invitrogen™ by life technologies™

BacMam Aequorin Cellular Assay User Guide

Catalog no. A13740 or A13741 **Shipping: Varies** **Storage: Varies**

Protocol part no. A13741.pps Rev. date: 02 Jun 2011

Table of Contents

Kit Contents and Handling

Component	SKU#	Amount	Storage	Handling
BacMam Aequorin Reagent	A13740 A13741	10 mL 100 mL	4°C	• Do not freeze • Avoid extended exposure to ambient room light • Use sterile technique • Aliquot into sterile containers to minimize handling, if necessary
BacMam Enhancer Solution (1000X)	PV6132 PV6133	400 µL 4000 µL	–20°C	Aliquot if necessary to avoid multiple freeze/thaw cycles

Overview

BacMam Aequorin utilizes BacMam technology to transiently deliver aequorin, a luminescent calcium sensor, into mammalian cells. BacMam technology is the use of a modified baculovirus (insect cell virus) to efficiently deliver and express genes in mammalian cells.

Aequorin

Aequorin, a luminescent calcium sensor, was originally isolated as the Fluorescent Resonance Energy Transfer (FRET) donor to GFP in the *Aequorea victoria* jellyfish and has been used extensively to monitor calcium changes in cultured, living mammalian cells.[1] More recently, aequorin has become a standard method for interrogating calcium second messenger pathways in a high-throughput screening format.[2] Upon binding of intracellular calcium ions, aequorin displays a "flash" style luminescence signal while consuming coelenterazine h as a substrate. Recently, aequorin has become a standard method for interrogating calcium second messenger pathways in high-throughput screening because luminescence signal from aequorin resolves any problems with autofluorescent compounds, as well as providing an exceptional signal-to-noise ratio.

References

1. Rizzuto, R. and Pozzan, T. (2006) *Physiol Rev* 86:369.
2. Brough, S.J. and Shah, P. (2009) *Methods Mol Biol.* 552:181.

BacMam Technology

In addition to the ready-to-use viral stocks, BacMam delivery technology has many advantages:

- High transduction efficiency across a broad range of cell types, including primary and stem cells
- Little to no microscopically observable cytopathic effects
- Highly reproducible and titratable expression
- Compatible with simultaneous delivery of multiple genes

Refer to Kost, T.A., et al. (2007) *Drug Disc Today* 12:396–403 for examples of BacMam gene expression in cells. For additional information on BacMam, visit **www.invitrogen.com/bacmam**.

Figure1 Illustration of Representative Aequorin Mechanism of Action. Aequorin Transduced cell lines are loaded with coelenterazine h (CTZ). Upon ligand-binding, intracellular calcium concentrations rise. The free calcium binds to the aequorin molecule leading to oxidization of CTZ to coelenteramide (CTA) producing CO_2 and emitting light.

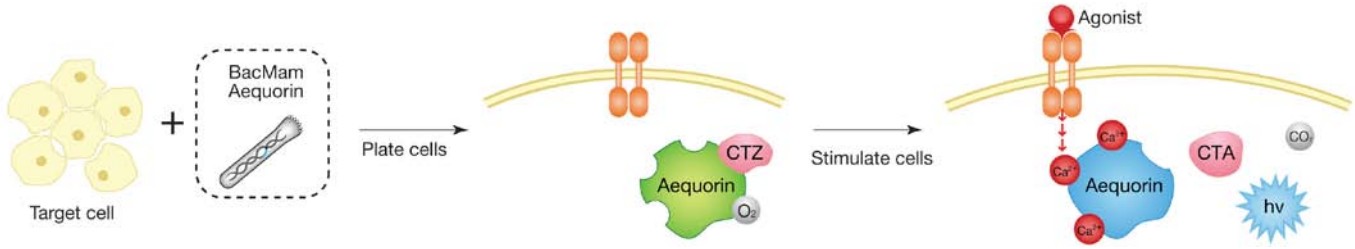
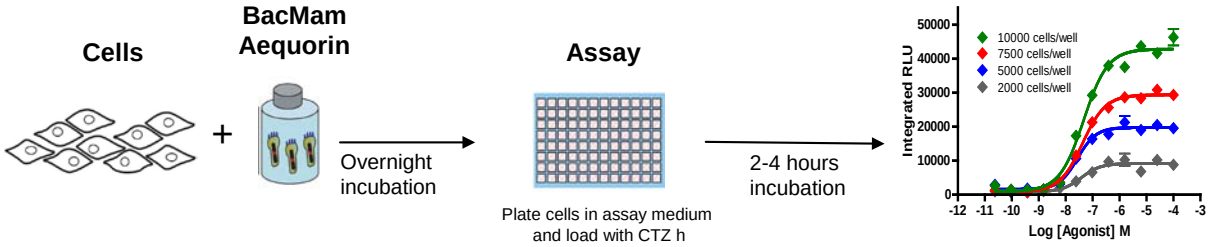


Figure 2 Illustration of Representative Assay Workflow. Cells are treated with the BacMam reagent encoding the aequorin protein and then plated in 384-well format. 24 hours post-transduction, the cells are loaded with coelenterazine h for 2–4 hours. Intracellular Calcium flux is analyzed on a Dispense and Imaging system equipped with luminescence read-out.

BacMam Aequorin Workflow



Before Starting

Materials Required but Not Provided

Materials	Recommended Source	Part No.
U-2 OS cells	ATCC	HTB-96
Cell line of interest	various	various
DMSO	Fluka	41647
Methanol	various	various
Coelenterazine h	Invitrogen	C6780
Black tissue culture-treated, 384-well assay plates	Corning	3712 (clear-bottom) 3571 (solid-bottom)
Dispense and Imaging system such as FLIPRTetra® system (with luminescence option), Hamamatsu FDSS 6000 or 7000 (with luminescence option), LumiLux®, LumiLux® CS, CyBi®-Lumax, Tecan GENios Pro™ or FLUOstar OPTIMA		

Reagents Required but Not Provided

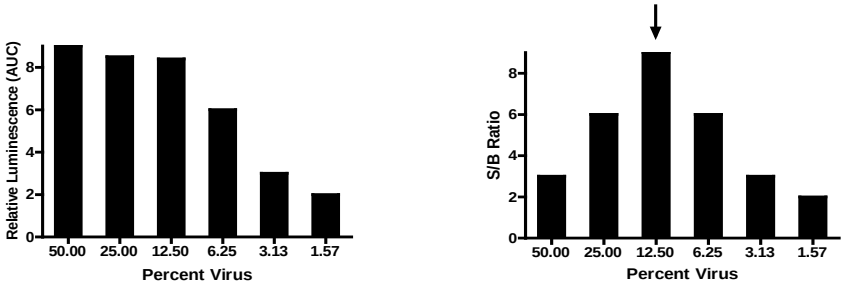
Media/Reagents	Recommended Source	Part No.
Positive Control : Receptor independent cellular calcium flux indicators such as:		
Ionomycin	Invitrogen	I24222
Calcium Ionophore A23187	Sigma	C7522
PMA	Sigma	P1585
Digitonin	Sigma	D141
Opti-MEM®-I Reduced-Serum Medium (with HEPES and L-glutamine, without Phenol Red)	Invitrogen	11058-021
Charcoal stripped Fetal Bovine Serum	Invitrogen	12676-011
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Invitrogen	11360-070
Penicillin-Streptomycin (antibiotic)	Invitrogen	15140-122
Dulbecco's Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}	Invitrogen	14190-136
Dulbecco's Phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}	Invitrogen	14040-133
Trypsin/EDTA	Invitrogen	25300-062
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010

Guidelines for Working with BacMam Reagent

- For first time users of BacMam reagent, we recommend including a control cell line that transduce exceptionally well, such as U2-OS (ATCC® number: HTB-96).
- Most cell types can be transduced efficiently using the U2-OS protocol described below.
- Some challenging cell types, such as CHO, require alternative protocols as described below.
- We recommend the following steps for optimizing the transduction and assay conditions for your cell background of interest.
- Performing optimization in small scale will minimize reagent consumption

Titration of BacMam Aequorin Reagent

We recommend performing a titration of the BacMam Aequorin Reagent to determine the optimal percentage of virus for the transduction in your cell background of interest. Select the lowest percentage of BacMam Aequorin reagent that yields the largest assay window (Signal/Background ratio). In the example below, 12.5% BacMam virus concentration would be recommended for further assay validation.



Quick Reference Protocols

The following agonist and antagonist mediated calcium flux protocols are generic to all cell lines. Conditions such as amount of BacMam Aequorin used for transduction, the number of cells per well and coelenterazine h loading time and temperature need to be optimized prior to screening test compounds. Ionomycin, Calcium ionophore A23187, PMA, or Digitonin can be used as a positive control for the receptor independent calcium flux assay.

Agonist Assay

		Unstimulated Control Wells	Stimulated Control Wells	Test Compound Wells	Positive Control (receptor independent calcium flux)
BacMam Transduction	**Step 1** Grow and transduce cells	• Grow cells in the appropriate Growth Medium to 80–90% confluence. • Remove media from the cells and add the diluted BacMam virus. • Incubate at room temperature or 37°C.			
	Step 2 Add Enhancer and incubate cells	• *Optional*: Remove virus and add Growth Medium plus 1X Enhancer solution, if required. • Incubate the plate at 37°C/5% CO_2 for 16–20 hours.			
Seeding and coelenterazine H loading	**Step 3** Harvest cells	• Harvest cells. • Wash once with Assay Medium.* • *Optional*: The cells can be cryopreserved and stored in liquid nitrogen for future use.			
	Step 4 Plate cells	• Plate 30 µL/well cells in Assay Medium with 5 µM coelenterazine h at optimal cell density. • Quick spin the plate at 300 rpm for 1 minute.			
	Step 5 Load coelenterazine h	Incubate the plate at 37°C/5% CO_2 for 5 hours			
Calcium Flux Assay	**Step 6** Prepare agonist	Prepare 4X compound dilution in Assay medium			
	Step 7 Add ligand on-line, read plate, and analyze data	Add 10 µL/well of 0.2% DMSO in Assay medium	Add 10 µL/well of 4X Positive control compound in Assay medium	Add 10 µL/well of 4X Test compound in Assay medium	Add 10 µL/well of 4X Positive compound in Assay medium

*** Assay Medium:** OptiMEM®-I with 1% cdFBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/100 µg/mL Streptomycin

Antagonist Assay

		Stimulated Control Wells	Control Compound wells	Test Compound Wells
BacMam Transduction	**Step 1** Grow and transduce cells	• Grow cells in Growth Medium to 80–90% confluence. • Remove media from cells and add the diluted BacMam virus. • Incubate at appropriate temperature.		
	Step 2 Add Enhancer and incubate cells	• *Optional*: Remove virus and add Growth Medium plus 1X Enhancer solution, if required. • Incubate the plate at 37°C/5% CO_2 for 16–20 hours.		
Seeding and coelenterazine H loading	**Step 3** Harvest cells	• Harvest cells. • Wash once with Assay Medium.* • *Optional*: The cells can be cryopreserved and stored in liquid nitrogen for future use.		
	Step 4 Plate cells	• Plate 30 µL/well cells in Assay Medium with 5 µM coelenterazine H at optimal cell density. • Quick spin the plate at 300 rpm for 1 minute.		
	Step 5 Load coelenterazine h	Incubate the plate at 37°C/5% CO_2 for 5 hours		
Calcium Flux Assay	**Step 6** Prepare antagonist	Prepare 4X compound dilution in Assay medium		
	Step 7 Add antagonist on-line	Add 10 µL/well of 0.2% DMSO in Assay Medium	Add 10 µL/well of 4X control antagonist in Assay Medium	Add 10 µL/well of 4X Test compound in Assay Medium
	Step 8 Incubate	Incubate the plate at 37°C/5% CO_2 for 10–15 minutes		
	Step 9 Add ligand on-line, read plate, and analyze data	Add 10 µL/well of 5X agonist in Assay Medium		

* **Assay Medium:** OptiMEM®-I with 1% cdFBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/100 µg/mL Streptomycin

Detailed Protocol for BacMam Aequorin Assay

The following provides detailed protocols for BacMam transduction, as well as agonist and antagonist assays.

Note: For first time users, it may be useful to work with 6-well plates to minimize reagent consumption during the transduction optimization process.

Day 1. BacMam Transduction

A. Cells types that are easy-to-transduce by BacMam (e.g., U-2 OS cells)

In this protocol, cells are incubated with virus at the time of plating.

1. Begin with cells grown to near confluence under normal growth conditions.

Note: For many cell types, such as U-2 OS, a cell seeding density of ~30,000 cells/cm^2 for 3 days with a harvest density of ~0.6 × 10^5 –0.8 × 10^5 cells/cm^2 is optimal.

2. Perform six 2-fold serial dilutions of the BacMam reagent in growth medium resulting in 50%, 25%, 12.5%, 6.2%, 3.1%, and 1.5% (v/v) aliquots. Use 3 mL of diluted BacMam reagent per well of a 6-well plate.
3. Remove media from cells and add the serial diluted BacMam virus. A typical final concentration of BacMam reagent is 1.5%–50% (v/v).
4. Incubate the cells in a 37°C/5% CO_2 incubator for 20–24 hours to allow for the transduction and expression of the Aequorin protein.

B. Cells types that are hard-to-transduce by BacMam (e.g., CHOK1 cells)

In this protocol, cells are allowed to adhere to the tissue-culture vessel before transduction with BacMam reagent.

1. Begin with cells grown to near confluence under normal growth conditions (e.g., CHO cells should be grown to 80–90% confluence, about 0.8 × 10^5–1.2 × 10^5 cells/cm^2).
2. Perform six 2-fold serial dilutions of BacMam reagent in PBS with Ca^{2+} and Mg^{2+} resulting in 50%, 25%, 12.5%, 6.2%, 3.1%, and 1.5% (v/v) aliquots.
3. Remove media from cells and add the serial diluted BacMam virus.
4. Incubate at room temperature for 3–4 hours in the dark.
5. Remove the BacMam reagent from the cells and add growth medium plus 1X Enhancer solution.
6. Incubate the cells in a 37°C/5% CO_2 incubator for 20–24 hours to allow for transduction and expression of the Aequorin protein.

C. Primary cells or stem cells (e.g., HUVEC, HASMC, rat neurons, human astrocytes, rat neural derived stem cells)

In this protocol, cells are pre-seeded before incubation with virus, usually 24–48 hours prior to re-plating onto the assay plate.

1. Begin with cell cultures grown to near confluence under normal growth conditions. Seed cells such that the monolayers will be approximately 50–80% confluent once attached and spread. Avoid plating the cells such that 80% confluence is exceeded 24 hours post-transduction.

Note: For first time users, it may be useful to work with 6-well plates to minimize reagent consumption during the transduction optimization process.

Tip: For most cell lines (with a doubling time of approximately 24 hours), a seeding density of 2×10^4–4×10^4 cells/cm^2 is optimal for BacMam transduction. It may be desirable to optimize the cell density for specific cell backgrounds.

2. After incubating the cells for 6–24 hours, add the desired amount of BacMam reagent to the cells. For initial optimization, we recommend testing a final concentration of BacMam reagent of 1.5%–50% (v/v).

3. Add Enhancer Solution at a 1X final concentration.

Note: For untested cell backgrounds, we recommend performing the transduction in the presence and absence of Enhancer Solution and then analyzing the expression of the BacMam target.

4. Place cells in a humidified 37°C/5% CO_2 incubator for 16–24 hours to allow for the transduction and expression of the Aequorin protein.

Day 2. Plating cells for assay

1. Harvest the transduced cells, being careful not to over-trypsinize the cells as this can result in poor viability and a decreased assay window.

Note: At this stage, cells can be cryopreserved in appropriate cryo-media and stored in liquid nitrogen for future use. We recommend using Recovery™ Cell Culture Freezing Medium

2. Resuspend the harvested cells in growth medium with serum to inactivate the trypsin. Centrifuge the cells at 200 × *g* for 5 minutes. Aspirate the growth medium and resuspend the cell pellet in assay medium (usually low serum). We recommend using Opti-MEM®-I with 1% cdFBS , 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/100 µg/mL Streptomycin

3. Centrifuge the cells at 200 × *g* for 5 minutes. Aspirate the assay medium and resuspend the cell pellet in assay medium at the desired density.

Tip: The number of cells per well will affect the assay window and can be optimized. We recommend starting with 5,000–20,000 cells per well seeded in 30 µL of assay medium with 5 µM coelenterazine h. Therefore, resuspend cells to 0.15×10^6–0.6×10^6 cells/mL.

4. Plate 30 µL of transduced cells in assay medium into black tissue culture-treated 384-well plates* and incubate the plates in a 37°C/5% CO_2 incubator (or appropriate) for approximately 5 hours.

   *A clear-bottom plate is recommended when using instruments with bottom-read capabilities and a solid-bottom plate is recommended when using instruments with top-read capabilities.

Tip: To optimize coelenterazine h loading time and temperature, testing 2, 4, 6, 8 and 24 hour loading at 37°C and room temperature is recommended. Some cell lines can tolerate an overnight incubation before significant signal intensity is lost.

5. Proceed with the following agonist or antagonist assay setup.

Agonist Assay Setup

Calcium assays are best read with on-line addition of the compounds.

1. Prepare a stock solution of 0.2% DMSO in assay medium.

Note: If you are using a solvent other than DMSO for the agonist, change the solvent used in the control wells accordingly. Be careful to keep the amount of solvent consistent in all wells.

2. Prepare 4X control agonist in assay medium.

Note: For best results perform a dose response curve to determine the EC_{100} for your control agonist solution.

3. Prepare 4X test compound in assay medium (if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 4X solution is 0.2%).

4. Prepare 4X ionomycin (40 µM) (or appropriate positive control compound like Digitonin, Calcium ionophore A23187, or PMA) in assay medium (used as positive control for receptor independent calcium flux).

5. Add 20 µL of assay medium with 0.4% DMSO to each unstimulated control well on the Ligand plate.

6. Add 20 µL of the 4X control agonist in assay medium to each stimulated control well on the Ligand plate.

7. Add 20 µL of the 4X test compound in assay medium to each test compound well on the Ligand plate.
8. Use this Ligand plate for online addition of 10 µL of solution to the Assay plate.
9. Proceed to Calcium flux assay on your Image and Dispense imaging instrument.

Antagonist Assay Setup

1. Prepare a stock solution of 0.2% DMSO in assay medium.

Note: If you are using a solvent other than DMSO for the agonist, change the solvent used in the control wells accordingly. Be careful to keep the amount of solvent consistent in all wells.

2. Prepare a 4X control antagonist in assay medium.

Note: For best results perform a dose response curve to determine the IC_{100} for your control inhibitor solution.

3. Prepare a 4X test compound in assay medium (if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 4X solution is 0.2%).
4. Add 20 µL of the assay medium with 0.2% DMSO to each unstimulated and stimulated control well on the Ligand plate.
5. Add 20 µL of the 4X control antagonist in assay medium to each control compound well on the Ligand plate.
6. Add 20 µL of the 4X test compound in assay medium to each test compound well on the Ligand plate.
7. Use this Ligand plate for online addition of 10 µL of ligands or DMSO control to the Assay plate.
8. Incubate the Assay plate at 37°C/5% CO_2 for 10–15 minutes.
9. Prepare a 4X stock of agonist serial dilutions in assay medium.

Note: For best results perform a dose response curve to determine the EC_{80} for your agonist solution.

10. Add 20 µL of the assay medium to each unstimulated control well on the Ligand plate.
11. Add 20 µL of 5X stock of known agonist to each stimulated control and test compound well on the Ligand plate.
12. Use this Ligand plate for online addition of 10 µL of agonist or DMSO control to the Assay plate.
13. Proceed to Calcium flux assay on your Image and Dispense imaging instrument.

Reading the Assay Plate and Data Analysis

Due to the "flash" type luminescence of aequorin, a luminometer with liquid handling injectors is needed. Depending on the instrument being used the measurements will be taken at room temperature and read from the top or bottom of the wells (Refer your instrument manual for top or bottom read capabilities of the instrument).

1. Let the assay plate warm to room temperature before reading, if necessary.
2. Follow the manufacturer's instructions for operating the plate reader. Two key requirements for aequorin read-out are
   (i) A sensitive detector/camera that enables luminescence read-out
   (ii) Emission filter at 465 nm.
3. Typically a calcium flux assay is performed by first collecting a baseline reading for 5-10 seconds before initiation of calcium flux via compound addition.
4. Add 10 µL of compound to 30 µL cells in the plate. The aequorin signal should appear immediately after compound addition and will only last for a few seconds.
5. Monitor the luminescent signal for 60 seconds post compound addition.
6. Data should be processed by computing the integrated value or area under the curve and plotting that value versus compound concentration.

Troubleshooting Guide

Observation	Potential Solutions
No response in calcium assay.	Confirm that your instrument is configured appropriately for detection of aequorin luminescence settings. For the bottom read instrument, be sure to use clear-bottom microtiter plates. For the top-read instruments, use solid-bottom microtiter plates.
	Perform a virus titration to find the optimal virus concentration for your cell background.
	Confirm that no contamination of the BacMam reagent has occurred.
	For first-time users, we recommend the standard transduction protocol using U-2 OS cells at 80–90% confluence (see page 7 for protocol). Overly confluent or unhealthy cells will not transduce efficiently.
Very low assay window or high background	Try serum starving the cells for 4 hours up to overnight.
	Perform a virus titration to find the optimal virus concentration for your cell background.
Following transduction with the BacMam reagent, cells have a rounded/unspread phenotype and appear to be in poor health.	Under low serum conditions, many cell types will appear rounded when imaged 24 or 48 hours post-transduction. This is common among cell types such as HEK293 and CHO. Under these conditions, cells are capable of mobilizing calcium, and should remain adhered despite poor cell spreading.
Day-to-day fluctuations in assay window are observed.	Be sure to use cells with the same growth conditions (e.g., same harvest density).

Purchaser Notification

Limited Use Label License: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use this product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License No. 21: Bac-to-Bac® and Bac-to-Bac® HT

This product is sold under patent license from Monsanto for research purposes only and no license for commercial use is included. Requests for licenses for commercial manufacture or use should be directed to Director, Monsanto Corporate Research, 800 N. Lindbergh, St. Louis, Missouri 63167.

Limited Use Label License No. 306: Baculovirus Vectors

This product is for research use only by those researchers in laboratories of academic, government, industrial and/or clinical institutions engaged in the investigation of biological or biochemical processes, or research and development of biological products. This product is not to be used in the manufacture, use or sale of human or animal diagnostic, therapeutic or prophylactic products.

Limited Use Label License No. 308: WPRE Element

This product contains the Woodchuck Post-transcriptional Regulatory Element ("WPRE") which is the subject of intellectual property owned by The Salk Institute for Biological Studies, and licensed to Life Technologies Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; and/or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. In addition, any use of WPRE outside of this product or the product's authorized use requires a separate license from the Salk Institute. Life Technologies will not assert a claim against the buyer of infringement of patents owned by Life Technologies and claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product or for a Commercial Purpose. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008, Phone (760) 603-7200. Fax (760) 602-6500, or The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, Attn.: Office of Technology Management, Phone: (858) 453-4100 extension 1275, Fax: (858) 546-8093.

Limited Use Label License No. 332: BacMam Virus Use

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer solely in accordance with the accompanying product literature or manual. Purchase of this product does not convey a license to expand, amplify, or otherwise propagate the provided viral particles or to otherwise modify or alter the virus by any means.

Trademarks

The trademarks mentioned herein are the property of Life Technologies Corporation or their respective owners.

ATCC and HTB-96 are trademarks of ATCC.
FLIPR Tetra is a trademark of Molecular Devices, Inc.
LumiLux is a trademark of PerkinElmer LAS, Inc.
CyBi is a trademark of CyBio AG.
Lumax is a trademark of Monobind, Inc.