CyQUANT® Cell Proliferation Assay Kit

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
CyQUANT® GR dye (Component A)	550 µL	400X in DMSO	• ≤–20°C * • Desiccate • Protect from light	When stored as directed, kit is stable for 6 months.
Cell-lysis buffer (Component B)	11 mL †	20X		
λ DNA standard	100 µL	100 µg/mL solution in TE (Tris/EDTA) buffer		

***** For short-term storage, components may be stored at 2–6°C, for convenience. **†** A 50 mL volume of this component is also available separately (C7027).

Number of Assays: The kit supplies sufficient material for performing 1,000 assays, using the protocol described in this manual. The 200 µL assay volume is suitable for fluorescence detection in microplates. The assay may be scaled up to 2 mL for detection in standard cuvettes; however, the number of assays possible will be reduced proportionally.

Approximate fluorescence excitation/emission maxima: 480/520 nm for CyQUANT® GR dye bound to nucleic acids.

Introduction

The CyQUANT® Cell Proliferation Assay Kit (C7026) provides a convenient, rapid, and sensitive procedure for determining the density of cells in culture. The assay has a linear detection range extending from 50 or fewer to at least 50,000 cells in 200 µL volumes using a single dye concentration (Figure 1). By increasing the dye concentration used in the assay, the linear range can be extended to 250,000 cells. The assay is ideal for cell proliferation studies as well as for routine cell counts and can be used to monitor the adherence of cells to surfaces. The CyQUANT® kit can detect much lower cell numbers than Neutral Red or methylene blue assays.[1-3] Unlike procedures that rely on the conversion of tetrazolium dyes to blue formazan [4] products or ^{3}H thymidine incorporation assays,[5] the CyQUANT® method is rapid and does not rely on cellular metabolic activity. Thus, cells can be frozen prior to assaying; time-course assays are facile and data obtained from samples taken at widely different time intervals can be directly compared.

The basis for the CyQUANT® kit is the use of a proprietary green fluorescent dye, CyQUANT® GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Frozen cells are simply thawed and lysed by addition of a buffer containing the CyQUANT® GR dye; fluorescence is then measured directly. There are no washing steps, growth medium changes, or long incubations. Also, unlike tetrazolium-conversion assays, serum components do not appreciably interfere with the assay. Our researchers have found that widely disparate cell types, including mouse fibroblasts (NIH 3T3 and CREBAG 2 cells), normal human umbilical vein endothelial cells (HUVEC), canine kidney cells (MDCK), chinook salmon embryo cells (CHSE), rat basophilic leukemia cells (RBL), rat glioma cells (C6), and mouse myeloma cells (P3X63A68) can all be reliably assayed using the CyQUANT® procedure. CyQUANT® GR dye may also prove useful for supplanting ^{51}Cr release measurements for monitoring

T-cell–mediated cytolysis and other cytolytic events.[6] Along with the CyQUANT® GR dye and concentrated cell-lysis buffer, the kit also includes a DNA solution to serve as a reference standard for assay calibration.

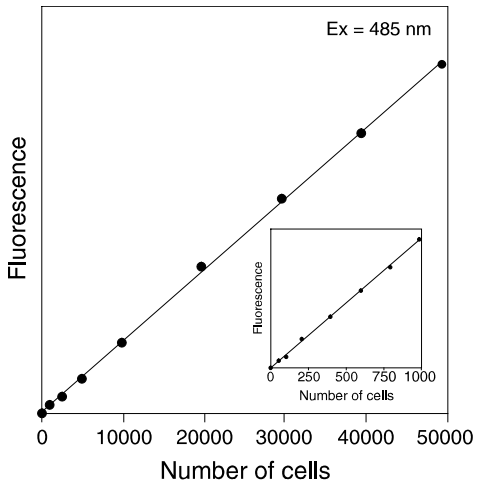

Figure 1. Quantitation of 3T3 fibroblasts using the CyQUANT® Cell Proliferation Assay Kit. Fluorescence measurements were made using a microplate reader with excitation at 485 ± 10 nm and emission detection at 530 ± 12.5 nm. The linear range of the assay under these conditions is from 50 to 50,000 cells per 200 µL sample.

Before You Begin

Caution

We must caution that no data are available addressing the mutagenicity or toxicity of CyQUANT® GR dye. Because this reagent binds nucleic acids, it should be treated as a potential mutagen and handled with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. Please dispose of dye in accordance with local regulations.

Preparing the Reagent

Allow the CyQUANT® GR stock solution in DMSO to warm to room temperature before opening the vial.

On the day of the experiment, dilute the concentrated cell-lysis buffer stock solution (Component B) 20-fold in distilled water. For each assay, 200 µL will be required. Just prior to running the experiment, dilute the CyQUANT® GR stock solution (Component A) 400-fold into the 1X cell-lysis buffer. For example, to prepare 20 mL of CyQUANT® GR working solution (enough for ~100 assays), first make the 1X cell-lysis buffer by mixing 1 mL of the 20X stock with 19 mL of nuclease-free distilled water; next add 50 µL of the CyQUANT® GR stock solution and mix thoroughly. Using this dilution of the CyQUANT® GR dye, the assay has a linear detection range extending from about 50 to 50,000 cells per microplate well. By increasing the dye concentration, the linear range can be extended up to about 250,000 cells.

Note: The linear detection range can be extended by using the CyQUANT® GR dye at a final concentration higher than 1X. For example, for detecting up to 100,000 cells in a 200 µL volume, use the CyQUANT® GR dye at 2X final concentration; dilute the CyQUANT® GR stock solution 200-fold into 1X cell-lysis buffer. For detecting up to 250,000 cells in a 200 µL volume, use the CyQUANT® GR dye at a 5X final concentration; dilute the CyQUANT® GR stock solution 80-fold into 1X cell-lysis buffer.

We recommend preparing the diluted solution in a plastic container, rather than in glass; the CyQUANT® GR reagent may adsorb to glass surfaces. Protect the working solution from light by keeping it in an opaque bottle, covering it with foil, or placing it in the dark to prevent photodegradation of the CyQUANT® GR dye. For best results, the solution should be used within a few hours of its preparation.

Creating a Cell Number Standard Curve

A reference standard curve can be created for converting sample fluorescence values into cell numbers. The cell type used for the standard curve should be the same as that which is used in the experiment. It is possible to assay either suspension cells or adherent cells, however, the latter must first be detached and suspended by treatment with trypsin. Note that some adherent cells are sensitive to trypsinization and some cell lysis might ensue.

1.1 Prepare a concentrated cell suspension in medium: ideally this should be ~1 mL total volume at a density of about 10^5–10^6 cells/mL. Determine the actual cell density by counting the cells using a hemacytometer.[7]

1.2 Centrifuge 1.0 mL of the concentrated cell suspension for 5 minutes at 200 × g (1500 rpm in a microcentrifuge). Carefully remove and discard the supernatant without disturbing the cell pellet, and freeze the cell pellet at –70°C.

Note: Frozen cells in microplates and cell pellets in centrifuge tubes can be stored at –70°C for up to four weeks. The freezing step is important for efficient cell lysis in the CyQUANT® assay.

Note: Phenol red may interfere with the fluorescence of the CyQUANT® GR dye. Either avoid the use of high concentrations of phenol red in the growth medium or remove as much growth medium as possible prior to freezing the cells. Alternatively, a wash step in PBS or growth medium lacking phenol red can be added, to ensure that all dye is removed.

1.3 Thaw the cell pellet at room temperature, add 1.0 mL of the CyQUANT® GR dye/cell-lysis buffer (prepared in *Preparing the Reagent*), and resuspend the cells by briefly vortexing.

1.4 Generate a dilution series in the wells of a microplate. Use CyQUANT® GR dye/cell-lysis buffer and make dilutions corresponding to cell numbers ranging from 50 to 50,000 in 200 µL volumes. Include a 200 µL sample with no cells as a control. Incubate the samples for 2–5 minutes at room temperature, protected from light.

1.5 Measure the fluorescence of the samples using a fluorescence microplate reader set up with appropriate excitation and emission filters. The excitation maximum is about 480 nm; the emission maximum is about 520 nm.

Creating a DNA Standard Curve

The CyQUANT® Cell Proliferation Assay Kit includes a 100 µg/mL sample of bacteriophage λ DNA (Component C) that can be used to prepare a standard curve for DNA content. The standard curve can serve to quantitate cellular DNA, provided the cell lysates are pretreated with DNase-free RNase to eliminate the RNA component of the fluorescent signal (see *Determination Based on DNA or RNA Alone*, below). Alternatively, the standard curve can be used to calibrate the assay for use of the same fluorometer or microplate reader at different times or on different days. Variation in the signal intensity of the standard curve is directly related to variation that will be observed for assaying cells on different days, and is instrument-dependent.

To generate a standard curve, prepare serially diluted 200 µL samples of bacteriophage λ DNA using CyQUANT® GR/cell-lysis buffer (as prepared in *Preparing the Reagent*) with concentrations ranging from 50 pg/mL to 1.0 µg/mL, as appropriate for your experiment, in the wells of a microplate. Include also a control well without DNA. Table 2 provides details for the preparation of a standard curve with DNA ranging from 10 ng/mL to 1.0 µg/mL. For this standard curve, the 100 µg/mL bacteriophage λ DNA standard is first diluted to 1.0 µg/mL by mixing 10 µL of the stock solution with 990 µL of CyQUANT® GR/cell-lysis buffer. If data points below 10 ng/mL are desired, the DNA standard can be diluted further and low-concentration samples prepared analogously. Measure the fluorescence using a filter combination for excitation at about 480 nm and emission at about 520 nm; for example, with filter sets typically used for fluorescein. Plot the DNA concentration versus the fluorescence of each sample, corrected for the background fluorescence determined for the no-DNA control.

Table 2. Preparation of a DNA standard curve for the CyQUANT® Cell Proliferation Assay.

Well No.	CyQUANT® GR/ Lysis Buffer (µL)	1 µg/mL DNA in CyQUANT® GR/ Lysis Buffer (µL)	Final DNA Concentration (ng/mL)
1	200	0	0
2	198	2	10
3	190	10	50
4	180	20	100
5	160	40	200
6	120	80	400
7	80	120	600
8	40	160	800
9	0	200	1000

Experimental Protocol for the Cell Proliferation Assay

Adherent Cells Grown in Microplates

2.1 Make a concentrated cell suspension in growth medium.

2.2 Prepare serial dilutions in the wells of a microplate such that 200 µL volumes of growth medium contain cell numbers ranging from 50 to 50,000. Include a control well with no cells. If a time course is desired, prepare duplicate dilutions in separate microplates.

2.3 Incubate the plate at 37°C for a time sufficient to allow the cells to attach (typically, 4–16 hours); incubate longer to follow cell proliferation. For long-term proliferation studies, 100 µL of medium should be removed from each well every other day and replaced with fresh medium.

2.4 At the desired time, gently invert the microplate, and blot it onto paper towels to remove medium from the wells. The wells may be washed carefully with phosphate buffered saline (PBS), but this is not essential, and is not recommended for very dense cultures where cells may dislodge. Freeze the cells in the microplate and store at -70°C until samples are to be assayed.

Note: Frozen cells in microplates and cell pellets in centrifuge tubes can be stored at -70°C for up to four weeks. The freezing step is important for efficient cell lysis in the CyQUANT® assay.

Note: Phenol red may interfere with the fluorescence of the CyQUANT® GR dye. Either avoid the use of high concentrations of phenol red in the growth medium or remove as much growth medium as possible prior to freezing the cells. Alternatively, a wash step in PBS or growth medium lacking phenol red can be added, to ensure that all dye is removed.

2.5 When you are ready to quantitate the samples, thaw the plates at room temperature, then add 200 µL of the CyQUANT® GR dye/cell-lysis buffer (prepared in *Preparing the Reagent*) to each sample well. Mix gently, if desired (generally not necessary). Incubate the sample for 2–5 minutes at room temperature, protected from light.

2.6 Measure the sample fluorescence using a fluorescence microplate reader with filters appropriate for ~480 nm excitation and ~520 nm emission maxima; for example, with filter sets typically used for fluorescein.

Suspension Cells Grown in Microplates

The above procedure (see *Adherent Cells Grown in Microplates*) can be adapted for suspension cells if a centrifuge capable of centrifuging microplates is available. Incubate the plates at 37°C on a shaker platform with constant agitation. Whenever changes of growth medium are required, centrifuge the plate at a speed and time sufficient to pellet the cells. Carefully

remove the old medium and replace it with fresh medium. At the desired time point, centrifuge the plate, invert, blot to remove the medium, and freeze at -70°C for up to four weeks. Analyze exactly as for adherent cells.

Experimental Protocol for Cell Number Determination

Cells Grown in Standard Culture Conditions

The CyQUANT® Cell Proliferation Assay Kit can be used to count the number of cells in a sample taken from a conventional cell culture. For cultures of adherent cells, the cells must first be detached and suspended. Cells grown in suspension can be assayed directly.

3.1 Transfer the sample of the suspended cells to a centrifuge tube and centrifuge for 5 minutes at 200 × g (e.g., 1500 rpm in a microcentrifuge). The sample should contain 50 to 50,000 cells. Remove and discard the supernatant without disturbing the cell pellet, and freeze the cell pellet at –70°C.

Note: Frozen cells in microplates and cell pellets in centrifuge tubes can be stored at –70°C for up to four weeks. The freezing step is important for efficient cell lysis in the CyQUANT® assay.

Note: Phenol red may interfere with the fluorescence of the CyQUANT® GR dye. Either avoid the use of high concentrations of phenol red in the growth medium or remove as much growth medium as possible prior to freezing the cells. Alternatively, a wash step in PBS or growth medium lacking phenol red can be added, to ensure that all dye is removed.

3.2 Thaw the cell pellet at room temperature and add 200 µL of CyQUANT® GR dye/cell-lysis buffer (prepared in *Preparing the Reagent*).

3.3 Transfer the entire 200 µL sample to a microplate and measure the fluorescence. Convert the observed fluorescence to cell number using a standard curve (see *Creating a Cell Number Standard Curve*). We have found that many different cell types can be assayed using this procedure, but the absolute signal is dependent upon the cell type. Thus it is advisable to use a standard curve generated from the same cell type that is being assayed, for comparison. Alternatively, a standard curve generated using pure DNA (see *DNA Standard Curve*) can be calibrated relative to an appropriate cell type.

Determination Based on DNA or RNA Alone

In the protocols described above, the CyQUANT® GR reagent is used to determine cell number by staining nucleic acids, both DNA and RNA. DNA to RNA ratios, however, may vary according to cell type and cell cycle. Fluorescence due to CyQUANT® GR dye binding to RNA can be eliminated by pretreating samples with DNase-free RNase. Figure 2 shows the results of an experiment in which lysed RBL cells were treated with RNase, treated with DNase, or untreated before CyQUANT® GR dye was added. RNase digestion caused a 30% decrease in sample fluorescence, while DNase digestion showed a 67% loss of fluorescence. Thus, the fluorescence observed for the untreated sample closely matches the sum of the fluorescent signals of DNA only (RNase-treated) and RNA only (DNase-treated) samples.

4.1 For determination of total cellular DNA or RNA, freeze a cell pellet containing 20,000–100,000 cells, being careful to remove as much medium as possible prior to freezing (see note in step 3.1 regarding phenol red and growth media). Thaw at room temperature. For RNase treatment, resuspend the pellet in a small volume (50–100 µL for microplate assays) of 1X cell-lysis buffer supplemented with 180 mM NaCl and 1 mM EDTA. For DNase treatment, resuspend the pellet in a small volume of 1X cell lysis buffer supplemented with 180 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$.

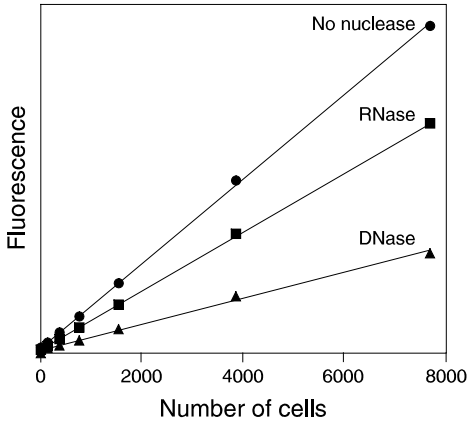

Figure 2. Quantitation of rat basophilic leukemia (RBL) cells before (●) and after treatment with RNase (■) or DNase (▲). Fluorescence measurements were performed as described in Figure 1.

4.2 Add DNase-free RNase A or RNase-free DNase I to a final concentration of about 1.35 Kunitz units/mL (RNase) or 45 Kunitz units/mL (DNase).[8,9] Incubate the samples for 1 hour at room temperature.

4.3 Add an equal volume of a 2X solution of CyQUANT® GR dye diluted in cell-lysis buffer (double the concentration of CyQUANT® GR dye used in *Preparing the Reagent*) to each sample. Incubate the samples for 2–5 minutes.

4.4 Measure the fluorescence as described above (step 1.5). It is suggested that controls be run for each digested sample, using the appropriate buffer, as the presence of salt and divalent cations slightly reduces the slope of the standard curve.

Experimental Protocol for Cell Adhesion Determination

The CyQUANT® Cell Proliferation Assay Kit can be used to determine the number of cells in a population that have adhered to a surface, such as a microplate well.

5.1 Prepare the surfaces, if desired, by treating the microplate wells with extracellular matrix proteins or antibodies. Wash thoroughly with PBS to remove unbound materials.

5.2 Prepare a cell suspension in growth medium. Generally, ~1–4 × 10^5 cells/mL is an appropriate concentration.

5.3 Apply 100 mL of suspended cells per microplate well, in two microplates (duplicates).

5.4 Incubate the cell suspensions at 37°C for a time that is sufficient to allow the cells to attach. Optimal incubation times vary widely according to the cell type and other experimental parameters, but generally 0.5–8 hours is sufficient.

5.5 At the desired time, centrifuge *one* of the plates to pellet the cells. Carefully remove the culture medium by gently inverting the microplate and blotting it onto paper towels. This plate will be used to determine the total cell number.

Note: Phenol red may interfere with the fluorescence of the CyQUANT® GR dye. Either avoid the use of high concentrations of phenol red in the growth medium or remove as much growth medium as possible prior to freezing the cells. Alternatively, a wash step in PBS or growth medium lacking phenol red can be added, to ensure that all dye is removed.

5.6 Carefully remove the medium from the *second* plate (do not centrifuge) by inverting and blotting or aspirating. Wash the wells gently with prewarmed (37°C) PBS or culture medium to remove nonadherent cells. Be careful to avoid dislodging adherent cells. If necessary, repeat the wash step up to four times. This plate will be used to determine the number of adherent cells.

5.7 Freeze the cells in both microplates at –70°C.

Note: Frozen cells in microplates and cell pellets in centrifuge tubes can be stored at –70°C for up to four weeks. The freezing step is important for efficient cell lysis in the CyQUANT® assay.

5.8 Quantitate the cells as described in steps 2.5 and 2.6 above.

References

1. In Vitro Toxicol 3, 219 (1990); **2.** Biotech Histochem 68, 29 (1993); **3.** Anal Biochem 213, 426 (1993); **4.** Cancer Res 48, 4827 (1988); **5.** Exp Cell Res 124, 329 (1979); **6.** Biochimie 76, 452 (1994); **7.** Methods Enzymol 58, 141 (1979); **8.** J Gen Physiol 24, 15 (1940); **9.** J Gen Physiol 33, 349 (1950).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
C7027	CyQUANT® cell-lysis buffer *20X concentrate*	50 mL
C7026	CyQUANT® Cell Proliferation Assay Kit *for cells in culture* *1000 assays*	1 kit

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