**High-throughput screening recommendations using the CyQUANT® Direct Cell Proliferation Assay (Catalog # C35011, C35012)**

The CyQUANT® Direct assay is based on a cell-permeant DNA-binding dye in combination with a background suppression reagent. Since DNA content is highly regulated, cell number estimates are very accurate. Accordingly, CyQUANT® Direct is commonly utilized as a fluorescence-based readout for cell proliferation and cytotoxicity assays. The addition-only, no-wash assay format also renders the CyQUANT® Direct assay suitable for high-throughput screening (HTS) applications. This technical note provides guidance for HTS applications using CyQUANT® Direct and mammalian cells. ***Importantly, since the CyQUANT® Direct assay readout involves a highly sensitive fluorescence measurement of cellular DNA content, proper plate setup and handling during HTS workflows is essential for optimal assay performance (Figure 1).***

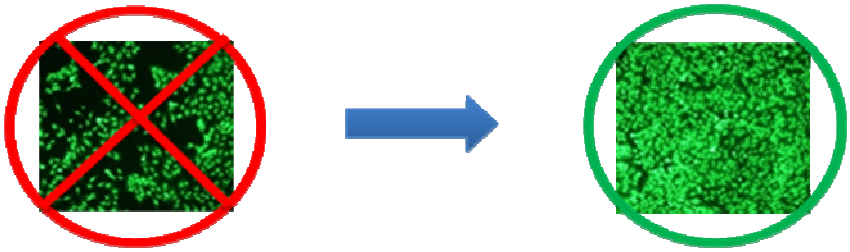

**Figure 1.** Even distribution of the cells in the wells is important for optimal CyQUANT® Direct assay performance. Left, image of cells stained with CyQUANT® Direct that were not setup properly and as a result grew unevenly during a cell proliferation time-course. Right, image of cells that were setup correctly, resulting in even distribution across the well.

**Part I: Cell plating setup recommendations:**

**Table 1. 10X HTS protocol using CyQUANT® Direct (CQD)**

| Plate format | Number of assay wells C35011 (10 plates) | C35012 (100 plates) | Plating volumes/well |
|---|---|---|---|
| 96-well | 960 | 9,600 | 100 µl cells/media<br>10 µl 10X CQD* |
| 384-well | 3,840 | 38,400 | 30 µl cells/media<br>3 µl 10X CQD* |
| 1536-well | 15,360 | 153,600 | 5 µl cells/media<br>0.5 µl 10X CQD* |

* Complete per plate 10X CyQUANT® Direct recipe (scale as needed):
1 ml PBS + 50 µl CQD nucleic acid stain + 250 µl CQD background suppressor dye

1. For HTS applications (see Table 1), use clear-bottom assay plates (e.g., Nunc #167008 or Corning #3603, 96-well format; Nunc #164688 or Corning #3712, 384-well format). Note that care must be taken during plate handling to ensure that the clear-bottom plates are kept clean and lint-free.

2. **Important:** to minimize well-to-well variability due to uneven distribution of the plated cells in the wells (Figure 1), it is important that the cells are dispensed such that the cell distribution in the wells is kept uniform. We recommend the following plate handling steps in order to ensure a uniform distribution of the cells in the assay plate wells:

    a. Option 1: Immediately following the addition of the cells to the assay plates (performed at room temperature), continue to incubate the assay plates at room temperature for 1 – 2 hours to allow the cells to settle to the bottom of the wells (and begin to adhere if using an adherent cell type) before returning them to a 37 °C incubator.

b. Option 2: Immediately following the addition of the cells to the assay plate, briefly spin the plates (e.g., 30 × *g* for 1 minute) to settle the cells to the bottom of the wells before returning them to a 37 °C incubator.

3. Avoid disrupting the cell monolayer during any downstream plate handling or manipulation step (e.g., compound addition, media exchange). The following recommendations may be helpful, depending on your workflow and experimental needs:

   a. Where practical, apply test compounds to assay plates first prior to adding the cells.

   b. Prepare test compounds at higher concentrations (e.g., 10X, not 2X) in order to add smaller volumes to the wells.

   c. Adjust dispense speed and/or dispense angle.

4. Optional: where practical, we recommend including control wells containing media only (i.e., cell-free controls) for signal-to-background assessment. These controls can also be used for fluorescence background subtraction during data analysis.

**Part II: CyQUANT® Direct detection recommendations:**

1. To minimize disrupting the cell monolayer with large volume additions, we recommend preparing 10X CyQUANT® Direct detection reagent (instead of 2X). For example (as per Table 1), to prepare enough 10X detection reagent to dispense 3 µL/well onto cells plated in 30 µL/well in one 384-well assay plate, combine the following (scale according to your needs):

   i. 1 mL PBS (without $CaCl_2$ or $MgCl_2$, Life Technologies catalog # 14190)

   ii. 50 µL CyQUANT® Direct nucleic acid stain

   iii. 250 µL CyQUANT® Direct background suppressor I

2. Avoid disrupting the cell monolayer when adding CyQUANT® Direct to the wells. Where practical, adjust dispense speed and/or angle.

3. Staining of the cells with 10X CyQUANT® Direct detection reagent can be performed at room temperature or 37 °C. For HTS applications, we recommend performing the detection step at room temperature as follows:

   i. Allow the assay plate(s) to equilibrate to room temperature before adding 10X detection reagent.

   ii. Apply 10X detection reagent (Table 1) and incubate a minimum of 2 hours (for optimal signal-to-background and plate-to-plate comparability) at room temperature before reading the fluorescence.

   Note: shorter incubation times (e.g., 30 minutes) can also be used, but signal-to-background will be smaller and plate-to-plate differences in signal intensity will be greater than with longer incubation times.

4. Perform bottom-read fluorescence using standard “green” filter sets (e.g., FITC filter set with a general 50/50 band-splitter mirror) or appropriate wavelengths (e.g., excitation at 480 nm, emission at 535 nm) if using a monochromater-based instrument. Use caution when moving plates to allow cells to remain settled or in contact with the bottom of the plate.

**Technical Support:**
Visit www.Lifetechnologies.com/support or email probestech@invitrogen.com or phone (800) 438-2209 or (541) 335-0353.