



Product Manual

NUCLEAR-ID® Green Cell Cycle Kit

Catalog #: ENZ-51014

100 Assays



Product Manual

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Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

Enzo Life Sciences' NUCLEAR-ID® Green Cell Cycle Kit provides a convenient approach for studying the induction and inhibition of cell cycle progression by flow cytometry. The kit is suitable for (1) determining the percentage of cells in a given sample that are in G₀/G₁, S and G₂/M phases, as well as to quantify cells in the sub-G₁ phase prior to apoptosis, and (2) DNA studies in live, permeabilized and fixed cells for normal cell lines and cell lines exhibiting multiple ploidy levels.

The progression of the cell cycle is controlled by a complex interplay among various cell cycle regulators that either stimulate or inhibit the cell from entering each stage of the cell cycle. These regulators activate transcription factors, which bind to DNA and turn on or off the production of proteins that result in cell division. Dysfunction of any step in this regulatory cascade causes abnormal cell proliferation which underlies many human pathological conditions, such as cancer. A crucial step to understanding these conditions is the ability to understand the mechanisms underlying alterations in cell cycle progression.

A control cell cycle perturbation agent, Nocodazole, is provided for monitoring changes in cell cycle dynamics. Potential applications for live-cell studies are in the determination of cellular DNA content and cell cycle distribution for the detection of variations in growth patterns, for monitoring apoptosis, and for evaluating tumor cell behavior and suppressor gene mechanisms.



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Avoid
freeze /
thaw cycles

REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at $\leq -20^{\circ}\text{C}$, protected from light. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for approximately 100 assays using either live, permeabilized or fixed cells.

Reagent	Quantity
NUCLEAR-ID® Green Cell Cycle Detection Reagent	100 μl
Nocodazole Control	10 μl
10X Assay Buffer	15 ml

OTHER MATERIALS NEEDED

- Flow cytometer
- Tubes appropriate for holding cells for the flow cytometer
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Deionized water
- Anhydrous DMSO (optional)
- Total growth medium suitable for cell type
- 70% Ethanol
- 10% Triton X-100 in water.



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SAFETY WARNINGS & PRECAUTIONS

**FOR RESEARCH USE ONLY. NOT FOR USE IN
DIAGNOSTIC PROCEDURES.**



Handle
with care

- The NUCLEAR-ID® Green Cell Cycle Detection Reagent contains DMSO which is readily absorbed through the skin. DMSO is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling these reagents.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.



Protect
from light



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METHODS AND PROCEDURES

The procedures described in this manual assume that the user is familiar with the basic principles and practices of flow cytometry and is able to run samples according to the operator's manual pertaining to the instrument being used.

NOTE: Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use, to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.

REAGENT PREPARATION

Positive Control

Nocodazole, a cell cycle perturbation agent, is an anti-neoplastic agent which exerts its effect by depolymerizing microtubules, causing arrest in the G₂/M phase of the cell cycle. It may be used as a positive control for cell cycle distribution changes.

The Nocodazole provided in the kit is supplied at a stock concentration of 1 mg/ml in DMSO. It is recommended that treatment with the agent be performed using 0.1 - 0.2µg/ml (1-2µl stock per 10ml medium) in order to observe changes in nuclear morphology and that the final DMSO concentration in the assay not exceed 0.2%.

To prepare a 0.1µg/ml solution, add 1µl stock Nocodazole Control per 10ml medium. Use 2µl stock Nocodazole Control per 10ml medium for a 0.2µg/ml solution. If small volumes are required, an intermediate dilution (e.g., 10µg/ml) can be made.

1X Assay Buffer

NOTE: The 1X Wash Buffer will be used for staining of fixed cells. See page 7.

Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the number of samples to be assayed by diluting each milliliter (ml) of the 10X Assay Buffer with 9ml of deionized water.



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DNA Staining Solutions

The concentration of NUCLEAR-ID® Green dye for optimal staining will vary depending upon the application. Suggestions are provided to use as guidelines, though some modifications may be required depending upon the particular cell type employed and other factors such as the permeability of the dye to the cells or tissues. To reduce potential artifacts from overloading the cells, the concentration of the dye should be kept as low as possible.

Refer to appropriate sections for details on the preparation of the staining solution for specific applications. Prepare sufficient amount of the staining solution for the number of samples to be assayed.

CELL PREPARATIONS

Cells should be maintained via standard tissue culture practices. Positive control cells should be pretreated with Nocodazole Control for 18-24 hours. Response to the perturbation agent, Nocodazole, is time and concentration dependent and may also vary significantly depending upon cell type and cell line. Negative control cells should be treated with a vehicle (DMSO, media or other solvent used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions.

STAINING LIVE CELLS

1. Grow cells overnight to log phase in a humidified incubator at 37°C, 5% CO₂.
2. Treat cells with or without experimental compounds.
3. Prepare positive control cells by incubating with pre-diluted Nocodazole (0.1-0.2µg/ml, see REAGENT PREPARATION, page 5) for 18-24 hours under normal tissue culture conditions.
4. At the end of treatment, trypsinize (adherent cells), or collect cells (suspension cells). Adjust cell count to 1x10⁵ to 1x10⁶ cells/ml.
5. Centrifuge at 400 x g for 5 min. to pellet the cells. Resuspend in media or other buffer of choice and centrifuge as before.



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6. Immediately prior to staining the live cell samples, prepare fresh DNA Staining Solution as follows:
***NOTE:** A concentration of 5-20µM NUCLEAR-ID® Green dye is recommended for live cell DNA analysis. The procedure described below is for preparation of 20µM dye solution.*
For each sample to be stained, dilute 2µl NUCLEAR-ID® Green Cell Cycle Detection Reagent to a final volume of 500µl with 1X Assay buffer.
7. Resuspend each live cell sample in 0.5ml of freshly prepared DNA Staining Solution (from step 6). Incubate for 30 minutes at room temperature (22°C) or 37°C in the dark. It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
8. Analyze the samples in the FL1/GREEN channel of a flow cytometer with a 488nm excitation laser.

STAINING ETHANOL FIXED CELLS

1. Grow cells overnight to log phase in a humidified incubator at 37°C, 5% CO₂.
2. Treat cells with or without experimental compounds.
3. Prepare positive control cells by incubating with pre-diluted Nocodazole (0.1-0.2µg/ml, see REAGENT PREPARATION, page 5) for 18-24 hours under normal tissue culture conditions.
4. At the end of treatment, trypsinize (adherent cells), or collect cells (suspension cells). Centrifuge at 400 x g for 5 min. to pellet the cells, washing twice with 1X Assay Buffer.
5. Resuspend the pellet in 100-200µl 1X Assay Buffer. This helps to prevent clumping.
6. Slowly add 5ml of ice cold 70% Ethanol (-20°C) drop-wise to each sample containing cells resuspended in 1X Assay Buffer while vortexing. At this point, cells may be stored at -20°C for several months before staining. Cells must be in fixative for at least one hour prior to staining.
7. When ready to stain the cells with NUCLEAR-ID® Green Cell Cycle Detection Reagent, centrifuge the fixed cells at 400 x g for 5 min. Decant fixative thoroughly.
8. Wash cells by adding 0.5ml of 1X Assay Buffer to each sample and gently resuspend the pellet. Centrifuge the cells for 5 min. at 500 x g and carefully remove the supernatant.
9. Immediately prior to staining the ethanol-fixed cell samples, prepare fresh DNA Staining Solution as follows:

NOTE: A concentration of 5-20 μ M NUCLEAR-ID® Green dye is recommended for ethanol-fixed cells. The procedure described below is for preparation of 20 μ M dye solution.

For each sample to be stained, dilute 2 μ l NUCLEAR-ID® Green Cell Cycle Detection Reagent to a final volume of 500 μ l with 1X Assay buffer.

10. Re-suspend each fixed cell sample in 0.5ml freshly prepared DNA Staining Solution (from step 9) to a final concentration of 1 x 10⁵ to 1 x 10⁶ cells/ml. Incubate for 30 min. at room temperature (22°C) or 37°C in the dark.
11. Analyze the samples in the FL1/GREEN channel of a flow cytometer with a 488nm excitation laser.

STAINING PERMEABILIZED CELLS

1. Grow cells overnight to log phase in a humidified incubator at 37°C, 5% CO₂.
2. Treat cells with or without experimental compounds.
3. Prepare positive control cells by incubating with pre-diluted Nocodazole (0.1-0.2 μ g/ml, see REAGENT PREPARATION, page 5) for 18-24 hours under normal tissue culture conditions.
4. At the end of treatment, trypsinize (adherent cells), or collect cells (suspension cells). Adjust cell count to 1x10⁵ to 1x10⁶ cells/ml.
5. Centrifuge at 400 x g for 5 min. to pellet the cells. Re-suspend in media or other buffer of choice and centrifuge as before.
6. Immediately prior to staining the cell samples, prepare fresh DNA Staining/Permeabilizing Solution as follows:

NOTE: A concentration of 5-20 μ M NUCLEAR-ID® Green dye is recommended for permeabilized cells. The procedure described below is for preparation of 20 μ M dye solution.

For each sample to be stained, dilute 2 μ l NUCLEAR-ID® Green Cell Cycle Detection Reagent and 10 μ l of 10% Triton X-100 to a final volume of 500 μ l with 1X Assay buffer of choice.

7. Re-suspend each fixed cell sample in 0.5ml freshly prepared DNA Staining/Permeabilizing Solution (from step 6) to a final concentration of 1x10⁵ to 1x10⁶ cells/ml. Incubate for 30 min. at room temperature (22°C) or 37°C in the dark.



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8. Analyze the samples in the FL1/GREEN channel of a flow cytometer with a 488nm excitation laser.

APPENDICES

A. Fluorescence Channel Selection for Data Collection

FL1/GREEN channel is recommended for imaging NUCLEAR-ID® Green Detection Reagent with 488nm excitation.

B. Expected Results

Propidium iodide staining of DNA in permeabilized cells and subsequent detection by flow cytometry has been widely employed to determine the percentage of cells in each phase of the cell cycle.^{1,2} While propidium iodide is impermeable to plasma membrane and nuclear membranes, NUCLEAR-ID® Green dye freely enters cells, allowing cell cycle analysis without the need for laborious permeabilization and fixation steps. Propidium iodide additionally has the disadvantage that it stains all double stranded nucleic acids, so cells must be incubated with RNase to remove any double stranded RNA. Since NUCLEAR-ID® Green dye intercalates exclusively into the base pairs of double-stranded DNA, no RNase treatment is required and the fluorescence intensity of a stained cell is directly proportional to the DNA content in the cell. Cells in the G₀/G₁ phase are either quiescent or preparing to enter the S phase (DNA synthesis) and contain one set of chromosomes. An exponentially growing population of cells displays a DNA content distribution containing two major peaks, a narrow peak representing G₀/G₁ phase cells and a second smaller peak representing G₂/M phase cells which contain four copies of each chromosome and thus twice the dye content of G₀/G₁ phase cells (Figure 1). Cells in S phase are in the process of DNA replication and hence their DNA content distribution will fall between the G₀/G₁ and G₂/M peaks.

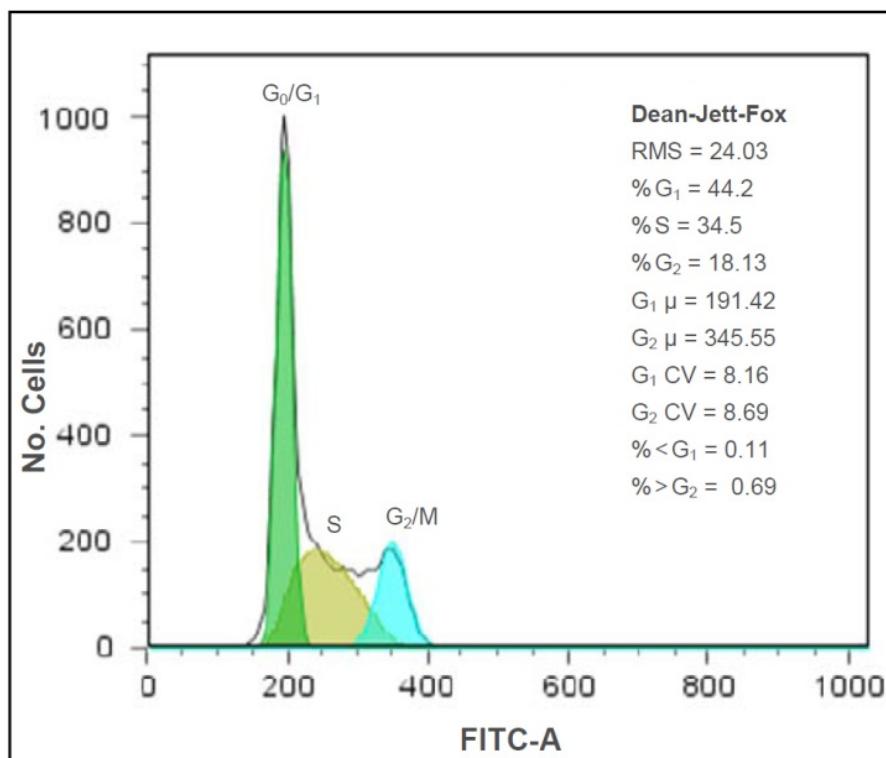


Figure 1. Typical DNA profile from asynchronously growing Jurkat cells. Normally, about 40% of the cells are in G₁ phase and about 34% in S and 20 % in G₂/M phases. Using standard 1-D flow cytometry, it is not possible to separate G₂ phase cells from mitotic cells.

The quality of a DNA histogram is estimated from the width of the peak of DNA from cells in G₁ of the cycle. This is measured by the coefficient of variation (CV) across the peak and is calculated from the standard deviation (SD).

$$\% \text{ } CV = \frac{SD}{\text{Peak Channel}} \times 100$$

In the above equation, the peak channel is the mean channel number of the peak.

The smaller the CV of the peaks in the DNA histogram, the more accurate is the measurement of ploidy and the better the estimation of the percentage of cells in the different parts of the cell cycle. It is essential that any unnecessary broadening of the peaks due to misalignment of the instrument or suboptimal dye-to-DNA ratio should be minimized. The number of cell clumps and the amount of debris present are also important factors influencing peak broadness.

The microtubule inhibitor, Nocodazole can arrest the cell cycle at the G₂/M phase. Flow cytometric analysis (Figure 2) below demonstrates the ability of the NUCLEAR-ID® Green dye to reflect the DNA content and cell cycle distribution.

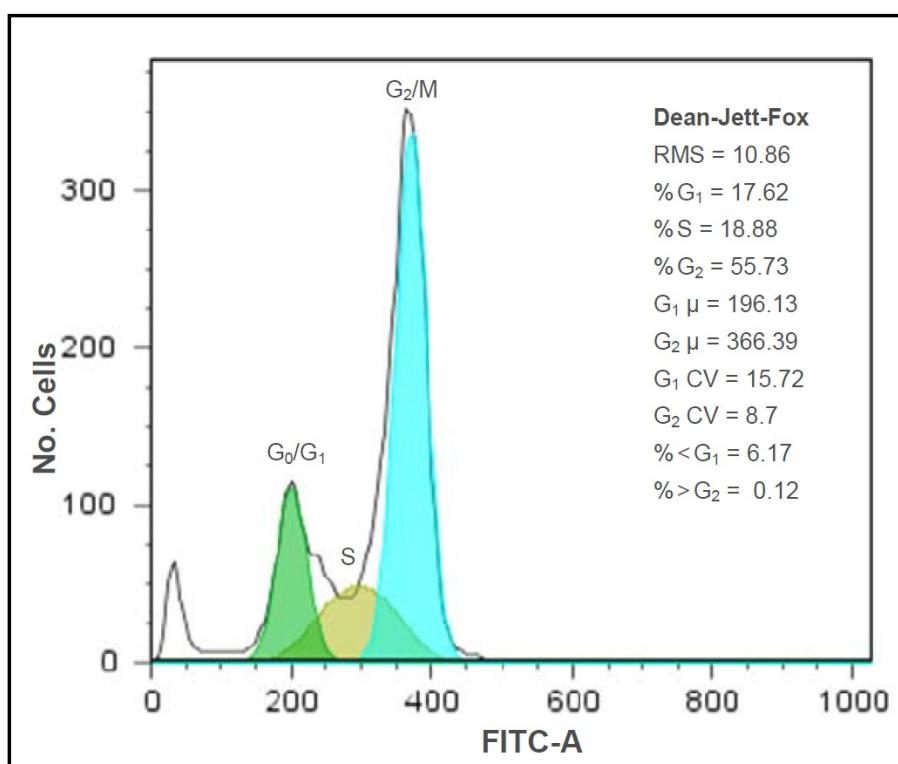


Figure 2: Effect of a cell cycle perturbation agent on DNA synthesis in Jurkat cells. The cells were treated with 0.1 μ g/ml Nocodazole for 20 hours. Cells were then washed and stained with NUCLEAR-ID® Green Cell Cycle Detection Reagent.



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REFERENCES

1. Darzynkiewicz, Robinson and Crissman (eds.) (1994) Flow Cytometry. Methods in Cell Biology, 41 & 42, Academic Press, Inc., San Diego.
2. Nunez (2001) “DNA measurement and cell cycle analysis by flow cytometry” Curr. Issues Mol. Biol. 3(3): 67-70.



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TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestions
DNA histograms are of poor overall quality.	The key elements in obtaining a histogram of high quality are good sample preparation, correct cell-to-dye ratio and proper instrument alignment. Live cell histograms are more challenging to generate than fixed cell ones.	<ul style="list-style-type: none">• Make sure the cell preparation protocol generates single cells with minimal clumping. Filtration or trituration of cell suspensions may be required.• Systematically vary the dye to cell ratio.• Optimize for cell type, medium or buffer used, time of incubation, temperature of incubation.• Gate on the live cells and increase the number of cycle events being analyzed.• Verify that the flow cytometer is correctly aligned, with stable fluidics, using calibrated fluorescent beads of known CV.• Verify that the cell concentration is 1×10^5 to 1×10^6 cells/ml.• For permeabilized cells ensure that sufficient Triton X-100 has been added to the staining solution to permeabilize the cells.• For ethanol fixed cells ensure that the cells were fixed and stored correctly.
No G ₂ block observed in cells treated with Nocodazole.	Incorrect drug concentration or time of incubation for cell type.	<ul style="list-style-type: none">• Optimize for cell type, medium or buffer used, time of incubation, temperature of incubation.



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