Guava Technologies

4600-0990
rev A

Guava® EasyCyte™ CellGrowth™ Kit

Cat. No. 4500-0270
200 tests

For the measurement of primary cell proliferation

Research Use Only
Not for Use in Diagnostic Procedures

1. PRODUCT DESCRIPTION AND INTENDED USE

Analysis of cell function is essential for investigations in oncology, pharmacology, infectious disease, and other areas of medical research. One of the functions of the immune system is to respond to mitogenic stimuli. In this regard, naïve or resting lymphocytes, which are exposed to a foreign antigen or chemical stimulus, are triggered to proliferate and differentiate into an active state with distinct regulatory or effector functions.[1]

Cellular proliferation has traditionally been measured using a ^{3}H-thymidine incorporation assay although recent work suggests this approach might underestimate the number of cells capable of proliferating due to radiation induced cell cycle arrest and apoptosis.[2] Alternative assays, including MTT[3] and AlamarBlue™[4] among others, have been used recently due to concerns about the handling and disposal of radioactive materials. Another approach to measure cell division involves the detection and quantification of proliferated cells by flow cytometry. Live and dead proliferated and resting cells can be distinguished through the use of differential staining by fluorescent dyes.[5-8]

The Guava CellGrowth assay uses two dyes, a cell permeant painting dye and a cell impermeant DNA binding dye, to distinguish live or dead proliferated cells from live or dead resting cells. The cell painting dye, carboxyfluorescein diacetate succinimidyl ester or CFSE (also known as CFDA SE), diffuses freely into cells where intracellular esterases cleave off the acetate groups, converting it to a fluorescent, membrane impermeant dye. The CFSE dye is equally distributed between daughter cells due to covalent crosslinking to proteins through its succinimidyl groups. The stain is long lived, allowing the resolution of at least three or four cycles of cell division. A membrane impermeant DNA intercalator, propidium iodide (PI), is then added to distinguish the live from the dead cells.

2. MATERIALS PROVIDED

Guava CellGrowth Kit (Cat No 4500-0270) includes the following reagents:

- Guava CFSE (Part No. 4700-0130, 100 µg)
- CellGrowth PI (Part No. 4700-0190, 1 mL)
- Anhydrous DMSO (Part No. 4300-0160, 0.5 mL)

3. HANDLING AND STORAGE

1. Always store Guava CFSE reagent (either powder or in solution) at (minus) –10° to –30°C in a desiccator.
2. Avoid repeated freezing and thawing of the reconstituted Guava CFSE reagent. Guava recommends that the reagent be aliquotted into smaller volumes to eliminate multiple freeze thaw cycles.
3. The Guava CFSE reagent is light sensitive. Shield from excessive exposure to light.
4. Store Guava CFSE reconstituted in DMSO at (minus) –10° to –30°C for up to 3 months. After 3 months, Guava CFSE reconstituted in DMSO is no longer stable.
5. Store the CellGrowth PI reagent at 2–8ºC and shield from excessive exposure to light.
6. Refer to expiration dates on component labels. Guava recommends using the Guava CFSE and CellGrowth PI before the expiration dates.

4. WARNINGS AND PRECAUTIONS

1. The Guava CellGrowth Kit is intended for research use only.
2. All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Avoid specimen contact with skin and mucous membranes
3. If using a frozen aliquot of Guava CFSE, make sure it is completely thawed before use by repeat pipetting.
4. Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
5. Avoid microbial contamination of the solutions, which may cause erroneous results.
6. Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product.
7. Dispose of all materials using proper precautions in accordance with federal, state, and local regulations.
8. Material Safety Data Sheets (MSDS) for kit reagents are available from our web site (www.guavatechnologies.com) or by contacting Guava Technologies Technical Support (support@guavatechnologies.com telephone 1-866-448-2827 or 510-576-1400).
9. The Guava CellGrowth Kit contains DMSO, which may transport toxic compounds directly through the skin. Avoid prolonged or repeated exposure.
10. The Guava CellGrowth PI reagent contains dye that may be carcinogenic and/or mutagenic. Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic and mutagenic reagents.

5. EQUIPMENT AND MATERIALS REQUIRED

- Guava EasyCyte system with CytoSoft™ software containing the CellGrowth assay module (0500-1300)
- Guava CellGrowth Kit (Cat. No. 4500-0270)
- Primary cell suspension such as peripheral blood mononuclear cells (PBMC)
- 1X sterile phosphate buffered saline (PBS), pH 7.2 to 7.4, or equivalent
- Sterile PBS/BSA—1X PBS supplemented with 0.1% (w/v) BSA (Sigma Cat. No. A-7284 or equivalent)
- Micropipettors, single- or multi-channel as needed
- Disposable micropipettor tips
- 8-channel microplate aspirator (VWR Cat. No. 29443-120 or equivalent)
- 96-well microplates, flat bottom (Falcon Cat. Nos. 353075 or 353915, or equivalent). Refer to the *Guava EasyCyte System User's Guide* for other compatible flat bottom microplates.
- Sterile reagent reservoirs, 55 mL (VWR Cat. No. 20901-921 or equivalent)
- Vortex mixer
- Disposable gloves
- Centrifuge with 96-well microplate holders
- 37°C CO_2 incubator
- Brown vials, 0.5 mL (VWR Cat. No. 20170-724 or equivalent)
- Guava ViaCount® reagent (Cat. No. 4000-0040) or Guava ViaCount Flex™ (Cat. No. 4500-0110), optional
- Deionized water
- Guava ICF™ Instrument Cleaning Fluid (Cat. No. 4200-0140), optional
- 20% bleach solution

6. REAGENT AND SAMPLE PREPARATION

Recommended Assay Conditions: Guava recommends using 25 x 10^6 cells/mL when painting cells. This corresponds to approximately 250 tests at 500 cells/µL in 200 µL for each mL of cells painted. If a lower concentration of cells is used per test, more tests can be performed; and if a higher concentration is used per test, fewer tests can be performed.

The Guava CellGrowth assay should be set up in flat bottom 96-well microplates to allow sufficient surface area for cell growth.

Time Considerations: Preparation of cells prior to painting them could take from 15 minutes (if just lysing and washing whole blood) to 2 hour (if isolating PBMC from whole blood) or longer (if purifying subsets of lymphocytes). Painting the cells with Guava CFSE requires about 1 hour. The incubation to stimulate cell growth of the painted cells could require between 3 days and 2 weeks, depending on the type of stimulus used. Once the incubation is complete, PI is added to the wells and after a 5-minute incubation, the samples are ready to acquire on the Guava EasyCyte. Acquiring data on the Guava EasyCyte system usually takes about 30 seconds per sample, but depends on the final cell concentration.

Reagent Preparation

2 mM Stock Guava CFSE Painting Solution

1. Add 89.7 µL anhydrous DMSO to the vial containing 0.1 mg of Guava CFSE.
2. **Mix thoroughly by repeatedly inverting the vial for 2–5 minutes.**

   IMPORTANT NOTE: The amount of solid Guava CFSE per vial is small and may be hard to see.

   NOTE: To recover all reconstituted CFSE, Guava recommends spinning the Guava CFSE vial for 10 seconds in a mini-microcentrifuge or 1 minute at 200 x g in a tabletop centrifuge.
3. Aliquot 10 to 45 µL of the resuspended Guava CFSE into brown vials and store at (minus) –10 to –30°C desiccated until needed.

   NOTE: Guava CFSE is stable for 3 months at (minus) –10 to –30°C when reconstituted in DMSO. Avoid more than two freeze/thaw cycles of the reconstituted Guava CFSE since the solvent will take up moisture and cause the dye to break down.

0.5 mM Working Guava CFSE Painting Solution

Prepare a 0.5 mM working solution using the freshly reconstituted or thawed 2 mM stock Guava CFSE painting solution.

IMPORTANT NOTE: If using a frozen aliquot of Guava CFSE, ensure it is completely thawed before use by repeat pipetting.

1. Pipette 50 µL of the 2 mM stock CFSE Painting Solution into a sterile 0.5-mL vial.
2. Add 150 µL sterile PBS and mix.

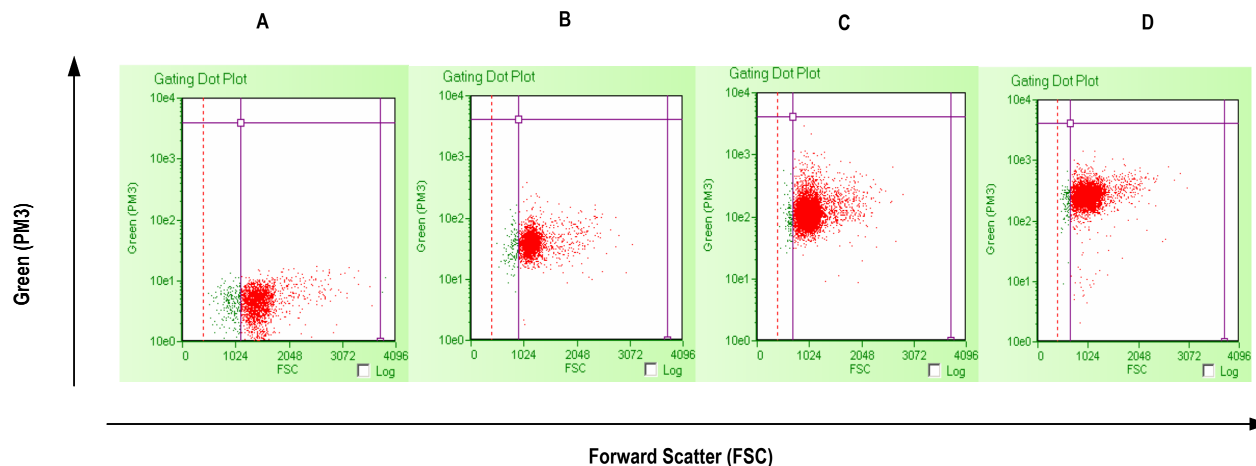

Figure 1 Titration of Guava CFSE.
A) This plot shows unpainted cells. For determining the optimal Guava CFSE concentration, the instrument was adjusted so that the unpainted cells were evenly distributed between 10e0 and 10e1 on the Green (PM3) scale.

B) This plot shows painted cells when the Guava CFSE concentration was too low (MFI ratio of 10). While there is a separation between the painted (B) and unpainted (A) populations, it is not optimal.

C) This plot shows painted cells when the Guava CFSE concentration was correct (MFI ratio of 40). Notice there is a very distinct separation between the painted (C) and unpainted (A) populations.

D) This plot shows painted cells when the Guava CFSE concentration was too high (MFI ratio of 64). While the separation here is even better than that shown in (C), this concentration of Guava CFSE (5 µM) was slightly toxic to cells.

IMPORTANT NOTE: Add PBS that contains no protein. Protein in the PBS will cause incomplete painting of the cells.

IMPORTANT NOTE: Prepare the 0.5 mM working solution immediately before staining, otherwise the dye will deteriorate.

Cell Preparation

Many different sources of primary lymphocytic cells can be used. Prepare leukocytes, peripheral blood mononuclear cells (PBMCs), or purified lymphocyte subsets following your laboratory's standard protocols.

Determining Optimal Cell Painting Concentration

Each cell preparation might require slightly different optimal painting concentrations. We have found that 2.5 µM Guava CFSE works best for human PBMCs. However, if you need to determine the optimal concentration for painting your cells, it is recommended to start with a concentration of between 1 and 5 µM Guava CFSE and titer the reagent either up or down as appropriate from there in steps of 0.5 or 1 µM. Optimal results are obtained when there is a 25- to 50-fold difference in the mean fluorescence intensity (MFI) between the painted cells and unpainted populations, and ensuring that the painted population is not saturating the detector. While the separation will be even better at higher concentrations of CFSE, the reagent at those concentrations might be toxic to the cells and thus inhibit their proliferation. See Figure 1 for examples of correct and incorrect cell painting patterns.

Cell Painting Protocol

For PBMCs

1. Prepare primary cells according to your laboratory's protocols. Cells should be thoroughly resuspended in either PBS or the buffer or medium that had been used in the last preparation step.
2. Count cells using Guava ViaCount reagent (for Guava PCA™ system) or Guava ViaCount Flex (for Guava EasyCyte or PCA-96 system). Refer to Guava ViaCount or Guava PCA-96 ViaCount Flex package insert for suggested protocols.
3. Centrifuge the cells at 300 x g for 5 to 7 minutes.
4. Aspirate the supernatant being careful not to disturb the cell pellet.
5. Add 1 mL PBS containing 0.1% BSA per 25 x 10^6 cells.
6. Gently resuspend cells by repeat pipetting. Avoid generating bubbles.

   NOTE: Painting the cells at 25 x 10^6 cells/mL is highly important to ensure consistent results with Guava CFSE. Changes in the cell concentration can change the amount of dye needed for optimal painting.
7. Reserve 1 mL of unpainted cells as a negative control. Treat these cells similarly to those being painted with CFSE, but do not add CFSE to this sample.
8. Add Stock Guava CFSE Painting Solution, as determined from your staining optimization study, to the tube containing the cells to be painted and mix gently. See Table 1 for suggested volumes.

Table 1. Volume (in µL) of 0.5 mM working Guava CFSE Painting Solution per Volume of Cells (in mL)

Final Guava CFSE Concentration (µM)	For 0.4 mL of cell suspension (100 tests)	For 0.8 mL of cell suspension (200 tests)	For 1.6 mL of cell suspension (400 tests)
1	0.8	1.6	3.2
2.5	2	4	6.4
4	3.2	6.4	12.8

NOTE: Assumes a cell concentration of 25 x 10^6 cells/mL.

9. Incubate cells at 37°C for 15 minutes in a tightly capped tube in the near horizontal position.
10. After incubation add 10 mL of complete medium containing at least 10% serum to the tube and mix gently. Incubate for 10 minutes at room temperature.

    NOTE: It is important to use medium with at least 10% serum to quench any remaining unbound dye.
11. Centrifuge the cells at 300 x g for 5 to 7 minutes.
12. Aspirate the medium.
13. Add 10 mL complete medium and resuspend by repeat pipetting.
14. Centrifuge the cells at 300 x g for 5 to 7 minutes.
15. Aspirate the medium.
16. Repeat steps 13 to 15 two more times for a total of three washes with complete medium.
17. Add 10 mL complete medium and resuspend the cells by repeat pipetting.
18. Count cells using Guava Viacount or Guava ViaCount Flex.
19. Adjust the cell concentration to 1 x 10^6 per mL with complete medium.

Stimulation

1. Set up in-vitro cell culture with different concentrations of mitogen, antigen, or other stimulant as desired. Add 100 µL per well of the diluted stimulant or 100 µL per well of medium only.
2. Dispense 100 µL or 1 x 10^5 cells from each cell suspension (unpainted or painted) into the appropriate wells, vortexing the cells just prior to dispensing.

   NOTE: Frequently change pipet tips to avoid cross contamination of samples with different stimulants and different labeled cells.
3. Incubate the plate for an appropriate length of time to stimulate proliferation in a humidified, 37ºC, 5% CO_2 incubator. For example, maximal responses to mitogens can occur within 3 to 4 days whereas maximal responses to antigens might require 7 to 10 days

Dead Cell Staining

1. After incubation, remove the plates from the 37°C CO_2 incubator.
2. Add 5 µL of the Guava CellGrowth PI reagent to each well and mix by repeat pipetting.

   NOTE: Maintain the sterility of the Guava CellGrowth PI reagent by dispensing the reagent in a laminar flow hood using sterile pipets.
3. Incubate the samples in the dark at room temperature for 5 minutes.

Samples are now ready for acquisition on the Guava EasyCyte.

Samples are stable for up to 7 hours at room temperature in the dark after the addition of the PI.

7. EXPECTED RESULTS

The Guava CellGrowth application displays acquired data with multiple options. Data can be displayed in one of two gating dot plots showing either FSC vs Green (PM3) or FSC vs Red (PM2), and an analysis dot plot showing Green (PM3) vs Red (PM2) or an analysis histogram showing Green (PM3). The results are displayed on the computer screen after each sample is acquired. During acquisition, the gate in the gating dot plot acts as a counting gate—when the number of events within the gate reaches the events to acquire, acquisition is

completed. This allows you to acquire a constant number of cells. During analysis, events to be analyzed can be gated to remove undesired debris or dead cells using the Gating Dot Plot. Events above the FSC threshold, falling within the rectangular gating region are displayed and analyzed on the Analysis dot plot. If **Enable Gating** is turned off (box is unchecked), all of the events collected above the FSC threshold are displayed and analyzed in the Analysis dot plot.

The Guava EasyCyte system calculates and presents results automatically in the results table. Results include the % of Live Cells Proliferating, as well as the Count (number of events falling in the indicated Dot Plot region), Cells/mL, % of Total (or, if gating is enabled, % of Gated), MFI values for PM3 and PM2 and FSC intensity for Live Proliferating, Live Resting, Dead Stimulated, and Dead Resting cells. The results table also displays the Gated Events (the number of events that are within the gate when enabled), Total Count, % Inside the Gate, and % Outside the Gate.

Analysis Results - Dot Plot

% of Live Cells Proliferating: 64.20 %

	Count	Cells/mL	% Gated	PM3 MFI	PM2 MFI	FSC Int
Proliferating Cells (LL)	1182	4.36e05	59.10	11.96	4.44	1822.83
Resting Cells (LR)	659	2.43e05	32.95	104.95	4.64	1554.96
Dead Resting Cells (UR)	93	3.43e04	4.65	167.31	143.72	1673.33
Dead Stimulated Cells (UL)	66	2.44e04	3.30	13.97	190.65	1586.61

Gated Events	Total Count	% Inside Gate	% Outside Gate
2000	2159	92.64	7.36

Figure 2 Analysis Results table in Guava CellGrowth software.

All data and instrument settings are automatically saved in FCS 3.0 file format. You can select the option to export data to a FCS 2.0 file. This allows you to analyze Guava files with other FCS 2.0 compatible programs.

Guava CellGrowth assay results can vary with the cell type and assay conditions used. Figure 3 and Figure 4 illustrate results that were obtained using the Guava CellGrowth Kit. In these examples, the cell proliferation cannot be detected in the unstimulated culture (Figure 3), whereas approximately 50% of the cells proliferated upon activation with 0.1875 µg/mL PHA (Figure 4).

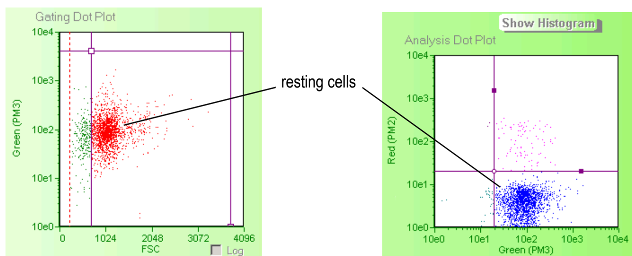

Figure 3 Painted, unstimulated sample.

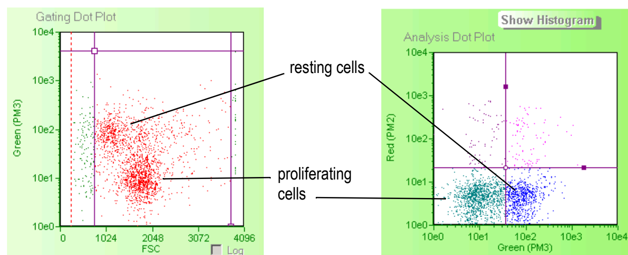

Figure 4 Painted, stimulated sample.
Painted, PI-negative, live, proliferating cells appear in the lower-left quadrant (teal green); painted, PI-negative, live, resting cells appear in the lower-right quadrant (blue); painted, PI-positive, dead, resting cells appear in the upper-right quadrant (pink); and painted, PI-positive, dead, stimulated cells appear in the upper-left quadrant (purple).

8. TROUBLESHOOTING TIPS

1. Dim or false negative staining obtained with the Guava CellGrowth Kit may indicate reagent degradation. Verify that the reagents are not past their expiration dates before using.
2. Dim staining may also be sign that the cells were not healthy enough to be properly painted. Be sure cells are healthy prior to painting.
3. Make sure that the frozen reagents are completely thawed and well mixed before using.
4. Acquire samples soon after addition of the PI dye. Although results were shown to be stable up to 7 hours post-PI addition, extended storage may adversely affect your results. Minimize exposure of the stained samples to light.
5. If the concentration of the stained cell sample for data acquisition is too low (<10 cells/µL), the accuracy of the results may be compromised. The experiment may need to be repeated at a higher cell density.
6. Be sure that samples are properly resuspended prior to acquisition. Check that the mixing option has been selected in the WorkEdit program when creating the Worklist file. Cells in the sample will settle quickly and your Guava CellGrowth results (percent of proliferating cells, MFIs, etc.) will be inaccurate unless the Guava EasyCyte mixes each sample prior to acquisition.
7. If the concentration of the stained cell sample for data acquisition is high (>500 cells/µL), the Guava EasyCyte systems will most likely not yield accurate results. Dilute the sample further with additional media to bring the cell concentration into an acceptable range.
8. The default number of events to acquire is 2000. You may input a different number; however, your statistical error will increase as you decrease the number of events for acquisition.
9. Run Guava Check™ (Guava Check Bead Kit Cat. No. 4500-0020) to verify proper instrument function and accuracy.
10. A Quick Clean will be performed at the end of every Worklist when using the Guava EasyCyte system. If your samples contain significant amounts of cellular debris that might build up in the flow system and cause a clog, you may want to select more frequent Quick Cleans from the WorkEdit program. Alternately, if your samples contain significant amounts of cellular debris, run Quick Clean with Guava ICF followed by water, to prevent clogs or blockage.
11. If you are acquiring data from a sample but the Cell Count number is not increasing and the acquisition progress bar is not moving, there is probably either insufficient volume to continue to acquire sample, or a blockage of the flow system. Check first for the lack of sufficient sample volume (on the Guava EasyCyte system, you must first pause the CytoSoft application and eject the tray). If the sample volume is below 50 µL, there is not enough sample for the instrument to acquire. Either add additional media to bring the sample volume up to greater than 50 µL, or proceed to the next sample. If the sample volume is more than 50 µL, then the lack of events acquired is probably due to a clog. A clog or blockage of the flow system can be caused by cell aggregates, cell debris, bleach crystals, or other particulates. Click **Pause**, then **Backflush** to flush out the clog into a tube containing 20% bleach. Then run Quick Clean to remove bleach residue. If this procedure does not alleviate the problem, consult the *Guava EasyCyte System User's Guide* or contact Technical Service for additional help.
12. For more troubleshooting tips, refer to the *Guava EasyCyte System User's Guide* or contact Technical Support.

9. LIMITATIONS

The Guava EasyCyte Systems yields optimal results when the stained cell sample for data acquisition is between 1 x 10^4 to 5 x 10^5 cells/mL. To obtain the most accurate assay results, adjust the concentration of the cell samples to within the recommended range.

10. REFERENCES

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