Application Note

Cancer Biology

Agilent

Trusted Answers

Automated Colony Formation Assay

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Abstract

The colony formation assay evaluates the proliferative capacity of a single cell. For applications such as cancer drug screening, it is important to distinguish cells that retain this proliferative capacity from those that do not. Conventional analysis of this assay involves scoring and quantifying colonies in each well of a multiwell format manually by eye, limiting its throughput capabilities. This application note presents an automated method for conducting and analyzing the colony formation assay in both a 6- and 96-well microplate using the upright color brightfield imaging capabilities of the Agilent BioTek Cytation 7 cell imaging multimode reader with a wide field of view.

Introduction

The colony formation assay is an essential method for cancer research, enabling drug screens and radiation dosing to be conducted.[1-5] The assay is performed by seeding cells at a low enough density such that individual cells can propagate to a sufficient colony area without impinging on a neighboring colony (Figure 1).[6,7] At a set time point, adherent colonies are fixed then stained with Crystal Violet colorimetric dye, which allows for visual inspection of the culture vessel and quantification of the number of colonies that expanded. A major drawback of this assay is that scoring colonies is typically carried out manually by a trained technician. This analytical approach is both labor-intensive and hinders the ability to carry out this assay in a high-throughput fashion using a format larger than 12- or 24-well plates. Furthermore, although the accepted criteria for what constitutes a colony is 50+ cells, a quantitative method to assign colony size cut-offs is not frequently adhered to, leaving such manually assessed cut-offs to be subjective.

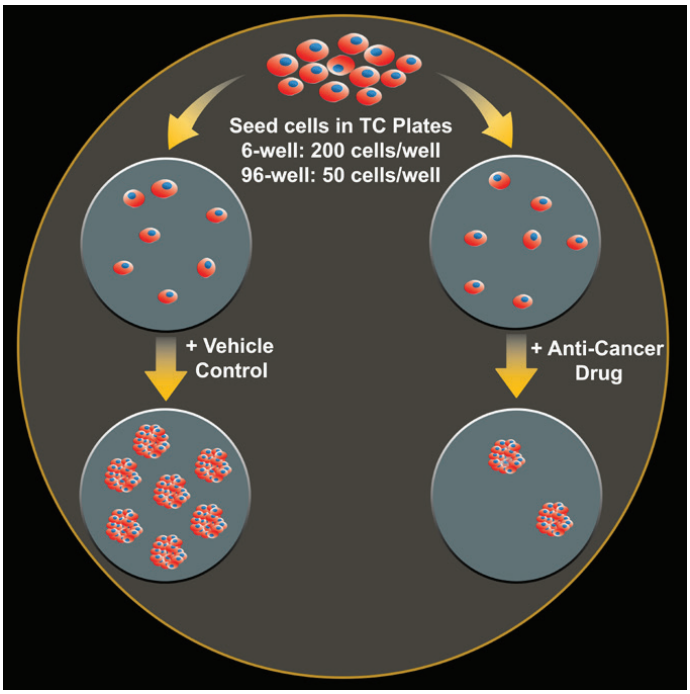

Figure 1. Schematic representation of the colony formation assay. Cells in suspension are seeded in tissue culture wells at a low enough density to enable single cells to proliferate into clonal populations. The potency of antiproliferative compounds can be assessed based on the number of surviving colonies relative to control.

This application note presents both an automated and high-throughput method for conducting a colony formation assay in a 96-well microplate using upright brightfield microscopy. The fluorescent properties of Crystal Violet are first used to define the colony area, while Hoechst 33342 is used to quantify the number of cells within the colony. Quantitative microscopy using the Agilent BioTek Cytation 7 cell imaging multimode reader enables an automated workflow to capture whole-well images, then identify, quantify, and characterize colonies on a large-scale format. This approach enables a more robust statistical sample set to be collected, both in terms of replicates, as well as a broader range of drug dosing.

Materials and methods

Reagents

Doxorubicin (50 mM in DMSO) (part number 2252) was purchased from Tocris Bioscience (Bristol, UK). PBS was made from tablets (Sigma P4417) dissolved in 1 tab/200 mL of de-ionized H_2O (dH_2O). 4% paraformaldehyde was prepared from powder (Sigma P6148) by heating to 60 °C in PBS with constant stirring for 1 hour or until completely dissolved and solution was clear, then clarified by passing through a 0.45 µm filter. Hoechst 33342 solution (20 mM) (part number 62249) was purchased from Thermo Fisher Scientific (Waltham, MA), then further diluted to a 10 mM stock with deionized H_2O. Crystal Violet (CV) (part number V5265) was purchased from Sigma-Aldrich (St. Louis, MO) as a 25 mM (1% w/v) aqueous solution.

Cell culture

HeLa (ATCC CCL-2) and Caco2 (ATCC HTB-37) cells were grown at 37 °C in Advanced Dulbecco's Modified Eagle's Medium (DMEM) (Gibco part number 12491) with 10% FBS (Gibco part number 10437) and 1x penicillin/streptomycin/L-glutamine (Gibco part number 10378).

Colony formation assay

Colony formation assays were carried out in two formats: 6-well culture plates (Costar part number 3516) and 96-well flat clear bottom black microplates (Costar part number 3904) with an initial seeding density of 200 cells/well and 50 cells/well, respectively. For initial seeding, cells cultured in T75 flasks were passaged with TrypLE (Gibco part number 12605) then transferred to a 15 mL conical tube and pelleted by centrifugation at 300 G for 5 minutes, followed by resuspension in 10 mL fresh media. Cell concentration was determined with a hemocytometer. For 96-well microplates, cells were diluted to 5.0×10^2 cells/mL then 100 µL were dispensed into

wells containing 100 µL media + 20 µL of 11x doxorubicin. For 6-well plates, cells were diluted to 2.0×10^2 cells/mL, then 1 mL was dispensed into wells containing 1 mL + 0.2 mL of 11x doxorubicin (see below for doxorubicin preparation). To ensure even distribution of cells across the well bottom by avoiding convection currents from rapidly warming media, plates containing freshly seeded cells were incubated for 30 minutes on a clean countertop at 25 °C before returning to a 37 °C incubator. Colony expansion of single cells were allowed to progress for 7 days, then washed 2x with PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes.

Drug treatment

An 11-point doxorubicin titration was set up as follows. Doxorubicin was diluted from stock to 11 µM, or 11x of the highest treatment concentration (1,000 nM), in Advanced DMEM. 11x dilutions were prepared from this 11x stock, then 20 µL of this was added to a final volume of 220 µL (96-well microplates) or 0.2 mL added to a final volume of 2.2 mL (6-well plate) containing seeded cells with following 1x final concentrations: 1,000, 100, 32, 10, 5.6, 3.2, 1.8, 1, 0.32, 0.1, and 0.01 nM. For the 96-well format, eight replicates of each concentration were set up column-wise, with the twelfth column including vehicle control (DMSO) at the highest concentration used in this study (0.2% v/v). For the 6-well format, concentrations were set up in triplicates across 6 plates. EC_{50} data are presented as % of colonies compared to the mean of vehicle control wells.

Crystal Violet staining

1% Crystal Violet (25 mM) was diluted to a working concentration of 250 µM in PBS containing 10 µM Hoechst 33342 nuclear stain. 100 µL of this was added to each well of a 96-well plate (1 mL for 6-well plates) containing fixed colonies and incubated for 30 minutes at room temperature. Dye was aspirated, and wells were washed 2x with PBS, then 3x with dH_2O. The last dH_2O wash was aspirated and remnant fluid was allows to evaporate before imaging.

Image capture

All images were captured using a Cytation 7 equipped with a wide-field-of-view (WFOV) camera with upright color brightfield set to 2x magnification. This imaging modality enabled single-frame whole-well imaging of 96-well microplates. To generate whole-well images of a 6-well microplate, 5 × 5 montages (no overlap) were captured, then stitched with the green channel as the reference channel. Autofocus and Capture binning, as well as "Crop image to size of well" were selected. In Advanced Options of the Imaging Read procedure, Delay after plate movement was set to 0 msec.

Cellular analysis

Table 1 describes the settings used to identify all objects in the well, and then apply a subpopulation to select for colonies that meet or surpass the area corresponding to the 50-cell threshold.

Table 1. Cellular analysis settings used for the colony formation assay in both the 6- and 96-well format.

Stitching (6-Well Microplates)	
Registration Channel	Green
Fusion Method	Linear blend
Cropped Borders	Checked
Downsize Image	50%
Cellular Analysis Parameters	
Primary Mask and Count	
Channel	Green
Threshold	
Value	5,000
Background	Light
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Advanced Detection Options	
Background Flattening	Auto
Image Smoothening Strength	10
Evaluate Background On	5% of lowest pixels
Primary Mask	Expand threshold mask by 10 µm
Object Selection	
Minimum Object Size	5 µm
Maximum Object Size	10,000 µm
Include Primary Edge Objects	Checked
Analyze Entire Image	Checked
Subpopulation	
Condition	Area
	Caco2: 103,000; HeLa: 90,000
Condition	Circularity
	≥0.2
Select Objects Where	All conditions are met
Calculate Metrics	Cell (colony) count
	Object (colony) area
	Object (colony) circularity

Results and discussion

Calibration of colony area based on cell number

An important criterion that qualifies a cluster of cells as a colony is the presence of 50+ cells, which is subjectively determined via stereoscope or by assessing directly by eye.[6,7] One goal was to establish an automated imaging analysis method based on colony area using the color brightfield signal from Crystal Violet. An automated approach was previously described to quantify colonies based on the fluorescence properties of Crystal Violet while verifying the number of cells using the spot counting module in the DAPI secondary mask.[8] To correlate colony area with its respective cell number, Caco2 and HeLa colonies stained with Crystal Violet and Hoechst 33342 were imaged with fluorescence microscopy (Figures 2A and 2D). From this data set, a linear regression was fit to correlate colony area with their respective number of cells (Figures 2B and 2D). The resulting linear equation enables an estimation of the area for a 50-cell colony, which in turn is used to calibrate the cellular analysis workflow where the subpopulation of colonies containing at least 50 cells can be quantified (Figures 2C and 2F).

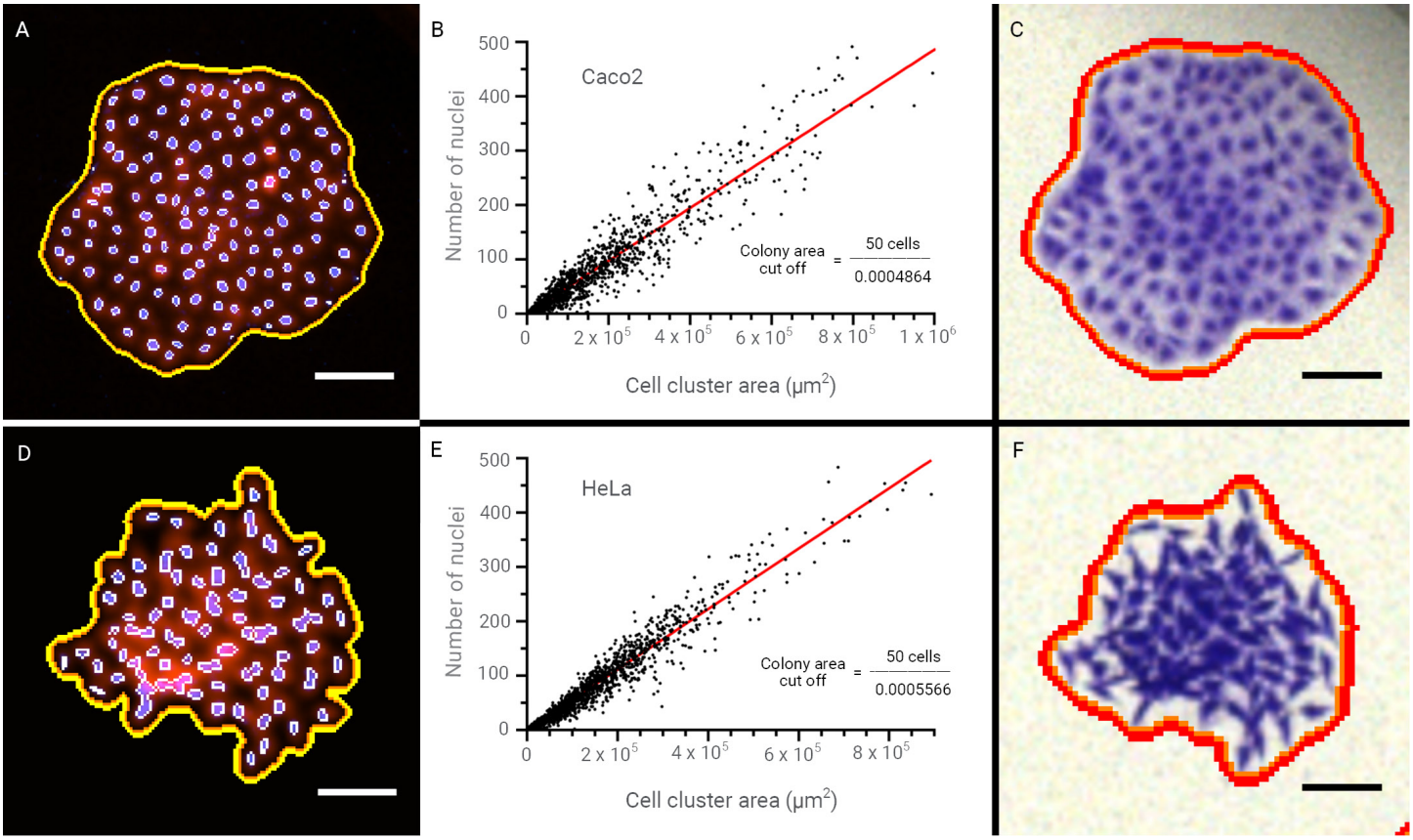

Figure 2. Correlation of colony size with cell number. Previously described method[8] to fluorescently image Crystal Violet and Hoechst 33342-stained Caco2 (A) and HeLa (D) colonies. A population of cell clusters was plotted and a linear regression was then fit to correlate colony area with cell number (nuclei) (B and E). The derived linear equation was reformatted to calculate the estimated area for a 50-cell colony as indicated. A 50-cell colony for Caco2 and HeLa cells corresponds to an area of 1.03×10^6 and 9.0×10^5 μm^2, respectively. These values were applied to color brightfield images of Crystal Violet-stained colonies (C and F).
Scale bar = 200 μm.

Automated color brightfield imaging and analysis of the colony formation assay

A common culture format to conduct the colony formation assay is the 6-well plate. Traditionally, plates containing colonies are stained with Crystal Violet, then the total number of colonies per well are counted manually by eye (Figure 3).[7] For example, a 3-replicate, 11-point drug titration that includes a vehicle control would require 36 wells, or six 6-well plates. For such an experimental set up, manual analysis can be extremely time consuming and laborious, which limits the throughput of the assay. Therefore, an automated workflow was configured to image entire wells of a 6-well plate, then set up a cellular analysis pipeline that identifies all colonies and isolates a subpopulation of colonies containing at least 50 cells based on colony area.

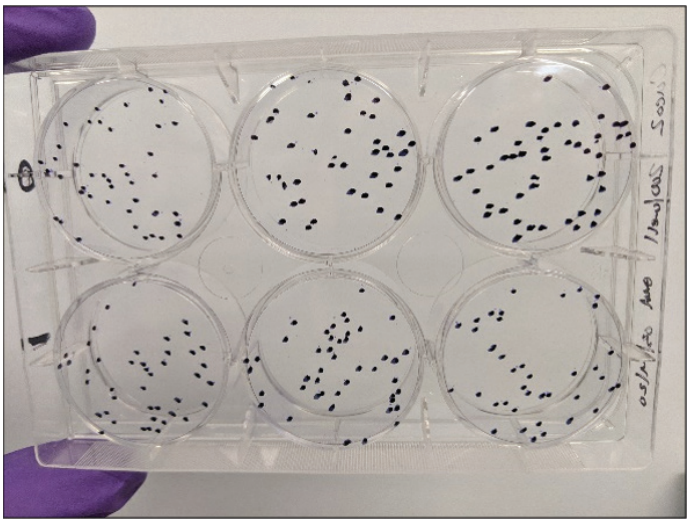

Figure 3. Caco2 colonies in a 6-well format. Traditionally, plates containing colonies are stained with Crystal Violet, then the total number of colonies per well are counted manually by eye.

Doxorubicin is an antitumor drug that disrupts cell division by intercalating DNA, inhibiting the progression of topoisomerase II, ultimately leading to an inhibition of macromolecular biosynthesis.[9-11] Using the automated imaging and cellular analysis pipeline, the EC_{50} of doxorubicin for the Caco2 and HeLa cell line in a 6-well plate format was determined. To establish an EC_{50} for doxorubicin, Caco2 or HeLa cells were seeded into 6-well plates (200 cells/well) in the presence of doxorubicin, or DMSO (vehicle control) with each concentration done in triplicates. After 7 days in culture, colonies were fixed, stained with Crystal Violet, and imaged with upright color brightfield microscopy. A subpopulation cellular analysis criterion was applied to consider only colonies within each well that reached an area cutoff that correlates to 50 cells. The area cutoff for Caco2 and HeLa was set to 1.03×10^5 mm^2 and 9.0×10^5 mm^2, respectively. The mean of qualifying colonies at each drug concentration was then plotted as a function of the log drug concentration and the EC_{50} was determined by fitting a 4-parameter dose-response curve. The doxorubicin EC_{50} for Caco2 and HeLa cells was determined to be 8.5 nM and 3.1 nM, respectively (Figure 4A and 4B).

High-throughput automated colony formation assay with upright color brightfield imaging

One disadvantage of performing the colony formation assay in a 6-well format is that deriving a multipoint drug titration requires multiple plates. Alternatively, a 96-well plate can accommodate the equivalent of sixteen 6-well plates. Successful recapitulation of an 11-point drug titration (vehicle control) across a 96-well microplate format allowed sample replicates to increase from 3 to 8. For the 96-well microplate format, Caco2 or HeLa cells were seeded at a density of 50 cells/well in the presence of doxorubicin, or DMSO (vehicle control) with 8 replicates of each concentration (one column per concentration). After 7 days in culture, colonies were fixed, stained with Crystal Violet, and imaged. The cellular analysis and subpopulation area cutoff parameters were set to the same values as for the 6-well format. The EC_{50} values derived in a 96-well microplate format are comparable to those obtained with the classic 6-well format: Caco2 = 7.5 nM and HeLa = 2.2 nM (Figures 4C and 4D).

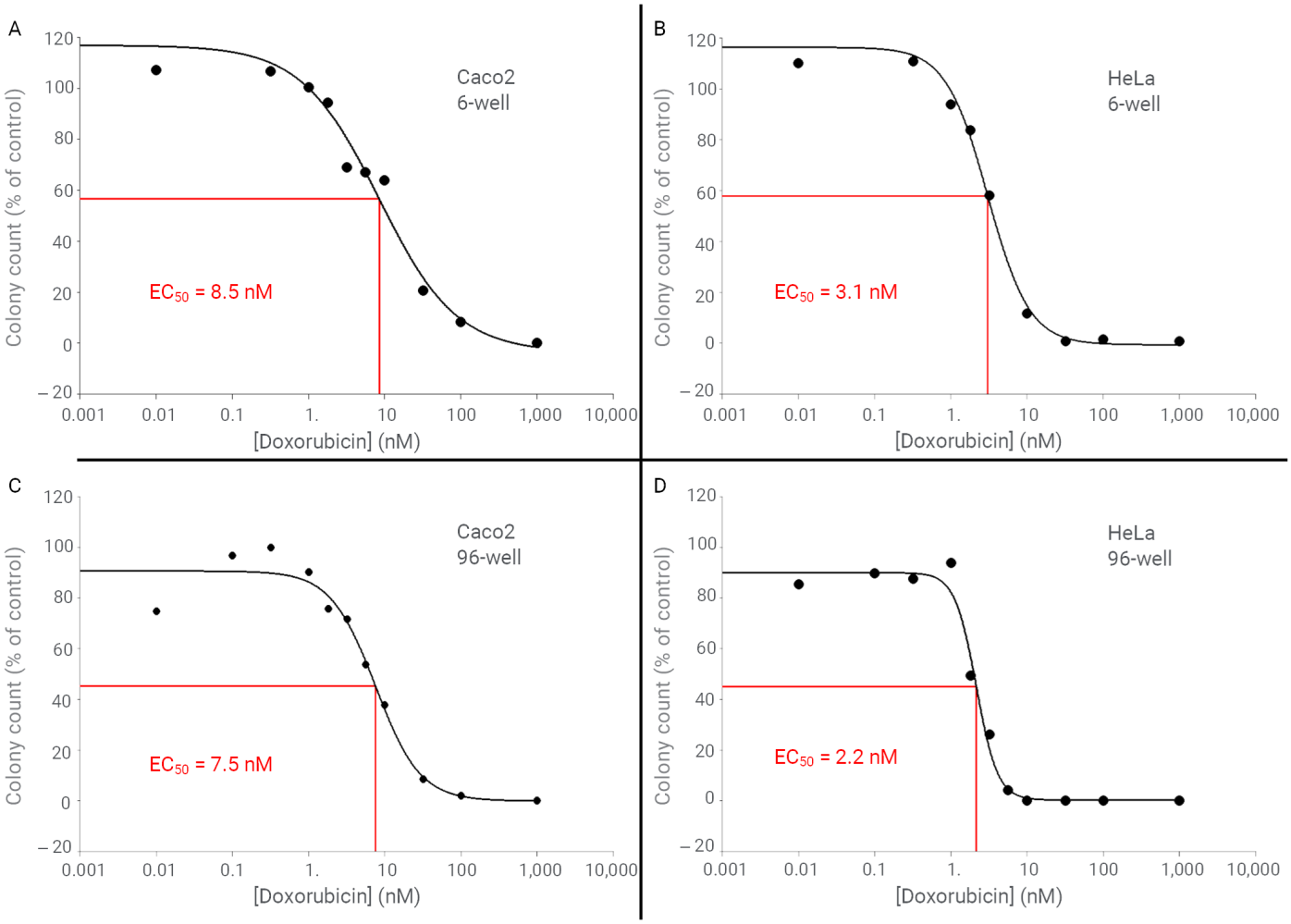

Figure 4. EC_{50} determination of doxorubicin in a 6- and 96-well format. The colony formation assay was conducted where Caco2 and HeLa cells were seeded in either 6-well plates (A and B) or 96-well microplates (D and C) and cultured for 7 days in the presence of increasing concentrations of doxorubicin. Automated upright color brightfield microscopy and cellular analysis of crystal violet-stained colonies was performed using the Agilent BioTek Cytation 7 cell imaging multimode reader with wide field of view. The EC_{50} of doxorubicin for Caco2 and HeLa cells cultured in 6-well plates was 8.5 nM and 3.1 nM, respectively, and for 96-well microplates, 7.5 nM and 2.2 nM, respectively.

Conclusion

The Agilent BioTek Cytation 7 cell imaging multimode reader with wide field of view enables automated upright brightfield imaging and analysis and is ideal for both 6- and 96-well microplates formats of the colony formation assay. In addition to providing a more quantitative image analysis pipeline, this automated format is exceptionally fast. The entire Augmented Microscopy workflow for a 96-well microplate from image capture to figure can be carried out in less than 5 minutes. An 11-point EC_{50} dose-response curve from samples spanning six 6-well plates can be built in 30 minutes, whereas the same sample set would take well over 4 hours if done manually (Table 2). In conclusion, a fast, quantitative, and reliable method to image and analyze the colony formation assay using the upright imaging power of the Cytation 7 cell imaging multimode reader with wide field of view has been developed. This method permits statistically robust data acquisition that is crucial for drug development applications such as cancer therapeutics.

Table 2. Augmented Microscopy timing for the colony formation assay in a 6- and 96-well format.

Format	Read	Stitching	Cellular Analysis	Total Time
96-Well Plate	2 min	–	1 min	3 min
6-Well Plate	3 min	1 min	1 min	5 min
6-Well Plates (6)	18 min	6 min	6 min	30 min
6-Well Plate (Manual) (6)	30+ min	–	>1 hour	At least 4 hours

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