INSTRUCTIONS

Thermo SCIENTIFIC

Cellomics® Cytoskeletal Rearrangement Kit

High-Content Screening Reagents

1986.2

Number	Description
8402401	**Cytoskeletal Rearrangement Kit – Whole Cell Stain, F-actin and Tubulin,** materials for 1 × 96 wells
8402402	**Cytoskeletal Rearrangement Kit – Whole Cell Stain, F-actin and Tubulin,** materials for 5 × 96 wells
8402501	**Cytoskeletal Rearrangement Kit – Whole Cell Stain and F-actin,** materials for 1 × 96 wells
8402502	**Cytoskeletal Rearrangement Kit – Whole Cell Stain and F-actin,** materials for 5 × 96 wells
8402601	**Cytoskeletal Rearrangement Kit – Whole Cell Stain and Tubulin,** materials for 1 × 96 wells
8402602	**Cytoskeletal Rearrangement Kit – Whole Cell Stain and Tubulin,** materials for 5 × 96 wells

Kit Contents:	8402401	8402402	8402501	8402502	8402601	8402602
Whole Cell Stain Green	50 µg	6 x 50 µg	50 µg	6 x 50 µg	50 µg	6 x 50 µg
DY554-phalloidin	10 units	50 units	10 units	50 units		
Tubulin Primary Antibody	8 µl	30 µl	--	--	8 µl	30 µl
DyLight™ 649 Conjugated Goat Anti-Mouse IgG	14 µl	72 µl	--	--	--	--
DyLight 549 Conjugated Goat Anti-Mouse IgG	--	--	--	--	8 µl	72 µl
DAPI Dye	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
Wash Buffer (10X Dulbecco's PBS)	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml
Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton® X-100)	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml
Blocking Buffer (10X)	85 ml	85 ml	85 ml	85 ml	85 ml	85 ml
Thin Plate Seal Assembly	7/pack	7/pack	7/pack	7/pack	7/pack	7/pack

Storage: Store DY554-phalloidin at -20°C. Store all other kit components at 4°C. Store the fluorescent antibodies and DAPI Dye solutions protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

Warning: Please completely read these instructions and the accompanying material safety data sheets before using this product. Cellomics Reagents are not for diagnostic use in humans or animals.

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Introduction

The Thermo Scientific Cellomics Cytoskeletal Rearrangement Kits are for the simultaneous quantitation of DNA content, cell morphology, and the intracellular arrangement of microfilaments and microtubules in the same cell (Figure 1). These kits allow direct measurements of cell and nuclear morphology, F-actin, and microtubule changes using a fixed end-point assay based on immunofluorescence detection in cells grown on standard high-density microplates. The primary antibody is specific for its target and has minimal cross-reactivity with other targets.

These kits have been optimized with the Thermo Scientific ArrayScan HCS Reader using the Morphology BioApplication Software Module. Thus, automated plate-handling, focusing, cell image acquisition/processing, and data analysis/ management are combined in one high-content screening (HCS) system to assay for test compounds. In addition to HCS instruments, cells labeled by the kit reagents can be viewed and analyzed by other fluorescence microscopes.

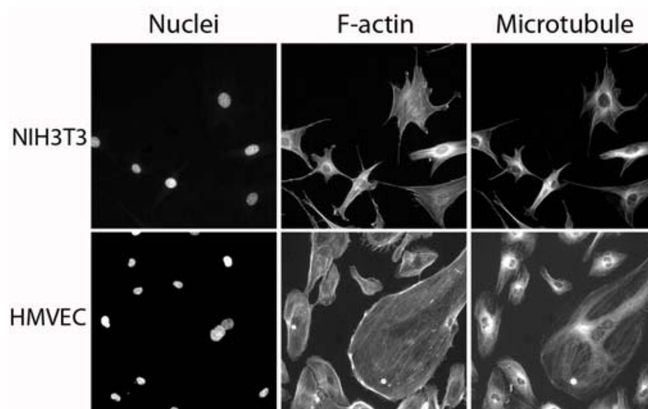

Figure 1. Staining of cytoskeleton structure in NIH 3T3 cells (upper panels, 20X objective) and HMVEC-L cells (lower panels, 20X objective). Cells were stained with DAPI (nuclei). Markers were detected according to the kit protocol. Cell images were acquired using the ArrayScan® HCS Reader.

Background

The intracellular meshwork of the cytoskeleton is responsible for maintaining cell shape, cell movement, cytokinesis and organelle organization.[1,2] The cytoskeleton network also facilitates proper function of other proteins by direct binding, transporting, repositioning and sequestering these proteins. The structure of cytoskeleton is controlled by cytoskeleton-associated proteins in response to the external signaling.[3] Therefore, defects in the ability to regulate the dynamics of cytoskeletal structure are likely to cause detrimental effects on other cell function.[4]

Cytoskeletal rearrangement is often associated with cellular toxicity, pathology and cell death.[4,5] Signaling defects in conjunction with cytoskeletal rearrangement can also contribute to cell proliferation and tumor cell activation, which result in metastasis.[6,7] An accurate assay for cytoskeletal rearrangement is essential to evaluate potential therapeutic targets.

High-content analysis (HCA) involves a fluorescence cell-based assay in which cells are automatically imaged and analyzed using fluorescence microscopy. The Multiplexed Cytoskeletal Rearrangement HCS Reagent Kits provide a highly effective tool for studying the cytoskeletal changes. The reagents combined with the Cellomics ArrayScan HCS Reader and the Morphology BioApplication Software Module allows accurate quantification of microfilaments and microtubules simultaneously within the same cell (Figure 2).

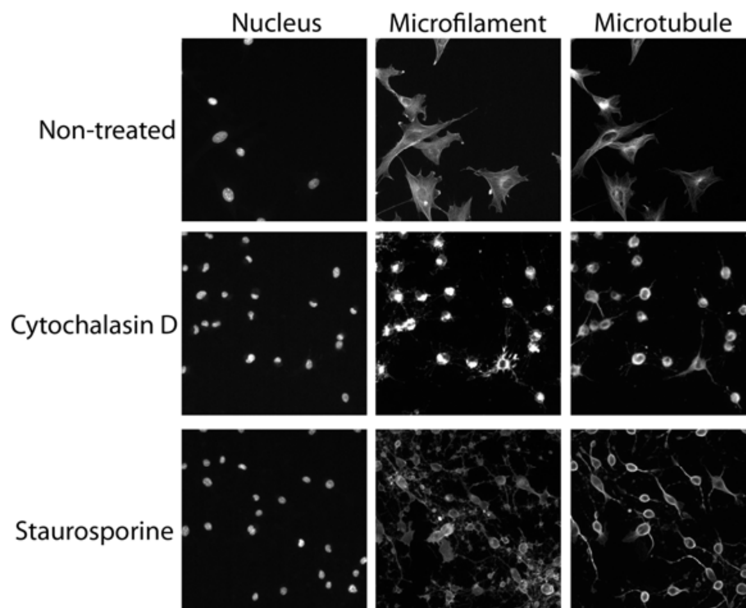

Figure 2. Staining of DAPI (nucleus), F-actin (microfilament) and tubulin (microtubule) in NIH 3T3 cells. Cells were treated with 10 µM cytochalasin D or 1 µM staurosporine for 3 hours or incubated only with media (non-treated). Markers were detected according to the kit protocol. Cytochalasin D and staurosporine dismantle cytoskeleton structure, which causes dramatic morphological change in cells. The cell images were acquired using a Cellomics ArrayScan HCS Reader.

Additional Material Required

- Cytochalasin D (or other cytoskeleton drugs)
- Paraformaldehyde (16%) (Thermo Scientific Product No. 28908 or 28906)
- Collagen-1 coated clear-bottom 96-well microplates (for example, BD BioCoat™ Plate Product No. 354407)
- DMSO
- Ultrapure water

Cell Preparation Information

- This protocol is optimized for NIH 3T3 (American Type Culture Collection # CRL-1658) cells; however, the assay will work on other cell types including C6 glioma, A549 and HMVEC primary endothelial cells. Please visit our website for more information related to these kits.
- For routine culture of NIH 3T3 cells, use Dulbecco's Minimum Essential Medium (DMEM) containing high glucose and the following supplements (= DMEM Complete Medium): 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin.
- Dilute cells when they reach 70-80% confluence (two to three times per week) at a dilution of 1:2 to 1:10 depending on the cell types. Use cells at a passage number ≤ 15.
- Harvest cells by trypsinization, dilute into DMEM Complete Medium and determine cell density. Dilute cells to a density of 5-10 × 10^4 cells/ml in DMEM Complete Medium and add 100 µl of the cell suspension per well of a BD Biocoat Collagen-1 96-well microplate. Incubate 16-24 hours at 37°C in 5% CO_2.

Procedural Notes

- Anti-tubulin antibody specifically binds to tubulin and it does not block microtubule assembly; it reacts to human, cow, pig, rat, mouse, guinea pig, gerbil, frog and chicken tubulins.
- Do not allow plate wells to become dry at any time during the protocol. Perform all steps at room temperature unless otherwise indicated. The protocol requires approximately 3 hours post-compound treatment to complete.
- Protocol optimized for NIH 3T3 cells cultured in 96-well plates. Using conditions other than those indicated may necessitate optimization.
- Please refer to the Compatible BioApplication Software Modules Section for Cellomics applications that can be used with this kit and the ArrayScan HCS instructions for optimal assay implementation on the ArrayScan HCS Reader.
- The absorption/emission maxima of the reagents are as follows:
  Whole Cell Stain Green: 491/518 nm
  DY554-phalloidin 551/572
  DyLight 549 Conjugates: 562/576 nm
  DyLight 649 Conjugates: 646/679 nm
  DAPI: 358/461 nm
- The total intensity from a DAPI-labeled nucleus, determined on an image analysis system such as on the Cellomics ArrayScan HCS Reader, is proportional to the nucleus' DNA content.
- Cells prepared and labeled according to these instructions can be analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multiwell chamber slides. Use image-processing software to quantify the targets.

Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 5 ml of 16% formaldehyde solution to 15 ml of 1X Wash Buffer and heat to 37°C in a water bath just before use. Pre-warming the Fixation Solution is critical for maintaining cell integrity. Prepare solution just before each assay.
1X Permeabilization Buffer	Add 2 ml of 10X Permeabilization Buffer to 18 ml of the 1X Wash Buffer. Store this buffer at 4°C for up to 7 days.
1X Blocking Buffer	Add 10 ml of the 10X Blocking Buffer to 90 ml of 1X Wash Buffer. Store buffer at 4°C for up to 7 days.
Primary Probe Solution	Reconstitute DY554-phalloidin (F-actin kits) by adding 110 µl for 10 units (550 µl for 50 units) of pure methanol and gently mixing. Use the DY554-phalloidin immediately and promptly store any unused portion at -20°C. **F-actin and tubulin kits (8402401, 8402402):** Add 100 µl of DY554-phalloidin and 6 µl of Tubulin Primary Antibody to 5.9 ml of 1X Blocking Buffer. Prepare solution just before each assay. **F-actin kits (8402501, 8402502):** Add 6 µl of DAPI, 100 µl of DY554-phalloidin to 5.9 ml of the 1X Blocking Buffer. Prepare solution just before each assay. **For the tubulin kits (8402601, 8402602):** Add 6 µl of Tubulin Primary Antibody to 6 ml of the 1X Blocking Buffer. Prepare solution just before each assay.
Staining Solution (Secondary Antibody)	**F-actin and tubulin kits (8402401, 8402402):** Add 6 µl of DAPI, 6 µl of the DyLight 649 Goat Anti-Mouse to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay. **For the tubulin kits (8402601, 8402602):** Add 6 µl of DAPI and 6 µl of the DyLight 549 Goat Anti-Rabbit to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Whole Cell Stain Solution	Just before use, reconstitute the Whole Cell Stain Green by adding 50 µl of DMSO to the tube and mixing. Add 12 µl of the stain in DMSO to 12 ml of 1X Wash Buffer. Prepare solution just before each assay. Use the Whole Cell Stain Solution immediately and discard the unused solution. Whole Cell Stain Green in DMSO may be stored at -20°C for < 1 week.

Protocol for the Cytoskeletal Rearrangement Kit

1. Dilute the cytochalasin D stock solution (e.g., 3 mM in DMSO) to 30 µM, or other test compounds to appropriate concentration, in culture medium. Add 50 µl of culture medium to the control wells. Add 50 µl of cytochalasin D in culture medium to each treatment well (150 µl final). Incubate for 3 hours at 37°C in 5% CO_2.
2. Aspirate culture medium and add 100 µl/well of warmed Fixation Solution. Incubate plate in a fume hood at room temperature for 15 minutes. Using warm (37°C) Fixation Solution is critical for maintaining cell integrity.
3. Aspirate Fixation Solution and wash plate twice with 100 µl/well of 1X Wash Buffer.
4. Aspirate Wash Buffer, add 100 µl/well of 1X Permeabilization Buffer and incubate for 15 minutes at room temperature.
5. Aspirate Permeabilization Buffer. Add 100 µl/well of 1X Blocking Buffer. Incubate at room temperature for 15 minutes.
6. Aspirate Blocking Buffer and add 50 µl/well Primary Probe Solution. Incubate for one hour at room temperature.

   Note: For whole cell stain and F-actin kits, skip step 7 and 8.
7. Aspirate Primary Probe Solution and then wash twice with 100 µl/well of 1X Blocking Buffer.
8. Aspirate Blocking Buffer and add 50 µl/well of Staining Solution (secondary antibody). Incubate for 30 minutes protected from light at room temperature.
9. Aspirate solution and then wash plate three times with 100 µl/well of 1X Wash Buffer.
10. Add 100 µl/well of Whole Cell Stain Solution. Incubate for 15 minutes protected from light at room temperature.
11. Aspirate Whole Cell Stain Solution and wash plate three times with 100 µl/well of 1X Wash Buffer.
12. Aspirate Wash Buffer and replace with 150 µl/well of 1X Wash Buffer.
13. Seal plate and evaluate on the ArrayScan HCS Reader. Store plates at 4°C.

Additional Information

A. Dose Response and Time Course Curves

Dose response curves and time course graph (Figure 3) were generated with staurosporine and cytochalasin D treatment in NIH 3T3 cells. Cells were stained with DY554-phalloidin and antibody against tubulin to detect F-actin and microtubules, respectively. Cytoskeletal rearrangement was measured using the difference in cell perimeter (micrometer), F-actin fiber (fiber number per cell) and microtubule fiber (fiber number per cell). IC_{50} values of staurosporine and cytochalasin D and the half time ($t_{1/2}$) of cytoskeleton rearrangement by cytochalasin D are indicated. Perimeter and cytoskeleton fiber results were normalized to non-treated values and are represented as percentage.

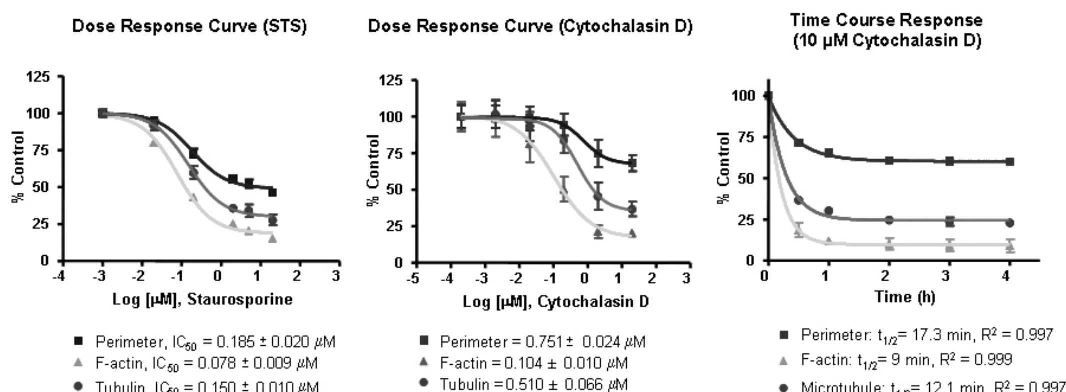

Figure 3. Response to the cytoskeleton drugs staurosporine and cytochalasin D. NIH 3T3 cells were maintained in DMEM complete media before drug treatment. In dose response experiments, staurosporine (STS) and cytochalasin D were treated for 3 hours. Data represents mean ±SD from three plates (16 wells per point in a plate).

B. Performance Robustness

The robustness of the multiplexed kit was ascertained by determining the Z′ for the average fiber numbers of F-actin and microtubules in non-treated wells (min, 0 µM) and wells treated with cytochalasin D (max, 10 µM). The mean ± SD of the Z′ was determined from three plates of NIH 3T3 cells that were treated identically. The Z′ values are as follows: Perimeter: 0.44 ± 0.06; F-actin: 0.43 ± 0.14; Microtubule: 0.36 ± 0.12.

C. Recommendations for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific Multidrop Combi or WellMate Dispensers.
- Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- Incubation: Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.
- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

Table 1. Suggested volumes to use for different cell culture plates.

Kit Component	96-Well Plates (µl/well)	384-Well Plates (µl/well)	24-Well Plates (µl/well)
Fixation Solution	100	25	400
1X Wash Buffer	100	25	400
Wash Buffer II	100	25	400
1X Permeabilization Buffer	100	25	400
1X Blocking Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

References

1. Furukawa R. and Fechheimer M. (1997). The structure, function, and assembly of actin filament bundles. *Int Rev Cytol* **175:** 29-90.
2. Glotzer, M. (2005). The molecular requirements for cytokinesis. *Science* **307:**1735-9.
3. Janmey, P.A. (1998). The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol Rev* **78:**763-81.
4. Gourlay, C.W. and Ayscough K.R. (2005). A role for actin in aging and apoptosis. *Biochem Soc Trans* **33(Pt 6):**1260-4.
5. White, S.R. *et al.* (2001). Initiation of apoptosis by actin cytoskeletal derangement in human airway epithelial cells. *Am J Respir Cell Mol Biol* **24:**282-94.
6. Yamazaki, D., *et al.* (2005). Regulation of cancer cell motility through actin reorganization. *Cancer Sci* **96:**379-86.
7. Dolle, L., *et al.* (2006). Anti-invasive/anti-metastasis strategies: new roads, new tools and new hopes. *Curr Cancer Drug Targets* **6:**729-51.

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