ab139475 –

CytoPainter Nucleolar Staining Kit - Green Fluorescence

Instructions for Use

Specifically designed for visualizing microscopically nucleoli in living cells.

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

ab139475 Nucleolar Staining Kit - Green Fluorescence Kit contains two proprietary dyes suitable for live cell staining of nucleoli. The dyes allow examination of nucleolar dynamic changes in intracellular distribution, trafficking and localization arising from biological processes such as the cell cycle and ribosome biogenesis. Historically, nucleolus imaging approaches have required much more laborious and time consuming methods, such as fluorescently labeled RNA microinjection, fluorescence *in situ* hybridization (FISH) or use of fluorescent protein-tagged RNA binding proteins (GFP or YFP constructs).

The nucleolus represents a highly dynamic nuclear domain arising from an equilibrium between the level of ribosomal RNA synthesis and the efficiency of ribosomal RNA processing. Although the nucleolus is primarily associated with ribosome biogenesis, several lines of evidence demonstrate that it has additional functions, such as regulation of mitosis, cell-cycle progression and proliferation, and many forms of stress response and biogenesis of multiple ribonucleoprotein particles. Ribosome biogenesis is regulated throughout interphase and ceases during mitosis. Thus, there is a direct relationship between cell growth and nucleolar activities. Nucleoli are well known to be dramatically modified in cancer cells. Additionally, a large number of key proteins from both DNA and RNA containing viruses are localized in the nucleolus, including the HIV-1 (Human immunodeficiency virus type 1) Rev and Tat proteins.

Targeting of viral proteins to the nucleolus not only facilitates virus replication, but may also be required for pathogenic processes. The nucleolus is also a sensor of stress due to the redistribution of the ribosomal proteins in the nucleoplasm through its disruption.

2. Product Overview

ab139475 is specifically designed for visualizing nucleoli in living cells by fluorescence microscopy. The dye in the kit is resistant to photobleaching, facilitating its use in imaging applications. This nucleolar stain is compatible with common live-cell nuclear stains. The kit includes a control nucleolus perturbation agent, Actinomycin D, for monitoring changes in nucleolar dynamics. Actinomycin D, an antibiotic, is a DNA-dependent RNA synthesis inhibitor. Nucleolar synthesis of ribosomal RNA is especially sensitive to Actinomycin D. Potential applications for this kit include monitoring of impaired ribosome biogenesis, inhibition of transcription, cell cycle dynamics, cellular stress, distribution, trafficking and dynamics of nucleolar proteins, distribution of viral proteins, and potentially, as an aid to identify cancer cells.

3. Assay Summary

Reconstitute positive control, dilute assay buffer and prepare Dual Detection Reagent. Prepare cells.

Pipette dual Detection Reagent onto cells, protect from light.

Incubate 15-30 minutes at 37°C.

Wash cells and add coverslip.

Analyze.

4. Components and Storage

A. Kit Contents

Item	Quantity	Storage Temperature
Green Detection Reagent	50 µl	-80°C
Actinomycin D Control	125 µg	-80°C
10X Assay Buffer	15 ml	-80°C

Reagents provided in the kit are sufficient for approximately 500 assays using either live, adherent cells or cells in suspension.

B. Storage and Handling

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at -80°C, protected from light. Avoid repeated freezing and thawing.

C. Additional Materials Required

- Standard fluorescence microscope.
- Calibrated, adjustable precision pipets, preferably with disposable plastic tips.
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Glass microscope slides
- Glass cover slips
- Deionized water
- Anhydrous DMSO (optional)
- Growth media (e.g. Dulbecco's Modified Eagle Medium, D-MEM).

5. Pre-Assay Preparation

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.

A. Reagent Preparation

1. Positive Control

The antibiotic, Actinomycin D is a DNA-dependent RNA synthesis inhibitor. Nucleolar synthesis of ribosomal RNA is especially sensitive to Actinomycin D. The rearrangement of the nucleolus due to Actinomycin D treatment is widely used to examine the localization of various nucleolar components, including nucleolar proteins. Typically, at higher doses of the drug (4-10 µg/ml for 4 hours), the nucleolus in mammalian cells disappears, or where still present, is dramatically reduced in amount, while at lower concentrations (1-4 µg/ml for 4 hours), less dramatic reduction in nucleolar amount is often observed.

The Actinomycin D provided in the kit may be used as a positive control for reducing nucleoli size and number. It is supplied lyophilized (125 µg) and should be centrifuged briefly to gather the material at the bottom of the tube. Reconstitute the lyophilized material in 250 µl deionized water for a 0.5 mg/ml stock solution. Vortex vigorously,

allow to sit for 15 minutes, then vortex again to completely bring it into solution. It is recommended that treatment with the agent be performed using 1-5 µg/ml final concentration in order to observe changes in nucleolar morphology. Unused stock Actinomycin D may be stored in small aliquots at -20°C for several weeks.

2. 1X Assay Buffer

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 9 ml of deionized water.

3. Green Detection Reagent

The concentration of the Green Detection Reagent 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.

Prepare sufficient amount of the Green Detection Reagent for the number of samples to be assayed as follows: To each ml of 1X Assay Buffer (see preparation in step 2) or cell culture medium containing serum, add 1 µl of Green Detection Reagent. Serum may be included, if preferred.

NOTE: If desired, other nuclear stains may be added to the diluted Green Detection Reagent at this time.

B. Cell Preparations

Cells should be maintained via standard tissue culture practices. Positive control cells should be pretreated with the Actinomycin D control for 2-6 hours. Response to Actinomycin D 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.

6. Assay Protocol

A. Staining of Live Adherent Cells

1. Grow cells on cover slips inside a Petri dish filled with the appropriate culture medium. When the cells have reached the desired level of confluence, carefully remove the medium.
2. Dispense sufficient volume of Green Detection Reagent (see Pre-Assay Preparation section 3), to cover the monolayer cells (~100 µl of labelling solution for cells grown on an 18 X 18 mm coverslip).
3. Protect samples from light and incubate for 15-30 minutes at 37°C.
4. Wash the cells with 100 µl 1X Assay Buffer. Remove excess buffer and place coverslip on slide.
5. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification is recommended). Use a standard FITC filter set for imaging the nucleolus.

B. Staining of Live Cells Grown in Suspension

1. Centrifuge cells for 5 minutes at 400 x g at room temperature (RT) to obtain a cell pellet.
2. Carefully remove the supernatant by aspiration and dispense sufficient volume of Green Detection Reagent (see Pre-Assay Preparation section 3), to cover the dispersed cell pellet.
3. Protect samples from light and incubate for 15 to 30 minutes at 37°C.
4. Wash the cells with 100 µl 1X Assay Buffer. Remove excess buffer. Resuspend cells in 100 µl 1X Assay Buffer, then apply the cells to a glass slide and overlay with a coverslip.
5. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard FITC filter set for imaging the nucleolus.

7. Data Analysis

A. Filter Set Selection

The selection of optimal filter sets for a fluorescence microscopy application requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope.

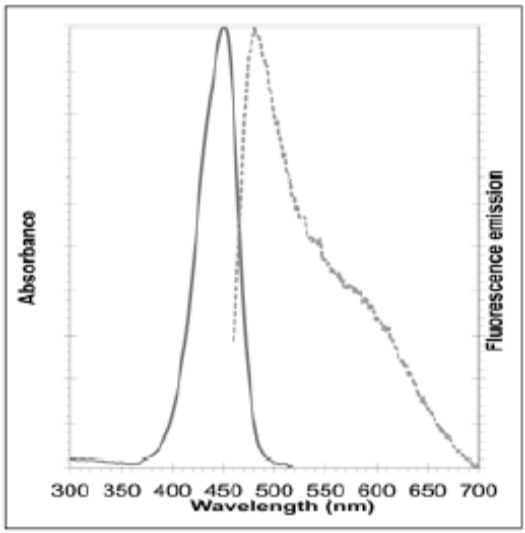

Figure 1. Absorption and fluorescence emission spectra for Green Detection Reagent dye. Spectrum was determined in 1X Assay Buffer.

B. Results

Ribosomal RNAs (rRNA's) are synthesized, processed and assembled with ribosomal proteins in the nucleolus. In mammalian cells, the nucleolus is disorganized in prophase and reassembled at the end of mitosis using the nucleolar machineries from the previous cell cycle. Ribosomal DNA (rDNA) transcription is maximal in the S and G2 phases, silent in mitosis, and slowly recovers in the G1 phase of the cell cycle. The nucleolus is a prominent nuclear structure in cycling cells but of limited size in the terminal stages of cell differentiation.

Cells stained with the Green Detection Reagent show maximal fluorescence signal within the nucleoli, and faint fluorescence throughout the nucleus. Weak fluorescence is also observed throughout the cytoplasm, predominantly associated with mitochondria. The dye displays high cellular plasma and nuclear membrane permeability, and is well tolerated by living cells.

The number of nucleoli in different cell types observed with the Green Detection Reagent will vary and they will be of different sizes as well. There appears to be an inverse relationship between size and number of nucleoli in mammalian cells. For example, HeLa Human cervical carcinoma cells, stained using the Green Detection Reagent typically display two prominent nucleoli per cell, while U2OS Human bone osteosarcoma epithelial cells are observed to contain a half dozen smaller nucleoli. Ribosomal DNA (rDNA) transcriptional arrest, induced by low doses of Actinomycin D, results

in loss of nucleolar staining in both cell lines as observed with the Green Detection Reagent in the kit. The dissipation of nucleolar signal induced by Actinomycin D is likely to coincide with rRNA degradation events known to occur during treatment with this drug. However, it is not definitively established whether the dye interacts with rRNA, with arginine/lysine-rich sequences in nucleolar proteins or with some other structural feature of nucleoli. Green Detection Reagent does bind nucleic acids in solution, but does not show significant selectivity towards RNA relative to DNA in such in vitro experiments.

8. Troubleshooting

Problem	Reason	Solution
Nucleoli are not sufficiently stained	Very low concentration of Green Detection Reagent was used or dye was incubated with the cells for an insufficient length of time.	Either increase the labeling concentration or increase the time allowed for the dye to accumulate in the nucleoli.
Green Detection Reagent fails to stain the nucleoli in fixed and/or permeabilized cells.	Green Detection Reagent provided in the kit is only suitable for live-cell staining.	Use the dye only for live cell analysis.
Precipitate is seen in the 10X Assay Buffer.	Precipitate forms at low temperatures.	Allow solution to warm to room temperature or 37°C, then vortex to dissolve all precipitate.

The Green Detection Reagent is too bright compared to the red nuclear stain.	Different microscopes, cameras and filters may make some signals appear very bright.	Reduce the concentration of the Green stain or shorten the exposure time.
Cells do not appear healthy.	Some cells require serum to remain healthy.	Add serum to the staining solution. Serum does not affect staining. Normal amounts of serum added range from 2% to 10%.
Actinomycin D Control does not go into solution.	The recommended volume to dissolve Actinomycin D is near the limit of solubility. Ambient temperature may affect solubility.	Warm the solution to 37°C to dissolve, or dissolve in a larger volume.
Actinomycin D treated cells appear dead or are no longer attached to the surface.	The EC_{50} of Actinomycin D may be different with different cell lines.	Lower the dose of Actinomycin D, or shorten the time of exposure.

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