Evaluation of Cryopreserved Bacmam Transduced Aequorin Cells in 384- and 1536-Well Suspension Cell Assays Using the Aequorin Option for the FLIPR^{TETRA}® System

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Abstract

Cryopreserved cells as reagents in Aequorin based calcium flux assays decouples the tissue culture process from high throughput screening while improving overall assay performance[1]. The need for culturing cells in plates is eliminated. Combined with the FLIPR^{TETRA}® system equipped with Aequorin options, cryopreserved cells are a powerful tool in the identification of lead compounds in drug discovery. The suspension cell distribution capability of the FLIPR^{TETRA}® system enables uniform delivery of cells directly to read-plates for luminescent detection in up to 1536-well format. This study demonstrates performance of cryopreserved Bacmam transduced Aequorin cells in 384-well and 1536-well formats. Aequorin-based suspension assays with frozen cells demonstrate both instrument and cell performance during extended assays without significant shift in EC_{50} or Z' factor.

Introduction

Aequorin based calcium mobilization assays are highly compatible with cryopreserved cells when screening in suspension format. The FLIPR^{TETRA}® system suspension cell option provides an effective method to perform Aequorin assays over an extended period of time enabling robust HTS. An enclosure surrounds the spinner flask to protect Coelenterazine loaded cells from light. Cells are pumped to the reservoir for pipetting during the assay and the remaining cells are returned to the cell flask to ensure an even cell suspension and consistent results during a six hour screening period.

FLIPR^{TETRA}® Cell System Suspension Option

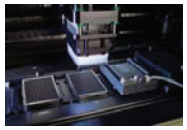


Figure 1. Cells are maintained in suspension in the external spinner flask until just prior to addition. At this time, cells are pumped into the user-installable cell reservoir located in source position 3 and then transferred to the read-plate using the system pipettor.

About Cryopreserved Bacmam Transduced Aequorin Cells

Cells were provided by GSK BRAD screening group, Stevenage, UK. Embryonic Kidney (HEK) cells, which stably express mitochondrial Aequorin, were cultured adherently and expanded into 6360cm² cell culture vessels. Cells were transduced to express the receptor of interest by the addition of Bacmam virus directly to the adherent cultures. Following a transduction period of 24 hours, cells were harvested and frozen to prepare 1 mL aliquots using standard GSK procedures.

Materials

1. Thawing media: DMEM/F12 (Invitrogen Cat# 11039-021) supplemented with 10% fetal bovine serum
2. Base buffer: Hanks' balanced salt solution without phenol red and sodium bicarbonate (Sigma Cat# H 8264 made up in sterile DI water, supplemented with 20 mM HEPES (Sigma Cat# H0887) and adjusted to pH 7.4 with NaOH.
3. Cell loading buffer: Base buffer supplemented with 0.1% Pluronic Acid F68 solution (Invitrogen Cat# 24040-032) and 0.1% BSA (Sigma Cat# A 3803)
4. Cell and compound diluent buffer: Base buffer with 0.1% Pluronic Acid F68
5. 500 µM Coelenterazine h stock: (Invitrogen Cat# C-6780) diluted in 100% ethanol and stored at -20°C protected from light

Methods

1. Cells were gently thawed, washed and the concentration was adjusted to 2.5 x10⁶/mL in loading buffer
2. Coelenterazine h was added at a final concentration of 5 µM and tubes were wrapped in foil to protect from light
3. Cells were loaded on a rotating mixer for 4-20 hours at room temperature (below 22°C for optimum Coelenterazine loading)
4. One hour before assay, cells were removed from the rotator and the concentration was adjusted as follows:
   a. 384-well suspension cell assays were performed at 2,500 cells/well in 20 µL
   b. 1536-well assays were performed at 2,000 cells/well in 3 µL
5. Cells were placed in the spinner flask under the cover on the suspension cell platform of the FLIPR^{TETRA}® and the stirring speed set to 7 (~60 RPM) for approximately 1 hour)
6. Read-plates were prepared with either an EC_{70} dose of reference compound or a concentration response curve (CRC) of reference compound using the AquaMax® DW4 dispenser
7. Plates were loaded onto the SynchroMax™ ET for FLIPR^{TETRA}® system and cycled into the instrument over time.
8. The FLIPR^{TETRA}® system added cells to the read-plate while detecting luminescent signal with the intensified camera option[2].

FLIPR^{TETRA} Suspension Cell Aequorin Assay Parameters

Read Mode	Luminescence		Luminescence	
Excitation/Emission				
Camera Gain	280,000		280,000	
LED Excitation				
Exposure Time	0.53 sec		0.53 sec	
Gate Open	100%		100%	
Read Interval	1 sec		1 sec	
Reads Before Pipetting	5		5	
Reads During first Interval	90		90	
	Fluid Transfer Settings			
	384-well		1536-well	
Single Aspirate, Single dispense	Aspirate	Dispense	Aspirate	Dispense
Cell Reservoir Rotation Speed	6	6	6	6
Source/Destination	Cell reservoir	Read plate	Cell reservoir	Read plate
Reservoir fill speed	5	-	5	-
Reservoir fill source	Cell flask	-	Cell flask	-
Reservoir drain speed	4	-	4	-
Reservoir drain destination	Cell flask		Cell flask	
Pipetting Volume	25 µL	25 µL	2 or 3 µL	2 or 3 µL
Pipetting Height	70 µL	10 µL	35 µL	0.5 µL
Pipetting Speed	20 µL/sec	50 µL/sec	5 µL/sec	10 µL/sec
Tip removal speed	10 mm/sec	10 mm/sec	10 mm/sec	10 mm/sec
Hold/Expel volume*	0	0	0	0
Pause in Well	-	0	-	0
Mix After dispense	-	None	-	None

* Using a hold/expel volume will cause an airgap resulting in a false luminescent signal

Table 1. Recommended setup parameters for luminescence assays using FLIPR^{TETRA}® intensified camera and cell suspension system.

Results

FLIPR^{TETRA}® System with Aequorin Option and Frozen Bacmam Transduced Aequorin Cells: Cell Titration and CRC Assay

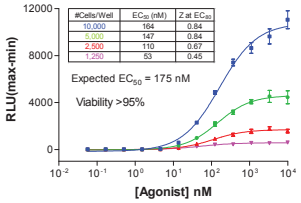
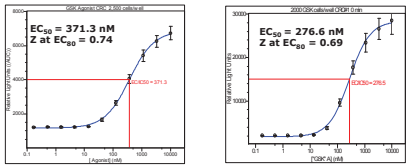


Figure 2. In a 384-well plate, cells were titrated against a CRC of target agonist. In this assay, cells were pre-plated in suspension prior to addition of compound.

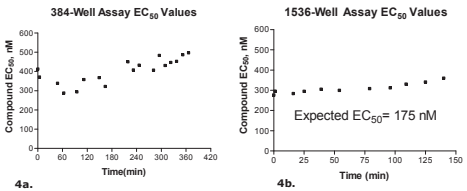
Comparison of EC_{50} Values Between 384- and 1536-Well Suspension Cell Aequorin Assays



3a. 3b.

Figure 3a. 384-well suspension cell assay at 2,500 cells/well. Figure 3b. 1536-well suspension cell assay at 2,000 cells/well. Both yield similar agonist EC_{50} values well within ½ log of the expected value.

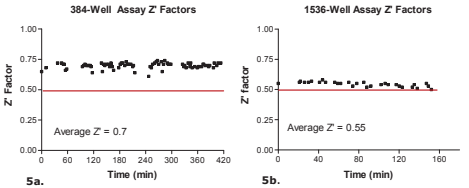
Change in EC_{50} During Frozen Bacmam Aequorin Suspension Cell Assays



4a. 4b.

Figure 4a. Reference compound EC_{50} values during a 6 hour 100 plate screening assay. The cell suspension system filled and emptied the reservoir 100 times. 384-well dose response plates were run every 5 cycles. During the assay, EC_{50} values remained within ½ log of expected value. Figure 4b. Compound A EC_{50} values during a similar 1536-well 2.5 hour 30 plate assay.

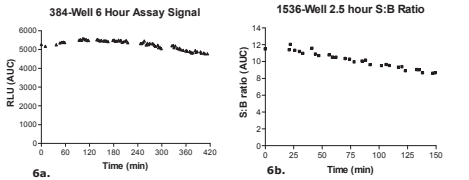
Comparison of Z' factor Over Time during Bacmam Aequorin Suspension Cell Assays



5a. 5b.

Figure 5. Recording of whole plate Z' factors during extended plate screening assays. A. 384-well plates contained 22 columns of EC_{80} reference compound and 2 columns of negative controls. Average Z' factor = 0.7. B. 1536-well plates contained 46 columns of EC_{80} reference compound and 2 columns of negative controls. Average Z' factor = 0.55.

Change in Signal Over time during Aequorin Suspension Cell Assays



6a. 6b.

Figure 6a. Change in RLU over time during 6 hour 384-well suspension cell experiment. Cells were added at 2,500/well. Figure 6b. Change in S:B ratio (auc) over time during 2.5 hour 1536-well suspension cell experiment. Cells were added at 2,000/well.

Summary

Cryopreserved Bacmam transduced Aequorin cell lines can be used to produce robust assay results in both 384-well and 1536-well formats on FLIPR^{TETRA}® instrument with Aequorin options. In both formats, EC_{50} values remain within one half log of expected results and there is little reduction in signal intensity over time. Average Z' = 0.7 in the 384-well 100 plate assay and average Z' = 0.55 in the 1536-well assay.

References

1. Wigglesworth, MJ, et. al., Use of Cryopreserved Cells for Enabling Greater Flexibility in Compound Profiling
J Biomol Screen, Jun 2008; 13: 354 - 362.

2. These assay methods are also described in the literature , e.g., in Boie et al., Eur. J. Pharmacology., 340(2-3):227-241 (1997), and in United States Patent 6,872,538 and European Patent 1,145,002. Users interested in the patented methods may wish to consult legal counsel in evaluating these patents.

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