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IN-VITRO ASSAY WITH HEPG2 CELLS ON THE CYRIS® ANALYSIS PLATFORM TO EVALUATE FERROPTOSIS AND APOPTOSIS

ABSTRACT

The following protocol describes the preparation, run and results of an in-vitro assay on the CYRIS® analysis platform to study ferroptotic or apoptotic processes based on the oxygen consumption rate (OCR) and morphological changes of a human hepatocarcinoma-derived cell line (HepG2). The automated CYRIS® platform measures OCR and acquires microscopic images of the cells label-free in parallel and in real time. With these parameters and optional down-stream analyses such as fluorescence staining and microscopy, it is possible to gain deeper insight into cell death processes.

HepG2 cells are measured for 12 hours without treatment for baseline, 24 hours under treatment with common ferroptosis or apoptosis inducers and/or inhibitors and additional 6 hours after treatment washout.

The results show that ferroptosis and apoptosis can be induced by different inducers such as Erastin or RSL3 (ferroptosis) and H_2O_2 (apoptosis). Furthermore, the ferroptosis inhibitor Ferrostatin blocks Erastin- and RSL3-induced ferroptosis. Microscopic images reveal different morphological changes of HepG2 cells after treatment with Erastin/RSL3 or H_2O_2. Correlations between the different cell parameters are clearly visible.

It is possible and may be necessary to adapt the protocol to other cell lines, cell numbers, and culture times. It is recommended to read the entire protocol once complete before the first test procedure to avoid

INTRODUCTION

A fine balance between new cell formation and death is required to maintain tissue homeostasis. To keep this balance, cells eliminate themselves by various cell death mechanisms such as apoptosis or necrosis. Distinct from this is ferroptosis, an iron-dependent form of cell death initiated and driven by the accumulation of lipid peroxides (Nirmala et al. 2019). Ferroptosis has been linked to a number of physiopathological processes and diseases such as Parkinson's disease, ischemia or several types of cancer (Mishra et al. 2018; Louandre et al. 2015; Eling et al. 2015). However, it is also associated with several common liver diseases including drug-induced liver injury (DILI) (Jing Wu et al. 2021). Although not all molecular mechanisms of ferroptosis are understood yet, key players in the initiation and progression of this form of cell death have already been identified (Song et al. 2018; Liu et al. 2018). To study this form of cell death, various inducers and inhibitors of ferroptosis have been developed. The most common inducers include Erastin and RSL3 (Cao and Dixon 2016; Hayano et al. 2016), both of which can be inhibited by the ferroptosis inhibitor Ferrostatin (Zilka et al. 2017). A better understanding of the molecular mechanisms of ferroptosis may contribute to the development of novel therapeutic strategies against proliferative and degenerative diseases.

Here we describe a protocol to study ferroptotic or apoptotic processes based on oxygen consumption rates and morphological changes of a human hepatocarcinoma-derived cell line (HepG2). Therefore, HepG2 cells were treated with common ferroptosis or apoptosis inducers and/or inhibitors. The assay is set up with a 12-hour pre-measurement without any active substance, a 24-hour treatment phase, and a 6-hour post-measurement.

ASSAY WORKFLOW

In preparation for the assay, the HepG2 cells are seeded into the sterile sensor plate and cultured for 5 hours under standard cell culture conditions. During this time, the CYRIS® analysis platform is set up for the assay. For this purpose, the deep-well plate (DWP) storage vessels for baseline/washout and treatment measuring are prepared with measuring media and the appropriate concentrations of ferroptosis or apoptosis inducers and/or inhibitors in measuring medium, followed by acclimatization in the CYRIS® climate chamber. The pipetting robot is equipped with sterile tips. After the cells have adhered sufficiently, the culture medium on the cells is replaced by fresh measuring medium, the special fluidic lid is closed, and the sensor plate is inserted into the platform. By starting the corresponding sequence program, the assay is performed automatically, and the acquired data is displayed in real time.

MATERIAL AND METHODS

Materials Required

Reagents

- DMEM high glucose
- Erastin (Biomol)
- Ferrostatin (Biomol)
- Fetal calf serum (FCS)
- Hydrogen peroxide solution (H_2O_2, 890.8 mM)
- Incyton measuring medium
- RSL3 (Biomol)

Devices and disposable materials

- Incyton CYRIS® flox
- Incyton metabolic sensor plate
- Pipettes and pipette tips (1-10 µl, 2-200 µl, 50-1000 µl)
- Standard cell incubator
- Vacuum pump, flexible tube, and glass pasteur pipettes
- 15 ml sterile conical tube
- 0.5 ml micro reaction tube

Method

Cell culture

HepG2 cells (a human hepatocarcinoma-derived cell line) are maintained in DMEM high glucose supplemented with 10 % FCS (culture medium). On the day of the assay, detach and count the cells. Seed a sample in 150 µl of culture medium containing 40,000 cells (266,666 cells/ml) into the wells (1-24) of a sterile sensor plate. Let the sensor plate rest in the workbench for 20 minutes to allow the cells to sediment evenly, then transfer the cells in the sensor plate to a standard cell incubator (37 °C, 5 % CO_2, 95 % humidity) for at least five hours to ensure proper attachment. It is possible and may be necessary to adjust the culture time to accommodate other cell types.

Reagents preparation

Prepare a stock solution of 10 mM Erastin in DMSO. Dissolve 1 mg Erastin in 182.8 µl DMSO, make aliquots of 20 µl and store the aliquots at -20 °C.
Prepare a stock solution of 10 mM RSL3 in DMSO. Dissolve 1 mg RSL3 in 182.8 µl DMSO, make aliquots of 20 µl and store the aliquots at -20 °C.
Prepare a stock solution of 38.1 mM Ferrostatin in DMSO. Dissolve 5 mg Ferrostatin in 500 µl DMSO, make aliquots of 10 µl and store the aliquots at -20 °C.

Prepare sterile measuring medium supplemented with 10 % FCS and prewarm to 37 °C in a water bath.

Preparation of DWP (Pos. 5) for the 12-hour baseline measurement and 6-hour post-measurement:
Fill each well of one sterile DWP with 7 ml warm measuring medium with 10 % FCS and place it directly into the CYRIS® incubator at position 5 for acclimatization. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP (Pos. 6) for the 24-hour treatment measurement:
Completely thaw an aliquot of the Erastin stock solution. Mix 28 ml of pre-warmed measuring medium containing 10 % FCS with 28 µl Erastin stock solution (10 mM) to create a final concentration of 10 µM Erastin in the measuring medium. Fill wells 8, 14 and 20 of a sterile DWP with 9 ml of this solution.
Completely thaw an aliquot of the RSL3 stock solution. Mix 28 ml of pre-warmed measuring medium containing 10 % FCS with 28 µl RSL3 stock solution (10 mM) to create a final concentration of 10 µM RSL3 in the measuring medium. Fill wells 10, 16 and 22 of a sterile DWP with 9 ml of this solution.
Completely thaw an aliquot of the Ferrostatin stock solution and prepare a pre-dilution of 381 µM Ferrostatin by diluting 2.3 µl Ferrostatin stock solution in 227.7 µl of PBS. Mix 28 ml of pre-warmed measuring medium containing 10 % FCS with 73.5 µl of the Ferrostatin pre-dilution to create a final concentration of 1 µM Ferrostatin in the measuring medium. Fill wells 12, 18 and 24 of a sterile DWP with 9 ml of this solution.

Mix 28 ml of pre-warmed measuring medium containing 10 % FCS with 28 µl Erastin stock solution (10 mM) and 73.5 µl of Ferrostatin pre-dilution to create a final concentration of 10 µM Erastin and 1 µM Ferrostatin in the measuring medium. Fill wells 9, 15 and 21 of a sterile DWP with 9 ml of this solution.
Mix 28 ml of pre-warmed measuring medium containing 10 % FCS with 28 µl RSL3 stock solution (10 mM) and 73.5 µl of Ferrostatin pre-dilution to create a final concentration of 10 µM RSL3 and 1 µM Ferrostatin in the measuring medium. Fill wells 11, 17 and 23 of a sterile DWP with 9 ml of this solution.
Mix 28 ml of pre-warmed measuring medium containing 10 % FCS with 15.7 µl H_2O_2 solution (890.8 mM) to create a final concentration of 500 µM H_2O_2 in the measuring medium. Fill wells 7, 13 and 19 of a sterile DWP with 9 ml of this solution.

Fill the wells 1-6 of a sterile DWP with 9 ml of warm measuring medium containing 10 % FCS for the untreated control group and place it directly into the CYRIS® incubator at position 6 for acclimatization. Cover it for transport from the sterile workbench to the incubator.

Prepare waste DWPs: Put empty DWPs in the CYRIS® incubator at positions 2 and 3 for wasted media.

Deep-well filling in list form:

Measured function	Substance	DWP position	Volume measuring medium for mix	Volume stock solution/ pre-dilution for mix	Wells to be filled with mix	Volume of mix per well
Baseline / post-measurement	None	5	168 ml	/	1-24	7 ml
Treatment	Erastin	6	28 ml	28 µl	8, 14, 20	9 ml
	RSL3	6	28 ml	2.8 µl	10, 16, 22	9 ml
	Ferrostatin	6	28 ml	73.5 µl	12, 18, 24	9 ml
	Erastin + Ferrostatin	6	28 ml	28 µl + 73.5 µl	9, 15, 21	9 ml
	RSL3 + Ferrostatin	6	28 ml	2.8 µl + 73.5 µl	11, 17, 23	9 ml
	H_2O_2	6	28 ml	15.7 µl	7, 13, 19	9 ml
	Untreated control	6	54 ml	/	1-6	9 ml
Waste	/	2, 3	/	/	/	/

Fluidic

Equip the robot head with 24 sterile pipette tips, center them using the pipette tip centering tool and attach it to the robot arm in the CYRIS® climate chamber.

RESULTS AND DISCUSSION

The test settings included a preincubation of HepG2 cells without any active substance for 12 hours, a treatment period with different inducers and/or inhibitors of ferroptosis and apoptosis for the next 24 hours and a post-measurement phase for 6 hours. After selecting and launching the appropriate program, the platform executed the test procedure autonomously. Throughout the experiment, the oxygen consumption rate of HepG2 cells was recorded every 20 minutes, as well as morphology based on regular microscope images.

Oxygen consumption rate

Raw measuring of OCR data can be exported directly to spreadsheet applications. This makes it possible to analyze the data according to one's own preferences. In this example, we evaluated OCR of HepG2 cells treated with different inducers and/or inhibitors of ferroptosis and apoptosis. We normalized each well to the point of treatment start (12 hours) and formed groups according to the distinct treatments. We calculated the mean value and standard deviation of OCR for each group and plotted them against time.

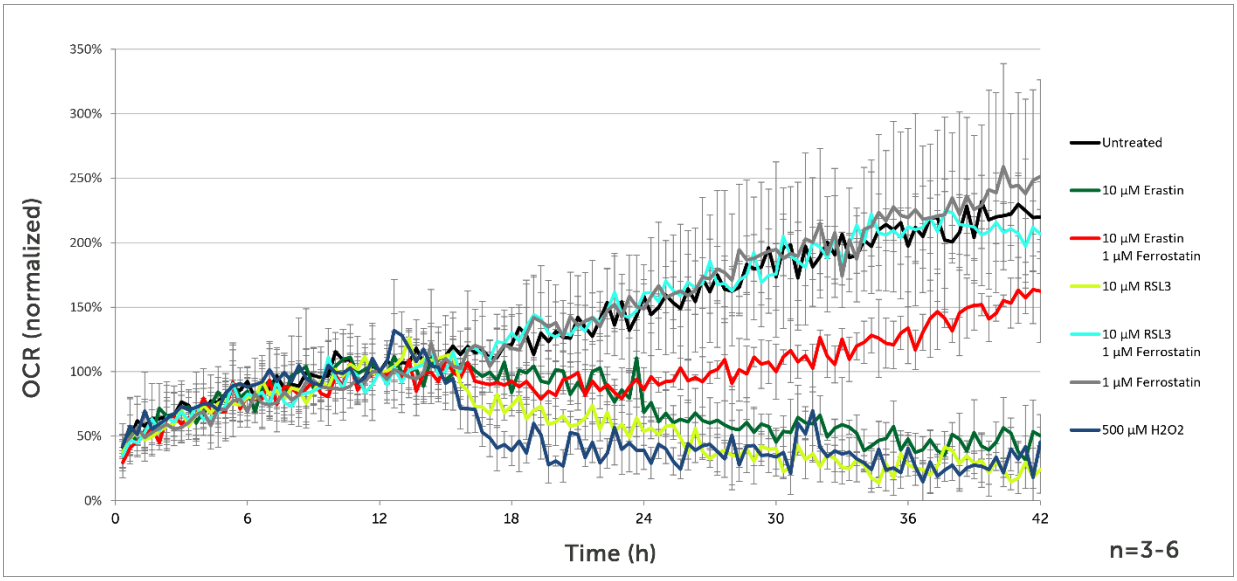

Figure 1. OCR of HepG2 cells treated with different inducers and/or inhibitors of ferroptosis or apoptosis.

Based on the oxygen consumption rates, it could be observed that HepG2 cells that were left untreated or treated only with the ferroptosis inhibitor Ferrostatin proliferated continuously during the entire experiment and the OCRs increased steadily. In contrast, the oxygen consumption rate of HepG2 cells treated with the ferroptosis inducers Erastin or RSL3 initially increased and then progressively decreased during treatment. This indicates that the cells first proliferated during the pre-measurement period and then entered cell death with the addition of the ferroptosis inducers. Interestingly, Erastin- or RSL3-induced ferroptosis could be prevented by simultaneous administration of the ferroptosis inhibitor Ferrostatin. When HepG2 cells were treated with Erastin and Ferrostatin in parallel, there was a moderate decrease in the oxygen consumption rate within the first 12 hours of the treatment.

Subsequently, the oxygen consumption rate increased steadily during the remaining treatment period and post-measurement phase. The co-treatment of HepG2 cells with RSL3 and Ferrostatin completely protected the cells from ferroptosis. In this case, the oxygen consumption rate reached the level of the untreated control group. The treatment of HepG2 cells with H_2O_2 induced rapidly apoptosis within a few hours after addition of H_2O_2. In this case, the oxygen consumption rate dropped quickly by about 60% in the first 6 hours of the treatment.

Imaging

Microscope images of all wells are taken every 20 minutes. This parallel imaging makes it possible to create time series and complements the metabolic data. In this example, the microscopic images of HepG2 cells treated with different inducers and/or inhibitors of ferroptosis or apoptosis at different time points are shown. In line with the OCR data, it can be seen in the imaging that untreated HepG2 cells or cells treated only with the ferroptosis inhibitor Ferrostatin or treated with RSL3 and Ferrostatin in parallel proliferated continuously during the entire experiment and did not show any signs of cell death. This indicates that the effect of the ferroptosis inducer RSL3 on HepG2 cells could be completely abolished by Ferrostatin. Based on the microscopic images, it can be seen that HepG2 cells co-treated with ferroptosis inducer Eratin and the ferroptosis inhibitor Ferrostatin also continuously proliferated during the entire experiment. However, compared to the group treated with RSL3 and Ferrostatin in parallel, these cells showed obvious morphological changes such as cellular inclusions (Figure 2, white arrows). This suggests that the ferroptotic effect of Erastin on HepG2 cells could not be completely reversed by Ferrostatin which is consistent with the metabolic data. Furthermore, microscopic images reveal that HapG2 cells treated with the ferroptosis inducers Erastin or RSL3 showed clear signs of cell death compared to the untreated control or the groups which were additionally treated with the ferroptosis inhibitor Ferrostatin. The cells were swollen and the membrane was disrupted after treatment with Erastin or RSL3 (Figure 2, black arrows). This was also reflected in the OCR data in terms of a steadily decreasing oxygen consumption rate. Interestingly, HepG2 cells treated with H_2O_2, which is known to induce apoptosis, also showed clear signs of cell death. However, compared to HepG2 cells treated the ferroptosis inducers Erastin or RSL3, a strong shrinking of the cells was observed due to the H_2O_2 treatment (Figure 2, green arrows). This was line with the OCR data revealing a fast decrease of the oxygen consumption rate of HepG2 cells within the first hours after treatment with H_2O_2.

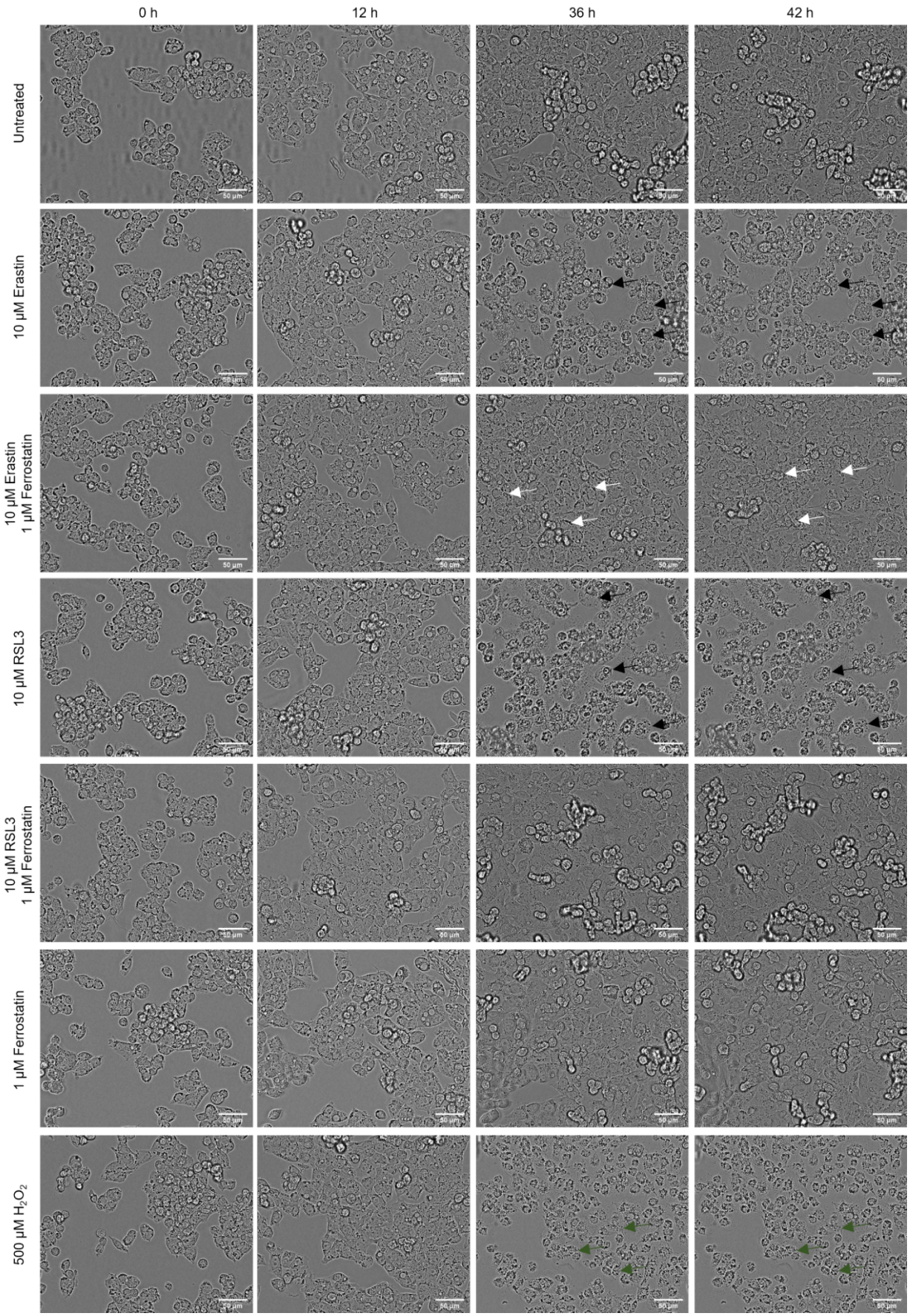

Figure 2. Microscopic images of HepG2 cells treated with different inducers and/or inhibitors of ferroptosis or apoptosis at the beginning of the measurement (0h), at the beginning of the treatment (12h), at the end of the treatment (36h) and at the end of the washout (42h).

CONCLUSION

This application note shows the exemplary performance of an in-vitro assay to investigate ferroptosis or apoptosis based on oxygen consumption rates and morphological changes of a human hepatocarcinoma-derived cell line (HepG2). Ferroptosis or apoptosis can be induced by different inducers such as Erastin or RSL3 (ferroptosis) and H_2O_2 (apoptosis). Ferrostatin is a ferroptosis inhibitor, which blocks Erastin- and RSL3-induced ferroptosis. Although HepG2 cells treated with RSL3 and Ferrostatin in parallel were completely protected from ferroptosis, but the ferroptotic effects induced by Erastin were only partially reversed by Ferrostatin. On the morphological level, different cell death effects on HepG2 cells after treatment with Erastin/RSL3 or H_2O_2 can be observed. A typical indication of apoptosis is cell shrinking, which could be observed after treatment with H_2O_2.
As possible downstream process, which might be requested by some users, you can show Chromatin condensation and DNA fragmentation, as typical signs in apoptotic cells, by Hoechst staining of the cell nuclei and fluorescence microscopy after a CYRIS® experiment (See INCYTONs protocol for post-experimental fluorescence cell staining in the sensor plate). Furthermore, Ferroptosis goes along with membrane rupture, lipid peroxidation, typical marker such as phosphorylated beclin-1 (Song et al. 2018), and smaller mitochondria, which can be analyzed by fluorescence staining and microscopy, too (See INCYTONs protocol for post-experimental fluorescence cell staining in the sensor plate).
The performed assay demonstrates the advantages of automated execution and multiparametric real-time detection on the CYRIS® platform, which can be enhanced by down-stream analyses.

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Published in Germany, June 2021, by:

INCYTON® GmbH

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