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Levels of cyclin B in THP-1 cells incubated in hibernating and non-hibernating bullfrog plasma, Lithobates catesbeianus

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LEVELS OF CYCLIN B IN THP-1 CELLS INCUBATED IN HIBERNATING AND

NON-HIBERNATING BULLFROG PLASMA, *Lithobates catesbeianus*.

An Abstract of a Thesis

Submitted

in Partial Fulfillment

of the Requirements for the Degree

Master of Science

Tharuka D. Pathmaperuma

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ABSTRACT

Hibernation is a process that occurs in nature where organisms undergo dormancy for long periods of time in order to improve their survival during extreme environmental conditions. During this time, organisms undergo physiological changes such as reduction in core body temperature and metabolic rate, as well as cells being impeded from going into mitosis. Alvarado et al. (2015) discovered that genomic DNA methylation is dynamic across torpor-arousal bouts during winter hibernation in thirteen lined ground squirrels (*Ictidomys tridecemlineatus*), indicating that physiological changes during hibernation are the results of more than simply cold temperatures slowing down metabolism and that there are cellular mechanisms that are responsible for such physiological changes. A study by Robbins (2017) found that THP-1 cells incubated in hibernating bullfrog plasma (*Lithobates catesbeianus*) stop undergoing cellular division, with THP-1 cells being restricted to the G2 stage of the cell cycle. This suggests that there may be one or more substances in hibernating bullfrog plasma that prevent THP-1 cells from progressing in cell division. In this study, I explored the effects of cyclin B levels on cellular division and its potential impact on confining THP-1 cells in the G2 phase, while maintained in hibernating bullfrog plasma. My results indicate that the levels of cyclin B in hibernating and non-hibernating bullfrog plasma do not vary, suggesting that the expression of cyclin B may not be responsible for confining THP-1 cells to the G2 stage of the cell cycle.

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This Study By: Tharuka D. Pathmaperuma

Entitled: Levels of cyclin B in THP-1 cells Incubated in Hibernating and

Non-Hibernating Bullfrog Plasma, *Lithobates catesbeianus.*

has been approved as meeting the thesis requirement for the

Degree of Master of Science

_____	_____
Date	Dr. David Saunders, Chair, Thesis Committee
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Date	Dr. David McClenahan, Thesis Committee Member
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Date	Dr. Ira Simet, Thesis Committee Member
_____	_____
Date	Dr. Jennifer Waldron, Dean, Graduate College

DEDICATION

I would like to dedicate this document to all the teachers, professors and mentors who have guided me along the way. It can be argued that every teacher, has made an impact over the years, however, there are those that stand above everyone. It is to them, that I direct most of my most sincere gratitude: Dr. David Saunders, Dr. David McClenahan, Dr. Ira Simet, Dr. Nina Rodriguez, Mary McDade, Dr. Tilahun Abebe, Dr. Carl Thurman, Dr. Kenneth Elgersma and Dr. James Jurgenson. Without your efforts, this document and much of my success would not have been possible. My best regards. Most especially, I would also like to dedicate this document to my loving fiancé, Hannah Grace Stone, who has given me the greatest support of all.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Some animals undergo hibernation to survive long-term stressful environmental conditions allowing them to live through periods of extreme cold and scarce availability of food. These animals are capable of undergoing physiological changes such as adjusting their body temperature to the ambient temperature and thereby dropping their metabolic rate during these periods of dormancy (Melvin & Andrews, 2009). Regulation of the cell cycle during these periods is extremely important, as carrying out certain processes might not be necessary for survival during these times, and provides for conserving energy allowing these organisms to return out of dormancy successfully (Carey et al., 2003).

The cell cycle, as shown in Figure 1, is the series of growth and development cells undergo from their formation by the division of a parent cell to create two new daughter cells. There are four stages of the cycle, Gap 1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis, which is the process of cell division. The G1, S, and G2 stages together are known as Interphase as it takes place between one mitosis stage and the next. During the G1 stage, cells increase in size, cellular organelles are being duplicated and the cell makes the molecular building blocks it will need in the later stages. During the S stage, the cell synthesizes a complete copy of the DNA in its nucleus. The cell also duplicates the centrosome, a microtubule-organizing structure that helps separate chromosomes during the M stage. During the G2 stage, the cell increases more in size and makes

proteins and cellular organelles, and begins to reorganize its contents and prepares for mitosis.
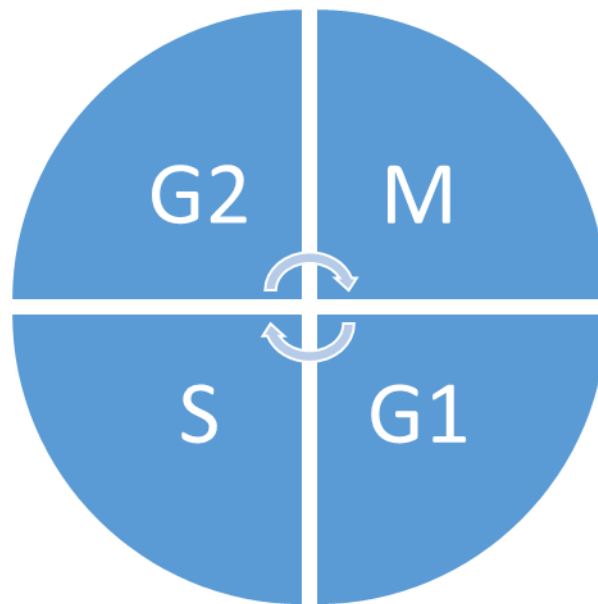

Figure 1: The different phases of the cell cycle. Gap 1 (G1),

Synthesis (S), Gap 2 (G2) and Mitosis (M).

During the M stage, two events take place, mitosis and cytokinesis. In mitosis, the nuclear DNA within the cell condenses into visible structures known as chromosomes and is pulled apart by a specialized structure made out of microtubules, known as the mitotic spindle. Mitosis takes place in four stages: prophase (which can be further divided into early prophase and prometaphase), metaphase, anaphase, and telophase. During cytokinesis, the parent cell's cytoplasm divides into two, making two new daughter cells. Cytokinesis generally takes place towards the end of mitosis. Once the

cell cycle is complete, the cell can take one of two possible paths: it can repeat the cell cycle, starting from G1, or exit the cell cycle through G0. G0 exists outside the cell cycle, where cells can undergo terminal differentiation, based on the characteristics of that cell's final function and destination.

Cyclins are vital core regulators of the cell cycle, consisting of a group of regulatory proteins. In most eukaryotes, four basic types of cyclins are found: cyclin A, cyclin B, cyclin D and cyclin E. Each of these cyclins plays an important role with the progression from various stages, or set of stages in the cell cycle, and helps move the cell through these stages.

Levels of each of these cyclins vary considerably across the cell cycle, as shown in Figure 2 below. Typically, a cyclin is kept at low levels for most of the cycle, but as the cell approaches the stage where cyclins are needed, the levels of specific cyclins increase. For example, levels of cyclin B increase dramatically at the progression from the G2 stage to the M stage.
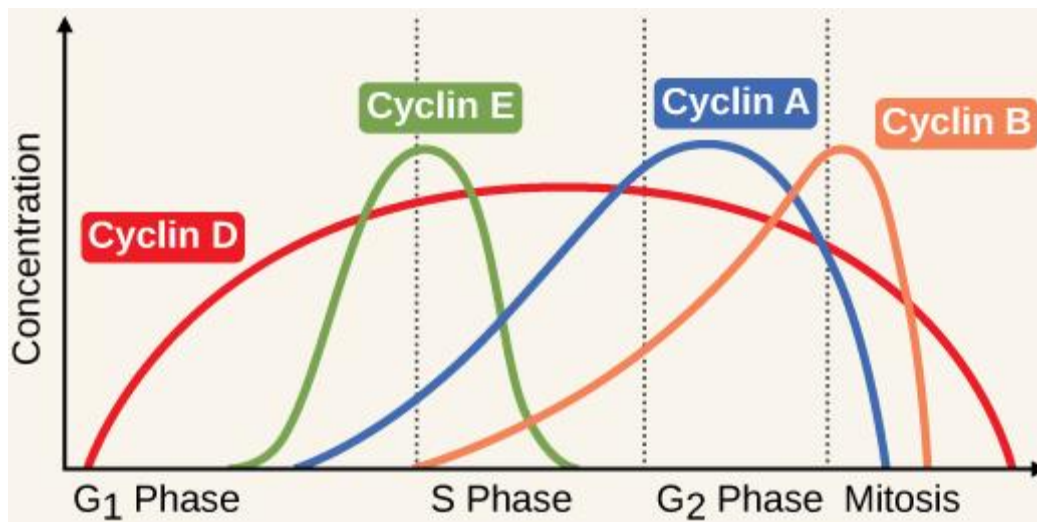
Figure 2: Change in levels of cyclin in relation to the various stages of the cell cycle.

There is a sharp decrease in levels of cyclins following each transition (the transition between

stages of the cell cycle), as cyclins are degraded by cytoplasmic enzymes (adapted from College

of the Redwoods, & Northern Virginia Community College, n.d.).

Cyclin Dependent Kinases (CDKs) are enzymes that phosphorylate (attach

phosphate groups to) a specific target once a particular CDK is activated. CDKs are

responsible for the progression from G1 to S stage and from G2 to M stage, by

phosphorylating specific substrates. Attachment of phosphate groups acts like a switch,

which increases or decreases the activity of a target protein. Cyclins attach themselves to

CDKs at various stages of the cell cycle which plays two important roles. First, it

activates the ability for a CDK to act as a kinase, and second it directs the CDK to

phosphorylate target proteins. These proteins are ones that are appropriate to that stage of

the cell cycle which is controlled by the cyclin. For example, the binding of cyclin B to

its CDK sends CDK1 to M phase targets (e.g., proteins that are responsible for breaking down the nuclear membrane).

In mammalian cells, the progression from G1 to S stage is specifically guided by the activity of two CDKs, which are comprised of the complexes between cyclin D and either CDK4 or CDK6 and between cyclin E (and possibly cyclin A) and CDK2. The progression from G1 to S stage is regulated by a variety of internal and external signals, including the availability of stage specific cyclin, the levels of CDK inhibitory (CDKI) proteins and the phosphorylation status of CDKs. Many of the components of the cell cycle regulatory machinery, including CDKs, CDKIs and CDK substrates, are important targets of mutations that lead to human cancer (Reed, 1997).

CDK1 and CDK2 both can bind with their choice of cyclin partners and can bind to cyclins A, B, D and E, whereas CDK4 and CDK6 only partner with cyclin D. Cyclin D and CDK4 or CDK6 regulate events during the early G1 phase, CDK2-cyclin E complex allows progression into the S stage, CDK2-cyclin A complex and CDK1-cyclin A complex regulate the completion of S stage, and CDK1–cyclin B complex is responsible for mitosis. Based on the results of the cyclin and CDK-knockout studies, a minimal threshold model of the cell cycle control has emerged, suggesting that either CDK1-cyclin A or CDK2-cyclin A is sufficient to control interphase, whereas CDK1-cyclin B is essential to take cells into mitosis (Reed, 1997).

Activation of the CDK1-cyclin B complex initiates the onset of mitosis and is tightly regulated. CDK1-cyclin B complex is a serine/threonine protein kinase composed of the catalytic subunit CDK1 and its positive regulatory subunit cyclin B (Hochegger et

al., 2008). Binding of CDK1 to cyclin B is essential for activating CDK1.

Phosphorylation at Threonine 161 (T161) positive regulatory site, is required for

activation of the CDK1-cyclin B complex and is mediated by the CDK activating kinase

(CAK) (Hochegger et al., 2008).

During the early G2 phase, as shown in Figure 3 below, the CDK1-cyclin B

complex is held in an inactive state by phosphorylation of CDK1 at the two negative

regulatory sites, Threonine 14 and Tyrosine 15 (T14 and Y15), by CDK1 protein kinases

Myt1 and Wee1, respectively (Hochegger et al., 2008; Squire et al., 2005). Removing

these phosphate groups from T14 and Y15 by cell division cycle (CDC25) protein

phosphatase (a protein that removes phosphate groups) in the late G2 phase activates the

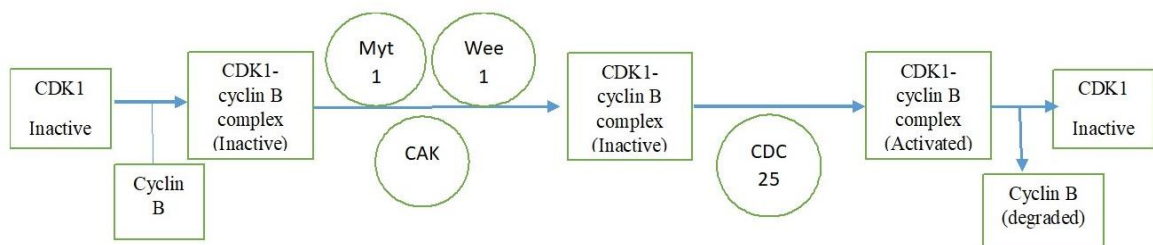CDK1-cyclin B complex and triggers the initiation of mitosis.

Figure 3: The different phases that CDK1 undergoes throughout the different stages of the cell
cycle from late S stage to mid M stage (Adapted from Pollard et al., 2017).

Zhang and Storey (2012), studied the effects of cell cycle arrest in the ability of

wood frogs (*Rana sylvatica)* to survive freezing. Freezing is multi-component stress that

includes ischemia due to decreased blood flow and dehydration that cells undergo due to

the accumulation of ice in extracellular spaces. To endure these stresses, cells need to reprioritize energy expenditure for cellular function. Expression of CDK2, CDK4, CDK6, cyclin A, cyclin B, cyclin D, and cyclin E and the phosphorylation states of CDKs (T14 and Y15), the phosphatase CDC25 and the CDKI p21 in the livers of these wood frogs were studied using immunoblotting techniques. Responses of these wood frogs to 24 hours of freezing and anoxia, along with 40% dehydration and recovery from these stresses were evaluated. Their results showed reduced levels of CDK2, 4 CDK6, and cyclins A and B in the liver cells of wood frogs after 24 hours of exposure to freezing. This supports the idea that the cell cycle is suppressed under these stresses and this suppression is reversed during recovery, finding substantially lower expression of cyclin B in frogs exposed to these stresses as compared to the levels in the wood frogs not exposed to stress.

Wu and Storey (2012) analyzed the cell cycle in the liver cells of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus)* during hibernation. They examined the different expression levels of regulators of the cell cycle over six stages of a cycle of torpor-arousal, again using immunoblotting techniques. They found that expression levels of cyclin D and cyclin E were reduced in the liver cells of hibernating ground squirrels. Using PCR analysis, they showed upregulation of cyclin D, cyclin A and cyclin B levels by about 1.5-fold, 1.57-fold, and 2.44-fold, respectively, during arousal. Also observed was suppression of cyclin B levels during entry into torpor; however, levels did not differ during long-term torpor and early torpor, but during early arousal, cyclin B increased greatly. Overall, this study suggested a pattern of how cyclins are expressed, with

expression of cyclin D and cyclin E inducing G1 to S stage arrest during torpor; and cyclin B and cyclin A expression upregulated during arousal. Similarly, previous research on sea cucumbers, *Apostichopus japonicus*, found methylation levels of cyclin B genes, and resulting suppression of gene expression, during aestivation, compared to non-aestivation (Zhu et al., 2016). Expression of cyclin B transcripts was significantly down regulated during deep aestivation.

Harley et al. (2010) found that the levels of the human anti-apoptotic (anti-cell death) protein Mcl-1 are regulated during the cell cycle and its levels increase at mitosis. The Mcl-1 protein is phosphorylated at two sites during mitosis, Serine64 and Threonine92 (S64 and T92). Phosphorylation of T92 by CDK1–cyclin B complex initiates the breakdown of Mcl-1 in cells that cannot perform mitosis due to damage by microtubule poisons resulting in apoptosis of these cells. Proteasome activity is required for the breakdown of the Mcl-1protein when mitosis cannot be performed and this is dependent on CDC20. CDC20 regulates the identification of mitotic substrates by the anaphase-promoting complex/cyclostome (APC/C) E3 ubiquitin ligase. This suggests that the regulation of apoptosis is highly connected to transition through mitosis and is regulated by timed mechanisms that distinguish between normal mitosis and prolong arrested mitosis. Addition of a phosphate group to the Mcl-1protein by CDK1–cyclin B complex and its APC/C^{Cdc20}-mediated destruction initiates apoptosis if a cell fails to transition into mitosis. This study shows that a lack of CDK1-cyclin B, or its activity, would prevent apoptosis in cells that have arrested in mitosis (Harley et al., 2010). This may be important in maintaining cells confined in the G2 phase in hibernating organisms.

Kruman et al. (1988) studied mitotic activity in the ground squirrel *Citellus undulatus* to determine if their intestinal epithelial cells progress through the cell cycle during hibernation and if the cell cycle gets blocked during the G2/M stage during hibernation. The intestinal epithelial cells were blocked at the G2 phase of the cell cycle during hibernation and the number of cells confined in the G2 phase in these ground squirrels slowly increased throughout their hibernation (Kruman et al., 1988). Rieder and Cole (2002) summarized previous findings that also showed cells were confined in the G2-M stage of mitosis in animals that undergo hibernation and torpor and exposure to cold temperatures. Robbins (2017) found that THP-1 cells (a leukemia cancer cell line) incubated in hibernating American bullfrog plasma, *Lithobates catesbeianus*, also stopped in the G2 phase and did not proceed to the M phase, while THP-1 cells incubated in non-hibernating plasma were unaffected. This suggests that there are one or more compounds in hibernating bullfrog plasma that prevent THP-1 cells progressing from the G2 to M stage of the cell cycle. My research focus was on potential mechanisms that would prevent progress from the G2 to M phase, specifically investigating levels of cyclin B in THP-1 cells cultured in hibernating and non-hibernating American bullfrog plasma.

The aforementioned studies suggest a potentially important role of cyclin B in the arresting of THP-1 cells during incubation in hibernating bullfrog plasma. Robbins (2017) found that a vast majority of THP-1 cells grown in hibernating bullfrog plasma had decreased/inhibited cell division as compared to cells grown in plasma from bullfrogs maintained at room temperature. These cells grown in hibernating bullfrog plasma appear

to be confined to the G2 stage, and unable to progress further in the cell cycle. I suspect that, unlike plasma from bullfrogs kept at room temperature, there is a compound in hibernating bullfrog plasma that is preventing THP-1 cancer cells from dividing. My hypothesis is that the levels of cyclin B in THP-1 cells cultured in hibernating bullfrog plasma are significantly lower than those cultured in the plasma of bullfrogs kept at room temperature, and this is responsible for the lack of movement from the G2 stage to mitosis. If no significant difference in the levels of cyclin B in THP-1 cells cultured in hibernating and non-hibernating bullfrog plasma is found, I will accept the null hypothesis that levels of cyclin B have no effect on inhibiting/decreasing cell division of THP-1 cells in hibernating bullfrog plasma. This would suggest mechanisms other than the levels of cyclin B are involved in the stalling of THP-1 cells in the G2 phase while incubated in plasma from hibernating bullfrogs.

CHAPTER 2

MATERIALS AND METHODS

The following protocol was designed to minimize discomfort and pain. Twenty-two of the American bullfrogs used in this study, *Lithobates catesbeianus*, were purchased from Kons Direct (Germantown, WI 53022). The frogs were separated into four twenty-gallon transparent plastic storage containers with ventilated lids and placed in a temperature-controlled environmental chamber held at 28°C. Each container was filled to a level of 4cm of tap water. Four bullfrogs were randomly selected and placed in a separate container that was kept in the laboratory at room temperature. These bullfrogs were classified as active non-hibernating bullfrogs. The remaining bullfrogs were randomly placed in three containers and kept in the environmental chambers. These bullfrogs were classified as the hibernating bullfrogs. The temperature of the cold room was lowered to 24°C for one week, and subsequently every four days, the temperature of the chamber was lowered by 4°C until the final temperature reached 4°C.This temperature was maintained for a period of 3 months, and these frogs were used to extract hibernating bullfrog plasma for this study. Bullfrogs were not fed during hibernation.

Collecting Blood Plasma

The following experiment was conducted according to the protocol described in the "AVMA Guidelines for the Euthanasia of Animals: 2013 edition," American Veterinary Medical Association, 2013(Underwood et al., 2013). Non-hibernating bullfrogs were kept in the laboratory for 3weeks at room temperature. Blood was drawn from two frogs at a time. Frogs were anesthetized using 2.5g/L solution of tricaine methanesulfonate (MS-

222). Sodium bicarbonate was added to the solution to raise the pH to approximately 7-7.5 since MS-222 is acidic with an approximate pH 5. Frogs were immersed in the solution until they were unable to move, which took 8-12 minutes after immersion.

Once anesthetized, frogs were placed on their dorsal side and blood was drawn through a cardiac puncture. The heart was exposed, using a vertical incision on the ventral side, to the right of the sternum. To prevent coagulation of blood entering the syringe a 3.5% sodium citrate flushed sterile syringe with a 21G x 1 ½ inch needle was used to draw blood from the ventricle. Blood drawn in these syringes was placed into a tube containing 3.5% sodium citrate. Blood was separated into centrifuge tubes with 1.5ml of blood per tube, followed by centrifugation for 5minutes at 2500xg to separate the plasma from blood. Each sample of plasma was then pipetted into separate centrifuge tubes with 1mL of plasma per tube, labeled, and was stored at -80°C. This procedure was used for each bullfrog in the study. Blood from the other two non-hibernating bullfrogs was drawn within nine days of the first two.

Blood was drawn from the hibernating bullfrogs after one week of exposure to 4°C. Blood was drawn from frogs maintained at this temperature every 3 weeks between the first and sixth week that these animals were exposed to 4°C. The procedure for drawing blood from hibernating bullfrogs was the same as that from non-hibernating animals. Immersion time in MS-222 was longer in hibernating frogs, 10-15 minutes, compared to that of room temperature frogs.

Experimental Design

Cell Culture Media

THP-1 cells, a human monocyte cell lineage derived from a 1-year old human male with leukemia, were used in this study. Media for culturing THP-1 cells was prepared by combining 180mL Roswell Park Memorial Institute (RPMI) media, 20mL Fetal Bovine Serum (FBS) and 0.78µL 2-Mercaptoethanol. 9mL of media was pipetted into a 50ml conical tube and combined with 1mL of THP-1 cells and centrifuged for 5 min at 1325xg. After centrifugation, the media was decanted and 10mL of new media was added to the cells remaining in the conical tube. This mixture was placed in a T25 flask then incubated at 37^0C under 5% CO_2. Cell cultures were maintained by removing 5mL of old media and adding 5mL of fresh media, 2 times per week. Cells were counted by staining 10uL of the culture mixture with 10uL Trypan blue and running the cell suspension through a TC10^{TM} Automated Cell Counter, to ensure that more than 500 000 cells were present to perform the antibody assay and flow cytometry.

Culturing Cells in Plasma for Flow Cytometry

A volume of 1mL of the cultured cells was added to a 5ml Falcon tube and spun for 5 min at 1325xg. The fluid was decanted without removing the pellet. The pellet was washed in 1mL of phosphate-buffered saline (PBS) and vortexed for approximately 10 seconds. The cells were spun again for 5min and the fluid was decanted again without removing the pellet. 100uL PBS was added to the cells and vortexed for approximately 10 seconds. From the suspension, 30µL was added to 300µL of each of the following: hibernating plasma, non-hibernating plasma, and in the culture media, which served as the

control. All cultures were incubated for 72 hours in an incubator maintained at 37°C with 5 % CO2.

Antibody Staining

At the end of the 72-hour incubation period, from each of the three suspensions (hibernating plasma, non-hibernating plasma, and culture media), a volume of 2mL was spun down for 5 min at 1325xg and the fluid was decanted without removing the pellet. The cells were then re-suspended in 1mL PBS and vortexed gently for approximately 10 seconds. The cells were spun down again for 5min at 1325xg, followed by decanting of the fluid. The cells were again re-suspended in 1mL PBS and vortexed gently for approximately 10 seconds. A volume of 100µL of 4% paraformaldehyde was then added to the cells and left to sit for 15min. This was followed by the addition of 100µL of 0.1% Triton X-100 and the mixture allowed to sit for 15min, after which, 2mL of PBS was added to the cells. The cells were spun down for 5 min at 1325xg and the fluid was decanted, and the cells were re-suspended in 1mL of PBS and vortexed gently for approximately 10 seconds, followed by the addition of 1µL of cyclin B antibody (Cyclin B1 Rabbit anti-Human, FITC, Clone: 2061A, Novus Biologicals™). Immediately afterward the cells were incubated on ice for 30 minutes, vortexing gently for approximately 10 seconds every 10 minutes. Following the incubation period, 2mL of PBS was added to the cells, and left to sit for 5min. Cells were then spun down for 5 min at 1325xg and the fluid decanted. The remaining pellet was re-suspended in 1mL of PBS and vortexed gently for approximately 10 seconds.

Flow Cytometry

An Applied Biosystems Attune Flow Cytometer (Life Technologies) was used to detect the levels of cyclin B in the THP-1 cells that were incubated in hibernating bullfrog plasma, non-hibernating bullfrog plasma, and culture media. The flow cytometer measured the intensity of fluorescence of the fluorescent cyclin B antibody that was used to stain the THP-1 cells. Adjusting the forward scatter (FSC), green fluorescent channel (BL1), and side scatter (SSC) voltages allowed for the appropriate collection of events with the flow cytometer software, so that the appropriate THP-1 cells would be measured. The cell population was dispalyed in a dot diagram. The flow cytometer was set to analyze a maximum of 10,000 events, or 750μL of the sample, for each sample. Each sample was vortexed for approximately 10 seconds prior to running through the flow cytometer. A histogram was set up for comparison between BL1 and the event count for each sample after its flow run and a gate was set during flow cytometry to separate any debris from the cells of interest.

Statistical Analysis

The BL1 median values for the amounts of fluorescence detected from the flow cytometer assay were analyzed using the statistical software R Studio. An ANOVA test was used for comparison between the data for cells incubated in hibernating bullfrog plasma, non-hibernating bull frog plasma, and culture media to determine if any significant differences between the three groups existed. A statistical significance was recognized with a p value less than 0.05.

CHAPTER 3

RESULTS

<u>Flow Cytometry Results</u>

The data obtained through flow cytometry are displayed in Appendix 1, with the results displayed in the order of cells cultured in room temperature bullfrog plasma (RT), hibernating bullfrog plasma (H), and culture media (M), for each group. The fluorescent intensity of the cells represents the relative levels of cyclin B in that particular cell populations. By using the median value, the effects of outliers on both sides of the peak are minimized.

As seen from Figure 4, THP-1 cells grown in hibernating plasma had the lowest fold change for cyclin B relative to RT and M. The cells grown in RT plasma had a higher fold change for cyclin B than cells grown in H (14.6%), but a lower fold change for cyclin B relative to cells grown in M (19.7%). Cells grown in M had the highest fold change for cyclin B relative to RT (19.7%) and H (21%).
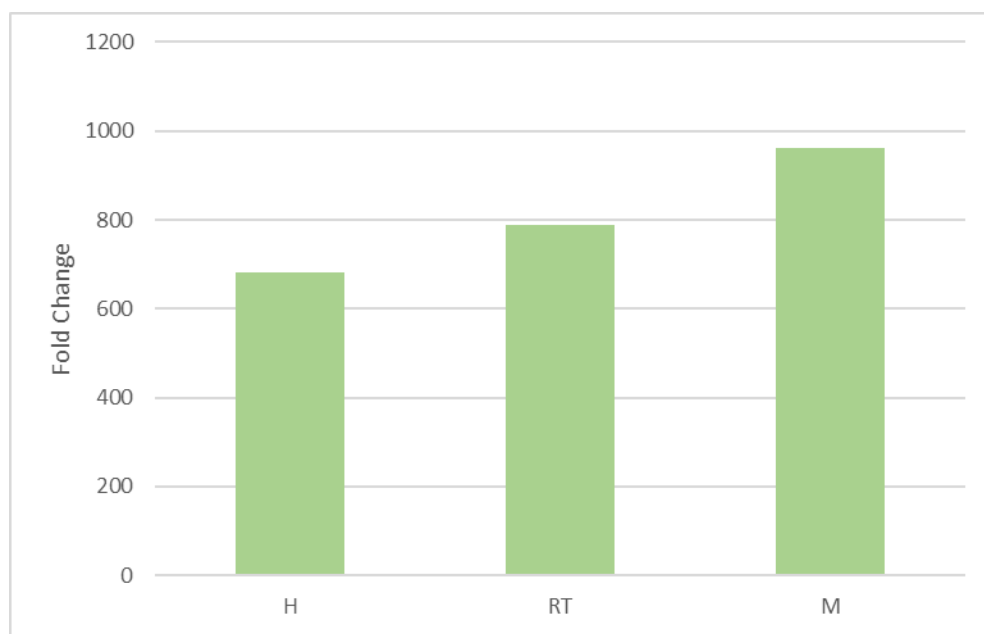
Figure 4: Average fold change in cyclin B for THP-1 cells incubated in hibernating

bullfrog plasma (H), room temperature bullfrog plasma (RT), and culture media (M).
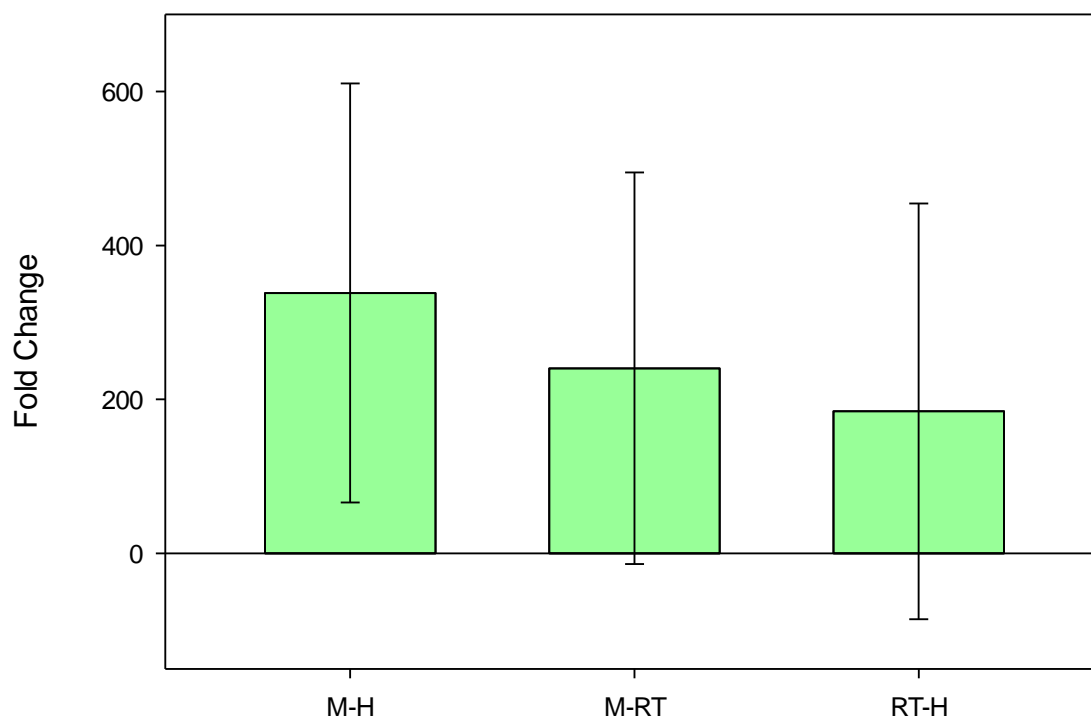
Figure 5: The difference in average fold change in THP-1 cell cyclin levels between those cultured in media and hibernating plasma (M-H), media and room temperature plasma (M-RT), and room temperature and hibernating plasma(RT-H).

The differences in fold change shown in Figure 5 indicates that there is a trend in cyclin B levels, with the highest fold difference occurring between THP-1 cells grown in media relative to those grown in hibernating frog plasma. This difference was 40.4% greater than the difference of THP-1 cells grown in media and room temperature plasma. The higher levels of cyclin B in media, shown by its greater fold change, was expected as FBS media is known to contain a large number of nutritional and macromolecular factors that are vital for cell growth. The positive difference in fold change between cells grown

in hibernating and room temperature frog plasma also indicates greater cyclin B levels in THP-1 cells cultured in room temperature plasma. However, as indicated by the error bars, there is no significant difference between these values.

<div align="center">ANOVA Results</div>

A statistical analysis of the data obtained from the flow cytometry was conducted using an ANOVA set at p value less than 0.05 to indicate significant differences between the levels of cyclin B in the hibernating and room temperature plasma. A p value of 0.259 showed no significant difference in fold change for levels of cyclin B between THP-1 cells cultured in plasma from hibernating and non-hibernating bullfrogs. No significant differences were found between cells cultured in either hibernating or non-hibernating frog plasma compared to cells grown in culture media.

CHAPTER 4

DISCUSSION

Decreasing the core body temperature during hibernation may lead to decreased enzymatic activity to a level in which cells are unable to carry out mitosis. Studies on the metabolic rate of hibernating mammalian models have concluded that the level of metabolic suppression cannot be explained by decrease in temperature alone, and there must be a physiological inhibition (Geiser, 2004). Carey et al. (2003) and Heldmaier et al. (2004) showed that physiological changes during hibernation are not just due to decreased temperature and metabolism, but there are cellular and molecular responses in play, including suppression of the cell cycle. Thus, there are several mechanisms that could be leading to suppression of cellular proliferation. Previous research by Kruman et al. (1988) found that intestinal epithelial cells of ground squirrels, *Citellus undulates,* are blocked at the G2 phase of the cell cycle during hibernation and the number of cells confined in the G2 phase slowly increases throughout their hibernation. Robbins (2017) found similar results in THP-1 cells incubated in hibernating bullfrog plasma in which a vast majority of cells had gone through the G1 and S stages but were held in the G2 stage, after DNA duplication has occurred. This suggest that one or more compounds in hibernating bullfrog plasma plays a role in controlling the cell cycle.

The CDK1-cyclin B complex regulates the progress of the G2 to M stage (Hochegger et al., 2008). My research found that the mechanism of cell cycle control in THP-1 cells incubated in hibernating bullfrog plasma is not due to the control of the levels of cyclin B although my results do show that there is a trend in fold change in

levels of cyclin B between THP-1 cells incubated in hibernating and non-hibernating bullfrog plasma, with a difference of 14.3% between the two groups. However this is not a significant difference as shown in Figure 4 with a p value of 0.259 from the ANOVA. If the levels of cyclin B were not altered in THP-1 cell grown in hibernating bullfrog plasma, other mechanisms must be involved.

Decreased levels of CDK1 may also be a potential reason as to why there is an inhibition in the G2 to M stage. If the levels of CDK1 are lower in THP-1 cells incubated in hibernating bullfrog plasma, it may cause inhibition of the cell cycle progression from the G2 stage. In order for progress from the G2 to M stage, cyclin B binding to CDK-1 is an essential part of activating the CDK1 kinase. Therefore even if the levels of cyclin B don't decrease, lower levels of CDK1 may prevent the formation of sufficient CDK1-cyclin B complexes that results in lower levels of kinase activity. Onufriev et al., (2016) found an increased expression of CDK1 levels in the hippocampus towards the end of winter hibernation in long tailed ground squirrel (*Spermophilus undulates).* This suggests that expression of CDK1 might be downregulated during hibernation and alternatively the active status of CDK1 might be altered without a change in expression.

The CDK1-cyclin B complex has been shown to partially phosphorylate Bcl-xL (an anti-apoptotic protein that plays an important role in the development, differentiation and clonal selection of B cells) during normal mitosis, causing its activation (Terrano et al., 2010). Terrano also showed that Bcl-xL is highly phosphorylated when mitosis occurs for a long period of time, suggesting that there is a change in the time period that CDK1 is activated, from being active for a short period of time during mitosis to

becoming highly active over a long period of time during mitotic arrest, which dramatically increases the amount of phosphorylation of Bcl-xL, deactivating its anti-apoptotic function. Detecting levels of phosphorylation of Bcl-xL could be an indication of how long CDK1 has been active or inactive in B cells. If a similar anti-apoptotic protein could be detected in THP-1 cells, its level of phosphorylation could potentially be used as an indication of CDK1 activity in THP-1 cells as the phosphorylation status of the CDK1-cyclin B complex also plays a crucial part in progression through the G2 to M stage of the cell cycle. Conducting further research on levels and activity of CDK1 may provide an explanation for this suppression in cellular proliferation.

The levels of cyclin activating kinase (CAK), Myt1, Wee1, and cell division cycle phosphatase (CDC25) also play significant roles in the control of the cell cycle. CAK is responsible for regulating phosphorylation of Threonine161 (Lolli & Johnson, 2005), which is required for activation of the CDK1-cyclin B complex. Lower levels of CAK in THP-1 cells cultured in hibernating bullfrog plasma might imply that the CDK1-cyclin B complex cannot be activated. This may stop further progression of the cell cycle after the G2 phase. Higher levels of Myt1 and Wee 1 in THP-1 cells cultured in hibernating bullfrog plasma might imply that a majority of the CDK1-cyclin B complexes are held inactive. Myt 1 and Wee1, two inhibitory protein kinases, are responsible for phosphorylating the CDK1-cyclin B complex at T14 and Y15, two negative regulatory sites on the CDK1-cyclin B complex (Squire et al., 2005; Hochegger et al., 2008; Fattaey & Booher, 1997). This may cause most of the CDK1-cyclin B complexes to stay

phosphorylated at the negative regulatory sites and stop further progress of the cell cycle after the G2 stage of the cell division.

CDC 25 is a protein phosphatase that is responsible for removing the phosphate groups of T14 and Y15 during the later stage of the G2 stage (Donzelli & Draetta, 2003). This activates the CDK1-cyclin B complex and triggers the initiation of mitosis. If the levels of CDC 25 are lower in THP-1 cells cultured in hibernating bullfrog plasma, it may inhibit dephosphorylation of the negative regulatory sites of the CDK1-cyclin B complex that results in a vast majority of the CDK1-cyclin B complexes remaining inactive, as they remain phosphorylated at their negative regulatory sites, preventing progress into mitosis (Hochegger et al., 2008).

Rieder and Cole (2002) suggests low temperatures, as low as 4º C, cause shock to cells and cellular programs. This can possibly trigger pathways, such as protein cascades, that can slow down and arrest cells from progressing through G2 stage. They suggest that the p53/p21 pathway (a pathway that is typically activated due to DNA damage, and causes the cell cycle to arrest) is active and is inhibiting CDC by p21 which is known to slow down the progression from the G2 to M stage. Rieder and Cole (2002) also suggest the presence of other pathways that are activated due to stress experienced during hibernation, and that can act as brakes to slow down progression of mitosis. MacDonald and Storey (2005) analyzed responses of mitogen-activated protein kinase (MAPK) family members (a type of protein kinase that is specific to the amino acids serine and threonine) during mammalian hibernation in five organs of Richardson's ground squirrels, *Spermophilus richardsonii.* They observed changes in extracellular signal-

regulated protein kinases (ERK), and c-jun NH$_2$-terminal kinases (JNK), all belonging to the family MAPK. These play an important role in cellular processes during hibernation such as regulating apoptosis. Their findings suggest that the signal transduction pathways of these MAPKs may cause transcription of genes that are associated with hibernation. MacDonald and Storey further infer that these kinase cascade pathways may execute specialized programs to survive conditions associated with hibernation. The THP-1 cells confined to the G2 stage when incubated in hibernating bullfrog plasma may be a result of such a program. However the initiation of such pathways in THP-1 cells in my study would be regulated, not by temperature, but by one or more compounds in hibernating plasma. Those possible compounds may have been a product of environmental stress.

A review study by Carey et al. (2003) suggests that for hibernating animals, seasonal changes are likely achieved by changing steady-state levels of proteins that serve a specific task by altering the abundance of the corresponding mRNA and protein. Changes in transcription, translation, and stability of both DNA and proteins may be brought about by rapidly reversible molecular switches, such as phosphorylation, and these changes regulate protein activity during hibernation. McMullen and Hallenbeck (2010) found phosphorylation levels of protein kinase B (a serine/threonine kinase that plays an important role in cellular processes) in 13-lined ground squirrels, *Ictidomys tridecemlineatus*, at two regulatory sites, threonine 308 and serine 473 to be about 2.0-fold and 3.2-fold higher, at the beginning of torpor and during arousal than when exposed to cold temperatures. This resulted in overall reduction in phosphorylation during the middle of the torpor.

The phosphorylation status of the CDK1-cyclin B complex may also result in its ability to phosphorylate Mcl1, an anti-apoptotic protein, which is degraded by phosphorylation in cells that cannot further progress in the cell cycle (Harley et al., 2010). This mechanism may prevent THP-1 cells that cannot further progress from G2 to M phase from undergoing apoptosis and to remain in the hibernating frog plasma even though they cannot progress further in the cell cycle. Thus, changes in kinase activity on proteins during hibernation may play an important role in the G2 stage for regulating the activity of essential proteins such as CDK1 which allows for progression into mitosis (MacDonald & Storey, 2005; McMullen & Hallenbeck, 2010).

Yan et al. (2008) studied differences in gene expression in seasonal and torpor-arousal cycles in the arctic ground squirrel *Spermophilus parryii*. Among their findings were differences in gene expression that are linked to apoptosis and protein protection. Using techniques of real time PCR, they found overexpression of genes involved in cell growth and apoptosis in non-hibernating arctic ground squirrels as compared to the arctic ground squirrels during torpor. They also observed a significant overexpression of genes involved in cell growth and apoptosis of arctic ground squirrels in arousal as compared with arctic ground squirrels in torpor. Their findings suggest a decrease in mRNA levels of genes involved in cell growth and apoptosis during torpor as compared to periods of non-hibernation and arousal.

Previous research on sea cucumbers, *Apostichopus japonicus*, suggests epigenetic changes during hibernation (Zhu et al., 2016). They found methylation levels of cyclin B genes to be significantly different during aestivation, compared to non-aestivation.

Methylation of DNA suppresses gene expression and they found expression of cyclin B transcripts to be down regulated during deep aestivation. Alvarado et al., 2015) also found DNA methylation to be altered during hibernation in liver and skeletal muscle cells of thirteen lined ground squirrels, *Ictidomys tridecemlineatus.* They observed hypomethylation of DNA in skeletal muscles in these animals during deep torpor, and global changes in methylation levels of DNA in liver cells. Yan et al. (2008) also discovered overexpression of genes responsible for molecular transport in arctic ground squirrels during torpor and arousal as compared to periods of non-hibernation that might result in an increase in cellular transportation of various substances, specific to hibernation.

For most mammals, lack of muscle use for long periods of time results in muscle atrophy. Previous research on green-striped burrowing frogs, *Cyclorana alboguttata*, found these animals suffer only minimal muscle damage after remaining dormant for several months (Society for Experimental Biology, 2014). Survivin is one of the genes identified in these burrowing frogs. The survivin protein appears to protect cells from apoptosis which normally removes damaged or diseased cells. Interestingly, this gene has also been shown to be highly active in human cancer cells. Tanaka et al., (2000) discovered the expression of survivin to inhibit apoptosis in cancer cells such as breast carcinomas. Immuno-histochemical analysis showed that survivin expression was positive in 118 of 167 (70.7%) cases of breast carcinomas of histological stages I to III. Under normal circumstances, cells impeded in mitosis undergo apoptosis (Harley et al., 2010), however in the presence of survivin apoptosis is inhibited. If survivin is expressed in THP-1 cells grown in hibernating bullfrog plasma, it has the potential to prevent

apoptosis in those cells that are held in the G2 stage. Positive cases of survivin were strongly associated with bcl-2 expression, a gene that encodes for proteins such as Bcl-xL that prevent apoptosis in cancer cells (Terrano et al., 2010). Their results suggest that inhibition of apoptosis by survivin, alone or in cooperation with bcl-2, is a significant prognostic parameter of worse outcomes in breast carcinomas. Cancer cells undergo uncontrolled mitosis, however, THP-1 cells in hibernating bullfrog plasma are confined in the G2 stage, and undergo controlled mitosis. As such, survivin may potentially be playing a similar mechanism in THP-1cells as it does in cancer cells.

Testing for levels of survivin in hibernating bullfrog plasma may allow us to see if there is a positive correlation between survivin and inhibition of the cell cycle progression from the G2 phase. If hibernating bullfrogs use survivin in order to prevent atrophy of their muscle, it may also be present in their plasma and prevent apoptosis of THP-1 cells. This could be supported by looking for levels of survivin in the plasma of hibernating bullfrogs

Further research in gaining a deeper understanding of how this mechanism that hibernating bullfrogs use to suppress progression of the cell cycle is very important and has significant applications in a wide array of fields. These underlying mechanisms can help make organ donations more viable. Extracting the substance responsible for the mitotic inhibition and decreasing the metabolic rate could increase the viability of a donated organ, and prevent it from undergoing damage, thereby increasing the success rate of organ transplantations. Further research on finding the underlying causes of this mechanism can also help find better treatments for cancer patients. If this substance that

is suppressing mitosis can be identified and extracted from hibernating bullfrog plasma, it has the potential to slow cancer cells from proliferating, and increase a patient's chances of survival by providing more time as better cancer treatments become available.

A potential weakness of my study was the use of human THP-1 cells rather than an ectothermic cell line. I used THP-1 cells (human cancer cells) in my study, and not frog cells, incubated in bullfrog plasma, since THP-1 cells are easier to manipulate and work with. The conditions of the incubator used in this study were 37°C and 5% carbon dioxide for all my experiments, including incubation of THP-1 cells in hibernating bullfrog plasma (plasma taken from bullfrogs exposed to 4°C). Previous studies have found that mammalian immortal cell lines, such as HeLa cells, incubated at 4°C for an hour and returned to 37°C, have mitotic activity that was reduced for approximately 17 hours, after which it increases for two hours, with 60-80% cells resuming mitosis (Manabe, 1969; Newton & Wildy, 1959). This study provides evidence that HeLa cells behaved similarly to frog cells under the same environmental conditions, which further supports using THP-1 cells in my study. I believe similar results of this study would have occurred had I used a frog cell line to study the levels of cyclin B in frog cell incubated in hibernating bullfrog plasma, compared to non-hibernating.

Previous research by Robbins (2017) found THP-1 cells incubated in hibernating bullfrog plasma, *Lithobates catesbeianus,* to be confined to the G2 stage of the cell cycle, unable to progress into mitosis, unlike the THP-1 cells cultured in non-hibernating bullfrog plasma. I tested for the levels of Cyclin B in THP-1 cells incubated in hibernating and non-hibernating bullfrog plasma, as it is essential for progression into

mitosis from the G2 stage, and previous studies have indicated that cyclin B expression may actually be decreased during hibernation (Zhu et al., 2016; Onufriev et al., 2016). My results show that there is no significant difference in cyclin B levels between THP-1 cells cultured in hibernating and non-hibernating bullfrog plasma (Figure 4).

Studying the activity of CDK1 can indicate if it is responsible for preventing THP-1 cells from undergoing mitosis, when cultured in hibernating bullfrog plasma, since activation of CDK1-cyclin B complex is essential for the progression of THP-1 cells from the G2 to M stage. Studying the activity of other proteins such as CAK and CDC25, which are responsible for phosphorylating CDK1-cyclin B complex at its stimulatory regulatory site and dephosphorylating its negative regulatory site respectively, can also show if there is a change in activity of CDK1-cyclin B complex due to phosphorylation status. Further, this could determine if they play a role in preventing cells from progressing into mitosis from the G2 stage. Looking for epigenetic changes, such as down regulation of genes that express proteins involved in the G2-M stage of mitosis and upregulation of genes expressing proteins that suppress the cell cycle would provide information regarding regulation of the cell cycle at the level of gene expression. Expression and activity of survivin in hibernating and non-hibernating bullfrog plasma might indicate a possible mechanism in which survivin is involved in preventing THP-1 cells from undergoing mitosis when cultured in hibernating bullfrog plasma.

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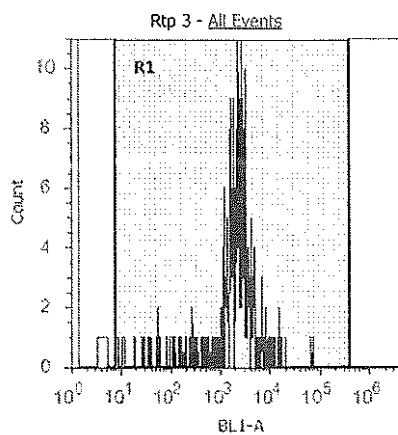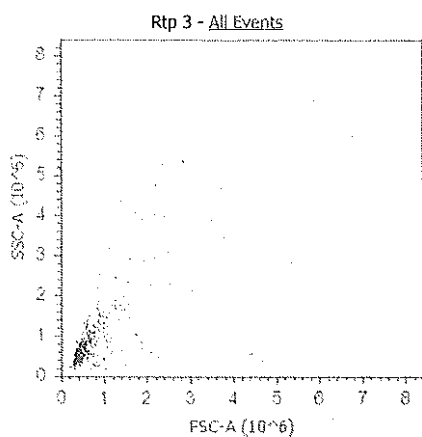
APPENDIX
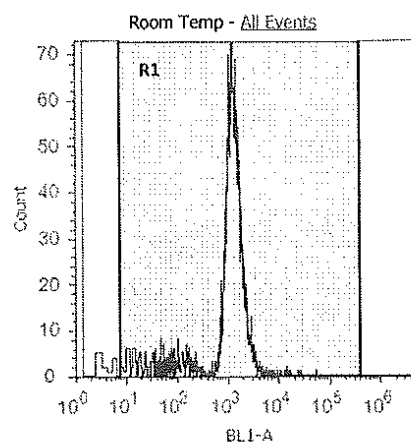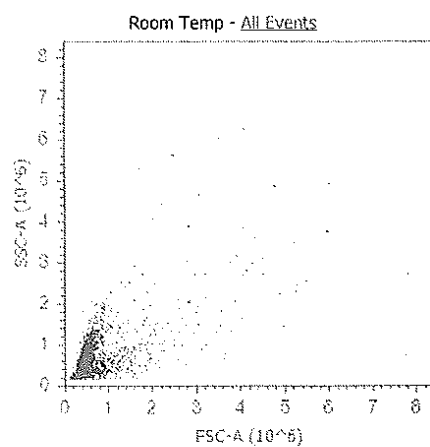
Room Temp - All Events
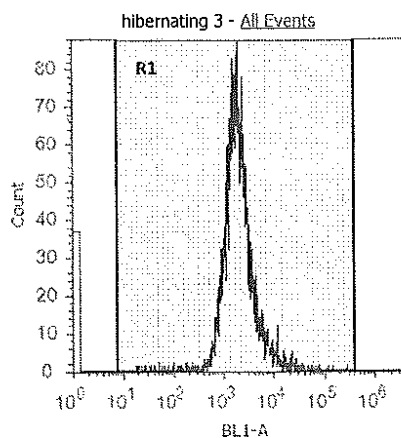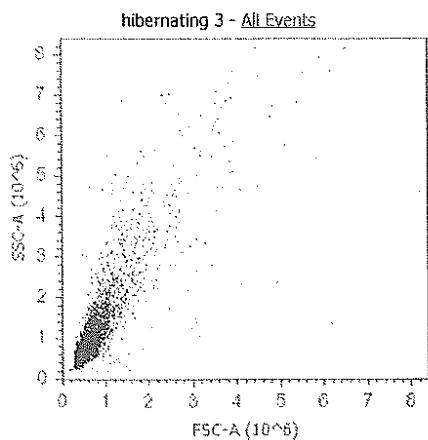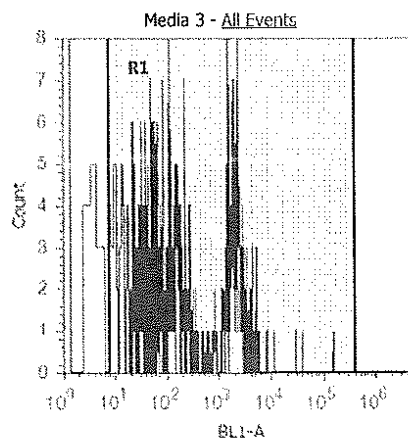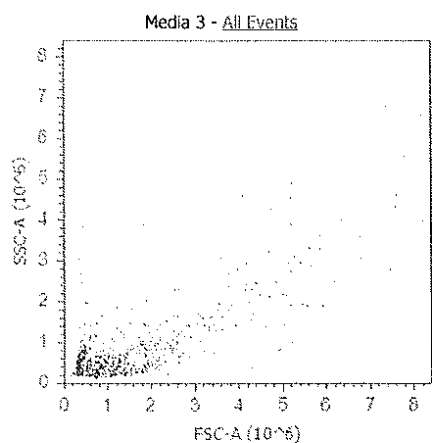

Room Temp - All Events

Parameters: **BL1-A vs Count**
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Room Temp**
Showing **4,200** of **4,200** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	4,200	100.000%	100.000%	1,260	1,220
R1	3,742	89.095%	89.095%	1,426	1,288
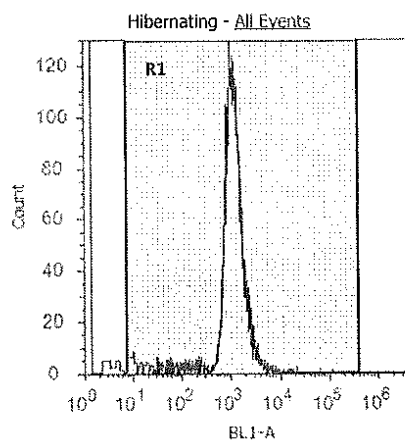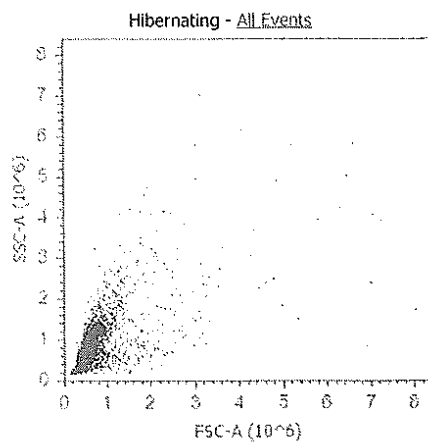
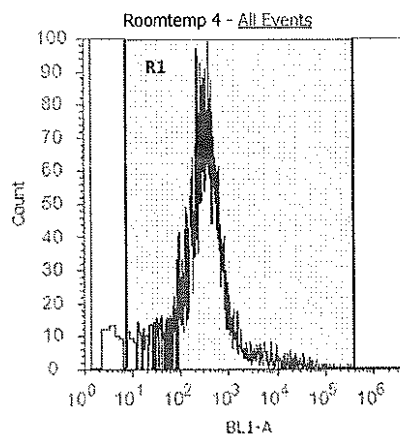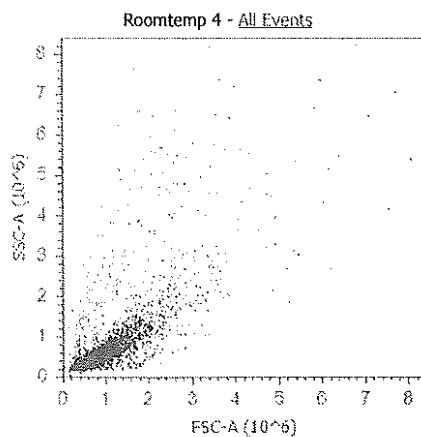### Hibernating - All Events

Hibernating - All Events

Parameters: **BL1-A vs Count**
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Hibernating**
Showing **7,856** of **7,856** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	7,856	100.000%	100.000%	1,253	1,113
R1	7,449	94.819%	94.819%	1,327	1,145
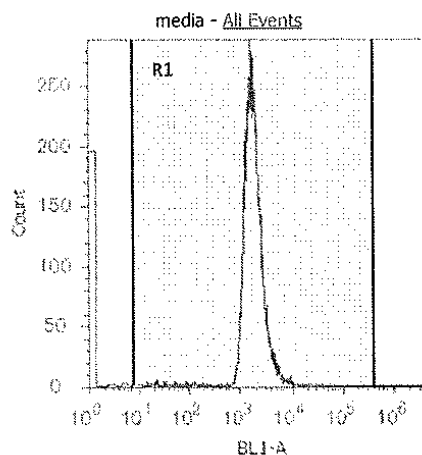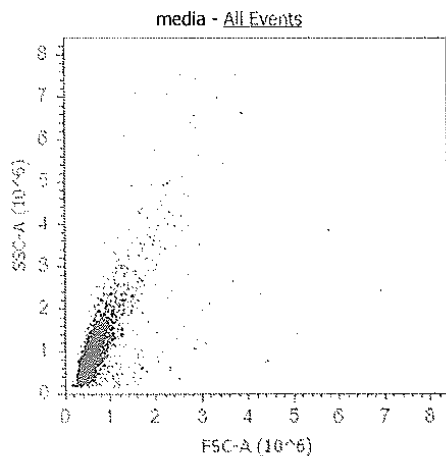
media - All Events

media - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **media**
Showing **14,648** of **14,648** events.

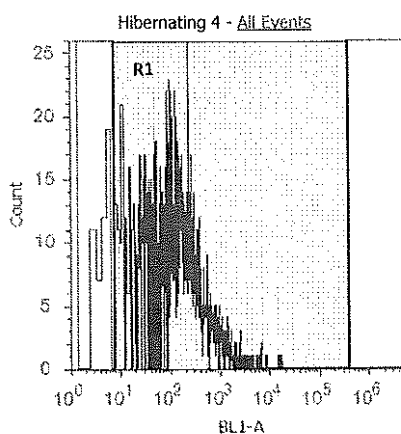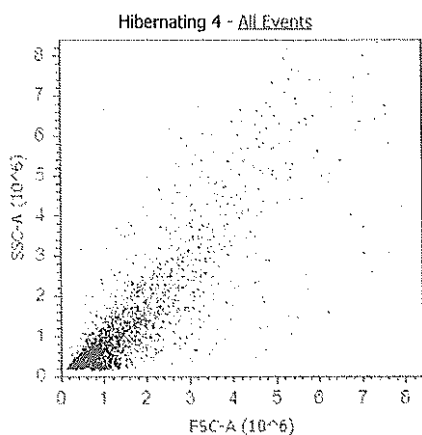| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	14,648	100.000%	100.000%	2,091	1,786
R1	14,447	98.628%	98.628%	2,121	1,799
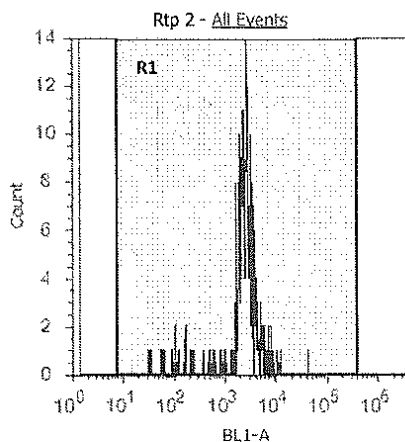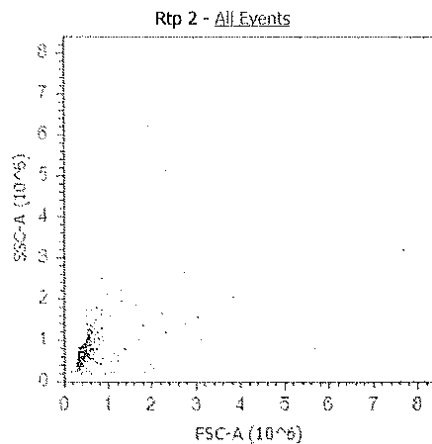
Rtp 2 - All Events

Rtp 2 - All Events
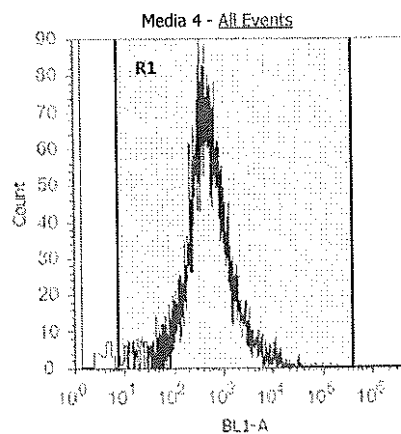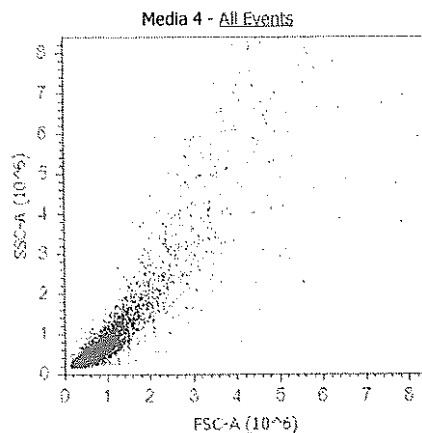

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Rtp 2**
Showing **496** of **496** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	496	100.000%	100.000%	2,719	2,555
R1	461	92.944%	92.944%	2,933	2,649
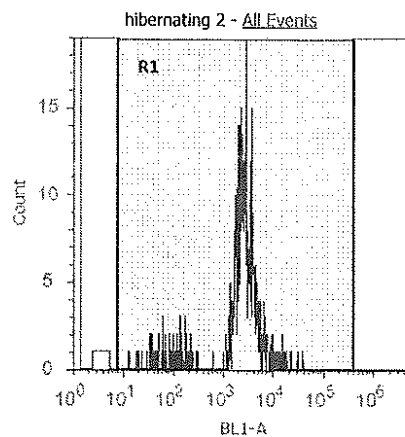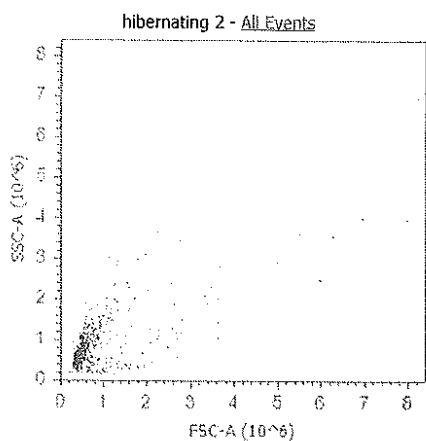
hibernating 2 - All Events

hibernating 2 - All Events

Parameters: BL1-A vs Count
Gate: Ungated
Experiment Name: Tharuka Cyclin B1
Specimen Name: Specimen
Sample Name: hibernating 2
Showing 864 of 864 events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Media
All Events	864	100.000%	100.000%	2,841	2,439
R1	781	90.394%	90.394%	3,154	2,595
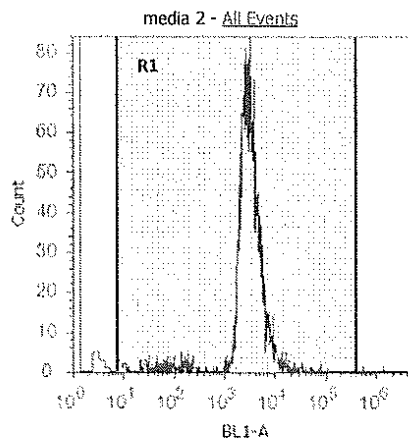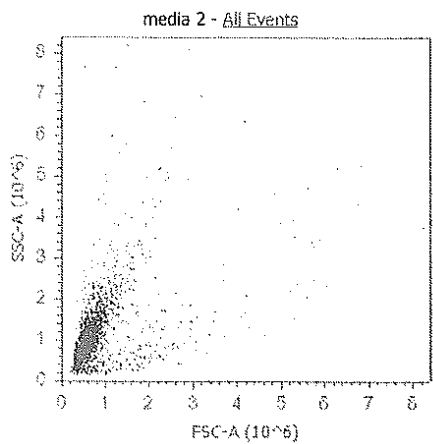
media 2 - All Events

media 2 - All Events

Parameters: BL1-A vs Count
Gate: Ungated
Experiment Name: Tharuka Cyclin B1
Specimen Name: Specimen
Sample Name: media 2
Showing 5,032 of 5,032 events.

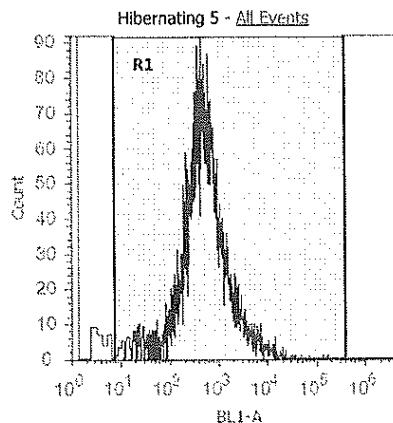| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Media
All Events	5,032	100.000%	100.000%	4,103	3,113
R1	4,827	95.926%	95.926%	3,895	3,190

Rtp 3 - All Events

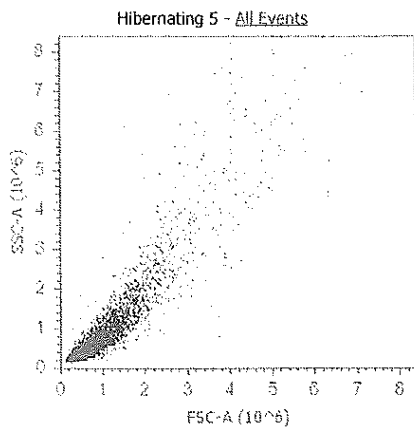Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Rtp 3**
Showing **528** of **528** events.

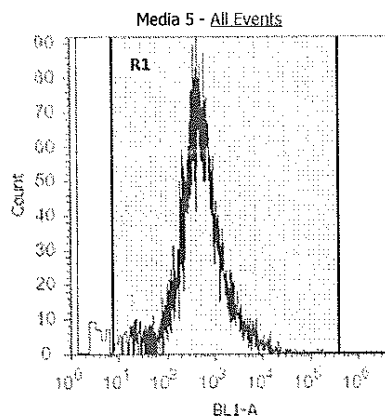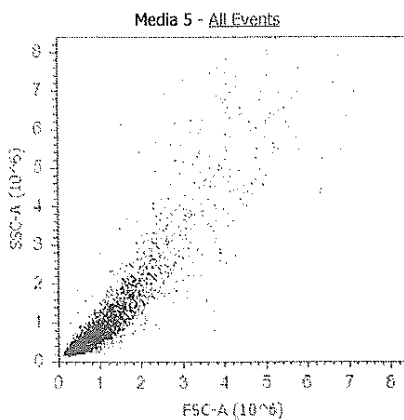| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	528	100.000%	100.000%	3,015	2,289
R1	498	94.318%	94.318%	3,202	2,337

hibernating 3 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **hibernating 3**
Showing **5,952** of **5,952** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	5,952	100.000%	100.000%	2,880	1,942
R1	5,915	99.378%	99.378%	2,899	1,949

Media 3 - All Events

Media 3 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Media 3**
Showing **1,024** of **1,024** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	1,024	100.000%	100.000%	731	51
R1	626	61.133%	61.133%	1,293	176

Roomtemp 4 - All Events

Roomtemp 4 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Roomtemp 4**
Showing **10,000** of **10,000** events.

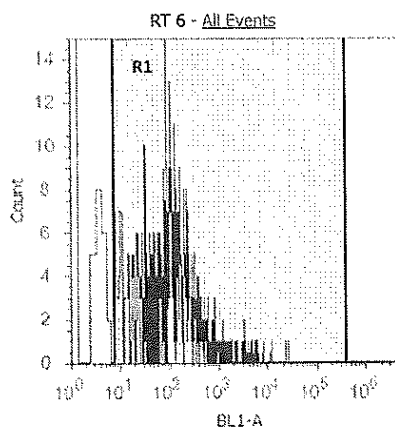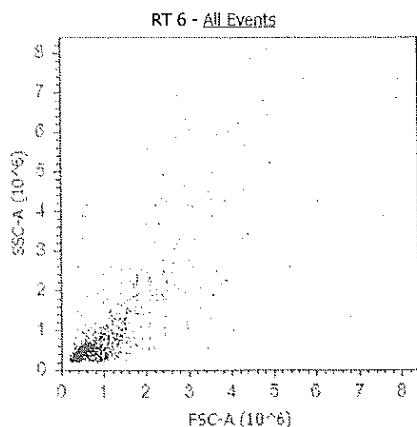| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	10,000	100.000%	100.000%	1,101	306
R1	9,214	92.140%	92.140%	1,220	331

Hibernating 4 - All Events
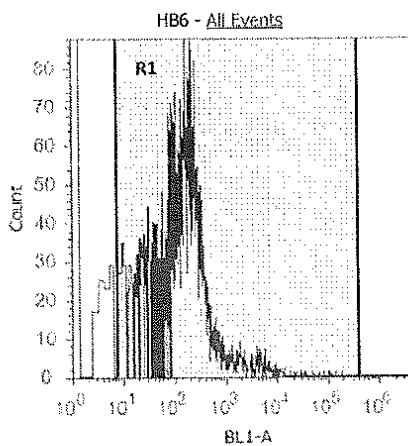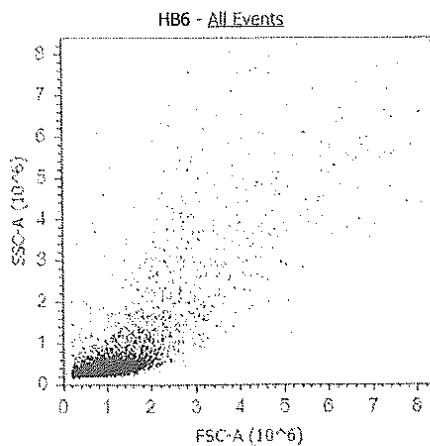
Hibernating 4 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Hibernating 4**
Showing **3,680** of **3,680** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	3,680	100.000%	100.000%	140	40
R1	2,144	58.261%	58.261%	318	135

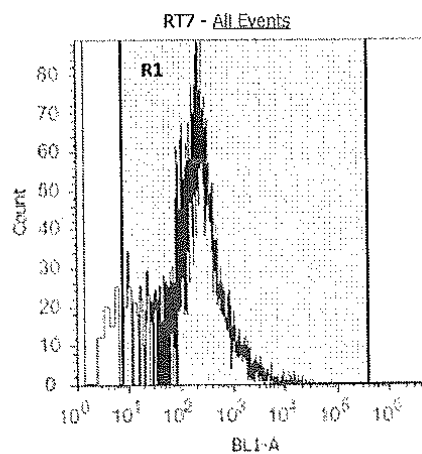Media 4 - All Events

Media 4 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Media 4**
Showing **10,000** of **10,000** events.

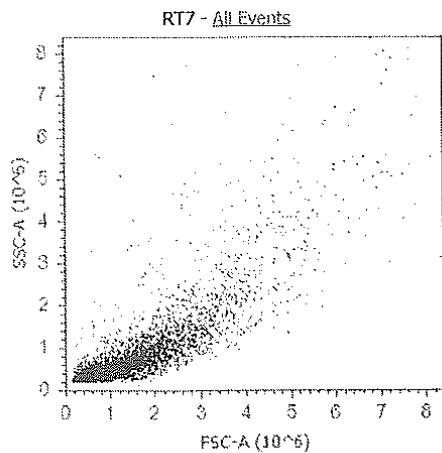| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	10,000	100.000%	100.000%	1,030	484
R1	9,518	95.180%	95.180%	1,087	513

Hibernating 5 - All Events

Hibernating 5 - All Events

Parameters: **BL1-A vs Count**
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Hibernating 5**
Showing **9,792** of **9,792** events.

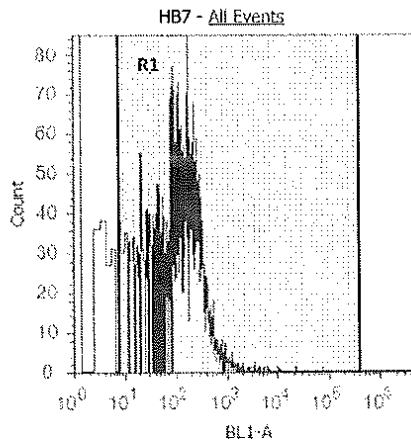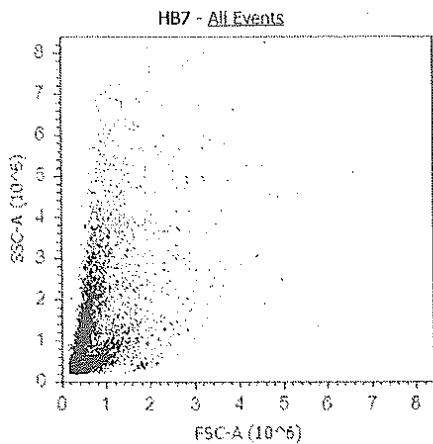| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	9,792	100.000%	100.000%	1,067	506
R1	9,410	96.099%	96.099%	1,114	529

Media 5 - All Events

Media 5 - All Events

Parameters: **BL1-A vs Count**
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Media 5**
Showing **9,792** of **9,792** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	9,792	100.000%	100.000%	1,067	506
R1	9,410	96.099%	96.099%	1,114	529

RT 6 - All Events

RT 6 - All Events
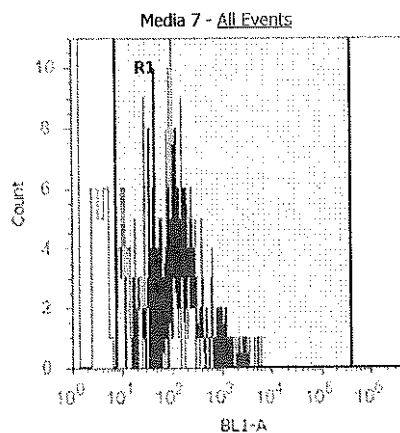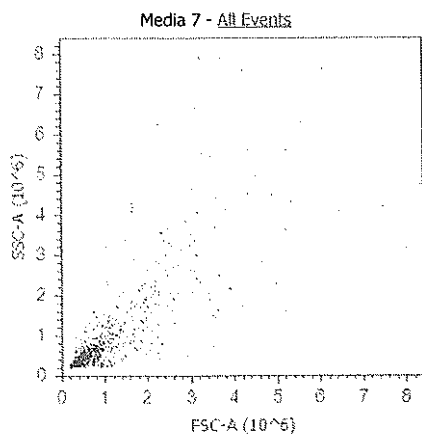

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **RT 6**
Showing **1,392** of **1,392** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	1,392	100.000%	100.000%	12	34
R1	780	56.034%	56.034%	358	130

HB6 - All Events

HB6 - All Events
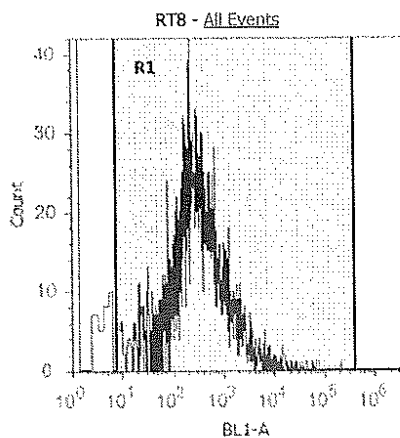

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **HB6**
Showing **10,000** of **10,000** events.

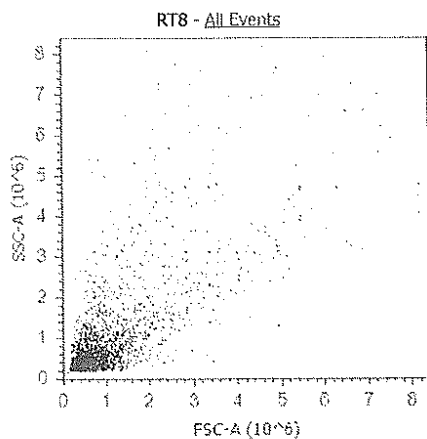| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	10,000	100.000%	100.000%	409	122
R1	7,853	78.530%	78.530%	541	168

RT7 - All Events

RT7 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **RT7**
Showing **10,000** of **10,000** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	10,000	100.000%	100.000%	399	194
R1	8,546	85.460%	85.460%	479	231

HB7 - All Events

HB7 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **HB7**
Showing **10,000** of **10,000** events.

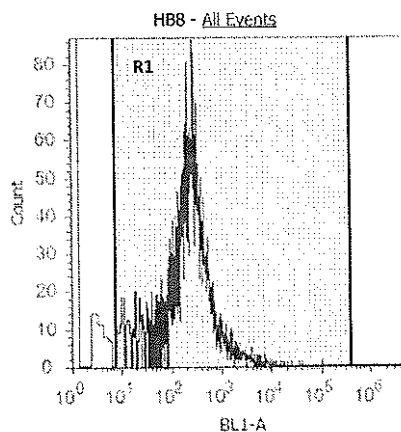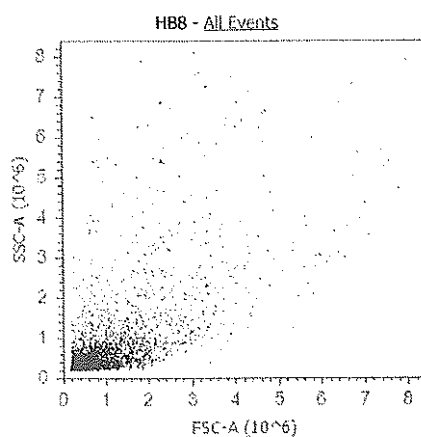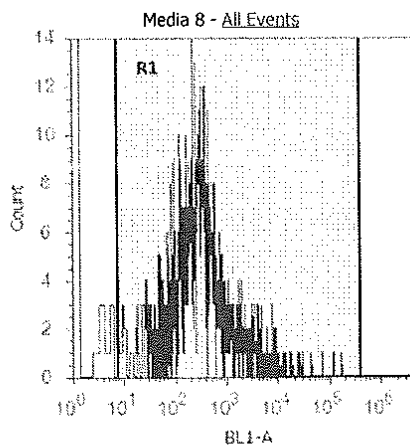| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	10,000	100.000%	100.000%	116	71
R1	6,679	66.790%	66.790%	218	139

Media 7 - All Events

Media 7 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Media 7**
Showing **1,032** of **1,032** events.

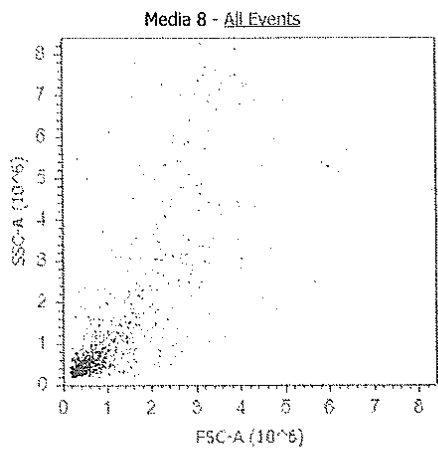| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	1,032	100.000%	100.000%	136	52
R1	638	61.822%	61.822%	280	131

RT8 - All Events

RT8 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **RT8**
Showing **4,432** of **4,432** events.

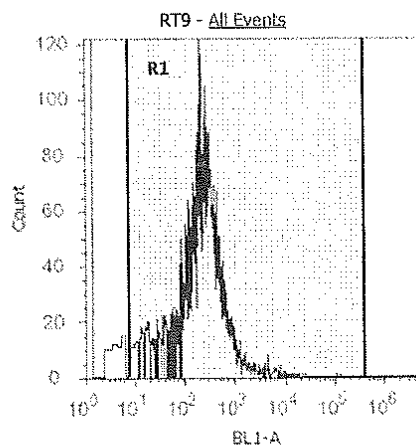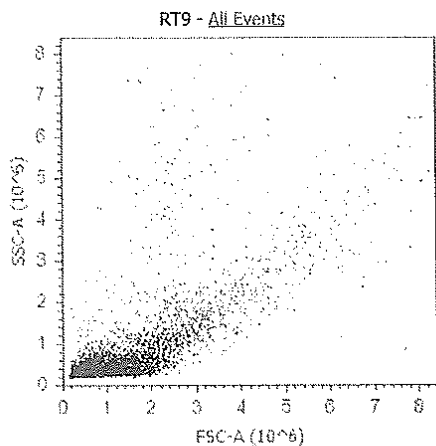| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	4,432	100.000%	100.000%	829	285
R1	3,986	89.937%	89.937%	931	329

HB8 - All Events

HB8 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **HB8**
Showing **7,128** of **7,128** events.

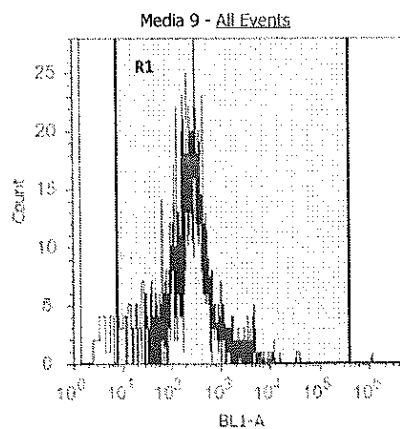| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	7,128	100.000%	100.000%	439	232
R1	6,409	89.913%	89.913%	496	258

Media 8 - All Events

Media 8 - All Events

Parameters: BL1-A vs Count
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Media 8**
Showing **1,512** of **1,512** events.

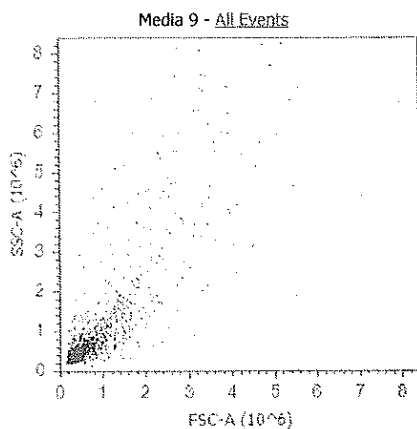| Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	1,512	100.000%	100.000%	1,091	212
R1	1,176	77.778%	77.778%	1,430	308

RT9 - All Events

RT9 - All Events

R1

Parameters: **BL1-A vs Count**
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **RT9**
Showing **10,000** of **10,000** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	10,000	100.000%	100.000%	426	227
R1	9,055	90.550%	90.550%	477	251

Media 9 - All Events

Media 9 - All Events

R1

Parameters: **BL1-A vs Count**
Gate: **Ungated**
Experiment Name: **Tharuka Cyclin B1**
Specimen Name: **Specimen**
Sample Name: **Media 9**
Showing **2,432** of **2,432** events.

Name	Event Count	% Parent	% Total	BL1-A Mean	BL1-A Median
All Events	2,432	100.000%	100.000%	947	227
R1	2,119	87.130%	87.130%	575	264