user manual

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G2M Cell Cycle Phase
Marker Assay

Amersham
Biosciences

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G2M Cell Cycle Phase Marker Assay

Amersham
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Front cover:

A field of U-2 OS cells expressing the G2M cell cycle phase marker with a single cell undergoing cell division. As the cell divides, the green fluorescent reporter initially moves from the cytoplasm to the nucleus (as the cell enters prophase) and then the fluorescence diminishes (as the cell completes metaphase).

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Chapter 1. Introduction

1.1 The Cell Cycle

The cell cycle (Figure 1.1) is the process by which cells replicate their DNA and divide, and is therefore one of the most fundamental processes occurring in eukaryotic cells (1,2). Literally a matter of life and death (2), the cell cycle is studied by scientists in a broad range of disciplines who are interested in understanding the mechanisms of this exquisitely regulated process or in elucidating targets for therapeutic intervention (3,4,5,6). The complexities of the cell cycle have been the subject of intense and varied study over the past century (7), and are likely to remain so for the foreseeable future. The G2M Cell Cycle Phase Marker assay allows researchers and screeners, to monitor the cell cycle phase of individual cells in real-time. In particular, the assay has been designed to resolve and quantify cells at the G2 to M transition point. The cell-based assay therefore has a number of potential applications, including screening for anti-proliferative compounds that maybe useful in treating cancer and other proliferative disorders and functional screening of orphan targets. The assay may also be used in secondary screening and lead optimization studies to detect undesirable toxic side-effects of lead compounds earlier in the drug development. In profiling studies, the assay could be used to investigate the cell cycle-dependence of key processes or pathways such as receptor-mediated signalling

Fig 1.1 The cell cycle. Cells that are not actively dividing are in G0 phase. When a signal is received to divide, the cell enters G1 phase. During G1 the cell becomes larger and prepares for DNA replication. In S-phase, a second copy of the genome is produced, thus doubling the amount of DNA within the cell. Once DNA replication is complete, the cell enters G2 phase, where proteins required for mitosis are synthesized and final checks on the integrity of the DNA are made. In mitosis (M phase), the cell divides to produce two daughter cells, each inheriting a copy of the entire genome.
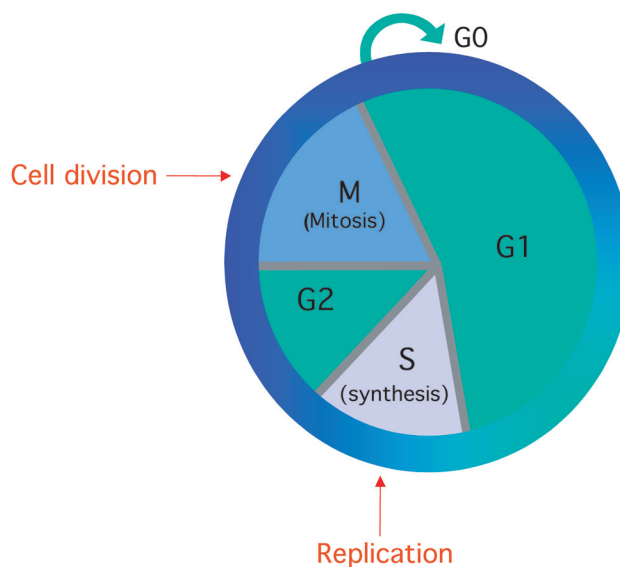
1.2. Cell Cycle Phase Markers

The G2M cell cycle phase marker (G2MCCPM) assay employs a non-destructive dynamic GFP-based probe to report the position of individual cells in the cell cycle (8). The probe (Figure 1.2) is comprised of cell cycle-dependent expression, destruction and localization elements from the gene for cyclin B1, a tightly-regulated cell cycle-dependent kinase that is expressed in late S-phase and is subsequently degraded during mitosis (Figure 1.3). By quantifying the location and fluorescence intensity of the expressed reporter molecule, the cell cycle position of individual cells can be pinpointed to one of four distinct phases of the cell cycle (Figure 1.4).

1.2.1. Cyclin B1 function

Cyclins are a family of proteins that bind to and activate cyclin-dependent kinases. Cyclins are produced at specific times during the cell cycle, and their expression levels and location are tightly controlled (9). Cyclin B1 was the first human cyclin identified, and is a 62 kDa protein encoded by a 1.6 kb mRNA (10). It is synthesized during the late S and G2 phases and complexes with the cell cycle dependent kinase p34cdc2. The key point in the transition of a cell from G2 to mitosis is the activation of a protein-serine/threonine kinase, which has been variously identified as MPF in frog eggs, as histone H1 kinase in sea urchin eggs and as growth-associated histone H1 kinase in mammalian cells. All these activities have a common component, a 32-34 kD protein, which in the yeast S. *pombe* is the product of the cdc2+ gene, and in S. *cerevisiae* is encoded by CDC28. The protein is sufficiently well conserved that antibodies raised against conserved epitopes cross-react with all the species tested. The human homolog of this protein p34cdc2 was identified by its ability to complement cdc2+ in yeast. The cdc2 kinase activity has been shown to vary through the cell cycle even though the level of the protein itself does not change. In HeLa cells cdc2 kinase activity is absent in G1 and increases through S, G2 and M phases in a manner that correlates with its association to cyclin B1 (11). The p34cdc2 binding site is located at the C-terminus of cyclin B1. We have deliberately excluded this region from our fusion protein so that the reporter molecule will not bind or activate p34cdc2 and therefore will act as a 'stealth sensor' that does not affect the cell cycle.

Fig 1.2. **G2M cell cycle phase marker design.** Location and expression of the fluorescent reporter are under the control of various cell cycle dependent elements. Synthesis is controlled by the cyclin B1 promoter which limits production to late S and G2 phases of the cell cycle. Destruction of the reporter is controlled by the cyclin B1 D-Box which mediates rapid degradation during mitosis. Location of the reporter is controlled by the cytoplasmic retention sequence (CRS) from Cyclin B1 which localizes the reporter to the cytoplasm until the start of mitosis, when it translocates to the nucleus.
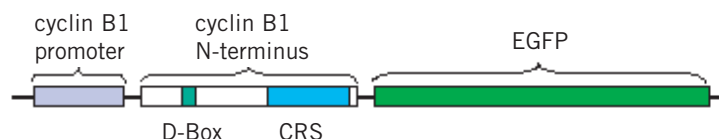
1.2.2. Cyclin B1 promoter

The human cyclin B1 promoter has been well characterized and the regions that are essential for cell cycle dependent expression have been identified (12). Human cyclin B1 mRNA appears at the end of the S phase and reaches its peak expression during G2. A 332-bp fragment upstream of the ATG codon is negatively regulated in quiescent cells, and its transcriptional activity depends on cell growth. Specifically, the region −150/-58 is required for promoter inactivation during quiescence. In the G2/M cell cycle marker technology the region from -150 to +180 is used to ensure S/G2-specific expression of the reporter molecule

1.2.3. *Cyclin B1 cytoplasmic retention sequence (CRS)*

Hagting *et al.* constructed a cyclin B1-GFP fusion protein encoding construct that was microinjected (either as pure protein or plasmid) into HeLa and fibroblast cells (13). The expressed fusion protein behaved identically to the constitutive cyclin B1 that was detected with an antibody (i.e. it was cytoplasmic for most of the cycle, moved into the nucleus during prophase and was rapidly degraded toward the end of mitosis). When the purified fusion protein was injected into the nucleus during S and G2 phases, the protein was rapidly and completely exported from the nucleus within 10 minutes after injection. The region containing the nuclear export signal was identified as an 11 amino acid hydrophobic stretch within the cytoplasmic retention sequence (CRS) (13).

Nuclear export has been reported to be mediated by at least two different "export factors": exportin1/CRM1, which is required for the export of the HIV Rev protein and IκBα, and exportin2/CAS which is responsible for the export of importin A. Nuclear export via exportin 1 is inhibited by the drug leptomycin B (13), and evidence implicates this pathway in the export of cyclin B1. When cells are treated with leptomycin B, cyclin B1 accumulates in the nucleus, showing that there is constitutive nuclear import occurring.The CRS of *Xenopus* cyclin B1 has been shown to bind to exportin 1 (14).

Phosphorylation of the CRS is required for nuclear trafficking of cyclin B1 (14). During S and G2 phase cyclin B1 shuttles between the nucleus and the cytoplasm because constitutive nuclear import is counteracted by rapid nuclear export. At M phase cyclin B1 is phosphorylated in the CRS. The nuclear export sequence (a region of the CRS) is then inactivated and the protein moves rapidly to the nucleus (Figure 1.3). The import of cyclin B1 into the nucleus occurs approximately 10 minutes before breakdown of the nuclear envelope as the cells enter prophase, the first stage of mitosis.

1.2.4. *Cyclin B1degradation box (D-box)*

Cyclin-dependent kinases (CDKs) promote progression through the cell cycle. By synthesizing and degrading CDK activators and inhibitors, the cell can be made to progress through the cell cycle and directly trigger the transition from metaphase to anaphase. King *et al* (15) describe how two distinct ubiquitin-conjugation pathways mediate proteolysis during the cell cycle. One pathway requires CDC34 and initiates DNA replication by degrading a CDK inhibitor. The second pathway involves a large protein complex called the "anaphase promoting complex" (APC) or "cyclosome" that initiates chromosome segregation and exit from mitosis by degrading anaphase inhibitors and mitotic

cyclins. The N-terminal domain of cyclin B1 contains a conserved 9 amino acid motif (RTALGDIGN) called the destruction box (D-box) that is necessary for cyclin B1 ubiquitination and subsequent degradation. Although deletion of the N-terminal region does not interfere with the capacity of mitotic cyclins to activate CDC2 and drive the cells into mitosis, these mutations dominantly arrest cell division in telophase. If the cyclin B D-box is grafted onto otherwise stable proteins, those proteins become unstable in mitosis (i.e. the D-box is portable). We have exploited this property of the cyclin B1 D-box to effect degradation of EGFP specifically at the end of metaphase.

Fig 1.3. **Cyclin B1 and the cell cycle.**
During G1 (lower right corner) cyclin B1 is absent, during S-phase (lower left corner) cyclin B1 is produced and begins to appear in the cytoplasm of the cell where it binds to CDC2. During G2 (upper right corner) the cyclin B1/CDC2 complex shuttles between the cytoplasm and the nucleus by a Crm1 dependent pathway. Because the rate of nuclear export is much faster than its import, the protein is localized predominantly in the cytoplasm. At the start of M-phase (prophase) (upper right corner) nuclear export is inhibited and the cyclin B1/CDC2 complex moves to the nucleus. As mitosis proceeds cyclin B1 is specifically degraded so that once the cells have re-entered G1-phase very little cyclin B1 is present (provided with permission from BioCarta, www.biocarta.com)
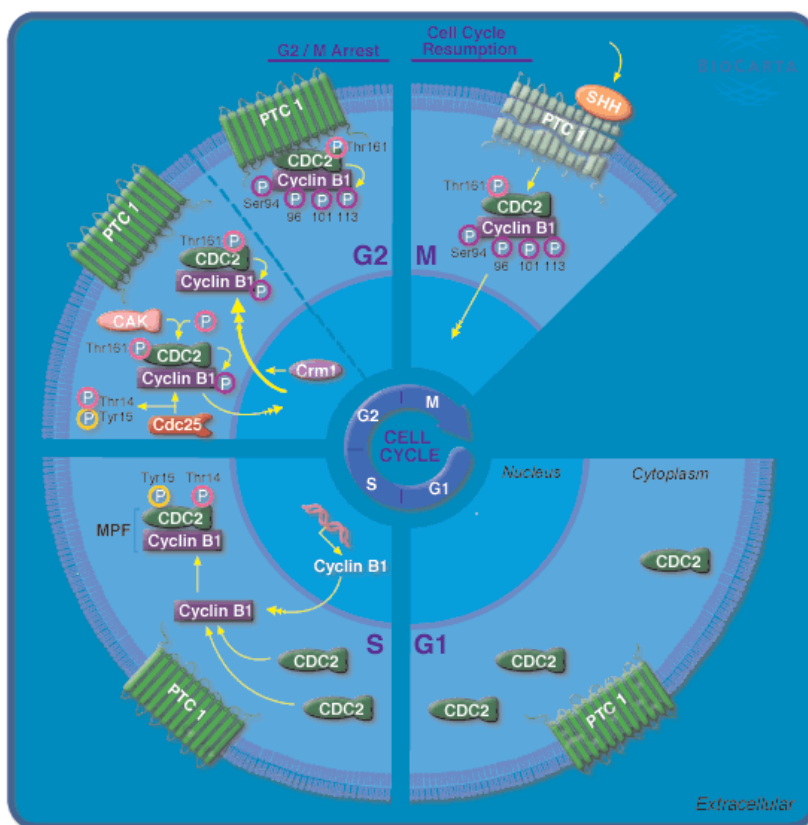
1.3. Applications in drug discovery

Since the CCPM reporter does not interfere with the cell cycle of the host cell, it allows non-destructive measurement of cell cycle position. The CCPM sensor has many potential applications in cultured cells and in more complex model systems. Perhaps most significantly, CCPM expression in stable cell lines allows continuous and individual monitoring of the cell cycle status of every cell in the culture and consequently removes the need to work with synchronized cells to identify cell cycle related phenomena.

One use of a stable cell line expressing the CCPM construct is to screen for anti-mitotic or anti-proliferative compounds that would be expected to arrest a proportion of cells in a certain phase of the cell cycle. Another potential

use is in toxicological screening during lead optimization. Earlier detection of toxic compounds that disrupt the cell cycle may be expected to reduce drug development costs.

Multiplexing with a second reporter may be valuable where it is suspected that an agent will exhibit cell cycle-dependent efficacy or toxicity. Use of the CCPM assay in conjunction with additional fluorescent probes for ligand binding, ion flux or other cellular processes will enable the cell cycle dependency of many signalling pathways utilized by drugs or other therapeutic regimes to be investigated.

Multiplexing the CCPM assay with other dynamic probes allows correlation of cellular events and processes with cell cycle position. A number of cell cycle-dependent responses to cell stimulation have been reported. For example, expression of endothelin receptors can be correlated with variation in endothelin-induced apoptosis (16). Vasopressin-induced calcium mobilization varies with cell cycle-dependent expression of different G-proteins (17). Cell cycle-dependent responses to the CCK-B/gastrin ligand CI-988 have also been observed (18). Cell cycle position can also significantly alter response to chemo-therapeutics and radiation. Wortmannin has been shown to preferentially radiosensitize cells in G1 (19) and similarly the result of combined taxol and radiation treatment have been shown to vary with the cell cycle (20).

Fig 1.4 Cell cycle-dependent CCPM reporter expression and location. There are four different patterns that can be distinguished: cells that are in G1/S (which are non-fluorescent or dimly fluorescent in the cytoplasm), cells that are in G2 (which are brightly fluorescent in the cytoplasm), cells in prophase (which are bright all over with nuclei brighter than the cytoplasm), cells in the rest of mitosis (which are rounded and intensely green in the entire cell.

G1/S   G2   Prophase   Mitosis
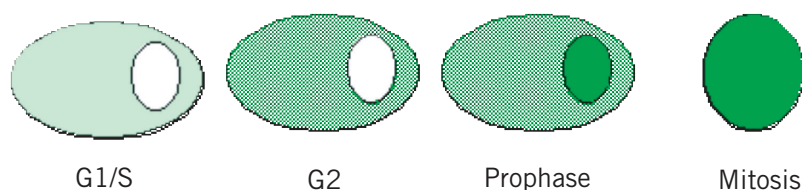
Chapter 2. Licensing considerations

2.1. Legal

Use of this product is limited as stated in the terms and conditions of sale. These vary in accordance with the product code purchased.

Description	Product Code
G2M Cell Cycle Phase Marker, Screening Applications	25-8010-50
G2M Cell Cycle Phase Marker, Research Applications	25-8010-51
G2M Cell Cycle Phase Marker, 6 month assay evaluation	25-8010-52
G2M Cell Cycle Phase Marker, 12 month assay evaluation	25-8010-53

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Chapter 3. Product contents

3.1. Components summary

● pCORON4004-CCEGFP expression vector (1 vial containing 10 μg DNA). Supplied in TE buffer (10 mM Tris, 1 mM EDTA pH8.0) NIF2034

● Cell cycle reporting cell line, U-2 OS derived (2 vials each containing 1 x 10^6 cells in 1 ml of fetal calf serum) NIF2033

● User manual

3.2. GFP- expression vector - pCORON4004-CCEGFP - NIF2034

The supplied plasmid, pCORON4004-CCEGFP, is 6.0 kb in length and contains a bacterial ampicillin resistance gene and a mammalian neomycin resistance gene. The cell cycle specific cyclin B1 promoter fragment can be excised from the vector using *Eco*RI and *Hind*III. The cyclin B1-N terminus-EGFP fusion protein can be excised using *Hind*III and *Sal*I. The sequence of the construct is available upon request; Please e.mail incellanalyzer@uk.amersham.com. A detailed restriction map is available in Chapter 11.

Fig 3.1. **Vector map of pCORON4004-CCEGFP expression vector**
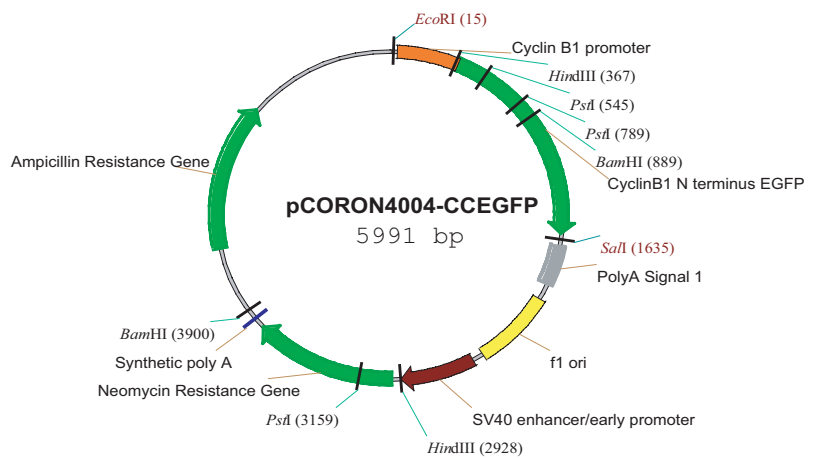
3.3. Cell cycle reporting, U-2 OS derived, cell line - NIF2033

3.3.1. U-2 OS parental cell line
The parental cell line U-2 OS (ATCC HTB-96) is a human osteosarcoma cell line derived from the thighbone of a15 year old Caucasian female (21,22,23). Unlike most human carcinoma cell lines U-2 OS is positive for p53 (24).

p53 is required to sustain the G2-phase arrest induced by DNA damage in tumor cells (25,26). Studies have shown that the p53 status of tumor cell lines is a crucial determinant of cellular sensitivity to chemotherapeutic agents because the growth of cell lines with mutant p53 is inhibited less than that of cells expressing wild type protein (27).

3.3.2. U-2 OS derived cell cycle reporting cell line

U-2 OS cells were transfected with the pCORON4004-CCEGFP vector using the FuGENE 6 Transfection Reagent method (Roche). Transfected cells were grown in the presence of Geneticin (G418, Sigma G-7034) at 1 mg/ml for approximately four weeks. The cells were then sorted on a high speed FACS into 96 well plates containing conditioned media (one fluorescent cell/well). After 10 days, the plates were imaged on the IN Cell Analyzer 3000 to determine which wells contained clonal cells. The clones were harvested, expanded and analyzed by flow cytometry. Clones that met our selection criteria were processed further and one of these lines C-8E6 is supplied. The cells are mycoplasma negative (details available on request).

3.4. Materials and equipment required

The following materials and equipment are required, but not provided.
- Microplates. For analysis using the IN Cell Analyzer 3000, Packard Black 96 Well ViewPlates (Packard Cat # 6005182) are recommended. For assays in 384 well format, please email incellanalyzer@uk.amersham.com for recommendations.
- A CASY 1 Cell Counter and Analyzer System (Model TT) (Schärfe System GmbH) is recommended to ensure accurate cell counting prior to seeding. Alternatively a hemocytometer may be used.
- Environmentally controlled incubator (5% CO_2, 95% relative humidity, 37 ℃)
- Imager/microscope (e.g. IN Cell Analyzer 3000 or IN Cell Analyzer 1000).
- Controlled freezing rate device providing a controlled freezing rate of 1 ℃ per min. (eg Nalgene "Mr Frosty", Sigma C1562)
- Standard tissue culture reagents and facilities (see also section 5.1.1.)

3.5. Software requirements

IN Cell Analyzer 3000: Images acquired using IN Cell Analyzer 3000 can be analyzed using Cell Cycle Trafficking Analysis Module product code 63-0050-71. Analyzed data are exported in the form of numerical files in ASCII format. These data can be utilized by Microsoft™ Excel, Microsoft™ Access, or any similar packages.

IN Cell Analyzer 1000: An analysis module for images acquired on IN Cell Analyzer 1000 is under development. Please contact your local representive for availability or e:mail incellanalyzer@amersham.com.

Other confocal or epifluorescence microscopes: Suitable software will be required for acquisition and analysis of images on these microscopes.

Chapter 4. Safety warnings, handling and precautions

4.1. Safety warnings

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

CAUTION! Contains genetically modified material

Genetically modified cells supplied in this package are for use in a suitably equipped laboratory environment. Users within the jurisdiction of the European Union are bound by the provisions of European Directive 98/81/EC which amends Directive 90/219/EEC on Contained Use of Genetically Modified Micro-Organisms. These requirements are translated into local law, which MUST be followed. In the case of the UK this is 'The GMO (Contained Use) Regulations 2000'. Information to assist users in producing their own risk assessments is provided in section 3.3.1 and 3.3.2 of 'The Genetically Modified Organisim (Contained Use) Regulations 2000'. http:/www.legislation,hmso.gov.uk/si/si2000/20002831.htm.

Risk assessments made under 'The GMO (Contained Use) Regulations 2000' for our preparation and transport of these cells indicate that containment 1 is necessary to control risk. This risk is classified as GM Class 1 (lowest category) in the United Kingdom. For handling precautions within the United States, consult the National Institute of Health's 'Guidelines for Research Involving Recombinant DNA Molecules'.

Instructions relating to the handling, use, storage and disposal of genetically modified materials:

1 These components are shipped in liquid nitrogen vapor. To avoid the risk of burns, extreme care should be taken when removing the samples from the vapour and transferring to a liquid nitrogen storage unit. When removing the cells from liquid nitrogen storage and thawing there is the possibility of an increase in pressure within the vial due to residual liquid nitrogen being present. Appropriate care should be taken when opening the vial.

2 Genetically modified cells supplied in this package are for use in a suitably equipped laboratory environment and should only be used by responsible persons in authorised areas. Care should be taken to prevent ingestion or contact with skin or clothing. Protective clothing, such as laboratory overalls, safety glasses and gloves should be worn whenever genetically modified materials are handled.

3 Avoid actions that could lead to the ingestion of these materials and NO smoking, drinking or eating should be allowed in areas where genetically modified materials are used.

4 Any spills of genetically modified material should be cleaned immediately

with a suitable disinfectant.

5 Hands should be washed after using genetically modified materials.

6 Care should be taken to ensure that the cells are NOT warmed if they are NOT being used immediately. To maintain viability DO NOT centrifuge the cells upon thawing.

7 Most countries have legislation governing the handling, use, storage, disposal and transportation of genetically modified materials. The instructions set out above complement Local Regulations or Codes of Practice and users of these products MUST make themselves aware of and observe the Local Regulations or Codes of Practice, which relate to such matters.

For further information, refer to the material safety data sheet(s) and / or safety statement(s).

4.2. Storage
The pCORON 4004-CCEGFP expression vector (NIF2034) should be stored at -15 ℃ to -30 ℃. The U-2 OS derived cells expressing the GFP fusion protein (NIF2033) should be stored at -196 ℃ in liquid Nitrogen vapour.

4.3. Handling
Upon receipt, the vector should be removed from the cryo-porter and stored at -15 ℃ to -30 ℃ until used.

The cells should be removed from the cryo-porter and transferred to a gaseous phase liquid nitrogen storage unit. Care should be taken to ensure that the cells are not warmed if they are not being used immediately. .

4.3.1. Vector
After thawing the DNA sample, centrifuge briefly to recover the contents.

4.3.2. Cells
Care should be taken to ensure that the cells are not warmed if they are not being used immediately. Do not centrifuge the cell samples upon thawing.

Chapter 5. Cell assay design

5.1. Culture and maintenance of U-2 OS derived cell cycle reporting cell line

5.1.1. Tissue culture media and reagents required

The following media and buffers are required to culture, maintain and prepare the cells for the assay.

● McCOYS 5A medium modified. Sigma M 8403
● Fetal Bovine Serum (FBS), Invitrogen life technologies 10099-141 or equivalent (Heat inactivated).
● Penicillin-Streptomycin (P/S), (5000 units/ml penicillin G sodium and 5000 µg/ml streptomycin sulfate), Invitrogen life technologies 15070-063 or equivalent
● Geneticin (G418), Sigma G-7034 or equivalent
● Trypsin-EDTA (1x) in HBSS w/o calcium or magnesium, Invitrogen life technologies 25300-054 or equivalent
● PBS Dulbecco's, w/o calcium, magnesium or sodium bicarbonate, Invitrogen life technologies 14190-094 or equivalent
● Dimethylsulfoxide (DMSO), Sigma D-2650 or equivalent
● L-Glutamine 200Mm (100 x). Gibco catalogue 25030-024
● L-Mimosine - Sigma M-0253
● Demecolcine (Colcemid; N-Deacetyl-N-methyl colchicine) – Sigma D1925 (10 ml sterile at 10 ug/ml)
● Standard tissue culture plastic-ware including tissue culture treated flasks (T-flasks), centrifuge tubes and cryo-vials

5.1.2. Reagent preparation

NOTE : the following reagents are required, but not supplied

● Growth-medium McCOYS 5A medium modified supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin, 1% (v/v) L-Glutamine and 1% (v/v) Geneticin (working concentration 1 mg/ml).
● Cryopreservation medium McCOYS 5A medium modified supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin , 1% (v/v) L-Glutamine and 10% (v/v) DMSO
● Nuclear-stain DRAQ5 (BioStatus). Prepare working solution of 10 µM DRAQ5 by diluting a 5 mM stock solution 1/500 in growth medium.
● Mimosine – Prepare a 20 mM stock solution by dissolving 25 mg in 6.25 ml of warmed growth media and place at 37 °C for 1 hour. Roller mix until dissolved (1-1.5 hours) and filter sterilize. The solution can be stored at 4-8 °C for up to 4 weeks. Dilute 1/10 in warm growth media before use.
● Demecolcine stock at 10 µg/ml. Dilute 1/100 in warm growth media before use.
● 4% Formalin solution Sigma HT 50-1-2.

5.1.3. Cell thawing procedure

Two cryo-vials, each containing 1 x 10^6 cells in 1 ml of Cryopreservation medium, are included with this assay. The vials are stored frozen in vapor phase liquid Nitrogen.

1. Remove a cryo-vial from storage.

2. Thaw the cells by holding the cryo-vial in a 37°C water bath for 1–2 min. Do not thaw the cells for longer than 3 min as this decreases viability.

3. Remove the cryo-vial from the water bath and wipe it with 70% (v/v) Ethanol. Immediately transfer the cells under asceptic conditions to a T flask containing 10-20 ml Growth-medium (depending on size of T-flask) at 37 °C.

NOTE: To ensure maximum cell viability, do not allow the cells to thaw at room temperature and do not thaw the cells using your hand to warm the vial.

5.1.4. Cell sub-culturing procedure
Incubation: 5% CO_2, 95% humidity, 37 °C.

Passage ratio: 1:5 to 1:20, twice a week. The cells should be passaged when they reach 70% to 95% confluence.

All reagents should be warmed to 37 °C.

1. Aspirate the medium from the cells and discard.

2. Wash the cells with 10-20 ml PBS depending on flask size. Take care not to damage the cell layer while washing, but ensure that the entire cell surface is washed.

3. Aspirate the PBS from the cells and discard.

4. Add trypsin-EDTA (2 ml for T-75 flasks and 5 ml for T-175 flask) ensuring that all cells are in contact with the solution. Remove the trypsin solution. Wait for 3 - 10 min for the cells to round up / loosen. Check on an inverted microscope.

5. When the cells are loose, dislodge the cells by adding Growth-medium (5 ml for T-75 and 10 ml for T-175), and resuspend by gentle agitation with a 10 ml pipette until all clumps have dispersed.

6. Aspirate the cell suspension and dispense the cells into a new culture vessel containing the amount of Growth-medium required to obtain the desired passage ratio.

5.1.5. Cell seeding procedure
This procedure is for cells grown in a standard T-175 flask and seeded into microplates. All reagents used for seeding cells should be pre-warmed to 37 °C.

1. Aspirate the medium from the cells and discard. Wash once with PBS.

2. Add 5 ml trypsin-EDTA to the cell surface and leave at 37 °C for approximately 3-10 min or until the cells loosen easily. Tap the flask gently to dislodge the cells.

3. Add 5 ml Growth-medium and gently resuspend the cells using a 10 ml pipette.

4. Spin the cell suspension at 1000rpm for 5 minutes and decant off the supernatant.

5. Resuspend cell pellet in 5-10 ml growth media.

6. Count the cells using either a CASY1 Cell Counter and Analyzer System (Model TT) or a hemocytometer.

7. Using fresh Growth-medium, adjust the cell density so that it will deliver the desired number of cells to each well. For example, to plate 3000 cells per well in 100 µl of suspension, the suspension is adjusted to 3×10^4 cells per ml.

8. Incubate the plated cells for 24 h at 37 °C, +5% CO_2 before starting the assay.

9. If the cells are near confluence prior to trypsinization, they should be passaged into two T-flasks. They will then be ready for seeding the following day.

5.1.6. Cell cryopreservation procedure

1. Harvest the cells as described in section 5.1.4 and prepare a cell suspension containing 1×10^6 cells per ml.

2. Pellet the cells at approximately 1000-g for 5 min. Aspirate the medium from the cells.

3. Resuspend the cells in cryopreservation-medium until no clumps remain and transfer into cryo-vials. Each vial should contain 1×10^6 cells in 1 ml of Freeze-medium.

4. Transfer the vials to a cryo-freezing device and freeze at -80 °C for 16–24 h.

5. Transfer the vials to the vapor phase in a liquid nitrogen storage device.

5.1.7. Growth characteristics

Under standard growth conditions, the cells should maintain an average size of 16.5 µm as measured using a CASY1 Cell Counter and Analyzer System (Model TT). The doubling time of the stably expressing cell line in exponential growth phase has been determined to be approximately 24h under standard conditions. This is identical to wild-type U-2 OS cells grown at the same time and under the same conditions, confirming that expression of the reporter molecule does not affect the normal cell cycle (Fig. 5.1).

Fig 5.1. **Growth curve of U-2 OS-derived cell lines**. Untransfected cells and cells expressing the cell cycle position marker have indistinguishable growth characteristics. Both cell lines have a doubling time of 24 hours $R^2 > 0.999$.
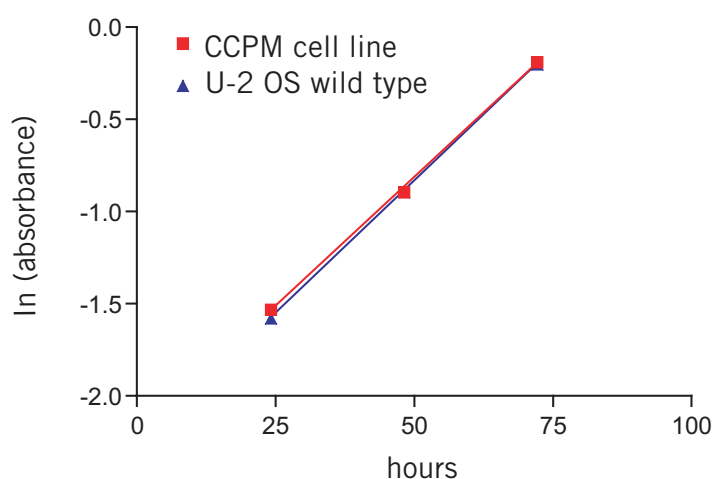
5.2. Assay set up

5.2.1. General Assay Set up

The cell cycle reporting cell line is an extremely flexible tool. It is important to decide how to perform the assay to get maximum benefit from the product. The assay can be performed as an endpoint or as a kinetic procedure and the cells can be imaged while still live or after fixation.

5.2.1.1. End point method

To facilitate analysis a nuclear marker is added. This should be added at the end of the assay test period as many nuclear stains will prevent normal cell division and completion of M phase.

5.2.1.2. Advantages of end point method

● Relatively fast; multiple images of the same cells are not required.
● Since each cell is only imaged once, bleaching of the sample is not a concern. This allows relatively high laser/lamp powers and longer imaging times to be used. This results in better image quality.
● Image analysis algorithms are available if using an IN Cell Analyzer 1000 or IN Cell Analyzer 3000.

5.2.1.3. Disadvantages of end point method

● When fine resolution of cell cycle position is required the relatively small proportion of prophase cells present necessitates imaging of over 1000 cells per condition to ensure statistically significant results.
● It can be difficult to differentiate cells undergoing mitosis and necrosis when only one image at a single time point is captured. Drugs that arrest cells in mitosis can be mistaken for toxic compounds and *vice versa,* leading to the potential for false positive or negative results.

5.2.1.4. Kinetic method

No nuclear marker is used and the only signal measured is from EGFP itself. The absence of nuclear marker allows the cells to divide repreatedly.

5.2.1.5. Advantages of kinetic method

● As the progress of cells is followed over time it is possible to obtain a large amount of information from a relatively small number of cells. For example, it is possible to determine whether a rounded cell is in mitosis (because it should become dim and split into two daughter cells) or whether it is dying. It is also possible to observe cells in prophase by following a relatively small number of cells but imaging frequently.
● Processes that can not be accurately spotted in an end-point assay (e.g. unusual drug-induced appearance or disappearance of the reporter molecule) are observable.

5.2.1.6. Disadvantages of kinetic method

● Automatic image analysis algorithms are not currently available.
● Photo-bleaching of the reporter or phototoxicity can result as a consequence of repeated exposure of the cells to high energy light sources. The consequent use of neutral density (ND) filters may reduce the

sensitivity of the assay.

● The use of intercalating nuclear dyes (Hoechst or DRAQ5) is not possible because these dyes inhibit mitosis.

5.2.2. Seeding and preparation of cells for a screen of cell cycle inhibiting compounds

Seed 3000 cells per well in a 96 well microplate or 0.8×10^3 cells per well in a 384 well microplate. Place in a humidified 37 ºC + 5% CO_2 incubator for 24 h as described in section 5.1.5.

It is recommended that cells are in log-phase growth and are maintained at 37 ºC during the assay. Reagents used during the assay should be pre-warmed to 37 ºC. It is essential that the number of cells per well in the assay plates is consistent in order to minimize assay variability. The following assay protocol is configured for 96 well microplates.

The cells should be seeded in the appropriate microplate the day before the experiment. Decant the culture medium and replace with Growth-medium containing 2mM mimosine, 0.1 µg/ml colchicine, test compound or Growth medium only (for controls). A typical 96-well microplate map containing control and test wells in a recommended configuration is shown in Fig 5.2.

Fig 5.2. A typical plate map for a cell cycle inhibitor screen

Control = Untreated cells
Colc Control = Cells treated with a compound that inhibits cells in mitosis e.g. 0.1µg/ml colchicine
Mimo Control = Cells treated with a compound that inhibits cells at the G1/S boundary e.g. 2mM Mimosine
Cmpd = Test Compound

	1	2	3	4	5	6	7	8	9	10	11	12
A	Colc Control	Cmpd #1	Cmpd #9	Cmpd #17	Cmpd #25	Cmpd #33	Cmpd #41	Cmpd #49	Cmpd #57	Cmpd #65	Cmpd #73	Control
B	Colc Control	Cmpd #2	Cmpd #10	Cmpd #18	Cmpd #26	Cmpd #34	Cmpd #42	Cmpd #50	Cmpd #58	Cmpd #66	Cmpd #74	Control
C	Colc Control	Cmpd #3	Cmpd #11	Cmpd #19	Cmpd #27	Cmpd #35	Cmpd #43	Cmpd #51	Cmpd #59	Cmpd #67	Cmpd #75	Mimo Control
D	Mimo Control	Cmpd #4	Cmpd #12	Cmpd #20	Cmpd #28	Cmpd #36	Cmpd #44	Cmpd #52	Cmpd #60	Cmpd #68	Cmpd #76	Mimo Control
E	Mimo Control	Cmpd #5	Cmpd #13	Cmpd #21	Cmpd #29	Cmpd #37	Cmpd #45	Cmpd #53	Cmpd #61	Cmpd #69	Cmpd #77	Mimo Control
F	Mimo Control	Cmpd #6	Cmpd #14	Cmpd #22	Cmpd #30	Cmpd #38	Cmpd #46	Cmpd #54	Cmpd #62	Cmpd #70	Cmpd #78	Colc Control
G	Control	Cmpd #7	Cmpd #15	Cmpd #23	Cmpd #31	Cmpd #39	Cmpd #47	Cmpd #55	Cmpd #63	Cmpd #71	Cmpd #79	Colc Control
H	Control	Cmpd #8	Cmpd #16	Cmpd #24	Cmpd #32	Cmpd #40	Cmpd #48	Cmpd #56	Cmpd #64	Cmpd #72	Cmpd #80	Colc Control

As explained in the IN Cell Analyzer 3000 user manual, each run must contain a flat field (FF) well to compensate for variations in fluorescence intensity across each image. It is possible to prepare a microplate solely for this purpose. Alternatively, a designated well on each plate can contain FF solution. When seeding the plate, this well must not contain any cells if the auxiliary flat field correction tool is to be applied in the analysis module. The plate map shown does not contain a FF well. Users should modify their plate accordingly, for example by replacing a compound well with the FF well.

5.2.3. End point screen

1. Seed 3000 cells per well in 100 µl of Growth-medium and incubate for 24 hours at 37 ºC, 5% CO_2.

2. Remove media from the plates by inversion and blotting onto a pad of sterile tissues. Prepare control wells containing either Growth medium, 2 mM mimosine (see section 5.1.2) or 0.1 µg/ml colchicine in Growth medium. Dilute the test compounds and dispense onto the cells. Each well should contain a final volume of 100 µl.

3. Incubate the microplates at 37 ℃, 5% CO_2 for 24 hours. In this time, the majority of untreated cells would be expected to complete a cell cycle. In the presence of a cell cycle perturbing drug the normal ratio of cells in each phase will be altered.

4. To assist in object identification it is necessary to stain the cell nuclei prior to imaging. Dilute nuclear stain (DRAQ5 or Hoechst 33342) to 10 µM and add 10 µl/well using a multi-channel pipette or automatic dispenser and mix.

5. Incubate for 15-20 minutes at 37 ℃ before imaging the plate.

5.2.4. Fixed cell screen

1. Seed 3000 cells per well in 100 µl of Growth-medium and incubate for 24 hours at 37 ℃, 5% CO_2.

2. Remove media from the plates by inversion and blotting onto a pad of sterile tissues. Prepare control wells containing either Growth medium, 2 mM mimosine (see section 5.1.2) or 0.1 µg/ml colchicine in Growth medium. Dilute the test compounds and dispense onto the cells. Each well should contain a final volume of 100 µl.

3. Incubate the microplates at 37 ℃, 5% CO_2 for 24 hours. In this time, the majority of cells would be expected to complete a cell cycle. In the presence of a cell cycle perturbing drug the normal ratio of cells in each phase will be altered.

4. Remove contents of wells by inverting plate onto a pad of sterile tissues and blot gently.

5. Add 200 µl /well of 4% formalin and incubate at room temperature in the dark for 30 minutes.

6. Remove contents of wells and wash plate once with 100 µl PBS.

7. Dilute the nuclear stain (DRAQ5 or Hoechst) to 1 µM and add 100 µl/well. Incubate at 37 ℃ for 30 minutes.

8. Remove contents of wells and wash plate twice with 100 µl PBS.

9. Leave the cells in 100 µl of PBS and seal the plate (to prevent evaporation). Store the plate at 4-8 ℃ in the dark until ready to image. Plates stored in this manner are stable for at least 2 weeks.

5.2.5. Kinetic assay for cell cycle perturbing drugs

1. Seed 3000 cells per well in 100 µl of Growth-medium and incubate for 24 hours at 37 ℃, 5% CO_2.

2. Remove media from the plates by inversion and blotting onto a pad of sterile tissues. Prepare control wells containing either Growth medium, 2 mM mimosine (see section 5.1.2) or 0.1 µg/ml colchicine in Growth medium. Dilute the test compounds and dispense onto the cells. Each well should contain a final volume of 100 µl.

3. Incubate the microplates at 37 ℃, 5% CO_2 for the desired period of time and image at defined time points. Temporal effects of cell cycle inhibiting drugs can be evaluated visually in the resulting time lapse movies of each well. For this assay, it is important to use high performance microimaging

equipment capable of returning to the same position in a well reliably. Care should be taken to optimize the excitation conditions to minimize detrimental effects on the cell (e.g. DNA damage by UV light) and to prevent photo-bleaching of the cells.

5.3. IN Cell Analysis System

The cell cycle position reporting assay has been designed as part of the IN Cell Analysis system and optimal results are obtained if the assay is performed on an IN Cell Analyzer 3000 or an IN Cell Analyzer 1000 instrument. Further advice on performing this assay as part of this system is included in the user manuals for the IN Cell Analyzer 1000, the IN Cell Analyzer 3000 and the Cell Cycle Trafficking Analysis Module.

5.3.1. IN Cell Analyzer 3000

When performing the assay on an IN Cell Analyzer 3000 in 96-well format it is recommended to use a Packard 96-well Viewplate. If planning to perform the assay in 384-format please contact incellanalyzer@uk.amershambiosciences.com for microplate details.

5.3.1.1. Kinetic assay using the IN Cell Analyzer 3000

Figure 5.3 shows selected frames of the same cell captured on the IN Cell Analyzer 3000. The cells were plated on a Packard Viewplate and imaged every 10 minutes for 8 hours. The figure shows a cell that is dividing normally. The six frames show a cell that was initially in G2 and that divides into two daughter cells.

Time-lapse movies showing the cell cycle position reporter in kinetic mode can be viewed by visiting http://www.amersham.com/drugscreening.

Fig 5.3. Cell cycle phase reporting U-2 OS cells imaged during a typical assay run performed on the IN Cell Analyzer 3000. Frames shown are a fraction ($1/75$ th) of the entire images captured.

G2   Prophase   Metaphase   Telophase   Cytokinesis   G1 phase
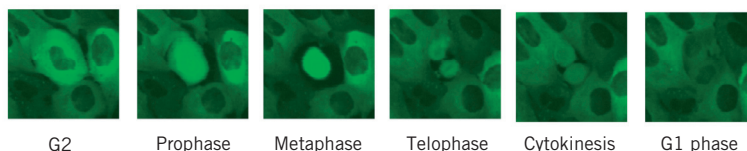
5.3.1.2. End point assay using the IN Cell Analyzer 3000

Images obtained from the IN Cell Analyzer 3000 are shown in Figure 5.4. The control image shows a few cells in mitosis. The number of mitotic cells is increased by treatment with colchicine and reduced by treatment with mimosine.

Fig 5.4. End-point assay images. Cells were seeded onto a Packard Viewplate at 3000 cells per well and incubated at 37 °C 5% CO2 for 24 hours before adding 2 mM mimosine or 0.1 µg/ml colchicine to the wells. After 24 hours, the wells were supplemented with 10 µM DRAQ5 (10 µl/well) and following a 37 °C incubation for 15 minutes the cells were imaged on an IN Cell Analyzer 3000. Cells in a range of different phases of the cell cycle are observed in the control wells. Predominantly mitotic cells are observed in the colchicine treated sample (bright rounded and in pairs). Evenly stained flattened cells predominante in the mimosine treated sample (almost all G1).

Control cells

Colchicine treated cells

Mimosine treated cells
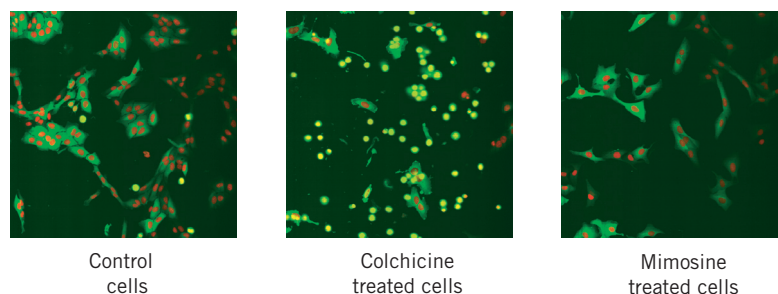
5.3.1.3. End point assay analysis using the IN Cell Analyzer 3000

On the IN Cell Analyzer 3000 the location and intensity of the cell cycle phase marker fusion protein can be determined using the Cell Cycle Trafficking analysis module. The number of cells in each of four cell cycle stages is then reported. A detailed description of this algorithm is provided in the Cell Cycle Trafficking Analysis Module user manual. The following is a brief description of the algorithm and its output.

The algorithm identifies the intensity of a region of the cytoplasm (Icyt), the intensity of the nucleus (Inuc) and the ratio between the nuclear and cytoplasmic intensities (nuc/cyt) (Figure 5.5).

Fig 5.5. Initial measurements for the cell cycle trafficking module analysis. The signal channel nuclear intensity, the cytoplasmic ring intensity and the ratio of the two values are determined.

Average pixel intensity in nuclear region gives Inuc

Ratio of nuclear to cytoplasmic intensities gives nuc/cyt

Average pixel intensity in the ring around the nuclear gives Icyt
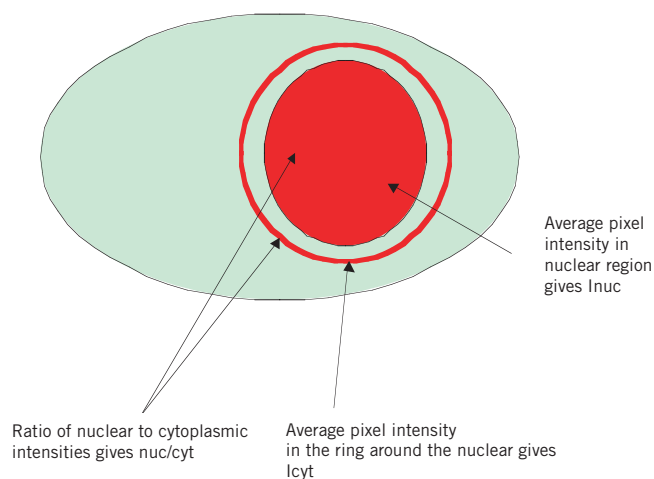
Fig 5.6. Four different phases of the cell cycle are distinguished by examining Inuc and I1-ratioI.

Once the nuclear/cytoplasmic ratio is determined it is used to calculate the modulus of one minus the ratio. As shown in Figure 5.6, four different phases in the cell cycle can be determined by analyzing Inuc and a I1-ratioI.

G1/S	G2	Prophase	Mitosis
Inuc - very low	Inuc - very low	Inuc - high	Inuc - very high
Icyt - Low	Icyt - High	Icyt - high	Icyt - high
Nuc/Cyt - low	Nuc/Cyt - very low	Nuc/Cyt - high	Nuc/Cyt - very high
I 1 - ratio I - low	I 1 - ratio I - high	I 1 - ratio I - low	I 1 - ratio I - high

Example numbers

Inuc	93	Inuc	261	Inuc	435	Inuc	738
Icyt	108	Icyt	669	Icyt	366	Icyt	295
Nuc/Cyt	0.87	Nuc/Cyt	0.39	Nuc/Cyt	1.19	Nuc/Cyt	2.5
I 1 - ratio I	0.13	I 1 - ratio I	0.61	I 1 - ratio I	0.19	I 1 - ratio I	1.5

Following image analysis, the population data are exported into a comma delimited text file which can be imported into Microsoft™ Excel (Figure 5.8)

Fig 5.7. **Data from a representative experiment exported to Microsoft Excel.**
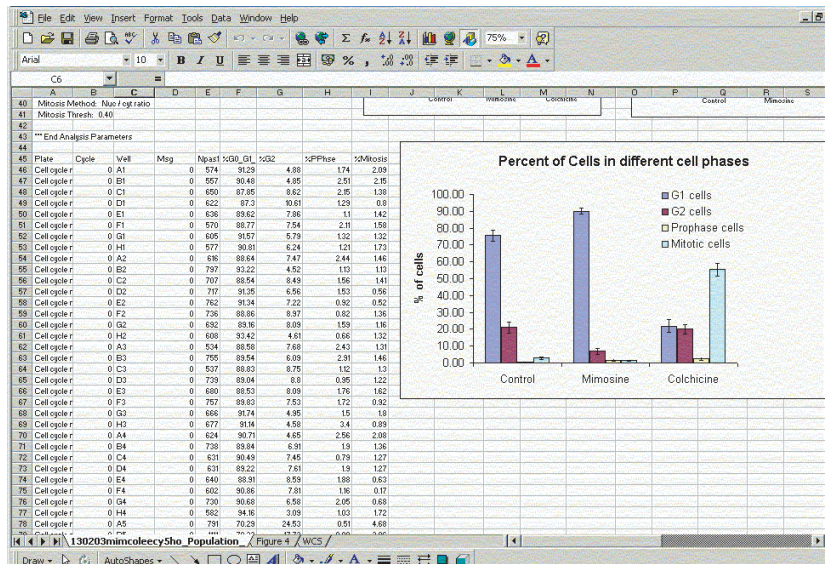
An example of the population distribution of cells in various phases of the cell cycle as determined by the Cell Cycle Trafficking Analysis algorithm is shown in Fig 5.8.

Fig 5.8 Population distribution of control cells in various phases of the cell cycle analyzed by the Cell Cycle Trafficking Analysis Module. IN Cell Analyzer 3000 assay.
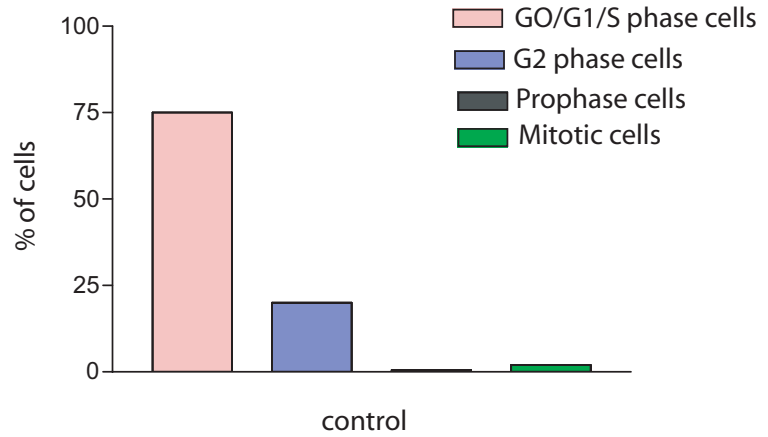
5.3.2. IN Cell Analyzer 1000

It is also possible to use the G2M cell cycle phase marker assay on the IN Cell Analyzer 1000 instrument and other non-confocal and non-laser based epifluorescent microscopes. (Figure 5.9)

An analysis module for images acquired on IN Cell Analyzer 1000 is under development.

Fig 5.9. Cell cycle position reporting cell line imaged on the IN Cell Analyzer 1000. Cells were treated as described in Figure 5.4 but were washed in PBS prior to imaging.
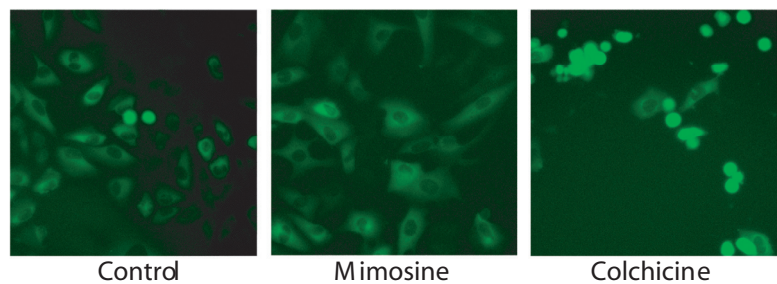
5.4. Cell cycle position reporting on epifluorescence microscopes

For speed of screening and quality of the images obtained, we recommend performing the cell cycle reporting assay on either the IN Cell Analyzer 1000 or the IN Cell Analyzer 3000. However, it is possible to adapt the assay to be read and analyzed on alternative imaging platforms.

Laboratory grade inverted epifluorescence microscopes such as the Nikon Diaphot or Eclipse models or the Zeiss Axiovert model are suitable for image acquisition. A high-quality objective (Plan/Fluor 40x 1.3 NA or similar) and epifluoresence filter sets compatible with GFP and the desired nuclear dye (if used) will be required. A motorized stage with multi-well plate holder and an environmental chamber are also recommended for assays performed on epifluorescence microscopes, and a suitable software package will be required for image analysis.

Fig 5.10 shows the analysis from a kinetic assay where an individual G2MCCPM cell was imaged repeatedly over 60 hours and three mitotic divisions on an Axiovert 100 microscope (Carl Zeiss, Welwyn Garden City, UK). The microscope was fitted with an environmental chamber capable of maintaining the stage at 37 °C, +/- 1°C and 5% CO_2 maintenance (Solent Scientific, Portsmouth, UK), and an ORCA-ER 12-bit, CCD camera (Hamamatsu, Reading, UK). Illumination was controlled by means of a shutter in front of the transmission lamp. An x,y positioning stage with separate z-focus (Prior Scientific, Cambridge, UK) was used to control multi-field acquisition. Image capture was controlled by AQM 2000 (Kinetic Imaging Ltd). All images were collected with a 40x 0.75 NA air apochromat objective lens providing a field size of 125x125 μm. Following collection of the images the average intensity of an area of the cell was determined at each time point using the Lucida software package (Kinetic Imaging). The level of reporter protein fluorescence was recorded in the cytoplasm from a region of interest (ROI) drawn in an area adjacent to the nucleus. The movement of the cell was compensated for during the time course. The intensity readout was background subtracted and normalized against basal levels of cyclin B1 and plotted against time. At mitosis, a large increase in average fluorescence is observed. The fluorescence is rapidly degraded as the cells enter G1 phase. This cycle is repeated each time the cell divides over the 60 - hour time-course shown in Fig. 5.11

Fig 5.10. **The average fluorescence intensity of a G2M CCPM-expressing U-2 OS cell followed over 60 hours**. During this time the cell undergoes three mitotic divisions. An increase in fluorescence is observed as the cell progresses through the cell cycle and a rapid reduction is seen immediately following each round of cell division.
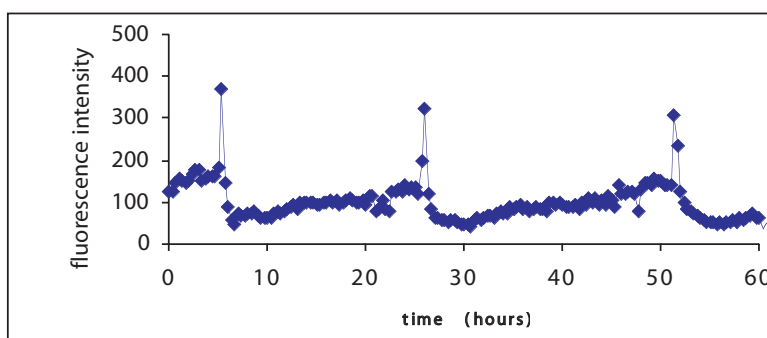
5.5. Cell cycle position reporting using flow cytometry

The G2M CCPM phase marker assay can also be used to report cell cycle using a flow cytometer. In Fig 5.11 the reporting cell line was grown in the presence or absence of either 2 mM mimosine or 100 ng/ml colchicine for 24 hours. The cells were then trypsinized and counted with a haemocytometer. 1 x10^6 cells were fixed and permeabilized using the cytofix/cytoperm kit (BD Pharmingen) following the manufacturer's instructions. The samples were incubated in 5 µg/ml propidium iodide, 0.4% Triton, 50 µg/ml RNase for 10 minutes prior to analysis on the FACScalibur flow cytometer (Becton Dickenson). The left side of the trace shows the histograms of the propidiumiodide (red) fluorescent channel and confirms that the mimosine and colchicine drug treatment have exerted the expected effects. The dot plots on the right hand side of Fig 5.11 show the green reporter fluoresence plotted against the red propidium iodide fluorescence. The two fluorescence signals are tightly correlated, showing that the GFP-based assay can be used as an alternative to flow cytometry analysis.

Fig 5.12. **Flow cytometry on fixed and propidium iodide stained cell cycle reporting cell line**.
a) Histogram of red fluorescence
b) Dot plot of red versus green fluorescence

a)

b)

CONTROL CELLS

Diagonal pattern confirms that cells with more GFP are in G2/M part of the cell cycle

Majority of cells are in G2/M

COLCHICINE TREATED CELLS

The majority of colchicine treated cells have a high green fluorescence

Majority of cells are in G1

MIMOSINE TREATED CELLS

The majority of mimosine treated cells have a low green fluorescence
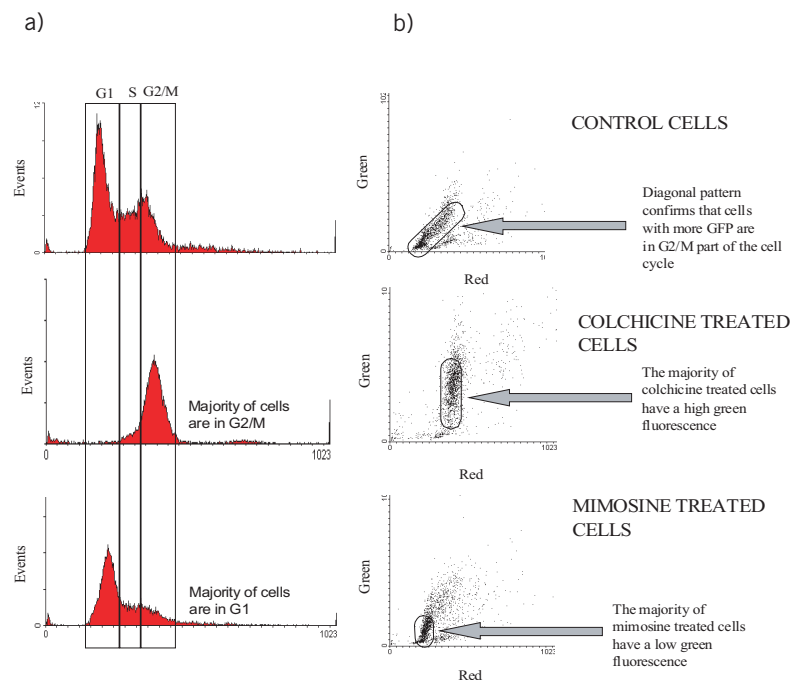
5.6. Assay characterization

5.6.1. Cell cycle validation

As part of the assay and image analysis validation process, G2M CCPM assay results obtained using the IN Cell Analyzer 3000 system and software were compared with results obtained by flow cytometry analysis of the G2M CCPM cell line . Sample preparations for each experiment were prepared in parallel from the same batch of G2M CCPM cells. For analysis using the IN Cell Analyzer 3000, cells were incubated in the absence or presence of cell cycle inhibiting compounds (colchicine or mimosine) as described in section 5.5. Following the incubation period, cells were imaged live and analyzed using the Cell Cycle Trafficking Analysis Module. for flow cytometry analysis, cells were treated, fixed and stained as descirbed in section 5.5 . The percentage of cells classified as G2M using the IN Cell analyzer 3000 and Cell Cycle Trafficking Analysis Module was 24.3% for control populations, 9.9% for mimosine-treated cells, and 78% for colchicine-treated cells (Fig 5.12).

These percentages closely agree with results obtained using flow cytometry analysis (Fig 5.13), where the corresponding values were 23.6% for control populations, 10.4% for mimosine-treated cells, and 67.8% for colchicine-treated cells. The results confirm that the compound treatment regimes specified in the G2M CCPM assay protocol are effective and that the image analysis module correctly identifies cells in G2/M phase.

Figure 5.12. Analysis using the IN Cell Analyzer 3000 system and software.
Mimosine treatment increases the percentage of cells in G0, G1 and S phases, and decreases the number of cells in G2/M. Colchicine treatment increases the percentage of cells in G2/M, but decreases the percentage of cells in G0, G1 and S phases. To allow comparison of IN Cell Analyzer 3000 results with flow cytometry data, the percentage of cells in G2/M was defined as the sum of the percentages of cells classified as G2, prophase or mitosis by the Cell Cycle Trafficking Analysis Module. Data bars represent the mean values obtained from 32 wells, with error bars showing standard deviation.
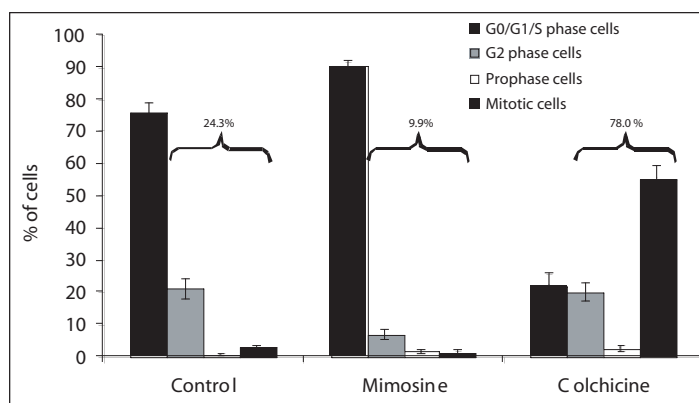
Figure 5.13. Flow cytometry analysis.
Following treatment for 24 h in the
absence or presence of cell cycle
inhibiting compounds, cells were fixed
and stained with propidium iodide. Flow
cytometry was performed as described
in section 5.5. Results are consistent
with those obtained using the IN Cell
Analysis system and software.

Sample	G2/M % of gate d
Control cells	23.6%
Mimosine treated cells	10.4%
Colchicine treated cells	67.8%
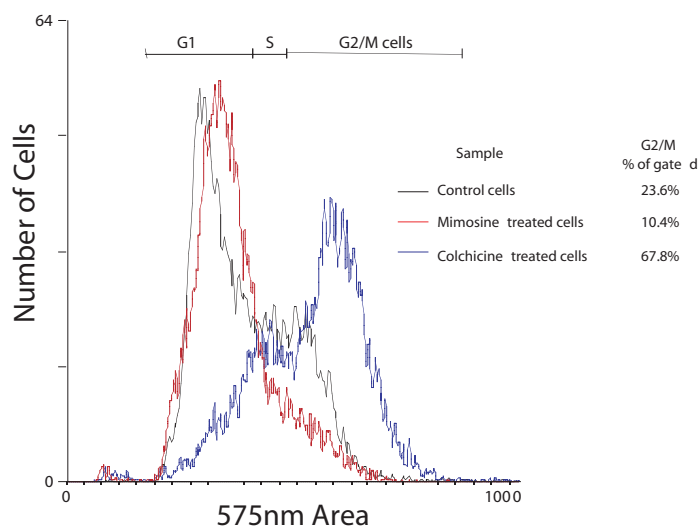
5.6.2. Colchicine dose response

Figure 5.15 shows a colchicine dose-response curve for the G2M CCPM
assay. Image data were acquired 24 hours after addition of the inhibitor. An
EC_{50} of 32 ng/ml was calculated from the dose-response curve. Equivalent
EC_{50} values were obtained from analysis of the increase in mitotic cells and
analysis of the disappearance of G0/G1/S phase cells with increasing
colchicine.

**Figure 5.14. Colchicine dose-response
using the supplied cell cycle phase
marker cells.** The data were collected
24 hours after addition of the drug,
yielding an EC50 of 32 ng/ml Error
bars indicate SD, n=8 replicates per
data-point and R2 > 0.96
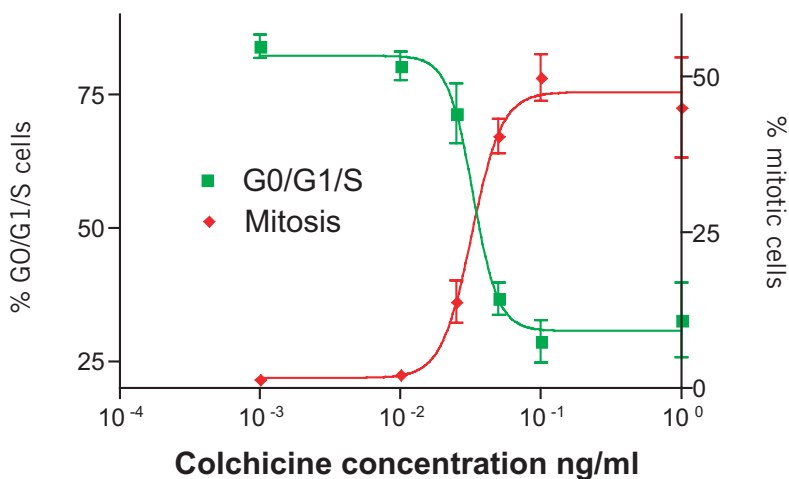
5.6.3. Leptomycin inhibition of nuclear export

Leptomycin B is an unsaturated, branched-chain fatty acid, and is an important tool in the study of nuclear export. It is a specific inhibitor of proteins containing a nuclear export signal (28) It has been reported that cyclin B1 translocation is inhibited by leptomycin B (29). The inhibition is thought to involve direct binding to CRM1 which prevents binding of CRM1 to proteins containing the nuclear export signal (30,31). Figure 5.16 shows the effects of 20 nM leptomycin B on the cell cycle position reporter cell line. The compound inhibits the export of the reporter molecule, confirming that the reporter molecule is continuously shuttling between the nucleus and cytoplasm.

Fig 5.15. Cells were imaged on the IN Cell Analyzer 3000 following a 2 hour treatment with 20 nM leptomycin B (or untreated as a control). Leptomycin B causes the reporter molecule to accumulate in the cell nuclei.

Control   Leptomycin treated
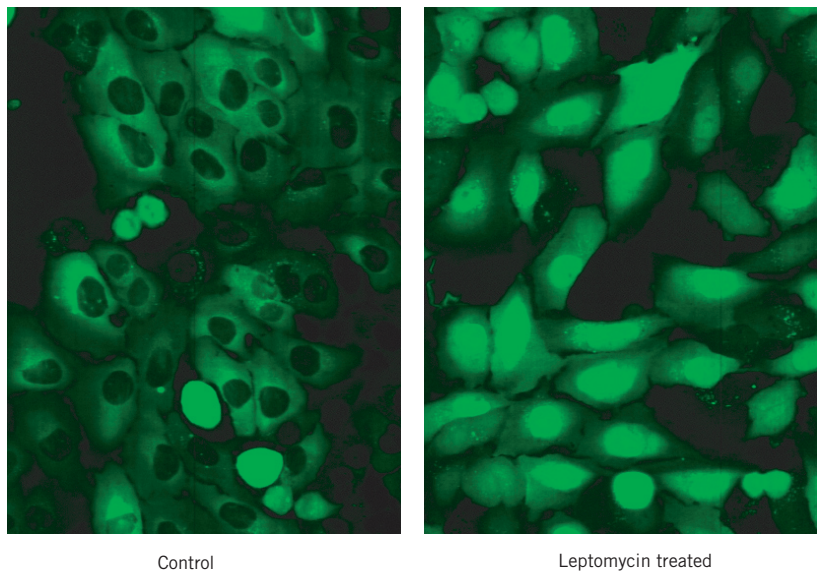
Chapter 6. Vector use details

The plasmid vector pCORON4004-CCEGFP (Fig 3.1.) can be used to transiently or stably express the cell cycle reporting fusion protein in the cell line of choice.

6.1. General guidelines for vector use

pCORON4004-CCEGFP has been used successfully to express the cell cycle reporting fusion protein transiently in MCF7, HeLa, A431 and U-2 OS cell lines and stably in the U-2 OS derived cell line. Expression levels and other assay parameters may vary depending on the chosen parent cell line and transfection procedure.

6.2. Transient transfection with pCORON4004-CCEGFP

Transfection protocols must be optimised for the cell type of choice. Both choice of transfection reagent and cell type will affect efficiency of transfection. FuGENE 6 Transfection Reagent (Roche) has produced successful results with pCORON4004-CCEGFP for a variety of cell lines. The following standard in-house protocols for adherent cells may serve as useful guidelines for establishing an appropriate protocol. For more information, refer to manufacturer's guidelines for the desired transfection reagent.

6.2.1. FuGENE 6 Transfection Reagent protocol
Day 1:
Seed cells so that the density will be 50–80% the next day.

Day 2:
● Add serum-free McCOYS 5A media to an empty tube.
● Add FuGENE 6 Transfection Reagent directly into this medium dropwise. Mix by gentle pipetting.
● Add the FuGENE 6 Transfection Reagent medium mix to the tube containing the DNA. Mix by gentle pipetting.
● Incubate for a minimum of 15 min at room temperature.
● Add transfection mixture directly to the cells dropwise without changing the medium, and mix by swirling gently.

Day 3
Change media to a complete McCOYS 5A without washing the cells.

Day 3/4
Cells are ready for use.

Stable cell lines may be obtained by sub-culturing 1:10 and selecting for resistant cells using Geneticin (Sigma G-7034).

6.3. Stable cell line generation with pCORON4004-CCEGFP

The process of establishing stable cell lines involves a large number of variables, many of which are cell-line dependent. Standard methods and guidelines for the generation of stable cell lines are widely available in the public domain (32).

pCORON4004-CCEGFP has been used to generate stably transfected cell populations. The magnitude of the response with different cell lines are unknown, and may deviate considerably from the values specified in this manual.

Selection with flow cytometer. Multiple selections of fluorescent and non-fluorescent cell lines sequentially will tend to select cells with the desired characteristics

Chapter 7. Quality control

7.1. Cell cycle position reporting cell line

The cell cycle position reporting cell line is supplied at a concentration of 1×10^6 cells per ml in fetal calf serum containing 10% (v/v) DMSO. The cell line has the characteristics detailed in Table 7.1.

Table 7.1.: Quality control information for cell cycle position reporting cell line

Property	Value	Measurement method
Viability from frozen	> 80 %	CASY1 Cell Counter and Analyzer System (Model TT)
Cell diameter (µm)	15–18	CASY1 Cell Counter and Analyzer System (Model TT)
Asynchronous cells fluorescence at 3×10^4 cells per ml (RFU)	> 40 000 for 20 passages after dispatch	FARCyte (Gain 61)

Flow Cytometry analysis

The supplied cell line was analyzed by flow cytometry to look at the variation in the intensity of GFP in the stable cell population compared to untransfected control cell population (Figure 7.1).

Fig.7.1. The intensity of GFP in the customer stocks of the stable cell line was compared to that in stable cells in culture at passages 9 and 24. There is no variation in the GFP intensity of the stable cells.
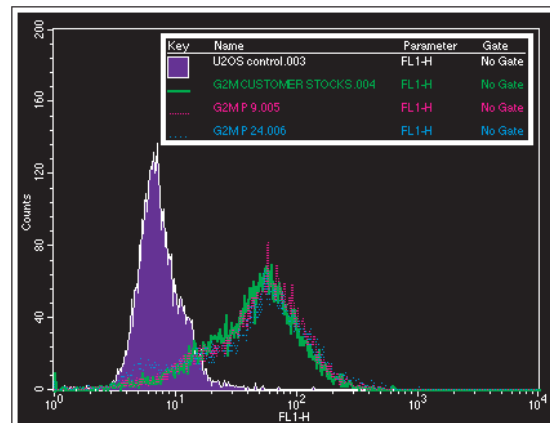
7.2. pCORON4004-CCEGFP expression vector

The pCORON4004-CCEGFP vector is supplied in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 250 µg/ml. The vector should have the characteristics outlined in Table 7.2.

Table 7.2.: Quality control information for the pCORON4004-CCEGFP expression vector

Property	Value	Limits	Measurement method
Concentration	250 µg/ml		UV Absorbance @ 260 nm in water
Purity - Minimal contamination of the DNA construct by RNA or protein	A_{260}/A_{280} ratio	Between 1.8–2.2	UV/Vis Absorbance @ 260 nm and 280 nm
Expected restriction	The restriction		Agarose gel

pattern digests should give electrophoresis
fragments of the
sizes shown in
Table 7.3.

Table 7.3.: Expected restriction pattern for the pCORON4004-CCEGFP expression vector

Enzyme(s)	# of cuts	Fragment(s) size (bp)
EcoRI	1	5991
BamHI	2	3011, 2980
SalI	1	5991
PstI	3	3377, 2370, 244
KpnI	3	4059, 999, 933
HindIII	2	3430,.2561

Chapter 8. Troubleshooting guide

8.1 Troubleshooting guide

❶

Low assay response. (positive vs. negative controls)

❷

Low nuclear intensity.

❸

Image is out of focus. (IN Cell Analyzer 3000 only).

❹

Cells do not adhere to well bottom in plate.

Possible causes and remedies

Possible cause

1.1. Passage number too high.
1.2. Cell density too low or too high.
1.3. Incorrect selection of analysis parameters.
1.4. Incorrect assay/incubation conditions.
1.5. Reagents were not stored properly or they are out of date.
1.6. Cells have been stressed during assay.

Remedy

1.1. Start a fresh batch of cells from an earlier passage number. Cells should be expanded, and additional vials should be frozen down from the vials delivered with the kit.
1.2. Verify density of cell plating; adjust plating density to values that yield optimal assay response.
1.3. Check that the primary parameters are correct and suitable for the cells currently in use.
1.4. Ensure that proper incubation is maintained as consistently as possible during the assay. When plates are out of the CO_2 incubator for extended periods, it is essential that HEPES buffer is added to the medium to maintain proper pH.
1.5. Repeat assay with fresh reagents.
1.6. Use actively growing cells maintained at 37 ºC. Pre-warm reagents to 37 ºC.

Possible cause

2.1. Nuclear stain concentration too low.
2.2. Nuclear stain incubation time too short.

Remedy

2.1. Adjust Nuclear stain concentration to recommended level.
2.2. Adjust Nuclear stain incubation time to recommended length.

Possible cause

3.1. Autofocus Offset is chosen incorrectly or the system may need to be realigned.

Remedy

3.1. Alignment and calibration of instrument. Perform Z-stack on cells. Change Autofocus Offset.

Possible cause

4.1. Plate is not treated correctly.
4.2. Plating density too high.
4.3. Cell cycle drugs block in mitosis.

Remedy

4.1. Poly-L-Lysine coat the plate if required.
4.2. Seed cells at a lower density.
4.3. Mitotic and rounded cells have a smaller surface area in contact with the

❺

Shading across image field. (IN Cell Analyzer 3000 only)

❻

Low cell number when using mitotic inhibitors (e.g. colchicine)

plate. Poly-L-Lysine coat the plate to increase adherence.

Possible cause
5.1. Flat field correction not applied or flat field solution too weak.
Remedy
5.1. Apply flat field correction or adjust flat field solution.

Possible cause
6.1. Cells lost when mixing contents of well with a pipette after addition of nuclear marker (e.g. after DRAQ5 addition).
6.2. Decanted contents of plate after treatment with a mitotic inhibitor (e.g. colchicine).
Remedy
6.1. Do not mix cells treated with a mitotic inhibitor (e.g. colchicine) with a pipette after nuclear dye (e.g. DRAQ5) addition. Mix these wells by tapping or swirling the plate gently.
6.2. After cells have been treated with a mitotic inhibitor (e.g. colchicine) do not remove contents of well prior to reading the assay.

Chapter 9. References

9.1. References

1. Nurse, P. (2000) The Incredible Life and Times of Biological Cells. Science 289, 1711-1716

2. Evan, G. and Littlewood, T. (1998) A matter of life and cell death. Science 281, 1317-22

3. Malumbres, M. and Barbacid, M. (2001) To cycle or not to cycle: a critical decision in cancer. Nat. Rev. Cancer 1, 222-31

4. Walker, M.G. (2001) Drug target discovery by gene expression analysis: cell cycle genes. Curr. Cancer Drug Targets 1, 73-83

5. Carnero, A. (2002) Targeting the cell cycle for cancer therapy. Br. J. Cancer 87, 129-337

6 Sampath, D. and Plunkett, W. (2001) Design of new anticancer therapies targeting cell cycle checkpoint pathways. Curr. Opin. Oncol. 13, 484-90

7. Nurse, P. (2000) A Long Twentieth Century of the Cell Cycle and Beyond. Cell 100, 71-78.

8. Thomas, N and Goodyer, I.D., 2003, Stealth sensors: real-time monitoring of the cell cycle, Targets, 2 (1), pp26-33

9. Pines, J., 1999, Four-dimensional control of the cell cycle, Nature Cell Biology, **1**, E73-E79

10. Pines, J. and Hunter A., 1989, Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2, Cell, **8**, 833-846

11. Clute, P and Pines, J., 1999, Temporal and spatial control of cyclin B1 destruction in metaphase, Nature Cell Biology, **1**, 82-87

12. Paiggio, G., Farina, A., Perrotti, D., Manni, I., Fuschi, P., Sacchi, A., and Gaetano, C., 1995, Structure and Growth-Dependent regulation of the Human Cyclin B1 promoter, Exp. Cell Res, **216**, 396-402

13. Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J., 1998, MPF localization is controlled by nuclear export, EMBO J., **17**, 4127-4138

14. Hagting, A., Jackman, M., Simpson, K. and Pines, J., 1999, Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal, Current Biology, **9** ,680-689

15. King, R.W., Deshaies, R.J., Peters, J.M., and Kirschner, M.W., 1996, How Proteolysis Drives the Cell Cycle, Science, 274, 1652-1659

16. Okazawa, M. et al. (1998) Endothelin-induced apoptosis of A375 human melanoma cells. J Biol. Chem. 273(20), 12584-92

17. Abel, A. *et a*l. (2000) Cell cycle-dependent coupling of the vasopressin V1a receptor to different G proteins. J. Biol. Chem. 275(42), 32543-51

18. Bestervelt, L. *et al.* (2000) Divergent proliferative responses to a gastrin receptor ligand in synchronized and unsynchronized rat pancreatic AR42J

tumour cells. Cell. Signal. 12(1), 53-61

19. Chernikova, S.B. *et al*. (2001) Cell cycle-dependent effects of wortmannin on radiation survival and mutation. Radiat. Res. 155(6), 826-31

20. Gorodetsky, R. *et al*.(1998) Paclitaxel-induced modification of the effects of radiation and alterations in the cell cycle in normal and tumor mammalian cells. Radiat. Res. 150(3), 283-91

21. Ponten J , *et al*. Two established in vitro cell lines from human mesenchymal tumours. Int. J. Cancer 2: 434-447, 1967

22. Heldin C.H , *et al*. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature 319: 511-514, 1986.

23. Raile K , *et al*. Human osteosarcoma (U-2 OS) cells express both insulin-like growth factor-I (IGF-I) receptors and insulin-like growth factor-II/mannose-6- phosphate (IGF-II/M6P) receptors and synthesize IGF-II: autocrine growth stimulation by IGF-II via the IGF-I receptor. J. Cell. Physiol. 159 : 531-541 , 1994.

24. Landers JE, *et al*. Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. Cancer Res. 57: 3562-3568, 1997

25 Bunz, F. et al., 1998, Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science, 282, 1497-1501

26 Flatt, P.M, et al, 2000, p53 Regulation of G2 checkpoint is retinoblastoma protein dependent, Mol Cell Biol, 20, 4210-4223

27 O'Commor, P.M. et al, 1997, Characterisation of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth inhibitory potency of 123 anticancer agents. Cancer Research, 57, 4285-4300

28 Ullman, K.S., Powers, MA and Forbes, D.J, 1997, Nuclear export receptors: from importin to exportin, Cell, 90(6), 967-970

29 Yang, J, Bardes E.S, Moore J.D, Brennan J, Powers M.A, Kornbluth S., 1998, Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1, Genes Dev, 12 (14), 2131-43

30 Nishi K, Yoshida M, Fujiwara D, Nishikawa M, Horinouchi S, Bepp T., 1994, Leptomycin B targets a regulatory cascade of CRM1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression, JBC, 269 (9), 6320-4

31 Henderson B.R, Eleftheriou A., 2000, A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals, Exp Cell Research, 256 (1), 213-224

32 Freshney, R.I. Cloning and selection of specific cell types in culture of animal cells, 3rd edition, Wiley-Liss Inc, chapter 11, pp161-178 (1994).

Chapter 10. Related products

10.1. Related products

Product Name: *Code:*

GFP Assays*
GFP-PLCδ-PH domain Assay   25-8007-26
GFP-Rac1 Assay   25-8007-27
GFP-MAPKAP-k2-Assay   25-8008-82
AKT1-EGFP Assay   25-8010-17
EGFP-2x FYVE Assay   25-8010-21

*Use of these products is limited in accordance with the type of license
purchased. Please contact your local representative for more details.

IN Cell Analysis System
IN Cell Analyzer 3000   25-8010-11
Cell Cycle Trafficking Analysis Module   63-0050-71
IN Cell Analyzer 1000   25-8010-26

Chapter 11. Appendix

11.1. Restriction map of pCORON4004-CCEGFP

The following enzymes do not cut the vector: *AccIII, AflIII, ApaI, AscI, BclI, BglII, BseAI, BsiWI, Bsp120I, BspEI, Bst1107I, BstEII, Bsu36I, Ecl136II, Eco47III, EcoRV, KspI, MluI, MroI, NdeI, NheI, NruI, PacI, PflMI, PmeI, SacI, SacII, SgrAI, SnaBI, SpeI, Sse8387I, SwaI, Van91I, XbaI.*

Enzyme	# of cuts	Positions (c) indicates the complementary strand
AatI	2	82 2911
AatII	1	4163
Acc65I	3	630 1629 2562
AccI	1	1636
AciI	73	11(c) 26(c) 109 169 223(c) 267(c) 270(c) 340 1104 1145 1212 1251 1389 1502 1562 1565 1645(c) 1649 1949 2010(c) 2024(c) 2027(c) 2055 2082 2460(c) 2486(c) 2499 2507(c) 2575(c) 2760 2772 2781 2793 2803 2814 2860 3015 3078 3172(c) 3236(c) 3337(c) 3340(c) 3580 3620(c) 3625 3675(c) 3691 3717 3773(c) 3832 3904 3942 3968 3978 4017 4191(c) 4238 4337(c) 4446(c) 4523(c) 4567 4688(c) 4734 4925(c) 5016(c) 5378 5387(c) 5522 5632(c) 5753(c) 5772(c) 5899(c) 5927(c)
AcsI	4	15 1757 2411 2422
AcyI	4	3106 3808 4160 4542
AflII	1	2960
AgeI	1	1603
AluI	31	32 347 369 505 583 859 906 939 1011 1044 1260 1308 1419 1593 1796 2141 2398 2588 2876 2930 3212 3670 4031 4050 4729 4792 4892 5413 5670 5716 5806
Alw44I	3	3913 4410 5656
AlwI	22	856(c) 884(c) 897 1380(c) 1579 1914(c) 1923 2517 3285 3350(c) 3531 3895(c) 3908 4443 4447(c) 4764 5227(c) 5228 5324(c) 5326 5412 5977(c)
AlwNI	5	586 658 682 694 5561
AosI	4	1977 2516 3208 4859
ApaLI	3	3913 4410 5656
ApoI	4	15 1757 2411 2422
AseI	2	407 4907
AsnI	2	407 4907
Asp700	2	124 4482
Asp718	3	630 1629 2562
AspEI	1	5082
AspHI	7	1495 3219 3409 3917 4414 4499 5660

Enzyme	# of cuts	Positions (c) indicates the complementary strand
AspI	1	3224
AsuII	1	3788
AvaI	4	1 36 1623 1640
AvaII	5	301 1542 3622 4718 4940
AviII	4	1977 2516 3208 4859
AvrII	1	2912
BamHI	2	889 3900
BanI	9	630 642 921 1629 2187 2562 3105 3140 5129
BanII	2	2157 3471
BbrPI	1	65
BbsI	1	800
BbvI	30	19(c) 41 177 251(c) 334(c) 337(c) 456(c) 1031(c) 1137 1421 1428 1454(c) 1457(c) 1783(c) 1990 2058 2529 3053(c) 3179 3221 3237(c) 3330(c) 3742 4037(c) 4648(c) 5039 5342(c) 5548(c) 5551(c) 5641
BcgI	2	1010 4544(c)
BfaI	10	92 242 1724 2075 2913 2967 4889 5224 5477 5987
BfrI	1	2960
BglI	3	1987 2865 4964
BlnI	1	2912
BmyI	15	783 926 1055 1304 1495 2157 3052 3145 3219 3409 3471 3917 4414 4499 5660
BpmI	3	1330 1570 5013
Bpu1102I	1	343
BpuAI	1	800
BsaAI	3	65 2228 3410
BsaBI	2	1914 3899
BsaHI	4	3106 3808 4160 4542
BsaI	1	5016
BsaJI	21	139 304 893 914 1054 1217 1241 1296 1623 1624 1640 2523 2624 2696 2819 2854 2863 2912 3269 3538 5810
BsaWI	6	321 1603 3137 4786 5617 5764
BsgI	3	1014(c) 1111 1435
BsiEI	8	136 162 1649 1958 3015 4564 4713 5636
BsiHKAI	7	1495 3219 3409 3917 4414 4499 5660
BsiYI	18	102 175 199 491 804 1055 1218 1568 2009 2335 2820 3087 3631 4044 5492 5771 5937 5955
BslI	18	102 175 199 491 804 1055 1218 1568 2009 2335 2820 3087 3631 4044 5492 5771 5937 5955

Enzyme	# of cuts	Positions (c) indicates the complementary strand
BsmAI	5	2957 4045 4087(c) 4240(c) 5016
BsmFI	6	526 2606(c) 2678(c) 2742(c) 3257 3789
BsmI	3	149(c) 1733 1826(c)
Bsp1286I	15	783 926 1055 1304 1495 2157 3052 3145 3219 3409 3471 3917 4414 4499 5660
BspDI	2	1918 3887
BspHI	3	4137 4242 5250
BspMI	3	2993(c) 3374 3824
BspWI	37	32 155 247 315 449 469 977 1037 1050 1094 1103 1957 1987 2019 2021 2063 2090 2120 2657 2729 2780 2859 2865 3097 3181 3204 3343 3349 3466 3502 3549 3816 3912 4964 5352 5924 5972
BsrBI	4	2084(c) 3719(c) 3773 4240(c)
BsrDI	3	3339 4848 5022(c)
BsrFI	7	161 1036 1603 2123 3425 3606 4997
BsrGI	1	1595
BsrI	17	591 646(c) 652(c) 664(c) 1497(c) 2317 2797(c) 3050 3251 4437 4607(c) 4876 4919 5037 5443 5555(c) 5568(c)
BssHII	1	3503
BstBI	1	3788
BstNI	17	74 79 141 355 393 931 1056 1168 1243 1297 2626 2681 2698 3493 5811 5824 5945
BstUI	23	26 130 132 452 1214 1532 2000 2024 2044 2420 2507 3172 3473 3505 3906 3986 4089 4091 4191 4523 5016 5346 5927
BstX	I	13827
BstYI	13	889 1385 2509 3277 3523 3900 4435 4452 5220 5232 5318 5329 5982
CeIII	1	343
CfoI	36	45 51 132 381 445 452 1175 1216 1532 1978 2002 2015 2024 2046 2072 2080 2517 3100 3108 3172 3209 3475 3505 3507 3735 3988 4091 4191 4523 4860 4953 5346 5455 5629 5729 5796
Cfr10I	7	161 1036 1603 2123 3425 3606 4997
ClaI	2	1918 3887
Csp45I	1	3788
Csp6I	8	631 1314 1596 1630 2563 3411 3924 4600
DdeI	19	236 343 370 490 659 683 695 725 1489 1507 2570 2872 3769 3920 4155 4581 5121 5287 5696
DpnI	31	135 430 863 891 1387 1535 1573 1917 1921 1957 2511 3279 3357 3438 3447 3525 3902 4401 4437 4454 4712 4758 4776 5117 5222 5234 5312 5320 5331 5406 5984

Enzyme	# of cuts	Positions (c) indicates the complementary strand
DpnII	31	133 428 861 889 1385 1533 1571 1915 1919 1955 2509 3277 3355 3436 3445 3523 3900 4399 4435 4452 4710 4756 4774 5115 5220 5232 5310 5318 5329 5404 5982
DraI	5	231 1873 4504 5196 5215
DraII	2	301 4102
DraIII	1	2231
DrdI	5	2275 2949 3133 3999 5868
DsaI	3	2523 2819 3538
DsaV	3	272 77 139 353 391 483 913 929 1054 1166 1241 1295 1567 1622 1623 1639 1640 1922 2624 2679 2696 3108 3268 3491 4008 4043 4544 4895 5591 5809 5822 5943
EaeI	10	75 957 1346 1646 3012 3186 3577 3604 3829 4689
EagI	2	1646 3012
Eam1105I	1	5082
EarI	6	257 355(c) 1936(c) 3450(c) 3660(c) 4283(c)
EclXI	2	1646 3012
Eco57I	8	563(c) 1039 1083(c) 1282 3252 3684 4416 5428(c)
EcoNI	1	802
EcoO109I	2	301 4102
EcoRI	1	15
EcoRII	17	72 77 139 353 391 929 1054 1166 1241 1295 2624 2679 2696 3491 5809 5822 5943
Esp3I	2	4045 4087(c)
EspI	1	343
Fnu4HI	55	27 30 33 166 169 265 268 348 351 470 1045 1104 1126 1410 1417 1468 1471 1565 1646 1649 1797 1979 2011 2025 2047 2518 2860 3015 3067 3078 3168 3173 3210 3251 3338 3341 3344 3580 3676 3717 3731 3832 3942 4051 4338 4567 4662 4689 5028 5356 5562 5565 5630 5773 5928
FnuDII	23	26 130 132 452 1214 1532 2000 2024 2044 2420 2507 3172 3473 3505 3906 3986 4089 4091 4191 4523 5016 5346 5927
FokI	12	387 788 856 913(c) 1279(c) 2763(c) 3430 3455 4000(c) 4643 4930 5111
FspI	4	1977 2516 3208 4859
HaeII	5	382 2073 2081 3109 5730
HaeIII	35	77 82 90 106 203 318 463 495 547 959 1059 1348 1460 1648 1947 2236 2378 2528 2853 2859 2868 2911 3014 3188 3579 3606 3831 4104 4691 4958 5038 5496 5930 5948 5959
HgaI	6	2006 3816 3992 4550 5280(c) 5858(c)
HgiAI	7	1495 3219 3409 3917 4414 4499 5660

Enzyme	# of cuts	Positions (c) indicates the complementary strand
HhaI	36	45 51 132 381 445 452 1175 1216 1532 1978 2002 2015 2024 2046 2072 2080 2517 3100 3108 3172 3209 3475 3505 3507 3735 3988 4091 4191 4523 4860 4953 5346 5455 5629 5729 5796
HinP1I	36	43 49 130 379 443 450 1173 1214 1530 1976 2000 2013 2022 2044 2070 2078 2515 3098 3106 3170 3207 3473 3503 3505 3733 3986 4089 4189 4521 4858 4951 5344 5453 5627 5727 5794
HincII	3	1609 1637 1812
HindII	3	1609 1637 1812
HindIII	2	367 2928
HinfI	12	21 386 1615 2276 2298 2934 3591 3725 3777 3884 5083 5600
HpaI	2	1609 1812
HpaII	32	87 162 322 485 914 977 1037 1568 1604 1624 1641 1923 2124 3011 3088 3110 3138 3269 3359 3426 3607 4010 4044 4545 4787 4897 4964 4998 5402 5592 5618 5765
HphI	15	369 381(c) 903(c) 1233 1257 1386 2228 3284(c) 4062(c) 4071(c) 4355(c) 4390 4596(c) 5012 5239
ItaI	55	27 30 33 166 169 265 268 348 351 470 1045 1104 1126 1410 1417 1468 1471 1565 1646 1649 1797 1979 2011 2025 2047 2518 2860 3015 3067 3078 3168 3173 3210 3251 3338 3341 3344 3580 3676 3717 3731 3832 3942 4051 4338 4567 4662 4689 5028 5356 5562 5565 5630 5773 5928
KasI	1	3105
KpnI	3	634 1633 2566
Ksp632I	6	257 355(c) 1936(c) 3450(c) 3660(c) 4283(c)
MaeI	10	92 242 1724 2075 2913 2967 4889 5224 5477 5987
MaeII	14	64 950 1163 1334 2117 2227 2270 2282 3222 3409 4160 4480 4853 5269
MaeIII	18	387 520 1068 1557 1782 2038 2050 3226 3532 4033 4421 4609 4762 4820 5151 5434 5550 5613
MamI	2	1914 3899
MboI	31	133 428 861 889 1385 1533 1571 1915 1919 1955 2509 3277 3355 3436 3445 3523 3900 4399 4435 4452 4710 4756 4774 5115 5220 5232 5310 5318 5329 5404 5982
MboII	24	244(c) 372 438 573 721 802 805 862 1128(c) 1173(c) 1176(c) 1371 1953 2089(c) 2929(c) 3467 3677 3757(c) 4300 4409 4487 5242 5313(c) 5465(c)
McrI	8	136 162 1649 1958 3015 4564 4713 5636
MfeI	1	1821
MluNI	2	77 3188
MnlI	48	10 47(c) 106 124 205(c) 231(c) 329 356(c) 485(c) 486 629 759 894(c) 975(c) 981(c) 1075 1212(c) 1224(c) 1275(c) 1395(c) 1657(c) 1857(c) 1897 1937(c) 2201 2541(c) 2549 2565(c) 2843(c) 2849(c) 2873 2879 2886(c) 2889(c) 2901(c) 3021(c) 3157(c) 3514(c) 3707 4056(c) 4115 4709(c) 4915(c) 5062 5143 5543 5793(c) 5867

Enzyme	# of cuts	Positions (c) indicates the complementary strand
MscI	2	77 3188
MseI	2	5230 407 704 1608 1669 1811 1872 2018 2289 2387 2404 2415 2427 2438 2961 3950 4131 4503 4868 4907 5142 5195 5209 5214 5266
MslI	11	774 916 1066 1243 1372 3543 3825 3864 4311 4670 4829
MspA1I	8	32 342 2588 3212 3980 4446 5387 5632
MspI	32	87 162 322 485 914 977 1037 1568 1604 1624 1641 1923 2124 3011 3088 3110 3138 3269 3359 3426 3607 4010 4044 4545 4787 4897 4964 4998 5402 5592 5618 5765
MunI	1	1821
MvaI	17	74 79 141 355 393 931 1056 1168 1243 1297 2626 2681 2698 3493 5811 5824 5945
MvnI	23	26 130 132 452 1214 1532 2000 2024 2044 2420 2507 3172 3473 3505 3906 3986 4089 4091 4191 4523 5016 5346 5927
MwoI	37	32 155 247 315 449 469 977 1037 1050 1094 1103 1957 1987 2019 2021 2063 2090 2120 2657 2729 2780 2859 2865 3097 3181 3204 3343 3349 3466 3502 3549 3816 3912 4964 5352 5924 5972
NaeI	2	2125 3608
NarI	1	3106
NciI	15	485 915 1569 1624 1625 1641 1642 1924 3110 3270 4010 4045 4546 4897 5593
NcoI	3	2523 2819 3538
NdeII	31	133 428 861 889 1385 1533 1571 1915 1919 1955 2509 3277 3355 3436 3445 3523 3900 4399 4435 4452 4710 4756 4774 5115 5220 5232 5310 5318 5329 5404 5982
NgoMI	2	2123 3606
NlaIII	25	438 1122 1152 1347 1542 1587 1689 2527 2660 2732 2823 2980 3325 3511 3542 3568 4057 4141 4246 4639 4675 4753 4763 5254 5974
NlaIV	24	107 464 632 644 891 923 1461 1631 2156 2168 2189 2564 2630 2702 3107 3142 3902 4195 4785 4996 5037 5131 5903 5942
NotI	1	1646
NsiI	2	2662 2734
NspI	4	2660 2732 3511 4057
NspV	1	3788
PaeR71	1	1
PinAI	1	1603
PleI	6	394 2284 2292(c) 3771(c) 5091 5594(c)
PmaCI	1	65
PmlI	1	65
Ppu10I	2	2658 2730

Enzyme	# of cuts	Positions (c) indicates the complementary strand
PpuMI	1	301
Psp1406I	2	4480 4853
PstI	3	545 789 3159
PvuI	3	136 1958 4713
PvuII	3	32 2588 3212
RcaI	3	4137 4242 5250
RsaI	8	632 1315 1597 1631 2564 3412 3925 4601
RsrII	1	3622
SalI	1	1635
SapI	3	257 3450(c) 3660(c)
Sau3AI	31	133 428 861 889 1385 1533 1571 1915 1919 1955 2509 3277 3355 3436 3445 3523 3900 4399 4435 4452 4710 4756 4774 5115 5220 5232 5310 5318 5329 5404 5982
Sau96I	15	105 301 316 462 1058 1459 1542 1946 2234 3622 4102 4718 4940 4957 5036
ScaI	1	4601
ScrFI	32	74 79 141 355 393 485 915 931 1056 1168 1243 1297 1569 1624 1625 1641 1642 1924 2626 2681 2698 3110 3270 3493 4010 4045 4546 4897 5593 5811 5824 5945
SexAI	1	2679
SfaNI	22	834(c) 984(c) 1262 1277 1376 1755(c) 2447(c) 2487 2669 2741 3064(c) 3319(c) 3405 3469 3535(c) 3744 3928(c) 4022 4381(c) 4630 4821(c) 5873(c)
SfcI	7	541 785 2005 3155 4836 5514 5705
SfiI	1	2865
SfuI	1	3788
SmaI	2	1625 1642
SnoI	3	3913 4410 5656
SphI	3	2660 2732 3511
SspBI	1	1595
SspI	2	2436 4277
StuI	2	82 2911
StyI	6	304 893 2523 2819 2912 3538
TaqI	24	2 19 118 400 935 1229 1256 1271 1400 1636 1677 1918 2193 2955 3219 3375 3399 3435 3597 3788 3887 4428 5872 5977
TfiI	6	21 1615 2934 3591 3725 3884
ThaI	23	26 130 132 452 1214 1532 2000 2024 2044 2420 2507 3172 3473 3505 3906 3986 4089 4091 4191 4523 5016 5346 5927

Enzyme	# of cuts	Positions (c) indicates the complementary strand
Tru9I	25	230 407 704 1608 1669 1811 1872 2018 2289 2387 2404 2415 2427 2438 2961 3950 4131 4503 4868 4907 5142 5195 5209 5214 5266
Tsp509I	15	6 15 404 821 1757 1821 2411 2422 2448 2666 2738 2830 4649 4904 5210
Tth111I	1	3224
XhoI	1	1
XhoII	13	889 1385 2509 3277 3523 3900 4435 4452 5220 5232 5318 5329 5982
XmaI	2	1623 1640
XmaIII	2	1646 3012
XmnI	2	124 4482