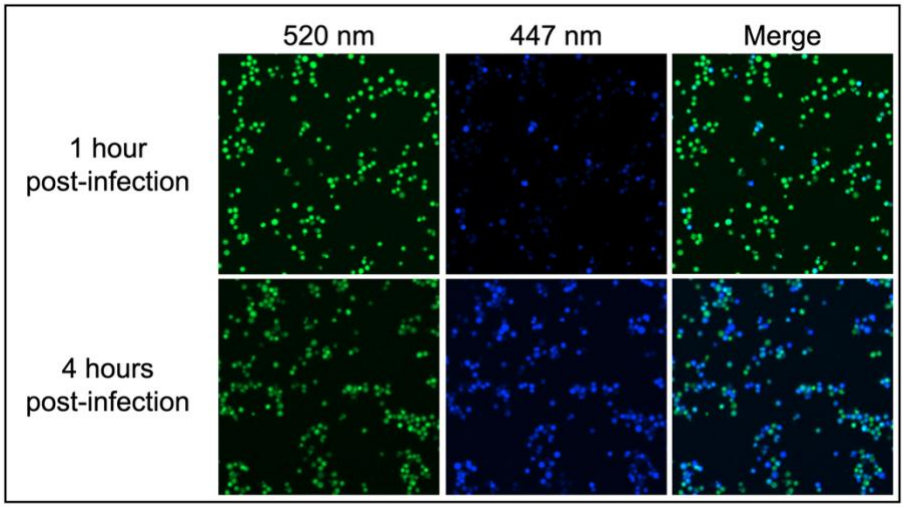
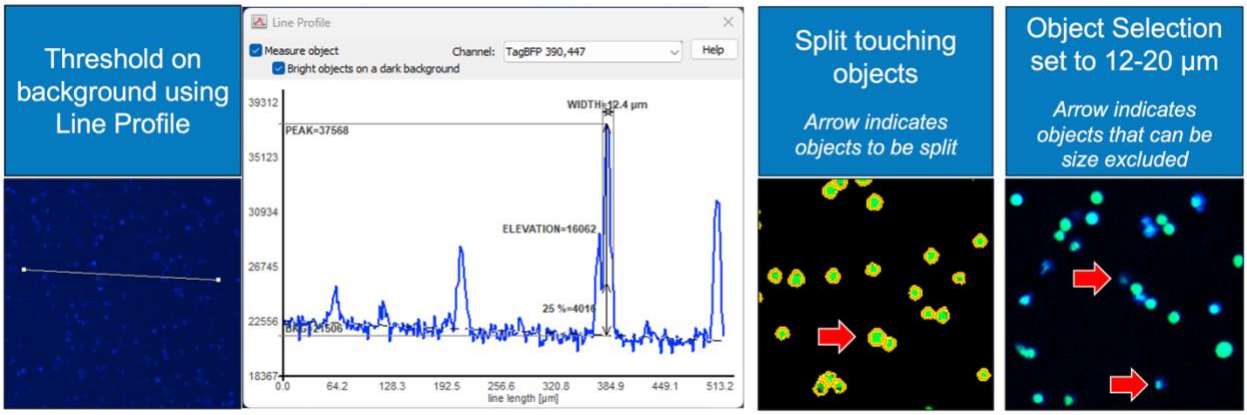



*Figure 4. Representative Images Corresponding to Cell Count Analysis Settings*




Examples of Gen5 software features for setting up Cell Count Analysis. Line Profile may be used to determine appropriate background and thresholding settings (left). Electing to split touching objects enables more accurate cell counting (middle). Selecting objects or Regions of Interest based on a size between 12-20 µm eliminates most debris in the field (right).