Application Note

Cancer Biology and Drug Discovery

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A Homogeneous FRET-Based HTS Assay for Quantification of pRb in Cancer Cell Lines to Monitor Inhibition of G_0/G_1 Cell Phase Transition

Author

Peter J. Brescia, MSc

Agilent Technologies, Inc.

Abstract

The field of cancer biology remains one of the most rapidly expanding areas of investigation in both industry and academia. Understanding disruptions that control pathways and checkpoints leading to uncontrolled tumor progression are key to understanding disease progression. Presented here is a novel HTRF cell-based assay that quantifies endogenous phosphorylated retinoblastoma protein as a readout of the G_0/G_1 cell phase transition.

Introduction

Cancer remains a global concern given the significant number of new cases reported annually. Recent advances in understanding the underlying biology of disease progression at the molecular level have proved beneficial for identification of new targets and agents against tumor cell growth.[1] A key factor of cancer progression in general is a disruption of cell cycle control leading to unobstructed cell proliferation. The ability to identify control pathways and checkpoints to target may provide points of intervention through novel therapeutics.[2]

During normal cell cycle control the distinct phases of the cell cycle are conserved progressing from G_0 (quiescence) followed by G_1 (preDNA synthesis), S (DNA synthesis), G_2 (predivision), and M (cell division). Of those, the progression from G_1 to S provides a sentry point that restricts cell proliferation via the interaction between the cyclin-dependent kinases (CDKs) and cyclin proteins.[3] One key role of a subgroup of CDKs, serine/threonine kinases, is the hyperphosphorylation of the retinoblastoma (Rb) gene product, pRb, in early G_1 by CDK4 and CDK6 interacting with cyclin D1 resulting in inactivation and the subsequent release of a number of transcription factors necessary for passage into S phase.[4] Previous observations suggest that CDK4/6 inhibition may prevent tumor growth and may help return cells to a near normal phenotype.[2]

This study demonstrates a novel HTRF cell-based assay that simply and accurately quantifies endogenous phosphorylated retinoblastoma protein at Ser807/811 as a readout of the G_0/G_1 cell phase transition. The dose response and IC_{50} concentration were determined for a representative kinase inhibitor in a high-throughput 384-well, homogeneous cell-based assay format using a representative cancer cell line.

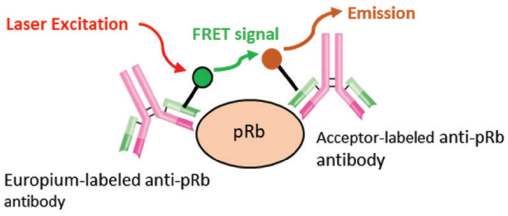

Figure 1. The HTRF assay. The HTRF proximity assay relies on two different specific antibodies targeting phosphorylated Rb. One antibody is labeled with a europium cryptate (donor) and the second with an acceptor. When the labeled antibodies come into close proximity, a FRET signal is detectable.

Materials and methods

HTRF Reader Control Kit (part number 62RCLPEA), Human TNF Alpha Kit (part number 62HTNFAPET) and Phospho-Rb (Ser807/811) Cellular Kit (63ADK105PEG) were from Cisbio (Bedford, MA). HCT116 cells (part number ATCC CCL-247) from ATCC (Manassas, MD) were cultured using standard tissue culture methods as per the manufacturers' recommendations. The phospho Rb assay was performed using the one-plate assay protocol. Briefly, the cells were harvested at 80 to 90% confluence using TrypLE dissociation reagent (part number 12605036) from Thermo Fisher Scientific (Waltham, MA) with gentle handling. The cells were collected by centrifugation and resuspended at the desired cell density in the appropriate growth medium followed by plating 10,000 cells in 8 µL into a small-volume, white, 384-well microplate (part number 3826) from Corning (Tewksbury, MA) and allowed to attach during incubation at 37 °C, 5% CO_2 overnight.

Palbociclib (part number 4786) from Tocris Bioscience (Minneapolis, MN) was prepared as a 3x stock as a half-log dilution series and added in a volume of 4 µL of the appropriate cell growth medium to the cells with mixing and incubated as above for six hours. Lysis reagent was prepared as per the manufacturers' recommendation and added in a volume of 4 µL with mixing for 1 hour. Antibody premix was prepared as per the manufacturers' recommendation for the single plate protocol. The antibody premix was added in a volume of 4 µL and incubated at room temperature overnight.

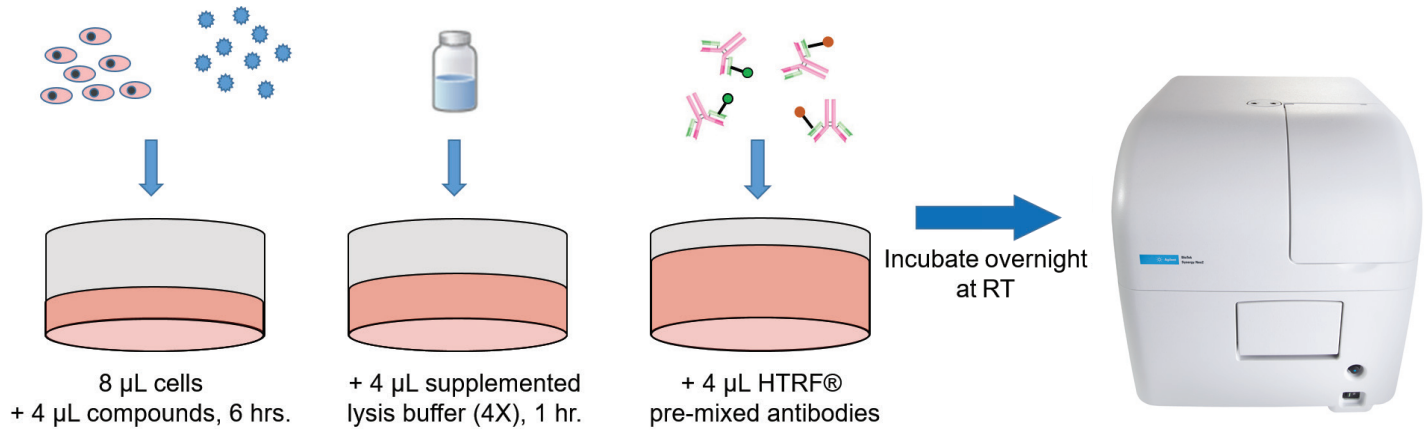

Figure 2. Phosho-Rb assay workflow.

Instrumentation

HTRF measurements for the Reader Control Kit and TNFα assay were taken using optimized parameters determined during reader certification. HTRF measurements for Rb detection were taken using the optimized settings shown in Table 1. Briefly, the Agilent BioTek Synergy Neo2 hybrid multimode reader was fitted with TRF laser reader cubes for Europium/Red acceptor (cubes 18 and 41). The read mode selected was TRF Laser in the top position to take advantage of the dual-PMT optical pathway. Dual photo-multiplier tube (PMT) gain settings were optimized using the auto-gain feature. Read height was set to 6.75 mm using auto adjust plate height calibration for use with a 384-well, small-volume plate. The remainder of the settings were based on parameters determined during performance of the HTRF reader certification assays.

Table 1. Agilent BioTek Synergy Neo2 hybrid multimode reader equipped with TRF laser cubes, with the following settings, was used to rapidly capture dual-emission HTRF measurements.

Agilent BioTek Synergy Neo2 Read Parameters	
Mode	Time-resolved fluorescence (laser)
Filter Sets	Dual PMT
Excitation/Emission	Ex 337/Em 620/665
Gain (PMT1, PMT2)	Auto
Read Speed	Normal
Delay After Plate Movement	0
Measurements Per Data Point	20
Read Height	6.75 mm
Dynamic Range	Standard
Light Source	TRF laser
TRF Parameters	
Delay	100 µsec
Data Collection Time	500 µsec

Results and discussion

Data normalization to account for cell plating differences was achieved using a calculated signal ratio by dividing acceptor signal by donor signal and multiplying the value times 10,000 for each well as seen in the following equation:

$$\text{Ratio} = \frac{\text{Signal}_{665\ \text{nm}}}{\text{Signal}_{620\ \text{nm}}} = \times 10^4$$

The Delta F (DF%) represents the signal-to-background of the assay using internal assay controls to compare day-to-day variability. The DF% is calculated by dividing the difference between the signal and background ratios by the background ratio as seen in the following formula:

$$\frac{\text{Ratio}_{\text{control or sample}} - \text{Ratio}_{\text{negative control}}}{\text{Ratio}_{\text{negative control}}} = \times 100$$

HTRF reader control kit

The HTRF reader control kit was used to validate laser parameters required to exceed the norms provided by Cisbio (Table 2). The data collected surpassed the typical norms of assay performance with as little as 1 flash with a standard 0 CV = 6.5%, low calibrator DF = 39%, high calibrator DF = 1,115%, and S/N = 412 (Table 3).

Table 2. HTRF reader control kit norms.

Norms	– Standard 0 Cv% ≤10% – Low calibrator ≥15% – High calibrator ≥550% – S/N ≥40

Table 3. Reader control kit performance data. Agilent BioTek Synergy Neo2 hybrid multimode reader fitted with laser was used in conjunction with an HTRF reader control kit for validation of reader parameters necessary to meet or exceed typical norms of assay performance.

	Reader Control Kit S/N Versus Laser Flashes (Low-Volume, White Plate)											
	1 Flash			5 Flashes			10 Flashes			20 Flashes		
Ratio	Mean	CV (%)	DF%	Mean	CV (%)	DF%	Mean	CV (%)	DF%	Mean	CV (%)	DF%
Std 0	2,188	6.5		2,378	3.33		2,378	2.8		2,405	2.8	
Low Calibrator	3,046	5.1	39	3,245	2.9	36	3,291	1.9	38	3,311	1.8	37
High Calibrator	26,576	3.3	1,115	27,985	1	1,077	27,968	0.8	1,060	27,892	0.8	1,052
S/N (620 nm/ blank)	412			378			381			406		

Human TNFα assay

The Human TNFα assay kit was used to validate the laser parameters required to meet or exceed assay norms for LOD seen below (Table 4). The Synergy Neo2 was able to surpass the typical assay norms with as little as 10 flashes with ratio CVs ≤2% and LOD = 6.6 pg/mL (Table 5 and Figure 3).

Table 4. Human TNFα assay norms.

Norm	– Detection limit (2SD) <12.5 pg/mL

Table 5. Human TNFα assay data.

	10 Flashes							
	665 nm		620 nm		Ratio			DF
	Mean	CV (%)	Mean	CV (%)	Mean	Std Dev	CV (%)	
Calibrator 0	34,285	4.4%	46,821	3.1%	7,320	141.59	1.9%	
Calibrator 1	38,768	6.1%	47,617	2.2.%	8,057	143.91	1.8%	10%
Calibrator 2	41,362	3.3.%	46,625	2.8%	8,871	159.75	1.8%	21%
Calibrator 3	47,525	3.1%	46,714	2.6%	10,173	150.35	1.5%	39%

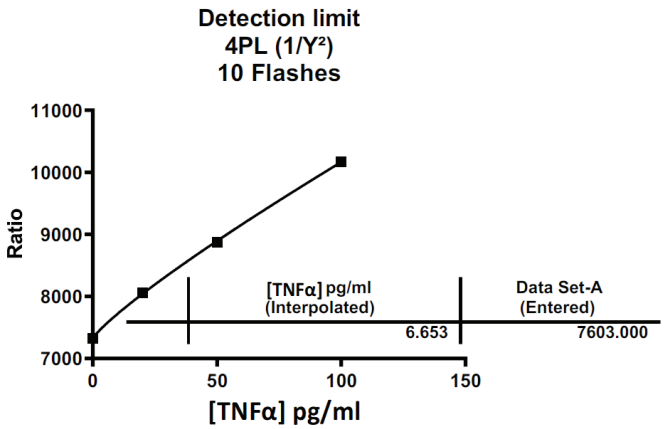

Figure 3. Human TNFα assay.

Phospho-Rb detection

Cells were plated as four replicates for each drug concentration in a high-throughput 384-well, solid white, small-volume microplate and allowed to attach overnight with incubation. An 11-point, half-log serial dilution series of the kinase inhibitor palbociclib, including a zero compound addition, was added to each well and allowed to incubate for six hours prior to addition of lysis reagent. The HTRF signal was read following a 1-hour incubation period at room temperature. The IC_{50} concentration was determined using a four-parameter dose-response curve fit in Prism software (GraphPad Software, Inc., La Jolla, CA) (Figure 4). The calculated IC_{50} value correlates well with previously reported data.[2]

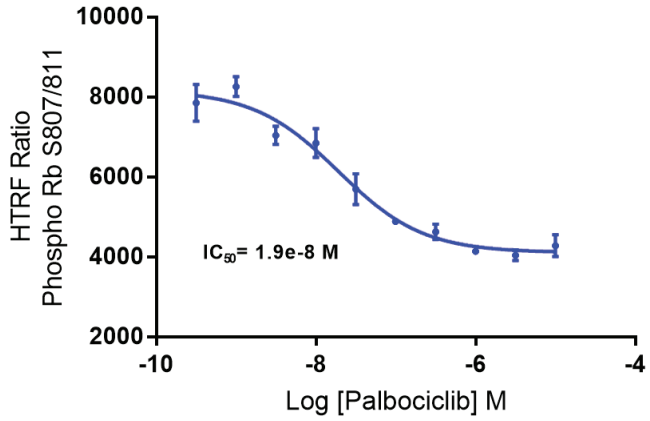

Figure 4. Inhibitor titration. Palbociclib dose response for HCT116 cells generated by the homogeneous, HTS one-plate protocol in a 384-well microplate.

The z'-factor was calculated using eight replicate measurement of ± palbociclib (10 µM). The assay resulted in z' factor of 0.63 indicative of very robust assay performance with low variability in a high-throughput microplate format (Table 6).

Table 6. Z'-factor.

Z'-Factor	
384-Well Assay	0.63

Conclusion

The Agilent BioTek Synergy Neo2 hybrid multimode reader configured with TRF laser and up to four PMTs allows high-throughput assay development and screening. A dose-response titration of palbociclib against a representative cancer cell line resulted in an IC_{50} value which correlates well with previously reported values.[2] The Synergy Neo2 provides rapid detection which is necessary for high-throughput assay formats. Read time was ~2m 42s for 384-wells with 10 flashes per well. The improved sensitivity provided by the laser excitation allows simplified workflows to be performed as indicated by the ability to execute the one-plate protocol in a 384-well assay format. The combination of assay and instrumentation provides an ideal solution for high-throughput detection for a variety of applications.

References

1. Finn, R. S. *et al.* PD 0332991, A Selective Cyclin D Kinase 4/6 Inhibitor, Preferentially Inhibits Proliferation of Luminal Estrogen Receptor-Positive Human Breast Cancer Cell Lines *In Vitro*. *Breast Cancer Research* **2009**, *11(5)*, R77. DOI: 10.1186/bcr2419.
2. Fry, D. W. *et al.* Specific Inhibition of Cyclin-Dependent Kinase 4/6 by PD 0332991 and Associated Antitumor Activity in Human Tumor Xenografts. *Molecular Cancer Therapeutics* **2004**, *3(11)*, 1427–1437.
3. Sage, J. *et al.* Targeted Disruption of the Three Rb-Related Genes Leads to Loss of G(1) Control and Immortalization. *Genes & development* **2000**, *14(23)*, 3037–3050. DOI: 10.1101/gad.843200.
4. Hsu, J. *et al.* E2F4 Regulates Transcriptional Activation in Mouse Embryonic Stem Cells Independently of the RB Family. *Nature communications* **2019**, *10(1)*, 2939. DOI: 10.1038/s41467-019-10901-x.

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