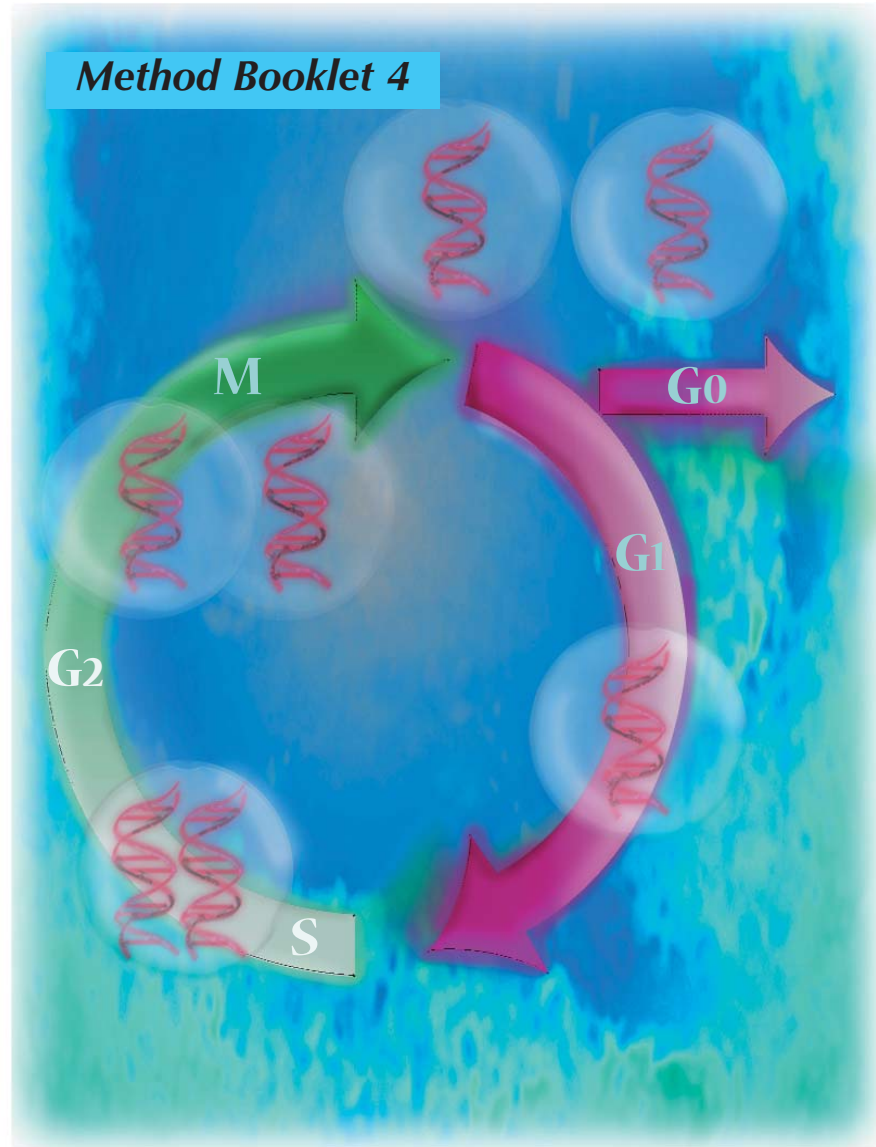


Method Booklet 4



Cell Cycle Methods



Cell Cycle Methods

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Introduction

The cell cycle is the universal process by which cells reproduce, and it underlies the growth and development of all living organisms. The most important events of the cell cycle are those concerned with the copying and partitioning of the hereditary material i.e., replicating chromosomal DNA during S phase, and separating the replicated chromosomes during mitosis. The controls operate by regulating the onset of these events and compensating for errors in their execution. The precision with which cell cycle events are executed ensures the survival of living organisms, while loss of this precision increases genomic instability, usually resulting in the formation of cancer. It is in cancer research where investigators determine the processes involved in the regulation of the cell cycle which can lead to more effective treatment of tumors and ultimately lead to a cure for the disease.

The known events involved in regulating the mammalian cell cycle are depicted in diagram 1:

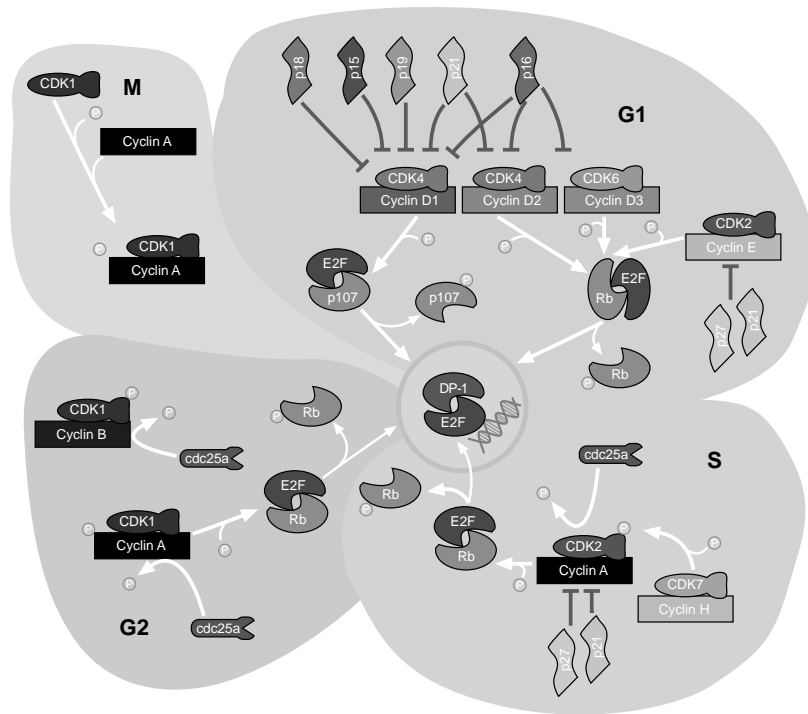


Diagram provided by BioCarta (www.biocarta.com)

Briefly, it was observed that the levels of a family of proteins, now known as cyclins, fluctuated through the cell cycle of marine invertebrates. It was reasoned that these proteins were important for the progression of the cell cycle. This led to the discovery of the kinase complexes, known as cyclin dependent kinases (CDKs), which have a catalytic protein kinase subunit and a regulatory cyclin subunit. These were shown to act as universal regulators of the cell cycle from yeast to mammals. Different CDKs control the onset of S phase and M phase and increasing activity of these CDKs can advance both events. CDKs are regulated by various mechanisms including the availability of the cyclin regulatory subunit, the phosphorylation of the catalytic subunit and by the presence of inhibitory subunits. As stated above, the expression of the cyclin subunits fluctuate throughout the cell cycle. This is also true of the inhibitory subunits such as p16INK4a and p21CIP. The expression of these proteins is regulated through transcription/translation. Degradation is regulated through phosphorylation "marking" and ultimately by ubiquitin-mediated proteolysis. The events regulating the phosphorylation of the catalytic subunits are described further in chapter IV.

This booklet describes the basic methods used to study cell proliferation and effective methods used to synchronize cells. In addition, methods using traditional antibody technology are described to study protein expression as well as the use of phosphorylation state-specific antibodies which can determine the regulatory state of cell cycle proteins.

Cell Proliferation

For many cell cycle researchers, the ability to maintain and manipulate cells in culture is a valuable tool. Cell culture models permit the study of a single cell type. Cell culture models also permit measurement of cell responses to conditions which are more tightly regulated than the complex environment presented by an entire organism. Cell responses, which are measured using cells in culture, include alteration in gene expression, production of chemical signaling agents (e.g., nitric oxide), and chemotaxis, as well as determination of proliferation and viability.

Cell proliferation assays are of particular use in cell cycle research as they allow direct quantitation of the ability of natural or synthetic signaling molecules (e.g., EGF, PDGF, VEGF etc.) to stimulate cell growth. In addition, these assays are valuable tools for drug discovery in the area of cancer research. Proliferation assays enable the researcher to measure the efficacy of anti-cancer agents at inhibiting cell division, thus facilitating the discovery process.

Methods of Assessing Cell Proliferation

To study proliferation, several methods are available. Researchers can either detect an antigen which is present in proliferating cells but absent in non-proliferating cells, measure DNA synthesis, or monitor the reducing environment of the cells. The following is a brief summary of these methods:

A. Detection of Antigens Associated with Proliferation

The appearance of several antigens during cell proliferation have been noted. A frequently cited antigen used to monitor proliferation of human cells is the antigen recognized by the monoclonal antibody Ki-67. Because this antigen is expressed during S, G₂, and M phases of the cycle but absent in G₀ and G₁, this monoclonal antibody is also suitable for examination of the cell cycle. Another such marker is proliferating cellular nuclear antigen (PCNA) which is essential for DNA synthesis. The monoclonal antibody, clone PC10, can be used to detect PCNA in tissue sections as well as at the single cell level using flow cytometry.

Antibodies directed to cyclins, proteins which are expressed during specific phases of the cell cycle, also have utility in monitoring cell proliferation. For example, cyclin E is expressed as cells move from the G₁ phase to the S phase. Cyclin E is observed to bind to a cyclin dependent protein kinase 2 (CDK2) subunit. The interaction of cyclin E with the CDK2 complex regulates a serine/threonine kinase activity which in turn regulates the progression of the cell cycle.

The use of monoclonal antibodies to detect proliferation requires that the cells under examination be processed for immunostaining. Because of this requirement, use of monoclonal antibodies can only be performed at pre-determined time points;

therefore, no continuous monitoring of a single sample can be performed with this method.

For a description of immunostaining methods frequently used to detect these antigens see section III, pages 22-45.

B. There are two methods of measuring DNA synthesis: (a) quantitation of ³H-thymidine incorporation and (b) quantitation of 5-bromodeoxyuridine (BrdU) incorporation.

1. ³H-Thymidine Incorporation

When supplied to proliferating cells, ³H-thymidine is incorporated into nascently synthesized DNA. This easy method provides an accurate indicator of DNA synthesis. Quantitation typically involves scintillation counting of labeled cells collected by aspiration upon membrane filters. Because this method is sample destructive, continuous monitoring of a single sample is not possible. This method will not be presented.

2. Bromodeoxyuridine Incorporation

Like ³H-thymidine, bromodeoxyuridine (BrdU) is incorporated into nascently synthesized DNA and provides a measurement of cell proliferation. The incorporation of BrdU is detected by an indirect method, most commonly with BrdU-specific antibody conjugated to a reporter system such as a fluorochrome or an enzyme suitable for use in immunohistochemistry. The major advantage of this method is that animals can be injected with BrdU which is incorporated into proliferating cells. Tissue sections from the animal can be easily analyzed by immunostaining. The demonstration that BrdU is incorporated into hippocampal cells of patients receiving aggressive chemotherapy disproved the long-held assertion that cells in the adult human brain are not able to divide. As with ³H-thymidine, the use of BrdU requires processing of samples at predetermined time points; therefore, no continuous monitoring of a single sample can be performed.

C. alamarBlue™ Reduction

The internal environment of proliferating cells is more reduced than that of non-proliferating cells. Specifically, the ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD increase during proliferation. Compounds such as alamarBlue™, which can be reduced by these metabolic intermediates, can be used to monitor cell proliferation. The oxidation-reduction potential of alamarBlue™ is +380 mV at pH 7.0, 25°C. alamarBlue™, therefore, can be reduced by NADPH (E_o=-320 mV), FADH (E_o=-220 mV), FMNH (E_o=-210mV), NADH (E_o=-320 mV), as well as the cytochromes (E_o=290 mV to +80 mV). As alamarBlue™ accepts electrons from these compounds, it changes from the oxidized indigo blue, non-fluorescing state to the reduced fluorescent pink state. Proliferation can then be monitored spectrophotometrically either by color measurement or fluorescence.

Since alamarBlue™ is non-toxic, it allows continuous monitoring of cells in culture. For example, cells grown in the presence of alamarBlue™ and subsequently analyzed by flow cytometry for CD44, CD45RB, CD4, and heat stable antigen, are found to produce similar numbers of viable cells and antigen expressing cells as non-exposed cells. Because alamarBlue™ is non-toxic, the cells under study can be returned to culture or used for other purposes including histological studies.

a. Method for use of alamarBlue™

The cells under consideration can be adherent or non-adherent. All procedures with alamarBlue™ should be performed under aseptic conditions because microbial contaminants are also able to reduce alamarBlue™. For this reason, media are usually supplemented with antibiotics such as penicillin G (1 unit per milliliter) and amphotericin B (0.0025 micrograms per milliliter).

The compatibility of several common reagents with alamarBlue™ has been determined. Generally, the pH of the medium should be buffered to pH 7.0 to 7.4, since the major redox indicator in alamarBlue™ is pH sensitive. Phosphate buffer is recommended. The presence of 10% fetal bovine serum in the cell culture medium has no effect on the results obtained with alamarBlue™ when reduction is measured spectrophotometrically; however, inclusion of 10% fetal bovine serum causes some quenching of fluorescence. Therefore, comparison of fluorescence of control solutions which also contain 10% BSA is indicated when reduction is measured by fluorescence. The presence of the pH indicator phenol red only minimally interferes with the assay. Phenol red is only observed to shift the apparent percent alamarBlue™ reduced upward by 0.03%. Generally, non-reducing media should be used such as RPMI 1640, Hank's modified Eagle medium, or Dulbecco's modified Eagle medium.

The conditions for use of alamarBlue™ in a particular system should be determined by performing initial studies in which factors such as cell density, incubation times, media composition, and concentrations of agents under investigation are systematically optimized.

To use alamarBlue™, the researcher harvests the cells of interest and plates them out in fresh medium supplemented with 10% (v/v) alamarBlue™ to the desired density. An example procedure is as follows:

1. Cells are grown in RPMI medium with HEPES, 10% fetal bovine serum, and 1% glutamine, supplemented with the desired antibiotics.
2. When cells reach 80% confluence, they are trypsinized and plated in micro-well plates at various densities of between 625-80,000 cells per well.
3. The cells are allowed to reattach for one hour, at which time alamarBlue™ is added. The cells are incubated at 37°C with 5% CO₂.
4. The reduction of alamarBlue™ is monitored at time points determined by the researcher.

Note: If insufficient reduction is observed, the researcher has the option of allowing the incubation to proceed for a longer period of time. This feature is a distinct advantage over many of the other methods of measuring proliferation.

For studying specific drugs that interfere with cell cycle progression (e.g., roscovitine), cells can be trypsinized, harvested and replated in new media in the presence of varying concentrations of the drug of interest. Cells are allowed to incubate for up to two days at 37°C at 5% CO₂. The spent media is removed and new media with 10% (v/v) alamarBlue™ is added. The reduction of alamarBlue™ is monitored at time points determined by the researcher.

b. Measurement of alamarBlue™ Reduction

i. Express as % alamarBlue™ reduction

There are two ways to monitor alamarBlue™ reduction: by measuring absorbance spectrophotometrically or by measuring fluorescence. Because the absorption spectra of the oxidized (blue) and the reduced (red) forms of alamarBlue™ overlap, the absorbance is measured at two wavelengths. These wavelengths are 570 and 600 nm, where the reduced and oxidized forms absorb, respectively. Alternatively, 540 and 630 nm may be used (see website for this method at www.biosource.com). In measuring alamarBlue™ reduction spectrophotometrically, the plate reader is first blanked on a well containing medium only at the two wavelengths. The absorbance of the medium without cells plus alamarBlue™ is read at the two chosen wavelengths. Then the absorbance of the medium with cells containing alamarBlue™ is read at the two chosen wavelengths.

In monitoring alamarBlue™ reduction spectrophotometrically, reduction is expressed as a percentage reduced alamarBlue™.

The calculation of % Reduced is as follows when the samples are read at 570 nm and at 600 nm wavelengths

$$\% \text{ Reduced} = \frac{117,216 \times (T_{570}) - 80,586 \times (T_{600})}{155,677 \times (C_{600}) - 14,652 \times (C_{570})} \times 100$$

Where:

155,677 = Molar extinction coefficient of reduced alamarBlue™ at 570 nm

14,652 = Molar extinction coefficient of reduced alamarBlue™ at 600 nm

80,586 = Molar extinction coefficient of oxidized alamarBlue™ at 570 nm

117,216 = Molar extinction coefficient of oxidized alamarBlue™ at 600 nm

T₅₇₀ = Absorbance of test wells at 570 nm

T₆₀₀ = Absorbance of test wells at 600 nm

C₅₇₀ = Absorbance of negative control wells which contain medium plus alamarBlue™ but to which no cells have been added at 570 nm.

C₆₀₀ = Absorbance of negative control wells which contain medium plus alamarBlue™ but to which no cells have been added at 600 nm.

Example:

T₅₇₀ = 0.61 with C₅₇₀ = 0.44
 T₆₀₀ = 0.64 with C₆₀₀ = 0.42

$$\frac{117,216 \times 0.61 - 80,586 \times 0.64}{155,677 \times 0.42 - 14,652 \times 0.44} \times 100 = 34\% \text{ reduction}$$

The second method of monitoring reduction of alamarBlue™ is by measuring the fluorescence. Fluorescence measurements are made by exciting at 530-560 nm and measuring emission at 590 nm. In reporting alamarBlue™ reduction by monitoring fluorescence, data are expressed as fluorescence emission intensity units as a function of time of incubation.

ii. LD₅₀ determination.

A common measure of cytotoxicity is LD₅₀ determination. To determine LD₅₀ with alamarBlue™, cell growth in the presence and absence of the compound under consideration is compared. Measurements are made either with the spectrophotometer at two wavelengths or by fluorescence. To determine LD₅₀ by spectrophotometric measurement, first calculate the percent inhibition of alamarBlue™ reduction at each concentration with the formula:

$$\frac{117,216 \times I_{570} - 80,586 \times I_{600}}{117,216 \times T_{570} - 80,586 \times T_{600}} \times 100$$

where

I₅₇₀ and I₆₀₀ being the absorbance at 570 nm and 600nm of the wells with inhibitor added.

T₅₇₀ and T₆₀₀ being the absorbance of the untreated positive growth control.

To determine LD₅₀ by fluorescent measurement, first calculate the percent inhibition of alamarBlue™ reduction at each concentration with the formula:

$$\frac{Fl_{590} \text{ of test agent dilution}}{Fl_{590} \text{ of untreated control}} \times 100$$

Data obtained from this comparison are plotted as percent inhibition on the y axis and log of concentration of the compound under consideration on the x axis. The LD₅₀ is determined from this curve corresponding to the concentration at which 50% inhibition is observed.

Figure 1: Effect of Addition of Growth Promoters and Growth Inhibitors on Cells in Culture using Alamar Blue™.

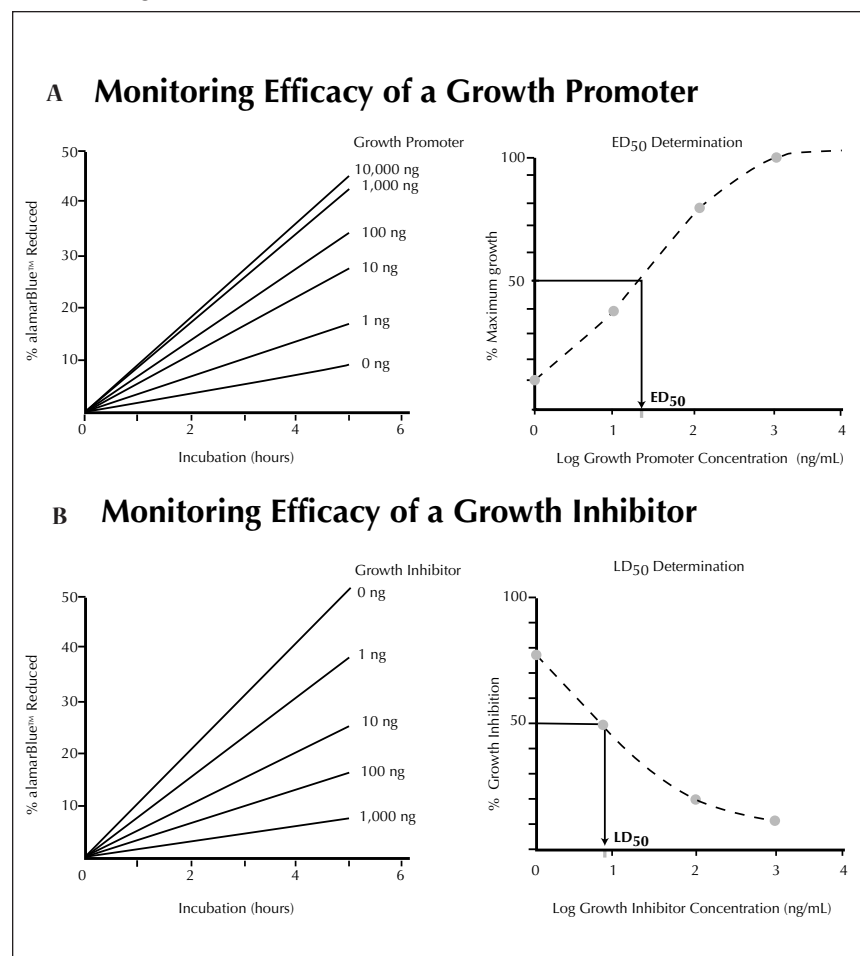


Figure 1A. Monitoring the Efficacy of a Growth Promoter. Addition of increasing concentrations of a compound which promotes cell growth and proliferation results in increased rates of an Alamar Blue™ reduction. The data obtained from the cell growth curves can be used to determine the ED₅₀ (the 50% effective dose) value for the compound under investigation.

Figure 1B. Monitoring the Efficacy of a Growth Inhibitor. Addition of increasing concentrations of a compound which is toxic to cells results in decreased rates of Alamar Blue™ reduction. The data from the cell growth curves can be used to determine the LD₅₀ (the dose which leads to the death of 50% of the test group) for the compound under investigation.

Products Available

Product Description	Part #	Size
ApoBrdU Kit	KHO1001	50 tests
Alamar Blue	DAL1025	25 mL
	DAL1100	100 mL

Growth Factors

Protein	Species	Part #	Size
EGF	Hu	PHG0064	200 µg
		PHG0062	500 µg
FGFa	Hu	PHG0014	10 µg
		PHG0015	50 µg
FGFb	Hu	PHG0024	10 µg
		PHG0026	50 µg
FGF-9	Ms	PMG014	10 µg
IGF-I	Hu	PHG0074	50 µg
		PHG0075	200 µg
IGF-II	Hu	PHG0084	50 µg
		PHG0085	200 µg
KGF	Hu	PHG0094	10 µg
		PHG0095	25 µg
NGF-β	Hu	PHG0124	10 µg
PDGF-AA	Hu	PHG0034	5 µg
		PHG0035	10 µg
PDGF-BB	Hu	PHG0044	5 µg
		PHG0045	10 µg
PDGF-AB	Hu	PHG0134	10 µg
TGF-α	Hu	PHG0054	10 µg
		PHG0051	100 µg
TGF-β1 (Natural)	Hu	PHG0104	1 µg/50 µL
VEGF	Hu	PHG0114	2 µg
		PHG0115	10 µg
	Ms	PMG0024	5 µg

Antibodies to Phase Specific Cell Cycle Analysis

Antibody	Clone	Part #	Size
BrdU	85-2C8	ANN0032	100 µg
Cyclin A	BF683	AHF0012	100 µg
	E23	AHF0022	100 µg
	E72	AHF0032	100 µg
Cyclin B1	Polyclonal	AHF0042	500 µg
	V152	AHF0052	100 µg
	Polyclonal	AHF0062	500 µg
Cyclin C	Polyclonal	AHF0072	500 µg
Cyclin D1	DCS-6	AHF0082	100 µg
	DCS-11	AHF0092	100 µg
	Polyclonal	AHF0102	500 µg
Cyclin D2	DCS-3.1	AHF0112	100 µg
	DCS-5.2	AHF0122	100 µg
Cyclin D3	DCS-22	AHF0132	100 µg
	DCS-28.1	AHF0142	100 µg
	Polyclonal	AHF0152	500 µg
Cyclin E	Polyclonal	AHF0162	500 µg
Ki 67	MIB-1	AHF0241	7.0 mL
PCNA/Cyclin	PC10	AHF0232	100 µg

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Cell Synchronization

In order to study many of the control mechanisms involved in cell cycle regulation, it is critical to synchronize the cells so that the distinct phases of the cycle are being studied. Synchronizing cells allows the precise study of regulatory mechanisms which determine cell cycle regulation at the level of gene expression and protein phosphorylation, facilitating drug discovery.

To synchronize cells, several methods are used. Commonly, researchers induce cells to quiescence by serum starvation before releasing them from this state, or treat cells with chemical inhibitors which arrest cells in distinct phases of the cycle. The following is a brief summary of these methods.

A. Serum starvation

In order to ensure that cells progress through the cell cycle in a synchronous manner, it is common to grow cells in serum-free media. This deprives the cells of the nutrients required to proliferate and forces the cells into quiescence (G0). Depending on the doubling time for a specific cell population, cells may be maintained in serum-free media from 12-48 hours. In order to synchronize cells in this way, it is important to ensure that cells are not at confluence when the serum is removed. The cells can be released from quiescence by adding back serum or by treating with specific growth factors. The major disadvantage of this method is that cells with high doubling times may require longer periods without media to enter quiescence. This has the disadvantage of stressing the cells, which may induce apoptosis or alter cellular responses. One way to circumvent this problem is to slowly decrease the serum content of the media, to acclimatize the cells to this serum-free environment. For example, cells that are normally maintained in 10% fetal calf serum are maintained in 5% serum for 24 hours. This amount of serum is reduced to 1% for a further 24 hours before finally moving cells into serum-free conditions.

B. Use of chemical inhibitors to synchronize cells

Several chemicals can be added to proliferating cells to arrest them in certain stages of the cell cycle. The inhibitor can then be removed from the media and the cells can progress through the cell cycle in a synchronous manner. There are many inhibitors available that can be used to arrest cells in various stages of the cell cycle; however, since the major aim is to release the cells in order to synchronize them, it is important that reversible inhibitors are used. Two commonly used drugs are (1) aphidocholin that arrests cells in G1/S phase by inhibiting DNA polymerase α and δ and (2) nocozadole which promotes tubulin depolymerization that blocks mitosis, thereby arresting the cells in G2/M.

The effectiveness of these drugs in synchronizing cells is based on a number of factors including cell type, concentration used, and the amount of time the cells are exposed to the inhibitor. Since these drugs interfere with the cell cycle machinery, it should also be cautioned that they may have unknown effects on the cells and can induce apoptosis if conditions are not optimal. Outlined below is a recommended procedure for incubating Jurkat cells with chemical inhibitors and a table giving the effective concentrations of commonly used drugs that arrest cells in specific phases of the cell cycle.

Method

1. Grow cells as recommended, making sure that they are not confluent.
2. Add chemical inhibitor to the cells at the effective concentration listed in the table. Most of these drugs can be dissolved in DMSO. Therefore, the appropriate DMSO control should be carried out on cells without treatment.
3. Grow cells in the presence of inhibitor for optimized time. It is important to optimize time as the drug may induce apoptosis if cells are left in these conditions for too long. The apoptotic effects of the drugs on a specific cell population can be assessed by using Annexin-V FITC/propidium iodide staining for suspension cells or using anti-PARP cleavage site specific antibody conjugated to FITC for either suspension or adherent cells (see www.biosource.com for these additional protocols).
4. Check that cells are all in the same phase of the cycle by taking an aliquot of cells and staining with propidium iodide (method outlined on page 15).
5. Remove media with the inhibitor present and place cells in fresh media.
6. The cells now start to grow in synchronicity and can be harvested at various time points for appropriate studies.

Table 1:

Inhibitor	Effective concentration	Phase arrested
Aphidocholin	1 µg/mL	G1/S
hydroxyurea	2 mM	G1/S
nocozadole	400 ng/mL	G2/M

Methods for detecting the synchronization of cells by flow cytometry.

Two methods are commonly used to detect synchronization by flow cytometry: (1) measuring DNA content of cells using DNA binding dyes such as propidium iodide; or (2) studying antigen expression patterns to determine cell position within the cell cycle.

Propidium iodide staining

1. Wash cells of interest twice in 5 mL PBS without Mg²⁺ and Ca²⁺.
2. Fix cells in 25 mL ice-cold Fixation Buffer. Cells should be fixed in Fixation Buffer at -20°C for at least 2 hours prior to staining and can be stored at -20°C in this buffer for up to 1 month prior to use.
3. Just prior to staining, remove Fixation Buffer by spinning at 300 x g for 10 minutes and wash cells twice in 25 mL PBS.
4. Resuspend in 0.5 mL Propidium Iodide Buffer to stain DNA.
5. Incubate cells at 37°C for 20 minutes in the dark prior to flow cytometry analysis.

Propidium iodide staining of synchronized cells

Figure 2:

CRF-CEM cells were grown for three days in RPMI 1640 +10% FBS prior to starvation in RPMI 1640 without FBS for 24 hours. Cells were released with 10% FBS, and cells were removed and stained with propidium iodide at the time points listed below and on the next page. The % of cells in each phase of the cell cycle was determined using the DNA staining analysis program ModFit™.

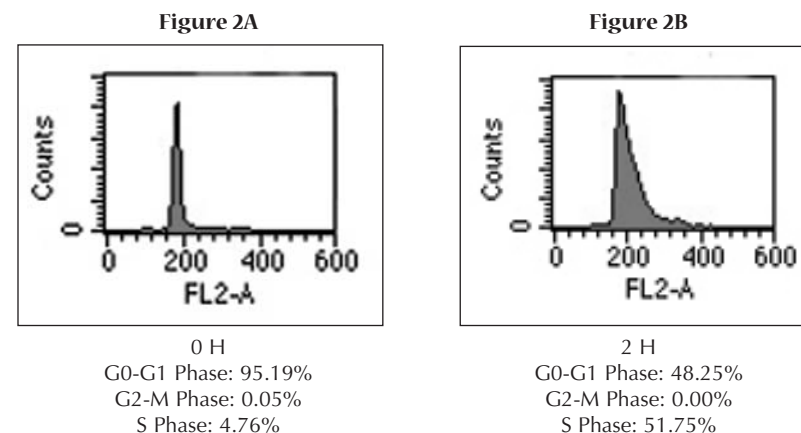
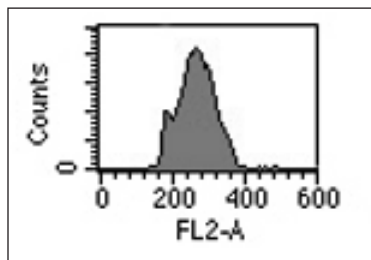
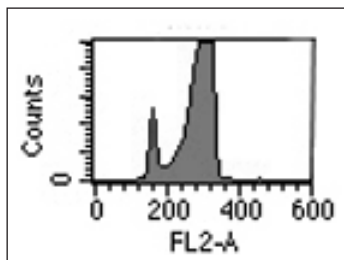


Figure 2C



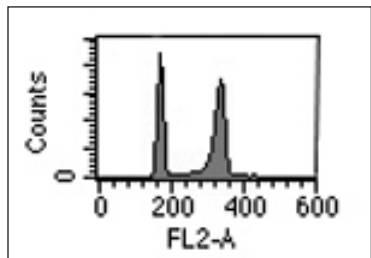
4 H
G0-G1 Phase: 0.00%
G2-M Phase: 0.00%
S Phase: 100.00%

Figure 2D



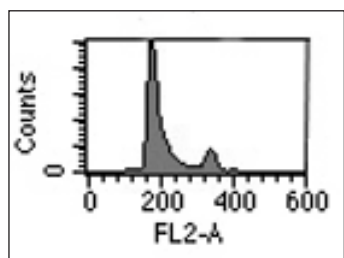
6 H
G0-G1 Phase: 0.93%
G2-M Phase: 70.20%
S Phase: 28.87%

Figure 2E



8 H
G0-G1 Phase: 34.55%
G2-M Phase: 52.48%
S Phase: 12.98%

Figure 2F



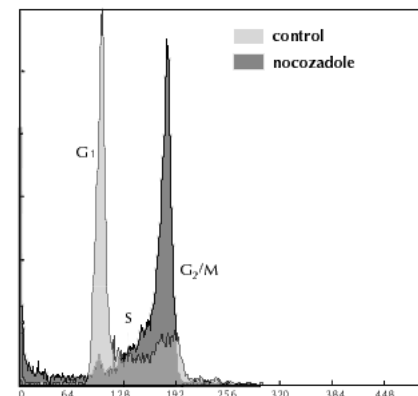
10 H
G0-G1 Phase: 75.74%
G2-M Phase: 3.37%
S Phase: 20.89%

The cells can clearly be seen moving through the cell cycle. In figure 2A (previous page) most of the cells are in G0-G1, the beginning of the cell cycle. In figure 2B (previous page), 2 hours later, half of the cells have entered S phase and by figure 2C, all of the cells are in S phase. In the 6th hour (figure 2D) most of the cells are in G2-M. Finally, in figures 2E and 2F the cells are moving through G2-M and re-entering the cell cycle at G0-G1 for their next division.

Expression of cyclins in human Jurkat cells

Figure 3:
Cells analyzed after nocozadole treatment to arrest them in G2/M.

Flow cytometry profile of PI stained cells



Notes on propidium iodide staining

1. Ethanol or methanol is the preferred method for fixing cells when using DNA dyes such as propidium iodide. Other fixatives (e.g., paraformaldehyde) lead to reduced binding of dyes to cellular DNA thereby reducing intensity of fluorescence and raising the measured coefficient of variation. This compromises accurate ploidy or S-phase measurements.
2. Since propidium iodide can also bind to RNA, it is recommended that propidium iodide buffer be prepared with DNase-free RNase at a concentration of 1 mg/mL. This can be obtained from Sigma-Aldrich (Part #R6513) and the PI buffer can be prepared as outlined below.

Detecting intracellular antigens by flow cytometry

Because of the phasic alteration in protein levels of certain cell cycle proteins, such as the cyclins and cyclin-dependent inhibitors, their expression patterns can be analyzed at the single cell level by flow cytometry. Together with DNA staining, this expression pattern can be used to verify the cell's position in the cycle.

Even though many high quality antibodies are available which recognize cyclins and cyclin-dependent inhibitors, not all of these antibodies work in flow cytometry applications. This is mostly due to the fact that not all antibodies recognize epitopes on native proteins or the epitope is altered during cell fixation. Because of this we

have screened all of these antibodies for use in this application and the majority of these antibodies are available in the FITC-conjugated format. This allows for a more rapid and convenient method to analyze multiple samples. Other antibodies are available in an unconjugated format which necessitates a two-step assay using a labeled secondary antibody. Here we describe both methods and provide a list of monoclonal antibodies recommended for use in flow cytometry.

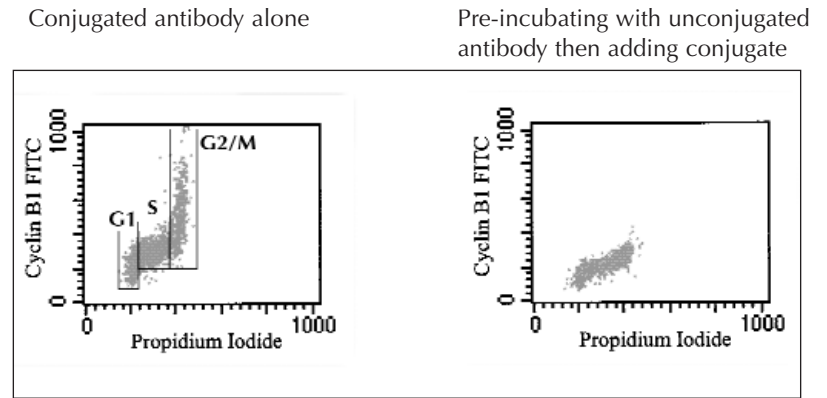
1. Method when using FITC-conjugated antibody:

1. Wash cells of interest twice in 5 mL PBS without Mg²⁺ and Ca²⁺.
2. Fix cells in 25 mL ice-cold Fixation Buffer. Cells should be fixed in Fixation Buffer at -20°C for at least 2 hours prior to staining and can be stored at -20°C in this buffer for up to 1 month prior to use.
3. Just prior to staining, remove Fixation Buffer by spinning at 300 x g for 10 minutes and wash cells twice in 25 mL PBS.
4. Resuspend pellet in 10 mL of Wash Buffer with 0.25% Triton X-100 added and incubate cells at 4°C.
5. After 10 minutes, bring volume to 50 mL with Wash Buffer and spin cells at 1500 rpm for 10 minutes. Resuspend pellet in Wash Buffer to give a final concentration of 1 x 10⁶ cells/50 µL. Aliquot 50 µL of the cells into 12 x 75 mm tubes for staining.
6. Add FITC-conjugated antibody to cells at desired concentration (typically 0.5–0.1 µg antibody stains 10⁶ cells) and incubate at 4°C for 30 minutes.
7. After incubating, wash cells three times in 2 mL Wash Buffer and resuspend in 0.5 mL Propidium Iodide Buffer to stain DNA.
8. Incubate cells at 37°C for 20 minutes in the dark prior to flow cytometry analysis.
9. At least one of the following specificity controls is recommended: 1) Isotype control matched to the monoclonal antibody used in the procedure; or 2) pre-incubating with excess unconjugated antibody prior to addition of conjugated antibody (typically 10–20 µg of unconjugated antibody is recommended to block for flow cytometry applications).

Table 2: Buffers

Fixation Buffer	75% ethanol in 50 mM phosphate buffered saline, pH 7.3, stored at -20°C.
Wash Buffer	50 mM phosphate buffered saline, pH 7.3, 2% (v/v) fetal calf serum, 0.1% (w/v) sodium azide at 4°C.
Propidium Iodide Buffer	10 µg/mL propidium iodide in 1.1% sodium citrate buffer with 1 mg/mL RNase A added.

Figure 4: Proliferating Jurkat Cells stained with anti-Cyclin B1 FITC



Jurkat cells were fixed in Fixation Buffer and stained with 250 ng/10⁶ cells of the anti-Cyclin B1 FITC and propidium iodide using the Suggested Staining Protocol for Flow Cytometry described above (left-hand figure). Jurkat cells in the right-hand figure were incubated with 10 mg unconjugated anti-Cyclin B1 prior to the addition of the FITC conjugated antibody.

2. Method when using unconjugated antibody:

1. Wash cells of interest twice in 5 mL PBS without Mg²⁺ and Ca²⁺.
2. Fix cells in 25 mL ice-cold Fixation Buffer. Cells should be fixed in Fixation Buffer at -20°C for at least 2 hours prior to staining and can be stored at -20°C in this buffer for up to 1 month prior to use.
3. Just prior to staining, remove Fixation Buffer by spinning at 1500 rpm for 10 minutes and wash cells twice in 25 mL PBS.
4. Resuspend pellet in 10 mL of Wash Buffer with 0.25% Triton X-100 added and incubate cells at 4°C.
5. After 10 minutes, bring volume to 50 mL with Wash Buffer and spin cells at 1500 rpm for 10 minutes. Resuspend pellet in Wash Buffer to give a final concentration of 1 x 10⁶ cells/50 µL. Aliquot 50 µL of the cells into 12 x 75 mm tubes for staining.
6. To cells, add unconjugated antibody at desired concentration (typically 0.5–0.1 µg antibody stains 10⁶ cells) and incubate at 4°C for 30 minutes.
7. Wash cells twice in 2 mL wash buffer and resuspend cells in 50 µL wash buffer.
8. Add 10 µL 1:100 dilution of Goat F(ab')₂ anti-mouse IgG FITC (or desired conjugate) and incubate cells for 30 minutes at 4°C.
9. After incubating, wash cells three times in 2 mL Wash Buffer and resuspend in 0.5 mL Propidium Iodide Buffer to stain DNA.
10. Incubate cells at 37°C for 20 minutes in the dark prior to flow cytometry analysis.

Antibodies recommended for flow cytometry:

Antigen	Clone	Part #	Size
Cyclin A, Unconj.	BF683	AHF0012	100 µg
Cyclin B1, Unconj.	V152	AHF0052	100 µg
Cyclin B1, FITC	V152	AHF0058	100 µg
Cyclin E, FITC	HE12	AHF0318	100 µg
Cyclin D1, Unconj.	DCS-6	AHF0082	100 µg
Cyclin D1, FITC	DCS-6	AHF0088	100 µg
Cyclin D2, Unconj.	DCS-5.2	AHF0122	100 µg
Cyclin D3, Unconj.	DCS-22	AHF0132	100 µg
p21waf1, Unconj.	HZ52	AHZ0412	100 µg
p21waf1, FITC	HZ52	AHZ0418	100 µg
p27kip1, Unconj.	DCS-72.F6	AHZ0452	100 µg
p27kip1, FITC	DCS-72.F6	AHZ0458	100 µg
p53, Unconj.	DO-7	AHO0142	100 µg
p53, FITC	DO-7	AHO0148	100 µg

Secondaries:

Goat F(ab) ₂ Anti-Mouse Immunoglobulins, Human Ig Adsorbed				
Specificity	Format	Part #	mL	mg/mL
Mouse Igs Gamma and Light chains	FITC	AMI4408	1	0.7
Mouse IgM Mu chain.	FITC	AMI4608	1	0.7
Mouse IgG & IgM: Gamma, Mu and Light chains	FITC	AMI4708	1	1.0

Notes on staining for flow cytometry:

1. Although the fixation method does not appear to be a critical factor in the detection of cell cycle related proteins, researchers may need to optimize this step depending on the protein being studied. It has been cited that ethanol is desirable for Cyclin A, B1 and E detection, whereas methanol yields the best results for the D-type cyclins (ice-cold 100% methanol is recommended). (Other issues with fixation of cells, results from the clumping of the cells when adding fixative.) It is recommended that the cells be well dispersed before adding the fixation buffer and that the fixative be added slowly while cells are being gently vortexed.
2. Even though the major aim of using reagents that arrest cells in distinct phases of the cell cycle is to study cell cycle regulatory mechanisms, it must also be cautioned that these drugs may also have an effect on the expression patterns of the some of the cell cycle proteins, where "unscheduled" expression of proteins occurs.

References:

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Immunological Methods

Introduction

As described in the previous chapters, regulation of cell cycle proteins involves many complex processes. In order to study the regulation of these proteins, many immunological methods have been developed that are relatively easy to perform, but yield a vast amount of information. Western blotting can be performed on cell extracts to determine the presence and phosphorylation state of a protein, kinase activity can be measured from an extract when the kinase of interest is immunoprecipitated, and the localization of a protein can be determined by immunohistochemistry. All of these methods can be used to diagnose the pathogenesis of a tumor and can be utilized by drug companies to develop more specific and effective cancer treatments.

Outlined below are easy to follow methods for immunoprecipitation, Western blotting, and immunostaining that are routinely used to study cell cycle proteins.

Preparation of Cell Extracts

The procedure that is used to extract cells and prepare lysates for studying cell cycle proteins is of the utmost importance. Since regulation of these proteins often involves the formation of complexes, methods must maintain the conformation of proteins. In addition, cellular events such as protein phosphorylation must be preserved to ensure that kinase activity can be measured. This procedure for cell extraction has been used at BioSource International for Western blotting and when antigens were immunoprecipitated, phosphorylation and kinase activities were preserved.

Extraction of Cells

Materials and Equipment

PBS without Ca ⁺⁺ and Mg ⁺⁺ , 4°C	Ice
Centrifuge	Vortex mixer
Freezer (-80°C)	Extract Buffer
50 mL conical tubes	

Table 3:

Reagent:	Extract Buffer:
	10 mM Tris, pH 7.4
	100 mM NaCl
	1 mM EDTA
	1 mM EGTA
	1 mM NaF
	20 mM Na ₄ P ₂ O ₇
	2 mM Na ₃ VO ₄
	1% Triton X-100
	10% glycerol
	0.1% SDS
	0.5% deoxycholate
	1 mM PMSF (stock is 0.3 M in DMSO)
	Protease inhibitor cocktail from Sigma
	(made according to manufacturer's guidelines)
	(add 250 µL per 5 mL extract buffer)

This buffer is good for 2-3 weeks at 4°C or aliquoted (without protease inhibitors and PMSF added) at -20°C for 6 months (thaw on ice). Important: add the protease inhibitors just before using. Stability = 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

1. Add ice cold PBS without calcium or magnesium (**PBS**) to stop stimulation. Place cells immediately on ice and remove buffer. If suspension cells, add ice cold **PBS**, spin at 4°C for 5 minutes at 3000 rpm, then remove buffer.
2. Wash cells this way in ice cold **PBS** two more times.
3. For adherent cells, add 10 mL of ice cold **PBS** and scrape cells on the ice. Put into 50 mL conical tubes and bring volume to 50 mL, pellet cells and resuspend in appropriate volume of **Extract Buffer**. Typically 500-1000 µL of **Extract Buffer** is used to resuspend pellet or 5 x 10⁶ cells.
4. Place cells in microfuge tubes on ice. Vortex extract vigorously every 2 minutes for 10 minutes, leaving the extract on ice in between times.
5. Centrifuge the extract at 14,000 rpm for 15 minutes at 4°C.
6. Remove supernatant and discard pellet. Freeze supernatant at -80°C until ready to use. It is better to aliquot cells since freeze thawing can destroy or interfere with the phosphorylation state of the protein.

Protein Assay for Cell Extracts

Before an IP or Western can be run on the extract, the protein concentration of the fractions needs to be assessed by a Bradford Assay. This can be done by using the Bio-Rad protocol using frozen BSA standards.

Materials and Equipment

Bio-Rad Protein Assay Kit	Microtiter plates
1 mg/mL BSA	Spectrophotometer

BioRad Protein Assay Standard Procedure for Microtiter Plates:

1. Prepare protein standards; 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL BSA in water.
2. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts deionized, distilled water.
3. Prepare the unknown protein solutions.
4. Protein concentrations will vary; however, dilutions of 1:2 to 1:10 in water typically result in concentrations that will fall within the specificity range of the assay.
5. Pipette 10 μ L of each standard and diluted unknown sample solution into separate microtiter plate wells.
6. Add 200 μ L of diluted dye reagent to each well. Mix the samples and reagent thoroughly, using a microplate mixer. At this point, you should be able to visually determine whether the unknown dilutions used are within the specificity range of the assay. If visually within the range, proceed to Step 7; if not, dilute the unknowns accordingly and add 10 μ L to wells, then proceed to Step 6.
7. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
8. Measure absorbance at 595 nm. Configure the template, accounting for dilutions, and label the assay, read the plate, and analyze the data curvilinearly.

Continue Westerns or Immunoprecipitations on the samples according to the following protocols.

Immunoprecipitation

Purpose: To separate the target antigen from a complex mixture of cellular components using a target specific antibody for the purpose of purification, quantification, or identification.

Materials and Equipment

Extraction Buffer	2X Sample Buffer
Microcentrifuge	Incubator/Water Bath (95°C)
Primary antibody	³² P
Protein A/G	Substrate
Rocker	P81 Filters
Refrigerator (4°C)	Scintillation counter

Table 4:

Reagent:	2X Sample Buffer: (SB)	125 mM Tris, pH 6.8 4% SDS, 20% Glycerol 10% β -Mercaptoethanol 0.0025% Bromphenol Blue
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2. Immunoprecipitation Protocol: Validated using cell cycle antibodies to retain protein complexes and kinase activity. Phosphorylation of proteins can also be studied using this method.

1. Prepare protein extracts in **Extract Buffer (see page 23)**.
2. Prepare Protein A/G beads by washing beads in **Extract Buffer** 3 times to equilibrate.
3. Determine protein concentration of extracts (page 24).
4. Use 200-500 μ g of total protein from cell extracts.
5. Add approximately 1.0 μ g of antibody. A typical antibody to extract ratio is 1-5 μ g to 500 μ g extract. (You can "preclear" the extract by incubating for 30 minutes at 4°C with an aliquot of Protein A/G beads, if desired, prior to this step). Alternatively, Protein G can be incubated with antibody for 30 minutes at 4°C prior to addition of precleared cell lysate.
6. Rotate tube for at least 1 hour at 4°C.
7. Add 50-60 μ L of Protein A/G beads. Beads should be at 1:1 volume with buffer.
8. Rotate for \geq 30 minutes at 4°C.
9. Spin for 5 seconds at RT and discard the supernatant.
10. Wash beads 4 times with 500 μ L cold **Extract Buffer**.

11. Vortex, spin for 5 seconds at RT and discard the supernatant. At this point, samples can either be processed for Western blotting or for kinase activity assays (see following protocol).
12. For Western blotting, resuspend beads in 10-30 μ L of **2X SB** and mix well. (For non-denatured samples, prepare Sample Buffer without SDS and freeze beads after addition of buffer.)
13. Heat to $\geq 95^\circ$ for 4-5 minutes.
14. Spin for 1-2 minutes at RT to pellet the beads.
15. Run supernatant on gel. (Supernatant can be frozen at $\leq -20^\circ\text{C}$ for future use.)
16. Follow Western blot procedure if desired; for IP/Western (see page 29).

IP Illustration:

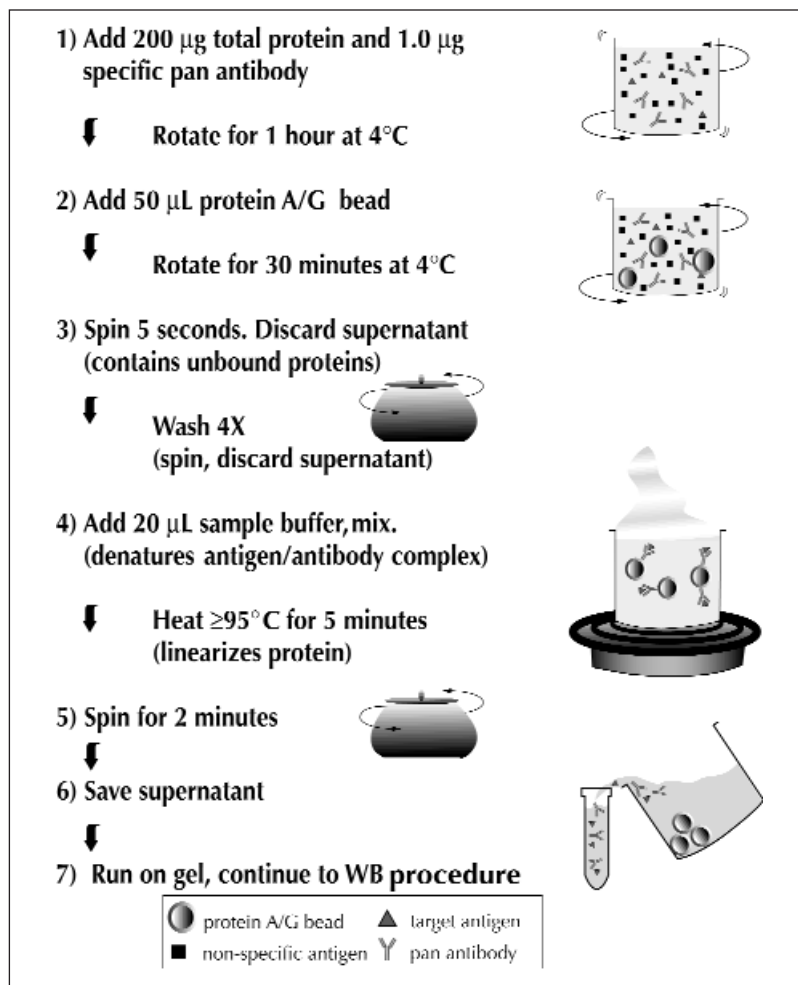
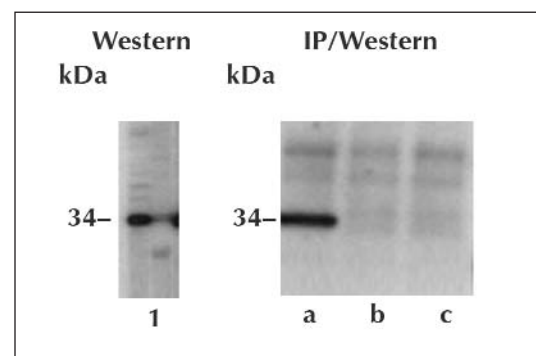


Figure 5:



1) shows HeLa cell extracts resolved by SDS-PAGE. Immunoprecipitations were performed on HeLa cells as outlined in the protocol using a) cdk1 pan (clone A17.1.1) b) cdk2 pan (clone 2B6) and c) cdk5 pan CD34. All antibodies were used at 1 μ g per 250 μ g protein extract. Westerns were performed on both blots using a cdk1 polyclonal at 1:500 on HeLa extracts resolved by SDS-PAGE on the immunoprecipitates above.

Kinase activity assay:

This method describes the kinase assay performed on whole cell extracts previously immunoprecipitated with Cyclin D monoclonal antibody to preserve the Cyclin D/cdk4 (or cdk6) complex. In this assay, Retinoblastomic protein is used as a substrate. In these assays, it is recommended to use recombinant cyclin-dependent kinase complex as a control for activation. In addition, histone may also act as a substrate for activated complexes.

After immunoprecipitation step:

1. Aspirate the final wash and elute the activated cyclin-cdk complex by resuspending the resin in 50 μ L kinase assay buffer containing 10 mM glutathione.

Table 5: Buffers

Kinase assay buffer (+ glutathione):			
94 mL Distilled H ₂ O	to final		100 mL
5 mL 1 M HEPES, pH 7.0	to final		50 mM
1 mL 1 M MgCl ₂	to final		10 mM
0.5 mL 1 M MnCl ₂	to final		5 mM
0.3 g glutathione	to final		10 mM
Add to each 1 mL immediately before use:			
1 μ L 1 M DTT	to final		1 mM
0.5 μ L 10 mM ATP	to final		5 μ M

Buffers: (cont.)**DDT Stock solution:**

10 mM ATP (adenosine 5'-triphosphate, Boehringer Mannheim): 60.5 mg in 10 mL distilled H₂O, dispense into small aliquots and store at -20°C.
 10 mM glutathione (Sigma): 30.7 mg in 10 mL 50 mM Tris-HCL, pH 8.0.

- Use 5 µL of the activated cyclin-cdk complex for kinase assays. Bring volume up to 25 µL with kinase assay buffer (which contains DTT to 1 mM and ATP to 5µM but which does not contain glutathione) and proceed to the kinase assay.

Kinase Assay

- Start the reaction by adding to each tube 5 µL of mixture:
 2 µL (approximately 0.5-2 µg) GST-pRb/p107/p130 fusion protein substrate
 0.5 µL [gamma-³²P]ATP (10 µCi.µL; 300 Ci/nmol)
 2.5 µL kinase assay buffer
 Incubate for 30 minutes at 30°C in a water bath designed for radioactive samples. There are three substrates available to measure the *in vitro* kinase activity of the cyclin D-associated kinase cdk4 and cdk6; pRb, p107 and p130. The most commonly used substrate is a fusion protein between GST and the C-terminal 137 amino acids (792-928) of human pRb. GST fusion proteins containing the pocket region and C terminus (amino acids 252-936) of human p107 or the spacer region of p130 can also serve as cdk4 and cdk6 substrates *in vitro*. As a negative control, the GST protein can be used as a substrate.
- Stop the reactions by adding 20 µL 2X SDS-DTT sample buffer to each tube. Boil the samples for 3 minutes.
- Load 20 µL of each sample onto a denaturing polyacrylamide gel (10-12.5%) being careful to avoid lane-to-lane leakage. The remaining sample may be stored at -20°C.
- Run the gel until the dye front has reached the bottom. Cut the upper left corner of the gel (for ease of orientation) and stain it in Coomassie dye (50% (v/v) methanol, 7% (v/v) acetic acid, 0.2% (w/v) Coomassie Brilliant Blue) for approximately 10-15 minutes. Destain for 30 minutes to 1 hour in destain solution (37.5% (v/v) methanol, 7% (v/v) acetic acid, 0.75% (v/v) glycerol). Observe the relative levels of both the IgG heavy chain for each sample and the substrate. If the loading was unequal, the remaining stored samples can be used to repeat the experiment.
- Dry the gel for 1 hour at 80°C. After drying, cover the gel with Saran Wrap and expose the gel either on a phosphorimage plate (about 2 to 4 hours) or on X-ray film (overnight to several days) with an intensifying screen.

Western Blot

Several procedures outlining Western blotting are given below. Specific details for performing both colorimetric and chemiluminescent detection are provided. In most cases, cell or tissue extract samples (1-30 µg of total protein) can be analyzed by conventional SDS-PAGE using mini-gels with a 10-12 well comb format.

Procedure I: This has been validated using our pan antibodies with either recombinant or native proteins.

1.1 Antigen Preparation

In a microcentrifuge tube, dilute antigen to 20 ng/µL with PBS, if using recombinant or purified protein. Alternatively, if cell extracts are being used, pipette amount of cells needed into clean centrifuge tube, typically 1-30 µg cell lysate is required per lane. Dilute sample with nonreducing buffer; or if reducing buffer is used, boil samples for 5 minutes.

Materials and Equipment

10%, 14%, or 16% , 4-20% Novex Tris-Glycine precast gels
 2 sponges (approximately 11 cm²)
 25 cm² container, or larger
 Electrophoresis equipment (mini-gel)*
 Microcentrifuge
 Power supply
 Prestained molecular weight marker
 Primary antibody
 Polyvinylidene Fluoride (PVDF) membrane (Fisher)
 Rocker
 Weigh boats
 Secondary antibody, IgG, Alkaline Phosphatase conjugate (See section table for appropriate antibody selection)
 Western blotting apparatus
 Whatman #1 filter paper
 Staining and Destaining Solutions
 Developing solution (BioRad Alkaline Phosphatase Conjugate Substrate Kit for colorimetric developing or Tropix Western CDP-Star Ready-to-Use Substrate Solution)

*** It is recommended that investigators follow the manufacturer's instructions for the apparatus used.**

Note: All buffers are shown in bold type, and formulations are given beginning on page 31.

1.2 Polyacrylamide Gel Electrophoresis (PAGE)

1. Set up gel in electrophoresis chamber with **1X Running Buffer** for PAGE.
2. Use 10% gel for proteins > 30 kDa; 14% gel for proteins ≤ 30 kDa; and 16% gels for proteins ≤10 to 4 kDa. Alternatively, a gradient gel of 4-20% may be used for cell extracts.
3. Load 10 µL prestained molecular weight markers.
4. Load sample : For recombinant protein, load 20 µL of antigen into appropriate lanes/wells. For cell extracts, typically load 1-30 µg of cell extract/lane or 200-500 µg of cell extract on a one lane gel.
5. Run gel at 120 volts for 10 minutes, followed by 130 volts for 90 minutes, until the dye front is at the bottom of the resolving gel.
6. Remove gel from cast.

1.3 Immunoblotting

1. Before PAGE is finished running, cut PVDF membrane to size slightly larger than that of the gel. Bookmark one edge where the top of lane one will be represented.

Note: Wear gloves whenever handling PVDF membrane.

2. Soak membrane in methanol for 1 minute, then rinse with diH₂O for 5 minutes.
3. Cut 2 pieces of Whatman filter paper slightly larger than size of the gel (At least as large as the membrane).
4. Gather together 2 sponges, the 2 pieces of Whatman paper, and the PVDF membrane and soak in **Transfer Buffer** for 2 minutes prior to setting up apparatus.
5. Lay electroblotting apparatus in a container that is at least 25 cm² containing 250 mL **Transfer Buffer**.
6. Open the sandwich apparatus with the black side down in the container.
7. Make a sandwich. Start by laying a sponge on the one side, then layering in the following order:

Sponge	-
Whatman filter paper	
gel	
PVDF membrane (smooth side facing gel)	
Whatman filter paper	
Sponge	+

8. It is important that no bubbles form between the PVDF membrane and the gel.
9. Close the sandwich apparatus making sure that the clasps are shut tight.
10. Pour 1 liter of **Transfer Buffer** into tank and place sandwich apparatus in one of the slots of the tank. (Use the cassette position nearest the center.)
11. Connect the black electrode to the terminal nearest the black side of the

sandwich apparatus. The red electrode should be placed in the other terminal. This ensures that the proteins run from the gel onto the membrane towards the red terminal.

12. Run the gel at 140 mA with the voltage at the highest point (500V) for 60-90 minutes at room temperature. This may require longer time if more than 2 gels are being transferred. Put transfer apparatus into container with ice.
13. Prepare the **Wash Buffer (tTBS)**
14. Prepare **Western Blocking Buffer**.

Table 6:

Buffers:	
Transfer Buffer:	2.4 g Tris base 14.2 g Glycine 200 mL Methanol Q.S. to 1 Liter and add 1 mL 10% SDS, cooled to 4°C prior to use
5X Running Buffer:	15 g Tris base 72 g Glycine 5 g SDS Adjust pH to 8.8 and Q.S. to 1 Liter. Dilute 1:4 for 1X Running Buffer
Wash Buffer (tTBS):	9.68 g Tris base 32 g NaCl Q.S. to 4 liters. Adjust to pH 7.6 and add 2 mL Tween-20
Western Blocking Buffer:	5 g fat-free dried milk 100 mL Wash Buffer

1.4 Staining Procedure (optional)

1. Remove the PVDF membrane from apparatus and place in large weigh boat. If transfer was successful, the prestained molecular weight markers should now be on PVDF membrane, not on the gel. The higher molecular weight standards (blue and orange bands) are more difficult to transfer, so some of the color may still be present on the gel. This is all right if the rest of the bands have transferred onto the PVDF.
2. Rinse the PVDF membrane with 10 mL **tTBS** for 5 minutes, 3 times. Membranes can be dried at this point for storage.
3. To store membranes: Once transfer is complete, wash membrane with wash buffer and put membrane into **Amido Black stain solution** for 30 seconds.
4. To destain, place in **Destain Solution** until protein bands are clearly visible and membrane is not too dark.

Table 7 Stain/Destain Solutions:

Amido Black stain solution:
1 g Amido Black
100 mL methanol
100 mL glacial acetic acid
Q.S. to 1 L with diH ₂ O
Destain Solution:
100 mL glacial acetic acid
100 mL isopropanol
Q.S. to 1 L with diH ₂ O

- At this point, membrane can be dried and stored for up to 1 year at room temperature. If using right away, don't dry. Wash membrane several times in **Wash Buffer**. If using dried membrane, wet in 100% methanol before using and wash with **Wash Buffer** several times.
- Place the PVDF membrane in 10 mL **Western Blocking Buffer** for at least 30 minutes at room temperature, rocking constantly. This may be left overnight at 4°C with constant rocking.
- Pour out **Western Blocking Buffer**. Pour 10 mL of fresh **Western Blocking Buffer** onto PVDF membrane. Add 10 µL of primary antibody at 1 mg/mL to the solution for a 1:1000 dilution. Place on rocker for 1 to 2 hours at room temperature. (The amount of the primary antibody may be modified according to the specific experiment setup, 1:500, 1:1000, or 1:2000.)
- Pour off primary antibody solution and rinse PVDF membrane with 10 mL **Wash Buffer** for 10 minutes, with constant rocking.
- Repeat wash step 3-4 more times.
- Select the secondary antibody.
The secondary antibody used is dependent upon the animal in which the primary antibody is produced. For example, if the primary antibody is a mouse anti-human antibody, the secondary antibody must be a goat anti-mouse IgG. Alkaline phosphatase conjugates are used for the colorimetric development and for the enhanced chemiluminescence (ECL) development. (ECL is more sensitive than colorimetric and should be used whenever possible.) See Table 8 on the next page.

Table 8: BioSource Secondary Antibodies

Primary	Secondary (AP)
Goat anti-	Swine Whole Molecule Anti-Goat Ig's, AP, Human Ig Adsorbed (Catalog #ACI3405)
Human anti-	Goat F(ab') ₂ Anti-Human IgG, AP, (Catalog #AHI1305)
Mouse anti-	Goat F(ab') ₂ Anti-Mouse Ig's, AP, Human Ig Adsorbed (Catalog #AMI4705)
Swine anti-	Goat Anti-Swine Ig's, AP (Catalog #ACI3405)
Rat anti-	Goat F(ab') ₂ Anti-Rat Ig's, AP, Mouse and Human Ig Adsorbed (Catalog #ARI4405)
Rabbit anti-	Goat F(ab') ₂ Anti-Rabbit Ig's, AP, Mouse and Human Ig Adsorbed (Catalog #ALI4405)

- Pour 20 mL of **Western Blocking Buffer** onto PVDF membrane. Add 4 µL of secondary antibody to the solution, for a 1:5000 dilution. (The amount of the secondary antibody may be modified according to the specific experiment setup. The secondaries have been shown to produce consistent results between 1:5000-1:20,000 dilution.)
- Place on rocker for 45 minutes to 1 hour at room temperature. Pour off secondary antibody solution and rinse PVDF membrane with 10 mL **Wash Buffer** for 5 minutes, with constant rocking.
- Repeat wash step 3-4 more times.

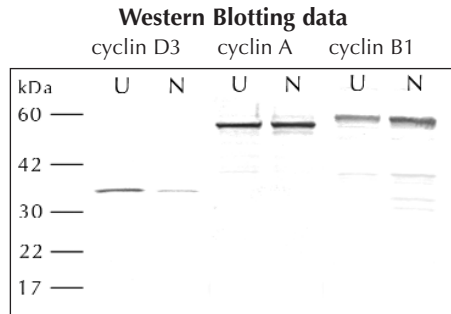
1.5 Blot Development

During final washing, prepare developing solution:

For Colorimetric Development:

- Combine the following:
 - 9.8 mL alkaline phosphatase developing buffer (0.1M Tris, pH 9.5)
 - 100 µL alkaline phosphatase Color Reagent A
 - 100 µL alkaline phosphatase Color Reagent B
- Pour off **Wash Buffer** from final wash and add 10 mL developing solution.
- Incubate in developing solution until bands appear on membrane (usually between 2 and 10 minutes). If no bands appear in this time, incubate for 30 minutes.
- Pour off developing solution and rinse with 20 mL of diH₂O 2 times for 5 minutes.
- Air dry and laminate.

Figure 6:



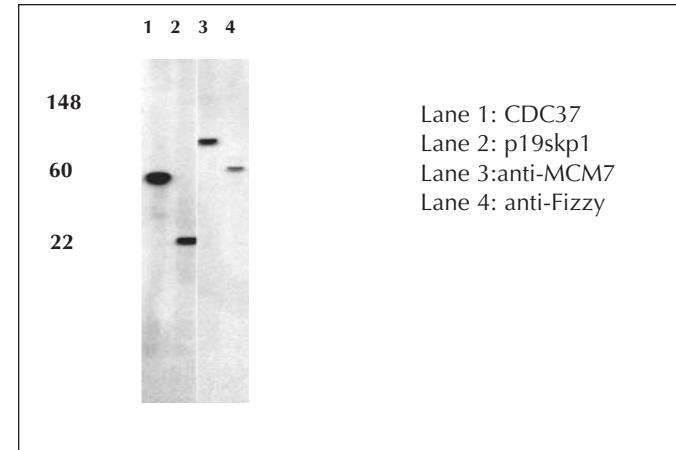
Western blot performed on untreated and nocozadole treated cells. (See pages 15-17 for protocol and diagram of PI staining.) The proteins were detected using monoclonal antibodies specific to the cyclins shown followed by incubation with Goat F(ab')₂ anti-Mouse IgG AP. The proteins were then treated using the colorimetric Bio-Rad detection unit.

For ECL Development:

- Carefully remove membrane from washing solution, sliding it along the side of the dish to remove excess **Wash Buffer** (DO NOT let membrane dry!).
- Place membrane in a small dish or large weigh boat. Add 3-5 mL of CDP-Star Ready-to-Use substrate solution (Tropix) from kit and incubate for 5 minutes.
- Remove membrane from dish by sliding it along side to remove excess solution. Place it on a sheet of plastic film and carefully wrap. Gently rub out air bubbles.
- Expose blot to film for ≥15 seconds. Exposure times may vary according to the antibody.
- Develop film.
- Dispose of blot unless otherwise required for further testing. If retained, store blot in plastic film at 4°C. (Note: the signal will be stable for up to 18 hours after incubation with substrate.)

We have found that the preceding protocol gives low background and clean signal when used with our primary antibodies. Sometimes the protocol will have to be modified depending on the primary antibody used, the amount of target protein present in the extract, whether the phosphorylation state of the protein is being determined and the way in which the extract was prepared. Procedure II has been used successfully with antibodies that showed high background or where no signal was detected with Procedure I.

Figure 7:



Western blot demonstrates the use of the cell cycle antibodies using the enhanced chemiluminescent detection method.

Alternate Protocol with Phosphorylation State-Specific Antibodies

2. Procedure II: This has been validated using our phosphorylation state-specific antibodies.

The supplied protocol is based on the use of Nitrocellulose membranes, an Alkaline Phosphatase (AP) labeled Goat Anti-Rabbit secondary antibody (Catalog #ALI4405), prequalified for use with the anti-polyclonal antibody, and Western Blue Stabilized Substrate for alkaline phosphatase. Other procedures including the use of Nylon membranes (e.g., PVDF, Immobilon, etc.) or other detection methods such as ¹²⁵I-Protein A or non-radioactive/chemiluminescent detection using the AP secondary conjugate or Horseradish Peroxidase (HRP)-conjugated secondary antibodies can also be used. The cell extracts used below were prepared from pheochromocytoma (PC12) cells; however, similar results have been obtained with many experimental systems including several cell lines (e.g., REF52, NIH3T3, Swiss3T3, F111, CHO, INS1 and 293 cells) and tissues (mammalian muscle or brain and Drosophila).

Equipment:

- heating block at 95°C.
- electrophoresis unit (Hoeffer Mighty Small or equivalent)
- electroblotting unit (BioRad or equivalent)
- microcentrifuge (Eppendorf or equivalent)
- oscillating platform shaker (Labline or equivalent)
- power supply (capable of delivering 125V, constant voltage)

Table 9:

Reagents:
1x TBS Buffer: Add 200 mL of 10x TBS to 1.8 L of nanopure water.
TBST Buffer (TBS + 0.05% Tween-20): Add 0.5 mL of Tween-20 to 1 liter of 1x TBS Buffer.
Blocking Buffer (TBS +1% BSA): Dissolve 1g of IgG-free and protease-free BSA (Jackson Laboratories) per 100 mL TBS.
Antibody sample buffer (TBST +0.1% BSA): TBST containing 0.1% BSA.
1x Running Buffer: 24 mM Tris base 0.19 M glycine 10% SDS
1x Western Transfer Buffer : Mix 1000 mL Gel Transfer Buffer 200 mL methanol 700 mL deionized water Store at 4°C!! Can be used twice before it needs to be replaced.
Gel Transfer Buffer: 12 mM Tris base 96 mM glycine 10% SDS

2.1 Polyacrylamide Gel Electrophoresis (PAGE)

Note: Wear gloves when handling gels and nitrocellulose membranes.

Commercial 10% PAGE gels are rinsed with Nanopure water to eliminate azide storage buffer.

1. Assemble SDS PAGE discontinuous gel apparatus and fill upper and lower buffer chambers.
2. In a microcentrifuge tube, mix protein samples with **2X Sample Buffer** containing reducing agent. Note: Sample buffer containing DTT should be made fresh on the day of the experiment.
3. Heat samples for 2 minutes in 95°C heating block.
4. Spin samples for 5 seconds in a microcentrifuge and carefully load gel with each sample.
5. Run the gel at 125V (constant voltage) until the dye front reaches the end of the gel (approximately 1.5- 2 hours).

2.2 Immunoblotting

1. Remove gel from the apparatus and soak in transfer buffer for 15 minutes. While this is occurring, cut nitrocellulose to fit the entire gel and 3 sheets of blotting paper. Soak nitrocellulose, blotting paper, and transfer apparatus sponges in transfer buffer as well.
2. Assemble transfer apparatus as follows, being careful not to introduce any air bubbles between layers:

black half of apparatus	-
sponge	
1 sheet blotting paper	
gel	
nitrocellulose paper	
1 sheet blotting paper	
sponge	
white half of apparatus	+

3. Insert assembled blot into the blotting apparatus, with orientation of nitrocellulose closest to positive electrode (transferred proteins have net negative charge from SDS). Add the ice finger pack and fill the tank with cold **Western Transfer Buffer**.
4. Transfer the entire assembled unit to 4°C and electroblot at 100 V for 1 hour.

2.3 Staining Procedure

1. Remove nitrocellulose blot and check that the prestained markers did indeed transfer.
2. Block membrane with TBS + 1% BSA for a minimum of 1 hour at 37° or overnight at 4°C.
3. Lay blot onto blotting paper and using a fresh razor blade, carefully cut the membrane into two strips.
4. Using **Antibody Sample Buffer**, dilute the primary antibody to 0.2-1 µg/mL in a final volume of 20 mL. Transfer to a suitable container and add blot. In addition, probing replicate blots with a corresponding antibody that recognizes both active and inactive forms (i.e., total enzyme) is a valuable control.
5. Using an oscillating platform shaker, incubate Blot #1 for 2 hours at ambient temperature.
6. Transfer and wash with wash buffer 3X 5 minutes (approximately 75 mL each wash).
7. Dilute the Goat Anti-Rabbit Ig's * AP (Catalog #AL14405) conjugate 1:10,000 in **Antibody Sample Buffer** and add to blot.
8. Incubate 1 hour at ambient temperature using oscillating platform.

9. Wash both blots with wash buffer 3X 5 minutes (approximately 75 mL each wash).
10. Wash blots with TBS alone 2X 1 minute (approximately 30-40 mL each).
11. Add both blots to plastic vessel containing Western Blue Stabilized NBT/BCIP. Watch as bands begin to materialize.
12. Stop color development by transferring strips to deionized water and rinse.

Note: NBT bands are stable as long as they are kept out of the light. Data can be photographed via AMBIS for long term storage.

2.4 Chemiluminescent Detection Using the ECL Western blot Kit:

Notes: Read through this entire next section before proceeding since it is necessary to work quickly once the blots have been exposed to the detection solution. It is therefore helpful to actually conduct all steps in the dark room so as to minimize any delays. Use gloves for this stage onward to prevent hand contact on film or detection reagents. Note that the color development will continue even after removing the blots from the development solution. Do not leave in solution too long as background will become increasingly darker with time.

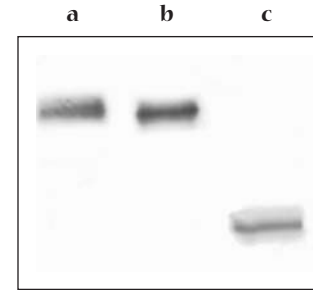
1. Prepare the ECL detection solution by mixing equal volumes of Solution 1 with Solution 2, as supplied with the ECL detection kit, to yield sufficient volume to cover the membrane (this requires ~ 0.125 mL/cm² of membrane). Most standard miniblots require 0.5 mL of each solution (1 mL final volume). Mix the detection solution immediately after combining stock Solutions 1 and 2.
2. Add the detection solution directly to the blot on the surface to which the protein was transblotted. Do not allow the blots to dry out.
3. Incubate the membrane for precisely 1 minute at room temperature.
4. Drain off excess detection solution by holding the blot vertical and touching the edge of the blot against a piece of tissue paper.
5. Gently place the blot, protein side down, on to a piece of plastic wrap. Fold over the surrounding plastic wrap to form an envelope that encloses the blot. Avoid applying excess pressure as this can cause high backgrounds.
6. Gently smooth out the air pockets. Again, avoid applying excess pressure as this can cause high backgrounds.
7. Place the blot, protein side up and surrounded by plastic wrap, into the film cassette making sure that no detection solution leaks out into the film cassette since the film must remain dry.
8. Switch off the white light and work under the film-safe red lights. Carefully place a sheet of autoradiography film (Kodak BioMAX Chemiluminescence film) on top of the blot, close the cassette and apply sufficient pressure to hold in place. Expose for 15 seconds and do not move the film while it is being exposed.
9. Remove the film and develop in the automatic film developer.

10. Place a second piece of film onto the blot, close the cassette and start a timer.
11. On the basis of the appearance of the first piece of film following development, estimate how long to continue the exposure of the second piece of film. Second exposures typically vary from 30 to 60 seconds, depending on the intensity of the signal.
12. Compare each experimental blot with the corresponding 'secondary conjugate alone control', especially if there are additional bands other than those corresponding to isoforms for the specific enzyme being detected.

Note: If background signals on the film are high, the blot can be rewashed twice for 10 minutes with wash buffer and the detection steps 1-10 repeated.

Figure 8:

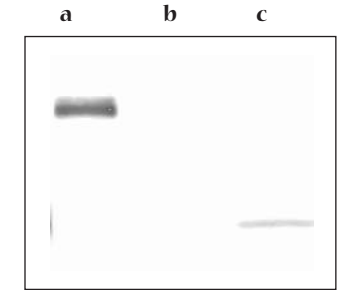
Western blotting data of anti-p53 monoclonal antibody at 1:1000 on extracts from CCRF-CEM cells.



8a-phosphorylated p53 fusion protein
8b-non-phosphorylated p53 fusion protein
8c-CCRF-CEM cells grown in 10% serum

Figure 9:

Western blotting data of anti-p53 [pS392] polyclonal antibody at 1:2000 on extracts from CCRF-CEM cells.



9a-phosphorylated p53 fusion protein
9b-non-phosphorylated p53 fusion protein
9c-CCRF-CEM cells grown in 10% serum

Immunohistochemistry

Introduction

Immunohistochemistry is used to identify the location and distribution of target antigens in cells or tissues by staining with a specific antibody. The antibody is conjugated to either a fluorescent or colorimetric label, and the location of the label seen through a microscope approximates the position of the target antigen. We present here a method that can be used for the staining of tissues and cell lines.

Purpose: Procedure for immunohistochemical staining of prepared frozen or paraffin-embedded sections and cultured cells.

Materials and Equipment

Prepared microtome-sectioned tissue slides, and chamber slides	Incubator box--large plastic tupperware type container with lid, bottom lined with moist paper towels
Glass adhesion microscope slides	ABC kit (Vectstain kit-Elite PK-6100 Standard, Vector Labs or equivalent)
Grease pencil	DAB substrate (DAKO #3465 or equivalent)
Cold acetone (store at -20°C)	Hematoxylin stain-Gill's Formulation #1 (Fisher #SP15-500; Cytoseal 60, Stephen Scientific or equivalent)
Small plastic funnel	Lab timer
Phosphate Buffered Saline (PBS) Buffer #1002	Fume hood
PBS with 2% BSA (Blocking Buffer)	Ethanol, 200 proof
Lab tissues (Kimwipes or equivalent)	Xylene, mixed (Sigma #X2377, or equivalent)
30% Hydrogen peroxide (Fisher/Acros #20246-5000 or equivalent)	Glass microscope cover slips
3M Sodium Azide stock solution	Sally Hansen™ Hard as Nails™ with nylon (Prod.#2700-95, nude)
Glass slide holder bottles (will accommodate 10 slides)-for a small number of slides	Light microscope
Large multiple slide holder (will accommodate 30 slides) with matching glass reservoir and lid-for a large number of slides	
Slide mounting medium (Permount, Fisher #SP15-500; Cytoseal 60, Stephens Scientific or equivalent)	

Preliminary Notes:

- Clearly label each slide with either a grease pencil or a regular graphite pencil in the frosted glass section, being sure to include a Negative Control for each different secondary antibody used.
- Draw a circle around the tissue sections with a grease pencil approximately 1 cm larger than the section itself before starting the staining procedure; this will help to contain the staining reagents in the area of interest.

- The acetone used to fix the tissues must be kept cold (-20°C) prior to use.
- Do not allow the tissue sections to dry out between steps, or at any time during the procedure, to help keep background staining to a minimum.

Safety Notes: 30% Hydrogen Peroxide, Sodium Azide and Ethanol: Wear gloves to prevent skin irritation/contamination. Acetone, Hematoxylin Stain, Xylene and Permout: wear gloves and utilize a fume hood.

Procedure:

Sample Preparation:

For paraffin-embedded tissue sections;

- Soak slides in 100% xylene for 10 minutes; decant the xylene into a vessel to re-use one time. Then repeat this 10 minute soak with fresh xylene; decant xylene, and save to re-use one time.
- Soak slides in 100% ethanol, then in 90% ethanol (in water), then in 70% ethanol for 3 minutes each, then soak in water for 5 minutes.
- Now go to **Blocking of Endogenous Peroxidase Activity** step.

For cultured cells:

- Use cells at a concentration such that 20 to 50 µL will deliver 1 x 10⁶ cells; dilute the cells accordingly with PBS.
- Apply 20 to 50 µL of the cell suspension to the centers of the appropriately-labeled glass adhesion microscope slides.
- Let stand for 5 to 10 minutes at room temperature.
- Rinse each slide with PBS three times.
- Now go to the **Fixation** step.

For frozen (-20° or -80°C) OCT-embedded tissue sections: proceed to **Fixation** step

Fixation:

- Place the slides into the appropriately-sized slide holder container. Pour in enough cold acetone (stored at -20°C) to cover the tissue sections; let stand for 2 minutes, then pour the acetone back into the same stock container for cold storage.
- Add enough PBS to cover the tissue sections on cells or slides; let stand for 10 minutes. Remove the slides and carefully remove excess PBS from around the tissue sections and from the bottom of the slides by wiping with a lab tissue.

Blocking of Endogenous Peroxidase Activity:

- Prepare Blocking Solution.

Table 10: Blocking Solution

	For 1 to 10 Sections	For Up to 40 Sections
PBS	1.0 mL	100 mL
30% Hydrogen Peroxide	10 µL	1.0 mL
3M Sodium Azide stock	5 µL	0.5 mL

2. For a few slides, lay the slides on a bench top and add enough Blocking Solution (approx. 100 µL) to cover the tissue sections.
3. For a larger number of slides, place the slides into a metal multiple holder, place into the rectangular glass vessel that the holder fits into, then add enough Blocking Solution (approx. 100 mL) to cover the tissue sections.
4. Block for 10 minutes at room temperature.
5. Place the slides in the appropriately-sized slide holder container.
6. Wash slides clean of Blocking Solution by filling the slide holder container with PBS, then decant; repeat for a total of 3 PBS washes.
7. Remove excess PBS from each slide by wiping edges and bottom of slide with a lab tissue.
8. Set up slides, if few in number, in a moist incubator box. With a small transfer or Pasteur pipette, add enough Blocking Buffer (PBS +2% BSA) to cover tissue sections. Place the cover onto the incubator box and incubate at room temperature for at least 30 minutes (this helps to minimize non-specific binding).
9. If working with a large number of slides, set them up in the large metal slide holder, place it into its matching glass container, then gently add enough Blocking Buffer poured down one side of the container to cover the tissue sections. Incubate at room temperature for 30 minutes.
10. Rinse slides with PBS one time.

Addition of Primary Antibody:

1. The most commonly used primary antibody concentrations are: 20, 10, 5, 1 µg/mL; dilute primary antibodies accordingly, using Blocking Buffer (PBS +2% BSA) as a diluent.
2. Add 100 µL of the diluted primary antibodies to each tissue section; incubate in a covered moist incubator box at room temperature for 1 hour.
3. Wash primary antibodies from the slides with PBS 2 times, allowing the slides to sit in the PBS for 5 minutes each time.

Addition of Secondary Antibody:

1. Dilute the secondary antibodies to be used 1:200 using Blocking Buffer (PBS +2% BSA) as a diluent.
2. Add 100 µL of diluted secondary antibody to each tissue section; incubate at room temperature in a moist incubator box for 40 to 60 minutes.

Note: This is now a good time to mix the ABC reagents together, as this must be done at least 30 minutes prior to use.

3. Wash slides free of excess antibody with PBS three times, 5 minutes each wash.

Immunoperoxidase Staining:

1. Preparation of ABC Reagents.

Table II:

	For 1 to 10 Slides	For Up to 40 Slides
PBS	1.0 mL	5 mL
Reagent A	10 µL	2 drops
Reagent B	10 µL	2 drops

2. Add 100 µL of the ABC solution to each slide; incubate at room temperature in a covered moist incubation box for 30 minutes.
3. Remove excess reagents by washing with PBS two times, 3 minutes each wash; wipe slides clean of excess PBS.
4. Prepare DAB Substrate: (Handle DAB reagent very carefully as it is highly toxic): 1 Drop (20 µL) DAB Chromogen per 1.0 mL Buffered Substrate.
5. Add 100 µL of freshly-prepared DAB Substrate to each tissue section; allow to react at room temperature for 5 minutes or until color develops (up to 15 minutes).
6. Rinse slides of excess reagents one time with PBS; then, wash slides under running tap water for 5 minutes to remove any residual DAB.

Hematoxylin Staining:

1. Set lab timer for 45 seconds.
2. Add enough hematoxylin to each slide to completely cover the tissue sections; stain for 45 seconds. Using a funnel, pour hematoxylin stain back into its stock container for re-use.
3. Remove excess stain by washing slides under running tap water for 10 minutes.

Serial Ethanol/Xylene Dehydration:

Note: This step to be performed under a Fume Hood.

1. For a small number of slides, set up a slide holder bottle and have each of the following reagents prepared in its own separate container:

Table 12:

	Ethanol	Xylene
Ethanol I	90% EtOH/10% H ₂ O	Xylene I 100% xylene
Ethanol II	100% EtOH	Xylene II 100% xylene
Ethanol III	100% EtOH	Xylene III 100% xylene

2. Soak the slides in Ethanol I for 3 minutes, then Ethanol II for 3 minutes, then Ethanol III for 3 minutes; in a similar manner, serially-expose the slides to Xylene I, II, and III, each for 3 minutes.

- For a larger number of slides, use the larger slide holder with its matching glass container. Pour in enough Ethanol I to cover the tissue sections, and let stand for 5 minutes to allow the dehydrant to fully penetrate the small spaces between slides. Raise the slide holder, pour off and save the Ethanol I, replace the slide holder, pour in enough Ethanol II to cover the tissue sections, and let stand for 5 minutes; continue this process in a similar manner using Ethanol III, and Xylenes I, II and III.
- The Ethanol and Xylene I, II and III reagents may be re-used again and again.

Permanent Mounting of Stained Tissue Sections for Long-Term Storage:

- Apply one drop of Permout or Cytoseal 60 to each stained tissue section.
- Apply a glass cover slip to each and gently press to expel excess mounting medium; carefully wipe away any excess medium with a lab tissue. Allow to air dry at room temperature. After the slides have fully-dried, seal the edges around the cover slip with a clear finger nail polish (or equivalent) to improve long-term storage; allow to air dry at room temperature. Slides mounted in this manner may be considered usable for an indefinite period of time.
- Examine slides under light microscope; 16X, then 40X.

Antibody	Clone	Part #	Size
bcl-2	124	AHO0042	200 µg
	100/D5	AHO0272	100 µg
BrdU	85-2C8	ANN0032	100 µg
cdk1/p34cdc2	A17.1.1	AHZ0122	100 µg
	POH-1/cdc2	AHZ0132	100 µg
cdk2	8D4	AHZ0152	100 µg
cdk4	DCS-35	AHZ0192	100 µg
	DCS-31	AHZ0202	100 µg
cdk6	K6.83	AHZ0232	100 µg
	K6.90	AHZ0242	100 µg
cdk7/CAK	MO-1.1	AHZ0262	100 µg
c-myc	9E10.3	AHO0062	100 µg
Cyclin A	BF683	AHF0012	100 µg
	E23	AHF0022	100 µg
	E72	AHF0032	100 µg
Cyclin B1	V152	AHF0052	100 µg
Cyclin D1	DCS-6	AHF0082	100 µg
Cyclin D3	DCS-22	AHF0132	100 µg
	DCS-28.1	AHF0142	100 µg
E2F-2	TFE22	AHF0192	100 µg
E2F-3	TFE31	AHF0202	100 µg
	TFE36	AHF0212	100 µg
E2F-4	TFE42	AHF0222	100 µg
Ki 67	MIB-1	AHF0241	7.0 mL
p16 INK4a	DCS-50.1/A7	AHZ0312	100 µg
	ZJ11	AHZ0332	100 µg

Antibody	Clone	Part #	Size
p21 WAF1	DCS-60.2	AHZ0392	100 µg
	HJ21	AHZ0422	100 µg
p27 kip1	DCS-72.F6	AHZ0452	100 µg
p53	Pab 240	AHO0112	100 µg
	Pab 1801	AHO0122	100 µg
	DO-7	AHO0142	100 µg
	DO-1	AHO0152	100 µg
	BP53-12	AHO0032	100 µg
PCNA/Cyclin	PC10	AHF0232	100 µg
Rb (AA 703-772)	1F8	AHO0172	100 µg
Rb (AA 444-621)	XZ55	AHO0182	100 µg
Rb (AA 444-535)	XZ91	AHO0192	100 µg
Rb (AA 393-572)	XZ104	AHO0202	100 µg
Rb (AA 620-665)	XZ133	AHO0212	100 µg

Phospho-Site Specific Antibodies

Antibody	Clone	Part #	Size
cdk1 [pTpY14/15]	Polyclonal	44-686	100 µL
c-Raf [pS621]	Polyclonal	44-504	25 µg
c-Raf [pYpY340/341]	Polyclonal	44-506	25 µg
ERK1/2 [pTpY185/187]	Polyclonal	44-680	100 µL
JNK/SAPK [pTpY183/185]	Polyclonal	44-682	100 µL
p38 [pTpY180/182]	Polyclonal	44-684	100 µL
p53 [pS392]	Polyclonal	44-640	25 µg
Rb [pT821]	Polyclonal	44-582	100 µL
Rb [pSpT249/252]	Polyclonal	44-584	100 µL

References:

- Boulton, T.G., et al., (1991) Purification and properties of extracellular signal regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. *Biochemistry* 30:278-286.
- Lange-Carter, C.A., et al., (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* 260:315-319.
- Burnette, W.N., (1981) "Western Blotting:" electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112:195-203.
- Laemmli, V.K., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Regulation of Protein Phosphorylation: Applications for Cell Cycle Research: Case Studies

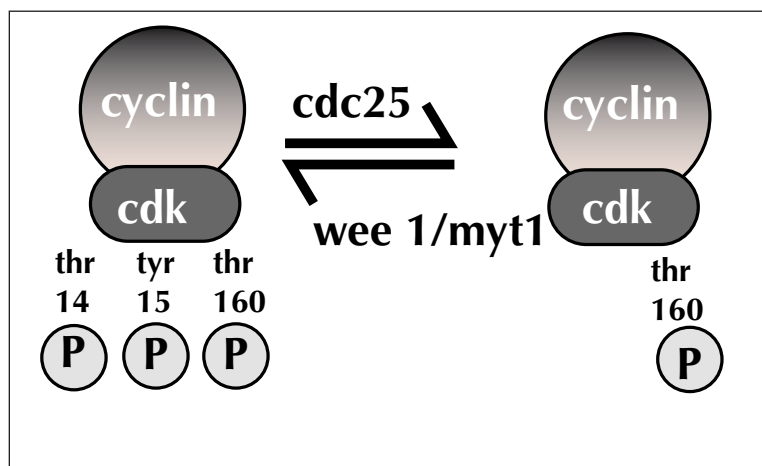
Introduction

Protein phosphorylation is required in many cellular processes, including division and growth. Traditionally, studying phosphorylation relied on labeling with ³²P-ATP, immunoprecipitating with a non-specific anti-phospho-tyrosine antibody or mutagenesis of recombinant protein. An alternative to these methods uses antibodies that specifically recognize the phosphorylated residue(s). Here we characterize rabbit polyclonal antibodies that recognize mammalian cyclin dependent kinase 1 (cdk1) phosphorylated on threonine 14 and tyrosine 15 and Rb phosphorylated on different residues.

Case Study 1:

Regulation of Cyclin B1/cyclin dependent kinase 1 complex

Cdk1, also known as cdc2 in yeast, is the catalytic portion of the cyclin B1/cdk complex [for review, see 1 (and ref. within)]. Upon activation, cdk 1 induces cells to progress from the G2 phase of the cell cycle through mitosis. This occurs when threonine 14 and tyrosine 15 are dephosphorylated in the presence of the phosphatase, CDC25C (see diagram)[2,3,4]. In *Xenopus* and humans, myt1 and wee1 phosphorylate threonine 14 and tyrosine 15 at the end of mitosis, inhibiting the complex activity [4,5].



Materials and Methods:

All antibodies used in this study were from BioSource International, Inc., The phosphorylation-state specific rabbit anti-cdk1 [pTpY14/15] antibody was generated against a synthetic phosphopeptide derived from the region of the human cyclin-dependent kinases that contain threonine 14 and tyrosine 15. The antibody was negatively preadsorbed using a column coupled to the non-phosphorylated peptide corresponding to the site of phosphorylation. This removed antibody reactive with non-phosphorylated cyclin-dependent kinase. The final product was generated by affinity chromatography using a column coupled to the phosphorylated peptide.

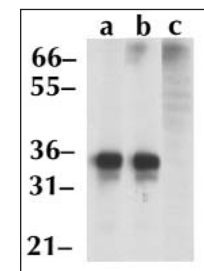
Immunoprecipitation and Western blotting experiments were performed using Jurkat cell extracts. Cells were grown in RPMI 1640 medium with 10% fetal bovine serum, supplemented with 4 mM glutamine and antibiotics. Cell extracts were prepared by lysing cells for 10 minutes on ice in buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% SDS, 1% triton-X100, 0.5% deoxycholate plus phosphatase and protease inhibitors. Extracts were clarified by centrifuging lysates at 14,000 x g, for 20 minutes at 4°C. For immunoprecipitation, cell extracts were incubated with the appropriate monoclonal antibody overnight at 4°C and antibody associated-proteins were precipitated using Protein A/G resin (Pierce). For immunoblotting, proteins were resolved by SDS-PAGE on a 4-20% tris-glycine gel and immobilized on PVDF membranes. After incubation with the primary antibodies, membranes were washed and incubated with goat F(ab')₂ anti-rabbit IgG alkaline phosphatase. Bands were detected using the Tropix WesternStar' detection method.

Results and Discussion

The anti-cdk1 [pTpY14/15] antibody specifically recognizes a 34 kDa band in proliferating Jurkat cells (Figure 1; lane a). Blocking studies with excess non-phosphorylated and phosphorylated peptide show that this antibody specifically recognizes the cdk1 phosphorylated on threonine 14 and tyrosine 15 and has no reactivity to cdk1 which is not phosphorylated at these residues (Figure 1; lanes b and c).

Figure 1:

Membranes were incubated with a) 0.25 µg/mL antibody alone; b) 0.25 µg/mL antibody pre-incubated with 10 µg non-phosphopeptide; c) 0.25 µg/mL antibody pre-incubated with 10 µg phosphopeptide.



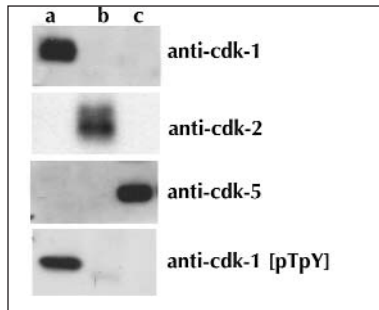
As Figure 2 shows, the region surrounding the threonine and tyrosine residues in human cdk1 are highly conserved in the other cyclin-dependent kinases, cdk2 and cdk5. Because of the homology of this region, it is presumed that an antibody that recognizes cdk1 [pTpY14/15] would also recognize cdk2 and 5 phosphorylated on these residues. To test the selectivity of the antibody for phosphorylated cdk1, immunoprecipitations were performed using monoclonal antibodies specific for cdk1 (Figure 3; lane a [clone #A17.1.1]), cdk2 (Figure 3, lane b [clone # 2B6]) and cdk5 (Figure 3; lane c [clone #DC17]). As the immunoblot in Figure 3 shows, the anti-cdk1 [pTpY14/15] antibody only recognizes the protein precipitated by the cdk1 antibody. These results are consistent with published observations, showing that cdk5 is not phosphorylated by wee1 on tyrosine 15 and wee1 only weakly phosphorylates cdk2 at this site [5,6]. In addition, cdk2 is not a substrate for phosphorylation by myt1, the kinase that phosphorylates cdk1 on both threonine 14 and tyrosine 15 [7].

Figure 2:

Alignment of human cyclin-dependent kinases.

Y T	K I E K I G E G T Y G V V Y K	G R H	human cdk1
F Q	K V E K I G E G T Y G V V Y K	A R N	human cdk2
Y E	K L E K I G E G T Y G T V F K	E K N	human cdk5

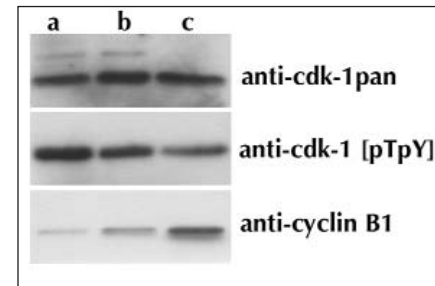
Figure 3:



Jurkat cell extracts were immunoprecipitated with pan monoclonal antibodies to a) cdk1; b) cdk2; or c) cdk5. All antibodies were used at 1 µg per 200 µg of extract.

To demonstrate the usefulness of PSSAs, immunoblotting was performed with the anti-cdk1 [pTpY14/15] antibody on serum-starved cells, proliferating cells and cells arrested in G2/M with nocozadole. The percentage of cells in each phase of the cell cycle was confirmed by flow cytometry (Figure 4a). Figure 4 demonstrates that as more cells enter into G2/M, there is an increase in the expression of Cyclin B1 protein. This increase correlates with a decrease in the amount of cdk1 phosphorylated on threonine 14 and tyrosine 15. These data are consistent with the role of the active Cyclin B1/cdk1 complex in mitosis and confirm early mutagenesis studies describing the phosphorylation state of cdk1 at various phases of the cell cycle [8].

Figure 4:



Jurkat cells were a) serum-starved; b) proliferating, and c) treated with 1 µg/mL nocozadole. All treatments were done for 18 hours.

In conclusion, the data demonstrates the specificity of PSSAs and offers an alternative to the use of radiolabeling, anti-phospho-tyrosine antibodies and mutagenesis to study protein phosphorylation.

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**Case Study 2:
Regulation of Retinoblastoma protein phosphorylation**

Retinoblastoma Protein, also know as RB, Rb or pRb, is a tumor suppressor gene which functions as a negative regulator of the cell cycle by interacting with transcription factors including E2F-1, PU.1, ATF-2, UBF, Elf-1 and c-abl. This ability to alter transcription is regulated by phosphorylation catalyzed by the cyclin-dependent protein kinases (cdks). pRb contains at least 16 consensus sequences for cdk phosphorylation (see figure for specific sites). The significance of all these sites is unclear. These sites are phosphorylated by different cyclin-dependent kinases. Phosphorylation of threonine 821 is catalyzed by cdk2 complexes such as Cyclin E-cdk2 and Cyclin A-cdk2. It has been demonstrated that phosphorylation of threonine 821 disrupts interaction with proteins containing the sequence LXCXE and also disrupts binding to E2F. Phosphorylation of serine 249 and threonine 252 is catalyzed by cdk4 complexes such as Cyclin D-cdk4. Cdk4 becomes active prior to cdk2 during G1, and recent evidence suggests that phosphorylation of Rb by cdk4 may be required for its subsequent phos-

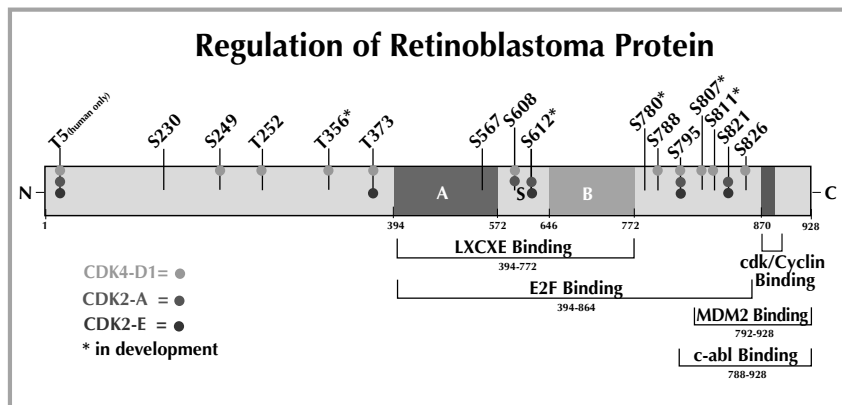
phorylation by cdk2. It has been shown that cdk4 phosphorylation inhibits HDAC binding which is involved in DNA acetylation and active repression by Rb.

In this section, we examined the regulation of Rb phosphorylation using synchronized cells to determine at which phase of the cycle specific Rb residues are phosphorylated. One of the major targets for drug research is to prevent Rb phosphorylation through the inhibition of cyclin-dependent kinase (CDK) activation. Therefore, a test of the effect of a generic CDK inhibitor on the phosphorylation of Rb on either a cdk2 site (T821) or a cdk4 site (S249/T252) follows.

Materials and Methods

For synchronization experiments, cells were serum starved for over 12 hours, then released by addition of 10% serum to the media (RPMI 1640 medium supplemented with 4 mM glutamine and antibiotics). For the inhibitor studies, cells were serum starved for 12-16 hours. The inhibitor (1 μM) was added at the same time as cells were placed in fresh media containing 10% serum. For both experimental conditions, cells were grown for the times indicated in the figures then harvested. Cell extracts were prepared by lysing cells for 10 minutes on ice in a buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% SDS, 1% triton-X100, 0.5% deoxycholate plus phosphatase and protease inhibitors. Extracts were clarified by centrifuging lysates at 14,000 x g for 20 minutes at 4°C. Proteins were resolved by SDS-PAGE on a 4-20% tris-glycine gel and immobilized on PVDF membranes. After incubation with the primary antibody [1F8 pan Rb antibody at 1:300 dilution and anti-[pSpT249/252] and the anti-[pT821] PSSAs at 1 μg/mL], membranes were washed and incubated with either goat F(ab')₂ anti-rabbit or anti-mouse IgG alkaline phosphatase. Bands were detected using the Tropix Westernstar detection method.

Regulation of Rb and E2F by cyclin/cdk complex throughout the cell cycle



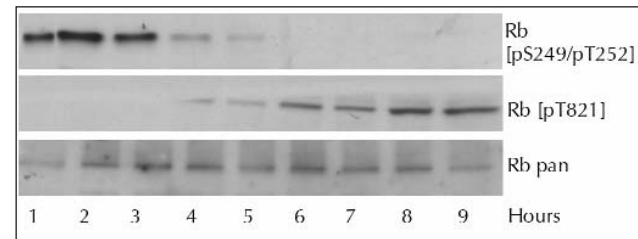
Results and Discussion

Since Rb phosphorylation is involved in cell cycle progression and various cyclin-dependent kinases phosphorylate different residues on the protein, it can be presumed that this is time dependent and coincides with the expression patterns of the CDKs. To demonstrate this, cell extracts were made each hour after cells were synchronized and the resulting blots were probed using either Rb [pSpT249/252], Rb [pT821] or a pan Rb antibody (figure 1). The propidium iodide staining profiles are shown on pages 15-16.

The results show early phosphorylation of the serine 249 and threonine 252 site. This is consistent with this site being regulated by the Cyclin D/cdk4 complex, which is active in early G1. The threonine 821 site is phosphorylated later on where more cells are in S, G2, and M phases of the cell cycle. This site is reported to be phosphorylated by the Cyclin E/cdk2 complex, which is necessary for the progression through S phase. However, this data shows that when 100% of the cells are in S phase there is minimal phosphorylation of this site (time point 4H). This may indicate that this site is also regulated by other CDKs such as the Cyclin B1/cdk1 complex.

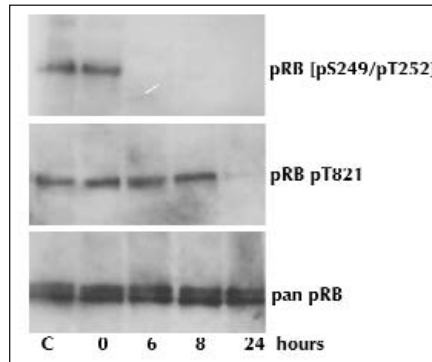
Another use of the antibodies directed towards the phosphorylation sites on Rb is to screen drug targets. During the cell cycle, Rb is phosphorylated by a number of CDKs. These complexes have been implicated in various cancer pathologies i.e. Cyclin D1/cdk4 and Cyclin E/cdk2 in breast cancer. Therefore, a major focus of drug discovery is to target these complexes and find effective inhibitory molecules. One downstream effect of inhibition is to prevent Rb phosphorylation and this can effectively be used to screen these drugs. In figure 2, we show the results obtained when cells are treated with a generic CDK inhibitor. The data indicate that the cdk4 activity is effectively inhibited before 6 hours, whereas the kinases that phosphorylate threonine 821 are inhibited later, at the 24 hour time point. This data show that this drug effectively inhibits Rb phosphorylation at different sites and appears to differentially inhibit CDKs.

Figure 1



Cell extracts were prepared from CRF-CEM cells synchronized through 24 hour serum starvation. Cells were grown for three days in RPMI 1640 + 10% FBS prior to starvation in RPMI 1640 without FBS for 24 hours. Cells were released with 10% FBS, and cell extracts were made at the above time points following release with serum. Cell extracts were resolved on SDS PAGE and transferred to PVDF membrane. Membranes were incubated with 1 mg/mL of either RB [pS249/pT252] PSSA, RB [pT821] PSSA, or RB pan (clone 1F8) antibody. After washing, membranes incubated with PSSA polyclonal antibodies were incubated with goat F(ab')₂ anti-rabbit IgG alkaline phosphatase 1:5000 dilution (cat. # ALI4405). The monoclonal pan RB clone 1F8 was incubated with goat F(ab')₂ anti-mouse IgG alkaline phosphatase 1:5000 dilution. Bands were detected using Tropix Western Star.

Figure 2



Cell extracts were prepared from human FSF cells that were treated with a generic cyclin-dependent kinase inhibitor at the time points indicated (C-24 hour vehicle control).

In conclusion, phosphorylation state specific antibodies targeted to an important regulatory molecule such as Rb can give important information as to how these molecules are differentially regulated and can be used to effectively screen new drugs improving the drug discovery progress.

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